Journal of Chromatography, 497 (1989) 17–37
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4981

STUDIES ON ANABOLIC STEROIDS

III. DETECTION AND CHARACTERIZATION OF STANOZOLOL URINARY METABOLITES IN HUMANS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY[a]

ROBERT MASSÉ*, CHRISTIANE AYOTTE, HONGGANG BI and ROBERT DUGAL

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

(First received May 17th, 1989; revised manuscript received July 28th, 1989)

SUMMARY

The metabolism of stanozolol (17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole), an androgenic–anabolic steroid widely used in sport for the purpose of enhancing performance, was investigated in humans. The analysis method was based on the use of solid-phase extraction on the Sep-Pak™ C_{18} cartridge, enzymic hydrolysis of steroid conjugates and high-resolution gas chromatograph–mass spectrometric (GC–MS) analysis of trimethylsilylated steroid extracts. After administration of a single 20-mg oral dose, twelve metabolites including unchanged stanozolol were recovered predominantly from the conjugated steroid fraction and characterized by GC–MS. In the unconjugated fraction, 16α-hydroxystanozolol, 17-epistanozolol, stanozolol and 3′-hydroxy-17-epistanozolol were the most abundant metabolites. In the aglycone fraction, 16α- and 16β-hydroxystanozolol, stanozolol and 3′-hydroxystanozolol were the most abundant metabolites. Other metabolites resulted from regioselective hydroxylation of stanozolol at C-4, whereas other were 17-epimers of 3′- and 16α-hydroxystanozolol. Further hydroxylation leading to the formation of four isomeric dihydroxylated metabolites was also observed. They were tentatively assigned the structures of 3′,16α-, 4β,16α-, 3′,16β- and 4β,16β-dihydroxystanozolol. The mass spectral features of their bis-N,O-trimethylsilyl derivatives obtained under electron-impact ionization are presented. The effect of pH on the relative recovery of some of these metabolites is also presented. The usefulness of this analytical methodology for the detection and identification of stanozolol urinary metabolites in doping-control situations is demonstrated. The metabolism of stanozolol is also discussed, and metabolic pathways accounting for the formation of its biotransformation products are proposed.

[a]For part II see ref. 13.

0378-4347/89/$03.50

INTRODUCTION

With the advent of sophisticated instrumental techniques and newer isolation and separation methods [1–3], remarkable progress has been achieved in the analysis and structural characterization of anabolic–androgenic steroid metabolites in humans [3]. Many of these steroids, which are of therapeutic interest, are also used in sports in order to improve athletic performance. The past decade has witnessed an accelerating growth in the use of anabolic steroids in sports, and this has necessitated the development of integrated analytical methods for the detection and characterization of their urinary metabolites [3–13].

A long-standing research programme in our laboratory has been based on the investigation of anabolic steroid metabolism in humans, and the development of gas chromatographic–mass spectrometric (GC–MS) methods for the detection of their urinary metabolites. Our studies have been extended to the challenging problems of the detection and characterization of stanozolol (17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole) urinary metabolites. The structure of this synthetic anabolic steroid [14], which bears a pyrazole moiety attached on its A-ring, differs from that of endogenous steroid hormones and most commercially available anabolic steroids. Consequently, it was anticipated that stanozolol biotransformation in humans would follow some metabolic pathways that are not commonly encountered in the metabolism of the latter steroids.

Although GC–MS of the N,O-bis-trimethylsilyl (N,O-bis-TMS) ether derivative of stanozolol has been reported, its analysis in biological fluids was nevertheless difficult to achieve, owing mainly to the fact that the derivative was thermally unstable on the packed chromatographic column used [8,15,16]. This analytical problem, and other chemical and methodological considerations [3,16,17], may account for the lack of information about the biotransformation and urinary excretion of stanozolol and its metabolites in humans. Conflicting data regarding the urinary excretion of stanozolol or some of its metabolites in humans were reported in previous studies. Whereas Lantto et al. [17] observed that unchanged stanozolol was mainly isolated from the conjugated steroid fraction, Schanzer and co-workers [18,19] recently reported the isolation of stanozolol metabolites hydroxylated at the C-3′ and C-4 positions from the free steroid fraction.

However, we recently reported that the use of fused-silica capillary column could greatly improve the GC analysis of stanozolol as its N,O-bis-TMS derivative. This enabled us to develop a specific and sensitive GC–MS screening method to detect and characterize this steroid in urine [3,4]. In developing our analytical strategy, careful consideration was given to extraction efficiency, enzymic hydrolysis, chemical derivatization and GC of stanozolol. This meth-

odology was further extended to the systematic investigation of stanozolol metabolism in humans.

This paper reports the detection of stanozolol and eleven urinary metabolites following administration of the steroid to humans. Attention was also given to the separation and identification of the free and conjugated metabolites. Their identity was assessed by comparison with authentic reference compounds, when available, and on the basis of the GC–MS features of their N,O-bis-TMS derivatives. The GC–MS data also illustrate that, in humans, the biotransformation of stanozolol is characterized by the formation of a diversity of mono- and dihydroxylated metabolites, most of which are excreted in urine in the form of conjugates. To the best of our knowledge, this is the first extensive report describing the major urinary metabolites of stanozolol in humans.

EXPERIMENTAL

Chemicals and reagents

Stanozolol and stanozolol tablets (Winstrol®) were obtained from Winthrop Labs. (Aurora, Canada), 3′-, 4β-, and 16β-hydroxystanozolol were kindly provided by Professor Manfred Donike (Deutsche Sporthochschule, Köln, F.R.G.) and 5α-androstan-17-one and β-glucuronidase type H-2 from *Helix pomatia* were purchased from Sigma (St. Louis, MO, U.S.A.). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Regis (Morton Grove, IL, U.S.A.) and dithioerythritol and trimethyliodosilane (TMSI) were from Aldrich (Milwaukee, WI, U.S.A.). Sep-Pak C_{18} cartridges were from Waters Assoc. (Milford, MA, U.S.A.).

Inorganic salts (J.T. Baker, Phillipsburg, NJ, U.S.A.) were of analytical grade. Anhydrous diethyl ether (reagent grade) was distilled in glass before use. Other solvents (HPLC grade, Caledon Labs., Georgetown, Canada) were used as provided.

Urine samples

Stanozolol metabolites were isolated from urine samples collected from healthy human volunteers to whom a single oral dose of 20 mg was administered. Urine samples were then collected for a period of seven days and stored at −20°C immediately after voiding. Urine specimens collected prior to stanozolol administration were used as blank samples. Stanozolol-positive urine samples collected during doping-control situations were used as reference specimens.

Steroid extraction

Free steroids. Urine (2 ml) was passed through a Sep-Pak C_{18} cartridge (previously washed with 5 ml of methanol and 5 ml of water) at a flow-rate of 20 ml/min. The cartridge was then washed with 5 ml of water. Steroids were

eluted with 5 ml of methanol. The solvent was evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 1.0 ml of 0.2 *M* acetate buffer (pH 5.2), and free steroids were extracted with 5 ml of diethyl ether containing 100 ng/ml 5α-androstan-17-one as external standard. After evaporation of the solvent, the residue was derivatized as described below and analysed by GC–MS.

