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Investigations on carbon isotope ratios and concentrations of urinary formestane

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The aromatase inhibitor formestane (4-hydroxy-androst-4-ene-3,17-dione, F) is prohibited in sports by the World Anti-Doping Agency (WADA). F possesses only weak androgenic properties and is presumed to be employed in order to suppress estrogen production during the illicit intake of anabolic steroids by athletes.

Former studies additionally showed that F is an endogenous steroid produced in low amounts. According to the regulations of WADA, urinary concentrations above 100 ng/ml are assumed to be due to ingestion of F. To distinguish between endogenous or exogenous sources of urinary F, isotope ratio mass spectrometry (IRMS) is the method of choice.

Therefore, a method to determine the carbon isotope ratio (CIR) of F in urine samples was developed and validated. Routine samples (n = 42) showing concentrations of F above 5 ng/ml were investigated and enabled elucidation of the CIR of endogenous F and subsequent the calculation of a reference limit. A reference population encompassing n = 90 males and females was investigated regarding endogenous concentrations of F.

An excretion study with one male volunteer was conducted to test and validate the developed method and to identify possible impact of F administration on other endogenous steroids.

By CIR determination of F it is clearly possible to elucidate its endogenous or exogenous source. Taking into account the CIR of other target analytes like testosterone, a differentiation between F and androstenedione intake is possible.

In 2011, the first exogenous F below the WADA threshold could be detected by means of the developed IRMS method.

Keywords: carbon isotope ratio; formestane; doping control; excretion study; reference population

Introduction

Formestane (4-hydroxy-androst-4-ene-3,17-dione, F) is a potent aromatase inhibitor. During investigations on inhibition properties of several steroids in the 1970s, F was found to be one of the drug candidates with high inhibition efficiency and low toxicity.[1,2] Therefore, it became a clinically, well-investigated, so-called second-generation aromatase inhibitor mainly used in the treatment of estrogen-dependent breast cancer.[3,4] In this context, the pharmacokinetics and metabolism of different F formulations were under investigation.[5–11]

For athletes, aromatase inhibition can be an advantageous issue during the misuse of anabolic steroids like testosterone (T) to minimize side effects of steroids abuse caused by peripheral conversion of anabolic steroids to estrogens.

Besides other aromatase inhibitors, F is explicitly mentioned on the World Anti-Doping Agency (WADA) prohibited list since 2005 as an agent with anti-estrogenic activity.[12] As it is also an endogenously produced urinary steroid metabolite,[13,14] WADA established a threshold for urinary F excreted as glucuronide of 100 ng/ml in 2010.[15]

In doping control analysis samples below this urinary threshold shall be submitted to isotope ratio mass spectrometry (IRMS) determination in order to prove either the exogenous or the endogenous origin of urinary F. Therefore, the carbon isotope ratios (CIR) of endogenous reference compounds (ERC) like pregnanediol (PD) are compared to the CIR of the target compound (TC) F. This is common procedure for many other endogenously occurring steroids like T or T prohormones,[16–22] epitestosterone,[23,24] norandrosterone,[25] and boldenone.[26] CIR are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on Equation (1):

$$\delta^{13}C[\ ‰\ ] = \frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{std}}{\left(^{13}C/^{12}C\right)std} * 1000 \qquad (1)$$

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard.[27]

Differences between ERC and TC are expressed as Δ values based on Equation (2):

$$\Delta[\ ‰\ ] = \delta^{13}C_{ERC} - \delta^{13}C_{TC} \qquad (2)$$

According to the effectual WADA document for IRMS determinations, a Δ value above 3‰ indicates the exogenous source of the TC.[28] Several recent publications pointed out that this threshold is inapplicable to some pairs of ERC and TC, as investigations on reference populations (RefPop) showed some Δ values to be influenced by endogenous isotopic fractionation

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which results in a different threshold value for those steroids.[21,22,24,29,30]

One aim of this study was to elucidate the natural abundance of CIR for endogenous F by means of a RefPop encompassing n = 42 urine samples with slightly elevated concentrations of F chosen from routine doping control samples.

Furthermore, urinary concentrations of endogenous F were determined in another RefPop comprising n = 90 healthy male and female volunteers in order to confirm the earlier findings on urinary concentrations and resulting thresholds of F.[14,31]

An excretion study with one healthy male volunteer administered F orally was conducted to test the developed method for determination of CIR and to investigate possible influence of F intake on other endogenous steroids usually under investigation in doping control analysis. If the intake of F is only reflected by a change in CIR of urinary excreted F and not by any other endogenous TC, this will allow for distinction between F or T prohormone administration like 4-androstenedione, which also influences the $\delta^{13}C$ values of urinary excreted F.[14] In the context of doping control this constitutes a relevant issue as the aromatase inhibitor F is listed in another class of substances than T prohormones, and is therefore punished by different disciplinary measures.[32]

Since the middle of 2010, several routine doping control samples showing elevated concentrations of F were investigated by the new method. And in 2011, the first finding of F in an athlete's urine beneath the threshold of 100 ng/ml but with CIR clearly showing the exogenous origin of F was reported.

Experimental

Method development and validation for urinary concentrations and CIR took place in the Cologne laboratory. Afterwards, the developed method was implemented in the sports drug testing of the Swiss Laboratory. As this implementation was accompanied by several small changes to the method it necessitates re-validation. So this section subdivides into Cologne, Germany (CG) and Epalinges, Swiss (ES) parts to support ease of reading.

Chemicals and steroids (CG)

Chromabond® C18 cartridges were obtained from Macherey and Nagel (Düren, Germany). Acetone (for gas chromatography), pyridine and acetic anhydride (distilled before use) were purchased from Merck (Darmstadt, Germany). *Tert.*-butyl methyl ether (TBME, distilled before use) was from KMF Laborchemie (St Augustin, Germany), β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany), and steroid reference material (3α-hydroxy-5α-androstan-17-one (A); 3α-hydroxy-5β-androstan-17-one (E); 3α,11β-dihydroxy-5α-androstan-17-one (OHA); 3α,11β-dihydroxy-5β-androstan-17-one (OHE); 5β-pregnane-3α,20α-diol (PD); 3β-hydroxy-androst-5-en-17-one (DHEA); 17β-hydroxy-androst-4-en-3-one (T); 17α-hydroxy-androst-4-en-3-one (EpiT), F and 3β − hydroxy-5α-androstane (RSTD)) was supplied by Sigma-Aldrich (Steinheim, Germany). 5α-androstane-3α,17β-diol (5a) and 5β-androstane-3α,17β-diol (5b) were purchased from Steraloids (Newport, RI, USA). β-Estradiol-3,17-diacetate (EST) was from Riedel-de Haen (Seelze, Germany). All solvents and reagents were of analytical grade.

