Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

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Sport und Buch Strauß, Köln, 2004

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 129-138

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Analytical Properties of 4-Hydroxysteroids and some Esters

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Introduction

The 4-hydroxysteroids 4-hydroxytestosterone (4OH-T), oxymesterone (4-hydroxy-17α-methyltestosterone) and oxabolone (4-hydroxy-19-nortestosterone, structures in Fig. 1) are classified as anabolic agents and are therefore prohibited in sports due to the list of WADA and IOC. 4-Hydroxyandrostenedione (formestane, 4OH-A, structure in Fig. 1) is introduced in the therapy of breast cancer because of its aromatase inhibiting properties. This results in a classification as prohibited substance for male athletes according to current WADA regulations [1].

Recently several preparations containing 4-hydroxysteroids are available by internet purchase mainly as esters. They are promoted as orally active preparations with high anabolic and reduced androgenic effects.

The analytical properties of those compounds are investigated with respect to GC-MS analysis.

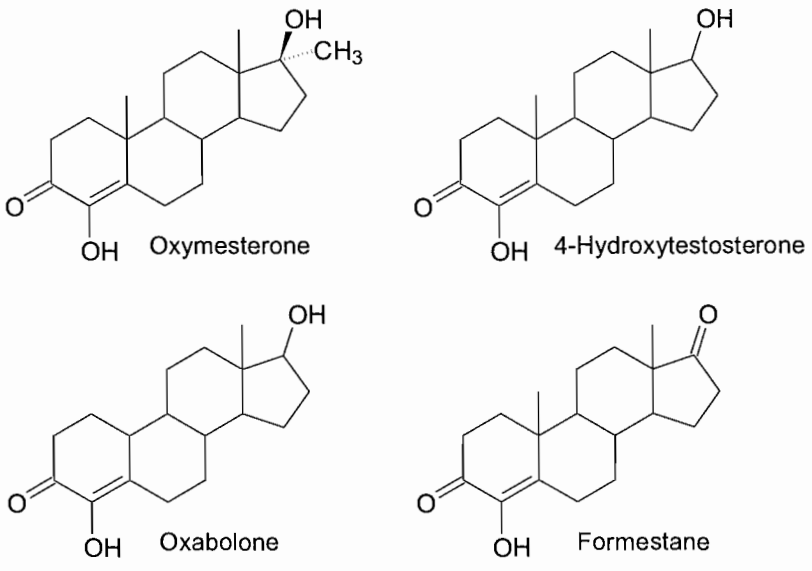


Fig. 1: Structures of the 4-hydroxysteroids oxymesterone, oxabolone, 4-hydroxytestosterone and formestane

Experimental

Chemicals

4-Hydroxytestosterone (androst-4-ene-4,17β-diol-3-one) and formestane (androst-4-ene-4-ol-3,17-dione = 4-hydroxyandrostenedione) were purchased from Steraloids (Wilton, USA), oxymesterone (17α-methylandrostene-4,17β-diol-3-one = 4-hydroxymethyltestosterone) and oxabolone cypionate (17β-(3-cyclopentyl-1-oxopropoxy)-4-hydroxyestr-4-ene-3-one) were gifts from Farmitalia (Milan, Italy). N,O-bis-(Trimethylsilyl)-acetamide (BSA) was purchased from Sigma (Deisenhofen, Germany), N,O-bis-($^2H_9$-trimethylsilyl)-acetamide (d_{18}-BSA) from Cambridge Isotope Laboratories (Andover, USA) and N-methyl-N-trimethylsilyl-trifluoro-acetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Other reagents and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Supplements

Two supplements were purchased in the Internet. The labelled contents were formestane acetate (Primobolan™) resp. 4-hydroxytestosterone THP (tetrahydropyranyl) ether (Testobol™).

Excretion study with Testobol™

Two tablets of Testobol™ were taken orally by a male volunteer. Urine samples were collected before and after the application up to 48 hours.

