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SPECIAL FEATURE: TUTORIAL

The application of carbon isotope ratio mass spectrometry to doping control

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The administration of synthetic steroid copies is one of the most important issues facing sports. Doping control laboratories accredited by the World Anti-Doping Agency (WADA) require methods of analysis that allow endogenous steroids to be distinguished from their synthetic analogs in urine. The ability to measure isotope distribution at natural abundance with high accuracy and precision has increased the application of Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry (GC–C–IRMS) to doping control in recent years. GC–C–IRMS is capable of measuring the carbon isotope ratio (δ^{13}C) of urinary steroids and confirm their synthetic origin based on the abnormal ^{13}C content. This tutorial describes some of the complexities encountered by obtaining valid δ^{13}C measurements from GC–C–IRMS and the need for careful interpretation of all relevant information concerning an individual's metabolism in order to make an informed decision with respect to a doping violation. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: endogenous steroids; doping control; carbon isotope ratio (δ^{13}C); gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS)

THE ENDOGENOUS STEROID CHALLENGE

Drug use and abuse is an unfortunate feature of sports. The fight against doping in sports is a challenge faced by governments, sports authorities, laboratories, coaches and athletes who wish to contribute to fair, healthy and legal competition. The abuse of synthetic steroid copies is one of the most important issues for doping control. Before 1982, only the use of xenobiotic steroids had been banned, with a positive result requiring unequivocal proof provided by techniques such as GC–MS that the banned substance and/or its metabolites were present in a urine sample. Following the prohibition of testosterone (T; androst-4-ene-17β-ol-3-one) administration by the International Olympic Committee and subsequently by the World Anti-Doping Agency (WADA),[1] new areas of research have been established within doping control laboratories.

Methods for the detection of administered synthetic steroids by molecular spectrometry techniques such as GC–MS are limited by their ability to identify and quantify. The GC–MS steroid profiles are, however, an important means of screening all urine samples entering the laboratory to eliminate those of nonsuspicious nature. The objective for doping control laboratories is, therefore, to maximize the detection of synthetic steroid doping violations while minimizing the additional resources required from stakeholders. This can be achieved with advanced GC–MS steroid profiling strategies based on the analysis of several urinary steroids that can originate from the endocrine system.[2–7] Control of any endogenous substance requires the establishment of 'normal' reference intervals and subsequent statistical determination of what constitutes an 'abnormal' state. The challenge for doping control is presented by steroid administration altering the human endocrine system in ways that are not yet fully understood, since they are largely dependent on the individual athlete.

Synthetic copies of endogenous steroids, namely, dehydroepiandrosterone [(DHEA); androst-5-ene-3β-ol-17-one], androstenedione (ADIONE; androst-4-ene-3,17-dione), 4-androstenediol (4-ADIOL; androst-4-ene-3β,17β-diol), 5-androstenediol (5-ADIOL; androst-5-ene-3β,17β-diol) and T are chemically and pharmacologically identical to their endogenous analogs (Fig. 1[8]). The mere excretion of these steroids in the urine of an athlete obviously cannot constitute proof of administration. To meet the endogenous

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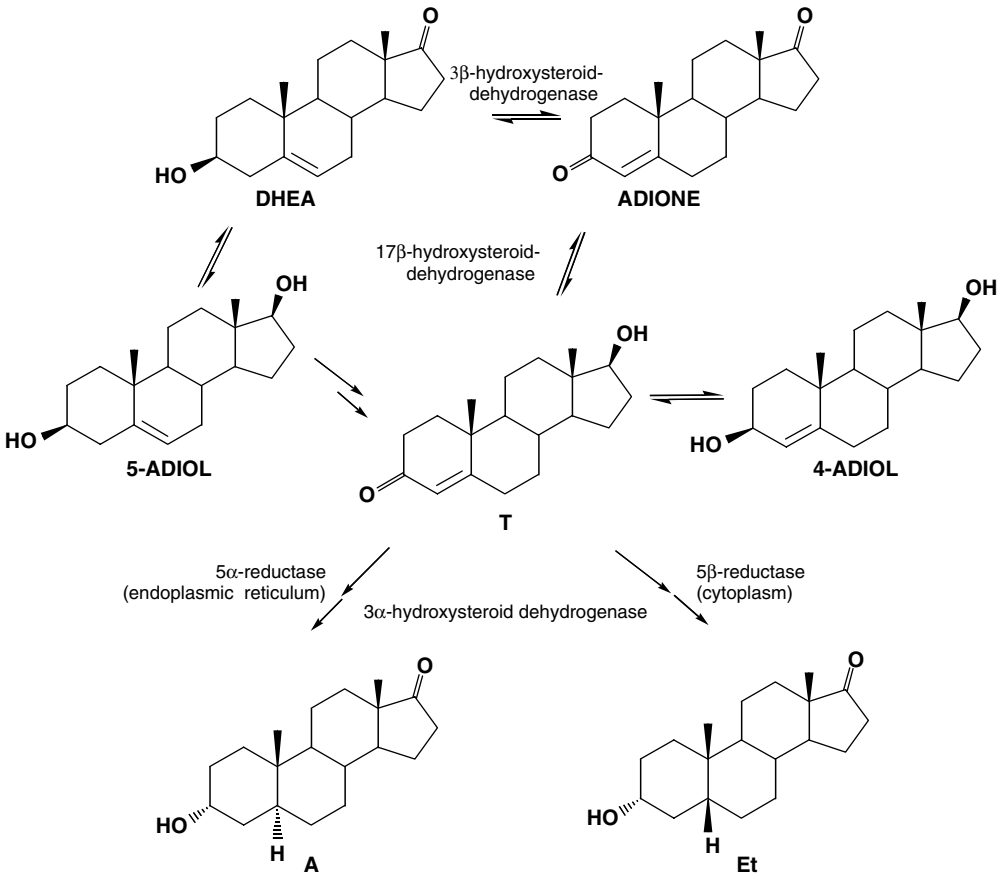

