INFLUENCE OF ETHYNODIOL DIACETATE ON THE FORMATION OF A-HOMO-3-OXA-5α-PREGNANE-4,20-DIONE IN FEMALE RATS

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Objective. To give more insight in the progesterone metabolism in rat after the treatment with the progestin ethynodiol diacetate.

Methods. Urinary excretion of the metabolites of subcutaneously administred $(4-^{14}C)$-progesterone was studied in female rats. After an acid hydrolysis and extraction of urine the metabolites were analysed by thin layer chromatography and by gas chromatography-mass spectrometry.

Results. The most of radioactivity was excreted during the first 24 h, and total of 8.36 % has been recovered within four days. The excreted metabolites in urine were found as glucuronides and free steroids (80.72 %), and 19.28 % were determined as sulphates. Among detected metabolites, 5α-pregnane-3,20-dione, 3α-hydroxy-5α-pregnan-20-one and A-homo-3-oxa-5α-pregnane-4,20-dione were determined in the urinary extracts. The last one has not yet been identified before in rat urine.

Conclusions. Consecutive injections of progestin ethynodiol diacetate (6 mg/kg b.w. daily) to adult female rats during 10 days (short-term treatment), or during 70 days (long-term treatment), starting on the 21st day of life, caused significant differences in the amounts of excreted 3α-hydroxy-5α-pregnan-20-one and A-homo-3-oxa-5α-pregnane-4,20-dione. Significant increase in the weights of pituitary, liver and kidneys were noted in rats treated with ethynodiol diacetate. The short-term treatment caused an increase, while after the long-term treatment a decrease of the ovarian weight was observed.

Key words: Progesterone metabolites - Rat - Urine - Ethynodiol diacetate - Gas chromatography - Mass spectrometry

Both natural and synthetic steroids undergo the enzymatic conversions in liver. Reduction of Δ^4-3-keto steroids into their corresponding saturated 3-alcohols, as the major metabolic pathway, is followed by hydroxylations at various positions of the molecule (DESGRES et al. 1984; ERIKSSON and ENEROTH 1987; SWINNEY 1990). Following the reduction and/or hydroxylation reactions, pregnane metabolites mostly form sulphates or glucuronides. The resulting conjugated metabolites are partially recirculated in the organism, and partially excreted into bile, faeces and urine.

Synthetic progestational and estrogenic agents, like derivatives with either steroid (EINARSSON et al. 1974; FREUDENTHAL et al. 1974; ARAKAWA et al. 1989) or other structures (KNIEWALD et al. 1991; SIMIC et al. 1994), can affect the activities of steroid converting enzyme systems, either by interfering with endogenous steroids as concurrent substrates, or by influencing the hypothalamo-pituitary control of hormone biotransformations.

In this study we were interested to give more insight in the progesterone metabolism in rat after the treatment with the progestin ethynodiol diacetate.

Materials and Methods

Chemicals: (4-^{14}C)-progesterone (specific activity 2.25 GBq/mmol) and (1,2,6,7-^{3}H)-androst-4-ene-3,17-dione (specific activity 2.18 GBq/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks, UK). Unlabeled progesterone, obtained from Fluka (Buchs, Switzerland), was purified by TLC before use.

Ethynodiol diacetate (3ß,17ß-diacetoxy-17α-ethynyl-4-estrene, ED) was purchased from Farmila (Settimo Milanese, Italy). Other steroids used in this study were obtained as a gift from Medical Research Council - Steroid Reference Collection (London, UK). All other chemicals were analytical grade commercial preparations.

Animals and treatment: Fischer strain female rats were maintained under standard laboratory conditions with a lighting schedule of 12 h light:12 h darkness. Food and water were given *ad libitum.* Body weight was recorded throughout the experimental procedures. Vaginal smears were taken daily between 8.00 and 10.00 h and classified as diestrous (primarily leucocytes), proestrous (nucleated epithelial cells), or estrous (cornified epithelial cells).

Daily doses of 6 mg ED/kg b.w., dissolved in 0.2 ml olive oil/ethanol (9:1 v/v), were injected subcutaneously to female rats. Rats were treated from the 90th to the 100th day (short-term treatment), or from the 21st to the 90th day of life (long-term treatment). Control animals were injected with vehicle only. On the 1st day after the termination of short-term treatment, or on the 10th day after the termination of long-term treatment, animals were injected sc. with (4-^{14}C)-progesterone (16.65 kBq per animal, in 0.2 ml of propylene glycol) and urine was collected during 24 hours. Unlabeled progesterone in the dose of 6 mg/kg b.w. was added to the injection solution when the metabolites were isolated from urine for the purpose of identification.

