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Characterisation of Steroid Metabolites Recently Detected in Doping Control Analyses

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Introduction

Recently new steroid metabolites were detected in routine doping control. The proposed structures were confirmed by chemical synthesis as the relevant steroids were not commercially available. The products of synthesis were characterised by mass spectrometry and nuclear magnetic resonance. The inference on the administered steroid was established by the analysis of post-administration (p.a.) urines.

17-Epimer of long-term metabolite of metandienone

As reported by Schänzer et al. (Schänzer *et al.* 2006) 17 β -hydroxymethyl-17 α -methyl-18-norandrosta-1,4,13-trien-3-one was detected as long-term metabolite in human urine after the administration of metandienone. Additionally its 17-epimer is excreted as minor metabolite after metandienone or “18-normetandienone” (17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one) application (Figure 1). Both epimers show similar mass spectra.

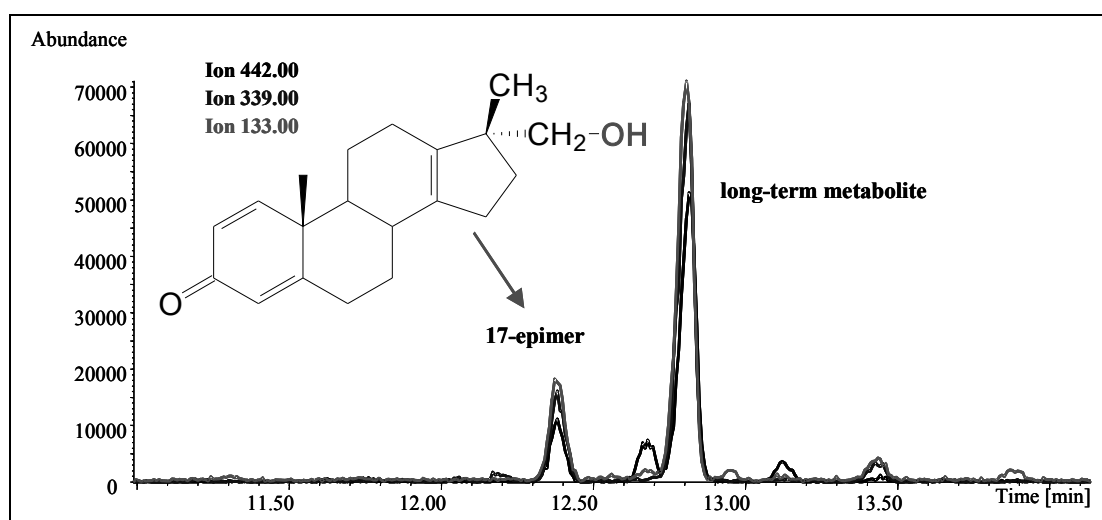


Figure 1: Chromatogram of urine after administration of metandienone

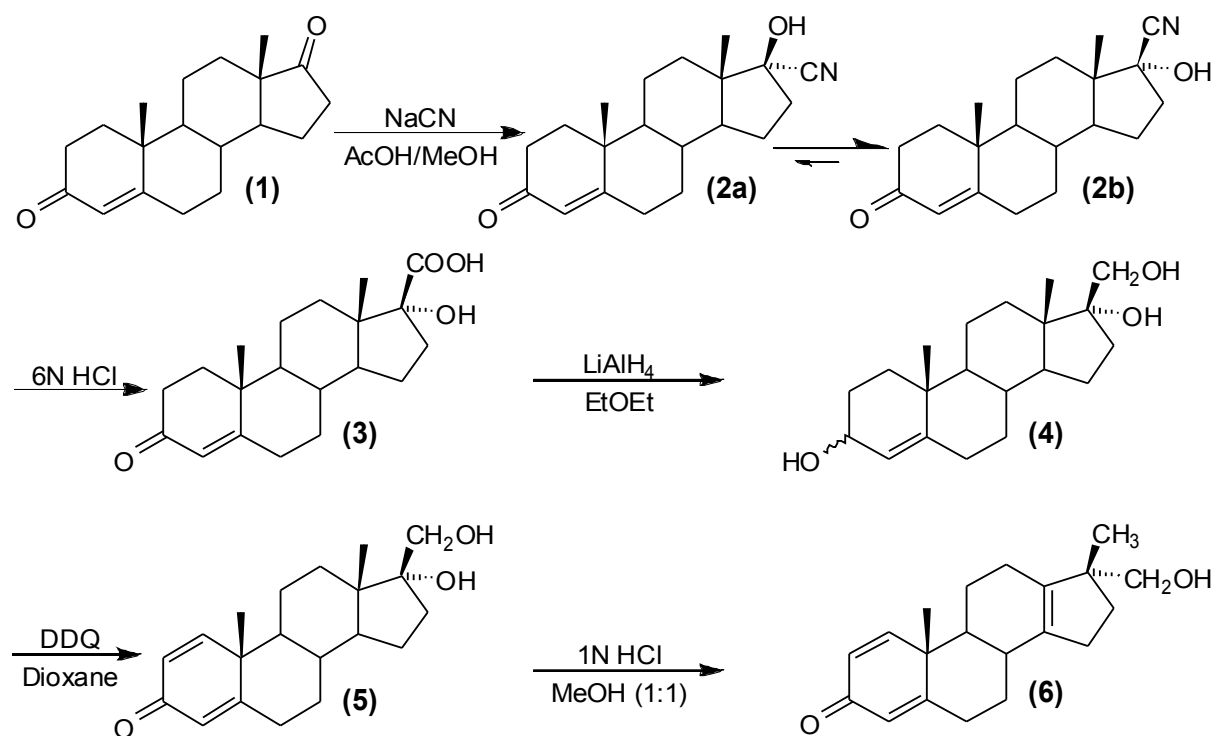


Figure 2: Synthesis of 17 α -hydroxymethyl-17 β -methyl-18-norandrosta-1,4,13-trien-3-one (**6**)

