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Determination of $^{13}C/^{12}C$ ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one

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Boldenone (androsta-1,4-dien-17β-ol-3-one, Bo) is an anabolic steroid known to have been used in cattle breeding or equine sport as a doping agent for many years. Although not clinically approved for human application, Bo or its main metabolite 5β-androst-1-en-17β-ol-3-one (BM1) were detected in several doping control samples. For more than 15 years the possibility of endogenous Bo production in human beings has been discussed. This is a challenging issue for doping control laboratories as Bo belongs to the list of prohibited substances of the World Anti-Doping Agency and therefore the chance for false positive testing is significant. By GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) it should be possible to analyze the $^{13}C/^{12}C$ ratio of either Bo or BM1 and to distinguish whether their source is endogenous or exogenous. Therefore a method was developed to determine the $^{13}C/^{12}C$ ratios of Bo, BM1, pregnanediol, androsterone, etiocholanolone, and testosterone from a single urine specimen. The validity of the method was ensured by repeated processing of urine fortified with 2–50 ng/mL Bo and BM1. The specificity of the method was ensured by gas chromatography/mass spectrometry determinations. Out of 23 samples investigated throughout the last four years, 11 showed $^{13}C/^{12}C$ ratios of Bo or BM1 inconsistent with an exogenous origin. Two of these samples were collected from the same athlete within a one-month interval, strongly indicating the chance of endogenous Bo production by this athlete.

Keywords: boldenone; isotope ratio mass spectrometry; doping control; carbon isotope ratio

Introduction

Boldenone (androst-1,4-dien-17β-ol-3-one, Bo) was first synthesized in 1940[1] and is a well-known anabolic steroid used in cattle breeding or equine doping for many years.[2–4] Although it is not clinically approved for human application, there were several cases of athletes testing positive for Bo or its main metabolite BM1 (5β-androst-1-en-17β-ol-3-one).[5–7] While testing cattle in order to detect the misuse of forbidden growth hormones, several false-positive Bo results occurred due to faecal cross-contamination during sampling.[8,9] In the urine of male horses (stallions) Bo is found quite regularly at the low ng-level, mainly excreted as sulfate derivative. In a population consisting of n = 63 horses, 26 showed concentrations of Bo-sulfate larger than 1 ng/mL.[10]

In 1994, only two years after the complete metabolism of Bo in humans was elucidated,[7] the first urine samples were investigated arousing suspicion of endogenous Bo production in man.[11] In urine specimens of two laboratory staff members not treated with Bo or any other anabolic steroid, small amounts of BM1, Bo, and another metabolite 3α-hydroxy-5β-androst-1-en-17-one were identified. Incubation of faeces with testosterone (T) or androst-4-en-3,17-dion (AD) yielded androsta-1,4-dien-3,17-dion (ADD) in the urine of one volunteer. Five years later this experiment was successfully repeated by another anti-doping laboratory.[12] The ability of faeces to 1-dehydrogenate the steroid nucleus has recently been investigated carefully using a large intestinal simulation model.[13] Again, the formation of ADD could be shown, unfortunately with limitation to abnormal physiologically aerobic conditions. Under physiologically anaerobic conditions, no Δ^1-dehydrogenase activity could be confirmed. Hence, the endogenous production of Bo is still not proven unambiguously.

The most likely source for endogenous Bo is the enterohepatic circulation, with the human gut working as an 'endocrine' active side organ.[14,15] Besides cholesterol and bile acids, up to 2 mg of steroid hormones enter the gut daily via bile. Here, numerous enzymatic transformations take place, such as hydrolysis, side-chain cleavage, isomerization, dehydroxylation and oxidation-reduction of both hydroxyl functions and the steroid nucleus. Excretion studies with labeled steroids revealed the presence of T, AD and dehydroepiandrosterone (DHEA) in bile.[16] After being dehydrogenated at position 1, T or AD can be reabsorbed as Bo or ADD into the portal vein, transported back to the liver, be further metabolized, and then excreted renally.

The first human intestinal bacteria with Δ^1-dehydrogenase activity was described by Aries *et al*. in 1971.[17] A strain of *Clostridium paraputrificum* showed 3-oxo-5β-steroid Δ^1-dehydrogenase activity on different bile acids and incubation experiments with 5β-androstane-3,17-dion yielded ADD. This was observed under borderline physiological conditions at a pH of 8.5 and at high concentrations of Vitamin K_2 as electron acceptor. Due to its specificity to 5β-steroids neither T not AD were 1-dehydrogenated by this enzyme.

