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VARIATIONS IN BILIARY METABOLITES OF ANDROSTERONE IN FEMALE RATS

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SUMMARY

The fate of [^{3}H]-androsterone was studied in female rats with biliary fistulas. The major portion of the intraperitoneally administered radioactivity was recovered in the bile within 24 h. Large variations were observed in the excretion and metabolism of androsterone. In the rat with a high rate of biliary excretion of steroids, androsterone glucuronide was the preponderant metabolite present in the bile, whereas monosulphates of 3α,7α- and 3α,11β-dihydroxy-5α-androstan-17-one were the major metabolites in rats with a low rate of biliary excretion of steroids.

INTRODUCTION

In previous papers dealing with the biliary metabolites of testosterone and testosterone conjugates in the rat, we reported that androsterone was the major metabolite of testosterone in the female [1, 2]. However, little is known about the *in vivo* metabolism of androsterone in the rat. In the present investigation, [^{3}H]-androsterone was administered intraperitoneally into female rats and the biliary metabolites were isolated and identified by gas chromatography–mass spectrometry.

EXPERIMENTAL

Materials. [1, 2-^{3}H]-Androsterone (44.5 Ci/mmol) was purchased from New England Nuclear Corp., and radiochemical purity was confirmed by t.l.c. shortly before use. Androsterone and 3α,11β-dihydroxy-5α-androstan-17-one were obtained from Sigma Chemical Co. Preparation of 5α-androstane-3α,15α,17β-triol was described previously [3]. 3α,17β-Dihydroxy-5α-androstan-16-one was prepared as described by Huffman *et al.* [4], and 5α-androstane-3α,7α,17β-triol by alkaline hydrolysis of 3α,17β-diacetoxy-5α-androstan-7α-ol [5]. Sodium borohydride reduction of androsterone gave 5α-androstane-3α,17β-diol.

Conditions of animal experiments. The common bile duct was cannulated in female rats of the Wistar strain weighing 200–250 g (Matsumoto Experimental Animal Lab., Tokyo, Japan) as described previously [1]. After operation, the animals were kept in a restraining cage with free access to water and food pellets. [^{3}H]-Androsterone (14.6 μCi) was mixed with carrier androsterone (66 μmol) and dissolved in 1.0 ml of ethanol. Fifty μl of ethanol solution of [^{3}H]-androsterone (0.73 μCi, 3.3 μmol) was diluted with 0.1 ml of ethanol and 0.15 ml of saline and injected intraperitoneally 18–20 h after operation into each of seven female rats. Bile was collected at 0–1, 1–2, 2–4, 4–6, 6–24, and 24–48 h. In the similar way, control biles were collected following injection of the same quantity of the vehicle into three female rats.

Extraction and purification of biliary metabolites. Bile samples obtained from each rat at 0–4 and 4–24 or 0–24 h were processed separately. The bile was extracted with ether, and the ether extract washed with water, dried, and evaporated *in vacuo* on a rotary evaporator to give the free steroid fraction. The aqueous fraction was evaporated *in vacuo* on a rotary evaporator to a vol. of 20 ml and then passed through a column (700 × 20 mm I.D.) packed with Amberlite XAD-2 resin (100 g). The column was washed with 100 ml of water, followed by 400 ml of methanol [6]. The methanol effluent was evaporated *in vacuo* on a rotary evaporator to afford the conjugate fraction. The conjugate fraction was dissolved in 3 ml of chloroform–methanol (1:1 v/v), containing 0.01 M NaCl, and applied on Sephadex LH-20 column (20 g, 800 × 15 mm I.D.) [7]. The column was eluted with 200 ml of the same solvent system, followed by 200 ml of methanol to give the monoglucuronide, monosulphate and diconjugate fractions.

