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Isotope ratio mass spectrometry at the London 2012 Olympic and Paralympic Games - Experience and Conclusions

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Abstract

The approach used to confirm the administration of endogenously produced anabolic steroids during the London 2012 Olympic and Paralympic Games using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is described. In partnership with GlaxoSmithKline, a satellite laboratory was established in Harlow, UK for the analysis of around 5000 urine, and 1500 blood samples. Typically, 3% of urine samples exceed criteria such as T/E ratio (4:1) and steroid concentrations that initiate IRMS analysis. Therefore, 150 urine samples were anticipated for GC-C-IRMS analysis. Methods for endogenous anabolic and androgenic steroids (testosterone and metabolites), 19-norandrosterone (2 ng/mL), boldenone (2 ng/mL, 10 ng/mL boldenone metabolite) and formestane (50 ng/mL) were accredited to ISO 17025 at the new facility.
Decision parameters used to initiate IRMS analysis during the Games are outlined, and their effectiveness and implications for doping control discussed. An overall summary of the data obtained is provided, with cases highlighted which are considered of particular interest to the anti-doping community.
In total, 109 GC-C-IRMS analyses were undertaken. One case of 19-norandrosterone was analysed prior to the Olympics and found not to show a difference from endogenous production. Seven boldenone cases (2 cases from pre-games testing and 5 cases during the Olympics and Paralympics) were analysed, with all cases found to be negative by IRMS. No formestane (> 50 ng/mL) was observed in any sample. Two GC-C-IRMS positive cases were reported and the athletes disqualified.

Introduction

Proving the administration of endogenously produced anabolic steroids such as testosterone (T) remains a challenge in sports drug testing. WADA requires its accredited laboratories to use GC-C-IRMS to help distinguish whether urinary steroids are of exogenous or endogenous origin [1]. Only urine samples meeting criteria considered indicative of endogenous steroid administration (i.e. elevated steroid concentrations and/or steroid ratios) are selected for IRMS analysis [2]. The GC-C-IRMS approach taken during the 2012 London Olympic and Paralympic Games is described. The effect of the decision criteria used to initiate IRMS analysis and the results are discussed.

Experimental

In brief, phosphate buffer (1 mL, 0.1 M, pH 6.2) and ethylacetate (5 mL) were added for up to 5 mL of urine, with the organic layer discarded after vortexing. Samples were incubated (1 h, 50 ±5 °C) after adding 1.5 mL *E. coli* working solution.
Isolute (C8) SPE cartridges were conditioned (3 mL, methanol, 3 mL H_2O) before loading the urine hydrolysate. Washes of 1 mL $NaHCO_3$ (0.1 %), 1 mL H_2O and 2 mL methanol/H_2O (20:80, v/v) were performed, before elution with 3 mL ethylacetate/methanol (90:10, v/v). Following evaporation (60 ±5 °C) samples were reconstituted in 50 µL acetonitrile: H_2O (40:60, v/v, containing trenbolone 40 mg/L) prior to LC fraction collection using an Agilent 1200 HPLC system (Agilent Poroshell® C18, 15 cm x 4.6 mm, 2.7 µm). Five fractions were collected per sample, F1 = 11-ketoetiocholanolone (11-K), F2 = testosterone (T), F3 = 5α- and 5β-androstanediol (5α- and 5β-diol), F4 = etiocholanolone (Et) and androsterone (A), F5 = pregnanediol (PD).

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Fractions were evaporated to dryness under nitrogen (60 ±5 °C) before derivatisation (60 ±5 °C, 1 h) with pyridine (50 µL) and acetic anhydride (50 µL). Samples were then evaporated (60 ±5 °C), and dependent on the steroid concentration, reconstituted in varying volumes of ethylacetate. To limit the number of injections, fractions were combined (F1+F4 and F3+F5), prior to analysis.
The programmable temperature vaporiser (Gerstel CIS 4) was operated in solvent vent mode with 40 µL injected [3]. The GC (Agilent 7890 equipped with a HP50+ 30 m x 0.25 mm x 0.25 µm column) conditions were 80 ˚C for 1 min, 25 ˚C to 270 ˚C (11 min), 15 ˚C to 280 ˚C (5 min) and 15 ˚C to 300 ˚C for 2 min. IRMS analysis was perfomed using a Thermo Scientific Delta V Plus, eqiupped with a GC Isolink and Conflo IV.
Methods for 19-norandrosterone (2 ng/mL) [4], boldenone parent (2 ng/mL) and boldenone metabolite (10 ng/mL) [5] were adapted from the literature.