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The first endogenous ADD production was found with a strain of *Escherichia coli* isolated from the faeces of a colon-cancer patient by Owen *et al*. in 1977.[18,19] Here, cholest-4-en-3-one and AD were 1-dehydrogenated yielding cholest-1,4-dien-3-one and ADD under anaerobic physiological conditions without any constraints. Interestingly, this strain of *E. coli* is not found regularly in human faeces. Later on, other bacteria like *Pseudomonas* sp. NCIB 10590 were isolated; showing the ability to produce ADD and 6-OH-ADD from bile acids.[20] Again, this transformation took place under anaerobic physiological conditions.

To summarize these findings, the following statements can be made: The endogenous production of the Bo-precursor ADD in the colon can be proven. The further transport of ADD into the liver via the portal vein can be demonstrated for other steroids and is therefore very likely for ADD, too.[16] Steroids entering the hepatic tissue via the portal vein are further metabolized and preferentially excreted by the kidney. As for the production of ADD, either borderline physiological conditions or bacteria strains not found regularly are necessary. The described temporary incidence of endogenous Bo production is plausible.[11,12] Unfortunately, these facts cannot prove the endogenous production of Bo unambiguously.

By means of GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) it is possible to ascertain the $^{13}C/^{12}C$ ratios of sample amounts as small as 10 ng of carbon per compound. So it should be possible to measure either Bo or BM1, if urinary concentrations fall between 1 and 100 ng/mL. To extract the required amount of steroid from the urine, a sufficiently large specimen (up to 20 mL) and an effective clean-up method are essential.

To clarify the origin of Bo, it is necessary to compare the measured $\delta^{13}C$ values of the target compounds (TC) Bo and BM1 to the $\delta^{13}C$ values of endogenous reference compounds (ERC), here etiocholanolone (E), androsterone (A), pregnanediol (PD) and T. $^{13}C/^{12}C$ ratios are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on Equation (1):

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

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard.[21]

Differences between TCs and ERCs are expressed as Δ values:

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

Expected $\delta^{13}C$-values for athletes range from $-17‰$ to $-25‰$, depending on the provenance of the athlete.[22] Bo preparations available on the market should exhibit values of $-27‰$ or even lower, comparable to other available synthetic steroids.[23] This difference should allow for a clear discrimination between endogenous and synthetic Bo.

Experimental

Chemicals and steroids

Chromabond® C18 cartridges were obtained from Macherey-Nagel (Düren, Germany). Acetone (for gas chromatography), pyridine, n-hexane, diethyl ether, acetic acid (glacial), chloroform, potassium hydroxide, and acetic anhydride (distilled before use) were purchased from Merck (Darmstadt, Germany). Glass plates (TLC Silica gel 60) were also 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, T and 5α-androstane-3β-ol (RSTD)) was supplied by Sigma (Steinheim, Germany). 11β-hydroxy-androsterone (OHA) and 5-androstene-3β,17α-diol (5EN) were purchased from Steraloids (Newport, RI, USA) and β-estradiol-3,17-diacetate (EST) and Bo from Riedel-de Haen (Seelze, Germany). BM1 was synthesized in our laboratory.[7] All solvents and reagents were of analytical grade.

Sample preparation

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

A detailed description of the extensive sample preparation method including two-fold HPLC clean-up has been published elsewhere [24] and will herein only be described in brief. Depending on the amount of urinary steroids, up to 20 mL of urine was 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 hydrolyzed 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 (high performance liquid chromatography) purification.

HPLC clean-up

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 (Darmstadt, Germany) analytical column (LiChrospher® 100 RP^{18}, 250 × 4 mm i.d., 5 µm particle size).

For the first run, a linear gradient increasing from 30/70 acetonitrile/water to 100% acetonitrile in 25 min was used. After 5 min at 100% acetonitrile, the column was re-equilibrated for 5 min; 50 µL injection volume (MeOH) and flow rate of 1 mL/min were used. Before each batch of samples, a standard solution containing approximately 100 µg/mL of OHA, Bo, BM1, E, A, PD, and EST each was injected twice to determine the retention times for fraction collection. The automatic fraction collector Foxy 200 from Isco (Lincoln, NE, USA) was programmed to prepare five fractions as illustrated in Figure 1. The different fractions were collected in conical test-tubes and evaporated to dryness under a stream of nitrogen. Fraction IV (E, A) was immediately subjected to GC/C/IRMS. All other fractions were acetylated due to considerably improved separation of steroids on both the HPLC and the GC column.

