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STUDIES ON ANABOLIC STEROIDS—6. IDENTIFICATION OF URINARY METABOLITES OF STENBOLONE ACETATE (17β-ACETOXY-2-METHYL-5α-ANDROST-1-EN-3-ONE) IN HUMAN BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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Summary—The metabolism of stenbolone acetate (17β-acetoxy-2-methyl-5α-androst-1-en-3-one), a synthetic anabolic steroid, has been investigated in man. Nine metabolites were detected in urine either as glucuronic or sulfuric acid aglycones after oral administration of a single 50 mg dose to a male volunteer. Stenbolone, the parent compound, was detected for more than 120 h after administration and its cumulative excretion accounted for 6.6% of the ingested dose. Most of the stenbolone acetate metabolites were isolated from the glucuronic acid fraction, namely: stenbolone,3α-hydroxy-2-methy-5α-androst-1-en-17-one, 3α-hydroxy-2ξ-methyl-5α-androstan-17-one; 3 isomers of 3ξ,16ξ-dihydroxy-2-methyl-5α-androst-1-en-17-one; 16α and 16β-hydroxy-2-methyl-5α-androst-1-ene-3,17-dione; and 16ξ,17β-dihydroxy-2-methyl-5α-androst-1-en-3-one. Only isomeric metabolites bearing a 16α or a 16β-hydroxyl group were detected in the sulfate fraction. Interestingly, no metabolite was detected in the unconjugated steroid fraction. The steroids identities were assigned on the basis of their TMS ether, TMS enol-TMS ether, MO-TMS and d_9-TMS ether derivatives and by comparison with reference and structurally related steroids. Data indicated that stenbolone acetate was metabolized into several compounds resulting from oxidation of the 17β-hydroxyl group and/or reduction of A-ring δ-1 and/or 3-keto functions with or without hydroxylation at the C_{16} position. Finally, comparison of stenbolone acetate urinary metabolites with that of methenolone acetate shows similar biotransformation pathways for both δ-1-3-keto anabolic steroids. This indicates that the position of the methyl group at the C_1 or C_2 position in these steroids has little effect on their major biotransformation routes in human, to the exception that stenbolone cannot give rise to metabolites bearing a 2-methylene group since its 2-methyl group cannot isomerize into a 2-methylene function through enolization of the 3-keto group as previously observed for methenolone.

INTRODUCTION

Stenbolone acetate (Anatrofin®) is a synthetic anabolic steroid bearing a double bond and a methyl group at the C_1 and C_2 positions, respectively. These specific structural features seem responsible for its anabolic activity in humans, which was reported to be higher than that displayed by testosterone [1].

To our knowledge, no information has been reported in the literature about the biotransformation of this steroid in humans. We recently reported [2] a detailed study on the urinary metabolites of methenolone acetate, an isomeric analog of stenbolone acetate. We showed that methenolone metabolism was characterized by regioselective hydroxylations at the C_2, C_6 and C_{16} positions with concomitant oxidation of the 17β-hydroxyl group. Furthermore, we showed that reduction of the δ-1 group was not a major metabolic reaction, whereas reduction of the 3-keto function was a preponderant reaction in only one of methenolone biotransformation route.

This study demonstrated that the presence of a δ-1-1-methyl moiety in methenolone A-ring has a marked effect on its biotransformation routes with respect to testosterone, the metabolism of which is being characterized by stereoselective reductions of its δ-4-3-keto moiety with concomitant oxidation of the 17β-hydroxyl group to yield androsterone, etiocholanolone and epiandrosterone as major urinary metabolites.

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Our interest here was motivated by the close structural resemblance between methenolone and stenbolone, the premise being that such a shift of the methyl group from the C_1 to the C_2 position in the later steroid could bring about subtle conformational changes or alter the redistribution of electron density in the δ-1-3-keto moiety which, in turn, could be reflected by some specific modifications of its biotransformation routes.

In this paper we report the isolation and the identification of 9 metabolites of stenbolone acetate from the steroid aglycone fractions. The urinary metabolites were detected and characterized by comparison with authentic or structurally related steroids and on the basis of the GC/MS features of their TMS ether, TMS enol-TMS ether, MO-TMS and corresponding perdeuterated (d_9-TMS) derivatives. Metabolic pathways accounting for their formation are proposed and the urinary excretion profile of unchanged stenbolone is presented. The metabolic routes of methenolone and stenbolone are compared and discussed on the basis of their respective structural features.

EXPERIMENTAL

Chemical and reagents

All chemicals and reagents used in this study were described in a previous paper [2].

Steroids

Stenbolone acetate was kindly supplied by Syntex (Palo Alto, CA, U.S.A.). 17β-Hydroxy-1-methyl-5α-androst-1-en-3-one (methenolone) was kindly supplied by Schering AG (Berlin, Germany). 5α-Androstan-17-one was purchased from Steraloids Inc. (Wilton, NH, U.S.A.).

Biological samples

Urine samples were collected in sterile plastic containers during the 6 days after oral administration of a single 50 mg dose of stenbolone acetate to a normal and healthy nonsmoking volunteer. All samples were kept at $-20°C$ until analysis. Blank urine samples were collected from the same volunteer during the 24 h preceding the ingestion of the anabolic steroid.

Hydrolysis of stenbolone acetate

Stenbolone, which was not commercially available, was obtained by hydrolysis of stenbolone acetate. 100 mg of stenbolone acetate was dissolved in 2 ml of 25% (w/v) sodium methoxide in methanol (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). The resulting solution was left at room temperature for 2 h. Then 2 ml of water were added to the mixture and stenbolone was extracted with 5 ml of diethylether. The etheral layer was dried over anhydrous sodium sulfate and evaporated to dryness at 40°C under a nitrogen stream. Recrystallization of the residue from a mixture of chloroform, diethylether and petroleum ether (30–60°C) afforded the title steroid as a crystalline and chromatographically homogenous product, its melting point was 165–167°C (m.p. 155–158°C, *The Merck Index*, 11th edn, No. 8763, 1989). The stenbolone acetate melting point was 155–157°C (m.p. 146–149°C, *The Merck Index*). Melting points are uncorrected. The purity of stenbolone (>98.6%) was determined by GC/MS analysis of its TMS enol-TMS ether derivative.

Stock solutions

Stock solutions of stenbolone (1 mg/ml), methenolone (1 mg/ml) and 5α-androstan-17-one (1 mg/ml) were prepared in anhydrous methanol and stored at 4°C.

Standard curve

A standard curve for the quantitation of stenbolone in urine samples was prepared. Serial dilutions of the stock solutions were carried out so as to prepare 7 solutions with stenbolone concentrations in the range of 1–20 and 10 ng/μl for methenolone which was used as external standard. After evaporation of the solvent, the steroids were derivatized as TMS enol-TMS either derivatives in 100 μl of a mixture of MSTFA and TMSI as previously described [2]. Each sample was prepared in duplicate and 1 μl was injected 3 times into the chromatograph.

The samples were analysed by GC/MS in the selected ion monitoring (SIM) mode. Quantitation was performed by measuring the peak area ratios of the molecular ions of stenbolone and methonolone at m/z 446. Linearity was obtained in the range 1–20 ng/μl. The corresponding regression equation was:

$$C_S = 4.926\,Ar + 0.211 \quad (r^2 = 0.996)$$

where C_S is the stenbolone concentration (ng/μl) and Ar is the area ratio of stenbolone to methenolone. A HP-5 (cross-linked 5% phenyl, methyl silicone) fused-silica capillary column

(25 m × 0.2 mm i.d., 0.33 μm film thickness) was used for quantitation.

Isolation of urinary steroids and GC/MS analysis

Conditions and methods used for extraction, hydrolysis, fractionation of steroid conjugates, derivatization and GC/MS analysis of stenbolone acetate urinary metabolites, as well as the method used to determine stenbolone recovery from urine, were as previously described [2] with slight modifications. To prevent overloading of the chromatographic column and inaccuracy in stenbolone quantitation, all urinary samples were submitted to a preliminary screening so as to determine the general features of the excretion profile of stenbolone acetate metabolites. Consequently, 1 ml of urine was used to quantify stenbolone in the samples collected during the first 24 h after administration and 2 ml aliquots were used for all other samples collected after that time. Urinary extracts were transferred to 0.3 ml Reactivials® containing 10 ng/μl methenolone and derivatization was carried out using: 50 μl of MSTFA-TMSI for samples 10–12, 15–17 and 21–25; 100 μl for samples, 1, 13, 14 and 18–20; and 200 μl for samples 2–7 in which the highest concentrations of stenbolone were encountered. The mean recovery of stenbolone from urine was 89.3% (cv. = 1.14%, $n = 6$). Finally, the free and conjugated steroids were isolated from 3 and 5 ml aliquots of urine, respectively.

Enzymatic oxidation of 3-hydroxy metabolites

In order to determine the α-stereochemistry of the 3-hydroxy metabolites of stenbolone acetate, namely 3-hydroxy-2-methyl-5α-androst-1-en-17-one and 3-hydroxy-2-methyl-5α-androstan-17-one, the latter steroids were extracted from volunteer's urine and oxidized with a specific 3α-hydroxysteroid dehydrogenase enzyme from Pseudomonas Testosteroni (Sigma Co., St Louis, MO, U.S.A.).

