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# STUDIES ON ANABOLIC STEROIDS—4. IDENTIFICATION OF NEW URINARY METABOLITES OF METHENOLONE ACETATE (PRIMOBOLAN®) IN HUMAN BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

DANIELLE GOUDREAULT and ROBERT MASSÉ*
Institut National de la Recherche Scientifique, INRS-Santé, Université du Québec, 245, Hymus Blvd, Pointe-Claire, Québec, Canada H9R 1G6

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**Summary**—The metabolism of methenolone acetate (17β-acetoxy-1-methyl-5α-androst-1-en-3-one), a synthetic anabolic steroid, has been investigated in man. After oral administration of a 50 mg dose of the steroid to two male volunteers, twelve metabolites were detected in urine either in the glucuronide, sulfate or free steroid fractions. Methenolone, the parent steroid was detected in urine until 90 h after administration. Its cumulative urinary excretion accounted for 1.63% of the ingested dose. With the exception of 3α-hydroxy-1-methylen-5α-androstan-17-one, the major biotransformation product of methonolone acetate, metabolites were excreted in urine at lower levels, through minor metabolic routes.

Most of methenolone acetate metabolites were isolated from the glucuronic acid fraction, namely methenolone, 3α-hydroxy-1-methylen-5α-androstan-17-one, 3α-hydroxy-1α-methyl-5α-androstan-17-one, 17-epimethenolone, 3α,6β-dihydroxy-1-methylen-5α-androstan-17-one, 2ξ-hydroxy-1-methylen-5α-androstan-3,17-dione, 6β-hydroxy-1-methyl-5α-androst-1-en-3,17-dione, 16α-hydroxy-1-methyl-5α-androst-1-en-3,17-dione and 3α,16α-dihydroxy-1-methyl-5α-androst-1-en-17-one.

Interestingly, the metabolites detected in the sulfate fraction were isomeric steroids bearing a 16α- or a 16β-hydroxyl group, whereas 1-methyl-5α-androst-1-en-3,17-dione was the sole metabolite isolated from the free steroid fraction. Steroids identity was assigned on the basis of the mass spectral features 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. The data indicated that methenolone acetate was metabolized into several compounds resulting from oxidation of the 17-hydroxyl group and reduction of A-ring substituents, with or without concomitant hydroxylation at the $C_6$ and $C_{16}$ positions.

## INTRODUCTION

Methenolone acetate (Primobolan®), unlike most anabolic steroid 17-ester derivatives is hydrolyzed, slowly enough by liver esterases to be orally active [1]. It has been preferred to other androgenic-anabolic steroids in the treatment of aplastic anemia for its therapeutic efficiency [2, 3] and lower hepatic toxicity compared to that exhibited by its 17α-alkylated analogs [3, 4]. When orally administered to patients suffering of primary hypogonadism, methenolone acetate was shown to be six times less androgenic than testosterone proprionate and about three time more anabolic than orally active 17α-methyltestosterone [5].

As many other androgenic-anabolic steroids, methenolone acetate has been used in sports in the last two decades in view of increasing strength and improving performance [6]. Concerned by the potential incidence of adverse health effects and for ethical reasons, the International Olympic Committee and other International Sport governing bodies have prohibited the use of these drugs in sport. In this context, we recently reported an integrated methodological approach developed in our laboratory for the gas chromatographic–mass spectrometric (GC/MS) analysis of anabolic steroid metabolites in human urine [7, 8]. An essential prerequisite for the implementation of such a methodology is a thorough knowledge of anabolic steroids biotransformation routes and urinary excretion profiles. Thus, a long-standing

*Author to whom correspondence should be addressed.

research program in our laboratory has been aimed at the study of anabolic steroids metabolism [9–11].

In early studies by Langecker [12] and Gerhards *et al.* [13], it has been shown that the major urinary metabolite of methenolone acetate in human is the glucuronide of 3α-hydroxy-1-methylen-5α-androstan-17-one (**1**). More recently, Björkhem and Ek reported an isotope-dilution GC/MS method for the detection and quantitation of this steroid in man urine [14].

The objective of the present study was to further characterize the biotransformation routes of methenolone acetate in human. Attention was given to the fractionation and identification of the metabolites isolated from the free and aglycone fractions. Twelve 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 urinary excretion profiles of unchanged methenolone in two human volunteers are presented.

## EXPERIMENTAL

### *Chemical and reagents*

Water was purified by reverse osmose and filtered over a Milli-Q system (Millipore, Mississauga, Ont., Canada) before use. The Sep Pak $C_{18}$® cartridges were from Waters Associates (Milford, Mass, U.S.A.). Helix Pomatia digestive juice (H-1-type), β-glucuronidase (IX-A type) from *E. coli* and methoxyamine hydrochloride were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Aryl sulfatase from *Helix pomatia* was purchased from Boehringer (Montreal, Qc, Canada). Dithioerythritol and trimethylsilyliodosilane (TMSI) were purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). *N*-methyl-*N*-(trimethyl)-trifluoroacetamide (MSTFA) was obtained from Regis Chemical Co. (Morton Grove, Ill., U.S.A.). Trimethylchlorosilane (TMCS) and silylation grade pyridine were from Pierce Chemical Co., (Rockford, Ill., U.S.A.). Trimethyl-$d_9$ chlorosilane ($d_9$-TMCS) and N,O-bis (trimethyl-$d_9$-silyl) acetamide ($d_9$-BSA) were purchased from MSD Isotopes (Pointe-Claire, Qc, Canada). Diethyl ether distilled on glass and stabilized with 0.25% of ethanol was restabilized with 1% of deionized water before use. Other solvents (HPLC) grade, Caledon Laboratories Ltd (Georgetown, Ont, Canada) were used as such. Inorganic salts were of analytical grade (J. T. Baker Chemical Co., Philipsburg, N.J., U.S.A.).

### *Steroids*

17β-hydroxy-1-methyl-5α-androst-1-en-3-one (methenolone), 1-methyl-5α-androst-1-en-3,17-dione and Primobolan (methenolone acetate) were kindly supplied by Schering AG (Berlin, F.R.G.). 17β-hydroxy-androsta-1,4-dien-3-one and 5α-androstan-17-one were purchased from Steraloids Inc. (Wilton, N.H., U.S.A.). 16α-hydroxy-4-androstene-3,17-dione was kindly supplied by Dr D. N. Kirk (Steroids Reference Collection, London, England).

### *Biological samples*

Urine samples were collected in sterile plastic containers during seven days after oral administration of a single 50 mg dose of methenolone acetate (Primobolan®) to two normal and healthy male volunteers. All samples were kept frozen at −20°C until analysis. Blank urine samples were collected from both volunteers during the last 24 h before ingestion of the steroid.

### *Stock solutions*

Stock solution of methenolone (1 mg/ml), 17β-hydroxy-1,4-androstadien-3-one (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 determination of methenolone in the range of 30–2000 ng/ml was prepared as follows: dilutions were made from the stock solutions and transferred to 0.3 ml vials so as to obtain 1–20 ng/μl of methenolone and 10 ng/μl of 17β-hydroxy-1,4-androstadien-3-one. After evaporation of the solvent at 40°C under a nitrogen stream, the samples were derivatized in 100 μl of a mixture of MSTFA and TMSI as described below to prepare the TMS enol–TMS ether derivatives.

Each sample was prepared in duplicate and 1 μl was injected three times in the gas chromatograph. The samples were analyzed by GC/MS in the SIM (selected ion monitoring) mode. Quantitation was performed by measuring peak area ratio of ions of *m/z* 195 (methenolone) and *m/z* 206 (17β-hydroxy-1,4-

androstadien-3-one). Linearity was obtained in the range of 1–20 ng/$\mu$l. The data can be fitted by the regression equation $C_m = 2.65\,Ar + 1.09$ ($r^2 = 0.994$) where $C_m$ is the concentration of methenolone in ng/$\mu$l and Ar the peak ratio of methenolone to 17$\beta$-hydroxy-1,4-androstadien-3-one.

*Sample preparation for methenolone quantitation*

In a typical experiment, 3 ml of urine was applied over a Sep Pak $C_{18}$® cartridge (previously washed with 5 ml of MeOH and 10 ml of water) and washed successively with 10 ml of water and 5 ml of hexane to remove the excess of water in the cartridge. The free and conjugated steroids were recovered by elution with 5 ml of MeOH. The solvent was evaporated to dryness at 40°C under a nitrogen stream. The residue was dissolved in 1 ml of 0.2 M sodium acetate buffer (pH 5.2) and 200 $\mu$l of *Helix pomatia* juice ($\beta$-glucuronidase 60,000 Fishman units/ml, aryl sulfatase 3435 Fishman units/ml) was added. After incubation at 37°C for 16 h, the hydrolysate was cooled to room temperature and 100 mg of a mixture of $NaHCO_3$:$Na_2CO_3$ (10:1, w/w) (pH 9) and 5 ml of diethyl ether were added. The mixture was shaken in a Eberback shaker for 10 min and centrifuged at 1260 *g* for 10 min. The etheral phase was dried over anhydrous sodium sulfate and evaporated to dryness at 40°C under a nitrogen stream. The residue was dissolved in 0.3 ml of MeOH.

The urinary extract was then transferred to a 0.3 ml Reactivial® containing 10 ng/$\mu$l of 17$\beta$-hydroxy-1,4-androstadien-3-one. The steroidal extract was evaporated to dryness and derivatized in 100 $\mu$l of a mixture of MSTFA:TMSI (100:2, v/v) as described below to prepare the corresponding TMS enol–TMS ether derivatives. It should be noted that, to avoid column overloading and enable accurate quantitation of methenolone, only 2 ml of urine instead of 3 ml was used for the analysis of the sample from volunteer B with the highest urinary level of 1.34 $\mu$g/ml.

