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Investigations in carbon isotope ratios of seized testosterone and boldenone preparations

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Abstract

In order to detect the misuse of testosterone (T) or boldenone (Bo) in doping control analysis, the confirmation of atypical findings employing the determination of carbon isotope ratios (CIR) is mandatory for issuing adverse analytical findings. Elevated concentrations of T (or elevated T/epitestosterone ratios) may result from confounding factors such as ethanol intake, and the presence of low urinary concentrations of Bo can originate from endogenous or urinary in situ production of small amounts of the steroid. As pharmaceutical preparations of Bo and T are generally depleted in ^{13}C, their CIR differ significantly from the ^{13}C-enriched endogenous steroids. Some rare cases have been reported on pharmaceutical preparations showing ^{13}C-enriched isotope ratios that complicate the current application of CIR in sports drug testing. Therefore, the CIR of a subset of $n = 157$ T preparations and $n = 39$ Bo preparations seized in Switzerland and Germany between 2013 and 2018 was analyzed in order to estimate the possible impact of steroid preparations showing ^{13}C-enriched isotope ratios on the current approach to detect their misuse. All investigated Bo preparations showed CIR in the expected range between – 26.7 and −30.3‰. Within the T samples, 95% showed the expected values below −26‰ while six samples fall between −25 and −26‰ and one sample was indistinguishable from endogenously produced T with a CIR of −23.3‰.

KEYWORDS
boldenone, carbon isotope ratio, doping, isotope ratio mass spectrometry, testosterone

1 | INTRODUCTION

The administration of testosterone (T) and boldenone (Bo) is strictly forbidden according to the List of Prohibited Substances issued by the World Anti-Doping Agency (WADA).[1] For both steroids, doping controls are complicated for different reasons. Regarding the detection of the endogenously produced T, doping control laboratories rely on urinary quantification of T and T-related metabolites. Elevated urinary concentrations or concentration ratios like T divided by epitestosterone trigger further investigations on such an atypical sample. As several confounding factors were described that impact these concentrations or calculated ratios, like, for example, ethanol intake, these atypical findings cannot directly be linked to a T administration.[2,3]

Regarding Bo, low concentrations (i.e., up to 20 ng/ml) may be produced in urine in situ due to bacterial contamination or during the enterohepatic circulation in the gut and subsequently be excreted into urine.[4,5] In order to unambiguously prove the exogenous source of urinary T or Bo, isotope ratio mass spectrometry (IRMS)-based

measurements have been implemented into sports drug testing.[6] Comparing the carbon isotope ratios (CIR) of endogenous reference compounds (ERC) such as pregnanediol to T and Bo or direct metabolites of T and Bo serving as target analytes (TC) enables to differentiate between an endogenous production and the illicit administration. CIR values are given in ‰ or mUr with reference to the primary isotopic reference material Vienna Pee Dee Belemnite (VPDB)-based on Equation 1[7]:

$$\delta^{13}C_{VPDB} = \frac{\left(^{13}C/^{12}C\right)_{SAMPLE}}{\left(^{13}C/^{12}C\right)_{VPDB}} - 1. \quad (1)$$

The differences between TC and ERC are given as Δ values:

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

As the calculated Δ values have to exceed a certain threshold to substantiate the administration, δ^{13}C values of exogenous preparations should differ significantly from endogenous values, which commonly fall between −17 and −25‰.[6,8]

While the first published values on CIR of pharmaceutical T-preparations always showed values more depleted than −28‰, already in 2001, the first value of −25.9‰ was reported.[9–11] A comprehensive investigation on seized T preparations in Australia showed a significant overlap with the endogenous CIR range for approximately 9% of $n = 266$ samples.[12] This finding, albeit with a lower percentage of 4% of preparations in the endogenous range, was substantiated 4 years later.[13] In-between, samples investigated in Austria showed a significantly higher percentage of T-preparation with CIR > −25.9‰, albeit referring to a relatively small subset of samples.[14] In Northern Europe, four out of 39 T-preparations were found enriched while the eight investigated Bo samples were all found depleted with CIR < −27.8‰.[15] Regarding Bo, the published CIR values for standards and preparations is scarce. An additional seven preparations, which were investigated in 2010, showed depleted values except for one specimen that exhibited an outstandingly enriched value of −22.7‰.[5]

Based on these data, it deemed necessary to investigate the CIR of seized T and Bo preparations present at the Cologne doping control laboratory and collected between 2013 and 2018 in order to substantiate the earlier findings on doping agents that reported on the presence of drug formulations with endogenous CIR signatures and to elucidate possible trends in more recently sold products.

