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Uniform isotopic standards for gas chromatography combustion isotope ratio mass spectrometry of steroids

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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 versions of normally endogenous steroids such as testosterone. However, there are currently no universally used steroid isotopic standards (SIS). We developed a protocol to prepare isotopically uniform steroids for use as a calibrant in GCC-IRMS. These SIS can be analyzed under the same conditions as used to analyze 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. CU/USADA 33-1 contains 5α-androstan-3β-ol acetate, 5α-androstan-3α-ol-17-one acetate, 5β-androstan-3α-ol-11,17-dione acetate and 5α-cholestane. CU/USADA 34-1 contains 5β-androstan-3α-ol-17-one, 5α-androstan-3α-ol-17-one, and 5β-pregnane-3α, 20α-diol. Each mixture was prepared and dispensed into a large set of ampoules using a protocol carefully designed to minimize isotopic fractionation and contamination. The ampoules were subsequently flame sealed. A natural gas reference material, which is traceable to the international standard NIST RM 8559, was used to calibrate the SIS. Eight ampoules were randomly selected from both SIS CU/USADA 33-1 and SIS CU/USADA 34-1 batches for analysis by GCC-IRMS. The absolute $\delta^{13}C_{VPDB}$ and the $\Delta\delta^{13}C_{VPDB}$ values measured for the steroids in both CU/USADA 33-1 and CU/USADA 34-1 indicate uniformity of steroid isotopic composition within measurement reproducibility among the ampoule sets prepared.

Introduction

After the use of anabolic androgenic steroids (AAS) was prohibited by the International Olympic Committee (IOC) in 1976, several techniques were developed to

detect these compounds (Bowers 1997). Donike et al (Donike *et al.* 1983) proposed an approach, which measures testosterone/epitestosterone (T/E) excretion ratio using benchtop gas chromatography-mass spectrometry (GC-MS). A T/E ratio greater than 4:1 (formerly 6:1) is used as a confirmation of exogenous T administration. This test was first employed in Olympic doping control during the 1984 Los Angeles Olympic Games (Cawley *et al.* 2006). There are several issues with the technique. It has been found that normal inter-individual variations related to urinary steroid excretion may give a T/E ratio > 4:1 in some cases, and a high ratio due to exogenous (doped) T use can be adjusted downward by simultaneous administration of E (Shackleton *et al.* 1997). In addition, tests of endocrine function have to be performed to rule out high T/E ratios due to T secreting tumors or other hormonal disorders (Aguilera *et al.* 2000). A major caveat of GC-MS is that it does not establish whether T is of exogenous origin.

Becchi et al (Becchi *et al.* 1994) first reported synthetic testosterone detection by compound specific isotope analysis (CSIA) using high precision GC combustion isotope ratio mass spectrometry (GCC-IRMS) in 1994. The principle of this method is based on the relative difference between the $^{13}C/^{12}C$ ratios of endogenous steroid compared with the $^{13}C/^{12}C$ ratio of the corresponding synthetic steroid (Farquhar 1983; Kleemann and Roth 1983; Vogel 1980). By this principle, the use of exogenous T can be detected by measuring the isotope ratio of an endogenous reference compound (ERC) in the precursor pathway to T, along with T metabolites. An isotope ratio difference of 3‰ has been used as a threshold level in sports legislation (WADA 2004). In the year 2000, the International Olympic Committee first authorized the antidoping labs to detect testosterone misuse by GCC-IRMS.

The Carbon Isotope Ratio (CIR) analysis of urinary steroids using GCC-IRMS is now a recognized tool for detection of doping with synthetic steroids (Aguilera *et al.* 2000); however, 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 (USADA 2003). 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 (Caimi *et al.* 1994), and 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. We introduce a procedure for isotopic calibration of GC peaks that uses an external CO2 tank but is immune to differential fractionation that may occur in the flow path between the volume containing the standard CO2 gas and the IRMS, compared to the flow path between the GC and the IRMS.

Materials and Methods

Chemicals and Standard Mixtures. High purity He, high purity N_2, ultra high purity O_2, and instrument Coleman minimum purity CO_2 were purchased from Airgas East (Salem, NH). The steroid isotopic standard coded CU/USADA 33-1 was made with the following components: 5α-androstan-3β-ol acetate (5α-andro-AC), 5α-androstan-3α-ol-17-one acetate (andro-AC), 5β-androstan-3α-ol-11,17-dione acetate (11-keto-AC), and 5α-cholestane (chol). The steroid isotopic standard coded CU/USADA 34-1 was made with the following components: 5β-androstan-3α-ol-17-one (etio), 5α-androstan-3α-ol-17-one (andro), 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).