Chemicals and steroids (ES)

Bakerbond™ SPE Octadecyl columns were purchased from J.T. Baker (Deventer, the Netherlands). Pyridine and acetic anhydride were from Sigma-Aldrich (Buchs, Switzerland) and β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany). TBME was obtained from Acros (Geel, Belgium), methanol (MeOH) from Merck (Darmstadt, Germany) and acetonitrile from Biosolve (Valkensward, the Netherlands). All solvents and reagents were of analytical grade. Steroid reference material E, A, PD, RSTD and 3α-hydroxy-5α-androst-16-ene (16EN) were supplied by Steraloids (Newport, RI, USA) while F was from Sigma-Aldrich (Buchs, Switzerland).

Reference population collected for urinary concentrations

The reference population included 90 subjects (n = 49 females and n = 41 males); all were students or employees of the German Sport University, Cologne. All participants were aged between 20 and 54 years and exercised 2 to 6 times per week. All subjects provided single spot urine samples collected before noon. The specimens were stored at 4°C until analysis for six months. For possible sample degradation has been tested by monitoring the ratios of E/5β-androstanedione and A/5α-androstanedione. Urines showing values below 100 were excluded. No participant declared the use of prohormones or dietary supplements. The study was approved by the local ethical committee, and written consent was given by all participants.

Reference population CIR

In 2010, routine doping control samples investigated at the Cologne laboratory found with elevated concentrations of F (range from 6–23 ng/ml, n = 37) and not suspicious for the intake of any doping relevant substance were investigated regarding their CIR of F and PD. An additional five samples collected at the Swiss laboratory for validation purposes showed sufficient F concentration to determine the CIR at natural abundance. These samples were used to calculate an upper reference limit for Δ(PD-F).

Excretion study

One healthy male volunteer (35 years, 77 kg, 171 cm) administered 100 mg F dissolved in ethanol/water (40/60 *v/v*; 50 ml) orally. Prior to the administration study the $\delta^{13}C$ value of F was determined to be -30.3 ± 0.16 ‰ (n = 6).

One blank urine was collected directly before the administration and then all urine samples were collected for the following two days. Subsequent only the morning and one evening urine were sampled for three days. This protocol resulted in a total number of 21 urine specimens. All specimens were stored frozen until preparation.

The ethical committee of the German Sport University approved the study, and the participant gave written consent.

Sample preparation steroid profile (CG+ES)

An aliquot of each specimen was prepared according to routine doping control sample preparation procedures to determine the amount of different endogenous steroids.[33] This allows for determination of urine volume requisite for IRMS.

GC-MS measurements (CG)

In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in some fractions, it was necessary to scan samples on a GC-MS (gas chromatography-mass spectrometry) system using equivalent chromatographic conditions

to the IRMS setup whilst method development. For this purpose, a GC Agilent 6890 coupled to a mass selective detector MSD Agilent 5973 was used. The GC system was equipped with a Macherey and Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 μm). The injections (2 μl) were performed splitless at 300°C. The initial oven temperature of 60°C was held for 1.5 min, increased at 40°C/min to 240°C, followed by a ramp at 2°C/min to 260°C, and 40°C/min to the final temperature of 300°C. A constant flow of 1.2 ml/min with helium as carrier gas was used. The MSD acquired data in scan mode from *m/z* 40 to 400 and mass spectral data were compared to those from standards.

GC-MS/MS measurements (CG)

Due to improved sensitivity in contrast to GC-MS single ion monitoring techniques, the RefPop for urinary concentration was analyzed using an Agilent GC7890A coupled to an Agilent MS7000B Triple Quad. The GC column was a J&W Scientific Ultra I (OV-1) (length 17 m; inner diameter 0.2 mm and 0.11 μm film thickness), injections were performed in split mode (1:10) at 300°C. Injection volume was 2.5 μl and helium was used under constant pressure of 14.6 psi. Temperature program started from 185°C with 4°C/min to 234°C and then with 40°C/min to 310°C held for 2 min. Data was acquired in MRM operation mode using nitrogen as collision gas and electron impact ionization (70 eV). F was detected and quantified using MRM 518.3 to 169.0 (Figure 1).

Sample preparation CIR (CG)

Analytes have to be efficiently isolated and purified before GC/C/IRMS analysis in order to avoid co-elution of compounds and to keep in readiness the ability to measure differently concentrated urinary steroids in comparable amounts. Both aspects are necessary for valid $^{13}C/^{12}C$ determinations. Therefore, extensive sample preparation followed by two-fold high performance liquid chromatography (HPLC) clean-up was employed.

A detailed description of sample preparation was published elsewhere [22] and will herein only be described in brief: 5–20 ml of urine were applied on a conditioned C18 solid-phase extraction cartridge, washed with 2 ml of water and eluted triply with 1 ml of MeOH; the dried residue was dissolved in 1 ml of sodium phosphate buffer and extracted with 5 ml of TBME to separate unconjugated steroids; the aqueous residue was hydrolysed with β-glucuronidase, adjusted to pH 9.6 with 0.5 ml of potassium carbonate buffer and again extracted with 5 ml TBME; the organic layer (containing formerly glucuronidated steroids) was transferred into a conical test tube and evaporated to dryness, re-dissolved in 200 μl of acetone, transferred into a HPLC auto-sampler vial and evaporated.

Sample preparation CIR (ES)

Only some minor adaptations to sample preparation were employed in contrast to the abovementioned procedure. The adjustment of the pH after hydrolysis with potassium carbonate buffer was omitted and the sample was directly extracted with 2 times 4 ml TBME. The organic layer was transferred into a test tube and evaporated to dryness, redissolved with 2 times 100 μl of MeOH, transferred into a HPLC auto-sampler vials and evaporated.

HPLC clean-up (CG)

The clean-up methods for steroids excreted glucuronidated has already been published elsewhere.[22] For F the existing method could be used, only the fraction collection times were adjusted (for F from 13.4 to 15.0 min and for FAc (acetylated formestane) from 12.5 to 14.3 min).

HPLC clean-up (ES)

In order to remove all interfering or co-eluting compounds prior to GC/C/IRMS measurements, two consecutive HPLC fractionation steps were employed. Both were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) with a Waters XBridge™ Shield RP18 3.5 μm (4.6 x 150 mm) column protected with a XBridge™ Shield RP18 3.5 μm (4.6 x 20 mm) guard column.