Conjugated steroids. Any trace of residual solvent was removed from the acetate buffer solution by spraying a flow of nitrogen over the aqueous mixture at 40 °C. Then 100 μl of a crude β-glucuronidase/sulphatase enzymic preparation from *H. pomatia* (type 2) were added to the buffered urinary extract. The resulting mixture was incubated for 16 h at 37 °C and cooled at room temperature prior to the addition of ca. 100 mg of potassium carbonate. The steroids were extracted with 5 ml of diethyl ether containing 100 ng/ml 5α-androstan-17-one as external standard. The organic phase was decanted, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was then derivatized and analysed by GC–MS as described below. *H. pomatia* β-glucuronidase and sulphatase activities were assessed using testosterone glucuronide and testosterone sodium sulphate (Steraloids, Wilton, NH, U.S.A.). Hydrolysis yields were quantitative, as determined by GC–MS analysis of hydrolysed and unhydrolysed aglycone solutions.

Improved extraction method for stanozolol urinary metabolites

As mentioned above, stanozolol and its metabolites are structurally characterized by the presence of either a pyrazole or a 3′-hydroxylated pyrazole moiety, which accounts for their relative acidity or basicity in urine. Consequently, it was expected that, during elution of urine on the Sep-Pak C_{18} cartridge, some of these compounds would be partially retained or adsorbed owing to specific interaction with the silica core of the chromatographic phase. Higher recoveries of stanozolol metabolites were obtained when urine samples were processed as follows. Urine (2 ml) was eluted through the Sep-Pak C_{18} cartridge as mentioned above. Then, the cartridge was washed successively with 5 ml of 0.1 *M* ammonium hydroxide and 5 ml of water. The steroids were recovered by elution with 5 ml of methanol. After evaporation of the solvent, the residue was dissolved in 1 ml of 0.2 *M* acetate buffer, and the free steroid was extracted with diethyl ether. Overall recoveries of free steroids can be increased if the resulting aqueous phase is made alkaline with potassium carbonate (pH 11) and reextracted with 5 ml of diethyl ether. The two organic phases were combined, dried over anhydrous sodium sulphate and processed as described above prior to GC–MS analysis. Relative recoveries were determined by comparison of metabolite/external standard ratios measured by selected-ion monitoring (SIM) GC–MS analysis of derivatized steroid extracts obtained from both extraction methods. The molecular ions of the steroids of

interest, and the external standard ion at *m/z* 331 $[M-15]^+$, were monitored with a dwell time of 100 ms.

Derivatization of steroids

Both free and conjugated steroid fractions were trimethylsilylated as follows. To the dried residue were added 100 μl of MSTFA and 1 μl of a dichloromethane solution of TMSI and triethylamine (858:142:2, v/v). The resulting mixture was heated at 70°C for 30 min, and 1 μl was injected into the gas chromatograph for GC–MS analysis. The preparation of N,O-bis-TMS derivatives of stanozolol and its metabolites was quantitative using this method. Incomplete derivative formation can occur if the derivatization mixture is heated for a shorter time. Deuterated ($[^2H_9]$TMS) derivatives were also prepared using a mixture of N,O-bis($[^2H_9]$trimethylsilyl)acetamide (BSA-deuterated, MSD Isotope, Pointe-Claire, Canada), pyridine and trimethylchlorosilane (1:1:0.1, v/v). The mixture was heated at 70°C for 30 min, and the solvent was evaporated to dryness under a nitrogen stream at 40°C. The residue was then dissolved in 50 μl of hexane, and 1 μl was injected for GC–MS analysis.

Detection limits of stanozolol

Detection limits were determined using standard solutions and urine extracts fortified with stanozolol. The steroid was analysed as the N,O-bis-TMS derivative. Analyses were performed by SIM of ions at *m/z* 143, (dwell time 50 ms), 472, 457, 382 and 342 (dwell time 100 ms) under the GC–MS condition described below.

The detection limit of stanozolol determined from standard solutions was 50 pg (injected) with a signal-to-noise ratio of 2.5:1 for ion of *m/z* 472 ($M^{+\bullet}$). In fortified urinary extracts, the detection limit was estimated at 100 pg (injected) with a signal-to-noise ratio of 3.5:1 for ion of *m/z* 472 ($M^{+\bullet}$). A full mass spectrum of the stanozolol N,O-bis-TMS derivative was obtained from urinary extracts spiked with 100 ng/ml (or 1 ng injected) of stanozolol. Urinary extracts were obtained from a blank urine specimen previously hydrolysed and processed as described above. Stanozolol was added to the dried extract prior to derivatization. As the volume of the derivatization mixture was 100 μl, the actual amount of stanozolol added to the urinary extracts was 100-fold the detection limits reported for SIM and full scan GC–MS.

Gas chromatography–mass spectrometry

Steroid analysis was carried out using a HP-5970 mass-selective detector, (Hewlett-Packard, Palo Alto, CA, U.S.A.), equipped with a HP-5890 gas chromatograph fitted with an HP Ultra-5 (methyl 5% phenyl, silicone) fused-silica capillary column (25 m × 0.2 mm I.D., 0.33 μm film thickness). The injections were done in the splitless mode using helium as carrier gas at a flow-rate of 0.8

ml/min. the oven temperature was programmed from 100°C (1 min hold) at 16°C/min to 220°C and then at 3.8°C/min to 300°C (maintained for 10 min). The GC injector and GC–MS transfer line temperatures were set at 270 and 310°C, respectively.

The mass spectrometer was operated in the full-scan mode for semi-quantitation of stanozolol metabolites, and it was assumed that all metabolites had the same response factor to the mass spectrometer. Mass spectra of the N,O-bis-TMS derivatives of the steroid of interest were also recorded at a scanning speed of 1 s per decade over the mass range 50–700 a.m.u.

RESULTS AND DISCUSSION

Profiles of the free and conjugated metabolites

Typical profiles of the urinary metabolites of stanozolol obtained by GC–MS analysis of the free and conjugated steroid fractions are shown in Fig. 1. They demonstrate clearly that stanozolol and its metabolites are mainly excreted in urine as conjugates. Semi-quantitative data from other volunteer studies and stanozolol-positive urine samples provided by athletes in drug-testing situations [20] corroborated this finding, since stanozolol metabolites recovered in the free steroids fraction accounted for only 2–10% of the overall amount of urinary metabolites. The relative abundance and distribution of the metabolites in both steroid fractions will be discussed hereafter. From an analytical point of view, the total-ion current (TIC) chromatograms shown in

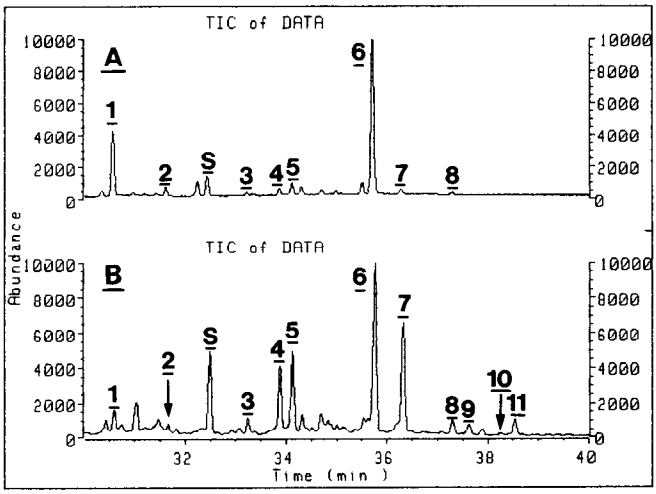

Fig. 1. Total ion current (TIC) chromatograms from (A) free and (B) conjugated steroid fractions obtained from a volunteer 15 h after the administration of a single 20-mg oral dose of stanozolol. The chromatograms are normalized with respect to the most abundant metabolite, **6**. Peaks as in Table I. Analytical conditions as in Experimental.

