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Determination of D/H ratios of endogenous urinary steroids: Method validation and first results

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Abstract

The development and application of a combined gas chromatography/thermal combustion/isotope ratio mass spectrometry (GC/TC/IRMS) method for D/H ratio determination of endogenous urinary steroids is presented. The key element in sample preparation is the consecutive clean up with high performance liquid chromatography (HPLC) of foremost free and then acetylated steroids. So it is possible to achieve sufficient clean up of all analytes and to determine their D/H ratio. Ten steroids (11β-hydroxy-androsterone **OHA**, 5α-androst-16-en-3α-ol **16EN**, pregnanediol **PD**, androsterone **A**, etiocholanolone **E**, testosterone **T**, epitestosterone **EpiT**, 5α-androstan-3α,17β-diol **5a**, 5β-androstan-3α,17β-diol **5b** and dehydroepiandrosterone **DHEA**) are measured from a single urine specimen.

Depending on the biological background the determination limit for all steroids ranges from 10 to 15 ng/mL for a 20 mL specimen. The method is validated by application of linear mixing models on each steroid which covers repeatability and reproducibility. The specificity for each steroid was ensured by gas chromatography/mass spectrometry (GC/MS) determinations. Within sample preparation no isotopic fractionation was observed and no amount-depending shift for the D/H ratios during measurement was noticed. Possible memory effects occurring during IRMS measurements were corrected for by applying a simple rule of proportion.

In order to determine the naturally occurring D/H ratios of all the implemented steroids a reference population of n = 18 subjects was established. By means of this it is possible to calculate preliminary reference limits for relevant Δ values among the steroids and to test the method for its possible application for doping control purposes.

Introduction

$^{13}C/^{12}C$ ratio analysis of androgenic steroids, their corresponding pro-hormones and metabolites in urine is a routine method in doping control. By application of this method it is often possible to clearly recognise the primary carbon source of these compounds and thereby to detect the abuse of synthetic androgenic steroids. The criteria are based on the comparison of $^{13}C/^{12}C$ in the target analytes (TC) and of $^{13}C/^{12}C$ in steroids that exhibit biosynthetic pathways independent from the androgens (endogenous reference compounds, ERC).

Besides the great potential of this method, two main problems became apparent within the last few years. On the one hand more and more samples with depleted ERCs were detected. Those samples show endogenous values down to -25‰[1,2] and the intake of testosterone with a typical value of -27‰[3] would not lead to an adverse analytical finding as the Δ values would not increase beyond the WADA threshold of 3‰. Δ values are the differences between the $^{13}C/^{12}C$ ratios of ERCs minus TCs.

On the other hand available steroid preparations with isotopic signatures equal to usual endogenous values (epitestosterone –20.2‰, boldenone -23.2‰) were found in the Cologne laboratory and others reported such values even for testosterone and 1-testosterone.[4] So it can not be excluded that such preparations are used by athletes or that such preparations might be produced exclusively for doping purposes.

A promising approach to detect a doping offence despite the described limitations of carbon isotope ratios would be the examination of the isotope ratio of the other abundant element in the steroidal backbone – hydrogen.[5] Similar to the $^{13}C/^{12}C$ ratio the deuterium/hydrogen (D/H) ratio of endogenous steroids is assumed to be mainly influenced by the diet, especially the δD value of drinking water. D/H ratios are expressed as δD values against the international standard Vienna Standard Mean Ocean Water (VSMOW) based on the equation:

$$\delta D[‰] = \frac{\left(D/H\right)_{sample} - \left(D/H\right)_{std}}{\left(D/H\right)_{std}} * 1000$$

where D/H refers to the isotopic composition of sample or standard.

As the relative mass difference between D and H is larger than between ^{13}C and ^{12}C, it can be expected that the isotopic signature for D/H is more pronounced as the belonging fractionation factors are greater than for carbon. For example, the δD values of precipitation throughout Europe differ for more than 100‰[6] and during the syntheses of steroids depletion of more than 150‰[7] can be expected. So, based on the large variability of both endogenous

and exogenous steroids, it can be assumed that the Δ values for D/H will differ more and should be influenced in another way than the carbon isotopes. In the end the combination of both isotopic ratios should allow for the detection of the abuse of synthetic steroids even in those cases were the $^{13}C/^{12}C$ ratio is not practical because of the above mentioned reasons.

Method

In parallel to the development of a new clean up procedure for carbon isotopes[8] the acetylation of urinary steroids prior to D/H determinations was tested. By this derivatization it is not only possible to enhance the clean up of different steroids but also to substitute the exchangeable hydrogen atoms for ones with a known isotopic composition. For the added hydrogen can easily be corrected afterwards.

