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THE EXCRETION OF FIVE DIFFERENT 2-HYDROXYOESTROGEN MONOMETHYL ETHERS IN HUMAN PREGNANCY URINE*

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SUMMARY

The excretion pattern of different 2-hydroxyoestrogen monomethyl ethers (2-methoxyoestrone, 2-hydroxyoestrone 3-methyl ether, 2-methoxyoestradiol, 2-hydroxyoestradiol 3-methyl ether and the sum of the isomeric monomethyl ethers of 2-hydroxyoestriol) was quantitatively determined in urines of pregnant women. The analytical procedure involved several chromatographic purification steps and microchemical reactions, *i.e.* oxidative demethylation of the monomethyl ethers to the catechol compounds (2-hydroxyoestrone, 2-hydroxyoestradiol and 2-hydroxyoestriol) and conversion of 2-hydroxyoestrone and 2-hydroxyoestradiol to the corresponding phenazine derivatives. The phenazines were quantitated by U.V.-spectrometry and 2-hydroxyoestriol was determined by gas-chromatography. The specificity of the procedure was established by various spectrometric, chromatographic and microchemical methods. The amounts of the different oestrogens varied within the following ranges: 2-methoxyoestrone = 20–360 μg/24 h; 2-hydroxyoestrone 3-methyl ether = 5–50 μg/24 h; 2-methoxyoestradiol = $<$5–25 μg/24 h; 2-hydroxyoestradiol 3-methyl ether = $<$5 μg/24 h; monomethyl ethers of 2-hydroxyoestriol = 10–240 μg/24 h.

INTRODUCTION

Many investigators have demonstrated that aromatic hydroxylation is of major importance in the metabolism of oestrogens in man (for a review see [1]). Furthermore, evidence has been obtained that 2-hydroxyoestrogens may participate in hormonal regulation mechanisms. Thus, Knuppen and co-workers showed that the enzymatic inactivation of catecholamines is strongly inhibited by 2-hydroxylated oestrogens [2–4], while Parvizi and Ellendorff observed a decrease of the plasma LH-levels after microinfusion of 2-hydroxyoestradiol into the amygdala of the orchidectomized miniature pig [5]. Recently, Davies *et al.*, demonstrated an interaction between 2-hydroxyoestrogens and oestrogen receptors of the pituitary and hypothalamus of the rat which could be biologically significant [42].

These findings prompted intensive quantitative investigations on 2-substituted oestrogens in the human and until now exact data are only available for the urinary concentrations of 2-hydroxyoestrone (2-OHE$_1$†) [6], 2-hydroxyoestradiol (2-OHE$_2$) [7] and 2-hydroxyoestriol (2-OHE$_3$) [8] in pregnant and of 2-OHE$_1$ [9] in nonpregnant subjects. Further metabolic transformation of 2-hydroxyoestrogens by enzymatic methylation is well documented [10] and the monomethyl ethers derived from 2-OHE$_1$ [11], 2-OHE$_2$ [12] and 2-OHE$_3$ [13] have been identified in human urine. Nevertheless, apart from 2-methoxyoestrone (2-OHE$_1$ 2Me) [14–19], quantitative data have not been given for the excretion of 2-hydroxyoestrogen monomethyl ethers in pregnancy.

The present investigation was undertaken in order to establish a complete pattern of the different 2-hydroxyoestrogen monomethyl ethers (2-OHE$_1$ 2Me, 2-OHE$_1$ 3Me, 2-OHE$_2$ 2Me, 2-OHE$_2$ 3Me, 2-OHE$_3$ Me‡) in the urine of pregnant women.

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† The following abbreviations and trivial names are used in this paper:

2-OHE$_1$ = 2-hydroxyoestrone = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one;
2-OHE$_2$ = 2-hydroxyoestradiol = 1,3,5(10)-oestratriene-2,3,17β-triol;
2-OHE$_3$ = 2-hydroxyoestriol = 1,3,5(10)-oestratriene-2,3,16α,17β-tetrol;
2-OHE$_1$ 2Me = 2-methoxyoestrone = 3-hydroxy-2-methoxy-1,3,5(10)-oestratrien-17-one;
2-OHE$_1$ 3Me = 2-hydroxyoestrone 3-methyl ether = 2-hydroxy-3-methoxy-1,3,5(10)-oestratrien-17-one;
2-OHE$_2$ 2Me = 2-methoxyoestradiol = 2-methoxy-1,3,5(10)-oestratriene-3,17β-diol;
2-OHE$_2$ 3Me = 2-hydroxyoestradiol 3-methyl ether = 3-methoxy-1,3,5(10)-oestratriene-2,17β-diol;
2-OHE$_3$ Me = sum of the isomeric monomethyl ethers of 2-OHE$_3$;
PhE$_1$ = phenazine of 2-OHE$_1$ = 1,3,5(10)-oestratrien-17-one-[2,3-b]-quinoxaline;
PhE$_2$ = phenazine of 2-OHE$_2$ = 1,3,5(10)-oestratrien-17β-ol-[2,3-b]-quinoxaline.