Sample preparation

Reference standards

The reference compounds were derivatised with TMIS reagent (MSTFA/ ammonium iodide/ ethanethiol, 1000:2:3, v:w:v) and injected into GC-MS resp. GC-MS/MS.

For the identification of the esters formestane resp. 4-hydroxytestosterone were derivatised with equimolar amounts of acetic anhydride with pyridine as catalyst. After evaporation the residue was derivatised with TMIS reagent. Additionally 4-hydroxytestosterone is derivatised with BSA followed by acetylation with a mixture of acetic anhydride and pyridine. After evaporation the residue is finally derivatised with TMIS reagent.

For the confirmation of the fragmentations oxymesterone was first derivatised with d_{18}-BSA followed by derivatisation with TMIS reagent. In addition it was deuterated by H/D-exchange and derivatised with TMIS reagent.

Cleavage of the esters

For the hydrolysis of the ester 10 µg of oxabolone 17-cypionate were incubated overnight with different reagents: 5 ml of KOH (1 M in H_2O) 60°C resp. 80°C, 5 ml of KOH (1 M in H_2O/EtOH, 1:1) 80°C, 5 ml of KOH (1 M in H_2O/MeOH, 1:1) 60°C, 5 ml of HCl (1 M in H_2O) 80°C, 4 ml of HCl (1 M in H_2O) + 4 ml of MeOH, 60°C. After adjusting pH <10 the samples were extracted with n-pentane and the dried n-pentane layer was derivatised with TMIS reagent.

pH-profiles of the extraction

To 5 ml of aqueous buffer solutions (pH 1 to 14) the analytes were added and the mixture was extracted with 5 ml of n-pentane. After centrifugation the organic layer was evaporated to dryness and the residue was derivatised with TMIS reagent.

Supplements

The supplements (0.1 g) were extracted with 5 ml of methanol. 10 µl of the extract were evaporated to dryness and derivatised with 100 µl of TMIS reagent. Additionally the evaporated extracts were derivatised with 100 µl of MSTFA. For the cleavage of the esters 4 ml of aqueous hydrochloric acid were added to 4 ml of the methanolic extracts of the supplements. After 60°C overnight the methanol was evaporated, the pH of the remainder was adjusted to 9.6 with solid carbonate buffer, extracted with 5 ml of n-pentane and the dried residues were derivatised with 100 µl of TMIS reagent.

Urine samples

The sample preparation was carried out according to routine screening analysis for anabolic androgenic steroids, including enzymatic hydrolysis with β-glucuronidase from E.coli and liquid-liquid extraction with t-butyl methyl ether (TBME). After derivatisation with TMIS reagent the samples were injected into the GC-MS [2] resp. GC-MS/MS. Additionally the unconjugated steroids were determined after similar sample preparation but skipping the enzymatic cleavage.

Instrumentation

For the analyses of urine samples the GC-MS was operated with the following parameters:

GC-MS:	GC: Hewlett Packard (HP) 6890, MSD: HP 5973
Injection param.:	Volume: 3 µl, Temp.: 300°C
Column:	HP Ultra-1 (OV 1); 17 m; 0.2 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:10, head pressure 89.6 kPa
Oven temp.:	177°C with 3°C/min to 226°C, with 40°C/min to 310°C, 2 min hold
Ionisation:	70 eV, electron impact (EI)
Data aqu.:	SCAN

For the analyses of the supplements and the experiments with the reference material the following parameters were applied:

GC-MS:	GC: Hewlett Packard (HP) 6890, MSD: HP 5973
Injection param.:	Volume: 2 µl, Temp.: 300°C
Column:	HP 5 MS; 16.5 m; 0.25 mm i.d.; 0.25 µm film thickness
Carrier gas:	Helium, splitless, head pressure 89.6 kPa
Oven temp.:	100°C with 40°C/min to 190°C, with 5°C/min to 240°C, with 40°C/min to 320°C, 3 min hold
Ionisation:	70 eV, electron impact (EI)
Data aqu.:	SCAN

In case of GC-MS/MS analyses the following parameters were applied:

GC-MS/MS:	GC Finnigan, GCQ
Injection param.:	Volume: 2 µl, Temp.: 325°C
Column:	HP Ultra-1 (OV 1); 14 m; 0.25 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:10, head pressure 68.9 kPa
Oven temp.:	100°C with 40°C/min to 190°C, with 5°C/min to 240°C, with 40°C/min to 320°C, 3 min hold
Ionisation:	70 eV, electron impact (EI)
Data aqu.:	Daughter ion scan
Coll. Energy:	1.2 V

Results and Discussion

Reference standards

After derivatisation with TMIS reagent all 4-hydroxysteroids investigated form two tris-TMS derivatives resulting from different positions for enolisation with the 3,5-dienol as the main product. The mass spectra of these dominant 3,5-dienol TMS derivatives of 4-hydroxy-testosterone, formestane, oxymesterone and oxabolone are presented in Fig. 2. All spectra show M^+ as base peak and the non-specific m/z 73 and 147. More diagnostic information is available from daughter ion spectra (M^+ as parent, e.g. formestane tris-TMS Fig. 3) or TMS derivatives obtained after derivatisation with pure MSTFA.

For oxymesterone fragmentation pathways confirmed by deuteration are proposed in Tab. 1.

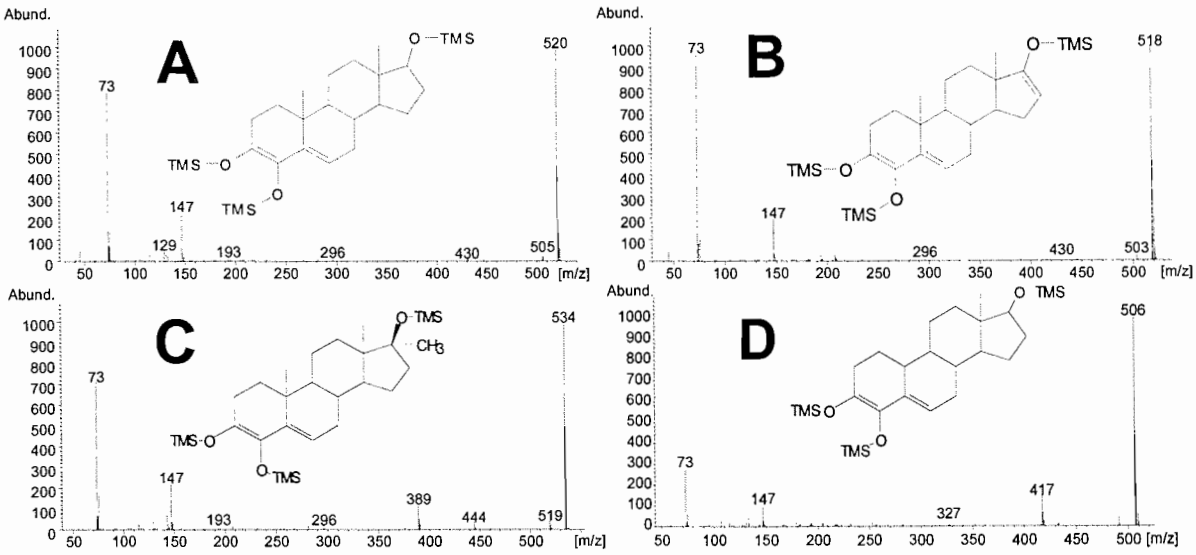


Fig. 2: Mass spectra of the enol-TMS derivatives of 4-hydroxytestosterone (A), formestane (B), oxymesterone (C), and oxabolone (D)

