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$^{13}C/^{12}C$ Ratios of endogenous urinary steroids investigated for doping control purposes

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In order to detect the misuse of endogenous anabolic steroids such as testosterone by athletes a total of n = 1734 suspicious urine samples were investigated by gas chromatography/combustion/isotope ratio mass spectrometry throughout the years 2005, 2006 and 2007. The $^{13}C/^{12}C$ ratio of a target substance (androsterone, a testosterone metabolite) was compared to the $^{13}C/^{12}C$ ratio of an endogenous reference compound (11β-hydroxyandrosterone).

N = 1340 samples were investigated due to elevated testosterone/epitestosterone ratios, with n = 87 (6.5%) exceptional findings regarding their isotopic ratios. An additional n = 164 samples were investigated because of elevated dehydroepiandrosterone concentrations, with n = 2 (1.2%) exceptional findings. The remainder were subjected to isotope ratio analysis because of elevated androsterone levels or because this was requested by sports federations.

Significant differences between female and male samples were found for the $^{13}C/^{12}C$ ratios of androsterone and 11β-hydroxyandrosterone but not for samples taken in or out of competition.

A further n = 645 samples originating from other World Anti-Doping Agency accredited laboratories, mainly throughout Europe as well as South America, South Africa and Southeast Asia, were investigated. The $^{13}C/^{12}C$ ratios of the urinary steroids differ significantly for each geographical region, reflecting the dietary status of the individuals.

The system stability over time has been tested by repeated injections of a standard solution and repeated processing of frozen stored blank urine. Despite a drift over time in absolute $^{13}C/^{12}C$ ratios, no significant change in the difference of $^{13}C/^{12}C$ (11β-hydroxyandrosterone) minus $^{13}C/^{12}C$ (androsterone) could be observed. **Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: isotope ratio mass spectrometry (IRMS); anabolic steroids; carbon isotopes; doping control

Introduction

The ratio between testosterone and epitestosterone (T/EpiT) – both excreted as glucuronides – is the main parameter for detecting the misuse of testosterone or testosterone prohormones by athletes.[1] This ratio varies naturally in a range between 0.1 to 3[2,3] and, in case of values $\geq$4, an atypical analytical finding is reported according to the rules of the World Anti-Doping Agency (WADA).[4] As there are numerous athletes with a naturally elevated T/EpiT ratio,[2,3] this criterion alone is not sufficient to prove a doping violation. In order to identify the naturally elevated T/EpiT ratios, gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) is the method of choice to verify the natural elevation or to provide evidence for a doping offence.[5–12]

$^{13}C/^{12}C$ ratios are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on the equation

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

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

As pharmaceutically produced anabolic steroids are not synthesized *de novo* but are derived from plant material,[14] they usually exhibit depleted $\delta^{13}C$ values in comparison to the values found for endogenously produced steroids.[15] After application of an exogenous anabolic steroid the $\delta^{13}C$ values of the steroid itself or of its metabolites exhibit depleted $\delta^{13}C$ values while the endogenous reference compounds (ERCs) are not affected. ERCs are urinary steroids derived from metabolic pathways independent from anabolic steroids like the corticoid metabolite 11β-hydroxyandrosterone (OHA).

The WADA has established a minimum threshold of 3‰ difference between an ERC and a target compound (TC).[16] These differences are expressed as Δ values based on the equation:

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

In addition to the T/EpiT ratio, the WADA has established several concentration thresholds for urinary steroid glucuronides. If dehydroepiandrosterone (DHEA) is present at more than 100 ng/mL, T or EpiT at more than 200 ng/mL or androsterone (A) or etiocholanolone (E) at more than 10 000 ng/mL, GC/C/IRMS measurements are recommended. All thresholds are effective for density-corrected urinary concentrations.

From a total of 1734 urine specimens investigated by IRMS in the time period from 2005 to 2007, 1340 samples (77.3%) were forwarded for isotope ratio determination because of elevated T/EpiT ratios, n = 164 samples (9.4%) were investigated because of elevated DHEA concentration and the remainder of

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n = 231 samples (13.3%) were under investigation because of elevated A concentrations or because GC/C/IRMS was requested by federations or doping authorities.

With the results obtained from these samples the influence of gender, of in or out of competition sampling, and the influence of the different sports on the $\delta^{13}C$ values, were studied.

