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METABOLISM OF 2-METHOXY- AND 4-METHOXYOESTRONE IN MAN *IN VIVO*

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SUMMARY

Radioactive 2-OHE_1 2-Me* and 4-OHE_1 4-Me was orally administered to one healthy male subject. The total radioactivity excreted within 5 days was 80 and 90%, respectively. About 0.5% and 2.7% of the urinary radioactivity consisted of free steroids. They were identified as 2-OHE_1 (0.2%) and 2-OHE_2 (0.1%) and 4-OHE_1 (0.8%) and 4-OHE_2 (0.7%), respectively. The remaining conjugate fraction consisted of steroid-sulphates and steroid-glucuronides. The steroid moieties, released by acid-hydrolysis, were identified as 2-OHE_1 (27.9%), 2-OHE_2 (3.4%), 2-OHE_1 2-Me (25.2%), 2-OHE_1 3-Me (1.2%), 2-OHE_2 2-Me (2.8%) and 2-OHE_3 2-Me (0.3%) and 4-OHE_1 (13.6%), 4-OHE_2 (4.2%), 4-OHE_1 4-Me (43.0%) and 4-OHE_2 4-Me (4.0%), respectively. Ring-A polyhydroxylated compounds were not detected in the urine.

INTRODUCTION

It is well established now that the 2-hydroxyoestrogens are the main products of the metabolism of oestradiol and oestrone in mammals [1–3]. Recently the isomeric catecholoestrogens, i.e. the 4-hydroxyoestrogens, were also detected as metabolites *in vivo* and *in vitro* [4, 5]. However, from the quantitative point of view, the 4-hydroxyoestrogens were found to be of minor importance [4, 5]. A great number of *in vitro* and *in vivo* experiments have demonstrated that the 2- as well as the 4-hydroxyoestrogens undergo further metabolism [2, 3, 6, 7]. One of the most important *in vitro* reactions is the attack of the catechol *O*-methyltransferase leading to the formation of mainly 2-monomethyl ethers in the case of 2-hydroxyoestrogens and almost exclusively 4-monomethyl ethers in the case of 4-hydroxyoestrogens. Nevertheless, 2-monomethyl ethers are *in vivo* of minor quantitative importance. This may be deduced from the data available on the urinary excretion of endogenous 2-methoxyoestrogens [8, 9], as well as from the data on the urinary excretion of radioactive 2-methoxyoestrogens biosynthesized from exogenous precursors [3, 10]. 4-Monomethyl ethers, to the best of our knowledge, have not been detected *in vivo*. In principal, two reasons could account for this: firstly, a rapid further metabolism of the 2- and especially the 4-monomethyl ethers and/or secondly, an extensive demethylation of the monomethyl ethers, i.e. a reversal of the last step of their biogenesis. Both possibilities have been shown to be valid *in vitro*. Stubenrauch *et al.*[11] reported on the formation of pyrogallol oestrogens and Hoppen *et al.*[12] demonstrated that monomethyl ethers may be demethylated.

EXPERIMENTAL

[4-^{14}C]2-OHE_1 2-Me and [4-^{14}C]4-OHE_1 4-Me were obtained by oxidation of oestrone (specific radioactivity 58 Ci/mol) with Fremy's salt [13] and subsequent enzymatic methylation of the reaction products with a catechol *O*-methyltransferase preparation from rat liver using S-adenosyl methionine [7]. Both the 2- and 4-monomethyl ethers were purified on formamide impregnated papers in the cyclohexane system.

Detailed information on non-radioactive steroids and chemicals, buffers and solutions is given l.c. [2].

The subject, a 45 year old man (79 kg), received orally 10 μCi (0.34 nmol) 2-OHE_1 2-Me and 10 μCi