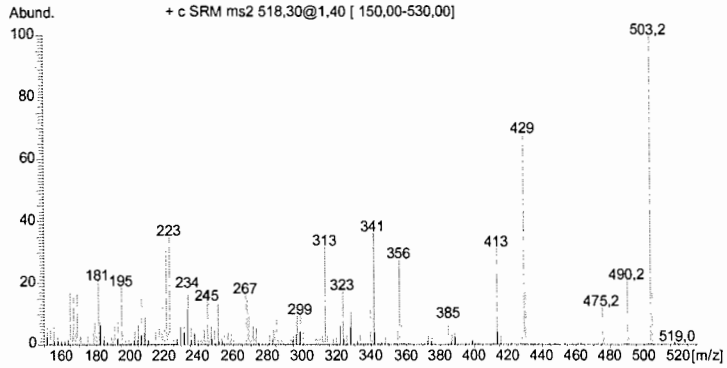
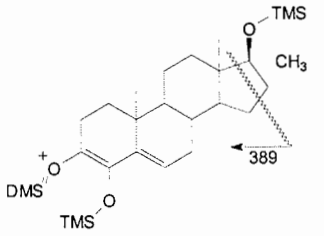
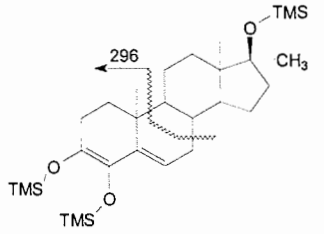
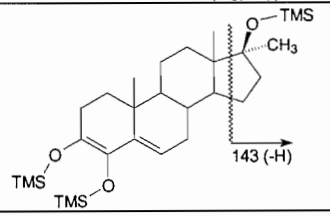


Fig. 3: Daughter ion spectrum of formestane, tris-TMS, parent m/z 518

Tab. 1: Proposed fragments of oxymesterone, tris-enol-TMS

m/z (d_0)	m/z (4-d_9-TMS)	m/z (4,17-bis-d_9-TMS)	m/z (2,2,6-d_3)	fragmentation
534	543	552	537	M^+
519	528	537	522	M^+-CH_3
444	453	453	447	M^+-TMS-OH (D-Ring)
389	398	398	392	
296	305	305	299	
147	153/156	153/156	147	TMS-O-DMS^+ (d_6, d_9)
143	143	152	143	

After reaction of 4-hydroxytestosterone with acetic anhydride followed by derivatisation with TMIS reagent a mixture of the mono- and bis-acetylated derivatives (again 2,4 and 3,5-dienol TMS) was obtained, after reaction with BSA followed by acetylation a mixture of the mono-acetylated and the non-acetylated compound could be derived. For formestane the mono-acetylated derivatives could be obtained with acetylation followed by silylation with TMIS reagent. The mass spectra of the mono-acetates (3,5-dienol) are presented in Fig. 4. Additionally the mass spectrum of the per-TMS derivative of oxabolone 17-cypionate (3,5-dienol) is included in Fig. 4.

Comparing these mass spectra the 17-esters provide only little diagnostic information (M^+, M^+-15, m/z 147, and m/z 73 and a fragment for the acid moiety of the ester, e.g. m/z 43 for the acetate).

The mass spectra of the 4-acetates show abundant ions at M^+, M^+-42 (CH_2=C=O) and M^+-58, in addition to M^+-15, m/z 193 and m/z 73.