The human diet is a varying mixture composed of C_3- and C_4-plants, where C_4-plants exhibit a more enriched ^{13}C signature. Due to different compositions of both plants in the diet, different carbon isotope ratios are reflected in different individuals and in particular by their urinary steroids.[17] As the eating habits in different regions are quite similar but distinguishable from each other, this should be reflected by the $\delta^{13}C$ values of urinary steroids. With the 645 samples sent to the Cologne laboratory originating from all over the world throughout the last three years this hypothesis was tested.

In order to demonstrate the stability of the IRMS method over time, repeated processing of blank urine was accomplished. The GC/C/IRMS system itself was tested by repeated determinations of a standard mixture.

With the large number of samples investigated it was possible, on the one hand, to show the power and reliability of GC/C/IRMS determination in the context of detecting steroid abuse by athletes and, on the other hand, to test which factors influence the $\delta^{13}C$ values of athletes. This will be helpful for the evaluation of future doping control samples.

Experimental

Sample preparation

All samples were processed according to our routine sample-preparation procedure; a detailed description is given by Flenker, Güntner and Schänzer.[12] In brief, 2 to 20 mL of urine were applied on a conditioned solid-phase extraction cartridge (500 mg, Chromabond™ C18, Macherey & Nagel, Düren, Germany), washed with distilled water and eluted with 2 mL methanol. The eluate was evaporated to dryness, reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and extracted with 5 mL of *tert*-butyl methyl ether (TBME) from VWR (Darmstadt, Germany). The organic layer was discarded and 100 µL β-glucuronidase from *E. coli* (Roche Diagnostics GmbH, Mannheim, Germany) was added. After 60 min of incubation at 50 °C 500 µL carbonate buffer (K_2CO_3/$KHCO_3$ 1:1, w/w, 200 g/L) were added and the deconjugated steroids were extracted with 5 mL of TBME. Further sample clean-up was performed on a semi-preparative HPLC (Agilent 1100 HPLC system, Waldbronn, Germany) with a Merck analytical column (LiChrospher® 100 RP^{18}, 250 × 4 mm i.d., 5 µm particle size). Afterwards OHA, A and E were sufficiently cleaned up for GC/C/IRMS determination.

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 a Macherey & Nagel OPTIMA δ 3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). 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 with 30 °C/min up to 250 °C, then with 2 °C/min to 270 °C, finally with 15 °C/min to 295 °C and maintained for 2 min. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.2 mL/min. The combustion furnace was operated at 940 °C and oxidized once every week for 15 min. Additionally, a short oxygen pulse of 2 s was admitted into the furnace prior to each measurement to keep the reactor on an appropriate level of oxygen saturation, which resulted in improved repeatability.[18] This oxidation protocol was started in 2007. Before this the reactor was oxidized once or twice a week, depending on the amount of measured samples.

In order to achieve similar peak heights, all samples were reconstituted in the necessary volume of a solution containing 5α-androstan-3β-ol (RSTD) in acetone (100 ng/µL). The amount of analyte was adjusted to 100 to 150 ng on column absolute.

Blank urine

A pooled urine sample was collected over a period of 5 days (total amount of 10 L) from two healthy male volunteers who declared that they had not used any prohibited substance or any nutritional supplements. The urine was bottled in 1 L containers and stored at −20 °C until analysis. The blank urine (BW) was processed with every batch of samples resulting in a total of n = 159 independent preparations and measurements in the time period of 2006 and 2007; providing the data necessary to determine the repeatability of the method.

In 2005 a different standard mixture and a different blank urine from another individual with different $\delta^{13}C$ values were used and therefore not included in the consideration of system stability over time.

Standard mixture

In order to test the readiness of the GC/C/IRMS system prior each batch of samples a standard mixture (S05) containing RSTD, E, A and OHA at 100 ng/mL in acetone was injected. It was therefore possible to fix retention times of the analytes of interest, to estimate the GC performance (peak tailing and peak separation) and to check the conditions for sample combustion. The RSTD was a sensitive marker for incomplete combustion, resulting in strongly depleted $\delta^{13}C$ values. Overall n = 191 standard determinations were performed throughout 2006 and 2007.

