STEROIDS 72 (2007) 278–286

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/steroids

Metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone: Mass spectrometric identification of urinary metabolites

Maxie Kohler[a,b,*], *Maria K. Parr*[a], *Georg Opfermann*[a], *Mario Thevis*[a], *Nils Schlörer*[c], *Franz-Josef Marner*[b], *Wilhelm Schänzer*[a]

[a] *Institute of Biochemistry, German Sport University of Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany*
[b] *Institute of Biochemistry, University of Cologne, Zülpicher Straße 47, 50939 Cologne, Germany*
[c] *Institute of Organic Chemistry, University of Cologne, Greinstraße 4, 50939 Cologne, Germany*

ARTICLE INFO

Article history:
Received 23 October 2006
Received in revised form
20 November 2006
Accepted 22 November 2006
Published on line 17 January 2007

Keywords:
4-Hydroxylated steroids
Formestane
4-Hydroxytestosterone
Metabolism
Gas chromatography–mass spectrometry

ABSTRACT

4-Hydroxyandrost-4-ene-3,17-dione is a second generation, irreversible aromatase inhibitor and commonly used as anti breast cancer medication for postmenopausal women. 4-Hydroxytestosterone is advertised as anabolic steroid and does not have any therapeutic indication. Both substances are prohibited in sports by the World Anti-Doping Agency, and, due to a considerable increase of structurally related steroids with anabolic effects offered via the internet, the metabolism of two representative candidates was investigated.

Excretion studies were conducted with oral applications of 100 mg of 4-hydroxyandrostenedione or 200 mg of 4-hydroxytestosterone to healthy male volunteers. Urine samples were analyzed for metabolic products using conventional gas chromatography–mass spectrometry approaches, and the identification of urinary metabolites was based on reference substances, which were synthesized and structurally characterized by nuclear magnetic resonance spectroscopy and high resolution/high accuracy mass spectrometry.

Identified phase-I as well as phase-II metabolites were identical for both substances. Regarding phase-I metabolism 4-hydroxyandrostenedione (**1**) and its reduction products 3β-hydroxy-5α-androstane-4,17-dione (**2**) and 3α-hydroxy-5β-androstane-4,17-dione (**3**) were detected. Further reductive conversion led to all possible isomers of 3ξ,4ξ-dihydroxy-5ξ-androstan-17-one (**4**, **6–11**) except 3α,4α-dihydroxy-5β-androstan-17-one (**5**).

Out of the 17β-hydroxylated analogs 4-hydroxytestosterone (**18**), 3β,17β-dihydroxy-5α-androstan-4-one (**19**), 3α,17β-dihydroxy-5β-androstan-4-one (**20**), 5α-androstane-3β,4β,17β-triol (**21**), 5α-androstane-3α,4β,17β-triol (**26**) and 5α-androstane-3α,4α,17β-triol (**28**) were identified in the post administration urine specimens. Furthermore 4-hydroxyandrosta-4,6-diene-3,17-dione (**29**) and 4-hydroxyandrosta-1,4-diene-3,17-dione (**30**) were determined as oxidation products.

Conjugation was diverse and included glucuronidation and sulfatation.

* *Corresponding author*. Tel.: +49 22 149826311; fax: +49 22 14973236.
E-mail address: maxie@biochem.dshs-koeln.de (M. Kohler).
0039-128X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2006.11.018

1. Introduction

4-Hydroxyandrost-4-ene-3,17-dione (4-hydroxyandrostenedione, formestane, **1**) is a second generation, irreversible aromatase inhibitor and is structurally related to androstenedione, the natural substrate of the enzyme aromatase. Because of its aromatase inhibiting activity formestane is used as anti breast cancer medication for postmenopausal women.

4-Hydroxyandrostenedione (**1**) is extensively metabolized. The main metabolite is 4-hydroxyandrost-4-ene-3,17-dione-4-glucuronide [1,2]. In women 20% of applied formestane is excreted as glucuronide within the first 24 h [3]. Men transform an even higher amount of formestane (35%) into its glucuronide [4]. Phase-I metabolism is manifold and mainly reductive. Conjugation includes glucuronidation as well as sulfatation. Studies were conducted with different forms of application, male and female subjects and different methods of analysis of either blood or urine [5–10]. Formestane was excreted as glucuronide and sulfate after oral administration of 500 mg by women [7,8] while it was identified as glucuronide only after intramuscular application to men [7]. In addition, 4-hydroxytestosterone was identified as metabolite of formestane as glucuronide in women's urine [8].

3α-Hydroxy-5β-androstane-4,17-dione (**3**) has been found in urine samples of men and women as glucuronide [5,7,8]. Its isomer, 3β-hydroxy-5α-androstane-4,17-dione (**2**), has not been detected in humans but only in rats [9].

The 17-hydroxylated analogs 3β,17β-dihydroxy-5α-androstan-4-one (**19**) and 3α,17β-dihydroxy-5β-androstan-4-one (**20**) were identified as glucuronidated products [5,7,8]. Sulfatation was only observed for metabolite **20** [7].

Further reduction leads to the 3ξ,4ξ-dihydroxy-5ξ-androstane products. Out of the eight possible isomers 3β,4β-dihydroxy-5α-androstan-17-one (**4**) and 3α,4β-dihydroxy-5α-androstan-17-one (**9**) were detected. Metabolite **4** was excreted as glucuronide by women [6] and the sulfates of **4** and **9** were found in blood samples after intravenous application [8]. Furthermore the oxidation product 4-hydroxyandrost-4,6-diene-3,17-dione (**29**) was identified in women's urine specimens [8].

4-Hydroxytestosterone (4,17β-dihydroxyandrost-4-en-3-one, **18**) is the 17-hydroxylated analog to formestane. It is commercially available on the internet as anabolic steroid for oral self-administration and does not have any therapeutic indication. Hence, only little information is available about its metabolism. So far, most studies dealt with 4-hydroxytestosterone as metabolite of formestane while one study investigated the glucuronic acid conjugates of metabolic products of 4-hydroxytestosterone [11]. After oral application to healthy men 4-hydroxyandrostenedione and 4-hydroxytestosterone as well as the reduction products **2**, **3** and **9** were identified. Detected oxidation products were 4-hydroxyandrost-4,6-diene-3,17-dione (**29**) and 4-hydroxyandrost-1,4-diene-3,17-dione (**30**) [11].

