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## **Metabolism, excretion and detection of androst-4-ene-3,6,17-trione**

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### **Introduction**

6-OXO®, a new nutritional supplement commercially available on the internet, is sold as an aromatase-inhibitor and contains androst-4-ene-3,6,17-trione (6-oxo-androstenedione) as active ingredient. This anabolic steroid is a prohibited substance in sports. It is produced by Ergopharm, the company that introduced other notorious products like 4-androstenedione, 4-androstenediol, 19-nor-4-androstenediol and 1-androstenediol.

Based upon its structure and its claimed pharmacological effects, 6-OXO® should be regarded as a prohibited substance by the WADA.

Unfortunately, this product does not appear on the list of substances mentioned in the recently signed "Anabolic steroid Control Act of 2004 and will hence most probably remain to be sold as an over-the-counter product under the 1994 US Dietary Supplement Health and Education Act in the United States.

Because most anabolic steroids are completely metabolised and no parent steroid is excreted, detection methods for these metabolites need to be developed (Schänzer and Donike, 1993).

At present no data on the in-vivo metabolism of 6-oxo-androstenedione in humans has been published. Based upon its structure it is highly unlikely that misuse of this steroid will be detected, using routine screening methods for anabolic steroids in WADA-accredited laboratories.

Therefore, the metabolism and excretion of 6-oxo-androstenedione after ingestion of 6-OXO® was investigated.

## Material and methods

The materials and methods used are similar to those described by Van Thuyne et al. (2005) and Deventer et al. (2005) for GC-MS and LC-MS respectively.

Two groups of volunteers participated in the study. Four volunteers (group 1) each took 1 capsule, while two other volunteers (group 2) each took the minimum recommended daily dose (3 capsules) for three consecutive days. Urine samples were collected three times per day (morning, noon, evening) one week before administration. Directly before administration a sample was collected (0h). Urine was collected quantitatively 2, 4, 6, 8, 10 and 12 h after intake during the administration period. Additional samples were three times per day (morning, noon, evening) for another week after the last administration.

## Results

Analysis of the supplement in full scan GC-MS according to the method of Van Thuyne and Delbeke (2004) revealed the presence of 6-oxo-androstenedione and minor amounts of 6 $\zeta$ -OH-androstenedione.

As described by van de Kerkhof et al. (2000) trimethylsilylation in a mixture of MSTFA/NH<sub>4</sub>I/ethanethiol, as routinely used in doping control laboratories, cannot be applied for the selective analysis of the steroids androst-4-ene-6 $\alpha$ -ol-3,17-dione and androst-4-ene-6 $\beta$ -ol-3,17-dione because the stereochemical integrity at C<sup>6</sup> is lost due to 3,5-dienol formation. Therefore derivatisation using MSTFA, potassium acetate and imidazole was used to retain the stereochemical information at the C<sup>6</sup> atom (Schänzer et al., 1995), resulting in the formation of 2,4-dienol-TMS-derivatives.

Using the second derivatisation technique it was shown that the capsules contained trace amounts of 6 $\beta$ -OH-androstenedione.

Analysis of the urine samples using GC-MS resulted in the identification of the parent drug 6-oxo-androstenedione, 6 $\alpha$ -OH-androstenedione and 6 $\alpha$ -OH-testosterone via direct comparison

with authenticated reference standards. Additionally, several major metabolites were detected. The ion chromatograms ( $m/z=522$ , 507 and 327) in an excretion urine sample collected after 24 h from a volunteer which ingested 1 capsule are shown in Fig. 1. One of the detected substances was identified as 6 $\alpha$ -OH-etiocholanolone via comparison with an authentic reference standard obtained from Steraloids. The retention times, monitored  $m/z$ -values and relative retention times of 6-oxo-androstenedione and its metabolites are given in Table 1. Based upon comparison with the ion chromatograms and mass spectra published by Lévesque et al. (1999), the other substances are most probably 6 $\alpha$ -OH-androsterone and 6 $\alpha$ -OH-epiandrosterone.

