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THE METABOLIC FATE OF ESTRADIOL BENZOATE IN FEMALE DOG

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SUMMARY

The metabolic fate of estradiol benzoate (estradiol 3-benzoate) was studied in intact and biliary fistula dogs. The steroid used was labeled with ^3H at position 6 and 7 of the steroid nucleus and with ^{14}C at the carbonyl carbon of the benzoyl group, thus affording the opportunity to ascertain the loss of the benzoyl group and the fate of both labels.

The averages of the radioactivity excreted, given as a percentage of the amounts injected, and the standard deviations were as follows: 11.9 ± 1.1% of the ^3H and 40.5 ± 3.5% of the ^{14}C were recovered in the urine of intact animals after 7 h. In dogs with biliary fistulas, the total radioactivity excreted was 6.3 ± 0.3% of the ^3H and 27.5 ± 2.5% of the ^{14}C in the urine, and 16.0 ± 0.5% of the ^3H and 1.05 ± 0.05% of the ^{14}C in the bile after 7 h. The concentration of estradiol benzoate in the blood was also investigated.

The dominant conjugates of estrogens in the urine were the sulfates. Hippuric acid, benzoic acid, 17-epiestradiol sulfate and estrone sulfate were detected in the urine. The dominant conjugates in the bile were the glucosiduronates and the sulfates. Estradiol 3-glucosiduronate, estrone glucosiduronate, 17-epiestradiol 3-sulfate and estrone 3-sulfate were detected.

These results indicate the immediate elimination of 3-benzoyl group of estradiol benzoate after injection. They also indicate that the resulting hippuric acids and benzoic acids are excreted mostly in the urine, and the enterohepatic circulation of the hydrolyzed steroid is observed to some extent.

INTRODUCTION

Estradiol 3-benzoate has been useful with regard to the slower rates of absorption, disintegration and excretion in comparison with those of estradiol [1, 2], and used widely in obstetrics and gynecology clinics. But little is known about its *in vivo* metabolism, and especially the elimination of the benzoyl group of this synthetic estrogen. To explore the metabolic fate of estradiol benzoate, the following study was performed. The study was done in four steps: (1) [6,7-^3H, 7'-^{14}C]-estradiol 3-benzoate was synthesized from [6,7-^3H]-estradiol. (2) The determination of the urinary and biliary excretions of radioactivity for 7 h was done in dogs with biliary fistulas, and in intact dogs following the intravenous injection of [6,7-^3H, 7'-^{14}C]-estradiol 3-benzoate. (3) Identification of the biliary and urinary metabolites of estradiol 3-benzoate was performed. (4) The disappearance of estradiol 3-benzoate from the blood was measured.

Systematic names of steroids used in this study are: 1. estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one. 2. estradiol = 1,3,5(10)-estratrien-3,17β-diol. 3. estriol = 1,3,5(10)-estratrien-3,16α,17β-triol. 4. 17-epiestradiol = 1,3,5(10)-estratrien-3,17α-diol. 5. 15α-hydroxyestrone = 3,15α-dihydroxy-1,3,5(10)-estratrien-17-one. 6. 15α-hydroxyestradiol = 1,3,5(10)-estratrien-3,15α,17β-triol. 7. 16-epiestriol = 1,3,5(10)-estratrien-3,16β,17β-triol. 8. 17-epiestriol = 1,3,5(10)-estratrien-3,16β,17α-triol. 9. estrone 3-glucosiduronate = 3-hydroxy-1,3,5(10)-estratrien-17-one 3-glucosiduronate. 10. estradiol 3-glucosiduronate = 1,3,5(10)-estratrien-3,17β-diol 3-glucosiduronate. 11. estradiol 17-glucosiduronate = 1,3,5(10)-estratrien-3,17β-diol 17-glucosiduronate. 12. estrone 3-sulfate = 3-hydroxy- 1,3,5(10)-estratrien-17-one 3-sulfate. 13. 17-epiestradiol 3-sulfate = 1,3,5(10)-estratrien-3,17α-diol 3-sulfate. 14. estriol 3-sulfate = 1,3,5(10)-estratrien-3,16α-17β-triol 3-sulfate. 15. estradiol 3-benzoate = 3-benzoyloxy-1,3,5(10)-estratrien-17β-ol. 16. estradiol 3-methyl ether = 3-methoxy-1,3,5(10)-estratrien-17β-ol. 17. medroxyprogesterone acetate = 17α-acetoxy-6α-methyl-4-pregnene-3,20-dione. 18. chlormadinone acetate = 17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione. 19. ethynodiol diacetate = 3β,17β-diacetoxy-17α-ethynyl-4-estrene.