Figure 1. Proposed biosynthesis of endogenous steroids.[5].

steroid challenge, stable carbon isotope ratio ($\delta^{13}C$) analysis of urinary steroids may be performed to satisfy the rigorous medicolegal demands of doping control.

$\delta^{13}C$ NOTATION USED IN CARBON ISOTOPE RATIO MEASUREMENTS

Stable isotope analysis concerns the variation of isotopic content arising from fractionation, a process in which one isotope of an element is discriminated against, physically or chemically, in relation to another. Isotopic signatures can be a source of information about reaction mechanisms, reaction environment and/or the source or fate of a compound. In terms of ^{13}C, photosynthesis is an important fractionation process where plants absorb carbon dioxide (CO_2) from the atmosphere. Variations in isotopic content are generally very small, so to avoid cumbersome figures with the reporting of isotopic variations, δ-notation[9] is used (**1**), which expresses the $\delta^{13}C$ value of a given sample relative to the strata of marine sediment called the 'Peedee' formation (*Belemntella Americana*, Cretaceous, South Carolina). The original Pee Dee Belemnite (PDB) limestone was assigned the value of zero on the corresponding $\delta^{13}C_{PDB}$ scale. Most biogenic carbon compounds were ^{13}C depleted in relation to it, and therefore, had negative $\delta^{13}C_{PDB}$ values. The exhaustion of PDB led to the use of a secondary standard, Vienna Pee Dee Belemnite (VPDB), the $\delta^{13}C$ value of which was determined to be +1.95‰ relative to its predecessor. Today, the $\delta^{13}C_{VPDB}$ scale is used to define $\delta^{13}C$ values as part-per-thousand differences from VPDB.[10] A difference of one unit on this per-mille (‰) scale corresponds to a change in 0.001099 atom% of natural ^{13}C abundance.[11]

$$\delta^{13}C = \left(\frac{r_{sample}}{r_{standard}} - 1\right) \times 1000 \quad r = \left(\frac{13_C}{12_C}\right) \qquad (1)$$

DERIVATION OF $\delta^{13}C$ VALUES – A PLANTS-TO-PILLS STORY

Natural variations in the abundance of ^{13}C reflect its passage through chemical and/or biological systems in which transformations are accompanied by isotopic fractionation that results in slight enrichment or depletion of ^{13}C due to the difference in mass. Carbon isotope fractionation results in lowering $\delta^{13}C$ values by approximately 20‰ for land plants and 10‰ for marine plants.[12] There are two methods by which plants can reduce the CO_2 absorbed from the atmosphere, and three categories of plants that employ these methods. The most common of these are called C-3 plants that derive their name from the enzyme reaction using ribulose *bis*-phosphate carboxylase oxygenase to produce 3-phosphoglyceric acid, a compound containing three carbon atoms. The reversible nature of this reaction, called the Calvin–Benson cycle, results in isotopic fractionation of carbon that produces a plant largely depleted in ^{13}C ($\delta^{13}C$ = −24‰ to −34‰). C-3 plants constitute about 90% of all plants that include wheat, rice and soy. The second pathway, called the Hatch–Slack cycle for C-4

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plants, uses the phosphoenolpyruvate carboxylase enzyme to facilitate CO_2 fixation and produce oxaloacetate, a compound containing four carbon atoms. This nonreversible process is less discriminating against $^{13}CO_2$ resulting in C-4 plants being less depleted (i.e. enriched) in ^{13}C ($\delta^{13}C = -6‰$ to $-19‰$). Examples of C-4 plants include hot-region grasses, and crops such as corn and sugarcane. The third category, called the Crassulacean Acid Metabolism (CAM) plants, uses either the C-3 or C-4 pathway depending on the environment, and includes cacti and pineapple.[12,13]

GAS CHROMATOGRAPHY–COMBUSTION–ISOTOPE RATIO MASS SPECTROMETRY (GC–C–IRMS)

The ability to measure isotopic distribution at natural abundance with high accuracy and precision has increased the application of Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry (GC–C–IRMS) in recent years.[14] The roots of GC–C–IRMS are traceable to the first mass spectrograph of Aston in 1919, which definitively showed that m/z 22 observed by Thompson in 1912 was indeed a minor isotope of Ne[15,16] – leading to IRMS being called the first form of analytical mass spectrometry.[17] Continuing developments led to the 1940 Nier IRMS design that included a 60° magnetic sector,[18] thereby allowing high-precision IRMS to become the standard technique for determining $^{13}C/^{12}C$ with pure CO_2 gas employed as a standard. It is the sample/standard comparison – based on the difference of $^{13}C/^{12}C$ values – that, in fact, provides the high precision and accuracy of IRMS, rather than the absolute $^{13}C/^{12}C$ value.

