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Drug testing data from the 2007 Pan American Games: δ ^{13}C values of urinary androsterone, etiocholanolone and androstanediols determined by GC/C/IRMS

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ABSTRACT

The main purpose of this article is to show the application of the CG/C/IRMS in real time during competition in the steroid confirmation analysis. For this reason, this paper summarizes the results obtained from the doping control analysis during the period of the 2007 Pan American Games held in Rio de Janeiro, Brazil. Approximately 5600 athletes from 42 different countries competed in the games. Testing was performed in accordance to World Anti-Doping Agency (WADA) technical note for prohibited substances. This paper reports data where abnormal urinary steroid profiles, have been found with the screening procedures. One 8 mL urine sample was used for the analysis of five steroid metabolites with two separate analyses by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Urine samples were submitted to GC/C/IRMS for confirmation analysis to determine the ^{13}C/^{12}C ratio of selected steroids. Fifty-seven urine samples were analyzed by GC/C/IRMS and the δ ^{13}C values (‰) of androsterone, etiocholanolone, 5β-androstane-3α, 17β-diol (5β-diol), 5α-androstane-3α, 17β-diol (5α-diol) and 5β-pregnane-3α, 20α-diol (5β-pdiol), the endogenous reference compound are presented. One urine sample with a testosterone/epitestosterone (T/E) ratio of 4.7 was confirmed to be positive of doping by GC/C/IRMS analysis. The δ values of 5β-diol and 5α-diol were 3.8 and 10.8, respectively, compared to the endogenous reference compound 5β-pdiol, which exceeded the WADA limit of 3‰. The results obtained by CG/C/IRMS confirmation analyses, in suspicious samples, were conclusive in deciding whether or not a doping steroid violation had occurred.

1. Introduction

The primary aim of this article is not only to report the IRMS data obtained during the games, but also to make obvious the significance of the IRMS measurements for the confirmation steroid abuse analyses. The 2007 Pan American Games were held in Rio de Janeiro, Brazil with the participation of 5500 athletes originating from 42 different countries. The analysis of all doping control samples was performed at the Anti-Doping Laboratory Rio de Janeiro, Brazil under the World Anti-Doping Agency (WADA) specifications [1].

The use of anabolic steroids was first prohibited by the International Olympic Committee (IOC) at the Olympic Games, held in Montreal, Canada in 1976. Since that time the detection of doping with exogenous androgenic steroids has long been an analytical challenge because the main technique used to detect androgenic steroids, gas chromatography/mass spectrometry (GC/MS), cannot distinguish between pharmaceutical (exogenous) and endogenous androgenic steroids. The IOC Medical Commission banned the use of testosterone (T) in 1982, and stated that a urinary T/E ratio above 6 was sufficient proof of T-abuse, based upon population studies [2,3]. However, it became evident that certain individuals had T/E ratios naturally elevated above 6 [4]. The IOC stated that follow-up longitudinal studies would be needed to prove that the suspicious urinary steroid excretion patterns (steroid profiling) were either due to natural physiological/pathological conditions or T-abuse [5]. Typically the T/E ratio increases significantly following T administration [6] and this ratio still represents the most important indication of T-abuse. Further studies indicated that Asian individuals excrete lower amounts of T glucuronide (TG) and hence have lower T/E ratios in urine, thereby increasing the risk of false-negative doping test results [7,8]. As a consequence the WADA cut-off limit was lowered to 4.0 in 2004 [9]. According to the WADA guidance, urine samples should be submitted to

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gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) to confirm steroid abuse if the steroid concentrations are greater than fixed cut-off values: T/E ratio greater than 4, T or E (equivalent to the glucuronide) greater than 200 ng mL^{-1}, androsterone or etiocholanolone (equivalent to the glucuronide) greater than 10,000 ng mL^{-1}, DHEA (equivalent to the glucuronide) greater than100 ng mL^{-1} [9]. If the confirmational GC/C/IRMS analysis does not indicate exogenous administration of steroids, the result is reported as inconclusive and further longitudinal studies may be performed. The GC/C/IRMS technique was used for the first time as an official method for confirmation of exogenous steroids abuse during the Olympic Games held in Nagano, Japan in 1998 Olympic Games [10].

Synthetic (pharmaceutical grade) steroids are generally produced by semi-synthesis from starting materials such as diosgenin and stigmasterol, which are derived from plants [11]. These pharmaceutical steroids contain less ^{13}C than their endogenous homologs found in man [11]. Therefore, it was postulated if any urinary steroids are found with a lower $^{13}C/^{12}C$ ratio than normal, this would indicate the presence of a steroid originating from a pharmaceutical source [12,13].

After a decade of research, GC/C/IRMS has become one of the most powerful tools to detect doping in sports. IRMS can measure the $^{13}C/^{12}C$ ratio in compounds with high accuracy and precision [14–16]. The GC/C/IRMS instrumentation used for determining the $^{13}C/^{12}C$ ratio of urinary steroids consists of a chromatographic separation by GC, on-line combustion to CO_2 and subsequent analysis by IRMS [15]. The $^{13}C/^{12}C$ ratio of urinary steroids, expressed in δ ^{13}C values (‰) versus VPDB (Vienna Pee Dee Belemnite, an international standard), are determined for T or its metabolites and compared to an endogenous reference compound (ERC) within the same urine sample. The ERC is used as an individual marker, which is also subject to the natural variation of the $^{13}C/^{12}C$ ratio in the endogenous steroids due to the athlete's diet and metabolism [17]. The $^{13}C/^{12}C$ ratio of these ERCs should not be affected by steroid administration for it to be a true reference compound [18]. According to the WADA guidance the result will be reported as consistent with the administration of a steroid, if a difference of 3.0‰ or more is determined between the δ ^{13}C values of the T metabolites and the ERC, or if the δ ^{13}C value of underivatized testosterone metabolite(s) is below −28‰ [9].