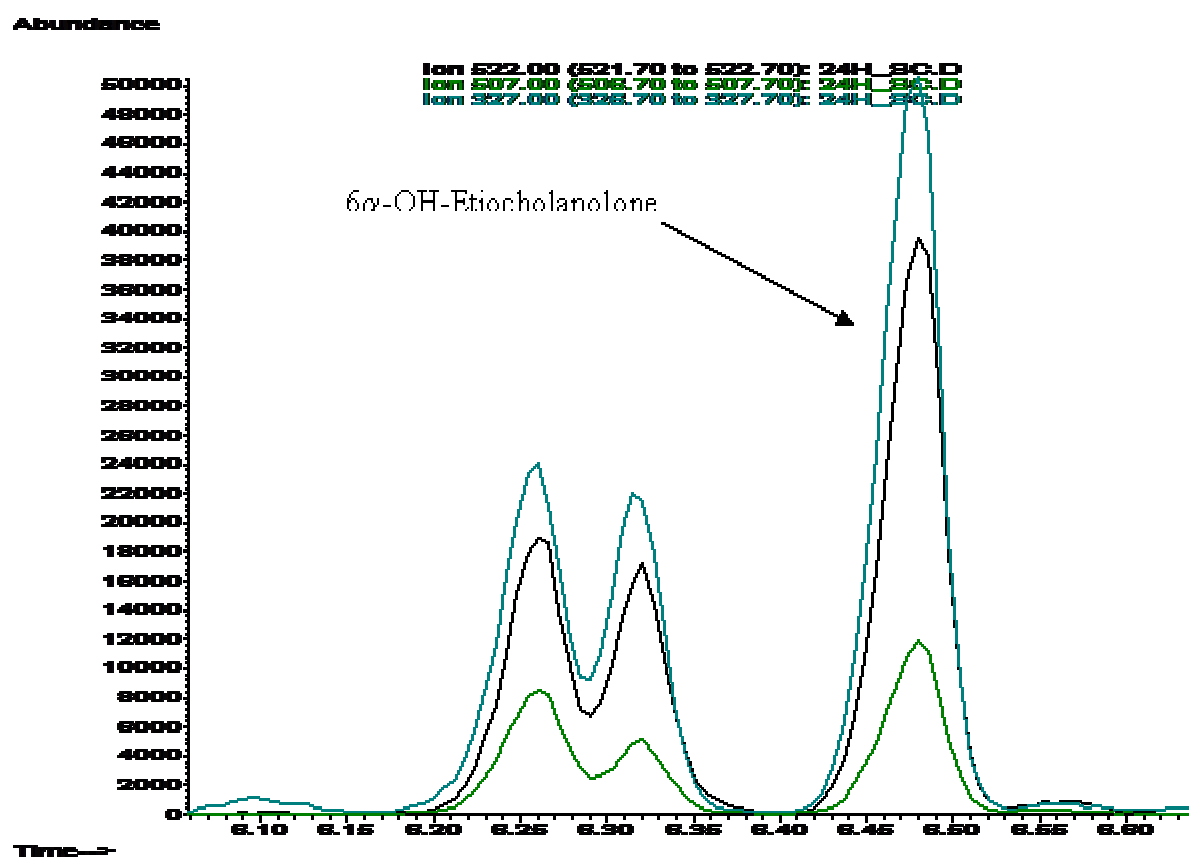


Figure 1. Ion chromatograms ( $m/z$  522,  $m/z$  507 and  $m/z$  327) in an excretion urine sample, 24 h post administration.

Using the GC-MS in SIM, detection limits of 5 ng/ml could be reached for 6-oxo-androstenedione, 6 $\alpha$ -OH-testosterone and 6 $\alpha$ -OH-etiocholanolone and 10 ng/ml for 6 $\alpha$ -OH-androstenedione.

Table 1: Retention time (RT), relative retention time (RRT), diagnostic ions (relative abundance) for the 3,5-dienol-trimethylsilylated compounds analysed in the selective ion monitoring mode.

Compound	RT (min)	RRT	Diagnostic ions (relative abundance %)
6 $\alpha$ -OH-etiocholanolone	6.48	0.90	327 (100), 522 (75), 507 (21)
6-oxo-androstene-dione	7.16	1.05	516 (100), 501 (20), 411 (12)
6 $\alpha$ -OH-androstdione	7.50	1.10	518 (100), 503 (6), 319 (6)
6 $\alpha$ -OH-testosterone	7.77	1.14	520 (100), 505 (11), 319 (5)

The detection times by GC-MS SIM are given in Table 2.