Steroid profiling

Approximately 35 800 urine samples were screened for their urinary steroid profile in the Cologne doping control laboratory in the years 2005 to 2007. Between 300 and 350 specimens annually exhibited a suspicious or abnormal steroid profile and were forwarded to IRMS measurement. A detailed description of sample preparation can be found in the literature.[19,20] A great advantage for the IRMS determination was the knowledge of the exact concentration of each steroid of interest which allowed the selection of necessary urine volumes to provide purified steroids in the appropriate amount of solvent prior to each measurement for comparable peak heights between the different analytes to improve the IRMS accuracy.

Statistical analysis

Possible presence of steroid abuse within the negative samples (Δ values <3‰) was investigated. The distribution of the Δ values (OHA-A) of this subpopulation was compared to a reference population.[21] The distributions were compared visually by a QQ plot. In addition, the Kolmogorov–Smirnov test was performed, where the distribution of the test samples was compared to a Gaussian with mean and standard deviation of the reference distribution.

The possible effects of sex and type of control (in-competition, IC; out-of-competition, OOC) on the $\delta^{13}C$ values of the different compounds were investigated by the general linear model. Like 'sex' and 'control', the identity of the compound was considered a predictor variable. Analysis started from the saturated model, including all possible main and interaction effects. Insignificant terms were eliminated stepwise and the respective models were compared by analysis of variance.

The possible effect of different sports disciplines on the Δ values was investigated by a single factor ANOVA. Only disciplines with sample sizes of at least 20 were considered. Females were excluded due to the grossly differing numbers. As before, positive cases were ignored. Tukey's honest significant differences were calculated *post hoc* at a confidence level of 0.95.

Significant differences in geographical region or stability over time was tested by application of generalized linear models.

Results and Discussion

Testosterone/epitestosterone ratio

Figure 1 shows a scatter plot of all samples with known T/EpiT ratios. Included are n = 1340 samples investigated because of elevated T/EpiT and n = 79 samples with a lower T/EpiT than 4, investigated because of the above-mentioned thresholds. Figure 2 presents a closer view of the samples with a T/EpiT up to 10. The majority of samples (93.5%) came out to be unsuspicious. According to the data, T/EpiT ratios up to 27 can occur naturally and values up to 15 are found quite regularly. This is in good agreement with the known fact that the T/EpiT distribution is positive skewed and that therefore values larger than 4 can occur naturally.[22] Usually low EpiT concentrations are responsible for these elevated ratios.

All T/EpiT > 27 were due to the intake of anabolic steroids. One sample with a T/EpiT of 76 was below the 3‰ threshold because of depleted endogenous values (see below), but a doping offence could be proven by analysing different target compounds.[21] Only four samples (0.3%), with a T/EpiT ranging from 4 to 6, exhibit values beyond the WADA IRMS threshold; the two samples with T/EpiT 1.7 and 1.8 respectively were both forwarded for IRMS determination because of A concentrations above the threshold of 10 000 ng/mL and were supposed to be due to the intake of testosterone prohormones.

A careful examination of the data presented in Fig. 2 gives the impression that there are a couple of samples lying above the conglomeration of the negative samples but beneath the 3‰ line. This raised the question of whether there are false negative samples in the distribution – samples that are not part of the normal negative distribution but not above the defined threshold. To test for this, the data of the T/EpiT samples were compared with the data obtained for the DHEA samples and with the results of a reference population later on.

Dehydroepiandrosterone

From n = 164 samples investigated because of elevated DHEA concentrations (>100 ng/mL), only two showed suspicious Δ values belonging to the intake of DHEA. The remainder did not differ significantly in Δ values for OHA-A from the independent reference population determined with the described IRMS method.[21] Further investigations on the topic of normal distribution of DHEA concentrations in urine showed that this distribution is positive skewed and therefore all samples determined with IRMS fit perfectly in this distribution and indeed should not be considered as suspicious.[23] Hence, after testing for different gender distribution between the DHEA and the T/EpiT samples (p = 0.8; no difference could be found) the DHEA samples were used as a reference population with the benefit that these samples and the T/EpiT samples were measured over the same period of time under the same conditions while the mentioned reference population only covers a short time period of four months.