Separation of sulphates, glucuronides and free steroids: Progesterone metabolites in urine, which appeared as sulphates, glucuronides or free steroids, were separated according to the procedure described earlier (GOLDZIEHER and AXELROD 1969).

Hydrolysis and extraction of urine: Hydrolysis and extraction were performed according to the method of WOTIZ and CHATTORAJ (1967). The urine (5 ml on the average per animal in 24 hours) was diluted with double volume of distilled water, covered with toluene and brought to a boil. After the addition of 1.5 ml of conc. HCl refluxing was continued for 10 min. The mixture was rapidly cooled and the toluene layer was removed. The residual urine was extracted with a new portion of toluene. Combined toluene extract was washed twice with 25 % NaCl in 1 N NaOH, and twice with distilled water. Washed extract was dried over the anhydrous sodium sulphate and evaporated to dryness under the reduced pressure. The procedural losses were evidenced by the addition of (^{3}H)-androstene-3,17-dione (16.65 Bq) to the collected urine as internal standard, and accounted in quantitative measurements.

Thin layer chromatography: Dry residues of the urinary extracts were dissolved in ether and transferred to the silica gel GF$_{254}$ plates for TLC. The plates were developed in n-heptane to remove impurities, and for a second time in a mixture chloroform/acetone/n-heptane (4:1:3 v/v/v). Chromatograms were autoradiographed with an exposing time of 7 days. The areas corresponding to progesterone metabolites were determined from the x-ray film and were scraped off, and transferred to counting vials. After the addition of 10 ml of PPO-POPOP scintillation fluid, the radioactivity was measured in a Mark II Nuclear Chicago liquid scintillation counter. Corrections were done for the counting efficiency and for the procedural losses.

Chemical conversions of steroids: Steroid reduction with sodium borohydride in cold methanol, oxidation with chromic acid, acetylation in acetic anhydride/pyridine mixture, and the reaction with ketonic reagent 2,4-dinitrophenylhydrazine in methanol in the presence of acetic acid, were carried out as described earlier (VAN DER MOLEN et al. 1968; ICHIKAWA et al. 1971).

Gas chromatography and gas chromatography - mass spectrometry: Perkin Elmer Model 881 GC was used for the steroid analysis. Glass column fitted with 2.5 % Silicone Gum Rubber E 301 on AW-DMCS Chromosorb G was used as a separating media under isothermal conditions of 225 ºC with the nitrogen carrier flow 30 mL/min and FID. Gas chromatography - mass spectrometry analyses were performed on an LKB 9000 instrument.

Data analysis: Student's t-test was applied to evaluate the significance of differences between the means (P values less than 0.05 were considered as significant).

Results

Progesterone metabolites in urine: The excretion of radioactivity in urine after sc. injection of 16.65 kBq (4-^{14}C)-progesterone to female rats is shown in Tab. 1. Most of the radioactivity recovered in the urine was excreted within the first 24 h. After the third day the radioactivity was under the detectable level (set at 50 dpm). In the urine, collected during the first 24 h after sc. injection of (4-^{14}C)-progesterone, 19.28 % of total excreted radioactivity was bound as sulphates (Tab. 2). Remaining of the radioactivity (80.72 %) was determined as glucuronides or free steroids.

The effects of ethynodiol diacetate on urinary progesterone metabolites in glucuronide and free steroid fraction were analyzed. Three metabolites were chosen as markers: 5α-pregnane-3,20-dione, 3α-hydroxy-5α-pregnan-20-one and A-homo-3-oxa-5α-pregnane- 4,20-dione.

The identities of metabolites were confirmed by TLC, GC, and by chemical conversions of extracted substances. Further analyses were performed to prove the structure of metabolite characterized as A-homo-3-oxa-5α-pregnane-4,20-dione. After purification and recrystallization, it has been shown that this metabolite can be reduced with sodium borohydride, and that the formation of dinitrophenyl-hydrazone is possible, while it does not undergo oxidation and acetylation. Mass spectrum obtained by GC-MS analysis of the extracted metabolite after the recrystallization is shown in Fig. 1. The metabolite showed a molecular ion (M) at m/e 332. Following the reduction with lithium aluminium hydride the trimethylsilyl (TMS) ether of the original compound gave rise to spectrum shown in Fig. 2. The TMS ether derivative gave a molecular ion (M) at m/e 554 suggesting the binding of three trimethylsilyl groups. The corresponding peaks are at m/e 464 (M-90), m/e 374 (M-90-90) and m/e 284 (M-90-90-90) indicating the structure of the derivative. The loss of 116 mass units (fragment at m/e 348: peak at m/e 464-116) indicate the presence of a C-21 side-chain with a trimethylsilyl ether group at C-20, as it was expected. Remaining two trimethylsilyl ether groups were bound to the ring A of A-homo-3-oxa-5α-pregnane-4,20-dione. Since the reduction of the lactone with lithium aluminium hydride gives a secotriol (NACE and WATTERSON 1966), the binding of TMS groups is enabled.

