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BILIARY AND URINARY METABOLITES OF ESTRONE IN THE GUINEA PIG

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SUMMARY

The biliary and urinary metabolites of [6,7-^{3}H]-estrone in guinea pigs with biliary fistulas were analyzed. In the bile, estrone-3-glucosiduronate was a major metabolite; and estrone-3-sulfate and polar conjugates (probably, diglucosiduronates and disulfates) were minor metabolites. In the urine, estrone-3-glucosiduronate and 17β-estradiol-3-glucosiduronate were detected. Moreover, polar conjugates (probably diglucosiduronates) were also found, but estrone-3-sulfate could not be detected. The possibility exists that estrone-3-glucosiduronate is derived from the liver and is partly converted to 17β-estradiol-3-glucosiduronate extrahepatically.

INTRODUCTION

The metabolic fate of estrone (E_1)† in the guinea pig has been studied by several groups of investigators. Jellinck *et al.* reported that most of the urinary conjugates of [^{14}C]-E_1 could be hydrolyzed with β-glucuronidase [1]. When [6,7-^{3}H]-E_1 was incubated with guinea pig liver homogenate and uridine diphosphoglucuronic acid (UDPGA), the biosynthesis of estrone-3-glucosiduronate (E_1-3G) and 17β-estradiol-3-glucosiduronate (17βE_2-3G) was observed [2]. Moreover, perfusion studies in the guinea pig carried out by Quamme *et al.* revealed that the liver was capable of biosynthesizing E_1-3G and 17βE_2-3G from [6,7-^{3}H]-E_1 [3]. The biliary excretion of metabolites of [4-^{14}C]-E_1 in the guinea pig was studied by Sandberg *et al.*[4]; it was found that about 59% of the administered dose was excreted in the bile during 4 h of collection. However, the exact identification of biliary metabolites was not performed in their studies. Therefore, in order to obtain more complete information about estrone metabolism in the guinea pig, we have studied the biliary and urinary metabolites of this estrogen in female guinea pigs.

EXPERIMENTAL

Compounds. [6,7-^{3}H]-E_1 (40 Ci/mmol) was purchased from New England Nuclear Corporation, Boston, MA. Purity was checked by paper chromatography (PC) in system III (see Table 1). E_1 was purchased from BDH Biochemicals Ltd. 17β-estradiol (17βE_2) was a generous gift of Dr. K. Yasuda, Teikoku-Zōki Pharmaceutical Co., Tokyo. Estrone-3-sulfate (E_1-3S) and 17β-estradiol-17-glucosiduronate (17βE_2-17G) were purchased from Sigma Chemical Co., St., Louis, MO. Chemical purity of these standard steroids was ascertained by submitting these steroids to PC in the appropriate systems. Saccharo-1,4-dilactone was a gift of Chūgai Pharmaceutical Co., Tokyo.

Enzymic hydrolysis. All incubations were performed at 37°C.

1. Beef liver β-glucuronidase (Tokyo Zōki Chemical Co., Tokyo). The sample was dissolved in 0.1 M acetate buffer at pH 4.6. The enzyme concentration was 300 u/ml and the incubation time 48 h. Inhibition by saccharo-1,4-dilactone was carried out at a concentration of 2.5×10^{-2} M.

2. Sulfatase (Type V, Sigma Chemical Co.). The sample was dissolved in 0.1 M acetate buffer at pH 4.8. The enzyme concentration was 2 mg/ml and the incubation time 48 h.

3. Bovine testicular hyaluronidase (Type IV, Sigma Chemical Co.). The sample was dissolved in 0.05 M citrate buffer at pH 4.2. The enzyme concentration was 3 mg/ml and the incubation time 48 h.

Solvolysis. The sample was dissolved in 10 ml of 2N H_2SO_4; then 4g of NaCl and 20 ml of ethyl acetate were added. After the solution was allowed to

