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# STUDIES ON ANABOLIC STEROIDS

# I. INTEGRATED METHODOLOGICAL APPROACH TO THE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF ANABOLIC STEROID METABOLITES IN URINE

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## SUMMARY

The analytical and methodological imperatives for large-scale and routine gas chromatographic–mass spectrometric screening of anabolic steroid urinary metabolites are described. Several aspects of their isolation, enzymatic hydrolysis, derivatization and metabolism in humans are discussed. Gas chromatographic–mass spectrometric data illustrating artifacts arising from enzymatic hydrolysis of 3$\beta$-ol-5-en steroids, and describing new metabolites of boldenone, methanedienone and stanozolol, as well as the conversion of norethisterone into 19-nortestosterone metabolites through de-ethylation at C-17, are presented. The analytical approach developed for gas chromatographic–mass spectrometric screening of anabolic steroids is based on the sequential selection-ion monitoring of specific and discrete ion groups characteristic to the steroids of interest under high-resolution chromatographic conditions. The major analytical and methodological requirements necessary to provide irrefutable evidence, in the case where the presence of a synthetic anabolic steroid or a testosterone to epitestosterone ratio higher than 6:1 is suspected in a given urine specimen, are also discussed.

## INTRODUCTION

The use of anabolic steroids in athletics as adjuvants to improve strength and performance was first reported in the 1950s [1]. Since then, these drugs have become increasingly popular among athletes, to such an extent that their misuse has apparently reached epidemic proportion in some sporting disciplines.

Concerned by the prospects of adverse health effects and on the basis of sport ethics, the International Olympic Committee (IOC) has categorically prohibited the use of anabolic steroids in athletics [2]. Conversely, the scientific community has devoted considerable research efforts to document the biological functions and mechanisms of action of anabolic steroids, as well as their physical, physiological and psychological consequences and adverse effects, and their efficiency

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in increasing athletic performance. These topics have been reviewed by several authors [3–9] and will not be further discussed here.

The ban of anabolic steroid misuse in the athletic community has prompted several workers to develop analytical methodologies which would allow a large number of samples to be reliably and economically screened in relatively short periods of time. In the late 1960s and early 1970s, the combined technique of gas chromatography–mass spectrometry (GC–MS), which was making use of packed columns, did not encompass, at that time, the analytical requirements for large-scale and routine analysis of anabolic and endogenous steroid urinary metabolites, namely (a) chromatographic resolution and separation and (b) specific detection and quantitation. Brooks et al. [10] and Lawson and Brooks [11] reported the first GC–MS investigation with some anabolic steroids, and Ward et al. [12] proposed a GC–MS method based mainly on single-ion monitoring and repetitive scanning for the detection of nandrolone, methanedienone, norethandrolone and orabolin urinary metabolites in humans. Shortly after, we reported the first integrated electron ionization–chemical ionization (EI–CI) GC–MS method for anabolic steroid screening using the selected-ion monitoring (SIM) mode, whereby several groups of two or three specific ions were recorded and changed during the analysis according to the retention times of the steroids of interest [13].

At that time radioimmunoassay (RIA) was the sole practical method available to analyse for several anabolic steroids in a large number of samples. This analytical approach was used at the 1976 Montreal Olympic Games where assays developed by Brooks et al. [14] and further evaluated in our laboratory [15] were implemented for the screening of 17$\alpha$-methyl, 17$\alpha$-ethyl and 19-nor anabolic steroids. In addition, GC–MS was also used to validate the specificity of the immunoassays in cases where positive results were obtained after preliminary RIA screening [13].

The introduction of glass capillary columns with superior resolving power, combined with the development of more sophisticated data systems, has increased the sensitivity and specificity of steroid detection and quantitation as well as the capacity of GC–MS systems to perform large-scale steroid analysis under routine conditions. Likewise, the remarkable progress which was achieved in the area of GC and MS in the last fifteen years [16–18] was paralleled by significant advances in the analysis of steroids in biological materials [19–21]. These analytical advances have also been beneficial in the area of anabolic steroids analysis. Several studies describing the detection, quantitation, identification and/or MS features of urinary metabolites of some anabolic steroids were reported in the last decade [22–32]. Although a few authors [33–35] have presented GC–MS methodologies for the routine screening of anabolic steroids in humans, no comprehensive study describing the analytical, metabolic and methodological imperatives and requirements for their selective detection in urinary samples has been reported. We recently presented a preliminary report in which some of these topics were briefly discussed [36].

The purposes of this paper are: (a) to report the major aspects of the general analytical approach to anabolic steroid isolation and detection in urine developed

in our laboratory; (b) to survey some of the analytical problems encountered in GC–MS routine analysis of anabolic steroids in athletics; (c) to review some of the features of their metabolism in humans; and (d) to show that a reliable, reproducible and steroid-specific analytical system can be set up through the proper integration of relatively simple extraction, purification and derivatization procedures with high-resolution chromatographic separation and selective MS detection.

## EXPERIMENTAL

### *Chemicals, glassware and solvents*

All glassware was silanized with a solution of dimethyldichlorosilane in toluene before use. Inorganic salts and solvents (Caledon Labs., Montreal, Canada) were of analytical grade. Water was purified by reverse osmosis and filtered over a Milli-Q water purification system (Millipore, Mississauga, Ontario, Canada) before use. Diethyl ether was analysed for the presence of peroxides and acidic contaminants using American Chemical Society (ACS) recommended procedures [37]. All solvents were redistilled in an all-glass distillation system and kept refrigerated in the dark until use. Solvents were also checked for the presence of contaminants: a 100-ml aliquot was evaporated to dryness and the residue was dissolved in 100 $\mu$l of the same solvent. This solution was then analysed by GC–MS using anabolic steroids SIM procedures. In the case where interfering peaks were detected at a level equal to or higher than steroid peak intensity corresponding to a concentration of ca. 500 pg/$\mu$l of the steroid, the solvent was repurified by distillation. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Regis, Morton Grove, Il, U.S.A.), iodotrimethylsilane (TMSI), trimethylchlorosilane (TMCS), and dithioerythritol (Aldrich, Milwaukee, WI, U.S.A.) were used as supplied. Sep-Pak $C_{18}$ cartridges were obtained from Waters Scientific (Mississauga, Canada). Anhydrous sodium sulfate was washed with anhydrous diethyl ether before use.

### *Enzymatic hydrolysis assays*

*Helix pomatia* digestive juice (Sigma, St. Louis, MO, U.S.A.) containing $\beta$-glucuronidase (100 000 Fishman U/ml) and sulfatase (1000–5000 Fishman U/ml) (referred to as extract A in the text) and $\beta$-glucuronidase (200 U/ml) from *Escherichia coli* K-12 (Boehringer Mannheim, Montreal, Canada) were used as supplied. The activity of each enzymatic solution was tested using testosterone $\beta$-D-glucuronide and dehydroepiandrosterone-3-sulfate (Sigma) and urine specimens obtained from human studies with selected anabolic steroids.

*Helix pomatia* digestive juice (Boehringer Mannheim, F.R.G.) kindly supplied by Dr. M. Ueki (Mitsubishiyuka Laboratory of Medical Science, Tokyo, Japan), is referred to as extract B in the text. The specific activity of this enzymatic preparation was tested towards androstenedione, androstenediol and dihydroepiandrosterone and compared with that of *Helix pomatia* extract A used in our laboratory and with $\beta$-glucuronidase from *E. coli*. Incubations with free reference steroids and urine samples were carried out with 5-ml aliquots of 0.2 *M* acetate

buffer fortified with 1 μg of the steroid of interest. *Helix pomatia* extracts or *E. coli* β-glucuronidase (100 μl) were added and the mixtures were incubated at 37°C for 16, 24 and 48 h or at 55°C for 3 h. The free steroids were extracted as described below. Steroids were measured by SIM GC–MS analysis using 5α-androstan-17-one as external standard. The trimethylsilyl (TMS) enol–TMS ether derivatives were prepared as described below.

*Steroids*

Reference anabolic steroids, some of their urinary metabolites and their pharmaceutical preparations were kindly supplied by pharmaceutical manufacturers. Reference endogenous steroids and some anabolic steroids were obtained from Sigma and Steraloids (Wilton, NH, U.S.A.). Steroids and steroid conjugates were kept refrigerated in the dark until use. Prior to use, steroids were derivatized to TMS ethers and analyzed by GC–MS. No trace of any isomeric form of the steroid and/or other contaminant was detected.

*Human studies*

The GC–MS screening method was set up using the data obtained from metabolic excretion studies carried out with healthy male volunteers using single and multiple therapeutic doses of the steroids. Urine specimens were collected in sterile bottles over a period of 24 h before steroid administration and about every 6 h after administration for at least seven days. The samples ($\simeq$75 ml) were frozen at 20°C shortly after voiding to prevent bacterial degradation and artifact formation.

*Steroid extraction*

*Free and conjugated steroids.* Urine (2 ml) was applied over a Sep-Pak $C_{18}$ cartridge (previously washed with 5 ml of methanol and 5 ml of water). The cartridge was then washed with 5 ml of water to eliminate most of the water-soluble urinary constituents, which are not adsorbed on the solid support. The steroids (free and conjugated) were then eluted with 5 ml of methanol. The entire effluent was evaporated to dryness under a nitrogen stream at 40°C and the residue was dissolved in 1.0 ml of 0.2 *M* acetate buffer (pH 5.2). Following addition of 100 μl of the crude enzyme solution (*Helix pomatia* extract A or *E. coli* β-glucuronidase), the mixture was incubated for 3 h at 55°C or 16 h at 37°C. The hydrolysate was then cooled to room temperature and ca. 100 mg of potassium carbonate were added.

The mixture was gently vortexed for a few seconds and extracted with 5 ml of diethyl ether containing 100 mg/ml 5α-androstan-17-one as external standard. The ethereal phase was decanted, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was then dissolved in 200 μl of methanol and transferred portion-wise to a 200-μl conical vial. The methanolic solution was kept at 4°C in the dark and evaporated to dryness under a nitrogen stream prior to derivatization.

