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GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC STUDIES ON OESTROGENS IN BILE—2. MEN AND NON-PREGNANT WOMEN

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SUMMARY

Oestrogens in bile, collected by T-tube drainage of the main bile duct, from fertile and post-menopausal non-pregnant women and men were studied, after conjugate hydrolysis, by gas chromatography-mass spectrometry. Oestriol, oestradiol-17β, 16-epioestriol, 17-epioestriol, 16α-hydroxyoestrone and 16-oxo-oestradiol-17β were identified in bile from fertile subjects. In addition, oestrone, 2-methoxyoestrone, oestradiol-17α, 11-dehydro-oestradiol-17α, 16β-hydroxyoestrone, 15α-hydroxyoestrone and 15α-hydroxy-oestradiol-17β were detected in these samples and mass-fragmentographic evidence for their presence obtained. Quantitative analysis of these 13 oestrogens by mass fragmentography showed the excretion of 4.1–21.3, 3.7–13.0 and 6.4–49.4 μg/24 h in bile from men (n = 8), post-menopausal women (n = 4) and fertile women (n = 2), respectively. The wide range of oestrogen concentration in the three groups is due, in particular, to great individual variation in the concentrations of the 3 principal biliary oestrogens: oestriol, 16α-hydroxyoestrone and oestrone. The quantitative oestrogen pattern in non-pregnancy bile differs significantly from that reported for urine showing greater relative amounts of oestriol and 16α-hydroxyoestrone and smaller concentrations of oestrone, oestradiol and 2-methoxyoestrone.

INTRODUCTION

The studies of Gsell-Busse[1] established that compounds exhibiting oestrogenic activity are excreted in human bile, while Cantarow *et al.*[2], using a bioassay, demonstrated the presence of relatively large amounts of oestrogen in human late pregnancy bile. Later, Sandberg and Slaunwhite[3] and Twombly and Levitz[4], by following the fate of injected radioactive oestrogens in human subjects showed that radioactive metabolites were secreted in the bile and reabsorbed from the intestine, suggesting an enterohepatic recirculation.

Endogenous concentrations of the three classical oestrogens oestrone (OE_1), oestradiol (OE_2) and oestriol (OE_3) in non-pregnancy bile were first determined, using a spectrophotometric method [5]. In the same study evidence for the identity of these oestrogens was obtained by various color reactions, thin-layer chromatography and counter-current distribution. Later OE_1, OE_2, OE_3, 2-methoxy-oestrone (2-MeOE_1), 16α-hydroxyoestrone (16α-$OHOE_1$), 16-oxo-oestradiol-17β (16-oxoOE_2) and 16-epioestriol (16-epiOE_3) were identified by gas chromatography–mass spectrometry in bile from an adult male with T-tube drainage of the main bile duct who had received a 25 mg intramuscular injection of OE_2 [6]. Further development of this method [7] led to the identification and quantitation of 12 oestrogens in human pregnancy bile [8]. A mass fragmentographic method based on the original gas chromatographic procedure but with a sensitivity up to $\times 10^3$ greater has allowed measurement of 11 oestrogens in non-pregnancy urine and bile [9, 10]. In this communication the oestrogen levels in bile from a group of adult men, post-menopausal and fertile non-pregnant women are reported in addition to some further mass spectrometric data on the chemical nature of the various oestrogens found in non-pregnancy bile.

EXPERIMENTAL

Patients

Bile was collected by T-tube drainage of the main bile duct from 8 adult men (29–70 years), 4 post-menopausal women (61–72 years) and 4 fertile non-pregnant women (34–37 years). All the patients had been operated on for the removal of gallstones (cholelithiasis and/or choledocholithiasis); none of them had jaundice due to biliary obstruction. They were not treated either pre- or postoperatively with antibiotics and in none was liver dysfunction apparent. Bile collection was commenced no sooner than three days postoperatively and the subjects were maintained on a diet low in fat. Bile was collected into plastic containers over 4–12 h intervals and was immediately frozen and stored at −20°C until processed.

Steroids

OE_1 (oestrone): 3-hydroxy-1,3,5(10)-oestratrien-17-one; OE_2 (oestradiol): 1,3,5(10)-oestratriene-3,17β-

diol; OE_3 (oestriol): 1,3,5(10)-oestratriene-3,16α,17β-triol; OE_2-17α (oestradiol-17α): 1,3,5(10)-oestratriene-3,17α-diol; 11-Dehydro-OE_2-17α (11-dehydro-oestradiol-17α): 1,3,5(10),11-oestratetraene-3,17α-diol; 2-$MeOE_1$ (2-methoxyoestrone): 3-hydroxy-1,3,5(10)-oestratrien-17-one-2-methyl ether; 16-epiOE_3 (16-epioestriol): 1,3,5(10)-oestratriene-3,16β,17β-triol; 17-epiOE_3 (17-epioestriol): 1,3,5(10)-oestratriene-3,16α,17α-triol; 16,17-epiOE_3 (16,17-epioestriol): 1,3,5(10)-oestratriene-3,16β,17α-triol; 16α-$OHOE_1$ (16α-hydroxyoestrone): 3,16α-dihydroxy-1,3,5(10)-oestratrien-17-one; 16β-$OHOE_1$ (16β-hydroxyoestrone): 3,16β - dihydroxy - 1,3,5(10) - oestratrien - 17 - one; 16-oxoOE_2 (16-oxo-oestradiol): 3,17β-dihydroxy-1,3,5(10)-oestratrien-16-one; 15α-$OHOE_1$ (15α-hydroxyoestrone): 3,15α-dihydroxy-1,3,5(10)-oestratrien-17-one; 15α$OHOE_2$ (15α-hydroxyoestradiol): 1,3,5(10)-oestratriene-3,15α,17β-triol; OE_1-SO_4 (oestrone sulphate): 3-hydroxy-1,3,5(10)-oestratrien-17-one-3-yl sulphate.