* 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; 4-OHE_1 = 4-hydroxyoestrone = 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHE_2 = 4-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,4,17β-triol; 2-OHE_1 2-Me = 2-hydroxyoestrone 2-methyl ether = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 2-methyl ether; 2-OHE_1 3-Me = 2-hydroxyoestrone 3-methyl ether = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 3-methyl ether; 2-OHE_2 2-Me = 2-hydroxyoestradiol 2-methyl ether = 1,3,5(10)-oestratriene-2,3,17β-triol 2-methyl ether; 2-OHE_3 2-Me = 2-hydroxyoestriol 2-methyl ether = 1,3,5(10)-oestratriene-2,3,16α, 17β-tetrol 2-methyl ether; 4-OHE_1 4-Me = 4-hydroxyoestrone 4-methyl ether = 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one 4-methyl ether; 4-OHE_2 4-Me = 4-hydroxyoestradiol 4-methyl ether = 1,3,5(10)-oestratriene-3,4,17β-triol 4 methyl ether; 2,4-$(OH)_2E_1$ 2-Me = 2,4-dihydroxyoestrone 2-methyl ether = 2,3,4-trihydroxy-1,3,5(10)-oestratrien-17-one 2-methyl ether; 2,4-$(OH)_2E_1$ 3-Me = 2,4-dihydroxyoestrone 3-methyl ether = 2,3,4-trihydroxy-1,3,5(10)-oestratrien-17-one 3-methyl ether; 2,4-$(OH)_2E_1$ 4-Me = 2,4-dihydroxyoestrone 4-methyl ether = 2,3,4-trihydroxy-1,3,5(10)-oestratrien-17-one 4-methyl ether.

(0.17 nmol) 4-OHE_1 4-Me dissolved in 20 ml water/ethanol 3:1 2 months apart. Drugs were withheld from the subject for at least 10 days prior to and throughout the experiments. The gastro-intestinal as well as liver and kidney function were normal.

Radioactivity accumulating in the blood was measured at 15 min intervals for 90 min starting immediately after ingestion of the steroid. Urine was collected in portions. Aliquots were monitored for radioactivity

50% Of each portion of urine was extracted three times with 1.5 times the urine volume of ether–chloroform 3:1 (v/v). To the extracts (free steroid fraction) 30 μg amounts of 2-OHE_1, 2-OHE_2, 2,4-$(OH)_2E_1$ 2-Me and 2,4-$(OH)_2E_2$ 2-Me or 4-OHE_1, 4-OHE_2, 2,4-$(OH)_2E_1$ 4-Me and 2,4-$(OH)_2E_2$ 4-Me were added. Ascorbic acid solution was added and the solvent evaporated.

20% Of the aqueous phase (conjugate fraction) of each urinary portion was purified on XAD-2 columns and subjected to high-voltage paper electrophoresis (for details see [2]).

80% Of the aqueous phase (conjugate fraction) was treated with acid (for details cf. [2]) and then handled exactly as described for the free steroid fraction.

Chromatography of the residues was performed on formamide–ascorbic acid impregnated papers in the chlorobenzene or chlorobenzene–ethyl acetate 3:1 system (PC I), followed by chloroform–ethyl acetate 5:1 (PC II) or chloroform (PC III) or cyclohexane–ethyl acetate 3:1 (PC IV) or cyclohexane (PC V).

For the measurement of radioactivity in solutions an Intertechnique liquid scintillation spectrometer, Model SL 36, was used. For aqueous solutions Insta-Gel, and for organic solutions a scintillation fluid containing 4 g of 2.5-diphenyl oxazole and 0.3 g of 1.4-bis-2-(5-phenyloxazolyl) benzene in 1 l of toluene were used. Radioactive blood samples were burned (Packard Tricarb Sample Oxidizer) prior to liquid scintillation counting in a solution of 3 ml ethanolamine, 9 ml methanol, and 7 ml scintillation fluid used for organic solutions. Radioactivity on paper chromatograms was measured with a Berthold paper strip scanner equipped with a dual ratemeter.

RESULTS

On oral administration of 10 μCi (53 μg) 4-OHE_1 4-Me plasma radioactivity began to rise 5 min after ingestion and continued to rise for 90 min (Fig. 1).

After administration of 2-OHE_1 2-Me ("2-Me experiment") as well as 4-OHE_1 4-Me ("4-Me experiment") the total urinary excretion of radioactivity was about 80 and 90%, respectively (cf. Table 1 and 2). In the "4-Me experiment" more radioactivity was observed in the second urinary portion (6–12 h) which accounted for the 10% greater overall excretion. In the following portions (12–120 h) the rates of excretion of the radioactivity were very similar in both experiments

About 0.5% ("2-Me experiment") and 2.7% ("4-Me experiment"), (Table 3) of the urinary radioactivity could be extracted with ether–chloroform 3:1 (lipophilic = free steroid fraction). The free steroids were characterized by multiple paper chromatography (for details see Experimental) and identified by microchemical reactions and recrystallization to constant specific radioactivity. In the free steroid fraction, in both experiments, no monomethyl ethers were found. Only catecholoestrogens, in the "2-Me experiment" 2-OHE_1 (0.2%) and 2-OHE_2 (0.1%) and in the "4-Me experiment" 4-OHE_1 (0.8%) and 4-OHE_2 (0.7%), could be identified with certainty. They were found only from the twelfth hour on, i.e. in the urinary portions 3, 4 and 5 (6)

