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Preliminary results of the implementation of some metabolites of the anabolic androgenic testolactone in the screening procedures

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Introduction

Testolactone (Figure 1) is a steroid, which is not present on the doping list of the IOC as an example of the forbidden anabolic agents. However, it can be classified according to that same list as an androgenic anabolic steroid. Moreover, in the U.S Code of Federal Regulations, Title 21 Part 1308.13, as defined in Part 1308.02 (1995) testolactone is explicitly indicated as a controlled substance (anabolic steroid) [1]. On some Internet websites it is occasionally mentioned as one the androgenic anabolic steroids also. Although there are no direct records of athletes using this steroid, the "Laboratório de Análises de Dopagem e Bioquímica" decided to implement the detection of use of testolactone in its screening procedures.

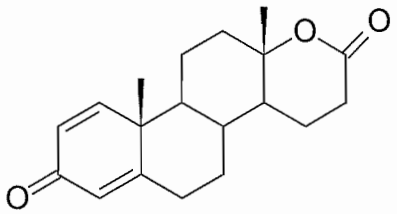
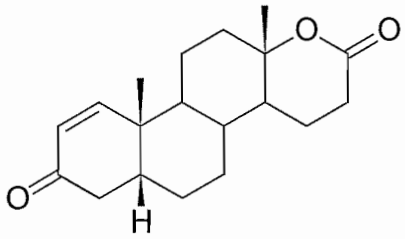


Figure 1: Structure of testolactone

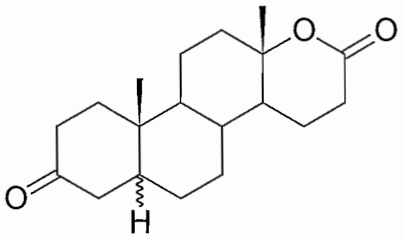
Testolactone can be obtained by microbial transformation of progesterone or testosterone [1]. From a therapeutic point of view it has been classified as antineoplastic. Although it may have some anabolic properties, its abuse may also result in several side effects.

Testolactone is, like other 1,4-diene-3-one-like steroids (methandienone and boldenone) extensively metabolised with modifications in the A-ring [2,3]. The main

metabolites reported in the literature are 4,5β-dihydrotestolactone (Figure 2, structure A), 1,2,4,5-tetrahydrotestolactone (Figure 2, structure B) and D-homo-17α-oxa-3-hydroxy-5β-androst-1-ene-17-ene (Figure 2, structure C). In the literature however, no mass spectrometric data are available. We will present some mass spectrometric data of two metabolites that have been found in urine samples after performing an excretion study with 50 mg orally applied.



A



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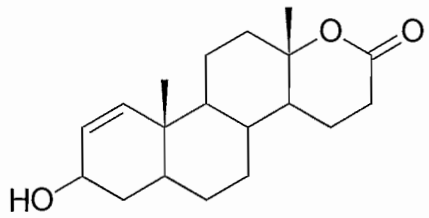

C

Figure 2: Structures of A: 4,5β-dihydrotestolactone; B: 1,2,4,5-tetrahydrotestolactone; C: D-homo-17α-oxa-3-OH-5β-androst-1-ene-17-one

Experimental

Extraction

To 2 ml of urine samples were added 20 µl of internal standard working solution (50 ppm), 750 µl of 0.8 M phosphate buffer pH 7 and 5 ml of *tert.*-butyl methyl ether. The samples were shaken during 5 min on the shaker and afterwards centrifuged during 5 min at 3000 rpm. The organic layer (free fraction) was separated from the aqueous layer and the organic solvent removed by a rotating evaporator. The

was put under a stream of nitrogen to remove the organic solvent and afterwards 50 µl of β-glucuronidase were added and the mixture incubated for 60 min at 55 °C. After cooling to room temperature 0.5 ml of $K_2CO_3/KHCO_3$ in water (1:1:8; w/v/v) and 5 ml of *tert.*-butyl methyl ether were added. The samples were shaken during 5 min and afterwards centrifuged during 5 min at 3000 rpm. The organic layer (conjugated fraction) was separate from the aqueous layer, and the solvent removed by the rotating evaporator. The residue of the conjugated fraction was dried in vacuum over di-P_2O_5/KOH for least 3 h also.

