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Detection of new 17-alkylated anabolic steroids on WADA 2006 list

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Introduction

As per 2006 WADA list [1] Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one), Methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-androst-1-en-3-one) and Methyl-nortestosterone (17β-hydroxy-17α-methyl-estr-4-en-3-one) are explicitly listed as anabolic androgenic steroids. All these steroids are promoted in the Internet and actually Methasterone is available on the nutritional supplement market. Also products containing Methyl-1-testosterone are available on Internet supplement market. It is advertised to be highly anabolic and moderately androgenic and not to convert to estrogens. In February 2006 Health Canada (Federal Department) warned consumers not to use these products because of potentially serious health risks as liver disorders and hardening of the arteries [2].
17α-Methyl-19-nortestosterone (Methylestrenolone, Normethandrolone, or Normethandrone) is widely advertised in the Internet, esp. as bulk material, on Chinese websites.

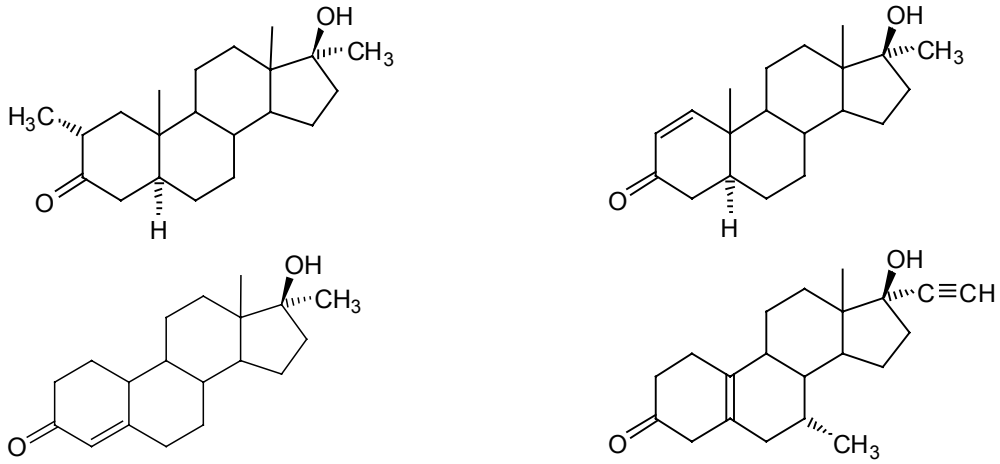

Fig. 1: Structure formulas of Methasterone (upper left), Methyl-1-testosterone (upper right), 17α-Methyl-19-nortestosterone (lower left) and Tibolone (lower right)

Likewise Tibolone (7α-methyl-17α-ethinyl-17β-hydroxyestr-5(10)-en-3-one) is explicitly listed since January 2006 under the category 'other anabolic agents'. It is therapeutically used for the treatment of menopausal disorders such as in the approved drug Liviella®. Anabolic-androgenic effects are well known besides the estrogenic activity [3,4].

Only little information is available on the gas chromatographic / mass spectrometric behaviour of these compounds (structure formulas in Fig. 1) with respect to supplement and/ or drug analysis. Also little is known about their urinary excretion. Only in case of Tibolone 3α- and 3β-reduction products are described as metabolites in literature [3,4].

In order to implement the above mentioned steroids in doping test routines, post-administration (p.a.) urines were analysed for possible metabolites. After cleavage of phase-II metabolites and derivatisation with TMIS reagent (MSTFA/NH_4I/ethanethiol, 1000:2:3, v:w:v) the samples were analysed by GC-MS and GC-MS/MS.
For confirmation of positive results in doping control reference compounds are required. As these metabolites are not commercially available they were synthesised from the parent compound by chemical reduction.

