Research article

Drug Testing and Analysis

Received: 27 June 2011 Revised: 15 September 2011 Accepted: 16 September 2011 Published online in Wiley Online Library: 18 November 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.373

Carbon isotope ratio determination and investigation of seized testosterone preparations

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In the present study, the content of a number of black market testosterone products collected in Austria has been analyzed. Additionally, $^{13}C/^{12}C$ ratios were measured for testosterone in the products after cleavage of the testosterone ester. The aim was to determine whether some of these products had similar $^{13}C/^{12}C$ ratios to those normally found for endogenous testosterone, which could prevent a positive isotopic ratio mass spectrometric (IRMS) finding in doping control. Moreover, it was investigated to what extent the preparations contained the masking agent epitestosterone, in order to lower the testosterone/epitestosterone (T/E) ratio in urinary steroid profiles. Out of 30 analyzed products, the declared ingredients differed from the actual content in 10 cases. Epitestosterone, however, could not be found in any of the products. The products displayed $\delta^{13}C_{VPDB}$ values between −23.6 and −29.4‰. For more than half of these products, the values were within a range reported for endogenous urinary steroids. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: IRMS; testosterone; doping; black market products

Introduction

Determining the origin of testosterone and other steroids in human urine is a major issue in doping control. According to the latest published laboratory statistics of 2009 from the World Anti-Doping Agency (WADA),[1] 65% of reported adverse and atypical analytical findings by the accredited anti-doping laboratories belonged to the substance group of anabolic agents. Out of these, testosterone is by far the most common agent reported, constituting as much as 70% of the findings.

An atypical analytical finding of testosterone, however, must not be confused with an anti-doping rule violation. The detection of the administration of testosterone is primarily based on a population reference interval of a urinary ratio of testosterone to epitestosterone, excreted as glucuronide, referred to as the T/E ratio.[2,3] Since it is well known that natural outliers of a normal steroid profile exist,[4,5] a further investigation is mandatory after a finding of an elevated T/E ratio. For this reason, samples with a T/E ratio that equals or exceeds 4 are amongst other parameters recommended to be submitted to isotope ratio mass spectrometric (IRMS) analysis.[6]

The IRMS analysis is based on differences in isotope ratio between endogenous and synthetic testosterone and provides the basis for confirmatory analysis.[7–15] The $^{13}C/^{12}C$ ratio in natural compounds, such as steroids, is determined by the pathway by which they were produced.[16,17] Consequently, synthetic testosterone is generally less enriched in ^{13}C and shows a different $^{13}C/^{12}C$ ratio than human endogenous testosterone.

Out of the samples with T/E ratios above 4 submitted to IRMS analysis in our laboratory, about 8% are reported as adverse analytical findings for the application of synthetic testosterone or testosterone prohormones. One possible explanation for some of the negative findings might be the use of testosterone with endogenous-like delta values. The isotopic ratio depends on the manufacturing process and on the carbon feed stocks of the starting materials. Hence, it could be possible to produce testosterone products with carbon isotope ratios in, or close, to the endogenous range, by using ^{13}C enriched starting materials. Thus, one objective of the presented investigation was to estimate the extent of the availability of such preparations.

A further source to circumvent the detection of testosterone doping could be the addition of epitestosterone to doping preparations in order to lower the T/E ratio after their application. Epitestosterone is analytically used as an indirect marker of testosterone administration. Conventional anabolic steroid screening includes the determination of the T/E ratio as part of the urinary endogenous steroid profile. Epitestosterone is a steroid with no anabolic activity, but since its administration will lower the urinary T/E ratio and mask testosterone administration, it is prohibited in sport.[18] Epitestosterone is not available as an approved pharmaceutical; it can, however, be bought in bulk from chemical companies.

In this work, the analysis of the content of 30 different black-market testosterone products collected by the Austrian police as part of seized doping substances are described. Additionally, the $\delta^{13}C_{VPDB}$ values of the free testosterone after cleavage of the ester are reported.