Firstly, the specificity of the enzyme was assayed with androsterone and epiandrosterone. 2 μg of each steroid were incubated at 37°C for 1 h with a mixture of 150 μl of 3α-hydroxysteroid dehydrogenase (0.1 U/ml), 1 ml of 0.1 M phosphate buffer pH 8.5 and 140 μl of NAD^+ 4 mM. The steroids were extracted with 5 ml of diethylether and derivatized in 100 μl of a mixture, MSTFA–TMSI (100:2) to prepare the corresponding TMS enol-TMS ether derivatives as described in a previous paper [2]. GC/MS analysis showed that androsterone was quantitatively transformed into 5α-androstane-3,17-dione, whereas the 3β-hydroxy group of epiandrosterone was not oxidized by the enzyme since it was recovered from the reaction mixture and no trace of 5α-androstane-3,17-dione was detected.

For stenbolone metabolites, 2 ml of urine collected 4.25 h after stenbolone acetate administration were extracted as previously described [2]. The steroidal extract, dissolved in 100 μl of methanol, was incubated for 3 h at 37°C with 1 ml of 3α-hydroxysteroid dehydrogenase (2 U/ml) in 2 ml of 0.1 M phosphate buffer (pH 8.5), to which 250 μl of NAD^+ 12 mM were added. The steroid was extracted and derivatized as described above. 1 μl was injected into the gas chromatograph and the GC/MS analysis was performed as described above.

RESULTS AND DISCUSSION

Urinary excretion of stenbolone acetate metabolites

The cumulative urinary excretion of unchanged stenbolone (**3**) and its urinary excretion profile (inset) after oral administration of a single 50 mg dose of Anatrofin® to a healthy volunteer are shown in Fig. 1. A peak level of 7.7 μg/ml occurred 4.25 h after administration. Then, stenbolone concentration rapidly decreased to a level of 0.10 μg/ml after 32 h. It is interesting to note that the urinary level of the steroid then raises periodically within the following time intervals: 37–57 and 57–82 h to levels of 1.0 and 0.8 μg/ml, respectively. This phenomenon probably reflects the enterohepatic circulation of stenbolone, although the retention of stenbolone acetate and/or stenbolone in specific tissues and their subsequent redistribution may also contribute, to a certain extent, to those punctual increases of stenbolone urinary levels. A similar excretion profile was observed for methenolone [2], but urinary level variations were much less intense since methenolone cumulative urinary excretion is only 25% that of stenbolone, the urinary excretion of which accounted for 6.6% of the administered 50 mg oral dose.

Fractionation of stenbolone acetate metabolites revealed that conjugation to glucuronic acid was the major urinary excretion route. This fraction contains 9 metabolites (Fig. 2A) that were clearly distinguished from endogenous

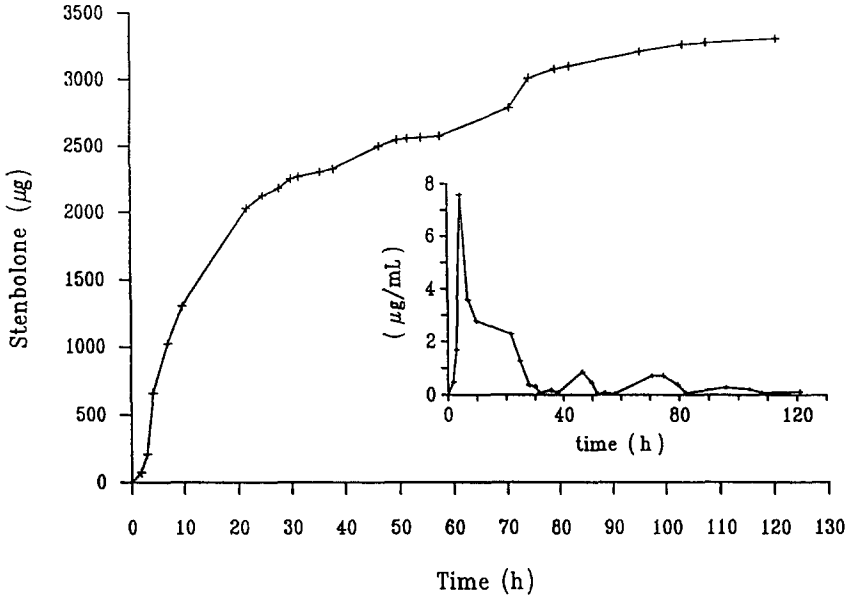


Fig. 1. Cumulative urinary excretion and excretion profile (inset) of stenbolone following administration of a 50 mg oral dose of stenbolone acetate.

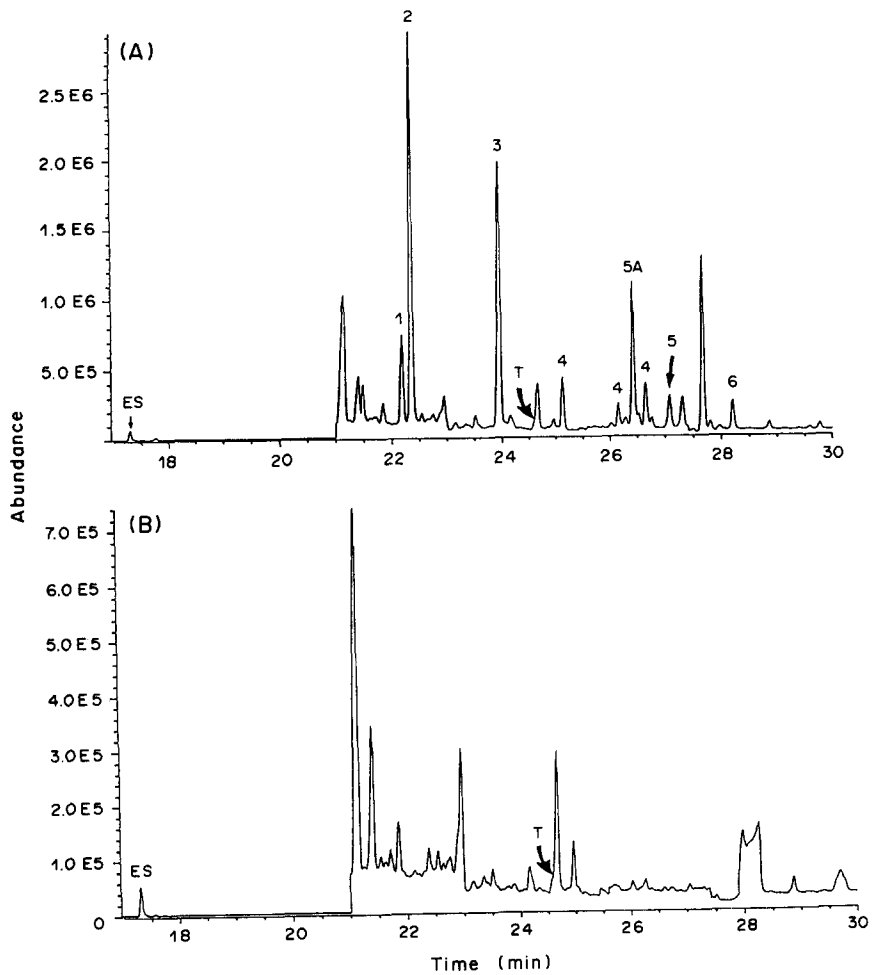


Fig. 2. Reconstructed total ion current chromatogram from SIM GC/MS analysis of the TMS ether derivative of stenbolone metabolites isolated from the glucuronide fraction of (A) a urine sample collected 4.25 h after Anatrofin® ingestion and (B) a blank urine sample. The identities of the numbered peaks are given in Table 1. Other labeled peaks are external standard (ES), 5α-androstan-17-one and testosterone (T).

