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Determination of $^{13}C/^{12}C$ ratios of urinary epitestosterone and its main metabolites 5α- and 5β-androstane-3α, 17α-diol

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Epitestosterone (17α-hydroxy-androst-4-en-3-one, EpiT) belongs to the list of prohibited substances of the World Anti-Doping Agency (WADA). Although it possesses no anabolic effect, it is presumed to be misused by athletes in order to mask administration of testosterone (T) by lowering the urinary T/EpiT ratio.

To improve detection, an excretion study with 40 mg of orally administered EpiT was conducted focusing on the metabolites of EpiT: 5α- and 5β-androstane-3α,17 α-diol (5aEpiD and 5bEpiD). A reference population of n = 74 volunteers was investigated to elucidate the urinary concentrations of these steroids.

In order to prove whether an unusual finding in urinary concentrations or ratios is due to an illicit intake of steroids or due to physiological elevation, determination of carbon isotope ratios is advisable. A method for isotope ratio determination was developed to enable $^{13}C/^{12}C$ ratios of EpiT, 5bEpiD, 5aEpiD, pregnanediol and androsterone and etiocholanolone to be measured from a single urine specimen. The method's validity was tested by applying linear mixing models and specificity was ensured by means of gas chromatography/mass spectrometry analysis. $\delta^{13}C$ values at natural levels were determined with a reference population and both Δ values and corresponding reference limits were calculated.

Considering the implemented EpiT-metabolites, a more than twofold extension of the detection time of EpiT administration was achieved with both the urinary concentration thresholds and the $^{13}C/^{12}C$ ratios.

Keywords: carbon isotope ratio; epitestosterone; isotope ratio mass spectrometry (IRMS); doping control; reference population; excretion study

Introduction

The routine screening method for the detection of testosterone (T) or testosterone prohormone misuse is based on the determination of the T/EpiT (epitestosterone) ratio – the ratio between the concentrations of testosterone and epitestosterone-glucuronide[1,2] – which is complemented by additional steroid profile parameters. The T/EpiT ratio naturally varies in the range of 0.1 to 3,[3,4] and in case of values $\geq$ 4 an atypical analytical finding is reported according to the rules of the World Anti-Doping Agency (WADA).[5] Since the 1980s, preparations combining T and EpiT have been available and therefore allow for the intake of T without significant alteration of the T/EpiT ratio.[6,7]

Several attempts were conducted to reveal EpiT administration,[8,10,17] for example the ratio of EpiT to androst-5-ene-3β,17α-diol (5EN17a), both excreted in urine as glucuronides-was investigated. WADA established a urinary threshold of 200 ng/mL for urinary EpiT after correction for specific gravity. Unfortunately, the detection time for both tools is considered to be less than 24 hours after an EpiT administration.[8]

Hence, the immediate metabolites of EpiT, 5α- and 5β-androstane-3α,17β-diol (5aEpiD and 5bEpiD)[9–13] were investigated regarding their urinary concentrations and their $^{13}C/^{12}C$ ratios so as to elucidate their potential to confirm the illicit intake of EpiT by athletes.

In order to determine urinary concentrations and carbon isotope ratios at naturally occurring levels, a reference population of n = 74 volunteers was investigated. As urinary concentrations of steroids exhibit large inter- and intra-individual variability, ratios of urinary concentrations tend to be more stable (for example, the ratio of T/EpiT). So, a similar approach was chosen for the detection of EpiT administration and different ratios of EpiT, 5EN17a, 5aEpiD and 5bEpiD were calculated and tested for their power of discrimination against EpiT intake. Subsequently, thresholds were calculated non-parametrically to allow for identification of urine samples with atypical steroid concentrations.

In order to prove whether an unusual concentration is due to an illicit intake of EpiT or elevated physiologically, determination of carbon isotope ratios is advisable. Gas chromatography/combustion/isotope ratio mass spectrometry (GG/C/IRMS) has been applied to doping controls for more than 10 years.[14–21] It allows endogenous and exogenous steroids to be distinguished by comparison of their $^{13}C/^{12}C$ ratios with that of endogenous reference compounds (ERCs). $^{13}C/^{12}C$ ratios are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee

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Belemnite (VPDB) based on the equation:

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

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard.[22]

As pharmaceutically produced anabolic steroids are not synthesized *de novo* but are derived from plant material,[23] they usually exhibit depleted $\delta^{13}C$ values[21,24] in comparison to the values found for endogenously produced steroids. After application of an exogenous anabolic steroid the steroid itself or its metabolites exhibit depleted $\delta^{13}C$ values while the ERCs are not affected. These differences are expressed as Δ values based on the equation:

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

Target compounds (TCs) in this study were EpiT, 5aEpiD and 5bEpiD. Pregnanediol (PD), etiocholanolone (E) and androsterone (A) were used as ERCs. The calculated reference limits (RLs) for PD-Δ values were found to exceed the established WADA threshold of 3 ‰.[25]

An excretion study with one male volunteer receiving a single oral application of 40 mg EpiT was also performed to probe for the established RLs. Thirty-four routine samples from 2008 with elevated urinary concentrations of 5aEpiD and 5bEpiD were investigated with the new method. According to the results a more than twofold prolonged detection time of EpiT abuse should be possible.

Experimental

Chemicals and steroids

Chromabond® C18 cartridges were obtained from Macherey-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 VWR (Darmstadt, Germany), β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany), and steroid reference material (A, E, PD, EpiT and 5α-androstane-3β-ol (RSTD)) was supplied by Sigma (Steinheim, Germany). 5aEpiD, 5bEpiD and 5-androstene-3β,17α-diol (5EN) were purchased from Steraloids (Newport, USA) and β-estradiol-3,17-diacetate (EST) from Riedel-de Haen (Seelze, Germany). All solvents and reagents were of analytical grade.

Reference population

The reference population included 25 female and 49 male subjects (students and employees of the German Sport University, Cologne) aged between 18 to 46 years. Body weights ranged from 50–100 kg, body heights from 160–203 cm. All participants exercised up to six times per week. Oral contraceptives were used by 17 out of the 25 investigated females. All subjects provided single spot urine samples and the specimens were stored at −20 °C until analysis. No participant declared the use of prohormones, dietary supplements or the need for particular medications, except for one subject who administered a sertraline preparation and four subjects who used antibiotics within the two weeks prior to sampling. As the analysis of their urine samples did not yield exceptional $\delta^{13}C$ values, they were retained within the reference population. A standardized questionnaire was completed by each individual. It surveyed eating habits, amount of time spent on sport daily and general state of health. The study was approved by the local ethical committee and written consent was given by all participants.