*Unconjugated steroids.* Some anabolic steroids' urinary metabolites are mainly excreted unchanged and/or unconjugated in urine. These steroids can be selec-

tively isolated by extraction with diethyl ether of the acetate buffer solution containing both the free and the conjugated steroids previously extracted by adsorption on a Sep-Pak $C_{18}$ cartridge. The free steroids were extracted as described above for the conjugated steroid fraction. This extraction step is not performed on a routine basis for large-scale GC–MS screening. It is specifically done to confirm and validate preliminary GC–MS data indicating the presence of a steroid in a given sample and of those metabolites known to be excreted unconjugated in urine.

*Preparation of derivatives*

*Trimethylsilyl enol–trimethylsilyl ether derivatives.* These derivatives are suitable for the GC–MS screening of steroids containing enolizable keto and hydroxyl functional groups. They were prepared as follows. Under a dry nitrogen stream, ca. 0.5–1 mg of dithioerythritol was added to the dried steroidal extract and the vial was stoppered under nitrogen. Next, 100 $\mu$l of MSTFA and 1 $\mu$l of a solution of TMSI in dichloromethane and triethylamine (142:858:2, v/v) were added. The mixture was heated at 70°C for 30 min, and 1 $\mu$l was injected into the gas chromatograph.

*Trimethylsilyl ether derivatives.* The dried steroidal residue was treated with a mixture of pyridine, MSTFA and TMCS (90:10:1, v/v) as described above and heated at 70°C for 30 min. A 1-$\mu$l aliquot was injected for GC–MS analysis.

*N-Trifluoroacetyl derivatives.* Stanozolol and its metabolites, which bear a pyrazole moiety, yield N,O-bis-TMS derivatives upon trimethylsilylation. The corresponding N-TFA, O-TMS derivatives were prepared in situ by adding 20 $\mu$l of MBTFA to 50 $\mu$l of the TMS enol–TMS ether derivatization mixture and heating at 70°C for 40 min. A 1-$\mu$l aliquot of the resulting solution was injected for GC–MS analysis.

*Gas chromatography–mass spectrometry*

The steroid extracts were analysed using an HP-5970 mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) linked to an HP-5890 gas chromatograph equipped with an HP Ultra-2 (5% phenyl, methyl silicone) fused-silica capillary column (25 m×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 on-line dried helium (zero-grade) as carrier gas at a rate of 0.6–0.8 ml/min. The oven temperature was maintained at 100°C for 1 min and programmed at 16°C/min to 220°C and then at 3.8°C/min to 300°C and maintained for 10 min. The injector and transfer line temperatures were set at 270 and 310°C, respectively. For screening purposes, the mass spectrometer was operated in the SIM mode where selected ions were monitored within nine discrete ion groups (Table I). Each ion group was set up for the detection of specific anabolic steroids on the basis of the chromatographic retention and MS features of their metabolites of interest. These data were obtained from the GC–MS analysis previously carried out on urinary samples collected from human excretion studies. Mass spectra were recorded in the repetitive scanning mode to establish the identity of a chro-

TABLE I

SCREENING OF ANABOLIC STEROIDS BY SIM GC-MS

| Steroid[a] | RRT[b] | Ions monitored[c] (dwell time) | Ion group[d] | Steroid fraction[e] |
|---|---|---|---|---|
| 5α-Androstane-3-one | 1.00 | 331(50) | 1 | n.a. |
| 19-Nortestosterone* (nandrolone) | 1.13 | 420(100), 405(100), 315(50) | 1 | C |
| | 1.17 | 420(100), 405(100), 315(50) | 1 | C |
| | 1.20 | 420(100), 405(100), 315(50) | 1 | C |
| Methenolone* | 1.28 | 446(100), 431(50) | 2 | C |
| Dehydroepiandrosterone | 1.29 | 432(50) | | |
| Methyltestosterone* | 1.31 | 450(100), 435(50), 143(50) | 2 | C |
| Mesterolone* | 1.33 | 448(100), 433(50) | 2 | C |
| Epitestosterone | 1.32 | 432(100) | 3 | C |
| 11-β Hydroxyandrosterone and hydroxyetiocholanolone | 1.34 | 522(50), 432(100) | 3 | C |
| Mesterolone | 1.35 | 448(100), 433(50) | 3 | C |
| Boldenone | 1.36 | 446(100), 206(50) | 3 | C |
| Testosterone | 1.37 | 432(100) | 3 | C |
| Bolasterone* | 1.39 | 449(100), 374(50), 143(50) | 4 | C |
| 4-Chlorotestosterone | 1.41 | 466(100), 451(100) | 4 | C |
| Methenolone | 1.41 | 446(100), 431(50), 195(50) | 4 | C |
| Fluoxymesterone* | 1.51 | 642(100), 552(50), 462(50), 143(50) | 5 | C, U |
| Norethandrolone* and orabolin* | 1.53 | 538(100), 421(50), 245(50) | 5 | C |
| Oxandrolone | 1.57 | 378(100), 363(100), 143(50) | 6 | U |
| Methanedienone* | 1.58 | 532(100), 517(50) | 6 | C, U |
| Oxymesterone | 1.65 | 534(100), 143(50) | 7 | C |
| Oxymetholone | 1.67 | 640(100), 550(50), 143(50) | 7 | C |
| Oxymesterone* | 1.69 | 538(100), 523(50), 143(50) | 7 | C |
| Turinabol* | 1.75 | 494(100), 315(50), 243(50), 143(50) | 8 | C, U |
| Stanozolol | 1.84 | 472(100), 457(100), 143(50) | 8 | C, U |
| Stanozolol* | 1.96 | 560(100), 545(100), 254(100), 143(50) | 9 | C, U |

[a]Anabolic steroids detected by GC-MS labelled with an asterisk indicate that metabolites of the corresponding parent steroid are detected.

[b]Retention times relative to that of 5α-androstane-3-one (17.80 min).

[c]Dwell times are expressed in milliseconds.

[d]GC-MS analysis starts after a 16.5-min solvent delay. Ion groups then sequentially changed at 22.00, 23.40, 24.60, 25.20, 27.50, 28.60, 30.80 and 33.50 min after injection. Overall analysis time was 38.00 min.

[e]Steroid fraction (C) conjugated and (U) unconjugated from which the urinary metabolites were extracted. See text for analysis conditions.

matographic peak or to verify homogeneity from ion current chromatograms obtained from SIM GC–MS analysis.

*Testosterone/epitestosterone (T/ET) ratio*

Epitestosterone is used here as an endogenous reference urinary metabolite to detect previous administration of exogenous testosterone. As shown by data obtained from human studies, the epitestosterone urinary level remains fairly constant after testosterone administration, so that any increase of the T/ET ratio will result in a positive test for testosterone, if the ratio value is greater than 6:1. According to our findings and that of other researchers, the mean value of the T/ET ratio in normal and healthy humans is ca. 1.10, the ratio values ranging from 0.10 to 5.0 according to a Gaussian distribution [38]. A threshold value of 6 was set by the IOC Medical Commission to discriminate negative from positive results on the basis of human studies performed with testosterone and on that of GC–MS data obtained from the analysis of more than 10 000 urine samples in several IOC accredited laboratories.

Testosterone and epitestosterone were detected by GC–MS monitoring of their common and intense molecular ion of $m/z$ 432. The corresponding peak areas and that of the external standard are computer-integrated and the T/ET ratio is then normalized with respect to a testosterone–epitestosterone calibration curve accounting for a ratio range from 1.0 to 20 ng/$\mu$l. Peak homogeneity was checked for the presence of any coeluting steroids, by monitoring ions of $m/z$ 344, 430, 434 and 522 pertaining to 11$\beta$-hydroxy steroids and androstenedione, which elute between epitestosterone and testosterone, whereas the androstenediol peak is baseline-resolved with that of epitestosterone in the chromatographic conditions used. If the presence of testosterone was suspected, a repeat analysis was carried out with five aliquots of the urine sample, and peak areas were both manually and computer-integrated. The T/ET ratio was finally determined using testosterone–epitestosterone standard solutions with T/ET ratio values slightly lower and higher (bracketing values) than that measured from the urine sample of interest.

## RESULTS AND DISCUSSION

The method described above was developed for large-scale and routine GC–MS analysis of anabolic steroids and some endognous steroids, including testosterone, which is misused in athletics for its anabolic properties. It also provides information regarding the urinary profile of some endogenous steroids, which reflects to a certain extent the endocrine status of the individual and the effects of anabolic steroids on both their secretion and metabolism.

*Extraction of steroids*

The Sep-Pak $C_{18}$ cartridge was preferred to Amberlite XAD-2 polystyrene resin [39,40] and liquid–liquid extraction. Contrary to the XAD-2 resin, whose capacity is low (0.5 g of resin is used per ml of urine) and which has to be eluted at a flow-rate of ca. 0.2 ml/cm$^2$/min to maximize steroid extraction [41], the Sep-

Pak $C_{18}$ cartridge has a much higher capacity (100 ml of urine) and can be used at a flow-rate as fast as 30 ml/min [42]. To our knowledge, this solid support offers the most rapid and versatile method for the extraction of urinary metabolites of anabolic steroids [36].

Both conjugated and unconjugated metabolites of anabolic steroids are quantitatively adsorbed from urine and recovered by elution with 5 ml of methanol. This single step extraction maintains the level of co-extractable urinary compounds at a reasonable level thus leading to cleaner steroid-specific ion current chromatograms and facilitating the detection and identification of the steroids of interest either by SIM or repetitive scanning GC–MS analysis. In addition, the Sep-Pak $C_{18}$ cartridge does not discriminate for the extraction of some steroids conjugates as it was reported for XAD-2 resin [40,43], thus providing improved extraction efficiency for the analysis of small amounts of anabolic steroids. This property of the Sep-Pak $C_{18}$ cartridge is used with advantage for the profiling of anabolic steroid metabolites in urine which is an important step in the confirmation procedure set up to assess and validate the MS data from GC–MS analysis of steroidal extracts in which an anabolic steroid is suspected. This matter will be further discussed below.

