In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (14). Sport und Buch Strauß - Köln 2006

Maxie Kohler, Maria Kristina Parr, Georg Opfermann, Wilhelm Schänzer

Metabolism of 4-Hydroxyandrostenedione and 4-Hydroxytestosterone

Institute of Biochemistry, German Sport University, Cologne, Germany

Introduction

4-Hydroxyandrost-4-ene-3,17-dione (4-hydroxyandrostenedione, formestane) and 4-hydroxytestosterone (4,17β-dihydroxyandrost-4-en-3-one) as well as other 4-hydroxylated steroids like Oxymesterone (4-hydroxy-17α-methyltestosterone) or Oxabolone (4-hydroxy-19-nortestosterone) are prohibited in sports by the World Anti-Doping Agency [1]. While 4-hydroxyandrostenedione is applied in the therapy of advanced breast cancer because of its aromatase inhibiting activity, 4-hydroxytestosterone is not therapeutically used but is advertised as anabolic agent. Different 4-hydroxylated steroids are more and more offered on the internet as steroids with anabolic properties.

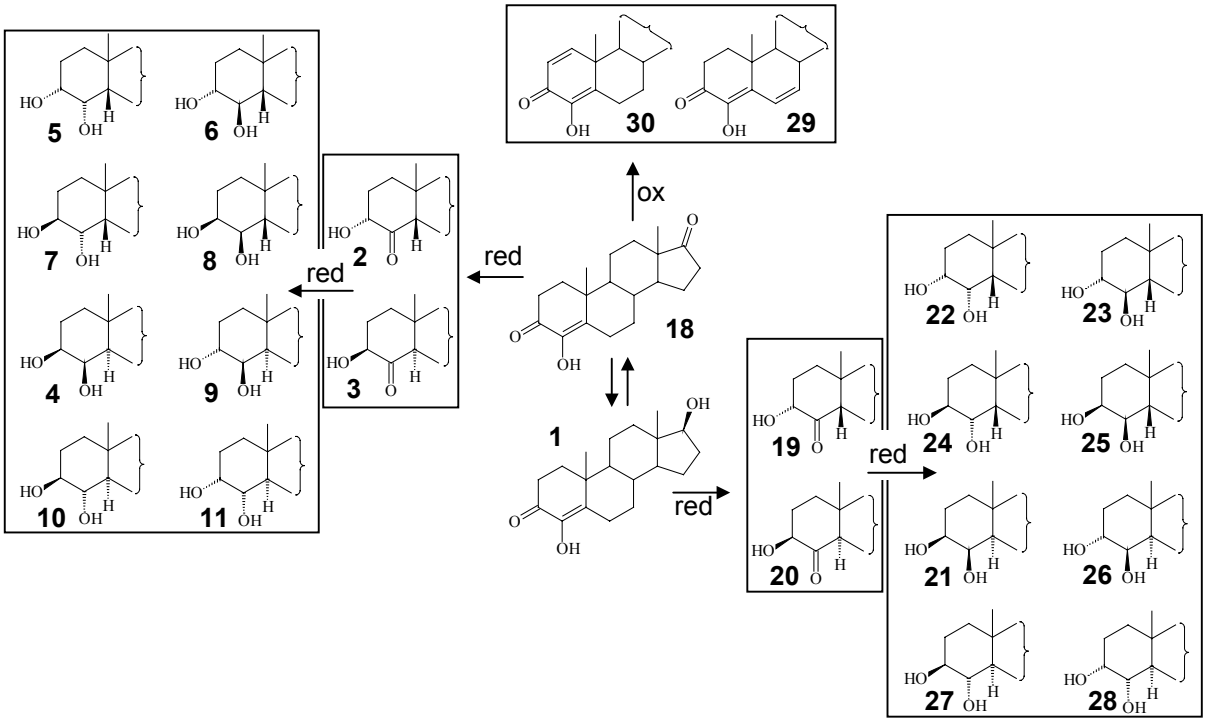

Fig. 1 Main ways of metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone

Human metabolic processes convert 4-hydroxyandrostenedione to 4-hydroxytestosterone and *vice versa*. Their main pathway of phase-I metabolism comprises reductions which lead to 3-hydroxy-4-oxo or 3,4-dihydroxylated compounds. In addition, the 17-oxo residue has also been subject of reductive activity. Another known way of transformation is dehydrogenation yielding metabolites such as 1,2- and 6,7-dehydroformestane [2-5] (Fig. 1).
For the detection of the misuse of 4-hydroxyandrostenedione or 4-hydroxytestosterone in sports their metabolism was investigated providing information on renally excreted metabolic products. Several possible metabolites were synthesised and compared with compounds found in urine after single application of one of the two substances. Analysis was performed by gas chromatography-mass spectrometry.