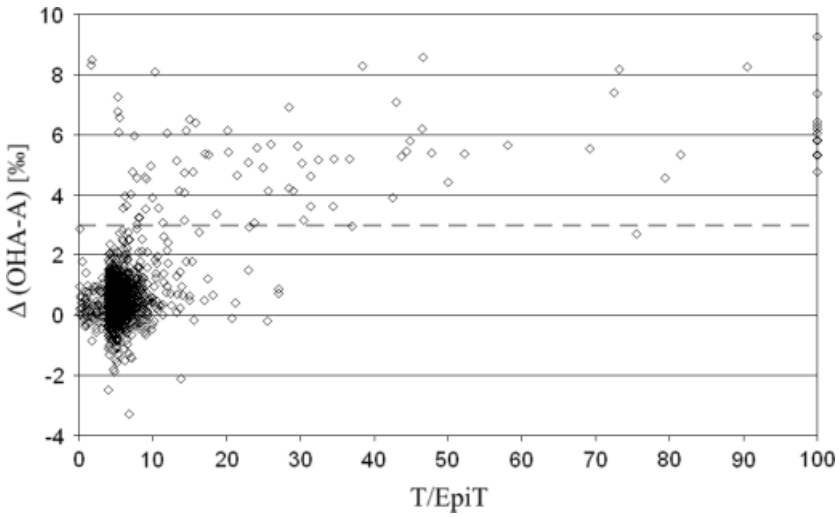

Figure 1. Scatter plot of 1419 samples with known T/EpiT ratios against the determined Δ value of (OHA-A). The samples with a higher T/EpiT than 100 were arbitrarily set to this value The maximum T/EpiT was determined as 419. The dashed line represents the WADA threshold of 3‰.

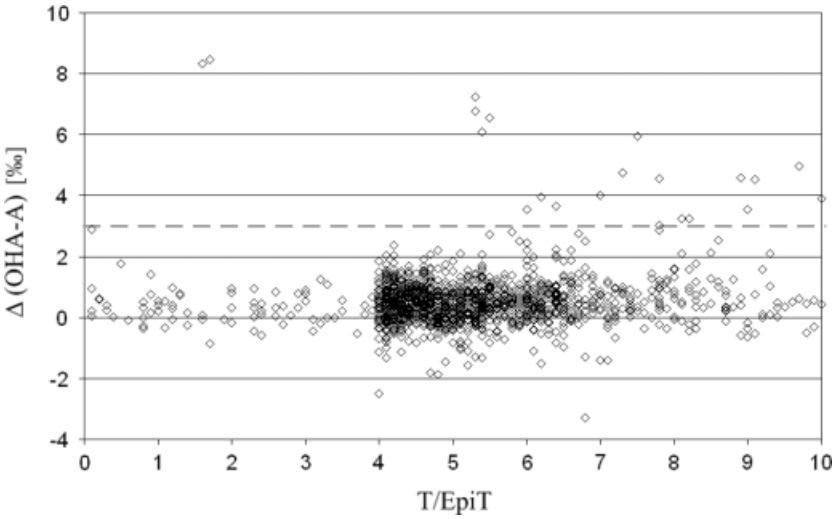

Figure 2. Scatter plot of samples with a T/EpiT up to 10 against the determined value of Δ (OHA – A).

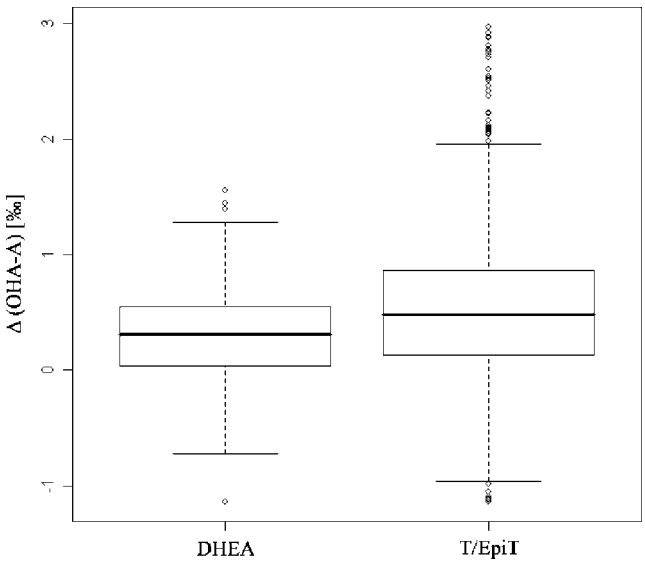

Figure 3. Boxplot of the Δ values of both the DHEA and the T/EpiT groups. Samples above threshold of 3‰ were excluded; samples below −1.2‰ were not depicted.

