J. Steroid Biochem. Molec. Biol. Vol. 42, No. 5, pp. 533–546, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

STUDIES ON ANABOLIC STEROIDS—12. EPIMERIZATION AND DEGRADATION OF ANABOLIC 17β-SULFATE-17α-METHYL STEROIDS IN HUMAN: QUALITATIVE AND QUANTITATIVE GC/MS ANALYSIS*

HONGGANG BI and ROBERT MASSÉ†
Institut National de la Recherche Scientifique, INRS-Santé, Université du Québec, 245 Boulevard Hymus, Pointe-Claire, Québec, Canada, H9R 1G6

(Received 18 November 1991)

Summary—The epimerization and dehydration reactions of the 17β-hydroxy group of anabolic 17β-hydroxy-17α-methyl steroids have been investigated using the pyridinium salts of 17β-sulfate derivatives of methandienone **1**, methyltestosterone **4**, oxandrolone **7**, mestanolone **10** and stanozolol **11** as model compounds. Rearrangement of the sulfate conjugates in buffered urine (pH 5.2) afforded the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes in a ratio of 0.8:1. These data indicated that both epimerization and dehydration of the 17β-sulfate derivatives were not dependent upon the respective chemical features of the steroids studied, but were instead inherent to the chemistry of the tertiary 17β-hydroxy group of these steroids. Interestingly, *in vivo* studies carried out with human male volunteers showed that only methandienone **1**, methyltestosterone **4** and oxandrolone **7** yielded the corresponding 17-epimers **2**, **5** and **8** and the 18-nor-17,17-dimethyl-13(14)-enes **3**, **6** and **9** in ratios of 0.5:1, 2:1 and 2.7:1, respectively. No trace of the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes derivatives of mestanolone **10** and stanozolol **11** was detected in urine samples collected after administration of these steroids. These data suggested that the *in vivo* formation of the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes derivatives of 17β-hydroxy-17α-methyl steroids is also dependent upon phase I and phase II metabolic reactions other than sulfation of the tertiary 17β-hydroxyl group, which are probably modulated by the respective chemical features of the steroidal substrates. The data reported in this study demonstrate that the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes are not artifacts resulting from the acidic or microbial degradation of the parent steroids in the gut as previously suggested by other authors, but arise from the rearrangement of their 17β-sulfate derivatives. Unchanged oxandrolone **7** was solely detected in the unconjugated steroid fraction whereas unchanged steroids **1**, **4** and **11** were recovered from the glucuronide fraction. These data are indirect evidences suggesting that the glucuronide conjugates of compounds **1** and **4** are probably enol glucuronides and that of compound **11** is excreted in urine as a *N*-glucuronide involving its pyrazole moiety. The urinary excretion profiles of the epimeric and 18-nor-17,17-dimethyl-13(14)-ene steroids are presented and discussed on the basis of their structural features.

INTRODUCTION

The epimerization of the tertiary 17β-hydroxy group in anabolic 17β-hydroxy-17α-methyl steroids appears to be an important biotransformation route in humans. The occurrence of this reaction was first reported by Rongone and Segaloff [1] who isolated 17-epimethandienone **2** from the urine of a cancer patient who had been administered methandienone **1**. However, their proposed structural assignment of **2** was only confirmed in 1971 when MacDonald *et al.* [2] reported its chemical synthesis and isolation from human urine. These findings were further investigated by Dürbeck *et al.* [3, 4] who performed a comprehensive GC/MS analysis of methandienone urinary metabolites and reported the characterization of 17-epi-4-chloro-methandienone, an abundant metabolite of 4-chloro-methandienone in humans [5]. In previous metabolic studies from this laboratory, we reported the characterization of the 17-epimers of methandienone, oxandrolone and stanozolol following administration of the parent steroids to human male volunteers [6–8].

*Part of this paper will be included in the Ph.D. thesis of H.Bi.

†To whom correspondence should be addressed.

More recently, Harrison and Fennessey [9] also reported the presence of **2** as a metabolite of **1** in urine samples collected from body builders who were given a relatively large dose of **1** [9]. Epimerization at C_{17} is not a reaction which is specific to the parent 17β-hydroxy-17α-methyl steroids. Indeed, some authors reported lately the characterization of the 17-epimers of other major urinary metabolites of methandienone **1**, methyltestosterone **4**, mestanolone **10** and stanozolol **11** [8, 10–12].

Other authors reported that 18-nor-17,17-dimethyl-13(14)-ene steroids are the major products resulting from the retropinacol rearrangement of 17-hydroxy-17-methyl steroids in acidic conditions [13, 14]. The mechanism of the 17-epimerization of 17β-hydroxy-17α-methyl anabolic steroids in humans remained unclear until Edlund *et al.* [15] reported the formation of **2** and **3** as major degradation products of the 17β-sulfate conjugate of **1** in equine urine. These authors showed that the 17β-sulfate derivative of methandienone undergoes spontaneous solvolysis in equine urine to give **2** and **3**. Although sulfation of steroids is much more active in horse than in human [15], this conjugation reaction is of prime importance in the biosynthesis and/or metabolism of endogenous and exogenous steroids in humans.

Biological glucuronidation and sulfation of steroids are effected by the transfer of glucuronic acid and sulfate moieties from uridine diphosphoglucuronic acid (UDP-glucuronic acid) and 3-phosphoadenosine-5-phosphosulfate (PAPS), respectively [16]. Examination of molecular models of 17β-hydroxy-17α-methyl steroids and UDP-glucuronic acid shows that one of the critical aspects of the conjugation reaction is the steric hindrance from both the tertiary 17β-hydroxy group and the UDP moiety, which appears to prevent the formation of glucuronic acid conjugate at the C_{17} position. This hypothesis is supported by data previously reported about methandienone, oxandrolone and stanozolol urinary excretion [7, 8, 10]. On the other hand, the sulfate moiety in PAPS is much less hindered and relatively vulnerable to nucleophilic attack by the tertiary 17β-hydroxy group of 17β-hydroxy-17α-methyl steroids so that sulfation at that position is more likely to occur than glucuronidation. Conversely, this also suggests that glucuronidation in compounds **1**, **4** and **11** may occur with other functional groups, namely the enol function of the 3-keto-4-ene steroids **1** and **4** and the pyrazole moiety of stanozolol **11**.

The objective of this study was to investigate the mechanistic aspects of the epimerization reaction which appears to be characteristic of anabolic 17β-hydroxy-17α-methyl steroids in humans. The secondary goal was to establish relationships between the occurrence in urine and the relative amounts of the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes and the chemical structures of the studied steroids. The formation of 17-epimeric and 18-nor-17,17-dimethyl-13(14)-ene steroids was investigated by GC/MS analysis of urine specimens collected after administration of compounds **1**, **4**, **7**, **10** and **11**. Excretion profiles of the parent, 18-nor-17,17-dimethyl-13(14)-ene and 17-epimeric

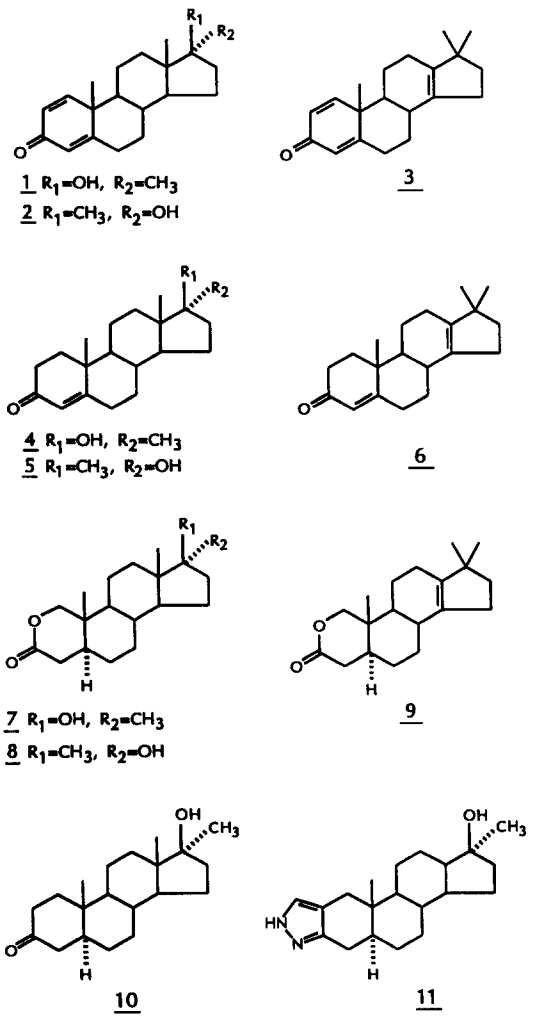

Fig. 1. The chemical structures of five 17β-hydroxy-17α-methyl anabolic steroids (**1**, **4**, **7**, **10** and **11**) and corresponding urinary 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes. See Experimental and Table 1 for the names of particular steroids.

steroids are presented as well as their mass spectral features.

