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$^{13}C/^{12}C$ Isotope ratio MS analysis of testosterone, in chemicals and pharmaceutical preparations

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Abstract

The $^{13}C/^{12}C$ ratio can be used to detect testosterone misuse in sport because (semi)-synthetic testosterone is supposed to have a ^{13}C abundance different from that of endogenous natural human testosterone. In this study, gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis for the measurement of the δ ^{13}C ‰ value of testosterone from esterified forms of 13 pharmaceutical preparations, six reagent grade chemicals and three bulk materials (raw materials used in pharmaceutical proarations) obtained world-wide was investigated after applying a strong acidic solvolytic procedure. Mean δ ^{13}C‰ values of non esterified (free) testosterone from chemicals and bulk materials of several testosterone esters were in the range: $-25.91/-32.82$‰ while the value obtained for a (semi)-synthetic, reagent grade, free testosterone was -27.36‰. The δ ^{13}C‰ results obtained for testosterone from the pharmaceuticals investigated containing testosterone esters were quite homogeneous (mean and S.D. of δ ^{13}C‰ values of free testosterone: 27.43 ± 0.76‰), being the range between -26.18 and -30.04‰. Values described above were clearly different from those reported by several authors for endogenous natural human testosterone and its main metabolites excreted into the urine in non-consumers of testosterone (δ ^{13}C‰ range: from -21.3 to -24.4‰), while they were similar to those of urinary testosterone and metabolites from individuals treated with testosterone esters and testosterone precursors. This finding justifies the fact that administration of these pharmaceutical formulations led to a statistical decrease of carbon isotope ratio of urinary testosterone and its main metabolites in treated subjects.

Keywords: Synthetic testosterone; GC/C/IRMS analysis; δ ^{13}C‰ value; Pharmaceutical preparations; Sports drug testing

1. Introduction

Recent studies indicate that (semi)-synthetic testosterone seems to present a different $^{13}C/^{12}C$ ratio, when compared to human endogenous compound [1]. This observation is based on the fact

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that endogenous testosterone reflects an average of all the carbon from vegetal and animal material eaten by humans, while synthetic testosterone is usually elaborated from a single plant species starting material (phytosterols) with a defined value of $^{13}C/^{12}C$ ratio fixed from its own mechanism of discrimination from atmospheric CO_2. The plant species commonly used is soy, which exhibits lower ^{13}C content as compared with human produced testosterone [2].

Differences in $^{13}C/^{12}C$ ratio can be determined by carbon isotope ratio mass spectrometry (IRMS) [3,4]. For complex mixtures, this technique can be coupled with a gas chromatograph via a combustion interface (GC/C). Separated organic analytes are combusted to CO_2 and the analysis of the $^{13}CO_2$ and $^{12}CO_2$ molecules allows the calculation of carbon isotope ratios for each compound [5]. Variations of the heavier isotope are of the order of 0.001–0.05 atoms % [4]. These minute differences in carbon isotope ratios are expressed as a per mil deviation compared to a designated isotopic standard using the δ $^{13}C‰$ notation:

$$^{13}C‰ = [(R_{sample}/R_{standard}) - 1] \times 1000$$ where R_{sample} is the measured $^{13}C/^{12}C$ isotope ratio for the sample and $R_{standard}$ is the measured $^{13}C/^{12}C$ isotope ratio for a defined standard. The international standard for C is a sample of $CaCO_3$, obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite, PDB) with an accepted value of $R_{standard} = 0.0112372 \pm 0.000009$ [2]. In practice, calibrated CO_2 gas or substances (e.g. alcanes) with a certified δ values are used as a reference standard.

In recent years, the use of GC/C/IRMS has been investigated for the detection of the misuse of testosterone and other endogenous steroids in sport [6,7]. Indeed, different studies showed that following the administration of testosterone and related compounds (androstenedione, dehydroepiandrosterone or androstendiols), carbon isotope ratio of urinary testosterone and its main metabolites declined significantly with differences of more than 3‰ units on the δ-scale [8–10]. These results assumed that synthetic sources of testosterone presented a lower ^{13}C content than naturally occurring steroids.