IRMS measurements incorporating $^{13}C/^{12}C$ values require carbon contained in a sample to be quantitatively converted by combustion into CO_2 prior to introduction to the ion source. Purification of a sample to selectivity combusting individual carbon-containing compounds has provided significant challenges. The first reports coupling GC to on-line combustion of GC separated compounds to yield CO_2 for isotope ratio analysis used single collector mass spectrometers.[19,20] Few articles were published that made use of this new technique until Barrie *et al*.[21] coupled a duel collector mass spectrometer to a GC via an on-line combustion interface, thus permitting continuous recording of $(m+1)/m$ values by detecting two successive masses at the same time. This development represented the first 'continuous flow' isotope ratio monitoring the GC–MS technique, later called GC–C–IRMS. It was not until 1990, however, that GC–C–IRMS instruments became commercially available.

Modern GC–C–IRMS instruments are designed for compounds to elute from the capillary column of the GC into a combustion interface consisting of an oxidation furnace, reduction furnace, water remover and open split. Figure 2 illustrates the components of a typical GC–C–IRMS system used today. The oxidation furnace converts all the carbon in each compound to CO_2. It consists of an alumina ceramic capillary tube containing one each of a Cu, Ni and Pt wire.[22] Oxidation in the furnace is achieved by the reaction of Cu with high purity O_2 gas to form CuO at 600–650 °C. Pt then acts as a catalyst for the reaction of Ni and O_2 to form NiO at 940 °C.[22] The reduction furnace removes excess O_2 from the gas stream and reduces potentially formed nitrous oxides to $N_2 - N_2O$ will be problematic due to isobaric interference with $^{12}C^{16}O^{18}O$. It consists of an alumina ceramic capillary tube containing three Cu wires at 620 °C to form CuO. After combustion of the compounds to CO_2 and H_2O, the latter may be removed using either a low-temperature trap (e.g. capillary immersed in acetone/dry-ice slurry) or a Nafion dryer. Nafion is a fluorinated polymer that acts as a semipermeable membrane through which H_2O passes freely without removing CO_2.[23] The outside of the Nafion is constantly purged with helium to maintain its drying capacity. The final component of the interface is the open split that allows the IRMS to sample the gas stream.

For GC–C–IRMS, the high-precision measurement of transient signals requires a high degree of linearity in the IRMS. EI at potentials of up to 130 eV is used for its excellent ionization yield and avoidance of isotopic fractionation during the decay of CO_2^+ ions. The ions formed are accelerated to energies of 3 keV before they are separated according to m/z in a magnetic sector. For the analysis of CO_2, three Faraday cups are positioned in the ion beam to collect the ions m/z 44, 45 and 46, representing $^{12}C^{16}O^{16}O^{+\bullet}$, $^{13}C^{16}O^{16}O^{+\bullet}$ and $^{12}C^{16}O^{18}O^{+\bullet}$, respectively. The ratio of m/z 45 to m/z 44 is determined for each target analyte by peak definition relative to the background. An algorithm correct m/z 45 for isotopic abundances contributed from $^{12}C^{16}O^{17}O^{+\bullet}$ by assumption that a fixed relationship exists between ^{18}O and ^{17}O. The abundance of ^{17}O is calculated from the m/z 46/44 ratio and this number is subtracted from the m/z 45 signal before calculating the ^{13}C abundance.[24]

$\delta^{13}C$ ANALYSIS IN DOPING CONTROL – IS IT FROM THE *BODY* OR THE *BOTTLE*?

The application of GC–C–IRMS extends to many disciplines of chemical analysis that aim to determine the origin of a given organic compound by measuring its characteristic isotope 'fingerprint'. The natural abundance of stable isotopes is not a fixed constant, rather a considerable variable. The variation of the natural abundance of ^{13}C can be as high as 0.1 atom%, reflecting the varying degree of mass discrimination associated with carbon assimilation and other metabolic processes.[11] The major impact of stable isotopes in drug metabolism has been in the detection and identification of metabolites, mechanistic studies concerned with metabolic pathways, and indirectly in the development of sensitive and specific methods for the analysis of drugs and metabolites in biological fluids.[11,14,16,25,26]

Lipid research, in particular, has benefited from stable isotope ratio techniques.[14,16,26] Steroids are, by definition, a variety of lipids, so it is no coincidence that advances in lipid research facilitated by GC–C–IRMS analysis have provided doping control with the potential to discriminate, based on ^{13}C content, between endogenous steroid metabolites and their synthetically derived analogs. Southan *et al*.[27] first demonstrated the distinctly different ^{13}C content of gonadal T and pharmaceutical T. The basis for this was provided by Coppen[28] who described a 'plants-to-pills' picture for synthesized steroid preparations that are

