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Metabolism of androsta-1,4,6-triene-3,17-dione and detection by gas chromatography/mass spectrometry in doping control

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The urinary metabolism of the irreversible aromatase inhibitor androsta-1,4,6-triene-3,17-dione was investigated. It is mainly excreted unchanged and as its 17β-hydroxy analogue. For confirmation, 17β-hydroxyandrosta-1,4,6-trien-3-one was synthesized and characterized by nuclear magnetic resonance (NMR) in addition to the parent compound. In addition, several reduced metabolites were detected in the post-administration urines, namely 17β-hydroxyandrosta-1,4-dien-3-one (boldenone), 17β-hydroxy-5β-androst-1-en-3-one (boldenone metabolite), 17β-hydroxyandrosta-4,6-dien-3-one, and androsta-4,6-diene-3,17-dione. The identification was performed by comparison of the metabolites with reference material utilizing gas chromatography/mass spectrometry (GC/MS) of the underivatized compounds and GC/MS and GC/tandem mass spectrometry (MS/MS) of their trimethylsilyl (TMS) derivatives. Alterations in the steroid profile were also observed, most obviously in the androsterone/testosterone ratio. Even if not explicitly listed, androsta-1,4,6-triene-3,17-dione is classified as a prohibited substance in sports by the World Anti-Doping Agency (WADA) due to its aromatase-inhibiting properties. In 2006 three samples from human routine sports doping control tested positive for metabolites of androsta-1,4,6-triene-3,17-dione. The samples were initially found suspicious for the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one. Since metabolites of androst-4-ene-3,6,17-trione were also present in the urine samples, it is presumed that these findings were due to the administration of a product like 'Novedex Xtreme', which could be easily obtained from the sport supplement market. Copyright © 2008 John Wiley & Sons, Ltd.

Several steroids have been available on the internet as sport supplements for some time, often sold through bodybuilding sites. In addition to classical anabolic androgenic steroids like metandienone or stanozolol, more preparations that appear on the market contain steroids that have never been marketed legally.[1–8] Recently, steroidal aromatase inhibitors were also marketed as supplements.[9–12] Products labeled as containing androsta-1,4,6-triene-3,17-dione have also been found on the market.[13–15] Androsta-1,4,6-triene-3,17-dione has been reported to effectively reduce estrogen biosynthesis by irreversible aromatase inhibition.[16,17] Willoughby *et al*.[13] investigated the effect of the supplement Novedex XT (labeled as containing 6,17-keto-etiocholeve-3-ol tetrahydropyranol, 3,17-keto-etiochol-triene and *Camellia sinensis*) on the serum concentrations of endogenous steroids in eugonadal men. Significant increases in dihydrotestosterone, free and total testosterone, and a decreased fat mass were reported during the 8-week administration. In addition to the aromatase-inhibiting properties, Motta *et al*. reported an effect on the 3-hydroxysteroid dehydrogenase system.[18] No preparation containing androsta-1,4,6-triene-3,17-dione has yet been approved for medical use.

According to the regulations of the World Anti-Doping Agency (WADA), steroidal aromatase inhibitors (as well as other aromatase inhibitors and anti-estrogens) are prohibited substances for use in sports.[19] Despite the coverage by the WADA list of prohibited substances by the wording ''...included, but not limited to ...'' an explicit inclusion may be helpful for clarification.

In the present study the excretion of urinary metabolites was investigated in order to detect a misuse in doping control. Three urine samples from a 2006 routine doping control were found to contain androsta-1,4,6-triene-3,17-dione.

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EXPERIMENTAL

The gas chromatography/mass spectrometry (GC/MS) analyses of the derivatized compounds were performed on a 6890N GC system (Agilent, Waldbronn, Germany) coupled to a 5973N quadrupole mass-selective detector (MSD) (Agilent) using electron ionization (EI, 70 eV). The analyses were carried out as routinely performed in our laboratory for screening for anabolic androgenic steroids[20] using an Ultra-1 column (Agilent; 17 m, 0.20 mm i.d., 0.11 μm film thickness). The oven temperature was 183–232°C by 3°C/min, 232–310°C by 40°C/min, 2 min hold. The sample solutions were injected in split mode (1:16), with a volume of 3 μL at a temperature of 300°C. Helium was used as the carrier gas with a constant head pressure of 1 bar. Data acquisition was performed in selected ion monitoring (SIM)[20] and full scan mode (*m/z* 40–800). In addition, the derivatized extracts from the urines were analyzed by gas chromatography/tandem mass spectrometry (GC/MS/MS) on a GCQ ion trap (Finnigan, Bremen, Germany) with EI (70 eV) using an Ultra-1 column (Agilent; 16.5 m, 0.20 mm i.d., 0.11 μm film thickness). The oven temperature was 100–190°C by 40°C/min, 190–240°C by 5°C/min, 240–320°C by 40°C/min, 2 min hold. The sample solutions were injected in split mode (1:10), with a volume of 2 μL at a temperature of 300°C. Helium was used as the carrier gas with a constant head pressure of 0.7 bar. Data was acquired in product ion scan mode (precursor ion *m/z* 430 (M^+ of the tetrahydro metabolites), scan range 110–440, collision energy 1.0 eV).

The underivatized steroids were analyzed on a 6890 gas chromatograph (Agilent) coupled to an 5973N MSD (Agilent) using EI (70 eV). The column used was an Optima δ3 (Macherey-Nagel, Düren, Germany; 20 m, 0.25 mm i.d., 0.25 μm film thickness) with an oven temperature of 60°C for 1.5 min, 60–240°C by 40°C/min, 240–260°C by 2°C/min, 260–300°C by 40°C/min, 1.5 min hold. The injection volume was 2 μL at a temperature of 300°C in splitless mode. Helium was used as the carrier gas (constant flow, 1.2 mL/min). Data was acquired in full scan mode (*m/z* 40–400).

The high-performance liquid chromatography (HPLC) fractionation of the extracts was carried out on a 1100 system (Agilent) equipped with a LiChroCART 250-4 LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany; 4.0 × 250 mm, particle size 5 μm). As mobile phase H_2O (as solvent A) and acetonitrile (solvent B) were used, running a linear gradient from 30% B to 100% B within 25 min at a flow rate of 1 mL/min. The detector wavelength used was 200 nm. The fractions were collected from 14.8 to 16.8 min and from 16.8 to 18.0 min.[21]

The nuclear magnetic resonance (NMR) analyses were performed in $CDCl_3$ (~4 mM solute) at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) on an Avance II 600 instrument (Bruker, Rheinstetten, Germany) at 298 K. The spectra were recorded as ^{1}H; H,H COSY; APT; H,C HMQC; H,C HMBC and NOESY using tetramethylsilane as the internal standard.

