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REVIEW

GAS—LIQUID CHROMATOGRAPHY—FREQUENCY PULSE-MODULATED ELECTRON-CAPTURE DETECTION IN THE DIAGNOSIS OF INFECTIOUS DISEASES*

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*The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the U.S. Navy Department or the Naval Service at large or the Centers for Disease Control. The use of trade names is for identification purposes and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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1. INTRODUCTION

The rapid diagnosis of infectious diseases continues to be of great importance to the clinician and the diagnostic laboratory. Even with advances in the development of diagnostic methods, however, many diagnostic problems still exist such as in pneumonia, lymphocytic meningitides, arthritis, pleural effusions, and parasitic and viral diseases. Moreover, the cultural, biochemical, immunochemical, and serologic diagnostic techniques used in these areas are sometimes slow or unreliable.

Gas—liquid chromatography (GLC) offers a very sensitive and rapid technologic concept that has been applied to the study of microorganisms and to the diagnosis of infectious diseases [1—8]. The term GLC—chemotaxonomy [9] has been used to describe the application of GLC methods to the study of microbial cells, extracts, and/or metabolic products found in culture media to identify or classify a microorganism on the basis of chromatographic profiles of carboxylic acids, hydroxy acids, alcohol, and amines or fingerprints.

Although specific GLC analytical schemes have been proposed and demonstrated for use in specific diagnostic microbiology and infectious diseases studies, we believe that the GLC methodology described holds the greatest promise for use in the differentiation and identification of the etiology of infectious diseases caused by bacteria, viruses, rickettsiae, fungi, and parasites by the analysis of a variety of body fluids or excretion products of the infected host. In addition, the technique has been used [10] to identify bacterial fatty acids and metabolic products from in vitro cultures for use in taxonomic and epidemiologic studies [10—15, 30].

The technique relies on (1) the extraction of metabolic products of the microbe, the host response to the microbe, or a combination of the two; (2) the preparation of functional-group specific electron-capturing derivatives for GLC examination; (3) GLC analysis using a very specific and sensitive frequency pulse-modulated (constant current) electron-capture detector (FP-ECD); (4) high resolution columns; and (5) manual and automated analysis of the chromatographic fingerprints to establish the identity of the etiologic agent or the infectious process. Our purpose in this presentation is not to evaluate the work of other scientists in relation to the identification of bacteria through the GLC analysis of spent media or cellular materials, but to review a promising technique for aiding in the rapid diagnosis of disease by GLC—ECD analysis of body fluids, to briefly discuss its potential for application to the analysis of spent culture media and cellular materials, and to discuss in detail some aspects of the procedure.

2. EXPERIMENTAL

2.1. Source material

Synovial (SF), pleural (PF), and cerebrospinal (CSF) fluids; blood, plasma, and serum; purulent exudates; urine from well documented and culture-proven cases of infections, and spent bacteriologic, rickettsial, fungal, or viral cell culture media served as the experimental source material. Organic compounds were then extracted, concentrated, derivatized, and analyzed. Fig. 1 summarizes the extraction technique, and it should be noted that as little as 1 ml of body fluid can be adequate for many analyses.

Microbial structural components were studied. Washed bacterial cells were saponified with 5% sodium hydroxide in 50% aqueous methanol for 30 min at 90—100°C [8, 10]. After saponification, the methanolysates were acidified to approximately pH 2 with 8 *N* sulfuric acid and then extracted immediately or neutralized and stored at −20°C for further study. Carboxylic acids were transesterified [10] to trichloroethanol esters, Fig. 1, method 1, and hydroxy acids

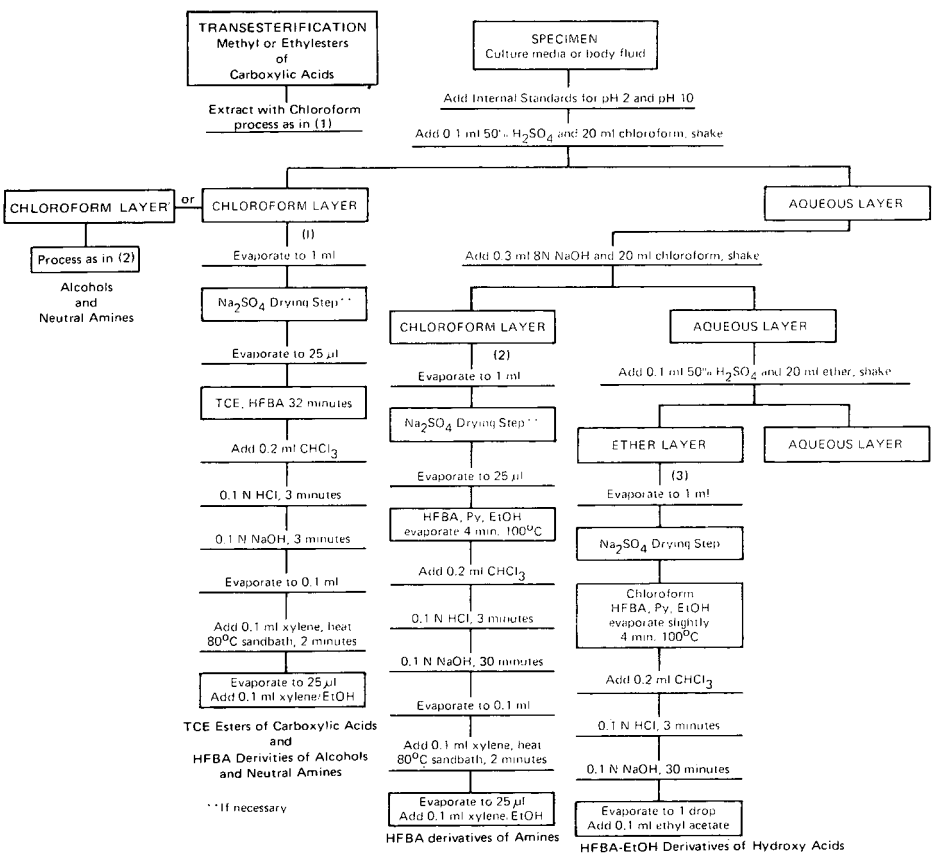


Fig. 1. Flow-chart for preparing specimens for GLC—ECD analysis. There should be *three* derivatives to be analyzed from one specimen.

TABLE 1

DETAILED PROCEDURE FOR PREPARATION OF ELECTRON-CAPTURING DERIVATIVES OF FATTY ACIDS, HYDROXY ACIDS AND ALCOHOLS, AND AMINES

I. 2,2,2-Trichloroethanol (TCE) derivatives of extracted organic acids or transesterification of esters

A. Chloroform layer (pH 2), 20 ml, in a 50-ml beaker.
B. Evaporate to 1 ml with clean, dry air.
C. Transfer 1 ml to a new, clean, 12 × 75 mm tube (can be stored at —20°C, cork stoppered, for future derivatization).
D. Remove any water by taking the 1 ml of chloroform up in a Pasteur pipette and depositing the chloroform in a clean, dry tube.
E. Evaporate to one drop with clean, dry air.
F. Add one drop TCE—chloroform (1:9) and six drops HFBA, cork.
G. Let the mixture stand 32 min at room temperature.
H. Add 0.1 ml chloroform and six drops 0.1 *N* hydrochloric acid, shake, cork, let the mixture stand 3 min.
I. Take up chloroform—hydrochloric acid mixture in a Pasteur pipette and redeposit chloroform layer in the same tube.
J. Add six drops of 0.1 *N* sodium hydroxide, shake, cork, let the mixture stand 3 min.
K. Take up the chloroform—sodium hydroxide mixture in a Pasteur pipette and redeposit chloroform layer in a clean, dry tube and discard the sodium hydroxide layer (repeat twice to remove all traces of water).
L. Add 0.1 ml xylenes and place tube in an 80°C sand bath or heating block for 2 min, uncorked, to remove all traces of TCE and most of the chloroform.
M. Evaporate to one drop, but *not to dryness.*
N. Add 0.1 ml xylenes—ethanol (50:50) as a final solvent.
O. For GLC—FP-ECD analysis use 1.4 μl (cultures) or 2.0 μl (body fluids).

II. Heptafluorobutyric anhydride (HFBA) derivatives of extracted hydroxy acids and alcohols and extracted amines

A. Diethyl ether (hydroxy acids and alcohols) extract (pH 2), 20 ml; or chloroform (amines) extract (pH 10), 20 ml, each put in a 50-ml beaker.
B. Evaporate to 1 ml with clean, dry air.
C. Transfer the 1 ml to a new, clean 12-X 75-mm tube (can be stored at —20°C, cork stoppered, for future derivatization).
D. Dry with anhydrous sodium sulfate, centrifuge, decant the ethyl or chloroform layer, add 1 ml of diethyl ether or chloroform, recentrifuge, and combine the diethyl ether or chloroform washes.
E. Evaporate to one drop with clean, dry air (for diethyl ether extract add 0.1 ml chloroform and reevaporate to one drop with clean, dry air).
F. Add one drop pyridine—chloroform—ethanol (2:4.4:1.6) solution and six drops of HFB.
G. Evaporate to one drop (to remove chloroform), cork, heat in a boiling water bath for 4 min and cool in running tap water.
H. Add 0.1—0.2 ml chloroform, six drops of 0.1 *N* hydrochloric acid, shake gently, cork, and let the mixture stand at room temperature for 3 min.
I. Take up the chloroform—hydrochloric acid mixture in a Pasteur pipette and redeposit the chloroform layer in the same tube, discard the aqueous layer.
J. Add six drops 0.1 *N* sodium hydroxide to the chloroform, shake gently, cork, and let the mixture stand for 30 min at room temperature.
K. Take up the chloroform—sodium hydroxide mixture in a Pasteur pipette and redeposit the chloroform layer in a clean dry tube; discard the aqueous layer (repeat several times to remove all traces of water).

TABLE 1 (continued)

L.	Add 0.1 ml xylenes, place tube in an 80°C sand bath or heating block for 2 min, uncorked, to remove most residual chloroform (for amines only).
M.	Evaporate to one drop (amines) or near dryness (hydroxy acids and alcohols) with clean, dry air.
N.	Final solvent for the pH 2 diethyl ether extract, 0.1 ml ethyl acetate. Final solvent for the pH 10 chloroform extract, 0.1 ml xylenes–ethanol (50:50).
O.	For GLC–FP-ECD analysis use 1.4 μl (cultures) or 2.0 μl (body fluids).

were further esterified [13] with heptafluorobutyric anhydride, Fig. 1, method 2.

2.2. Extraction and derivatization

Regardless of the source, all samples were either first esterified then extracted or extracted with appropriate solvents to yield fractions that contained organic acids. The samples of body fluids, exudates, urine or spent media were further extracted to yield fractions that contained hydroxy acids, alcohols, and amines. The process is summarized in Fig. 1. Organic acids alcohols or esters were extracted from the acidified (pH 2) sample in a 50-ml centrifuge tube equipped with a PTFE-lined screw cap by adding 20 ml of nanograde chloroform (Mallinckrodt) and shaking on a wrist-action shaker for 5 min. To obtain amines, we made the residual sample (pH 10) and again extracted with 20 ml of nanograde chloroform as stated for acids. The residual sample was reactified (pH 2) and extracted for a third time with 20 ml of reagent grade diethyl ether (Baker or Fisher) stabilized with butylated hydroxytoluene to obtain hydroxy acids. This particular acid–base–acid extraction is the beginning of the selective process to detect specific chemical components. The extraction methodology is summarized in Fig. 1.

Electron-capturing derivatives, one of the two critical components of the procedure, were then made after the samples had been extracted. The extracting solvent was reduced in volume by evaporation with clean dry air, and any residual water was removed with anhydrous sodium sulfate. Trichloroethanol–heptafluorobutyric anhydride (TCE), derivatives of carboxylic acids, and heptafluorobutyric anhydride–pyridine–ethanol (HFBA–EtOH), derivatives of hydroxy acids, and alcohols and amines were the specific electron-capturing species formed. The final solvent for the TCE and HFBA-amine derivatives is 0.1 ml of xylene–ethanol (50:50), and 0.1 ml of ethyl acetate was used as a final solvent for the HFBA–EtOH esters of hydroxy acids. It should be noted that if alcohols or neutral amines are present in the initial acid fraction extracted with chloroform to obtain carboxylic acids, they will be derivatized with the HFBA. Hydroxy acids which are obtained in the second acid fraction extracted with diethyl ether will form a diester derivative with the HFBA and ethanol in the derivatization reagent mixture. The derivatization scheme is listed in Fig. 1 and Table 1.

2.3. Gas—liquid chromatography

The second critical component of this analytical scheme was a gas—liquid chromatograph equipped with a frequency-pulsed modulated ECD and high resolution columns. We have used the Perkin-Elmer Model 3920B instrument with dual columns and temperature programming capability, but instruments of other manufacturers have also been used successfully, e.g. Hewlett-Packard Model 5830. The instrument was equipped with either one or two 7.3 m × 2 mm I.D. coiled glass columns packed with 3% OV-101 liquid phase coated on Chromosorb W (80—100 mesh, acid washed, and dimethylsilane treated; Applied Science Labs., College Station, PA, U.S.A.).

The following operating conditions apply to all analyses: (1) injection port temperature, 225°C; (2) detector temperature, 275°C; (3) detector attenuation, 512 with a standing current of 2 or less, but in actual use response obtained from standard mixtures will determine optimum setting of the standing current, with attenuation set at 512 or lower; (4) manifold temperature setting (Perkin-Elmer 3920B only), 250°C. The 5% methane in 95% argon carrier gas mixture was regulated to 50 ml/min, as measured at the detector vent with a soap-bubble flow meter, and an additional 17 ml/min of flush or purge carrier gas was introduced between the column exit fitting and before the detector (Perkin-Elmer 3920B and 900 only).

The following column oven operating temperature programming parameters were used. For the TCE (fatty acid) derivatives, the initial oven temperature was 100°C with a 4°C/min program to 265°C with a final hold of 16—32 min. The HFBA (hydroxy acid, alcohol, and amine) derivatives were analyzed with an initial oven temperature of 90°C held for 8 min, 4°C/min increase to 265°C, and the final temperature was held for 32 min or less.

In addition to the use of high resolution packed glass columns, fused-silica capillary columns are available in lengths up to 100 m with the liquid phase coated directly on the inside wall. These capillary columns, used in conjunction with the purged-splitless injectors, are now being used for specialized analyses and are also being evaluated as a replacement for the packed glass columns [16]. These fused-silica capillary columns improve component separation and can be used to shorten the analysis time by up to 50%. The carrier gas used for the capillary columns is helium and the purge or flush gas was 5% methane in 95% argon. The column flow was 3 ml/min and the combined column and flush gas flow-rate through the detector was 50 ml/min.

2.4. Chromatogram analysis and interpretation

Data generated from the GLC—FP-ECD analysis of body fluids, excretion products, and spent culture media, are complex and interpretation requires specific skills and acumen. However, it does not take long to master the skills necessary for interpretation and with completion of computer programs, now under development, interpretation of data should be made easier. However, when new columns are used, several instruments are employed or columns are repacked in the existing instruments, retention times, separation characteristics, and response characteristics will differ between instruments. In our studies

normalization of these differences has been addressed. Routine procedures were used by the Perkin-Elmer PEP-2 (or Sigma 10) microprocessor equipped with Modular Software System (MS-16 revision B) to accumulate data from the instrument, analyze the data according to a stored method, and prepare a report. Then these stored data from several instruments with differing retention characteristics were transmitted by telephone to a larger time-sharing computer. The data were stored and manipulated by this larger computer, and the data points were standardized through the analysis of standard mixtures which were then processed by a spline-fit programming technique on the time-shared system. This procedure then changed retention times to straight-chain hydrocarbon equivalence numbers for standardization of data between instruments.

A second software program is under final development and testing that will take the normalized or standardized data from individual samples and analyses with a known etiology and create a representative profile for each particular infection or organism, and report an identification match for an unknown sample based on the best possible correlation with the representative GLC—FD-ECD computer profile. In the future we hope to make this software system available to diagnostic laboratories and other interested investigators.

3. RESULTS AND DISCUSSIONS

Early developmental studies by Brooks and colleagues introduced a derivatization and high resolution flame ionization GLC technique as an alternative GLC technologic component in the anaerobic bacteria identification scheme developed by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University [17]. Brooks and co-workers [11, 18] continued this work later at the Centers for Disease Control and examined clostridia further by flame ionization and nonfrequency-pulsed mode GLC—ECD. In subsequent studies the taxonomic usefulness of the GLC—FP-ECD technology was demonstrated in the differentiation of additional *Clostridium sporogenes* [12].

Two main events contributed to the perfection of the GLC—FP-ECD technique presently in use: (1) advances in ECD development (frequency-pulsed mode), and (2) the development of specific derivatives to exploit the extreme sensitivity and selectivity of the FP-ECD.

3.1. Septic arthritis

Septic arthritis was the first infectious process to be examined by GLC—FP-ECD. Examination of synovial fluids (SF) from infected patients and uninfected controls showed conclusively that the chromatographic profile of fluid from an infected patient differed significantly from that of an uninfected one [7, 19]. In addition, GLC—FP-ECD profiles of SF obtained from patients with different types of infecting bacterial agents were distinguishable from each other, Fig. 2.

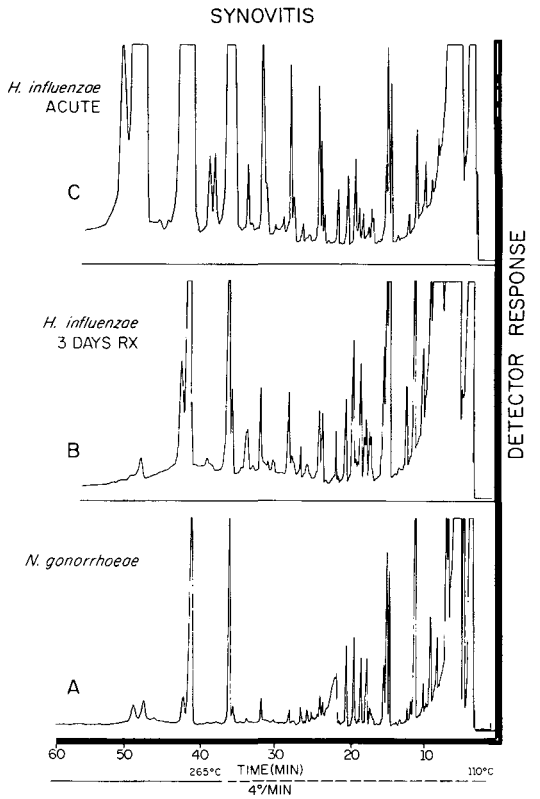


Fig. 2. GLC—FP-ECD profiles of TCE derivatized acidic chloroform extracts of synovial fluids. Etiologies of synovites are indicated in the figure.

3.2. Cystitis

Cystitis was studied concurrently with septic arthritis and a remarkable array of compounds were present in urine samples from infected patients, Fig. 3 [20]. It was also shown that some of the compounds that were present in the urine from patients were produced by the bacterium isolated from the urine sample. When the etiologic agent, *Proteus mirabilis*, was introduced into a sterile urine sample from a previously infected and antibiotic-treated patient, the GLC—FP-ECD profile was very similar to the pattern seen in the natural infection. In addition, this study also showed that *P. mirabilis* produced N-nitrosodimethylamine, a member of the highly carcinogenic nitrosamine group of compounds, both in vivo and in vitro [14, 20].

Urine was suspected to be a less than ideal body fluid or excretion product for study by GLC—FP-ECD because there was a possibility that it would undergo unpredictable composition changes due to diet, medications, etc. This proved to be partially true, and subsequent studies on urinary tract infections were temporarily suspended. Efforts were redirected toward finding a more homogeneous body fluid with which to demonstrate the diagnostic potential of the GLC—FP-ECD analytical scheme. Based on earlier work utilizing non-

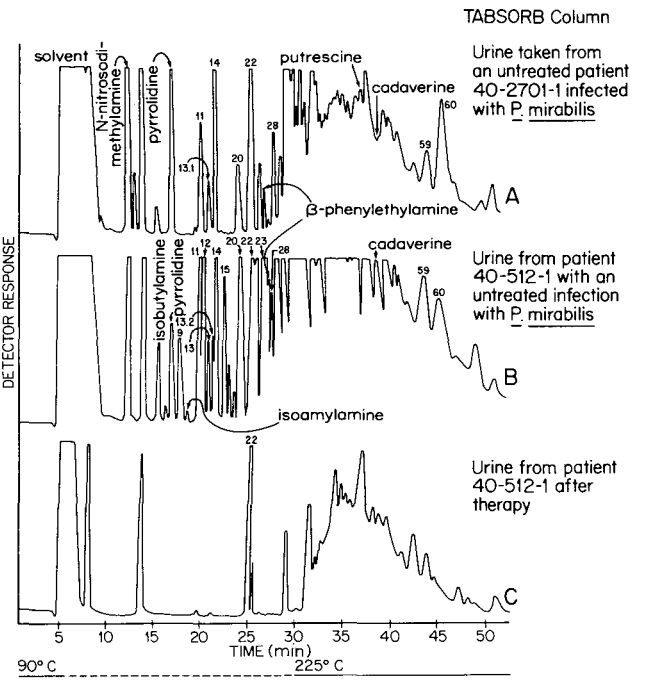


Fig. 3. Gas chromatograms of amines extracted from basic chloroform extracts of urine and derivatized with heptafluorobutyric anhydride (HFBA). (From ref. 20 with permission.)

selective detectors, such as the flame ionization detector, concerns were also raised about the composition and changes in whole blood, plasma, or serum, and these were not extensively examined in early GLC studies [16].

3.3. Meningitis and encephalitis

Attention was subsequently focused on a body fluid whose composition in the healthy individual is controlled within very rigid bounds, specifically cerebrospinal fluid (CSF). The choice of CSF and changes in CSF in meningitis cases as subjects of study was considered very relevant because meningitis and encephalitis are infections where a specific and rapid diagnosis is desirable and in some cases essential for the well-being of the patient. When conventional microscopic, cultural, or immunochemical diagnostic techniques are negative for bacterial meningitis and there is a central nervous system (CNS) infection which tests show to have a lymphocytic pleocytosis and an equivocal CSF glucose, the physician would benefit greatly from a diagnostic technique that would give a rapid definitive diagnosis.

Lymphocytic meningitides, which include tuberculous meningitis (TBM), and fungal, parasitic, and asceptic meningitis, and viral meningoencephalitis, were infections chosen to exploit. Control CSF specimens were obtained from patients receiving myelograms studies or spinal epidural anesthesia. These specimens from uninfected patients served as controls; they were relatively free

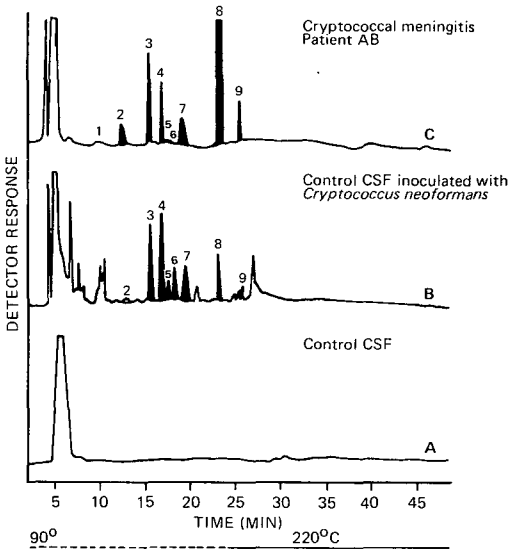


Fig. 4. GLC—ECD of HFBA derivatives from the basic chloroform extracts of CSF from *Cryptococcus neoformans*, and a normal uninfected CSF specimen obtained from a patient undergoing a diagnostic myelogram. (From ref. 21 with permission.)

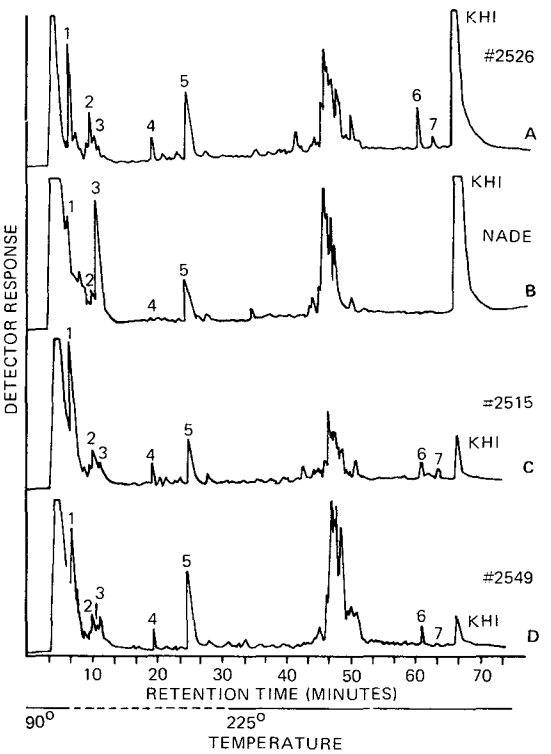


Fig. 5. GLC—FP-ECD of HFBA derivatives of the basic chloroform extracts (pH 10) from four patients with tuberculous meningitis. The peak labeled KHI has been identified by mass spectrometry as 3-(2′-ketohexyl)indoline and is a novel amine compound found in many bacterial infections. (From ref. 22 with permission.)

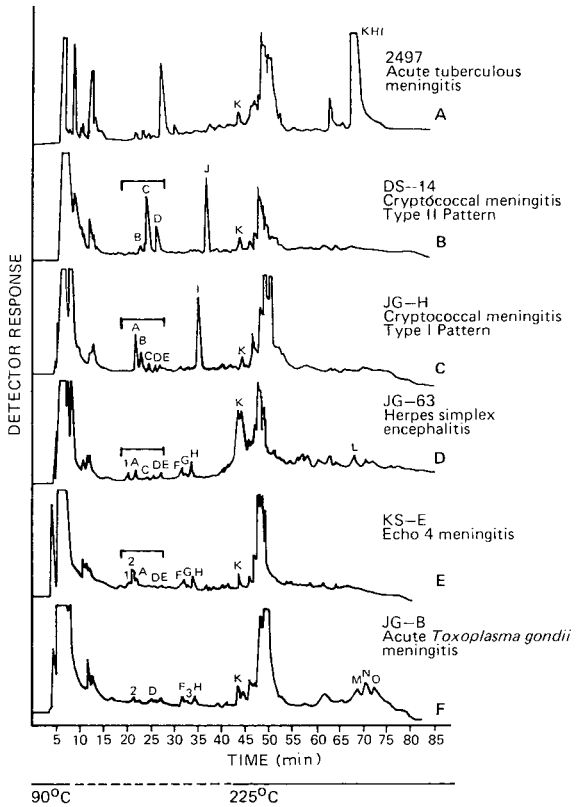


Fig. 6. Comparison of GLC—FP-ECD profiles of HFBA derivatized basic chloroform extracts of CSF samples taken from patients with the diseases indicated in the figure. (From ref. 22 with permission.)

of compounds capable of being detected by the GLC—FP-ECD procedure, and yielded a clean GLC—FP-ECD profile.

The initial infections examined were from culture-proven cases of cryptococcal (*Cryptococcus neoformans*) meningitis and aseptic meningitis caused by various echoviruses [21]. The cryptococcal specimens, though limited in number, were easily distinguishable from the echoviral ones when the basic chloroform extract HFBA derivatives (amines) were examined. Many of the compounds detected in the clinical specimens were also present when *C. neoformans* was inoculated into a sterile CSF specimen containing a supplement and incubated for seven days at 35° C. These findings suggested the possibility that some of the compounds detected were microbial metabolic products, Fig. 4.

Studies of lymphocytic meningitides were expanded with the major efforts directed toward the rapid diagnosis of TBM. Additional studies were made of cryptococcal, and various viral CNS infections [22]. A reproducible GLC—FP-ECD pattern of amines was obtained when culture-proven *M. tuberculosis* CSF specimens from Egypt were examined, Fig. 5, and this pattern was different from the patterns of the cryptococcal meningitides and viral CNS infections,

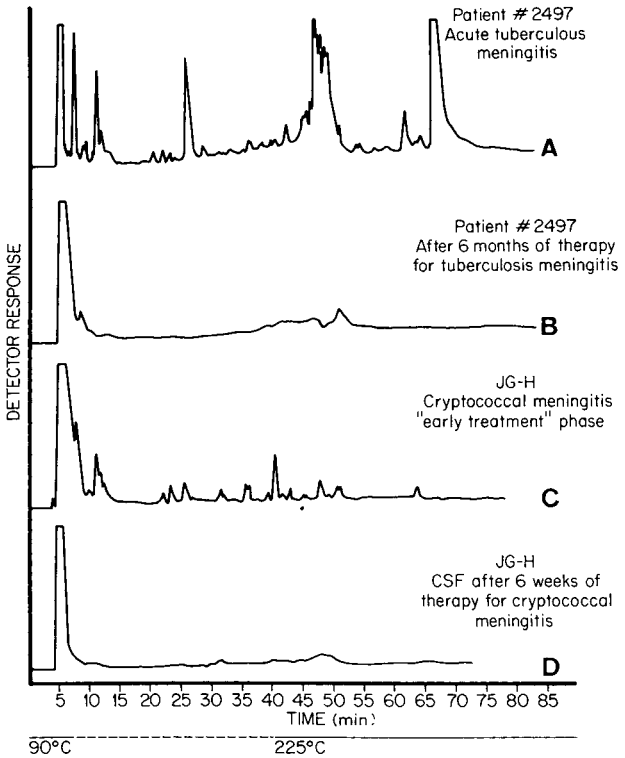
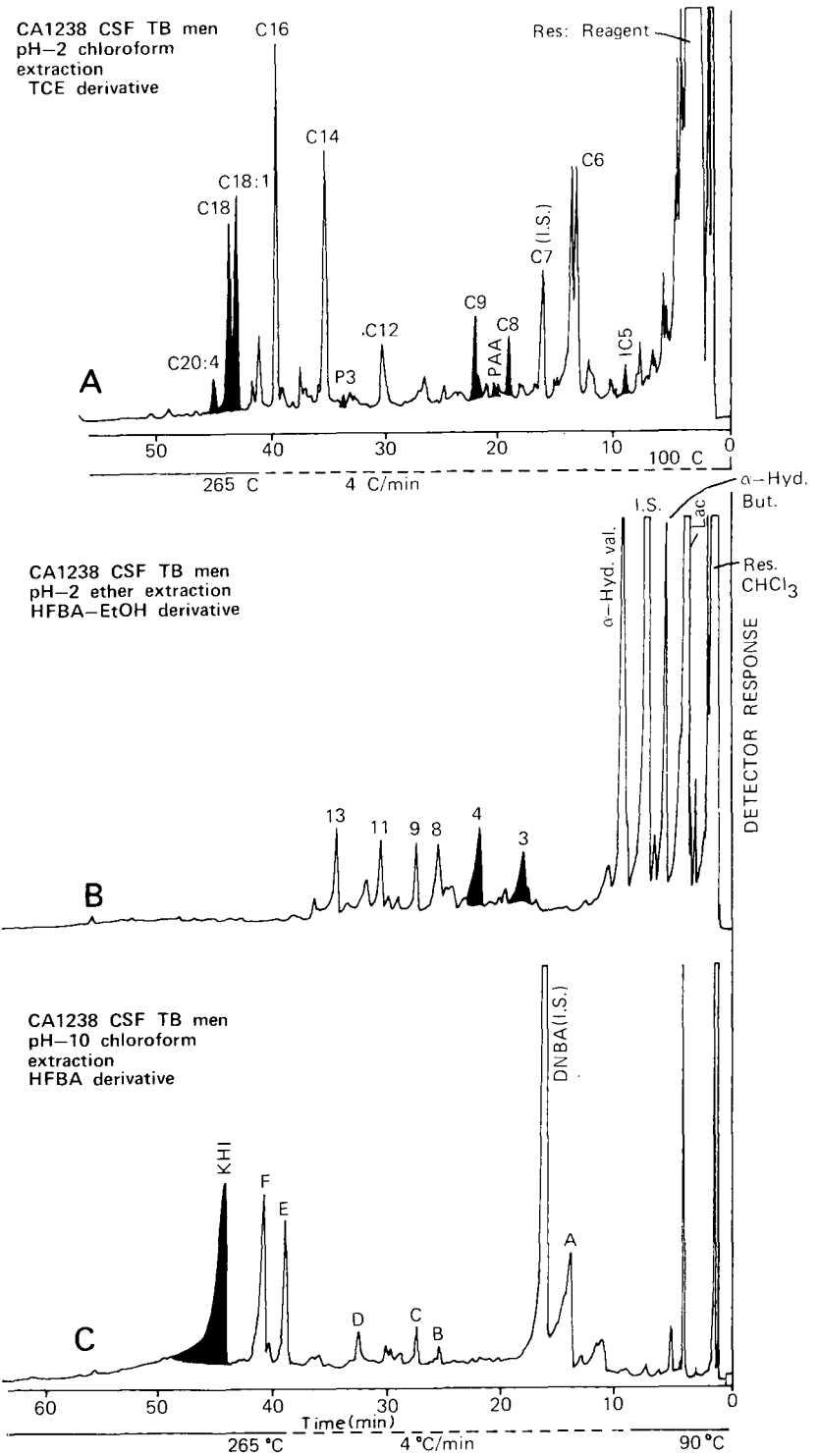


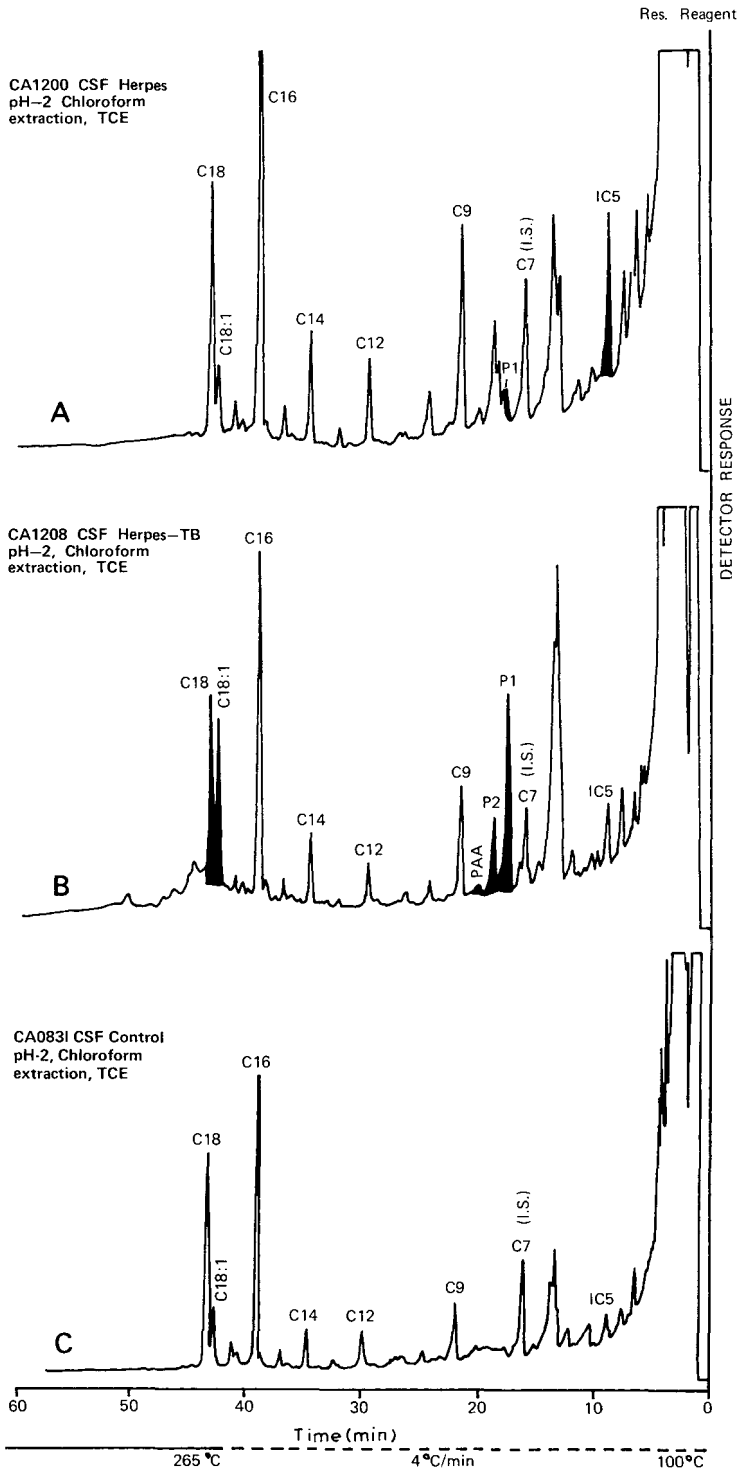
Fig. 7. GLC—FP-ECD profiles of HFBA derivatized basic chloroform extracts of CSF samples taken from patients with the diseases indicated in the figure. (From ref. 22 with permission.)

Fig. 6. In addition, when serial CSF specimens from Egyptian patients with TBM were obtained and analyzed during specific antituberculous therapy, the compounds initially detected diminished and eventually disappeared with time, Fig. 7.

The CSF specimens from acutely ill Egyptian patients yielded rather large amounts of a new amine compound which disappeared after effective therapy. Gas chromatography—mass spectrometry was eventually used to identify this amine as 3-(2'-ketohexyl)-indoline (KHI) [23]. KHI has not been previously reported in biologic materials nor had it been synthesized chemically. Interestingly, in subsequent studies KHI was not detected in CSF samples from patients with TBM in the United States [24]. The function, origin, and significance of KHI has yet to be established, but its structure is similar to certain neurotransmitters and it may play a role in the sequelae frequently seen in meningitis patients. Initially, KHI was thought to be unique to TBM and as

Fig. 8. GLC—FP-ECD profiles from the tuberculous meningitis CSF samples: (A) TCE derivatives of acidic chloroform extracts; (B) HFBA—EtOH derivatives of acidic diethyl ether extracts (third extraction); and (C) HFBA derivatives of basic chloroform extracts. (From ref. 24 with permission.)


CA1238 CSF TB men
pH–2 chloroform
extraction
TCE derivative
C16
Res: Reagent
C14
C18:1
C18
C6
C7 (I.S.)
C9
C8
C12
PAA
IC5
C20:4
P3
A
50
40
30
20
10
0
100 C
265 C
4 C/min
α–Hyd.
But.
I.S.
Lac
α–Hyd. val.
Res.
CHCl3
CA1238 CSF TB men
pH–2 ether extraction
HFBA–EtOH derivative
13
11
9
8
4
3
B
DETECTOR RESPONSE
CA1238 CSF TB men
pH–10 chloroform
extraction
HFBA derivative
DNBA(I.S.)
KHI
F
E
A
D
C
B
C
60
50
40
30
20
10
0
Time (min)
265 °C
4 °C/min
90 °C


Res. Reagent
CA1200 CSF Herpes
pH–2 Chloroform
extraction, TCE
C16
C18
C18:1
C14
C12
C9
P1
C7
(I.S.)
IC5
A
DETECTOR RESPONSE
CA1208 CSF Herpes–TB
pH–2, Chloroform
extraction, TCE
C16
C18
C18:1
P1
C9
P2
C7
(I.S.)
IC5
C14
C12
PAA
B
CA083I CSF Control
pH-2, Chloroform
extraction, TCE
C16
C18
C18:1
C14
C12
C9
C7
(I.S.)
IC5
C
60
50
40
30
20
10
0
Time (min)
265 °C
4°C/min
100°C

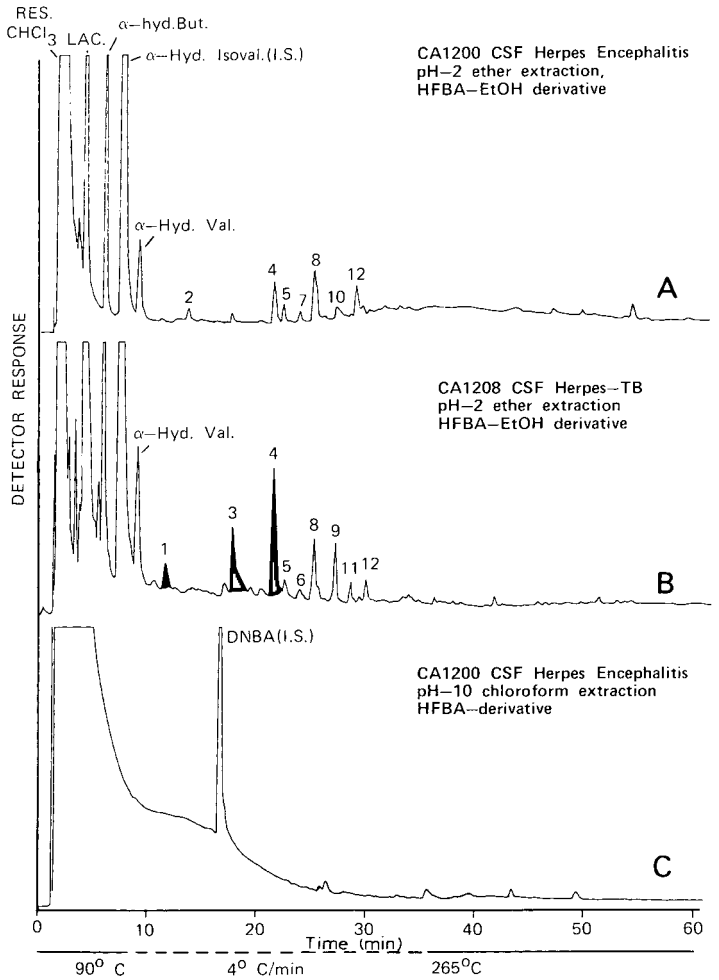


Fig. 10. GLC—FP-ECD chromatograms of: (A en B) HFBA—EtOH derivatized diethyl ether extracts of CSF taken from patients with the disease indicated in the figure, and (C) from an HFBA derivatized basic chloroform extract of CSF taken from a patient with herpes encephalitis. (From ref. 24 with permission.)

such it might be of diagnostic significance. This has not held true because KHI has since been found in CSF samples from Egyptian patients with meningitides caused by some common bacterial agents, i.e., *Haemophilus influenzae* [25], *Streptococcus pneumoniae* [16], and *Neisseria meningitidis* [16]. KHI has also been shown (Brooks et al., unpublished data) to be present in the sera of Egyptian patients with the above type infections in the United States. KHI has been detected in the serum of a child with a bacterial infection and in the serum and synovial fluid of a race horse with an acute bacterial infection. The

Fig. 9. GLC—FP-ECD profiles of TCE derivatized acidic chloroform extracts of CSF taken from patients with the diseases indicated in the figure. (From ref. 24 with permission.)

compound has thus far only been detected in bacterial infections, never in viral or parasitic infections.

Subsequent studies of additional cases of lymphocytic meningitides established that certain carboxylic and hydroxy acids and selected easily obtainable clinical data can be used effectively to rapidly diagnose TBM even in the absence of KHI, Fig. 8 [24]. This study also showed that in a well documented case the GLC—FP-ECD patterns of herpes virus meningitis/encephalitis (HVM), mixed TBM—HVM, were different, Figs. 9 and 10. This study established that KHI is not responsible for the positive color reaction in the tryptophan color test used for many years in the diagnosis of TBM. In our laboratory more than 400 specimens have been analyzed for tuberculous meningitis, and based on final reports obtained from the physician, the test has been accurate over 90%.

3.4. Pleural effusions

During the studies of meningitides, similar GLC—FP-ECD analyses were performed on a series of pleural effusions (PF) from patients with sepsis,

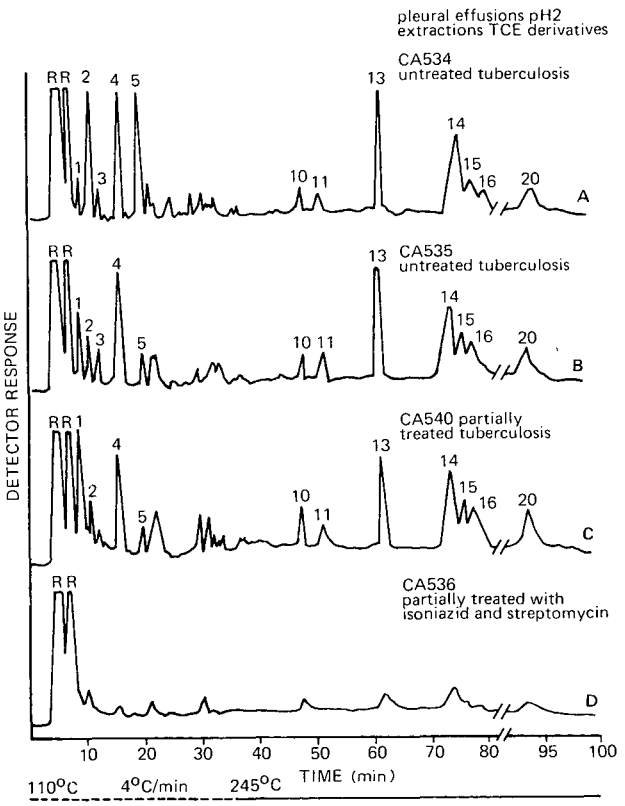


Fig. 11. GLC—FP-ECD chromatograms of TCE derivatized acid chloroform extracts of pleural fluids taken from patients with the type of disease indicated in the figure. (From ref. 26 with permission.)

congestive heart failure, malignancies, uremia, and systemic lupus erythematosus [26]. GLC—FP-ECD profiles were assigned to various groups before we received clinical information and then later we compared the profiles with the tentative diagnoses. GLC—FP-ECD analysis detected some errors made in the tentative diagnosis. The GLC—FP-ECD profiles from different types of effusions differed markedly and they may be of diagnostic value, Fig. 11 [26].

3.5. Diagnostic potential of serum

In all the above studies CSF, SF, PF, or urine were used for GLC—FP-ECD analysis. It would be very useful, however, to use a body fluid that is more easily obtainable than CSF, SF, or PF. Serum can be obtained in larger amounts with less discomfort to the patient.

A recent study used GLC—FP-ECD to analyze serum specimens from human controls and patients with the following diseases: (1) Rocky Mountain spotted

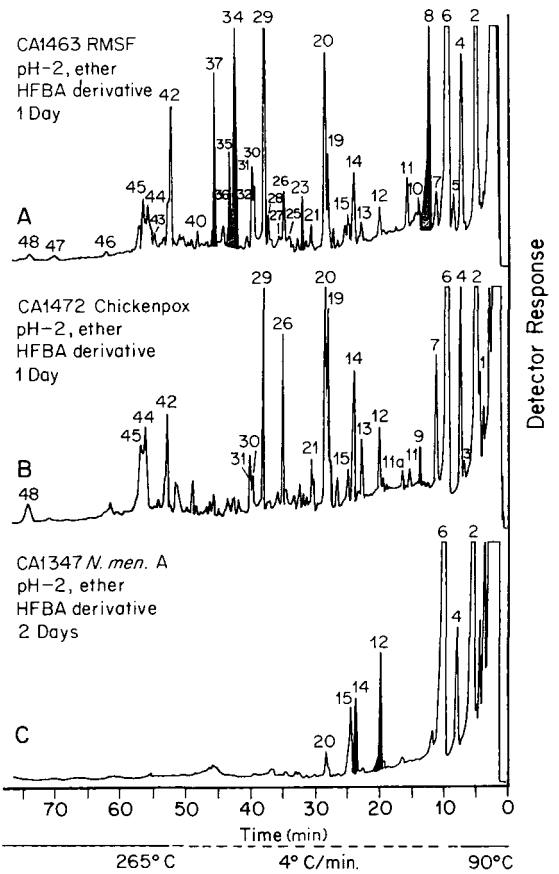


Fig. 12. GLC—FP-ECD chromatograms of HFBA—EtOH derivatized acidic diethyl ether extracts of sera taken from patients with the type of disease indicated in the figure. (From ref. 27 with permission.)

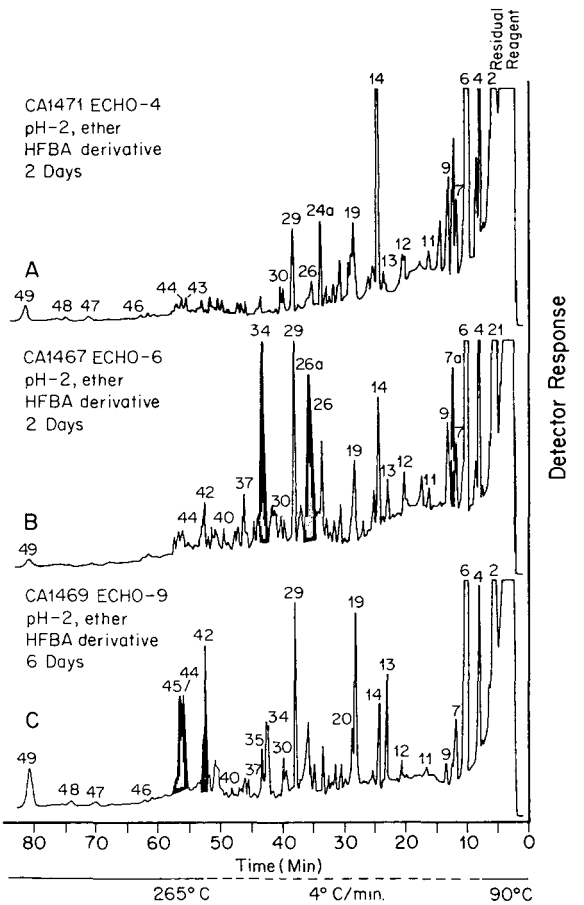


Fig. 13. GLC—FP-ECD chromatograms of HFBA—EtOH derivatized acidic diethyl ether extracts of sera taken from patients with the indicated ECHO viral infections. (From ref. 27 with permission.)

fever (RMSF), a rickettsial disease caused by *Rickettsia rickettsii*, (2) chickenpox caused by herpes virus, (3) measles, rubeola and rubella, enterovirus infections, and (4) *Neisseria meningitidis* infection. All of these diseases produce a rash and can be confusing diagnostically. GLC—FP-ECD profiles of hydroxy acids were most useful for rapidly differentiating these clinically similar diseases during the early stages (1—5 days) of infection, Figs. 12—14 [27]. A prospective study is necessary to determine the diagnostic efficacy of the procedure; however, the fact that early and acute phase sera are best for analysis is fortuitous because early diagnosis is essential in treating and managing RMSF patients.

Recent studies of sera from Egyptian patients with both *Schistosoma mansoni* and *S. hematobium* infections showed that body chemistry changes were detectable by GLC—FP-ECD using capillary columns and splitless injection [28]. Further studies are in progress to determine the reproducibility of these changes and to detect other changes in body chemistry that might

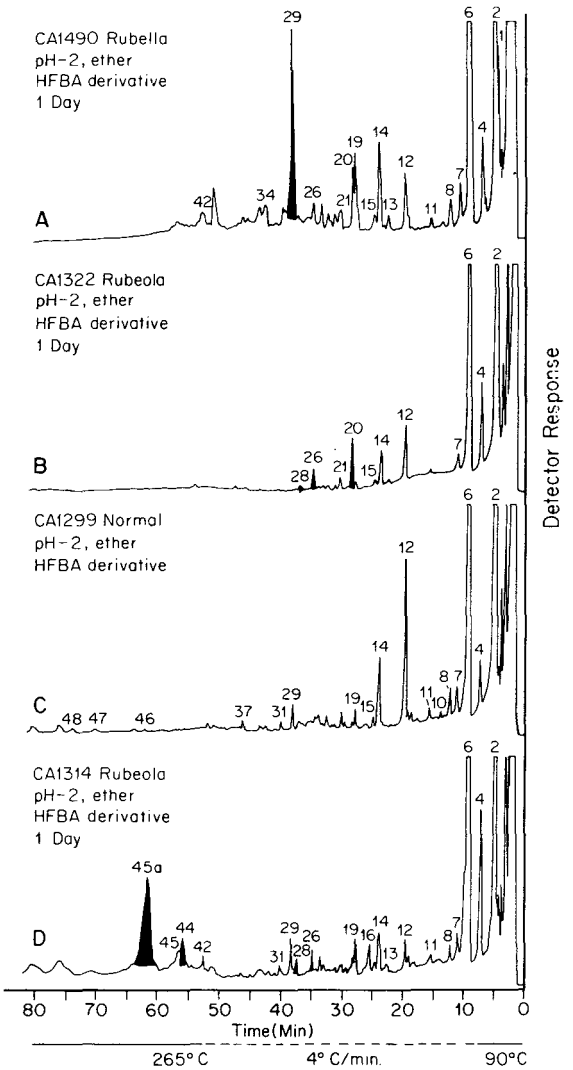


Fig. 14. GLC—FP-ECD chromatograms of HFBA—EtOH derivatized acidic diethyl ether extracts of sera taken from patients with the disease indicated in the figure. (From ref. 27 with permission.)

occur during schistosomiasis infection. These findings are important since this parasitic disease affects millions of the world population.

In addition to the analysis of CSF and sera from patients with the viral infections reported above have focused on the GLC—FP-ECD examination of monkey kidney cell culture supernatants infected with recent serum isolates of dengue virus and also stock isolates of the various dengue virus serotypes. The resulting GLC-FP-ECD fingerprints of the derivatives from the extracts of the infected cell culture medium were definitive enough to suggest GLC—FP-ECD could be used as an aid in the grouping of these viruses [29]. Further

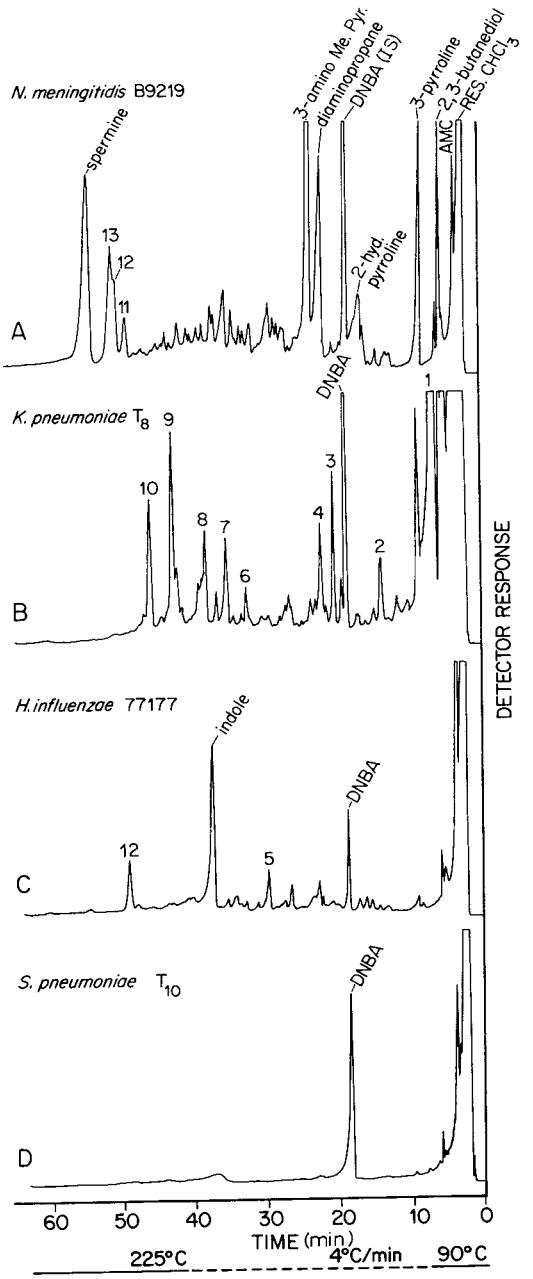


Fig. 15. GLC—FP-ECD chromatograms of HFBA derivatized basic chloroform extracts of spent bacterial culture medium, Neisseria defined medium. The type of organisms are indicated in the figure. Peaks: RES, residual; AMC, acetylmethylcarbinal; hyd, hydroxy; DNBA (IS), di-*n*-butylamine (internal standard); Me. Pyr., methylpyrrolidine. (From ref. 30 with permission.)

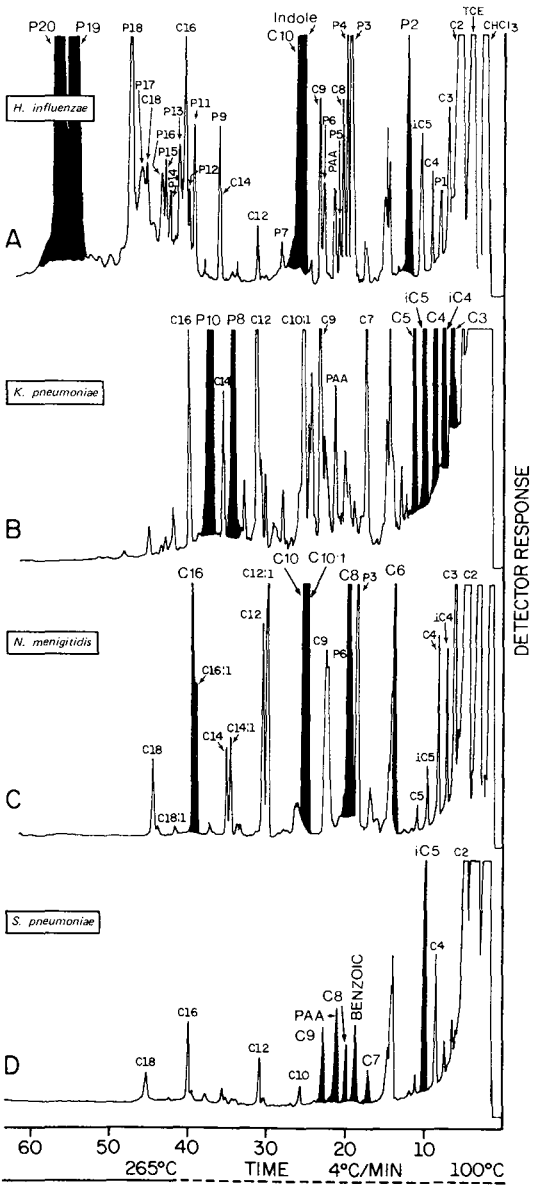


Fig. 16. GLC—FP-ECD chromatograms of TCE derivatized acidic chloroform extracts of spent Neisseria defined medium. The types of organisms are indicated in the figure. Most important peaks are blackened. C followed by a number indicates a straight chain carboxylic acid with the chain length indicated by the number. i indicates iso, and a colon between two numbers indicates unsaturation. (From ref. 15 with permission.)

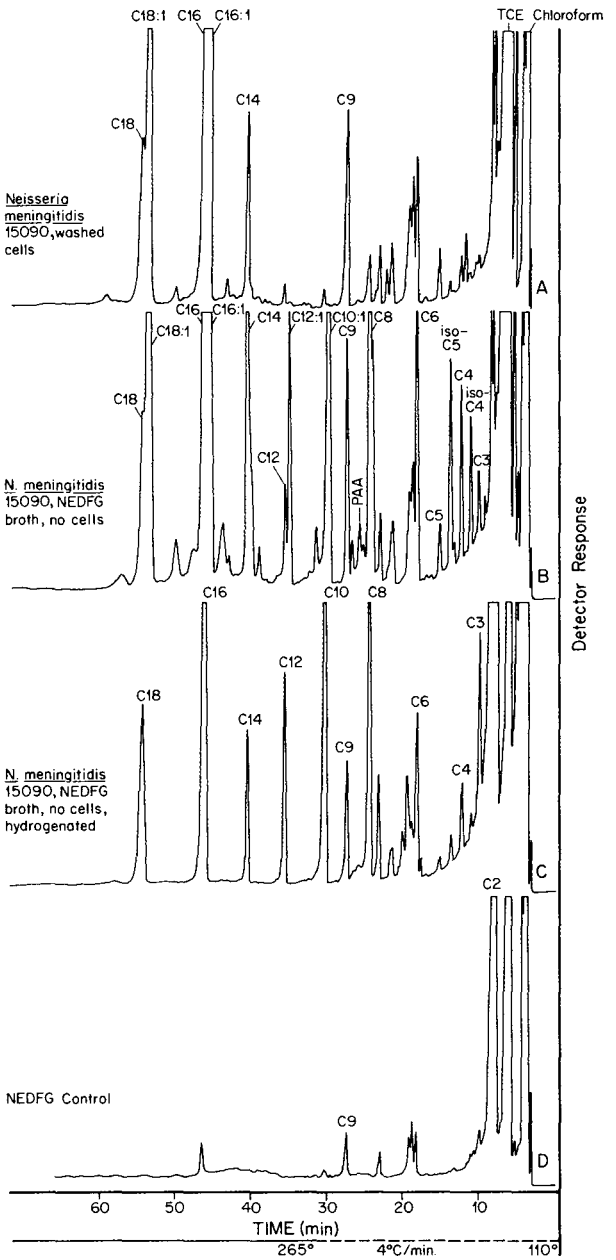


Fig. 17. GLC—FP-ECD chromatograms of TCE derivatized and TCE transesterified acidic chloroform extracts of methyl esters and free carboxylic acids prepared from (A) saponified washed cells, (B) spent culture medium with cells removed after hydrogenation; and (D) Neisseria defined medium control. The organism is indicated in the figure. (From ref. 10 with permission.) PAA = phenyl acetic acid.

studies on dengue virus infected cell culture supernatants and sera from patients with dengue fever are in progress to determine if GLC—FP-ECD has any diagnostic potential in dengue virus infections.

3.6. Metabolic and structural analysis of microorganisms

Although the GLC—FP-ECD techniques described here were developed primarily as a rapid diagnostic tool, the analytical scheme is not limited to body fluid, exudate, or excretion product analyses. It has proved to be a very useful tool for examining the metabolic activity of microorganisms in culture [10—15, 30], and for determining some of the carboxylic acid components of the microbial cell envelope [10], e.g. Figs. 15—17.

4. TECHNICAL NOTES

It is important to reiterate that the GLC—FP-ECD procedure was designed to make easy the preparation of electron-capturing derivatives of fatty acids, hydroxy acids, alcohols, and amines, to exploit the FP-ECD, and take advantage of the high temperature stability of liquid phases such as OV-101. Many established techniques, although effective for certain types of analyses, require several types of column packing materials and liquid phases, very different chromatographic conditions and detectors, or vastly different and frequently hard to prepare derivatives to be able to detect the classes of compounds that can be examined by the GLC—FP-ECD technique [1]. Even then, the selectivity and sensitivity of those procedures and instruments do not approach that of the GLC—FP-ECD procedure or the FPEC detector. We feel that the selectivity, and extreme sensitivity, to 10^{-12} M or greater, and potential for high resolution of chemical mixtures make the GLC—FP-ECD technique very versatile for examining chemical changes in living systems.

Secondly, the introduction of the purged splitless injector for capillary column trace analyses and the fused-silica capillary column, which can withstand stress without breakage in conjunction with the FP-ECD will allow even better separation and will reduce analysis times. It should be noted that peak retention times can vary with splitless injection if the amount of sample injected is not kept fairly constant. Also, column overloading is a factor that cannot be overlooked when capillary columns are used.

5. CONCLUSIONS

The data acquired to date indicate that the very sensitive and selective process of extraction, derivatization, and analysis by GLC—FP-ECD can detect changes in body fluids during the diseased state. These changes seem reproducible enough, to aid in the rapid identification of certain diseases. The rationale of the GLC—FP-ECD technique is different from past approaches to disease diagnosis where only one or several preselected components, such as toxins, antigens, antibodies, or a narrow spectrum of microbial or host derived products are sought and detected by a variety of frequently diverse biochemical

and immunologic procedures. Although the GLC—FP-ECD procedure relies on the detection of limited classes of organic molecules, it is used in an identical manner in all diagnostic applications, and provides data to construct a composite profile representing a particular etiology. It will probably be used initially as an adjunct for difficult diagnostic problems for which the research indicates application.

The change to the use of GLC—FP-ECD can and will be made more attractive, less expensive, and less labor intensive by the use of automated derivative preparation equipment, automatic injectors, and microprocessor based instrument controllers and data analyzers. Considerable developmental work remains to be done before many of the potential applications of the GLC—FP-ECD are standardized and the limits of the procedure are known.

Care must be taken in acquiring and handling the specimens before analysis. For example, samples should be taken before therapy is initiated; CSF should not be contaminated with blood as evidenced by red blood cells, and the sample should be frozen in a clean sterile container, preferably glass, if there is a delay in immediate analysis. One should be aware that more than one type of disease may be present and disease combinations, infectious or noninfectious, may affect or even change the GLC—FP-ECD profiles. In addition, the quality control of the GLC—FP-ECD instrumentation should be assessed at least once a month for resolution and sensitivity by analysis of standard mixtures.

If the scope of GLC—FP-ECD analyses can be limited to specific diseases, the data analysis and diagnostic usefulness are more meaningful and simple. For example, in the study of RMSF we limited the study to diseases associated with a rash which often makes early detection of RMSF difficult. We are confident that the contributions of other scientists in the utilization, testing, and improvement of the various components of the GLC—FP-ECD procedure will play a major role in its further development, acceptance, and usefulness in the diagnostic laboratory.

6. SUMMARY

The extremely sensitive and selective gas—liquid chromatography—frequency pulsed-modulated electron-capture detection (GLC—FP-ECD) procedure has been applied to the diagnosis of bacterial, fungal, viral, rickettsial, and parasitic diseases by the examination of various body fluids, effusions and exudates, and excretion products. Carboxylic acid and alcohol, hydroxy acid, and amine product profiles of microbial or host-response origin, have been used to establish specific etiologies, these profiles are reproducible, and can be used to aid in the diagnosis of infections. In addition, we have used the GLC—FP-ECD procedure to analyze microbial metabolic products in vitro and to provide data for identification and classification. We also explored computer time-sharing for data analysis, profile library comparison, and eventual profile matching for diagnosis.

REFERENCES

1 B.M. Mitruka, in Gas Chromatographic Applications in Microbiology and Medicine, John Wiley & Sons, New York, 1975.
2 B.M. Mitruka, in C-G. Heden and T. Illeni (Editors), New Approaches to the Identification of Microorganisms, John Wiley & Sons, New York, 1975, p. 123.
3 B.M. Mitruka, Methods of Detection and Identification of Bacteria, CRC Press, Boca Raton, FL, 1976, p. 141.
4 L. Larsson and P-A. Mardh, Acta Pathol. Microbiol. Scand., Sect. B., Suppl., 259 (1977) 5.
5 J.B. Brooks, in D. Schlessinger (Editor), Methods in Microbiology-1975, American Society for Microbiology, Washington, DC, 1975, p. 45.
6 J.B. Brooks, in J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown (Editors), Advances in Chromatography, Vol. 15, Marcel Dekker, New York, 1977, p. 1.
7 D.C. Edman, R.B. Craven and J.B. Brooks, CRC Critical Rev. Clin. Lab. Sci., 14 (1981) 133.
8 C.W. Moss, J. Chromatogr., 203 (1981) 337.
9 D.B. Drucker, in J.R. Norris (Editor), Methods in Microbiology, Vol. 9, Academic Press, New York, 1976, p. 52.
10 C.C. Alley, J.B. Brooks and D.S. Kellogg, Jr., J. Clin. Microbiol., 9 (1979) 97.
11 J.B. Brooks and W.E.C. Moore, Can. J. Microbiol., 15 (1969) 1433.
12 J.B. Brooks, M.J. Selin and C.C. Alley, J. Clin. Microbiol., 3 (1976) 180.
13 J.B. Brooks, C.C. Alley and J.A. Liddle, Anal. Chem., 46 (1974) 1930.
14 L. Thacker and J.B. Brooks, Infect. Immun., 9 (1974) 648.
15 J.B. Brooks, D.S. Kellogg, Jr., M.E. Shepard and C.C. Alley, J. Clin. Microbiol., 11 (1980) 45.
16 J.B. Brooks, R.B. Craven and D.C. Edman, personal communication.
17 L.V. Holdeman, E.P. Cato and W.E.C. Moore (Editors), Anaerobe Laboratory Manual, Southern Printing Co., Blacksburg, VA, 4th ed., 1977, p. 134.
18 J.B. Brooks, C.W. Moss and V.R. Dowell, J. Bacteriol., 100 (1969) 528.
19 J.B. Brooks, D.S. Kellogg, Jr., C.C. Alley, H.B. Short, H.H. Handsfield and B. Huff, J. Infect. Dis., 129 (1974) 660.
20 J.B. Brooks, W.B. Cherry, L. Thacker and C.C. Alley, J. Infect. Dis., 126 (1972) 143.
21 D. Schlossberg, J.B. Brooks and J.A. Shulman, J. Clin. Microbiol., 3 (1976) 239.
22 R.B. Craven, J.B. Brooks, D.C. Edman, J.D. Converse, J. Greenlee, D. Schlossbert, T. Furlow, J.M. Gwaltney, Jr. and W.F. Miner. J. Clin. Microbiol., 6 (1977) 27.
23 J.B. Brooks, G. Choudhary, R.B. Craven, C.C. Alley, J.A. Liddle, D.C. Edman and J.D. Converse, J. Clin. Microbiol., 5 (1977) 625.
24 J.B. Brooks, D.C. Edman, C.C. Alley, R.B. Craven and N.I. Girgis, J. Clin. Microbiol., 12 (1980) 208.
25 D.C. Edman, J.D. Converse, J.B. Brooks and R.B. Craven, Abstracts, Annual Meeting, Amer. Soc. Microbiol., Washington, DC, 1978, Abst. No. C155.
26 J.B. Brooks, R.B. Craven, D. Schlossberg, C.C. Alley and F.M. Pitts, J. Clin. Microbiol., 8 (1978) 203.
27 J.B. Brooks, J.D. McDade and C.C. Alley, J. Clin. Microbiol., 14 (1981) 165.
28 J.B. Brooks, M.T. Basta and A.M. El Kholy, personal communication.
29 J.B. Brooks, G. Guno, R.B. Craven, C.C. Alley and B.M. Wycoff, personal communication.
30 J.B. Brooks, D.S. Kellogg, Jr., M.E. Shepard and C.C. Alley, J. Clin. Microbiol., 11 (1980) 52.

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HEAD-SPACE GAS—LIQUID CHROMATOGRAPHY FOR THE RAPID LABORATORY DIAGNOSIS OF URINARY TRACT INFECTIONS CAUSED BY ENTEROBACTERIA

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(First received October 11th, 1982; revised manuscript received December 28th, 1982)

SUMMARY

Urine specimens were analysed in parallel in a hospital laboratory by routine methods which were regarded as the standard of correct diagnosis and by the rapid test already developed in a research laboratory. Technical modifications made to the rapid test ensured that its results agreed with those from routine methods and increased its rapidity so that a result was possible 4 h after receipt of the specimen. When 382 urine specimens were analysed by the modified test which is described in detail, there were neither false negatives nor false positives for infections with *Escherichia, Klebsiella, Citrobacter* or *Proteus* species.

INTRODUCTION

A test using head-space gas—liquid chromatography (HS-GLC) has been proposed for the rapid detection of bacteria in urine specimens [1]. The test depends on the production of ethanol from lactose or arabinose by *Escherichia coli* and some related species, and of methyl mercaptan from methionine by *Proteus* spp. Methyl mercaptan oxidizes spontaneously to dimethyl disulphide. Ethanol, methyl mercaptan and dimethyl disulphide appear early in growth and can be detected in one HS-GLC analysis. When the rapid test was applied in a research laboratory to urine specimens from patients, both *E. coli* and *Proteus* spp. were detected in specimens cultured in lactose methionine medium which was incubated and analysed after 3 and 5 h [2]. This was confirmed by Coloe [3] who used an arabinose methionine medium.

This paper reports technical modifications that were made to the rapid test when urine specimens were analysed in parallel by the rapid test and by routine methods in a hospital laboratory. Three trials were done in sequence, difficulties in the earlier trials being overcome in the final trial.

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EXPERIMENTAL

Gas chromatography and mass spectrometry

In HS-GLC the liquid to be analysed for volatile compounds is sealed in a container and the supernatant vapour (HS) is injected into the gas chromatograph. In the present investigation vapour pressures of volatile compounds in HS were increased by adding potassium carbonate to liquids for analysis (salting-out) and by heating the sealed containers to 60°C. Potassium carbonate salts out neutral and alkaline compounds into the HS. To prepare HS samples, 2 ml of each liquid for analysis were added to 3 g of potassium carbonate in a glass vial provided with the automatic head-space injector, sealed immediately, shaken on a Vortex mixer to disperse the salt in the liquid and held in the injector turntable at 60°C for injection.

The gas chromatograph was a Perkin-Elmer F45 with an automatic head-space injector, a flame ionization detector and a stainless-steel column, 2 m × 3 mm, packed with 0.4% Carbowax 1500 on graphite 60—80 mesh. The injector needle temperature was 150°C, the oven temperature 115°C and the injector and detector temperatures 140°C. Nitrogen carrier gas pressure was 180 kPa, hydrogen 400 kPa and air 380 kPa. The injection time was 3 sec and the analysis time 1.8 min.

Aqueous solutions of ethanol that had been redistilled over potassium hydroxide to constant boiling point, dimethyl disulphide (Eastman Organic Chemicals, New York, NY, U.S.A.), and S-methylthiourea which yielded methyl mercaptan were added to potassium carbonate in glass vials and analysed with each day's samples to check retention times for the identification of products from urine cultures.

The concentrations of ethanol, dimethyl disulphide and methyl mercaptan in urine cultures necessary for a positive response against the background of noise due to volatile compounds in the urine culture medium and urine specimens were 0.00022 *M*, 0.00048 *M* and 0.0024 *M*, respectively.

The mass spectrometer was a 30-cm radius 60° magnetic deflection instrument coupled to the gas chromatograph by a Watson—Bieman frit [4].

Specimens of urine

Specimens of urine (382) were refrigerated after voiding and later analysed by the HS-GLC rapid test in parallel with the routine laboratory test.

Method of HS-GLC rapid test

A basal methionine yeast-extract peptone medium was prepared by dissolving 37.5 g L-methionine (BDH), 25 g proteose peptone (Difco) and 7.5 g yeast extract (Oxoid) in 1 l 0.25 *M* sodium phosphate buffer, pH 7.2, dispensing in presterilized screw-capped bottles of 28-ml capacity, autoclaving at 115°C for 15 min and storing at 4°C. A 10% aqueous solution of L-arabinose was sterilized by membrane filtration and stored at 4°C. Within 24 h of use, medium for the HS-GLC rapid test was prepared by adding one part of arabinose solution to four parts of basal medium.

Urine cultures were inoculated in duplicate by mixing 1.1 ml of the urine specimen with 1.1 ml medium for the HS-GLC rapid test in screw-capped

bottles of 28-ml capacity. These volumes provided 2 ml of culture for HS-GLC analysis. The final concentrations of the ingredients were 1% (0.07 *M*) L-arabinose, 1.5% (0.1 *M*) L-methionine, 1% peptone, 0.3% yeast extract, 0.1 *M* phosphate buffer and 50% urine. Cultures were incubated for 3.5 h. Two degrees of aeration were provided, lower aeration in an unshaken culture sloping at an angle of 12° from the horizontal, and higher aeration in a culture standing in a shaker operating at 200 horizontal 20-mm oscillations per min.

Medium for the HS-GLC rapid test, diluted with an equal volume of sterile water, was incubated and analysed with each day's urine cultures. This analysis was to detect volatile substances originally present in the medium (medium blank). A 1-ml aliquot of each urine specimen was diluted with 1 ml water and analysed without incubation. These analyses were to detect volatile substances originally present in the urine specimens (non-incubated urine).

Method of routine laboratory analysis

MacConkey agar without salt was prepared as directed by the manufacturer (Oxoid). Firm blood agar [5] contained 6% equine blood and 3% agar (Oxoid) in a nutrient broth base. For the routine laboratory analysis of urine, the number of colony-forming units (CFU) per ml urine was determined on MacConkey agar and on firm blood agar after incubation for 18—24 h at 37°C. If more than one colony type was distinguished, each was counted and recorded separately. If the count for an organism was significant, that is $\geqslant 10^5$ CFU per ml urine [6], it was isolated and identified by standard methods [7] taking at least another 18—24 h. Species of the genera *Escherichia*, *Klebsiella* and *Citrobacter*, all of which were detected by ethanol production from arabinose in the HS-GLC analysis, are grouped together for the purposes of this paper and called "coliforms".

RESULTS

Technique of HS-GLC analysis

For the final trial, modifications were made to the technique of the earlier trials. The analysis time was reduced and operator time was saved so that results could be reported earlier. The accuracy of analysis was improved and the volume of urine specimen required was reduced.

Analysis time. The column was shorter and contained a lower concentration of the stationary phase on a coarser support, and the oven temperature was higher than in previous investigations. Consequently retention times (t_R) were short, 0.275 min, 0.325 min and 1.7 min for methyl mercaptan, ethanol and dimethyl disulphide, respectively. The analysis time of 1.8 min, compared with 2.5 min in earlier trials, was a significant saving when 50 or more analyses were to be carried out in a hospital laboratory. The increased throughput of analyses enabled a larger number of urine specimens to be tested in a given time and the results of all of them to be reported earlier. No further peaks eluted after the analysis time of 1.8 min.

Operator time. Proportionately more potassium carbonate was used to

prepare HS compared with previous investigations, sufficient to saturate the solution. This ensured that the solutions were always at the same concentration without the time-consuming need for a high degree of accuracy in weighing potassium carbonate.

The automatic HS injector was important in saving operator time. After the turntable had been loaded with sample vials, chromatograms were interpreted by the operator while analyses were proceeding and results could be reported earlier than with manual injection in which the operator could not start interpreting chromatograms until the last sample had been injected.

When manual injection was used it was difficult to detect partial blockage of the syringe needle impeding the passage of vapour from HS to the barrel of the syringe. This led to errors and duplicate analyses were essential. By contrast, results from automatic injection were highly reproducible and only one analysis was done on each culture. This halved operator time in preparation of culture medium, inoculation of cultures, and analysis of cultures by HS-GLC.

Precision. Automatic recording of times of injection enabled the t_R of each volatile compound in a chromatogram to be measured with a high degree of precision. The identification of these volatile compounds was therefore made with more confidence than was possible with manual injections.

When a 1-m column was used in preliminary work before the start of the final trial, the t_R of a compound that was expected to be methyl mercaptan was noted to be 0.025 min longer than the t_R of the known sample of methyl mercaptan. This small difference would not have been detected with manual injection. Analysis by mass spectrometry (MS) showed that the 1-m column had failed to separate two compounds, methyl mercaptan and trimethylamine. The mass spectrum of trimethylamine has distinctive peaks, at *m/e* 58, 42, 59 and 30 in order of intensity, which were clearly distinguishable from methyl mercaptan in the mass spectrum of the composite peak. The two compounds were separated from each other and from ethanol with the 2-m column.

Ethanol, methyl mercaptan and dimethyl disulphide, the volatile products that indicated coliforms and *Proteus* spp., were previously identified by MS [1–3,8].

Volume of urine specimen. In the preparation of HS for each analysis, only a small volume of urine culture and therefore of urine specimen was required. This is an advantage because patients with urinary tract infections often suffer from frequency or oliguria and are unable to provide large specimens for diagnostic tests. A further halving of the volume of urine specimen required for each analysis was achieved because results were highly reproducible (see above) and only one analysis was required on each culture.

The volume of urine required for the HS-GLC rapid test was 3.2 ml, 1 ml for the analysis of nonincubated urine and 2.2 ml for the urine cultures.

Technique of culture of urine specimens

Modifications were made to improve agreement with routine methods which were regarded as the standard of correct diagnosis. Results from HS-GLC rapid tests of urine specimens in the final trial (Table I) were divided into Groups 1 to 4 on the basis of routine results. To arrive at the HS-GLC results, the chromatograms from urine cultures (viz. Figs. 1 and 2) were interpreted in

TABLE I

COMPARISON BETWEEN RESULTS OF ROUTINE LABORATORY AND HS-GLC RAPID TESTS OF URINE

Sig. = Significant, i.e. $\geqslant 10^5$ colony-forming units (CFU) per ml urine.

Group	Routine laboratory result	No. of specimens	HS-GLC rapid test				
			Aeration of culture	HS-GLC result			
				Sig. coliform	Sig. *Proteus*	Sig. coliform + *Proteus*	Not sig. coliform or *Proteus*
1	Sig. coliform	111	Unshaken	111	0	0	0
	10^4 CFU coliform per ml urine	14	Unshaken	0	0	0	14
2	Sig. *Proteus*	22	Shaken	0	22	0	0
3	Sig. coliform + *Proteus*	11	One unshaken and one shaken	0	4	7	0
4	Not sig. coliform or *Proteus*	224	One unshaken and one shaken	0	0	0	224

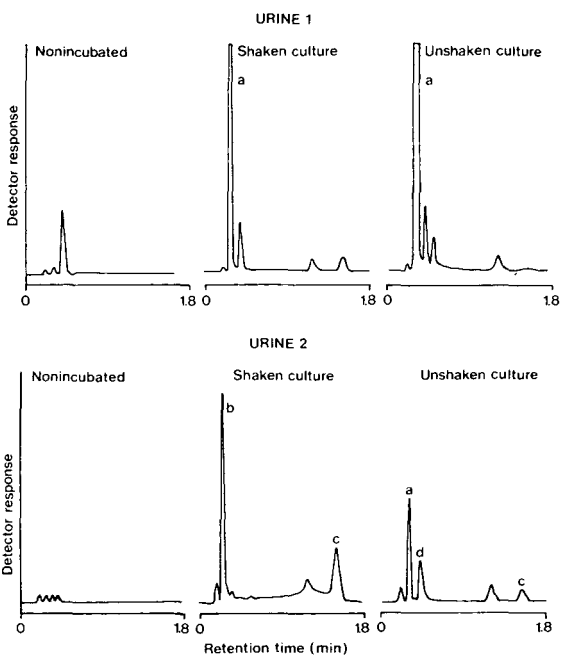

Fig. 1. Chromatograms of urine specimens diluted with an equal volume of water (nonincubated) or diluted with an equal volume of medium for the HS-GLC rapid test and incubated either shaken or unshaken for 3.5 h. Urine 1 contained $>10^5$ CFU *E. coli* per ml. Urine 2 contained $>10^5$ CFU *P. mirabilis* per ml. Peaks: a = ethanol, b = methyl mercaptan, c = dimethyl disulphide, d = trimethylamine.

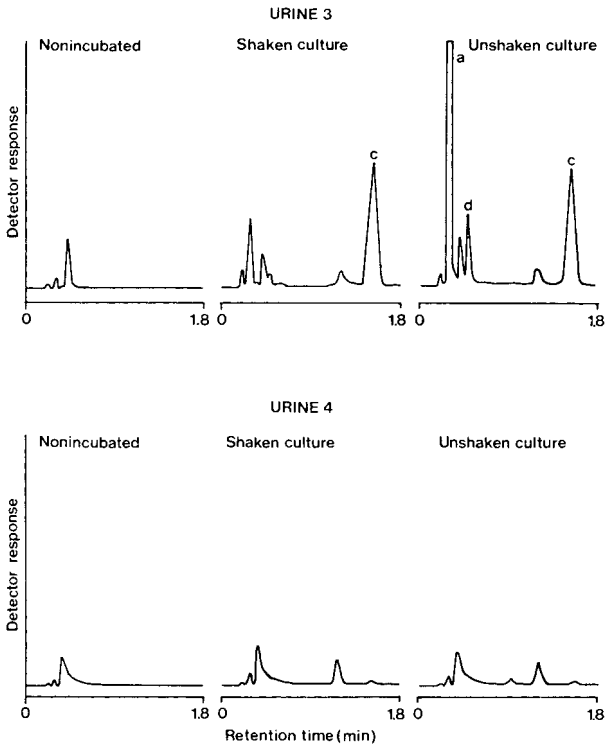

Fig. 2. Chromatograms of urine specimens diluted with an equal volume of water (nonincubated) or diluted with an equal volume of medium for the HS-GLC rapid test and incubated either shaken or unshaken for 3.5 h. Urine 3 contained $>10^5$ CFU *E. coli*, $>10^5$ CFU *P. vulgaris* and $>10^5$ CFU *P. morganii* per ml. Urine 4 did not contain $>10^5$ CFU per ml of any microorganism. Peaks: a = ethanol, c = dimethyl disulphide, d = trimethylamine.

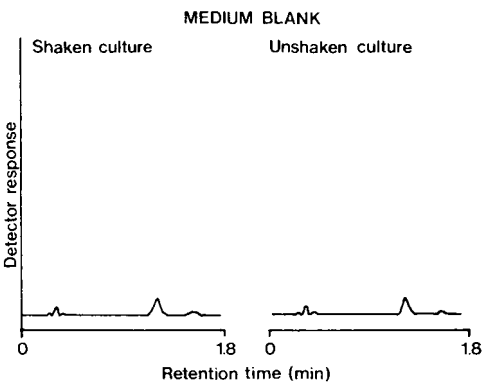

Fig. 3. Chromatograms of medium for the HS-GLC rapid test diluted with an equal volume of water and incubated either shaken or unshaken for 3.5 h.

the light of the chromatograms of the medium blank (viz. Fig. 3) and of non-incubated urine specimens (viz. Figs. 1 and 2).

Effect of period of incubation of urine cultures on detection of significant numbers of coliforms (Group 1). The incubation period set the quantitative

parameter of the HS-GLC test. In an early trial it was 3 h. Ethanol was detected by HS-GLC only in unshaken cultures of urine specimens with higher counts and not in cultures of specimens with counts at or near 10^5 CFU coliforms per ml urine. When the incubation period was increased to 4 h, ethanol was detected in unshaken cultures of all urine specimens containing 10^5 CFU coliforms per ml urine but also in cultures of specimens containing 10^4 CFU coliforms per ml urine, a count that is not generally regarded as significant.

In the final trial the incubation period was 3.5 h. Ethanol was detected in unshaken cultures of all 111 urine specimens containing $\geqslant 10^5$ CFU coliforms per ml urine (viz. Urine 1, Fig. 1). The 111 coliforms included 84 *E. coli*, 7 A—D group, 10 *K. aerogenes*, 2 *K. oxytoca*, 5 *C. freundii* and 3 *C. koseri*. Ethanol was not detected in cultures of 14 urine specimens containing 10^4 CFU coliforms per ml urine.

The incubation period of 3.5 h was suitable for the detection of *Proteus* spp. (Group 2).

Effect of aeration of urine cultures on detection of significant numbers of Proteus spp. (Group 2). Oxygen promoted the production of dimethyl disulphide by *Proteus* spp. After incubation for 3.5 h, dimethyl disulphide and sometimes also methyl mercaptan, was detected by HS-GLC in shaken cultures of all 22 urine specimens containing $\geqslant 10^5$ CFU *Proteus* spp. per ml (viz. Urine 2, Fig. 1). The 22 *Proteus* spp. included 17 *P. mirabilis*, 1 *P. vulgaris*, 2 *P. morganii* and 2 *P. stuartii*. The yield of dimethyl disulphide was usually small in the unshaken culture, despite being incubated at a slope to increase its surface area and consequently its aeration. By contrast, coliforms produced higher yields of ethanol in unshaken culture (viz. Urine 1, Fig. 1). It was concluded that two cultures of each urine specimen were necessary, one shaken and the other unshaken.

The detection of trimethylamine in most unshaken cultures of urine specimens containing significant numbers of *Proteus* spp. was a new observation. It was usually associated with a small ethanol peak.

Detection of significant numbers of both coliform and Proteus spp. occurring together in urine specimens (Group 3). After incubation for 3.5 h, ethanol was detected by HS-GLC in unshaken cultures and dimethyl disulphide in shaken cultures of 7 out of 11 urine specimens containing $\geqslant 10^5$ CFU coliform and *Proteus* spp. per ml urine (viz. Urine 3, Fig. 2). Out of the 7 urine specimens, 4 contained *E. coli* and *P. mirabilis*, 2 contained *K. aerogenes* and either *P. mirabilis* or *P. morganii*, and 1 contained *E. coli*, *P. vulgaris* and *P. morganii*. Ethanol was not detected in unshaken cultures but dimethyl disulphide was detected in shaken cultures of the remaining 4 polymicrobic specimens, 2 containing *E. coli* and *P. mirabilis*, 1 *K. oxytoca* and *P. mirabilis*, and 1 *E. coli* and *P. vulgaris*.

Results of rapid test on urine specimens that did not contain significant numbers of either a coliform or a Proteus sp. (Group 4). Neither ethanol nor dimethyl disulphide was detected by HS-GLC in 188 cultures of urine specimens that did not contain significant numbers of any organism (viz. Urine 4, Fig. 2) nor in 36 cultures of urine specimens containing significant numbers of an organism not detected by the HS-GLC rapid test. The organisms included *Staphylococcus epidermidis* (9), *Streptococcus faecalis* (7), *Pseudomonas*

aeruginosa (11), *Candida* sp. (3), *S. aureus* (1), *Serratia marcescens* (2), *P. aeruginosa* and *S. faecalis* (2), and *S. epidermidis* and *S. faecalis* (1).

Summary of results

The HS-GLC rapid test gave results that agreed with those from routine methods in the hospital laboratory. All urine specimens containing significant numbers of either a coliform or a *Proteus* sp. were detected. All polymicrobic urine specimens containing both a coliform and a *Proteus* sp. were detected as infections, both organisms being found in the majority of them. Thus the test did not yield any false negative result for urine specimens containing a coliform or a *Proteus* sp.

All urine specimens containing no organism in significant numbers or an organism that was neither a coliform nor a *Proteus* sp. were negative. Thus the test did not yield any false positive results.

DISCUSSION

The laboratory diagnosis of urinary tract infections requires both a quantitative and a qualitative microbiological analysis of urine specimens. Almost all urine specimens contain contaminating microorganisms added during voiding in addition to pathogenic microorganisms from the bladder. The contaminants and the pathogens are generally the same species and the distinction between them is quantitative. Contaminants should not exceed 10^4 CFU per ml urine were thought to be significant in a particular patient, for example a patient receiving antibiotic therapy, this lower concentration of bacteria could be detected by incubating the urine culture longer than 3.5 h.
urine cultures for the HS-GLC test. This was long enough to detect *Escherichia*, *Klebsiella*, *Citrobacter* and *Proteus* spp. in significant numbers. If $<10^5$ CFU per ml urine were thought to be significant in a particular patient, for example a patient receiving antibiotic therapy, this lower concentration of bacteria could be detected by incubating the urine culture longer than 3.5 h.

Analysis of urine cultures by HS-GLC separated specific chemical markers for the commonest causes of urinary tract infections. In this way the HS-GLC test was qualitative and pointed to the identity of the infecting bacterium on the day the specimen was received in the laboratory instead of the next day, the time required for routine laboratory results.

Several factors contributed to the rapidity of the HS-GLC test. Chemical analysis by GLC is very sensitive and when used to detect bacteria by analysis for their metabolic products it was possible to detect them in the earliest stages of growth of cultures of urine specimens. The advantage of this short period of incubation was reinforced by the rapid preparation of urine cultures. Coloe [3] used the centrifuged sediment from 60 ml of urine for each culture. This step causes a delay of about 20 min. In the present investigation whole urine was simply mixed with culture medium, a step that took less than 1 min before incubation of the cultures could commence. The use of HS (in preference to other kinds of sample such as solvent extracts) for GLC analysis of the culture after incubation further ensured the rapidity of the whole test. The preparation of HS samples was simple and compounds in HS were few and highly

volatile so that the GLC analysis time was short. Consequently bacteria could be detected by HS-GLC in a given urine specimen within 4 h. Urine specimens are among the most numerous processed in diagnostic microbiological laboratories. A gas chromatograph with an automatic head-space injector was invaluable for the early reporting of large numbers of analyses of urine cultures done in sequence on a single gas chromatograph.

It has appeared possible that a sloping unshaken culture might provide a level of aeration that would permit the detection of both coliforms and *Proteus* spp. in a single culture and obviate the need for two cultures from each urine specimen. However the detection of *Proteus* spp. was often uncertain in the unshaken culture. A shaken culture is necessary if methyl mercaptan production from methionine is the marker for *Proteus* spp. The observation that trimethylamine was produced by *Proteus* spp. in unshaken cultures merits further investigation because this marker might allow the detection of both coliforms and *Proteus* spp. in a single unshaken culture.

In this hospital laboratory trial of the HS-GLC rapid test the theoretical concept on which it is based [1] was fully supported. With technical modications it was rapid and reliable for the diagnosis of urinary tract infections caused by enterobacteria.

ACKNOWLEDGEMENTS

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REFERENCES

1 N.J. Hayward, T.H. Jeavons, A.J.C. Nicholson and A.G. Thornton, J. Clin. Microbiol., 6 (1977) 195.
2 N.J. Hayward and T.H. Jeavons, J. Clin. Microbiol., 6 (1977) 202.
3 P.J. Coloe, J. Clin. Pathol., 31 (1978) 365.
4 W. Stark, J.F. Smith and D.A. Forss, J. Dairy Res., 34 (1967) 123.
5 N.J. Hayward, G.M. Incledon and J.E. Spragg, J. Med. Microbiol., 11 (1978) 155.
6 E.H. Kass, Trans. Assoc. Amer. Physicians, 69 (1956) 56.
7 S.T. Cowan, Cowan and Steel's Manual for the identification of medical bacteria, Cambridge University Press, London, 2nd ed., 1974.
8 N.J. Hayward, T.H. Jeavons, A.J.C. Nicholson and A.G. Thornton, J. Clin. Microbiol., 6 (1977) 187.

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GAS CHROMATOGRAPHIC ANALYSIS OF BACTERIAL AMINES AS THEIR FREE BASES

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SUMMARY

Columns of Chromosorb 103, Tenax-GC, Amine 220 plus potassium hydroxide on Chromosorb W, and Carbowax 20M plus potassium hydroxide on Chromosorb W were compared for their ability to separate bacterial amines as their free bases in aqueous solution. A 1.52 m × 0.6 cm O.D. column of Chromosorb 103 separated eleven amines when operated isothermally at 185°C. A further four high-boiling amines could be separated at 240° C. The other packings separated only eight amines isothermally, except for Tenax-GC which separated seven of the free bases. Chromosorb 103 performed less well than Carbowax 20 M plus potassium hydroxide with respect to number of plates or peak resolution. The maximum number of amines separated, thirteen, required Chromosorb 103 programmed from 170° C to 230° C at 3° C min^{-1} after an initial holding time of 20 min. It was possible tentatively to identify amines in culture supernatant fluid of *Proteus mirabilis*, viz. ethylamine, isobutylamine and isoamylamine, after direct injection of culture supernatant fluid.

INTRODUCTION

The trace analysis of amines is important because they are commonly found in foodstuffs [1], tobacco leaf [2], human urine [3] and blood [4] and also in micro-organisms [5]. The production of amines by micro-organisms and their subsequent release into the growth medium has been known for many years and several mechanisms for amine production have now been described [5—7].

However, the gas chromatographic (GC) separation of complex mixtures of free amines at very low concentrations in aqueous solutions is hampered by technical difficulties associated with the analysis of highly polar solutes; for example, adsorption and decomposition of the compounds in the column,

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ghosting phenomena, badly tailed peaks and very low detector response. A common method of overcoming these problems is to convert such polar compounds to relatively non-polar derivatives that are characteristically more suitable for GC analysis. Several derivatives such as Schiff bases [8], carbethoxyamines [9], dinitrophenyl amines [10], trimethylsilylated [11] and acetylated [12] derivatives have been used for this purpose. Another successful approach has been to employ less reactive column packing materials to reduce the interaction with solutes. Examples of this approach include the use of porous polymers [13], and the deactivation of supports by treatment with alkali [14]. Wall-coated open tubular [15], and support-coated open tubular columns [12] which minimise column-solute interactions have also been used, though much less widely than packed columns. Where large numbers of samples are to be analysed, a simple and rapid technique is required. Therefore, the object of the present study was to compare various analytical conditions and column packings to determine the optimum GC parameters for analysis of complex mixtures of bacterial amines as their free bases, in aqueous solution.

EXPERIMENTAL

Reagents

Methylamine, ethylamine, isopropylamine and *n*-propylamine were the gift of W. Goddard. All other amines were purchased from BDH (Liverpool, Great Britain) and were analytical-grade reagents. Glass columns, liquid phases and supports were obtained from Phase Separations (Clwyd, Great Britain).

Column packings

The following packings were prepared and evaluated; 10% (w/w) Amine 220 plus 10% (w/w) potassium hydroxide, 10% (w/w) Carbowax 20M plus 2% (w/w) potassium hydroxide, both coated onto Chromosorb W acid washed DMCS treated support (85—100 mesh). The porous polymers, Tenax-GC and Chromosorb 103, were used as supplied.

All packings were tested as packed glass columns of 1.52 m × 0.6 cm O.D. For potassium hydroxide packings, the support was treated with aqueous alkali prior to treatment with a solution of organic stationary phase in methanol. All columns were conditioned by heating at 8°C min^{-1} to 20°C below the maximum recommended temperature for the stationary phase, then held at that temperature with the carrier gas flowing through the column for 24 h. An exception was Amine 220 plus potassium hydroxide which was heated at 2°C min^{-1} to 160°C.

Bacterial cultures

In order to confirm the applicability of various techniques to the analysis of bacterial amines, actual cultures were examined. *Proteus mirabilis* was grown in 50 ml broth under an atmosphere of carbon dioxide at 35°C for 24 h. After centrifugation at 3000 *g* for 15 min, the culture supernatant fluid was made basic with 1.5 g potassium carbonate and 0.1 ml 10 *N* sodium hydroxide. GC analysis was by direct injection of 5-μl samples onto the column being tested.

GC analysis

Initially all packings were tested isothermally using the following oven temperatures: Amine 220 plus potassium hydroxide, 60°C and 100°C; Carbowax 20M plus potassium hydroxide, 60°C and 150°C; Chromosorb 103, 185°C and 240°C; Tenax-GC, 120°C and 165°C.

Subsequently, the most satisfactory packing (see Results), Chromosorb 103, was evaluated in dual 2.74 m × 0.6 cm O.D. columns heated from 170°C to 230°C at 3°C min^{-1} after an initial holding time of 20 min.

All analyses were performed in a PYE 104 gas chromatograph equipped with dual flame ionization detectors. Peak times and areas were recorded by a minigrator computing integrator (Spectra-Physics, Luton, Great Britain). Gas chromatograms were recorded using a 10-mV f.s.d. W + W flat bed chart recorder operated at 30 cm h^{-1}. Gas flow-rates were as follows: air, 600 ml min^{-1}; hydrogen, 40 ml min^{-1}, nitrogen, 45 ml min^{-1}.

Methods for evaluation and comparison of columns

The evaluation of gas chromatograms was carried out by determining the following (1) number of theoretical plates; (2) peak resolution factors; (3) maximum number of amines resolved in an isothermal analysis; (4) total analysis time per elution temperature, with regard to the maximum and minimum operating temperatures of the liquid phases.

TABLE I

COMPARISON OF COLUMN PACKINGS FOR ANALYSIS OF BACTERIAL AMINES

Column packing	Operating temperature* (°C)	Maximum numbers of amines separated	Number of plates per meter**	Peak resolution***
10% (w/w) Amine 220 plus 10% (w/w) potassium hydroxide	60 100	8 3	450	2.0
10% (w/w) Carbowax 20M plus 2% (w/w) potassium hydroxide	60 150	8 5	850	2.0
Chromosorb 103	185 240	11 4	650	1.6
Tenax-GC	120 165	7 5	450	1.4
Chromosorb 103 (temperature programmed)	At 170°C for 20 min then to 230°C at 3°C/min, final holding time of 5 min	13	—	—

*Isothermal, except for temperature programmed Chromosorb 103. Higher temperatures were used to separate only the less volatile amines.

**Calculated as the mean number of plates for all peaks.

***Calculated as the mean resolution for all peak pairs.

TABLE II

RETENTION DATA RELATIVE TO *n*-BUTYLAMINE FOR AMINES ANALYSED AS FREE BASES IN AQUEOUS SOLUTION

Amine	Synonym	Column packings				
		Amine 220 plus potassium hydroxide (60°C isothermal)	Carbowax 20M plus potassium hydroxide hydroxide (60°C isothermal)	Tenax GC (120°C isothermal)	Chromosorb 103	
					185°C isothermal	Temperature programmed from 170 to 230°C (see Table I)
Methylamine	Aminomethane	0.18	0.29	–	0.19	0.18
Ethylamine	Aminoethane	0.26	0.36	0.14	0.29	0.25
Isopropylamine	2-Aminopropane	–	–	–	0.42	0.37
n-Propylamine	1-Aminopropane	0.45	0.55	0.36	0.54	0.51
tert.-Butylamine	–	–	–	0.35	0.54	–
sec.-Butylamine	–	–	–	0.63	0.84	0.75
Isobutylamine	–	0.69	0.74	0.64	0.84	0.82
n-Butylamine	1-Aminobutane	1.00	1.00	1.00	1.00	1.00
Triethylamine	–	–	–	–	–	1.32
2-Methylbutylamine	–	–	–	–	–	1.69
Isoamylamine	3-Methylbutylamine	1.68	1.51	1.26	1.65	–
n-Amylamine	Pentylamine	2.33	1.74	2.08	1.87	1.93
Pyrrolidine	Tetrahydropyrrole	1.90	1.98	2.51*	1.36	–
n-Hexylamine	1-Aminohexane	–	–	–	–	3.00
Ethanolamine	1-Aminoethanol	–	–	2.54*	2.19	–
1,3-Diaminopropane	1,3-Propanediamine	–	–	–	2.66	2.80
Di-*n*-butylamine	–	–	–	–	–	3.78
Putrescine	1,4-Diaminobutane	–	–	–	–	3.78
Cadaverine	1,5-Diaminopentane	–	–	–	–	4.72

*These two amines are not resolved.

RESULTS

The results of comparing different column packings, with respect to analysis of free amines in aqueous solution, are shown in Table I.

Resolution could be improved by use of lower temperatures but only at the expense of peak broadening and longer analysis time under isothermal conditions. The data in Table I, therefore, represent a compromise. On the basis of the isothermal tests, Chromosorb 103 proved the most satisfactory packing since it permitted adequate separation of eleven amines at 185°C; also it did not suffer from the ghosting observed with alkali-washed support-packings. When Chromosorb 103 was used as the packing in 2.74 × 0.6 cm O.D. columns with temperature programming, the number of amines resolved was increased to thirteen. Individual retention data relative to *n*-butylamine are presented in Table II. A separation of thirteen standard amines as their free bases by temperature programmed GC on Chromosorb 103 is depicted in Fig. 1. An additional two bases, putrescine and cadaverine, could be separated provided that di-*n*-butylamine was excluded from the standard mixture (Table II). The use of longer columns resulted in increased analysis time which could not be reduced by use of a higher final temperature owing to excessive column bleed. The application of the method to a culture of *Proteus mirabilis* yielded the chromatogram shown in Fig. 2. Peaks were tentatively identified as ethylamine, isobutylamine and isoamylamine with the largest peak having the retention time of the neutral fermentation end-product, ethanol. Fig. 3 shows a gas

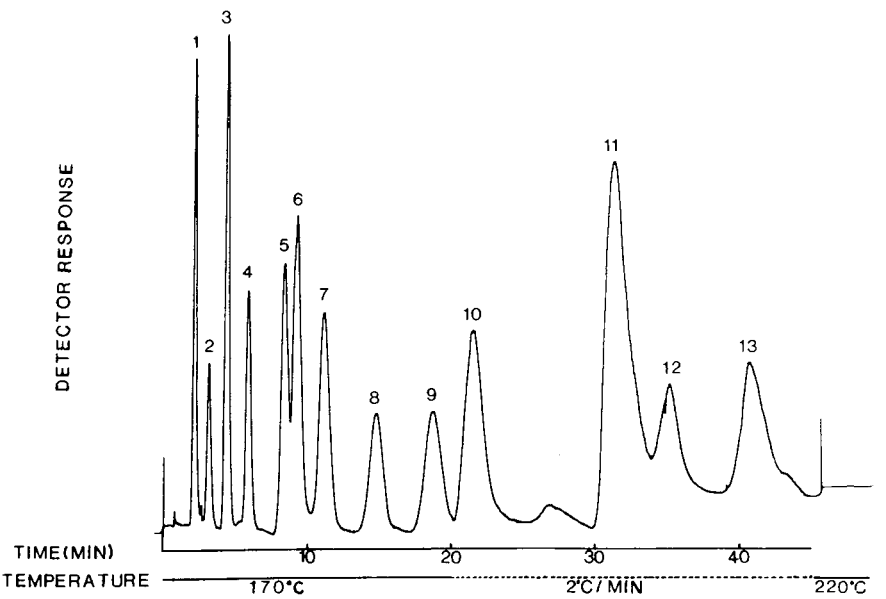

Fig. 1. Thirteen amines separated as their free bases in aqueous solution by analysis on 2.74 m × 0.6 cm O.D. columns of Chromosorb 103 using temperature programming. Peaks: 1 = methylamine; 2 = ethylamine; 3 = isopropylamine; 4 = *n*-propylamine; 5 = *sec*-butylamine; 6 = isobutylamine; 7 = *n*-butylamine; 8 = triethylamine; 9 = 2-methylbutylamine; 10 = *n*-amylamine; 11 = 1,3-diaminopropane; 12 = *n*-hexylamine. 13 = di-*n*-butylamine. Data for a separation programmed at 3° C/min are shown in Table II.

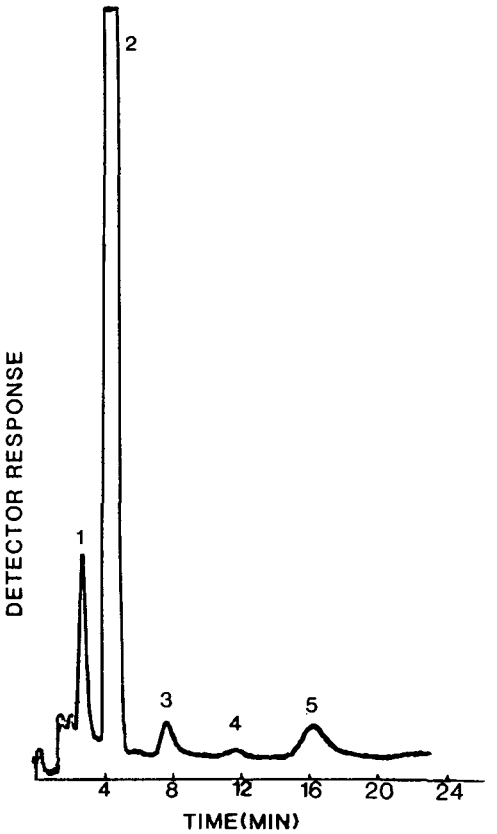

Fig. 2. Amines of *Proteus mirabilis* analysed as their free bases on a column of Chromosorb 103 (1.52 × 0.6 cm O.D.), isothermally at 165° C. Peaks: 1 = ethylamine; 2 = ethanol; 3 = isobutylamine; 4 = unknown peak; 5 = isoamylamine.

chromatogram for a mixture of standards of ethylamine, ethanol, isobutylamine, isoamylamine and *n*-amylamine added to un-inoculated culture medium. No such peaks were observed when an un-inoculated medium was analysed as a negative control.

DISCUSSION

The occurrence of amines in a number of micro-organisms has led to the suggestion that their analysis in head-space gas samples might assist microbial identification [13]. However, the lack of really satisfactory methods for analysis of amines has delayed detailed taxonomic studies on bacterial amines despite the far-sighted studies of Brooks et al. [16] who separated thirteen amines after extraction and formation of their less-polar HFBA derivatives. In a previous study, Dunn et al. [17] assessed the performance of ten column packings for the separation of a small number of primary, secondary and tertiary amines. In their hands, Chromosorb 103 proved "inconsistent" and "difficult to pack". This porous polymer does tend to expand on heating,

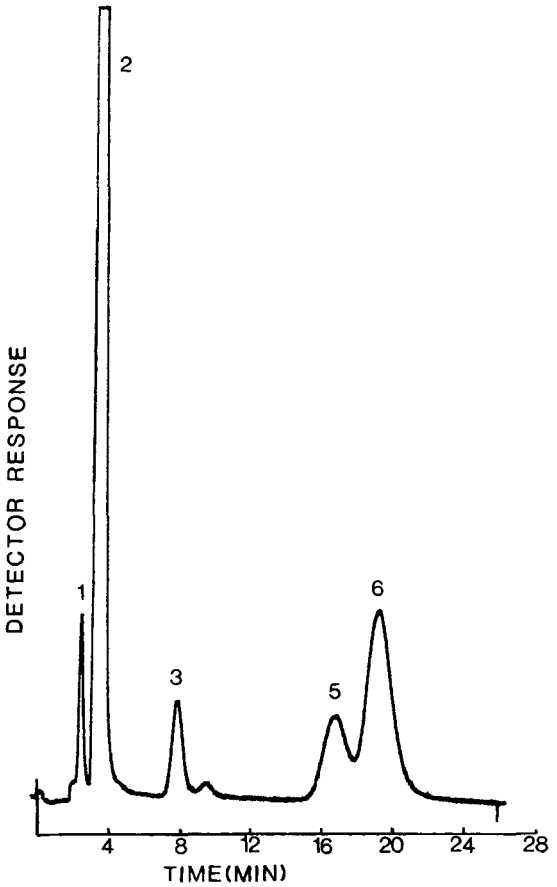

Fig. 3. Standard gas chromatogram. Ethanol and free amines analysed on Chromosorb 103 at 165°C. Peaks: 1 = ethylamine; 2 = ethanol; 3 = isobutylamine; 5 = isoamylamine; 6 = *n*-amylamine.

leaving gaps in the column upon cooling. This effect was minimised in the present investigation by paying scrupulous attention to packing of columns. Whereas Amine 220 plus potassium hydroxide was excellent for separating primary, secondary and tertiary amines in the Dunn et al. [17] study, it proved less satisfactory for the purpose of separating a large number of bacterial amines. Direct analysis of free amines in aqueous samples offers the advantages of simplicity and rapidity of analysis since it obviates the need for complex and time-consuming extraction and derivatization procedures. Results of the present study indicate that although the tailing of amines is largely reduced on the packings tested, the number of amines separated remains relatively low. The ghost peaks observed with alkaline supports have been described by other workers [18]. The phenomenon of ghosting complicated peak identification. The major disadvantage of alkali-washed packings lies in the thermal instability of the liquid phases tested which prevents the temperature programmed analysis that is required for the complete separation of complex mixtures of bacterial amines. Nevertheless, alkaline packings seemed useful for the separa-

tion of homologues of simple aliphatic amines, in agreement with other studies [19]. When amines of *Proteus mirabilis* were analysed isothermally on Chromosorb 103, ethylamine was found in addition to the two amines previously reported [13] when a head space gas sample was analysed. The separation of putrescine and cadaverine from the other amines tested is of particular significance. The diamines are commonly found in cultures of Gram-negative bacteria where they are believed to be synthesized by direct decarboxylation of ornithine and lysine respectively [20]. Putrescine may also be formed by decarboxylation of L-arginine to agmatine, followed by the cleavage of the resultant agmatine by the enzyme urea hydrolase [21]. It has been suggested that putrescine along with other polyamines may participate in regulatory mechanisms involved in transcription and cell division [22]. The diamines are reportedly present in dental plaque [23] and the urine of cancer patients [24]. The techniques described in this study permit the direct analysis of bacterial cultures and body fluids for thirteen amines including putrescine, cadaverine and other urinary monoamines.

REFERENCES

1 T.A. Gough, K.S. Webb and R.F. Coleman, Nature (London), 272 (1978) 161.
2 W.J. Irvine and M.J. Saxby, Phytochemistry, 8 (1969) 473.
3 T.L. Perry and W.A. Schroeder, J. Chromatogr., 12 (1963) 358.
4 Y. Ishitoya, S. Baba and I. Hashimoto, Clin. Chim. Acta, 46 (1973) 55.
5 E.F. Gale, Advan. Enzymol., 6 (1946) 1.
6 R.V. Golovnya, I.L. Zhuravleva and S.G. Kharatyan, J. Chromatogr., 44 (1969) 262.
7 M. Luckner, Secondary Metabolism in Plants and Animals, Chapman and Hall, London, 1969, p. 186.
8 Y. Hoshika, Anal. Chem., 48 (1976) 1716.
9 T. Gejvall, J. Chromatogr., 90 (1974) 157.
10 T. Walle, Acta Pharm. Suecica, 5 (1968) 367.
11 J.F. Klebe, H. Finkbeiner and D.M. White, J. Amer. Chem. Soc., 88 (1966) 3390.
12 B. Crathorne and M.J. Saxby, J. Chromatogr., 82 (1973) 373.
13 L. Larsson, P.A. Mårdh and G. Odham, Acta Pathol. Microbiol. Scand., 86 (1978) 207.
14 E.G. Smith and R.D. Radford, Anal. Chem., 33 (1961) 1160.
15 W.J. Irvine and M.J. Saxby, J. Chromatogr., 43 (1969) 129.
16 J.B. Brooks, W.B. Cherry, L. Thacker and C.C. Alley, J. Infect. Dis., 126 (1372) 143.
17 S.R. Dunn, M.L. Simenhoff and L.G. Wesson, Anal. Chem., 48 (1976) 41.
18 R.A. Simonaitis and G.C. Guvernator, J. Gas Chromatogr., 5 (1967) 49.
19 G.R. Umbreit, R.E. Nygren and A.J. Testa, J. Chromatogr., 43 (1969) 25.
20 M.A.S. Lambert and C.W. Moss, Appl. Microbiol., 26 (1973) 517.
21 H. Tabor and C.W. Tabor, Advan. Enzymol., 36 (1972) 203.
22 R.W. McGilvery, Biochemistry — A Functional Approach, W.B. Saunders Company, Philadelphia, 2nd ed., 1979, p. 628.
23 A.T. Hyatt and M.L. Hayes, Arch. Oral Biol., 20 (1975) 203.
24 D.H. Russel, C.C. Levy, S.C. Schimpff and I.A. Hawk, Cancer Res., 31 (1971) 1555.

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CHROMBIO. 1595

POSTCOLUMN DERIVATIZATION OF CATECHOLAMINES WITH 2-CYANOACETAMIDE FOR FLUORIMETRIC MONITORING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Catecholamines in the eluates of high-performance liquid chromatography were reacted with 2-cyanoacetamide in borate buffer in flow analysis mode, and the intensity of fluorescence developed was recorded. Under the optimum conditions (column: Hitachi 3011 C, 25 cm × 2.6 mm I.D., 45°C; eluent: 0.05 *M* KH_2PO_4 containing 0.05% H_3PO_4, 0.60 ml/min; reagent solution: a mixture of 1% 2-cyanoacetamide, 0.50 ml/min, and a 0.60 *M* H_3BO_3—KOH buffer, 1.0 ml/min; size of reaction coil: 5 m × 0.5 mm I.D.; reaction temperature: 100°C; wavelengths for detection: 383 nm for excitation and 486 nm for emission), this method allowed simultaneous determination of 5—500 pmol catecholamines with high reproducibility. The lower limits of detection (signal-to-noise ratio = 2) for epinephrine, norepinephrine and dopamine were 0.28, 0.11 and 0.098 pmol, respectively. Some applications of the analysis of urinary and serum catecholamines are also presented.

INTRODUCTION

Biochemical and clinical investigations of catecholamines have made great progress owing to the development of sensitive high-performance liquid chromatography (HPLC). Although electrochemical detection is currently used for monitoring catecholamines [1], fluorimetric detection based on postcolumn derivatization still plays an important role, because of its stability and the durability of the monitoring system. The most widely used method for fluorimetric detection is the method based on postcolumn oxidation to trihydroxyindole (THI) derivatives and their fluorimetric monitoring [2]. However, this method has the disadvantage that its sensitivity for dopamine (DA) is approximately two orders of magnitude lower than for epinephrine (E) and norepinephrine (NE). The ethylenediamine (ED) method [3] shows rather uniform sensitivity to all catecholamines, but it is less sensitive than the THI method.

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Recently, we found that the condensation reaction with 2-cyanoacetamide is also applicable to fluorimetric determination of catecholamines. The results obtained for manual determination [4] demonstrated its high, uniform sensitivity, as well as simplicity of procedure. Therefore, we have applied this reaction to fluorimetric monitoring of catecholamines in HPLC.

EXPERIMENTAL

Materials

A sample of 2-cyanoacetamide was obtained from Kanto Kagaku (Tokyo, Japan) and used without further purification. Reagent grade samples of E (bitartrate), NE (bitartrate) and DA (hydrochloride) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), Wako (Osaka, Japan) and Nakarai (Kyoto, Japan), respectively. All other chemicals were of the highest grade commercially available.

The samples of urine and serum were collected from a 24-year-old healthy man and were immediately subjected to clean-up treatment with aluminium oxide.

Apparatus

Fluorescence spectra were recorded on a Shimadzu RF-500 spectrofluorimeter using a 1 × 1 cm quartz flow cell.

Separation of catecholamines by HPLC

A Hitachi 635 A high-performance liquid chromatograph, equipped with a jacketed stainless-steel column (25 cm × 2.6 mm I.D.) packed with Hitachi 3011 C resin (particle size, 10—25 μm), was used for the separation of catecholamines. The column was maintained at 45°C by circulating warm water and eluted with 0.05 *M* dipotassium hydrogen phosphate containing 0.05% phosphoric acid at a flow-rate of 0.60 ml/min. This separation system is a modification of the system used for Hitachi catecholamine analyzers. The solutions of catecholamine samples were introduced onto the column via a Rheodyne 20-μl loop injector.

Postcolumn derivatization

Atto SF-2396 twin-piston pumps were used to supply the reagent solution. One pump head was used for pumping an aqueous 1% solution of 2-cyanoacetamide and the other for pumping a borate buffer. These solutions were mixed by a three-way connector, and the resultant reagent solution was introduced into the stream of the eluate via another three-way connector. The flow-rates of the aqueous reagent solution and the buffer were 0.50 and 1.0 ml/min, respectively.

The postcolumn derivatization was performed in a PTFE coil with an inner diameter of 0.5 mm, which was immersed in a glycerol bath thermostated at 100±1°C. The fluorescence intensity was measured with a Hitachi 650 LC fluorimetric detector equipped with a 90-μl quartz flow cell. All tubing in the derivatization system was PTFE with an inner diameter of 0.5 mm.

Clean-up of urine samples

To a 5-ml portion of urine was added 2 *M* hydrochloric acid (5.3 ml), and the mixture was heated for 20 min at 100°C. After the mixture was cooled to room temperature, 0.05 *M* disodium ethylenediaminetetraacetate (1 ml) was added, followed by ammonia water to adjust the pH to 8.5. Aluminium oxide (Wako, 200 mesh, 500 mg) was added to the resultant solution, and the mixture was shaken for 10 min. Then the mixture was filtered by a pencil column (5 cm × 8 mm I.D.) carrying a Millipore filter. The aluminium oxide in the column was subsequently washed with water (10 ml), followed by 0.3 *M* acetic acid (5 ml). The acetic acid eluate was analyzed for catecholamines.

Clean-up of serum samples

The procedure of Itano [5] was slightly modified. Activated aluminium oxide (100 mg) was suspended in 1 *M* Tris—HCl buffer (pH 8.7, 1 ml), and to this suspension was added a serum sample (1 ml). The mixture was stirred and allowed to stand for 10 min. The supernatant was discarded, and the precipitates were washed three times with 5-ml portions of water, followed by methanol (3 ml). The precipitates finally obtained were dried under reduced pressure, and catecholamines adsorbed on the precipitates were percolated with 4 *M* acetic acid (3 ml). The percolate (2.7 ml) was evaporated to dryness under reduced pressure, and the residue was dissolved in water (90 μl). A 10-μl portion of the solution was subjected to catecholamine analysis.

RESULTS AND DISCUSSION

Characteristics of the fluorescence reaction

The generation of fluorescence from catecholamines and 2-cyanoacetamide is considered to be due to the production of nitrogenous bicyclic compounds by condensation of the diphenol group with the reagent, followed by dehydrative cyclization [6]. The presence and absence of the hydroxyl and N-methyl groups in the aliphatic chain are responsible for slight variation of the optimum pH and wavelengths of excitation and emission maxima among catecholamines. In the established procedure for manual determination [4] an aqueous solution of a catecholamine sample, an aqueous 1% solution of 2-cyanoacetamide and a 0.3 *M* borate buffer were mixed and the mixture was heated to generate fluorescence. The optimum pH of the buffer to be added was 12.0, 10.0 or 11.0 for E, NE or DA, respectively. The wavelengths for detection were (excitation/emission) 380/483, 361/460, or 362/435 nm, respectively.

pH dependence of postcolumn derivatization

Fig. 1 shows the relationship between pH of waste fluid and peak response for each catecholamine species. Each catecholamine was detected at three different wavelengths mentioned above for manual determination.

The maximum point for E was observed at about pH 11, when monitored at 380/483 nm. For other wavelengths the peak response continued to increase with increasing pH values, but it did not exceed the maximal value for 380/483 nm over the whole pH range. Although there was a slight difference of optimum pH between HPLC (approx. 11) and manual determination (12.0),

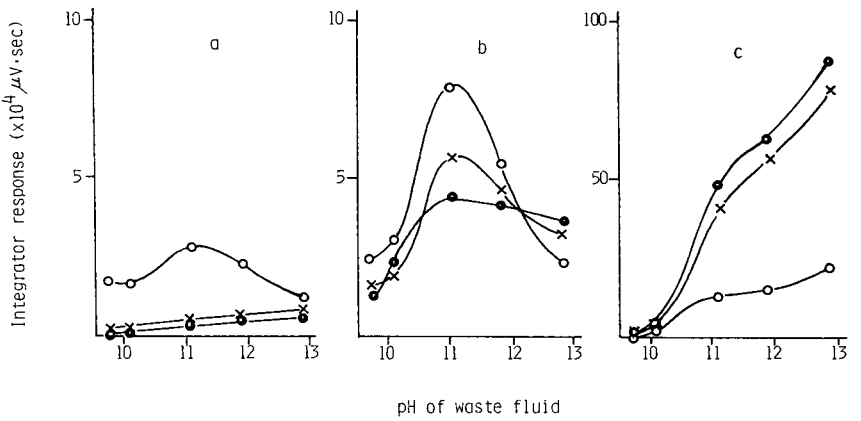

Fig. 1. Relationship between pH of waste fluid and peak response for (a) epinephrine, (b) norepinephrine and (c) dopamine. (○) Obtained at 380 (excitation)/483 (emission) nm; (×) obtained at 361/460 nm; (●) obtained at 362/435 nm. Sample scale: 50 pmol of each.

it should be noted that the pH for HPLC was that of the waste fluid and the value for manual determination was that of the buffer to be added. Because the HPLC eluate was acidic this would explain the difference of pH.

For monitoring of NE, unexpected phenomena were observed. Firstly, the peak response obtained at 361/460 nm, where the maximum intensity was obtained for NE in the manual method, was smaller than that obtained at 380/483 nm. Secondly, the optimum pH (approx. 11) was higher than that (10.0) for manual determination. The value for HPLC should be somewhat lower considering the acidity of the eluate. In order to clarify these discrepancies, NE was reacted with 2-cyanoacetamide both in a borate buffer and a mixed borate—phosphate buffer having an identical pH value. These systems correspond to manual determination and HPLC, respectively. The result was that the former system gave only one fluorescence peak at 460 nm with the excitation maximum at 361 nm, as expected, but in the latter system two fluorescence peaks occurred at 451 and 466 nm, with excitation maxima at 362 and 371 nm, respectively. This diversity of fluorescence formation may account for the unexpected pH—peak response profile of NE.

The peak response of DA increased rapidly up to a pH of approx. 11. Thereafter, the rate of increase was rather reduced. The curve obtained at 362/435 nm, the wavelengths of DA for manual determination, was the uppermost over this pH range. These observations conform to the results obtained for manual determination.

Length of reaction coil

Reaction coils of various lengths were examined for derivatization, and the best result was obtained for the length of 5 m, when a PTFE coil with an inner diameter of 0.5 mm was used. It took 35 sec for a sample to pass through this coil when the flow-rates of the eluate and the reagent solution were controlled at 0.60 and 1.50 ml/min, respectively. With longer coils separation of peaks was incomplete due to spreading. With shorter coils peaks were smaller.

Recommended conditions for monitoring catecholamines

Although pH and wavelength for detection can be selected for each catecholamine species in manual determination, they should be fixed to common values in HPLC. We chose a value of 11 for the pH of the waste fluid. This value could be obtained by using a borate buffer containing 0.60 *M* boric acid and 0.75 *M* potassium hydroxide as reagent buffer. The selected wavelengths were 383 nm (excitation) and 486 nm (emission). Fig. 2 shows the chromatogram obtained for an equimolar mixture of authentic catecholamines under these conditions.

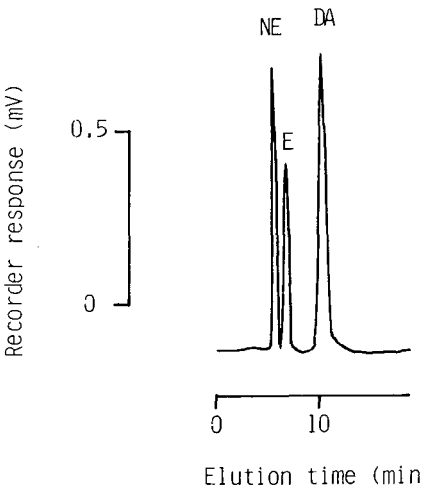

Fig. 2. Chromatogram of an equimolar mixture of epinephrine (E), norepinephrine (NE) and dopamine (DA), obtained by fluorimetric monitoring with 2-cyanoacetamide. Column, Hitachi 3011 C (25 cm × 2.6 mm I.D.); column temperature, 45°C; flow-rate of eluate, 0.60 ml/min; reagent solution for postcolumn derivatization, a mixture of an aqueous 1% solution (0.50 ml/min) of 2-cyanoacetamide and 0.60 *M* H_3BO_3—0.75 *M* KOH buffer (1.0 ml/min); wavelengths for detection, 383 nm (excitation)/486 nm (emission); sample scale, 20 pmol.

Calibration curves

As can be seen in Fig. 3, the calibration curves for all catecholamines showed good linearity for sample amount in the range 5 to at least 500 pmol.

Sensitivity

The lower limits of detection of E, NE and DA were 0.28, 0.11 and 0.098 pmol, respectively, for a signal-to-noise ratio of 2. The sensitivity for DA was approximately 100 times higher than with the THI method, and the sensitivity for E and NE was approximately the same as with the THI method.

Reproducibility

The coefficient of variation obtained for ten determinations of E, NE and DA at the 50 pmol level was 3.1%, 3.9% and 3.2%, respectively, indicating that the present method is satisfactorily reproducible. The values at the 5 pmol level were rather high (6.8%, 6.6% and 4.9%, respectively), but they were within the limits for practical analysis of biological samples.

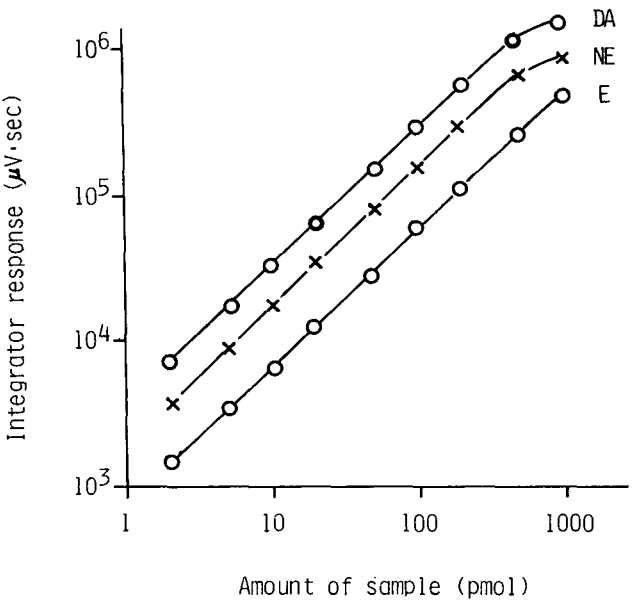

Fig. 3. Calibration curves of epinephrine (E), norepinephrine (NE) and dopamine (DA).

Interference

In the fluorescence reaction of catecholamines with 2-cyanoacetamide there was interference from the presence of reducing carbohydrates. The relative molar intensities of aldoses to DA were in the range 0.01—0.04 when measured by the manual method using a borate buffer of pH 11.0. However, the interference in HPLC was not very serious when the molar ratio of total reducing carbohydrates to catecholamines was less than 100, because the peak of reducing carbohydrates eluted fast and was well separated from those of catecholamines. Interference by other biological substances such as amino acids, carboxylates, proteins, nucleic acids and related compounds, as well as inorganic salts in body fluids, was negligible at equimolar levels.

The detailed data obtained for manual determination have been reported [4].

Analysis of urinary catecholamines

Direct injection of a urine sample gave a sharp peak of DA, but the peaks of E and NE were superimposed on a fast-eluting tailing peak arising from reducing carbohydrates, since the molar ratio of total urinary reducing carbohydrates to urinary catecholamines was in the range 100—1000 [6]. Therefore, urine samples were pretreated by a simple procedure with commercial aluminium oxide. Fig. 4 shows an example of chromatograms obtained for a pretreated urine sample. The volume of the sample injected onto the column was equivalent to 10 μl of untreated urine. The major urinary catecholamine was shown to be DA (276 ng/ml). The concentrations of E and NE were 14 and 24 ng/ml, respectively. All these values were consistent with the reported values of normal urinary catecholamine levels [7].

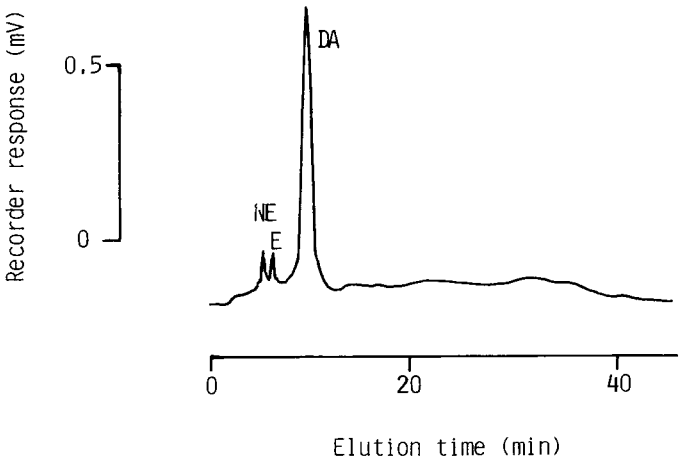

Fig. 4. Analysis of urinary catecholamines of a normal man by fluorimetric monitoring with 2-cyanoacetamide. Injected sample volume was equivalent to 10 μl of urine. E = epinephrine; NE = norepinephrine, DA = dopamine.

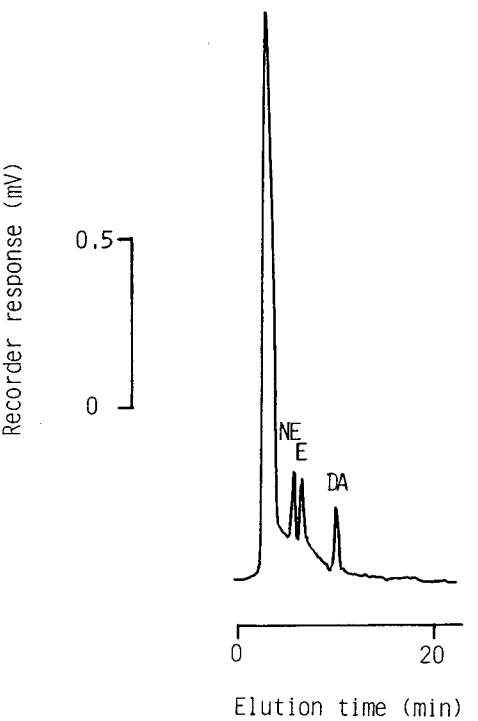

Fig. 5. Analysis of serum catecholamines of a normal man by fluorimetric monitoring with 2-cyanoacetamide. Injected sample volume was equivalent to 100 μl of serum. E = epinephrine, NE = norepinephrine, DA = dopamine.

Analysis of serum catecholamines

Because the serum D-glucose level (approx. 5 μmol/ml) was much higher than the catecholamine level (1—10 pmol/ml), the interference by this aldose was serious. Accordingly, serum samples were pretreated by a modification of the method of Itano [5] by using activated aluminium oxide. Fig. 5 shows an example of chromatograms obtained for pretreated serum samples.

The injected volume was equivalent to 100 μl of serum. Peaks of E, NE and DA were easily recognized and quantitated, the concentrations being 0.9, 2.6 and 2.6 ng/ml, respectively. All these values were slightly higher than those reported in the literature [7] (E = 0.77±0.09 ng/ml; NE = 1.69±0.20 ng/ml; DA = 1.16±0.06 ng/ml), probably because the blood sample was collected from a patient in a rather excited state.

The foregoing results indicate that the postcolumn derivatization of catecholamines to fluorescent products with 2-cyanoacetamide may offer a simple method for their monitoring by HPLC. The high and uniform sensitivity of the method for all naturally occurring catecholamines should be evaluated for the simultaneous analysis of biological samples, especially body fluids.

REFERENCES

1 P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Riggins and R.E. Shoup, Clin. Chem., (1977) 1449.
2 Y. Yui, T. Fujita, T. Yamamoto, Y. Itokawa and C. Kawai, Clin. Chim. Acta, 26 (1980) 194.
3 T. Seki, J. Chromatogr., 155 (1978) 415.
4 S. Honda, Y. Araki, M. Takahashi and K. Kakehi, Anal. Chim. Acta, in press.
5 Y. Itano, unpublished results.
6 S. Honda, unpublished results.
7 A. Mori and H. Kishikawa, Jap. J. Clin. Chem., 4 (1975) 34.

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CHROMBIO. 1611

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOR THE SIMULTANEOUS DETERMINATION OF THE METHOXYLATED AMINES, NORMETANEPHRINE, METANEPHRINE AND 3-METHOXYTYRAMINE, IN URINE

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SUMMARY

A simple method for the simultaneous analysis of normetanephrine, metanephrine and 3-methoxytyramine (both free and conjugated) in human urine by reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection has been developed. Existing methods have been optimized for extraction by study of analytical parameters. The hydrolysed urines are purified and concentrated by successive passages on two ion-exchange resins and ammoniacal elution to eliminate interference from pigments or related chemical compounds. The methoxyamines are separated by high-performance liquid chromatography on a reversed-phase column. Detection and quantitation are achieved with an electrochemical detector using a vitreous carbon electrode. Samples can be injected at 25-min intervals. Reference values of adults and children are given.

INTRODUCTION

The need for rapid analysis of 3-O-methylated catecholamines arises from their important biological role. Various methods have been developed for their detection; however, some lack the necessary sensitivity and others are too complicated for routine analysis. The relatively low concentrations of monoamine metabolites have necessitated the use of sensitive analytical techniques such as gas chromatography—mass spectrometry [1] which requires expensive equipment, or high-performance liquid chromatography (HPLC) with fluorescence detection [2] or electrochemical detection (ElCD) [3—8].

We report here a procedure for urinary metabolites, which has the adequate sensitivity and accuracy otherwise exhibited by the more complex HPLC

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separation and ElCD systems. Reversed-phase chromatography is eminently suitable for separation of 3-O-methyl derivatives [9]. HPLC on microparticulate reversed-phase columns [10] offers a powerful technique for separating related compounds which are water-soluble but with some hydrophobic character. ElCD offers a major increase in sensitivity and is also somewhat selective for these metabolites [11—13]. A large peak was encountered in certain urine samples which interfered with the accurate measurement of 3-methoxytyramine; to eliminate all interference, we have made appropriate modifications to the extraction procedure [14—20] from a small quantity of acidified urine and under varying chromatographic conditions. Also, nanogram levels of 3-O-methylated amines have been determined in urine. The simplicity of the procedure favours multiple sample analysis.

EXPERIMENTAL

Apparatus

The analyses were performed on a Hewlett-Packard 1080 A chromatograph with an electrochemical detector (Tacussel Systems) consisting of: Faraday cage enclosing a cell-Tacussel DELC with a glassy carbon electrode (working electrode), a Pt electrode (auxiliary electrode), a Ag/AgCl electrode (reference electrode) and polarographic analyser Tacussel PRG-E.

The chromatograph comprises an analytical column (300 mm × 3.9 mm I.D.) packed with μBondapak C_{18} (10 μm particle size), and combined with a 30 × 3.9 mm I.D. precolumn filled with μBondapak C_{18}/Corasil, all from Waters Assoc. (Milford, MA, U.S.A.). Peak areas were obtained using a Hewlett-Packard integrator.

Chromatographic conditions

The flow-rate was adjusted to 1.5 ml/min, and the temperature of the column compartment and the eluents was 25°C. Eluent A contained a buffer consisting of 2 vols. of 0.02 *M* citric acid and 1 vol. of 0.02 *M* $NaHPO_4$ $2.5 \cdot 10^{-3}$ *M* sodium octylsulfonate and $5 \cdot 10^{-5}$ *M* disodium EDTA were added to 100 ml of buffer [13]. The mixture was filtered under vacuum through a 0.40-μm Millipore HA type filter before use. Eluent B was methanol (special for chromatography) from E. Merck (Darmstadt, G.F.R.). The mobile phase contained 90% of buffer (eluent A) and 10% of methanol (eluent B).