Synthetic testosterone is usually present in pharmaceutical preparations as esterified forms, which are administered orally or intramuscularly for primary male hypogonadism or breast cancer therapy and can be obtained by medical prescription.

To date, only δ $^{13}C‰$ values of a limited number of pharmaceutical preparations of synthetic testosterone esters have been reported but no detailed information was given about the products (type of esterified compound, name of the formulation, etc), or whether the measurements were carried out directly on the esters or using the eventual hydrolysis of esterified forms to obtain free testosterone, which is the substance of interest to be analysed by GC/C/IRMS [11,12].

In this work, the δ $^{13}C‰$ value of free testosterone obtained after cleavage of different esterified forms from 13 pharmaceutical preparations, six reagent grade chemicals and three bulk materials (raw materials used in pharmaceutical preparations) obtained worldwide was investigated using GC/C/IRMS analysis.

2. Experimental

2.1. Materials

Testosterone esters reagent grade chemicals were obtained from Sigma (St Louis, MO) and Research Plus (Bayonne, NJ). Testosterone esters bulk materials were from Organon Española S.A. (Sant Boi de Llobregat, Barcelona, Spain). Testosterone esters pharmaceutical preparations were obtained from China, Japan, Germany, Greece, Sweden, Portugal, Italy, Norway and Czech Republic by local authorised dealers (see Table 2 for specific information). Standards of *n*-octadecane and *n*-tricosane with a certified δ $^{13}C‰$ value were purchased from Chiron (Trondheim, Norway). Testosterone and 5α-androstane-3β-ol reagent grade chemicals were purchased from Sigma (St Louis, MO). All other reagents were of analytical grade.

2.2. Treatment of testosterone esters in chemicals, bulk materials and pharmaceutical preparations for GC/C/IRMS analysis of non esterified (free) testosterone

Reagent grade chemicals and bulk materials were directly subiected to strong acidic solvolysis. A 400 μl volume of testosterone esters methanolic solution (10 μg/ml) were mixed with 1 ml ethyl acetate–methanol–concentrated sulphuric acid 100:20:5 v/v/v and incubated at 55°C overnight under nitrogen atmosphere. After incubation, samples were added with two drops of 20% ammonia agueous solution, 1 ml acetate buffer (final pH 5.2) and 2 μg 5α-androstane-3β-ol as internal standard and then extracted with three aliquots of 2 ml *n*-hexane. In the case of pharmaceutical formulations, which contained testosterone esters mixed with oily excipients (e.g. ricin oil, arachid oil, oleic acid, benzyl benzoate), an appropriate volume was sampled and diluted with *n*-hexane in order to have stock solutions of 100 μg/ml esters. A 40 μl volume of this solution underwent strong acidic solvolysis as described above. After solvolysis, samples were treated and extracted as reported above. Final extracts (from chemicals, bulk materials or pharmaceutical preparations) were evaporated under nitrogen stream at 45°C, dried out under vacuum (600 mbar) over phosphorous pentoxide for at least 20 min and redissolved in 70 μl ciclohexane. Two microlitres of this non-derivatised solution were directly injected into the gas chromatograph/combustion/carbon isotope ratio mass spectrometer system (GC/C/IRMS).

2.3. GC/C/IRMS analysis

Carbon isotope ratio measurements were performed using a Finnigan MAT Delta Plus instrument (Finnigan MAT GmbH, Bremen, Germany). The GC (Hewlett–Packard HP 6890) was equipped with an HP1 column (17 m, 0.2 mm i.d., 0. 11 μm film thickness, Hewlett–Packard) with helium as the carrier gas and injections were made in splitless mode using an open liner with a 5 mm plug of silanised glass wool inside. Constant flow through the column was set at 2 ml/min. The GC oven was programmed from 150 (1 min) to 260°C (30°C/min), then to 290°C (1.5°C/min) and finally increased to 300°C (5°C/min). A glass three-way connector (BGB analytik AG, Anwil, Switzerland) was used to connect the GC column to the fused silica capillaries leading to the combustion oven and backflush valve. Oxidation and reduction reactors operated respectively at 940 and 600°C.