Chemicals and reagents

Testosterone (17β-hydroxyandrost-4-en-3-one, T), epitestosterone (17α-hydroxyandrost-4-en-3-one, epiT), etiocholanolone (3α-hydroxy-5β-androstan-17-one, ETIO), androsterone (3α-hydroxy-5α-androstan-17-one, AND), methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), toluene-4-sulfonic acid, and triethyl orthoformate were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). Androsta-1,4,6-triene-3,17-dione, androsta-4,6-diene-3,17-dione, 17β-hydroxyandrosta-4,6-dien-3-one, 3-hydroxyestra-1,3,5(10),6-tetraen-17-one, 5α-androstane-3α,17β-diol (Adiol), 5β-androstane-3α,17β-diol (Bdiol), and 3α,6α-dihydroxy-5β-androstan-3-one were purchased from Steraloids (Wilton, NH, USA). 17β-Hydroxy-5β-androst-1-en-3-one (metabolite of boldenone) was synthesized in our laboratory as described by Schänzer and Donike.[22]

β-Glucuronidase from *E. coli* (>140 U/mL) was obtained from Roche Diagnostics (Mannheim, Germany), and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

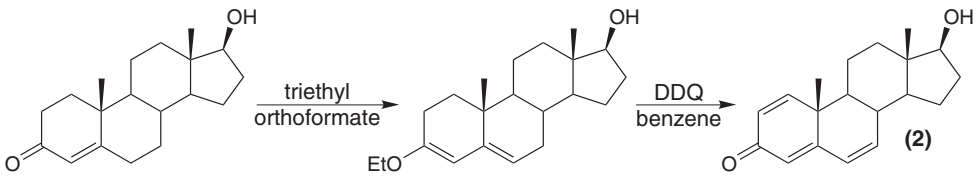
Preparation for GC/MS measurements

The final residues of sample preparations were either reconstituted in acetone or derivatized with trimethyliodosilane (TMIS) reagent (MSTFA/NH_4I/ethanethiol, 1000:2:3, v/w/v) by heating for 20 min at 60°C and injected into the GC/MS instrument. As reference, solutions of the respective steroids in methanol were evaporated to dryness and processed in the same manner.

Synthesis of 17β-hydroxyandrosta-1,4,6-trien-3-one

For use as reference 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**) was synthesized as shown in Scheme 1.

A solution of testosterone (270 mg, 0.94 mmol) in dioxane (3 mL) was stirred with toluene-4-sulfonic acid (44 mg) and triethyl orthoformate (1 mL, 6 mmol). After 3 h at ambient



Scheme 1. Synthesis of 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**).

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temperature, the solution was evaporated almost to dryness and dissolved in 50 mL of *tert*-butyl methyl ether (TBME). The mixture was washed with 20 mL of aqueous carbonate buffer ($K_2CO_3/KHCO_3/H_2O$, 1:1:8, w/w/v, pH 9.6). Following evaporation of the ethereal layer, the residue (311 mg of raw material containing 3-ethoxyandrosta-3,5-dien-17β-ol) was re-dissolved in benzene (50 mL) and added to a solution of DDQ (510 mg, 2.25 mmol) in benzene (30 mL) while stirring for 10 min. After dilution with dichloromethane and filtration of the mixture, the filtrate was washed with sodium hydroxide solution (4 × 50 mL; 1% in H_2O) followed by water (50 mL). Evaporation of the organic layer yielded 190 mg of raw material, which was purified by column chromatography (silica gel, eluents n-hexane/ethyl acetate, 60:40, v/v). After crystallization from n-hexane/ethyl acetate (6.5:1, v/v) 55 mg of pure 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**) were obtained.

Supplement analysis

The product 'Novedex Xtreme' of the brand name Gaspari Nutrition was purchased from the internet sport supplement market. The labeled ingredients were: 'Proprietary Blend (6,17-keto-etiocholeve-3-ol tetrahydropyranol, 3,17-keto-etiochol-triene, *Camellia sinensis*) 50 mg', thereby indicating steroidal ingredients.

The homogenized content of one capsule was suspended in 5 mL of methanol. After shaking for 5 min and centrifugation for 5 min at 800 *g*, the methanolic layer was separated, aliquots were evaporated to dryness and analyzed by GC/MS (underivatized and as TMS derivatives). For characterization of the second steroid labeled to be present as tetrahydropyranyl (THP) ether the residue was hydrolyzed for 1 h in refluxing aqueous acetic acid (30%) prior to GC/MS analysis.

Administration study

An administration study with 50 mg of androsta-1,4,6-triene-3,17-dione was performed in a healthy male volunteer. It was approved by the Human Research Ethics Committee of the Russian Federal Agency for Physical Culture and Sports, Moscow. Urine samples were collected for 23 h. The samples were prepared according to the routine steroid screening procedure.[20] In brief, after addition of the internal standard, 2 mL of urine were incubated at pH 7 with β-glucuronidase from *E. coli* at 50°C for 1 h. The steroids were extracted with 5 mL of TBME at pH 9.6 and the organic layer was evaporated to dryness.

For the separation of the phase II metabolites, 2 mL of urine were applied to solid-phase extraction (SPE) cartridges (Chromabond C18; Macherey-Nagel, Düren, Germany). After washing with 2 mL of water the analytes were eluted with 2 mL of methanol. Following evaporation the remainder was reconstituted in 1 mL of phosphate buffer ($NaH_2PO_4xH_2O/Na_2HPO_4$, 1:2, w/w, 0.2 M, pH 7.0) and extracted twice with 5 mL of TBME. The combined ethereal layers were evaporated to dryness and analyzed for their content of unconjugated steroid metabolites. The aqueous layer was incubated at 50°C for 2 h with 50 μL of β-glucuronidase from *E. coli*. After the addition of 500 μL of aqueous carbonate buffer ($K_2CO_3/KHCO_3/H_2O$, 1:1:8, w/w/v, pH 9.6), the liberated aglycons were extracted twice with 5 mL of TBME. The combined ethereal layers were evaporated to dryness.

For the cleavage of steroid sulfates the remaining aqueous layer was applied to SPE cartridges (Chromabond C18). After washing with 2 mL of water the cartridges were predried in a flow of air and stored overnight in a desiccator over P_4O_{10}. After elution with 1 mL of methanol and addition of 5 mL of ethyl acetate/sulfuric acid (2500:2, v/w) the sulfates were cleaved within 1 h at 55°C. After addition of 0.75 mL of aqueous potassium hydroxide solution (1 M) the mixture was evaporated almost to dryness, 0.5 mL of potassium hydroxide (1 M) was added, and the steroids were extracted with 5 mL of TBME. The organic layer was separated and evaporated to dryness.

Analysis of doping control urine samples

Three urine samples from human sports doping control in 2006, that had already tested positive for the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one, were reanalyzed in order to confirm the exogenous origin of boldenone as described by Piper *et al*.:[23] A volume of 10 mL of urine was applied to SPE cartridges (Chromabond C18). After washing with 2 mL of water the analytes were eluted with 2 mL of methanol. The dried eluate was redissolved in 1 mL of sodium phosphate buffer (0.2 M, pH 7.0) and incubated with β-glucuronidase from *E. coli* at 50°C for 1 h. The steroids were extracted twice with 5 mL of TBME each at pH 9.6 and the combined organic layers were evaporated to dryness. The residue was reconstituted in 60 μL of methanol and submitted to HPLC fractionation. The relevant fractions were evaporated to dryness and analyzed by GC/MS.