Fig. 1 also provided irrefutable evidence demonstrating that the methodology used here was successful in resolving the analytical problems encountered in previous studies [8,15–17,19,21]. The use of the Sep-Pak C_{18} cartridge [1–3] in place of Amberlite XAD-2 resin [15,16,19,21] and of organic solvent extraction [8,17] has contributed to the improvement in the recovery of stanozolol metabolites from urine, whereas the proper selection of derivatization and GC–MS conditions [3] were the determinating factors in the successful GC–MS analysis of such minute amounts of these steroids.

Characterization of urinary metabolites

Metabolites 1 and S. Fig. 2 shows typical ion chromatograms of the molecular ion of the N,O-bis-TMS derivative of stanozolol ($M^{+\bullet}$, 472) obtained from GC–MS analysis of the free and conjugated steroid fractions. The peak labelled S was identified as stanozolol by comparison of its mass spectrum with that of the TMS derivative of an authentic standard (Fig. 3B). The mass spectra of stanozolol and its urinary metabolites are summarized in Table I. The stanozolol mass spectrum is characterized by an intense molecular ion at *m/z* 472 and a prominent fragment ion of *m/z* 143 (Fig. 4) arising from D-ring cleavage [22]. A structurally informative fragment ion arising from cleavages of the bond from C-1 to C-10 and the bond from C-4 to C-5 with concomitant migration of two hydrogen atoms at C-1 and C-4 is observed at *m/z* 168 [18]. This latter ion is characteristic of stanozolol metabolites with no hydroxyl group either on the pyrazole moiety or the A-ring. Interestingly, the mass spectrum of compound **1** TMS derivative (Fig. 3A), eluting 1.9 min before stano-

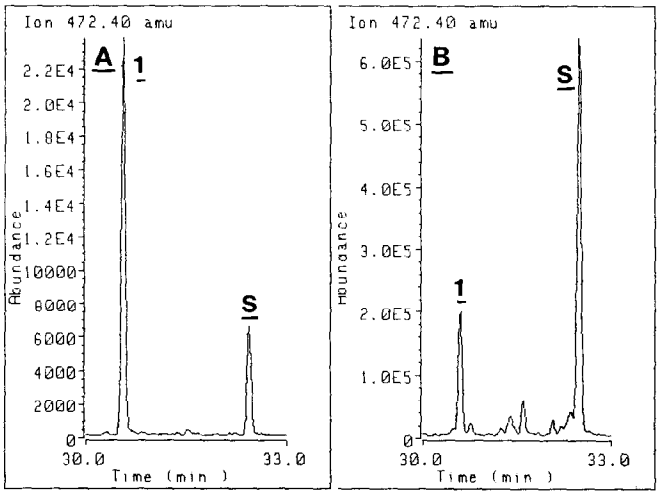

Fig. 2. Reconstructed ion chromatograms of *m/z* 472 obtained from GC–MS analysis of (A) free and (B) conjugated steroid fractions. Peaks: **1** = 17-epistanozolol; S = stanozolol. Note that the abundance scale is not identical in the two parts. Analytical conditions as in Fig. 1.

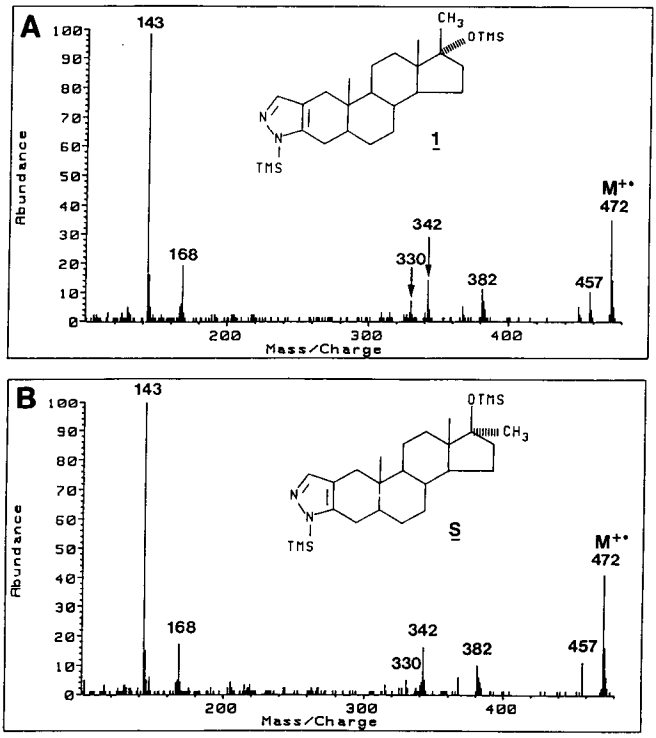

Fig. 3. Electron-impact (EI) mass spectra of the N,O-bis-TMS derivatives of (A) 17-epistanozolol (**1**) and (B) stanozolol (S) isolated from the free and conjugated steroid fractions.

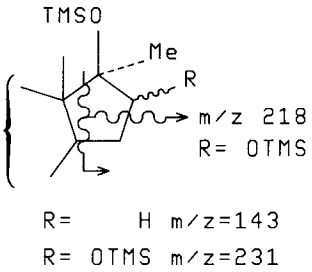

Fig. 4. Formation of ions of *m/z* 143, 218 and 231 from D-ring cleavage following electron-impact ionization.

zolol, was virtually identical with that of stanozolol (Fig. 3B). We know that some anabolic steroids bearing 17β-hydroxy and 17α-methyl groups are epimerized into the corresponding 17α-hydroxy-17β-methyl configuration in humans. This is especially the case for methanedienone [7,23], 4-chloromethanedienone [11] and oxandrolone [13]. We observed that the 17α-hydroxy-17β-methyl epimers of methanedienone, 6β-hydroxymethanedi-

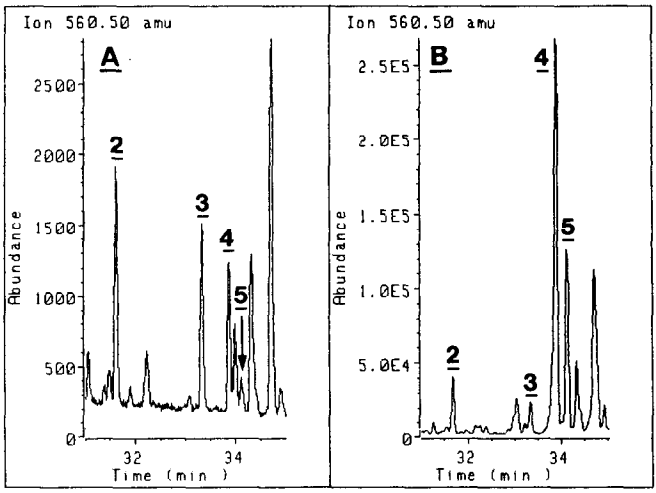

Fig. 5. Reconstructed ion chromatograms of *m/z* 560 obtained from GC–MS analysis of (A) free and (B) conjugated steroid fractions. Peaks: **2** = 3′-hydroxy-17-epistanozolol; **3** = 16α-hydroxy-17-epistanozolol; **4** = 3′-hydroxystanozolol; **5** = 4β-hydroxystanozolol. Mass spectral analysis indicates that unlabelled peaks are not stanozolol metabolites. Analytical conditions as in Fig. 1.

enone [3] and oxandrolone [13] elute in a range of ca. 1.8–2.5 min before their corresponding 17β-hydroxy-17α-methyl analogues, and that the mass spectra of their TMS derivatives are virtually identical. Compound **1** was thus identified as 17-epistanozolol on the basis of its specific GC–MS properties. To the best of our knowledge, this metabolite has never been reported previously.