In the last decade, GC/C/IRMS has been established to detect for the use of T [19–27] DHEA [13,23,28–31], Epi-T [29,32], DHT [29,28], nandrolone [33], corticosteroids [34], pregnenolone [28,35] and GHB [36]. As recently stated by Piper et al. [37] the methods can be roughly subdivided into two classes, those focusing on 5β-pdiol together with 5β-diol, and 5α-diol and others focusing on androsterone and etiocholanolone.

The data presented here are based upon a modification of the procedure previously developed by Aguilera et al. to analyze for 5β-diol, 5α-diol and 5β-pdiol [19,26]. Briefly, in the final solid phase extraction (SPE) step the derivatized steroids are sequentially washed off the SPE column, the fraction containing androsterone and etiocholanolone was previously not analyzed. In the modified SPE procedure, androsterone, and etiocholanolone are washed off in one fraction, 5β-diol, 5α-diol and 5β-pdiol in a second fraction, and both analyzed by GC/C/IRMS. Saudan et al. has recently used the same procedure to analyze the same steroids [28,35,38]. A recent review of GC/C/IRMS in doping control was published by Cawley and Flenker [39].

The data presented were obtained from the Brazilian Anti-Doping WADA accredited laboratory during the 2007 Pan American Games. The screening procedure detected 54 urine samples, which were subsequently analyzed by GC/C/IRMS for confirmation. One urine sample with a T/E ratio of 4.7 was confirmed to be positive by GC/C/IRMS.

2. Experimental

2.1. Urine sample collection

A total of 1274 urine samples (234 out of competition and 1049 in competition) were collected and analyzed from athletes during the Pan American Games held in Rio de Janeiro, Brazil from July 13th to 29th 2007. Urine samples from competing athletes were collected according to WADA guidance [1]. One urine sample from an individual known not to be taking steroids was used as negative quality control sample (QC-Neg). The positive control urine sample was formed by spiking, androsterone, etiocholanolone, 5β-diol, 5α-diol and 5β-pdiol into a urine sample (QC-Pos). These two quality control samples were used for batch criteria acceptance.

2.2. Chemicals

Etiocholanolone, 5α-androstane-3α, 17β-diol (5α-diol) and, 5β-androstane-3α, 17β-diol (5β-pdiol), 5β-pregnane-3α, 20α-diol (5β-diol), and 19-norandrosterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Androsterone was purchased from Fluka (Buchs, Switzerland). Pyridine was purchased from Merck (Darmstadt, Germany). 17α-Methyltestosterone was purchased from Serva (Heidelberg, NY, USA). [2,2,4,4-^{2}H] etiocholanolone and [2,2,3,4,4-^{2}H] androsterone-glucuronide, [16,16,17-^{2}H] testosterone and [16,16,17-^{2}H] epitestosterone were a kind gift from the Institute of Biochemistry, Germany Sports University, Cologne, Germany. HPLC grade methanol and acetonitrile were purchased from Tedia (Fairfield, OH, USA). Solid phase extraction (SPE) cartridges C_{18}, 500 mg columns and VF-17ms factor four fused silica column (50% phenil–50% methylsiloxane) were obtained from Varian Inc. (Palo Alto, CA, USA). Fused silica capillary columns: HP-1 100% methylsiloxane (17 m × 0.20 mm i.d., 0.11 μm film thickness) was purchased from Hewlett Packard (Palo Alto, CA, USA). A 24-port vacuum manifold used to house the SPE cartridges was purchased from Fisher Chemicals (Fairlawn, NJ, USA). β-Glucuronidase from *Escherichia coli* was purchased from Boehringer Mannheim (Düsseldorf, Germany). *N*-Methyl-*N*-trimethylsilyltrifluroacetanide (MSTFA) was purchased from Chem Fabrik (Waldstetten, Germany), NH_4I and ethanethiol from Sigma (St. Louis, MO, USA). Acetic anhydride and ethyl acetate were purchased from Spectrum Chemical Co. (Torrance, CA, USA). A hydrocarbon mix containing C_{16}–C_{30} alkanes with certified δ ^{13}C values was purchased from Indiana University (Bloomington, IN, USA).

2.3. Sample preparation urinary steroid analysis (screening procedure)

A standard urinary steroid "screening procedure" was used to determine the concentration of androsterone, etiocholanolone, E, T, DHEA and the T/E ratio. The urine sample preparation included addition of 17α-methyltestosterone, [2,2,4,4-^{2}H] etiocholanolone, [16,16,17-^{2}H] testosterone, [16,16,17-^{2}H] epitestosterone, [2,2,3,4,4-^{2}H] androsterone-glucuronide as internal standards for quantitation, recovery and evaluation of glucuronide hydrolysis, respectively. Deconjugation was carried out with β-glucuronidase. To each tube was added 1 mL of 0.2 M phosphate buffer pH 7.0 and 100 μL of β-glucuronidase from *E. coli*. Hydrolysis was carried out for 1 h at 65 °C. The hydrolysate was alkalinized with 500 μL of 20% potassium carbonate solution, and the pH adjusted to 9.0. To the hydrolysate 5 mL of tertiary butyl dimethyl ether (TBME) was added, and the tube capped and shaken for 5 min. The tube was then subsequently centrifuged at 3000 rpm for 5 min. The TBME layer (containing the free steroids) was then transferred into a screw-cap glass test tube (100 mm × 16 mm) and evaporated to dryness

under nitrogen at 40 °C. The residue was dried in a desiccator over phosphorous pentoxide/potassium hydroxide for at least 40 min. Trimethylsilyl derivatives were formed by the addition of 50 μL of a mixture of MSTFA/NH_4I/2-mercaptoethanol and heating for 60 °C for 15 min, and then transferred to a 1.5 mL auto-sampler vial for GC/MS analysis.