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† *Trivial names*; The following trivial names and abbreviations are used: 2-methoxyestrone = 2-methoxy-3-hydroxy-1,3,5(10)-estra-trien-17-one; estrone(E_1) = 3-hydroxy-1,3,5(10)-estra-trien-17-one; 17α-estradiol = 1,3,5(10)-estra-triene-3,17α-diol; 17β-estradiol(17βE_2) = 1,3,5(10)-estra-triene-3,17β-diol; 2-hydroxy-estrone = 2,3-dihydroxy-1,3,5(10)-estra-trien-17-one; 16α-hydroxyestrone = 3,16α-dihydroxy-1,3,5(10)-estra-trien-17-one; 16-epiestriol = 1,3,5(10)-estra-triene-3,16β,17β-triol; estriol(E_3) = 1,3,5(10)-estra-triene-3,16α,17β-triol; estrone-3-sulfate(E_1-3S) = 3-sulfato-1,3,5(10)-estra-trien-17-one; 17β-estradiol-17-glucosiduronate (17βE_2-17G) = 3-hydroxy-1,3,5(10)-estra-trien-17β-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-glucosiduronate (17βE_2-3G) = 17β-hydroxy-1,3,5(10)-estra-trien-3-yl-β-D-glucopyranosiduronate; estrone-3-glucosiduronate (E_1-3G) = 17-oxo-1,3,5(10)-estra-trien-3-yl-β-D-glucopyranosiduronate; estriol-3-16α-disulfate = 3,16α-disulfato-estra-1,3,5(10)-trien-17β-ol; 17α-estradiol-3-glucosiduronate-17-N-acetylglucosaminide (17αE_2-3G-17NAG) = 1,3,5(10)-estra-trien-3-yl-β-D-glucopyranosiduronate-17α-yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside.

Table 1. Solvent systems used for analysis

Countercurrent distribution (CCD)	
System A;	*n*-butanol–water (1:1, v/v)
System B;	*n*-butanol–ethyl acetate–0.2% NH_4OH (3:1:4, by vol.)
System C;	*n*-butanol–ethyl acetate–0.2% NH_4OH (1:1:2, by vol.)
System D;	*n*-butanol–ethyl acetate–0.2% NH_4OH (1:9:10, by vol.)
Paper chromatography (PC)	
System I;	benzene–*n*-butanol–methanol–water (9:1:5:5, by vol.)
System II;	benzene–methanol–water (100:55:45, by vol.)
System III;	benzene–petroleum ether–methanol–water (4:6:7:3, by vol.)
System IV;	acetic acid–water–ethylene dichloride–tert-butanol (6:14:15:5, by vol.)
System V;	*n*-butanol–toluene–10% NH_4OH (1:1:2, by vol.)
System VI;	*n*-butanol–ethyl acetate–0.01 M phosphate buffer (pH 7.4) (1:1:2, by vol.)
System VII;	*n*-butanol–10% NH_4OH (1:1, v/v)

stand for 48 h at room temperature, the ethyl acetate layer was separated and washed twice with 2 ml of 9% $NaHCO_3$ solution. The washings were discarded and the ethyl acetate layer was evaporated to dryness and a portion removed for radioactive counting.

Radioactivity measurements. Radioactivity was measured by a liquid scintillation spectrometer. Bray's reagent [5] was used as the scintillation fluid and the counting efficiency for tritium was more than 25%. c.p.m. Was usually converted to d.p.m. by external standardization or channel ratio method.

Separation of the metabolites. The separation of the conjugates was performed by countercurrent distribution (CCD). Radiochemical purity of the separated conjugate (especially, monoconjugate) was checked by PC. Aglycones were separated by PC. Radioactivity on paper was located by a radiochromatogram scanner. The solvent systems used for CCD and PC are shown in Table 1.

Synthesis of [6,7-^{3}H]-17β-estradiol-3-glucosiduronate. When [6,7-^{3}H]-E_1 was incubated with guinea pig liver homogenate and UDPGA (Sigma Chemical Co.) a mixture of [6,7-^{3}H]-E_1-3G and [6,7-^{3}H]-17βE_2-3G was obtained. This mixture was then reduced by sodium borohydride and the reduced product purified by CCD in system C ($K = 0.85$). Radiochemical purity of this product was checked by PC in systems IV and VI. In both cases, a single peak of radioactivity was observed [R_F in system IV, 0.74; R_F in system VI, 0.41]. In system VI, the mobility was slightly less than that of standard 17βE_2-17G ($R_F = 0.44$), whereas in system VII ($R_F = 0.63$) it was slightly greater than that of standard 17βE_2-17G ($R_F = 0.59$). This reduced product could be hydrolyzed with β-glucuronidase (97% hydrolysis); the hydrolysis was inhibited (>80%) in the presence of saccharo-1,4-dilactone. Hyaluronidase effected only 10% hydrolysis. The aglycone had the same mobility as that of standard 17β-estradiol on PC (system III). Thus, the identification of the reduced substance as [6,7-^{3}H]-17βE_2-3G was established.

Animals with biliary drainage. The experiments were performed on three female guinea pigs weighing 200–300 g. Catheterization of the hepatic and common bile ducts was performed under pentobarbital sodium (Nembutal sodium 20 mg/kg, subcutaneously) anesthesia. [6,7-^{3}H]-E_1 was dissolved in 0.2 ml of ethanol and diluted to 5 ml with 0.9% (w/v) saline. 1 ml of the latter solution was injected intramuscularly into each animal; bile and urine were collected for 5 h.