*Recovery of methenolone from urine*

The accuracy of the method used to quantify methenolone in urine was determined by adding known amounts of the steroid (250, 500, 750 and 1000 ng/ml) to blank urine samples. The mixtures were equilibrated for 16 h at 37°C. Duplicate samples were then extracted, hydrolyzed and derivatized as described above. Each extract was analyzed twice by SIM GC/MS and mean recovery was 70.5% (CV 1.8%).

*Fractionation of free and conjugated metabolites*

The unconjugated steroids were extracted as follows: 5 or 10 ml of urine were passed through a Sep Pak $C_{18}$® cartridge as described above. The residue obtained after evaporation of MeOH was dissolved in 1 ml of 0.2 M phosphate buffer (pH 6.9). The extraction of the free steroids was carried out twice with 5 ml of diethyl ether as described previously. The organic phase was decanted and evaporated to dryness. The residue which contains the free steroids was then derivatized as described below. Any trace of residual diethyl ether was removed by spraying a flow of nitrogen over the aqueous phase at 40°C (to prevent potential denaturation of the enzyme). Then, 50 $\mu$l (71700 Fishman units/ml) of $\beta$-glucuronidase from *E. coli* were added. The mixture was incubated for 16 h at 37°C or for 3 h at 55°C to cleave the glucuronide aglycones. The corresponding neutral steroids were then extracted with diethylether and the resulting organic phase was processed as described above prior to GC/MS analysis. The remaining aqueous phase was eluted through a Sep Pak $C_{18}$® cartridge according to the method described above. To the resulting residue was successively added 1.0 ml of 0.2 sodium acetate buffer (pH 5.2) and 50 $\mu$l ($1.45 \times 10^6$ Roy Units/ml) of aryl sulfatase. The mixture was then incubated for 16 h at 37°C or for 3 h at 55°C, and the neutral steroid were then extracted as described above.

## PREPARATION OF DERIVATIVES

*Trimethylsilyl enol-trimethylsilyl ether derivatives*

The steroidal extract was transferred to a 0.3 ml Reactivial® and 10 ng/$\mu$l of 5$\alpha$-androstan-17-one (external standard) was added. After evaporation of the solvent at 40°C under a nitrogen stream, 0.5–1 mg of dithioerythritol was added and the vial stoppered under nitrogen. Following the addition of MSTFA:TMSI (100:2 v/v), the mixture was heated at 70°C for 30 min and 1 $\mu$l was injected into the gas chromatograph.

*Trimethylsilyl ether and $d_9$-trimethylsilyl ether derivatives*

The urinary extract was treated with a mixture of MSTFA:pyridine:TMCS (25:25:

2.5 v/v) as described above and the resulting mixture heated at 70°C for 30 min. The sample was then cooled to room temperature and the solvent removed at 60°C under a nitrogen stream. The dry residue was dissolved in 50 $\mu$l of hexane and 1 $\mu$l was injected into the gas chromatograph. The corresponding per-deuterated $d_9$-TMS derivatives were prepared using a mixture of $d_9$-BSA:pyridine: $d_9$-TMCS (25:25:2.5 v/v) under the conditions mentioned above.

*$d_0$-trimethylsilyl enol–$d_9$-trimethylsilyl ether derivatives*

These mixed derivatives were prepared as follows: firstly, the hydroxyl groups were selectively reacted with $d_9$-BSA as described above to prepare the $d_9$-TMS ether derivatives; the solvent was removed under a nitrogen stream at 40°C and the residue dissolved in hexane. The resulting solution was transferred in another Reactivial® and evaporated to dryness. The keto functions were then selectively transformed into their corresponding $d_0$-TMS enol derivative using a mixture of MSTFA:TMSI as described above.

*0-methyloxime trimethylsilyl ether (MO–TMS) and (MO–$d_9$-TMS)*

The dry steroidal extract was dissolved in 50 $\mu$l of a 5% (w/v) solution of methoxyamine hydrochloride in pyridine and heated at 70°C for 30 min. The trimethylsilyl and $d_9$-TMS ether derivatives were then prepared by adding 25 $\mu$l of a mixture of MSTFA:TMCS (25:2.5 v/v) or a mixture of $d_9$-BSA:$d_9$-TMCS (25:2.5 v/v). The resulting mixture was heated at 70°C for 30 min. The solvent was evaporated at 60°C under a stream of nitrogen and hexane (50 $\mu$l) was added to the dry residue. 1 $\mu$l of the resulting solution was injected in the gas chromatograph for GC/MS analysis.

## GAS CHROMATOGRAPHY/MASS SPECTROMETRY

The steroidal extracts were analyzed using a HP-5970 mass-selective detector (Hewlett–Packard, Palo Alto, Calif. U.S.A.) linked to a HP-5890 gas chromatograph equipped with HP-5 (cross-linked 5% phenyl, methyl silicone) fused-silica capillary column (25m × 0.2 mm i.d., 0.33 $\mu$m film thickness). The injections were made in the splitless mode (30 s delay before the splitter is opened) using helium as a carrier gas at a rate of 0.8 ml/min. The oven temperature was maintained at 100°C for 1 min and programmed at 16°C/min to 220°C and then 3.8°C/min to 300°C and maintained for 10 min.

For the quantitation of methenolone, a DB-5 (polymethyl 5% phenyl) siloxane fused capillary column (30 m × 0.25 mm i.d., 0.25 $\mu$m film thickness) from Chromatographic Specialities Inc. (Brockville, Ont., Canada) was used The oven temperature was held at 100°C for 1 min, programmed at 16°C/min to 220°C and then at 3.8°C/min to 300°C and maintained for 10 min.

The injector and the transfer line temperatures were set at 270°C and 310°C respectively. The mass-selective detector (MSD) was used in the SIM (selected ion monitoring) and repetitive scanning modes for the specific detection and recording of the full mass spectra to establish the identity of chromatographic peaks of interest.

## RESULTS AND DISCUSSION

*Urinary excretion of methenolone acetate metabolites*

Figure 1 shows the cumulative urinary excretion and excretion profiles (inset) of unchanged methenolone **5** following oral administration of a 50 mg single does of Primobolan® to two healthy male volunteers. Methenolone was detected in urine from 3 h until 90 h after administration. During that period of time, 808 $\mu$g (1.62%) and 825 $\mu$g (1.65% of the ingested dose) was excreted in urine as unchanged methenolone in volunteer A and B respectively. Although the cumulative excretion of methenolone was almost identical in both volunteers,

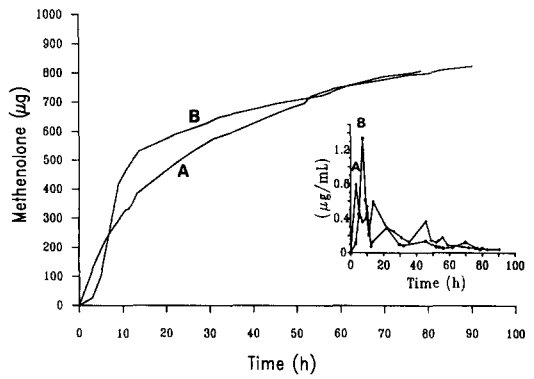


Fig. 1. Cumulative urinary excretion and excretion profile (inset) of methenolone following administration of a 50 mg oral dose of methenolone acetate.

Fig. 1 shows that absorption and distribution of this steroid did proceed according to slightly different kinetic parameters. Indeed, excretion of methenolone in volunteer A rapidly reached its maximum level of 0.79 $\mu$g/ml only 3 h after administration, whereas in volunteer B, a maximum level of 1.34 $\mu$g/ml was attained 7 h after Primobolan® ingestion. Two distinct phases can be distinguished from these excretion profiles. First a rapid elimination phase occurring between 1 and 12 h was followed by a slower excretion phase, which probably corresponds to the distribution and elimination phases of the steroid.

Fractionation of the urinary steroids demonstrated that most metabolites were excreted as glucuronic acid aglycones, and nine metabolites were detected in this fraction (Fig. 2). On the other hand, sulfatation was a minor conjugation and excretion route. Indeed, we observed that only three metabolites, all bearing a hydroxyl group at the $C_{16}$ position, were excreted as sulfuric acid aglycones (Fig. 3A). These metabolites could be easily differentiated from the endogeneous glucuronide sulfate steroids by comparison with the corresponding total ion-current chromatograms (TIC) obtained from a blank urine sample (Figs 2B and 3B).