‡ 2-OHE$_3$ Me represents the sum of 2-OHE$_3$ 2Me and 2-OHE$_3$ 3Me, since a suitable chromatographic system was not available for the separation of these isomeric methyl ethers.

EXPERIMENTAL

Steroids. 2-Hydroxyoestrogens were prepared according to Stubenrauch and Knuppen [20] and their monomethyl ethers were synthesized by the method of Fishman [21,22]. [4-^{14}C]-Labeled 2-hydroxyoestrogens [8,23] (specific radioactivity 45–55 mCi/mmol) were converted to their monomethyl ethers by enzymatic methylation as described by Ball *et al.* [24]. The isomeric monomethyl ethers of [4-^{14}C]2-OHE$_1$ were separated by paper chromatography in system B for 16 h and those of [4-^{14}C]2-OHE$_2$ in system B for 48 h. An appropriate chromatographic system was not available for the separation of 2-OHE$_3$ 2Me and 2-OHE$_3$ 3Me.

Chemicals and solutions. All chemicals were purchased from E. Merck, Darmstadt, Germany, and were of analytical grade, except for o-phenylenediamine (grade: for synthesis) and chloroform (grade: pure) used for the preparation of the phenazine derivatives.

The standard ascorbic acid solution contained ascorbic acid (15 g) and glacial acetic acid (4 ml) in methanol (400 ml). The ascorbic acid solution of pH 10.5 was prepared by dissolving ascorbic acid (30 g) and $NaHCO_3$ (45 g) in water (600 ml); shortly before use 15 ml of 15 M NaOH were added.

Chromatographic methods. Paper chromatography (2043 b Mgl paper, Schleicher & Schüll) and silica gel column chromatography (Kieselgel 60, particle size 0.063–0.2 mm; E. Merck) were carried out on ascorbic acid-impregnated stationary phases as described by Gelbke and Knuppen [25]. The phenazine derivatives were chromatographed on papers impregnated with formamide–dimethylformamide–methanol (2:3:5, by vol.). Alumina t.l.c. was performed on MN-Polygram Alox N/U.V.$_{254}$ precoated plates (Machery & Nagel, Germany), silica gel t.l.c. on DC-Fertigplatten, Kieselgel F-254 (E. Merck), and alumina for column chromatography (Aluminium Oxide, basic, activity grade I) was obtained from Woelm, Germany, and desactivated with water (3 ml/100 g). Solvent systems used for paper and thin-layer chromatography are given in Table 1.

Table 1. Solvent systems for paper (A-J) and t.l.c. (M-O).

A:	Fa-asc/benzene–cyclohexane (2:1 v/v)
B:	Fa-asc/cyclohexane
C:	Eg-asc/chlorobenzene–ethyl acetate (1:1 v/v)
D:	Fa-asc/chlorobenzene–ethyl acetate (3:1 v/v)
E:	Fa-asc/chloroform–ethyl acetate (5:1 v/v)
F:	asc/water–acetic acid–1,2-dichloroethane (3:7:10 by vol)
G:	Fa-DmFa (2:3)/methylcyclohexane
H:	Fa-asc/cyclohexane–benzene (5:1 v/v)
J:	Fa-DmFa (2:3)/methylcyclohexane–chlorobenzene (4:1 v/v)
M:	silica gel/benzene–ethanol (3:1 v/v)
N:	alumina/benzene–ethanol (3:1 v/v)
O:	alumina/benzene–ethyl acetate (2:1 v/v)

Fa = formamide; asc = ascorbic acid; Eg = ethylene glycol; DmFa = dimethylformamide.