*Hydrolysis of steroid conjugates*

In most methods for steroids analysis, this particular step is often time consuming. The digestive juice of *Helix pomatia* is certainly the most versatile enzymatic preparation for steroid glucuronic and sulfate conjugates hydrolysis. Under normal hydrolysis conditions, the buffered urinary extract is usually incubated with *Helix pomatia* digestive juice at 37 °C for 12–48 h. However, in order to cope with the time constraints of large-scale analysis of anabolic steroids, hydrolysis can be conducted at 55 °C for 3 h with no apparent losses of the steroids of interest. However, enzymatic activities of the crude *Helix pomatia* digestive juice should be systematically tested prior to use in order to detect any selective loss of steroids due to unforseeable enzyme reactivities and selectivities. In this context, Vanluchene et al. [44] reported the conversion of free 3$\beta$-hydroxy-5-ene steroids of the pregnene series into the corresponding 4-en-3-oxo analogues. Our findings and those reported by Donike [38] indicate that similar transformations are observed in the androstene series. To this effect, we have compared the respective ability of two *Helix pomatia* extracts (A and B) obtained from different sources and that of $\beta$-glucuronidase from *E. coli* to convert androstenedione, androstenediol and dehydroepiandrosterone (DHEA) into the corresponding 4-en-3-oxo, or other steroids. The data are summarized in Table II.

One notes with interest that DHEA is converted into androstenedione only to a very small extent in both enzymatic preparations, although extract B appears to be five-to-six-fold more reactive than the other in transforming this steroid. Androstenedione is apparently not an ideal substrate for the enzymes of both *Helix pomatia* extracts since it was converted into testosterone in very minute amounts solely when incubations were carried out at 55 °C with extract B. On the other hand, androstenediol was extensively and rapidly transformed into testos-

TABLE II

ENZYMATIC CONVERSION OF 4- AND 5-ENE STEROIDS DURING INCUBATION WITH *HELIX POMATIA* DIGESTIVE JUICES AND *E. COLI* β-GLUCURONIDASE

See text for incubation conditions. Steroids were analysed by SIM GC–MS.

| Steroid incubated[a] | Incubation conditions and steroid detected[b] | | | | | |
|---|---|---|---|---|---|---|
| | *Helix pomatia* A | | *Helix pomatia* B | | *E. coli* | β-Glucuronidase |
| | 3 h/55 °C | 16 h/37 °C | 3 h/55 °C | 16 h/37 °C | 3 h/55 °C | 16 h/37 °C |
| DHEA | $A^4$-dione (0.2%) | N.D.[c] | $A^4$-dione (1.2%) | $A^4$-dione (0.9%) | N.D. | N.D. |
| $A^4$-dione | T (0.1%) | N.D. | N.D. | N.D. | N.D. | N.D. |
| $A^5$-diol | T (0.2%) $A^4$-dione (0.05%) | T (0.1%) | T (70%) $A^4$-dione (1.5%) | T (50%) $A^4$-dione (2.0%) | N.D. | N.D. |

[a]DHEA = dehydroepiandrosterone; $A^4$-dione = 4-androstene-3,17-dione; $A^5$-diol = 5-androstene-3β,17β-diol; T = testosterone, 17β-hydroxy-4-androsten-3-one.

[b]Steroids detected during incubation of steroidal substrates with enzymatic preparation are expressed as a percentage of substrate initial concentration. Enzymes used are from Sigma (*Helix pomatia* A) and Boehringer Mannheim (*Helix pomatia* B, and *E. coli* β-glucuronidase).

[c]No steroid other than the original steroid substrate was detected during incubation.

terone, irrespectively of incubation conditions, by *Helix pomatia* extract B, but was barely affected by the other.

A typical GC–MS analysis of androstenediol after incubation with *Helix pomatia* extract A and B is illustrated in Fig. 1. When incubated with *Helix pomatia* extract B, the decrease of androstenediol is paralleled by the concomitant formation of testosterone and minor amounts of androstenedione (Fig. 1A and B). The transformation was nearly quantitative after 24 h of incubation. On the other hand, only trace amounts of the 4-en-3-oxo steroid were detected by SIM GC–MS when incubations were carried out with extract A (Fig. 1C). It is of interest to note that contrary to data reported previously [44] decrease of the incubation time did not reduce the amount of testosterone produced from the 5-ene-diol steroid when the temperature was increased to 55 °C.

These data indicate that there may exist a great variability in *Helix pomatia* extracts reactivities and selectivities during incubation with free steroids. In addition, these data provide further evidence for the presence in *Helix pomatia* extracts of various steroid dehydrogenases and oxidases, which are essential to the observed conversions [44]. This hypothesis was strengthened by evidence from the electrophoretic profiles of both *Helix pomatia* extracts which revealed the presence in extract B of proteins which were not detected or were in very low concentrations in extract A. These proteins could account for the capacity of extract B to transform androstenediol into testosterone.

In another set of experiments, the capacity of *Helix pomatia* to produce the

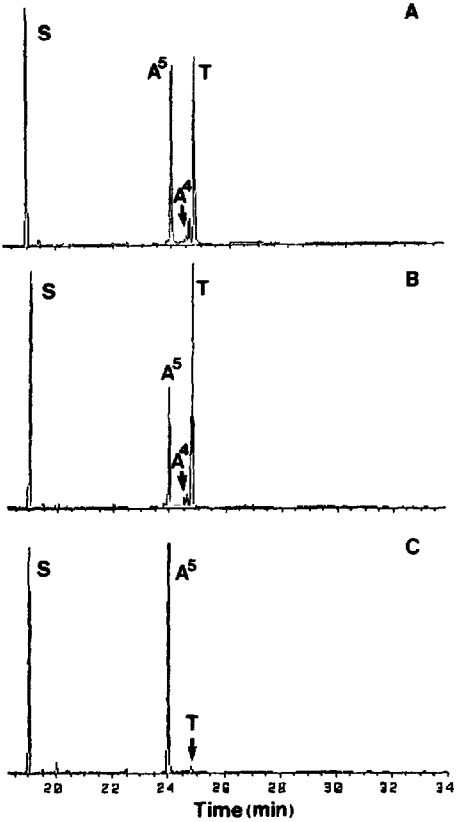


Fig. 1. Reconstructed total ion current chromatograms from SIM GC–MS analysis of androstenediol incubated in 0.2 *M* acetate buffer with *Helix pomatia* extract B: (A) at 37°C for 16 h, (B) at 55°C for 3 h and (C) with *Helix pomatia* extract A at 55°C for 3 h. See Experimental for incubation and analysis conditions. Peaks: S = 5α-androstane-17-one (standard); $A^5$ = androstenediol; $A^4$ = androstenedione; T = testosterone.

above-mentioned conversions was assessed with urine specimens from healthy subjects. The SIM GC–MS profiles obtained after hydrolysis of two aliquots of the same urine sample by *Helix pomatia* extracts A and B are shown in Fig. 2.

The most striking difference between the two profiles is the significant increase of the testosterone peak in the total ion current (TIC) chromatogram from the urine aliquot hydrolyzed with *Helix pomatia* extract B. Whereas the level of epitestosterone remains unchanged in both hydrolyzates, that of testosterone was raised by 2.9-fold in the B extract, thus increasing the T/ET ratio to 7.8:1 from its original and normalized value of 2.7:1, as previously determined after hydrolysis with *Helix pomatia* extract A and *E. coli* β-glucuronidase. The formation of testosterone was paralleled by a concomitant decrease of androstenediol in the urinary extract. It is of importance to mention that none of the steroidal substrates was converted into epitestosterone by the enzyme extracts used. This example shows how inadequate and uncontrolled hydrolysis conditions may lead to false positive results since a sample is declared positive when the T/ET ratio is above 6:1.

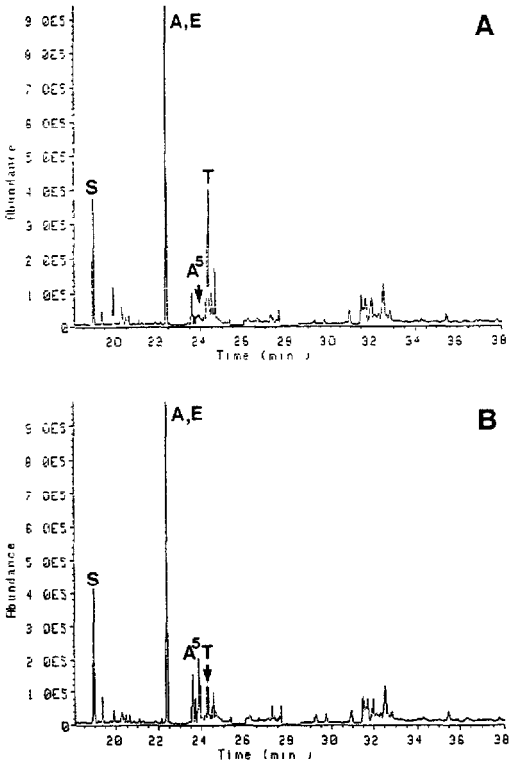


Fig. 2. Reconstructed total ion current chromatograms from SIM GC–MS analysis of a urinary extract previously hydrolyzed (A) with *Helix pomatia* extract B at 55°C for 3 h and (B) with *Helix pomatia* extract A at 55°C for 3 h. See Experimental for hydrolysis and analysis conditions. Peaks: S = 5α-androstane-17-one (standard); A, E = androsterone and etiocholanolone; T = testosterone; $A^5$ = androstenediol. Steroids were analyzed as TMS enol–TMS ether derivatives.