The synthesis of 17 α -hydroxymethyl-17 β -methyl-18-norandrosta-1,4,13-trien-3-one (**6**) was performed as displayed in Figure 2 starting with androst-4-ene-3,17-dione (**1**). The formation of androstenedione cyanhydrine (**2a and b**) was achieved as described (Nitta *et al.* 1985). They reported that under the conditions applied 17 β -cyano-17 α -hydroxy-androst-4-en-3-one (**2b**) precipitates from the mixture. After hydrolysis and reduction, two isomers of 17 β -hydroxymethyl-androst-4-ene-3 ξ ,17 α -diol (**4**) were obtained. Dehydrogenation with 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) in dioxane resulted in 17 α -hydroxy-17 β -hydroxymethyl-androsta-1,4-dien-3-one (**5**). After Wagner-Meerwein rearrangement 17 α -hydroxymethyl-17 β -methyl-18-norandrosta-1,4,13-trien-3-one (**6**) was obtained. After purification its structure was confirmed by means of NMR (¹H, H,H COSY, APT, H,C HMQC, H,C HMBC and H,H NOESY).

6 α -Methylandrost-4-ene-3,17-dione and its metabolites

In recent years products containing 6 α -methylandrost-4-ene-3,17-dione appeared on the sport supplement market. First reports were published by Kazlauskas (Kazlauskas 2006). Scientific studies have proven aromatase inhibition (Numazawa *et al.* 1996; Numazawa and Oshibe 1994) and 6 α -methyl-androst-4-enes are described to show higher myotrophic and similar

androgenic activity compared to their non-methylated analogues (Campbell *et al.* 1958; Ringold *et al.* 1957). However no preparation was approved for medical use up to now. In sports, 6 α -methylandroster-4-ene-3,17-dione has to be classified as prohibited substance according to the regulations of the World Anti-Doping Agency (WADA).