RESULTS AND DISCUSSION

Analyses of androsta-1,4,6-triene-3,17-dione and 17β-hydroxyandrosta-1,4,6-trien-3-one

Androsta-1,4,6-triene-3,17-dione (**1**) and 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**) were analyzed by NMR (assignments in Table 1), confirming the successful synthesis. The findings for **1** are in accordance to the ^{1}H-NMR data reported by Kirk *et al*.[24]

The mass spectra (GC/MS) of underivatized androsta-1,4,6-triene-3,17-dione and 17β-hydroxyandrosta-1,4,6-trien-3-one are shown in Fig. 1. Explanations for the generation of some fragment ions are also indicated. They are also found after direct insertion EI-MS as reported by Smith and Smith.[25]

The derivatization of both androstatrienes at 60°C with TMIS reagent (usually used in steroid doping control analysis) resulted in two major products in a ratio of 4:1. The mass spectra of the mono-TMS derivatives (main product) and artifacts (minor product), probably obtained by a loss of the 19-methyl group, are displayed in Figs. 2 and 3. The artifact from androsta-1,4,6-triene-3,17-dione showed

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Table 1. ^{1}H and ^{13}C NMR spectral data of androsta-1,4,6-triene-3,17-dione (1) and 17β-hydroxyandrosta-1,4,6-trien-3-one (2) (coupling constants are given in parentheses, signals indicated as *m* were unresolved or overlapped multiplets)

	Androsta-1,4,6-triene-3,17-dione (**1**)		17β-Hydroxyandrosta-1,4,6-trien-3-one (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	152.4	7.09 *d (10)*	153.0	7.10 *d (10)*
2	128.4	6.29 *dd (10/2)*	128.1	6.28 *m*
3	186.2	–	186. 4	–
4	124.3	6.06 *dd (3/2)*	123.9	6.03 *m*
5	161.7	–	162.7	–
6	128.6	6.34 *dd (10/3)*	128.2	6.26 *m*
7	135.7	6.13 *dd (10/2)*	137.7	6.04 *m*
8	37.6	*β*: 2.47 *m*	38.3	*β*: 2.35 *dd (11/11)*
9	48.4	*α*: 1.49 *m*	48.5	*α*: 1.46 *m*
10	41.1	–	41. 3	–
11	21.2	*α*: 1.95 *m*	21.6	*α*: 1.87 *m*
		β: 1.70 *m*		*β*: 1.68 *dddd (13/13/13/4)*
12	31.2	*α*: 1.36 *ddd (13/13/4)*	36.5	*α*: 1.17 *ddd (13/13/4)*
		β: 1.95 *m*		*β*: 1.95 *ddd (13/3/3)*
13	47.9	–	43.5	–
14	48.9	*α*: 1.54 *m*	48.5	*α*: 1.23 *m*
15	21.4	*α*: 2.17 *m*	23. 1	*α*: 1.84 *m*
		β: 1.77 *m*		*β*: 1.54 *m*
16	35.6	*α*: 2.17 *m*	30.5	*α*: 1.52 *m*
		β: 2.55 *m*		*β*: 2.16 *m*
17	219.1	–	81.3	*α*: 3.71 *dd (8/8)*
18	13.8	1.03 *s*	11. 2	0.90 *s*
19	20.8	1.25 *s*	20.9	1.24 *s*

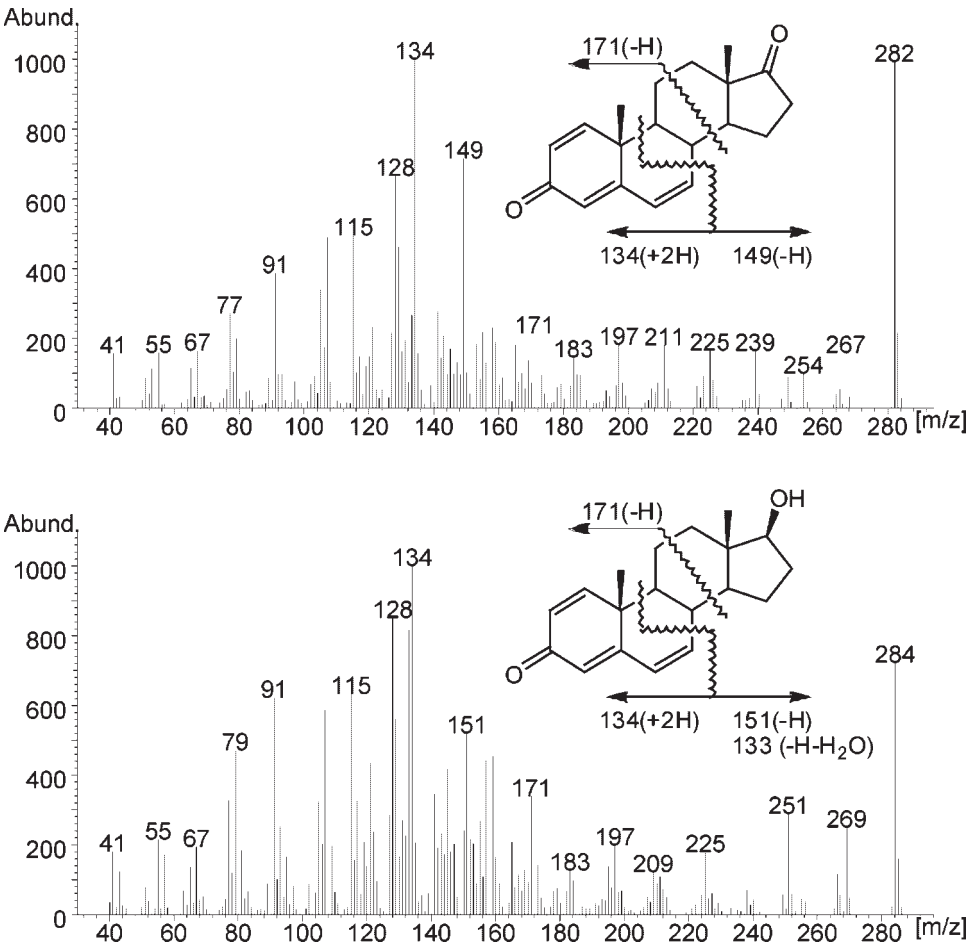

Figure 1. Mass spectra (EI) of androsta-1,4,6-triene-3,17-dione (**1**), underivatized, $M^+ = 282$ (top) and 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**), underivatized, $M^+ = 284$ (bottom).

