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IN VITRO METABOLISM OF CATECHOL ESTROGENS BY HUMAN FECAL MICROFLORA

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Summary—Intestinal bacterial metabolism of the catechol estrogens, 2-hydroxyestrone (2-OHE1), 2-methoxyestrone (2-MeOE1) and 2-hydroxyestradiol (2-OHE2), was studied by incubation of the steroid with mixed fecal flora. 3-Methoxyestrone (3-MeOE1) was included in order to evaluate whether demethylation occurs also with regard to noncatecholic estrogens. The purification of the samples and separation into fractions was carried out by ion exchange chromatography and the metabolites formed were identified by combined gas chromatography–mass spectrometry (GC–MS). Mixed human fecal flora was able to interconvert 2-OHE1 and 2-OHE2 both in aerobic and anaerobic conditions. Demethylation occurred both for 2-MeOE1 and 3-MeOE1.

INTRODUCTION

It has been repeatedly demonstrated that the intestinal tract plays a significant physiological role in overall steroid metabolism, particularly in estrogen metabolism [1]. During their enterohepatic circulation modifications of the estrogens occur which affect their biological activity and degree of reabsorption from the intestine. Intestinal bacterial interconversion of estrone (E1) and estradiol (E2), reduction of 16α-hydroxyestrone to estriol, of 16-oxoestradiol to 16-epiestriol and of 15α-hydroxyestrone to 15α-hydroxyestradiol was observed previously [2, 3]. Recently bacterial demethylation was demonstrated for 2-MeOE1 with rat cecal content [4]. This would suggest that biliary 2-MeOE1 [5] may be demethylated during the enterohepatic circulation resulting in 2-OHE1, a biologically more active estrogen, which in turn may be reduced to 2-OHE2 [2, 6]. Both these estrogens may then be reabsorbed and again enter the circulation. In order to throw some light on intestinal bacterial metabolism of these compounds in man the effect of human fecal microflora on the *in vitro* metabolism of three catechol estrogens and 3-MeOE1 was investigated.

EXPERIMENTAL

Steroids

2-Hydroxyestrone and 2-hydroxyestradiol were from Steraloids (Wilton, N.H., U.S.A.) and 2-methoxyestrone from Ikapharm (Rhamat-Gan, Israel]. 3-Methoxyestrone was kindly donated by Organon (Oss, The Netherlands), 6,7[3H]-2-Hydroxyestrone (50 Ci/mmol) was from New England Nuclear, U.K. A mixture of 6,7[3H]-2-methoxyestrone and 6,7[3H]-2-hydroxy-3-methoxyestrone was prepared from 6,7[3H]-2-OHE1 as described earlier by Fotsis *et al.* [7]. The methylation reaction results in a mixture of tritiated 2-methoxy-estrone and 2-hydroxy-3-methoxyestrone [8]. These radioactive methoxy-catechol estrogens are well suited for use in the experiments showing demethylation reactions. Stigmasterol, used as an internal standard in GC, was from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Ion exchangers

All three ion exchangers (DEAE-Sephadex A-25 Cl^--form, QAE-Sephadex A-25 Cl^--form, SP-Sephadex C-25 Na^+ form) were from Pharmacia (Uppsala, Sweden). Before being converted to the form used for chromatography the ion-exchangers were washed successively with 20%, 50%, pure and 70% methanol in water (v/v) and stored at 4°C [9].

Conversion of SP-Sephadex-Na^+ to the H^+ form

The washed cation exchanger was slowly eluted in a sintered glass funnel with 10 bed vol of 0.3 mol/l HCL in 70% methanol and 10 bed vol. 70% methanol and stored in 70% methanol at 4°C [10].

Conversion of DEAE-Sephadex-Cl^- to the acetate form

The washed ion exchanger was slowly washed with 10 bed vol. of 0.1 mol/l NaOH in 70% methanol, 10 bed vol. of 70% methanol, 10 bed vol. of 0.5 mol/l acetic acid and 10 bed vol. 70% methanol. The gel was stored in 70% methanol at 4°C [9].