Interestingly, the sole metabolite that we detected in the free steroids fraction was 1-methyl-5$\alpha$-androst-1-ene-3,17-dione **4** which arises from oxydation of methenolone 17-hydroxyl group. The latter observation was not in agreement with a study of Björkhem and Ek [14] who did not report the presence of compound **4** in the free steroid fraction but instead, that about 30% of the total amount of compound **1** (1-methylenandrosterone) excreted in urine was recovered from the free steroid fraction. Their observation was somewhat surprising since one would have expected compound **1** to be excreted in urine as aglycones of glucuronic and/or sulfuric acid, as it is the case for androsterone, its structurally related endogeneous analog. Although we cannot rationalize these different

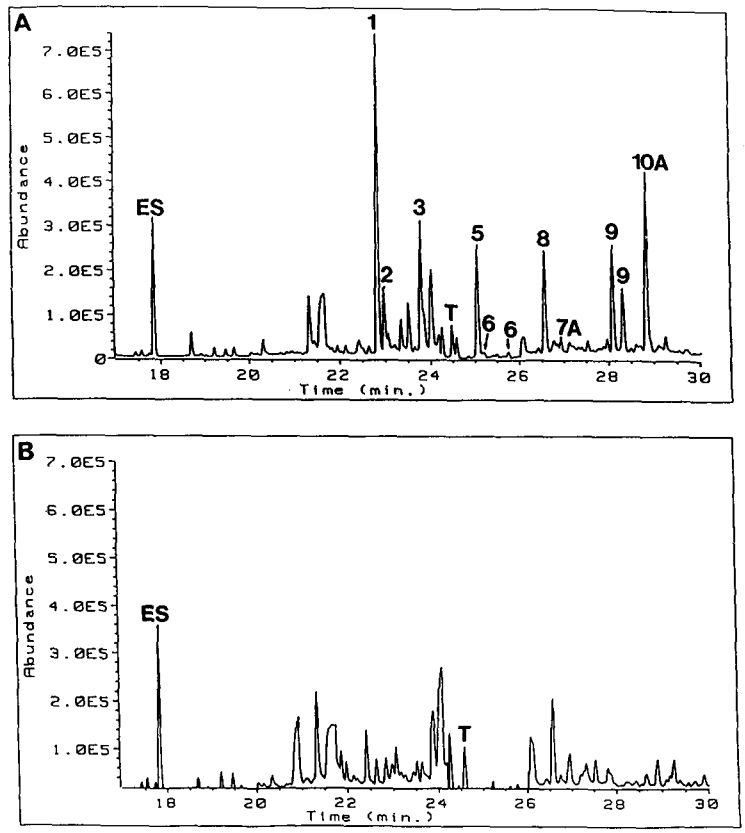


Fig. 2. Reconstructed total ion-current chromatogram from SIM GC/MS analysis of the TMS enol–TMS ether derivatives of methenolone metabolites isolated from the glucuronide fraction of (A) a urine sample collected 3 h after Primobolan® ingestion and (B) a blank urine sample. Labelled peaks (numbered) identity is given in Table 1. Other labelled peaks are: external standard (ES), 5$\alpha$-androstan-17-one and testosterone (T). Compound **6** and **9** were detected as two distinct isomeric forms when analyzed as TMS enol–TMS ether derivatives. See Experimental for analytical conditions.

observations on the basis of documented metabolic rationales, it should be mentioned here that the methodology used by these authors to isolate 1-methyleneandrosterone **1** from urine was very different from that used in this study and could account for the reported [14] presence of **1** in the free steroid fraction.

The TIC presented in Figs 2A and 3A were obtained from a urine sample collected 3 h after ingestion. They are typical of the urinary excretion profile of Primobolan® metabolites in human, since all 12 metabolites reported in this study could be detected in urine samples collected between 3 and 7 h after ingestion. In fact, some metabolites were detected only during specific period of time after ingestion. Thus, minute amounts of of 1-methyl-5α-androst-1-ene-3,17-dione **4** and 3α,6β-dihydroxy-1-methylen-5α-androstan-17-one **6** were only detected in the first 24 h. The absence of compound **4** in urine samples collected between 24 and 90 h after ingestion probably results from an increase of its biotransformation rate by hepatic enzymes, because as demonstrated below, this metabolite is the immediate precursor of compound **1** and of the isomeric 16-hydroxylated metabolites **10a** and **10b** which are detected until 90 h after ingestion.

Interestingly, metabolites **2**, **3**, **6**, **7a**, **7b**, **8** and **9** were only detected in the first 70 h after Primobolan® ingestion. This observation can be rationalized by the fact that these metabolites are produced through minor metabolic routes and are excreted in relatively low amounts in urine (Figs 2A and 3A), contrarily to methenolone **5** and metabolites **1**, **10a** and **10b** which are excreted in much larger amounts. Consequently, the latter metabolites provide for a longer retrospectivity with regard to the detection of Primobolan® use. Only trace amounts of these major metabolites were detected in urine samples collected between 90 and 120 h after ingestion. However, their respective urinary levels were too low (i.e. less than 1 ng/ml) to permit quantitation with adequate reliability since we did not use an isotope-

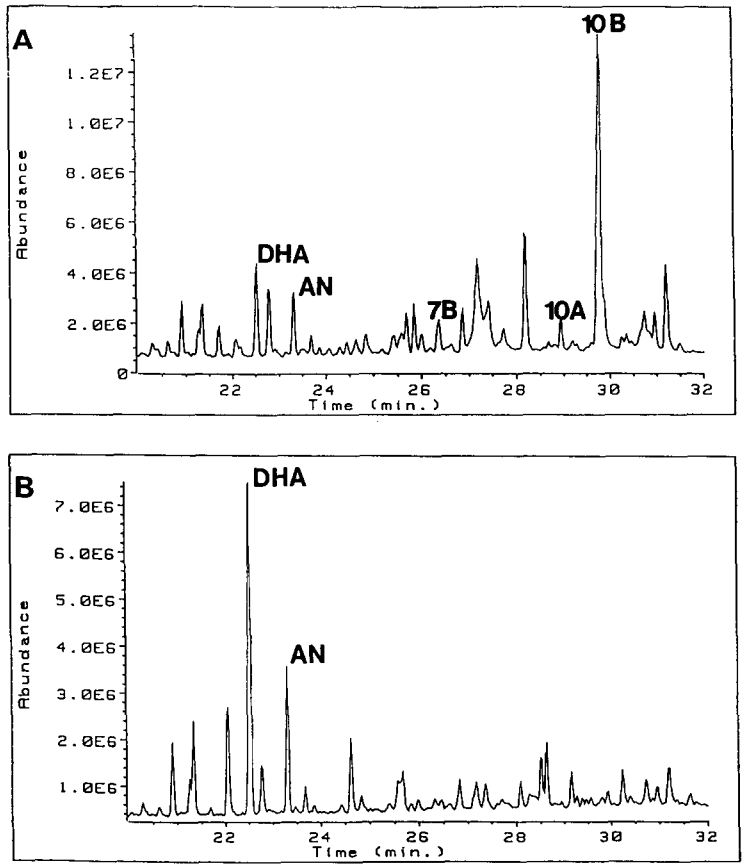


Fig. 3. Reconstructed total ion-current chromatogram from GC/MS analysis of the TMS ether derivatives of steroids isolated from the sulfate fraction of (A) a urine sample collected 3 h after methenolone ingestion and (B) a blank urine sample. For labelled peak identity, see Table 1. Other steroids are: DHA, dehydroepiandrosterone and AN: androstenediol. Analytical conditions were as in Fig. 2.

dilution method to carry out this work. The sole metabolite which was excreted in urine in amounts larger than methenolone **5** was 1-methylenandrosterone **1**. It was estimated by SIM GC/MS analysis that its cumulative excretion during the first 90 h was about 2.4% of the ingested Primobolan® dose. This data is in accordance with Björkhem and Ek [14] study who reported that about 3.0% of an ingested 10 mg dose of Primobolan® was excreted as compound **1** during the first 120 h.

## IDENTIFICATION OF METABOLITES

Table 1 summarizes the partial mass spectra and chromatographic methylene units of the TMS ether, TMS enol–TMS ether and/or MO–TMS derivatives of the metabolites which were detected in the free, glucuronic and sulfuric acid fractions (Figs 2A and 3A). Pathways accounting for the formation of the metabolites of interest are proposed in Figs 5, 11 and 12.

### *3α-hydroxyl-1-methylen-5α-androstan-17-one* **1**

This compound has been previously described as the major metabolite of methenolone acetate in human [13, 14]. The mass spectrum of its TMS ether derivative (Fig. 4A) shows a molecular ion at $m/z$ 374 which is in accordance with the proposed structure. Structurally informative ions at $m/z$ 318 $(M-56)^+$ and 303 $(M-71)^+$ arising from D-ring cleavage with concomitant elimination of a methyl radical, indicate the presence of a 17-keto group [15, 16]. Further evidences were obtained by the preparation of the corresponding MO–TMS derivative (Fig. 4b) which mass spectrum exhibits characteristic ions at $m/z$ 403 $(M^+)$, 388 $(M-15)^+$, 372 $(M-31)^+$, 313 $(M-TMSOH)^+$, 298 $(M-15-TMSOH)^+$ and 282 $(M-31-TMSOH)^+$. Interestingly enough, the prominent ions at $m/z$ 142 ($m/z$ 151, $d_9$-TMS) and 155 ($m/z$ 164, $d_9$-TMS) bearing the 3α-hydroxyl group are likely formed by cleavage of the A-ring according to a