Both substances are prohibited in sports by the World Anti-Doping Agency. Formestane is categorized as anti-estrogenic agent and 4-hydroxytestosterone is listed as anabolic steroid [12].

In Fig. 1, an overview on the main metabolic pathways of formestane and 4-hydroxytestosterone is illustrated. Because the only difference of the two substances is the oxo- and hydroxy-group at C-17, metabolism is supposedly very similar.

Identification and comparison of phase-I and phase-II metabolites of **1** and **18** may give a better understanding of

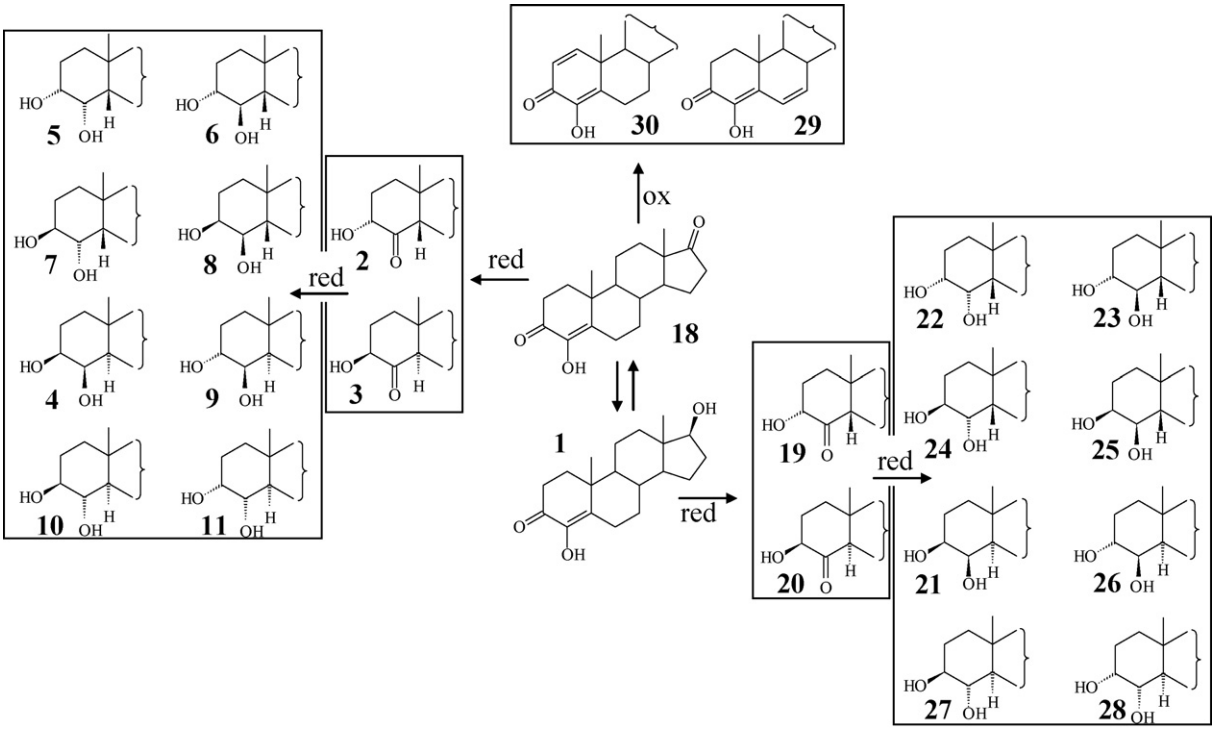

Fig. 1 – Main ways of metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone.

the metabolic fate of these 4-hydroxylated steroids in humans, and for doping control purposes it may allow to identify the applied substance by analyzing informative metabolites and to describe a long term metabolite.

Therefore, the metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone was investigated. Reference substances were synthesized for identification of urinary metabolites. Structures and configurations were verified by nuclear magnetic resonance spectroscopy and high resolution/high accuracy mass spectrometry. Identification of metabolites was performed by gas chromatography–mass spectrometry (GC–MS) analysis after per-trimethylsilylation.

2. Experimental

GC–MS spectra were recorded on an Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector with electron impact ionization (70 eV). The column used was a HP5MS (15.5 m, 0.25 mm inner diameter, 0.25 μm film thickness). Oven temperature was 100–190 °C by 40 °C/min; 190–245 °C by 5 °C/min; 245–320 °C by 40 °C/min, 3 min hold. Injection volume was 2 μL in split mode (1:10) for reference substances and in splitless mode for samples from the administration studies. For calculation of retention indices an almost linear oven temperature was applied: 160–280 °C by 5 °C/min, 280–330 °C by 40 °C/min, 2.5 min hold. Helium was used as carrier gas (1.5 ml/min).

A total of 50 μg of reference compounds was per-trimethylsilylated (per-TMS) for GC–MS measurements using 100 μL of a mixture of MSTFA/NH_4I/ethanethiol (1000:2:3, v/w/v) by heating 15 min at 80 °C [13].

Structure confirmation of synthesized material was obtained by nuclear magnetic resonance (NMR) spectroscopy and high resolution/high accuracy mass spectrometry.

NMR analyses were performed on a Bruker DRX 500 instrument, equipped with a 5 mm inverse probehead with z-gradient coil. Samples were referenced to tetramethylsilane as internal standard. An amount of 5 mg of each compound was dissolved in deuterated chloroform and spectra were recorded at room temperature. Conducted experiments verifying the presumed structures were ^1H, H_2 COSY, H,C HMQC, H,C HMBC and NOESY.

High resolution/high accuracy mass spectrometry for verification of elemental composition was accomplished using a Thermo LTQ Orbitrap mass spectrometer in positive mode with ionization by electrospray. Analytes were dissolved in acetonitrile/water (1:1, v/v) containing 0.1% formic acid at concentrations of 50 μg/mL and introduced into the mass spectrometer using a syringe pump. The ionization voltage was +3.5 kV, the capillary temperature was set to 300 °C. Damping gas in the linear ion trap was helium, and gas supplied to the curved linear ion trap was nitrogen. Spectra were acquired at a resolution of 100,000.