2.4. Urinary steroid analysis by GC/MS (screening procedure)

A Hewlett Packard 6890 Series (Palo Alto, CA, USA) GC equipped with a Hewlett Packard 7673 auto-sampler was coupled to an Agilent quadrupole MS (5973 Network). The GC carrier gas was helium with a constant flow rate of 1 mL min^{-1} The GC inlet temperature was 280 °C. Injection mode: split 1/10, pulse pressure 50 psi 0.80 min^{-1}. A split/splitless glass single-taper liner from Hewlett Packard (79 mm × 7 mm i.d., Cup 6 mm length × 1 mm hole) with an internal volume of 0.9 mL was used (in-house deactivated). Inside the liner, 0.017 mg of deactivated glass wool was compacted to a distance of between 23 and 33 mm measured from the top of the liner. The injection volume was 3 μL.

A HP-1 fused silica capillary column (Hewlett Packard, 100% methylsiloxane, 17-m long, 0.20 mm i.d., and film thickness 0.11 μm) was used for analytical chromatography. The GC temperature programming rates were as follows: initial oven temperature 140 °C, then raised to 180 °C at 40 °C min^{-1}, then to 240 °C at 3 °C min^{-1} and to 300 °C at 40 °C min^{-1} (held for 3 min). The GC column transfer line was set at 280 °C.

Mass spectrometer operating conditions—ion source temperature: 220 °C, quadrupole temperature: 150 °C, accelerating voltage: 200 eV higher than the manual tune, and ionization voltage: 70 eV. Mass spectra were obtained in the selective ion monitoring (SIM) mode. Three characteristic ions were monitored for each analyte. The dwell time was 20 ms for each analyte and each internal standard ion.

Samples prepared for GC/C/IRMS were subject to GC/MS analysis for specificity determination. The GC capillary column and inlet conditions used for the specificity determinations were the same as those as listed below for GC/C/IRMS analyses.

2.5. Sample preparation for GC/C/IRMS analysis (confirmation procedure)

A total of 57 urine samples were selected for IRMS confirmation analyses after steroid profile interpretation which included free and glucuronide fractions. The samples were extracted, purified and derivatized as shown in Fig. 1. The urine sample was divided into two 4 mL aliquots, and placed in two different test tubes. To each tube was added 1 mL of 0.2 M phosphate buffer pH 7.0 and 100 μL of β-glucuronidase. Hydrolysis was carried out for 1 h at 65 °C. A solid phase extraction (SPE) column was used to separate the hydrolyzed steroids from the combined hydrolysates. The SPE column was conditioned with 6 mL methanol and 6 mL distilled water, respectively. The combined hydrolysate was applied onto the SPE column. The eluate was evaporated to dryness under a gentle stream of nitrogen (Turbo Vap LV evaporator Zymark, Hopkinton, MA, USA) and subsequently dried over phosphorous pentoxide for 30 min. Acetylated derivatives of the steroids were prepared by adding 50 μL of pyridine and 50 μL of acetic anhydride to the dried residue and heating for 1 h at 60 °C. The derivatized extract was evaporated to dryness under nitrogen; the derivatized steroids were then redissolved in 3 mL of acetonitrile/water (50:50, v/v). The mixture was applied onto a pre-conditioned SPE column. Fraction 1 was eluted with 6 mL of a mixture acetonitrile/water (50:50, v/v). After that, 6 mL of acetonitrile/water (75:25, v/v) was used to elute Fraction 2. Finally, 6 mL of acetonitrile was added to the SPE cartridge and Fraction 3 collected. Fractions 2 and 3 were dried under a nitrogen flow at 60 °C. After evaporation the residues of each Fractions 2 and 3 were redissolved with 400 and 30 μL of cyclo-