Experimental

Chemicals, solvents and materials: 4-Hydroxyandrostenedione was purchased from Thinker Chemical (Hangzou, China), [2,2,4,4-$^{2}H_4$]-11β-hydroxyandrosterone was synthesised in our laboratory [6].
β-Glucuronidase was purchased from Roche Molecular Diagnostics (Mannheim, Germany), and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) from Chemische Fabrik Karl Bucher (Waldstetten, Germany). Potassium carbonate, potassium hydrogen carbonate, potassium hydroxide, methanol, n-hexane, hydrochloric acid and t-butyl methyl ether (TBME) were purchased from KMF (St. Augustin, Germany), ethanethiol and ammonium iodide were supplied by Fluka (Bucks, Schweiz). Other reagents and solvents were bought from Merck (Darmstadt, Germany). All solvents and reagents were of analytical grade. All solutions and buffers were prepared using deionised water (Water Lab System, Millipore, Eschborn, Germany). Cartridges for solid phase extraction (Chromabond C18, 6 mL, 500 mg) were purchased from Macherey-Nagel (Düren, Germany).

Synthesised reference substances: For the identification of putative metabolites the following substances out of the different classes of possible metabolites have been synthesised:

- 4-hydroxytestosterone
- 3-hydroxy-4-oxo compounds: 3α-hydroxy-5β-androstane-4,17-dione and 3β-hydroxy-5α-androstane-4,17-dione plus respective 17β-hydroxylated analogues

- 3,4-dihydroxylated compounds (all combinations of configuration): 3β,4β-dihydroxy-5α-androstan-17-one, 3α,4β-dihydroxy-5α-androstan-17-one, 3β,4α-dihydroxy-5α-androstan-17-one, 3α,4α-dihydroxy-5α-androstan-17-one, 3β,4β-dihydroxy-5β-androstan-17-one, 3α,4β-dihydroxy-5β-androstan-17-one, 3β,4α-dihydroxy-5β-androstan-17-one, 3α,4α-dihydroxy-5β-androstan-17-one plus respective 17β-hydroxylated analogues
- 1,2- and 6,7-dehydroformestane

Excretion studies: The Promatrix products [7] Primobolan™ containing 100 mg of 4-hydroxyandrostenedione-4-acetate per tablet and Testobol™ containing 100 mg of 4-hydroxytestosterone-17-acetate [8] were used for the excretion studies. Either one tablet of Primobolan™ or two tablets of Testobol™ were taken orally by male volunteers.

Sample preparation:
A volume of 2 mL of urine was spiked with 60 µL of the internal standard ([2,2,4,4-$^2H_4$]-11β-hydroxyandrosterone (12 µg/mL in methanol)).
Hydrolysis of glucuronides: Samples were diluted with 1 mL of sodium phosphate buffer (0.8 M, pH 7). After addition of 50 µL of β-glucuronidase from *E.coli* hydrolysis was performed by heating at 50°C for 1 h. After addition of 0.75 mL of K_2CO_3/$KHCO_3$-solution (20 %, 1:1, pH 9.6) extraction was performed with 5 mL of TBME. The organic layer was separated and evaporated to dryness.
Cleavage of sulphates: The remaining aqueous layer was extracted using solid phase extraction (SPE) cartidges. SPE-columns were dried in a vacuum desiccator over P_4O_{10} and analytes were eluted with 1 mL of methanol. Solvolysis was accomplished after addition of 5 mL of ethyl acetate/H_2SO_4 (250 mL/200 µg) at 55°C for 1 h. A volume of 0.75 mL of potassium hydroxide (1 M) was added, samples were evaporated to dryness, reconstituted in 1 mL of potassium hydroxide (1 M) and extracted with 5 mL of TBME. The organic layer was separated and evaporated to dryness.
Preparation for GC-MS: All evaporated samples were treated with 100 µL of MSTFA/ ammonium iodide/ethanethiol (1000:2:3 / v:w:v) and heated for 15 min at 60°C for per-trimethylsilylation.

