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# A Method to Determine $\delta^{13}C_{VPDB}$-Values of Trace Amounts of Boldenone and its main Metabolite

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**Abstract**

For more than 10 years the possibility of endogenous boldenone production in human beings has been discussed. This is an interesting issue for doping control as boldenone is a prohibited substance according to the WADA list.

Boldenone (androsta–1,4–dien–17β–ol–3–one, Bo) is an anabolic steroid known to be used in cattle mast or equine doping for many years. While testing cattle in order to detect the misuse of forbidden growth hormones, it became obvious that false-positive boldenone results may occur due to faecal cross-contamination during sampling. Further investigations demonstrated the capability of different intestinal bacteria to produce enzymes witch exhibit $\Delta^1$-steroid dehydrogenase activity. Bacteria of this species can also be found in the human intestine.
Although Bo is not clinically approved for human application, there were several cases of athletes tested positive for boldenone or its main metabolite 5β-androst-1-en-17β-ol-3-one (BM1).
By GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) it is possible to analyse either Bo or BM1 and to distinguish whether the source is endogenous or exogenous. Both steroids exhibit urinary concentrations between 1-100 ng/mL. To obtain the required amount of steroid a large specimen and an effective method are unavoidable to fulfil the requirements for a reliable IRMS – measurement.
The developed method together with first results concerning doping control samples will be presented.

## 1. Introduction

Boldenone is a well known anabolic steroid used in cattle mast or equine doping for many years.[1,2] Although it is not clinically approved for human application, there were several cases of athletes tested positive for boldenone or its main metabolite BM1[3]. More than 10 years ago the suspicion raised that trace amounts of boldenone and its metabolites may be of endogenous origin in humans[4] as it could be demonstrated for cattle before.[5]

The possible sources of Bo or BM1 may be testosterone or androst-4-en-3,17-dione entering the intestine via the bile ("enterohepatic route"). There they are 1-dehydrogenated by rare bacteria[6]. The ability of bacteria to produce enzymes with $\Delta^1$-steroid-dehydrogenase activity has been shown in the literature as example for *Arthrobacter simplex*[7] or *Corynebacterium simplex*[8], both can transform hydrocortisone to prednisolone. Especially the second one converts testosterone directly to Bo (personal communication, M. Parr). Reabsorption of the formed 1,4-dien-steroid into the circulatory system followed by common metabolic clearance lead to boldenone- or BM1-glucuronide in urine specimens.

Several attempts were made to confirm the endogenous origin of boldenone in some human beings, but unfortunately there isn´t a clear proof for these cases yet.[4,9]

With GC/C/IRMS it is nowadays possible to ascertain the $^{13}C/^{12}C$-ratios of sample amounts as less as 10ng of carbon per compound. So it should be possible to measure either Bo or BM1, if urinary concentrations fall between 1-100 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 inevitable.

To clarify the origin of Bo it is necessary to compare the measured $^{13}C/^{12}C$-ratio of the target compounds (TC) Bo and BM1 to the $\delta^{13}C$-values of endogenous reference compounds (ERC). $^{13}C/^{12}C$-ratios are expressed as $\delta^{13}C$-values:

$$\delta^{13}C_{VPDB} = \left( \frac{R_{SPL} - R_{VPDB}}{R_{VPDB}} \right) * 10^3 \qquad \text{where} \qquad R = {}^{13}C / {}^{12}C$$

Differences between compounds are expressed as $\Delta\delta$-values:

$$\Delta\delta = \delta^{13}C_{TC} - \delta^{13}C_{ERC}$$

As Bo is neither metabolized to androsterone (A), etiocholanolone (E), 11β-OH-androsterone (11OHA), pregnanediol (PD) or 16-androstenol (16EN)[3], all of these steroids may be used as ERC. And if really testosterone is the precursor for endogenous Bo, this would represent an interesting ERC, too.