For the detection of its misuse the metabolism was studied following the administration of the preparation Methyl-1-Pro of the brand name ProLine. It was obtained from the Internet (<http://www.masterpiecefitnesssupplements.com>) and was labelled to contain: "Anabolic agent (17-hydroxy-6 α -ethyl-cholesterol-3,20-dione): 150 mg, estrogen control complex (6 α -methyl-cholesterol-3,17-dione): 30 mg". As indicated by these non-approved names the analysis of the product confirmed the presence of 6 α -methylandroster-4-ene-3,17-dione and 5 β -dihydroprogesterone (17 α -hydroxy-6 α -methyl-5 β -pregnane-3,20-dione) in addition to non-labelled androst-4-ene-3,17-dione. Several metabolites as well as the parent compounds were synthesized and the structures of 3 α -hydroxy-6 α -methyl-5 β -androstane-17-one, 6 α -methylandroster-4-ene-3,17-dione, and 5 β -dihydroprogesterone were confirmed by NMR. The main metabolite, 3 α -hydroxy-6 α -methyl-5 β -androstane-17-one, was found to be excreted as glucuronide and was still detectable in $\mu\text{g/mL}$ -amounts until urine collection was stopped (after 25 hours). The mass spectrum of its bis-TMS derivative is shown in Figure 3.

Screening analysis can be easily performed by the existing screening procedure for anabolic steroids (Geyer *et al.* 1998) using 3 α -hydroxy-6 α -methyl-5 β -androstane-17-one as target substance (limit of detection < 10 ng/mL of urine). It almost coelutes with the drostanolone metabolite 3 α -hydroxy-2 α -methyl-5 α -androstane-17-one showing a very similar mass spectrum.

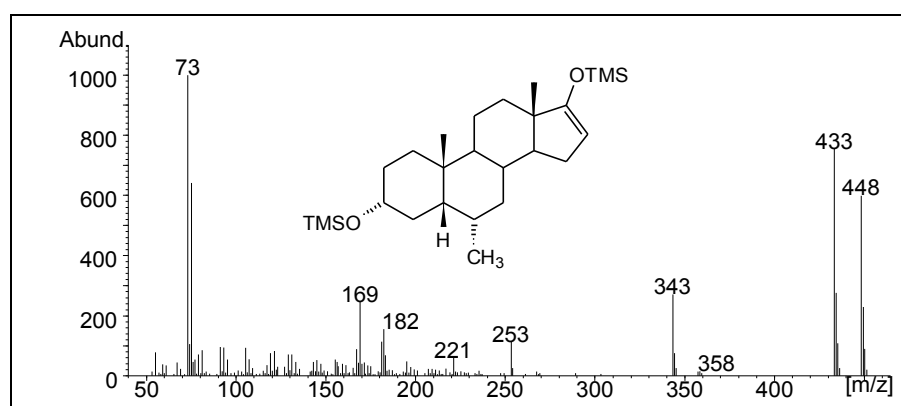


Figure 3: Mass spectrum (EI) the bis-TMS derivative of 3 α -hydroxy-6 α -methyl-5 β -androstane-17-one, $M^+ = 448$, the main metabolite of 6 α -methylandroster-4-ene-3,17-dione

Their discrimination is possible using the mono-TMS derivative formed using pure MSTFA for derivatisation. The resulting mass spectra are shown in Figure 4 and Figure 5.

In 2006 two samples collected for human routine sports doping control were already tested positive for the presence of metabolites of 6 α -methylandrosta-4-ene-3,17-dione. Due to the additional detection of metabolites of 5 β -dihydromedroxyprogesterone together with an elevated T/E ratio in the samples (most likely due to the androst-4-ene-3,17-dione in the products), it is presumable that the athletes providing the samples also administered a product like Methyl-1-Pro.

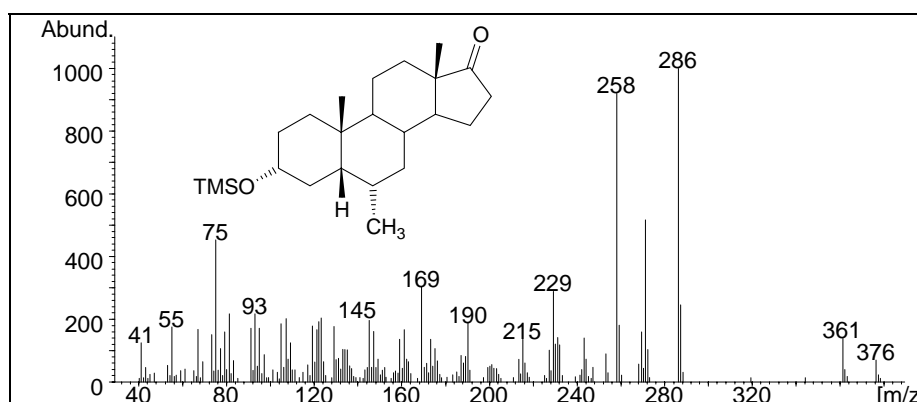


Figure 4: Mass spectrum (EI) of 3 α -hydroxy-6 α -methyl-5 β -androstane-17-one (8), mono-TMS, $M^+ = 376$

