Journal of Chromatography, 562 (1991) 323–340
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5555

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

ROBERT MASSÉ*, HONGGANG BI, CHRISTIANE AYOTTE, PING DU, HUGUETTE GÉLINAS and ROBERT DUGAL

Institut National de la Recherche Scientifique, INRS-Santé, Université du Québec, 245 Hymus Boulevard, Pointe-Claire, Québec H9R 1G6 (Canada)

ABSTRACT

The biotransformation of methandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one) in human adults, more particularly the sequential reduction of its A-ring substituents, was investigated by gas chromatography–mass spectrometry. Two pairs of 17-epimeric tetrahydro diols resulting from the stereoselective reduction of the Δ^4- and 3-oxo groups and of the Δ^1-function were characterized. The major diols were 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol, which were both excreted in the conjugate fraction in a 1:3.8 ratio. The immediate metabolic precursors of the 5β-diol, namely 17β-hydroxy-17α-methyl-5β-androsta-1-en-3-one and 17α-methyl-5β-androsta-1-en-3α,17β-diol and their corresponding 17-epimers, were also identified in post-administration urine samples. These data indicated that reduction of methandienone A-ring substituents proceeds according to the sequence. Δ^4-, 3-oxo- and Δ^1-. The A-ring reduction products of the structurally related steroids mestanolone, 17α-methyltestosterone and oxymethone were also characterized and provided further analytical and metabolic evidence supporting the proposed route of methandienone A-ring reduction. It was also demonstrated using synthetic 17β-sulfate conjugates of methandienone and 17α-methyltestosterone that their corresponding 17-epimers are formed by nucleophilic substitution by water of the labile sulfate moiety. The steroidal metabolites were identified on the basis of their characteristic mass spectral features and by comparison with authentic reference standards. Metabolic pathways accounting for the occurrence of the metabolites of interest in post-administration urine samples are proposed.

INTRODUCTION

The sequential reduction of the Δ^4- and 3-oxo functions are key steps in the biotransformation of several endogenous and exogenous steroids in humans [1–6]. It is well known that the stereoselective 5α-reduction of testosterone to yield 5α-dihydrotestosterone is of major importance in the expression of androgenic activity in tissue of the male reproductive tract [4]. Among the four tetrahydro

0378-4347/91/$03.50

urinary metabolites which are produced in the course of testosterone catabolism through the concerted action of 5α- and 5β-reductases and 3α- and 3β-hydroxysteroid dehydrogenases (3-OHSDH), androsterone (3α,5α) and etiocholanolone (3α,5β) are the most abundant and excreted in urine in a mean ratio of about 1:0.9 in healthy men [6].

It is of interest that the relative affinity of 5α- and 5β-reductases towards steroidal substrates can be significantly modulated by slight but specific chemical modifications at positions either close or remote from the Δ^4-function. A typical example of the enzymatic select action of these enzymes is given by the reductive metabolism of 19-nortestosterone (19-NT) and 17α-methyltestosterone (17α-MT), which are catabolized into tetrahydro metabolites analogous to androsterone and etiocholanolone. We previously reported that the metabolic profile of 19-NT 5α- and 5β-tetrahydro metabolites was markedly influenced by the absence of the 19-methyl group, as 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) were shown to be always excreted in urine in a ratio larger than 2:1 [7]. The preponderance of 19-NA was observed not only after 19-NT ester administration, but also in positive urine samples obtained from athletes in the context of drug-testing activities [8,9].

These findings corroborated those of Engel *et al.* [10] and Floch *et al.* [11], who reported that 19-NA was the predominant urinary metabolite of 19-NT in humans. Further evidence for the preponderance of 5α-reduction in 19-NT metabolism was recently obtained in man [12] in the course of the oxidative de-ethynylation of norethisterone into 19-NT, which then undergoes reduction to yield 19-NA and 19-NE. Interestingly, the latter isomeric steroids were also excreted in urine in a ratio larger than 2:1.

Conversely, Rongone and Segaloff [13] showed that 17α-MT was mainly transformed into two isomeric tetrahydro metabolites with 3α,5α- and 3α,5β-configurations, which were excreted in urine in a 1:10 ratio after oral administration of 1 g of 17α-MT to a female patient with advanced breast cancer. These data indicated that the introduction of a 17α-methyl group in testosterone seems to promote 5β-reduction, whereas the absence of a 19-angular methyl group favours 5α-reduction of the Δ^4-function.

Several 17α-methyl anabolic steroids bearing Δ^4- and/or 3-oxo functions are likely to be metabolized into similar if not identical tetrahydro metabolites [2]. This could be the case with methandienone (Δ^1-17α-MT), 17α-MT, mestanolone and oxymetholone (2-hydroxymethylenemestanolone), which are differentiated by the structural features of their A-ring.

We therefore undertook a comprehensive investigation of the urinary metabolites which are likely to arise from the reductive metabolism of their A-ring substituents in order to elucidate the sequence of reactions through which methandienone is partially transformed into tetrahydro compounds, determine the ratio of the corresponding tetrahydro 5α- and 5β-metabolites in urine and characterize the structures of the metabolites of interest. Here, mestanolone, 17α-MT and

oxymetholone were used as model steroids so as to obtain reference steroids with known 5α-configuration and 5α- and 5β-steroids with no Δ^1-function that we subsequently used to characterize and assess the identity of the metabolites produced from methandienone.

In this paper we report the gas chromatographic–mass spectrometric (GC–MS) characterization of two isomeric tetrahydro metabolites and their corresponding 17-epimers which are excreted in urine following methandienone and 17α-MT ingestion. In the case of methandienone, the metabolic intermediates of the tetrahydro metabolites were also isolated and characterized. Reference tetrahydro 17-epimers with a 3α,5α-configuration were obtained form mestanolone and oxymetholone human studies. 17α-MT sulfate pyridinium salt was synthesized and used as a model substrate to demonstrate that the 17α-hydroxy-17β-methyl-epimeric metabolites are produced from the 17β-sulfate aglycone of the parent steroid or any other metabolic intermediate. Mass spectral data supporting the proposed identity of the urinary metabolites are presented and metabolic pathways accounting for their formation are proposed.

EXPERIMENTAL

Steroids

Authentic 17α-methyl-5α-androstan-3α,17β-diol, 17-α-methyl-5β-androstan-17β-ol-3-one, 17α-methyltestosterone, mestanolone (17α-methyl-5α-androstan-17β-ol-3-one) and oxymetholone (2-hydroxymethylene-17α-methyl-5α-androstan-17β-ol-3-one) were purchased from Steraloids (Wilton, NH, U.S.A.) and Sigma (St. Louis, MO, U.S.A.); tablets of methandienone and 17α-methyltestosterone were obtained from Ciba-Geigy Canada (Dorval, Québec, Canada); tablets of oxymetholone were from Syntex (Montréal, Québec, Canada). Reference 17α-methyl-5β-androstan-3α,17β-diol was prepared by sodium borohydride reduction of 17α-methyl-5β-androstan-17β-ol-3-one.