*Derivatization of the steroids*

The urinary metabolites of the anabolic and endogenous steroids of interest bear a wide variety of structural features, including hindered hydroxyl and keto functions. For the specific purpose of GC–MS screening, where specificity and sensitivity are the major analytical requirements, TMS enol–TMS ether derivatives were prepared using trimethylsilyl iodide (TMSI) as catalyst [36,45]. TMSI is a versatile synthetic agent in which silicon is a hard acid and iodide a soft base. This reagent therefore reacts very readily with organic compounds containing oxygen (a hard acid) forming silicon–oxygen bond. The applications of TMSI in organic synthesis were reviewed by Olah and Narang [46]. Here, TMSI is used as a catalyst to promote the regioselective formation of TMS enol–ether of ketonic groups and trimethylsilylation of hydroxyl functions at hindered positions using MSTFA as silylation agent. The reaction appears to be initiated by the reaction of TMSI with MSTFA to form the quaternary ammonium salt (Fig. 3). The latter readily reacts with hydroxyl and keto groups to yield the corresponding TMS ether and TMS enol–ether derivatives. The catalyst is regenerated in situ by nucleophilic displacement on silicon of MSTFA by iodide. Contrary to previ-

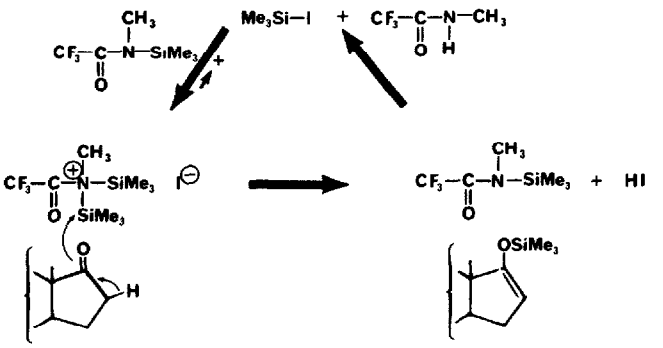

Fig. 3. Probable mechanism accounting for the catalytic action of TMSI during trimethylsilylation of steroid using MSTFA as silylation agent.

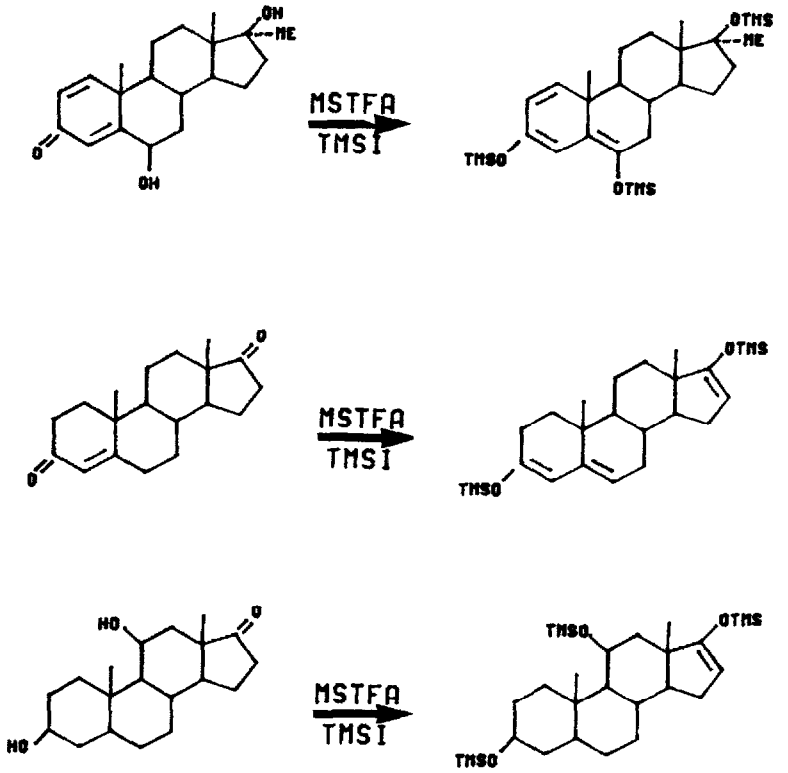

Fig. 4. Trimethylsilylation of hydroxyl and ketonic group of steroids.

ously published methods in which TMS enol derivatives were prepared under drastic conditions, the use of TMSI as catalyst has provided the most rapid and versatile method for TMS enols and ethers preparation.

The versatility of TMSI as catalyst is illustrated in Fig. 4. Indeed, steroids with conjugated or unconjugated keto groups, hindered 11-keto and 11-hydroxy functions and/or tertiary hydroxyls are converted into their corresponding TMS enol and/or TMS ether derivatives under very mild conditions. The derivatization

method was successfully designed for GC–MS screening purposes since most anabolic and endogenous steroids of interest give single derivatives exhibiting prominent molecular ($M^+$) and/or $(M-15)^+$ ions. These general MS features are illustrated in the mass spectra of 6β-hydroxy-methanedienone TMS enol–ether and TMS ether derivatives (Fig. 5). It is of interest to note that the TMS enol–ether derivative provides less structural information than its TMS ether analogue but is much more useful for screening purposes due to the absence of extensive fragmentation upon EI. However, the reaction is not applicable to the analysis of corticosteroids with a 20-oxo group due to the formation of isomeric TMS enol–ethers giving rise to several peaks of variable intensities in GC–MS. Due to analytical and methodological imperatives, the silylation reagents are not evaporated before GC–MS analysis. As a consequence, repeated injections of derivatized urinary extracts are often necessary in order to achieve optimum chromatographic conditions when a new capillary column is used. In addition, the use of MSTFA as solvent increases derivative stability in the reaction vial as well as in the chromatographic system, probably because MSTFA prevents loss of TMS groups from the steroidal derivatives which could arise through hydrolysis and exchange with hard base hydroxyls from active sites in the chromatographic phase. This is further demonstrated by the fact that derivatized extracts can be kept at room temperature for ca. 48 h and five to six days at 4 °C without noticeable degradation of the derivatives and alteration of the GC–MS steroidal profile.

However, repeated injections of the derivatization mixture in the splitless mode has the disadvantage of gradually washing-out the chromatographic phase, giving rise to poor peak shape (tailing) due to the presence of active sites. One of the most striking effects is the irreversible adsorption of stanozolol TMS derivative, a basic steroid which bears a pyrazole moiety, due to the formation of acidic active sites in the cold-trapping area of the capillary column. Removal of the first 50–100 cm from the injector usually restores column performance for ca. two weeks when 20–25 injections are performed daily. This process is repeated once or twice

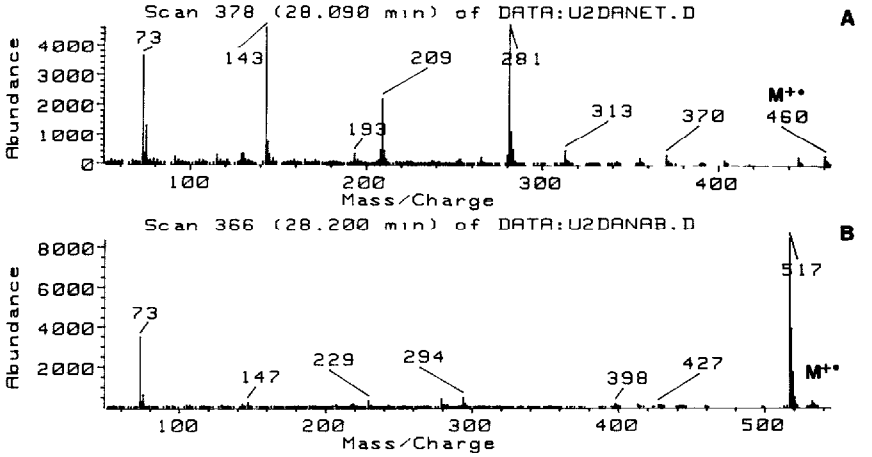


Fig. 5. Mass spectra of 6β-hydroxymethanedienone as (A) TMS ether and (B) TMS enol-ether derivatives.

until column performance cannot be restored. Thus, column stability and chromatographic status are checked daily using a mixture of anabolic and endogenous steroids including stanozolol. According to our experience and providing that 20–25 samples are injected on a daily basis, the column usually maintains optimal efficiency and performances for ca. 30–60 days.

*Urinary metabolites of anabolic steroids*

Most synthetic anabolic steroids were originally designed and synthesized after the endogenous anabolic–androgenic testosterone in order to produce steroids with strong anabolic property and low androgenicity. As shown in Fig. 6, this was achieved by the design of compounds bearing new functional groups mainly at those positions (C-2, C-3, C-4 and C-17) which are of importance for both the expression of testosterone androgenic properties in humans and its metabolic transformation. Consequently, the metabolic profiles of these synthetic steroids differ substantially from that of testosterone and are modulated to a large extent by the nature and the position of the functional groups in rings A and D. A thorough knowledge of both anabolic and endogenous steroids metabolism and urinary excretion profiles in humans is a fundamental prerequisite for implementing a selective and specific GC–MS screening method for anabolic steroids. Our purpose here is not to make an extensive review of their metabolism in humans, but to show with selected examples the wide diversity of their urinary metabolic profiles. Furthermore, we demonstrate how the introduction of high-resolution fused-

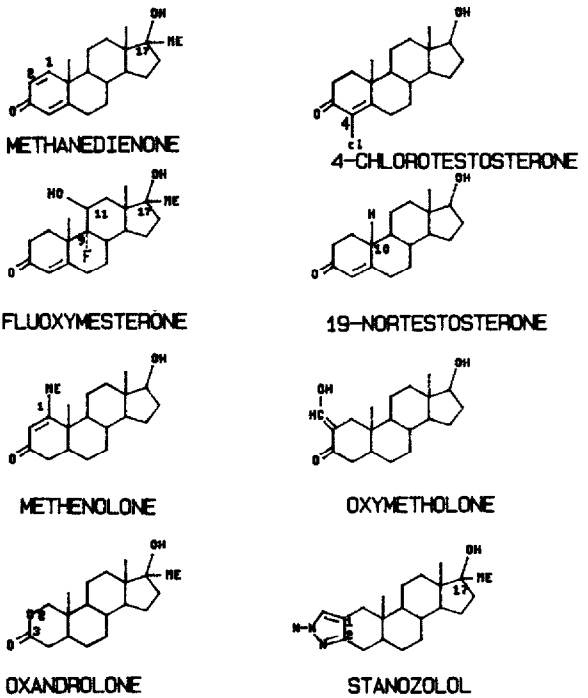


Fig. 6. Typical structural features of anabolic steroids.

silica capillary columns has improved our knowledge of their metabolism in humans.