The radioactivity remaining in the aqueous phase was purified on XAD-2 columns and then separated by high-voltage paper electrophoresis into essentially two radioactive zones corresponding in mobility to steroid-sulphates and steroid-glucuronides. As may be seen from Table 4 in the "2-Me experiment" more glucuronides than sulphates were found whereas in the "4-Me experiment" the reverse was true. Steroid-thioethers were not detected.

Following acid-hydrolysis of the conjugates of the different urinary portions 80–90% of the steroid moieties were released (Table 3). Subsequently the steroids were characterized by multiple paper chromatography and identified as described for the free steroid fraction. After ingestion of 2-OHE_1 2-Me: 2-OHE_1, 2-OHE_2, 2-OHE_1 2-Me, 2-OHE_1 3-Me, 2-OHE_2 2-Me and 2-OHE_3 2-Me, and after ingestion of 4-OHE_1 4-Me: 4-OHE_1, 4-OHE_2, 4-OHE_1 4-Me and 4-OHE_2 4-Me were found as urinary metabolites. Ring-A polyhydroxylated compounds as 2,4$(OH)_2E_1$ 2-Me, 3-Me and 4-Me were not detected in the urines, at least not in amounts exceeding 0.5% of the ingested radioactivity.

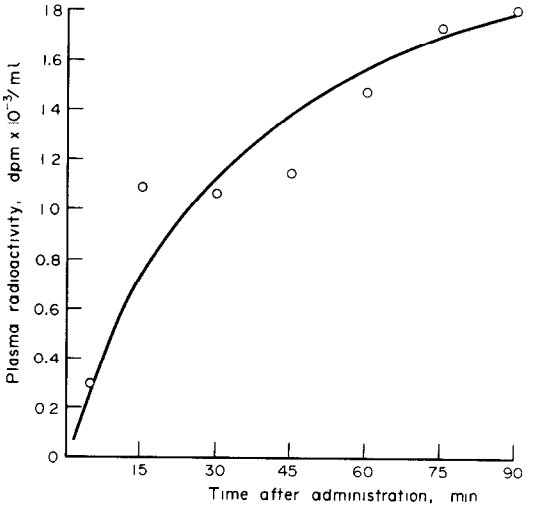

Fig. 1. Plasma radioactivity after oral administration of [4-^{14}C]4-OHE_1 4-Me.

Table 1. Excretion of radioactivity in the urine after oral administration of radioactive 2-OHE_1 2-Me to man; percentages given refer to ingested radioactivity

Urinary portion	Hours after adminstr.	Radioactivity excreted		
		in portion	in 24 h	accumulated
1	0–6	27.8	—	27.8
2	6–12	14.3	—	42.1
3	12–24	18.2	60.3	60 3
4	24–48	13.2	13.2	73.5
5	48–72	5.7	4.0	77.5
	72–96		1.7	79.2
6	96–120	0.5	0.5	79.7

Table 2. Excretion of radioactivity in the urine after oral administration of radioactive 4-OHE_1 4-Me to man; percentages given refer to ingested radioactivity

Urinary portion	Hours after adminstr.	Radioactivity excreted		
		in portion	in 24 h	accumulated
1	0–6	29.5	—	29.5
2	6–12	22.1	—	51.6
3	12–24	20.0	71.1	71.1
4	24–48	12.5	12.5	83 6
5	48–72	4.5	3.4	86.9
	72–96		1.1	88.1

Table 3. Distribution of radioactivity on the lipophilic and water-soluble fractions after oral administration of 4-OHE_1 4-Me to man; percentages given refer to ingested radioactivity

Urinary portion	Lipophilic fraction	Water-soluble fraction	
		total	acid-hydrolizable
1	0.95	28.5	27.1
2	0.32	21 8	19.8
3	0.65	19.4	15.5
4	0.52	12.0	10.1
5	0.28	4.2	3.1

Table 4. Distribution of radioactivity on the different conjugate fractions after administration of 2-OHE_1 2-Me and 4-OHE_1 4-Me, respectively, to man; percentages given refer to relative radioactivity within the respective urinary portion, percentages in brackets refer to ingested radioactivity

