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Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography–high-resolution mass spectrometry

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Abstract

The misuse of anabolic androgenic steroids (AAS) in human sports is controlled by gas chromatography–mass spectrometric analysis of urine specimens obtained from athletes. The analysis is improved with modern high-resolution mass spectrometry (HRMS). The detection and identification of metabolites of stanozolol (I) [3′-hydroxystanozolol (II) and 4β-hydroxystanozolol (III)] and metandienone (IV) [17β-methyl-5β-androst-1-ene-3α,17α-diol (V) and 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol (VI)] with GC–HRMS at 3000 resolution yielded a large increase in the number of positive specimens. A total of 116 anabolic steroid positives were found in this laboratory in 1995 via GC–MS and GC–HRMS screening of 6700 human urine specimens collected at national and international sporting events and at out-of-competition testing. Of the 116 positive cases, 41 were detected using conventional (quadrupole) GC–MS screening. The other 75 positives were identified via GC–HRMS screening. To confirm the HRMS screening result, the urine sample was reanalyzed using a specific sample workup procedure to selectively isolate the metabolites of the identified substance. II and III were selectively isolated via immunoaffinity chromatography (IAC) using an antibody which was prepared for methyltestosterone and shows high cross reactivity to II and III. V and VI were isolated using high-performance liquid chromatography (HPLC) fractionation.

Keywords: Metandienone; Stanozolol; Anabolic androgenic steroids; Steroids

1. Introduction

The use of anabolic androgenic steroids (AAS) in sports has been banned since 1976 by the International Olympic Committee (IOC) and National and International Sport Federations. This ban is controlled by the analysis of urine samples obtained from athletes, where the urinary excreted anabolic steroids and their metabolites are identified by gas chromatography (GC)–mass spectrometry (MS) [1–4]. In order that a large number of samples can be analyzed in a short time period, a general screening method which encompasses most AAS and their metabolites is used. In sample isolation, unconjugated and conjugated steroids with varying chemical properties are extracted from the urine. Following isolation, the steroids are derivatized to yield trimethylsilyl (TMS) derivatives which are well suited for GC–MS analysis with electron impact (EI) ionization. In the case of a positive screening result, meaning that a suspicious signal for an AAS or its metabolite is observed, the sample is worked up a

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second time using a method which is specific for its isolation, derivatization and GC–MS identification.

As AAS are mainly misused during training periods it is expected that athletes will discontinue their use at sometime prior to competition. This minimizes the chance that AAS or their metabolites can be detected in the urine of the athlete when controlled at a sporting event. To overcome this dilemma the analyst must increase the sensitivity and specificity of the analytical procedure. A second strategy, out-of-competition testing, has been introduced and has had some acceptance. Overall, however, out-of-competition testing is not uniform and international harmony has not been achieved, even for control of top level athletes. Unannounced tests are difficult to organize and the absence of an athlete, for example, training out of the country, prolongs the time between controls.

Improvement in the sensitivity and specificity of the GC–MS screening analysis, which is normally performed using a benchtop quadrupole mass spectrometer, is achieved by selected ion monitoring (SIM) for specific AAS and their metabolites. Distinct ions are recorded for each steroid and selected ion chromatograms are plotted over short time intervals around the elution time of the forbidden substance. This is the method used by all IOC accredited laboratories performing doping control analysis in human sports. The use of modern high-resolution mass spectrometry (HRMS) for the detection and identification of AAS and their metabolites in a general SIM screening procedure was introduced by Horning and Donike [5] in 1993. Prior to this time, HRMS was used in drug testing analysis for confirming results obtained by quadrupole mass spectrometers, e.g., by precise measurement of selected ions, and for obtaining mass spectra. The use of SIM with HRMS for routine analysis has recently become possible because of significant improvements in the design (ion optics) of double focusing mass spectrometers and the controlling electronics.

In the past year the advantage of HRMS as a routine screening technique for controlling the misuse of AAS became evident, especially for the identification of metabolites of stanozolol (I) and metandienone (IV) (Fig. 1). This is demonstrated by the large increase in the number of positive samples found in the Cologne laboratory in 1995 for 6700 urine specimens collected at national and international sporting events and at out-of-competition testing. In 116 positive cases for AAS misuse, 75 were identified solely using HRMS screening. Upon screening identification, confirmation of a positive case is done by reanalysis of the urine, where a specific workup procedure is used to isolate the substance in question. Here we present the methods implemented for HRMS screening and identification of stanozolol and metandienone metabolites.

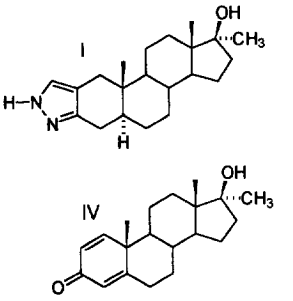

Fig. 1. Structures of stanozolol I and metandienone IV.

2. Experimental

2.1. Steroids and reagents

3′-Hydroxystanozolol [6], 4β-hydroxystanozolol [6], 4α-hydroxystanozolol [6], 17β-methyl-5β-androst-1-ene-3α,17α-diol [7], 17α-hydroxy-17β-methyl-5β-androst-1-en-3-one [7], *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) [8], and *N*-methyl - *N* - tertiarybutyldimethylsilyltrifluoroacetamide (MTBSTFA) [9] were synthesized in our laboratory. All other reagents and solvents were of analytical grade.

2.2. Isolation of steroids and their metabolites for GC–MS screening

Screening of AAS and their metabolites includes the analysis of conjugated and unconjugated excreted steroids, the so called total fraction. Steroids were isolated from urine (typically 2 ml) as described by Schänzer and Donike [4]. The isolated steroids were dried and derivatized (see Section 2.6).

2.3. Isolation of stanozolol and metandienone metabolites for GC–HRMS identification

To confirm the identification of stanozolol and metandienone metabolites, urine (4–16 ml) from a suspicious specimen was absorbed on an Amberlite XAD-2 column and the unconjugated steroids separated from the conjugated steroids [4]. After enzymatic hydrolysis of the conjugated steroids with β-glucuronidase from *Escherichia coli* (Boehringer, Mannheim, Germany), the extract was alkalized with 250 μl of an aqueous 20% buffer solution of K_2CO_3–$KHCO_3$ (1:1, w/w) to approximately pH 9.6. Stanozolol metabolites II and III were extracted with 5 ml of *tert.*-butylmethyl ether, whereas the metandienone metabolites V and VI were extracted with 5 ml of *n*-pentane. The organic layer was evaporated to dryness under reduced pressure and the residue dried. Stanozolol metabolites were further isolated via immunoaffinity chromatography (IAC) and metandienone metabolites V and VI isolated via HPLC.

2.4. Isolation of stanozolol metabolites II and III via immunoaffinity chromatography (IAC)

Stanozolol metabolites II and III were isolated from the urine extract using immunoaffinity chromatography. A 1-ml volume of immunoaffinity gel (binding capacity to methyltestosterone 250 ng) was added to an Econo column (Biorad, Munich, Germany) and the column stored at 4°C in PBS buffer (120 mmol NaCl, 2.7 mmol KCl, 10 mmol $Na_2HPO_4 \cdot 12H_2O$, adjusted to pH 7.4 with concentrated HCl) containing 0.05% of NaN_3. The following scheme was used for sample isolation: (i) the column was washed with 5 ml PBS buffer; (ii) the urine extract was dissolved in 5 ml PBS buffer, after first dissolving the dry residue in 50 μl methanol, and applied to the column; (iii) the column was washed once with 15 ml of a 15% methanol–water solution (15 g methanol plus bidistilled water to reach 100 ml total volume); (iv) the steroids were eluted from the column with 3 ml of a 60% methanol–water solution. Afterwards the column was washed with 5 ml of the 60% methanol–water solution, equilibrated with 10 ml PBS buffer containing 0.05% NaN_3 and stored.