Excretion study

One healthy male volunteer administered a single oral dose of 40 mg EpiT ($\delta^{13}C_{VPDB} = -33.2 \pm 0.1‰$ (n = 6)) dissolved in ethanol. Two blank urine samples were collected 3 hours and immediately before administration. A further 13 urine samples were collected for the following three days. All specimens were investigated with respect to their steroid concentrations and $\delta^{13}C$ values. The study was approved by the ethical committee of the National Institute for Sport Research (Bucharest, Romania) and written consent was given by the participant.

Urinary steroid concentrations

An aliquot of each of the abovementioned specimens was prepared to determine the amounts of different steroids according to routine sample preparation procedures[26] in order to probe for normal steroid profiles and to determine the urine volume requisite for GC/C/IRMS. The steroids of interest, 5aEpiD, 5bEpiD and 5EN17a (all excreted as glucuronides), were therefore implemented in the routine procedure to determine the steroid profile by gas chromatography/mass spectrometry (GC/MS). The sample preparation was maintained without changes. The characteristic ions were m/z 434 for 5EN17a and m/z 241 for 5aEpiD and 5bEpiD, all measured as bis-TMS derivatives.

Sample preparation GC/C/IRMS

Analytes have to be efficiently isolated and purified before GC/C/IRMS analysis in order to avoid co-elution of compounds and to be ready to measure differently concentrated urinary steroids in comparable amounts. Both aspects are necessary for valid $^{13}C/^{12}C$ determinations.

A detailed description of the extensive sample preparation method including twofold HPLC clean up was published elsewhere[21] and it will only be described in brief here. Figure 1 presents a flow scheme of the sample preparation. Depending on the amount of urinary steroids, up to 20 mL of urine were applied on a C18 solid-phase extraction cartridge, washed with 2 mL of water and eluted twice with 1 mL of methanol (MeOH). The dried residue was dissolved in 1 mL of sodium phosphate buffer and extracted with 5 mL of TBME, then hydrolysed with β-glucuronidase, adjusted to pH 9.6 and again extracted twice with TBME. The combined organic layers were dried, reconstituted and forwarded to HPLC purification.

High-performance liquid chromatography cleanup

In order to remove all interfering or co-eluting compounds prior to GC/C/IRMS measurements, two consecutive HPLC isolation steps were employed. Both were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a Merck analytical column (LiChrospher® 100 RP^{18}, 250×4 mm i.d., 5 µm particle size).

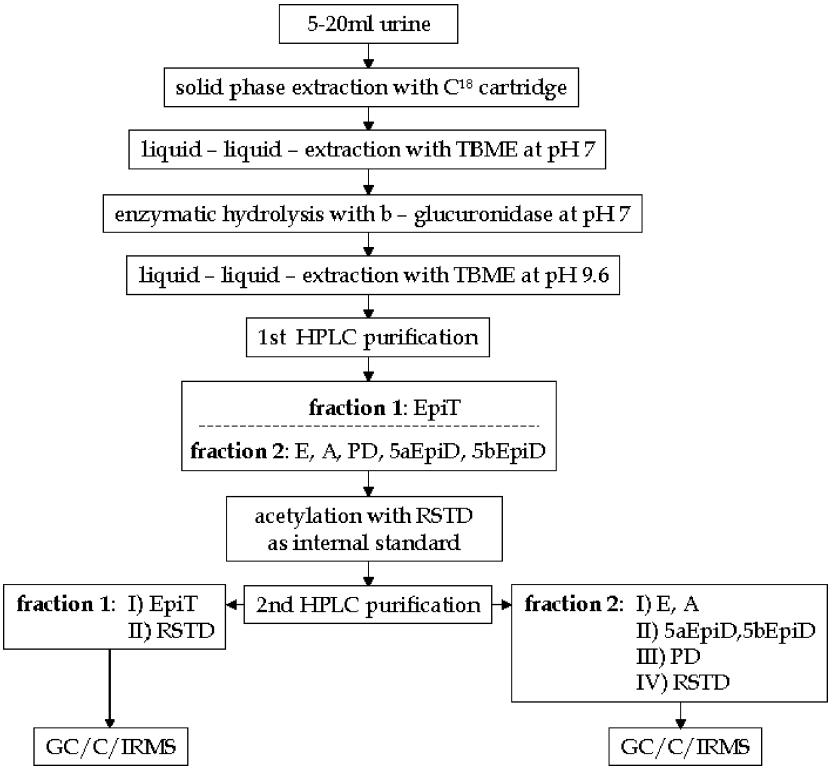

Figure 1. Flow scheme of sample preparation, further information in the text.

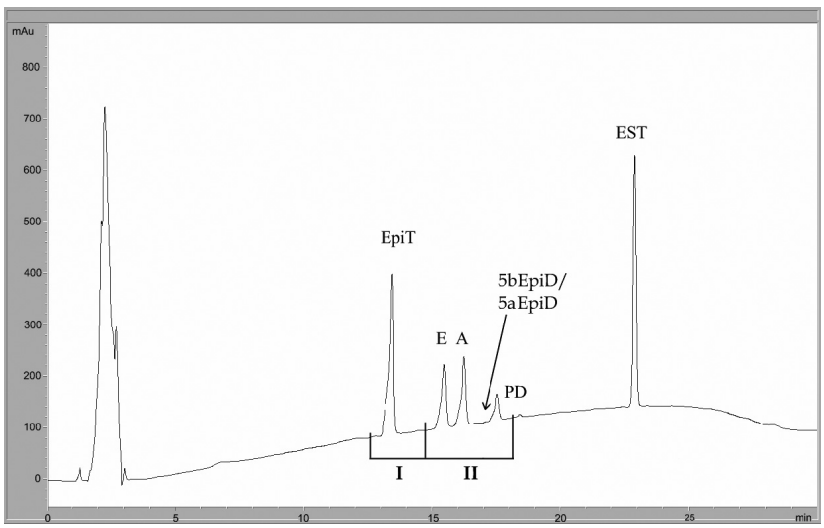

Figure 2. HPLC chromatogram of a standard containing EpiT, E, A, 5aEpiD, 5bEpiD and EST (wavelength 192 nm). The fractions were collected as demonstrated.