Comparison of T/EpiT and DHEA Samples

In order to investigate the difference between the DHEA and the T/EpiT samples, positive samples, i.e. samples above the 3‰ threshold for Δ (OHA-A), were removed from both groups. The T/EpiT group results in a mean of 0.49 ± 0.68‰ for $n = 1332$ and the DHEA group results in 0.33 ± 0.41‰ for $n = 162$ samples. The difference between the mean values was significant ($p < 0.001$) and, as can be seen in Fig. 3, there are numerous samples in the T/EpiT population showing Δ values between 2 and 3‰. In order to ensure that these differences are due to false negative samples, the T/EpiT group was further tested against a reference population of $n = 61$ subjects.[21]

Figure 4 shows the comparison of the Δ values of the negative test samples with the Δ values of the reference population. Large Δ values are much more abundant among the T/EpiT samples than would be expected from the reference population. The difference

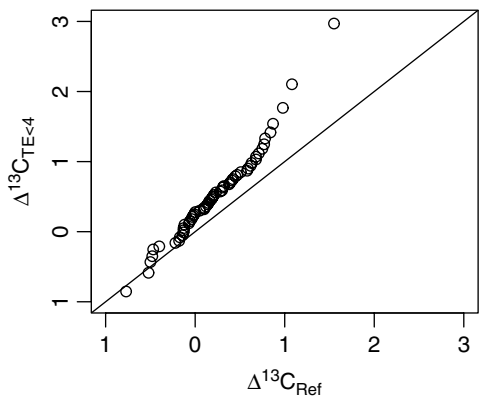

Figure 4. QQ-plot of a reference population (Ref) and the T/EpiT samples (T/E < 4).

between the cumulative distributions rapidly increases towards higher quantiles. The corresponding one-sided Kolmogorov-Smirnov test is highly significant ($D = 0.217$, $p < 0.001$). These results clearly suggest that steroid application frequently goes undetected by stable isotope analysis.

For the further statistical investigations on the IRMS results samples above Δ (OHA-A) = 2‰ were excluded in order to ensure the comparability of the data and to minimize bias evoked by Δ values that did not occur naturally. A threshold of 2‰ represents the combination of the 99.9% reference limit found for the reference population and a 90% confidence interval.

Comparison of sex and type of control

In order to investigate possible differences between both genders, their Δ (OHA-A), δ^{13}C A and δ^{13}C OHA mean values were compared. Included were 154 female and 769 male samples from the T/EpiT group. Samples with unknown gender were excluded. The results are listed in Table 1. There was a significant difference for both δ^{13}C values of A and OHA together with the associated Δ value in the T/EpiT group ($p < 0.001$). This is in good agreement with the results of a reference population consisting of $n = 55$ subjects investigated by Flenker, Güntner and Schänzer in 2003.[12] A difference in the absolute δ^{13}C values of A and OHA might be

Table 1. Comparison of female and male samples of both groups; samples with a Δ (OHA – A) value above 2‰ were excluded – the mean values of each group are listed

	T/EpiT		DHEA	
	Female	*Male*	*Female*	*Male*
A (‰)	−22.7	−22.0	−22.0	−21.8
OHA (‰)	−22.5	−21.5	−21.7	−21.5
Δ(OHA – A) (‰)	0.18	0.49	0.23	0.34

Table 2. Comparison of in- and out-of-competition samples of both groups; samples with a Δ (OHA – A) value above 2‰ were excluded – the mean values of each group are listed

	T/EpiT		DHEA	
	IC	*OOC*	*IC*	*OOC*
A (‰)	−22.1	−22.2	−21.9	−22.0
OHA (‰)	−21.7	−21.8	−21.5	−21.6
Δ(OHA-A) (‰)	0.41	0.47	0.33	0.33

explained by different diets of males and females (see below) but this cannot elucidate the difference in the Δ value of both steroids. If looking at the DHEA group with n = 23 women and n = 131 men the same trend is visible. The difference in the $\delta^{13}C$ values and the Δ value is not significant but shifted in parallel to the T/EpiT group. The fact that these differences are present in three different groups investigated independently can be taken as proof for a basic dissimilarity between the genders. More likely than a different diet might be a difference in isotopic fractionation factors for enzymatic reactions during the biosyntheses of steroids *de novo*. This would result in slightly unequal $\delta^{13}C$ values. The assumption is supported by the fact that both genders exhibit – in part – quite different pathways for steroid synthesis,[24] from which discrete isotopic discrimination is likely.

