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External calibration in Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry measurements of endogenous androgenic anabolic steroids in sports doping control

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ABSTRACT

An alternative calibration procedure for the Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry (GC–C–IRMS) measurements of the World Antidoping Agency (WADA) Accredited Laboratories is presented. To alleviate the need for externally calibrated CO_2 gas for GC–C–IRMS analysis of urinary steroid metabolites, calibration using an external standard mixture solution of steroids with certified isotopic composition was investigated. The reference steroids of the calibration mixture and routine samples underwent identical instrumental processes. The calibration standards bracketed the entire range of the relevant $\delta^{13}C$ values for the endogenous and exogenous steroids as well as their chromatographic retention times. The certified $\delta^{13}C$ values of the reference calibrators were plotted in relation to measured m/z $^{13}CO_2/^{12}CO_2$ (i.e. $R(45/44)$) mass spectrometric signals of each calibrator. $\delta^{13}C$ values of the sample steroids were calculated from the least squares fit through the calibration curve. The effect of the external calibration on $\delta^{13}C$ values, using the same calibration standards and set of urine samples but different brands of GC–C–IRMS instruments, was assessed by an interlaboratory study in the WADA Accredited Laboratories of Sydney, Australia and Athens, Greece. Relative correspondence between the laboratories for determination of androsterone, etiocholanolone, 5β-androstane-3α,17β-diacetate, and pregnanediacetate means were SD($\delta^{13}C$) = 0.12‰, 0.58‰, −0.34‰, and −0.40‰, respectively. These data demonstrate that accurate intralaboratory external calibration with certified steroids provided by United States Antidoping Agency (USADA) and without external CO_2 calibration is feasible and directly applicable to the WADA Accredited Laboratories for the harmonization of the GC–C–IRMS measurements.

1. Introduction

The detection of testosterone (T) or prohormones abuse in human urine samples is an analytical challenge in doping control because the main analytical technology in use, Gas Chromatography–Mass Spectrometry (GC–MS), cannot discriminate mass spectral signals of pharmaceutical from endogenous androgens. At present, longitudinal monitoring of the steroid profile [1–3] and GC–C–IRMS studies [4–8] are used complementarily in order to prove the application of exogenous androgens. During the last two decades, GC–C–IRMS methods have contributed greatly to the elucidation of whether an analytical finding, regarding abnormal endogenous steroids concentrations and/or increased testosterone to epitestosterone ratio, is due to an individual physiological steroids profile or has resulted from the exogenous application of a T-like prohibited substance [9–18]. The methods are based on the different ^{13}C abundance between pharmaceutical T and endogenous human T and similarly between synthetic precursors or metabolites, and endogenous reference compounds (ERC), which are not affected by the administration of synthetic androgens [5,6,10,19–21].

Carbon isotope ratios are not absolute values, but are reported as δ values ($\delta^{13}C$) relative to a reference material, such as $CaCO_3$ obtained from the Vienna Pee Dee Belemnite (VPDB) [17,22,23]. The $^{13}C/^{12}C$ ratio is defined as parts per thousand [24]. Currently, calculation of the $\delta^{13}C$ value of steroids and therefore, isotopic calibration depends on the reference gas, CO_2. This isotopically

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calibrated gas is inserted as pulses from a gas cylinder, via an independent secondary capillary, directly into the ion source of the IRMS instrument [22,25,26].

Isotopic calibration is an essential process for reliable GC–C–IRMS and has attracted considerable notice in the 1990s [25–31]. Several groups have carried out research into isotopic calibration by (a) adding the reference compounds to the sample, (b) introducing reference gas pulses directly into the ion source, and (c) introducing reference gas pulses to the carrier gas stream via a low dead volume T-piece, placed between column end and combustion furnace. In the absence of systematic error, such as incomplete combustion, the first two methods of isotopic calibration are equivalent [26].

WADA Laboratories use a variety of GC–C–IRMS instruments and methodologies. However, it is desirable for all laboratories to produce data with minimum deviations. In order to achieve harmonization, it is necessary to have common reference standards available for GC–C–IRMS analysis and a common calibration procedure.

In this study, the use of external reference standards, such as steroids with certified $\delta^{13}C$ value, for $\delta^{13}C$ value calibration is proposed. The reference compounds can be the same for all WADA accredited laboratories, in order to achieve consistency in measurements. Thanks to a USADA research project [32] conducted by Cornell University, Ithaca, USA, steroids with certified $\delta^{13}C$ values have become available. Sydney and Athens WADA Accredited Laboratories collaborated on developing the external calibration method. The method was also examined in the same urine samples using the different GC–C–IRMS instruments of Sydney and Athens WADA Accredited Laboratories, providing interlaboratory data.