Hydrolysis of conjugate fractions. Details of hydrolysis of conjugate fractions were described previously [1]. The glucuronide fraction was hydrolyzed by incubation with β-glucuronidase (Ketodase), the monosulphate fraction was solvolyzed in acidified ethyl acetate, and the diconjugate fraction was hydrolyzed by solvolysis and by incubation with β-glucuronidase.

Thin-layer chromatography (t.l.c.). The liberated steroids were purified by t.l.c. on plates coated with silica gel GF (Merck), using chloroform–acetone (29:1 v/v) as solvent. Radioactive zones were detected with a Packard Model 7201 autoscanner, scraped, and eluted with methanol as previously described [1].

Polar steroid fractions remaining near the starting line on the t.l.c. plates were further separated with the solvent system cyclohexane–ethyl acetate (2 : 3 v/v). In general, t.l.c. plates were developed three or four times in the same solvent system.

Sodium borohydride reduction. A portion of the purified metabolite, which was tentatively identified as 17-oxo-$C_{19}O_3$ steroid, was dissolved in 1 ml of methanol and reduced with 20 mg of $NaBH_4$ at 0°C. After 1 h the solution was poured into water, and extracted with chloroform. The extract was washed with water and dried over Na_2SO_4. Evaporation gave 5α-androstanetriol which was submitted to gas chromatographic and gas chromatography–mass spectrometric analyses.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Metabolites from t.l.c. plates were trimethylsilylated [8] and analyzed by GC and GC–MS. GC was performed on a Shimadzu GC-4BM chromatograph with a flame ionization detector using 0.5% CHDMS (2.0 m × 3 mm; column 200°C; detector and flash heater 240°C) and 1.5% SE-30 (1.5 m × 3 mm; column 230°C; detector and flash heater 250°C) as the stationary phase. N_2 flow-rate was 40 ml/min. Relative retention times were calculated relative to 5α-cholestane. In general, the peaks were quantitated by peak-height measurement on the CHDMS and SE-30 columns, employing known amount of 5α-cholestane as internal standard. Quantitation of the steroids which had similar relative retention times to 5α-cholestane on the CHDMS column was performed on the SE-30 column. GC–MS was carried out on a JEOL JMS-D100 spectrometer using 1.5% SE-30 column (2.0 m × 3 mm; column 260°C; detector and flash heater 270°C). Helium gas flow-rate was about 30 ml/min. The temperatures of the molecular separator and ion source were 200°C. Mass spectra were recorded with a bombarding electron energy of 24 eV and filament current of 300 μA.

Measurement of radioactivity. Radioactivity was counted in an Aloka LSC-502 liquid scintillation spectrometer in a toluene medium as previously described [1]. Efficiency of ^3H counting was about 40%. Results are expressed in d.p.m.

RESULTS

Distribution of radioactivity. The mean recovery of the administered dose in the bile was about 75% during the first 24 h. Less than 1% of the injected radioactivity was found in the 24–48 h bile fraction. Rats provided a considerably constant bile flow, but variation was observed in the biliary excretion rate of the metabolites (Table 1). Rats could be classified into two groups based on the excretion rate of the radioactivity in the bile. One group (the HE rat) excreted about 44% of the radioactivity during the first h and approximately 96% of the injected dose was found in the bile during 24 h. In contrast, another group (the LE rat) eliminated only 9% of the radioactivity in the first h, about 61% of the dose appearing in the bile during 24 h. Biles obtained at 0–4 and 4–24 or 0–24 h were processed separately.

Extraction of each bile fraction with ether resulted in the quantitative recovery of the radioactivity in the aqueous fraction, which was then passed through Amberlite XAD-2 column, followed by elution with methanol. The recovery of the radioactivity in the methanol effluent (conjugate fraction) was about 98%. There were significant quantitative differences in the constitution of these conjugates separated on Sephadex LH-20 between the HE and LE rats (Table 2). In the HE rat, glucuronide was the main conjugate present in the 0–24 h bile fraction, whereas monosulphate predominated in the LE rat. There was little variation in the conjugate pattern between the 0–4 and 4–24 h bile fractions in the LE rat. In contrast to the 0–4 h bile, the 4–24 h bile showed an intermediary pattern in the HE rat, smaller proportion of glucuronide and increased amount of the monosulphate being excreted. However, the overall pattern in the 0–24 h bile was dominated by that of the 0–4 h bile, which accounted for more than 80% of the radioactivity in the 0–24 h bile.