2.5. Isolation of metandienone metabolites V and VI via HPLC

The dried urine extract was dissolved in 50 μl of methanol and injected onto a Hewlett-Packard (HP) Hypersil ODS precolumn (5 μm, 20×4 mm I.D.) connected to a Merck LiChrospher RP 18 (5 μm, 125×4 mm I.D.) column. HPLC (1090 HP) separation was performed as follows: solvent, (A) water, (B) acetonitrile; flow, 1.0 ml/min with a linear gradient increasing from 30% to 100% in concentration of B in 17.5 min; photodiode detector 190–600 nm, wavelength: 200 nm. Fractions eluting between 11.9 and 12.4 min, containing metabolite V (retention time 12.0 min), and between 17.5 and 18.2 min, containing metabolite VI (retention time 18.0 min) were collected in separate glass tubes, evaporated under reduced pressure, dried in a vacuum desiccator over KOH/P_4O_{10}, and derivatized for GC–MS analysis. To determine the elution times over which urine fractions were collected, 50 μl of a methanolic solution containing 200 ng of V and VI was analyzed. Prior to injection of a urine extract, two 50-μl methanol injections and one urine blank injection were made to eliminate the possibility of sample carryover.

2.6. Derivatization for GC–MS analysis

2.6.1. GC–MS screening

For GC–MS screening N-TMS and O-TMS derivatives were formed. The dried sample was dissolved in 100 μl of MSTFA–NH_4I–ethanethiol (100:0.2:0.6, v/w/v), which is equivalent to a mixture of MSTFA–trimethyliodosilane (TMIS) (1000:2, v/v) [9], and heated at 60°C for 15 min.

2.6.2. GC–HRMS identification of stanozolol metabolites II, III and metandienone metabolite V

Derivatization of II, III and V yielding per-TMS derivatives was achieved by dissolving the dried sample in 50 μl of MSTFA–imidazole (Imi) (100:2, v/w) and heating for 15 min at 60°C [1]. For analysis of the stanozolol metabolites II and III, an internal standard, 4α-hydroxystanozolol, was added to the derivatizing reagent at a concentration of 0.1 ppm (5 ng in 50 μl).

2.6.3. GC–HRMS identification of metandienone metabolite VI

Derivatization of VI yielding the tertiarybutyldimethylsilyl (TBS) ether was carried out by dissolving the dried sample in 50 μl of MTBSTFA–imidazole (100:2, v/w) and heating at 60°C for 15 min.

2.7. GC–MS screening analysis

GC–MS screening analysis was performed with a HP 5971A quadrupole mass spectrometer coupled to a HP 5890 gas chromatograph. A HP Ultra 1 crosslinked methyl silicone capillary column was employed; length 17 m, I.D. 0.2 mm, film thickness 0.11 μm, helium carrier gas at a flow of 1.0 ml/min. A 3-μl aliquot of sample was injected into the GC system which was operated in the split (1:10) mode. The GC temperature was ramped as follows: initial temperature 180°C, program rate 3°C/min to 229°C, program rate 40°C/min to 310°C. The injection port and transfer line were heated to 300°C and 320°C, respectively.

The steroid TMS derivatives were analyzed using selected ion monitoring (SIM) with 70 eV EI ionization. Seven groups, containing between 16 and 20 ions, were recorded with scan cycle times between 0.55 and 0.66 s. Metandienone metabolite V was registered in the group from 7.75 to 11.45 min with the ions m/z 216, 358 and 448 (20 ms dwell times). Stanozolol metabolites II and III were registered in the group from 18.1 to 20.0 min with the ions m/z 254, 545 and 560.

2.8. GC–HRMS screening analysis

High-resolution mass spectrometry analysis was performed with a reverse geometry double focusing mass spectrometer (Finnigan MAT 95, Bremen, Germany) coupled to a HP 5890 gas chromatograph. A HP Ultra 1 column or SGE (Weiterstadt, Germany) OV1 (crosslinked methyl silicone capillary column, length 17 m, I.D. 0.25 mm, film thickness 0.1 μm) was employed with helium carrier gas (flow 1.0 ml/min). A 2-μl volume of sample was injected into the GC operated in the split (1:20) mode. The GC temperature was ramped as follows: initial temperature 185°C, program rate 5°C/min to 245°C, then program rate 20°C/min to 310°C. The injection port and transfer line were heated to 300°C.

Ions were formed by 65 eV EI ionization (1 mA emission current). The ion source was held at 220°C. High-resolution selected ion monitoring was performed by electric field switching (multiple ion detection in the ESCAN mode) using a reference gas (perfluorophenanthrene) for mass locking and mass calibration. The instrument was tuned so that there was less than 30% loss in the ion signal as the acceleration voltage was decreased by a ratio of 1.5. In the ESCAN a narrow mass range (mass ratio smaller than 1.6) was analyzed to attain maximum sensitivity. Four groups containing between 6 and 12 ions were registered with scan cycle times of 0.45 s. The mass resolution was 3000. The electron multiplier voltage was set to 1.7 kV (corresponding to a gain of 10^6) and the conversion dynode to −18 kV.

2.9. GC–HRMS identification of stanozolol metabolites II and III

Stanozolol metabolites II and III were confirmed using a specific analysis procedure. The GC temperature was ramped as follows: initial temperature 200°C, program rate 15°C/min to 320°C. At a mass resolution between 3000 and 5000 the following ions were monitored via electric field switching (lock mass 454.9728) m/z 560.3650, 545.3415, 520.3462, 472.3305 and 471.3227. Following elution of the internal standard, 4α-hydroxystanozolol, the ions m/z 218.1158 and 231.1236 (for 16β-hydroxystanozolol) were monitored (lock mass 180.9888). The scan cycle time was 0.4 s. All other instrument parameters were the same as above.

To record mass spectra the same GC temperature program was used but injection was made in the splitless mode. The magnet was scanned exponentially from m/z 65 to 650 in 0.7 s (total scan cycle time 0.9 s). Data were collected in the centroid mode at 1200–1500 resolution. One or two scans were averaged and background signal from the GC injection port and column subtracted.

2.10. GC–HRMS identification of metandienone metabolites V and VI

Metandienone metabolites V and VI were confirmed using specific analysis procedures. In both analyses the GC temperature was ramped as follows: initial temperature 130°C, program rate 10°C/min to 240°C, then 60°C/min to 320°C. Splitless injection was used. For identification of metandienone metabolite V the following ions were monitored via electric field switching (lock mass 366.9792) *m/z* 448.3192, 433.2958, 358.2692 and 343.2457 at a scan cycle time of 0.4 s. For metabolite VI (TBS derivative) the ions *m/z* 400.3161 385.2927, 343.2457, 267.2113 and 253.1956 were monitored (lock mass 242.9856). Mass spectra were recorded using the same GC temperature program (splitless injection). The magnet was scanned exponentially from *m/z* 65 to 550 in 0.7 s (total scan cycle time 0.9 s).