Gas–liquid chromatography was carried out with a Pye–Unicam gas chromatograph, series 104, equipped with a flame ionization detector. For silylation the material was treated with 30 μl of the silylating reagent (N,O-bis(trimethylsilyl)-trifluoroacetamide containing 5% of trimethylchlorosilane) for 2 h at 120°C. The gas chromatographic conditions have been described previously for PhE$_1$ [26], PhE$_2$ [7] and 2-OHE$_3$ [8].

Demethylation of 2-hydroxyoestrogen monomethyl ethers. [4-^{14}C]2-OHE$_1$ 2Me (20 μg, 50,000 d.p.m.) in 0.5 ml of glacial acetic acid was oxidized to the 2,3-quinone by treatment with $NaIO_3$ (10 mg in 0.5 ml of water) for 1 min at room temperature. After extraction with chloroform (3 times with 1 ml) the combined organic extracts were washed twice with water containing 10% of acetic acid. 2 ml of glacial acetic acid were added and the 2,3-quinone was reduced with a few crystals of KI. After 2 min the reaction mixture was treated with 2 ml of an aqueous solution containing 1% of sodium disulfite for reduction of iodine. The organic layer was separated and the aqueous phase was twice extracted with ethyl acetate. The combined organic extracts were washed twice with a 10% aqueous acetic acid solution and evaporated *in vacuo* after addition of 0.2 ml of the standard ascorbic acid solution. The yield of [4-^{14}C]2-OHE$_1$ was determined by liquid scintillation counting and radio paper chromatography (system D). The purity of the radioactive 2-OHE$_1$ was also occasionally confirmed by reverse isotope dilution.

[4-^{14}C]2-OHE$_3$ Me (20 μg, 50,000 d.p.m.) was converted to [4-^{14}C]2-OHE$_3$ accordingly, except that all aqueous phases were saturated with Na_2SO_4 before extraction. The purity of the 2-OHE$_3$ was checked by radio paper chromatography in system F.

Best yields were obtained, when the 2,3-quinones were reduced *in situ* without prior extraction as will be described below.

Determination of 2-hydroxyoestrogen monomethyl ethers in pregnancy urine. Complete 24-h urine specimens were analyzed directly or stored at −15°C. A portion of 100 ml was used for the measurement of 2-OHE$_1$ [26] and of the total oestrogens [27], while 2-OHE$_1$ 2Me, 2-OHE$_1$ 3Me, 2-OHE$_2$ 2Me, 2-OHE$_2$ 3Me and 2-OHE$_3$ Me were determined in the remaining urine as follows:

To each one hundred ml of urine 20 ml of conc. HCl (37%) were added and the solution was refluxed for 1 h. After cooling to room temperature [4-^{14}C]2-OHE$_1$ 2Me, [4-^{14}C]2-OHE$_1$ 3Me, [4-^{14}C]2-OHE$_2$ 2Me, [4-^{14}C]2-OHE$_2$ 3Me and [4-^{14}C]2-OHE$_3$ Me (100 000 d.p.m. of each) were added and the hydrolyzed urine was extracted 3 times with half the volume of benzene-ethyl acetate (1:1). The combined organic extracts were once washed with a buffer of pH 10.3 (30 g of NaOH and 80 g of $NaHCO_3$ dissolved in 1000 ml of water) and evaporated *in vacuo*. The residue was dissolved in benzene (150 ml), cyclohexane

(450 ml) was added and the solution was extracted 3 times with 300 ml of 2 M NaOH–methanol (1:1). The combined aqueous phases were acidified with 200 ml of HCl (32%) and reextracted 3 times with 500 ml of benzene–ethyl acetate (1:1). The organic extracts were washed once again with the buffer of pH 10.3 (400 ml), once with a 10% aqueous acetic acid and evaporated *in vacuo*.

Separation of the monomethyl ethers with different substitution pattern of ring D was achieved by paper chromatography in system A. After localization the radioactive steroids (2-OHE_1 2Me and 2-OHE_1 3Me: $R_F = 0.9$; 2-OHE_2 2Me and 2-OHE_2 3Me: $R_F = 0.5$; 2-OHE_3 Me: no migration) were eluted with methanol. The solutions were evaporated and the residues were partitioned between 10% aqueous acetic acid and benzene–ethyl acetate (1:1). Final separation and further purification of the isomeric monomethyl ethers was carried out by paper chromatography:

(a) 2-OHE_1 2Me and 2-OHE_1 3Me: system B, 16 h (migration of 2-OHE_1 2Me = 33 cm./16 h, and of 2-OHE_1 3Me = 26 cm./16 h).