Hydrolysis of conjugates. After β-glucuronidase hydrolysis of the glucuronide fraction, about 70% of the radioactivity in the HE rat fraction appeared in the ether extract. The corresponding value for the LE rat was 35%. By solvolysis of the monosulphate fraction, about 79 and 83% of the radioactivity in the HE and LE rat fractions were extracted with ethyl acetate, respectively. The diconjugate fraction was solvolyzed to remove sulphate group. By this, about 50 and 68% of the radioactivity in the HE and LE rat fractions were extracted with ethyl acetate, respectively. After solvolysis, the aqueous phase was then hydrolyzed with β-glucuronidase and about 33% of the radioactivity in the aqueous fraction of the HE and LE rats was extracted with ether. When β-glucuronidase hydrolysis of the diconjugate was done first, only a few % of the radioactivity was extracted with ether. These results demonstrated that the diconjugates mainly consisted of disulphates and to a minor extent sulphoglucuronides.

Identification of steroids. The liberated steroids were subsequently separated by t.l.c. and separated meta-

Table 1. Biliary excretion of radioactivity (% dose) following intraperitoneal injection of [^3H]-androsterone into female rats*

Bile (h)	HE rat† Bile (g)	HE rat† ^3H(%)	LE rat‡ Bile (g)	LE rat‡ ^3H(%)
0–1	0.63 ± 0.08§	43.7 ± 7.6	0.50 ± 0.12	9.2 ± 3.5
1–2	0.55 ± 0.08	18.5 ± 0.7	0.50 ± 0.10	12.7 ± 3.5
2–4	1.12 ± 0.14	18.8 ± 2.0	0.99 ± 0.23	14.9 ± 3.4
4–6	0.94 ± 0.09	5.5 ± 1.1	0.95 ± 0.26	8.7 ± 3.5
6–24	8.17 ± 0.42	9.4 ± 4.1	7.91 ± 1.33	15.9 ± 9.8
Total	11.42 ± 0.72	96.0 ± 4.5	10.79 ± 2.05	61.4 ± 18.6

* Dose: [^3H]-androsterone 0.73 μCi, 3.3 μmol. † Rats with high rate of biliary excretion of steroids (n = 3). ‡ Rats with low rate of biliary excretion of steroids (n = 4). § Mean ± S.D.

Table 2. Distribution of biliary radioactivity (% dose) in various fractions

Rat	Fraction	Bile (h) 0–4	4–24	0–24
HE*	Monoglucuronide	62.3 (75.6)‡	4.9 (49.4)	69.0 ± 3.3† (73.6 ± 6.4)
	Monosulphate	15.7 (19.0)	4.2 (42.4)	17.9 ± 7.4 (19.1 ± 7.0)
	Diconjugate	4.4 (5.3)	0.8 (8.0)	6.8 ± 1.4 (7.3 ± 1.4)
LE§	Monoglucuronide	5.0 ± 1.1 (13.9 ± 7.4)	2.2 ± 1.5 (9.1 ± 6.0)	7.2 ± 2.4 (12.0 ± 5.8)
	Monosulphate	24.7 ± 7.1 (68.8 ± 5.0)	19.3 ± 11.0 (80.0 ± 5.1)	44.0 ± 14.8 (73.4 ± 3.3)
	Diconjugate	6.2 ± 3.3 (17.2 ± 5.1)	2.6 ± 2.0 (10.7 ± 2.7)	8.7 ± 5.1 (14.5 ± 3.8)

* Rats with high rate of biliary excretion of steroids. The 0–4 and 4–24 h biles of one rat and the 0–24 h biles of two rats were analyzed separately. † Mean ± S.D. ‡ Figures in parentheses indicate % of the total radioactivity excreted in each bile fraction. § Rats with low rate of biliary excretion of steroids. The 0–4 and 4–24 h biles of four rats were analyzed separately.