Materials

D,L-Metanephrine hydrochloride (B grade; MN), D,L-normetanephrine hydrochloride (B grade; NMN) and 3-methoxytyramine hydrochloride (A grade; MT) were all from Calbiochem (San Diego, CA, U.S.A.). D,L-α-methyl DOPA (3,4-dihydroxyphenylalanine) was from Sigma (St. Louis, MO, U.S.A.). Water was demineralized and distilled in an all-glass apparatus. Stock solutions of standards and external standard (100 μg/ml) were prepared in 0.01 *N* hydrochloric acid. Octane sulfonic acid sodium salt was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Ethylenediaminetetraacetic acid (EDTA) disodium salt was from E. Merck (Darmstadt, G.F.R.). All other reagents were reagent grade from E. Merck: 4 *N* and 5 *N* ammonium hydroxide, 0.1 *M*

sodium acetate, and borate buffer containing 3.11% (w/v) of boric acid adjusted to pH 8.8 with 0.5 *N* sodium hydroxide. Millipore filters type GS, pore size 0.22 μm, were from Millipore Corporation (Bedford, MA, U.S.A.). The resins used are Dowex 50W-X2 (100—200 mesh) obtained from Fluka (Buchs, Switzerland) and Bio-Rex 70 (50—100 mesh, Na^+) from Bio-Rad (Richmond, CA, U.S.A.).

The resin Dowex 50W-X2 was first washed with numerous baths of glass-distilled water, then regenerated by washing with successive volumes of 4 *M* hydrochloric acid, 2 *M* sodium hydroxide and 4 *M* hydrochloric acid. The pH of the resin was rigorously adjusted to pH 5 and then washed with numerous baths of glass-distilled water just before use. The resin was poured into a Pyrex glass column.

The resin Biorex 70 may be cycled [13] by washing with successive volumes of 3 *M* hydrochloric acid, 3 *M* sodium hydroxide, 3 *M* acetic acid, 1.0 *M* ammonium acetate (pH 6.5) and 0.1 *M* ammonium acetate (pH 6.5). The pH was adjusted to 6.5 during the last wash if necessary. Just before use the resin was poured into a polypropylene Bio-Rex column (Bio-Rad).

Urine samples were collected over hydrochloric acid and acidified to pH 1 [21]. Methoxyamines are less sensitive to oxidation than catecholamines [22], thus acidified urine (pH 1) may be stored for at least a month without addition of EDTA or ascorbic acid.

Method

Urine samples (10 ml) were adjusted to pH 1 with 6 *N* HCl and hydrolysed at 100°C for 20 min [15, 21, 23, 24]. The hydrolysed urines were diluted to 40 ml with distilled water [16], the pH was readjusted to 6, and the amines were adsorbed on a 10 × 1 cm column of Dowex W-X2 H^+ resin. Then the column was washed with 10 ml of 0.1 *N* sodium acetate and 20 ml of water. The methoxyamines were eluted with 18 ml of 5 *N* ammonium hydroxide. The eluate was evaporated to dryness under vacuum at 40°C. The dry deposit was dissolved in 4 ml of borate buffer (pH 8.8). This eluate can not be used directly because it contains a large amount of urinary pigments. A second purification was necessary. The solution was diluted to 45 ml with 1% (w/v) EDTA solution, adjusted to pH 6.5 and passed through a short column (3.2 × 0.7 cm of Bio-Rex 70 resin). The column was washed with 10 ml of distilled water. The 3-O-methoxylated amines were eluted with 30 ml of 4 *N* ammonium hydroxide. Then 50 μl of α-methyl DOPA solution as an external standard were added. The eluate was evaporated to dryness under vacuum at 40°C. The dry deposit was dissolved in 2 ml of borate buffer (pH 8.8) and filtered on Millipore filters; 40 μl of this solution were injected onto the column. All experiments were carried out with citrate—phosphate buffer—methanol (90:10, v/v) by isocratic elution at a flow-rate of 1.5 ml/min. The present compounds were determined by an external standard method.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

The aim of this work has been to separate the three amines from a single

urine sample and to determine them with minimal interference from pigments or related chemical compounds. By coupling Dowex 50W-X2 and Bio-Rex 70 resins a better purification was achieved than with the use of one resin. One essential condition for quantitative adsorption of 3-O-methylated amines on Dowex 50W-X2 and good exchange of organic compounds on the resin was a low saline content of the sample [4]. The separation and the peak heights or areas of catecholic compounds on reversed-phase packed columns depended strongly on the methanol composition and the pH of the mobile phase, and the oxidizing potential (vs. Ag/AgCl).

In order to obtain the required separation the pH was optimised. Fig. 1 shows the variation of the retention times of 3-O-methylated amines with the pH of the eluent when the percentage of methanol was held constant. A pH of 3.2 seems to be suitable to achieve a good separation of the three compounds.

The effect of percentage of methanol on the retention times (Fig. 2A) and peak heights (Fig. 2B) was studied. Standards of NMN, MN and MT were

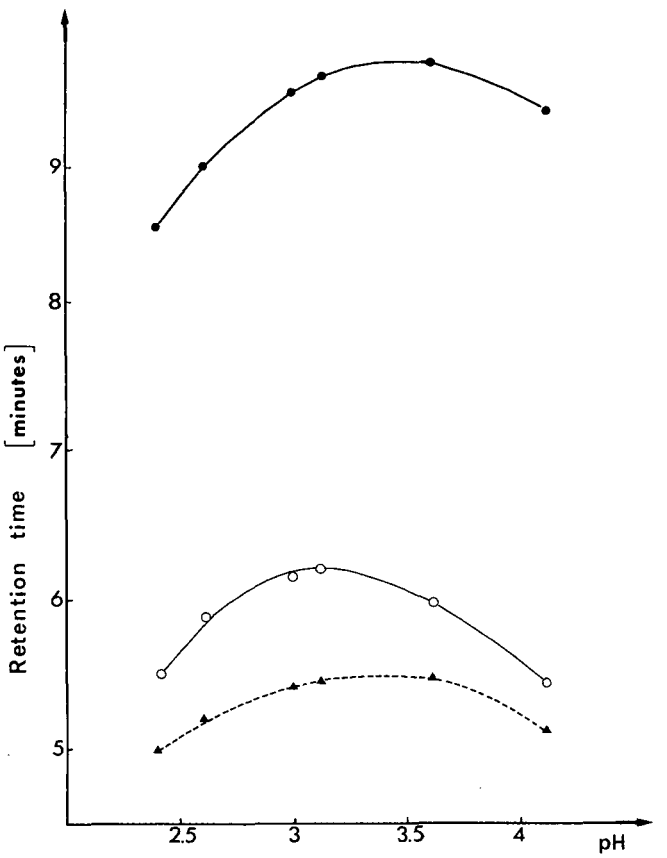

Fig. 1. Relationship between the retention times and the pH of the mobile phase. Column μBondapak C_{18}, mobile phase 90:10 (v/v) mixture of the 0.02 *M* citrate—phosphate buffer and methanol with octane sulfonic acid ($2.3 \cdot 10^{-3}$ *M*); pH adjusted to the different values with phosphoric acid or sodium hydroxide; flow-rate 1.5 ml/min; temperature 25°C; electrode potential +0.9 V vs. Ag/AgCl reference electrode. Symbols: ▲, NMN; ○, MN; ●, MT.

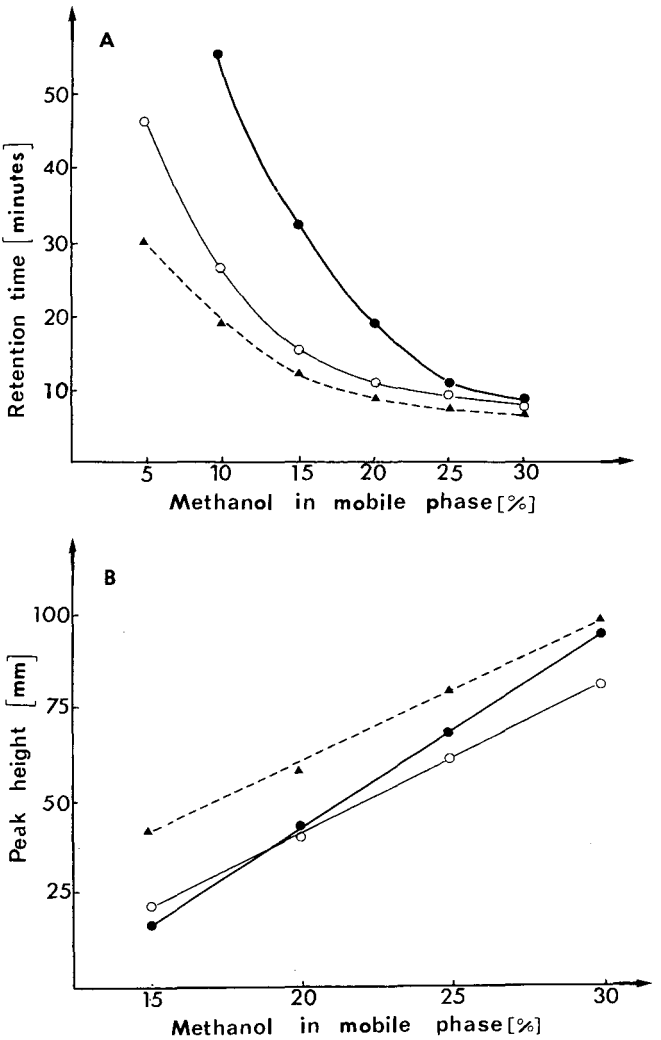

Fig. 2. Retention times and peak heights as a function of the percentage of methanol in the mobile phase. Symbols as in Fig. 1.

chromatographed using methanol—citrate—phosphate buffer pH 3.2 with increasing concentrations of methanol, as indicated in Fig. 2A and B. The addition of methanol to the buffer was necessary to reduce retention times. In our system 10% of methanol was found to be sufficient to achieve adequate resolution of the 3-O-methylated compounds.

Fig. 3 shows the relative detector response for assumed NMN, MN and MT peaks as a function of the oxidizing potential vs. Ag/AgCl reference electrode. The response at 0.9 V was set at 100% for each standard. Figs. 4 and 5 show that under the conditions outlined above the three O-methylated amines could be resolved within less than 25 min, at a flow-rate of 1.5 ml/min. The retention times for NMN, MN and MT were 8, 10 and 20 min, respectively. A constant

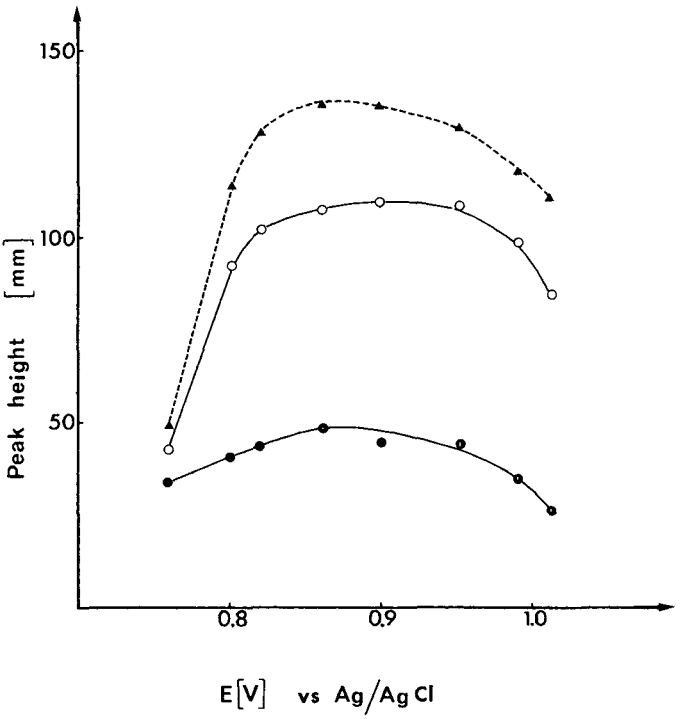

Fig. 3. Relationship between peak heights and the oxidizing potential, vs. Ag/AgCl. Symbols as in Fig. 1.

column temperature (25°C) was found to be necessary to obtain constant retention times. This result shows that different chromatographic conditions can lead to similar separations. A further consequence was that one can compensate for the loss of resolution of a column by decreasing the methanol concentration, or by changing both and adjusting the pH. In this case peak broadening occurs.

The linearity of both the extraction procedure and detector response (determined from peak area) was verified for each methoxyamine over the anticipated range of assay. The former was investigated by assaying pooled urine to which known amounts of NMN, MN and MT had been added and determining the peak areas obtained for each compound. Calibration curves were plotted for each compound (Fig. 6). In each case a linear relationship between methoxyamine concentration and peak area was observed over the concentration ranges studied. The equations for the calibration curves obtained were as follows:

NMN: $y = 0.166x - 0.78$

MN: $y = 0.153x - 0.05$

MT: $y = 0.165x - 0.01$

Each point on the calibration curve was established from the mean of five determinations. The linearity of detector response was confirmed by the injec-

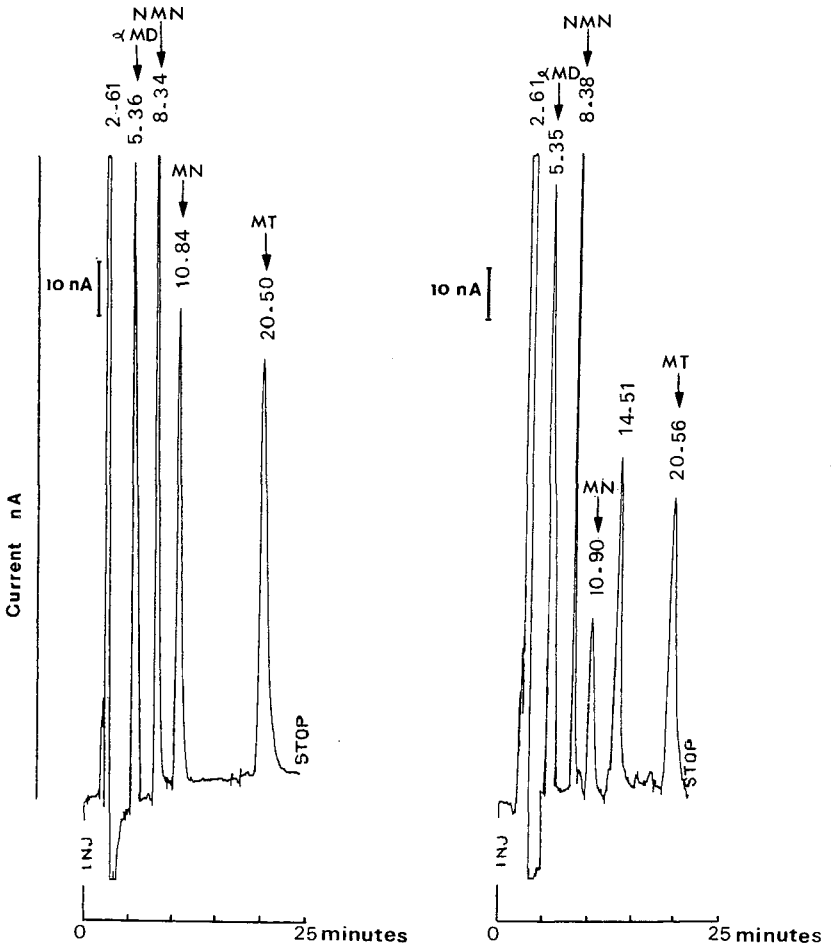

Fig. 4. Chromatogram of a standard mixture of 40 ng of normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MT), with α-methyl DOPA (α-MD) as an external standard.

Fig. 5. Chromatogram of a urine extract from a normal subject.

tion of known amounts of methoxyamine standards directly onto the chromatograph. Response for each compound was found to be linear over the range investigated (0—0.4 nmol).

Reference values

Forty urine specimens from normal children (twenty boys and girls) between the ages of 2 and 6 years and (twenty boys and girls) between the ages of 6 and 13 years were analysed. The reference values are presented in Table I. Thirty urine specimens from normal volunteers (healthy laboratory staff) (fifteen males and fifteen females) between the ages of 20 and 50 years were analyzed and found to yield a mean NMN excretion of 85.2 μmol/mol creatinine, a mean

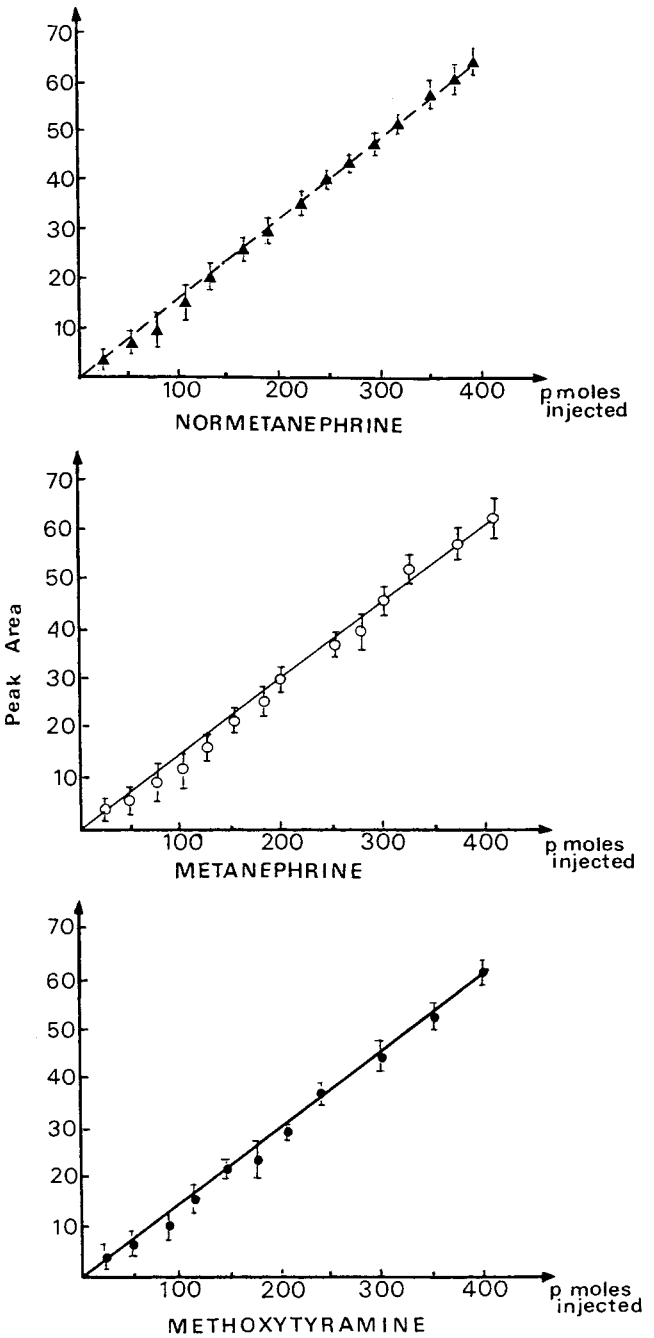

Fig. 6. Calibration curves for the determination of normetanephrine, metanephrine and methoxytyramine in urine by the assay procedure described.

TABLE I

URINARY NORMETANEPHRINE, METANEPHRINE AND METHOXYTYRAMINE IN 40 NORMAL CHILDREN AND 30 NORMAL ADULTS

Values are given in μmol/mol creatinine, mean ± S.D.

	Age group		
	2—6 years (n = 20)	6—13 years (n = 20)	20—50 years (n = 30)
Normetanephrine	203 ± 41	93.5 ± 8.5	85.2 ± 38.9
Metanephrine	204 ± 32	109 ± 29	73.2 ± 19.2
Methoxytyramine	183 ± 30	88.5 ± 18.5	59.6 ± 15.9

TABLE II

EXCRETION OF NORMETANEPHRINE, METANEPHRINE AND METHOXYTYRAMINE IN PATIENTS WITH PHEOCHROMOCYTOMA AND ESSENTIAL HYPERTENSION

Values are given in μmol/mol creatinine.

	Normetanephrine	Metanephrine	Methoxytyramine
Pheochromocytoma (n = 4)	3322	2562	174
	4018	2067	219
	4375	3346	145
	5026	3596	221
Essential hypertension* (n = 22)	200.5 ± 87.1	153 ± 69	112 ± 87

*Values expressed as mean ± S.D.

MN excretion of 73.2 μmol/mol creatinine and a mean MT excretion of 59.6 μmol/mol creatinine (Table I).

Medications were suppressed. Bananas, tea, coffee, tomatoes and vanilla-containing food were omitted.

A typical chromatogram for a normal urine is illustrated in Fig. 5.

Pathology

The excretion patterns of four patients with pheochromocytoma prior to therapy are presented in Table II. NMN, MN and MT excretions are respectively 49.1, 39.5 and 3.2 times higher than the reference values.

Twenty-two urine specimens from patients with essential arterial hypertension (twelve males, ten females) without other pathology able to modify catecholamine metabolism and who were on a normal diet, were analysed and found to yield a mean NMT excretion of 200.5 μmol/mol creatinine, a mean MN excretion of 204 μmol/mol creatinine and a mean MT excretion of 183 μmol/mol creatinine. NMN, MN and MT excretion are respectively 2.3, 2.7 and 3.07 times higher than the reference values.

CONCLUSION

In conclusion, the present method for analysis of urinary 3-O-methylated amines appears to be relatively simple and reliable. This technique is applicable to studies of derangements in catecholamine metabolism such as is found in patients with pheochromocytoma as well as to studies of hypertensive patients whose catecholamine metabolism may be minimally different from normal. Although this technique can at present be used for the determination of urinary NMN, MN and MT, it is being extended to the quantitation of other biologically important amines such as octopamine and tyramine. This methodology should find wide application in both clinical and research laboratories.

REFERENCES

1 P.M. Lax, G.S. King, B.R. Pettit and M. Sandler, Clin. Chim. Acta, 96 (1979) 269.
2 G.P. Jackman, Clin. Chem., 27 (1981) 1202.
3 L.M. Bertani-Dziedzic, A.M. Krstulović, S.W. Dziedzic, S.E. Gitlow and S. Cerqueira, Clin. Chim. Acta, 110 (1981) 1.
4 P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Riggin and R.E. Shoup, Clin. Chem., 23 (1977) 1449.
5 A.M. Krstulović, L. Bertani-Dziedzic, S. Bautista-Cerqueira and S.E. Gitlow, J. Chromatogr., 227 (1982) 379.
6 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
7 A.M. Krstulović, J. Chromatogr., 229 (1982) 1.
8 G. Wenk and R. Greenland, J. Chromatogr., 183 (1980) 261.
9 I. Molnar and C. Horvath, Clin. Chem., 22 (1976) 1497.
10 M.H. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
11 P.T. Kissinger, L.J. Felice, R.M. Riggin, L.A. Pachia and D.C. Wenke, Clin. Chem., 20 (1974) 992.
12 P.T. Kissinger, R.M. Riggin, R.L. Alcorn and L.D. Rau, Biochem. Med., 13 (1975) 299.
13 P.T. Kissinger and R.M. Riggin, Anal. Chem., 49 (1977) 2109.
14 Y. Kakihoto and M.D. Amstrong, J. Biol. Chem., 237 (1962) 208.
15 H. Greer, T.J. Spinkle and C.M. Williams, Clin. Chim. Acta, 27 (1970) 203.
16 M.C. Goodall and H. Alton, Biochem. Pharmacol., 17 (1968) 905.
17 H. Kaser and E. Thomke, Clin. Chim. Acta, 27 (1970) 203.
18 F. Geissbuhler, Clin. Chim. Acta, 30 (1970) 43.
19 Y. Dalmaz and L. Peyrin, J. Chromatogr., 116 (1976) 379.
20 E. Kempf and P. Mandel, Anal. Biochem., 112 (1981) 223.
21 Y. Dalmaz and L. Peyrin, J. Neural Transm., 46 (1979) 153.
22 L. Peyrin, Rev. Fr. Etud. Clin. Biol., 13 (1968) 88.
23 T.W. Fendley and C. Frings, Clin. Biochem., 9 (1976) 106.
24 P. Riederer and G.P. Reynolds, J. Chromatogr., 225 (1981) 179.

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QUANTITATION OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK USING A HIGH-SPEED AMINO ACID ANALYZER WITHOUT PURIFICATION OF THE DIPEPTIDE

APPLICATION TO ENZYMATIC DIGESTED MIXTURES OF KERATIN AND THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

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SUMMARY

ε-(γ-Glutamyl)lysine in an enzymically digested mixture of keratin and the membranous fraction of human stratum corneum was directly quantitated using a high-speed amino acid analyzer without purification of the dipeptide. The analytical conditions were improved so that ε-(γ-glutamyl)lysine clearly separated from other amino acids and eluted directly after tyrosine. The enzymatically digested mixtures were filtered through an Ultra Free membrane and deammoniated before analysis. By our present method, keratin and the membranous fraction of human stratum corneum were analyzed and 5.8 and 43.5 nmol/mg of ε-(γ-glutamyl)lysine, respectively, were detected.

INTRODUCTION

ε-(γ-Glutamyl)lysine is known to be one of the cross-links within and between molecules [1]. It is formed by the catalytic action of transglutaminase, which is a Ca^{2+}-dependent acyl transfer reaction between peptide-bound glutamine residues and peptide-bound lysine residues. The role of this

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covalent cross-link is to maintain gross forms of structure and limits of degrees of extensiveness. Pisano et al. [2] detected the ϵ-(γ-glutamyl)lysine cross-link in Factor XIII (plasma transglutaminase) polymerized fibrin after chemical and enzymatic treatments. Mosher [3] reported that fibronectin is a substrate for Factor XIII and can be cross-linked to collagen and the α-chain of fibrin. Birckbicher et al. [4] reported that there was less transglutaminase activity, fibronectin and ϵ-(γ-glutamyl)lysine in malignant hepatoma, virus-transformed human and hamster cells than in normal counterparts. Abernethy et al. [5] found ϵ-(γ-glutamyl)lysine cross-links in human stratum corneum and determined the dipeptide.

Following these analytical studies, many authors attempted to detect the cross-link in numerous tissues, organelles and proteins. Conventional methods are as follows. High molecular weight proteins or cell membranes which might contain ϵ-(γ-glutamyl)lysine cross-links were digested by many kinds of exo- and endopeptidases to determine the dipeptide. After digestion, the cross-linked dipeptide was purified using column liquid chromatography or electrophoresis. Finally, the dipeptide that consisted of glutamyl and lysyl residues was identified using amino acid analysis after acid hydrolysis. These purification and identification processes were complicated and time-consuming.

In this paper, we demonstrate ϵ-(γ-glutamyl)lysine cross-links in enzymically digested mixtures of keratin and the membranous fraction of human stratum corneum using a high-speed amino acid analyzer without purification of the dipeptide.

EXPERIMENTAL

Reagents

ϵ-(γ-Glutamyl)lysine was purchased from Vega-Fox Biochemicals (lot No. 10883) and α-aminobutyric acid from Sigma. A standard solution of ninhydrin-positive compounds was prepared as described in a previous paper [6]. Subtilisin, pronase, carboxypeptidase A-diisopropylfluorophosphate (DFP), leucine aminopeptidase and prolidase were the products of Sigma. Carboxypeptidase B was purchased from Boehringer Mannheim.

TABLE I

EXPERIMENTAL CONDITIONS FOR ϵ-(γ-GLUTAMYL)LYSINE

Column: 2.6 × 250 mm stainless-steel column packed with Hitachi ion-exchange resin No. 2619.									
Buffers: (1) 0.155 *N* lithium citrate pH 3.0 (lithium citrate 9.80 g/l, LiCl 2.12 g/l, citric acid 35 g/l, ethanol 40 ml/l, thiodiglycol 5 ml/l, and 25% Brij-35 4 ml/l). (2) 0.255 *N* lithium citrate pH 3.7 (lithium citrate 9.80 g/l, LiCl 6.36 g/l, citric acid 13 g/l, ethanol 30 ml/l, thiodiglycol 5 ml/l, and 25% Brij-35 4 ml/l). (3) 0.200 *N* LiOH (LiOH 8.40 g/l, and 25% Brij-35, 4 ml/l).									
Flow-rates: Buffer pump 0.275 ml/min, 190 kg/cm². Ninhydrin pump 0.300 ml/min, 30 kg/cm².									
Programs: Buffer change times	0		70		140		148		180 min
		Buffer 1		Buffer 2		Buffer 3		Buffer 1	
Column temperature	0		32		81				180 min
		34°C		43°C		34°C			

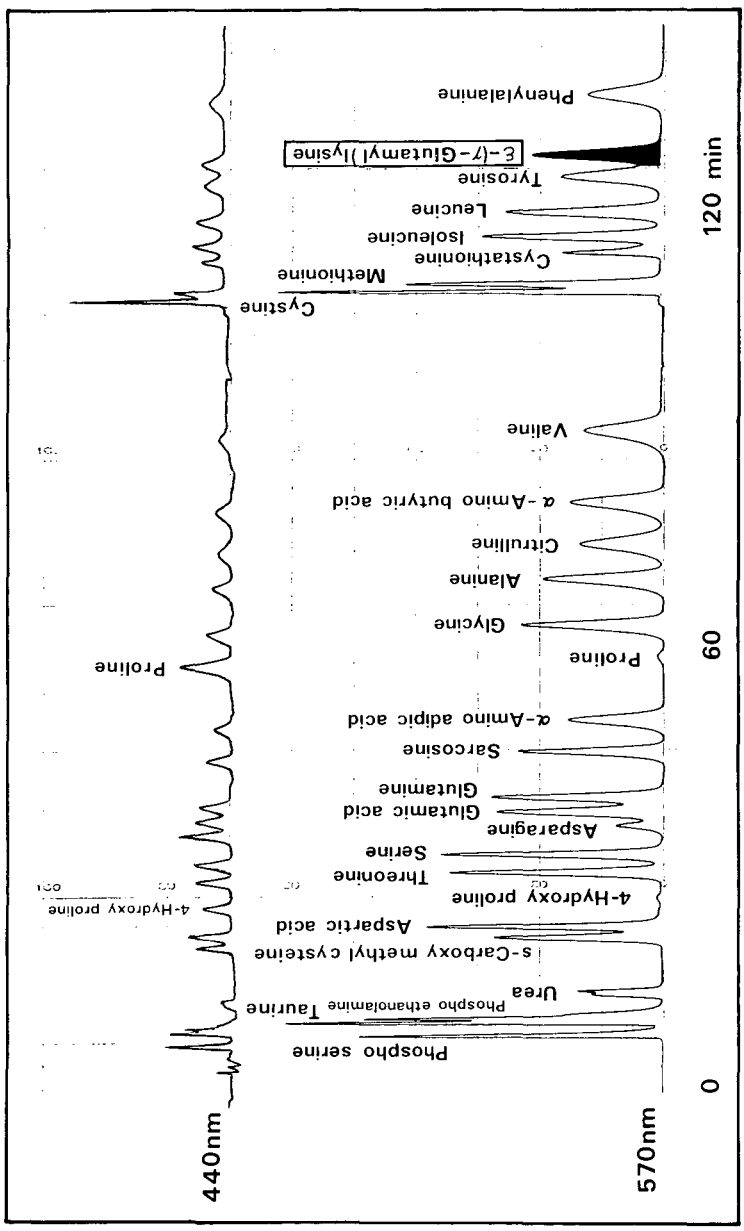

Fig. 1. Chromatogram of ε-(γ-glutamyl)lysine and amino acids using a high-speed amino acid analyzer. The concentration of ε-(γ-glutamyl)lysine was 1.463 nmol and that of other amino acids was 2.5 nmol except for cystathionine (1.0 nmol), S-carboxymethylcysteine (1.25 nmol), proline (5.0 nmol), sarcosine (5.0 nmol) and urea (250 nmol).

Analysis of amino acids and ε-(γ-glutamyl)lysine

Previously we reported an improved method for analyzing physiological amino acids using an Hitachi 835 high-speed amino acid analyzer [7]. Based on this analytical method, further modifications were made in order to identify ε-(γ-glutamyl)lysine. The detailed conditions are given in Table I.

Purification of ε-(γ-glutamyl)lysine as a standard

We had purchased ε-(γ-glutamyl)lysine from Vega-Fox Biochemicals; however, many peaks besides the main peak emerged on the amino acid chromatogram. Therefore, the ε-(γ-glutamyl)lysine was purified from this commercial source by thin-layer chromatography (TLC). The TLC plate pre-coated with silica gel was purchased from Merck. *n*-Butanol—acetic acid—water—ethyl acetate (1:1:1:1) was the solvent. The amino acids and ε-(γ-glutamyl)lysine were detected by spraying with a ninhydrin reagent. There were two spots, with R_F values of 0.25 and 0.20. The upper spot (R_F 0.25) was collected and extracted with 0.01 *N* hydrochloric acid. This spot was a single peak on the amino acid chromatogram and was found to contain equimolar amounts of glutamic acid and lysine after hydrolysis with 6 *N* hydrochloric acid at 110°C for 24 h.

Enzymic digestion of keratin and the membranous fraction of human stratum corneum

Keratin was prepared from human stratum corneum according to the method of Ogawa and Hattori [8]. The membranous fraction was isolated from the same materials, as described in our previous paper [9]. Sulfide bonds were modified by carboxymethylation as described by Abernethy et al. [5]. Each 25 mg of the carboxymethylated keratin and membranous fraction was digested with, sequentially, subtilisin (2%), pronase (2%), carboxypeptidase A (2%) and B (0.5%), leucine aminopeptidase (2%), prolidase (2%) and leucine

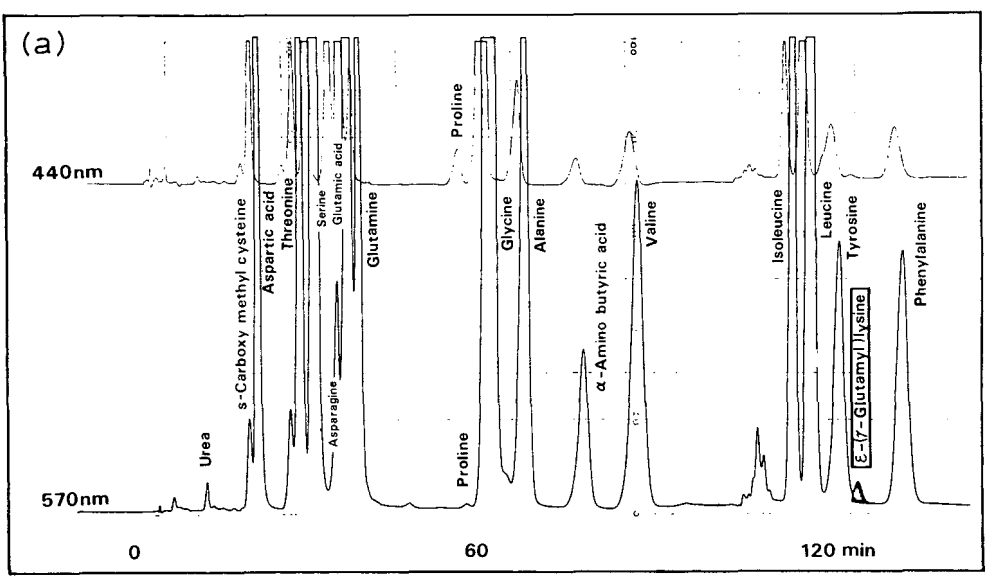

Fig. 2.

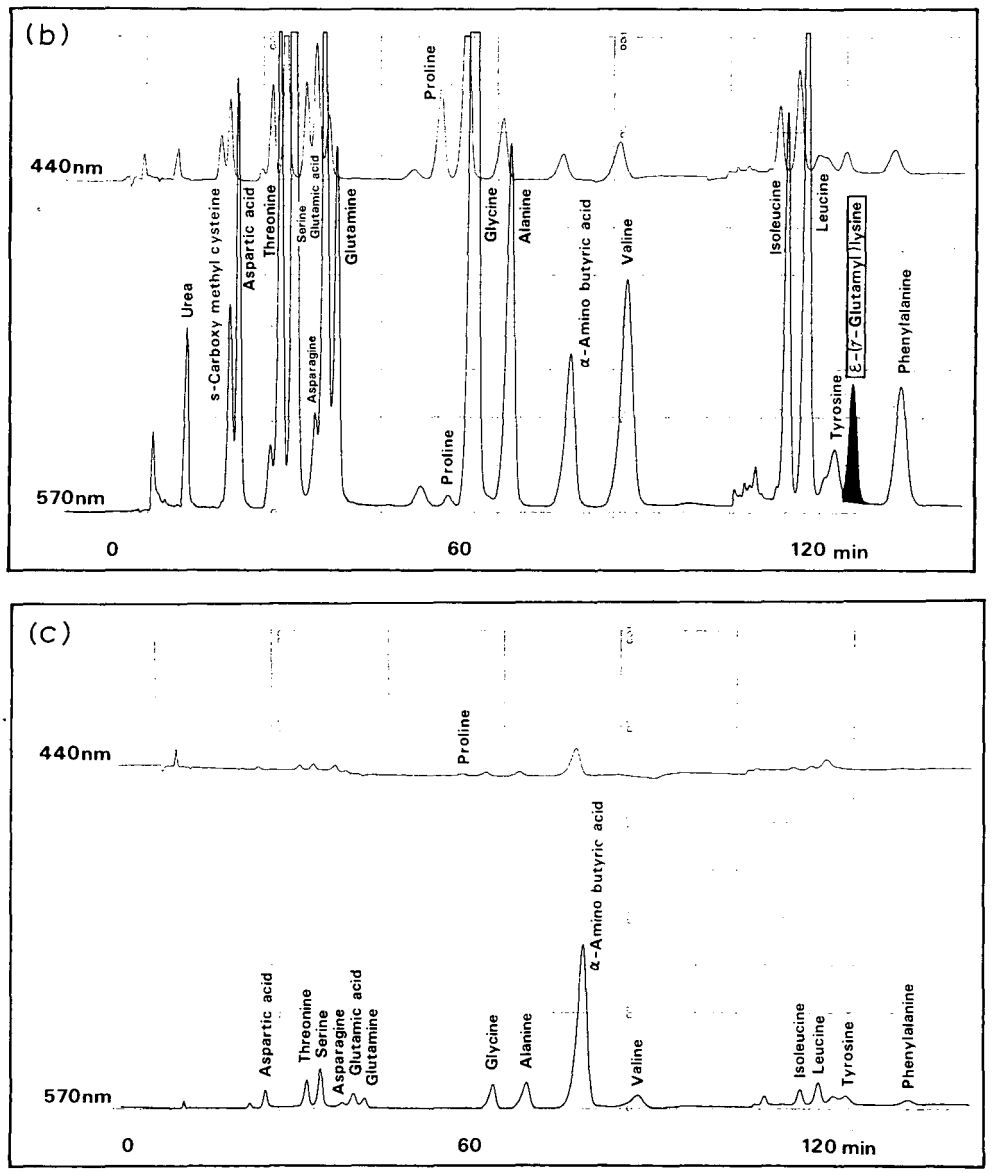

Fig. 2. Chromatograms of keratin (a) and the membranous fraction (b) of human stratum corneum for ϵ-(γ-glutamyl)lysine. Chromatogram (c) was obtained from the enzyme mixture as a control (10.8 μl). The amounts of keratin and the membranous fraction were 39.9 and 39.5 μg, respectively.

aminopeptidase (2%) using a modification of the method of Abernethy et al. [5]. We performed each enzymic digestion for 24 h at 37°C. Finally, digested mixtures were filtered through an Ultra Free membrane (Millipore), adjusted to pH 12 with 1 *N* sodium hydroxide solution and deammoniated under vacuum for about 12 h.

RESULTS

Separation of ε-(γ-glutamyl)lysine on the amino acid chromatogram

A sample was made by mixing purified ε-(γ-glutamyl)lysine (as described under Experimental) with the amino acid solution. Then, we improved the experimental conditions of amino acid analysis for clear detection of ε-(γ-glutamyl)lysine using a high-speed amino acid analyzer. Fig. 1 shows the chromatogram of ε-(γ-glutamyl)lysine and the other amino acids. ε-(γ-Glutamyl)-lysine clearly separated from the other amino acids and appeared just behind the tyrosine peak. It took 126.22 min. The resolution between tyrosine and the dipeptide was more than 90%. Following this result, ε-(γ-glutamyl)lysine in keratin and the membranous fraction was analyzed using the high-speed amino acid analyzer. Fig. 2 shows the chromatograms obtained with the enzymically digested mixtures of these fraction.

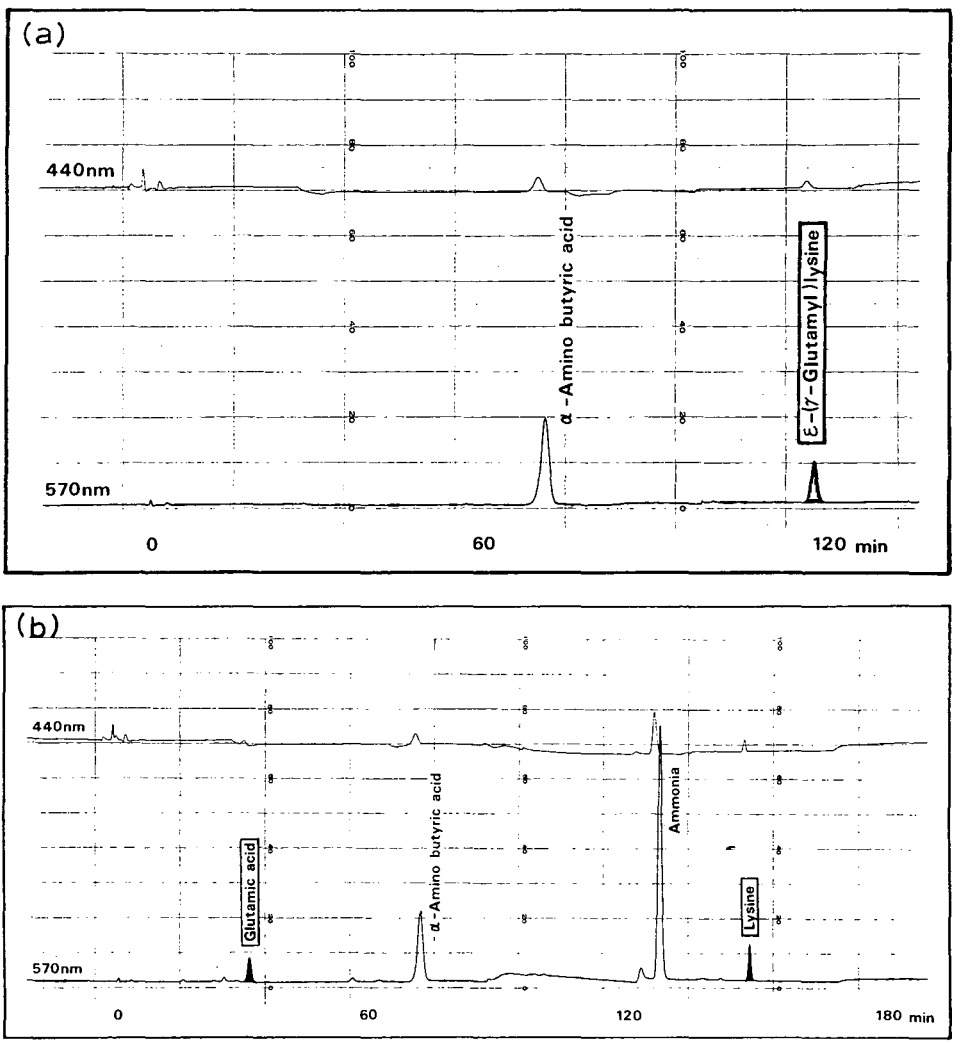

Fig. 3.

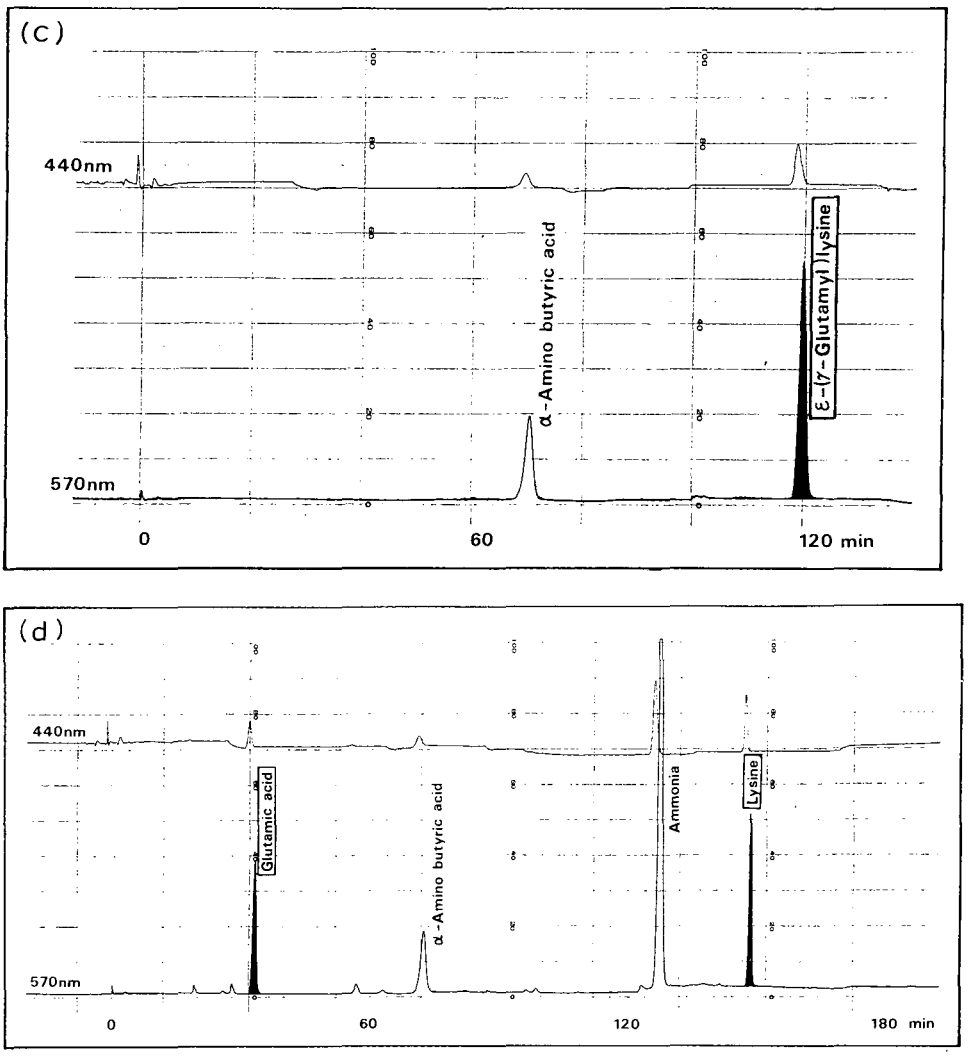

Fig. 3. (a) Chromatograms of ϵ-(γ-glutamyl)lysine obtained from the membranous fraction of human stratum corneum; and (b) after hydrolysis. (c) Chromatogram of ϵ-(γ-glutamyl)-lysine obtained from the standard solution (2.5 nmol); and (d) after hydrolysis.

Keratin contained a small amount of the dipeptide and its dipeptide peak was resolved about 50% from the tyrosine peak. The membranous fraction contained much more dipeptide and its dipeptide peak was completely resolved from the tyrosine peak.

Further identification of ϵ-(γ-glutamyl)lysine in the membranous fraction

We ascertained whether or not the peak behind tyrosine on the chromatogram of the membranous fraction of human stratum corneum was only ϵ-(γ-glutamyl)lysine. We used a manual sampler attached to a Hitachi 835 high-

speed amino acid analyzer. A 100-μl volume of the enzymatic digested mixture (362 μg of membranous fraction) was charged on the column. The effluent corresponding to the retention time of ϵ-(γ-glutamyl)lysine was collected before addition of ninhydrin reagent. α-Aminobutyric acid (10 nmol per 100 μl) as an internal standard was added to part (100 μl) of that effluent. Seventy microliters of the mixture were directly charged on the column again. The other 100 μl were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 24 h and the amino acid composition determined. Fig. 3 shows the chromatograms of ϵ-(γ-glutamyl)lysine eluted from the membranous fraction with (Fig. 3a) and without (Fig. 3b) hydrolysis. There are two major peaks on the chromatogram before hydrolysis. The faster peak is the internal standard and the later one is ϵ-(γ-glutamyl)lysine. After hydrolysis, glutamic acid and lysine emerged in almost equimolar amounts and ϵ-(γ-glutamyl)lysine disappeared (Fig. 3b). We calculated the molar ratios of ϵ-(γ-glutamyl)lysine/glutamic acid/lysine as 1:0.95:0.91. Large amounts of ammonia and another small peak appeared on the chromatogram after hydrolysis. They were also found on the chromatogram where ϵ-(γ-glutamyl)lysine as the standard substance was eluted from the column and hydrolyzed under the same conditions (Fig. 3d).

Pre-treatment of the enzymically digested mixture before amino acid analysis

A large amount of ammonia was produced during the enzymic digestions. The following pre-treatments were carried out before amino acid analysis: the digested mixture was (A) centrifuged at 1050 *g* for 5 min, (B) centrifuged under the same conditions and deammoniated at pH 12 under vacuum, (C) filtered through an Ultra Free membrane, and (D) filtered through the membrane and deammoniated by the same method. Fig. 4 shows the chromatograms of the samples treated by procedures A, B, C and D. The peak areas of methionine, tyrosine, phenylalanine and ϵ-(γ-glutamyl)lysine from each chromatogram are shown in Table II.

During deammoniation, the peak areas of tyrosine and methionine decreased markedly, whereas those of phenylalanine and ϵ-(γ-glutamyl)lysine were not so

TABLE II

COMPARISON OF PRE-TREATMENTS ON THE ENZYMICALLY DIGESTED MIXTURE OF THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

Pre-treatment	nmol/mg membranous fraction			
	(A) Centrifugation	(B) Centrifugation + deammoniation	(C) Filtration	(D) Filtration + deammoniation
Methionine	23.0	2.1	24.4	4.9
Tyrosine	146.0	92.7	140.9	37.1
Phenylalanine	114.1	118.8	118.3	121.8
ϵ-(γ-Glutamyl)lysine	40.1	39.1	40.4	38.7
Total amino acids after enzymic digestion	4269 (87%)	4195 (86%)	4306 (88%)	4191 (87%)
Total amino acids after hydrolysis*	4906 (100%)	4898 (100%)	4900 (100%)	4838 (100%)

*6 *N* Hydrochloric acid at 110°C for 24 h.

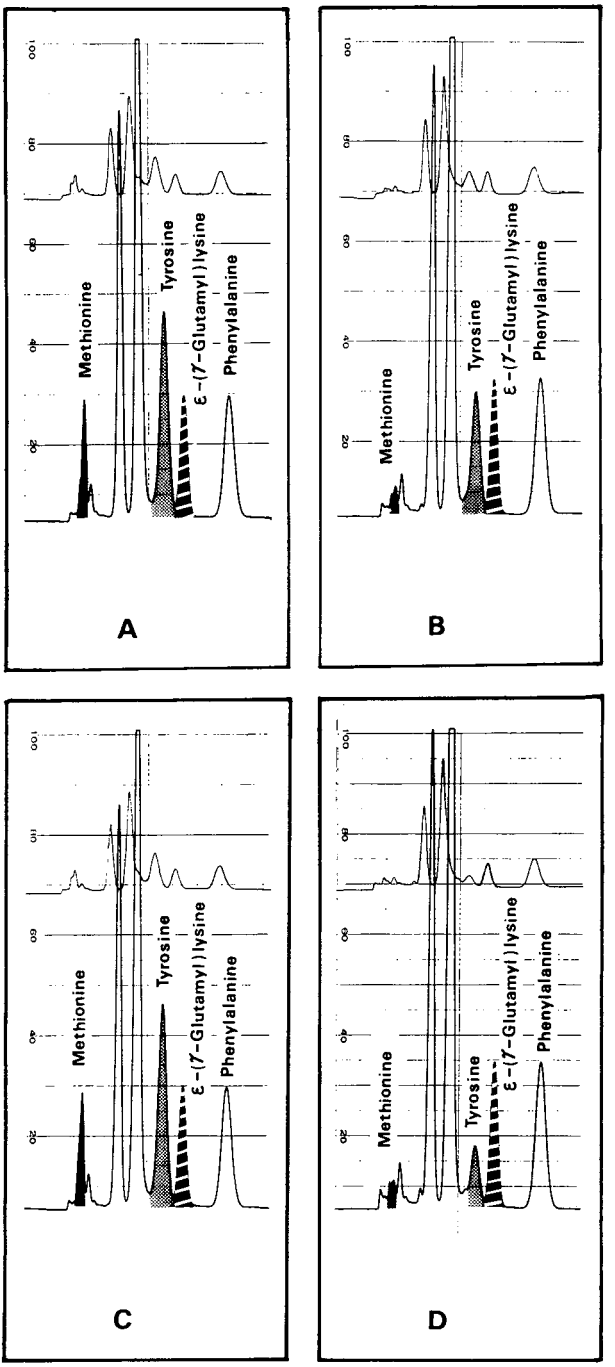

Fig. 4. Comparison of pre-treatments for ϵ-(γ-glutamyl)lysine in the membranous fraction. (A) Centrifugation, (B) centrifugation + deammoniation, (C) filtration, and (D) filtration + deammoniation.

variable. The former two peaks overlapped the ammonia peak which derived from the sample during the enzymic digestion.

The quantitative analysis of ε-(γ-glutamyl)lysine was affected by tyrosine. It was necessary, therefore, for the enzymically digested mixture to be pre-treated by deammoniation. The mixture was also filtered through the Ultra Free membrane to reject a small number of large molecules.

The total amino acid values of each pre-treated sample with and without acid hydrolysis are compared in Table II. These values were not so variable in all cases. In the membranous fraction, the amounts of amino acids released by enzymic digestion were over 85% (86—88%) of the acid hydrolyzed one.

Quantitation of ε-(γ-glutamyl)lysine in keratin and the membranous fraction of human stratum corneum

We determined ε-(γ-glutamyl)lysine in keratin and the membranous fraction of human stratum corneum digested by various enzymes according to a modified method of Abernethy et al. The results are shown in Table III. There were small amounts of ε-(γ-glutamyl)lysine in keratin, whereas the membranous fraction contained about seven times as much as keratin did.

TABLE III

QUANTITATION OF ε-(γ-GLUTAMYL)LYSINE IN KERATIN AND THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

	nmol/mg material	
	Keratin	Membranous fraction
Experiment 1	4.9	43.3
Experiment 2	6.7	43.6
Average	5.8	43.5

DISCUSSION

We have modified the conditions of a high-speed amino acid analyzer to identify ε-(γ-glutamyl)lysine, which overlapped the leucine peak when we followed our previous method [7]. The procedure was improved (Table I) so that the first and second buffer change times were longer than in the original method [7] and the column temperature was altered from 34 to 43 to 34°C. Under these conditions, ε-(γ-glutamyl)lysine appeared between tyrosine and phenylalanine (Fig. 1).

In order ascertain whether that peak contained other peptides, the ε-(γ-glutamyl)lysine fraction eluted from the high-speed amino acid analyzer was hydrolyzed with 6 *N* hydrochloric acid and measured with the same analyzer. Small amounts of ninhydrin-positive compounds, apart from glutamic acid, lysine and the internal standard, appeared after hydrolysis. They were also detected only in the buffer solution eluted from the same instrument after acid hydrolysis. Thus it seems that these peaks were derived from the citrate buffers

or the column used with the high-speed amino acid analyzer. Even if polypeptides were included in that peak, they did not appear on the chromatogram, because the time taken for reaction with the ninhydrin reagent, 1.70 min, was too short for the polypeptides to react.

It was necessary for the enzymically digested mixture to be deammoniated. Ammonia was produced from glutamine and asparagine during enzymic digestion. Large amounts of ammonia overcharged the column and overlapped the tyrosine peak on the amino acid chromatograms.

The membranous fraction of human stratum corneum contained a large amount of ϵ-(γ-glutamyl)lysine in comparison with keratin. It also had a number of disulfide bonds [9]. It seems that the membranous fraction of the stratum corneum has the strict structure necessary for these cross-links.

REFERENCES

1 J.E. Folk and J.S. Finlayson, Advan. Prot. Chem., 31 (1977) 1.
2 J.J. Pisano, J.S. Finlayson and M. Peyton, Biochemistry, 8 (1969) 871.
3 D.F. Mosher, J. Biol. Chem., 250 (1975) 6614.
4 P.J. Birckbicher, H.A. Carter, G.R. Orr, E. Conway and M.K. Patterson, Jr., Biochem. Biophys. Res. Commun., 84 (1978) 232.
5 J.L. Abernethy, R.L. Hill and L.A. Goldsmith, J. Biol. Chem., 252 (1977) 1837.
6 K. Murayama and N. Shindo, J. Chromatogr., 143 (1977) 137.
7 K. Murayama and T. Sugawara, J. Chromatogr., 224 (1981) 315.
8 H. Ogawa and M. Hattori, Arch. Dermatol. Res., 268 (1980) 71.
9 T. Hirotani, M. Manabe, H. Ogawa, K. Murayama and T. Sugawara, Arch. Dermatol. Res., 274 (1982) 169.

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SIMULTANEOUS DETERMINATION OF PLASMA AND URINARY URIC ACID, XANTHINE, HYPOXANTHINE, ALLOPURINOL, OXIPURINOL, OROTIC ACID, OROTIDINE AND CREATININE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new high-performance liquid chromatographic procedure is described for the simultaneous determination of plasma and urinary uric acid, xanthine, hypoxanthine, allopurinol, oxipurinol, orotic acid and orotidine whose quantities are varied by allopurinol treatment in man. Creatinine was also measurable. The method was established by high-performance liquid chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

Allopurinol, 4-hydroxypyrazolo[3,4-d]pyrimidine, a xanthine oxidase (E.C. 1.2.3.2) inhibitor [1], is a most widely used purine analogue in clinical practice, and is converted mainly to a metabolite, oxipurinol, 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine [2]. Treatment of gouty and hyperuricemic patients with the drug results in decreased uric acid production and a concomitant increase of hypoxanthine and xanthine [3], which are substrates of xanthine oxidase. The importance of monitoring the purine analogue and purines in blood and urine is obvious in handling these diseases.

There are many methods (see, for example, refs. 4—8) for estimating allopurinol, oxipurinol and oxipurines, but their combined use to estimate all these compounds in plasma and urine is time-consuming. It is therefore convenient if plasma and urinary allopurinol, its metabolite and the variable purines described above can be simply measured. In establishing such an assay by high-performance liquid chromatography (HPLC) we found a few unidentified

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materials in the HPLC profiles of urine samples of normal and gouty subjects. These unidentified materials were obtained in the HPLC eluates and subjected to identification by ultraviolet (UV) spectrometry and gas chromatography—mass spectrometry (GC—MS), and together with confirmation by the retention times of authentic compounds their estimation became possible.

This paper describes those identification studies and the simultaneous HPLC assay procedure for plasma and urinary allopurinol, oxipurinol, uric acid, xanthine, hypoxanthine, creatinine, orotic acid and orotidine. The latter two pyrimidines are known [9] to be increased in urine following allopurinol ingestion. Therefore, the amount of compounds varying following allopurinol therapy can be estimated by the present systems, as well as creatinine, an essentially invariable indicator.

EXPERIMENTAL

Chemicals

Uric acid, hypoxanthine, xanthine, orotic acid, orotidine and creatinine were obtained commercially (Wako, Kohjin, and Calbiochem-Behring). Allopurinol was our own product. Oxipurinol was synthesized as described previously [2]. All other chemicals were of analytical reagent grade.

HPLC apparatus

The HPLC apparatus used was a Waters Model ALC/GPC-204 liquid chromatograph consisting of a Model 6000A high-performance pump and a Model 440 absorbance detector at a wavelength of 254 nm, equipped with either a U6K injector and a National pen recorder VP-6511W or a Waters WISP 710A sample processor and Waters data module.

GC—MS apparatus

The GC—MS apparatus used was a JMS-D300 mass spectrometer equipped with a JGC-20K gas chromatograph and JMA-2000 data anlysis system (JEOL).

Procedure for HPLC

A 30 cm × 3.9 mm μBondapak C_{18} (particle size 8—10 μm) column (Waters Assoc.), connected to a pre-column (Waters guard column) packed with μBondapak C_{18}, was used for HPLC. The mobile phase was 4 m*M* sodium phosphate buffer (pH 5.0—8.0). For routine work, the pH of the buffer was 6.0. The mobile phase was pumped at a flow-rate of 1.0 ml/min. Column pressure ranged between 70 and 84 bars. Separation was done at ambient temperature. Sample solutions of 1—100 μl were introduced through the injector or the processor. Peak heights were measured either manually or by an on-line computer in the data module equipment.

Procedure for GC—MS

A glass column (1 m × 2 mm I.D.) containing 3% OV-17 on Chromosorb W AW DMCS (80—100 mesh) was used under the following conditions: oven temperature 150°C to 260°C programmed at 8°C/min, injection port tempera-

ture 300°C, helium as carrier gas at 0.9 kg/cm^2, ionization voltage of 70 V, trap current of 300 μA, and ionization chamber temperature of 150°C.

Direct mass spectra were also measured under conditions similar to above using the direct inlet system.

Sample preparation for HPLC

Urine. Human urine (0.5 ml) was mixed with 5 ml of 0.02 *M* sodium phosphate buffer (pH 8.0). Ten to fifty microlitres of the mixture were injected for HPLC.

Plasma. Plasma (0.5 ml) was mixed with 0.4 ml of water and then with 0.1 ml of 20% perchloric acid in an ice-bath for deproteinization. The mixture was centrifuged at 1300 *g* at 4°C for 10 min. An aliquot (0.5 ml) of the supernatant solution was combined with 0.5 ml of 0.2 *M* disodium phosphate and 50 μl of the mixture were injected for HPLC.

Sample storage. Urine and plasma were stored at −20°C until analyzed. Urine yielded precipitates which contained no significant amounts of interfering compounds except in the case of the Lesch-Nyhan [10] patient. The precipitates were soluble in 0.1 *N* sodium hydroxide and the solution was processed in the same way as the urine samples.

Sample processing for mass spectrometry

The aqueous samples separated and recovered from HPLC, which contained the material to be identified and an appreciable amount of sodium phosphate, were freeze-dried. The residue was divided into two portions, one of which was introduced directly into the mass spectrometer via the direct inlet system; the other portion was dissolved in 50 μl of 0.05 *M* trimethylphenylammonium hydroxide (TMPAH) in methanol for analysis by GC—MS. TMPAH was successfully used for methylation of the samples in these studies; the usual trimethylsilylation procedure [11] failed to derivatize the samples, possibly due to the presence of phosphate.

Urine and plasma samples of human subjects

Urine and plasma of normal human subjects were supplied to our laboratory during a bioavailability test of our allopurinol tablets. Normal healthy males ingested a single oral dose of 200 mg of allopurinol tablets with informed consent. Samples of a gouty subject,who is a colleague, were kindly supplied by him in our laboratory. Urine of a Lesch-Nyhan syndrome patient [10,12] was kindly supplied by the Department of Urology, Tokushima University Hospital, School of Medicine, Tokushima University, Tokushima, Japan. The patients had been under therapy with a daily dose of 200 mg of allopurinol.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Authenthic compounds. After examination of columns and solvents it was decided to use the HPLC systems described in the Experimental section based on the retention time of uric acid, hypoxanthine, xanthine, oxipurinol and allopurinol which were around 4.5 min, 11.2 min, 12.8 min, 16.0 min and 18.8

min, respectively. Besides these compounds, orotic acid, orotidine and creatinine were identified in the identification studies described in the latter part of this paper. Their retention times were around 2.9 min (orotidine), 3.4 min (orotic acid) and 6.1 min (creatinine). Fig. 1 is an HPLC profile of a mixture of authentic samples of these compounds, 5—20 ng of each. The resolution was satisfactory under the present conditions.

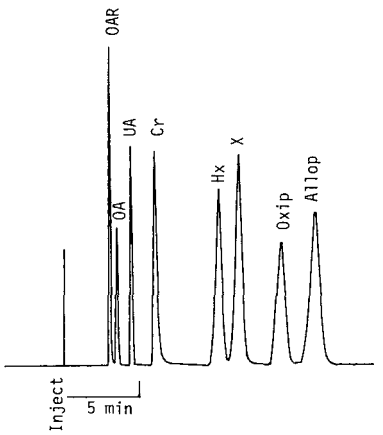

Fig. 1. HPLC profile of a standard mixture. Abbreviations: OAR = orotidine; OA = orotic acid; UA = uric acid; Cr = creatinine; Hx = hypoxanthine; X = xanthine; Oxip = oxipurinol; Allop = allopurinol.

Urine. In chromatograms of urine of normal subjects, a significant peak of uric acid was seen as well as smaller peaks of hypoxanthine and xanthine (a typical chromatogram is shown in Fig. 2a). Addition of authentic hypoxanthine and xanthine to the urine resulted in the corresponding increase and appearance of each compound, confirming that for urine they are recovered from the HPLC column with good resolution. When the man ingested 200 mg of allopurinol, peaks of the unchanged allopurinol and metabolite oxipurinol were seen in urine pooled 0—8 h after ingestion (Fig. 2b). The identity and resolution of these compounds were also confirmed by the addition of authentic compounds to the urine, and GC—MS as described below.

Peak I seen in the urine of the normal subject was identified as creatinine by mass spectrometry as described below, UV spectra and then co-elution with the authentic compound. Peak II may be adenine on the basis of retention time and that of authentic adenine determined separately, but no further confirmation was made.

As above, it is possible to estimate uric acid, xanthine, hypoxanthine, allopurinol, oxipurinol and creatinine in the present systems.

Fig. 2c is the HPLC profile of urine of a gouty subject where xanthine and hypoxanthine were more significant than those in normal subject's urine. Their identity was confirmed by co-elution with authentic purines and GC—MS as described later. As well as peaks of purines and the drug and its metabolite, unidentified peaks III and IV were also seen in his urine. They were identified as orotidine and orotic acid, respectively, as described later.

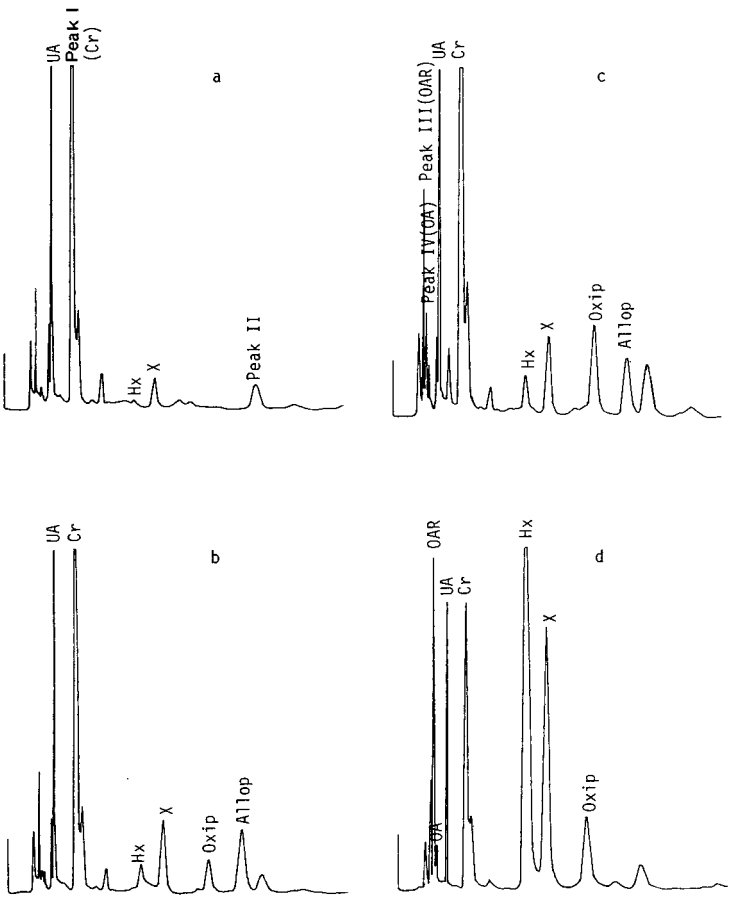

Fig. 2. HPLC profiles of (a) a normal subject's urine, (b) 0—8-h pooled urine after he had ingested 200 mg of allopurinol, (c) 0—8-h pooled urine after a gouty subject under allopurinol therapy (200 mg/day) had ingested the drug, and (d) 24-h pooled urine of a Lesch-Nyhan syndrome patient under allopurinol therapy (200 mg/day). Abbreviations as in Fig. 1.

It is now possible to estimate two pyrimidines, three purines and allopurinol including its metabolite, all of whose amounts are varied by allopurinol ingestion. Besides these, creatinine is also measurable, whose amount is essentially unrelated to allopurinol ingestion. The amount of creatinine excreted in the urine within a given time interval is virtually constant in man. Therefore, the ratio of peak height in one HPLC chromatogram of the purines and pyrimidines to that of creatinine is variable, and dependent on allopurinol ingestion alone. The creatinine peak in an HPLC chromatogram can thus be a measure for comparing the relative quantity of those variable compounds; i.e. the HPLC profile can be used as a follow-up of allopurinol therapy to provide the biochemical status of patients for purines and pyrimidines.

For instance, the HPLC profile of the Lesch-Nyhan patient's urine (Fig. 2d) shows unusually large peaks of hypoxanthine and xanthine relative to creatinine due to his lack [10] of hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8) and partly due to allopurinol therapy, the latter possibly

causing the elevated orotidine and orotic acid excretion seen in the figure. The identity of the compounds appearing in the HPLC chromatogram was confirmed by the following GC—MS studies. Precipitates occurring in his urine during storage at −20°C were found to contain uric acid, xanthine, hypoxanthine and oxipurinol when processed and analysed as described in the Experimental section.

Plasma. HPLC profiles of normal human plasma gave distinct peaks of creatinine and uric acid (e.g., Fig. 3a) and smaller ones of two oxipurines. Allopurinol and oxipurinol were seen as well in this subject's plasma 2 h after he had ingested 200 mg of allopurinol (Fig. 3b).

Two hours after the gouty subject ingested 200 mg of allopurinol, peaks of creatinine, uric acid, hypoxanthine, xanthine, allopurinol and oxipurinol were significant (Fig. 3c). Oxipurinol and two oxipurine peaks were distinct relative to those in the normal subject's urine, possibly because he had been repeatedly taking the drug. The elimination rate of oxipurinol in plasma is known [2] to be much longer than that of unchanged allopurinol.

The identity of the compounds in plasma was confirmed first by their retention times and by the addition of authentic compounds to plasma and the appearance of the corresponding peak in the HPLC chromatogram.

Calibration and recovery. Aqueous mixtures containing known amounts of uric acid, xanthine, hypoxanthine, creatinine, orotic acid and orotidine were prepared and injected into the HPLC apparatus. Plots of their peak heights on chromatograms versus their quantity gave a straight calibration line (usually, correlation coefficient > 0.99) for each compound. Known amounts of the compounds were added to a known volume of urine or plasma and HPLC was carried out according to the standard procedure. The increment of peak height

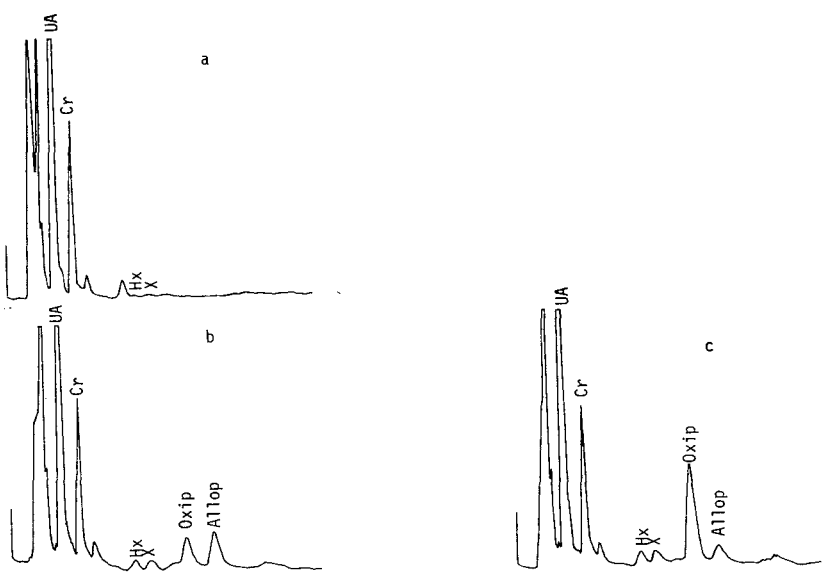

Fig. 3. HPLC profiles of (a) a normal subject's plasma (b) plasma 2 h after he had ingested 200 mg of allopurinol, and (c) a gouty subject's plasma 2 h after he had ingested his daily 200 mg of allopurinol for therapy. Abbreviations as in Fig. 1.

gave their recoveries on chromatography (Table I). The precision was satisfactory when judged from the reproducibility, indicated by standard error means in Table I. The reliable limits of estimation of compounds were different due to their different molar extinction coefficients and were virtually in the range of 3—9 ng (approximately 0.2—0.7 μg/ml plasma and 0.7—2 μg/ml urine). When values were expressed as the mean ± S.E.M. of five normal men, the urinary excretion of uric acid and creatinine determined by the present HPLC procedure was 440 ± 53 and 1350 ± 84 mg/day, respectively. Their plasma levels were 5.86 ± 0.50 and 0.83 ± 0.06 mg per 100 ml, respectively, in the morning. The values are in the well-known normal range in clinical chemistry.

Peak heights of xenobiotic allopurinol and oxipurinol added in a known volume of normal urine or plasma were linear (correlation coefficients > 0.99) when plotted against the concentration in the specimen. Detection of these compounds was possible at > 0.1 μg/ml of plasma and at > 0.3 μg/ml of urine.

Identification of peak components by mass spectrometry

Peak I. The component of peak I gave a molecular ion at *m/z* 113, and fragments at *m/z* 112, 84 and 69 in the direct electron-impact (EI) mass spectrum (Fig. 4), suggesting that it was either creatine or creatinine. Since creatine is easily dehydrated at low temperature to give creatinine, it is inadequate to distinguish the two compounds by EI mass spectrometry where the sample is vaporized by heat. However, peak I was finally identified as creatinine by comparison with the retention time and UV spectrum of authentic creatinine.

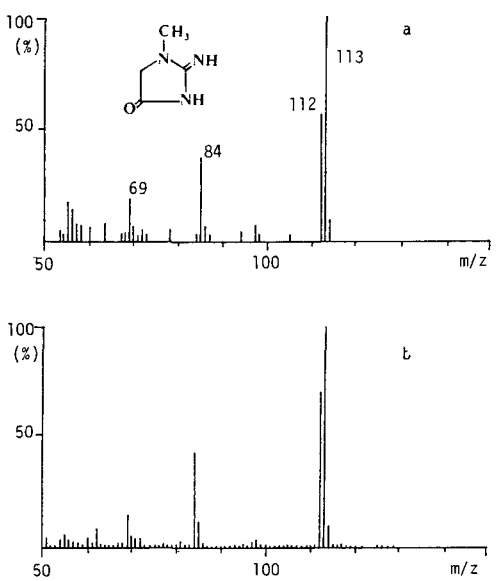

Fig. 4. Mass spectra of (a) creatinine and (b) peak I (EI, 70 V, direct inlet system).

TABLE I

RECOVERIES OF STANDARDS FROM URINE AND PLASMA SPECIMENS

Sample	Urine				Plasma			
	Amount added* (ng)	Amount recovered (ng)	Recovery (%)	*n*	Amount added* (ng)	Amount recovered (ng)	Recovery (%)	*n*
Orotidine	20.0—79.0	18.6—87.3	103 ± 4	8	62.5—500	59.1—372	83 ± 5	6
Orotic acid	100 —400	91.3—381	94 ± 1	8	250 —500	212 —302	73 ± 7	4
Uric acid	217 —1740	189 —1590	92 ± 4	6	125 —1000	104 —802	82 ± 1	6
Creatinine	217 —1740	217 —1840	102 ± 2	6	15.6—62.5	16.4—59.9	95 ± 6	6
Hypoxanthine	63.0—189	58.4—189	90 ± 2	8	3.1—21.0	2.9—21.3	90 ± 3	6
Xanthine	91.0—364	106 —385	112 ± 2	8	3.1—100	3.1—99.5	92 ± 3	9

*Absolute amount injected for HPLC. Therefore, 100 ng for urine and plasma is equivalent to 23 μg/ml of urine and 8 μg/ml of plasma when 50 μl of sample solution prepared as described in the text were injected.

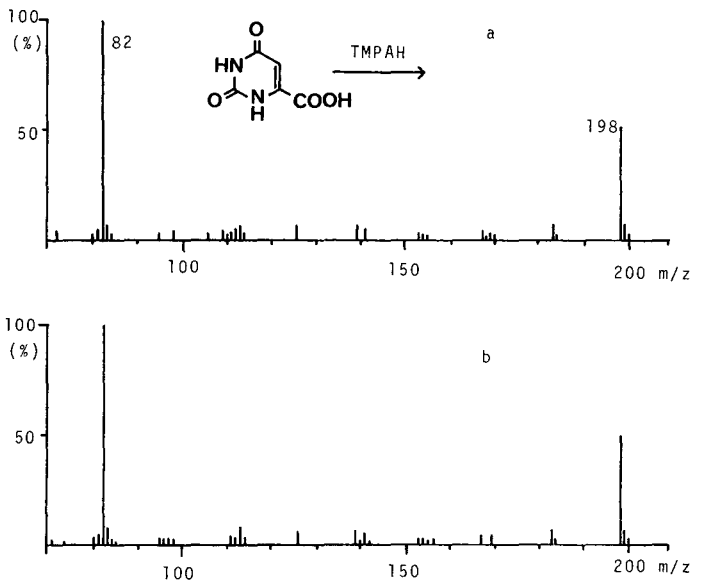

Fig. 5. Mass spectra of (a) orotic acid and (b) peak III after co-injection with TMPAH (EI, 70 V, GC—MS).

Peaks III and IV. The direct mass spectra of peaks III and IV were essentially the same as each other, giving rise to prominent ions at *m/z* 112 and 69 attributable to uracil. However, their behaviour in HPLC was quite different from that of uracil. This suggested that uracil was their thermal decomposition product in the sample probe of the mass spectrometer.

For further clarification, GC—MS was carried out after derivatization. Under the conditions described in the Experimental section, peak III gave a response at 3 min in the total ion monitor chromatogram and the mass spectrum showing ions at *m/z* 198 and 82 attributable to trimethylated orotic acid. Peak III was confirmed as orotic acid by agreement of the mass spectrum with that of authentic orotic acid as shown in Fig. 5. The retention time of peak III in HPLC also agreed with that of the authentic compound.

Peak IV also gave a response at 3 min, the mass spectrum being identical to that of peak III, trimethylorotic acid. The orotic acid detected was thought to be a product by heat yielded from a non-volatile derivative of the acid, i.e., orotidine, since methylation by TMPAH is a flash-heater reaction involving pyrolysis of the quarternary ammonium salt which often causes decomposition of samples [13]. Finally, peak IV was confirmed as orotidine by coelution with the authentic compound in HPLC.

Other peaks. The peak components, the behaviour of which in HPLC was identical to uric acid, xanthine, hypoxanthine, allopurinol and oxipurinol, were also confirmed by selected ion monitoring (SIM). Each ion selected was the molecular ion of the respective derivative: *m/z* 224 corresponds to that of tetramethylated uric acid, *m/z* 194 to trimethylated xanthine and oxipurinol, and *m/z* 164 to dimethylated allopurinol and hypoxanthine. Typical SIM chromatograms are shown in Fig. 6, indicating fair agreement of urinary

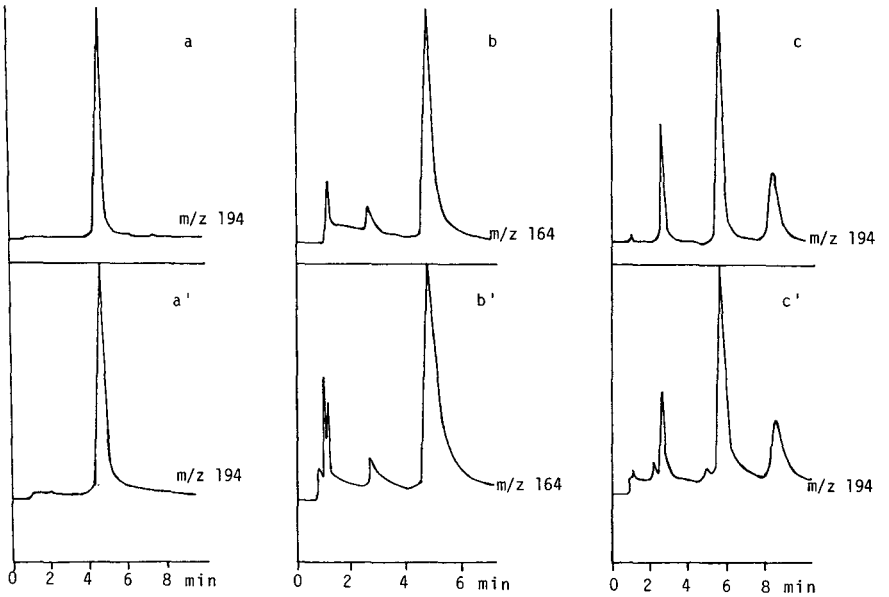

Fig. 6. Identification of xanthine, hypoxanthine and oxipurinol: SIM of authentic (a) xanthine, (b) hypoxanthine and (c) oxipurinol and HPLC eluate corresponding to each compound (a', b' and c', respectively) after co-injection with TMPAH at 300° C and isothermal analysis at 190° C. Each ion selected was the molecular ion of the corresponding derivative.

xanthine, hypoxanthine and oxipurinol with the authentic compounds. Multiple peaks in the chromatograms indicate that, under the conditions employed, methylation did not result in a single product of each purine and analogue due to the formation of either the O-methyl or the N-methyl isomer.

CONCLUDING REMARKS

In an attempt to establish a system to determine simultaneously uric acid, xanthine, hypoxanthine, allopurinol and its metabolite by HPLC, two pyrimidines and creatinine were identified with the aid of GC—MS, and became measurable. As pointed out, since the quantity of creatinine excreted following allopurinol treatment does not change, and can be determined as well as the variable purines and pyrimidines, the HPLC profile, particularly that of urine, could help us to know the biochemical status of patients receiving the drug. Combination of HPLC with GC—MS as described in this study is conceivably of great importance for developing a new assay system for multiples of compounds.

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REFERENCES

1 G.B. Elion, S. Callahan, H. Nathan, S. Bieber, R.W. Rundles and G.H. Hitchings, Biochem. Pharmacol., 12 (1963) 85.
2 G.B. Elion, A. Kovensky and G.H. Hitchings, Biochem. Pharmacol., 15 (1966) 863.
3 T.-F. Yü and A.B. Gutman, Amer. J. Med., 37 (1964) 885.
4 N.D. Brown, J.A. Kintzios and S.E. Koetitz, J. Chromatogr., 177 (1979) 170.
5 W. Voelter, K. Zech, P. Arnold and G. Ludwig, J. Chromatogr., 199 (1980) 345.
6 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704.
7 V. Notsche and H. Mascher, Arzneim.-Forsch., 30 (1980) 1855.
8 S. Sved and D.L. Wilson, Biopharm. Drug Dispos., 1 (1980) 111.
9 R.M. Fox, D.R. Smith and W.J. O'Sullivan, Science, 168 (1970) 861.
10 M. Lesch and W.L. Nyhan, Amer. J. Med., 36 (1964) 561.
11 V. Miller, V. Pacáková and E. Smolková, J. Chromatogr., 119 (1976) 355.
12 K. Hiraishi, A. Yamamoto, H. Yamamoto and H. Tsujimura, Nyosan (Uric Acid Res.), 4 (1980) 77 (in Japanese).
13 R.G. Wien and F.S. Tanaka, J. Chromatogr., 130 (1977) 55.

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CHROMBIO. 1590

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR CYTOSINE ARABINOSIDE, URACIL ARABINOSIDE AND SOME RELATED NUCLEOSIDES

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SUMMARY

A novel, dual-column high-performance liquid chromatographic method for determination of the anti-cancer drug cytosine arabinoside (Ara-C) and its major metabolite uracil arabinoside (Ara-U) has been developed. The analytical procedure is sensitive (25 ng/ml) and specific for Ara-C, Ara-U and the endogenous nucleosides that may influence response to Ara-C therapy, cytidine and deoxycytidine. Conventional and high dose calibration curves were linear and the method precise with the assay coefficient of variation for Ara-C and Ara-U not greater than 9.1% over the range of 0.1—10 μg/ml. Accuracy was determined to be within ± 3 to 9% over this concentration range. Using this method, patient plasma samples from both conventional dose (100—200 mg/m² per day) and high dose (3500—6500 mg/m² per day) Ara-C can be simultaneously analyzed for Ara-C, Ara-U and nucleosides so that comparative pharmacokinetic and pharmacodynamic studies can be conducted.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine; Ara-C) is a pyrimidine analogue that is effective in the treatment of acute myelogenous leukemia and acute lymphocytic leukemia [1—4].

After activation by deoxycytidine kinase and further intracellular phosphorylation to arabinosyl-CTP [5,6], the anti-metabolite selectively inhibits DNA synthesis.

Ara-C is rapidly deaminated to an inactive metabolite, uracil arabinoside (Ara-U) by cytidine deaminase [7,8]. Due to this rapid deamination, Ara-C has a short plasma elimination half-life (12—200 min) when administered by intravenous bolus injection [9,10]. The short half-life of Ara-C and its S-phase

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(cell cycle-dependent) specificity have led to clinical trials of continuous intravenous or subcutaneous (S.Q.) infusions of conventional dose (CD = 100—200 mg/m^2 per day) or high doses (HD = 1000—6500 mg/m^2 per day) of Ara-C, over a period of several days [10—15]. Clinically, variation in the pharmacokinetics (drug metabolism and disposition) of Ara-C may be a potentially important determinant of its efficacy and toxicity [16]. The interindividual variation in the endogenous nucleoside and nucleotide concentrations may also be an important determinant in cytotoxic effects during Ara-C treatment [17,18]. To investigate the human disposition and biochemical effects after administration (pharmacodynamics) of Ara-C, a sensitive and specific method for quantitating Ara-C, Ara-U, cytidine and deoxycytidine is required.

Several techniques have been employed for the elucidation of Ara-C pharmacokinetics, including microbiology [19,20], radioimmunology [21,22], ultraviolet (UV) spectroscopy [23] and gas chromatography—mass spectrometry [12] or gas—liquid chromatography—flame ionization—mass spectrometry [24]. Some of these techniques are sensitive enough for the detection of Ara-C when administered at conventional dosages. However, the assays may not be specific for quantitation of both Ara-C and Ara-U, or they may require specialized handling of radioactive materials.

High-performance liquid chromatography (HPLC) with UV detection offers a sensitive and specific method to monitor concentrations of Ara-C and its metabolites in biological fluids of patients receiving the drug. There have been numerous publications of HPLC methods for determination of Ara-C [15, 25—29]; however, most of the previously described procedures cannot separate Ara-C, Ara-U and the structurally similar endogenous nucleosides (cytidine and deoxycytidine), which may influence the anti-cancer effects of Ara-C (Fig. 1). Therefore, we have developed a sensitive and specific dual-column HPLC method to monitor plasma, urine and cerebrospinal fluid (CSF) levels of Ara-C,

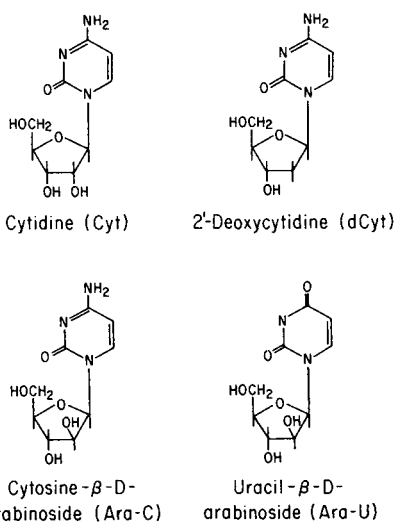


Fig. 1. Comparative structures of cytidine (Cyt), deoxycytidine (dCyt), cytosine arabinoside (Ara-C) and uracil arabinoside (Ara-U).

its major metabolite Ara-U and the endogenous nucleosides, deoxycytidine and cytidine during continuous infusions of conventional and high doses of Ara-C in children with cancer.

EXPERIMENTAL

Materials

Ara-C, Ara-U, adenine arabinoside (Ara-A), tetrahydrouridine (THU), nucleosides and deoxyribonucleosides including cytidine (Cyt) and deoxycytidine (dCyt) were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium phosphate (monobasic) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). HPLC-grade methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was deionized, distilled and filtered for HPLC use. The MPS-1 micropartition system (Amicon, Lexington, MA, U.S.A.) was used to filter plasma samples before HPLC analysis. Other reagents and glassware were of standard laboratory quality.

Instrumentation and chromatography

The chromatographic conditions used a mobile phase of 2.5 m*M* KH_2PO_4 (pH 3.2) with 2.5% methanol, a Waters Assoc. (Milford, MA, U.S.A.) Model M-45 HPLC pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 100-μl sample loop, and a 5-μm reversed-phase Ultrasphere-ODS column, 15 cm × 4.6 mm, Altex, Berkeley, CA, U.S.A.), connected in series with a 10-μm cation-exchange column (Partisil PXS 10/25 SCX; 25 cm × 4.6 mm; Whatman, Clifton, NJ, U.S.A.).

The column effluent was monitored for UV absorbance at 280 nm with a Spectra-Physics (Houston, TX, U.S.A.) 8300 UV monitor. Detector output was integrated by a Spectra-Physics SP4000 chromatography data system with graphical display on a Fisher Recordall strip chart recorder attenuated to 10 mV. The mobile phase flow-rate was about 0.8 ml/min at a pressure of 17 MPa (2500 p.s.i.).

Assay procedure

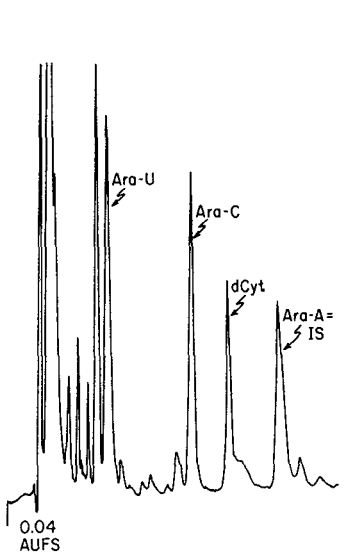
Blood and CSF samples (2—3 ml) were collected in heparinized tubes containing the deaminase inhibitor, tetrahydrouridine (0.1 mmole) and immediately placed on ice. Urine was collected in four 12-h intervals in sterile containers and refrigerated immediately. After the whole blood was centrifuged (800 *g* for 7 min), the plasma was collected and frozen at −70°C until analyzed. After appropriate addition of internal standard (Ara-A, 1 μg/ml), up to 25 plasma samples (1 ml) were filtered with the MPS-1 Micropartition System for 15—20 min at 800 *g* in a clinical centrifuge. Aliquots (100 μl) of the plasma ultrafiltrates were injected in duplicate. CSF samples (100 μl) were injected directly into the system and urine was appropriately diluted with deionized-distilled water (1:5 or 1:10) before HPLC injection (100 μl). Stability of Ara-C after sample collection was assessed by incubation of Ara-C (0.1 and 5 μg/ml) in fresh plasma, urine and CSF in tubes with and without 0.1 mmole THU. The samples were incubated at 25°C and 0°C (on ice) and the concentration of Ara-C was determined at 0 h and at the end of the incubation (1 h).

Results are expressed as the percent Ara-C remaining relative to the 0-h concentration.

In order to assess the linearity, precision and accuracy at low and high plasma concentrations of Ara-C and Ara-U, multilevel calibration curves were constructed. The peak areas of the conventional and high-dose patient samples were thereafter converted to absolute quantities using the respective predetermined calibration curves. Linear regression analysis was used to determine the best-fit line through the peak area or peak height versus concentration plot. Replicate analysis of low, medium and high calibrators were used to determine the assay precision (intra-assay $n = 10$ and inter-assay, $n = 5$) with three different operators and the same chromatographic system. Accuracy was determined by analysis of four operator-blinded "unknowns" at low to high concentrations of Ara-C, Ara-U, Cyt and dCyt (range of 0.05—10 μg/ml). Potential loss of the five compounds during the filtration process was attained by comparing filtrate of spiked samples in water, mobile phase and plasma to the equivalent amount injected directly in the system.

RESULTS AND DISCUSSION

There have been a number of reversed-phase or cation-exchange HPLC methods published for the determination of Ara-C [15,25—29], however, the ability to quantitate Ara-C, Ara-U, Cyt, dCyt and Ara-A using a novel reversed-phase cation-exchange dual-column method has not been previously described. Figs. 2 and 3 illustrate a patient's plasma ultrafiltrate obtained at 72 h during an intravenous infusion of high-dose Ara-C and at 24 h during a conventional



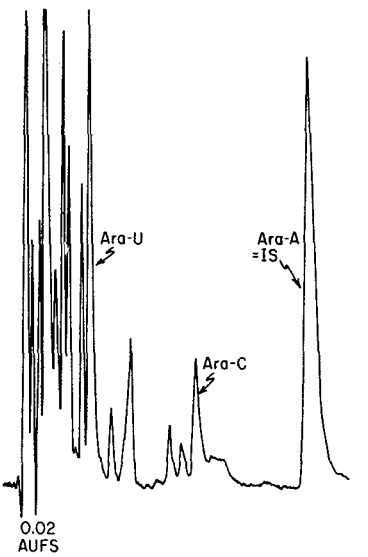


Fig. 2. Patient's plasma sample separation (high-dose Ara-C). High dose = 5 g/m² per day; 72-h during infusion sample. Ara-C = 2.6 μg/ml; Ara-U = 42.6 μg/ml.

Fig. 3. Patient plasma separation (conventional-dose Ara-C). Conventional dose = 100 mg/m² per day; 24-h during infusion sample. Ara-C = 0.045 μg/ml; Ara-U = 0.99 μg/ml.

dose infusion of Ara-C, respectively. The retention times and capacity factors (k' values) of Ara-U, Cyt, Ara-C, dCyt and Ara-A (used as internal standard) are 13.4 min (1.25), 22.0 min (4.5), 25.7 min (5.0), 28.5 min (6.25) and 35.7 min (8.0), respectively. The coefficient of variation for retention times over a 3-day period for all four compounds was less than 1%. The quantitative limit of detection for Ara-C and Ara-U was 20 ng/ml, which is comparable to other HPLC methods [29] but less sensitive than radioimmunoassay methods [21,22]. Loss of any of the five compounds during the filtration process was less than 8% in water, mobile phase and plasma and not considered significant.

Ara-C stability studies verified the need for THU-containing tubes for plasma sample collection. These studies showed no loss of parent drug at room temperature or on ice when the deaminase inhibitor was added. Samples that did not contain THU showed substantial conversion on ice (70% remaining) and at room temperature (only 40% remaining) after 1-h incubation in fresh plasma. Ara-C was stable in urine and CSF for at least 48 h at room temperature and on ice.

The high-dose and conventional-dose calibration curves were linear over their respective concentration range, as illustrated in Figs. 4 and 5. The intra-assay (n = 10) coefficients of variation for Ara-U and Ara-C, respectively, were 8.1% and 9.1% for 0.1 μg/ml samples, 4.4% and 4.8% for 1 μg/ml and 3.4% for 10 μg/ml samples. The inter-assay (day to day, n = 5) variability was less than 8% for all compounds of interest. Accuracy at the lowest concentration (0.05 μg/ml) was within ± 9% (91—109%) and accuracy improved to ± 3% (97—103%) at the higher concentrations (⩾ 5 μg/ml).

Blood samples (2—3 ml) from two patients who received conventional-dose Ara-C and two patients administered high-dose Ara-C were taken daily during an intravenous infusion of the drug in four children with acute myelogenous

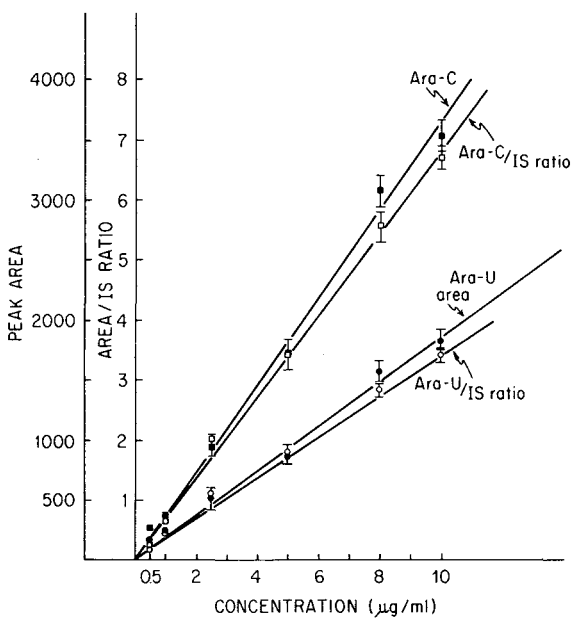
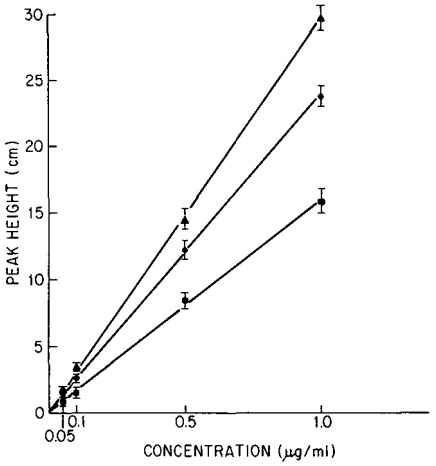


Fig. 4. High-dose calibration curve.



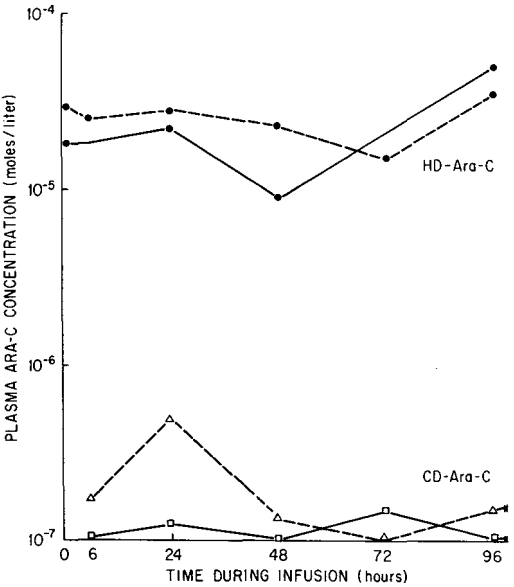


Fig. 5. Conventional-dose calibration curve. ● = Ara-C; ▲ = Ara-U; ■ = dCyt.

Fig. 6. Comparative concentration versus time curves of two patients administered high-dose (HD) Ara-C (3.5 g/m^2 per day) and two patients administered conventional-dose (CD) Ara-C (100 mg/m^2 per day.

leukemia (AML). As shown in Fig. 6, there appears to be significant variation in the steady-state plasma concentrations (Cp_{ss}) and the plasma concentration versus time profiles. Plasma clearance of Ara-C (K_0/Cp_{ss}) calculated for the two patients given high-dose Ara-C average 323 ml/min/m^2, substantially slower than that observed in the two patients given conventional-dose Ara-C (1543 ml/min/m^2) or previously reported in adults [13] following conventional-dose Ara-C (942 ml/min/m^2). This suggests the potential saturation of Ara-C clearance at the high plasma concentrations of Ara-C and/or Ara-U obtained after continuous infusion of high-dose Ara-C. This observation remains to be confirmed by additional pharmacokinetic studies in a larger number of patients.

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REFERENCES

1 G.P. Bodey, E.J. Freireich, R.W. Monto and J.S. Hewlett, Cancer Chemother. Rep., 53 (1969) 59—66.

2 R.R. Ellison, J.F. Holland and M. Weil, Blood, 32 (1968) 507—523.
3 B. Goodell, B. Leventhal and E. Henderson, Clin. Pharmacol. Ther., 12 (1971) 599.
4 W.A. Creasey, R.J. Papac, M.E. Markiw, P. Calabresi and A.D. Welsh, Biochem. Pharmacol., 15 (1966) 1417—1428.
5 J.J. Furth and S.S. Cohen, Cancer Res., 28 (1968) 2061—2067.
6 D. Kessel, T.C.C. Hall and I. Wodinsky, Science, 156 (1967) 1240—1241.
7 G.W. Comiener and C.G. Smith, Biochem. Pharmacol., 14 (1965) 1405—1416.
8 J.Z. Finklestein, J. Scher and M. Karon, Cancer Chemother. Rep., 54 (1970) 35—39.
9 D.H.W. Ho and E. Frei, Clin. Pharmacol. Ther., 12 (1971) 944—954.
10 R. van Prooijen, E. van der Kleijn and C. Haahen, Clin. Pharmacol. Ther., 1 (1977) 744—750.
11 D.H.W. Ho, N.S. Brown, J. Benvenuto, K.B. McCredie, D. Buckels and E.J. Freireich, Clin. Pharmacol. Exp. Ther., 22 (1977) 371—375.
12 M.L. Slevin, E.M. Piall, G.W. Aherne, A. Johnston, M.C. Sweatman and T.A. Lister, Brit. J. Clin. Pharmacol., 12 (1981) 507—510.
13 S.H. Wan, D.H. Huffman, D.L. Azarnoff, B. Hoogstraten and W.E. Larsen, Cancer Res., 34 (1974) 392—397.
14 S.A. Rudnick, E.C. Codman, R.L. Capizzi, R.T. Skeel, J.R. Bertino and S. McIntosh, Cancer, 44 (1979) 1189—1193.
15 K.R. Hande, R.S. Stein, D.A. McDonough, F.A. Greco and S.N. Wolff, Clin. Pharmacol. Ther., 31 (1982) 669—674.
16 T. Lincoln, T. Morrison, J. Aroesty and G. Carter, Cancer Treat. Rep., 60 (1976) 1723—1739.
17 L.L. Danhauser and Y.M. Rustum, Cancer Res., 40 (1980) 1274—1280.
18 A.L. Harris and D.G. Grahame-Smith, Cancer Chemother. Pharmacol., 5 (1981) 185—192.
19 B.M. Mehta, M.B. Meyers and D.J. Hutchison, Cancer Chemother. Rep., 59 (1975) 515—522.
20 H.C. van Prooijen, G. Vierwinder, J. van Egmond, J.M.C. Wessels and C. Haaner, Eur. J. Cancer, 12 (1976) 899—905.
21 I. Okabayashi, S. Mihara, D.B. Repke and J.G. Moffat, Cancer Res., 37 (1977) 619.
22 E.M. Piall, G.N. Aherne and V.M. Marks, J. Cancer, 40 (1979) 548—556.
23 R.E. Notari, J. Pharm. Sci., 56 (1967) 804.
24 J. Boutagy and D.J. Harvey, J. Chromatogr., 146 (1978) 283.
25 W. Kreis, C. Gordon, C. Gizoni and T. Woodcock, Cancer Treat. Rep., 61 (1977) 643.
26 H. Breithaupt and J. Schick, J. Chromatogr., 225 (1981) 99—106.
27 P. Linssen, A. Drenthe-Schonk, H. Wessels and C. Haanen, J. Chromatogr., 223 (1981) 371—378.
28 R.W. Bury and P.J. Keary, J. Chromatogr., 146 (1978) 350—353.
29 M.G. Pallavicini and J.A. Mazrimas, J. Chromatogr., 183 (1980) 449—458.

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CHROMBIO. 1617

REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC PROCEDURE WITH ELECTROCHEMICAL DETECTION FOR THE ANALYSIS OF URINARY THIOSULPHATE

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SUMMARY

A method using high-performance liquid chromatography with mercury-based electrochemical detection has been developed for the determination of thiosulphate in urine. The chromatographic separation is based upon ion-pair formation between thiosulphate and tetrabutylammonium and reversed-phase chromatography. The method was compared with an earlier reported colorimetric assay and found to be superior with respect to specificity and sensitivity.

INTRODUCTION

Urine from human subjects contains low concentrations of thiosulphate [1], which is a metabolic conversion product of sulphite [2]. Moreover, very high urinary concentrations of thiosulphate are found in subjects with sulphite oxidase deficiency [3], a rare hereditary disorder. An acquired form of sulphite oxidase deficiency was recently found in a patient on prolonged total parenteral nutrition [4] as demonstrated by a heavy thiosulphaturia.

However, studies on the excretion of thiosulphate under normal and pathological conditions have been hampered by a lack of reliable analytical methods. An early method [5] for the determination of urinary thiosulphate based on the precipitation of the nickel—ethylenediamine complex of thiosulphate apparently gives too high results. A more specific colorimetric method based on cyanolysis of thiosulphate to thiocyanate in the presence of cupric ions [6] was adopted for determinations on human urine as a screening test for sulphite oxidase deficiency [7]. However, this method is not sufficiently sensitive for

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determination of normal or moderately raised urinary thiosulphate concentrations. A more sensitive method for the determination of urinary thiosulphate was developed [8] by combination of ion-exchange techniques and the original cyanolysis method [7], but it is fairly laborious and time-consuming. This challenged us to investigate alternative methods and we now describe a procedure whereby urinary thiosulphate is determined by ion-pair high-performance liquid chromatography (HPLC) followed by electrochemical detection.

EXPERIMENTAL

Materials

Sep-Pak C_{18} cartridges were obtained from Waters (Milford, MA, U.S.A.). Tetrabutylammonium hydrogen sulphate (TBA) was a product of E. Merck (Darmstadt, G.F.R.) and methanol HPLC grade was from Rathburn Chemicals (Walkerburn, Great Britain). A stock standard solution of thiosulphate, 100 mmol/l, was prepared from a Titrisol ampoule (E. Merck) and working standards, 100 μmol/l or less, were prepared daily with the mobile phase as diluent.

Urine collection and preparation

Urine was collected for 24 h with thymol—isopropanol [9] as a preservative. A 2.5-ml aliquot of urine was passed through a Sep-Pak C_{18} cartridge, and the first 0.5 ml was discarded. After five-fold dilution with the mobile phase used for HPLC, the sample was ready for chromatography.

Chromatographic conditions

We used a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.) and a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop. All separations were performed on a RSiL C_{18} HL column (10 μm, 250 × 4.6 mm) from Alltech (Deerfield, IL, U.S.A.). The mobile phase was prepared from 85 vols. of a solution containing sodium phosphate (2 m*M*), disodium EDTA (0.1 m*M*) and TBA (17.6 m*M*) adjusted to pH 6.0 with NaOH, and 15 vols. of methanol. The mobile phase was suction-filtered through a 0.5-μm cellulose acetate filter, type EH (Millipore, Bedford, MA, U.S.A.) before use and delivered to the column at a flow-rate of 1 ml/min at room temperature. A mercury-based electrochemical detector was constructed according to the description of Rabenstein and Saetre [10] and operated at a potential of 0.0 V of the working electrode vs. saturated calomel electrode. The polarographic current was registered by an LC-2A Amperometric Controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.) connected to a recorder via an electronic filter and amplifier (Model 1021 A, Spectrum, Newark, DE, U.S.A.). Peak heights were measured for quantitative evaluation of thiosulphate. Capacity ratios, k', were calculated from the retention time, t_R, and the time for the non-sorbed peak, t_0, by $k' = (t_R - t_0)/t_0$. The time for the non-sorbed peak was determined by injecting 100 μl of water and measuring the time from injection to the first deviation of the recording from baseline.

Comparison method

The thiosulphate assay reported by Sörbo and Öhman [8] is based on cyanolysis of thiosulphate to thiocyanate in the presence of cupric ions followed by colorimetric determination of thiocyanate as the ferric thiocyanate complex. Preformed thiocyanate and compounds interfering with cyanolysis are removed by chromatography on ion-exchange resins. However, the original method had to be modified as the manufacturer of the resin Lewatit MP 7080 (Bayer, Leverkusen, G.F.R.) suddenly changed the manufacturing process for this resin. Whereas earlier batches of this resin had a high affinity for thiocyanate [11], later batches showed a very low affinity for this ion. We have now found that another weakly basic anion-exchange resin Amberlyst A 21 (Rohm & Haas, Philadelphia, PA, U.S.A.) can replace MP 7080 in the method for thiosulphate assay. Furthermore, we observed that compounds inhibiting the cyanolysis reaction were removed by chromatography on Amberlyst A 21. This permitted the omission of the initial chromatography step on the resin AG 3 necessary in the original method. As Amberlyst A 21 is delivered as fairly coarse particles, it should be ground in a laboratory mill before use.

Our modified method for thiosulphate assay was as follows. Urine was adjusted to pH 4.0 with acetic acid and a 25-ml aliquot taken for analysis. To another 25-ml aliquot of urine was added 0.10 ml sodium thiosulphate (10 m*M*) as an internal standard. Each sample was then applied to a 5 × 0.7 cm column of Amberlyst A 21 (Cl^-) which was washed with 10 ml of water. Elution of thiosulphate was then performed with 20 ml of a solution containing ammonia (2.5 *M*) and ammonium sulphate (0.2 *M*) and the column was washed with 10 ml of water. To the combined effluent and washing was added 1.0 ml of KCN (0.5 *M*) followed by 0.5 ml of $CuCl_2$ (1.0 *M*). The reaction mixture was transferred to a 2.5 × 0.7 cm column of Amberlyst A 21 (free base) which was washed with 10 ml of water and 10 ml of nitric acid (0.3 *M*). The washings were discarded and the thiocyanate formed in the cyanolysis reaction was eluted with 5.0 ml of a solution containing ferric nitrate (0.25 *M*), nitric acid (3.25 *M*) and methanol (30%). The absorbance of the eluate was then determined at 470 nm in a 1-cm cuvette. A blank correction was obtained by addition of 50 μl of mercuric nitrate (1 *M*) in nitric acid (4.8 *M*) and determination of the resulting blank absorbance. We have verified on a number of human urine samples that the modified method gives results in close agreement with those of the original procedure.

RESULTS

Chromatography of standard solutions

Due to the ionic nature of thiosulphate, an ion-pairing agent (TBA) must be present in the mobile phase in order to obtain retention during reversed-phase chromatography. The mobile phase also contained phosphate buffer, a small amount of EDTA to prevent on-column oxidation of thiosulphate and methanol as an organic modifier in order to obtain suitable retention times. We verified in this connection that addition of methanol up to 20% final concentration had no noticeable effect on the sensitivity of the detector. In

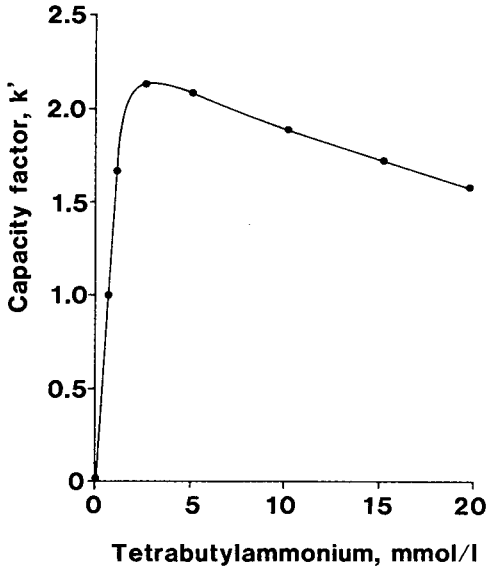

Fig. 1. Effect of TBA on the retention of thiosulphate. Other chromatographic conditions as in the standard procedure.

preliminary experiments we studied the effect of pH between 3 to 7 and found that k' for thiosulphate slightly decreased from pH 3 to pH 7, but that at the lower pH the thiosulphate peak was broad and tailing. Satisfactory peaks were obtained at pH 6.0 which was consequently used in further experiments. The effect of TBA on the retention of thiosulphate was then studied and a k'-maximum at about 3 m*M* TBA was observed (Fig. 1). A definite maximum and not a plateau value was obtained with increasing concentration of the ion-pairing agent. This is often obtained in ion-pair chromatography and the underlying mechanism has been discussed, for example by Knox and Hartwick [12].

Chromatography of urine

Urine samples were initially analyzed using a TBA concentration of 5 m*M*. The thiosulphate peak was identified from its retention time determined with a standard solution and by the peak enhancement technique. However, the thiosulphate peak obtained at this TBA concentration was tailing (Fig. 2a), suggesting the presence of an unresolved peak. In fact, when the TBA concentration was raised to 15 m*M*, the shape of the thiosulphate peak improved and a minor peak appeared close to the thiosulphate peak (Fig. 2b). As higher TBA concentrations did not improve the results, a concentration of 15 m*M* was used in the final procedure. The standard curve (Fig. 3) was slightly non-linear. We thus found it convenient to calculate the thiosulphate concentrations of unknown samples from the standard curve using non-linear regression calculations with a desk-top computer [13]. Before chromatography, urine samples were passed through Sep-Pak columns to remove non-polar constituents, which otherwise might irreversibly bind to the reversed-phase column. Although not proven, we surmise that the Sep-Pak treatment of urine results in a prolonged

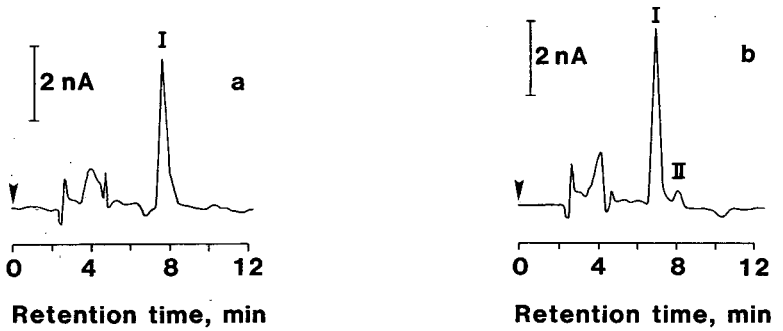

Fig. 2. Representative chromatograms of urine. (a) Mobile phase containing 0.05 *M* TBA. (b) Mobile phase containing 0.15 *M* TBA. Peak I = thiosulphate. Peak II = unknown component, resolved from thiosulphate at higher TBA concentrations.

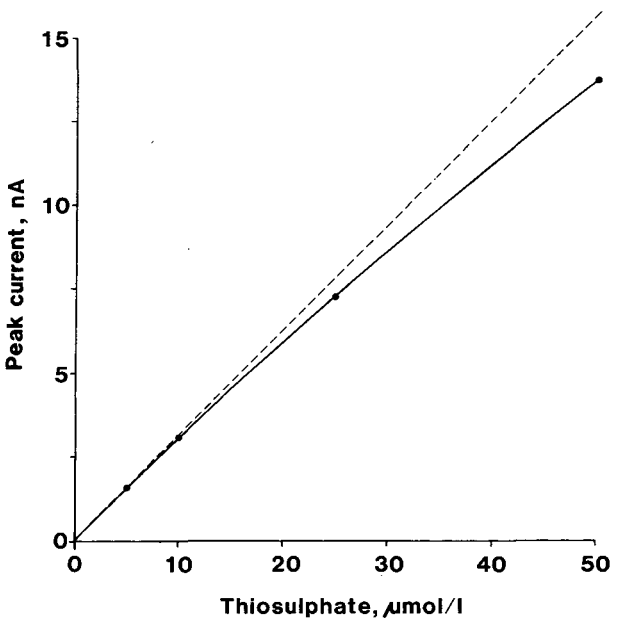

Fig. 3. Standard curve for thiosulphate.

life of the analytical column. As verified by experiments with standard solutions, the recovery of thiosulphate in this step was quantitative.

Precision and sensitivity

The intra-assay precision of the method was evaluated by analyzing ten aliquots of a urine sample. The result, 19.9 ± 0.82 μmol/l (mean ± S.D.), corresponds to a coefficient of variation of 4.2%. Similarly, the inter-assay precision was determined by analyzing the same urine on ten different days, giving 21.4 ± 1.1 μmol/l (mean ± S.D.), or a coefficient of variation of 5.3%. The sensitivity of the method was evaluated by ten injections of "simulated urine" [14] devoid of thiosulphate. The blank values thus obtained had an S.D. value corresponding to a thiosulphate concentration of 0.16 μmol/l, and the

detection limit was thus 0.3 μmol/l if defined as twice the S.D. of blank determinations.

Recovery

Thiosulphate was added to eleven urine samples of known thiosulphate concentrations (range 3.0—27.6 μmol/l) to increase the latter by 20 μmol/l. Renewed thiosulphate determinations on these samples gave a recovery of thiosulphate corresponding to 88.4 ± 4.2% (mean ± S.D.). When similar experiments were conducted with "simulated urine" a recovery of 98.0 ± 2.2% (mean ± S.D.) was obtained. The somewhat lower recovery obtained with authentic urine is thus probably due to a matrix effect. As the effect was small it was neglected in routine determinations.

Stability

When five freshly voided urine samples were analyzed for thiosulphate and then kept for 24 h at room temperature with thymol—isopropanol as a preservative, the repeated determination of thiosulphate gave 95.2 ± 6.0% (mean ± S.D.) of the original value. Furthermore, when aliquots of a normal urine sample were stored at −20°C, no significant change of the initial value was observed after 45 days of storage.

Method comparison

When urine samples from healthy subjects were analyzed by the present method and by an earlier described colorimetric method [8], the latter was found to give somewhat higher values (Fig. 4). In the light of the satisfactory recovery of thiosulphate obtained with the HPLC method, the colorimetric method apparently overestimates urinary thiosulphate, especially in the lower normal range.

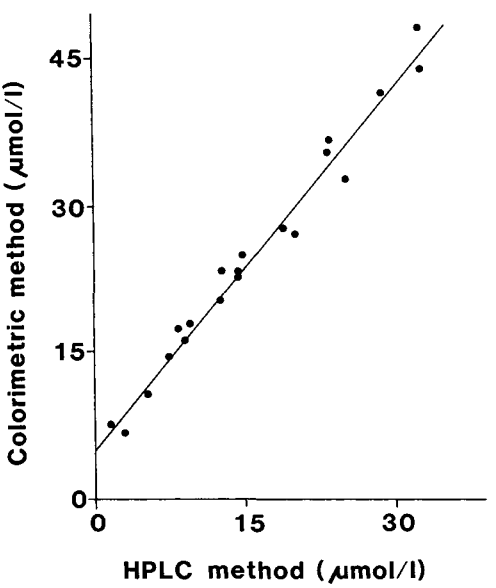

Fig. 4. Correlation between present method and colorimetric method for determination of thiosulphate in urine. Regression line: $Y = 1.23X + 5.23$ (r=0.99).

TABLE I

THIOSULPHATE EXCRETION IN TRAUMA PATIENTS

Diagnosis	Thiosulphate (μmol per 24 h)	
	Colorimetric method	HPLC
Burn + septicemia	146	1.1
Burn + septicemia	278	5.5
Multiple trauma + septicemia	167	2.1
Multiple trauma + meningitis	112	6.9

The unspecificity of the colorimetric method was dramatically illustrated by results obtained on certain patient samples where the colorimetric method gave considerably higher values than the HPLC method (Table I). Apparently, these patients excreted an unknown compound which behaved as thiosulphate in the colorimetric method. Furthermore, we observed that this compound in contrast to thiosulphate was not precipitated as a nickel—triethylenediamine complex [5] and was not retained on an organomercurial adsorbent [15]. However, its chemical identity remains to be established. The (few) patients who excreted this compound suffered from thermal or mechanical trauma complicated by a serious bacterial infection (Table I) and were treated with heavy doses of antibiotics. However, the mechanism behind the excretion of the thiosulphate-mimicking compound is unknown.

Urinary excretion in normal subjects

The excretion of thiosulphate in twelve healthy males and eight healthy females on a free diet was found to be 27.3 ± 21.5 μmol per 24 h and 18.1 ± 13.1 μmol per 24 h (mean ± S.D.), respectively. There was no significant difference between the sexes and the results were consequently combined giving an overall mean as 23.6 ± 18.7 μmol per 24 h. This is somewhat lower than the previously reported value of 31.7 ± 12.8 μmol per 24 h obtained with the colorimetric method [8].

DISCUSSION

Thiosulphate is detected in the present HPLC method with a mercury electrode, which is unfortunately not commercially available. Recently, an HPLC method for the determination of thiosulphate was reported [16] which used a commercially available glassy carbon working electrode. However, the method was only applied to the analysis of aqueous solutions of thiosulphate. We found in preliminary experiments that although a glassy carbon detector may be used for thiosulphate analysis of aqueous solutions, it was not applicable to the direct analysis of urine due to interference from a number of electroactive substances.

The present HPLC method is apparently more specific than the earlier colorimetric method. This is supported by the fact that it gives lower values for normal urine than the earlier colorimetric method and the demonstration of a

compound in urine from certain patients which interferes in the colorimetric determination. On the other hand, with the HPLC method we have confirmed (unpublished observations) a previous report [17] that subjects with the metabolic disorder mercaptolactatedisulphiduria excrete normal amounts of thiosulphate. This is of special importance with respect to the suggested biochemical abnormality underlying this condition [17].

The HPLC method has other advantages over the colorimetric method such as better precision and higher sensitivity. Furthermore, a smaller amount of urine is required for the HPLC method, which is an advantage when urine from newborn infants needs to be analysed. The HPLC method is also less time-consuming and consequently a higher number of samples can be processed in one working day.

It should be pointed out that thiosulphate in human urine is sufficiently stable to permit 24 h urine collection at room temperature, as demonstrated by the present investigation. On the other hand, we have confirmed (unpublished results) a previous observation by Gunnison et al. [18] that thiosulphate is fairly unstable in rat urine. This may be explained by a heavy contamination with faecal bacteria which is difficult to avoid during collection of rat urine in a metabolic cage.

ACKNOWLEDGEMENTS

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REFERENCES

1 A Royer and C. Fromageot, Enzymologia, 11 (1945) 361.
2 B. Sörbo, Biochim. Biophys. Acta, 24 (1957) 324.
3 F. Irrevere, S.H. Mudd, N.D. Heizer and L. Laster, Biochem. Med., 1 (1967) 187.
4 N.N. Abumrad, A.J. Schneider, P. Steel and L.S. Rogers, Amer. J. Clin. Nutr., 34 (1981) 2551.
5 J.H. Gast, K. Arai and F.L. Aldrich, J. Biol. Chem., 195 (1952) 875.
6 B. Sörbo, Biochim. Biophys. Acta, 23 (1957) 412.
7 V.E. Shih, M.M. Carney and R. Mandell, Clin. Chim. Acta, 95 (1979) 143.
8 B. Sörbo and S. Öhman, Scand. J. Clin. Lab. Invest., 38 (1978) 521.
9 L. Naftalin and L.R. Mitchell, Clin. Chim. Acta, 3 (1958) 197.
10 D.L. Rabenstein and R. Saetre, Anal. Chem., 49 (1977) 1036.
11 P. Lundquist, J. Mårtensson, B. Sörbo and S. Öhman, Clin. Chem., 25 (1979) 678.
12 J.H. Knox and R.A. Hartwick, J. Chromatogr., 204 (1981) 3.
13 W.K. Bates and D.F. McAllister, Anal. Biochem., 59 (1974) 190.
14 A. Hodgkinson, Clin. Chim. Acta, 109 (1981) 239.
15 L.A.AE. Sluyterman and J. Widjenes, Biochim. Biophys. Acta, 200 (1970) 593.
16 T. Imanari, K. Ogata, S. Tanabe, T. Toida, T. Kawanishi and M. Ichikawa, Chem. Pharm. Bull., 30 (1982) 374.
17 U. Hannestad, J. Mårtensson, R. Sjödahl and B. Sörbo, Biochem. Med., 26 (1981) 106.
18 A.F. Gunnison, T.J. Farruggella, G. Chiang, L. Dulak, J. Zaccardi and J. Birkner, Food Cosmet. Toxicol., 19 (1981) 209.

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DETERMINATION OF THE *R*(—)- AND *S*(+)-ENANTIOMERS OF γ-VINYL-γ-AMINOBUTYRIC ACID IN HUMAN BODY FLUIDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

An analytical procedure, which allows the determination and quantitation of the *R*(—)- and *S*(+)-enantiomers of γ-vinyl-γ-aminobutyric acid (γ-vinyl-GABA; MDL 71.754) in body fluids was developed. The method is based on a combined gas chromatographic—mass spectrometric technique. A glass capillary column coated with a chiral phase enabled the separation of the enantiomers of γ-vinyl-GABA as their N-trifluoroacetyl-O-methyl ester derivatives. This was followed by quantitation using a selected ion monitoring technique in the electron-impact mode of ionization. The internal standard, γ-acetylenic GABA, was included throughout the work-up of the samples. The assay was shown to be reproducible, specific and sensitive. No interferences were encountered from plasma, urine or cerebrospinal fluid constituents. The method was applied to the analysis of plasma samples obtained from a human volunteer who had received racemic γ-vinyl-GABA. Significant differences in the plasma concentrations and plasma half-lives of the two enantiomers were seen, clearly illustrating the need for a specific assay technique capable of distinguishing between the enantiomers of this drug.

INTRODUCTION

Increasing the concentration of γ-aminobutyric acid (GABA) in the central nervous system has possible therapeutic potential for a number of neurologic disorders including epilepsy [1], tardive dyskinesia [2], Huntington's chorea [3], Friedreich's ataxia [4, 5] and Parkinsonism [6]. γ-Vinyl-GABA (Fig. 1) has been shown to be a potent and selective enzyme-activated inhibitor of the GABA-degrading enzyme, GABA-α-oxoglutarate aminotransferase (GABA-T) in vitro [7]. When administered orally to patients suffering from various neurologic disorders it was shown to cause dose-dependent increases of GABA in their cerebrospinal fluid [8, 9].

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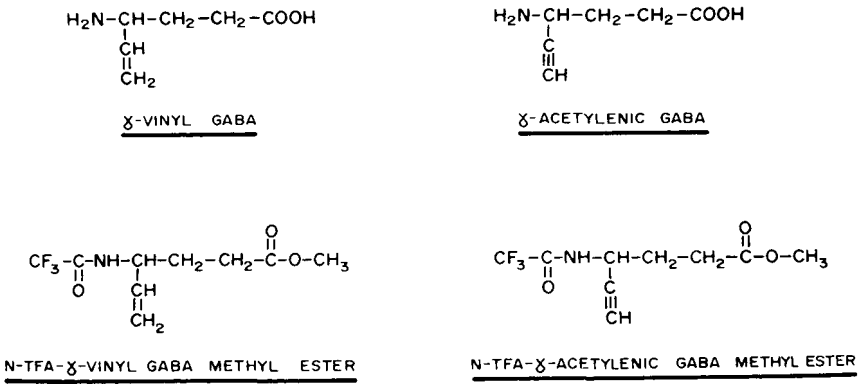

Fig. 1. Structures of γ-vinyl-GABA and γ-acetylenic GABA and of their N-trifluoroacetyl- (N-TFA-) and methyl ester derivatives.

Due to its synthetic route [10], the drug is presently supplied as the racemic *R,S* mixture. In animal experiments, however, it has been shown that only the *S*(+)-enantiomer of γ-vinyl-GABA is an inhibitor of mouse brain GABA-T, whereas the *R*(—)-enantiomer is devoid of any activity [11]. It is well known that the administration of enantiomeric mixtures of drugs such as propranolol [12—15], hexobarbital [16], methadone [17], *p*-chloroamphetamine [18], metoprolol [19], alprenolol [19], disopyramide [20] and oxaprotiline [21] can result in differing blood levels and pharmacokinetics of the enantiomers. These pharmacokinetic differences may result in differing concentration—effect relationships and, thus, be of clinical importance.

Our objective was to develop an analytical method capable of separating the *R*(—)- and *S*(+)-enantiomers of γ-vinyl-GABA. Successful separations of amino acid enantiomers have been described by Frank et al. [22, 23] by capillary gas chromatography using a chiral phase. The inclusion of an internal standard in the procedure makes it possible to quantitate the enantiomers in biological fluids and thus makes future pharmacokinetic studies feasible.

EXPERIMENTAL

Reagents

R,S-γ-Vinyl-GABA (MDL 71.754; Merrell-Dow Pharmaceuticals) and the internal standard *S*(+)-γ-ethynyl-GABA (*S*(+)-γ-acetylenic GAMA; MDL 71.667; Merrell-Dow Pharmaceuticals) (Fig. 1) were supplied as crystalline powders. For oral use, hard gelatin capsules containing 250 mg of drug per capsule were provided. Reagent-grade trifluoroacetic anhydride (E. Merck, Darmstadt, G.F.R.) and diazomethane prepared from N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich-Europe, Beerse, Belgium) were used in the derivatization.

Gas chromatography—mass spectrometry

Mass spectrometric analysis was carried out on a combined gas chromatograph—mass spectrometer—computer system (Ribermag R-10-10, Nermag S.A., France).

Separation of the derivatized enantiomers of *R,S*-γ-vinyl-GABA and of the internal standard *S*(+)-γ-acetylenic GABA (Fig. 1) was achieved using a 25 m × 0.92 mm O.D. capillary column coated with L-valine—*tert.*-butylamide coupled to an alkylsiloxane polymer (Chirasil-Val; Applied Science Labs., Oud-Beijerland, The Netherlands).

The sample was injected onto the column using a Ros injector, allowing solvent-free injections (Girdel, Suresnes, France). The gas chromatograph (Serie 32, Girdel) was coupled directly to the mass spectrometer without the use of a molecular separator. The injector port temperature was maintained at 270°C, the interphase temperature was 270°C and the ion-source temperature was kept at 180°C. Separation of the compounds was achieved using helium as carrier gas at a column pressure of 0.8 bar. The initial column temperature of 90°C was maintained for 1 min after the injection and then increased at 4°C/min to 130°C. Mass spectra were acquired in the electron-impact mode of ionization using an electron energy of 70 eV.

Work-up of samples

To 100 μl of plasma were added 20 μl of an aqueous solution containing the internal standard *S*(+)-γ-acetylenic GABA. Protein was precipitated by addition of 200 μl of methanol. After centrifugation the supernatant was evaporated to dryness with a stream of nitrogen. The residue was redissolved in 200 μl of dichloromethane and 200 μl of trifluoroacetic anhydride and left to react for 1 h at room temperature. The solvent was then evaporated with a stream of nitrogen and the residue redissolved in 100 μl of methanol. To this solution was added, dropwise, a solution of diazomethane in diethyl ether until the sample solution had a distinctly yellow colour. Excess diazomethane was destroyed by addition of a droplet of acetic acid, resulting in a colorless solution which was evaporated to dryness. The dry residue was partitioned between 1 ml of water and 2 ml of benzene and the organic phase transferred to another vial for evaporation to dryness. Before analysis, the sample was redissolved in 10—100 μl of benzene of which 1—2 μl were deposited on the injection capillary of the Ros injector. After evaporation of the solvent the sample was brought into the heated injector port and thus injected into the column.

Calibration curves

Calibration curves were obtained for stock solutions of the products to determine linearity of response and for spiked human plasma, urine and cerebrospinal fluid.

RESULTS AND DISCUSSION

Reproducibility

Spiking of human plasma with 200 nmol/ml of racemic *R,S*-γ-vinyl-GABA plus 200 nmol of the internal standard, *S*(+)-γ-acetylenic GABA, and subsequent analysis yielded good reproducibility (Table I).

On separate occasions, distributed over a 45-day period, six different sets of

TABLE I

ASSAY REPRODUCIBILITY AND DAY-TO-DAY VARIABILITY IN HUMAN PLASMA SPIKED WITH 200 nmol/ml OF *R,S*-γ-VINYL-GABA AND 200 nmol OF *S*(+)-γ-ACETYLENIC GABA

Each time 100 μl of plasma were used. Values are expressed in nmol/ml ± standard deviation.

	γ-Vinyl-GABA		
	R(—)-Enantiomer	*S*(+)-Enantiomer	
Reproducibility	104.2 ± 4.0	103.7 ± 4.3	(n = 5)
Day-to-day variability (45-day period)	100.6 ± 16.6	99.6 ± 16.3	(n = 6)

plasma samples with two samples in each set were spiked with the same quantity of *R,S*-γ-vinyl-GABA and internal standard. This day-to-day variability is also presented in Table I.

Calibration curves

A calibration curve for human plasma was obtained using as internal standard 20 nmol of *S*(+)-γ-acetylenic GABA added per 100 μl of plasma, while the *R,S*-γ-vinyl-GABA concentration varied from 2.5 to 20 nmol. By linear regression analysis the line was calculated to be $C = -0.01926 + 0.006179 \cdot$ ng *R*(—)-γ-vinyl-GABA and $C = -0.01363 + 0.006165 \cdot$ ng *S*(+)-γ-vinyl-GABA with a correlation coefficient for both curves of —0.9992. Since the two curves were essentially identical a mean calibration curve of $C = 0.01652 + 0.006172 \cdot$ ng with a correlation coefficient of —0.9993 may be used to determine either the *R*(—)- or the *S*(+)-enantiomer. Expanding the calibration to higher plasma concentrations of γ-vinyl-GABA yielded a lesser degree of linearity; such concentrations thus require dilution before analysis or, alternatively, a smaller amount of plasma may be used. Calibration curves for urine and cerebrospinal fluid also showed excellent linearity for the same concentration range.

Mass spectrometry

Fig. 2 shows the electron-impact mass spectra of N-trifluoroacetyl(TFA)-γ-vinyl-GABA methyl ester and of the corresponding derivative of γ-acetylenic GABA. The molecular ions, *m/e* 239 for N-TFA-γ-vinyl-GABA-OCH_3 and *m/e* 237 for N-TFA-γ-acetylenic GABA-OCH_3 were both weak. In the following discussion, ions for the corresponding γ-acetylenic GABA derivative are shown in parentheses. Loss of $CH_3O^{\bullet}$ from the molecular ions leads to *m/e* 208 (206) and elimination of CH_3OH to *m/e* 207 (205). Expulsion of $HCOOCH_3$ leads to *m/e* 179 (177). A McLafferty rearrangement yields the ions at *m/e* 165 (163) and *m/e* 74 (74). The base peak of the spectrum is produced through a loss of $^{\bullet}CH_2—CH_2—COOCH_3$ to give the ion at *m/e* 152 (150). The fragmentation scheme of the two derivatives is presented in Fig. 3.

For quantitative determination of γ-vinyl-GABA in human body fluids, a selected ion monitoring (SIM) technique was employed. For this purpose, the ions at *m/e* 152 (γ-vinyl-GABA) and *m/e* 150 (γ-acetylenic GABA) were

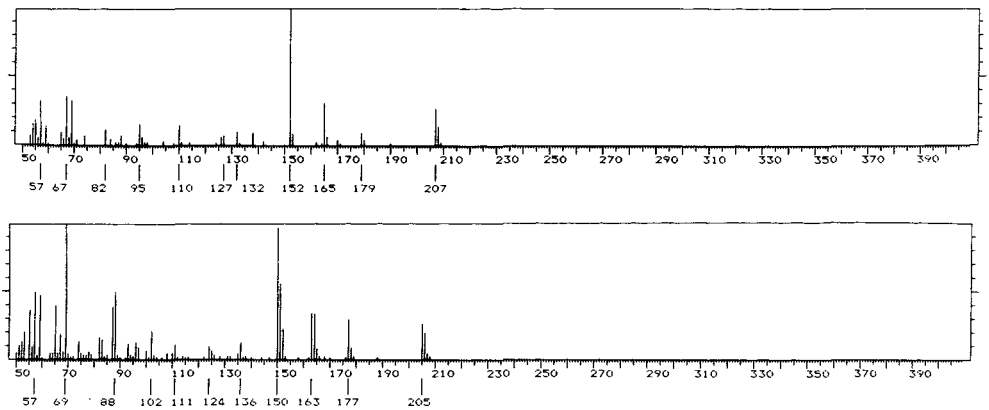

Fig. 2. 70 eV mass spectra of N-trifluoroacetyl-γ-vinyl-GABA methyl ester (top) and of the corresponding derivative of γ-acetylenic GABA (bottom). Column, 25 m Chirasil-Val; temperature, 130°C isothermal; pressure, 0.8 bar.