*Metabolites **2**, **4** and **5***. GC–MS data were further investigated for the presence of monohydroxylated metabolites of stanozolol. Their molecular ions were expected to be increased by 88 a.m.u. with respect to that of stanozolol to *m/z* 560. The ion chromatograms of *m/z* 560 ($M^{+\cdot}$) (Figs. 5 and 6) indicated the possible presence of six isomeric monohydroxylated metabolites, which were mainly recovered from the conjugated fraction.

As illustrated in Figs. 7 and 8, the mass spectra of the TMS derivatives of compounds **2**, **4** and **5** showed striking similarities. Their common molecular ion at *m/z* 560 (shifted to *m/z* 587 in the mass spectra of their [$^2H_9$]TMS derivatives) and other fragment ions at *m/z* 545 $[M-15]^+$, 470$[M-90]^{+\cdot}$, 455 $[M-15-90]^+$ and 370 $[M-90-90]^{+\cdot}$ indicate that these metabolites arise from monohydroxylation of stanozolol. Compounds **4** and **5** were identified as 3′-hydroxystanozolol (3′ refers to the carbon atom of the pyrazole ring) and 4β-hydroxystanozolol by comparison of their GC–MS properties with those of the TMS derivatives of authentic reference compounds. Interestingly, the GC–MS features of compound **2** (Fig. 7 and Table I), which are consistent with the general structure of compound **4**, also suggest that the C-17 substituents were epimerized as in compound **1** (Figs. 2 and 3). Careful examination

TABLE I

PARTIAL MASS SPECTRA OF THE N,O-BIS-TMS DERIVATIVES OF STANOZOLOL URINARY METABOLITES

Peak No.	Metabolite	RRT[a]	$M^{+\bullet}$[b]	Major ions[c]						
1	17-Epistanozolol	0.94	472(35)	457(9)	382(10)	367(7)	342(13)	330(8)	168(19)	143(100)
2	3′-Hydroxy-17-epistanozolol	0.97	560(35)	545(51)	470(10)	455(21)	416(12)	254(89)	143(100)	
S	Stanozolol	1.00	472(42)	457(11)	382(10)	367(6)	342(14)	330(5)	168(17)	143(100)
3	16α-Hydroxy-17-epistanozolol	1.02	560(26)	545(4)	470(13)	455(6)	380(11)	365(6)	353(13)	231(56)
				218(100)	168(14)	147(15)				
4	3′-Hydroxystanozolol	1.04	560(22)	545(32)	470(6)	455(6)	416(13)	254(76)	143(100)	
5	4β-Hydoxystanzolol	1.05	560(42)	545(31)	471(10)	470(9)	455(6)	380(14)	254(64)	143(100)
6	16α-Hydroxystanozolol	1.10	560(50)	545(8)	470(18)	455(5)	445(11)	444(10)	353(19)	328(14)
				231(69)	218(100)	168(15)	147(20)			
7	16β-Hydroxystanozolol	1.12	560(34)	545(6)	470(13)	455(5)	445(8)	444(7)	381(20)	380(10)
				353(17)	328(10)	231(57)	218(100)	168(16)	147(43)	
8	3′,16α-Dihydroxystanozolol	1.15	648(19)	633(4)	558(8)	543(6)	468(6)	453(3)	379(8)	378(6)
				254(9)	231(42)	218(100)	147(25)	117(23)		
9	4β,16α-Dihydroxystanozolol	1.16	648(23)	633(5)	558(7)	543(3)	468(6)	453(3)	379(10)	378(5)
				254(18)	231(35)	218(100)	147(8)	117(25)		
10	3′,16β-Dihydroxystanozolol	1.18	648(17)	633(3)	558(5)	543(4)	468(7)	453(4)	379(5)	378(5)
				254(12)	231(42)	218(100)	147(8)	117(25)		
11	4β,16β-Dihydroxystanozolol	1.19	648(16)	633(2)	558(9)	543(2)	468(11)	453(3)	379(4)	378(4)
				254(26)	231(32)	218(82)	147(5)	73(100)		

[a]Retention time relative to that of stanozolol N,O-bis-TMS derivative (t_R = 32.43 min).

[b]Molecular ion m/z values.

[c]Values in parentheses refer to relative intensity given in terms of % base peak. Ion abundances have not been corrected for ^{13}C or ^{29}Si isotopic contribution.

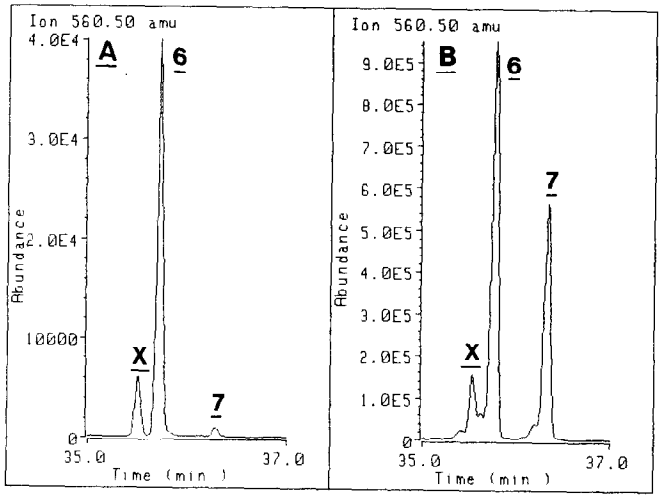

Fig. 6. Reconstructed ion chromatograms of *m/z* 560 obtained from GC-MS analysis of (A) free and (B) conjugated steroid fractions. Peaks: **6** = 16α-hydroxystanozolol; **7** = 16β-hydroxystanozolol. Peak X seems to be a metabolite of stanozolol but could not be identified. Analytical conditions as in Fig. 1.

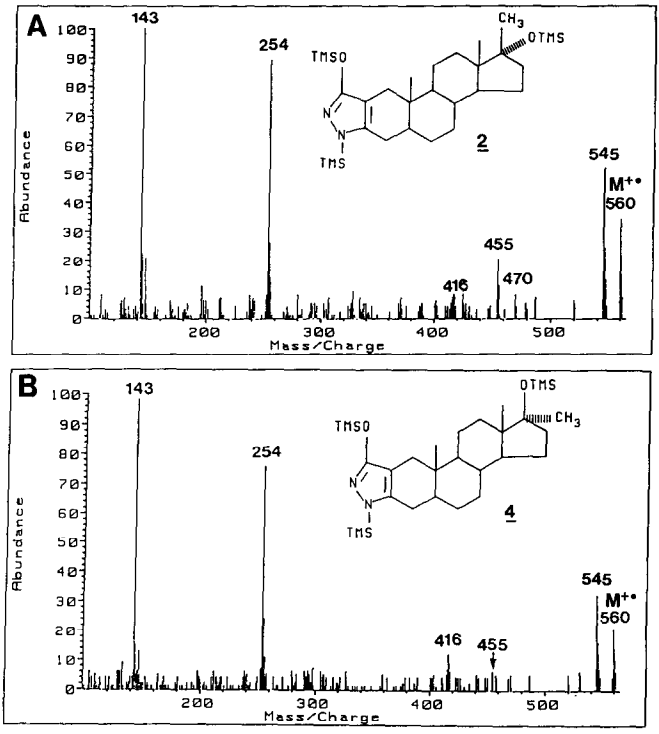

Fig. 7. Electron-impact mass spectra of (A) 3′-hydroxy-17-epistanozolol (**2**) and (B) 3′-hydroxystanozolol (**4**) as their N,O-bis-TMS derivatives.

