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THE METHYL-5α-DIHYDROTESTOSTERONES MESTEROLONE AND DROSTANOLONE; GAS CHROMATOGRAPHIC/MASS SPECTROMETRIC CHARACTERIZATION OF THE URINARY METABOLITES

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Summary—Before including the detection of the methyl-5α-dihydrotestosterones mesterolone (1α-methyl-17β-hydroxy-5α-androstan-3-one) and drostanolone (2α-methyl-17β-hydroxy-5α-androstan-3-one) in doping control procedures, their urinary metabolites were characterized by gas chromatography/mass spectrometry. Several metabolites were found after enzymatic hydrolysis and conversion of the respective metabolites to their trimethylsilyl-enol-trimethylsilyl ether derivatives. The major metabolites of mesterolone and drostanolone were identified as 1α-methyl-androsterone and 2α-methyl-androsterone, respectively. The parent compounds and the intermediate 3α,17β-dihydroxysteroid metabolites were detected as well. The reduction into the corresponding 3β-hydroxysteroids was a minor metabolic pathway. All metabolites were found to be conjugated to glucuronic acid.

INTRODUCTION

For years steroids with anabolic properties have been abused in sport [1]. For reasons of unfairness and health risks the use of these steroids by athletes is forbidden by the International Olympic Committee (IOC) [12]. Most of the sport organizations are following this policy.

The need for reliable anabolic androgen steroid doping control has led to screening and confirmation procedures either by gas chromatography/mass spectrometry (GC/MS) [3–5] or by immunoassays followed by GC/MS [6, 7]. Immunoassays are practical for the rapid screening of a large number of samples. However early applications of these techniques proved not to be successful due to the observed significant number of false positives and negatives.

5α-Dihydrotestosterone (I), mesterolone (II; 1α-methyl-17β-hydroxy-5α-androstan-3-one or 1α-methyl-5α-dihydrotestosterone) and drostanolone, also called dromostanolone (III_a; 2α-methyl-17β-hydroxy-5α-androstan-3-one or 2α-methyl-5α-dihydrotestosterone), are steroids with no obvious anabolic properties [8]. Mesterolone is used in the treatment of hypogonadism and of infertility [9]. A physical performance study regarding the effect of mesterolone showed no significant improvement compared to a placebo group [10]. The propionate ester of drostanolone (III_b) has been applied in the treatment of breast carcinoma [11]. Despite the lack of a marked anabolic effect and unclear influence on the performance enhancement, the IOC has reported that athletes using these structurally related steroids [1] were found positive after sports events.

In order to detect the administration of anabolic steroids the urinary metabolites must be characterized by GC/MS. Although some urinary metabolites of mesterolone were reported [12] no specific GC/MS details were given. As the metabolism of drostanolone was only studied in rabbits [13], human urinary metabolites have not been identified at all. Therefore we investigated the metabolism of mesterolone and drostanolone. This study describes a detailed GC/MS characterization of the human urinary metabolites of both methyl-5α-dihydrotestosterones.

EXPERIMENTAL

Materials and reagents

Sephadex LH-20 was obtained from Pharmacia (Woerden, The Netherlands). Amberlite

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XAD-2 was obtained from Serva (Heidelberg, Germany) and was washed successively with acetone, methanol and several times with distilled water until the washing solvent was clear. It was stored at room temperature as a slurry in distilled water. The *Helix pomatia* enzyme solution in water (No. 23867), containing β-glucuronidase and arylsulfatase, was also obtained from Serva. The *Escherichia coli* K12 enzyme solution was prepared by adding 50 μl of a suspension of β-glucuronidase (E.C. 3.2.1.31) from Boehringer Mannheim (Almere, The Netherlands) to 1 ml phosphate buffer at pH 7.0. 3α-Hydroxysteroid dehydrogenase (3α-HSD, E.C. 1.1.1.50) from *Pseudomonas* species was also from Boehringer Mannheim. *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). Trimethylsilyl iodide (TMSI) and dithioerythritol (DTE) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). *N*-hepta-fluorobutyryl-imidazole (HFBI) was purchased from Pierce Europe B. V. (Oud-Beijerland, The Netherlands). Mesterolone was obtained from Organon (Oss, The Netherlands). Proviron® tablets, containing 25 mg mesterolone, were purchased from Schering (Berlin, Germany). To obtain drostanolone, the propionate ester of drostanolone from Upjohn (Kalamazoo, MI, U.S.A.) was refluxed for three days in a 10% sodium hydroxide solution in water containing 2 mg/ml sodium laurylsulfate. After refluxing the steroid was extracted with diethyl ether and the solvent was removed in a Büchi rotating evaporator (Flawil, Switzerland). All other steroids were obtained from Sigma. Working steroid standard solutions (the internal standard, 5α-androstan-17β-ol, at a concentration of 50 μg/ml methanol and all other steroid solutions at concentrations of 1 mg/ml methanol) were kept at −20°C. Sodium borohydride and lithium aluminium deuteride were obtained from Merck (Amsterdam, The Netherlands). Glass wool pretreated with dimethyldichlorosilane (DMCS) was obtained from Alltech Europe (Eke, Belgium). All solvents and other reagents were of analytical grade.

Collection of urine specimens

Athletes, self-administering steroids, were asked to supply us with urine specimens before and after injecting themselves with drostanolone propionate. Urine specimens containing mesterolone metabolites were obtained by giving a male volunteer 1 tablet of Proviron®. The urine specimens were stored at −20°C.