2.11. Synthesis of 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol VI

Metabolite VI was synthesized as a reference substance for GC–HRMS analysis and to determine the retention time for HPLC fractionation: 5 mg of 17α-hydroxy-17β-methyl-5β-androst-1-en-3-one was dissolved in 1 ml of acetonitrile–trifluoroacetic acid (90:10, v/v) and heated for 1 h at 60°C. The sample was evaporated to dryness under reduced pressure. The reaction yielded more than 95% single product (GC–MS analysis), 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3-one. This product was identical with the main rearrangement product in the 17-epimerization of 17β-hydroxy-17α-methyl-5β-androst-1-en-3-one [10]. The dried reaction product was not further purified and dissolved in 1 ml of absolute dry tetrahydrofurane.

Addition of 20 mg of lithium aluminum hydride yielded reduction of the 3-keto group with over 90% to the 3α-hydroxy configuration. The reaction mixture was diluted with 2 ml of water and extracted with 5 ml of *tert.*-butylmethyl ether. The organic layer was separated and dried over sodium sulphate. The reaction yielded with 90% 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol VI.

3. Results and discussion

3.1. Stanozolol

3.1.1. Stanozolol metabolism

GC–MS screening and identification of stanozolol is based on the main excreted metabolites which are detected for a much longer period of time in urine than the parent steroid itself. The metabolism of stanozolol in human was investigated [6,11] and the main excreted metabolites were synthesized as reference compounds [6]. Metabolites which are detected for the longest time after a single oral administration are 3′-hydroxystanozolol (II), 4β-hydroxystanozolol (III) and 16β-hydroxystanozlol (Fig. 2).

3.1.2. Isolation of stanozolol metabolites from urine, sample purification via IAC

The main stanozolol metabolites are excreted as glucuronide conjugates which can be hydrolyzed with β-glucuronidase from *Escherichia coli.* The metabolites are extracted from urine at a moderately alkaline pH of 9–10 with *tert.*-butylmethyl ether. The pH should not be higher than 10 as the 3′-hydroxy group is acidic (pyrazol ring) and extraction yields are decreased at higher pH values. For additional sample cleanup the urine extract is separated via immunoaffinity chromatography (IAC) [12,13]

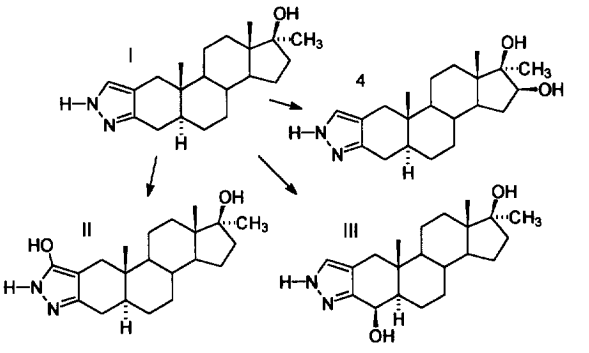

Fig. 2. Metabolism of stanozolol (I) to its main excreted metabolites: 3′-hydroxystanozolol (II), 4β-hydroxystanozolol (III) and 16β-hydroxystanozolol (4). All metabolites are excreted as conjugates which are hydrolyzed with β-glucuronidase from *E. coli.* Positions of the glucuronide conjugates are unknown.

with an antibody raised against methyltestosterone. The antibody was raised in rabbits and was directed towards methyltestosterone-3-carboxymethyloxime-bovine serum albumin. After isolation the antibodies were immobilized on a chemically modified agarose gel and filled in columns. The antibodies show high cross reactivity to other 17β-hydroxy-17α-methyl steroids [14] and can therefore be used for isolation of stanozolol and its metabolites. The immunoaffinity column, when properly handled, is very stable and up to now has been used for several years without any noticeable loss in performance.

3.1.3. Derivatization of stanozolol metabolites and GC analysis

In the general screening analysis the extracted steroids are derivatized with MSTFA–TMIS generating also TMS enol-ethers. For stanozolol metabolites II and III, steroids without a keto group, full derivatization can be accomplished with MSTFA–imidazole reagent. The advantage of this reagent is the long term stability of the derivatized sample. The TMIS catalyzed reaction mixture should be protected from light and additional secondary reactions, resulting from iodine reaction products, can occur over time. For GC–HRMS analysis of II and III after IAC isolation, an internal standard, 4α-hydroxystanozolol, is added to the MSTFA–imidazole reagent.

GC analysis of stanozolol and its metabolites using a crosslinked methyl silicone capillary column shows that the TMS-derivatives of II and III are more stable than I and 16β-hydroxystanozolol. Fig. 3 depicts a GC–HRMS chromatogram of a standard mixture (0.1 ng/μl each) of pertrimethylsilylated I, II, III and 16β-hydroxystanozolol recorded under ideal GC conditions. Under normal GC conditions 16β-hydroxystanozolol is not detected at this concentration and the response of stanozolol (*m/z* 472.3305) is much lower than that of II and III (*m/z* 560.3650). As the performance of the GC column diminishes, the response of II drops with respect to III. The stability of the stanozolol metabolites as their TMS derivatives is still a great problem. For this reason reference substances are of high importance to monitor GC conditions.

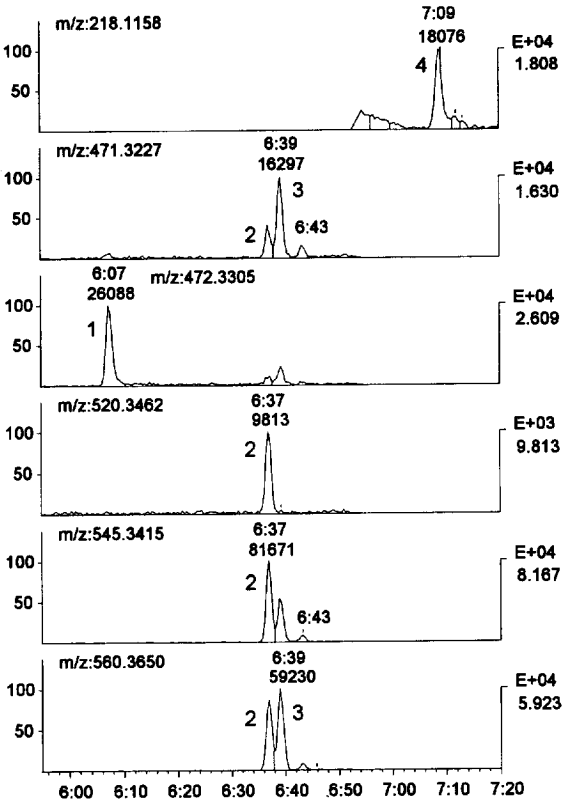

Fig. 3. GC–HRMS ion chromatograms of stanozolol (1) and its metabolites 3′-hydroxystanozolol (2), 4β-hydroxystanozolol (3) and 16β-hydroxystanozlol (4) recorded by ESCAN in the multiple ion detection mode at 3000 resolution. A 200-pg amount of each compound was injected in the split mode (1:20), meaning 10 pg of each compound on column. The peak at 6:43 min corresponds to the internal standard (4α-hydroxystanozolol, at 0.01 ng/μl).