(b) 2-OHE_2 2Me and 2-OHE_2 3Me: system B, 48 h (migration of 2-OHE_2 2Me = 22 cm./48 h, and 2-OHE_2 3Me = 16 cm./48 h).

(c) 2-OHE_3 Me: system E, 10 h (migration = 30 cm./10 h).

The radioactive steroids were eluted with methanol, subjected to a solvent partition (see above) and were demethylated subsequently to the corresponding 2-hydroxyoestrogens (Fig. 1a):

The monomethyl ethers of 2-OHE_1 and 2-OHE_2 were dissolved in glacial acetic acid (0.5 ml) and oxidized with $NaIO_3$ (10 mg in 0.5 ml of water) for 1 min at room temperature. 200 μl of a solution was added, containing sodium sulfite (2 g) and KI (2.5 g) in 10 ml of water. After 2 min the reaction mixture was extracted 3 times with chloroform (1.5 ml each) and the organic extract was washed once with a 10% aqueous acetic acid solution and once with water containing 1% of sodium disulfite. Standard ascorbic acid solution (0.4 ml) was added and the organic phase was evaporated *in vacuo*.

2-OHE_3 Me was converted to 2-OHE_3 correspondingly, except that all extractions were carried out with ethyl acetate and NaCl-saturated aqueous phases.

The 2-hydroxyoestrogens thus obtained were further processed and quantitated as described previously.

(a) 2-OHE_1 [26]: paper chromatography in system D, column chromatography on ascorbic acid-impregnated silica gel, conversion to the phenazine derivative (PhE_1; Fig. 1b), column chromatography of PhE_1 on basic alumina, and quantitative determination by liquid scintillation counting and U.V.-spectrometry.

(b) 2-OHE_2 [7]: as under (a).

(c) 2-OHE_3 [8]: paper chromatography in system E (72 h), column chromatography on ascorbic acid-impregnated silica gel, quantitative determination of 2-OHE_3 by liquid scintillation counting and gas chromatography.

RESULTS

Stability of 2-hydroxyoestrogen monomethyl ethers during hot acid hydrolysis and chromatography. To investigate the stability in the course of urine hydrolysis, $[4\text{-}^{14}C]2\text{-OHE}_1$ 2Me or $[4\text{-}^{14}C]2\text{-OHE}_3$ Me (10 or 1 μg of each) were added to 10 ml portions of 4 different pregnancy urines which were subsequently subjected to hot acid hydrolysis. After extraction with benzene–ethyl acetate (1:1) the recovery and the purity of the radioactive steroids was determined by liquid scintillation counting and radio paper chromatography (system H for 2-OHE_1 2Me; system C for 2-OHE_3 Me) or reverse isotope dilution. 86–93% of the original $[4\text{-}^{14}C]2\text{-OHE}_1$ 2Me and 71–78% of $[4\text{-}^{14}C]2\text{-OHE}_3$ Me were recovered undecomposed. These figures closely resembled to those reported by Scheike *et al.* [19] for similar experiments with unlabeled 2-OHE_1 2Me.

Similarly, the stability of $[4\text{-}^{14}C]$-labeled 2-hydroxyoestrogen monomethyl ethers was investigated during paper chromatography. In comparison to stan-

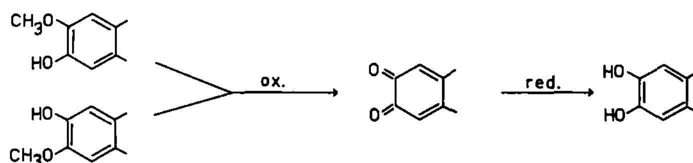

Fig. 1a. Reaction scheme for the conversion of 2-hydroxyoestrogen monomethyl ethers to 2-hydroxyoestrogens.

