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A simplified and accurate method for the analysis of urinary metabolites of testosterone-related steroids using gas chromatography-combustion-isotope ratio mass spectrometry

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Abstract

The determination of the exogenous origin of urinary metabolites through the measurement by GC-C-IRMS of their carbon isotopic signature ($\delta^{13}C$) is widely used to confirm the administration of steroids related to testosterone and prohibited in sports. The rigorous analytical conditions required to perform accurate measurements have often dictated the selection of the final most intense metabolites, androsterone (A) and etiocholanolone (Etio), potentially limiting its efficacy. In fact, their alteration is less marked and persistent than for testosterone (T) itself and 5β-androstane-3α,17β-diol (5β-Adiol) or 5α-androstane-3α,17β-diol (5α-Adiol), which on the other end are present in much lower amount and hence, more difficult to test reliably. We have developed a one-step HPLC purification of seven diagnostic urinary metabolites (target steroids: TS, hydrolyzed glucuronides): T, DHEA, 5β-Adiol, 5α-Adiol, epitestosterone (E), A, Etio and two endogenous reference compounds (ERC), pregnanediol (pgdiol) and 5α-androst-16-en-3β-ol (16-enol). These steroids are pooled in three fractions and analyzed without derivatization. With regards to the GC-C-IRMS analysis, a multi-level isotopic calibration using the 'identical treatment' principle was found to provide results for purified reference steroids with a precision ≤ 0.17 and accuracy of ≤ 0.30 ‰ (between assay, n=26). Compared to other common endogenous reference compounds, those selected in this study have $\delta^{13}C$ values close to the testosterone metabolites which, along with the proposed isotopic calibration, produced reference intervals within ± 3 ‰ for most diagnostic TS-ERC pairs, in compliance with the requirements of the World Anti-Doping Agency.

Introduction

The administration of an AAS like testosterone is demonstrated when the isotopic signature of one diagnostic urinary metabolite (TS) is below -28 ‰ or when the difference between its $\delta^{13}C$ value and that of the endogenous reference compound (ERC), is larger than three units [1]. However, several publications in the field have described larger isotopic differences between urinary testosterone metabolites and selected ERCs in reference populations [2-4]. When further considering the relatively close $\delta^{13}C$ values measured between the urinary metabolites in Europe and some ^{13}C enriched synthetic testosterones from black market, the diagnostic efficacy of the technique could be compromised if the analytical precision and accuracy cannot be improved [5,6]. To that end, we have opted for the following conditions: i) analysis of underivatized steroids; ii) isotopical correction of the CO_2 with certified standards of the same analytes; iii) HPLC purification of hydrolyzed urinary steroids. In this context, we wish to report an efficient and simple pre-analytical purification as well as a precise and accurate GC-C-IRMS method for the routine analysis of seven urinary metabolites and two endogenous reference compounds. The application of this method to the analysis of volunteers and athletes' samples resulted in the description of reference ranges for each TS-ERC pair that supports reporting abnormal analytical findings (AAF) with differences that are lower than previously reported in the literature.