Table 1.
The recovery of radioactivity in urine after sc. injection of (4-^{14}C)-progesterone to female rats[a].

Time after the injection (hr)	Excreted radioactivity (dpm)
0 - 24	81468 ± 6168.5^{b}
24 - 48	9558 ± 566.8
48 - 72	1889 ± 500.1
72 - 96	<50
Total:	92916 ± 6401.9

[a]Fischer strain rats (n = 5) aged 100 days, injected with 16.65 kBq of (4-^{14}C)-progesterone.
[b]Mean + S.E.

Table 2.
The distribution of free and conjugated metabolites in urine following sc. injection of (4-^{14}C)-progesterone[a] to female rats.

C[14]-labeled metabolites	Radioactivity (dpm)	% of excreted radioactivity
Total	81468 ± 6168.5^{b}	
Sulphates	15707 ± 1802.7	19.28
Free + glucuronides	65761 ± 3368.0	80.72

[a]16.65 kBq of (4-^{14}C)-progesterone was injected sc., and urine was collected during 24 hr.
[b]Mean + S.E. of three samples.

The possibility that A-homo-3-oxa-5α-pregnane-4,20-dione is an artifact arised during the acid hydrolysis, or during the procedure of isolation, was excluded experimentally. The animals were injected with labeled progesterone and the acid hydrolysis was omitted in the extraction procedure. TLC of the extract showed the presence of this metabolite. Further, labeled progesterone was added into urine of intact animals, and the complete method of hydrolysis and extraction was performed. TLC of urinary extract showed the presence of added substrate only. This suggests that the compound is endogenously formed progesterone metabolite.