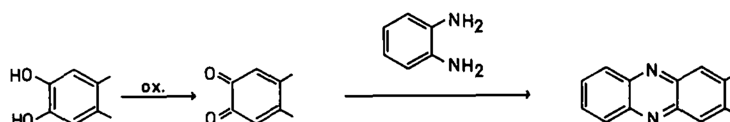

Fig. 1b. Reaction scheme for the conversion of 2-hydroxyoestrogens to their phenazine derivatives.

dard chromatographic methods the recovery of undecomposed monomethyl ethers was markedly improved when using ascorbic acid-impregnated paper chromatograms. These findings are in good accordance with reports of Doerr [28] and Morreal and Dao [29] who demonstrated autoxidative decomposition of classical oestrogens during t.l.c. in the absence of antioxidants. Since 80–90% of even 0.2 μg of [4-^{14}C]2-OHE$_1$ 2Me and [4-^{14}C]2-OHE$_3$ Me were recovered from ascorbic acid-loaded paper chromatograms, this procedure was selected for the purification and separation of the urinary 2-hydroxyoestrogen monomethyl ethers.

Conversion of 2-hydroxyoestrogen monomethyl ethers to 2-hydroxyoestrogens. As demonstrated in Fig. 1a 2-hydroxyoestrogen monomethyl ethers can be easily converted to the corresponding catechol compounds by oxidation to 2,3-quinones and subsequent reduction. The oxidation and reduction steps of this sequence were separately investigated.

For the first step various oxidants can be used [30], but highest yields were obtained by oxidation with $NaIO_4$ [31–33] or $NaIO_3$ in aqueous acetic acid. The ratio of water and acetic acid could be varied between 1:1 and 4:1 without impairment of the yield. Exposure of 2-OHE$_1$ 2Me and 2-OHE$_1$ 3Me to $NaIO_4$ or $NaIO_3$ for 1 or 2 min afforded optimal yields, but after 10 min an increased decomposition of approx. 10% (oxidation with $NaIO_4$) and 5% (oxidation with $NaIO_3$) was observed. After treatment of 2-OHE$_3$ Me with $NaIO_4$ the recoveries were found to be approx. 10% lower than after treatment with $NaIO_3$. Therefore, $NaIO_3$ was finally selected as oxidant.

Several reductants (ascrobic acid, sodium thiosulfate, sodium disulfite) were used for the second part of the reaction sequence, but best results were obtained with KI. Iodine, generated in the course of this reaction, was subsequently reduced with sodium disulfite.

The recovery of the total reaction sequence (Fig. 1a) was investigated as described in the experimental section. The yields were approx. 80% for 2-OHE$_1$ 2Me and 65% for 2-OHE$_3$ Me. When the 2,3-quinones were reduced *in situ* directly after the oxidation without prior extraction, yields were increased by another 5% for 2-OHE$_1$ 2Me and 10% for 2-OHE$_3$ Me. Also with respect to the simplicity, this *in situ* procedure was finally used for the urine analyses.

Quantitative determination of 2-hydroxyoestrogen monomethyl ethers in pregnancy urine was carried out as outlined in Fig. 2. Besides intensive chromatographic purification, the procedure included the microchemical formation of the catechol (Fig. 1a) and phenazine (Fig. 1b) compounds.

Reliability of the analytical procedure. (a) *2-OHE$_1$ 2Me, 2-OHE$_1$ 3Me*: Specificity of the analytical procedure was examined by a microchemical reaction as described previously [26]: After quantitation of 2-OHE$_1$ 2Me and 2-OHE$_1$ 3Me by U.V.-spectrometry of PhE$_1$, this phenazine derivative was reduced to the corresponding 17β-hydroxy compound (PhE$_2$). PhE$_2$ was purified by another column chromatography and the urinary amounts of 2-OHE$_1$ 2Me and 2-OHE$_1$ 3Me were independently calculated by U.V.-spectrometry of PhE$_2$. The results obtained with several urines for the determination of 2-OHE$_1$ 2Me and 2-OHE$_1$ 3Me *via* PhE$_1$ and PhE$_2$ were in good agreement (difference less than 10%).

The identity of PhE$_1$ and PhE$_2$ was established by U.V.-spectrometry, mass spectrometry and gas chromatography on silicone OV-101 and OV-17 as described previously [7,26]. The radiochemical purity of PhE$_1$ and PhE$_2$ was better than 95% as checked by radio thin-layer (system M, N, and O) and radio paper chromatography (system G and J).

(b) *2-OHE$_2$ 2Me, 2-OHE$_2$ 3Me*: Specificity was investigated by the microchemical oxidation of PhE$_2$ to PhE$_1$ described previously [7]. The amounts of the urinary 2-OHE$_2$ 2Me and 2-OHE$_2$ 3Me calculated by spectrometry of PhE$_2$ and PhE$_1$ differed less than 10%. To investigate the specificity of the determination of 2-OHE$_2$ 3Me, 10 or 25 μg of 2-OHE$_2$ 3Me were added to the pregnancy urines after hydrolysis.