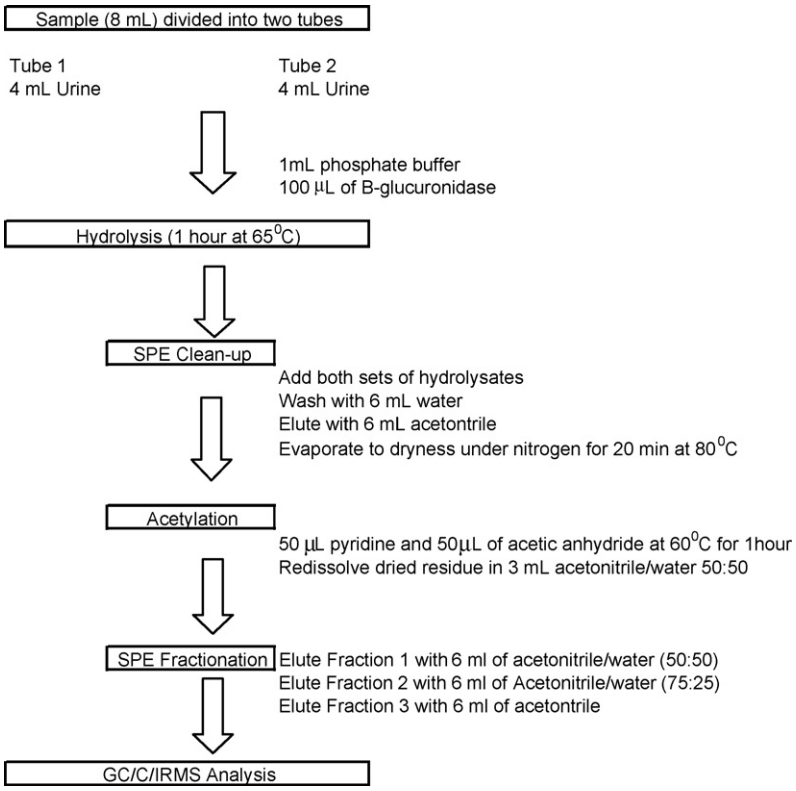

Fig. 1. Flow chart for sample preparation used for the 2007 Pan American Games.

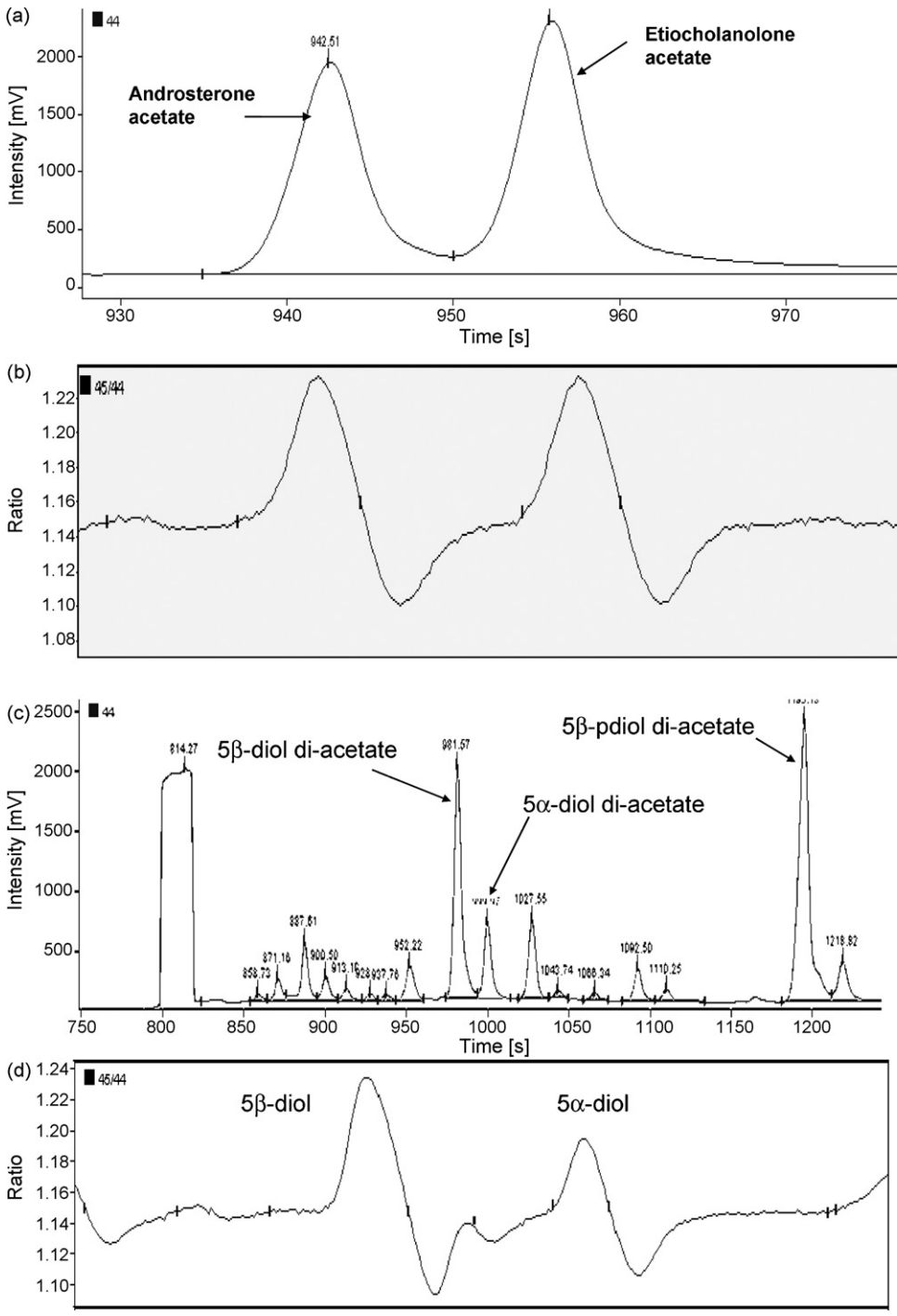

Fig. 2. A typical GC/C/IRMS chromatograms of Fractions 2 and 3 from the method applied for the games.

hexane, respectively. The separate reconstituted fractions were transferred to separate auto-sampler vials. The samples were analyzed by GC/C/IRMS. The androsterone acetate and etiocholanolone acetate derivatives were contained in Fraction 2. The 5α-diol, 5β-diol and 5β-pdiol di-acetate derivatives were contained in Fraction 3.