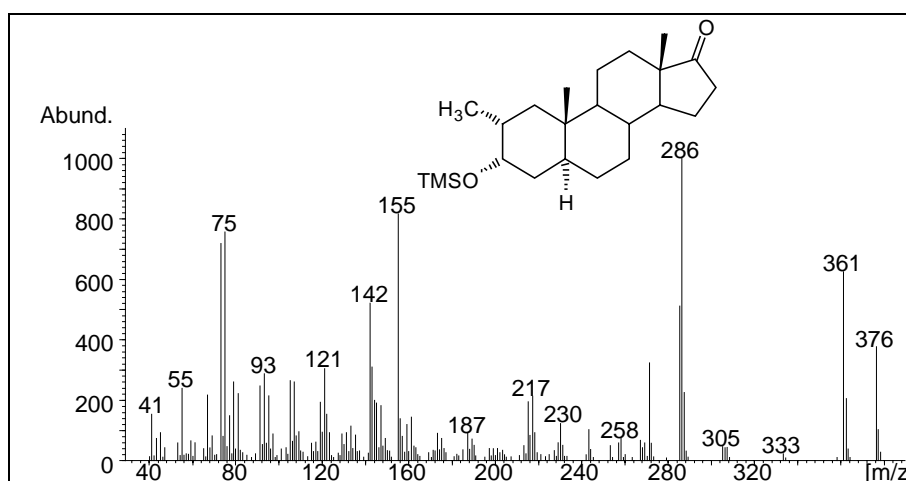


Figure 5: Mass spectrum (EI) of 3 α -hydroxy-2 α -methyl-5 α -androstane-17-one (drostanolone metabolite), mono-TMS, $M^+ = 376$

Androsta-1,4,6-triene-3,17-dione and 17-hydroxy metabolite

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 17 β -hydroxy analogue. For confirmation 17 β -hydroxy-androsta-1,4,6-triene-3-one was synthesised by dehydrogenation of testosterone and NMR analyses were performed for both 1,4,6-trienes. Additionally several reduced metabolites were detected in the post-administration urines, namely 17 β -hydroxy-androst-4,6-diene-3-one, 17 β -hydroxy-androst-1,4-diene-3-one (boldenone) and 17 β -hydroxy-5 β -androst-1-ene-3-one (boldenone metabolite).

For androsta-1,4,6-triene-3,17-dione and its 17-hydroxy analogue the derivatization, using TMIS reagent (MSTFA/ NH₄I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60 °C, resulted in two products in a ratio of 4:1. The mass spectra of the mono-TMS derivative (main product) and an artifact obtained by a loss of the 19-methyl group are displayed in Figure 6 and Figure 7. The structure of the artifact could be confirmed by GC-MS comparison of the bis-TMS derivative of commercially available 3-hydroxy-estra-1,3,5(10),6-tetraen-17-one. GC-MS of the underivatized compounds and LC-MS/MS analysis was successfully performed. The mass spectra are shown in Figure 8 and Figure 11.