Expected $\delta^{13}$C-values for athletes are in the range from –17 ‰ to -25 ‰, depending on the provenience of the athlete[10]. Boldenone preparations available on the market should exhibit values of -27 ‰ or even lower, if testosterone used as precursor in chemical synthesis.

## 2. Method

Analytes have to be cleaned up carefully 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 requirements have to be met for a valid $^{13}C/^{12}C$ determination.[11, 12]

Hence sample preparation encompasses a solid phase extraction, an enzymatic hydrolysis with β-glucuronidase followed by a liquid-liquid-extraction with TBME (detailed description of the method can be found elsewhere).[13]

To achieve the necessary purification and separation of the relevant steroids by HPLC, two consecutive and different runs are required. The first separation is performed on a RP18-column from Merck ( LiChroCART®250-4, LiChrospher®100, 5 µm), gradient acetonitril/ water 30/70 to 100 % acetonitril (Merck, HPLC grade) in 25 min, hold for 5 min and reequilibration, flow 1 mL/min. As visible in figure 1, within this clean up step it is possible to separate Bo together with 11OHA and 11β-OH-etiocholanolone (11OHE) and BM1 together with 5α- and 5β-androstane-3α,17β-diol (5α-, 5β-Diol).

The reference material for Bo was obtained from Sigma-Aldrich (VETRANAL®) and the BM1 was synthesized in our laboratory.[3]

### *2.1 HPLC-Fraction I*

As Bo and 11OHE show the same retention time on the chosen GC setup (parameters see below), a second HPLC run is unavoidable. For this separation a NP-HPLC run was performed: Macherey-Nagel EC250/4.6 Nucleosil 100-5 $N(CH_3)_2$, gradient n-hexane/isopropanol 96/4 to 80/20 in 20 min, 20/80 for 5 min and reequilibration for 6 min,

flow 1.3 mL/min (both solvents from Merck, HPLC grade). Under these conditions Bo is well separated from 11OHE. Afterwards Bo is ready for IRMS-measurement; however 11OHA is still present in the Bo-fraction. As the concentration of 11OHA is often more than 20-fold higher than that of Bo, there is always a huge overload of the column (see peak shape in figure 2). But as far as we could see, this has no effect on the δ-values of Bo.

*2.2 HPLC-Fraction II*

The clean up for BM1 turned out to be more complicated. In fraction II of the RP-HPLC run there are many different steroids. One is still unidentified and coelutes with BM1 on the GC-column. Even the NP-HPLC was not sufficient to separate BM1 from all co-elutions. More promising was the acetylation of fraction II with 50 µL pyridine and 50 µL acetic anhydride (both Merck, analytical grade) at 70°C for 45 min. Afterwards the former mentioned co-elution disappeared, but the acetates of 5α- and 5β-diol have retention times like BM1-acetate on the GC-column. By a subsequent RP-HPLC run (same column and gradient as above) these compounds could be separated without difficulty. The following IRMS-measurements showed no coeluting peak (figure 3). This was confirmed by GC/MS-analysis.