Table 2. Detection time (h) after last ingested capsule by GC/MS SIM according to substance and ingested dose (group 1: 1 capsule; group 2: 3 capsules for 3 consecutive days)

substance	Group 1	Group 2
6-oxo-androstenedione	12	24
6 $\alpha$ -OH-androstenedione	29	37
6 $\alpha$ -OH-testosterone	12	24
6 $\alpha$ -OH-etiocholanolone	37	48

The samples were also analyzed quantitatively by LC-MS<sup>2</sup> using an APCI interface operated in the positive mode. Unfortunately, the quantitative LC-MS analysis only resulted in the detection of 6-oxo-androstenedione, 6 $\alpha$ -OH-androstenedione and 6 $\alpha$ -OH-testosterone. Using the current instrumental settings 6 $\alpha$ -OH-etiocholanolone could not be detected via LC-MS, most probably because the proton affinity of this substance is too low for ionization. As shown in Fig. 2, this LC-MS analysis resulted in the direct separation of the 6 $\alpha$ - and 6 $\beta$ -isomers of the investigated substances.

The LC-MS method was a quantitative method which was linear in the range of 5 ng to 1  $\mu$ g/ml in urine. Accuracy, repeatability and reproducibility were tested at 5 ng/ml, 100 ng/ml and 1  $\mu$ g/ml and complied with generally accepted criteria. Selectivity and specificity of the method was tested by analysing a large number of blank urine samples and the reference solutions of several of our methods that are used for the analysis of samples for anabolic steroids.

