Analytical Methods for the Determination of Urinary 2,4-Dichlorophenoxyacetic Acid and 2-Methyl-4-Chlorophenoxyacetic Acid in Occupationally Exposed Subjects and in the General Population

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Abstract

Two methods for the quantitative analysis of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) in urine were compared. The first was an high-performance liquid chromatography method using a C_8 column with ion suppression and diode array detection. The urine extracts were first purified by solid-phase extraction (SPE) on silica capillary columns. The detection limit of the method was 15 µg/L for both compounds. The percentage coefficient of variation of the whole analysis evaluated at a concentration of 125.0 µg/L was 6.2% for 2,4-D and 6.8% for MCPA. The mean recovery of analysis was 81% for 2,4-D and 85% for MCPA. The second was a gas chromatographic (GC) method in which the compounds were first derivatized with pentafluorobenzylbromide to pentafluorobenzyl esters, which were determined with a slightly polar capillary column and electron capture detection. Before GC analysis, the urine extracts were purified by SPE on silica capillary columns. This method had a detection limit of 1 µg/L for both compounds and a percentage coefficient of variation of the whole analysis, evaluated at a concentration of 30.0 µg/L, of 8% for 2,4-D, and of 5.5% for MCPA. The mean recovery was 87% for 2,4-D and 94% for MCPA. The low detection limit made the second method suitable for assaying the two herbicides in the general population. Duplicate analysis of ten urine samples from occupationally exposed subjects by the two methods gave identical results for a wide range of concentrations.

Introduction

The phenoxyacetic herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) are widely used to eliminate weeds from cereal and grass crops. There are limited data available on occupational poisoning of farmers by these substances. Exposure to 2,4-D is reported to be associated with irritation of the mucous membranes and skin, gastrointestinal symptoms, headache, and possibly polyneuritis. Workers exposed to 2,4-D and MCPA may also be at risk for allergic dermatitis (1). Exposure to mixtures of phenoxyacetic, chlorophenol, and chlorinated dibenzodioxin herbicides seems to be associated with an increase in the incidence of malignant lymphoma and sarcoma of the soft tissues (2). Because many cases of tumors have been associated with exposure to phenoxy herbicides and their contaminants or other chemical compounds, it is uncertain if exposure to 2,4-D and MCPA is specifically related to the development of sarcoma of the soft tissues.

Studies of volunteers have shown that phenoxyacetic herbicides (2,4-D and MCPA) are not metabolized in any substantial way and are excreted in the urine (and in traces in the feces), mostly in free form with only a small percentage in conjugated form (3–5). Urinary levels of 2,4-D and MCPA as free acids can therefore be used as indicators of exposure to these compounds and their salts (6).

Various methods of determining 2,4-D and MCPA in urine have been developed. High-performance liquid chromatographic (HPLC) procedures (3,7,8) have involved assay of nonderivatized acids but have had quite high detection limits when reported (200 µg/L [3], 50 µg/L [7]). The high polarity of these compounds makes it impractical to analyze them directly by gas chromatography (GC), and they must first be derivatized to stable, more volatile compounds. The derivatives most commonly prepared for the determination of phenoxyacetic herbicides in urine are alkyl esters: methylation with dimethylsulfate was proposed by Vural (9); other authors have used diazomethane (10) and diazoethane (11). Pentafluorobenzylbromide is another derivatizing agent used to increase the response of electron capture detectors in the GC analysis of the two herbicides (12). These procedures rarely have detection limits less than 20–30 µg/L, except the procedure used by Holler (11) which had a detection limit of 1 µg/L. Recently, a radioimmunoassay (RIA) with a detection limit of 1 µg/L was used for the analysis of 2,4-D in urine (13,14).

In the present study, we compared two methods of analysis of urinary residues of 2,4-D and MCPA, one using HPLC and the other using gas–liquid chromatography (GLC). The methods were extensively tested, had good precision, excellent recovery, detection limits of 15 and 1 µg/L, respectively, and were suitable for determining the two phenoxyacetic herbicides in professionally exposed subjects (15) and in the general population (unpublished data).