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Experimental

Urine samples Reference population: Canadian healthy human male (n=25) and female (n=25) volunteers (18 to 55 year-old) plus North American and International athletes' samples received for testing (n=125). Negative control: urines (4 L) from a male volunteer. Positive control: urines (2.5 L) from a male volunteer treated with testosterone enanthate (i.m. 250 mg) for medical purpose. Informed consent was provided.
The first steps of sample preparation (5 to 15 mL) are the isolation by SPE (tC18 cartridges, Waters), enzymatic hydrolysis of glucuroconjugated steroids (*E. coli* type IXA, Sigma) and liquid-liquid extraction with hexane (pH 9) as described previously [7]. The dry residue dissolved in 20 µL of MeOH, was injected on a HPLC (Agilent 1100 HPLC-UV) coupled to a fraction collector (Gilson FC 203B). Urinary metabolites were purified in five fractions at 40°C on two coupled Agilent Zorbax SB-C18 columns (4.6 mm x 250 mm x 5 µm particle size). With the methanolic content kept constant at 6%, the composition of ACN in water and the flow rate were gradually increased to 55 % at 0.76 mL/min, 67 % at 1.00 mL/min and to 94 % at 1.00 mL/min after 20, 36 and 39 min respectively. The steroids were combined in three fractions to which androstanol (25 ng/µL) was added (retention time): 1) T and DHEA; 2) 5β-Adiol, 5α-Adiol, E and pgdiol; 3) Etio, A and 16-enol.
GC-C-IRMS analyses Two GC-C-IRMS (Agilent HP7890-Isoprime) systems were used with the following conditions: i) GC column DB-5MS, 25 m x 0.20 mm ID, 0.33 µm film thickness; ii) GC injector temperature set to 270°C in a pulsed (40 psi) splitless mode (1 min) and oven temperature set at 80°C for 1 min, increased to 250°C, 275°C and 320°C at a rate of 15°C, 5°C and 20°C, respectively, then kept at 320°C for 4.8 min (same conditions for GC-MSD and GC-C-IRMS analyses); iii) temperature of transfer line and furnace set to 350°C and 850°C respectively; iv) current of the IRMS source fixed at 400 nA.
Isotopic calibration The $\delta^{13}C$ values of the reference standards (A, 5α-Adiol, 5β-Adiol, androstanol, cholesterol, DHEA, 16-enol, E, Etio, pgdiol, T) ranging from -17.1 to -33.5 ‰, were externally certified as well as the reference CO_2 gas against VPDB (GEOTOP, UQAM). The isotopic calibration was done with two solutions of the certified standards by plotting their certified vs. measured $\delta^{13}C$ values. The resulting linear (y = mx + b) curve was used in the correction of the data where y represents the isotopically corrected value, m is the slope, x is the measured isotopic value calibrated against the reference CO_2 and b is the deviation of the measurements at the intercept (Figure 1).

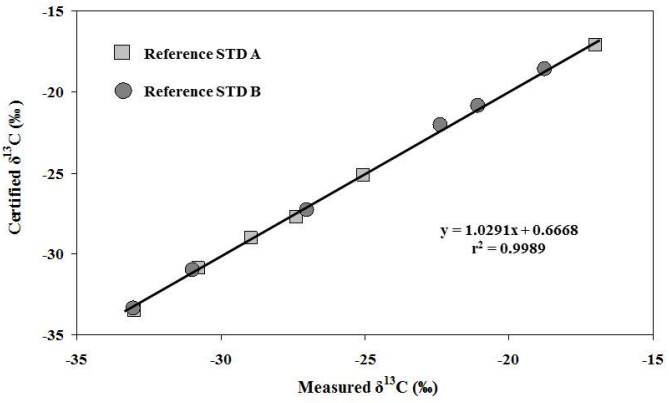

Figure 1: Typical calibration curve built from the GC-C-IRMS analysis of solutions A and B (Table 1), each containing 6 reference standards (certified vs. measured $\delta^{13}C$ values).

The accuracy of this correction was verified in each sequence with an external reference, USADA Cu 34-1. The results were judged acceptable when the deviation from the certified values did not exceed 0.3 ‰. The efficiency of the combustion was verified by the injection of certified reference alkanes in four different concentrations (Indiana Mix C3).

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Results and Discussion

Since we analyze underivatized steroids, urinary pgdiol and 16-enol [8] showing similar $\delta^{13}C$ values were preferred to 11-ketoetiocholanolone, 11-hydroxyandrosterone and 11-hydroxyetiocholanolone which furthermore could not be sufficiently purified under our conditions.
Careful monitoring of analytical conditions (inert injection port, column) allows the direct analysis of steroids with excellent chromatographic behavior, therefore bypassing the acetylation and subsequent isotopic correction. The single-run HPLC method on two coupled analytical columns enabled the purification of all steroids and was found to be suitable for accurate GC-C-IRMS routine analyses (Figure 2). The metabolites and ERCs were combined taking into account their concentration and GC elution, in order to fit within their linearity range. Fraction 1 (T and DHEA) only, showed the presence of other small peaks when analyzed (Figure 2F), and the impact was negligible (Table 2).