Effects of ethynodiol diacetate: Influence of ED on the progesterone metabolism was investigated in female rats. Compared to the control animals quantitative changes were determined (Tab. 3) after ED treatment. The short-term treatment caused statisti-
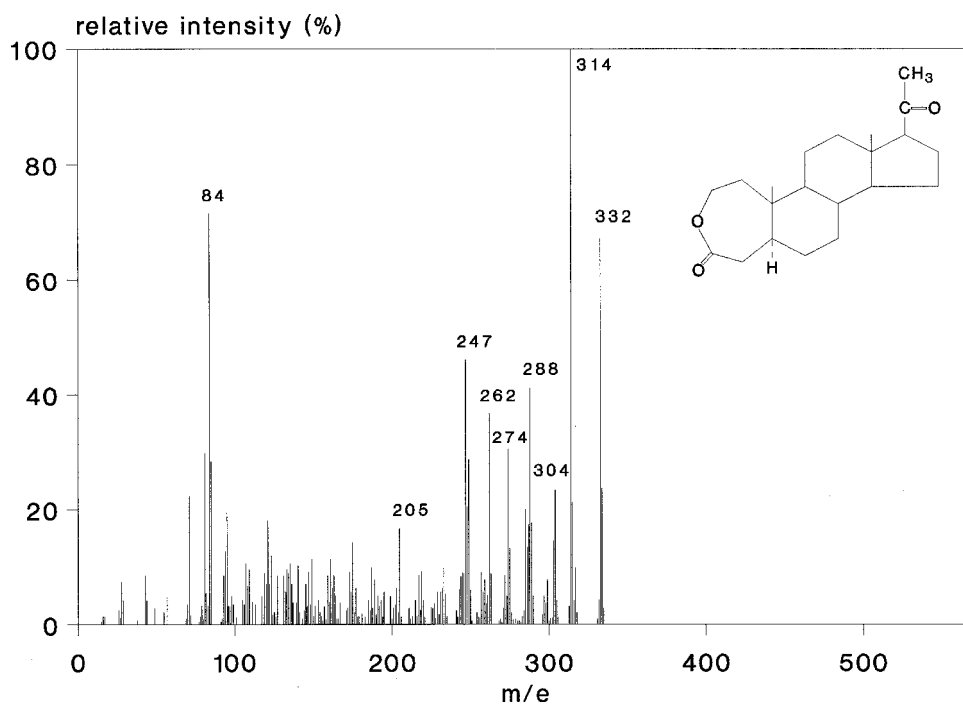
Fig. 1.
Mass spectrum of the tentatively identified urinary progesterone metabolite A-homo-3-oxa-5α-pregnane-4,20-dione.
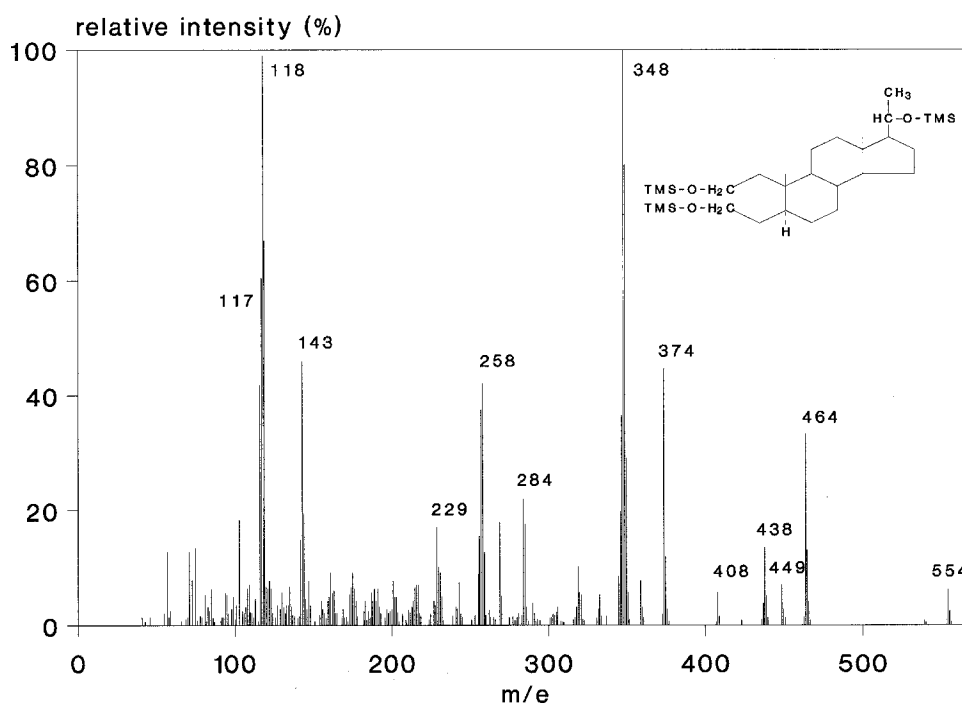

Fig. 2.
Mass spectrum of the TMS ether derivative of the progesterone metabolite identified as A-homo-3-oxa-5α-pregnane-4,20-dione.

Table 3.
The effects of ethynodiol diacetate[a] on the excretion of (4-^{14}C)-progesterone metabolites in female rat urine.

Metabolite	dpm in extract		
	Control	Short-term treatment	Long-term treatment
	(21)	(10)	(10)
3α-hydroxy-5α-pregnan-20-one	9293 ± 541.4[b]	8361 ± 809.4	6941 ± 604.0**
A-homo-3-oxa-5α-pregnane-4,20-dione	6788 ± 448.5	9250 ± 837.0*	10547 ± 695.9***
5α-pregnane-3,20-dione	3879 ± 331.3	3771 ± 444.7	3876 ± 171.3

[a]Rats were injected sc. with 6 mg ED / kg bw., daily, from the 90th to the 100th day (short-term treatment), or from the 21st to the 90th day of life (long-term treatment). Urine was collected during 24 hr after sc. injection of 16.65 kBq of (4-^{14}C)-progesterone.
[b]Mean ± S.E.; ( ) = Number of animals.
Statistical evaluation was done according to the Student's t-test: ***P<0.001; **P<0.01; *P<0.02 vs. control.

Table 4.
Effect of ethynodiol diacetate (ED) on relative organ weights of female rat.