Chemicals

N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Regis Chemical (Morton Grove, IL, U.S.A.), sodium borohydride ($NaBH_4$), dithioerithritol and trimethyliodosilane (TMSI) from Aldrich (Milwaukee, WI, U.S.A.) and Sep-Pak C_{18} cartridges form Waters Assoc. (Milford, MA, U.S.A.). Inorganic salts (J. T. Baker, Philipsburg, NJ, U.S.A.) were of analytical-reagent grade. All the solvents were high-performance liquid chromatography grade (Caledon Labs., Georgetown, Ontario, Canada) and used as received.

Urine samples

Blank urine samples were collected before administration of anabolic steroids to healthy male volunteers (24–40 years of age). The volunteers were given one

oral dose of mestanolone (10 mg), methandienone (25 mg), 17α-methyltestosterone (10 mg) or oxymetholone (50 mg). Urine samples were than collected for seven days in order to obtain comprehensive excretion profiles of the parent steroids and their metabolites and were immediately frozen at −20°C after collection until analyzed.

Extraction of urinary steroids and their metabolites

A urine sample (3–5 ml) was passed through a Sep-Pak C_{18} cartridge (prewashed with 5 ml of methanol and 5 ml of water). The cartridge was then washed with 5 ml of water and 2 ml of hexane (to eliminate residual water in the cartridge). Steroids and their metabolites were finally eluted with 5 ml of methanol and the solvent was evaporated under a stream of nitrogen below 60°C. The resulting dried residue was dissolved in 1 ml of 0.2 *M* acetate buffer (pH 5.2) and 100 μl of β-glucuronidase sulfatase (type H-2) from *Helix pomatia* (Sigma) were added to the solution. The mixture was incubated for 16 h at 37°C or 3 h at 55°C. After hydrolysis, 100 mg of solid buffer [$KHCO_3$–K_2CO_3 (9:1)] were added and the aqueous phase was extracted with 5 ml of diethyl ether. After evaporation of the organic solvent under nitrogen, the residue was derivatized as described below. The crude preparation of *Helix pomatia* used was tested for potential side activities (*e.g.*, oxidation of steroids) prior to use as reported previously [12].

Derivatization and GC–MS analysis

The derivatization method using MSTFA and N,O-bis(d_9)trimethylsilylacetamide as reagents and TMSI (trimethylsilyl iodide), TMCS (trimethylchlorosilane) and d_9-TMCS as catalysts and the GC–MS conditions were as reported previously [12,14,15]. The repetitive scan mode was used to obtain full mass spectra of relevant GC peaks for identification of the metabolites of interest. Capillary columns (30 m) with methylsilicone (HP-1 and DB-1) and methylphenylsilicone (HP-5 and DB-5) stationary phases were used in order to achieve the separation of the isomeric tetrahydrodiols **1** and **2**. Unfortunately, all attempts to resolve these steroids by chromatography were unsuccessful.

Synthesis of model steroids

17α-MT sulfate pyridinium salt (17α-MTSPS) and fast atom bombardment MS analysis. This steroid conjugate was prepared by the reaction of 17α-MT with chlorosulfonic acid in pyridine. 17α-MTSPS was characterized by ^{13}C NMR and fast atom bombardment (FAB) mass spectrometry in the negative ion mode. A detailed synthetic procedure and spectral characterization of 17α-MTSPS and of a series of other 17α-methyl anabolic steroid sulfate salts, together with epimerization conditions, will be reported elsewhere. 17α-MTSPS was characterized by negative ion FAB-MS using a Kratos MS-50 TC mass spectrometer. The bombarding atom beam was 6-kV xenon atoms of 1 mA current. Glycerol was used as the sample-supporting matrix and the xenon flow-rate was 0.5 ml/min. The FAB

mass spectrum of 17α-MTSPS was measured at a resolution of 10 000 and showed a base peak at *m/z* 381.173 ($C_{20}H_{29}O_5S$) corresponding to the 17α-$MTSO_3^-$ ion.

Synthesis of the 17-epimer **4**. 17α-MTSPS was epimerized in aqueous potassium carbonate and 17-epimethyltestosterone was recovered in a 50% yield by extraction with diethyl ether and characterized by GC–MS. This steroid was identical with 17-epiMT isolated from 17α-MT urine samples [9]. Reduction of the latter 17-epimeric steroid with $NaBH_4$ in isopropanol using a method adapted from that of Barton [16] preponderantly afforded compound **4** and trace amounts of 17β-methyl-5α-androstan-3β,17α-diol. Synthetic **4** was shown to be identical with metabolite **4** isolated from 17α-MT and methandienone urine samples. Epimerization of 17α-MTSPS also gave several dehydration products, namely 18-*nor*-17,17-dimethyl-4,13(14)-androstadien-3-one ($M^{+\cdot}$ 284) and 4,16-androstadien-3-one ($M^{+\cdot}$ 284). These compounds are homologous with those reported by Edlund *et al.* [17] from the decomposition of methandienone sulfate.

Synthesis of compound **2**. This steroid was prepared by $NaBH_4$ reduction of 17α-methyl-5β-androstan-17β-ol-3-one in isopropanol according to a method adapted from that of Barton [16].

RESULTS AND DISCUSSION

Mestanolone and oxymetholone

Reconstructed ion chromatograms illustrating typical urinary profiles of the tetrahydro metabolites resulting from the stereoselective reduction of the Δ^4- and/or 3-oxo functions of mestanolone, methandienone, 17α-MT and oxymetholone are shown in Fig. 1. The identity and methylene unit values of the tetrahydro metabolites are given in Table I. There is a striking similarity between the ion profiles of mestanolone, oxymetholone and methyltestosterone urinary exracts in that they are dominated by a prominent peak at 23.35 min. Peak 1 in Fig. 1A and B was identified as 17α-methyl-5α-androstan-3α,17β-diol by comparison of the mass spectrum of its TMS ether derivative (Fig. 2A) with that of an authentic reference compound. Further examination of the mass spectral data showed the presence of a minor compound at 20.45 min and the mass spectrum of which (Fig. 3A) was virtually identical with that of **1**. On the basis of its GC–MS features and comparison with those of other epimeric 17-methyl steroids [14,15,17–19], **3** was identified as 17β-methyl-5α-androstan-3α,17β-diol. A scheme illustrating the route of formation of **1** and **3** from mestanolone is proposed in Fig. 4. It is of interest that **1** and **3** were also detected in human urine after administration of oxymetholone (2-hydroxymethylenemestanolone), as shown by the characteristic ion chromatogram in Fig. 1B. Here, the catabolic transformation was probably initiated by oxidation of the 2-hydroxymethylene group to yield the corresponding β-keto acid intermediate, which then undergoes decarboxylation to give mestanolone (Fig. 4). Further reduction of mestanolone by 3α-OHSDH with con-