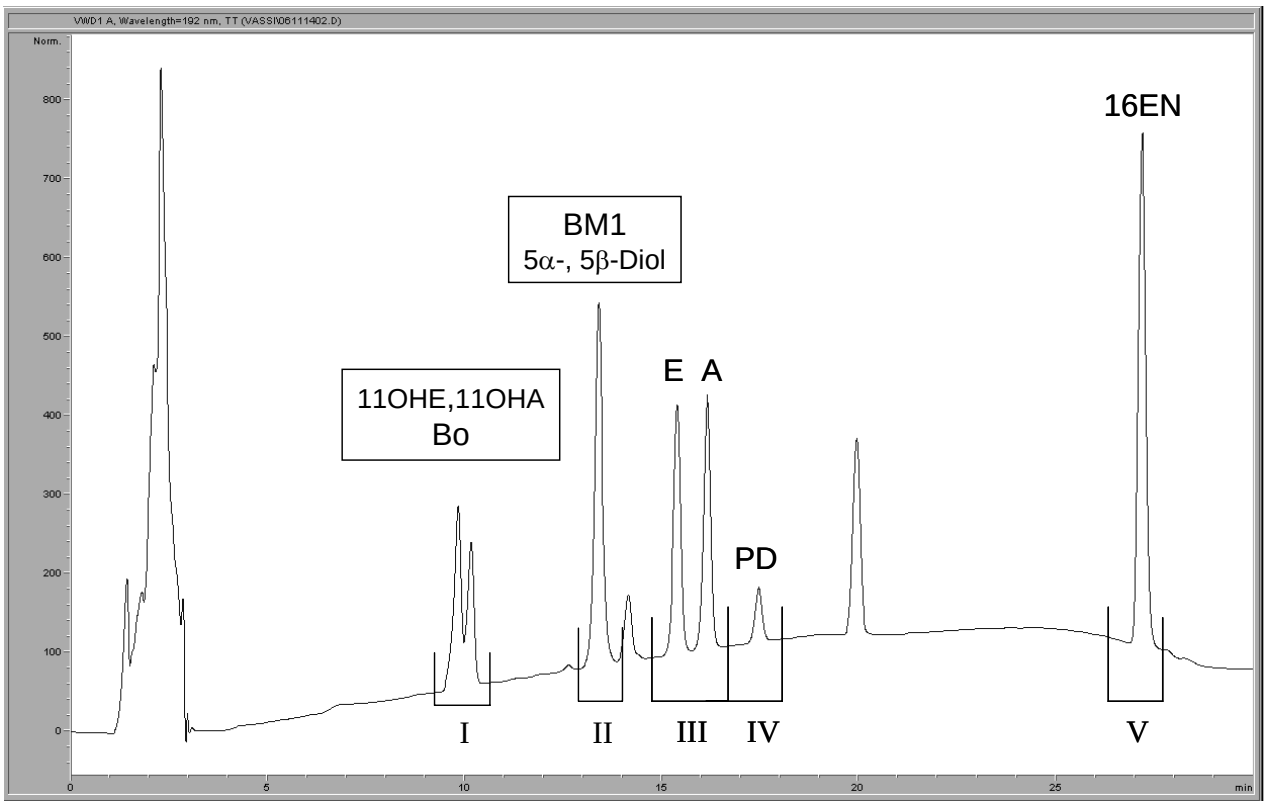


Figure 1: HPLC-UV chromatogram of a standard mixture measured to ascertain the retention times of different steroids. Five fractions are collected, the unlabelled peak is the reference standard.

*2.3 GC/C/IRMS*

All samples were measured on a HP5890 Series II Gas Chromatograph (Agilent Technologies) coupled to a Delta C gas isotope ratio mass spectrometer (ThermoElectron) via the GC Combustion Interface II (ThermoElectron). The GC-system was equipped with an OPTIMA δ3 (Macherey & Nagel) column (length 20 m, an i. d. 0.25 mm, film thickness 0.25 µm). Injection was performed cool on-column.A a retention gap of 1 m length (0.53 mm inner diameter, HMDS deactivated, BGB Analytik) was used. The very low concentrated Bo and BM1 samples were dissolved in 5 µL of acetone (GC grade, Merck). Out of this solution 3 µL were injected. The higher concentrated samples were dissolved in 10 µL and 2 µL were injected at a temperature of 50°C. The cool on-column injection unit is always held on a temperature 3°C above that of the GC-oven. Hereby the transfer from the point of injection into the GC for all analytes is ensured independent from there boiling point. After holding the initial temperature for 0.5 min it is increased with 30°C/min up to 250°C, then with 2°C/min to 270°C, then with 15°C/min to final 295°C, hold for 2 min. Carrier gas is He 5.0 with a constant flow of 2.2 mL/min.