EXPERIMENTAL

Steroids and materials

Methandienone **1**, methyltestosterone **4**, oxandrolone **7**, mestanolone **10**, 5α-androstan-17-one (internal standard 1, IS1) and 17α-methyl-5α-androstan-3β,17β-diol(internal standard 2, IS2) were purchased from Steraloids (Wilton, NH); stanozolol **11** was obtained from Winthrop Labs. (Aurora, Canada). The 17β-sulfate derivatives of **1**, **4**, **7**, **10** and **11**, the corresponding 17α-hydroxy-17β-methyl and 18-nor-17,17-dimethyl-13-ene steroids were synthesized in our laboratory according to methods reported previously [17]. The chemical structures of parent steroids and related urinary 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes are presented in Fig. 1.

Sep-Pak C_{18}® cartridges were supplied by Waters Assoc. (Milford, MA); β-glucuronidase (from *E. Coli*) and arylsulfatase (from *Helix Pomatia*) were purchased from Boehringer Mannheim (Laval, Canada); *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL); dithioerythritol and trimethylsilyl iodide (TMSI) were purchased from Aldrich Chemical Co. (Milwaukee, WI); *N,O*-bis([$^2H_9$]trimethylsilyl)-acetamide(BSA-d_9) and [$^2H_9$]trimethylsilyl chloride(TMSCl-d_9) were obtained from MSD-Isotope (Pointe-Claire, Canada); inorganic salts were of analytical grade (J. T. Baker, Phillipsburg, NJ or Caledon Labs., Georgetown, Canada); all the organic solvents (HPLC grade, Caledon Labs.) were used as provided.

Steroid administration and collection of urine samples

Blank urine samples were collected prior to steroid administration to healthy male volunteers (aged 24 to 35 years old). A single oral dose of methandienone **1** (25 mg), 17α-methyltestosterone **4** (10 mg), oxandrolone **7** (10 mg), mestanolone **10** (10 mg) and stanozolol **11** (20 mg) was administered. Each volunteer received only one steroid. The post-administration urine samples were collected at regular time intervals up to 72 h after administration. Urine samples were stored at −20°C immediately after voiding until analyzed.

*Quantitation of steroids **1** to **9** in urine samples*

Stock solution and standard curves. Stock solutions of each steroid (**1** to **9**, 50 μg/ml and 1.0 mg/ml), IS1 (50 μg/ml) and IS2 (25 μg/ml) were prepared in absolute methanol and stored at 4°C until used. All standard curves (5 to 500 ng/ml) were prepared as follows: aliquots of stock solutions were transferred to a 300 μl conical vial so as to obtain 25 to 2500 ng of the corresponding steroid in each vial, to which 500 ng of IS1 and 250 ng of IS2 were added. The solvent was evaporated to dryness under a N_2 flow at 40°C and the residue was treated with 50 μl of a mixture of MSTFA/TMSI to prepare the TMS-enol-TMS-ether derivatives. For each concentration point, samples were prepared in triplicate and 1 μl was injected for GC/MS analysis (duplicate injections for each sample). Quantitation of the steroids was performed by selective ion monitoring (SIM) using the most abundant and characteristic ions of each steroid TMS derivatives. Peak area ratios of the selected ions to those of the two internal standards (*m/z* 331 and 143 for IS1 and IS2, respectively) were measured for each concentration point (Table 1 lists the major ions of each steroid as TMS-ether and TMS-enol-TMS-ether derivatives and ions selected used for quantitation are indicated). Standard curves were linear for each steroid in the 5–500 ng/ml range. The following regression equations were obtained:

Steroid	Equation		ISTD
1	$C = 87.0\ A - 0.61$	($r^2 = 0.9995$)	IS1
2	$C = 80.0\ A - 0.07$	($r^2 = 0.9995$)	IS1
3	$C = 990.0\ A + 2.63$	($r^2 = 0.9995$)	IS1
4	$C = 111.0\ A - 2.35$	($r^2 = 0.9995$)	IS1
5	$C = 114.0\ A - 1.74$	($r^2 = 0.9985$)	IS1
6	$C = 62.5\ A - 2.53$	($r^2 = 0.9995$)	IS1

7	$C = 239.0\ A + 4.16$	$(r^2 = 0.9995)$	IS2
8	$C = 227.0\ A + 1.07$	$(r^2 = 0.9995)$	IS2
9	$C = 79.4\ A - 6.39$	$(r^2 = 0.9985)$	IS1

In these equations, C is the concentration of the steroid in ng/ml and A is the peak area ratio of the selected ions from the measured steroid and the internal standard.

Quantitation of unconjugated steroids. In a typical experiment, a urine sample (2–5 ml) was passed through a Sep-Pak C_{18} cartridge (prewashed successively with 5 ml of MeOH and water). The cartridge was then washed with 5 ml of water and 2 ml of hexane to remove residual water in the cartridge. Unconjugated and conjugated steroids were eluted with 5 ml of MeOH. The methanolic solution was evaporated under a stream of N_2 at 40°C. The residue was dissolved in 1 ml of pH 5.2 acetate buffer (0.2 M) and extracted with 2 × 5 ml of diethyl ether. The organic phase was decanted, dried over Na_2SO_4 and evaporated to dryness under N_2 (the aqueous solution was used to quantitate conjugated steroids). The residue was dissolved in 200 μl of MeOH and transferred to a 300 μl vial. Internal standards (500 ng of IS1 and 250 ng of IS2) were added and the solvent was evaporated under N_2. The residue was then derivatized with 50 μl of a mixture of MSTFA and TMSI and 1 μl of the resulting solution was analyzed by GC/MS.

Quantitation of conjugated steroids. Residual traces of diethyl ether were removed from the above aqueous solution with a stream of nitrogen and 100 μl of a β-glucuronidase solution was added and the resulting mixture was incubated at 37°C for 16 h. The hydrolysate was then cooled to room temperature and extracted with 2 × 5 ml of ether. The organic phase was decanted, dried over sodium sulfate and evaporated to dryness. The residue was transferred to a 300 μl vial and solutions of both IS1 and IS2 standards were added. The solvent was evaporated. The dried residue was derivatized and analyzed as above.

Residual traces of diethyl ether were removed from the remaining aqueous phase with a stream of nitrogen and 100 μl of an aryl sulfatase solution was added. The mixture was incubated for 16 h at 37°C and the neutral steroids were extracted with 2 × 5 ml of ether. The ether layer was analyzed as above.

Recoveries and detection limits. Aliquots (5 ml) of blank urine samples were fortified with steroids **1** to **9** so as to obtain 10, 50 and 250 ng/ml. Three aliquots were prepared for each concentration. The resulting mixtures were equilibrated at 37°C for 4 h, and successively extracted, derivatized and analyzed as described above. Duplicate analyses of each sample were carried out. Recoveries are listed in Table 2.

Detection limits were also determined using the selected ion monitoring and repetitive scanning modes. These experiments were performed as follows: extracts from a blank urine specimen were fortified with increasing amounts of each steroid so as to obtain concentration ranging from 0.25 to 20 ng/ml. TMS-enol-TMS-ether derivatives were prepared to achieve a higher sensitivity. Selected ions used to determine the detection limits were also those used for quantitative analysis. Table 3 lists the detection limits obtained and gives the corresponding urinary concentrations for each of the steroids studied.

In vitro tests of degradation of the 17β-sulfates in human urine

Five aliquots of blank urine (5 ml) were buffered to pH 5.2 and fortified with various amounts of the synthetic 17β-sulfates equivalent to 200 ng/ml of the parent steroids **1**, **4**, **7**, **10** and **11**. The resulting solutions were incubated at 37°C for 16 h. Each sample was then extracted with diethyl ether (2 × 5 ml). The ether extract was processed as described above and the derivatized residue was analyzed by GC/MS. The solvolysis reactions afforded the corresponding 17-epimers and dehydration products (18-nor-17,17-dimethyl-13(14)-enes) in a 0.8:1 ratio from each of the sulfate derivatives studied.