The dried residue was dissolved in 50 μl of a mixture containing acetonitrile/water (50/50, *v/v*), the injection volume was 50 μl and the flow rate 1 ml/min. A linear gradient was used increasing from 40/60 acetonitrile/water to 60% acetonitrile in 16 min, then within 1 min to 98%, than in 5 min to 100% acetonitrile. After 3 min at 100%, the column was re-equilibrated for 5 min. Before each batch of samples, a standard solution containing approximately 40 μg/ml of F and 16EN, 200 μg/ml of PD and 100 μg/ml of E and A each was injected twice to determine the retention times for fraction collection. The automatic fraction collector Agilent 1200 was programmed to prepare 5 fractions as illustrated in Figure 2a. The different fractions were collected in small test tubes and evaporated to dryness under a stream of air. All fractions were acetylated due to considerably improved separation and peak shape of steroids on both the HPLC and the GC column.

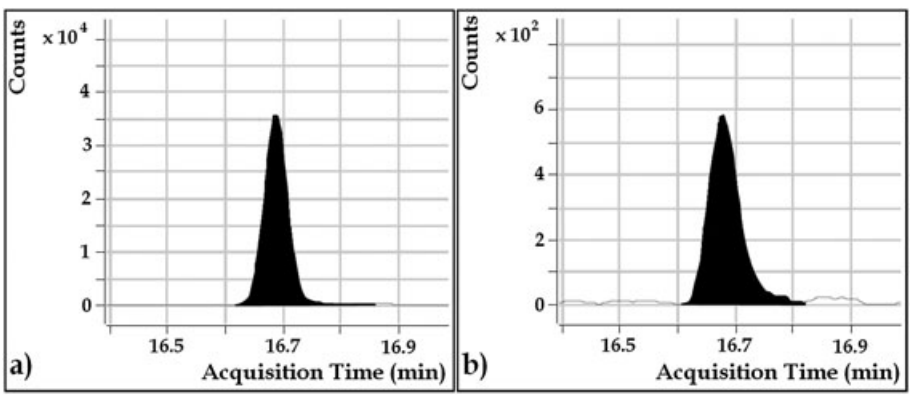

Figure 1. GC-MS/MS chromatograms of F. a) Standard containing 40 ng/mL F, b) sample containing 0.8 ng/mL F. Depicted is the MRM 518.3 −> 169.0.

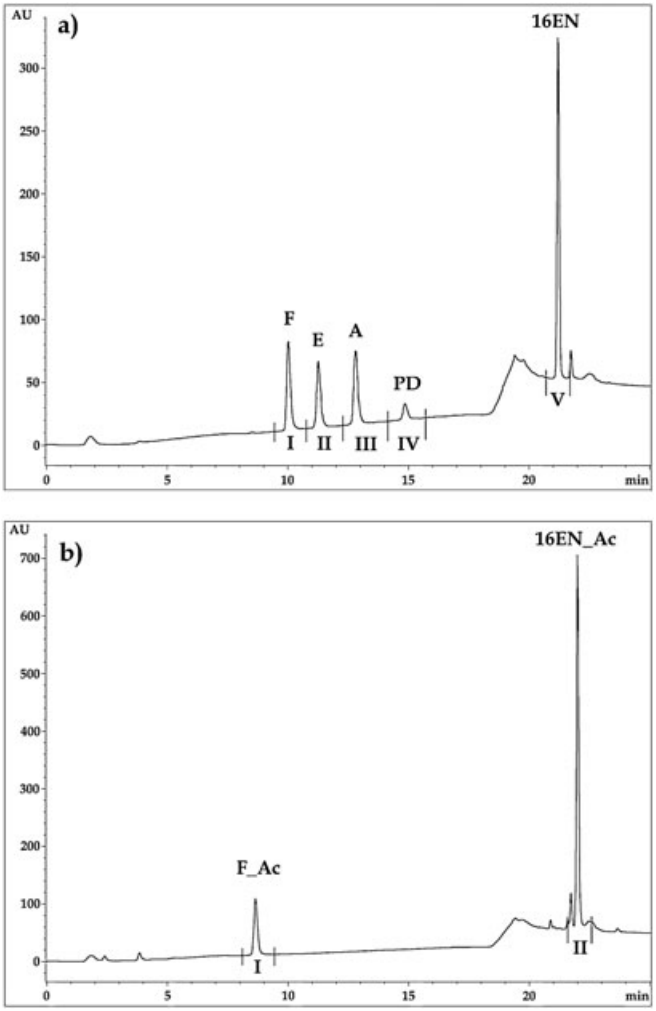

Figure 2. a) HPLC chromatogram of a standard containing F, E, A, PD and 16EN and b) HPLC chromatogram of a standard containing F_Ac and 16EN_Ac (wavelength 195 nm). The fractions were collected as demonstrated.

Therefore, 50 µl of pyridine and 50 µl of acetic anhydride were added. The mixture was incubated for 60 min at 70°C and evaporated to dryness under a stream of air and the dried residue was transferred to either GC or LC autosamplervials.

Before acetylation 1 µg of 16EN (now used as internal standard, 16EN_IS) was added to the test tube containing F to enable supervising of the acetylation afterwards to ensure no unexpected isotopic fractionation has taken place.

Fraction I containing F_Ac and 16EN_IS_Ac was further purified by an additional HPLC fractionation using the same gradient as above. Again, the mixture containing acetonitrile/water was used as solvent for injection with 50 µl. A standard solution containing approximately 40 µg/ml of F_Ac and 16EN_IS_AC each was injected twice to determine the retention times for fraction collection (Figure 2b). Two fractions were collected as demonstrated and evaporated to dryness.

The effectiveness of this laborious sample preparation is demonstrated in Figure 3 showing the absence of any co-eluting biological compound after sample clean-up.

GC/C/IRMS measurements (CG)

All samples were measured on an Agilent 6890 Gas Chromatograph (Waldbronn, Germany) coupled to a Delta plus XP gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via a modified GC combustion interface (GCC III, ThermoElectron).[34] The GC system was equipped with a Macherey and Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). Injection was performed with a Gerstel (Mühlheim an der Ruhr, Germany) KAS unit at 50°C in solvent vent mode with a helium flow of 100 ml/min. Injection volumes ranged from 2 to 5 µL of TBME, injection speed was 3 µl/s. The initial temperature was maintained for 3 min and increased at 40°C/min up to 260°C, then at 2°C/min to 280°C and then at 40°C/min up to 295°C and kept for 3 min. For the F_Ac measurements the GC temperatures were increased to 270°C, 290°C, and 300°C, respectively. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.4 ml/min. The combustion furnace was operated at 950°C. Data was acquired using ISODAT® NT 2.0 software (ThermoElectron).