Experimental

Steroids and Preparations

17α-Methyl-19-nortestosterone, provided by Organon (Oberschleißheim, Germany), and Methyl-1-testosterone, from Steraloids (London, UK), were analysed as per-TMS derivatives. 17α-Methyl-5α-androstane-3α,17β-diol (3α,5α-THMT) and the 3β-analogue (3β,5α-THMT) were synthesised in our laboratory [5] and also analysed as per-TMS derivatives.
The "supplements" Methyl 1-Testosterone™ Xtreme from IDS (Oviedo, USA) and Superdrol™ from Anabolic Xtreme (Charlotte, USA) were purchased on the Internet. They were analysed for their steroid content by GC-MS after extraction with methanol and derivatisation as per-TMS derivatives.
Liviella®, a licensed pharmaceutical preparation containing 2.5 mg of Tibolone from Organon (Oberschleißheim, Germany), was obtained in a German pharmacy. After extraction with methanol it was analysed as per-TMS derivative.

Synthesis of reference compounds

The 3-oxo groups of the parent steroids were reduced using an excess of $NaBH_4$ in methanol/H_2O (10:1, v:v). After 1 h at ambient temperature 1 N HCl was added until hydrogen production ceased. The solvent was evaporated and the residue was extracted at alkaline pH (>12) with n-pentane. Methyl-1-testosterone was also reduced using K-Selectride

in absolute diethylether. After 1 h at ambient temperature 1 N HCl was added and the mixture was extracted with t-butylmethyl ether (TBME) at alkaline pH.
In case of 17α-Methylnortestosterone catalytic hydrogenation was performed in methanol using PtO_2 as catalyst.
The products of all reactions were analysed as per-TMS derivatives.

Metabolism studies

Excretion study
A female patient regularly administering Liviella® (one tablet containing 2.5 mg Tibolone per day) provided a 17-20 hour p.a. urine. Males who reported to use 17α-Methylnortestosterone or Methasterone (Superdrol™), respectively, also provided p.a. urines.
A routine doping control sample was found to contain Methyl-1-testosterone. Therefore it was used for the detection of possible metabolites.

Isolation of steroids from the urines
In order to separate the different conjugate fractions of the excreted steroids 10 ml aliquots of the urine samples were applied to SPE cartridges (Chromabond C18). They were washed with water and the steroids were eluted with methanol. After evaporation to dryness the residues were redissolved in Na-phoshate buffer (pH 7, 0.2 M) and the unconjugated compounds were extracted with TBME. For cleavage of the glucuronides the aqueous remainder was incubated with β-glucuronidase from *E.coli* at 37°C overnight. The liberated aglycons were extracted with n-pentane at pH 9.6.
For solvolysis the aqueous layer obtained after separation of the glucuronide fraction was again applied to SPE cartridges (Chromabond C18). After washing with 2 ml of water the cartridges were pre-dried in a flow of air and stored overnight in a desiccator. After elution with 1 ml of methanol and addition of 5 ml of ethylacetate/ sulphuric acid (1250:1, v:w) the sulphates were cleaved within 1 h at 55°C. After addition of 0.75 ml of KOH (1 M) the mixture was evaporated almost to dryness, 0.5 ml of KOH (1 M) were added, and the steroids were extracted with 5 ml of TBME.
For the implementation of the title compounds in procedures routinely used to screen for doping agents the screening method for anabolic steroids used in our laboratory [6] was applied to the p.a. urines. In brief, after addition of the internal standard and adjusting the pH to 7, 2 ml of urine were incubated 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.

GC-MS analyses

Derivatisation for GC-MS analyses

The per-TMS derivatives of the steroids were formed by redissolving the dry residue in 100 µl of TMIS reagent (MSTFA/NH_4I/ethanethiol, 1000:2:3, v:w:v) and heating for 15 min at 60°C.