The identity and radiochemical purity of PhE$_2$ and PhE$_1$ (before and after the microchemical oxidation) was checked as described above; the radiochemical purity again was better than 95%.

(c) *2-OHE$_3$ Me*: In order to assess the specificity of the determination of 2-OHE$_3$ Me, the material obtained after paper chromatography of 2-OHE$_3$ was divided into two parts. One part was analyzed according to the flow diagram (Fig. 2) and the other part was subjected to a further paper chromatography (system F) before silica gel column chromatography. The results obtained without and with this additional paper chromatographic purification step differed less than 10%. Similarly, differences of less than 5% were found, when the final quantitation was carried out by gas chromatography on silicone OV-101 and OV-17 using methyl cholate as internal standard [8].

The identity of 2-OHE$_3$, isolated after urine analysis of 2-OHE$_3$ Me, was also established by mass spectrometry.

Quantitative results. The excretion patterns of 2-hydroxyoestrogen monomethyl ethers in the urine of different subjects with normal pregnancies are listed in Table 2. For comparison, the amounts of the total oestrogens and of 2-hydroxyoestrone are given, too.

In the course of the first analyses it was found that urinary 2-OHE$_2$ 3Me could not be detected by the procedure described here. In additional studies 10 μg of 2-OHE$_2$ 3Me were added to 7 urines. The amounts of 2-OHE$_2$ 3Me analyzed thereafter varied between 8.7 and 10 μg (mean 9.3 μg), indicating the detection limit of this procedure to be approx. 5 μg of 2-OHE$_2$ 3Me/24 h.