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

| | Steroid | Fraction[a] | M.U.[b] | Derivative[c] | $M^+$ | Characteristic ions |
|---|---|---|---|---|---|---|
| **1** | 3α-hydroxy-1-methylen-5α-androstan-17-one | G | 26.44 | TMS enol–ether | 446 (31) | 169 (12), 431 (44), 341 (7) |
| | | | 26.27 | TMS ether | 374 (44) | 142 (90), 155 (31), 284 (41) |
| | | | 26.75 | MOTMS | 403 (12) | 142 (39), 155 (18), 282 (50) |
| **2** | 3α-hydroxy-1α-methyl-5α-androstan-17-one | G | 26.50 | TMS enol–ether | 448 (6) | 169 (20), 343 (23), 433 (82) |
| | | | 26.30 | TMS ether | 376 (8) | 145 (87), 286 (52), 361 (16) |
| | | | 26.81 | MOTMS | 405 (4) | 145 (20), 284 (71), 374 (100) |
| **3** | 17-epimethenolone | G | 26.93 | TMS enol–ether | 446 (16) | 179 (12), 195 (60), 208 (30) |
| **4** | 1-methyl-5α-androst-1-ene-3,17-dione | F | 26.93 | TMS enol–ether | 444 (4) | 179 (10), 195 (36), 208 (7) |
| **5** | Methenolone | G | 27.68 | TMS enol–ether | 446 (18) | 179 (14), 195 (100), 208 (45) |
| | | | 28.43 | TMS ether | 374 (10) | 129 (69), 136 (61), 284 (10) |
| | | | 28.35 | MOTMS | 403 (32) | 153 (20), 166 (61), 284 (10) |
| | | | 28.60 | MOTMS | 403 (32) | 153 (20), 166 (61), 284 (10) |
| **6** | 3α,6β-dihydroxy-1-methylen-5α-androstan-17-one | G | 27.42 | TMS enol–ether | 534 (17) | 191 (12), 230 (4), 297 (5) |
| | | | 28.17 | TMS enol–ether | 534 (6) | 191 (9), 230 (5), 297 (5) |
| | | | 27.83 | TMS ether | 462 (20) | 191 (23), 230 (25), 297 (12) |
| | | | 28.30 | MOTMS | 491 (6) | 191 (17), 230 (13), 197 (7) |
| **7a** | 3α-16α-dihydroxy-1-methyl-5α-androst-1-en-17-one | G | 28.67 | TMS enol–ether | 534(21) | 429 (4), 519 (30) |
| | | | 27.75 | TMS ether | 462 (3) | 155 (49), 210 (29), 318 (16) |
| **7b** | 3α,16β-dihydroxy-1-methyl-5α-androst-1-en-17-one | S | 28.62 | TMS enol–ether | 534 (18) | 429 (3), 519 (16) |
| | | | 28.37 | TMS ether | 462 (4) | 155 (81), 210 (37), 318 (23) |
| **8** | 6β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione | G | 28.53 | TMS enol–ether | 532 (96) | 193 (8), 281 (6), 295 (6) |
| | | | 29.04 | TMS ether | 388 (4) | 223 (6), 373 (9) |
| | | | 29.51 | MOTMS | 446 (82) | 252 (34), 294 (21), 325 (38) |
| **9** | 2ξ-hydroxyl-1-methylen-5α-androstane-3,17-dione | G | 29.38 | TMS enol–ether | 532 (20) | 191 (42), 283 (78), 295 (4) |
| | | | 29.51 | TMS enol–ether | 532 (20) | 283 (42), 295 (4), 517 (4) |
| | | | 29.63 | TMS ether | 388 (4) | 142 (36), 170 (22), 373 (100) |
| **10a** | 16α-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione | G | 29.85 | TMS enol–ether | 532 (25) | 195 (6), 517 (100) |
| | | | 29.89 | TMS ether | 388 (10) | 136 (100), 244 (9), 317 (80) |
| | | | 29.58 | MOTMS | 446 (48) | 174 (55), 415 (100), 273 (2) |
| | | | 29.74 | MOTMS | 446 (48) | 174 (55), 415 (100), 273 (2) |
| | | S | 29.86 | TMS enol–ether | 532 (21) | 195 (4), 517 (100) |
| | | | 28.86 | TMS ether | 388 (4) | 136 (100), 244 (15), 317 (87) |
| **10b** | 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione | S | 29.86 | TMS enol–ether | 532 (21) | 195 (4), 517 (100) |
| | | | 30.17 | TMS ether | 388 (3) | 136 (100), 244 (6), 317 (70) |
| | | | 30.20 | TMS ether | 388 (28)[d] | 129 (90), 156 (56), 373 (90) |
| | | | 30.19 | MOTMS | 446 (23) | 174 (53), 273 (83), 415 (100) |
| | | | 30.31 | MOTMS | 446 (23) | 174 (53), 273 (83), 415 (100) |

[a]Steroid fractions: (F) free, (G) glucuronide and (S) sulfate.
[b]Methylene unit (M.U.) values were calculated by linear interpolation of the retention time of the steroid derivative relatively 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 isomeric forms as TMS ether or TMS enol–TMS ether derivatives.
[d]This TMS ether derivatives is that of the 17-hydroxy-16-keto tautomer of compound **10b**.

mechanism analogous to that accounting for the formation of the ion of *m/z* 129 in $\Delta^5$-3-hydroxy and 3-hydroxy-5α-steroids [17]. Finally, the corresponding TMS enol–TMS ether derivative showed a molecular ion shifted by 72 a.m.u. to *m/z* 446 (*m/z* 455, in the mass spectrum of the mixed $d_0$-TMS enol–$d_9$-TMS ether derivative) thus confirming the presence of one hydroxyl and one keto groups in compound **1**. The presence of an exocyclic 1-methylene function can be rationalized as shown in Fig. 5 by enolization of methenolone 3-keto group to the corresponding 3-keto-1-methylene intermediate **5a** which then undergoes reduction to yield compound **1**. As we will discuss below, the *in vivo* formation of **5a** which was not detected in post-administration urine samples, was further demonstrated by the formation of compounds **6** and **9**.

*3α-hydroxy-1α-methyl-5α-androstan-17-one* **2**

The mass spectrum of the TMS enol–TMS ether derivative of 2 (Fig. 6A) showed strong similarities with that of compound **1** (Table 1) suggesting that they were structurally related. Theoretically, three compounds with a molecular weight of 448 a.m.u. (as TMS ether or TMS enol–TMS ether derivatives) can be formed from methenolone; two could arise from either the reduction of the 3-keto or 1-ene group whereas the third one could originate from the reduction of both A-ring substituents with concomitant oxydation of the 17-hydroxyl function. The TMS ether derivative (Fig. 6B) of **2** showed a molecular ion at *m/z* 376 (*m/z* 385, $d_9$-TMS), thus indicating the presence of one hydroxyl and one keto group. The presence of a prominent ion at *m/z* 145 (*m/z* 154, $d_9$-TMS) and comparison with ion of *m/z* 142 in the mass spectrum of methenolone TMS ether derivative (Fig. 4A) demonstrate that both A-ring substituents were reduced in compound **2**, since the former ion comprises the 3-hydroxyl group as well as the carbon atoms 1, 2, and 3 and the 1-methyl group. This implies that the keto group is located at the C-17 position. This hypothesis

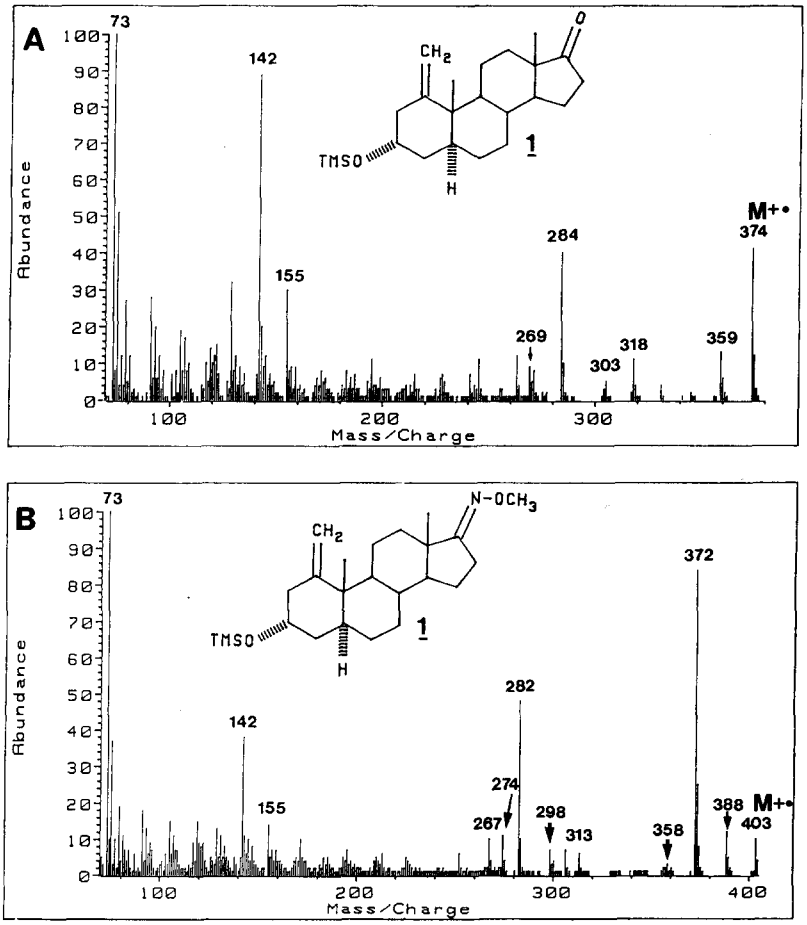


Fig. 4. Mass spectra of 3α-hydroxy-1-methylene-5α-androstan-17-one **1** as (A) TMS ether and (B) MO–TMS derivatives.