RESULTS

The excretion of radioactivity in bile and urine is shown in Table 2. The biliary excretion of radioactivity during 5 h of collection ranged from 41 to 42% of the administered dose, whereas only 10–16% appeared in the urine during the same period. All the biliary or urinary samples were pooled and submitted for the identification of biliary and urinary metabolites.

Guinea pig I, bile

The biliary sample was distributed in system B for 24 transfers. Three distinct peaks were present (Fig. 1):

Peak A: 11%, $K < 0.04$
Peak B: 73%, $K = 1.67$
Peak C: 16%, $K = 11.00$

Table 2. Biliary and urinary excretion of radioactivity following the administration of [6,7-^{3}H]-estrone to guinea pigs with biliary fistulas (expressed as % of the administered dose)

		Guinea pig-I	Guinea pig-II	Guinea pig-III
Bile	0–1 hr	16.3%	19.3	19.6
	1–2	10.1	10.7	11.2
	2–3	6.3	7.2	4.6
	3–4	5.1	3.0	3.3
	4–5	3.2	1.8	3.0
	0–5 hr	41.0%	42.0	41.7
Urine	0–5 hr	15.9%	16.4	9.6

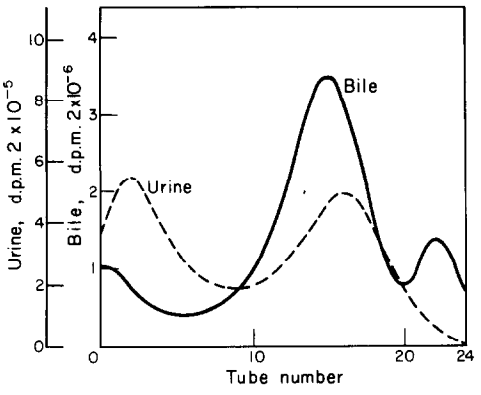

Fig. 1 CCD patterns in system B of guinea pig—I urine and bile following the injection of [6,7-^{3}H]-estrone.

Analysis of peak A substance

Upon redistribution of this material in system A for 24 transfers, K was 0.04. This substance was considered as consisting of diconjugates.

When an aliquot of this substance was incubated with β-glucuronidase, 58–62% of the radioactivity became ethyl acetate extractable. This hydrolysis was significantly inhibited (>80%) in the presence of saccharo-1,4-dilactone. When the aglycones thus obtained were chromatographed on paper in system I or system II, a number of radioactive peaks were observed. The final identification of these aglycones was not successful.

When another aliquot of peak A substance was submitted to solvolytic procedure, some 30% of hydrolysis was observed. Therefore, peak A substance was considered to be a mixture of diglucosiduronates and possibly disulfates.

Analysis of peak B substance

When this substance was redistributed in system C for 24 transfers, a single peak with a K of 0.78 was observed.

When an aliquot of this material was subjected to PC in system IV, a single peak with an R_F of 0.81 was observed, which was slightly more mobile than standard 17βE_2-3G (R_F = 0.72) or standard 17βE_2-17G (R_F = 0.74).

When another aliquot of this material was incubated with β-glucuronidase, 94% of radioactivity became ether extractable. This hydrolysis was inhibited (>85%) in the presence of saccharo-1,4-dilactone. The aglycones thus obtained were chromatographed on paper (system III). Two distinct peaks were obtained.

Aglycone B-1: 2%
Aglycone B-2: 98%

Aglycone B-1 had the same mobility as that of standard 17βE_2 (R_F = 0.42). Because of low radioactivity available, no further identification was carried out. Aglycone B-2 had the same mobility as that of standard E_1 (R_F = 0.78). The latter was mixed with 25.7 mg of E_1 and crystallized from ethanol to constant S.A. The results were as follows; calculated S.A.: 1277 c.p.m./mg, S.A. of the second crystal: 1205 c.p.m./mg, S.A. of the third crystal: 1210 c.p.m./mg. Thus, identification of aglycone B-2 as E_1 was established.

Another aliquot of peak B substance was reduced by sodium borohydride and then subjected to CCD (system B, n = 24). A single peak with a K of 2.69 was obtained. An aliquot of this peak was chromatographed on paper in system IV and a single peak of radioactivity observed; the mobility of radioactivity was identical with that of standard 17βE_2-3G (R_F = 0.74). The reduced material was then hydrolyzed with β-glucuronidase and 96% of radioactivity became ether extractable. In the presence of saccharo-1,4-dilactone, hydrolysis was significantly inhibited (>90%). When the aglycones were chromatographed on paper in system III, two distinct peaks were obtained.