2. Experimental

2.1. Materials and methods of Athens

2.1.1. Chemicals and reagents

All solvents used were of analytical grade and were purchased from Labscan, Ireland. Ultra pure water (MilliQ) was from Millipore (Billerica, MA, USA) and acetonitrile (CH_3CN) of HPLC grade. β-Glucuronidase from *Escherichia coli* (Type IX-A, lyophilized power, 1,000,000 units/g protein, Part Number G-7396, Sigma–Aldrich, Germany) was used for the enzymatic hydrolysis of endogenous glucuronated steroids. N-tricosane (0.15 mg/mL in cyclohexane) with certified $\delta^{13}C$ value −26.71‰ was supplied from Chiron AS, Norway. Acetic anhydride (Part Number 11,004-3, Lot 27220-040) and pyridine were supplied from Sigma–Aldrich, Germany and were of 99% and 99.8% grade respectively. 5α-Androstane-3β-ol (CU/USADA 30-1) with a $\delta^{13}C$ value −29.7‰ [32]. As reference gas was used carbon dioxide of 99.7% purity, from Air Liquide Hellas, Greece.

2.1.2. Instrumental conditions

Sample clean up was performed on a High Pressure Liquid Chromatograph (HPLC) HP 1090 (Agilent Technologies, Germany) with a Merck analytical column (LiChrospher 100RP, 125 mm × 4 mm i.d., 5 μm particle size) and an automatic injection system. The injection volume was 100 μL, the flow rate was set to 1 mL/min and the oven temperature at 40 °C. The mobile phase was a mixture of solvent A, H_2O:CH_3CN (90:10) and solvent B, CH_3CN, starting at a proportion of 20% solvent B. A linear gradient was used, increasing from the initial proportion to 55% solvent B (acetonitrile) in 10 min, held for 5 min, and then increased to 100% solvent B in 10 min. The fractions collection was performed on a Waters Fraction Collector II. ^{13}C fractionation was monitored by the GC–C–IRMS analysis of each HPLC fraction for the presence or absence of the target metabolites.

Carbon isotope measurements were performed on an Isoprime IRMS instrument (Isoprime Ltd., Cheadle Hulme, UK) coupled to a 6890N Gas Chromatograph (Agilent, Santa Clara, USA) and combustion system. Injections were performed in splitless mode at 250 °C. The fused silica capillary column Supelco SPB™-50 (Sigma–Aldrich, Germany) was of 30 m length, 250 μm internal diameter and 0.25 μm film thickness. Helium was used as carried gas. The initial oven temperature was set at 120 °C and held for 3 min, then increased at 40 °C/min to 280 °C and held for 10 min, then increased at 40 °C/min to 300 °C and held for 2 min. The interface and the furnace temperatures were set to 350 °C and 850 °C, respectively. The combustion gases, CO_2 and H_2O, passed through a capillary made of Nafion, for water removal. Two reference carbon dioxide gas pulses were introduced in each analysis. For the analysis of CO_2, three Faraday cups were positioned in the ion beam to collect the ions m/z 44, 45, 46 representing $^{12}C^{16}O^{16}O^{\bullet+}$, $^{13}C^{16}O^{16}O^{\bullet+} + {}^{12}C^{17}O^{16}O^{\bullet+}$ and $^{12}C^{16}O^{18}O^{\bullet+}$ respectively. A Masslynx data system, version 4.0 was used for analysis and data evaluation.

2.1.3. Sample preparation

Ten millilitres of urine sample, after the removal of the free steroid fraction, were hydrolysed with 1.0 mL phosphate buffer (pH 7.0) and 100 μL β-glucuronidase for 1.5 h at 50 °C or overnight at 37 °C. At pH 9–10, adjusted by addition of carbonate buffer 30% (w/v), the deconjugated steroids were extracted with 5.0 mL tert-butylmethylether (TBME). 50 μL of dexamethasone, 200 μg/mL, were added as internal standard for HPLC-clean up step. After evaporation and reconstitution, samples were subjected to HPLC to collect six fractions. Fraction 3, containing etiocholanolone and androsterone, was dissolved in 50 μL acetonitrile and was transferred to a vial, where 10 μL 5α-androstane-3β-ol, 250 μg/mL, were added. Etiocholanolone and androsterone fractions were immediately subjected to GC–C–IRMS analysis, without derivatisation. To fractions 1, 2 and 4 contained the steroids of interest, 20 μL of the internal standard 5α-androstane-3β-ol (75 μg/mL) were added and evaporated in order to be derivatised. The dry residues of fraction 1 (11-keto-etiocholanolone, 11β-OH-etiocholanolone, 11β-OH-androsterone), fraction 2 (epitestosterone, testosterone, 5β-androstane-3α-17β-diol, 5α-androstane-3α-17β-diol) and fraction 4 (pregnanediol) were acetylated with 100 μL of dry pyridine and 100 μL of acetic anhydride at 60 °C for 1 h. Evaporation of the reaction mixture was followed by reconstitution in 10–100 μL of 5α-androstane-3β-ol (75 μg/mL).