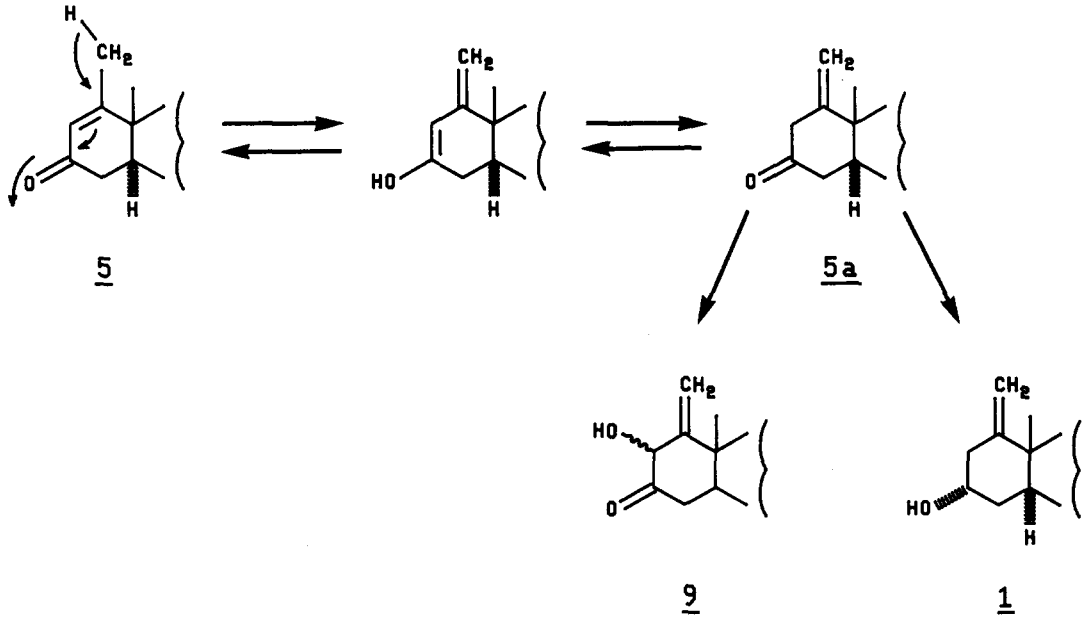


Fig. 5 Proposed pathway accounting for the formation of 1-methylene androsterone **1** from methenolone **5** in human.

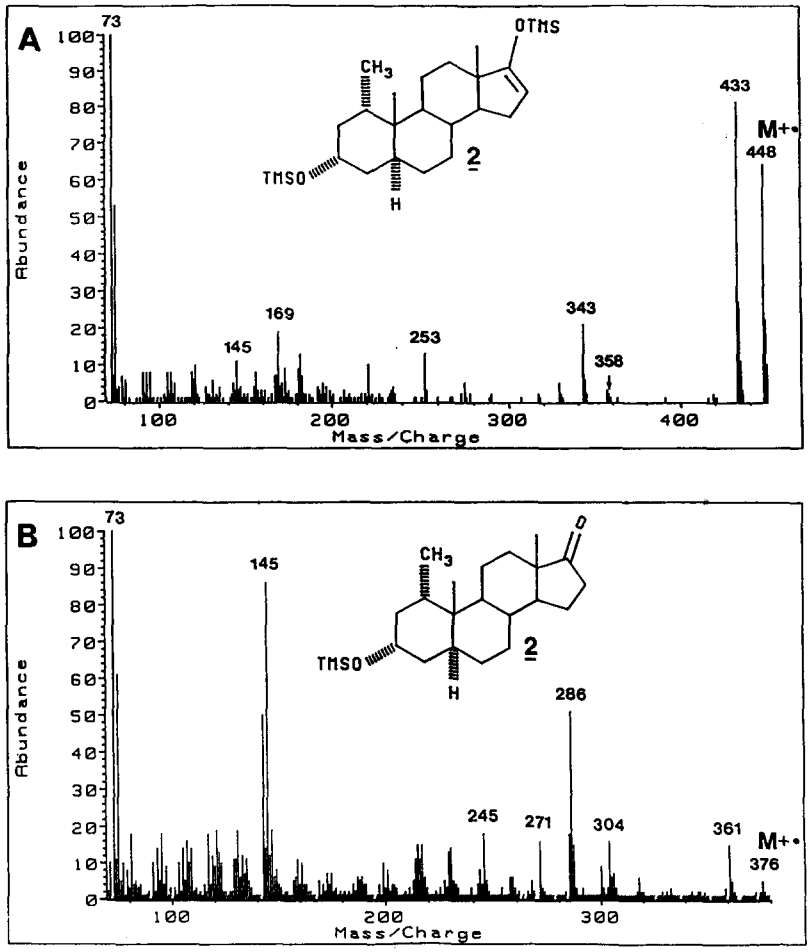


Fig. 6. Mass spectra of 3α-hydroxy-1α-methyl-5α-androstan-17-one **2** as (A) TMS enol–TMS ether and (B) TMS ether derivatives.

was corroborated by the mass spectrum of the corresponding MO–TMS derivative (Table 1) which exhibited a molecular ion at $m/z$ 405 ($m/z$ 414, $d_9$-TMS) and prominent ion at $m/z$ 372 $(M\text{-}31)^+$ and 145 (A-ring cleavage). Further analytical evidences were obtained by chemical reduction of methenolone with excess sodium borohydride to yield a mixture of isomeric steroids having the general structure of 1ξ-methyl-5α-androstane-3α,17β-diol, which TMS derivative showed a molecular ion at $m/z$ 450 ($m/z$ 468, $d_9$-TMS) and a prominent ion at $m/z$ 145 ($m/z$ 154, $d_9$-TMS) as observed in the mass spectrum of compound **2** (Fig. 6B).

The *in vivo* reduction of methenolone 1-ene and 3-keto groups to yield compound **2** is not a metabolic route specific to this steroid. Indeed, Ungar and Dorfman [18] reported that 1-androstene-3,17-dione, a model compound of methenolone, is transformed into androsterone and isoandrosterone by reduction of the 1-ene and 3-keto groups. Thus, compound **2** was identified as 3α-hydroxy-1α-methyl-5α-androstan-17-one.

*17-epimethenolone 3 and methenolone 5*

As shown in Table 1, methenolone was characterized by GC/MS analysis as various TMS and MO–TMS derivatives and by comparison with an authentic reference standard. A thorough mass spectral analysis of these derivatives and those of other metabolites of methenolone will be reported elsewhere.

Interestingly enough, a compound eluting about 1.7 min before methenolone TMS enol–TMS ether derivative (Fig. 7A) and exhibiting identical mass spectral features (Fig. 7B) was detected in minute amounts in the glucuronide fraction. This compound was identified as 17α-hydroxy-1-methyl-5α-androst-1-ene-3-one **3** (17-epimethenolone) on the basis of its GC/MS properties. Epimerization of the 17β-

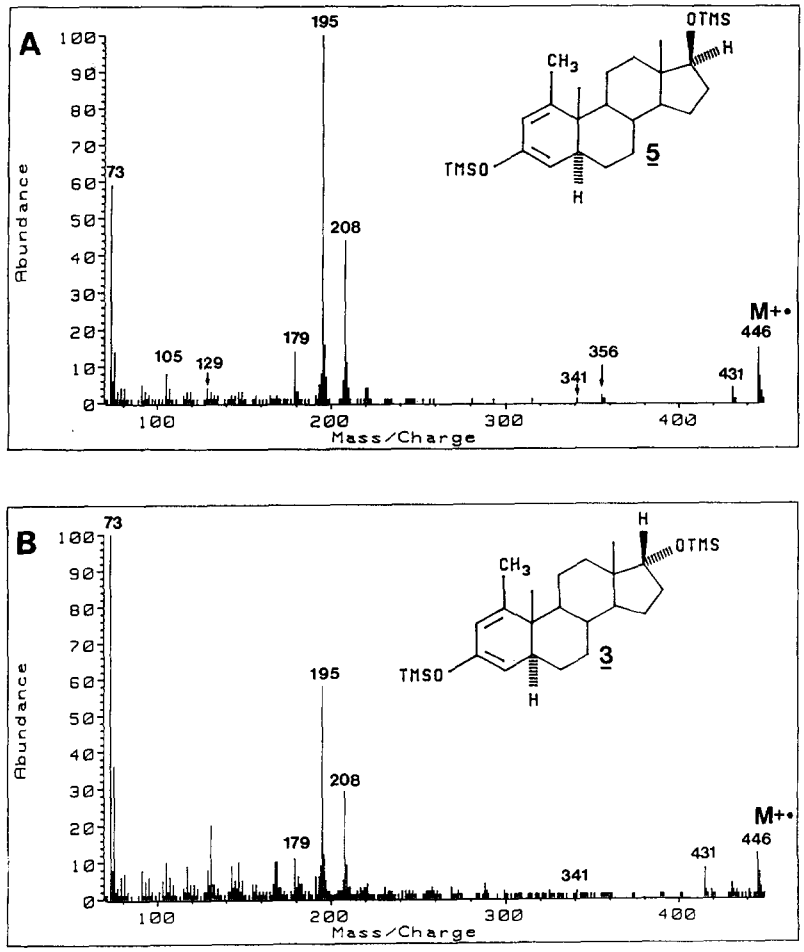


Fig. 7. Mass spectra of (A) methenolone **5** and (B) 17-epimethenolone **3** as TMS enol–TMS ether derivatives.

hydroxy group is also a characteristic feature of the 17β-hydroxy-17α-methyl steroids [9, 10, 19]. Edlund *et al.* [19] have proposed that epimerization at C-17 occurs through the nucleophic attack by water of the labile sulfate conjugate. We have recently synthetized the sulfate conjugate of several 17β-hydroxy-17α-methyl anabolic steroids, and demonstrated that their 17-epi analogs could be readily formed under specific conditions (results to be published). It is thus likely that 17-epimethenolone originated from the corresponding sulfate aglycone, although no trace of its was detected in the sulfate fraction. The occurrence of 17-epimethenolone in the glucuronide fraction suggested that epimerization of methenolone likely occurs in hepatic tissues where its corresponding epimer can be readily conjugated with glucuronic acid prior to elimination in urine.

*1-methyl-5α-androst-1-ene-3,17-dione* **4**

It was mentioned in two studies reported in the 1960a that compound **4** was excreted in the glucuronide fraction through enolization of the 3-keto group [13, 20]. Consequently, we gave special attention when performing the SIM GC/MS analysis of the urinary extracts to the monitoring of **4**, but no trace of this steroid could be detected in the glucuronide fraction of any of the urine samples tested in the course of this study. As we could have expected, this 3,17-dioxo steroid was instead recovered from the free steroid fraction. Its identity was confirmed (Table 1) by comparison with authentic reference compound prepared by Jones oxydation of methenolone 17-hydroxyl group.