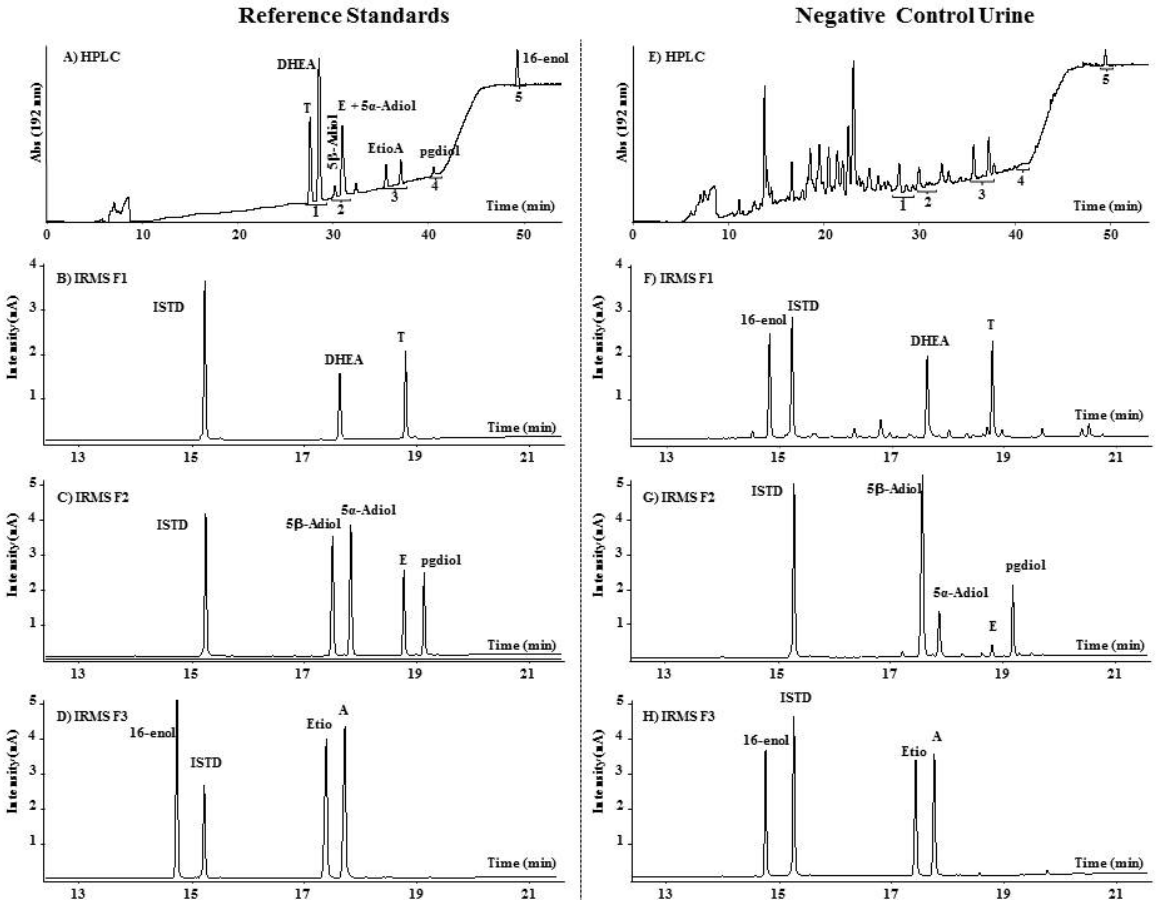

Figure 2: Chromatograms of the HPLC purification (UV detection at 192 nm) of A) reference standards and E) negative control sample and GC-C-IRMS chromatograms of the three combined fractions of purified steroids in the reference standards (panels B,C and D) and negative control urine (panels F, G and H). Abbreviations given in the text (ISTD: androstanol).

Isotopic calibration The application of the GC-C-IRMS technique in the doping control context implies measuring $\delta^{13}C$ values over a relatively wide range, e.g. from -16 to -32 ‰. Since the determination of an AAF is made from the comparison of exogenous metabolites vs. endogenous ERC, depleted and enriched values are diagnostically crucial and must be determined with the same accuracy. To that end, isotopic calibration curves built before every sequence ensures day to day isotopic linear measurements with compensation for the overall system noise, column bleeding and contaminations.

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This approach presents a definitive advantage over the absence of calibration of the CO_2 or the correction against certified standards that have values in the synthetic range only. Following the analysis of 74 different batches of samples, the slope and intercept of the linear isotopic correction curves were found to vary from 1.00 to 1.03 and from 0.0 to 0.9 ‰, respectively (Figure 1). The results obtained for the USADA Cu 34-1 standards used for verification were also accurate and precise. The comparison of the $\delta^{13}C$ values measured with isotopic correction from the calibration curve or directly from the CO_2 reference gas is presented in Table 1, while the results obtained for the longitudinal analysis of the negative, positive controls and reference standards routinely included within the sequences of analyses are shown in Table 2. The between-assay precision for urinary steroids was ranging from 0.1 (e.g. androsterone) to 0.5 ‰, while the reference standards were measured with a standard deviation of ≤ ± 0.17 ‰ and accuracy ≤ ± 0.32 ‰.