2.6. GC/C/IRMS confirmation analysis

Analysis was performed on a Thermo Scientific Delta Plus IRMS system coupled to a Trace GC system and a Thermo Scientific A200S auto-sampler via a Thermo Scientific GC Combustion III interface (Bremen, Germany). A Thermo Scientific ISODAT data system Version NT 2.0 was used for analysis and data reduction. Chromatographic separation of the steroids was achieved on a Hewlett Packard cross-linked 50% phenyl methyl siloxane fused silica capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness). The GC injector temperature was 280 °C. The splitless valve time was 0.6 min. The GC flow rate was 1.0 mL min^{-1}. The initial oven temperature was set at 70 °C and held for 1 min, then increased at 30 °C min^{-1} to 271 °C and held for 0 min, then raised to 280 °C at 0.6 °C min^{-1} then raised to 300 °C at 5 °C min^{-1}. The combustion oven temperature was set at 940 °C.

Table 1
Within-assay precision for four aliquots of the QC-Neg urine sample.

Sample	Etio	Andro	5β-Diol	5α-Diol	5β-Pdiol
1	−23.2	−22.4	−24.4	−25.5	−22.8
2	−23.4	−22.5	−24.6	−24.0	−23.5
3	−23.1	−22.3	−25.2	−24.0	−24.1
4	−23.6	−22.6	−25.1	−23.9	−24.1
Mean	−23.3	−22.4	−24.8	−24.3	−23.6
SD	0.21	0.15	0.41	0.77	0.64
CV (%)	0.92	0.65	1.64	3.18	2.70

Table 2
Between-assay precision for four aliquots of the QC-Neg urine samples analyzed over 4 days.

Day	Etio	Andro	5β-Diol	5α-Diol	5β-Pdiol
1	−23.4	−21.5	−24.4	−24.3	−23.4
2	−25.5	−22.8	−24.4	−25.5	−22.9
3	−23.2	−22.4	−24.4	−25.5	−22.8
4	−23.8	−22.9	−25.6	−24.3	−24.5
Mean	−24.0	−22.4	−24.8	−24.7	−23.4
SD	1.03	0.66	0.67	0.69	0.77
CV (%)	4.28	2.97	2.71	2.78	3.31

2.7. Quality control samples and system suitability of the GC/C/IRMS measurements

The QC-Neg and QC-Pos urine samples were prepared each time a batch of samples was prepared and analyzed by GC/C/IRMS. Each fraction from the two QC samples was injected three times. The mean and standard deviation (SD) of each set of data were calculated for the δ ^{13}C values of androsterone, etiocholanolone, 5α-diol, 5β-diol and the 5β-pdiol derivatives from both the positive and negative quality control urine samples. The individual values, mean and 2-sigma limits were plotted on the quality control or Shewhart Charts [40]. Data from each subsequent analytical sequence would then be compared against the control chart data. If any two of the new QC values were outside the 2-sigma limits no sample data from the analytical run would be accepted. This is based upon probabilistic considerations that the 2-sigma limits should contain 19/20 of the points [40]. In these cases, where data were outside the 2-sigma limits, the entire set of samples and QC samples would be re-prepared and analyzed. Causes of the out-of-control data would be sought, such as bad chromatography, and low analyte response. If the data for the QC samples were statistically acceptable, the new set of accumulated data would be pooled to obtain limits based upon the increased number of degrees of freedom, i.e. a new mean and limits based upon re-calculated SDs, and new 2-sigma limits set.

The within-assay precision was determined by analyzing four aliquots of the QC-Neg urine sample in 1 day. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated. The between-assay precision was determined by analyzing one aliquot of the QC-Neg sample per day for 4 days. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated.

The system suitability of the isotope ratio mass spectrometric system was determined by injecting the alkane C_{21} three times according to established procedures [22,26]. The means of the three δ ^{13}C values for C_{21} were calculated and plotted on a QC chart, which was updated every day with each successive set of alkane analyzed during the games. A standard steroid acetate solution was also included to serve as system suitability to control peak shape and retention time.

3. Results

3.1. GC/MS identification and GC/C/IRMS analysis of Fractions 2 and 3

The same samples prepared for GC/C/IRMS analysis were also analyzed by GC/MS. The GC/MS chromatograms showed that all the compounds had symmetrical peaks, no tailing, and no evidence of co-eluting compounds. The relative abundances of the main ion ratios in the compounds extracted from urine were within 15% of those of standards, indicating unambiguous identification of the correct compounds and that no co-eluting compounds were present.

Fig. 2a shows the GC/C/IRMS trace at m/z 44 are illustrated for Fraction 2. The acetylated derivatives of etiocholanolone and androsterone from Fraction 2 eluted at 943 and 956 s, respectively and were baseline separated. The lower panel (Fig. 2b) shows the characteristic "S-shaped" m/z 45/m/z 44 ratio [41], due to chromatographic isotope effects where the m/z 45 ($^{13}CO_2$) signals precedes the m/z 44 ($^{12}CO_2$) signal by about 150 ms [42]. Reference carbon dioxide gas pulses were introduced at 328, 428, 815, 1333, 1375 and 1427 s. The reference carbon dioxide gas pulses were used to calculate the δ ^{13}C values of the steroids. The reference carbon dioxide gas had a known δ ^{13}C value.

Fig. 2c shows the GC/C/IRMS trace at m/z 44 for Fraction 3, where the acetylated derivatives of 5β-diol, 5α-diol and 5β-pdiol, eluted at 982, 1000 and 1195 s, respectively. Reference carbon dioxide gas pulses were introduced at 328, 428, 806, 1341, 1381 and 1428 s. Fig. 2c and d show the baseline separation of 5β-diol and 5α-diol acetate derivatives.

3.2. System suitability and quality control

The C_{21} alkane, measured over 3 weeks had a mean value of −30.1‰ (n = 26), a SD of 0.58‰ and a CV of 1.91%.