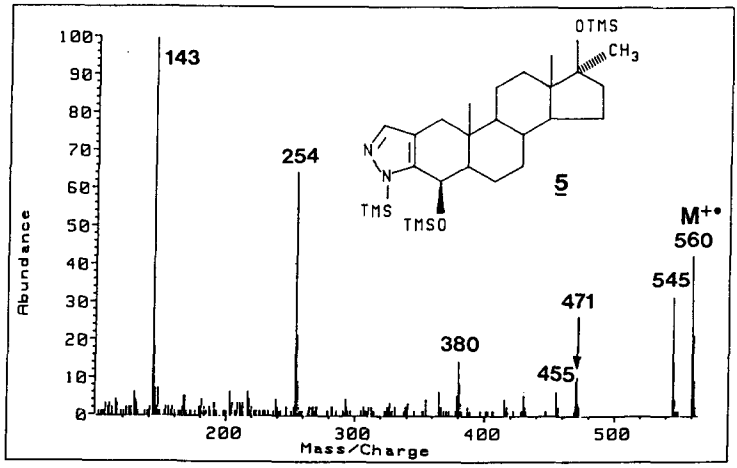

Fig. 8. Electron-impact mass spectrum of 4β-hydroxystanozolol (**5**) as its N,O-bis-TMS derivative

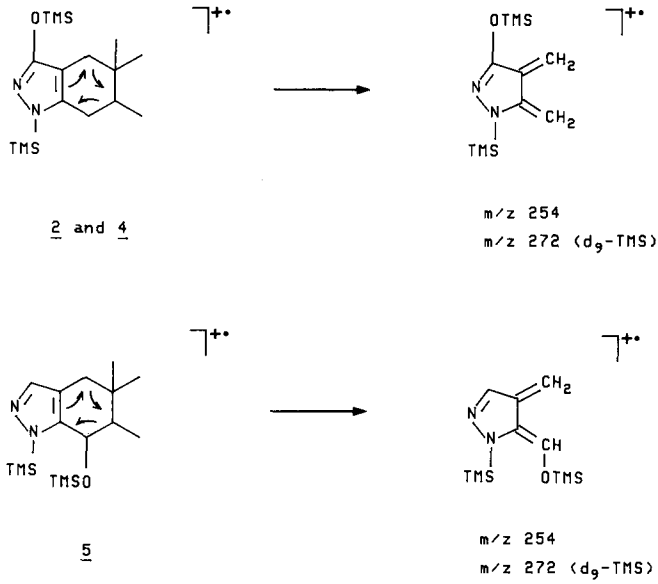

Fig. 9. Formation of ion of *m/z* 254 from A-ring retro Diels-Alder fragmentation following electron-impact ionization of the TMS derivatives of compounds **2**, **4**, **5** and **8**–**11**.

of the mass spectra of **2**, **4** and **5** indicates that compounds **2** and **4** possess similar mass spectral features, which differ somewhat from that of 4β-hydroxystanozolol (**5**), in which the ions at *m/z* 380 $[M-90-90]^{+\bullet}$ and 470 $[M-90]^{+\bullet}$ are relatively more abundant. Accordingly, we propose that com-

pound **2**, which eluted 2.1 min before its analogue **4**, has the structure of 3′-hydroxy-17-epistanozolol.

The mass spectra of the TMS derivatives of compounds **2**, **4** and **5** also provided additional information regarding their respective sites of hydroxylation. Indeed, their mass spectra exhibited a prominent fragment ion at *m/z* 254, which was shifted by 18 a.m.u. to *m/z* 272 in the mass spectra of their corresponding [$^2H_9$]TMS derivatives. This is a clear indication that this ion bears two TMS groups, one being located on the pyrazole ring (N-TMS) whereas the second one is attached to the newly introduced hydroxyl group. It is very probable that the latter ion results from a retro Diels–Alder fragmentation with concomitant cleavage of the bond from C-1 to C-10 and the bond from C-4 to C-5 (Fig. 9).

Here, the fragmentation of the A-ring may be attributed to the presence of the pyrazole moiety, which can exist in several tautomeric forms. The location of one of the pyrazole double bonds from C-2 to C-3 in A-ring (Fig. 9) induces the retro Diels–Alder fragmentation leading to the formation of an ion at *m/z* 254. It is worth noting that Schanzer et al. [18] have isolated and characterized three tautomeric forms of 3′-hydroxystanozolol (**4**) as their N,O- and N,N-bismethyl derivatives. However, these tautomeric forms could not be detected when compound **4** was transformed into its N,O-bis-TMS derivative. This observation can be rationalized on the basis of the probable co-elution of the tautomeric TMS derivatives as a single chromatographic peak or, alternatively, by the possibility that only one tautomeric form of compound **4** was formed under the derivatization conditions used.

*Metabolites **3**, **6** and **7**.* Three other metabolites eluting as well resolved chromatographic peaks (Figs. 5 and 6) and exhibiting a common molecular ion at *m/z* 560 and similar mass spectral features (Fig. 10) were detected, mainly in the conjugated steroid fraction following stanozolol administration. The compound with the longest retention time was identified as 16β-hydroxystanozolol by comparison with an authentic reference compound. The mass spectrum of its TMS derivative is dominated by two prominent ions at *m/z* 218 and 231, arising from D-ring cleavage as shown in Fig. 4. These ions are formed by fission of the bond from C-13 to C-17 and/or that from C-15 to C-16 and/or that from C-14 to C-15. The shift of these ions by 18 a.m.u. to *m/z* 236 and 249, respectively, in the mass spectrum of the corresponding [$^2H_9$]TMS derivative provided further evidence supporting their proposed structures and mechanisms of formation. The ion at *m/z* 231 is produced by the same mechanism that gives rise to the ion at *m/z* 143 in the mass spectra of the TMS derivatives of 17-hydroxy-17-methyl steroids [22,24].

The ions at *m/z* 218 and 231, which are highly characteristic of 16-hydroxylated analogues of the latter steroids [3,13] are also observed in the mass spectra of compounds **3** and **6**. It was thus reasonable to propose that compound **6** has the structure of 16α-hydroxystanozolol whereas compound **3**,

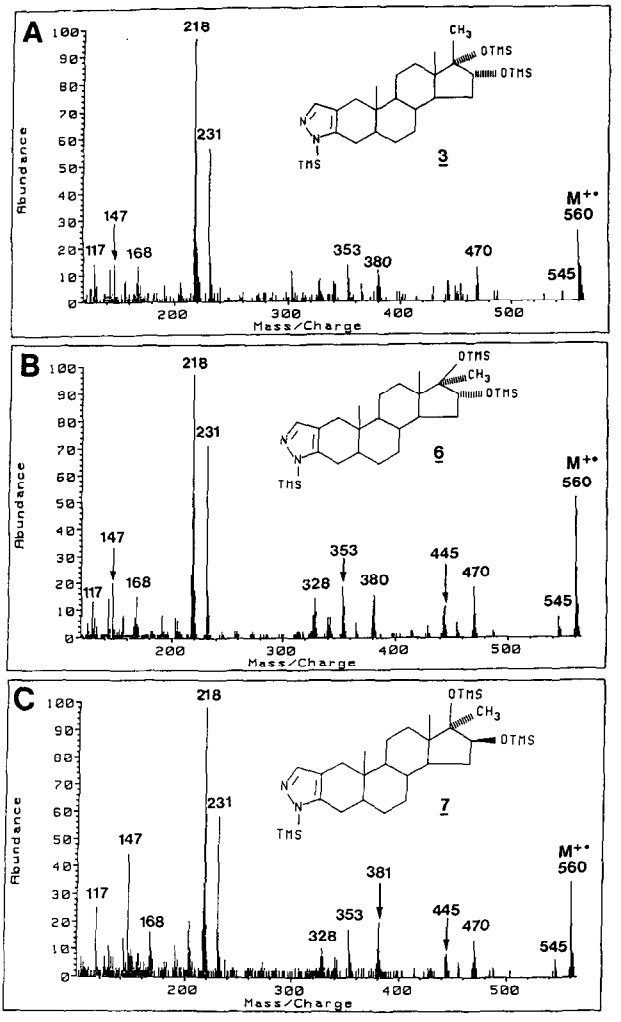

Fig. 10. Electron-impact mass spectra of (A) 16α-hydroxy-17-epistanozolol (**3**), (B) 16α-hydroxystanozolol (**6**), and (C) 16β-hydroxystanozolol (**7**) as their N,O-bis-TMS derivatives.