Substrate administered	Urinary portion	Conjugates			Total
		Sulphates	Glucuronides	Thioethers	
2-OHE_1 2-Me	0–9 h	12 (4.4)	75 (27)	<0 1	97 (35)
2-OHE_1 2-Me	9–48 h	20 (7.3)	65 (24)	<0.1	98 (36)
4-OHE_1 4-Me	0–6 h	82 (24)	15 (4.3)	<0.1	96 (27)
4-OHE_1 4-Me	6–12 h	79 (17)	17 (3 7)	<0.1	99 (22)
4-OHE_1 4-Me	12–24 h	35 (6.8)	60 (12)	<0 1	97 (19)

Table 5. Excretion of radioactive metabolites in the urine after oral administration of 2-OHE_1 2-Me to man; percentages given refer to ingested radioactivity; percentages in brackets refer to relative radioactivity within the respective urinary portions

Urinary portion	Metabolites found					
	2-OHE_1	2-OHE_2	2-OHE_1 2-Me	2-OHE_1 3-Me	2-OHE_2 2-Me	2-OHE_3 2-Me
0–9 h	6.5 (22)	0.89 (3.0)	18.6 (63)	0.30 (1.0)	2.1 (7.0)	0.12 (0.4)
9–48 h	17.9 (61)	2.15 (7.3)	5.9 (20)	0.79 (2.7)	0.7 (2.4)	0.15 (0.5)
48–120 h	3.5 (67)	0.40 (8.0)	0.7 (15)	0.13 (2.7)	<0.1 (<1)	<0.10 (<1)
0–48 h	24.4	3.04	24.5	1.09	2.8	0.27

Table 6. Excretion of radioactive metabolites in the urine after oral administration of 4-OHE_1 4-Me to man; percentages given refer to ingested radioactivity, percentages in brackets refer to relative radioactivity within the respective urinary portions

Urinary portion	Metabolites found			
	4-OHE_1	4-OHE_2	4-OHE_1 4-Me	4-OHE_2 4-Me
0–6 h	1.7 (5.7)	0.15 (0.5)	21.8 (74)	1.9 (6.4)
6–12 h	2.2 (10)	0.55 (2.5)	12.4 (56)	1.2 (5.5)
12–24 h	5.4 (27)	1.84 (9.2)	6.0 (30)	0.6 (3.0)
24–48 h	4.3 (34)	1.63 (13)	2.8 (22)	0.3 (2.0)
0–48 h	13.6	4.17	43.0	4.0

The quantitative distribution of the steroids released from the water-soluble fraction is given in the Tables 4 and 5. In both experiments the following trends are seen: 1. The contribution of the ingested monomethyl ethers to the excreted radioactivity steadily decreases and that of the corresponding catecholoestrogens steadily increases. 2. The ratio of C-17 oxo to C-17 hydroxy compounds remains approximately the same. This is true for the monomethyl ethers as well as for the corresponding catecholoestrogens.

The only isomeric monomethyl ether found was the 3-monomethyl ether of 2-OHE_1 after ingestion of 2-OHE_1 2-Me. Its relative contribution to the different urinary portions was a function of the amount of catecholoestrogen intermediary formed. The final (urinary portion 48–120 h) ratio of the 2- to the 3-monomethyl ether was approx. 6:1. In the "4-Me experiment" no 3-monomethyl ether of 4-OHE_1 was detected, this was not surprising as it is well known from *in vitro* experiments with the catechol *O*-methyltransferase from human liver [7] that the ratio of 4- to 3-methylation is about 95:1.

DISCUSSION

In the present paper it is unambiguously demonstrated that radioactive 2-methoxyoestrone and 4-methoxyoestrone when administered to man *in vivo* are extensively metabolized. The excretion of glucuronidated and sulphated 2-OHE_1, 2-OHE_2, 2-OHE_1 3-Me, 2-OHE_2 2-Me, 2-OHE_3 2-Me ("2-Me experiment") and 4-OHE_1, 4-OHE_2, 4-OHE_2 4-Me ("4-Me experiment") in the urine indicates that the catecholoestrogen monomethyl ethers undergo well known metabolic reactions, i.e. conjugation with sulphuric and glucuronic acid, demethylation, oxidoreduction at C-atom 17 and hydroxylation at C-atom 16. It should be mentioned that in spite of careful search, no pyrogalloloestrogens could be detected in either experiment, although 2,4-$(OH)_2E_1$ 4-Me has been identified as main metabolite of 4-OHE_1 4-Me in rat liver slices by Stubenrauch *et al.* [11].