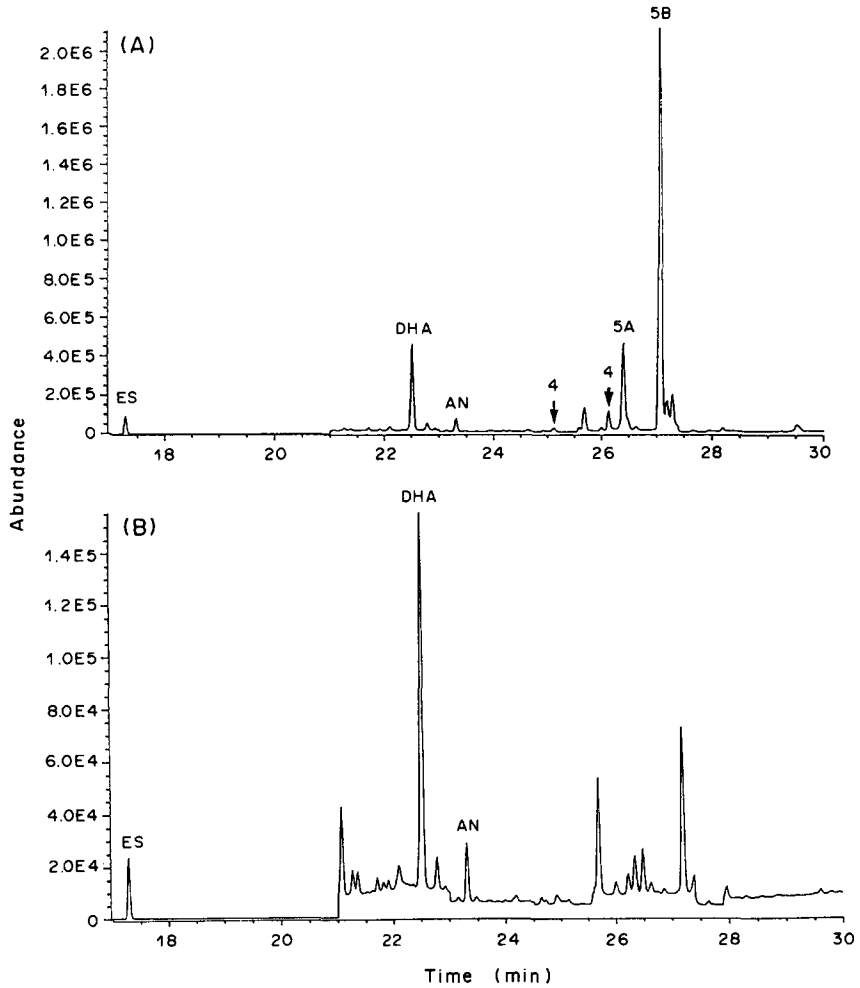


Fig. 3. Reconstructed total ion current chromatogram from SIM GC/MS analysis of the TMS ether derivative of stenbolone metabolites isolated from the sulfate fraction of (A) a urine sample collected 4.25 h after Anatrofin® ingestion and (B) a blank urine sample. The identities of the numbered peaks are given in Table 1. Other labeled peaks are external standard (ES), 5α-androstan-17-one, dehydroepiandrosterone (DHA) and androstenediol (AN).

glucuronide steroids by comparison with the corresponding chromatogram from a blank urine sample (Fig. 2B). As previously observed in the excretion profile of methenolone metabolites [2], sulfation was a minor conjugation route for Anatrofin® metabolites. Indeed, only 4 metabolites bearing a hydroxyl group at the C_{16} position were excreted as sulfuric acid aglycones (Fig. 3A). These steroids were also easily differentiated from the endogenous sulfated steroids as shown in Fig. 3B.

We also investigated the free steroid fraction specifically for 2-methyl-5α-androst-1-ene-3,17-dione, an intermediary metabolite expected to arise from the oxidation of the 17β-hydroxyl group and which is likely a precursor of metabolites **2**, **5A** and **5B**. However, no trace of this steroid was detected neither in the free nor in the conjugated steroid fractions. It is of interest to note that the corresponding isomeric 1-methyl steroid was characterized in the free steroid fraction after methenolone acetate ingestion [2].

Total ion current chromatograms presented in Figs 2A and 3A were obtained from a urine sample collected 4.25 h after steroid ingestion. They are both typical of the urinary excretion profile of Anatrofin® metabolites in humans, since all compounds detected in this sample were also found in urine, in variable concentration, until 121 h; to the exception of 16ξ,17β-dihydroxy-2-methyl-5α-androst-1-en-3-one (**6**), a minor metabolite which was detected only until 95 h after administration. A striking similarity between stenbolone acetate and methenolone acetate metabolism is the formation of the prominent metabolite **2** (Fig. 2A) resulting from 3α-reduction of the 3-keto group with concomitant oxidation at C_{17}. Further reduction of

Table 1. Identity and partial GC/MS data of stenbolone acetate urinary metabolites in human

Steroid	Fraction[a]	M.U.[b]	Derivative[c]	$M^{+\cdot}$	Characteristic ions
1 3α-Hydroxy-2ξ-methyl-5α-androstan-17-one	G	26.30	TMS E-E	448 (48)	169 (14), 343 (36), 433 (42)
		26.04	TMS ether	376 (23)	142 (28), 155 (44), 286 (44)
		26.60	MOTMS	405 (2)	142 (10), 155 (15), 284 (47)
2 3α-Hydroxy-2-methyl-5α-androst-1-en-17-one	G	26.41	TMS E-E	446 (67)	169 (9), 275 (46), 290 (31)
		26.15	TMS ether	374 (82)	141 (100), 156 (32), 359 (12)
		26.71	MOTMS	403 (47)	141 (86), 156 (33), 388 (8)
3 Stenbolone	G	27.17	TMS E-E	446 (100)	193 (34), 208 (46), 221 (15)
		27.03	TMS ether	374 (21)	129 (95), 136 (36), 276 (35)
		27.51	MOTMS	403 (77)	129 (30), 282 (21), 372 (28)
4 3ξ,16ξ-Dihydroxy-2-methyl-5α-androst-1-en-17-one	G	27.24	TMS E-E	534 (6)	147 (19), 429 (1), 519 (19)
		28.63	TMS E-E	534 (13)	147 (15), 429 (3), 519 (46)
		27.68	TMS ether	462 (17)	141 (57), 157 (54), 318 (64)
		28.25	TMS ether	462 (10)	141 (27), 157 (27), 318 (37)
		28.54	TMS ether	462 (19)	141 (72), 157 (59), 318 (73)
		27.54	MOTMS	491 (5)	141 (20), 157 (16), 174 (5)
		27.64	MOTMS	491 (5)	141 (15), 157 (12), 174 (5)
		28.53	MOTMS	491 (13)	141 (29), 157 (17), 460 (7)
		28.62	MOTMS	491 (8)	141 (37), 157 (22), 174 (6)
	S	27.20	TMS E-E	534 (7)	147 (19), 429 (1), 519 (2)
		28.61	TMS E-E	534 (10)	147 (22), 429 (3), 519 (44)
		27.67	TMS ether	462 (1)	141 (18), 157 (22), 318 (23)
		28.25	TMS ether	462 (21)	141 (61), 157 (59), 318 (97)
5A 16α-Hydroxy-2-methyl-5α-androst-1-ene-3,17-dione	G	29.28	TMS E-E	532 (13)	193 (8), 207 (19), 517 (46)
		28.41	TMS ether	388 (2)	136 (100), 244 (12), 317 (42)
		28.82	MOTMS	446 (17)	174 (24), 325 (11), 415 (50)
		28.93	MOTMS	446 (12)	174 (23), 325 (4), 415 (35)
	S	29.28	TMS E-E	532 (20)	193 (7), 207 (22), 517 (71)
		28.40	TMS ether	388 (2)	136 (100), 244 (15), 317 (58)
		28.82	MOTMS	446 (11)	174 (22), 325 (7), 415 (37)
		28.92	MOTMS	446 (11)	174 (38), 325 (5), 415 (35)
5B 16β-Hydroxy-2-methyl-5α-androst-1-ene-3,17-dione	G	29.28	TMS E-E	532 (13)	193 (8), 207 (19), 517 (46)
		28.76	TMS ether	388 (2)	136 (41), 244 (7), 317 (22)
		28.92	TMS ether	388 (5)	129 (61), 156 (12), 373 (25)[d]
	S	29.28	TMS E-E	532 (20)	193 (7), 207 (22), 517 (71)
		28.76	TMS ether	388 (3)	136 (100), 244 (12), 317 (60)
		28.93	TMS ether	388 (9)	129 (83), 156 (12), 373 (50)[d]
		29.44	MOTMS	446 (16)	174 (57), 273 (61), 415 (100)
		29.49	MOTMS	446 (3)	174 (25), 273 (31), 415 (51)
6 16ξ,17β-Dihydroxy-2-methyl-5α-androst-1-en-3-one	G	29.42	TMS E-E	534 (3)	147 (15), 191 (4), 193 (15)
		29.24	TMS ether	462 (10)	147 (30), 191 (24), 372 (20)
		29.83	MOTMS	491 (6)	147 (27), 191 (13), 401 (4)

[a] Steroid fractions: free (F), glucuronide (G), sulfate (S).
[b] Methylene units (M.U.) values were calculated by linear interpolation of the relative retention time of the steroid derivative to that of C_{26}, C_{27}, C_{28}, C_{29}, C_{30} and C_{32} hydrocarbons.
[c] The syn and anti forms of some MO-TMS derivatives were chromatographically resolved, whereas some metabolites afforded their isomeric forms upon derivatization. TMS E-E = TMS enol-TMS ether derivatives.
[d] This TMS ether derivative is that of the 17-hydroxy-16-keto tautomer of compound **5B**.

the δ-1 group yields 3α-hydroxy-2ξ-methyl-5α-androstan-17-one (**1**).