In order to monitor any occurring isotopic fractionation caused by the acetylation step, it was necessary to add a control standard prior to the acetylation and to determine its $\delta^{13}C$ value afterwards. To fraction I and V 10 µL of a solution containing 50 µg/mL 5EN, to fraction II 10 µL of a solution containing 50 µg/mL RSTD and to fraction III 10 µL of a solution containing 50 µg/mL T in acetone

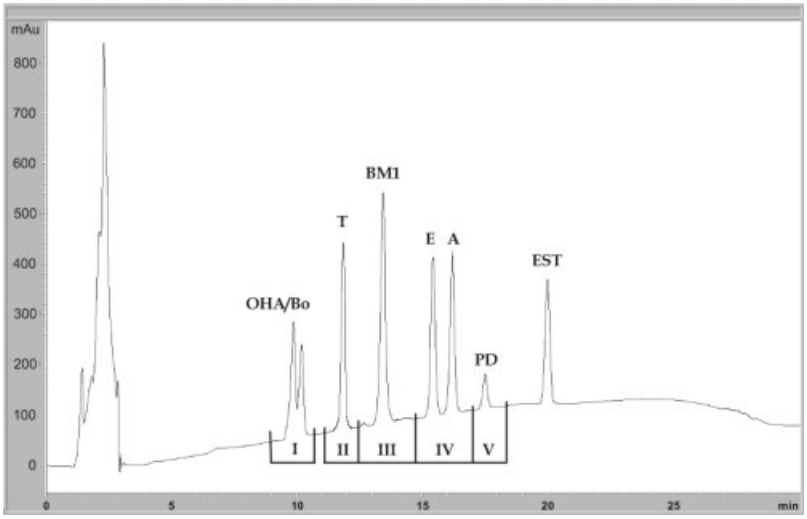

Figure 1. HPLC chromatogram of a standard containing OHA, Bo, T, BM1, E, A, PD and EST (wavelength 192 nm). The fractions were collected as demonstrated.

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. Afterwards, fraction V was submitted to GC/C/IRMS.

For the second HPLC clean-up, the same gradient as before was used for fractions I and III. 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 2. Fractions Ia and Ib were combined and all fractions were evaporated to dryness under a stream of nitrogen and subjected to GC/C/IRMS.

The isolation of T (fraction II) has already been described in detail.[24]

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 (gas chromatography/mass spectrometry) system using equivalent chromatographic conditions to the IRMS set-up whilst method development. Additionally, all routine samples were measured to ensure peak purity. The small amounts of Bo and BM1 investigated were prone to interfering co-elutions strongly influencing the determined $\delta^{13}C$ value. 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 δ 3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). The injections were performed splitless at 300 °C. The initial oven temperature of 60 °C was held for 1.5 min, increased at 40 °C/min to 240 °C, followed by a ramp at 2 °C/min to 260 °C, and 40 °C/min to the final temperature of 300 °C. A constant flow of 1.2 mL/min with helium as carrier gas was used. The MSD acquired data in scan mode from *m/z* 40 to 400 and mass spectral data were compared with those from 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, Bremen, Germany). The GC system was equipped with the same column as mentioned earlier. 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, Boeckten, Switzerland). 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 and maintained for 2 min. 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.

GC/C/IRMS chromatograms of both TCs are depicted in Figures 3 and 4 demonstrating the absence of any disturbing co-elution, which improved the validity of $\delta^{13}C$ determinations.[25]

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) can not be used as kinetic isotope effects (KIE) during acetylation are probable.[26] Consequently, a slightly adapted mass balance formula (Equation 4) was applied, which takes possibly occurring KIE into account:[27]

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

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

with 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 utilized for urinary steroids.