Elemental compositions were measured with a maximum error of 2.5 ppm. Purity of the synthesized substances was determined from GC–MS data.

2.1. Chemicals, solvents and materials

4-Hydroxyandrostenedione was purchased from Thinker Chemical ((purity > 99.5%) Hangzou, China), 5β-dihydrotestosterone from Sigma (St. Louis, MO, USA), [2,2,4,4-$^2H_4$]-11β-hydroxyandrosterone was synthesized in our laboratory [14].

β-Glucuronidase from *E. coli* 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, acetone, ethyl acetate and *t*-butyl methyl ether (TBME) were purchased from KMF (St. Augustin, Germany). Ethylene glycol, pyridine and zinc were supplied by Aldrich (Deisendorf, Germany), ammonium iodide and ethanethiol by Fluka (Bucks, Schweitz). Other reagents and solvents were bought from Merck (Darmstadt, Germany). All solvents and reagents were of analytical grade, all solutions and buffers for sample preparation were prepared using deionized water (Water Lab System, Millipore, Eschborn, Germany). Stock solutions of reference substances were prepared in methanol. Cartridges for solid phase extraction (Chromabond C18, 6 mL, 500 mg) were purchased from Macherey-Nagel (Düren, Germany).

2.2. Synthesis of reference compounds

2.2.1. Synthesis of 4-hydroxytestosterone (18)

4-Hydroxytestosterone (**18**) was prepared according to a preparation of Marsh et al. [9,15] by oxidation of 5β-dihydrotestosterone with oxygen in alkaline medium. Briefly, 200 mg (0.7 mmol) of 5β-dihydrotestosterone were diluted in 25 mL of *t*-butanol (45 °C). After addition of 10 mL of 962 mg of potassium-*t*-butylate in *t*-butanol the mixture was stirred at 45 °C for 6.5 h. Hydrochloric acid (7 mL, 1 M) was added and the solution was evaporated to dryness. A volume of 20 mL of water was added and extraction was performed three times with 20 mL of TBME each. The combined organic layers were washed with water and evaporated to dryness. 4-Hydroxytestosterone was purified by column chromatography (silica gel, eluents *n*-hexane/ethyl acetate 40:60) and recrystallized from *n*-hexane/ethyl acetate (6:1).

4-Hydroxytestosterone (**18**): 35.5 mg (0.11 mmol, 16%), purity 98%; ^1H NMR (500 MHz, $CDCl_3$) δ: 2.56/2.53 (*m*, 2H, H-2), 3.67 (t, 1H, H-17), 0.81 (s, 3H, H-18), 1.21 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 31.6 (C-2), 193.5 (C-3), 140.4 (C-4), 140.0 (C-5), 81.5 (C-17), 10.9 (C-18), 16.9 (C-19). Elemental composition (protonated): $C_{19}H_{29}O_3$, *m*/*z* (theor.): 305.2111, *m*/*z* (exp.): 305.2106, error: −1.6 ppm.

2.2.2. Synthesis of 3-hydroxy-4-oxo compounds

2.2.2.1. Synthesis of 3β-hydroxy-5α-androstane-4,17-dione (2) and 3α-hydroxy-5β-androstane-4,17-dione (3). The synthesis was performed by hydrogenation of 4-hydroxyandrostenedione with Pd/C as catalyst as described elsewhere [11]. NMR and HRMS data of **2** are as follows.

3β-Hydroxy-5α-androstane-4,17-dione (**2**): ^1H NMR (500 MHz, $CDCl_3$) δ: 1.64/2.43 (*m*, 2H, H-2), 4.15 (dd, 1H, H-3), 2.23 (dd, 1H, H-5), 0.89 (s, 3H, H-18), 0.77 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 35.7 (C-2), 64.6 (C-3), 212.4 (C-4),

56.9 (C-5), 220.8 (C-17), 13.7 (C-18), 13.8 (C-19). Elemental composition (protonated): $C_{19}H_{29}O_3$, *m/z* (theor.): 305.2111, *m/z* (exp.): 305.2111, error: −0.2 ppm.

*2.2.2.2. Synthesis of 3β,17β-dihydroxy-5α-androstan-4-one (**19**) and 3α,17β-dihydroxy-5β-androstan-4-one (**20**).* For the synthesis of the 17-hydroxylated analogs to the substances **2** and **3** 600 mg (1.97 mmol) of testosterone were diluted in 20 mL of methanol and hydrogenated with 48 mL (2 mmol) of hydrogen using Pd/C as catalyst. The solution was filtered and the filtrate was evaporated to dryness. Products were separated and purified by column chromatography (silica gel, *n*-hexane/ethyl acetate 50:50) and dried in a vacuum desiccator over P_4O_{10} and potassium hydroxide.

3β,17β-Dihydroxy-5α-androstan-4-one (**19**): 110 mg (0.36 mmol, 18%), purity 97%; ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.64/2.42 (*m*, 2H, H-2), 4.14 (dd, 1H, H-3), 2.20 (dd, 1H, H-5), 3.67 (t, 1H, H-17), 0.76 (s, 3H, H-18), 0.76 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 32.3 (C-2), 74.6 (C-3), 212.5 (C-4), 56.9 (C-5), 81.9 (C-17), 11.2 (C-18), 13.9 (C-19). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2260, error: −2.4 ppm.

3α,17β-Dihydroxy-5β-androstan-4-one (**20**): 62 mg (0.20 mmol, 10%), purity 92%; ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.59/1.99* (*m*, 2H, H-2), 4.04 (dd, 1H, H-3), 2.30 (dd, 1H, H-5), 3.63 (t, 1H, H-17), 0.74 (s, 3H, H-18), 1.16 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 33.5* (C-2), 75.2 (C-3), 212.3 (C-4), 54.6 (C-5), 82.0 (C-17), 11.2 (C-18), 23.1 (C-19) (* was not identified unambiguously). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2262, error: −2.0 ppm.

2.2.3. Synthesis of 3,4-dihydroxy-5-androstan-17-ones

*2.2.3.1. Synthesis of 3β,4β-dihydroxy-5β-androstan-17-one (**8**) and 3α,4α-dihydroxy-5α-androstan-17-one (**11**).* 5α- and 5β-androst-3-en-17-one were synthesized as described by Da Silva et al. [16].