Purification, separation and quantitation of bile oestrogens by gas chromatography

This was done according to the method of Adlercreutz and Luukkainen[6] with some modifications [7]. Details of the quantitation of these oestrogens in pregnancy bile by gas chromatography were recently reported [8]. All assays were carried out in duplicate and losses incurred during purification were corrected for as described [8].

Gas chromatography–mass spectrometry

Mass spectra were recorded on a LKB 9000 gas chromatograph–mass spectrometer (GC-MS) combination instrument (LKB-Produkter AB, Bromma, Sweden) linked to a Hewlett-Packard minicomputer (HP 2100A); this system was described in detail [10]. Operating conditions were as published [9] except in most instances 3% OV-210 was used instead of 1% QF-1 as column stationary phase.

Quantitative mass fragmentography (MF) was carried out on the LKB 9000, on a Varian MAT CH7 (GC–MS) equipped with a peak-matching system allowing the measurement of two ions or on a Varian MAT 112 (GC–MS) with a multiple ion detection (MID) system. The analyses on the Varian instruments were carried out in the application laboratory of Varian MAT in Bremen, Germany. Some measurements were also made on an LKB 2091 GC–MS fitted with an MID system at the LKB application laboratory in Stockholm, Sweden. Details of the MF procedure have been published [9, 10]. The results were calculated as described previously [9] but using peak height instead of peak area. All assays were carried out in duplicate and losses incurred during extraction and purification were corrected for as described [9]. The levels of 11-dehydro-oestradiol-17α were calculated using oestradiol-17β as standard despite its different fragmentation pattern, as no standard is available.

Criteria used for establishing the identity of the unknown steroids with reference standards

Identification, based on the comparison of the gas chromatographic properties and mass spectra of the biliary oestrogens with those of reference standards was discussed in detail in Part I of this study [8]. However, some of the oestrogen fractions, despite the use of 200–400 ml sample volumes, contained such small amounts of one or several of the steroids that mass spectra could not be obtained. In these cases gas chromatographic and mass fragmentographic evidence for identity was obtained by monitoring the molecular ion and other prominent or characteristic ions of the oestrogens during GC–MS. If their behaviour was identical with those of the reference standards the steroids were regarded as identified with reservation.

RESULTS

Gas chromatographic and mass spectrometric studies were carried out on the oestrogens isolated from bile obtained from two fertile women and led to the identification of OE_3, OE_2-17β, 16-epiOE_3, 17-epiOE_3, 16α-$OHOE_1$ and 16-oxoOE_2 and the detection of 16β-$OHOE_1$ and 15α-$OHOE_1$ (Table 1). In addition mass fragmentographic evidence was obtained for the presence of OE_1, 2-$MeOE_1$, OE_2-17α, 11-Dehydro-OE_2-17α and 15α-$OHOE_2$ in non-pregnancy bile and bile from male subjects (Table 1). The concentration of oestrone would have permitted a mass spectrum of the peak to be taken but we omitted to take the spectrum, in error; there is, however, little doubt of its presence on the basis of the MF evidence and previous findings [5]. Attempts to identify oestrogens other than those listed in Table 1 were not made.

The oestrogens in two samples of bile obtained from fertile non-pregnant women were quantitated by gas chromatography (Table 2). Four consecutive 6-h collections of bile from a third fertile woman were analyzed by mass fragmentography, three on a Varian MAT 112 instrument and one on the LKB 2091. In addition, two 12-h bile samples collected on consecutive days from a male patient were quantitated on the LKB 9000. The variation in biliary oestrogen excretion in these two subjects is illustrated in Fig. 1.