MATERIALS AND METHODS

Steroids. [6,7-^3H]-estrone 3-glucosiduronate (E_1-3G) and [4-^{14}C]-E_1-3G were prepared according to the method of Sa'at and Slaunwhite[3]. They were reduced with $NaBH_4$ to give [6,7-^3H]-estradiol 3-glucosiduronate (E_2-3G) and [4-^{14}C]-E_2-3G. [4-^{14}C]-estriol 16-glucosiduronate (E_3-16G) and [6,7-^3H]E_3-16G were prepared according to the method of Boon *et al.*[4] and [6,7-^3H]-E_3-3G by the method of Goebelsmann *et al.*[5]. [6,7-^3H]-E_2-17G (54.4 Ci/mmol) was purchased from the New England Nuclear Corp (Boston, MA U.S.A.). Non radioactive

estrogens were obtained from the Sigma Chemical Company (St. Louis, MO U.S.A.). [7-^{14}C]-benzoic acid (14.29 mCi/mmol) and [6,7-^{3}H]-estradiol (49.3 Ci/mmol) were purchased from the New England Nuclear Corp. [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate was synthesized in three stages as follows.

Synthesis of [7'-^{14}C]-estradiol 3-benzoate. Thionyl chloride (0.05 ml) was added to [7'-^{14}C]-benzoic acid (8.56 mCi/lmg) at room temperature. After 1 h. the excess thionyl chloride was evaporated under heat, resulting in an oily residue, which was mixed with a solution of estrone (5 mg) in pyridine (0.05 ml). The mixture was heated for 2 h at 70°C, and then poured into ice-water. The product was extracted with ether–dichloromethane mixture, and the organic layer was washed with 5% H_2SO_4, 4% NaOH, and then with water. After drying with Na_2SO_4, the solvent was removed under vacuum to give a crystalline product, which was submitted to preparative thin layer chromatography on Silica Gel (Merch GF_{254}, benzene–acetone (19:1, v/v)). Estrone benzoate obtained from the main fraction was recrystallized from an acetone–hexane mixture to give colorless flakes (2.32 mg, 1.90×10^8 d.p.m., mp. 216–220).

To a solution of [7'-^{14}C]-estrone benzoate (1.89×10^8 d.p.m. ^{14}C, 2.30 mg) in dichloromethane (0.58 ml), a mixture of sodium borohydride (1.39 mg: in ethanol (0.58 ml) was added dropwise at 5°C. The mixture was stirred at room temperature for 2 h, and then poured into water. The product was extracted with ether, and the organic layer was washed with 5% H_2SO_4, 4% NaOH and then water. After drying with Na_2SO_4, removal of the solvent gave an oil, which was also submitted to preparative thin layer chromatography on Silica Gel (benzene–acetone (19:1, V/V)). From the more polar fraction, estradiol 3-benzoate (1.50 mg, 1.27×10^8 d.p.m. ^{14}C) was obtained, and the starting material (0.36 mg, 2.72×10^7 d.p.m. ^{14}C) was recovered from the less polar fraction.

Synthesis of [6,7-^{3}H]-estradiol 3-benzoate. To a solution of [6,7-^{3}H]-estradiol (2.5 mCi/1.0 mg) in 2% NaOH solution (1 ml), benzoyl chloride (0.1 ml) was added while stirring at room temperature. The mixture was allowed to stand at room temperature overnight. The product was extracted with ether, and the ether layer was washed with 5% Na_2CO_3, and then water, and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was submitted to preparative thin layer chromatography on Silica Gel (benzene–acetone (19:1, V/V)). Estradiol 3-benzoate (1.07 mg) was obtained from the main fraction with a radioactivity of 4.30×10^9 d.p.m. for ^{3}H.

Preparation of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate. [7'-^{14}C] and [6,7-^{3}H]-estradiol 3-benzoate obtained above were mixed in a portion of 1.14×10^8 d.p.m. ^{14}C/1.35 mg and 3.90×10^8 d.p.m. ^{3}H/0.14 mg, respectively. The mixture was purified twice by preparative thin layer chromatography on Silica Gel (benzene–acetone (19:1, V/V)), and [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate (1.25 mg) was obtained with a radioactivity of 3.88×10^8 d.p.m. for ^{3}H and 0.98×10^8 d.p.m. for ^{14}C. The weight was calculated by ultraviolet absorption spectrum data. The identification and purity were confirmed by recrystallization after the addition of carrier steroid.

