DISULPHATES OF 16-OXYGENATED KETONIC C_{19} STEROIDS AS BILIARY METABOLITES OF ANDROSTERONE SULPHATE IN FEMALE RATS

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ABSTRACT

The chemical synthesis of 16β-hydroxyandrosterone was described preparatory to studies of the disulphates of the 16-oxygenated ketonic C_{19} steroids present in the bile of female rats dosed with [^{3}H]androsterone sulphate. The biliary metabolites were separated by chromatography on Sephadex LH-20 to afford monosulphate and diconjugate fractions. After solvolysis of the diconjugate fraction, the liberated steroids were separated by partition chromatography on Celite 545 and analyzed by gas chromatography-mass spectrometry. In addition to 3α, 17β-dihydroxy-5α-androstan-16-one isolated previously, 16β-hydroxyandrosterone was identified as a disulphate.

INTRODUCTION

It is well established that the 16β-hydroxy-17-oxosteroid is readily rearranged to the stable epimer, the 17β-hydroxy-16-oxosteroid, by means of alkali or acid (1, 2), and by isolation procedures from urine (3). In a paper dealing with biliary metabolites of androsterone conjugates in female rats, we reported that androsterone sulphate was metabolized to the disulphate of 3α,17β-dihydroxy-5α-androstan-16-one (4). But, we did not preclude the artifact formation by rearrangement of 16β-hydroxyandrosterone to 3α,17β-dihydroxy-5α-androstan-16-one during the isolation procedure, because 3β,16β-dihydroxy-5-androsten-17-one, a model compound, was entirely converted into 3β,17β-dihydroxy-5-androsten-16-one by thin-layer chromatography (TLC) on silica gel plate. Very recently, Mattox et al. purified synthetic 3β,16β-dihydroxy-5-androsten-17-one by partition chromatography on Celite 545 (2). These studies

prompted us to investigate the occurrence of 16β-hydroxyandrosterone in the 16-oxygenated ketonic steroid metabolites of androsterone sulphate by employing the partition chromatography to the separation of the solvolyzed metabolites.

In this report, we describe the synthesis of 16β-hydroxyandrosterone and the identification of 16β-hydroxyandrosterone disulphate as a biliary metabolite of androsterone sulphate in female rats.

MATERIALS AND METHODS

Melting points were determined with a Kofler hot-stage apparatus. Proton magnetic resonance spectra (PMR) were measured for solutions in deuteriochloroform with tetramethylsilane as internal standard on a JEOL JNM-MH-100 spectrometer. Chemical shifts are expressed in δ (ppm): s, singlet; m, multiplet.

The following trivial names are used in this paper: androsterone = 3α-hydroxy-5α-androstan-17-one; androsterone sulphate = 3α-sulphooxy-5α-androstan-17-one; 16α-hydroxyandrosterone = 3α,16α-dihydroxy-5α-androstan-17-one; 16β-hydroxyandrosterone = 3α,16β-dihydroxy-5α-androstan-17-one; 16β-hydroxyandrosterone disulphate = 3α,16β-disulphooxy-5α-androstan-17-one.

Materials. Preparation of [1,2-^{3}H]androsterone sulphate, 5α-androstane-3α,17β-diol, and 16α-hydroxyandrosterone was described previously (4). 3α,17β-Dihydroxy-5α-androstan-16-one and 5α-androstane-3α,16β,17β-triol were prepared by the procedures of Huffman et al. (5) and Lieberman et al. (6), respectively. All other reagents were of analytical grade.

16β-Hydroxyandrosterone. Following the procedure described by Johnson et al. (1), 5α-androst-16-ene-3α,17-diol diacetate (1.24 g) (7) was treated with $Pb(OAc)_4$ (2.0 g) in acetic acid (30 ml) and acetic anhydride (3 ml), and crystallized from methanol to afford 3α,16β-diacetoxy-5α-androstan-17-one (1.0 g), m.p. 169-173°; PMR δ : 0.82 (3H, s, 19-H_3), 0.96 (3H, s, 18-H_3), 2.04 (3H, s, 3-$OCOCH_3$), 2.10 (3H, s, 16-$OCOCH_3$), 4.90-5.10 (2H, m, 3-H & 16-H). Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 70.45; H, 8.76. The removal of the acetate group of 3α,16β-diacetoxy-5α-androstan-17-one was done according to the procedure of Mattox et al. (2). A solution of 3α,16β-diacetoxy-5α-androstan-17-one (500 mg) in chloroform (6 ml) and methanol (60 ml) was added to a solution of semicarbazide hydrochloride (1.5 g) and $NaHCO_3$ (0.7 g) in water (3 ml) and allowed to stand at 20°C for 18 hr. The reaction mixture was evaporated in vacuo to give a residue, which was washed with water. The residue was crystallized from methanol to yield 3α,16β-diacetoxy-5α-androstan-17-one semicarbazone (560 mg), m.p. 148-157°. Anal. Calcd. for $C_{24}H_{37}O_5N_3 \cdot 0.25H_2O$: C, 63.76; H, 8.36; N, 9.29. Found: C, 63.67; H, 8.42; N, 9.10. To a solution of 3α,16β-diacetoxy-5α-androstan-17-one semicarbazone (500 mg) in chloroform (4 ml) was added 0.4N NaOH-methanol (16 ml). After standing at 20°C for 20 hr, the mixture was neutralized with 2N acetic acid-methanol and evaporated in vacuo to afford a crysta-