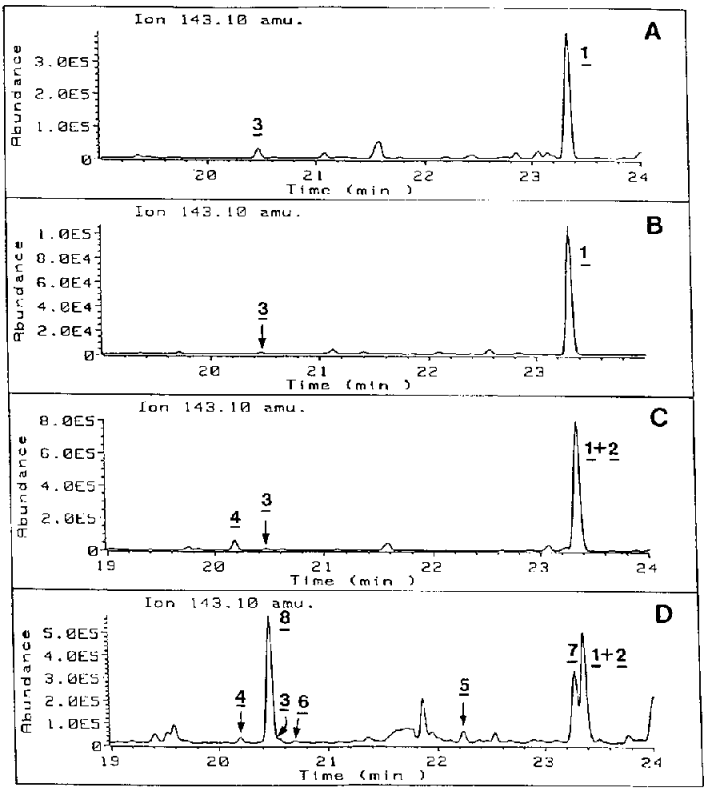

Fig. 1. Reconstructed ion chromatograms (*m/z* 143) from conjugated steroid fractions illustrating the detection of the tetrahydro metabolites produced from (A) mestanolone, (B) oxymetholone, (C) 17α-methyltestosterone and (D) methandienone. Note that compounds **1** and **2** (peaks 1 and 2) are not chromatographically resolved. The identity of compounds **1–4** (peaks 1–4) is given in Table I. Other labelled peaks: 5 = 17β-hydroxy-17α-methyl-5β-androsta-1-en-3-one (**5**) and 6 = its 17-epimer (**6**), 7 = 17α-methyl-5β-androsta-1-en-3a-17β-diol (**7**) and 8 = its 17-epimer (**8**). See Experimental for GC–MS conditions.

TABLE I

IDENTITY AND METHYLENE UNIT VALUES OF THE TETRAHYDRODIOLS DETECTED IN HUMAN URINE

Urinary metabolite	No.	Steroid[a]			
		MSTL	MHDN	17α-MT	OXYM
17α-Methyl-5α-androstan-3α,17β-diol	**1**	26.65	26.65	26.65	26.65
17α-Methyl-5β-androstan-3α,17β-diol	**2**	n.d.[b]	26.65	26.65	n.d.
17β-Methyl-5α-androstan-3α,17α-diol	**3**	25.06	n.d.	25.06	25.06
17β-Methyl-5β-androstan-3α,17α-diol	**4**	n.d.	24.95	24.95	n.d.

[a] Steroid abbreviations: MSTL, mestanolone; MHDN, methandienone; 17α-MT, 17α-methyltestosterone; OXYM, oxymetholone. Methylene unit values were determined by linear interpolation of the retention time of the steroid TMS ether derivative between the retention times of C-24, C-25, C-26 and C-27 alkanes.

[b] Not detected.

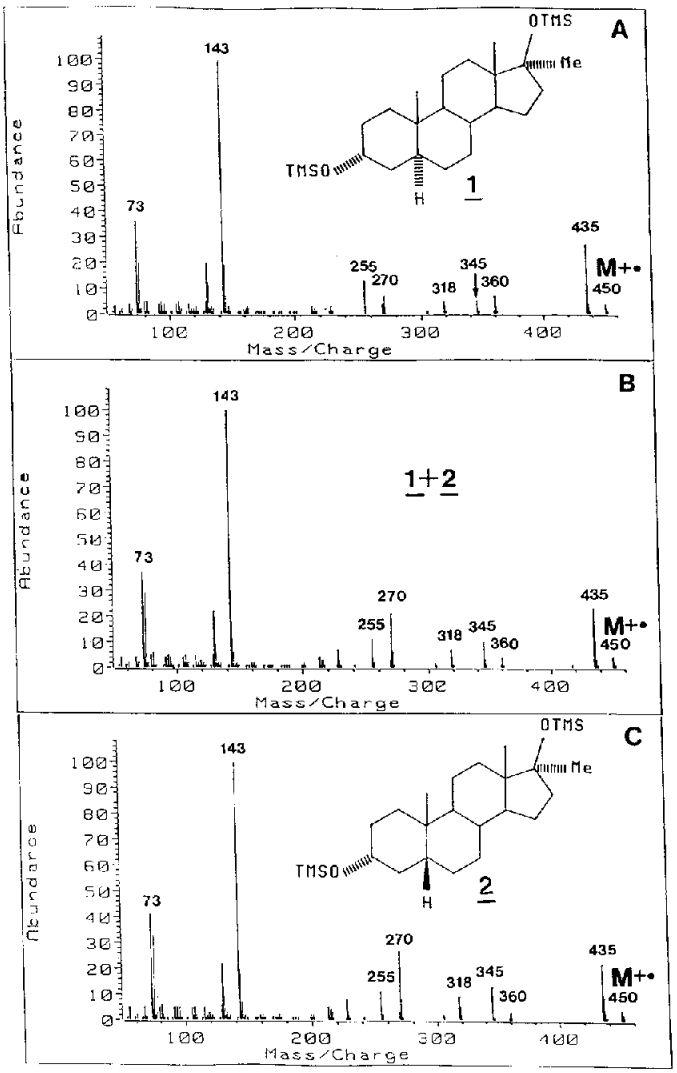

Fig. 2. Mass spectra of (A) 17α-methyl-5α-androstan-3α,17β-diol (1) isolated in human urine after administration of mestanolone and oxymetholone, (B) a mixture of compounds 1 and 2 isolated from 17α-MT post administration urine samples and (C) authentic 17α-methyl-5β-androstan-3α,17β-diol (2) as the TMS ether derivatives.

comitant epimerization at the C-17 position afforded **1** and **3**. It is worth noting that, in addition to decarboxylation, the above-mentioned β-keto acid intermediate (17β-hydroxy-17α-methyl-5α-androstan-3-oxo-2-carboxylic acid) was shown to be also further oxidized to its 3α-hydroxy analogue and 17α-methyl-17β-hydroxy-2(3)-*seco*- 5α-androstane-2,3-dicarboxylic acid through a pathway reminiscent of β-keto acid oxidation [20]. The formation of the epimeric steroid **3** will be discussed below.