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Conversion of QAE-Sephadex-Cl⁻ to borate or bicarbonate form

Borate form. The exchanger was washed slowy with 10 bed vol. of 0.1 mol/l NaOH in 70% methanol, 10 bed vol. of 70% methanol, 10 bed vol. of 0.5 mol/l H_3BO_3 in 70% methanol and with 10 bed vol. of 70% methanol. It was stored in 70% methanol at 4°C [9].

Bicarbonate form. QAE-Sephadex-Cl⁻ was converted to its acetate form as described for DEAE-Sephadex. The acetate form was washed with 10 bed vol. of 0.2 mol/l $NaHCO_3$ in 40% methanol, 10 bed vol. of 40% methanol and 10 bed vol. of methanol. The bicarbonate form should be used immediately [9].

Preparation of glassware

All glassware including Pasteur pipettes was silanized with 1% dimethylchlorsilane (Fluka AG, Bucks, Switzerland) in toluene, because of known creepage of estrogens and subsequent decomposition [11].

Bacteria and incubation conditions

Microbial cultures were maintained in metal capped test tubes containing 10 ml of broth (Lab. Lemco Broth. Oxoid, England). The steroid (60–100 μg) was added in a volume of 100–500 μl methanol. In addition, ascorbic acid was added to give a final concentration of 1 mmol/l. In the incubations with 2-OHE1 and 2-MeOE1 the radioactive standards described above were also added.

After careful mixing, the tubes were inoculated with a loopful of fresh human feces obtained from healthy omnivorous adults. The cultures were incubated both aerobically and anaerobically at 37°C for 24 h. In addition, 2-MeOE1 was incubated for 72 h, because the structure is more stable than that of 2-OHE1.

Control experiments

All steroids were also incubated without bacteria using the same conditions. Broths with feces but without steroids were also incubated. All these fractions were handled in the same way as the steroid test samples in order to record the background in the GC–MS analysis.

Purification of the samples before GC–MS

After centrifugation of the incubated samples, the supernatant was sterilized by filtration through a Millipore filter (0.22 μ). The supernatant was extracted 3 times with an equal volume of ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness under nitrogen. The microbial mass of the fecal cultures was extracted with 10 ml of methanol mixing 1 min with a Vortex mixer. The extract was added to the dry residue remaining after extraction of the supernatant and evaporated to dryness. This extract was dissolved in 1 ml 70% methanol and used for purification by ion-exchange chromatography.

Removal of organic acids stronger than estrogens and some basic compounds

The DEAE-Sephadex A-25 anion exchanger in the acetate form and the SP-Sephadex C-25 cation exchanger in the H^+ form were prepared in the same Pasteur pipette column. On the bottom (with a cotton plug) a 2.5 cm column of the DEAE-Sephadex suspended in 70% methanol was packed followed by a similar column of the cation exchanger also suspended in 70% methanol, giving a final column dimension of 0.55 × 5 cm. One half of each sample, prepared as described above, was applied in 0.5 ml 70% methanol on the ion-exchange column. The column was then eluted with 4.5 ml 70% methanol. The whole fraction was collected in the same tube and evaporated to dryness.

Separation of catechol estrogens from other steroids

QAE-Sephadex in the borate form suspended in 70% methanol was packed in a Pasteur pipette column to a height of 2.5 cm and washed with methanol [9]. The evaporated residue from the previous column was dissolved in 0.5 ml of methanol and applied to the column. This was eluted with 2.5 ml of methanol and 2 ml of 0.1 mol/l acetic acid in methanol. The combined fractions contains all other steroids except those with vicinal *cis*-hydroxyls [10]. After that cathecol estrogens were eluted with 3 ml of 0.1 mol/l acetic acid in methanol, which was evaporated to dryness. After derivatization (trimethylsilyl ethers = TMS) this fraction was ready for GC–MS analysis.