Derivatization and GC/MS analysis

TMS-enol-TMS-ether derivatives were prepared using a method described previously [7, 8, 18]. Perdeuterated(d_9-) TMS-ether derivatives were prepared to obtain further information about fragmentation routes and structural features of the steroids of interest and were prepared as follows: a mixture containing 50 μl of BSA-d_9 and TMSI mixture (100:1, v/v) was added to dried reference steroids or urinary extracts (containing 0.5 mg of

Table 1. Identity and partial GC/MS data of urinary steroids 1 to 9

Steroids	Deriv.[a]	RT[b]	MU[c]	M+·[d]	Characteristic ions
1. Methandienone	t	26.90	28.72	372 (1)	282 (28), 219 (14), 194 (11), 161 (18), 143 (100).
	e	25.79	28.09	444 (54)	339 (25), 229 (14), 206 (100)*, 191 (16), 143 (25).
2. 17-Epimethandienone	t	24.58	27.41	372 (1)	282 (39), 219 (15), 194 (11), 161 (24), 143 (100).
	e	23.81	26.98	444 (42)	339 (56), 229 (10), 206 (100)*, 191 (17), 143 (14).
3. 8-Nor-17,17-dimethyl-androsta-1,4,13(14)-triene-3-one	un	19.94	24.80	282 (71)	267 (55), 171 (31), 161 (95), 122 (72), 91 (100).
	e	19.06	24.29	354 (40)	339 (62)*, 206 (21), 194 (19), 148 (56), 133 (100).
4. Methyltestosterone	t	26.30	28.38	374 (2)	359 (7), 317 (15), 304 (16), 284 (60), 143 (100).
	e	26.15	28.29	446 (67)	356 (11), 314 (11), 301 (100)*, 208 (5), 143 (8).
5. 17-Epimethyltestosterone	t	24.11	27.14	374 (2)	359 (8), 317 (15), 304 (18), 284 (68), 143 (100).
	e	24.28	27.24	446 (77)	356 (12), 314 (11), 301 (100)*, 208 (7), 143 (7).
6. 18-Nor-17,17-dimethyl-androsta-4,13(14)-diene-3-one	un	19.38	24.48	284 (38)	269 (100), 251 (7), 161 (11), 105 (16), 91 (27).
	e	19.37	24.47	356 (79)	341 (11), 208 (100)*, 194 (13), 193 (18), 133 (15).
7. Oxandrolone	t	27.86	29.27	378 (3)	363 (18), 321 (12), 308 (21), 176 (5), 143 (100)*.
8. 17-Epioxandrolone	t	25.75	28.07	378 (6)	363 (21), 321 (16), 308 (23), 176 (8), 143 (100)*.
9. 18-Nor-17,17-dimethyl-2-oxa-5α-androst-13(14)-ene-3-one	un	20.80	25.28	288 (9)	273 (100)*, 161 (6), 148 (3), 133 (5), 105 (9), 91 (12).

[a] Derivatives are: t, TMS-enol-TMS-ether and un, underivatized steroid.
[b] Retention time.
[c] Methylene units were determined by linear interpolation of the retention time of the steroid derivatives between the retention time of C_{24} to C_{30} *n*-alkanes.
[d] Molecular ions.
*Selected ions monitored for quantitation and determination of detection limits.

Table 2. Recovery of steroids 1 to 9 from human urine

Steroids[a]	Concentration[b] (ng/ml)	Recovery[c] (%)	CV[d] (%)
1	10	63.2 ± 3.3	5.2
	50	73.9 ± 2.5	3.5
	250	79.8 ± 2.9	3.6
2	10	65.7 ± 3.4	5.2
	50	71.5 ± 3.0	4.2
	250	79.1 ± 2.5	3.2
3	10	67.9 ± 3.9	5.7
	50	71.8 ± 3.7	5.2
	250	77.1 ± 4.2	5.4
4	10	62.5 ± 3.4	5.4
	50	68.7 ± 3.2	4.7
	250	74.8 ± 2.8	3.7
5	10	63.9 ± 3.4	5.3
	50	69.3 ± 2.5	3.6
	250	75.1 ± 2.8	3.7
6	10	63.6 ± 3.8	6.0
	50	66.0 ± 3.1	4.7
	250	68.8 ± 3.1	4.5
7	10	71.1 ± 3.3	4.6
	50	76.8 ± 3.1	4.0
	250	79.4 ± 2.5	3.1
8	10	71.4 ± 2.7	3.8
	50	74.9 ± 3.4	4.5
	250	79.2 ± 1.8	2.3
9	10	68.1 ± 3.9	5.7
	50	72.6 ± 4.0	5.5
	250	79.4 ± 3.4	4.3

[a] See Table 1 for steroid identity.
[b] 5 ml aliquots of urine were spiked with a specific amount of the steroid.
[c] Samples were prepared in triplicate for each concentration and each sample was analyzed in duplicate so that the determined recoveries are the mean of 6 analyses.
[d] Coefficient of variation.

dithioerythritol) and the resulting mixture was heated at 70°C for 30 min. The methods used to prepare the TMS-ether (MSTFA:TMSCl) and the corresponding d_9-TMS-ether (BSA-d_9:TMSCl-d_9) derivatives were also reported in previous papers [18, 19]. GC/MS conditions were as previously reported [7, 8, 18].

RESULTS

Derivatization and mass spectral analysis of compounds 1–9

The analytical approach used for the selective detection and quantitation of the 17-epimeric and 18-nor-17,17-dimethyl-13(14)-ene steroids in human urine was based on the preparation of their TMS-enol-TMS-ether or TMS-ether derivatives and the monitoring of their characteristic and intense fragment ions which are produced upon electron-impact ionization (EI) (Table 1). Their chromatographic and mass spectrometric properties were determined using reference compounds previously synthesized in our laboratory [17]. The TMS-enol-TMS-ether

Table 3. Detection limits for steroids 1 to 9[a]

Steroids	SIM[b] On column (pg)	SIM[b] In urine (ng/ml)	Scan[c] On column (ng)	Scan[c] In urine (ng/ml)
1	25	0.25	0.5	5
2	25	0.25	0.5	5
3	50	0.50	1.0	10
4	25	0.25	0.5	5
5	25	0.25	0.5	5
6	50	0.50	0.5	5
7	100	1.00	2.0	20
8	100	1.00	2.0	20
9	100	1.00	2.0	20

[a]TMS-enol-TMS-ether derivatives were used to determine detection limits for compounds 1, 2, 4 and 5; TMS-enol derivatives were used for 4 to 6; TMS-ether derivatives were used for 7 to 8; and 9 was analyzed without derivatization.

[b]See Table 1 for the selected ions for each steroid. Detection limits were measured at a signal to noise (S/N) ratio of 3.5:1.

[c]At the detection limit, for each steroid, reasonable mass spectrum were obtained and proper ratios were maintained for the major ions listed in Table 1.

derivatives were selected for the detection and quantitation of the 3-keto steroids **1–6** because they afforded mass spectra with intense and structurally informative high-mass ions. In addition, these derivatives are produced with total enolization of the 3-keto group, which is an essential prerequisite for quantitation purposes. As shown in Table 1, the corresponding TMS-ether derivatives do not provide similar advantages because they exhibit low intensity molecular ions and prominent ions at low m/z, to the exception of compounds **3** and **6**, the mass spectra of which show intense molecular and $[M-15]^+$ ions. On the other hand, compounds **7** and **8** which are not keto steroids were quantitated as TMS-ether derivatives, and steroid **9** was analyzed underivatized.

Recovery and detection limit of compounds 1–9

Recoveries for each pair of the 17-epimeric steroids **1**, **2**, **4**, **5**, **7** and **8** were virtually identical and progressively increased from 63 to 79% with increasing concentration of the steroids. Recoveries for the 18-nor-17,17-dimethyl-13(14)-enes **3**, **6** and **9** were of the same order of magnitude and varied accordingly. It was also of interest to determine the detection limit of these steroids in urine both in the SIM and repetitive scanning modes (Table 3). The epimeric steroids **1**, **2**, **4** and **5** were analyzed as the TMS-enol-TMS-ether derivatives were detected at concentrations as low as 25 and 500 pg (amounts injected on column) in the SIM and repetitive scanning mode, respectively.