Isolation of urinary steroids

The isolation was based on a method described by Donike *et al.* [3]. Respective extraction columns were prepared by pouring an Amberlite XAD-2 slurry into a pasteur pipette (150 × 5 mm), fitted with a small glass ball (dia 2.5 mm), up to a height of 2 cm. First the columns were washed with 5 ml of distilled water. Then 5 ml of a urine specimen were passed through the columns, followed by a second wash with 5 ml of distilled water. The adsorbed steroids were eluted with 3 consecutive portions of 0.9 ml methanol. The collected methanol fractions were removed by a gentle stream of nitrogen at 55°C. The residue and some traces of water were resolved in 1 ml of sodium acetate buffer at pH 5.2 (if to be hydrolyzed by *Helix pomatia* enzyme solution) or 1 ml of phosphate buffer at pH 7.0 (if to be hydrolyzed by *Escherichia coli* enzyme solution). The non-conjugated steroids were isolated by extraction with 5 ml of diethyl ether. To the remaining buffer 50 μl of enzyme solution were added to hydrolyze the conjugated steroids. Hydrolysis was performed at 50°C for 3 h. After cooling to room temperature the solution was made basic by adding 50 mg of potassium bicarbonate. Diethyl ether (5 ml), 50 μl of the internal standard solution and 3 g of anhydrous sodium sulfate were added consecutively and the mixture was shaken for 30 s. The diethyl ether layer containing the deconjugated steroids was separated. The consecutive diethyl ether fractions containing the non-conjugated or deconjugated steroids were evaporated by a gentle stream of nitrogen at 40°C. The residues were dried under reduced pressure over phosphorus pentoxide and potassium hydroxide for at least 1 h.

Sodium borohydride reduction

The dry residues containing the steroids were dissolved in 375 μl ethanol. Volumes of standard solutions of steroids in ethanol were adjusted with ethanol to 375 μl. Water (125 μl) and 0.66 M sodium borohydride (500 μl) in ethanol were added to the 375 μl ethanol solutions [14]. After 30 min at room temperature the ethanol was removed by a gentle stream of nitrogen. Chloroform (3 ml) and 0.5 g sodium sulfate were added to isolate the reduced steroids and the samples were shaken for 30 s.

The chloroform layer containing the reduced steroids was separated. To the remaining salt layer another 3 ml of chloroform were added. The samples were shaken again and the second chloroform layer was combined with the first. The combined chloroform fractions were evaporated by a gentle stream of nitrogen at 40°C. The obtained residues were dried under reduced pressure over phosphorus pentoxide and potassium hydroxide for at least 1 h.

Preparing deuterium labeled steroids

The 3α-*d*-3b-hydroxy- and 3β-*d*-3α-hydroxy isomers of 5α-androstan-3,17β-diol, 1α-methyl- and 2α-methyl-5α-androstan-3,17β-diol were synthesized by reducing 17β-hydroxy-5α-androstan-3-one, mesterolone and drostanolone, respectively. 50 μg of a steroid was dissolved in 500 μl water free diethyl ether. A suspension of 500 μl of 0.66 M lithium aluminium deuteride in dry diethyl ether was added, successively. After shaking 1 h the reaction was stopped by pouring 1 ml of a saturated sodium sulfate water solution into the mixture. The deuterated steroids were isolated by extraction with 5 ml of diethyl ether. The diethyl ether fraction was evaporated by a gentle stream of nitrogen at 40°C. The residues were dried under reduced pressure over phosphorus pentoxide and potassium hydroxide for at least 1 h.

Enzymatic oxidation by 3α-HSD

The residues were dissolved in a 100 mM sodium glycine buffer, pH 9.0, with acetonitrile as a co-solvent. The steroids were oxidized by 3α-HSD according to Ricigliano and Penning [15] with the exception that the reaction time applied was 1 h.

Derivation of steroids

Trimethylsilyl-enol-trimethylsilyl (TMS-enol-TMS) ether derivatization. A derivatization solution was prepared of MSTFA–TMSI (1000:2, v/v) and 2 mg/ml DTE [3]. To the respective dry residue containing the steroids 50 μl of the reagent mixture was added. The mixture was heated for 15 min and, after cooling to room temperature, it was used directly for GC/MS analysis.

Trimethylsilyl (TMS) ether derivatization. To the respective dry residue containing the steroids 50 μl of MSTFA was added. The mixture was incubated at room temperature for 15 min. Then the reaction was stopped and the excess of derivatization reagent was removed by filtration through a column (20 × 5 mm) of Sephadex LH-20 slurry packed in a pasteur pipette using chloroform–hexane (1:1) as an eluant. The steroid derivatives were eluted in the first 2 ml of eluant and the solvent was removed under a gentle stream of nitrogen. The residue was redissolved in 50 μl of ethyl acetate–isooctane (1:4, v/v) for GC/MS analysis.

Heptafluorobutyryl (HFB) ester derivatization. To the respective dry residue containing the steroids 50 μl of HFBI was added. The mixture was incubated at room temperature for 15 min. Then the reaction was stopped by removing the reagents on a LH-20 column (see TMS derivatization). The residue was redissolved in 50 μl of ethyl acetate–isooctane (1:4, v/v) for GC/MS analysis.

GC/MS

GC/MS analysis was performed on a 5790 Gas Chromatograph (Hewlett Packard, Palo Alto, CA, U.S.A.) coupled to a 5970 Mass Selective Detector (MSD, Hewlett Packard). The GC was equipped with a fused silica capillary column DB1 (J & W Scientific, Folsom, CA, U.S.A.), 30 m long, 0.32 mm i.d. and 0.25 μm film thickness. A multilevel oven program was used to achieve optimum separation. The temperature was maintained at 120°C for 0.5 min, programmed up to 180°C at 30°/min, programmed up to 230°C at 4°/min, programmed up to 233°C at 1°/min and then up to 265°C at 9°/min and maintained for 10 min. The temperatures of the injection port and the transfer line were 250 and 270°C, respectively. Helium was used as a carrier gas at a flowrate of 1.8 ml/min. A specimen volume of 1 μl was injected by the splitless mode according to Grob [16, 17]. The split valve was opened 30 s after the injection. To avoid discrimination against high boilers the splitless liner contained a 0.5 cm plug of DMCS treated glass wool centered in the liner.