Reagents and chemicals. D-saccharic acid 1,4-lactone and sulfatase were purchased from the Sigma Chemical Company, and β-glucuronidase from the Tokyo Zoki Chemical Company (Tokyo, Japan). Ketamine hydrochloride (57.6 mg/ml), and Diazepam (10 mg/ml) were purchased from the Sankyo Co., Ltd. (Tokyo, Japan) and the Takeda Co., Ltd (Osaka, Japan).

Animals. Seven female adult mongrel dogs were used for this experiment.

Administration of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate. The dogs were anesthetized using Ketamine hydrochloride and Diazepam, and the common bile duct was catheterized. The bilateral ureters were also catheterized. After confirmation of a smooth urinary flow from each catheter and of a smooth bile flow, [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate (^{3}H:10.0 μCi, ^{14}C:2.53 μCi, ^{3}H/^{14}C = 3.95, 4.96×10^{-2} mg) in 3 ml of physiological saline containing a small amount of ethanol was injected into the saphenous vein. The syringes and containers used for the injection were washed with methanol and aliquots of the washings were counted in order to determine the exact amount of radioactivity injected.

Collection of urine, bile and blood. After injection of the steroid, urine and bile were collected through the catheters for various time intervals over a period of 7 h. The collected urine and bile samples were immediately adjusted to pH 9 with ammonium hydroxide and kept at 4°C until analyzed. Blood from the femoral artery was also collected from a previously inserted catheter and kept as above.

Radioassay of samples. Aliquots of the urine samples were transferred to counting vials containing 10 ml of dioxane scintillator (contents of 1000 ml dioxane scintillator: toluene 333 ml, dioxane 333 ml, cellosolve 333 ml, DPO 4 g, POPOP 100 mg and naphtalen 75 g, from the Wako Junyaku Chemical Co. Ltd (Osaka, Japan)). Aliquots of bile and blood were absorbed with 100 mg of Cellulose Powder (Whatman, England) in Combusto-Cone™ (Packard, U.S.A.), and were then oxidized in a Packard Tri-Carb Sample Oxidizer Model 306. The urinary, biliary and blood radioactivities were determined in Packard instruments Tri-Carb Spectrometers Model 3375 or 3390 to a relative standard error of 2% and corrected for background and quenching.

Chromatographic method. The collected samples were applied to DEAE Sephadex A-25 column chromatography (column:0.9 × 60 cm, DEAE Sephadex A-25: from the Pharmacia Fine Chemicals AB, Uppsala, Sweden). DEAE Sephadex A-25 was mixed with distilled water, and packed into the columns to a height of 55 cm by gravity and allowed to equilibrate

overnight at room temperature. The column was eluted with 100 ml of water. This was then eluted with a linear gradient obtained by mixing 400 ml of 0.2 M NaCl with 400 ml of water, and then with 200 ml of 0.6 M NaCl, and finally with 150 ml of 2 M NaCl. A flow rate of approximately 80 ml per h was maintained. Fractions of 10 ml were collected and radioassayed as described above. The elution patterns from these columns were such that the water fractions contained non-charged compounds, whereas the latter fractions contained charged compounds. Namely, glucosiduronates of estrogen appear at about 0.1 M NaCl concentration, sulfates of estrogen appear at about 0.6 M NaCl concentration and polyconjugates of estrogen are washed out at 2 M NaCl solution [6, 7]. Enzymatic hydrolysis was performed on glucosiduronate fractions and sulfate fractions. The sample of glucosiduronate fractions, dissolved in 0.1 M acetate buffer (pH 5), was incubated at 37.0°C for 48 h with 500 units of β-glucuronidase/ml buffer. Inhibition by D-saccharic acid 1,4-lactone was tested at a concentration of 10^{-2} M. Extractions were done three times with water saturated ether. The sample in the sulfate fraction was dissolved in 0.1 M acetate buffer (pH 5). This was incubated at 37.0°C for 36 h with 2.0×10^{-4} units of sulfatase/ml buffer. Extractions were carried out three times with ether. Radioactivity extracted by ether from uninhibited incubates were chromatographed by thin layer chromatography (in chloroform-ethyl ether, 1:4 for separation of estrogens and in chloroform-methanol, 9:1 for separation of benzoic acids). The recovery experiments with glucosiduronate standards were carried out only with human and baboon material; details were described elsewhere [6, 7, 13].

RESULTS

Excretion of radioactivities

Six female adult mongrel dogs were used for this experiment. Bilateral ureterostomy was performed in each dog to collect urine. A biliary fistula was prepared in dogs 1–3 to collect bile. Injected radioactivities of ^{3}H, ^{14}C and mass were the same in all experiments.