Experimental

Reagents and standards

Pentafluorobenzylbromide (PFB-Br), a powerful lachymatory compound, was obtained from Aldrich Chimica (Milan, Italy). Acetone, methanol, hexane, dichloromethane, LiChrosolv-type acetonitrile, analytical-grade sodium chloride, analytical-grade anhydrous potassium carbonate, pure anhydrous sodium sulfate (fine powder), analytical-grade monobasic potassium phosphate, pure 85% orthophosphoric acid, analytical-grade 37% hydrochloric acid, and pure 100% glacial acetic acid were from Bracco Merck (Milan, Italy).

Solid-phase extraction (SPE) silica columns (3-mL column reservoir, 500-mg sorbent) were obtained from Supelchem (Milan, Italy). They were eluted at 1 mL/min on an SPE vacuum manifold (Supelchem). Ultrapure water was obtained with a Milli Q system (Millipore, Milan, Italy). 2,3-Dichlorophenoxyacetic acid (2,3-D) (98.4% purity) and 98% pure 4-chlorophenoxyacetic acid (4CPA) (Lab Service Analytica, Bologna, Italy) were used as internal standards. The samples were filtered on Millex HV 13 filters (Millipore) before injection onto the HPLC column.

Reference standards of 2,4-D (99.77% purity) and MCPA (99.7% purity) were obtained from Lab Service Analytica. Standard 1 mg/mL solutions of 2,4-D and MCPA were prepared by dissolving appropriate quantities of standard in methanol. The working solutions used to construct the calibration curves for urine were prepared daily by diluting the standard solutions with methanol.

Apparatus

The HPLC system consisted of a Perkin-Elmer (Perkin-Elmer Italia, Milan, Italy) 250 liquid chromatographic (LC) pump with an LC 235 diode-array detector (DAD) and a 1020 integrator. The injection loop had a volume of 10 µL. A detector wavelength of 230 nm was selected. The column was a Supelco LC8 (25 cm × 4.6-mm i.d., 5-µm grain size) (Supelchem, Milan, Italy). The flow of pure helium used for degassing the mobile phase was 0.7 L/min.

The GC was a Carlo Erba (Milan, Italy) HRGC 5300 Mega series with ECD 400 and a Perkin-Elmer 1020 integrator. The chromatographic columns were Chromopack (Milan, Italy) CP Sil 5 (column 1, 30 m × 0.32-mm i.d., 0.4-µm film thickness) and Chromopack CP Sil 8 (column 2, 50 m × 0.32-mm i.d., 0.4-µm film thickness). The detector temperature was 300°C. The carrier gas was ultra-pure helium at a flow rate of approximately 2 mL/min; the makeup gas was argon–methane (5% methane) at a flow rate of 65 mL/min. Injection (1 µL) was performed by the splitless technique. The injector temperature was 250°C.

The urine extracts were evaporated in vacuo with a Buchi 011 rotating evaporator and a Buchi 461 water bath. The vacuum was maintained with a Jet 1 Automatic Vacuum System recycling water pump (Genser Scientific Instruments, Rothenbury, Germany).

Analytical procedure

Extraction of 2,4-D and MCPA from urine. Urine aliquots (20 mL) spiked with internal standard (400 µL of 4CPA solution at 20 ppm for HPLC and 400 µL of 4CPA and 2,3-D solutions at 5 ppm for GC) and saturated with sodium chloride were acidified with 200 µL concentrated HCl and extracted twice with 12 mL dichloromethane. The organic extracts were pooled, dehydrated with anhydrous sodium sulfate, and evaporated to dryness in a rotating vacuum evaporator at 30°C.

HPLC method. After the initial extraction, the residue was purified by SPE on silica capillary columns. The residue was made up with 1 mL of dichloromethane solution acidified with acetic acid (1 mL acetic acid was diluted to 100 mL with dichloroethane) and deposited on the column, which was previously conditioned with 6 mL dichloromethane. The eluate was not collected. The column was washed with 1 mL of the same solution, and the eluate was collected with the subsequent 3 mL dichloromethane. The organic phase obtained was evaporated to dryness under a gentle nitrogen stream. The residue was made up with 0.5 mL methanol, filtered, and injected into the HPLC column.