Experimental

Materials

Confiscated testosterone products were kindly provided by a special police force of Lower Austria (LKA Niederösterreich, St Pölten, Austria). All products were oily solutions for intramuscular (i.m.) application. Testosterone esters for confirmation of the

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ingredients of the black market products, trimethylsilyl-ethanthiol and methyl-t-butyl-ether (TBME) were supplied by Sigma (St Louis, MO, USA), whereas epitestosterone, testosterone and d3-testosterone were obtained from the National Measurement Institute (Sydney, Australia). 5α-androstane-3β-ol (androstanol) was obtained from Steraloids (Newport, RI, USA) and 17β-estradiol-3,17-diacetate (estradioldiacetate) was supplied by Honeywell Riedel-de Haen (Seelze, Germany). Hydrochloric acid (HCl), potassium hydroxide (KOH), ethyl acetate, isopropanol (all analytical grade), diphosphorus pentoxide (P_2O_5) (extra pure) and all solvents used for HPLC analysis (HPLC grade) were purchased from Merck (Darmstadt, Germany). Methanol (analytical grade) was obtained J.T. Baker (Deventer, Netherlands) and N-methyl-N-trimethylsilylftrifluoroacetamide (MSTFA) was supplied by Marchery and Nagel (Düren, Germany). Purified water was obtained by a Milli-Q reagent grade water system (Millipore, Bedford, MA, USA).

Reagents and solutions

Working solutions of the testosterone ester ampoules were prepared by diluting an aliquot of each ampoule (1:50 – 1:200) in isopropanol. A high performance liquid chromatography (HPLC) solution was prepared by dissolving estradioldiacetate in methanol at a concentration of 20 μg/ml. For GC-C-IRMS analysis a solution of androstanol (25 μg/ml; injection standard) was prepared in TBME. An internal standard (IS) working solution for gas chromatography-mass spectrometry (GC-MS) preparations containing 50 μg/ml d3-testosterone was prepared in methanol. All solutions were stored at 4 °C.

A trimethylsilyl iodide (TMSI) stock solution was prepared by adding 5 ml MSTFA to 100 mg NH_4I and 300 μl trimethylsilyl-ethantriol. A TMSI working solution was then obtained by mixing 1 mL of the TMSI stock solution with 9 ml MSTFA. The mixture was stored in the dark at room temperature.

Sample preparation for GC-MS analysis

Two different sample preparations were performed on the products. On one side the esters were hydrolyzed prior to extraction, whereas on the other side testosterone esters were directly analyzed without any hydrolysis. All preparations were performed in duplicate.

Hydrolysis was performed by adding 0.5 ml 1 M methanolic KOH to 5 μl of the testosterone ester working solutions and 50 μl of the IS working solution. After 2 h at 60 °C, 0.5 ml of 1 M HCl and 1 ml 20% carbonate buffer pH 9.8 were added. A liquid-liquid extraction was performed with 7 ml, of TBME. After evaporation and drying over P_2O_5, 100 μL TMSI working solution were added, and the mixtures were heated for 20 min at 60 °C.

The non-hydrolyzed samples were prepared by derivatizing 5 μl aliquots of the testosterone ester working solutions after evaporation of the solvent. Derivatization was performed as described above. Additionally, underivatized extracts of the products were prepared by adding 100 μl ethyl acetate to 5 μL of the testosterone ester working solutions.

All samples were analyzed with GC-MS in scan mode as described in the next section.

GC-MS analysis of the content of the confiscated products

GC-MS analysis was carried out using a Trace 2000 gas chromatograph coupled to a DSQ mass spectrometer (Thermo Quest, Austin, TX, USA). Separation was performed on a Rtx-1MS fused silica capillary column, 15 m x 0.25 mm ID, 0.1 μm film thickness (Restek, CP-Analytica, Mistelbach, Austria). Helium was used as a carrier gas, and the inlet pressure was set to 60kPa. Oven temperatures were programmed as follows: 175 °C initial column temperature, 3 °C/min to 210 °C, 45 °C/min to 305 °C, held for 3 min. The injection port temperature was set to 270 °C, and all the injections were performed in the split mode (1:15 split ratio), using an injection volume of 3 μl.