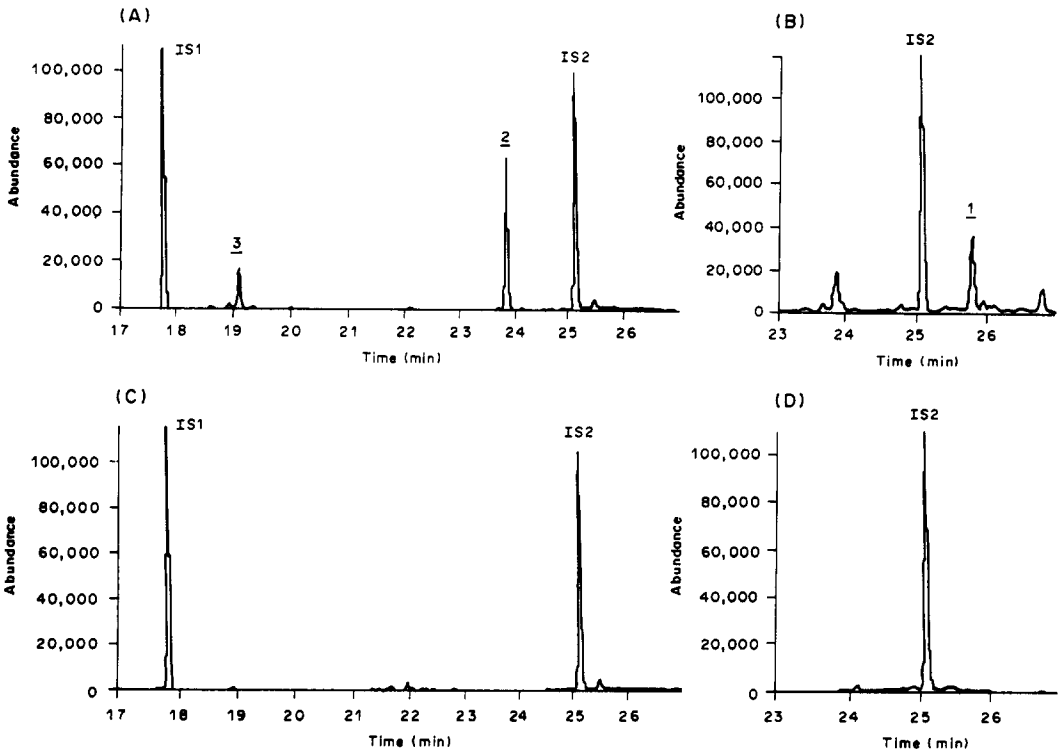

Fig. 2. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of (A) unconjugated and (B) glucuronide fraction of the urine sample 6.5 h after methandienone administration. (C) and (D) are the corresponding chromatograms obtained from analysis of a blank urine. See Table 1 for the identification of labeled peaks. IS1 and IS2 are the internal standards. See Experimental for analytical conditions.

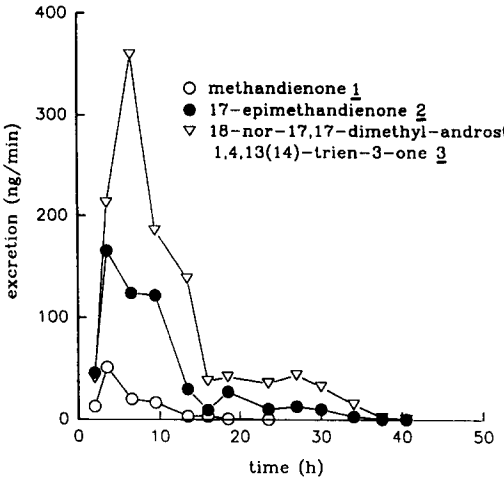

Fig. 3. Excretion curves of 1 (glucuronide fraction) and two metabolites 2 and 3 (unconjugated fraction) after oral administration of 25 mg of 1.

Table 4. Total urinary excretion of steroids 1 to 9

Steroids	Total excretion (μg)	% Dose	Ratios[a]	Ratio[b]
1	14.15	0.06		
2	85.68	0.34	2/3 = 0.54 ± 0.06:1	0.8:1
3	157.7	0.63		
4	9.31	0.09		
5	9.53	0.10	5/6 = 2.0 ± 0.3:1	0.8:1
6	4.64	0.05		
7	2646	26.5		
8	385.9	3.90	8/9 = 2.7 ± 0.8:1	0.8:1
9	157.3	1.60		

[a]Relative ratios of corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes.
[b]Relative ratios obtained from the solvolysis of the 17β-sulfate derivatives of steroids 1, 4 and 7 [18].

On the other hand, steroids devoid of a 17-hydroxyl or 3-keto group such as compounds 3, 6, 7, 8 and 9 were detected with less sensitivity. The lowest concentrations detected with

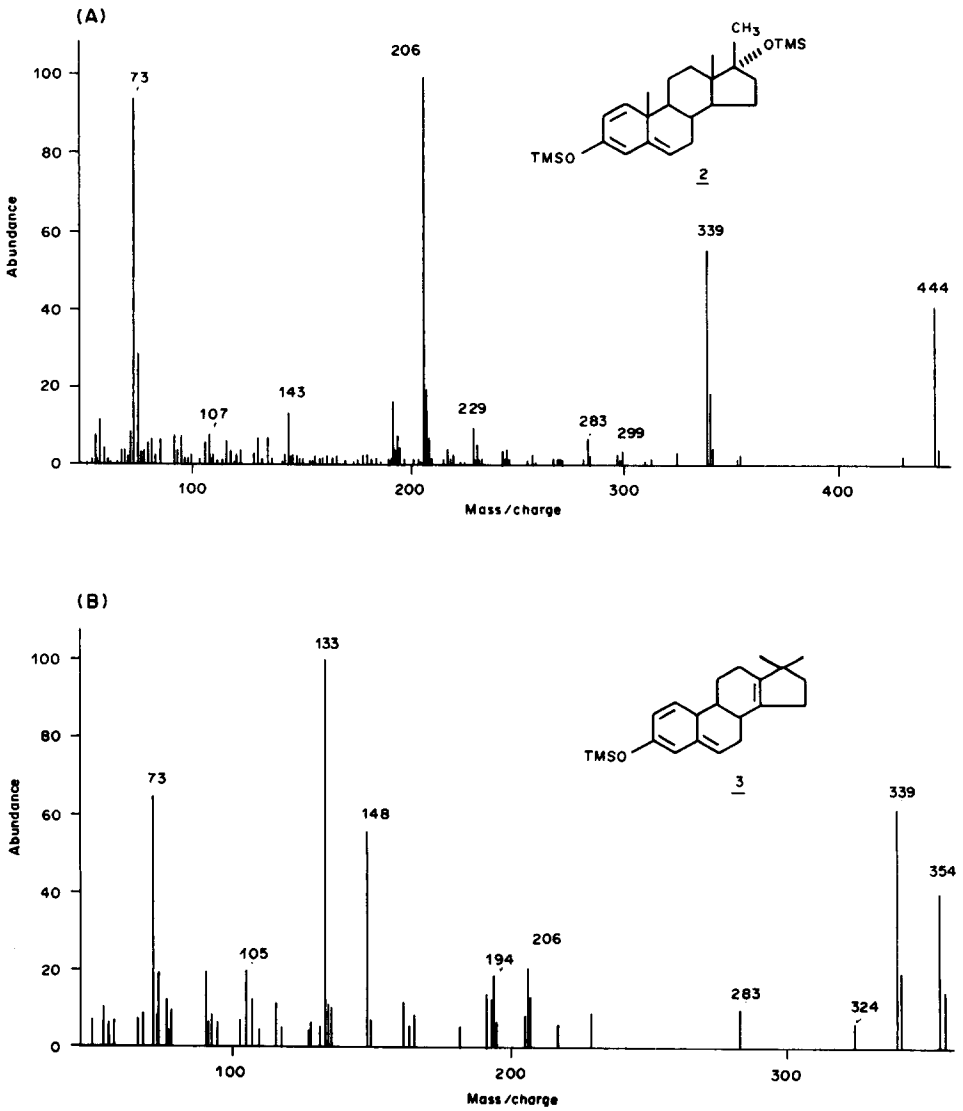

Fig. 4. Mass spectra of the TMS-enol-TMS-ether derivatives of (A) 17-epimethandienone 2 and (B) 18-nor-17,17-dimethyl-androsta-1,4-13(14)-trien-3-one 3.