Table 3. Gas chromatographic and mass spectrometric data of trimethylsilyl derivatives of metabolites obtained by hydrolysis of biliary conjugates

Metabolite	Conjugate*	GC (RRT)† CHDMS	SE-30	GC-MS (*m/e*)‡ M^+	BP	Other prominent ions							
androsterone	G,S	0.75	0.39	362	272	271	347	215	257	129	155		
5α-androstane-3α,17β-diol	D	0.29	0.49	436	241	256	129	346	215	331	148		
3α,7α-dihydroxy-5α-androstan-17-one	G,S	0.76	0.57	450	270	360	271	255	213	243	129	121	332	243
3α,11β-dihydroxy-5α-androstan-17-one	S	1.10	0.70	450	156	199	184	186	157	360	394			
3α,15α-dihydroxy-5α-androstan-17-one	S	0.91	0.64	450	143	270	145	216	106	360				
3α,17β-dihydroxy-5α-androstan-16-one	D	1.02	0.76	450	129	215	216	435	117	173				
5α-androstane-3α,7α,17β-triol§		0.33	0.62	524	393	344	254	255	394	434	239	213		
5α-androstane-3α,15α,17β-triol¶		0.48	0.71	524	217	191	218	219	169	434	254	344	228	

* G = monoglucuronide, S = monsulphate, D = diconjugate. † Gas chromatographic conditions are described in "Experimental". RRT = relative retention time. ‡ Gas chromatography-mass spectrometric conditions are described in "Experimental". M^+ = molecular ion. BP = base peak. § Metabolite, 3α,7α-dihydroxy-5α-androstan-17-one was reduced with $NaBH_4$ and subjected to GC–MS. ¶ Metabolite, 3α,15α-dihydroxy-5α-androstan-17-one was reduced with $NaBH_4$ and subjected to GC MS.

bolites were trimethylsilylated and analyzed by GC and GC–MS (Table 3). The identified metabolites had relative retention times and mass spectra identical with those of the respective reference steroids. 3α,7α- and 3α,15α-Dihydroxy-5α-androstan-17-ones were not available in this laboratory. The metabolites characterized as such had similar relative retention times on SE-30 and mass spectra to these steroids described by Gustafsson *et al.* [9, 10]. Definitive identification was carried out by sodium borohydride reduction of these metabolites and by comparison of the resulting androstanetriols with authentic 5α-androstane-3α, 7α,17β-triol and -3α,15α,17β-triol as shown in Table 3. Quantification of these metabolites were done after reduction to androstanetriols.

Table 4 gives the percentage conversions of the identified metabolites, which were calculated from the injected dose. In the HE rat, androsterone glucuronide was the predominant metabolite. In addition to this, small amounts of 3α,7α-dihydroxy-5α-androstan-17-one monoglucuronide and monosulphates of 3α,7α-, 3α,11β- and 3α,15α-dihydroxy-5α-androstan-17-ones and androsterone were identified. Major metabolites in the LE rat were monosulphates of 3α,7α-, 3α,11β- and 3α,15α-dihydroxy-5α-androstan-17-ones and androsterone. As minor metabolites, androsterone and 3α,17β-dihydroxy-5α-androstan-16-one were isolated in the monoglucuronide and diconjugate fractions of the LE rat, respectively. Very small amounts of 5α-androstane-3α,17β-diol were identified in the diconjugate fraction of both HE and LE rats.

Analysis of the 0–24 h control biles did not give any identifiable steroids.