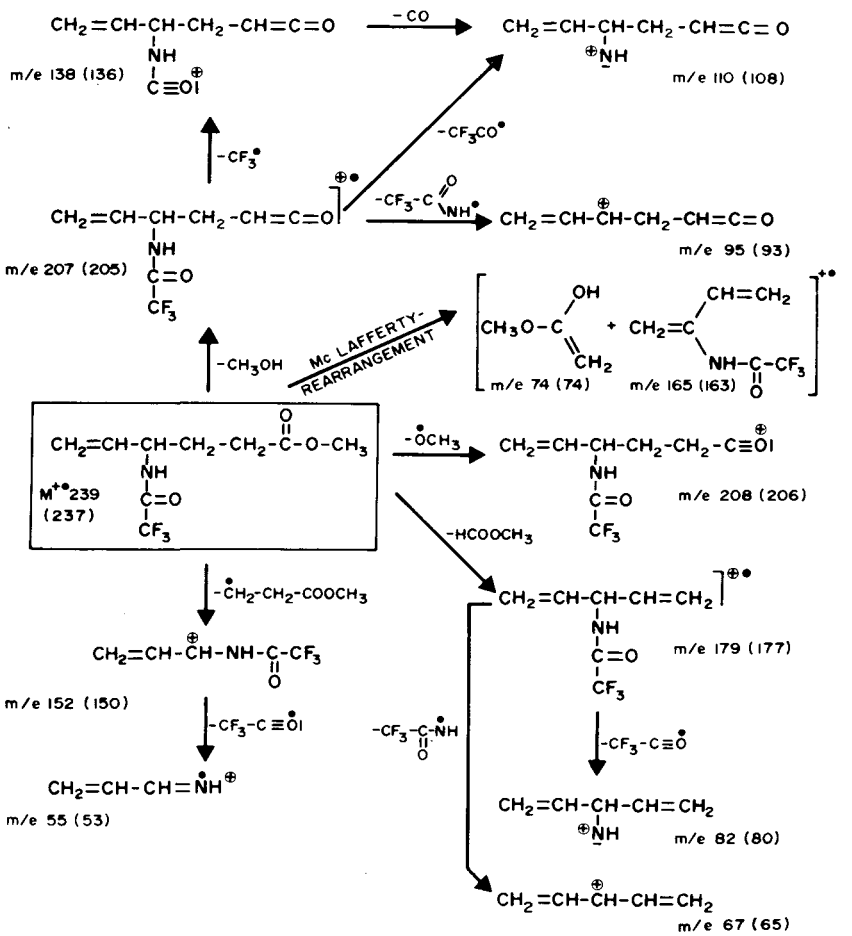

Fig. 3. Mass spectrometric fragmentation scheme of the derivatives of γ-vinyl-GABA and γ-actylenic GABA.

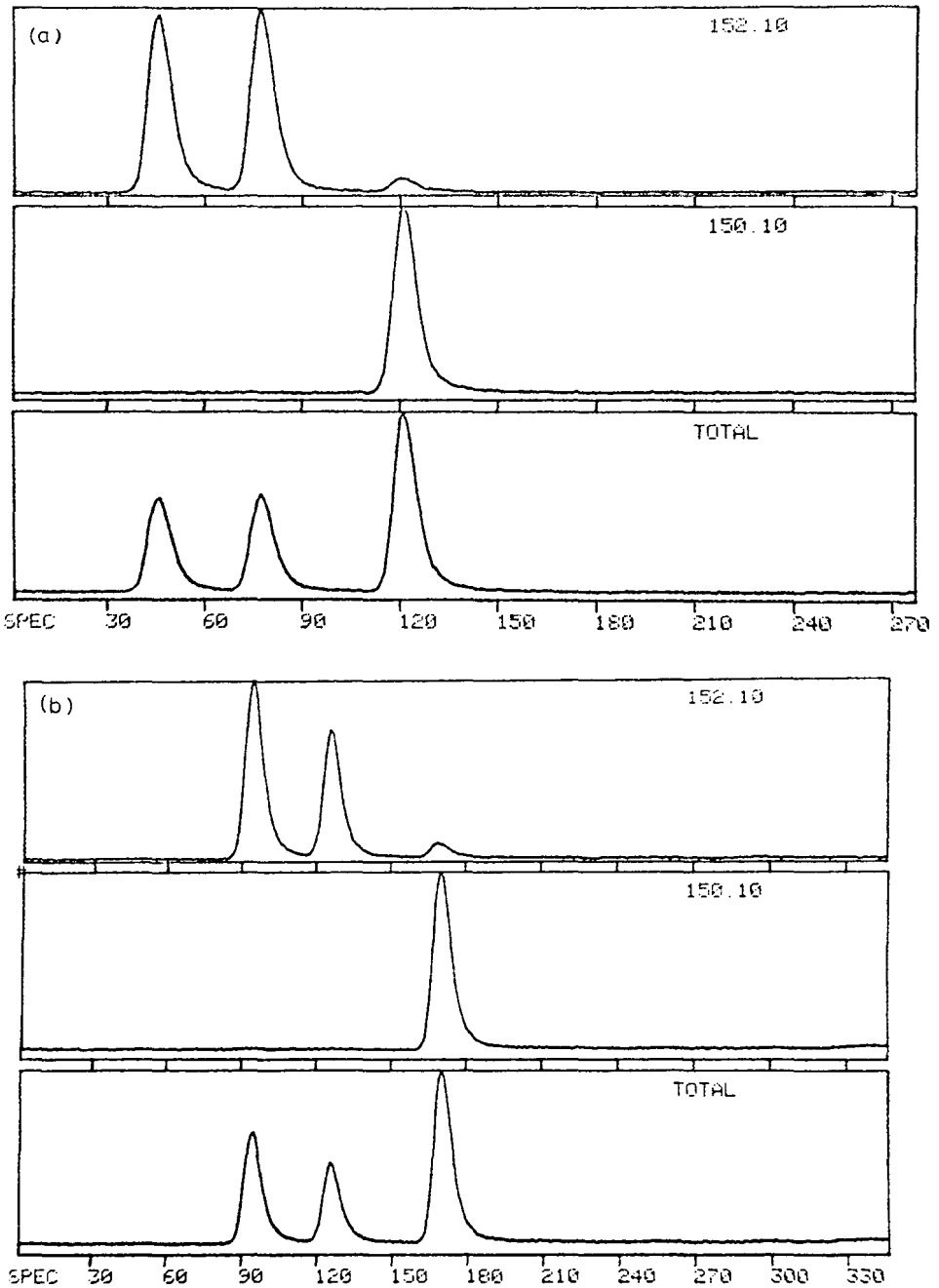

Fig. 4. SIM chromatograms of *m/e* 152 for γ-vinyl-GABA and *m/e* 150 for the internal standard: (a) standard mixture; (b) urinary sample obtained from a volunteer after a single oral dose of 500 mg of *R,S*-γ-vinyl-GABA.

chosen. A SIM chromatogram of a standard mixture is shown in Fig. 4a and that of a urinary sample of a volunteer who had received a single oral dose of 500 mg of *R,S*-γ-vinyl-GABA is presented in Fig. 4b. No interferences from body constituents were encountered.

Time course of R(—)- and S(+)-γ-vinyl-GABA in a human volunteer

A healthy, male volunteer received five doses of 500 mg of racemic *R,S*-γ-vinyl-GABA given at 12-h intervals. The drug sample was shown to contain equal amounts of the two enantiomers. Blood samples were obtained just prior to the last dose and at 30 min, 45 min, 1, 2, 3, 4, 6, 8 and 12 h after the last dose and the enantiomers of γ-vinyl-GABA were determined quantitatively. The result is shown in Fig. 5. It is evident that γ-vinyl-GABA was rapidly absorbed, peak plasma concentrations being observed at 1 h post dose. Plasma concentrations of the two enantiomers differ substantially from one another for the first 4 h post dose, and subsequently seem to approach equal levels. The

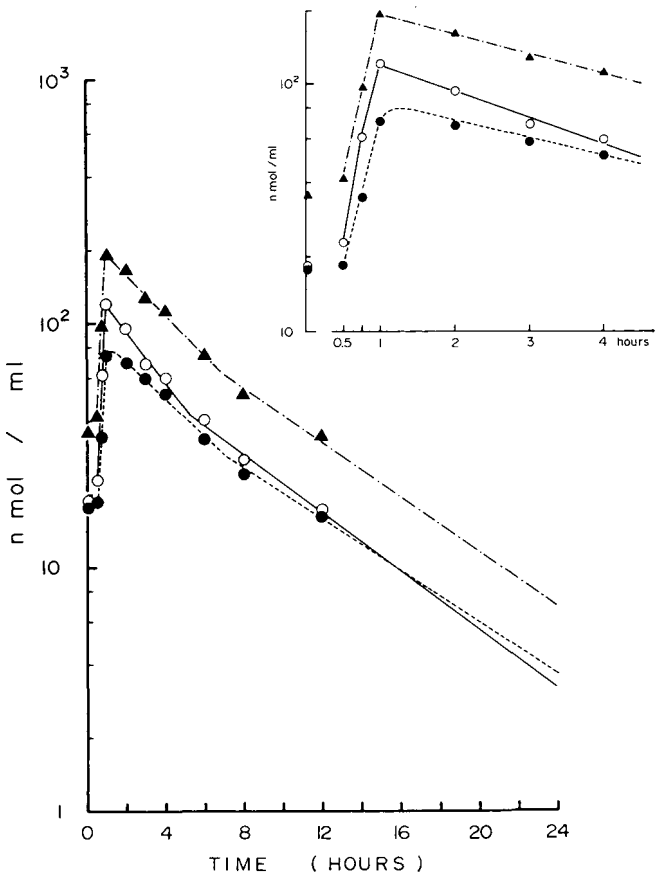

Fig. 5. Time course of *R*(—)- and *S*(+)-γ-vinyl-GABA in the plasma of a volunteer who had ingested a total of five doses each of 500 mg of racemic γ-vinyl-GABA at 12-h intervals when followed after the last dose. (▲), Sum of *R*- and *S*-enantiomers; (○), *R*(—)-enantiomer; (●), *S*(+)-enantiomer. Half-lives: *R*(—): $t_{½\alpha}$ = 2.8 h, $t_{½\beta}$ = 5.0 h; *S*(+): $t_{½\alpha}$ = 3.9 h, $t_{½\beta}$ = 5.7 h; *R* + *S*: $t_{½\alpha}$ = 3.6 h, $t_{½\beta}$ = 5.3 h.

peak plasma concentration of the biologically inactive *R*(−)-enantiomer was 85.1 nmol/ml, whereas the concentration of the biologically active *S*(+)-enantiomer was only 40.7 nmol/ml. Thus, of a total of 125.8 nmol/ml of γ-vinyl-GABA present in this sample less than one third corresponded to the biologically relevant form of this GABA-transaminase inhibitor.

These results clearly demonstrate the necessity of a specific assay technique capable of separating the enantiomeric forms of drugs. Such a technique becomes obligatory in the case where the enantiomers of a drug administered as the racemic mixture possess different pharmacological properties. The described method will be applied to future pharmacokinetic studies.

REFERENCES

1 B.S. Meldrum, Lancet, i (1978) 304.
2 G. Tell, P.J. Schechter, J. Koch-Weser, P. Cantiniaux, J.P. Chabannes and P.A. Lambert, N. Engl. J. Med., 305 (1981) 581.
3 S.J. Enna, E.D. Bird, J.P. Bennett, D.B. Bylund, H.I. Yamamura, L.L. Iversen and S.H. Snyder, N. Engl. J. Med., 294 (1976) 1305.
4 R.J. Huxtable, J. Azart, T. Reisine, P. Johnson, H.I. Yamamura and A. Barbeau, Canad. J. Neurol. Sci., 6 (1979) 255.
5 Y. De Smet, J.Y. Mear, G. Tell, P.J. Schechter, F. Lhermitte and Y. Agid, Can. J. Neurol. Sci., 9 (1982) 171.
6 A. Barbeau, Lancet, ii (1973) 1499.
7 M.J. Jung, B. Lippert, B.W. Metcalf, P. Böhlen and P.J. Schechter, J. Neurochem., 29 (1977) 797.
8 J. Grove, G. Tell, P.J. Schechter, J. Koch-Weser, J.M. Warter, C. Marescaux and L. Rumbach, Lancet, ii (1980) 647.
9 J. Grove, G. Tell, P.J. Schechter, J. Koch-Weser, A. Sjoerdsma, J.M. Warter, C. Marescaux and L. Rumbach, Life Sci., 28 (1981) 2431.
10 B. Lippert, B.W. Metcalf, M.J. Jung and P. Casara, Eur. J. Biochem., 74 (1977) 441.
11 M.J. Jung, private communication.
12 S. Caccia, G. Guiso, M. Ballabio and P. de Ponte, J. Chromatogr., 172 (1979) 457.
13 J. Hermansson, J. Chromatogr., 221 (1980) 109.
14 J. Hermansson, Acta Pharm. Suecica, 19 (1982) 11.
15 C. von Bahr, J. Hermansson and K. Tawara, Brit. J. Clin. Pharmacol., 14 (1982) 79.
16 K. Miyano, T. Ota and S. Toki, Drug Metab. Dispos., 9 (1981) 60.
17 K. Nakamura, D.L. Hachey, M.J. Kreek, C.S. Irving and P.D. Klein, J. Pharm. Sci., 71 (1982) 40.
18 M.M. Ames and S.K. Frank, Biochem. Pharmacol., 31 (1982) 5.
19 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113.
20 C.S. Cook, A. Karim and P. Sollman, Drug Metab. Dispos., 10 (1982) 116.
21 W. Dieterle and J.W. Faigle, J. Chromatogr., 242 (1982) 289.
22 H. Frank, G.J. Nicholson and E. Bayer, Angew. Chem., 90 (1978) 396.
23 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr., 146 (1978) 197.

Journal of Chromatography, 274 (1983) 111—120
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CHROMBIO. 1585

DETERMINATION OF CHLOROQUINE AND ITS MAJOR METABOLITE IN BLOOD USING PERFLUOROACYLATION FOLLOWED BY FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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SUMMARY

Tandem fused-silica capillary gas chromatographic methods for the determination of chloroquine and its major metabolite, desethylchloroquine, are described. Method A employs a single extraction step and internal standardization to permit rapid, precise analyses for chloroquine in whole blood. Method B, employing derivatization with pentafluoropropionic anhydride, can then be applied to the extract to allow qualitative and quantitative confirmation of chloroquine and sensitive, precise quantification for desethylchloroquine. The detection limit for chloroquine in blood is 5 ng/ml by both methods; the limit for desethylchloroquine is 15 ng/ml. Excellent precision is achieved by the methodology, partly due to the use of separate internal standards for the two analytes, each internal standard being a close analogue of the corresponding analyte. Data are presented which demonstrate the increase over time of metabolite relative to unchanged chloroquine found in the blood of a volunteer undergoing a chemoprophylactic regimen of chloroquine.

INTRODUCTION

A variety of analytical approaches have been employed to quantify chloroquine in body fluids [1—12] in support of investigations into its use as an antimalarial and antirheumatic drug. Four of these methods permit quantitation of both chloroquine and its major metabolite, desethylchloroquine, in blood, three using high-performance liquid chromatography (HPLC) [6,10,11] and the other gas—liquid chromatography [9]. In the present work we report a procedural sequence which permits the analyst to rapidly, sensitively, and precisely determine chloroquine concentration in blood and then, if desired,

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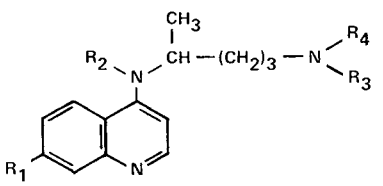
employ derivatization with pentafluoropropionic anhydride to confirm chloroquine identity and quantity while quantifying desethylchloroquine.

EXPERIMENTAL

Standards

Chloroquine diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.)*. This material was dried under vacuum over phosphorus pentoxide and the melting point determined using a Fisher—Johns apparatus calibrated with Fisher TherMetric standards before its use as a standard. The melting point range was 192.1—193.3°C. Desethylchloroquine base and iodoquine diphosphate were supplied by the Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). The isopropyl analogue of desethylchloroquine was provided by Mr. William Ellis of the Walter Reed Army Institute of Research (Washington, DC, U.S.A.).

The internal standard mixture referred to in the analysis (see below) contains roughly 6.6 μg/ml iodoquine (as base) and 5.1 μg/ml isopropyl analogue of desethylchloroquine (Fig. 1, compounds IIa and IVa, respectively) in 0.002 *M* aqueous hydrochloric acid.



Designation	R_1	R_2	R_3	R_4	t_R(min)
Ia	Cl	H	CH_2CH_3	CH_2CH_3	8.6
Ib	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	CH_2CH_3	7.0
IIa	I	H	CH_2CH_3	CH_2CH_3	11.6
IIb	I	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	CH_2CH_3	9.0
IIIa	Cl	H	H	CH_2CH_3	8.0
IIIb	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	7.8
IVa	Cl	H	H	$CH(CH_3)_2$	8.3
IVb	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$CH(CH_3)_2$	8.1

Fig. 1. Structures of chloroquine, desethylchloroquine, internal standard compounds, and their pentafluoropropionyl derivatives together with retention times (t_R) under the chromatographic conditions employed in this study. The final column temperature is 230°C for compounds Ia—IVa, 220°C for compounds Ib—IVb.

*Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Pentafluoropropionylchloroquine was synthesized by adding 100 μl of pentafluoropropionic anhydride and 25 μl of triethylamine to a 10.3-mg quantity of chloroquine base in a 15-ml screw-cap centrifuge tube. The tube was capped and sealed using PTFE tape and the mixture heated to 90°C for 30 min. The mixture was cooled, diluted with 3 ml of benzene, and partitioned with 0.5 ml of 5% ammonia. The benzene layer was transferred to a separate centrifuge tube and this stock diluted as necessary for qualitative chromatographic studies. Gas chromatographic—mass spectrometric (GC—MS) analysis showed the presence of a small amount of unreacted chloroquine and an earlier eluting peak due to a large quantity of material containing an apparent molecular ion at *m/e* 465 together with major fragments at *m/e* 450 (loss of CH_3), *m/e* 318 (loss of $CF_3CF_2CO^{\bullet}$) and *m/e* 86 [$CH_2{=}\overset{+}{N}(CH_2CH_3)_2$]. This pattern is definitive for the proposed compound.

Bis-pentafluoropropionyldesethylchloroquine was similarly synthesized. The benzene extract contained primarily the compound sought together with a small quantity of the expected monoacylated product. GC—MS analysis showed no peaks for the molecular ion expected, but prominent peaks at *m/e* 436 (loss of $CF_3CF_2CO^{\bullet}$), *m/e* 351 [loss of $^{\bullet}CH_2CH_2CH_2N(CH_2CH_3)(COCF_2CF_3)$], *m/e* 260 [$CH_3\overset{+}{C}HCH_2CH_2CH_2N(CH_2CH_3)(COCF_2CF_3)$], and *m/e* 204 [$CH_2{=}\overset{+}{N}(CH_2CH_3)(COCF_2CF_3)$]. These fragments establish the structure of the compound proposed.

Reagents

Hexane was glass-distilled, available from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The octane and *n*-decylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Human blood used for standards was freshly collected using acid—citrate—dextrose (ACD) as an anticoagulant. Pentafluoropropionic anhydride was supplied by Pierce (Rockford, IL, U.S.A.). Pyridine was from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and was distilled over sodium hydroxide. All other chemicals used were of reagent grade or better.

Equipment

Gas chromatography—mass spectrometry. Pentafluoropropionyl derivatives of chloroquine and desethylchloroquine were characterized with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 5992 gas chromatograph—mass spectrometer—computer system. Studies were performed using a 1.83 m × 2.0 mm I.D. glass column packed with 5% OV-101 on 100—120 mesh Gas Chrom Q and a helium flow-rate of 16 ml/min; injector, column, jet separator, and source temperatures were 250, 250, 250, and 150°C, respectively, and electron-impact ionization was performed at 70 eV.

Gas chromatography. A Hewlett-Packard Model 5880A gas chromatograph equipped with a capillary injector and a nitrogen-specific detector was used in this study. A 25 m × 0.3 mm I.D. fused-silica capillary column was used which had been siloxane deactivated and coated with OV-1 by the manufacturer (Part No. 19091-62325; Hewlett-Packard). On-column injection was used. The front 1.5-m portion of the column was stripped of liquid phase using methylene chloride. When the injection of a large number of samples had degraded column performance, removal of a 25-cm portion of the front of the

column restored efficiency. After four to five such "surgeries," the front end of the column was again stripped of liquid phase. The injector heater was not turned on, so that the injector temperature remained below the column initial temperature of 125°C. Detector temperature was 290°C. The column temperature remained at the 125°C initial temperature for 1 min and then was programmed to increase at 20°C/min to 230°C for Method A and 220°C for Method B. Helium carrier gas flow-rate was in the range of 3—4 ml/min. The syringe used for on-column injection was Part No. 9301-0562 (Hewlett-Packard), which is fitted with a needle 125 mm in length.

Method A

Conical centrifuge tubes (15 ml) were fortified with 100-μl volumes of 0.001 *N* aqueous hydrochloric acid solutions containing the following quantities of chloroquine and desethylchloroquine expressed in ng as base: 0.0, 0.0; 99.1, 119.7; 198.2, 239.4; 247.7, 359.2; 495.4, 478.9; and 743.1, 598.6. To each tube were added 100 μl of the internal standard mixture (see standards), 1 ml of ACD blood, and 2 ml of deionized water. A 1-ml quantity of each (heparinized) blood sample was pipetted into a 15-ml centrifuge tube to which a 100-μl quantity of internal standard mixture had been added. A 2-ml rinse of deionized water served to complete the transfer of blood sample into the centrifuge tube. Sample and standard alike were mixed using a 5-sec vortex. To each tube were added 3 ml of hexane and 0.5 ml of 5 *N* sodium hydroxide solution. Each tube was vortexed for 30 sec, followed by 20 min shaking on a reciprocal shaker. Two drops of isoamyl alcohol were added, followed by 5 min centrifugation, to aid in the separation of layers. The bulk of each hexane layer was transferred to a silanized [5] 15-ml centrifuge tube fitted with a cap containing a PTFE insert. The hexane was evaporated using a 60°C water bath and a stream of dry nitrogen. Each sample and standard were reconstituted using 25 μl of *n*-octane. On-column injections were made after drawing up 1.2 μl of 200 ng/ml *n*-decylamine in octane, about 1.8 μl of air, and 1.4 μl of sample or standard. After injection an 0.8-μl quantity of liquid remained in the syringe.

Method B

To the remainder of each of the concentrated extracts left after the injection of Method A, about 2 ml of benzene were added and then evaporated using a stream of dry nitrogen. This benzene addition—evaporation step was repeated to ensure dryness of the residue. To each tube were added 50 μl of pentafluoropropionic anhydride and 10 μl of dry pyridine. The tubes were sealed using PTFE tape and heated to 60°C for 30 min. Each tube was then cooled and 2 ml of hexane added, followed by 5-sec vortexing. A 2-ml quantity of 1 *N* ammonia was added and each tube vortexed for 30 sec. The bulk of the hexane layer was transferred by a Pasteur pipet to a 3-ml Reacti-vial. The hexane was evaporated using an aluminum block at 60°C with hood air-flow passing over the vials. Each sample and standard was then reconstituted in 25 μl of *n*-octane with vortexing, and a 1—2 μl quantity was injected onto the capillary column.

RESULTS AND DISCUSSION

Characterization of Methods A and B

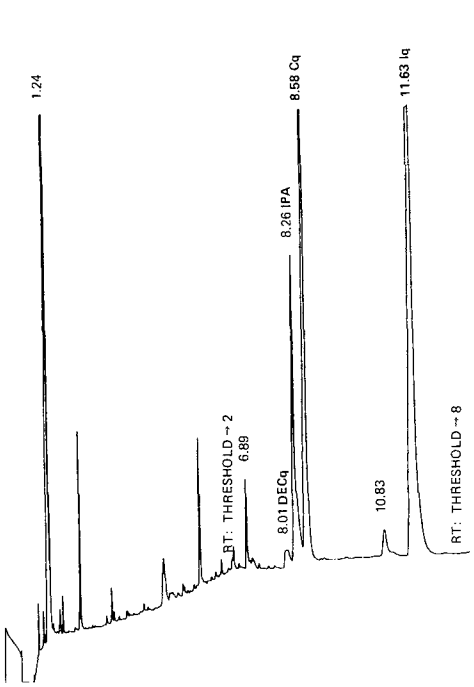
The methodology presented herein is designed to provide a rapid, accurate value for chloroquine concentration in whole blood and blood fractions while presenting the option for confirmation of the chloroquine value and quantification of desethylchloroquine, all using a single 1-ml sample. The first phase of the analysis, referred to as Method A, is a modification of our previously presented procedure [12]. Iodoquine has been substituted for bromoquine as internal standard, since pentafluoropropionylbromoquine (PFP-bromoquine) and bis-PFP-desethylchloroquine (Fig. 1, compound IIIb) would coelute in the second phase of the determination. Also, *n*-decylamine is not added directly to the *n*-octane solvent for the concentrated extract but is instead used in an *n*-octane solution to provide a "solvent flush" [13], as described in the Experimental section, to minimize adsorption both within the syringe and on the front of the capillary column. Failure to use the solvent-flush technique appreciably lowered sensitivity of the method.

Method B consists of a derivatization and extraction step performed subsequent to Method A. The perfluoropropionated compounds exhibit no evidence of adsorption under the GC conditions employed in the present study. Comparison of the results from the two methods can be made by perusal of Figs. 2 and 3 and Tables I and II.

The column performance was less than optimal when the chromatograms in Fig. 2 were run. Tailing is evident not only for compound IVa but also for iodoquine (IIa). Chloroquine peak shape is good, with chloroquine eluting on the tail of IVa. Removal of 25 cm of the solvent-stripped front end of the column followed by reinstallation yielded the much-improved column performance seen in Fig. 3. The discrimination of the column against desethylchloroquine relative to chloroquine is evident in Figs. 2 and 3. On the other hand, as might be expected, the peak shapes of the PFP derivatives, seen in the chromatographic traces on the right half of Fig. 2, are not adversely affected by the factors which cause tailing in the underivatized compounds. The internal standard peak heights for the chromatogram of the blank standard in Fig. 2 are less than for the standard containing both chloroquine and desethylchloroquine. Some losses of amine analytes and standards occur, presumably by adsorption, during the analysis, and such losses have the greatest impact on internal standard peak heights when no analyte compounds are present to share adsorptive losses.

The small chloroquine peak seen for the blank by Method A is due to non-negligible but reproducible "ghosting," the magnitude of which is only slightly affected by the quantity of chloroquine in the immediately preceding injection [12].

Results in Table I show that Method B exhibits marginally better linearity and precision than Method A. The slopes of the two regression lines are nearly identical. The relative standard deviation (R.S.D.) at the 247.7 ng/ml level for chloroquine is better than 2% for both methods. The detection limit for both methods is conservatively calculated to be 5 ng/ml of chloroquine in blood. It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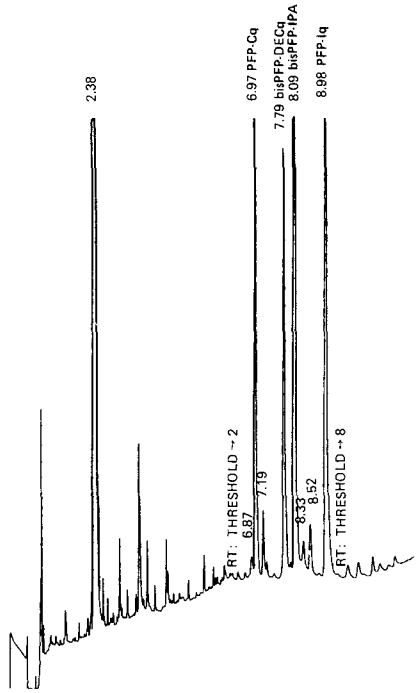
8.01 DECq
8.26 IPA
8.58 Cq
10.83
11.63 Iq
RT: THRESHOLD → 8


2.38
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6.87
7.19
6.97 PFP-Cq
7.79 bisPFP-DECq
8.09 bisPFP-IPA
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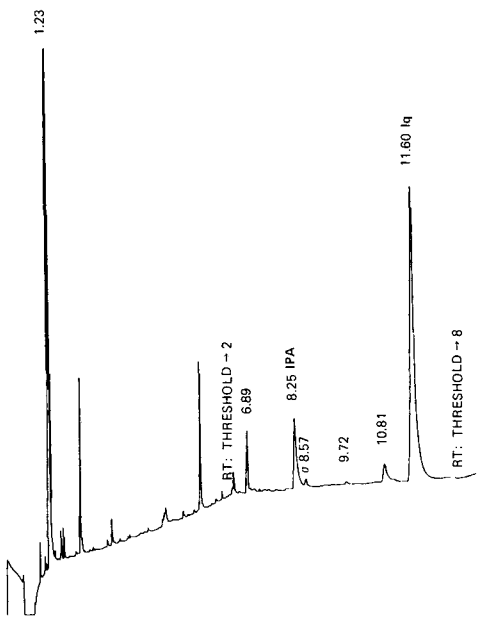
8.98 PFP-Iq
RT: THRESHOLD → 8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8.25 IPA
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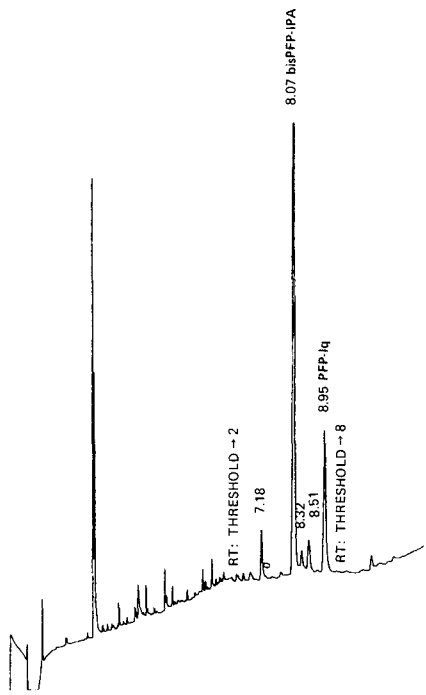
9.72
10.81
11.60 Iq
RT: THRESHOLD → 8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7.18
8.07 bisPFP-IPA
8.32
8.51
8.95 PFP-Iq
RT: THRESHOLD → 8

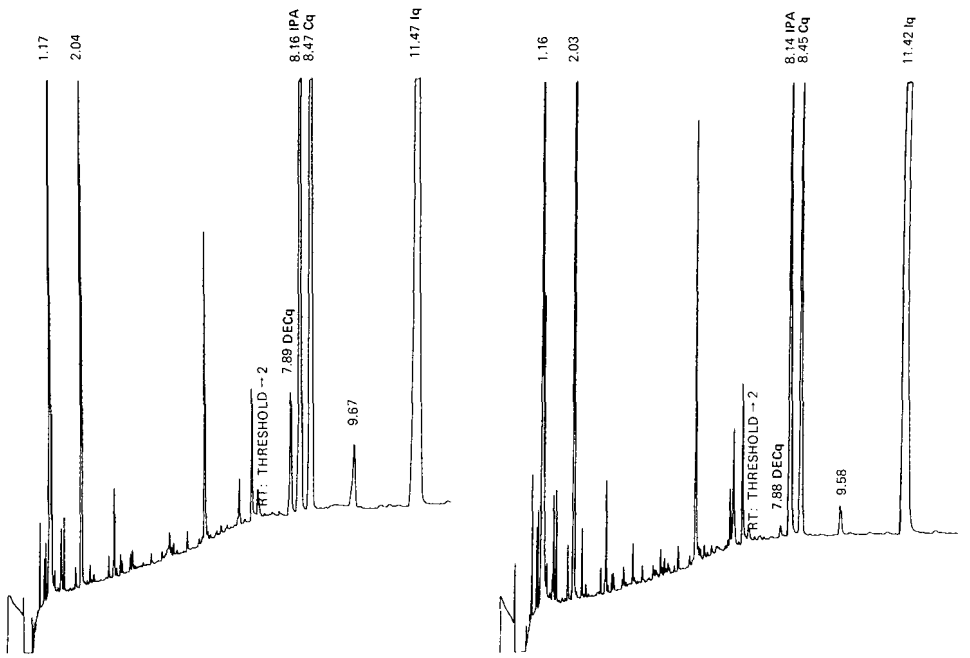

Fig. 3. Chromatograms resulting from the application of Method A to a blood standard and a serum sample. Note the efficiency and lack of activity of the column exhibited by these chromatograms. The left trace corresponds to a blood standard containing 247.7 ng of chloroquine, 359.2 ng of desethylchloroquine, and internal standard compounds. The right trace corresponds to a serum sample found to contain 77.2 ng/ml of chloroquine. Because of a marginally higher flow-rate, retention times are slightly shorter than those in Figs. 1 and 2. Abbreviations are as in Fig. 2.

should be emphasized that the same standards were carried first through Method A and then through Method B so that all corresponding values are directly comparable. Compound IVa is central to the tandem methodology in that it serves as a carrier desorber and improves chloroquine chromatography in Method A and then serves as an analogue internal standard for desethylchloroquine in Method B.

Data in Table II illustrate excellent linearity for the desethylchloroquine determination. Four replicate injections at the 359.2 ng/ml standard level show that repeatability of the chromatography is excellent. The relative standard deviation found for four standards at the 359.2 ng/ml level was 4.17%. It is to be expected that the precision would be less for this more basic, more readily adsorbed analyte than for chloroquine. The detection limit is 15 ng/ml of desethylchloroquine in blood.

Fig. 2. Chromatograms resulting from the application of Method A and Method B to standards. Upper chromatograms correspond to a standard containing 247.7 ng/ml of chloroquine (Cq) and 359.2 ng/ml of desethylchloroquine (DECq) in blood together with added internal standards. Lower chromatograms correspond to a blank sample containing added internal standards only. Left chromatograms result from Method A, right chromatograms from Method B. Peaks: IPA = isopropyl analogue of desethylchloroquine; Iq = iodoquine; and PFP = the pentafluoropropionyl derivative of a given compound.

TABLE I

STANDARD CURVE DATA FOR DETERMINING CHLOROQUINE IN BLOOD BY METHOD A AND METHOD B

Cq added x (ng)	Method A		Method B	
	Cq/internal standard peak area ratio	Cq calculated* x' (ng)	Cq/internal standard peak area ratio	Cq calculated*** x' (ng)
0.0	0.0103	7.00	0.0000	−2.20
99.1	0.2215	99.4	0.2253	98.6
198.2	0.4278	189.6	0.4587	203.1
247.7	0.5422	239.6**	0.5615	249.1§
	0.5634	248.9	0.5536	245.6
	0.5425	239.8	0.5468	242.6
	0.5445	240.6	0.5522	245.0
495.4	1.1456	503.5	1.1144	496.6
743.1	1.6904	741.7	1.6620	741.7

*Calculated from the least-squares line, $y=mx + b$ (m = 0.002287; b = −0.005710); r_6^2 = 0.99942.
**R.S.D. (interstandard, n = 4) =1.84%.
***Calculated from the least-squares line, $y=mx + b$ (m = 0.002234; b = 0.004914); r_6^2 = 0.99990.
§ R.S.D. (interstandard, n = 4) = 1.09%; R.S.D. (intrastandard, 4 injections) = 0.94%.

TABLE II

STANDARD CURVE DATA FOR DETERMINING DESETHYLCHLOROQUINE (DECq) IN BLOOD BY METHOD B

DECq added x (ng)	DECq/internal standard peak area ratio	DECq calculated* x' (ng)
0.0	0.0000	1.37
119.7	0.1275	125.0
239.4	0.2380	232.2
359.2	0.3681	358.4**
	0.3634	353.9
	0.3848	374.6
	0.3476	338.5
478.9	0.4911	477.8
598.6	0.6202	603.0

*Calculated from the least-squares line, $y=mx + b$ (m = 0.001031; b = −0.001409); r_6^2 = 0.99956.
**R.S.D. (interstandard, n=4) = 4.17%; R.S.D. (intrastandard, 4 injections) = 0.63%.

Should studies indicate that chloroquine is, in general, substantially more effective than its metabolite against malaria parasites, the very precise blood-level value for chloroquine rapidly available using Method A might well provide sufficient information for most purposes. In cases where confirmation of chloroquine presence and quantity and/or measurement of desethylchloroquine levels is of interest, Method B provides values with excellent precision.

The precision of the analytical results within the concentration range of application of Methods A and B is better than most, if not all, chloroquine assays published to date. This combination of methods employs a separate close-analogue internal standard for each of the two analytes so that compensation during prechromatographic steps is highly effective. The precision for replicate injections of a given standard demonstrates that the structural similarities between internal standards and analytes together with the inert chromatographic system conditions provide a high order of internal compensation during chromatography.

Application of the methodology to clinical studies

The demonstration of an appreciable chloroquine level in the serum of a patient who contracted falciparum malaria while under chloroquine chemoprophylaxis (Fig. 3) showed, in concert with other evidence, that the parasites causing the disease were indeed chloroquine resistant. In a volunteer study, the percentage of desethylchloroquine relative to total determined, chloroquine-derived species in the subject increased substantially over the first eight weeks of a chemoprophylactic regimen. In general, however, the quantity of chloroquine in the blood at each of the observed time intervals subsequent to weekly tablet ingestion increased over the eight-week period. The data are summarized in Table III.

In summary, the methodology described above provides a valuable set of tools for continuing studies of chloroquine chemoprophylaxis and chemotherapy, particularly as they apply to chloroquine-resistant strains of *Plasmodium falciparum*.

TABLE III

WHOLE BLOOD CONCENTRATIONS OF CHLOROQUINE (Cq) AND DESETHYLCHLOROQUINE (DECq) AT INTERVALS DURING THE FIRST EIGHT WEEKS OF A CHLOROQUINE DIPHOSPHATE CHEMOPROPHYLACTIC REGIMEN (5 mg/kg AS BASE/WEEK)

Week	Time (h)	Cq (ng/ml)	DECq (ng/ml)	$\frac{\text{DECq (100)}}{\text{Cq + DECq}}$ (%)
1	8	343	57.6	14.4
	24	243	54.0	18.2
	72	131	15.5	10.6
	168	61	6.8	10.0
3	8	586	286	32.8
	24	352	198	36.0
	72	213	110	34.1
	168	122	90.5	42.6
8	8	631	377	37.4
	24	349	269	43.5
	72	226	193	46.1
	168	164	119	42.0

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REFERENCES

1 J.L. Holtzman, Anal. Biochem., 13 (1965) 66.
2 L. Kazyak, in I. Sunshine (Editor), Manual of Analytical Toxicology, CRC Press, Cleveland, OH, 1971, pp. 86—88.
3 C.W. Vogel and E. Konigk, Tropenmed. Parasit., 26 (1975) 278.
4 A. Viala, J.P. Cano and A. Durand, J. Chromatogr., 111 (1975) 299.
5 M.A. Staiger, P. Nguyen-Dinh and F.C. Churchill, II, J. Chromatogr., 225 (1981) 139.
6 Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119.
7 M. Bonini, F. Mokofio and S. Barazi, J. Chromatogr., 224 (1981) 332.
8 A. Viala, E. Deturmeny, M. Estadieu, A. Durand and J.P. Cano, J. Chromatogr., 224 (1981) 503.
9 Y. Bergqvist and S. Eckerbom, J. Chromatogr., 226 (1981) 91.
10 G. Alván, L. Ekman and B. Lindström, J. Chromatogr., 229 (1982) 241.
11 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 229 (1982) 248.
12 F.C. Churchill, II and M.A. Staiger, Analytical Division, 183rd National Meeting of the American Chemical Society, Las Vegas, Nevada, March 31, 1982, Abstract No. 92.
13 H. Brötell, J. Chromatogr., 196 (1980) 489.

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CHROMBIO. 1608

QUANTITATIVE ANALYSIS OF MELPERONE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED ION MONITORING

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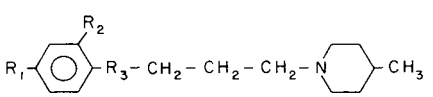
SUMMARY

A sensitive, specific and reliable assay utilizing a combined gas chromatography—mass spectrometry—selected ion monitoring technique is described for the quantitative determination of melperone, a neuroleptic agent, in human plasma. Using a 2-ml plasma sample, the method is sensitive and has acceptable precision in the range of 1 to 100 ng/ml. The method is applied in a limited bioavailability study and found to be adequate. A plasma metabolite was isolated and identified in the clinical samples.

INTRODUCTION

Melperone, 1-(4-fluorophenyl)-4-(4-methyl-1-piperidinyl)-1-butanone (I) (Fig. 1) is an experimental butyrophenone that showed a wide spectrum of neuroleptic properties [1]. Preliminary clinical trials indicated that it is particularly effective in the treatments of senile dementia [2—6]. In order to fa-



melperone (I) $R_1 = F$; $R_2 = H$; $R_3 = C{=}O$

internal standard (II) $R_1 = H$; $R_2 = Cl$; $R_3 = C{=}O$

alcohol metabolite (III) $R_1 = F$; $R_2 = H$; $R_3 = CHOH$

Fig. 1. Chemical structure of melperone, internal standard and alcohol metabolite.

cilitate the studies of bioavailability and pharmacokinetics of this compound, a sensitive and reliable analytical method is needed to measure plasma concentrations of melperone. Although there have been quite a few published assays for measuring plasma butyrophenone levels [7—9], these methods could not be applied directly to measure melperone due to the fact that there is only one halogen atom in the molecule which affords insufficient sensitivity for the gas chromatographic electron-capture detection. The present paper describes a fast and reliable assay, using a combined gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM) technique and demonstrates sensitivity down to the low nanogram range using 2 ml of plasma sample.

EXPERIMENTAL

Glassware treatment

All glassware used in this assay was cleaned by normal laboratory procedures. They were then rinsed with reagent-grade acetone and air dried prior to use.

Reagents and chemicals

Hexane, methanol and acetone were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Reagent-grade sodium hydroxide and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Melperone (I), internal standard (II, MDL 17,673) and the alcohol metabolite (III, MDL 18,664) (Fig. 1) were obtained from Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). [^{14}C]-Melperone was obtained from A.B. Ferrosan (Malmö, Sweden).

Preparation of standard curve

Standard solutions of I were prepared in methanol and an appropriate amount of 0.1 ml was added to 2 ml of drug-free human plasma, capped and frozen until analyzed.

Validation study

To test the precision and accuracy of the assay a four-day validation study was carried out. A nine-point standard curve in duplicate together with ten duplicate unknown spiked plasma samples were analyzed each day.

Feasibility study

To investigate the sensitivity and specificity of the assay it was applied to a human bioavailability study. Four healthy male volunteers were given a single oral dose of melperone. Two volunteers received a 25-mg melperone tablet and the other two received three 25-mg melperone tablets. Predose plasma and twelve post-dose plasma samples were collected from each subject and blind coded before it was sent to the analyst. The analyst was unaware of the dose or sample times. The code was not broken until all samples were analyzed.

Extraction procedure

Standard and unknown plasma samples were carried through the following extraction procedure: In a 25-ml screw cap extraction tube, 0.1 ml (70 ng) internal standard solution in methanol, 0.5 ml 1.5 *N* sodium hydroxide and 11 ml hexane were added to a 2.0-ml sample. The compounds were extracted into the hexane by mixing for 20 min on a horizontal reciprocating shaker. After centrifuging at ca. 600 *g* for 10 min, 10 ml of the hexane phase were transferred to another 25-ml extraction tube containing 2 ml 0.5 *N* hydrochloric acid. The phases were mixed for 20 min then centrifuged for 10 min. The hexane phase was removed by aspiration and discarded. After adding 1 ml 1.5 *N* sodium hydroxide and 11 ml hexane, the phases were mixed for 20 min, then centrifuged for 10 min. A 9-ml aliquot of the hexane phase was transferred to a 15-ml conical tube which was then placed in a heating block set at 50—55°C. The solvent was evaporated to dryness under a slow stream of nitrogen gas. The residue was dissolved in 100 μl of acetone and 5 μl of the resulting solution was taken for GC—MS analysis.

Instrumentation

All analyses were performed on a Finnigan 3300 mass spectrometer coupled with a Finnigan 9500 gas chromatograph. A 1.5 mm × 2 mm I.D. glass column was packed with 5% SE-30 on Chromosorb W HP (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.). To reduce peak tailing, the column was first base loaded with 10 μl of a 25% tetraethylene pentamine solution in methanol, and one base loading would last about two weeks. The column oven temperature was set at 190°C. The injector port temperature was 230°C. Helium was used as the carrier gas and the flow-rate was adjusted so that the column head pressure gave a reading of 1.7 bar. The mass spectrometer was operated in the electron impact (EI) mode and the ion selected for monitoring was the base peak of both melperone and internal standard II at *m/z* 112 (Fig. 2). The signal was connected to a Hewlett-Packard strip chart recorder, Model 7127A. The recorder sensitivity was set at 0.5 V. Emission current was set at 0.5 mA with an electron energy source of 30 eV. The electron multiplier was set at 20 kV and the preamp sensitivity set at $1 \cdot 10^{-8}$ A for full scale deflection.

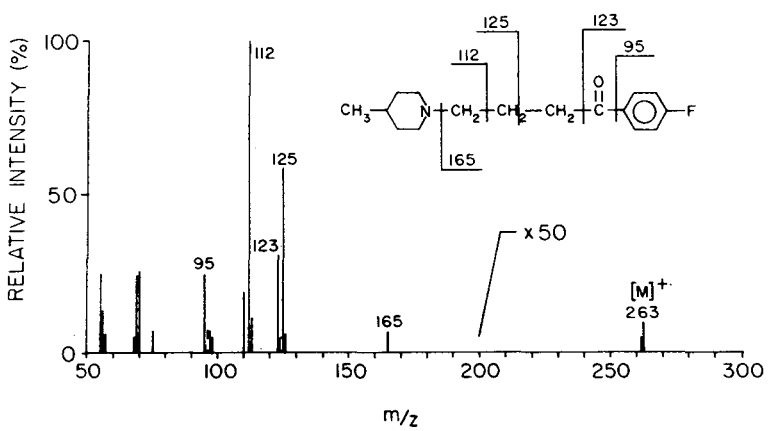


Fig. 2. EI mass spectrum of melperone.

Calibration and calculation

The peak height ratios (expressed as percent) of melperone divided by the internal standard (II) were plotted against melperone concentrations. The peak height ratio of each unknown was applied to this curve to determine the concentration. Alternatively the equation of the line was found by linear regression and the concentration of the unknown calculated.

RESULTS AND DISCUSSION

Extraction efficiency

Using the extraction procedure described above, the extraction efficiency of melperone from plasma at 100 ng/ml was 78.5% by using [^{14}C]-melperone.

GC–MS considerations

Under the GC conditions described above, the retention time for melperone was about 2.4 min and for the internal standard was 4.1 min. Samples could be injected every 6 min. There was no interfering peak extracted from the control drug-free plasma. Fig. 3 shows some typical chromatograms.

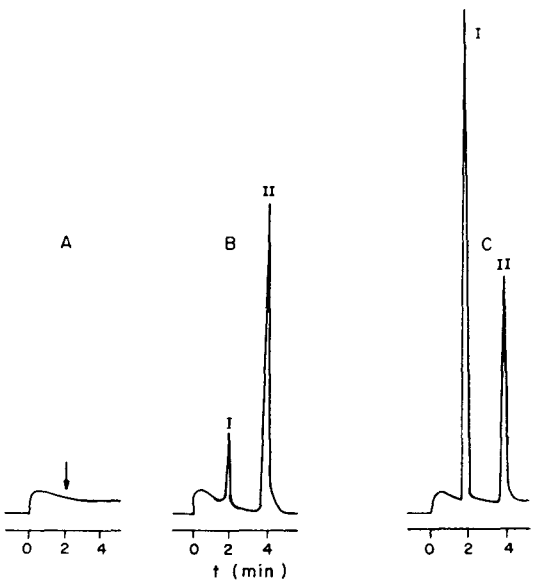


Fig. 3. Chromatograms of extracted plasma standard: (A) blank (↓ is where melperone emerges), (B) 5 ng/ml, (C) 50 ng/ml. Peaks: I = melperone, II = internal standard.

Precision and accuracy

The mean percent peak height ratios for the four-days validation study are tabulated in Table I. The calibration was linear over the entire range, as evidenced by a linear regression analysis of 0.9994 for correlation coefficiency. The slopes of the lines for the four days remained practically constant at 3.259 ± 0.0477 (mean ± standard deviation).

The precision and accuracy of the method were demonstrated by analyzing 40 unknown spiked plasma samples in a random coded fashion. The

TABLE I

PERCENT PEAK HEIGHT RATIO OF MELPERONE/INTERNAL STANDARD

Concentration (ng/ml)	Mean response ± S.D. (n = 8)
1	5.3 ± 1.2
2.5	9.1 ± 1.0
5.0	17.8 ± 1.7
10	34.2 ± 1.3
25	79.1 ± 3.0
50	160.6 ± 8.3
75	244.2 ± 5.5
100	300.0 ± 9.9

TABLE II

ANALYSIS OF CODED UNKNOWN PLASMA SAMPLES

Added	n	Found (ng/ml) (mean ± S.D.)	Recovery (%)
0	4	0 ± 0	—
2.5	6	3.0 ± 0.5	120
10	6	10.7 ± 1.4	107
15	6	15.3 ± 1.1	102
50	6	49.7 ± 2.5	99.4
75	6	73.4 ± 3.7	97.9
100	6	101.8 ± 4.4	101.8

results are shown in Table II. The relative standard deviations ranged between 4.3% (100 ng/ml added) to 17% (2.5 ng/ml added). Recoveries ranged from 97.9% (75 ng/ml added) to 120% (2.5 ng/ml) with a mean recovery of 105% (± 8% S.D.) across all concentrations, excluding 0 ng/ml. Blank samples all gave 0 ng/ml.

Clinical samples

The plasma concentrations of melperone following a single 25- or 75-mg dose to human subjects are plotted in Fig. 4.

Plasma metabolite

During the course of analyzing human clinical samples, it was noticed that there was a drug-related peak that had a retention time of 2.6 min (Fig. 5). The sizes of this peak corresponded closely with the concentrations of melperone. Since detection was based on monitoring the ion at m/z 112, this component must contain an intact piperidine ring; and the GC retention time suggested that the unknown compound was closely related to melperone in molecular weight and structure. Therefore it was assumed that the unknown metabolite could quite possibly be the secondary alcohol that would result from the reduction of the ketone group of melperone. Com-

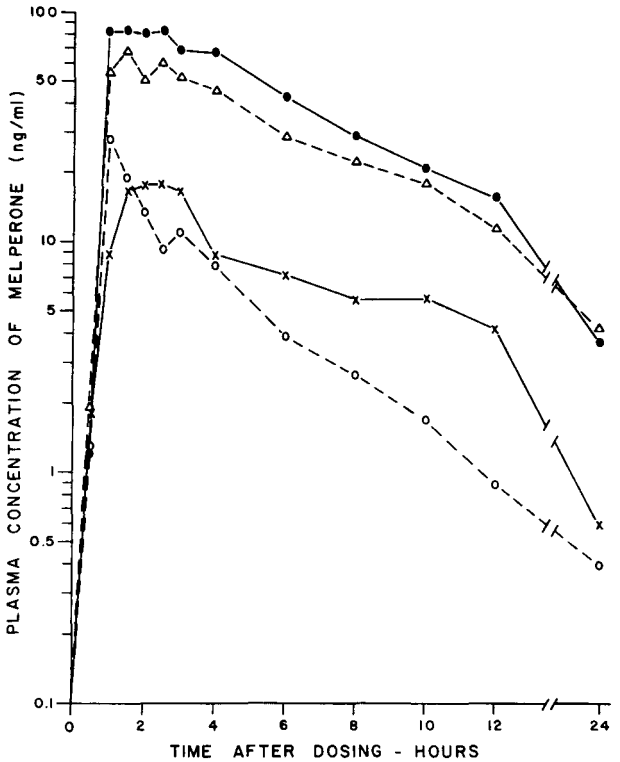


Fig. 4. Plasma concentration of melperone in four subjects following either a 25- or 75-mg oral dose. (●—●, Subject 1 and △— —△, subject 2; 75 mg), (×—×, subject 3 and ○— —○, subject 4; 25 mg).

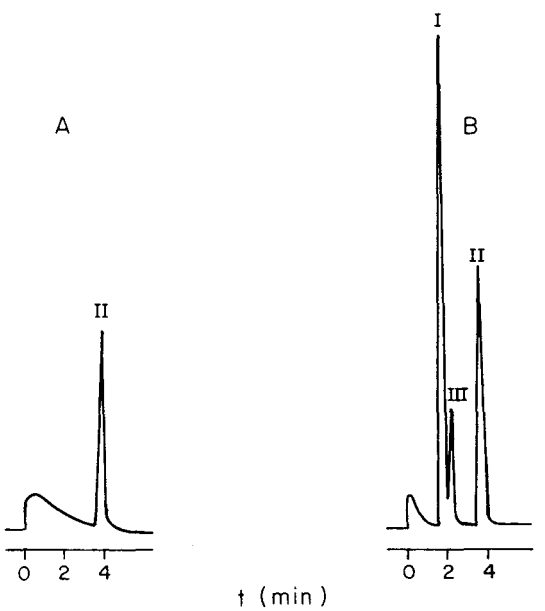


Fig. 5. GC—MS chromatograms from subject 2: (A) predose sample, (B) 2.5-h post-dose sample. Peaks: I = melperone, II = internal standard, III = alcohol metabolite.

paring the reference alcohol (III) with the plasma metabolite confirmed that they had identical GC retention times. By GC—MS—SIM analysis of the ions at *m/z* 112, 125 and 265 it was indicated that the structure of this plasma metabolite was most likely the reduced alcohol of melperone. The alcohol III has been identified as a human urinary metabolite [10].

In conclusion, the results indicated that the GC—MS—SIM assay of melperone in human plasma is a fast, sensitive and reliable method. Besides its use in studying the bioavailability of the parent compound, it could also be used to monitor the alcohol metabolite level in the plasma sample.

REFERENCES

1 J.A. Christensen, S. Hernestam, J.B. Lassen and N. Sterner, Acta Pharmacol. Toxicol., (KBH) 23 (1965) 109.
2 E. Engstrand, Nord. Psykiatr. Tidsskr., 21 (1967) 234.
3 A. Norgard, P. Tingsgaard and I.L. Nillsen, Nord. Psykiatr. Tidsskr., 21 (1967) 240.
4 H. Simmelsgaard, M. Mellergaard and A. Vangtorp, Nord. Med., 77 (1967) 221.
5 J. Beck-Nielsen and J. Clausen, Nord. Med., 83 (1970) 659.
6 P. Viskam, Nord. Med., 84 (1970) 1298.
7 I.A. Zingales, J. Chromatogr., 54 (1971) 15.
8 F. Marcucci, L. Airoldi, E. Mussini and S. Garattini, J. Chromatogr., 59 (1971) 174.
9 A. Forsman, E. Martensson, G. Nyberg and R. Ohman, Naunyn Schmiedeberg's Arch. Pharmacol., 286 (1974) 113.
10 F.A. Wiesel, L. Bjerkenstedt and P. Skett, Acta Pharmacol. Toxicol., 43 (1978) 129.

Journal of Chromatography, 274 (1983) 129—138
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GAS—LIQUID CHROMATOGRAPHIC RESOLUTION AND ASSAY OF TOCAINIDE ENANTIOMERS USING A CHIRAL CAPILLARY COLUMN AND STUDY OF THEIR SELECTIVE DISPOSITION IN MAN

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SUMMARY

Tocainide is a new antiarrhythmic agent that is used clinically as the racemic mixture. In order to study the disposition of the individual enantiomers in man, a gas—liquid chromatographic assay was developed based on the resolution of the *R*-(—)- and *S*-(+)-enantiomers as their heptafluorobutyryl derivatives on a capillary column coated with a chiral stationary phase. Two healthy male volunteers ingested an oral solution dose of racemic tocainide hydrochloride at a dose of 3 mg/kg and plasma and urine were collected at intervals for up to 54 h. Analysis of the plasma samples revealed a stereoselective disappearance of the *R*-(—)-enantiomer, such that the apparent half-life for the *S*-(+)- and *R*-(—)-enantiomers were 25.6 and 20.5 h, respectively in one subject and 11.1 and 9.0 h in the second subject. Similar relationships were observed in urine, where the ratio of the *S*-(+)- to *R*-(—)-enantiomers varied from 0.98 in 1 h to 3.03 in the 54-h samples in one subject over the same time period.

INTRODUCTION

Tocainide, 2-amino-2′,6′-propionoxylidide (I), is a relatively new antiarrhythmic agent presently undergoing clinical trials in Canada and the United States. Tocainide is a structural analogue of lidocaine (II), but unlike the latter agent is effective by the oral route. Several studies [1—4] have reported the pharmacokinetic parameters of tocainide in humans and have clearly demonstrated its high bioavailability by the oral route (90—100%), its rapid peak plasma levels (90 min) and its comparatively long apparent elimination half-life ranging from 11.7 to 29.1 h among subjects. Tocainide has been reported [4, 5] to undergo a novel biotransformation to tocainide carbamoyl-O-β-D-glucuronide (III) and 2-hydroxy-2′,6′-propionoxylidide (IV). Urinary excretion of the unchanged drug varies between 28—55% after an oral dose [4].

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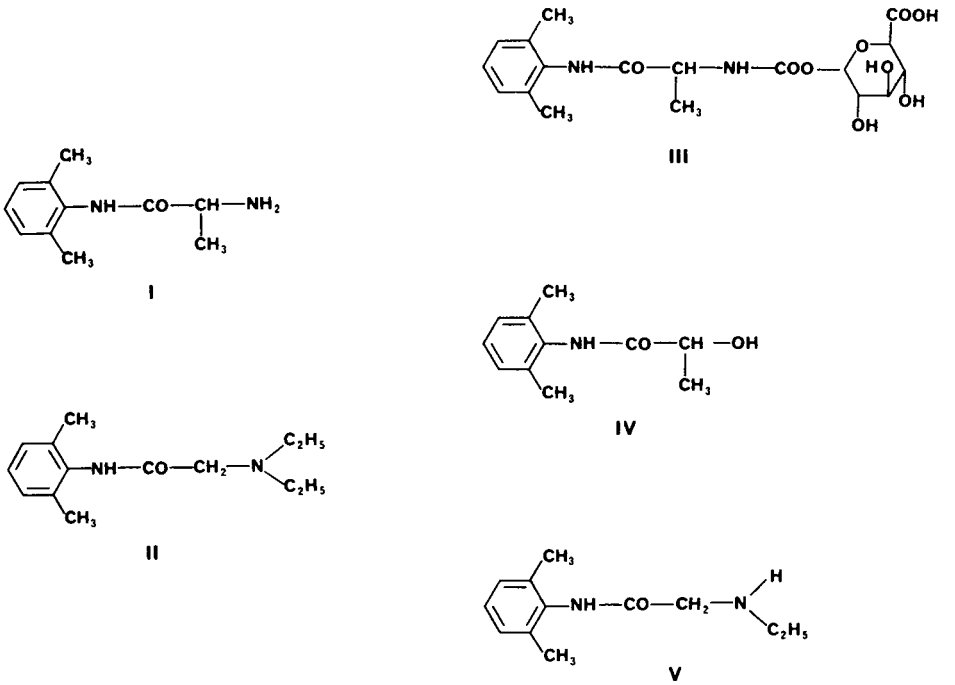

Tocainide has a chiral centre and is used therapeutically as the racemic mixture. To date, the antiarrhythmic activity of the individual *R*-(—)- and *S*-(+)-enantiomers have only been tested in dogs and mice [6]. The results indicated that the *S*-(+)-isomer exhibits higher antiarrhythmic activity than its antipode. The possibility that the two enantiomers exhibit different pharmacokinetic parameters has been investigated in rats and mice [7] and the findings indicate that a preferential excretion of one isomer occurs, and that this is species dependent. In a preliminary study [8] conducted on two human subjects, plasma level ratios of *R*- and *S*-tocainide were found to differ in one of the volunteers. Unfortunately, the levels of the two enantiomers were only measured at 2 h and at 3 days following drug administration; hence firm conclusions could not be drawn on the stereoselective disposition of tocainide enantiomers in humans.

The present study was therefore undertaken to determine if sequential sampling of the absorption, distribution and elimination phases of tocainide enantiomers would reveal distinct differences in the ratios of these enantiomers in plasma and urine after oral administration of the racemic drug to human volunteers.

EXPERIMENTAL

Materials

R,S-Tocainide hydrochloride (Astra Pharmaceuticals, Mississauga, Canada), 2-ethylamino-2′,6′-acetoxylidide hydrochloride (Astra Pharmaceuticals Products, Worcester, MA, U.S.A.), carbobenzyloxy-D-alanine, N-*tert.*-butoxycarbonyl-L-alanine (Sigma, St. Louis, MO, U.S.A.), 2,6-dimethylaniline, N,N′-

dicyclohexylcarbodiimide, (+)-di-*p*-toluoyl-*d*-tartaric acid monohydrate, 30—32% hydrogen bromide in acetic acid (Aldrich, Milwaukee, WI, U.S.A.) and heptafluorobutyric anhydride (Pierce, Rockford, IL, U.S.A.) were used without further purification. Water and *n*-hexane were of HPLC grade (Fisher Scientific, Vancouver, Canada) and dichloromethane and benzene were distilled in glass quality (Caledon, Georgetown, Canada). All other chemicals and reagents were of analytical quality. Supplies required for blood collection were obtained from Becton-Dickinson and Co. (Mississauga, Canada).

Melting points were determined on a capillary tube melting point apparatus, Model 6406 (Thomas Hoover, Philadelphia, PA, U.S.A.) and are uncorrected. Optical rotation studies were conducted on a Perkin-Elmer Model 142 polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) in a 10-cm tube at 25°C.

All analyses were carried out on a Model 5830A reporting gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with split/splitless injection modes and a ^{63}Ni electron-capture detector operated in the pulsed mode (150 μsec). Helium carrier gas flow-rate was maintained through the capillary columns at 1 ml/min with 25 ml/min passing through the split vent. Argon—methane (95:5) was used as the make-up gas at the detector at a flow-rate of 60 ml/min. The injection port and detector temperatures were 240°C and 350°C, respectively and the oven was operated isothermally at the conditions required for each assay procedure.

The 50 m × 0.2 mm fused silica capillary column coated with Carbowax 20M was prepared in the laboratory by a procedure reported earlier [9]. The oven temperature for this column was maintained at 180°C and samples were introduced in the split injection mode of the gas chromatograph.

The Chirasil-Val® 25 m × 0.25 mm borosilicate capillary column (Applied Science, State College, PA, U.S.A.) was operated at an oven temperature of 180°C for urine samples and 183°C for plasma samples. The latter temperature was found useful to facilitate the resolution of a small peak due to an endogenous substance in plasma from the two peaks for the enantiomers of tocainide.

Synthesis of R-(—)-tocainide hydrochloride

To a solution of 13.38 g (0.06 mole) of carbobenzyloxy-D-alanine and 7.3 g (0.06 mole) of 2,6-dimethylaniline in 150 ml of dichloromethane were added 13.6 g (0.066 mole) of N,N′-dicyclohexylcarbodiimide in 60 ml of dichloromethane. After the solution was left standing at room temperature for 1 h, the precipitated N,N′-dicyclohexylurea was filtered off and the solvent was evaporated from the filtrate under reduced pressure leaving 12.3 g of white solid, m.p. 167—168°C.

To remove the carbobenzyloxy-group, 70 ml of a solution of 30—32% hydrogen bromide in acetic acid were added to 12.3 g of the above reaction product, and the mixture was stirred until dissolved. To this solution, 200 ml of dry diethyl ether were added and the precipitated tocainide hydrobromide was filtered off and dried, yielding 8.2 g of white solid, m.p. 267°C. This material was converted to a hydrochloride salt and recrystallized from ethanol—diethyl ether to yield *R*-(—)-tocainide hydrochloride, m.p. 265—266°C, and $[\alpha]_D$ —42.16° (*c*, 2.63 in methanol). Literature [6] gave $[\alpha]_D$ —44.1° (*c*,

2.63 in methanol). A sample of this material was converted to its base and reacted with heptafluorobutyric anhydride as described below. Evaluation of the enantiomeric ratio using the Chirasil-Val column indicated that this material had a 95:5 ratio of the *R*:*S* enantiomers.

Synthesis of S-(+)-tocainide hydrochloride

To a solution of 18.9 g (0.1 mole) of N-*tert.*-butoxycarbonyl-L-alanine and 12.1 g (0.1 mole) of 2,6-dimethylaniline in 200 ml of dichloromethane were added 20.6 g (0.1 mole) of N,N'-dicyclohexylcarbodiimide. After the mixture was stirred at room temperature for 1 h, the precipitated N,N'-dicyclohexylurea was filtered off and the solvent was evaporated from the filtrate under reduced pressure, leaving 16.1 g of a creamy white solid, m.p. 131°C.

To remove the N-*tert.*-butoxycarbonyl group, 50 ml of 30—32% hydrogen bromide in acetic acid was added to 10 g of the above reaction product and the mixture was stirred until dissolved. To this solution 200 ml of dry diethyl ether were added and the precipitated tocainide hydrobromide was filtered off and dried, yielding 7.8 g of white solid, m.p. 274°C. This material was converted to a hydrochloride salt and recyrstallized from ethanol—diethyl ether to yield *S*-(+)-tocainide hydrochloride, m.p. 266°C. A sample of this material was evaluated as its heptafluorobutyryl derivative on the Chirasil-Val column and was found to consist of a 81:19 ratio of the *S*:*R* enantiomers.

To increase the optical purity of this product, the tocainide base obtained from 1 g of the above material was added to a warm solution of 1.4 g of di-*p*-toluoyl-*d*-tartaric acid in 10 ml of 95% ethanol. The diastereoisomeric salt was crystallized five times at room temperature to yield fine needles. This material was converted to its hydrochloride salt and recrystallized from ethanol—diethyl ether to yield *S*-(+)-tocainide hydrochloride, m.p. 266°C, $[\alpha]_D$ +42.35° (*c.* 2.64 in methanol). Literature [6] gave m.p. 264.5—265.5°C, $[\alpha]_D$ +41.7° (*c.* 2.64 in methanol). An evaluation of the enantiomer ratio of the heptafluorobutyryl derivative on the Chirasil-Val column indicated a ratio of 91:9 of the *S*:*R* enantiomers.

Stock solutions

Racemic tocainide hydrochloride and the internal standard, 2-ethylamino-2',6'-acetoxylidide hydrochloride (V), were dissolved in HPLC grade water at concentrations of 1 μg/ml and 10 μg/ml, respectively.

In-vivo study

Two healthy male volunteers (38 years, 68 kg and 39 years, 98 kg) fasted overnight for 12 h and were administered 100 ml of an oral solution of racemic tocainide hydrochloride at a dose of 3 mg/kg body weight under the supervision of a physician. Food was allowed after 3 h and water was allowed ad libitum. An indwelling butterfly cannula was inserted in the cubital vein of the arm by a medical laboratory technologist and was used to collect blood samples for the first 7 h. Subsequent blood samples were collected in 10-ml heparinized Vacutainers®. Blood samples (approximately 8 ml) were collected at 15-min intervals for the first 2 h and at 3, 5, 7, 24, 48 and 72 h thereafter. The cannula was flushed with sterile isotonic heparin solution (50 units in 1 ml) after each

collection). Blood samples were centrifuged at 2300 *g* and the plasma was separated and stored at −20°C until required for assay. Urine samples were collected in polyethylene bags at 0, 1, 2, 3, 5 and 7 h and as convenient for the subject thereafter up to 54 h. Urine samples were stored at −20°C without treatment until required for assay. Samples of plasma and urine were obtained before drug ingestion to serve as blanks and for determination of calibration curves.

Gas chromatographic assay

To 0.5 ml of plasma or 0.1 ml of urine were added, 0.1 ml of the internal standard solution, 0.2 ml of 0.1 *N* sodium hydroxide solution and 5 ml of dichloromethane. The tubes were tumbled for 15 min on a Model 343 Roto-Rak tube tumbler (Fisher Scientific). After centrifugation at 1600 *g* for 10 min, 4 ml of the organic phase were transferred to a 10-ml PTFE-lined screw-capped culture tube and the contents were evaporated under a gentle stream of clean dry nitrogen which had been passed through a gas purifier, Model 451 cartridge (Matheson, Edmonton, Canada). The residue was dissolved in 100 μl of *n*-hexane, 20 μl of heptafluorobutyric anhydride were added and the tightly capped tubes were heated at 55°C for 30 min in an aluminum block (Thermolyne Dri-Bath, Fisher Scientific). The excess reagent and solvent were removed under a stream of clean dry nitrogen and the residue was reconstituted in 200 μl of *n*-hexane. A 1—2 μl aliquot was used for chromatographic assay on the Chirasil-Val column. The procedure was repeated for assay on the Carbowax column. The stability of the derivatives stored in hexane at 4°C were tested over four weeks and evidenced no detectable change in peak area ratios between enantiomers and their relationship to the internal standard.

Determination of calibration curve and detector linearity

To 0.5 ml of blank plasma were added 100, 250, 500, 750, 1000, 1500 ng of racemic tocainide hydrochloride contained in the stock solution and 0.1 ml of (1000 ng) the internal standard solution. In a similar fashion, 100, 200, 400, 1000, 2000 ng of the racemic tocainide hydrochloride stock solution were added to 0.1 ml of urine along with 0.1 ml of the internal standard solution. Triplicate samples of plasma and urine thus prepared, were extracted and derivatized as described above.

RESULTS AND DISCUSSION

Direct resolution of enantiomers of chiral amino compounds on optically active stationary phases offers several advantages compared to other methods which require the preparation of diastereoisomers by reaction with asymmetric reagents such as N-trifluoroacetyl-(*S*)-prolyl chloride [9,10] or (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride [7, 8]. In the latter methods, however, the optical purity of the resolving agent, its stability during synthesis, storage or derivatization, and volatility of the diastereoisomers thus formed, are frequent problems in the routine and accurate assay of enantiomeric amines by the use of such reagents. The readily prepared and stable monoheptafluorobutyryl derivative of tocainide enantiomers has excellent chromatographic

properties and the resulting isomer derivatives are well resolved on a chiral column coated with N-*tert.*-butyl-L-valine-*tert.*-butylamide. This chiral phase, also known as Chirasil-Val, has been reported to resolve *R,S*-mixtures of amino acids [11]. The mechanism of resolution of such optical isomers on this phase appears to be due to the transient formation of diastereoisomers in the column by hydrogen bonding [12].

In the resolution of the optical isomers of tocainide using the Chirasil-Val phase, the identities of the enantiomers were established by comparison with retention times of these enantiomers prepared by a stereoselective synthesis starting from *S*-(+)- or *R*-(—)-alanine based on the method originally described by Byrnes et al. [6]. The synthetic approach is similar to that used in peptide synthesis, and from examination of the resulting products by their optical rotation, and comparison with literature values [6], it was observed that the optical purity was retained to a large extent. Accordingly, the first peak to elute from the Chirasil-Val column was designated the *S*-(+)-enantiomer.

For purposes of analysis of human plasma and urine samples, detector linearity and assay precision were determined from calibration curves constructed from blank plasma and urine samples obtained from the two volunteers prior to ingestion of the drug. Accordingly, linearity was established over the range of 0.2 to 3 μg/ml of *R,S*-tocainide hydrochloride in plasma and from 1.0 to 20 μg/ml in urine. Each calibration curve was constructed from triplicate samples of each of five concentrations of *R,S*-tocainide hydrochloride. The data given in Table I show the mean slopes and correlation coefficients. The mean correlation coefficients for plasma and urine were 0.9998 and 0.9989, respectively.

TABLE I

CALIBRATION CURVE DATA FOR PLASMA AND URINE

Quantity of each tocainide enantiomer (ng)*	Plasma area ratios (± S.D.)**		Urine area ratios (± S.D.)**	
	S-(+)	*R*-(—)	*S*-(+)	*R*-(—)
50	0.0449 ± 0.0026	0.0383 ± 0.0075	0.0719 ± 0.0100	0.0487 ± 0.0116
100	—	—	0.0965 ± 0.0141	0.0965 ± 0.0141
125	0.1129 ± 0.0136	0.1048 ± 0.0150	—	—
200	—	—	0.2339 ± 0.0509	0.2092 ± 0.0573
250	0.2181 ± 0.0284	0.2088 ± 0.0273	—	—
375	0.3404 ± 0.0030	0.3115 ± 0.0074	—	—
500	0.4515 ± 0.0136	0.4110 ± 0.0165	0.6823 ± 0.0811	0.6318 ± 0.0789
750	0.6748 ± 0.0090	0.6121 ± 0.0080	—	—
1000	—	—	1.2987 ± 0.1444	1.2833 ± 0.1746
Correlation coefficient	0.9998	0.9998	0.9989	0.9996
Mean slope	0.9024	0.8171	1.3210	1.3161
Y-Intercept	—0.0012	0.0018	—0.0108	—0.0330

*Tocainide hydrochloride used as the racemate mixture.

**Area ratio determined for drug/internal standard. Standard deviations calculated for three aliquots for each weight of *R,S*-tocainide hydrochloride.

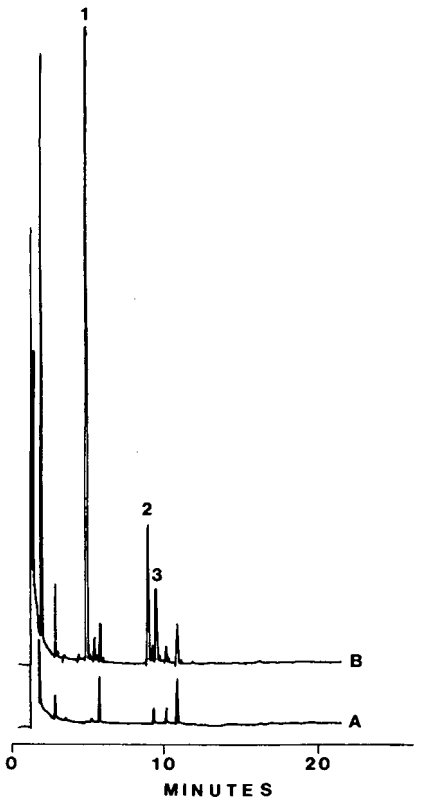

Fig. 1. B = Chromatogram of the heptafluorobutyryl derivatives of tocainide enantiomers extracted from plasma 24 h after oral administration of a racemic mixture. Peaks: 1 = internal standard; 2 = *S*-(+)-tocainide; 3 = *R*-(—)-tocainide. A = Blank plasma chromatogram.

TABLE II

PLASMA CONCENTRATION—TIME DATA

Concentrations are expressed in μg/ml.

Time (h)	Subject 1		Subject 2			
	Chiral column		Chiral column			Carbowax
	S(+)	*R*(—)	*S*(+)	*R*(—)	Total	*R,S*
0.25	0.585	0.536	0.080	0.083	0.163	0.281
0.50	0.842	0.741	0.287	0.220	0.507	0.565
0.75	0.802	0.728	0.372	0.317	0.689	0.613
1.00	0.793	0.708	0.308	0.248	0.556	0.563
1.50	0.768	0.670	0.425	0.345	0.770	0.897
1.75	0.781	0.696	0.355	0.301	0.656	0.657
2	0.844	0.742	0.403	0.352	0.755	0.519
3	0.629	0.538	0.368	0.325	0.693	0.545
5	0.618	0.508	0.300	0.206	0.506	0.545
7	0.481	0.375	0.289	0.252	0.541	0.533
24	0.234	0.125	0.176	0.118	0.294	0.270
48	0.071	0.028	0.113	0.076	0.189	0.137
72	—	—	0.049	0.032	0.081	0.086

The application of the method in human studies was shown by analysis of plasma and urine samples collected at intervals from two subjects given oral solutions of *R,S*-tocainide hydrochloride at a dose of 3 mg/kg. The chromatogram shown in Fig. 1 is representative of the isomer separation on the chiral phase and shows the differing enantiomer ratios seen in plasma at 24 h after the initial dose.

The data given in Table II for plasma concentrations, at each sample time, show the more rapid disappearance of the *R*-(—)-enantiomer in both subjects. In order to compare the values obtained on the chiral phase for the individual enantiomers to those obtained by analysis of the total racemate, samples were also analysed by the use of a Carbowax 20M capillary column. This procedure had earlier been found [13] to be suitable for the analysis of racemic tocainide in rat plasma and urine. It is also found equally suitable for human studies. Accordingly, the same plasma samples from one subject

TABLE III

APPARENT PLASMA HALF-LIFE (h) OF *S*-(+)-, *R*-(—)- AND *R,S*-(±)-TOCAINIDE IN TWO VOLUNTEERS

	Subject 1	Subject 2
R,S-(±)-Tocainide	22.1	9.2
S-(+)-Tocainide	25.6	11.1
R-(—)-Tocainide	20.5	9.0

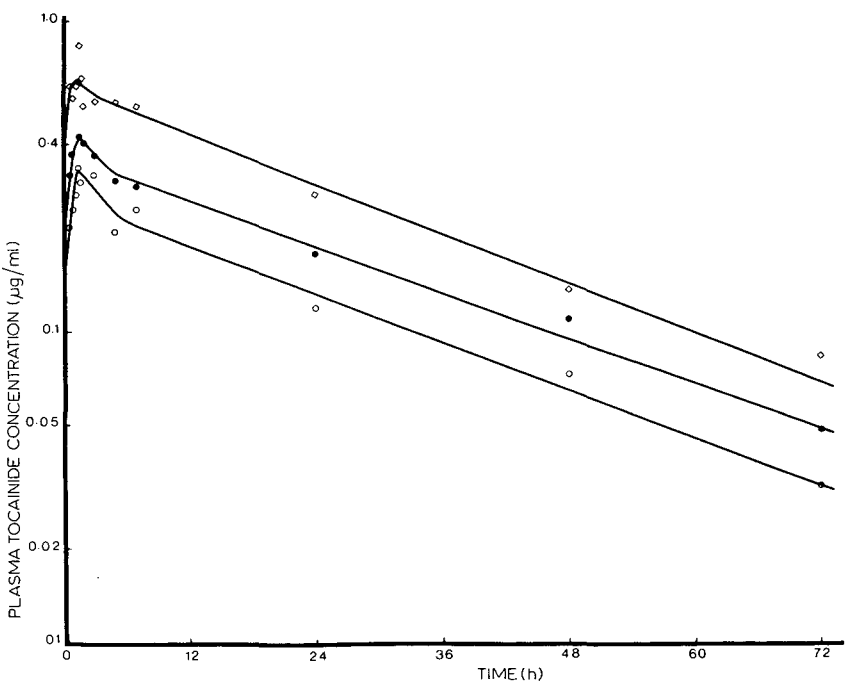

Fig. 2. Log plasma concentration—time curves for tocainide enantiomers and racemic mixture. (▫—▫) racemate; (●—●) *S*-(+)-tocainide; (○—○) *R*-(—)-tocainide.

TABLE IV

QUANTITIES OF *S*-(+)- AND *R*-(—)-TOCAINIDE EXCRETED IN URINE

Quantities are given in μg/ml of urine.

Time (h)	Subject 1			Subject 2		
	(*S*)-(+)	(*R*)-(—)	Ratio *S*/*R*	(*S*)-(+)	(*R*)-(—)	Ratio *S*/*R*
1	6.29	6.30	0.99	5.72	5.83	0.98
2	6.74	6.53	1.06	16.35	14.77	1.12
3	3.73	3.61	1.09	11.48	10.62	1.14
5	6.63	6.17	1.10	73.72	63.6	1.15
7	7.93	7.33	1.11	60.89	53.5	1.17
14			—	28.76	23.23	1.27
15	4.70	4.27	1.11			—
22	14.36	11.8	1.24			—
24	—	—	—	10.66	7.75	1.45
30	—	—	—	63.53	32.66	1.94
31	5.22	3.68	1.50			—
38	—	—	—	10.28	6.34	1.62
42	1.90	1.22	1.88			—
48	18.16	9.61	1.88	10.21	4.62	2.36
54	4.56	2.60	1.91	14.48	4.96	3.03

were assayed by the two procedures. The data for the analysis using Carbowax 20M are included in Table II. It can be observed that the totals for the individual *R*- and *S*-enantiomers observed on the chiral phase are in close agreement with racemate of tocainide eluting from the Carbowax phase. The apparent plasma half-lives ($t_{1/2}$) for the terminal elimination phase of the two enantiomers were calculated by an iterative non-linear least squares regression computer program [14] and are summarized in Table III along with the apparent $t_{1/2}$ of the total racemate. The two subjects were noted to exhibit significantly different $t_{1/2}$ values for the two enantiomers, however, in both cases the *R*-(—)-enantiomer of tocainide is eliminated from plasma at a faster rate. The log plasma concentration—time curves obtained from one subject for the individual enantiomers as well as the racemate are presented graphically in Fig. 2. The values for the apparent $t_{1/2}$ for the racemate in both subjects lies between that observed for the individual enantiomers. This would be anticipated since the apparent $t_{1/2}$ for the racemate is made up by contributions for each enantiomer at every data point.

The urinary excretion of *R*- and *S*-tocainide was also studied and as shown in Table IV, the two subjects evidenced more rapid excretion of the *S*-(+)-enantiomer. The higher levels of *S*-(+)-tocainide (up to 3.03 times the *R*-(—)-enantiomer at 54 h in one subject) are likely due to a more rapid metabolism of the *R*-(—)-enantiomer. This is consistent with the plasma data, which showed a more rapid disappearance of the *R*-(—)-enantiomer.

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REFERENCES

1 D.G. McDevitt, A.S. Nies, G.R. Wilkinson, R.F. Smith, R.L. Woosley and J.A. Oates, Clin. Pharmacol. Ther., 19 (1976) 396.
2 D. Lalka, M.B. Meyer, B.R. Duce and A.T. Elvin, Clin. Pharmacol. Ther., 19 (1976) 759.
3 C. Graffner, T.B. Conradson, S. Hofvendahl and L. Ryden, Clin. Pharmacol. Ther., 27 (1980) 64.
4 A.T. Elvin, J.B. Keenaghan, E.W. Byrnes, P.A. Tenthorey, P.D. McMaster, B.H. Takman, D. Lalka, C.V. Manion, D.T. Baer, E.M. Wolshin, M.B. Meyer and R.A. Ronfeld, J. Pharm. Sci., 69 (1980) 47.
5 R.A. Ronfeld, E.W. Wolshin and A.J. Block, Clin. Pharmacol. Ther., 27 (1980) 282.
6 E.W. Byrnes, P.D. McMaster, E.R. Smith, M.R. Blair, R.N. Boyes, B.R. Duce, H.S. Feldman, G.H. Kronberg, B.H. Takman and P.A. Tenthorey, J. Med. Chem., 22 (1979) 1171.
7 J. Gal and T.A. French, The Pharmacologist, 23 (1981) 196.
8 J. Gal, T.A. French, T. Zysset and P.E. Haroldsen, Drug Metab. Disp., 10 (1982) 399.
9 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109.
10 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643.
11 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr. Sci., 15 (1977) 174.
12 C.H. Lochmüller and R.W. Souter, J. Chromatogr., 113 (1975) 283.
13 G.K. Pillai, J.E. Axelson and K.M. McErlane, J. Chromatogr., 229 (1982) 103.
14 C.M. Metzler, G.L. Elfring and A.J. McEwan, Biometrics, 30 (1974) 562.

Journal of Chromatography, 274 (1983) 139—148
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GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF TOLOXATONE IN HUMAN PLASMA

ROUTINE ANALYSIS OF A WIDE RANGE OF DRUG CONCENTRATIONS USING A NITROGEN-SELECTIVE DETECTOR

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SUMMARY

A selective and sensitive gas—liquid chromatographic (GLC) method has been developed for the measurement of toloxatone at therapeutic concentrations in plasma. The technique is based on a single extraction from plasma at pH 10, the preparation of a trimethylsilyl derivative and detection by a nitrogen-selective detector. The traditional calibration curve, peak-area ratio of toloxatone to internal standard versus toloxatone plasma concentration, was slightly concave for the wide concentration considered (10—3000 ng/ml). As a consequence, the linear least-squares regression analysis gave a negative intercept on the *y*-axis which affected quantitation accuracy of low plasma concentration values. A calibration method taking into consideration the non-linearity of the calibration curve is proposed.

The characteristics of the detector were examined in order to analyse response linearity and sensitivity. A linear relationship was found between background current and detector sensitivity.

INTRODUCTION

Toloxatone, 5-(hydroxymethyl)-3-(3-methylphenyl)-2-oxazolidinone is a reversible inhibitor of type A monoamine oxidase both in vitro and ex vivo in the rat [1] and has been shown to possess an antidepressant activity in man [2—4]. Two thin-layer chromatographic (TLC) methods for the determination of toloxatone in plasma have been reported [5, 6]. In the first technique [5] [^{14}C]toloxatone was measured by liquid scintillation counting after TLC separation of the drug from metabolites. The technique was therefore unsuitable for studies with unlabelled drug. The second analytical method [6] was based on TLC of a dichloroethane—amyl alcohol plasma extract followed

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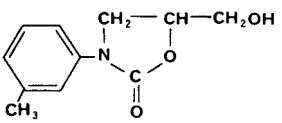
by densitometric quantitation. The detection limit of this method was 10 ng/ml with a coefficient of variation of 12% at a concentration of 250 ng/ml. This technique was time-consuming and required a large volume of plasma (5 ml).

The aim of the present investigation was to improve the precision and the sensitivity of the quantitative analysis of toloxatone in plasma using a simple gas—liquid chromatographic (GLC) method.

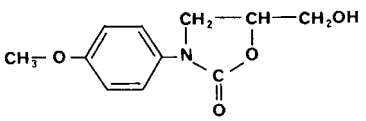
EXPERIMENTAL

Standards and reagents

Toloxatone and the internal standard MD700075, 5-(hydroxymethyl)-3-(4-methoxyphenyl)-2-oxazolidinone, were synthesized in the Chemistry Department of Delalande Research Centre. The chemical structures are given in Fig. 1. All reagents were of analytical grade purity. Toluene and ethyl acetate were RPE-ACS grade, and diethyl ether was RPE grade (Carlo Erba, Milan, Italy). Ethyl acetate was dried over anhydrous Na_2SO_4 (Merck, Darmstadt, G.F.R.); sodium hydroxide (Prolabo, Paris, France) and phenazine (Merck) were used. Trimethylsilyl derivatives were prepared with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Pierce, Rockford, IL, U.S.A.).



TOLOXATONE



MD 700075

Fig. 1. Structures of toloxatone and internal standard.

Gas—liquid chromatographic conditions

A 5880A Hewlett-Packard gas chromatograph (with a Model Level 4 Terminal) equipped with a nitrogen—phosphorus detector (NPD) was used. A glass column (2 m × 2 mm I.D.) was silanized and packed with 3% OV-101 coated on Supelcoport 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.). A second column of the same geometry was packed with 3% SP-2250 coated on Supelcoport 80—100 mesh (Supelco). Both columns were conditioned as follows: a temperature program was run from 50°C to 300°C at 5°C/min flushing the column with nitrogen at a flow-rate of 40 ml/min, then the column was held isothermally at 300°C for 2 h without gas flow and for 48 h with a nitrogen flow-rate of 40 ml/min.

The operating conditons were: column temperature 200°C (OV-101) or 235°C (SP-2250), with injection port and detector temperature at 300°C. Helium was used as carrier gas at a flow-rate of 35 ml/min.

Gas chromatography—mass spectrometry

Identification of the gas chromatographic peaks was made by gas chromatography—mass spectrometry (GC—MS) under the electron-impact mode with a Nermag R10-10 mass spectrometer coupled with a Sidar data system and with a Girdel gas chromatograph (Model 32). Operating conditions were: electron beam energy 70 eV, filament current 200 μA, pressure of the ion source $2 \cdot 10^{-2}$ Torr and ion-source temperature of 170°C. Data were acquired for ions in the mass range of 50—550 a.m.u. A glass column (2 m × 2 mm I.D.) packed with 3% SP-2250 coated on Supelcoport 100—120 mesh (Supelco) was coupled to the mass spectrometer. The temperature of the interface was 260°C.

Sample preparation

A standard solution of internal standard MD700075 (10 ng/μl) was prepared in distilled water. For the calibration curves, aqueous solutions of toloxatone (10, 50, 100, 500, 1000, 2000 and 3000 ng per 200 μl) were prepared using the aqueous solution of the internal standard MD700075 (10 ng/μl).

The extraction recovery of toloxatone was determined by addition of the internal standard after the extraction of the drug from plasma (reference sample was the same sample without extraction).

Among the solvents tested (toluene, ethyl acetate and diethyl ether) toluene at pH 10 provided the cleanest plasma extract with a recovery of 73 ± 2% (n = 3).

To a conical tapered tube, 200 μl of internal standard solution (for the calibration curve 200 μl of internal standard—toloxatone solution), 1 ml of plasma and 110 μl of 0.2 N sodium hydroxide solution (to adjust pH to 10) were added and vigorously shaken on a Vortex mixer. The sample was extracted with 6 ml of toluene for 30 min on a rock-and-roll extractor. The two phases were then separated by centrifugation at 4°C (1000 g for 10 min). The aqueous phase was discarded and the toluene phase was transferred to another tube and evaporated to dryness at 55°C in a water bath. Then 100 μl of a solution of BSTFA—ethyl acetate (1:5) were added. After 30 sec shaking on a Vortex mixer and 30 min reaction time at 60°C the excess reagent was evaporated to dryness at 40°C under a stream of nitrogen. The dry extract was then dissolved in 100 μl of ethyl acetate (shaking on a Vortex mixer for 1 min); 2 μl of this sample were injected into the gas chromatograph. No significant degradation of the trimethylsilyl derivatives of toloxatone and its internal standard was noticed after ten days at 4°C.

Statistical analysis

Calibration curves were tested using a programme for regression analysis [7] with a Tektronix 4052 desk computer. In the routine assay a Texas TI-51-III calculator was used.

RESULTS AND DISCUSSION

Gas chromatographic separation

Typical chromatograms of plasma extracts of toloxatone and its internal standard are shown in Figs. 2 (OV-101) and 3 (SP-2250).

Peak symmetry and resolution for both compounds were improved by formation of trimethylsilyl (TMS) derivatives. The retention time of the TMS derivatives of toloxatone (Tx-TMS) and the internal standard (IS-TMS) were adjusted approximately to 4 and 8 min on the SP-2250 column and to 2.5 and 4.5 min on the OV-101 column, respectively. Under these conditions the peaks corresponding to Tx-TMS and IS-TMS were well resolved. Overall time of analysis was 10 min on the SP-2250 column and 6 min on the OV-101 column.

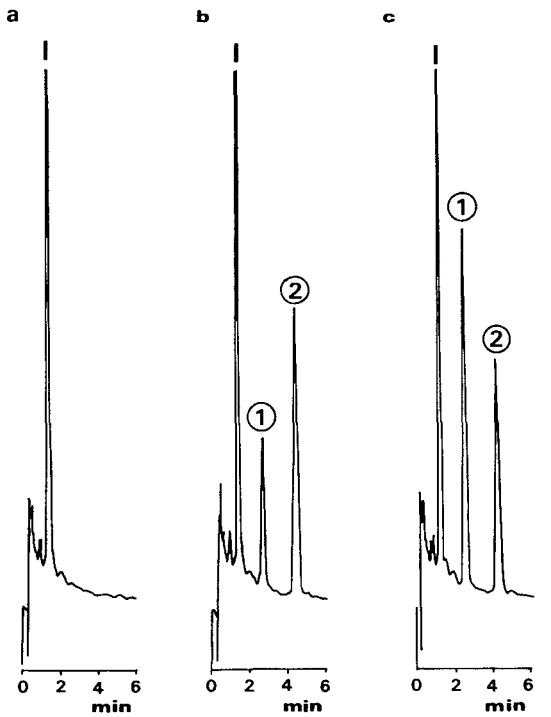


Fig. 2. Chromatograms obtained with the 3% OV-101 column at 200°C. (a) Plasma extract of a blank sample; (b) plasma extract of a sample containing spiked concentrations of toloxatone (500 ng/ml) and MD700075 (2000 ng/ml); (c) typical chromatogram after oral administration of toloxatone. 1 = Tx-TMS; 2 = IS-TMS; I = caffeine.

Derivatisation was checked by GC—MS. The mass spectra of the gas chromatographic peaks corresponding to Tx-TMS (molecular ion *m/z* 279) and IS-TMS (molecular ion *m/z* 295) demonstrated that both compounds were monosilylated. As shown in Fig. 2 and 3, an unknown compound was extracted from plasma samples (compound I). This peak was identified as caffeine using the library search programme of the Sidar data system.

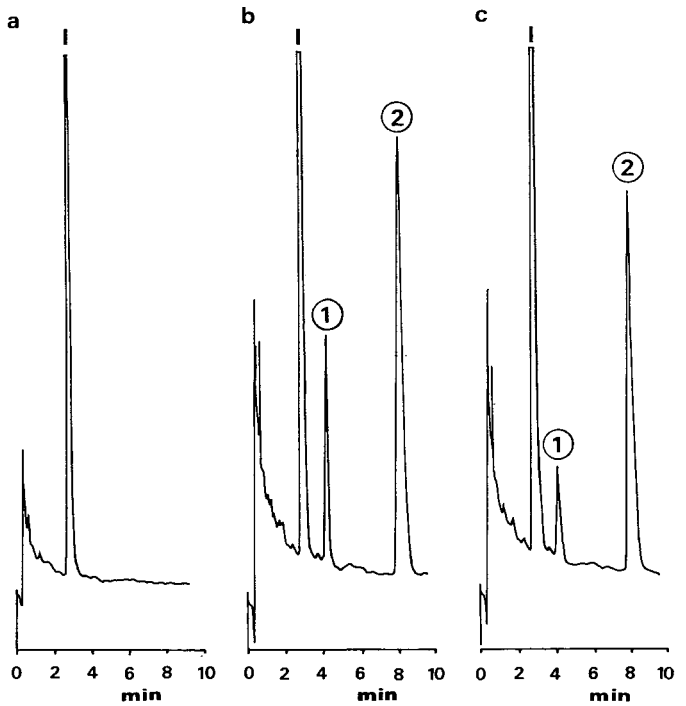


Fig. 3. Chromatograms obtained with the 3% SP-2250 column at 235°C. (a) Plasma extract of a blank sample; (b) plasma extract of a sample containing spiked concentrations of toloxatone (500 ng/ml) and MD700075 (2000 ng/ml); (c) typical chromatogram after oral administration of toloxatone. 1 = Tx-TMS; 2 = IS-TMS; I = caffeine.

Evaluation of the NPD

Since the first description by Kolb and Bischoff in 1974 [8], the NPD has been extensively used for quantitative analysis of drugs in body fluids and in tissues by GLC. However, in spite of its widespread use, its mechanism has not been yet fully elucidated [9, 10]. The NPD is known to have a relatively high baseline and sensitivity drift. In addition, there is a loss of sensitivity of the rubidium ceramics with time and so it is not easy to maintain the same sensitivity [11]. These problems can be minimised using an internal standard assuming the same change of sensitivity for the drug measured and for the reference compound. This problem was investigated by measuring the detector response of toloxatone/internal standard TMS derivatives at different heating power values.

Detector background current but not heating power was linearly related to detector response (Fig. 4). A practical consequence of this linear relationship of background current against detector response is that, with an internal standard, the heating power of the NPD may be increased occasionally for analysis of low concentrations. Relative detector response for toloxatone and the internal standard was determined from the slope of the two curves and gave a value of 1.03, the theoretical value calculated from their molecular weights being 1.08.

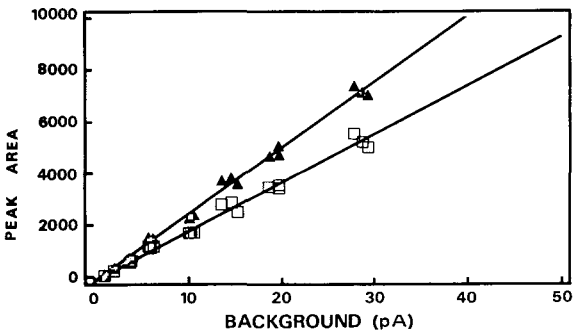


Fig. 4. Relationship between detector background current (pA) and detector response (peak area). Injection of Tx-TMS (▲, 40 ng toloxatone) and IS-TMS (□, 30 ng internal standard). Intercept values are not significantly different from the detector zero current. ▲: $y = 258.1x - 197.3$, $r = 0.9971$, $x_{(y=0)} = 0.76$ pA. □: $y = 188.0x - 123.1$, $r = 0.9948$, $x_{(y=0)} = 0.65$ pA.

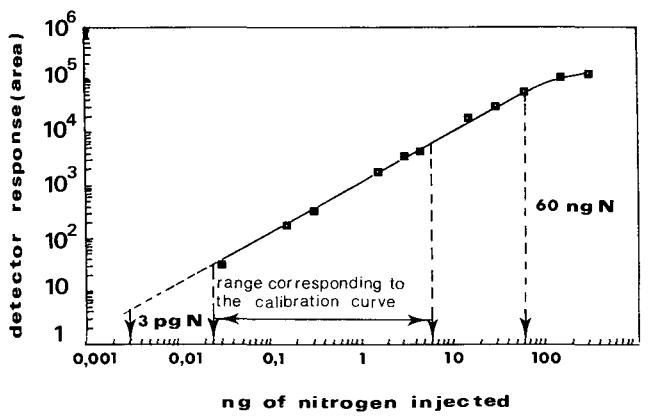


Fig. 5. NPD response linearity with phenazine (for conditions, see text).

NPD sensitivity was tuned by repeated injections of phenazine (SP-2250 column at 190°C). Optimum sensitivity and the same time-stable function of the detector was obtained with a hydrogen flow rate of approx. 2 ml/min and an air flow rate of approx. 45 ml/min. The lower detection limit using phenazine was estimated to be 3 pg of injected nitrogen. The linear dynamic range was approx. 10^4 (Fig. 5).

Calibration curves

Quantitative analysis was carried out using the area ratio method. Area ratios (y = Tx-TMS/IS-TMS) were plotted against concentration of toloxatone (x). Slope and intercept of the calibration curves were determined by linear regression analysis. The precision (relative standard deviation) and the accuracy (relative error) of the method were established in the range of toloxatone concentrations from 10 to 3000 ng/ml. Each sample was analysed in triplicate. Tables I and II show the results obtained with the same samples using SP-2250 and OV-101.

The negative y-intercept using the OV-101 column made this calibration

TABLE I

PRECISION AND ACCURACY OF THE GLC METHOD FOR THE MEASUREMENT OF TOLOXATONE

Column: 3% OV-101, 200°C. n = 3.

	Spiked concn. (ng/ml) (x)	Relative S.D. (%) (y)	Found concn. (ng/ml) (x')	S.D. (ng/ml) (x')	Relative S.D. (%) (x')	Mean error $\Delta x = x' - x$ (ng)	Relative error $\Delta x/x$ (%)
Straight-line fit	10	4.2	73.2	0.5	0.7	+ 63.2	+ 632.0
$y = 0.001067x - 0.06913$	50	4.4	102.0	1.8	1.8	+ 52.0	+ 104.0
($r = 0.9967$)	100	4.4	138.6	5.0	3.6	+ 38.6	+ 38.6
	500	3.7	449.0	14.5	3.2	— 5.1	— 10.2
	1000	0.8	876.0	5.9	0.7	— 124.0	— 12.4
	2000	4.5	1924.0	84.0	4.4	— 76.0	— 3.8
	3000	2.7	3098.0	84.0	2.7	+ 98.0	+ 3.2
Power-function fit	10	4.2	11.3	0.6	5.5	+ 1.3	+ 13.0
$y = 0.00073x^{1.0330}$	50	4.4	47.5	2.2	4.6	— 2.5	— 5.0
($\log y = 1.0330 \log x - 3.1342$)	100	4.4	91.9	4.2	4.5	— 8.1	— 8.1
($r = 0.9988$)	500	3.7	456.2	16.4	3.6	— 43.8	— 8.7
	1000	0.8	940.4	7.1	0.7	— 59.6	— 5.9
	2000	4.5	2099.5	92.0	4.4	+ 99.5	+ 5.0
	3000	2.7	3372.0	89.6	2.6	+ 372.0	+ 12.4

TABLE II

PRECISION AND ACCURACY OF THE GLC METHOD FOR THE MEASUREMENT OF TOLOXATONE

Column: 3% SP-2250, 235°C. n = 3.

	Spiked concn. (ng/ml) (x)	Relative S.D. (%) (y)	Found concn. (ng/ml) (x')	S.D. (ng/ml) (x')	Relative S.D. (%) (x')	Mean error $\Delta x = x' - x$ (ng)	Relative error $\Delta x/x$ (%)
Straight-line fit	10	6.0	36.2	0.5	1.4	+ 26.2	+ 262.0
$y = 0.00090x - 0.02491$	50	3.1	70.6	1.3	1.9	+ 20.6	+ 41.2
($r = 0.9991$)	100	5.0	113.4	4.3	3.8	+ 13.4	+ 13.4
	500	1.9	479.3	8.5	1.8	— 20.7	— 4.1
	1000	2.1	950.8	19.7	2.0	— 49.2	— 4.9
	2000	4.9	1971.6	96.0	4.8	— 28.4	— 1.4
	3000	1.6	3037.6	48.4	1.6	+ 37.6	+ 1.2
Power-function fit	10	6.0	10.3	0.6	5.8	+ 0.3	+ 3.0
$y = 0.00070x^{1.0283}$	50	3.1	49.7	1.5	3.0	— 0.3	— 0.6
($\log y = 1.0283 \log x - 3.1572$)	100	5.0	97.3	4.7	4.8	— 2.7	— 2.7
($r = 0.9998$)	500	1.9	489.7	9.0	1.8	— 10.3	— 2.0
	1000	2.1	981.1	20.3	2.0	— 18.9	— 1.9
	2000	4.9	2023.7	96.8	4.8	+ 23.7	+ 1.2
	3000	1.6	3096.3	48.9	1.6	+ 96.3	+ 3.2

curve unsuitable for the low toloxatone concentrations, as this linear regression would severely overestimate the lowest concentration values (Table I). The situation was improved when using the SP-2250 column; however, even this linear regression gave unacceptable errors for low concentrations (Table II). Note that the correlation coefficients calculated from all experimental data points were 0.9991 and 0.9967 for SP-2250 and OV-101, respectively. These values emphasize the caution needed when this parameter is used to judge linearity.

TABLE III

CHARACTERISTICS OF THE CALIBRATION CURVE BY INCREASING THE RANGE OF TOLOXATONE PLASMA CONCENTRATIONS

Column: 3% SP-2250, 235°C.

Range of calibration (ng/ml)	Sraight-line fit ($y = ax + b$)				Power-function fit $y = ax^b$ (log y = log a + $b$log x)*			
	$a \cdot (10^{-4})$	$b \cdot (10^{-4})$	$x_{(y=0)}$ (ng)	r	log a	$a \cdot 10^{-4}$	b	r*
10— 50	7.74	0.72	0.09	0.9988	—3.1207	7.57	1.0052	0.9988
10— 100	7.72	0.07	0.01	0.9977	—3.1180	7.62	1.0029	0.9992
10— 500	8.16	22.3	2.7	0.9997	—3.1340	7.35	1.0143	0.9997
10—1000	8.31	40.5	4.9	0.9997	—3.1397	7.25	1.0180	0.9998
10—2000	8.72	141.9	16.0	0.9985	—3.1493	7.09	1.0238	0.9998
10—3000	9.00	249.1	27.7	0.9991	—3.1572	6.96	1.0283	0.9998

*Linear regression after logarithmic transformation.

Table III shows that slope and intercept on the y-axis increased by increasing the range of concentration values, indicating that calibration curves were slightly concave.

The possible cause of this non-linearity could be: loss by adsorption in the glassware, adsorption in the chromatographic system and/or in the extraction procedure, non-linear detector signal and integration errors. Some of these factors can be controlled; however, it is not always possible to determine the step which causes the non-linearity.

The comparison between the results obtained from the two different columns suggests an important contribution of the GLC system to the non-linearity of the calibration curve.

In spite of a better peak symmetry when using OV-101 column, the SP-2250 column gave a better linearity, probably because of a better deactivated column packing.

Non-linear calibration curves can be used by dividing them into linear subranges [12] or by polynomial fitting [13]. With our data a power function through the origin ($y = ax^b$) linearized by a logarithmic transformation (log y = log a + $b$log x) was attempted. The advantage of this fitting is that the

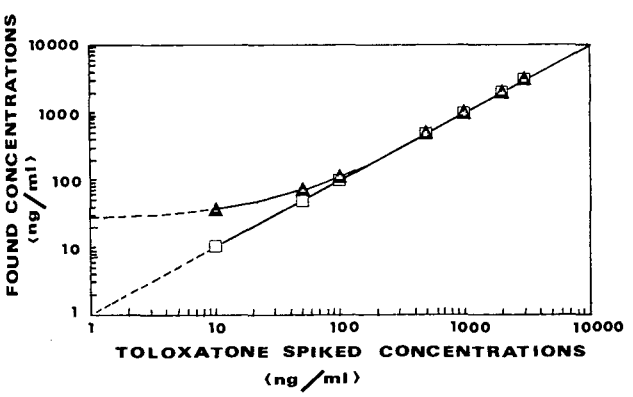


Fig. 6. Accuracy of the calibration (SP-2250 column). (▲), linear calibration curve; (□), power-function fit calibration curve with logarithmic transformation.

logarithmic transformation introduces a weighting factor in the least-squares computations, which is inversely proportional to the observed value. This weighting factor seems to be reasonable, because it corresponds to the hypothesis of approximately constant relative standard deviation of the observed area ratio values (S.D.(y)/$\bar{y}$).

The comparison between results from the two methods of calculation (Tables I and II) showed that the power function fitting linearized by logarithmic transformation considerably improved the accuracy of the low concentrations (Fig. 6).

The day-to-day reproducibility of the calibration curves was investigated during one month and the overall relative standard deviation was $\leqslant$ 6% for toloxatone plasma concentrations between 10 and 3000 ng/ml.

Application of the method

This method has been extensively applied in our laboratory to investigating the pharmacokinetics of toloxatone in man following single and multiple doses. A typical drug plasma concentration—time plot after oral administration of a 200-mg capsule to a healthy adult is shown in Fig. 7.

Toloxatone plasma concentrations were determined in the 0—12 h interval using both the linear and the logarithmic calibration curves. No difference of toloxatone plasma concentrations was observed above 100 ng/ml. However, when plasma concentrations were below 100 ng/ml the linear calibration curve gave a significant overestimation of the concentration values and this would considerably change the interpretation of the experimental data.

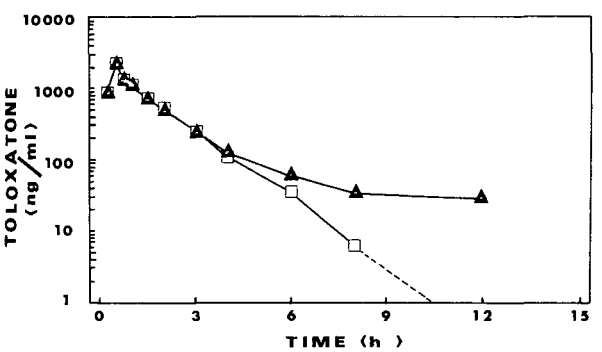


Fig. 7. Plasma toloxatone concentrations from a healthy adult following a single oral administration of the drug (200 mg, capsule). (▲), linear calibration curve; (□), power-function fit calibration curve with logarithmic transformation.

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REFERENCES

1 J.P. Kan, A. Malnoë and M. Strolin Benedetti, J. Pharm. Pharmacol., 30 (1978) 190.
2 M. Martin, l'Information Psychiatrique, 49 (1973) 1023.
3 R. Suttel and J. Duplan, Actualités Psychiatriques, 2 (1975) III.
4 S.J. Dencker and A. Nagy, Nord. Psykiatr. Tidsskr., in press.
5 A. Malnoë and M. Strolin Benedetti, Xenobiotica, 9 (1979) 281.
6 M. Strolin Benedetti, V. Rovei, S.J. Dencker, A. Nagy and R. Johansson, Arzneim.-Forsch., 32 (1982) 276.
7 G.W. Snedecor and W.G. Cochran, Statistical Methods, Iowa State University Press, 1967, p. 135.
8 B. Kolb and J. Bischoff, J. Chromatogr. Sci., 12 (1974) 625.
9 B. Kolb, M. Auer and P. Pospisil, J. Chromatogr. Sci., 15 (1977) 53.
10 K. Olah, A. Szöke and S. Vajta, J. Chromatogr. Sci., 17 (1979) 497.
11 J.A. Lubrowitz, J.L. Glajch, B.P. Semonian and L.B. Rogers, J. Chromatogr. Sci., 133 (1977) 37.
12 U. Timm, M. Zell and L. Herzfeld, J. Chromatogr., 229 (1981) 111.
13 K.L. Reed, M.E. Young, L.J. Hutchinson, J.J. Warsh and H.C. Stancer, Progr. Neuro-Psychopharmacol., 5 (1981) 311.

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ANALYSIS OF N-*n*-PROPYLNORAPOMORPHINE IN PLASMA AND TISSUE BY CAPILLARY GAS CHROMATOGRAPHY—ELECTRON-CAPTURE DETECTION

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SUMMARY

Capillary gas chromatography combined with electron-capture detection (GC—ECD) was applied to the detection and quantitation of N-*n*-propylnorapomorphine (NPA) and related compounds in serum and tissue using trifluoroacetyl (TFA) derivatives. The detection limits for NPA using GC—ECD of TFA derivative extend into the subpicogram level, but quantitation in serum was limited to levels of 100 ng/ml due to matrix interferences. The method was applied to the analysis of NPA in rat serum after administration of a moderate dose of the drug and was applied to the detection of NPA in rat brain after the peripheral administration of (—)10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA). These results support previous proposals that MDO-NPA is a prodrug of NPA, which acts at cerebral dopamine-receptors.

INTRODUCTION

Apomorphine (APO) and N-*n*-propylnorapomorphine (NPA) have potent and selective actions at central and other dopamine (DA) receptors [1]. They have been used clinically especially in neurological and psychiatric disorders [2—5] although their clinical use has been limited by their poor oral bioavailability and short duration of action [1,6]. A prodrug of NPA, (—)10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA), has recently been described [7,8] as a unique, orally effective and long-acting apomorphine derivative that exerts activity at DA receptors in the brain.

In view of the clinical significance of APO, NPA and MDO-NPA (structures in Fig. 1) the detection and quantitation of these drugs in biological fluids are of

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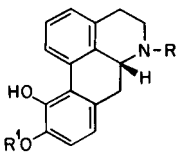

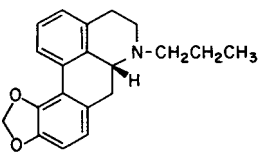

(-) Apomorphine (APO), R = CH_3, R^1 = H

(-) N-n-propylnorapomorphine (NPA), R = $CH_2CH_2CH_3$, R^1 = H

(-) Apocodeine, R = R^1 = CH_3

(-) N-n-propylnorapocodeine (10-O-Me-NPA), R = $CH_2CH_2CH_3$, R^1 = CH_3

(-)10,11-Methylenedioxy-N-n-propylnoraporphine (MDO-NPA),

Fig. 1. Structures of apomorphine (APO), N-*n*-propylnorapomorphine (NPA), apocodeine N-*n*-propylnorapocodeine (10-O-Me-NPA), and 10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA).

interest. In order to establish more closely the correlation between tissue levels of these drugs and their pharmacological effects in vivo, several analytical methods for the assay of APO and NPA have been investigated. Techniques such as thin-layer chromatographic fluorescence quenching [9], spectrophotometry [10], paper chromatography [11], fluorometry [12], gas chromatography using flame ionization or thermal conductivity detectors [13,14] and high-performance liquid chromatography using UV or electrochemical detectors [15—19] have reported sensitivities ranging from 5 ng/ml to a few hundred ng/ml of biological fluid without the need for chemical derivatization. In a recent publication, Watanabe et al. [20] have reported detection limits of 30 ng/ml for APO using trimethylsilyl derivatives and gas chromatography—mass spectrometry (GC—MS) in the single ion monitoring mode. A less sensitive radioenzymatic assay of APO has also been developed [21].

While many of the techniques summarized above offer specific advantages, a number of drawbacks may also be noted. For example, most of the GC work was conducted with conventional packed columns and flame ionization detectors. Under these conditions detection limits of, at best, 1 ng have been reported. On the other hand, even though GC—MS single ion monitoring is capable of detection at the picogram level, the technique is costly and not generally accessible. In view of the increasing clinical significance of NPA and MDO-NPA we investigated the utility of fused silica capillary GC—electron-capture detection (GC—ECD) for the analysis of these compounds in plasma and urine. While packed column GC—ECD has been used previously for the determination of APO in equine plasma [22], it was reasoned that the fused silica capillary GC would provide us with superior chromatographic resolution for identification of the parent drugs or their metabolites with a much higher degree of confidence based on retention times. Moreover, it was anticipated that the well established sensitivity of the electron-capture detector should permit detection and quantitation at the picogram level or lower. It was felt that use of the relatively simple capillary GC—ECD combination should make available a simple and inexpensive method for assaying these compounds in a clinical laboratory. Our studies reported here focused on the analysis of NPA and related compounds

in plasma and tissues, after the periphenal administration of both NPA and MDO-NPA.

Our approach was based on the use of a three-step extraction procedure for isolation of the alkaloids, formation of trifluoracetyl derivatives to enhance the electron affinity and volatility of the solutes and finally analysis by capillary GC—ECD. This methodology was applied to in vivo studies in rats. The enzymatic transformation of MDO-NPA to NPA in rat brain was confirmed by the detection and quantitation of NPA in the brain tissue, following administration of the parent drug. The results of this study are reported below.

EXPERIMENTAL

Materials

N-*n*-propylnorapomorphine (NPA), apomorphine (APO), 10-O-methyl-N-*n*-propylnorapomorphine (10-O-Me-NPA) and 10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA) were synthesized in these laboratories as their hydrochloride or hydrobromide salts. The MDO-NPA was analyzed for its NPA content by GC—ECD. The NPA contamination of this material was found to be 0.01%. Radioactive [^{3}H] NPA was provided by New England Nuclear, Boston, MA, U.S.A. (0.25 μg, specific activity 60.0 Ci/mmole). All organic solvents were chromatography grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). The other reagents were of analytical research grade. The glassware was treated with a hot solution of 5% (v/v) hexamethyldisilazane in toluene for 15 min to reduce surface adsorption.

Preparation of standards

A standard stock solution of N-*n*-propylnorapomorphine-HCl (NPA) (equivalent to 1 mg free amine per 10 ml) was made up in methanol and stored under refrigeration for no more than one week. The working standard was diluted to a final free amine concentration of 1 μg/ml. Triplicate standards containing increasing concentrations of NPA·HCl were prepared by measuring 100, 200, 400, 600, 800 and 1000 μl of working standard solution into screw-cap vials. The solvent was removed under nitrogen and the residues derivatized as described below.

Standard solutions of the other two compounds, APO and 10-O-Me-NPA, and the mixture of the three were prepared in a similar fashion.

Extraction

Biological samples of the three-component mixture containing 1 μg or 10 μg of each solute per ml of plasma were prepared by spiking 1 ml of a rat plasma with the appropriate amount of the drug. The plasma mixture (1 ml) was mixed with sodium triphosphate buffer (1.0 ml, pH 7.4). This mixture was fortified with 1 mg of dithiothreitol to prevent oxidation of the amines [14] and extracted by shaking with three 5-ml portions of ethyl acetate. The dithiothreitol was included in every step of the extraction process. To minimize interference with the GC analysis from any coextracted plasma constituents, the ethyl acetate extracts were further purified by a back extraction as follows: 5 ml of 0.2 *M* aqueous hydrochloric acid was added to the combined ethyl acetate extracts and the amine solutes extracted into the aqueous layer as their hydro-

chloride salts. The aqueous layer was washed once with 5 ml *n*-hexane to remove any lipophilic impurities and the pH was adjusted to near 7.4 by addition of 1 *M* sodium hydroxide and 2 ml of pH 7.4 buffer. The free amines were re-extracted into three 5-ml portions of ethyl acetate. After each extraction the tubes were centrifuged for 10 min to define better the separation of the two phases. A comparison of the GC profiles of a plasma extract before and after back extraction will be shown later in Fig. 4, indicating the advantage obtained by introducing a second purification step.

The efficiency of the extraction procedure was determined using tritium labeled NPA (specific activity 60 Ci/mmole) [23]. Plasma samples (1.0 ml) were each spiked with 10 μg, 1.0 μg and 0.1 μg of NPA. To each 1.0-ml plasma sample 0.12 ng of radioactive NPA was added. The results, indicating the percent recovery in each of the three extraction steps, are summarized in Table I. The average of six determinations are given along with the percent standard deviation in each set of six. It is apparent that, while the bulk of the NPA can be recovered following the first extraction, at least a second extraction step is necessary to assure near full recovery. It should be noted, however, that even after a single extraction, a very large fraction of the lipophilic constituents of the plasma was co-extracted. Back-extraction, as described above, is therefore necessary in order to obtain a sample relatively free of coeluting interferences for the GC—ECD analysis of the NPA. As a result of these additional cleanup steps, final sample recovery corresponded to approximately 75% of the original sample.

TABLE I

EXTRACTION EFFICIENCY OF NPA FROM PLASMA INTO ETHYL ACETATE

For details of analysis see Experimental section. Values are the average of six determinations; values in parentheses refer to percent standard deviation in each group of six determinations.

NPA (μg/ml plasma)	Recovery* (%)			
	Extraction 1	Extraction 2	Extraction 3	Total
10.0	94.0 (5.9)	8.7 (0.4)	1.4 (0.2)	104.3 (6.0)
1.0	94.0 (4.4)	8.6 (1.2)	1.4 (0.1)	103.9 (4.6)
0.1	90.5 (7.2)	7.7 (0.5)	1.3 (0.1)	99.4 (7.6)

*Total recovery for the group of all 18 samples correspond to 102.5% with a standard deviation of 6.3%.

Preparation of derivatives

TFA derivatives of NPA and other related compounds were prepared according to procedures described in a previous publication [24]. The ethyl acetate extracts of the free amines, or the combined ethyl acetate plasma extracts, were evaporated under a nitrogen stream. To the residue in each vial, test or standard, was added excess of redistilled trifluoroacetic anhydride (TFAA). The vials were tightly capped with PTFE-lined screw-caps, placed in a vacuum dessicator and exposed to an infra-red heating lamp. The reaction temperature was maintained at 60—70°C for 60 min. This procedure allowed only minimal exposure to atmospheric moisture which may cause decomposi-

tion of the derivatives. After completion of the reaction, the excess reagent was removed under vacuum. The residue of the derivatized sample in each vial was then reconstituted in 1 ml toluene containing 1% TFAA which was added to prevent hydrolysis of the derivative. Volumes of 1 μl were injected into the gas chromatograph.

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with both a flame ionization detector (FID) and a ^{63}Ni pulsed ECD and a Varian Model 9176 1-mV recorder were used. A Varian CDS 111 chromatography data system was employed for measurement of peak retention times and peak areas. The injector port was maintained at 270°C and the detector at 320°C. A fused-silica column (15 m × 0.025 mm I.D.) coated with SE 54 (J. & W. Scientific) was used. The initial column temperature was 170°C for 1 min, then programmed to a final temperature of 285°C at a rate of 6°C/min. Ultra high purity nitrogen was used as the carrier gas at a flow-rate of 5.0 ml/min. Nitrogen with a flow-rate of 30 ml/min was used as the make-up gas.

RESULTS AND DISCUSSIONS

Our evaluation of the practical utility of fused silica capillary GC—ECD for the analysis of NPA using TFA derivatives was conducted in three stages. For the first stage, standard samples of pure NPA, APO, and 10-O-Me-NPA were analyzed. This was followed by an examination of plasma and urine samples spiked with NPA. Finally, the methodology developed in the first two stages was applied to the analysis of a number of biologically derived samples.

Analysis of pure samples by GC—ECD

This portion of the study was necessary to establish a basis for the analysis of NPA by GC—ECD, specifically the detection limits for its trifluoroacetyl (TFA) derivatives and criteria for its quantitation. Construction of a standard curve for NPA, indicated a linear dynamic range covering more than three orders of magnitude (5 to 10,000 pg). The capability of the technique for trace level detection was further demonstrated by the fact that detection limits of 0.20 pg were determined for the TFA derivative of NPA. A signal-to-noise ratio of ca. 5:1 was obtained for a standard sample of 0.20 pg injected into the gas chromatograph. It should be emphasized, however, that to ensure reproducibility of the results at these levels, daily replacement of the glass-lined insert, used in the injection port of the capillary gas chromatograph, is necessary.

A chromatogram showing the separation of the three components of a standard mixture (20 pg injected of each) of NPA, APO and 10-O-Me-NPA is given in Fig. 2.

The 10-O-Me-NPA was included in the chromatogram because the latter compound is a potential metabolite of NPA, and it was important to compare its chromatographic retention with that of NPA.

Analysis of spiked serum

Serum samples spiked with standard quantities of NPA, APO and 10-O-Me-

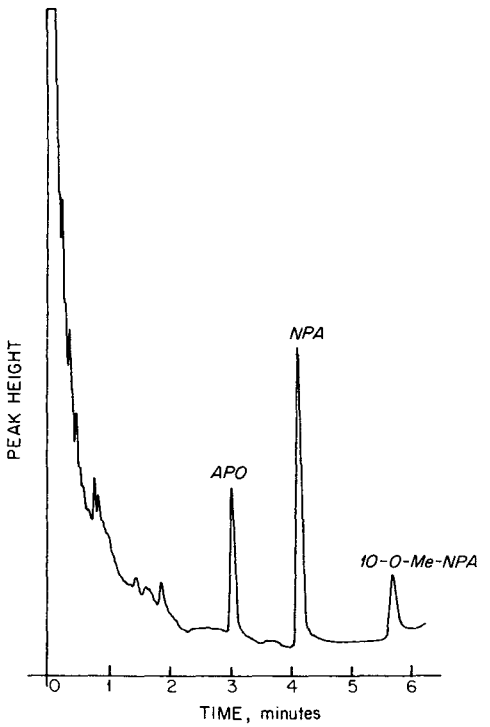

Fig. 2. Chromatogram of TFA derivatives of a standard mixture (20 pg each) of APO, NPA, and 10-O-Me-NPA. Column: 15 m fused-silica capillary, SE-54, 170°C (1 min), programmed to 285° C (6° C/min).

NPA were examined in order to establish the extraction conditions necessary for effective analysis of these compounds by capillary GC—ECD. The results obtained from a serum sample spiked with 1 μg of each compound per ml of serum are summarized in Fig. 3. A chromatographic profile of the ethyl acetate

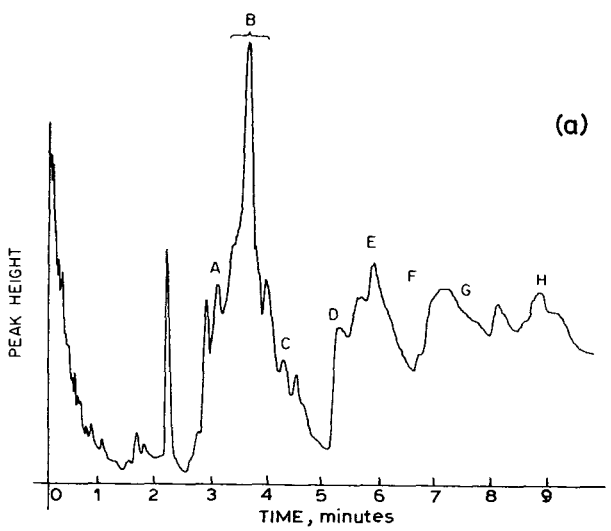

Fig. 3.

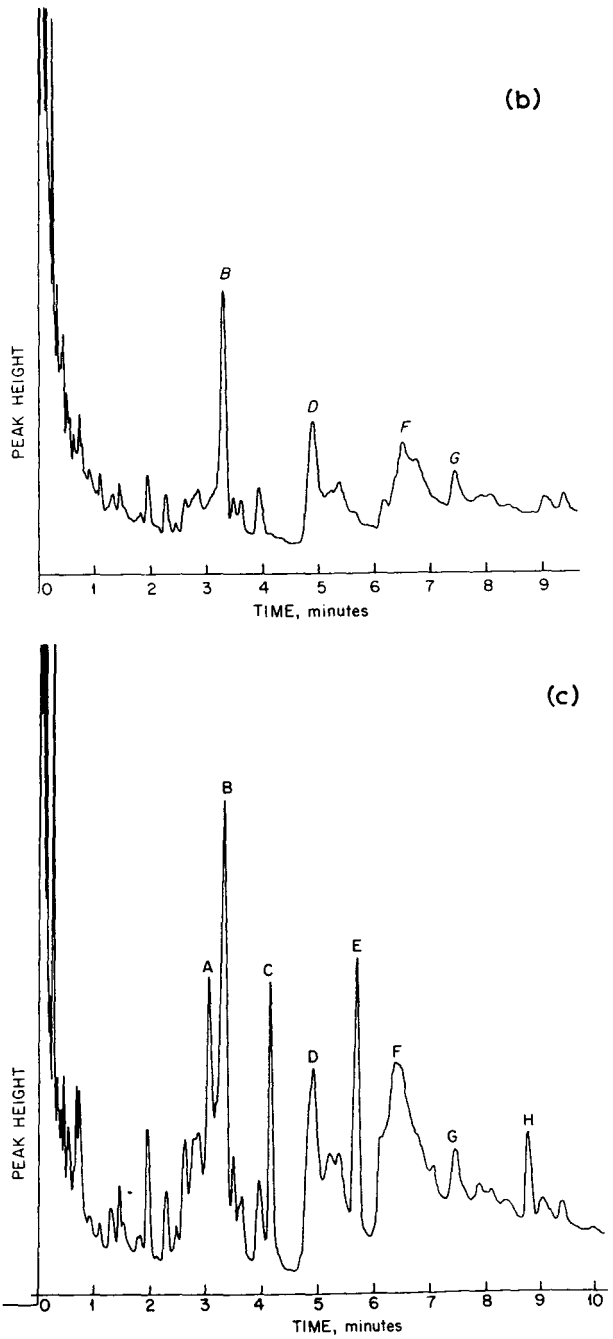

Fig. 3. GC—ECD recordings of derivatized serum extracts spiked with 1 μg/ml each of APO (A), NPA (C) and 10-O-Me-NPA (E). GC conditions as in Fig. 2. (a) spiked rat serum without back extraction; (b) serum control with back extraction; (c) spiked serum with back extraction.

extract following trifluoroacetylation is shown in Fig. 3a. Peaks A, C, and E correspond to the retention times of APO, NPA and 10-O-Me-NPA respectively. Clearly, the contributions from the serum prohibit quantitation of NPA under these conditions. This problem, however, was rectified by further clean-up of the sample via back extraction (see Experimental section). Fig. 3b shows the GC profile of the serum (control) sample after back extraction and this is compared with the corresponding profile of the spiked sample in Fig. 3c. The peaks of interest (A, C, and E) are now sharply defined permitting facile determination of the respective compounds. This is particularly the case for NPA, which elutes in a region relatively free of interferences and is thus most suitable for quantitation.

According to the radioactivity measurements discussed in the Experimental section, the back extraction process results in the retrieval of approximately 75% of the NPA added to rat serum. This value was also confirmed by the GC experiments and comparison of the NPA signal in Fig. 3c to that in the standard curve.

These results, therefore, confirmed the compatibility of the extraction conditions discussed in the Experimental section with the analysis of NPA in serum using capillary GC—ECD. Some applications of these procedures in vivo and in vitro are discussed in the following section.

Biological experiments

Two different types of in vivo applications were considered in this study in order to establish the general applicability of the method described above. The first sample (Fig. 4a) shows the chromatogram resulting from a serum sample taken 30 min after intraperitoneal administration of the drug [0.84 mg NPA; (5 mg/kg)]. Comparison with a control sample (Fig. 4b) shows good definition of the peak (A) corresponding to the retention time of NPA. The signal corresponds to 6.2 ng of NPA or 0.41 μg of NPA per ml serum after correcting for the 75% recovery in extraction. A second peak (B) in the chromatogram of Fig. 4a is presumably an unidentified metabolite of NPA. Studies using GC—MS are currently in progress to determine the nature of this and other possible metabolites.

In the second example we applied capillary GC—ECD to determine whether enzymatic conversion of MDO-NPA to NPA in the body may be responsible for the biological activity of that compound. Brain tissues from rats administered 10.5 mg of MDO-NPA per kg intraperitoneally were homogenized, extracted and derivatized as previously described. Chromatograms of extracts resulting from the treatment of control sample and samples after treatment with MDO-NPA are shown in Fig. 5. The signal arising from the NPA peak corresponds to 1.1 ng or 21 ng/g of brain tissue after correcting for the 75% recovery in extraction. The results are, in general, in agreement with those of Sperk et al. [25] who independently measured NPA levels ranging from 10—20 ng NPA per g of brain tissue for similar rat samples using high-performance liquid chromatography—electrochemical detection.

In conclusion, the data presented in this paper show that capillary GC combined with ECD provides a reliable technique for the detection and quantitation of NPA and related compounds in serum and tissue using trifluoroacetyl deriva-

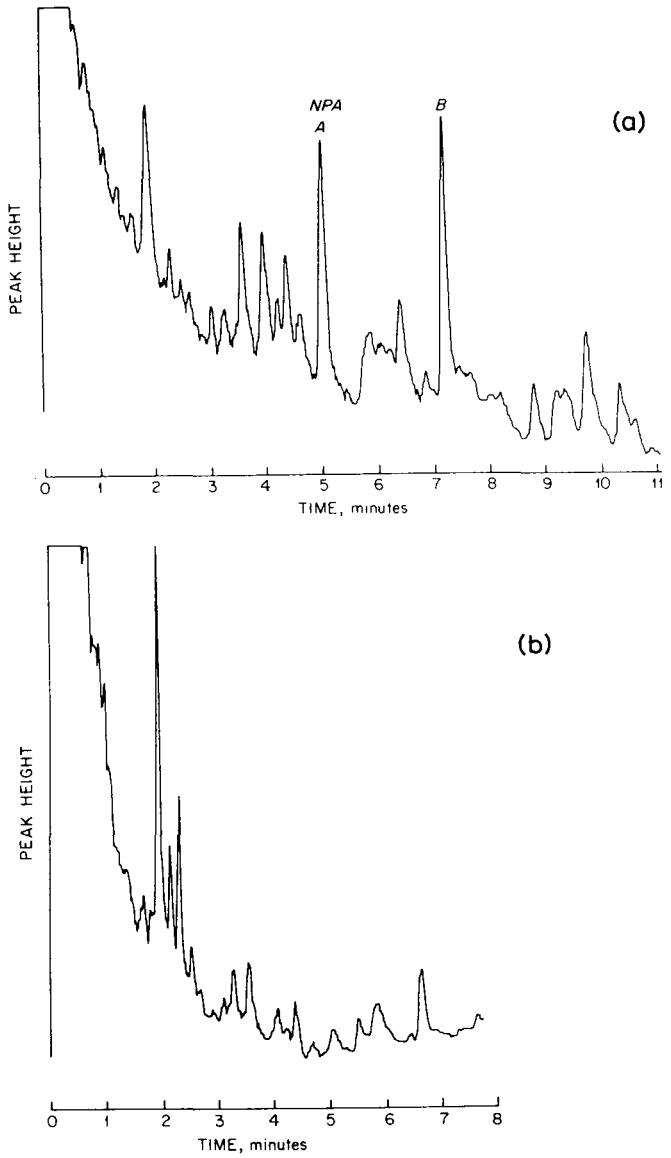

Fig. 4. GC—ECD recording of derivatized serum extract from (a) in vivo sample of rat administered 5 mg/kg NPA; (b) control sample. GC conditions as in Fig. 2.

tives. The use of a back extraction step was an effective means for cleanup of the sample prior to derivatization and GC analysis. The present results further support previous proposals that the activity of MDO-NPA in vivo may be related to its function as a prodrug to liberate NPA [7,8,25].

The detection limits for NPA using the capillary GC—ECD approach have been shown to extend into the subpicogram level while quantitation was routinely conducted to levels lower than 5 pg of sample injected into the gas chromatograph. While on an absolute basis these values compare favorably with

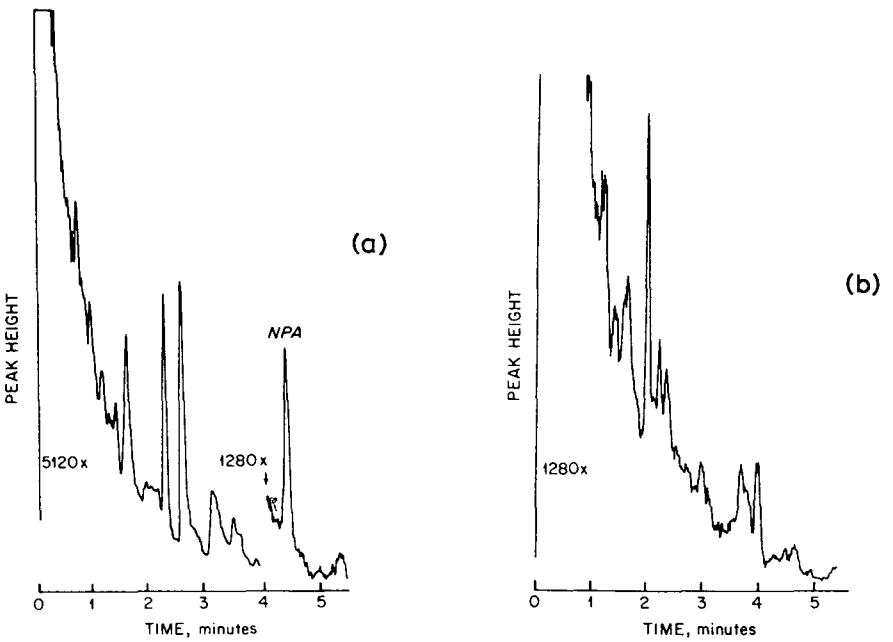

Fig. 5. GC—ECD recordings of derivatized brain tissue extracts from (a) in vivo sample of rat administered MDO-NPA (10.5 mg/kg); (b) control sample. GC conditions as in Fig. 2.

radioenzymatic (REA) or radioreceptor (RRA) assays where detection limits of 750 pg and 50 pg, respectively, are reported [6], the requirements for sample clean-up are considerably higher for the GC—ECD method. On the basis of our present data, it is estimated that the detection limits possible with the methodology developed here are, at best, in the range of 100 ng/ml of serum due to matrix interferences. This compares much less favorably to the corresponding detection limits of RRA (0.150 ng/ml) and REA (5 ng/ml). On the other hand, the GC method is not necessarily limited to the analysis of a given compound (APO or NPA), but can be applied to other constituents, such as metabolites, containing at least one derivatizable hydroxy group. Moreover, further improvements in the detection limits are anticipated via the use of more selective chromatographic detectors (e.g. mass spectrometric). Studies to that effect are currently in progress.

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REFERENCES

1 J.L. Neumeyer, S. Lal and R.J. Baldessarini, in G.L. Gessa and G.U. Corsini (Editors), Apomorphine and Other Dopaminomimetics, Vol. 1, Basic Pharmacology, Raven Press, New York, 1981, pp. 1—17.
2 S. Lal, in G.U. Corsini and G.L. Gessa (Editors), Apomorphine and Other Dopaminomimetics, Vol. 2, Clinical Pharmacology, Raven Press, New York, 1981, pp. 1—11.
3 G.U. Corsini, G.F. Pitzalis, F. Bernardi, A. Bochetta and M. Del Zompo, Neuropharmacology, 20 (1981) 1309—1313.
4 C.A. Tamminga, E.G. DeFraites, M.D. Gotts and T.N. Chase, in G.U. Corsini and G.L. Gessa (Editors), Apomorphine and Other Dopaminomimetics, Vol. 2, Clinical Pharmacology, Raven Press, New York, 1981, pp. 49—58.
5 A. Argiolas, G. Mere, G. Serra, M.R. Melis, F. Fadda and G.S. Gessa, Brain Res., 231 (1982) 109—116.
6 R.J. Baldessarini, G.W. Arana, N.S. Kula, A. Campbell and M. Harding, in G.L. Gessa and G.U. Corsini (Editors), Apomorphine and Other Dopaminomimetics, Vol. 1, Basic Pharmacology, Raven Press, New York, 1981, pp. 219—228.
7 R.J. Baldessarini, J.L. Neumeyer, A. Campbell, G. Sperk, V.J. Ram, G.W. Arana and N.S. Kula, Eur. J. Pharmacol., 77 (1982) 87—88.
8 A. Campbell, R.J. Baldessarini, V.J. Ram and J.L. Neumeyer, Neuropharmacology, 21 (1982) 953—961.
9 R.V. Smith, M.R. Cook and A.W. Stocklinski, J. Chromatogr., 87 (1973) 294—297.
10 P.N. Kaul, E. Brochmann-Hanssen and E.L. Way, J. Amer. Pharm. Assoc. Sci. ed., 48 (1959) 638—641.
11 K. Missala, S. Lal and T.L. Sourkes, Eur. J. Pharmacol., 22 (1973) 54—58.
12 W.K. Van Tyle and A.M. Burkman, J. Pharm. Sci., 60 (1971) 1736—1738.
13 R.V. Smith and A.K. Stocklinski, Anal. Chem., 47 (1975) 1321—1325.
14 D.M. Baaske, J.E. Keiser and R.V. Smith, J. Chromatogr., 140 (1977) 57—64.
15 R.V. Smith, J.C. Glade and D.W. Humphrey, J. Chromatogr., 172 (1976) 520—523.
16 R.V. Smith, D.W. Humphrey, S. Szeinbach and J.C. Glade, Anal. Lett., 12 (1979) 371—379.
17 R.V. Smith, A.E. Klein, A.M. Clark and D.W. Humphrey, J. Chromatogr., 179 (1979) 195—198.
18 R.V. Smith, A.E. Klein and D.V. Thompson, Mikrochim. Acta, I (1980) 151—157.
19 B.-M. Eriksson, B.-A. Persson and M. Lindberg, J. Chromatogr., 185 (1979) 575—581.
20 H. Watanabe, S. Nakano, N. Ogawa and T. Suzuki, Biomed. Mass Spectrom., 7 (1980) 160—163.
21 J.W. Kebabian, J. Neurochem., 30 (1978) 1143—1148.
22 J.R. Miller, J.W. Blake and T. Tobin, Research Commun. Chem. Pathol. Pharmacol., 15 (1976) 447—455.
23 C.N. Filer, D. Ahern, F.E. Granchelli and J.L. Neumeyer, J. Org. Chem., 45 (1980) 3465—3467.
24 J.F. Green, G.N. Jham, J.L. Neumeyer and P. Vouros, J. Pharm. Sci., 69 (1980) 936—942.
25 G. Sperk, A. Campbell, R.J. Baldessarini, A. Stoll and J.L. Neumeyer, Neuropharmacology, 21 (1982) 1311—1316.