which elutes 2.5 min faster than **6**, has probably the structure of 16α-hydroxy-17-epistanozolol. The structure assignment proposed for compound **3** is in agreement with that previously proposed for the epimeric pairs **1** and S, and **2** and **4**. Structure assignment of compound **6** was facilitated by the fact that a reference standard of 16β-hydroxystanozolol (**7**) was available to us. Further analytical evidence was provided by the chromatographic retention of **6**, which eluted before its 16β-hydroxy analogue (**7**). This seems to be a general feature

of the isomeric 16-hydroxy derivatives of anabolic steroids bearing 17β-hydroxy and 17α-methyl groups [24].

Metabolites 8, 9, 10 and 11. In addition to the monohydroxylated metabolites **2–7**, a series of four metabolites resulting from dihydroxylation of stanozolol were detected by monitoring of their common molecular ion at *m/z* 648 (Fig. 11). It is worth noting that, among these four metabolites, only **8** and trace amounts of **9** were detected in the free steroid fraction, whereas more than 98% of the total amount of compounds **8–11** was recovered from the conjugated steroid fraction. Investigation of their mass spectra (Figs. 12 and 13 and Table I) suggested that these compounds originated from the same regiospecific hydroxylations of stanozolol that previously led to the formation of their monohdyroxylated analogues **2–7**. Indeed, their mass spectra were dominated by an intense and characteristic ionic doublet at *m/z* 218 and 231 (Fig. 4), indicating that compounds **8–11** bear a 16-hydroxy group. Most interesting was the presence of a diagnostically important ion at *m/z* 254, which strongly suggests that these metabolites also possess a second hydroxyl group at the C-3′ or C-4 position.

Although no authentic reference compound with hydroxyl groups at C-16 and C-3′ or C-4 was available, the probable structure of compounds **8–11** was tentatively assigned on the basis of their relative chromatographic retention with respect to that of their monohydroxylated analogues **2–7**. The GC–MS data presented above showed that metabolites with a 3′- or a 16α-hydroxyl

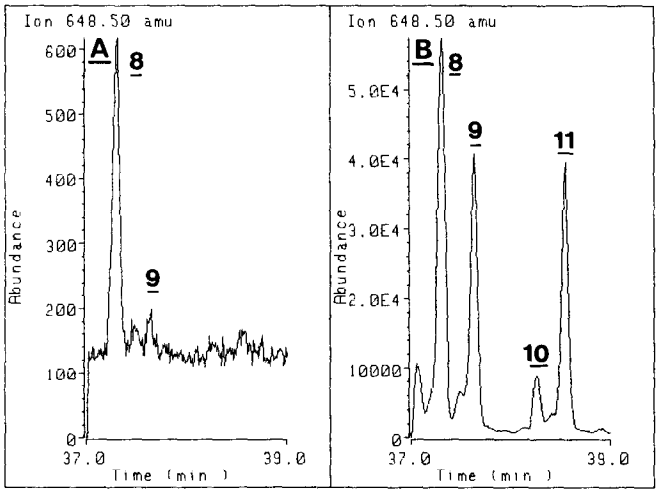

Fig. 11. Reconstructed ion chromatogram of *m/z* 648 obtained from GC-MS analysis of (A) free and (B) conjugated steroid fractions. Structures were tentatively assigned to the labelled peaks as follows: **8** = 3′,16α-dihydroxystanozolol; **9** = 4β,16α-dihydroxystanozolol; **10** = 3′,16β-dihydroxystanozolol; **11** = 4β,16β-dihydroxystanozolol. Analytical conditions as in Fig. 1.

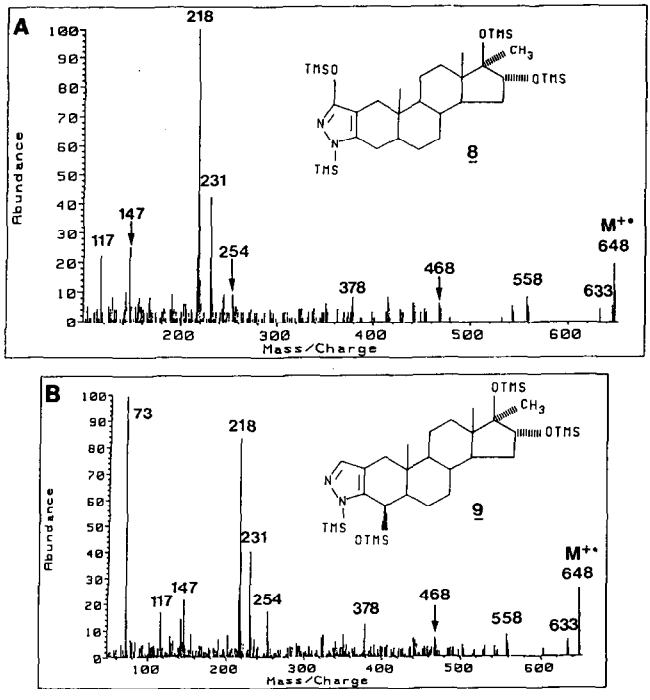

Fig. 12. Electron-impact mass spectra of (A) 3′,16α-dihydroxystanozolol (**8**) and (B) 4β,16α dihydroxystanozolol (**9**) as their N,O-bis-TMS derivatives.

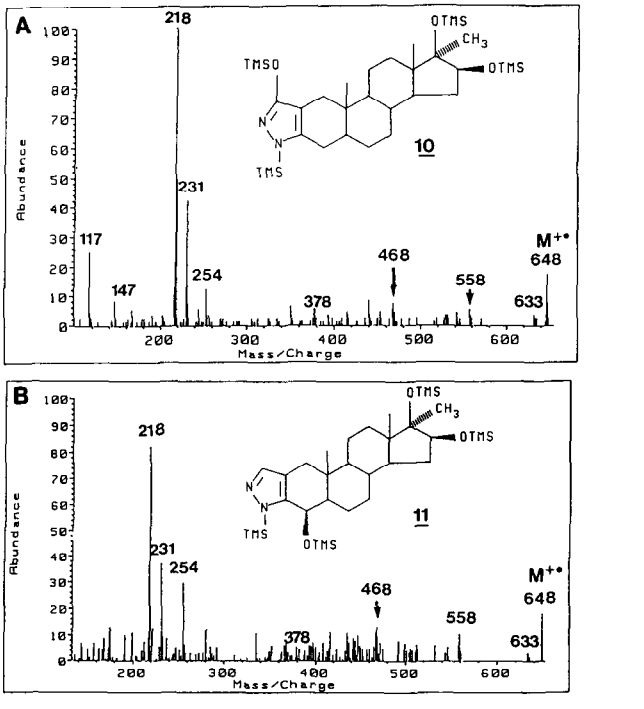

Fig. 13. Electron-impact mass spectra of (A) 3′,16β-dihydroxystanozolol (**10**) and (B) 4β,16β-dihydroxystanozolol (**11**) as the N,O-bis-TMS derivatives.

group elute before their 4β- and 16β-hydroxylated analogues, respectively (Table I).