Cis-hydroxylation of the 3,4-double bond was achieved with osmium tetroxide in analogy to literature data [17,18].

To a stirring solution of 67.5 mg (0.25 mmol) of 5α-androst-3-en-17-one in 0.9 mL of pyridine 63.7 mg of OsO_4 (0.25 mmol) were added. After stirring for 2 h at room temperature 100 mg of sodium bisulfite diluted in 1.8 mL of water and 1.2 mL of pyridine were added. The mixture was stirred for another 5 min and then extracted four times with 5 mL of dichloromethane each. The combined dichloromethane layers were evaporated to dryness, recrystallized from acetonitrile and dried in a vacuum desiccator over P_4O_{10} and potassium hydroxide.

3α,4α-Dihydroxy-5α-androstan-17-one (**11**): (44 mg (0.14 mmol, 58%), purity > 99.5%), ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.71/1.83 (*m*, 2H, H-2), 4.00 (ddd, 1H, H-3), 3.47 (dd, 1H, H-4), 1.45 (ddd, 1H, H-5), 0.88 (s, 3H, H-18), 0.85 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 27.0 (C-2), 69.1 (C-3), 71.6 (C-4), 45.6 (C-5), 221.5 (C-17), 13.8 (C-18), 12.7 (C-19). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2260, error: −2.5 ppm.

3β,4β-Dihydroxy-5β-androstan-17-one (**8**) was prepared as described above using 13.9 mg (0.05 mmol) of 5β-androstenone and 13.0 mg (0.05 mmol) of OsO_4 (9.5 mg (0.03 mmol, 62%), purity > 99.5%). The resulting amount was not adequate for NMR analysis. Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2263, error: −1.7 ppm.

*2.2.3.2. Synthesis of 3β,4α-dihydroxy-5α-androstan-17-one (**10**).* The synthesis of 3β,4α-dihydroxy-5α-androstan-17-one (**10**) required the protection of the 17-oxo residue of the starting material. Hence, 1 g (3.5 mmol) of androstenedione was dissolved in 30 mL of benzene. After addition of 400 μL of ethylene glycol and 36 mg of *p*-toluene sulfonic acid the mixture was refluxed for 1 h. After cooling to ambient temperature the solution was diluted with 60 mL of water and extracted four times with 50 mL of TBME each. The combined organic layers were washed with 60 mL of water, evaporated to dryness and recrystallized from *n*-hexane/ethyl acetate (15:2) (androstenedione-17-ethylene-ketal: 553 mg (1.8 mmol, 52%), purity 90%).

Hydroboration of the double bond and reduction of 3-oxo residue was achieved in accordance to the preparation by Schänzer et al. [19]. Crystals of androstenedione-17-ethyleneketal (1.8 mmol) were dissolved in 90 mL of diethyl ether. Under argon as cover gas 1.98 mL (15.9 mmol) of boron trifluoride etherate were added. A suspension of 275 mg of $LiAlH_4$ (7.6 mmol) in diethyl ether was added dropwise within 20 min. After stirring at room temperature for 70 min the mixture was transferred into a saturated sodium sulfate solution. Extraction was performed three times with 60 mL of diethyl ether each, and the combined organic layers were evaporated to dryness. Oxidation of the BH_3-complex was performed by dissolving the dried residue in 50 mL of methanol, adding 3.3 mL of sodium hydroxide (6 M) and 10.1 mL of hydrogen peroxide and stirring at room temperature for 1 h. After dilution with 100 mL of water the preparation was extracted three times with 150 mL of TBME each, and the combined etheral layers were evaporated to dryness. Finally, for removal of the protection group the dry product was dissolved in 100 mL of acetic acid (30%) and refluxed for 30 min. After addition of 70 mL of water, it was extracted three times with 100 mL of TBME each, and the organic layers were evaporated to dryness. The product was purified by column chromatography (silica gel, *n*-hexane/ethyl acetate 60:40) and recrystallized from acetonitrile.

3β,4α-Dihydroxy-5α-androstan-17-one (**10**): 15.5 mg (0.05 mmol, 1.4%), purity 98%; ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.91/1.55 (*m*, 2H, H-2), 3.37 (ddd, 1H, H-3), 3.31 (dd, 1H, H-4), 1.09 (ddd, 1H, H-5), 0.89 (s, 3H, H-18), 0.88 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 28.5 (C-2), 76.9 (C-3), 75.3 (C-4), 50.8 (C-5), 216.3 (C-17), 13.7 (C-18), 13.7 (C-19). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2263, error: −1.5 ppm.

*2.2.3.3. Synthesis of 3β,4β-dihydroxy-5α-androstan-17-one (**4**), 3α,4α-dihydroxy-5β-androstan-17-one (**5**), 3α,4β-dihydroxy-5β-androstan-17-one (**6**), 3β,4α-dihydroxy-5β-androstan-17-one (**7**) and 3α,4β-dihydroxy-5α-androstan-17-one (**9**).* The synthesis of these five 3,4-dihydroxy compounds was described elsewhere [11] and NMR and HRMS data of **4** and **9** are shown below.

3β,4β-Dihydroxy-5α-androstan-17-one (**4**): ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.59/1.62 (*m*, 2H, H-2), 3.92 (ddd, 1H, H-3), 3.65 (dd, 1H, H-4), 1.54 (ddd, 1H, H-5), 0.88 (s, 3H, H-18),

1.07 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 35.0 (C-2), 70.1 (C-3), 75.8 (C-4), 43.9 (C-5), 221.6 (C-17), 13.8 (C-18), 14.3 (C-19). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2263, error: −1.6 ppm.

3α,4β-Dihydroxy-5α-androstan-17-one (**9**): ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.79/1.64 (m, 2H, H-2), 3.60 (ddd, 1H, H-3), 3.79 (dd, 1H, H-4), 1.11 (ddd, 1H, H-5), 0.88 (s, 3H, H-18), 1.07 (s, 3H, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 26.1 (C-2), 72.5 (C-3), 74.9 (C-4), 49.0 (C-5), 221.7 (C-17), 13.7 (C-18), 14.5 (C-19). Elemental composition (protonated): $C_{19}H_{31}O_3$, *m/z* (theor.): 307.2268, *m/z* (exp.): 307.2263, error: −1.5 ppm.