GC/C/IRMS measurements (ES)

All samples were measured on an Agilent 7890 Gas Chromatograph (Waldbronn, Germany) coupled to a Delta V gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via the GC combustion interface (GCC III, ThermoElectron). Injections were performed in the splitless mode at 280°C with injection volumes ranging from 1 to 3 µl cyclohexane. The GC column was a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 µm) from Agilent. The initial oven temperature of 70°C was maintained for 2 min and increased at 30°C/min up to 270°C, then at 2°C/min to 290°C and then at 30°C/min up to 300°C and kept for 3 min. For the F_Ac measurements the GC temperature started identically but from 270°C on the ramp was 3°C/min up to 300°C and kept for 3 min. Carrier gas was purified He (purity grade 4.9) with a constant flow of 1.4 ml/min. The combustion furnace was operated at 940°C. Data was acquired using ISODAT® 3.0 software (ThermoElectron).

Correction for the acetate moiety

All determined values were corrected for the influence of the acetate moiety as described in the literature.[22,35] All $\delta^{13}C$ values reported within this article are for the underivatized steroid.

Method validation (CG)

The specificity of the method was ensured by GC-MS measurements under equivalent conditions to the GC/C/IRMS. Repeatability of the whole method was tested by repeated preparations of blank urine fortified with 10, 20, and 40 ng/ml F. Reproducibility was tested with repeated preparations of a QC urine over the time period of four months in which the RefPop was investigated. The obtained $\delta^{13}C$ values were compared to the $\delta^{13}C$ values of the added standard.

Method validation (ES)

The developed method for F was validated by means of a linear mixing model using Equation (3):[22,36,37]

$$\delta^{13}C_m = \left(\delta^{13}C_e - \delta^{13}C_a\right)\frac{c_e}{c_m} + \delta^{13}C_a \qquad (3)$$

with c_x = corresponding concentration and $\delta^{13}C_x$ = corresponding $\delta^{13}C$ value; $_m$ stands for mixture, e for endogenous and a for added standard.

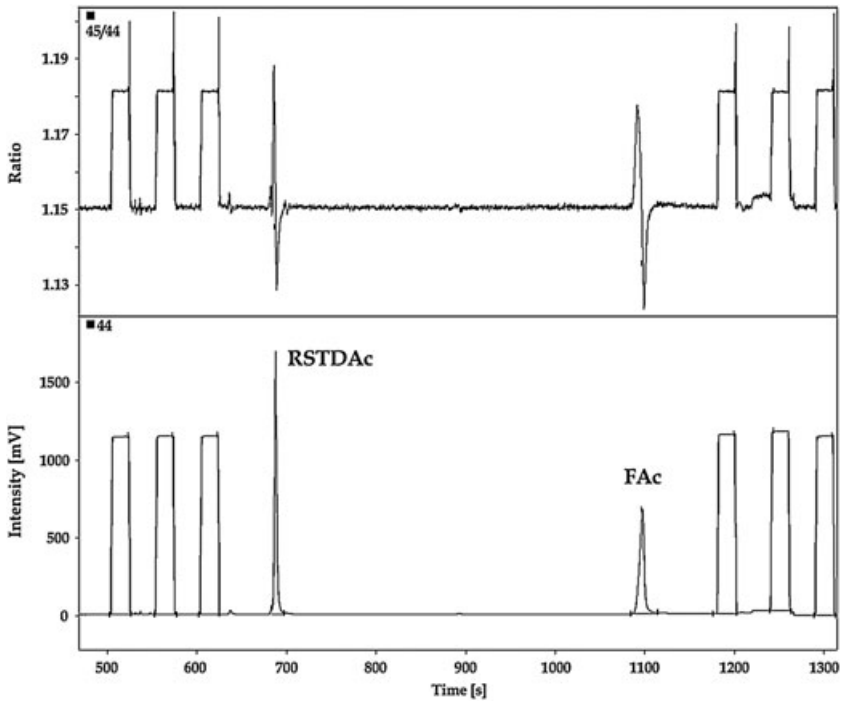

Figure 3. GC/C/IRMS chromatogram of a sample containing 12 ng/mL F. The rectangular peaks are pulses of calibrated tank gas, RSTDAc act as reference standard.

A comparison with the linear equation ($y = a*x + b$) shows that the corresponding equation of the resulting line of best fit represents the difference in $\delta^{13}C$ values between the endogenous steroid and the added standard ($\delta^{13}C_e - \delta^{13}C_a$) as its slope. The absolute $\delta^{13}C$ value of the standard is represented by the intercept on the y-axis ($b = \delta^{13}C_a$).

For this approach, five different blank urine samples containing between 3 to 6 ng/ml F were fortified at three different levels with up to 400 ng F to yield the necessary concentration ratios between endogenous and exogenous steroid.[22] So over all 20 measurements were accomplished for this validation.

Results and discussion

Method validation

Both approaches used for method validation could clearly demonstrate the validity of the developed method. The results obtained for fortified blank urines in CG are summarized in Table 1. Obviously, there is no isotopic fractionation taking place during sample preparation influencing the measured values. No co-elutions with FAc could be detected in the measurements.

In Table 2, the results obtained for the linear mixing model are listed. Again, no isotopic fractionation could be detected. Over all, the standard deviations (SD) were found to be larger in ES, maybe due to the different GC injection system used for IRMS determination (hot splitless instead of KAS).

The repeated preparation of a spiked QC urine resulted in a mean of -30.4 ± 0.20 ‰ for six samples over the time period of four months.

F concentrations of the RefPop

In all of the 90 investigated urine samples, F could be detected at a range from 0.5 to 13 ng/ml. The distribution of the raw data is visualized in Figure 4. As expected for urinary steroid concentrations the distribution was found right skewed.[38] As females and males exhibit slightly different urinary concentrations of F (Figure 5), the distribution is additionally broadened. Nevertheless, after log-normal-transformation of the data it was possible to calculate a parametrical 99.9% upper reference limit of the distribution to be 50 ng/ml. Additionally, a non-parametric approach was chosen and twice the far outside limit was calculated to be 24 ng/ml.[39,40] Hence, the herein investigated RefPop showed similar results to the already published data where n = 200 and n = 3031 subjects were investigated and limits of 40 and 36.5 ng/ml were found.[14,31] All three investigations propose a lower threshold value for urinary F as the actual WADA-defined

Table 1. Mean $\delta^{13}C_{VPDB}$ values (n = 6) of fortified blank urines processed with the described method. The F standard used has a $\delta^{13}C_{VPDB}$ value of -30.3 ± 0.16 ‰ (n = 6). SD stands for standard deviation

conc [ng/ml]	$\delta^{13}C_{VPDB}$ [‰]	SD
10	−30.1	0.27
20	−30.4	0.43
40	−30.2	0.29