The results for the comparison of type of control are listed in Table 2. Again, if the type of sampling was unknown, samples were excluded. In the T/EpiT group were n = 581 in-competition samples and n = 364 out-of-competition samples, in the DHEA group were n = 147 IC and n = 15 OC samples included. No significant difference was found ($p > 0.05$). In order to test for confounded variables in between the groups, a general linear model approach was applied and the relationships of sex, different compounds and $\delta^{13}C$ values were investigated. All other factors were insignificant and successively had been removed from the model. In particular, the type of control did not contribute significantly to the variability of the $\delta^{13}C$ values. Males exhibit significantly ($p < 0.001$) higher $\delta^{13}C$ values of A than females. In males, but not in females, OHA is significantly ($p < 0.001$) enriched in ^{13}C *versus* A, which explains the difference in Δ values between the genders.

Comparison of sports

Differences in Δ (OHA-A) values of sports with n > 20 samples were investigated; female samples were excluded because of the reason mentioned above. The ten sports fulfilling this criterion are listed in Table 3. The largest group, 'soccer' with more than 200 samples, exhibits the lowest Δ value and the group 'powerlifter' with n = 59 samples is afflicted with the largest Δ value. The

Table 3. Summary of the Δ (OHA – A) values for different sports

Sport	n	OHA-A [‰]	SD [‰]
Soccer	211	0.36	0.47
Cycling	78	0.41	0.55
Weightlifting	62	0.52	0.65
Powerlifting	59	0.74	0.69
Athletics	52	0.33	0.63
Canoe	24	0.42	0.51
Ice skating	24	0.50	0.49
Skiing	24	0.47	0.51
Rowing	23	0.34	0.62
Basketball	20	0.41	0.64

n = number of male samples in each sport and SD for the standard deviation.

sports discipline has significant influence on the Δ values. This was tested for using ANOVA. All differences that exceed the critical value are represented by the powerlifting group. This suggests that testosterone abuse is much more abundant in this group than in any other of the disciplines considered. It is notable that nearly 1/3 (5 out of 17) of the samples with a Δ value between 2‰ and 3‰, which have been excluded as mentioned above, belong to powerlifters.

Comparison of geographical regions

Not all of the WADA-accredited doping laboratories operate a GC/C/IRMS system. Hence it is usual procedure for those laboratories to forward suspect samples. The Cologne laboratory received n = 645 samples in this way throughout the years 2005 to 2007.

The $\delta^{13}C$ values of the endogenous steroids should reflect the corresponding values of the individual diet; and the diet is, in the end, a composition of C_3-, C_4- and CAM (crassulacean acid metabolism) plants. These plants exhibit different $\delta^{13}C$ values because of different biosynthetic pathways, namely in CO_2 fixation.[25] According to the percentage of each plant type in diet results a specific carbon isotope ratio of the body and consequently of the cholesterol as the main precursor of all steroids. During the biosynthesis of steroids, small but measurable fractionation is taking place which results in the significant difference of OHA and A. For other urinary steroids these small distinctive differences are known, too.[11,21,26] As this difference is part of human steroid biosynthesis it should be found comparable for all individuals regardless of their diet and, hence, the absolute $\delta^{13}C$ values. This hypothesis was tested for with the doping control samples from seven different geographical regions. The group 'South America' encompasses samples from Bolivia, Cuba and Brazil (n = 12), Bloemfontein ('South Africa') sent n = 38 samples and 'Southeast Asia' combines n = 14 samples from Malaysia and Korea. Europe was subdivided into 'South' (Romania, Italy, Austria and Switzerland with n = 181 samples), 'Middle' (Poland, Czech Republic and Belgium with n = 174) and 'North' (Sweden and Norwegian with n = 180 samples). As the n = 47 samples from Finland differ significantly, they were retained as a group on their own. For the other groups it should be stated that the different countries did not differ significantly from each other and therefore were classified as one group, of course taking into account the geographical distribution too.