RESULTS AND DISCUSSION

Steroid profile analysis

GC/MS steroid profiles of the urine specimens taken in the time interval of 3 to 12 h after administration were compared with those of the blank specimen taken before administration. Several metabolites were observed and the GC characteristics are summarized in Table 1. All metabolites were found in the conjugated

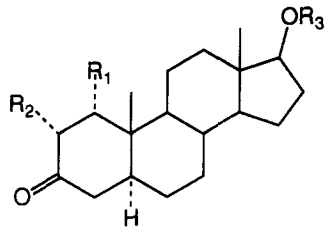

Fig. 1. The structures of some 5α-dihydrotestosterones. I, 5α-dihydrotestosterone ($R_1 = H$; $R_2 = H$; $R_3 = H$); II, mesterolone ($R_1 = CH_3$; $R_2 = H$; $R_3 = H$); III_a, drostanolone ($R_1 = H$; $R_2 = CH_3$; $R_3 = H$); and III_b, drostanolone propionate ($R_1 = H$; $R_2 = CH_3$; $R_3 = CO\text{-}CH_2\text{-}CH_3$).

fraction of the specimens after hydrolysis using either an enzyme solution of *Helix pomatia*, containing β-glucuronidase and sulfatase activity, or an enzyme solution of *Escherichia coli*, containing only β-glucuronidase activity. As the steroids were found in the same concentration using both enzyme solutions the metabolites must have been conjugated to glucuronic acid.

Parent compounds

The parent compounds for both mesterolone and drostanolone were observed as glucuronides. The mass spectra of the TMS-enol-TMS ether derivatives of these compounds are shown in Fig. 2. By comparing these spectra with those of authentic reference steroids, compound M_{IV} was identified as mesterolone and D_{IV} as drostanolone. Molecular ions and the ions $[M\text{-}15]^+$ were observed at *m/z* 448 and 433, respectively. The loss of 15 a.m.u. is typical for TMS ether steroid derivatives and arises from the loss of a methyl radical [18]. The sequential loss of one or two trimethylsilanol molecules from the $M^{+\cdot}$ and $[M\text{-}15]^+$ ions, being typical for TMS ether steroid derivatives, were not observed. Instead an abundant A-ring fragmentation was observed resulting in ions at *m/z* 405, 157 and 141 (Fig. 3). The TMS-enol-

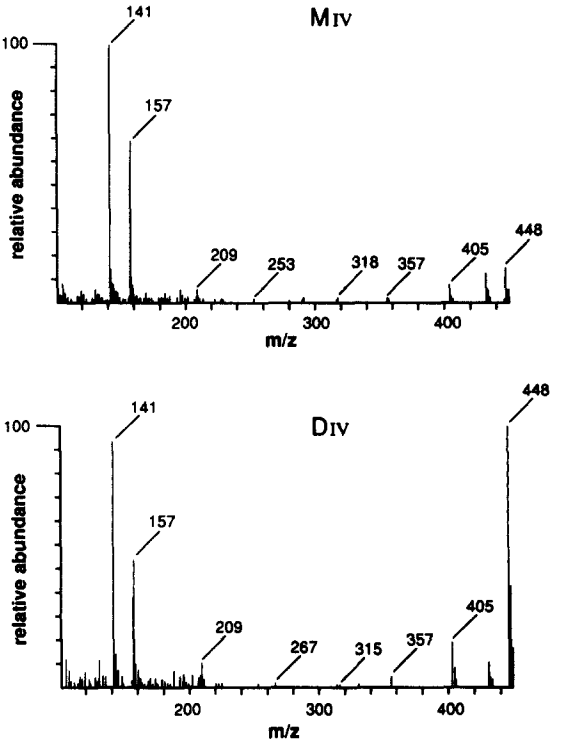

Fig. 2. The electron impact mass spectra of the TMS-enol-TMS ether derivatives of mesterolone (M_{IV}) and drostanolone (D_{IV}).

TMS ether derivative of mesterolone showed two not completely resolved GC peaks (Table 1) with identical mass spectra. As the GC/MS analysis of both the TMS ether and the HFB ester derivative of mesterolone resulted in one single GC peak, the parent compound analyzed must have been pure. The two GC peaks must therefore correspond to the 2- and 3-enol-TMS ether isomers, being formed from the tautomers of mesterolone (Fig. 4) separated under the GC conditions used. Separation of the enol-TMS ether isomers of testosterone has been described

Table 1. Gas chromatographic characteristics of the TMS-enol-TMS derivatives of urinary metabolites of mesterolone and drostanolone

Metabolite	RRT[a]	Significance[b]
Drostanolone		
D_I	1.689	Major metabolite
D_{II}	1.757	Minor metabolite
D_{III}	1.759	Minor metabolite
D_{IV}	2.167	Minor metabolite
Mesterolone		
M_I	1.896	Major metabolite
M_{II}	1.953	Minor metabolite
M_{III}	1.959	Minor metabolite
M_{IV}	2.036 and 2.056	Minor metabolite
M_V	2.081	Minor metabolite

[a]RRT = relative retention time to the internal standard 5α-androstan-17β-ol.
[b]Based on relative intensity of the gas chromatographic peak.