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SIMULTANEOUS ASSAY OF TRIAZOLAM AND ITS MAIN HYDROXY METABOLITE IN PLASMA AND URINE BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A gas—liquid chromatographic method for the simultaneous determination of triazolam and its major hydroxy metabolite (1-hydroxymethyltriazolam) in human plasma and urine is described. After addition of two internal standards to the biological fluid, extraction at pH 9, acid washing, back-extraction, and derivatization, the analysis was performed on a wall-coated superior capacity open-tubular (WSCOT) CP-Sil 5 capillary column with electron-capture detection. The detection limit was 0.1—0.2 ng/ml; reproducibility was about 6—7% for plasma concentrations below 1 ng/ml. No interference from other possible minor hydroxy metabolites of triazolam was found. Gas chromatography coupled with mass spectrometry validated the chromatographic results. The method was successfully applied to plasma specimens collected from healthy human volunteers following a single intravenous administration of 1 mg of triazolam or 1-hydroxymethyltriazolam.

INTRODUCTION

Triazolam is a triazolobenzodiazepine derivative with sedative—hypnotic properties [1]. This compound is extensively metabolized in man, mainly by hydroxylation. The main metabolites (Table I) are 1-hydroxymethyltriazolam and 4-hydroxytriazolam [2, 3]. The 1-hydroxymethyl metabolite is reported to have 50—100% of the pharmacological activity of the parent compound [4].

Several methods have been described for the analysis of triazolam in biological fluids, including gas—liquid chromatography (GC) [5, 6], high-performance liquid chromatography (HPLC) [7, 8], radioimmunoassay (RIA) [9] and radioreceptorassay (RRA) [10]. RIA and RRA lack specificity as far as active metabolites are concerned, and GC and HPLC methods allow the

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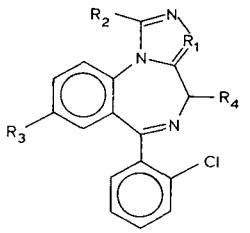
TABLE I

CHEMICAL STRUCTURES OF TRIAZOLAM, ITS HYDROXYLATED METABOLITES AND INTERNAL STANDARDS USED



Compound	Peak No.*	R_1	R_2	R_3	R_4
Triazolam	I	N	CH_3	Cl	H
1-Hydroxymethyltriazolam	II	N	CH_2OH	Cl	H
4-Hydroxytriazolam	III	N	CH_3	Cl	OH
1-Hydroxymethyl-4-hydroxytriazolam	IV	N	CH_2OH	Cl	OH
Estazolam	V	N	H	H	H
Ro 21-6962	VI	C-CH_2OH	CH_3	Cl	H

*Refers to labelled peaks in Figs. 1 and 3.

determination of triazolam only. It was considered important for the elucidation of the global pharmacokinetic behaviour of such a benzodiazepine to be able to determine both the parent drug and the 1-hydroxy active metabolite.

In this paper, a method is described for the simultaneous assay of triazolam and 1-hydroxymethyltriazolam in plasma and urine by GC. The method was validated by GC—mass spectrometry (MS), and was applied to pharmacokinetic studies in healthy volunteers.

EXPERIMENTAL

Reagents

Reagent-grade chemicals were used to prepare the following: phosphate buffer, 1.7 *M*, pH 10.5, prepared by dissolving 30 g of anhydrous K_2HPO_4 (Merck, Darmstadt, G.F.R.) in 100 ml of water and adjusting the mixture to pH 10.5 with KOH; buffer pH 9, obtained by a 15% dilution of concentrated buffer Titrisol (Merck); sulphuric acid 0.1 *N* from H_2SO_4 Ultrex. All these aqueous reagents were prepared with deionized and quartz-glass redistilled water, and then washed with diethyl ether agitation for approximately 2 h before use. Other reagents included diethyl ether, hexane, acetonitrile and toluene, all Pestipur brands supplied from SDS (Peypin, France). Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.).

Instrumentation

The GC apparatus employed was a Girdel (Suresnes, France) Model 330, equipped with a solid injection system (glass moving needle injector from

Girdel) and a 15 mCi electron-capture detector (ECD) operating with a pulse interval of 200 μsec. The column was a glass wall-coated superior capacity open-tubular (WSCOT) capillary column (25 m × 0.32 mm) with a polydimethylsiloxane (CP-Sil 5) as stationary phase (Chrompack, Orsay, France). The operating temperatures were injection port and detector 320°C, oven 280°C. Helium was used as carrier gas at a column head pressure of 0.8 bar. Argon—methane (95:5), at a flow rate of about 20 ml/min, was auxiliary gas. New capillary columns were conditioned before use by slow temperature programming (2°C/min) from 150 to 300°C and repeated injections of 2—3 μl of a 1% solution of cholesterol in toluene over 24—28 h. For GC—MS an HP 5985 connected to a data system HP 1000 (Hewlett-Packard, Les Ulis, France) was used with the same injection port and column model.

Standards

The compounds used were triazolam (T), 1-hydroxymethyltriazolam (1-HMT), 4-hydroxytriazolam (4-HT) and 1-hydroxymethyl-4-hydroxytriazolam (1-HM-4-HT) (Table I). All these compounds were supplied from Hoffmann-La Roche (Basle, Switzerland). Stock solutions were prepared by carefully weighing about 10 mg of each compound into a 10-ml volumetric flask and dissolving in methanol (Uvasol, Merck). Working solutions containing about 10 and 1 ng/ml were obtained by sequential dilutions of the respective stock solutions in methanol.

Internal standards

Estazolam (ET), supplied from Cassenne (Paris, France), was used for the triazolam assay, and Ro 21-6962, a hydroxylated imidazobenzodiazepine (Table I), supplied by Hoffmann-La Roche, was used for the 1-hydroxymethyltriazolam assay. For estazolam, stock and working solutions were prepared as described above for standards.

Ro 21-6962 after silylation of its hydroxy group by BSTFA gave two resolved peaks on a CP-Sil 5 capillary column. This compound was purified by HPLC as follows. Injections of stock solutions were performed on a Hewlett-Packard 1084 LC instrument equipped with an RP-18 column and UV detection (254 nm). The mobile phase was methanol—water (70:30) at a flow-rate of 1.5 ml/min. The two peaks observed under these conditions were collected separately and an aliquot was derivatized by BSTFA, analysed by GC, and identified by GC—MS in the chemical-ionization (CI) mode. The second HPLC peak corresponded to pure Ro 21-6962 and this fraction was used to prepare the stock solution. Working solutions were 0.5% solutions in methanol.

Preparation of samples

To 50 μl of methanol containing both internal standards estazolam and Ro 21-6962, 1 or 2 ml of buffer pH 9 and 0.5 or 1 ml of plasma, depending on expected concentrations, were added. After homogenization, the sample was extracted for 10 min with 10 ml of diethyl ether, then centrifuged for 5 min at 2500 *g*. The organic phase was evaporated to dryness under a stream of nitrogen and reconstituted in 1.5 ml of 0.1 *N* sulphuric acid. This was then washed for 2 min with 3 ml of hexane. The acid layer was adjusted to pH 9

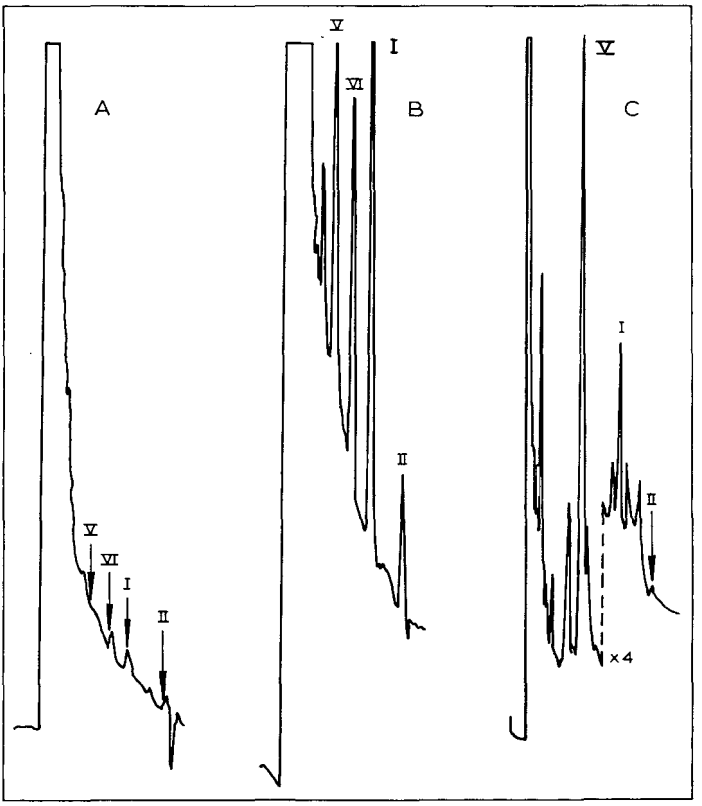


Fig. 1. Chromatograms obtained by GC—ECD after extraction and derivatization from: (A) control subject plasma; (B, C) subject plasma 1.5 h (9.3 ng triazolam; 1.2 ng 1-hydroxymethyltriazolam) and 12 h (0.18 ng triazolam; 1-hydroxymethyltriazolam not detectable) following a 1-mg intravenous bolus of triazolam. For peak labelling see Table I.

with phosphate buffer (approx. 1.5 ml), back-extracted for 10 min with 10 ml of diethyl ether and centrifuged for 15 min at 2500 *g*. The ether layer was concentrated to 500 μl, transferred to a 1.0-ml minivial (from Pierce) and evaporated to dryness under a gentle stream of pure nitrogen at 30—35°C, taking care that all water was removed. The residue was dissolved in 100 μl of acetonitrile; 10 μl of BSTFA were added as silylating agent and the minivial heated at 65°C for 15 min with continuous stirring (Reacti-Therm Heating Stirring module supplied from Pierce). After evaporation to dryness and reconstituting the residue in 50 μl of toluene, 2—3 μl were injected for GC analysis. Fig. 1 shows typical chromatograms, for control plasma extract (A), extracts of human plasma 1.5 h (B) and 12 h (C) following intravenous administration of 1 mg of triazolam.

Quantitation of unknown samples

Along with each set of unknowns, one blank plasma and two control plasmas to which had been added different concentrations of T and 1-HMT in the range to be determined were assayed. These calibration standards were used to establish daily a least-squares linear regression curve (from the peak area ratios

of T/ET and 1-HMT/Ro 21-6962 versus the respective plasma concentrations of T and 1-HMT. Peak areas were computed by means of a Hewlett-Packard 3388 A integrator. This internal standard curve was then used to interpolate unknown concentrations of triazolam and its hydroxylated metabolite in biological samples. Same calculations could be performed using peak height ratios instead of peak area ratios.

RESULTS AND DISCUSSION

Recovery, reproducibility, linearity, and sensitivity

The recovery of T and 1-HMT was calculated by comparing peak area ratios of T/ET and 1-HMT/Ro 21-6962 before extraction and after extraction of T and 1-HMT from human plasma. Then, the internal standards were added to the last extract. The extraction yield (Table II) was found to be satisfactory taking into account the extraction complexity, glass adsorption at low concentrations, and compound losses during the washing step due to the formation of an emulsion.

The reproducibility of the assay for T and 1-HMT is presented in Table III. The analysis precision given by the confidence interval of the mean value of each tested concentration was satisfactory for both compounds over the concentrations investigated. The analysis accuracy was obtained from the difference between expected and found concentrations, referring to a mean of three calibration curves established in the same period of time. These results, which appeared less acceptable than precision data, especially for low concentrations, reflect the variation of column conditioning and then of the column response from one day to another day. Thus, it seemed necessary, as indicated above, to undertake each day a limited two-point calibration curve with one blank in addition for the calculation of unknown plasma concentrations.

On the other hand, the linearity of the method was satisfactory for both compounds over the concentration ranges studied. In each case, one blank plasma and eight control plasmas, to which had been added four different concentrations (in duplicate) of T and 1-HMT, were simultaneously extracted

TABLE II

EXTRACTION YIELD OF TRIAZOLAM AND 1-HYDROXYMETHYLTRIAZOLAM FROM HUMAN PLASMA

$n = 6$.

Triazolam			1-Hydroxymethyltriazolam		
Concentration (ng/ml)	Recovery (%)	C.V.* (%)	Concentration (ng/ml)	Recovery (%)	C.V.* (%)
17.0	76	4	16.0	66	2
6.8	68	3	6.4	65	4
3.4	55	4	3.2	58	3
1.7	59	3	1.6	54	4

*C.V. = coefficient of variation.

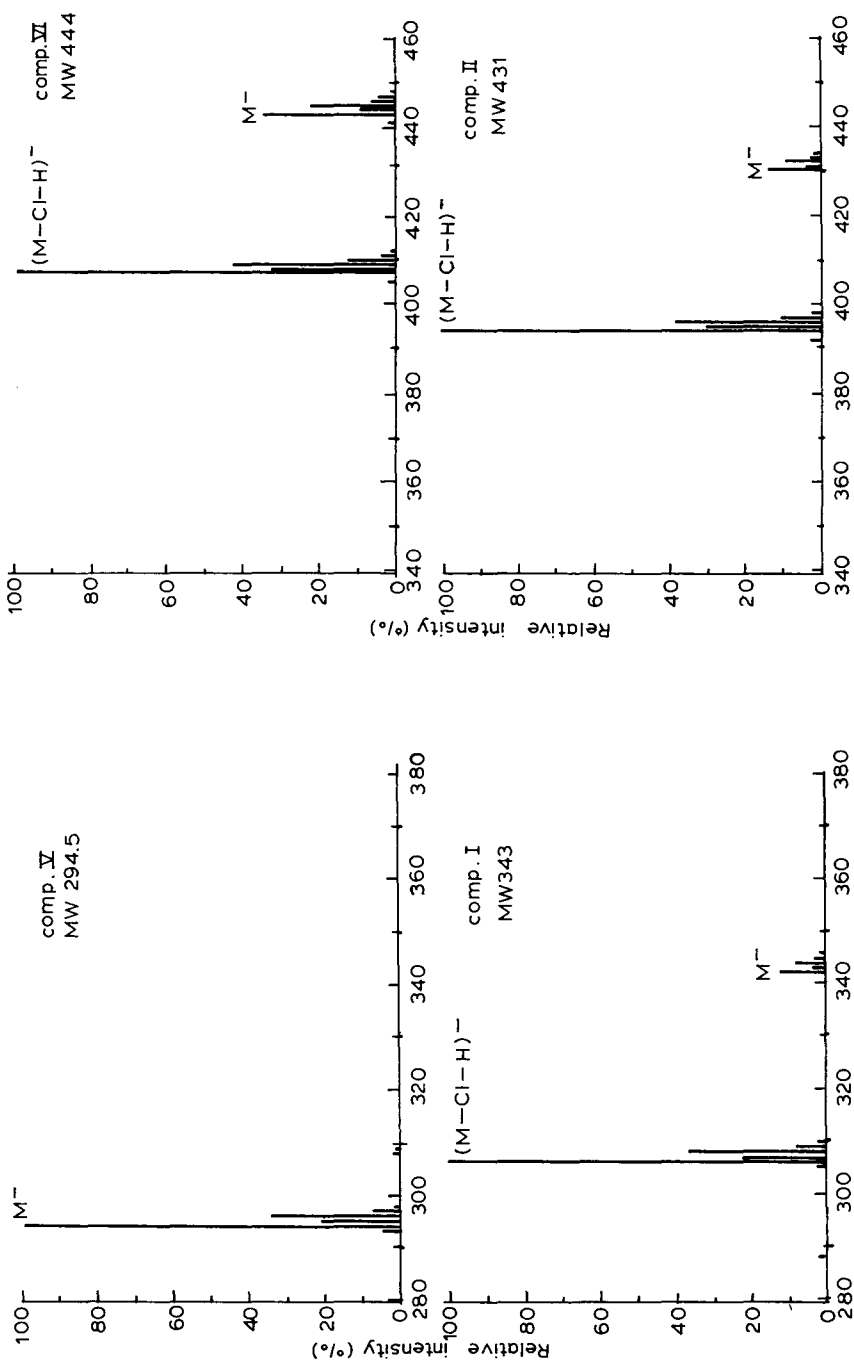


Fig. 2. Molecular mass spectra of analyzed compounds (T and 1-HMT) and respective internal standards (ET and Ro 21-6962).

TABLE III

INTRA-DAY REPRODUCIBILITY OF TRIAZOLAM AND 1-HYDROXYMETHYLTRIAZOLAM PLASMA ASSAY

n varies from 6 to 8.

Triazolam			1-Hydroxymethyltriazolam		
Concentration added (ng/ml)	Concentration found (ng/ml)	C.I.M.* (%)	Concentration added (ng/ml)	Concentration found (ng/ml)	C.I.M.* (%)
5.40	5.18	3	4.30	4.14	3.3
2.70	2.83	4.7	2.15	2.40	3.4
2.15	2.42	6.1	1.07	1.28	3.6
1.07	1.19	6.1	0.53	0.62	7.5
0.53	0.60	7.0			

*C.I.M. = confidence interval of mean (significance level = 0.05).

and derivatized according to the described procedure and chromatographed in triplicate. From the mean of peak area ratios T/ET and 1-HMT/Ro 21-6962 obtained for each investigated concentration, the equation of a typical calibration curve for triazolam concentrations of 2.15—21.5 ng/ml (estazolam: 11.6 ng) was $y = 0.8493 \cdot 10^{-2}x - 0.003$ (correlation coefficient $r = 0.9995$) and for 1-hydroxymethyltriazolam concentrations of 1.07—8.56 ng/ml, $y = 0.3530x + 0.005$ ($r = 0.9999$) (Ro 21-6962: 50 μl of a 0.5% methanol dilution of working solutions).

The sensitivity limit (Fig. 1) for plasma determinations, defined by a signal/noise ratio of about 3, was about 0.1—0.2 ng/ml with a 1.0-ml plasma specimen (this corresponds to an absolute amount of 10 pg of T or 1-HMT per injection). At this concentration, the relative error of the measurements, determined by analysing six control plasmas containing 0.2 ng/ml of standards, was about 15%.

Specificity

In relation to other hydroxylated metabolites of triazolam, the interference of 1-HM-4-HT can be neglected since this compound is, at most, present in trace amounts, while the formation of 4-HT seems to be a minor pathway of triazolam metabolism in man. This latter compound was incompletely derivatized using the conditions described. The retention time was slightly lower than that of triazolam and the detection limit higher. Moreover, during routine determinations on subject plasma, no interference near to the triazolam peak was observed. The best evidence for specificity came from the good agreement between the results obtained from the analysis of samples by both GC and GC—MS (Fig. 4), as mentioned below.

Validation of assay by GC—MS

The validity of the assay developed for plasma determinations of T and its main hydroxylated metabolite 1-HMT was confirmed by GC combined with an

identification of peak compounds by mass spectrometry. The GC—MS apparatus was operated in the negative CI mode. GC separations were carried out using a fused-silica wall-coated open tubular capillary column (12 m × 0.2 mm) with OV-101 as stationary phase. Oven temperature was programmed during analysis from 180°C to 280°C at a step rate of 15°C/min. The solid injector, ion source, and GC—MS interface were held at 300°C, 150°C and 270°C, respectively. Helium was used as carrier gas at a head column pressure of 0.5 bar. Methane was the reagent gas with an ion source pressure of 1 Torr. The mass spectrometer was operated with an emission current of 300 μA and an electron energy of 150 eV.

Under these conditons, mass spectra of T and 1-HMT with their respective internal standards, ET and Ro 21-6962, were obtained as presented in Fig. 2.

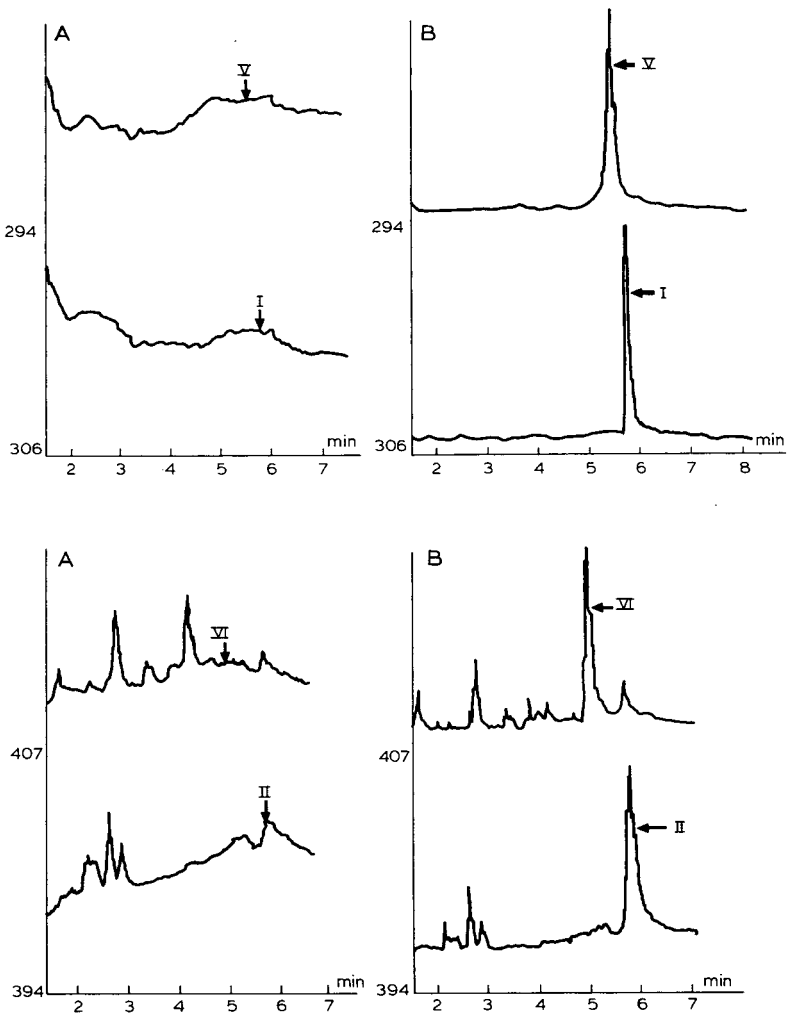


Fig. 3. Chromatograms obtained by GC—MS—CI after extraction and derivatization from (A) control subject plasma; (B) subject plasma 1.5 h following a 1-mg intravenous bolus of triazolam (same concentrations as in Fig. 1B). For peak labelling see Table I.

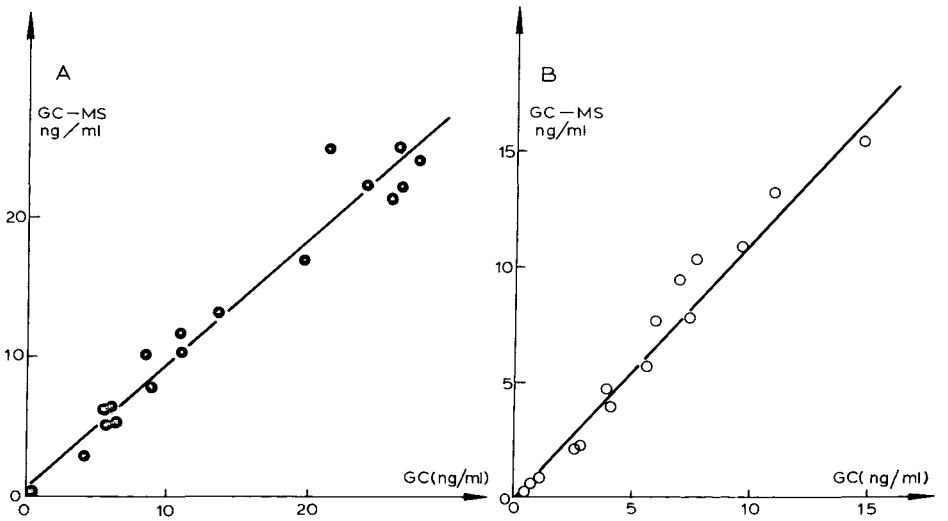


Fig. 4. Correlation between GC and GC—MS—CI results for triazolam (A) and 1-hydroxymethyltriazolam (B) plasma measurements.

These mass spectra were characterized by base isotopic peaks $(M - Cl - H)^-$ at 306, 394 and 407, respectively, for T and the TMS derivatives of 1-HMT and Ro 21-6962, and $(M)^-$ at 294 for ET. These ions, being the most intense, were used for quantitative determinations by mass fragmentography.

Typical chromatograms obtained following extraction and derivatization of subject plasmas, before administration of triazolam (blank), and 1.5 h after intravenous administration of 1 mg of triazolam are presented in Fig. 3. The same extracts were simultaneously analyzed by GC with ECD and MS detection. Correlations between GC—ECD and GC—MS—CI for 20 measurements are reported in Fig. 4A for triazolam determinations (regression curve $y = 0.8798x + 0.695$, $r = 0.978$) and in Fig. 4B for 1-HMT determinations (regression curve $y = 0.9319x - 0.166$, $r = 0.974$). The experimentally investigated concentrations ranged from 0.2 to about 20 ng/ml for each of these two compounds.

Discussion of application

The sensitivity of detection by ECD or MS operating in the negative CI mode, combined with the high efficiency of capillary columns, is sufficient to allow the pharmacokinetics in man of a benzodiazepine such as triazolam, under a very low dose administration (0.5—1.0 mg), to be followed. If only triazolam is to be determined, then the extraction procedure described may be used without derivatization and with different chromatographic conditions such as 290°C oven temperature and 0.9 bar column head pressure. This should allow a lower detection limit for plasma assay, the sensitivity limiting factor being glass adsorption during extraction and column adsorption. However, if simultaneous determination of T and 1-HMT is required, an extraction procedure with an acid-wash step and derivatization is imperative to prevent plasma extract contamination.

Application

Preliminary pharmacokinetic studies in six healthy volunteers following cross-over intravenous bolus of 1 mg of triazolam and 1 mg of 1-hydroxymethyltriazolam, with a one-week interval between the two administrations, were performed. Extensive treatment of these experimental data will be the subject of a further publication.

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REFERENCES

1 M. Monti, Meth. and Find. Exp. Clin. Pharmacol., 3 (1981) 303.
2 F.S. Eberts, Y. Philopoulos, L.M. Reineke and R.W. Vliek, Clin. Pharmacol. Ther., 29 (1981) 81.
3 F.S. Eberts, Y. Philopoulos and R.W. Vliek, Pharmacologist, 21 (1979) 168.
4 D.D. Breimer, R. Jochemsen and H.H. Von Albert, Arzneim.-Forsch., 30 (1980) 875.
5 R. Jochemsen and D.D. Breimer, J. Chromatogr., 223 (1981) 438.
6 D.J. Greenblatt, M. Divoll, L.J. Moschitto and R.I. Shader, J. Chromatogr., 225 (1981) 202.
7 W.J. Adams, U.M. Rykert and P.A. Bombardt, Anal. Lett., 13 (1980) 149.
8 W.J. Adams, Anal. Lett., 12 (1979) 657.
9 H. Ko, M.E. Royer, J.B. Hester and K.T. Johnston, Anal. Lett., 10 (1977) 1019.
10 J. Lund, Scand. J. Clin. Lab. Invest., 41 (1981) 275.

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CHROMBIO. 1580

DETERMINATION OF PROPRANOLOL IN PERITONEAL DIALYSIS FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITHOUT EXTRACTION

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SUMMARY

A rapid, sensitive method for the high-performance liquid chromatographic determination of propranolol in peritoneal dialysis fluid is described. An extraction step is replaced by the use of a C_{18} Sep-Pak® cartridge for sample preparation. The procedure offers an acceptable alternative to sample extraction and will allow for pharmacokinetic studies of propranolol in patients undergoing peritoneal dialysis for chronic renal failure.

INTRODUCTION

Propranolol·HCl is a well known beta antagonist having a wide variety of indications in medical practice [1]. Its use in the therapy of hypertension in normal individuals and in patients with end-stage renal disease is well documented [2—5]. Propranolol pharmacokinetics have been studied in patients undergoing hemodialysis [6—9], but no information is available concerning kinetics of the drug in patients undergoing peritoneal dialysis.

Analytical procedures utilizing high-performance liquid chromatography (HPLC) have been described for the determination of propranolol·HCl in plasma and urine [10—16]. These procedures call for extraction of the drug from the biological fluid, at a basic pH, into an organic solvent. While some published procedures then use an additional acid extraction from the organic solvent [10—13], most procedures call for evaporation of the organic solvent followed by reconstitution of the residue with another solvent followed by injection into an HPLC system. One published report utilizes protein precipitation rather than extraction [17]. Almost all published HPLC procedures use a fluorescence detector with a variety of wavelengths for excitation

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and emission [10—17]. No analytical procedures have been described for the determination for propranolol in peritoneal dialysis fluid.

This paper describes a new procedure for the HPLC determination of propranolol in peritoneal dialysis fluid obtained from patients undergoing continuous ambulatory peritoneal dialysis [18] for end-stage renal disease. Previously described extraction procedures were found to give unreliable results when used with dialysis fluid. Consequently the extraction step was replaced by the use of a C_{18} Sep-Pak cartridge (supplied by Waters Assoc., Milford, MA, U.S.A.). The stability of propranolol in dialysis fluid was also studied.

EXPERIMENTAL

Instrumentation

A high-performance liquid chromatograph equipped with an M-45 pump, U6K injector and reversed-phase C_{18} μBondapak column (all from Waters Assoc.) was used. The detector was a Spectra/Glo fluorometer (Gilson Electronics, Middleton, WI, U.S.A.) equipped with a 45-μl quartz flow-through cell and a 280-nm light source and filter for excitation. An emission filter of 330—380 nm was used. Detector output was recorded with a single-pen 25-cm strip-chart recorder (Linear Instrument, Irvine, CA, U.S.A.).

Chemicals and reagents

Glacial acetic acid and propyl-paraben were reagent grade. Acetonitrile and methanol were HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Propranolol·hydrochloride was supplied courtesy of Ayerst Laboratories (New York, NY, U.S.A.). Peritoneal dialysis fluid (Dianeal-137® with 4.25% dextrose, Travenol Labs., Deerfield, IL, U.S.A.) was obtained from renal failure patients undergoing continuous ambulatory peritoneal dialysis as part of their normal medical therapy.

Mobile phase

The mobile phase was acetonitrile—methanol—glacial acetic acid—deionized water (30:5:1:64), which is a modification of that reported by Pritchard et al. [12]. After filtration and sonification for 15 min the degassed mobile phase was pumped through the column at a flow-rate of 2 ml/min.

Assay standards

A stock solution of propranolol·HCl in methanol (1.00 mg/ml) was prepared. For assay work the stock solution was diluted with methanol so as to contain 4.00 ng/ml. Both stock solution and dilution were prepared fresh monthly and stored at 4°C. The internal standard, propyl-paraben, was prepared in methanol (0.499 mg/ml). It was also prepared monthly and stored at 4°C. No changes in the chromatograms, or extra peaks, were noted during use of the standard solutions over a period of 30 days.

Analytical procedure

Dry C_{18} Sep-Pak cartridges were prepared for use by passing through approx-

imately 2 ml of methanol followed by approximately 4 ml of deionized water. Sep-Pak cartridges were used only once and then discarded because Sep-Pak cartridges which had been cleaned and re-used gave unreliable results.

Dialysis fluid

To 6 ml of peritoneal dialysis fluid were added 100 μl of internal standard solution and an aliquot of the dilute propranolol standard containing 40—400 ng of drug as the hydrochloride salt (10—100 μl of standard dilute solution). Each sample was then vortexed for 10 sec and a 5-ml aliquot was passed through a prepared Sep-Pak at a rate of approximately 100 drops per min. Drug and internal standard were then eluted from the Sep-Pak by passing 2 ml of a wash solution through the Sep-Pak. The wash solution was acetonitrile—methanol—deionized water—glacial acetic acid (40:20:39:1). Injections of 200 μl were made from this wash solution using a 500-μl syringe (Hamilton, Reno, NV, U.S.A.). The chromatograms were recorded at a chart speed of 15 cm/h and separation was adequate for measurement of peak heights (see Fig. 1). Peak height ratios (propranolol to internal standard) were calculated and plotted versus propranolol concentration expressed as ng/ml.

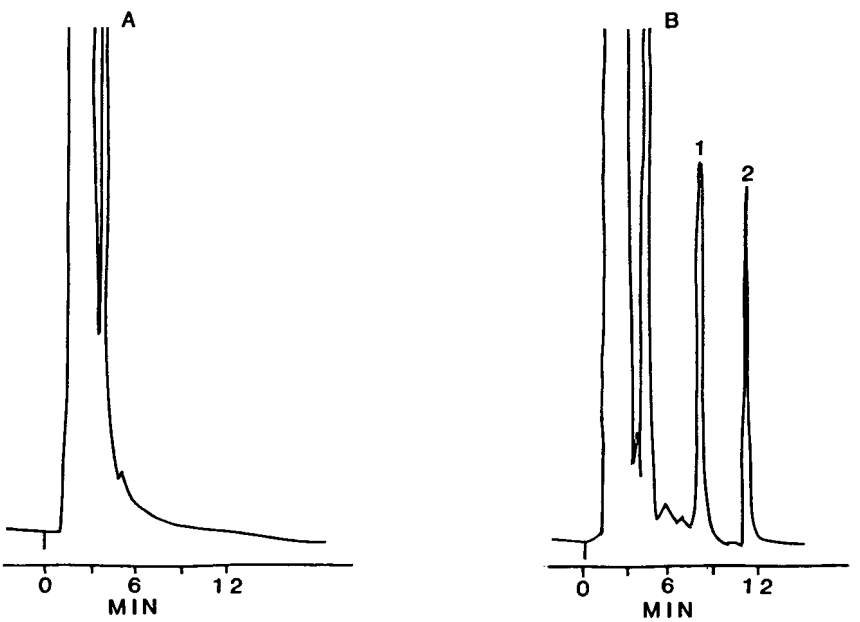

Fig. 1. Assay of propranolol in peritoneal dialysis fluid obtained from renal failure patients undergoing continuous ambulatory peritoneal dialysis. (A) Propranolol-free dialysis fluid; (B) dialysis fluid with added propranolol. Peaks: 1 = propranolol, 32 ng/ml; 2 = propylparaben (internal standard).

Stability study

Volumes of 500 ml of dialysis fluid containing 16 and 64 ng/ml of propranolol were prepared and divided into thirty, 15-ml samples for each concentration. The samples were placed in 50-ml, glass, screw-top bottles and

fifteen samples of each concentration were stored at refrigerator temperature (5°C) and −60°C. Three samples at each concentration and storage condition were assayed at days 0, 3, 7, 10 and 14.

Recovery study

Recovery of both propranolol and internal standard was studied in dialysate. The procedure used for the study is shown in Table I. Sample 2 represents 100% recovery of drug since the propranolol was added to the wash solution after the wash was passed through the Sep-Pak. Likewise sample 3 represents 100% recovery of internal standard. The procedure followed for sample 1 follows that used for the standard curves as run in dialysis fluid. Mean peak height ratios were determined for each sample and percent recovery of propranolol and internal standard determined by comparing samples 1 and 2 and samples 1 and 3, respectively.

TABLE I

PROCEDURE FOR RECOVERY STUDY, PERITONEAL DIALYSIS FLUID

Samples 2 and 3 represent 100% recovery of drug and internal standard, respectively. Sample 1 represents the standard curve procedure.

Sample No.		
1	2	3
1 ml I.S.* + 500 µl propranolol** + dialysate to a final volume of 50 ml	1 ml I.S.* + 500 µl methanol + dialysate to a final volume of 50 ml	1 ml methanol + 500 µl propranolol** + dialysate to a final volume of 50 ml
↓	↓	↓
5 ml aliquot*** through Sep-Pak	5 ml aliquot*** through Sep-Pak	5 ml aliquot*** through Sep-Pak
↓	↓	↓
2 ml wash§ through Sep-Pak	2 ml wash§ through Sep-Pak	2 ml wash§ through Sep-Pak
↓	↓	↓
add 100 µl methanol	add 50 µl propranolol** + 50 µl methanol	add 100 µl I.S.*
↓	↓	↓
200 µl injected	200 µl injected	200 µl injected

*Internal standard, 0.499 ng/ml.
**Dilute standard solution, 4 ng/ml.
***Repeated five times with new Sep-Paks.
§ Acetonitrile—methanol—deionized water—glacial acetic acid (40 : 20 : 39 : 1).

RESULTS AND DISCUSSION

Recovery of both propranolol and internal standard from dialysate was 100%. Using Student's t test there was no significant difference in peak height ratios when comparing samples 1 and 2 and samples 1 and 3 ($P > 0.05$). Reproducibility of recovery was good. The percent coefficient of variation in peak height ratios for sample 1 (five replications) was 3.1%. Recovery of propranolol from plasma using various extraction techniques has been reported as 80±5% [10,19] to 90% [11].

The pH of the propranolol solution does not seem to effect binding of the drug to the Sep-Pak. When 2 ml of a solution of propranolol in sodium carbonate (pH 11.4) was passed through the Sep-Pak no drug was detected in the effluent. When 2 ml of wash solution was then passed through the Sep-Pak recovery of propranolol was 100%. Likewise, when 2 ml of a solution of propranolol·HCl in dilute hydrochloric acid (pH 3.1) was passed through another new Sep-Pak no drug was detected in the effluent. However, when 2 ml of wash solution was passed through the Sep-Pak recovery of drug was approximately 80%. The reason for this change in binding is unknown. Studies of the effect of pH on binding of internal standard were not done.

Five standard curves (6.28–65.7 ng/ml) in dialysate were run over a period of fourteen days. Linear regression analysis was used to calculate the slope and intercept for each curve. Mean percent of theory for each curve was calculated using the technique of inverse estimation [20]. These results are shown in Table II. Curves 4 and 5 were run on a new C_{18} μBondapak column which may account for the change in slope and intercept. Mean percent of theory for the five curves was 100.9% with a mean coefficient of variation of 5.1%. Day-to-day accuracy and precision of the assay are shown by the data in Table III. Mean retention times for propranolol and internal standard were 7.5 and 10.5 min, respectively. It was possible to decrease the lower limit of the assay to 1.79 ng/ml by doubling the volume of dialysate passed through the Sep-Pak from 5 to 10 ml. Three standard curves were run at approximately 30-day intervals using this procedure. Mean percent of theory was 98.9% with a mean coefficient of variation of 5.0% for these curves. Each curve included four

TABLE II

STANDARD CURVE DATA FOR ASSAY OF PROPRANOLOL·HCl IN PERITONEAL DIALYSIS FLUID

Five points (6.28—65.7 ng/ml) included for each curve. Data collected over fourteen days at approximately 2-day intervals.

Curve	Correlation coefficient (r)	Slope	Intercept	Mean percent of theory	Coefficient of variation (%)
1	0.999	0.0448	−0.0655	100.3	3.0
2	0.999	0.0432	−0.0645	101.5	4.5
3	0.999	0.0415	−0.0502	101.2	6.3
4	0.997	0.0335	−0.0918	103.3	9.8
5	0.999	0.0315	0.0203	99.3	2.0

TABLE III

PRECISION AND ACCURACY FOR ASSAY OF PROPRANOLOL·HCl IN PERITONEAL DIALYSIS FLUID

Data obtained from standard curves run over a period of fourteen days at approximately 2-day intervals. Each experimental concentration is the mean of five determinations.

Actual concentration (ng/ml)	Mean experimental concentration (ng/ml)	Mean percent of theory	Coefficient of variation (%)
6.28	6.74	107.3	8.5
16.55	16.66	100.7	3.7
32.87	31.86	96.9	2.7
49.15	49.12	99.9	3.0
65.70	66.15	100.7	1.9
Mean ± S.D.		101.1 ± 3.8	3.96 ± 2.6

TABLE IV

STABILITY DATA FOR PROPRANOLOL·HCl IN PERITONEAL DIALYSIS FLUID

Values represent the mean percent remaining ± S.D. of three determinations at the times indicated.

Time (days)	Percent remaining			
	5°C		−60°C	
	16 ng/ml	64 ng/ml	16 ng/ml	64 ng/ml
0	116.8 ± 1.0	105.9 ± 1.7	116.8 ± 1.0	105.9 ± 1.7
3	104.3 ± 6.07	105.4 ± 2.84	107.7 ± 3.03	107.3 ± 2.06
7	100.0 ± 3.92	105.0 ± 1.33	105.0 ± 0.50	105.6 ± 0.61
10	111.3 ± 2.55	98.9 ± 1.87	111.0 ± 4.45	100.5 ± 2.48
14	103.3 ± 2.51	104.3 ± 1.79	107.2 ± 5.31	115.2 ± 1.85

points and ranged in concentration from 1.79—33.12 ng/ml. The correlation coefficient was 0.999. These results in dialysate are comparable to previously published urine and plasma studies using extraction for sample preparation [12—14].

Propranolol, at a concentration of both 16 and 64 ng/ml, was stable in dialysis fluid for up to fourteen days when stored at either 5°C or −60°C (see Table IV). Previously published studies have shown propranolol to be stable in plasma for up to three days when frozen [7,21].

The use of C_{18} Sep-Pak cartridges for sample preparation offers an acceptable alternative to extraction for the determination of propranolol in peritoneal dialysis fluid. The availability of a procedure to measure propranolol in peritoneal dialysis fluid will allow for studies of propranolol clearance in renal failure patients undergoing peritoneal dialysis as part of their medical therapy.

ADDENDUM

The use of a C_{18} Sep-Pak cartridge for determination of propranolol in

plasma was investigated although this was not the primary purpose of this project. Protein precipitation was accomplished by adding acetonitrile containing internal standard to the plasma sample. After mixing and centrifugation an aliquot of supernatant was passed through a prepared Sep-Pak. Drug and internal standard were then eluted from the Sep-Pak as described for dialysate samples. Recoveries of drug and internal standard were 71.2% and 72.8%, respectively. The recovery study for plasma was similar to that described for dialysis fluid. Although incomplete, recovery was reproducible. The percent coefficient of variation in peak height ratios (five replications) was 4.4%. A five-point standard curve ranging from 18 to 190 ng/ml was run following the procedure described above. The correlation coefficient, mean percent of theory and coefficient of variation were 0.995, 104% and 7.3, respectively. It appears that this technique may be suitable for the determination of propranolol in plasma. Narasimhachari [22] recently evaluated C_{18} Sep-Pak cartridges in the determination of tricyclic antidepressants in plasma and urine. He concluded that the cartridges were quite useful and provided a considerable saving in time.

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REFERENCES

1 Physicians' Desk Reference, Medical Economics Co., New Jersey, 1982, p. 64.
2 F.O. Simpson, Drugs, 7 (1974) 85.
3 F.D. Thompson and A.M. Joekes, Brit. Med. J., 2 (1974) 555.
4 O.B. Holland and N.M. Kaplan, N. Engl. J. Med., 294 (1976) 930.
5 F.A. Finnerty, Jr., Clin. Ther., 1(2) (1977) 135.
6 F.D. Thompson, A.M. Joekes and D.M. Foulkes, Brit. Med. J., 2 (1972) 434.
7 D.T. Lowenthal, W.A. Briggs, T.P. Gibson, H. Nelson and W.J. Cirksena, Clin. Pharmacol. Ther., 16 (1974) 761.
8 G. Bianchetti, G. Graziani, D. Brancaccio, A. Morganti, G. Leonetti, M. Manfrin, R. Sega, R. Gomeni, C. Ponticelli and P.L. Morselli, Clin. Pharmacokin., 1 (1976) 373.
9 D.T. Lowenthal, Amer. J. Med., 62 (1977) 532.
10 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 70 (1981) 688.
11 G. Nygard, W.H. Sheluer and S.K. Khalil, J. Pharm. Sci., 68 (1979) 379.
12 J.F. Pritchard, D.W. Schneck and A.H. Hayes, Jr., J. Chromatogr., 162 (1979) 47.
13 R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 145 (1978) 429.
14 D.W. Schneek, J.F. Pritchard and A.H. Hayes, Jr., Res. Comm. Chem. Path. Pharmacol., 24 (1979) 3.
15 A. Taburet, A.A. Taylor, J.R. Mitchell, D.E. Rollins and J.L. Pool, Life Sci., 24 (1979) 209.
16 W.D. Mason, E.N. Amick and O.H. Weddle, Anal. Lett., 10 (1977) 515.
17 M. Lo and S. Riegelman, J. Chromatogr., 183 (1980) 213.
18 R.D. Popovich, J.W. Moncrief, K.D. Nolph, A.J. Ghods, Z.J. Twardowski and W.K. Pyle, Ann. Intern. Med., 88 (1978) 449.

19 J.W. Black, W.A.M. Duncun and R.G. Shanks, Brit. J. Pharmacol., 25 (1965) 577.
20 J.W. Ayres, E. Sakmar, M.R. Hallmark and J.G. Wagner, Res. Comm. Chem. Path. Pharmacol., 16 (1977) 475.
21 D.G. Shand, E.M. Nuckolls and J.A. Oates, Clin. Pharmacol. Ther., 11 (1970) 112.
22 N. Narasimhachari, J. Chromatogr., 225 (1981) 189.

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DETERMINATION OF HEPTAMINOL IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Heptaminol was measured in plasma and urine following pre-column derivatisation with *o*-phthalaldehyde and reversed-phase high-performance liquid chromatography employing fluorescence detection. The limits of detection were sufficient for pharmacokinetic studies of the drug after clinically-used doses. Plasma concentrations of heptaminol reached peak levels (2.19 μg/ml) at 0.75 h after single oral doses (0.47 g of heptaminol) and declined with a half-life of 2.1 h (± 0.5 S.D.). Heptaminol was well absorbed and excreted rapidly, mainly unchanged in urine, 82% dose (± 10 S.D.).

INTRODUCTION

Heptaminol (6-amino-2-methyl-2-heptanol) hydrochloride (Fig. 1a) has been used for many years as a cardiotonic agent with a positive inotropic action [1]. Heptaminol has been measured in human urine by gas—liquid chromatography [2, 3] and in rat plasma and urine using radiochemical procedures [4]. Two further methods have been reported incorporating derivatisation of heptaminol to form fluorescent adducts [5, 6] but were not applied to biological samples.

The reaction of primary amines with *o*-phthalaldehyde (OPA) in alkaline medium in the presence of 2-mercaptoethanol to form highly fluorescent adducts is well established [7]. Pre-column fluorescence derivatisation of

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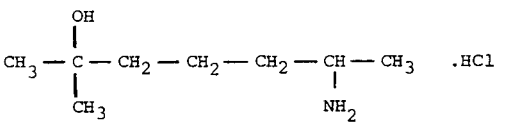
(a)



(b)

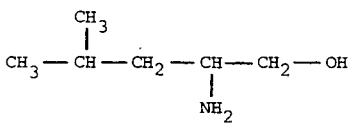


Fig. 1. Chemical structures of (a) heptaminol hydrochloride and (b) leucinol.

amino acids with OPA followed by high-performance liquid chromatography (HPLC) in a reversed-phase mode has been previously used [8].

This paper describes a simple and rapid procedure for the measurement of heptaminol in plasma and urine using pre-column OPA derivatisation and HPLC with fluorescence detection. L-Leucinol (2-amino-4-methyl-1-pentanol) (Fig. 1b) is incorporated as an internal standard.

No sample pre-treatment is required other than the removal of plasma proteins. The method is capable of measuring circulating levels of heptaminol in plasma and urine after administration of clinically-used doses of the drug.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Acetonitrile was HPLC-far UV grade (Fisons Scientific Apparatus, Loughborough, Great Britain). *o*-Phthalaldehyde (OPA, Fluoropa®) was purchased from Pierce (Gillingham, Great Britain). Heptaminol hydrochloride was supplied by Delalande (Courbevoie, France). OPA derivatising solution (40.3 m*M*) was prepared as previously described [8]. The solution was stored at room temperature and regenerated every alternate day by adding 2-mercaptoethanol (20 μl). The activity of this solution was checked daily by measuring the chromatographic—fluorimetric response of a standard mixture containing heptaminol and leucinol. An aliquot of a standard mixture (40 μl, containing 200 ng of each compound in acetonitrile) was mixed with the derivatising solution (20 μl) using a vortex mixer for 30 sec and an aliquot (10 μl) of this mixture injected into the chromatograph. Response of this standard mixture showed a coefficient of variation of ± 3% during a four-week period.

Standard solutions of heptaminol and leucinol were prepared at concentrations of 1 mg free base per ml and 10 μg free base per ml in acetonitrile and stored at 4°C in the dark.

Plasma derivatisation procedure

Conical centrifuge tubes (10 ml capacity) were spiked with internal standard solution (10 μl, containing 100 ng leucinol in acetonitrile). The total volume of acetonitrile was adjusted to 100 μl and plasma (100 μl) was slowly added so

that precipitation of the proteins occurred. The tubes were vortex mixed for 30 sec and then centrifuged at 2000 *g* for 10 min. Aliquots (50 μl) of the acetonitrile layer were removed to clean conical tubes and derivatising solution (20 μl) was added with mixing using a vortex mixer for 30 sec. Aliquots (40 μl) of this solution were then immediately injected into the liquid chromatograph.

Urine derivatisation procedure

Aliquots (10 μl or 100 μl) of urine samples diluted 1 in 100 with glass-distilled water were transferred into conical tubes, spiked with internal standard solution (10 μl, containing 100 ng leucinol in acetonitrile). The total volume of acetonitrile was adjusted to 100 μl and the tubes mixed for 10 sec using a vortex mixer. Derivatising solution (20 μl) was added to the tubes with mixing using a vortex mixer for 30 sec and aliquots (30 μl) of this solution immediately injected into the liquid chromatograph.

Calibration procedures

Samples of control (drug-free) plasma (100 μl) were spiked with amounts equivalent to 10, 20, 40, 80, 120 and 200 ng of heptaminol free base and taken through the plasma derivatisation procedure. Samples of control urine (10 μl) which had been diluted 1 in 100 with glass-distilled water were spiked with amounts equivalent to 5, 10, 20, 40, 60, 80, 100, 120 and 150 ng of heptaminol free base and taken through the urine derivatisation procedure.

Instrumentation

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Cheshire, Great Britain) connected to an LC1000 fluorescence detector (Perkin-Elmer, Beaconsfield, Great Britain) using a 338-nm excitation filter and an emission wavelength of 445 nm. Injection was performed manually using a U6K universal injector (Waters Assoc.). Chromatograms and peak area measurements were recorded using a 3380A computing integrator (Hewlett-Packard, Hitchin, Great Britain).

Chromatography

Chromatography was performed in a reversed-phase mode using a mobile phase of 45% (v/v) acetonitrile in aqueous sodium acetate buffer (0.05%, w/v) with a final pH adjustment to 6.1 using glacial acetic acid. The column used for the analysis was constructed of stainless-steel (25 cm × 0.46 cm I.D.) and packed with Zorbax® C_8 (mean particle diameter 6 μm) (Dupont, Stevenage, Great Britain). A pre-column constructed of stainless steel (7 cm × 0.2 cm I.D.) and dry-packed with Co:Pell® ODS (particle diameter 25—37 μm) (Whatman, Maidstone, Great Britain) was installed in series in front of the main analytical column to protect it from contamination and could be changed if the back-pressure in the system increased beyond reasonable limits (> 280 bar). A mobile phase flow-rate of 2.5 ml/min was maintained, and under these conditions the adducts of heptaminol and leucinol had retention times of about 6.5 min and 7 min, respectively (Fig. 2). Samples could be injected every 10 min.

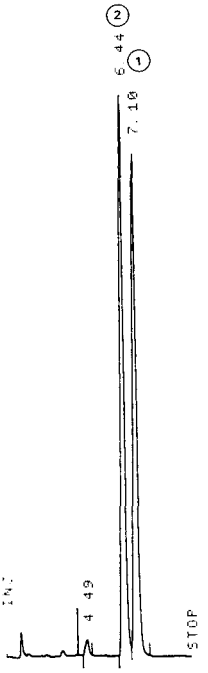


Fig. 2. Chromatogram of OPA-adducts of leucinol (peak 1) and heptaminol (peak 2). Column: 25 cm × 0.46 cm I.D., containing Zorbax C_8; flow-rate: 2.5 ml/min; solvent system: 45% (v/v) acetonitrile in aqueous sodium acetate, pH 6.1; detector: fluorescence, excitation wavelength = 338 nm, emission wavelength = 445 nm, scale expansion × 50.

Studies in human subjects

Plasma and urine samples were obtained from five human volunteer subjects dosed orally with a syrup formulation containing 0.47 g heptaminol and analysed by the foregoing procedures. These volunteer studies were conducted under conditions similar to those described by Brodie et al. [9].

RESULTS AND DISCUSSION

Heptaminol shows negligible UV—visible spectral properties but does possess a primary amine group which can be readily reacted with derivatising agents to form fluorescent adducts. Of the more popular fluorescence derivatising procedures, dansylation required elevated temperature and rather long reaction times, fluorescamine reacted rapidly at room temperature but in common with amino acids resulted in the formation of two fluorescent derivatives which separated during chromatography [10]. OPA gave the most satisfactory results. The derivatisation occurred rapidly at room temperature, was reproducible and the resultant adducts could be chromatographed with short analysis times.

Precision and accuracy of measurement

Derivatisation and measurement were repeated on five occasions at each concentration over the calibration ranges during a three-day period. The

TABLE I

BETWEEN-ASSAY PRECISION MEASUREMENTS OF HEPTAMINOL IN PLASMA

Concentration of heptaminol (μg/ml)	Peak area ratio $\frac{\text{heptaminol}}{\text{internal standard}}$					Mean (± S.D.)	Coefficient of variation (%)
0.1	0.10	0.10	0.12	0.09	0.08	0.10 (0.01)	10
0.2	0.18	0.21	0.18	0.19	0.20	0.19 (0.01)	5
0.4	0.35	0.37	0.38	0.37	0.37	0.37 (0.01)	3
0.8	0.74	0.73	0.74	0.77	0.72	0.74 (0.02)	3
1.2	1.07	1.09	1.11	1.11	1.13	1.10 (0.02)	2
2.0	1.76	1.73	1.83	1.77	1.82	1.78 (0.04)	2

TABLE II

BETWEEN-ASSAY PRECISION MEASUREMENTS OF HEPTAMINOL IN URINE

Concentration of heptaminol (μg/ml)	Peak area ratio $\frac{\text{heptaminol}}{\text{internal standard}}$					Mean (± S.D.)	Coefficient of variation (%)
0.5	0.06	0.03	0.06	0.05	0.05	0.05 (0.01)	20
1	0.09	0.09	0.10	0.09	0.11	0.10 (0.01)	10
2	0.19	0.18	0.20	0.19	0.19	0.19 (0.01)	5
4	0.36	0.36	0.38	0.38	0.39	0.37 (0.01)	3
6	0.54	0.55	0.56	0.59	0.59	0.57 (0.02)	4
8	0.72	0.74	0.73	0.75	0.77	0.74 (0.02)	3
10	0.92	0.93	0.91	0.92	0.95	0.93 (0.02)	2
12	1.06	1.10	1.10	1.10	1.20	1.11 (0.05)	5
15	1.31	1.35	1.32	1.40	1.45	1.37 (0.06)	4

between-day precision of the method for the plasma assay, as indicated by the coefficient of variation of peak area ratio measurements of drug to internal standard ranged from ± 10% at 0.1 μg/ml to ± 2% at 2.0 μg/ml (Table I). The precision of the method for the urine assay was ± 20% at 0.5 μg/ml to ± 4% at 15 μg/ml (Table II).

The calibration line for the determination of heptaminol in plasma constructed from five replicate measurements at six concentrations in the range 0.1—2 μg/ml, was linear (Y = 0.017 + 0.884X). The accuracy of the method as indicated by the standard error of the fitted least squares regression line was ± 0.027 μg/ml.

The calibration line for the estimation of heptaminol in urine, constructed from five replicate measurements at nine concentrations over the range 0.5—15 μg/ml of diluted urine was also linear (Y = 0.009 + 0.0913X). The standard error of this fitted line was ± 0.3 μg/ml.

Recovery and limit of detection

Heptaminol was completely recovered from plasma and urine. No interfering peaks with the same retention time as heptaminol were present in control plasma and urine samples, although a small peak was sometimes present with a similar retention time to that of the internal standard (Fig. 3). The concentration of internal standard was selected so that the contribution of this occasional component was negligible.

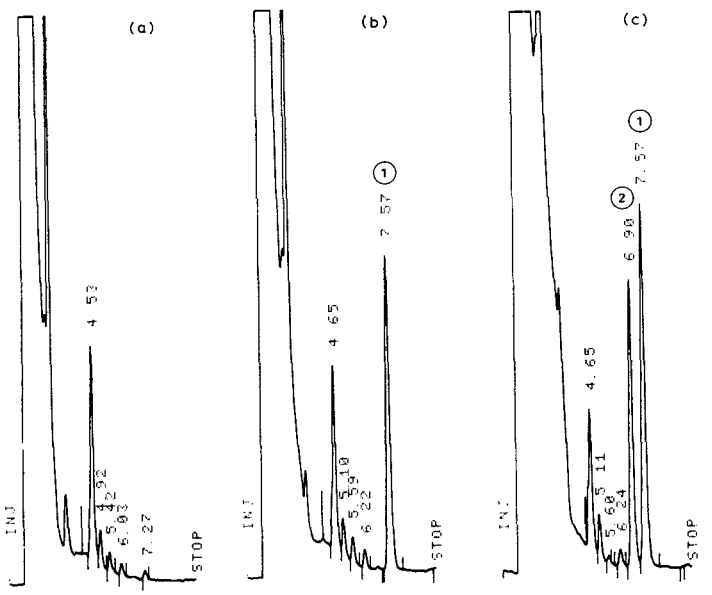


Fig. 3. Chromatograms of (a) control plasma (b) control plasma containing internal standard and (c) plasma containing heptaminol at a concentration of 0.8 μg/ml. Peaks: (1) = internal standard adduct; 2 = heptaminol adduct. Chromatography conditions as for Fig. 2.

Concentrations of heptaminol in plasma could be measured down to 0.1 μg/ml when assaying 100 μl plasma and using a final injection volume of 40 μl. This represents < 2 ng of heptaminol on column. The sensitivity of the method could be improved if necessary by analysing larger volumes of plasma or injecting larger volumes of derivatised solution.

Concentrations of heptaminol in urine could be measured down to 0.05 μg/ml of diluted urine when assaying 100 μl and using a final injection volume

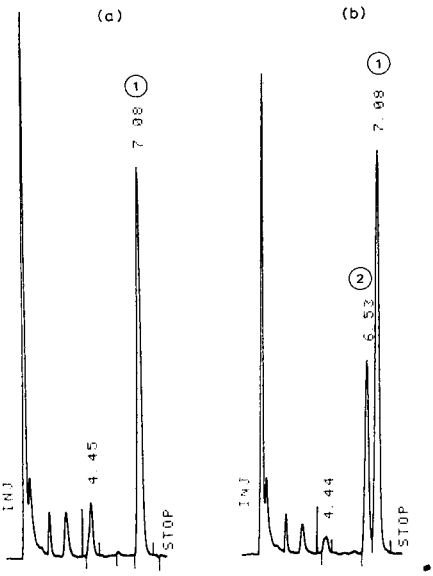


Fig. 4. Chromatograms of (a) pre-dose human urine and (b) 0—12 h diluted human urine containing heptaminol at a concentration of 4.8 μg/ml. Peaks: 1 = internal standard adduct; 2 = heptaminol adduct. Chromatography conditions as for Fig. 2.

of 30 μl. The sensitivity of the method is more than adequate since urinary concentrations of heptaminol are high (Fig. 4). As a highly polar compound, heptaminol is not metabolised to any extent, and between 75 and 95% of an oral dose is eliminated as unchanged drug within 24 h of dosing [2].

Concentrations of heptaminol in human plasma and urine

After single oral doses of a syrup formulation containing 0.47 g heptaminol, a peak of mean plasma heptaminol concentrations of 2.19 μg/ml occurred at 0.75 h and remained at about this level for about 2 h (Table III), indicating that heptaminol was relatively rapidly absorbed from the gastrointestinal tract of humans. Thereafter plasma levels of heptaminol declined monoexponentially with a half-life of 2.1 h (± 0.5 S.D.). Assuming complete absorption of heptaminol, the mean clearance and volume of distribution of heptaminol were 48.5 l/h (± 14.5 S.D.) and 145 l (± 45 S.D.), respectively. This clearance is numerically similar to the renal plasma flow-rate indicating that the renal extraction ratio for heptaminol is close to unity.

Urinary excretion of unchanged heptaminol was almost complete (77 ± 11% S.D.) within 12 h of dosing as might be expected from the plasma drug clearance since heptaminol is eliminated mainly, if not totally, by renal excretion. A mean of 82 ± 10% S.D. (range 68—92%) of the administered dose was excreted in the urine as unchanged drug. These data indicate that heptaminol was well absorbed from the gastrointestinal tract of humans. Similar results have been obtained by Chanoine et al. [3] who also reported a biexponential decline of plasma concentrations (half-lives 1.8 and 6.6 h) in contrast to the monoexponential decline obtained in the present study over the same time-course. Nonetheless, consideration of the urinary excretion data indicates that

TABLE III

PLASMA CONCENTRATIONS (μg/ml) OF HEPTAMINOL AFTER SINGLE ORAL DOSES OF 0.47 g HEPTAMINOL TO HUMAN SUBJECTS

For calculation of means, the concentration is taken as zero if < 0.10 μg/ml.

Time (h)	Subject No.					Mean	S.D.
	1	2	3	4	5		
0.25	0.45	0.28	0.63	2.37	1.48	1.04	0.87
0.50	1.40	2.00	1.20	2.16	2.11	1.77	0.44
0.75	2.68	2.45	1.59	2.37	1.87	2.19	0.45
1.0	2.22	2.33	1.32	2.19	1.80	1.97	0.42
1.5	2.01	2.56	1.41	2.24	1.56	1.96	0.48
2.0	2.63	2.16	1.78	2.51	1.47	2.11	0.49
2.5	2.37	1.94	1.42	2.14	1.49	1.87	0.41
3.0	2.13	1.86	1.31	1.94	1.47	1.74	0.34
4.0	1.83	1.22	0.88	1.33	0.82	1.22	0.41
5.0	1.16	1.03	0.51	1.02	0.61	0.87	0.29
6.0	0.93	0.60	0.40	0.82	0.40	0.63	0.24
7.0	0.60	0.57	0.31	0.66	0.30	0.49	0.17
8.0	0.21	0.44	0.12	0.45	0.19	0.28	0.15
10.0	0.12	0.25	<0.10	0.27	<0.10	0.13	0.13
12.0	<0.10	0.14	<0.10	0.18	<0.10	0.06	0.09

the half-life of about 2 h probably accounts for elimination of most of the administered dose.

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REFERENCES

1 B. Pourrias, M. Sergant and G. Raynaud, Therapie, 24 (1969) 451.
2 J. Rabiant, M. Sergant and A.F. Gaudin, Ann. Pharm. Fr., 29 (1971) 331.
3 F. Chanoine, V. Rovei and M. Strolin Benedetti, Proceedings of the First European Congress of Biopharmaceutics and Pharmacokinetics, Clermont-Ferrand, 1981, Vol. 1, Libraires Lavoisier, Paris, 1981, p. 125.
4 F. Chanoine, M. Strolin Benedetti, J-F. Ancher and P. Dostert, Arzneim.-Forsch., 31 (1981) 1430.
5 W. Messerschmidt, Arzneim.-Forsch., 20 (1970) 726.
6 A. Nicolas, P. Leroy, A. Moreau and M. Mirjolet, J. Chromatogr., 244 (1982) 148.
7 M. Roth, J. Clin. Chem. Clin. Biochem., 14 (1976) 361.
8 P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
9 R.R. Brodie, L.F. Chasseaud, T. Taylor, D.A. O'Kelly and A. Darragh, J. Chromatogr., 146 (1978) 152.
10 W. McHugh, R.A. Sandmann, W.G. Haney, S.P. Sood and D.P. Wittmer, J. Chromatogr., 124 (1976) 376.

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DETERMINATION OF TRIMETHOPRIM, SULPHAMETHOXAZOLE AND ITS N^4-ACETYL METABOLITE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A normal-phase high-performance liquid chromatographic method was developed to determine therapeutic concentrations of trimethoprim, sulphamethoxazole, and its N_4-acetyl derivative in biological fluids. The compounds are extracted at pH 6.2 using ethyl acetate—chloroform in a single extraction. The detection limit is 15 ng/ml for trimethoprim, 20 ng/ml for sulphamethoxazole, and 10 ng/ml for its N^4-acetyl metabolite. The method is rapid, sensitive, precise, and well suited to clinical pharmacokinetic investigations.

INTRODUCTION

Cotrimoxazole, an association of trimethoprim (TMP) and sulphamethoxazole (SMZ) in a 5:1 ratio, is a powerful broad-spectrum antimicrobial agent used clinically for the treatment of a variety of infections in man [1, 2]. In order to study its pharmacokinetic parameters in the elderly, we decided to re-examine determination methods for the constituents of cotrimoxazole and the metabolite, N^4-acetyl sulphamethoxazole (N^4SMZ) in biological fluids.

SMZ determination has been described using gas—liquid chromatography (GLC) with flame-ionisation detection (FID) [3—5] and by high-performance

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liquid chromatography (HPLC) with spectrophotometric detection [6—11]. TMP determination was carried out using GLC [12, 13] with thermoionic detection and, more easily and with more sensitivity, using HPLC [14]. Determinations using GLC—mass spectrometry [15], polarography [16, 17], and microbiological [18, 19] or isotopic [20, 21] assay have also been described.

TMP and SMZ determinations in the cotrimoxazole association can be done using spectrofluorimetry [22], but the method is long, requires two extractions, and is not free from interference. GLC determination with thermionic detection [23] also requires two extractions and preparation of a methyl derivative for separation of SMZ and N^4SMZ. The method is long and not free of interfering peaks after plasma extraction.

Different authors have reported HPLC methods requiring either two extractions and two chromatographic columns [24], or deproteinisation followed by a reversed-phase separation after an unsuitable dilution for TMP pharmacokinetic determinations [25], or separation using two elution solvents and two successive chromatographic operations [26]. A single extraction method proposed by Ascalone [27] with reversed-phase HPLC separation allows the simultaneous determination of TMP, SMZ, and N^4SMZ, with detection limits compatible with pharmacokinetic studies. In our experience, we were not able to obtain acceptable and reproducible plasma extracts using this method, or to reproduce the extraction yields described, which can be prejudicial for a technique developed without an internal standard.

More recently, Ascalone [28] proposed the simultaneous determination of TMP, sulfadiazine and its N^4-acetyl metabolite by normal-phase HPLC but without an internal standard. Gochin et al. [29] described the simultaneous determination of TMP, SMZ and its N^4-acetyl metabolite with reversed-phase HPLC and one internal standard for the sulfonamide derivatives. This method requires two different extractions for serum and urines samples and is time-consuming.

Taking all these facts into account, and also the adequate method described by Weinfeld and Macasieb [14] for TMP determination only, we described a normal-phase HPLC method for the simultaneous determination of TMP, SMZ and N^4SMZ.

The purpose of this study was to determine the solvent composition and optimum pH providing the best compromise for simultaneous extraction and efficient elution of the three products. This new method was applied to determinations in human plasma for therapeutic concentrations of cotrimoxazole.

EXPERIMENTAL

Chemicals

Trimethoprim, sulphamethoxazole, N^4-acetylsulfamethoxazole, and 2,4-diamino-5-(3,5-dimethoxy-4 methylbenzyl) pyrimidine, used as internal standard for TMP (IS-TMP), were supplied by Hoffmann-La Roche (Basle, Switzerland). Sulphamoxol (Justamil®) used as internal standard for the sulphonamides (IS-SMZ) was supplied by Amphar-Rolland (Paris, France). Chloroform, ethyl acetate, methanol suprapur, and 5 μm LiChrosorb Si-60 (Merck 9388) were purchased from E. Merck (Darmstadt, G.F.R.), or a ready-

to-use column Merck Hibar (ref. 50388); ammonia solution of 28–30% was from Prolabo (Paris, France). Phosphate buffer 0.2 *M*, pH 6.2, was prepared from potassium phosphate, monobasic (27.24 g/l) and sodium phosphate, dibasic dodecahydrate (71.6 g/l) (81.5:18.5, v/v), and purchased from Prolabo. Internal standards and calibration solutions were prepared and then diluted in methanol from the free bases.

Chromatography

The chromatography was performed on a Varian Model 5000 instrument with a fixed-wavelength detector (280 nm) and a 50-μl fixed-volume injector. The column was a stainless-steel tube (25 cm × 4 mm) filled with 5 μm LiChrosorb Si-60 (Merck 9388) using the balanced-density slurry packing

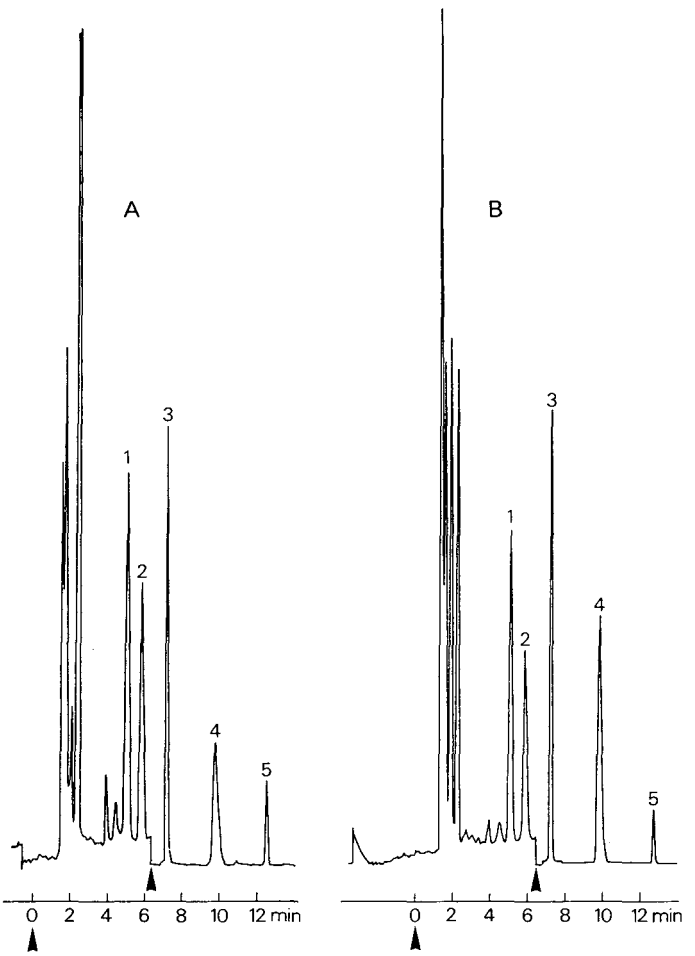

Fig. 1. Chromatograms corresponding to the extract of: (A) 1 ml of spiked plasma, with 1 = IS-TMP 2 μg, 2 = TMP 2.5 μg, 3 = IS-SMZ—N^4SMZ 40 μg, 4 = SMZ 25 μg, and 5 = N^4SMZ 12.5 μg; (B) 1 ml of patient plasma, with 1 = IS-TMP, 2 = TMP, 3 = IS-SMZ, 4 = SMZ, and 5 = N^4SMZ. Retention times are (min): peak 1 = 5, peak 2 = 5.8, peak 3 = 7, peak 4 = 9.6, peak 5 = 12.6.

technique. The slurry, 3.6 g of 5 μm LiChrosorb Si-60 dispersed in 15.6 ml of carbon tetrachloride, was forced into the column with methanol. Alternatively, a Merck Hibar column (ref. 50388) was used. The precolumn was a stainless-steel tube (4 cm × 4 mm) filled with 25—40 μm LiChroprep Si-60 (Merck 9390). The degassed mobile phase used at a flow-rate of 2 ml/min is a mixture of chloroform—methanol—distilled water—ammonia solution (94.5:5.0:0.25:

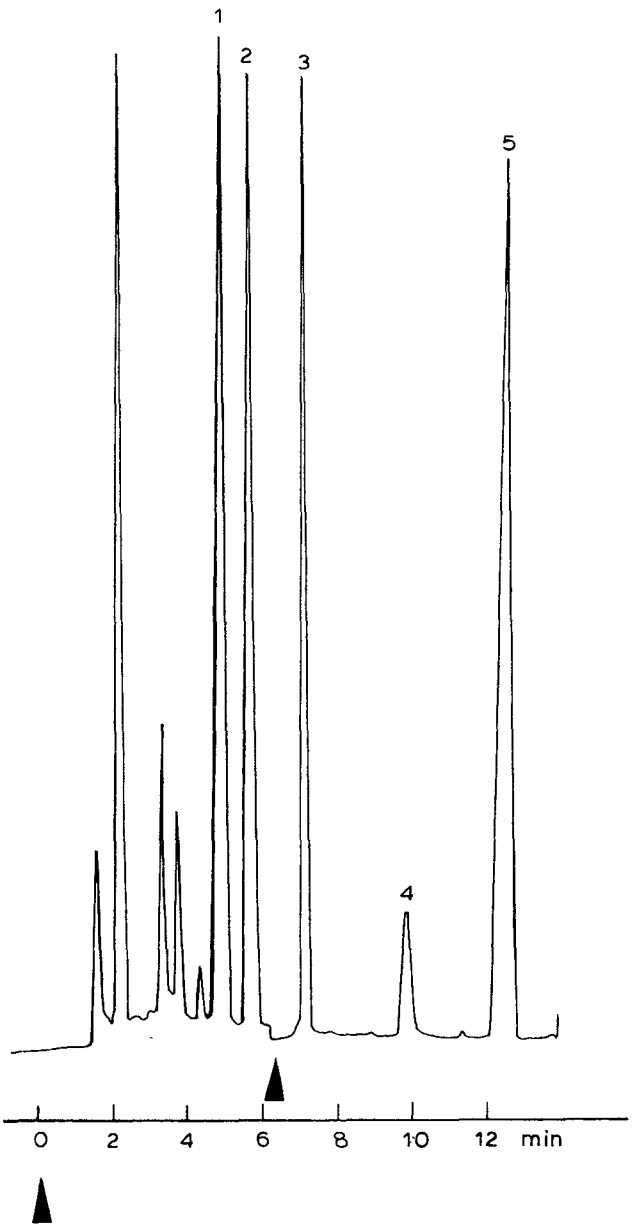

Fig. 2. Chromatogram of an extract of urine (subject 2) containing IS-TMP (1), TMP (2), IS-SMZ (3), SMZ (4), and N^4SMZ (5).

0.19, v/v, solvent A) during the first 3 min and (79:20:1:0.15, solvent B) during the following 8 min. Programming is: 0—3 min, solvent A; 3.5 min, solvent A + solvent B (50:50); 4—12 min, solvent B; 12.5 min, solvent A + solvent B (50:50); 13—16 min, solvent A.

TMP and its internal standard were eluted between 0 and 6 min; SMZ, N^4SMZ and their internal standard were eluted between 7 and 13 min.

The detector sensitivity is 0.02 a.u.f.s. between 0 and 6 min, and 0.5 a.u.f.s. beyond, which allows all five peaks that are being looked for to show on the same chromatogram, the differences in concentration to be detected between TMP and the sulphonamides being taken into account. Retention times are shown in Figs. 1 and 2. Maximum pressure was about 180 bars. After about 500 injections, the pressure had not increased, and the retention times were not modified. The mobile phase and the column were at room temperature.

Sample preparation

To 1 ml of plasma or urine (usually as a 1/2 to 1/5 aqueous dilution) in a 30-ml glass tube were added 20 μl of internal standard solutions (IS-TMP, 2 μg; IS-SMZ—N^4-SMZ, 40 μg), 5 ml of phosphate buffer, 0.2 *M* pH 6.2, and 12 ml of chloroform—ethyl acetate (75:25). The stoppered tube was shaken mechanically for 15 min (Kahn vibrator), and centrifuged at 3000 *g* for 10 min. Then 10 ml of the chloroform phase were transferred into another tube and evaporated to dryness under nitrogen at 50°C. The residue was dissolved in 300 μl of mobile phase A. After mixing for 20 sec on a Vortex mixer, 50 μl were injected.

Calibration curves

Plasma samples were prepared by adding 20 μl of each internal standard solution and 100 μl of methanolic solutions of increasing concentrations of TMP (0.5—10 μg/ml), SMZ (5—100 μg/ml) and N^4SMZ (2.5—50 μg/ml) to 1 ml of plasma from drug-free patients. Urine calibrations were established with 1 ml of dilute sample usually spiked with TMP, SMZ (2.5—50 μg/ml) and N^4SMZ (5—100 μg/ml). These samples were then worked up according to the procedure described above. The calibration curves were obtained by plotting the peak area ratio for each substance with its reference, against concentration.

RESULTS AND DISCUSSION

Choice of extraction solvent

This choice was guided by previous work. Weinfeld [14] extracted TMP, pH 10, in chloroform. Ascalone [27, 28] extracted TMP and the sulphonamides, pH 6.8, in ethyl acetate.

We have verified experimentally that TMP in a neutral or alkaline condition is more soluble in chloroform than in ethyl acetate, contrary to SMZ and its acetyl derivative. The influence of the proportions of the two constituents of the extraction solvent (chloroform and ethyl acetate) was studied for each product.

For each extraction solvent, 3—6 extractions were carried out on 1 ml of plasma loaded with 2 μg of TMP, 2 μg of IS-TMP, 40 μg of SMZ, 20 μg of

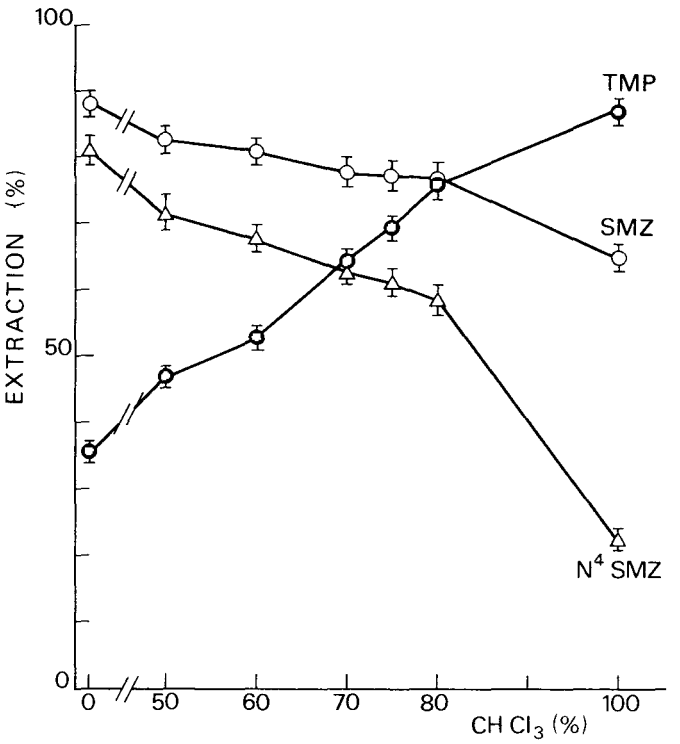

Fig. 3. Influence of the relative proportions of the two constituents of the extraction solvent (chloroform—ethyl acetate) on the percentage extraction of TMP (2 μg/ml), SMZ (40 μg/ml), and N^4SMZ (20 μg/ml) using a pH 6.2 buffer before extraction (mean ± S.E.M., n = 3—6).

N^4SMZ, and 40 μg of IS-SMZ. The extraction was worked up according to the procedure described above using a phosphate buffer, pH 6.2, and 12 ml of the extraction solvent. The mean extraction percentage results were obtained by comparing the amount added to the plasma with the same amount without extraction.

Fig. 3 shows that TMP extraction is considerably improved by an increased proportion of chloroform, while that of the sulphonamides is improved by an increased proportion of ethyl acetate.

In view of the relative proportions of the different products, TMP being present only in low concentrations must be determined more accurately. Only the range of the proportion of chloroform from 70 to 80% at pH 6.2 permits the acceptable simultaneous extraction of 60—80% of the three products and justifies the choice of the 75:25 proportions in the proposed method.

Choice of buffer pH before extraction

The effect of the pH of the buffer used before extraction has been studied for each product. Phosphate buffer solutions (0.2 *M*, at pH 5.4, 5.8, 6.2, 6.6, 7.0, 8.0, and 11) were prepared according to Sörensen's method [30].

For each pH, four extractions were carried out on 1 ml of plasma loaded

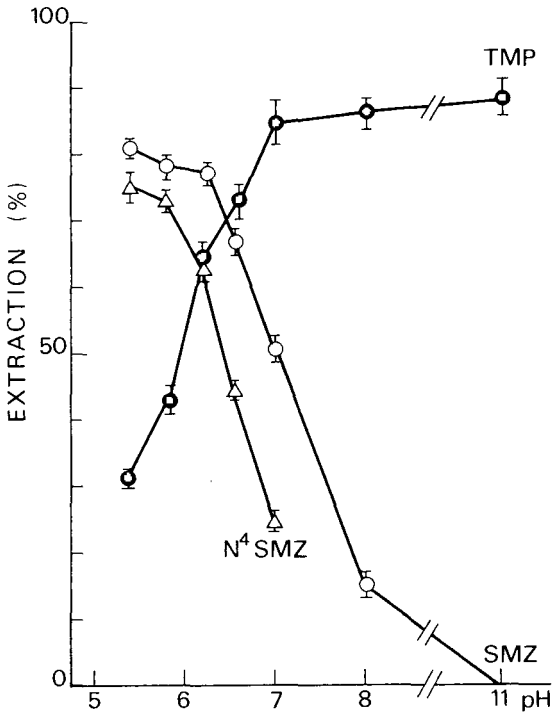

Fig. 4. Influence of buffer pH on the extraction of TMP (2 μg/ml), SMZ (40 μg/ml), and N^4SMZ (20 μg/ml) using 12 ml of chloroform—ethyl acetate (70:30) as the extraction solvent (mean ± S.E.M., $n = 4$).

with 2 μg of TMP, 2 μg of IS-TMP, 40 μg of SMZ, 20 μg of N^4SMZ, and 40 μg of IS-SMZ. The extraction was worked up according to the procedure described above using 12 ml of chloroform—ethyl acetate (70:30) as the extraction solvent. The mean extraction percentage results were obtained by comparing the amount added to the plasma with the same amount without extraction.

The choice of pH for the aqueous phase at the moment of extraction is also the result of a compromise that takes into account the differences in concentration being researched for TMP and SMZ. The extraction of SMZ is maximal at pH < 5.6, and that of TMP at pH > 8.0 (Fig. 4).

The choice of pH 6.2 ensures simultaneous extraction suitable for SMZ, its metabolite, and TMP. It should be noted that, in these experimental conditions, the choice of an extraction pH of 6.8, as recommended by Ascalone [27], would be prejudicial to the extraction of SMZ.

Optimal conditions for each product

The extraction yield of TMP was evaluated using the same procedure, but with a buffer solution of pH 11 and chloroform (100%) for the extraction of five series of three doses (1,2 and 4 μg/ml) of TMP, giving extraction efficiencies of 96.6, 98 and 95.4% with coefficients of variation of 1.4, 0.7 and 0.9%, respectively.

TABLE I

ACCURACY AND DAY-TO-DAY REPRODUCIBILITY FOR TMP, SMZ AND N⁴SMZ (SPIKED HUMAN PLASMA SAMPLES, 1 ml)

	TMP			SMZ			N⁴SMZ		
Amount added (μg/ml)	1	2	4	20	40	80	10	20	40
Amount found (μg/ml)	0.70	1.34	2.71	15.4	31.1	64.8	6.6	12.1	25.2
Replicates (*n*)	6	6	6	6	6	6	6	6	6
Coefficient of variation (%)	2.9	0.7	1.2	2.0	1.1	0.5	2.8	0.4	0.5
Mean recovery (%)	70.8	67.2	67.8	81	78.8	83.8	65.5	61	64.2

TABLE II

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR TMP, SMZ AND N⁴SMZ (SPIKED HUMAN PLASMA SAMPLES, 1 ml)

	TMP/IS-TMP				SMZ/IS-SMZ				N⁴SMZ/IS-SMZ			
Amount added (μg/ml)	1	2	3	4	20	40	60	80	10	20	30	40
Mean ratio product/IS	0.24	0.45	0.66	1.0	0.87	1.35	1.99	2.53	0.34	0.63	0.93	1.15
n replicates	8	7	7	3	7	6	6	3	8	7	11	4
Coefficient of variation (%)	0.78	1.3	0.28	6.4	0.6	1.75	1.1	1.25	1.8	1.5	3.4	2.4

The extraction yields of SMZ and its metabolite were assessed with the same methodology, but using a buffer solution of pH 5.4 and ethyl acetate (100%) for the extraction of a series of 4—6 doses of SMZ (20, 40, and 80 μg/ml) and of N^4SMZ (10, 20, and 40 μg/ml). The extraction percentages were 97, 98.5 and 99.1% for SMZ and 99.3, 98.2 and 98.8% for N^4SMZ, respectively, with coefficients of variation of 0.8, 2.4 and 1.1% for SMZ and 1.4, 2.7 and 0.9% for N^4SMZ, respectively. In view of the analytical characteristics of these molecules, associated in cotrimoxazole, it would be illusory to envisage a quantitative yield for intermediate pH values. The choice of an intermediate pH is therefore a compromise that allows a simpler and more rapid investigation. It was validated by using two internal standards added before extraction insofar as these internal standards are homologous or structurally analogous to the two structures with similar analytical characteristics.

It should be noted that Weinfeld [14] for the determination of TMP alone, and Gochin et al. [29] for cotrimoxazole association, use this procedure, but the latter using only one internal standard for the sulfonamide derivatives.

Plasma interference

Theophylline and an unknown metabolite of caffeine were identified as interfering compounds with the same retention time (5.0 min) as the internal standard for TMP (IS-TMP), while caffeine has a shorter retention time (less than 2 min).

The ingestion of tea or coffee should therefore be prohibited at the time of biological measurements in humans.

Chromatographic conditions

Chromatographic separation is developed without an elution gradient, but with only one change in the polarity of the elution solvent. This solvent contains chloroform and two different proportions of polar solvent (methanol—water, 20:1) providing also a variable basicity (NH_4OH).

The increase in proportion of the polar solvent accelerates the elution of all the peaks on the chromatogram.

The solvent A used is the one proposed by Weinfeld [14] for TMP determination. Its basicity allows the separation of TMP and IS-SMZ. The more polar solvent B accelerates the elution of SMZ and N^4SMZ.

Linearity

The calibration curves were established as described above. A good linear relationship was obtained in the range of 0.1—20 μg/ml for TMP (r = 0.999), 2—150 μg/ml for SMZ (r = 0.999), and in the range of 1—100 μg/ml for N^4SMZ (r = 0.999).

For routine analysis, a calibration curve is established each day. The linear regression equations are $y = 0.049 + 0.28x$ for TMP, $y = 0.089 + 0.025x$ for SMZ, and $y = 0.043 + 0.012x$ for N^4SMZ.

Accuracy, precision and sensitivity

The day-to-day reproducibility for several series is shown in Table I. The variation coefficients are satisfactory. Extraction yields are weak but were expected in view of the opposing optimal conditions of the different products for the pH of the plasma buffer and for the extraction solvent.

The study of intraserial reproducibility is shown in Table II. The variation coefficients observed are satisfactory.

The sensitivity limit in 1 ml of plasma is 15 ng/ml for TMP, 20 ng/ml for SMZ, and 10 ng/ml for N^4SMZ, using the detector set at 0.01 a.u.f.s. for TMP and 0.05 a.u.f.s. for SMZ and N^4SMZ and a 100-μl sample of the evaporation residue instead of 300 μl. This corresponds to a signal-to-noise ratio of three for TMP and two for SMZ and N^4SMZ.

TABLE III

PHARMACOKINETIC PARAMETERS FOR TMP AND SMZ IN THREE NORMAL VOLUNTEERS

$K_{abs.}$ = Apparent absorption rate constant; K_e = elimination rate constant; $t_{½\beta}$ = elimination half-life; AUC = area under the curve; V_d = volume of distribution; and Cl_T = total plasma clearance.

	TMP	SMZ
$K_{abs.}$ (h^{-1})	2.36 ± 0.98	2.53 ± 1.38
K_e (h^{-1})	0.093 ± 0.023	0.068 ± 0.063
$t_{½\beta}$ (h)	8.74 ± 2.58	10.25 ± 0.50
AUC ($mg\ l^{-1}\ h^{-1}$)	27.1 ± 6.2	826 ± 102
V_d ($l\ kg^{-1}$)	72.2 ± 5.7	14.6 ± 1.4
Cl_T ($l\ h^{-1}$)	6.44 ± 1.26	0.99 ± 0.11

Selectivity

N^4-Acetylation is a major hepatic pathway of the biotransformation of sulphonamides in man. The derivative N^4SMZ is usually found in the proportions of 30% in the blood and 60% in urine [31]. We were interested in being able to quantify it simultaneously with SMZ and TMP.

The biotransformation of TMP is less important, the proportion of its metabolites in the blood being of the order of 10% and of the order of 20—40% in urine [31].

Stability

The dry residue was dissolved in 300 μl of the mobile phase just before injection to ensure good reproducibility. The dry evaporation residue remains stable for 2 days when stored in the refrigerator; upward of two days was not tested.

Application

Pharmacokinetic constants of TMP, SMZ and N^4SMZ were studied in three elderly volunteers (two males and one female) aged 74 ± 3.6 years and weighing 58.6 ± 7.6 kg. They were all healthy subjects, gave their informed consent, and were not taking concurrent major medication.

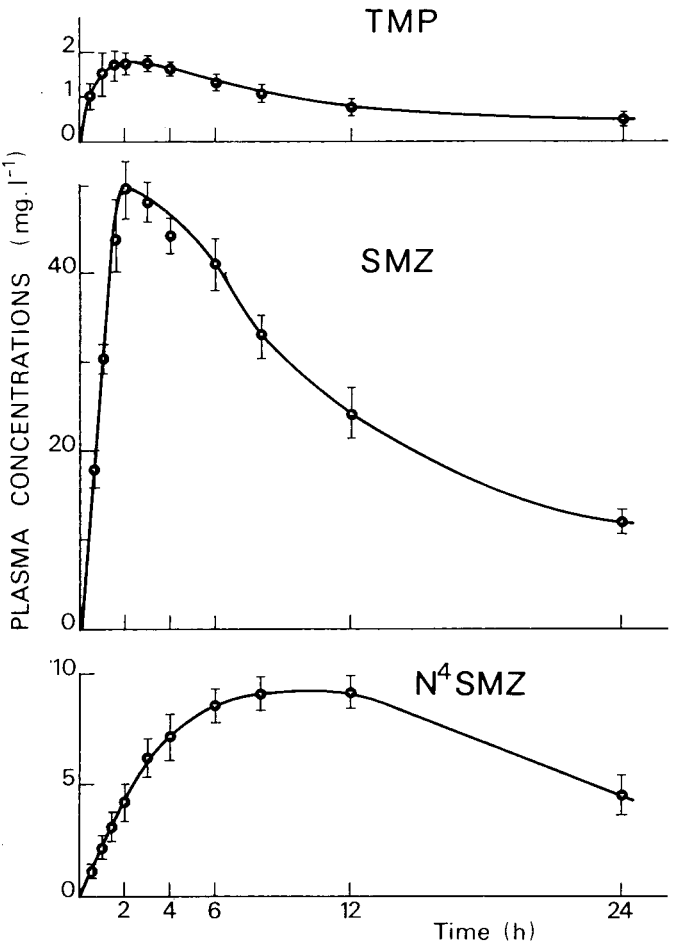

Fig. 5. Plasma TMP, SMZ and N^4SMZ concentrations (± S.E.M.) against time, after oral administration of TMP 160 mg, and SMZ 800 mg to three volunteers.

Each subject received orally, in a fasting state, TMP (160 mg) and SMZ (800 mg) (Bactrim forte®) and blood samples were collected 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after administration. Plasma was immediately separated, frozen and kept at −15°C until analysed.

The plasma levels vs. time data following this administration are shown in Fig. 5, and the resultant pharmacokinetic parameters using a one-compartment model are represented in Table III. These pharmacokinetic data observed for TMP and SMZ are in good agreement with those reported in the literature [31].

Urine elimination of TMP, SMZ and N^4SMZ in the three patients represented in Table IV appears to be less than those reported in the literature for young adults (respectively, 58—71% and 70—84% for TMP and total SMZ) [32—35].

These preliminary results validate the analytical method. A further pharmacokinetic study in healthy and infected elderly patients will be published later.

TABLE IV

URINARY ELIMINATION (0—48 h) OF TRIMETHOPRIM (TMP), FREE SULFAMETHOXAZOLE (SMZ) AND N^4-ACETYLSULFAMETHOXAZOLE (N^4SMZ) IN THREE SUBJECTS

Subject	TMP		SMZ		N^4SMZ	
	Total mg excreted	Percentage dose excreted	Total mg excreted	Percentage dose excreted	Total mg excreted	Percentage dose excreted
1	22.5	14.0	3.8	0.4	112.6	14.3
2	41.7	26.1	42.8	5.4	181.9	22.9
3	53.0	33.1	37.1	4.6	395	49.1
Mean	39.0	24.4	27.9	3.4	229.8	28.7
S.E.M.	9.1	5.7	12.4	1.6	86.6	10.6

CONCLUSION

The present method with normal-phase HPLC permits pharmacokinetic studies and drug monitoring. The limit of detection, the use of internal standards for each of the studied structures and a rapid and identical protocol for the extraction of both urine and plasma samples [29] differentiate it from previously published methods [27—29].

REFERENCES

1 S.R.M. Bushby and G.H. Hitchings, Brit. J. Pharmacol., 33 (1968) 72.
2 A. Kucers and N. McK. Bennett, The Use of Antibiotics, William Heinemann Medical Books Ltd., London, 3rd ed., 1979, p. 687.
3 R.J. Daun, J. Ass. Offic. Anal. Chem., 54 (1971) 1277.
4 E. Roder and W. Stuhe, Z. Anal. Chem., 271 (1974) 281.
5 N. Nose, S. Kobayashi, A. Hirose and A. Watanabe, J. Chromatogr., 123 (1976) 167.
6 S.C. Su, A.V. Hartkopf and B.L. Karger, J. Chromatogr., 119 (1976) 523.
7 G.W. Peng, M.A.F. Gadulla and W.L. Chiou, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 233.
8 K.L. Johnson, D.T. Jetter and R.C. Clairbone J. Pharm. Sci., 64 (1975) 1657.
9 J.P. Sharma, E.G. Perkins and R.F. Bevill, J. Pharm. Sci., 65 (1976) 1606.
10 M.H. Penner, J. Pharm. Sci., 64 (1975) 1017.
11 T.C. Kram, J. Pharm. Sci., 61 (1972) 254.
12 A.P. Lotter and A.P. Goossens, S. Afr. Pharm. J., 44 (1977) 282.
13 G. Land, K. Dean and A. Bye, J. Chromatogr., 146 (1978) 143.
14 R.E. Weinfeld and T.C. Macasieb, J. Chromatogr., 164 (1979) 73.
15 W.J. Irwin and J.A. Slack, J. Chromatogr., 139 (1977) 364.
16 M.A. Brooks, J.A.F. de Silva and L. D'Arconte, Anal. Chem., 45 (1973) 263.
17 M.A. Brooks, J.A.F. de Silva and L. d'Arconte, J. Pharm. Sci., 62 (1973) 1395.
18 S.R.M. Bushby, Postgrad. Med. J., 45 (Suppl.) (1969) 10.
19 J.C. Pechere, Pathol. Biol., 18 (1970) 343.
20 D.E. Schwartz, W. Wetter and G. Englert, Arzneim.-Forsch., 20 (1970) 1867.
21 P. Nielsen and R. Folke, Acta Pharmacol. Toxicol., 37 (1975) 309.
22 D.M. Lichtenwalner, B. Suh, B. Lorberg and A.M. Sugar, Antimicrob. Agents Chemother., 16 (1979) 579.

23 V. Ascalone, Boll. Chim.-Farm., 117 (1978) 176.
24 A. Bye and M.E. Brown, J. Chromatogr. Sci., 15 (1977) 365.
25 T.B. Vree, Y.A. Hekster, A.M. Baars, J.E. Damsma and E. van der Kleijn, J. Chromatogr., 146 (1978) 103.
26 R.W. Bury and M.L. Mashford, J. Chromatogr., 163 (1979) 114.
27 V. Ascalone, J. High Resolut. Chromatogr. Commun., 3 (1980) 261.
28 V. Ascalone, J. Chromatogr., 224 (1981) 59.
29 R. Gochin, I. Kanfer and J.M. Haigh, J. Chromatogr., 223 (1981) 139.
30 S.P.L. Sörensen, Ergeb. Physiol., 12 (1912) 393.
31 R.B. Patel and P.G. Welling, Clin. Pharmacokin., 5 (1980) 405.
32 D.E. Schwartz and J. Rieder, Chemotherapy, 15 (1970) 337.
33 S.A. Kaplan, R.E. Weinfeld, S. Cotler, C.W. Abruzzo and K. Alexander, J. Pharm. Sci., 59 (1970) 358.
34 P.G. Welling, A. Graig, G.L. Amidon and C.M. Kunin, J. Infect. Dis., 128 (Suppl.) (1973) 556.
35 H. Nolte and H. Büttner, Chemotherapy, 18 (1973) 274.

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CHROMBIO. 1622

SIMULTANEOUS DETERMINATION OF AMINOPYRINE HYDROXYLATION AND AMINOPYRINE N-DEMETHYLATION IN LIVER MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Aminopyrine and its metabolites, including 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one which is a hydroxylated metabolite of aminopyrine, were separated on a reversed-phase (C_8) Radial-Pak column using a mobile phase of methanol—triethylamine—water (30:1:69) adjusted to pH 5.40 with acetic acid. Detection of the peak was performed by an ultraviolet detector at 254 nm. By the rapid and simple method, aminopyrine hydroxylation as well as aminopyrine N-demethylation in liver microsomes can be examined simultaneously.

INTRODUCTION

Aminopyrine (AM) has been widely used as an analgesic and antipyretic drug or a model substrate for in vitro and in vivo investigation of drug metabolism. Brodie and Axelrod [1] have demonstrated that the major metabolic route is two sequential N-demethylations to give first 4-monomethylaminoantipyrine (MAA) and then 4-aminoantipyrine (AA). This route in liver microsomes was confirmed by Gram et al. [2] in the rat and rabbit. Recently, Iguchi et al. [3, 4] demonstrated that 4-formylaminoantipyrine (FAA) is a noticeable metabolite of aminopyrine in man and animals. Nigam et al. [5] found that FAA is formed from AM in the liver microsomes of phenobarbital-treated rats. Another route of AM disposition is the oxidation of the 3-methyl group to give 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one (AM-OH), which was demonstrated by Yoshimura et al. [6]. This is a minor but very important route because the aldehyde intermediate of

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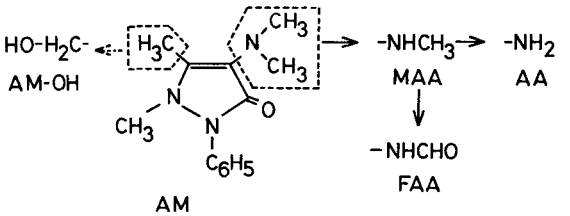

Fig. 1. Illustration of aminopyrine metabolic pathway found and postulated in liver microsomes. The abbreviations are described in the Introduction.

the further metabolite of AM-OH binds irreversibly to tissue macromolecules or proteins and forms stable complexes [7]. It has not been confirmed that AM-OH is formed in liver microsomes.

Although several methods have been described for monitoring AM and its metabolites by high-performance liquid chromatography (HPLC) [5, 8, 9], none has attempted to assay AM-OH as well as MAA, AA, and FAA simultaneously. Fig. 1 shows the metabolic pathway of aminopyrine found and postulated (the left pathway) in liver microsomes.

In this paper we report a rapid and simple assay method that separates the metabolites of aminopyrine, and determines AM-metabolizing enzyme activity in liver microsomes.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system and Model U6K injector. Separations were carried out on a 10 cm × 5 mm I.D. Radial-Pak C_8 (10 μm particle size) column (Waters Assoc.). The mobile phase was methanol—triethylamine—water (30:1:69) adjusted to pH 5.40 with acetic acid. The flow-rate was 1 ml/min. Detection was performed by a Model 440 UV detector (Waters Assoc.) at 254 nm. Areas of peaks were calculated by a Waters data module.

Materials

Aminopyrine was purchased from Daiichi Seiyaku (Tokyo, Japan). AM-OH was kindly supplied by Professor Yoshimura (Kyushu University). FAA and MAA were kindly supplied by Dr. Yoshioka and Dr. Sakai (National Institute of Hygienic Sciences). All other chemicals used were of reagent grade.

Procedure

Rat liver microsomes were obtained according to the method described previously [10, 11]. The standard incubation mixture consisted of the following components in a total volume of 0.5 ml: NADPH 4 m*M*, $MgCl_2$ 5 m*M*, microsomal suspension 0.1 ml, 0.2 *M* sodium phosphate buffer (pH 7.4), and AM 4 m*M*. Incubation was carried out at 37°C; the reaction was stopped by mixing with 1 ml of cold chloroform, then the mixture was centrifuged at 1800 *g* for 5 min. Aqueous and chloroform layers were applied to Extrelut 1 (E.

Merck, Darmstadt, G.F.R.). After 5 min, 9 ml of chloroform were poured onto the column. The eluate was evaporated to dryness in vacuo and dissolved in 100 μl of methanol. Then 10 μl of the solution was injected into the HPLC system.

RESULTS

A typical chromatogram from a single 10-μl injection of a standard solution of AM and four metabolites is presented in Fig. 2. The separation and baseline assay could be achieved within 20 min.

Linearity was evaluated at the concentration range described in Table I. Calibration curves of all compounds were fitted to each regression line and the correlation coefficients were larger than 0.9990. In the case of AM, which is a

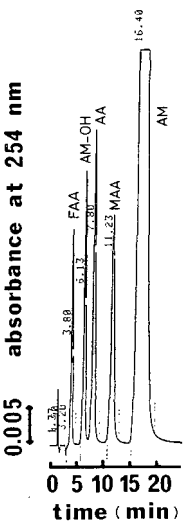

Fig. 2. Chromatogram of a standard solution containing AM, MAA, AA, FAA, and AM-OH.

TABLE I

CALIBRATION CURVE OF AMINOPYRINE AND ITS METABOLITES

The relative area (y) calculated by Waters data module and concentration (x, nmol per 10 μl) were fitted to a function of the regression line $y = ax + b$.

Compound	Concentration range of calibration curve (nmol per 10 μl)	Parameter a	Parameter b	Coefficient of correlation
FAA	0.14— 4.6	2533.2	68.1	0.9993
AM-OH	0.31— 9.9	1947.4	22.3	0.9994
AA	0.26— 8.4	2681.1	25.2	0.9996
MAA	0.26— 16.7	2535.0	20.8	0.9995
AM	0.32— 10.3	2076.1	—21.0	0.9995
	25.0 —200.0	2268.1	—663.2	0.9990

TABLE II

REPRODUCIBILITY OF THE ASSAY

Reproducibility was calculated by the equation

$$\frac{\text{amount found (nmol)}}{\text{amount taken (nmol)}} \times 100 = \text{reproducibility (\%)}.$$

Each value represents the mean ± S.E. of three determinations.

Compound	Amount taken (nmol)	Reproducibility (mean ± S.E. %)		Coefficient of variation (%)
FAA	0.14	95.3	6.08	11.1
	0.29	102.8	3.90	6.6
	0.58	107.2	0.82	1.3
	1.15	110.3	0.96	1.5
	2.30	104.4	1.76	2.9
	4.60	100.0	1.71	3.0
AM-OH	0.14	90.1	4.63	8.9
	0.31	85.9	4.17	8.4
	0.62	91.6	0.63	1.2
	1.24	96.1	0.86	1.6
	2.48	105.4	0.44	0.7
	4.96	101.4	1.52	2.6
	9.91	99.4	1.32	2.3
AA	0.26	83.7	4.45	9.2
	0.52	95.1	2.15	3.9
	1.05	95.8	2.05	3.7
	2.10	105.1	1.02	1.7
	4.19	101.2	1.63	2.8
	8.38	99.5	0.90	1.6
MAA	0.26	82.1	1.21	2.6
	0.52	83.6	2.92	6.0
	1.04	92.2	1.76	3.3
	2.08	96.0	1.79	3.2
	4.17	105.7	0.69	1.1
	8.33	101.3	1.48	2.5
	16.67	99.4	0.98	1.7
AM	0.32	80.6	0.95	2.0
	0.65	81.0	4.00	8.6
	1.29	92.6	3.85	7.2
	2.58	104.2	0.55	0.9
	5.16	99.2	1.70	3.0
	10.32	99.6	0.33	0.6
	25.0	98.9	0.21	0.4
	50.0	100.8	0.89	1.5
	100	99.9	1.60	2.8
	200	98.5	1.71	3.0

TABLE III

RECOVERY

Compounds were added to incubation mixtures without NADPH, incubated for 10 min, and extracted as described in the procedure.
Recovery was calculated by the equation

$$\frac{\text{(amount found at single injection)} \times 10}{\text{amount added to incubation mixture}} \times 100 = \text{recovery (\%)}.$$

Compound	Amount added to incubation mixture (nmol)	Recovery (mean ± S.E. %)		Coefficient of variation (%)
FAA	5 (n = 5)	92.4	2.28	6.0
	20 (n = 6)	97.0	2.34	5.4
AM-OH	10 (n = 5)	95.0	1.85	4.8
	40 (n = 6)	101.4	3.02	6.7
AA	10 (n = 5)	82.0	3.84	10.5
	40 (n = 6)	81.2	2.81	8.5
MAA	10 (n = 5)	83.4	2.13	5.7
	40 (n = 6)	81.6	2.07	6.2
AM	500 (n = 5)	98.3	3.16	7.2
	1500 (n = 6)	97.8	2.67	6.7

substrate, the concentration of unchanged AM is so high that we have to use the parameters a and b in the range (25—200 nmol per 10 μl) to calculate the concentration.

Reproducibility is as described in Table II. The coefficients of variation of all compounds were very small. When FAA 0.14 nmol was injected, the coefficient of variation was 11.1%. But at higher amounts the precision improved.

Recovery studies were carried out by the addition of AM and metabolites to incubation mixtures which contained liver microsomal fraction as described in the procedure. The result is given in Table III. The amount of AM added to incubation mixtures was larger than that of metabolites because it must remain at high concentration. Recoveries of all compounds were constant as shown in Table III.

Fig. 3 shows a typical chromatogram of the chloroform extract of the incubation mixture with liver microsomal enzymes of untreated rat. The main metabolites were MAA (retention time: t_R = 11.36 min), AA (t_R = 8.06 min) and AM-OH (t_R = 6.23 min). The peak at t_R = 5.06 min was not FAA since FAA added to this extract gave both the FAA peak and the peak at 5.06 min.

Fig. 4A shows the relationship between the formation of these three metabolites and incubation time. Linearity was observed from 0.5 to 10 min. AM disposition rate in liver microsomal fractions as a function of enzyme concentration is shown in Fig. 4B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

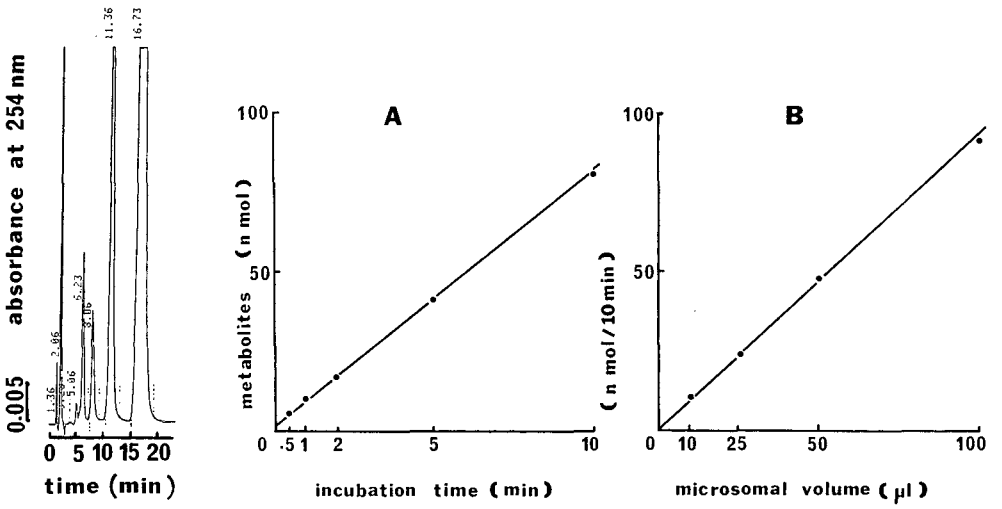

Fig. 3. Chromatogram of the chloroform extract of the incubated mixture containing AM and its metabolites with liver microsomal enzymes of untreated rat. Volume of the extract injected was 10 μl.

Fig. 4. (A) Relationship between the amount of metabolites (MAA + AA + AM-OH) in the liver microsomal enzymes measured by the HPLC assay and incubation time. Incubation mixture contained 100 μl of liver microsomal suspension of rat treated with phenobarbital (60 mg/kg intraperitoneally, 72, 48 and 24 h before sacrifice). Incubations were carried out at 37°C. (B) Relationship between the amount of metabolites and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

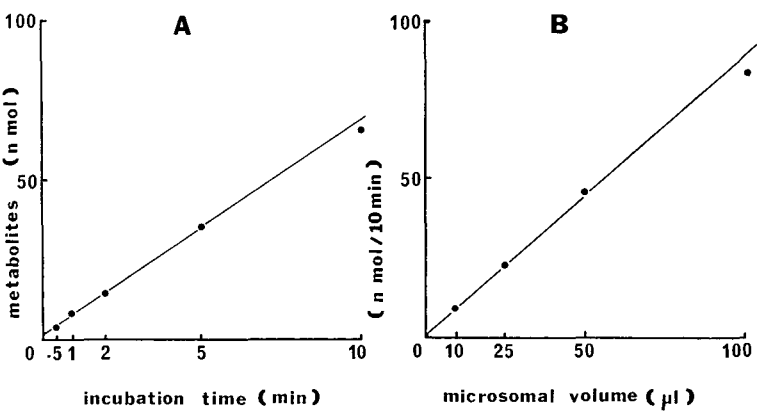

Fig. 5. (A) Relationship between the amount of MAA + AA formed and incubation time. The incubation conditions were the same as in Fig. 4A. (B) Relationship between the amount of MAA + AA formed and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

Fig. 5A shows the relationship between the total formation of MAA and AA, and incubation time. Linearity was observed from 0.5 to 10 min. The formation of MAA and AA under these conditions is regarded as a result of AM N-demethylation. The AM N-demethylation rate in liver microsomal fractions as a function of enzyme concentration is shown in Fig. 5B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

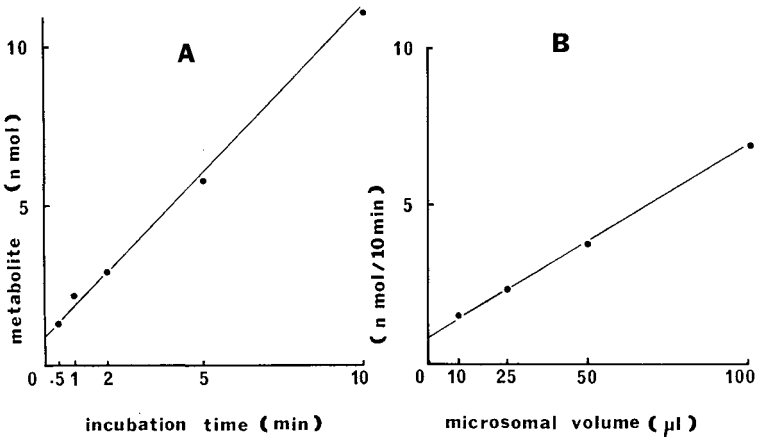

Fig. 6. (A) Relationship between the amount of AM-OH formed and incubation time. The incubation conditions were the same as in Fig. 4A. (B) Relationship between the amount of AM-OH formed and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

Fig. 6A shows the relationship between the formation of AM-OH and incubation time. Linearity was observed from 0.5 to 10 min. AM hydroxylation activity in liver microsomes as a function of enzyme concentration is shown in Fig. 6B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

DISCUSSION

A multi-faceted approach to the analysis of AM metabolism in liver microsomes has been presented. The primary advantage of this method is to determine simultaneously AM hydroxylation as well as AM N-demethylation activity.

The hydroxylation pathway has been thought to be the minor one in AM disposition [6, 7]. But the percentage of AM-OH of the total metabolites was higher than 15% in liver microsomes of phenobarbital-treated rats (calculated from Figs. 4 and 6). This value can not be ignored in the study of AM disposition. Moreover, the further metabolite of AM-OH binds irreversibly to macromolecules. This phenomenon is very important because the stable complex formed is thought to be the main factor behind AM allergy [7]. It is possible that this metabolite is concerned with other toxicities of AM. Therefore the examination of AM-hydroxylation is very necessary and the HPLC method to separate AM-OH has proved to be very useful.

The simultaneous separation of AM-OH, AA and/or MAA has not been previously reported. The separation of AM-OH from AA and/or MAA depends on the property of the column and the pH of the mobile phase. We used a Radial-Pak C_8 column and a mobile phase consisting of methanol—triethylamine—water (30:1:69) at pH 5.40. A good separation was achieved by these conditions.

This method is very simple and rapid, and will be a useful tool for an all-round examination of AM metabolism in liver microsomes.

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REFERENCES

1 B.B. Brodie and J. Axelrod, J. Pharmacol. Exp. Ther., 99 (1950) 171.
2 T.E. Gram, J.T. Wilson and J.R. Fouts, J. Pharmacol. Exp. Ther., 159 (1968) 172.
3 S. Iguchi, T. Goromaru and A. Noda, Chem. Pharm. Bull., 23 (1975) 932.
4 S. Iguchi, T. Goromaru, A. Noda and N. Tsubone, Chem. Pharm. Bull., 23 (1975) 1889.
5 S. Nigam, B. Raack, I. Roots, G. Heinemeyer and A.G. Hildebrandt, in M.J. Coon, A.H. Conney and R.W. Estabrook (Editors), Microsomes, Drug Oxidation, and Chemical Carcinogenesis, Vol. 2, Academic Press, New York, 1980, p. 869.
6 H. Yoshimura, H. Shimeno and H. Tsukamoto, Yakugaku Zasshi, 90 (1970) 1406.
7 H. Shimeno and H. Yoshimura, Xenobiotica, 2 (1972) 461.
8 G.F. Lockwood and J.B. Houston, J. Pharm. Pharmacol., 31 (1979) 787.
9 K. Shimada and Y. Nagase, J. Chromatogr., 181 (1980) 51.
10 K. Inoue, A. Takanaka, K. Mizokami, K. Fujimori, M. Sunouchi, Y. Kasuya and Y. Omori, Toxicol. Appl. Pharmacol., 59 (1981) 540.
11 K. Mizokami, K. Inoue, M. Sunouchi, K. Fujimori, A. Takanaka and Y. Omori, Biochem. Biophys. Res. Commun., 107 (1982) 6.

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CHROMBIO. 1581

QUANTITATIVE DETERMINATION OF THE γ-AMINOBUTYRIC ACID AGONIST, 4,5,6,7-TETRAHYDROISOXAZOLO[5,4-*c*]PYRIDIN-3-OL, IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method was developed to determine pharmacologically relevant concentrations of the γ-aminobutyric acid agonistic compound THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) in serum. THIP is extracted from serum by a cation-exchange column, derivatized by dansyl chloride, and further separated and quantitated by reversed-phase high-performance liquid chromatography using measurement of ultraviolet absorption at the optimal wavelength (265 nm). The lower detection limit of the method is 0.07 μmol l^{-1} (10 ng ml^{-1}) when 2 ml of serum are used for extraction. This sensitivity is sufficient for pharmacokinetic studies in man following administration of a presumably therapeutic dose of THIP. Blood samples collected from 28 patients treated with one or more of 26 drugs relevant for co-administration with THIP did not contain substances which would affect the reliable quantitation of THIP, if present.

INTRODUCTION

The compound 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) (Fig. 1) is a heterocyclic compound related to γ-aminobutyric acid (GABA). It has been shown to be a GABA-receptor agonist [1, 2], and the assessment of the possible therapeutic value of the substance is at present in progress.

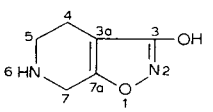

Fig. 1. Structure of THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol).

A reversed-phase ion-pair high-performance liquid chromatographic (HPLC) method for determination of THIP in aqueous solution has been reported [3]. It provides a sensitive detection of THIP, especially when an electrochemical detector is used. However, the application of this method for quantitation of THIP in extracts from biological material, in particular serum, has not been successful.

The procedure described in the present paper is related to a method for the determination of THIP in urine [4]. The inclusion of an extraction step for concentration of THIP from the sample prior to HPLC analysis has decreased the lower detection limit several hundred times. This makes the quantitative determination of pharmacologically relevant serum concentrations of THIP possible, and the method has been used in human and animal studies of THIP pharmacokinetics. The extraction procedure used is inspired from a method for GABA purification [5] which in turn is a modification of a separation method for biogenic amines [6].

EXPERIMENTAL

Instrumentation

HPLC was carried out using a Pye Unicam LC-XPD pump (Cambridge, Great Britain) delivering a constant flow of 2.00 ml min^{-1}, a Rheodyne 7125

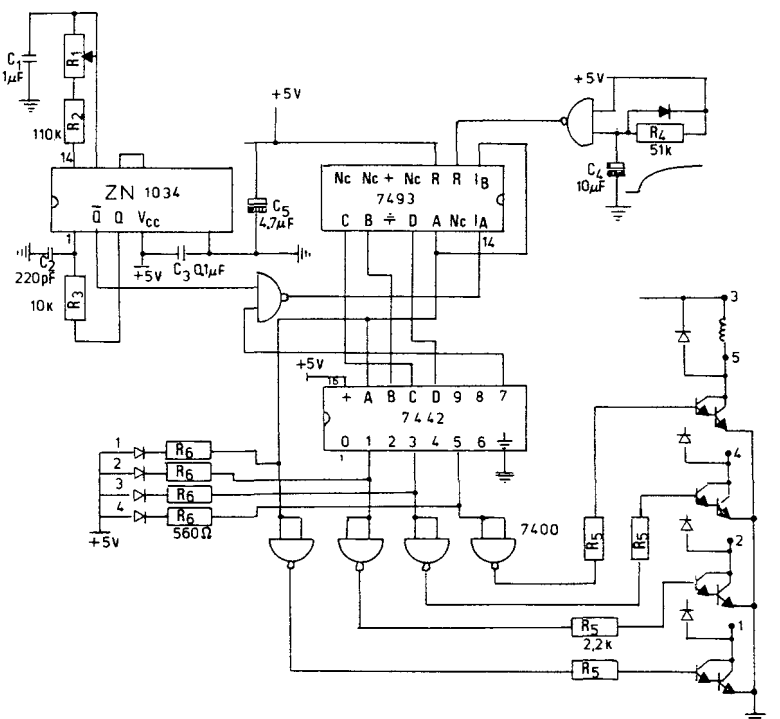

Fig. 2. Diagram for device for unattended rinsing of extraction columns. R_1 is varied (approx. 200 Ω) for time (volume) adjustment. The four electromagnetic valves are driven from the Darlington couplings in the right side of the diagram. A conventional power supply (5 V for the timer electronics, 20 V for the electromagnetic valves) is not shown.

injection valve (Berkeley, CA, U.S.A.) equipped with a 80-μl loop, and a Uvikon 725 variable-wavelength spectrophotometric detector (Kontron, Zürich, Switzerland) operated at a wavelength of 265 nm. The detector signal was recorded and processed by a Kipp & Zonen Model BD 9 recorder (Delft, The Netherlands) and a Hewlett-Packard Model 3390 A reporting integrator (Avondale, PA, U.S.A.). The column consisted of a 40 mm long precolumn and a 120 mm main column, both of them packed with Spherisorb S5-ODS (5 μm particle size) by a slurry technique using the Pye Unicam pump. Both column tubes were made by Knauer (Oberursel, G.F.R.), internal diameter 4.6 mm, connected end to end by a socket. Ultraviolet (UV) absorbance spectra of the components eluted from the HPLC column were recorded by a Uvikon LC 720 detector. Some experiments on alternative fluorescence monitoring were carried out using a Kontron SFM-23 LC fluorescence detector.

A device has been constructed for unattended rinsing of cation-exchange columns before use. Four electromagnetic valves (Gemü type 102, EPDM for water and PTFE for hydrochloric acid, sodium hydroxide, and buffer solution, made by Gebr. Müller, Ingelfingen, G.F.R.) were controlled by a timer including a counting circuit (diagram in Fig. 2) for delivery of the flushing solutions to a sixteen-pronged manifold as given under Extraction.

Materials

Chemicals used were AG 50W-X4 (200—400 mesh) cation exchanger (Bio-Rad Labs., Richmond, CA, U.S.A.), ammonium bicarbonate (certified grade, Fisher, Fair Lawn, NJ, U.S.A.), Dns chloride (5-dimethylamino-1-naphthalene sulfonyl chloride) (Sigma, St. Louis, MO, U.S.A.), ethanol 96% or (for protein precipitation) absolute (DDSF, Copenhagen, Denmark), muscimol base (purum grade, Fluka, Buchs, Switzerland), Spherisorb S 5-ODS (Phase Sep, Queensferry, Great Britain), tetrahydrofuran (HPLC grade, Rathburn, Walkerburn, Great Britain), THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol, monohydrate; synthesized by our company), water redistilled in an all-glass apparatus (used for all solutions), and acetone (dried by anhydrous sodium sulphate), citric acid 1-hydrate, disodium ethylene diamine tetraacetate (EDTA), hydrochloric acid (fuming), methanol, sodium hydroxide, disodium tetraborate 10-hydrate, sodium dihydrogen phosphate 1-hydrate and disodium hydrogen phosphate 2-hydrate (all pro analysi grade, E. Merck, Darmstadt, G.F.R.). Minisorb® 70 × 11 mm (3 ml) tubes with stoppers, and plain polyethylene tubes 100 × 16 mm (11 ml) (Nunc, Roskilde, Denmark). Vacutainer® tubes (siliconized, without anticoagulant; B-D, Ireland). Serological glass pipettes, disposable, 5.5 mm I.D., total length 150 mm (Bilbate, Daventry, Great Britain).

Buffer solutions

Buffer solutions were prepared from the amounts given below by dissolving in the appropriate volume of water required for 1000 ml of solution: 0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA: 8.83 g of sodium dihydrogen phosphate 1-hydrate, 6.40 g of disodium hydrogen phosphate 2-hydrate, 1.00 g of EDTA. Citrate buffer pH 3.0: 8.47 g of citric acid 1-hydrate, 80.6 ml of 1 *N* sodium hydroxide, 30.0 ml of hydrochloric acid.

0.025 *M* sodium citrate buffer pH 4.5: 5.25 g of citric acid 1-hydrate, 40.0 ml of 1 *N* sodium hydroxide, adjusted to pH 4.50 ± 0.05 if necessary. 0.05 *M* sodium citrate buffer pH 5.35: 10.50 g of citric acid 1-hydrate, 110 ml of 1 *N* sodium hydroxide, adjusted to pH 5.35 ± 0.05 if necessary. 0.05 *M* sodium borate buffer pH 8.5: 9.53 g of disodium tetraborate 10-hydrate were dissolved in 430 ml of water, pH adjusted to 8.50 ± 0.02 by 2 *N* hydrochloric acid; and finally made up to 500 ml with water.

Final version of the procedure

Extraction. A 75-g portion of cation-exchange resin was rinsed in a Büchner funnel with 300 ml of 0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA, followed by 150 ml of water, 300 ml of 60% aqueous methanol, 300 ml of 2 *N* hydrochloric acid, 300 ml of 2.4 *N* ethanolic (60%) hydrochloric acid, and 150 ml of water in the order stated. The washed resin was packed from a slurry into serological glass pipettes fitted with a plug of glass wool at the shoulders of the pipette. The height of the resin bed was 75 mm.

The newly packed columns (and columns used once or twice before) were mounted in the column rinsing apparatus and treated with a sequence of 2 *N* sodium hydroxide containing 1% EDTA—water—2 *N* hydrochloric acid—water—0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA—water, 20 ml of each. During the extraction of a sample, the column (washed within three days) was connected through a three-way stopcock to a vertically mounted 20-ml glass syringe. The plunger was loaded with a 165 g weight.

Before application onto the column, a 2.00-ml sample of serum was pipetted into a Minisorb® tube, the internal standard, (2.1 μg of muscimol) was added, dispensed as 60 μl of a 35 μg ml^{-1} solution, and proteins were precipitated by addition of 1.0 ml of ethanol. Sixteen samples were prepared at the same time. The tubes were stoppered and the contents mixed by turning. Then the samples were centrifuged (18,000 *g* at 5°C for 20 min). The supernatant from each sample was decanted into an 11-ml polypropylene tube, 6.0 ml of citrate buffer pH 3.0 were added, and finally the pH of the mixture was adjusted to 3.0 by the addition of approximately 70 μl of 2 *N* hydrochloric acid. This mixture was centrifuged (18,000 *g* at 5°C for 20 min).

The clear supernatant was injected by means of a polypropylene syringe through the side-arm of the three-way stopcock into the glass syringe and thereupon passed through the cation-exchange column. When no solution remained in the glass syringe, the following solvents were passed through in the same manner: water (8.0 ml), 0.025 *M* sodium citrate buffer pH 4.5 (8.0 ml), 0.05 *M* sodium citrate buffer pH 5.35 (4.0 ml), water (8.0 ml), 0.05 *M* ammonium bicarbonate (0.9 ml) (freshly prepared). Then the weights were removed, the column tips rinsed with water, and each column was eluted with 2.0 ml of 0.05 *M* ammonium bicarbonate. The eluate was collected in a 4-ml glass tube, which had been rinsed and deactivated by ethanol in an ultrasonic bath. The tube was closed by a perforated stopper, and the eluate was deaerated by standing 5 min in an ultrasonic bath. Then the samples were frozen at −18°C and freeze-dried at 0.02—0.05 Torr for about 16 h. If less than 4.0 ml serum were

available for the duplicate determination, the extraction procedure was carried out using an aliquot of serum of at least 650 μl without any modification of the procedure.

Derivatization. The freeze-dried residue from each sample was dissolved in 45 μl of sodium borate buffer pH 8.5 and 90 μl of a 7 mg ml^{-1} solution of dansyl chloride in dry acetone were added. The tube was stoppered and placed in a 40°C water bath for 40 min, centrifuged (1500 *g* at 5°C for 10 min), and stored in ice water until analysis.

High-performance liquid chromatography. Eighty microlitres of the reaction mixture, corresponding to almost the entire liquid phase of the prepared sample, were injected into the HPLC system. The mobile phase used in the HPLC system was a tetrahydrofuran—water (50.3:49.7) mixture, deaerated by helium purging. The flow rate was 2.00 ml min^{-1}. Sample concentrations of THIP were calculated from the chromatograms using a known relation between THIP serum concentration and the THIP-Dns/muscimol-Dns peak height ratio. This relation was established by linear regression analysis of data obtained by extraction and HPLC analysis of standard samples. These were made by adding known amounts of THIP to human blank serum to produce concentrations in the range 0—4 μmol l^{-1} (0—561 ng of non-aqueous THIP per ml).

In the characterization of the performance of the method the coefficient of variation (C.V.) and the limit of sensitivity were estimated from analysis of six 2-ml aliquots of each of six standard serum samples representing the above range.

Experiments on analytical interferences

Serum was prepared from blood samples collected from in total 28 patients of both sexes undergoing antineoplastic, antiinflammatory or antihypertensive treatment, and the serum samples were analysed by the method described above, using detection by UV-absorption measurement. The only deviation from the established procedure was the omission of internal standard addition. The drugs administered were the following: acetylsalicylic acid, atenolol, bendroflumethiazide, bleomycin, CCNU, cholestyramine, cisplatin, cyclophosphamide, dactinomycin, diclofenac, digoxin, doxorubicin, 5-fluorouracil, ibuprofen, indomethacin, melphalan, methotrexate, misonidasol, mitopodozide, naproxen, paracetamol, penicillamine, prednisone, tolmetin, vinblastine, and vincristene. Also studied was the compatibility of the analytical method with the use of Vacutainers®.

RESULTS

Chromatography

The chromatogram of a serum sample obtained from a human volunteer 2 h after peroral administration of 10 mg of THIP (representing the lower part of the presumed therapeutic dose range) is reproduced in Fig. 3. Detection was made by measurement of UV absorbance and fluorescence (in series). Also shown is the chromatogram of a sample of human blank serum, without muscimol added, detected by UV-absorption measurement at 265 nm. The arrows indicate the position of peaks referring to THIP and muscimol, if present.

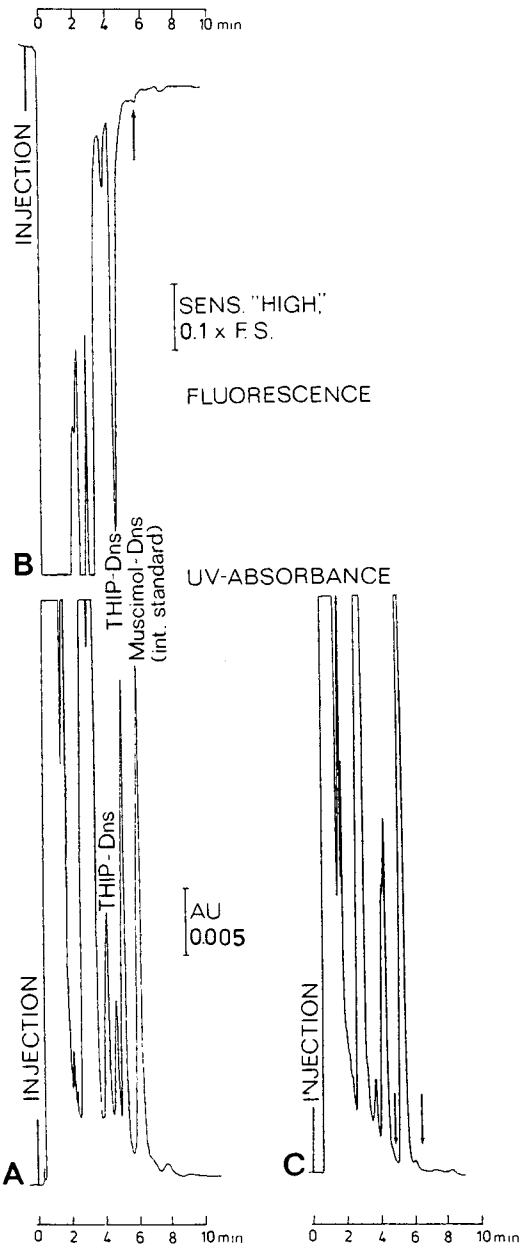

Fig. 3. Chromatograms of (A) a serum sample obtained from a human volunteer 2 h after peroral administration of 10 mg of THIP, detection by measurement of UV absorbance at 265 nm; (B) the same sample, monitored by fluorescence measurement, excitation wavelength 346 nm, emission wavelength 514 nm, the arrow indicates the position of muscimol-Dns; (C) human blank serum (without muscimol added), UV detection, arrows indicate the positions where THIP-Dns and muscimol-Dns would elute.

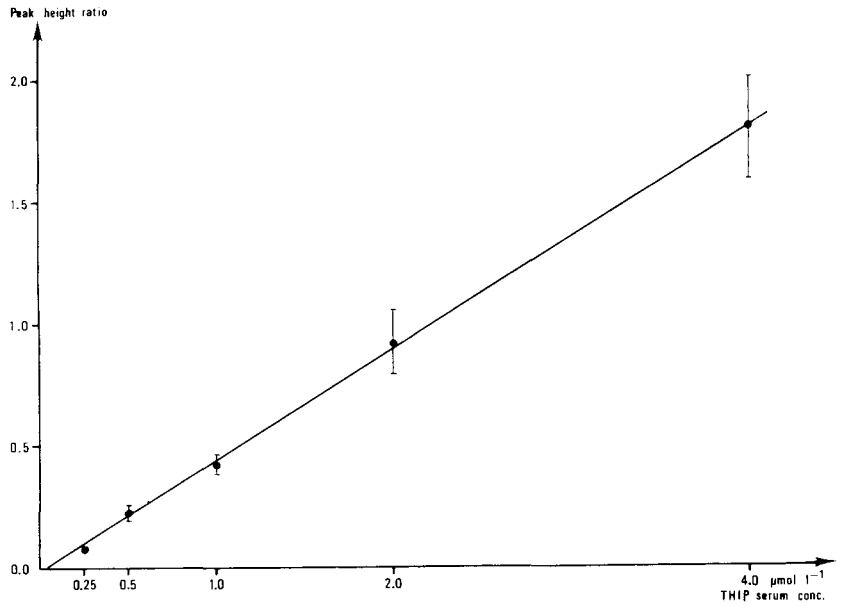

Fig. 4. Calibration curve constructed from peak height ratios. Mean of six determinations at each concentration. Error bars indicate the standard deviation.

Calibration curves, precision and linearity

Fig. 4 shows graphically the relation between THIP serum concentration and the peak height ratio. Each point represents the mean of six determinations, performed on the same day. The correlation coefficient in linear regression analysis was 0.9857.

A variance ratio test revealed the experimental points to belong to a straight line at the 0.90 probability level. The following values of C.V. were calculated in the five groups of THIP-spiked samples (the numbers in parentheses are the corresponding nominal THIP concentrations): 8.5 (0.25 μM); 13.7 (0.50 μM); 9.8 (1.0 μM); 14.3 (2.0 μM); 11.9 (4.0 μM). No correlation between C.V. and concentration was apparent.

Limit of sensitivity

From the data referring to the calibration curve shown (Fig. 4) it can be estimated that the lower limit of sensitivity is about 0.07 μmol l^{-1} (10 ng ml^{-1}). The intercept of the calibration curve with the abscissa axis was at 0.04 μmol l^{-1}, but the concentration must be 0.07 μmol l^{-1} (0.067 μmol l^{-1}) to separate the calculated peak height ratio from zero by twice the standard deviation (estimated from the C.V. of the 0.25 μM group).

A series of experiments on determination of THIP in serum samples spiked with relatively low THIP concentrations, 0.02 μmol l^{-1}, 0.03 μmol l^{-1}, or 0.04 μmol l^{-1}, revealed 0.03 μmol l^{-1} to be the lowest concentration able to produce an integrator signal exceeding the blank sample baseline at the position of THIP-Dns in the chromatogram.

Analytical interferences

In the analysis of the serum samples collected from patients receiving other drugs, no peaks occurred in the chromatograms, with one exception, at the positions where THIP or the internal standard, muscimol, would elute (in the form of Dns derivatives) if present in the serum sample. The single sample which contained an interfering substance, eluting at the position of THIP-Dns, had been obtained from a patient treated with indomethacin and mitopodozide. The size of the peak would correspond to a THIP serum concentration of about 0.15 μM. The interfering substance is not likely to be mitopodozide or a metabolite of this drug since it did not appear in any of the other samples from the several patients treated with this drug. Nor does indomethacin obviously account for the interfering peak, since the peak was absent in the chromatograms of the sample collected from the other indomethacin-treated patient.