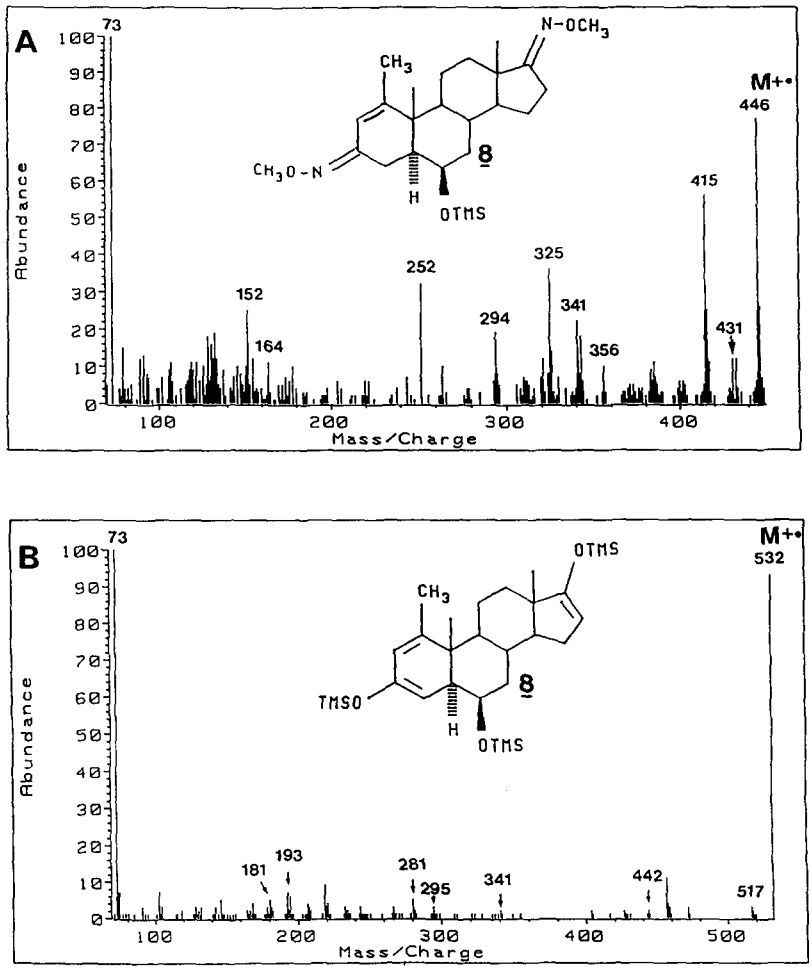


Fig. 8. Mass spectra of 6β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione **8** as (A) MO–TMS and (B) TMS enol–TMS ether derivatives.

*3α-6β-dihydroxy-1-methylen-5α-androstan-17-one* **6** *and 6β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione* **8**

The mass spectra of the TMS ether ($M^+$ 462; *m/z* 480, $d_9$-TMS), TMS enol–TMS ether ($M^+$ 534; *m/z* 552, $d_0$-TMS enol–$d_9$-TMS ether mixed derivative) and MO–TMS derivative ($M^+$ 491, *m/z* 509 MO–$d_9$-TMS) derivatives indicated the presence of 2 hydroxyls and one keto groups in compound **6**. Two isomeric derivatives were detected when **6** was analyzed at the TMS enol–TMS ether derivative (Fig. 2A). We did not further investigate this observation since the corresponding TMS ether and MO–TMS derivatives provided each a single derivative. This observation probably indicates that the TMS enol isomeric pair is due to the isomerization of the methylene group double bond upon derivatization rather than from two different isomeric steroids with α- and β-configurations. Further evidence for the presence of hydroxyl groups at the C-3 and C-6 positions was obtained from a diagnostically important ion at *m/z* 297 (*m/z* 315, $d_9$-TMS) in the mass spectra of all the above mentioned derivatives (Table 1), and which is analogous to the fragment ion of *m/z* 285 observed in the mass spectra of 3,6-dihydroxy $C_{19}$-steroids [21]. These data also indicate that the keto group is in the $C_{17}$ position. Although of low intensity, the ion of *m/z* 191 (*m/z* 209, $d_9$-TMS) observed in all derivatives (Table 1) arises from a rearrangement involving the 3- and 6-OTMS groups and carbon atom 3 according to a fragmentation route previously proposed by Harvey and Vouros [21]. Examination of the mass spectral features of compound **8** derivatives also provided evidence supporting compound **6** structural assignment. Indeed, the ion of *m/z* 295 in the mass spectrum of compound **8** TMS enol–TMS ether derivative (Fig. 8B) is analogous to the ion of *m/z* 297 in compound **6** and comprise the A-ring and carbon 6 [21]. Additional evidence for the presence of a 6-hydroxy group in compound **8** was obtained by selective $d_9$-TMS labelling of the

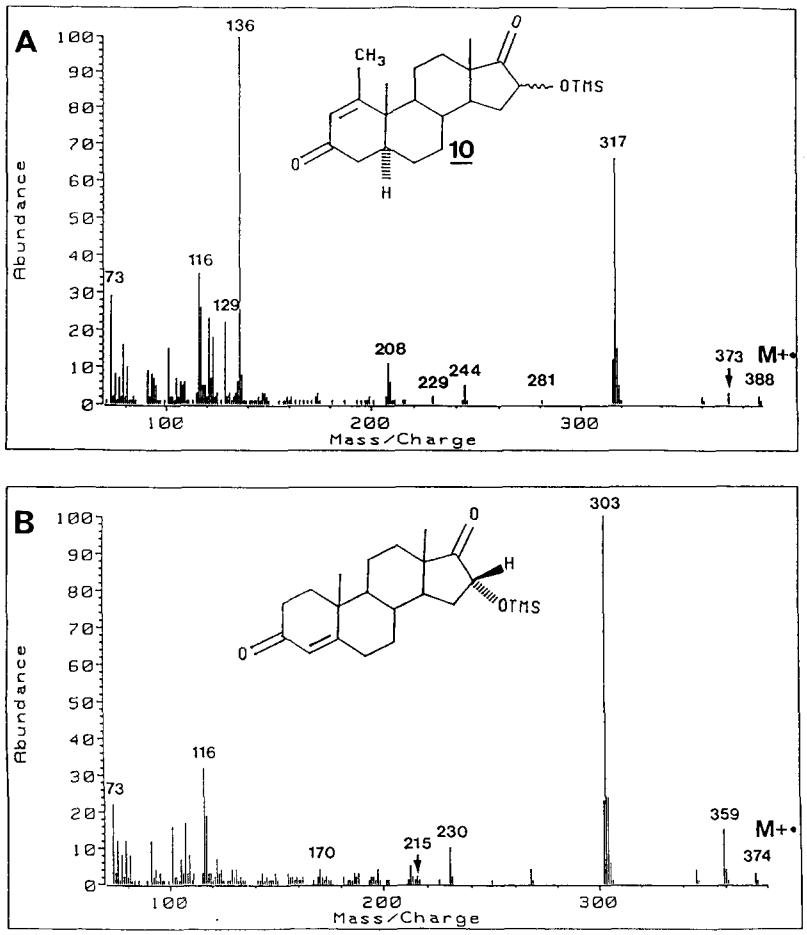


Fig. 9. Mass spectra of (A) 16α and 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione **10a** and **10b** and (B) authentic 16α-hydroxy-4-androstene-3,17-dione as TMS ether derivatives.

hydroxyl and keto functions, which shifted ion of $m/z$ 295 to $m/z$ 304 in the $d_0$-TMS enol–$d_9$-TMS ether derivative thus confirming the presence of the 3-keto and 6-hydroxy groups. Preparation of the MO–TMS derivative (Fig. 8A) demonstrated that **8** was a 3,17-dione steroid. Its mass spectrum also exhibit an ion at $m/z$ 252 ($m/z$ 261, $d_9$-TMS) which is analogous to the ions of $m/z$ 295 and 297 discussed above.

Finally, comparison of the mass spectrum of the TMS ether derivative of compound **8** with that of 6β-hydroxy-5α-androst-1-en-3-one, a model reference steroid, provided additional evidence that further assessed the proposed structure of compounds **6** and **8** [21, 22]. A 6β-configuration can be reasonably assigned to the 6-hydroxyl group in both compounds **6** and **8** on the basis of the mass spectral data presented above the published metabolic data which indicate that C-6β-hydroxylation is one of the major route for the metabolism of several synthetic anabolic steroids [23, 25] and testosterone [26] in the mammalian liver.