lline residue, which was washed with water to give 3α,16β-dihydroxy-5α-androstan-17-one semicarbazone (410 mg), m.p. 215-221°. Anal. Calcd. for $C_{20}H_{33}O_3N_3 \cdot H_2O$: C, 62.96; H, 9.25; N, 11.02. Found: C, 62.93; H, 8.82; N, 10.75. To a solution of 3α,16β-dihydroxy-5α-androstan-17-one semicarbazone (400 mg) in acetic acid (15 ml) was added 0.5M aqueous pyruvic acid (4 ml). After standing at 20°C for 24 hr, the reaction mixture was poured into water and extracted with chloroform. The organic layer was washed with cold 5% aqueous $NaHCO_3$, water, and dried. Evaporation of the solvent in vacuo gave a residue (362 mg), which was chromatographed on a column of Celite 545 (100 g mixed with 40 ml of formamide) using benzene-chloroform (3:1, v/v) saturated with formamide as solvent (hold-back volume 190 ml). Hold-back volumes 1.5-3.0 were combined, washed with water, dried, and evaporated in vacuo. The crystalline residue (225 mg) was crystallized from acetone to afford 3α,16β-dihydroxy-5α-androstan-17-one (170 mg), m.p. 169-176°; PMR δ : 0.81 (3H, s, 19-H_3), 0.93 (3H, s, 18-H_3), 3.85-4.10 (2H, m, 3-H & 16-H). Anal. Calcd. for $C_{19}H_{30}O_3 \cdot 0.25H_2O$: C, 73.39; H, 9.89. Found: C, 73.65; H, 9.75. Sodium borohydride reduction of 3α,16β-dihydroxy-5α-androstan-17-one yielded 5α-androstane-3α,16β,17β-triol, identical (mixed m.p. and gas chromatography) with an authentic sample.

Animal experiments. The common bile duct was cannulated in female rats of the Wistar strain weighing 200-230 g (4). After operation, the rats were kept in a restraining cage with free access to water and food pellets. Ethanol solution of [^{3}H]androsterone sulphate (0.25 ml, 0.45 μCi, 8.6 μmole) was diluted with 0.15 ml of saline and injected intraperitoneally 18-20 hr after operation into female rats. Bile was collected at 0-1, 1-2, 2-4, 4-6, 6-24, and 24-48 hr.

Extraction and purification of biliary metabolites. Bile samples obtained from three rats at 0-24 hr were combined and treated as described previously in detail (4). The bile samples were diluted with water and extracted with ether. The aqueous fraction was processed with a column of Amberlite XAD-2 resin, followed by chromatography on Sephadex LH-20 to yield monosulphate and diconjugate fractions. The diconjugate fraction was solvolyzed in acidified ethyl acetate to afford the liberated steroids. The liberated steroids were chromatographed on a column of Celite 545 (40 g mixed with 16 ml of formamide) with benzene-chloroform (3:1, v/v) saturated with formamide as solvent (hold-back volume 75 ml). Twenty ml fractions were collected. Each fraction was washed with water, dried, and evaporated in vacuo and stored as ethanol solution. Fractions 1-2, 3-4, and 5-9 contained mainly 5α-androstane-3α,17β-diol, 3α,17β-dihydroxy-5α-androstan-16-one (and 16α-hydroxyandrosterone), and 16β-hydroxyandrosterone, respectively.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Metabolites separated by chromatography on Celite 545 were analyzed as trimethylsilyl (TMS) and 0-methyloxime-trimethylsilyl (MO-TMS) derivatives (8). GC was performed on a Shimadzu GC-4BM chromatograph with a flame ionization detector using 0.5% CHDMS and 1.5% SE-30 as stationary phases, while GC-MS was performed with a JEOL JMS-D100 spectrometer using 1.5% SE-30 column as previously described (4).

Radioactivity measurements. Radioactivity was counted in an Aloka LSC-502 liquid scintillation spectrometer in a toluene medium (9).