3.1.4. GC–HRMS analysis of stanozolol metabolites II and III

In the GC–HRMS screening for AAS and their metabolites characteristic ions (molecular and/or fragment ions) are registered and the ion intensities are plotted over short time windows to simplify the detection of a banned substance. For stanozolol metabolites II and III the ions *m/z* 560.3650 [M^+ $C_{20}H_{25}N_2O{\cdot}(SiC_3H_8)_3$], 545.3415 [$M^+-CH_3$] and 520.3462 [$M^+-C_2H_2N$] are recorded and plotted, as shown in Fig. 4. Here the result of a screening analysis for a routine urine sample found positive for stanozolol is shown for screening with the HRMS instrument (A) and the quadrupole mass spectrometer

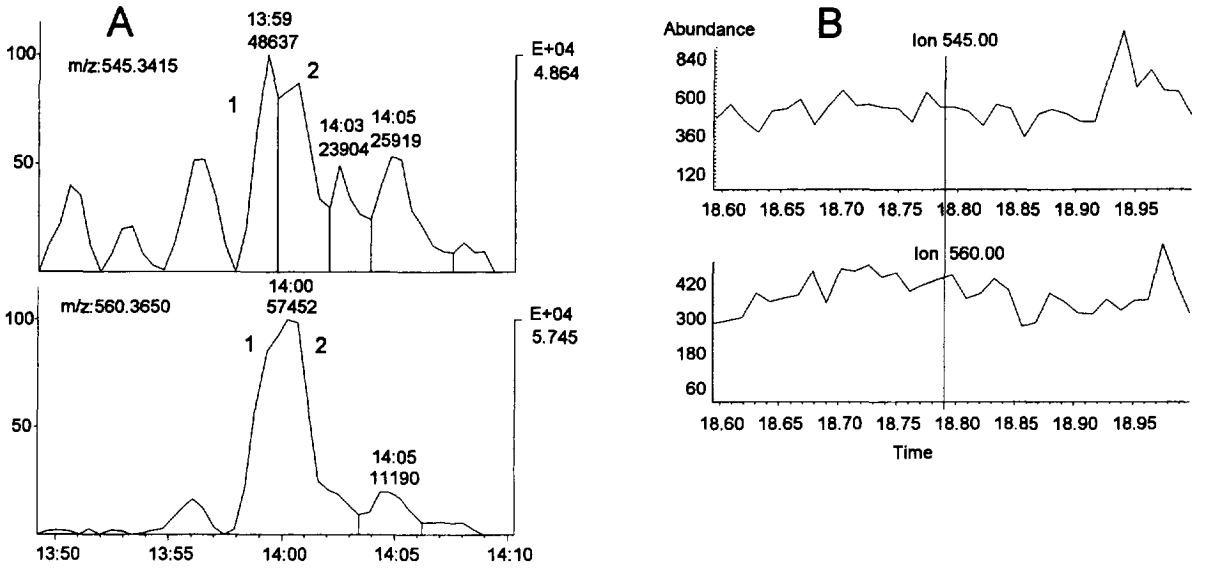

Fig. 4. GC–HRMS (A) and GC–MS (B) screening results of a urine sample (2 ml) containing stanozolol metabolites 3′-hydroxystanozolol (1) and 4β-hydroxystanozolol (2), eluting at 14:00 min (A) and expected at 18.79 min (B), as indicated by the solid line.

(B). The GC temperature program used in the routine screening analysis does not allow for separation of II and III. When a suspicious sample is identified, the sample is reanalyzed using a fast ramping GC temperature program which enables separation of the two metabolites and gives more information on the likelihood of stanozolol metabolites being present in the urine. This is illustrated in Fig. 5A. Interestingly, in all urine samples found positive for stanozolol, metabolites II and III have been detected in nearly equal amounts. Never has a case been found where only one metabolite was present.

To identify and confirm the presence of II and III, the urine sample is reanalyzed by immunoaffinity chromatography preparation to selectively isolate the steroids. As evident in Fig. 5B, the only ion traces registered following IAC isolation are those due to the stanozolol metabolites. Biological material which leads to the background ion signals in Fig. 5A is removed. An interesting feature is the presence of the fragment ion m/z 520.3462 for 3′-hydroxystanozolol (loss of C_2H_2N), which is a very specific indicator of the presence of II.

Mass spectra recorded for II and III after extraction from urine and following IAC isolation are shown in Fig. 6 for 3′-hydroxystanozolol tris–TMS and Fig. 7 for 4β-hydroxystanozolol tris–TMS. Without IAC isolation it is not possible to confirm the presence of the stanozolol metabolites because of the high level of biological background which coelutes with the stanozolol metabolites. After IAC isolation, the coeluting biological background is removed and the mass spectra obtained unambiguously demonstrate the presence of the metabolites.

3.2. Metandienone

3.2.1. Metandienone metabolism

The metabolism of metandienone was published in 1963 by Segaloff and Rongone [15] who confirmed 6β-hydroxymetandienone and 17-epimetandienone as the main excreted metabolites in human. In 1980 Dürbeck and Bücker [16] presented a GC–MS method for the determination of unconjugated excreted metandienone metabolites, including the 6β-hydroxy and 17-epi products already described. Further studies by Schänzer et al. [7] confirmed the presence of conjugated excreted metabolites, such as V (Fig. 8), which could be detected for a much

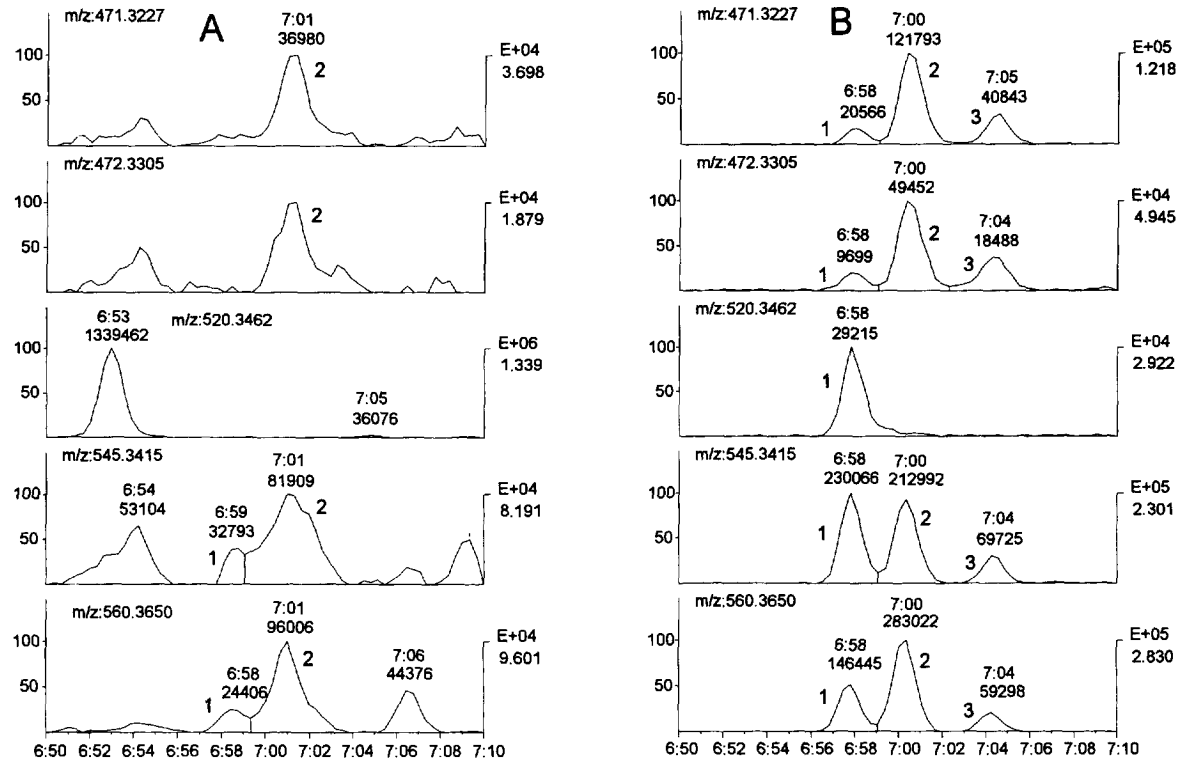

Fig. 5. GC–HRMS analyses of a urine sample containing stanozolol metabolites II (1) and III (2). (A) Liquid–liquid extraction (2 ml urine) and (B) IAC isolation (10 ml urine). The sample used to obtain the data shown in A was the same sample as in Fig. 4. Analyses were performed using a fast ramping GC temperature program (split mode, 1:20). Peak 3, eluting at 7:04 min in B corresponds to the internal standard (4α-hydroxystanozolol) added to the derivitization mixture (0.1 ng/μl).

longer period of time than the unconjugated metabolites. Based on metabolic studies, V has been shown to be a long term excreted metabolite. By examining the presence of V in urine, metandienone administration can be retrospectively monitored over a long time period.