Results and Discussion

A DHEA concentration exceeding 100 ng/mL was found in 399 samples, (8.1 % of total), Figure 1. IRMS analysis of this number of samples would have proved extremely difficult despite the number of instruments available (3 instruments), therefore a review system of DHEA cases between 100 and 200 ng/mL in conjunction with accompanying luteinizing hormone (LH) and steroid data prior to analysis was agreed.
DHEA concentrations >200 ng/mL occurred more (29 cases, 0.59 % of total, of which 1 AAF) than T concentrations >200 ng/mL (1 case, 1 AAF).

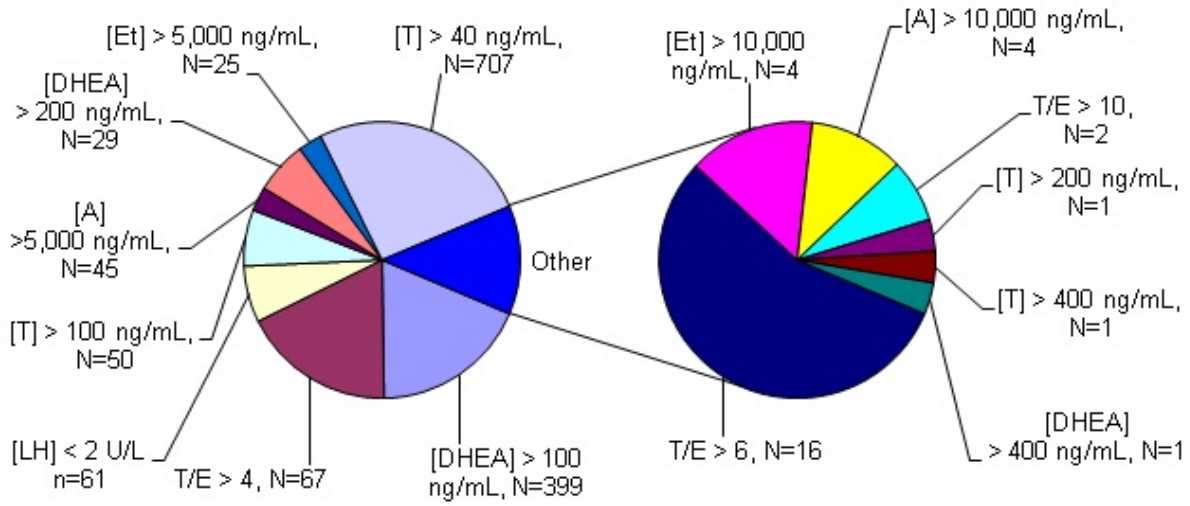

Figure 1. GC screen data meeting IRMS criteria during the London 2012 Olympic and Paralympic Games (total number of urine samples = 4913; samples not exceeding any threshold are not shown). All concentrations have been adjusted to SG 1.020.

Linking T/E and T concentration criteria (Table 1) reduced the number of GC-C-IRMS analyses required on samples with a raised T/E due to low E concentrations (<10 ng/mL). However, T concentration and the T/E ratio should not be the only markers used to indicate the administration of exogenous androgenic anabolic steroids (EAAS), as demonstrated by 'Positive 2', Figure 2.
LH was below 2 U/L in 61 cases (1.2 % of total, Figure 1) with careful consideration of SG adjusted data (particularly when <1.005) advised before commencing IRMS analysis. Also, depending on the administered EAAS, LH may not be suppressed, 'Positive 2', Figure 2.

Compound	GC-C-IRMS decision criteria
Testosterone (T)	T/E > 4 and T > 40 ng/mL
Etiocholanolone (Etio, Et)	>10,000 ng/mL[1]
Androsterone (Andro, A)	>10,000 ng/mL[1]
DHEA	>100 ng/mL[1]
Formestane	>50 ng/mL[1]
19-norandrosterone	>2 ng/mL[1]
Boldenone	>2 ng/mL[1]
Luteinizing hormone (LH)	<2 U/L[1] and SG > 1.005

Table 1. GC-C-IRMS decision criteria used during the London 2012 Olympic and Paralympic Games. [1]Adjusted to SG 1.020.