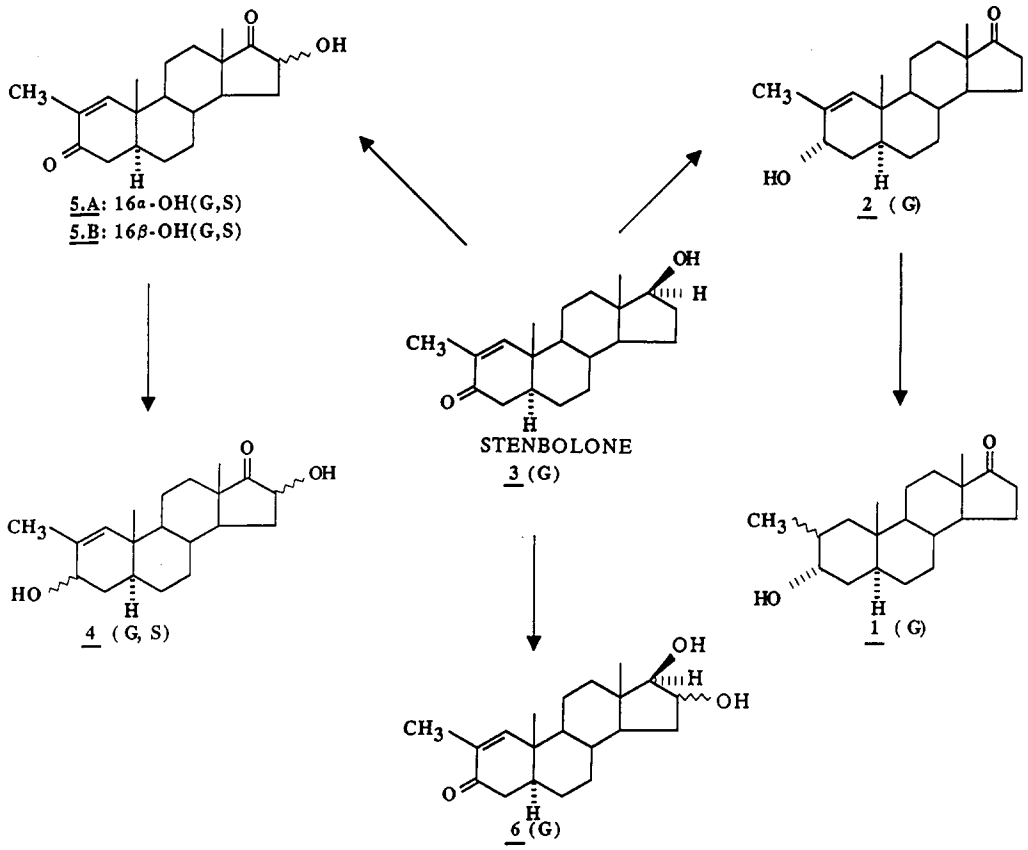
Interestingly, the ratio (based on peak height) of metabolites **2** and **1** urinary levels was 4.5:1, whereas it was 4.8:1 for the corresponding methenolone metabolites [2]. This indicates that the presence of a methyl group either at the C_1 or C_2 (stenbolone) position has little influence on this metabolic route. It is also of interest to note that this chemical feature has virtually no effect on the stereoselectivity of the hydroxylation at the C_{16} position and on the conjugation route of the resulting 16-hydroxylated steroids. Indeed, as previously observed in methenolone metabolism [2], the 16α-hydroxylated metabolite **5A** was mainly recovered from the glucuronic fraction whereas its prominent 16β-isomer **5B** was found predominantly in the sulfate fraction. The same general pattern of metabolites was obtained from a second volunteer (corresponding quantitative data are not shown in Fig. 1) to which stenbolone acetate was administered. The relative abundance of the metabolites in the glucuronide and sulfate fractions was also very similar to that observed in Figs 2 and 3.

Identification of metabolites

Table 1 summarizes the partial mass spectra and methylene units of the TMS ether, TMS enol-TMS ether and/or MO-TMS derivatives of the metabolites which were detected in the glucuronide and sulfate fractions. The pathways accounting for the formation of the metabolites are proposed in Scheme 1.

3α-Hydroxy-2-methyl-5α-androst-1-en-17-one (2)

The mass spectra of TMS either and MO-TMS derivatives of **2** (Fig. 4B and 4C) show



Scheme 1. Proposed structures of stenbolone metabolites resulting from oxidation of the 17-hydroxyl group and reduction of A-ring substituents, with or without concomitant hydroxylation at the C_{16} position. See Table 1 for further details.

molecular ions at m/z 374 (m/z 383, d_9-TMS) and m/z 403 (m/z 412, d_9-TMS), respectively, thus indicating the presence of only one hydroxyl group on the molecule. Comparison of their common fragment ions at m/z 141 (m/z 150, d_9-TMS) and m/z 156 (m/z 165, d_9-TMS) with those observed at m/z 142 and 155 in the mass spectrum of the TMS ether derivative of 3α-hydroxy-1-methylen-5α-androstan-17-one [2] clearly indicates that the hydroxyl function is at the C_3 position. Both ions arise from A-ring cleavages according to a mechanism analogous to that accounting for the formation of the m/z 129 ion in the mass spectrum of the TMS ether derivatives of δ-5-3-hydroxy steroids [3].

Additional information was obtained from the TMS enol-TMS ether mass spectrum (Fig. 4A) which exhibited a molecular ion at m/z 446 ($M^{+\cdot}$455, d_0-TMS enol-d_9-TMS ether mixed derivative). Structurally informative ions at m/z 290 (m/z 290, d_0-TMS enol-d_9-TMS ether mixed derivative) and m/z 275 (m/z 275, d_0-TMS enol-d_9-TMS ether mixed derivative) resulting from the cleavage of the C_1—C_{10} and C_4—C_5 bonds provided further mass spectral evidence for the presence of 3-hydroxyl and 17-keto functions in compound **2**. A 3α-hydroxy configuration was assigned to compound **2** on the basis of the data observed from the oxidation reaction performed with the enzyme 3α-hydroxysteroid dehydrogenase. GC/MS analysis of the resulting TMS enol-TMS ether derivative showed that compound **2** was totally transformed into 2-methyl-5α-androst-1-ene-3,17-dione, molecular ion of which at m/z 444 and the presence of characteristic ions of stenbolone A-ring at m/z 193, 208 and 221 (Fig. 3A). Besides the chromatographic and mass spectral properties of compound **2** are very similar to those of 3α-hydroxyl-1-methylen-5α-androstan-17-one [2], a prominent metabolite of methenolone [4]. The assignment of an α configuration for the 3-hydroxyl group in compound **2** is also in agreement with numerous studies which have shown that the reduction of 3-keto steroids predominantly results in the formation of 3α-hydroxy-steroids [5–7].

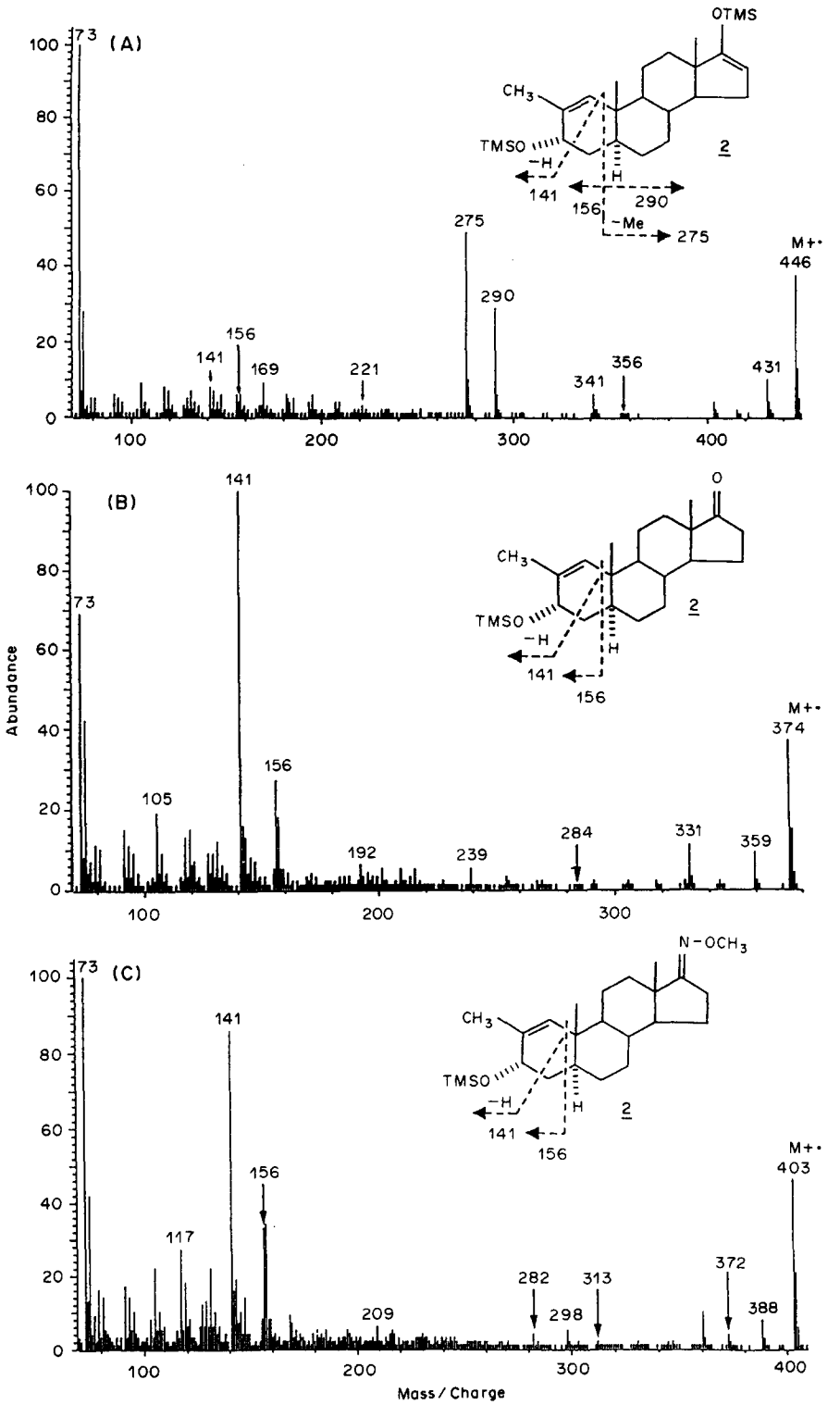


Fig. 4. Mass spectra of compound **2** as (A) TMS enol-TMS ether, (B), TMS ether and (C) MO-TMS derivatives.