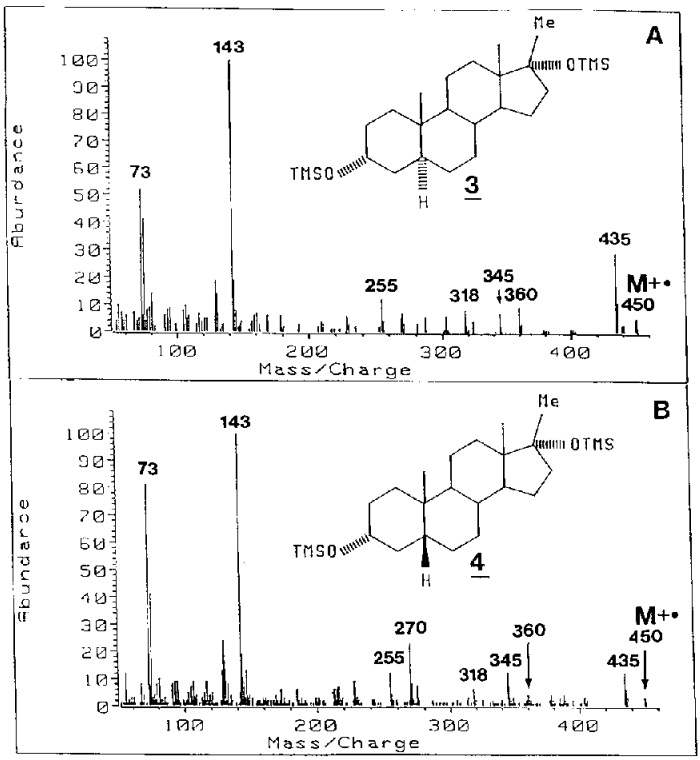

Fig. 3. Mass spectra of (A) 17β-methyl-5α-androstan-3α,17β-diol (**3**) isolated in human urine after administration of mestanolone and oxymetholone; and (B) 17β-methyl-5β-androstan-3α,17β-diol (**4**) isolated from 17α-MT and methandienone urine samples and synthesized from 17α-MT sulfate pyridinium salt as the TMS ether derivatives.

17α-Methyltestosterone

From the previous work of Rongone and Segaloff [13], it was expected that **2** (Fig. 4) would be the major tetrahyhdro metabolite arising from 17α-MT metabolism. Surprisingly, a prominent peak with the same retention time as that of **1** was first detected by GC–MS (Fig. 1C). However, comparison of the TMS ether derivatives of authentic reference steroids **1** and **2** showed that both compounds coeluted under the GC–MS conditions used (Table I). Attempts to resolve these isomers chromatographically by modifying GC conditions and using various stationary phases were unsuccessful. Indirect evidence for the presence of both **1** and **2** in this prominent peak was provided by the presence of trace amounts of epimeric steroid **3** (Fig. 1C and Table I) and of **4**, the TMS ether of which exhibited a mass spectrum (Fig. 3B) virtually identical with that of authentic 17α-methyl-5β-androstan-3α,17β-diol (**2**) (Fig. 2C). Thus, **4** was identified as 17β-methyl-5β-androstan-3α,17α-diol on the basis of the GC–MS rationales men-

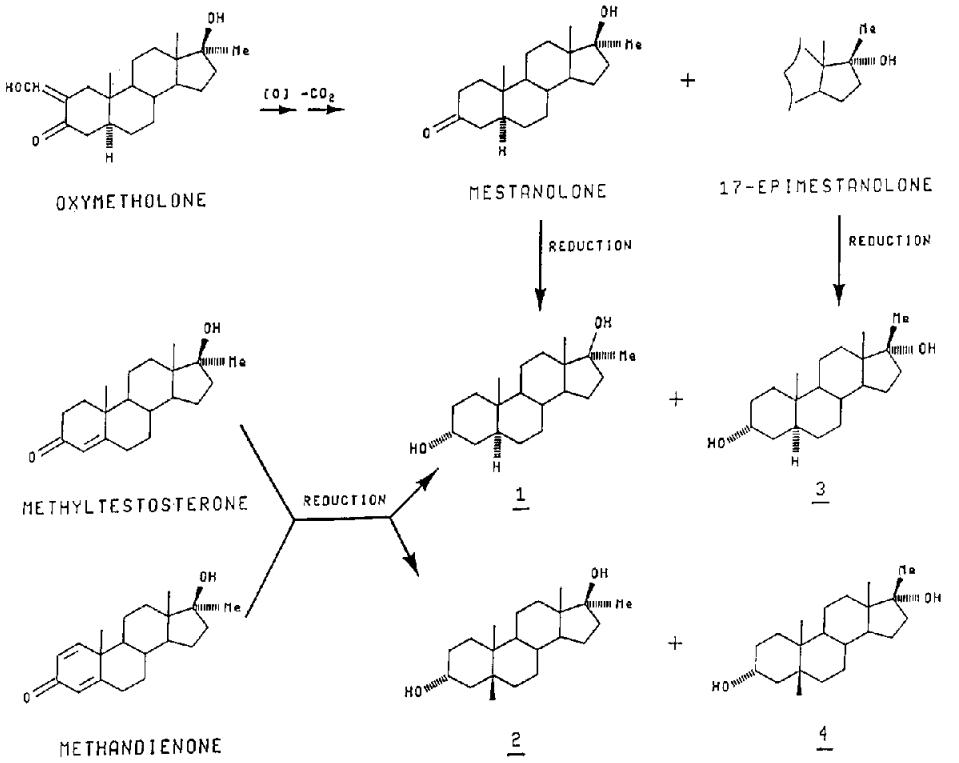

Fig. 4. Proposed pathways accounting for the formation of tetrahydro diols **1-4** from oxymetholone, mestanolone, 17α-MT and methandienone metabolism in human. Me = Methyl.

tioned above. Further examination of the mass spectral data from authentic steroids **1** and **2** showed characteristic differences in the relative intensities of several of their common fragment ions (Table II). Indeed, ions of *m/z* 345 and 370 are about two and three times more intense, respectively, in the mass spectrum of the 5β-steroid **2** than in its 5α-isomer **1**. Conversely, ions of *m/z* 435 and 360 are about two and three times more prominent, respectively, in the mass spectrum of **1**. It was then possible to use these mass spectral features not only to differentiate **1** from its isomer **2**, but also to determine their respective amounts in the chromatographic peak in which they coelute. This was achieved by analyzing the TMS ether derivative of authentic steroids **1** by selected ion monitoring (SIM) GC–MS. The results are presented in Table II. From the general equations $ax + by = c$ and $x + y = 100$, where x and y are the percentages of steroids **1** and **2**, respectively, in the coelution peak, we calculated, using the relative intensities of ions of *m/z* 270, 345, 360 and 435, that metabolites **1** (17%) and **2** (83%) were excreted in urine in a 1:4.9 ratio [relative standard deviation (R.S.D.) = 1.36%, $n = 3$]. This ratio is half that reported by Rongone and Segaloff [13] in a woman with ad-

TABLE II

RELATIVE INTENSITIES OF CHARACTERISTIC FRAGMENT IONS OR URINARY METABOLITES 1 AND 2 AND OF CORRESPONDING AUTHENTIC STEROIDS AS TMS ETHER DERIVATIVES

Metabolite[a]	Relative intensity (%)[b]							
	m/z 143	*m/z* 255	*m/z* 270	*m/z* 345	*m/z* 360	*m/z* 435	*m/z* 450	5α/5β[c]
1 (MSTL)	100.0	15.4	8.9	7.0	9.1	35.4	5.2	–
1 (OXYM)	100.0	15.3	8.9	6.9	9.2	34.9	5.2	–
1 + **2** (17α-MT)	100.0	14.4	25.7	11.1	4.4	23.0	3.9	1:4.9
1 + **2** (MHDN)	100.0	14.3	24.4	10.9	4.5	23.6	3.9	1:3.8
Authentic **1**[d]	100.0	14.9	9.3	6.7	9.4	36.4	5.4	–
Authentic **2**[e]	100.0	14.0	28.9	12.1	3.5	20.3	3.5	–

[a] Compounds **1** and **2** refer to the urinary metabolites detected in mestanolone (MSTL), oxymetholone (OXYM), 17α-methyltestosterone (17α-MT) and methandienone (MHDN) post-administration urine samples.