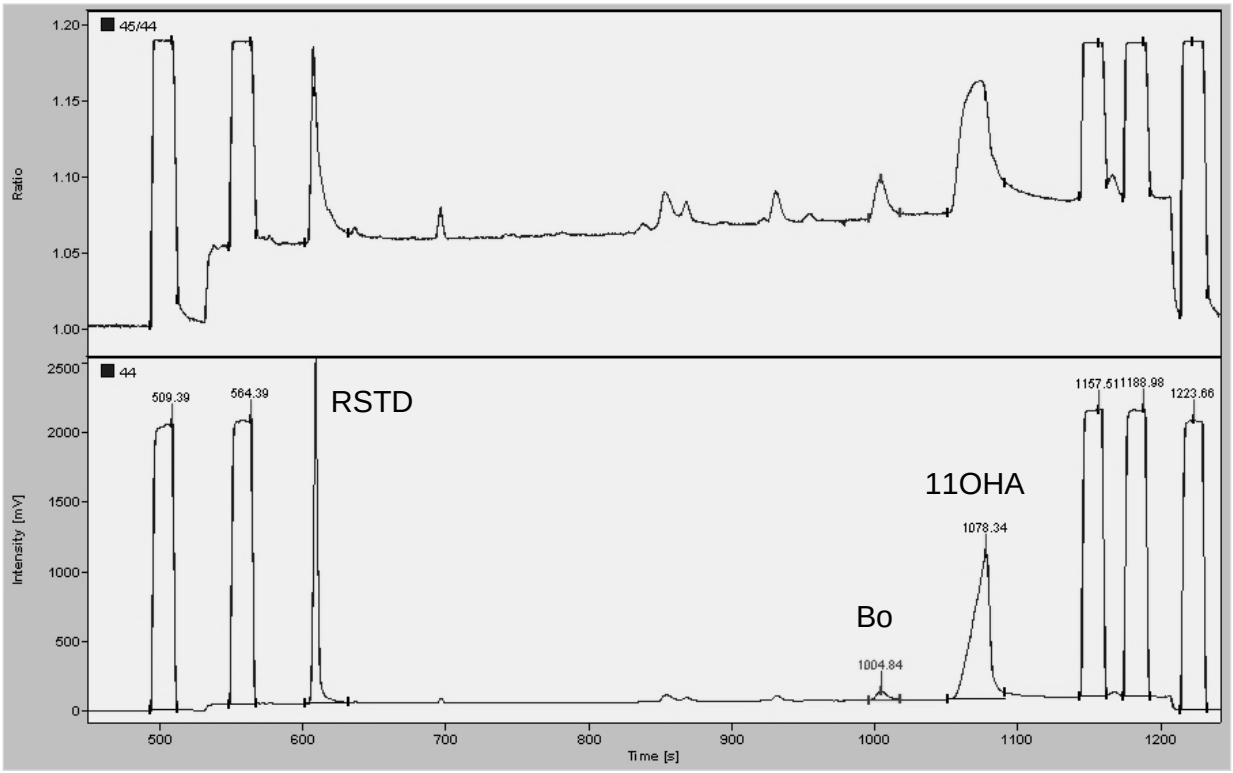


Figure 2: GC/C/IRMS chromatogram of the Bo-fraction of a QC-sample spiked with 8ng/ml Bo.