Instrumentation

The GC-MS analyses were performed with the following parameters:

GC-MS system:	GC: Hewlett Packard (HP) 6890N coupled to mass selective detector HP 5973
Injection parameters:	Volume: 3 µl, Temp.: 300°C
Column:	HP Ultra-1 (OV 1); 17 m; 0.20 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:16, head pressure 14 psi
Oven temp.:	0 min 183°C, +3°C/min, 0 min 232°C, +40°C/min, 2 min 310°C
Ionisation:	70 eV, electron impact (EI)
Data aquisition:	SCAN, 40-800 amu, sampling rate 2^2

Additionally GC-MS/MS analyses were performed using the following conditions:

GC-MS/MS system:	GC Finnigan, GCQ
Injection parameters:	Volume: 2 µl, Temp.: 325°C
Column:	HP Ultra-1 (OV 1); 14 m; 0.20 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:10, head pressure 10 psi
Oven temp.:	0 min 100°C, +40°C/min, 0 min 190°C, +5°C/min, 0 min 240°C, +40°C/min, 3 min 320°C
Ionisation:	70 eV, electron impact (EI)
Data aquisition:	Product Ion Scan, Parent Methasterone m/z 449 and m/z 464, SCAN range 120-470, Tibolone m/z 443, SCAN range 110-445
Collision Energy:	1.0 volts

Chemicals and solvents

Chromabond C18 (6 ml, 500 mg) were supplied by Macherey-Nagel (Düren, Germany), Platinum dioxide, K-Selectride (Potassium tri-sec-butylborohydride, 1 M in THF) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Chem. Fabrik

Karl Bucher (Waldstetten, Germany). Other reagents and solvents were of analytical grade and provided by Merck (Darmstadt, Germany).

Results and Discussion

Methyl-1-testosterone

The mass spectrum of Methyl-1-testosterone as per-TMS derivative is displayed in Fig. 2.

The reduction with an excess of $NaBH_4$ resulted in 17α-methyl-5α-androst-1-ene-3β,17β-diol and the saturated 17α-methyl-5α-androstane-3β,17β-diol (3β,5α-THMT), whereas the reduction with K-Selectride (Fig. 3) resulted in the two isomeric 17α-methyl-5α-androst-1-ene-3ξ-17β-diols (ratio 3α:3β = 16:84). The mass spectrum of the 3α-isomer is shown in Fig. 2.

The analysis of the product Methyl 1-testosterone™ Xtreme confirmed the presence of Methyl-1-testosterone as active ingredient.

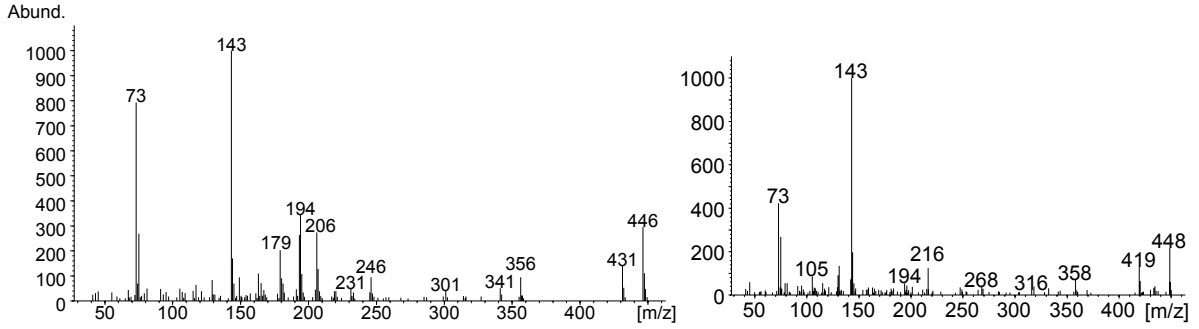

Fig. 2: Mass spectra (EI) of Methyl-1-testosterone (left) and its metabolite 17α-methyl-5α-androst-1-ene-3α,17β-diol (right)