Aglycone B-1′: 95%
Aglycone B-2′: 5%

Aglycone B-1′ moved at the same rate as standard 17βE_2 (R_F = 0.51).

Analysis of peak C substance

This substance was redistributed in system D for 24 transfers. The substance with a K of 0.50 was well separated from small amounts of other contaminants. When this material was chromatographed on paper in system V, only a single peak which moved at the same rate as standard E_1-3S (R_F = 0.70) was observed.

When this substance was incubated with β-glucuronidase, only 6% of hydrolysis was attained, whereas solvolysis released 80% of the aglycones. Incubation of this material with sulfatase rendered 91% of radioactivity ether extractable.

The aglycone liberated by aryl sulfatase was subjected to PC (system III). A single peak was obtained, the mobility of which was identical with that of standard E_1 (R_F = 0.85). The aglycone was mixed with 21.5 mg of standard E_1 and crystallized from methanol to constant S.A. The following results were obtained; calculated S.A.: 677 c.p.m./mg, S.A. of the first crystal: 616 c.p.m./mg, S.A. of the second crystal: 603 c.p.m./mg, S.A. of the third crystal: 609 c.p.m./mg. Thus, the aglycone was identified as E_1.

Guinea pig-I, urine

The urinary sample voided during 5 h of collection was diluted to 10 ml with distilled water and extracted with ether (1 vol., three times); 91% of radioactivity remained in the aqueous phase. This water-soluble material was subjected to CCD (system B, n = 24). Two distinct peaks were observed (Fig. 1).

Peak A: 45%, K = 0.09
Peak B: 55%, K = 2.0

Analysis of peak A substance

When this material was distributed in system A for 24 transfers, a radioactive peak with a K of 0.12 was observed and considered to consist of diconjugates.

When this material was incubated with β-glucuronidase, 69% of radioactivity became ethyl acetate extractable. The hydrolysis was inhibited (>80%) in the presence of saccharo-1,4-dilactone. When the aglycones thus obtained were submitted to PC in system I or II, a number of radioactive peaks were observed. Final identification of these aglycones could not be established.

Analysis of peak B substance

This material was subjected to CCD (system C, $n = 24$). A single peak with a K of 0.71 was observed.

An aliquot of this material was hydrolyzed with β-glucuronidase and 90% hydrolysis attained. This hydrolysis was inhibited (>85%) in the presence of saccharo-1,4-dilactone. When the aglycones thus obtained were submitted to PC (system III), two distinct peaks were observed:

Aglycone B-1: 44%
Aglycone B-2: 56%

The R_F values of aglycone B-1 and aglycone B-2 were identical with those of standard 17βE$_2$ ($R_F = 0.65$) and E$_1$ ($R_F = 0.85$), respectively.

Another aliquot of this material was reduced by sodium borohydride and the reduced substance subjected to CCD (system B, $n = 24$). A single peak with a K of 2.69 was observed. When an aliquot of this reduced material was subjected to PC (system VI), a single peak with an R_F of 0.23 was observed, which was slightly less mobile than standard 17βE$_2$-17G ($R_F = 0.25$). When the reduced substance was incubated with β-glucuronidase, 92% of hydrolysis was attained. The hydrolysis was inhibited (>80%) in the presence of saccharo-1,4-dilactone. The aglycones were chromatographed on paper (system III). Two distinct peaks were observed.

Aglycone B-1′: 97%
Aglycone B-2′: 3%

The mobilities of aglycone B-1′ and aglycone B-2′ were identical with those of standard 17βE$_2$ ($R_F = 0.42$) and E$_1$ ($R_F = 0.72$), respectively.

Guinea pig II and guinea pig III

The biliary and urinary samples in these cases were similarly analyzed, as in the case of guinea pig I. Only the distribution pattern of radiometabolites in system B will be shown:

Guinea pig II

Bile

Peak A: 16%, $K < 0.05$
Peak B: 68%, $K = 1.53$
Peak C: 16%, $K = 23.0$

Urine

Peak A: 50%, $K = 0.09$
Peak B: 50%, $K = 2.0$

Guinea pig III

Bile

Peak A: 11%, $K = 0.09$
Peak B: 71%, $K = 1.53$
Peak C: 18%, $K = 11.0$

Urine

Peak A: 32%, $K = 0.09$
Peak B: 68%, $K = 1.67$

DISCUSSION

The biliary excretion of the metabolites of [4-^{14}C]-E$_1$ in rodents and human was studied by Sandberg *et al*; it was found that a preponderant amount of radioactivity appeared in the bile [4, 6].