3α-Hydroxy-2ξ-methyl-5α-androstan-17-one (1)

The mass spectra of the TMS ether ($M^{+\cdot}$376; *m/z* 385, d_9-TMS) and MO-TMS derivatives ($M^{+\cdot}$405; *m/z* 414, d_9-TMS) (Figs 5B and 5C) indicated the presence of only one hydroxyl group in compound **1**. The shift of the molecular ions by 2 a.m.u. with respect to those of compound **2** TMS ether and MO-TMS derivatives (Figs 4B and 4C) suggested that the δ-1

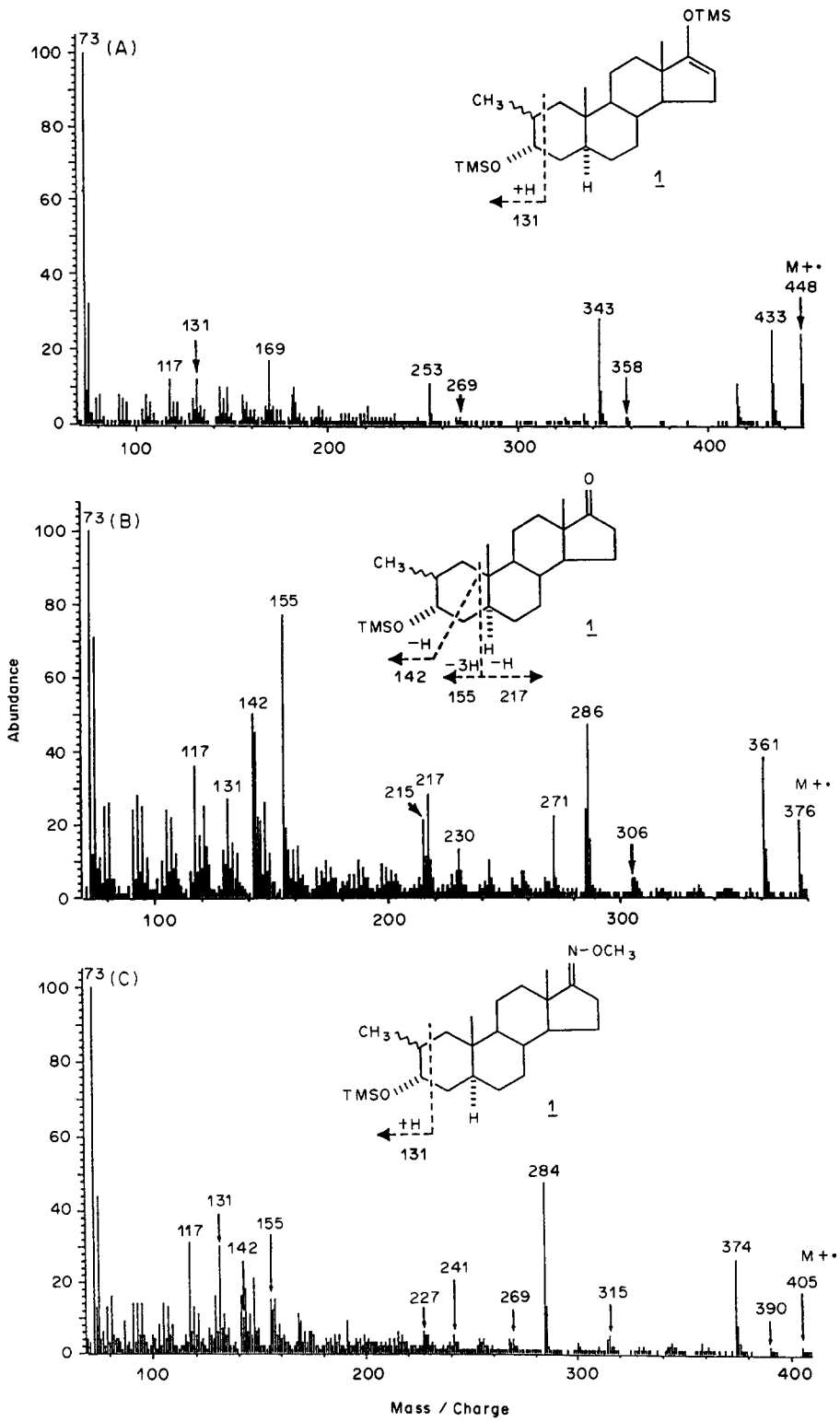


Fig. 5. Mass spectra of compound 1 as (A) TMS enol-TMS ether, (B) TMS ether and (C) MO-TMS derivatives.

function was also reduced. This hypothesis was assessed by the presence of the structurally informative ions at m/z 142 (m/z 151, d_9-TMS) and m/z 155 (m/z 164, d_9-TMS). These ions arise from A-ring cleavages as illustrated in Figs 5B and 5C and both bear the 3-hydroxyl group as evidenced by d_9-TMS labeling.

The presence of a 17-keto group was well demonstrated by comparison of the mass spectral features of the TMS-ether and MO-TMS derivatives. The later structural feature was further characterized in the mass spectrum of the TMS enol-TMS ether derivative (Fig. 4A). The ion of m/z 131 (m/z 140, d_9-TMS) which is present in all 3-OTMS derivatives (Figs 5A–5C) provides further evidence for the reduction of A-ring functional groups in compound **1**. This ion which results from cleavage of the C_1—C_2 and C_3—C_4 bonds with concomitant migration of a hydrogen atom likely have the structure of CH_3—CH_2—$CH = O^+TMS$. The formation of this ion is not favored in metabolites such as compound **2** in which the δ-1 function is not reduced (Fig. 4). Thus, metabolite **1** was assigned the structure of 3α-hydroxy-2ξ-methyl-5α-androstan-17-one. A 3α-configuration was proved for the 3-hydroxyl group of compound **1** on the basis of the experimental data obtained from the oxidation reaction as for compound **2**. The GC/MS analysis revealed that compound **1** was totally converted into 2ξ-methyl-5α-an-