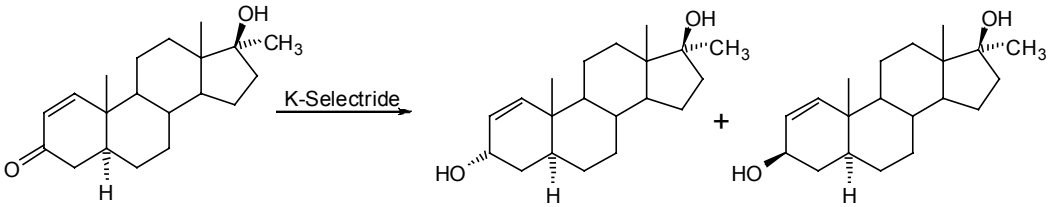

Fig. 3: Reaction scheme of the reduction of Methyl-1-testosterone with K-Selectride

In a routine doping control sample of the year 2006, Methyl-1-testosterone was detected besides its main metabolites 17α-methyl-5α-androst-1-ene-3α,17β-diol and 17α-methyl-5α-androstane-3α,17β-diol (3α,5α-THMT). Additionally several other compounds, presumably the 17-epimers, 17β-hydroxymethyl-17α-methyl-18-nor-5α-androsta-1,13-dien-3-one and other metabolites, were detected. The elucidation of their structures is still in progress.

Tibolone

The derivatisation of Tibolone (17β-hydroxy-7α-methyl-17α-ethinylestr-5(10)-ene-3-one) with TMIS reagent resulted in a loss of two mass units probably due to aromatisation in the injector (mass spectrum in Fig. 4). Almost no peak corresponding to Tibolone-bis-TMS could be detected.

The reduction of Tibolone with $NaBH_4$ yielded the two isomeric 7α-methyl-17α-ethinylestr-5(10)-ene-3ξ,17β-diols (Fig. 5, ratio 3α:3β = 20:80). Both show similar full scan mass spectra and product ion spectra of m/z 443 (Fig. 6). The corresponding retention times were determined at $RT_{3\alpha}$ = 12.09 min and $RT_{3\beta}$ = 13.14 min using the GC-MS method, and $RT_{3\alpha}$ = 8.66 min and $RT_{3\beta}$ = 9.30 min using the GC-MS/MS method.

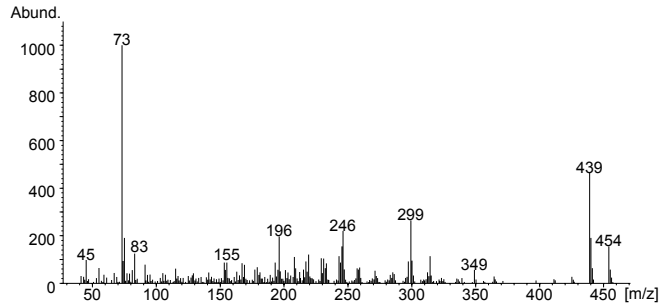

Fig. 4: Mass spectrum obtained after derivatisation of Tibolone with TMIS reagent