Removal of neutral steroids from estrogens

First fraction from the QAE-Sephadex A25 was evaporated to dryness, dissolved in 0.5 ml of methanol and applied to a Pasteur pipette containing DEAE-Sephadex in the bicarbonate form (0.55 × 5 cm). Neutral steroids were eluted in 0.5 ml + 4.5 ml methanol and estrogens with 6 ml 80% methanol. All fractions were evaporated to dryness and derivatized.

Measurement of radioactivity

Tritium activity of each fraction was measured with an automatic liquid scintillation counter (Rackbeta 1215 > LKB-Wallac, Turku, Finland).

Formation of trimethylsilyl ethers

The TMS ether derivatives were prepared as described previously [9].

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

After addition of the stigmasterol internal standard all fractions were first analyzed by capillary GC with

Table 1. Summary of semiquantitative recovery results obtained with radioactive estrogen standards in the different ion exchange chromatography steps used in the purification of steroids incubated with fecal microflora

Steroid	Bacteria	Incubation time		DEAE-Sephadex-Ac⁻ SP-Sephadex-H⁺	QUA-Sephadex borate⁻ Total	Part of Fr. 1	Part of Fr. 2	QUA-Sephadex-bicarbonate⁻ Total	Part of Fr. 1	Part of Fr. 2
2-MeOE1[a]	—	Aerobic	72 h	100	Over 100	99	1	87	4	96
2-MeOE1[a]	—	Anaerobic	72 h	89	Over 100	92	8	89	6	94
2-MeOE1[a]	Feces	Aerobic	72 h	83	Over 100	31	69	91	8	92
2-MeOE1[a]	Feces	Anaerobic	72 h	87	Over 100	69	31	89	6	94
2-MeOE1[a]	Feces	Aerobic	24 h	89	Over 100	83	17	36	5	95
2-MeOE1[a]	Feces	Anaerobic	24 h	85	Over 100	77	23	47	6	94
2-OHE1	—	Aerobic	24 h	96	95	1	99	NR[b]		
2-OHE1	—	Anaerobic	24 h	90	Over 100	1	99	NR		
2-OHE1	Feces	Aerobic	24 h	81	65	1	99	NR		
2-OHE1	Feces	Anaerobic	24 h	77	Over 100	0	100	NR		

[a]Mixture of 2-MeOE1 and 2-hydroxy-3-methoxy estrone; [b]No radioactivity.

a Carlo Erba model 2300 AC instrument equipped with a flame ionization detector and a 25 m wall-coated open tubular glass capillary column containing OV-210 or SE-30. After comparisons with the blank samples, fractions with metabolites were analyzed by GC–MC using the Hewlett-Packard 5995 GC–MS instrument equipped with a SE-30 capillary column. Identification of the metabolites formed was based on the relative retention times both in GC and GC–MS and on the identity of the spectra obtained with those of the authentic reference standards.

RESULTS AND DISCUSSION

Analytical method

The purification and fractionation of steroids in the incubation media were carried out by ion exchange chromatography. It proved to be a very useful method in this work, because steroids are divided into fractions according to their A-ring structure [12]. In addition, the chromatographic behaviour of the steroids gave further support to structural information obtained by GC–MS. The first ion exchange step with anion and cation resins in the same column removed organic acids stronger than estrogens and some basic compounds. Estrogens and also neutral steroids will pass straight through the column. The recoveries in this purification step were reasonable for these labile steroids (77–100%) (Table 1). No antioxidants were added to the eluants. The QAE-Sephadex column in borate form will retain steroids with vicinal *cis*-hydroxyls and protect them from oxidative decomposition [10]. Recoveries calculated using the radioactive standards added were almost quantitative (95–100%), with one exception (see Table 1). The recoveries in experiments with the bicarbonate column varied from 36 to 91%. On two occasions the recoveries were below 80% (Table 1). The reason for this is unknown. The fractions obtained were clean enough for the GC–MS analysis. The combined anion–cation exchanger worked especially well. It is a suitable purification step for many types of steroid analysis because only bases and strong acids are retained in the resin.