The mobile phase was acetonitrile and phosphate buffer. The buffer was obtained by dissolving 1.36 g KH_2PO_4 and 500 µL acetic acid in 1 L water; the pH was adjusted to 3.2 by adding a few drops of phosphoric acid. The two-step elution gradient used 25 and 33% acetonitrile, each for 5 min. The velocity of the increase in the percentage of solvent was 1.14%/min. The concentration of acetonitrile was then brought back to 25% at a velocity of 2.67%/min. After 10 min of equilibrium, the system was ready for the next injection. Elution flow was 2 mL/min.

GC method. After the previous extraction, the residue was derivatized by adding 200 µL of a solution of pentafluorobenzylbromide (PFB-Br) in acetone (1:100 dilution of pure PFB-Br), 15 µL of a 60% aqueous solution of K_2CO_3, and 4 mL acetone. The mixture was shaken in a vortex mixer and left to react overnight at ambient temperature. After the reaction, 3 mL water and 10 mL dichloromethane were added. The mixture was shaken for 5 min in a mechanical shaker and then centrifuged. The organic phase was removed, dried with anhydrous Na_2SO_4, and evaporated to dryness in a rotating vacuum evaporator. The residue was made up with 1 mL hexane and purified on an SPE silica column that was previously conditioned with dichloromethane and hexane. The column was washed with 2 mL hexane–dichloromethane (70:30) and then eluted with 4 mL hexane–dichloromethane (60:40), which was collected and evaporated to approximately 0.5 mL before injection onto the GC.

The three-step temperature program for column 1 was as follows: 50, 200, and 250°C for 1, 5, and 10 min, respectively; the rates of temperature increase were 30 and 5°C/min, respectively. The two-step temperature programs for column 2 were 50 and 270°C for 1 and 30 min, respectively; the rate of temperature increase was 35°C/min.

Calibration curves. 2,4-D and MCPA were determined by means of calibration curves constructed with solutions prepared by adding appropriate quantities of the working solutions to 20-mL aliquots of urine from a subject who was not occupationally exposed to phenoxyacetic herbicides. A blank of the same urine (not spiked with standard 2,4-D and MCPA) was also prepared. The additions were in the range of 0 to 500 µg/L of urine for each of the two compounds.

Evaluation of reproducibility and recovery. To evaluate analytical reproducibility, two pools of urine from subjects not occupationally exposed to 2,4-D and MCPA were spiked with the working solutions of the two herbicides to obtain concentrations of 125 µg/L (for the HPLC method) and 30 µg/L (for the GLC method). Ten 20-mL aliquots were obtained from each pool and analyzed by both methods.

To evaluate the reproducibility of the HPLC and GC measurements, two of the urine extracts obtained for the evaluation of analytical reproducibility were injected onto the respective instruments.

Recovery was evaluated by comparing the results obtained by analyzing the solutions used for evaluating analytical reproducibility with those of standard solutions at the same concentration.

Comparison of the two methods. The two methods were compared by duplicate analysis of 10 urine samples from subjects occupationally exposed to 2,4-D and MCPA.

Results and Discussion

In the procedure of extracting 2,4-D and MCPA from urine, dichloromethane was used as extraction solvent. As reported by Nidasio et al. (7) for chloroform, this solvent enables approximately 99% of the two compounds to be recovered by double extraction from the aqueous matrix acidified with HCl. Saturation of the urine with NaCl gives fast separation of the organic phase from the aqueous phase and almost complete recovery of the organic solvent. Other workers used toluene (7), chloroform (3), and benzene (9,11) as extracting solvents; we excluded these a priori because of their high toxicity. Other authors isolated phenoxyacetic herbicides from urine with Sep-Pak C_{18} (12) and amberlite XAD-2 columns (10), extracting the eluate from the columns with organic solvents such as ether (10).