Modified sample preparation for detection of minor metabolites: For the first urine samples collected after administration a modified sample preparation was used. Compared with the "standard" sample preparation the following changes were made:

- 10 mL of urine were used and concentrated by solid phase extraction
- no internal standard was added
- before hydrolysis unconjugated substances were separated by TBME extraction (pH 7)
- extraction after hydrolysis was done with n-pentane instead of TBME; TBME was used afterwards to assure complete extraction of metabolites using n-pentane
- an additional clean up by HPLC fractionation was performed with all extracts

HPLC: For HPLC-fractionation an Agilent 1100 with a Merck LiChroCart 250-4 LiChrospher 100 RP_{18} EC (5 µm) column and a Merck LiChroCart 25-4 LiChrospher 100 RP_{18} EC (5 µm) pre-column was used. The mobile phases were water (A) and acetonitrile (B) with a linear gradient from 30 to 100 % B within 20 min. The flow rate was 1 mL/min, reequlibration was conducted with 30 % B for 5 min.

GC-MS: Analysis of the conjugate fractions and reference substances was performed on an Agilent 6890 GC system coupled to an Agilent 5973 mass selective detector. The column used was an HP5MS (SE 54, 15.5 m, 0.25 mm inner diameter, 0.25 µm film thickness) with the following oven temperature: 100°C to 190°C by 40°C/min; 190°C to 245°C by 5°C/min; 245°C to 320°C by 40°C/min, 3 min hold. The injection volume was 2 µL splitless for urine samples and split (1:10) for reference substances. Detected masses ranged from 40 to 800 u using electron impact ionisation (70 eV). Helium was used as carrier gas (1.5 mL/min).

Results and Discussion

Qualitative consideration of metabolism:
The transformation of 4-hydroxyandrostenedione and 4-hydroxytestosterone by male volunteers after single oral application resulted in identical metabolites. Phase-I metabolism as well as conjugation of the metabolites were similar in both excretion studies.

Phase-I metabolism: Besides 4-hydroxyandrostenedione (**1**) and 4-hydroxytestosterone (**18**) many reduction products were detected. 3-Hydroxy-4-oxo compounds with 3α,5β- and 3β,5α-configurations were identified as 17β-hydroxylated and 17-oxo steroids each (**2**, **3**, **19**, **20**). The further reduced 3,4-dihydroxylated compounds were mainly excreted as 17-oxo steroids. Except for 3α,4α-dihydroxy-5β-androstan-17-one (**5**) all possible combinations of

configuration were identified (**4**, **6**-**11**). Interestingly, only three 17β-hydroxylated analogues all having 5α-configuration were determined (3β,4β (**21**), 3α,4β (**26**), 3α,4α (**28**)). Furthermore the oxidation products 1,2- (**30**) and 6,7-dehydroformestane (**29**) were identified. The above mentioned 3,4,17-trishydroxylated compounds as well as 3β-hydroxy-5α-androstan-17-one and its 17β-hydroxylated analogue were detected only using the modified sample preparation because of comparably small concentrations.

For the assignment of all configurations of the 3,4-dihydroxylated compounds mass spectra of the per-trimethylsilylated (per-TMS) derivatives were utilized.