Sample preparation

Chromabond® C18 cartridges (500 mg, 6 mL) were conditioned with 2 mL of methanol (**MeOH**) and 2 mL of water. Depending on the urinary steroid concentration, 10 – 30 mL of urine were applied to the column. After washing with 2 mL of water the residue was eluted with 2 x 1 mL of MeOH and evaporated to dryness under a stream of nitrogen. The dried eluate was dissolved in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 5 mL of TBME were added. After shaking for 5 min and centrifugation (1200 *g*, 5 min) the organic layer was discarded. One hundred µL of β-glucuronidase were added and the sample was incubated for 1 h at 50°C. After cooling to room temperature 500 µL of potassium carbonate buffer ($K_2CO_3/KHCO_3$ 1:1, w/w, 200 g/L) were added. The aqueous layer was extracted with 2 x 5 mL of TBME, shaken for 5 min and centrifuged (1200 *g*, 5 min), and the organic layers were combined in a conical test-tube and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 2 x 100 µL of MeOH, transferred to a HPLC vial with insert, dried in a dessiccator and stored there until further cleanup.

HPLC-cleanup

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

For the first run the dried residue was dissolved in 50 µL of MeOH and 1 µL of a solution containing 500 mg/mL β-estradiol-3,17-diacetate (**EST**) in MeOH was added as reference standard. The injection volume was 50 µL and the flow rate 1 mL/min. 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 reequilibrated for 5 min. Before each batch of 12 samples, a standard solution containing approximately 100 µg/mL of OHA, T, EpiT, E, A, PD, 16EN 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 six fractions. The different fractions were collected in conical test-tubes and evaporated to dryness under a stream of nitrogen.

In order to monitor any occurring isotopic fractionation whilst acetylation it was necessary to add a control standard prior to the acetylation and to determine its δD value afterwards. To fractions II (T), V (PD) and VI (16EN), 10 µL of a solution containing 50 µg/mL of 5-androstene-3β,17α-diol (**5EN**) in MeOH and to fraction I (OHA), III (EpiT, DHEA, 5a and 5b) and IV (E, A) 10 µL of a solution with 50 µg/mL androstanol (**RSTD**) in acetone were added and evaporated in a dessiccator. Different standards were utilized because selected standards and analytes coelute from the GC column. 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. After solving the residue in 10-100 µL of acetone, fractions I, IV, V and VI were analyzed by GC/TC/IRMS.

Fraction II containing T [now the monoacetate (TAc)] was further purified by an additional HPLC fractionation (column and gradient as above). Also here, two standards containing approximately 100 µg/mL of TAc, 5EN as acetate (5ENAc) and EST in MeOH were injected prior to each batch to characterize retention times and, thus, collection windows. The fractions were collected, combined and evaporated.

For fraction III containing EpiTAc, DHEAAc, 5aAc, 5bAc and RSTDAc a different gradient was used. From 70/30 acetonitrile/water a linear increase to 100% acetonitrile was accomplished in 33 min and maintained for 5 min. Subsequently, the column was re-equilibrated for 5 min. The flow rate was set to 1 mL/min. In order to optimise peak shape and separation especially of 5aAc and 5bAc, a mixture containing acetonitrile/water (70/30, v/v) was used instead of MeOH as solvent for injection. Again, EST functioned as reference standard. The fractions of DHEAAc and RSTDAc were combined. All fractions were evaporated to dryness.

GC/TC/IRMS

All samples were measured on an Agilent 6890 Gas Chromatograph coupled to a Delta plus XP gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via the TC Combustion Interface. The GC system was equipped with a HP50+ column (length 30m, ID 0.25mm, film 0.5µm). Injection was performed with a Gerstel KAS unit[9] at 40°C. After keeping the initial temperature for 3 min it was increased with 40°C/min up to 250°C, then with 3°C/min to 300°C and maintained for 5 min. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.0 mL/min. The thermal combustion furnace was operated at 1450°C. Initially the pyrolysis reactor was conditioned with 3-5 injections of a standard solution containing RSTDAc, EAc and AAc at 200 ng/mL in acetone. Two GC/TC/IRMS chromatograms are depicted in Figure 1, one for PDAc and one for TAc. Both show the absence of any disturbing co-elution, which improved the validity of δD determinations.