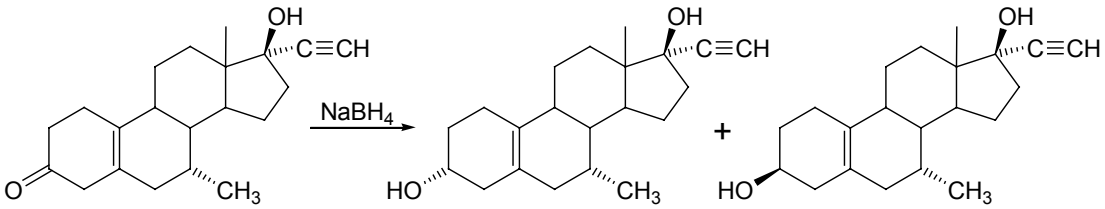

Fig. 5: Reaction scheme of the reduction of Tibolone with $NaBH_4$

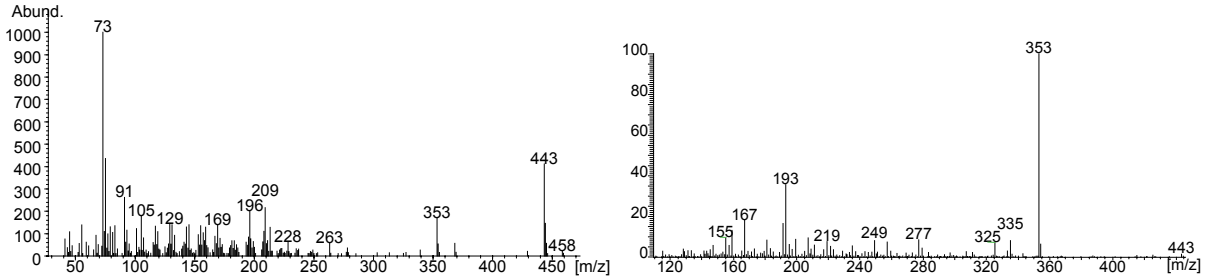

Fig. 6: Mass spectra of the Tibolone metabolite 7α-methyl-17α-ethinylestr-5(10)-ene-3α,17β-diol, left: EI, full scan; right: product ion spectrum of m/z 443

In the 17-20 hour urine of a female patient, who regularly administered 2.5 mg of Tibolone per day, 7α-methyl-17α-ethinylestr-5(10)-ene-3α,17β-diol could be detected in the glucuronide fraction using GC-MS/MS. In the sulphate fraction both isomers were detectable (chromatograms in Fig. 7). Also using routine screening sample preparation, the administration of Liviella® was detectable applying GC-MS/MS measurement.

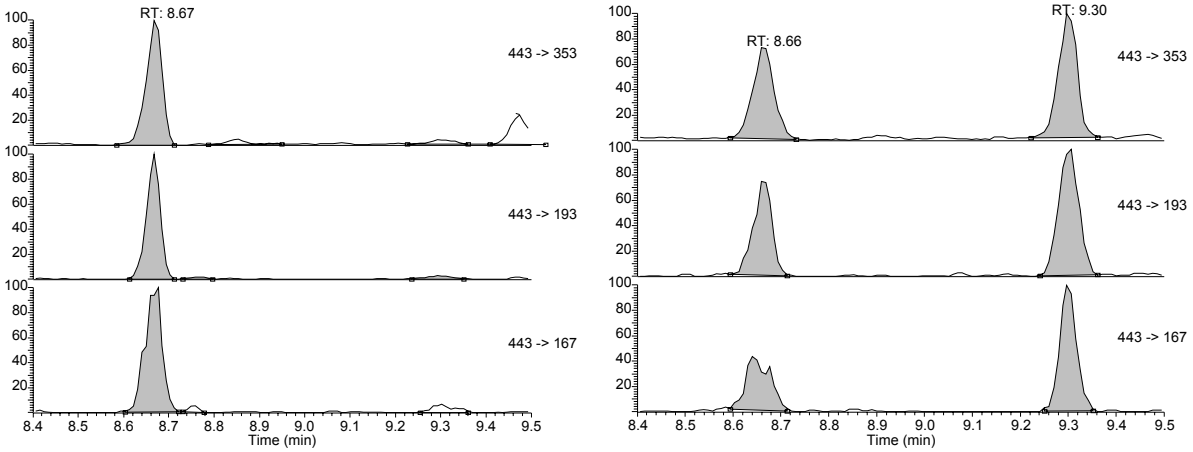

Fig. 7: Chromatograms (selected reaction monitoring, SRM) of 17-20 h urine, after administration of Tibolone, 10 ml aliquot, left: glucuronide fraction, right: sulphate fraction

Methasterone

The GC-MS analysis of the methanolic extract of the product Superdrol™ confirmed that it contained Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstane-3-one = 17α-methyl-drostanolone) as active ingredient in high amounts. The derivatisation with TMIS reagent resulted in two derivatives with analogue mass spectra, the 3- and 2-enol-TMS (ratio 3-enol:2-enol = 2:98). In contrast to 5α-dihydrotestosterone, the 3-enol-TMS derivative was clearly separated from the 2-enol-TMS (GC-MS: $RT_{3\text{-enol}}$ = 14.36min, $RT_{3\text{-enol}}$ = 15.40 min). The mass spectrum of Methasterone, 2-enol-bis-TMS, is presented in Fig. 8.