Generation of V is shown in Fig. 8. In excreted urine the 17β-sulfate of 17α-methyl-5β-androst-1-ene-3α,17β-diol-3-β-glucuronide (3) undergoes spontaneous desulfonation. Loss of sulfate and consequent epimerization (or rearrangement) occurs for all 17β-sulfate conjugates and is an energetically favored process since it relieves steric strain at the tertiary C-17 atom. This reaction process was first described by Edlund et al. [17] in 1989 in the metabolism of metandienone in the horse. Further studies confirming the proposed mechanism were published [10,18,19]. In the generation of the 17β-epimer, a process proceeding via a carbonium ion intermediate formed upon desulfonation and subsequent attack by water, dehydrogenation products accompanied by rearrangement are also formed. One of the major rearrangement products is the 18-nor-17,17-dimethyl steroid VI (18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol). This 18-nor product of metandienone metabolism is present in all urine samples in which metabolite V is found. The concentration of VI cannot be estimated exactly, but as expected it is approximately the same as that of metabolite V.

3.2.2. Isolation of metandienone metabolites from urine, sample preparation via HPLC

Metandienone metabolites V and VI (formed in excreted urine via spontaneous desulfonation as illustrated in Fig. 8) are conjugated as glucuronides

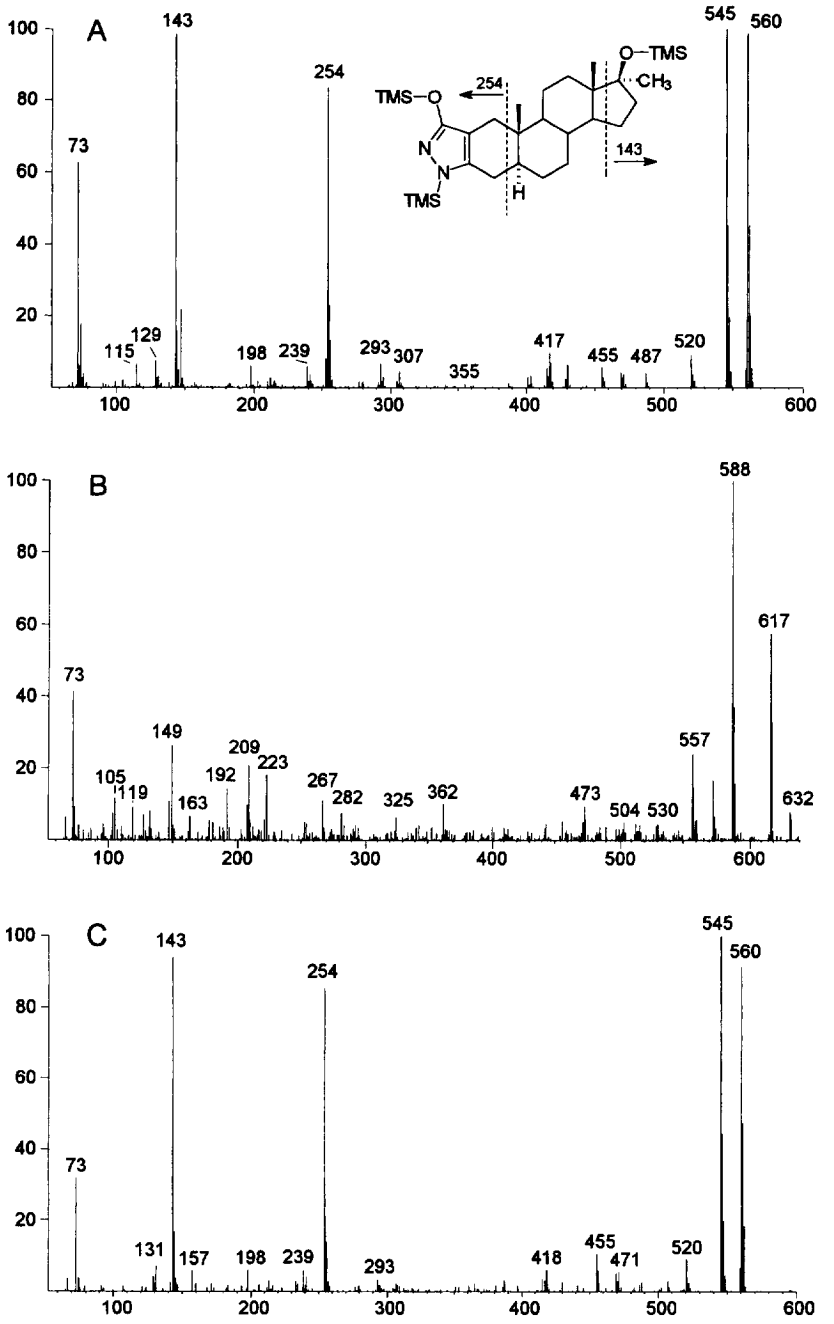

Fig. 6. EI mass spectra of 3′-hydroxystanozolol tris–TMS (II) extracted from urine by liquid–liquid extraction (B) and by IAC isolation (C). Synthesized reference standard (M^+ 560) is shown in (A). Analyses were performed using a fast ramping GC temperature program. Injection in B (split mode 1:20) and C (splitless).