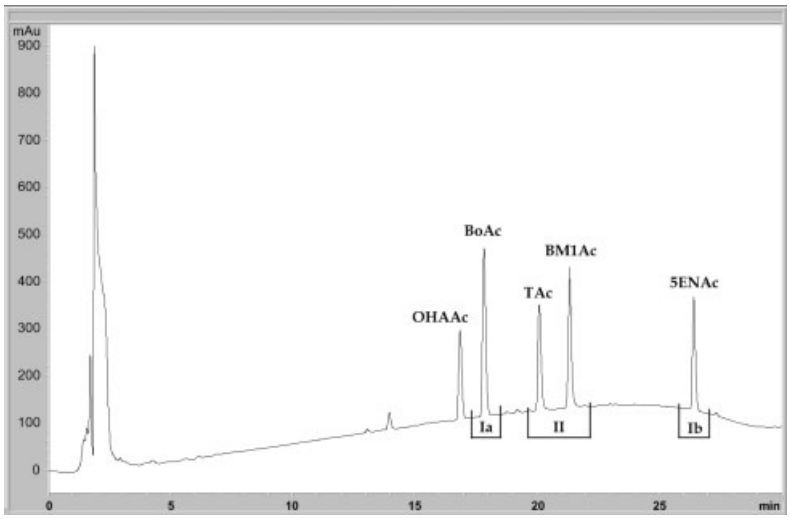

Figure 2. HPLC chromatogram of a standard containing OHA, Bo, T, BM1 and 5EN as acetate (wavelength 192 nm). The fractions were collected as demonstrated.

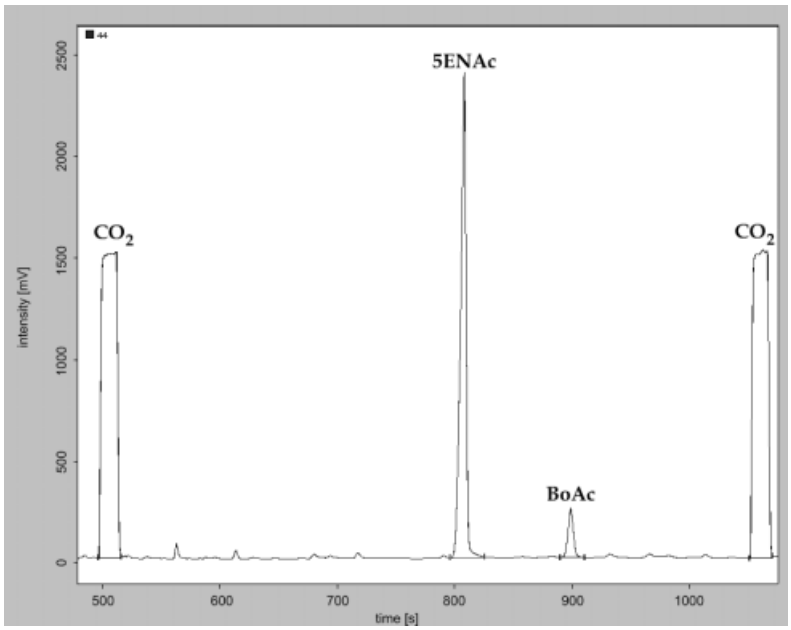

Figure 3. GC/C/IRMS chromatogram of BoAc. The rectangular peaks are pulses of the calibrated tank gas CO_2. 5ENAc acts as reference standard.

Blank and quality control urine

A pooled urine sample was collected over a period of five days (total amount of 10 L) from two healthy male volunteers who declared no use of any prohibited substance or any nutritional supplement. The urine was bottled in 1 L containers and stored at −20 °C until analysis. In parallel with each doping control sample, a blank urine (BW) and a quality control urine (QC) were processed. Before sample preparation, the QC was prepared by fortifying 10 mL of BW with 100 ng of Bo and 100 ng of BM1 absolute.

Method validation

The specificity of the method was ensured by GC/MS measurements under equivalent conditions to the GC/C/IRMS. Repeatability of the whole method was tested by repeated preparations of BWs fortified with 2 to 50 ng/mL Bo (n = 32) and BM1 (n = 22). The different concentrations used were 2, 4, 6, 8, 15, 30 and 50 ng/mL. Reproducibility was tested with repeated preparations of the QC over a time period of 20 months. The obtained $\delta^{13}C$ values were compared to the $\delta^{13}C$ values of the added standards.

Steroid profiling

An aliquot of each specimen was prepared to determine the amount of different steroids according to routine sample preparation procedure and to estimate the amount of Bo and BM1 in order to determine the urine volume requisite for GC/C/IRMS.[28]

Purification of boldenone solutions suitable for injection

Bo preparations for horses or preparations available on the black market are usually solutions in oil suitable for injection fortified