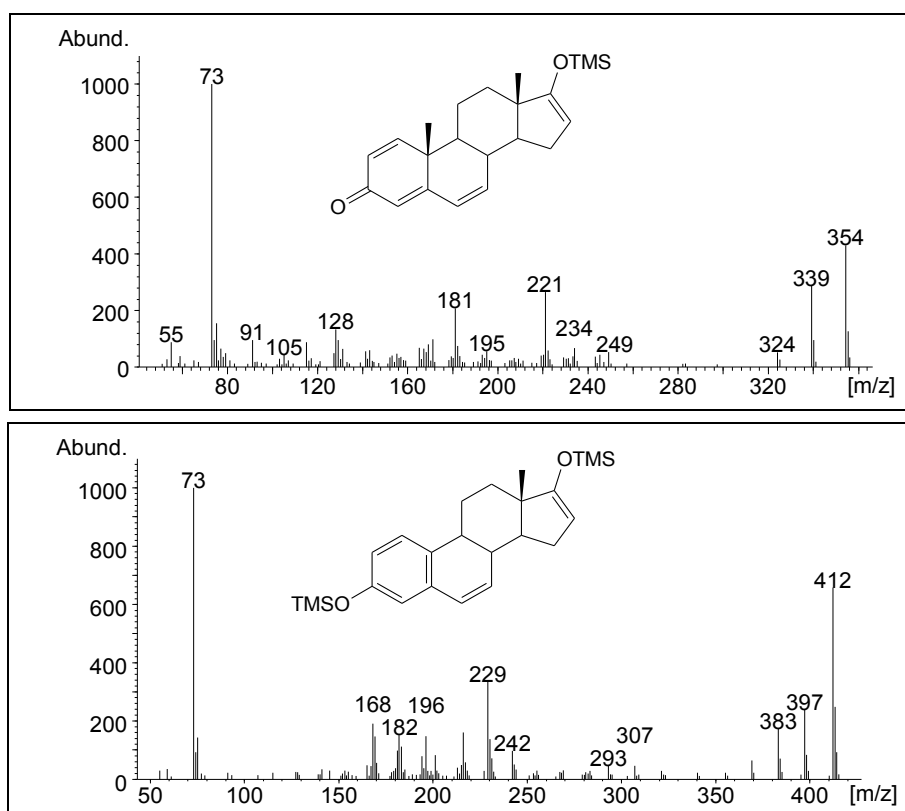


Figure 6: Mass spectrum (EI) of androsta-1,4,6-triene-3,17-dione, mono-TMS derivative, $M^+ = 354$ (upper), artifact, bis-TMS, $M^+ = 412$ (lower)