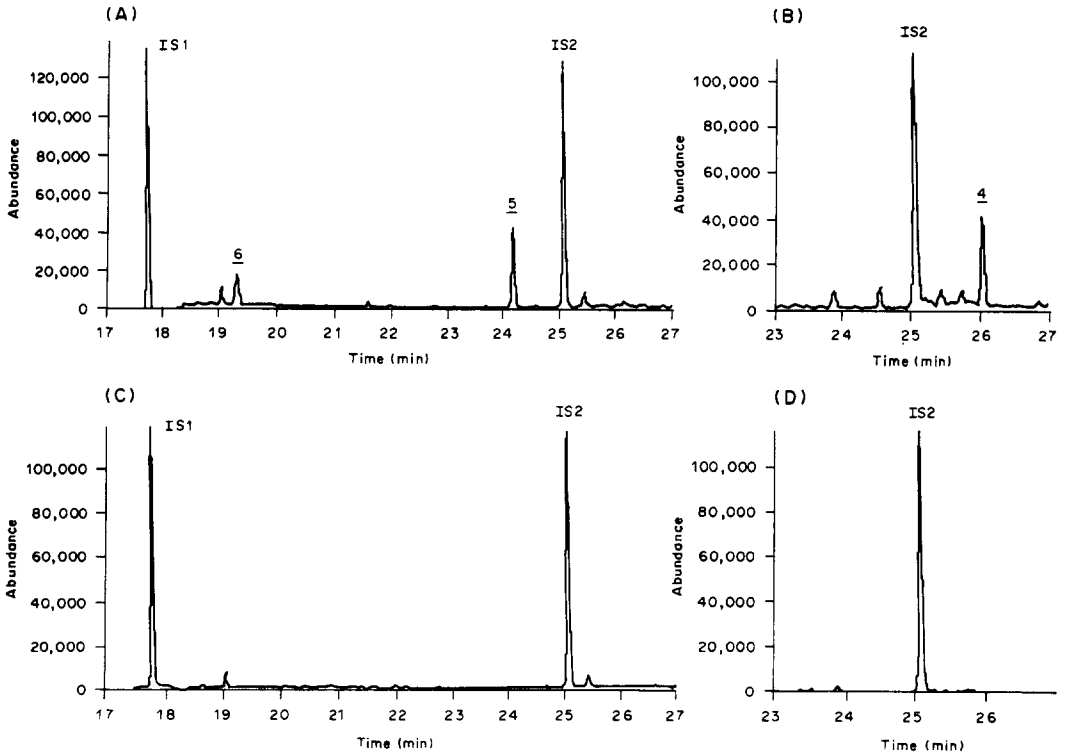

Fig. 5. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of (A) unconjugated and (B) glucuronide fraction of the urine sample 3 h after the administration of methyltestosterone. (C) and (D) are the chromatograms obtained from the analysis of a blank urine. See Table 1 for peak identification. Other conditions as in Fig. 2.

a signal-to-noise ratio of 3.5:1 were in the 50–100 pg in the SIM mode and 1–2 ng in the repetitive scanning mode.

Methandienone 1

Figure 2 presents typical ion chromatograms illustrating the detection of unchanged methandienone **1** in the glucuronide fraction and that of epimethandienone **2** and dehydration product **3** in the unconjugated steroid fraction. Their urinary excretion curves (Fig. 3) indicate that they can be detected for about 24 to 41 h after methandienone administration. Excretion rates of these steroids in urine were maximum between 2 and 6 h after administration. The above excretion profile of epimethandienone **2** was in agreement with the data recently reported by Schänzer *et al.* [12]. The cumulative urinary excretion of compound **3** which has been previously reported as an artifact by Dürbeck *et al.* [3, 4], corresponded to 0.63% of methandienone dose (Table 4). It is of interest to note that **2** and **3** were excreted in a 0.5:1 ratio whereas they were produced in a 0.8:1 ratio by hydrolysis of methandienone 17β-sulfate derivative. This suggests that the *in vivo* formation of **2** and **3** is not only dependent upon the intrinsic reactivity of the tertiary 17β-sulfate group but also on biochemical factors as further discussed below.

The identity of compounds **1–3** was determined by comparison with reference steroids. The mass spectrum of the TMS-enol-TMS-ether

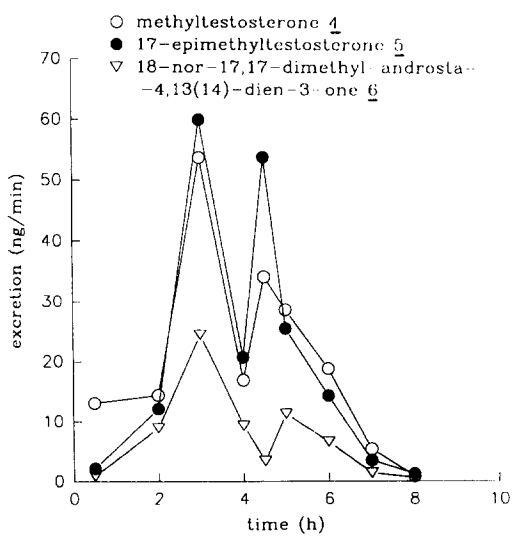

Fig. 6. Excretion curves of **4** (glucuronide fraction) and two metabolites **5** and **6** (unconjugated fraction) after oral administration of 10 mg of **4**.

derivative of compound **2** [Fig. 4(A)] which is dominated by a prominent ion at m/z 206 arising from the cleavage of the C_9–C_{10} and C_7–C_8 bonds, comprises the A-ring and the C_6 and C_7 carbons [10]. The mass spectrum of compound **3** TMS-enol derivative show an intense ion at m/z 148 which results from the cleavage of the same chemical bonds and comprises the C- and D-rings. Subsequent elimination of a methyl radical gives rise to the ion of m/z 133 [Fig. 4(B)]. These mass spectral data are in accordance with those of the corresponding reference steroids.

Methyltestosterone **4**

This steroid which is structurally similar to methandienone (Fig. 1) was also partially excreted unchanged in the glucuronide fraction [Fig. 5(B)], whereas its epimer **5** and dehydration product **6** were isolated from the unconjugated steroid fraction [Fig. 5(A)]. Contrary to methandienone, compounds **4**–**6** were excreted in urine only over a period of 8 h, with maximum excretion rates observed 3 h after methyltestosterone administration. An interesting feature of the excretion profile of compound **5** and **6** is that they were steadily excreted in a 2:1 ratio throughout their 8 h excretion period (Table 4). In addition, the cumulative excretion of compounds **4**–**6** was relatively small with respect to that of methandienone metabolites **1**–**3** (Fig. 6).

The mass spectrum of the TMS-enol-TMS-ether and TMS-enol derivative of compounds **5** and **6** are presented in Figs 7(A) and (B), respectively. The mass spectrum of **5** is