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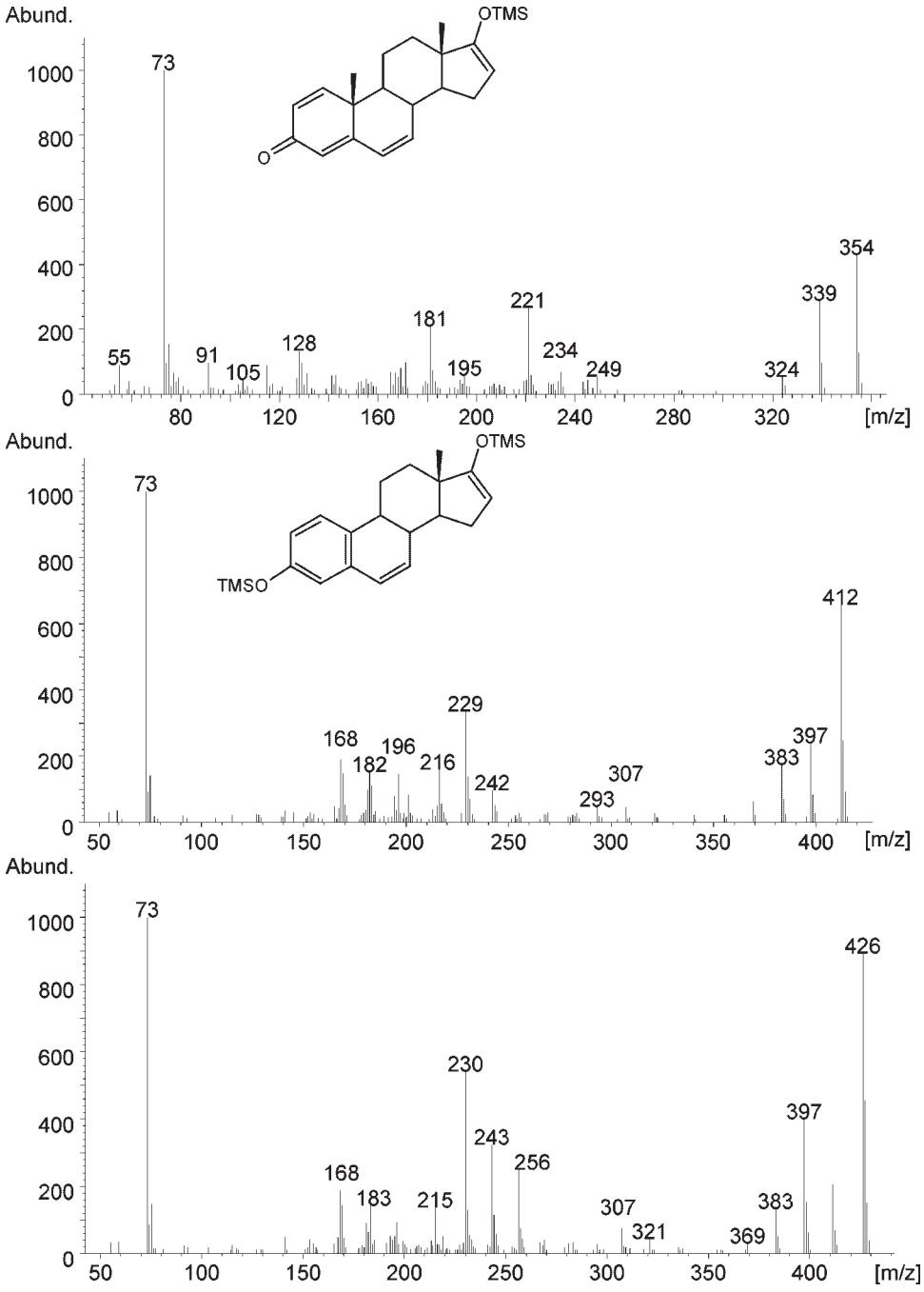

Figure 2. Mass spectra (EI) of androsta-1,4,6-triene-3,17-dione (**1**), top: mono-TMS derivative, $M^+ = 354$; center: artifact from TMIS derivatization, $M^+ = 412$; bottom: bis-TMS reaction product, $M^+ = 426$.

the same retention time (RT) and mass spectrum as were obtained from 3-hydroxyestra-1,3,5(10),6-tetraen-17-one, bis-TMS (mass spectra available as Supporting Information), thus confirming the postulated structure of the artifact. In addition, traces (<1%) of bis-TMS derivatives were also obtained when applying the above mentioned conditions for derivatization. Their structures may be assigned to the enol-TMS derivatives (androsta-1,3,5,7,16-pentaene-3,17-diol, bis-O-TMS and androsta-1,3,5,7-tetraene-3,17-diol, bis-O-TMS). However, after comparison of the fragment ions with the data from the artifacts a migration of the 19-methyl group to an A/B-ring position is more favorable. Proof of these structures has to be addressed in future studies.

Supplement analysis

The methanolic extract of the product 'Novedex Xtreme' was analyzed by GC/MS. The ingredients on the label of the

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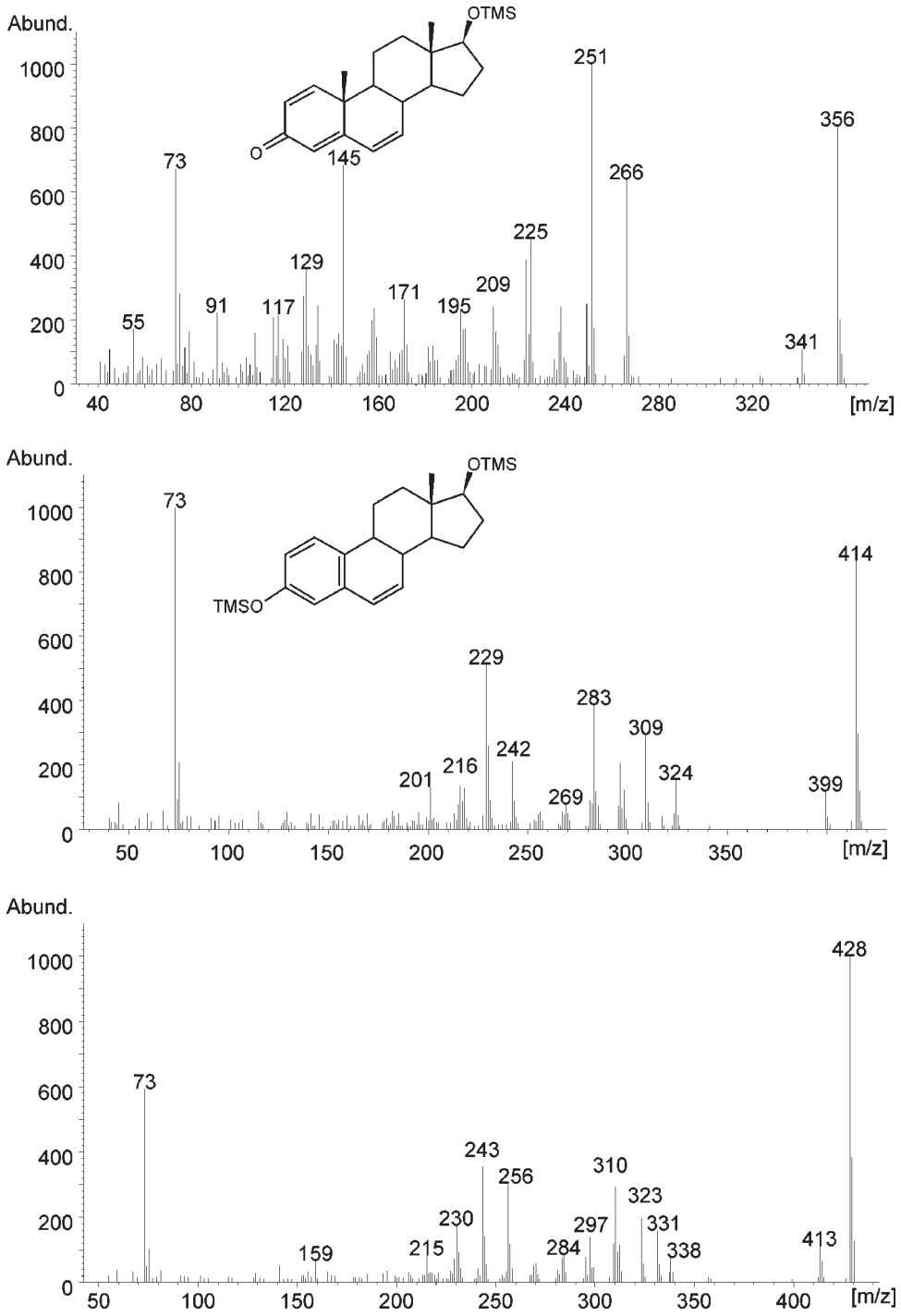

Figure 3. Mass spectrum (EI) of 17β-hydroxyandrosta-1,4,6-trien-3-one (**2**), top: mono-TMS derivative, $M^+ = 356$; center: artifact from TMIS derivatization, $M^+ = 414$; bottom: bis-TMS reaction product, $M^+ = 428$.