The descriptive statistics for the within-assay and between-assay precision of the QC-Neg urine sample are shown in Tables 1 and 2, respectively. The within-assay CVs ranged from 0.32 to 3.2% and from 2.7 to 4.3%, respectively.

A total of 19 batches spanning 3 weeks were analyzed during the Pan American Games. The mean and 2-sigma limits from each steroid analyzed in the QC-Neg and QC-Pos urine sample, for each individual steroid are listed in Table 3. The overall CVs for androsterone and etiocholanolone, ranged from 2.8 to 5.6% and the diols ranged from 0.6 to 1.0%.

Table 3
Statistical data obtained for QC-Neg and QC-Pos urines measured during the games.

	δ ^{13}C values (‰)									
	Andro		Etio		5β-Diol		5α-Diol		5β-Pdiol	
	QC-Neg	QC-Pos	QC-Neg	QC-Pos	QC-Neg	QC-Pos	QC-Neg	QC-Pos	QC-Neg	QC-Pos
Mean	−23.2	−32.7	−24.5	−31.9	−25.1	−33.1	−25.8	−35.7	−23.5	−33.5
SD	0.68	0.92	1.37	1.71	0.75	1.76	0.61	0.62	0.76	1.00
CV (%)	2.95	2.81	5.59	5.37	2.99	5.33	2.37	1.74	3.22	2.99
2 SD (+)	−21.8	−30.8	−21.7	−28.5	−23.6	−29.6	−24.5	−34.4	−22.0	−31.5
2 SD (−)	−24.6	−34.5	−27.2	−35.4	−26.6	−36.6	−27.0	−36.9	−25.0	−35.5

Table 4
Steroid profile data and $\delta^{13}C$ values for all athletes' samples analyzed in the 2007 Pan American Games. The samples were submitted to GC/C/IRMS confirmation according to the four WADA criteria (those that exceed the criteria are in bold).

Descriptions			Concentration (μg/L)					$\delta^{13}C$ values (‰)				
Athlete	Sex	T/E	Andro	Etio	Epit	Testo	DHEA	Andro	Etio	5α-Diol	5β-Diol	5β-Pdiol
1	M	**8.3**	964	1412	5	40	16	−24.0	−25.3	N.A.	−27.4	−24.3
2	M	**7.3**	4794	2258	12	82	26	−23.9	−24.9	−26.6	−26.1	−25.9
3	M	**6.4**	2365	1883	15	88	82	−21.2	−22.6	N.A.	−25.9	−23.1
4	M	**6.4**	2523	1374	11	66	57	−24.0	−24.9	−24.8	−23.9	−22.5
5	M	**6.0**	3419	2068	24	138	76	−21.6	−21.9	−26.8	−27.5	−25.2
6	M	**5.9**	1408	1368	11	59	44	−23.4	−25.1	−26.3	−26.2	−23.5
7	M	**5.9**	2588	1940	17	90	48	−23.2	−24.3	−26.1	−24.7	−24.1
8	M	**5.5**	297	437	2	8	8	−21.2	−22.5	N.A.	N.A.	−22.4
9	M	**5.0**	2562	1530	6	29	34	−23.4	−24.5	N.A.	N.A.	−24.5
10	M	**4.7**	2296	3006	12	53	83	−24.3	−25.5	−28.0	−25.1	−24.0
11	M	**4.6**	2063	2075	14	60	25	−22.1	−23.1	−25.9	−25.3	−23.3
12	M	**4.6**	2680	1968	23	104	61	−21.6	−22.6	−25.9	−25.1	−22.7
13	M	**4.5**	318	193	1	6	5	−24.6	−24.2	N.A.	N.A	−23.4
14	M	**4.4**	494	481	3	10	12	−22.2	−21.6	N.A.	−23.3	−21.2
15	M	**4.4**	2099	2044	10	40	78	−24.3	−24.2	N.A.	−25.4	−24.7
16	M	**4.4**	1349	1902	13	54	43	−20.5	−21.1	−25.0	−24.2	−22.9
17	M	**4.3**	6833	5106	59	172	85	−23.6	−25.5	−23.9	−24.2	−23.6
18	M	**4.3**	2214	2216	21	83	58	−20.3	−21.6	−25.0	−24.9	−25.7
19	M	**4.3**	283	204	2	6	5	−20.3	−23.4	N.A.	N.A.	N.A.
20	M	**4.2**	1264	6148	22	77	58	−22.0	−24.7	−25.4	−26.8	−25.2
21	M	**4.2**	296	497	6	22	12	−25.2	−25.5	−21.1	−26.2	−24.8
22	M	**4.2**	3188	2887	7	30	47	−22.9	−25.1	N.A.	−26.0	−22.7
23	M	**4.2**	9209	4585	17	64	98	−22.4	−25.2	−26.0	−26.7	−23.9
24	M	3.9	3440	3383	14	49	**110**	−23.3	−24.4	−26.3	N.A.	−24.2
25	M	2.0	3779	2162	29	55	**133**	−24.0	−25.3	N.A.	−27.2	−23.7
26	M	1.7	7535	5584	48	72	**289**	−23.9	−24.4	−22.1	−27.0	−25.8
27	M	1.7	4944	**14220**	58	90	**136**	−21.9	−22.4	N.A.	−25.6	−22.8
28	M	1.7	6033	9880	128	168	**197**	−24.4	−26.3	−24.9	−25.9	−25.4
29	M	1.5	2653	2803	70	98	**126**	−23.2	−24.4	−26.7	−26.7	−24.7
30	M	1.2	4549	2401	70	79	**137**	−23.1	−24.0	−25.9	−24.7	−22.8
31	M	1.0	5214	9113	82	72	**116**	−24.3	−26.3	−24.7	−26.5	−25.3
32	M	1.0	6752	6341	101	90	**122**	−22.8	−24.9	−24.3	−26.1	−24.3
33	M	1.0	3029	3727	59	55	**152**	−22.9	−24.5	−26.8	−26.6	−24.0
34	M	0.9	6446	8097	113	100	**206**	−25.1	−26.8	−27.1	−27.6	−27.2
35	M	0.7	8962	3337	**195**	119	**147**	−23.7	−25.2	−26.3	−26.6	−24.6
36	M	0.6	**15224**	9817	167	89	**127**	−21.1	−24.1	−25.2	−26.8	−23.6
37	M	0.2	3617	3041	76	11	**154**	−23.7	−25.5	N.A.	N.A.	−25.7
1	F	**7.2**	58	109	0	2	4	−24.9	−25.7	N.A.	N.A.	N.A.
2	F	**4.8**	189	334	0	2	12	−23.5	−26.0	N.A.	N.A.	−24.9
3	F	**4.7**	2265	2591	4	17	106	−23.0	−23.1	N.A.	−26.5	−24.8
4	F	**4.0**	1901	1990	2	8	45	−24.1	−26.4	−24.4	−25.4	−23.4
5	F	2.3	8017	5845	18	39	**203**	−23.8	−25.9	N.A.	N.A.	−26.2
6	F	1.9	2516	4360	12	20	**220**	−22.4	−22.8	−25.4	−22.4	−25.6
7	F	1.7	3059	3666	8	13	**135**	−24.7	−26.7	N.A	N.A.	−27.9
8	F	1.6	9377	5676	25	37	**196**	−22.9	−26.1	−25.3	−25.6	−23.6
9	F	1.5	2614	2296	13	17	**137**	−21.2	−23.1	−24.3	−24.8	−22.7
10	F	1.4	2357	2982	29	35	**202**	−23.2	−22.9	−23.6	−25.5	−24.1
11	F	1.3	5787	9346	43	47	**388**	−25.6	−25.7	−27.5	−27.0	−26.1
12	F	1.2	7277	9852	29	32	**189**	−23.2	−27.6	−26.8	−27.8	−25.7
13	F	1.1	4805	6972	31	31	**234**	−25.6	−29.3	N.A.	N.A.	−25.3
14	F	1.0	**12821**	4265	22	21	**144**	−22.5	−26.4	−28.7	−27.7	−22.6
15	F	0.8	3683	3557	16	12	**143**	−22.0	−22.6	N.A.	N.A.	−23.8