The reduction of Methasterone with $NaBH_4$ yielded the two isomers of 2α,17α-dimethyl-5α-androstane-3ξ,17β-diol in a ratio of 3α:3β = 27:73. Both show almost the same mass spectra (Fig. 8) but different retention times ($RT_{3\alpha}$ = 12.93 min, $RT_{3\beta}$ = 14.84 min). The product ion spectra of m/z 464 and m/z 449 of 2α,17α-dimethyl-5α-androstane-3α,17β-diol are presented in Fig. 9 (left). In the p.a. urines of the Superdrol™ excretion study this metabolite was detectable for at least 120 hours using GC-MS/MS (chromatogram in Fig. 9, right) in the free + glucuronide fraction (f+g fraction) using the sample preparation routinely used in our laboratory for screening of anabolic androgenic steroids [6].

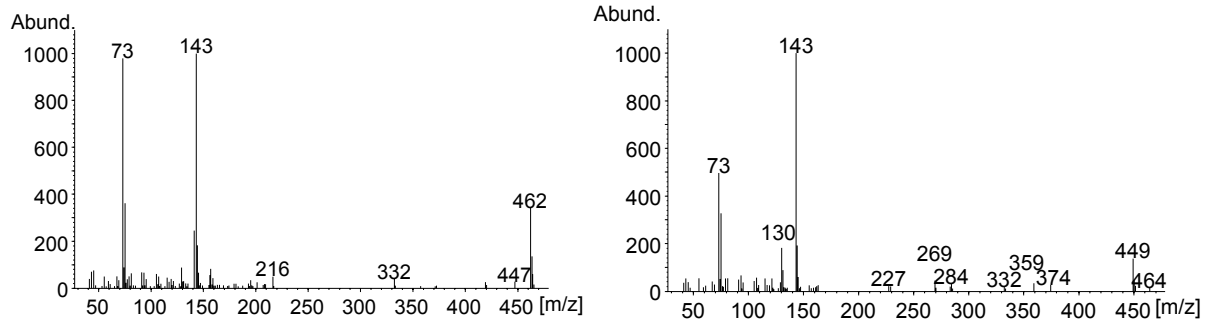

Fig. 8: Mass spectra (EI) of Methasterone (left) and its metabolite 2α,17α-dimethyl-5α-androstane-3α,17β-diol (right)