2.2. Materials and methods of Sydney

2.2.1. Chemicals and reagents

The solvents hexane, TBME and ethylacetate were of AR grade and methanol of HPLC grade or nanograde (Merck, Darmstadt, Germany). Water was obtained from a Milli-Q purification system capable of 10 MΩ/cm^3 or better. The enzyme β-glucuronidase isolated from *E. coli*, was purchased from Boehringer-Mannheim, Germany (Solution in 50% glycerol, Part Number 03 707 601 001). 5α-Androstane-3β-ol was of the same source as for the Athens Laboratory. Acetic anhydride (Part Number A-6404, Lot #19H0460) and pyridine re-distilled from AR grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BondElut C18 cartridges were purchased from Varian (Harbor City, CA, USA). Phosphate buffer was prepared by dissolving 28.4 g (0.2 M) disodium hydrogen phosphate and 27.2 g (0.2 M) potassium dihydrogen phosphate in 1.0 L water. A cylinder of CO_2 gas obtained from BOC Gases (Sydney, Australia), contained the reference gas with isotope ratio ($\delta^{13}C_{VPDB} = -21.3 \pm 0.1$‰) determined relative to NBS-19 via NBS-

22 by dual-inlet IRMS analysis (Environmental Isotopes, Sydney, Australia).

2.2.2. Instrumental conditions

Automated solid phase extraction was performed using a Gilson ASPEC XL4 apparatus. HPLC purification prior GC–C–IRMS analysis was performed on an Agilent 1090 separations module coupled with a single wavelength detector operating on Chemstation® software. Steroid separation was achieved with a Gemini C18 column (250 mm × 4.6 mm, 3 μm, 110 Å) protected by a Gemini C18 SecurityGuard cartridge (4 mm × 3.0 mm) (Phenomenex, Torrence, CA, USA) at 40 °C and 0.8 mL/min flow rate. The injection volume was 90 μL. The mobile phase was a mixture of solvent A, H_2O:CH_3CN (90:10) and solvent B, CH_3CN, starting at a proportion of 100% solvent A. Gradient elution was used, increasing from the initial proportion to 55% solvent B (acetonitrile) in 10 min, held for 5 min, and then increased to 100% solvent B in 3 min. The fractions were collected with Gilson FC203B fraction collector (John Morris Scientific). ^{13}C fractionation was monitored by the GC–C–IRMS analysis of each HPLC fraction for the presence or absence of the target metabolites.

All samples were measured on an Agilent 6890 GC coupled to a Thermo GC–Combustion–III interface and Delta^{PLUS} IRMS (ThermoElectron, Bremen, Germany). The GC was equipped with an A200S autosampler and the system was operated by ISODAT software, NT version 2.0 on a PC computer. Chromatographic separation of steroids was achieved on a J&W Agilent DB17-MS (50% phenyl/50% methylsiloxane cross-linked) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The injections (1 μL) were made in splitless mode at 280 °C, using helium as carrier gas at a constant flow of 1.2 mL/min. The injector pressure was 29.7 kPa. The GC separation began at 180 °C, where the oven was held for 1 min, then the temperature increased to 250 °C at a rate of 12 °C/min, then increased to 280 °C at 3 °C/min, finally increased at 15 °C/min to 300 °C and maintained for 4 min.

The combustion interface consisted of a thermo Cu/Ni/Pt ceramic capillary reactor operated at 940 °C, and a reduction furnace consisted of a thermo Cu ceramic capillary reactor, operated at 620 °C. Water removal is accomplished with a Nafion tube (0.3 mm i.d.) which is a semipermeable membrane. The final component of the interface is the open split that allows the IRMS to sample the gas stream. Electron ionization at 70 eV was used for optimal ionization yield. The ions formed were accelerated to energies of 3 keV before they were separated according to m/z in a magnetic sector. For the analysis of CO_2, three Faraday cups were positioned in the ion beam to collect the ions m/z 44, 45, 46 representing $^{12}C^{16}O^{16}O^{\bullet+}$, $^{13}C^{16}O^{16}O^{\bullet+} + {}^{12}C^{17}O^{16}O^{\bullet+}$, and $^{12}C^{16}O^{18}O^{\bullet+}$ respectively.

2.2.3. Sample preparation

Steroid glucuronides were isolated from urine (10 mL) using BondElut C18 solid phase extraction cartridges. The methanolic extract was evaporated to dryness before the addition of pH 7 phosphate buffer (0.2 M, 1.5 mL) to facilitate enzyme hydrolysis with β-glucuronidase (50 μL) for 1.5 h in a water bath at 50 °C. The hydrolysate was adjusted to pH 9.8 with carbonate buffer (20% (w/v), 250 μL) before liquid–liquid extraction with TBME (2 × 4 mL). The combined organic supernatants were evaporated to dryness before being reconstituted in acetonitrile/water (35:65, 100 μL). The extract was filtered through a 0.45 μm membrane before HPLC purification to collect six fractions. Fractions 1 (blank) and 2 (11-keto-etiocholanolone, 11βOH-etiocholanolone and 11βOH-androsterone) were fortified with 5α-androstane-3β-ol (25 μg/mL, 50 μL) and evaporated to dryness under nitrogen. The steroid residue was reconstituted in 50 μL ethyl acetate and transferred to a vial for GC–C–IRMS analysis, without derivatisation. Fraction 4, containing etiocholanolone and androsterone, was fortified with 5α-androstane-3β-ol (100 μg/mL, 50 μL) and evaporated to dryness under nitrogen. The steroid residue was reconstituted in 100 μL ethyl acetate and transferred to a vial for GC–C–IRMS analysis, without derivatisation. Fractions 3 and 5 containing the 5β-androstane-3α-17β-diol and 5α-androstane-3α-17β-diol were combined and fortified with 5α-androstan-3β-ol (25 μg/mL, 50 μL) before being acetylated using acetic anhydride (100 μL) and re-distilled pyridine (100 μL) at 70 °C for 1.5 h. Evaporation of the reaction mixture was followed by addition of 5α-androstane-3β-ol (25 μg/mL, 50 μL), evaporation to dryness and reconstitution in acetonitrile/water (35:65, 100 μL) for a second HPLC purification to obtain 5α-androstane-3α,17β-diacetate, 5β-androstane-3α,17β-diacetate and 5β-pregnane-3α,20α-diacetate in fraction 4 and 5α-androstane-3β-acetate in fraction 5. The fractions were fortified with 5α-androstane-3β-ol (25 μg/mL, 50 μL) and evaporated to dryness under nitrogen. The steroid residue was reconstituted in 50 μL ethyl acetate and transferred to a vial for GC–C–IRMS analysis.