3.3. GC/C/IRMS analysis and $\delta^{13}C$ values from athlete's urine samples

Table 4 shows the results from the urinary steroid "screening procedure" and "confirmation procedure" GC/C/IRMS for a total of 53 samples. Twenty-four males samples were submitted to GC/C/IRMS confirmation due to a T/E ≥ 4. The T/E values ranged from 4.2 to 8.3. Four urine samples from females met these criteria and the T/E values ranged from 4.0 to 7.2. With DHEA, a total of 25 samples met the criteria > 100 ng mL^{-1}, the concentrations ranged from 110 to 289 ng mL^{-1} for the urine samples from males (n = 14) and from 135 to 388 ng mL^{-1} for the urine samples from females (n = 11). A total of three samples exceeded the criteria for androsterone or etiocholanolone of >10,000 ng mL^{-1}, androsterone with

Table 5
Summary of corrected $\delta^{13}C$ values obtained from the confirmed positive case.

Sample	T/E	Andro	Etio	Epit	Testo	DHEA	Corrected $\delta^{13}C$ values (‰)				
							Andro	Etio	5α-Diol	5β-Diol	5β-Pdiol
Athlete	4.7	1935.3	1776.1	9.1	42.0	32.9	−25.3	29.9	−33.2	−26.3	−22.5
QC-Neg	1.6	4000	4000	28	45	N.A.	−22.9	−24.0	−25.9	−24.4	−23.2
QC-Pos	N.A.	1500	1500	N.A.	N.A.	N.A.	−32.7	−32.0	−34.3	−30.5	−33.2

values of 12,821 (female) and 15,224 ng mL^{-1} and etiocholanolone at 14,220 ng mL^{-1} (male).

3.4. Adverse analytical findings

According to the WADA guidance for any athlete to be reported positive for exogenous steroid abuse the δ ^{13}C values obtained by GC/C/IRMS the urinary steroid must differ by 3‰ units or more from that of the urinary endogenous reference compound (9). 5β-Pdiol was the endogenous reference compound (ERC).

With the male athletes, subjects 1, 10, 22, 30 and 36 had δ-values of more than 3 units. However, the data were "inconclusive" according to the WADA guideline (9) as the absolute δ ^{13}C data were not below −28‰ based on non-derivatized steroid values. Subject 36 also had DHEA and androsterone levels above the cut-off criteria. Subject 14 had δ-values for 5β-diol, 5α-diol of 6.1 and 5.0, respectively; once again these were re-calculated as non-derivatized values they were inconclusive. Interestingly the DHEA and androsterone levels were also above cut-off levels for this subject.

The one male athlete with a T/E of 4.7 was confirmed as positive. The corrected δ ^{13}C values for 5β-diol, 5α-diol and 5β-pdiol were −26.3, −33.2 and −22.5‰, respectively (Table 5). The δ-values exceeded the 3-unit difference for 5β-diol, 5α-diol and were 3.3 and 9.2 units, respectively. This was therefore "consistent with the administration of a steroid" according to the WADA guidance [9].