*16α-hydroxyl-1-methyl-5α-androst-1-ene-3,17-dione* ***10a*** *and 16β-hydroxyl-1-methyl-5α-androst-1-ene-3,17-dione* ***10b***

We estimated by SIM GC/MS analysis that about 92% of the prominent metabolite **10a** was excreted as glucuronide (Fig. 2A) and 8% as sulfate conjugates (Fig. 3A). Interestingly, its 16β-analog **10b** was exclusively detected in the sulfate fraction. These isomeric compounds were excreted in a 1:2 ratio as determined by GC/MS analysis on the glucuronide and sulfate fractions. Their TMS ether derivatives (Table 1 and Fig. 9a) were very similar and in accordance with the proposed structures as indicated by their molecular ion at $m/z$ 388 ($m/z$ 397, $d_9$-TMS) and diagnostic ions at $m/z$ 136 ($m/z$ 136, $d_9$-TMS), $m/z$ 244 (M-144; $m/z$ 244, $d_9$-TMS) and $m/z$ 317 (M-71; $m/z$ 326, $d_9$-TMS) which demonstrated that both **10a** and **10b** possess 1-en-3-one and 16-hydroxy-17-keto substituents in the A and D rings respectively [17, 27]. The ion at $m/z$ 317 $(M-71)^+$ was of diagnostic importance since it was shown to be characteristic of 16-hydroxy-3,17-dione steroids as illustrated by comparison with the analogous ion ($m/z$ 303; $m/z$ 312, $d_9$-TMS) observed in the mass spectrum of reference 16α-hydroxy-4-androstene-3,17-dione TMS derivative (Fig. 9B). The mechanism accounting for its formation has been previously proposed by several authors [15, 16, 28]. The structural features of **10a** and **10b** were further assessed by their MO–TMS derivatives (Table 1). Thus, ions at $m/z$ 153 and 166 arised from A-ring fragmentation as observed for methenolone MO–TMS derivative (Table 1) whereas ions at $m/z$ 174 ($m/z$ 183, $d_9$-TMS and $m/z$ 273 (M-173; $m/z$ 273 $d_9$-MO–TMS) were characteristic of the 16-hydroxy-17-keto groups in the D-ring [29]. As one would have expected from the tautomerism of the latter adjacent functional groups, the TMS enol–TMS ether derivatives of both **10a** and **10b** provided identical mass spectral features and GC retention time. More interesting was the capacity of the 16β-isomer **10b**, to tautomerize into the corresponding 17-hydroxy-16-keto tautomer which provided characteristic GC/MS features, namely ions at $m/z$ 129, and 156 arising from D-ring fragmentation [17]. The formation of this tautomer can be rationalized on the basis of intramolecular hydrogen bonding of the 16β-hydroxyl with the 17-keto group, which is not possible in the corresponding 16α-isomer. This type of hydrogen bonding promoted the transfer of the 16β-hydroxyl hydrogen atom to the oxygen atom of the 17-keto group upon enolization, leading to the formation of the 17-hydroxy-16-keto tautomer. This reaction could either occur during derivatization or extraction of the neutral steroids at pH 9 as previously reported by Shackleton *et al.* [30] in the case of 16β-hydroxy DHA. The tendency of such D-ring ketols to rearrange in acidic media as well, was also reported by Waxman *et al.* [31] who observed that solely 16β-hydroxy androstenedione and not its 16α-isomer was rearranged into 16-keto testosterone upon exposure to acidic silica gel. The α- and β-configuration of the 16-hydroxyl groups in compounds **10a** and **10b** was further ascertained by comparison with the retention data obtained from model 16-hydroxy-17-keto steroids [30, 32].

*3α, 16α-dihydroxy-1-methyl-5α-androst-1-en-17-one* ***7a*** *and 3α-16β-dihydroxy-1-methyl-5α-androst-1-en-17-one* ***7b***

These metabolites were detected in the glucuronide and sulfate fractions respectively and originated from the stereoselective reduction of the 3-keto group of compounds **10a** and **10b** by 3α-reductase. Their prominent structural features were clearly illustrated in the mass spectra of the TMS ether derivatives (Table 1).

Diagnostic ions at $m/z$ 318 (M-144, $m/z$ 327, $d_9$-TMS), and 228 (M-144-TMSOH; $m/z$ 228 $d_9$-TMS) are characteristic of the presence 3,16-dihydroxy and 17-keto groups [17, 27, 29]. On the other hand, the ion of $m/z$ 155 ($m/z$ 164, $d_9$-TMS) which comprises carbon atoms $C_1$–$C_4$, and ion of $m/z$ 210 ($m/z$ 219, $d_9$-TMS) arising from the cleavage of the $C_9$–$C_{10}$ and $C_6$–$C_7$ bonds are both characteristics of A-ring substituents. The corresponding TMS enol–TMS ether derivatives (Table 1) provided further evidence ascertaining their proposed structure.

*2ξ-hydroxy-1-methylen-5α-androstane-3,17-dione* **9**

The molecular ions of the TMS enol–TMS ether ($M^+$ 532, Fig. 10A), $d_0$-TMS enol–$d_9$-TMS ether ($M^+$ 541, Fig. 10B) and TMS ether ($M^+$ 388, Fig. 10C; $m/z$ 397, $d_9$-TMS) derivatives indicated the presence of one hydroxyl and 2 keto groups in compound **9**. The absence of diagnostic ions similar to those observed in the mass spectra of the 16-hydroxylated metabolites (Table 1) indicated that the hydroxyl group was not located in the D-ring. It is of interest to note

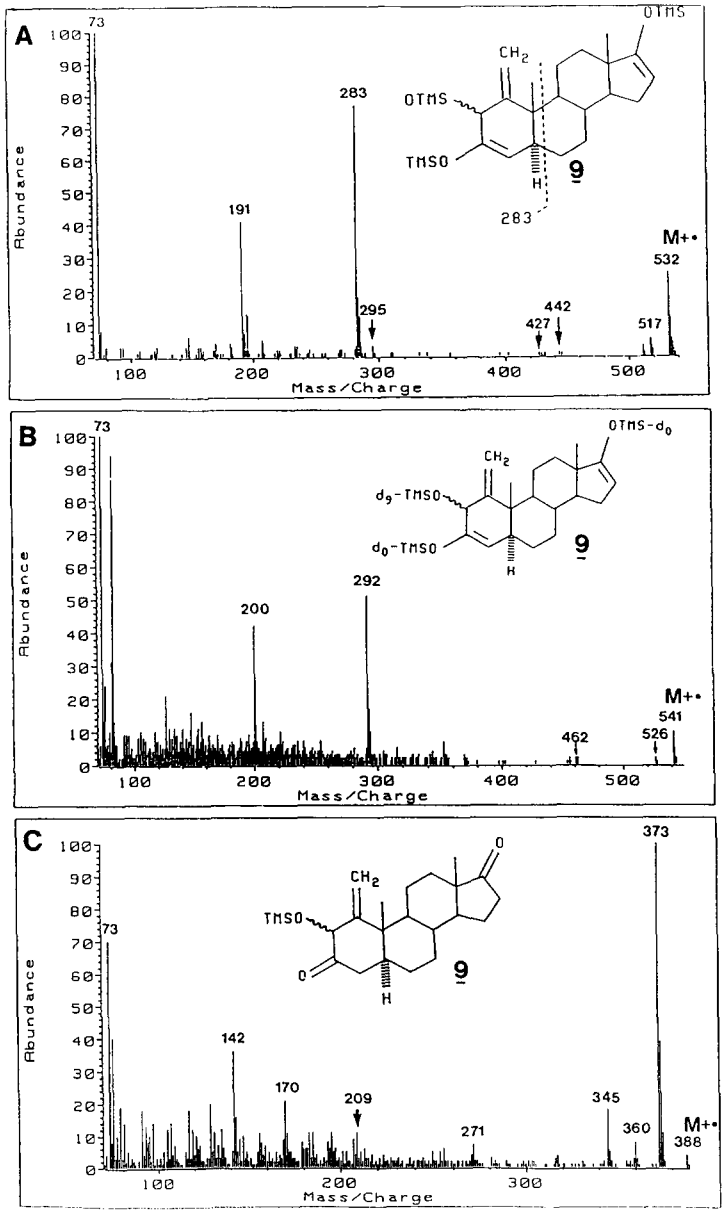


Fig. 10. Mass spectra of 2ξ-hydroxy-1-methylene-5α-androstane-3,17-dione **9** as (A) TMS enol–TMS ether, (B) $d_0$-TMS–$d_9$-TMS ether and (C) TMS ether derivatives.

that the ion of $m/z$ 283 ($m/z$ 195 + 88 a.m.u.) (Fig. 10A) which is analogous to that observed at $m/z$ 195 in the mass spectra of compounds **3** and **5** TMS enol–TMS ether derivatives, suggested that the hydroxyl group was located on the A-ring either at the $C_2$ or $C_4$ position. This hypothesis was further evidenced by the occurrence of an ion at $m/z$ 191 ($m/z$ 200, $d_9$-TMS) (Fig 10A and B) which has been previously shown to arise from the rearrangement of steroids bearing vicinal-OTMS groups [33]. As expected, this ion was not observed in the mass spectrum of the TMS ether derivative (Fig. 10C) thus suggesting that the hydroxyl group was adjacent to the 3-keto group. This hypothesis was supported by diagnostically important ions at $m/z$ 142 ($m/z$ 151, $d_9$-TMS), 170 ($m/z$ 179, $d_9$-TMS) and 209 ($m/z$ 218, $d_9$-TMS) which are homologous to ions of $m/z$ 142, 185 and 195 in the mass spectra of compound **1** (Figs 4A and B). The presence of 1-methyl-1-ene-functional groups was unlikely since it would have prevented the formation of the ion of $m/z$ 191 which requires the presence of a >CH-OTMS group [33]. Thus, it seems reasonable to propose that compound **9** has the structure of 2ξ-hydroxy-1-methylen-5α-androstane-3,17-dione. Configuration of the 2-hydroxy group could not be assigned due to the lack of reference compounds and to the probable tautomerism of A-ring substituents from which four tautomeric forms of **9** can be produced. Hydroxylation at the $C_2$ position, which is in an allylic relationship with the 1-methylene group is metabolically favoured. Hydroxylation at the $C_4$ position would have theoretically provided a metabolite with similar mass spectral features. However, comparison with the mass spectral features of reference 4-hydroxy-3-keto and 3,4-dihydroxy steroids indicated that hydroxylation at $C_2$ was more likely.