Determination of the THIP concentration in six aliquots of THIP-spiked serum which had been contained in Vacutainer blood collection tubes revealed the recovery of THIP from these tubes to amount on an average 97.4% of the recovery of THIP from plain glass tubes. Recoveries were assessed from the areas under the peaks corresponding to THIP. Serum blank samples from Vacutainers did not contain interfering substances. In conclusion, Vacutainers (without anticoagulant) can be used for blood sample collection without affecting the determination of THIP quantitatively or qualitatively.

Application to pharmacokinetic studies

The proposed method for quantitative determination of THIP in serum has been applied for pharmacokinetic studies in man, dog, baboon, and rat. A

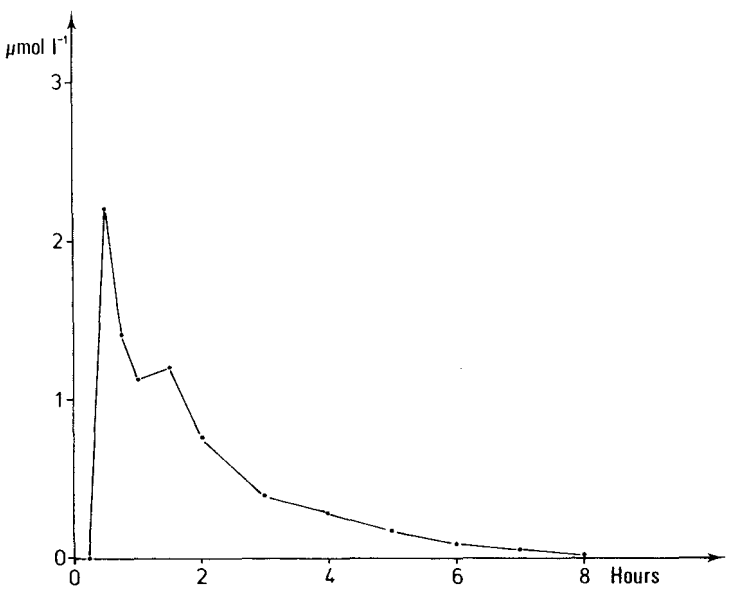

Fig. 5. Time course of THIP serum concentration after administration of a capsule containing 15 mg of THIP to a healthy, human volunteer.

representative observation on the time course of serum THIP is shown in Fig. 5. The curve refers to a healthy, human volunteer who received an oral dose of 15 mg of THIP.

DISCUSSION

The procedure for THIP serum concentration determination may seem troublesome and time-consuming, but experience has shown a capacity of duplicate determination of twelve samples per working day to be a realistic long-term schedule for one technician.

During the development of the present method, the use of alternative procedures and materials was of course considered:

Extraction

The efficient extraction of THIP from biological samples implied considerable trouble. Irrespective of the pH of the solvent, THIP is too polar to be extractable by solvents less polar than water, so extraction of THIP by means of an anion or cation exchanger, or as an ion-pair, was attempted. For ion-pair extraction the counter-ions trifluoroacetic acid or perfluorooctanosulfonic acid were used; chromatography did not reveal THIP to be extracted in this way.

Extraction of THIP by means of an ion-exchange material was attempted using strong as well as weak anion or cation exchangers. The influence of application buffer pH and resin bed height on column binding efficiency, and the suitability of various buffer solutions for selective elution of substances other than THIP, was studied using ^{14}C-labelled THIP, which was quantitated by liquid scintillation counting. This method was also used for evaluation of serum sample pretreatment (deproteinization), choice of solution for effective elution of THIP, and choice of eluate fraction to be collected for HPLC. The recovery of THIP in the extraction procedure was found to be 101.1 ± 2.3% when determined by [^{14}C]THIP.

Evaporation of water and ammonium bicarbonate from the extracts is necessary before derivatization and HPLC analysis. Freeze-drying was preferred to the use of a rotary evaporator because the former procedure resulted in a more complete ammonium bicarbonate sublimation, was less time-consuming, and allowed the use of smaller test tubes for eluate collection and evaporation.

Derivatization

The concentration of Dns chloride in the reagent used has been observed to influence the ratio of derivatives from THIP and muscimol detected in the HPLC analysis. A concentration of 7 mg ml^{-1} was found most suitable since in the range 5—10 mg ml^{-1} the ratio was rather constant, whereas the use of a great excess of dansyl chloride produced potentially interfering substances.

High-performance liquid chromatography

Nucleosil 5 C_{18} was compared with Spherisorb S5-ODS for use as column packing material. When tested by a benzene—naphthalene—anthracene mixture, the Nucleosil column was the more efficient one, but the opposite was the case when tested with authentic samples in the HPLC system described in the present paper.

The optimum wavelength setting for the UV-absorbance detector was found by means of a Uvikon LC 720 detector, which is able to record a UV-absorbance spectrum of the components anywhere in the chromatogram when eluent flow has been temporarily stopped. The UV absorbance of the Dns derivatives from THIP and muscimol is maximum at 254 nm in the medium used as mobile phase, but a wavelength of 265 nm was found to offer a more favourable proportion between the absorbance of the THIP and muscimol derivatives on the one hand and the absorbance of components eluting close to THIP-Dns on the other hand. However, monitoring at 254 nm will be satisfactory if only a detector equipped with this wavelength is available.

Detection by fluorescence monitoring was attempted using the excitation/emission wavelength combinations 254/514 nm or 346/514 nm (selected on the basis of absorption and fluorescence spectra). As is seen from Fig. 3, the component eluting just after THIP-Dns was not detected in fluorescence measurement. However, probably owing to the noise of the fluorescence detector, application of this technique was not found to improve the sensitivity of the method. In addition, muscimol-Dns was not detected by the fluorescence detector, neither did any of 30 other substances plausible for use as internal standard possess the desired combination of extractability, ability to form a fluorescent Dns derivative, favourable retention characteristics in the HPLC system, and stability in solution.

Interference from metabolites

THIP is known to undergo conjugation with glucuronic acid in mouse, rat, and man [7]. Conjugation at the enolic oxygen atom as well as at ring nitrogen in position 6 is imaginable; an O-monoglucuronide may extract in a similar way as unconjugated THIP in the cation-exchange extraction procedure. However, the conjugate will not form the same Dns derivative as free THIP does.

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REFERENCES

1 P. Krogsgaard-Larsen, T. Honoré and K. Thyssen, GABA-Neurotransmitters, Munksgaard, Copenhagen, 1978, pp. 201—216.
2 S.J. Enna, Biochem. Pharmacol., 30 (1981) 907.
3 S.M. Madsen, J. Chromatogr., 238 (1982) 509.
4 B. Schultz and S.H. Hansen, J. Chromatogr., 228 (1982) 279.
5 E. Carmona, C. Gomes and G. Trolin, Acta Pharmacol. Toxicol., 46 (1980) 235.
6 C. Atack and T. Magnusson, Acta Pharmacol. Toxicol., 42 (1978) 35.
7 B. Schultz, T. Aaes-Jørgensen, K.P. Bøgesø and A. Jørgensen, Acta Pharmacol. Toxicol., 49 (1981) 116.

Journal of Chromatography, 274 (1983) 219—230
Biomedical Applications
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SIMULTANEOUS DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYL METABOLITE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure is described for the determination of disopyramide and its mono-N-dealkyl metabolite which offers simplicity of extraction with excellent selectivity, sensitivity and reproducibility. The drug and metabolite, following basic diethyl ether extraction and back-extraction with acetic acid, are injected into a reversed-phase high-performance liquid chromatographic column and the absorbance of the eluate measured at 254 nm. Detectability limits of 0.05 μg/ml were obtained with both compounds, and studies of the reproducibility, precision, recovery, stability during storage and effect of time in separating plasma from erythrocytes are described. Applications of this high-performance liquid chromatographic procedure to plasma samples from patients on disopyramide therapy and to plasma and urine from a healthy dog administered single doses are reported.

INTRODUCTION

Disopyramide (I), [4-diisopropylamino-2-phenyl-2-(2-pyridyl) butyramide] (Fig. 1), is an antiarrhythmic agent which is active at the atrial as well as the ventricular level. It has pharmacological effects on the heart that are qualitatively similar to those of quinidine and procainamide. The major metabolite in man is the mono-N-dealkyl disopyramide (II, Fig. 1) [1] which is also found in dog. Species differences in the metabolism of I have also been described [2—4].

Many of the gas—liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) procedures applied to analysis of I in biological fluids have been reviewed by Duchateau and Hollander [5]. Since that time, HPLC procedures by Ahokas et al. [6] and Flood et al. [7] have been described, as well as a new enzyme immunoassay by Johnston and Hamer [8]. However,

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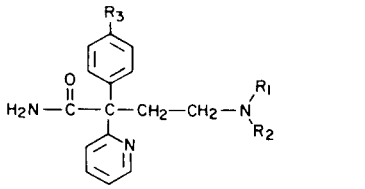
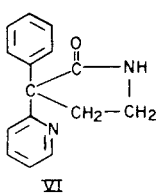


	R_1	R_2	R_3
I	$CH(CH_3)_2$	$CH(CH_3)_2$	H
II	$CH(CH_3)_2$	H	H
III	$CH(CH_3)_2$	$COCH_3$	H
IV	H	H	H
V	$CH(CH_3)_2$	$CH(CH_3)_2$	Cl



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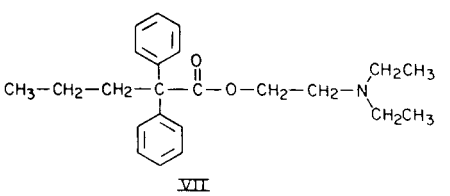

VII

Fig. 1. Chemical structures of disopyramide (I), mono-N-dealkyl disopyramide (II), acetylated mono-N-dealkyl disopyramide (III), di-N-dealkyl disopyramide (IV), *p*-chloro-disopyramide or SC 13068 (V), 3-phenyl-3-(2-pyridyl)-2-pyrrolidone or SC 32046 (VI) and β-diethylaminoethyl-2,2-diphenylpentanoate or SKF-525A (VII).

most of these methods do not allow simultaneous estimation of I and II. Methods which describe this capability include thin-layer chromatography (TLC) [9—11], GLC [12—14] and HPLC [15—17], but these either include cumbersome extraction procedures or do not attain the required sensitivity.

This paper describes a new HPLC procedure, involving a simple extraction, with system conditions to provide increased selectivity, sensitivity and reproducibility for estimation of I and II as well as some minor biotransformation products. Further, some comparison with GLC is given together with applications to patient and animal studies.

EXPERIMENTAL

HPLC

An HPLC system was assembled from an Altex Model 110A pump (Beckman Instruments, Toronto, Canada), a Model U6K injector (Waters Scientific, Mississauga, Canada), a Waters 202 UV detector operated at 254 nm and an Altex Ultrasphere ODS 5-μm stainless-steel column 150 mm × 4.6 mm I.D. and was employed for all analyses. The mobile phase was pumped at a flow-rate of 2.0 ml/min at room temperature producing a back pressure of 138 bars. A Brownlee Labs. (T.M.A. Scientific Supply, Mississauga, Canada) RP-8 column, 250 mm × 4.6 mm I.D., 10 μm, operated at 2.0 ml/min (back pressure of 55 bars) was used as an alternative.

The chromatographic mobile phase consisted of aqueous 0.05 *M* acetic acid—aqueous 0.05 *M* ammonium formate—distilled water—acetonitrile (9:13.5:22.5:55) and was prepared daily.

Gas—liquid chromatography with alkali flame ionization detector and with mass spectrometry

A gas chromatograph (Model 5730A; Hewlett-Packard, Avondale, PA, U.S.A.) equipped with an alkali flame ionization detector (AFID) was used. The 1.83 m × 2 mm I.D. coiled-glass column was packed with 5% OV-17 on Gas-Chrom Q, 100—120 mesh (Chromatographic Specialities, Brockville, Canada). The injection port and detector temperatures were maintained at 300°C and the column at 250°C. The detector bead current was adjusted to obtain maximum sensitivity. The carrier gas, helium, was maintained at a flow-rate of 30 ml/min and the air and hydrogen flow-rates were adjusted for optimum sensitivity.

A Hewlett Packard 5985 GLC—mass spectrometry (MS)—data system, operated in the electron impact mode with 70 eV as the ionization beam energy, was used for GLC—MS analysis with source maintained at 200°C. The chromatographic column was coiled glass, 1.22 m × 2 mm I.D., packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh. The injection port and detector temperatures were 275°C. The column oven temperature was maintained at 240°C for 1 min after the injection, then temperature programmed at 5°C/min to a final temperature of 280°C which was held for 5 min. Helium was used as carrier gas with a flow-rate of 30 ml/min.

Standards

Disopyramide (SC-7031), mono-N-dealkyl disopyramide (SC-24566), pyrrolidone metabolite (SC-32046) and *p*-chlorodisopyramide (SC-13068) were kindly supplied by Searle Pharmaceuticals (Oakville, Canada) and throughout this report will be identified as I, II, VI and V, respectively (Fig. 1).

Stock solutions (100 μg/ml) of I, II and V were prepared by dissolving appropriate amounts of the base in 0.01 *N* aqueous hydrochloric acid. Working plasma solutions containing I (0.5—4.0 μg/ml) and II (0.125—3.0 μg/ml) were prepared fresh daily by appropriate dilution of stock solutions with blank plasma. Working solutions of the internal standard V (4 μg/ml) were obtained by diluting the stock solution with distilled water.

Chemicals and reagents

Diethyl ether (Mallinckrodt, Montréal, Canada) was glass-distilled prior to use. HPLC-grade acetonitrile, chloroform and methanol (Fisher Scientific, Ottawa, Canada), distilled in glass ethyl acetate (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), Analar grade amyl acetate and acetic anhydride (BDH, Toronto, Canada) and all other analytical-grade chemicals were purchased from commercial sources. Compound SKF 525A [β-diethylaminoethyl-2,2-diphenylpentanoate] (Smith, Kline & French Labs., Philadelphia, PA, U.S.A.) was used as an internal standard for the GLC procedure and identified as VII (Fig. 1).

Patients

All plasma samples were obtained from patients admitted to the cardiac unit of a local hospital and treated with disopyramide (Rythmodan capsule).

Dog

Separate doses of disopyramide phosphate capsules (Norpace) equivalent to 150 mg (1 capsule) and 750 mg (5 capsules) base respectively were given orally to a healthy male Labrador dog (33.1 kg) with 28 days between doses. The animal was placed in a metabolism cage and total urine was collected for the 24 h prior to ingestion and for 24-h periods up to 72 h. The urine was divided in two: one portion being stored at 4°C and the other at −15°C prior to extraction. Blood samples (10 ml) were taken from the saphenous vein into heparinized tubes at appropriate time intervals up to 72 h. The tubes were centrifuged, plasma was separated and stored at 4°C till analysis. For both doses, the dog was fasted overnight, no food was given till the 7-h sample post dose, but water was permitted ad libitum after drug administration.

Extraction procedure

HPLC analysis. To a plasma sample (1 ml) was added 0.5 ml of an aqueous solution of the internal standard V (4 μg/ml). The contents were mixed (Vortex Genie, Fisher Scientific) for 15 sec before adding 0.1 ml of concentrated ammonium hydroxide and 6 ml diethyl ether. The drug and the metabolite, along with V, were extracted into the ethereal phase by shaking on a rotary mixer (Roto-Rack, Fisher Scientific) for 15 min followed by centrifugation. A portion of the organic layer (5.0 ml) was transferred to a conical tube containing 0.2 ml of an 0.1 *M* aqueous acetic acid solution. The contents were mixed and centrifuged for 5 min each. Aliquots, 25—100 μl, of the aqueous layer were analysed by HPLC.

Urine was similarly processed; 9-ml aliquots of urine being basified with 1.5 ml concentrated ammonium hydroxide and extracted with 9 ml diethyl ether on the rotary mixer. The ethereal layer, separated by centrifugation, was then evaporated to dryness under a light stream of nitrogen at 55°C, the residue dissolved in 300 μl acetonitrile and appropriate aliquots injected onto the chromatograph.

GLC—AFID and GLC—MS analysis. The extraction procedure for plasma analysis was essentially that of Johnston and McHaffie [18]. The urine samples were extracted as described previously for HPLC. Acetylation of plasma or urine extracts was accomplished by dissolving the dry residue from the ethereal

extract with 3 ml of chloroform. To this solution, 25 μl of acetic anhydride were added before mixing on a Vortex mixer. This organic solution was evaporated in a dry bath at 55°C under light stream of nitrogen, the residue dissolved with 300 μl of ethyl acetate or methanol and aliquots (1—5 μl) injected into the GLC or GLC—MS system.

Calibration curves

Peak height ratios were calculated by dividing the height of the peak due to the drug or the metabolite by the height of the peak due to the internal standard. Calibration curves were assembled from the results obtained by analysing spiked control plasma by plotting the peak height ratios against the concentration of I or II. Calibration curves were run with every set of unknowns.

RSULTS AND DISCUSSION

HPLC analysis

Under the conditions described in the Experimental section, analysed on a Ultrasphere ODS column, the compounds I, II, and V gave sharp and symmetrical peaks with retention times of 4.2, 3.1 and 5.4 min, respectively. The total analysis time for one injected sample was approximately 8 min. Fig. 2 shows chromatograms obtained by processing a 1-ml sample of blank plasma

TABLE I

HPLC CALIBRATION CURVE OF DISOPYRAMIDE AND MONO-N-DEALKYL DISOPYRAMIDE EXTRACTED FROM PLASMA

Concentration (μg/ml)	*n*	Mean ratio	R.S.D. (%)
A: Disopyramide			
0.50	24	0.43	5.7
1.00	24	0.84	3.2
2.00	16	1.70	3.1
3.00	14	2.50	1.5
4.00	6	3.27	1.3
		Mean	3.0
Slope = 0.822, intercept = 0.027 and coefficient of determination r^2 = 0.998			
B: Mono-N-dealkylated disopyramide			
0.125	3	0.13	7.7
0.25	14	0.28	3.6
0.50	20	0.64	4.7
1.00	14	1.26	3.2
1.50	7	1.86	4.8
2.00	4	2.54	3.9
3.00	2	3.88	—
		Mean	4.8
Slope = 1.285, intercept = —0.024 and coefficient of determination r^2 = 0.996			

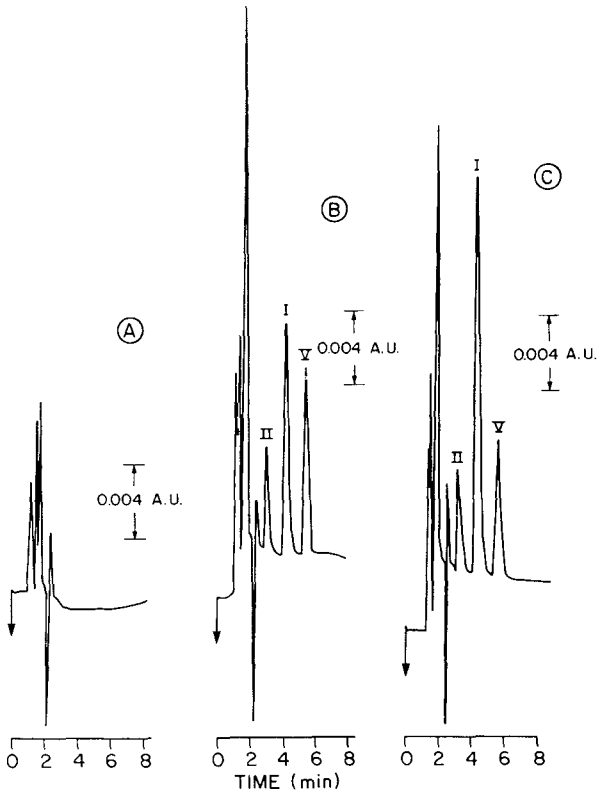

Fig. 2. HPLC chromatograms from human plasma. A, blank plasma; B, plasma spiked with 0.50 μg of mono-N-dealkyl disopyramide and 1.00 μg of disopyramide; C, plasma from a patient estimated to contain 0.78 μg of the mono-N-dealkyl and 2.58 μg of disopyramide. Peaks: I = disopyramide; II = mono-N-dealkyl disopyramide; V = *p*-chlorodisopyramide, the internal standard.

(A), plasma containing added drug and metabolite (B) and plasma from a patient treated with disopyramide (C). The peaks observed in blank plasma did not interfere with the peaks due to I, II and V.

The typical calibration curves obtained were linear with negligible intercepts over the range studied: 0.50—4.00 and 0.125—3.00 μg/ml for I and II, respectively. The accuracy and precision of the HPLC procedure are demonstrated in Table I. The overall mean relative standard deviations for I and II were 3.0 and 4.8% over their respective ranges.

The overall mean recoveries (± S.D.) of I, II and V from plasma were 72.8 ± 2.9%, 55.8 ± 1.5% and 73.8 ± 3.1%, respectively. The percentages recovered from different plasma concentrations for the drug and its metabolite, and the internal standard are demonstrated in Table II. There was no marked change in recovery over the concentration ranges investigated.

Results of the application of the present method to the determination of plasma I and II concentrations in samples from patients treated with disopyramide are shown in Table III. Those from dog experiments are

TABLE II

RECOVERY OF DISOPYRAMIDE, MONO-N-DEALKYL DISOPYRAMIDE AND *p*-CHLORODISOPYRAMIDE FROM PLASMA (HPLC EXTRACTION)

Compound	Amount added to 1 ml of plasma (μg)	*n*	Mean percentage recovered	R.S.D. (%)
Disopyramide	0.50	7	73.0	6.1
	1.00	10	76.7	4.7
	2.00	5	71.5	3.6
	3.00	6	69.8	5.1
	mean	—	72.8	4.9
Mono-N-dealkyl disopyramide	0.25	7	55.1	5.2
	0.50	6	56.2	5.0
	1.00	6	57.6	5.1
	1.50	5	54.2	5.2
	Mean	—	55.8	5.1
p-Chlorodisopyramide	2.00	25	73.8	4.2

TABLE III

PATIENT PLASMA LEVELS OF DISOPYRAMIDE AND MONO-N-DEALKYL DISOPYRAMIDE BY HPLC

Patient	Collection time (h)		Plasma concentration (μg/ml)	
			I	II
1	08:00		2.15	0.69
	11:00		1.79	0.75
2	08:00		2.36	0.84
3	13:00		1.35	0.64
4	unknown		0.95	0.54
5	22:00		2.55	0.72
6	11:00		2.99	0.78
7	09.30		2.24	3.74
8	11:00		2.62	0.99
9	11:00		2.85	0.83
10	11:00		2.90	1.02
11	11:00		2.00	1.88
12	11:00		1.84	0.37
13	19:00		1.72	1.56
14	11:00		1.83	0.31
15	08:00	dosed 0 h	no sample	
	10:00	2 h	3.33	0.94
	13:00	5 h	3.01	0.87
	15:00	7 h	2.85	0.80
	19:00	11 h	1.70	0.66
16	08:00		3.56	1.35
17	11:00		7.87	1.58
18	11:00		3.77	0.95

illustrated in Fig. 3. Each quoted estimation is the mean from replicate analysis.

The HPLC conditions used for plasma level determinations are also applicable to urine analysis and chromatograms are shown in Fig. 4. The urine extraction was judged to be adequate because no endogenous peaks eluted at the retention times of I, II and VI (Fig. 4A). This procedure readily allows quantitation of the drug and the mono-N-dealkyl (II) and di-N-dealkyl (VI) metabolites.

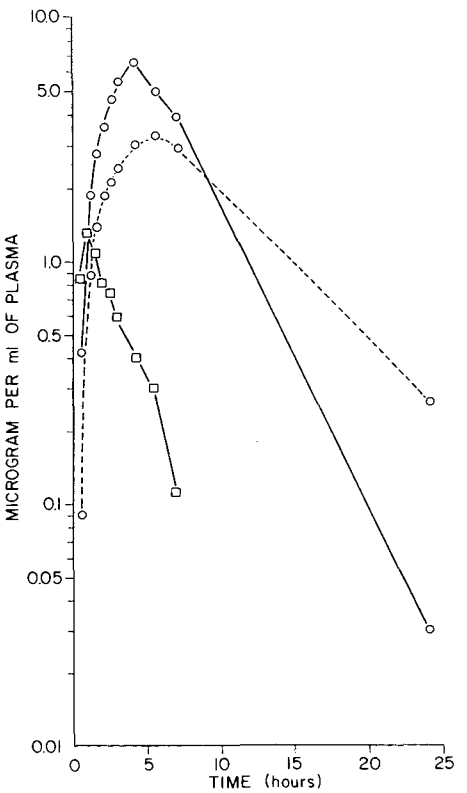

Fig. 3. Plasma concentration profiles obtained from the same dog following ingestion of 150 mg Norpace capsule and five 150-mg Norpace capsules. □——□, disopyramide (150 mg); ○——○, disopyramide (750 mg); ○— — —○, mono-N-dealkyl disopyramide (750 mg).

The RP-8 column sorbent was selective for the analysis of I, II, V and VI and gave sharp and symmetrical peaks eluting at 4.1, 3.1, 5.3 and 2.1 min, respectively under the conditions described in the Experimental section. It appears to compare favorably with the Ultrasphere ODS column in terms of sensitivity and stability.

Although the GLC—AFID procedure was adequate for the determination of I in plasma (0.15—2.00 μg/ml), problems were encountered with the determination of II (Fig. 5). Derivatization of II with acetic anhydride was required prior to GLC—AFID analysis because otherwise the metabolite did not elute as a single peak. Although this additional step did not affect the determination

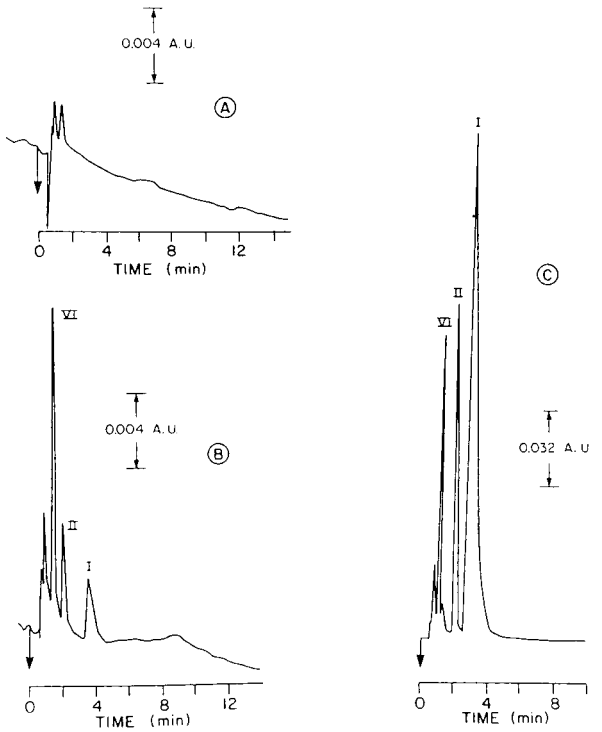

Fig. 4. Typical HPLC chromatograms of dog urine after oral dose of Norpace capsule(s). A, blank control urine; B, 24—48 h urine collection, 150 mg; C, 0—24 h urine collection 750 mg. Peaks: I = disopyramide, II = mono-N-dealkyl disopyramide and VI = cyclic di-N-dealkyl disopyramide (SC 32046).

of I, it did not prove possible to obtain reproducible calibration curves for II and the significant negative intercepts noted may indicate instability. This problem was not overcome despite modification of various conditions such as injector and detector temperatures, column phases, solvents, temperature and time for reaction. At higher concentrations, i.e. greater than 4 μg of II per ml plasma, this problem would possibly be less important. Because of these difficulties, the simultaneous determination of I and II by GLC—AFID was abandoned in favor of the HPLC method.

Stability of I and II in plasma

A stability study was undertaken over a 31-day period to determine the effect of storage on the concentration of I and II. A control human blank plasma was spiked with I and II giving a final concentration of 1.8 and 1.0 μg/ml, respectively with aliquots being stored at —20°C and at 4°C until analysis time. There was no difference in the estimated levels for either I or II under either conditions of storage over the 31 days as found from triplicate HPLC analysis at various time intervals (mean I, 1.75 μg/ml ± 5%, mean II, 0.98 μg/ml ± 6%) indicating that the substances are stable in plasma for at least 1 month under those conditions.

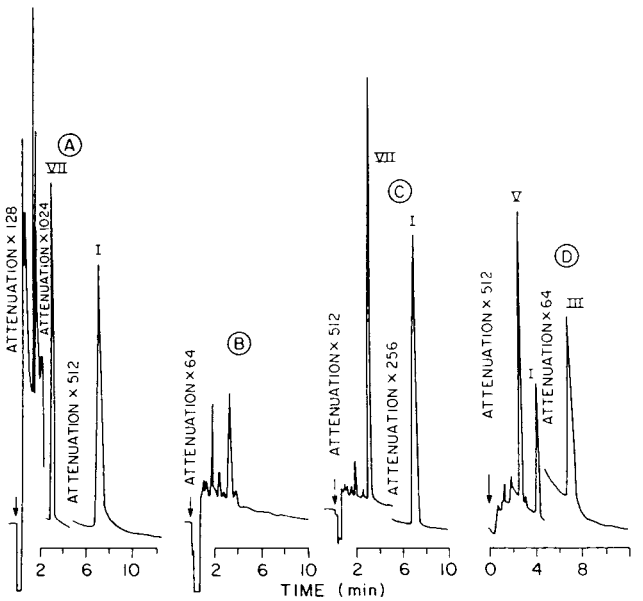

Fig. 5. Typical GLC—AFID chromatograms of I and VII when injected as a methanolic standard solution containing 20 and 15 μg/ml of I and VII respectively (A), blank plasma (B), spiked plasma at 1 μg/ml of I to which 1 μg of VII (internal standard) was added (C), of I, II acetylated (III) and V acetylated obtained at column temperature of 270° C from a 1-ml methanolic standard solution containing 3 μg I, 1.5 μg II and 2 μg V after derivatization (D). Peaks: I = disopyramide; III = monoacetylated derivative of mono-N-dealkyl disopyramide; V = *p*-chlorodisopyramide (internal standard); VII = SKF 525A (internal standard).

Effect of blood collection method

Blood was withdrawn from healthy subjects using heparinized evacuated glass tubes (Vacutainer, green rubber stopper) avoiding contact with the stopper. The samples were divided into two aliquots and spiked with I and II to obtain concentrations of 2 μg I and 1 μg II per ml and 1.5 μg I and II per ml. Equal volumes of each spiked blood sample were transferred to a green rubber stopper Vacutainer and to a polypropylene tube respectively. The tubes were stoppered and placed on a Roto-Rack for 2 min at 5 rpm, centrifuged, then plasma was removed and analysed in duplicate by HPLC. Ratios of the concentrations found from the Vacutainer tube over the polypropylene tube were 0.96 and 1.02 for disopyramide at 2.00 and 1.50 μg/ml levels and 1.01 for II at the 1.5 μg/ml concentration values which indicated no substantial difference.

A similar experiment was carried out during the dog study (750 mg) with blood collected by glass syringe and by Vacutainer on three separate occasions. Again there were no substantial differences in the values from the two collection procedures as determined by HPLC.

The above experiments showed that these materials used for blood collection did not affect the plasma drug or metabolite concentrations.

Effect of the pre-analysis storage time of blood samples

Blood from a healthy human subject was spiked with I and II to give a final

concentration of 2.0 and 1.0 μg/ml, respectively. The blood was divided into three aliquots and the tubes kept at room temperature. One tube was centrifuged immediately (0 h) and plasma was analysed in duplicate by HPLC. The second aliquot was centrifuged and the plasma analysed after standing 1.5 h at room temperature. The percentages found compared to the 0-h sample were 100.4% for I and 89.4% for II. After standing 2.45 h, the third aliquot was centrifuged and analysis of plasma yielded 101.9% and 62.8% of the 0-h value for I and II, respectively. This experiment demonstrated that while the time of standing before centrifugation and transfer of plasma did not affect the disopyramide plasma level, the mono-N-dealkyl disopyramide plasma concentration decreased significantly, i.e. 37.2% in 2.45 h.

In contrast to the above result, Daniel and Subramanian [19] using GLC–FID reported a loss of 30% for disopyramide in 1 h when blood (spiked with 5.5 μg/ml) was stored at ambient temperature. Johnston and McHaffie [18], however, again with GLC—FID, could not confirm that latter observation in plasma from a volunteer treated with 400 mg of I.

Knowing that I and II are stable in plasma, as demonstrated previously, the experiment completed with spiked blood indicated the importance of minimizing the time between blood sampling and centrifugation, particularly when the mono-N-dealkyl metabolite is being estimated.

Dog studies

The plasma concentrations of I found following the 150 and 750-mg doses of disopyramide to the dog are illustrated in Fig. 3. With the lower dose, the drug was not detectable after 7 h nor was metabolite II detectable at any time, while with the higher dose II was also followed over 24 h. The elimination half-life was about 1.8—2.5 h for I and 5.0 h for II, values in agreement with Karim et al. [4]. The dog was unaffected by the low dose but was noticeably docile and vomited about 7 h after the larger dose. The peak for I was also delayed with the higher dose (from 1 to 4 h).

Typical HPLC chromatograms obtained from urine extracts are shown in Fig. 4. Collection of fractions from the liquid chromatograph followed by mass fragmentography indicated that VI was the cyclic stable product formed after di-N-dealkylation as described by Karim et al. [2]. GLC—MS analysis of the acetylated urine extracts also confirmed the presence of I, II and VI with retention times of 3.6, 7.4 and 2.1 min, respectively from comparison with the mass spectra obtained from authentic samples. In the HPLC examination of the urine extracts (Fig. 4) the amount of VI increased with time relative to I and II and persisted even with the low dose to 48 h. With the higher dose only small amounts of I and II were detectable in the 48—72 h collection, while VI again persisted.

Patients

Results of the application of the HPLC method to patient plasma samples are given in Table III. Values for I ranged widely from 0.95 to 7.87 μg/ml and II for 0.31 to 3.74 μg/ml with means of 2.65 and 1.04 μg/ml, respectively. These ranges although wide are in agreement with values in various studies in the literature [12, 20].

CONCLUSION

The described HPLC procedure, for the simultaneous determination of disopyramide and mono-N-dealkyl disopyramide in plasma, is rapid, sensitive, specific and easily reproducible. It has adequate sensitivity for plasma level determination with detectability limit as low as 0.05 μg/ml of either I or II. Storage of plasma at 4°C and −20°C for a period as long as 31 days did not affect the amount of I and II recovered. However, blood should be centrifuged immediately after collection because loss of II but not I occurs on standing at room temperature. The different tubes used for blood collection had no apparent influence on the plasma levels of I and II. This HPLC method is adequate for plasma level determinations of disopyramide and mono-N-dealkyl disopyramide after single or therapeutic doses in pharmacokinetic studies.

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REFERENCES

1 R.C. Heel, R.N. Brogden, T.M. Speight and G.S. Avery, Drugs, 15 (1978) 331.
2 A. Karim, R.E. Ranney and S. Kraychy, J. Pharm. Sci., 61 (1972) 888.
3 A. Tatematsu, T. Nadai, H. Yoshizumi, M. Suzuki, N. Nagahama, Y. Nakajima, E. Tajima and K. Mochizuki, Shitsuryo Bunseki, 21 (1973) 259.
4 A. Karim, C. Kook, R.L. Novotney, J. Zagarella and J. Campion, Drug Metab. Disp., 6 (1978) 338.
5 A.M.J.A. Duchateau and J.M.R. Hollander, in F.W.H.M. Merkus (Editor), Proceedings of a symposium on the occasion of the 50th anniversary of the Netherlands Association of Hospital Pharmacists, Amsterdam, December 15, 1979, Excerpta Medica, Amsterdam, Oxford, Princeton, 1980, p. 162.
6 J.T. Ahokas, C. Davies and P.J. Ravenscroft, J. Chromatogr., 183 (1980) 65.
7 J.G. Flood, G.N. Bowers and R.B. McComb, Clin. Chem., 26 (1980) 197.
8 A. Johnston and J. Hamer, Clin. Chem., 27 (1981) 353.
9 R.N. Gupta, F. Eng and D. Lewis, Anal. Chem., 51 (1979) 455.
10 R. Mallein, M. Boucherat, J. Rondelet, M. Pignat and H. Ksavrelof, Ann. Pharm. Fr., 36 (1978) 655.
11 M.G. Simona and E.M. Grandjean, J. Chromatogr., 224 (1981) 532.
12 T.C. Hutsell and S.J. Stachelski, J. Chromatogr., 106 (1975) 151.
13 M.-L. Aitio, J. Chromatogr., 164 (1979) 515.
14 J.E. Bredesen, Clin. Chem., 26 (1980) 638.
15 P.J. Meffin, S.R. Harapat and D.C. Harrison, J. Chromatogr., 132 (1977) 503.
16 J.J. Lima, Clin. Chem., 25 (1979) 405.
17 G. Nygard, W.H. Shelver and S.K.W. Khalil, J. Pharm. Sci., 68 (1979) 1318.
18 A. Johnston and D. McHaffie, J. Chromatogr., 152 (1978) 501.
19 J.W. Daniel and G. Subramanian, J. Int. Med. Res., 4 (1976) 2.
20 M.L. Aitio and T. Vuorenmaa, Brit. J. Clin. Pharmacol., 9 (1980) 149.

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ANALYSIS OF ACENOCOUMARIN AND ITS AMINO AND ACETAMIDO METABOLITES IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Acenocoumarin and its acetamido metabolite, after extraction at pH 4.4, were analysed by isocratic reversed-phase high-performance liquid chromatography using aqueous acetonitrile (pH 4.90) as eluent. Warfarin was used as internal standard. The amino metabolite, after back-extraction into 0.5 *N* HCl, was derivatized by diazotization and heat treatment. The resulting product was analysed by the same reversed-phase system.

The sensitivity of the method for acenocoumarin and its amino metabolite was in the range of 20 ng/ml. To achieve this sensitivity for the analysis of the acetamido compound, the acetonitrile content of the eluent had to be lowered. The assay was applied to the analysis of plasma samples of patients under acenocoumarin therapy. The disposition of the amino compound in rats was investigated.

INTRODUCTION

Acenocoumarin (Sintrom^{R}) belongs to the 4-hydroxycoumarin congeners in use clinically as oral anticoagulants. Pharmacologically acenocoumarin is classified as a short-acting anticoagulant [1]. The compound differs chemically from warfarin by its 4′-nitro group (Fig. 1). Potentially, this nitro group is vulnerable to biotransformation (i.e. reduction) and, by using ^{14}C-labeled acenocoumarin Dieterle et al. [2] could show the formation of the amino and the corresponding acetamido metabolite in man. In mice these metabolites appeared to be more potent as anticoagulants than acenocoumarin [2]. In rats the amino metabolite was the most potent [3]. The significance of these metabolites for the pharmacodynamics of acenocoumarin in man, although stressed recently by Godbillon et al. [4], has not yet been established. Further investigation of the clinical relevance of the formation of these metabolites in

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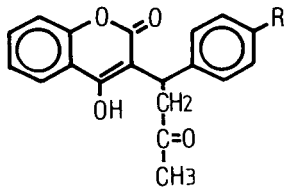

R = NO_2	acenocoumarin
R = NH_2	amino
R = $NHCOCH_3$	acetamido
R = H	warfarin

Fig. 1. Chemical structures of acenocoumarin, the amino and acetamido metabolites, and warfarin.

a broader group of subjects [5] requires an easy and reliable assay method for acenocoumarin and its amino and acetamido metabolites. This paper describes the application of high-performance liquid chromatography (HPLC) to analyse the compounds in body fluids.

EXPERIMENTAL

HPLC instrumentation and chromatographic conditions

The HPLC system consisted of a Waters M-6000A pump equipped with a U6K injector. Ultraviolet (UV) absorbance at 303 nm was monitored with a Pye-Unicam LC UV detector, and the signal was processed with a Waters computing integrator. A LiChrosorb RP-8 column (particle size 5 μm, 150 mm × 4.6 mm I.D.; Chrompack, Middelburg, The Netherlands) was used in combination with a guard column (ODS pellicular material; 50 mm × 2.1 mm I.D.). The mobile phase consisted of 0.1% acetic acid—acetonitrile—ethyl acetate (100:90:1, v/v) adjusted to pH 4.90 with 1 *N* ammonia. The flow-rate was 1.5 ml/min.

Materials

Acenocoumarin was a gift of Ciba-Geigy, The Netherlands. The amino derivative was prepared by catalytic (Pt) hydrogenation of acenocoumarin. The acetamido derivative was prepared by treating the amino derivative with acetic anhydride. The identity of the products was verified by comparison with authentic material obtained from Ciba-Geigy, Basel, Switzerland. Warfarin was obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

To 1 ml of plasma in a centrifuge tube, 500 ng of warfarin (10 μl of a 50 μg/ml solution in water), and 1 ml of concentrated citric acid—phosphate buffer, pH 4.40, were added. Extraction was made twice with 5 ml of dichloromethane—*n*-pentane (1:1, v/v). The combined organic phase was extracted with 0.2 ml of 0.5 *N* hydrochloric acid. Collection of the organic phase was performed by freezing the water phase.

The organic phase, containing acenocoumarin, acetamido derivative, and warfarin, was evaporated to dryness at 35°C under a stream of nitrogen. The residue was dissolved in 100 μl of eluent. The acid phase, containing the amino compound, was processed as follows: after removing remnants of organic phase by a stream of nitrogen at 35°C (takes about 5 min), 500 ng of warfarin (10 μl of a 50 μg/ml solution in water) and 200 μl of sodium nitrite (2% solution in

water) were added. After standing for 5 min at room temperature to complete the diazotization, the mixture was heated at 65°C for 30 min.

Together with the amino sample, calibration points were processed as follows: to 200 μl of 0.5 *N* hydrochloric acid containing 50, 100, 500 and 1000 ng of the amino compound in centrifuge tubes, warfarin (500 ng) and 200 μl of the nitrite solution were added. The mixtures were heated for 30 min at 65°C. Following the heat treatment, the reaction mixtures were extracted with 5 ml of the dichloromethane—*n*-pentane extraction solvent. After evaporation to dryness, the residue was dissolved in 100 μl of eluent. Of both residues a 20-μl volume was injected.

RESULTS AND DISCUSSION

Chromatography

Because of the acidic character of the 4-hydroxycoumarins, the reversed-phase system described in the literature for these drugs, i.e. warfarin [6], phenprocoumon [7], and acenocoumarin [8], basically consists of an acidic eluent. The amino metabolite, however, also posesses a basic function. This

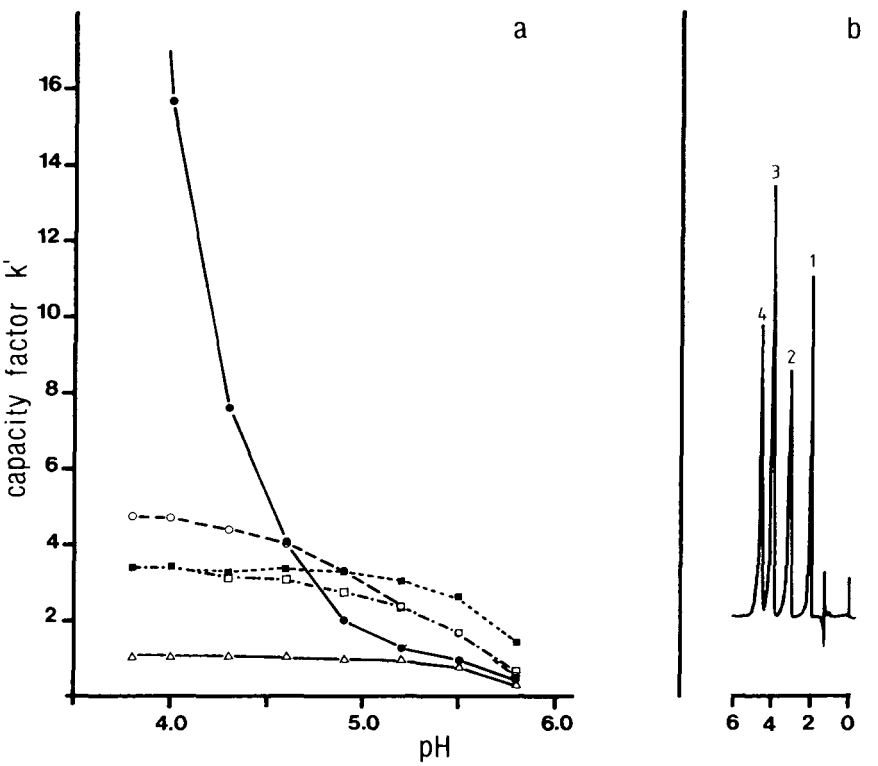

Fig. 2. (a) The dependency of the retention time (capacity factor *k'*) on the pH of the eluent. (□), acenocoumarin; (●), amino metabolite; (△), acetamido metabolite; (○), phenprocoumon; (■), warfarin. (b) Chromatogram of a mixture of acetamido metabolite (1), amino metabolite (2), acenocoumarin (3), and warfarin (4). The peaks represent 500 ng. Detector sensitivity, 0.08 a.u.f.s. Times scale in min.

makes the elution of the compound strongly pH-dependent. Fig. 2a shows that by raising the pH of the eluent the capacity factor of the amino metabolite decreased from "infinity" at pH 3.80, i.e. the pH of the eluent without addition of ammonia, to $k' = 0.4$ at pH 5.80. The same was observed when 0.01 *M* citric acid was used instead of 0.1% acetic acid. With sulfuric acid it was found that the retention time varied inversely with the concentration of the sulfuric acid in the eluent. These observations suggest ion-ion interactions between the stationary phase (LiChrosorb 5 RP-18) and the protonated amino metabolite. At pH 4.9 optimal separation was obtained for acenocoumarin its metabolites and warfarin, the latter being chosen as internal standard (Fig. 2b).

Sample preparation

Having once obtained optimal chromatographic conditions it was found that the recovery of the amino metabolite following straightforward sample preparation (i.e. extraction followed by evaporation of the organic phase) was erratic, apparently due to decomposition of the compound during evaporation of the organic phase. Several experimental variations were tried to solve the problem. Acid back-extraction of the amino metabolite from the organic phase followed by diazotization and heat treatment gave reliable results. Under the conditions used (see Methods) only one acid-extractable reaction product was formed (Fig. 3).

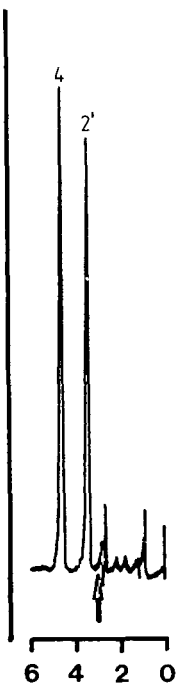

Fig. 3. Chromatogram of the extract of the diazotization reaction of the amino compound. Peak 2' represents the reaction product, peak 4 represents warfarin. The reaction was performed with 500 ng of the amino compound. (For reaction details, see Methods). The arrow indicates the position of the amino compound in the chromatogram. Detector sensitivity, 0.02 a.u.f.s. Time scale in min.

The precise identity of the product is not yet clear. In view of the way aromatic diazonium salts react, it is reasonable to think of either 4′-chloro- or 4′-hydroxywarfarin. The UV absorption spectrum of the compound showed a resemblance to that of warfarin. The reaction conditions, especially the reaction temperature, appeared to be critical. Temperatures above 75°C resulted in the formation of other products at the cost of the product just discussed. At temperatures lower than 60°C the reaction rate was slow. At a reaction temperature of 65°C about 60% of the amino derivative was converted to the product within 30 min; at 90 min this amounted to about 80%. Because of the specificity of the procedure, and because calibration points were always processed in parallel with the samples, a 30-min reaction time was found to be sufficient for the assay. The calibration curves (peak area ratio of product to warfarin vs. the amount of amino derivative) were found to be linear over the entire concentration range tested; up to 5 μg of amino metabolite. A typical regression is given by $y = (13.4 \cdot 10^{-4})x - (0.1 \cdot 10^{-4})$, $s = 0.01$, $r^2 = 0.999$, where y is the peak area ratio and x is the amount (ng) of the amino metabolite in the reaction mixture.

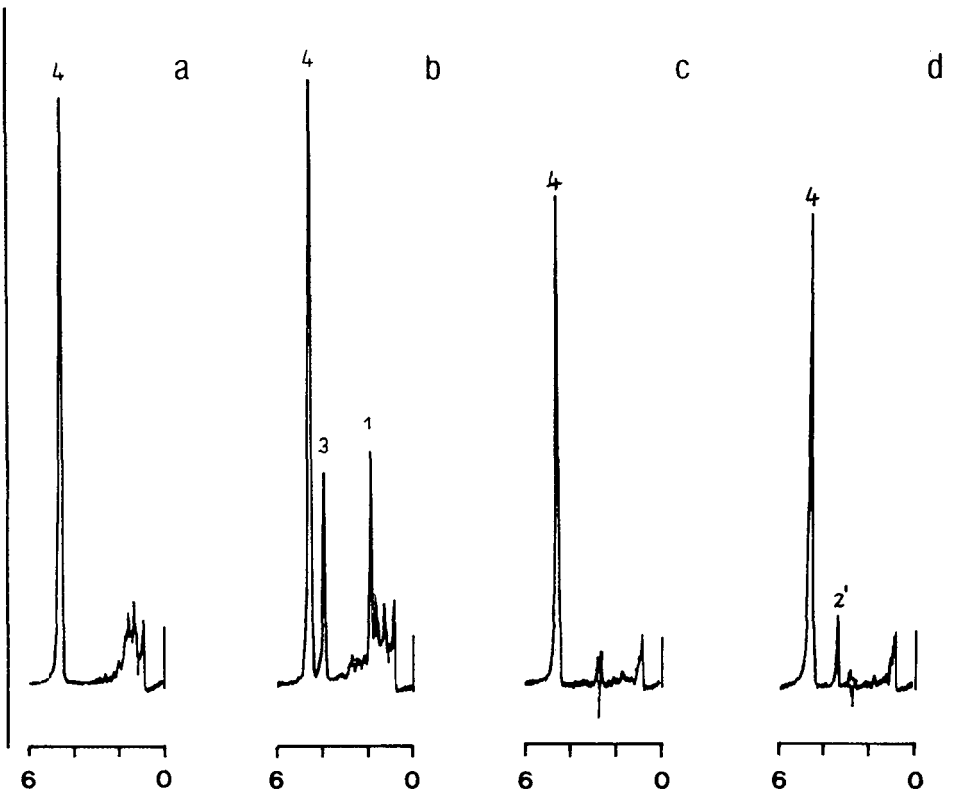

Fig. 4. Chromatograms of blank human plasma and of a plasma sample spiked with 100 ng of acenocoumarin and its amino and acetamido metabolites. (a) The organic phase of the blank. (b) The organic phase of the spiked plasma. (c) The "amino phase" of the blank. (d) The "amino phase" of the spiked plasma. The peaks represent: acetamido metabolite (1); the diazotization produce (2′); acenocoumarin (3); warfarin (4). Detector sensitivity, 0.02 a.u.f.s. Time scale in min.

Specificity and reproducibility

Fig. 4 shows examples of chromatograms of extracts obtained from pooled human plasma containing 100 ng/ml of acenocoumarin and its metabolites. For acenocoumarin and the amino derivative the procedure appeared to be highly specific, allowing the quantitation of concentrations of at least 25 ng/ml. For the acetamido metabolite, at low plasma concentrations (less than about 100 ng/ml) the system appeared to be less reliable because the compound eluted in the front where plasma constituents may also be present (Fig. 4b).

This problem can be surmounted by enhancing the resolution of the chromatographic system for the acetamido derivative, i.e. by decreasing the acetonitrile content of the eluent to 50 volume parts. Results of experiments testing the accuracy and precision of the assay are given in Table I. Using the internal standard procedure, the recoveries of acenocoumarin and the acetamido compound were about quantitative. Absolute recoveries of both the compounds and the internal standard warfarin, as measured by using ^{14}C-labeled compounds, were found to be about 90%. For the amino derivative, as calculated from the calibration points, about 80% recovery was obtained (Table I).

TABLE I

RECOVERY FOR ACENOCOUMARIN AND ITS METABOLITES

Results are expressed as mean ± S.D. (n = 4).

Plasma conc. (ng/ml)	Acenocoumarin	Amino metabolite*	Acetamido metabolite
50	96 ± 5	74 ± 3	92 ± 6**
100	98 ± 4	82 ± 10	95 ± 10**
200	103 ± 7	80 ± 2	92 ± 8
500	90 ± 3	76 ± 5	100 ± 8

*Calculated from calibration points processed along with the diazotization reaction (see methods).
**By analysis with the "slow" eluent (see text).

To test the applicability of the procedure for the amino and acetamido compounds, the former was given to three rats in a 2 mg/kg dose subcutaneously. Blood samples (0.2 ml) were obtained via an indwelling catheter in the femoral artery. Urine was collected for 48 h. The blood concentration—time profiles are shown in Fig. 5. Acetylation of the amino compound was rapid and extensive, the first blood sample (10 min after administration) already showed the presence of the acetamido metabolite. In urine about 10% of the dose was excreted unchanged and about 40% as the acetamido metabolite. The blood half-life was about 8 h for the amino compound.

Plasma samples of 27 patients anticoagulated with acenocoumarin were analysed (the patients were under control of the Thrombosis Department of the University Hospital). In all but two samples acenocoumarin was analysed, concentration range 14—154 ng/ml. In none of the samples could the

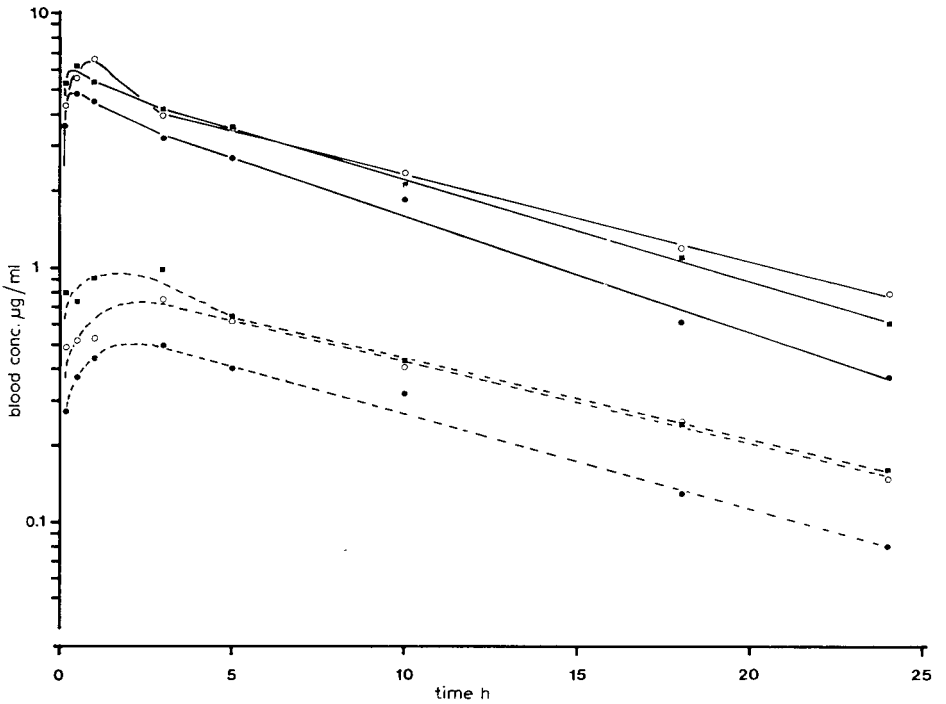

Fig. 5. Individual blood concentration — time profiles of the amino compound (——) and its acetylated derivative (- - - - -) in three rats. The amino compound (2 mg/kg) was administered subcutaneously.

acetamido metabolite be detected, and in three samples only was the amino metabolite analysed. These data do not fully agree with the results of Dieterle et al. [2], who for two volunteers showed plasma levels of the amino metabolite to exceed those of acenocoumarin. However, as optimal time schedules of dosing and sampling were not under control in our patients, the results do not give a correct insight into the clinical relevance of the formation of both the metabolites. Controlled clinical pharmacokinetic and pharmacodynamic studies are in progress at the moment.

CONCLUSIONS

The procedure presented permits the analysis of body fluids (plasma, urine) for acenocoumarin and its amino and acetamido metabolites with sufficient accuracy necessary for clinical pharmacological studies of the oral anticoagulant.

REFERENCES

1 R.A. O'Reilly, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The pharmacological Basis of Therapeutics, Macmillan, New York, 6th edn., 1980, p. 1353.
2 W. Dieterle, J.W. Faigle, C. Montigel, M. Sulc and W. Theobald, Eur. J. Clin. Pharmacol., 11 (1977) 367.

3 H.H.W. Thijssen, L.G. Baars and M.J. Reijnders, Naunyn-Schmiedeberg's Arch. Pharmacol., 319S (1982) 19.
4 J. Godbillon, J. Richard, A. Gerardin, T. Meinertz, W. Kasper and E. Jähnchen, Brit. J. Clin. Pharmacol., 12 (1981) 621.
5 H.H.W. Thijssen and L.G. Baars, manuscript in preparation.
6 M.J. Fasco, M.J. Cashin and L.S. Kaminsky, J. Liquid Chromatogr., 2 (1979) 565.
7 J.X. De Vries, J. Harenberg, E. Walter, R. Zimmermann and M. Simon, J. Chromatogr., 231 (1982) 83.
8 F.A. de Wolff, C.A.M. Tetteroo-Tempelman and P.M. Edelbroek, J. Anal. Toxicol., 4 (1980) 156.

Journal of Chromatography, 274 (1983) 239—253
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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF REPROTEROL IN PLASMA USING ON-LINE TRACE ENRICHMENT AND AMPEROMETRIC DETECTION WITH A ROTATING WORKING ELECTRODE

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SUMMARY

A method is described to determine nanogram quantities of reproterol in plasma. It consists of deproteinization of the plasma samples, on-line trace enrichment, and liquid chromatographic separation of the compounds brought on the analytical column, coupled with amperometric detection. Reliable quantitation can be done down to levels of 1 ng/ml.

INTRODUCTION

Reproterol (Bronchospasmin® in G.F.R.; Bronchodil® in Great Britain) is a new drug in the treatment of chronic aspecific respiratory affections. It has β_2-sympathicomimetic activity [1, 2] and its structure is depicted in Fig. 1.

So far, information on the fate of this drug in the body has only been obtained from experiments involving radioactively labeled material [3]. Therapeutically effective oral doses are reported to be 20 mg, three times daily [4, 5] which may lead to plasma concentrations in the order of 3—30 ng/ml or less, since total radioactivity was measured [3].

To investigate its pharmacokinetic properties in man in detail, a suitable method to measure low concentrations of unlabeled reproterol in plasma was necessary. Until now, only analytical methods consisting of sample clean-up, gas chromatography and mass spectrometric (GC—MS) detection have been used to investigate the pharmacokinetic properties of β_2-sympathicomimetic

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drugs like terbutaline and salbutamol [6—9]. The reason for this was that only GC—MS methods seemed to offer sufficient sensitivity and selectivity to measure therapeutic levels of these drugs in plasma.

From 1973 on, investigations have been published in which catecholamines were measured in very low concentrations by analytical methods based on high-performance liquid chromatography (HPLC) and amperometric detection. Because reproterol contains two phenolic hydroxy groups, it seemed worthwhile to try amperometric detection, especially because ultraviolet and fluorescence detection of underivatized reproterol does not offer sufficient sensitivity.

By using HPLC, some advantages over GC may be obtained with regard to stability, selectivity (both stationary and mobile phase can be altered) and sensitivity, as relatively large amounts of sample can be injected without overloading the column. The latter aspect opens the possibility to use on-line trace enrichment to purify the plasma samples and to concentrate the sample with regard to the reproterol concentration. Especially when compounds like reproterol need to be analyzed, which are difficult to extract from the biological matrix and which are present in very low concentrations, the on-line enrichment procedure may be of great advantage.

We now report a method based on on-line trace enrichment, reversed-phase HPLC and amperometric detection capable of detecting reproterol in plasma down to 0.5 ng/ml.

EXPERIMENTAL

Chemicals

Reproterol hydrochloride and the structural analogs, D4908 and D4959, used as internal standard (see Fig. 1) were a gift of Homburg/Degussa Pharma Gruppe, Frankfurt/M., G.F.R.

Disodium hydrogen phosphate dihydrate, tetrabutylammonium hydrogen sulphate ($TBAHSO_4$), propanol-1, perchloric acid 70% in water, citric acid and sodium hydroxide were of pro analysi quality and obtained from E. Merck (Darmstadt, G.F.R.).

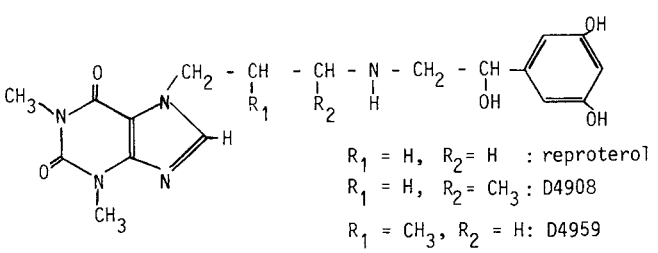

Fig. 1. The chemical structure of reproterol, D4908 and D4959.

Freshly glass-distilled deionized water was used for the preparation of all stock solutions of reproterol, D4908, D4959 and perchloric acid, as well as for the preparation of the mobile phases.

Carbon powder was purchased from Ringsdorff Werke (Ringsdorff Spektral-

kohle, RW-A höchster Reinheit; Bonn-Bad Godesberg, G.F.R.), high vacuum grease from Dow Corning (Seneffe, Belgium) and carbon paste (EA 267 c) from Metrohm (Herisau, Switzerland).

On-line trace enrichment

The set-up for the on-line trace enrichment procedure is schematically represented in Fig. 2. By injecting the sample in valve 1 (V_1) the loop L is filled. By switching V_1, the contents of loop L are pumped into V_2 on the concentration column CC. The wash mobile phase S_1 is made in such a way and the flush time t_1 chosen such that reproterol and D4908 are retained on the stationary phase present in CC whereas a great many accompanying compounds present in the sample are flushed away to waste. After a given time t_1, V_2 is switched and mobile phase S_2 is pumped through CC in the same direction as was S_1. Reproterol and D4908 are now flushed onto the analytical column AC and, after separation, enter the amperometric detector DC.

Deproteinized plasma samples can easily be handled this way.

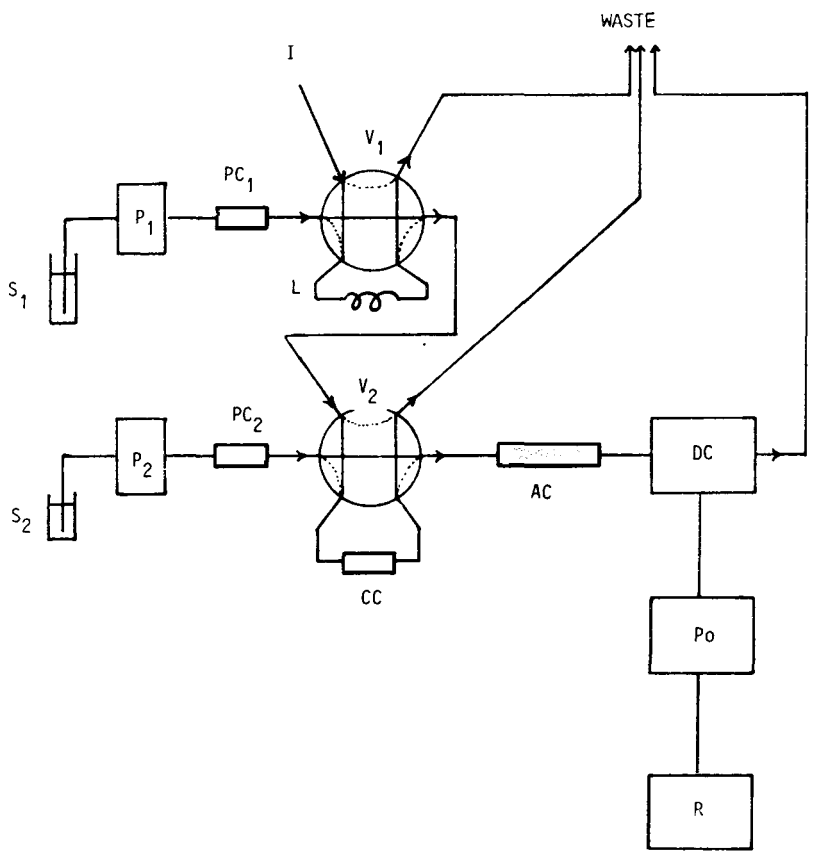

Fig. 2. A schematic representation of the set-up for the on-line trace enrichment procedure. S_1, S_2 = solvent reservoirs. P_1 = Model 740 pump (Spectra Physics, Eindhoven, The Netherlands). P_2 = Model 3500 B pump (Spectra Physics). PC_1, PC_2 = precolumns. I = injection. V_1, V_2 = 6-port valves (Model 7010, Rheodyne, Cotati, CA, U.S.A.). L = loop. CC = concentration column. AC = analytical column. DC = detector cell. Po = potentiostat. R = recorder BD40, 04/05 (Kipp & Zn, Delft, The Netherlands).

Chromatography

All columns were filled with Nucleosil 5 C_{18}, mean particle size 5 μm (Macherey, Nagel, Düren, G.F.R.).

The analytical column (150 × 4.6 mm I.D.) was slurry-packed according to a method described recently [10], which was modified slightly.

The concentration column CC was home-made, by filling a piece of stainless steel tubing (35 × 4.6 mm I.D.) with Nucleosil 5 C_{18} to a height of 20 mm. The remaining part of the tubing is filled with a cylinder of tight-fitting PTFE (polytetrafluoroethylene) through which a capillary stainless steel (type 316) tubing with an external diameter of 1/16 in. is extended. On both sides of the Nucleosil column stainless steel frits are placed. The CC is depicted schematically in Fig. 3. This design was especially suited to be used in on-line trace enrichment of plasma samples with foreward flushing. The CC was packed by preparing a slurry as for the analytical column and this slurry was poured into the column under tapping with a rod.

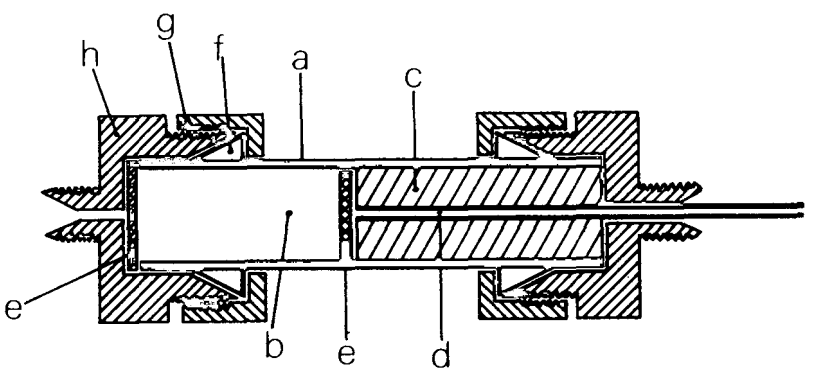

Fig. 3. A schematic representation of the concentration column. a = tubing. b = stationary phase. c = hollow cylinder. d = capillary tubing. e = frits. f = ferrules. g = nuts. h = snubber.

The precolumns were dry-packed with Nucleosil 5 C_{18}; they are taken up to provide on-line purification of the mobile phases. PC_1 and CC were cleaned each day by flushing with 15 ml of water—methanol (1:1). PC_2 and AC were flushed with 15 ml of water—methanol (2:1) each day.

The mobile phases were prepared from a mother buffer solution made by dissolving 7.12 g of disodium hydrogen phosphate dihydrate and 8.4 g of citric acid in 1970 ml of water and titrating this solution with 4 *N* sodium hydroxide solution to pH 7.0. This solution was diluted 1:1 with distilled water to give mobile phase S_1. Mobile phase S_2 was prepared by adding propanol-1 (to a concentration of 2.2—3.0%) and $TBAHSO_4$ (to a concentration of $4 \cdot 10^{-3}$—$6 \cdot 10^{-3}$ *F*) to the mother buffer (phosphate—citrate buffer, pH 7.0, 0.02 *F*).

The flow of S_1 was 1.0 ml/min and that of S_2 1.2 ml/min.

The mobile phases were degassed by ultrasonication under vacuum and filtered through a cellulose acetate membrane filter (Schleicher and Schüll, Dassel, G.F.R., OE 67, 0.45 μm).

Amperometric detection

A home-made amperometric detector was used which has been described previously [11—13].

Some small modifications were made to improve the detector response. (1) The rotation speed was continuously adjustable as was the distance between working electrode and bottom of the cell. This improved the reproducibility of the detector at high oxidation potentials. (2) The diameter of the channel connecting the compartment containing the working electrode with that in which the reference electrode is present was enlarged to 5 mm. This lowered the internal resistance of the detector and increased the linear range.

The modified detector is depicted in Fig. 4. In the cell block (a) holes are drilled for the working electrode (b), the reference electrode (c) and the auxiliary electrode (d). Via the inlet (e) the eluent enters the cell and impinges on the working electrode, then streams along the carbon paste (f), through a channel (o) into a compartemt (p) containing the reference and auxiliary electrode and then to waste. The inlet consists of a stainless steel cylinder with a hole drilled in it (1.0 mm) which is screwed into the bottom of the working electrode compartment.

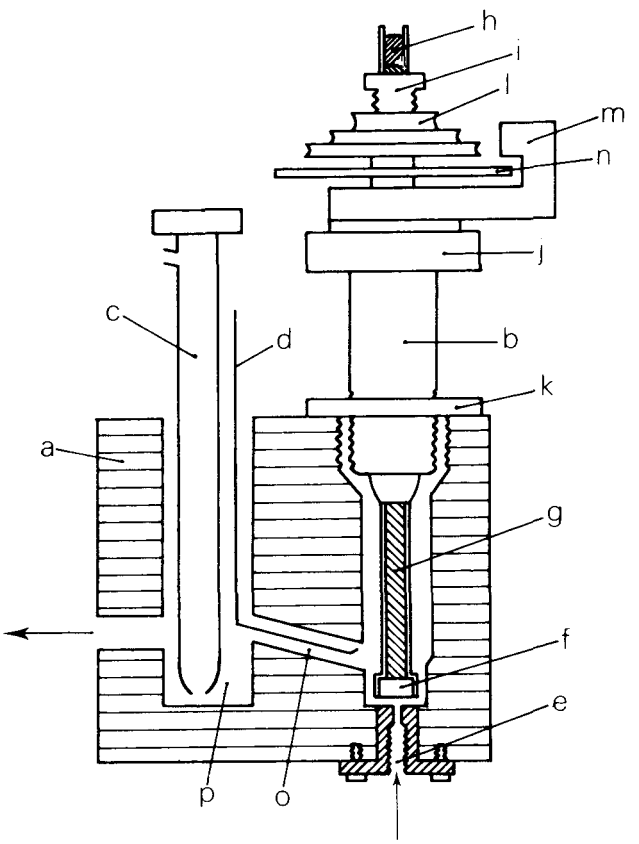

Fig. 4. Amperometric detector with rotating-disc working electrode, continuously adjustable nozzle height and rotation speed. a = cell block. b = working electrode holder. c = reference electrode. d = auxiliary electrode. e = inlet. f = carbon paste. g = brass rod. h = mercury. i, j, k = screws. l = pulleys. m = optical plate. n = plate with slieves in it.

The working electrode consists of a holder (h), which contains a piece of Kel-F tubing with a brass rod (g) in it to connect the carbon paste (g) to the mercury (h) on top of the rod in which the wire to the potentiostat is placed. The rod is placed in the holder with the screw (i). The holder is screwed into the cell block with screw (j) and fixed into place with screw (k). In this way the distance between the surface of the carbon paste and the surface of the metal bottom can be continuously and accurately adjusted. The rod is rotated in the holder with the aid of the pulleys (l) constructed on the top of the rod which are connected with an elastic string to a stepper motor. The rotation speed is measured by the optical cell (m) and the circle (n) screwed on the rod. With an external device the rotation speed is measured and the rotation velocity of the stepper motor regulated.

The electrode material consisted of carbon paste prepared as follows: carbon powder (0.66 g) and vacuum grease (0.33 g) were mixed in an agate mortar with a pestle until a homogeneous paste was obtained. Then Metrohm carbon paste (0.2 g) was pounded through the mixture in the same way. The auxiliary electrode was a platinum wire of diameter 0.6 mm. The oxidation potential was chosen to be 0.90 V vs. a Ag/AgCl—3 *M* KCl reference electrode.

The potentiostat was home-made and comparable to a LC-2A electronic controller (Bioanalytical Systems, Lafayette, IN, U.S.A.).

Plasma sample preparation

To investigate the properties of the method, spiked plasma samples were analyzed. In the experiments several lots of single donor human plasma and bovine plasma were used.

To 2.2 ml of plasma are added 50 μl of a stock solution of reproterol in water to give the concentrations desired. In some experiments also 50 μl of a freshly prepared stock solution of the internal standard were added. After 15 min equilibration time 1.8 ml of a solution of perchloric acid in water (6%) are added. After shaking on a Vortex mixer at maximum speed for three times 10 sec, the tubes are centrifuged at 0°C for 15 min at 2000 *g*. Then the supernatant is decanted into another tube and centrifuged for 5 min at 0°C at 4500 *g*. In this way a clear supernatant is obtained which is injected directly into V_1.

Total procedure

The total procedure can be taken together as follows: (1) A 2.2-ml volume of (spiked) plasma is deproteinized by adding 1.8 ml of a 6% perchloric acid solution, and shaking on a Vortex mixer at maximum speed for three times 10 sec. Then the tubes are centrifuged at 0°C for 15 min at 2000 *g*. The supernatant is decanted into another tube and centrifuged for 5 min at 0°C and 4500 *g*. (2) The loop L of the chromatographic system depicted in Fig. 2 is filled with 2.1 ml of the clear supernatant. Then the positions of the valves are changed as described in Table I. The exact time of injecting the next sample in loop L (in Scheme 1 marked by an asterisk) depends on the composition (origin) of the plasma sample. In some cases, late-eluting peaks were observed in the chromatograms and the moment of injection was postponed until the last compounds eluted from AC. Before this procedure was applied to the

TABLE I

TIME SCHEDULE FOR THE SWITCHING OF THE VALVES IN THE ON-LINE TRACE ENRICHMENT OF REPROTEROL-SPIKED PLASMA SAMPLES WHEN THE SET-UP DEPICTED IN FIG. 2 IS USED

Time (min)	Position V_1	Position V_2	Function
			CC is equilibrated with S_1, AC and DC are stable
0.00	Load	Load	Injection sample in L
2.00	Inject	Load	Sample loaded onto CC
10.00	Inject	Inject	Reproterol is flushed onto AC; the HPLC separation (and the chromatogram) starts
10.05	Load	Inject	V_1 is cleaned
14.00	Load	Load	CC is equilibrated with S_1
17.00	Load*	Load	L is filled with the next sample
19.00	Inject	Load	Sample loaded onto CC

*Indicates injection of the next sample if no late-eluting peaks are observed.

samples, three aliquots of a spiked water sample (10—20 ng/ml) and a test plasma sample were injected.

A chromatographic run takes between 13 and 75 mins, and 3—6 plasma samples plus standards can be analyzed in duplicate within one day depending on the origin of the plasma sample.

The quantitative evaluation was done by measuring the peak height by hand.

RESULTS AND DISCUSSION

Amperometric detection

Reproterol could be oxidized at a carbon paste electrode in an aqueous medium; for example in the mobile phase used to elute reproterol from the concentration column, the drug was oxidized at potentials higher than 650 mV vs. an Ag/AgCl—3 *M* KCl reference electrode.

The choice of the potential used to oxidize reproterol depends on the reproducibility, selectivity and sensitivity needed. The reproducibility of the results is influenced by the amperometric detector itself. For example, if a potential is chosen at which no diffusion-limited current is obtained, if the applied voltage does not remain constant, if the electrode surface changes with time, or if poisoning of the electrode occurs. To investigate these factors, voltammograms were recorded on-line after an aqueous solution of reproterol or reproterol in plasma was led through the entire procedure. A current—voltage plot is given in Fig. 5.

Repetitive injection of the same aqueous solution at a constant potential of around 850 mV showed a decrease of the response in the first three injections; thereafter the response remained constant, indicating that the electrode surface reacted in a constant way. This behaviour is also observed in the amperometric detection of catecholamines [14]. Therefore all quantitative results mentioned in this article were obtained after three initial injections of an aqueous solution of reproterol.

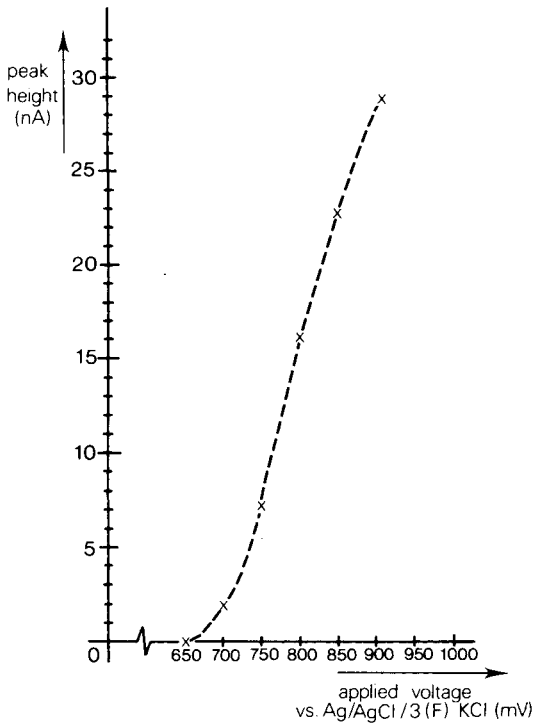

Fig. 5. Peak height (nA) measured at different potentials between working and reference electrode, when aliquots of an aqueous solution of reproterol (21 ng/ml, 3% perchloric acid) are injected in the set-up described in Fig. 2. S_2 consisted of phosphate–citrate buffer (pH 7.0, 0.01 *F*), propanol-1 (2.4%) and $TBAHSO_4$ ($5 \cdot 10^{-3}$ *F*). Nozzle height 1.0 mm. Rotation speed: 30 r.p.s.

In the voltammogram no flattening of the curve is observed.

At potentials higher than 950 mV, the residual current becomes too high, caused by the oxidation of water.

If the coefficient of variation (C.V.) is determined at 900 mV, by injecting five times a solution of reproterol in distilled water (2 ng/ml) a C.V. was found of 2.1% (i.e. including the on-line trace enrichment device).

The lower detection limit of reproterol in aqueous solution is 0.1–0.2 ng/ml and depends for the largest part on the amount of interfering compounds present in the sample, in the wash mobile phase or on the column, and not so much on high-frequency noise factors like electronic noise. This means that the signal-to-noise ratio (S/N ratio), which fixes the lower detection limit, may vary with the origin of the (plasma) sample. That is why no exact S/N ratios can be given. For plasma samples, the quantitation limit was usually found to be around 1.0 ng/ml. On the basis of the above observations, detection at a potential between 850 and 950 mV was routinely chosen, allowing quantitation of (sub)nanogram quantities of reproterol.

Trace enrichment and chromatography

Reproterol appeared to be very difficult to extract from plasma, with

recoveries below 40% using a variety of organic solvents. This is due to its rather polar character which results in a partition coefficient of 0.39 in the system *n*-octanol—0.02 *F* phosphate buffer, pH 7.4 [3].

Ion-pair extraction procedures with di-(2-ethylhexyl) phosphoric acid (DEHP) which appeared to be a suitable counter ion for resorcinol-type sympathicomimetics [6, 15] were also investigated. Though extraction from water could be performed, recoveries from plasma remained more than 40% lower.

By using on-line trace enrichment, several of the problems occurring with solvent extraction are circumvented. (1) The method is easy to apply: often only a deproteinization step is necessary for plasma. (2) The procedure is amenable to automation. (3) Possible losses due to adsorption (to glass during evaporation, for example) are minimized as well as losses which may occur during the transfer of small volumes. (4) By making an adequate choice of the stationary and mobile phases, a high degree of purification of the sample and a high selectivity can be obtained [16]. (5) Sensitivity can be increased by concentration effects (for example, by using a step gradient).

On-line trace enrichment has been used successfully for several years in the analysis of various compounds in different matrices and more recently also in the analysis of drugs in a complex matrix like biological fluids (serum [17, 18], plasma [18—23], urine [23]).

In our studies on trace enrichment, Nucleosil 5 C_{18} appeared to be a suitable material to trap reproterol when an aqueous phosphate—citrate buffer (0.01 *F*, pH 7.0) is used as mobile phase.

When using this stationary phase for the enrichment procedure, the only plasma clean-up required is deproteinization, to prevent clogging of the concentration column. Satisfactory deproteinization was obtained by adding 1.8 ml of a solution of 6% perchloric acid [24] in distilled water to 2.2 ml of plasma. More than 100 injections of 2.1 ml of deproteinized plasma can be done on the same concentration column, without significant damage.

Several compositions of mobile phase to elute reproterol into the analytical column were tried for best performance. By using an elution mobile phase based on mixtures of phosphate—citrate buffer, sodium perchlorate and propanol-1, tailing peaks and bad efficiencies on various C_{18} reversed-phase analytical columns resulted. (Altex Ultrasphere-ODS, 5 μm, 25.0 × 0.46 cm; μBondapack C_{18}; Nucleosil 5 C_{18}, 15.0 × 0.46 cm; Hibar LiChrosorb RP-18, 10 μm, 25.0 × 0.40 cm).

When, instead of perchlorate, other ion-pairing agents were used like heptane sulphonate, high concentrations of organic modifier were necessary in order to obtain a reasonable retention time. This disturbs the performance of the carbon paste working electrode.

We found the most suitable mobile phase to be a solution of $TBAHSO_4$ in an aqueous phosphate—citrate buffer, containing a small amount of propanol-1, with which a highly efficient column behaviour was obtained, using Nucleosil 5 C_{18} as stationary phase [25].

The retention time of reproterol in such a system can be governed in several ways. (1) By changing the pH of the buffer (and keeping the other constituents constant: propanol-1 5.0%, $TBAHSO_4$ 5×10^{-3} *F*) from 6.5 to 8.0, the retention time of reproterol is increased from 4.6 to 8.4 min and the number of

effective plates decreased by 15%. (2) By changing the propanol-1 content in the mobile phase from 2.5 to 5.0% ($TBAHSO_4$ $5 \cdot 10^{-3}$ *F*) the retention time of reproterol changed from 12.8 to 5.0 min. (3) By increasing the $TBAHSO_4$ concentration from $5 \cdot 10^{-3}$ to $6 \cdot 10^{-3}$ *F* (2.6% propanol, $1 \cdot 10^{-2}$ *F* McIlvaine buffer pH 7.18), the retention time of reproterol decreases from 14.8 to 12.0 min. This would seem to be in contrast to the behaviour expected when ion-pair formation between reproterol and $TBAHSO_4$ is a major factor in the chromatographic behaviour of reproterol on a C_{18} stationary phase. (4) The ionic strength also influences retenton time in the way that increasing ionic strength causes increasing retention times. For example, when the phosphate–citrate buffer (pH 7.0) is made $1.5 \cdot 10^{-2}$ *F* instead of $1.0 \cdot 10^{-2}$ *F* (propanol-1 2.8%; $TBAHSO_4$ $8.0 \cdot 10^{-3}$ *F*) a retention time of 8.5 min instead of 7.1 min is found.

With these parameters the retention of reproterol can be properly adjusted to the most suitable position in the chromatogram. We found that, for plasma samples, a mobile phase composition of phosphate–citrate buffer, pH 7.0–7.2, 0.01 *F*, propanol-1, 2.2–2.6%, and a $TBAHSO_4$ concentration of $5 \cdot 10^{-3}$ to $6 \cdot 10^{-3}$ *F* provided good chromatographic results coupled with an excellent clean-up and enrichment of the sample, as can be observed in Fig. 6.

The addition of $TBAHSO_4$ to the above mobile phase system decreased the retention time (t_R) of reproterol and diminished tailing of the peak.

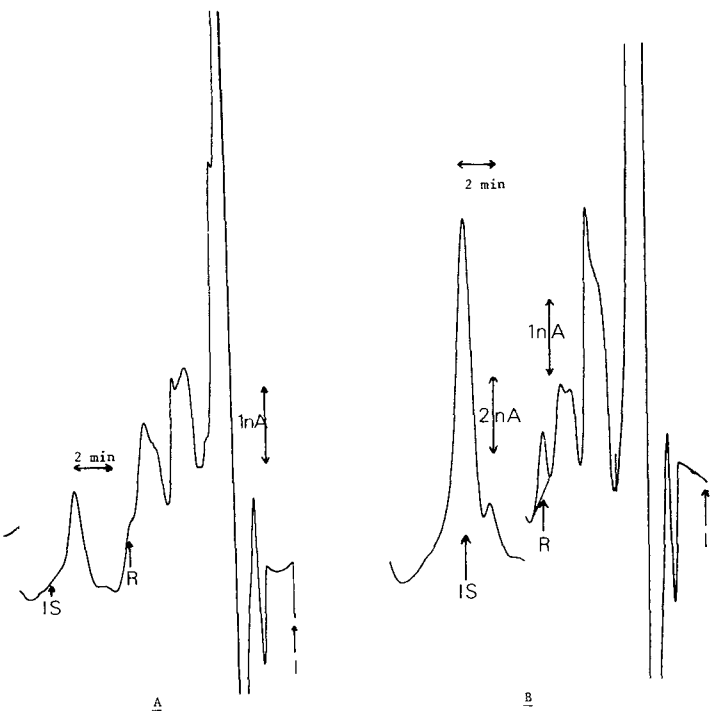

Fig. 6. Chromatograms of (A) a blank plasma sample and (B) a spiked plasma sample (0.55 ng reproterol HCl per ml of plasma). I represents the time of injection, R represents the reproterol peak and IS the peak of the internal standard D4908.

The choice for this elution mobile phase also has the advantages that reproterol is electrochemically very reactive in this medium and that the chance for isoquinoline formation (Pictet-Spengler reaction) is very small due to the absence of aldehydes or reactive aldehyde-forming agents [26].

With the latter chromatographic system, the properties of the analytical procedure were investigated. The enrichment method appeared to be dependent on two factors: (1) If the concentration column (CC) was in equilibrium with the wash eluent, before the next sample was injected. When 2.0 ml were used to equilibrate the CC, a 10% lower peak height was measured as compared to flushing with 16 ml of wash eluent. When 4.0 ml are used, CC approaches complete equilibrium. (2) The time the CC is flushed with the wash eluent, after the introduction of the sample. The peak height is inversely related to increasing volumes of S_1 used to wash the CC. When a complex sample like plasma is analyzed, a wash volume of 8 ml proved to be a good compromise.

It should be noticed that these relatively large flushing volumes put high demands upon the purity of the mobile phase S_1. The impurities present in S_1 will accumulate on top of the CC and (partly) be eluted when the CC is flushed with S_2. Thus, when the phosphate–citrate buffer is prepared, salts of highest quality must be used, together with freshly distilled water. Despite these precautions it appeared that impurities remained in S_1 and S_2 and that they originated from the salts (determined by making a gradient from 100% buffer to 100% methanol and measuring the eluent by ultraviolet detection at 254 nm). Therefore on-line purification of S_1 and S_2 was used.

Internal standards

D4908 and D4959 are closely related to reproterol and showed good chromatographic behaviour. However, we found that the two drugs did not improve the precision of the method. Coefficients of variation using peak height ratios of reproterol to internal standard were considerably larger than when using peak height of reproterol alone. This may be due to lower and less reproducible recoveries of the internal standards, which were found to be around 50%. Other factors may be differences in response at the electrode.

No further studies into these phenomena were undertaken nor did we investigate other potential candidates for internal standard. Instead, peak height of reproterol was measured and used as described below.

Response, calibration, recovery and precision

As mentioned above, when aqueous samples of reproterol were analyzed, the response decreased during the first three samples, and then remained constant during the rest of the day. Yet, when working with plasma samples we observed that after the first three aqueous samples the response continued to decrease slowly but steadily during the day. This may indicate that plasma contains compounds that influence the behaviour of reproterol in the system and/or the electrode surface.

In order to correct for this decrease in response and to obtain insight in the recoveries, a correction scheme as depicted in Fig. 7 was devised. The first three peaks represent the peak heights obtained when an aqueous solution of 5.0

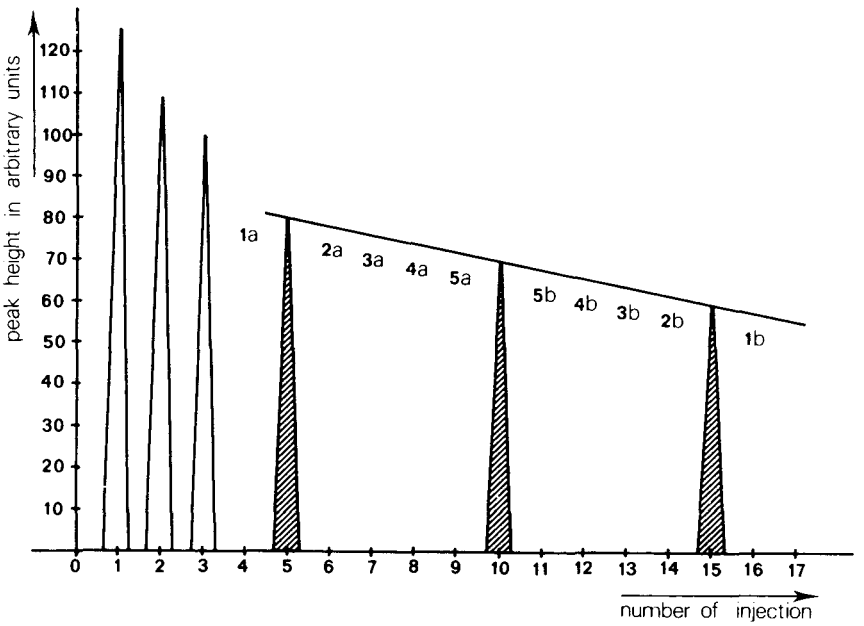

Fig. 7. A typical example of decrease of response when several plasma samples are injected.

ng/ml reproterol is injected. After the first three injections, the peak height for aqueous samples remains constant at H_3 (which represents 100% recovery). The shaded peaks at the numbers 5, 10 and 15 represent the peak heights obtained when spiked plasma samples (5.0 ng/ml) are injected during the day. From the latter the decrease in response can be calculated. The standards are made up in plasma from the same source as the plasma samples with the unknown concentration of reproterol. Taking into account the decrease in response, the relative recovery can be calculated by using H_5, H_{10} and H_{15} versus H_3. The injections numbered 1a—5a and 5b—1b represent plasma samples with an unknown quantity of reproterol. The "b" numbers are duplicates of the "a" numbers. Thus, by analyzing the plasma samples according to this scheme, the peak height can be corrected for the gradual decrease during the day. Of course the number of injections in the scheme can be enlarged by injecting more samples and standards.

When the above injection scheme was used for the analysis of plasma samples (spiked reproterol in the concentrations 0.55, 1.10, 1.84, 3.68 ng/ml), the linear calibration curve depicted in Fig. 8 was obtained. The mean values lie on a straight line of the equation $H_{repr.} = 1.42$ (conc. repr. in plasma) + 0.068, with a correlation coefficient of $r = 0.995$, indicating that the decay in response is accurately corrected for by the described procedures.

In Fig. 6 the chromatograms of blank plasma and of spiked plasma (0.55 ng/ml) are reproduced. These chromatograms, combined with the calibration curve, give an impression of the detection limit of the procedure. Because of the response changes during the day and between days, the lower limit of accurate determination is about 1 ng/ml in plasma.

The procedure is linear to at least concentrations up to 40 ng/ml. For the

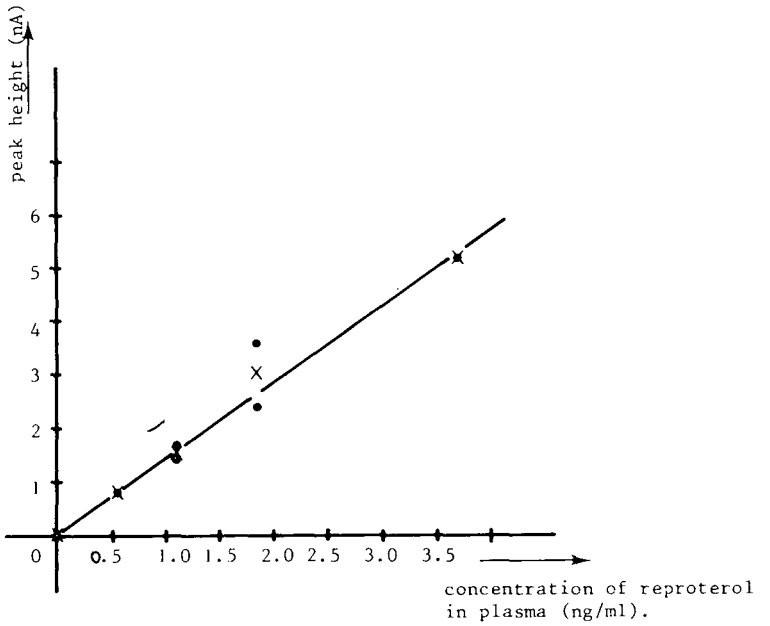

Fig. 8. Standard curve. Peak heights found when reproterol spiked plasma samples are analyzed according to the procedure described in the text. The crosses (x) represent the mean values of the two single determinations (•). No peak at t_R = 8.3 min (= reproterol) was detectable in the blank plasma sample.

range 3—40 ng/ml a straight line was found with the equation $H_{repr.}$ = 1.01 (conc. repr. in plasma) — 0.74 with a correlation coefficient of r = 0.998.

If the method of Haefelfinger and Wall [27] is used to combine the values of C.V. found for experiments at different concentrations on different days, a weighted mean C.V. of 7.8% was found for the concentration range 1—40 ng/ml. No significant difference in the value of C.V. was observed when plasma samples with a concentration of reproterol in the range 1—3 ng/ml or in the range 3—40 ng/ml, were analyzed.

Because of the decrease in response also no exact value of the recovery can be given. This is illustrated in Fig. 7. When the relative recovery (%) is defined as

$$\frac{\text{peak height reproterol in spiked plasma (ng/ml)} \times 100}{\text{peak height reproterol in spiked water (ng/ml) third injection}}$$

in our experiments recoveries were found in the range 60—90%. Since each plasma sample serves as its own recovery control (as indicated in Fig. 8) reliable quantitation is possible.

No significant differences were noted in recovery when plasma samples of different origin were analyzed.

It appeared that the plasma deproteinization procedure was a major factor in influencing the recovery. When 2.2 ml of blank plasma were spiked to a concentration of 3.0 ng/ml and deproteinized by adding 1.8 ml of a solution of 6% perchloric acid in distilled water and analyzed, a mean recovery of 72%

was found (n = 3). When 2.2 ml of blank plasma were deproteinized in the same way and the supernatant was spiked, a mean recovery for the on-line trace enrichment device of 94% (n = 3) was found. Higher concentrations of perchloric acid decreased recoveries, so as a compromise the 6% solution was used.

When plasma samples of unknown reproterol concentration have to be analyzed, blank plasma samples of the same person should be used as standards to correct for possible differences in recovery that may be present.

Interferences

We investigated the possible interference of some structurally related drugs in the quantitation of reproterol. It appeared that terbutaline, salbutamol, fenoterol, theophylline and the major metabolite of reproterol in humans (the isoquinoline derivative of reproterol [28] did not interfere.

Applicability

The applicability of the developed procedure is shown in Fig. 9, which depicts the plasma concentration—time curve when two tablets of Bronchospasmin® (containing 20 mg of reproterol HCl each) were given to a young healthy male volunteer. The tablets were swallowed together with 200 ml of water, and at certain times blood samples were collected in Vacutainer® tubes (with heparin). The tubes were stored in ice until centrifugation (4800 g, 0°C, 10 min). After centrifugation, two times 2.2 ml of the supernatant were brought to separate glass tubes (Sovirel® 15) and stored in the freezer (at

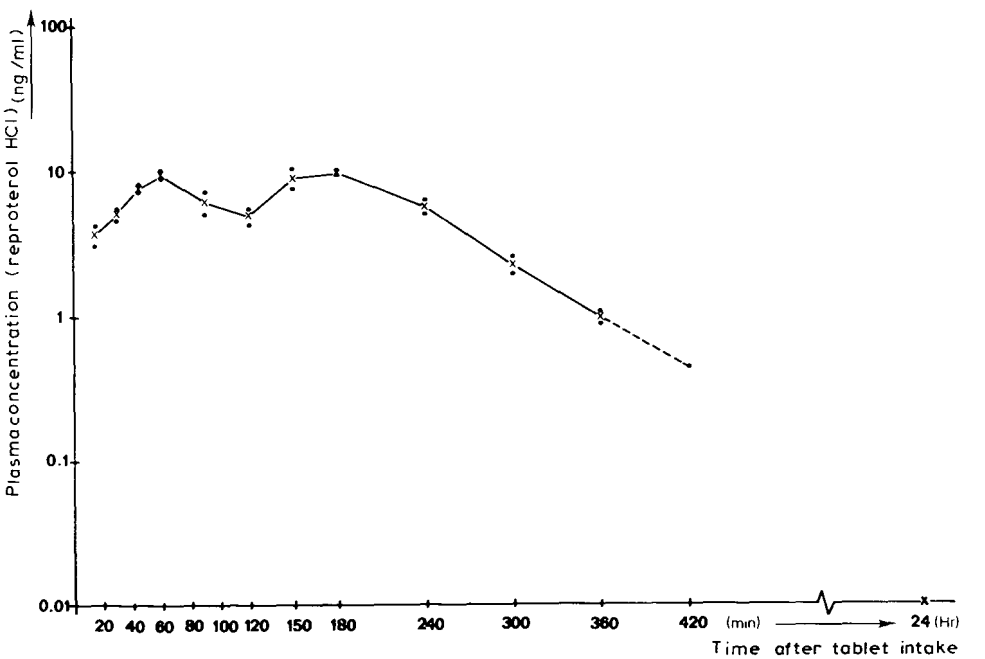

Fig. 9. Plasma concentration—time curve after oral administration of two tablets of Bronchospasmin® to a young healthy male volunteer. The crosses (x) represent the mean value of two determinations (•).

−20°C) until analysis. Details of the kinetic properties of reproterol in humans will be published elsewhere.

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REFERENCES

1 S. Habersang, F. Leuschner, F. Stroman, A. Domenico and A. von Schlichtegroll, Arzneim.-Forsch., 27 (1977) 22—35.
2 D. Zecević, D. Tabori, S. Mijatovic, S. Mirković, V. Todić, B. Čončić and R. Stadler, Arzneim.-Forsch., 27 (1977) 53—55.
3 G. Niebch, K. Obermeier, H. Vergin and K. Thiemer, Arzneim.-Forsch., 27 (1977) 37—45.
4 D. Tabori, B. Čonkić, V. Todić, M. Mijatović, S. Mirković, D. Zečević and D. Čamprag, Arzneim.-Forsch., 27 (1977) 55—60.
5 D. Nolte, G. Galgóczy, H. Lode, A. Mándi, H. Matthys and E. Stresemann, Deut. Med. Wochenschr., 102 (1977) 619—623.
6 J.G. Leferink, Ph.D. Thesis, State University Utrecht, 1979.
7 D.S. Davies, C.F. George, E. Blockwell, M.E. Connolly and C.T. Dollerey, J. Pharmacol., 1 (1974) 129—136.
8 R.A. Clare, D.S. Davies and T.A. Baillie, Biomed. Mass Spectrom., 6 (1979) 31—38.
9 S.E. Jacobsson, S. Jönsson, C. Lindberg and L.A. Svensson, Biomed. Mass Spectrom., 7 (1980) 265—269.
10 K. Kuwata, M. Uebori and Y. Yamazaki, J. Chromatogr., 211 (1981) 378—382.
11 B.H.C. Westerink and T.B.A. Mulder, J. Neurochem., 36 (1981) 1449—1462.
12 K. Brunt, C.H.P. Bruins, D.A. Doornbos and B. Oosterhuis, Anal. Chim. Acta, 114 (1980) 257—266.
13 B. Oosterhuis, K. Brunt, B. Westerink and D.A. Doornbos, Anal. Chem., 50 (1980) 203—206.
14 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren, E. Rosengren and L.-E. Edholm, J. Chromatogr., 162 (1979) 7—22.
15 C. Hoogewijs and D.L. Massart, Anal. Chim. Acta, 106 (1979) 271—277.
16 D.H. Freeman, Anal. Chem., 53 (1981) 2—5.
17 G.C. Davies and P.T. Kissinger, Anal. Chem., 51 (1979) 1960—1965.
18 W. Voelter, T. Kronbach, K. Zech and R. Huber, J. Chromatogr., 239 (1982) 475—482.
19 A. Yamatodani and H. Wada, Clin. Chem., 27 (1981) 1983—1987.
20 F. Erni, H.P. Keller, C. Morin and M. Schmitt, J. Chromatogr., 204 (1981) 65—76.
21 R.A. Hux, H.Y. Mohammed and F.F. Cantwell, Anal. Chem., 54 (1982) 113—117.
22 J. Lankelma and H. Poppe, J. Chromatogr., 149 (1978) 587—598.
23 J.A. Apffel, T.V. Alfredson and R.E. Majors, J. Chromatogr., 206 (1981) 43—57.
24 J. Blanchard, J. Chromatogr., 226 (1981) 455—460.
25 K.T. Muir, J.H.G. Jonkman, D.-S. Tang, M. Kunitani and S. Riegelman, J. Chromatogr., 221 (1980) 85—95.
26 N. Kucharczyk, personal communication, 1982.
27 P. Haefelfinger and M. Wall, Z. Anal. Chem., 307 (1981) 271—276.
28 G. Niebch, G.H. Klingler, G. Eikelmann and N. Kucharczyk, Arzneim.-Forsch., 28 (1978) 765—767.

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ANALYSIS AND QUANTITATION OF A METABOLITE OF DOXYCYCLINE IN MICE, RATS, AND HUMANS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A metabolite of doxycycline has not previously been isolated. In this paper it is demonstrated that doxycycline is metabolized in mice, rats, and humans. By means of high-performance liquid chromatography and consecutive gel chromatography one metabolite of doxycycline was isolated from animal organs and human urine. The metabolite was tentatively identified as N-monodemethyldoxycycline by mass spectral and spectrophotometric analyses. The rate of metabolism could be enhanced by pretreatment of the animals with phenobarbital, an inducing agent of the drug-metabolizing enzymes.

INTRODUCTION

Except for the lipophilic tetracycline derivative minocycline [1], metabolites of tetracycline antibiotics have not previously been isolated from humans or experimental animals. A decrease in the half-life of the lipophilic tetracycline derivative doxycycline was observed following coadministration of enzyme-inducing substances such as barbiturates [2], anti-epileptics [3, 4] and also of ethanol [5]. These findings could not be related to an increased metabolism of doxycycline since a metabolite of doxycycline could not be detected in human urine or feces [6].

In the present paper the analysis and isolation of a metabolite of doxycycline is described. It is performed by high-performance liquid chromatography (HPLC) and gel chromatography.

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MATERIALS AND METHODS

Animals

Female mice weighing 24—26 g and female albino Wistar rats weighing 180—220 g were used for the experiments. They were housed in plastic cages on soft wood bedding at 25° C room temperature under a 12-h dark—light rhythm. The animals had free access to standard laboratory diet (Herilan, Eggersmann, Rinteln, G.F.R.) and tap water.

Treatment of animals

To induce the drug-metabolizing enzymes the animals were pretreated intraperitoneally with phenobarbital (35 μg/g in 10 μl) twice a day for three days. Control animals received the same volume of saline. At the fourth day each animal received 50 μg/g doxycycline in 10 μl of 1.5 mmol/l $MgSO_4$. The mice were killed after 6 h (ten mice per group) and the rats after 10 h (four animals per group). Livers and kidneys were rapidly dissected and kept frozen at −70° C until preparation. The organs for HPLC analysis were prepared as described earlier [7, 8].

Human experiment

For analysis of doxycycline from human urine 2 × 100 mg doxycycline capsules were given to a healthy volunteer (male, 36 years, 72 kg) in the evening. Urine was collected for the following 16 h (980 ml); it was kept cold and freeze-dried. The residue of 200 ml urine was dissolved in 2 ml of water, filtered and prepared for HPLC analysis as described for the analysis of serum [8].

Chemicals

All chemicals used were of analytical grade except for acetonitrile which was of the grade "for residue analysis" (Merck, Darmstadt, G.F.R.). Doxycycline and 4-epi-doxycycline were kindly provided by Pfizer (Karlsruhe, G.F.R.).

Chromatography

For analytical HPLC a reversed-phase system as previously reported [8] was used with slight modifications. The HPLC apparatus consisted of a Waters 6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.) with the automatic sample injection system ASI 45 (Kontron Analytik, Eching, G.F.R.) with a 0.05-ml sample loop. The separation was performed on a column (25 × 0.4 cm) packed with Nucleosil C_8, 10 μm (Macherey & Nagel, Düren, G.F.R.) with a flow-rate of 1.9 ml/min (about 140 bars) at room temperature. The column was conditioned for at least 6 h with 3.5 mmol/l NaH_2PO_4 in water—acetonitrile (65:35) with the pH adjusted to 2.7 with H_3PO_4. The separation was performed with 3.5 mmol/l NaH_2PO_4 in water—acetonitrile (70:30) with the same pH and at the same flow-rate. After each separation the column was equilibrated again with the conditioning eluent for at least 10 min. The eluent was monitored in an LC 720 liquid chromatography ultraviolet (UV) detector (Kontron). The absorption was read at 344 nm (one absorption maximum of doxycycline), 370 nm, or 380 nm (67% and 41% of

the absorption at 344 nm, respectively) to eliminate the absorption of interfering endogenous substances from the organs and from urine. The absorption was automatically recorded on a C-RIA printer plotter (Shimadzu, Kyoto, Japan).

For semipreparative HPLC separations a column (25 × 1 cm) was packed with Nucleosil C_8, 10 μm. The column was conditioned and run with the same eluents as described for analytical separations at a flow-rate of 5 ml/min (about 120 bars). Samples of 1 ml were applied to the column.

Further purification of the docycycline metabolite was performed by gel chromatography (Sephadex G-15, 30 × 1.5 cm; Pharmacia, Uppsala, Sweden). Pooled fractions of doxycycline and its metabolite from 30—40 separations by the semipreparative HPLC were collected and freeze-dried. The residue was dissolved in 1—2 ml water and applied to the column. The column was conditioned and eluted with double-distilled water at 4°C with a flow-rate of 45 ml/h. The effluent was monitored at 280 nm in a UV absorbance monitor Model UA 5 (Isco, Lincoln, NE, U.S.A.) and automatically recorded. The peak fractions were combined and freeze-dried. The residue was used for UV analysis, fluorimetric analysis and for mass spectrometry.

UV analysis

A double-beam spectrophotometer (Beckman Model 5230; Beckman Instruments, Irvine, CA, U.S.A.) was used. UV spectra of doxycycline and its metabolite isolated from organs or urine were recorded in 0.1 mol/l HCl, alkali [addition of 0.05 ml of 32% (w/v) NaOH to 1 ml of the acidic solution] and alkali plus calcium ions [addition of 0.02 ml of a 1% (w/v) $CaCl_2$ solution to the basic solution].

Fluorimetry

Fluorimetric analyses were performed with an SFM 23 LC spectrofluorimeter (Kontron) fitted with a Phillips 150-W xenon arc lamp, and grating excitation and emission monochromators. Fluorescence spectra were obtained according to the method of Kohn [9].

Mass spectrometry

A Varian Model MAT 312 (Varian, Bremen, G.F.R.) was used to obtain direct probe mass spectra. In the electron impact mode the mass spectrometer was operated at 70 eV.

The residues of the freeze-dried fractions from gel chromatography were dissolved in small volumes of methanol and transferred into the analytical system. The mass spectrometric analyses were performed at the Institute of Organic Chemistry (Prof. Dr. G. Spiteller) of the University of Bayreuth (G.F.R.).

RESULTS AND DISCUSSION

In Fig. 1 the analytical separation of a liver extract of a control mouse (Fig. 1A) and a phenobarbital-pretreated mouse (Fig. 1B) is shown 6 h after application of 0.05 mg/g doxycycline. At 0.28 min prior to doxycycline a substance

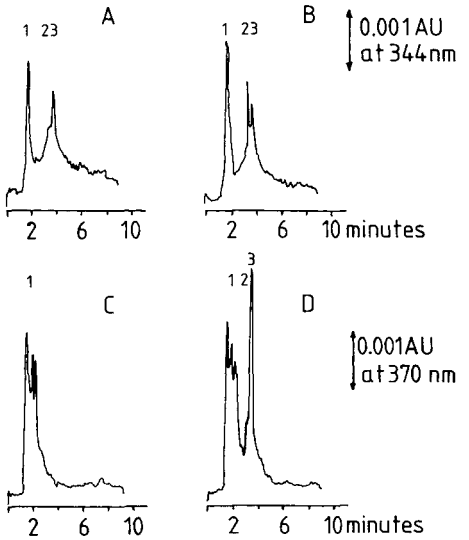

Fig. 1. Chromatographic analysis of doxycycline and its metabolite on RP C_8, 10 μm. Chromatographic conditions: column 25 × 0.4 cm; eluent, 3.5 mmol/l NaH_2PO_4 in water—acetontirile (70:30), pH 2.7; flow-rate, 1.9 ml/min; sample volume, 0.05 ml; detection, at 344 nm (A and B) and 370 nm (C and D); detection limit for doxycycline = 2—3 ng per injection. (A) Liver extract of a control mouse, 6 h after intravenous application of 50 μg/g doxycycline. (B) Liver extract of a phenobarbital pretreated mouse, 6 h after intravenous application of 50 μg/g doxycycline. (C) Human blank urine, collected before application of the drug. (D) Human urine, 16 h after ingestion of 2 × 100 mg doxycycline capsules. Peaks: 1 = solvent front; 2 = metabolite of doxycycline; 3 = doxycycline.

was eluted. Its amount increased ten times after phenobarbital pretreatment compared to the control. The mean values of the organ concentration are given in Table I. When female rats received doxycycline the metabolite could also be identified and its amount increased also after induction of the drug-metabolizing enzymes.

When human urine was analyzed after a single oral dose of 200 mg of doxycycline, 15.5 mg of doxycycline were excreted during the first 16 h. By HPLC analyses at different wavelengths (344 nm, 370 nm, 380 nm) the occurrence of the metabolite could be demonstrated (Fig. 1C and D).

The chromatographic and spectrophotometric behaviour of the doxycycline metabolite from mice, rats, and human urine were identical.

To isolate the metabolite of doxycycline the extracts of livers and kidneys of phenobarbital-pretreated mice were chromatographed on a semipreparative reversed-phase column. Doxycycline and its metabolite were eluted after 4.03 ± 0.08 min (doxycycline) and 3.77 ± 0.07 min (metabolite), respectively.

Since endogenous substances were eluted from the column together with doxycycline and its metabolite further purification was necessary for consecutive analyses. It was performed by gel chromatography (Fig. 2A). When re-chromatographed on the analytical HPLC system the substance of peak 3 of the gel chromatography coincided with doxycycline and the compound of peak 2 of the gel chromatography coincided with the metabolite (Fig. 2B).

TABLE I

CONCENTRATION OF DOXYCYCLINE AND ITS METABOLITE IN LIVER AND KIDNEY OF FEMALE NMRI MICE AND FEMALE WISTAR RATS AFTER INTRAVENOUS INJECTION OF 0.05 mg/g DOXYCYCLINE IN A CONTROL EXPERIMENT AND AFTER INDUCTION OF THE DRUG-METABOLIZING ENZYMES WITH PHENOBARBITAL

The data, expressed as mean ± S.D. (n = 10 for mice and n = 4 for rats), are corrected for the organ's blood content. The variance of the data is due to the biological variance because the method has a standard deviation of between 4 and 5% only [8]

		Doxycycline (μg/g)		Metabolite (μg/g)	
		Control	Phenobarbital	Control	Phenobarbital
Mouse, 6 h post injection	Liver	44.3 ± 16.5	33.6 ± 18.3	2.9 ± 2.8	48.1 ± 23.6
	Kidney	88.6 ± 21.3	43.4 ± 18.9	10.9 ± 2.1	100.8 ± 26.1
Rat, 10 h post injection	Liver	174.6 ± 32.4	92.2 ± 24.4	2.6 ± 2.5	7.8 ± 4.1
	Kidney	304 ± 62.9	168.3 ± 33.5	4.9 ± 3.8	70.7 ± 24.6

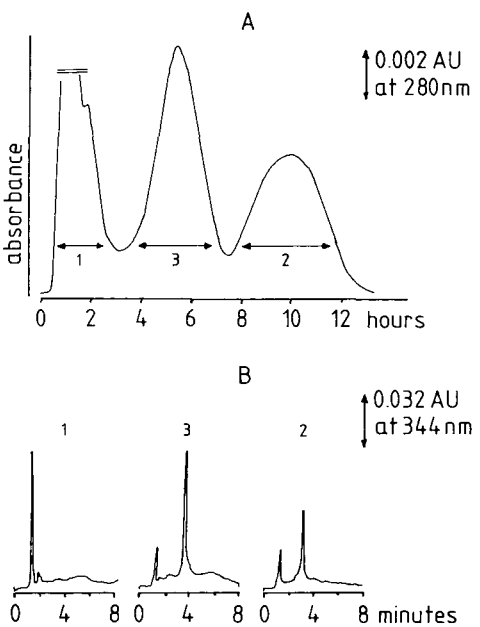

Fig. 2. Purification of doxycycline and its metabolite from livers of mice pretreated with phenobarbital, by gel chromatography (A) and analytical control by HPLC (B). (A) Gel chromatography: column 30 × 1.5 cm, packed with Sephadex G-15, particle size 40—120 μm; eluent, double-distilled water; flow-rate, 45 ml/h; detection at 280 nm. Separation of freeze-dried fractions of doxycycline and its metabolite after semipreparative HPLC of liver extracts of phenobarbital-pretreated mice, 6 h after application of 50 μg/g doxycycline. Peak 3 corresponds to 30 μg of doxycycline and peak 2 to 15 μg of metabolite (spectrophotometric quantitation after freeze-drying of the fractions indicated). (B) HPLC conditions were as described in the legend of Fig. 1 and in Materials and methods. The residues of peak 2 and peak 3 material were dissolved in water and 50 μl were applied to the analytical HPLC column.

The metabolite is not the 4-epi-doxycycline because this compound showed nearly the same retention time as doxycycline in this analytical system and a conversion between doxycycline and its metabolite did not occur in the acidic eluent even after 2 h. Additionally, the formation of the metabolite was enhanced by phenobarbital pretreatment, a typical inducing agent of the drug-metabolizing enzymes, and epimerization is normally not catalyzed by enzymes.

The metabolite displayed UV and fluorescence characteristics nearly identical with those of doxycycline (Table II). Addition of sodium hydroxide or calcium ions to the acidic solution of doxycycline and its metabolite, respectively, resulted in a bathochromic shift of the 344 nm peak. These effects are highly characteristic for tetracyclines. They are associated with enolization of the chromophore located on rings B, C and D (Fig. 3) [10, 11]. Both the metabolite and doxycycline itself showed an excitation maximum at 398 nm and an emission maximum at 536 nm in the fluorescence analysis.

TABLE II

UV CHARACTERISTICS OF DOXYCYCLINE AND ITS METABOLITE, EXTRACTED FROM LIVER AND KIDNEY OF MICE PRETREATED WITH PHENOBARBITAL

	λ_{max} (nm) in:		
	0.1 mol/l HCl	NaOH	NaOH—Ca^{2+}
Doxycycline*	267, 344	374	390
Metabolite	265, 344	371	380

*Reference standard as well as extracted from liver and kidneys.

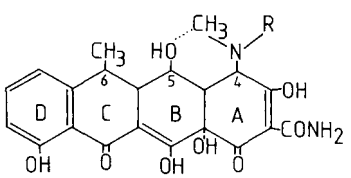

Fig. 3. Configuration of doxycycline (R = CH_3) and its metabolite (R = H). The methyl group (R = CH_3) becomes susceptible to enzymatic cleavage because the structure is fixed by a hydrogen bond between the hydroxyl group at position 5 and one methyl group of the dimethylamino group (indicated by the dotted line). By this means the methyl group at positon 6 is sterically hindered against enzymatic cleavage.

The metabolite is most probably a demethylated derivative of doxycycline as evidenced from its mass spectrum. The electron-impact (EI) spectrum (Fig. 4C) yielded a molecular ion (M^+) at *m/z* 430 compared to a molecular ion (M^+) at *m/z* 444 for the standard, which means loss of a methyl group. Nearly identical mass spectra were obtained from peak 3 material of the gel chromatography (Fig. 4B) and the doxycycline standard. The maxima of molecular ions were observed at the same temperature in the programme-controlled evaporation of the sample for doxycycline and its metabolite.

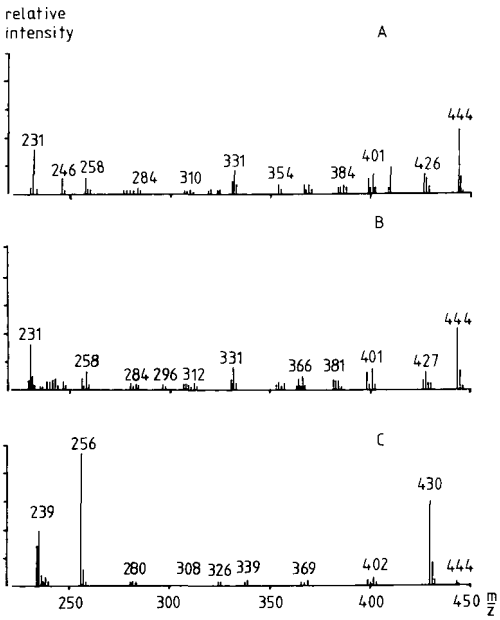

Fig. 4. EI mass spectral analysis of doxycycline standard (A), doxycycline extracted from liver (B), and the metabolite of doxycycline extracted from liver (C). The sensitivity of (C) is ten times the sensitivity of (A) and (B). The molecular ion M^+ *m/z* 444 of doxycycline accounts for about 25% relative intensity in (A) and (B) and about 3% in (C) attributable to impurities. For further details see Materials and methods.

Further analytical investigations were not possible because of the limited amounts of material available and the presence of impurities.

Since the UV and fluorescence characteristics of doxycycline and its metabolite were nearly the same, the absorption at 344 nm was taken for quantitation of the metabolite. Based on the assumption of comparable extinction coefficients the concentration of the metabolite was determined in livers and kidneys of mice and rats, and in human urine.

In the kidneys of mice the concentration of the metabolite was about twice the concentration in liver after phenobarbital pretreatment. In the kidneys of rats the concentration of the metabolite was about ten times the concentration in liver (Table I). In control mice about 5—10% of "doxycycline" was analyzed as metabolite while in phenobarbital-pretreated mice at the same time after application 50—70% of the totally analyzed drug was found as metabolite.

In human urine a total of 50 μg of metabolite could be detected together with 15.5 mg of unchanged doxycycline (i.e. 7.75% of the dose applied). From the present analytical data it is concluded that one of the methyl groups of the dimethylamino group is enzymatically cleaved off. The methyl group at position 6 is protected against enzymatic cleavage by steric hindrance because the configuration of the doxycycline molecule is stabilized by a hydrogen bond between the hydroxyl group at position 5 and one methyl group of the dimethylamino group [12] (Fig. 3). In this configuration one methyl group of the dimethylamino group becomes susceptible to enzymatic cleavage.

This appears to be the first time that a metabolite of doxycycline has been isolated. The results are in some contrast to observations by Nelis and De Leenheer [6] who stated that doxycycline was metabolically inert. In the chromatographic system used by these authors two peaks were analyzed as "unidentified by-products of doxycycline" and it might be possible that one of those peaks corresponds to N-monodemethyldoxycycline.

On the other hand, the occurrence of an N-monodemethyl derivative would be in good accordance with the recently described metabolism of minocycline, a tetracycline derivative which is nearly as lipophilic as doxycycline [1].

The method presented is suited for studies of doxycycline metabolism in animals and humans, especially to clarify the importance of doxycycline demethylation for shortening the half-life of doxycycline in man after premedication with drugs which induce the drug-metabolizing enzymes [2—4].

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REFERENCES

1 H.J.C.F. Nelis and A.P. De Leenheer, Drug Metab. Dispos., 10 (1982) 142.
2 P.J. Neuvonen and O. Penttilä, Brit. Med. J., i (1974) 535.
3 O. Penttilä, P.J. Neuvonen, K. Aho and R. Lehtovaara, Brit. Med. J., ii (1974) 470.
4 P.J. Neuvonen, O. Penttilä, R. Lehtovaara and K. Aho, Eur. J. Clin. Pharmacol., 9 (1975) 147.
5 P.J. Neuvonen, O. Penttilä, M. Roos and J. Tirkkonen, Int. J. Clin. Pharmacol., 14 (1976) 303.
6 H.J.C.F. Nelis and A.P. De Leenheer, J. Pharm. Sci., 70 (1981) 226.
7 R. Böcker and C.-J. Estler, Arzneim.-Forsch., 31 (1981) 2116.
8 R. Böcker, J. Chromatogr., 187 (1980) 439.
9 K.W. Kohn, Anal. Chem., 33 (1961) 862.
10 C.R. Stephens, K. Murai, K.J. Brunings and R.B. Woodward, J. Amer. Chem. Soc., 78 (1956) 4155.
11 J.R.D. McCormick, S.M. Fox, L.L. Smith, B.A. Bitler, J. Reichenthal, V.E. Origoni, W.H. Muller, R. Winterbottom and A.P. Doerschuk, J. Amer. Chem. Soc., 79 (1957) 2849.
12 D.A. Hussar, P.J. Niebergall, E.T. Sugita and J.T. Doluisio, J. Pharm. Pharmacol., 20 (1968) 539.

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CHROMBIO. 1599

LIQUID CHROMATOGRAPHIC DETERMINATION OF MITOMYCIN C IN HUMAN PLASMA AND URINE

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SUMMARY

A method is given for the determination of the antineoplastic drug mitomycin C in plasma and urine samples. Mitomycin is isolated from the biological matrix with the aid of a Sep-Pak C_{18} extraction column and eluted with methanol. The methanol is evaporated and the residue is redissolved in the chromatographic mobile phase (methanolic phosphate buffer). Mitomycin C is separated from coextracted compounds by reversed-phase liquid chromatography on a LiChrosorb RP-8 column. A high detection sensitivity and selectivity was obtained by photometric measurements at 365 nm. The precision of the determinations was better than 6% relative standard deviation for plasma samples within the range 2—1000 ng/ml, and for urine samples within the range 0.5—4.4 μg/ml. The pH-dependent stability of mitomycin in buffer solutions has been studied.

INTRODUCTION

Mitomycin C (Fig. 1), an antitumour antibiotic, was discovered in the late 1950's. Early clinical trials, however, revealed severe toxicity and the impor-

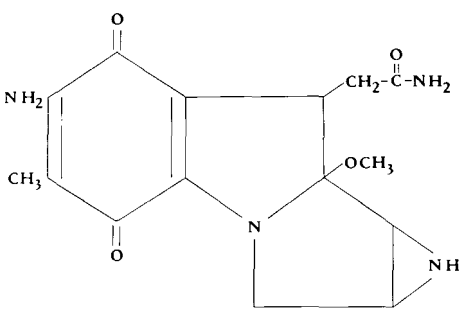

Fig. 1. Structural formula of mitomycin C.

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tance of proper dosage schedules to reduce toxicity has been emphasized. The mechanism of action of mitomycin C has been extensively studied in model systems [1—3]. Mitomycin C behaves as a bifunctional "alkylating" agent upon chemical or enzymatic reduction [2]. Only limited data of the clinical pharmacokinetics of mitomycin C are available, due to the lack of suitable assay methods. The microbiologic assay methods [4, 5] suffer from low selectivity and sensitivity. High-performance liquid chromatography (HPLC) has been used for separation of mitomycin from impurities [6] as well as for studies of the stability of mitomycin in aqueous solutions [7]. Recently, HPLC methods have been used for quantitation of mitomycin in serum [8—10].

Physicochemical investigations of porfiromycin, which differs from mitomycin C by having a methyl substituent on the aziridine nitrogen, have indicated that the chemical degradation of this compound is complex and strongly pH-dependent, as studied by photometric technique [11, 12]. These findings were recently confirmed also to be valid for mitomycin C [7].

The present paper gives a liquid chromatographic method for the analysis of mitomycin C in plasma and urine samples including a simplified extraction method.

The stability of mitomycin C was investigated in the present study with the aim of finding conditions under which the degradation of mitomycin C was negligible for a proper handling of biological samples as well as for the use of a suitable liquid chromatographic isolation procedure.

EXPERIMENTAL

Apparatus

Photometric measurements were performed with a Zeiss PMQ III Spectral photometer. An Orion Research Model 701/digital pH meter equipped with an Ingold combined electrode type 401 was used for pH measurements.

Chromatographic system

The pump was of the LDC 711 Solvent Delivery System type, and the columns were of stainless steel (length 150 mm, I.D. 4 mm, O.D. 6.35 mm). The LiChrosorb RP-8 support (E. Merck, Darmstadt, G.F.R.) had a mean diameter of 5 μm, and the chromatographic system was operated at ambient temperature (25 ± 2°C). A Rheodyne (Model 7125) injection valve with a sample loop of 150 μl was used. The chromatographic detectors used were an Altex 253 photometric detector measuring at 253.7 nm or an LDC Spectromonitor III, measuring at 365 nm. Both detectors had cells with a 10-mm path length and a volume of 8 μl.

Chemicals

All chemicals used were of analytical grade and used without further purification. Mitomycin C was kindly supplied by Bristol Laboratorier AB (Solna, Sweden). Desipramine chloride was obtained from AB Hässle, (Mölndal, Sweden). The mobile phases were prepared from acetonitrile (Uvasol, E.

Merck), methanol (p.a., E. Merck), phosphate buffer (pH 7.0, μ = 0.1) or citrate buffer (pH 5.1, μ = 0.1), and distilled water.

The buffers used in studies of the stability of mitomycin C were prepared from phosphoric acid, citric acid and sodium hydroxide to give pH values within the range 2.0—8.0. Unless otherwise stated the buffer solutions had an ionic strength of 0.1. Sep-Pak C_{18}® extraction columns were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Chromatographic technique

The chromatographic columns were packed by slurry packing [13] using glycerol—methanol (1:3) as the suspension medium. The slurry was forced into the column at a flow-rate of 9 ml/min or a pressure of 34 bar, whichever was the limiting factor. The mobile phases were passed through the chromatographic system until constant retention was obtained. Usually less than 50 ml were required. The interstitial volume, V_m, of the columns was determined by the injection of 0.1 *M* phosphoric acid (10 μl).

The lifetime of the separation column was considerably increased by the use of a 7-cm pre-column, packed with the same support as the separation column, inserted between the pump and the injector.

All capacity factors (k') given are averages from at least three determinations. The mobile phase flow-rate was 0.7 ml/min throughout this study.

Drug administration

A solution containing 0.5 mg/ml mitomycin C in sterile water was used. A dose of 0.15—0.35 mg/kg body weight was given intravenously as a bolus injection over a period of 3 min. Intrahepatically 0.35 mg/kg body weight was injected into the arteria hepatica, the injection time being 3—5 min.

Plasma samples

Blood samples (5—7 ml) were taken by venipuncture from mitomycin-treated cancer patients at various time intervals. The samples were collected in 10 ml test tubes (Vacutainer®) containing 250 IU of heparin (freeze-dried). The plasma fractions were isolated by centrifugation at 325 *g* for 10 min. The separated plasma fractions were stored at —70°C until analysis.

Urine samples

Aliquots of urine samples from mitomycin-treated patients were collected in glass test tubes and stored at —70°C until analysis.

Spiking of urine and plasma samples

Appropriate amounts of mitomycin C were dissolved in methanol. To each millilitre of blank urine or plasma sample were added 100 μl of the methanolic solution.

Degradation of mitomycin C

(1) A stock solution of mitomycin C in distilled water (1.56 mg/ml) was diluted five times with citrate or phosphate buffer (final ionic strength 0.08).

The degradation of mitomycin C was followed by photometric measurement at 355 nm.

(2) A stock solution of mitomycin C in distilled water (36.8 μg/ml) was diluted five times with citrate or phosphate buffer (final ionic strength 0.08). The degradation of mitomycin C was followed by reversed-phase liquid chromatography with photometric detection at 253.7 nm. The mobile phase used was a mixture of 10% acetonitrile in citrate buffer pH 5.1.

Unless otherwise stated the degradation experiments were performed at 25.0 ± 0.1°C.

Analytical method

Extraction procedure

The Sep-Pak columns were pretreated, according to instructions from the manufacturer, by passage of 2 ml of methanol followed by 5 ml of distilled water with the aid of a glass syringe. A 1-ml volume of plasma or urine sample was mixed with 100 μl of desipramine chloride solution (100 μg/ml) and forced through the Sep-Pak cartridge. The cartridge was washed with 3 ml of water (discarded) and mitomycin was eluted with 4 ml of methanol. The methanol was evaporated to dryness under nitrogen flow. The residue was dissolved in 200 μl of mobile phase with the aid of a vortex-type mixer. After centrifugation, 100 μl of the clear liquid was injected into the liquid chromatograph.

Liquid chromatography

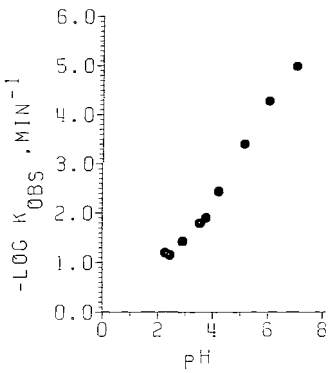
Mitomycin was separated from coextracted compounds on a LiChrosorb RP-8, 5 μm, column, eluted with an aqueous mobile phase containing 10% of phosphate buffer (pH 7.0 μ = 0.1) and methanol, 20 and 25% for urine and plasma samples, respectively. The absorbance of the eluate was measured at 365 nm. Quantitation was based on peak area measurements and the molar absorptivity of mitomycin. All plasma and urine levels of mitomycin were corrected for recoveries.

RESULTS AND DISCUSSION

Stability of mitomycin C

The chemical degradation of mitomycin C was studied by the photometric technique previously used by Garrett et al. [11, 12] for degradation studies of porfiromycin, and also by reversed-phase liquid chromatography [7]. The results (Fig. 2) show that the stability of mitomycin C increases with increasing pH within the pH range 2—7, the half-lives being about 10 min and 50 days at pH 2.3 and 7.0, respectively. The constants determined by the photometric technique were about 30% lower than the constants determined from chromatographic data, which may be the result of difficulties in determination of the absorbance values at infinite time for the first degradation step [11]. The data for the degradation of mitomycin C presented in Fig. 2 are very close to those found by Garrett et al. for porfiromycin [11, 12].

The stability of mitomycin C was strongly temperature-dependent. The



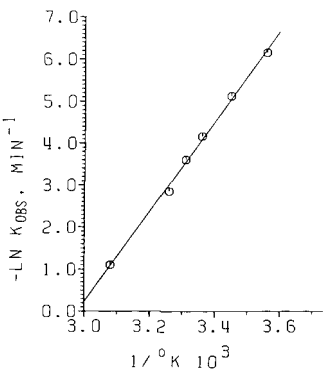


Fig. 2. Degradation of mitomycin C — influence of pH.

Fig. 3. Temperature dependence of rate constants. pH 3.51; chromatographic data.

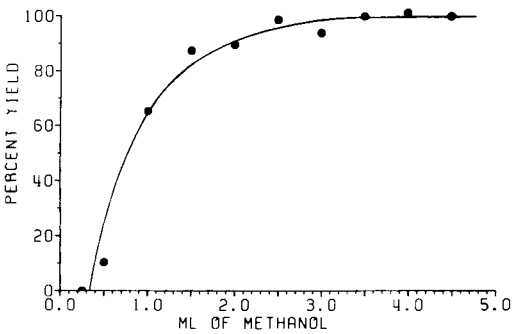
Arrhenius activation energy, calculated from the slope of the plot in Fig. 3, was 98 kJ/mol in citrate buffer pH 3.51 (liquid chromatographic data). A value of 81.1 kJ/mol has been found in unbuffered medium [7]. The rate constants for the degradation of mitomycin C in 10^{-2} *M* phosphoric acid were almost unaffected by the addition of NaCl (10^{-2} to 1 *M*), i.e. the rate constants were essentially unaffected by the ionic strength.

Extraction procedure

In the previously published HPLC analytical methods for mitomycin C [8, 9] the drug was isolated from the biological matrix by liquid—liquid extraction. Since mitomycin C has a low partition coefficient into the organic extractants used, a large ratio of organic phase to aqueous phase was necessary to obtain quantitative transfer of the drug from the plasma. Since large amounts of organic extractants with high boiling points were used and an evaporation temperature not exceeding 40°C is recommended to avoid decomposition of mitomycin C [9], the extraction procedure seemed too time-consuming for routine analysis. Isolation of mitomycin C from plasma with the aid of a non-ionic resin, Porapak Q, gave greatly varying recoveries (65—85%) [10].

In the analytical procedure for mitomycin C proposed in the present paper the drug was isolated from the biological matrix with the aid of a Sep-Pak C_{18} cartridge. The polar constituents of the biological matrix were removed by a wash with distilled water. Mitomycin C was eluted from the extraction column with methanol. A quantitative recovery of mitomycin C was obtained when the amount of methanol used for the elution exceeded 3.5 ml (Fig. 4).

To avoid decomposition of mitomycin C the evaporation of the methanol extract was performed under a nitrogen stream at a temperature not exceeding 40°C [9]. Hartigh et al. [9] found that the dissolution of mitomycin C from evaporated plasma extracts using a mobile phase containing 25% of methanol was neither complete nor reproducible, which necessitated the use of pure methanol for the dissolution of the drug. However, injection of samples dissolved in a liquid of a higher solvent strength than the mobile phase used often gives rise to disturbances of the chromatographic behaviour [14, 15] and should



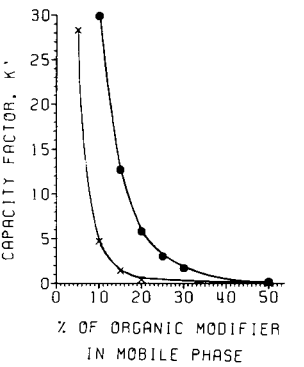


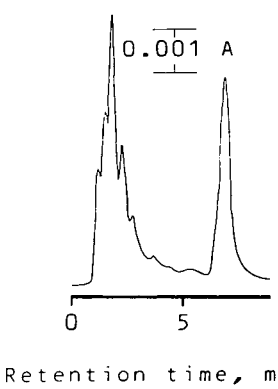
Fig. 4. Elution of mitomycin C from Sep-Pak C_{18} extraction columns.

Fig. 5. Capacity factors and concentration of organic modifier in mobile phase. Mobile phase: organic modifier in phosphate buffer pH 7.0 with a final ionic strength of 0.01. Organic modifier: X = acetonitrile; • = methanol.

accordingly be avoided. In the present paper complete dissolution of mitomycin C in the chromatographic mobile phase was obtained after the addition of desipramine, previously shown to prevent adsorption on glass surfaces effectively [14—17].

Chromatographic isolation

To avoid decomposition of mitomycin C during the liquid chromatographic procedure too low a pH of the mobile phase should be avoided; cf. Fig. 2. The retention of mitomycin C was strongly dependent upon the concentration of organic modifiers in the mobile phase (Fig. 5). Acetonitrile was the most suitable modifier for the separation of mitomycin and its degradation products. However, in the proposed analytical method, aimed for routine analysis of mitomycin in biological samples, methanol was preferred as modifier due to



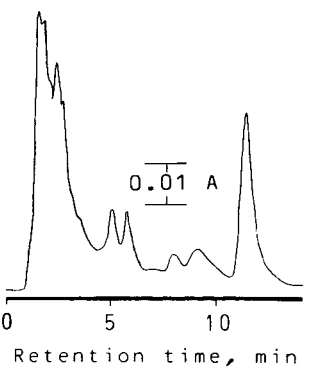


Fig. 6. Chromatogram of plasma sample from a mitomycin-treated cancer patient. The chromatographic peak corresponds to 62 ng of mitomycin per ml of plasma.

Fig. 7. Chromatogram of a urine sample from a mitomycin-treated cancer patient. The chromatographic peak corresponds to 7 μg of mitomycin per ml of urine.

its lower toxicity. Mitomycin in plasma extracts was completely isolated from endogenous compounds using a mobile phase containing 25% of methanol (Fig. 6). For the analysis of mitomycin in urine samples a decrease of the methanol concentration in the mobile phase to 20% was necessary to avoid interference (Fig. 7).