A series of nine bile samples from 8 adult men, four bile samples from post-menopausal women and a further sample from another fertile woman were also analyzed by the MF method. The results of these determinations are presented in Table 3. In Table 4, 24-h biliary oestrogen excretion values calculated from the data presented in Table 3 are given. The mean and range of biliary excretion of the three classical oestrogens in this patient series, as determined by MF and GC, is compared in Table 5 to that determined for a similar patient series in 1962

Table 1. Oestrogens identified and detected in bile from fertile non-pregnant woman by gas chromatography–mass spectrometry: the nature of the oestrogen derivative studied and references to the appropriate published mass spectra are also included

Oestrogen	Derivative	Comment	Published spectra (reference)
OE_3	TMS	identified	[31]
OE_1	TMS	mass fragmentographic data only	[6]
2-MeOE_1	TMS	mass fragmentographic data only	[6]
OE_2	3-Methylated-TMS	identified	[32]
OE_2-17α	3-Methylated-TMS	mass fragmentographic data only	[29]
11-Dehydro-OE_2-17α	3-Methylated-TMS	mass fragmentographic data only	[30]
16-epiOE_3	16,17-Acetonide, TMS	identified	[33]
17-epiOE_3	16,17-Acetonide, TMS	identified	[33]
16α-$OHOE_1$	TMS	identified	[6]
16β-$OHOE_1$	TMS	detected	
16-oxoOE_2	TMS	identified	[6]
15α-$OHOE_1$	TMS	detected, but spectrum contaminated	[29]
15α-$OHOE_2$	TMS	mass fragmentographic data only	[11]

[5] using a colorimetric technique. Three oestrogens: 15α-$OHOE_2$, OE_2-17α and 11-Dehydro-OE_2-17α were detected and quantitated in a limited number of bile samples, only. Their concentration and approximate 24-h excretion are documented in Table 6.

DISCUSSION

In Part I of this study [8] 11 of these oestrogens (except 15α-$OHOE_2$) and 15α-$OHOE_3$ were identified and quantitated in human pregnancy bile; their biosynthesis and occurrence in human pregnancy plasma and urine were reviewed. The identification and quantitation of these oestrogens in human pregnancy faeces has also been reported [11]. In the present study OE_3, OE_1-17β, 16-epiOE_3, 17-epiOE_3, 16α-$OHOE_1$ and 16-oxoOE_2 were identified and less complete evidence obtained for the presence of OE_1, 2-MeOE_1, OE_2-17α, 11-Dehydro-OE_2 and 15α-$OHOE_2$ (Table 1). The failure to identify OE_1 arose because of an oversight—no spectra of this fraction were taken. The three classical oestrogens have previously been identified in non-pregnancy and male bile by a combination of counter-current distribution, thin-layer chromatography, colour reactions and UV-spectroscopy [5]. Some evidence for the presence of 16-epiOE_3 was also obtained. Of the present group of oestrogens the formation of 16α-$OHOE_1$, 2-MeOE_1, 15α-$OHOE_2$, OE_3 and 16-epiOE_3 from OE_1 or OE_2 [12, 13], 16β-$OHOE_1$ and 17-epiOE_3 from 16α-$OHOE_1$ [14, 15], 16-oxoOE_2 from 16-oxoOE_1 [16, 17] and 15α-$OHOE_1$ and 15α-$OHOE_2$ from 15-oxoOE_2 [18] by human liver preparations, *in vitro*, have been reported.

Table 2. Oestrogen concentrations in bile from two fertile non-pregnant women as determined by gas chromatography

Oestrogen	Subject A.M. (37 years)	Subject A.S. (43 years)
Oestriol	19.8	14.2
Oestrone	5.6	2.0
2-Methoxyoestrone	0.4	2.6
Oestradiol-17β	1.3	1.6
16-Epioestriol*	5.6	5.7
17-Epioestriol*	1.0	—
16α-Hydroxyoestrone	25.0	40.6
16β-Hydroxyoestrone	9.7	10.7
16-Oxo-oestradiol-17β	10.0	8.6
15α-Hydroxyoestrone*	5.0	3.8

* Peaks probably impure with resultant overestimation. Values are expressed as μg/l.

In the present series of nine male (eight subjects), four post-menopausal female and five fertile female (two subjects) bile samples, analyzed by the mass fragmentographic procedure, large variation in total oestrogen excretion was seen (Tables 3 and 5). The wide range of OE_3 excretion is the major quantitative contributor to this variation (Tables 3 and 4). Similar variation was also apparent for some of the other oestrogens. The classical oestrogens were previously measured in bile from similar groups of subjects [5] and wide variation in the OE_3 levels in the post-menopausal series, for instance, noted (Table 5). The source of these large amounts of OE_3 in some patients remains unexplained and can lead to the remarkable finding of higher total biliary oestrogens in some males as compared to some fertile females (Tables 2, 3 and 4). In a study on the influence of age on the urinary excretion of the classical oestrogens in post-menopausal women with atrophic endometrium and normal liver function [19] it was found that despite the concentration constancy shown by each age group there was considerable interindividual variation, the highest values being some 10-fold greater than the lowest values. In this connection it must be emphasized that the present material, which was obtained from patients with T-tube drainage of the main bile duct post-operatively, cannot be regarded as normal. Some of the variation in biliary oestrogen levels observed may have been caused by the influence of drugs given to these patients during their illness on the steroid metabolizing enzyme systems. The calculation of 24-h excretion of oestrogens