Steroid Isotopic Standard (SIS) Creation. 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 steroid 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. For both CU/USADA 33-1 and CU/USADA 34-1, all steroids were allowed to dissolve completely overnight at room temperature in capped containers to prevent solvent evaporation. 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 making them suitable for storage and shipment. For each standard, approximately 100 ampoules were made.

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-propanol and allowed to sit over 3 hours to fully dissolve steroid components. After full dissolution, the SIS solution was then removed from the ampoule into a clean working glass container. Before analysis with GCC-IRMS, the SIS was diluted to approximately 100 ng/µL in the case of CU/USADA 33-1 and 94 ng/µL in the case of CU/USADA 34-1. A 1 µL sample was analyzed in each GCC-IRMS run.

GCC-IRMS Setup. An HP 5890 or 6890 GC with a split/splitless inlet (Agilent Technologies, Palo Alto, CA) with autoinjector were coupled to a Thermo MAT 253 IRMS (Bremen, Germany) via a home-built combustion interface. The IRMS was tuned for high linearity. The IRMS was operated at a source pressure of 2.3×10^{-6} Torr, and high voltage of 9.511 kV with a measured sensitivity of 830 molecules/ion detected. Data were collected from the IRMS and analyzed using ISODAT 2.5.

The GC column was connected to an online micro-combustion reactor via a four-way rotary valve which permitted solvent diversion. The micro-combustion reactor was constructed with a 30cm × 0.5mm i.d. alumina tube hand-packed with three 20cm x 0.1mm wires (i.e. 1 Cu, 1 Pt, and 1 Ni wire). The tube was maintained at 950°C using a 30 cm Thermcraft tube furnace (Winston Salem, NC). Water generated due to combustion was removed from the system using a Nafion® water trap (dimensions = 10 cm × 0.8 mm i.d.) upstream of the IRMS. An open-split consisted of a 1m × 0.075mm IRMS inlet sampling capillary that was directly inserted into the post-water trap transfer line. The same combustion interface design was used for all measurements.

GC Parameters for SIS CU/USADA 33-1 Analysis. A 30 m × 0.25 mm × 0.25 µm Varian VF-5ms column (5% phenyl, 95% dimethyl polysiloxane) was used. The GC conditions were: head pressure 21 psi, inlet 280 °C, splitless injection, total flow 25.4 mL/min, purge flow 10 mL/min. The oven parameters were: 80°C (initial, hold 1 min) ramped to 270 °C (30 °C/min, hold 12 min), and ramped to 300 °C (10 °C /min hold 4.67 min).

GC Parameters for CU/USADA 34-1 Analysis. A 30 m × 0.25 mm × 0.25 µm J & W Scientific DB-17ms (50% diphenyl, 50% dimethyl polysiloxane) was used. The GC conditions were

same as those for CU/USADA 33-1 analysis, except that the head pressure was 20 psi. The oven parameters were: 70°C (initial, hold 1 min) ramped to 270 °C (30 °C/min, hold 11 min), and ramped to 300 °C (10 °C/min hold 3 min).

GC Parameters for NIST RM 8559 Analysis. A 30 m × 0.32 mm GS-GasPro column (Agilent Technologies, Palo Alto, CA) was used. The GC conditions were: head pressure 12 psi, inlet 200 °C, split injection at 50:1, and purge flow at 10 mL/min. The oven parameters were: 25 °C (initial, hold 4 min) and ramped to 80 °C (10 °C /min, hold 1 min). A 100 µL gas tight syringe (Hamilton) was used for manual injections of gas on both setups for CU/USADA 33-1 and CU/USADA 34-1 calibration.