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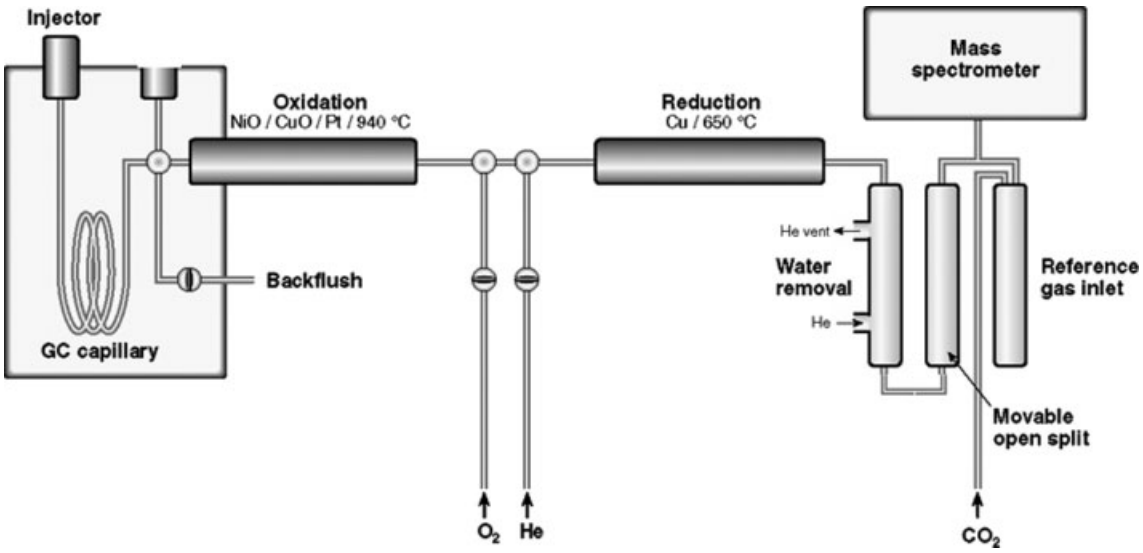

Figure 2. Schematic of a typical GC–C–IRMS instrument used for doping control (Courtesy of Thermo Electron Corporation, Bremen, Germany).

largely derived from *stigmasterol* and *sitosterol*, plant sterols obtained from ^{13}C depleted soy (*Glycine spp.*). Additionally, reference intervals reported by Ueki *et al*.[29] and de la Torre *et al*.[30] for endogenous steroid metabolites ($\delta^{13}C = -15‰$ to $-25‰$) and commercially available steroids ($\delta^{13}C = -26‰$ to $-36‰$) have substantiated the ability for GC–C–IRMS analysis to confirm endogenous steroid abuse. These differences, although potentially small, are statistically significant and measurable.

Early research carried out by doping control laboratories demonstrated the use of GC–C–IRMS analysis to show a decrease in the ^{13}C content of the steroid diol metabolites – 5α-androstane-3α,17β-diol ($\alpha\alpha\beta$-diol) and 5β-androstane-3α,17β-diol ($\beta\alpha\beta$-diol) – following administrations of endogenous steroids, particularly testosterone, toward the value of the isotope ratio in the synthetic material.[31–36] There are, however, considerable analytical requirements for successful $\delta^{13}C$ determination of urinary steroid diols. First, the urinary excretion of $\alpha\alpha\beta$-diol and $\beta\alpha\beta$-diol in concentrations as low as 10 ng/ml, may require large sample volumes ($\geq$10 ml) to achieve sufficient sensitivity for GC–C–IRMS. Second, high-resolution GC necessitates high specificity for the isolation of steroid diols from the urine matrix, often requiring High Performance Liquid Chromatography (HPLC) purification. These factors may restrict the ability of a laboratory to perform comprehensive $\delta^{13}C$ analysis of steroid diols for all samples that are deemed to be suspicious on the basis of discrete GC–MS profiling parameters.

A two-stage GC–C–IRMS analysis strategy can be implemented to overcome this impediment for doping control laboratories:

1. A preliminary screen can be implemented to measure the $\delta^{13}C$ value of androsterone (A; 5α-androstane-3α-ol-17-one) and etiocholanolone (Et; 5β-androstane-3α-ol-17-one) – the terminal androgen metabolites (Fig. 1) that are excreted in urinary concentrations typically ranging from 700 ng/ml to 6000 ng/ml.[6] GC–C–IRMS screening using such methods as reported by Aguilera *et al*.[37] can provide a resource-efficient analysis procedure from as little as 2 ml of urine to identify genuinely suspicious samples with $\delta^{13}C$ values lower than a reference interval established for the laboratory.
2. Should laboratory criteria classify a urine sample as potentially originating from endogenous steroid administration, GC–C–IRMS confirmation can be carried out using HPLC purification procedures to enable valid $\delta^{13}C$ determination of $\alpha\alpha\beta$-diol, $\beta\alpha\beta$-diol and/or T. For its time and expense, the advantage of such confirmation methods over the measurement of A and Et is twofold: First, the specificity that it provides can potentially identify which endogenous steroid was administered. Second, the postadministration detection period can be significantly greater due to a lower degree of endogenous dilution.