2.2.4. *Synthesis of 5ξ-androstane-3ξ,4ξ,17β-triols (21–28)*

5ξ-Androstan-3ξ,4ξ,17β-triols were prepared by reduction of respective 17-oxo analogs using $NaBH_4$. To a solution of 1 mg of each compound in 1.1 mL of methanol/water (11:1) 2 mg of $NaBH_4$ were added. After 1 h at room temperature, samples were evaporated to dryness, dissolved in 1 mL of hydrochloric acid (0.06 M) and extracted with 5 mL of TBME.

Synthesis of 1,2- (**30**) and 6,7-dehydroformestane (**29**) was performed as described by Marsh et al. [9].

2.3. *Administration studies*

Administration studies were conducted with four healthy male volunteers who gave their written consent after ethical approval by the ethical committee of the German Sport University Cologne. Two volunteers applied 100 mg of 4-hydroxyandrostenedione using the Promatrix product Primobolan™. 4-Hydroxytestosterone was taken as 200 mg Testobol™ by two volunteers. Analysis of the tablets was performed prior to respective application [20]. Urine samples were collected continuous for a period of 1 day and additionally 48 h post administration of 4-hydroxytestosterone. In case of 4-hydroxyandrostenedione urine specimens were collected over a period of 72 h and additional morning urine aliquots were sampled for the following 6 days.

2.4. *Preparation of urine samples*

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)).

2.4.1. *Hydrolysis of glucuronides*

Samples were diluted with 1 mL of sodium phosphate buffer (0.8 M, pH 7). After addition of 50 μL (7 units) of β-glucuronidase from *E. coli* hydrolysis was performed for 1 h at 50 °C. Resulting unconjugated metabolites were extracted after addition of 0.75 mL of K_2CO_3/$KHCO_3$-solution (20%, 1:1, pH 9.6) with 5 mL of TBME. The organic layer was separated and evaporated to dryness.

2.4.2. *Cleavage of sulfates*

The remaining aqueous layer was extracted using solid phase cartidges. SPE-columns were dried in vacuum desiccator, and analytes were eluted with 1 mL of methanol. Solvolysis was perfomed after addition of 5 mL of ethyl acetate/H_2SO_4 (250 mL/200 μg) at 55 °C for 1 h. 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 TMBE. The organic layer was separated and evaporated to dryness.

2.4.3. *Preparation for GC–MS*

Residues were treated with 100 μL of MSTFA/ammonium iodide/ethanethiol (1000:2:3, v/w/v, 80 °C for 15 min) for pertrimethylsilylation and analyzed as such.

3. Results and discussion

3.1. *Qualitative consideration of metabolism*

The transformation of 4-hydroxyandrostenedione and 4-hydroxytestosterone by male volunteers after single oral application resulted in the same metabolites. Phase-I metabolism as well as conjugation of the metabolites were identical for both administration studies.

3.1.1. *Phase-I metabolism*

Beside 4-hydroxyandrostenedione and 4-hydroxytestosterone many reduction products were detected as illustrated in Table 1. Out of the reduced metabolites with 3-hydroxy-4-oxo residues the 3α,5β- and 3β,5α-configurations were identified. These compounds were detected as 17-hydroxylated and 17-oxo steroids each (**2**, **3**, **19**, **20**). The further reduced 3,4-dihydroxylated compounds were mainly detected as 17-oxo steroids. Except for 3α,4α-dihydroxy-5β-androstan-17-one (**5**) all possible combinations of configuration were identified. Interestingly, only three 17-hydroxylated analogs with 5α-configuration were determined, namely **21** (3β,4β), **26** (3α,4β) and **28** (3α,4α). Furthermore the oxidation products 1,2- (**30**) and 6,7-dehydroformestane (**29**) were identified.

The above mentioned 3,4,17-trihydroxylated metabolites as well as 3β-hydroxy-5α-androstane-4,17-dione (**2**) and its 17-hydroxy analog (**19**) were detected only in minor amounts and thus excretion times demonstrated in Fig. 5 were not determined for these metabolites.

Reduction of 3-oxo-4-ene compounds like the substances **1** and **18** is common in steroid metabolism [21]. In contrast, 1,2- and 6,7-dehydrogenated products are rarely observed. 1,2-dehydrogenation is known from fluoxymesterone and as bacterial transformation of 3-oxo-4-ene steroids such as testosterone. 6,7-dehydrogenation, which has also been found by Poon et al. [8] as metabolite of 4-hydroxyandrostenedione, has further been observed in the metabolism of metandienone [21].

For separation and identification of all the configurations of 3,4-dihydroxylated compounds mass spectra of the pertrimethylsilylated derivatives were utilized.

As shown in Fig. 2 the 5α-steroids produce a very abundant ion at *m/z* 393. In contrast, the 5β-compounds generate intense ions at *m/z* 303 and 327 (Fig. 3). The origin of *m/z* 393 as indicated in the EI mass spectrum of 3α,4α-dihydroxy-5α-androstan-17-one (Fig. 2) was substantiated by deuterium labelling experiments [11]. Accordingly, derivation of the ion at *m/z* 303 was assigned to the same A-ring cleavage plus an additional loss of TMSOH (−90 u).

The mass spectra of the 3,4,17-trihydroxylated steroids showed resemblances. For the diagnostic ions at *m/z* 303 and

Table 1 – Identification of metabolites

Compound	Molecular weight (per-TMS)	Retention index		Identified in administration studies	
				4-Hydroxy-androstenedione	4-Hydroxy-testosterone
1	518	2866	2793*	X	X
2	520	2846	2829*	X	X
3	520	2596	2740*	X	X
4	522	2827		X	X
5	522	2657			
6	522	2754		X	X
7	522	2581		X	X
8	522	2697		X	X
9	522	2692		X	X
10	522	2901		X	X
11	522	2757		X	X
18	520	2887	2811*	X	X
19	520	2867	2848*	X	X
20	520	2603	2754*	X	X
21	524	2845		X	X
22	524	2659			
23	524	2776			
24	524	2582			
25	524	2712			
26	524	2707		X	X
27	524	2919			
28	524	2767		X	X
29	516	2823		X	X
30	516	2835		X	X

* Two retention indices due to isomers resulting from per-silylation.