Interestingly, compound **8** elutes before and is relatively more abundant than its analogues **9–11** in the free steroid fraction. This led us to assume that this metabolite probably has the structure of 3′,16α-dihydroxystanozolol, since its mass spectral features and chromatographic retention are in accordance with those of compounds **4** and **6**, which bear a hydroxyl group at the C-3′ and C-16 position, respectively. This structural assignment was further supported by the characteristic fragmentation of the molecular ion at *m/z* 648 ($M^{+\cdot}$ 684, [$^2H_9$]TMS), which give rise to a series of fragment ions at *m/z* 633 $(M-15)^+$, 558 $(M-90)^{+\cdot}$, 468 $(M-2\times90)^{+\cdot}$ and 378 $(M-3\times90)^{+\cdot}$.

Comparison of the retention times of compounds **8–11** shows that they can be grouped into two distinct isomeric pairs: **8** and **9**, and **10** and **11**. The retention time difference between **8** and **9**, and between **10** and **11**, was 0.33 min in both cases, whereas that between **8** and **10**, and between **9** and **11**, was 0.96 min. In addition, the shorter retention time increment (0.33 min) observed between **8** and **9**, and between **10** and **11** can be associated with the retention time difference of 0.22 min observed between 3′- and 4β-hydroxystanozolol (**4** and **5**) (Fig. 5). This observation was further supported by the fact that 16α- and 16β-hydroxystanozolol (**6** and **7**) were resolved by a retention time increment of 0.58 min, which is about three times that observed between compounds **4** and **5**. Interestingly, these retention time differences are of the same magnitude as those measured between compounds **8–11**. These data indicate that the hydroxy group substitution patterns in compounds **8–11** are very similar and originate from a series of regio- and stereoselective hydroxylations of stanozolol at the C-3′, C-4 and C-16 positions. This hypothesis is further evidenced by the characteristic mass spectral properties of their TMS derivatives (Figs. 12 and 13 and Table I), which reflect the major structural features of 3′-, 4β-, 16α- and 16β-hydroxystanozolol (**4–7**) (Figs. 7, 8 and 10).

Taking into account these chromatographic properties and mass spectral features of compounds **8–11**, compounds **9**, **10** and **11** were tentatively assigned the structures of 4β,16α-hydroxystanozolol, 3′,16β-dihydroxystanozolol and 4β,16β-dihydroxystanozolol, respectively.

Biotransformation and urinary excretion of stanozolol metabolites

The proposed structures of stanozolol metabolites illustrated in Fig. 14 show that the steroid is hydroxylated with a high degree of regioselectivity, since only the C-3′, C-4 and C-16 positions were hydroxylated.

Interestingly, the presence of hydroxyl and methyl groups in the D-ring and of a pyrazole moiety attached to A-ring exerts a dominant directing influence, which results in the introduction of hydroxyl functions in the latter steroidal rings (Fig. 14). It appears also that pyrazole ring inhibits hydroxylation at C-

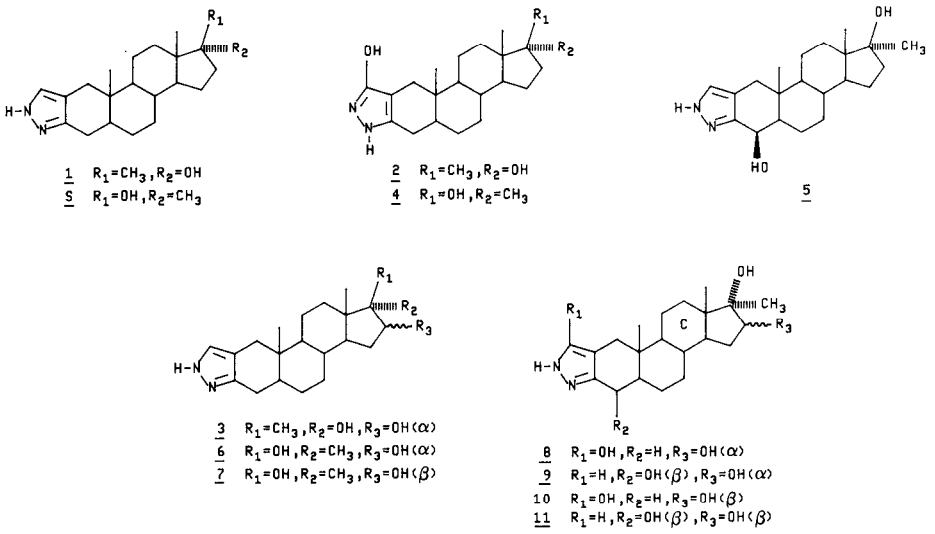

Fig. 14. Proposed structures of stanozolol metabolites in humans.

6, since several steroids with 17β-hydroxy-17α-methyl substituents and a Δ^4-3-one group are hydroxylated at C-6 in humans [3,7,11,20,23].

In addition, the data indicate that a specific relationship may exist between the relative positions of the substituents and the site of the hydroxylation reactions. It was reported by several authors that in mammals [25] and microorganisms [26] steroid hydroxylation occurs at a position ca. 5.5–7.5 Å away from an electron-rich substituent. This suggests that stanozolol is attached by a chelation-type interaction with the heme moiety of the P-450 cytochromes. We may speculate that when the electron-rich pyrazole moiety is the anchor point, the stanozolol molecule would lie over the plane of the porphyrin ring so that C-16 can be readily hydroxylated. Conversely, if the 17-hydroxyl group serves as the point of attachment, then the C-3′ and C-4 would be hydroxylated. Since hydroxylation at the 16α and 16β positions is the major biotransformation route (Table II), we may assume that the pyrazole moiety markedly enhances the interaction of stanozolol with ferric cytochrome P-450, probably through coordination with the propionic acid residue of the heme moiety. This proposed mechanism of stanozolol hydroxylation at C-16 is supported by a study recently reported by Rendic and Ruf [27]. These authors demonstrated that stanozolol interacts with cytochrome P-450 from rat liver microsomes by forming a high-affinity ligand complex involving the pyrazole moiety as the coordinating ligand. This observation is in accordance with the metabolic profile of stanozolol presented above, where hydroxylation occurs regioselectively at the C-16 position. The α-epimer is formed normally, although in some subjects 16β-hydroxylation appears to be the major route. This observation could

TABLE II

RELATIVE RECOVERY OF STANOZOLOL AND SOME OF ITS METABOLITES IN DIFFERENT pH CONDITIONS

Steroid	Relative recovery[a] (%)			
	pH 5+pH 11	pH 5	pH 11	pH 9
Stanozolol (S)	100	63	61	56
3′-Hydroxystanozolol (**4**)	100	78	50	68
4β-Hydroxystanozolol (**5**)	100	77	102	83
16α-Hydroxystanozolol (**6**)	100	100	101	83
16β-Hydroxystanozolol (**7**)	100	102	97	88
3′,16α-Dihydroxystanozolol (**8**)[b]	100	52	58	68
4β,16α-Dihydroxystanozolol (**9**)[b]	100	84	73	81

[a]Optimal recoveries (100%) were obtained by combining the organic extracts from hydrolysis carried out at pH 5 and 11 (see Experimental for conditions). Recoveries obtained from a single extraction of the hydrolysates at pH 5, 9 or 11 are expressed as a percentages of the optimal recovery. Data are semi-quantitative since authentic reference compounds were not available for all metabolites. The numbers are the mean of triplicate measurement of three urinary aliquots. Standard deviations ranged from 2.5 to 6.8%. Peak areas were integrated from molecular ion chromatograms obtained by SIM GC–MS analysis, with respect to the peak area of the ion m/z 331 $(M-15)^+$ of 5α-androstan-17-one (external standard). See Experimental for further details.
[b]Proposed structure.

not be rationalized on the basis of environmental factors, such as smoking, diet or drug use prior to stanozolol administration.