Photometric detection

The absorption spectra of mitomycin in the mobile phase used in the analytical procedures (containing 20 and 25% of methanol, respectively) were identical and showed that the commonly used fixed-wavelength ultraviolet detectors measuring at 254 nm are unsuitable for the analysis of mitomycin in the low concentration range. The high detection sensitivity as well as the high detection selectivity in the present method was obtained by photometric measurement of the eluate at 365 nm; cf. ref. 18. The signal-to-noise ratio was better than 10 for an injected amount of 1 ng of mitomycin C.

Quantitative determination

Quantitation was based on peak area measurement and the molar absorptivity of mitomycin C in the mobile phase ($\epsilon = 1.65 \cdot 10^4$) according to the principles given in ref. 19.

The amount of sample (in mmol), M, can be calculated from the equation

$$M = Y \times u \times b \times \epsilon^{-1}$$

where Y = peak area in mm^2, u = ml/mm chart paper, b is absorbance/mm chart paper. From the equation it follows that the peak area is not dependent on chromatographic parameters such as column efficiency and capacity factors of the solutes, as well as length and diameter of the chromatographic column. Hence, once the molar absorptivity has been determined no calibration graph need be constructed.

Recovery and precision

The recovery and precision of the method for the determination of mitomycin in plasma and urine samples are presented in Table I. The precision of the determinations was better than 6% relative standard deviation for plasma

TABLE I

RECOVERY AND PRECISION FROM SPIKED PLASMA AND URINE SAMPLES

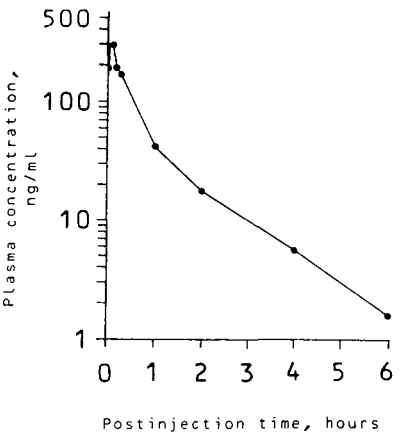
Biological sample	Drug concentration (ng/ml)	Recovery* (%)
Plasma	2.13	71.5 ± 5.0
Plasma	4.94	94.6 ± 4.3
Plasma	12.6	97.2 ± 1.5
Plasma	105	101.9 ± 0.7
Plasma	1050	102.9 ± 2.5
Urine	450	87.0 ± 1.5
Urine	930	89.8 ± 0.4
Urine	2120	82.2 ± 5.9
Urine	4370	84.6 ± 4.1

*Recovery ± relative standard deviation ($n = 6$).

samples within the range 2—1000 ng/ml and for urine samples within the range 0.5—4.4 μg/ml.

Clinical applications

Plasma levels of mitomycin after an intrahepatic dose of 0.35 mg/kg are given in Fig. 8, the terminal half-life being 1.1 h. About 12% of mitomycin was excreted unchanged in the urine after an intravenous dose of 0.15 mg/kg (Fig. 9).



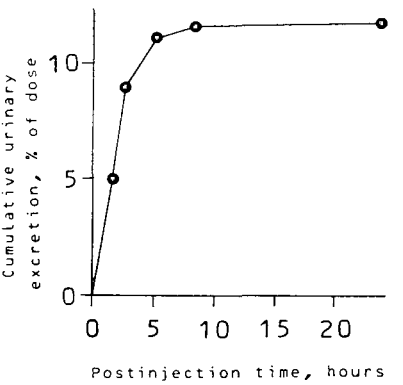


Fig. 8. Plasma concentration—time curve after intrahepatic administration of mitomycin C. Mitomycin, 0.35 mg/kg body weight, was given as a bolus injection in the arteria hepatica.

Fig. 9. Urinary excretion of mitomycin C. Mitomycin, 0.15 mg/kg body weight, was given as an intravenous bolus injection.

REFERENCES

1 V.N. Iyer and W. Szybalski, Science, 145 (1964) 55.
2 M. Tomasz and R. Lipman, J. Amer. Chem. Soc., 101 (1979) 6063.
3 G. Rodighiero, S.M. Magno, F. Dell'Acqua and D. Vedaldi, Farmaco, 33 (1979) 651.
4 H.S. Schwartz and F.S. Philips, J. Pharmacol. Exp. Ther., 133 (1961) 335.
5 H. Fujita, Jap. J. Clin. Oncol., 12 (1971) 151.
6 S.C. Srivastava and U. Hornemann, J. Chromatogr., 161 (1978) 393.
7 D. Edwards, A.B. Selkirk and R.B. Taylor, Int. J. Pharm., 4 (1979) 21.
8 A. Kono, Y. Hara, S. Eguchi, M. Tanaka and Y. Matsushima, J. Chromatogr., 164 (1979) 404.
9 J. den Hartigh, W.J. van Oort, M.C.Y.M. Bocken and H.M. Pinedo, Anal. Chim. Acta, 127 (1981) 47.
10 G.A. van Hazel and J.S. Kovach, Cancer Chemother. Pharmacol., 8 (1982) 189.
11 E.R. Garrett, J. Med. Chem., 6 (1963) 488.
12 E.R. Garrett and W. Schroeder, J. Pharm. Sci., 53 (1964) 917.
13 R.P.W. Scott and P. Kucera, J. Chromatogr., 142 (1977) 213.
14 P.J. Naish, M. Cooke and R.E. Chambers, J. Chromatogr., 163 (1979) 363.
15 D.A. Jowett, Clin. Chem., 27 (1981) 1785.
16 H. Brötell, J. Chromatogr., 196 (1980) 489.
17 S. Eksborg, H. Ehrsson, B. Andersson and M. Beran, J. Chromatogr., 153 (1978) 211.
18 S. Eksborg, H. Ehrsson and U. Lönroth, J. Chromatogr., 185 (1979) 583.
19 S. Eksborg and G. Schill, Anal. Chem., 45 (1973) 2092.

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CHROMBIO. 1614

AN INTERNALLY-STANDARDIZED ASSAY FOR AMPHOTERICIN B IN TISSUES AND PLASMA

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SUMMARY

A high-performance liquid chromatographic (HPLC) method with *p*-nitrophenol as internal standard is described for the rapid analysis of amphotericin B recovered by methanolic extraction from tissues and plasma. Programmed, gradient elution of the ODS column was used with detection by tungsten light at 388 nm. Standard curves were derived based on the peak height ratios. The lowest reproducible limit of the assay was 0.04 μg/ml with plasma. The extraction and chromatographic procedures recovered 53—71% of the amphotericin B from each of these sources. The coefficient of variation of the recovery ratios was less than 18% from plasma over a range of concentrations of amphotericin B from 0.08 to 10.0 μg/ml. Recovery from tissues, studied over a narrower concentration range, showed a similar degree of precision. Variations in precolumns apparently resulting in selective binding of the amphotericin B were found to have a systematic but important influence on recovery efficiency. No substances were detected which interfered with the assay procedures as described. By incorporating an internal standard we have enhanced the reliability and flexibility of the HPLC assay for amphotericin B especially for assay of tissues.

INTRODUCTION

Amphotericin B, a non-aromatic heptaene compound, is the drug of choice for most systemic fungal infections. Its antimycotic properties are related to its ability to form strong hydrophobic bonds to sterols, especially ergosterol, in the membranes of fungal cells [1, 2]. Binding to sterols in the membranes of animal cells may contribute to the prolonged tissue retention of the drug [3]. Amphotericin B has been detected in the serum and bile of dogs for more than 7 days, in urine for more than 20 days, and in kidney tissue for 50 days, after a single dose [4]. However, many of the pharmacological characteristics of this compound remain poorly understood [5].

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The major limitation to the use of amphotericin B is its toxicity. Most patients treated with this agent develop adverse reactions, particularly impairment of renal function [6—8]. Although toxicity is generally dose-dependent, there is considerable interindividual variability. It has been difficult to establish a clear relation between blood levels of amphotericin B and its efficacy or toxicity.

Many of the difficulties encountered in studying the pharmacology and toxicity of amphotericin B arise because of problems in assaying the compound. Microbiological assays are satisfactory for many purposes [9], but generally lack the precision, sensitivity and speed of chromatographic procedures. Direct spectrophotometric assay at 405 nm may be affected by products of hemolysis or by other drugs [10]. Both types of assay are difficult to apply to tissue specimens.

High-performance liquid chromatographic (HPLC) procedures have been used to determine serum or plasma antibiotic levels in the microgram to nanogram range which would be crucial for assays of the drug in whole tissues [10—13]. However, methods described so far for amphotericin B have failed to incorporate an internal standard [10, 14]. The internal standard corrects for inadvertent changes in drug concentration incurred during extraction, dilution or concentration of plasma or tissue homogenates. We report here a sensitive and precise HPLC assay for amphotericin B incorporating an internal standard.

MATERIALS AND METHODS

Apparatus and reagents

An Altex (Berkeley, CA, U.S.A.) high-performance liquid chromatograph was used. The instrument was equipped with two Model 110A pumps, a Rheodyne Model 7125 injector (50-μl load loop), a Model 420 Microprocessor System Controller, and a reversed-phase Ultrasphere-ODS column (15 cm × 4.6 mm I.D.; 5 μm average particle size). One chromatographic column was used for all experiments. A 3.0-cm C_{18} precolumn was used (Brownlee Labs., Santa Clara, CA, U.S.A.). The precolumns were either RP-18 LiChrosorb (10 μm) or RP-18 Spheri-5 (5 μm). Eluents were monitored by absorbance of tungsten light at 388 nm on a 0.05 a.u.f.s. scale using a Hitachi Model 100-30 spectrophotometer. Flow-rate was maintained at 1.0 ml/min. HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethylene dinitrilotetraacetic acid disodium salt (EDTA) and *p*-nitrophenol (PNP, the internal standard) were from Sigma (St. Louis, MO, U.S.A.). Amphotericin B type 1 reference powder was generously supplied by E.R. Squibb (Princeton, NJ, U.S.A.). The drug was kept in stock at a concentration of 2500 μg per ml of methanol—dimethyl sulfoxide (1:1) and stored at —20°C. *p*-Nitrophenol was stored at 5°C as 3.0 mg per ml methanol. Two separate pools of the drug and of the internal standard were maintained. These stock solutions were alternately sampled to determine standard values. New pools were introduced every three to four weeks and verified against the old pools for their concentration of amphotericin B.

Chromatography

The mobile phase components consisted of 50% methanol in 0.01 *M* EDTA (phase A) and 50% acetonitrile in 0.01 *M* EDTA (phase B). For the programmed elution gradient, the system was maintained in phase A for 6 min, then shifted to phase B over a span of 1 min. After 15 min, the mobile phase was progressively shifted back to phase A over a span of 0.5 min. A new specimen could be introduced every 19 min.

The recovery value for amphotericin B from individual samples was calculated as the ratio of the peak height of the deflection produced by amphotericin B to that produced by PNP (internal standard, 150 μg). Calculations based on the ratios of areas under the curve were generally less precise than those based on peak height ratios. Standard curves were derived by linear regression analysis of specimens containing known concentrations of amphotericin B over a range of values.

Tissue extraction procedures (liver, kidney, spleen)

The recovery of amphotericin B was studied in samples to which known quantities of the drug were added before homogenization (spiked homogenates) or in which the drug was added to methanol extracts of tissue homogenates (spiked extracts). The former preparations were expected to simulate the tissues of treated animals; the latter were compared with these to determine whether there was a difference in recovery of drug attributable to the presence of whole tissue. In each instance, 150 μg PNP (50 μl of methanolic stock) were mixed and equilibrated with the tissues before homogenization.

Approximately 650 mg of kidney were used (range 620—770 mg), 500 mg of liver (range 420—650 mg) and 350 mg of spleen (range 250—490 mg). The extraction process was carried out by adding 1.5—2.0 ml of methanol to tissues which were then finely minced in a microhomogenizer cup (Sorvall, Norwalk, CT, U.S.A.). The cup was suspended in ice, and the mixture was homogenized at high speed for three 30-sec periods. The homogenate together with two methanol rinses (0.5—1.5 ml) of the cup was decanted into a 15-ml cork-stoppered Corex tube and heated (50°C) in a shaking water bath for 15 min. The extraction mixture was then centrifuged (12,000 *g* for 10 min) and the clear supernatant was collected. The pellet was reextracted and the clear supernatants were pooled. On occasion, it was necessary to clarify the extracts by further centrifugation or by filtration (0.5 μm Millex SR, Millipore, Bedford, MA, U.S.A.).

Plasma extraction procedures

A 150-μg amount of PNP (50 μl of methanolic stock) and 3.0 ml of methanol were added to 1.0 ml of plasma containing known quantities of amphotericin B. The opaque mixture was vigorously agitated and then centrifuged at 12,000 *g* for 10 min. The clear supernatant was collected for HPLC analysis.

RESULTS

Amphotericin B had a retention volume of 14.0 ml and PNP a retention volume of 5.4 ml in the chromatographic system described. The small amounts

of detectable background from tissue extracts and plasma or serum did not interfere with quantitation of the drug. Fig. 1 shows chromatograms of methanolic extracts of mouse liver homogenates (left) and human plasma (right) without amphotericin B. There were no significant chromatographic bands in the elution regions of the internal standard or of amphotericin B. The chromatograms which resulted from adding 5.0 μg of amphotericin B to 500 mg of mouse liver homogenate or 2.5 μg of amphotericin B to 1.0 ml of human plasma, together with PNP, are also depicted in Fig. 1 (lower panels). Similar results were obtained with spleen and kidney. The small chromatographic band eluting at 13.0—13.2 ml is recovered only in the presence of the drug. A similar

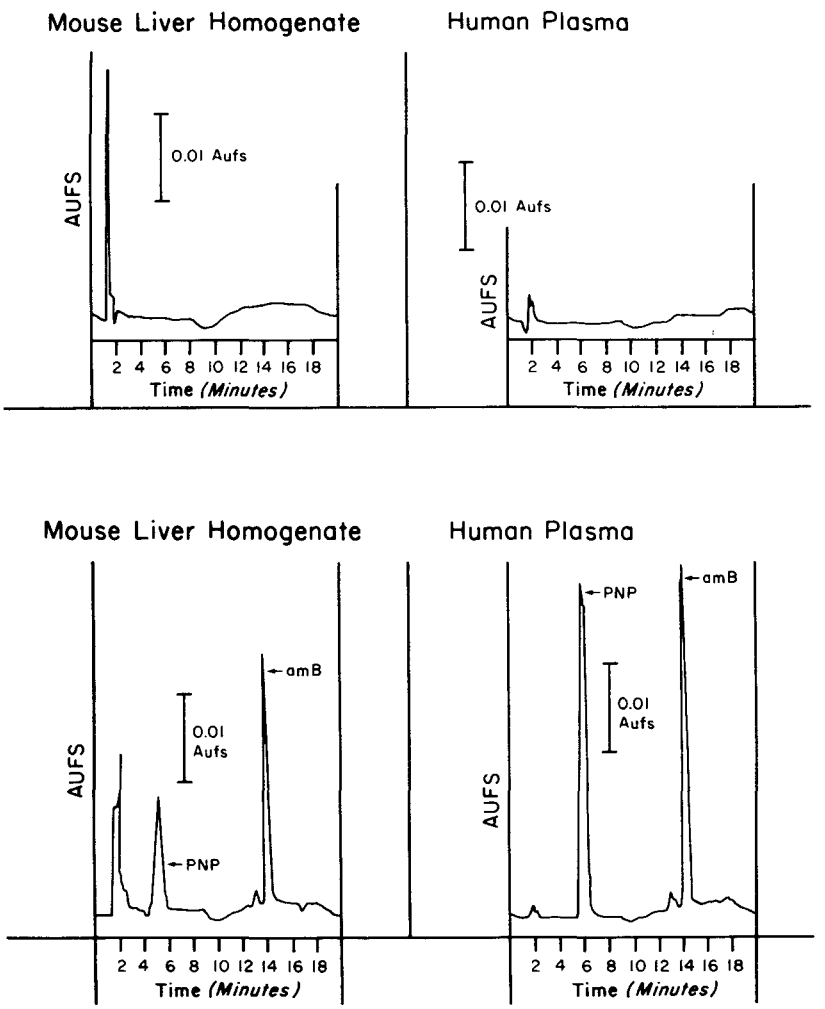

Fig. 1. Top panels: chromatograms of a methanolic extract of homogenized mouse liver and of human plasma; bottom panels: chromatograms of the same substances with amphotericin B (amB) and internal standard (*p*-nitrophenol, PNP) added before the extraction procedure. A.u.f.s. signifies absorbance units full scale.

band which may represent a separate component of amphotericin B has been previously reported [10, 14].

A primary source of analytical variation can be found in the chromatographic system itself. Changes in the slope of the ratio recovery curves can be linked to the state of the guard or precolumn (age, previous use, lot number). To verify this conclusion, a methanolic plasma extract (5 μg amphotericin B per ml plasma) supplemented with PNP was repeatedly chromatographed. The mean height ratios (amphotericin B:PNP) varied strikingly from column to column as shown in Table I. These discrepancies were generally statistically significant. For example, the values for column L_1 exceeded those for all other columns ($p < 0.01$ by unpaired t-test). In contrast, within-column variations were slight as shown by the small standard deviations. The absolute peak heights for the internal standard, PNP, were not significantly different among precolumns, varying from 25—30 mm in 24 tests. However, the peak heights for amphotericin B ranged from 18—83 mm, suggesting that the precolumn selectivity primarily affected the drug and not the internal standard. The precolumn was routinely used as a protective device; however, in the few instances in which it was omitted, height ratios near 3.0 were encountered. When the results for each precolumn are considered separately, the coefficients of variation (C.V.) were very small (Table I).

TABLE I

THE CHROMATOGRAPHIC RECOVERY OF AMPHOTERICIN B FROM PLASMA USING FIVE DIFFERENT PRECOLUMNS

Multiple aliquots from a single methanolic extract of human plasma containing 5 μg amphotericin B per ml were chromatographed for each precolumn.

Precolumn*	Mean recovery ratio ± S.D.**	C.V. (%)
L_1	2.56 ± 0.18 (8)	7.0
L_2	1.40 ± 0.04 (5)	3.0
L_3	1.90 ± 0.11 (5)	6.0
S_1	0.76 ± 0.06 (5)	7.0
S_2	2.80 (1)	

*L (LiChrosorb), S (Spherisorb); L_1, L_3, S_2 new precolumns with no or very limited use; L_2 and S_1 in use for approximately one month.

**Values are the mean ± standard deviation (S.D.) of the ratios of the peak height of amphotericin B:PNP. Values in parentheses indicate the number of trials.

In order to assure that the assay was functioning properly each day, an initial run was made using pools of methanolic standards (PNP + 5 μg amphotericin B in methanol) maintained at −20°C. When the elution characteristics were abnormal or peak height ratios of these standards departed by more than 2 S.D. from previous values, the equipment was rechecked for mechanical problems; however, such variations often indicated the need for replacement of the precolumn.

Estimates of the effect of the biological matrix upon recovery of amphotericin B were made by comparing the height ratios observed with methanol-diluted standards to those from drug-supplemented tissues (Table II) or plasma

TABLE II

THE RELATIVE EFFICIENCY OF AMPHOTERICIN B RECOVERY FROM MOUSE TISSUE HOMOGENATES

Amphotericin B (μg)	Mean recovery ratio		Relative recovery (Mean tissue—mean methanol standard)
	Tissue*	Methanol standard	
2.5	0.98 (9)	1.50 (6)	0.65
5.0	1.83 (9)	2.85 (6)	0.63
10.0	4.24 (7)	5.90 (5)	0.71

*Values are the mean of the peak height ratio of amphotericin B to that of PNP in separate analyses of five liver homogenates, two kidney homogenates, and two spleen homogenates. Each sample contained 350—650 mg of tissue and was supplemented with known quantities of amphotericin B and PNP. Values in parentheses are the number of separate preparations analyzed.

TABLE III

THE RELATIVE EFFICIENCY OF AMPHOTERICIN B RECOVERY FROM HUMAN PLASMA

Amphotericin B (μg)	Mean recovery ratio		Relative recovery (Mean tissue—mean methanol standard)
	Plasma*	Methanol standard	
2.5	0.93 (10)	1.70 (4)	0.53
5.0	1.99 (10)	3.30 (4)	0.61
10.0	4.61 (10)	7.50	0.61

*Values are the mean of the peak height ratio of amphotericin B to that of PNP in separate analyses of ten different human plasma pools supplemented with known quantities of amphotericin B and PNP. Values in parentheses are the number of separate preparations analyzed.

(Table III). There were no observable differences in the efficiency of recovery among liver, kidney, and spleen homogenates; therefore, the data for these tissues were pooled in Table II. The relative recovery of amphotericin B from tissue was 63—71% and from human plasma was 53—61% of the corresponding values for methanol standards. Recovery did not appear to be correlated with the absolute quantity of the drug. These data indicate that as much as 47% of amphotericin B is either not readily extractable from tissues and plasma or is in a bound or complexed form which does not fractionate efficiently in the chromatographic system. Only another 1—2% of the amphotericin B could be recovered by reextraction of pellets. The overall extraction efficiency of PNP was 85—90%. When amphotericin B was added to methanolic supernatants of tissue extracts, recovery ratios were statistically indistinguishable from those for methanolic standards. Thus, the interfering substances were either not methanol soluble or were unable to interact with amphotericin B in methanol.

Regression equations were calculated for the recovery of known quantities

of amphotericin B from the tissue homogenates. Two different precolumns, L_1 and L_2, were used. The recovery slope and the intercept for the L_1 set were 0.66 (r^2 = 0.99) and 0.075, respectively, and for the L_2 set were 0.44 (r^2 = 0.99) and —0.23, respectively. The relationship between the recovery ratio and the quantity of amphotericin B is linear. The coefficients of variation for the two sets of data ranged from 10—20%.

The precision of recovery of amphotericin B added to plasma at concentrations in the range 0.01—10.0 μg/ml is shown in Table IV. The minimum reproducible limit of the assay as described was 0.04 μg/ml. Lower values, 0.02 and 0.01 μg/ml appeared as barely discernible chromatographic bands which were too small for precise measurement. Within the range 0.04 to 5.0 μg/ml, the recovery slope (0.37; r^2 = 0.96) was linear. The coefficient of variation for

TABLE IV

THE PRECISION OF AMPHOTERICIN B RECOVERY FROM HUMAN PLASMA*

Amphotericin B (μg/ml)	Mean recovery ratio ± S.D.	C.V. (%)
0.01	0.010 ± 0.000 (5)	**
0.02	0.010 ± 0.000 (5)	**
0.04	0.015 ± 0.002 (6)	36.5
0.08	0.034 ± 0.003 (5)	16.1
0.16	0.058 ± 0.005 (4)	16.6
0.31	0.134 ± 0.008 (5)	12.5
0.63	0.255 ± 0.007 (4)	5.0
1.25	0.460 ± 0.031 (7)	17.9
2.50	0.93 ± 0.13 (10)	14.0
5.0	1.99 ± 0.16 (10)	8.0
10.0	4.61 ± 0.55 (10)	11.9

*See footnote for Table III.
**Peaks were too small for precise measurement.

TABLE V

AMPHOTERICIN B RECOVERY FROM THE LIVER AND KIDNEYS OF MICE

Mice were given a single i.v. dose of amphotericin B and were killed at the indicated time. The liver and kidneys were processed to determine drug concentrations.

i.v. Dose (mg/kg)	Time after injection (h)	Mouse No.	Liver (μg drug per g tissue)	Kidney (μg drug per g tissue)
1.5	1	0	14.58	3.48
		1	20.91	2.95
		2	14.15	0.65
1.5	42	6	10.03	1.42
		7	12.56	2.19
		8	13.30	2.70
2	72	12	14.39	2.52
		13	10.54	2.92
		14	36.43	5.38

the results ranged from 36.5% at 0.04 μg/ml to 5% at 0.625 μg/ml. Accordingly, the precision of drug recovery from plasma was very similar to that for tissues.

Nine mice were given single intravenous (i.v.) injections of amphotericin B in distilled water, after which drug levels in the liver and kidneys were determined by the chromatographic method (Table V). Although the three groups are not fully comparable because the dosages and timing of study were different, the values for each tissue within a group were fairly similar except for mouse 2 (low levels in kidney) and mouse 14 (high levels in both tissues). Hepatic levels were 3—20 fold greater than those recovered from the kidney.

DISCUSSION

Amphotericin B remains the most useful of agents for the therapy of most deep-seated fungal infections. However, its use is complicated by the almost universal development of toxic reactions, particularly those affecting the kidney. The pharmacokinetic behavior of amphotericin B is unusual. The drug is extensively bound to sterol-containing membranes and is excreted slowly and incompletely in the urine and bile [4, 15]. Investigations into the pharmacokinetic properties and mechanisms of toxicity of the compound have been hampered by lack of a simple, rapid and precise assay method capable of being applied to tissues and in the presence of other drugs.

A variety of microbiological assays have been described [16]. These are convenient for certain clinical purposes [9]. In comparison with the HPLC assay, however, bioassays are somewhat less precise, are difficult to standardize, and pose problems in interpretation especially at low drug concentrations [10]. Microbiological bioassays require 16—18 h of incubation. Natural antifungal activity in the blood of normal individuals may interfere with the assay as may other drugs given concomitantly. Direct spectrophotometric and microbiological assays may be difficult to interpret because of heme and other colored substances present in plasma and tissues [16].

The present study is the first report of an internally-standardized chromatographic assay for amphotericin B in tissues and plasma. The methanolic extraction used in our laboratory recovers 53—71% of the amphotericin B present in tissues and plasma. The data suggest that the avidity of binding to tissues and plasma are nearly equivalent.

The internal standard acts as a correction for procedural losses which might be incurred during extraction, dilution, filtration or chromatographic manipulations particularly when one is working with tissues. This has been especially helpful when concentration of the methanolic extract by evaporation is desirable in order to quantitate very low levels of drug in tissues. PNP, of course, is chemically very unlike amphotericin B. However, our efforts to find a useable congener of amphotericin B to serve as an internal standard were unsuccessful. N-acetylamphotericin B and the methyl ester of amphotericin B were initially considered; however, the former was difficult to acquire as a pure standardized powder and the latter had undesirable chromatographic elution characteristics (avid association with the RP column). PNP was an attractive choice because of its solubility and its chromatographic and light-

absorption properties. The linearity of the recovery curves over a wide range of concentrations and the low coefficients of variation at each concentration suggest that the extractability of amphotericin B and *p*-nitrophenol are similar under the circumstances of the assay. Although amphotericin B has a striking absorption peak at 405 nm, detection at 388 nm (visible) slightly enhanced the recovery precision for both compounds in these studies.

The data presented in Table I demonstrate a marked effect of the precolumn on the analytical yield. The recovery of PNP as determined by peak height measurement was unaffected. In contrast, the absolute recovery of amphotericin B was strikingly dependent upon the precolumn. Methanol-soluble substances from the biological matrix may bind avidly to certain precolumns and may in turn bind amphotericin B. The slow elution of such a complex or of the drug from the complex might not produce a discrete chromatographic band which could be distinguished from the background.

The lowest reproducible limit of the assay as described is 0.04 μg/ml (C.V. 36%). As in the case of most chromatographic assays, this one can be refocused to a lower range. Reduction in the quantity of PNP, concentration of methanolic extracts by evaporation, increase of the injection volume, and increase of the absorbance scale all may contribute to increased sensitivity of the assay. We have not fully explored the minimum concentration of drug in tissue at which the signal-to-noise ratio would limit sensitivity. Preliminary tests indicate the critical point to be less than 0.01 μg/ml plasma and 0.02 μg/g tissue. Even in tissue extracts concentrated 8-fold by evaporation, we have failed to find interfering chromatographic bands which elute in the region of PNP or amphotericin B.

REFERENCES

1 R.Y. Cartwright, J. Antimicrob. Chemother., 1 (1975) 141.
2 W.C. Chen, D-L Chou and D.S. Feingold, Antimicrob. Ag. Chemother., 13 (1978) 914.
3 J.E. Bennett, New Engl. J. Med., 290 (1974) 914.
4 P.C. Craven, T.M. Ludden, D.J. Drutz, W. Rogers, K.A. Baegele and H.B. Skrdlant, J. Infect. Dis., 140 (1979) 329.
5 G. Medoff and G.S. Kobayashi, New Engl. J. Med., 302 (1980) 145.
6 W.R. Butler, J.E. Bennett and D.W. Alling, Ann. Intern. Med., 61 (1964) 175.
7 R.P. Miller and J.H. Bates, Ann. Int. Med., 71 (1969) 1089.
8 J.L. Burgess and R. Birchall, Amer. J. Med., 53 (1972) 77.
9 R.B. Bannatyne, R. Cheung and E.R. Devlin, Antimicrob. Ag. Chemother., 11 (1977) 44.
10 I. Nilsson-Ehle, T.T. Hoshikawa, J.E. Edward, M.C. Schotz and L.B. Guze, J. Infect. Dis., 135 (1977) 414.
11 J.S. Wold, Antimicrob. Ag. Chemother., 11 (1977) 105.
12 J.P. Anhalt and S.D. Brown, Clin. Chem., 24 (1978) 1940.
13 I. Nilsson-Ehle, G. Kahlmeter and P. Nilsson-Ehle, J. Antimicrob. Chemother., 4 (1978) 169.
14 W. Mechlinski and C.P. Schaffner, J. Chromatogr., 99 (1974) 619.
15 A.J. Atkinson Jr. and J.E. Bennett, Antimicrob. Ag. Chemother., 13 (1978) 271.
16 T.B. Platt, J.D. Levin, J. Gentile and M.A. Leitz, in F. Kavanagh (Editor), Amphotericin B and Nystatin, Analytical Microbiology, Vol. 2, Academic Press, New York, 1972, pp. 147—185.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR PHARMACOKINETIC STUDIES ON THE NEW ANTHRACYCLINE 4-DEMETHOXYDAUNORUBICIN AND ITS 13-DIHYDRO DERIVATIVE

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SUMMARY

The anthracyclines are a group of antitumoral antibiotics with significant clinical efficacy. Among the new anthracycline derivatives, 4-demethoxydaunorubicin showed interesting biological properties in terms of both spectrum of activity and therapeutic index and was recently introduced in clinical trials. The present paper describes the analytical method developed to investigate the pharmacokinetics of this derivative.

The method consists of the extraction of 4-demethoxydaunorubicin and its 13-dihydro metabolite from plasma with chloroform—1-heptanol (9:1) and re-extraction with 0.3 *M* phosphoric acid, separation by high-performance liquid chromatography and quantification by sensitive fluorescence detection.

Plasma level curves obtained from cancer patients treated with the drug are shown.

INTRODUCTION

The anthracycline antibiotics are a group of chemotherapeutic agents with significant antitumor activity.

Daunorubicin and doxorubicin are the best known drugs of this family. In the Research and Development Laboratories of Farmitalia Carlo Erba, new anthracycline analogues have been synthesized and tested in an attempt to obtain new anticancer agents with improved efficacy and therapeutic index. 4-Demethoxydaunorubicin (I) (Fig. 1) showed interesting biological properties [1—3] and is currently under extensive clinical investigation. In order to investigate the pharmacokinetic properties of this new compound in man, a specific, sensitive and accurate analytical method is required to measure con-

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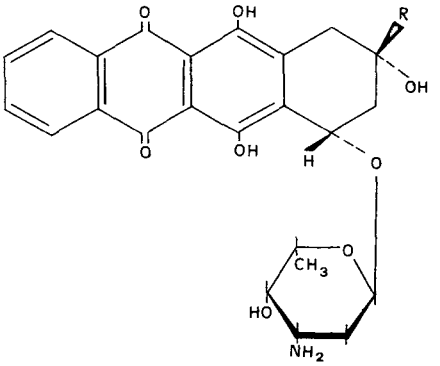

4-demethoxydaunorubicin (I) $R = COCH_3$

13-dihydro-4-demethoxydaunorubicin (II) $R = CH(OH)CH_3$

Fig. 1. Structure of 4-demethoxydaunorubicin (I) and of 13-dihydro-4-demethoxydaunorubicin (II).

centrations of the unchanged drug and of its main metabolite, the 13-dihydro derivative, in biological fluids.

This paper describes in detail this method and presents some results of its application on biological samples obtained from cancer patients treated with the drug at different dosage schedules.

The method is based on previous procedures reported by other workers [4, 5] in this field, using high-performance liquid chromatography (HPLC) coupled with a highly sensitive detection system such as ultraviolet (UV) or fluorimetric detection. The combination of highly efficient separation and highly sensitive detection proved capable of overcoming the difficulties of determining even very low concentrations of drug at long intervals after administration.

EXPERIMENTAL

Chemicals

Compound I, its 13-dihydro derivative (II) and doxorubicin (internal standard) were supplied by the Chemical Research and Development Laboratories of Farmitalia Carlo Erba. Desipramine chloride was from Prodotti Gianni (Milan, Italy); all other chemicals and solvents were from Farmitalia Carlo Erba (Analytical Division), used without further purification.

Instrumentation

A Spectra-Physics (Santa Clara, CA, U.S.A.) high-performance liquid chromatograph, Model SP 3500, equipped with a Rheodyne injection system, Model 7120, and a 170-μl sample loop (laboratory made) was used. The chromatographic detectors were a Laboratory Data Control (Riviera Beach, FL, U.S.A.) UV Monitor III, Model 1203 (cell volume 10 μl, path length 10 mm) at a fixed wavelength, and a Schoeffel (Westwood, NJ, U.S.A.) fluorimetric detector, Model FS 970 (5 μl cell volume). They were interfaced to a Spectra-

Physics laboratory data system, Model SP 4000, and the data were recorded on a terminal printer plotter SP 4050 (Spectra-Physics).

The column used was a μBondapak Phenyl (30 cm × 2 mm I.D., particle size 10 μm) (Waters Assoc., MA, U.S.A.). The column and the mobile phase were thermostatically controlled at 23 ± 0.5° C. A pellicular ODS precolumn (Whatman, Clifton, NJ, U.S.A.) was used.

All glassware was silanized before use by treatment with dichlorodimethylsilane—toluene (7:93) followed by washing with methanol and chloroform.

Drug administration

The drug was administered in distilled water intravenously, over a period of 1—5 min, or orally in capsules at doses of 15 mg/m^2 and 45 mg/m^2 body surface area, respectively.

Plasma samples from cancer patients

Samples of 5—7 ml (heparinized) of venous blood were collected before and at appropriate times after drug administration. These were immediately centrifuged, and the plasma was separated and frozen at —20° C until analysed.

Spiking of plasma samples

To determine the precision and accuracy of the method, blank plasma samples were spiked with appropriate amounts of the drug, its 13-dihydro derivative and the internal standard. These substances were dissolved as hydrochloride salts in distilled water containing 10 μg/ml desipramine. The solutions were prepared weekly and stored at 4° C. The stability of the substances was tested daily by HPLC analysis.

The samples were submitted to the analytical procedure described below.

Analytical procedure

Extraction. To 1-ml plasma samples, 10—40 ng of doxorubicin (internal standard) in 0.1 ml of water were added. The samples were carefully mixed with 2 ml of borate buffer, pH 8.4 (ionic strength, μ = 0.05) in a 15-ml glass-stoppered test tube and extracted with 10 ml of a chloroform—1-heptanol (9:1) mixture by mechanical shaking for 40 min. After centrifugation at 1200 *g* for 10 min the upper aqueous layer was removed by aspiration. The lower organic phase was transferred to another test tube and re-extracted with 0.3 ml of 0.3 *M* phosphoric acid, containing 10 μg/ml desipramine, in order to avoid adsorption losses, for 10 min. The aqueous phase was again transferred to a centrifuge test tube containing 2 ml of hexane and centrifuged. A 170-μl portion of the aqueous phase was injected into the chromatographic column.

Chromatographic procedure and quantitation. The mobile phase consisted of an isocratic mixture of acetonitrile—0.05 *M* KH_2PO_4 (35:65). The flow-rate was 0.4 ml/min. The fluorimeter was set at 254 nm excitation wavelength and 550 nm (filter) emission wavelength. Compounds I and II can also be determined using an UV detector with high sensitivity at a fixed wavelength of 254 nm.

Quantitation was obtained by peak area measurement with the computer integrator.

RESULTS AND DISCUSSION

Extraction procedures

The extraction of anthracyclines using chloroform and 1-pentanol or heptanol has been extensively studied [6, 7]. The degree of extraction depends greatly on the pH of the aqueous phase. For compounds I and II we found the optimum extraction was at pH 8.4, close to the optimum value (pH 8.6) for doxorubicin, used as internal standard. Interference from aglycone metabolites is avoided since these compounds are not re-extracted into the acidic phase.

Chromatographic separation

Separation of anthraquinone glycosides by liquid chromatography has already been described in detail [8]. On the basis of these studies and our own we found that the chromatographic system used gave good results regarding selectivity and efficiency.

We analyzed about 250 samples without any loss in the efficiency of the column. This long column life is due to the use of a precolumn which was refilled with new pellicular ODS every week.

To obtain the maximum detector response, we found that the best mobile phase was a mixture with a relatively high concentration of acetonitrile and a correspondingly low flow-rate.

Fig. 2A shows the chromatogram of a plasma extract from a cancer patient treated with compound I, whereas Fig. 2B shows the chromatogram of a blank plasma extract. No peaks corresponding to the retention times of the two compounds are present in this latter chromatogram.

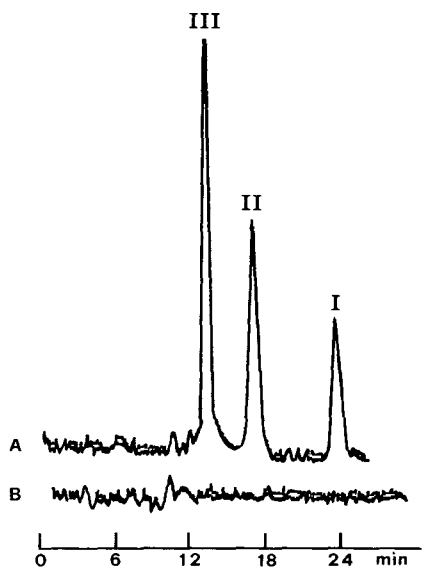

Fig. 2. Chromatograms of (A) plasma extract from a cancer patient 2 h after an intravenous dose (15 mg/m^2) of 4-demethoxydaunorubicin, and (B) blank plasma extract. Peaks: I = 4-demethoxydaunorubicin (8 ng/ml), II = 13-dihydro-4-demethoxydaunorubicin (12 ng/ml), III = doxorubicin (internal standard) (40 ng/ml). Chromatographic conditions are detailed in the text.

Selectivity and sensitivity

Compounds I and II can be determined by using both UV and fluorescence detectors because of their high absorbance at 254 nm. Thus, with a UV detector at a fixed wavelength of 254 nm we obtained the same results as with a fluorimetric detector in terms of sensitivity. We tried the UV detector for the determination of plasma levels, but the chromatograms of some samples showed peaks that interfered with the drug and its metabolite. These interferences were probably due to other drugs administered to patients together with compound I. To avoid these interferences we preferred the fluorimetric detector since it proved more selective.

The excitation wavelength used, 254 nm, gave better results in terms of response/noise ratio than the wavelength usually used, 470 nm.

Quantitative determination

The evaluation of compounds I and II is based on the use of the internal standard (doxorubicin) added in known amounts to the plasma samples. The relative weight ratio (RWR) values were obtained by assaying blank plasma samples spiked with known amounts of the two compounds, by the equation

$$\mathrm{RWR} = \frac{A_s}{A_{is}} \cdot \frac{C_{is}}{C_s}$$

where

A_s = peak area of I or II
A_{is} = peak area of internal standard
C_{is} = amount of internal standard in the sample (ng/ml)
C_s = amount of I or II in the sample (ng/ml).

The RWR values are constant for each compound in a given chromatographic condition and are used for the determination of unknown plasma samples according to the equation

$$C_s = \frac{A_s}{A_{is}} \cdot \frac{C_{is}}{\mathrm{RWR}}$$

The RWR values were 2.43 and 2.61, respectively, for compounds I and II. The coefficients of variation of the RWR values provide an estimate of the precision of the analytical method and were 5.95 and 6.3% (n = 20). The linearity gave regression coefficients of r = 0.995 and r = 0.997 for compounds I and II,

TABLE I

ACCURACY OF 4-DEMETHOXYDAUNORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
40.0	4	40.76	3.6
20.0	4	19.67	4.0
10.0	4	9.64	5.69
5.0	4	4.98	6.8
		Average:	5.1

TABLE II

ACCURACY OF 13-DIHYDRO-4-DEMETHOXYDAUNORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
40.0	4	40.21	3.3
20.0	4	20.13	2.41
10.0	4	9.94	3.44
5.0	4	5.01	8.58
		Average:	4.53

respectively, in the concentration range 5—30 ng/ml. The data for the accuracy test are given in Tables I and II; the coefficients of variation were 5.1% and 4.5%, for compounds I and II, respectively.

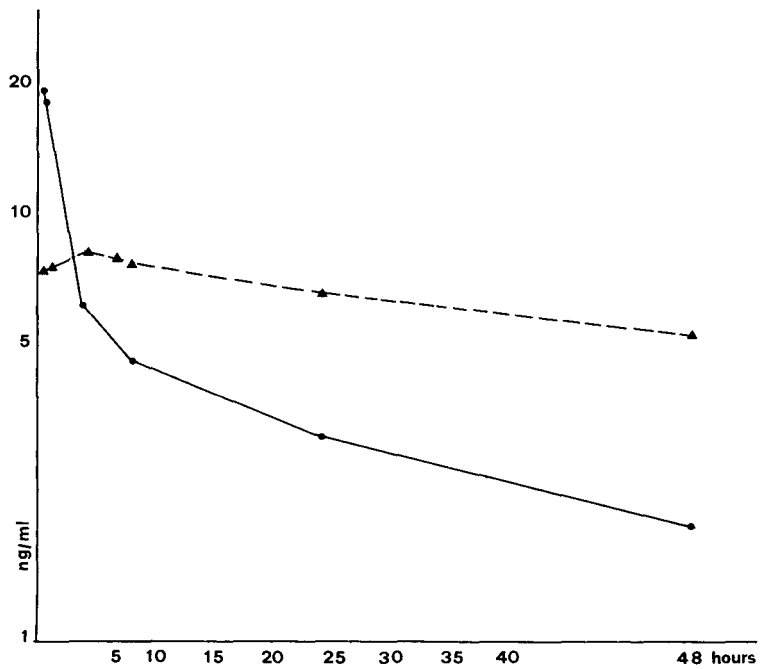

Fig. 3. Plasma levels of compounds I (•) and II (▲) in a cancer patient after intravenous administration of 15 mg/m² 4-demethoxydaunorubicin (I).

Plasma samples from patients

The method described here was applied to several plasma samples from cancer patients who received compound I. The plasma levels of the unchanged drug and of its 13-dihydro metabolite after intravenous (15 mg/m²) and oral (45 mg/m²) administration are given in Figs. 3 and 4, respectively. With this method the aim of measuring plasma levels of compounds I and II with a good degree of accuracy and high sensitivity has been achieved. Generally the sensiti-

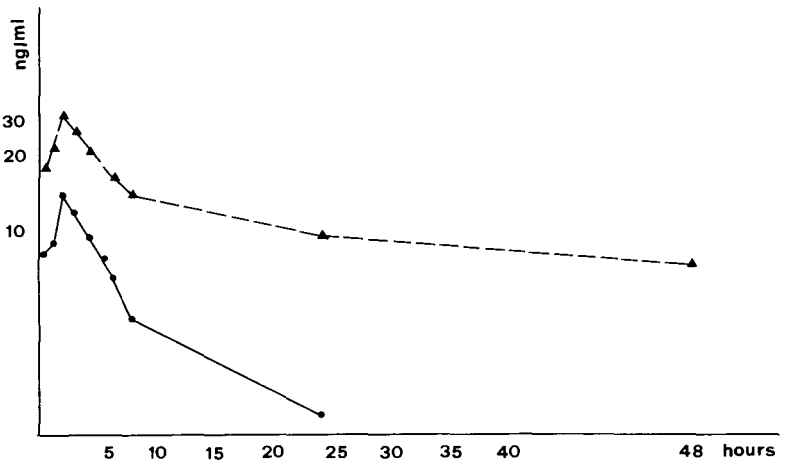

Fig. 4. Plasma levels of compounds I (•) and II (▲) in a cancer patient after oral administration of 45 mg/m² 4-demethoxydaunorubicin (I).

vity of the analytical method for anthracyclines is particularly important because of the unusual plasma level profiles of these drugs, which are characterized first by a rapid decrease to values below 10 ng/ml, followed by a very slow terminal phase with plasma half-live of about 1—2 days. This problem is particularly important with very potent anthracyclines such as compound I administered at doses substantially lower than doxorubicin, daunorubicin and 4′-epidoxorubicin.

REFERENCES

1 F. Arcamone, L. Bernardi, P. Giardino, B. Patelli, A. Di Marco, A.M. Casazza, G. Pratesi and P. Reggiani, Cancer Treatm. Rep., 60 (1976) 829.
2 A. Di Marco, A.M. Casazza and G. Pratesi, Cancer Treatm. Rep., 61 (1977) 893.
3 R. Supino, A. Necco, T. Dasdia, A.M. Casazza and A. Di Marco, Cancer Res., 37 (1977) 4523.
4 S. Eksborg, H. Ehrsson, B. Andersson and M. Beran, J. Chromatogr., 153 (1978) 211.
5 S. Eksborg, H. Ehrsson and I. Andersson, J. Chromatogr., 164 (1979) 479.
6 S. Eksborg, Clinical Pharmacology of Antineoplastic Drugs, Elsevier/North-Holland Biomedical Press, Amsterdam, 1978, p. 193.
7 E. Moro, M.G. Jannuzzo, M. Ranghieri, S. Stegnjaich and G. Valzelli, J. Chromatogr., 230 (1982) 207.
8 S. Eksborg, J. Chromatogr., 149 (1978) 225.

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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF MAJOR METAMIZOLE METABOLITES IN SERUM AND URINE

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SUMMARY

Thin-layer chromatographic (TLC) methods were developed for pharmacokinetic studies of major metamizole metabolites in serum and urine. These methods proved practicable, selective, accurate and sensitive with detection limits as follows: 4-methylaminoantipyrine 0.6 μg/ml serum and 2.0 μg/ml urine; 4-aminoantipyrine 0.16 μg/ml serum and 1.0 μg/ml urine; 4-acetylaminoantipyrine 0.14 μg/ml serum and 0.6 μg/ml urine; 4-formylaminoantipyrine 0.12 μg/ml serum and 1.0 μg/ml urine.

Samples of 250 μl suffice for TLC analysis. Following chromatographic separation, detection is performed in the ultraviolet range at 265 nm. Serum and urine levels were determined following a single oral dose of 1 g of metamizole sodium to eight volunteers. These results were compared with those following an accidental overdose of 49 g.

INTRODUCTION

Metamizole (Fig. 1) is an effective and widely used analgesic drug. The drug and its fate within the body have been characterized mainly by using radiolabelled material. Metamizole in solution rapidly undergoes hydrolysis [1] to yield 4-methylaminoantipyrine, which in man [2] is further metabolized to 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formylaminoantipyrine. Known metabolites account for approx. 77% of total radioactivity in serum

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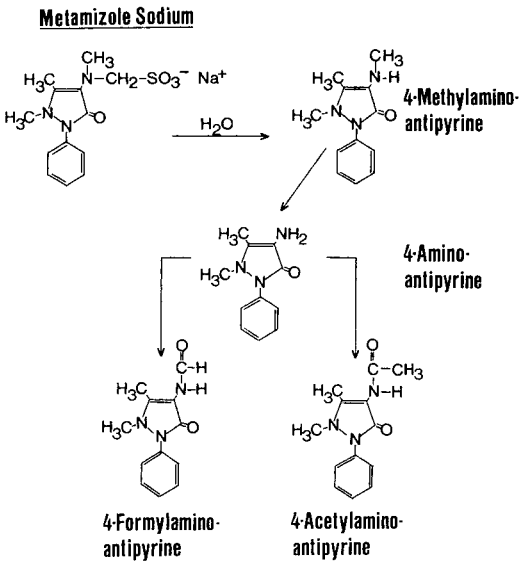

Fig. 1. Structure of metamizole sodium and metabolites.

(2—8 h post administration) and approx. 66% of total radioactivity in urine (0—24 h).

Biopharmaceutical and pharmacokinetic data in rat, dog and man were reported by Christ et al. [3]. Single oral doses of 480 mg of [^{14}C]metamizole administered to eleven human volunteers were rapidly and almost completely absorbed. The serum half-life was calculated from total radioactivity data as 7 ± 1 h up to 8 h post administration. The urinary half-life was 10 ± 1 h up to 4 days post administration. During that period, 90 ± 5% of the dose was renally excreted.

Further, it would be valuable to characterize the pharmacokinetics of commercial, i.e. non-labelled, preparations of the drug. Since for such purposes, large numbers of individual blood samples are usually required, it would be desirable to use small serum aliquots per analysis.

The present report describes selective thin-layer chromatographic (TLC) assays for 4-methylaminoantipyrine, 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formylaminoantipyrine in serum and urine. In one single extraction step, 250-μl samples are cleaned-up for chromatographic analysis.

EXPERIMENTAL

Reagents

The reagents used were buffer pH 10 AR, trichloroacetic acid AR (100 g/l), dichloromethane AR, diethyl ether AR, chloroform AR (freshly distilled), methanol AR and concentrated ammonia solution (25%) AR. The solvent system was chloroform—methanol—diethyl ether—concentrated ammonia (25:5:6:1).

Equipment

A Zeiss KM3 chromatogram spectrophotometer with microoptics and a Servogor^{R} 210 (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates F 254* (No. 5642, E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (No. 25254). For sample clean up and spotting, a Vortex^{R} mixer, a centrifuge, glass-stoppered tubes (approx. 8 ml), conical glass-stoppered tubes (approx. 8 ml) and a Desaga Autospotter^{R}** were used.

Sample preparation

Serum. In a glass-stoppered tube, 250 µl of serum were deproteinized with 50 µl of trichloroacetic acid solution. Following centrifugation (15 min), 100 µl of deproteinized serum were transferred to a second tube and treated with 1 ml of buffer pH 10. The mixture was extracted with 5 ml of dichloromethane for 30 sec on a Vortex mixer. The phases were separated by centrifugation and 4 ml of the organic phase were transferred to a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 µl of chloroform. Using the Autospotter, 70 µl were transferred onto the HPTLC plate as a series of small consecutive droplets, approx. 100 nl each.

Urine. In a glass-stoppered tube, 250 µl*** of urine were treated with 1 ml of buffer pH 10. The urine was extracted with 5 ml of chloroform for 30 sec on the Vortex mixer. The phases were separated by centrifugation (5 min) and 70 µl were transferred onto the HPTLC plate.

Chromatography

The twin-trough HPTLC developing chamber contained 20 ml of solvent in one compartment. The plate was developed in the dark without previous saturation over a distance of 7 cm. R_F values were: 4-methylaminoantipyrine 0.80, 4-aminoantipyrine 0.65, 4-acetylaminoantipyrine 0.50, and 4-formylaminoantipyrine 0.40.

Measurements were carried out in the direction of the solvent flow with an effective slit (microoptics) of 4.5 mm × 0.15 mm at a wavelength of 265 nm (Fig. 2), scanning speed 50 mm/min and paper speed 150 mm/min. Peak heights were evaluated and quantified by means of a calibration graph based on parallel analyses of standards on the same plate (Figs. 3 and 4).

RESULTS

Serum

The compounds were admixed to blank serum in five concentrations over the analytical ranges indicated in Table I. Each admixture was split into six

*As an advantage, the fluorescence indicator allows a fast overview of the chromatographic result before scanning. The baseline quality, as we experienced, is not markedly affected when extracts from biological samples are used.

**Modified version, Tygon^{R} tubes of larger diameter (Technicon, flow-rated, code 116-0549-09, white) and 60-cm long Hostaflon^{R} tubes were used.

***Samples of 100 µl were used for concentrations greater than 50 µg/ml urine.

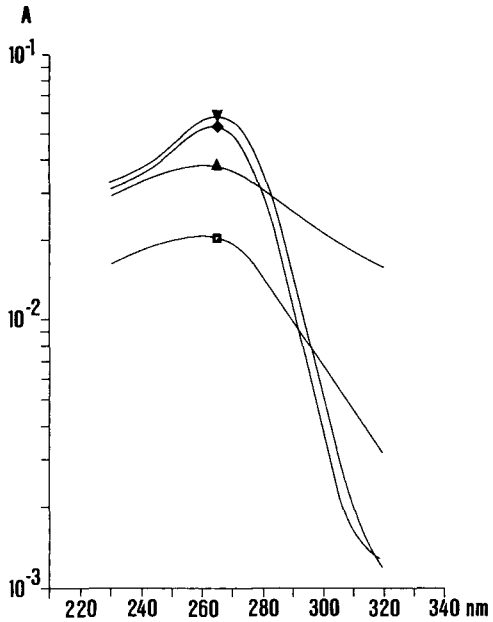

Fig. 2. In situ ultraviolet spectra on an HPTLC plate, 70 ng/spot: (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine; (◆), 4-acetylaminoantipyrine; (▼), 4-formylaminoantipyrine.

TABLE I

DETERMINATION OF METAMIZOLE METABOLITES IN SERUM BY TLC: RECOVERY AND ASSAY PRECISION

n = 6 determinations, concentration in μg/ml.

4-Methylaminoantipyrine		4-Aminoantipyrine		4-Acetylaminoantipyrine		4-Formylaminoantipyrine	
Added	Found	Added	Found	Added	Found	Added	Found
20.0	19.9 ± 0.2	5.00	5.1 ± 0.06	5.0	5.0 ± 0.06	5.0	5.0 ± 0.05
10.0	10.3 ± 0.4	2.0	1.9 ± 0.11	2.0	2.0 ± 0.08	2.0	2.1 ± 0.08
5.0	5.1 ± 0.3	1.00	0.95 ± 0.07	1.00	0.97 ± 0.11	1.00	1.07 ± 0.10
2.0	2.1 ± 0.3	0.50	0.53 ± 0.08	0.50	0.52 ± 0.04	0.50	0.51 ± 0.05
1.0	0.7 ± 0.2	0.25	0.26 ± 0.08	0.25	0.24 ± 0.03	0.25	0.22 ± 0.04
Blank	0	Blank	0	Blank	0	Blank	0

portions of 250 μl, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of an analytical method are selectivity, accuracy, precision and sensitivity. The corresponding parameters were derived from the analytical results given in Table I. As regards selectivity, the assay is free from interferences for all substances (Fig. 3). Accuracy was considered to be the deviation (bias) of the mean value of the results from the amount added. In the case of all substances, the average accuracy was < 30 ng/ml. Regression coefficients were greater than 0.999. Assay precision was defined in terms of the standard deviation (S.D.), which was constant in the concentration range considered. Sensitivity was expressed by the detection limit (D.L.) and was taken

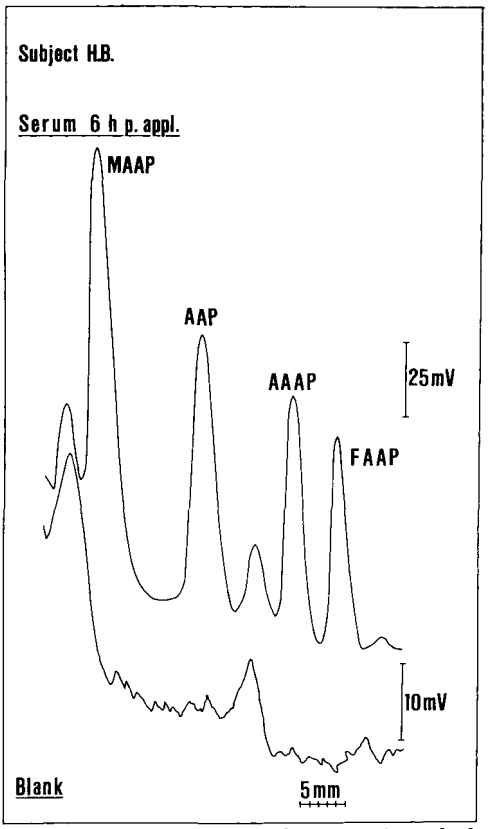

Fig. 3. Determination of serum 4-methylaminoantipyrine (MAAP) (3.2 μg/ml), 4-aminoantipyrine (AAP) (1.2 μg/ml), 4-acetylaminoantipyrine (AAAP) (1.2 μg/ml) and 4-formylaminoantipyrine (FAAP) (1.0 μg/ml) compared to a blank.

as precision × 2. Thus, for
4-methylaminoantipyrine, precision = 0.3 ± 0.1 μg/ml, D.L. = 0.6 μg/ml;
4-aminoantipyrine, precision = 0.08 ± 0.02 μg/ml, D.L. = 0.16 μg/ml;
4-acetylaminoantipyrine, precision = 0.07 ± 0.04 μg/ml, D.L. = 0.14 μg/ml;
4-formylaminoantipyrine, precision = 0.06 ± 0.03 μg/ml; D.L. = 0.12 μg/ml.

Urine

The compounds were admixed to blank urine in concentrations over the range 1—50 μl/ml urine. Each admixture was split into six portions of 250 μl, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of the method for urine were defined by the corresponding parameters abstracted from the analytical results given in Table II. As regards selectivity, the assay is free from interferences for all substances (Fig. 4). In the case of all substances, the average accuracy was < 0.2 μg/ml. Regression coefficients were greater than 0.999. Precision as defined in terms of the standard deviation (S.D.) was constant in the concentration range considered. Sensitivity was expressed by the detection limit (D.L.), i.e. precision × 2.

TABLE II

DETERMINATION OF METAMIZOLE METABOLITES IN URINE BY TLC: RECOVERY AND ASSAY PRECISION

n = 6 determinations, concentration in μg/ml.

4-Methylaminoantipyrine		4-Aminoantipyrine		4-Acetylaminoantipyrine		4-Formylaminoantipyrine	
Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)
50.0	50.3 ± 0.7	50.0	49.7 ± 0.5	50.0	49.5 ± 0.3	50.0	49.2 ± 0.3
20.0	19.6 ± 2.0	20.0	20.5 ± 1.1	20.0	20.0 ± 0.5	20.0	20.7 ± 1.3
10.0	9.3 ± 0.5	10.0	9.4 ± 0.6	10.0	10.4 ± 0.5	10.0	10.3 ± 0.5
5.0	5.4 ± 0.8	5.0	4.7 ± 0.5	5.0	5.3 ± 0.3	5.0	5.2 ± 0.6
2.0	2.2 ± 0.9	2.0	1.9 ± 0.2	2.0	2.0 ± 0.3	2.0	2.1 ± 0.4
1.0	0	1.0	1.1 ± 0.2	1.0	0.8 ± 0.2	1.0	0.8 ± 0.2
Blank	0	Blank	0	Blank	0	Blank	0

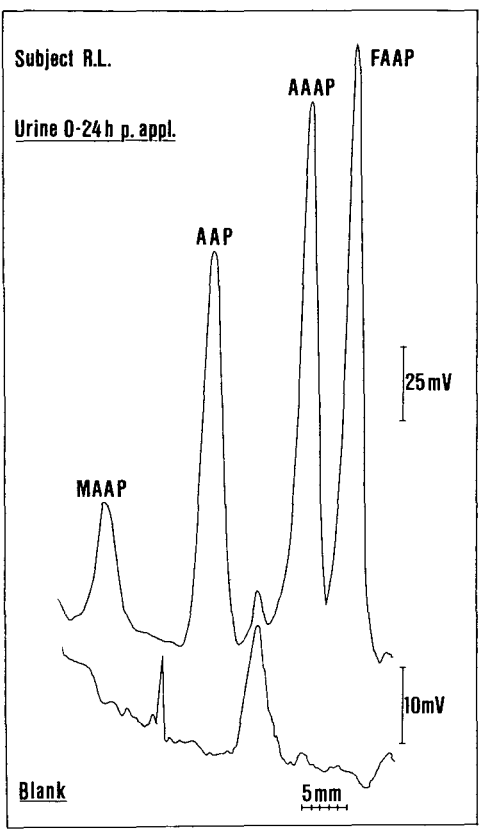

Fig. 4. Determination of urinary 4-methylaminoantipyrine (MAAP) (6.8 μg/ml), 4-aminoantipyrine (AAP) (23 μg/ml), 4-acetylaminoantipyrine (AAAP) (53 μg/ml) and 4-formylaminoantipyrine (FAAP) (58 μg/ml) compared to a blank.

Thus for

4-methylaminoantipyrine, precision = 1.0 ± 0.6 μg/ml, D.L. = 2.0 μg/ml;
4-aminoantipyrine, precision = 0.5 ± 0.3 μg/ml, D.L. = 1.0 μg/ml;
4-acetylaminoantipyrine, precision = 0.3 ± 0.1 μg/ml, D.L. = 0.6 μg/ml;
4-formylaminoantipyrine, precision = 0.5 ± 0.4 μg/ml, D.L. = 1.0 μg/ml.

Pharmacokinetics

Metamizole sodium was administered as a single oral dose of 1 g to eight human volunteers★. This dosage corresponds to that generally recommended in therapeutic use. Serum samples were obtained before and at 2, 6 and 24 h following treatment; urine was collected for 24 h.

Mean serum and urine concentrations are presented in Tables III and IV, respectively. The overall serum concentration up to 6 h post administration was dominated by levels of methylaminoantipyrine, later by those of acetyl- and formylaminoantipyrine.

TABLE III

MEAN SERUM CONCENTRATIONS FOLLOWING A SINGLE ORAL DOSE OF 1 g OF METAMIZOLE SODIUM

n = 8 volunteers, concentration in μg/ml, mean ± S.D.

h post dose	4-Methylamino-antipyrine	4-Amino-antipyrine	4-Acetylamino-antipyrine	4-Formylamino-antipyrine	Overall concentration (drug)
0	0	0	0	0	0
2	9 ± 2	1.0 ± 0.3	0.8 ± 0.6	1.0 ± 0.5	19 ± 3
6	5 ± 1	1.4 ± 0.8	1.8 ± 1.0	1.7 ± 0.7	15 ± 2
24	0.1 ± 0.1	0.2 ± 0.6	1.5 ± 0.6	0.8 ± 0.3	4 ± 1

TABLE IV

MEAN URINARY CONCENTRATIONS FOLLOWING A SINGLE ORAL DOSE OF 1 g OF METAMIZOLE SODIUM, 0—24 h COLLECTION PERIOD

n = 8 volunteers, concentration in μg/ml, mean ± S.D.

4-Methylamino-antipyrine	4-Aminoantipyrine	4-Acetylamino-antipyrine	4-Formylamino-antipyrine
7 ± 3	18 ± 15	95 ± 55	51 ± 19

From serum data, metabolite half-lives of approx. 3 h for 4-methylamino-antipyrine and approx. 6 h for 4-aminoantipyrine were calculated. Half-lives of 4-acetyl- and 4-formylaminoantipyrine were estimated as approx. 10—15 h (Fig. 5). An overall half-life of approx. 10 h was in accordance with ^{14}C-data from Christ et al. [3].

★This study was performed by Drs. W. Rupp and M.J. Badian, Hoechst AG.

Mean areas under the serum curves up to 24 h post administration are given in Table V.

Within 24 h post administration, 35 ± 4% of the dose was accounted for in urine as the sum of excreted metabolites, predominantly acetyl- and formyl-

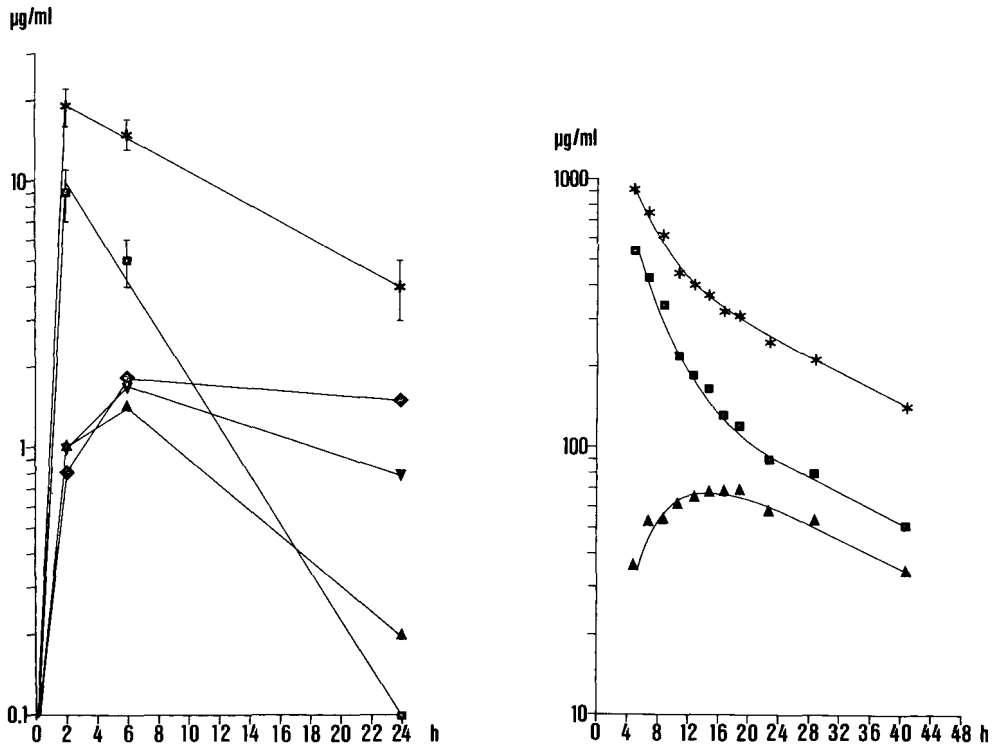

Fig. 5. Serum pharmacokinetics following a single oral dose of 1 g of metamizole sodium, mean serum levels of eight volunteers. (*), Molar sum expressed as metamizole sodium; (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine; (◆), 4-acetylaminoantipyrine; (▼), 4-formylaminoantipyrine.

Fig. 6. Serum pharmacokinetics following an accidental overdose of 49 g of metamizole sodium. (*), Molar sum expressed as metamizole sodium; (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine.

TABLE V

MEAN AREAS UNDER SERUM CURVES (AUC) 0—24 h, 1 g DOSE

n = 8 volunteers.

	AUC (μg h ml^{-1}) (mean ± S.D.)
4-Methylaminoantipyrine	85 ± 23
4-Aminoantipyrine	20 ± 11
4-Acetylaminoantipyrine	36 ± 17
4-Formylaminoantipyrine	29 ± 11
Overall metamizole sodium	255 ± 34

aminoantipyrine (Table VI). These findings correspond with data from Volz and Kellner [2].

Further, the analytical methods were applied in the case of attempted suicide by an 18-year-old girl. The extreme overdose of 49 g of metamizole sodium was well tolerated [4]. In contrast to the 1 g oral dosage, overall concentrations in serum and urine were dominated by methylaminoantipyrine and aminoantipyrine levels. The overall serum concentration of approx. 1000 μg/ml 5 h post administration decreased in two phases with half-lives of 2.5 h and 20 h (Fig. 6). Methylaminoantipyrine serum levels of 540 μg/ml 5 h post administration decreased with half-lives of 2.5 h and approx. 20 h reaching approx. 50 μg/ml after 48 h.

TABLE VI

MEAN CUMULATIVE URINARY EXCRETION 0—24 h, 1 g DOSE

n = 8 volunteers; mean ± S.D.

	Amount excreted (mg)	Percentage of dose
4-Methylaminoantipyrine	10 ± 4	1.5 ± 0.6
4-Aminoantipyrine	27 ± 23	4.4 ± 3.8
4-Acetylaminoantipyrine	135 ± 60	18.3 ± 8.2
4-Formylaminoantipyrine	76 ± 24	11.0 ± 3.5
Overall excretion as metamizole sodium	354 ± 44	35 ± 4

TABLE VII

CUMULATIVE URINARY EXCRETION 0—24 h, 49 g OVERDOSE

1 patient.

	Approx. amount excreted (g)	Percentage of dose
4-Methylaminoantipyrine	2.7	8.8
4-Aminoantipyrine	2.5	8.8
4-Acetylaminoantipyrine	0.9	2.7
4-Formylaminoantipyrine	0.7	2.2
Overall excretion as metamizole sodium	11	22

Maximum aminoantipyrine serum levels of 68 μg/ml were observed between 15 and 19 h post administration. They declined by the same terminal half-life of approx. 20 h, reaching approx. 35 μg/ml after 48 h. Acetylaminoantipyrine

and formylaminoantipyrine serum levels were below 6 μg/ml. Within 24 h post administration, approx. 22% of the dose was accounted for in urine as the sum of excreted metabolites (Table VII).

The overall urinary excretion up to 24 h may be assumed to contain at least the same percentage of dose absorbed as after the 1 g dose. Therefore in the case of the 49 g overdose, an absorbed fraction of at least 30 g of metamizole sodium may be assumed.

CONCLUSION

Metamizole assay in serum and urine was carried out to demonstrate quantitative TLC as a practicable, selective and reliable analytical tool adequate for drug/metabolite research in the therapeutic range and for handling samples from acute overdosing, where unexpected effects may occur and fast results are needed.

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REFERENCES

1 F. Pechtold, Arzneim.-Forsch., 14 (1964) 1056.
2 M. Volz and H.M. Kellner, Brit. J. Clin. Pharmacol., 10 (1980) 299S.
3 O. Christ, H.M. Kellner, G. Ross, W. Rupp and A. Schwarz, Arzneim.-Forsch., 23 (1973) 1760.
4 H. Wolhoff, G. Altrogge, W. Pola and N. Sistovaris, Deut. Med. Wochenschr., in press.

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Note

Direct analysis of free fatty acids in bacteria by gas chromatography

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Fatty acids in the range $C_{4:0}$ to $C_{12:0}$ are synthesized in the mammary glands; fatty acids with longer chain-lengths are present in animal tissues, especially in brain and the nervous system, and in bacteria. In bacteria, whole cells are increasingly being used for chemotaxonomic purposes. Within bacterial cells the fatty acids may occur free or in the form of glycerides. Most acids, however, are linked to large molecules such as phospholipids, glycolipids, lipoproteins, lipopolysaccharides, lipotechoic acid. Unfortunately, there is no universally accepted method for extracting fatty acids from bacteria, and a series of techniques, some of them quite complex, are being used [1]. In extraction, a dilemma is presented to the biochemist: if the technique is gentle enough, the lipids obtained will probably have undergone relatively minor degradation; however, a certain portion of the total lipids may be missing. On the other hand, if the extraction technique is rigorous enough to obtain all or most of the total cellular lipids, most of them may have become degraded [2].

At present, "bound" fatty acids, which require acid or alkaline hydrolysis to be released, are most commonly used in chemotaxonomy. After hydrolysis, these acids are derivatized before gas chromatographic analysis. Since both the hydrolysis and derivatization procedures may produce artifacts, we feel that free fatty acids should be increasingly used for taxonomic purposes. These acids may be quite specific and differ markedly from "bound" lipids in bacteria such as *Pseudomonas*, *Alcaligenes*, *Moraxella*, and *Neisseria* [2].

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In the present study, the free fatty acids of whole cells of the taxonomically closely related *Haemophilus aphrophilus* and *Actinobacillus actinomycetemcomitans* were extracted with hexane in a Soxhlet apparatus and analyzed directly without derivatization in a gas chromatograph. In general, free fatty acids are difficult to analyze directly by gas chromatography due to the broad and often tailing peak shape. Even if this can be overcome by esterification, the derivatization step is time-consuming and in some cases may result in non-quantitative recovery [3]. So far, the best peak symmetry of free fatty acids seems to have been obtained by using glass microbeads covered with FFAP (free fatty acid phase) stationary phase, but even on this column the higher acids gave relatively broad peaks with low resolution [4].

In the present paper emphasis will be placed on methodology, while comparative fatty acid profiles of all the bacterial strains investigated will be detailed elsewhere.

MATERIAL AND METHODS

Bacteria

Two reference strains (ATCC 33389, 19415) and two laboratory strains (FDC 655, 654) of *H. aphrophilus* and two reference strains (ATCC 33384, 29523) of *A. actinomycetemcomitans* were cultivated in Brain Heart Infusion® broth (Difco Laboratories, Detroit, MI, U.S.A.) in air plus 10% carbon dioxide for 5 days at 37° C.

Synthetic fatty acids

The following synthetic fatty acids were chromatographed: caproic ($C_{6:0}$) and caprylic ($C_{8:0}$) acid (E. Merck, Darmstadt, G.F.R.), nonanoic ($C_{9:0}$) acid (Koch-Light Laboratories, Colbrook, Great Britain), capric ($C_{10:0}$) and undecanoic $C_{11:0}$) acid (Fluka, Buchs, Switzerland), and lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and stearic ($C_{18:0}$) acid (Sigma Co., St. Louis, MO, U.S.A.).

Extraction of free whole-cell fatty acids

Bacterial cells were harvested by centrifugation, washed three times with deionized distilled water and lyophilized. Extraction of lyophilized material was performed two times with fresh *n*-hexane (Merck) in an all-glass Soxhlet-type apparatus furnished with a refluxing Liebig water condenser, each time for 3–4 h. Extended extraction times did not result in higher yields. The extracts were pooled, dried and stored at −20° C in an oxygen-free atmosphere. The reproducibility of the total analysis was determined by the reproducibility of the extraction steps, which was found to be ± 5%. By standard addition of $C_{16:0}$ to the *H. aphrophilus* 654 cell strain, a recovery of 90% was obtained.

Gas chromatography

A Carlo Erba 4200 (Carlo Erba, Milan, Italy) gas chromatograph equipped with a CP-Sil 5 glass capillary column was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 (polydimethylsiloxane) column was 25 m × 0.22 mm

I.D., 0.14 μm film thickness and 0.25 mm HETP. Helium was used as carrier gas at 2 ml/min. The pressure at the inlet of the column was 151.5 kPa.

RESULTS

The gas chromatogram of a series of saturated and unsaturated synthetic fatty acids is shown in Fig. 1. Analogous acids exist as cellular constituents, or are produced as metabolic products by a wide spectrum of bacteria. The synthetic fatty acids were eluted successively according to increasing chain length. The $C_{16:0}$ acid followed after $C_{16:1}$. In Fig. 2 separation of these two major bacterial fatty acids are shown at various constant temperatures. The techniques described above provided excellent separation of free whole-cell fatty acids recovered from *H. aphrophilus* and *A. actinomycetemcomitans*. The fatty acid profiles of two representative strains are presented in Fig. 3. A high temperature (210°C) was chosen in order to be able to include higher fatty acids, if present.

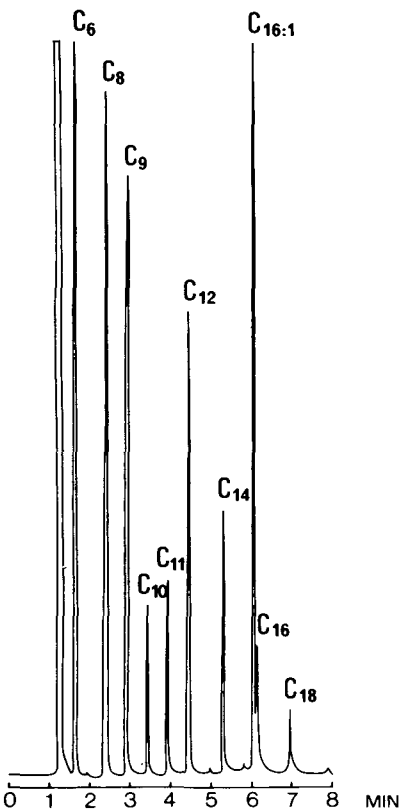

Fig. 1. Gas chromatogram of a mixture of saturated and unsaturated synthetic fatty acids. Program: hold 1 min at 110°C, then 110 to 290°C at 30°C/min. Injector, 240°C. Flame ionization detector, 320°C. Split injection, 1/100. Paper speed, 10 mm/min. Sample injected in hexane, 0.4 μl. Attenuator, 8.

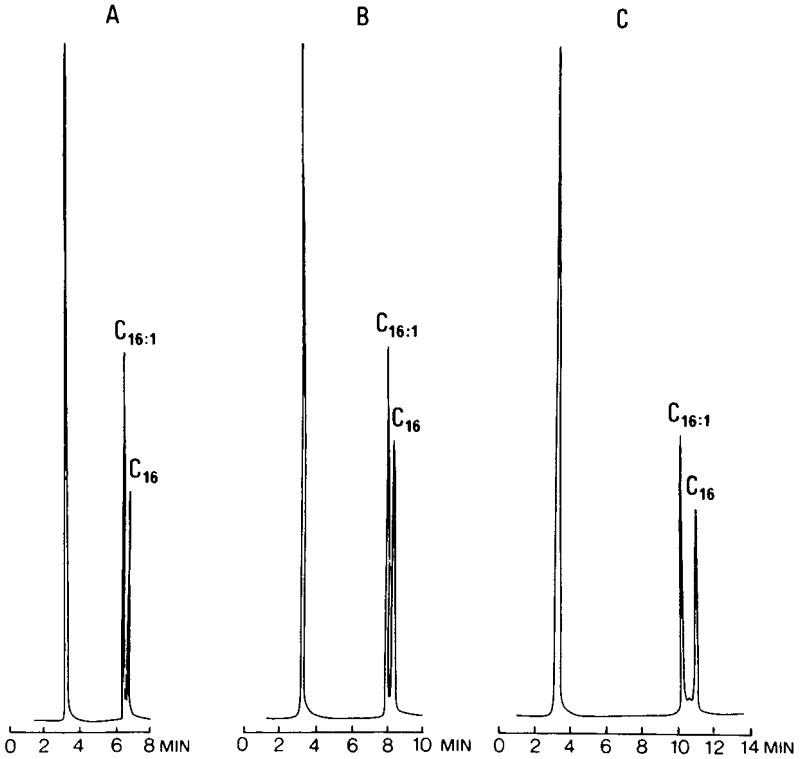

Fig. 2. Gas chromatograms showing separation of $C_{16:1}$ and $C_{16:0}$ fatty acids. (A) Isothermal, 190°C; (B) isothermal, 180°C; (C) isothermal, 170°C. Otherwise chromatographic settings were as given in Fig. 1, except the paper feed rate (5 mm/min).

DISCUSSION

In bacteriology, Soxhlet extraction has previously been performed with *Sarcina lutea* using benzene—methanol as solvent [5] and with *P. maltophilia* using chloroform—methanol [6]. The present study with *H. aphrophilus* and *A. actinomycetemcomitans* suggested that Soxhlet extraction is a simple method for removing taxonomically important long-chain fatty acids from whole bacterial cells, and that it deserves wider application in chemotaxonomy. The type of association between the lipids and the other cellular constituents determines the solvent to be used. Thus, non-polar solvents are used for extraction of neutral lipids, polar solvents for polar lipids, and after hydolysis appropriate solvents are used to extract "bound" lipids. The purpose of using a nonpolar solvent such as hexane was to provide a relatively simple extract which included the free fatty acids to be compared. The possible presence of neutral lipids in the extract did not interfere with the determination of the free fatty acids in the bacterial strains examined.

The general gas chromatographic problem of broad tailing peaks of free fatty acids was solved by using a thin film of a high-temperature non-polar stationary phase (CP-Sil 5). Extracted saturated ($C_{14:0}$—$C_{16:0}$) and unsaturated ($C_{16:1}$) whole-cell fatty acids, as well as a mixture of synthetic acids ($C_{6:0}$—

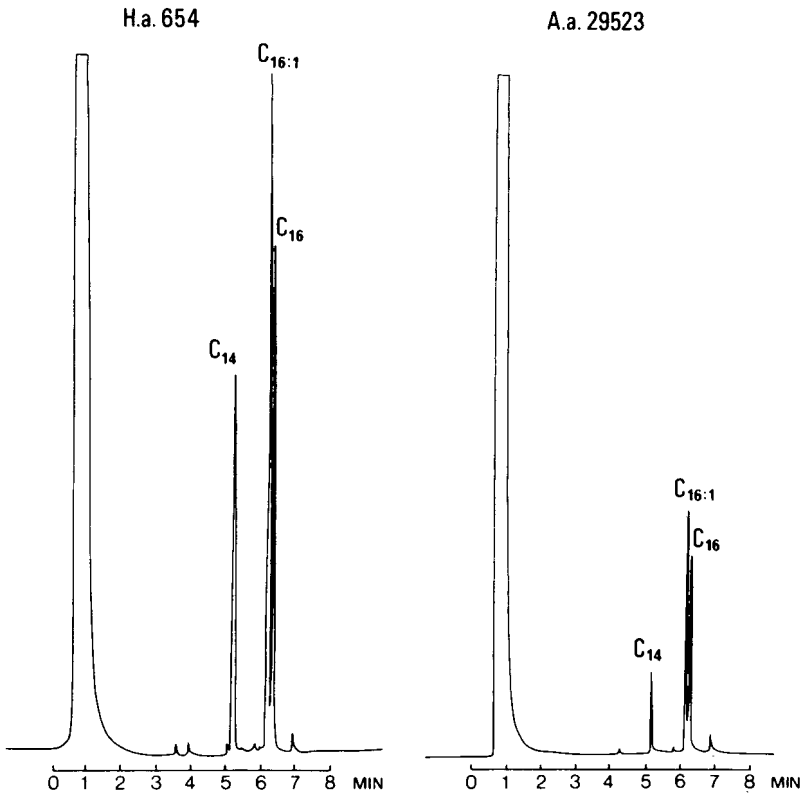

Fig. 3. Typical gas chromatograms of free fatty acids in *Haemophilus aphrophilus* (H.a.) and *Actinobacillus actinomycetemcomitans* (A.a.). Isothermal, 210°C. Injector temperature, 240°C. Flame ionization detector, 320°C. Split injection, 1/100. Sample injected in hexane, 0.4 µl. Paper speed, 10 mm/min. Attenuator, 8.

$C_{18:0}$), could be analyzed directly without derivatization. This may represent a significant improvement to or supplement current procedures of gas chromatography of fatty acids as applied in microbial taxonomy. Our method has now been used for fatty acid analysis of a series of strains of *H. aphrophilus* and *A. actinomycetemcomitans* (Brondz and Olsen, unpublished results) and should be well fitted for routine use in clinical microbiological laboratories due to its simplicity and high sensitivity. The wide spectrum of acids separated suggests that the present method is also applicable to acidic fermentation products. It may also be used to determine fatty acids in other living matter such as animals and plants.

CONCLUSIONS

Whole cells of *H. aphrophilus* and *A. actinomycetemcomitans* were Soxhlet extracted with hexane and the content of free fatty acids determined directly, without derivatization, by gas chromatography on a non-polar fused-silica column. A series of authentic standards ($C_{6:0}$—$C_{18:0}$, $C_{16:1}$) was also analyzed. Both saturated and unsaturated acids were recovered and determined with good

precision, without measurable interference. The present method may represent an important improvement on or supplement current procedures of gas chromatography of bacterial fatty acids as applied in chemotaxonomy.

REFERENCES

1 D.B. Drucker, Microbial Applications of Gas Chromatography, Cambridge University Press, Cambridge, London, 1981, p. 183.
2 M.P. Lechevalier, Crit. Rev. Microbiol., 5 (1977) 109.
3 G.C. Cochrane, J. Chromatogr. Sci., 13 (1975) 440.
4 L. Zoccolillo and M. Rinchetti, J. Chromatogr., 245 (1982) 321.
5 T.G. Tornabene, E. Gelpi and J. Oró, J. Bacteriol., 94 (1967) 333.
6 C.W. Moss, S.B. Samuels, J. Liddle and R.M. McKinney, J. Bacteriol., 114 (1973) 1018.

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Note

Separation of *erythro* and *threo* forms of alkane-2,3-diols from the uropygial gland of the quail by glass capillary column gas chromatography

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The uropygial or preen gland is the unique but large sebaceous gland which is present in birds. Its lipidic secretion is spread all over the plumage by the bill to prevent water penetration. It seems that the preen waxes play a role in the bacteriostatic and fungistatic protection of the bird's skin against micro-organisms. In addition, it has been found that the uropygial gland produces pheromones involved in sexual behaviour in ducks [1, 2].

Intensive chemical investigations of the composition of the lipidic secretion have been performed. It is now well established that the secretion consists of mono or diester waxes and is species-specific [3, 4]. In the quail and the Galliformes the lipidic secretion is mainly composed of alkane-2,3-diol diesters [5—8].

The present paper describes the separation and identification of *erythro* and *threo* forms of the alkane diols from the Japanese quail *Coturnix coturnix*

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Japonica by capillary gas–liquid chromatography (GLC) and mass spectrometry (MS).

MATERIALS AND METHODS

Animals

Male Japanese quails (*Coturnix coturnix Japonica*) were purchased from R. Salaun (Lannilis, France) and killed when 6—7 weeks old. Food (Guyomarc'h, Vannes, France) and water were offered ad libitum. Temperature and humidity were constant and lights were on a long-day cycle (18 h light—6 h darkness).

Reagents

The extraction solvents were obtained from E. Merck (Darmstadt, G.F.R.). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rotterdam, The Netherlands). 2-Bromododecane was obtained from Sigma (St. Louis, MO, U.S.A.). C_{14} to C_{34} *n*-alkanes were purchased from Fluka (Buchs, Switzerland).

Isolation and derivatization of diols

Animals were killed by cervical decapitation and the glands were quickly removed. Each gland was opened and washed with 20 ml of hexane. The separation into main lipid classes was performed by column chromatography. The extracts were applied directly to a 200 × 15 mm silica gel (70—200 mesh) chromatographic column. Three eluent fractions were obtained with 100 ml of hexane, *n*-hexane–diethyl ether (95:5, v/v) and diethyl ether–methanol (50:50, v/v). The homogeneity of all fractions was checked by thin-layer chromatography (TLC) on silica gel using precoated plates (HF 60/254 Merck) with *n*-hexane–diethyl ether–acetic acid (80:20:1, v/v) as mobile phase. The wax fraction was found in the hexane–diethyl ether eluate and its yield was 85% by weight of the total extract. Subsequently the wax fraction was subjected to alkaline hydrolysis in potassium hydroxyde and ethanol–benzene–water (5:2:1, v/v) at 80°C for 2 h. The unsaponifiable alcoholic fraction was extracted by 200 ml of hexane. TMS (trimethylsilyl ethers) derivatives were prepared by heating the alcoholic fraction with the mixture BSTFA–pyridine (20:1, v/v) for 1 h at 60°C and subjected to gas chromatography without further purification.

Synthesis of threo-2,3-dodecanediol from 2-bromododecane

2-Dodecene was prepared from 2-bromododecane according to the method of Sturtz and Rio [9]. After distillation, proton nuclear magnetic resonance (NMR) revealed the presence of two compounds: 15% of 1-dodecene and 85% of *trans*-2-dodecene. The olefins were oxidized without any further purification using osmium tetraoxide according to the following procedure: 100 mg of olefins were added to an equal weight of OsO_4 in the presence of *tert.*-butanol; a brown coloration appeared immediately and after 1 h 2 ml of H_2O_2 were added; the mixture was allowed to stand overnight, 35 ml of diethyl ether and 2 g of sodium sulfite were added and the mixture was allowed to stand 1 h; the aqueous layer was then discarded and the diethyl ether layer was evaporated.

The diols were submitted to TMS derivatization as described above and subjected to GLC and MS.

Gas—liquid chromatography

Analyses were carried out with a Carlo Erba 2150 (Milan, Italy) gas chromatograph equipped with a flame ionization detector.

The glass capillary column (SE-30 film thickness 0.4 μm; 39 m × 0.3 mm I.D.) was prepared in our laboratory as described by Berthou et al. [10, 11]. The carrier gas was hydrogen at a flow-rate of 2 ml/min. The gas chromatograph was operated under the following conditions: injector and detector temperatures 270°C, column temperature programmed from 130°C to 270°C at a rate of 4°C/min. Injections were carried out according to the split—splitless mode.

Analysis on a packed column was run with a glass column packed with Gas-Chrom Q with 2% OV-1 (3 m × 3 mm I.D.). Conditions for chromatography were the same as described for the capillary column.

Mass spectrometry

Mass spectra were run on a Ribermag R-10-10B (Rueil Malmaison, France) apparatus. Sample introduction was via the GC inlet OV-101 on a glass capillary column (50 m × 0.4 mm I.D.). The temperature was programmed from 130°C to 280°C at 4°C/min. The mass spectra were recorded at an ionization voltage of 70 eV.

RESULTS

Thin-layer chromatography on silica gel of preen secretion of the Japanese quail showed that the major component (85—90%) consisted of waxes and revealed the presence of more polar compounds identified as free fatty acids (unpublished data). The unsaponifiable fraction was converted to TMS ethers and submitted to gas chromatography. It was found to contain nine major doublets and a wide range of minor peaks (Fig. 1). Their structures were determined by mass spectrometry. The two components of doublets exhibited the same spectrum (Fig. 2); in the electron ionization a base peak at *m/z* M—117 resulted from the elimination of CH_3-CH_2-O-TMS. The other fragment ion produced from this cleavage was found at *m/z* 117 but was much weaker than M—117. The molecuar ion was never detected; the heaviest ion observed was M—15 which indicated loss of a CH_3 from the molecular ion. The mass spectrum showed a strong peak at *m/z* 73 characteristic of silyl ethers. These structural data, which are in agreement with those of Sawaya and Kolattukudy [8], suggested that such components were alkane-2,3-diols and that each doublet consisted of diastereoisomers.

In order to confirm the stereochemistry of the diols, a diastereoisomer was obtained by unequivocal synthesis. Oxidation of *trans*-2-dodecene, whose structure was ascertained by proton NMR, provided the *threo* isomer of dodecane-2,3-diol. When compared to the two dodecane diols from the biological extract, the synthetic isomer gave the same retention time as the second compound of the chromatographic doublet.

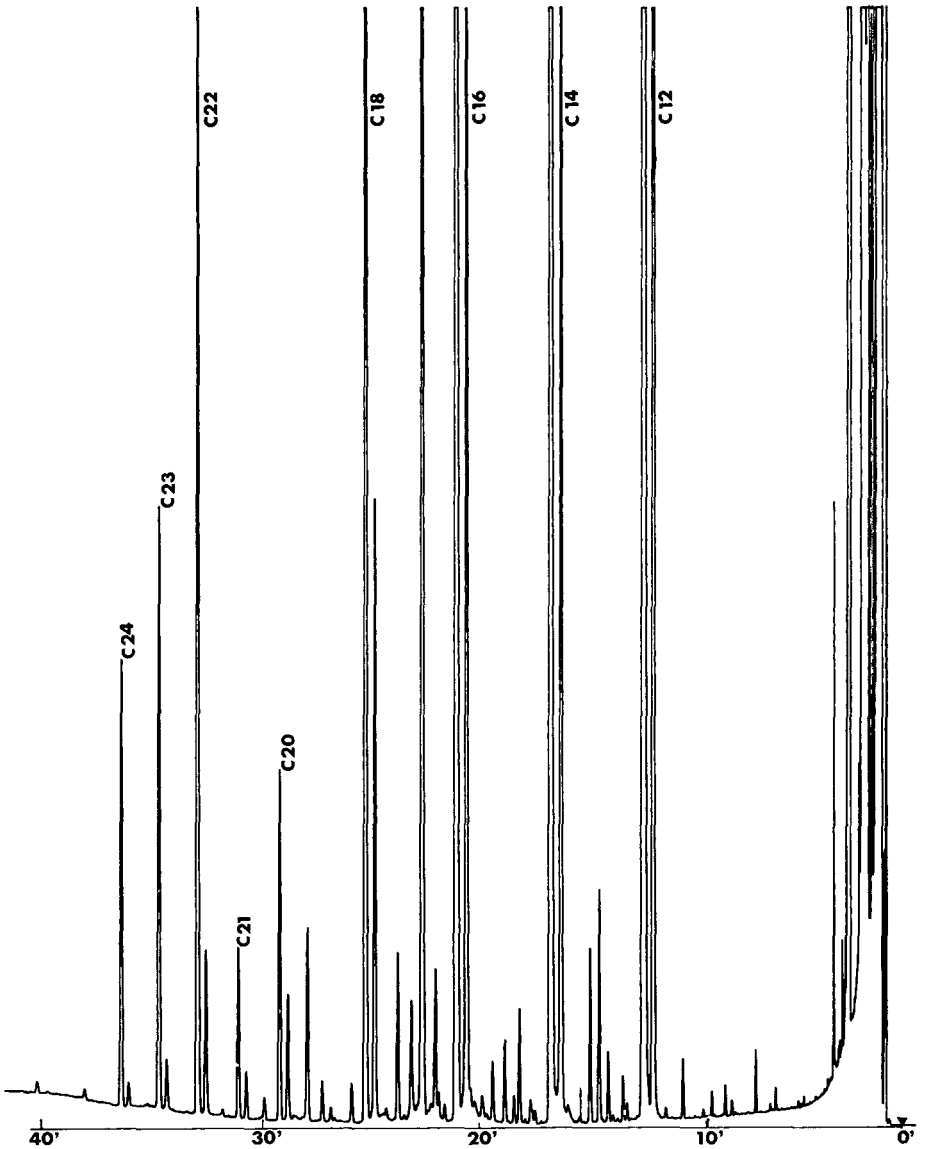

Fig. 1. Separation of alkane-2,3-diols as TMS derivatives by GLC with a glass capillary column. GLC conditons: SE-30 capillary column (39 × 0.3 mm I.D.), temperature programming from 130°C to 270°C at 4°C/min.

The methylene unit values were measured on the capillary column according to the method described by VandenHeuvel et al. [12]. Plots of methylene unit values against alkyl chain length are linear from diols C_{12} to C_{24}. The *erythro* and the *threo* forms of the diols gave two parallel lines. The difference in retention index between the *threo* and *erythro* isomers was shown to be about 20 units on SE-30.

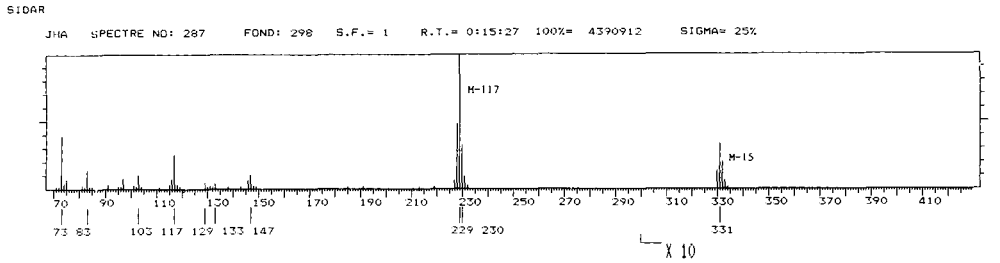

Fig. 2. Mass spectra of TMS ether of C_{12} diol (for experimental conditions see text).

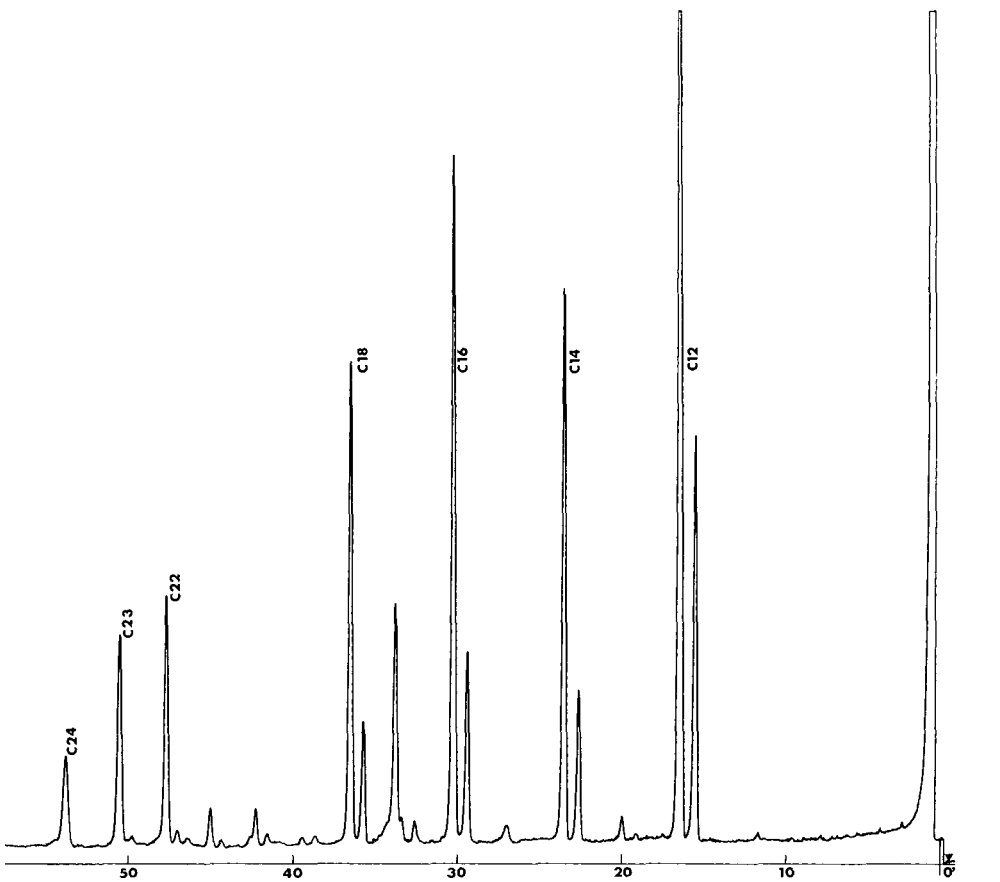

Fig. 3. Gas chromatogram of TMS derivatives on a packed column. GLC conditions: OV-1, 2% packed column (3 m × 3 mm I.D.), temperature programming from 130°C to 270°C at 4°C/min.

Ettre [13] has shown that the separation number TZ was related to the number of effective theoretical plates N by $TZ = a\sqrt{N} - 1$. The separation number between *erythro* and *threo* isomers was 5.4 for a complete resolution of 1.5. Accordingly the required N for $a = 1.5$ was only 1300 theoretical plates for a capacity factor of 5. Such an efficiency could easily be obtained on a

TABLE I

COMPOSITION (PEAK AREA %) OF TMS ETHER DIOLS FROM THE UROPYGIAL GLAND OF THE JAPANESE QUAIL

Values are means of observations on a group of 20 animals ± S.D. ($p < 0.05$).

Chain length	Percentage composition		
	Total	*Erythro* form	*Threo* form
C_{12}	37.75 ± 2.62	10.17 ± 0.77	27.58 ± 2.00
C_{13}	0.47 ± 0.05	0.13 ± 0.02	0.34 ± 0.04
C_{14}	12.80 ± 0.85	3.29 ± 0.30	9.51 ± 0.55
C_{15}	0.26 ± 0.03	0.08 ± 0.02	0.18 ± 0.05
C_{16}	14.84 ± 1.07	3.68 ± 0.30	11.16 ± 0.82
C_{17}	0.39 ± 0.04		0.39 ± 0.04
C_{18}	9.85 ± 0.96	2.24 ± 0.20	7.61 ± 0.79
C_{19}	0.32 ± 0.02	0.09 ± 0.01	0.23 ± 0.02
C_{20}	1.16 ± 0.12	0.30 ± 0.03	0.86 ± 0.10
C_{21}	0.69 ± 0.07	0.13 ± 0.02	0.56 ± 0.07
C_{22}	6.71 ± 0.76	0.49 ± 0.10	6.22 ± 0.67
C_{23}	4.45 ± 0.64	0.20 ± 0.05	4.25 ± 0.45
C_{24}	3.15 ± 0.46	0.17 ± 0.04	2.98 ± 0.43

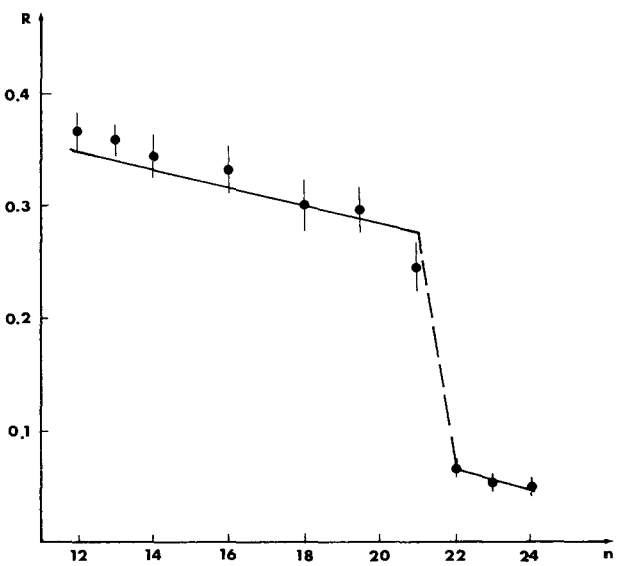

Fig. 4. Ratio of *erythro* to *threo* for each diol present in the wax esters from the uropygial gland of Japanese quail. Each value represents the mean ± S.E.M. of observations on a group of 20 male quails.

packed column. The *threo/erythro* separation was actually satisfactory as shown in Fig. 3. We found that the major advantage of using a capillary column was the shorter time required for the separation of all the diastereoisomers.

Table I shows the diol composition in the wax esters from the Japanese quail. The C_{12} diol was the major component followed by C_{14}, C_{16}, C_{18}, C_{22}, C_{23} and

C_{24} diols. Contents of odd-numbered diols were very small. Primary alcohols from C_{13} to C_{21} represented less than 5% of the unsaponifiable fraction. The percentage of erythro and threo diastereoisomers for each doublet was analyzed (Fig. 4). The ratio of *erythro* and *threo* forms was about 0.35 for the shorter-chain diols between C_{12} and C_{21}; but for chain lengths of C_{22} to C_{24} this ratio became very low; the *erythro* form of the diol was almost undetectable.

DISCUSSION

The TMS ethers of diols appeared to be very convenient derivatives for analysis by GLC. The preparation was simple and quick, no purification was required prior to injection and results could be reproduced easily.

As the *threo* and *erythro* isomers are not available, the assignment of the stereochemistry of the doublet was based upon the synthesis of the *threo* dodecane-2,3-diol. Moreover, the structure was confirmed by the chromatographic behaviour.

The *erythro* isomer of the diol had a retention time shorter than that of the *threo* form. The two diastereoisomers would not be expected to be well separated on a non-polar liquid phase such as SE-30. Advances towards understanding the separation mechanisms for diastereoisomers have shown that the chromatographic resolution depends upon the conformational immobility around a C—C bond with asymmetric centers [14]. The transformation of alkane-2,3-diols into TMS derivatives maximized the geometric differences between the two isomers. We assumed that the *threo* isomer of the diol presented a greater spatial bulk than the *erythro* form and that the surface interaction of the *threo* isomer with the stationary phase was then stronger than that of the *erythro* form and its retention index was therefore higher.

The major diols in the preen gland of the Japanese quail were found to be C_{12}, C_{14}, C_{16} and C_{18}. They were very different from those obtained by Sawaya and Kolattukudy [8], who found C_{22}, C_{23} and C_{24} as major components in the uropygial gland of the Japanese quail. Such a difference between our results and those of others could be due to the fact that those authors used GC in an isothermal mode (205°C) and that our results were obtained with a temperature programme from 130°C to 270°C. We assumed that the separation of *erythro* and *threo* forms would be better performed with a programmed temperature and that the shorter chains would be detected at lower temperatures.

Hansen et al. [15] suggested that the *erythro* and *threo* forms of the diols were synthesized by two distinct enzyme systems each producing one stereoisomeric series. According to the hypothetical pathway for biosynthesis of alkane-2,3-diols, a fatty aldehyde was condensed with hydroxyethylthiamine pyrophosphate to give an acyloin which was reduced to a diol [16]. We found that the proportion of the *erythro* form in each diol up to a chain length of C_{21} remained more or less constant; but for longer chains this proportion decreased dramatically. It was assumed that for long chains (more than C_{21}) the reduction step became difficult because of the chain length, and only the *threo* form could be synthesized. This would indicate that diols were produced by one enzyme system which was not stereospecific.

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REFERENCES

1 J. Jacob, J. Balthazart and E. Schoffeniels, Biochem. Syst. Ecol., 7 (1979) 149.
2 J. Balthazart and E. Schoffeniels, Naturwissenschaften, 66 (1979) 55.
3 J. Jacob and J. Poltz, Lipids, 10 (1975) 1.
4 J. Jacob, in P.E. Kolattukudy (Editor), Chemistry and Biochemistry of Natural Waxes, Elsevier, Amsterdam, 1976, p. 93.
5 E.R.O. Haahti and H.M. Fales, J. Lipid Res., 8 (1967) 131.
6 E. Edkins and I.A. Hansen, Comp. Biochem. Physiol., 39B (1971) 1.
7 M. Gamo and K. Saito, Comp. Biochem. Physiol., 39B (1971) 151.
8 W.N. Sawaya and P.E. Kolattukudy, Biochemistry, 11 (1972) 4398.
9 G. Sturtz and M.Y. Rio, Bull. Soc. Chim. Fr., (1974) 2180.
10 F. Berthou, D. Picart, L.G. Bardou and H.H. Floch, J. Chromatogr. Sci., 12 (1974) 662.
11 F. Berthou, Thèse de Doctorat en Sciences Physiques, Université de Bretagne Occidentale, Brest, 1977, pp. 18 and 63.
12 W.J.A. VandenHeuvel, W.L. Gardiner and E.C. Horning, J. Chromatogr., 26 (1967) 387.
13 L.S. Ettre, Chromatographia, 8 (1975) 281.
14 B.L. Karger, R.L. Stern, W. Keane, B. Halper and J. Westley, Anal. Chem., 39 (1967) 229.
15 I.A. Hansen, B.K. Tang and E. Eokins, J. Lipid Res., 10 (1967) 267.
16 P.E. Kolattukudy and T.J. Walton, Prog. Chem. Fats Other Lipids, 13 (1972) 119.

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Note

A new assay of monoamine oxidase by gas chromatography

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The enzymatic oxidation of amines in the monoamine oxidase (MAO) reaction is accompanied by the production of ammonia, hydrogen peroxide and the corresponding aldehyde from the amine under consumption of oxygen. It is still required to establish a rapid and specific method for the assay of MAO activity, which is generally applicable to all kinds of substrates. Polarographic measurement of oxygen consumption has been used, but it is not sensitive. Measurement of ammonia formed by colorimetric or fluorometric techniques has also been applied for this purpose [1]. But this method is not applicable for secondary amines such as epinephrine and metanephrine, since ammonia is not formed during the deamination by MAO, and methylamine is produced instead of ammonia. It is necessary, therefore, that the formation of hydrogen peroxide is measured.

Many methods are available for measurement of hydrogen peroxide by enzymatic colorimetry [2] or fluorometry [3] using peroxidase. The colorimetric method is generally not very sensitive. It would be desirable to have a more sensitive method for the determination of hydrogen peroxide in the study of biological systems. Fluorometric determination of hydrogen peroxide formed is sensitive, but is not applicable for catecholamines and serotonin which are preferential substrates for type A MAO [4, 5]. It is known that catalase converts methanol to formaldehyde through the action of hydrogen peroxide formed in this oxidation reaction [6]. This principle was applied to a gas chromatographic (GC) determination of hydrogen peroxide

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[7] and to a colorimetric determination of uric acid in serum and urine with uricase—catalase system [8], but has never been used for the MAO assay.

Pentafluorobenzyloxylamine (PFBOA) hydrochloride has been found to be an excellent reagent for derivatization of low-molecular-weight carbonyl compounds in GC [9, 10]. A condensation reaction of PFBOA and formaldehyde proceeds readily even in an aqueous solution at room temperature. We are not aware of any report dealing with catalase reaction for the determination of MAO activity. The present paper describes a reliable and sensitive GC method for assay of MAO activity by connecting the catalase reaction and the derivatization of formaldehyde with PFBOA.

EXPERIMENTAL

Reagents

PFBOA hydrochloride (melting point 115°C) was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) [9]. Iodobenzene was used as an internal standard. A 0.3% hydrogen peroxide solution was prepared by diluting a 30% stock solution of hydrogen peroxide (Merck Sharp and Dohme, Rahway, NJ, U.S.A.) with distilled water. The peroxide concentration was assayed by iodometry. The solution was stable for a month in a refrigerator.

Enzymes

Catalase (hydrogen-peroxide:hydrogen-peroxidase oxidoreductase, EC 1.11.1.6) (270,000 U/ml) was obtained from Boehringer, Mannheim, G.F.R. A stock solution (20,000 U/ml) was prepared by diluting it with distilled water.

For preparations MAO (monoamine:O_2 oxidoreductase, EC 1.4.3.4) male Wistar rats (150—200 g) were decapitated and their livers were homogenated in 6 volumes of pH 7.5, 50 m*M* phosphate buffer. The mitochondrial fraction was prepared by differential centrifugation by the method of Hogeboom et al. [11]. The mitochondria were washed once and resuspended in 50 m*M* potassium phosphate buffer and used as the MAO preparation. The enzyme protein content was estimated by the method of Lowry et al. [12] and was prepared to a concentration of 10 mg/ml.

Apparatus and conditions

A Shimadzu GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector (ECD) and a 2-m glass column packed with 3% XE-60 (Wako Junyaku, Osaka, Japan) on 80—100 mesh Celite 545 (AW DMCS) was used, with a column temperature of 90°C, a detector temperature of 150°C, and an injection temperature of 150°C.

Standard procedure for the assay of MAO activity

To a 10-ml test tube are added 0.25 ml of 8 m*M* substrate, 0.10 ml of 50 m*M* phosphate buffer pH 6.8, 0.20 ml of methanol, 0.10 ml of catalase aqueous solution (20,000 U/ml), 0.25 ml of PFBOA hydrochloride aqueous solution (1 mg/ml), and 0.10 ml of MAO preparation containing an amount of enzyme to give a peak height ratio to the internal standard of between

one and two. The reagents were added in this order and the reaction mixture (total volume 1.0 ml) was shaken for 30 min at 37°C in air. The standard incubation mixture contained 2 m*M* substrate, 20% methanol, 2000 units of catalase and about 30 times as much PFBOA as would be needed for binding 1 μg of formaldehyde formed. After incubation, one drop of 18 *N* sulfuric acid and 1 ml of *n*-hexane containing 3.5 μg of iodobenzene as internal standard were added to the reaction solution, followed by saturation with sodium chloride, and the resulting O-pentafluorobenzyloxime (O-PFBO) was extracted. An aliquot of the extract was diluted 25-fold with *n*-hexane, and 1 μl of the solution was injected on to the column.

RESULTS AND DISCUSSION

The principle of this method using methanol and catalase is as follows:

$$\text{Substrate} \xrightarrow[O_2,\ H_2O]{\text{MAO}} \text{oxidation product} + NH_3\ (\text{or } RNH_2) + H_2O_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{catalase}} HCHO + 2\,H_2O$$

$$HCHO + \text{PFBOA} \longrightarrow \text{F-C}_6\text{F}_4\text{-CH}_2\text{ON=CH}_2 \longrightarrow \text{GC (ECD)}$$

In a preliminary experiment, benzylamine as substrate was incubated with MAO preparation, and then methanol, catalase and PFBOA were added to the reaction solution, and the mixture was allowed to stand for 1 h at room temperature. The resulting O-PFBO derivative of formaldehyde was extracted with *n*-hexane and an aliquot of the extract was subjected to GC. But no peak corresponding to formaldehyde appeared on the gas chromatogram. The cause of the failure was found to be a small amount of catalase existing in the crude MAO preparation used, which had decomposed the hydrogen peroxide formed by MAO reaction before the addition of methanol and catalase.

When methanol and catalase were added to a sample solution containing substrate before starting the MAO reaction, the hydrogen peroxide formed by MAO reaction immediately reacted with methanol to convert it to formaldehyde, and we succeeded in obtaining a sharp peak corresponding to formaldehyde on the gas chromatogram. Thus, a one-step procedure was established to permit the three reactions, MAO reaction, catalase reaction and condensation reaction with PFBOA, simultaneously.

The MAO activities in the rat liver mitochondria were measured according to this standard procedure, using benzylamine or β-phenylethylamine for type B MAO, norepinephrine and serotonin for type A MAO, and dopamine for the two types of MAO. The peak height formed on the gas chromatogram showed a linear relationship with up to 60 min of MAO incubation time, using each of the substrates. The calibration curves of MAO activity against the

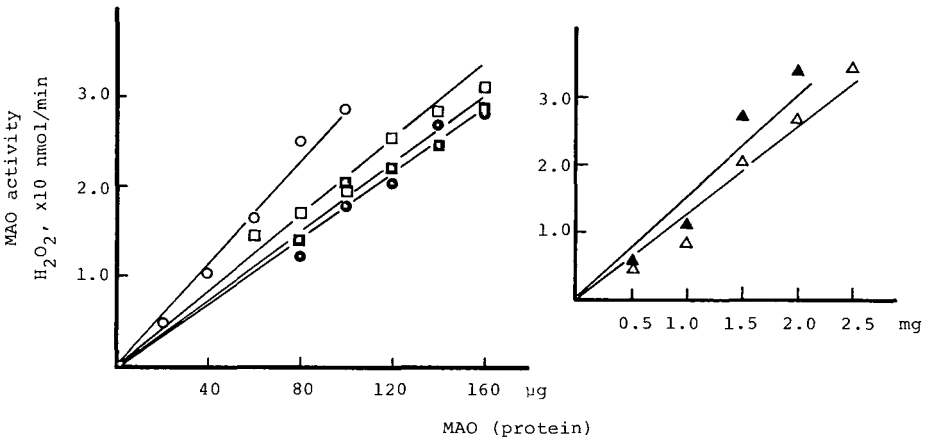

Fig. 1. Relationship between the amount of hydrogen peroxide formed by MAO and the enzyme concentration. Benzylamine (○——○), β-phenylethylamine (●——●), serotonin (□——□), normetanephrine (■——■), norepinephrine (△——△), or dopamine (▲——▲) was used as substrate. Incubations were carried out for 30 min at 37°C with increasing amounts of rat liver mitochondria.

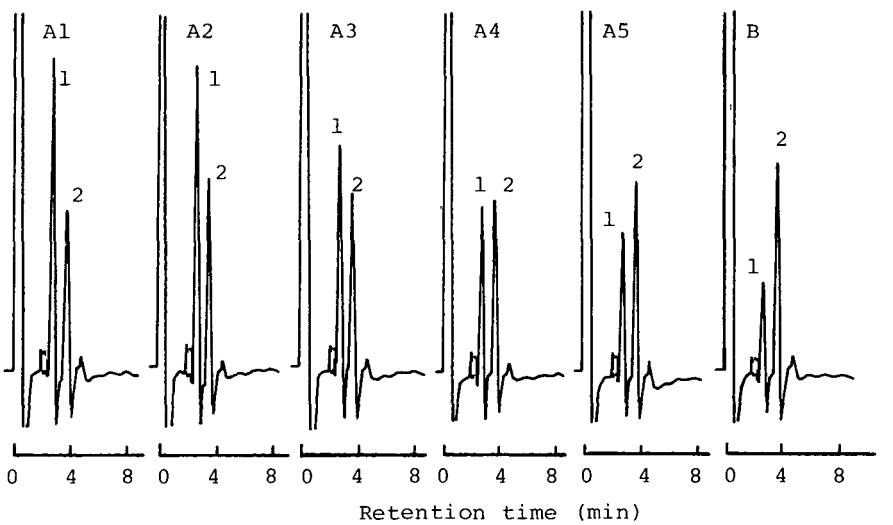

Fig. 2. Typical gas chromatograms obtained by assays using five different MAO concentrations (A1 = 100, A2 = 80, A3 = 60, A4 = 40, A5 = 20 µg of protein; B = no-enzyme blank) and benzylamine as substrate. Peaks: 1 = HCHO-O-PFBO, 2 = internal standard (3.5 µg of iodobenzene). Conditions: 3% XE-60, 2 m, 90°C, ECD.

enzyme concentration are shown in Fig. 1. Linear curves passing through the origin were obtained with all substrates. Fig. 2 illustrates a set of six chromatograms obtained for the calibration curve using benzylamine as substrate. Chromatogram B is of the blank, the product in an incubation without MAO preparation. Iodobenzene was used as a suitable internal standard. The O-PFBO derivative of formaldehyde was very volatile, and the separation could be carried out at a low temperature of 90°C. The O-PFBO derivative was also very sensitive to ECD. The peak in chromatogram A5 (Fig. 2) corresponded

to about 50 ng of hydrogen peroxide formed in the MAO reaction (plus about 50 ng of the blank value in B equals about 100 ng) in the reaction solution, which corresponded to an injection of about 2 pg of formaldehyde.

The precision of the method was determined by five repeated assays on an identical MAO preparation. Standard deviations were 5.9% for benzylamine using 60 μg of protein, 2.9% for β-phenylethylamine using 120 μg of protein, 5.0% for normetanephrine and 4.4% for serotonin using 120 μg of MAO protein. As compared to the substrates mentioned above, the results with dopamine or norepinephrine as substrate were worse in accuracy and sensitivity, probably due to oxidative decomposition by hydrogen peroxide of the catechol ring in the catecholamines. However, it was possible to determine MAO activity using catecholamine or serotonin as substrate. We are now investigating an effective way to protect the catecholamines against oxidation by hydrogen peroxide during MAO incubation.

There is one more problem in the present assay, namely that blank values were found to be slightly high and to correspond to about 50 ng of hydrogen peroxide in the reaction mixture (see B in Fig. 2), so the sensitivity of this MAO assay was about 2 nmol of hydrogen peroxide formed, though the O-PFBO derivative of formaldehyde was extremely sensitive to ECD. The origin of the high peak in the blank is not evident. It may be caused by formaldehyde in air and solutions derived from smoking. As a matter of fact, the blank value was reduced to about half by prohibiting smoking in the laboratory. Reducing blank values is also one of the subjects for a future study.

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REFERENCES

1 M. Harada and T. Nagatsu, Anal. Biochem., 56 (1973) 283.
2 G.G. Guilbault and D.N. Kramer, Anal. Chem., 36 (1964) 2494.
3 G.G. Guilbault, D.N. Kramer and E. Hackley, Anal. Chem., 39 (1967) 271.
4 G.G. Guilbault, P.J. Bringnac, Jr. and M. Juneau, Anal. Chem., 40 (1968) 1256.
5 S.H. Synder and E.D. Hendley, J. Pharmacol. Exp. Ther., 163 (1968) 386.
6 P. Nicholls and G.R. Schonbaum, in P.D. Boyer, H. Lardy and K. Myrback (Editors), The Enzymes, Vol. 8, Academic Press, New York, 1963, p. 147.
7 K. Kobayashi and S. Kawai, J. Chromatogr., 245 (1982) 339.
8 N. Kageyama, Clin. Chim. Acta, 31 (1971) 421.
9 K. Kobayashi, M. Tanaka and S. Kawai, J. Chromatogr., 187 (1980) 413.
10 K. Kobayashi, E. Fukui, M. Tanaka and S. Kawai, J. Chromatogr., 202 (1980) 93.
11 G.H. Hogeboom, W.C. Schneider and G.E. Pallade, J. Biol. Chem., 172 (1948) 619.
12 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.

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Note

Reversed-phase ion-pair chromatography of amino acids

Application to the determination of amino acids in plasma samples and dried blood on filter papers

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High-performance liquid chromatographic separation of amino acids has been mainly performed by the use of ion-exchange chromatography [1] coupled to reaction with ninhydrin [2], fluorescamine [3] and *o*-phthalaldehyde, followed by ultraviolet absorbance or fluorescence detection. In comparison with partition chromatography, however, ion-exchange chromatography entails problems with column efficiency, the long equilibrium time until the next analysis, the high cost of columns and the necessity of a complicated stepwise gradient elution technique using different kinds of solvents for good separation.

Reversed-phase chromatography has been also used for the same purpose. But this method requires pre-column derivatization with phenylisothiocyanate [4], or 1-N,N-dimethylaminonaphthalene-5-sulfonyl chloride [5, 6].

Ion-pair chromatography has been recently used for the separation of ionic compounds as well as ion-exchange chromatography. Radjai and Hatch [7] applied this chromatographic system to separate amino acids in conjunction with post-column derivatization using *o*-phthalaldehyde—2-mercaptoethanol. Their study showed good results similar to those obtained using the ion-exchange system. With this method, however, the complicated stepwise gradient elution technique must have been applied, and its application to biological samples such as serum was not shown.

This paper deals with a simpler reversed-phase ion-pair chromatographic method for the separation of amino acids and with their determination in plasma and dried blood on filter papers using this method.

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EXPERIMENTAL

Chemicals

o-Phthalaldehyde was purchased from Tokyo Chemical Industry (Tokyo, Japan), 2-mercaptoethanol and acetonitrile were from Kanto Chemicals (Tokyo, Japan). Sodium lauryl sulfate and monochloroacetic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Redistilled water was used for all reagent preparation. Other reagents and solvents were of analytical grade.

o-Phthalaldehyde–2-mercaptoethanol reagent solution was prepared as follows: 40 mg of *o*-phthalaldehyde were dissolved in 20 ml of ethanol and then 0.1 ml of 2-mercaptoethanol and 200 ml of 0.1 *M* borate buffer (pH 9.0) were added to this ethanolic solution.

Apparatus

All parts were obtained from Japan Spectroscopic (Tokyo, Japan). The high-performance liquid chromatographic system consisted of a Tri Rotor I high-performance liquid chromatograph connected with a Model GP-A30 solvent programmer and a Model FP-110 fluorescence spectrofluorometer with a Model RC strip chart recorder. A 250 × 4.0 mm stainless steel column packed with LiChrosorb RP-8 (particle size 5 μm, E. Merck, Darmstadt, G.F.R.) was used for the separation of amino acids. The eluate from the column was successively mixed with *o*-phthalaldehyde–2-mercaptoethanol reagent solution using a Model LCP 150 liquid chromatographic pump and introduced into a fluorescence detector after being passed through a mixing coil made of stainless steel tube (1 m × 0.25 mm). The operating conditions are shown in Fig. 1.

Procedure for high-performance liquid chromatographic separation of amino acids in plasma and dried blood on filter paper

The solution of amino acids (50 μl) was injected onto the column in the form of a solution prepared in the starting mobile phase solvent: 0.05 *M* sodium lauryl sulfate solution acidified with concentrated monochloroacetic acid at pH 3.0.

A 10-μl volume of human plasma was mixed with 500 μl of 70% ethanol containing γ-aminobutyric acid (0.5 mg/dl) as an internal standard. The mixture was vigorously shaken and the supernatant was evaporated to dryness after centrifugation. The residue was dissolved with 100 μl of the starting solvent and then 50 μl of the resulting solution were injected onto the column.

In the case of the dried blood on filter paper, a 3 mm diameter disk of dried blood was placed into a small test tube. A 500-μl volume of 70% ethanol containing γ-aminobutyric acid (0.5 mg/dl) was added to the tube and kept at 4°C overnight. The resulting ethanolic solution was prepared in the same manner as that of the plasma sample.

RESULTS AND DISCUSSION

Fig. 1 shows a typical high-performance liquid chromatogram of a standard mixture of amino acids, each at a concentration of 2 mg/dl. These conditions

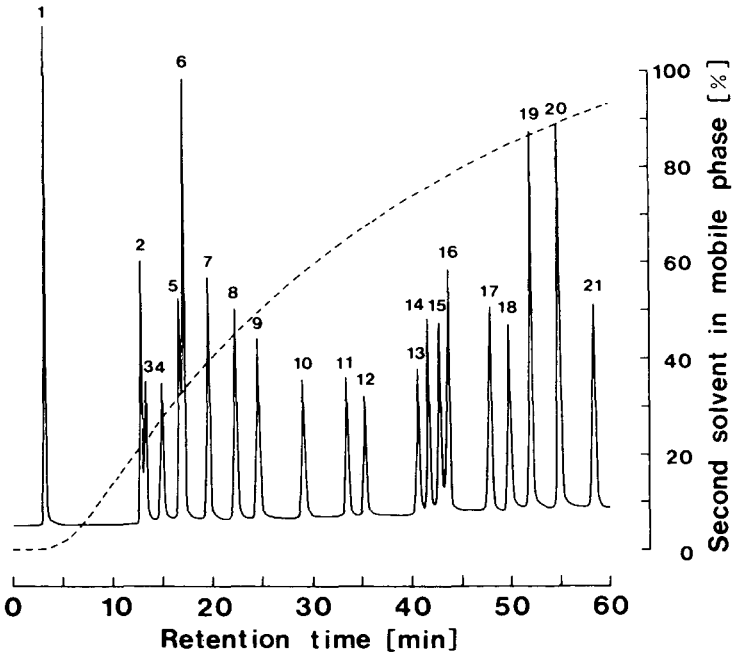

Fig. 1. High-performance liquid chromatogram of a standard mixture of amino acids (dotted line shows the percentage of the second solvent in the mobile phase). Peaks: 1 = taurine, 2 = aspartic acid, 3 = serine, 4 = glutamic acid, 5 = threonine, 6 = glycine, 7 = citrulline, 8 = alanine, 9 = γ-aminobutyric acid (internal standard, IS), 10 = tyrosine, 11 = valine, 12 = methionine, 13 = alloisoleucine, 14 = isoleucine, 15 = phenylalanine, 16 = leucine, 17 = tryptophan, 18 = histidine, 19 = ornithine, 20 = lysine, 21 = arginine. Operating conditions: column, 250 × 4.0 mm LiChrosorb RP-8 (5 μm); mobile phase first solvent, 0.05 *M* sodium lauryl sulfate (pH 3.0), second solvent 0.05 *M* sodium lauryl sulfate—acetonitrile (55:45). The gradient was prepared using a Model GP-A30 solvent programmer (Convex 1, 64 min); mobile phase flow-rate, 0.7 ml/min; reagent solution flow-rate, 1.2 ml/min; column and mixing coil temperature, room temperature; fluorescence detector, excitation 365 nm, emission 455 nm.

did not offer sufficient separation between aspartic acid and serine, and threonine and glycine. The separation of 21 amino acids was achieved within about 60 min. Flowing the first solvent for 10 min after the end of analysis made it possible to inject the next sample.

Radjai and Hatch [7] used acetic acid to adjust the pH of the starting mobile phase to 2.85 in order to inhibit the ionization of the carboxyl group of amino acids and obtain better separation. However, considering that the pK_a of acetic acid is 4.75, it might not be a suitable means of adjusting the pH of the solvent to about 2.8. Therefore, monochloroacetic acid, the pK_a value of which is 2.85, was used to adjust the pH in this study, instead of acetic acid.

Radjai and Hatch [7] also applied a four-step gradient elution technique using two kinds of counter-ion solutions to achieve separation of the amino acids. This technique is very complicated and can not easily be applied to the routine analysis of amino acids. The technique adopted for our method was the simpler gradient elution technique, whereby 0.05 *M* sodium lauryl sulfate—acetonitrile (55:45) is successively added to 0.05 *M* sodium lauryl sulfate (pH

3.0) according to the solvent program (Convex 1, 64 min). The percentage of the second solvent in mobile phase is shown by a dotted line in Fig. 1.

The temperature of the column markedly influenced the separation efficiency. Within the temperature range investigated (15—50° C), the lower the temperature the better the separation obtained. This was especially true of the separation of isoleucine, phenylalanine and leucine. On the basis of these results the column was maintained at 20° C.

Investigations of the flow-rate of mobile phase from 0.6 to 1.0 ml/min showed that the separation efficiency of amino acids hardly varied within this range. The need to protect the column from high pressure and also to shorten the analytical time led us to select a flow-rate of 0.7 ml/min.

The reagent blank from *o*-phthalaldehyde—2-mercaptoethanol reagent solution gave rise to a serious drift in the baseline. To overcome this problem, the pH of the reagent solution was adjusted to 9.0 according to Roth's [8] suggestion about the reaction conditions of *o*-phthalaldehyde with amino acids. This step suppressed the drift as shown in Fig. 1. Further, the flow-rate of the reagent solution was arranged at 1.2 ml/min for the same purpose.

Figs. 2 and 3 show the chromatograms obtained from a normal human plasma sample and from a sample from a patient with maple syrup urine disease (MSUD), respectively. On the chromatogram from the MSUD patient it can be observed that there were remarkable increases in the amounts of the branched-chain amino acids valine, isoleucine and leucine. Also, a peak corresponding to alloisoleucine was detected, which could not be found on the chromatogram from normal plasma.

The analytical results of this method are summarized in Table I. The recovery and reproducibility of aspartic acid, serine, glycine and threonine were relatively low, compared with other amino acids.These results might be due to inferior separations.

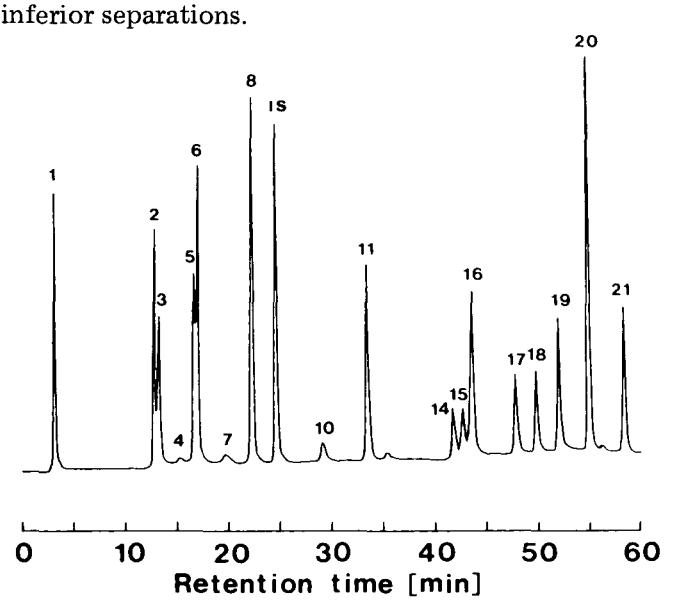

Fig. 2. High-performance liquid chromatogram obtained from a normal human plasma sample. Peak numbering and operating conditions as in Fig. 1.

TABLE I

RECOVERY TEST OF AMINO ACIDS FROM HUMAN PLASMA

Values are given in mg/dl.

Sample		Tau	Asp Ser	Glu	Thr	Gly	Cit	Ala	Tyr
Spiked plasma	1	2.67	15.43	4.94	4.69	2.18	2.50	7.86	3.56
	2	2.63	15.43	5.41	4.69	2.04	2.50	7.72	3.56
	3	2.56	16.29	5.18	4.41	2.04	2.50	7.86	3.33
	4	2.63	16.14	5.18	4.28	1.98	2.50	7.65	3.33
	5	2.74	14.00	5.65	4.55	2.11	2.63	8.21	3.56
	6	2.49	12.71	5.18	3.86	1.98	2.50	7.72	3.33
	7	2.58	13.86	4.94	3.72	2.04	2.50	7.58	3.33
Mean		2.61	14.84	5.21	4.31	2.05	2.52	7.80	3.43
C.V. (%)		3.1	9.0	4.8	9.0	3.5	1.9	2.7	3.6
Plasma blank		0.51	9.28	0.71	1.80	0.85	0.44	3.69	1.33
Added		2.00	2.00 2.00	4.00	4.00	2.00	2.00	4.00	2.00
Recovery (%)		105	139	113	63	60	104	103	105

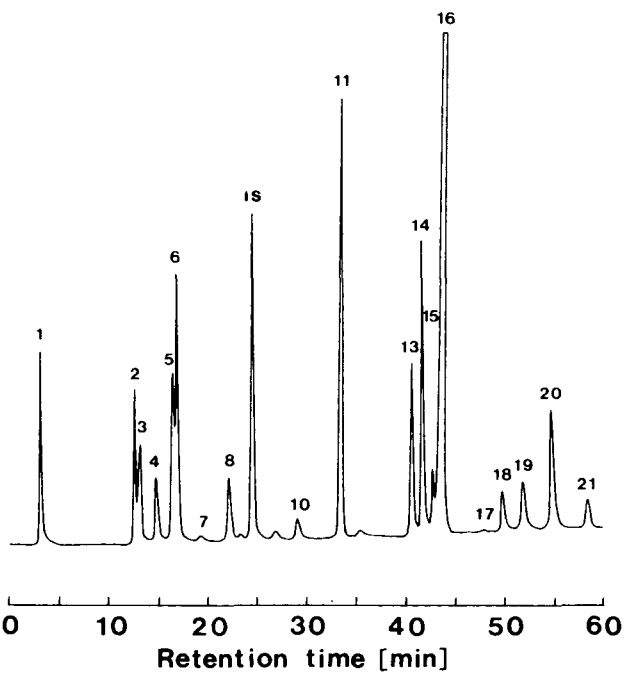

Fig. 3. High-performance liquid chromatogram obtained from the plasma sample of a MSUD patient. Peak numbering and operating conditions as in Fig. 1.

Dried blood on filter paper has been widely used for screening newborns throughout the world [9, 10]. Our method was applied to the analysis of amino acids in dried blood samples. Fig 4 shows a chromatogram obtained from a 3 mm diameter disk of dried blood from a patient with phenyl-

Val	alloIle	Ile	Phe	Leu	Try	His	Orn	Lys	Arg
7.00	2.20	3.18	2.88	3.57	2.89	3.38	2.68	6.92	5.87
6.88	2.20	3.06	3.13	3.71	3.00	3.25	2.75	6.79	6.04
6.75	2.20	3.06	2.88	3.64	3.00	3.50	2.79	7.24	5.96
6.63	2.00	2.94	2.88	3.43	2.78	3.25	2.75	7.06	5.96
7.25	2.20	3.18	3.00	3.79	3.22	3.50	2.98	7.64	6.13
6.75	2.20	3.06	3.00	3.64	3.00	3.38	3.02	7.60	6.13
6.50	2.20	3.06	3.00	3.64	3.00	3.38	3.06	7.78	6.40
6.82	2.17	3.08	2.97	3.63	3.00	3.38	2.86	7.29	6.07
3.6	3.5	2.7	3.2	3.1	4.7	3.0	5.4	5.3	2.9
2.94	—	1.06	1.07	1.75	1.00	1.25	0.72	2.92	1.83
4.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	4.00	4.00
99	109	101	95	94	100	107	107	109	106

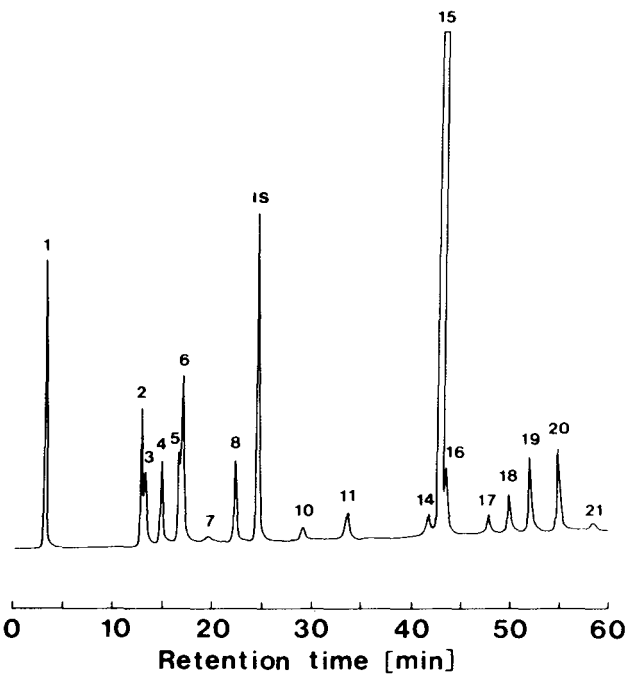

Fig. 4. High-performance liquid chromatogram obtained from 3 mm disk of dried blood of a patient with phenylketonuria. Peak numbering and operating conditions as in Fig. 1.

ketonuria. The results of the determination agree well with those obtained by means of a conventional amino acid analyzer. These data suggest that one 3 mm disk of dried blood is sufficient to analyze the amino acids.

CONCLUSION

This study has demonstrated that the reversed-phase ion-pair chromatographic method can be effectively used for the determination of amino acids in plasma samples and dried blood on filter paper. Relatively good recovery and reproducibility were obtained from the investigations using plasma samples. This method appears to be superior to conventional ion-exchange chromatography in terms of the equilibrium time until the next sample injection, the cost of the column and the simplicity of the elution technique, etc. The present results suggest that this method could be used for screening newborns by means of dried blood on filter paper.

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REFERENCES

1 D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem., 30 (1958) 1190.
2 P.B. Hamilton, Anal. Chem., 35 (1963) 2055.
3 S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leingruber and M. Weigele, Science, 178 (1982) 871.
4 A. Hagg and K. Langer, Chromatographia, 7 (1974) 659.
5 H. Engelhardt, J. Asshauer, U. Neue and N. Weigand, Anal. Chem., 46 (1974) 336.
6 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, Anal. Chem., 48 (1976) 1106.
7 M.K. Radjai and R.T. Hatch, J. Chromatogr., 196 (1980) 319.
8 M. Roth, Anal. Chem., 43 (1971) 880.
9 E. Tokita, Y. Ohbayashi and H. Naruse, Biomed. J., 3 (1979) 827.
10 H. Naruse, E. Tokita, K. Inoue, E. Kishimoto and M. Irie, in Y. Shimazono (Editor), Report on Biological Research on Psychiatric Disorders, National Center for Nervous, Mental and Muscular Disorders, Tokyo, 1979, p. 83.