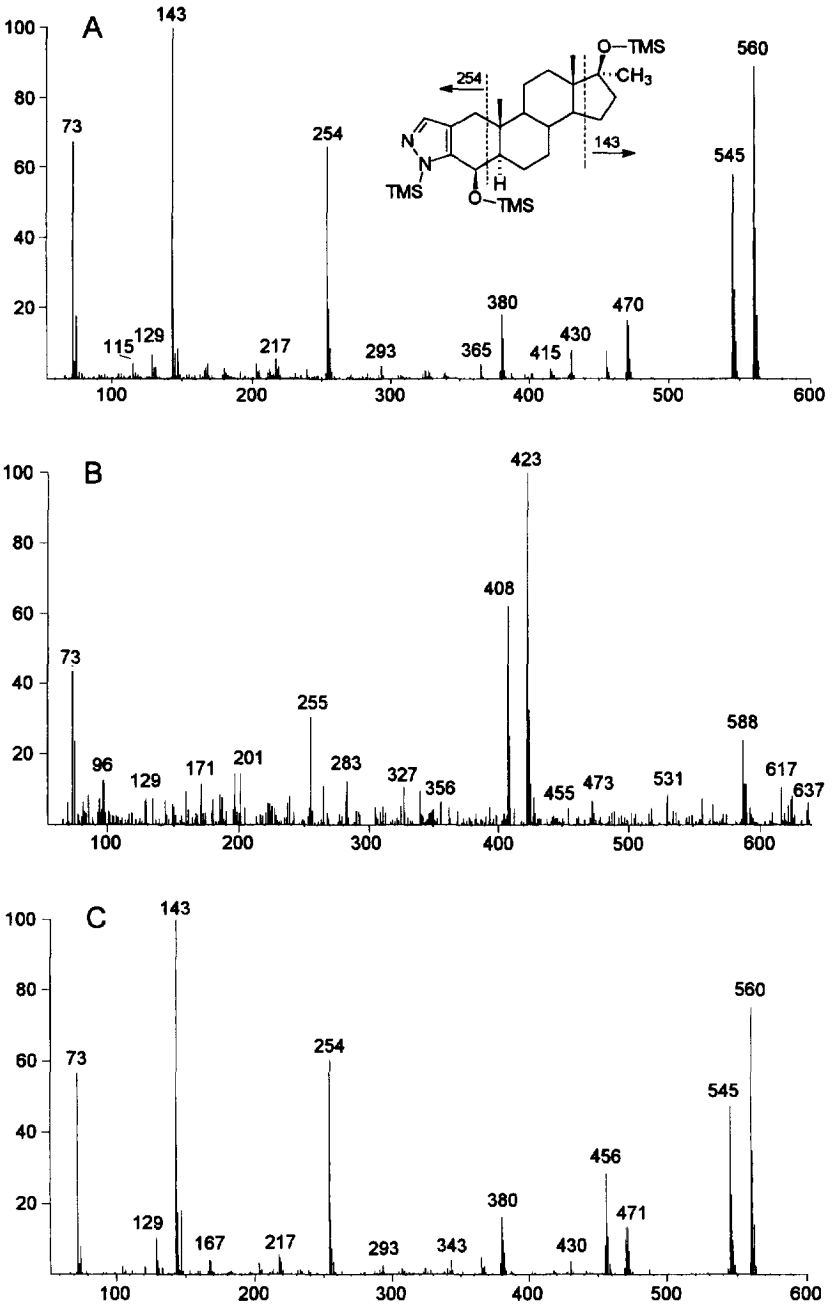

Fig. 7. EI mass spectra of 4β-hydroxystanozolol tris–TMS (III) extracted from urine by liquid–liquid extraction (B) and by IAC isolation (C). Synthesized reference standard (M^+ 560) is shown in (A). Analyses were performed using a fast ramping GC temperature program. Injection in B (split mode 1:20) and C (splitless).

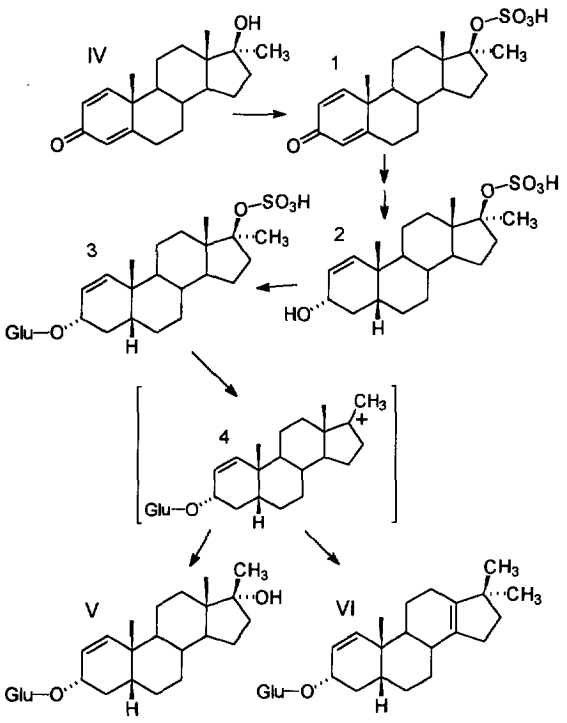

Fig. 8. Metabolism of metandienone IV to long-term excreted metabolites: 17β-methyl-5β-androst-1-ene-3α,17α-diol (V); 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (VI), Glu=glucuronide.

at the 3α-hydroxy position and must be hydrolyzed with β-glucuronidase from *Escherichia coli*. After hydrolysis they can be isolated quantitatively by liquid–liquid extraction with *n*-pentane. The extract is further separated by HPLC using an analytical reversed-phase C_{18} column.

HPLC analysis of V and VI is hampered by the weak UV adsorption (200 nm) of the allylic 3-hydroxy group. To determine the elution times a large amount of standard (200 ng of V and VI) must be injected onto the HPLC column. The standard is injected in triplicate to determine the exact retention times of V and VI for the subsequent fractionations. Slight contamination from sample carryover is eliminated by injection of successive methanol blanks. After injection of three methanol solutions, the last of which is fractionated and analyzed by GC–HRMS, a urine blank (known not to contain metabolites of metandienone) is fractionated and finally the suspicious urine sample is injected onto the HPLC column.

3.2.3. Derivatization of metandienone metabolites and GC analysis

Derivatization of V and VI for GC–MS screening analysis is performed using MSTFA–TMIS. This derivatization procedure was discussed for stanozolol. For confirmation of V and VI derivatization can be accomplished using MSTFA–imidazole. This works well for metabolite V; however, for the TMS derivative of VI there is a large interference from stearic acid which elutes prior to VI. The high concentration of stearic acid normally found in the sample (even after HPLC fractionation) makes it difficult, if not impossible, to record mass spectra. To circumvent this problem, the TBS derivative of VI is formed using MTBSTFA–imidazole. Note that in the general screening analysis the stearic acid TMS signal is observed in the ion trace for metabolite VI (*m/z* 358.2692) and is a useful biological marker for identifying VI.

3.2.4. GC–HRMS analysis of metandienone metabolite V

A GC–HRMS screening analysis for V using ions *m/z* 448.3192 [M^+ $C_{20}H_{30}O_2 \cdot (SiC_3H_8)_2$] and 358.2692 [$M^+ - C_3H_{10}OSi$] at 3000 resolution is depicted in Fig. 9A. Shown is a urine sample (2 ml) spiked with 2 ng/ml each of V and VI. The selected ion monitoring ion chromatograms for V recorded with the quadrupole mass spectrometer are shown in Fig. 9B. The 4-ng sample (4 pg injected onto the GC column) leads to a well defined signal at *m/z* 358.2692 which can easily be distinguished from biological background. The long retrospectivity following metandienone administration is in part due to the low level of biological background at the *m/z* 358.2692 ion. It is of interest to note that a signal is sometimes observed in the low mass resolution GC–MS analysis at the same retention time as V (due to a metabolite of diclofenac), but when the sample is analyzed using GC–HRMS no signal is observed, illustrating that GC–HRMS can be used to negating a finding by quadrupole GC–MS.