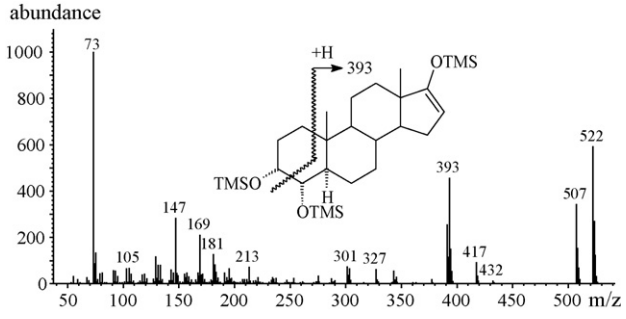

Fig. 2 – Typical per-TMS mass spectrum of a 3,4-dihydroxy-5α-androstan-17-one (3α,4α-dihydroxy-5α-androstan-17-one).

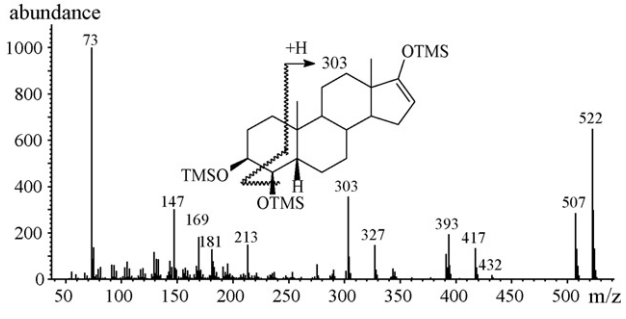

Fig. 3 – Typical per-TMS mass spectrum of a 3,4-dihydroxy-5β-androstan-17-one (3β,4β-dihydroxy-5β-androstan-17-one).

393 corresponding counterparts were observed at *m*/z 305 and 395 resulting from the additional two hydrogen atoms in position 17. The 17-hydroxylated steroids show a characteristic ion at *m*/z 215 which is assigned to be an additional loss of 90 u (TMSOH) from *m*/z 305.

Consideration of retention indices demonstrated that elution of the 17-oxo steroids was similar to the retention of the corresponding 17-hydroxylated analogs. As shown in Table 1 all 17-hydroxylated compounds elute slightly later in comparison to their 17-oxo analogs which results from the slightly higher molecular weight.

Comparison of retention indices of the 3,4-dihydroxylated steroids with the known order of elution of androstanediols [22] with different configurations at positions 3 and 5 shows the contribution of the additional 4-hydroxy group, which first of all results in an adjustment of retention indices to higher values for all configurations as shown in Fig. 4. Houghton et al. showed that androstanediols elute in the order 3β,5β, 3α,5α, 3α,5β and 3β,5α. While the first three configurations elute with a comparably small difference, the value for retention of the 3β,5α-compound is much higher [22]. 4-Hydroxylated analogs also show the highest retention indices for the 3β,5α-configuration.

Conversion of 5α-androstane-3α,17β-diol into its 3β-isomer results in a positive difference in retention index of 84. The same conversion of the 5β-steroid leads to a difference of −10. For the 4-hydroxylated compounds these differences have the same tendencies but higher values. Independently from the configuration of 4-hydroxylation conversion of the 3α-hydroxy-5α-androstanes into their 3β-analogs leads to

Table 2 – Characteristic GC–MS data of 3,4-dihydroxyandrostan-17-ones (per-TMS) (*m/z* 522 was set to 100%)

Compound		*m/z* 303 (%)	*m/z* 327 (%)	*m/z* 391 (%)	*m/z* 393 (%)	*m/z* 507 (%)	*m/z* 522 (%)
3β4β5α	**4**	11.5	10.1	46.8	78.4	67.9	100
3α4α5β	**5**	40.7	38.6	12.5	19.1	51.4	100
3α4β5β	**6**	58.5	35.7	13.9	24.8	41.2	100
3β4α5β	**7**	42.1	50.6	12.0	17.0	45.9	100
3β4β5β	**8**	54.9	22.6	16.6	29.7	44.0	100
3α4β5α	**9**	8.5	13.7	36.3	42.5	65.7	100
3β4α5α	**10**	12.5	8.7	54.1	86.3	58.6	100
3α4α5α	**11**	11.3	10.8	43.2	77.2	58.0	100

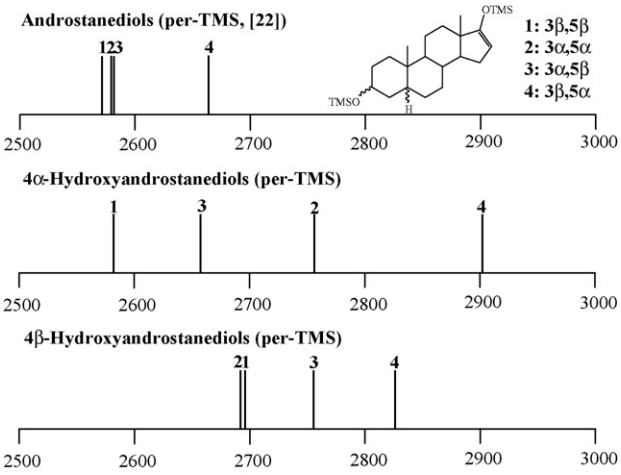

Fig. 4 – Demonstration of retention indices of 3,4α- and 3,4β-Dihydroxyandrostan-17-ones as per-TMS derivatives in comparison with retention of per-trimethylsilyated androstanediols.