The mass spectrometer was operated in the electron impact (EI) mode at an ionization energy of 70 eV. The ion source temperature was 270 °C, and the GC-MS interface was set to 280 °C. Analyses were performed in scan mode (70–600 *m/z*).

Sample preparation for GC-C-IRMS analysis

All samples were hydrolysed by adding 2 ml 1 M methanolic KOH to 200 μl of the testosterone working solutions. Hydrolysis and extraction of all samples were performed as described under section *Sample preparation for GC-MS analysis*, except for using larger aliquots (200 μl testosterone ester working solutions, 2 ml 1 M KOH, 2 ml 1 M HCl and 2 ml carbonate buffer pH 9.8). The samples were evaporated to dryness before being reconstituted in 200 μl TBME and transferred to HPLC autosampler vials. After evaporation of the solvent 100 μl of HPLC solution were added.

Subsequently, a HPLC purification was performed on a C18 column (MZ-AquaPerfekt RP-18, 5 μm, 200 x 4,6 mm, MZ-Analysentechnik, Mainz, Germany) with a Thermo Scientific-Surveyor HPLC (Thermo Quest, Austin, TX, USA) coupled to a Thermo Scientific-Surveyor PDA detector (Thermo Quest, Austin, TX, USA). The flow rate was set to 1 ml/min and the eluate monitored at 190 nm. The fraction collector Gilson FC 204 (Gilson, Middelton,WI, USA) was programmed to dispense column effluent into separate test tubes at given intervals. Testosterone was collected between 12.2 and 13.7 min. The purified fraction was evaporated to dryness, dried over P_2O_5 in vacuum and finally reconstituted in 100 μl GC-C-IRMS solution.

After the hydrolysis step, one aliquot of sample extract was derivatized and analyzed by full scan GC-MS under the conditions described under section *GC-MS analysis of the content of the confiscated products*. This was to ensure completeness of basic hydrolysis of the esters.

GC-C-IRMS analysis

An Agilent 7890 GC instrument (Hewlett Packard, Palo Alto, CA, USA) was equipped with a RTX-35Sil MS capillary column, 30 m x 0.25 mm ID, 0.25 μm film thickness (Restek, CP-Analytica, Mistelbach, Austria) with helium as carrier gas. Injections were made using a PTV injector in solvent vent mode. The injector temperature was increased from 30 °C (0.15 min) to 300 °C (2.5 min) at 11 °C/s, then to 340 °C (3 min) at 10 °C/s. The GC oven was programmed from 130 °C (1 min) to 240 °C (30 °C/min), and finally increased to 300 °C (4 °C/min), held for 2 min. The injection volume was set to 2 μl.

The outlet of the GC column was connected to a GC Isolink interface and a Delta V isotope ratio mass spectrometer (Bremen, Germany). The combustion furnace was operated at 940 °C.

Reference carbon dioxide gas pulses were used to determine the $\delta^{13}C_{VPDB}$ values. The reference gas was calibrated using a testosterone reference standard (National Measurement Institute,

Sydney, Australia) with a $\delta^{13}C_{VPDB}$ value assigned by independent elemental analysis (−29.8‰).

Quality control

As part of the quality control of the entire analysis, positive and negative control samples were included in every batch of samples. Control samples included testosterone esters for the analyses using GC-MS, as well as blank oil as negative control.

In the case of GC-C-IRMS analysis, stability and reproducibility of the measurements were monitored by determining the $\delta^{13}C_{VPDB}$ values of the testosterone reference standard described in the section above. The mean $\delta^{13}C_{VPDB}$ – value of 9 determinations of the testosterone reference substance analysed before, during and after the sequence of analysis was determined to be −29.8 ‰ +/− 0.2‰. The measured $\delta^{13}C_{VPDB}$ – value of the testosterone reference substance after the sample preparation procedure using blank sesame oil as matrix was determined to be −29.6‰ +/− 0.1‰.