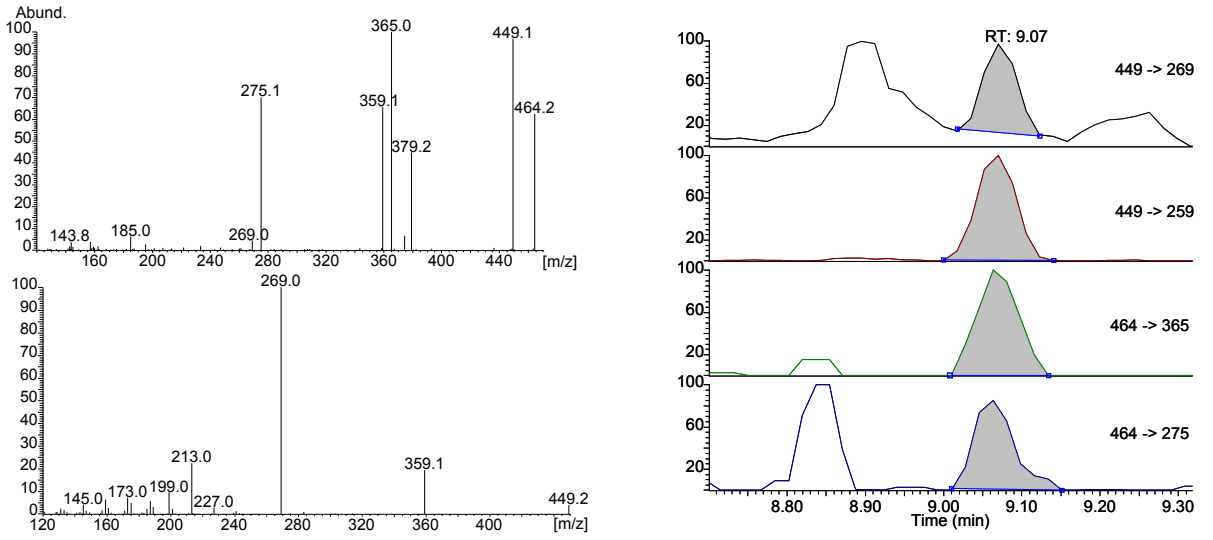

Fig. 9: Methasterone metabolite 2α,17α-dimethyl-5α-androstane-3α,17β-diol, upper left: product ion spectrum of m/z 464 lower left: product ion spectrum of m/z 449, right: chromatogram (GC-MS/MS) of 120 h p.a. urine, after application of 10 mg Methasterone (2 ml aliquot f+g fraction, [6])

17α-Methyl-19-nortestosterone

The mass spectrum of the per-TMS derivative of 17α-Methyl-19-nortestosterone (Fig. 11) is dominated by the fragment ion m/z 287, which can be explained by a loss of the D-ring (C-15, C-16, C-17, and C-20). M/z 300 is most likely generated by the loss of C-16, C-17, and C-20, m/z 417 by a loss of a methyl group and m/z 342 and m/z 327 by (additional) losses of TMSOH. M/z 143 corresponds to the D-ring fragment (with additional H abstraction) and m/z 194 to the A/B-ring fragment which is generated by a retro-Diels-Alder rearrangement.

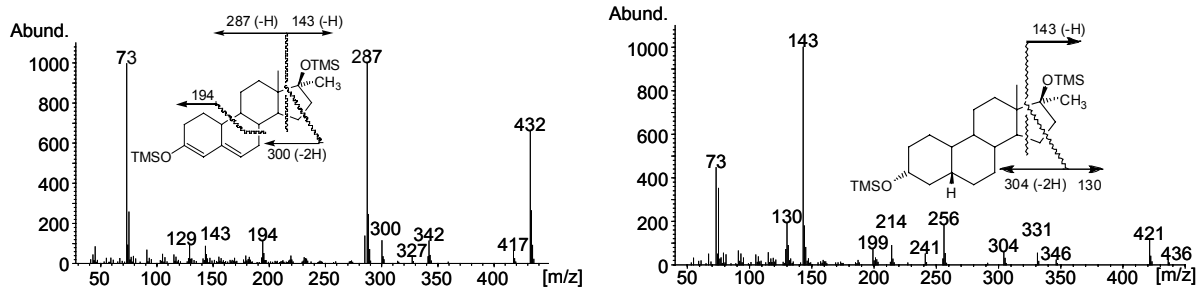

Fig. 10: Mass spectra (EI) of 17α-Methylnortestosterone (left) and its metabolite 17α-methyl-5β-estrane-3α,17β-diol (right)