2.3. Working solutions

Stock solutions were prepared, according to USADA guidelines, from steroids with known $\delta^{13}C$ values. The CU/USADA-33-1 contained 5α-androstane-3β-acetate ($\delta^{13}C = -30.61‰$), 5α-androstane-3α-acetate-17-one ($\delta^{13}C = -33.04‰$), 5β-androstan-3α-acetate-11,17-dione ($\delta^{13}C = -16.69‰$), 5α-cholestane ($\delta^{13}C = -24.74‰$) in cyclohexane, at concentration of 200 μg/mL and was used for the construction of the curve of the external calibration. The CU/USADA-34-2 contained androsterone, 401 μg/mL ($\delta^{13}C = -27.06‰$), etiocholanolone, 401 μg/mL ($\delta^{13}C = -28.90‰$) and pregnanediol, 402 μg/mL ($\delta^{13}C = -31.48‰$) in isopropanol. The CU/USADA 35-1 contained 5β-androstane-3α,17β-diol, 396 μg/mL ($\delta^{13}C = -28.98‰$), 5α-androstane-3α,17β-diol, 399 μg/mL ($\delta^{13}C = -30.52‰$), pregnanediol, 398 μg/mL ($\delta^{13}C = -18.43‰$) and 5α-cholestane, 399 μg/mL ($\delta^{13}C = -24.92‰$). CU/USADA-34-2 and CU/USADA 35-1 were used as accuracy check for the derivatised and the underivatised steroids.

2.4. Calibration curve

The external calibration method for the GC–C–IRMS, that does not need reference gas CO_2, comprises a linear calibration curve in which the analytical information is $\delta^{13}C$ value of steroids (y-axis) and the measured parameter is the isotopic ratio $R(m/z\ 45/44)$ (x-axis). The $\delta^{13}C$ values of analytes in urine samples were calculated by means of the measured isotope ratios $R(45/44)$, obtained from GC–C–IRMS analysis, through the calibration equation. The steroids of CU/USADA-33-1 were used for the calibration curve. The four reference compounds have certified $\delta^{13}C$ values that cover the entire range of the expected $\delta^{13}C$ values for the exogenous and endogenous steroids [18,28–31,33,34] as well as an extended range of chromatographic retention times. A simplification in the external calibration was to not introduce to the calculations the m/z 46 signal, which is described in the discussion session below.

2.5. Administration studies analyzed as interlaboratory tests

Testosterone was obtained from the Chemical Reference Materials section of the National Measurement Institute (Pymble, NSW, Australia). Androstenedione was obtained in capsule form from ONE-LIFE (Santa Monica, CA, USA [Lot #569]). 4-Androstenediol was obtained as a reference material from Steraloids Inc. (Newport, RI, USA). Prior to administration the identity, purity and $\delta^{13}C$ value of each substrate was determined by GC–MS and GC–C–IRMS analysis on 10 mg × 2 mg portions.

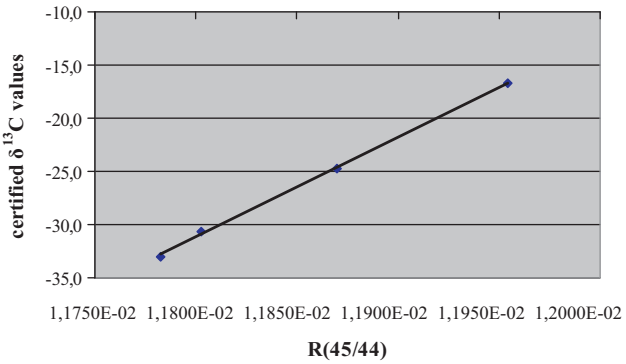

Fig. 1. Calibration curve for the data in Table 1, certified $\delta^{13}C$ against $R(45/44)$ from GC–C–IRMS analysis $y = 93893x - 1139.1$, $R^2 = 0.9988$.