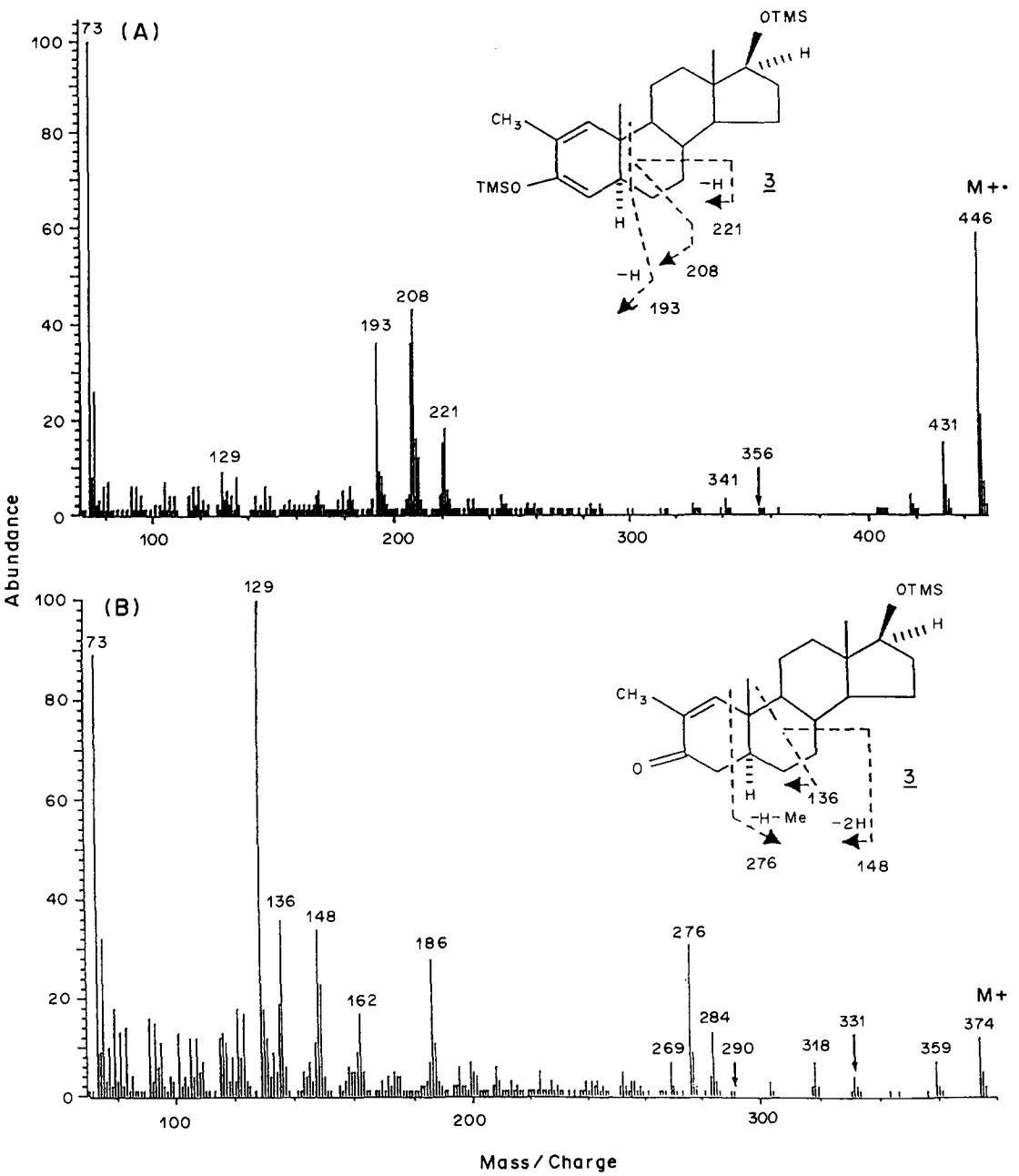


Fig. 6. Mass spectra of compound **3** as (A) TMS enol-TMS ether and (B) TMS ether derivatives.

drostane-3,17-dione, the TMS enol-TMS ether derivative of which exhibited a molecular ion at m/z 446 and characteristic ions at m/z 141 and 157. Although not demonstrated in this study, it is likely that the *in vivo* reduction of the δ-1 function gave rise to the 2α-isomer, as previously reported for methenolone acetate [2, 4].

Stenbolone (3)

The identity of urinary stenbolone was assessed by comparison of its TMS ether, TMS enol-TMS ether and MO-TMS mass spectra (Table 1) with those of authentic stenbolone. The mass spectrum of stenbolone TMS enol-TMS ether derivative (Fig. 6A) exhibited 3 ions of diagnostic importance at m/z 193, 208 and 221 (m/z 193, 208 and 221, d_0-TMS enol-d_9-TMS ether mixed derivative). As illustrated in Fig. 6A and assessed by d_0- and d_9- mixed-TMS labeling, these ions, likely arise from specific B-ring cleavages according to mechanisms analogous to those accounting for the fragmentation of δ-1,4-3-keto steroids [8]. The prominent ion at m/z 129 (m/z 138, d_9-TMS) in the mass spectrum of the TMS ether derivative (Fig. 6B) is typical of several 17-hydroxy steroids [3, 9, 10]. However, it is of low intensity in the mass spectrum of the TMS enol-TMS ether derivative (Fig. 6A) where fragmentation is predominantly directed at the B-ring. The mass spectrum presents other structurally informative ions at m/z 136 (m/z 136, d_9-TMS), m/z 148 (m/z 148, d_9-TMS), m/z 276 (m/z 285, d_9-TMS) and m/z 331 (m/z 340, d_9-TMS), which are characteristic of δ-1-3-keto steroid [11, 12]. Their fragmentation route and complementary mass spectral data rationalizing their formation will be reported elsewhere.

*16α-Hydroxy-2-methyl-5α-androst-1-ene-3,17-dione (**5A**); 16β-hydroxy-2-methyl-5α-androst-1-ene-3,17-dione (**5B**)*

The occurrence and relative abundance of metabolites **5A** and **5B** (Figs 2A and 3A) in both the glucuronide and sulfate fractions, and comparison with the corresponding profiles of methenolone urinary metabolites [2] suggested that these compounds were 16-hydroxysteroids. This hypothesis was further supported by the fact that the mass spectrum of its TMS ether derivative (Fig. 7A) was virtually identical to that of 16-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione [2]. The prominent ion at m/z 136 (m/z 136, d_9-TMS) was characteristic of stenbolone A-ring (Figs 6B and 7A), whereas the low intensity ion at m/z 244 was indicative of a steroid with 16-hydroxy and 17-keto groups [3, 13] as previously reported for methenolone metabolites bearing a hydroxyl group at C_{16} and keto functions at the C_3 and C_{17} positions [2]. The presence of a 16β-hydroxyl group in **5B** was demonstrated by the formation of its 17-hydroxy-16-keto tautomer, the mass spectrum of which (Table 1) exhibited characteristic ions at m/z 129 (m/z 135, d_9-TMS) and m/z 156 (m/z 165, d_9-TMS) arising from D-ring cleavage [3]. It is of interest to note that 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione, a prominent metabolite of methenolone acetate, displayed similar tautomerism upon derivatization with MSTFA [2].

Although the molecular ion (m/z 532) of the TMS enol-TMS ether derivative was in accordance with the proposed structure for both **5A** and **5B**, very little information about their structural features was obtained from the GC/MS analysis of this derivative (Table 1). However, these isomeric steroids were clearly differentiated as MO-TMS derivatives, as shown in Figs 7B and 7C. The syn- and anti-isomeric form of the MO-TMS derivative of **5A** and **5B** were chromatographically separated and afforded similar mass spectra (Table 1). The molecular ion was at m/z 446 (m/z 455, d_9-TMS), indicating the presence of two ketonic functions. Both steroids showed diagnostic ions at m/z 150 (m/z 150, d_9-TMS) and 164 (m/z 164, d_9-TMS) characteristic of A-ring structural features, whereas the ion at m/z 174 (m/z 183, d_9-TMS), is typical of the MO-TMS derivative of 16-hydroxy-17-keto steroids [14]. Thus, these mass spectral properties were consistent with the proposed structure.

Interestingly, the 16β-isomer **5B** could be easily distinguished from its 16α-analog **5A** by the specific formation of two prominent ions at m/z 273 (m/z 273, d_9-TMS) and m/z 242 (273-OMe; m/z 242, d_9-TMS) which are observed only in the former steroid (Fig. 7B). Retention of the positive charge on the steroid nucleus in compound **5B** rather than the D-ring moiety can be accounted on the stability of the respective ions produced. Alternatively, charge retention on the D-ring moiety accompanied by hydrogen transfer from the steroid nucleus gave rise to peak at m/z 174 (Figs 7B and 7C). Interestingly Vouros and Harvey [13] reported that the mass spectrum of the MO-TMS derivative of 3β,16α-dihydroxy-5-androsten-17-one shows virtually no elimination of the D-ring $(M-173)^+$ as