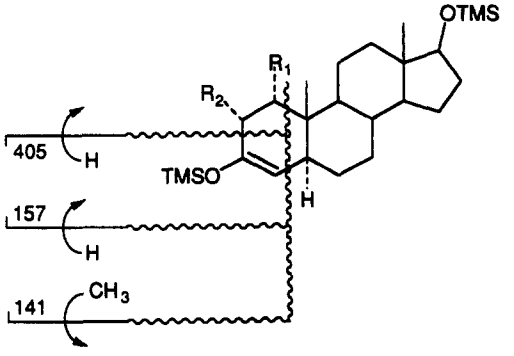

Fig. 3. A-ring fragmentation of the TMS-enol-TMS ether derivatives of some methyl-5α-dihydrotestosterones. M_{IV}, mesterolone ($R_1 = CH_3$; $R_2 = H$); and D_{IV}, drostanolone ($R_1 = H$; $R_2 = CH_3$).

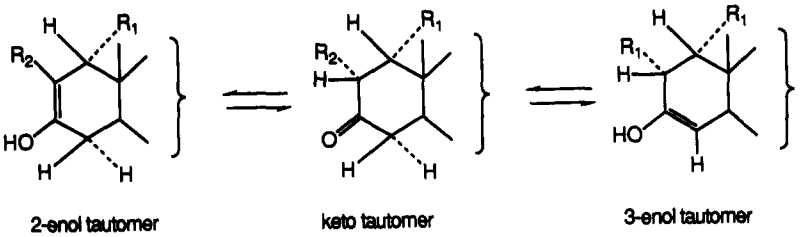

Fig. 4. Keto-enol equilibrium in some methyl-5α-dihydrotestosterones. M_{IV}, mesterolone ($R_1 = CH_3$; $R_2 = H$); and D_{IV}: drostanolone ($R_1 = H$; $R_2 = CH_3$).

Table 2. Partial GC/MS data of the TMS ether derivatives of the 3-hydroxy isomers of 5α-androstan-3,17β-diol, 5β-androstan-3,17β-diol, 1α-methyl- and 2α-methyl-5α-androstan-3,17β-diol

Isomer	RRT[a]	m/z Values of ions (ion intensities normalized on base peak)								
		$M^{+\cdot}$	$[M-15]^+$	$[M-90]^{+\cdot}$	$[M-105]^+$	$[M-180]^{+\cdot}$	$[M-195]^+$	Other ions		
5α-Androstan-3,17β-diol										
3α-Hydroxy	1.645	436(25)	421(21)	346(38)	331(43)	256(71)	241(100)	379(2)		129(75)
3β-Hydroxy	1.859	436(19)	421(54)	346(42)	331(29)	256(36)	241(71)	379(6)		129(100)
1α-Methyl-5α-androstan-3,17β-diol										
II	1.953	450(25)	435(18)	360(23)	345(42)	270(29)	255(40)	379(6)	145(100)	129(63)
V	2.081	450(11)	435(15)	360(15)	345(9)	270(16)	255(12)	379(11)	145(100)	129(67)
2α-Methyl-5α-androstan-3,17β-diol										
II	1.757	450(31)	435(26)	360(47)	345(64)	270(65)	255(100)	379(3)		129(87)
V	2.046	450(21)	435(38)	360(41)	345(26)	270(38)	255(79)	379(6)		129(100)
5β-Androstan-3,17β-diol										
3α-Hydroxy	1.659	436(4)	421(9)	346(33)	331(5)	256(100)	241(65)	—		129(5)
3β-Hydroxy	1.618	436(8)	421(14)	346(30)	331(25)	256(100)	241(57)	379(15)		129(7)

[a]RRT = retention time relative to internal standard.

previously [19]. For the TMS-enol-TMS ether derivative of drostanolone only a single GC peak was observed. Probably the formation of the 2-enol tautomer of drostanolone, and thus the 2-enol-TMS ether isomer, is favored due to the methyl group on the 2α-position. Another possibility is that the enol-TMS ethers of drostanolone were not separated.

3,17β-Dihydroxysteroid metabolites

The mass spectrometic data of the TMS-enol-TMS ether derivatives of the metabolites M_{II}, M_V and D_{II} indicated the existence of 3,17β-dihydroxy metabolites. In order to confirm the structures of these metabolites the 3-hydroxy isomers of 1α-methyl- and of 2α-methyl-5α-androstan-3,17β-diol were synthesized by reducing mesterolone and drostanolone using sodium borohydride. The reduction of the 3-keto-group was not stereospecific as mixtures of both 3-hydroxy isomers were formed. The GC/MS data of the TMS ether derivatives of these isomers (II and V) are shown in Table 2. In contrast to the parent compounds these 3,17β-dihydroxysteroid analogs showed, besides the ions $[M]^{+\cdot}$ and $[M-15]^+$, the typical ions $[M-90]^{+\cdot}$, $[M-105]^+$, $[M-180]^{+\cdot}$ and $[M\ 195]^+$, respectively. The ion at *m/z* 129 originates from the D-ring [18] (Fig. 5). The ion at *m/z* 145 in the isomers' spectrum of 1α-methyl-5α-androstan-3,17β-diol results from A-ring fragmentation as confirmed by the mass shift to *m/z* 146 in the deuterium labeling experiments with a deuterium atom at the 3α- or the 3β-position.