The percent recoveries of ^{3}H and ^{14}C in the urine from the biliary fistula dogs (dogs 1–3) in 7 h were $6.3 \pm 0.3\%$ and $27.5 \pm 2.3\%$, respectively. Those in the bile from the same animals during the same interval were $16 \pm 0.5\%$ and $1.05 \pm 0.05\%$, respectively. Those in the urine from the intact dogs (dogs 4–6) in 7 h were $11.9 \pm 1.10\%$ and $40.5 \pm 3.5\%$, respectively.

These cumulative excretions are shown in Fig. 1 and Fig. 2. The ^{3}H labeled compounds were excreted more dominantly in the bile than in the urine. The amount of ^{3}H in the urine from the intact dogs was larger than that from the biliary fistula dogs. These results indicate that the ^{3}H labeled metabolites undergo an enterohepatic circulation to some extent. The ^{14}C labeled compounds were excreted predominantly in both the intact and biliary fistula dogs' urine, but little was found in the biliary fistula dogs' bile. The $^{3}H/^{14}C$ ratios were very low in the urine and extremely high in the bile in comparison with the initial ratio. These results indicate that the ^{14}C labeled benzoyl group was quickly eliminated from the steroid skeleton following injection, and the ^{14}C labeled metabolites did not circulate enterohepatically and were essentially excreted into the urine.

Elution of the sample on DEAE Sephadex A-25 column chromatography

Collected urine and bile samples were initially processed on DEAE Sephadex A-25 column. Figures 3–5 are elution patterns of the chromatography in dogs (dogs 1–4). Five radioactive peaks were obtained from the chromatography. They were tentatively identified according to their chromatographic characteristics. These were benzoic acid (hippuric acid) (P-I), estrone 3-glucosiduronate (P-II), and estradiol 3-glucosiduronate (P-III) in 0–0.2 M NaCl gradient and estrone 3-sulfate (P-IV) and 17-epiestradiol sulfate (P-V) in 0.6 M NaCl. No peak was eluted after a change of NaCl molarity from 0.6 M to 2.0 M. P-I mainly consisted of ^{14}C, but P-II to P-V contained largely ^{3}H.

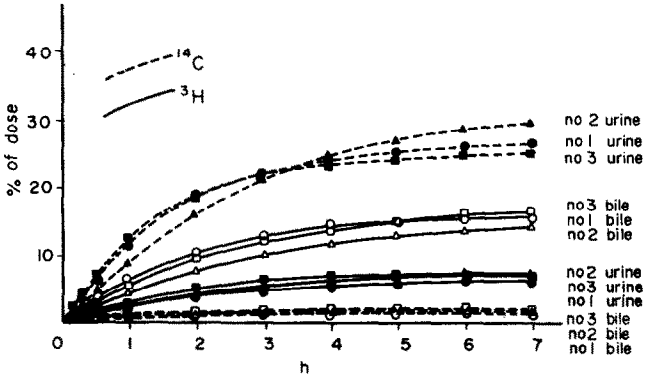

Fig. 1. Cumulative excretion of radioactivity in the urine and bile from the biliary fistula dogs following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate.

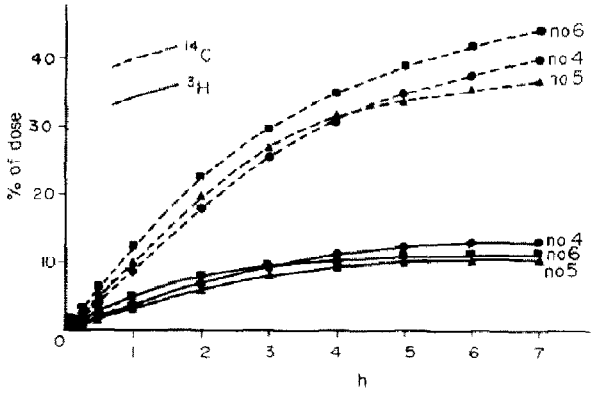

Fig. 2. Cumulative excretion of radioactivity in the urine from the intact dogs following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate.