During routine operation, determination of δ ^{13}C‰ values was done using pulses of CO_2 gas (δ ^{13}C‰ -37.50), previously calibrated against *n*-octadecane and *n*-tricosane certified standards (δ ^{13}C‰ of -30.71 and -26.71, respectively). CO_2 gas was pulsed directly into the ion source during the backflush period (solvent elution) for five times. The first two were used to stabilise the system, the third one was taken for the calibration and the last two to back-check for the calibration values.

2.4. Statistics

Experimental data were statistically analysed with common statistical packages using one factor ANOVA. Results were considered statistically different when $P < 0.05$.

3. Results and discussion

Testosterone is usually administered as a prodrug (e.g. as 17-β-hydroxy-esters). Nonetheless, for the purpose of sports drug testing, a study on δ ^{13}C‰ values of testosterone in esterified form, as reported by some authors [12], appeared to be of minor relevance since, physiologically, free testosterone is the active compound and the usual target analyte in extracts of human urine. The knowledge of δ ^{13}C‰ value of non-esterified (free) testosterone obtained from testosterone esters of synthetic origin was considered essential as only free compound participated in the catabolic pathway of endogenous steroids.

3.1. Solvolysis procedure

Different procedures to obtain free testosterone were evaluated (data not shown) on testosterone esters reagent grade chemicals: an alkaline hydrolysis (20% KOH in absolute ethanol), an enzymatic hydrolysis with β-glucuronidase enzyme, a mild acidic solvolysis (ethyl acetate–methanol–concentrated sulphuric acid 100:20:1 v/v/v) and a strong acidic solvolysis (ethyl acetate–methanol–concentrated sulphuric acid 100:20:5 v/v/v). A GC/MS analysis with a standardised methodology [13] showed that consistently higher hydrolysis yields (> 97%) were obtained with strong acidic solvolysis. Thus, that procedure was chosen, for the treatment of testosterone esters chemicals, bulk materials and pharmaceutical preparations before GC/C/IRMS analysis.

3.2. GC/C/IRMS analysis

3.2.1. δ ^{13}C‰ values of free testosterone obtained after solvolysis of different testosterone esters (chemicals and bulk materials)

Table 1 reports δ ^{13}C‰ values of free testosterone obtained after strong acidic solvolysis of several testosterone esters (chemicals and bulk materials) as well as the value obtained for testosterone (reagent grade chemical) concurrently injected. Values obtained (range from -25.91 to -32.82‰; mean $\pm$ S.D. -28.81 ± 2.10‰), were always lower (more negative) than -25‰ in agreement with values reported by other authors for commercially available testosterone [7,11]. From the manufacturers checked, only Research Plus showed a statistically significant difference from the others. Probably such differences can be explained by the different phytosteroids coming from plants of different species or geograhical areas used as a starting material for synthesis. Even though these differences could account for such inter-supplier statistical variations, differences among various testosterone esters were also found for each manufacturer.

3.2.2. δ ^{13}C‰ values of free testosterone obtained after solvolysis of testosterone esters from pharmaceutical preparations

Table 2 shows the δ ^{13}C‰ values of free testosterone obtained from testosterone esters in pharmaceutical preparations. A narrower range of values (from -26.80 to -28.45 ‰ with mean $\pm$ S.D. -27.43 ± 0.76‰) was found as compared with chemicals and bulk materials of testosterone esters. Nevertheless, in case of testosterone enanthate, statistically significant differences were found between batches of the same product (i.e. Testoviron Depot from Schering, Germany) and among the different manufacturers. Conversely, for testosterone undecanoate, batches from the

Table 1
δ ^{13}C‰ value of free testosterone obtained after solvolysis of testosterone esters as chemicals and bulk materials

Substance	Batch number	Supplier	δ ^{13}C‰ value (free testosterone)[a]
Reagent grade chemicals			
Testosterone cypionate	128FO620	Sigma	-26.77 ± 0.41
Testosterone enanthate	35FO663	Sigma	-28.43 ± 1.40
Testosterone propionate	10H0308	Sigma	-28.67 ± 1.11
Testosterone hemisuccinate	7494	Research Plus	-29.12 ± 0.58
Testosterone undecanoate	14389	Research Plus	-32.82 ± 1.03
Testosterone valerate	3-125	Research Plus	-31.07 ± 0.44
Pure free testosterone	126F-0426	Sigma	-27.36 ± 0.60
Bulk materials			
Testosterone decanoate	06586	Organon	-25.91 ± 0.47
Testosterone isocaproate	06626	Organon	-27.68 ± 0.76
Testosterone phenylpropionate	06587	Organon	-28.81 ± 1.14