Stereospecific assignment to 3-hydroxy-androstane isomers can be based on the combination of their GC and MS characteristics using ion ratios as shown by Houghton *et al.* [20]. Typical characteristics of the 3-hydroxy isomers of 5α-androstan-3,17β-diol are shown in Table

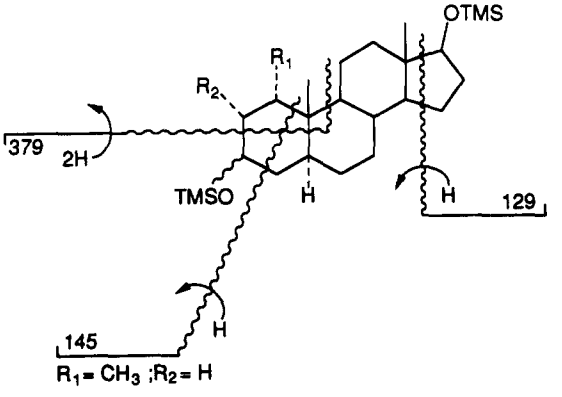

Fig. 5. Fragmentation of the A-, B- and D-ring of the TMS ether derivatives of the 3-hydroxy isomers of 1α-methyl-5α-androstan-3,17β-diol ($R_1 = CH_3$; $R_2 = H$) and 2α-methyl-5α-androstan-3,17β-diol ($R_1 = H$; $R_2 = CH_3$).

Table 3. Stereospecific ion ratios of the TMS ether derivatives of the 3-hydroxy isomers of 5α-androstan-3,17β-diol, 1α-methyl- and 2α-methyl-5α-androstan-3,17β-diol

Isomer	$M^{+\cdot}/[M-15]^+$	$[M-90]^{+\cdot}/[M-105]^+$
5α-Androstan-3,17β-diol		
3α-Hydroxy	1.2	0.9
3β-Hydroxy	0.4	1.4
1α-Methyl-5α-androstan-3,17β-diol		
II	1.4	0.5
V	0.7	1.7
2α-Methyl-5α-androstan-3,17β-diol		
II	1.2	0.7
V	0.6	1.6

2. The TMS ether derivative of the 3α-hydroxy isomer of 5α-androstan-3,17β-diol eluted earlier than the 3β-isomer. The 3-hydroxy isomers of 5α-androstan-3,17β-diol showed the same differences between the intensities of the ions $M^{+\cdot}$, $[M-15]^+$, $[M-90]^{+\cdot}$ and $[M-105]^+$ as reported by Houghton *et al.* [20]. Typical differences can be seen for the ion ratios of the 3α- and 3β-isomer (Table 3). The spectrum of the 3α-hydroxy isomer shows a ratio of $M^{+\cdot}$ to $[M-15]^+ > 1$ and of $[M-90]^{+\cdot}$ to $[M-105]^+ < 1$, while those data for the 3β-hydroxy isomer were reversed. Tables 2 and 3 also show the GC/MS characteristics of the 3-hydroxy isomers (II and V) of 1α-methyl- and 2α-methyl-5α-androstan-3,17β-diol. The retention time sequence and the stereospecific ratios indicate that the earlier eluted isomers (II) of both methyl-5α-androstan-3,17β-diols are the 3α-hydroxy isomers and the later eluted compounds (V) the 3β-hydroxy isomers.

We tried to assign definite stereospecificity by performing an enzymatic reaction using 3α-HSD. Although the 3α-HSD enzyme is known to be highly specific [21], we have checked the specific activity of the commercially available 3α-HSD preparation on the 3-hydroxy isomers of 5α-androstan-3,17β-diol. The 3α-HSD preparation did not prove to be specific under all conditions. After 1 h the preparation converted the 3α-hydroxy isomer into its 3-ketosteroid analog, but the oxidation was incomplete. However when the reaction was performed for 3 h, the 3-ketosteroid analog was converted into a steroid similar to a 3-hydroxy-5α-androst-1-en-17-one isomer. In order to avoid unwanted reactions the 1 h reaction time was chosen to oxidize the mixtures obtained after the sodium borohydride reduction reaction.

The earlier eluted 3-hydroxy isomer in the 1α-methyl-5α-androstan-3,17β-diol mixture was converted by 3α-HSD into its 3-ketosteroid analog, mesterolone. Therefore that result confirms the statement that the earlier eluted isomer is the 3α-hydroxy isomer. However when the reaction was performed on the 2α-methyl-5α-androstan-3,17β-diol mixture a steroid was observed, which could not be identified. Probably the 2α-position of the methyl group prevents the 3α-oxidation and combined with the unwanted enzymatic activity, the 3α-HSD preparation lead to a steroid with an unknown structure.

Although the 3α-HSD reaction could not verify stereospecificity of the drostanolone 3,17β-dihydroxysteroid metabolites, all other data obtained confirm that the earlier eluted 3-hydroxy compound (II) is the 3α-hydroxy isomer and the later eluted isomer (V) the 3β-hydroxy isomer. Therefore the M_{II} and M_V metabolites were identified as 1α-methyl-5α-androstan-3α,17β-diol and 1α-methyl-5α-androstan-3β,17β-diol, respectively and the D_{II} metabolite as 2α-methyl-5α-androstan-3α,17β-diol.

17-Ketosteroid metabolites

Partial mass spectrometric data of the TMS-enol-TMS derivative of the metabolites M_I, M_{III}, D_I and D_{III} are summarized in Table 4. Abundant ions were observed at *m/z* 448 and 433, the molecular ion $M^{+\cdot}$ and the ion $[M-15]^+$, respectively. Also the typical TMS ether

Table 4. Partial GC/MS data of the TMS-enol-TMS ether derivative of the 3-hydroxy isomers of 3-hydroxy-5α-androstan-17-one and the metabolites D_I, D_{III}, M_I and M_{III}

Isomer	*m/z* Values of ions and ion intensities normalized on base peak						Miscellaneous	
	$M^{+\cdot}$	$[M-15]^+$	$[M-90]^{+\cdot}$	$[M-105]^+$	$[M-180]^{+\cdot}$	$[M-195]^+$		
3-Hydroxy-5α-androstan-17-one								
3α-Hydroxy	434(55)	419(100)	344(1)	329(18)	254(1)	239(19)	169(28)	129(7)
3β-Hydroxy	434(74)	419(100)	344(2)	329(48)	254(1)	239(25)	169(35)	129(9)
Drostanolone metabolites								
D_I	448(81)	433(100)	358(3)	343(40)	268(4)	253(20)	169(37)	129(9)
D_{III}*	448(100)	433(95)	—	343(80)	—	253(38)	169(51)	129(45)
Mesterolone metabolites								
M_I	448(81)	433(100)	358(2)	343(27)	268(1)	253(14)	169(29)	129(8)
M_{III}	448(66)	433(100)	—	343(20)	—	253(14)	169(30)	129(9)

*The signal in the total ion chromatogram had a low intensity.