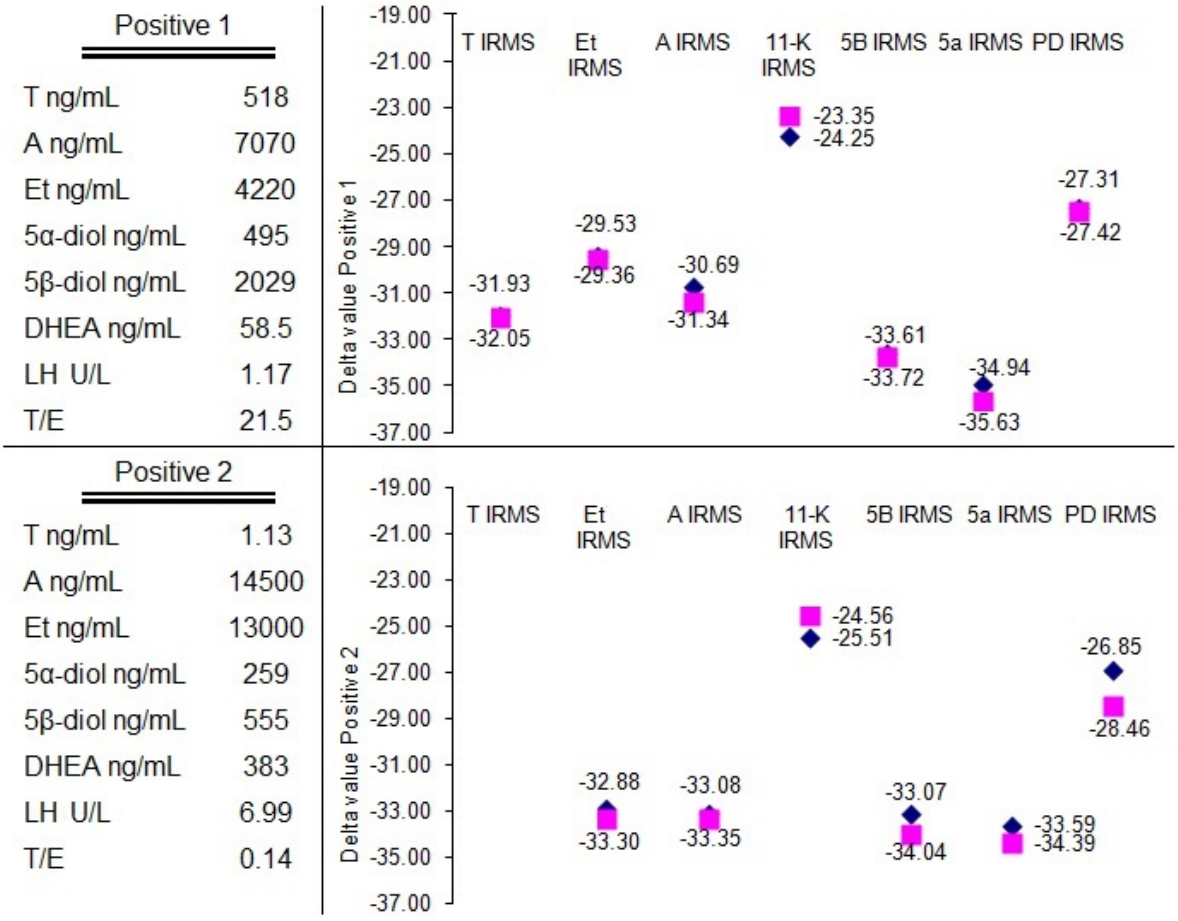

Figure 2. Summary of GC data, and IRMS data, From two positive cases during London 2012 (2 analyses per sample). LH values adjusted to SG 1.020 and measured by Immulite 1000

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Overall 89 cases (98 % of IRMS samples) were found to be negative by IRMS. A summary including measured delta values and delta-delta values is presented in Figure 3. Boldenone cases in our laboratory are usually rare (less than 1 a year), but 7 cases (0.14 %, all IRMS negative samples, boldenone and metabolite, both <20 ng/mL) were found during the Olympic period, suggesting such cases may be more prevalent in individuals from other regions. No case of formestane (>50 ng/mL) or 19-norandrosterone (>2 ng/mL) was found during the Games although a 19-norandrosterone case was found pre-games and found to be negative on IRMS.