*Methanedienone*

Dürbeck et al. [25] reported the determination of methanedienone and its major metabolites 6$\beta$-hydroxymethanedienone, 6$\beta$-hydroxyxepimethanedienone and epimethanedienone in human urine specimens by GC–MS analysis. In the course of a study (unpublished results) on methanedienone metabolism in humans, we detected by GC–MS analysis of hydrolyzed urine extracts 17$\alpha$-methyl-3$\alpha$,17$\beta$-dihydroxy-5$\beta$-androstane, the major urinary metabolite of 17$\alpha$-methyltestosterone. This metabolite originates from the reduction of all A ring functions. This result indicated that methanedienone metabolism was apparently much more complex and extensive than previously reported [25]. Indeed, in addition to previously reported 6$\beta$-hydroxylation and epimerization at C-17, we found that methanedienone was also hydroxylated at C-16 and that the A ring was extensively metabolized through a cascade of reactions including sequential reduction of the double bonds at C-1 and C-4 and 3-oxo group to yield several metabolites which afforded a very characteristic ion fragmentographic profile when monitoring ring D cleavage ion at *m/z* 143 as shown in Fig. 7. The partial structures of some of these metabolites are illustrated in Fig. 8. Thus, the detection of such a characteristic metabolic profile by GC–MS provides the analyst with irrefutable evidence assessing that methanedienone was administered in fairly large amounts prior to urine collection.

*Boldenone*

This steroid is structurally similar to methanedienone but lacks a 17$\alpha$-methyl group. To our knowledge, the determination of boldenone metabolites urinary profile in humans by GC–MS has never been reported. Contrary to methanedienone, 6$\beta$-hydroxylation is a very minor route in boldenone metabolism. In addition to unchanged boldenone, several metabolites resulting from sequential reduction of ring A substituents and oxidation at C-17 were detected in urine. Fig. 9 shows the GC–MS analysis of unchanged boldenone and 3-hydroxy-1-androsten-17-one. It is of interest to note that the latter elutes between androsterone and etiocholanolone TMS ether derivatives. The stereochemistry at C-3 and C-5 was not determined yet when this text was written, but is presumably 3$\alpha$ and 5$\alpha$ on the basis of its GC retention and MS features. A detailed report will be presented elsewhere.

*Stanozolol*

The metabolism of this steroid has been investigated by Donike and Schänzer recently [47]. They reported the formation of 4$\beta$-hydroxy, 16$\beta$-hydroxy and 3′-hydroxy (pyrazole ring hydroxylation) metabolites of stanozolol and the 17$\alpha$-hydroxy-17$\beta$-methyl epimer of the latter metabolite. Stanozolol presents unique structural features, such as the presence of a pyrazole moiety and the absence at C-3 of an oxygen atom, which in testosterone and other steroids is essential to the expression of their androgenic and anabolic properties. It was of interest to

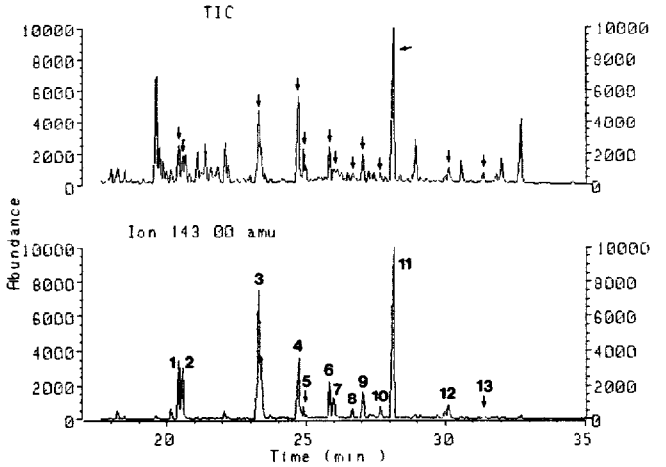


Fig. 7. Reconstructed total ion current chromatograms (above) and ion fragmentogram of ion *m/z* 143 (below) from a urine sample collected 18 h after administration of methanedienone (50 mg). Steroids were analyzed as TMS ether derivatives. Peaks: 1 = 17$\beta$-methyl-5$\xi$-1-androsten-3$\xi$,17$\alpha$-diol ($M^{+\cdot}$ 448); 2 = 17$\alpha$-methyl-5$\beta$-androstan-3-one ($M^{+}$ 376, tentative assignment); 3 = 17$\alpha$-methyl-5$\beta$-androstan-3$\alpha$,17$\beta$-diol ($M^{+\cdot}$ 450); 4 = epimer of compound 1 ($M^{+\cdot}$ 448); 5 = 18-nor-17,17-dimethyl-1,4-13(14)-androstatrien-3-one ($M^{+\cdot}$ 282); 6 = 6$\beta$-hydroxy-17-epimethanedienone ($M^{+\cdot}$ 460); 7 = 17$\alpha$-methyl-6$\beta$,17$\beta$-diol-androstan-3-one ($M^{+\cdot}$ 464, base peak *m/z* 143, tentative assignment); 8 = 17$\alpha$-methyl-6$\beta$,17$\alpha$-diol-1-androsten-3-one ($M^{+\cdot}$ 462, base peak *m/z* 143, tentative assignment); 9 = isomer of compound 12 ($M^{+\cdot}$ 548); 10 = isomer of compound 8 ($M^{+\cdot}$ 462, base peak *m/z* 143); 11 = 6$\beta$-hydroxymethanedienone ($M^{+\cdot}$ 460, base peak *m/z* 143); 12 = hydroxylated analogue of 6$\beta$-hydroxymethanedienone ($M^{+\cdot}$ 548, *m/z* 361, 281, 209, 170 and 143); 13 = 6$\beta$,16-dihydroxy-methanedienone ($M^{+\cdot}$ 548, *m/z* 281, 231, 218, 209 and 143).

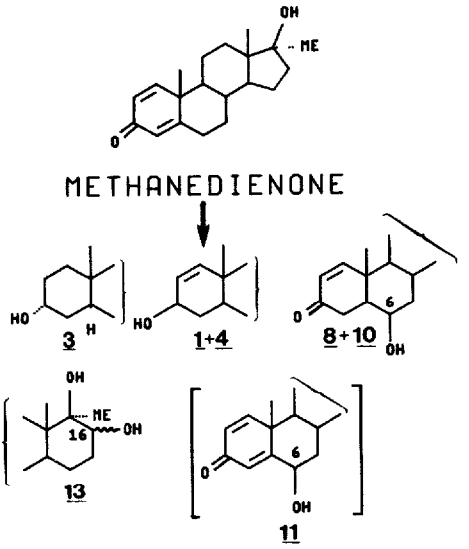


Fig. 8. Partial structures of some methanedienone metabolites in humans. Refer to Fig. 7 for compound identity.

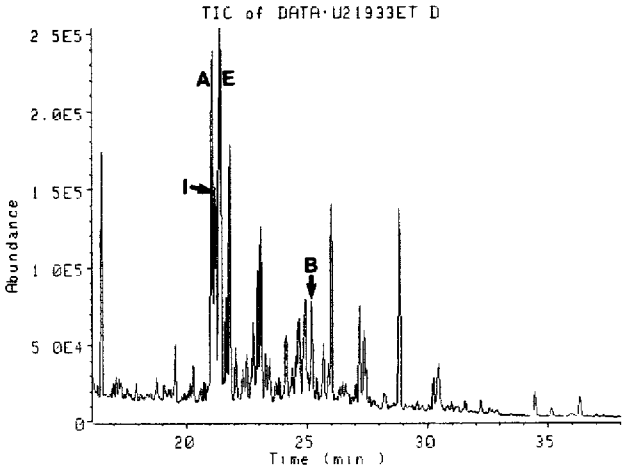


Fig. 9. Reconstructed total ion current chromatogram from GC–MS analysis of a urinary extract obtained after administration of 20 mg of boldenone. Peaks: I = 3-hydroxy-1-androsten-3-one ($M^{+\cdot}$ 360, *m/z* 291, 290, 195, 143, 142, 127); B = boldenone ($M^+$ 358, *m/z* 268, 194, 122); A = androstenone; E = etiocholanolone. Steroids were analyzed as TMS ether derivatives.

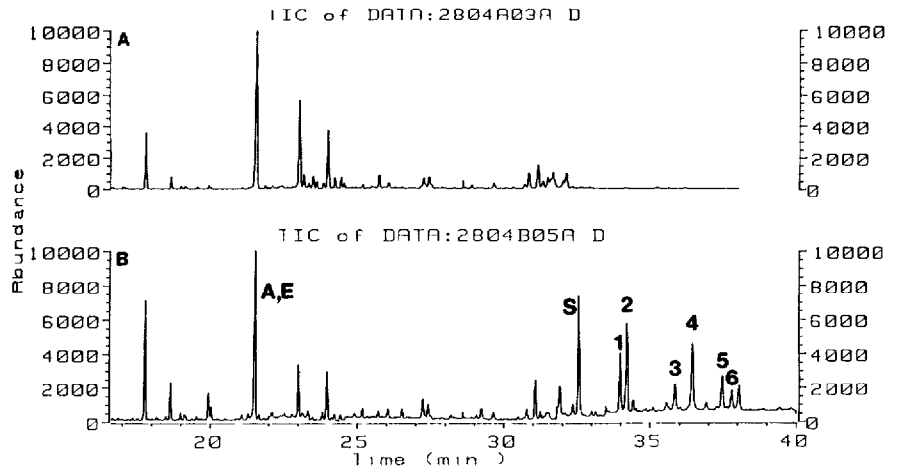


Fig. 10. Reconstructed total ion current chromatograms from GC–MS analysis of (A) a blank urine sample and (B) a urine sample after administration of 20 mg of stanozolol. See Table III and Fig. 9 for peak identification. Steroids were analyzed as TMS ether derivatives.

further study this steroid in order to determine the effect of the pyrazole moiety on its metabolism in humans and to compare its urinary metabolic profile with that of other anabolic steroids which are structurally related to testosterone. Fig. 10 compares the TIC chromatograms from a blank urine sample with that from a subject to whom stanozolol was administered. It is of interest to note that stanozolol metabolites are scattered in about a 6-min elution range in a region of the chromatogram where potential interferences from the endogenous steroids are

TABLE III

PARTIAL MS AND GC RETENTION DATA OF STANOZOLOL URINARY METABOLITES IN HUMANS

| Steroid[a] | Hydroxylation site | RRT[b] | $M^{+\cdot}$ |
|---|---|---|---|
| 1 | C-3′ | 1.043 | 560 |
| 2 | C-4 | 1.051 | 560 |
| 3 | C-16 | 1.100 | 560 |
| 4 | C-16 | 1.118 | 560 |
| 5 | C3′ or C-4[c] and C-16 | 1.150 | 648 |
| 6 | C-4 or C-3′[c] and C-16 | 1.160 | 648 |

[a]Refer to Fig. 10 for chromatographic separation of the steroids.
[b]Retention time relative to that of stanozolol N,O-bis-TMS derivative: 32.58 min.
[c]Hydroxylation at C-16 was ascertained by MS analysis; the second hydroxylation site in both metabolites 5 and 6 could not be undoubtedly assessed by MS, but can be assigned to the C-4 or C-3′ position.

minimal. These steroids elute into a very characteristic profile dominated by unchanged stanozolol.