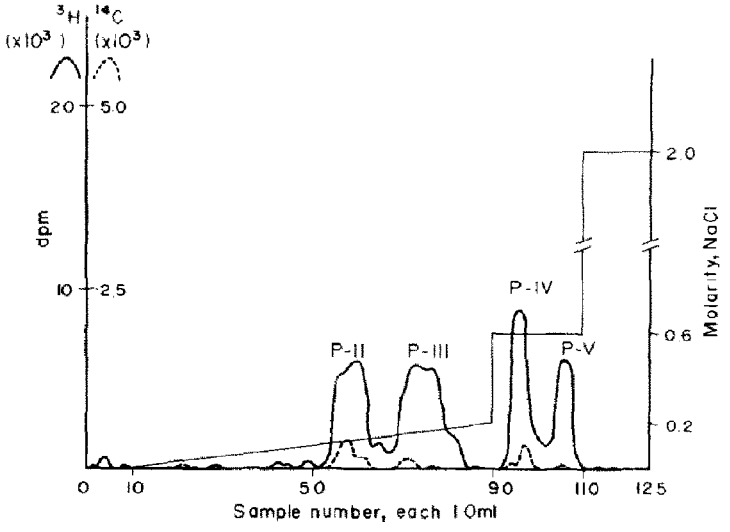

Fig. 3. Elution pattern on DEAE Sephadex A-25 column chromatography of the 2–4 h bile collection from the no. 1 biliary fistula dog following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate.

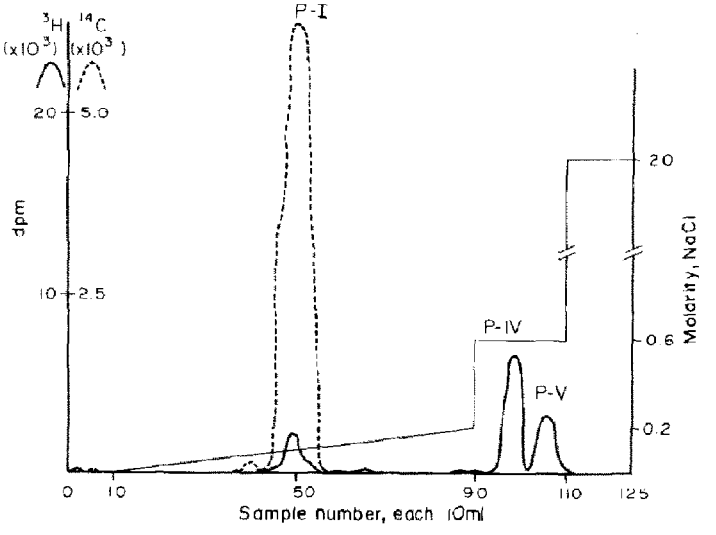

Fig. 4. Elution pattern on DEAE Sephadex A-25 column chromatography of the 2–4 h urine collection from the no. 1 biliary fistula dog following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate.

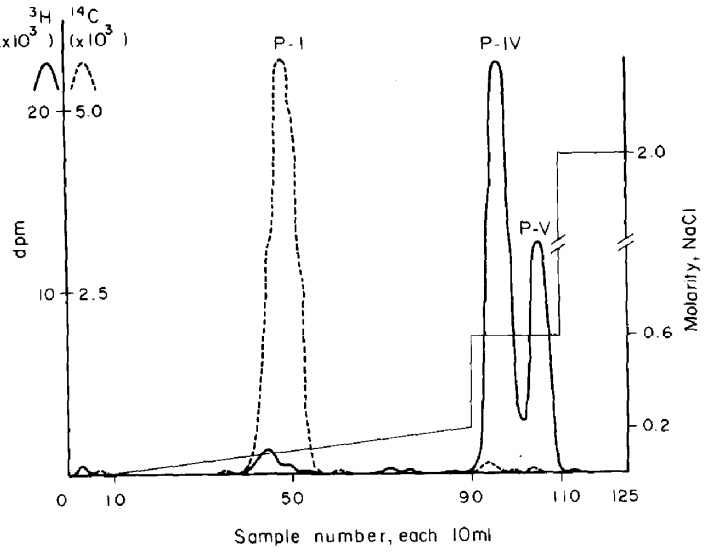

Fig. 5. Elution pattern on DEAE Sephadex A-25 column chromatography of the 2–4 h urine collection from the no. 4 intact dog following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate.

^{3}H in P-I and ^{14}C in P-II to P-V were negligible. P-I was detected only in the urine, and both P-II and P-III were detected only in the bile.

Non-conjugated estrogens which appeared in the initial water fraction were scarcely detectable in this experiment.