product do not have correct accepted nomenclature. The main ingredient indicated as '3,17-keto-etiochol-triene' was found to correspond to androsta-1,4,6-triene-3,17-dione. The second steroid ingredient indicated as '6,17-keto-etiocholeve-3-ol tetrahydropyranol' was identified as 6-hydroxyandrost-4-ene-3,17-dione in 'Novedex XT' by Kazlauskas.[14] On derivatization with TMIS, 6β-hydroxyandrost-4-ene-3,17-dione yields mainly the 3,5-dienol-TMS derivative ($RT_{tris\text{-}TMS} = 16.29$ min). Minor amounts of 2,4-dienol-TMS ($RT_{tris\text{-}TMS} = 13.83$ min) were also obtained (mass spectra in Fig. 5). However, in our case two isomeric steroids with almost identical mass spectra ($RT_{tris\text{-}TMS} = 14.28$ and 15.71 min, mass spectra in Fig. 4) were released from the THP ether by acidic hydrolysis. Their GC/MS properties did

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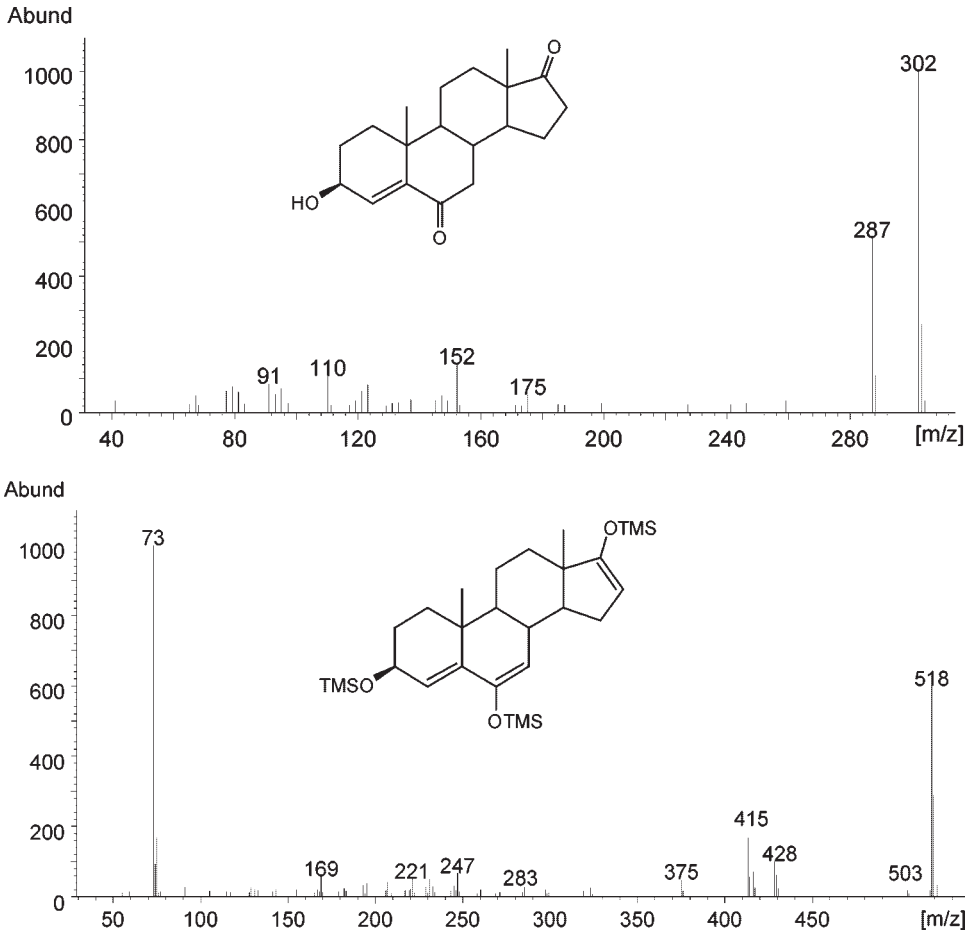

Figure 4. Mass spectra of the second steroid ingredient in Novedex Xtreme (3ξ-hydroxyandrost-4-ene-6,17-dione, proposed), top: underivatized, $M^+ = 302$; bottom: tris-TMS, $M^+ = 518$.

not match those of 6ξ-hydroxyandrost-4-ene-3,17-dione. Based on the mass spectrometric data, isomers of 3ξ-hydroxyandrost-4-ene-6,17-dione are most likely. According to literature reports,[26,27] the 3β-hydroxy isomers have longer retention times than the 3α-hydroxy isomers.

Administration study

Following the administration of androsta-1,4,6-triene-3,17-dione (**1**) several metabolites were identified in human urine. The structures are displayed in Fig. 6 and their retention times are listed in Table 2.

In all urine samples collected (up to 23 h after administration) the parent compound **1** was detected unconjugated and its 17β-hydroxy analogue **2** in the glucuronide fraction, both in high amounts (μg/mL range). The highest concentrations were obtained in the 2–4.5 h urine (about 30 μg/mL for both compounds). A significant decrease in the urinary steroid profile ratio of AND/T was observed together with increases in the ratio of T/epiT, Adiol/Bdiol and AND/ETIO, indicating either the formation of testosterone as an additional metabolite or its accumulation due to the inhibition of the aromatase.

17β-Hydroxy-5β-androst-1-en-3-one (**5**), the metabolite routinely used in doping control for the detection of boldenone misuse,[22] was detectable as the glucuronide in all samples in relatively low concentrations (3–150 ng/mL) with the highest concentration in the 4.5–14 h urine (morning urine). The kinetics are displayed in Figs. 7–10 (concentrations not adjusted from density). In addition, minor amounts of boldenone (17β-hydroxyandrosta-1,4-dien-3-one (**4**)) and 17β-hydroxyandrosta-4,6-dien-3-one (**3**) were detected in the glucuronide fraction of all samples. The derivatized compounds (**3** and **4**) coelute with the artifact obtained from androsta-1,4,6-triene-3,17-dione (**1**). Separation of the unconjugated fraction removed **1** leaving **3** and **4** in the glucuronide fraction. Subsequently, GC/MS/MS allowed their identification. The GC/MS spectra and product ion spectra of the bis-TMS derivatives of **3** and **4** are shown in Figs. 11 and 12.

The analyses of the underivatized extracts resulted in the coelution of both steroids, **3** and **4**, with a high concentration of the metabolite 17β-hydroxyandrost-1,4,6-trien-3-one (**2**) even after cleanup by HPLC. Thus, isotope ratio mass spectrometry (IRMS) analyses of boldenone could not be performed successfully.