Table 2. Calculated values for the linear mixing models referring to the equation $y = a*x + b$. a represents the Δ value (endogenous steroid minus standard) and b the $\delta^{13}C$ value of the standard. For comparison the estimated values of the standard (n = 6) is listed, too. All values in $\delta^{13}C_{VPDB}$[‰]

Steroid	a[‰]	SD[‰]	b[‰]	SD[‰]	Std[‰]	SD[‰]
F	7.3	0.41	−30.3	0.19	−30.5	0.61

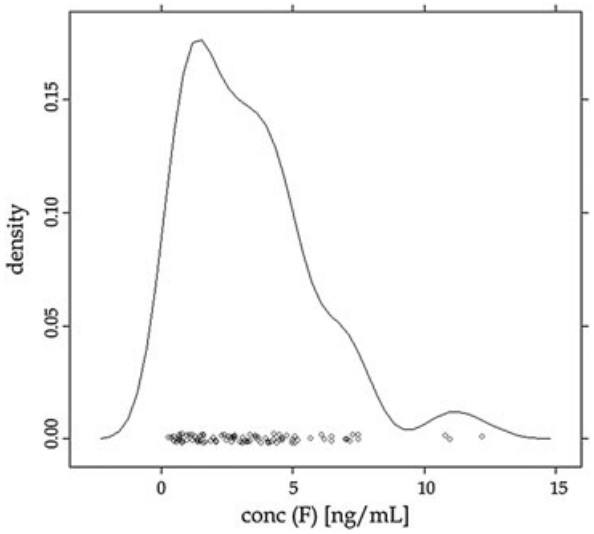

Figure 4. Density plot of the urinary concentrations of F found in a RefPop of n = 90 males and females.

limit of 100 ng/ml. Lowering the threshold would not increase the probability for a false positive finding, as urines above the limit would first be investigated by IRMS before a decision is made.

CIR of urinary F

All 42 investigated urine samples showed similar values in their CIR Δ values for PD - F. A scatter plot of PD versus F is depicted in Figure 6. As the Δ values showed a Gaussian distribution (Shapiro-Wilk test at p = 0.05) a reference limit could be calculated parametrically by adding the three-fold SD to the mean value. This resulted in a suggested limit for PD – F of 2.9 ‰. This value – by chance – fits perfectly with the actual WADA threshold of 3 ‰. The mean difference between the $\delta^{13}C_{VPDB}$ values of PD and F was 0.5 ‰ and the largest difference found within the RefPop was 2.2 ‰.

The main drawback of the investigated population is the fact that it cannot completely be excluded that a false negative sample is included. Within the 37 specimens taken from routine doping control, there might have been one of an athlete using F. This might have influenced the Δ value as could be demonstrated for PD – A.[41] But as this inclusion of a false negative sample can only elevate the suggested reference limit, we do not foresee any problems in applying the determined threshold in future sports drug testing.

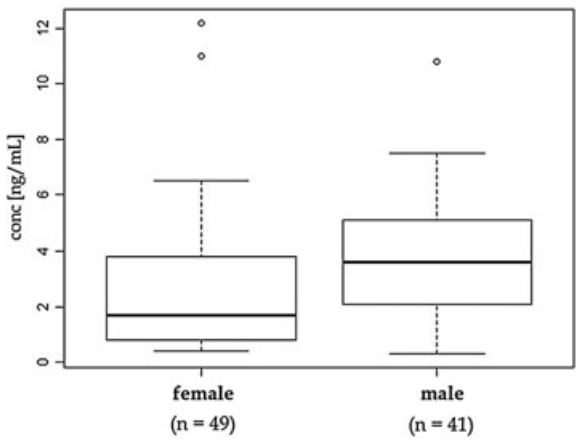

Figure 5. Box plot of the urinary concentrations of F found in a RefPop of n = 41 males and n = 49 females. The mean values differ highly significantly (p < 0.01, Wilcoxon rank sum test).

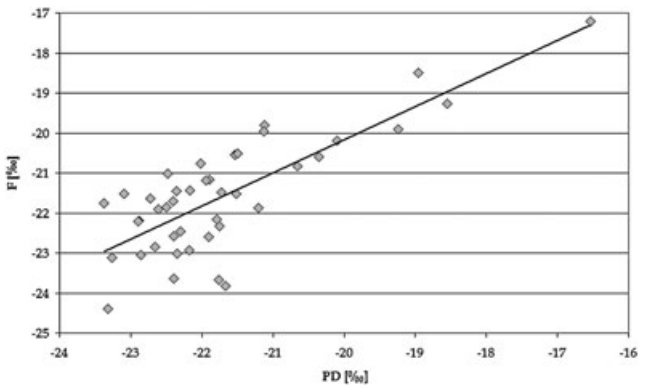

Figure 6. Scatter plot of $\delta^{13}C_{VPDB}$ values for all pairs of PD and F found in the investigated RefPop of n = 42 individuals. The bold line demonstrates the linear correlations between the samples (Pearson r = 0.81, p < 0.001).

Excretion study – urinary concentrations

In the case of F administration nearly all investigated steroids showed no influence in their urinary concentrations. Besides the so called steroid profile consisting of seven steroids excreted glucuronidated into urine (A, E, T, EpiT, DHEA, 5α- and 5β-androstane-3α,17β-diol)[28] several other endogenous steroids were monitored: F, dihydrotestosterone, PD, OHE OHA and 4-androstene-3,17-dione.

As depicted in Figure 7, only F itself and interestingly, OHE showed significant changes in their urinary concentrations (further explanation can be found later on). High amounts of orally administered F seem to be directly eliminated during their first path through the liver by glucuronidation and subsequent renal excretion. Five hours after application a peak concentration of nearly 40 μg/ml glucuronidated F is found in urine and 24 h after application the urinary concentrations of F are back in the normal range but remain slightly elevated until 40 h. As already reported in the literature,[5,8] F is solely excreted into urine as glucuronide and no F was found unconjugated.

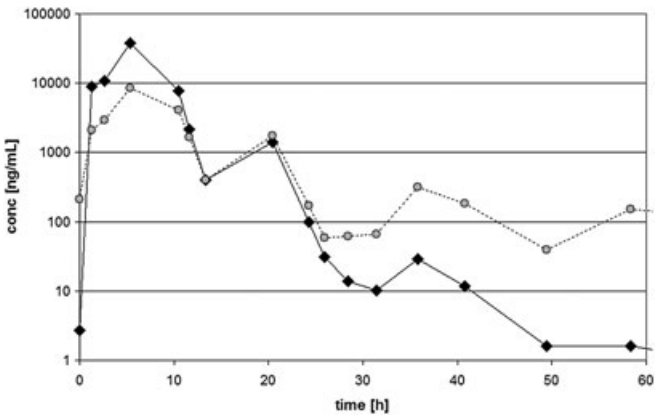

Figure 7. Semi-logarithmic plot of urinary concentrations over the course of time after oral administration of 100 mg F at t = 0h. Black diamonds represent F excreted glucuronidated, grey circles the sum of OHE and a co-eluting steroid, both excreted glucuronidated. Further information in the text.