Five urine samples were used in this study to assess the consistency of $\delta^{13}C$ results between the Athens and Sydney Laboratories. Human Ethics Approval (ECN-05-99) was obtained from Southern Cross University (Lismore, NSW, Australia) for single oral administrations of steroid preparations. Samples A and B were collected from a male volunteer at 6 and 9 h, respectively following oral (100 mg) testosterone ($\delta^{13}C = -30.0‰$) administration. Samples C, D and E were collected from a different male volunteer than for A and B. Samples C and D were collected at 0 and 12 h, respectively before and after oral (100 mg) 4-androstenedione administration ($\delta^{13}C = -35.0‰$). Sample E was collected 42 h following 4-androstenediol ($\delta^{13}C = -30.5‰$) administration.

The candidate freeze-dried human urine reference material MX005, National Measurement Institute (NMI), Sydney, Australia, was also used for the accuracy test after the following preliminary reference values for etiocholanolone $\delta^{13}C = -24.1‰$, androsterone $\delta^{13}C = -27.2‰$ and pregnanediol $\delta^{13}C = -23.1‰$ [35].

Athens and Sydney analyzed the same urine samples using the same certified reference compounds for calibration. The analytes of the current study were androsterone, etiocholanolone, 5β-androstane-3α,17β-diol and pregnanediol (ERC).

3. Results and discussion

3.1. External calibration method

The calibration curve was plotted with the instrumental signals $R(45/44)$ on the horizontal (x) axis and the $\delta^{13}C$ values of the steroids on the vertical (y) axis. The certified $\delta^{13}C$ values of the calibrators were plotted in relation to the corresponding mean ratios $R(45/44)$ derived from GC–C–IRMS analysis to form a linear regression of least squares equation. Fig. 1 illustrates a typical calibration curve with equation $y = 93893x - 1139.1$ and R^2 value 0.9988. Table 1 summarizes the experimental data, where the second column comprises the theoretical certified $\delta^{13}C$ values of CU/USADA-33-1 and the last two columns the experimental $\delta^{13}C$ as estimated from CO_2 calibration and calibration curve, respec-

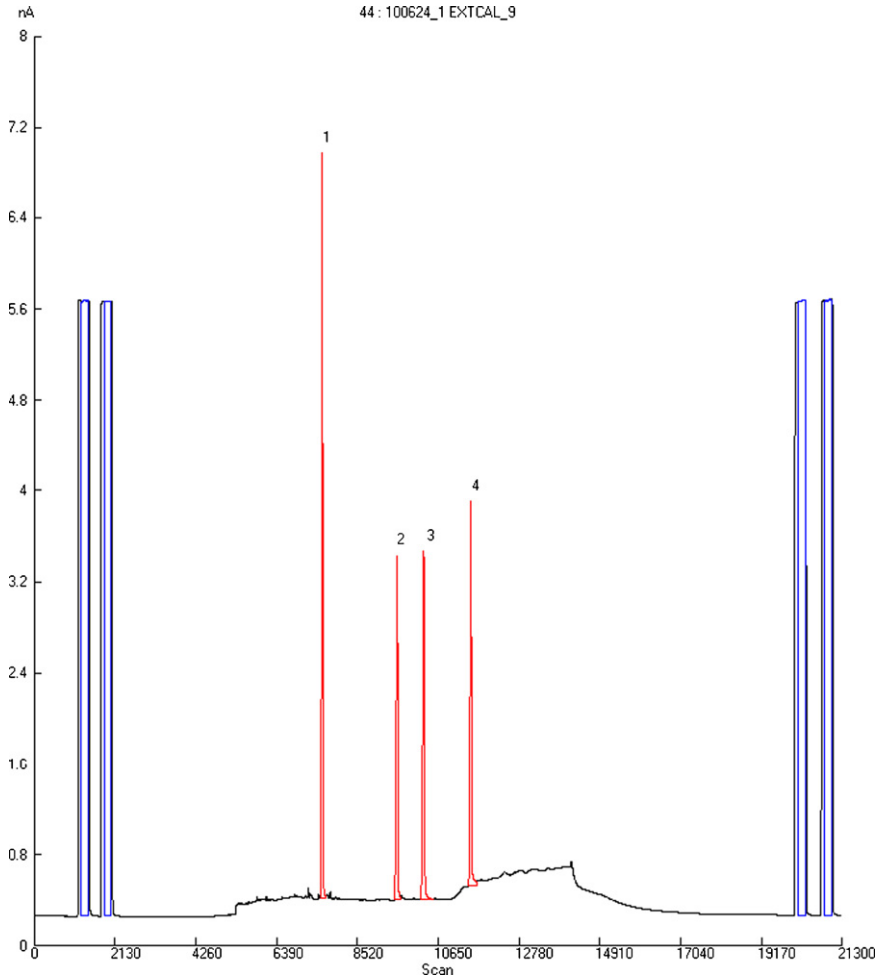

Fig. 2. Reference chromatogram of external mixture. Peaks refer to (1) 5α-androstane-3β-ol acetate, (2) 5α-cholestane, (3) androsterone acetate, and (4) 11-keto-etiocholanolone acetate.

Table 1
Data for calibration curve construction after three repeated measurements.