To assist in the identification and confirmation of V, the urine (typically 8 ml) is reanalyzed using HPLC fractionation to selectively isolate V as well as

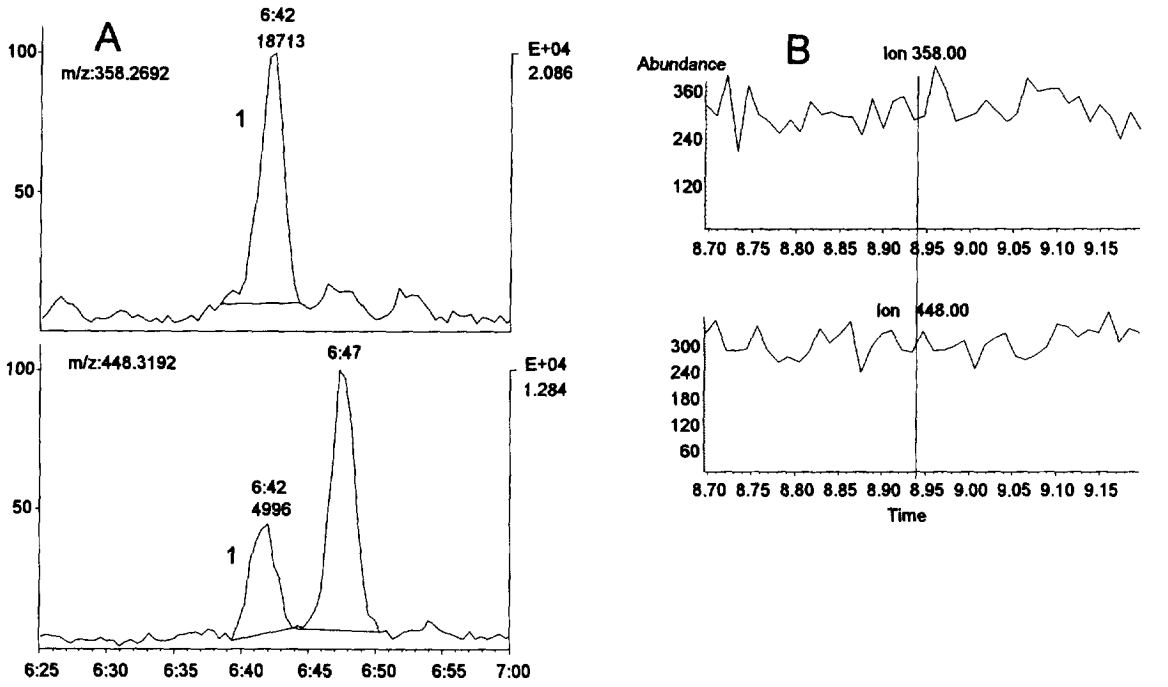

Fig. 9. GC–HRMS (A) and GC–MS (B) screening results of a urine sample (2 ml) containing metandienone metabolite (V) 17β-methyl-5β-androst-1-ene-3α,17α-diol (2 ng/ml), eluting at 6:42 min (A, peak 1) and expected at 8.94 min (B), as indicated by the solid line.

VI. When a large portion of the biological matrix is removed it is possible to analyze the sample using splitless injection. As expected, the signal increase is at least a factor of 20 (typically 30) when splitless injection is used. The suspicious urine sample, after HPLC isolation, is analyzed via selected ion monitoring to confirm the presence of V.

Mass spectra recorded for V extracted from urine using a liquid–liquid extraction (2 ml urine, GC split 1:20) and following HPLC isolation (8 ml, GC splitless) are shown in Fig. 10B and 10C, respectively. The major fragment ions (*m/z* 143 and 358) for V (Fig. 10B) are observed in the 2-ml urine extract at this concentration (2 ng/ml) but the mass spectrum is not indicative of the presence of V. There is a dramatic improvement seen in Fig. 10C, where, due to the removal of biological matrix with HPLC fractionation, the sample can be injected in the splitless mode. The mass spectrum unambiguously demonstrates the presence of the metandienone metabolite V (Fig. 10C), which is identical with the mass spectrum of the reference compound shown in Fig. 10A.

3.2.5. GC–HRMS analysis of metandienone metabolite VI

The TMS derivative of metandienone metabolite VI elutes shortly after the TMS derivative of stearic acid ($M^{+\cdot}$, *m/z* 356.3110). At 3000 resolution an isotope for stearic acid is observed in the ion trace for VI (M^{+} *m/z* 358.2692). Observation of VI during routine GC–HRMS screening assists in the identification of metabolite V. Since the inclusion of VI in the screening analysis more than 85% of the suspicious samples which are then isolated using HPLC fractionation are found to contain metandienone metabolites.

As the TMS derivative of VI elutes shortly after stearic acid and this interferes in the mass spectral analysis, a TBS derivative is used. The TBS derivatives of VI and stearic acid are well separated using the corresponding GC temperature program. Mass

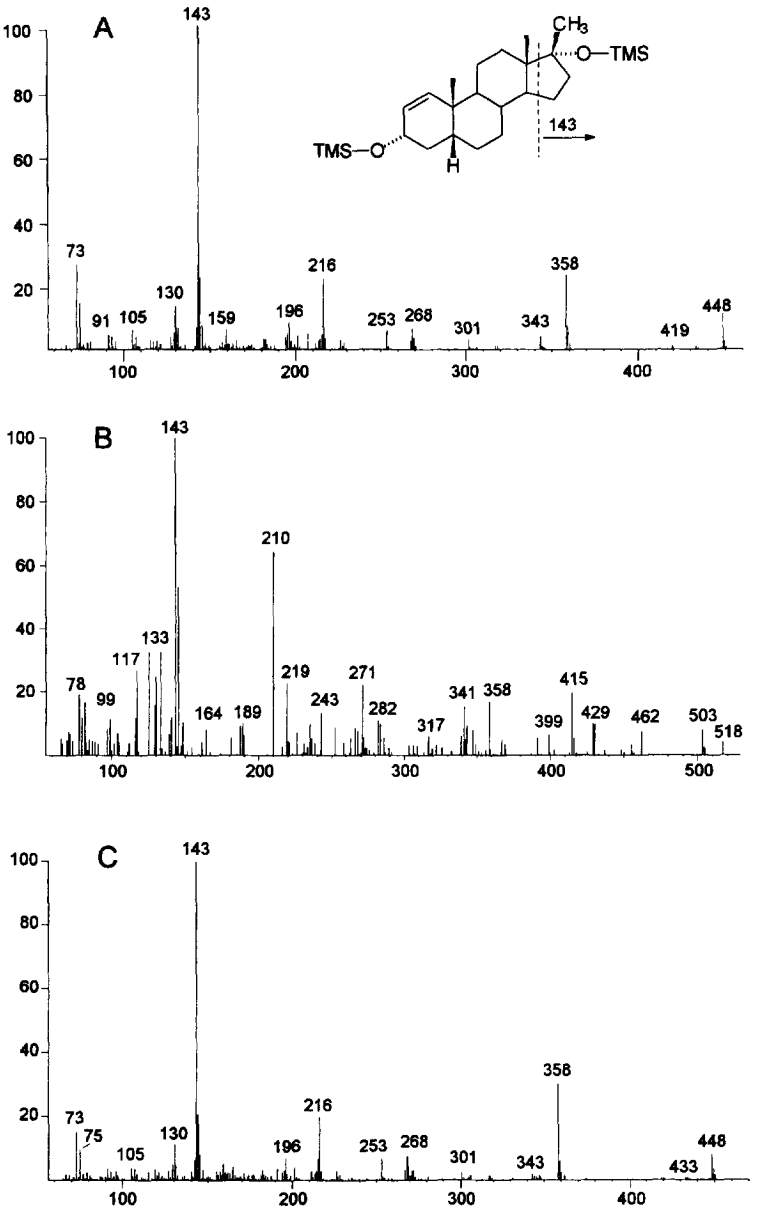

Fig. 10. EI mass spectra of 17β-methyl-5β-androst-1-ene-3α,17α-diol bis-TMS (V). Urine (2 ml) liquid–liquid extraction (B) and urine (8 ml) extraction followed by HPLC fractionation (C). Synthesized reference standard (M^+ 448) is shown in (A). Injection in B (split mode 1:20) and C (splitless).

spectra of the TMS and TBS derivatives of VI are shown in Fig. 11.