2 | EXPERIMENTAL

2.1 | Chemicals and steroids

All solvents and reagents were of analytical grade. Potassium hydroxide (KOH), methanol (MeOH), *tert*-butyl methyl ether (TBME), and cyclohexane were purchased from Merck (Darmstadt, Germany). Pyridine was from Sigma-Aldrich (Steinheim, Germany), and acetic anhydride was a blend of reagents purchased from Sigma-Aldrich and Merck and distilled in-house before use. Helium, oxygen, and CO_2 were from Linde (Pullach, Germany). The CO_2 tank gas was calibrated against a secondary reference material USADA 33-1 provided by Cornell University (Ithaca, NY, USA).[16]

Steroid reference material T was supplied by Sigma-Aldrich, Bo by LGC (Wesel, Germany), and 3β-hydroxy-5α-androstane (RSTD) by Steraloids (Newport, RI, USA).

2.2 | Samples under investigation

The majority of samples was seized at the Swiss border in the years 2013 and 2014 ($n = 147$) and have already been described in detail.[17] Additional samples were seized at the German border or during German police investigations in the years 2017 and 2018 ($n = 12$).

A total of 159 T samples was included in the study encompassing mainly propionate and enanthate derivatives or non-esterified T. A significant number of samples contained two or all different chemical forms of T. For these samples, a combined CIR was determined, and no differentiation between the different forms was carried out. Different chemical formulations were investigated encompassing oily solutions, gels, and tablets. Regarding Bo, $n = 39$ oily solutions containing Bo-undecylenate were included in this study.

2.3 | Sample preparation

Fifty microliters of oily solutions containing either T-esters or Bo-esters were placed in a test tube and fortified with 1 ml of methanolic KOH (1 N), vortexed and placed in a heating block for 1 h at 80°C. After cooling, the samples were dried under a stream of air at 50°C and reconstituted with 5 ml of H_2O. After adding 5 ml of TBME, the samples were shaken for 5 min and centrifuged, and the organic layer was transferred to a new test tube and evaporated to dryness. All samples were acetylated by adding 75 μl of acetic anhydride and 75 μl pyridine and incubating the sample for 45 min at 70°C. Afterwards, all samples were dried and reconstituted with 200 μl of cyclohexane and diluted by a factor of 1 to 10,000 depending on the amount of analyte. The diluted samples were first injected into a gas chromatograph (GC) coupled to a time-of-flight mass spectrometer (QTOF) in order to ensure appropriate dilution before being forwarded to IRMS determinations. Samples containing only the non-esterified form of T were not subjected to the alkaline hydrolysis. Tablets were pestled and extracted with 5 ml of MeOH, and after centrifugation, 0.5 ml of the methanolic solution was placed in a test tube for further processing. For T gel, the content of a single bag was dissolved in 5 ml of MeOH and centrifuged, and again, 0.5 ml of the supernatant was further processed.

2.4 | Gas chromatography-time of flight mass spectrometry set up

All samples were injected on an Agilent 7890A GC coupled to an Agilent 7200 Accurate-Mass Q-TOF (Santa Clara, CA) system in order

to check sample dilution and purity prior to the IRMS determinations. Based on the results the final dilution was established. Two out of 159 T samples showed significant co-elutions that hindered valid CIR determinations.

The GC was equipped with an Agilent J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm) column, and the temperature program started at 100°C held for 2 min. Then the oven temperature was ramped at 40°C/min to 260°C and then with 3°C/min to 289°C and finally with 40°C/min to 320°C and held for 5 min. Samples were injected in pulsed splitless mode at 280°C and an injection pulse pressure of 25 psi. The helium column flow was set constant at 1.2 ml/min, and the injection volume was 1 μl. The QTOF acquired data in full scan mode from *m/z* 50 to 800 at an acquisition rate of 5 spectra/s using MassHunter software (version B.06, Agilent). The instrument was mass calibrated prior to each sequence in order to achieve mass accuracy in the range of ±5 ppm.

2.5 | Gas chromatography-combustion-isotope ratio mass spectrometry setup

The gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) system employed consisted of a Delta V Plus isotope ratio mass spectrometer coupled to a Trace GC 1310 equipped with a TriPlus RSH Autosampler (ThermoFisher, Bremen, Germany). Both systems were connected via the GC IsoLink CNH combustion unit operated at 950°C and the ConFlo IV interface (ThermoFisher). Isodat 3.0 (ThermoFisher) was used for data acquisition and evaluation. To ensure specificity and peak purity, a Thermo ISQ single quadrupole mass spectrometer was hyphenated by a micro channel device (SGE, Sydney, Australia) and a restriction capillary (length 5 m, i.d. 0.15 mm, SGE) to the GC column effluent. The mass spectrometer was operated in electron ionization (EI) mode, and total ion chromatograms were recorded from *m/z* 50 to 500 using Thermo Xcalibur (version 2.2).