Reference compounds	Certified $\delta^{13}C$ (‰)	GC–C–IRMS analysis		
		R_{mean}(45/44)	$\delta^{13}C$ (‰) (instrument-CO_2)	$\delta^{13}C$ (‰) (curve)
5α-Androstane-3β-acetate	−30.6	1.1802E−02	−30.3	−31.4
5α-Cholestane	−24.7	1.1870E−02	−24.5	−25.1
Androsterone acetate	−33.0	1.1783E−02	−32.0	−33.3
11-Keto-etiocholanolone acetate	−16.7	1.1954E−02	−17.0	−17.1

Table 2
Calibration curves and statistics of Athens IRMS analysis.

Day	Curve	R^2	r	S_α	S_β	$S_{y/x}$
	$y = 92{,}471x - 1123.4$	0.9990	0.9995	2034	24.1	0.28
1	$y = 93{,}469x - 1134.6$	0.9984	0.9992	2617	31.0	0.35
	$y = 93{,}186x - 1131.3$	0.9988	0.9994	2244	26.6	0.30
	$y = 94{,}370x - 1145.2$	0.9988	0.9994	2286	27.1	0.31
2	$y = 91{,}349x - 1108.9$	0.9986	0.9993	2421	28.7	0.33
	$y = 93{,}893x - 1139.1$	0.9988	0.9994	2285	27.1	0.31
	$y = 93{,}249x - 1133.4$	0.9989	0.9994	2222	26.4	0.30
3	$y = 92{,}200x - 1121.0$	0.9973	0.9986	3400	40.4	0.46
	$y = 95{,}168x - 1156.3$	0.9995	0.9997	1554	18.5	0.21
	$y = 93{,}241x - 1133.4$	0.9986	0.9993	2463	29.2	0.33
4	$y = 92{,}659x - 1126.5$	0.9976	0.9988	3184	37.8	0.43
	$y = 93{,}572x - 1137.5$	0.9963	0.9982	4021	47.8	0.54

Table 3
Calibration curves and statistics of Sydney IRMS analysis.

Curve	R^2	r	S_α	S_β	$S_{y/x}$
$y = 103{,}764x - 1237.3$	0.9991	0.9996	2181	25.4	0.26
$y = 98{,}247x - 1173.7$	0.9968	0.9984	3908	45.6	0.50
$y = 101{,}528x - 1211.3$	0.9978	0.9989	3400	39.7	0.42

Table 4
Comparison between Sydney and Athens $\delta^{13}C$ after (A) external calibration and (B) CO_2 calibration for underivatised androsterone and etiocholanolone.

Sample	Androsterone $\delta^{13}C$			Etiocholanolone $\delta^{13}C$		
	Sydney	Athens	$\Delta\delta$	Sydney	Athens	$\Delta\delta$
A						
CU/USADA 34-2	−27.0	−27.4	0.4	−29.5	−29.5	0.0
NMI CRM Urine	−27.9	−27.8	−0.1	−24.0	−24.8	0.8
Urine A	−29.2	−28.3	−0.9	−29.3	−29.1	−0.3
Urine B	−25.4	−25.6	0.1	−28.0	−28.6	0.6
Urine C	−18.9	−19.3	0.4	−20.2	−20.7	0.5
Urine D	−27.6	−27.6	0.0	−28.2	−28.9	0.8
Urine E	−20.0	−21.0	1.0	−24.7	−26.0	1.3
B						
CU/USADA 34-2	−26.7	−26.4	−0.3	−29.0	−28.4	−0.6
NMI CRM Urine	−27.2	−26.7	−0.5	−24.0	−23.9	−0.1
Urine A	−28.4	−27.9	−0.4	−28.5	−28.6	0.1
Urine B	−25.3	−25.4	0.1	−27.5	−28.2	0.8
Urine C	−19.9	−19.3	−0.6	−21.0	−20.6	−0.4
Urine D	−27.1	−26.8	−0.3	−27.5	−28.0	0.5
Urine E	−20.8	−20.3	−0.5	−24.7	−25.0	0.4

tively. The chromatogram of the reference mixture CU/USADA-33-1 is presented in Fig. 2. Tables 2 and 3 list the standard deviation of the residuals ($s_{y/x}$), the standard deviation for the slope (s_a) and for the intercept (s_b) for Athens and Sydney curves, respectively. The r-values are in the range $0.9982 \leq r \leq 0.9997$. An inference for the stability of the calibration curves can be extracted, estimating the relative standard deviation of the slopes of Table 2 to be 1.08% after four (4) experimental days using the same combustion reactor, and of Table 3 to be 2.74%. The relative standard deviation of the slopes of both Athens and Sydney calibration curves is 3.76%. Moreover, the $\delta^{13}C$ values of the external mixture compounds were calculated and compared with corresponding certified $\delta^{13}C$ values for both Tables 2 and 3, where the error values fluctuate between −0.4 and 0.5 and standard deviation between 0.04 and 0.33.

3.2. Urine analysis of the interlaboratory test

The protocols of the Sydney–Athens interlaboratory test comprised the following features, as described in the experimental part: LC clean up, separate analysis of the fractions, common derivatisation and derivatives, same ERC compared to the same steroid

Table 5
Comparison between Sydney and Athens $\delta^{13}C$ after (A) external calibration and (B) CO_2 calibration for 5β-androstane-3α,17β-diol (diacetate) and pregnanediol (diacetate).