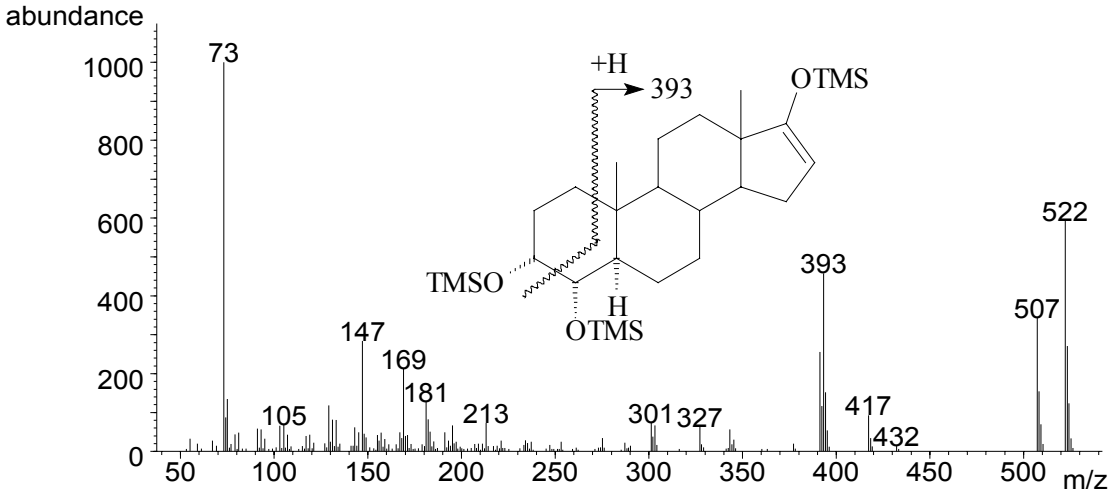

Fig. 2 EI-mass spectrum of a 3α,4α-dihydroxy-5α-androstan-17-one, per-TMS

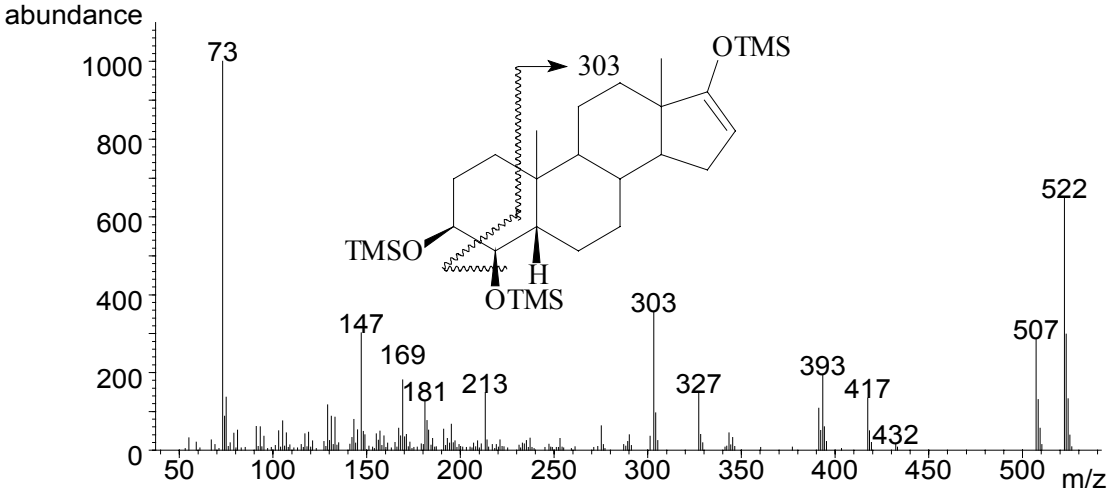

Fig. 3 EI-mass spectrum of 3β,4β-dihydroxy-5β-androstan-17-one, per-TMS

As demonstrated in Fig. 2 the 5α-steroids produce a very intensive ion at *m/z* 393. In contrast, for the 5β-compounds the ions at *m/z* 303 and 327 are more abundant (Fig. 3). The generation of *m/z* 393 was substantiated by deuterium labelling experiments [5]. Accordingly, derivation

of the ion at *m/z* 303 was assigned to result from the same A-ring cleavage and an additional loss of TMSOH.

Phase-II metabolism: The conjugation of the identified metabolites comprises glucuronidation and sulphatation and cannot be deduced from metabolic routes known from testosterone. Obviously, the 4-hydroxy group has a strong influence on conjugation of the investigated drugs.