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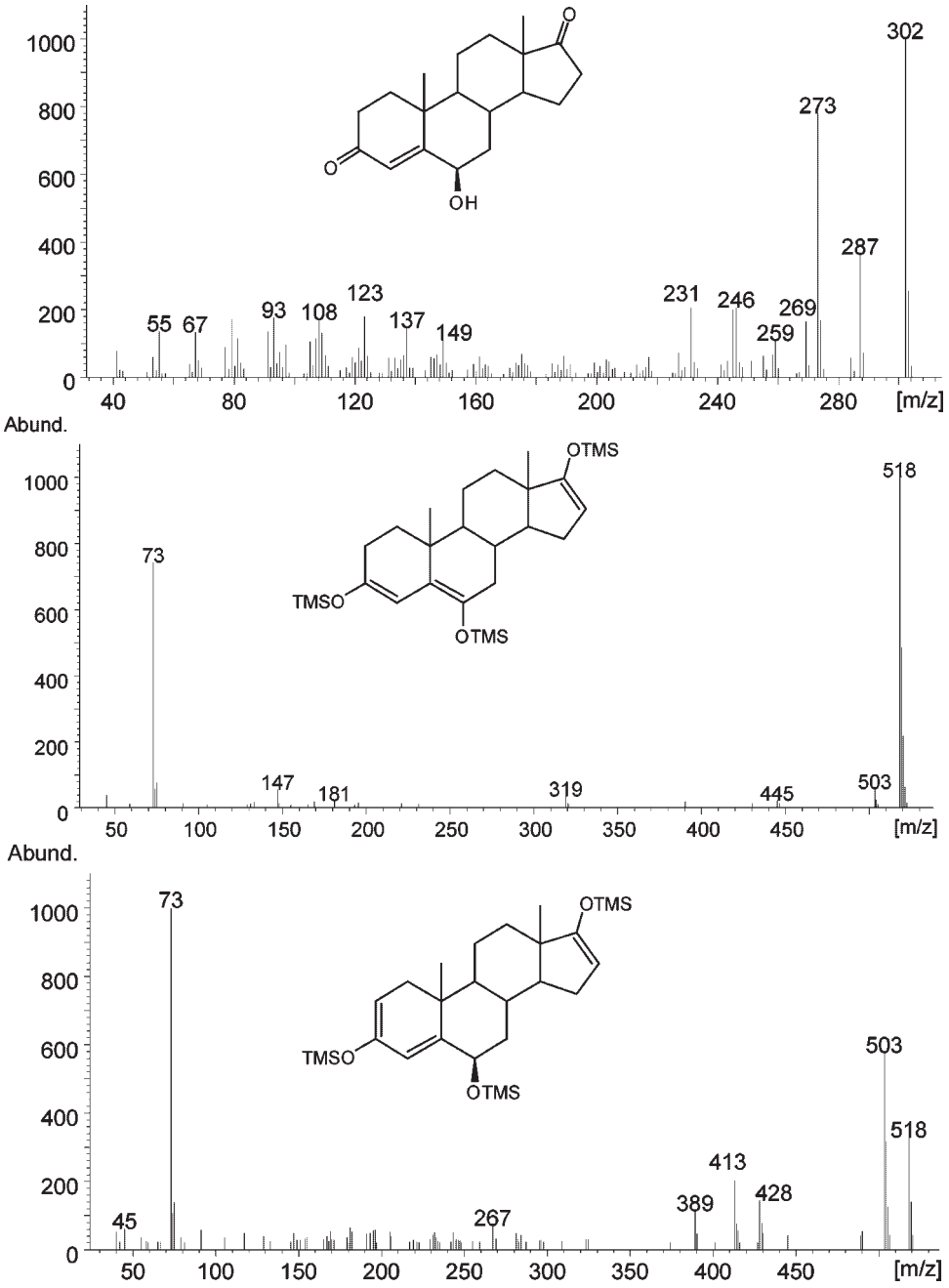

Figure 5. Mass spectra (EI) of 6β-hydroxyandrost-4-ene-3,17-dione, top: underivatized, $M^+ = 302$; center: 3,5-dienol, tris-TMS, $M^+ = 518$; bottom: 2,4-dienol, tris-TMS, $M^+ = 518$.

The sulfate fractions of all samples were found to contain an additional metabolite. Following solvolysis the aglycon yielded a TMS derivative with the same GC/MS properties as androsta-4,6-diene-3,17-dione (mass spectrum in Fig. 13).

Analysis of doping control urine samples

The urine samples from human routine doping control, initially positive with low amounts of boldenone and its metabolite 17β-hydroxy-5β-androst-1-en-3-one (**5**), were afterwards found to contain androsta-1,4,6-triene-3,17-dione (**1**) and its 17β-hydroxy analogue **2**.

Because of the aromatase-inhibiting properties of androsta-1,4,6-triene-3,17-dione, the World Anti-Doping Agency (WADA) classified its administration in sports as doping.[19,28]

Metabolites of androst-4-ene-3,6,17-trione as reported by van Eenoo *et al.*[12] were present in the doping control urines (mass spectrum of the metabolite 3α,6α-dihydroxy-5β-androstan-17-one is shown in Fig. 14), also likely after

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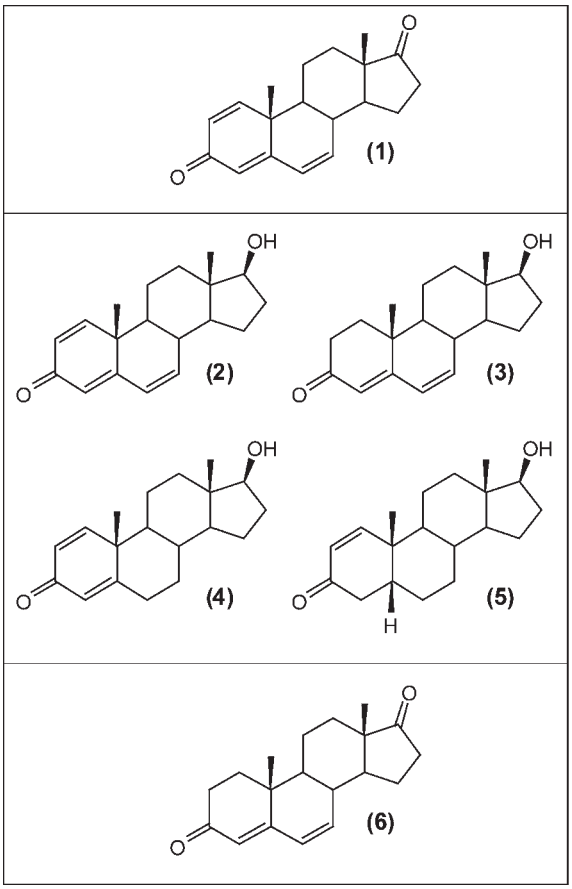

Figure 6. Structural formulae of metabolites detected in human urine after the administration of androsta-1,4,6-triene-3,17-dione (**1**), excreted unconjugated (top); as glucuronide (center); and as sulfate (bottom).

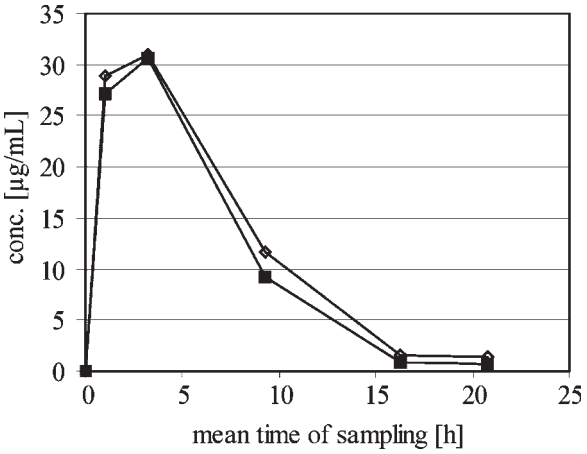

Figure 7. Urinary excretion of parent compound (◇) and 17β-hydroxyandrosta-1,4,6-trien-3-one (■) after ingestion of 50 mg of androsta-1,4,6-triene-3,17-dione (**1**).