For the first run a linear gradient was used increasing from 30/70 acetonitrile/water to 100% acetonitrile in 25 min. After 5 min at 100% acetonitrile, the column was re-equilibrated for 5 min; 50 µL injection volume and flow rate 1 mL/min were used. Before each batch of 12 samples, a standard solution containing approximately 100 µg/mL of EpiT, E, A, 5aEpiD, 5bEpiD, PD and EST each was injected twice to determine the retention times for fraction collection. The automatic fraction collector Foxy 200 from Isco (Lincoln, Nebraska, USA) was programmed to prepare two fractions as illustrated in Figure 2.

In order to monitor any isotopic fractionation occurring during acetylation it was necessary to add an internal standard prior to the acetylation and to determine its $\delta^{13}C$ value afterwards. To both fractions 10 µL of a solution containing 50 µg/mL RSTD in acetone were added and evaporated to dryness. Then, 50 µL of pyridine and 50 µL of acetic anhydride were added. The mixture was incubated for 45 min at 70 °C and evaporated to dryness under a stream of nitrogen.

For the second HPLC clean up a different gradient was used. From 60/40 acetonitrile/water a linear increase to 100% acetonitrile was accomplished in 33 min and maintained for 5 min.

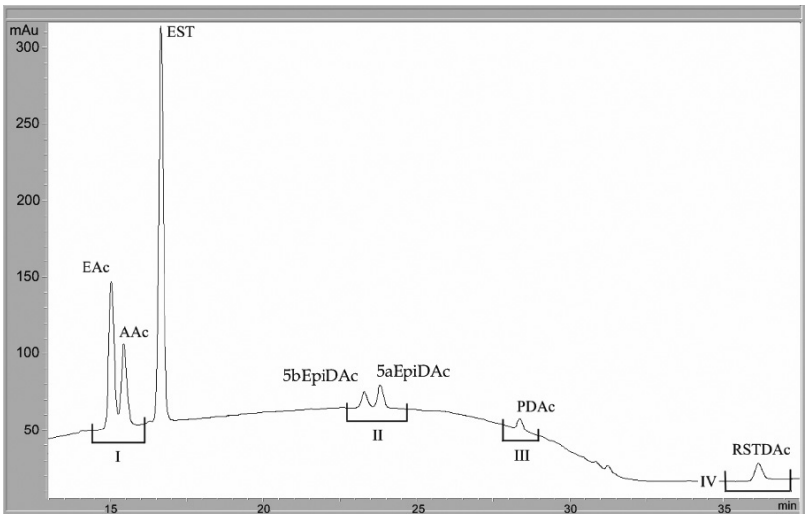

Figure 3. HPLC chromatogram of a standard containing E, A, 5aEpiD, 5bEpiD and RSTD as acetate plus EST (wavelength 192 nm). The fractions were collected as demonstrated.

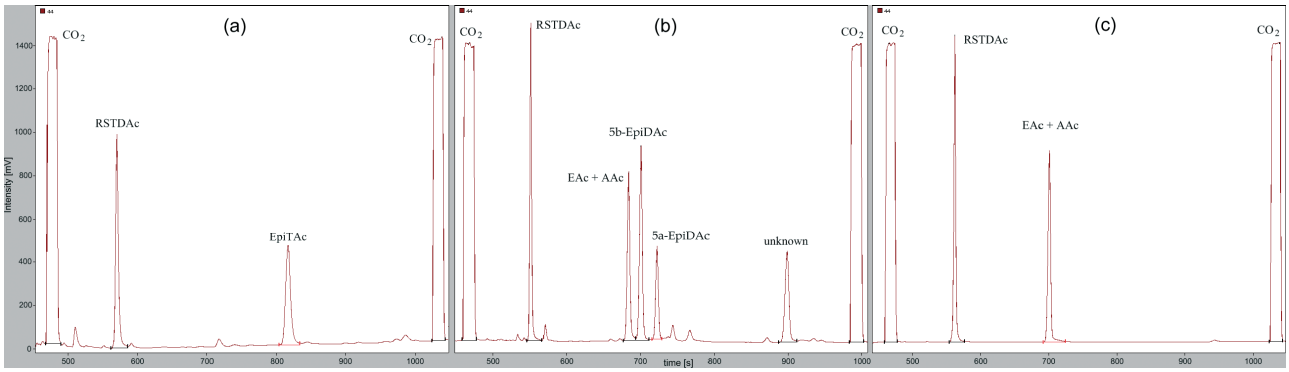

Figure 4. GC/C/IRMS chromatograms of acetylated compounds demonstrating the high performance of sample clean up. a) EpiT, b) 5aEpiD and 5bEpiD and c) E and A. RSTD acts as reference standard, the rectangular peaks are pulses of the calibrated tank gas.

Subsequently, the column was re-equilibrated for 5 min. The flow rate was set to 1 mL/min. In order to optimize peak shape and separation a mixture containing acetonitrile/water (60/40, v/v) was used instead of MeOH as solvent for injection, injection volume was again 50 µL. Fractions were collected as depicted in Figure 3, Fractions 2 and 4 were combined and all fractions were evaporated to dryness under a stream of nitrogen.

The isolation of EpiT has already been described in detail.[21]

GC/MS measurements

In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in all fractions it was necessary to scan all samples on a GC/MS system using chromatographic conditions equivalent to the IRMS setup during method development. Afterwards, only samples containing unknown peaks were measured. 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 & Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). The injections were splitless and were performed 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 was compared to standards.

GC/C/IRMS measurements

All samples were measured on a Hewlett-Packard HP5890 Series II Gas Chromatograph (Böblingen, Germany) coupled to a Delta C gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via the GC Combustion Interface II (ThermoElectron). The GC system was equipped with the same column as mentioned above. A retention gap of 1 m length (0.53 mm i.d., HMDS deactivated) from BGB Analytik (Boeckten, Switzerland) was used. All connections within the GC were prepared by means of deactivated press fit connectors (BGB Analytik). Injection was performed cool-on-column at 50 °C, and the injection unit temperature was maintained 3 °C above that of the GC oven. After keeping the initial temperature for 0.5 min it was increased at 30 °C/min up to 250 °C, then at 2 °C/min to 270 °C, finally at 15 °C/min to 295 °C, which was maintained for 2 min. The carrier gas was purified He (purity grade 5.0) with a constant flow of 2.4 mL/min. The combustion furnace was operated at 940 °C.

Three GC/C/IRMS chromatograms are depicted in Figure 4 demonstrating the absence of any disturbing co-elution, which improved the validity of δ^{13}C determinations.[27] Especially for

samples containing only low ng-amounts of steroid this is of utmost importance.

Due to the partly low amount of TC in the specimens, some measurements fall beneath the linearity range of the IRMS (500–5000 mV). The validity of measurements down to 100 mV could be proven with help of the linear mixing models covering this low range of urinary steroid concentration.

