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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-