In the present HPLC method, purification of the urine extract on an SPE silica column was necessary before chromatographic analysis with spectrophotometric detection at 230 nm. Without this preliminary phase, chromatograms obtained under our analytical conditions showed a lot of interference. The chromatograms (Figure 1) of urine extracts obtained without adding the analytes to the samples were without interference in the area of interest after purification. Dichloromethane used as the eluant during the purification gave a selective migration of the two compounds and the internal standard (4CPA) through the silica column and left behind other components of the urine extract that could interfere with the analysis. The addition of acetic acid to dichloromethane dissolved the two phenoxycarboxylic acids present in the residue of the urine extract and favored subsequent extraction of the two compounds from the silica, which increased the reproducibility of the procedure.

After purification, the sample was ready for HPLC that was performed as reported by Nidasio et al. (7), with an inverse-phase column and ion suppression. Fjellstad and Wannag (3) used direct-phase columns.

The high polarity and low volatility of phenoxyacetic herbicides made direct GC analysis impractical and made derivatization to more volatile compounds necessary. The formation of pentafluorobenzyl esters is useful with electron capture detection because it substantially increases the analytical response and improves the detection limit. The derivatization procedure used in this communication was the procedure reported by Chau and Terry (16). In the original method that was developed for standard solutions rather than for urine, the reaction time for maximum yield was 5 h. We preferred to leave the mixture to react overnight because no changes or degradation occurred after the first 5 h. In this manner, the reaction proceeded without the chemist, who found the samples ready for analysis in the morning. Because standards of the pentafluorobenzyl esters of the two herbicides were not available, the yield of the derivatizing reaction could not be evaluated.

In our opinion, pentafluorobenzylbromide is preferable to other derivatizing agents. The diazoalkanes (10,11) are explosive and carcinogenic and produce highly volatile derivatives that are easily lost during the concentration step.

In our GC method, purification on SPE silica columns after extraction and derivatization eliminated interference by urinary and derivatization products. The eluants used were less polar than those used in the purification procedure of the HPLC method. The first step of washing with a hexane–dichloromethane (70:30) mixture eliminated certain apolar components from the samples. Subsequent elution with 4 mL of hexane–dichloromethane (60:40) enabled quantitative and selective recovery of the pentafluorobenzyl esters of the two compounds

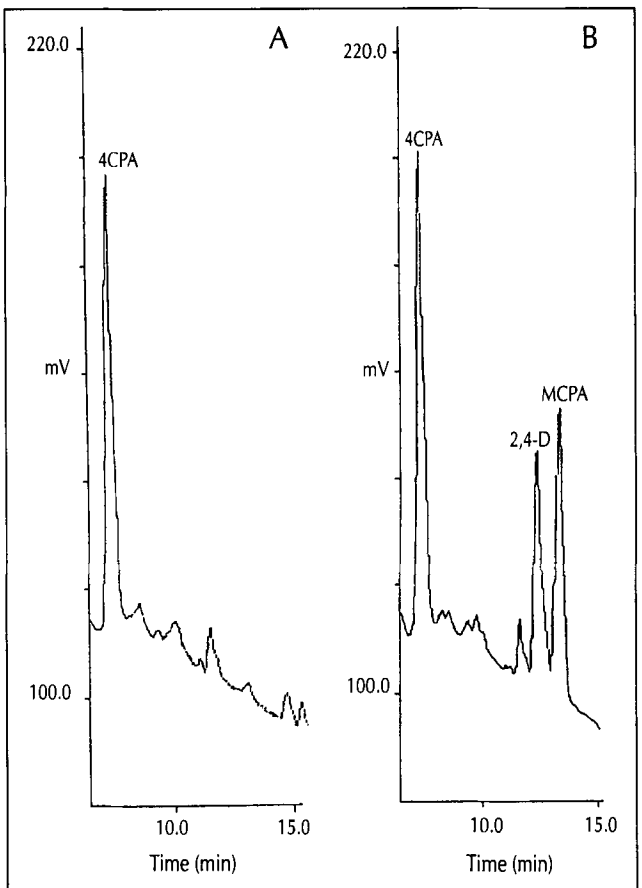

Figure 1. Chromatograms of urine extracts obtained by the HPLC method. A, blank urine; B, urine spiked with 125.0 µg/L of 2,4-D and MCPA.

and the internal standards (2,3-D and 4CPA), leaving the more polar components, which did not elute, on the silica.