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Note

Rapid high-performance liquid chromatographic method for the assay of glutamine in human cerebrospinal fluid

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Glutamic acid (Glu) and glutamine (Gln) are the amino acids occurring in the highest concentration in the central nervous system [1]. There is strong evidence that Glu is an excitatory transmitter in some corticostriatal neurons [2, 3]. A possible role of Glu in the pathophysiology of mental disorders such as schizophrenia and depression has been discussed [4, 5]. In the central nervous system Glu and Gln take part in the glutamine cycle [6], where Gln can be both a precursor and an end-product to the transmitter Glu. The extracellular concentration of Gln is in the same order as the total brain concentration while the extracellular concentration of Glu is low. Gln is the dominating amino acid in the cerebrospinal fluid (CSF), while the concentration of Glu is just above the detection limit [7].

Gln in CSF has previously been measured with an amino acid analyzer [7] or using an enzymatic procedure [8]. The first of these methods is time-consuming. With the enzymatic procedure it is difficult to detect interference from other components. Lindroth and Mopper [9] have recently developed a method for the analysis of amino acids using high-performance liquid chromatography (HPLC) with fluorescence detection. The method, which involves derivatization of the amino acids with *o*-phthaldialdehyde, is extremely sensitive.

On the basis of the method of Lindroth and Mopper [9] we have developed a new procedure for the analysis of Gln in CSF. The principle is similar but there are differences in the preparation of the CSF. The introduction of an internal standard (cysteic acid) and isocratic elution instead of gradient elution of the column has shortened the time of analysis considerably and made it possible to analyze as many as 50 samples per day with high accuracy and specificity.

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EXPERIMENTAL

Materials

Methanol for liquid chromatography was obtained from May and Baker (Dagenham, Great Britain). Glutamine and cysteic acid were from Sigma (St. Louis, MO, U.S.A.). Water was distilled over active carbon in a glass apparatus. Other chemicals were of analytical grade.

HPLC was performed with a Spectra-Physics System 3500 B equipped with a Valco rotary valve injector with a 10-μl loop for syringe injection. The fluorescence detector was a SFM 22 Model from Kontron (Stockholm, Sweden) equipped with a 10-μl flow-through cell. The recorder was a W + W 600 Tarkan.

A 150 mm × 4 mm reversed-phase column packed with C_{18} Nucleosil 5 μm (Macherey, Nagel & Co, Düren, G.F.R.) was used. The packing of the column was performed principally according to the procedure of Bristow et al. [10].

Buffers. The phosphate buffer was prepared from 1/15 *M* KH_2PO_4 and 1/15 *M* Na_2HPO_4 which were mixed to give pH 6.0. The borate buffer was prepared from boric acid solution (0.4 *M*) which was adjusted to pH 9.5 with 1 *M* NaOH.

Reagent. *o*-Phthaldialdehyde reagent (OPT) was prepared and handled according to the method of Lindroth and Mopper [9] with the exception that the concentration of *o*-phthaldialdehyde was doubled.

Sampling of CSF

Fresh CSF was obtained from healthy volunteers (age 21—36 years), 12 ml being collected by lumbar puncture. The samples were mixed and centrifuged for 15 min at 2000 *g* at +4°C within 1 h after sampling. The technique used for sampling is described in detail by Sedvall et al. [11]. The fresh samples were processed immediately.

A pool of frozen CSF taken for diagnostic purposes was obtained from the Department of Neurology at the Karolinska Hospital.

Sample preparation

Ethanol (300 μl) was added to a sample of CSF (100 μl). The sample was mixed, stored for 10 min in a refrigerator at +4°C and centrifuged for 10 min at 2000 *g*. A part of the supernatant (300 μl) was transferred to a plastic tube which was stored at −80°C pending analysis. On the day of analysis 50 μl of the supernatant were added to borate buffer (500 μl), pH 9.5, containing the internal standard cysteic acid (2 μg/ml). Part of the sample (20 μl) was reacted with OPT reagent (50 μl) at room temperature. After exactly 4 min 10 μl of the reaction mixture were injected into the chromatograph.

Preparation of standard samples

A stock solution of glutamine (20 mg per 100 ml) in water—methanol (1:1) was prepared every month. It was stored at −20°C. Standard concentrations in the range 100—1000 nmol/ml were prepared by dilution of the stock solution with water—ethanol (1:3). The standard solutions were handled as the supernatant of the CSF sample deproteinized with ethanol.

Liquid chromatography

The chromatograph was run in an isocratic mode using methanol—phosphate buffer, pH 6.0 (45:55) as the mobile phase. The flow-rate was 1.2 ml/min; 10 μl of the sample were injected. About 3 min after the injection, the next sample was mixed with reagent. Shut-down procedures were performed according to the procedure of Lindroth and Mopper [9]. The detector settings were excitation 330 nm, emission 455 nm.

RESULTS AND DISCUSSION

A typical chromatogram of Gln in lumbar CSF from a healthy human subject is shown in Fig. 1. The retention times of cysteic acid and Gln were less than 5 min but the interval between each injection had to be 8 min due to the occurrence of some peaks after the Gln peak. The concentration of Gln in human CSF has been reported to be about 600 nmol/ml [7, 12]. We found a Gln concentration of 512 ± 109 nmol/ml (mean ± S.E.) in fresh lumbar CSF from 16 female volunteers. Naturally occurring amino acids that may interfere with the analysis are serine, asparagine and Glu. These amino acids together have a mean concentration of less than 40 nmol/ml, i.e. about 6% of the Gln level [7]. Asparagine and Glu eluted together in the small peak seen between the internal standard and the Gln peak (Fig. 1). Serine did not separate from the Gln peak, but addition of serine to CSF samples to a concentration of 30 nmol/ml resulted in only a 2% increase of the Gln level.

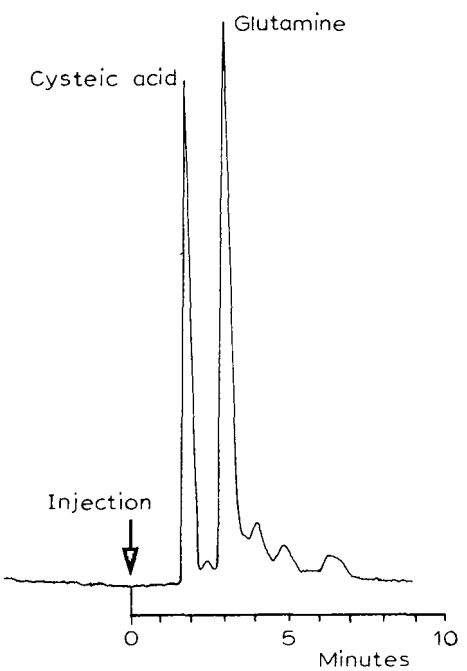

Fig. 1. Chromatogram of glutamine in human CSF. Cysteic acid was added as an internal standard. Conditions as described in the text.

TABLE I

EXPERIMENTAL ERROR AND RECOVERY OF HPLC METHOD FOR THE ANALYSIS OF GLUTAMINE

	Number of samples	Mean Gln concentration (nmol/ml)	± S.D. (%)	Recovery (%)
Frozen pool of CSF	5	568	8	—
Frozen pool of CSF*	6	98	4	—
Individual fresh CSF samples	9	516	8	—
Frozen pool of CSF + Gln 600 nmol/ml CSF	6	1198	10	98

*Frozen pool of CSF diluted 1:6 (v/v) with distilled water containing NaCl (9 mg/ml) and bovine albumin (300 μg/ml).

The standard curve was linear in the range 100—1000 nmol/ml. The recovery of Gln added to a pool of frozen CSF was 98% and the standard deviation was 8% (Table I).

The use of an internal standard was necessary as the peak heights from identical samples varied considerably during the day. This may be due to the accumulation of reagent-consuming material in the upper part of the column. Cysteic acid seems to be a suitable standard since it is apparently not present in human CSF and since its retention time interferes only with that of aspartic acid which is present in human CSF in a concentration below 1 nmol/ml [7]. The OPT derivative of cysteic acid did not reach its fluorescence maximum until after 4 min [9]. Therefore the reaction time should be at least 4 min.

The importance of rapid deproteinization of CSF samples to prevent enzymatic hydrolysis of Gln to Glu has been emphasized by several authors [7, 12]. We studied the stability of Gln in CSF by keeping samples at room temperature for different time intervals after sampling (Table II). No significant change of the Gln concentration was seen for up to 6 h without deproteinization.

The stability of Gln in CSF during storage for up to two months was also studied (Fig. 2). CSF samples from two healthy volunteers were pooled within 1 h after sampling. One part of the sample (control) was deproteinized within 1 h of sampling. It was analysed on the same day as it was sampled. The rest of the sample was divided into four fractions which were processed as described in Fig. 2. After two weeks there was no difference between the samples. After storage for one or two months the Gln concentration had decreased about 15% in the fraction which was stored at −20°C without deproteinization. The low Gln concentration found for all four fractions after two weeks' storage as compared to the control illustrates that the coefficient of variation between assays may be larger than within assays. Values of 20—30% were reported for the coefficient of variation for between-assay analysis of amino acids with HPLC in another investigation [13]. The deproteinization was made with ethanol, as perchloric acid (3%) caused a minor hydrolysis (< 1%) of Gln. Deproteinization with ethanol will be of importance if Glu, too, is analysed in the samples as the

TABLE II

GLUTAMINE CONCENTRATION IN CSF STORED AT ROOM TEMPERATURE

The samples were deproteinized at 0, 3 or 6 h after lumbar puncture. Otherwise they were handled as described in the Experimental section. Data respresent mean of duplicate samples.

Time of storage (h)	Gln concentration (nmol/ml)
0	470
3	493
6	503

Glu concentration in CSF is only about 0.2% of the Gln level [7] (Alfredsson, unpublished observation).

The present method has been used to determine Gln in CSF from patients with an acute psychosis of schizophrenic type. Samples were taken before and after treatment with chlorpromazine. Before treatment there was a tendency to a positive correlation between Gln concentrations in CSF and the severity of the illness. During treatment the reduction in psychotic symptoms was significantly correlated to a decrease in Gln levels in CSF [14].

The advantages of the present procedure for measuring Gln in CSF are its

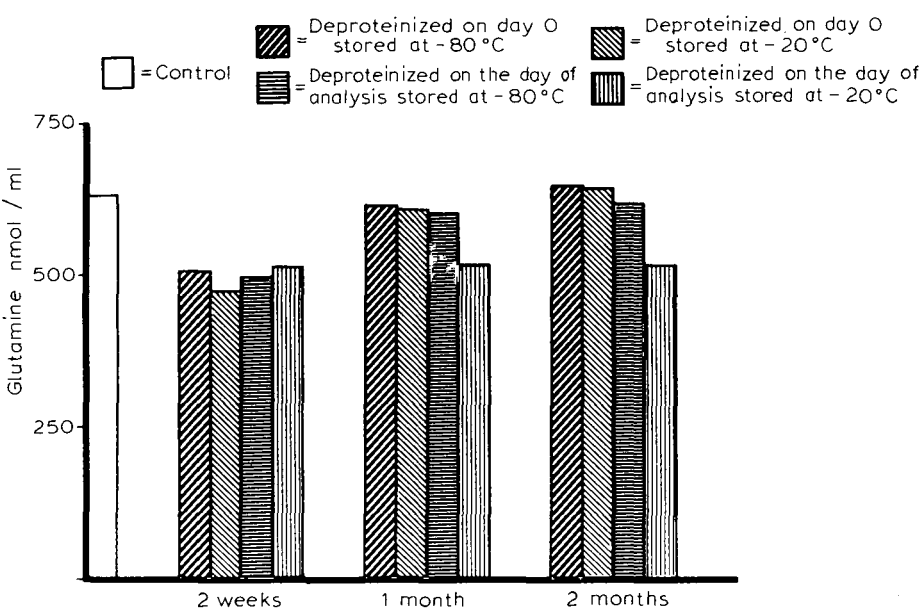

Fig. 2. CSF samples from two healthy volunteers were pooled within 1 h after sampling and deproteinized and analysed on the day of sampling (control). The rest of the sample was divided into four fractions. Two of the fractions were immediately deproteinized and thereafter stored at —20°C or —80°C. The other two fractions were stored at —20°C or —80°C without deproteinization pending analysis. All samples were run in duplicate.

accuracy, rapidity and simplicity, allowing the analysis of up to 50 samples per day. With an amino acid analyzer each sample takes several hours. The method is suitable for the analysis of Gln levels in CSF from patients with neuropsychiatric disorders in order to elucidate the role of Gln in the pathophysiology of such conditions.

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REFERENCES

1 T.L. Perry, K. Berry, S. Hansen, S. Diamond and C. Mok, J. Neurochem., 18 (1971) 513.
2 A.C. Hamberger, G. Han Chiang, E.S. Nylén, S.W. Scheff and C.W. Cotman, Brain Res., 168 (1979) 513.
3 J.C. Reubi and M. Cuenod, Brain Res., 176 (1979) 185.
4 J.S. Kim, H.K. Kornhuber, W. Schmid-Burgk and B. Holzmüller, Neurosci. Lett., 20 (1980) 379.
5 P.J. Goodnick, H.E. Evans, D.L. Dunner and R.R. Fieve, Biol. Psychiat., 15 (1980) 557.
6 R.P. Shank and M.H. Aprison, Life Sci., 28 (1981) 837.
7 T.L. Perry, S. Hansen and J. Kennedy, J. Neurochem., 24 (1975) 587.
8 S.R. Nahorski, Anal. Biochem., 42 (1971) 136.
9 P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
10 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.
11 G. Sedvall, B. Fyrö, B. Gullberg, H. Nybäck, F.-A. Wiesel and B. Wode-Helgodt, Brit. J. Psychiat., 136 (1980) 366.
12 E.H.F. McGale, I.F. Pye, C. Stonier, E.C. Hutchinson and G.M. Aber, J. Neurochem., 29 (1977) 291.
13 M.H. Fernström and J.C. Fernström, Life Sci., 29 (1981) 2119.
14 L. Bjerkenstedt, B. Gullberg, C. Härnryd, G. Oxenstierna, G. Sedvall and F.-A. Wiesel, in preparation.

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Note

A highly sensitive assay for non-specific N-methyltransferase activity in rat tissues by high-performance liquid chromatography with electrochemical detection

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Phenylethanolamine N-methyltransferase (PNMT) and non-specific N-methyltransferase (EC 2.1.1.28) catalyze the N-methylation of aromatic amines. PNMT is specific for phenylethanolamines such as noradrenaline (NA), and catalyzes the last step in catecholamine biosynthesis, forming adrenaline (AD) from NA. PNMT activity is high in adrenal gland [1,2], whereas non-specific N-methyltransferase is distributed in various tissues such as the lung [3]. Borchardt et al. [4] first reported a method to detect PNMT activity by high-performance liquid chromatography with electrochemical detection (HPLC—ElCD), which could demonstrate the activity only in the adrenal medulla and hypothalamus. Recently, Trocewicz et al. [5] reported a highly sensitive assay method for PNMT using HPLC—ElCD by which the activity in all regions of rat brains could be measured. The activity of non-specific N-methyltransferase in brain regions and peripheral tissues of the rat could be detected by a radioassay [6]. However, there has been no report on an assay method for non-specific N-methyltransferase using HPLC—ElCD.

In this paper, we describe a highly sensitive assay procedure for the activity of non-specific N-methyltransferase by high-performance reversed-phase ion-pair chromatography with electrochemical detection. By this method the non-specific N-methyltransferase activity could be determined in various rat brain regions and peripheral tissues.

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EXPERIMENTAL

Materials

The chemicals used were as follows: pargyline HCl, dopamine (DA), N-methyldopamine (N-CH_3-DA) (Sigma, St. Louis, MO, U.S.A.), S-adenosylmethionine hydrogen sulfonate (SAM) (Boehringer, Mannheim, G.F.R.), sodium N-pentanesulfonate (PSS) (Regis, Norton Grove, IL, U.S.A.), and 3,4-dihydroxybenzylamine (DHBA) (Eizai, Tokyo, Japan).

Sample preparation

The rats were decapitated, and brain and peripheral tissues were removed immediately. The brain regions were cut on glass plate placed over ice according to the method of Carlsson and Lindqvist [7]; hypothalamus, pons plus medulla oblongata, septum, cerebral cortex, striatum, cerebellum, olfactory lobe, and limbic brain were dissected out. The peripheral tissues examined were the lung, liver, stomach, heart, adrenal gland, and salivary gland. The brain and peripheral tissues were homogenized in 5 vols. of 0.32 *M* and 0.25 *M* sucrose, respectively, in a glass Potter homogenizer.

Apparatus

The chromatograph used was a Yanaco Model L-2000 with a Yanaco VMD-101 electrochemical detector and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} (particle size, 7.5 μm).

Experimental procedure

The HPLC column (stainless-steel, 25 cm × 0.4 cm I.D.) was packed with Nucleosil 7 C_{18} using the slurry technique [8] with the slurry column packing apparatus Model 124 (Chemco Scientific, Osaka, Japan) as described before [5].

The incubation mixture for non-specific N-methyltransferase assay consisted of the following components in a total volume of 250 μl (final concentrations in parentheses): 10 μl of 0.01 *M* pargyline HCl (0.4 m*M*), 25 μl of 1 *M* Tris—HCl buffer, pH 8.0 (0.1 *M*), 20 μl of 0.3 m*M* SAM (24 μ*M*), 25 μl of 0.2 m*M* DA (20 μ*M*), 100 μl of 0.32 *M* sucrose for the brain or 0.25 *M* sucrose for the peripheral tissues, containing homogenized tissues as enzymes, and distilled water. No enzyme was added to the blank and standard, but the latter contained 30 pmol of N-CH_3-DA. The control was incubated without enzyme, which was then added after incubation.

After incubation for 60 min at 37°C, reaction was stopped by adding 600 μl of 0.42 *M* perchloric acid which contained 1.55 mg of Na_2EDTA, 3.12 mg of $Na_2S_2O_5$ and 30 pmol of DHBA as an internal standard. The reaction mixtures were left in an ice bath for 10 min, then 200 μl of 0.8 *M* potassium carbonate were added to remove excess perchloric acid, and 1 ml of 0.5 *M* Tris—HCl buffer, pH 8.5, to adjust the pH of the solution at 8.0—8.5. The mixture was then centrifuged at 1900 *g* for 10 min at 4°C. The supernatant was loaded on a column containing 100 mg of aluminium oxide, then the column was washed with 4 ml of 0.05 *M* Tris—HCl buffer (pH 8.5), 5 ml of distilled water

twice, then 100 μl of 0.5 *M* HCl. The adsorbed product, N-CH_3-DA, and internal standard (DHBA) were eluted with 200 μl of 0.5 *M* HCl.

A 50-μl volume of eluent was injected into the high-performance liquid chromatograph equipped with an electrochemical detector and a column packed with Nucleosil 7 C_{18}. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.6) containing 5 m*M* PSS and 0.25% (v/v) acetonitrile, at a flow-rate of 0.9 ml/min; the detector potential was set at 0.6 V vs. a Ag/AgCl electrode. The chromatography was performed at a temperature between 25°C and 28°C. Under these conditions the retention times were: solvent front 2.0 min, NA 4.4 min, AD 6.8 min, DHBA 8.0 min, DA 12.0 min, and N-CH_3-DA 16.4 min.

The N-CH_3-DA formed enzymatically by non-specific N-methyltransferase was calculated by the following equation:

$$\frac{R(\mathrm{E}) - R(\mathrm{C})}{R(\mathrm{S}) - R(\mathrm{B})} \times 30 \text{ (pmol)}$$

where R is the ratio of peak height (peak height of N-CH_3-DA/peak height of DHBA), R(E) being that from the experimental incubation, R(C) from the control incubation, R(S) from the standard, and R(B) from the blank.

RESULTS

The calibration curve indicated a linear relationship between the peak height and the amount of N-CH_3-DA from 0.1 to 10 pmol. The sensitivity was 1 pmol of N-CH_3-DA formed by the enzyme reaction. The chromatographic patterns of the non-specific N-methyltransferase reaction of rat whole brain is shown in Fig. 1. The experimental incubation with 10 mg of rat whole brain (Fig. 1A) showed significant formation of N-CH_3-DA during the reaction at 37°C for 1 h, as compared with a small peak in the control incubation without enzyme (Fig. 1B). Figs. 1C and D show no-enzyme blank without and with 30 pmol of N-CH_3-DA as a standard. The optimum pH for non-specific N-methyltransferase obtained using Tris–HCl buffer was pH 8.0. Enzyme activity was linear with the incubation period for at least 90 min (Fig. 2A) and with the amount of enzyme (Fig. 2B).

The kinetics of non-specific N-methyltransferase activity of the whole brain homogenate are shown in Fig. 3. Lineweaver-Burk plots [9] were obtained with DA (Fig. 3A) and with SAM (Fig. 3B) as substrates. The K_M values for DA and SAM calculated from the Lineweaver-Burk plots by Wilkinson's programme [10] were 32 μM and 14 μM, respectively. The maximum plateau activity was obtained when the concentration of SAM was between 20 and 40 μM, and the activity was inhibited when the concentration of SAM was higher than 40 μM (Fig. 3B).

Table I shows the activity of non-specific N-methyltransferase in brain regions and peripheral tissues of the rat. Non-specific N-methyltransferase activity can be detected in various brain regions and peripheral tissues. The highest activity in the brain was detected in the hypothalamus and cerebral cortex. Various peripheral tissues gave high activity.

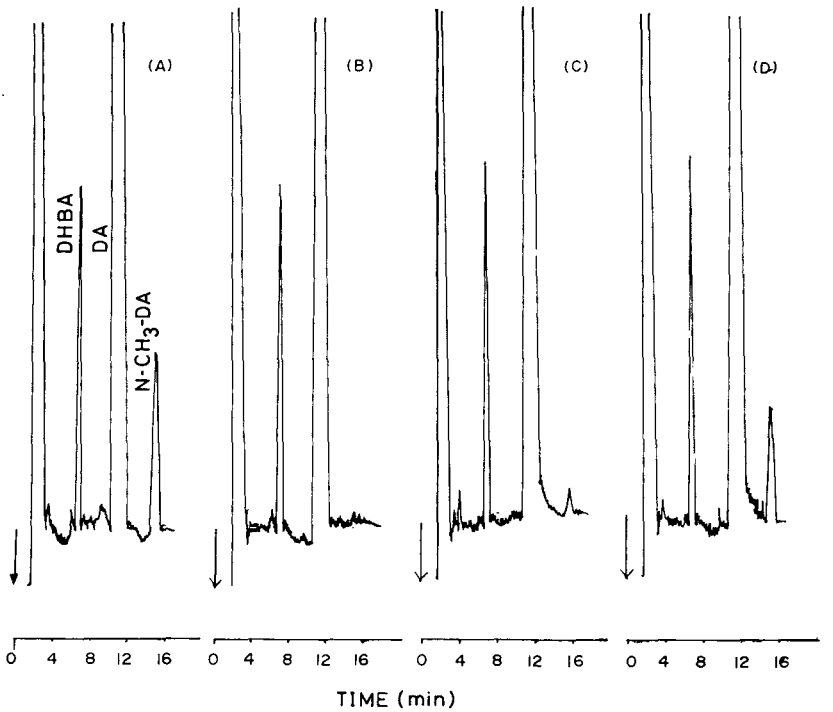

Fig. 1. A typical elution pattern of non-specific N-methyltransferase incubation mixtures with the homogenate of rat whole brain as enzyme. The conditions are described in Experimental procedure. The incubation mixture contained 10 mg of rat whole brain as enzyme and 20 μM dopamine (DA) and 24 μM S-adenosyl-L-methionine (SAM) as substrates. (A) Experimental incubation with homogenate of 10 mg of rat whole brain. (B) Control incubation without enzyme, and with addition of enzyme after incubation. (C) Blank without enzyme. (D) Standard without enzyme and with 30 pmol of N-methyldopamine (N-CH_3-DA). DHBA = dihydroxybenzylamine.

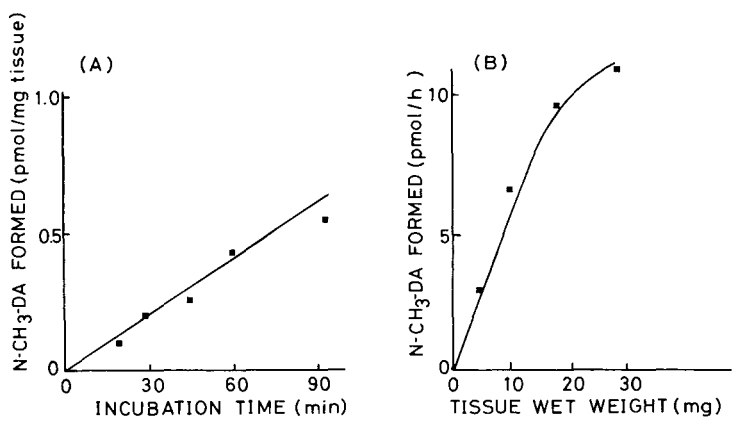

Fig. 2. (A) The rate of N-methyldopamine (N-CH_3-DA) formation using an homogenate of rat whole brain as enzyme at 37°C. Standard incubation system containing 10 mg of rat whole brain was used as described in Experimental procedure. (B) Relationship between the amount of N-CH_3-DA formed by non-specific N-methyltransferase and the tissue concentration as measured by HPLC—ElCD assay. Incubations were carried out for 60 min at 37°C with increasing amounts of rat whole brain.

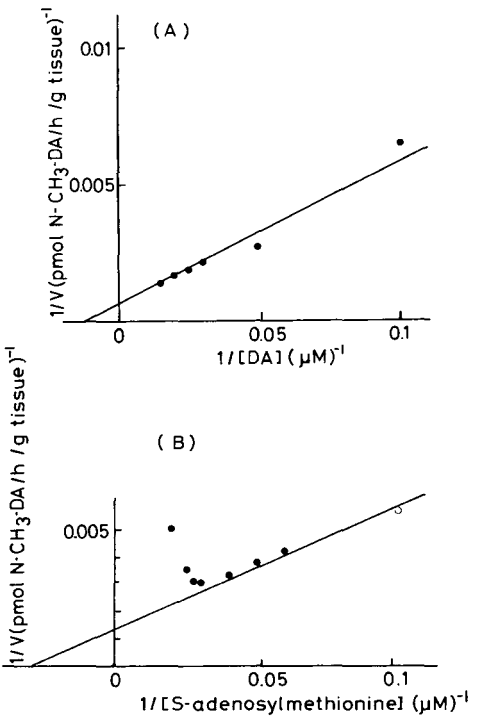

Fig. 3. Lineweaver-Burk plot of non-specific N-methyltransferase activity as function of (A) dopamine (DA) concentration and (B) S-adenosylmethionine (SAM) concentration. Standard incubation system containing 24 μM SAM or 20 μM DA and 10 mg of whole rat brain as enzyme was used as described in Experimental procedure.

TABLE I

NON-SPECIFIC N-METHYLTRANSFERASE ACTIVITY IN VARIOUS RAT BRAIN REGIONS AND PERIPHERAL TISSUES

Tissues were dissected out and processed as described under Materials. Results represent mean ± S.E.M. for a group of five animals. Activity is expressed in pmol N-methyldopamine formed per g of tissue per h of incubation.

	Non-specific N-methyltransferase activity (pmol per g per h)
Hypothalamus	736 ± 97
Pons plus medulla oblongata	435 ± 111
Septum	337 ± 100
Cerebral cortex	661 ± 178
Striatum	135 ± 44
Cerebellum	322 ± 126
Olfactory lobe	122 ± 51
Limbic brain	754 ± 31
Lung	699 ± 162
Liver	1170 ± 171
Stomach	276 ± 108
Heart	719 ± 154
Adrenal gland	885 ± 224
Salivary gland	240 ± 145

DISCUSSION

Saavedra et al. [6] reported the presence and properties of non-specific N-methyltransferase in the brain by a radioassay method using tryptamine and radiolabeled SAM as substrates. They found that the activity of non-specific N-methyltransferase is generally distributed throughout the rat brain, and that the regional and subcellular distribution of non-specific N-methyltransferase in rat brain was different from that reported for PNMT.

The present methods permit the activity of non-specific N-methyltransferase to be measured using DA as substrate and using HPLC—ElCD. PNMT can also be measured simultaneously by the previously reported HPLC—ElCD method [5]. The activity of non-specific N-methyltransferase was uniformly distributed in both brain and peripheral tissues, whereas PNMT activity was unevenly distributed [5]. These results agree with those by Saavedra et al. [6].

The present method is highly sensitive, simple and rapid. It is economical since a labeled substrate and liquid scintillation spectrometer are not needed. Since the endogenous substrate, DA, is used, this method should be useful to study changes in non-specific N-methyltransferase activity in physiological and pharmacological studies.

REFERENCES

1 N. Kirshner and M. Goodall, Biochim. Biophys. Acta, 24 (1957) 658.
2 J. Axelrod, J. Biol. Chem., 237 (1962) 1657.
3 J. Axelrod, J. Pharmacol. Exp. Ther., 138 (1962) 28.
4 R.T. Borchardt, W.C. Vincek and G.L. Grunewald, Anal. Biochem., 82 (1977) 149.
5 J. Trocewicz, K. Oka and T. Nagatsu, J. Chromatogr., 227 (1981) 407.
6 J.M. Saavedra, J.T. Coyle and J. Axelrod, J. Neurochem., 20 (1973) 745.
7 A. Carlsson and M. Lindqvist, J. Pharm. Pharmacol., 25 (1973) 437.
8 T.J.N. Webber and E.H. McKerrell, J. Chromatogr., 122 (1976) 243.
9 H. Lineweaver and D.L. Burk, J. Amer. Chem. Soc., 56 (1934) 658.
10 G.N. Wilkinson, J. Biochem., 80 (1961) 324.

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Note

Studies on multiple molecular forms of transferrin

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The clinical significance of the iron-transport protein transferrin has been known for more than 30 years. Interpretation of its function and variability has been published [1]. With isoelectric focusing this protein has been found to occur in multiple molecular forms [2]. These forms differ in the glycoside residues attached to the polypeptide and/or in the amount of iron bound to the protein [3]. The presence of seven multiple molecular forms in human serum and nine in cerebrospinal fluid has been demonstrated with immunofixation after isoelectric focusing as described by Stibler [2]. One of these multiple molecular forms, having an isoelectric point (p*I*) of 5.7 ($Tf_{5.7}$), has attained a special interest. The ratio of the $Tf_{5.7}$ concentration and the total transferrin (Tf_{tot}) concentration, i.e. ($Tf_{5.7}/Tf_{tot}$) × 100%, has been proposed to be inversely correlated to hepatocyte membrane function and might be used as a marker of damage of the membrane function such as is caused by alcoholic abuse [3, 4].

In order to study this further we have developed an analytic procedure using isoelectric focusing and zone immunoelectrophoretic assay [5] for quantitation of the concentration of $Tf_{5.7}$ and the relation of the $Tf_{5.7}$ concentration to the Tf_{tot} concentration.

MATERIAL AND METHODS

Serum samples were obtained at the Karolinska Hospital, Stockholm, Sweden, from healthy blood donors with ages in the range 21—65 years (39 male, 11 female). The serum samples were frozen within 1 h in small aliquots at −24°C and analyzed within one month. The method used has been presented in ref. 5. In addition, we now incorporate (as a spacer) glycylglycine

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(free base, Sigma, St. Louis, MO, U.S.A.) at 1% (w/v) in the gel, which improves the separation of $Tf_{5.7}$ and the component anodal to this by about 1 mm. This facilitates the isolation of $Tf_{5.7}$.

RESULTS AND DISCUSSION

Absolute concentrations of Tf_{tot} and $Tf_{5.7}$ are summarized in Tables I and II, respectively. The ratio $Tf_{5.7}/Tf_{tot}$ in 50 sera ranged from 2% to 7%. The frequency distribution is shown in Fig. 1. Some additional calculations are given in Table III. Plotting Tf_{tot} against $Tf_{5.7}$ (not shown) displays a regression coefficient (r) of only 0.47.

As can be seen in Table III there appears to be quite a large biological variability, especially for the $Tf_{5.7}$ concentrations. The relative standard deviation (R.S.D.) is 28%. These figures include the method error, which for $Tf_{5.7}$ was typically about 10%. The biological variability is more truly reflected by the R.S.D. of the Tf_{tot} concentration (biological 16%, method 3%). The $Tf_{5.7}/Tf_{tot}$ ratio consequently has an R.S.D. of 24%, including these errors.

TABLE I

THE FREQUENCY DISTRIBUTION OF THE Tf_{tot} CONCENTRATION IN 50 NORMAL SERUM SAMPLES

i	Classes (Tf_{tot}, g/l)	n_i
1	1.75—2.00	2
2	2.00—2.25	11
3	2.25—2.50	8
4	2.50—2.75	3
5	2.75—3.00	9
6	3.00—3.25	12
7	3.25—3.50	1
8	3.50—3.75	3
9	3.75—4.00	1
10	4.00—4.25	1

TABLE II

THE FREQUENCY DISTRIBUTION OF THE ABSOLUTE $Tf_{5.7}$ CONCENTRATION IN 50 NORMAL SERUM SAMPLES

i	Classes ($Tf_{5.7}$, mg/l)	n_i
1	62.5— 75.0	5
2	75.0— 82.5	7
3	82.5—100.0	6
4	100.0—112.5	7
5	112.5—125.0	10
6	125.0—132.5	4
7	132.5—150.0	3
8	150.0—162.5	4
9	162.5—175.0	4
10	175.0—182.5	1

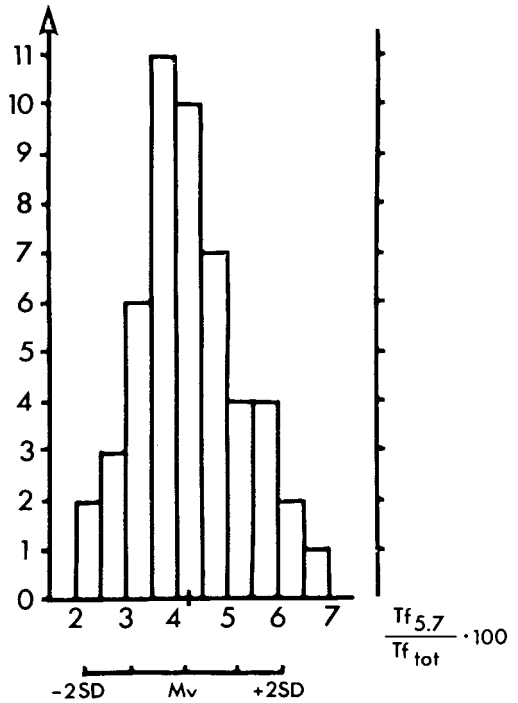

Fig. 1. Histogram presentation of the $Tf_{5.7}$ concentration frequency distribution. The mean value (Mv) and the ± 2S.D. range are also indicated. The denomination is per cent and the division is 0.5%.

TABLE III

THE MEAN VALUES, ± S.D. AND ± 2S.D. RANGES AND THE RELATIVE STANDARD DEVIATION OF THE PARAMETERS SUMMARIZED IN TABLES I AND II

	Mean (g/l)	Range of mean ± S.D.	Range of mean ± 2S.D.	R.S.D. (%)
Tf_{tot}	2.73	2.21 —3.25	1.67 —3.77	19
$Tf_{5.7}$	0.11	0.080—0.140	0.650—0.170	28
$Tf_{5.7}/Tf_{tot}$%	4.2	3.2 —5.2	2.2 —6.2	24

Some controversy is introduced as there seems to be no direct correlation between $Tf_{5.7}$ and Tf_{tot}. Probably, $Tf_{5.7}$ has lost some of its capping sialic acid residues [3] and thus should be cleared from the blood with a certain normal capacity per time unit. The proposed usage of the $Tf_{5.7}/Tf_{tot}$ ratio as an indicator of alcohol abuse supposes a change in one of these parameters, i.e. $Tf_{5.7}$ [3]. Damaged hepatocytes are supposed to have a reduced capacity for removal of this transferrin species, thus increasing the $Tf_{5.7}/Tf_{tot}$ ratio. In normal sera a high concentration of Tf_{tot} should therefore be accompanied by a high $Tf_{5.7}$ concentration, thereby not significantly altering the ratio between synthesis and removal. The possibility to detect damage in this system required a correlation between these parameters. Normally, a correlation coefficient greater than 0.5 is said to validate correlation. We are quite surprised at the low

correlation obtained (0.47). The understanding of the $Tf_{5.7}/Tf_{tot}$ ratio as a marker of hepatic (mal-)function requires additional studies. The 50 values for the $Tf_{5.7}/Tf_{tot}$ ratio is published to serve as a reference for such purposes.

REFERENCES

1 E.B. Brown, in Iron Metabolism, Ciba Foundation Symposium 51, Elsevier/North-Holland, Amsterdam, 1977, pp. 125—138.
2 H. Stibler, J. Neurol. Sci., 41 (1979) 275—281.
3 H. Stibler, S. Borg and C. Allgulander, Acta Med. Scand., 206 (1979) 275—281.
4 H. Stibler and O. Sydow, Acta Med. Scand., 204 (1978) 49—50.
5 O. Vesterberg and U. Brieg, J. Immunol. Methods, 46 (1981) 53—62.

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Note

Determination of aprindine in plasma by gas chromatography—mass spectrometry

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Aprindine hydrochloride [N-phenyl-N-(3-diethylaminopropyl)-2-indanylamine hydrochloride] (Fig. 1), an effective antiarrhythmic drug against a variety of supraventricular and ventricular arrhythmias, has been used for several years in Europe [1] and is currently undergoing clinical evaluation in Japan. As this drug has a narrow therapeutic index [1], monitoring of plasma levels of the drug is of great importance in assessing drug therapy.

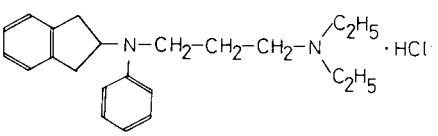

Fig. 1. Structure of aprindine hydrochloride.

Several assay methods using gas chromatography (GC) with a flame ionization detector [2,3] and with a nitrogen-sensitive detector [3,4] as well as high-performance liquid chromatography [5] have been applied for the measurement of aprindine in biological fluids. A radioimmunoassay procedure has also been reported [6].

The present report describes a rapid and sensitive gas chromatography—mass spectrometry (GC—MS) method for the measurement of aprindine concentrations in plasma and its application to the analysis of plasma from subjects who received the drug.

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EXPERIMENTAL

Standards and reagents

Aprindine hydrochloride was supplied by A. Christiaens S.A. (Brussels, Belgium) and amitriptyline hydrochloride was purchased from Kodama Chemicals (Tokyo, Japan). Stock standard solutions of aprindine hydrochloride and amitriptyline hydrochloride were prepared in distilled water and stored at 4°C throughout the experiments. The hydrochloride salt of aprindine was used and the concentrations, when expressed as μg/ml, have been calculated as the salt. All other reagents were of analytical grade and obtained from usual commercial sources.

Instrumentation

An MS-DC 05/06 automated gas chromatograph—JMS-DX300 mass spectrometer coupled with a JMA-3500 data system (JEOL, Tokyo, Japan) was used.

The coiled glass column (2 m × 1.5 mm I.D.) packed with 3% silicone OV-1 on Chromosorb 750 (80—100 mesh) (Nihon Chromato Works, Tokyo, Japan) was used for the gas chromatography. The temperatures of the column and injector were maintained at 260°C and 280°C, respectively. The flow-rate of the carrier gas (helium) was set at 40 ml/min.

The mass spectrometer was operated in the chemical ionization (CI) mode under the following conditions; ionization energy, 200 eV; ionization current, 300 μA; accelerating voltage, 3 kV; reagent gas, ammonia at a pressure of 0.5 torr. Under these conditions the ions selected for monitoring were the quasi-molecular ions, $[M+1]^+$, of *m/z* 323 and 278 for aprindine and amitriptyline, respectively, and the retention times were aprindine 0.84 min and amitriptyline 0.5 min.

Sample preparation and extraction

To a 15-ml screw-cap glass test tube were added in succession human plasma (1 ml), 100 μl of the internal standard solution containing 100 ng of amitriptyline as hydrochloride salt, 1 ml of 0.2 *M* sodium carbonate buffer (pH 10.2) and 6 ml of diethyl ether. The samples were shaken for 15 min. Following centrifugation at 2000 *g* for 5 min, the upper organic phase was transferred to another tube, and the aqueous phase was extracted with 6 ml of ether. The combined organic phase was evaporated to dryness under nitrogen gas flow at 40°C. Then 1 ml of 0.1 *N* HCl and 2 ml of ether were added to the residue, followed by shaking for 5 min. After a brief centrifugation, the organic phase was discarded by aspiration. The aqueous phase was made alkaline with 1 ml of 0.2 *M* sodium carbonate buffer and extracted with 6 ml of ether. The organic phase was evaporated to dryness under nitrogen. The residue was dissolved in chloroform (50—100 μl), and an aliquot (2 μl) was injected into the gas chromatograph—mass spectrometer.

Calibration curve and quantitative analysis

A calibration curve was constructed with every analysis by adding various amounts of aprindine hydrochloride (10—600 ng) and a constant amount of

internal standard (100 ng as salt) to 1 ml of control plasma and analyzing the samples as described above. To assess linearity, the peak area ratio (PAR) of aprindine hydrochloride to the internal standard was plotted against the amount of aprindine hydrochloride added.

The amount of aprindine in each plasma sample from subjects was calculated from a calibration curve on the basis of the PAR value.

Recovery and precision

Recoveries were calculated by comparing the PAR values of spiked plasma standards with those obtained when the internal standard was added to the plasma and the known amounts of aprindine hydrochloride (50, 100 and 300 ng) were added to the aqueous phase prior to the last extraction with ether. The precision of the method was estimated by analyzing spiked plasma standards that had been prepared on three different days.

Application of the method

Five healthy volunteers (male, 58—69 kg, age 20—40 years) each received a 25-mg capsule of aprindine hydrochloride. Venous blood was collected with heparinized syringes at various intervals for 72 h after dosing and centrifuged at 2000 *g* for 15 min. Plasma samples were stored at −20°C until taken for assay.

RESULTS AND DISCUSSION

To obtain suitable GC—MS conditions for the determination of aprindine and amitriptyline, a comparative evaluation of ionization modes [electron impact (EI) and CI modes] was carried out, and the results are shown in Fig. 2. The EI mass spectra of both compounds showed some fragment ions but no M^+ ion (Fig. 2A). The CI spectra of aprindine and amitriptyline using ammonia as a reagent gas, on the other hand, showed $[M+1]^+$ ion with additional small fragment ions (Fig. 2B). The use of diethylamine as a reagent gas gave similar results, while other reagent gases such as methane and isobutane gave no $[M+1]^+$ ion.

To avoid possible interference and to improve the sensitivity with a view to the analysis of low levels of aprindine in plasma, an ion peak with high intensity should be selected for monitoring. For this reason, mass fragmentation by CI using ammonia as a reagent gas and the detection of the $[M+1]^+$ ion seem to be adequate for the determination of the compounds of interest.

The mass fragmentograms of the plasma samples are presented in Fig. 3. The drug-free control plasma extract gave no interfering peaks at *m/z* 323 and 278, selected for monitoring aprindine and amitriptyline, respectively.

The calibration graph obtained from the analysis of plasma samples containing various amounts of aprindine gave a straight line (correlation coefficient, r=0.96) over the concentration range of 50—600 ng/ml.

The overall recoveries from plasma determined in triplicate at three different concentrations of aprindine (50, 100 and 300 ng) were over 89%. The lower detection limit was 5 ng/ml in the case of a plasma volume of 1 ml.

The overall within-day coefficients of variation were 4.4%, 11.2% and 7.7%

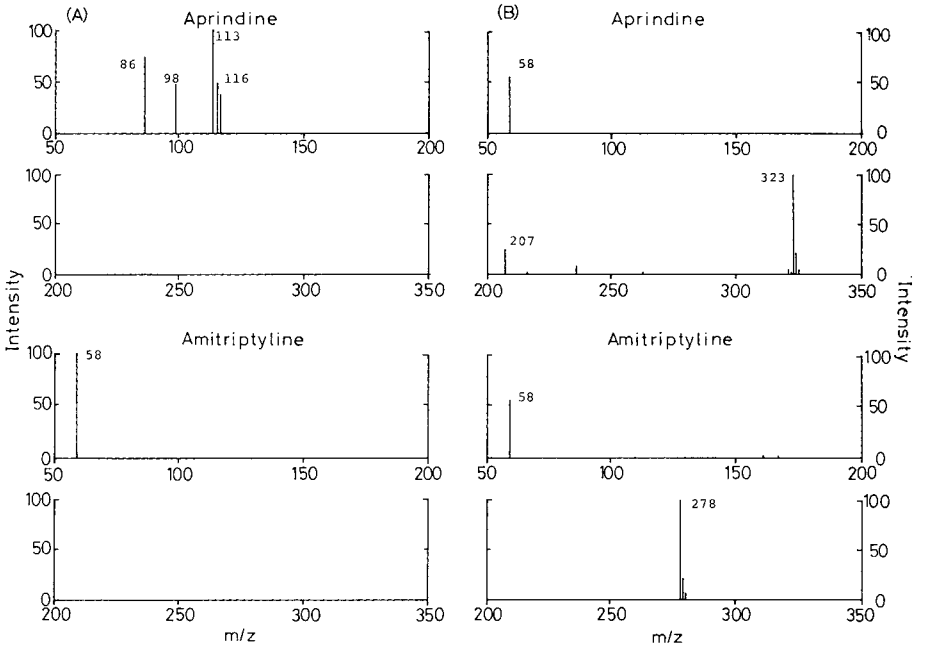

Fig. 2. Mass spectra of aprindine and amitriptyline: (A) electron impact mode; (B) chemical ionization mode with ammonia.

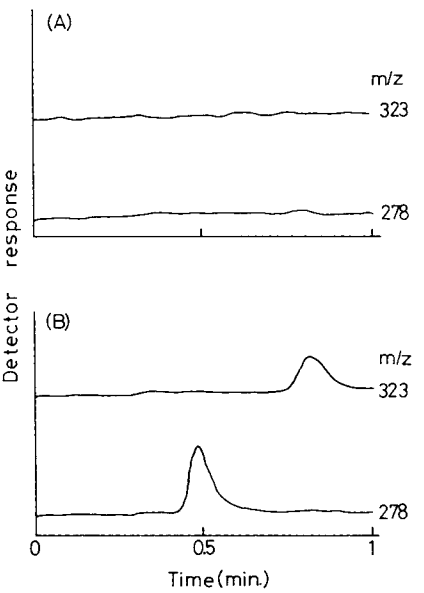

Fig. 3. Chromatograms of (A) plasma blank and (B) plasma standard with aprindine hydrochloride (100 ng) and internal standard (100 ng).

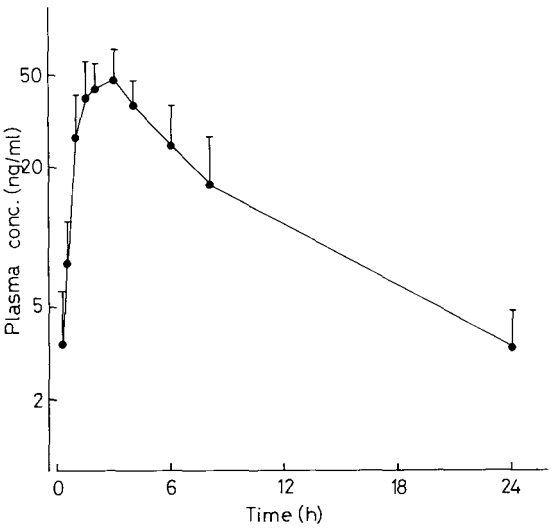

Fig. 4. Plasma concentrations of the unchanged drug after oral administration of aprindine hydrochloride (25 mg) to healthy volunteers. Each point represents the mean ± S.D. from five subjects.

for 100, 200 and 400 ng/ml aprindine, respectively. The overall between-day coefficient of variation ranged from 15.4% to 22.1% at the same concentrations. These wide between-day variations are most likely due to day-to-day changes in ionization reaction conditions and/or column conditions. However, this problem could be overcome by running a calibration curve with every analysis.

The present method is rapid and sufficiently sensitive to be satisfactory for the determination of aprindine in plasma; it is applicable to routine measurement of plasma levels occurring in clinical studies, and one person can analyze 100—150 samples during an average working day.

Application of the method for measuring mean plasma concentrations of aprindine in healthy volunteers who received 25 mg of aprindine hydrochloride orally is illustrated in Fig. 4. Plasma concentrations reached a maximum at about 2 h after dosing, and then declined biexponentially, with a terminal half-life of 8.0 h. These studies will be reported in detail elsewhere.

REFERENCES

1 D.P. Zipes and F.P.J. Troup, Amer. J. Cardiol., 41 (1978) 1005.
2 B.S. Rutherford and R.H. Bishara, J. Pharm. Sci., 65 (1976) 1322.
3 J.F. Nash and R.H. Carmicheal, J. Pharm. Sci., 69 (1980) 1094.
4 F. Andreasen, S. Husted, P. Jakobsen and E.B. Jensen, Acta Pharmacol. Toxicol., 46 (1980) 105.
5 P.-O. Lagerström and B.-A. Persson, J. Chromatogr., 149 (1978) 331.
6 M. Lesne and R. Dolphen, J. Immunol. Methods, 17 (1977) 189.

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Note

Gas chromatographic assay of pramiracetam in human plasma using nitrogen specific detection

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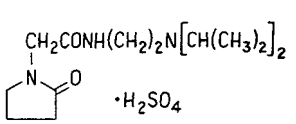
Pramiracetam, N-[2-[bis(1-methylethyl)amino] ethyl] -2-oxo-1-pyrrolidine-acetamide sulfate (1:1), is a new orally active cognition activator [1] currently undergoing clinical evaluation. To facilitate pharmacokinetic and bioavailability studies, a simple and sensitive gas chromatographic method has been developed for the determination of pramiracetam at therapeutic concentrations. The assay entails a one-step extraction with chloroform and subsequent quantitation using a nitrogen—phosphorus detector.

EXPERIMENTAL

Materials

Pramiracetam (I) and the internal standard (II) were synthesized in the Warner-Lambert/Parke-Davis Research Labs. (Ann Arbor, MI, U.S.A.). Glass distilled chloroform with 1% ethanol as preservative was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Triethylamine (TEA) was obtained from Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.); 3% OV-225 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). Working standards containing 2, 1.5, 1, and 0.5 μg/ml of



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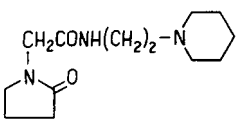

II

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pramiracetam free base were prepared in 0.05 *N* hydrochloric acid. An internal standard solution (2 μg/ml) was likewise prepared.

Extraction procedure

Aliquots of plasma (0.5—1 ml) and 0.5 ml of internal standard solution (1 μg) were pipetted into 150 × 16 mm glass-stoppered tubes containing 8 ml of the extraction solvent, chloroform—TEA (99.5:0.5). After the addition of 0.3 ml of 1 *N* sodium hydroxide and ca. 0.5 g of Na_3PO_4, the tubes were shaken for 10 min in a mechanical shaker. Following centrifugation, the aqueous phase and the plasma protein plug were aspirated off and discarded. The tubes were centrifuged again briefly, and the organic extract was transferred into 100 × 13 mm tubes by pouring smoothly, avoiding any transfer of the residual aqueous phase. The samples were evaporated to dryness under a stream of nitrogen and the residue was taken up in 25 μl of isopropanol—methanol (80:20) containing 0.5% TEA.

Calibration standards containing 0.125, 0.25, 0.5, 0.75, and 1 μg of pramiracetam in 1 ml of drug-free control human plasma were processed as described above with each set of unknown samples.

Apparatus

A Perkin-Elmer Sigma 2 gas chromatograph equipped with a nitrogen—phosphorus detector and a coiled glass column (1.22 m × 2 mm I.D.) packed with 3% OV-225 on 100—120 mesh Gas-Chrom Q were used. The injector, oven, and detector temperatures were maintained at 275, 225, and 275°C, respectively. The gas flow-rates were: hydrogen, 2.5 ml/min; air, 100 ml/min; nitrogen (carrier), 28 ml/min. The peak areas and retention times of pramiracetam and the internal standard were obtained using a Perkin-Elmer Sigma 10 data systems.

Calculations

A calibration curve was constructed by plotting the ratio of the peak area or peak height of pramiracetam to that of the internal standard as a function of the amount of pramiracetam added to control human plasma. The best fit straight line was determined using the method of least squares. The concentrations of pramiracetam in the unknown samples were calculated by interpolation from the standard curve.

RESULTS AND DISCUSSION

The retention times of pramiracetam and the internal standard were 4.65 and 6.76 min, respectively. The chromatograms of a control human plasma extract and of the same plasma spiked with 1 μg each of pramiracetam and the internal standard are shown in Fig. 1. There were no interferences from the normal components of the plasma extract. The total analysis time required for each run was less than 12 min.

The extraction recovery of pramiracetam from control human plasma was 98%. Triethylamine was added to the extraction solvent to minimize drug adsorption to glassware.

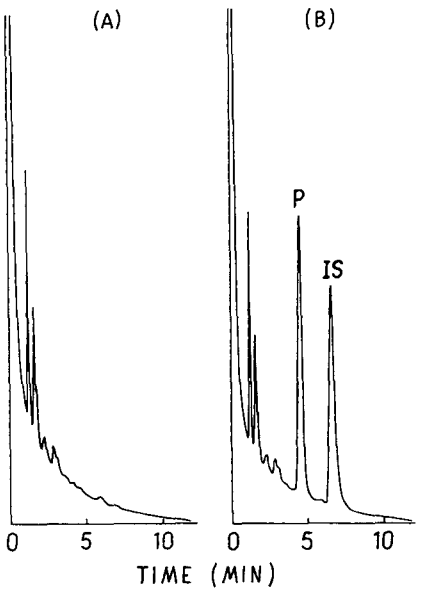

Fig. 1. Chromatograms of human plasma extracts. (A) Control human plasma; (B) control plasma containing 1 μg each of pramiracetam (P) and internal standard (IS).

The peak area or peak height ratios were linearly related to drug concentrations over a range of 0.125—1.0 μg/ml. The best fit linear regression line was $y = 0.9725x - 0.0198$ ($r = 0.9999$) for the peak area ratios, or $y = 1.3356x - 0.0272$ ($r = 0.9997$) for the peak height ratios, where y is the peak area or peak height ratio, and x is the amount of pramiracetam in human plasma. The slopes of four calibration curves constructed over a two-month period showed a relative standard deviation (R.S.D.) of 1.4% and 5% for the peak area and peak height methods, respectively. The lower limit of detection was estimated to be 0.02 μg/ml.

The precision and accuracy of the method were evaluated by extracting and analyzing replicate control human plasma samples containing 0.125—1 μg/ml of pramiracetam. The R.S.D. ranged from 0.95 to 3.8% for the peak area ratios, and from 1.4 to 4.9% for the peak height ratios (Table I). The average measured drug concentrations showed close agreement with the expected values by either the peak area or peak height methods. Column reproducibility following five sequential injections of the same sample had a relative standard deviation of 2.8% and 1.9% for peak area ratios and peak height ratios, respectively.

A number of nootropic drugs, including piracetam, hydergine, and vincamine, were chromatographed under the same conditions to test the specificity of the method, and no interferences were observed.

To demonstrate the utility of the method, plasma samples obtained from a human volunteer receiving a single 400-mg oral dose of pramiracetam capsule were assayed, and the resulting plasma concentration versus time profile is shown in Fig. 2. The apparent elimination half-life was 4.9 h. Details of the clinical pharmacokinetic studies will be reported elsewhere. Recent studies also indicate that the same procedure can be applied to human urine without further modification. However, it should be noted that pramiracetam concen-

TABLE I

PRECISION AND ACCURACY OF REPLICATE ANALYSIS OF PRAMIRACETAM ADDED TO CONTROL HUMAN PLASMA (n = 4)

Expected value (μg/ml)	Peak area ratios		Peak height ratios	
	Assayed value (μg/ml)	Relative standard deviation (%)	Assayed value (μg/ml)	Relative standard deviation (%)
1.0	1.007	0.95	1.001	1.4
0.75	0.743	2.1	0.734	1.7
0.50	0.492	3.1	0.491	1.5
0.25	0.250	2.4	0.249	1.6
0.125	0.132	3.8	0.125	4.9

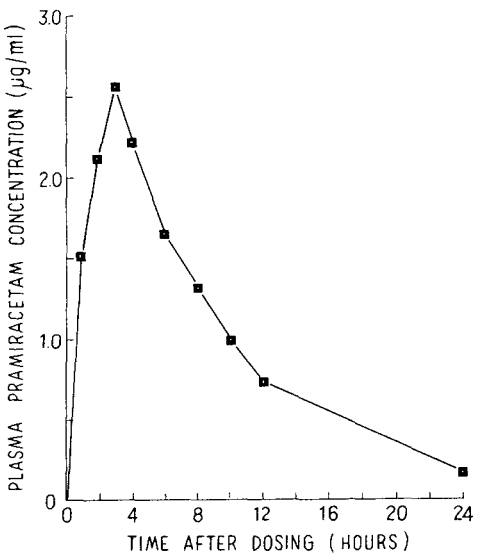

Fig. 2. Plasma concentrations of pramiracetam in a human volunteer following a single 400-mg oral dose.

tration in human urine is far greater than in plasma, and appropriate calibration standards covering a higher concentration range should be used.

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REFERENCE

1 B.P.H. Poschel, J.G. Marriott and M.I. Gluckman, Psychopharmacol. Bull., (1983) in press.

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Note

Determination of desmethyl nortriptyline in plasma by gas chromatography before and after treatment with salicylaldehyde

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During routine monitoring of patients' plasma levels of amitriptyline (AT) and nortriptyline (NT) by gas chromatography (GC) without derivatization [1], the corresponding primary amine desmethyl nortriptyline (DMNT) was found to have a retention time indistinguishable (within 1%) from that of NT, on OV 17 columns at 230°C. The extraction procedure (specific for lipophilic bases, see *Method* section below) extracted DMNT quantitatively from plasma and the primary amine was therefore being estimated as NT.

Fredricson Overø [2] used salicylaldehyde (SA) to eliminate interference by DMNT in the estimation of NT by radioactive acetylation. We found that SA, added to the final heptane extract of plasma, would react with DMNT and the SA, being phenolic, could then be removed by extraction with aqueous alkali. Chromatography before and after this treatment enabled DMNT to be determined by difference.

EXPERIMENTAL

Materials

DMNT hydrochloride and the *cis* and *trans* forms of 10-hydroxy-AT and 10-hydroxy-NT were kindly provided by Dr. A. Jørgensen of H. Lundbeck & Co (Copenhagen, Denmark). Salicylaldehyde (BDH, Poole, Great Britain), general purpose reagent grade, was used without further purification. A fresh solution was prepared daily, containing 0.75 ml salicylaldehyde in 4.25 ml *n*-heptane.

Method

To each plasma sample (1—10 ml, usually about 4 ml) the following in-

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ternal standards were added: 2.5 μg dextromethorphan, 1.0 μg clomipramine, 1.0 μg desmethyl clomipramine. The compounds were extracted [1] into 10 ml heptane, thence into 2 ml 0.1 *M* hydrochloric acid and finally into 50 μl heptane containing diethylamine (0.5%, v/v) and tetracosane (5 μg/ml). A 10-μl aliquot of this extract was chromatographed as previously described [1] and the AT and apparent NT concentrations calculated, using dextromethorphan as internal standard. To the remaining extract, from which all of the original aqueous layer had been carefully removed by Pasteur pipette, 5 μl of the SA solution were added. After standing for 20 min, 100 μl of an aqueous solution containing 0.15 ml diethylamine in 10 ml 0.17 *M* sodium hydroxide solution were added. The mixture was vibrated on a whirlimixer, centrifuged briefly with the stopper in place and the aqueous layer removed by Pasteur pipette. A 10-μl aliquot of the organic phase was injected into the chromatograph.

RESULTS

Extracts of drug-free plasma without added compounds gave no peaks on the chromatogram before or after SA treatment. No new peak appeared after SA treatment of extracts of plasma samples containing tetracosane, dextromethorphan, clomipramine, desmethyl clomipramine, AT, NT, or the *cis* or *trans* forms of the metabolites 10-hydroxy-AT and 10-hydroxy-NT. These compounds were tested separately and/or in various appropriate combinations.

DMNT hydrochloride, added to plasma and extracted by the usual technique, gave its peak at the same retention time as NT, together with a small extra peak at a retention time 1.12 times that of NT. This minor peak did not appear when DMNT hydrochloride was simply dissolved in chloroform and injected; it must therefore have been formed during the extraction procedure. Its peak area was 3—5% of that of the main peak. On SA treatment both peaks disappeared completely.

The peak area of NT, when present without DMNT, decreased on SA treatment by about 3% relative to internal standards. The only other secondary amine usually present, desmethyl clomipramine, decreased by about 5%. The relative peak areas of clomipramine, dextromethorphan, tetracosane and amitriptyline were unchanged by SA treatment. However, treatment greatly reduced both the *cis* and *trans* isomers of 10-hydroxy-NT. Plasma extracts from patients taking AT or NT usually contain small amounts of the hydroxy metabolites, which interfere with the clomipramine and desmethyl clomipramine peaks. Consequently dextromethorphan and tetracosane were chosen as internal standards for calculation of the effect of SA treatment on the apparent peak area of NT. Using the average of these two internal standards, the ratio F of apparent NT concentrations before and after treatment was calculated from

$$F = \tfrac{1}{2} \frac{N_1}{N_2} \left(\frac{dM_2}{dM_1} + \frac{T_2}{T_1} \right)$$

where dM_1 and dM_2 are the dextromethorphan peak areas before and after SA treatment, respectively, and T_1 and T_2 the corresponding peak areas of tetracosane. N_1 and N_2 are the NT peak areas, before and after treatment, corrected for chromatographic losses as previously described [1].

A total of 164 assays were carried out on drug-free plasma samples to which various amounts of NT and/or DMNT were added. Table I shows that, at a constant ratio of DMNT to NT, F was independent of the concentration of either component. In Fig. 1 the graph of F against the DMNT:NT ratio is shown to be a straight line. Thus both the relative extraction yield of DMNT and NT and the relative chromatographic response to these two compounds must be independent of concentration.

TABLE I

F VALUES DETERMINED ON PLASMA SAMPLES CONTAINING KNOWN AMOUNTS OF NORTRIPTYLINE AND DESMETHYL NORTRIPTYLINE

F is the ratio of apparent NT concentrations, before SA treatment/after SA treatment, using both dextromethorphan and tetracosane for internal standardization.

NT concentration (ng/ml in 5 ml plasma)		Ratio DMNT/NT			
		0	0.1	0.2	0.5
200	Mean F	1.029	1.109	1.189	—
	S.D.	0.022	0.015	0.020	
	n	10	10	10	
100	Mean F	1.032	1.105	1.185	1.434
	S.D.	0.021	0.020	0.018	0.027
	n	10	21	9	10
40	Mean F	1.029	1.101	1.192	—
	S.D.	0.011	0.020	0.025	
	n	10	10	9	
20	Mean F	1.028	1.113	1.175	1.424
	S.D.	0.034	0.043	0.026	0.048
	n	15	10	10	20
Total	Mean F	1.029	1.107	1.185	1.427
	S.D.	0.024	0.025	0.023	0.042
	n	45	51	38	30

Let the ratio of the weight of DMNT to that of NT in the plasma sample be r and let the ratio of the peak area of DMNT to that of NT, for equal weights of the two compounds in the sample, be a. Then the apparent amount of NT present before SA treatment is $(1 + ar)$ times the true amount. Let the peak area of NT itself be reduced by the factor $1/f$ by SA treatment. Then

$$F = \frac{1 + ar}{1/f} = f + afr$$

The graph of F against r is therefore linear, with intercept f and slope af; r may be determined as $(F - f)/af$ and the true amount of NT in the plasma

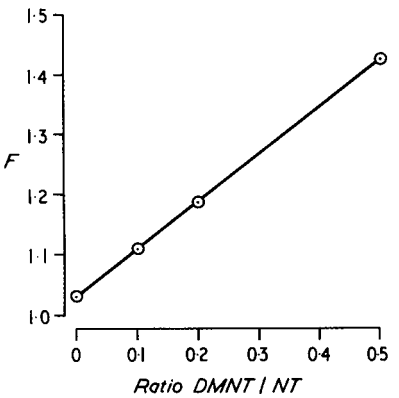

Fig. 1. Mean values of F (from Table I) plotted against r, the ratio of DMNT/NT in the plasma sample. The straight line represents the equation $F = 1.03 + 0.79r$.

is equal to the apparent amount as estimated before SA treatment, multiplied by the factor f/F. From Fig. 1, $f = 1.03$ and $af = 0.79$.

The S.D. of determinations of F (Table I) was approximately constant at 0.02 for NT concentrations of 200, 100 and 40 ng/ml at DMNT:NT ratios of 0, 0.1 and 0.2. This represents a 2% coefficient of variation for the process of SA treatment and rechromatography. At 20 ng/ml NT this coefficient of variation rose to about 3.5%. At a DMNT:NT ratio of 0.5 the S.D. was slightly greater than when less DMNT was present, presumably because of an appreciable contribution from the variance of the proportion of DMNT extracted.

Stability of DMNT

In aqueous solution in 0.01 *M* hydrochloric acid in the refrigerator, DMNT was found to be stable over a period of six months. As a base, however, in solution in heptane containing 0.5% of diethylamine at room temperature, breakdown was appreciable in 24 h. The main DMNT peak diminished, the minor peak of later retention time increased and various new peaks formed.

Drug-free human plasma samples were freshly prepared from heparinized blood, spiked with NT and DMNT and frozen. Samples thawed and estimated at intervals for six months showed no change in drug content.

Plasma levels of DMNT

Sixty-four plasma samples were analysed from 54 different patients being treated with AT. AT concentrations ranged from 8 to 207 ng/ml and NT levels from 8 to 270 ng/ml. The mean value of F was 1.097 ± 0.041 (S.D.) and no correlation could be discerned between F and either drug level. These values of F correspond to a DMNT:NT ratio of 0.084 ± 0.051, and an apparent NT concentration 1.065 ± 0.040 times the true concentration. Plasma DMNT levels ranged from < 1 to 20 ng/ml.

DISCUSSION

Plasma DMNT concentrations were determined by Borgå et al. [3] using

GC with mass spectrometry, a technique not available in many laboratories. In eight patients being treated with NT, NT plasma levels were 14 to 182 ng/ml and the mean DMNT:NT ratio may be calculated as 0.085 ± 0.046, a result closely similar to our own. No information was given on the precision of the method for DMNT, but for NT the coefficient of variation was 5.8%. Hence it seems that most if not all of the observed variation in the DMNT:NT ratio is accounted for by the assay variation and no estimate is possible of the true variation between plasma samples.

In the present study, an appreciable part of the overall variance is attributable to experimental error. Considering only those samples containing at least 200 ng of NT, the mean value of F was 1.095 ± 0.031 (S.D.) (n = 35) while in Table I the S.D. of such determinations was found to be approximately 0.020. Comparison of variances leaves a value of 0.024 for the S.D. of the true variation of F between plasma samples, equivalent to 0.030 in the ratio of DMNT to NT.

Both the average plasma content of DMNT and its variation between subjects are thus small compared with NT levels. GC without derivatization is in common use [4] for the determination of plasma NT and OV-17 (or the closely-related SP2250) are frequently-used liquid phases. However, since the amount of DMNT present averages only 8.4% of the NT, interference is unlikely to be of clinical significance in the estimation of NT levels unless the sensitivity of detection is heavily weighted in favour of DMNT. Our system, with flame-ionization detectors, gave a somewhat lower response to DMNT than to NT (a = 0.77) reducing the average interference to 6.5%.

The salicylaldehyde method also allows DMNT to be determined separately. Whether this metabolite has significant pharmacological action is not known. However, if the hypothesis of the action of tricyclic antidepressants by the inhibition of amine uptake is accepted, then current evidence [5, 6] on the relative activity of AT, NT and their various metabolites suggests that DMNT is unlikely to contribute significantly when present in the low relative concentrations found in patients treated with AT or with NT.

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REFERENCES

1 J.E. Burch, M.A. Raddats and S.G. Thompson, J. Chromatogr., 162 (1979) 351.
2 K. Fredricson Overø, Acta Pharmacol. Toxicol., 31 (1972) 433.
3 O. Borgå, L. Palmér, A. Linnarsson and B. Holmstedt, Anal. Lett., 4 (1971) 837.
4 S. Jones and P. Turner, Postgrad. Med. J., 56 (Suppl. 1) (1980) 94.
5 L. Bertilsson, B. Mellström and F. Sjöqvist, Life Sci., 25 (1979) 1285.
6 J. Hyttel, A.V. Christensen and B. Fjalland, Acta Pharmacol. Toxicol., 47 (1980) 53.

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Note

A simplified method for the gas chromatographic determination of pethidine and norpethidine after derivatization with trichloroethyl chloroformate

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Several analytical procedures have been developed for the assay of plasma samples of the narcotic analgesic drug, pethidine. The majority of these are based on gas chromatography with flame ionization detection without prior derivatization [1—4]. The sensitivity of the methods is low and does not enable the determination of the major plasma metabolite, norpethidine. The simultaneous determination of pethidine and norpethidine has been achieved using gas chromatography—mass spectrometry with selected ion monitoring after acylation of the secondary amine [5, 6]. Recently, an analytical method for the assay of the two amines from plasma samples was presented which used gas chromatography with thermionic detection [7]. The poor gas chromatographic properties of pethidine may limit both precision and sensitivity of the method.

We have previously developed a procedure for the simultaneous assay of pethidine and norpethidine in plasma after conversion to the corresponding carbamate with trichloroethyl chloroformate [8, 9]. The method was extremely sensitive and made analysis possible in 0.1-ml plasma samples. Norpethidine could also be determined after a single therapeutic dose of pethidine [9]. However, since both pethidine and norpethidine form the same derivative with trichloroethyl chloroformate, the two amines must be separated before derivatization. This was accomplished by partition chromatography in a simple column separation.

The separation step was time-consuming and the success of analysis was dependent on the quality of cellulose used in the separation column. Therefore an easier method was required for the processing of a high number of samples [10].

This present paper presents a simplified procedure, where pethidine and norpethidine are separated in the extraction procedure. The secondary amine reacts readily with alkyl chloroformate at room temperature in a two-phase

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system, whereas the tertiary amine only forms carbamate in organic solvent [11, 12].

EXPERIMENTAL

Gas chromatography

A Pye GCV gas chromatograph with flame ionization and electron-capture detectors was used. The glass column (150 cm × 0.2 cm I.D.) was filled with 3% OV-17 on Gas-Chrom Q 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.). The column temperature was 260°C, while injector and detector temperatures were 300°C and 290°C, respectively. Flow of nitrogen carrier gas was 30 ml/min.

Reagents and chemicals

Trichloroethyl chloroformate was purchased from EGA Chemie, Steinheim bei Hedidenheiml, G.F.R. Ethyl chloroformate was from Fluka, Buchs, Switzerland. Alcoholic alkali consisted of 2.8 g of potassium hydroxide in a mixture of 75 g of methanol and 22 g of water. Saturated alcoholic alkali was a saturated solution of potassium hydroxide in methanol. Tetrabutylammonium iodide was from Labkemi, Stockholm, Sweden. *n*-Butanol, methylene chloride and methanol (E. Merck, Darmstadt, G.F.R.) were used without purification, whereas toluene (E. Merck) was distilled before use. Sodium hydroxide 0.5 *M* and phosphate buffers (μ = 0.1) were also used.

Internal standard in the assay of pethidine was the O-butyl analogue (butyl-1-methyl-4-phenyl-4-piperidine carboxylate as hydrochloride) [8]. A solution in water containing 4 μg/ml was used. In the analysis of norpethidine, the O-propyl analogue, propyl-4-phenyl-4-piperidine carboxylate [9], was used in a concentration of 2 μg/ml in water.

Methods

Evaluation of reaction conditions. The derivatization of pethidine and norpethidine was studied as described previously [8]. To 0.25 ml of amine (2×10^{-3} *M*) in toluene, trichloroethyl chloroformate was added in a concentration of 0.4—10% together with about 10 mg of tetrabutylammonium iodide. Hexaeicosane, 0.5 mg/ml, was present as internal marker. The mixture was heated in a metal block and the reaction was quenched by washing with 0.1 *M* sulphuric acid. Analysis was performed by gas chromatography with flame ionization detection and the peak height ratio of formed carbamate to internal marker was calculated.

Determination of pethidine in plasma samples. To a 0.5-ml plasma sample, 0.1 ml of internal standard solution, 0.5 ml of 1 *M* phosphate buffer pH 8.3, and 2 ml of water were added. This mixture was gently shaken for 20 min with 5 ml of toluene containing 50 μl of ethyl chloroformate. After centrifugation at 500 *g* for 10 min, the organic phase was transferred to another tube. One ml of 0.1 *M* phosphate buffer pH 1.9 was added and the tube shaken for 10 min and centrifuged. The aqueous phase was made alkaline, 0.25 ml of toluene was added and the mixture was shaken for 15 min and thereafter centrifuged for 5 min. The organic phase was transferred to another tube and

10 μl of trichloroethyl chloroformate and about 10 mg of tetrabutylammonium iodide were added. The reaction tube was heated for 1 h at 100°C in a metal block. Alcoholic alkali solution, 1.0 ml, was added to the reaction mixture and shaken for 10 min. After that, 1 ml of water was added and the mixture shaken for another 10 min. After centrifugation, the aqueous phase was discarded followed by the addition of 0.5 ml of saturated alcoholic alkali. The tube was shaken vigorously for 15 sec and 1 ml of water was added. A 1—2 μl volume of the organic phase was taken to analysis with electron-capture gas chromatography.

Determination of norpethidine in plasma. A 0.5 ml plasma sample containing norpethidine was taken to analysis. To the sample 0.1 ml of the internal standard solution, 0.5 ml of 0.5 *M* sodium hydroxide and 2 ml of water were added and shaken with 5 ml of a mixture of *n*-butanol—methylene chloride (1:4) for 10 min. After centrifugation, the organic phase was transferred to another tube and 1 ml of 0.1 *M* sulphuric acid was added. The mixture was shaken for 10 min and after centrifugation the aqueous phase was transferred to another tube containing 0.5 ml of 1 *M* phosphate buffer pH 7.3, and thereafter 0.3 ml of toluene containing 1% trichloroethyl chloroformate was added. The mixture was shaken for 20 min and excess reagent was removed as described above. Analysis was made as for pethidine.

Standard curves for pethidine and norpethidine, respectively, were prepared in parallel by treating known concentrations of pethidine and norpethidine in blank plasma according to the procedures above.

RESULTS AND DISCUSSION

Separation of pethidine and norpethidine

Since both pethidine and its N-demethylated metabolite, norpethidine, form the same carbamate with trichloroethyl chloroformate, the two amines must be quantitatively separated before derivatization. This was easily achieved in the first extraction step from plasma, as the secondary amine readily reacted with ethyl chloroformate in the two-phase system, while the tertiary amine was left underivatized. A quantitative removal of norpethidine with this method was performed even with a very low reagent concentration, e.g. $> 2 \times 10^{-5}$ *M*. pH 8.3 was chosen in order to obtain a quantitative extraction of pethidine as base into the organic phase [8]. At pH > 12, hydrolysis of the ester function of both pethidine and norpethidine occurred giving the corresponding ethoxycarbonyl esters on reaction with ethyl chloroformate.

The ethyl carbamate of norpethidine has a high distribution to organic phase while pethidine was quantitatively extracted to acidic aqueous phase in the second extraction step.

The different reactivity between a secondary and a tertiary amine to chloroformate has been used previously for separation before derivatization [13]. This method is very time-saving compared to the previous separation by partition chromatography [8]. The number of samples processed in one day is increased to twenty samples plus the standard samples.

Trichloroethyl chloroformate reaction with pethidine

Pethidine reacts with chloroformate reagent to the formation of the corresponding carbamate and methylamine. The reaction proceeds via a reactive intermediate ion [12]. The reaction is promoted by the addition of anhydrous sodium carbonate. The effect of anhydrous sodium carbonate is most likely to be a removal of hydrolytic compounds, which will compete in the reaction with the intermediate ion.

The formation rate of carbamate with trichloroethyl chloroformate was low, probably due to the degradation of the intermediate ion by a competing and rapid hydrolysis or by other mechanisms [12]. By addition of a strong nucleophilic agent such as iodide in the form of tetrabutylammonium iodide to the reaction mixture, the reaction rate for the carbamate formation was increased at the expense of the hydrolysis reaction [11].

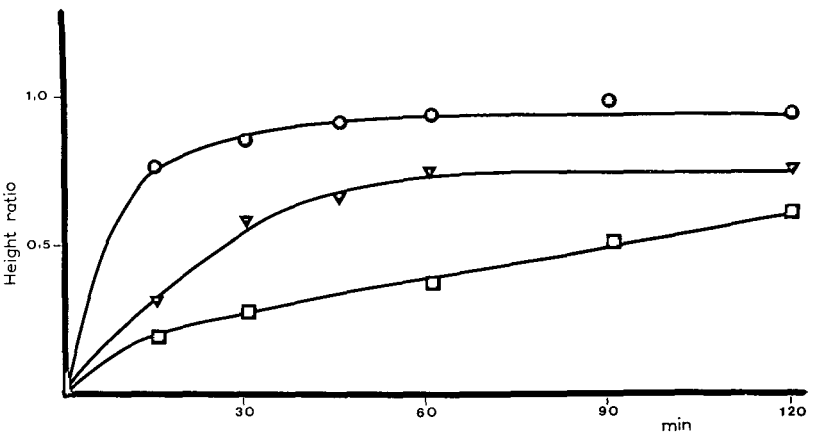

Fig. 1. Time dependence of trichloroethyl carbamate formation from pethidine. Sample concentration 2 × 10^{-3} *M*. Temperature 80°C. (•——•), 10% reagent with tetrabutylammonium iodide present; (▾——▾), 10% reagent with sodium carbonate present; (▪——▪), 1% reagent with tetrabutylammonium iodide present.

The favourable effect of iodide on the reaction rate as compared to anhydrous sodium carbonate is shown in Fig. 1, where the formation of carbamate was studied at 80°C. Although the reaction rate was increased after the change of catalyst, a quantitative formation of carbamate required a reaction time of 1 h at 100°C. The reagent concentration was lower, 0.3 *M* compared to 0.7 *M* used previously. In the concentration range 0.03—0.3 *M* of reagent, the time for quantitative reaction was almost identical. At higher concentrations of trichlorethyl chloroformate the reaction rate decreased. This is probably due to the liberation of acid in the reaction which may hamper the carbamate formation. This low reagent concentration increased both sensitivity and selectivity of the method owing to less disturbance in the chromatogram. Purification of the reaction mixture was performed with alcoholic alkali solution in order to hydrolyze bis(trichloroethyl) carbonate formed in the reaction [8].

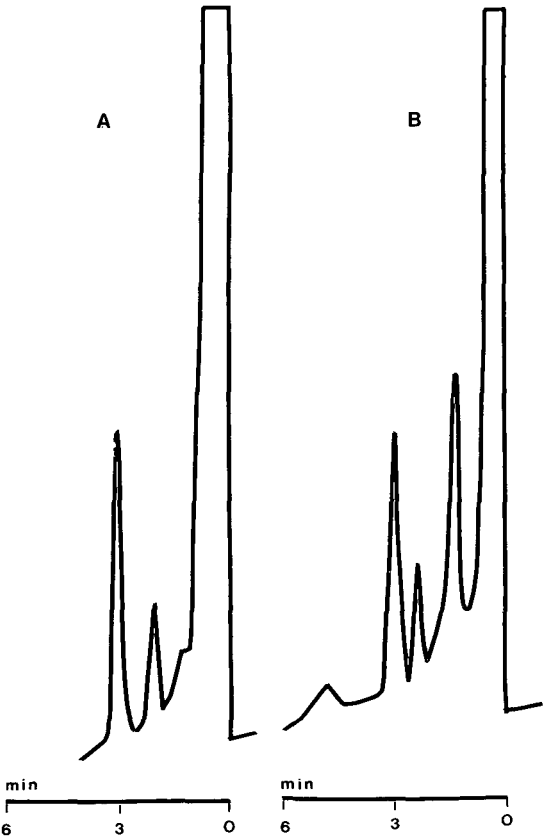

Fig. 2. Gas chromatograms of plasma samples with: (A) pethidine as trichloroethyl carbamate. Internal standard: O-butyl analogue of pethidine. (B) norpethidine as trichloroethyl carbamate. Internal standard: O-propyl analogue of norpethidine.

Application to the analysis of plasma samples of pethidine and norpethidine

The simplified method has now been used for the determination of pethidine and norpethidine in patient plasma samples for more than two years [10]. Plasma norpethidine can be determined in a separate run after selective isolation from plasma by solvent extraction.

Derivatization of norpethidine with trichloroethyl chloroformate was achieved using a two-phase procedure with an aqueous phase of pH 8.3. It was shown that pethidine did not interfere in the assay of norpethidine. Norpethidine is the only metabolite from pethidine which has been found in plasma. On continuous therapy with pethidine for several days, the plasma concentrations of norpethidine are low.

Pethidine and norpethidine could be detected below 10 ng in a 0.5-ml plasma sample and quantitative determinations were performed above 20 ng/ml with a relative standard deviation less than 10% (n = 8) for each compound. A chromatogram is shown in Fig. 2. The absolute recovery for pethidine and norpethidine through the respective methods was 92% and 86%, respectively. A comparison between this simplified method and the previous method with

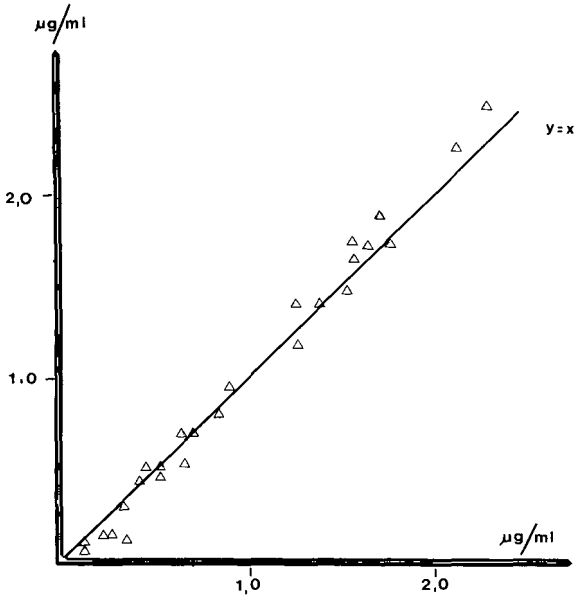

Fig. 3. Plasma concentration of pethidine obtained by the method with chromatographic separation (ordinate) and the present simplified method (abscissa). Line of best fit $Y = 1.09X - 0.07$ ($n = 27$, $r = 0.994$).

chromatographic isolation of pethidine was carried out in the analysis of a number of patient plasma samples. After plotting the results of the present method against those from the old method (Fig. 3), a line of best fit $Y = 1.09X - 0.07$ ($n = 27$, $r = 0.994$) was obtained. Thus, the agreement between the two methods was close.

REFERENCES

1 J.A. Knowles, G.R. White and H.R. Ruelius, Anal. Lett., 6 (1973) 281.
2 T.J. Goehl and C. Davidson, J. Pharm. Sci., 62 (1973) 907.
3 K. Chan, M.J. Kendall and M. Mitchard, J. Chromatogr., 89 (1974) 169.
4 L.E. Mather and G.T. Tucker, J. Pharm. Sci., 63 (1974) 306.
5 C. Lindberg, M. Berg, L.O. Boréus, P. Hartvig, K.E. Karlsson, L. Palmér and A.M. Thörnblad, Biomed. Mass Spectrom., 5 (1978) 540.
6 R.K. Verbeeck, R.C. James, D.F. Taber, B.J. Sweetman and G.R. Wilkinson, Biomed. Mass Spectrom., 7 (1980) 58.
7 P. Jacob, III, J. Rigod and J.M. Pond, J. Pharm. Sci., 71 (1982) 166.
8 P. Hartvig, K.-E. Karlsson, L. Johansson and C. Lindberg, J. Chromatogr., 121 (1976) 235.
9 P. Hartvig, K.E. Karlsson, C. Lindberg and L.O. Boréus, Eur. J. Clin. Pharmacol., 11 (1977) 65.
10 B. Zarén, P. Hartvig and A. Tamsen, Anaesthesiol. Intensive Care Med., in press.
11 K.E. Karlsson and P. Hartvig, Acta Pharm. Suecica, 18 (1981) 337.
12 K.E. Karlsson and P. Hartvig, Acta Pharm. Suecica, 17 (1980) 249.
13 T. Yamaguchi, S. Yoshikawa, Y. Sekine and M. Hashimoto, J. Chromatogr., 173 (1979) 147.

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Note

A simple and sensitive method for the determination of perhexiline in plasma using gas—liquid chromatography with nitrogen—phosphorus detection

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Perhexiline is a calcium agonist used in the treatment of angina pectoris. The use of this drug is limited to patients who do not respond to conventional drug therapy and who are considered unsuitable for surgery. The reason for the poor general acceptance of perhexiline is the incidence of serious side effects that have been reported to occur during treatment with the drug [1].

Although it has been suggested that the kinetics of the drug are non-linear, very little is known of the disposition of perhexiline [2]. To a large extent this is due to poor analytical methods available for the measurement of perhexiline. Available methods have used laborious extraction techniques and then measurement by gas—liquid chromatography (GLC) with either flame ionization or electron-capture detection [3—5]. Detection by electron capture requires derivatisation with trifluoroacetic anhydride or heptafluorobutyric anhydride. Similarly, a high-performance liquid chromatographic (HPLC) method has recently been developed which also involves an extraction procedure and then derivatisation (with Dns chloride) to form a fluorescent product [2]. While this method has good sensitivity, the chromatography time is long.

The GLC method described here is precise, reproducible and requires only a protein precipitation step followed by a back extraction into solvent. No solvent evaporation or derivatisation procedure is required and the use of nitrogen—phosphorus detection provides good specificity and sensitivity.

EXPERIMENTAL

Analyses were performed on a Hewlett-Packard 5730A gas chromatograph fitted with a nitrogen—phosphorus detector. The following chromatographic conditions were employed: glass column (1.6 m × 2 mm I.D.) containing 3%

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OV-101 on Gas-Chrom Q, 100—120 mesh (Applied Sciences Labs., State College, PA, U.S.A.), column temperature 218°C, injector port and detector temperatures 250°C and nitrogen carrier gas flow-rate 20 ml/min.

Reagents and standards

A pure sample of perhexiline maleate was supplied by the William S. Merrell Company (Sydney, Australia) and benzhexol, the internal standard, was extracted from a tablet preparation (Artane, Cyanamid Australia, Sydney, Australia). Silyl-8 was supplied by Pierce (Rockford, IL, U.S.A.).

A standard solution of perhexiline was prepared by dissolving perhexiline maleate (0.14188 g) in 100 ml of ethanol giving a concentration of 1000 mg/l perhexiline. A 10-ml aliquot of this was further diluted to 100 ml with 1.0 *N* hydrochloric acid giving a standard solution containing 100 mg/l perhexiline. Working standard solutions containing 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l perhexiline were prepared daily by making dilutions from the 100 mg/l stock solution with distilled water. Plasma standard solutions containing 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/l perhexiline were prepared by taking 0.1-ml aliquots of the working standards and adding 0.9 ml of drug-free plasma.

The internal standard was prepared by extracting a tablet containing benzhexol hydrochloride (5 mg) with 30 ml of methanol. The extraction was aided by breaking up the tablet with a spatula while shaking with methanol. The insoluble tablet matrix was removed by filtration through a Whatman No. 1 filter. The filtrate was made up to 100 ml with methanol. A 1.0-ml aliquot of this solution was then diluted to 50 ml with methanol to give a final concentration of approximately 1.0 mg/l.

Extraction

To a 5-ml disposable plastic tube fitted with a cap was added 1.0 ml of plasma (patient sample or standard) followed by 1.0 ml of methanol containing the internal standard, and then 0.5 ml of 5 *N* hydrochloric acid. The mixture was vortex mixed for 3 min and the samples allowed to settle for 5 min before centrifuging at 1500 *g* for 15 min. The supernatant solutions were decanted into conical centrifuge tubes followed by the addition of 0.5 ml 8 *N* sodium hydroxide. The tubes were allowed to cool and then 0.1 ml of 1,2-dichloroethane—hexane (8:2) was added. The contents of the centrifuge tubes were vortex mixed for 1 min and then centrifuged at 1500 *g* for 2 min to partition the aqueous and organic phases. The vortex mixing and centrifugation steps were repeated and finally 3.0 μl of the lower organic phase taken for injection into the gas chromatograph.

RESULTS AND DISCUSSION

Protein precipitation with reagents such as acetonitrile, methanol and aqueous solutions of trichloroacetic acid or mineral acids is frequently used in the preparation of plasma samples for analysis by HPLC [6]. The procedure is simple and rapid but nevertheless provides a matrix of suitable purity for analysis by this technique. However, injection of the plasma supernatant into a gas—liquid chromatograph after a protein precipitation step produces problems.

Firstly, endogenous material from plasma may interfere by giving an excessively large solvent front and pyrolysis products of plasma material could give a noisy baseline and extraneous peaks. Secondly, the small volume capacity of GLC (usually 10 μl) prevents the injection of larger volumes to improve poor sensitivity resulting from sample dilution by the protein precipitating agent. These two problems can be solved by extraction into a smaller volume of organic solvent after the protein precipitation procedure. The organic phase is then cleaner and has a higher concentration of analyte, thus making it suitable for analysis by GLC. Protein precipitation is quick and involves relatively small amounts of sample and reagent in comparison with other published methods. Although the use of a preliminary protein precipitation step in a GLC method is unusual, extremely clean chromatograms can be obtained. Fig. 1A is the chromatogram of a blank plasma sample showing it to be free of endogenous peaks which may interfere with perhexiline or the internal standard.

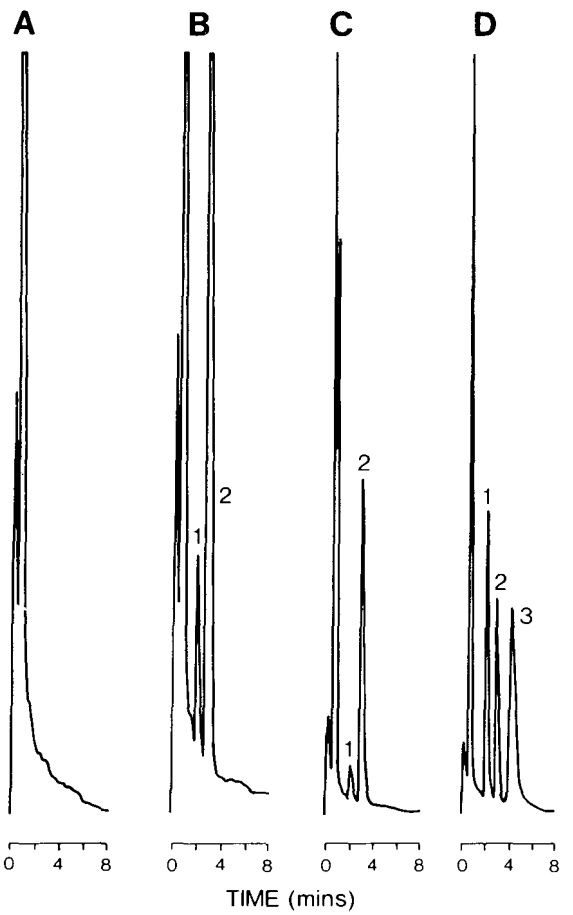

Fig. 1. GLC tracings of extracted plasma samples containing perhexiline and the internal standard, benzhexol. A, plasma blank; B, plasma perhexiline standard at 0.1 mg/l; C, plasma perhexiline standard at 0.1 mg/l but recorded at a 5-times reduced sensitivity of B; D, patient sample. Peaks: 1 = perhexiline, 2 = the internal standard, benzhexol, and 3 = an extraneous substance in the plasma.

Initially, a double extraction technique was investigated in order to obtain a clean and more concentrated sample for analysis. Various organic solvents were evaluated in extracting perhexiline from an alkaline sample of plasma and it was found that the following solvents extracted perhexiline in order of increasing recovery, diethyl ether < hexane < toluene < ethyl acetate. However, the quantitative back extraction of perhexiline from these organic solvents into a smaller volume of acid solution was not possible because the salts of perhexiline are very soluble in organic solvents. Of the following acids, hydrochloric, perchloric, nitric, sulphuric and phosphoric, only phosphoric acid gave a salt that would back extract into an aqueous phase to any significant extent. Attempted isolation of the acid extract by freezing, decanting of the organic phase then alkalinisation and extraction into chloroform also gave low recoveries and was not reproducible.

An alternative approach was to recover the perhexiline after extraction from plasma with an organic solvent. Although this technique is successful, as shown by Cooper and Turnell [3], the time involved in processing large numbers of samples was considered unacceptable.

In the method described in this paper, methanol and hydrochloric acid are used to precipitate protein. Protein precipitation using only concentrated acid gave very low recoveries (10%), presumably because the perhexiline binds to the protein pellet. Methanol is essential for high recoveries and the ratio of reagents has been optimised to give the best results. The following acids, 10% trichloroacetic, 10% perchloric and 5 *N* nitric were also tried in conjunction with methanol as precipitating agents and all were inferior to 5 *N* hydrochloric acid and methanol.

In the extraction procedure, dichloromethane and chloroform were tested but both are unsuitable because they evaporate easily during vortex mixing in the final extraction step. The less volatile 1,2-dichloroethane works well and the specificity of the extraction is improved by adding hexane (20%).

All the chromatopgraphic data were recorded on a dual-pen recorder with the pens set at 1 and 5 mV for full scale deflection. The attenuation of the GLC was set at 128 and with this arrangement the peak heights for perhexiline could be measured over the concentration range of 0.02—1.00 mg/l. Figs. 1B and C are chromatograms obtained for a 0.10 mg/l standard where B has 5 times the sensitivity of C. Fig. 1D is the chromatogram of a typical patient sample having a perhexiline concentration of 1.08 mg/l. The retention times of perhexiline and benzhexol (internal standard) are 2 min 24 sec and 3 min 30 sec, respectively.

The calibration curve is linear over the range 0.02—1.00 mg/l when peak height ratios for the standards are plotted against concentration. Although peak symmetry and linearity deteriorate with time, this can be corrected by injection of Silyl-8 and all new columns should be silanised with the reagent during the conditioning period.

Blank plasma was spiked with perhexiline to give solutions containing 0.05 and 0.50 mg/l. Each test solution was analysed in replicate on a between-day and within-day assessment. For the between-day analysis at a concentration of 0.05 mg/l (n = 8), the mean was 0.0484 ± 0.0026 S.D. with a coefficient of variation of 5.4%. At 0.50 mg/l (n =8), the mean was 0.4673 ± 0.0282 S.D. with a coefficient of variation of 6.0%. For the within-day analysis at the 0.05

mg/l concentration (n =10), the mean was 0.0463 ± 0.0013 S.D. with a coefficient of variation of 2.8%. Similarly, within-day analysis at the 0.50 mg/l sample gave a mean of 0.4861 ± 0.0072 S.D. with a coefficient of variation of 1.5%.

The recovery of perhexiline was determined by comparing (in triplicate) the peak heights for perhexiline obtained from plasma standards containing 0.1, 0.5 and 1.00 mg/l with standards made up in dichloromethane obtaining 1.0, 5.0 and 10.0 mg/l. Using this procedure the recovery of perhexiline was shown to be essentially quantitative, with the mean recovery over the 0.1 to 1.00 mg/l range being 94%. The recovery for the internal standard was determined in the same way (in triplicate) and was 103%.

Drugs that are used to supplement perhexiline therapy do not interfere with this assay procedure. These are beta-adrenoreceptor antagonists, metoprolol, pindalol, propranolol, labetalol, alprenolol, timolol, practolol and antenolol, the antiarrhythmic drugs mexiletine, procainamide, disopyramide and quinidine; and other drugs used in the treatment of angina, sorbide nitrate, nitroglycerine and verapamil.

Drugs that are extracted and interfere are the tricyclic antidepressants, amitriptyline, nortriptyline and doxepin. These are not used in the treatment of angina but may be encountered occasionally.

REFERENCES

1 M. Chevais, Rev. Med., 22 (1981) 335.
2 J.D. Horowitz, P.M. Morris, O.H. Drummer, A.J. Goble and W.J. Louis, J. Pharm. Sci., 70 (1981) 320.
3 J.D.H. Cooper and D.C. Turnell, Ann. Clin. Biochem., 17 (1980) 155.
4 G.J. Wright, G.A. Leeson, A.V. Zeiger and J.F. Lang, Postgrad. Med. J., April Suppl. (1973) 8.
5 E. Singlas, M.A. Ganjet and P. Simon, Eur. J. Clin. Pharmacol., 14 (1978) 195.
6 P.J. Meffin and J.O. Miners, in J.W. Bridges and L.F. Chasseaud (Editors), Progress in Drug Metabolism, Vol. 4, Wiley Interscience, New York, 1980, pp. 261—307.

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Note

Rapid and sensitive method for the determination of salicylic acid in serum by reversed-phase ion-pair high-performance liquid chromatography

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We have undertaken several studies which involve antithrombotic and non-steroidal anti-inflammatory agents. Most of these studies required patient abstinence from ingestion of pharmaceutical preparations containing salicylic acid. We required a method of salicylate determination suitable for application to the large number of serum specimens to be screened during the course of these projects. The short serum half-life of salicylate in man [1] complicated this requirement, since sensitivity far greater than that necessary for therapeutic salicylate level determination would be essential for detection of salicylate in serum more than 24 h after ingestion. The serum salicylate assay methods currently available include colorimetric methods [2—4], gas chromatographic [5, 6], thin-layer chromatographic [7], and high-performance liquid chromatographic (HPLC) procedures [8—18]. The colorimetric methods are limited by both a lack of specificity for salicylic acid [2—4] and marked inter-laboratory variation in results [19]. Gas chromatography of salicylic acid requires sample derivatization prior to separation [5, 6] which limits its convenience in handling large numbers of samples. High-performance thin-layer chromatography is less sensitive than modern HPLC determination methods and suffers from poor reproducibility [7]. Some authors describe HPLC assay methods which provide detection limits for salicylate in serum of 1 μg/ml, which would be adequate for screening applications [9, 12—15, 17, 18].

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However, none of these methods is adequately rapid for routine application to large numbers of samples. We also noted in preliminary trials with some of these separation procedures that salicylate often was unresolved from comparatively large quantities of endogenous interfering serum substances.

This paper presents a method for determination of serum salicylate involving extraction of salicylate and an internal standard from acidified serum, separation from interfering compounds by reversed-phase ion-pair HPLC, and absorbance detection at 301 nm. The detection limit corresponds with 45 ng injected into the chromatograph, and the total chromatographic separation time is 4.2 min. The reversed-phase ion-pair separation mechanism provides excellent chromatographic selectivity for salicylic acid and the internal standard with respect to both endogenous interference and 27 therapeutic drugs tested.

EXPERIMENTAL

Materials

Oxalic acid, tetrabutylammonium hydrogen sulfate, salicylic acid, and 3-methylsalicylic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sulfuric acid, sodium hydroxide, and ethyl acetate were purchased from Fisher Scientific (Cleveland, OH, U.S.A.). Acetonitrile (non-spectro grade) and methanol were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Equipment

The liquid chromatograph consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model M6000A pump, WISP 710B automatic sample injector, and RCM-100 radial compression module. A precolumn constructed from zero dead volume chromatographic unions (Crawford Fitting Co., Solon, OH, U.S.A.) and packed with the reversed-phase pellicular chromatographic medium Co:Pell-ODS (Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column against particulate sample contamination. A Perkin-Elmer (Norwalk, CT, U.S.A.) Model LC-75 variable-wavelength absorbance detector was used to monitor the absorbance of the eluent stream; the detector output was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. All peak height and area measurements and quantitative calculations were performed by a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354 chromatographic data system.

The analytical separation was carried out on a 10 cm × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm nominal particle diameter, Waters Assoc.). The chromatographic mobile phase was aqueous 0.1 *M* oxalic acid + 0.005 *M* tetrabutylammonium hydrogen sulfate (pH 4.00)—acetonitrile (75 : 25). The pH of the aqueous portion of the mobile phase was adjusted with solid sodium hydroxide prior to the addition of acetonitrile. The solvent pump was operated at a flow-rate of 5.0 ml/min.

Sample preparation

Standard solutions were prepared over a concentration range of 0.6—20 μg/ml by addition of measured quantities of salicylic acid stock solution to

chromatographically proven salicylate-free human serum. Serum aliquots of 500 μl were pipetted into 150 × 16 mm glass culture tubes. To these were added 50 μl of a 50 μg/ml solution in methanol—water (50:50) of the 3-methylsalicylic acid internal standard and 1 ml of 1 *M* sulfuric acid. The tube contents were vortexed briefly, 5 ml of ethyl acetate added, and the tubes vortexed continuously for 2 min. The tubes were centrifuged for 10 min at 1500 *g* to facilitate phase separation. The organic phase was transferred to clean 150 × 16 mm test tubes and evaporated to dryness at room temperature under a stream of dry, oil free compressed air. The residues were reconsituted in 200 μl of methanol—water (50 : 50) and 30-μl aliquots of this solution were injected into the chromatograph.

Reproducibility and recovery demonstration

Injection reproducibility was demonstrated by ten replicate injections of a solution containing both salicylic acid and the internal standard. Extraction efficiency and recovery experiments were performed by addition of 5-μg quantities of salicylic acid to the usual 500-μl serum aliquot, extraction, reconstitution in 200 μl of methanol—water (50 : 50) containing 5 μg 3-methylsalicylic acid, and injection of a 30-μl aliquot into the chromatograph. The salicylic acid: 3-methyl-salicylic acid peak height and area ratios obtained with experimental specimens were compared with the ratios obtained upon injection of a 30-μl aliquot of a methanol—water (50 : 50) solution containing 25 μg/ml each of salicylic acid and 3-methyl-salicylic acid; thus, this reference solution aliquot contained the absolute quantity of salicylic acid expected to be present upon complete extraction of salicylate from the experimental serum specimens. Apparent variations in extraction efficiency among the solvents investigated for experimental suitability were considered to be insignificant when smaller than the demonstrated injection reproducibility.

RESULTS AND DISCUSSION

We were unable to duplicate the recovery and reproducibility claimed by the authors of several published liquid chromatographic serum salicylate determination procedures. Experiments designed to investigate the dependence of extraction efficiency upon the identity and concentration of the mineral acid used for sample acidification were conducted. No significant differences in recovery were noted among experimental groups of six specimens acidified with 2 *M* sulfuric, 1 *M* sulfuric, 0.6 *M* hydrochloric, 1.2 *M* hydrochloric, 3.5 *M* phosphoric, 7% perchloric, and 14% perchloric acids and subsequently extracted with any one of the organic solvents used. The use of perchloric acid resulted in a flocculent precipitation of serum protein which was difficult to separate cleanly from the organic phase after sample extraction. Significant variation in the recovery of salicylic acid with the identity of the organic solvent used for extraction of serum aliquots acidified with 1 *M* sulfuric acid was observed. These results are presented in Table I. The recovery of salicylic acid from acidified serum containing 10 μg/ml of salicylate and extracted according to the outlined procedure was 95 ± 4%.

TABLE I

SALICYLIC ACID RECOVERY WITH SELECTED ORGANIC SOLVENTS

Recovery of 10 μg/ml salicylic acid from 500-μl serum aliquots acidified with 1 ml of 1 *M* sulfuric acid and extracted with 5 ml of solvent.

Solvent	Recovery (%) (n = 6)
Diethyl ether	17.9 ± 3.4
Toluene	41.1 ± 2.7
Butyl acetate	36.2 ± 7.8
Ethyl acetate	94.7 ± 3.6
Chloroform	72.4 ± 4.9
Isooctane—isopropanol (85 : 15)	37.8 ± 6.3

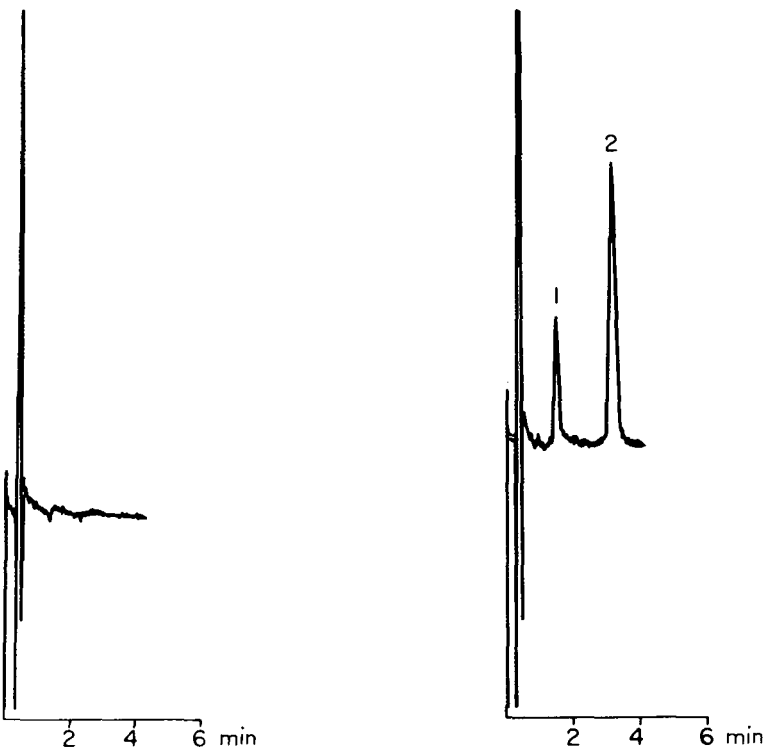

Fig. 1. Chromatogram obtained upon extraction of a salicylate-free serum specimen without added internal standard according to the outlined experimental procedure. The column was a 10 cm × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm nominal particle diameter). The chromatographic eluent was aqueous 0.1 *M* oxalic acid + 0.005 *M* tetrabutylammonium hydrogen sulfate (pH 4.00)—acetonitrile (75 : 25) and was pumped at a flow-rate of 5.0 ml/min. The absorbance detector was operated at 301 nm. The full scale of the ordinate is 0.005 absorbance units.

Fig. 2. Chromatogram obtained upon preparation of a serum aliquot containing 1.25 μg/ml salicylic acid and internal standard according to the described method. The chromatographic conditions were as described in Fig. 1. Salicylic acid (1) was eluted at 1.6 min; the internal standard 3-methyl-salicylic acid (2) was eluted at 3.2 min.

The chromatographic eluent system used was a slight modification of one developed by Waters Assoc. for quality assurance of an over-the-counter pharmaceutical preparation [20]. A chromatogram of a processed salicylate-free serum specimen is shown in Fig. 1. Fig. 2 is a chromatogram of a processed serum sample containing 1.25 μg/ml salicylic acid and added internal standard. The detection limit for salicylate (at a signal-to-noise ratio greater than 5 : 1) was found to correspond with 45 ng injected into the chromatograph. Especially noteworthy are the complete chromatographic separation time of 4.2 min, the extreme stability of the instrument signal baseline at maximum detector sensitivity, and the selectivity of the eluent and column combination for the compounds of interest relative to endogenous or medicinal interference (Table II). We infrequently noticed a chromatographic peak of an endogenous compound present in some serum specimens; this was eluted prior to salicylate and was completely resolved from the salicylate peak when both were present. The column has been used for assay development and routine determinations of more than 1600 serum specimens without undergoing noticeable deterioration in performance.

TABLE II

DRUGS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Duplicate 30-μl injections of each drug at a concentration of 1 mg/ml in methanol—water (50 : 50).

Non-steroidal anti-inflammatory agents	Anti-neoplastic agents	Miscellaneous
Phenylbutazone	5-Fluorouracil	Allopurinol
Naproxen	Doxorubicin	Theophylline
Fenoprofen	Vincristine	Procainamide
Ibuprofen	Vinblastine	Quinidine
Tolmetin	Cyclophosphamide	Furosemide
Zomepirac	Mutamycin	Phenytoin
Sulindac	Vindesine	Phenobarbital
Indomethacin	Cytosine arabinoside	Carbamzepine
Aspirin(O-acetylsalicylic acid)	Carmustine (BCNU)	Dipyridamole

Standard curves of salicylic acid : internal standard peak height ratios vs. sample salicylate concentration were linear (r^2 = 0.993) with a slightly positive y intercept of 0.05; this intercept value was less than 20% of the peak height ratio obtained for the standard solution containing the smallest salicylate concentration used for establishment of standard curves. The relative standard deviation for ten replicate injections of a single prepared serum specimen containing 10 μg/ml salicylate was 1.7%. The relative standard deviation in ten replicate determinations performed upon a serum specimen containing 10 μg/ml salicylate was 3.9%.

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REFERENCES

1 G. Levy, Drug. Metab. Rev., 9 (1979) 3.
2 B.B. Brodie, S. Udenfriend and A.F. Coburn, J. Pharm., 80 (1944) 114.
3 P. Trinder, Biochem. J., 57 (1954) 301.
4 L.A. Muni, J.L. Leeling, R.J. Helms, W. Johnson, J.J. Bare and B.M. Phillips, J. Pharm. Sci., 67 (1978) 289.
5 M.J. Rance, B.J. Jordan and J.D. Nichols, J. Pharm. Pharmacol., 27 (1975) 425.
6 R.N. Galante, J.C. Egoville, A.J. Visalli and D.M. Patel, J. Pharm. Sci., 70 (1981) 167.
7 G. Drehsen and P. Rohdewald, J. Chromatogr., 223 (1981) 479.
8 D. Blair, B.H. Rumack and R.G. Peterson, Clin. Chem., 24 (1978) 1543.
9 B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff and M. Rowland, Clin. Chem., 25 (1979) 1420.
10 E. Wahlin-Boll, B. Brantmark, A. Hanson, A. Melander and C. Nilsson, Eur. J. Clin. Pharmacol., 20 (1981) 375.
11 C.P. Terweij-Groen, T. Vahlkamp and J.C. Kraak, J. Chromatogr., 145 (1978) 115.
12 C.P. Terweij-Groen, S. Heemstra and J.C. Kraak, J. Chromatogr., 181 (1980) 385.
13 L.Y. Lo and A. Bye, J. Chromatogr., 181 (1980) 473.
14 R.H. Rumble, M.S. Roberts and S. Wanwimolrak, J. Chromatogr., 225 (1981) 252.
15 G.W. Peng, M.A.F. Gadalla, V. Smith, A. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 710.
16 V. Das Gupta, J. Pharm. Sci., 69 (1980) 113.
17 L.I. Harrison, M.L. Funk and R.E. Ober, J. Pharm. Sci., 69 (1980) 1268.
18 J.N. Buskin, R.A. Upton and R.L. Williams, Clin. Chem., 28 (1982) 1200.
19 K.D. Mutchie, G.H. Saunders, A.S. Hanissian and T.E. Poe, J. Rheumatol., 7 (1980) 737.
20 Waters Assoc., personal communication.

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Note

Determination of therapeutic concentrations of codeine by high-performance liquid chromatography

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Various analytical methods have been reported for the determination of codeine, a narcotic analgesic and antitussive drug. Gas—liquid chromatographic methods were often used to determine codeine in various media [1—3], sometimes in combination with thin-layer chromatography [4] or mass spectrometry[5]. Several high-performance liquid chromatographic (HPLC) methods have been described using reversed-phase or ion-pair systems [6—9]. In addition, a radioimmunoassay procedure has been reported to study the disposition of codeine in man [10]. However, not all these methods are always available, and some of them are not sensitive enough for measuring therapeutic plasma levels (10—200 ng/ml) after a single dose of codeine phosphate (60 mg).

In order to study the biopharmaceutics of codeine after oral and rectal administration in man, we developed a rapid and accurate determination of codeine by straight-phase HPLC with a detection limit of 5 ng/ml. Methadone hydrochloride was used as an internal standard.

EXPERIMENTAL

Chromatographic system

The analyses were performed on a Waters liquid chromatograph consisting of a 6000A pump, a U6K injector and Model 440 UV detector set at 254 nm and operating at 0.005 AUFS (Waters Assoc., Milford MA, U.S.A.). A μPorasil (10 μm) straight-phase column (30 cm × 3.9 mm I.D.) was used (Waters Assoc.) guarded with a pre-column of Vydac 101 SC, 10 cm × 2.1 mm I.D. (Chrompack, Middelburg, The Netherlands). The eluent was dichloromethane—

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methanol—ammonia solution (33%) (90:10:0.1) with a flow-rate of 1.5 ml/min resulting in a back-pressure of 6.9 MPa (1000 p.s.i.).

Materials

Reagent-grade dichloromethane, methanol and ammonia solution (33%) were obtained from Merck (Darmstadt, G.F.R.); codeine phosphate (Ph.Eur.) and methadone hydrochloride (Ph.Eur.) were from Brocacef (Maarssen, The Netherlands). Standard solutions of 2 μg/ml codeine phosphate in dichloromethane and 80 ng/ml methadone hydrochloride in dichloromethane were made.

Methods

To 1.0 ml plasma in a 20-ml glass-stoppered tube, 1.0 ml of ammonia solution (10%) and 4.0 ml of the methadone hydrochloride solution were added. After mixing for 1 min on a Vortex mixer and centrifugation for 5 min at 2500 *g*, most of the upper layer was removed. The tube was held in liquified nitrogen to freeze the rest of the upper layer. The dichloromethane layer was poured into a smaller tube and the volume was reduced to 200—300 μl by evaporation. The whole residue was injected into the liquid chromatograph.

The retention times were: codeine 6.3 min and methadone 7.2 min. Peak height ratios were used to calculate codeine concentrations, based on standard curves prepared from spiked plasma samples (Table I).

With 1.0-ml plasma samples this method is accurate to concentrations as low as 5 ng/ml codeine.

RESULTS AND DISCUSSION

A typical chromatogram from a plasma extract is shown in Fig. 1. For a good separation the amount of ammonia in the eluent turned out to be very critical. During storage and the analysis ammonia is gradually lost from the mobile phase mixture. Therefore fresh solvent should be used daily in order to keep retention times constant and to maintain effective separation.

The standard curve was linear over the range 10—160 ng/ml ($y = 0.0061x - 0.0028$; $r = 0.9993$). From Table I it can be calculated that the mean recovery of extraction from plasma is 99.8% ± 5.3%.

TABLE I

REPRODUCIBILITY OF THE MEASUREMENT OF CODEINE AT VARIOUS PLASMA CONCENTRATIONS

Codeine (ng/ml)		*n*	± S.D. (ng/ml)	Coefficient of variation (%)	Recovery (%)
Added	Found				
10	10.9	7	0.9	8.3	109.0
20	18.6	7	1.7	9.1	93.0
40	38.9	7	1.9	4.9	97.3
80	81.0	7	4.0	4.9	101.3
120	118.8	7	4.6	3.9	99.0
160	158.6	7	5.0	3.2	99.1

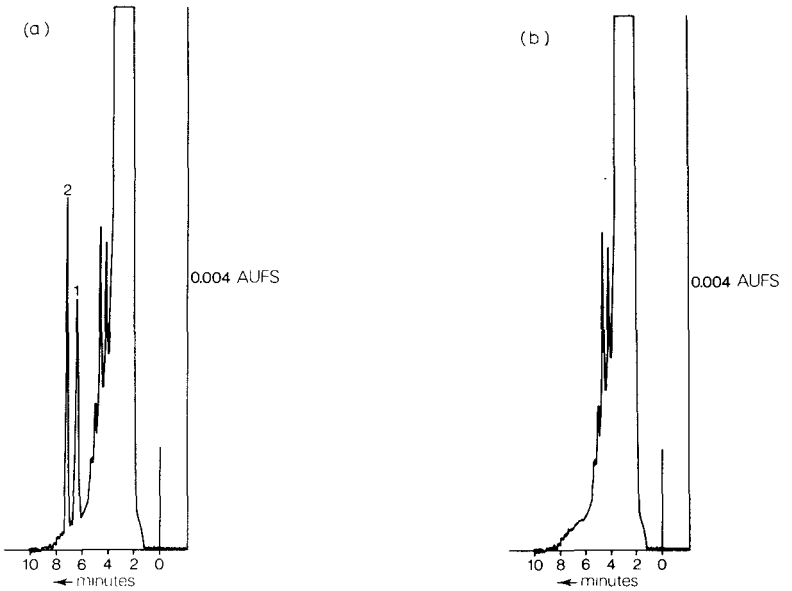

Fig. 1. (a) HPLC separation of 100 ng of codeine (1) and 320 ng of methadone (2) after extraction from plasma. (b) Chromatogram of a blank human plasma after extraction.

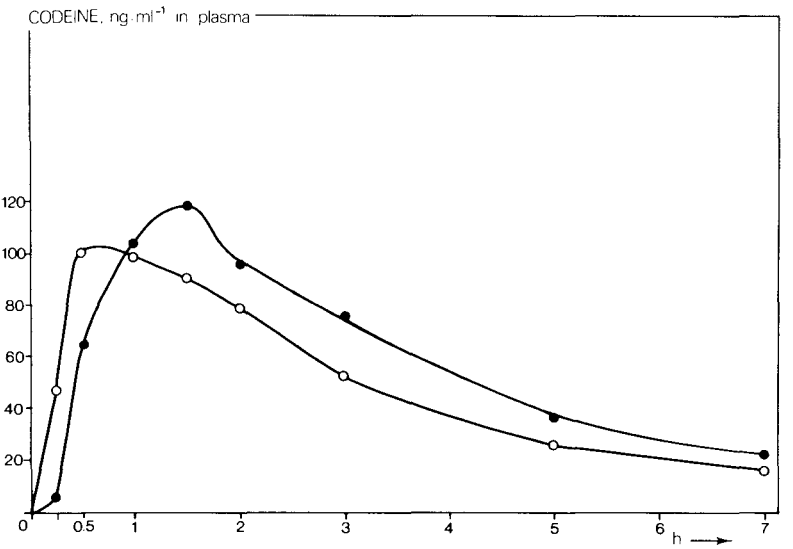

Fig. 2. Typical plasma concentration curves of codeine after administration of 60 mg of codeine phosphate in one subject as an oral solution (○) and as a rectal suppository (●).

It is important to note that the accuracy and precision depends on the volume to which the dichloromethane layer is reduced by evaporation. This volume should not be less than 200 μl, since the methadone peak in that case is reduced significantly.

Although it is possible to detect morphine concentrations in the same run

(retention time is about 15 min), the plasma concentrations of this active metabolite produced after an oral dose of 60 mg of codeine phosphate are too low (< 10 ng/ml) to allow quantitative determination, since the detection limit of morphine following the described procedure is about 50 ng/ml of plasma.

Fig. 2 shows typical plasma concentration curves for codeine after oral and rectal administration of 60 mg of codeine phosphate to a human subject. Subsequent experiments with seven volunteers have been carried out to determine the absorption profiles after administration of various dosage forms [11].

The methods of Zweidinger et al. [1] and Tsina et al. [9] are also useful for measuring therapeutic plasma concentrations of codeine. However, these procedures require 2-ml plasma samples. Besides, HPLC with UV detection is probably the most widely used technique available in clinical laboratories.

In conclusion, it can be said that our HPLC method is a simple, fast and accurate procedure and can be used to study biopharmaceutics of codeine in man.

REFERENCES

1 R.A. Zweidinger, F.M. Weinberg and R.W. Handy, J. Pharm. Sci., 65 (1976) 427.
2 M.K. Brunson and J.F. Nash, Clin. Chem., 21 (1975) 1956.
3 M.J. Kogan and M.A. Chedekel, J. Pharm. Pharmacol., 28 (1976) 261.
4 K.D. Parker, J.A. Wright and C.A. Hine, Forensic Sci. Soc. J., 7 (1967) 162.
5 W.O.R. Ebbighausen, J.H. Mowat, P. Vestergaard and N.S. Kline, Adv. Biochem. Psychopharmacol., 7 (1973) 135.
6 C. Olieman, L. Maat, K. Waliszewski and H.C. Beyerman, J. Chromatogr., 133 (1977) 382.
7 C.Y. Ko, F.C. Marziani and C.A. Janicki, J. Pharm. Sci., 69 (1980) 1081.
8 L. Ulrich and P. Rüegsegger, Arch. Toxicol., 45 (1980) 241.
9 I.W. Tsina, M. Fass, J.A. Debban and S.B. Matin, Clin. Chem., 28 (1982) 1137.
10 J.W.A. Findlay, R.F. Butz and R.M. Welch, Clin. Pharmacol. Ther., 22 (1977) 439.
11 F. Moolenaar, G. Grasmeijer, J. Visser and D.K.F. Meijer, Biopharm. Drug Dispos., 4 (1983) in press.

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Note

Determination of apomorphine and N-*n*-propylnorapomorphine in plasma using high-performance liquid chromatography and fluorescence detection

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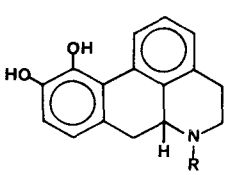
Apomorphine (I) is a drug that has been used as an emetic for many years. There is an increasing interest in I and related aporphines such as N-*n*-propylnorapomorphine (II) in the treatment of neurological disorders (e.g. Gilles de la Tourette's syndrome, Huntington's chorea, and Parkinsonism) which result from dopamine imbalances in the brain [1—7]. These studies have stimulated the development of gas chromatographic (GC) [8—10], spectrophotometric [11], thin-layer chromatographic [12], fluorometric [13], radioenzymatic [14—16], radioreceptor assay [17, 18], and high-performance liquid chromatographic (HPLC) (UV detection [19—21] and electrochemical detection [22]) methods for the determination of I in biological fluids. It was felt, however, that an HPLC procedure using fluorescence detection might provide advantages in sensitivity and/or convenience relative to methods developed earlier.

This paper describes a sensitive and selective procedure for the determination of I and II in plasma using HPLC with fluorometric detection. The devised method provides good accuracy and precision in the concentration range of 100—1000 ng/ml.



(I) Apomorphine: R = CH_3
(II) N-*n*-Propylnorapomorphine: R = $CH_2CH_2CH_3$

MATERIALS AND METHODS

R-(—)-Apomorphine hydrochloride hemihydrate was obtained from McFarland-Smith (Edinburgh, Great Britain). *R*-(—)-N-*n*-Propylnorapomorphine

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was supplied by the Sterling Winthrop Research Institute (Rensselaer, NY, U.S.A.). The aporphines were essentially homogeneous following HPLC [19, 21] and found to have greater than 98% purity based on UV spectroscopy [23]. Organic solvents were distilled-in-glass grade. Water was distilled and deionized. All other chemicals were reagent-grade or better. The diethyl ether used for extraction was distilled on the same day in which analyses were performed. Recently outdated human plasma was obtained through a local blood bank.

HPLC

The HPLC system used throughout consisted of an Altex 100A pump and Altex 210 injector, Schoeffel FS 970 LC fluorometric detector, and Altex integrator (Model C-RIA). The integrator was operated with the chart speed set at 2 cm/min and attenuation at 4. The detector was set at an excitation wavelength of 281 nm and fitted with filters 7-54 (bandpass; entrance) and 418 nm (cut-off; emission). Separations were achieved with an Altech μBondapak-phenyl column (15 cm × 4 mm I.D., particle size 10 μm) (Arlington Heights, IL, U.S.A.). The mobile phase consisted of 40% methanol in buffer (0.02 *M* sodium acetate—0.03 *M* acetic acid, pH 3.25) and was filtered through a Millipore PTFE-filter and degassed by ultrasonication before use. The flow-rate was set at 1.0 ml/min.

Extraction and recovery studies

In a 10-ml screw-capped culture tube, a mixture of 1 ml of human plasma and 100 μl of standard solution (I and II) was adjusted to pH 2.0 using 100 μl of 1 *N* hydrochloric acid. A 3-ml portion of toluene was added and the mixture vortexed for 30 sec at minimum speed (Vortex mixer, Scientific Industries, Bohemia, NY, U.S.A.) and centrifuged at ca. 800 *g* for 10 min. The toluene layer was discarded. A 200-μl portion of 1 *M* $NaHCO_3$ and a 100-μl portion of 15 m*M* of Tris—HCl [24] were added to adjust the pH to 7.0. The mixture was vortexed for 15 sec and a 3-ml portion of diethyl ether was added. After vortexing for 1 min at minimum speed, the mixture was centrifuged at ca. 800 *g* for 10 min to separate the layers. The diethyl ether was taken to dryness under a nitrogen stream at room temperature and the residue dissolved in 1 ml of mobile phase containing 0.5 mg/ml of $NaHSO_3$ [25]. A 100-μl portion of the solution was injected into the HPLC system.

Plasma standard curve

Apomorphine assay. Solutions of 0.1 mg/ml of I and 0.1 mg/ml of II (internal standard) were freshly prepared in 0.1 *N* hydrochloric acid containing 100 mg/ml of ascorbic acid [25]. These solutions were used to prepare standard solutions containing 1.0, 2.5, 7.5, 10.0 μg/ml of I and 5.0 μg/ml of II in 0.1 *N* hydrochloric acid.

N-n-Propylnorapomorphine assay. Solutions of 0.1 mg/ml of II and 0.1 mg/ml of I (internal standard) were freshly prepared in 0.1 *N* hydrochloric acid containing 100 mg/ml of ascorbic acid. These solutions were used to prepare standard solutions containing 1.0, 2.5, 7.5, 10.0 μg/ml of II and 5.0 μg/ml of I in 0.1 *N* hydrochloric acid.

Four 1-ml portions of plasma are spiked with drug (I or II; 100, 250, 750, and 1000 ng/ml) and internal standard (II or I; 500 ng/ml) by addition of 100-μl portions of aporphine standard solutions. Peak area ratios (drug/internal standard) are determined for each injection and plotted versus the concentration of drug (I or II).

Accuracy and precision of assay

Plasma test samples containing an equivalent of 150, 300, 450, 900 ng/ml of I or II and 500 ng/ml of I or II (Serving as internal standard) were extracted and analyzed in replicate as described above. Peak area ratios (drug/internal standard) were determined for each injection. Concentrations of I and II were calculated using a standard curve generated from the standards for each day of analysis. The means and relative standard deviations of these values were calculated.

RESULTS AND DISCUSSION

A satisfactory separation of I and II was achieved using a solvent system consisting of 40% methanol in acetate buffer, pH 3.25 (40 : 60) and a μphenyl reversed-phase HPLC column (capacity factor, k', I = 2.3; II = 3.3). The mobile phase is similar to ones previously described [19, 21] for these aporphines but is less complicated and permits complete chromatographic development within 10 min. A typical chromatogram resulting from the analysis of I in plasma is depicted in Fig. 1.

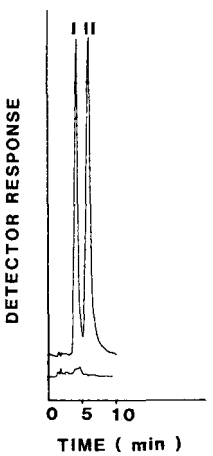

Fig. 1. Chromatograms of blank human plasma (lower trace) and human plasma spiked with apomorphine (I), 300 ng/ml and N-*n*-propylnorapomorphine (II), 500 ng/ml (upper trace).

The cleanup procedure incorporates a toluene pre-extraction of acidified plasma which removes potentially interfering fluorescent endogenous substances. After pre-extraction, the plasma is extracted with diethyl ether at pH 7.0 which has been shown previously to give quantitative recoveries of I and II in the μg/ml range [20].

The absolute mean recovery of I from plasma was 86.0 ± 10.1% (n = 15) at

levels of 250—1000 ng/ml; recovery of II averaged 83.6 ± 12.8 (n = 15) for samples containing 500 ng/ml. Consistently linear calibration curves for I/II peak area ratios (determination of I) in the concentration range of 100—1000 ng apomorphine per ml were typically obtained (slope = 0.021, y intercept = 0.059, r = 0.997) along with the II/I peak area ratios (determination of II) in the concentration range of 100—1000 ng N-n-polynorapomorphine per ml (slope = 0.002, y-intercept = —0.045, $r > 0.999$).

The results of replicate analyses of I and II in plasma are given in Tables I and II. The accuracy and precision of the assays for I and II are good.

Attempts to measure levels of I below 100 ng/ml revealed apparent endogenous interferences which affected accuracy adversary though R.S.D. values were about ± 10%. The detection limit of the method is approximately 50 ng/ml of I or II (signal-to-noise ratio ≈ 7).

The HPLC method described provides convenient measurement of nanogram quantities of I and II in plasma and improved sensitivity by a factor of approximately ten compared to previous chromatographic methods [19—21]. The method shows complete chromatographic development within 10 min and good accuracy and precision in the concentration range of 100—1000 ng/ml. This compares favorably with all literature HPLC procedures for apomorphine in plasma [19—21]. Westerink and Horn [22] report higher sensitivities for determinations of apomorphine in brain tissue using electrochemical detection, however, their method has not been extended to plasma. No comparable methods have been published for N-n-propylnorapomorphine.

TABLE I

ACCURACY AND PRECISION OF HPLC ASSAY FOR APOMORPHINE (I) IN HUMAN PLASMA

I concentration prepared (ng/ml)	Mean recovery (%)	R.S.D. (%)	n
150.0	104.4	8.2	8
300.0	105.0	7.0	8
450.0	102.4	6.6	7
900.0	92.9	8.7	8

TABLE II

ACCURACY AND PRECISION OF HPLC ASSAY FOR N-n-PROPYLNORAPOMORPHINE (II) IN HUMAN PLASMA

II concentration prepared (ng/ml)	Mean recovery (%)	R.S.D. (%)	n
150.0	104.8	4.9	6
300.0	97.2	4.2	5
450.0	102.8	5.6	6
900.0	102.8	4.1	6

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REFERENCES

1 F.C. Colpaert, W.F.M. Van Bever and J.E.M.F. Leysen, Int. Rev. Neurobiol., 19 (1976) 225.
2 C.A. Tamminga, M.H. Schaffer, R.C. Smith and J.M. Davis, Science, 200 (1978) 567.
3 I. Shoulson and T.N. Chase, Ann. Rev. Med., 26 (1975) 416.
4 E.S. Tolosa, G.C. Cotzias, P.G. Burckhardt, L.C. Tang and K.E. Dahl, Exp. Neurol., 55 (1977) 56.
5 G.C. Cotzias, P.S. Papavasiliou, E.S. Tolosa, J.S. Mendez and M. Bell-Midura, New Engl. J. Med., 294 (1976) 567.
6 M.K. Menon, W.G. Clark and J.L. Neumeyer, Eur. J. Pharmacol., 52 (1978) 1.
7 W.H. Riffee, R.E. Wilcox and R.V. Smith, Eur. J. Pharmacol., 54 (1979) 273.
8 R.V. Smith and A.W. Stocklinski, Anal. Chem., 47 (1975) 1321.
9 J.R. Miller, J.W. Blake and T. Tobin, Res. Commun. Chem. Path. Pharmacol., 15 (1976) 447.
10 D.M. Baaske, J.E. Keiser and R.V. Smith, J. Chromatogr., 140 (1977) 57.
11 P.N. Kaul, E. Brochmann-Hanssen and E. Leong Way, J. Amer. Pharm. Assoc., Sci. Ed., 48 (1959) 638.
12 P.W. Erhardt, R.V. Smith, T.T. Sayther and J.E. Keiser, J. Chromatogr., 116 (1976) 218.
13 W.K. Van Tyle and A.M. Burkman, J. Pharm. Sci., 60 (1971) 1736.
14 R.J. Baldessarini, in E. Usdin, J.D. Barchas and I.J. Kopin (Editors), Catecholamines: Basic and Clinical Frontiers, Vol. 2, Pergamon Press, New York, 1979, pp. 1596—1598.
15 A. Campbell, N.S. Kula, B. Jeppson and R.J. Baldessarini, Eur. J. Pharmacol., 67 (1980) 139.
16 J. Kebabian, J. Neurochem., 30 (1978) 1143.
17 A. Campbell, M. Herschel, B.M. Cohen and R.J. Baldessarini, Life Sci., 27 (1980) 633.
18 B.M. Cohen, B. Herschel, E. Miller, H. Mayberg and R.J. Baldessarini, Neuropharmacology, 19 (1980) 663.
19 R.V. Smith, J.C. Glade and D.W. Humphrey, J. Chromatogr., 172 (1979) 520.
20 R.V. Smith, D.W. Humphrey, S. Szeinbach and J.C. Glade, Anal. Lett., 12 (1979) 371.
21 R.V. Smith, A.E. Klein and D.O. Thompson, Mikrochim. Acta, I (1980) 151.
22 B.H.C. Westerink and A.S. Horn, Eur. J. Pharmacol., 58 (1978) 39.
23 W.H. Soine, P. Salgo and R.V. Smith, J. Labelled Compd. Radiopharm., 16 (1979) 597.
24 R.V. Smith and S.P. Sood, Anal. Lett., 5 (1972) 273.
25 R.E. Wilcox, D.W. Humphrey, W.H. Riffee and R.V. Smith, J. Pharm. Sci., 69 (1980) 974.

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Note

Determination of noscapine in serum by high-performance liquid chromatography

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Noscapine [1-α-methyl-8-methoxy-6,7-methylenedioxy-1-(6,7-dimethoxy-3-phthalidyl)-1,2,3,4-tetrahydroisoquinoline] (Fig. 1), which is one of the alkaloids in opium, has been known for more than 100 years, and several methods for its quantitative determination are therefore available. In recent years gas chromatographic methods [1, 2] and high-performance liquid chromatographic methods (HPLC) [3—7] have been applied in separating and determining the opium alkaloids.

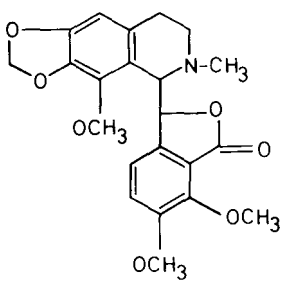

Fig. 1. Formula of noscapine.

Pharmaceutical preparations containing noscapine for its antitussive effect, have been analysed for noscapine by thin-layer chromatography with in situ scanning [8], or by HPLC [9]. In forensic laboratories HPLC is now the method most commonly used for identification of noscapine [10, 11].

In biological fluids noscapine has been determined by a fluorimetric method [12, 13] or by gas and thin-layer chromatography [14, 15]; and, finally, an HPLC method has been described for the determination of noscapine in serum [16].

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This study describes a simple HPLC method which is sensitive enough for the determination of noscapine concentrations in serum following therapeutic doses of the compound.

EXPERIMENTAL

Material and reagents

Hexane and dichloromethane were specially purified (nanograde) from Mallinckrodt (St. Louis, MO, U.S.A.). Acetonitrile (E. Merck, Darmstadt, G.F.R.) was special HPLC grade. All other chemicals were analytical grade. The tablets used contained ion-exchange resin bound noscapine (Longatin®) 25 mg, and were produced by Dumex Ltd.

HPLC conditions

The apparatus consisted of a solvent delivery system, Model 6000A (Waters Assoc., Milford, MA, U.S.A.), a loop injection system U6K (Waters Assoc.) a UV detector Model 440 with fixed wavelength of 254 nm (Waters Assoc.), and a variable-wavelength detector, Model 450, of wavelength 230 nm (Waters Assoc.). The column was μBondapak C_{18} (30 cm × 3.9 mm, particle size 10 μm). The temperature was 25°C.

The mobile phase consisted of 45% acetonitrile in 55% potassium dihydrogen phosphate (0.6%, w/v) adjusted to pH 3 with phosphoric acid. The flow-rate was 0.9 ml/min. The mobile phase was membrane-filtered (pore size 0.45 μm) and kept in an ultrasonic bath for 15 min immediately before use.

Printing of the chromatograms was performed by an electronic integrator (No. 3080) from Hewlett-Packard, Avondale, PA, U.S.A. The peak heights were measured manually.

Procedure

To 2.00 ml of serum add 200 μl of a 0.5 *M* solution of sodium hydroxide and 10.00 ml of a mixture of *n*-hexane—dichloromethane (9:1). Rotate for 15 min (30 rpm) in a rotating device and, after centrifuging (ca. 950 *g*) for 5 min at 5°C, remove a 7.00 ml volume from the organic phase. Evaporate to dryness at 20°C with a gentle stream of nitrogen. The residue is dissolved in 100 μl of mobile phase in an ultrasonic bath and 50 μl are injected into the chromatograph.

Sampling

One female volunteer, who fasted overnight, received four tablets of noscapine 25 mg (Longatin®). Blood was collected immediately before, and 15, 35, 45, 60, 75, 90, 105, 120, 150 and 180 min after administration. Serum was centrifuged off and kept at −18°C until analysis.

RESULTS AND DISCUSSION

This study describes a liquid chromatographic method in which noscapine is determined without previous derivatization of the compound.