Fecal bacterial metabolism of catechol estrogens

The reactions seen were demethylations, reductions and oxidations. 2-OHE1 was reduced to 2-OHE2, and 2-OHE2 was oxidized to 2-OHE1. Both 3-MeOE1 and 2-MeOE1 were demethylated to E1 and 2-OHE1, and then reduced to E2 and 2-OHE2, respectively. All conversions observed are summarized in Table 2. The recovery results agreed well with the qualitative information obtained in the GC–MS analyses. Of 2-MeOE1 17 and 23% was converted, in aerobic and anaerobic conditions respectively, to estrogens with *cis*-hydroxyl groups (Table 1). GC–MS analysis showed that the major metabolite was 2-OHE1 and the minor one was 2-OHE2. The longer incubation time gave more effective conversion (69 and 31%, in aerobic and anaerobic conditions respectively). When 2-OHE1 was incubated with fecal bacteria all radioactivity was found in the catechol estrogen fraction. Also the GC–MS analysis showed that the only metabolite was 2-OHE2.

After incubation with 2-OHE2 the only metabolite found both in aerobic and anaerobic conditions was 2-OHE1. Incubation of 3-MeOE1 demonstrated that demethylation took place also for this compound followed by reduction to E2.

Earlier studies have shown that human intestinal microflora can oxidize and reduce estrogens [2, 3]. In addition, Axelson *et al.* showed that isolated cecum from rat is capable of demethylation and reduction. They found a reduction of 2-MeE1 to 2-MeOE2. In the present study we did not find this metabolite, at least not in detectable amounts. The only estrogen

Table 2. Metabolites formed upon incubation of catechol estrogens and 3-MeOE1 with human fecal microflora in aerobic and anaerobic conditions

Steroid substrate	Incubation time	Metabolite
2-Hydroxyestrone	24 h	2-Hydroxyestradiol
2-Hydroxyestradiol	24 h	2-Hydroxyestrone
2-Methoxyestrone	24 h	2-Hydroxyestrone 2-Hydroxyestradiol
2-Methoxyestrone	72 h	2-Hydroxyestrone 2-Hydroxyestradiol
3-Methoxyestrone	72 h	Estrone Estradiol

in the noncatecholic fraction proved to be unmetabolized 2-MeOE1. It has been shown earlier that rat and human liver is capable to demethylate 3-MeOE2 and 2-MeOE2 [13, 14]. The work of Axelson *et al.* [4] with rat cecal content and the present one support the view that intestinal bacteria by demethylation are able to transform the less active ring-A-methylated estrogens to biologically more active compounds. Thus, intestinal bacterial metabolism leads also in the case of catechol estrogens to the formation of biologically more active estrogens as shown for other estrogens [6]. These estrogens may subsequently be absorbed and may exert biological effects.

Recently nutritional studies have shown that diet components can alter the metabolism of estrogens, including catechol estrogens. High fat diet increase oxidation at C-16 and decrease at C-2 for estradiol-17β [15] and low fat diet seemed to have the opposite effect [16]. A protein-rich diet increases 2-hydroxylation of estradiol-17β but does not affect 16-hydroxylation [17]. Adlercreutz *et al.* [18, 19] found that in young women a high carbohydrate/protein ratio in the diet is associated with low urinary excretion of 2-hydroxylated estrogens and 2-OHE1/4-OHE1 ratio. In addition, it has been shown that diet alters fecal enzyme activity [20] and thus may affect these metabolic reactions quantitively.

However the site of action of the various macronutritiens on catechol estrogen metabolism is unknown, but it is likely that this occurs both via an influence on intestinal and liver metabolism of these steroids. Metabolic conversions obtained with mixed fecal flora may not be representative for intestinal metabolism [21]. However, this and other [3] studies with mixed fecal flora and the experiments with rat cecal contents [4] have all produced similar results, suggesting that these experiments reflect true intestinal metabolic events.

It is concluded that intestinal bacterial metabolism of catechol estrogens may play a significant role in overall metabolism of catechol estrogens. The biological role of the metabolic events described remains unknown.

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