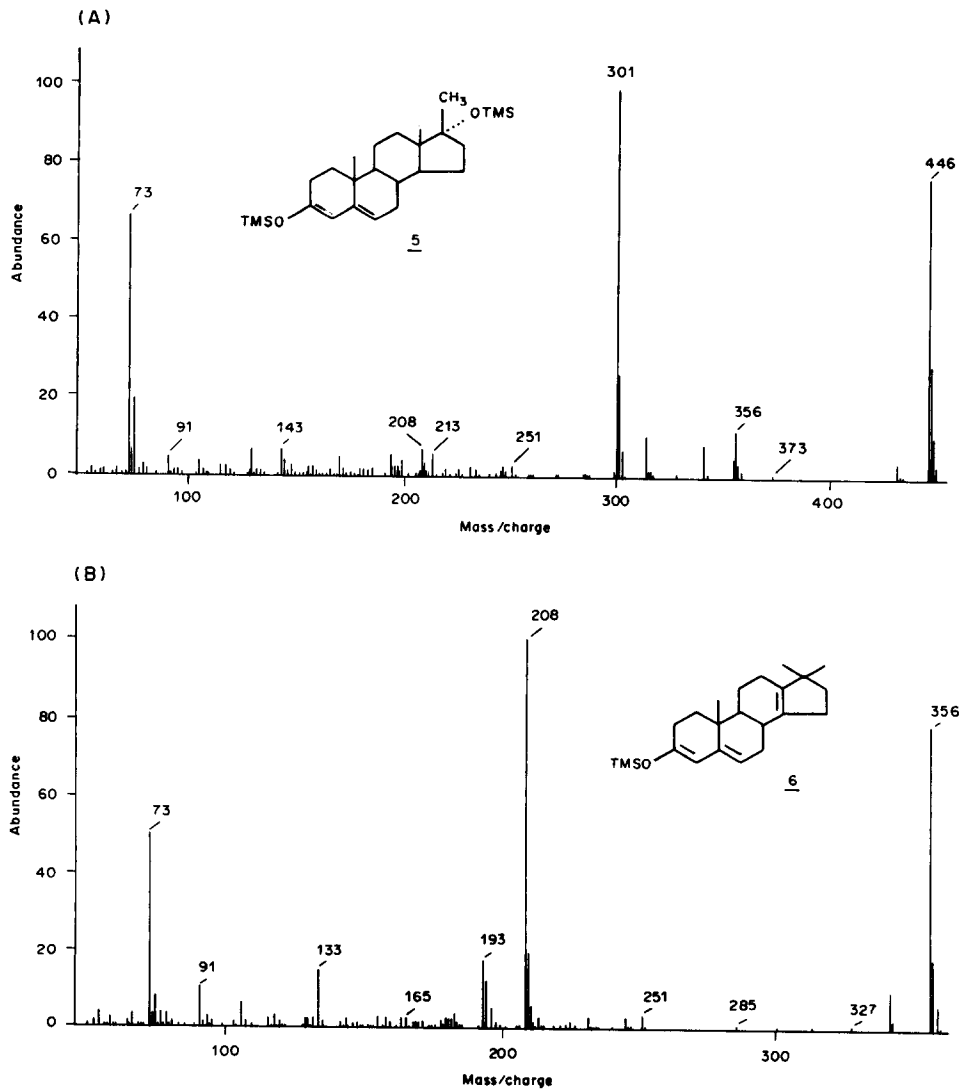

Fig. 7. Mass spectra of the TMS-enol-TMS-ether derivatives of (A) 17-epimethyltestosterone **5** and 18-nor-17,17-dimethyl-androsta-4,13(14)-dien-3-one **6**.

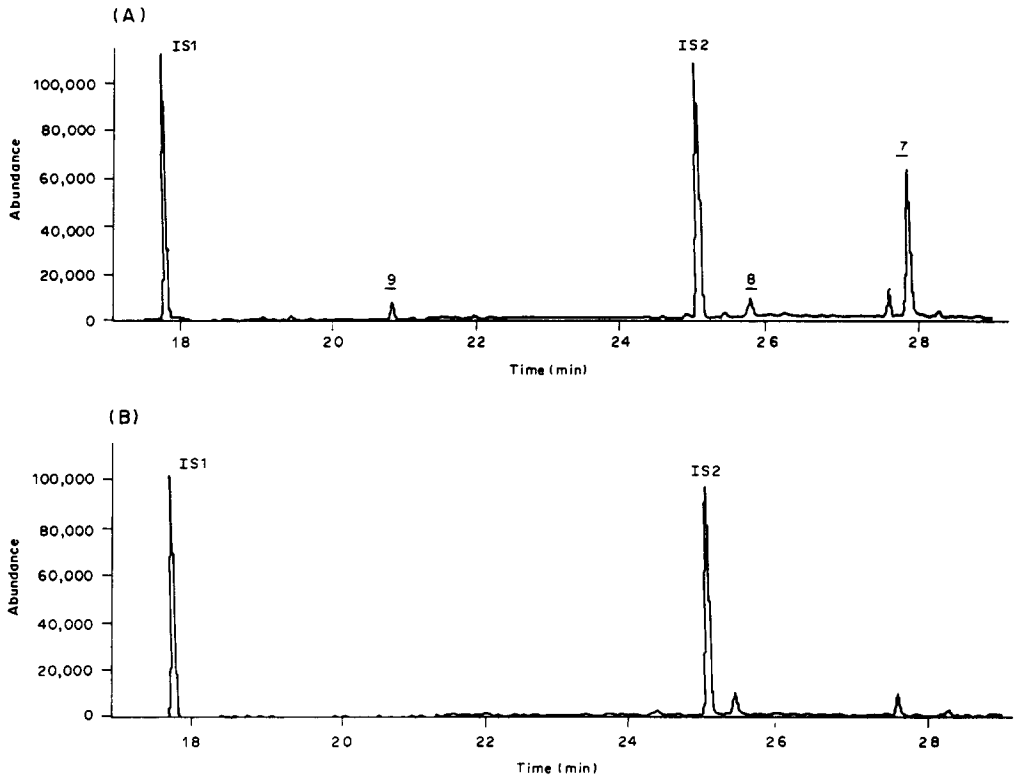

Fig. 8. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of unconjugated fraction of (A) the urine sample 7 h after oxandrolone administration and (B) a blank urine. See Table 1 for peak identification. Other conditions as in Fig. 2.

characterized by a prominent ion at m/z 301 produced from the cleavage of the D-ring with concomitant elimination of a hydrogen radical, most probably from C_8. The resulting radical can be readily stabilized by migration to C_7 and resonance with the 3,5-diene group of the TMS-enol moiety to yield an even-numbered TMS oxonium ion. The high degree of conjugation between this ion at C_3 and the resulting 4,6-diene function may account for its intensity and absence of other prominent ions in the mass spectrum of **5** [Fig. 7(A)].

The mass spectrum of compound **6** [Fig. 7(B)] showed an abundant ion at m/z 208 which is produced according to a fragmentation route similar to that giving rise to the ion of m/z 206 in the mass spectrum of the TMS derivatives of **1** and **2** [Fig. 4(A) and Table 1]. A low intensity ion characteristic of the 13(14)-ene group is observed at m/z 133. These data are in accordance with the mass spectral features of the reference steroids.

Oxandrolone 7

The excretion profiles of oxandrolone and its metabolites **8** and **9** were different from those of methandienone and methyltestosterone in that all three steroids were isolated from the unconjugated steroids fraction (Fig. 8). Their excretion curves indicate that they can be detected until 48 h after oxandrolone administration (Fig. 9). Maximum excretion rates were observed 7 h after administration, which is consistent with the fact that these steroids were not conjugated prior to excretion in urine.

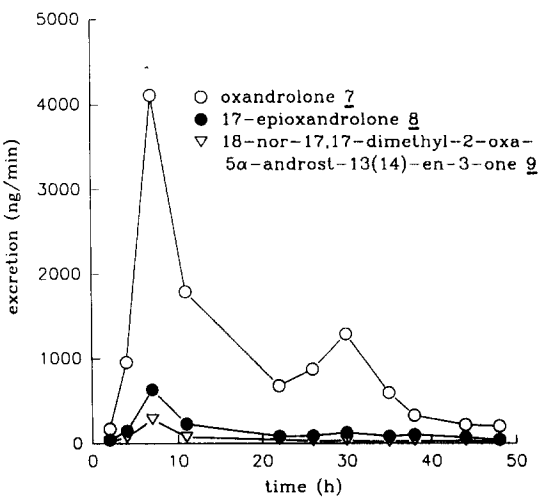

Fig. 9. Excretion curves of **7**, **8** and **9** (unconjugated fraction) after oral administration of 10 mg of **7**.

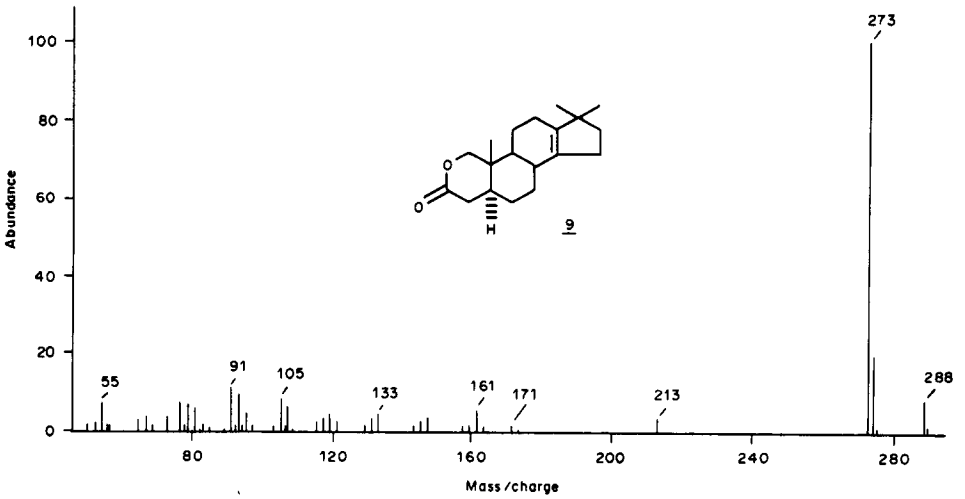

Fig. 10. Mass spectrum of 18-nor-17,17-dimethyl-2-oxa-5α-androst-13(14)-en-3-one **9**.