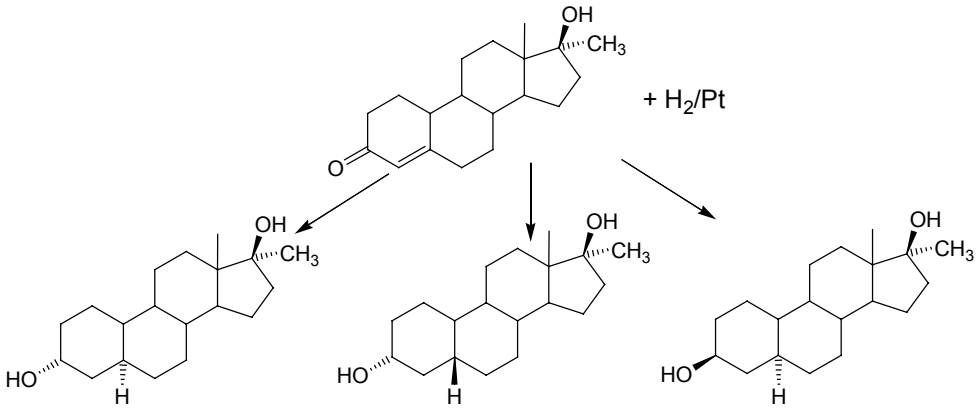

Fig. 11: Reaction scheme of the catalytic hydrogenation of 17α-Methylnortestosterone with PtO_2 as catalyst

The catalytic hydrogenation of 17β-hydroxy-17α-methylestr-4-ene-3-one using PtO_2 as catalyst resulted in three isomers of 17α-methyl-5ξ-estrane-3ξ,17β-diol: 3α,5α-, 3β,5α- and 3α,5β- (Fig. 12) in a ratio of 19:57:24. 17α-Methyl-5β-estrane-3β,17β-diol was not obtained during this reaction, most likely due to sterical hindering. The mass spectrum of 17α-methyl-5β-estrane-3α,17β-diol is shown in Fig. 11.

After the administration of 17α-Methylnortestosterone, 17α-methyl-5β-estrane-3α,17β-diol and the 3α,5α-isomer (GC-MS: $RT_{3\alpha,5\beta}$ = 11.57 min, $RT_{3\alpha,5\alpha}$ = 10.73 min) were detectable in the glucuronide fraction besides the parent compound, whereas in the sulphate fraction no parent steroid was detectable. The main metabolite 17α-methyl-5β-estrane-3α,17β-diol was detectable in the glucuronide fraction up to 31 hours after administration.

Summary

The metabolites found to be suitable for screening in doping control are

after Tibolone application: 7α-methyl-17α-ethinylestr-5(10)-ene-3α,17β-diol,

after Methylnortestosterone application: 17α-methyl-5β-estrane-3α,17β-diol,

after Methasterone application: 2α,17α-dimethyl-5α-androstane-3α,17β-diol and

after Methyl-1-testosterone application: 17α-methyl-5α-androst-1-ene-3α,17β-diol.

These metabolites were synthesised by reduction of the 3-oxo group with $NaBH_4$, K-Selectride or catalytic hydrogenation using platinum as catalyst.

Mass spectra and retention times of the above mentioned steroids and metabolites are presented.

References

[1] World Anti-Doping Agency. The 2006 prohibited list. 2006; online at http://www.wada-ama.org/rtecontent/document/2006_LIST.pdf, 17.01. 2006

[2] Health Canada. Health Canada warns consumers not to use supplements containing methyl-1-testosterone due to potential serious health risks. 2006; online at http://www.hc-sc.gc.ca/ahc-asc/media/advisories-avis/2006/2006_06_e.html, 11.10. 2006

[3] Organon GmbH. Fachinformation Liviella. Oberschleißheim, 2005. pp. 1-5.

[4] Markiewicz L, Gurpide E. Invitro evaluation of estrogenic, estrogen antagonistic and progestagenic effects of a steroidal drug (org od-14) and its metabolites on human endometrium. J Steroid Biochem Mol Biol 1990;35:535-41.

[5] Schänzer W, Donike M. Metabolism of anabolic-steroids in man - synthesis and use of reference substances for identification of anabolic-steroid metabolites. Anal Chim Acta 1993;275:23-48.

[6] Geyer H, Schänzer W, Mareck-Engelke U, Nolteernsting E, Opfermann G. Screening procedure for anabolic steroids -the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent advances in doping analysis (5). Cologne: Sport und Buch Strauss, 1998: 99-102.