Figures 2 and 3 show the chromatograms of the urine extracts obtained with and without the addition of the analytes in question to the samples. One column was apolar (dimethylpolysiloxane, 30 m, CP Sil 5) and the other was slightly polar (dimethyl [95%] diphenyl [5%] polysiloxane, 50 m, CP Sil 8). Although the short apolar column gave good separation of the compounds with respect to each other and the other matrix components, the longer, slightly polar column gave more efficient separation of 2,3-D (internal standard) from 2,4-D and narrower, more symmetrical peaks with an increase in run time of only 6 min. Manninen et al. (12) reported using an apolar dimethylsilicon capillary column (12.5 m) to determine phenoxyacetic herbicides in urine after transformation to pentafluorobenzyl esters. The use of slightly polar 30-m dimethyl (95%) diphenyl (5%) polysiloxane columns enabled a series of compounds, including 2,4-D and 2,3-D, to be determined in urine after conversion to the respective ethyl esters (11). Packed columns have been used for the determination of 2,4-D after transformation into its methyl ester (9,10).

In our HPLC procedure, analysis was carried out with the addition of the internal standard 4CPA, which was suitable for the assay of both compounds. In the GC method, two internal standards, 4CPA and 2,3-D, were used because they behave identically to MCPA and 2,4-D, respectively, during extraction and chromatography. This increased the reproducibility of the assay. In calculating the results of the GC method, the respective standard was used for each to give one result for 2,4-D and one result for MCPA.

The calibration curves were constructed with aliquots from a urine pool spiked with standards of the two herbicides. These aliquots were treated as samples of unknown concentration. For both methods, the curves were linear ($r > 0.990$) for 2,4-D and MCPA in the interval of concentrations between the detection limit and 500 µg/L.

The time required to analyze 20 samples by the HPLC method was approximately 18 h. The GC method required 24 and 26 h with columns 1 and 2, respectively, for the same number of samples.