Etiocholanolone and A as acetates co-elute from the OPTIMA δ3 column used and therefore only one $\delta^{13}C$ value representing a summation of both steroids could be obtained.

Small amounts of EA were always detected in the subsequent collection of 5aEpiD and 5bEpiD, probably due to small dead volumes in the fraction collector.

Correction for the acetate moieties

During acetylation carbons with a different $^{13}C/^{12}C$ ratio are incorporated into the steroidal backbone. In order to compare free, mono- and diacetylated steroids, correction of the obtained $\delta^{13}C$ values for the acetate moieties was necessary. The simple mass balance formula (Equation 3) cannot be used because kinetic isotope effects (KIEs) during acetylation are probable.[28] Consequently, a slightly adapted mass balance formula (Equation 4) was used, which takes possibly KIEs into account:[29]

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (3)$$

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_{dcorr} \quad (4)$$

where n = number of moles of carbon, c = compound of interest, d = derivative group, and cd = derivatized compound. As $\delta^{13}C_d$ is not known, $\delta^{13}C_{dcorr}$ is estimated empirically by consecutive measurements of both the native and the derivatized steroid. Subsequently, the $\delta^{13}C$ value for the acetate moiety can be determined and used for urinary steroids.

Blank urine

A pooled urine sample was collected over a period of 48 h (total amount of 2 L) from a healthy male volunteer who declared that he was not using any prohibited substance or any nutritional supplement. The urine was stored at −20 °C until analysis. During the sample preparation for reference values the blank was processed with every batch, resulting in a total of seven independent preparations and measurements providing the data necessary to determine the repeatability of the method over a period of six months. The urine was also used to conduct the experiments concerning the linear mixing models for all steroids.

Linear mixing models

The approach used here to test for the method's validity is essentially a Keeling plot.[30] The approach has already been described in detail.[21,31] The application of a two-pool mixing model results in equation 5:

$$\delta^{13}C_m = (\delta^{13}C_e - \delta^{13}C_a)\frac{c_e}{c_m} + \delta^{13}C_a \quad (5)$$

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

A comparison with the linear equation ($y = a \times x + b$) 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 ($b = \delta^{13}C_a$).

As the sample preparation and the measurements for the two batches were performed on different days it was possible to test for both repeatability and reproducibility. By calculating the least-squares fit it is possible to evaluate standard deviations (SDs) for the described method, if assumed that errors for the ratio of concentrations are negligible.

Statistical analyses

All values obtained from the reference population were tested for Gaussian distribution using the Shapiro Wilk test. No significant discrepancies were detected for the $\delta^{13}C$ values ($a = 0.95$). Hence, it was possible to estimate reference values parametrically. This treatment is in accordance with the International Federation of Clinical Chemistry (IFCC).[32] For urinary concentrations and the corresponding ratios Gaussian distributions were only observed after the elimination of outliers. A non-parametric approach was therefore also calculated.[33,34] The lines of best fit were determined with the Gaussian method of least squares.

Results and Discussion

Method

As every step of sample preparation might cause isotopic fractionation, the validity of a method has to be carefully investigated. Established guidelines how to probe for the validity, for example of a GC/MS method, cannot readily be adapted to IRMS analysis. For instance, the recovery of an analyte is not as important as the fact that the analyte was isolated without isotopic fractionation.

Linear mixing models

The linear mixing models (LMM) approach was chosen to study the method's validity. In Table 1, the determined steroid concentrations for the blank urine are listed together with the added amount of each steroid in order to test the method by means of LMM.

Table 2 shows the results obtained for the lines of best fit. Except for 5aEpiD, all steroids passed the test, yielding good standard deviations (SD) and perfect agreement with the added standards.

Table 1. Endogenous steroid concentrations of the blank urine and added amounts of steroid standards prepared for the linear mixing model

		Amount added				
Steroid	Endogenous [ng/mL]	S1 [ng/mL]	S2 [ng/mL]	S3 [ng/mL]	S4 [ng/mL]	S5 [ng/mL]
PD	120	40	80	120	200	300
5bEpiD	4.5	1	2	4	7	10
5aEpiD	2.8	1	2	3	4	5
EpiT	11	4	8	12	16	20
EA	2387	800	1600	2400	3200	4000

Table 2. Calculated values for the linear mixing models referring to the equation y = a × x + b, where 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 standards are listed, too (all values in $\delta^{13}C_{VPDB}$[‰])

Steroid	a [‰]	SD [‰]	b [‰]	SD [‰]	Std [‰]	SD [‰]
PD	10.0	0.41	−32.1	0.25	−32.4	0.03
5bEpiD	10.7	0.30	−33.1	0.19	−33.2	0.18
5aEpiD	3.9	0.58	−26.6	0.36	−26.7	0.40
EpiT	8.4	0.57	−32.6	0.36	−33.2	0.24
EA	7.8	0.38	−31.4	0.23	−30.9	0.21

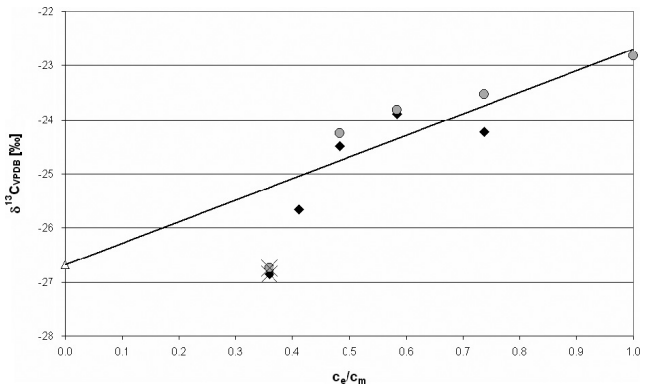

Figure 5. Linear mixing model for 5aEpiD; the determined d13C values were plotted against the ratio of c_e/c_m. The black diamonds represent the first sample preparation; the grey circles the second one and the open triangle the value of the added standard. The solid line represents the best fit after removal of the crossed values.