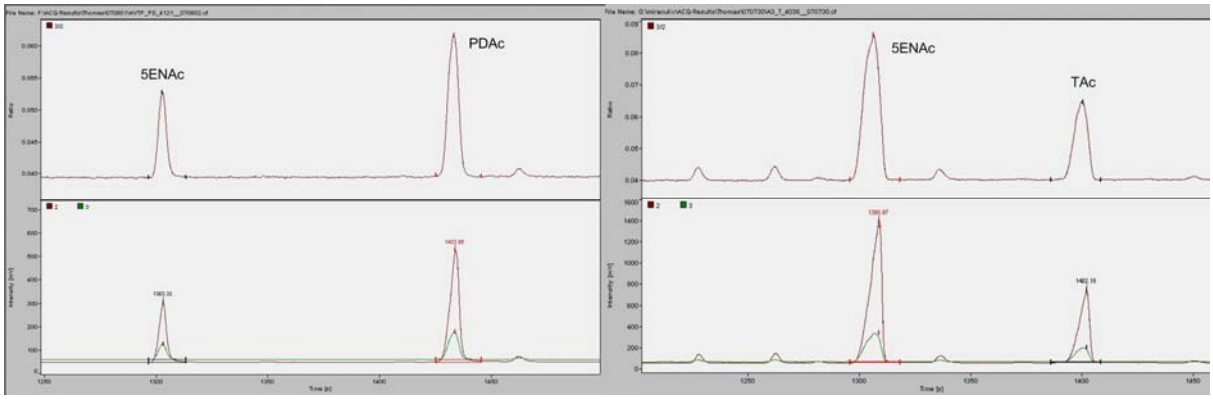

Figure 1: GC/TC/IRMS chromatograms of one ERC (PDAc) on the left side and one TC (TAc) on the right side

Results and Discussion

First different standards were measured underivatized and acetylated in order to determine the δD value of the acetate moiety. The results are listed in Table 1. The underlying formula is equal to the one used for correction of carbon isotope ratios:

$$n_{cd}\,\delta D_{cd} = n_c\,\delta D_c + n_d\,\delta D_{dcorr}$$

with n = number of moles of hydrogens, c = compound of interest, d = derivative group, and cd = derivatised compound. δD_{dcorr} is estimated empirically by consecutive measurements of both the native and the derivatised steroid.

Steroid	Mean (n=3) [‰]	SD [‰]
OHA	-183.8	2.2
PD	-213.6	1.0
16EN	-288.5	0.6
E	-259.8	1.8
A	-329.9	0.5
T	-283.5	1.3
EpiT	-180.9	2.3
DHEA	-262.7	0.9
5a	-332.9	1.9
5b	-265.8	2.4

Steroid (Ac)	Mean (n=3) [‰]	SD [‰]
OHA	-167.0	0.6
PD	-191.4	1.6
16EN	-273.7	0.6
E	-250.7	0.6
A	-312.5	0.4
T	-266.3	3.5
EpiT	-174.0	1.7
DHEA	-247.2	1.6
5a	-298.9	1.7
5b	-240.7	0.3

Table 1: δD values of standards, measured as free compound (left hand) and as acetate (right hand)

Over all the standard deviations (SD) decrease for the acetates and are excellent with the exception of testosterone. The δD value for the acetate moiety was calculated to –2.4‰. As can be seen the arbitrarily chosen standards from our laboratory cover a range of more than 140‰ which supports the assumption that the δD value will spread over a broader range than the values of the carbon isotope ratios.

In order to correct for possible memory effects namely occurring during pyrolysis, all determined δD values where recalculated using a simple rule of proportion formula:

$$\delta(XAc)_{corr} = \delta(XAc)\frac{\delta(StdAc)_{corr}}{\delta(StdAc)}$$

Where X stands for the steroid of interest, δ for the according δD value and the numerator represents the empirically predetermined value of the added standard while the denominator represents the actual measured value. The underlying correlation of the added reference standard and the target compound during pyrolysis with each measurement seems to be quite similar for all steroids under investigation. So it was possible to treat all steroids in the same manner with an improved repeatability and reliability for all measurements. Especially for small sample sizes this correction was essential. The correction was able to reduce the SD for endogenous steroids by a factor of two for EpiT and 5a for example.

The repeatability of steroids extracted from urine has been tested with six consecutive preparations of one blank urine; the results are listed in Table 2.

Steroid	Mean [‰]	SD [‰]
OHA	-330.3	7.4
PD	-249.0	5.8
16EN	-258.7	16.4
E	-307.2	5.6
A	-308.4	4.8

Steroid	Mean [‰]	SD [‰]
T	-207.5	5.5
EpiT	-179.9	6.9
DHEA	-243.0	12.9
5a	-252.8	6.3
5b	-267.5	4.8

Table 2: Corrected δD values of a blank urine, n = 6 preparations

The over-all standard deviation is 7.6‰ and therefore quite near to the achievable value of 5‰ according to literature.[10] The method's validity was tested by the application of linear mixing models[8] and - as can be seen in Table 3 - none of the steroids failed this test. Again, the standard deviations are excellent.