YOU ARE WHAT YOU EAT!–ENDOGENOUS REFERENCE COMPOUNDS TO ACCOUNT FOR DIET

Diets can vary greatly with culture and sport, and this may be accounted for in GC–C–IRMS analysis by measuring the $\delta^{13}C$ value of a 'background marker' steroid – an endogenous reference compound (ERC) – that is not involved in the androgen metabolic pathway. The use of an ERC was addressed in the studies headed by Becchi[31] and Shackleton[33,34] that determined the $\delta^{13}C$ value of pregnanediol (PD; 5β-pregnane-3α,20α-diol). The difference of an ERC $\delta^{13}C$ value from that of selected androgen metabolites is proposed to be the most effective reporting method of normalizing GC–C–IRMS results in relation to the diet of the individual athlete. These $\Delta\delta^{13}C$ values provide a sound basis to identify doping cases,[38] although the magnitude of the subsequent doping criteria may be dependent on what ERC is used. For marker steroids, such as PD, that closely reflect the ^{13}C content of the androgen metabolites, a 3.0‰ limit enforced by the WADA appears suitable.[39] ERCs, such as 11keto-etiocholanolone (11keto-Et; 5β-androstane-3α-ol-11,17-dione) – a product of

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the cortisol–cortisone metabolic pathway – that are relatively ^{13}C-enriched, may require a higher confidence limit up to 4.0‰.[40] Of course, in the ongoing antidoping battle between the scientists and the cheats, the weaknesses of any analysis method need to be considered. Just as the use of T/E values raised the possibility of offenders coadministering epitestosterone (E; androst-4-ene-17α-ol-3-one) with T, GC–C–IRMS analysis methods based on $\Delta\delta^{13}$C values may be susceptible to coadministration of an ERC. To this effect, Saudan *et al*.[41] have demonstrated ^{13}C depletion of PD following oral administration of its precursor, pregnenolone. This investigation led to 5α-androst-16-ene-3α-ol being proposed as a suitable ERC, when precursors to PD have been administered.[42]

GC–C–IRMS AT THE OLYMPIC GAMES

GC–C–IRMS for doping control was first introduced to the Olympics during the Atlanta 1996 Summer Games, albeit for demonstration purposes only. Implementation of GC–C–IRMS analysis into the Nagano 1998 Winter Olympic Games testing program represented a real milestone for the technique in doping control. At this event, 450 samples providing suspicious GC–MS steroid profiles were analyzed by GC–C–IRMS to confirm that there was no endogenous steroid abuse. This exercise provided the first incompetition elite athlete δ^{13}C reference intervals for $\alpha\alpha\beta$-diol (−10.5‰ to −24.6‰) and $\beta\alpha\beta$-diol (−15.2‰ to −26.2‰) corrected for the measurement of diacetate derivatives.[29] In contrast to most studies investigating GC–C–IRMS analysis of steroid metabolites from the glucuronide fraction following liquid–liquid extraction, the Japanese antidoping laboratory employed solid-phase extraction techniques with pH gradient elution to include δ^{13}C values of DHEA (−15.6‰ to −24.1‰) obtained from the sulfoconjugate fraction.

GC–C–IRMS analysis was conducted on 223 urine samples as part of the Sydney 2000 Olympic Games doping control program on the basis of abnormal GC–MS steroid profiles, with at least one of the following conditions being satisfied:

- T/E >4
- A and/or Et >3000 ng/ml*
- DHEA glucuronide >100 ng/ml* * Corrected for specific gravity of 1.020[39]

This represented an 8.6% subset of the 2585 urine samples collected from elite athletes in September 2000. No sample was found to be representative of synthetic administration on the basis of δ^{13}C values satisfying the following criteria:

- δ^{13}C Et and/or δ^{13}C A < −27.0‰
- $\Delta\delta^{13}$C > 4.0‰

Since 2004, the WADA has subsequently set δ^{13}C and $\Delta\delta^{13}$C limits of < −28.0‰ and >3.0‰, respectively.[39] There were two samples determined by GC–C–IRMS to have originated from synthetic steroid administration during the Athens 2004 Olympic Games, the first incompetition Olympic cases resulting in sanctions against the athletes involved. The number of prospective samples for GC–C–IRMS is increasing further for each Olympic Games. In Beijing, there will be three GC–C–IRMS instruments available to analyze an estimated 450 samples, approximately 10% of the total sample collection program.

METHODS FOR URINARY STEROID ANALYSIS

Hydrolysis and isolation of urinary steroids from their glucuronide moieties has been derived from reported methods used for GC–MS steroid screening in doping control[2–7] and adapted for the application of GC–C–IRMS analysis.[43–45] There has also been limited development of sulfoconjugate isolation methods for GC–C–IRMS purposes, targeted to the detection of DHEA abuse.[29,46] In addition, HPLC purification of urinary steroids is becoming increasingly important to satisfy the specificity requirements of GC–C–IRMS.[47] An optimized HPLC purification procedure is provided by Table 1 with the corresponding fraction collection strategy in Table 2.

Prior to the GC–C–IRMS analysis of steroid extracts, a background mass scan (m/z 10 to m/z 50) should be performed with the GC in split injection mode and an oven temperature of 180 °C to verify the leak-free integrity of the system by ensuring the levels of background gases –N_2, H_2O, O_2, Ar and CO_2– are below the limits established within each laboratory. The sensitivity of the instrument is shown to be satisfactory by saturating the source with reference to CO_2 while monitoring m/z 44, m/z 45 and m/z 46. Zero enrichments demonstrate that the source is conditioned prior to beginning a sequence and to verify the repeatability of δ^{13}C measurements. Up to 10 reference CO_2 pulses, each of the 20-s duration, may be flushed into the source over an acceptable time period. The ratio of ^{13}C to ^{12}C is measured for the reference peaks and the standard deviation then determined to be within a laboratory limit.