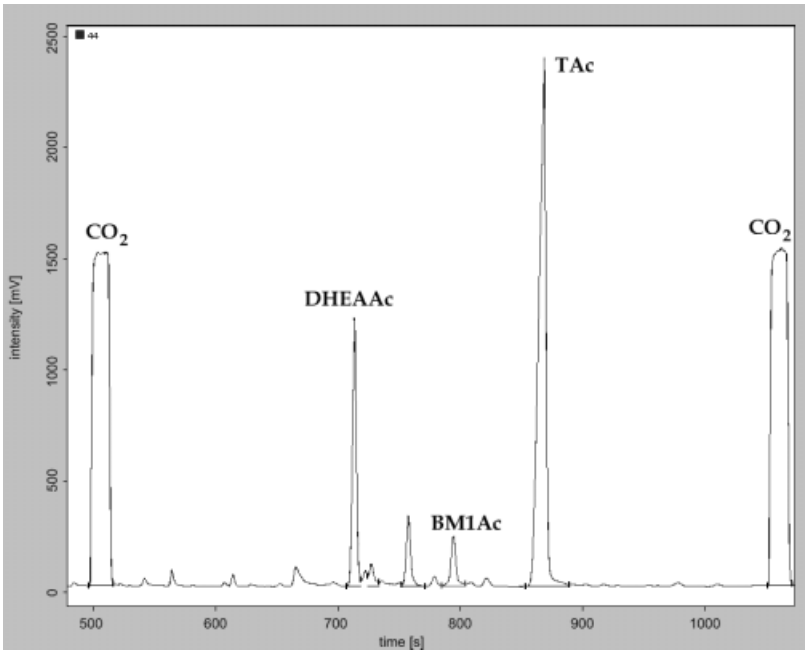

Figure 4. GC/C/IRMS chromatogram of BM1Ac. The rectangular peaks are pulses of the calibrated tank gas CO_2. TAc acts as reference standard. Due to similar retention times on the HPLC, DHEAAc (dehydroepiandrosterone-acetate) is always found together with BM1Ac.

with the undecylenate ester of Bo (Bo-17-(10-undecenate), Bo-ester). Separation of Bo-ester from oil was obtained with thin layer chromatography using n-hexane, diethyl ether and acetic acid (70/30/1; v/v/v) as mobile phase. The spot containing Bo-ester was transferred into a test tube and extracted with 2 mL of MeOH/chloroform (1/1; v/v). The organic layer was transferred into another test tube, dried under a stream of nitrogen, and the residue saponified with 1 mL of methanolic KOH (1M) for 1 h at 80 °C. Afterwards, the methanolic KOH was dried, reconstituted with 1 mL H_2O, and extracted with 5 mL n-hexane. The organic layer containing underivatized Bo was transferred into a new test tube and dried under a stream of nitrogen and then forwarded to GC/C/IRMS measurement.

Results and Discussion

Method validation

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, 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 routine samples were investigated in order to ensure peak purity and identity for both the TCs and the ERCs.

Repeatability

In order to check the repeatability of the method, BWs were fortified with different amounts of Bo and BM1, respectively. The concentration ranged from 2 to 50 ng/mL per compound. 10 mL of urine were spiked, prepared as described and analysed. For Bo, 32 specimens were analyzed. During method development, the HPLC clean-up of Bo was investigated for Bo itself and for BoAc and no significant differences were found. Contrary to the first evaluation,[29] BoAc came out to be more robust and afflicted with less co-elutions than the procedure for free Bo. Therefore the sample preparation for BoAc described herein was selected for routine doping control purposes. The results are listed in Table 1.

Table 1. Repeated measurements of standards (n = 6) and urines fortified with 2–50 ng/mL BM1 (n = 21) and Bo (n = 32). All values in $\delta^{13}C_{VPDB}$

	Std BM1 [‰]	BM1 [‰]	Std Bo [‰]	Bo [‰]
Mean	−27.7	−27.6	−30.3	−30.3
SD	0.5	0.6	0.2	0.9

Obviously, there is no isotopic fractionation taking place during sample preparation while the standard deviation (SD) is raising up to 0.9‰in contrast to the measured standard. This is probably due to the low amount of steroid compared to the standard analyzed, resulting in a low signal intensity which is known to raise the SD of isotope measurements.[30] The low concentrated samples in particular show a larger SD (1.0‰for 2–8 ng/mL vs. 0.7‰for 15–50 ng/mL). Despite the increasing SD, no bias was found in the data (r = 0.06), so the measured $\delta^{13}C$ value was independent of the signal intensity. This corresponds with a very good linearity of the IRMS (Isotope Ratio Mass Spectrometry) in the low signal range.

Comparable results were obtained for the 21 samples fortified with BM1 (Table 1). The data exhibited no bias (r = 0.12) and again the $\delta^{13}C$ values of the lower concentrated specimens were afflicted with a larger SD (0.6‰for 2–8 ng/mL vs. 0.4‰for 15–50 ng/mL).