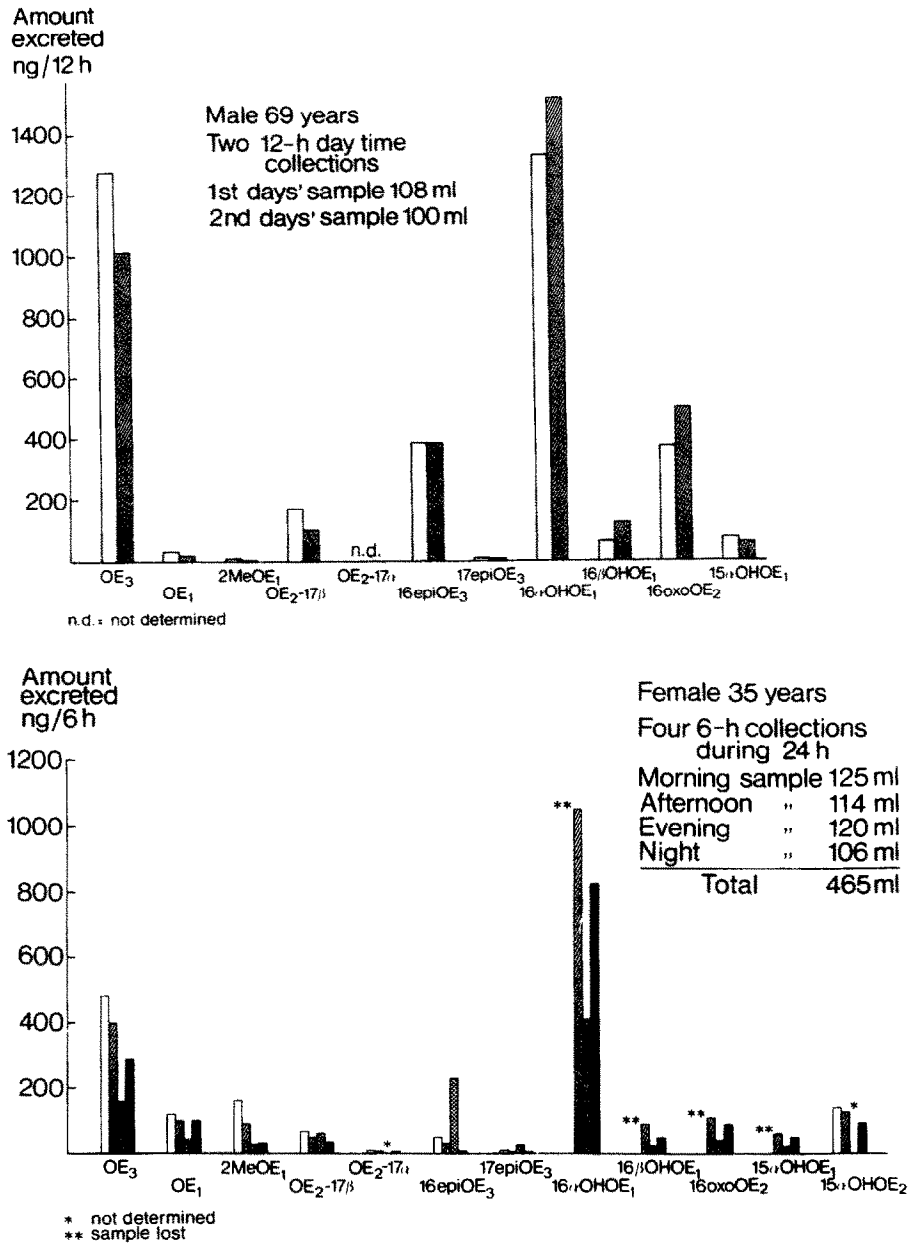

Fig. 1. Upper panel: Oestrogens excreted in two consecutive 12-h bile samples in a 69-year old male subject. Lower panel: Oestrogens excreted in four consecutive 6-h bile samples in a 35-year old female subject.

from samples collected during shorter periods seems reasonably valid because of the rather limited variation in excretion seen during the day (Fig. 1). The large difference in values for the fertile women may be dependent on the day of the menstrual cycle on which the samples were collected. In this study and that of Adlercreutz[5], utilizing colorimetric determination, compared in Table 5, the order of magnitude of the values obtained by mass fragmentography, GLC or colorimetry are reasonably constant.

The major portion of OE_1 in plasma is in the form of OE_1-SO_4 [20]. Mean concentrations of OE_1-SO_4 in plasma from normal men of 0.33 ($n = 30$) [21] and 0.72 μg/l ($n = 5$) [22] have been reported. The mean biliary OE_1 level in the male group was 2.2 μg/l suggesting a plasma to bile concentration gradient of the order of 4. In pregnancy a corresponding value of 5 was calculated. In contrast, a concentrating effect of up to 111-fold was seen for the 16-oxygenated oestrogens in pregnancy [18]. OE_1 is secreted in bile, for the most part, as OE_1-SO_4 [5].