Derivatisation

The derivatisation was performed by adding 50 µl of MSTFA/NH4I/dithioerythritol (1000:5:5; v/w/w) to the free and the conjugated fraction and heating the mixture for 30 min at 60 °C. The derivatisation mixture was injected and analysed according to Table 1.

Analytical parameters

Table 1: Conditions and specifications of GC and MS

MSD 5973	GC 6890
MS Quad – 150 °C	Initial Temperature – 190 °C
MS Source – 230 °C	Rate 1– 2° C/min
Interface – 310 °C	Temperature level 1 – 240 °C
Ionisation mode – EI	Rate 2– 15° C/min
Acquisition mode – Scan	Temperature level 1 – 300 °C
Multiplier voltage – Autotune Voltage	Time level 2 – 2 min
	Column
Injection parameters	
	Brand – Hewlett Packard
Injection mode – 1:10 split	Type – HP1 Length– 18 m
Injection volume – 2 µl	Inner diameter – 0.2 mm
Injector temperature – 250 °C	Film Thickness– 0.11 µm

Results

Two metabolites were found using the screening IV procedure for detection free and conjugated fraction of anabolic steroids in urine samples. Etiocholanolone was used as the internal standard to calculate the relative retention times, because the traditional internal standards could co-elute with possible metabolites.

The metabolite 1 was found in the free fraction and the major ion observed was at *m/z* 446 (Figure 3). The relative retention time of the peak was 1.09. Our interpretation is that the ion at *m/z* 446 is the molecular ion. The ion at *m/z* 431 subsequently corresponds to the loss a methyl group. The clusters of ions in the range of *m/z* 165-206 represents fragmentations at the A- and B-ring, respectively (Figure 3). This cluster is typically for the 3-O-enolTMS derivatives of 1-ene-3-one steroids [5].

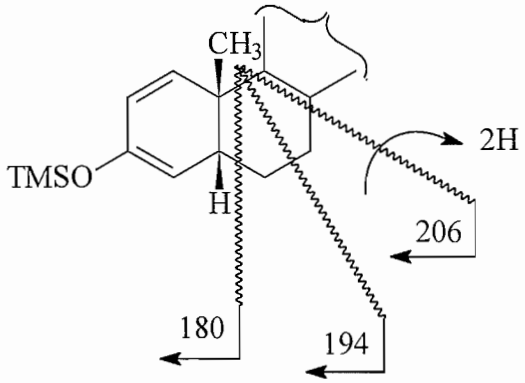

Figure 4: Interpretation of the mass fragmentation of the O-TMS-O-enolTMS derivative of metabolite 1

The metabolite 2 was found in the conjugated fraction. The relative retention time of the peak was 1.27. The ion observed at *m/z* 448 is apparently the molecular ion (Figure 5). Another significant ion was at *m/z* 129. This ion however, is a non-specific ion, which can originate in general from a A-ring as well as a D-ring [6]. Therefore, with the information available we could not assign definitely a structure to this metabolite. There are however, in principal two possible compounds 1,2,4,5-tetrahydrotestolactone or D-homo-17α-oxa-3-OH-5β-androst-1-ene-17-one that could fit this structure. Most likely it is D-homo-17α-oxa-3-OH-5β-androst-1-ene-17-one as

other 1,4-diene-3-one steroids like boldenone and methandienone are know to be transformed into androst-1-ene-17-one like metabolites [4,5]

Conclusion

Metabolite 1, present in the free fraction, was identified based on its mass spectrum of the O-TMS-O-enolTMS derivative as being 4,5β- dihydrotestolactone.

For metabolite 2, present in the conjugated fraction, it was not possible to assign the structure with certainty. However based on the metabolism of other 1,4-diene-3-one-like steroids, it can be predicted that the most probable structure is D- homo-17α-oxa-3-OH-5β-androst-1-ene-17-one.