Organ	mg/100 g of body weight			
	Short-term treatment*		Long-term treatment**	
	control	ED	control	ED
	(6)	(12)	(5)	(10)
Pituitary	5.8 ± 0.31	8.4 ± 0.28[b]	5.2 ± 0.42	8.0 ± 0.42[b]
Ovaries	44.8 ± 2.11	53.2 ± 1.66[a]	42.8 ± 3.20	35.5 ± 1.56
Uterus	166.2 ± 10.01	257.0 ± 9.88[b]	173.3 ± 16.48	215.4 ± 11.75
Liver	3568 ± 63.2	4892 ± 143.5[b]	3915 ± 220.0	5292 ± 115.3
Kidneys	785 ± 26.4	957 ± 20.0[b]	828 ± 24.4	1111 ± 17.3[b]

Ethynodiol diacetate was injected sc. in daily dose of 6 mg/kg b.w. *from the 90th to the 100th day, or **from the 21st to the 90th day of life. ( ) = Number of animals. Statistical significance ([a]P<0.01; [b]P<0.001 - vs. corresponding control values) was evaluated by Student's t-test.

cally significant increase of A-homo-3-oxa-5α-pregnane-4,20-dione (P<0.02). After the long-term treatment the increase of A-homo-3-oxa-5α-pregnane-4,20-dione (P<0.001), and the decrease of 3α-hydroxy-5α-pregnan-20-one (P<0.01) were observed.

The animals of control groups showed regular ovarian cycling throughout the experiments. During the short-term treatment with ED normal ovulatory estrous cycle was inhibited. Vaginal cytology showed continuous diestrus in all animals. In the long-term treated group the estrous cycle was not monitored during the whole period of the treatment, but during ten days following the cessation of treatment continuous diestrus was found in 70 % of the animals, and in the other animals the irregular cycling with prolonged leucocytic phase was observed.

The increased pituitary weights above 45 % (P<0.001) were determined after both regimens of treatment with ED. The weights of uterus and ovaries were increased in short-term ED treated animals, compared to their control group (P<0.001 and P<0.01, respectively). After the long-term treatment, decreased ovarian weights compared to controls were found, but the difference was not statistically significant (Tab. 5). Both after the short-term and after the long-term treatment with ED, the weight of liver, calculated per 100 g b.w., increased by 35 % (P<0.001), and the weight of kidneys increased by 18 % (P<0.001) as compared to the control animals.

Discussion

After sc. injection of 16.65 kBq of (4-^{14}C)-progesterone to female rats only 8.36 % of the administered radioactivity was excreted in urine. It means

that the major way of steroid elimination was by feces which is in aggreement with the previously published data (Björkhem et al. 1972). In the early period of life the urinary excretion of (4-¹⁴C)-pregnenolone is the major way of elimination, while the excretion by feces predominates in the later period of life. Also, the amount of free steroids exceeds the amount of conjugated forms in the later phase of life. In female rat urine, the progesterone metabolites are excreted mainly as glucuronides or free steroids. Only a small part of metabolites is excreted as sulphates.

Among the metabolites extracted from the free steroid and glucuronide fraction of female urine A-homo-3-oxa-5α-pregnane-4,20-dione (Fig. 1) was identified. Although this compound has been characterized earlier (Nace and Watterson 1966) as a synthetic product, it has not yet been referred as urinary progesterone metabolite.

It has been reported that ethynodiol diacetate, which undergoes the biotransformation to norethindrone, affects the hepatic microsomal enzyme systems *in vitro*, but not *in vivo* (Freudenthal et al. 1974). Our findings suggest that ED influences also the hepatic progesterone metabolism *in vivo*. In the immature gonadectomized female rats norethindrone suppresses FSH and LH levels in serum, and induces the increase in uterine weight (McPherson et al. 1974). Such effect is more estrogenic than progestogenic, due to the possible inherent estrogenic activity of norethindrone, like of some other 19-nortestosterone progestins, or to the conversion of the compound into metabolites with estrogenic activity (McPherson et al. 1974; Darney 1995). This could explain the dual effects of progestational substances, while inhibitory effect of progesterone on pituitary LH release is estradiol-dependent (Girmus and Wise 1992). After the treatment with ED, we have determined the tendency in an increase of uterine weights, and also a significant increase of pituitary weights. Suppression of gonadotrophins secretion and their accumulation in pituitary under the influence of ED (Suzuki et al. 1972) could increase the size of gonadotrophs, and consequently the increase of tissue weight.

Increased ovarian weight after the short-term treatment probably is due to the progestinic activity of ED. Vaginal cytology showed continuous diestrus in those animals. Such effect of ED was described earlier (Suzuki et al. 1972). At the same time, signif-icantly increased concentrations of A-homo-3-oxa-5á-pregnane-4,20-dione, the main progesterone metabolite out of those which were estimated, suggest the induced gestagenic events in female rat treated with ED. Progesterone is decreasing the growth rate of ovarian follicles, but it does not inhibit follicle ripening (Buffler and Roser 1974). Low secretion of gonadotropins, as the consequence of chronic exposure to ED in the long-term treatment, caused the reduction of ovarian weight and disruption of the regularity of estrous cycle, leading to the persistent diestrus.

Acknowledgements

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