[b] Relative intensities were measured by SIM GC–MS of the ions listed.

[c] These values refer to the ratio of compounds **1** and **2** as measured by SIM GC–MS of 17α-MT and methandienone urine samples.

[d] Authentic 17α-methyl-5α-androstan-3α,17β-diol.

[e] Authentic 17α-methyl-5β-androstan-3α,17β-diol.

vanced breast cancer. In addition to analytical and methodological differences, the higher 5α/5β ratio which they observed can be rationalized by the facts that 5β-reductases are generally more active in females than in males [5], the disease state of the subject could have hindered or reduced the number of steroid receptors associated with microsomal fractions, where the 5α-reductases are located [5,21,22], thus promoting the activity of 5β-reductases which are found in the soluble fraction of liver homogenates [5], and the large doses (1 g daily for 4 days) ingested could have saturated the 5α-reductase receptors, thus promoting 5β-reduction. However, the marked affinity of some 17α-methyl-Δ^4-3-one steroids for 5β-reductases [23] appears to be the key factor accounting for the predominance of **2** in 17α-MT catabolism.

Methandienone

It was then of interest to study the effect that the introduction of a Δ^1-function in 17α-MT would have on the stereoselectivity of the Δ^4-group reduction. Preliminary evidence obtained by GC–MS analysis of urine specimens collected after oral administration of methandienone (Fig. 1D) indicated that the biotransformation of methandienone A-ring was substantially different from that of 17α-MT (Fig. 1C).

The relative intensities of diagnostic ions (Table II) determined by SIM GC–

MS of the prominent peak eluting at 23.35 min indicated the presence of both **1** (21%) and **2** (79%) in a ratio of 1:3.8 (R.S.D. 2.7%, $n = 3$). This ratio is very similar to that measured for the corresponding metabolites of 17α-MT, thus suggesting that the presence of a Δ^1-function in methandienone does not significantly influence its relative affinities for 5α- and 5β-reductases with respect to 17α-MT affinities for the same enzymes.

Further examination of the ion chromatogram (Fig. 1D) shows a minor compound (**5**), the TMS ether mass spectrum of which (Fig. 5A) exhibited a molecular ion at m/z 374 (m/z 383, d_9-TMS), and diagnostic ions at m/z 317 (M − 57; m/z 326, d_9-TMS), m/z 304 (M − 70; m/z 313, d_9-TMS), which are formed according to mechanisms reported previously [24], and m/z 143 (m/z 152, d_9-TMS) which are consistent with the proposed structure. The mass spectrum of the corresponding TMS enol–TMS ether derivative (Fig. 5B) confirmed the presence of a Δ^1-3-oxo moiety in **5** by its molecular ion at m/z 446 (m/z 455, d_0-TMS

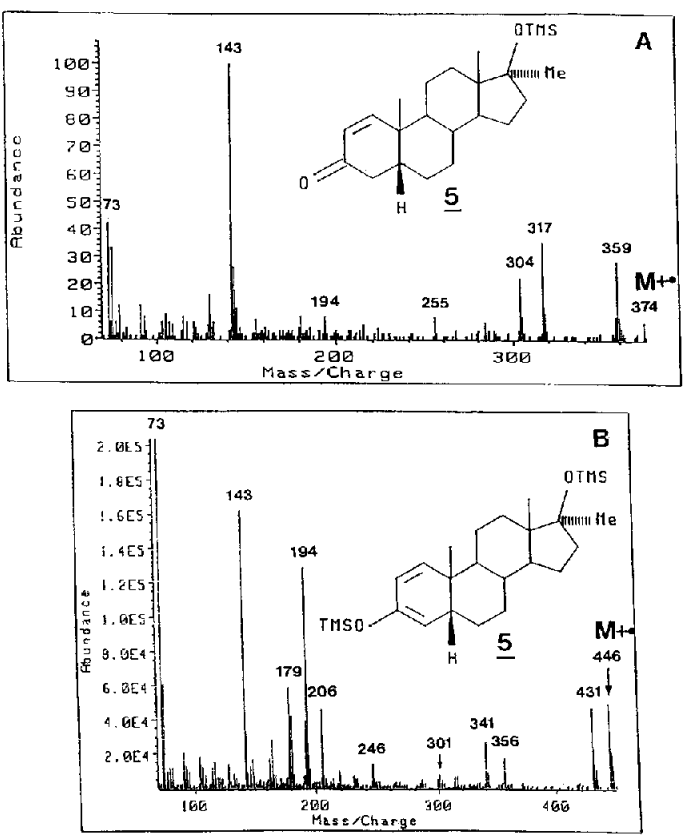

Fig. 5. Mass spectra of compound **5** as (A) TMS ether and (B) TMS enol–TMS ether derivatives isolated from methandienone post-administration urine samples.

enol–d_9-TMS ether). Structurally informative ions at m/z 194 and 206 arising from B-ring cleavage provided further evidence for the identity of **5**. As evidenced by selective d_9-TMS labelling (Fig. 6), the formation of the ion at m/z 194 in the mass spectrum of the TMS ether derivative of **5** (Fig. 5A) was probably initiated by cleavage of the B-ring [25] with concomitant elimination of two hydrogen atoms and long-range migration of the C-17 TMS group to the 3-oxo function. Similar long-range TMS group migration has been described by Gaskell *et al.* [26]. We also observed a similar long-range TMS group migration in the mass spectrum of the TMS ether derivative of 6β-hydroxymethandienone [9], where diagnostic ions at m/z 209 (cleavage of the C-9–C-10 and C-6–C-7 bonds) and m/z 281 (cleavage of the C-9–C-10 and C-6–C-7 bonds with concomitant migration of the 17-OTMS group to the 3-oxo function) were shifted by 9 and 18 u, respectively, to m/z 218 and 299 in the mass spectrum of the corresponding d_9-TMS ether, thus confirming TMS group migration from C-17 to C-3. Durbeck and Buker [24] previously proposed that the ion at m/z 281 arises from the consecutive losses of trimethylsilanol and a TMSO$^{\cdot}$ radical from the molecular ion $[M-90-89]^+$ without supporting their hypothesis by a d_9-labelling experiment. In the mass spectrum of **5** TMS enol–TMS ether derivative (Fig. 5B), the formation of a similar ion was strongly favored in addition to that of the ion at m/z 206