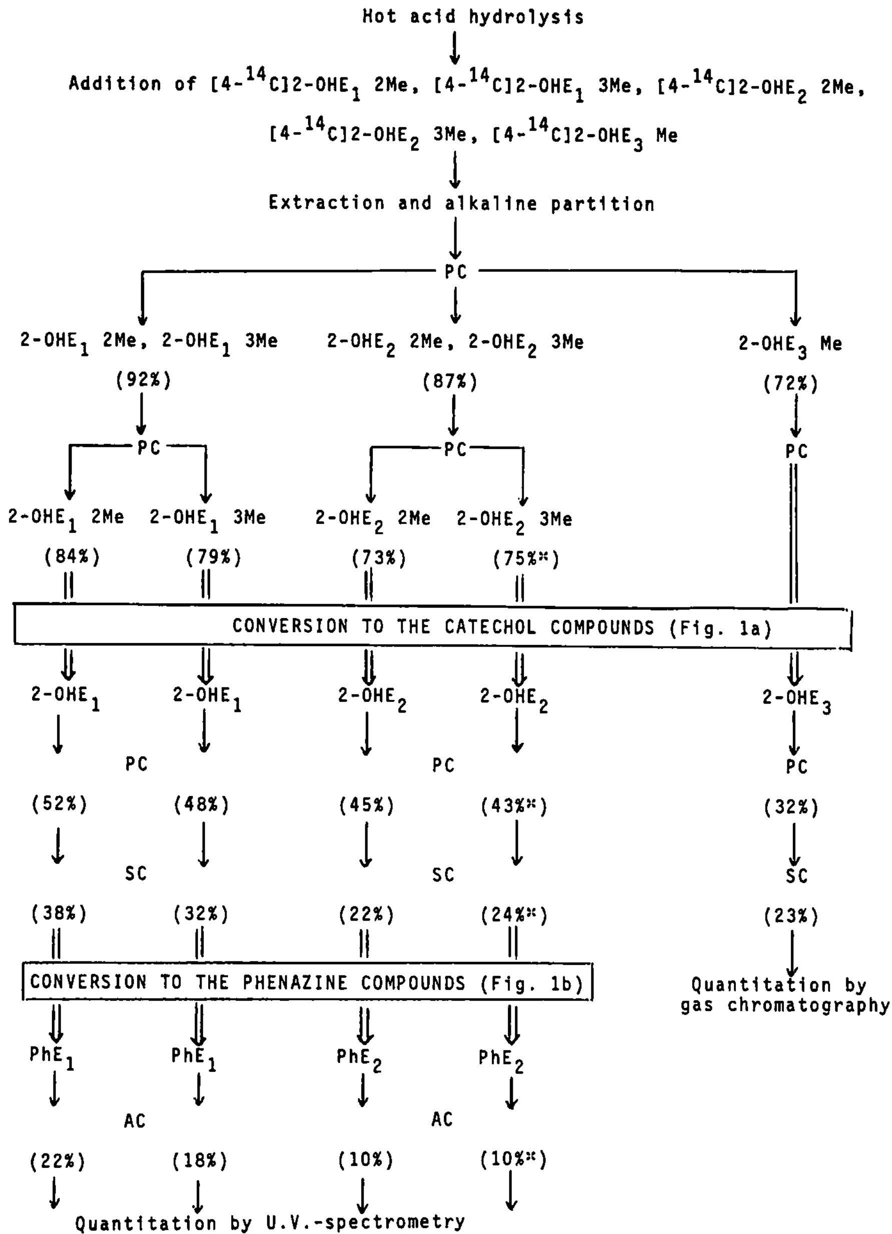

Fig. 2. Flow diagram for the quantitative determination of 2-hydroxyoestrogen monomethyl ethers in pregnancy urine. PC = paper chromatography; SC = silica gel column chromatography; AC = alumina column chromatography. The recoveries are given in parenthesis; they are related to the originally added radioactive internal standards and represent the mean of 5 determinations.

* These recoveries were obtained, when 10 μg of 2-OHE$_2$ 3Me were added to the urine after hydrolysis.

DISCUSSION

In this paper for the first time exact quantitative data are presented for the excretion of 2-hydroxyoestrogen monomethyl ethers in the urine of pregnant women. For the determination of these phenolic steroids a very laborious procedure was used employing highly specific microchemical reactions (formation of the corresponding catechol, Fig. 1a, and phenazine, Fig. 1b, compounds), besides several chromatographic purification steps. The paper chromatographic systems enabled a complete and reliable separation even of the isomeric monomethyl ethers of 2-hydroxyoestrone (2-OHE$_1$) and 2-hydroxyoestradiol (2-OHE$_2$). Thus, among the large amounts of "classical" oestrogens excreted in pregnancy, quantities of even less than 10 μg/24 h of the 2-hydroxyoestrogen monomethyl ethers could be measured with a high specificity as checked by various chromatographic, spectroscopic and microchemical methods. Radioactive internal standards guaranteed the exact calculation

Table 2. Excretion of 2-hydroxyoestrogen monomethyl ethers [μg/24 h], 2-OHE$_1$ [μg/24 h] and total oestrogens [mg/24 h] in the urine during normal pregnancies. 2-OHE$_1$ was determined by the method of Gelbke *et al.* [26] and the total oestrogens according to Kuss [27]. nd = not detcted

Subject	Week of pregnancy	2-OHE$_1$ 2Me	2-OHE$_1$ 3Me	2-OHE$_2$ 2Me	2-OHE$_2$ 3Me	2-OHE$_3$ Me	2-OHE$_1$	Total oestrogens
G	11	30	6	3	nd	18	200	1,1
E	15	49		nd	nd	10	280	1,5
Si	24	23	15	1	nd	37	250	8,7
	31	60	16	8,5	2	63	155	9,0
J	27	127	14	7,5	nd	41	530	9,4
Su	35	248	8	16	nd	52	550	10,4
	37	364	27	nd	nd	120	430	12,2
Sa	36	172	26	22	8	80	800	13,6
M	37	176	3	9	nd	133	400	27
	38	188	19	nd			610	38,1
R	39	144	16	16	nd	120	530	17,3
L	39	268	13	9,5	nd	236	590	16
	40	284	28	17	nd	222	790	22,7
A	39	146	45	25	nd		550	24,7
	40	200	46		nd	104	640	35,4
F	40	268	9	14	nd	190	610	17,3

of precedural losses. Since so far no information is available concerning the conjugates of 2-hydroxyoestrogen monomethyl ethers, the unconjugated radioactive steroids were used as internal standards. With regard to the fact that definite amounts of the unconjugated oestrogens are present only at the end of the hydrolytic procedure, the internal standards were added after hydrolysis of the urine. Obviously, any losses incurring during hydrolysis could not be assessed, but it was demonstrated that only small amounts of unconjugated 2-hydroxyoestrogen monomethyl ethers were decomposed in the course of hot acid hydrolysis.