Literature

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[2] Segaloff *et al.*, The metabolism of 4-^{14}C-Δ-Testolactone, Steroids, 7, 321 (1966)

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[5] Schänzer *et al.*, Metabolism of anabolic steroids in the man : synthesis and use of reference substances for identification of anabolic steroid metabolites, Anal. Chim. Acta, 275, 23 (1993)

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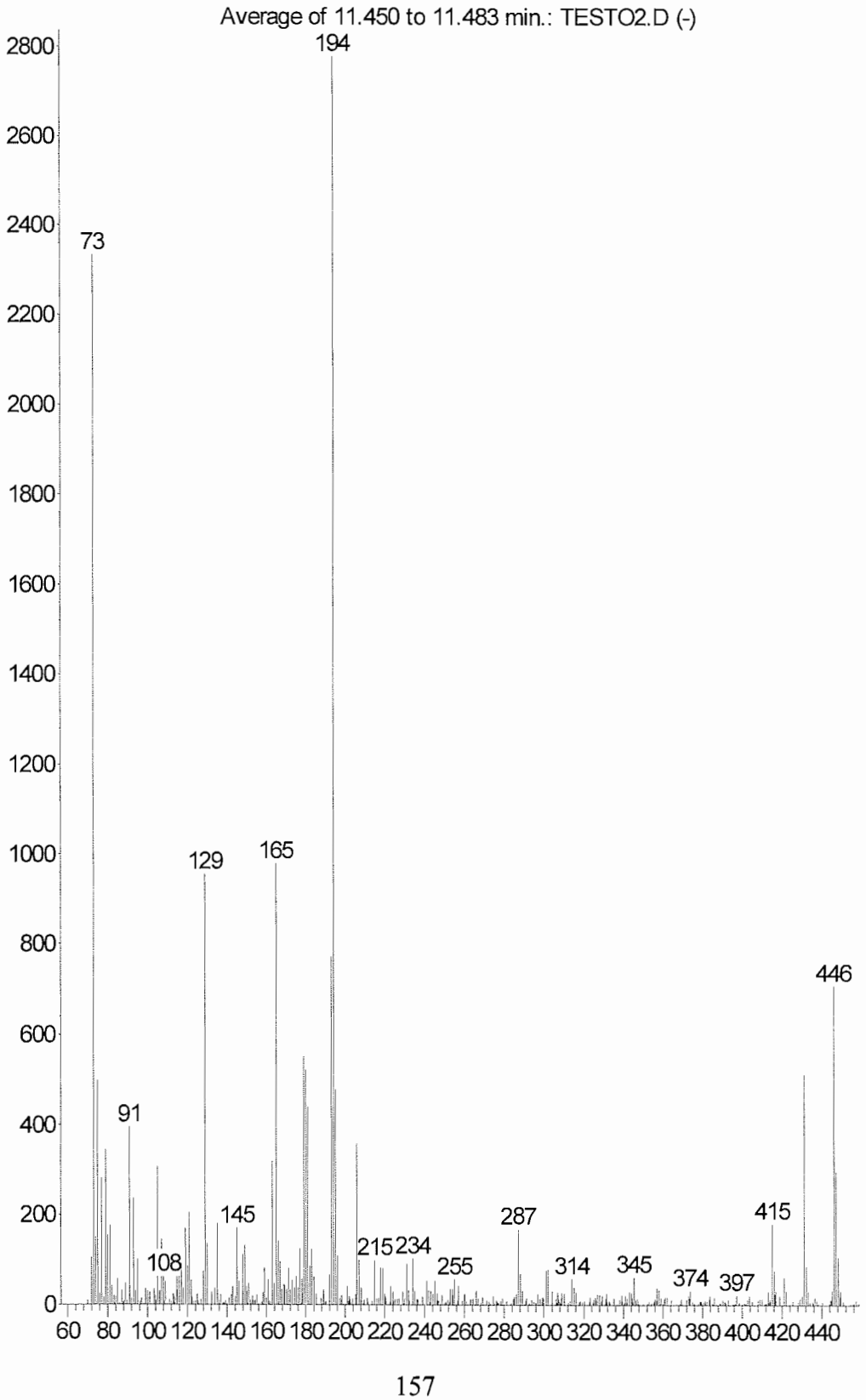