Table 2. Repeated measurements (n = 14) of standards and the QC urine fortified with 10 ng/mL Bo and BM1, respectively. All values in $\delta^{13}C_{VPDB}$

	Std BM1 [‰]	QC BM1 [‰]	Std Bo [‰]	QC Bo [‰]
Mean	−28.2	−27.9	−30.3	−30.7
SD	0.4	0.6	0.4	0.4

Reproducibility

After implementing the described method into doping control routine analysis (at the beginning of 2008), a defined QC urine was prepared with each investigated sample which resulted in a total of n = 14 independent preparations (Table 2). In addition, both standards were measured in parallel to each preparation.

Over the covered time period of 20 months, a small systematic drift over time was visible (approx. 1‰, r = 0.24). This drift was comparable to other results obtained for the used instrument and both the pure standards and the QCs were affected.[22] Within measurement precision, no significant difference between the results of the experiments concerning repeatability and reproducibility were found.

Doping control samples

Throughout the years 2006 to autumn 2009, a total of 23 samples were investigated for Bo and its metabolite with GC/C/IRMS. The results are listed in Tables 3 and 4. $\delta^{13}C$ values of Bo and BM1 were considered exogenous if the Δ values exceeded the 3‰threshold established by the World Anti-Doping Agency (WADA).[31] Additionally, all of these samples show $\delta^{13}C$ values of −27‰ or even more depleted for both TCs strongly indicating an exogenous origin as pharmaceutical preparations usually have $\delta^{13}C$ values between −27 and −33‰.[23]

In general, samples with exogenous values for Bo or BM1 usually exhibited higher concentration for both TCs. But even samples with as low as 2 ng/mL Bo or 6 ng/mL BM1 exhibited exogenous values. In contrast, samples with up to 23 ng/mL Bo or 20 ng/mL BM1 were found unsuspicious. Therefore, a concentration threshold for urinary Bo or BM1 does not seem to be helpful for a definitive discrimination between endogenous or exogenous Bo.

By contrast, with GC/C/IRMS a clear separation of the investigated samples concerning the origin of Bo and BM1 is possible. The difference between the largest Δ value for an unsuspicious finding (PD-Bo = 1.6‰, PD-BM1 = 2.2‰) and the lowest Δ value for a suspicious finding (PD-Bo = 6.3‰, PD-BM1 = 6.5‰) differ

Table 3. Summary of all investigated doping control samples exhibiting exogenous values. Listed are the estimated concentrations of Bo and BM1, the $\delta^{13}C_{VPDB}$ values of all ERCs and the $\delta^{13}C_{VPDB}$ values of both TCs (all values corrected for the acetate moiety). nd – not determined

	Bo [ng/mL]	BM1 [ng/mL]	PD [‰]	E [‰]	A [‰]	T [‰]	Bo [‰]	BM1 [‰]
#1/06	50	50	−18.0	−19.6	−18.1	nd	−28.7	−29.2
#6/07	5	6	−18.7	−20.0	−18.4	nd	−27.0	−30.0
#8/08	10	30	−18.4	−20.3	−18.4	−19.5	−30.1	−28.7
#9/08	nd	50	−18.7	−23.5	−20.4	−21.5	nd	−28.6
#10/08	2	12	−20.3	−21.4	−19.9	−21.5	−28.9	−30.8
#11/08	2	12	−19.3	−21.7	−19.9	−21.4	−31.3	−28.7
#13/08	40	17	−22.5	−24.9	−22.5	−24.2	−29.2	−29.1
#14/09	10	30	−19.4	−20.0	−19.3	−19.7	−29.6	−28.7
#15/09	50	120	−20.6	−23.1	−21.2	−24.2	−27.4	−29.1
#16/09	nd	25	−19.5	−23.1	−21.7	−21.4	−29.6	−29.4
#19/09	5	6	−22.8	−23.9	−21.9	−22.8	−29.2	−29.3
#23/09	170	170	−21.8	−23.8	−21.9	nd	−28.1	−28.2

Abbr.: Bo – boldenone; BM1 – boldenone metabolite; PD – pregnanediol; E – etiocholanolone; A – androsterone; T – testosterone.