Steroid	a [‰]	SD [‰]	b [‰]	SD [‰]
OHA	-133.4	8.9	-173.4	5.6
PD	-23.8	12.0	-214.0	7.4
16EN	6.7	9.7	-291.9	6.8
E	-39.3	2.4	-269.3	1.5
A	63.9	5.8	-343.8	3.6

Steroid	a [‰]	SD [‰]	b [‰]	SD [‰]
T	15.6	4.5	-288.3	2.7
EpiT	-66.7	6.0	-195.6	3.7
DHEA	-41.8	3.5	-261.3	2.2
5a	43.1	3.9	-328.6	2.5
5b	-26.4	3.3	-266.7	2.0

Table 3: Summarized results of the linear mixing models; **a** is the slope of the calculated line of best fit and **b** the intercept with the y-axis

Afterwards a reference population of n = 18 healthy young men was investigated in order to ascertain the naturally occurring δ values of all steroids and the resulting Δ values. All 18 volunteers collected their entire urine in aliquots over a time period of 48 h. The urine was stored at -20°C until sample preparation.

As all participants were students or employees of the German Sport University, their δ values were quite similar. This is in accordance with the assumption that the urinary steroids should reflect the D/H ratio of the drinking water. So the δD values are reported as mean values with standard deviations in Table 4.

sample	mean [‰]	SD [‰]
OHA	-301	17.4
PD	-240	12.6
16EN	-255	30.1
E	-302	10.6
A	-282	7.9

sample	mean [‰]	SD [‰]
T	-278	11.9
EpiT	-244	17.3
DHEA	-300	20.6
5a	-321	26.4
5b	-303	17.3

Table 4: Corrected δD values of a group of n = 18 men.

But the in part quite large standard deviations show that even for residents of the same city the δ values are not equal. Therefore in parallel to carbon isotope ratios again the Δ values are the more promising approach to detect the misuse of anabolic steroids.

The results (Table 5) show clearly the potential of reference based Δ values for doping control purposes.

	PD-E [‰]	PD-A [‰]	PD-T [‰]	PD-EpiT [‰]	PD-DHEA [‰]	PD-5a [‰]	PD-5b [‰]
mean	61,6	42,2	38,0	3,4	59,9	80,6	62,9
SD	10,6	11,0	10,7	14,6	15,3	26,9	13,3
RL up	93,3	75,1	70,2	47,1	105,9	161,4	102,8
RL down	30,0	9,3	5,8	-40,4	14,0	-0,2	23,1

Table 5: Δ values of a group of n = 18 men; RL up is calculated by adding the threefold standard deviation and RL down by subtracting

In comparison to the repeatability of the blank urine (Table 2), the standard deviations increases as here not only the deviations belonging to sample preparation and measurement but also the natural occurring deviations between different individuals contribute. But, in comparison to the δD values (Table 4) the standard deviations decrease, as expected. Especially for E, A and T the results are excellent, the measurements of 5a seem to be complicated in a manner we were not able to identify till now.

At the moment the calculated reference limits (RL) are preliminary as a population of n = 18 persons is too small for parametrical mathematical calculations. In contrast to $^{13}C/^{12}C$ measurements, where the administered steroid is always assumed to be more depleted for D/H determinations both RLs are of interested. Exogenous steroids can be more or less enriched than the ERCs.

Finally the $^{13}C/^{12}C$ ratios of the reference population were determined to check the feasibility of combined isotope ratio measurements – not only to detect the misuse of steroids but also to track steroidal pathways. Figure 2 shows the mean value of each steroid ± 1 SD.

These first results suggest in general a depletion in hydrogen for the steroids throughout their metabolic pathway. The first metabolites PD and 16EN show the highest content of deuterium and for both the androgens (E, A) and the corticoids (11OHA) the depletion seems to be quite similar. However, the carbon isotopes are fractionated differently as there seems to take place depletion for the androgens and enrichment for the corticoids. Also interesting are the quite large differences between the "Diols" (5a and 5b) and T, presumably due to the introduction of depleted hydrogen via NADPH.[11] Outstanding are the values of EpiT suggesting a completely different pathway for this steroid.