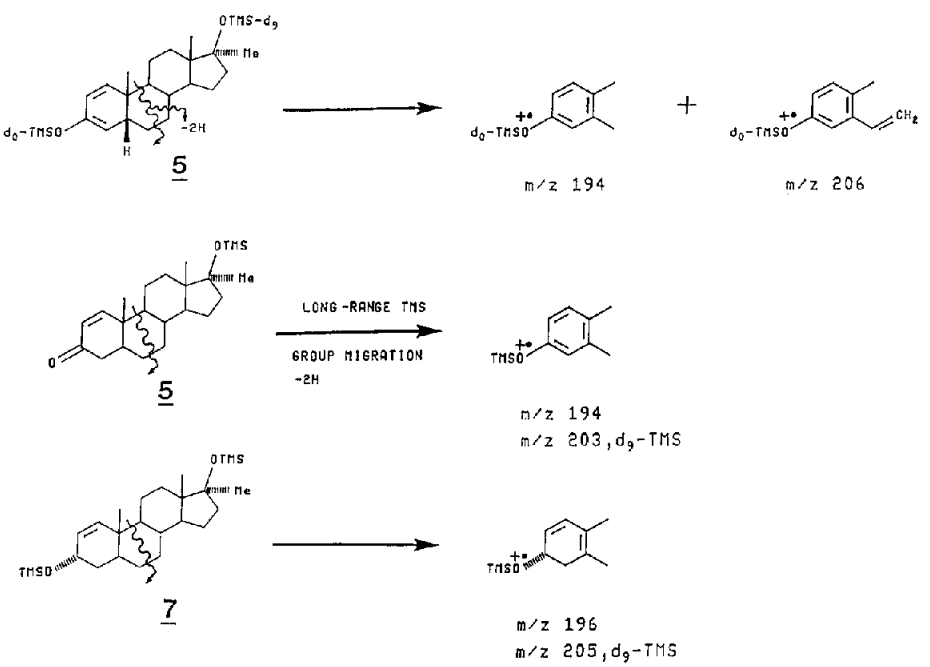

Fig. 6. Proposed structure of diagnostically important ions of m/z 194, 196 and 206 observed in the mass spectra of compounds **5**, **6**, **7** and **8** TMS derivatives.

which arises from the cleavage of the C-9–C-10 and C-7–C-8 bonds. Here selective d_9-TMS labelling demonstrated that ions of m/z 194 and 206 comprise the A-ring and the C-6 and C-7 atoms, respectively (Fig. 6) and not the C-ring and C-7 and C-17 atoms as proposed by Durbeck and Buker [24]. Compound **5** was thus identified as 17β-hydroxy-17α-methyl-5β-androsta-1-en-3-one. This structural assignment was also in accordance with that of the prominent tetrahydrodiol **2** resulting from further reductive metabolism of **5** as shown below.

The faster eluting compound **6** (20.70 min), which exhibited mass spectral features identical with those of **5**, was assigned the structure of 17α-hydroxy-17β-methyl-5β-androsta-1-en-3-one.

Finally, the prominent peaks 7 and 8 (Fig. 1D) provided mass spectra which were virtually identical (Fig. 7A and B), thus suggesting that compound **8** was the 17-epimer of **7**. Their TMS ether derivative showed a molecular ion at m/z 448 (m/z 466, d_9-TMS), indicating that the Δ^4- and 3-oxo groups were probably reduced. This hypothesis was supported by the fact that attempts to prepare their corresponding MO-TMS derivatives afforded two compounds with mass spectra identical with those shown in Fig. 7A and B. Interestingly, the low-intensity ion at

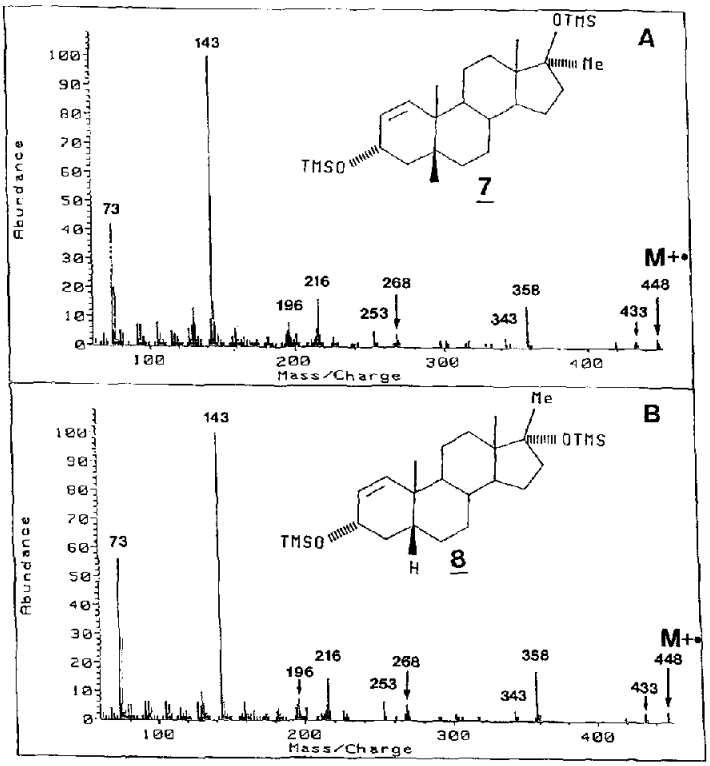

Fig. 7. Mass spectra of (A) compound **7** and (B) compound **8** as the TMS ether derivatives isolated in human urine after administration of methandienone.

m/z 196 (*m/z* 205, d_9-TMS) is analogous to that observed at *m/z* 194 in the mass spectra of **5** and **6** (Fig. 5A and B), and probably arises, as shown by d_9-TMS labelling, from B-ring cleavage (Fig. 6). Thus, **7** was assigned the structure of 17α-methyl-5β-androsta-1-en-3α,17β-diol and **8** was identified as 17β-methyl-5β-androsta-1-en-3α,17α-diol.

The data presented above provided strong evidence that the sequential reduction of methandienone A-ring functional groups could proceed as proposed in Fig. 8. First, methandienone is converted into **5** and its 17-epimer **6** by the action of 5β-reductases with concomitant epimerization at C-17. Compounds **5** and **6** did not accumulate in large amounts in urine because their 3-oxo group can be rapidly reduced by 3α-OHSDH to yield the epimeric Δ^1-steroids **7** and **8**, respectively. This observation is supported by the fact that the rate of 3α-OHSDH is generally faster than that of 4-ene-5α- and 5β-reductases in several tissues [5]. Higher rates of 3α-OHSDH could also account for the absence in urine of 17β-hydroxy-17α-methyl-5β-androstane-3-one (Fig. 8), the intermediate metabolite resulting from the 5β-reduction of 17α-MT. In all probability, this metabolite was gradually converted by 3α-OHSDH into **2**, as it was produced from the parent steroid. The proposed sequence of methandienone A-ring reduction is also in accordance with the reduction patterns of Δ^4-3-oxo endogenous steroids reported previously [1,4,6]. Although the data presented above showed that small amounts of the 5α-steroids **1** and **3** were generated by methandienone biotransformation, no trace of their corresponding metabolic precursors, analogous to **5** and **7**, were detected in urine. This probably reflects the low production rate of **1** and **3** (Fig. 1), and consequently that of their Δ^1-3-oxo and Δ^1-3-hydroxy precursors. The metabolic route shown in Fig. 8 was proposed to account for the presence in urine of **1, 2, 5** and **7** and their corresponding epimers **3, 4, 6** and **8**. However, there

