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Steroid isotopic standards for gas chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS)

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ABSTRACT

Carbon isotope ratio (CIR) analysis of urinary steroids using gas chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS) is a recognized test to detect illicit doping with synthetic testosterone. There are currently no universally used steroid isotopic standards (SIS). We adapted a protocol to prepare isotopically uniform steroids for use as a calibrant in GCC-IRMS that can be analyzed under the same conditions as used for steroids extracted from urine. Two separate SIS containing a mixture of steroids were created and coded CU/USADA 33-1 and CU/USADA 34-1, containing acetates and native steroids, respectively. CU/USADA 33-1 contains 5α-androstan-3β-ol acetate (5α-A-AC), 5α-androstan-3α-ol-17-one acetate (androsterone acetate, A-AC), 5β-androstan-3α-ol-11, 17-dione acetate (11-ketoetiocholanolone acetate, 11k-AC) and 5α-cholestane (Cne). CU/USADA 34-1 contains 5β-androstan-3α-ol-17-one (etiocholanolone, E), 5α-androstan-3α-ol-17-one (androsterone, A), and 5β-pregnane-3α, 20α-diol (5βP). Each mixture was prepared and dispensed into a set of about 100 ampoules using a protocol carefully designed to minimize isotopic fractionation and contamination. A natural gas reference material, NIST RM 8559, traceable to the international standard Vienna PeeDee Belemnite (VPDB) was used to calibrate the SIS. Absolute $\delta^{13}C_{VPDB}$ and $\Delta\delta^{13}C_{VPDB}$ values from randomly selected ampoules from both SIS indicate uniformity of steroid isotopic composition within measurement reproducibility, SD($\delta^{13}C$) < 0.2‰. This procedure for creation of isotopic steroid mixtures results in consistent standards with isotope ratios traceable to the relevant international reference material.

1. Introduction

Anabolic androgenic steroid (AAS) use was prohibited by the International Olympic Committee (IOC) in 1976, and since various approaches to monitor compliance were developed [1]. Urinary testosterone (T) concentration is an unsuitable metric because of the large concentration range of inter-individual urinary steroid excretion, eliminating the possibility that T doping could be detected on the basis of concentration [2]. In 1983, Donike et al. [3] proposed the testosterone/epitestosterone (T/E) excretion ratio measured by benchtop gas chromatography–mass spectrometry (GC–MS) for this purpose. This test was first employed in Olympic doping control during the 1984 Los Angeles Olympic Games [4]. Currently, a T/E ratio greater than 4:1 is used as a screen for exogenous T administration. There are several issues with the use of T/E alone to confirm T use. Normal inter-individual variations related to urinary steroid excretion gives a T/E ratio > 4:1

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in some cases. Tests of endocrine function are necessary to rule out high T/E ratios due to T secreting tumors or other hormonal disorders [2]. Also, simultaneous T doping with exogenous E as a masking agent can prevent a high urinary T/E ratio [5]. Finally, a major caveat of GC–MS is that it does not establish whether urinary T in any particular sample is of exogenous origin.

High precision gas isotope ratio mass spectrometry (IRMS) is the classical technique for determination of isotope fractionation due to natural processes for C, H, N, O, and S [6,7]. High precision compound specific isotope analysis (CSIA) was introduced in 1978 by Matthews and Hayes [8] using previously established instrumental principles [9], and instrumentation has been available commercially since about 1990. Briefly, the effluent of a gas chromatography (GC) column is directed to a combustion (C) interface, and the CO_2 thus from analyte combustion is in turn directed to an IRMS for continuous isotope ratio monitoring. Modern GCC-IRMS instruments calibrate isotopic signals using isotopically calibrated CO_2 admitted to the IRMS from a separate, external volume.

Becchi et al. [10] first proposed synthetic testosterone detection by CSIA using high precision GCC-IRMS in 1994, now known as the Carbon isotope ratio (CIR) test. The method exploits the natural difference in $^{13}C/^{12}C$ between C3 and C4 plants the mix of which determine the $^{13}C/^{12}C$ of biomolecules excreted in urine. The $^{13}C/^{12}C$ of a steroid on a parallel metabolic pathway but unaffected T or E doping, known as an endogenous reference compound (ERC), is compared with the $^{13}C/^{12}C$ of T or one of its metabolites [11–13]. Synthetic T produced commercially entirely from the C3 plant soy has a significantly lower $^{13}C/^{12}C$ than the ERC which arises metabolically from mixed C3 and C4 precursors.