Because it is barely degraded, the total excretion of unchanged oxandrolone accounted for 26.5% of the dose, whereas that of epioxandrolone **8** and compound **9** was also relatively abundant with respect to that of their methandienone and methyltestosterone analogs **2**, **3**, **5** and **6**. The mass spectrum of compound **9** which is shown in Fig. 10 was identical to that of the reference steroid. This spectrum, which is characterized by a prominent ion at m/z 273 $(M\text{-}Me)^+$, shows low intensity and structurally informative ions at m/z 133 and 148 (C- and D-rings). The mass spectral data of **7** and **8** have been reported previously [7].

*Mestanolone **10** and stanozolol **11***

No trace of the 17 epimers and dehydration products of both mestanolone **10** and stanozolol **11** was detected in urine specimens collected after the administration of single dose of the steroids (10 and 20 mg, respectively) to human volunteers.

DISCUSSION

Epimerization at the C_{17} position

Results from our previous *in vitro* study [17] on the solvolysis of sulfate derivatives of 17β-hydroxy-17α-methyl steroids are consistent with the data from the *in vivo* study presented above. Our data are also in accordance with those of Edlund *et al.* [15], who used methandienone sulfate as a model substrate. These authors originally suggested that epimerization at the C_{17} position occurred through the formation of a tertiary carbonium ion resulting from the elimination of the sulfate group which is labile in aqueous media. Nucleophilic attack by water afforded 17-epimethandienone. The present study and previous *in vitro* investigations from our laboratory[17] provided further evidence for the occurrence of the above mechanism in the epimerization reaction of steroids **1**, **4** and **7**. Thus, the *in vivo* formation of the 17-epimers of 17β-hydroxy-17α-methyl steroids is dependent upon the previous biosynthesis of the corresponding 17β-sulfate conjugates.

However, sulfation, as glucuronidation, is a phase II metabolic reaction which is affected by the phase I reactions such as hydroxylation and reduction. From a mechanistic point of view, there appears to be some selectivity in the sulfation and glucuronidation reactions of 17β-hydroxy-17α-methyl steroids, which are probably subjected to "steric hindrance". Examination of molecular models of oxandrolone and uridine diphosphoglucuronic acid (UDP-glucuronic acid), the glucuronic acid donor in the enzymatic formation glucuronides, shows that there is a great deal of interference between the bulky uridine diphosphate moiety and the steroid molecule when the tertiary 17β-hydroxy group draws near to the anomeric center of the glucuronic acid moiety [16]. This interference appears to be strong enough to prevent glucuronidation at C_{17}. Conversely, the molecular model of PAPS, the sulfuric acid donor in the enzymatic formation of sulfate conjugates, shows that there is much less interference between the steroid molecule and the 3-phosphoadenosine moiety so that the tertiary 17-hydroxy group of oxandrolone can easily

come within bonding distance to the sulfate group. On the basis of these observations, it is reasonable to postulate that the C_{17} hydroxyl group of anabolic 17β-hydroxy-17α-methyl steroids is preferentially sulfated in the course of the phase II biotransformation reactions in human. This hypothesis is supported by the fact that oxandrolone **7**, which bears one hydroxyl group at C_{17}, is not excreted in urine as a glucuronic acid conjugate. Furthermore, the occurrence in urine of 17-epioxandrolone **8** and compound **9** demonstrates that oxandrolone was sulfated prior to excretion.

Metabolic factors can also affect the production of tertiary 17β-sulfate conjugates. Quantitative data presented in Table 4 indicate that the relative abundance of the 17-epimers produced parallels the overall rate of biodegradation of their respective precursors. Indeed, relatively low amounts of epimethyltestosterone **5** and compound **6** are produced from methyltestosterone **4** because the latter steroid is rapidly metabolized by reduction of its 3-keto and 4-ene functions [10, 20] and the resulting metabolites are readily glucuronidated. In such metabolic conditions, glucuronidation is the major phase II reaction which accounts for the rapid elimination of the parent steroid and its metabolites in urine and sulfation at C_{17} is a minor route of elimination.

The absence of the 17-epimers and the 18-nor dehydration products of mestanolone **10** and stanozolol **11** in the urine samples collected after oral administration of these steroids could presumably be the result of their rapid and extensive biodegradation, primarily through reduction of mestanolone 3-keto and hydroxylation at several sites of the stanozolol nucleus [8, 10, 11]. In addition, their hydroxylated metabolites are excreted mainly as glucuronides. Data about stanozolol were not in complete agreement with previous studies from this laboratory [8] and that of Schänzer *et al.* [11] reporting the isolation and characterization of 17-epistanozolol, 3′-hydroxy-17-epistanozolol and 16-hydroxy-17-epistanozolol from human urine. It is of interest to note that in the previous study [11], 17-epistanozolol was detected in the urine samples after administration of an oral 40 mg dose of stanozolol **11**. When substantial amounts of steroids are used, as is the case with chronic users, sulfation may then become an important complementary phase II reaction that accelerates the elimination of the parent steroids and its metabolites.

However, if the structure of the parent steroid is such that phase I reactions are hampered or their rates decreased, sulfation may then become a complementary and important elimination route of the parent steroid and/or its metabolites. This hypothesis is supported by quantitative data from the excretion of methandienone and oxandrolone presented in Table 4. The presence of an additional double bond at C_1 in methandienone has an important effect on its metabolic rate with respect to those of methyltestosterone so that methandienone is metabolized and eliminated at a slower rate. This in turn appears to promote the sulfation of methandienone at C_{17} which is reflected by the excretion of larger amounts of its 17-epimer **2** and compound **3**. In the case of oxandrolone which is barely degraded via phase I reactions [7] and which is not a substrate for glucuronidation, sulfation at C_{17} is a major phase II reaction, the importance of which is demonstrated by relatively high amounts of 17-epioxandrolone **8** and compound **9** excreted in urine. In conclusion, the 17β-sulfation of 17β-hydroxy-17α-methyl steroids become an important metabolic route when phase I reactions and glucuronidation are hampered due to particular structural features of these steroids.

The in vivo formation of the 18-nor-17,17-dimethyl-13(14)-enes

The occurrence in human urine of 18-nor-17,17-dimethyl-13(14)-ene steroids after administration of the parent 17β-hydroxy-17α-methyl steroids has been originally related to their retropinacol rearrangement under acidic conditions [9, 13, 14]. Other authors reported that compound **3** was an artifact produced by the decomposition of methandienone **1** by gastric acid [4]. According to this hypothesis, all 17β-hydroxy-17α-methyl steroids should give the corresponding 18-nor-13(14)-unsaturated steroids when ingested. However, the data presented above showed that no trace of the expected 13(14)-unsaturated dehydration products of mestanolone **10** and stanozolol **11** was detected in urine. This observation indicates that the unsaturated steroids **3**, **6** and **9** are not artifacts but dehydration products originating from the 17β-sulfate conjugates of their parent steroids. Our data are also in accordance with a study from Edlund *et al.* [15] who recently proposed that compound **3** arises from the

elimination of the sulfate group of methandienone **1** sulfate derivative through rearrangement of the resulting 17-carbonium ion. This study provided clear evidence that the 17-epimers **2**, **5** and **8** and their corresponding 18-nor-17,17-dimethyl-13(14)-ene analogs from unique precursors which are the 17β-sulfate derivatives of compounds **1**, **4** and **7**.

In vivo ratio of the 17-epimers to the 18-nor-17,17-dimethyl-13(14)-enes

The results presented in Table 4 clearly show that the *in vivo* rearrangement of the 17β-sulfate conjugates of compounds **1**, **4** and **7** leads to the formation of their corresponding 17-epimers and dehydration products in ratio of 0.54:1, 2:1 and 2.7:1, respectively. These results were somewhat surprising since the *in vitro* degradation of all three 17β-sulfates afforded the same products in an identical 0.8:1 ratio. This leads us to postulate that the *in vivo* formation of the 17-epimeric steroids and their corresponding dehydration products is not solely determined by the chemistry of the tertiary 17β-sulfate group, but also by biochemical factors such as binding of the sulfate conjugates with albumin, sex-hormone-binding globulin or other steroid-binding proteins.