Sample	5β-Androstane-3α,17β-diacetate $\delta^{13}C$			Pregnanediacetate $\delta^{13}C$		
	Sydney	Athens	$\Delta\delta$	Sydney	Athens	$\Delta\delta$
A						
CU/USADA 35-1	−32.9	−33.8	0.9	−24.4	−23.6	−0.8
Urine A	−33.1	−33.1	0.1	−27.5	−26.8	−0.6
Urine B	−32.8	−33.0	0.2	−27.5	−26.4	−1.0
Urine C	−23.9	−23.3	−0.7	−23.8	−23.6	−0.2
Urine D	−30.5	−29.3	−1.1	−24.2	−23.8	−0.4
Urine E	−31.1	−30.9	−0.2	−24.6	−24.8	0.2
B						
CU/USADA 35-1	−31.5	−32.5	1.0	−24.5	–	–
Urine A	−31.6	−32.3	0.7	−27.0	−26.6	−0.4
Urine B	−31.4	−32.0	0.7	−25.3	−26.2	0.9
Urine C	−24.1	−22.9	−1.2	−23.8	−23.5	−0.2
Urine D	−29.5	−28.4	−1.0	−24.3	−23.8	−0.5
Urine E	−30.0	−29.8	−0.2	−24.6	−24.6	0.0

Table 6
Comparison of the differences of $\delta^{13}C$ pregnanediacetate and 5β-androstane-3α,17β-diol diacetate obtained from Sydney and Athens.

Sample	Δ (‰) = $\delta^{13}C_{ERC} - \delta^{13}C_{analyte}$		$\Delta_{Syd} - \Delta_{Ath}$
	Sydney	Athens	
CU/USADA 35-1	8.5	10.2	−1.7
NMI CRM Urine	–	–	–
Urine A	5.6	6.3	−0.7
Urine B	5.3	6.6	−1.3
Urine C	0.1	−0.3	0.4
Urine D	6.3	5.5	0.8
Urine E	6.5	6.1	0.4

metabolites and same set of the external calibration compounds of CU/USADA-33-1.

In Table 4A, the differences between Sydney and Athens $\delta^{13}C$ of underivatised metabolites etiocholanolone and androsterone are presented. The sample Urine E comprises the highest disagreement between Athens and Sydney, 5.3%. In Table 5A, the respective data for the acetylated 5β-androstane-3α,17β-diol and pregnanediol are presented. The sample Urine D comprises the highest disagreement between Athens and Sydney, 4.1%. In Tables 4B and 5B, $\delta^{13}C$ and differences respective to Tables 4A and 5A after the regular routine CO_2 calibration as performed by the instruments' software are presented as a comparison and proof to the accuracy of the external calibration approach.

In Table 6, the differences of the same diacetates derivatives of pregnanediol and 5β-androstane-3α,17β-diol are presented [36]. The agreement between Sydney and Athens is considered satisfactory for GC–C–IRMS in anti-doping laboratories.

3.3. Test of accuracy

The use of certified reference materials in the GC–C–IRMS analysis allows the inclusion of accuracy quality control samples to the analytical protocol. The accuracy of the method was examined by the analysis of three different sets of certified steroids, obtained from different sources. Two accuracy control samples, having water as matrix, were spiked with the solutions USADA 35-1 for the derivatised fractions and USADA 34-2 for the underivatised fraction, respectively. The third accuracy control material was the NMI urine sample. The comparison between theoretical and experimental $\delta^{13}C$ estimated by the calibration curve is shown in Table 7. The certified δ-values of pregnanediol and 5β-androstan-3α,17β-diol were corrected with the Athens correction factor, because of the introduction of acetate moieties, during derivatisation [37–40]. The $\delta^{13}C$ values of the second column represent the average of duplicate analysis. If a ±10% target accuracy value is considered appropriate for antidoping quantitative measurements [41], then the results indicate good agreement, between certified and experimental $\delta^{13}C$.

3.4. Considerations of ^{17}O correction to R45

The *R*45 signal is composed of ion currents of $^{13}C^{16}O^{16}O^{\bullet+} + {}^{12}C^{17}O^{16}O^{\bullet+}$. Numerous methods have been proposed for subtraction of the $^{12}C^{17}O^{16}O^{\bullet+}$ contribution to the *R*45 signal to yield an R13(=$^{13}C/^{12}C$) [42]. Most require use of the *R*46 signal and the assumptions of a stochastic distribution of oxygen isotopes to derive a $^{18}O/^{17}O$ value. An adjustment factor can then be calculated for conversion of *R*45 to R13, for suitable calculation of $\delta^{13}C$. A deviation from existing methods is that our calibration method does not employ the *R*46 signal.

Most ^{17}O correction methods result in differences in the final calculated isotope ratio of <0.1‰. Distinctions among them are of value in the highest precision, dual inlet IRMS analyses that report SD($\delta^{13}C$) ~ 0.01‰, most relevant for samples of CO_2 occurring in nature where the $\delta^{13}C$ and $\delta^{18}O$ are both of interest and may vary independently from sample to sample. In contrast, the precision of GC–C–IRMS is about SD($\delta^{13}C$) ~ 0.3‰, and the O isotope ratio arises primarily from the combustion reactor, discussed below.