GC–C–IRMS parameters are required that provide optimal chromatographic resolution of steroid peaks, high-sample throughput and minimal maintenance of the relatively complex instrument. An example for effective GC–C–IRMS analysis of underivatized steroid extracts is

Table 1. HPLC parameters for the purification of urinary steroids

HPLC	HP 1090 LC with automatic injection system and PDA detector (Hewlett-Packard, Waldbronn, Germany)
Injection volume	90 µl
Column	LiChroCART (125 mm × 4 mm × 5 µm) reverse phase C18 protected by a LiChroCART (4 mm × 4 mm × 5 µm) C18 guard column (Merck, Darmstadt, Germany)
Flow rate	1 ml/min
Solvent gradient	20%CH_3CH_2CN/80% H_2O → 100%CH_3CH_2CN @ 4%/min (4 min)
Detector	λ = 345 nm (absorption maximum of β-trenbolone)
Data acquisition	HP Chemstation Rev. A.06.03
Collection	FOXY200 (ISCO, Lincoln, NE, USA)

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Table 2. Collection program for urinary steroids purified by HPLC

Fraction	Time (min : sec)	Steroid
1	7 : 40–8 : 20	–
2	8 : 20–9 : 00	11βOH-Et
3	9 : 00–9 : 40	11βOH-A; 11keto-Et
4	9 : 40–10 : 20	T
5	10 : 20–11 : 00	E; $\beta\alpha\beta$-diol
6	11 : 00–11 : 40	$\alpha\alpha\beta$-diol
7	11 : 40–12 : 20	Et
8	12 : 20–13 : 00	A
9	13 : 00–13 : 40	PD
10	13 : 40–14 : 20	–
11	19 : 00–19 : 40	–
12	19 : 40–20 : 20	Cholesterol
13	20 : 20–21 : 00	–
14	21 : 00–21 : 40	5α-androstane-3β-ol (international standard)
15	21 : 40–22 : 20	

Table 3. GC–C–IRMS parameters for the analysis of urinary steroids

GC–C–IRMS	Agilent 6890 GC (Palo Alto, USA) coupled to a Thermo Combustion III interface, Thermo Delta Plus IRMS and equipped with a Thermo A200S autosampler (Bremen, Germany)
Injection	Volume: 3 µl, splitless (0.5 min), temperature: 280 °C
Column	J&W Agilent HP 50+: 30 m, 0.25 mm I.D, 0.25 µm (cross-linked 50% phenyl-methyl siloxane) film thickness
Carrier gas	Helium, constant flow: 1.8 ml/min
Oven temperature	180 °C (1 min) → 250 °C @ 12 °C/min → 280 °C @ 3 °C/min → 300 °C @ 15 °C/min (4 min)
Timed events	0 to 430 s: Backflush on/open split out/three CO_2 pulses 430 to 1200 s: Backflush off/open split in/two CO_2 pulses 1200 to 1300 s: Backflush on/Open split out
Oxidation	Cu/Ni/Pt ceramic capillary reactor: 940–1000 °C
Reduction	Cu ceramic capillary reactor: 620 °C
Water removal	Nafion semipermeable membrane with He carrier gas, or cryogenic trapping with acetone/dry-ice slurry
Ionization	Electron impact (EI): up to 130 eV
Accelerating voltage	3 keV
Data acquisition	Thermo ISODAT NT 2.0 (Bremen, Germany)

provided in Table 3 and should include the following protocol:

1. A solvent blank that does not contain any steroid and/or unknown compounds should be the first injection of a sequence to verify that the system is void of contamination.
2. Injection(s) of reference standards that are not observed to coelute using the chosen conditions. The δ^{13}C of steroids are determined with careful definition of each peak relative to the background to ensure there is no systematic bias due to erroneous integration.
3. The δ^{13}C value of urinary steroids calculated by the software is relative to the δ^{13}C value of the CO_2 or steroid reference materials that have been determined by comparison to the NBS-19 standard described by the International Atomic Energy Agency to represent VPDB.
4. Stability and reproducibility of GC–C–IRMS measurements can be monitored by determining the δ^{13}C value of an internal standard such as 5α-androstane-3β-ol in each sample. This δ^{13}C value should lie within an estimated uncertainty from the δ^{13}C value measured by independent combustion ^{13}C analysis.
5. Quality controls analyzed with each sequence should demonstrate that the procedure is capable of identifying 'positive' urine samples that contain ^{13}C-depleted steroid peaks. The δ^{13}C values of steroid metabolites and the ERC determined for such samples should be within the range of the estimated measurement uncertainty from the means of cumulative analysis, else rejection rules need to be applied.
6. Similarly, a 'negative' quality-control urine sample that contains naturally derived steroid peaks should be analyzed to represent a typically normal result.
7. An extract from a water-blank sample extracted with the urine samples can be analyzed to demonstrate the absence of any peaks other than the internal standards, thereby eliminating contamination as contributing to the analysis.