[a] Mean $\pm$ S.D. of six replicates

Table 2
δ ^{13}C‰ value of free testosterone obtained after solvolysis of testosterone esters from pharmaceutical preparations

Active principle	Manufacturer	Authorised dealer	Commercial name	Batch number	Pharmaceutical form	δ ^{13}C‰. value (mean $\pm$ S.D.)
Testosterone enanthate	Schering (Germany)	Schepa (Greece)	Testoviron Depot	72496	Oily i.m. injectable	-26.80 ± 0.23
	Schering (Germany)	Schering Lusitana (Portugal)	Testoviron Depot	73502	Oily i.m. injectable	-28.45 ± 0.78
	Fuji Pharmaceuticals (Japan)	Fuji Pharmaceuticals (Japan)	Testron Depot-S	N.A.[a]	Oily i.m. injectable	-27.21 ± 0.34
	Jenapharm (Germany)	Jenapharm (Germany)	Testosteron Depot	73807	Oily i.m. injectable	-26.82 ± 0.41
	Rotexmedica (Germany)	Rotexmedica (Germany)	Testosteron Depot	70471	Oily i.m. injectable	-27.40 ± 0.28
Testosterone propionate	Spofa (Czech Republic)	Spofa (Czech Republic)	Agovirin	3020294	Oily i.m. injectable	-27.38 ± 0.55
	9th Pharmaceutical Factory (China)	9th Pharmaceutical Factory (China)	Tongyong	970104	Oily i.m. injectable	-27.00 ± 0.16
Testosterone undecanoate	N-V.Organon Oss (Holland)	Organon (Greece)	Restandol	070421	Oily capsules	-27.40 ± 0.45
	N.V.Organon Oss (Holland)	Organon (Portugal)	Andriol	113443	Oily capsules	-28.32 ± 1.05
	N.V.Organon Oss (Holland)	Organon A/S (Norway)	Androxon	A17511	Oily capsules	-27.46 ± 1.09
	N.V.Organon Oss (Holland)	Oy Organon Ab (Sweden)	Panteston	A20296	Oily capsules	-27.15 ± 0.46
	Organon (Germany)	Organon (Germany)	Andriol	4416101	Oily capsules	-27.50 ± 0.70
	Scherer (Italy)	Organon (Italy)	Andriol	957904	Oily capsules	-27.78 ± 0.80

[a] N.A., data not available.

same manufacturer (N.V.Organon Oss, Holland) did not present statistical differences and no other factors such as authorised dealer or country were found to significantly contribute to differences in δ ^{13}C‰ value. For testosterone propionate, no difference was obtained between the two products analysed. In any case, values of δ ^{13}C‰ obtained were in agreement with those reported by Shackleton and co-workers [10], once corrected for the contribution of the acetyl group introduced in the molecule as derivative by those authors. Interestingly, values presented in Tables 1 and 2 ('synthetic' testosterone) were different from those found for endogenous testosterone and its main metabolites extracted from urine in non consumers of testosterone (δ ^{13}C‰ range: $-21.3/-24.4$‰) as reported by several authors [7,11,14]. Conversely, δ ^{13}C‰ values of free testosterone obtained after solvolysis of testosterone esters as chemicals, bulk materials and from pharmaceutical preparations were similar to those of urinary testosterone and metabolites of individuals treated with testosterone esters and testosterone precursors [6–15].

In conclusion, carbon isotope mass spectrometry appeared to be an effective mean to distinguish synthetic testosterone coming from reagent grade chemicals, bulk materials or pharmaceutical preparations from human endogenous compound or its urinary metabolites. This finding justify the fact that administration of these pharmaceutical formulations led to a statistical decrease of carbon isotope ratio of urinary testosterone and its main metabolites in treated subjects.

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