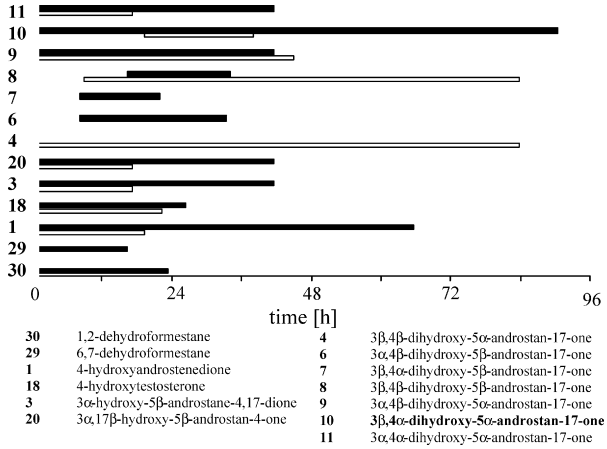

Fig. 5 – Conjugation and excretion times of the identified metabolites for the 4-hydroxy-androstenedione excretion study. Black bars show glucuonidated and white bars show sulfated metabolites. The long term metabolite is written in bold letters.

a positive difference of approximately 140, conversion of the 5β-analogs results in a negative difference of retention indices of approximately −65. Comparison of 4α- and 4β-compounds shows that for 4α-steroids 5β-compounds elute earlier than their 5α-anaolgs. In contrast, for 4β-steroids elution of 5β-compounds is adjusted to higher values which can be attributed to a more bulky shape. 5α-Compounds change to earlier retention indices as they are more compact than their 4α-analogs.

Assignment of the spectra with the configurations of 3- and 4-hydroxy groups was performed by retention indices and intensities of characteristic ions (Tables 2 and 3).

Spectra and characteristics of 4-hydroxyandrostenedione and 4-hydroxytestosterone as well as the 3-hydroxy-4-oxo compounds have been demonstrated earlier [11,20]. Retention indices of these substances are listed in Table 1.

3.1.2. *Excretion times of conjugated metabolites*

Excretion times and periods of traceability have been investigated for the 4-hydroxyandrostenedione excretion study as illustrated in Fig. 5. Most of the metabolites are excreted as glucuronide and sulfate conjugates. Comparing the excretion times of these metabolites (**1**, **18**, **3**, **20**, **6**, **7**, **8**, **9**, **10**, **11**) shows that glucuronides are excreted longer than sulfates except for 3β,4β-dihydroxy-5β-androstan-17-one (**8**) and 3α,4β-dihydroxy-5α-androstan-17-one (**9**). 3β,4α-Dihydroxy-5α-androstan-17-one (**10**) was detected for the longest period and found for about 90 h in urine samples as glucuronide. 4-

Table 3 – Characteristic GC–MS data of 3,4,17-androstantriols (per-TMS) (*m/z* 215 was set to 100%)

Compound		*m/z* 215 (%)	*m/z* 305 (%)	*m/z* 393 (%)	*m/z* 395 (%)	*m/z* 434 (%)	*m/z* 509 (%)	*m/z* 524 (%)
3β4β5α	**21**	100	17.8	83.7	97.7	14.9	20.1	–
3α4α5β	**22**	100	38.3	43.0	40.7	16.5	11.7	–
3α4β5β	**23**	100	67.6	85.2	58.2	33.0	–	32.1
3β4α5β	**24**	100	36.5	32.2	39.9	23.9	–	–
3β4β5β	**25**	100	40.9	26.8	39.9	32.2	10.2	–
3α4β5α	**26**	100	17.5	85.0	79.1	26.3	–	18.1
3β4α5α	**27**	100	–	140.0	105.5	24.3	–	21.2
3α4α5α	**28**	100	20.7	87.4	92.4	27.0	–	17.0

Hydroxyandrostenedione itself was found as glucuronide for 65 h.

Considering especially the 3,4-dihydroxylated steroids 5α-compounds were detected as glucuronic acid and sulfate except for 3β,4β-dihydroxy-5α-androstan-17-one (**4**), which was detected as sulfate only. In contrast, the 5β-compounds 3α,4β-dihydroxy-5β-androstan-17-one (**6**) and 3β,4α-dihydroxy-5β-androstan-17-one (**7**) have only been found as glucuronides while 3β,4β-dihydroxy-5β-androstan-17-one (**8**) could be identified in both conjugate fractions. The 5α-steroids were already found in the first samples post administration while the 5β-steroids were not detected until 8 h p.a. as demonstrated for substances **6**, **7** and **8** in Fig. 5.

3.1.3. *Phase-II metabolism*

The conjugation of the identified metabolites comprises glucuronidation and sulfatation. It cannot be deduced from metabolic routes known from testosterone or other well investigated steroids as the 4-hydroxy group has a strong influence on phase-II metabolism.

The metabolites 3β-hydroxy-5α-androstan-4,17-dione (**2**), its 17-hydroxylated analog 3β,17β-dihydroxy-5α-androstan-4-one (**19**) as well as 3β,4β-dihydroxy-5α-androstan-17-one (**4**) with its 17-hydroxylated analog 5α-androstane-3β,4β,17β-triol (**21**) and 5α-androstane-3α,4β,17β-triol (**26**) have only been detected in sulfate fractions, which means that they are excreted either sulfated or as mixed conjugates. Metabolites only found as glucuronides were the 3,4-dihydroxylated compounds 3α,4β-dihydroxy-5β-androstan-17-one (**6**) and 3β,4α-dihydroxy-5β-androstan-17-one (**7**) as well as the oxidation products **29** and **30**. The other metabolites, 4-hydroxyandrostenedione (1), 4-hydroxytestosterone (**18**), the 3-hydroxy-4-oxo compounds 3α-hydroxy-5β-androstane-4,17-dione (**3**) and its 17-hydroxylated analog 3α,17β-dihydroxy-5β-androstan-4-one (**20**) as well as the 3,4-dihydroxy-17-oxo steroids 3β,4β-dihydroxy-5β-androstan-17-one (**8**), 3α,4β-dihydroxy-5α-androstan-17-one (**9**), 3β,4α-dihydroxy-5α-androstan-17-one (**10**) and 3α,4α-dihydroxy-5α-androstan-17-one (**11**) were found in both conjugate fractions.

These results are in accordance with observations from further studies for most of the earlier detected metabolites (**1**, **18**, **3**, **20**, **29**) [5–8]. Metabolite **19** was found in the sulfate fraction, and Poon et al. [5,8] observed this metabolite as glucuronide. Further differences were observed for the 3,4-dihydroxylated metabolites **4** and **9**. 3β,4β-Dihydroxy-5α-androstan-17-one (**4**) was observed as glucuronide and sulfate in earlier studies [6,8]. In the present consideration, it was only detected in the fraction of glucuronides. Lonning et al. detected 3α,4β-dihydroxy-5α-androstan-17-one (**9**) as sulfate. In this study it was additionally identified as glucuronide. In none of the mentioned studies 4-hydroxyandrostenedione was applied orally to men which means that differences of conjugation may also result from different gender or form of application.