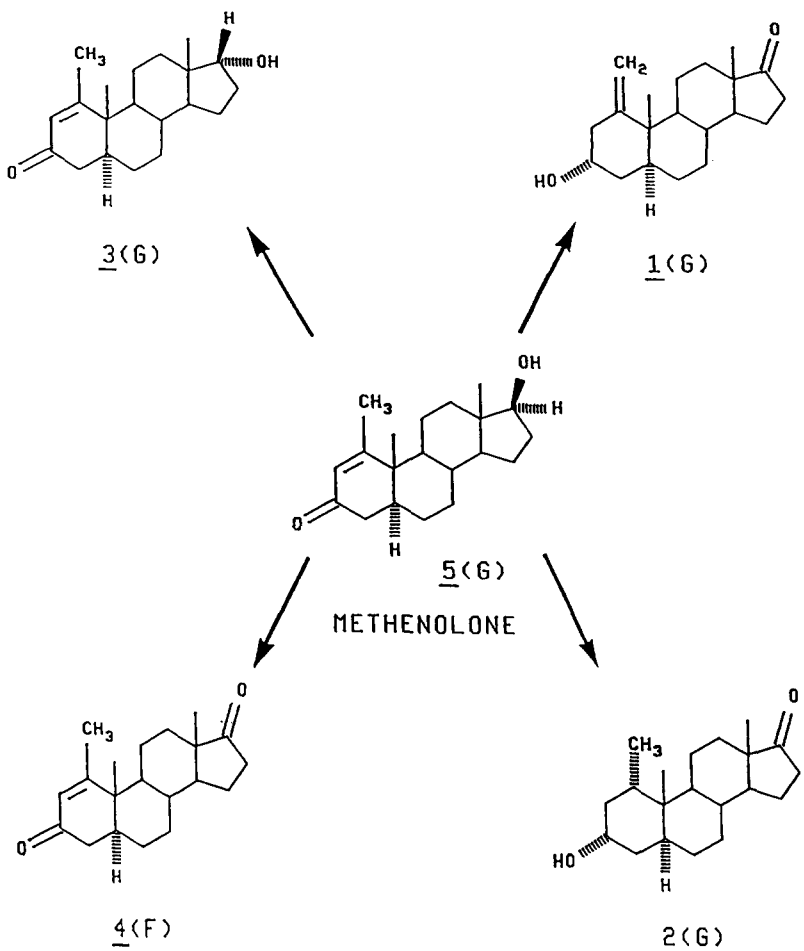


Fig. 11. Proposed structures of metabolites arising from methenolone epimerization and oxidation at the $C_{17}$ position with concomitant reduction of its A-ring functional groups. See Table 1 for further details.

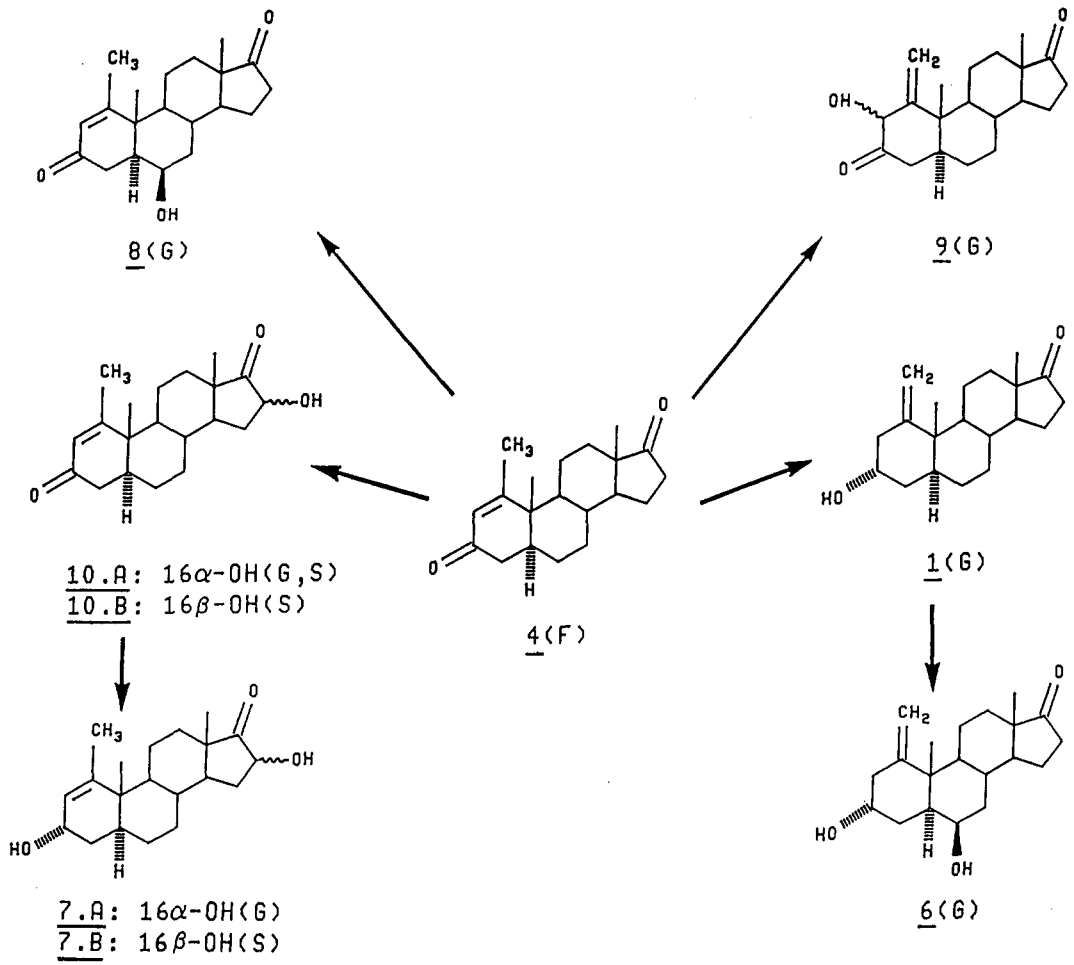


Fig. 12. Proposed structures of methenolone metabolites resulting from hydroxylation reactions at the $C_2$, $C_6$ or $C_{16}$ positions. See Table 1 for further details.

*Methenolone acetate metabolic routes*

As shown in Figs 11 and 12, oxydation by 17β-hydroxysteroid dehydrogenases was the prominent metabolic reaction in methenolone metabolism since all metabolites to the exception of 17-epimethenolone bear a 17-keto group. This reaction which is characteristic of 17β-hydroxy androgens metabolism in a number of mammalian species [34] is apparently not affected by the structural features of methenolone A-ring. The data provided above indicated that reduction of the 3-keto group did not precede oxidation at $C_{17}$ since no 3,17-dihydroxy metabolite was detected in urine. Thus, the biotransformation appears to be initiated by oxydation at $C_{17}$ to yield **4** (Fig. 11) and the 17-keto analog of **5a** (Fig. 5). Then, the latter metabolite can undergo stereoselective reduction at $C_3$ to give **1**. Alternatively, reduction of **4** 1-ene and 3-keto functions could likely account for the formation of **2**, although this metabolite can also arise from reduction of the 1-methylene group of compound **1**. It is interesting to note that among the metabolites shown in Fig. 11, only compound **4**, which does not bear any hydroxyl group, was excreted unconjugated in urine.

Compounds **1** and **4** are probably the metabolic precursors of the hydroxylated metabolites presented in Fig. 12. Hydroxylation of **4** at the $C_6$ and $C_{16}$ positions yielded compounds **8** and **10a** and **10b** respectively and subsequently to the formation of their glucuronide and/or sulfate conjugates. The latter isomeric 16-hydroxy steroids were then reduced by 3α-reductases to give **7a** and **7b** respectively.

On the other hand, the occurrence of compound **6** can be rationalized by 6β-hydroxylation of **1** and/or reduction of the 3-keto group

of **8** with concomitant isomerization of the 1-ene double bond to the corresponding 1-methylene group as shown in Fig. 5. Finally, the formation of the 2-hydroxy steroid **9** likely occurs by hydroxylation of the 3-keto 1-methylene tautomers of **4** and/or **5** as illustrated in Fig. 5. It is probable that the physiological importance of these reactions is to transform methenolone into more polar products susceptible to conjugation and ultimately to facilitate its excretion. However, possible biological activities of these hydroxylated steroids cannot be totally rejected. No pathway of sequential hydroxylation reactions, as those we previously reported for methandienone [8] and stanozolol [10], was observed in methenolone metabolism. The absence of a 17α-methyl group and the presence of a reducible 3-keto function in methenolone probably account for its characteristic hydroxylation profile. The 6β-hydroxylation of methenolone can be correlated with the 6β-hydroxylase reactions previously reported in the metabolism of synthetic [23–25] and endogeneous steroids [35] in human. Interestingly, 16-hydroxylation was quantitatively more important than 6β-hydroxylation, probably because of the absence of a 4-ene group [35] and the presence of a readily reducible 3-keto function. Owing to the fact that oxidation at $C_{17}$ increases the lipophilic character of the corresponding steroidal moiety, it is reasonable to assume that hydroxylation at $C_{16}$ would then be promoted. In this way, this moiety of the steroid becomes more polar and a site for conjugation. Moreover, compound **4**, the immediate precursor of the 16-hydroxysteroids **10a** and **10b** possess the necessary structural features to efficiently bind with microsomal cytochrome *P*-450 isozymes [36].

It has been shown that methenolone is transformed in human into a series of hydroxylated metabolites resulting either from hydroxylation at the $C_2$, $C_6$ or $C_{16}$ positions, or reduction of the 3-keto group with concomitant oxidation of the 17β-hydroxy group. The total excretion of both methenolone and its major metabolite **1** accounted for less than 5% of the ingested 50 mg dose of Primobolan®, thus suggesting that urinary excretion was not the major elimination route.

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