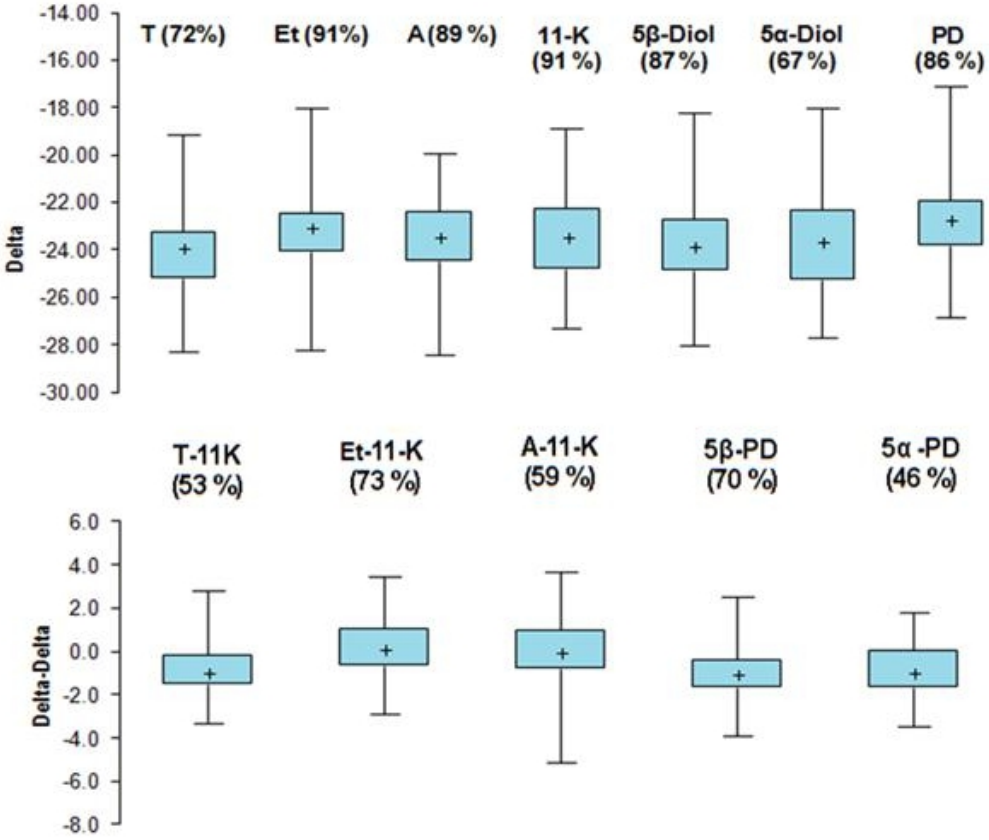

Figure 3. Box and Whiskers plots (median, upper and lower quartiles, minimum and maximum) of delta values and of delta-delta values for 89 samples considered to be negative by GC-C-IRMS analysis during the London 2012 Olympic and Paralympic Games. The percentage of samples containing peaks of adequate signal and chromatography is given in brackets. All samples corrected for acetylation and relative to Pee Dee Belemnite (PDB).

Conclusions

IRMS remains a valuable tool in providing evidence of the administration of synthetically derived endogenous androgens. Due to the time and costs associated with IRMS analysis, sample selection remains a crucial factor in an effective laboratory strategy. Multiple criteria were considered for sample selection with no individual measure proving critical. Careful consideration of all criteria for individual samples proved beneficial. Two positive cases were identified, one due to a high T/E (21.5) with T concentration (518 ng/mL) and suppressed LH. The second was not identified by any of these criteria but by elevated concentrations of androsterone and etiocholanolone (>10,000 ng/mL).

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