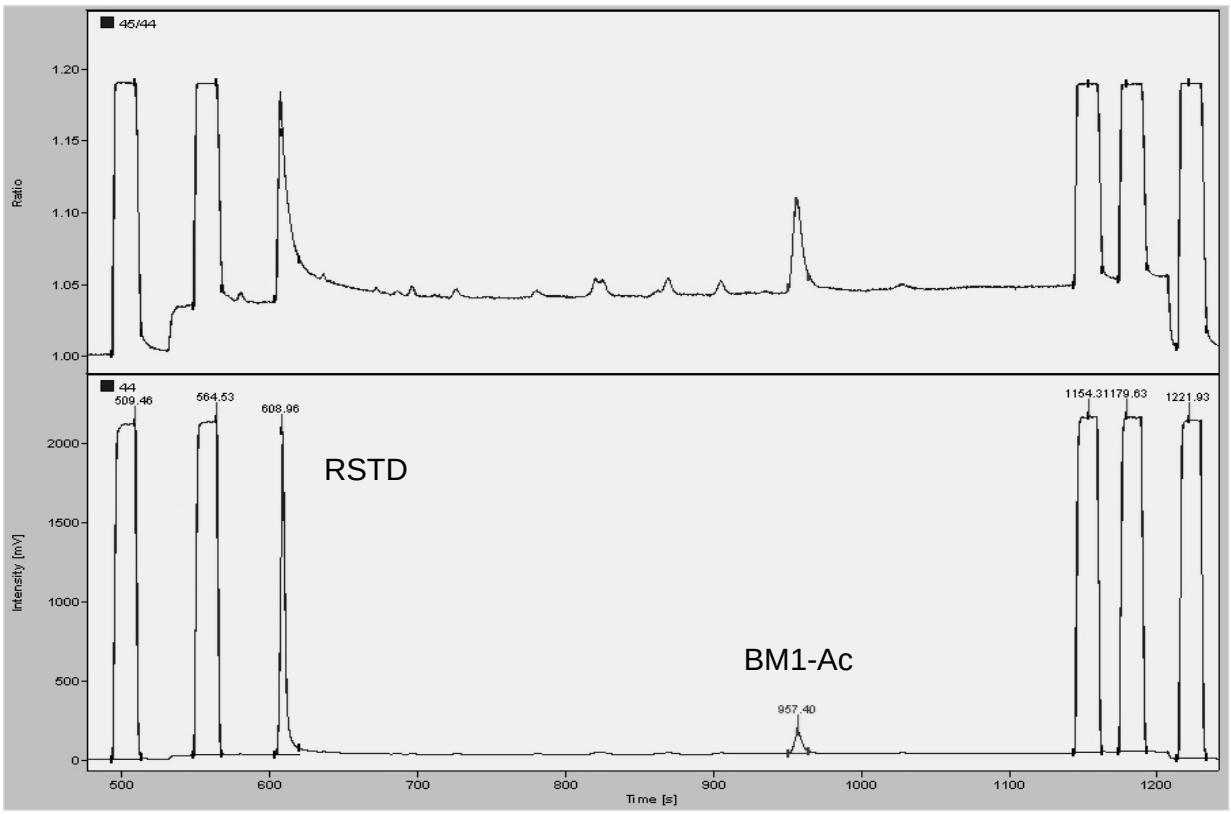


Figure 3: GC/C/IRMS chromatogram of BM1-acetate of a QC-sample spiked with 8ng/ml BM1.

*2.4 GC/MS*

In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in doping control samples, it is necessary to scan the samples on a GC/MS-system using settings comparable to the IRMS-setup. For this purpose we used an GC Agilent 6890 coupled to a MSD Agilent 5973. The column is the same as above, the injections are performed in splitless mode at 300°C. The initial oven temperature of 60°C is held for 1.5 min, than with a heating rate of 40°C/min to 240°C, followed by a ramp with 2°C/min to 260°C, than again with 40°C/min to final 300°C hold for 1.5 min, constant flow of 1.2 mL/min.

**3. Results**

Different steps of the method validation have already been conducted. The specificity of the method has been controlled throughout the whole method development by EI-GC/MS measurements by means of comparison to mass spectra of standards.