4. Discussion

The data from the "screening" and the GC/C/IRMS "confirmation" procedures collected during the 2007 Pan American Games to detect for exogenous androgenic steroid doping are presented. A modified GC/C/IRMS assay for determination urinary δ ^{13}C values of androsterone, etiocholanolone (Fraction 2), 5β-diol, 5α-diol and 5β-pdiol (Fraction 3) was used. The method was based upon previous extraction procedure developed by Aguilera et al. [19,26]. The GC/MS and GC/C/IRMS chromatograms of the acetylated derivatives exhibited symmetrical peak shapes and absence of co-eluting peaks. Peak purity was also confirmed by GC/MS analysis. The chromatograms appeared to have a superior separation of etiocholanolone from androsterone, and 5β-diol from 5α-diol, than chromatograms originally presented by Aguilera et al. [19] and Saudan et al. [28,38]. However, the precision data were a factor of two less than Saudan et al. [28,38], Shackleton et al. [29] and Flenker et al. [17] and our previous publications [22,26]. Nevertheless, the present data allowed the discrimination between positive and negative cases. The alkane precision data were similar to our previous data [26] indicating the discrepancy maybe steroid sample related and not the IRMS measurement process. Further work is necessary to investigate this.

The method of Saudan et al. [18,28], allowed both androstenol and 5β-pdiol to be used as ERCs. In our modified procedure one ERC 5β-pdiol was analyzed. 5β-Pdiol has been used as the ERC for T or DHEA administration [13,19]. If pregnenolone was administered then 5β-pdiol cannot be used as an ERC as it is an indicator of pregnenolone administration [28,35]. However, elevated 5β-pdiol δ ^{13}C values along with elevated 5β-diol, and 5α-diol values would indicate pregnenolone administration. A recent paper by Piper et al. [37] separated 10 urinary steroid analytes. The method was not intended as a confirmation procedure but as a means to allow retrospective discrimination and possible determination of which particular steroid was administered.

Examination of the δ ^{13}C values for androsterone, etiocholanolone 5β-diol, and 5α-diol indicated there were 0, 4, 4, and 4, values respectively above the 3-unit difference cut-off (Table 4). The low discriminating power of androsterone was recently indicated by Flenker et al. [17]. There the difference between androsterone and ERCs used to generate reference limits were slightly larger than 2‰, and symmetrically scattered (from a population of 56 subjects). Therefore, it is extremely unlikely that δ values of greater than 3‰ would occur. The identity of the steroid, sex, oral contraception, travel and physical activity, were shown to have an effect on δ ^{13}C values. The shift was in parallel for all steroids and therefore not expected to be a significant influence on the δ ^{13}C values [17].

Diet has been shown to affect the δ ^{13}C values of urinary steroids [38]. One subject spent a month in Africa, which resulted in an increase in the ^{13}C/^{12}C values in androsterone, etiocholanolone, 5β-diol, androstenol, and 5β-pdiol by 2.5‰. Therefore the use of δ values rather than δ ^{13}C values has been proposed [42].

The degree and rate of T-glucuronide excretion has a strong association with a deletion polymorphism in the *UGT2B17* gene [43]. Subjects devoid of the gene had a T/E ratio below 0.4 [43]. This polymorphism is considerably more common in Korean Asians than in a Swedish Caucasian population (66.7 and 9.3%, respectively). Therefore it has been proposed that there should be genotype based cut-off values for T/E ratios that greatly improve the sensitivity and specificity of the T/E test [44]. Interestingly, it has been recently proposed that the T/E ratio should be a subject-based threshold [45].

The screening procedure used at the 2007 Pan American Games presented 57 athletes with steroid profiles above the cut-off values for T/E ratios, testosterone, epitestosterone, androsterone, etiocholanolone or DHEA. One urine sample with a T/E ratio of 4.7 (limit ≥ 4) was confirmed positive by GC/C/IRMS. The δ values for 5α-diol and 5β-diol were above the 3-unit difference [9]. The sample was reported, with no dispute from the athlete. Moreover, the testosterone and epi-T were in the normal range concentration for normal population.

These important adverse analytical findings obtained from the IRMS measurement were conclusive, (according to WADA guidelines) in deciding whether or not doping steroid abuse has occurred. Despite the fact that only one positive case was confirmed by IRMS (one of the four positive cases found during the games) many other suspicious cases were close to the borderline cut-off value. Nevertheless, the δ-value exceeded the 3-unit difference between the endogenous reference compound and the metabolites and the absolute δ ^{13}C value below −28‰ could not be the only conclusive criteria values to decide adverse analytical findings. Furthermore, DHEA 100 ng mL^{-1} concentration criteria for choosing samples for IRMS confirmation have to be reviewed; because this is a normal low concentration, therefore it is not the best indicator for selecting suspicious samples [46].

5. Conclusion

The GC/C/IRMS confirmation method used to detect androgenic steroid abuse at the 2007 Pan American Games detected one positive male athlete. On-going research, with GC/C/IRMS methods, those areas related to T/E values and genotyping continue to aid the understanding of steroid metabolism and the subsequent detection of androgenic steroid abuse in sports.

Since the Winter Olympic Games in Nagano, Japan 1998, IRMS has become the incontestable technique to confirm steroid synthetic natural abuse. The confirmed positive case found during the games represents the ultimate balance of the CG/C/IRMS stage.

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