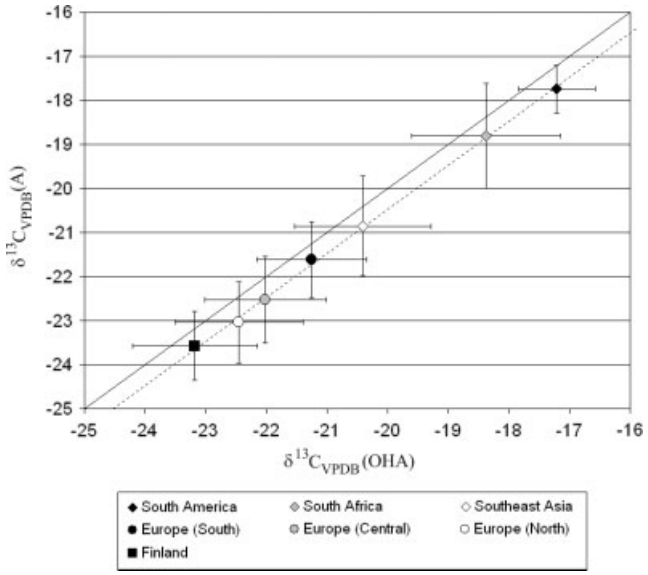

Figure 5. $\delta^{13}C$ mean values of OHA plotted against A for each geographic region. The error bars represent one standard deviation. The solid line represents the one-to-one correlation of OHA and A, the dashed line represents the mean Δ value of 0.47‰.

Table 4. Comparison of geographical regions

	OHA (‰)	A (‰)	OHA – A (‰)
South America	−17.21 **	−17.75 *	0.54
South Africa	−18.37 ***	−18.81 ***	0.44
Southeast Asia	−20.41 **	−20.85 **	0.44
Europe (South)	−21.26 ***	−21.62 ***	0.36
Europe (Middle)	−22.02 ***	−22.53 ***	0.51
Europe (North)	−22.45 **	−23.04 ***	0.59
Finland	−23.18	−23.57	0.39

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – the level of significance corresponds with both groups bracketing the shown significance.

The result of this classification is depicted in Fig. 5. The absolute $\delta^{13}C$ mean values for both OHA and A cover a range from −17‰ to −23‰. The most depleted values for OHA and A are −24.7‰ and −25.0‰, respectively, the most enriched values represent −16.5‰ and −16.9‰, respectively. This range of more than 8‰ must be considered as the naturally possible distribution of the $\delta^{13}C$ values of these two steroids. Despite this large range, the Δ values of OHA – A were found to be stable throughout all geographical regions as can be seen in Table 4. Every group was tested for statistically significant differences against the following group; the level of significance is shown, too. This implies that the proximate group – for example Europe (South) compared to Europe (North) – differs significantly, too. The different quantity of false negative samples in the different geographical groups is assumed to be responsible for the small variations in the Δ value together with probable different gender distributions.

Overall, $\delta^{13}C$ values are strongly influenced by the provenance of the athletes, most likely because of the varying composition of diet. Otherwise the Δ values are comparable, do not correlated with the absolute $^{13}C/^{12}C$ ratio of the diet or, hence, the steroidal precursor cholesterol. This finding supports the assumption of enzymatic induced fractionation during biosynthesis, independent from the isotopic composition of the precursor.

System and method stability over time

The considerations concerning method and system stability revert to the data of 2006 and 2007. As mentioned above, in 2005 a different standard mixture and a blank urine from a different person were used and are therefore not comparable as the $\delta^{13}C$ values were unequal.

RSTD

The RSTD values obtained by measuring the standard mixture and the values obtained from the blank urine measurements were combined to investigate the drift over time. In total the values shifted from −32.7 to −33.7‰. During this shift two gradations were visible (Table 5). The first discontinuity emerged in the context of changing the CO_2 tank gas. Afterwards this new tank gas was calibrated several times against the Indiana Mixture 'A'[27] and with this new calibration the RSTD exhibited a $\delta^{13}C$ value of −33.25‰. This value remained quite stable until March 2007. At this point in time the oxidation protocol for reoxidizing the reactor was changed as mentioned above. Again the Indiana Mixture was determined several times and the virtual value of the tank gas did not change. Despite this, the $\delta^{13}C$ value of the RSTD changed to −33.7‰, showing the sensitivity of this steroid towards the combustion process. As shown in Table 5, the standard deviation decreases slightly for this oxidation procedure.