The GC column was similar to the one employed in the GC-QTOF. The initial oven temperature was 100°C, held for 2 min, and increased with 40°C/min to 273°C, then with 2°C/min to 301°C, then with 40°C/min to 320°C, and finally held for 2 min. Carrier gas was helium with a programmable flow starting with 2.5 ml/min during injection and then with 2 ml/min during the analytical run. Injections were performed using the internal standard double method with 1 μl of a cyclohexane solution containing 40 μg/ml RSTD and 4 μl of sample reconstituted in cyclohexane. All samples were analyzed in duplicate to ensure valid CIR determinations.

3 | RESULTS AND DISCUSSION

All determinations were considered valid if the difference between the first and second injections was |<1.4‰|. Samples showing a larger

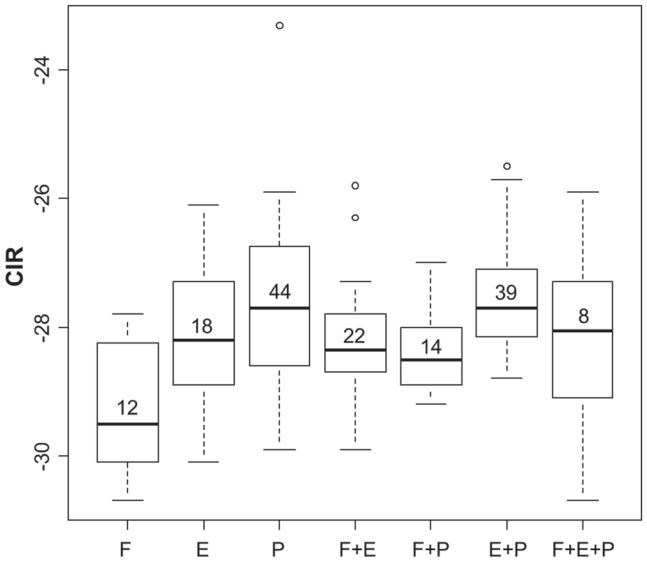

FIGURE 2 Box-plot of the found CIR depending on the chemical form of T and the mixtures of different forms. F stands for non-esterified T, P, for T-propionate esters and E for T-enanthate esters. The number of samples in each class is given above the median. All values in $\delta^{13}C_{VPDB}$ (‰)

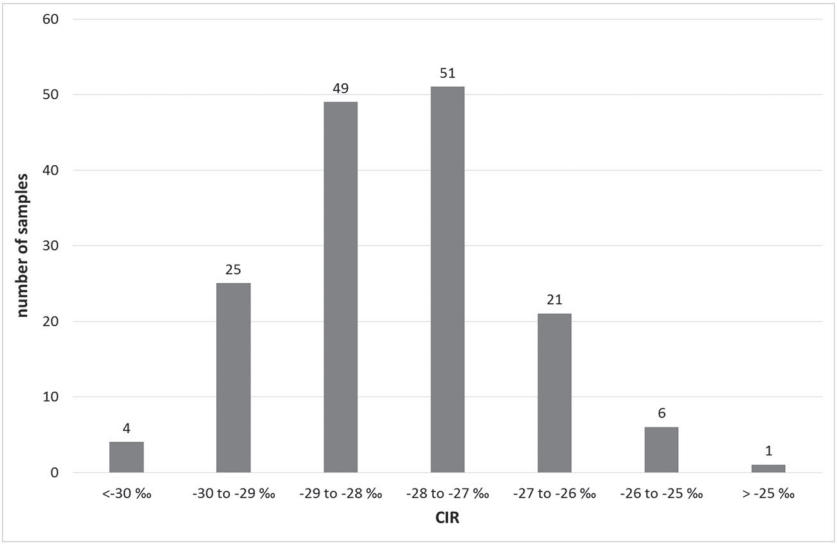

FIGURE 1 Frequency distribution of $\delta^{13}C_{VPDB}$-values for $n = 157$ testosterone samples