To achieve acceptable results for 5aEpiD, two measurements had to be removed as depicted in Figure 5 and no valid $\delta^{13}C$ values could be obtained regarding another two measurements. Obviously the removed values are not located on the line of best fit; their $\delta^{13}C$ values were found to be even more depleted than the added standard (−26.7‰). Different sources of errors result in the failure of $^{13}C/^{12}C$ determinations of this steroid. First, 5aEpiD is usually found at urinary concentrations below that of 5bEpiD, as demonstrated in Table 1 for the blank urine. Second, the amount of added steroid for the experiment of LMM is correlated with the starting value. If there is a difference in the endogenous concentrations this will become increasingly pronounced. Third, the 5bEpiD standard used is depleted by more than 6‰in contrast to the 5aEpiD standard (Table 2). This, together with the fact that 5aEpiD elutes from the GC column shortly after 5bEpiD (cf. Figure 4b), probably results in the failure of LMM for 5aEpiD. The increasing concentration ratio of 5bEpiD/5aEpiD overstrained in the end the separation capacity of the GC column so that small amounts of 5bEpiD co-elute with 5aEpiD and strongly influence the measured $\delta^{13}C$ value.

Despite the fact that the $\delta^{13}C$ values should be quite similar in urine samples containing only endogenous steroid, 5aEpiD showed the worst SD in all the experiments and therefore the RL calculated for Δ values of this steroid should be handled with care.

Specificity

The specificity of the method was ensured for all steroids by GC/MS measurements. Due to the comparable conditions to the GC/C/IRMS method, it was possible to exclude substances having the same retention time as the examined steroid and to identify co-elutions where required. All steroids were identified by mass spectral data and retention times of standards. All samples containing co-elutions were investigated in order to ensure peak purity and identity.

Table 3. Repeatability of the blank urine over a time period of six month; nd stands for not determined (all values in $\delta^{13}C_{VPDB}$[‰])

	PD [‰]	5bEpiD [‰]	5aEpiD [‰]	EpiT [‰]	EA [‰]
BW1	−23.3	−24.9	−22.6	−23.6	−23.7
BW2	−22.6	−24.5	−25.2	−23.3	−22.6
BW3	−22.3	−23.2	−24.5	−24.4	−23.3
BW4	−22.1	−22.9	−22.7	nd	−23.7
BW5	−22.7	−24.2	−24.8	−25.0	−24.1
BW6	−22.4	−23.3	−24.5	−24.8	−22.8
BW7	−22.3	−23.5	−23.7	−24.5	−22.9
Mean	**−22.5**	**−23.8**	**−24.0**	**−24.3**	**−23.3**
SD	**0.39**	**0.75**	**1.03**	**0.67**	**0.56**

Repeatability of the blank urine

One blank urine was prepared with every batch of samples belonging to the reference population (n = 7) in order to check for repeatability of the method over a long period of time (approximately six months). The results are listed in Table 3; no systematic drift over time was observed and the SD was good for most analytes (0.4–0.8‰) if taking into account the low urinary concentrations and the obtainable measurement precision of ±0.3‰. Again 5aEpiD showed the largest SD (1.0‰) and it was not possible to distinguish whether the small sample amount or co-elution of 5bEpiD were responsible for this observation.

Reference population

The reference population was investigated for both the urinary concentrations of steroids of interest and the corresponding $^{13}C/^{12}C$ ratios. According to the recommendations of the IFCC, mean values and SD were calculated after testing the investigated population-derived values for Gaussian distribution. For all Δ values RLs were calculated by adding the threefold SD to the mean value, resulting in 99.7% reference intervals for each parameter. Urinary concentrations and corresponding ratios showed non-Gaussian distributions and additional RLs were calculated by using the far outside limits (FO) approach.[33,34]

None of these limits is sufficient to prove the illicit use of EpiT if one value is found elevated above the defined threshold. In parallel to the procedural method for elevated T/EpiT ratios, it is suggested that urinary steroid concentrations be employed for screening purposes and the IRMS determination be used as a confirmation procedure. Thus, the chance for a false positive finding becomes negligible. Another possible improvement will be the collection of steroid profile longitudinal data resulting in individual reference ranges. In addition, population-derived values were investigated for differences between genders and other factors that might influence urinary concentrations or $\delta^{13}C$ values.[20,35,36]

Table 4. Urinary concentrations and calculated ratios for all steroids of interest. For each parameter the mean value, the median and the covered range are listed. RL-PA stands for parametrical calculated reference limit, RL-FO for non-parametrical calculated reference limit

	5bEpiD [ng/mL]	5aEpiD [ng/mL]	EpiT [ng/mL]	5EN17a [ng/mL]	EpiT/5EN17a	5bEpiD/5aEpiD	5bEpiD/EpiT
Mean	24.5	7.4	42.0	153.9	0.29	3.8	1.8
Median	11.4	4.1	23.8	113.8	0.23	3.1	0.5
Range	2.6–176.7	0.7–54.0	1.1–253.0	7.0–770.3	0.03–0.83	0.4–14.3	0.1–29.8
RL-PA	116.6	34.9	184.8	574.7	1.04	11.4	14.0
RL-FO	**153.9**	**41.0**	**319.2**	**1015.5**	**1.79**	**20.7**	**11.3**

Steroidal urinary concentrations

In Table 4 the urinary concentrations of 5bEpiD, 5aEpiD, EpiT and 5EN17a are summarized together with the calculated ratios of EpiT/5EN17a, 5bEpiD/5aEpiD and 5bEpiD/EpiT. As mentioned above, neither the urinary concentrations themselves nor the calculated ratios were Gaussian distributions. This was due to three or four outliers in each distribution. Excluding those outliers would contradict the idea of reference-based thresholds and therefore the values were maintained in the population for the parametrically calculated RLs (RL-PA).

The non-parametric RLs (RL-FO) were established by using twice the far outside value as the limit. Generally, the RL-FOs were found to fall above the RL-PAs (Table 4) and the non-parametric limits should be more robust if the underlying distribution cannot be identified unambiguously. Regarding urinary concentrations, even with the RL-FOs, there were one to three specimens for each steroid near or above the defined thresholds while for the calculated ratios only one subject had an elevated 5bEpiD/EpiT due to a low EpiT concentration. Again, as observed in the literature,[8,11,37] ratios demonstrate to be more robust and therefore we would suggest using the calculated RL-FO of the ratios for detection of illicit EpiT administration. The results obtained for the excretion study (see below) support this suggestion.

The data obtained for EpiT/5EN17a agreed well with data from a population investigated by Dehennin.[8,12] The agreement for 5bEpiD/5aEpiD was found to be worse, which might be explainable by an interchange of both steroids by Dehennin. In contrast to our results, 5aEpiD was found in higher concentrations in urine than 5bEpiD. But in accordance with Shackleton,[10] 5bEpiD shows the higher urinary concentration for the population investigated here. Taking this permutation into account, the results were approximately equal.