Watanabe reported that a considerable amount of radioactivity was excreted in the bile following the administration of [6,7-^{3}H]-17β-E$_2$ into the rat [7]. Similar findings were obtained when [4-^{14}C]-17βE$_2$ was injected into the cat or [6,7-^{3}H]-E$_1$ injected into the cat and the hen [8, 9].

In all of the species referred to above except guinea pigs, identification of the biliary metabolites of labeled E$_1$ or 17β-E$_2$ has been performed.

In guinea pigs, as shown in the present study, a major biliary metabolite of E$_1$ was E$_1$-G, which comprised about 70% of the biliary radioactivity. This E$_1$-G was, in all probability, E$_1$-3G, since the 17-oxo radical could be reduced to 17β-hydroxy radical by sodium borohydride. On the other hand, 17βE$_2$-G comprised only 1.5% of the biliary metabolites, probably as the 17βE$_2$-3G, though that it might be 17βE$_2$-17G could not be excluded.

Moreover, E$_1$-3S was detected as a biliary metabolite, comprising 7–11% of biliary radioactivity.

In addition, a more polar peak than E$_1$-3G was observed, which comprised 10–16% of the biliary metabolites. This polar peak is probably a mixture of diglucosiduronates and disulfates. In this connection, it is to be noted that guinea pig liver slices convert E$_1$-3S to disulfates of estriol (E$_3$) and of 16α-hydroxyestrone [10] and E$_3$ to estriol-3,16α-disulfate [11].

As major urinary metabolites we identified E$_1$-G and 17 E$_2$-G in a ratio of 1:1. E$_1$-G was, in all probability, E$_1$-3G, since 17-oxo radical could be reduced

to 17β-hydroxy radical by sodium borohydride. There was also observed a more polar peak than E_1-3G, which probably consisted of diconjugates and comprised about 30–50% of urinary metabolites. This conjugate could be readily hydrolyzed with β-glucuronidase. Unfortunately, identification of the aglycones could not be established.

When E_1 was incubated with guinea pig liver homogenate and UDPGA, biosynthesis of E_1-3G and 17βE_2-3G was obtained [2]. As mentioned above, very small amounts of 17βE_2-3G were detected as biliary metabolites. It is possible that 17βE_2-3G was formed in the liver *in vivo*, transported into the blood stream and excreted into the urine. However, the possibility that E_1-3G but not 17βE_2-3G was biosynthesized in the liver *in vivo* with part of E_1-3G being converted to 17βE_2-3G extrahepatically could not be excluded.

E_1-3S could not be detected as a urinary metabolite. Even if a part of E_1-3S, formed in the liver, might be transported in the blood stream, it would be extensively hydrolyzed extrahepatically, resulting in the absence of E_1-3S as a urinary metabolite.

Now, the nature of the biliary metabolites of E_1 or 17βE_2 in the other species should be mentioned. In the human, the major biliary metabolites of labeled E_1 were the diconjugates (sulfoglucosiduronate, sulfo-N-acetylglucosaminide and so on). The steroid aglycones identified in the bile included 15α-hydroxyestrogens, E_3, 16-epiestriol and E_1 [12]. In the rabbit, a major biliary metabolite of [6,7-^{3}H]-17βE_2 was 17α-estradiol-3-glucosiduronate-17-N-acetylglucosaminide. It is noted that no sulfate was detected in the bile [9].

The nature of the biliary metabolites of [6,7-^{3}H]-17βE_2 was investigated in the rat and the major metabolites were found to be glucosiduronates. The major aglycones identified were 2-methoxyestrone, 2-hydroxyestrone and E_1 [7].

Analysis of the biliary metabolites of [6,7-^{3}H]-E_1 in the cat and the hen was carried out by Quamme *et al.*[9]. In the cat, the biliary metabolites consisted predominantly of monosulfates and disulfates. On the other hand, the monoconjugates were the major biliary metabolites in the hen. Therefore, an outstanding feature derived from the above results is the diversity of the nature of the biliary metabolites in the various species.

Finally, the pattern of metabolism of E_1 in the guinea pig should be compared with that of E_3 in the same species [13]. 1. In either case, the preponderant amount of the steroid was excreted as a 3-glucosiduronate, both in bile and urine, whereas disulfate(s) was found only in the bile. 2. While a part of E_1 administered into the guinea pig was converted to 17βE_2 and the other aglycones, the E_3 nucleus remained unchanged during the conjugation processes.

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