Considering the present results sulfatation of metabolites **2** and **4** can be deduced from the conjugation of testosterone metabolites which are also sulfated if featuring a 3β-hydroxy group.

Conjugation of the oxidation products demonstrates that 4-hydroxy-4-ene structures are only glucuronidated. Metabolite **6** may be glucuronidated because of its 3α-hydroxy group which would be similar to testosterone metabolism. For metabolite **7** evaluation and comparison of phase-II metabolism is difficult because a testosterone metabolite with 3β,5β-configuration is barely formed.

Especially the structures found in both conjugate fractions show the exceptional conjugation of 4-hydroxylated steroids. Metabolites **9** (3α,4β) and **10** (3β,4α) as well as **8** (3β,4β) and **11** (3α,4α) have opposite configuration of the 3- and 4-hydroxy groups. 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.

Because the 17-hydroxy position is known to be conjugated in both ways, the 17-hydroxylated metabolites are not considered more closely.

In conclusion, qualitative consideration of phase-I metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone demonstrates that the mainly reductively produced urinary metabolites are identical. Phase-II metabolism including glucuronidation and sulfatation was also comparable for both administration studies. For inference on the applied substance a quantitative investigation and subsequent evaluation of ratios of selected metabolic products may be an opinion. The long term metabolite 3β,4α-dihydroxy-5α-androstan-17-one (**10**) was detected for about 90 h. Using selective ion monitoring mode, a much longer detection time should be possible and prolong the traceability of surreptitious misuse of 4-hydroxyandrostenedione and 4-hydroxytestosterone in sports drug testing.

Acknowledgement

The authors thank the Manfred Donike Institute for doping analysis for supporting the presented study.

REFERENCES

[1] Dowsett M, Coombes RC. Second generation aromatase inhibitor—4-hydroxyandrostenedione. Breast Cancer Res Treat 1994;30(1):81–7.
[2] Goss PE, Jarman M, Wilkinson JR, Coombes RC. Metabolism of the aromatase inhibitor 4-hydroxyandrostenedione in vivo. Identification of the glucuronide as a major urinary metabolite in patients and biliary metabolite in the rat. J Steroid Biochem 1986;24(2):619–22.
[3] Dowsett M, Cunningham DC, Stein RC, Evans S, Dehennin L, Hedley A, Coombes RC. Dose-related endocrine effects and pharmacokinetics of oral and intramuscular 4-hydroxyandrostenedione in postmenopausal breast cancer patients. Cancer Res 1989;49(5):1306–12.
[4] Dowsett M, Lloyd P. Comparison of the pharmacokinetics and pharmacodynamics of unformulated and formulated 4-hydroxyandrostenedione taken orally by healthy men. Cancer Chemother Pharmacol 1990;27(1):67–71.
[5] 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. J Chromatogr 1992;576(2):235–44.

[6] Lonning PE, Geisler J, Johannessen DC, Gschwind HP, Waldmeier F, Schneider W, Galli B, Winkler T, Blum W, Kriemler HP, Miller WR, Faigle JW. Pharmacokinetics and metabolism of formestane in breast cancer patients. J Steroid Biochem Mol Biol 2001;77(1):39–47.
[7] 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 Metab Dispos 1992;20(6):941–7.
[8] 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. J Chromatogr 1991;565(1-2):75–88.
[9] Marsh DA, Romanoff L, Williams KI, Brodie HJ, Brodie AM. Synthesis of deuterium- and tritium-labelled 4-hydroxyandrostene-3,17-dione, an aromatase inhibitor, and its metabolism in vitro and in vivo in the rat. Biochem Pharmacol 1982;31(5):701–5.
[10] Foster AB, Jarman M, Mann J, Parr IB. Metabolism of 4-hydroxyandrost-4-ene-3, 17-dione by rat hepatocytes. J Steroid Biochem 1986;24(2):607–17.
[11] 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, editors. Recent advances in doping analysis (13). Köln: Sport und Buch Strauß; 2005. p. 65–74.
[12] The 2005 prohibitied list. World Anti-Doping Agency 2004. www.wada-ama.org, access 27.12.06.
[13] Donike M, Zimmermann J. Preparation of trimethylsilyl, triethylsilyl and tert-butyldimethlsilyl enol ethers from ketosteroids for investigation by gas-chromatography and mass spectrometry. J Chromatogr 1980;202(3):483–6.
[14] Schänzer W, Donike M. Synthesis of deuterated steroids for GC/MS quantification of endogenous steorids. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke E, editors. Recent advances in doping analysis (2). Köln: Sport und Buch Strauß; 1995. p. 93–112.
[15] Camerino B. Oxidation of 3-keto steroids in alkaline medium. Tetrahedron Lett 1961:554–9.
[16] Da Silva EJ, Roleira FM, Sa e Melo ML, Neves AS, Paixao JA, de Almeida MJ, Silva MR, Andrade LC. X-ray and deuterium labeling studies on the abnormal ring cleavages of a 5 beta-epoxide precursor of formestane. Steroids 2002;67(3–4):311–9.
[17] Baran DB. Method for the cleavage of osmate esters. J Org Chem 1960:257.
[18] Fieser LF, Fieser M. Reagents for organic synthesis. New York: John Wiley & Sons, Inc; 1974. p. 759.
[19] Schänzer W, Opfermann G, Donike M. Metabolism of stanozolol: identification and synthesis of urinary metabolites. J Steroid Biochem 1990;36(1–2):153–74.
[20] Parr MK, Opfermann G, Schänzer W. Analytical properties of 4-hydroxysteroids and some esters. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, editors. Recent advances in doping analysis (12). Köln: Sport und Buch Strauß; 2004. p. 129–38.
[21] Schänzer W. Metabolism of anabolic androgenic steroids. Clin Chem 1996;42(7):1001–20.
[22] Houghton E, Ginn A, Teale P, Dumasia MC, Copsey J. Comparison of the use of mass spectrometry and methylene unit values in the determination of the stereochemistry of estranediol, the major urinary metabolite of 19-nortestosterone in the horse. J Chromatogr 1989;479(1):73–83.