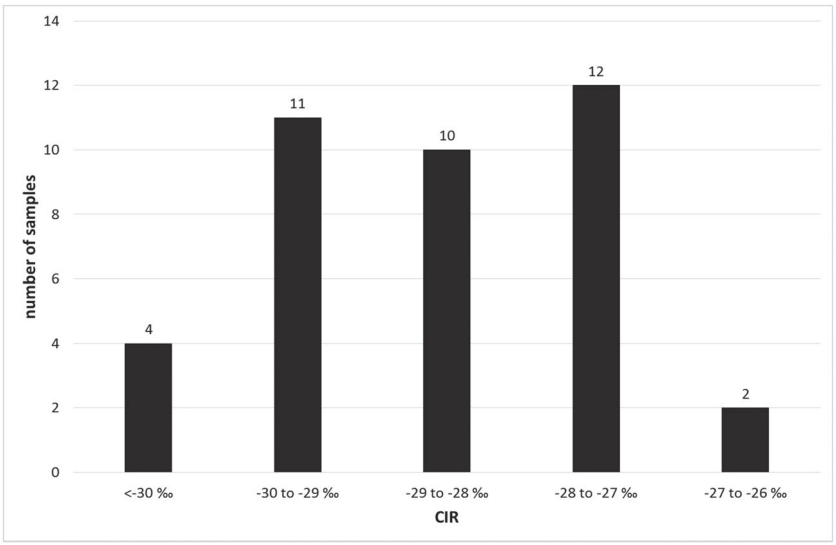

FIGURE 3 Frequency distribution of $\delta^{13}C_{VPDB}$-values for $n = 39$ boldenone samples

deviation ($n = 7$) were re-injected after further dilution that improved the repeatability. The final distribution of differences was found Gaussian distributed (Shapiro-Wilk test, $p < 0.05$) with a mean value of −0.01‰ and a standard deviation of 0.62‰. For all T gel preparations, it was necessary to add a solvent injection after each measurement due to high boiling compounds that otherwise did impact the subsequent analysis.

3.1 | Preparations containing testosterone

All results obtained on the $n = 157$ samples seized between 2013 and 2018 are summarized in Figure 1. The overall distribution of CIR was found highly comparable with the already published data encompassing $n = 283$ T preparations published by Brooker et al.[13] Again, the number of samples showing enriched CIR (i.e., $\delta^{13}C_{VPDB}$-values larger than −26‰) was below 5% encompassing only one T preparation with a clearly endogenous CIR at −23.3 ± 0.36‰ ($n = 3$). The relatively large percentage of enriched T preparations (more than 60%) reported by Forsdahl et al. may be due to the unique subset of samples investigated in this study and seems not to represent a common trend for black market products.[14] These are promising results for doping control analysis as in general in can be expected that illicit administration of T will be detectable employing IRMS. Especially as even in those cases where T preparations with a CIR signature close to the endogenous one have been administered, a detection of the misuse may be feasible in the near future.[18]

Differentiating the found CIR depending on the chemical form of T, that is, in non-esterified form (F), as propionate-ester (P), or as enanthate-ester (E), did not show a clear trend as depicted in Figure 2. A tendency to more depleted values found in those samples containing non-esterified T is visible, but taking into account the different number of samples in each group this finding should not be over interpreted.

Comparing the $n = 12$ samples collected in the years 2017 and 2018 to the much larger number of samples collected in 2013 and 2014 did not show any significant difference. Therefore, a time-dependent change in CIR for illicit T preparations has not been recognized during this study.

3.2 | Preparations containing boldenone

Within this first comprehensive investigation on CIR of a larger subset of Bo preparations, a relatively homogenous and depleted distribution of values was found as depicted in Figure 3. Even the two most enriched preparations showed values of −26.9 and −26.7‰ and should therefore be well distinguishable from endogenously produced steroids. Unfortunately, only one Bo sample seized 2018 was in the subset of Bo preparations, so no possible time-dependent trend could be established here as has been reported for nandrolone, another pseudo-endogenous steroid.[19,20] The already reported sample with a CIR of −22.7‰ dating back to 1985 was substantiated to be a rare exemption.[5]

4 | CONCLUSIONS

In order to prove the illicit administration of black market products, all available preparations beneficially employ a significantly different CIR compared to steroids produced inside the body. Usually, pharmaceutical preparations tend to show more depleted values in ^{13}C compared with endogenous steroids and for the vast majority of preparations investigated here this holds true. Only one T-propionate was found to fall perfectly into the endogenous range with a CIR value of −23.3‰, while the six samples found between −25 and −26‰ may only become problematic in athletes' samples from Northern Europe where endogenous CIR values of −24 to −25‰ are commonly observed. For the majority of athletes, an administration with such a preparation

might directly lead to an adverse analytical finding, or at least an atypical finding can be concluded. The overview gained on the Bo preparations was very promising from the anti-doping perspective as here none showed CIR in the endogenous range. A similar trend as recently described for nandrolone preparations was not reflected here.

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