A complete pattern of the excretion of the different 2-hydroxyoestrogen monomethyl ethers in pregnancy is presented here. So far, data have only been available for the urinary amounts of 2-methoxyoestrone (2-OHE$_1$ 2Me) [14–19], but the chromatographic systems described by these authors were unsuited for the separation of 2-OHE$_1$ 2Me and 2-hydroxyoestrone 3-methyl ether (2-OHE$_1$ 3Me), and no radioactive internal standards were used for correction of procedural losses. Furthermore, Breuer and Breuer [13] have estimated the excretion of the monomethyl ethers of 2-hydroxyoestriol (2-OHE$_3$ Me) in pregnancy to vary between 30 and 300 μg/24 h without reporting values for individual pregnancies. The urinary concentrations of 2-OHE$_1$ 2Me and 2-OHE$_3$ Me found in the present investigation are well within the order of magnitude given by the other authors [13–19]. However, until now no measurements have been carried out for the excretions of 2-OHE$_1$ 3Me, 2-methoxyoestradiol (2-OHE$_2$ 2Me) and 2-hydroxyoestradiol 3-methyl ether (2-OHE$_2$ 3Me).

The data on the urinary excretion of endogenous 2-substituted oestrogens directly confirmed several quantitative aspects of their biological formation, which so mostly had been deduced from metabolic experiments with radioactive precursors:

(1) Endogenous 2-hydroxyoestrogen 3-methyl ethers were of only minor importance as compared to the isomeric 2-methyl ethers. Thus, the excretion of 2-OHE$_1$ 3Me was 4–30 times smaller than that of 2-OHE$_1$ 2Me, and 2-OHE$_2$ 3Me was virtually not detectable in the pregnancy urines in contrast to 2-OHE$_2$ 2Me. A similar observation was reported by Knuppen *et al.* [34] after isolation of 2-OHE$_1$ 3Me from human pregnancy urine. Furthermore, several authors have demonstrated by *in vivo* experiments that radioactive monophenolic oestrogens are converted into 2-OHE$_1$ 3Me to an only negligible extent [35–38].

(2) Similar to the ratio of 2-OHE$_1$ and 2-OHE$_2$ [7], the monomethyl ethers of 2-OHE$_1$ by far exceeded those of 2-OHE$_2$ in pregnancy urines.

(3) After administration of radioactive oestradiol to men and non-pregnant women Fishman and coworkers have reported in a series of investigations that the urinary excretion of radioactive 2-OHE$_1$ was about 3 times higher than that of 2-OHE$_1$ 2Me (for example cf. [39,40]). Accordingly, a relation between 2:1 and 5:1 has now been found for the amounts of endogenous 2-OHE$_1$ and 2-OHE$_1$ 2Me in the urine of pregnant women. The excretions of 2-OHE$_2$ 2Me were definitely smaller than those of 2-OHE$_2$ [7].

(4) In contrast, the urinary concentrations of 2-hydroxyoestriol (2-OHE$_3$) [7,8] and 2-OHE$_3$ Me were found to be in the same order of magnitude. Thereby a statement of Ball *et al.* [41] was supported, which was deduced from incubation experiments with rat liver slices, that 2-OHE$_3$ Me not only stems from methylation of 2-OHE$_3$ but also from 16α-hydroxylation of 2-OHE$_1$ 2Me.

In connexion with investigations on 2-OHE_1 [6,26], 2-OHE_2 [7] and 2-OHE_3 [7,8] a complete pattern is now available for the urinary excretion of 2-substituted oestrogens in pregnancy. From the quantitative point of view among the group of these oestrogens 2-OHE_1 (100–2500 μg/24 h) is the most important one, followed by 2-OHE_1 2Me (20–360 μg/24 h), 2-OHE_3 35–250 μg/24 h), 2-OHE_3 Me (10–240 μg/24 h) and 2-OHE_2 (20–180 μg/24 h). On the other hand, only negligible amounts of 2-OHE_1 3Me (5–50 μg/24 h), 2-OHE_2 2Me (<5–25 μg/24 h) and 2-OHE_2 3Me (<5 μg/24 h) are found in pregnancy urines. Thus, for elucidation of the physiological meaning of 2-substituted oestrogens it seems justified to restrict further investigations to the quantitation of 2-OHE_1 and possibly of its monomethyl ether 2-OHE_1 2Me.

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