As a practical matter, the vast majority of GC–C–IRMS analyses, particularly in the antidoping field, employ one of two methods

Table 7
Comparison between estimated and certified $\delta^{13}C$ values (‰) for accuracy test of Athens data (differences between $\delta^{13}C$ values indicated with *).

Steroid	$\delta^{13}C$ experimental	$\delta^{13}C$ certified	Corrected $\delta^{13}C$ certified	$\Delta\delta$
Etiocholanolone (USADA 34-2)	−29.5 *	−28.9 *	–	0.6
Etiocholanolone (NMI CRM)	−24.8 *	−24.1 *	–	0.7
Androsterone (USADA 34-2)	−27.4 *	−27.1 *	–	0.3
Androsterone (NMI CRM)	−27.8 *	−27.2 *	–	0.6
Pregnanediacetate (USADA 34-2)	−33.8 *	−31.5	−34.4 *	−0.6
Pregnanediacetate (USADA 35-1)	−23.6 *	−18.4	−23.4 *	0.2
Pregnanediacetate (NMI CRM)	−26.8 *	−23.1	−27.3 *	−0.5
5β-Androstan-3α,17β-diol diacetate (USADA 35-1)	−33.8 *	−29.0	−32.5 *	1.3

available in instrument vendor-supplied software. Both the original Craig method [24] and the Santrock et al. method [43] rely upon assumptions about the average mass-dependent fractionation of ^{17}O in nature so to calculate ^{17}O based on a measurement of ^{18}O. The usual implementation of the Craig correction assumes a constant ratio of about $^{18}O/^{17}O = 5.47:1$ and fractionation of 0.5. Santrock et al. [43] make a similar assumption with a fractionation of 0.516. They present experimental data showing that ^{18}O selectively enriched waters produce incorrect results by many ‰. They show that this can be taken into account by either specifying $^{13}C/^{12}C$ or $^{17}O/^{16}O$; thus one or the other must be known, or assumed, to calculate the other. In the present case, $^{13}C/^{12}C$ is the unknown, thus $^{17}O/^{16}O$ cannot be calculated from it and an assumption must be made.

Corrections to *R*45 for ^{17}O depend (weakly) on the abundances of O isotopes in CO_2 derived from steroid analytes, and thus we consider their sources in steroid GC–C–IRMS analysis. The $\delta^{18}O$ originating in the combustion furnace is independent of the analyte and CO_2 isotopologues will be stochastically distributed due to the high reaction temperature. Consider the contribution of O from steroids and from the combustion furnace. Native steroids contain 19–23 C and 2–5 O. Taking the low extreme for C as 19, and the high for O as 5, we note that the final CO_2 entering the IRMS has 38 O, of which at most 5 are native to the steroid; a minimum of 33/38 = 87% of the O originate in the combustion furnace. As a general rule, we can thus estimate that 90% of the O in CO_2 analyzed by the IRMS originates in the oxidation furnace and thus is expected to be invariant from sample to sample and analyte to analyte.

An experimental verification of this latter assumption is found by regular analysis of QC samples which include a steroid with a stable $\delta^{13}C$. Observation of a constant $\delta^{13}C$, within experimental error, for a particular steroid analyzed by GC–C–IRMS is necessary and sufficient to conclude that the contribution of ^{17}O to the *R*45 signal is stable over the course of a series of GC runs. When this is the case, measurement of *R*46 is redundant, and an unbiased $\delta^{13}C$ within the precision of the measurement can be calculated from a calibration curve based on a plot of $\delta^{13}C$ vs *R*45/*R*44 as described here.

Finally, it is of interest to note that a now-defunct continuous flow IRMS manufacturer, PDZ Europa, used a slope-based method [44,45] that explicitly used only two signals to calculate isotope ratios. This procedure differs in form but is not unlike the procedure evaluated in detail here.

4. Conclusion

To our knowledge, this is the first literature presentation of interlaboratory data on IRMS measurements of steroids for the antidoping analysis. The proposed external calibration protocol for the GC–C–IRMS uses the same approach as the calibration protocols of the common quantitative procedures of the WADA Accredited Laboratories. A potential advantage over the CO_2 pulse method is that the reference steroids undergo all the processes of GC injection, separation and combustion that the steroids of the samples. Moreover, the $\delta^{13}C$ values and the retention times of the reference steroids are chosen to be at the respective range of the analytes of interest. The certified steroids can be provided to WADA Accredited Laboratories and therefore, a uniform calibration of GC–C–IRMS instruments is feasible and directly applicable. Moreover, the certified steroids offer the possibility to develop a quality control scheme for every batch of samples. External calibration has the capability of correcting the systematic drift, observed to GC–C–IRMS analysis over time.

The calibration curves are reproducible and linear in the range of $\delta^{13}C$ values of interest. The external calibration was applied to urine specimens and accuracy data proved its suitability. The Sydney and Athens Laboratories applied the new method to the same set of urine samples, using instruments from different vendors. The interlaboratory study showed a good agreement between the $\delta^{13}C$ values calculated by Sydney and Athens Laboratories. In conclusion, the current project enhances the harmonization of GC–C–IRMS analyses and the application of quality control for the WADA Accredited Laboratories.

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