Identification of peak-I from urine

P-I appeared at 0.125 M NaCl concentration (estrogen glucosiduronate appears in these fractions). An aliquot of the P-I fraction was submitted to enzymatic hydrolysis. It was, however, not hydrolyzed with β-glucuronidase (shown in Table 1). Standard ^{14}C labeled benzoic acid was added to the fractions designated as "pooled peak I" obtained from the urine of the biliary fistula dog or the intact animal, and the mixture was rechromatographed on DEAE Sephadex A-25 column. There was only one ^{14}C labeled peak on the chromatography. An aliquot of the pooled P-I fraction and [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate were mixed, and the mixture was submitted to DEAE Sephadex column chromatography. It clearly showed two separated peaks. The pooled P-I fraction was concentrated by evaporation and the resulting material was chromatographed on thin layer plates of Silica Gel (solvent system, chloroform–methanol (9:1, V/V)). On the chromatography in this solvent system, benzoic acid and its hydroxylated compounds (ex. *p*- and *m*-hydroxy benzoic acids) were effectively separated. The R_F value of standard benzoic acid, *p*-hydroxybenzoic acid and *m*-hydroxybenzoic acid were 0.41, 0.21 and 0.28, respectively. The pooled P-I fraction had the same R_F as standard hippuric acid and benzoic acid ($R_F = 0.41$). It has been further confirmed with another solvent system (chloroform–ether (1:4, V/V)).

Identification of peak-II and peak-III from bile

P-III fraction and standard [4-^{14}C]-estradiol 3-glucosiduronate were mixed and the mixture was chromatographed on a DEAE Sephadex A-25 column in a linear gradient of 0–0.2 M NaCl. ^{3}H and ^{14}C were eluted in the same pattern. Both P-II and P-III fractions were hydrolyzed with β-glucuronidase in over 80% of the yield (Table 1). These hydrolysis were significantly inhibited by D-saccharo-1,4-lactone. The hydrolyzed materials from P-II and P-III fractions were found to be estrone and estradiol, respectively,

Table 1. Percent yield of radioactivity from the peaks on a DEAE Sephadex column chromatography by hydrolysis; (Control group was treated without β-glucuronidase or sulfatase)

		Control (%)	β-Glucuronidase (%)	Sulfatase (%)	β-Glucuronidase + Inhibitor (%)
Pooled urine	P-I (^{14}C)	18.7	23.7		24.1
(Dog no. 1)	P-IV (^{3}H)	4.0		96.0	
	P-V (^{3}H)	0.5		98.0	
Pooled bile	P-II (^{3}H)	23.1	81.9		48.7
(Dog no. 1)	P-III (^{3}H)	12.6	82.3		43.9

Inhibitor: D-Saccharo-1,4-lactone.

Table 2. Time sequence of ^{14}C in P-I and ^{3}H in P-II to P-V as percentages of the injected dose

		P-I (^{14}C)/h. (%)	P-II (^{3}H)/h. (%)	P-III (^{3}H)/h. (%)	P-IV (^{3}H)/h. (%)	P-V (^{3}H)/h. (%)
	no. 1 Bile	—	0.18	0.16	0.32	0.16
0–0.5 h.	no. 1 Urine	0.80	—	—	0.64	0.32
	no. 4 Urine	1.36	—	—	1.08	0.82
	no. 1 Bile	—	1.56	1.20	1.76	0.80
0.5–1 h.	no. 1 Urine	2.76	—	—	2.34	0.90
	no. 4 Urine	4.93	—	—	6.58	3.66
	no. 1 Bile	—	1.00	1.03	1.15	0.55
1–2 h.	no. 1 Urine	5.48	—	—	1.33	0.65
	no. 4 Urine	4.93	—	—	1.23	0.63
	no. 1 Bile	—	0.68	0.52	0.84	0.42
2–4 h.	no. 1 Urine	4.61	—	—	0.78	0.50
	no. 4 Urine	4.31	—	—	4.07	2.67
	no. 1 Bile	—	0.09	0.16	0.14	0.10
4–7 h.	no. 1 Urine	0.59	—	—	0.23	0.16
	no. 4 Urine	0.61	—	—	0.29	0.15

no. 1: biliary fistula dog.
no. 4: intact dog.

by thin layer chromatography in the solvent system of chloroform-ether (1:4, v/v). Accordingly, P-II and P-III were thought to be estrone 3-glucosiduronate and estradiol 3-glucosiduronate, respectively.

Identification of peak-IV and peak-V from urine or bile

Standard radioactive estrone 3-sulfate was mixed with the fractions designated as "pooled peak-V" and the mixture was chromatographed on DEAE Sephadex A-25 column, and only a single peak appeared in the sulfate region (0.6 M NaCl). P-IV and P-V fractions were almost completely hydrolyzed with sulfatase. By thin layer chromatography in the solvent system of chloroform–ether (1:4, V/V), aglycones derived from P-IV and P-V had the same R_F as 17-epiestradiol and estrone, respectively. From these results, the components of P-IV and P-V were thought to be as 17-epiestradiol 3-sulfate and estrone 3-sulfate, respectively.