Analysis of the seized testosterone ester preparations was done in duplicate. The analytical results were accepted when the both values were within a range of 0.7 delta units. For the purpose of this publication the value of the first determination was used; the second value was regarded as confirmation of the first determination.

Blank sesame oil used as negative control sample showed no traces of testosterone.

Results and discussion

Content of the ampoules

The analysis of 30 testosterone products revealed that 10 (i.e. 33 %) had an actual content deviating from the declared ingredients. The analysis performed was qualitative, as the aim of the investigation was to identify the ingredients. For this reason deviations in the concentrations of the ingredients other than declared on the investigated products were not ruled out.

Out of 30 products, only 20 matched with the declared ingredients. In one product the declared substance was missing. Furthermore, seven of the products showed the presence of one or more testosterone esters or nandrolone decanoate, in addition to the substances declared. Two products were not labelled but contained testosterone esters. The results are summarized in Table 1.

Before starting the study, one hypothesis was that testosterone preparations to some extent might contain the masking agent epitestosterone, consequently preventing a further investigation of a doping control sample, by lowering the T/E ratio in urine steroid profiles. This could, however, not be confirmed in the present study. Epitestosterone was not found in any of the products investigated.

IRMS analysis

Testosterone esters are almost completely cleaved to the corresponding free testosterone in the human body, and are finally excreted in urine as testosterone glucuronide. After hydrolysis of the glucuronide, free testosterone is one of the markers for testosterone misuse. Hence, only the $\delta^{13}C_{VPDB}$ values of free testosterone after cleavage of the ester were of interest for the purpose of this study.

$^{13}C/^{12}C$ isotope ratios are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB). The $\delta^{13}C_{VPDB}$ value is calculated as follows:

$$\delta^{13}C_{VPDB}(‰) = \left(\left(R_{sample}/R_{standard}\right) - 1\right)x1000 \qquad (1)$$

where R_{sample} is the measured $^{13}C/^{12}C$ isotope ratio for the sample, whereas $R_{standard}$ is the measured $^{13}C/^{12}C$ isotope ratio for a defined standard.

IRMS methods have proven to be very useful in the detection of exogenous androgens. After ingestion of testosterone or a testosterone precursor, a decrease in the $\delta^{13}C_{VPDB}$ value for urinary testosterone and its metabolites is normally observed, due to the lower ^{13}C abundance in synthetic testosterone. Hence, the difference in $^{13}C/^{12}C$ isotope ratio can be effectively used to detect testosterone misuse.[7–15] The diagnostic metabolites selected are in most cases androstandiols, androsterone and etiocholanolone. They are isolated from urine together with an endogenous reference steroid, commonly 11β-hydroxyandrosterone, 16-androstenol or pregnandiol.[19–21] Endogenous reference steroids are compounds not affected by xenobiotics and reflect the diet composition of the athlete. According to the respective WADA Technical Document TD2004EAAS the administration of a synthetic steroid is proven when the $^{13}C/^{12}C$ value measured for testosterone or its metabolites differs by 3.0‰ or more from that of the endogenous reference steroid.[6] Thus, a shift in $\delta^{13}C_{VPDB}$ values in testosterone preparations towards endogenous values would be of great concern regarding detection of testosterone doping in sport.

Absolute $\delta^{13}C_{VPDB}$ values of endogenous steroids have been found to range between a large interval of −16‰ to −26‰, primarily depending on the composition of the diet.[15,16,21–23] Nevertheless, previously published literature has revealed typically $\delta^{13}C_{VPDB}$ values for synthetic testosterone preparations to be significantly lower than human endogenous androgens.[16,17] Often values of about −27‰ and lower are observed. There has, however, been one recent report on analysis of testosterone materials collected in Australia, showing values within the endogenous range.[24]

From the 30 black market products being in the scope of this investigation, only 29 contained one or more testosterone esters. Table 1 summarises the $\delta^{13}C_{VPDB}$ values obtained for all 29 products after sample preparation and analysis, as described under experimental.