Table 4. Summary of all investigated doping control samples exhibiting endogenous values. Listed are the estimated concentrations of Bo and BM1, the $\delta^{13}C_{VPDB}$ values of all ERCs and the $\delta^{13}C_{VPDB}$ values of both TCs (all values corrected for the acetate moiety). nd – not determined

	Bo [ng/mL]	BM1 [ng/mL]	PD [‰]	E [‰]	A [‰]	T [‰]	Bo [‰]	BM1 [‰]
#2/06	12	5	−20.1	−21.9	−20.7	nd	−21.6	−20.0
#3/06	23	5	−21.5	−21.7	−21.0	nd	−22.8	−20.2
#4/07	3	3	−20.7	−22.0	−21.2	nd	nd	−21.5
#5/07	2	4	−22.3	−22.7	−22.4	nd	nd	−21.8
#7/07	nd	1	nd	−23.0	−21.8	nd	nd	−23.2
#12/08	3	3	−20.7	−23.2	−21.6	−22.0	−20.8	−22.5
#17/09	5	6	−21.7	−23.2	−22.0	−22.4	−22.8	−23.9
#18/09	6	10	−21.6	−23.6	−22.4	−22.6	−22.1	−23.0
#20/09	15	15	−21.3	−22.4	−21.0	nd	−22.9	−21.6
#21/09	2	15	−21.9	−22.8	−21.8	nd	−22.2	−22.8
#22/09	20	20	−19.3	−21.0	−20.1	nd	−20.3	−21.0

Abbr.: Bo – boldenone; BM1 – boldenone metabolite; PD – pregnanediol; E – etiocholanolone; A – androsterone; T – testosterone.

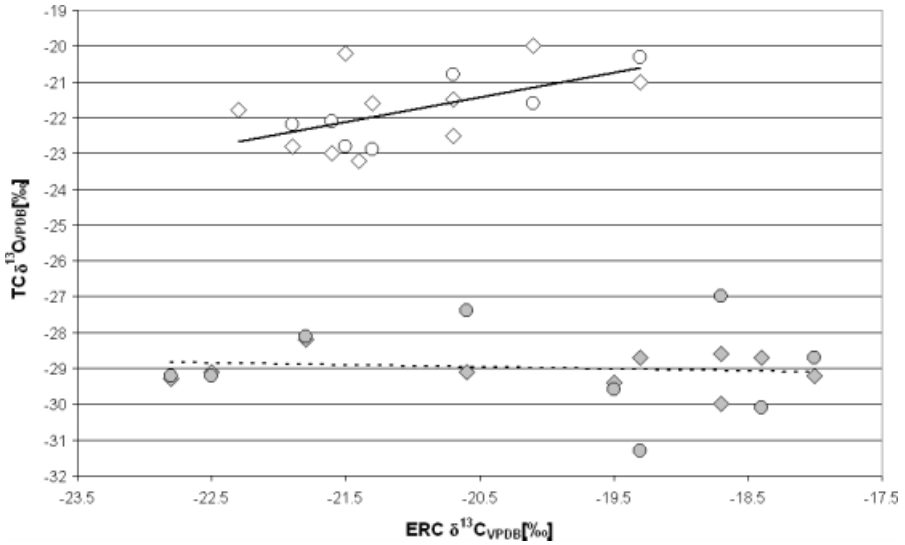

Figure 5. Scatter plot of $\delta^{13}C$ values for all pairs of PD (ERC) and TCs. Circles represent pairs of PD and Bo, diamonds of PD and BM1. Open symbols represent unsuspicious samples, grey symbols suspicious ones. The trendlines demonstrate the linear correlations between the suspicious (dashed) and unsuspicious samples (continuous). Further information in the text.

by more than 4‰. Taking into account the larger SD for small sample amounts a Δ value of 4‰should be favoured as threshold. The chance for a false-positive finding would be minimized and the power of discrimination against exogenous Bo would still be sufficient.

In Figure 5, pairs of PD $\delta^{13}C$ values and belonging TCs are depicted. For each sample the $\delta^{13}C$ value of BM1 is plotted against the corresponding value of PD. The same was done for Bo. The considerable separation of suspicious and unsuspicious sample is visible. Another interesting point is the correlation of the $\delta^{13}C$ values of ERC and TCs within the different samples. For all doping control samples with depleted TCs, no correlation was found at all (Pearson $r = 0.098$; $p = 0.690$). In contrast, in all samples with 'endogenous' $\delta^{13}C$ values, a significant correlation between the ERC and the TCs was found ($r = 0.584$; $p = 0.014$). This correlation indicates the endogenous production of Bo and BM1, and the missing correlation within the depleted samples indicates the administration of Bo. Usually all endogenous steroids excreted in urine show a very good correlation amongst each other.[24] This correlation is caused by the fact that all steroids are metabolized from a single precursor, cholesterol. So the correlation found between the $\delta^{13}C$ values of Bo and BM1 and the ERC indicates that both TCs reflect the individual endogenous value which only can be explained with an endogenous production of these steroids.