Although epimerization at C-17 is a reaction specific to several 17β-hydroxy-17α-methyl steroids [7,11,13,20], 17-epistanozolol (**1**) and 3′-hydroxy-17-epistanozolol (**2**) were detected 5 and 8 h, respectively, after stanozolol administration. However, their rates of excretion and formation vary greatly from one subject to another, so that in some cases, they are excreted in urine only 21–24 h after administration. Likewise 16α-hydroxy-17-epistanozolol (**3**), which is excreted in minute amounts, was not detected in the urine of some volunteers. This further illustrates that stanozolol metabolism in humans is subject to inter-individual variation although the major routes of hydroxylation are the same for all the subjects investigated in this study.

Detection limit of stanozolol

Although it has been reported [17] that the detection of stanozolol in urine was possible only if the drug was administered in very large doses, we demonstrated (Fig. 1) that this steroid and its metabolites can be detected with high sensitivity and specificity even if a low amount of the steroid is administered. Indeed, stanozolol can be detected by SIM GC–MS at a level as low as 1 ng/ml (ca. 50 pg injected) with a signal-to-noise ratio of 2.5:1 using a standard solution of the steroid. In urine, the detection limit was 2 ng/ml (ca. 100 pg

injected) with a signal-to-noise ratio of 3.5:1. This methodology presents undoubted advantages for the detection and the positive identification of stanozolol in the context of the control of drug abuse in athletics [3].

Extraction of stanozolol metabolites

Contrary to the majority of neutral endogeneous and synthetic anabolic steroids, whose extraction from urine is only slightly if at all, affected by the pH, the recovery of stanozolol is likely to be influenced by acidity of the extraction conditions. As shown in Table II, the best relative recoveries are obtained when both pH 5 and 11 extracts are combined.

Although the recoveries of the 16-hydroxylated metabolites **6** and **7** are barely affected at pH 5 and pH 11, that of compound **4** was reduced to 50% of the optimum relative recovery when it was extracted at pH 11. This can be rationalized on the basis that 3′-hydroxystanozolol is slightly acidic owing to the tautomerism of the pyrazole ring [18]. Similarly, compound **8**, which is proposed to carry a hydroxyl group at the C-3′ position, was also extracted with relatively low recoveries at pH 5 and 11. This provides indirect support for our tentative structural assignment for compound **8**.

We also investigated the effect of pH on the recoveries of stanozolol metabolites using the Sep-Pak C_{18} cartridge. Interestingly, recoveries were improved, by 4% for stanozolol to 26% for 4β,16α-dihydroxystanozolol (**8**) when the cartridge was washed with 2 ml of 0.1 *M* ammonium hydroxide after the elution of the urine sample or prior to the elution of the steroids from the cartridge with methanol.

When judiciously used either in combination or separately, these extraction methods were very useful for the specific detection of low amounts of stanozolol metabolites in urine, particularly in drug-testing situations for the purpose of their identification.

ACKNOWLEDGEMENTS

Financial support from the Sports Medicine Council of Canada, The Natural Sciences and Engineering Research Council of Canada (Grant GP-1310) and the National Collegiate Athletic Association is gratefully acknowledged. The authors are grateful to Professor Manfred Donike for the generous gift of 3′-, 4β- and 16β-hydroxystanozolol, to Mrs. Pauline Fournier and Mr. Alain Charlebois for technical assistance and to Mrs. Francine Sauvageau for typing the manuscript and Mrs. Diane Lacoste for drawings.

REFERENCES

1 C.H.L. Shackleton, J. Chromatogr., 379 (1986) 91.
2 J. Sjovall and M. Axelson, Vitam. Horm., 39 (1982) 31.

3 R. Massé, C. Ayotte and R. Dugal, J. Chromatogr., 489 (1989) 23.
4 R. Massé, C. Ayotte and R. Dugal, in G. Piemonte, F. Tagliaro, M. Marigo and A. Frigerio (Editors), Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences, Plenum, New York, 1987, pp. 183–190.
5 R.J. Ward, A.M. Lawson and C.H.L. Shackleton, J. Steroid Biochem., 8 (1977) 1057.
6 C.J.W. Brooks, A.R. Thawley, P. Rocher, B.S. Middleditch, G.M. Anthony and W.G. Stillwell, J. Chromatogr., Sci., 9 (1977) 35.
7 H.W. Durbeck, I. Buker, B. Scheulen and B. Telin, J. Chromatogr., 167 (1978) 117.
8 M. Bertrand, R. Massé and R. Dugal, Belg. Farm. Tijdschr., 554 (1978) 55.
9 I. Bjorkhem and H. Ek, J. Chromatogr., 17 (1982) 447.
10 I. Bjorkhem and H. Ek, J. Steroid Biochem., 18 (1983) 481.
11 H.W. Durbeck, I. Buker, B. Scheulen and B. Telin, J. Chromatogr. Sci., 21 (1983) 405.
12 R. Massé, C. Laliberté, L. Tremblay and R. Dugal, Biomed. Mass Spectrom., 12 (1985) 115.
13 R. Massé, H. Bi, C. Ayotte and R. Dugal, Biomed. Environ. Mass Spectrom., 18 (1989) 429.
14 R.O. Clinton, A.J. Manson, F.W. Stonner, A.L. Beyler, G.O. Potts and A. Arnold, J. Am. Chem. Soc., 81 (1959) 1513.
15 R.J. Ward, C.H.L. Shackleton and A.M. Lawson, Br. J. Sports Med., 9 (1975) 93.
16 R.J. Ward, A.M. Lawson and C.H.L. Shackleton, in A. Frigerio and E.L. Ghisalberti (Editors), Proceedings of the International Symposium in Mass Spectrometry in Drug Metabolism, Milan, 1976, Plenum, New York, 1977, p. 465.
17 O. Lantto, I. Bjorkhem, H. Ek and D. Johnston, J. Steroid Biochem., 14 (1981) 721.
18 W. Schanzer, G. Opferman and M. Donike, in Proceedings of the 36th American Society for Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, San Francisco, CA, June 5–10, 1988, pp. 397–398.
19 M. Donike, J. Zimmermann, K.R. Barwald, W. Schanzer, V. Christ, K. Klostermann and G. Opfermann, Dtsch. Z. Sport Med., 35 (1984) 14.
20 R. Massé, C. Ayotte and R. Dugal, unpublished results.
21 C.K. Hatton and D.H. Catlin, Clin. Lab. Med., 7 (1987) 655.
22 D.C. Mammamoto and G.A. Eadon, J. Org. Chem., 40 (1975) 1784.
23 B.S. Macdonald, P.J. Sykes, P.M. Adhikary and R.A. Harkness, Steroids, 18 (1971) 753.
24 B.S. Middleditch, P. Vouros and C.J.W. Brooks, J. Pharm. Pharmacol., 25 (1973) 143.
25 R.W. Estabrook, G. Martinez-Zedillo, S. Young, J.A. Peterson and J. McCarthy, J. Steroid Biochem., 6 (1975) 419.
26 H.L. Holland, Chem. Soc. Rev., 11 (1982) 371.
27 S. Rendic and H.H. Ruf, Biochem. Pharmacol., 37 (1988) 766.