RESULTS AND DISCUSSION

The diacetate of 16β-hydroxyandrosterone was prepared by lead tetra-

acetate oxidation (1) of 5α-androst-16-ene-3α,17-diol diacetate. Hydrolysis of the acetate group was done by formation of a C-17 semicarbazone, alkaline hydrolysis of the acetate group, and removal of the semicarbazone group (2). The configuration of the 16-hydroxy group was established by comparison of the sodium borohydride reduction product with 5α-androstane-3α,16β,17β-triol.

Table 1. Relative retention times (5α-cholestane = 1.00) of the TMS and MO-TMS derivatives of the 16-oxygenated ketonic C_{19} steroids.

Compound	CHDMS		SE-30	
	TMS	MO-TMS	TMS	MO-TMS
16α-Hydroxyandrosterone	0.86	0.57	0.66	0.64
16β-Hydroxyandrosterone	1.00	0.66	0.76	0.85
3α,17β-Dihydroxy-5α-androstan-16-one	1.01	0.82	0.76	0.92

Table 1 shows the GC analyses of the TMS and MO-TMS derivatives of 16α-hydroxyandrosterone, its 16β-epimer, and 3α,17β-dihydroxy-5α-androstan-16-one. The TMS ether of the 16β-hydroxysteroid was not separated from that of the 16-oxosteroid, while the MO-TMS derivatives provided good separation. Mass spectra of the MO-TMS derivatives are listed in Fig. 1A, 1B, and 1D. The molecular ion at m/e 479 is characteristic of the dihydroxy-monooxo C_{19} saturated steroid. The 16α- and 16β-hydroxysteroids give base peak at m/e 448, whereas the 16-oxosteroid affords base peak at m/e 158. Furthermore, several ion abundance differences can be observed among these spectra. The ions at m/e 433 and 343 are more abundant in the 16α-hydroxysteroid than in the other steroids, while the fragments at m/e 142 and m/e 133 are characteristic of the 16β-hydroxysteroid and the 16-oxosteroid, respectively.