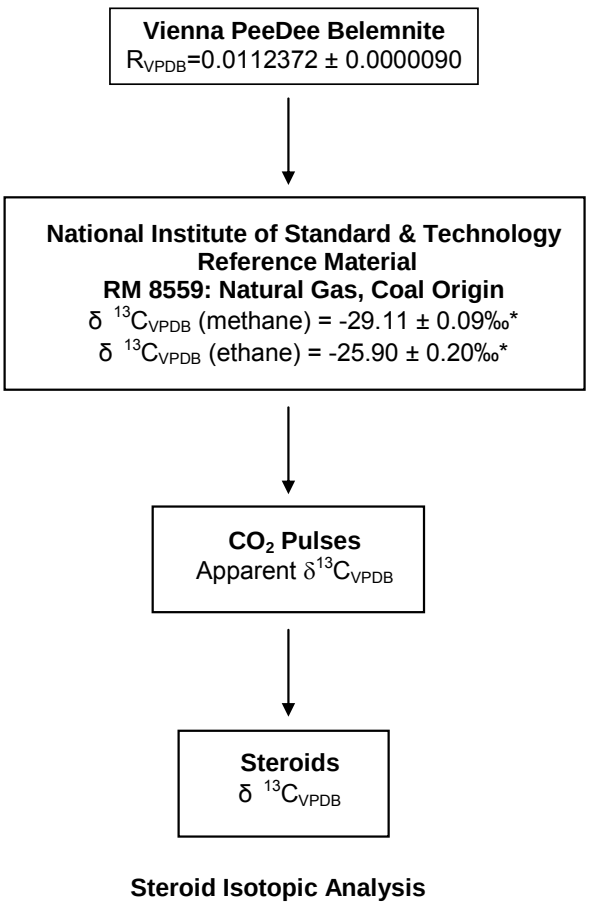

Figure 1: δ $^{13}C_{VPDB}$ Calibration traceability. *Uncertainty reported by NIST.

EA-IRMS Parameters for Analysis of Individual Steroids in CU/USADA 34-1. Four replicates of each steroid (1.25 mg each replicate) was analyzed using a NC 2500 elemental analyzer (Thermo Finnigan, Bremen, Germany) interfaced to a Delta Plus IRMS (Thermo Finnigan, Bremen, Germany) (Grassineau 2006). Data were collected from the IRMS and analyzed using ISODAT 2.0. Analysis was performed in the Cornell University Stable Isotope Laboratory (COIL).

GCC-IRMS Calibration for CU/USADA 33-1 and CU/USADA 34-1. A system for sampling reference material NIST RM8559 (natural gas, coal origin, >80% methane) was built. An aliquot of RM 8559 was expanded and equilibrated at ~1-2 atm into a previously evacuated 25 mL volume fitted with a septum sampling port. Approximately 2 µL, 30 µL, and 60µL volumes of RM8559 gas from the septum sampling port were injected into GCC-IRMS with 4 replicates of each volume for analysis. Figure 1 shows the calibration traceability of our

measurements for CU/USADA 33-1 and CU/USADA 34-1. Two of the major components in the RM8559, methane and ethane, have $\delta^{13}C_{VPDB}$ = -29.11‰ and $\delta^{13}C_{VPDB}$ = -25.9‰, respectively, and were used to calibrate the CO_2 gas pulses admitted into the IRMS from a high volume pressurized CO_2 tank. Three CO_2 gas pulses are admitted at the beginning and three pulses are admitted towards the end of each GCC-IRMS run for the SIS and the RM 8559. The value measured for the CO_2 is referred to as an "apparent" $\delta^{13}C_{VPDB}$ since it does not tranverse the same flow path as the GC analytes. The apparent $\delta^{13}C_{VPDB}$ assigned to the CO_2 is then used to subsequently calibrate CU/USADA 33-1 and CU/USADA 34-1 peaks. With this scheme, any differential fractionation attributable to the different flow paths cancels out. Steroid calibration is traceable to the international standard Vienna Pee Dee Belemnite (VPDB, R_{VPDB}=0.0112372 ± 0.0000090).

Results and Discussion

CIR Analysis of CU/USADA 33-1. The contents of eight randomly selected ampoules containing the SIS mixture CU/USADA 33-1 were analyzed by GCC-IRMS, with four replicate injections analyzed for each ampoule. Two sets of data were acquired identically on two separate days. The absolute $\delta^{13}C_{VPDB}$ data from all the measurements are presented in Table 1, with no outliers eliminated. The absolute $\delta^{13}C_{VPDB}$ measured for the four steroids ranged from -33.04‰ to -16.70‰ with an average standard deviation of 0.11‰ within ampoules and 0.08‰ between ampoules. The $\Delta\delta^{13}C_{VPDB}$ values between 11-keto-AC and the other three steroids ranged from 8.08‰ to 16.34‰ with an average standard deviation of 0.15‰ within ampoules and 0.09‰ between ampoules (calculation not shown). This indicates uniformity of steroid isotopic composition within measurement reproducibility among the ampoule set prepared. Using a least squares fit model, an analysis of variance confirmed that there was no ampoule effect on the absolute $\delta^{13}C_{VPDB}$ values and $\Delta\delta^{13}C_{VPDB}$ values for all the steroids in CU/USADA 33-1. The average deviation of isotope ratios of the four steroids was <0.13‰ between the two different days.