The principal disadvantage of GC–C–IRMS is its inability to provide structural information. In the case of steroid identity and/or purity being in doubt, a sample extract can also be analyzed by full-scan GC–MS with identical chromatographic conditions and EI spectral comparison to steroid reference standards. Results can be linked to the δ^{13}C values determined by GC–C–IRMS using relative retention rules with appropriate rejection criteria.[48] In addition, it is important that the preceding and following HPLC fraction relative to each steroid isolated by such methods is analyzed by full-scan GC–MS to ensure that there was no presence of the target steroid, thereby confirming fractionation from the HPLC process to be negligible.

DEVELOPMENTS IN GC–C–IRMS METHODOLOGY

Gas chromatographic conditions

High-resolution capillary chromatography is a prerequisite for high-precision compound-specific isotope analysis by online IRMS. Peak overlap and peak distortion have a detrimental effect on both accuracy and precision of isotope ratio measurements.[14] To eliminate these effects, in the first instance, basic gas chromatographic rules must be observed.

1. Oncolumn steroid amounts of between 10 and 300 ng may be effectively analyzed.

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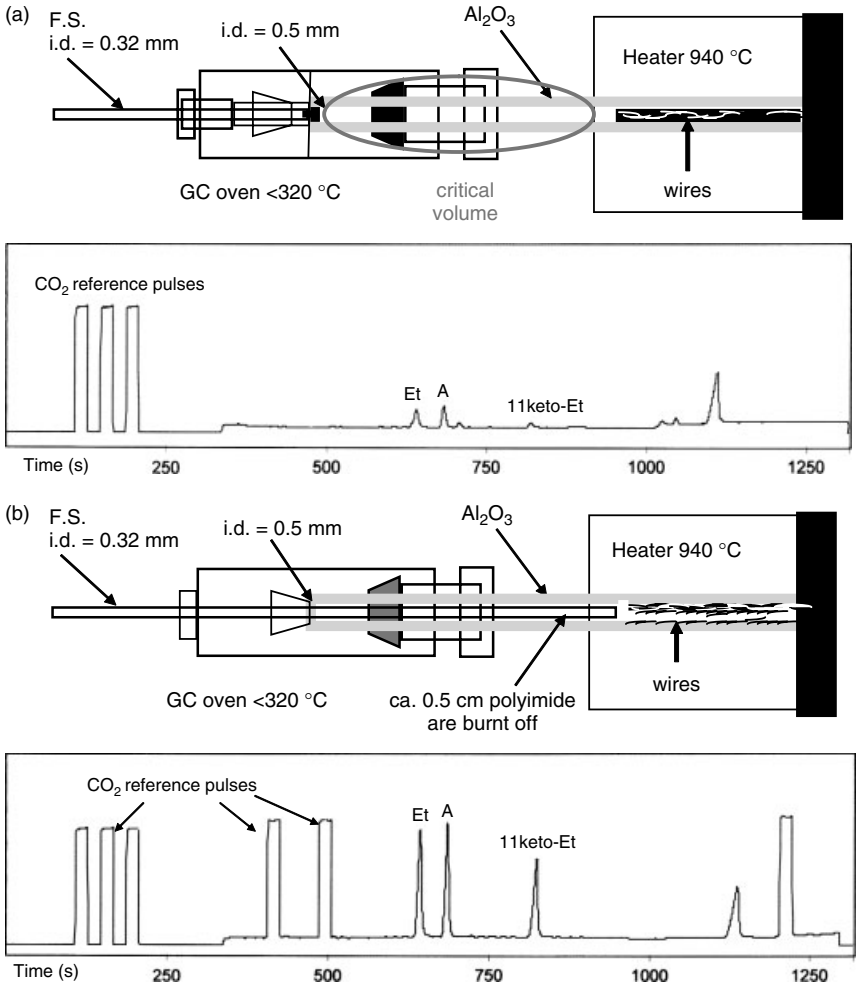

Figure 3. Connection of GC eluent to the combustion furnace with (a) dead volume adversely affecting signal response and (b) minimal dead volume allowing superior peak definition (interface diagrams, courtesy of Thermo Electron Corporation, Bremen, Germany).

2. Splitless or oncolumn injection is recommended – split injection can induce boiling point discrimination. True splitless injection may be performed with the use of a retention gap, and a solvent with high boiling point and small molar gas volume.
3. The polarity of the stationary phase should reflect the polarity of the steroids – low-bleed characteristics are preferable.
4. Constant flow conditions are recommended.
5. Burst-free capillary cuts.

Combustion interface

The GC–C–IRMS system includes numerous capillary connections and unions that represent small dead volumes. The ConFlo GC–C–III interface (Thermo Electron Corp., Bremen, Germany), for instance, possesses a backflush design with three tee junctions and six unions. In addition, four of the connections reside in the GC where thermal stress makes them suspect as being responsible for peak broadening and tailing. It was recognized by Goodman[49] that chromatography is adversely affected by the backflush design of GC–C–IRMS systems. In addition, peak shape and baseline separation are easily impaired during transfer to the combustion furnace and the subsequent passage through the interface. This, in particular, applies to steroids. On one hand, the high boiling points complicate chromatography, and often this problem is amplified by the presence of several hydroxyl or ketone functional groups. On the other hand, the condensed ring structure of the steroid backbone is very stable, which may limit the combustion process. Therefore, it is sometimes helpful to raise the temperature of the combustion interface up to 1000 °C. More frequent reoxidation will be necessary, however, as more oxygen is released.