$^{13}C/^{12}C$ ratios and Δ values

Both the $\delta^{13}C$ and the Δ values were found to form a Gaussian distribution and the results are listed in Tables 5 and 6. Out of the 370 determined $\delta^{13}C$ values only eight EpiT measurements could not be completed due to a HPLC failure.

Despite the fact that all individuals of the population should reflect a similar dietary status, the absolute $\delta^{13}C$ values covered a range of more than 4‰. As already ascertained for other steroids,[21] The Δ values were more robust and scatter less than the absolute values. Only the difference of PD-5aEpiD showed a large SD. For the reasons mentioned above, this reference limit should not be used for doping-control purposes.

No significant correlations between $\delta^{13}C$ and Δ values or between urinary concentrations and $\delta^{13}C$ values were detected.

The reference limits for PD-5bEpiD and PD-EpiT are above the WADA threshold of 3‰and therefore decisions concerning these steroids should be based on the calculated limits and not on the WADA criterion.

Table 5. $\delta^{13}C$ values of the investigated reference population; listed are the mean value and the respective minimum and maximum (all values in $\delta^{13}C_{VPDB}$[‰])

	PD [‰]	EA [‰]	5bEpiD [‰]	5aEpiD [‰]	EpiT [‰]
Mean	−22.5	−23.4	−24.2	−24.5	−24.0
Min	−23.8	−25.2	−26.3	−27.1	−26.7
Max	−21.0	−21.7	−22.2	−21.8	−22.2

Table 6. δ values of the investigated reference population with PD as ERC – listed are the mean value, the standard deviation and the resulting reference limit (all values in $\delta^{13}C_{VPDB}$[‰])

	PD-EA [‰]	PD-5bEpiD [‰]	PD-5aEpiD [‰]	PD-EpiT [‰]
Mean	1.0	1.7	2.0	1.5
SD	0.50	0.94	1.19	0.84
RL	2.4	4.5	5.6	4.0

Comparison to other reference populations

Regarding EpiT, two other reference population-based $\delta^{13}C$ values have been published.[14,21] Unfortunately, the data presented by Aguilera *et al.* were not complemented by $\delta^{13}C$ values of ERCs. So no comparison of Δ values and reference limits was possible. The $\delta^{13}C$ values for n = 43 males cover a range from −21.8‰ to −25.6‰ and show good agreement with the values obtained in this study (Table 5).

The reference population investigated in 2007 by the authors showed comparable but not equal results. Whereas the ranges of EpiT and PD were equal, both mean values in this study tend to be less depleted (PD_{old}-PD_{new} = −0.4‰; $EpiT_{old}$ − $EpiT_{new}$ = −1.1‰). This results in a lower Δ value and, due to a smaller SD, it also results in a considerably lower reference limit for the new population (old = 5.6; new = 4.0). The lower SD can be attributed to larger urinary EpiT concentrations within this study. The absolute amount of steroid available for IRMS measurement influences the repeatability,[38,39] but this cannot explain the different mean values. The composition of a reference population can influence the values obtained (see next section) but it is unlikely that this was the only reason for the variations found. Moreover, the small variations within the sample preparation seemed to influence the $\delta^{13}C$ values too. This was reflected by the determined values for the blank urine (Table 3) in comparison with the values obtained in 2007 for the same urine.[21] Again, the mean

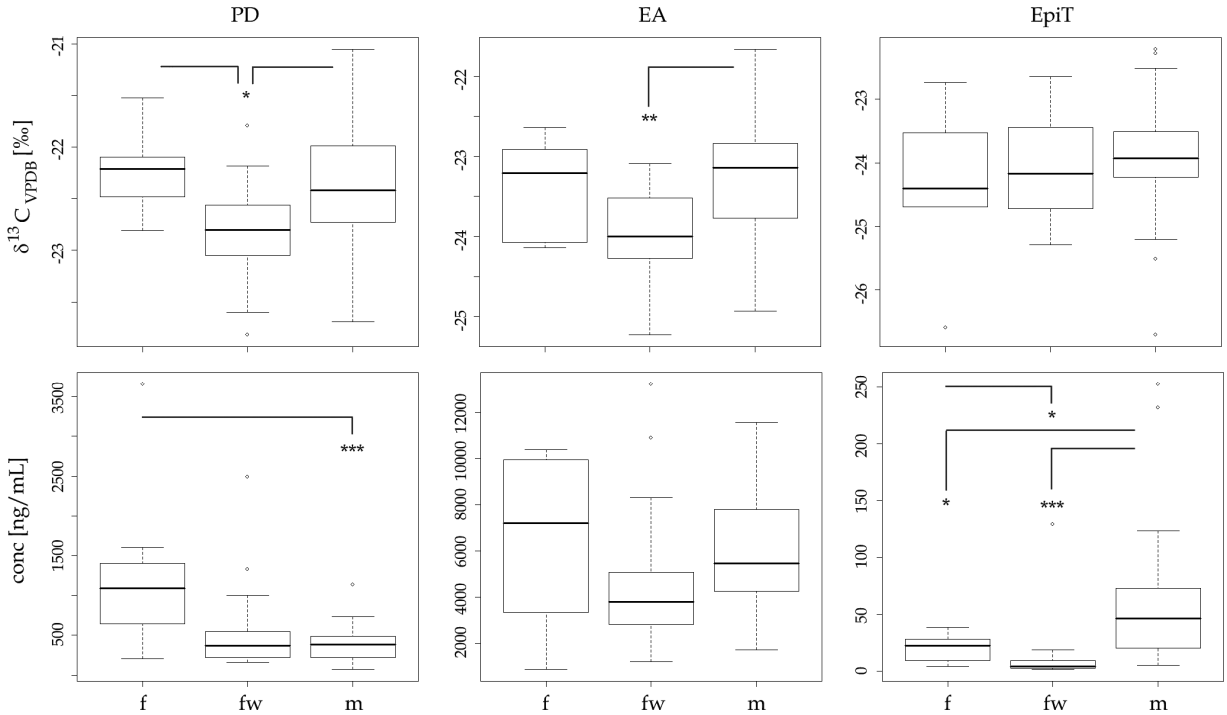

Figure 6. Box plots of urinary concentrations (base part) and $\delta^{13}C$ values (upper part) of PD, EA and EpiT. The reference population encompassed n = 48 males (m), n = 18 females administering oral contraceptives (fw) and n = 8 females without administration of oral contraceptives (f). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ after Bonferroni-correction. All other correlations were not significant.

values tend to be less depleted now (PD_{old}-$PD_{new} = -0.4‰$; $EpiT_{old} - EpiT_{new} = -0.9‰$).