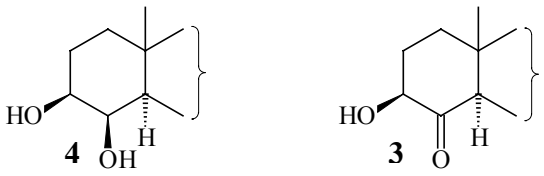

Fig. 4 17-Oxo metabolites only found in sulphate fraction

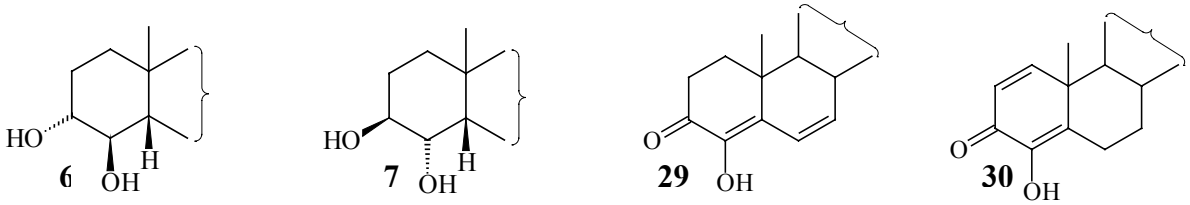

Fig. 5 17-Oxo metabolites only found in glucuronide fraction

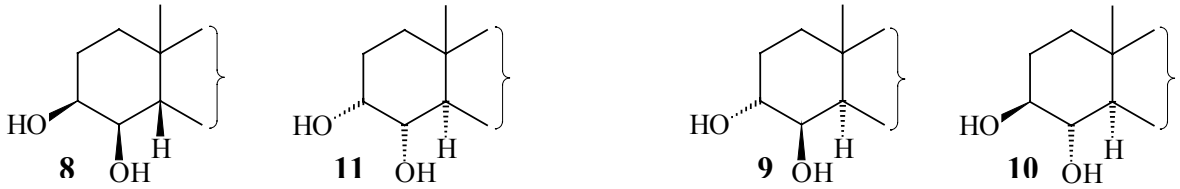

Fig. 6 Examples for 17-oxo metabolites found in both conjugate fractions

With testosterone metabolism in mind 3β-hydroxy groups should be sulphated whereas 3α-hydroxy groups are usually glucuronidated [9]. This applies to the 17-oxo metabolites which were detected only in sulphate fractions (Fig. 4).

For the metabolites only found as glucuronides (Fig. 5) a comparison to testosterone metabolites is more difficult. The 3β,5β-configuration does not appear in testosterone metabolism and its phase-II metabolism is therefore unknown. Because of the 3β-hydroxy group of the identified metabolites a sulphate-conjugation would have been proposed but

results of the present study suggest that at least one metabolite was also found as glucuronide (see Fig. 7, **8**).

Dehydrogenated products are supposably conjugated in 4-position. From literature 1,2-dehydrogenation is only known from fluoxymesterone [9] which has an 11- and a 17-hydroxy group for conjugation. 6,7-dehydrogenation is not common in testosterone metabolism and only known from metandienone.

The examples of glucuronidated and sulphated metabolites in Fig. 6 show two pairs of opposite configuration. If conjugation of the 3-hydroxy group would be similar to that of testosterone metabolites the 4-hydroxy position must be conjugated independently from configuration.

Excretion times of conjugated metabolites:

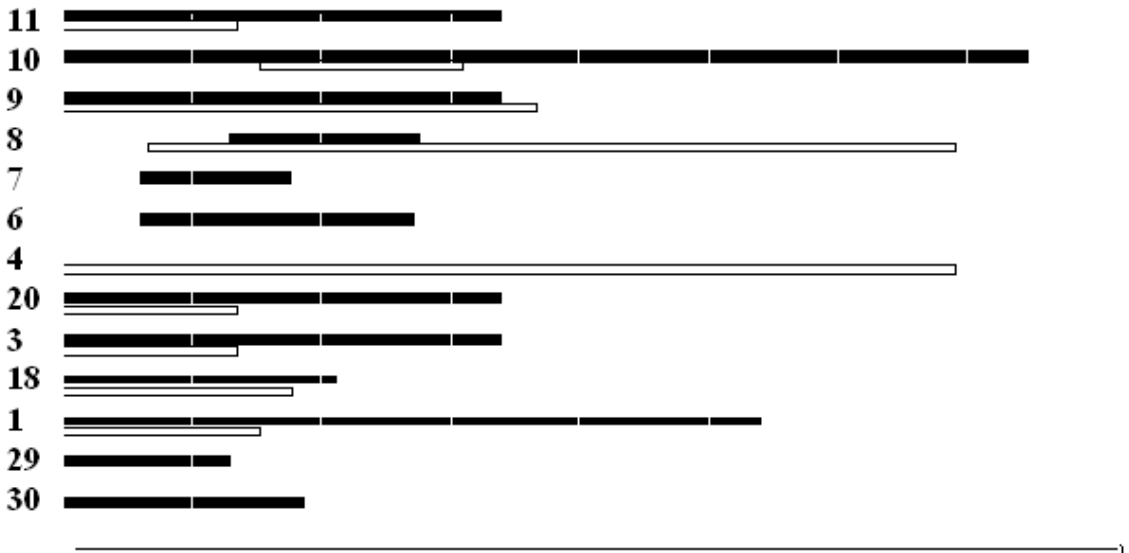

Fig. 7 Conjugation and excretion times of the identified metabolites in the 4-hydroxy-androstenedione excretion study. Black bars represent glucuronidated, white bars sulphated metabolites. The long term metabolite is written in bold letters.

Excretion times have been investigated for the 4-hydroxyandrostenedione administration study. Monitoring the excretion times of the conjugated metabolites (Fig. 7) shows that, except for 3β,4β-dihydroxy-5α-androstan-17-one (**4**), 3β,4β-dihydroxy-5β-androstan-17-one (**8**) and 3α,4β-dihydroxy-5α-androstan-17-one (**9**), glucuronides were detected longer than sulphates. 3β,4α-Dihydroxy-5α-androstan-17-one (**10**) was detected longest. It was found for about 90 hrs in urine samples as glucuronide. In general, the 3,4-dihydroxy-5α-steroids could usually be detected in both conjugation fractions while the 5β-compounds have often only been found as glucuronides. The 5α-steroids were already found in the first samples post administration while the 5β-steroids were not detected until 8 hours p.a. as demonstrated in Fig. 7 for substances **6**, **7** and **8**.

Semi-quantitative consideration of 4-hydroxyandrostenedione- and 4-hydroxytestosterone-glucuronide excretion:

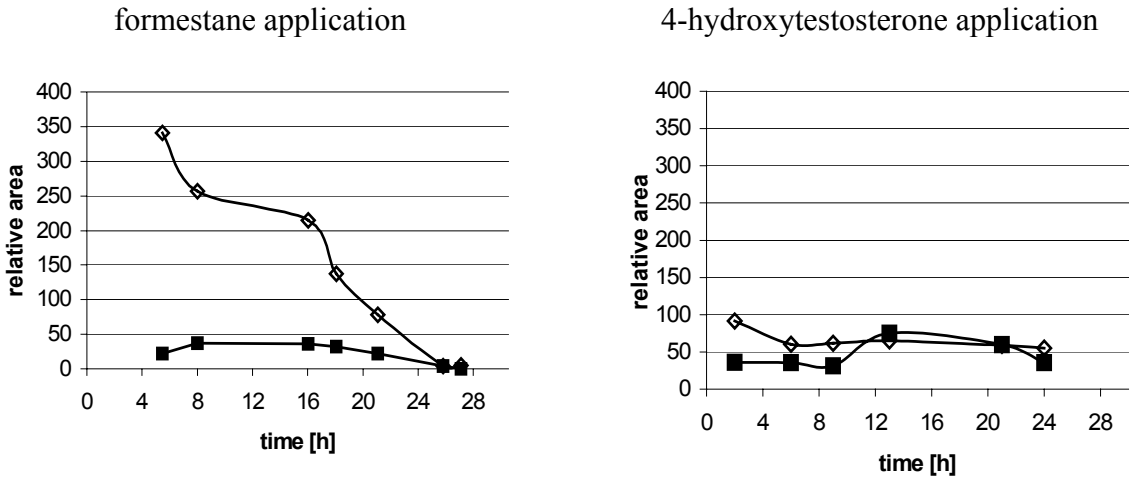

Fig. 8 Excretion of glucuronidated 4-hydroxyandrostenedione (◊) and 4-hydroxytestosterone (■), left: 4-hydroxyandrostenedione excretion study, right: 4-hydroxytestosterone excretion study. Areas normalised with m/z 526 of the internal standard $^{2}H_{4}$-11β-hydroxyandrosterone.