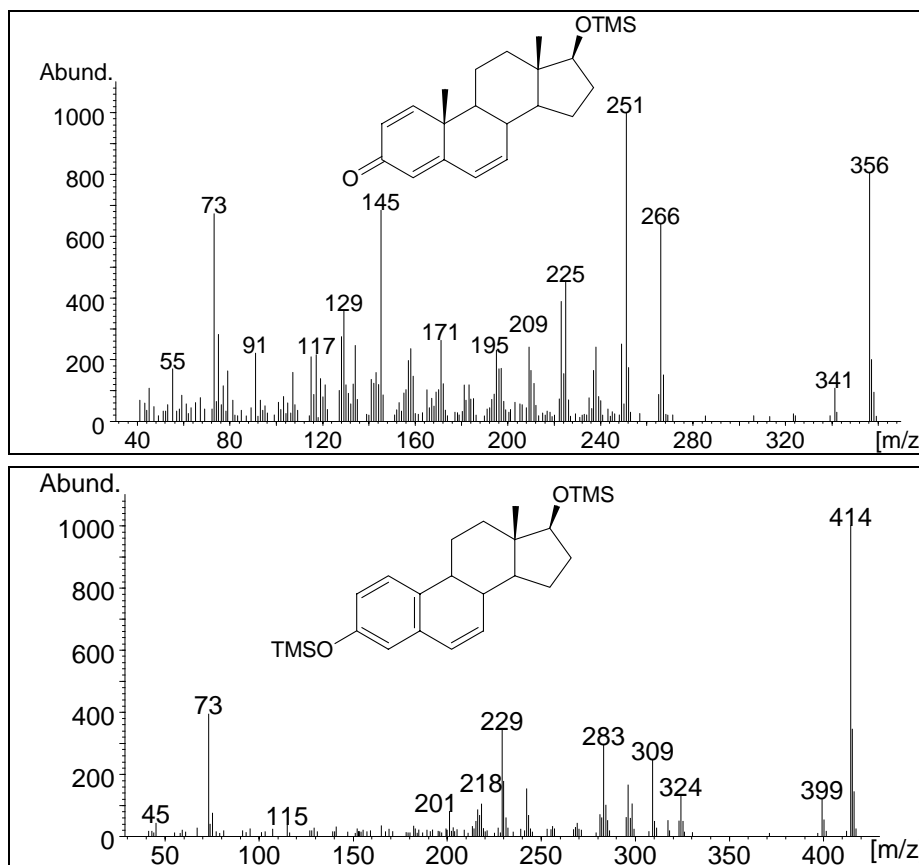


Figure 7: Mass spectrum (EI) of 17β-hydroxyandrost-1,4,6-triene-3-one, mono-TMS, $M^+ = 356$ (upper), artifact, bis-TMS, $M^+ = 414$ (lower)

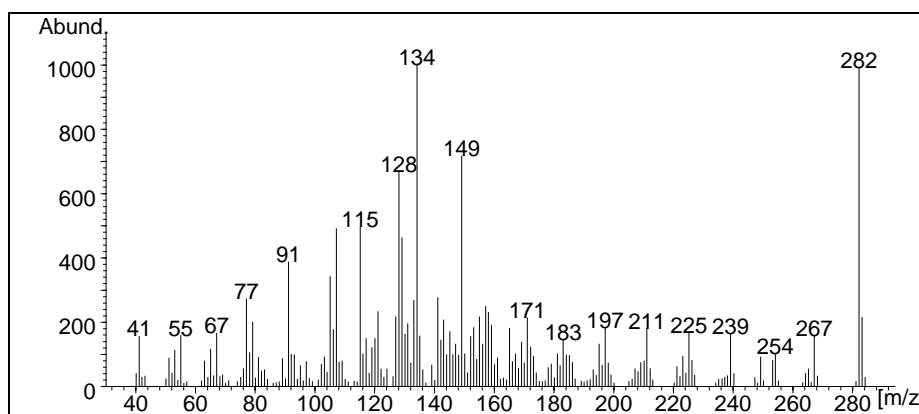


Figure 8: Mass spectrum of androst-1,4,6-triene-3,17-dione, GC-MS (EI) underivatized, $M^+ = 282$

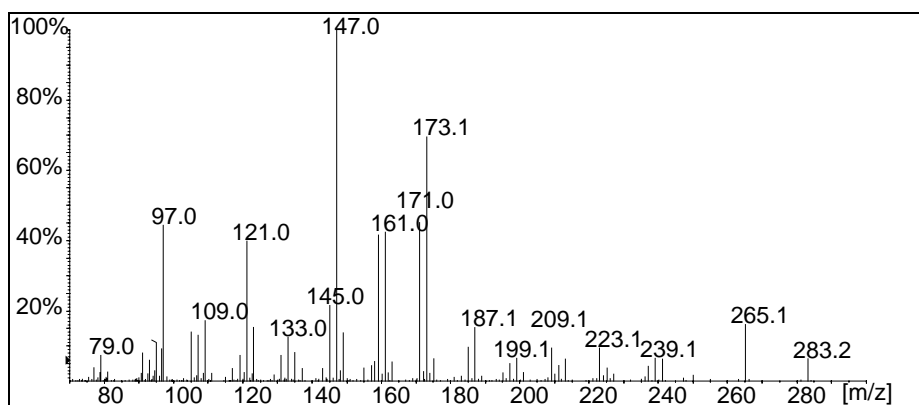


Figure 9: Product ion spectrum of androst-1,4,6-triene-3,17-dione, LC-ESI-MS/MS, $[M+H]^+ = 283$