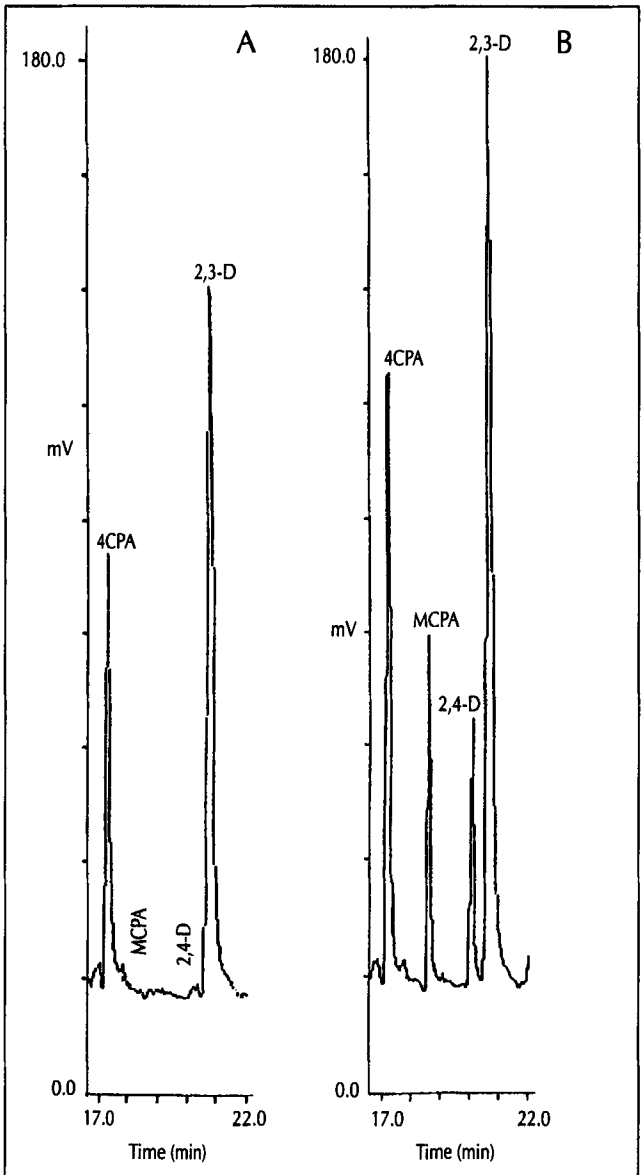

Figure 2. Chromatograms of urine extracts obtained by the GC method with a Chromopack CP Sil 5 column (30 m x 0.32 mm, 0.4 µm). A, blank urine; B, urine spiked with 30.0 µg/L of 2,4-D and MCPA.

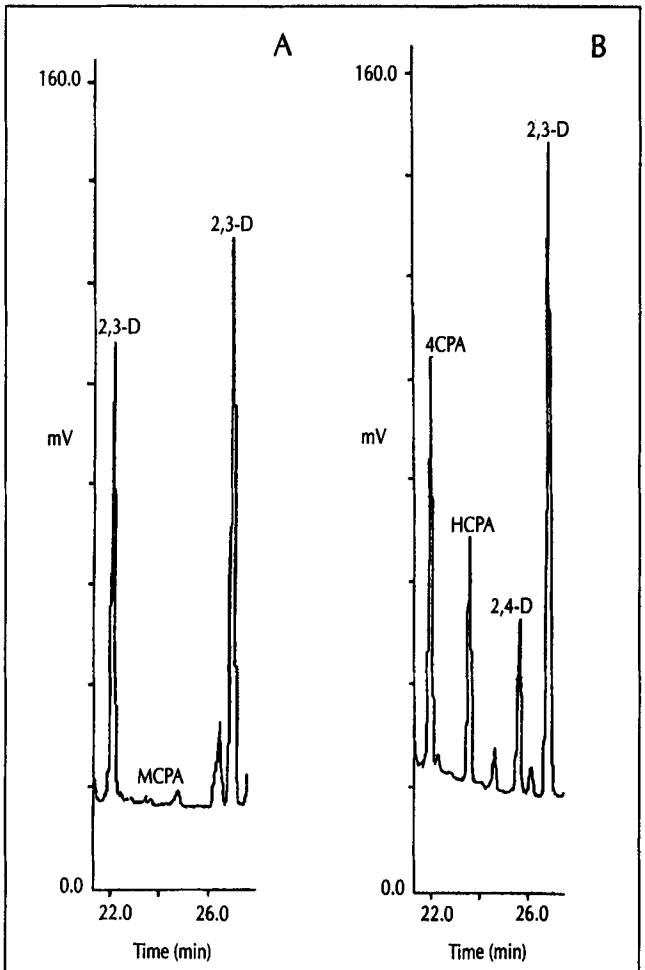

Figure 3. Chromatograms of urine extracts obtained by the GC method with a Chromopack CP Sil 8 column (50 m x 0.32 mm, 0.4 µm). A, blank urine; B, urine spiked with 30.0 µg/L of 2,4-D and MCPA.

Table I. Recovery, Detection Limits, and Precision of the Analytical Methods

	HPLC method				GC method			
	Mean recovery (%)*	CV† (1%)	CV‡ (2%)	Detection limits§ (µg/L)	Mean recovery (%)‖	CV† (1%)	CV‡ (2%)	Detection limits§ (µg/L)
2,4-D#	81.4	6.2	1.9	15.0	87.1	8.0	2.5	1.0
MCPA#	85.3	6.8	1.7	15.0	94.3	5.5	2.1	1.0

* Recovery was evaluated at a concentration of 125.0 µg/L of 2,4-D and MCPA.
† Coefficient of variation of the whole analysis.
‡ Coefficient of variation of chromatographic injection only.
§ The detection limit was calculated for a signal three times the background noise.
‖ Recovery was evaluated at a concentration of 30.0 µg/L of 2,4-D and MCPA.
Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; MCPA = 2-methyl-4-chlorophenoxyacetic acid.