Time sequence study of the metabolites

Table 2 shows the time sequence study of the amounts of radioactivities in peaks on the DEAE Sephadex A-25 column chromatography shown as a percentage of the injected dose per hour. P-I: hippuric acid (benzoic acid) was excreted essentially in the urine and its excretion reached a maximum at 1–2 h P-II, P-III, P-IV, and P-V: estrone 3-glucosiduronate, estradiol 3-glucosiduronate, 17-epiestradiol 3-sulfate and estrone 3-sulfate, respectively, were excreted with

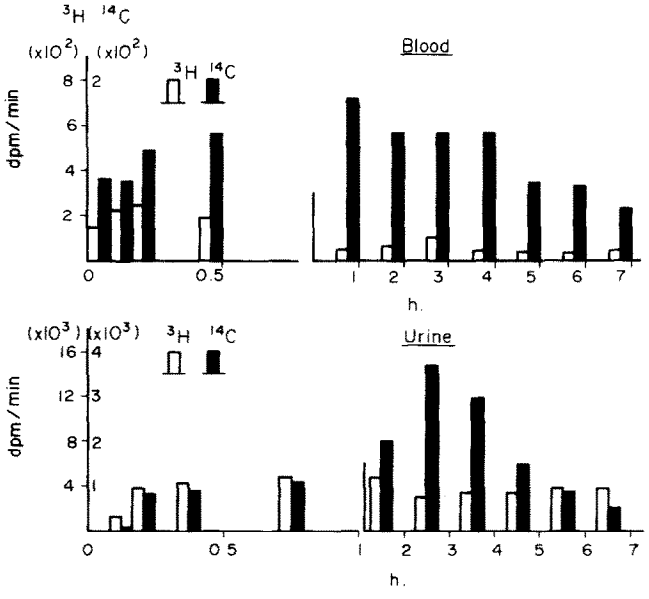

Fig. 6. The concentration of ^{3}H and ^{14}C in the blood of the intact dog following the I.V. injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate. Rate of excretions of ^{3}H and ^{14}C in the urine at the same intervals are also shown.

maximum levels at 0.5–1 h. It is evident from these results that the injected estradiol benzoate was hydrolyzed immediately and that the steroid was excreted earlier than the benzoic acids.

Glucosiduronates of estrogens were detected in the bile but were not detectable in the urine of the intact dog or in the biliary fistula dog. There may be some possibility that the glucosiduronates of the estrogens were hydrolyzed and then reconjugated to the sulfates during the enterohepatic circulation.

^3H and ^{14}C in the blood following I.V. injection of [6,7-^3H, 7'-^{14}C]-estradiol 3-benzoate

The same amount of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate (^{3}H:10.0 μCi, ^{14}C:2.53 μCi, 4.96×10^{-2} mg) was injected intravenously to no. 7 intact dog. The blood samples were collected from the right femoral artery at various time intervals over a period of 7 h. The radioactivities in blood and urine were shown in Fig. 6. A peak of ^{3}H in the blood was reached 15 min after injection, and a peak of ^{14}C was reached 1 h later. ^{3}H was excreted gradually into the urine and ^{14}C was excreted at a high ratio, 1–4 h later. It was also shown that estradiol 3-benzoate was immediately hydrolyzed and that the benzoic acids were retained longer than the estrogens in the blood.

DISCUSSION

This is the first report to describe the metabolism of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate. When [4-^{14}C]-estradiol was injected intravenously to adult dogs whose ureters and common bile duct were cannulated [8], an average of 43% of the injected radioactivity was recovered from the urine and bile of male dogs in 3 h. The recovered radioactivity in female dogs was only 35%. In another study [9], [6,7-^{3}H]-estradiol was injected into a mature dog and 29% of the injected dose was excreted into the first day's urine, and 2% into the second day's urine. In our study with injections of [6,7-^{3}H]-estradiol to adult dogs [10], the cumulative excretion of the ^{3}H in the urine was 9 ~ 23% of the injected dose over a period of 7 h. In the present study with the injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate, the total ^{3}H excreted from dogs with biliary fistulas was $6.3 \pm 0.3\%$ in the urine and $16.0 \pm 0.5\%$ in the bile after 7 h. The total ^{3}H excreted from intact animals was $11.9 \pm 1.1\%$ in the urine. Therefore, there appears to be no great difference in the excretion of estradiol and estradiol 3-benzoate after their administration, respectively.