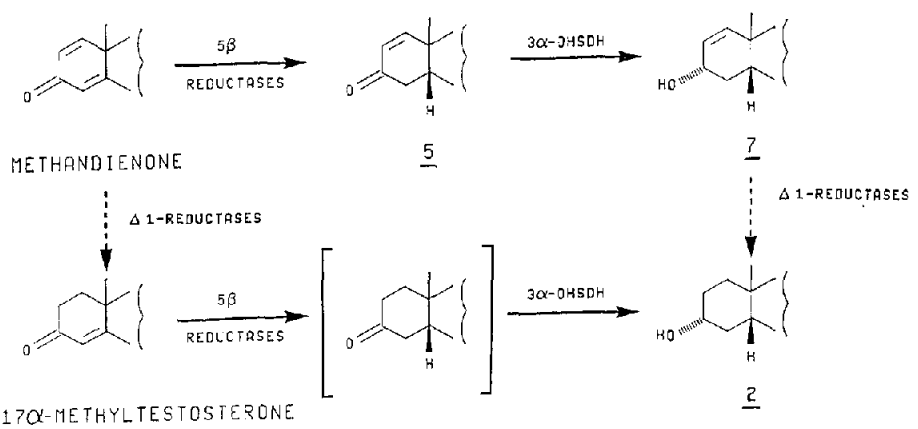

Fig. 8. Proposed metabolic pathways accounting for the formation of methandienone and 17α-methyltestosterone urinary metabolites resulting from the sequential reduction of their A-ring substituents.

could be another metabolic route whereby the Δ^1-group of methandienone would first be reduced to give 17α-MT, as the intermediate metabolite, instead of **5** (Fig. 8). Although 17α-MT was not detected in urine after administration of methandienone, its probable formation could probably have been demonstrated by GC–MS of plasma samples, which unfortunately were not available in this study.

Epimerization of 17α-methyl-17β-sulfate conjugate

The formation of 17-epimethandienone from methandienone was first reported by Macdonald *et al.* [18] and was later characterized by Durbeck *et al.* [19] in human urine by GC–MS. The mechanism underlying this reaction remained highly speculative until Edlund *et al.* [17] recently reported that epimethandienone was readily formed by nucleophic attack of water on the sulfate conjugate of methandienone. To investigate this reaction further and identify some of the 17-epimeric metabolites reported above, we synthetized the 17β-sulfate conjugates of several 17α-methyl-17β-hydroxy steroids, more particularly those of methandienone, 17α-MT and 17α-methyl-5β-androstan-17β-ol-3-one. The latter sulfates were characterized by ^1H and ^{13}C NMR and FAB-MS (to be reported separately). Epimerization reactions were carried out with the sulfate pyridinium salts in aqueous solutions at 55°C for 60 min. Under these conditions the corresponding 17α-hydroxy-17β-methyl epimers were produced in about 50% yield together with dehydration by-products. Fig. 9 illustrates the epimerization reaction of a steroidal sulfate pyridinium salt into its corresponding 17-epimer. The reduction of 17-epimethyltestosterone thus prepared with $NaBH_4$ preponderantly afforded the 17-epimer **4**. GC–MS of the synthetic 17-epimeric steroids

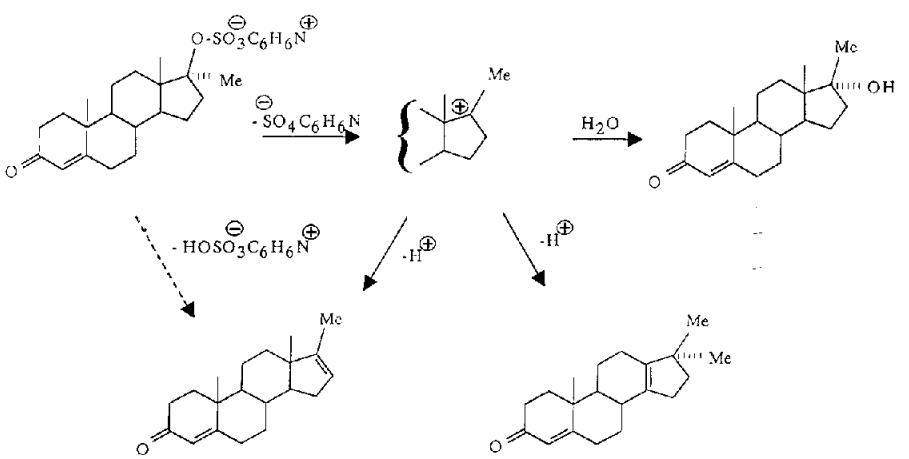

Fig. 9. Epimerization of the 17β-sulfate pyridinium salt of 17α-methyltestosterone into the corresponding 17α-hydroxy-17β-methyl epimers and rearrangement products through a transient carbocation.

demonstrated that they were identical with the 17-epimeric steroids previously isolated from post-administration urine samples. These results are in accordance with those reported by Edlund *et al.* [17] and indicate that epimerization does not proceed following an enzymatic pathway.

It should be noted that epimethandienone is recovered only from the free steroid fraction [17,19] of human urine. This indicates that this steroid is not reconjugated after epimerization, thus suggesting that epimerization could have occurred in the bladder, after excretion from the kidneys. However, it is also possible that epimerization could have occurred in the liver, the gut and/or in the course of the enterohepatic circulation of the 17-sulfate conjugate, as 17-epimethandienone does not bear chemical features favouring conjugation reactions. This hypothesis was strengthened by the fact that the 17-epimeric metabolites **3, 4, 6** and **8** were solely isolated from the glucuronide conjugate fraction. This suggests that sulfation, and subsequently epimerization, could occur in the hepatic tissue concomitantly with A-ring reduction, where the resulting 3α-hydroxy group could be readily conjugated with glucuronic acid prior to excretion in urine. As the biliary route of excretion of neutral steroids is of quantitative importance in humans [27], biliary excretion and enterohepatic circulation of methandienone and/or its metabolites could partially account for the presence of the epimeric metabolites in the glucuronic fraction. This hypothesis is supported by the fact that sulfate conjugation favours excretion via this route [28]. Thus, it is probable that epimerization of sulfate conjugates and reconjugation with glucuronic acid could have also occurred in the intestine, where the resulting glucuronides can be partly reabsorbed. However, reabsorption of glucuronides involves passive ionic diffusion, which is a slow process. Reabsorption of the free steroid, formed by bacterial enzymes, followed by glucuronidation in the intestinal wall or liver, seems more likely. The reabsorbed metabolites could also undergo further metabolism in the intestine wall and/or at hepatic sites and the products may again be excreted in bile or eliminated in urine [28,29]. An excretion route involving biliary excretion and enterohepatic circulation and the apparent low rate of Δ^1-reductases could account for the presence in urine of **8** during long period of time after methandienone ingestion, even when its epimer **7** and the tetrahydrodiols **1** and **2** become barely detectable. Retention of methandienone in certain cells or tissues could also account for this phenomenon, but is less likely as it does not provide any rationale for the progressive slowing down and stopping of the urinary excretion of the prominent metabolites **1** and **2** (Fig. 1D), while that of **8** was still observed. This hypothesis was further supported by the fact that metabolic precursors **5** and **7** (Fig. 8) of the tetrahydrodiol **2** were first detected in urine 3.5 h after methandienone ingestion, whereas the epimeric steroid **8** and compound **2** were first observed 6.5 and 10.0 h after steroid ingestion. Although the urinary level of **2** progressively increased during the next 48 h, those of its metabolic precursors **5** and **7** rapidly decreased, thus suggesting that the rates of the 5β-, 3α- and Δ^1-reductions were concomitantly increased so that **5** and **7** were