A more than twenty-fold increase was monitored for OHE directly after administration of F and normalization took place within the following 24 h.

Excretion study – CIR

In accordance with the findings for urinary concentrations, most of the investigated steroids did not show any influence in their CIR (E, A, T, EpiT, DHEA, 5a and 5b). The determined mean values and SDs are listed in Table 3.

In contrast to our expectations, OHE did not show any influence in CIR but we found a strong shift in isotopic values of OHA as can be seen in Figure 8. These contradictory results are discussed in the following section.

Figure 9 shows the results obtained for the TC F and the used ERC PD. As expected, the CIR of F were strongly influenced directly after administration showing the most depleted value after 5 h. Interestingly, the $\delta^{13}C_{VPDB}$ value of the administered steroid (−30.3 ‰) was not reached by urinary F. As the endogenous dilution of the CIR should be very small for F, this cannot be the explanation for this result. More likely an isotopic fractionation is taking place during the extensive metabolism of F evoking slightly enriched values for F itself whereas other metabolites should be more depleted than the starting material. As the numerous different metabolites of F were not investigated during this study, this hypothesis could not be proven.

As already demonstrated for other steroids like T or DHEA,[22,24,30] the CIR of F showed influenced values for a longer time period than the belonging urinary concentrations. The Δ value of F – PD remained above the threshold for approximately 60 h, more than twofold the time frame urinary concentrations were found suspicious.

PD remained stable throughout the complete investigation (mean equals -22.4 ± 0.23 ‰) and therefore proved its usefulness as an ERC.

Table 3. CIR mean values of all steroids apparently not influenced after administration of 100 mg F orally. Listed are the mean values for n = 15 specimens together with the belonging SD.All values in $\delta^{13}C_{VPDB}$[‰]

steroid	mean [‰]	SD [‰]
E	−23.9	0.15
A	−22.7	0.10
T	−23.6	0.35
EpiT	−24.8	0.49
DHEA	−21.6	0.44
5a	−23.4	0.48
5b	−22.0	0.43

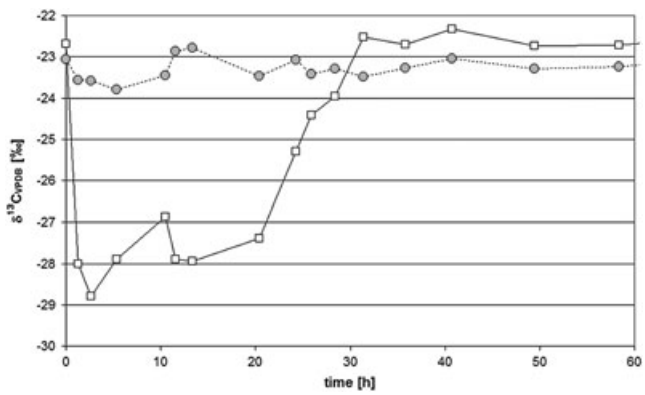

Figure 8. Changes of CIR over the course of time after oral administration of 100 mg F at t = 0h. Grey circles represent OHE, open squares the sum of OHA and a co-eluting steroid. Further information in the text.

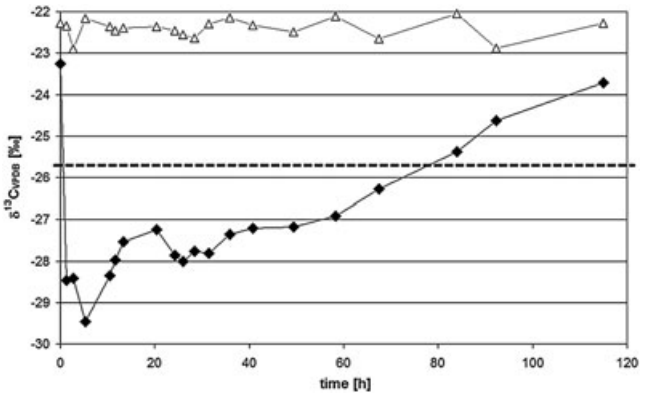

Figure 9. Changes of CIR over the course of time after oral administration of 100 mg F at t = 0h. Open triangles represent PD used as ERC and black diamonds F. The broken bold line represents the determined reference limit for F – PD.

Influences on OHA and OHE

As mentioned earlier, the found influences on urinary concentrations of OHE together with strong influences on CIR of OHA were in contradiction to the usually perfect correlation between both parameters. So further investigations were employed to identify the co-eluting substance disturbing the measurements of both steroids. Full-scan GC-MS experiments were carried out with both the aliquots for concentration determinations and GC/C/IRMS determinations. As an example, Figure 10 shows the GC-MS chromatogram and the relevant mass spectrum of the HPLC fraction obtained for determination of OHA and OHE.[22] While in the GC/C/IRMS chromatogram there was only little fronting of the OHA peak visible, a third steroid falling between OHE and OHA became visible on the GC/MS system. The mass spectrum of X was nearly similar to the ones of the 11-hydroxy-steroids. Thanks to extensive prior investigations on the metabolism of orally ingested F by Kohler *et al.*, we were enabled to compare the retention time and mass spectrum of X to standards.[42] This allowed for clear identification of the disturbing compound to be 3α,4α-dihydroxy-androstan-17-one.

With the used chromatographic conditions on the GC/MS/MS this dihydroxy-metabolite showed exactly the same retention time as OHE, explaining the presumed influence of F administration on this steroid. Due to the structural similarity between the 11-hydroxy-steroids and 3α,4α-dihydroxy-androstan-17-one it might be advisable not to use those steroid as ERC in the case of a suspicious sample for F misuse to avoid possible problems provoked by co-eluting peaks.