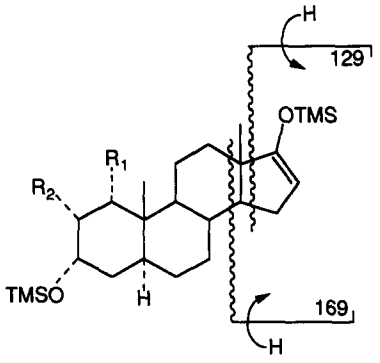

Fig. 6. Fragmentation of the D-ring of the TMS-enol-TMS ether derivatives of 1α-methyl-androsterone ($R_1 = CH_3$; $R_2 = H$) and 2α-methyl-androsterone ($R_1 = H$; $R_2 = CH_3$).

steroid ions, $[M-90]^{+\cdot}$, $[M-105]^+$, $[M-180]^{+\cdot}$ and $[M-195]^+$ were found. The ion at m/z 169 is a D-ring fragment characteristic for all 17-enol TMS ether steroid derivatives (Fig. 6). D-ring fragmentation also results in the ion at m/z 129 [18].

Table 4 also gives the mass spectrometric data of the TMS-enol-TMS ether derivative of androsterone (3α-hydroxy-5α-androstan-17-one) and epiandrosterone (3β-hydroxy-5α-androstan-17-one). Compared to those data the metabolites must be 3-hydroxy-17-ketosteroids too. However the stereospecificity of the 3-hydroxy group of these metabolites could not be derived from the mass spectra shown.

In order to elucidate the stereospecificity of the 3-hydroxy-17-ketosteroid metabolites, the respective steroids were converted into 3,17-dihydroxysteroids by reduction using sodium borohydride. The stereospecificity of this reduction of 17-ketosteroids was first tested by the reduction of 3α-hydroxy-5α-androstan-17-one and 3β-hydroxy-5α-androstan-17-one. Both steroids were completely converted into the 17β-hydroxysteroid analogs. Since the metabolites of mesterolone and drostanolone were not isolated before reduction, the complete urinary conjugated steroid fraction was reduced. By comparing the non-reduced GC/MS steroid profiles with the reduced profiles the metabolites M_I and D_I were observed to be converted into the 3α,17β-dihydroxysteroid analogs. Therefore metabolite M_I is 1α-methyl-androsterone (3α-hydroxy-1α-methyl-5α-androstan-17-one) and D_I is 2α-methyl-androsterone (3α-hydroxy-2α-methyl-5α-androstan-17-one). The reduced products of M_I and D_I found in the sodium borohydride treated urine specimens actually consisted of the reduced methyl-androsterone metabolite and the 3α,17β-hydroxysteroid metabolite. Because the last metabolite was only present in a small concentration it did not significantly influence the interpretation of the results of the reduction reaction.

A mass spectrum of the TMS-enol-TMS derivative of a 3α-hydroxy-2-methyl-5α-androstan-17-one isomer has been described [22] and compared to the spectrum of D_{II}, showed only one difference. The spectrum published showed a fragment, with significant intensity, in the m/z 410–420 region which was not characterized by the authors. For a correct comparison however the stereospecificity of the CH_3 on the C2-position in the described spectrum should be known.

The stereospecificity of the metabolites M_{III} and D_{III}, which are also methyl-androsterone isomers, could not be elucidated, because these metabolites were only present in small concentrations. For a correct assignment these metabolites should be isolated. However these metabolites must be the respective methyl-epiandrosterones, the only remaining possibility.

Mass spectrometric identification of stereoisomers

Table 3 describes stereospecific fragmentation characteristics of some androstandiols. The fragmentation mechanism for this loss of trimethylsilanol involves a hydrogen abstraction in which the hydrogen must be available within bonding distance [23]. The small differences between the ratios of $M^{+\cdot}$ to $[M-15]^+$ and of $[M-90]^{+\cdot}$ to $[M-105]^+$ of the TMS ether derivative of the 3-hydroxy isomers of 5α-androstandiols (Table 3) are probably also determined by the availability of hydrogen atoms. In the two different 3-hydroxy isomers these bonding distances are not the same, which could explain the observed differences in intensities of the characteristic ions. The availability of hydrogens and thus binding distance does not only depend on the stereospecificity of the intact steroid, but also on the stereospecific confirmation of the respective ions. It has been reported that the mechanism of loss of a water molecule in non-derivatized steroids, analog to the loss of trimethylsilanol in TMS-derivatized steroids, from the metastable $M^{+\cdot}$ is different from that from $[M-CH_3]^+$ ions [24].