Six hydroxylated metabolites were detected and partial GC–MS data are given in Table III. Peaks 1 and 2 (Fig. 10) correspond to the metabolites hydroxylated at the C-3′ and C-4 positions [47] whereas peaks 3 and 4 account for 16$\beta$- and 16$\alpha$-hydroxystanozolol, respectively. The mass spectra of the latter metabolites are illustrated in Fig. 11. The molecular ions at *m/z* 560 and the structurally informative ions at *m/z* 218 and 231 resulting from ring D cleavage indicate the presence of a hydroxyl group at C-16 in both compounds. In compounds 5 and 6 mass spectra (Fig. 12), the common molecular ion at *m/z* 648 indicates the presence of three hydroxyl groups. From the characteristics fragment ions at *m/z* 218, 231 and 254 (the last ion arising from the cleavage of ring A, as shown in Fig. 13), it is very likely that these metabolites bear a hydroxyl group at C-16, the third one being most probably at the C-4 and/or C-3′ position. The complete characterization of these metabolites will be presented in a separate communication.

*Anabolic steroids with 3-oxo-4-en functions*

Most anabolic steroids with a 3-oxo-4-en moiety are generally metabolized into mixtures of the corresponding isomeric tetrahydro steroids by enzymatic reduction of both functional groups as illustrated in Fig. 14. For example, the metabolites of 19-nortestosterone excreted in urine are the 19-nor analogues of androsterone, etiocholanolone and epiandrosterone [22]. Similarly, norethandrolone (17$\alpha$-ethyl-19-nortestosterone) is metabolized in humans by ring A redution and side-chain hydroxylation [32]. On the other hand, one would expect the presence of a substituent at the C-4 position to impede the enzymatic reduction of the 3-oxo-4-en group in man. Interestingly, several steroids with a chlorine atom or a hydroxyl group at C-4 are metabolized by reduction of ring A to yield four isomeric tetrahydro metabolites. Thus, oxymesterone (4-hydroxymethyltestoster-

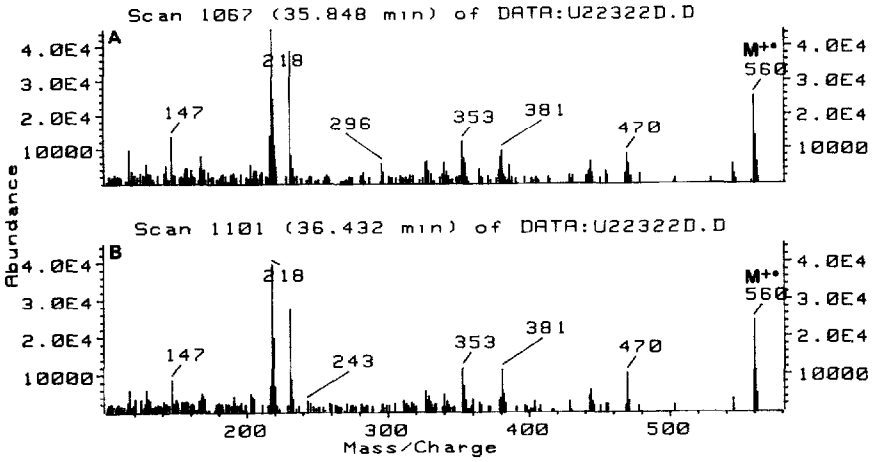


Fig. 11. Mass spectra of (A) 16$\beta$- and (B) 16$\alpha$-hydroxystanozolol as TMS ether derivatives.

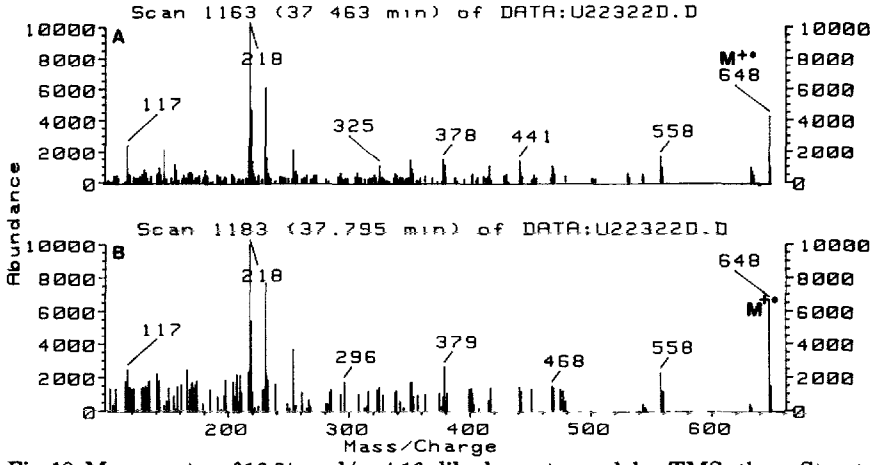


Fig. 12. Mass spectra of 16,3′- and/or 4,16-dihydroxystanozolol as TMS ethers. Structure assignment is tentative.

one) is transformed in humans into the four isomeric forms of 17$\alpha$-methyl-5$\xi$-androstan-3$\xi$,17$\beta$-diol [48].

In this context we also investigated the metabolism of 4-hydroxy-19-nortestosterone (steranabol) in humans. GC–MS analysis indicated that the steroid was converted into the three 4-hydroxy homologues of 19-nortestosterone metabolites, namely 4-hydroxyandrosterone, 4-hydroxyetiocholanolone and 4-hydroxyepiandrosterone [48]. The vicinal OTMS groups at C-3 and C-4 give rise to a characteristic and structurally informative ion at *m/z* 330, which arises from the successive loss of TMSOH and one molecule of tetramethylsilane from the rearrangement of the adjacent C-3 and C-4 OTMS groups.

Contrary to its 4-hydroxy analogue described above, clostebol's (4-chlorotestosterone) A ring is less extensively metabolized since only the 4-en function appears to be reduced to yield four isomeric forms corresponding most likely to

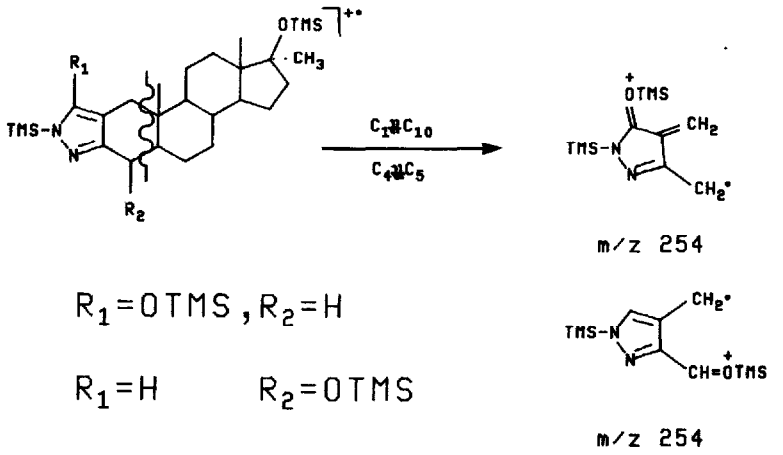


Fig. 13. Formation of the ion *m/z* 254 from stanozolol metabolites 1, 2, 5 and 6 (Fig. 7) upon electron impact ionization.

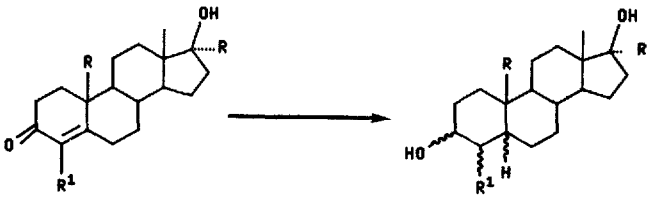


Fig. 14. General metabolic routes of 3-oxo-4-en anabolic steroids.

4-chloroandrosterone, 4-chloroetiocholanolone and their respective 4-chloro epimers [48]. The metabolism of this steroid is still under investigation in our laboratory. Fluoxymesterone (9$\alpha$-fluoro-11$\beta$-hydroxymethyltestosterone) is another steroid belonging to this structural group whose metabolism in humans has not been extensively investigated. It is of interest to note that its major urinary metabolites arise from 6$\beta$-hydroxylation and selective reduction of the 4-en group [48]. The proposed structures were ascertained by GC–MS analysis using TMS enol–ether ($M^{+\cdot}$ 642) and TMS ether ($M^{+\cdot}$ 570) derivatives. In addition, fluoxymesterone metabolites are somewhat sensitive to high temperature and exhibit a propensity to lose one molecule of HF upon injection in the gas chromatograph. In order to eliminate or minimize this chromatographic artifact, on-column injection should be performed in preference to the widely used split–splitless injections.