Data shown in Figs 3, 6 and 9 and in Table 4 strongly suggest that the degradation of the sulfate conjugates do not occur in urine. Indeed, we demonstrated that the epimerization and dehydration products are formed in a 0.8:1 ratio in urine at 37°C. This implies that these reactions probably occurred *in vivo*, shortly after sulfation of the 17β-hydroxyl group and that they are affected by biochemical factors, most probably by noncovalent interaction with proteins. Such interaction or binding between the 17β-sulfates and/or their 17-carbonium ion and plasma proteins could sterically hinder the C_{17} position and prevent, to a certain extent, nucleophilic attack of water which ultimately gives the corresponding 17-epimers. This in turn would favor the formation of dehydration products as observed in the case of methandienone. Conversely, interaction with proteins could favor the $S_{N}i$ nucleophilic attack of water at the carbonium ion to give the 17-epimeric steroids as observed in the case of methyltestosterone and oxandrolone. Although speculative, this hypothesis is in agreement with the underlying mechanistic aspects of the unimolecular $S_{N}i$ substitution and E_{i} elimination reactions which lead to the formation of 17-epimers and dehydration products, respectively. Moreover, this hypothesis is supported by quantitative data (Table 4) indicating that these steroids are produced in uneven ratios ranging from 0.54:1 to 2.7:1.

Glucuronidation of steroids 1, 4 and 11

Data presented above indicate that the studied 17β-hydroxy-17α-methyl steroids are not glucuronidated at the C_{17} position. This hypothesis is supported by the fact that oxandrolone, which only bears a tertiary 17β-hydroxyl group was not found in the glucuronide fraction but in the unconjugated steroid fraction. Although they also have an identical hydroxyl group at C_{17}, unchanged methandienone **1** and methyltestosterone **4** were isolated from the glucuronide fraction (Figs 2 and 5). This indicates that **1** and **4** are probably excreted as enol glucuronides. The formation of enol glucuronide of some endogenous 3-keto-4-ene steroids, particularly androstenedione and testosterone, has been previously reported [21]. Kjeld *et al.* [22] also provided analytical evidence demonstrating the occurrence of the 3-enol glucuronide of testosterone in human urine. Given the structural similarities of compounds **1** and **4** with those of testosterone, the occurrence of their 3-enol glucuronides is not surprising since their formation is mainly dependent upon the chemistry of the 3-keto-4-ene group. The detection of unchanged stanozolol **11** in the glucuronide fraction indicates that this nitrogen-containing steroid was excreted as a *N*-glucuronide involving its pyrazole moiety. Further investigation will be carried out to characterize these unusual glucuronides.

CONCLUSION

This study showed that 17β-sulfation is an important phase II reaction in the metabolism of some 17β-hydroxy-17α-methyl steroids in human, particularly when their biotransformation is hampered because of the presence of specific functional groups at positions of metabolic importance. The *in vivo* degradation of these sulfates gives rise to mixtures of the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes, which were excreted in the unconjugated fraction. The sulfation reaction at C_{17} appears to be affected by competitive phase I metabolic reactions which, as in the case of mestanolone **10** and stanozolol **11**, favor the elimination of the parent steroids and

their metabolites through routes other than sulfation at C_{17}. Quantitative data indicate that the *in vivo* degradation of 17β-sulfates could be affected by interaction with plasmatic proteins, the demonstration of which would require further investigations. The formation of enol glucuronides of steroids **1** and **4** and that of a *N*-glucuronide of stanozolol **11** was also proposed.

Acknowledgements—Financial assistance from the Natural Sciences and Engineering Research Council of Canada, the Sport Medicine Council of Canada and the National Collegiate Athletic Association are gratefully acknowledged. The authors thank Mrs D. Lacoste for drawings.

REFERENCES

1. Rongone E. L. and Segaloff A.: *In vivo* metabolism of Δ^1, 17α-methyltestosterone in man. *Steroids* **1** (1963) 179–184.
2. MacDonald B. S., Sykes P. J., Adhikary P. M. and Harkness R. A.: The identification of 17α-hydroxy-17-methyl-1,4-androstadien-3-one as a metabolite of the anabolic steroid drug 17β-hydroxy-17-methyl-1,4-androstadien-3-one in man. *Steroids* **18** (1971) 753–766.
3. Dürbeck H. W., Büker I., Scheulen B. and Telin B.: Gas chromatographic and capillary column gas chromatographic-mass spectrometric determination of synthetic anabolic steroids. *J. Chromat.* **167** (1978) 117–124.
4. Dürbeck H. W. and Büker I.: Studies on anabolic steroids. The mass spectra of 17α-methyl-17β-hydroxy-1,4-androstadien-3-one(dianabol) and its metabolites. *Biomed. Mass Spectrom.* **7** (1980) 437–445.
5. Dürbeck H. W., Büker I., Scheulen B. and Telin B.: GC and capillary GC/MS determination of synthetic anabolic steroids II. 4-chloro-methandienone (oral turinabol) and its metabolites. *J. Chromat. Sci.* **21** (1983) 405–410.
6. Massé R., Ayotte C. and Dugal R.: Studies on anabolic steroids I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromat.* **489** (1989) 23–50.
7. Massé R., Bi H., Ayotte C. and Dugal R.: Studies on anabolic steroids II—Gas chromatographic/mass spectrometric characterization of oxandrolone urinary metabolites in man. *Biomed. Environ. Mass Spectrom.* **18** (1989) 429–438.
8. Massé R., Ayotte C., Bi H. and Dugal R.: Studies on anabolic steroids III. Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography—mass spectrometry. *J. Chromat.* **497** (1989) 17–37.
9. Harrison L.M. and Fennessey P. V.: Methandrostenolone metabolism in humans: potential problems associated with isolation and identification of metabolites. *J. Steroid Biochem.* **36** (1990) 407–414.
10. Massé R., Bi H., Ayotte C., Du P., Gélinas H. and Dugal R.: Studies on anabolic steroids V. Sequential reduction of methandienone and structurally related steroid A—ring substituents in humans: gas chromatographic—mass spectrometric study of the corresponding urinary metabolites. *J. Chromat.* **562** (1991) 323–340.
11. Schänzer W., Opfermann G. and Donike M.: Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J. Steroid Biochem.* **36** (1990) 153–174.
12. Schänzer W., Geyer H. and Donike M.: Metabolism of methandienone in man: identification and synthesis of conjugated excreted urinary metabolites, determination of excretion rates and gas chromatographic—mass spectrometric identification of bis-hydroxylated metabolites. *J. Steroid Biochem. Molec. Biol.* **38** (1991) 441–464.
13. Segaloff A. and Gabbard R. B.: Antiandrogenic activity of 17,17-dimethyl-18-nor-androst-13-enes. *Steroids* **4** (1964) 433–443.
14. Smith D. M. and Steele J. W.: Anabolic steroids part 4. Chemical rearrangement of oxymetholone. *Can. J. Pharmac. Sci.* **16** (1981) 68–72.
15. Edlund P. O., Bowers L. and Henion J.: Determination of methandrostenolone and its metabolites in equine plasma and urine by coupled—column liquid chromatography with ultraviolet detection and confirmation by tandem mass spectrometry. *J. Chromat.* **487** (1989) 341–356.
16. Alvares A. P. and Pratt W. B.: Pathways of drug metabolism. In *Principles of Drug Action (3rd Edn)* (Edited by W. B. Pratt and P. Taylor). Churchill Livingstone, New York (1990) pp. 365–422.
17. Bi H., Massé R. and Just G.: Studies on anabolic steroids. 9. Tertiary sulfates of anabolic 17α-methyl steroids: synthesis and rearrangement. *Steroids*. In press.
18. Massé R., Bi H. and Du P.: Studies on anabolic steroids 7. Analysis of urinary metabolites of formebolone in man by gas chromatography/mass spectrometry. *Analyt. Chim. Acta* **247** (1991) 211–221.
19. Bi H., Du P. and Massé R.: Studies on anabolic steroids—8. GC/MS characterization of unusual acidic seco metabolites of oxymetholone in human urine. *J. Steroid Biochem. Molec. Biol.* **42**(2) (1992) 229–242.
20. Rongone E. L. and Segaloff A.: Isolation of urinary metabolites of 17α-methyltestosterone. *J. Biol. Chem.* **237** (1962) 1066–1067.
21. Hadd H. E. and Blickenstaff R. T.: *Conjugates of Steroid Hormones*. Academic Press, New York (1969) pp. 301–310.
22. Kjeld J. M., Puah C. M. and Joplin G. F.: Labile testosterone conjugate in human urine: further evidence. *Clin. Chim. Acta* **93** (1979) 227–233.