OE_2 is a relatively minor biliary oestrogen both in men and non-pregnant, post-menopausal (Table 3) and pregnant women [5]. However, the daily excretion of OE_2 in faeces during pregnancy is as great as in urine [11] and preliminary results show

Table 3. Oestrogen concentrations in bile from males and non-pregnant females with T-tube drainage of the main bile duct determined by mass fragmentography

Patient (age)	Oestriol	Oestrone	2-Methoxy-oestrone	Oestradiol-17β	16-Epi-oestriol	17-Epi-oestriol	16α-Hydroxy-oestrone	16β-Hydroxy-oestrone	16-Oxo-oestradiol-17β	15α-Hydroxy-oestrone	Instrument
Males											
L.B. (70)	8.95	2.83	0.14	0.25	0.19	0.01	8.64	1.96	1.07	0.99	Varian MAT 112
O.N. (70)	34.20	2.48	n.d.	0.67	0.83	0.77	3.52	0.75	1.19	0.62	LKB-9000
S.K. (69)	40.90	7.45	0.22	1.67	1.05	0.26	4.69	0.67	1.86	0.14	LKB-9000
J.J. (67)	5.64	0.12	0.04	0.67	1.89	0.05	7.21	0.45	2.21	0.31	LKB-2091
H.N. (53)	2.10	0.23	0.03	0.43	0.85	0.06	4.95	1.10	0.90	0.31	Varian MAT CH-7
Y.N. (47)	24.60	1.31	n.d.	1.01	1.09	0.53	2.53	1.37	2.36	0.52	LKB-9000
J.P. (36)	9.68	3.18	0.18	0.43	0.24	0.21	1.83	0.47	0.50	0.04	LKB-9000
E.T. (29)	1.57	0.09	0.02	0.39	0.57	0.02	3.43	0.22	0.49	0.05	LKB-9000
Mean	16.00	2.21	0.11	0.69	0.84	0.24	4.60	0.87	1.32	0.37	
Females (postmenopausal)											
A.W. (72)	20.40	2.87	n.d.	0.74	0.48	0.35	2.45	0.37	0.34	0.03	LKB-9000
A.S. (64)	1.63	0.60	0.27	0.11	0.50	0.04	3.14	0.51	0.95	0.28	Varian MAT CH-7
B.S. (62)	1.79	0.39	0.04	0.18	0.53	0.07	8.27	1.58	1.24	0.45	Varian MAT CH-7
H.M. (61)	1.86	1.53	0.38	0.87	1.22	0.06	16.30	3.34	1.43	0.97	Varian MAT CH-7
Mean	6.42	1.34	0.23	0.48	0.68	0.13	7.54	1.45	0.99	0.43	
Females (fertile)											
V.N. (37)	43.80	1.17	0.06	1.16	0.56	0.32	2.57	0.55	0.81	0.20	LKB-9000
B.B. (35)*	2.87	0.61	0.54	0.42	0.22	0.04	6.80	0.50	0.72	0.38	Varian MAT 112(3)† LKB-2091 (1)†
Mean	23.3	0.89	0.30	0.79	0.39	0.18	4.69	0.53	0.77	0.29	

* Mean of four 6-h samples collected over a 24-h period. † Number of samples analyzed from the same patient using each particular instrument. n.d. = Not determined. Values are expressed as μg/l and the instrument on which the measurements were made is recorded.

Table 4. Approximate 24-h excretion (μg/24 h) of various oestrogens in bile, calculated from analyses of 4-, 6-, 8-, 12-, 16- or 24-h bile collections

Patient (age)	Oestriol	Oestrone	2-Methoxy-oestrone	Oestra-diol-17β	16-Epi-oestriol	17-Epi-oestriol	16α-Hydroxy-oestrone	16β-Hydroxy-oestrone	16-Oxo-oestradiol-17β	15α-Hydroxy-oestrone	Total excretion of 10 oestrogens
Males											
L.B. (70)	2.5	0.78	0.04	0.07	0.05	0.004	2.40	0.54	0.30	0.28	7.0
O.N. (70)	10.9	0.78	n.d.	0.21	0.26	0.25	1.12	0.24	0.38	0.20	14.3
S.K. (69)	14.4	2.62	0.08	0.59	0.37	0.09	1.65	0.24	0.65	0.05	20.7
J.J. (67)	2.3	0.05	0.02	0.27	0.76	0.02	2.87	0.18	0.87	0.13	7.5
H.N. (53)	0.8	0.09	0.01	0.16	0.32	0.02	1.84	0.41	0.34	0.12	4.1
Y.N. (47)	14.9	0.79	n.d.	0.61	0.66	0.32	1.42	0.83	1.43	0.32	21.3
J.P. (36)	4.0	1.31	0.07	0.18	0.10	0.09	0.75	0.19	0.21	0.02	6.9
Mean	7.1	0.92	0.04	0.30	0.36	0.11	1.72	0.37	0.60	0.16	11.7
Females (postmenopausal)											
A.W. (72)	9.5	1.34	n.d.	0.34	0.22	0.16	1.14	0.17	0.16	0.01	13.0
A.S. (64)	0.76	0.28	0.13	0.05	0.23	0.02	1.46	0.24	0.44	0.13	3.7
B.S. (62)	0.53	0.12	0.01	0.05	0.16	0.02	2.43	0.46	0.37	0.13	4.3
H.M. (61)	0.75	0.62	0.15	0.35	0.49	0.02	6.55	1.34	0.58	0.39	11.2
Mean	2.9	0.59	0.10	0.20	0.28	0.06	2.97	0.55	0.39	0.17	8.1
Females (fertile)											
Mass fragmentography											
V.N. (37)	42.30	1.13	0.06	1.12	0.54	0.31	2.48	0.53	0.78	0.19	49.4
B.B. (35)	1.33	0.48	0.29	0.21	0.31	0.03	3.04	0.21	0.32	0.17	6.4
Mean	21.8	0.81	0.18	0.67	0.43	0.17	2.76	0.37	0.55	0.18	27.9
Gas chromatography											
A.M. (37)	11.9	3.36	0.24	0.78	*	*	15.0	5.82	6.00	*	
A.S. (34)	4.6	0.65	0.84	0.52	*	*	13.2	3.47	2.79	*	
Mean	8.25	2.0	0.54	0.65			14.1	4.65	4.4		
Mean of all values for fertile women	15.0	1.41	0.36	0.66	(0.43)†	(0.17)†	5.93	2.51	2.47	(0.18)†	29.1