Characteristic ion at m/z 379

In our studies we also observed an interesting ion at m/z 379, which showed stereospecificity,

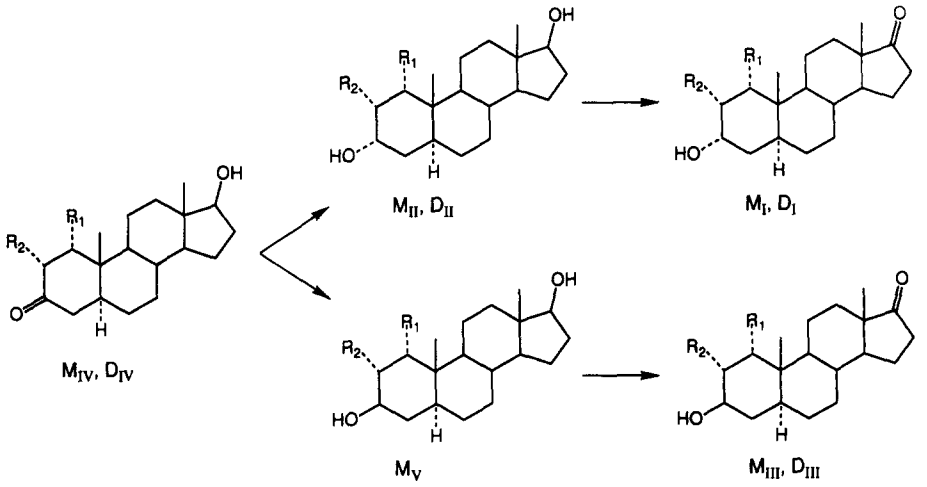

Fig. 7. The reduction into the corresponding 3α-hydroxy- and 3β-hydroxysteroids in the metabolism of mesterolone (M: $R_1 = CH_3$; $R_2 = H$) and drostanolone (D: $R_1 = H$; $R_2 = CH_3$). I, methyl-androsterone; II, methyl-5α-androstan-3α,17β-diol; III, methyl-epiandrosterone; IV, parent compound; and V, methyl-5α-androstan-3β,17β-diol.

but was not reported by Houghton *et al.* [20]. In the spectra of the 5α-androstan-3,17β-diol isomers a small difference between the intensities of the ion at *m/z* 379 was found. In the spectra of the isomers of 5β-androstane-3,17β-diol the difference was more obvious (Table 2). The formation of the ion at *m/z* 379 originates from A/B-ring fragmentation (Fig. 5) and is probably also determined by the availability of hydrogen atoms. We expected that a C9–C11 cleavage and a hydrogen shift from the C3- to a C9-position would be crucial for this fragmentation and would determine the difference in intensities observed for the ion at *m/z* 379. However incorporation of a deuterium atom at that position could not confirm that expectation. In the deuterium labeled 3α-*d*-3β-hydroxy and 3β-*d*-3α-hydroxysteroid isomers of 5α-androstan-3,17β-diol, 1α- and 2α-methyl-15α-androstan-3,17β-diol this A/B-fragment was observed at *m/z* 380. Further selective deuterium labeling studies are carried out in order to reveal this mechanism.

Metabolism of mesterolone and drostanolone

The biotransformations of mesterolone and drostanolone in man, as observed in our study, follow almost identical pathways (Fig. 7). We did not only observe the 2α-methyl-5α-androstan-3β,17β-diol metabolite for drostanolone in the urine specimens studied. For mesterolone our results are in agreement with those reported previously [12]. As for dihydrotestosterone [25] and testosterone [26, 27] two routes can be distinguished, the major pathway leading to 3α-hydroxysteroid metabolites and the minor to 3β-hydroxysteroids. In other synthetic 17β-hydroxy-3-ketosteroids [22, 28–30] 17-dehydrogenation was a dominant metabolic reaction. Compared to dihydrotestosterone [25] the 1α- or 2α-methyl substituents did not influence the metabolism. As for all 3α-hydroxy-5α-steroids in humans the metabolites found were excreted as 3-glucuronides [31]. For drostanolone 17-epimerization is an important biotransformation step in rabbits [4]. We did not observe 17α-hydroxysteroid metabolites for drostanolone at all. 17-Epimerization has been reported in humans, but is not a common metabolic pathway and is observed in the metabolism of 17α-methyl-17β-hydroxysteroids [32–35] and trenbolone [36]. For some steroids metabolites 17-epimerization is believed to be a urinary inversion of the respective conjugated metabolites and thus an *in vitro* process [37].

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REFERENCES

1. Dubin C. L.: Commission of inquiry into the use of drugs and banned practices intended to increase athletic performance. Canadian Government Publishing Centre, Ottawa (1990).
2. International Olympic Committee: International Olympic Charter against Doping in Sport. Lausanne (1990).