TABLE I

SLOPE, INTERCEPT AND CORRELATION COEFFICIENTS FOR FOUR DIFFERENT CALIBRATION GRAPHS

	Slope	Intercept	Correlation coefficient
I	0.487	15.6	0.988
II	0.496	13.5	0.996
III	0.540	9.2	0.993
IV	0.504	5.9	0.996

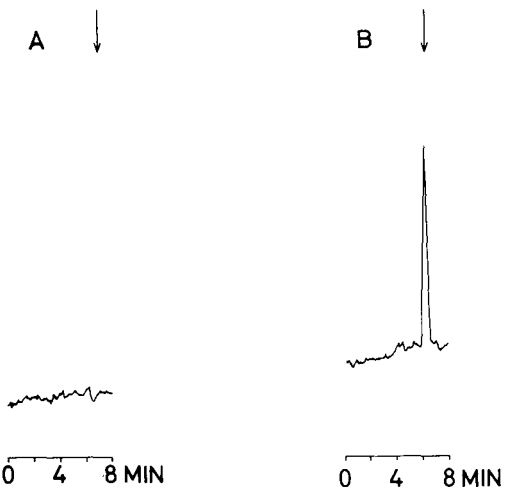

Fig. 2. Chromatograms of (A) mobile phase, and (B) mobile phase + noscapine (50 ng) (retention time: 5.9 min).

In Table I is shown slope, intercept and correlation coefficient for four calibration graphs from four different days.

Fig. 2 illustrates the chromatographic response to 50 μl of mobile phase and to 50 μl of mobile phase with 50 ng of noscapine added.

Chromatograms of control serum and of control serum with 25 ng/ml or 100 ng/ml noscapine added are shown in Fig. 3.

Fig. 4 depicts chromatograms of control serum measured at 254 nm (UV detector with fixed wavelength) and at 230 nm (detector with variable wavelength).

The recovery of noscapine from serum samples (10—250 ng/ml) by extraction with *n*-hexane or with dichloromethane added, was found to be 50% (*n*-hexane), 100% [*n*-hexane—dichloromethane (4:1)], and 90% [*n*-hexane—dichloromethane (9:1)]. The last mixture was preferred, because addition of more than 10% dichloromethane to *n*-hexane produced an unsatisfactory separation of the noscapine peak from other interferences.

The precision of the method was controlled by analysis of control serum with 75 ng/ml noscapine added. The coefficient of variation was found to be 3% (n = 10). Vedsø [12] reported the S.E.M. to be 5.4% (50—1360 ng/ml)

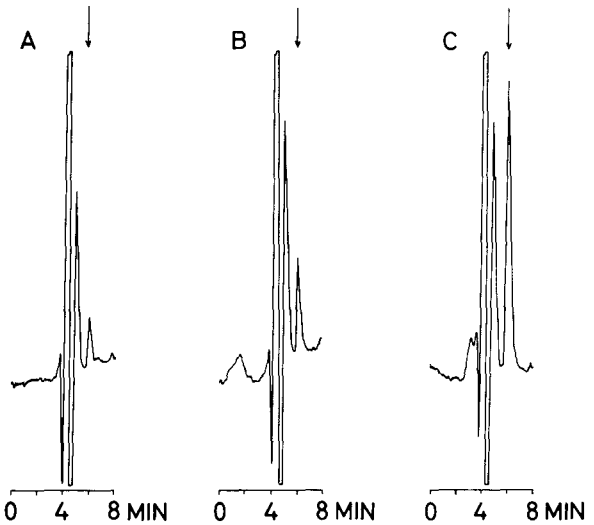

Fig. 3. Chromatograms of (A) control serum, (B) control serum with noscapine (25 ng/ml added), and (C) control serum with noscapine (100 ng/ml added) (retention time: 5.9 min).

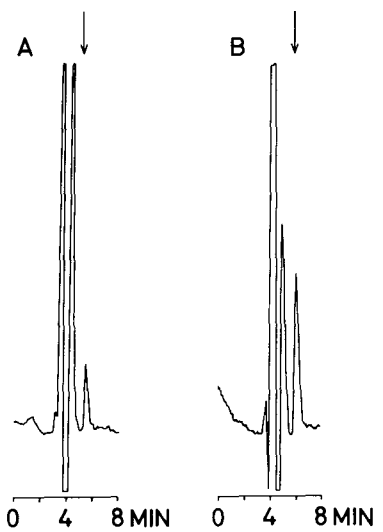

Fig. 4. Chromatograms of control serum with noscapine (50 ng/ml added) (retention time: 5.9 min), measured at (A) 254 nm and (B) 230 nm.

and according to the method by Johansson et al. [16] the coefficient of variation was 3.2% (89 ng/ml).

The minimal concentration detectable is 10 ng/ml of serum. In comparison, the detection limit reported by Vedsø [12] is 50 ng/ml and by Johansson et al. [16] 5 ng/ml.

The amount of sample volume injected into the chromatograph affects the peak heights. In this study a volume of 50 μl was chosen, because the low

serum concentrations obtainable necessitated injection of as big a fraction of the sample volume as possible.

It has been reported by Pawelczyk et al. [17—19] that an alkaline environment easily produces a lactone ring opening of the noscapine molecule, which is thereby transformed to noscapinic acid. Consequently, there is a risk that this transformation may take place during the extraction procedure, which involves alkalization of the serum (pH 10—11).

The stability of noscapine during the analysis procedure was examined. A 2-ml volume of serum with 200 μl of 0.5 *N* sodium hydroxide and 100 or 200 ng of noscapine added was left at room temperature for up to 24 h, and during this period no decomposition of the compound was observed.

Noscapinic acid, if present, is not determined by the prescribed method, and since no reduction in content of noscapine was observed in alkaline serum left at room temperature for 24 h, this may suggest that the noscapine molecule is protected in the serum against the opening of the lactone ring.

Various drugs which might interfere with the analysis were injected into the chromatograph. The retention times relative to noscapine are listed in Table II.

TABLE II

RELATIVE RETENTION TIMES FOR SOME COMPOUNDS

Compound	Relative retention time
Noscapine	1.00
Nitrazepam	1.38
Imipramine-N-oxide	1.51
Nortriptyline	1.52
Imipramine	1.57
Desmethylimipramine	1.62
Amitriptyline-N-oxide	1.66
Amitriptyline	1.69
Diazepam	2.25
2-Amino-5-nitrobenzophenone	2.45

Application to clinical samples

Chromatograms of serum drawn from one volunteer immediately before and 45 and 120 min after oral administration of 100 mg of noscapine are depicted in Fig. 5. Fig. 6 shows the serum concentration course in the same volunteer during the first 3 h after administration.

The peak serum concentration after 1 h was 135 ng/ml; the estimated half-life was 1.5 h (calculated on the basis of serum concentrations obtained 2, 2.5 and 3 h after administration). Vedsø [12] found serum concentrations of 300—1500 ng/ml after 1 h and 100 ng/ml after 4 h following oral administration of 250—300 mg of noscapine (Longatin®) to volunteers. Peak serum concentrations of an average 94 ng/ml after 2.5 h were reported by Dahlström et al. [21] following oral administration of 150 mg of noscapine (Longatin®). The half-life in the elimination phase was calculated to be approximately 2 h. However, great individual differences were observed, the same authors reporting an absolute bioavailability with a factor 4.8 variation among the volunteers.

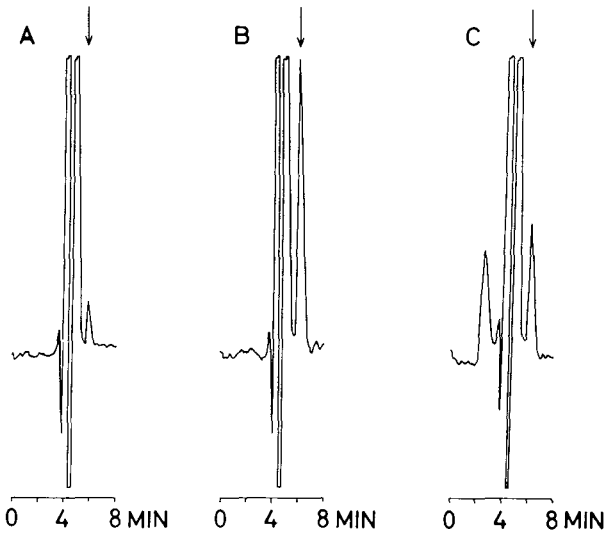

Fig. 5. Chromatograms of serum from a volunteer following an oral dose of noscapine, 100 mg: (A) before the dose, (B) 45 min after the dose, (C) 2 h after the dose (retention time of noscapine: 5.9 min).

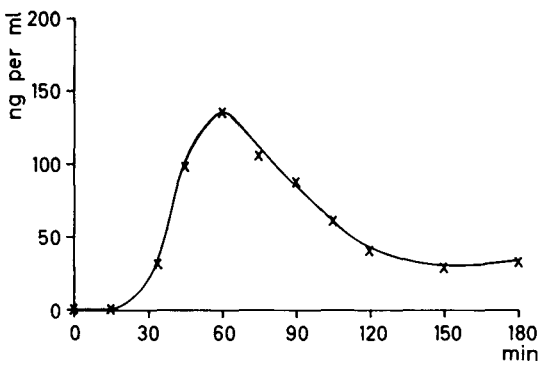

Fig. 6. Serum concentrations of noscapine in a volunteer following an oral dose of noscapine, 100 mg.

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REFERENCES

1 G. Fisher and R. Gillard, J. Pharm. Sci., 66 (1977) 421.
2 D. Furmanec, J. Chromatogr., 89 (1974) 76.
3 H.W. Ziegler, T.H. Beasley and D.W. Smith, J. Assoc. Offic. Anal. Chem., 58 (1975) 888.
4 C.Y. Wu and J.J. Wittick, Anal. Chem., 49 (1977) 359.
5 R. Gimet and A. Filloux, J. Chromatogr., 177 (1979) 333.
6 Y. Nobuhara, S. Hirano, K. Namba and M. Hashimoto, J. Chromatogr., 190 (1980) 251.

7 W. Lindberg, E. Johansson and K. Johansson, J. Chromatogr., 211 (1981) 201.
8 W. Schlemmer and E. Kammerl, J. Chromatogr., 82 (1973) 143.
9 M. Tatsuzawa, S. Hashiba and A. Ejima, J. Hyg. Chem., 23 (1977) 282.
10 I. Lurie, J. Assoc. Offic. Anal. Chem., 60 (1977) 1035.
11 J.K. Baker, R.E. Skelton and C.Y. Ma, J. Chromatogr., 168 (1979) 417.
12 S. Vedsø, Acta Pharmacol. Toxicol., 18 (1961) 119.
13 K.P. Nayak, E. Brochmann-Hanssen and E.L. Way, J. Pharm. Sci., 54 (1965) 191.
14 N. Tsunoda and H. Yoshimura, Xenobiotica, 9 (1979) 181.
15 N. Tsunoda and H. Yoshimura, Xenobiotica, 11 (1981) 23.
16 M. Johansson, A. Arbin and S. Eksborg, Poster at Farmacevtisk Årskongres, Stockholm, 12—13 Oct, 1981.
17 E. Pawelczyk, M. Zajac and M. Remisz, Pol. Pharmacol. Pharm., 26 (1974) 569.
18 E. Pawelczyk and M. Zajac, Pol. Pharmacol. Pharm., 27 (1975) 69.
19 E. Pawelczyk, M. Zajac and B. Matlak, Pol. Pharmacol. Pharm., 28 (1976) 69.
20 S. Vedsø, Acta Pharmacol. Toxicol., 18 (1961) 157.
21 B. Dahlström, T. Mellstrand, C.G. Löfdahl and M. Johansson, Eur. J. Clin. Pharmacol., 22 (1982) 535.

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Note

High-performance liquid chromatographic determination of tetrahydroaminoacridine in human and rat tissues using a rapid Sep-Pak C_{18} extraction

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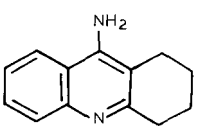
(Received September 28th, 1982)

Tetrahydroaminoacrin (THA, 9-amino-1,2,3,4-tetrahydroacridine), I, is a competitive cholinesterase inhibitor [1]. It has been used clinically for the treatment of intractable pain of terminal carcinoma [2], myasthenia gravis [3], and as a decurarizing agent [4]. The drug has also been utilized as a specific therapy for reversing the signs and symptoms of the central anticholinergic syndrome [5]. Particular interest in this compound derives from its possible efficacy in patients with Alzheimer's disease [6, 7].



I

THA has been administered in variable doses ranging from 0.25 to 1.5 mg/kg. It has a duration of action from 8 to 16 h [8]. There are considerable interindividual variations in adverse reactions. Hence, an assay of the plasma concentration of THA would be an useful adjunct to its use in elderly patients with Alzheimer's disease.

Yago et al. [9] used high-performance liquid chromatography (HPLC), utilizing a two-step extraction procedure for sample purification, to determine

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THA levels in spiked plasma samples. More recently, small packed (Sep-Pak) cartridges have been used [10, 11] for the purification of biological samples prior to HPLC.

In the present paper, we have employed our previously developed purification technique [10] for the isolation of THA from plasma and brain samples. THA was separated and quantified by reversed-phase HPLC with UV absorbance detection. This method has been successfully utilized in pharmacokinetic studies of rats administered THA intraperitoneally and is applicable for the determination of THA in human plasma.

EXPERIMENTAL

Apparatus

An LC-306 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.) was used throughout this work. A 30 cm × 4 mm μBondapak C_{18} reversed-phase column (Waters Assoc., Milford, MA, U.S.A.) was connected to a 3 cm × 4.6 mm guard column of 5-μm RP-18 (Brownlee Labs., Santa Clara, CA, U.S.A.). A Waters Model M420 fluorometric detector (excitation 385 nm/emission 425 nm) was used to confirm the identity of the THA peak observed in plasma and brain samples. The mobile phase was prepared by mixing 1600 ml of 0.1 *M* phosphoric acid solution (adjusted to pH = 2.8 by triethylamine) and 200 ml of acetonitrile. The flow-rate was fixed at 1.5 ml/min at ambient temperature and the UV wavelength set at 254 nm.

Reagents

THA standard was obtained from Sigma (St. Louis, MO, U.S.A.). C_{18} Sep-Pak cartridges were supplied by Waters Assoc. Acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All chemicals were reagent grade.

Sample preparation

Male, Sprague—Dawley rats (Charles River, MA, U.S.A.) weighing 200—300 g were used for the studies. THA was dissolved in saline and administered intraperitoneally in a dose of 20 mg/kg. Control animals were injected with saline.Blood was drawn at 5, 25, 30, 45 and 60 min following the THA injection by an open chest cardiac puncture. The blood was centrifuged at 2000 *g* (4°C) for 20 min and plasma carefully removed. Immediately upon completion of the cardiac puncture, each rat was decapitated by guillotine and the whole brain rapidly removed. Brain tissue samples were weighed and homogenized in propylene tubes with 0.2 *N* perchloric acid (1 : 2, g/ml). The homogenates were then centrifuged at 12,000 *g* for 20 min at 4°C. The clear supernatant was adjusted to a pH of 7.2 by adding 1 *N* sodium hydroxide. Human plasma samples were obtained from the hospital blood bank.

THA isolation procedure

The C_{18} Sep-Pak cartridge was activated by passing through 5 ml of water and then 5 ml of methanol under pressure using a glass syringe followed by 10 ml of water. A 1-ml aliquot of rat plasma or brain homogenate was passed

through the cartridge via a syringe at a flow-rate not greater than 2 ml/min. The cartridge was washed with 10 ml of water and 4 ml of the mobile phase. The residue solution was evacuated by pushing a plunger through the syringe. Methanol (0.3 ml) was added to the cartridge and the evacuation procedure repeated. An additional 0.7 ml of methanol was passed through the cartridge and the eluate collected in a 10-ml glass disposable tube. The methanol was evaporated to dryness under a stream of dry nitrogen at 35°C. The dried residue was dissolved in 100 μl of the mobile phase. Ten μl were injected into the chromatograph. The cartridge can be regenerated by flushing with 10 ml of methanol and 15 ml of water. The concentration of THA was calculated from the peak height using a standard curve.

RESULTS AND DISCUSSION

Representative chromatograms of rat plasma and brain samples are shown in Figs. 1 and 2 utilizing optimal chromatographic conditions. No interfering peaks were seen in control samples of rat plasma (Fig. 1A) and brain (Fig. 2A). The following drugs which may be administered concurrently with THA injection, did not interfere with the assay: L-dopa, guanidine, haloperidol, apomorphine, oxotremorine, physostigmine, and probenecid. Standard curves for THA in rat plasma and brain were linear over the concentration range 0.1—10 μg/ml or g, respectively. Over this concentration range, the within-day and day-to-day precision (R.S.D.) values were 3.8% (n = 15) and 6.9% (n = 15), respectively. The overall recovery of THA added to rat plasma per ml and brain per g with concentrations between 0.1 and 10 μg was 53 ± 3.6% (mean ± S.D., n = 25). The recovery could be increased to 70% when 1 ml of methanol was

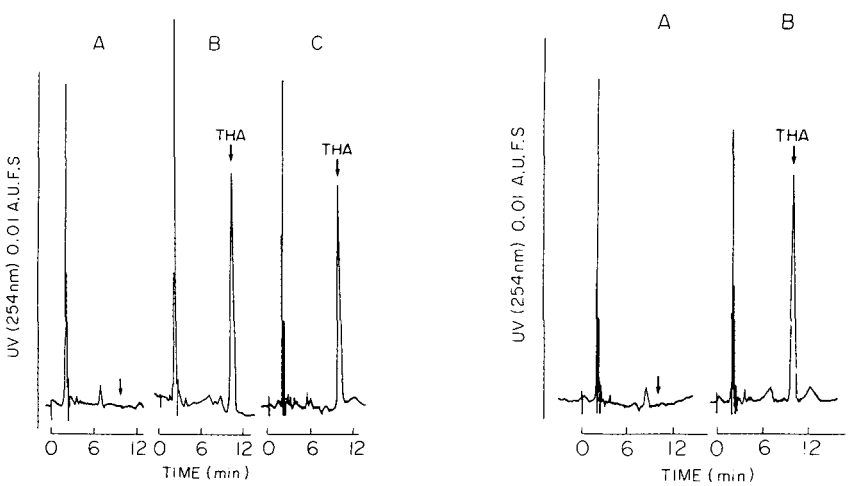

Fig. 1. Chromatograms of (A) blank rat plasma; (B) rat plasma containing 3.0 μg/ml of THA; and (C) human plasma containing 2.8 μg/ml of THA. Conditions were as given in the Experimental section.

Fig. 2. Chromatograms of (A) blank rat brain; and (B) brain containing 1.9 μg/g of THA. Conditions were as given in the Experimental section.

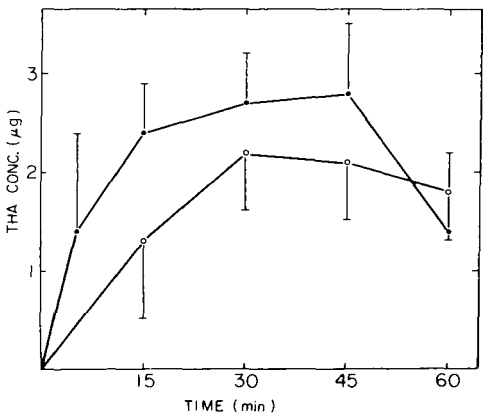

Fig. 3. Time—concentration curves for rat plasma in μg/ml (•——•) and brain in μg/g (○——○). Each point represents the mean value ± S.D. of five rats.

passed through the cartridge. However, under these circumstances, increasing noise was observed. The detection limit of THA was 100 ng/ml of plasma.

The addition of an ion-pairing reagent e.g. sodium dodecyl sulfate to rat plasma samples did not affect the recovery of THA. This behavior is unlike that of physostigmine in our previous report [10]. It is crucial to evacuate the cartridge before adding the washing fraction (0.3 ml) of methanol otherwise low recovery (< 30%) will be obtained.

The method described was applied to the quantitation of a series of rat plasma and brain samples obtained after intraperitoneal administration of THA (20 mg/kg). The time course of THA concentration in rat plasma and brain is illustrated in Fig. 3. The plasma curve reveals that the absorption of THA is very rapid and peak concentrations (2.4—2.6 μg/ml) are reached with 30—45 min. THA seems to cross the blood—brain barrier readily as shown in Fig. 3 and peak brain levels occur by 30 min after injection.

Human plasma samples spiked with various amounts of THA were also assayed. Fig. 1C shows a representative chromatogram of human plasma. No endogenous compounds of human plasma interfered with the detection of THA. Linearity between the detector response and the concentration of THA added was observed over the range of 0.1—20 μg. Precision and recovery data were similar to those of rat plasma and brain. This method is currently being utilized in pharmacokinetic studies in psychiatric patients receiving THA, and correlations of plasma concentrations with therapeutic response will be investigated.

The purification procedure using C_{18} Sep-Pak cartridge was found to be superior to the conventional, two-step extraction procedure [9]. It has the advantages of convenience, enhanced recovery and reproducibility, is less time-consuming, and more economical. The cartridge can be used at least three times without losing its isolation efficiency. One technician can easily prepare 60 samples per day.

Finally, the following compounds have been isolated, in our laboratory, utilizing this purification and enrichment method from the biological matrixes

including: physostigmine, probenecid, catecholamine and metabolites, and various polypeptides [12].

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REFERENCES

1 S. Maayani, H. Weinstein, N. Ben-Zui, S. Cohen and M. Sokolovsky, Biochem. Pharmacol., 23 (1974) 1263.
2 V. Stone, W. Moon and F.H. Shaw, Brit. Med. J., 1 (1961) 471.
3 C.R. Jones and M. Davis, Med. J. Aust., 2 (1975) 650.
4 S. Gershon and F.H. Shaw, J. Pharm. Pharmacol., 10 (1958) 638.
5 W.K. Summers, K.R. Kaufman, F. Altman, Jr. and J.M. Fischer, Clin. Toxicol., 16 (1980) 269.
6 K.L. Davis, R.C. Mohs and J.R. Tinklenberg, New Engl. J. Med., 301 (1979) 946.
7 W.H. Kaye, N. Sitaram, H. Weingartner, M.H. Ebert, S. Smallberg and J.C. Gillin, Biol. Psychiat., 17 (1982) 275.
8 W.K. Summers, J.O. Visselman, G.M. Marsh and K. Candelora, Biol. Psychiat., 16 (1981) 145.
9 L.S. Yago, W.K. Summers, K.R. Kaufman, O. Aniline and F.N. Pitts, Jr., J. Liquid Chromatogr., 3 (1980) 1047.
10 J.Y.K. Hsieh, R.K. Yang and K.L. Davis, J. Liquid Chromatogr., 5 (1982) 1691.
11 Sep-Pak Cartridge Applications Bibliography, Waters Assoc., Milford, MA, November, 1981.
12 J.Y.K. Hsieh, R.K. Yang and K.L. Davis, in preparation.

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CHROMBIO. 1583

Note

A rapid method for the simultaneous determination of the major metabolites of sulphasalazine in plasma

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(First received August 9th, 1982; revised manuscript received November 5th, 1982)

Sulphasalazine (SASP) is widely used in the treatment of Crohn's disease and ulcerative colitis [1,2]. After an oral dose 10—30% of the drug is absorbed unchanged while the remainder is cleaved by bacterial azo reductases in the large intestine to 5-aminosalicylate (AS) and sulphapyridine (SP) which are subsequently absorbed [3]. It is currently believed that AS is the active therapeutic species whereas most of the toxic side effects are due to SP and consequently SASP therapy is generally followed by monitoring plasma concentrations of SP [4].

AS in plasma has been assayed fluorimetrically [5] whilst SP in plasma has been determined using a spectrophotometric method [6]. More recently the pharmacokinetics of SASP and its metabolites have been studied by Klotz and co-workers using assay techniques based on high-performance liquid chromatography (HPLC) [7—10]. Overbach et al. [11] have compared the HPLC assay of SP with the spectrophotometric assay and found them comparable. However, the acetylated metabolite of SP has to be measured by difference (after hydrolysis) in the spectrophotometric assay whereas it is determined directly in the HPLC assay. Hansen [12] has recently described an improved HPLC assay for the determination of AS and its acetylated metabolite.

In the course of recent studies on the disposition of SASP in patients with large bowel disease we have developed a rapid, sensitive HPLC method for the determination of AS, SP and their acetylated metabolites (AcAS and AcSP). The advantage of the present assay over previous HPLC assays is that it measures all the metabolites simultaneously and consequently is more rapid.

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EXPERIMENTAL

Materials

All chemicals were of analytical grade. AcAS and AcSP were prepared by reacting AS and SP respectively with acetic anhydride followed by recrystallization.

Apparatus

Separations were performed on a 200 mm × 4.6 mm I.D. column packed with short alkyl chain bonded silica (Hypersil-SAS, 5 μm). The mobile phase was pumped through the column by a Waters Assoc. Model 6000A pump. Detection was achieved by coupling a UV monitor (Pye Unicam LC3, wavelength 290 nm) to a fluorimetric detector (Schoeffel LC fluorimeter FS970, excitation wavelength 320 nm; emission cut-off 389 nm) in series.

Chromatographic conditions

The mobile phase consisted of a mixture of methanol (22.5%) and 0.05 *M* phosphate buffer, pH 7.4, containing 0.1% tetrabutylammonium hydrogen sulphate and was pumped through the column at a flow-rate of 1 ml min^{-1} with a resulting back pressure of 150 bar. All assays were performed at ambient temperature.

Sample preparation

A 500-μl volume of methanol containing salicylic acid (SA, 30 mg l^{-1}) was added to either 500 μl of plasma obtained from a patient or a standard prepared in blank plasma. After vortexing, the solution was centrifuged at 650 *g* for 2 min. A 10-μl aliquot of the clear supernatant was then injected directly onto the column. Calibration plots of peak height ratio against concentration were then prepared.

RESULTS AND DISCUSSION

The concentrations of AS and AcAS observed after SASP administration are much less than those of SP and AcSP [8]. Consequently, it is necessary to use fluorimetry in order to detect AS and AcAS. However since AS and AcAS only fluoresce in their ionized form, to get them to run on a reversed-phase column a pairing ion [tetrabutylammonium (TBA)] had to be added.

The differential effect of the TBA concentration on the capacity factors of AS and SP is shown in Fig. 1. A TBA concentration of 1 g l^{-1} gave sufficient resolution of all components. SP and AcSP were determined on the UV detector and AS and AcAS on the fluorimetric detector. AcSP fluoresces but does not interfere with the determination of AS or AcAS.

The internal standard in this assay must be fluorescent as well as chromatographing in the right region. Salicylic acid proved to be an ideal internal standard but unfortunately there is a danger that patients may be taking aspirin which would invalidate the use of salicylic acid as an internal standard. Consequently plasma samples were run without the addition of internal standard to check for the presence of salicylate.

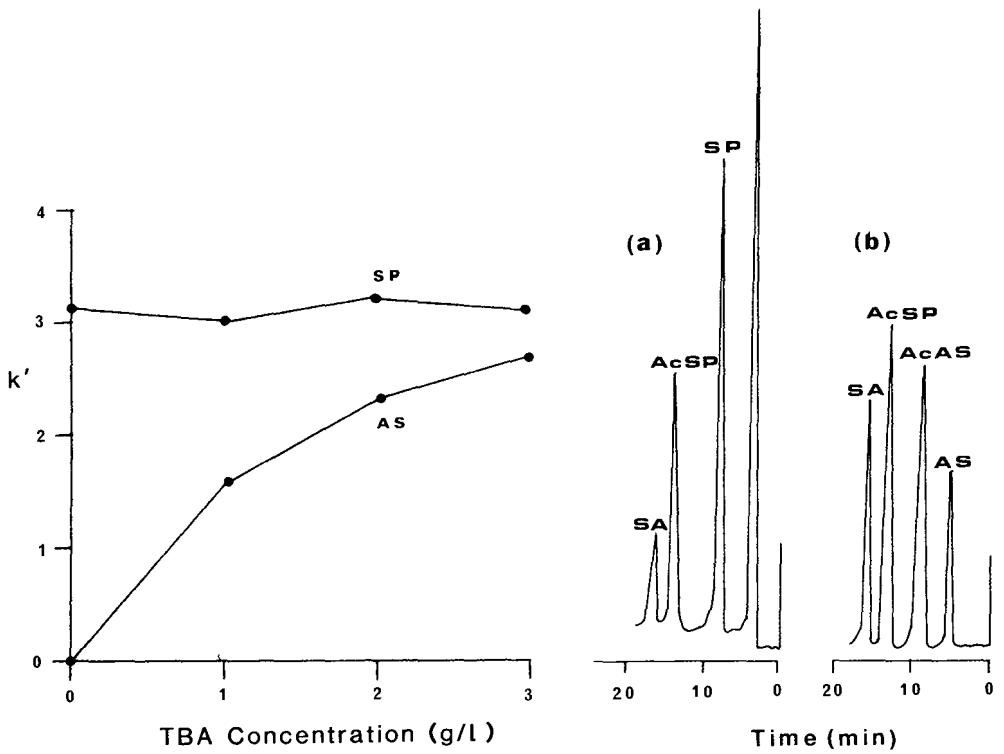

Fig. 1. The effect of counter-ion concentration (TBA) on the capacity factor (k') of sulphapyridine (SP) and 5-aminosalicylate (AS).

Fig. 2. Typical chromatograms of a standard solution prepared in plasma containing SP (20 mg l^{-1}), AcSP (20 mg l^{-1}), AS (5 mg l^{-1}), AcAS (5 mg l^{-1}) and SA (15 mg l^{-1}). (a) Recording from the UV monitor; (b) recording from fluorescence monitor. Apart from concentration, chromatograms obtained from patient samples were very similar to the ones shown above.

TABLE I

INTRA-DAY AND INTER-DAY VARIABILITY OF HPLC ANALYSIS OF SULPHASALAZINE METABOLITES

Intra-day values are the mean of five replicates. Inter-day values are the mean of assays carried out on five separate days.

	Intra-day				Inter-day			
	SP	AcSP	AS	AcAS	SP	AcSP	AS	AcAS
Actual concentration (mg l^{-1})	20	20	5	5	20	20	5	5
Estimated concentration (mg l^{-1})	20.4	19.7	4.8	5.2	20.8	20.7	5.0	5.2
Coefficient of variation (%)	3.9	3.6	3.1	1.9	4.8	5.3	3.0	2.9

A typical chromatogram is shown in Fig. 2. The retention times of AS, SP, AcAS and SA were 5.5, 7.5, 9.0, 13.5 and 16.0 min, respectively. Calibration plots were linear over the range 0.5—50 mg l^{-1} for SP and AcSP and 0.5—10 mg l^{-1} for AS and AcAS. Using the present assay it is easily possible to measure concentrations of each metabolite down to 0.5 mg l^{-1}. At concentrations of 20 mg l^{-1} of SP and AcSP and 5 mg l^{-1} of AS and AcAS the coefficient of variation of the assay was generally less than 5% (Table I).

The SASP metabolite time profiles in a subject suffering from Crohn's disease are shown in Fig. 3. The subject was receiving SASP 1 g every 12 h and the profile shown was measured on the 8th day of therapy.

Although the assay involves direct injection of diluted plasma, providing the column was thoroughly washed out with methanol—water (50 : 50) overnight, the column remained efficient for a period in excess of six months.

In summary the assay described in this report requires little sample preparation, is rapid and is sufficiently sensitive to monitor sulphasalazine therapy in patients with large bowel disease.

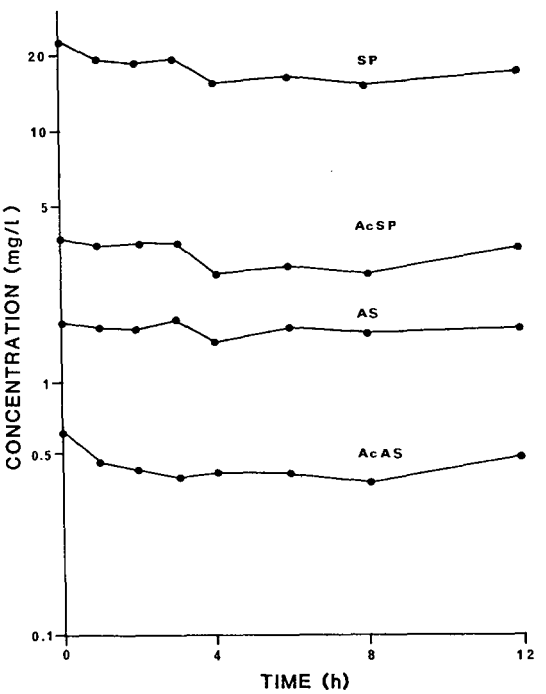

Fig. 3. Sulphasalazine metabolite time profiles in a subject taking 1 g of sulphasalazine every 12 h.

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REFERENCES

1 R.W. Summers, O.M. Switz, J.T. Sessions, J.M. Beckel, W.R. Best, F. Kern and J.W. Singleton, Gastroenterology, 77 (1979) 847.
2 A.Z. Azad Khan, D.T. Howes, J. Pris and S.C. Truelove, Gut, 21 (1980) 232.
3 H. Schröder and D.E.S. Campbell, Clin. Pharmacol. Ther., 13 (1972) 539.
4 K.M. Das and R. Dubin, Clin. Pharmacokin., 1 (1976) 406.
5 K.A. Hannson and M. Sandberg, Acta Pharm. Suecica, 10 (1973) 153.
6 K.A. Hannson and M. Sandberg, Acta Pharm. Suecica, 10 (1973) 87.
7 C. Fischer and U. Klotz, J. Chromatogr., 146 (1978) 157.
8 C. Fischer and U. Klotz, J. Chromatogr., 162 (1979) 237.
9 C. Fischer, K. Maier and U. Klotz, J. Chromatogr., 225 (1981) 498.
10 U. Klotz, K. Maier, C. Fischer and K. Heinkel, New Engl. J. Med., 303 (1980) 1499.
11 J. Overbach, N.F. Johnson, T.R. Bates, H.J. Pieniaszek and W.J. Jusko, J. Pharm. Sci., 67 (1978) 1250.
12 S.H. Hansen, J. Chromatogr., 226 (1981) 504.

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CHROMBIO. 1586

Note

Determination of biopterin and other pterins in tissues and body fluids by high-performance liquid chromatography

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(First received September 7th, 1982; revised manuscript received November 3rd, 1982)

Tetrahydrobiopterin serves as a cofactor in the hydroxylation of phenylalanine, tyrosine and tryptophan [1] as well as in the cleavage of glycerol ethers [2]. Further investigation of alterations of pterin metabolism associated with phenylketonuria [3], Parkinsonism and other neurological diseases [4, 5], and neoplastic diseases [6, 7] as well as investigations related to the biosynthesis and metabolism of the biopterin cofactor necessitate a method that is specific, rapid and reproducible for analyzing these compounds in tissues and body fluids.

Biopterin and/or its metabolites and related compounds have been determined by bioassay using *Crithidia fasciculata* [8], enzymatic assay using phenylalanine hydroxylase [9], conventional column and thin-layer chromatography [10, 11], radioimmunoassay [4], and high-performance liquid chromatography (HPLC) [12, 13]. However, limitations of these methods include a lack of specificity, the need for large amounts of material, long retention times, the inability to detect all precursors and metabolites of biopterin, or changes in the elution profile due to salt effects. The aim of this investigation was to develop an HPLC method for the separation of the pterins present in mammalian tissues and fluids that is rapid, specific and insensitive to salts present in the samples to be analyzed.

MATERIALS AND METHODS

Biopterin, neopterin, sepiapterin and 6-hydroxymethylpterin were purchased from Dr. B. Schircks (Wettswil, Switzerland); pterin, xanthopterin, isoxanthopterin and pterin-6-carboxylic acid were from Sigma (St. Louis, MO,

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U.S.A.). Acetonitrile and tetrahydrofuran were HPLC grade. All other chemicals were reagent grade.

Neuroblastoma N115 cells were grown in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum. Sample preparation was carried out as described by Fukushima and Nixon [12]. Urine samples were chromatographed directly following iodine oxidation in acid or base after excess iodine had been neutralized with ascorbic acid. Extracts of cells in culture were carried through the entire procedure. Creatinine was determined using a creatinine kit from Sigma according to the method of Jaffé [14] as modified by Heinegard and Tinderstrom [15].

Fluorescence spectra of the pterins were determined using a Perkin-Elmer MPF-2A scanning spectrofluorometer. The HPLC system consisted of a Tracor Model 995 isochromatographic pump, a Waters Model 710B WISP autoinjector and either a Perkin-Elmer Model 650-10LS or a Schoeffel Model FS-970 fluorometer. Data were collected and analyzed with a DS-80 microcomputer (Digital Specialties). A Whatman PXS 10/25 ODS 10-μm column (25 × 0.46 cm) fitted with a pre-column packed with CO:Pell ODS (7 × 0.21 cm) was used in all separations. The solvent system consisted of 0.5% acetonitrile and 0.1% tetrahydrofuran in water which was filtered using a 0.45-μm Ultipor NX membrane filter (Rainin), and degassed before use. Flow-rate was 1.5 ml/min. Pterins were detected with the Perkin-Elmer using an excitation wavelength of 360 nm with a 5-nm slit width and an emission wavelength of 450 nm with a 10-nm slit width. When the Schoeffel fluorometer was used, a filter with an emission cutoff below 418 nm was employed.

RESULTS AND DISCUSSION

The fluorescence spectra of the oxidized pterins were determined in the solvent system used for HPLC. The excitation and emission maxima are given in Table I. Based on these results an excitation wavelength of 360 nm was chosen for detection of pterins in this system. A chromatogram illustrating the separation of a mixture of standard pterins is illustrated in Fig. 1A. The elution profile was not affected by salts or organic solvents in the sample. All samples were acidified before being chromatographed since pterin-6-carboxylic acid would elute in the void volume if the samples were at neutral or alkaline pH. The k' values as well as the limits of detection of each of the pterins are also presented in Table I. The limits of detection for all the pterins except xanthopterin ranged from 50—200 pg. However, sensitivity could be increased by the use of wider slit widths on the Perkin-Elmer or by the selection of a different excitation wavelength if increased sensitivity for a specific pterin is desired. During the useful life of the column retention times did not significantly vary and repeated injections of standard solutions of biopterin indicated a variation in peak heights of 4%.

The limits of detection of xanthopterin are considerably higher than those of the other commonly occurring pterins, due to the fact that xanthopterin elutes as a broad peak and also has an excitation maximum considerably higher than the other pterins. Under the elution conditions employed in this study, sepiapterin is markedly retained on the column. Moreover the marked

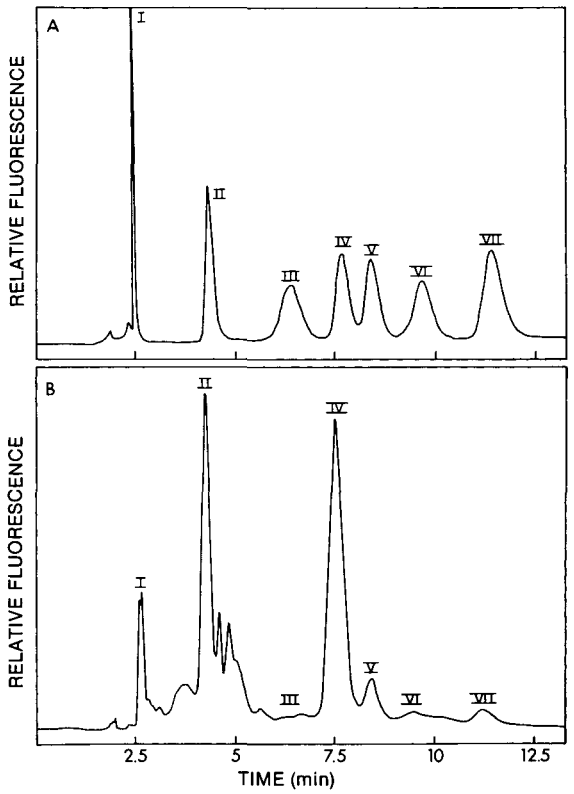

Fig. 1. HPLC separation of pterins. (A) Chromatogram of a standard mixture of pterins containing 56 ng of xanthopterin and 2.25 ng of all the other pterins. (B) Chromatogram of normal human urine that had been oxidized with iodine in acidic solutions and the excess iodine removed by addition of ascorbic acid. The equivalent of 10 μl of urine was chromatographed. Peaks: I = pterin-6-carboxylic acid; II = neopterin; III = xanthopterin; IV = biopterin; V = isoxanthopterin; VI = 6-hydroxymethylpterin; VII = pterin.

difference in excitation and emission maxima from those used in the present study renders all but large quantities of sepiapterin undetectable.

The chromatographic system described above was used for the analysis of pterin distribution and content in human urine and neuroblastoma cells in culture. A typical chromatogram of normal human urine that had been oxidized with iodine in acid solution is presented in Fig. 1B and the distribution of pterins in normal urine as well as in neuroblastoma cells is presented in Table II. The concentration of pterins in urine is high enough and interfering fluorescent material low enough so that urine can be chromatographed directly after iodine oxidation without being further treated on Dowex resins as described for tissues [12, 13]. However, the cells in culture must be carried through the entire procedure. Using the conditions described above the high concentration of salt present in urine samples does not affect the elution profile of the pterins, and all of the pterins which commonly occur in tissues and urine can be separated and quantitated.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF PTERINS

Pterin	λ max (nm)		k'*	Limits of detection** (pg)
	Excitation	Emission		
Pterin carboxylic acid	365	444	0.32	45
Neopterin	365	450	1.29	190
Xanthopterin	397	465	2.38	2700
Biopterin	364	447	3.05	185
Isoxanthopterin	347	405	3.47	90
Hydroxymethylpterin	357	443	4.13	185
Pterin	355	442	5.04	200
Sepiapterin	420	520	—	—

*$k' = \frac{V_e - V_0}{V_0}$ where V_e is the elution volume and V_0 is the void volume.

**Excitation 360 nm; emission 450 nm.

TABLE II

PTERIN CONTENT OF BIOLOGICAL SAMPLES

Pterin	Urine: ng/mg creatinine	Neuroblastoma N115: ng/10^6 cells	
		Cells	Medium
Pterin carboxylic acid	3.2 ± 0.5	n.d.*	n.d.
Neopterin	539 ± 90	0.079 ± 0.01	4.14 ± 0.88
Xanthopterin	258 ± 91	n.d.	n.d.
Biopterin	1148 ± 76	8.13 ± 0.88	20.1 ± 1.9
Isoxanthopterin	1007 ± 234	n.d.	n.d.
Hydroxymethylpterin	25.4 ± 4.7	0.257 ± 0.034	29.8 ± 7.5
Pterin	126 ± 31	1.57 ± 0.17	119 ± 16.7

*n.d. = Not detectable.

REFERENCES

1 T. Nagatsu, Trends Pharmacol. Sci., Oct. (1981) 276.
2 A. Tietz, M. Lindberg and E.P. Kennedy, J. Biol. Chem., 239 (1964) 4081.
3 J.C. Nixon, C.-L. Lee, S. Milstien, S. Kaufman and K. Bartholomé, J. Neurochem., 35 (1980) 898.
4 T. Nagatsu, T. Yamaguchi, T. Kato, T. Sugimoto, S. Matsuura, M. Akino, I. Nagatsu, R. Iizuka and H. Narabayashi, Clin. Chim. Acta, 109 (1981) 305.
5 A.C. Williams, R.A. Levine, T.N. Chase, W. Lovenberg and D.B. Calne, J. Neurol. Neurosurg. Psychiat., 43 (1980) 735.
6 A. Hausen, D. Fuchs, K. Grünewald, H. Huber, K. König and H. Wachter, Clin. Biochem., 15 (1982) 34.
7 K.N. Rao, S. Trehan and J.N. Noronha, Cancer, 48 (1982) 1656.
8 H. Baker, O. Frank, C.J. Bacchi and S.H. Hutner, Amer. J. Clin. Nutr., 27 (1974) 1247.

9 S. Kaufman, S. Berlow, G.K. Summer, S. Milstien, J.D. Schulman, S. Orloff, S. Spielberg and S. Pueschel, N. Engl. J. Med., 299 (1978) 673.
10 T. Fukushima and T. Shiota, J. Biol. Chem., 247 (1972) 4549.
11 H. Rembold and H. Metzger, Z. Naturforsch., 226 (1967) 827.
12 T. Fukushima and J.C. Nixon, Anal. Biochem., 102 (1980) 176.
13 B. Stea, R.M. Halpern and R.A. Smith, J. Chromatogr., 168 (1979) 385.
14 M. Jaffé, Hoppe-Seyler's Z. Physiol. Chem., 20 (1886) 391.
15 D. Heinegard and G. Tinderstrom, Clin. Chim. Acta, 43 (1973) 305.

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CHROMBIO. 1594

Note

Dosage de l'antipyrine dans le plasma et la salive par chromatographie liquide haute-performance chez l'homme

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(Reçu le 11 août 1982; manuscrit modifié reçu le 25 novembre 1982)

L'antipyrine est souvent utilisée comme indicateur du métabolisme hépatique. En effet, à faible dose, elle n'a pas d'effet pharmacologique important. Elle est rapidement absorbée et se distribue dans l'eau totale sans liaison importante aux protéines. Elle n'est que faiblement extraite au niveau du foie et son oxydation se fait complètement au niveau de cet organe; l'excrétion rénale de l'antipyrine non transformée est négligeable; sa mesure est donc un paramètre fiable reflétant le métabolisme hépatique. En effet la clairance de l'antipyrine est augmentée après administration répétée d'un inducteur enzymatique. En postulant la constance du volume de distribution, la comparaison des constantes d'élimination de l'antipyrine avant et après traitement évaluera l'effet inducteur.

Par ailleurs, la mesure de la concentration d'antipyrine est réalisable sur des prélèvements salivaires [1], ce qui évite les prélèvements veineux [2]. Différentes techniques analytiques ont été proposées: colorimétrie [3,4], chromatographie en phase gazeuse [5—7], spectrodensitométrie [8], chromatographie en phase liquide haute-performance (CLHP) [9], radio-immunologie [10]. La méthode radio-immunologique permet une détection à partir de 10 ng/ml mais s'avère lourde car elle nécessite la réalisation d'anticorps spécifiques difficiles à obtenir et la méthode spectrodensitométrique quoique sensible reste très longue à mettre en oeuvre. La chromatographie en phase gazeuse quant à elle, permet une détection de 2 à 5 ng/ml mais nécessite un volume plasmatique d'au moins 1 ml.

La méthode de chromatographie en phase liquide haute performance décrite récemment par Danhof et al. [9] permet d'effectuer les dosages dans le plasma ou la salive. Cette méthode est sensible mais a cependant l'inconvénient

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d'utiliser un tampon phosphate qui nécessite un rinçage prolongé pour la maintenance de l'appareillage.

La méthode proposée fait appel à la chromatographie liquide à haute performance en phase normale avec détection ultraviolette après extraction d'échantillons auxquels est ajouté un étalon interne.

MATERIELS ET METHODES

Réactifs et solvants

Antipyrine (Fig. 1) ou 2-3-dimethyl-1-phenyl-3-pyrazolin-5-one (Fluka, Buchs, Suisse), et étalon interne: déméthyldiazepam (Fig. 1) ou 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-2-one (Roche, Neuilly sur Seine, France) sont utilisés.

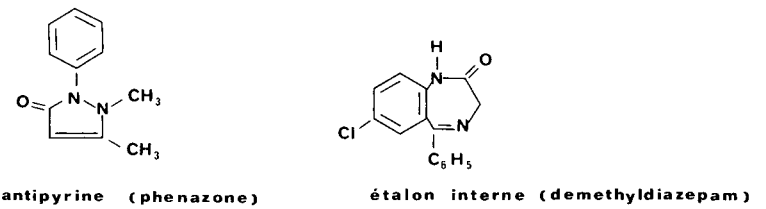

Fig. 1. Formules développées de l'antipyrine et de l'étalon interne.

La solution mère d'antipyrine est préparée dans le méthanol aux concentrations de 20 μg/ml ou 200 μg/ml et la solution mère de déméthyldiazepam est préparée dans le méthanol à la concentration de 125 μg/ml.

Les solvants utilisés sont de qualité pour analyse: chloroforme puriss. (Fluka), éthanol (Carlo-Erba, Italie), méthanol (Carlo-Erba), 1 *M* acide chlorhydrique (Prolabo, Paris, France), ammoniaque pure 20% (Merck, Darmstadt, R.F.A.), sulfate de sodium anhydre (Merck).

Conditions chromatographiques

Appareillage. Appareillage utilisé: Chromatographe liquide haute-performance Waters I 6000A muni d'un injecteur automatique Wisp 710 B (Waters Assoc., Milford, MA, U.S.A.) et d'un spectrophotomètre Lambda Max (Modèle 480, Waters), longueur d'onde 280 nm, sensibilité 0.02 unité D.O., pleine échelle relié à un enregistreur Omniscribe (Houston Instruments); déroulement du papier: 5 mm/min. La colonne est une LiChrosorb Si 60 de 5 μm (Merck), 15 cm × 4.8 mm I.D. et la phase mobile un mélange éthanol—ammoniaque—chloroforme (1.6:0.05:98.35) dégazé aux ultrasons (appareil Bromsonic 52) utilisé à un débit de 4 ml/min soit une pression d'environ 150 bars.

Extraction. L'extraction se fait en milieu acide par le chloroforme d'échantillons salivaires ou plasmatiques. A 500 μl de salive ou de plasma (échantillon à doser ou étalon) sont ajoutés 100 μl de 1 *M* acide chlorhydrique, 50 μl d'une solution méthanolique à 125 μg/ml de déméthyldiazepam, 500 μl de chloroforme; après agitation pendant 15 min au banc agitateur (Realis type 44-40), les tubes sont centrifugés à 1000 *g* pendant 10 min. La phase aqueuse surnageante est aspirée et rejetée. La phase organique inférieure est déshydratée

par du sulfate de sodium anhydre; 50 μl de cette phase sont ensuite injectés dans le chromatographe.

Etalonnage. Les courbes d'étalonnage sont obtenues en traitant dans les mêmes conditions soit des plasmas témoins (provenant du centre de transfusion) soit des salives témoins (provenant d'un recueil de salives de volontaires sains) surchargés en antipyrine à des concentrations de 0, 0.5, 2, 5, 10, 20, 30 μg/ml. On porte en ordonnée le rapport des hauteurs de pic d'antipyrine sur le pic d'étalon interne et en abscisse les concentrations d'antipyrine.

RESULTATS

Les chromatogrammes obtenus lors de cette étude sont représentés sur la Fig. 2. La substance précédant l'étalon interne sur le chromatogramme d n'a pu être identifiée.

Dans les conditions décrites les temps de rétention de l'étalon interne et de l'antipyrine sont respectivement de 1.2 min et 2.4 min (facteurs de capacité respectifs: 1 et 3.0).

La Fig. 3 montre les droites d'étalonnage obtenues dans la salive et le plasma pour des concentrations d'antipyrine de 0.5 μg/ml à 30 μg/ml.

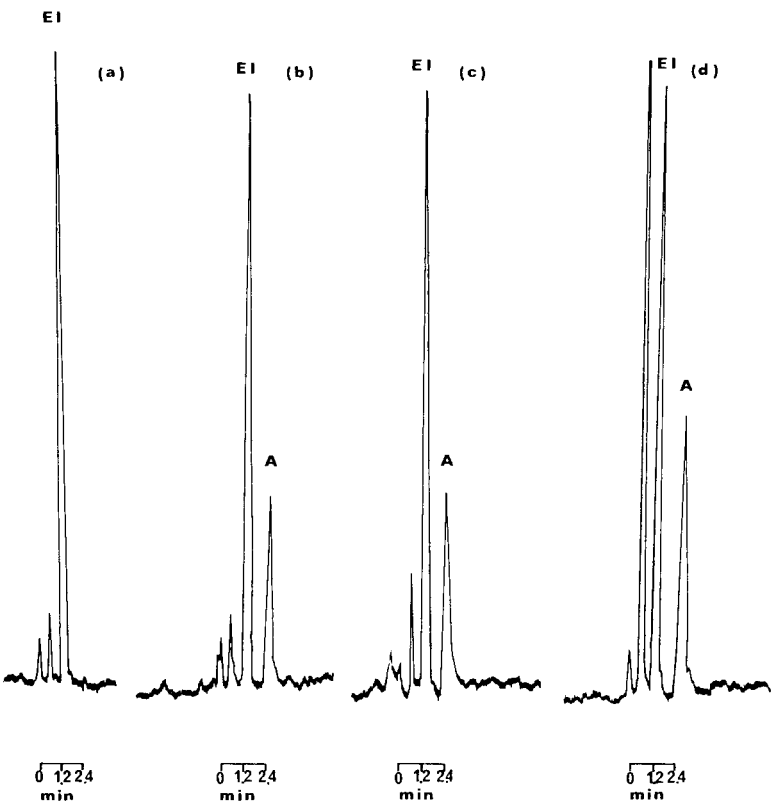

Fig. 2. Chromatogrammes obtenus après injection: (a) d'une solution plasmatique contenant uniquement l'étalon interne (EI); (b) d'un témoin plasmatique à 10 μg/ml d'antipyrine; (c) d'un témoin salivaire à 10 μg/ml d'antipyrine; (d) d'un échantillon salivaire prélevé chez un volontaire sain.

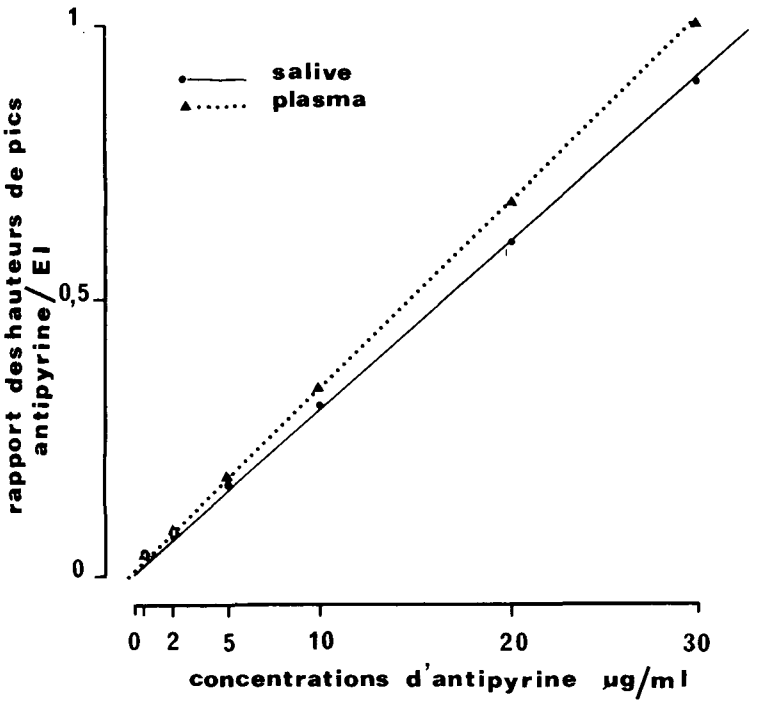

Fig. 3. Droite d'étalonnage: Rapport des hauteurs de pics d'antipyrine et d'étalon interne en fonction des concentrations plasmatiques ou salivaires d'antipyrine.

Précision

La reproductibilité de la méthode a été déterminée dans la salive et le plasma pour 3 concentrations 2, 5 et 10 µg/ml; chaque concentration ayant fait l'objet de 10 déterminations le même jour. Les coefficients de variation sont respectivement pour la salive de 5.1, 2.0 et 1.9% et pour le plasma de 4.2, 2.5 et 3.5% (Tableau I).

La répétabilité de la méthode a été controlée dans la salive et le plasma pour 3 concentrations 2, 5 et 10 µg/ml; chaque concentration ayant fait l'objet d'une détermination 6 jours de suite. Les coefficients de variation sont respectivement de 4.3, 5.9 et 6.3% pour la salive, de 5.3, 5.9 et 6.1% pour le plasma (Tableau I).

TABLEAU I

COEFFICIENTS DE VARIATION OBTENUS LORS DE LA REPRODUCTIBILITE ET DE LA REPETABILITE DES MESURES DANS LA SALIVE ET LE PLASMA

Antipyrine (µg/ml)	Coefficient de variation (%)			
	Reproductibilité		Répétabilité	
	Salive	Plasma	Salive	Plasma
2	5.1	4.2	4.3	5.3
5	2.0	2.5	5.9	5.9
10	1.9	3.5	6.3	6.1

Dans les conditions décrites la limite de détection est de 0.2 µg/ml. Il est possible d'abaisser cette limite soit en augmentant la sensibilité du détecteur (0.005 unité D.O., pleine échelle, le bruit de fond restant faible) soit en injectant la totalité de l'extractum. Dans ce cas, l'échantillon extrait sera congelé rapidement par immersion du tube dans l'azote liquide; la totalité de la phase organique est ainsi recueillie puis évaporée. Le résidu est repris par 50 µl de phase mobile qui sont injectés dans le chromatographe. La limite de détection est alors de 50 ng/ml.

Rendement

Le rendement de l'extraction a été calculé en comparant la hauteur du signal obtenu en injectant une quantité connue d'antipyrine à celle du signal obtenu après extraction d'échantillons salivaires ou plasmatiques surchargés de la même quantité d'antipyrine (Tableau II).

TABLEAU II

RENDEMENT D'EXTRACTION DE L'ANTIPYRINE DANS LA SALIVE ET LE PLASMA

Antipyrine (µg/ml)	Rendement en antipyrine (%)	
	Salive	Plasma
0.5	78	89
2	77	96
5	86	119
10	78	93
20	76	94
30	74	94
Moyenne	78.1	97.5

DISCUSSION

La méthode décrite a été utilisée pour déterminer les concentrations salivaires d'antipyrine après administration d'une dose de 1 g d'antipyrine per os chez des volontaires sains lors d'une étude d'induction enzymatique.

Cette méthode CLHP présente une limite de détection de 0.2 µg/ml susceptible d'être portée à 50 ng/ml par la technique de congélation-évaporation, ces concentrations étant très inférieures à celles retrouvées lors des études pharmacocinétiques chez l'homme (environ 4 µg/ml, 24 h après une prise de 1 g d'antipyrine per os sous inducteur enzymatique). L'utilisation d'un étalon interne permet une amélioration notable de la reproductibilité, surtout dans les valeurs moyennes où se trouvent la plupart des patients après prise de 1 g.

La phase mobile, solvant organique simple évite les précipitations de sels obtenus avec l'utilisation des tampons phosphate donc la détérioration de l'appareillage et raccourci les délais de manipulation.

Cette technique rapide permet en outre, à la différence de la méthode de chromatographie en phase gazeuse qui utilise des échantillons plasmatiques, d'effectuer les dosages d'antipyrine indifféremment dans le plasma ou la salive

et de surcroît sur des échantillons de très faible volume; le dernier milieu étant d'accès bien plus aisé pour les études pharmacocinétiques.

La méthode proposée se caractérise donc par sa rapidité, sa simplicité sa sensibilité et sa facilité d'application aux études pharmacocinétiques chez l'homme.

BIBLIOGRAPHIE

1 E.S. Vessel, G.T. Passananti, P.A. Glenwright et B.H. Dvorchick, Clin. Pharmacol. Ther., 18 (1975) 259.
2 P.M. Brooks, M.A. Bell et H. Burns, Br. J. Clin. Pharmac., 3 (1976) 945.
3 B.B. Brodie, J. Axelrod, R. Soberman et B.B. Levy, J. Biol. Chem., 179 (1949) 25.
4 D. Mendelsohn et N.W. Levin, S. Afr. J. Med. Sci., 25 (1960) 13.
5 D.H. Huffman, D.W. Shoeman et D.L. Azarnoff, Biochem. Pharmacol., 23 (1974) 197.
6 S. Lindgren, P. Collste, B. Norlander et F. Sjoquist, Eur. J. Clin. Pharmacol., 7 (1974) 381.
7 L.F. Prescolt, K.K. Adjepon-Yamoah et E. Roberts, J. Pharm. Pharmacol., 25 (1973) 205.
8 R.M. Welch, R.L. De Angelis, M. Wingfield et T.W. Farmer, Clin. Pharmacol. Ther., 18 (1975) 249.
9 M. Danhof, E. de Groot van der Vis et D.D. Breimer, Pharmacology, 18 (1979) 210.
10 R.L. Chang, A.W. Wood, W.R. Dixon, A.H. Conney, K.E. Anderson, J. Eiseman et A.P. Alvares, Clin. Pharmacol. Ther., 20 (1976) 219.

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CHROMBIO. 1601

Note

Determination of atracurium besylate in human plasma

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(First received October 11th, 1982; revised manuscript received December 7th, 1982)

Atracurium besylate, 2,2′-(3,11 dioxo-4,10-dioxatridecylene)-bis-[6,7 dimethoxy-1(3,4 dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzene sulphonate (Fig. 1) has been developed as a neuromuscular blocking agent which undergoes non-enzymic decomposition under physiological conditions, thus having a predictable duration of action [1]. Earlier studies had shown that the compound does undergo a facile decomposition at pH 7.4 by Hofmann elimination [2]. Analysis of atracurium therefore necessitated the

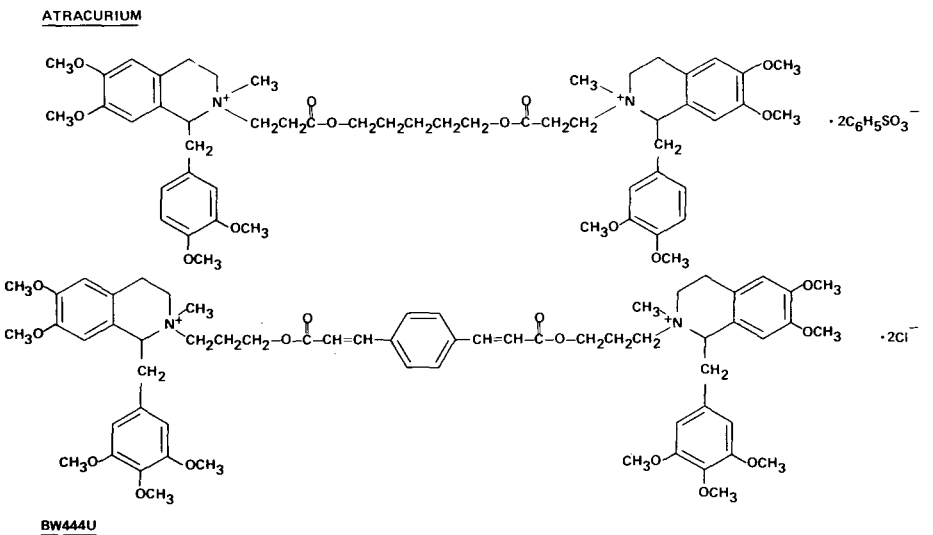

Fig. 1. Chemical structures of atracurium and BW444U.

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use of rapid sample preparation and relatively mild chromatographic conditions if the integrity of the molecule were to be preserved.

High-performance liquid chromatography (HPLC) was the method of choice, since this was readily applicable for fairly high molecular weight ionized compounds. Reversed-phase chromatography with a variety of mobile phases including ion-pairing reagents was investigated, but efficiency and capacity were low so a method was developed using cation-exchange chromatography.

EXPERIMENTAL

Chemicals

Atracurium besylate and compound BW444U were supplied by the Wellcome Foundation (Dartford, Great Britain). Acetonitrile, HPLC grade, was obtained from Rathburn Chemicals (Walkerburn, Great Britain); sodium sulphate and sulphuric acid, both AnalaR grade, were obtained from BDH Chemicals (Poole, Great Britain).

Equipment

Sep-Pak C_{18} cartridges (Waters Assoc., Northwich, Great Britain) were prewetted before use by successive passage of 2 ml acetonitrile, 2 ml acetonitrile—sulphuric acid, pH 2 (80:20) and 5 ml distilled water.

An Eppendorf centrifuge, Model 5414 was used for the rapid separation of blood samples.

HPLC equipment consisted of a Hewlett-Packard 1084B chromatograph linked to a Hitachi 650-LC fluorescence detector. The column used was 250 × 4.9 mm Partisil 10 SCX, particle size 10 μm, packed by Hichrom (Woodley, Great Britain).

Preparation of standards for calibration curve

Samples of atracurium were prepared at 0.01, 0.1 and 1 mg/ml in dilute sulphuric acid (pH 3.2) containing BW444U as a carrier at 5 μg/ml and were used to spike plasma for calibration purposes. An additional quantity of 2.5 μg BW444U was added to each ml of plasma to suppress adsorption.

Replicate 2-ml portions of the spiked plasma were dispensed into glass vials containing 200 μl of sulphuric acid (pH 1) and immediately frozen in solid carbon dioxide. These standards were prepared on the same day as blood samples were obtained.

Preparation of samples

Fresh heparinised blood samples (10 ml) were centrifuged for approximately 30 sec in 1.5-ml micro-test tubes. Plasma was dispensed as two 2-ml portions into glass vials containing 200 μl of sulphuric acid (pH 1) and 5 μg of BW444U and frozen as above.

Extraction of samples and standards

Plasma samples were extracted within 24 h of freezing. They were gently thawed and immediately centrifuged in the Eppendorf for approximately 30 sec to remove any precipitated protein. The supernatant (2 ml) was applied

through a Luer-tip syringe to a Sep-Pak cartridge and then washed with 1 ml sulphuric acid (pH 1) followed by 0.4 ml acetonitrile—sulphuric acid, pH 2 (80:20). These effluents were discarded. The retained atracurium was eluted with 1 ml acetonitrile—sulphuric acid, pH 2 (80:20) into a micro test tube. All samples were centrifuged for approximately 1 min to remove any precipitate which might have formed, 0.8 ml of the supernatant was transferred to a glass vial for HPLC and tightly capped. Extracted samples were found to be stable for overnight runs and could be kept in a refrigerator for several days.

HPLC conditions

The mobile phase was acetonitrile—sulphuric acid, pH 2 (50:50) containing 0.03 *M* sodium sulphate. At a flow-rate of 3.5 ml/min atracurium eluted in approximately 4 min with an oven temperature of 60°C. Normally 20 μl were injected.

Fluorimeter settings were 280 nm and 320 nm for excitation and emission, respectively. Under these conditions BW444U does not fluoresce and does not appear on the chromatogram.

RESULTS AND DISCUSSION

Atracurium is, by design, difficult to handle under physiological conditions, so the assay was developed to minimise possible degradation during sample processing. The small amount of acid added to plasma samples was sufficient to take the pH to approximately 6 without causing excessive protein precipitation. BW444U was added because it is sufficiently similar in structure to atracurium to reduce the extent of adsorption of atracurium on glass vials used for storage. These should preferably be of neutral glass, since problems of breakdown with solutions inadvertently stored in soda glass vials have occurred. Even at −20°C, Hofmann elimination can occur under mild basic conditions, so the 24-h limit on sample storage before extraction was established as a working practice.

No suitable internal standard was available for this assay. This caused no problems in practice but to allow for any extraction variability, duplicate samples were used. The coefficient of variation of the extraction and assay of atracurium from spiked control plasma was typically 10% over the range 0.05–10 μg/ml (3% at 3 μg/ml, 16% at 0.3 μg/ml and 19% at 0.05 μg/ml; $n=6$).

Fig. 2 shows a typical HPLC trace for extracted standards. Linearity was observed over the range 0.05—10 μg/ml; $r=0.9983$ for extracted standards; $r=0.9999$ for unextracted standards. Extraction efficiency was found to be approximately 70% by comparison with unextracted standards. Peak heights were measured as this was found to be more reliable than peak area integration, especially at low concentrations. About 1 ng is the limit of detection on column.

With several samples to extract, we found it more convenient to set up a succession of Sep-Pak cartridges inserted through pierced "Suba-seal" stoppers into tubes under vacuum suction. In this way a steady negative pressure could be applied.

In general this method was easy, convenient and reliable over many months of use. Occasional topping up of the column with, if necessary, a slight adjust-

Fig. 2. Sample traces from HPLC. (a) Unextracted standard equivalent to 300 ng/ml atracurium; (b) extracted plasma containing 300 ng/ml atracurium; (c) extracted plasma blank.

ment of the inorganic salt content was all that was required. With small injection volumes we were able to recycle the mobile phase during a run, since this did not cause a significant rise in the baseline and ensured steady run conditions. This method allowed separation of atracurium from all its known breakdown products except for the monoquaternary compound which was present only as a very minor impurity.

The wide range of standards was necessary to measure plasma concentrations of atracurium in man following the intravenous administration of a paralysing dose [3]. The assay has been found to be adequate for all the clinical studies so far undertaken, which at present exceeds 500 samples.

ACKNOWLEDGEMENTS

Our thanks are due to Ian Pratt and Paul Johnson for their expert technical assistance.

REFERENCES

1 R. Hughes and D.J. Chapple, Brit. J. Anaesthesia, 53 (1981) 45.
2 J.B. Stenlake, Advan. Pharmacol. Ther., 3 (1978) 303.
3 S. Ward, E.A.M. Neill, B.C. Weatherley and I.M. Corrall, Brit. J. Anaesthesia, (1983) in press.

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CHROMBIO. 1604

Note

Determination of oxypertine in human serum by high-performance liquid chromatography

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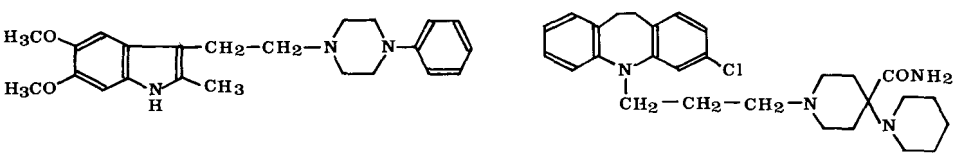
(First received September 6th, 1982; revised manuscript received December 6th, 1982)

Oxypertine is different from previous antipsychotic drugs in that it has a chemical structure similar to that of serotonin (Fig. 1).

It is presumed that the main mechanism of action of oxypertine is a marked depletion of the noradrenaline level at the nerve terminals by acting on the noradrenergic neurons with a slight depletion of serotonin and dopamine levels [1—3]. In general, antipsychotic drugs act primarily on dopaminergic neurons and decrease the dopamine level at the nerve terminals. There are also some reports that dopamine or serotonin levels in the rat brain are reduced as a result of oxypertine administration [4—6].



(1) (2)

Fig. 1. Structures of oxypertine (1) and clocapramine (2).

When oxypertine is administered to patients, there is a minute or no increase in the serum prolactin level [7], which reflects the dopamine receptor blocking activity of the antipsychotic drug in the central nervous system. To date the correlation between serum oxypertine concentration and prolactin levels in humans has not been established.

Several assays for oxypertine have been described [8, 9], but they were not applicable to our laboratory. Thus we developed a high-performance liquid chromatographic method for measuring oxypertine in human serum.

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EXPERIMENTAL

Materials

Oxypertine was obtained from Daiichi Seiyaku (Tokyo, Japan) and clocapramine from Yoshitomi Pharmaceutical (Osaka, Japan). Sodium carbonate, sodium acetate, acetic acid, isopropanol, diethyl ether and methanol were purchased from Wako Pure Chemical (Tokyo, Japan).

Oxypertine standards

Oxypertine (20 mg) is dissolved in methanol to a final volume of 20 ml. This solution is diluted to concentrations of 10, 25, 50 and 100 mg/l. The solutions were stored at 4°C.

Clocapramine standards

Clocapramine (20 mg) is dissolved in 20 ml of methanol. Store the solution at 4°C. An internal standard is prepared by diluting 1 ml of the stock solution to 10 ml with methanol when needed.

Instrumentation

A Hitachi 635 high-performance liquid chromatograph equipped with an ultraviolet detector (Hitachi, Tokyo, Japan) was used for the analysis. The column employed was 30 cm × 4 mm I.D. packed with Zorbax Sil 7–8 μm (Du Pont, Wilmington, DE, U.S.A.). The analysis was performed at room temperature. The effluent was monitored at 252 nm. The composition of the mobile phase was methanol–acetic acid–sodium acetate (200:0.3:0.1) with a flow rate of 0.8 ml/min. The chromatogram was recorded on an Hitachi 065 recorder and peak areas were calculated with a Chromatopac C-E1B (Shimadzu, Kyoto, Japan).

Procedure

In the case of an acute study, 50 mg of oxypertine were administered to a schizophrenic patient and blood samples were obtained at 0, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 h. Then, 150 mg of oxypertine were administered daily for eight weeks and blood samples were obtained at intervals of three or four days.

The serum concentration of prolactin was determined by radioimmunoassay using Prolactin Kit "Daiichi".

The method of measuring serum oxypertine levels was as follows. To 0.5 or 1 ml of serum in a glass centrifuge tube, was added 0.5 μg per 5 μl clocapramine (internal standard) along with 0.5 ml of 1 *M* sodium carbonate to make it basic. After the addition of 5 ml of diethyl ether containing 0.05 ml of isopropanol, the tube was shaken for 10 min. The organic layer was filtered through a cotton filter for dehydration into a pear-shaped flask then evaporated to dryness in vacuo at room temperature. The residue was dissolved in 50 μl of methanol and 5 μl were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Fig. 2 shows chromatograms of blank serum, serum spiked with 0.5 μg/ml

oxypertine and serum from a patient. Oxypertine was well separated from the internal standard and serum constituents. The retention times of oxypertine and internal standard were 6.4 and 8.8 min, respectively.

The calibration curve passes through the origin and is linear within the range 0—2 μg/ml. The lower limit of quantitation of oxypertine was 20 ng/ml in serum. The absolute recovery of oxypertine was 79.1 ± 3.5% (C.V. = 4.37%) at a concentration of 0.1 μg/ml. There was no remarkable increase in recovery of oxypertine when other solvents were used as extractants such as ethyl acetate, benzene, *n*-pentane or diethyl ether with a higher concentration of isopropanol. In the cases of ethyl acetate and diethyl ether with a higher concentration of isopropanol, additional peaks derived from serum were observed on the chromatograms.

Within-run and between-run precision values are summarized in Table I. These results indicate that the reproducibility of the procedure is satisfactory.

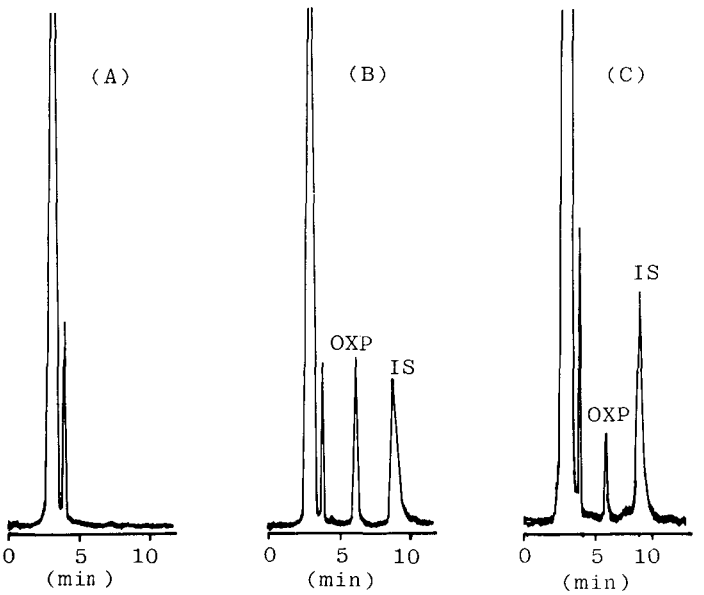

Fig. 2. Typical chromatograms of (A) blank serum, (B) serum spiked with 0.5 μg of oxypertine (OXP) and clocapramine (IS), (C) serum obtained from a patient 3 h after oral administration of oxypertine.

TABLE I

VALUES OF PRECISION

	Within-run		Between-run	
n	10	10	5	5
Mean (ng)	91.47	483.93	100.47	449.85
S.D. (ng)	4.27	18.96	4.66	17.97
C.V. (%)	4.43	3.92	4.63	3.99

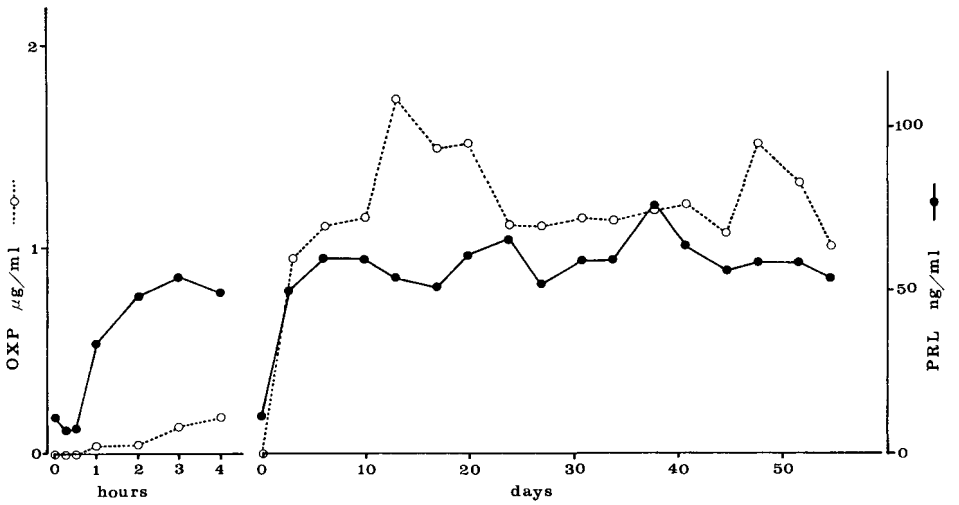

Fig. 3. Serum levels of oxypertine (OXP) and prolactin (PRL) after oral administration of oxypertine to a patient.

Some commonly prescribed antiparkinsonian drugs such as trihexyphenidyl and biperiden were chromatographed after extraction by the method described in this paper. No peaks interfered with the measurement of oxypertine.

The simplicity and precision of the extraction procedure leads us to conclude that the procedure is acceptable as a routine method for measuring oxypertine.

Fig. 3 shows serum levels of oxypertine and prolactin after acute and chronic administration of oxypertine. When oxypertine was administered acutely, increased serum oxypertine and prolactin levels were seen between 1 and 4 h. In the case of chronic treatment, the serum concentration of oxypertine reached a steady state within four days. In addition, parallel changes in serum prolactin levels were observed.

REFERENCES

1 S. Spector, K. Melmon and A. Sjoerdsma, Proc. Soc. Exp. Biol. Med., 3 (1962) 79.
2 M. Matsuoka, Jap. J. Pharmacol., 14 (1964) 181.
3 R. Hassler, J.I. Back and J.S. Kim, Nervenarzt, 41 (1970) 105.
4 K. Fuxe, H. Grobecker, T. Hökfelt, J. Jonsson and T. Malmfors, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 256 (1967) 450.
5 F. Nitsch, R. Hassler, J.S. Kim and I.J. Back, Nervenarzt, 47 (1976) 40.
6 R. Moroji, K. Takahashi, C. Ikeda, H. Murayama and R. Iizuka, Complete record of symposium in Japan on oxypertine by Prof. van Praag, Daiichi Seiyaku Co. Ltd., Tokyo, Japan, 1980, p. 105.
7 T. Miyagawa, Y. Aoba, A. Homma, H. Amamoto and K. Hasegawa, Complete record of symposium in Japan on oxypertine by Prof. van Praag, Daiichi Seiyaku Co. Ltd., Tokyo, Japan, 1980, p. 116.
8 M.M. Airaksinen, P.J. Neuvonen and I. Jaakonmäki, Biochem. Pharmacol., 19 (1970) 2181.
9 H. Yuki, C. Watanabe and H. Kawasaki, J. Pharm. Soc. Jap., 102 (1982) 1201.

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CHROMBIO. 1605

Note

Quantitative determination of oxcarbazepine

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(First received September 23rd, 1982; revised manuscript received December 6th, 1982)

Oxcarbazepine (10,11-dihydro-10-oxo-carbamazepine) (Ciba-Geigy, GP 47680) is a potential anticonvulsant drug. Studies suggest good efficacy and tolerability of the new compound, which is metabolized in humans to 10,11-dihydro-10-hydroxycarbamazepine (GP 47779) and 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine (CGP 10.000). GP 47779 possesses an anticonvulsant activity of its own [1–4].

This note describes a simple high-performance liquid chromatographic assay for monitoring the plasma of patients when therapeutic doses are administered.

MATERIALS AND METHODS

Reagents

Ammonia (25% NH_3; UCB 4747), ammonium nitrate (Merck 1188), isopropanol (Merck 9634), ethyl acetate (Merck 863) and ethanol (UCB 1115) were from UCB and E. Merck.

10,11-Dihydro-10-oxo-carbamazepine (oxcarbazepine) (GP 47680), 10,11-dihydro-10-hydroxycarbamazepine (metabolite) (GP 47779), 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine (metabolite) (GGP 10.000), and 9-hydroxymethyl-10-carbamyl-acridine (internal standard) (CGP 9955) were from Ciba-Geigy.

Carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and valproic acid were from our collection.

Apparatus

A Pye-Unicam liquid chromatograph type LCXPD was used with a dual-piston reciprocating pump, a Rheodyne injection valve (Model 7120, capacity

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200 μl) and a variable-wavelength detector with a Philips PM 8251/02 recorder. A Macherey-Nagel column (200 × 6 mm O.D. × 4 mm I.D.) packed with reversed-phase C_{18} silica gel, particle size 7.5 μm (Polygosil® 60-7C_{18}) was used.

Sample preparation

To 1 ml of plasma or serum in a 20-ml glass centrifuge tube were added 1 ml of internal standard solution (2 mg/l CPG 9955) and 2 ml of water. This mixture was extracted with 6 ml of ethyl acetate by mechanical shaking. The tube was then centrifuged at 3000 *g* for 5 min. The organic phase was transferred to an evaporation tube and the ethyl acetate layer evaporated to dryness on a water-bath under a stream of dry nitrogen [4]. The residue was redissolved in 1 ml of mobile phase of which 200 μl were injected into the chromatographic column.

Chromatography

The mobile phase consisted of 56% of solution A and 44% of solution B. Solution A: 6‰ (w/v) of ammonium nitrate and 0.15‰ (v/v) of ammonia solution in deionized and distilled water. Solution B: 50% solution A and 50% isopropanol.

Instrumental conditions were as follows: flow-rate, 1.0 ml/min; temperature, 22—26°C; detection wavelength, 250 nm.

Quantitative estimation was by measurement of peak height, relative to the internal standard.

RESULTS

Under the experimental conditions used the following retention times were recorded (Figs. 1 and 2): CGP 10.000 (metabolite) 4.0 min, GP 4779 (metabolite) 6.1 min, oxcarbazepine 8.7 min, CGP 9955 (internal standard) 10.6 min.

Carbamazepine, ethosuximide, phenytoin, primidone and valproic acid

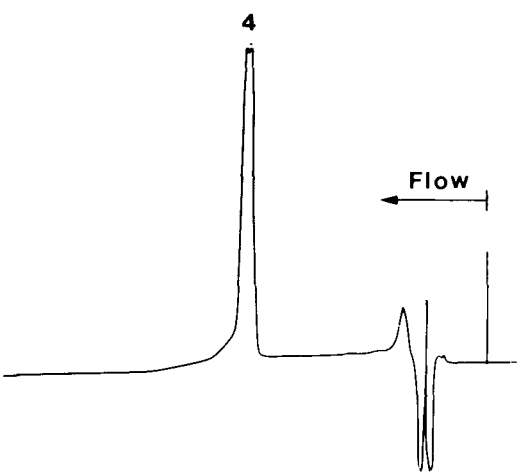

Fig. 1. Chromatogram of blank serum with internal standard (4). Sensitivity 0.32 a.u.f.s.

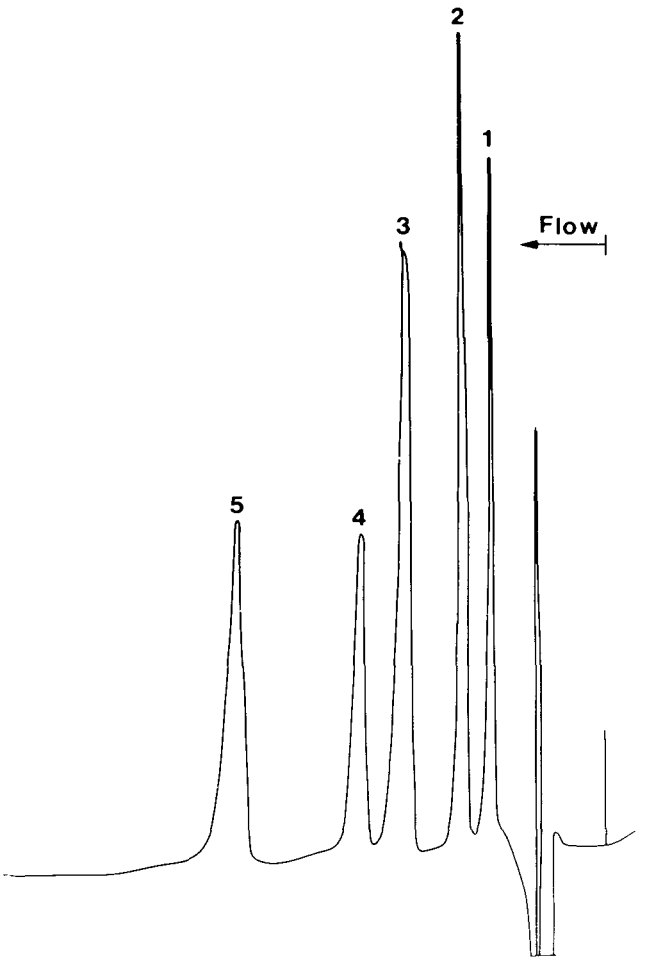

Fig. 2. Chromatogram of serum containing 4 mg/l of GCP 10.000 (1), GP 47779 (2), oxcarbazepine (3), internal standard (4) and carbamazepine (5). Sensitivity 0.16 a.u.f.s.

were found not to interfere in the analysis. For phenobarbital the retention time is 6.4 min.

The quantitative limits are about 150 ng/ml for oxcarbazepine and 1000 ng/ml for GP 47779 and CGP 10.000.

The recovery was found to be 99.99 + 4.75% for oxcarbazepine at a concentration of 4 mg/l. At 4 mg/l, the within-run variation is 101.60 ± 3.97% and over a long period (five months) 101.06 ± 5.34%.

The determination of serum levels of oxcarbazepine and its metabolites after administration of oxcarbazepine to patients is the objective of this study. These results will be published in due course, but at this stage it is apparent that the metabolite GP 47779 is the principal constituent present in serum and that the serum therapeutic level is probably the same as for carbamazepine (4—9 mg/l).

REFERENCES

1 K.F. Feldman, S. Brechbüler, J.W. Faigle and P. Inhof, in H. Meinardi and A.J. Rowan (Editors), Advances in Epileptology, Swets and Zeitlinger, Amsterdam, Lisse, 1977, p. 290.
2 V. Baltzer and M. Schmitz, in H. Meinardi and A.J. Rowan (Editors), Advances in Epileptology, Swets and Zeitlinger, Amsterdam, Lisse, 1977, p. 295.
3 K.F. Feldman, G. Dörhöfer, J.W. Faigle and P. Inhof, Acta Neurol. Scand. Suppl., 79 (1980) 62.
4 G. Dörhöfer, personal communication.

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Note

Determination by high-performance liquid chromatography of pipemidic acid in human serum and urine

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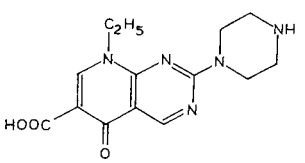
(First received July 28th, 1982; revised manuscript received December 13th, 1982)

Pipemidic acid is an antibacterial agent [1—3] used in the treatment of urinary tract infections. It is normally given as a 400-mg dose every 12 h. Our experience is, that at this dose, over this period of time, serum levels of 0—4 μg/ml and urine levels of 0—800 μg/ml may be expected (see Tables I and II).



Pipemidic acid: 8-ethyl-5,8-dihydro-5-oxo-2-(1-piperazinyl)-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid

Previously pipemidic acid has been determined in these two body fluids either by microbiological [4] or fluorescence methods [5].

Two recent papers have used high-performance liquid chromatography (HPLC) to determine the drug in biological fluids obtained from animal studies. The first [6] utilises a complex extraction procedure prior to normal-phase

TABLE I

SERUM LEVELS (μg/ml) OBTAINED FROM A TYPICAL PATIENT AFTER A SINGLE 400-mg DOSE OF PIPEMIDIC ACID

	Time (h)								
	0	1	2	3	4	5	6	7	8
Pipemidic acid (μg/ml)	<0.1	<0.1	2.76	3.64	3.60	3.19	2.95	1.90	1.13

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TABLE II

URINE LEVELS (mg) OBTAINED FROM A TYPICAL PATIENT AFTER A SINGLE 400-mg DOSE OF PIPEMIDIC ACID

	Time period (h)						Total	Percent dose
	0–2	2–4	4–6	6–8	8–12	12–24		
Pipemidic acid excreted (mg)	28.7	69.3	40.3	45.5	48.8	27.4	260.0	65.0

chromatography, whilst the second [7] uses reversed-phase chromatography followed by post-column derivatisation and fluorescence detection.

Our procedure offers a simple fast alternative for quantitative determination of the drug in both serum and urine and may be easily automated to cope with large sample numbers.

EXPERIMENTAL

Chromatography system

Reversed-phase chromatography was carried out on a Waters Assoc. (Milford, MA, U.S.A.) chromatography system consisting of a Waters pre-packed column (stainless-steel 30 cm × 3.9 mm I.D.) packed with μBondapak C_{18}, 10 μm, a Waters 6000A solvent delivery pump, a Waters 440 fixed-wavelength detector with 280 nm phosphor and a Waters Wisp 710A automatic sample injector. The analytical column was protected by a guard column, stainless-steel 5 cm × 5 mm I.D., packed with Co:Pell ODS C_{18} pellicular packing material, 30 μm (Whatman, Clifton, NJ, U.S.A.). A 10-mV recorder was used and peak integration was carried out using a Hewlett-Packard 3351 laboratory data system.

Mobile phase

The mobile phase for the urine analysis, mobile phase A, consisted of 46.8 g of sodium dihydrogen orthophosphate dihydrate, $NaH_2PO_4 \cdot 2H_2O$, (Analar) dissolved in a mixture of 275 ml of HPLC grade methanol and 725 ml of water.

For the serum analysis the mobile phase was altered to separate the pipemidic acid peak from background peaks due to the serum. Mobile phase B consisted of 46.8 g of $NaH_2PO_4 \cdot 2H_2O$ dissolved in a mixture of 75 ml methanol (HPLC grade), 75 ml of acetonitrile (HPLC grade) and 850 ml of water.

Both mobile phases were filtered through Whatman GF/F glass microfibre filters (Whatman, Springfield Mill, Maidstone, Great Britain) under vacuum to degas them before use.

Sample preparation and chromatography

Urine. A 1-ml volume of the urine sample under test was diluted to 50 ml with mobile phase A and filtered through a Millipore GS, 0.2-μm, filter (Millipore, London, Great Britain). A 50-μl sample of the resulting filtrate

was injected into the chromatograph. The flow-rate of mobile phase A was 1.5 ml/min. Chromatography was at ambient temperature and detection was at 280 nm with a detector sensitivity of 0.05 absorbance units full scale (a.u.f.s.).

The retention time of pipemidic acid under these conditions is approximately 7 min (see Fig. 1). Peak areas in the sample chromatograms were quantitated by the external standard technique using a standard solution of pipemidic acid trihydrate reference standard (Dainippon Pharmaceuticals, Osaka, Japan) dissolved in mobile phase A.

Serum. A 1-ml volume of the serum under test was diluted by addition of 3 ml of dilute acetic acid (60 ml of glacial acetic acid in 1 l of water). The resulting solution was filtered through a Millipore GS, 0.2-μm filter and 200 μl of the fitrate were injected. The flow-rate of mobile phase B was 1.8 ml/min. Chromatography was at ambient temperature with detection at 280 nm, 0.02 a.u.f.s.

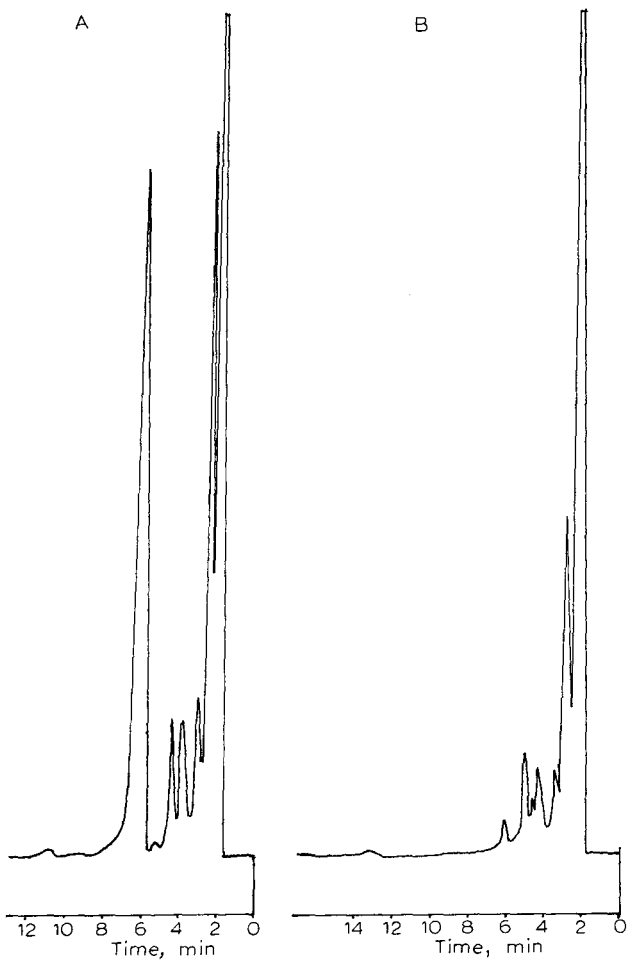

Fig. 1. Chromatograms of blank urine (B) and urine containing 324 μg/ml (A) of pipemidic acid obtained from a typical patient. Conditions as stated in the text (mobile phase A, 0.05 a.u.f.s.).

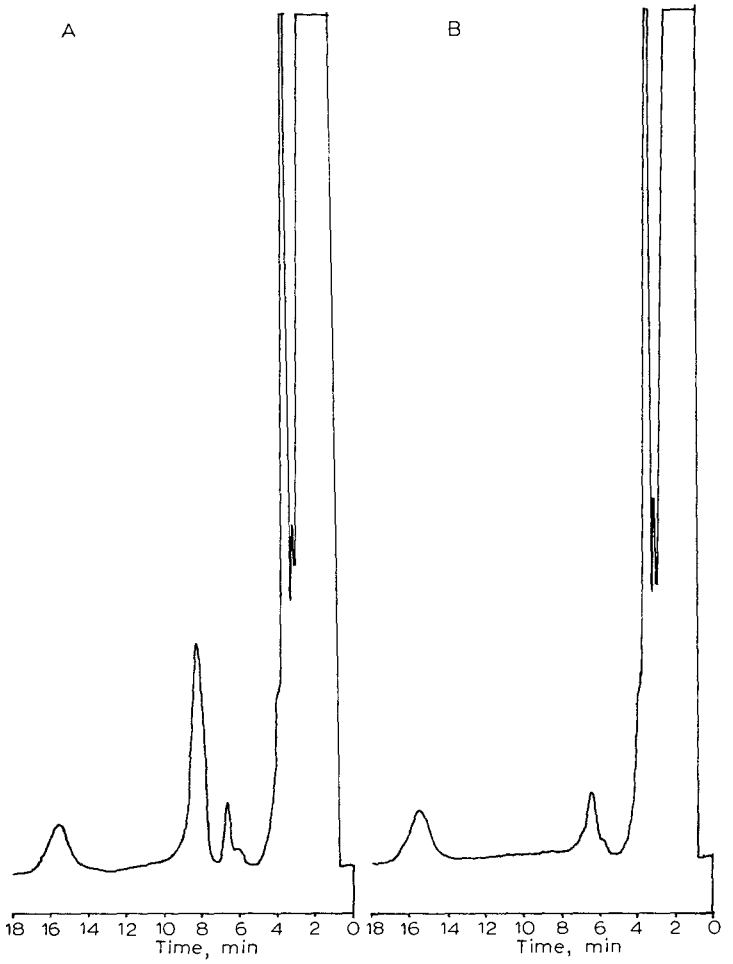

Fig. 2. Chromatograms of blank serum (B) and serum containing 1.40 μg/ml (A) of pipemidic acid obtained from a typical patient. Conditions as stated in the text (mobile phase B, 0.02 a.u.f.s.).

Quantitation was again carried out by comparison of peak areas with those from an external standard. The retention time of pipemidic acid under these conditions was approximately 9 min (see Fig. 2).

Recovery experiments were performed by spiking 1-ml aliquots of unmedicated human serum and urine with various amounts of pipemidic acid trihydrate reference standard dissolved in dilute acetic acid. These samples were then analysed as outlined above and a factor for the recovery of pipemidic acid from the two body fluids was obtained (see Tables III and IV).

The precision of the method was determined by analysis of six aliquots from one sample each of urine and serum containing pipemidic acid (see Table V).

TABLE III

RECOVERY OF PIPEMIDIC ACID FROM URINE

Pipemidic acid added, (μg/ml, x)	Pipemidic acid found, (μg/ml, y)	Recovery (%)
25.3	25.4	100.4
63.1	66.1	104.8
108	108	100.0
216	198	91.7
324	326	100.6
432	438	101.4
540	535	99.1
	Mean	99.7%

Slope = 0.9989; linear correlation coefficient = 0.9992; y intercept = −1.4.

TABLE IV

RECOVERY OF PIPEMIDIC ACID FROM SERUM

Pipemidic acid added, (μg/ml, x)	Pipemidic acid found, (μg/ml, y)	Recovery (%)
0.568	0.50	88.0
1.136	1.17	103.0
2.272	2.11	92.9
3.408	3.18	93.3
4.544	4.20	92.4
5.680	5.55	97.7
	Mean	94.6%

Slope = 0.959; linear correlation coefficient = 0.9986; y intercept = −0.028.

TABLE V

PRECISION OF THE ASSAYS OF PIPEMIDIC ACID IN BODY FLUIDS

Body fluid	Pipemidic acid found (μg/ml)	Mean ± relative standard deviation (%)
Urine	353 351 361 346 329 338	346 ± 3.29 (n = 6)
Serum	1.94 1.92 1.83 2.00 1.80	1.90 ± 3.99 (n = 6)

RESULTS AND DISCUSSION

Choice of chromatography systems

Pipemidic acid is amphoteric in nature and from consideration of its pK values [8] the best approach to developing a suitable mobile phase appeared to be an anionic ion-pairing system combined with a reversed-phase column.

Some of the mobile phases tried were: (1) methanol—water—acetic acid (45:55:0.1); (2) methanol—water—acetic acid (60:40:0.8) containing 0.005 *M* sodium lauryl sulphate; (3) methanol—water (30:70) containing sodium dihydrogen orthophosphate at different strengths.

The major problem with systems 1 and 2 was that the pipemidic acid peak was affected by very bad tailing. It was found that addition of sodium dihydrogen orthophosphate to the mobile phase produced sharper peaks and that increasing its concentration eventually eliminated tailing. The optimum concentration was found to be 0.3 *M*. Such a high salt concentration in the mobile phase can have a detrimental effect on the μBondapak C_{18} column packing over a sustained period of time, as it slowly dissolves away the silica support phase eventually resulting in a void space in the column which affects chromatographic efficiency. This may be overcome by use of a pre-column packed with 30-μm silica inserted between the pump and injector of the chromatograph. In this way the mobile phase becomes saturated with silica before entering the main guard column—analytical column system, and any void generated in the silica pre-column should not affect the chromatography.

Sample preparation

A 1-cm layer of a solution of pipemidic acid (1%, w/v) in aqueous acetic acid (1%, v/v) has been shown to have an absorbance (*A* 1 cm, 1%) of circa 1900 at a wavelength of 275 nm [9]. This strong absorbance meant that the drug could easily be estimated in urine by dilution followed directly by chromatography without any prior extraction step. It was decided to try a similar procedure for the serum samples. Due to the large number of analyses involved, avoidance of prior extraction would cut down the time required for each analysis. Using a direct dilution method on serum samples did have some disadvantages. Firstly, there were often a considerable number of background peaks in the chromatogram from the serum (see Fig. 2) and the mobile phase had to be carefully adjusted so that the pipemidic acid peak was well separated from them. Secondly, the continual injection of diluted serum onto the reversed-phase C_{18} chromatography column resulted in irreversible adsorption of non-polar compounds contained in the serum which eventually affected the chromatographic efficiency. For this reason the use of the guard column is very important and it was repacked with fresh Co:Pell ODS after every 50 sample injections to protect the main analytical column from contamination.

Sensitivity, linearity and precision

Using these methods the lower limit of detection for pipemidic acid in urine is estimated to be ca. 5 μg/ml, and in serum ca. 0.1 μg/ml. However, the peaks in the chromatogram become too small for accurate quantitation at urine levels below ca. 15 μg/ml and at serum levels below ca. 0.3 μg/ml. For this reason linearity has only been demonstrated over the range for which accurate determination of peak areas is possible. Below these levels, which are outside the range of interest for this particular investigation, the drug concentrations may only be approximated.

The data in Table III show the recovery of pipemidic acid from urine to be essentially quantitative with a linear detector response in the range 25—540 μg/ml. The precision of the method is satisfactory with a relative standard deviation of 3.29% (Table V).

The recovery of pipemidic acid from serum was slightly lower (Table IV), and detector response varied linearly with pipemidic acid concentration in the range 0.50—5.55 μg/ml. The precision of the assay for serum samples was also satisfactory, relative standard deviation = 3.99% (Table V).

The sensitivity, linearity and precision of the methods were found to be adequate for the determination of pipemidic acid in both serum and urine.

Applications

These methods have been used successfully to assay 200 serum samples and 130 urine samples during a bioavailability comparison of several pipemidic acid formulations.

The simple sample preparation steps and the use of an auto-injector on overnight runs, enabled one analyst to complete the analysis of 40 samples of serum or urine in less than the equivalent of one working day; this included the preparation of samples and standards, carrying out chromatography and calculation of results.

CONCLUSION

Pipemidic acid in body fluids may be determined rapidly and accurately using the reversed-phase HPLC methods described.

REFERENCES

1 P. Adamowicz, J.F. Delagneau, P. DeLajudie, M. Pesson and M. Regnier, Chemother. Proc. 9th Int. Congr. Chemother., 5 (1976) 379.
2 M. Shimizu, Y. Takase, S. Nakamura, H. Katae, A. Minami, K. Nakata, S. Inoue, M. Ishiyama and Y. Kuobo, Antimicrob. Ag. Chemother., 8 (1975) 132.
3 S. Goto, I. Dogasaki. Y. Kaneko, M. Ogawa, T. Takita, A. Tsuji, K. Takahasi and S. Kuwahara, Chemotherapy (Tokyo), 23 (1975) 2640.
4 Communication from Dainippon Pharmaceutical, December 14, 1977.
5 Communication from Dainippon Pharmaceutical, April 25, 1975.
6 C.A. Zio, G. Massarani and P. Pietta, J. Chromatogr., 200 (1980) 245.
7 M. Le Duff, D. Gibassier, P.A. Sado and R. Le Verge, in J.M. Aiache and J. Hirtz (Editors), 1st European Congress Biopharmaceutics and Pharmakinetics, Clermont Ferrand, 1—3 April 1981, Vol. 2, Technique Documentation, Paris, 1981, pp. 508—515.
8 F. Ficicchia, Farmaco, Ed. Pr., 30 (1974) 207.
9 J. Desbarres and H.O. El Sayed, C.R. Acad. Sci. Ser. C, Paris, 285 (1977) 431.

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CHROMBIO. 1609

Note

High-performance liquid chromatographic determination of iohexol in plasma, urine and feces

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(First received August 10th, 1982; revised manuscript received December 15th, 1982)

Iohexol, 5-[acetyl(2,3-dihydroxypropyl)amino]-N,N′-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide, Fig. 1, is a new non-ionic radiographic contrast agent which has sufficient stability for delivery to the X-ray laboratory as a sterilized solution. Like others of this class of diagnostic agents [1], it is not metabolized, but excreted in an unchanged form [2]. This paper describes a specific high-performance liquid chromatographic (HPLC) method for the quantitation of iohexol in spiked samples of human plasma, urine and feces.

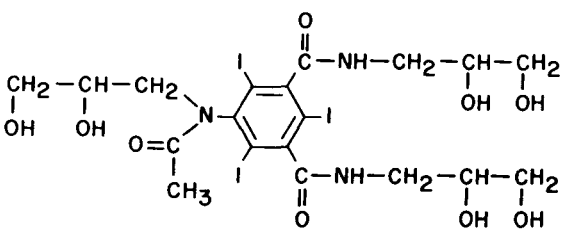

Fig. 1. Structural formula for iohexol.

EXPERIMENTAL

Chemicals

Iohexol and the internal standard for the assay, 5-acetylamino-2,4,6-triiodo-N,N′-bis-(2,3-dihydroxypropyl)-1,3-benzenedicarboxamide, were synthesized at the Sterling-Winthrop Research Institute. Other chemicals were obtained commercially (reagent grade) and used without further purification.

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Analysis of plasma samples

To a tube containing 1.0 ml of spiked plasma, 50 μl of internal standard solution (250 μg/ml in water) were added. The tube was mixed and a 100-μl aliquot was transferred to a clean tube and mixed with 850 μl of water, 50 μl of 0.2 *N* perchloric acid and 5 ml of chloroform. After centrifugation, a 400-μl aliquot of aqueous phase was transferred to a clean tube and mixed with 38 μl of 1.0 *N* sodium hydroxide. The sample was filtered with a centrifugal filtration apparatus (Bioanalytical Systems MF-1, W. Lafayette, IN, U.S.A.). A 100-μl aliquot was injected into the HPLC system. A set of standards, in duplicate, covering the range of zero and 2.8—27.8 μg/ml was prepared; both standards and samples were chromatographed under the following conditions: mobile phase, 0.01 *M* sodium phosphate buffer, pH 7.4—methanol (96:4, v/v); flow-rate, 1.75 ml/min; retention times, internal standard 4.9 min and iohexol 6.0 min.

Analysis of urine samples

Internal standard (150 μl of 3 mg/ml in water) was added to 850 μl of iohexol-containing urine. After mixing, a 50-μl aliquot was added to 2450 μl of water (1:50 dilution). A portion of the diluted sample was centrifugally filtered. A duplicate set of standards, covering the range of zero and 133—2670 μg/ml, was prepared. All samples were chromatographed under the following conditions: mobile phase, 0.01 *M* sodium phosphate buffer, pH 7.4—methanol (94:6, v/v); flow-rate, 1.75 ml/min; retention times, internal standard 4.4 min and iohexol 5.2 min.

Analysis of fecal samples

A fecal homogenate was prepared by adding 4 ml of iohexol-containing ethanol to 1 g of feces in a Waring blender. Internal standard (100 μl of 1 mg/ml in water) was added to a 1.0-ml aliquot of fecal homogenate. Ethanol, 10 ml, was added to each sample and the mixture was centrifuged. The supernatant was decanted into a clean silanized tube and dried in a 40°C water bath under a stream of nitrogen. The residue was dissolved in 1.35 ml of 0.05 *N* sodium hydroxide and passed through a 3.8 cm × 6 mm I.D. column packed with 50—100 mesh Dowex 50W-X4 resin (Na^+). The column was rinsed with 1.65 ml of 0.05 *N* sodium hydroxide. The combined eluate was adjusted to pH 3.8 with 1.5 *N* hydrochloric acid. The sample was centrifugally filtered and a 100-μl aliquot was injected onto the HPLC system. A set of standards, in duplicate, covering the range of zero and 125—2500 μg/g of feces, was prepared. Samples and standards were chromatographed under the following conditions: mobile phase, 0.01 *M* sodium phosphate buffer, pH 7.4—methanol (95:5, v/v); flow-rate, 1.5 ml/min; retention times, internal standard 8.5 min and iohexol 11 min.

HPLC system

The pump was a Constametric II (Laboratory Data Control, Riviera Beach, FL, U.S.A.) high pressure pump; the injector was a Rheodyne (Cotati, CA, U.S.A.) syringe-loaded valve; and the detector was an Altex (Berkeley, CA, U.S.A.) Model 153 UV detector with a 254-nm wavelength filter. The column

was a 5-μm Spherisorb ODS, Excalibar (Applied Science, State College, PA, U.S.A.), 25 cm × 4.6 mm I.D. column. Between the pump and the injector was a wide-bore precolumn, packed with bulk silica, which saturated the mobile phase with silica as a precautionary measure to protect the analytical column. A guard column (Waters, Milford, MA, U.S.A.) packed with glass beads was placed between the injector and the analytical column. The dimensions of the precolumn and guard column are not critical. The HPLC system was used at ambient temperature.

Data processing

The HPLC detector was interfaced with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3354 Laboratory Automation System which computed peak heights and peak height ratios, iohexol:internal standard, for each standard and sample. A least-squares regression analysis was performed and the linear regression model was used to determine the concentration of iohexol in the sample by inverse prediction [3]. The minimum quantifiable level (MQL) of the assay was estimated as that concentration at which the lower 80% confidence interval just encompassed zero [4].

The observed concentrations for the prepared, spiked samples were expressed as percent differences from the nominal values. The range of these percent differences was used to define the accuracy of the assay. Precision was estimated from the square root of the mean square error term (standard deviation) derived from the analysis of variance on percent differences.

RESULTS AND DISCUSSION

Plasma samples

Representative chromatograms of treated plasma are shown in Fig. 2 (left half). The assay was linear over the range of standards employed, 0 and 2.8—27.8 μg/ml.

The concentrations of the prepared plasma samples are summarized in Table I. The accuracy of the method, defined as the mean percentage difference from the nominal value, ranged from −3.2% to +5.3%. The estimated precision of the assay, from the square root of the mean square error term of the two-way analysis of variance, was ±4.2%. The mean (±S.E.) MQL was 0.54 (±0.23) μg/ml.

Urine samples

Fig. 2 (right half) shows representative chromatograms of processed urine standards. The assay was linear over the range of standards, 0 and 133—2670 μg/ml. The results of the analysis of the prepared urine samples are summarized in Table I. The urine samples at the lowest concentration were found to vary by more than 20% from the nominal concentration; they were reanalyzed against standards in the range of 0 and 66.7—667 μg/ml. Under these conditions, the mean percent differences were −1.59% and −2.54% for the 147 and 380 μg/ml samples, respectively, which were acceptable. The MQL obtained with the 66.7—667 μg/ml standards was 10.2 μg/ml; the mean (±S.E.) MQL from the full sets of standards was 26.5 (± 1.1) μg/ml. Truncating the

TABLE I

CONCENTRATIONS OF IOHEXOL IN PREPARED PLASMA, URINE AND FECAL SAMPLES

Plasma		Urine		Feces	
Nominal concentrations (μg/ml)	Assayed concentration (μg/ml)	Nominal concentrations (μg/ml)	Assayed concentration (μg/ml)	Nominal concentrations (μg/ml)	Assayed concentration (μg/ml)
0	<MQL*	0	<MQL**	0	<MQL***
	<MQL		<MQL		<MQL
	<MQL		<MQL		<MQL
3.1	3.1	147	135	138	133
	3.4		135		133
	3.1		136		131
Mean	3.2		135		132
S.E.M. (%)	3.1		0.25		0.50
Mean percent difference	3.2		−7.94		−4.11
5.7	6.0	380	378	263	254
	6.1		382		258
	5.7		379		254
Mean	5.9		380		255
S.E.M. (%)	2.0		0.32		0.52
Mean percent difference	4.1		−0.09		−2.92
15.3	15.5	971	990	540	543
	16.5		996		538
	15.2		1010		549
Mean	15.7		999		543
S.E.M. (%)	2.5		0.59		0.59
Mean percent difference	2.8		2.85		0.62
20.0	20.1	1800	1810	1430	1500
	20.6		1810		1460
	20.7		1830		1450
Mean	20.5		1820		1470
S.E.M. (%)	0.9		0.37		1.04
Mean percent difference	2.3		0.93		2.80
27.2	27.0	2400	2400	2300	2280
	27.9		2390		2290
	29.1		2440		2250
Mean	28.0		2410		2270
S.E.M. (%)	2.2		0.63		0.53
Mean percent difference	2.9		0.42		−1.16

*Mean MQL = 0.54 μg/ml.
**Mean MQL = 26.5 μg/ml.
***Mean MQL = 15.9 μg/g.

set of standards, which are run in duplicate, reduces the variability in the standard line; this results in lower estimated MQL values for the urine assay and reduces the mean percent differences for the samples containing low concentrations of iohexol. The accuracy of the urine assay ranged from −7.94% to 4.36% with an estimated precision of 1.35%.

Fecal samples

Representative chromatograms of processed fecal homogenates are shown in Fig. 3. The assay was linear over the range of 0 and 125—2500 μg/g of feces.

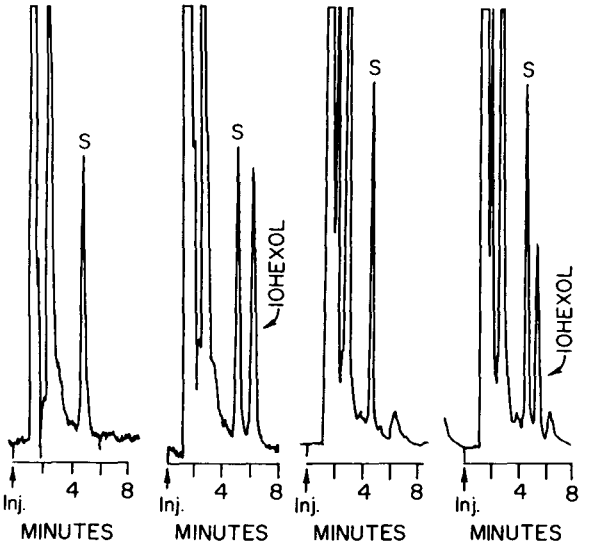

Fig. 2. Chromatograms of control human plasma (left half) containing the internal standard, S, and the same sample containing 16.7 μg/ml of iohexol. Chromatograms of control human urine (right half) containing the internal standard and the same sample containing 400 μg/ml of iohexol. Tallest peaks are full scale. Attenuation 0.01 a.u.f.s.; chart speed 0.5 cm/min.

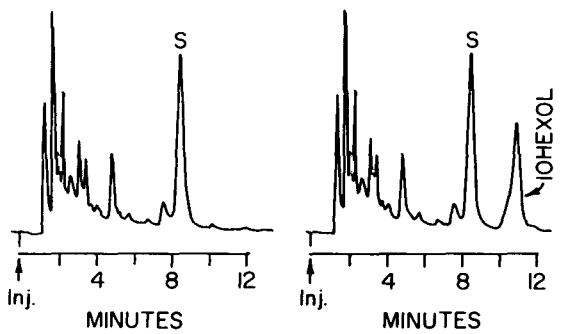

Fig. 3. Chromatograms of a processed fecal sample containing (left panel) the internal standard, S, and (right panel) the same sample containing 500 μg of iohexol, per g of feces. The internal standard peak is 18% of full scale. Attenuation 0.16 a.u.f.s.; chart speed 0.5 cm/min.

The observed concentrations for the prepared samples are shown in Table I. The accuracy ranged from —4.11% to +3.77%, with an estimated precision of ±1.13%. The mean (±S.E.) MQL was 15.9 (±3.70) μg/g of feces.

The extraction efficiencies of iohexol and the internal standard from feces, based on a comparison of peak heights of extracted versus direct standards, were 83.8% and 87.3%, respectively. Since neither plasma nor urine were subjected to an extraction procedure, recovery data were not needed.

Small changes in the composition of the mobile phase and the flow-rate are necessary to avoid the presence of small interference peaks. These can be seen in the urine samples (Fig. 2, right half), eluting after the iohexol peak and in the fecal samples (Fig. 3) eluting just before the internal standard.

Sets of spiked samples that had been stored, in the frozen state, for periods

up to 30 days were also analyzed. The assay results were not significantly different from the results that were obtained with samples that were analyzed immediately after preparation.

In summary, an accurate, selective, reproducible and precise HPLC assay has been developed for the measurement of iohexol concentration in human plasma, urine and feces. This method has proven useful for the analysis of specimens obtained during clinical trials; these results will be reported elsewhere.

REFERENCES

1 E.W. McChesney, in P.K. Knoefel (Editor), Radiocontrast Agents, Vol. 1, Pergamon Press, Oxford, 1971, p. 147.
2 T. Aakhus, S.C. Sommerfelt, H. Stormorken and K. Dahlstrom, in E. Lindgren (Editor), Iohexol, Acta Radiologica, Supplementum 362, Almqvist and Wiksell, Uppsala, 1980, p. 131.
3 R.R. Sokal and F.J. Rohlf, Biometry, W.H. Freeman, San Francisco, 1969.
4 R.W. Ross and H. Stander, Some Statistical Problems in Drug Metabolism, presented at the Princeton Conference on Applied Statistics, December, 1975.

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Note

Determination of razoxane by high-performance liquid chromatography

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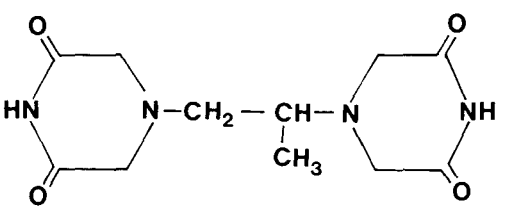
Pharmacokinetic studies of antineoplastic drugs have contributed to an understanding of the relationship between drug concentration, intensity of pharmacological effects and appearance of adverse effects.

The anticancer agent razoxane (ICRF 159; NSC 129943; (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane; Fig. 1) has been in clinical use for over a decade but to date no simple and accurate assay has been available to permit measurements of important pharmacokinetic parameters.



Razoxane

Fig. 1. Structure of razoxane.

Apart from assays using the radiolabelled drug [1, 2] the only sensitive technique reported for the measurement of razoxane has been the gas—liquid chromatography—mass fragmentography (GLC—MF) method of Sadeé et al. [3]. This assay however, involves a long and potentially explosive methylation step with diazomethane and is therefore unsuitable for extensive in-

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vestigations. These difficulties led us to examine the suitability of high-performance liquid chromatography (HPLC) to detect razoxane in plasma and serum samples from animals and patients.

An HPLC assay method has recently been developed for the *d*-enantiomer of razoxane, ICRF 187, by Earhart and Tutsch [4] and the method described below for razoxane was adapted from that for ICRF 187. It has been found to be simple, sensitive to nanogram levels of razoxane as well as accurate and reproducible. However it differs from the method of Earhart and Tutsch in the use of an ultrafiltration step for plasma and serum samples prior to HPLC analysis.

EXPERIMENTAL

HPLC separation was carried out on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph consisting of two Model 6000A solvent pumps, a 660 programmer, a U6K injector and an LKB Bromma 2138 UV detector. Signals from the detector were recorded by an LKB Bromma 2210 two-channel recorder. All separations were carried out on a Varian MCH-10 analytical column (C_{18} reversed-phase bonded on 10-μm silica gel; 30 cm × 4 mm) preceded by a guard column filled with Whatman ODS reversed-phase pellicular packing.

Materials

Millipore-filtered, degassed distilled water was used in all experiments. Potassium phosphate, sodium hydroxide (BDH, Poole, Great Britain) were all of analytical grade. Methanol (Rathburn Chemicals, Edinburgh, Great Britain) was HPLC grade. Razoxane was a gift from Imperial Chemical Industries (Macclesfield, Great Britain).

Animal studies

Rat plasma. Female Sprague—Dawley rats weighing 200 g were starved overnight and dosed with a single dose of 21.75 mg/kg razoxane in neutralized aqueous solution either orally or intravenously.

Blood was obtained by cardiac puncture at various times after razoxane administration using three rats per time point. The blood was centrifuged in heparin at 7000 *g* for 5 min at 4°C to obtain plasma which was assayed for razoxane.

Rat serum. Female Sprague—Dawley rats weighing 200 g were starved overnight and dosed with a single dose of 21.75 mg/kg razoxane orally or 10.9 mg/kg razoxane intravenously in a neutralised aqueous solution. Blood was taken by cardiac puncture at various times after razoxane administration and allowed to clot at 4°C. The pooled serum obtained at each time point was assayed for razoxane.

Rat cerebrospinal fluid. Razoxane (100 mg/kg) was administered intraperitoneally to rats of approx. 200 g weight. At various times after they were anaesthetised with sodium pentobarbital (40 mg/kg), blood and cerebrospinal fluid (CSF) were taken and the animals killed. Blood was removed and plasma prepared as described; CSF was obtained by percutaneous sampling from the cisterna magna [5] and placed in solid carbon dioxide prior to analysis.

Human studies

Patients with colorectal carcinoma received one tablet containing 125 mg razoxane and blood was taken via an indwelling intravenous cannula at 0, 1, 2, 4 and 24 h following razoxane administration. The blood was allowed to clot at 4°C to obtain serum, which was then assayed for razoxane.

Ultrafiltration procedure

Plasma and serum samples were filtered prior to HPLC analysis in Chemlab Ultrafiltration cells (Model C10) (Chemlab, Cambridge, Great Britain) through UM05 Diaflow membranes (Amicon, MA, U.S.A.) at 2 bar nitrogen pressure.

Rat CSF which contains minimal amounts of protein required no ultrafiltration prior to assay.

HPLC analysis

A 20-μl aliquot of the ultrafiltrate was injected onto the column. Samples were eluted in 20% methanol in 0.01 *M* phosphate buffer, pH 7.1 (helium degassed) at 98 bar pressure and a flow-rate of 1 ml/min. The absorbance of the eluate at 206 nm was recorded, razoxane peak heights were measured and values were read off standard curves constructed for plasma, serum and CSF samples.

RESULTS AND DISCUSSION

Quantitative aspects of the method

A typical separation of razoxane is illustrated in Fig. 2. Razoxane had a retention time of 5.4 min under these conditions of assay and the detection limit was 0.1 μg/ml. The Model 2138 UV detector response was linear over the range of razoxane concentrations studied in these experiments (0—250 μg/ml).

Recovery of razoxane was investigated by the addition of known concentrations of razoxane to 1-ml aliquots of rat serum or plasma samples immediately prior to ultrafiltration and prior to direct sample injection onto the HPLC column. This showed the razoxane loss during the ultrafiltration step to 38.6%. This poor recovery may be attributed to non-specific binding of the drug to the material of the membrane. Samples were assayed in quadruplicate and could be assayed with a reproducibility of ± 3.3%.

No loss of razoxane was seen following storage of the samples for up to 12 weeks at —70°C prior to analysis.

Animal studies

No razoxane was detectable in plasma, serum or CSF at time zero. Fig. 3 shows the plasma levels of razoxane following administration of 21.75 mg/kg razoxane by the oral and intravenous routes against time.

Following intravenous administration of the drug, plasma levels of razoxane appeared to follow a biphasic pattern of decay (Fig. 3). The initial and terminal phase half-lives were calculated to be 11.7 ± 2.4 min and 40.3 ± 3.8 min, respectively. These values correlated well with work of Sadeé et al. [3] who demonstrated a plasma elimination half-life of 40—45 min for razoxane, and

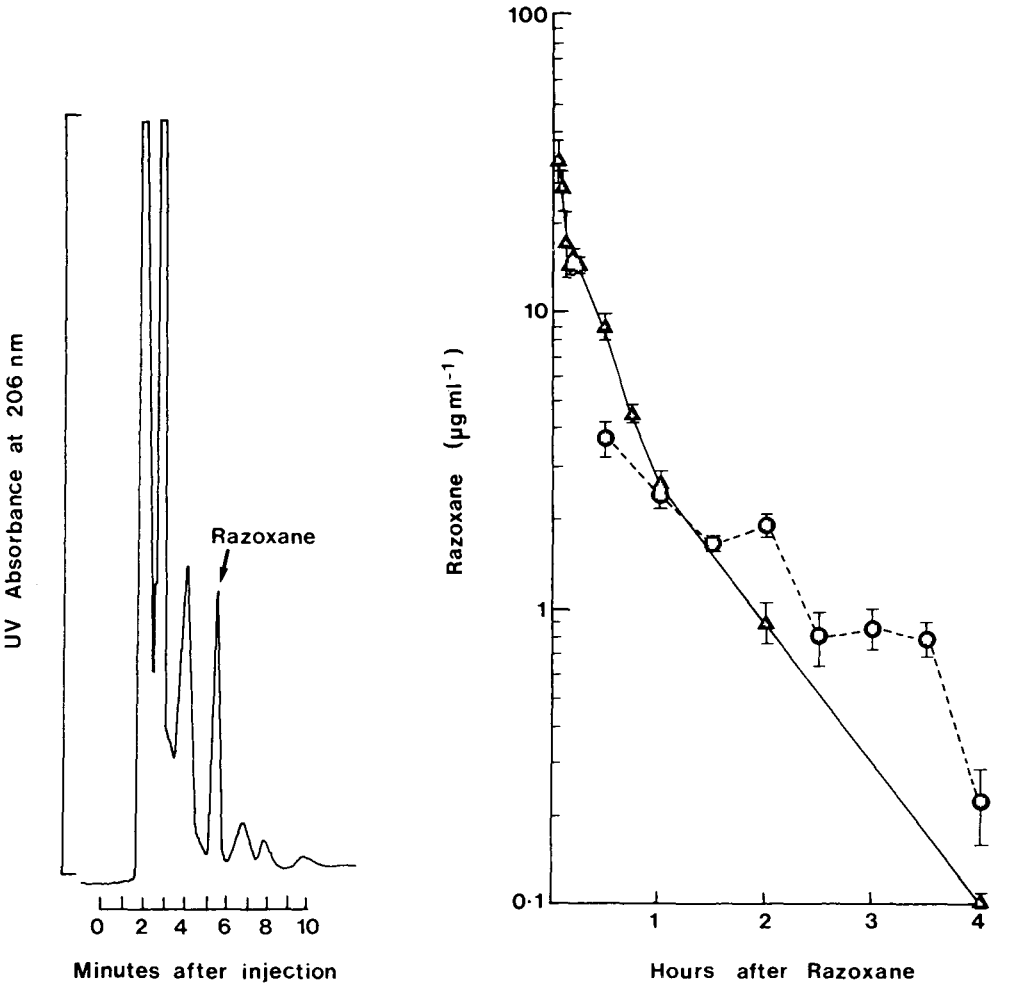

Fig. 2. Typical chromatogram of an ultrafiltrate of plasma/serum following razoxane administration.

Fig. 3. Plasma concentration of razoxane at various times after intravenous (▲) or oral (●) administration of 21.75 mg/kg razoxane in neutralised aqueous solution. The data presented are mean values ± S.E.M. for three rats per time point.

similar studies by Field et al. [2] in which a plasma half-life of about 30 min was found. Complete elimination of razoxane occurred by 8 h.

Following oral administration of an equal quantity of razoxane, a peak plasma level of 3.7 μg/ml was seen within 30 min of drug treatment. The plasma levels then declined monoexponentially with a half-life of 78.4 ± 6.8 min; complete elimination occurred by 8 h.

The ratio of the areas under the plasma concentration time curves for oral versus intravenous administration of razoxane is 0.53 suggesting that the bioavailability of oral razoxane is limited.

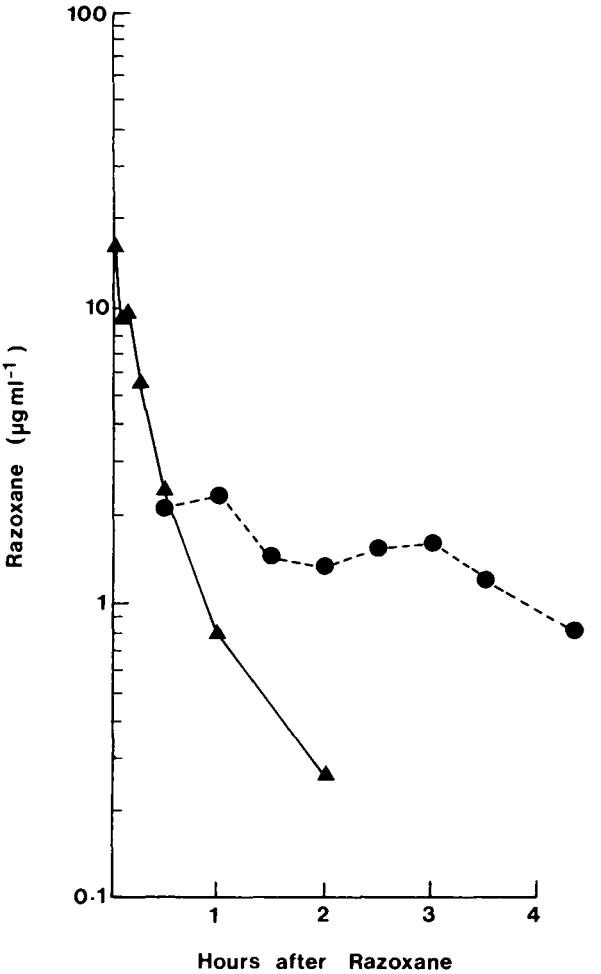

Fig. 4. Serum concentration of razoxane at various times after intravenous administration of 10.9 mg/kg razoxane (▲) or oral administration of 21.75 mg/kg razoxane (●) in neutralised aqueous solution. The data presented are values from pooled samples of four rats per time point.

A similar pattern was seen for razoxane levels in rat serum (Fig. 4). Following administration of 10.9 mg/kg razoxane intravenously values of $t_{½}$ α of 8.1 min and $t_{½}$ β of 42.2 min correlated well with the values of these parameters in plasma. Complete elimination occurred by 4 h.

Following oral administration of 21.75 mg/kg razoxane, a peak serum level of 2.3 μg/ml was achieved within 1 h. Serum levels then declined monoexponentially with a half-life of 157 ± 27 min.

Fig. 5 shows razoxane plasma and CSF concentrations following intraperitoneal administration of the drug. Razoxane was absorbed rapidly from the peritoneal cavity reaching a peak plasma concentration of 12 μg/ml within 15 min. Razoxane levels then declined monoexponentially with a half-life of 96 min; complete elimination occurred by 8 h.

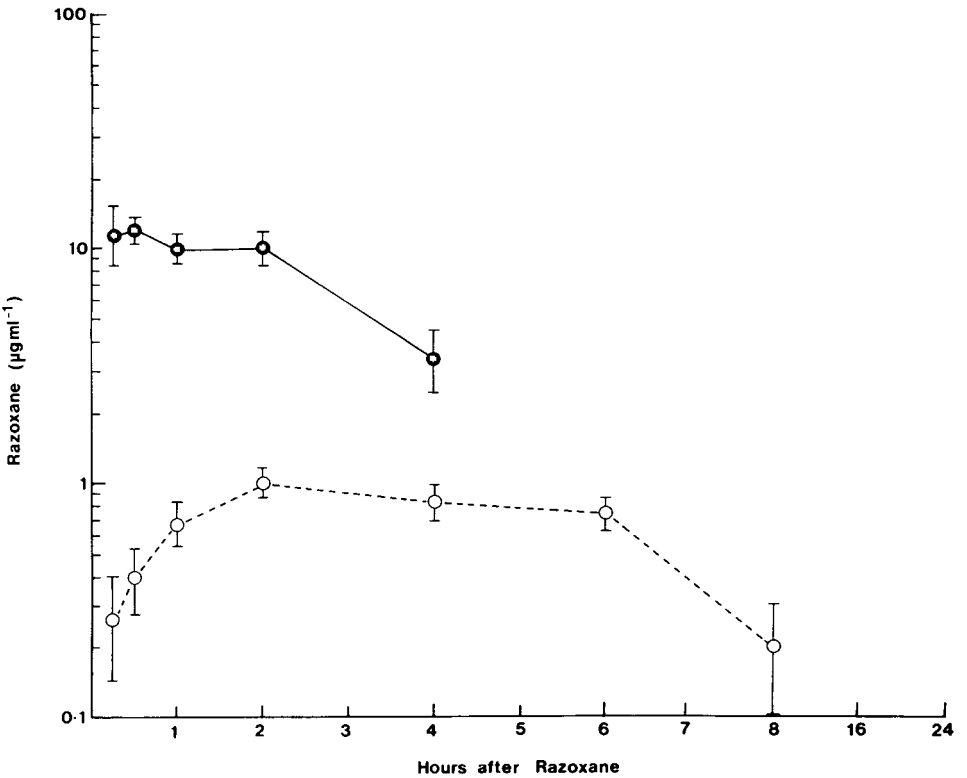

Fig. 5. Plasma (•) and cerebrospinal fluid (○) concentrations of razoxane after intraperitoneal administration of 100 mg/kg razoxane suspended in 0.5% carboxymethylcellulose in 0.9% sodium chloride. The data presented are mean values ± S.E.M. for 2—4 rats per time point.

Human studies

Preliminary experiments to measure razoxane levels in the serum of cancer patients treated with razoxane have so far been unsuccessful because the serum levels of razoxane seen were below the detection limit of the assay. Further attempts are being made to measure razoxane levels following administration of higher doses of razoxane under more controlled conditions, such as starving the patients and the use of patients with no prior drug therapy.

Thus, the use of a method involving HPLC and ultrafiltration has enabled the measurement of razoxane levels in the plasma, serum and CSF of rats. The method is easy to use, gives reproducible results and appears suitable for use in establishing pharmacokinetic data for razoxane. It also has potential for use in routine clinical investigations.

ACKNOWLEDGEMENT

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REFERENCES

1 P.J. Creaven, L.M. Allen and D.A. Alford, J. Pharm. Pharmacol., 27 (1975) 914.
2 E.O. Field, F. Mauro and K. Hellmann, Cancer Chemother. Rep., 55 (1971) 527.
3 W. Sadeé, J. Staroscik, C. Finn and J. Cohen, J. Pharm. Sci., 64 (1975) 990.
4 R.H. Earhart and K.D. Tutsch, Proc. Amer. Assoc. Cancer Res., 22 (1981) 350.
5 N. Greig, unpublished work.

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Book Review

Pharmaceutical analysis: modern methods, Part A, edited by J.W. Munson, Marcel Dekker, New York, Basel, 1981, XIII + 485 pp., price Sfr. 154.00, ISBN 0-8247-1502-0.

According to the Editor's statement the purpose of the book is to fill the gap between basic texts and highly specified journal articles in the field of pharmaceutical analysis. Part A of the series contains six chapters, out of which the first three are devoted to gas chromatography (GC), followed by three other chapters devoted to fluorescence and phosphorescence spectroscopy, liquid scintillation counting, and radioimmunoassay and related immunoassay techniques. In this review we shall focus our attention on the chromatographic part of the book.

The first chapter, by B.J. Kline and W.H. Soine, deals with the theory, instrumentation and pharmaceutical applications of GC. The first two general parts are on the level of a classical fundamental textbook about GC; pharmaceutical applications are presented in tabular form. The part devoted to techniques is very limited in figures and tables. It is very likely that the reader will miss a table giving a survey of stationary phases without which the appendix of applications loses much of its importance. Inadequate attention is paid to exploiting computers for data handling; also ancillary techniques are dealt with rather briefly. Out of 338 references (covering the literature up to 1978/1979) two-thirds cover the general part and the remaining one-third covers the pharmaceutical applications.

The other chapter, written by T.A. Roy and S.G. Schulman, describes the combination pyrolysis—GC. The chapter is clearly arranged; however, it is questionable whether this combination is of sufficient importance in pharmaceutical analysis to warrant a separate chapter. The examples presented are not witnessing this fact.

The most valuable chapter is that by J.E. Cone about the analysis of drugs in biological samples by combined gas chromatography—mass spectrometry (GC—MS). This chapter consists of a part dealing with GC—MS methodology, a part devoted to applications in drug analyses, like drug metabolism, toxicologic and forensic applications, diagnosis of diseases state and quantitation of drugs in biological samples.

In general it can be said that the concept of the book and the presentation of some chromatographic chapters lag considerably behind the original intention. The reader who is looking for the status quo and applicability of individual techniques in drug analysis would certainly be interested in a general

chapter that would compare individual techniques, demonstrate their advantages and drawbacks and that would offer a guideline for the selection of the most suitable technique for a given analytical task. In the chapter dealing with GC, the description of procedures for sample preparation mainly in the analysis of biological material is completely missing. Such a part is presented only in the chapter about GC—MS; even here some more recent fast procedures that make use of disposable columns are lacking. Also, the general chapters describing the identification of compounds (without MS) including the methods of systematic analysis should be dealt with in more detail. This holds also for expressing the precision and accuracy of results, for expressing limits of sensitivity and judging possible interference of other drugs, etc. It is surprising that in two chapters there are examples of application of GC for the diagnosis of disease states, which certainly is not a part of pharmaceutical analysis. On the other hand, however, the application of drug assay in plant material, which constitutes an important part of pharmaceutical applications, is missing completely. Further, the categorization of chapters into parts A and B is also obscure. It would be a matter of logic to publish chromatographic procedures in one volume and other procedures in the second volume.

From the typographical point of view the book is well presented and includes useful drug, author and subject indexes. Though this first volume does not fulfill all expectations, several chapters are likely to be useful for potential readers.

Prague (Czechoslovakia) KAREL MACEK

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