The occurrence of epimerization of the 16β-hydroxy-17-oxosteroid to

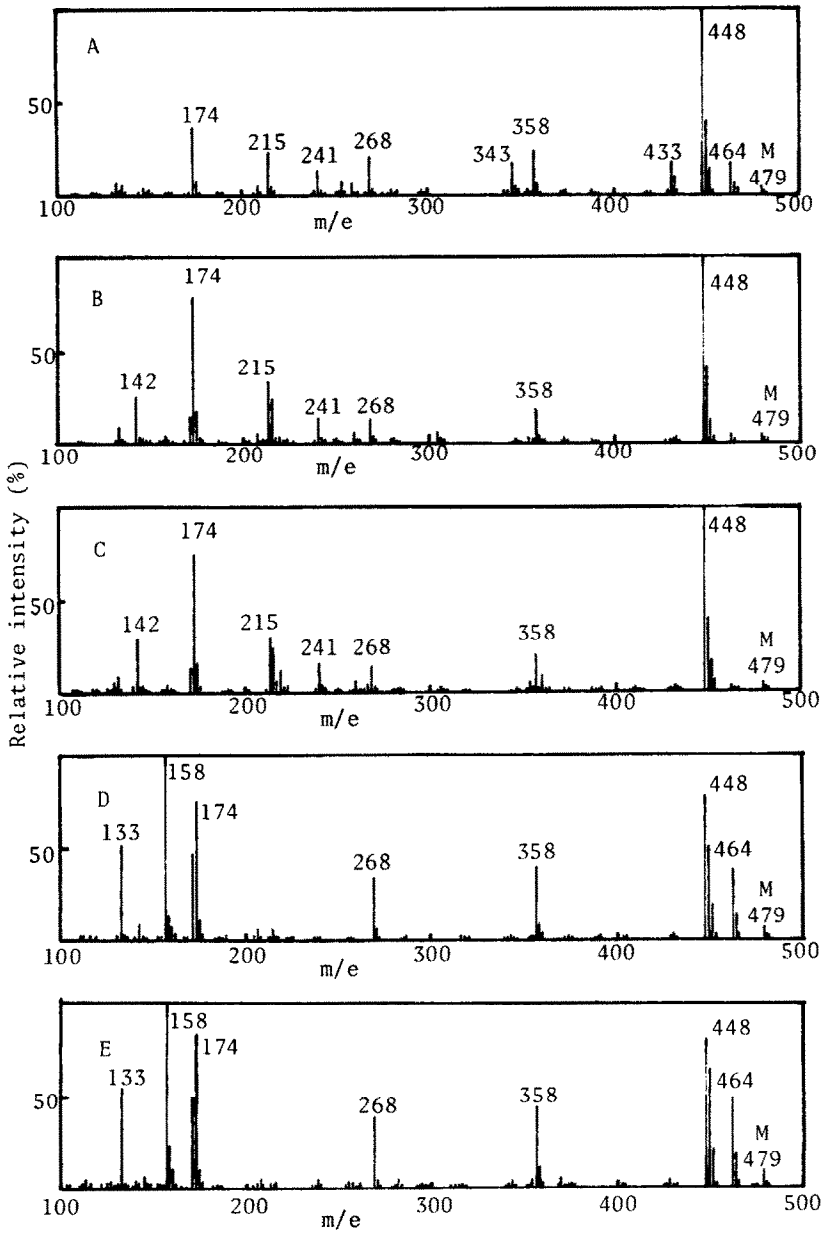

Fig. 1. Mass spectra of the MO-TMS derivatives of 16α-hydroxyandrosterone (A), 16β-hydroxyandrosterone (B), the metabolite identified as 16β-hydroxyandrosterone (C), 3α,17β-dihydroxy-5α-androstan-16-one (D), and the metabolite identified as 3α,17β-dihydroxy-5α-androstan-16-one (E).

the 17β-hydroxy-16-oxosteroid is of crucial problem in the analysis of the 16β-hydroxy-17-oxosteroid. In a previous paper, we reported the complete conversion of 3β,16β-dihydroxy-5-androsten-17-one to 3β,17β-dihydroxy-5-androsten-16-one by TLC on silica gel plate (4). However, successful application of partition chromatography to the purification of synthetic 16β-hydroxyandrosterone prompted us to employ the partition chromatography to the separation of solvolyzed metabolites. To check to what extent artifact formation occurred during the isolation procedure, 16β-hydroxyandrosterone was subjected to incubation in solvolytic medium, followed by partition chromatography. GC analysis revealed that only a few % of 16β-hydroxyandrosterone was epimerized to 3α,17β-dihydroxy-5α-androstan-16-one.

Following intraperitoneal injection of [^{3}H]androsterone sulphate into female rats with bile fistulas, the excretion of the radioactivity in the bile was about 76% during 24 hr. The biliary radioactivity consisted of monosulphate (65%) and diconjugate (35%) fractions. By solvolysis of the diconjugate fraction, 58% of the radioactivity appeared in the liberated steroid fraction. These figures were similar to those described in a previous paper (4). Partition chromatography of the liberated steroid fraction yielded 79, 2, and 4% of the radioactivity in 5α-androstane-3α,17β-diol, 3α,17β-dihydroxy-5α-androstan-16-one, and 16β-hydroxyandrosterone fractions, respectively. The production of large amounts of 5α-androstane-3α,17β-diol was a pronounced feature in this study. One explanation for this may be that we administered the increased amounts of androsterone sulphate into rats in this study. Eriksson et al. (10) reported the change of metabolic patterns with different concentrations of progesterone in perfused rat liver. The each fraction

obtained by partition chromatography was analyzed as the TMS and MO-TMS derivatives by GC and GC-MS. The identified metabolites provided relative retention times and mass spectra identical with those of the respective reference steroids. Details of the identification of 5α-androstane-3α,17β-diol were not given here, because it was already done previously (4). 16β-Hydroxyandrosterone and 3α,17β-dihydroxy-5α-androstan-16-one were identified in the respective fractions, though the 16β-hydroxyandrosterone fraction was contaminated with small amounts of 3α,17β-dihydroxy-5α-androstan-16-one. No other steroid metabolites (16α-hydroxyandrosterone, etc.) were identified in these fractions. The mass spectra of the MO-TMS derivatives of the identified metabolites are shown in Fig. 1C and 1E. Although our results do not preclude the occurrence of the disulphate of 3α,17β-dihydroxy-5α-androstan-16-one, the production of 16β-hydroxyandrosterone disulphate as a biliary metabolite of androsterone sulphate in female rats is firmly established. To our knowledge, this is the first identification of 16β-hydroxyandrosterone as a disulphate in rat bile. Thus, the major portion of 3α,17β-dihydroxy-5α-androstan-16-one identified in the previous study (4) must be the artifact formed from 16β-hydroxyandrosterone.

Several 17β-hydroxy-16-oxosteroids were isolated from the incubation of C_{19} steroids with rat liver (11) or human foetal liver (12), and from rat bile (13). The present study suggests the occurrence of the 16β-hydroxy-17-oxosteroids in these 16-oxygenated steroid metabolites. Recently, 16β-hydroxylating enzymes active on steroid sulphates were found in human foetal liver (12, 14). Thus, androsterone sulphate must undergo direct metabolism by liver microsomal 16β-hydroxylase to 16β-hydroxyandrosterone 3-sulphate, which must be consequently conjugated

with sulphuric acid to 16β-hydroxyandrosterone disulphate by the sulphotransferase located in the soluble fraction of the liver cell.

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