These results demonstrate the necessity of establishing reference limits for each Δ value by means of a reference population for each different method of sample preparation used in the context of doping control.

Differences within the population

The reference population was investigated for factors influencing $\delta^{13}C$ values and urinary concentrations such as gender or the physical activity of the individuals. The eating habits were similar for the whole population and therefore were not investigated further.

In Figure 6 the results obtained for PD, EA and EpiT are depicted. Both PD and EA were significantly depleted (Wilcoxon test) in females using contraceptives but no differences were found for the $\delta^{13}C$ values of EpiT although here a significant difference in urinary concentration was detected. Males excrete more than twice the amounts of EpiT which is in parallel to T (data not shown) and in good agreement with data obtained for a larger population.[37] Despite no significant difference being found, the trend for females using contraceptives to excrete lower amounts of urinary steroids was observed for all steroids, including 5EN17a, 5bEpiD and 5aEpiD (data not shown). As expected, females not using contraceptives excreted the largest amounts of PD.[40]

Interestingly, neither the presumed precursor of EpiT, 5EN17a nor the metabolites 5aEpiD and 5bEpiD showed a similar pattern to EpiT. Here, no significant variations in urinary concentrations were visible while the $\delta^{13}C$ values show more depleted values for females using contraceptives just like PD and EA. In summary, it seems to be the case that EpiT is not the only precursor of 5aEpiD and 5bEpiD and that it is not the only metabolite of 5EN17a; if it were then similar behaviour would have been expected of all steroids.

In contrast to another reference population,[20] the amount of physical activity had no influence on any $\delta^{13}C$ value.

Excretion study

One healthy male volunteer was administered 40 mg of EpiT orally and for the following three days relevant urinary steroids were investigated to ascertain their concentrations and $\delta^{13}C$ values. In order to estimate the individual values at natural abundance two blank urine samples were collected, one 3 h before and one directly before application.

Urinary concentrations and ratios

The results obtained for EpiT concentrations and for the previously investigated ratio of EpiT/5EN17a are depicted in Figure 7. The misuse of EpiT can only be detected using urinary concentrations for less than 24 h despite the fact that the administered amount of 40 mg is quite ample. The ratio of EpiT to 5EN17a could only slightly improve the detection time. The last urine sample with a suspicious ratio was collected after 25 h. This is in perfect agreement with the study conducted by Dehennin.[8]

In contrast to these findings, Figure 8 shows the novel ratios 5bEpiD/5aEpiD and 5bEpiD/EpiT, obviously reacting with a time lag. The ratio between both 'EpiDiols' is not influenced as quickly as the urinary concentration of EpiT or its ratio to 5EN17a. The first elevated value is found 12 h after application. This is in accordance with the assumption that both 'EpiDiols' are metabolites of EpiT. The ratio of 5bEpiD to EpiT does not increase until 20 h after application due to the fact that in

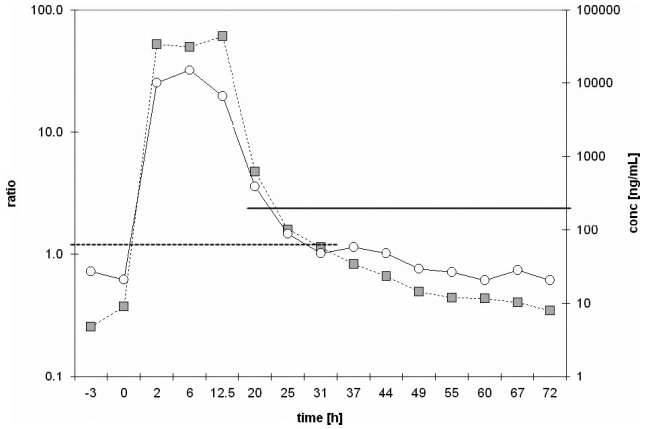

Figure 7. Urinary concentration of EpiT (open circles, solid line) and the ratio of EpiT/5EN17a (grey squares, dashed line) after application of 40 mg EpiT orally at 0 h. The horizontal lines represent the calculated RLs according to the investigated population.

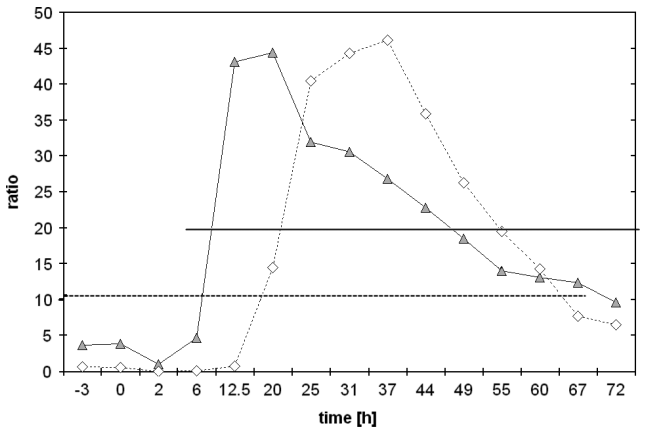

Figure 8. Ratios of 5bEpiD/5aEpiD (grey triangles, solid line) and 5bEpiD/EpiT (open diamonds, dashed line) after application of 40 mg EpiT orally at 0 h. The horizontal lines represent the calculated RLs according to the investigated population.

the first 20 h the excretion of EpiT outweighs the influence on 5bEpiD. After EpiT has returned to its basal value, the influence on the metabolite becomes noticeable, resulting in the strong increase of the calculated ratio. While 5bEpiD/5aEpiD is elevated for more than 44 h, the ratio of 5bEpiD/EpiT is influenced until 60 h after application. This prolongs the detection time more than twofold.

Urinary δ^{13}C values

The determined δ^{13}C values support this finding (Figure 9). All three TCs are strongly influenced directly after application. EpiT was found depleted to a minimum value of −31.8‰, indicating that endogenous EpiT production was not completely suppressed by the 40 mg dosage. If it had been completely suppressed, EpiT should have reflected the value of the administered steroid (−33.2‰). Despite the incomplete suppression of EpiT-production, the δ^{13}C values of EpiT were influenced beyond the threshold for more than 40 h after ingestion and did not reach their starting values within the investigated 72 h period.