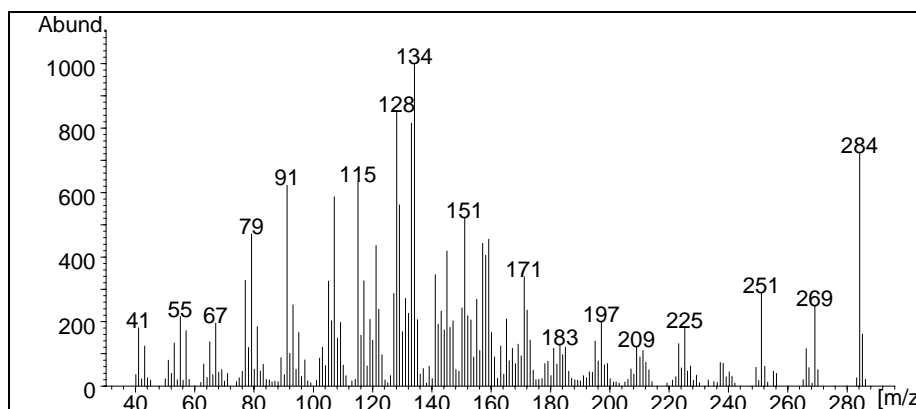


Figure 10: Mass spectrum (EI) of 17β-hydroxyandrosta-1,4,6-triene-3-one, underivatized, $M^+ = 284$

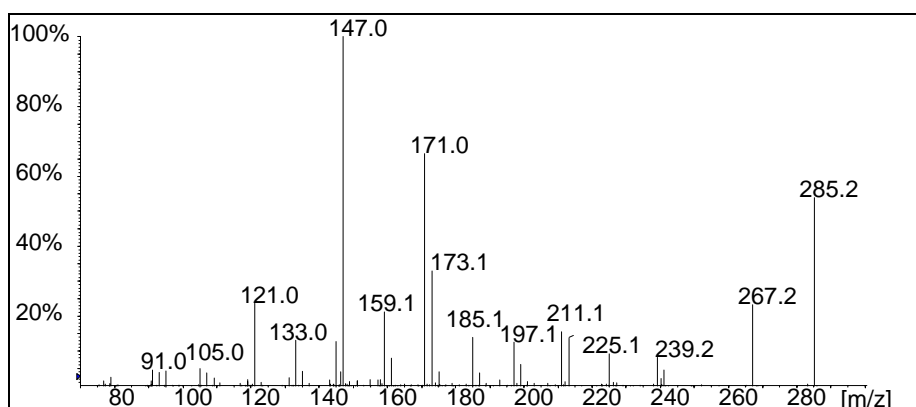


Figure 11: Product ion spectrum of 17β-hydroxyandrosta-1,4,6-triene-3-one, LC-ESI-MS/MS, $[M+H]^+ = 285$

Even if not explicitly listed the World Anti-Doping Agency (WADA) classifies the administration of androsta-1,4,6-triene-3,17-dione in sports as doping (Rabin 2006; World Anti-Doping Agency 2007) due to the aromatase inhibiting properties (Covey and Hood 1981; Schwarzel *et al.* 1973).

In three samples collected for human routine sports doping control metabolites of androsta-1,4,6-triene-3,17-dione were detected in 2006. The samples were initially found suspicious for the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one. It is presumable that these findings were due to the administration of a product like “Novedex Xtreme”. This product was obtained from the Internet sport supplement market (www.bodybuilding.com). As labelled it was found to contain a prohormone of androst-4-ene-3,6,17-dione (“6,17-keto-etiiocholeve-3-ol tetrahydropyranol”, 3-hydroxyandrost-4-ene-6,17-dione acc. to IUPAC) in addition to androsta-1,4,6-triene-3,17-dione. Metabolites of this so-called “6-Oxo” (structures proposed by van Eenoo *et al.* (Van Eenoo *et al.* 2005)) were also present in the urine samples.

For the main androst-4-ene-3,6,17-trione metabolite, 3 α ,6 α -dihydroxy-5 β -androstane-3-one (6 α -hydroxyetiocholanolone), this proposal could be confirmed by GC-MS comparison with reference material during our study (mass spectrum of the tris-TMS derivative in Figure 12).