Out of the 29 products investigated, 11 confirmed to have $\delta^{13}C_{VPDB}$ values below −26.6, and are in the expected range for synthetic testosterone preparations. As much as 18 samples, however, displayed $\delta^{13}C_{VPDB}$ values inside the interval reported for endogenous urinary metabolites. This constitutes 62 % of the testosterone products investigated. The endogenous $\delta^{13}C_{VPDB}$ values ranged between −23.6 and −25.9. The distribution of the $\delta^{13}C_{VPDB}$ values are illustrated in Figure 1.

Seized products derive from 3 different sources in Austria. Six pairs of products had the same producer and product name, but were seized from different locations. These are the product pairs: 1/22, 2/20, 3/25, 6/27, 15/23, and 16/24.

These products show similar delta values. Four product pairs are in the endogenous range, whereas two of these pairs are in the exogenous range. Subtracting one product from these pairs from the total number of analyzed products does not significantly change the overall percentage of products in the endogenous range. After this subtraction the percentage still is 60.8% in a total population of 23 products.

Table 1. Contents and $\delta^{13}C_{VPDB}$ values for seized black market testosterone products.

Product	Labelled	Determined Content	$\delta^{13}C_{VPDB}$(‰)
1	Testosterone Propionate 150 mg/ml	Testosterone Propionate Testosterone Enanthate	−29.0
2	Testosterone Cypionate 200 mg/ml	Testosterone Cypionate Nandrolone Decanoate	−27.7
3	Testosterone Blend 400 mg/ml	As labelled: Testosterone Cypionate Testosterone Propionate Testosterone Decanoate Testosterone Isocaproate Testosterone Phenylpropionate	−25.7
4	Testosterone Enanthate 250 mg/ml	As labelled	−27.7
5	Testosterone Cypionate 250 mg/ml	As labelled	−25.8
6	Testosterone Propionate 30 mg/ml Testosterone Phenylpropionate 60 mg/ml Testosterone Isocaproate 60 mg/ml Testosterone Decanoate 100 mg/ml	As labelled	−24.6
7	Not labelled	Testosterone Propionate	−23.8
8	Testosterone Propionate 100 mg/ml	As labelled	−23.6
9	Testosterone Enanthate 250 mg/ml	As labelled	−25.9
10	Testosterone Enanthate 250 mg/ml	As labelled	−25.3
11	Testosterone Propionate 30 mg/ml Testosterone Phenylpropionate 60 mg/ml Testosterone Isocaproate 60 mg/ml Testosterone Decanoate 100 mg/ml	As labelled	−28.5
12	Testosterone Enanthate 250 mg/ml	As labelled	−24.6
13	Testosterone Propionate 30 mg/ml Testosterone Phenylpropionate 60 mg/ml Testosterone Isocaproate 60 mg/ml Testosterone Decanoate 100 mg/ml	As labelled	−26.6
14	Testosterone Enanthate 250 mg/ml	As labelled	−25.6
15	Testosterone Propionate 100 mg/ml	As labelled	−23.7
16	Testosterone Enanthate 250 mg/ml	As labelled	−24.7
17	Testosterone Propionate 100 mg/ml	No doping substances detected	-
18	Not labelled	Testosterone Enanthate	−24.9
19	Testosterone Enanthate 250 mg/ml	As labelled	−25.4
20	Testosterone Cypionate 200 mg/ml	As labelled	−27.5
21	Testosterone Enanthate 300 mg/ml	Testosterone Enanthate Nandrolone Decanoate	−29.4
22	Testosterone Propionate 150 mg/ml	Testosterone Propionate Testosterone Enanthate	−28.8
23	Testosterone Propionate 100 mg/ml	As labelled	−23.4
24	Testosterone Enanthate 250 mg/ml	As labelled	−24.5
25	Testosterone Blend 400 mg/ml	As labelled: Testosterone Cypionate Testosterone Propionate Testosterone Decanoate Testosterone Isocaproate Testosterone Phenylpropionate	−25.8
26	Testosterone Propionate 100 mg/ml	Testosterone Propionate Testosterone Enanthate	−25.7
27	Testosterone Propionate 30 mg/ml Testosterone Phenylpropionate 60 mg/ml Testosterone Isocaproate 60 mg/ml Testosterone Decanoate 100 mg/ml	As labelled	−25.9
28	Testosterone Cypionate 250 mg/ml	Testosterone Cypionate Testosterone Enanthate	−28.8
29	Testosterone Enanthate 100 mg/ml*	Testosterone Enanthate Nandrolone Decanoate	−28.3
30	Testosterone Enanthate 100 mg/ml*	As labelled	−27.7