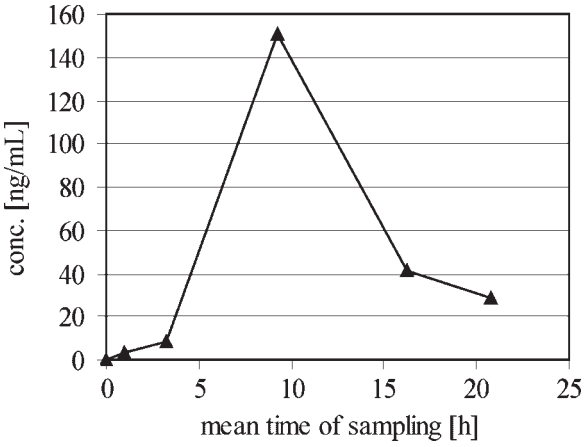

Figure 8. Urinary concentration of 17β-hydroxy-5β-androst-1-en-3-one (▲) after p.o. administration of 50 mg of androsta-1,4,6-triene-3,17-dione.

the consumption of 3ξ-hydroxyandrost-4-ene-6,17-dione. Comparing the data from the administration study with those obtained for the routine doping control samples, it is presumed that these findings are due to the administration of a product like 'Novedex Xtreme', which could be easily obtained from the sport supplement market.

Table 2. Retention times of parent compound and metabolites identified in human urine after the administration of androsta-1,4,6-triene-3,17-dione

Analyte	Retention time TMS derivative	Retention time underivatized
Androsta-1,4,6-triene-3,17-dione (**1**)	12.28 min (mono-TMS) 12.82 min (artifact) 15.01 min (bis-TMS)	11.27 min
17β-Hydroxyandrosta-1,4,6-trien-3-one (**2**)	12.81 min (mono-TMS) 13.26 min (artifact) 15.35 min (bis-TMS)	11.44 min
17β-Hydroxyandrosta-4,6-dien-3-one (**3**)	13.05 min (bis-TMS)	8.88 min
17β-Hydroxyandrosta-1,4-dien-3-one (**4**)	12.97 min (bis-TMS)	11.40 min
17β-Hydroxy-5β-androst-1-en-3-one (**5**)	9.21 min (bis-TMS)	9.87 min
Androsta-4,6-diene-3,17-dione (**6**)	12.61 min (bis-TMS)	8.00 min
Internal standard	methyltestosterone, bis-TMS 15.01 min	androstanol 7.16 min

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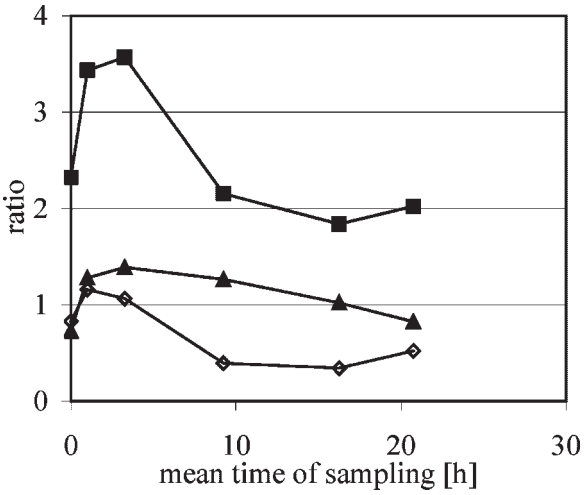

Figure 9. Influence on steroid profile data: ratio of Adiol/Bdiol (◇),T/epiT (▲) and AND/ETIO (■) after ingestion of 50 mg of androsta-1,4,6-triene-3,17-dione.

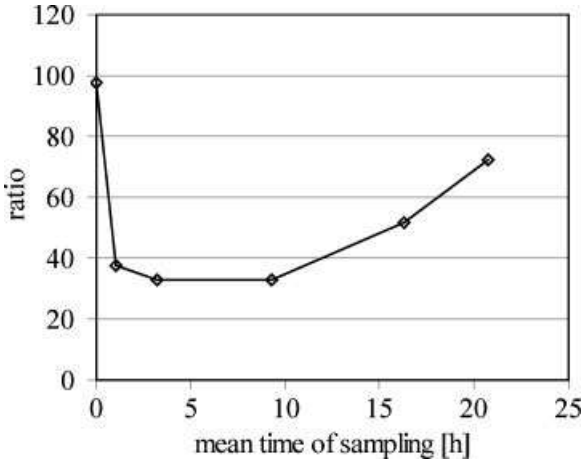

Figure 10. Ratio of AND/T (◇) after ingestion of 50 mg of androsta-1,4,6-triene-3,17-dione.

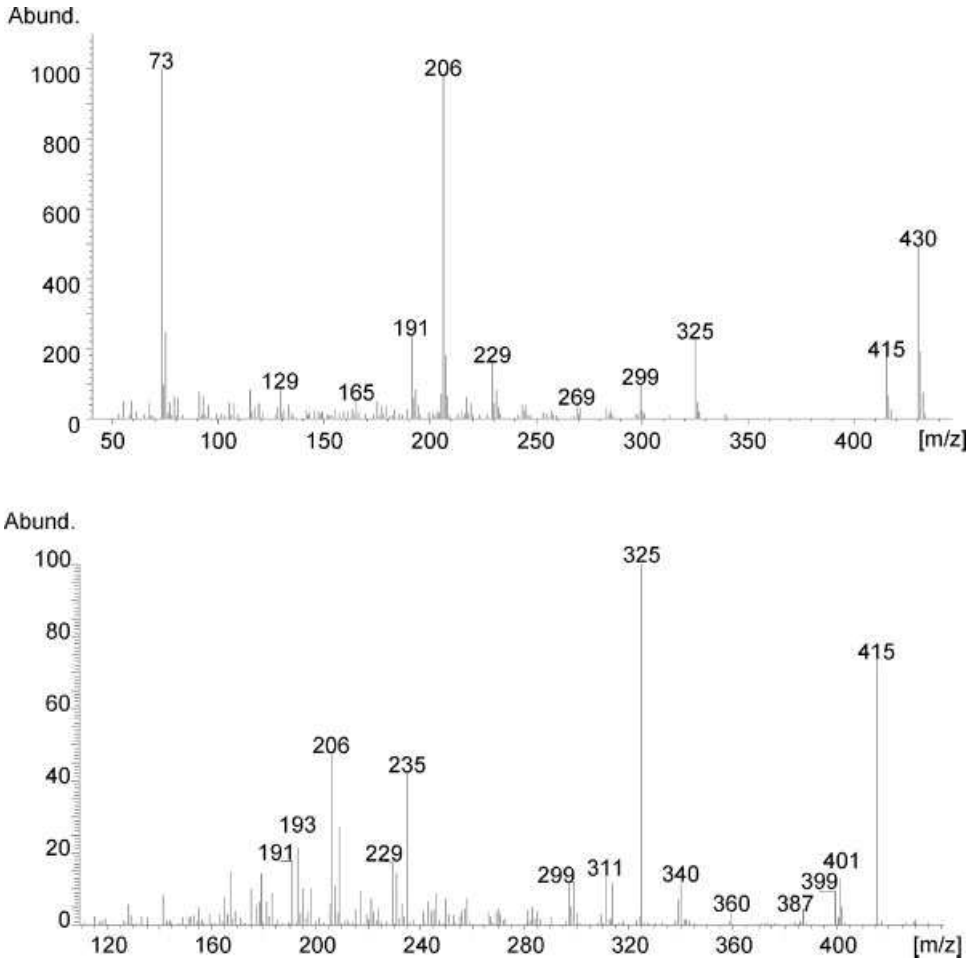

Figure 11. Mass spectra of boldenone (17β-hydroxyandrosta-1,4-dien-3-one (**4**)), bis-TMS, $M^+ = 430$, top: GC/MS (full scan), bottom: GC/MS/MS (product ion scan of *m/z* 430).