Besides conjugation and 17β-oxidoreduction quantitatively the most significant metabolic reaction consisted in the *O*-demethylation of the orally administered monomethyl ethers, i.e. a reversal of the biogenetic pathway. This presumably indicates that the methylation of phenolic hydroxy groups leads to a deadlock, the only way out of which is demethylation.

These findings are in accordance with our *in vitro* experiments on the metabolism of catecholoestrogen monomethyl ethers in the liver of the rat [12] and recent *in vivo* studies on the metabolism of 2-methoxyoestrone injected i.m. to 2 female subjects* (Ball and Knuppen, to be published). In both cases extensive *O*-demethylation was demonstrated and high amounts of catecholoestrogens were isolated. In the present investigation the *O*-demethylation of 2-methoxyoestrone in man *in vivo* is established by the identification of radioactive 2-OHE_1 3-Me since this isomeric monomethyl ether can arise only by *O*-methyla-

* After i.m. injection of 2-OHE_1 2-Me (5 mg in 0.5 ml of propylene glycole) 14 and 15% of the injected dose was excreted as 2-OHE_1 within 5 days; after direct i.m. injection of 2-OHE_1 (20 and 12 mg in 0.5 ml of propylene glycole) 17 and 21% of the injected dose was excreted as 2-OHE_1.

tion of the catechol 2-OHE_1. To the contrary, Yoshizawa and Fishman[14] reported that after intravenous administration of radioactive 2-OHE_1 2-Me and its isomer, 2-OHE_1 3-Me, to human subjects neither the 2- nor the 3-monomethyl ether were *O*-demethylated to any significant extent.

It seems noteworthy that the metabolic behaviour of both the monomethyl ethers administered is somewhat different with regard to the rate of excretion and the rate of *O*-demethylation. In the experiment with 2-methoxyoestrone 79.2% of the orally administered radioactivity was excreted within 96 h, a result which is in good accordance to the 76.7% recovery of Yoshizawa and Fishman[14], although these authors administered the radioactivity intravenously. When 4-methoxyoestrone was given 88.1% of the dose was excreted during the same period. This difference mainly resulted from the larger content of radioactivity in the urine portion collected 6–12 h post administration (cf. Table 1 and 2). The difference in *O*-demethylation even exceeded the differences in the excretion rates. Within 48 h 28.5% of the radioactive 2-methoxyoestrone administered was excreted as 2-hydroxyoestrogens, 24.5% of the dose was recovered unchanged. In the experiment with 4-methoxyoestrone only about 18% of the dose was excreted as 4-hydroxyoestrogens. 43% Of the 4-OHE_1 4-Me was recovered unchanged, the bulk of it being excreted within the first 12 h. These results seem to be significant since the same subject was involved in both studies.

It is difficult to evaluate whether the more rapid excretion of radioactivity within the first hours in the "4-Me experiment" and the higher rate of demethylation in the "2-Me experiment" are due to a different pattern or a different rate of conjugation. Although in the "4-Me experiment" much more sulphated than glucuronidated material was excreted and the reverse was true in the "2-Me experiment" (Table 4) it seems more likely that a different rate of conjugation is responsible for both observations. Although the time course of the ratio of free and conjugated metabolites in blood plasma was not measured it may be concluded that the 4-monomethyl ether of 4-OHE_1 is a better substrate for glucuronyl and especially sulpho-transferases than the 2-monomethyl ether of 2-OHE_1. This latter finding is in good accordance with our *in vitro* experiments demonstrating that 2-hydroxylated oestrogens are predominantly conjugated at C-atom 2. Only when conjugation of C-atom 2 is hindered by other substituents—as e.g. a methyl group—is conjugation at C-atom 3 possible but then only to a limited extent[2, 3].

The most important result presented in this paper is the fact that 2- and 4-methoxyoestrogens, orally administered to human subjects, are converted slowly but to a high extent to their corresponding catecholoestrogens. As these catecholoestrogens are excreted continuously in the urine the concentration of the catecholoestrogens in the body is maintained for a long time at a relatively high level. This will probably facilitate forthcoming investigations on the effect of catecholoestrogens in the human. I.m. or i.v. injections of laboriously sterilized solutions of the highly unstable catecholoestrogens may be replaced by the much more convenient oral administration using the much more stable monomethyl ethers.

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