* Not included because value is probably too high due to insufficient purification of the fraction. † Values used to calculate total excretion of 10 oestrogens. n.d. = Not determined.

Table 5. A comparison of classical oestrogen measurements in human bile by various techniques

	Males					Post-menopausal women					Fertile women				
	No. of		Mean and range			No. of		Mean and range			No. of		Mean and range		
	samples	subjects	Oestrone μg/l	Oestradiol μg/l	Oestriol μg/l	samples	subjects	Oestrone μg/l	Oestradiol μg/l	Oestriol μg/l	samples	subjects	Oestrone μg/l	Oestradiol μg/l	Oestriol μg/l
Adlercreutz[5]—Colorimetry	8	4	0.80 0.1–1.8	0.45 0–1.3	5.93 1.5–12.2	5	4	0.84 0.2–1.2	0.35 0–0.8	10.8 1.0–28	4	3	4.5 2–7.5	2.5 1.6–4.0	26.5 16.9–36.2
Present investigation—Gas chromatography											2	2	3.8 2–5.6	1.5 1.3–1.6	17 14.2–19.8
Present investigation—Mass fragmentography	9	8	2.21 0.1–7.5	0.69 0.4–1.7	16.0 1.6–40.9	4	4	1.34 0.4–2.9	0.48 0.11–0.87	6.4 1.6–20.4	5	2	0.89 0.24–1.17	0.79 0.25–1.16	23.3 1.3–43.8

Table 6. Biliary excretion of some oestrogens measured in a limited number of bile samples

Initials	Sex	Age	Time of collection	15α-Hydroxy-oestradiol (μg/l)	15α-Hydroxy-oestradiol (μg/24 h)	Oestradiol-17α (μg/l)	Oestradiol-17α (μg/24 h)	11-Dehydro-oestradiol-17α (μg/l)	11-Dehydro-oestradiol-17α (μg/24 h)
L.B.	M	70	06.00–22.00	1.91	0.53				
H.N.	M	53	07.00–15.00			0.06	0.022		
A.S.	F	64	24.00–08.00			0.02	0.009		
B.S.	F	62	06.00–14.00			0.02	0.006		
H.M.	F	61	06.00–14.00			0.07	0.028		
B.B.	F	35	06.00–12.00	1.13	0.57	0.024	0.012	0.062	0.031
			12.00–18.00	1.15	0.52	0.024	0.010	0.048	0.021
			24.00–06.00	0.88	0.37	0.022	0.009	0.074	0.031
B.B. (Mean)				1.05	0.49	0.023	0.010	0.061	0.028

Approximate 24-h excretion values were calculated from results obtained for 6-, 8- or 16-h collections of bile.

relatively high excretion also in fertile women (Adlercreutz, unpublished observations). The low biliary levels suggest the possibility of OE_2 formation in the intestine, for example, by bacterial transformation of other oestrogens [23] or neutral steroids [24]. This possible significant formation of biologically active oestrogen in the intestinal tract may have physiological or pathological significance which is as yet not understood and should be further investigated.

Of the non-classical oestrogens measured 16α-$OHOE_1$ was present in largest concentrations (Table 3) and again showed large variation in biliary excretion. Examination of the concentrations of the 13 oestrogens measured showed little difference in relative distribution between the male and post-menopausal female groups with the exception of a tendency for higher OE_3:16α-$OHOE_1$ secretion in males (Tables 3 and 4). A similar conclusion could be drawn from a comparison of relative concentrations in the men and fertile women. Using the MF procedure, this group of oestrogens have also been measured in the urine of a fertile woman on days 14, 17 and 20 of the menstrual cycle [9]. A comparison of this data with our present findings highlights the relative quantitative importance of 16α-$OHOE_1$ excretion in bile. This has previously been noted when studying the quantitative oestrogen pattern in body fluids during pregnancy [25]. The high 16-$OHOE_1$:OE_3 ratio in pregnancy bile as compared to urine and the increased urinary 16α-$OHOE_1$ excretion in diseases associated with impaired bile secretion led to the proposal that a significant formation of OE_3 from 16α-$OHOE_1$ occurred during its enterohepatic circulation [26]. The results of this study would tend to strengthen this hypothesis. However, a further possible fate of biliary 16α-$OHOE_1$ and other labile ring D α-ketolic oestrogens is degradation in the intestinal tract: A study of the oestrogen content of human pregnancy faeces [11] demonstrated the virtual absence of these steroids in pregnancy faeces. In addition, the oral administration of ampicillin, which by reducing the intestinal bacterial flora, altered the faecal oestrogen profile to near biliary character, failed to increase the concentration of the 16-ketolic oestrogens in faeces [23] suggesting that such degradation may not be dependent on bacterial enzymes. In this study the biliary excretion of 16-oxoOE_2 and 16β-$OHOE_1$ was in all cases some 5–10 times lower than that of 16α-$OHOE_1$.