Also interesting is the different behaviour of the 5β-configurated steroids 5b and E and the 5α-configurated steroids 5a and A. For E and A, E shows the more depleted values while for

the “Diols” 5a seems to be more depleted. Thus, the difference between 5α- and 5β-configurated steroids can not be explained by a different fractionation according to 5α- and 5β-reductase.

Besides the interesting distribution of the different steroids it becomes obvious that – with the data of an appropriate reference population – the calculation of 2-dimensional reference limits should be possible.

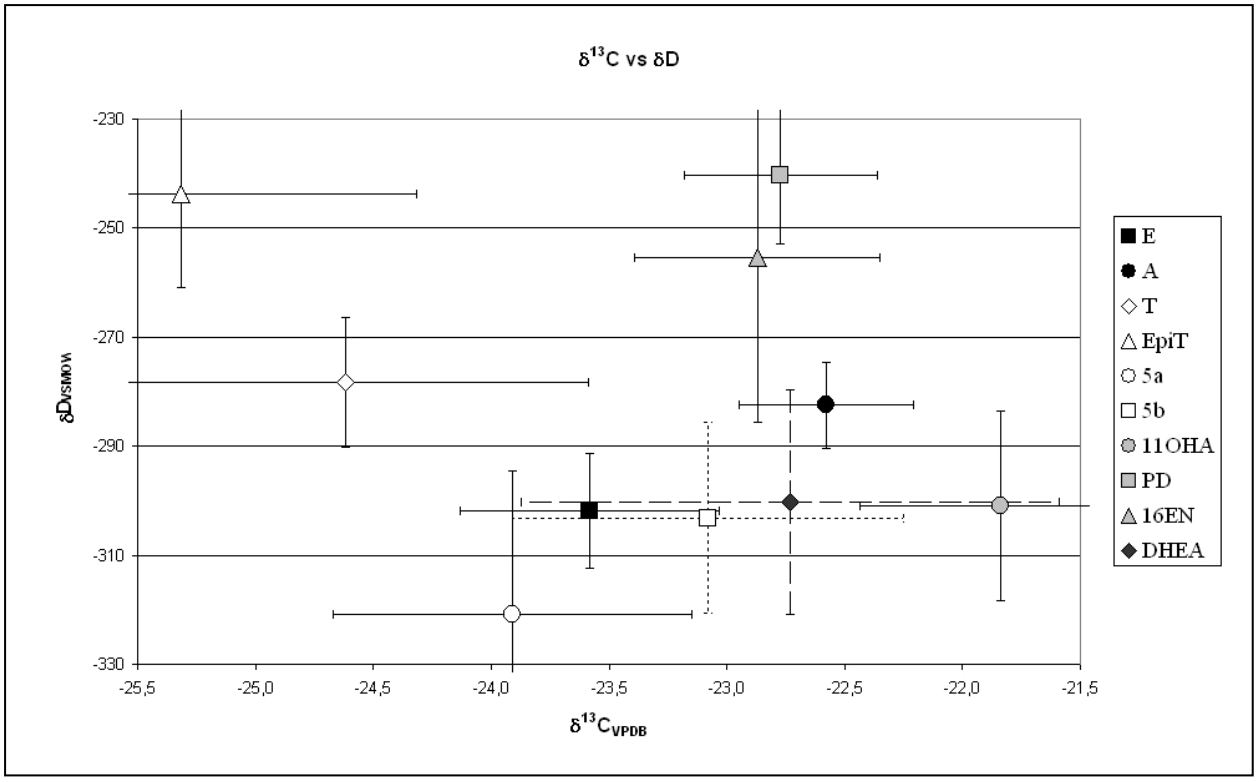

Figure 2: Corrected δD values plotted against corrected $\delta^{13}C$ values of n = 18 urine specimens

Conclusion

These first results clearly show the potential of D/H determinations to detect the misuse of anabolic steroids by athletes. But still several investigations will have to be completed prior this method is fit for purpose. First of all an appropriate reference population of at least 50 healthy volunteers has to be set up to achieve the necessary statistical power for reference limits. Furthermore it has to be proven that large variations in δ values are not accompanied by unexpected changes in the belonging Δ values.

Another interesting point will be the behaviour of the different metabolic pathways after a change of drinking water which will definitely go hand in hand with travelling. It is a well known fact that the body water equilibrates with drinking water within 2-3 hours. Hence the isotopic composition of the body water can change very fast and thus the composition of the hydrogen pool to which will be reverted to whilst biosynthesis of cholesterol. If the production and urinary excretion of – for example – PD is much faster than that for E and A, this might result in false positive findings as the Δ value can exceed the reference limit.

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