* The preparations were additionally declared to contain trenbolone enanthate which was not in the scope of this investigation.

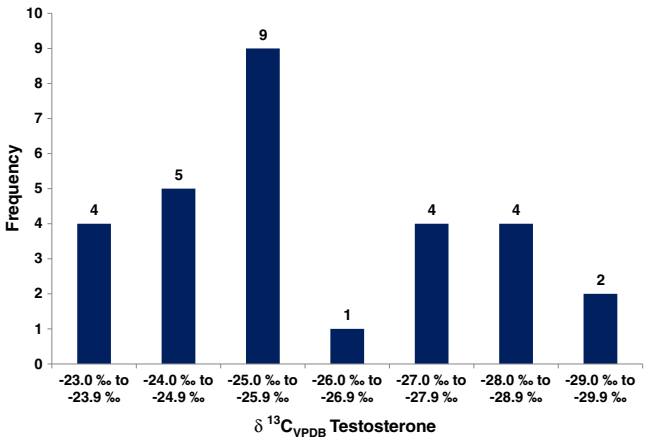

Figure 1. Distribution of the $\delta^{13}C_{VPDB}$ values for 29 seized testosterone products.

Finally, it must be pointed out that the sample size in this investigation is not representative for a global situation but reflects only a spot seizure at a certain time frame within one country. Nevertheless, it clearly shows that such products are on the market and their global availability has to be expected and taken into account for future anti-doping strategies.

Conclusion

In conclusion, the present study confirms that testosterone preparations not containing the ingredients as declared on the label are available on the black market in Austria (33% in the present study). Contamination, false labelling, and production not following good manufacturing processes (GMP) are factors contributing to non-foreseeable health risks to consumers of black market products. Hence, drugs from unauthorized sources represent a particular health threat to athletes. Moreover, anabolic steroid consumption is associated with serious physical side effects, and many consumers take anabolic steroids at doses that are much higher than normally prescribed for medical conditions.

IRMS can be a strong tool in elucidating the origin of xenobiotics, and is the method of choice to provide evidence of a doping offence involving testosterone and other endogenous steroids.

The current study, however, clearly shows the extent of availability of testosterone products with endogenous carbon isotope profiles on the black market. More than 60% of testosterone preparations seized by the Austrian Anti-Doping Police contained testosterone derivatives with $\delta^{13}C_{VPDB}$ values above $-26‰$.

Especially in cases where endogenous reference compounds in a urine sample of a test person show $\delta^{13}C_{VPDB}$ values in the upper endogenous range (close to $-26‰$), the use of the IRMS technique to detect testosterone doping is expected to be limited, if the carbon isotope ratio is solely used as analytical parameter. It is therefore of great importance to continue to monitor this development and intensify research on alternatives for the detection of testosterone misuse.

Acknowledgements

This project has been carried out with the financial support of WADA. The authors thank the police force of Lower Austria for providing the seized testosterone preparations.

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