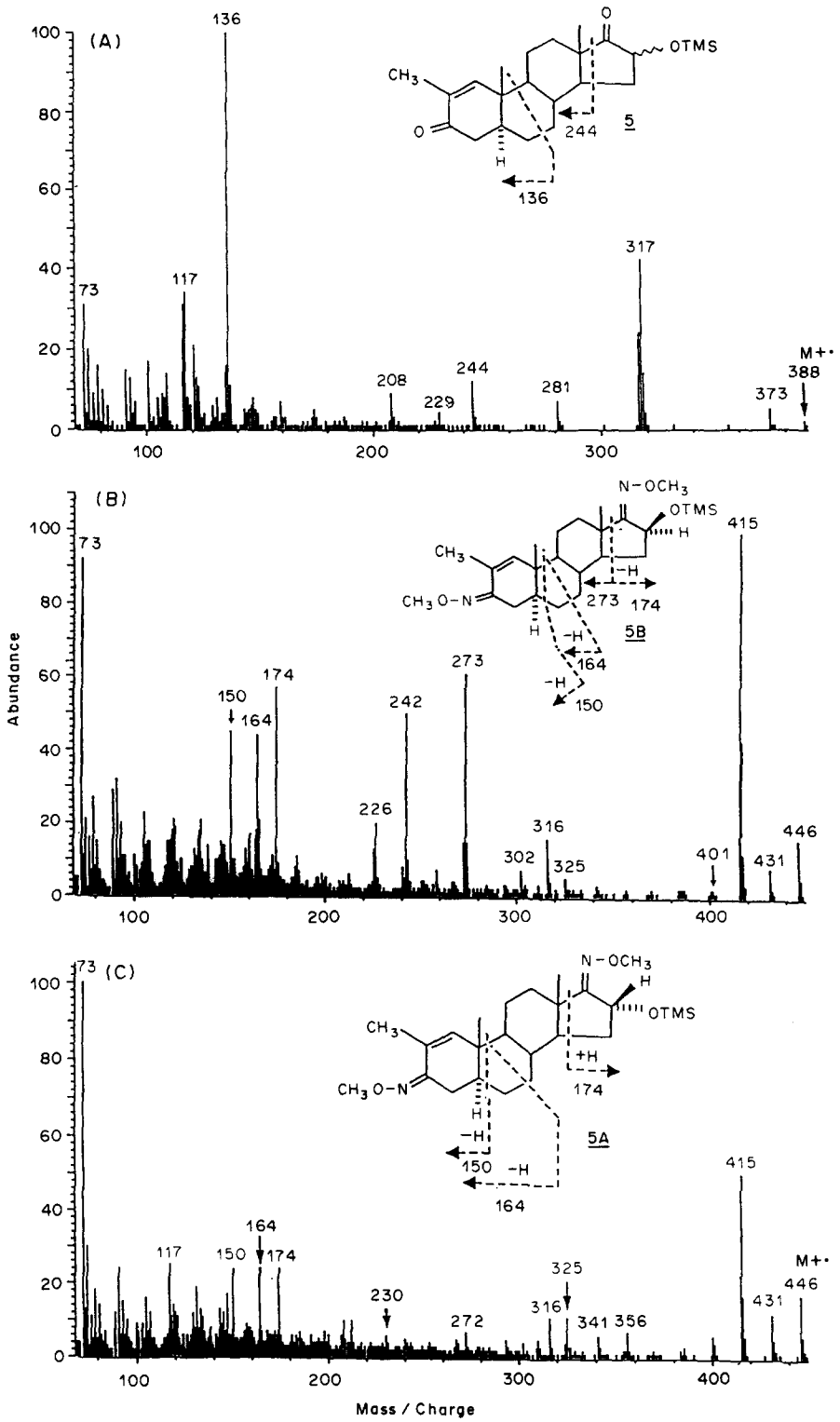


Fig. 7. Mass spectra of (A) compounds **5A** and **5B** as TMS ether derivative, (B) compound **5B** as MO-TMS derivative and (C) compound **5A** as MO-TMS derivative.

neutral species, as observed here for compound **5A** (Fig. 7C). Thus, it seems that the 16β configuration of the —OTMS group in compound **5B** MO-TMS derivative (Fig. 7B) promotes charge retention on the steroid nucleus to give prominent ions at m/z 273 and 242. These data are in accordance with mass spectral features of structurally related steroids previously

reported in the literature [3, 13, 14] and with those of the corresponding metabolites of methenolone [2].

The configuration at C_{16} in compounds **5A** and **5B** was further assessed by comparison with retention data reported for model 16-hydroxy-17-keto steroids [15, 16]. Compounds **5A** and **5B** were excreted in urine in a 1:1.5 ratio (**5A**/**5B**) as determined by SIM GC/MS analysis of the conjugated steroid fractions. Finally, it was estimated from these mass spectral data that 69 and 31% of **5A** and 11 and 89% of **5B** were excreted as glucuronide and sulfate conjugates, respectively. These data were different from those obtained for methenolone metabolites since the corresponding 16β-isomer was solely secreted as sulfate conjugate, whereas 92% of its 16α-analog was excreted in the form of glucuronide [2].

3ξ,16ξ-Dihydroxy-2-methyl-5α-androst-1-en-17-one (4)

Three chromatographic peaks (Fig. 2A), the TMS ether derivatives of which exhibited

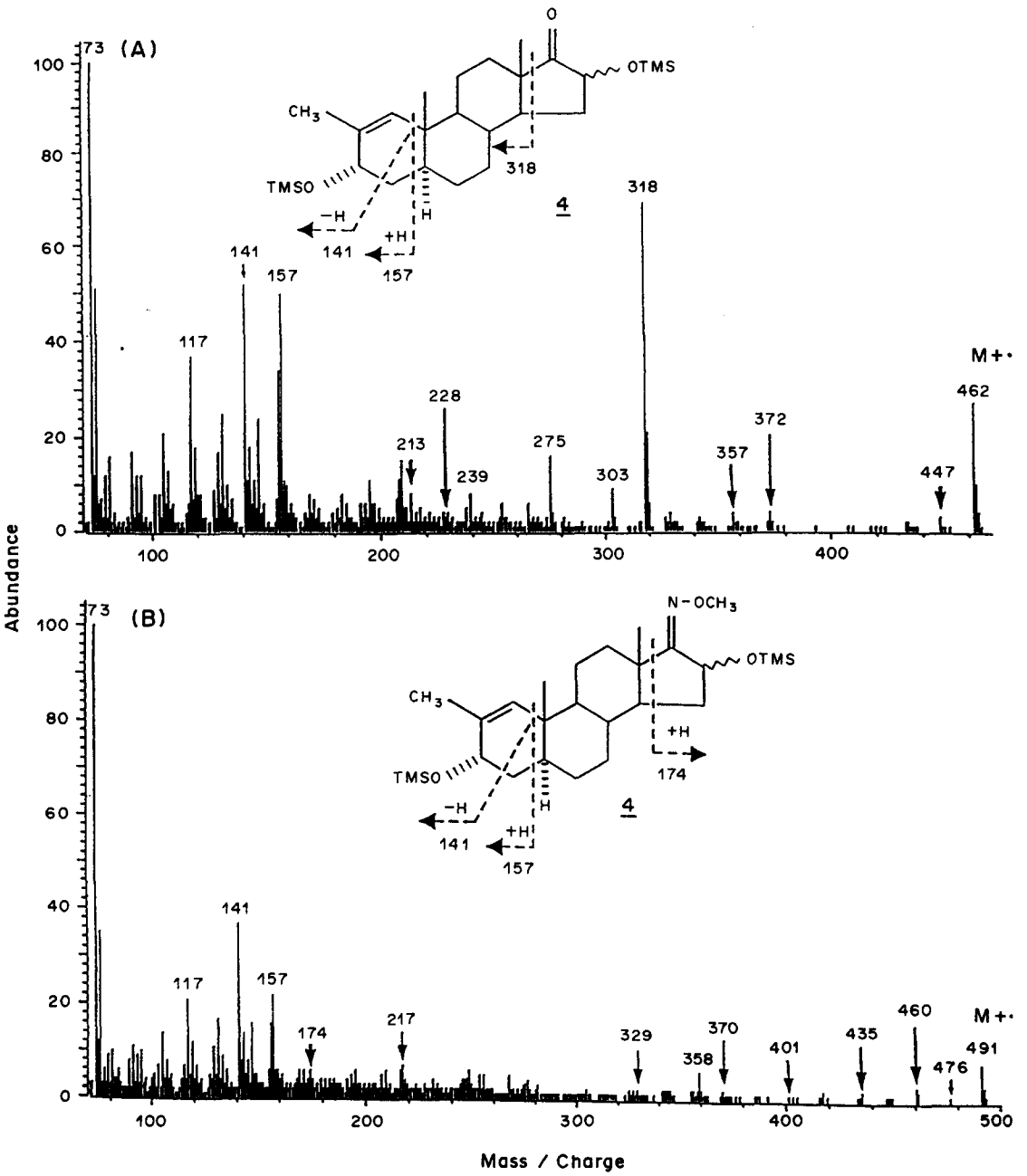


Fig. 8. Mass spectra of compound **4** as (A) TMS ether and (B) MO-TMS derivatives.

similar mass spectral features (Table 1) were detected in the glucuronide fraction and were assigned the general structure of compound **4**. Their mass spectra (Fig. 8A) showed a molecular ion at m/z 462 (m/z 480, d_9-TMS) and fragment ions at m/z 318 (M-144: comprising A-, B- and C-rings; m/z 327, d_9-TMS), m/z 157 (m/z 166, d_9-TMS; A-ring fragment via cleavage of the C_1—C_{10} and C_4—C_5 bonds with concomitant transfer of a proton) and m/z 141 (m/z 150, d_9-TMS; A-ring fragment via cleavage of the C_1—C_{10} and C_3—C_4 bonds with loss of a proton). These mass spectral properties were characteristic of isomeric 3,16-dihydroxy-17-keto steroids [2, 3, 13, 14]. This hypothesis was supported by GC/MS data from their corresponding TMS-enol-TMS ether derivatives which gave only two chromatographic peaks (Table 1). Since isomeric 16α- and 16β-hydroxy-17-keto steroids give a single derivative as the TMS enol-TMS ether derivative, these data indicate that two of the three metabolites probably have 3α,16α- and 3α,16β-hydroxyl groups, respectively. The proposed stereochemistry at C_3 was demonstrated by oxidation with 3α-hydroxysteroid dehydrogenase. Indeed, the first two isomers eluting at 25.20 and 26.15 min (Fig. 2A) were transformed into the corresponding 3-keto derivatives **5** (Fig. 7). Consequently, the third isomeric metabolite **4** probably bears a 3β-hydroxyl group. However, the stereochemistry at C_{16} was not determined in this compound. Additional information was obtained from the fragmentation data of their MO-TMS derivatives (Fig. 8B) which exhibited a molecular ion at m/z 491 (m/z 509, d_9-TMS) and fragments ions characteristic of A-ring cleavage at m/z 141 and 157. Thus, these mass spectral features provided further evidence for the presence of hydroxyl groups at the C_3 and C_{16} positions and a 17-keto group. Further studies have been undertaken to determine the stereochemistry of the 3- and 16-hydroxyl groups in these steroids.