In order to check the repeatability of the method boldenone-free urines were spiked with different amounts of Bo and BM1, respectively. The concentration range varied between 2-50 ng/mL per compound. 10 mL of urine were spiked, prepared as described and analysed. For Bo 34 specimens were spiked of which 32 were measurable, the LOD is 2 ng/mL. In figure 4 the scatter of the different measurements around the mean value is shown. During method development the acetylation of Bo after the second HPLC run has been tested, too. As this additional step exhibits no improvement for the measurements, it was skipped finally, but the obtained data are included in this presentation as they show no difference in their δ-values. The pure standard has a δ-value of -30.25 ± 0.19 ‰ (n = 6) and the mean value of the 32 specimens is -30.3 ± 0.9 ‰. So, on the one hand, there is no isotopic fractionation taking place during sample preparation while on the other hand the standard deviation (SD) is rising up to nearly 1 ‰. This is due to the low amount of steroid resulting in a low signal intensity which is known to raise the SD of isotope measurements. Especially the low concentrated samples show a large SD (1.0 ‰ for 2-8 ng/mL vs. 0.7 ‰ for 15-50 ng/mL). More interesting is the fact that there is no bias in the data, so the measured δ-value is independent from the signal intensity. This corresponds with a very good linearity of the IRMS in the low signal range.

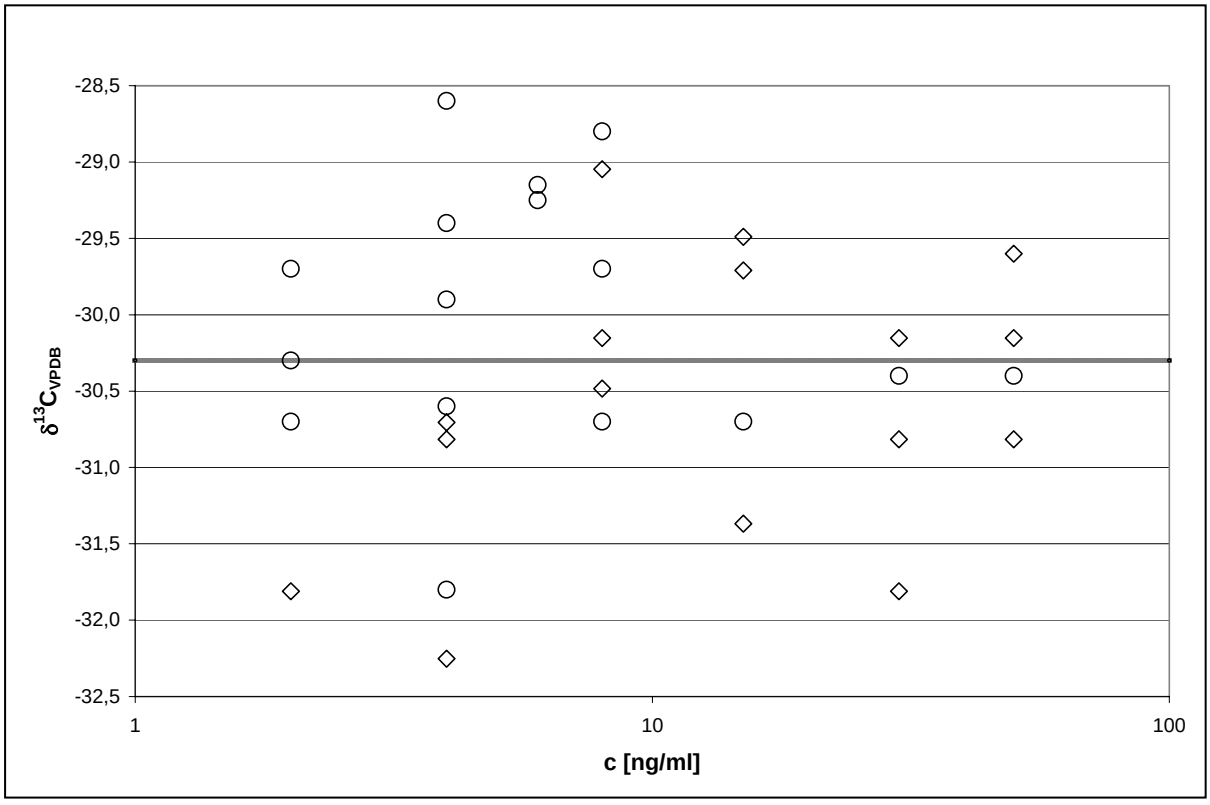


Figure 4: Scatter plot of the measurements referring to the repeatability of Bo. $\delta^{13}C_{VPDB}$ in ‰; c concentration in logarithmical scale; open circles represent Bo measured free, open diamonds acetylated Bo; the bold line points out the mean value.