	$\delta^{13}C$ ‰ (σ)						
Reference Standards	Androstanol	T	E	5β-Adiol	5α-Adiol	DHEA	A
Certified value	-31.0	-27.8	-33.3	-29.0	-30.9	-33.5	-20.9
vs. CO_2	-30.9 (0.2)	-27.3 (0.2)	-32.4 (0.2)	-28.9 (0.2)	-30.6 (0.2)	-32.8 (0.2)	-20.9 (0.2)
vs. calibration curves	-31.2 (0.1)	-27.6 (0.1)	-32.7 (0.2)	-29.2 (0.1)	-30.9 (0.2)	-33.2 (0.1)	-21.0 (0.1)

Reference Standards	Etio	16-enol	pgdiol (1)	pgdiol (2)	cholesterol
Certified value	-22.1	-27.3	-17.1	-18.6	-25.1
vs. CO_2	-22.1 (0.2)	-26.9 (0.2)	-16.8 (0.2)	-17.7 (0.2)	-24.9 (0.2)
vs. calibration curves	-22.2 (0.1)	-27.2 (0.1)	-16.8 (0.1)	-18.4 (0.1)	-25.1 (0.1)

USADA Cu 34-1	A	Etio	pgdiol
Certified value	-27.2	-28.8	-31.5
vs. CO_2	-26.8 (0.2)	-28.7 (0.2)	-31.3 (0.2)
vs. calibration curves	-27.1 (0.2)	-28.9 (0.1)	-31.6 (0.1)

Table 1: Mean $\delta^{13}C$ ‰ and standard deviation (σ) of the certified calibration reference standards (first and second rows, n=74) and USADA Cu 34-1 (third row, n= 54) measured vs. CO_2 and vs. calibration curves and compared with certified values (values rounded to one decimal).

	$\delta^{13}C$ ‰ (σ)								
	T	E	5β-Adiol	5α-Adiol	DHEA	A	Etio	16-enol	pgdiol
Negative Control	-21.5 (0.3)	-22.4 (0.4)	-20.9 (0.2)	-21.6 (0.5)	-19.7 (0.2)	-20.6 (0.2)	-21.1 (0.1)	-20.9 (0.3)	-20.9 (0.2)
Positive Control	-28.5 (0.3)	-	-27.5 (0.3)	-27.4 (0.3)	-19.9 (0.5)	-21.0 (0.1)	-26.3 (0.2)	-20.9 (0.3)	-21.0 (0.3)
Ref. Standards	-28.1 (0.2)	-33.3 (0.2)	-29.1 (0.1)	-30.9 (0.2)	-33.3 (0.1)	-30.7 (0.1)	-28.9 (0.1)	-27.2 (0.2)	-16.8 (0.1)
Certified value	-28.3	-33.6	-29.0	-30.9	-33.7	-30.8	-29.0	-27.3	-17.1

Table 2: Mean $\delta^{13}C$ ‰ and standard deviation (σ) for the metabolites and ERCs in the negative and positive control urine and in the certified reference steroids analyzed routinely (n=23).

Uncertainty of the measurement The combined uncertainty of the measurement (u_c) was estimated for each metabolite and ERC and TS-ERC pair. Consideration was given to the error of the determined certified value, the accuracy testing this certified reference material, the standard deviation of the measurements made on 23 different batches of the positive and negative control samples. Combined uncertainty results for pairs of TS-ERC were all lower than 1.1 ‰ (data not shown).
Reference population The differences between the $\delta^{13}C$ values for each TS-ERC pair in the different reference populations are presented in Table 3. The values measured in the athletes' samples ranged from -18.2 and -23.8 ‰, which reflects their origin and is consistent with literature describing values in reference athletes' populations of -17.2 to -25.8 ‰ [4].

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Pgdiol and 16-enol were very close in all four groups with Δ16-enol-pgdiol mean values at 0 ‰ (max: 0.9 ‰) and 0.13 ‰ (max: 1.8 ‰) in the volunteers' and athletes' samples respectively. As shown in Table 3, no significant difference was observed for the TS-ERC values between these two populations and consequently, the influence of athletic conditions do not seem to play a significant role. Slightly larger variations were recorded in the female groups for T and E, both present at much lower concentrations. A graphic representation of the mean ΔTS-pgdiol values and respective limits in the male and female combined populations is shown in Figure 3.