Of the 15-hydroxylated oestrogens, 15α-$OHOE_2$ was measured in only two cases and the calculated 24-h excretion of 15α-$OHOE_1$ was found to be about 0.5 μg (Table 6). In a series of reports [see, 27, 28] it has been shown that 15α-$OHOE_1$ and 15α-$OHOE_2$ are present in body fluids principally conjugated to N-acetylglucosamine. It is not known whether the *Helix pomatia* extract used in this study hydrolyzes N-acetylglucosaminides. The levels of 15α-$OHOE_1$ determined may thus be an underestimation of the real content. That the intestine may be a significant site of oestrogen 15α-hydroxylation is evidenced by the finding that 15α-$OHOE_1$ was the principle metabolite in portal venous blood of orally administered 16α-$OHOE_1$ [23]. It is of some interest, also, that 15α-$OHOE_2$ was one of the most significant oestrogens, quantitatively (125–180 μg/24 h), measured by this MF procedure in pregnancy faeces [11].

OE_2-17α was determined in bile samples from two male and four female subjects (Table 6). Only 6–28 ng/24 h were excreted, thus, this oestrogen is a very minor metabolite [see also 29]. The excretion of 11-dehydro-oestradiol-17α [30] was measured in three samples only from one of the fertile women. The concentrations were about three times higher than those of OE_2-17α, the mean being 28 ng/24 h (Table 6).

It may be concluded that the amount of oestrogens excreted in bile per 24 h is relatively high in all groups of subjects studied. This suggests that metabolic events in the intestinal tract can contribute significantly to the overall metabolism of these steroids and may influence their biological activity in the organism as the bulk of the biliary oestrogens are reabsorbed.