In previous reports using mixtures of two single labeled steroids, we demonstrated a significant deacetylation of medroxyprogesterone acetate [11] and of ethynodiol diacetate [12], but found less deacetylation of chlormadinone acetate [13] in baboons. Furthermore, it has been postulated that the side-chain cleavage of progesterone did not occur in the intestinal tract [14]. Williams *et al.*[15] administered a mixture of [4-^{3}H]- and [4-^{14}C]-mestranols orally to women and showed that reactions involving position 4 were no greater than 1.7 ~ 3% of the dosage. They also [16] administered a mixture of [2-^{3}H]- and [4-^{14}C]-estradiol 3-methyl ether to a man and to a woman *per os*, and showed prominent reactions involving position 2.

To our knowledge there has been no report to date on the metabolic fate of the benzoyl group in estradiol benzoate. It became clear in the present study using the mixture of two single labeled estradiol 3-benzoate that the loss of the benzoyl group occurred immediately after its intravenous injection. Falconi *et al.*[17] synthesized estradiol 3-benzoate 17-cyclopentyl ether as an oral long-lasting estrogen. They insisted on the role of the 17-cycloalkenyl ether group for long lasting effect, but not on the benzoyl group. It could be said that the conjunction of benzoic acid to estradiol did not significantly prolong its effects. In our results, most of the eliminated benzoic acid was excreted in the urine, but little was found in the bile. Namely, neither prominent enterohepatic circulation of the acid, nor derivatives of estradiol benzoate occurred. Bray *et al.*[18] and El Masry *et al.*[19] showed that benzoic acids were mainly excreted into urine as hippuric acids in dogs or rabbits. The benzoate was excreted as both hippuric acid and benzoic acid in the urine without any hydroxylation. It is clear that the ^{14}C peak appeared somewhat later than the ^{3}H peak in the blood following injection of [6,7-^{3}H, 7'-^{14}C]-estradiol 3-benzoate. Therefore, the excretion of the benzoate appears to be slower than that of estradiol.

Cantarow *et al.*[20] and Longwell *et al.*[21] showed that administered estrogens readily passed into the bile and small amounts of the dosage were excreted in the urine. Our results denote some enterohepatic circulation of the hydrolyzed steroid.

Siegel *et al.*[9] reported that the urinary estrogens were mainly the conjugates of estrogens with glucuronic acid after administration of estradiol in dogs. The identification of the conjugates was carried out with enzymatic hydrolysis with bacterial β-glucuronidase, solvolysis and acid hydrolysis. Collins[22] isolated estrone glucosiduronate as a major metabolite and estradiol 3-glucosiduronate and estrone sulfate as minor metabolites in dog plasma following administration of [6,7-^{3}H]-estrone. The estrogen conjugates were separated by DEAE Sephadex chromatography. In another study (10), [4-^{14}C]-estradiol was injected into a peripheral vein of dogs and the conjugates in the urine were predominantly sulfates and to a lesser extent glucosiduronates. Our present study showed that the main conjugate of estrogens in the bile was the sulfate and the minor one was the glucosiduronate. It also showed that in the urine the dominant conjugate was the sulfate, and that no glucosiduronates of estrogen were detected, either with or without the biliary fistula. At present, the absence of glucosiduronates in urine is difficult to explain. However

one possibility is the immediate sulfurylation of estradiol which occurs at the site of hydrolyzing estradiol 3-benzoate.

When estradiol was administered to dogs, the major metabolites in the urine were estrone and 17-epiestradiol [8, 9, 23]. When estrone was given, the major metabolite was estradiol [24] in the urine. Estrone glucosiduronate was detected as a major metabolite in plasma after the administration of estrone [22]. When estrone glucosiduronate was infused, the major metabolite in plasma was estradiol 3-glucosiduronate and the minor one was 17-epiestradiol 3-sulfate [25]. Batchelor *et al.*[26] investigated endogenous estrogens in beagle bitches and detected estrone and estradiol and a very small amount of estriol. Minor metabolites after administration of estrogen to dogs were 17-epiestriol [24] 15α-hydroxy estrogens [23], estriol [27] and 16-epiestriol [27]. The formation of estriol was questioned by Bell *et al.*[28]. In our present study, the chief metabolites were estrone and 17-epiestradiol in the urine and the bile. Thus, it seemed that administered estradiol benzoate was hydrolyzed immediately and that the separated estradiol was metabolized instantly as free estradiol, with the exception of the difference of the conjugation formation.

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