The $^{13}C/^{12}C$ values detected by IRMS are expressed in terms of relative parts per thousand compared to an international standard reference material (VPDB), $\delta^{13}C_{VPDB} = \{(R_S - R_{VPDB})/R_{VPDB}\} \times 1000$, where R_S is the $^{13}C/^{12}C$ of a steroid, and $R_{VPDB} = 0.0112372 \pm 0.0000090$. An isotope ratio difference of 3‰ has been used as a threshold level in sports legislation [14]. Although CIR analysis of urinary steroids using GCC-IRMS is in wide use worldwide for detection of T doping [2], GCC-IRMS continues to be a specialty technique that requires careful procedures for standardization. In 2003, a United States Antidoping Agency (USADA) research symposium recommended development of steroid isotopic internal standards to harmonize reported values and achieve more uniform results [15]. Ideally, these would be isotopically calibrated steroids that could be analyzed under GC conditions identical to sample steroids, and their GC retention time and isotope ratios would bracket the range of interest.

Here we report a protocol for creation of isotopically uniform steroid standards for calibration of GCC-IRMS data between the antidoping labs. This procedure adapted from Caimi et al. [16] ensures uniform stable isotopic composition among a prepared set of containers. The steroids were selected based on their isotopic composition, metabolic role, and GC retention times. Isotopic values are calibrated against a natural gas reference mixture to insure that the standard traversed the same analytical path as the steroids to be calibrated. We introduce a procedure for isotopic calibration of GC peaks that uses an external CO_2 tank but is immune to differential fractionation that may occur in the flow path between the volume containing the standard CO_2 gas and the IRMS, compared to the flow path between the GC and the IRMS.

2. Experimental

2.1. Chemicals and standards

High purity He, N_2, O_2, and CO_2 were purchased from Airgas East (Salem, NH). The steroid acetate isotopic standard coded CU/USADA 33-1 was made with the following components: 5α-androstan-3β-ol acetate (5α-A-AC), 5α-androstan-3α-ol-17-one acetate (androsterone acetate, A-AC), 5β-androstan-3α-ol-11, 17-dione acetate (11-ketoetiocholanolone acetate, 11k-AC) and 5α-cholestane (Cne). A second isotopic standard coded CU/USADA 34-1 was made with native (underivatized) steroids with the following components: 5β-androstan-3α-ol-17-one (etiocholanolone, E), 5α-androstan-3α-ol-17-one (androsterone, A), and 5β-pregnane-3α, 20α-diol (5βP). All steroids were 99% purity, and were purchased from Steraloids (Newport, RI) with the exception of 5βP which was purchased from Acros Organics USA (Morris Plains, NJ). The steroid isotopic standards (SIS) were created and prepared as described in the sections below. The natural gas reference material NIST RM 8559 (coal origin, >80% methane) was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

2.2. Creation of steroid isotopic standards (SIS)

All glass containers were used to prevent any possible contamination due to solvent extraction of polymeric materials associated with plastic containers. The glass containers were thoroughly solvent washed and dried before use. For CU/USADA 33-1, 60 mg of each of the four steroids were dissolved into a single volume of 300 mL 2-propanol. For CU/USADA 34-1, 100 mg of each of the three steroids was dissolved into separate volumes of 20 mL 2-propanol. All steroids were allowed to dissolve completely overnight at room temperature in capped containers. For CU/USADA 34-1, a 1.5 mL aliquot of each pure steroid solution (~7.5 mg) was taken for elemental analysis IRMS (EA-IRMS), while the remainder of the solutions was pooled to create a master solution containing all the steroids for the respective standards, and then diluted into 2-propanol to a total volume of 500 mL. For both CU/USADA 33-1 and CU/USADA 34-1, 2 mL aliquots of the master solution were dispensed into 2 mL amber glass ampoules (Fisher Scientific Inc.). High purity N_2 was used to evaporate solvent at 80 °C, leaving crystallized standard in the container. The ampoules were then N_2-flushed and flame-sealed for safe and convenient storage and shipment. For each standard, approximately 100 ampoules were made.

2.3. Steroid isotopic standard (SIS) preparation for GCC-IRMS analysis

Randomly selected ampoules for each SIS were prepared for GCC-IRMS analysis. Each amber glass ampoule was carefully cracked open at the score at the neck, filled with 2 mL of 2-