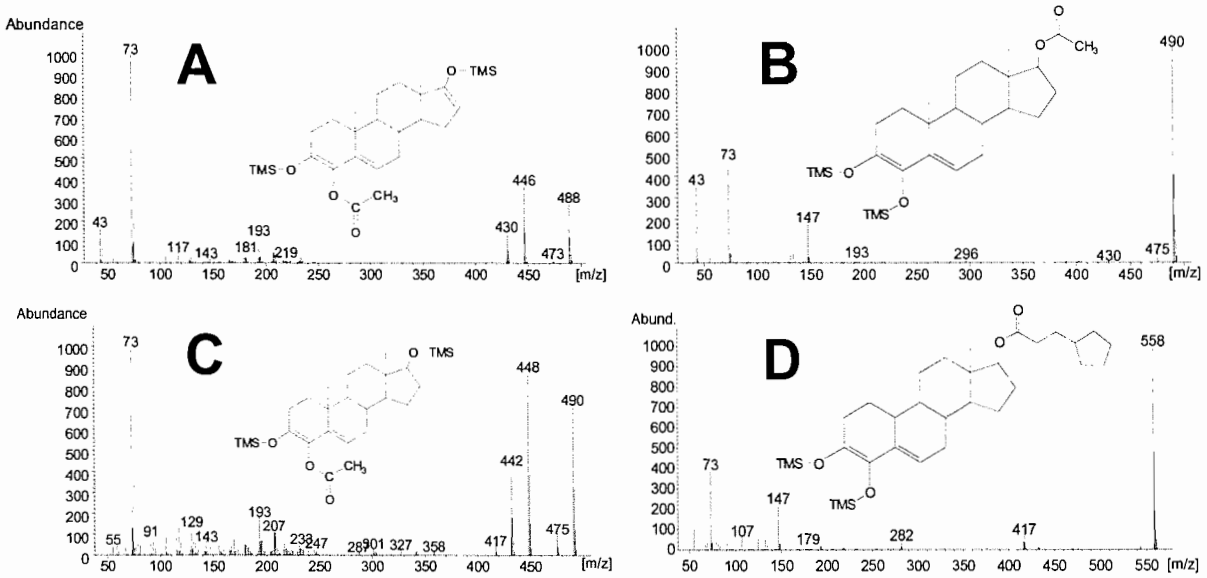


Fig. 4: Mass spectra of the mono-acetyl-bis-TMS derivatives of formestane (A: 4-acetate), 4-hydroxytestosterone (B: 17-acetate, C: 4-acetate), and bis-TMS derivative of oxabolone 17-cypionate (D)

Cleavage of the esters

After hydrolysis of oxabolone cypionate with the different reagents overnight no intact ester was detected. After hydrolysis with the reagents containing KOH at 80°C also nearly no oxabolone was detected because of decomposition.

Best results were obtained with HCl (1 M in H_2O) and HCl (0.5 M in H_2O/MeOH, 1:1) yielding high amounts of oxabolone. Hydrolysing oxabolone cypionate with KOH (1 M in H_2O/MeOH, 1:1) and KOH (1 M in H_2O) at 60°C resulted in detectable amounts of oxabolone. However the intensitiy was only ~1/20 of oxabolone obtained after HCl hydrolysis.

Therefore hydrolysis with HCl (0.5 M in H_2O/MeOH) was chosen for all future experiments where a cleavage of the esters is included.

pH-profiles

The extraction yields of the 4-hydroxysteroids (Fig. 5) are strongly influenced by the pH of the aqueous solution. The relative acidic OH-groups of the A-ring can be deprotonated resulting in low lipophilia at high pH values. Also the 17-esters of the 4-hydroxysteroids show a similar pH profile for liquid-liquid extraction (Fig. 6). The 4-acetate of formestane does not have an acidic OH group which might result in bad extraction yields at high pH values. However, the recovery of this ester at pH>9 is also very bad (Fig. 6). This can be explained by cleavage of the ester during the extraction. Formestane itself can be detected in those samples (in the aqueous or the n-pentane layer depending on the pH value).