	ΔTS-pgdiol (σ) / Range of urinary concentrations (ng/mL)[1]						
	T	E	5β-Adiol	5α-Adiol	DHEA	A	Etio
Volunteers M (n=25)	-0.6 ± 0.5	-1.2 ± 0.6	-0.7 ± 0.7	-1.3 ± 0.5	1.2 ± 0.6	0.4 ± 0.4	-0.5 ± 0.4
	4 - 134	4 - 187	22 - 602	8 - 270	3 - 108	400 - 6976	292 - 5227
Athletes M (n=90)	-0.6 ± 0.7	-1.1 ± 0.8	-0.8 ± 0.6	-0.9 ± 0.7	1.3 ± 0.7	0.5 ± 0.5	-0.5 ± 0.5
	4 - 310	0.5 - 262	22 - 1646	12 - 789	5 - 198	159 - 11418	151 - 11153
Volunteers F (n=25)	-0.7 ± 0.9	-1.1 ± 1.1	-1.0 ± 0.7	-1.2 ± 0.8	1.4 ± 0.5	0.4 ± 0.5	-0.5 ± 0.4
	0.1 - 25	0.3 - 23	12 - 305	2 - 75	2 - 83	121 - 3563	183 - 4112
Athletes F (n=35)	-0.2 ± 0.8	-1.0 ± 1.1	-0.9 ± 0.7	-0.8 ± 0.7	1.2 ± 0.6	0.4 ± 0.5	-0.4 ± 0.5
	0.2 - 15	0.2 - 51	7 - 406	3 - 50	4 - 151	33 - 12217	399 - 6629

[1] hydrolyzed glucuronides

Table 3: Mean ΔTS-pgdiol values, standard deviation (σ) and respective ranges of concentrations, in reference populations of male and female volunteers' and athletes' samples.

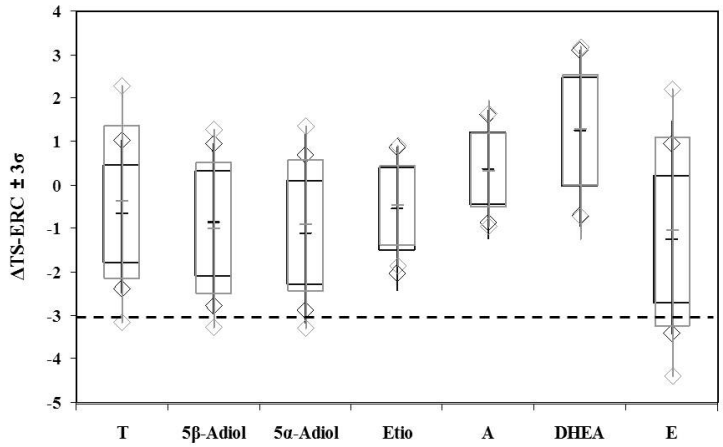

Figure 3: Mean TS-Pgdiol values with confidence intervals of 95% (2δ, boxes) and 99% (3δ, lozenges) in the male (black) and female (gray) reference populations.

These results show that for both genders, ΔTS-pgdiol values are below respectively 1 and 2 ‰ for DHEA, A and Etio and below 3 ‰ for T, 5α-Adiol or 5β-Adiol. Piper et al. [3] comprehensively described the reference ΔTS-ERC values of the metabolites of testosterone plus three ERCs; the reference limits for T vs. pgdiol, 16-enol or 11-hydroxyandrosterone were respectively, 4.4, 4.7 and 5.0 units, which shows a wider dispersion of values. In our conditions, the mean ΔEtio-A is close to 1 and the ΔE-pgdiol value plus 3σ does not exceed 2, in contrast to other reports [4,9]. Due to its low urinary level, epitestosterone is the only urinary metabolite in female samples with a larger limit close to -4.5 ‰, which does not typically represent a problem, epitestosterone being diagnostic only of its administration as a potential, rarely observed masking agent of testosterone.

Conclusions

Strictly controlled analytical conditions, calibration of the GC-C-IRMS instrument with certified steroids corresponding to the tested analytes and improved purification of urinary metabolites and reference compounds, permit accurate measurement of $\delta^{13}C$ with the accuracy required to efficiently detect the administration of testosterone-related steroids. The two ERCs that were selected have close $\delta^{13}C$ values, thus providing more homogenous results. The ΔTS-ERC values measured in the reference populations of male and female volunteers and athletes were described and provided support to the decision criteria recommended by the World Anti-Doping Agency.

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