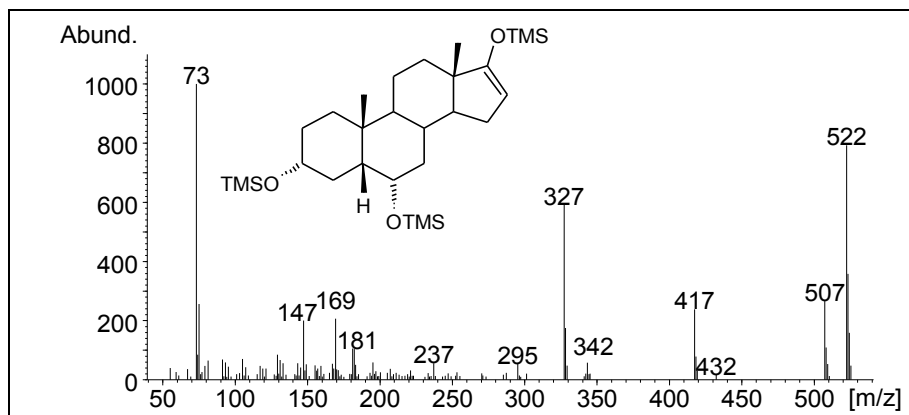


Figure 12: Mass spectrum (EI) of 3 α ,6 α -dihydroxy-5 β -androstane-3-one, tris-TMS, $M^+=522$

Remarks

The detailed results of the investigations will be published elsewhere.

The data for 6 α -methylandrost-4-ene-3,17-dione are available in:

Parr MK, Kazlauskas R, Schlörer N, Opfermann G, Piper T, Schulze G, Schänzer W (2008). 6 α -Methylandrostenedione: gas chromatographic mass spectrometric detection in doping control. Rapid Commun Mass Spectrom **22**, 321–329.

Acknowledgements

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References

Campbell, J.A., Babcock, J.C. and Hogg, J.A. (1958) 6-methyl steroids in the androstane series. *J Am Chem Soc* **80**, 4717-4721.

Covey, D.F. and Hood, W.F. (1981) Enzyme-generated intermediates derived from 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione cause a time-dependent decrease in human placental aromatase activity. *Endocrinology* **108**, 1597-1599.

Geyer, H., Schänzer, W., Mareck-Engelke, U., Nolteernsting, E. and Opfermann, G. (1998) 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. and Mareck-Engelke, U.(eds.) *Recent advances in doping analysis (5)*, Köln. pp 99-102.

Kazlauskas, R. (2006) Micellaneous projects in sports drug testing at the national measurement institute, australia, 2005. In: Schänzer, W., Geyer, H., Gotzmann, A. and Mareck, U.(eds.) *Recent advances in doping analysis (14)*, Köln. pp 129-140.

Nitta, I., Fujimori, S. and Ueno, H. (1985) The syntheses of the corticoid side-chain .1. An improved method for the preparation of 17-alpha-hydroxyprogesterone from androst-4-ene-3,17-dione. *B Chem Soc Jpn* **58**, 978-980.

Numazawa, M., Kamiyama, T., Tachibana, M. and Oshibe, M. (1996) Synthesis and structure-activity relationships of 6-substituted androst-4-ene analogs as aromatase inhibitors. *J Med Chem* **39**, 2245-2252.

Numazawa, M. and Oshibe, M. (1994) 6-alkyl- and 6-aryl androst-4-ene-3,17-diones as aromatase inhibitors - synthesis and structure-activity-relationships. *J Med Chem* **37**, 1312-1319.

Rabin, O. (2006) New steroid substance androsta-1,4,6-triene-3,17-dione. Email communication to Dezso, M., 22.06.

Ringold, H.J., Batres, E. and Rosenkranz, G. (1957) Steroids .84. Synthesis of 6-methyl hormone analogs. *J Org Chem* **22**, 99-100.

Schänzer, W., Geyer, H., Fußhoeller, H., Halatcheva, N., Kohler, M., Parr, M., Guddat, S., Thomas, A. and Thevis, M. (2006) Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine. *Rapid Commun Mass Spectrom* **20**, 2252-2258.

Schwarzel, W.C., Kruggel, W.G. and Brodie, H.J. (1973) Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. *Endocrinology* **92**, 866-880.

Van Eenoo, P., Mikulèíková, P., Deventer, K., VanThuyne, W. and Delbeke, F. (2005) Metabolism, excretion and detection of androst-4-ene-3,6,17-trione. In: Schänzer, W., Geyer, H., Gotzmann, A. and Mareck, U.(eds.) *Recent advances in doping analysis (13)*, Köln. pp 57-64.

World Anti-Doping Agency. The 2007 prohibited list City (2007) http://www.wada-ama.org/rtecontent/document/2007_List_En.pdf (access date 17.01.2007)