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REFERENCES

1. Gsell-Busse M. A.: Thelykinine in der Galle. *Klin. Wschr.* **7** (1928) 1606.
2. Cantarow A., Rakoff A. E., Paschkis K. E., Hansen L. P. and Walkling A. A.: Excretion of exogenous and endogenous estrogen in bile of dogs and humans. *Proc. Soc. exp. Biol. (N.Y.)* **52** (1943) 256–257.
3. Sandberg A. A. and Slaunwhite W. R. Jr.: Studies on phenolic steroids in human subjects. II. The metabolic fate and hepato-biliary-enteric circulation of [C^{14}]-estrone and [C^{14}]-estradiol in women. *J. clin. Invest.* **36** (1957) 1266–1278.
4. Twombly G. H. and Levitz M.: Metabolism of estrone-16-[C^{14}] sulfate in women. *Am. J. Obstet. Gynec.* **80** (1960) 889–897.
5. Adlercreutz H.: Studies on oestrogen excretion in human bile. *Acta endocr., Copenh. Suppl.* **72** (1962) 220.
6. Adlercreutz H. and Luukkainen T.: Biochemical and clinical aspects of the enterohepatic circulation of oestrogens. *Acta endocr., Copenh. Suppl.* **124** (1967) 101–140.
7. Adlercreutz H.: Determination of several estrogens in various biological fluids by gas–liquid chromatography. In *Methods of Hormone Analysis* (Edited by H. Breuer, D. Hamel and H. L. Krüskemper). Georg Thieme Verlag, Stuttgart; John Wiley, New York, Sydney, Toronto (1976) 480–499.
8. Adlercreutz H., Ervast H.-S., Tenhunen A. and Tikkanen M. J.: Gas chromatographic and mass spectrometric studies on oestrogens in bile. Part I. Pregnant women. *Acta endocr., Copenh.* **73** (1973) 543–554.
9. Adlercreutz H., Tikkanen M. J. and Hunneman D. H.: Mass fragmentographic determination of eleven estrogens in the body fluids of pregnant and nonpregnant subjects. *J. steroid Biochem.* **5** (1974) 211–217.
10. Adlercreutz H., Martin F., Wahlroos Ö. and Soini E.: Mass spectrometric and mass fragmentographic determination of natural and synthetic steroids in biological fluids. *J. steroid Biochem.* **6** (1975) 247–259.
11. Adlercreutz H. and Martin F.: Oestrogen in human pregnancy faeces. *Acta endocr., Copenh.* **83** (1976) 410–419.
12. Breuer H., Knuppen R. and Haupt M.: Metabolism of oestrone and oestradiol-17β in human liver *in vitro*. *Nature (Lond.)* **212** (1966) 76.
13. Dowben R. M. and Rabinowitz J. L.: Oxidation *in vitro* of radioactive oestradiol by preparations of human tissue. *Nature (Lond.)* **178** (1956) 696.
14. Breuer H., Nocke L. and Knuppen R.: Reduktion von 16α-Hydroxy-oestron und 16-Keto-oestradiol-(17β) durch menschliches Lebergewebe *in vitro*. *Hoppe-Seyler's Z. physiol. Chem.* **311** (1958) 275–278.
15. Dahm K., Lindlau M. and Breuer H.: Steroidepimerase—Ein neues Enzym des Oestrogenstoffwechsels. *Biochim. biophys. Acta* **159** (1968) 377–389.
16. Breuer H. and Knuppen R.: Metabolism of 16-oxo-oestrogens by human liver slices *in vitro*. *Nature (Lond.)* **182** (1958) 1512.
17. Breuer H., Knuppen R. and Pangels G.: Stoffwechsel von 16-Keto-oestron in menschlichen Geweben. *Acta endocr. Copenh.* **30** (1959) 247–258.
18. Knuppen R. and Breuer H.: Stoffwechsel von 15-substituierten Oestrogenen in der Leber des Menschen. *Naturwissenschaften* **53** (1966) 506–507.
19. Procopé B.-J. and Adlercreutz H.: Studies on the influence of age on oestrogens in post-menopausal women with atrophic endometrium and normal liver function. *Acta endocr., Copenh.* **62** (1969) 461–467.
20. Purdy R. H., Engel L. L. and Oncley J. L.: The characterization of estrone sulfate from human plasma. *J. biol. Chem.* **236** (1961) 1943–1950.
21. Loriaux D. H., Ruder H. J. and Lipsett M. B.: The measurement of estrone sulfate in plasma. *Steroids* **18** (1971) 463–472.
22. Hawkins R. A. and Oakey R. E.: Estimation of oestrone sulphate, oestradiol-17β and oestrone in peripheral plasma: concentrations during the menstrual cycle and in men. *J. Endocr.* **60** (1974) 3–17.
23. Adlercreutz H., Martin F., Pulkkinen M., Dencker H., Rimér U., Sjöberg N.-O. and Tikkanen M. J.: Intestinal metabolism of estrogens. *J. clin. Endocr.* **43** (1976) 497–505.
24. Goddard P. and Hill M. J.: Degradation of steroids by intestinal bacteria. IV. The aromatisation of ring A. *Biochim. biophys. Acta* **280** (1972) 326–342.
25. Adlercreutz H. and Tikkanen M. J.: Studies on the maternal contribution to estrogen metabolism in pregnancy. In *Hormonal Investigations in Human Pregnancy*. Proceedings of the International Symposium Paris-Fresnes (France) October 1973 (Edited by R. Scholler). Editions Sepe, Paris, Paris (1974) pp. 33–51.
26. Adlercreutz H., Tikkanen M. J., Wichmann K., Svanborg A. and Ånberg Å.: Recurrent jaundice in pregnancy. IV. Quantitative determination of urinary and biliary estrogens, including studies in pruritus gravidarum. *J. clin. Endocr.* **38** (1974) 51–57.
27. Jirku H. and Levitz M.: Biliary and urinary metabolites of estrone-6,7-^{3}H-sulfate -^{35}S in women. *J. clin. Endocr.* **29** (1969) 615–637.
28. Frey M. T., Jirku H. and Levitz M.: Levels of N-acetylglucosaminides and sulfo-N-acetylglucosaminides of 15α-hydroxyestradiol in human late pregnancy urine. *J. clin. Endocr.* **32** (1971) 532–538.
29. Adlercreutz H. and Luukkainen T.: Studies on estrogen metabolism in the adult human organism *in vivo*. In *Advances in the Biosciences* 3 (Edited by R. Raspé). Pergamon Press, Oxford (1969) pp. 53–70.
30. Luukkainen T. and Adlercreutz H.: Isolation and identification of 11-dehydro-estradiol-17α, a new type of urinary steroid, in the urine of pregnant women. *Biochim. biophys. Acta* **107** (1965) 579–592.
31. Luukkainen T. and Adlercreutz H.: Mass spectrometric studies in the specificity of estriol determination in urine by gas–liquid chromatography. *Ann. Med. exp. Fenn.* **45** (1967) 264–268.
32. Adlercreutz H., Johansson C.-J. and Luukkainen T.: Gas chromatographic and mass spectrometric studies in the influence of metabolites of bis(p-acetoxyphenyl)-cyclohexylidenemethane on the estimation of estrogens in urine. *Ann. Med. exp. Fenn.* **45** (1967) 269–276.
33. Adlercreutz H. and Luukkainen T.: Mass spectrometric studies on epimeric polyhydroxy oestrogens. In *Gas Chromatography of Hormonal Steroids* (Edited by R. Scholler and M. F. Jayle). Dunod, Paris; Gordon and Breach, New York (1967) pp. 93–103.