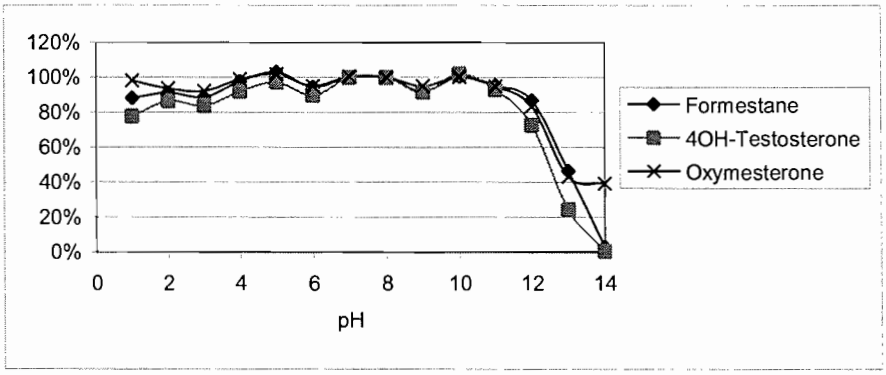


Fig. 5: Liquid-liquid extraction of 4-OH steroids, pH profiles

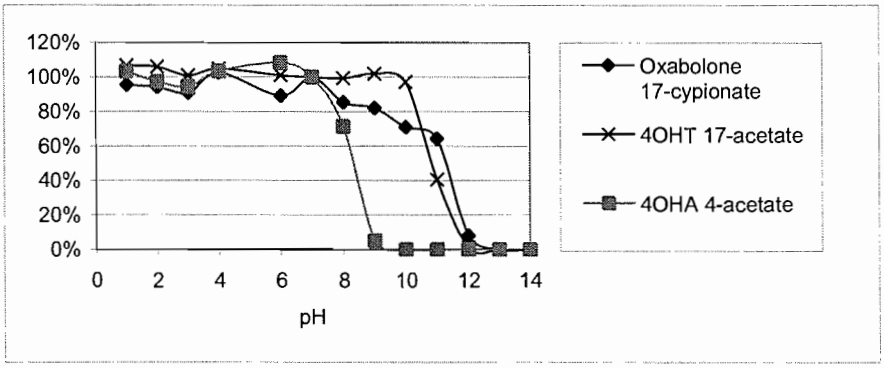


Fig. 6: Liquid-liquid extraction of oxabolone 17-cypionate, 4-OH-testosterone 17-acetate and formestane 4-acetate, pH profiles

Supplement analyses

The supplement Primobolan™ contained the 4-acetylated formestane as labelled. In the supplement Testobol™ the 17-acetylated 4-hydroxytestosterone could be confirmed. No THP ether was present despite the indication on the label.

Excretion study with Testobol™

In the urine samples obtained after oral administration of two tablets of the supplement which was found to contain the 17-acetate of 4-hydroxytestosterone several metabolites are present in the combined unconjugated + glucuronide (f+g) fraction. The chromatogram obtained after routine screening analysis for anabolic steroids (Scr. IV, [2], GC/MS in SCAN mode) is presented in Fig. 7 (ion traces m/z 518 and 520). The main metabolite could be identified as 4-hydroxyandrostenedione (M^+=518 as per-TMS) by means of GC-MS/MS (chromatogram of ion trace m/z 518 in Fig. 9) based on the comparison with reference material. It was present in all urines collected (up to 48 hours). Another metabolite (metabolite II) with a similar full scan mass spectrum almost co-elutes with it. The daughter ion scan of m/z 518 (Fig. 8) appears to be different from that of 4-hydroxyandrostenedione.

Additionally 4-hydroxytestosterone (M^+=520 as per-TMS) was found in the post administration (p.a.) samples after cleavage of the glucuronides by comparison with reference material. By means of GC-MS/MS it could be shown that also in this case another metabolite (metabolite III) with M^+=520 co-elutes. The daughter ion scan of metabolite III (m/z 520 as parent, Fig. 10) shows an analogue spectrum as metabolite II. Until now the structures of these metabolites as well as some other minor metabolites are still unidentified.

In the unconjugated fraction only 4-hydroxyandrostenedione could be confirmed in low concentration.

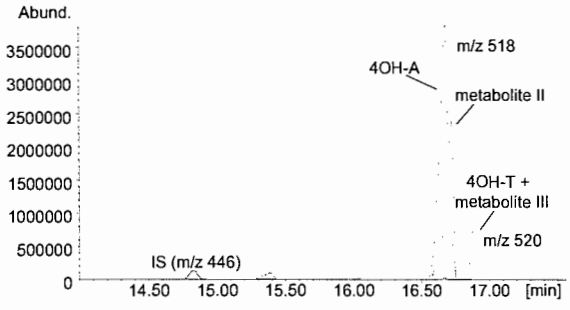


Fig. 7: Chromatogram of the p.a. urine (0-2h, Scr. IV analysis [2])