*Steroids with a 3-oxo group*

Some anabolic steroids bear a 3-oxo group with no double bond in the A ring. The metabolism of such steroids is dominated by reduction of the 3-oxo group to form one or two isomeric 3-ol steroids, as shown by the urinary metabolic profiles of androstanolone, mestanolone, dimethylandrostanolone and oxymetholone (Fig. 6). This last steroid, which bears a hydroxymethylene group at C-2, is further

metabolized by C-6 $\beta$-hydroxylation and reduction of the hydroxymethylene group. The specific oxidation of this group and its subsequent elimination in situ as carbon dioxide with concomitant reduction of the 3-oxo group yields 17$\alpha$-methyl-5$\beta$-androstan-3$\alpha$,17$\beta$-diol, a steroidal metabolite which is also formed from methyltestosterone and methanedienone metabolism in humans.

Anabolic steroids with 3-oxo-1-en functions and bearing a methyl group either at C-1 (methenolone, Fig. 6) or C-2 (stenbolone) are not metabolized very extensively in humans. Their major metabolites arise from reduction of the 3-oxo group with concomitant oxidation of the 17-hydroxyl group. However, our knowledge of their metabolism remains somewhat fragmentary and is worth further investigations.

Finally, oxandrolone (Fig. 6), a steroid with a lactone moiety at the C-2, C-3 positions is excreted unchanged and unconjugated in urine along with a small amount of its 17$\alpha$-hydroxy-17$\beta$-methyl epimer and two isomeric 16-hydroxy derivatives [49].

*Conversion of norethisterone into 19-norandrosterone in humans*

Norethisterone (NE) is used as the progestational component in a number of oral contraceptive preparations. Braselton et al. [50] described in detail its urinary metabolic profile by GC–MS analysis. The metabolites arise mostly from ring A reduction. Although de-ethynylation of ethynylestradiol was reported to occur in primate liver microsomes, at that time this reaction had not been documented in humans. This matter is of concern because de-ethynylation of NE could lead to the in vivo formation of 19-nortestosterone (Fig. 15), a banned anabolic steroid, and its metabolites.

Evidence indicating the probable de-ethynylation of NE in humans was recently presented. Indeed, Clausnitzer [51] reported the detection of 19-norandrosterone (19-NA), the major metabolite of 19-norestosterone in humans, after administration of 10 mg of NE to female subjects. Monitoring molecular ($M^{+\cdot}$) and $(M-15)^+$ ions of both 19-NA (*m/z* 420 and 405) and androsterone (A) (*m/*

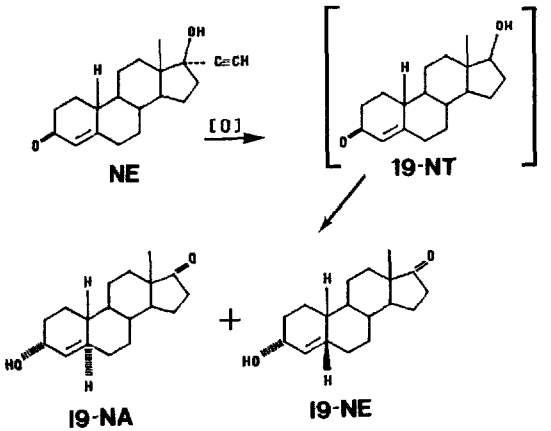


Fig. 15. De-ethynylation of norethisterone (NE) in humans: formation of 19-nortestosterone (19-NT) and its urinary metabolites 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE).

z 434 and 419) TMS enol-ether derivatives by SIM GC–MS, this author demonstrated that the ratio 19-NA/A in urine (which mean value was greater than 1:1000 before NE administration) increased to 1:175, 3 h after the administration of a 5-mg single dose of NE. Then, the 19 NA/A ratio progressively decreased to reach a value of 1:1250 24 h after NE administration.

In regard to this matter, Catlin and Hatton [52] have proposed a more integrated and systematic approach to distinguish between positive cases for 19-nortestosterone administration from those arising from NE administration in females. These authors submit the analytical data of potential positives to statistical analysis by comparing the frequency distribution of NE major urinary metabolite (NE-M) (17$\alpha$-ethynyl-5$\beta$-estrane-3$\alpha$,17$\beta$-diol) found in a population of normal women taking NE. The 19-NA/NE-M ratio is also compared to the corresponding frequency distribution in a population including normal women taking NE and women who did not declare an oral contraceptive and did not refute a positive test for 19-nortestosterone metabolites. Consequently, administration of NE results in low 19-NA/NE-M ratio whereas that of 19-nortestosterone gives rise to the excretion of large amount of 19-NA and a high 19-NA/NE-M ratio.

We investigated the metabolic transformation of 17$\alpha$-ethynyl steroids into 19-nortestosterone in humans using NE as model substrate. Fig. 16 compares the steroid profiles obtained from urine samples collected before and after the administration of NE. The administration of NE was clearly demonstrated by the specific detection of its most abundant metabolite (Fig. 16). On the other hand, 19-nortestosterone metabolites, which are exptected to arise from the previously mentioned metabolic conversion of NE, are barely detected. Further GC–MS

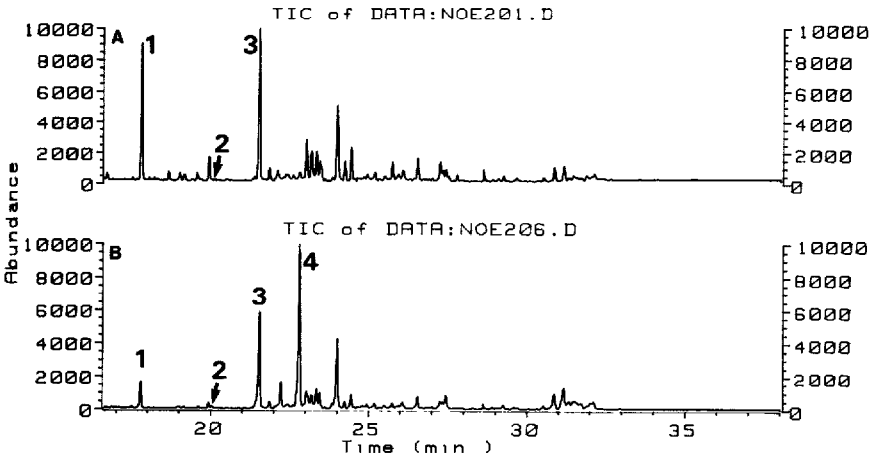


Fig. 16. Reconstructed total ion current chromatograms of urinary steroids detected by SIM GC–MS analysis of (A) a blank urine sample obtained prior norethisterone administration and (B) a urine sample collected 6 h after the administration of 25 mg of norethisterone. See text for analysis conditions. Peaks: 1 = external standard, 17-oxo-androstane; 2 = 19-norandrosterone; 3 = androsterone-etiocholanolone; 4 = 17$\alpha$-ethynyl-5$\beta$-estrane-3$\alpha$,17$\beta$-diol. Steroids were analyzed as TMS enol-ether derivatives.

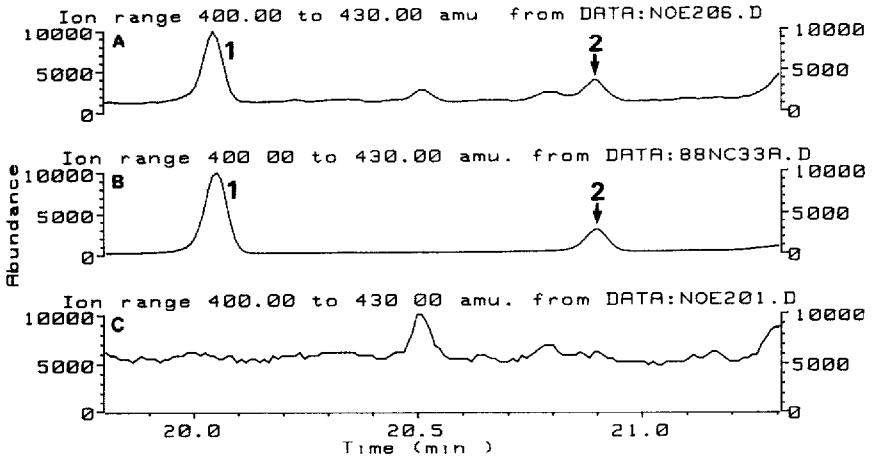


Fig. 17. Specific detection of 19-nortestosterone urinary metabolite following the administration of 25 mg of norethisterone to humans by repetitive scanning of ion range 400–430. Total ion current profiles are from: (A) urine sample collected 6 h after norethisterone administration, (B) positive urine sample to 19-nortestosterone metabolites, (C) urine sample collected prior to the administration of norethisterone. Peaks: 1 = 19-norandrosterone; 2 = 19-noretiocholanolone. Analysis conditions as in Fig. 16.

analysis was performed to detect and characterize these 19-nor steroids and assess the proposed metabolic transformation (Fig. 15). By the selective monitoring of their specific ions, the presence of 19-NA and 19-noretiocholanolone (19-NE) in urine samples collected after NE administration was demonstrated (Fig. 17). Although no trace of 19-nortestosterone was detected in urine after NE administration, these data indicate that 19-nortestosterone is most probably the metabolic intermediate through which NE is partially transformed into 19-NA and 19-NE. Further metabolic studies with labelled steroids are in progress in order to investigate specific metabolic aspects of NE de-ethynylation.

*Measurement of the T/ET ratio*

The endogenous steroid testosterone is also misused in athletics due to its anabolic properties. The misuse of testosterone can be detected by an increase of the ratio of testosterone to the pituitary gonadotropins (i.e. luteinizing hormone, LH; follicle-stimulating hormone, FSH) [53] and also, as mentioned above, by the increase of the T/ET ratio determined by SIM GC–MS analysis.