rapidly metabolized into **2** and consequently were excreted in urine in only minute amounts. On the other hand, the urinary excretion of **8** progressively increased during that period and was maintained for more than 4 days after steroid ingestion. This suggests that epimerization at C-17 is a major metabolic reaction in methandienone metabolism which appears to decrease the rate of Δ^1-reductases, as its corresponding epimer **7** did not accumulate in urine for a long period after steroid ingestion.

The epimerization of 17β-sulfate-17α-methyl steroids could be of toxicological importance and related to some of the liver dysfunctions known to be induced by these steroids [30–32]. Mechanistically, the 17β-sulfate group reacts through a transient carbocation, which could further react "*in vivo*" with phenol, amine and thiol groups of endogenous compounds, including biopolymers, to yield covalent adducts. This sequence of chemical reactions is compatible with the hypothesis that metabolic activation into electrophilic species and their interactions with macromolecular targets are critical events in the induction of toxic processes in living organisms [33].

CONCLUSION

Investigation of the sequential reduction of methandienone A-ring substituents demonstrated that they were reduced according to the sequence Δ^4-group, 3-oxo group and Δ^1-group. The corresponding Δ^1-3-oxo, Δ^1-3-hydroxy metabolic intermediates and the resulting tetrahydrodiols were characterized by GC–MS, whereas the structure of their diagnostically important ions was assessed by d_9-TMS labelling. Further analytical and metabolic evidence was obtained using mestanolone, 17α-MT and oxymetholone as model steroids. For each metabolite of interest, we detected and identified the corresponding 17-epimer. We have shown that these epimers were produced by nucleophilic substitution by water of the labile sulfate conjugates. This finding is in agreement with the study of Edlund *et al.* [17]. Epimerization of 17β-sulfate conjugates could be of toxicological importance owing to the formation of transient electrophilic species that could ultimately promote toxic effects.

ACKNOWLEDGEMENTS

Financial assistance from the Sports Medicine Council of Canada, the Natural Sciences and Engineering Research Council of Canada (Grant No. 1310 to R.M.) and the National Collegiate Athletic Association is gratefully acknowledged. We thank Mr. C. Laliberté for recording the FAB mass spectra, Mrs. F. Sauvageau for typing the manuscript and Mrs. D. Lacoste for drawings.

REFERENCES

1 R. I. Dorfman and F. Ungar, *Metabolism of Steroid Hormones*, Academic Press, New York, 1965, pp. 382–400.
2 H. L. J. Kruskemper, *Anabolic Steroids*, Academic Press, New York, 1968, pp. 21–30.
3 K. Fotherly and F. James, *Adv. Steroid Biochem. Pharmacol.*, 3 (1972) 21.
4 A. F. Clark, in R. Hobkirk (Editor), *Steroid Biochemistry*, Vol. 2, CRC Press, Boca Raton, FL, 1979, pp. 1–27.
5 D. B. Gower, in H. L. J. Makin (Editor), *Biochemistry of Steroid Hormones*, Blackwell, London, 1984, pp. 271–279, and references cited therein.
6 D. B. Gower, in H. L. J. Makin (Editor), *Biochemistry of Steroid Hormones*, Blackwell, London, 1984, pp. 349–382, and references cited therein.
7 R. Massé, C. Laliberté, L. Tremblay and R. Dugal, *Biomed. Mass Spectrom.*, 12 (1985) 115.
8 R. Massé, C. Ayotte and R. Dugal, in G. Piemonte, F. Tabliaro, M. Marigo and A. Frigerio (Editors), *Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences*, Plenum Press, New York, 1987, pp. 183–190.
9 R. Massé *et al.*, unpublished results.
10 L. L. Engel, J. Alexander and M. Wheeler, *J. Biol. Chem.*, 231 (1958) 159.
11 H. Floch, A. Crastes de Paulet, E. E. Naulieu, *C.R. Acad. Sci.*, 255 (1962) 2512.
12 R. Massé, C. Ayotte and R. Dugal, *J. Chromatogr.*, 489 (1989) 23.
13 E. L. Rongone and A. Segaloff, *J. Biol. Chem.*, 237 (1962) 1066.
14 R. Massé, C. Ayotte, H. Bi and R. Dugal, *J. Chromatogr.*, 497 (1989) 17.
15 R. Massé, H. Bi, C. Ayotte and R. Dugal, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 429.
16 D. H. R. Barton, *J. Chem. Soc.*, (1953) 1027.
17 P. O. Edlund, L. Bowers and J. Henion, *J. Chromatogr.*, 487 (1989) 341.
18 B. S. Macdonald, P. J. Sykes, P. M. Adhikary and R. A. Harkness, *Steroids*, 18 (1971) 753.
19 H. W. Durbeck, I. Buker, B. Scheulen and B. Telin, *J. Chromatogr.*, 167 (1978) 117.
20 R. Massé, H. Bi and P. Du, *J. Steroid Biochem.*, in press.
21 S. Liao, J. L. Tymoczko, T. Liang, K. M. Anderson and S. Fang, *Adv. Biosci.*, 7 (1971) 155.
22 R. J. Pietras and C. M. Szego, *J. Steroid Biochem.* 11 (1970) 1471.
23 R. E. Steele, F. Didato and B. G. Steinetz, *Steroids*, 29 (1977) 331.
24 H. W. Durbeck and I. Buker, *Biomed. Mass Spectrom.*, 7 (1980) 437.
25 F. J. Brown and C. Djerassi, *J. Am. Chem. Soc.*, 102 (1980) 807.
26 S. J. Gaskell, A. G. Smith and C. J. W. Brooks, *Biomed. Mass Spectrom.*, 2 (1975) 148.
27 T. Laatikainen, *Ann. Clin. Res.*, 2 (1970) 338.
28 K. Hartialla, *Physiol. Rev.*, 53 (1973) 496.
29 F. Martin, H. Adlercreutz, B. Lindstrom, H. Dencker, U. Rimer and N. O. Sjöberg, *J. Steroid Biochem.*, 6 (1975) 1371.
30 J. Schappler and P. Gunzel, *Adv. Pharmacol. Ther.*, 8 (1979) 159.
31 M. W. Kibble and M. B. Ross, *Clin. Pharm.*, 6 (1987) 686.
32 T. M. Creagh, A. Rubins and D. J. Evens, *J. Clin. Pathol.*, 41 (1988) 441.
33 F. P. Guengerich and D. C. Liebler, *Crit. Rev. Toxicol.*, 14 (1985) 259.