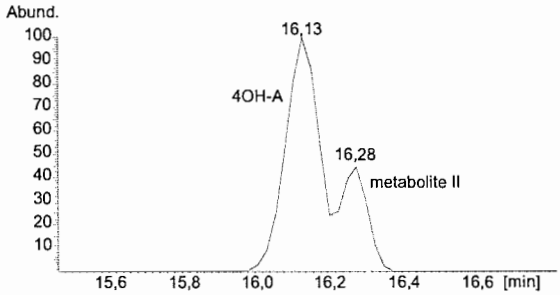


Fig. 9: GC-MS/MS chromatogram of m/z 518

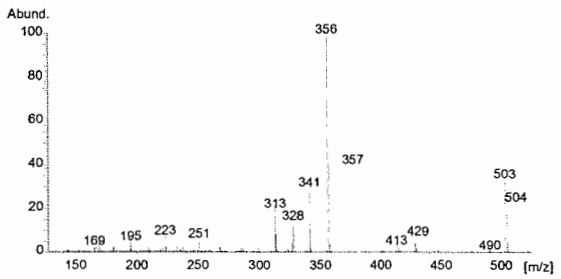


Fig. 8: Daughter ion spectrum of metabolite II parent m/z 518

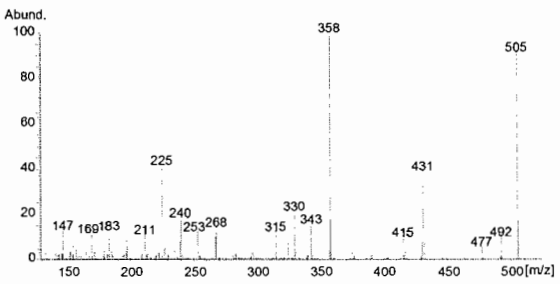


Fig. 10: Daughter ion spectrum of metabolite III parent m/z 520

Summary

4-Hydroxysteroids resp. their metabolites can be included in the screening procedure for anabolic steroids. As the obtained enol-TMS derivatives provide only little diagnostic information, for confirmation MS/MS or the formation of other derivatives should be used.

Because of acidic OH-groups in the A-ring they are extracted only with low recoveries at pH levels >10. The esters are easily cleaved under acidic conditions yielding the target compounds. Fragmentation pathways for oxymesterone are proposed and confirmed by some deuteration experiments. Analogue fragmentations are also likely for the other 4-hydroxysteroids investigated.

After oral administration of 4-hydroxytestosterone 17-acetate 4-hydroxyandrostenedione is found as the major metabolite after cleavage of the glucuronides. Additionally 4-hydroxy-testosterone was present in the urine. Both substances are co-eluting with two other metabolites which are still unidentified. Additionally several other unidentified metabolites are obtained. Data from the literature [3] show that also after the administration of formestane 4-hydroxyandrostenedione can be found as the main and 4-hydroxytestosterone as a minor metabolite in the fraction after enzymatic cleavage with glucuronidase. To avoid difficulties with the interpretation of 4-hydroxyandrostenedione findings (parent compound after formestane application or metabolite after 4-hydroxytestosterone application) in female athletes urines, also formestane should be explicitly listed as anabolic agent[1].

Acknowledgements

The Manfred-Donike-Society, Cologne, and the Bundesinstitut für Sportwissenschaften, Bonn, are acknowledged for supporting the study.

References

[1] World Anti-Doping Agency: The 2004 prohibited list. World Anti-Doping Agency, Montreal 2004, at http://www.wada-ama.org/rtecontent/document/list_standard_2004.pdf

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[3] Poon, G.K., Jarman, M., Rowlands, M.G., Determination of 4-hydroxyandrost-4-ene-3,17-dione metabolism in breast cancer patients using high-performance liquid chromatography-mass spectrometry. J. Chromatogr, 565 (1991), 75

[1] Remark: Since 2005 anti-estrogens are prohibited for female athletes as well (http://www.wada-ama.org/rtecontent/document/list_standard_2005.pdf).