Fig. 8 shows a first result on a semi-quantitative consideration of the excretion of 4-hydroxyandrostenedione and 4-hydroxytestosterone as glucuronides.

The excretion of the glucuronidated substances is different for the two excretion studies. In the 4-hydroxyandrostenedione administration study the amount of 4-hydroxyandrostenedione-glucuronide in the urine samples was very high in the beginning and decreased very fast.

4-hydroxytestosterone-glucuronide excretion was much more constant. In contrast, for the 4-hydroxytestosterone administration study the amounts of the two substances were similar.
An inference on the applied substance might be possible by the ratio of 4-hydroxy-androstenedione to 4-hydroxytestosterone-glucuronide. Since this is a first consideration these results have to be verified and tested for multiple administration.

Conclusion

The increase of 4-hydroxylated steroids with anabolic effects offered on the internet makes a closer investigation of their metabolism essential.
Regarding 4-hydroxyandrostenedione and 4-hydroxytestosterone phase-I as well as phase-II metabolism were found to be identical. Phase-I metabolism is mainly reductive and versatile, and for phase-II metabolism glucuronides and sulphates were identified. For inference on the applied substance the analysis of excretion times or ratios of amounts of the urinary metabolites might be useful. A first consideration of the amounts of excreted glucuronidated 4-hydroxyandrostenedione and 4-hydroxytestosterone gave an idea of a possible quantitative difference.

References

1. World Anti-Doping Agency, the 2006 Prohibited List.
2. Poon GK, Jarman M, McCague R, Davies JH, Heeremans CE, van der Hoeven RA, Niessen WM, van der Greef J. Identification of 4-Hydroxyandrost-4-ene-3,17-dione Metabolites in Prostatic Cancer Patients by Liquid Chromatography-Mass Spectrometry. Journal of Chromatography 1992; 576 (2): 235-244
3. Poon GK, Chui YC, Jarman M, Rowlands MG, Kokkonen PS, Niessen WM, van der Greef J. Investigation of Conjugated Metabolites of 4-Hydroxyandrost-4-ene-3,17-dione in Patient Urine by Liquid Chromatography-Atmospheric Pressure Ionization Mass Spectrometry. Drug Metabolism and Disposition 1992; 20 (6): 941-947
4. Poon GK, Jarman M, Rowlands MG, Dowsett M, Firth J. Determination of 4-Hydroxyandrost-4-ene-3,17-dione Metabolism in Breast Cancer Patients Using High-Performance Liquid Chromatography-Mass Spectrometry. Journal of Chromatography 1991; 565 (1-2): 75-88
5. Parr MK, Fusshoeller G, Opfermann G, Kohler M, Hebestreit M, Schänzer W. Synthesis of Reference Compounds for the Identification of Metabolites of 4-Hydroxytestosterone. In: Schänzer W., Geyer H, Gotzmann A, Mareck U (Eds.) Recent Advances in Doping Analysis (13). Sport und Buch Strauß, Köln, 2005: 65-74
6. Schänzer W, Donike M. Synthesis of Deuterated Steroids for GC/MS Quantification of Endogenous Steorids. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U (Eds.) Recent Advances in Doping Analysis (2). Sport und Buch Strauß, Köln, 1995: 93-112
7. www.promatrixtech.com/. access 29.09.05.
8. Parr MK, Opfermann G, Schänzer W. Analytical Properties of 4-Hydroxysteroids and Some Esters. In: Schänzer W., Geyer H, Gotzmann A, Mareck U (Eds.) Recent Advances in Doping Analysis (12). Sport und Buch Strauß, Köln, 2004: 129-138
9. Schänzer W. Metabolism of Anabolic Androgenic Steroids. Clinical Chemistry 1996; 42 (7): 1001-1020