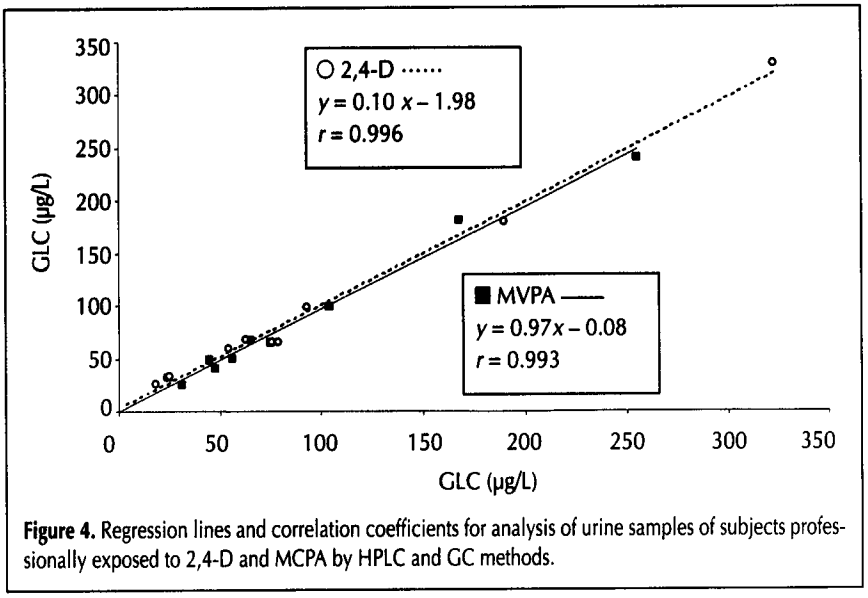

Figure 4. Regression lines and correlation coefficients for analysis of urine samples of subjects professionally exposed to 2,4-D and MCPA by HPLC and GC methods.

Table I shows the precision, recoveries, and detection limits of the methods based on 10 determinations. Because the large number of interfering compounds made it impossible to inject the GC or HPLC with unpurified urine extracts to evaluate recovery, we compared the results obtained with urine after purification and standard solutions of the same concentration.

Conclusion

The excellent reproducibility, recovery, and operational simplicity of the methods make them suitable for routine use in most laboratories. The detection limit of 15 µg/L means that the HPLC method can be used to determine 2,4-D and MCPA in occupationally exposed subjects. The GC method with a detection limit of 1 µg/L can also be used to assay the two herbicides in the general population. In subjects not occupationally exposed to these herbicides, low levels of urinary excretion are probably due to residues in food or environmental contamination (17).

The analytical procedures reported in the present paper have been extensively tested by us. In workers spraying wheat fields, the mean concentrations (maximum concentration in each subject) detected by HPLC were 121 ± 100 µg/L (mean plus or minus standard deviation) (124 determinations in 21 subjects) with a range of 35–400 µg/L for 2,4-D and 145 ± 184 µg/L (165 determinations in 34 subjects) with a range of 15–800 µg/L for MCPA. In a study over a 10-year period (1984–1994), urine excreted during the work shift and from the end of one shift to the beginning of the next was obtained for all the days of spraying. Extemporaneous samples were obtained before the start of the first day of spraying and 24-h urine samples were obtained for 3–4 days after the last day of spraying (15). The preliminary results of a study of elementary school children (unpublished data) show that concentrations of 2,4-D and MCPA were detectable in only 20% of the 100 children studied and never exceeded 2.5 µg/L for either compound (GC method). The urine specimens were extemporaneous morning samples.

The excellent agreement between the results obtained with the two methods in the duplicate analysis (Figure 4) shows that the methods are equivalent for determining these substances in occupationally exposed subjects.

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Manuscript received October 29, 1996;
revision received February 6, 1997.