DISCUSSION

The present study demonstrates that the major portion of androsterone metabolites was rapidly excreted in the bile. Some interesting features characterize the metabolism of androsterone in female rats, i.e., large variations in the biotransformation and biliary excretion. A relationship evidently existed between the rate of biliary excretion of steroids and the biliary conjugates. The HE rat excreted large amounts of glucuronides in bile, whereas monosulphates were the predominant conjugate in the LE rat. Analysis of the free steroids after hydrolysis revealed the marked difference between two groups. Androsterone was the major steroid identified in the glucuronide fraction of the HE rat. In contrast, 7α- and 11β-hydroxylated

Table 4. Biliary metabolites (% dose) of intraperitoneal administration of [^{3}H]-androsterone in the female rat

Fraction	Metabolite	Bile (h)	HE rat*	LE rat†
Monoglu-curonide	androsterone	0–4	36.9	trace‡
		4–24	1.7	—
		0–24	40.6 ± 4.2§	trace
	3α,7α-dihydroxy-5α-androstan-17-one	0–24	trace	
Monosulphate	androsterone	0–4	1.9	2.3 ± 0.7
		4–24	—	1.0 ± 0.4
		0–24	1.9 ± 0.8	3.3 ± 0.7
	3α,7α-dihydroxy-5α-androstan-17-one	0–4	1.1	6.5 ± 1.5
		4–24	—	7.8 ± 3.6
		0–24	2.4 ± 1.8	14.3 ± 3.9
	3α,11β-dihydroxy-5α-androstan-17-one	0–4	2.8	4.5 ± 2.1
		4–24	—	2.5 ± 1.8
		0–24	2.6 ± 1.4	7.0 ± 3.8
	3α,15α-dihydroxy-5α-androstan-17-one	0–4	trace	1.4 ± 0.6
		4–24	—	trace
		0–24	trace	1.4 ± 0.6
Diconjugate	5α-androstane-3α,17β-diol	0–4	trace	trace
		0–24	trace	trace
	3α,17β-dihydroxy-5-androstan-16-one	0–4	—	trace

* Rats with high rate of biliary excretion of steroids. The 0–4 and 4–24 h biles of one rat and the 0–24 h biles of two rats were analyzed separately. † Rats with low rate of biliary excretion of steroids. The 0–4 and 4–24 h biles of four rats were analyzed separately. ‡ Metabolites less than 1% of the injected dose. § Mean ± S.D.

metabolites of androsterone were the major metabolites in the monosulphate fraction of the LE rat. Gustafsson *et al.* reported the isolation of these metabolites from the pooled faeces of germfree rats [10] and the bile of female rats dosed with [^{14}C]-pregnenolone [11]. Thus, these metabolites seem to be present as normal constituents in rat bile, but the quantities of these steroids present in the 0–24 h control bile were under the limit of our analytical method.

Androsterone is metabolized by several different pathways of biotransformations such as hydroxylation at C-7, C-11, C-15 and C-16, reduction of 17-keto group, and conjugation with glucuronic acid and sulphuric acid. The rate at which each reaction proceeds, and its relative importance, may be affected by genetic and physiological factors, resulting in changes in the pattern of metabolism. Siiteri *et al.* [12] administered ^{3}H labelled androsterone glucuronide to humans and found that this conjugate was rapidly excreted in urine without undergoing further metabolism. It is of interest to speculate that UDP-glucuronyltransferase enzyme might be very active in the HE rat. Thus, the injected androsterone should be rapidly conjugated with glucuronic acid and eliminated in the bile. On the other hand, low activity of UDP-glucuronyltransferase or high activity of sulphotransferase in the LE rat might result in the further metabolism of androsterone or androsterone sulphate. A similar variation was observed in the metabolism of [^{14}C]-testosterone in female rats [13]. When the major portion of the radioactivity was rapidly eliminated in the bile, the predominant biliary metabolite of testosterone was androsterone glucuronide. Comparative studies on the metabolism of androsterone glucuronide and androsterone sulphate in female rats will be the subject of a future communication.

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