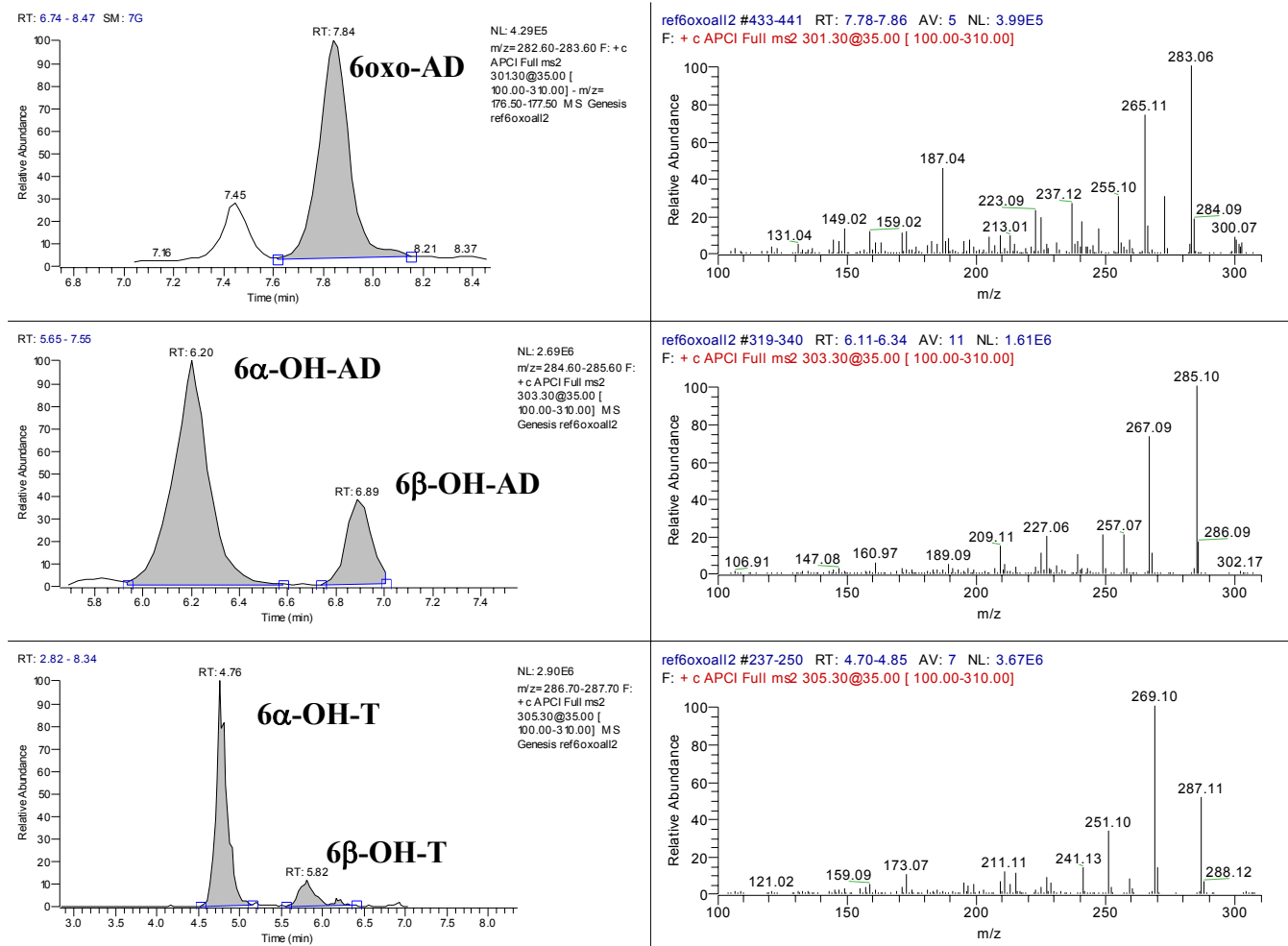


Figure 2: Extracted ion chromatograms and mass spectra of 6oxo-AD (m/z 283), 6α-OH-AD (m/z 285) and 6α-OH-T (m/z 287) in a reference mixture (100 ng/ml).

The limit of quantitation was defined as the lowest concentration where acceptable accuracy, repeatability and reproducibility could be guaranteed and was 5 ng/ml. The LOD is set arbitrarily at half the LOQ (2.5 ng/ml). All concentrations in the urine samples were adjusted to a standard urinary density of 1.020 kg/L.

An overview of the most important quantitative results for the volunteers which took 1 capsule is given in Table 3.

Table 3. Overview on urinary excretion data per substance after ingestion of 1 capsule of 6-OXO® (four volunteers)

Substance	First detection	Last detection	Time of Cmax	C max (µg/ml)
6-oxo-androstenedione	2 h	29 h	2-4 h	1,9
6α-OH-androstenedione	2 h	29 h	2-4 h	32
6α-OH-testosterone	2 h	24 h	2-4 h	0,5

Additional analyses revealed that only  $12.6 \pm 2.8$  % (n=6) of 6-oxo-androstenedione was excreted nonconjugated. All other metabolites were solely excreted as conjugates.

The detection of 6α-OH-testosterone and especially the parent drug, 6-oxo-androstenedione, in a urine sample can be used as a direct indication of the intake of 6-OXO® as these substances have not been reported elsewhere. However, Lévesque *et al.* (1999) and van de Kerkhof (2001) showed that orally administered androst-4-ene-3,17-dione is metabolised *in-vivo* to 6α-OH-androstenedione. Hence, screening of samples for 6α-OH-androstenedione will result in the detection of the administration of androstenedione as well as 6-oxo-androstenedione.

6-Oxo-androstendione is marketed as an aromatase-inhibitor. Therefore, the influence of the intake of 6-OXO® on the urinary steroid profile was investigated by comparison of the steroid profile, 1 week before until 1 week after administration of 6-OXO®. During the first 12 h post administration, we were unable to determine the steroid profile due to large interferences at the retention times of the monitored endogenous steroids caused by 6α-OH-etiocholanolone, 6α-OH-androsterone and 6α-OH-epiandrosterone.

In the samples collected 24 and 29 h post administration elevated levels of androstenedione were detected (concentration of androstenedione > 30 ng/ml). Based upon these preliminary results the aromatase inhibiting claims made by the manufacturer, which are based on in-vitro experiments might be true. Moreover based upon the initial preliminar results there might also be an age-related effect. This hypothesis is based on the results for the volunteers in group 2, where the effect was more drastical in the first volunteer compared to the second, younger volunteer. Nevertheless, the effects were minimal and 36 h post administration all endogenous steroids had returned to their basal levels.

Nevertheless, at present further work is done to determine the origin and nature of the interfering substances in the steroid profile and we will attempt to determine the exact magnitude of the effect of 6-oxo on the steroid profile.

## **Conclusion**

Administration of 6-oxo-androstenedione results in the detection of 6-oxo-androstenedione, 6 $\alpha$ -OH-testosterone (minor metabolite), 6 $\alpha$ -OH-etiocholanolone and 6 $\alpha$ -OH-androstenedione (major metabolite).

An increase in the urinary concentration of androstenedione was also noticed after intake of 6-OXO®, indicating a possible aromatase inhibition.

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