Testosterone levels can be increased by the administration of the drug itself or through the action of human chorionic gonadotropin (HCG) which, when administered to humans, has an action similar to LH (i.e. stimulates the testes to produce testosterone). For the specific purpose of detecting testosterone abuse in athletics, GC–MS is preferred to RIA techniques. The GC–MS approach allows for the specific, sensitive and reproducible detection and quantitation of both testosterone and epitestosterone. However, RIA can be used in positive cases to assess the hormonal status of the pituitary gland/testes axis so as to obtain

complementary information regarding the hormonal feedback effect of exogenous testosterone administration.

In large-scale and routine screenings, the T/ET ratio is measured by monitoring of the molecular ($M^{+\cdot}$ 432) ion of their TMS enol–ether derivatives, as shown in Fig. 18. The separation of testosterone from the 11$\beta$-hydroxyandrosterone and

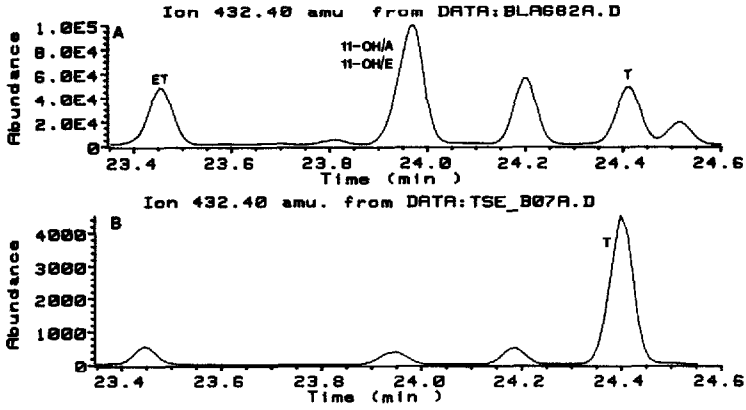


Fig. 18. Reconstructed ion fragmentograms of *m/z* 432 obtained by SIM GC–MS analysis of (A) a blank urine sample and (B) a urine sample positive to testosterone, T/ET = 8.53. Analysis conditions are given in the text. Peaks: ET = epitestosterone; 11-OH/A = 11$\beta$-hydroxyxandrosterone; 11-OH/E = 11$\beta$-hydroxyetiocholanolone; T = testosterone.

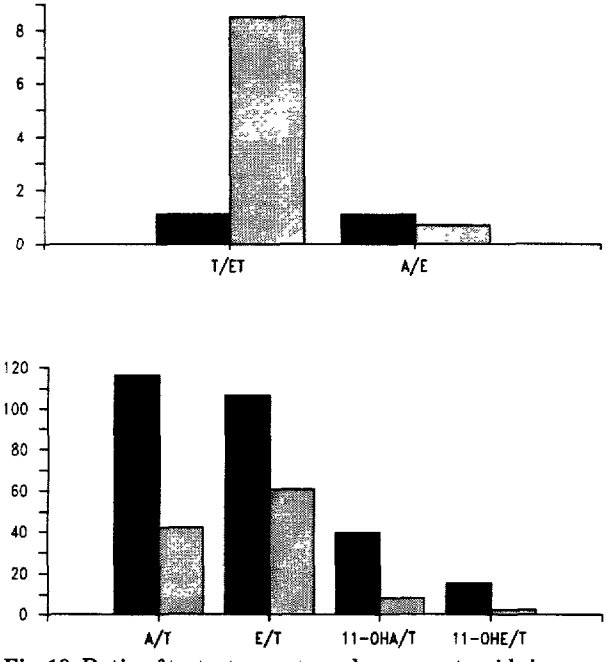


Fig. 19. Ratio of testosterone to endogenous steroids in normal urine (black) and in a sample positive to testosterone (gray). Steroids abbreviation as in Fig. 17. The normal ratio values are the mean of ratios from 200 urine samples from healthy males.

etiocholanolone ($M^{+\cdot}$ 522) peak centered at 23.95 min and epitestosterone yields chromatographically homogeneous T and ET peaks, thus permitting a highly specific and reproducible quantitation of both peaks.

The effect of exogenous testosterone on the metabolism of endogenous steroids is also very informative, in that it provides further evidence of the administration of the steroid. Fig. 19 compares the ratio of testosterone to several endogenous steroids which are urinary metabolites of testosterone both in a normal and a testosterone-positive urine sample. The increase of the T/ET ratio is accompanied by systematic decreases of A/T, E/T, 11-OHA/T and 11-OHE/T ratios due to testosterone upsurge following its administration. It is of interest to note that the A/E ratio in the positive sample is smaller than the normal ratio. This observation is in agreement with Hatton and Catlin's findings [54] to the effect that A/E ratios in high-risk-sport males are lower than in the normal population. Lower A/E ratios are apparently due to a decreased activity of 5$\alpha$-reductase, which transforms androstenedione into androsterone.

Samples for which a T/ET ratio greater than 6:1 was determined by preliminary GC–MS screening were submitted to a strict analytical protocol whereby chromatographic homogeneity of ET and T peaks was assessed and the T/ET ratio measured by SIM GC–MS on at least five aliquots of the urinary samples of interest. The details of the confirmation procedure have been described above.

*Drugs affecting the detection of urinary steroids*

It is of interest to note that other pharmaceutical agents are often taken in combination with anabolic steroids in order to elude a positive urinary test. In athletics, diuretics have been extensively misused, particularly in sports involving weight categories, to reduce weight prior to a competition or to deliberately dilute the urine specimen as an attempt to escape the drug test. It appears that diuretics do not exert any selective retention effect on anabolic steroids excretion in urine. The resulting effect of diuretic administration in humans appears to be a simple dilution of the urine specimen which is readily detected in GC–MS by a marked decrease of the urinary level of the steroids of interest. Studies in our laboratory indicate that diuresis does not have any significant effect on the relative excretion of epitestosterone and testosterone and the T/ET ratio remains fairly constant, varying within normal circadian ranges.

We also report in this issue a high-performance liquid chromatographic screening method for diuretic urinary metabolites which is currently used in our laboratory for doping control purposes [55].

It was reported recently [47] that probenecid, a uricosuric agent used for the treatment of hyperuricemia of gout, was used in athletics to escape the anabolic steroids test. In addition to its uricosuric effect, probenecid has the remarkable property to inhibit active tubular secretion of other organic acids by competing for some transport processes. The urinary excretion of steroids conjugated with glucuronic and sulfuric acid is also affected by probenecid, and a progressive decrease of the conjugated steroids is observed in urine following administration of the drug. The magnitude of this phenomenon parallels dosage and period of administration [47,48].

Urine specimens having a specific gravity lower than 1.010 are given special atttention. Larger volumes of the specimen (i.e. 10–50 ml) are usually extracted and derivatization is performed in smaller volumes of MSTFA (20–50 $\mu$l) in order to maximize detection sensitivity.

In view of their potential illicit uses in athletics, both diuretics and probenecid were recently added to the IOC Medical Commission list of banned drugs.

*Gas chromatographic–mass spectrometric screening of anabolic steroids*

The profile of urinary steroids in normal and healthy young adults is relatively complex [19,20]. In addition, the analytical imperatives of large-scale and routine GC–MS screening require that only a limited number of $m/z$ values specific to anabolic steroids and some endogenous steroids of interest should be monitored. GC–MS screening of anabolic steroids was thus based on SIM of ions which are highly specific to the steroids of interest. Since anabolic steroids elute within a range of ca. 20 min under the GC–MS conditions used, the selected ions were distributed into nine discrete groups according to the chromatographic retention of the TMS derivatives of the steroidal metabolites. Each ion group was then sequentially monitored over the corresponding elution range. Although not exhaustive, Table I summarizes some of the parameters used for GC–MS screening and lists some of the most widely used anabolic steroids. One will notice that ion fragmentograms are recorded for a small number of ions (one to four) per steroid in order to maximize screening sensitivity.

Data are first computer-analysed to locate the external standard, calculate relative retention time (RRT) and integrate peaks of interest at preselected $m/z$ and RRT values. This first step in data analysis provides information about the chromatographic and MS status of the GC–MS system used as well as on the level of the endogenous steroids of interest. The ion fragmentograms are then plotted in time-windows corresponding to those used for SIM screening, and searches for the potential presence of two, three or four peaks eluting in coincidence at proper RRT values are performed. The absence of coeluting peaks in a specific time-window is interpreted as the absence of the corresponding steroid in the urine specimen. On the other hand, the presence of coeluting peaks indicates the potential presence of a steroid, providing that their RRT values are in accordance with reference values obtained from human studies with the corresponding steroid. Ion peaks are then integrated and peak-ion ratios compared to those from reference compounds and urinary metabolites from human studies. Meanwhile, the sample is reinjected in both the SIM and repetitive scanning modes to assess identity by comparison of steroid profiles and to confirm data from preliminary screening. Next, two aliquots of the urine specimen are re-extracted, taking into account the features of the excretion profile of the steroid and the concentration of the metabolites in the specimen. Consequently the conjugated and/or unconjugated steroid fractions are preferentially isolated, larger volumes of urine are extracted if necessary and data acquisition is performed so as to increase sensitivity and specificity towards the steroids of interest. The profile of the urinary metabolites is then obtained using SIM and repetitive scanning of short and extended mass ranges and compared to that obtained from

human studies. The data are finally assessed and validated by comparison with data from reference compounds and urinary samples obtained from volunteers known to have taken a particular steroid.

In conclusion, we reviewed here the major aspects and underlying rationales of anabolic steroids urinary metabolites screening by GC–MS. Several methodoligal aspects regarding steroid extraction, hydrolysis and derivatization were described and potential artifacts involving the enzymatic hydrolysis of 5-en steroids and the chromatography of stanozolol metabolites were identified. The metabolism of several steroids was discussed and new metabolites of boldenone, methanedienone and stanozolol were reported.

The conversion of norethisterone into 19-nortestosterone was demonstrated and the effects of diuretics and probenecid on anabolic steroid detection in urine were briefly commented on. Finally, we described a specific GC–MS method used for large-scale and routine screening of anabolic steroids in urine, stressing the importance of unambiguous confirmation procedures for the assessment and validation of preliminary screening data.

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