16ξ,17β-Dihydroxy-2-methyl-5α-androst-1-en-3-one (6)

This metabolite was only detected in the glucuronide fraction. The molecular ions of the TMS enol-TMS ether ($M^{+\cdot}$534; $M^{+\cdot}$552, d_0-TMS enol-d_9-TMS ether mixed derivative) (Fig. 9A), TMS ether ($M^{+\cdot}$462; $M^{+\cdot}$480, d_9-TMS) (Fig. 9B) and MO-TMS ($M^{+\cdot}$491; $M^{+\cdot}$519, d_9-TMS) (Fig. 9C) derivatives indicated the presence of two hydroxyl and one keto groups. The occurrence of a derivative indicated that the hydroxyl groups were not on the A-ring or at the C_6 or C_7 positions. Interestingly enough, all TMS derivatives (Fig. 9) exhibited an ion at m/z 191 (m/z 209, d_9-TMS) which has been previously shown to arise from the rearrangement of vicinal-OTMS group in the D-ring [17]. The ion at m/z 147 (m/z 162, d_9-TMS) is often observed in the TMS derivatives of steroids bearing two or more hydroxyl groups [18]. Low intensity but diagnostically informative ions were observed at m/z 269 and 298 $(M-193)^+$ in the mass spectra of the TMS ether and MO-TMS derivatives. These ions are characteristic of 16,17-dihydroxy steroids and originate from the elimination of one molecule of trimethylsilanol (TMSOH) and a $^{\cdot}CH_2OTMS$ radical according to a mechanism previously reported by Vouros and Harvey [13]. On the basis of these data, metabolite **6** was proposed as the structure of 16ξ,17β-hydroxy-2-methyl-5α-androst-1-en-3-one. Owing to the lack of availability of isomeric reference steroids, its stereochemistry at the C_{16} position could not be determined.

Stenbolone acetate metabolic routes

In the present study we have shown that stenbolone acetate biotransformation in humans essentially follows the predominant routes previously reported for its isomeric analog methenolone acetate [2, 4, 19]. The dominant reaction was 17-dehydrogenation of the 17β-hydroxy group since all metabolites, to the exception of unchanged stenbolone and compound **6** bear a 17-keto function. Since testosterone is also extensively oxidized at C_{17} by hepatic 17β-hydroxy steroid dehydrogenases [20], this indicates that the presence of δ-1 and 2-methyl functions do not inhibit this reaction in stenbolone metabolism. Thus, oxidation at C_{17}, accompanied by subsequent reduction of the 3-keto group was, as for methonolone [2], a significant pathway in stenbolone biotransformation. Hydroxylation at C_{16} was also a major metabolic route that afforded the isomeric hydroxylated compounds **5A** and **5B**, whereas further reduction of their 3-keto group was shown to be a minor reaction.

Finally, another minor metabolic route was the direct hydroxylation of stenbolone to give compound **6**, which was solely isolated from the glucuronide fraction. As previously observed in methenolone biotransformation [2], reduction of the δ-1 function also was a minor reaction

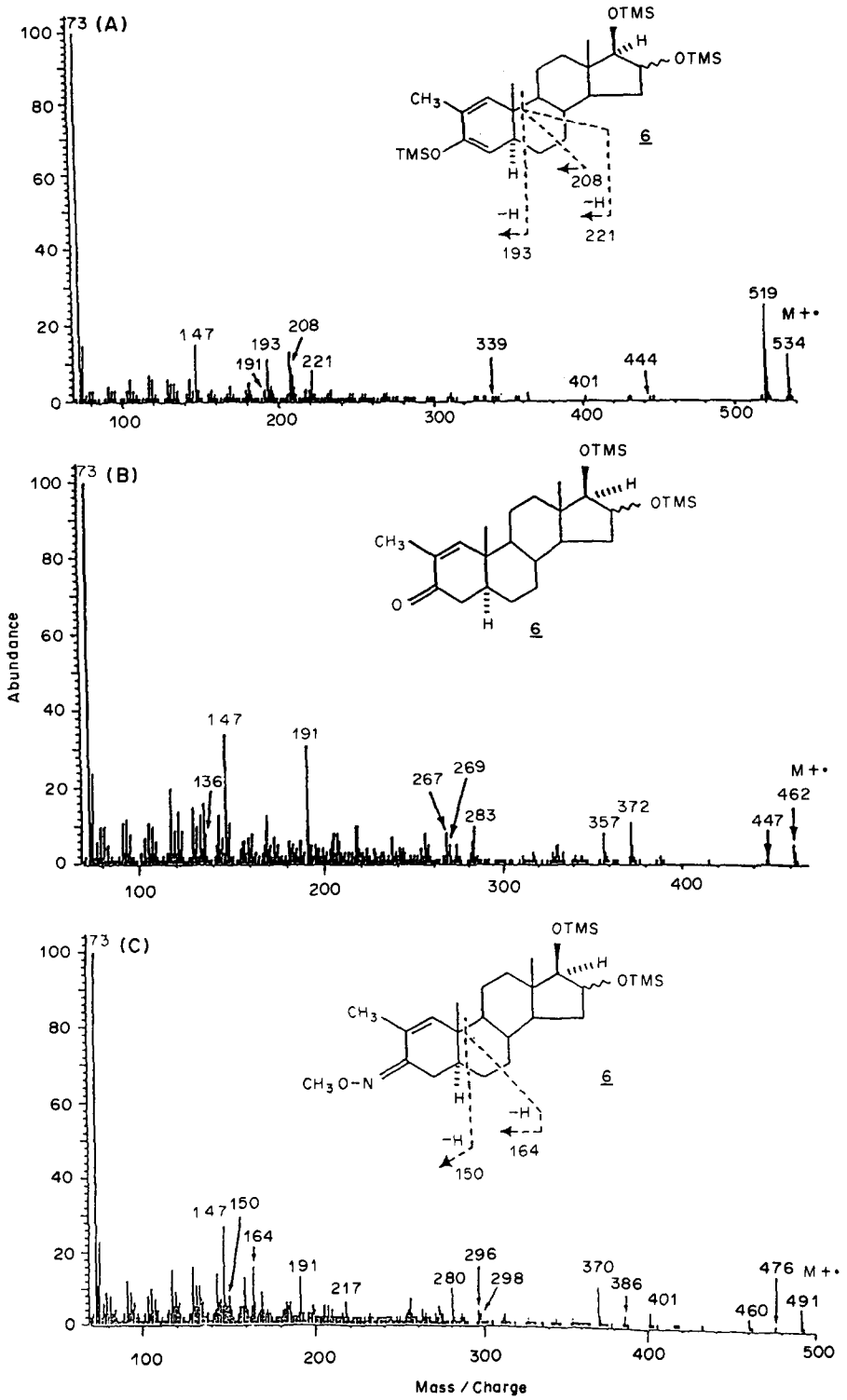


Fig. 9. Mass spectra of compound **6** as (A) TMS enol-TMS ether, (B) TMS ether and (C) MO-TMS derivative.

and only one tetrahydro metabolite was produced in low amounts. This observation is in agreement with the study of Galletti and Gardi [21] who showed that the δ-1 function was barely reduced during the metabolism of 17β-hydroxyandrosta-1,4-dien-3-one in man.

No trace of 2-methyl-5α-androst-1-ene-3,17-dione was detected in the free steroid fraction. As its methenolone analog, which was excreted

free in urine, this steroid is likely a key intermediate in stenbolone biotransformation and the immediate precursor of the major metabolites identified in this study. It is well known that 3,17-diketosteroids are good substrates for hepatic hydroxylases and reductases [22] and thus are rapidly transformed into more polar compounds which are then conjugated and excreted in bile or urine.

The conjugation pattern of stenbolone was also similar to that of methenolone metabolites which were preponderantly excreted as glucuronic acid conjugates. Only metabolites hydroxylated at C_{16} were excreted as sulfate conjugates and stereochemistry at this position have a marked effect on the nature of the conjugation mode. Thus, 16α-hydroxy stenbolone was predominant in the glucuronide fraction whereas nearly 90% of urinary 16β-hydroxy stenbolone was excreted as sulfate conjugate.

It was estimated by GC/MS analysis and quantitation of unchanged stenbolone that <20% of the ingested 50 mg oral dose of Anatrofin® was excreted in urine. This indicates, that urinary excretion was not the major elimination route of this steroid and its metabolites.

CONCLUSION

This study has shown that the general features of stenbolone metabolism in humans are similar to those of methenolone [2], thus indicating that the presence of a methyl group either at the C_1 or C_2 position has little effect on their major biotransformation routes. In contrast to the biotransformation of testosterone, the shift of the A-ring double bond to C_1 and the additional methyl group at C_2 promotes hydroxylation at C_{16} and decreases reduction of the δ-1 and 3-keto groups. Owing to the stability of stenbolone to reductive metabolism, which was also observed in methenolone biotransformation [2], the major metabolites either retained the δ-1-3-keto or δ-1 group. This demonstrates the greater inhibitory effect of the δ-1 over the δ-4 function on the reduction of the 3-keto group.

Finally, these new data about stenbolone metabolism will permit the development of an efficient analytical method to detect and identify the parent steroid and its urinary metabolites in the context of the control of the illegal use of anabolic steroids in sport.

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