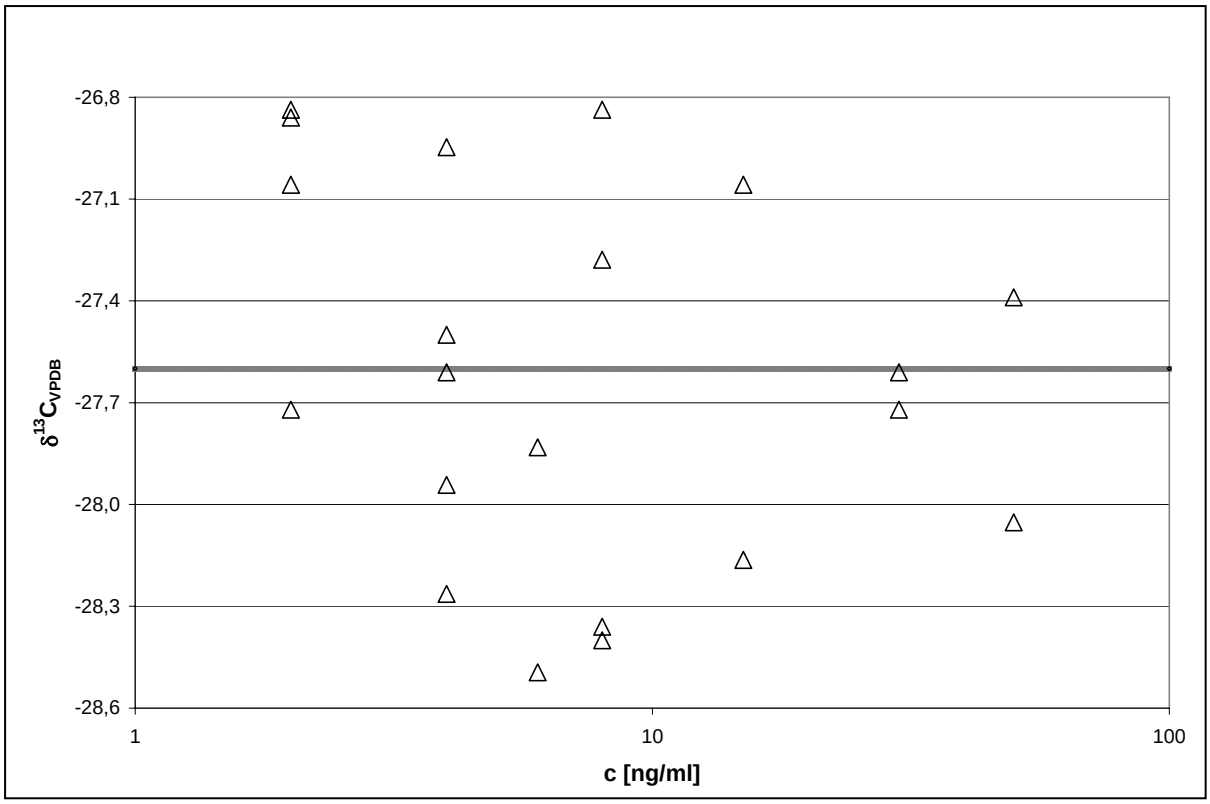


Figure 5: Scatter plot of the measurements referring to the repeatability of BM1. $\delta^{13}C_{VPDB}$ in ‰; c concentration in logarithmical scale; the bold line represents the mean value.