CIR Analysis of Steroid Standard CU/USADA 34-1. The contents of eight randomly selected ampoules containing the SIS mixture CU/USADA 34-1 were analyzed by GCC-IRMS, with four replicate injections analyzed for each ampoule. Full sets of data were acquired identically on two separate days. Sixteen replicates of the master solution were also analyzed using GCC-IRMS on a separate day. The absolute $\delta^{13}C_{VPDB}$ data from all the measurements are presented in Table 2, with no outliers eliminated. The absolute $\delta^{13}C_{VPDB}$ measured for the three steroids

Table 1: CU/USADA 33-1 measured $\delta^{13}C_{VPDB}$ values, and their respective standard deviations (SD). Eight randomly selected ampoules containing the SIS mixture were analyzed. Data set 1 and data set 2 were acquired identically on two separate days. The 95% confidence limits were calculated using Student's t distribution.

Ampoule Data Set 1

		5a-andro-AC		Andro-AC		11-keto-AC		Chol	
Ampoule	**n**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**
1	4	-30.56	0.06	-32.81	0.18	-16.66	0.09	-24.81	0.10
2	4	-30.73	0.07	-32.88	0.26	-16.64	0.06	-24.78	0.05
3	4	-30.61	0.04	-33.14	0.30	-16.60	0.08	-24.78	0.14
4	4	-30.74	0.17	-33.09	0.19	-16.78	0.04	-24.80	0.13
5	4	-30.71	0.10	-33.04	0.20	-16.78	0.06	-24.91	0.15
6	4	-30.77	0.02	-33.12	0.06	-16.89	0.09	-24.94	0.08
7	4	-30.82	0.07	-33.10	0.15	-16.74	0.01	-24.99	0.08
8	4	-30.75	0.06	-33.25	0.11	-16.86	0.06	-24.83	0.07
1-8	**8**	**-30.71**	**0.08**	**-33.05**	**0.14**	**-16.74**	**0.10**	**-24.86**	**0.07**

Ampoule Data Set 2

		5a-andro-AC		Andro-AC		11-keto-AC		Chol	
Ampoule	**n**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**	**$\delta^{13}C_{VPDB}$**	**SD**
1	4	-30.45	0.09	-32.91	0.17	-16.72	0.15	-24.62	0.08
2	4	-30.47	0.06	-32.85	0.30	-16.69	0.07	-24.62	0.11
3	4	-30.56	0.09	-33.00	0.23	-16.61	0.11	-24.68	0.07
4	4	-30.48	0.06	-33.09	0.13	-16.66	0.16	-24.71	0.07
5	4	-30.52	0.07	-32.93	0.24	-16.64	0.04	-24.67	0.06
6	4	-30.56	0.08	-32.99	0.36	-16.55	0.14	-24.68	0.06
7	4	-30.49	0.05	-33.20	0.16	-16.64	0.07	-24.68	0.08
8	4	-30.56	0.08	-33.18	0.08	-16.65	0.09	-24.72	0.07
1-8	**8**	**-30.51**	**0.04**	**-33.02**	**0.13**	**-16.65**	**0.05**	**-24.67**	**0.04**

ranged from -27.06‰ to -31.49‰ with an average standard deviation of 0.10‰ within ampoules and master solution, and 0.07‰ between ampoules and master solution. The $\Delta\delta^{13}C_{VPDB}$ values between 5βP and the other two steroids ranged from 2.58‰ to 4.43‰ with an average standard deviation of 0.12‰ within ampoules and master solution, and 0.09‰ between ampoules and master solution. This indicates uniformity of steroid isotopic composition within measurement reproducibility among the master solution and ampoule set prepared. Analysis of variance showed that there is no ampoule effect for absolute $\delta^{13}C_{VPDB}$ and $\Delta\delta^{13}C_{VPDB}$ measurements of the three steroids.

Comparison of CIR Analysis of CU/USADA 34-1 by Elemental Analysis IRMS (EA-IRMS) and GCC-IRMS. The $\delta^{13}C_{VPDB}$ and $\Delta\delta^{13}C_{VPDB}$ values for CU/USADA 34-1 components

Table 2: CU/USADA 34-1 analytical results, including 95% confidence limits. See caption to Table 1 for details.