Blank urine

In comparison with the highly sensitive RSTD, the other steroids determined from the blank urine exhibit a parallel shift over time but without showing definable gradations (Fig. 6) as here the randomly occurring errors may contribute to a higher degree. Consecutive numbers were chosen instead of date as the measurements were carried out very regularly and the proper allocation was not possible for each determination. Overall the shift results in −0.9‰ for the two years. The Δ (OHA-A) value remains constant for the whole time period at 0.6 ± 0.5‰ covering a range from −0.84 to 1.51‰. This value does not change significantly when correcting all values for the long term drift by a simple linear model (Table 6). In both cases – corrected and uncorrected – the Δ values show a normal distribution (Shapiro Wilk test, $W = 0.9952$ and 0.9946

Table 5. Comparison of the different gradations of RSTD

Time intervals	01/06 to 07/06	07/06 to 03/07	03/07 to 12/07
Mean	−32.7	−33.3	−33.7
SD	0.42	0.46	0.39

All mean values differ highly significant from each other.

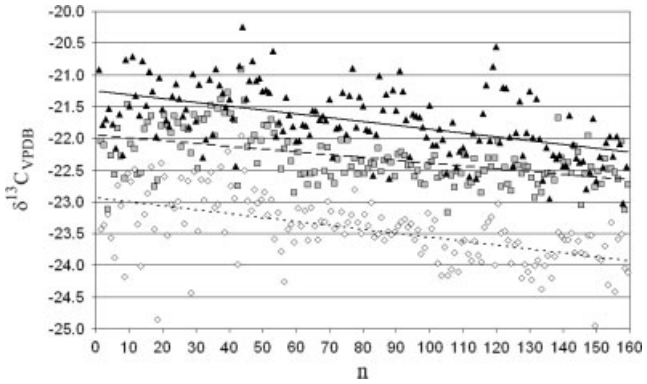

Figure 6. $\delta^{13}C$ values of E (open diamonds), A (grey squares) and OHA (black triangles) of the blank urine prepared in 2006 and 2007. The corresponding lines represent the Gaussian least-square fitted linear slope equations, see Table 6.

Table 6. Comparison of the different mean values uncorrected and corrected for the long-term drift

	E (‰)	A (‰)	OHA (‰)	OHA-A (‰)
BW	−23.4	−22.3	−21.7	0.6
SD	0.51	0.41	0.53	0.51
BW (corr.)	−22.9	−21.9	−21.3	0.7
SD	0.42	0.35	0.46	0.50

The corrections were carried out with linear equations $y = ax + b$. The fitted parameters were: E (−0.006; −22.9), A (−0.005; −21.9) and OHA (−0.006; −21.3).

and $p = 0.89$ and 0.82, respectively) and so the scattering can be related to randomly occurring measuring errors. Interestingly, the standard deviation of the Δ value with ±0.51‰ is smaller than the expected one calculated according to Gaussian error propagation for the values of A and OHA which results in ±0.67‰. This, in addition to the stability over time, shows the usefulness and robustness of Δ values for doping control purposes.

The SD for the corrected δ values decreases for all steroids (±0.35‰ to ±0.46‰), which is excellent for GC/C/IRMS determinations.

Conclusion

The IRMS data of more than 1700 urine samples investigated in the context of doping control was presented and interpreted considering different aspects relevant to doping and the geographical origin of the samples.

The comparison of samples investigated because of elevated DHEA concentrations and elevated T/EpiT ratios showed the likeliness of false negative samples within the latter group. This finding was substantiated by the comparison of the T/EpiT group to a reference population.

A distinctive difference between female and male steroids in their $^{13}C/^{12}C$ ratio could be shown. No such difference was found for IC and OOC sampling and for most sports. Only the 'powerlifting' group exhibit Δ values with significant variations. For all these findings has to be taken into account that the investigated population might not be representative as demonstrated by the comparison of the T/EpiT group and the DHEA group.

The hypothesis of significant different $\delta^{13}C$ values for geographical regions could be verified and no difference for the Δ (OHA-A) value was found despite the $\delta^{13}C$ values range of more than 7‰. This proves the reliability of Δ values in the detection of steroid misuse and demonstrates the unlikelihood of false positive testing.

The same holds true for method stability. Despite a drift over time – namely of the GC/C/IRMS instrument – the Δ (OHA-A) values of the determined blank urine remain stable without being correlated to the virtual depletion of the $\delta^{13}C$ values.

The data prove the repeatability, reliability and validity of GC/C/IRMS determinations for detecting the misuse of endogenously occurring anabolic steroids in sport.

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