Out of the 12 suspicious samples (Table 3) two were investigated twice. Samples #8/08 and #14/09 were the same test sample send to the Cologne Anti-Doping Laboratory in consecutive years. For both sample preparations and measurements, the results were identical, demonstrating the high performance of the method. Samples #10/08 and #11/08 were the A and the B sample, both tested in the Cologne laboratory. Within measurement precision, the results are in agreement, but not as good as for the test sample. This might be due to lower urinary concentrations, especially for Bo.

Out of the 11 samples (Table 4) not showing depleted $\delta^{13}C$ values, two specimens are very interesting (#17/09 and #18/09). Both samples were collected from the same athlete at a time interval of 1 month. The urinary concentrations and the measured $\delta^{13}C$ values are equal. As the half-life for orally administered Bo is approx. 10 h[7] and the estimated half-life for a depot preparation (usually Bo-ester) around 10 days, it is impossible to attribute the found Bo to one administration. Both samples were collected without informing the athlete in advance. So even with a Bo preparation exhibiting an 'endogenous' $\delta^{13}C$ value (vide infra), it is almost impossible for the athlete to provide two urine samples with the low equal concentrations found. This is further strong evidence for the possibility of endogenous Bo production.

Boldenone standards and preparations

Several different preparations and standards were investigated in order to elucidate the $\delta^{13}C$ values for Bo. Pharmaceutically produced anabolic steroids usually range from -27 to -33‰ in their $\delta^{13}C$ values.[23] In general, the expected values were found as can be seen in Table 5. Only one, a steroid preparation for intramuscular injection for horses produced in 1985, exhibits a $\delta^{13}C$ value of -22.7‰. It is therefore indistinguishable from the ERCs of a European athlete. Fortunately, this preparation is not available anymore and none of the current available pure substances or preparations exhibited $\delta^{13}C$ values are more enriched than -6.3‰.

In one solution (sample 6), bought on the black market via the World Wide Web, no Bo at all was present.

Conclusion

The described method enables the measurement of $\delta^{13}C$ values of urine specimens containing low amounts of Bo and BM1. The validity of the method was carefully investigated and urinary concentrations as low as 2 ng/mL could be determined with good repeatability and reproducibility. Throughout the sample preparation, no isotopic fractionation was detectable.

With help of this method, a definitive discrimination between endogenous and exogenous Bo and BM1 is possible. Bo production by athletes seems to be quite rare with annual occurence limited to 3 or 4 cases. With the constraint of one old Bo preparation

Table 5. $\delta^{13}C_{VPDB}$ of Bo standards and solutions suitable for injection (mean values of n = 3 measurements). * The Bo-sulfate was deconjugated before measurement by acidic solvolysis and 1,4-Androstadien-3-on-17-hydroxy-17-methyl was also found in this standard ($\delta^{13}C_{VPDB} = -32.0‰$).** This preparation did not contain any Bo

Sample	Name	Supplier	Year	Lot	$\delta^{13}C_{VPDB}$ [‰]	SD
1*	Androsta-1,4-dien-17ß-ol-3-one-sulfate	Steraloids	2005	TL1306	−27.5	0.4
2	1-Dehydrotestosterone	Sigma	unknown	29F-0410	−27.4	0.1
3	VETRANAL®	Riedel-de Haen	2005	3036X	−30.3	0.2
4	Eldorben	Squibb	1985	4H2890	−22.7	0.1
5	Ganabol	Laboratorios V.M. Ldta	2004	4Q179	−26.3	0.2
6**	Ganabol	Laboratorios V.M. Ldta	2009	1Z203		
7	Boldabolic	Asia Pharma	2008	AP-001B1	−27.1	0.1
8	Boldabol 200	British Dragon Pharmaceutics	2006	unknown	−27.7	0.1

found with 'endogenous' $\delta^{13}C$ value, which enables athletes to administer Bo and go undetected by GC/C/IRMS, the presented data are strong evidence of endogenous Bo production. The case of one athlete with low amounts of Bo and BM1 in his specimens collected at a time interval of 1 month and showing similar $\delta^{13}C$ values, supports a conclusion of endogenous Bo production in humans. The same holds true for the correlation found between Bo and BM1 and the ERC in unsuspicious samples.

This has to be taken into account for doping control samples to avoid false-positive test results. Application of GC/C/IRMS on samples with low urinary Bo or BM1 concentrations is highly recommended.

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