3. Donike M., Zimmerman J., Bärwald K.-R., Schänzer W., Christ V., Klosterman R. and Opfermann G.: Routine determination of anabolics in man. *Deutsch. Z. Sportmed.* **1** (1984) 14–24.
4. Massé R., Ayotte C. and Dugal R.: Studies on anabolic steroids. I Integrated methodological approach to the gas chromatographic/mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromat.* **489** (1989) 23–50.
5. Chung B.-C., Choo H.-Y. P., Kim T.-W., Eom K.-D., Kwon O.-S., Suh J., Yang J. and Park J.: Analysis of anabolic steroids using selected ion monitoring. *Analyt. Toxic.* **14** (1990) 91–95.
6. Bertrand M., Massé R. and Dugal R.: GC-MS approach for the detection and characterization of fluids at major international sporting events. *Farm. Tijdschr. Belg.* **55** (1978) 85–101.
7. Brooks R. V., Jeremiah G., Webb W. A. and Wheeler M.: Detection of anabolic steroid administration to athletes. *J. Steroid Biochem.* **11** (1979) 913–917.
8. Vida J.: *Androgens and Anabolic Agents.* Academic Press, New York (1969).
9. Martindale W. H.: *The Extra Pharmacopoiea* (Edited by J. E. F. Reynolds). Pharmaceutical Press, London (1989) p. 1403.
10. Strömme S. B., Meen H. D. and Aakvaag A.: Effect of an androgenic-anabolic steroid on strength development and plasma testosterone levels in normal subjects. *Med. Sci. Sports* **6** (1974) 203–208.
11. Straub G.: Die Behandlung des metastasierenden und rezidivierenden Mammakarzinoms mit Masterid®. Thesis München, Germany (1976).
12. Cartoni G. P., Ciardi M., Giarruso A. and Rosati F.: Capillary gas chromatographic-mass spectrometry detection of anabolic steroids. *J. Chromat.* **279** (1983) 515–523.
13. Templeton J. F. and Kim R. S.: Metabolism of 17β-hydroxy-2α-methyl-5α-androstan-3-one in the rabbit. *Steroids* **29** (1977) 371–381.
14. Vogel: *Vogel's Textbook of Practical Organic Chemistry.* Longman Group Ltd., London (1979) p. 466.
15. Ricigliano J. W. and Penning T. M.: Synthesis and evaluation of non-steroidal mechanism-based inactivators of 3α-hydroxy-steroid dehydrogenase. *Biochem. J.* **262** (1989) 139–149.
16. Grob K. and Grob G.: Splitless injection on capillary columns Part I; the basic technique: steroid analysis as an example. *J. Chromat. Sci.* **7** (1969) 584–586.
17. Grob K. and Grob G.: Splitless injection on capillary columns Part II; conditions and limits, practical realization. *J. Chromat. Sci.* **7** (1969) 587–591.
18. Diekman J. and Djerassi C.: Mass spectrometry in structural and stereochemical problems. CXXV. Mass spectrometry of some steroid trimethylsilyl ethers. *J. Org. Chem.* **32** (1967) 1005–1012.
19. Poole C. P.: Recent advances in the silylation of organic compounds for gas chromatography. In *Handbook of Derivatives for Chromatography* (Edited by K. Blau and G. S. King). Heyden & Son Ltd., London (1978) pp. 161–162.
20. Houghton E., Ginn A., Teale P., Minoo C. and Copsey J.: Comparison of the use of mass spectrometry and methylene unit values in the determination of the stereochemistry of estranediol, the major urinary metabolite of 19-nortestosterone in the horse. *J. Chromat.* **479** (1989) 73–83.
21. Skalhegg B. A.: On the 3α-hydroxysteroid dehydrogenase from Pseudomonas testosteroni. *Eur. J. Biochem.* **46** (1974) 117–125.
22. Goudreault D. and Massé R.: 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. *J. Steroid Biochem. Molec. Biol.* **38** (1991) 639–655.
23. Harvey D. J. and Vouros P.: Influence of the 6-trimethyl-silyl group on the fragmentation of the trimethylsilyl derivatives of some 6-hydroxy- and 3,6-dihydroxy-steriods and related compounds. *Biomed. Mass Spectrom.* **6** (1979) 135–143.
24. Zaretskii Z. V. I., Curtis J. M., Brenton A. G., Beynon J. H. and Djerassi C.: Translational energy release and stereochemistry of steroids. VIII—The mechanism of the eliminating of water from metastable ions in epimeric 3-hydroxy steroids of the 5α-series. *Org. Mass Spectrom.* **23** (1988) 453–459.
25. Träger L.: *Steroidhormone.* Springer-Verlag, Berlin (1977) pp. 191–192.
26. Baulieu E. E. and Mauvais-Jarvais P.: Studies on testosterone metabolism I. *J. Biol. Chem.* **239** (1964) 1569–1577.
27. Baulieu E. E. and Mauvais-Jarvais P.: Studies on testosterone metabolism II. *J. Biol. Chem.* **239** (1964) 1578–1584.
28. Galetti F. and Gardi R.: Metabolism of 1-dehydroandrostanes in man. *Steroids* **18** (1971) 39–50.
29. Massé R., Laliberté C., Tremblay L. and Dugal R.: Gas chromatographic/mass spectrometric analysis of 19-nortestosterone urinary metabolites in man. *Biomed. Mass Spectrom.* **12** (1985) 115–121.
30. Goudreault D. and Massé R.: Studies on anabolic steroids—4. Identification of new urinary metabolites of methenolone acetate (primobolan®) in human by gas chromatography/mass spectrometry. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 137–154.
31. Shackleton C. H. L.: Profiling steroid hormones and urinary hormones. *J. Chromat.* **379** (1986) 91–156.
32. Kammerer R. C., Merdink J. L., Jagels J. L. and Catlin D. H.: Testing for fluoxymesterone (Halotestin®) administration to man: identification of urinary metabolites by gas chromatography-mass spectrometry. *J. Steroid Biochem.* **36** (1990) 659–666.
33. Harrison L. M. and Fennessey P. V.: Methandrostenolone metabolism in humans: potential problems associated with isolation and identification of metabolites. *J. Steroid Biochem.* **36** (1990) 407–414.
34. Massé R., Bi H., Ayotte C. and Dugal R.: Studies on anabolic steroids. II Gas chromatographic/mass spectrometric characterization of oxandrolone urinary metabolites in man. *Biomed. Environ. Mass Spectrom.* **18** (1989) 429–438.
35. Massé R., Ayotte C., Bi H. and Dugal R.: Studies on anabolic steroids. III Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography/mass spectrometry. *J. Chromat.* **497** (1989) 17–37.
36. DeBoer D., Gainza Bernal M. E., VanOoijen R. D. and Maes R. A. A.: The analysis of trenbolone and the human urinary metabolites of trenbolone acetate by gas chromatography/mass spectrometry and gas chromatography/tandem mass spectrometry. *Biol. Mass Spectrom.* **20** (1991) 459–466.
37. Schänzer W., Geyer H. and Donike M.: Metabolism of metandienone in man: identification and synthesis of conjugated excreted urinary metabolites, determination of excretion rates and gas chromatographic-mass spectrometric identification of bis-hydroxylated metabolites. *J. Steroid Biochem. Molec. Biol.* **38** (1991) 441–464.