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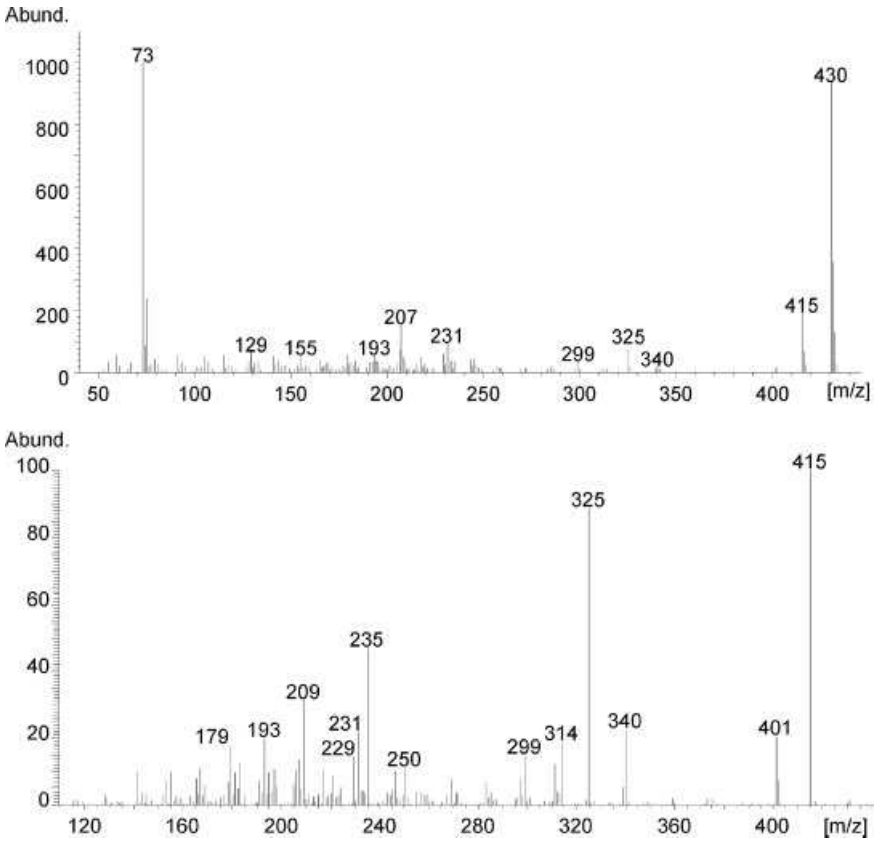

Figure 12. Mass spectra of 17β-hydroxyandrosta-4,6-dien-3-one (**3**), bis-TMS, $M^+ = 430$, top: GC/MS (full scan); bottom: GC/MS/MS (product ion scan of *m/z* 430).

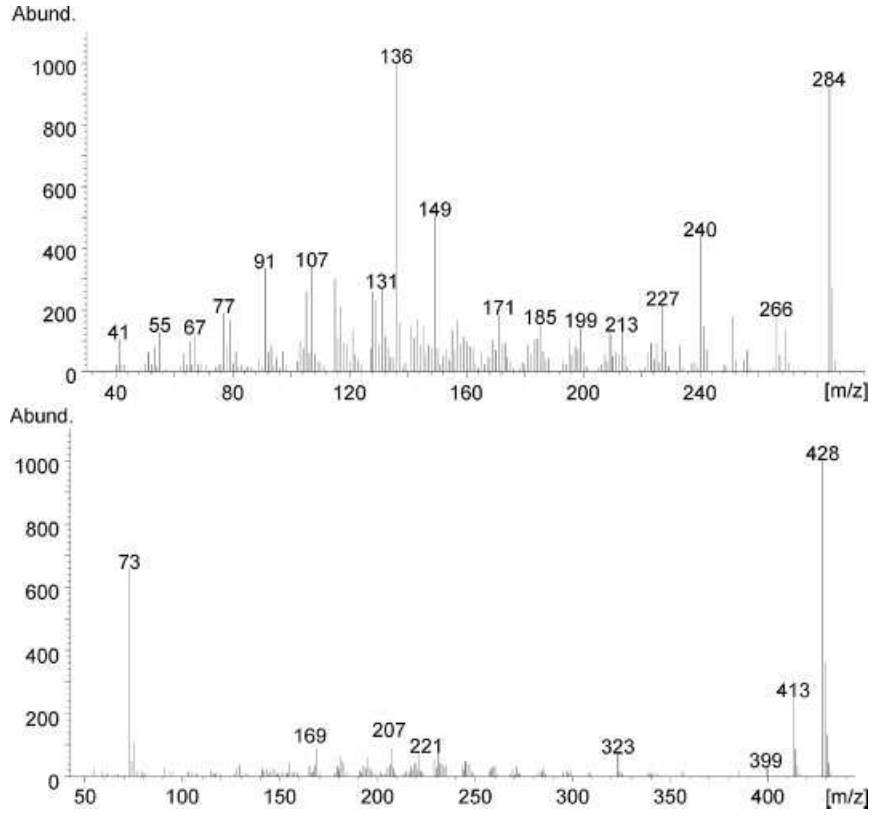

Figure 13. Mass spectra (EI) of androsta-4,6-diene-3,17-dione (**6**), top: underivatized, $M^+ = 284$; bottom: bis-TMS, $M^+ = 428$.

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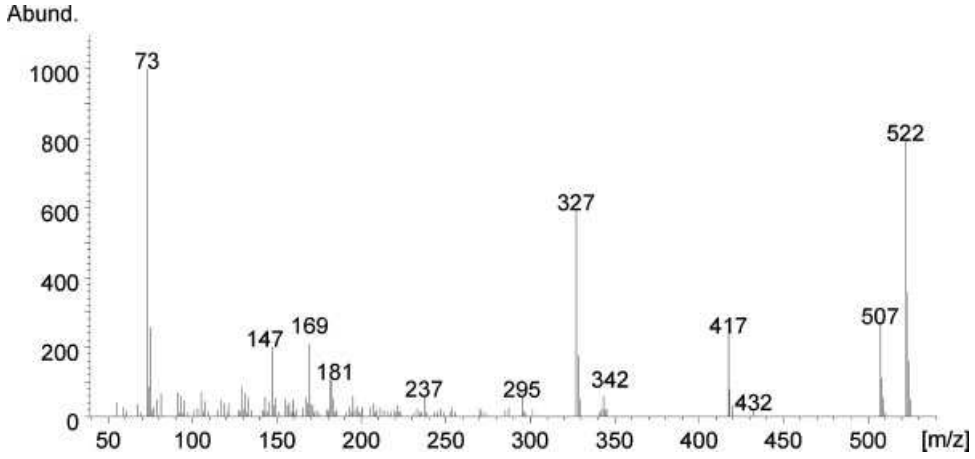

Figure 14. Mass spectrum (EI) of 3α,6α-dihydroxy-5β-androstan-17-one, tris-TMS, $M^+ = 522$, RT = 12.75 min.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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