Ampoule Data Set 1										
		Etio			Andro			5βP		
Ampoule	n	$\delta^{13}C_{VPDB}$	SD	95% CL	$\delta^{13}C_{VPDB}$	SD	95% CL	$\delta^{13}C_{VPDB}$	SD	95% CL
1	4	-29.02	0.11	0.11	-27.38	0.12	0.11	-31.62	0.16	0.16
2	4	-28.80	0.10	0.10	-27.29	0.10	0.10	-31.53	0.15	0.15
3	4	-28.71	0.19	0.19	-27.13	0.17	0.16	-31.57	0.09	0.09
4	4	-28.83	0.05	0.05	-27.29	0.05	0.05	-31.53	0.09	0.09
5	4	-28.89	0.07	0.07	-27.13	0.10	0.09	-31.54	0.16	0.16
6	4	-28.79	0.15	0.14	-27.10	0.09	0.09	-31.50	0.11	0.11
7	4	-28.79	0.10	0.10	-27.02	0.11	0.11	-31.47	0.08	0.08
8	4	-28.88	0.09	0.09	-27.19	0.06	0.06	-31.49	0.13	0.13
1-8	**8**	**-28.84**	**0.09**	**0.06**	**-27.19**	**0.12**	**0.08**	**-31.53**	**0.05**	**0.03**
Ampoule Data Set 2										
		Etio			**Andro**			**5βP**		
Ampoule	**n**	$\delta^{13}C_{VPDB}$	**SD**	**95% CL**	$\delta^{13}C_{VPDB}$	**SD**	**95% CL**	$\delta^{13}C_{VPDB}$	**SD**	**95% CL**
1	4	-28.99	0.07	0.07	-26.86	0.11	0.11	-31.43	0.10	0.10
2	4	-29.02	0.05	0.05	-26.90	0.10	0.09	-31.35	0.12	0.12
3	4	-28.95	0.02	0.02	-26.93	0.13	0.12	-31.41	0.11	0.10
4	4	-28.94	0.14	0.14	-26.80	0.14	0.13	-31.51	0.07	0.07
5	4	-28.98	0.03	0.03	-26.90	0.05	0.05	-31.33	0.12	0.11
6	4	-28.97	0.05	0.05	-27.02	0.08	0.08	-31.47	0.06	0.06
7	4	-28.95	0.03	0.03	-27.01	0.09	0.09	-31.52	0.06	0.06
8	4	-28.95	0.07	0.07	-26.94	0.09	0.08	-31.47	0.08	0.08
1-8	**8**	**-28.97**	**0.03**	**0.02**	**-26.92**	**0.07**	**0.05**	**-31.44**	**0.07**	**0.05**
Master Solution Data Set										
Master Solution	**16**	**-28.82**	**0.13**	**0.06**	**-26.98**	**0.12**	**0.06**	**-31.62**	**0.11**	**0.06**

determined by EA-IRMS for the individual steroid master solutions is compared to that determined by GCC-IRMS for the master solution and eight randomly selected ampoules contents, presented in Table 3. The results show that the absolute $\delta^{13}C_{VPDB}$ values and $\Delta\delta^{13}C_{VPDB}$ measured by EA-IRMS for the three steroids deviates by less than 0.2‰ on average from those measured with GCC-IRMS analysis. The results also confirm that there is no significant isotopic fractionation between the master solution and the prepared ampoules.

In summary, a protocol is demonstrated here for the creation of uniform steroid isotopic standards for use in calibration of GCC-IRMS and harmonization of results from the carbon isotopic analysis for the detection of synthetic steroid use in sport doping. The protocol used to prepare the steroid isotopic standards in ampoule containers maintains isotopic integrity to a uniformity within 0.09‰ from ampoule to ampoule. A limited quantity of the standards

Table 3. Comparison of the $\delta^{13}C_{VPDB}$ values determined for CU/USADA 34-1 components by EA-IRMS of the individual steroid master solutions and by GCC-IRMS of the master solution and eight randomly selected ampoules. *average of two days.

CU/USADA 34-1										
		Etio			Andro			5βP		
Technique	n	$\delta^{13}C_{VPDB}$	SD	95% CL	$\delta^{13}C_{VPDB}$	SD	95% CL	$\delta^{13}C_{VPDB}$	SD	95% CL
EA	4	-28.91	0.02	0.02	-27.06	0.04	0.04	-31.42	0.06	0.06
GCC-IRMS (master)	16	-28.82	0.13	0.06	-26.98	0.12	0.06	-31.62	0.11	0.06
GCC-IRMS (ampoule)	2*	-28.90	0.09	0.13	-27.06	0.19	0.27	-31.48	0.07	0.09

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