Figure 3: The mass spectrum of the O-TMS-O-enolTMS derivative of metabolite 1

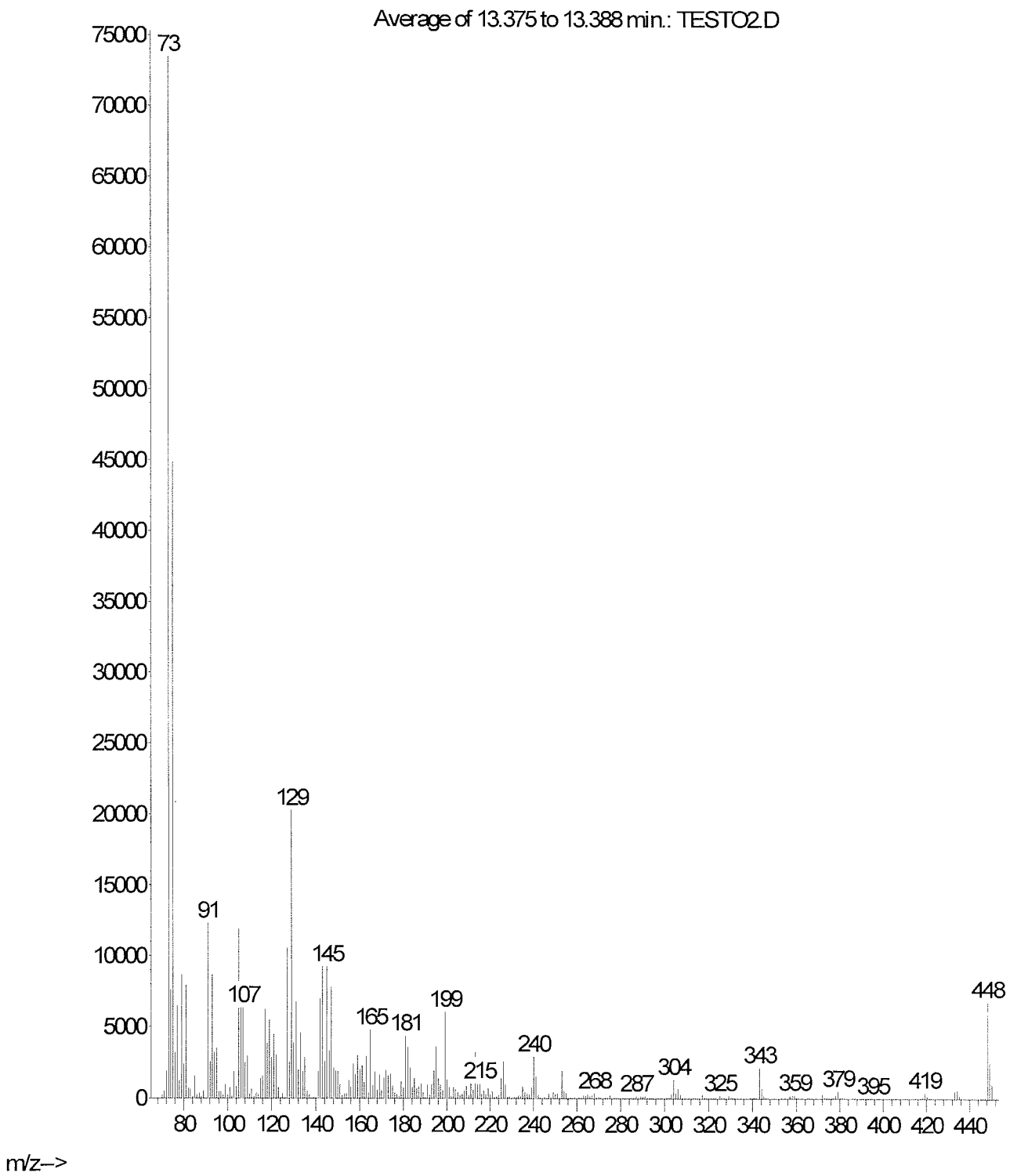

Figure 5: Mass spectrum of the O-TMS-O-enolTMS derivative of metabolite 2