Positive doping control sample

In 2010 and 2011, several samples showing elevated concentrations of urinary F were investigated with the developed IRMS method. Samples containing up to 49 ng/ml were investigated

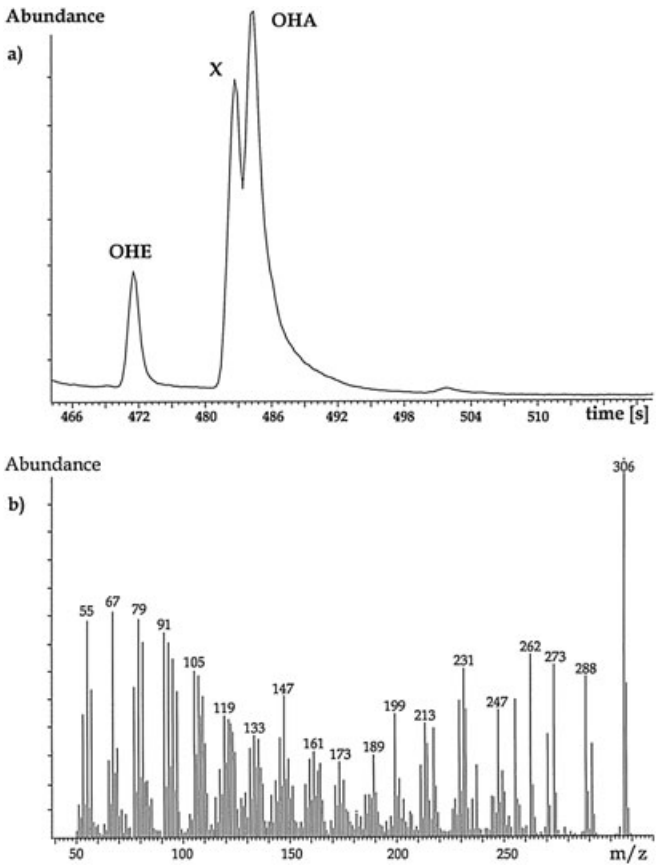

Figure 10. a) Total ion count chromatogram of the HPLC fraction containing OHA and OHE underivatized for IRMS determinations. 22 The co-eluting compound X strongly influenced the GC/C/IRMS measurements as under the employed GC and combustion conditions both peaks are not separated. b) Mass spectrum of compound X.

and showed clearly endogenous CIR for F. The largest difference between PD and F was found to be 1.6 ‰.

In 2011, one highly suspicious urine sample containing ca. 80 ng/ml of F was detected in the Swiss Laboratory for Doping Analysis within routine doping control samples. The sample was forwarded to IRMS analysis and the exogenous origin of F in this urine specimen could clearly be demonstrated. The found Δ value of PD – F was 5.0 ‰. On request of the athlete the B sample analysis was performed and confirmed the finding of the A sample. Additionally, a follow up sample of the same athlete was investigated showing completely unsuspicious results for PD – F of −1.3 ‰ despite still high endogenous concentrations of F of ca. 35 ng/ml.

This sample could prove the exogenous origin of urinary F below the WADA threshold and demonstrated the usefulness and validity of the developed GC/C/IRMS method. Additionally, it highlighted the necessity to investigate findings of urinary F below the actual WADA threshold with IRMS to prove the endogenous or exogenous origin of this steroid.

Conclusion

A method to determine the CIR of urinary excreted F was successfully developed, validated, and employed in sports drug testing. The investigated RefPop demonstrated the endogenous origin of F and proved that a Δ value between an ERC like PD and F can be used to unambiguously distinguish the source of urinary F. The found reference limit of 2.9 ‰ is in agreement with the actual WADA defined threshold.

The GC-MS/MS method used for investigations of another RefPop regarding the urinary concentrations had a detection limit below 0.5 ng/ml and could show that F is ubiquitous and can be found in every urine specimen. The upper reference limits calculated within this study where found to be 24 or 50 ng/ml respectively, depending on the used statistical approach. These limits are in agreement with the already published values of 36.5 and 40 ng/ml. All studies suggest to lower the threshold for urinary F, which in addition with the carefully employment of CIR will improve the sensitivity of F detection by doping control laboratories.

The developed GC/C/IRMS method could already prove its usefulness and identify urinary F in an athlete's specimen containing less than 100 ng/ml to be of exogenous origin. Without determination of CIR this sample would not have resulted in an adverse analytical finding.

As the administration of F did not influence the CIR of any usually employed TC it will be possible with the assistance of the developed method to distinguish between an administration of F and for example 4-androstene-3,17-dione. This T prohormone also results in elevated concentrations of urinary F but will definitely influence the CIR of T, 5a and 5b. The absence of this influence will have impact on the athletes punishment as disciplinary measures are different for anabolic steroid misuse than for aromatase inhibitor administration.

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References

[1] W.C. Schwarzel, W.G. Kruggel, H.J. Brodie. Studies on the mechanism of estrogen biosynthesis. VIII. The developement of inhibitors of the enzyme system in human placenta. *Endocrinology* **1973**, *92*, 866.
[2] A.M.H. Brodie, C. Longcope. Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3, 17-dione. *Endocrinology* **1980**, *106*, 19.
[3] R.C. Coombers, P. Goss, M. Dowsett, J.C. Gazet, A. Brodie. 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. *Lancet* **1984**, *324*, 1237.
[4] P.E. Goss, T.J. Powles, M. Dowsett, G. Hutchinson, A.M.H. Brodie, J.C. Gazet, *et al*. Treatment of advanced postmenopausal breast cancer with aromatase inhibitor, 4-hydroxyandrostenedione: Phase II report. *Cancer Res.* **1986**, *46*, 4823.
[5] P.E. Goos, M. Jarman, J.R. Wilkinson, R.C. Coombes. 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*, 619.
[6] M. Dowsett, D.C. Cunningham, R.C. Stein, S. Evans, L. Dehennin, A. Hedley, *et al.* Dose-related endocrine effects and pharmacokinetics of oral and intramuscular 4-hydroxyandrostenedione in postmenopausal breast cancer patients. *Cancer Res.* **1989**, *49*, 1306.
[7] M. Dowsett, P. Lloyd. Comparison of the pharmacokinetics and pharmacodynamics of unformulated and formulated 4-hydroxyandrostenedione taken orally by healthy men. *Cancer Chemother. Pharmacol.* **1990**, *27*, 67.
[8] G.K. Poon, M. Jarman, R. McCague, J.H. Davies, C.E.M. Heeremans, R.A.M. van der Hoeven, *et al*. Identification of 4-hydroxyandrost-4-ene-3,17-dione metabolites in prostatic cancer patients by liquid chromatography-mass spectrometry. *J. Chromatogr. Biomed. Appl.* **1992**, *576*, 235.