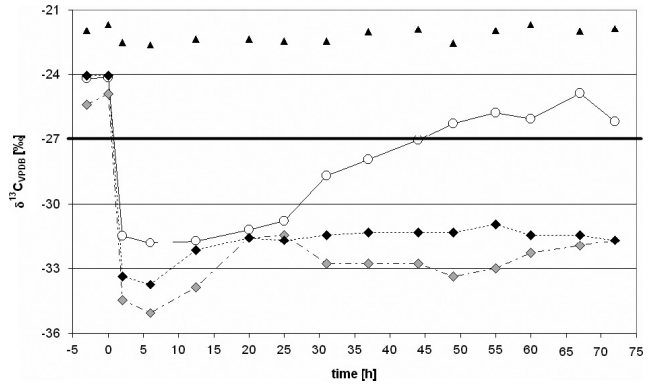

Figure 9. δ^{13}C values of PD (black triangles), EpiT (open circles), 5bEpiD (black diamonds) and 5aEpiD (grey diamonds) after application of 40 mg EpiT orally at 0 h. The solid line at −27‰ represents the average RL for EpiT and 5bEpiD according to the investigated population.

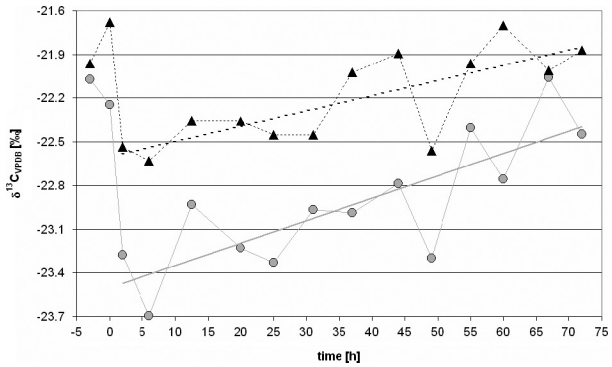

Figure 10. δ^{13}C values of EA (grey circles) and PD (black triangles) after application of 40 mg EpiT orally at 0h. The bold lines exemplify the influence on δ^{13}C values after application.

5bEpiD reflected the δ^{13}C values of the administered EpiT for the first 6h (−33.4‰), then showed a slight decrease to −31.4±0.2‰ and remained at this value over the whole sample period. So even 3 days after ingestion, the misuse of EpiT could be detected by the δ^{13}C value of this steroid. 5aEpiD showed similar results but due to the analytical difficulties with this steroid mentioned above, an interpretation of the data obtained might be ambiguous. Stronger effects on the 5α in contrast to the 5β isomers would be comparable with results obtained after T application.[21] This finding might be of interest for further studies of the metabolism of 3-oxo-4-ene-steroids.

For both, the novel ratios based on urinary concentrations and the δ^{13}C values, the excretion study clearly demonstrated the potential to improve the detection of illicit intake of EpiT.

Effects on other steroids

The values obtained for EA and PD showed an interesting trend after application of EpiT, too. For both urinary concentrations an obvious decrease was observed directly after ingestion (sample 2 h), returning to normal values within the next sample (6 h). The δ^{13}C values belonging to the steroids EA and PD in the samples collected after 2 h and 6 h, were depleted by 0.8‰for PD and 1.1‰for EA, respectively (Figure 10). This seemed to influence the steroid pools inside the body as the

Table 7. Summarized results of n = 34 routine doping samples from the year 2008 – the determined values do not differ significantly related to the investigated reference population and no value above the established RLs was found

	PD-EA [‰]	PD-5bEpiD [‰]	PD-5aEpiD [‰]
Mean	0.7	0.9	1.4
SD	0.63	0.94	1.80
Range	−0.6 to 1.8	−1.5 to 2.7	−2.4 to 4.1

reversion to starting values takes nearly three days in both cases.

The reduction in urinary concentrations can be explained by a feedback regulation on steroid production at the molecular level caused by a high EpiT concentration in circulating blood[41–43] or by a deficiency in renal excretion due to large amounts of EpiT. Only the first assumption can also explain the observed influence on $\delta^{13}C$ values. If the observed depletion was due to an isotopic fractionation during excretion, the values should be influenced for a short time period only. Taking into account possible isotopic fractionation at the production site of steroids, this would result in the observed slowly declining values. For EA it might be possible to explain the influence by the enzymatic properties of 17a-OH-steroid-dehydrogenase,[44,45] showing the ability to convert 17α-OH-steroids to 17-oxo-steroids. But this can definitely be excluded for PD as no enzymatic reaction converting C19-steroids back to C21-steroids is known.

At the moment, as no additional data on this phenomenon are available, it can be concluded that the ingestion of large amounts of steroid has a short, but distinctive, influence on the endogenous steroid production, which may be accompanied by isotopic fractionation. Further studies to investigate the mechanism of this fractionation seem advisable.

Doping control samples

During the year 2008, n = 34 routine doping control samples out of 7000 with elevated ratios of 5bEpiD/EpiT and 5bEpiD/5aEpiD were investigated by means of GC/C/IRMS. None of these samples showed suspicious $\delta^{13}C$ values (summarized in Table 7).

Of the 34 samples, 24 showed an elevated 5bEpiD/EpiT ratio with a maximum of 62.8 and 20 samples were suspicious regarding their 5bEpiD/5aEpiD ratio with a maximum value of approximately 1200 (in this sample almost no 5aEpiD was detectable). Only 15 samples showed an elevation in both ratios. Taking into account that these 34 samples were taken from 7000 investigated samples, the additional workload for IRMS measurement seems to be justified.

Despite the fact that, in the investigated reference population, no differences were found between female and male urinary concentrations for both 'EpiDiols' and the investigated ratios, more than 70% of the suspicious doping control samples were delivered by women. This finding is in contrast to the fact that only 28% of all routine samples were delivered by women. Maybe future investigations on this topic will reveal the factors influencing the ratios of interest and urinary concentrations.

Conclusion

The method presented enables the detection of EpiT misuse over long time periods and therefore complements previous approaches in both GC/C/IRMS and steroid profiling by means of GC/MS. The combination of both tools seems to be the most reliable way to detect a doping offence by reducing the probability of a false-positive finding to a negligible level. As biological parameters always show a large variability, physiologically elevated ratios will sometimes be found. Consecutive investigation of these samples by GC/C/IRMS facilitates investigation into whether natural elevation of ratios or a doping offence causes the suspicious finding.

As soon as more samples have been investigated, the possible influence of gender on the urinary amounts of 17α-OH-steroids might be elucidated. Further investigations of the related metabolism might then be helpful.

For doping-control purposes, an excretion study with simultaneous administration of T and EpiT would be of interest in demonstrating the ability of the new method to detect a doping offence in cases where the T/EpiT ratio is not elevated.

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