Compareable results were obtained for BM1. 21 out of 22 spiked samples were measurable (LOD 2ng/mL), the data exhibits no bias (figure 5) and again the δ-values of the lower concentrated specimens are afflicted with a larger SD (0.61 ‰ for 2-8 ng/mL vs. 0.41 ‰ for 15-50 ng/mL). The mean value for all 21 measurements is -27.6 ± 0.55 ‰ after correction for the acetate, the pure standard results in -27.7 ± 0.49 ‰ (n = 7). Over all the acetylated BM1 is characterised by a better repeatability and no isotopic fractionation is taking place during sample preparation and acetylation, too.

During screening for anabolic steroids within the last two years, a few samples exhibit a detectable amount of Bo and BM1. If the concentration exceeded a limit of 2 ng/mL, the δ-values of both target compounds and the ERC were determined. In table 1 the results for three samples out of 2006 and three samples from 2007 are listed. By comparing the results for the TC and the ERC, the samples can be divided into two groups. The measured values for sample #1/06 with a relatively high concentration of both TCs suggest an intake of exogenous Bo, as the Δδ-value by far exceeds 3 ‰, it averages 10 ‰. Sample #3/07 shows the same characteristics. The TCs are depleted by more than 8 ‰, so despite the low concentration an exogenous source is likely.

| sample | Bo / BM1 [ng/ml] | E [‰] | A [‰] | PD [‰] | 11OHA [‰] | Bo [‰] | BM1cv [‰] |
|---|---|---|---|---|---|---|---|
| QC | 8 / 8 | -23.2 | -22.6 | -22.1 | -21.9 | -29.7 | -27.8 |
| BW | - / - | -23.4 | -22.6 | -21.9 | -22.3 | - | - |
| #1/06 | ~50 / ~50 | -19.6 | -18.1 | -18.0 | -17.4 | -28.7 | -29.2 |
| #2/06 | 12 / 5 | -21.9 | -20.7 | -20.1 | -20.5 | -21.6 | -20.0 |
| #3/06 | 23 / 5 | -21.7 | -21.0 | -21.5 | -22.0 | -22.8 | -20.2 |
| #1/07 | 3 / 3 | -22.0 | -21.2 | -20.7 | -21.1 | - | -21.5 |
| #2/07 | 2 / 4 | -22.7 | -22.4 | -22.3 | -22.2 | - | -21.8 |
| #3/07 | 5 / 6 | -20,0 | -18,4 | -18,7 | -18,2 | -27,0 | -30,0 |

Table 1: Summary of the results of suspicious samples containing both Bo and BM1. Column 2: estimated concentration of the TC; columns 3-6: δ-values of ERC; columns 7-8: δ-values of the TC.

The other four Bo findings seem to be due to endogenous Bo production. The measured δ-values for the ERC and the TC are equal within the accuracy of the method. Unfortunately this can not be taken as proof for endogenous origin of Bo, because it might be possible, that exogenous Bo exhibits a δ-value similar to that of the ERC.

In order to check for this, different available Bo-preparations were measured. Untill now we found five different preparations. Four obtained the expected values between -27 ‰ to -30 ‰. One of them, a steroid preparation for intramuscular injection for horses produced in the late 80´s, exhibits a δ-value of -23.2‰. It is therefore indistinguishable from ERCs of a European athlete.

## Discussion

The described method enables the measurement of δ-values of urine samples containing low amounts of Bo and BM1. The LOD so far is 2 ng/mL, by utilising larger specimens it might be even lower, especially for BM1. With this tool it is possible to elucidate the origin of these steroids and to distinguish between an endogenous Bo-production and an intake of prohibited substances. As there is the possibility for artificial Bo with δ-values close to the endogenous ones, this is not a clear proof for endogenous Bo-production, but it can be taken as a hint for this kind of abnormal steroid metabolism. Further preparations will have to be analysed in order to minimize the chance of Bo-preparations with a δ-value near to the ERC.

Another interesting object of research would be an athlete who produces Bo over a period of time. By measuring both the concentration and the δ-values of Bo and BM1 over the time it should be possible to demonstrate endogenous Bo-Production.

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