[9] M. Dowsett, A. Mehta, N. King, I.E. Smith, T.J. Powles, R.C. Stein, *et al*. An endocrine and pharmacokinetic study of four oral doses of formestane in postmenopausal breast cancer patients. *Eur. J. Cancer* **1992**, *28*, 415.
[10] M. Dowsett, R.C. Coombes. Second generation aromatase inhibitors – 4-hydroxyandrostenedione. *Breast Cancer Res. Treat.* **1994**, *30*, 81.
[11] P.E. Looning, J. Geisler, D.C. Johannessen, H.P. Gschwind, F. Waldmeier, W. Schneider, *et al*. Pharmacokinetics and metabolism of formestane in breast cancer patients. *J. Steroid Biochem.* **2001**, *77*, 39.
[12] World Anti-Doping Agency. The 2005 Prohibited List. International Standard. Available at: www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2005_EN.pdf [6 May 2011].
[13] D.H van de Kerkhof. Steroid Profiling in Doping Analysis, PhD thesis. Utrecht University, Utrecht, **2001**.
[14] A.T. Cawley, G.J. Trout, R. Kazlauskas, A.V. George. The detection of androstenedione abuse in sport: a mass spectrometry strategy to identify the 4-hydroxyandrostenedione metabolite. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 4147.
[15] World Anti-Doping Agency. TD2010MRPL. Available at: www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010MRPLv1.0_Minimum%20Required%20Performance%20Levels_Sept%2001%202010_EN.pdf [6 May 2011].
[16] M. Becchi, R. Aguilera, Y. Farizon, M.M. Flament, H. Casabianca, P. James. Gas chromatograhy/combustion/isotope ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 304.
[17] R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, *et al*. Improved method of detection of testosterone abuse by gas chromatography/ combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **1996**, *31*, 169.
[18] C.H.L. Shackleton, E. Roitman, A. Phillips, T. Chang. Confirming testosterone administration by isotope ratio mass spectrometry analysis of urinary androstenediols. *Steroids* **1997**, *62*, 379.
[19] C. Saudan, N. Baume, P. Mangin, M. Saugy. Urinary analysis of 16(5α)-androsten-3α-ol by gas chromatography/combustion/isotope ratio mass spectrometry: Implications in anti-doping analysis. *J. Chromatogr. B* **2004**, *810*, 157.
[20] A.T. Cawley, R. Kazlauskas, G.J. Trout, J.H. Rogerson, A.V. George. Isotopic fractionation of endogenous anabolic androgenic steroids and its relationship to doping control in sports. *J. Chromatogr. Sci.* **2005**, *43*, 32.
[21] U. Flenker, U. Güntner, W. Schänzer. δ^{13}C-values of endogenous urinary steroids. *Steroids* **2008**, *73*, 408.
[22] T. Piper, U. Mareck, H. Geyer, U. Flenker, M. Thevis, P. Platen, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2161.
[23] R. Aguilera, C.K. Hatton, D.H. Catlin. Detection of epitestosterone doping by isotope ratio mass spectrometry. *Clin. Chem.* **2002**, *48*, 629.
[24] T. Piper, P. Riemann, G. Opfermann, U. Mareck, H. Geyer, G. Vajiala, *et al*. Determination of $^{13}C/^{12}C$ ratios of urinary epitestosterone and its main metabolites 5α- and 5β-androstane-3α,17α-diol. *Drug Test. Analysis* **2009**, *1*, 576.
[25] M. Hebestreit, U. Flenker, G. Fußhöller, H. Geyer, U. Güntner, U. Mareck, *et al*. Determination of the origin of urinary norandrosterone traces by gas chromatography combustion isotope ratio mass spectrometry. *Analyst* **2006**, *131*, 1021.
[26] T. Piper, H. Geyer, V. Gougoulidis, U. Flenker, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one. *Drug Test. Analysis* **2010**, *2*, 217.
[27] H. Craig. Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochim. Cosmochim. Acta* **1957**, *12*, 133.
[28] World Anti-Doping Agency. TD2004EAAS. Available at: www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA_TD2004EAAS_Reporting_Evaluation_Testosterone_Epitestosterone_TE_Ratio_EN.pdf [6 May 2011].
[29] A.T. Cawley, G.J. Trout, R. Kazlauskas, C.J. Howe, A.V. George. Carbon isotope ratio (δ^{13}C) values of urinary steroids for doping control in sport. *Steroids* **2009**, *74*, 379.
[30] T. Piper, G. Opfermann, M. Thevis, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids excreted as sulpho conjugates. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3171.
[31] P. van Renterghem, P. van Eenoo, H. Geyer, W. Schänzer, F.T. Delbeke. Reference ranges of urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. *Steroids* **2010**, *75*, 154.
[32] World Anti-Doping Agency. The 2011 Prohibited List. International Standard. Available at: www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_-Prohibited_List_2011_EN.pdf [6 May 2011].
[33] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schänzer. Factors influencing the steroid profile in doping control analysis. *J. Mass Spectrom.* **2008**, *43*, 877.
[34] U. Flenker, M. Hebestreit, T. Piper, F. Hülsemann, W. Schänzer. Improved performance and maintenance in gas chromatography/isotope ratio mass spectrometry by precolumn solvent removal. *Anal. Chem.* **2007**, *79*, 4162.
[35] G. Docherty, V. Jones, R.P. Evershed. Practical and theoretical considerations in the gas chromatography/combustion/isotope ratio mass spectrometry δ^{13}C analysis of small polyfunctional compounds. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 730.
[36] C.D. Keeling. The concentration and isotopic abundance of atmospheric carbon dioxide in rural areas. *Geochim. Cosmochim. Acta* **1958**, *13*, 322.
[37] D.E. Pataki, J.R. Ehlering, L.B. Flanagan, D. Yakir, D.R. Bowling, C.J. Still, *et al*. The application and interpretation of Keeling plots in terrestrial carbon cycle research. *Global Biogeochem. Cy.* **2003**, *17*, 1022.
[38] S. Rauth. Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten, PhD Thesis. German Sport University Cologne, Cologne, **1994**.
[39] P. Laidler, D.A. Cowan, R.C. Hider, A.T. Kicman. New decision limits and quality-control material for detecting human chorionic gonadotropin misuse in sports. *Clin. Chem.* **1994**, *40*, 1306.
[40] A.T. Kicman, S.B. Coutts, C.J. Walker, D.A. Cowan. Proposed Confirmatory procedure for detecting 5α-dihydrotestosterone doping in male athletes. *Clin. Chem.* **1995**, *41*, 1617.
[41] T. Piper, U. Flenker, U. Mareck, W. Schänzer. $^{13}C/^{12}C$ ratios of endogenous urinary steroids investigated for doping control purposes. *Drug Test. Analysis* **2009**, *1*, 65.
[42] M. Kohler, M.K. Parr, G. Opfermann, M. Thevis, N. Schlörer, F.J. Marner, *et al*. Metabolism of 4-hydroxyandrostenedione and 4-hydroxytestosterone: Mass spectrometric identification of urinary metabolites. *Steroids* **2007**, *72*, 278.