To identify and confirm the presence of VI in urine HPLC fractionation is used. Normally both metabolites V and VI are automatically collected during the HPLC analysis. Fig. 12 depicts selected ion chromatograms for the TBS derivative of VI (2 ng/ml) isolated from 8 ml of urine using HPLC fractionation. GC–HRMS screening for metandienone metabolite VI utilizes the ions m/z 400.3161

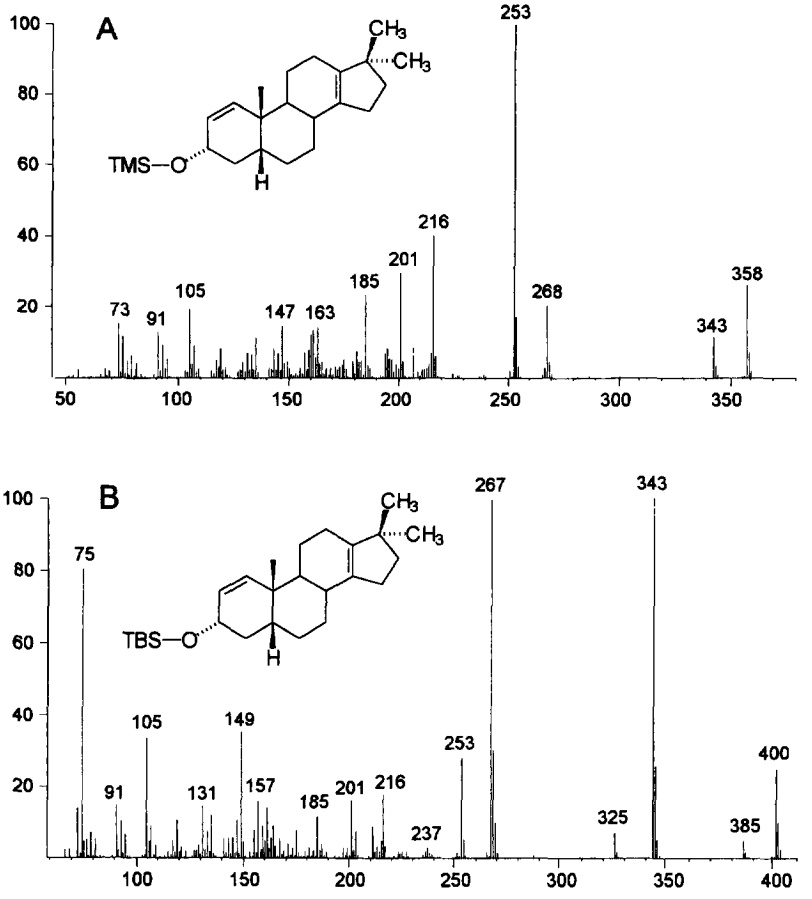

Fig. 11. EI mass spectra of 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (VI) as its (A) TMS (M^+ 358) and (B) TBS derivative (M^+ 400).

$[M^+C_{20}H_{30}O{\cdot}SiC_6H_{14}]$, 385.2927 $[M^+-CH_3]$ 343.2457 $[M^+-C_4H_9]$, 267.2113 $[M^+-C_6H_{15}SiOH_2]$ and 253.1956 $[M^+-C_7H_{18}SiOH]$. An advantage of the TBS derivative of VI is that there are several fragment ions present and at high abundance which assist in discriminating from the biological background that is present even after HPLC fractionation.

4. Conclusion

Identification of anabolic steroids in human urine is greatly improved using modern high resolution mass spectrometry (GC–HRMS) as a routine screening method, as compared to conventional quadrupole GC–MS. GC–HRMS selected ion analyses are performed at 3000 resolution using multiple ion detection in the electric scan mode with continuous mass locking and calibration using fragment ions generated from a fluorocarbon reference compound. Screening identification of the stanozolol metabolites [3′-hydroxystanozolol (II) and 4β-hydroxystanozolol (III)] and metandienone metabolites [17β-methyl-5β-androst-1-ene-3α,17α-diol (V) and 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol (VI)] with GC–HRMS resulted in a large increase in the number of positive samples in the Cologne Laboratory. In 116 positive cases for AAS misuse reported in 1995, 75 were identified solely using HRMS screening.

Upon screening identification, the initial finding is

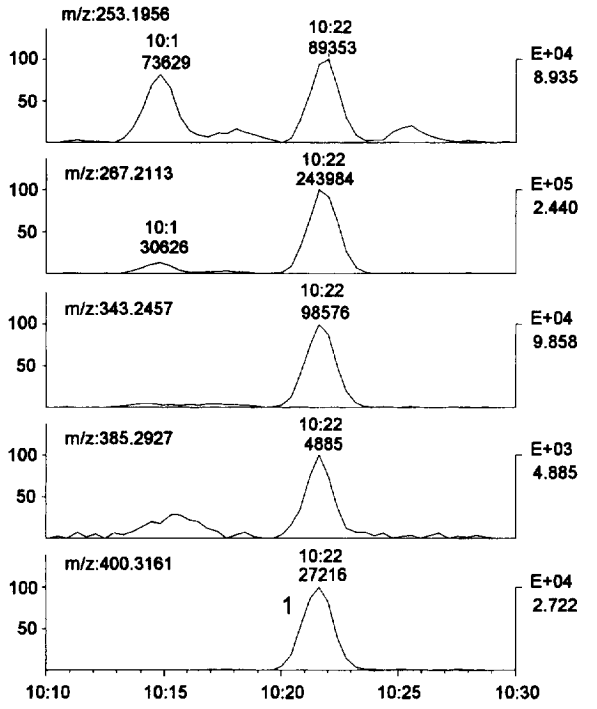

Fig. 12. GC–HRMS analysis of a urine sample containing metandienone metabolite (2 ng/ml) 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (VI), eluting at 10:22 min. VI extracted from urine (8 ml), fractionated using HPLC and derivatized to form the TBS derivative. Injection made in the splitless mode.

confirmed using a specific workup procedure to isolate the suspicious substance. The stanozolol metabolites are isolated from the hydrolyzed urine extract via immunoaffinity chromatography (IAC) with an antibody raised against methyltestosterone that show high cross reactivity to 17β-hydroxy-17α-methyl steroids. The immunoaffinity column, when properly handled, storage at 4°C in PBS buffer containing 0.05% of NaN_3, is very stable and can be reused several times. Metandienone metabolites are isolated from the hydrolyzed conjugated steroids using HPLC fractionation. Following isolation from the urine extract, the sample is sufficiently clean so that GC analyses can be carried out in the splitless mode. This is especially advantageous for obtaining mass spectra of the steroid metabolites.

It is important to note that the samples screened using GC–HRMS are not specially prepared and are the same as those analyzed by conventional GC–MS. The sample extraction procedure is a general method which encompasses unconjugated and conjugated steroids, as well as substances with similar chemical properties. Only upon identification of a suspicious sample a selective isolation procedure is utilized.

To enhance the sensitivity of the general screening procedure, selective isolation procedures could be implemented, especially in the case of stanozolol metabolites where detection is hampered by high biological background. An ideal screening method would be the use of a series of immunoaffinity gels with antibodies against all anabolic steroids and their metabolites in combination with GC–HRMS analysis.

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