Changes in tubing diameter and frequent use of unions lead to a loss in peak definition. In an attempt to circumvent this doping, control groups have provided advances in GC–C–IRMS hardware configurations with particular emphasis surrounding the connection of capillary GC columns to the combustion interface.[50] These unions represent the greatest source of dead volume in GC–C–IRMS systems, thereby distorting peak shape (Fig. 3(a)) and increasing errors associated with peak definition. An important aim of doping control laboratories has been to increase the molecular sensitivity of the GC–C–IRMS technique by reducing the dead volume effects that a multiconnection device provides (Fig. 3(b)).[51]

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The interface efficiency is critical for providing valid $\delta^{13}C$ values. Complete conversion of steroids to CO_2 is required to minimize incomplete combustion that results in the formation of CO. This byproduct, containing a C–O triple bond of smaller length, is ^{13}C-enriched in relation to CO_2 produced from steroid combustion, thereby resulting in lower $\delta^{13}C$ values for the analyzed steroids. Regular reoxidation of the combustion furnace, therefore, constitutes preventative maintenance to ensure optimum performance of the interface for $\delta^{13}C$ analysis.[22] The best interval for oxidation largely depends on the respective conditions and has to be found empirically. Often, peak tailing or poor resolution indicate incomplete combustion rather than poor chromatography. Careful tracking of the $\delta^{13}C$ values of a steroid internal standard will be helpful here.

Software considerations for $\delta^{13}C$ determinations

Data processing operations, including definition of beginning and end points for chromatographic peaks and the determination of background levels, have been shown to play a critical role in providing accurate $\delta^{13}C$ values.[54] Modern computational techniques have facilitated direct reporting of isotope ratios, without the need for approximations required from previously used mass ratios.[9] The measured ratios pertain to combinations of isotopic species, for example, m/z 45/44 is represented by $(^{13}C^{16}O^{16}O^{+\bullet} + {}^{12}C^{16}O^{17}O^{+\bullet})/(^{12}C^{16}O^{16}O^{+\bullet})$. Resolution of the individual isotopic contributions is required for the calculation of $\delta^{13}C$ values, and this is based on the consideration of statistical relationships governing the distribution of individual isotopes among the molecular species listed as follows:

Measurement gas	m/z	Species	Corrections
CO_2	44	$^{12}C^{16}O^{16}O^{+\bullet}$	
	45	$^{13}C^{16}O^{16}O^{+\bullet}$	$^{12}C^{16}O^{17}O^{+\bullet}$
	46	$^{12}C^{16}O^{18}O^{+\bullet}$, $^{13}C^{17}O^{16}O^{+\bullet}$, $^{12}C^{17}O^{17}O^{+\bullet}$	

Water removal

Avoidance of protonation artifacts in CO_2 IRMS is achieved using a Nafion dryer, which when operated at ambient temperature, can remove water from a saturated gas stream with up to 99.96% efficiency.[23] This efficiency is maintained by constant purging of the Nafion membrane with inert carrier gas in order to avoid saturation. The effectiveness of the Nafion dryer is ultimately dependent on the dryness of the helium carrier gas. Consideration also needs to be given to the fact that construction of the dryer inevitably results in two additional unions being required. To avoid problems with Nafion dryers, water removal can alternatively be achieved by cryogenic trapping in an acetonitrile/dry-ice slurry.

Signal intensity

At low signal intensities, $\delta^{13}C$ measurements become suitably dependent. One reason for this phenomenon, as outlined earlier, is the presence of H_2O in the ion source. The bias toward ^{13}C enrichment increases with smaller relative CO_2 concentration.[52] Effective GC–C–IRMS method validation requires a limit of measurement to be estimated that represents a signal intensity, below which valid $\delta^{13}C$ values would not be expected.[53] This presents a challenge to doping control laboratories that aim to determine $\delta^{13}C$ values for urinary steroids such as T and the respective $\alpha\alpha\beta$-diol and $\beta\alpha\beta$-diol metabolites at low natural abundance. Valid $\delta^{13}C$ measurements for these steroids necessitate larger urine sample volumes (up to 10 ml) and rigorous preanalysis concentration steps.

Another issue to be considered is column bleeding. Theoretically, the algorithms used to calculate $\delta^{13}C$ values from transient IRMS signals subtract any background. However, at smaller peak intensities, a larger proportion of the column bleed inevitably contributes to the calculated $\delta^{13}C$ value. Hence, the magnitude of the column bleed is important as well as its $\delta^{13}C$ value. As a consequence, not every column is comparably suited to GC–C–IRMS.

Contributions of doubly substituted isotopologs to m/z 46 are neglected, which is well justified by the rareness of these species.

Chromatographic resolution of isotopic species is sufficient to observe variation in the $^{13}C/^{12}C$ values. This 'chromatographic isotope effect' needs to be considered when processing GC–C–IRMS data. While peak start and end times are intuitively derived from the m/z 44 trace, the time disparity of the m/z 45/44 ion ratio trace needs to be taken into account to minimize ^{13}C fractionation that would otherwise discriminate against the heavier isotope. Peak definitions should, therefore, start and end with reference to the m/z 45/44 and m/z 44 traces, respectively (Fig. 4). Typically, a timeshift correction for the m/z 45 signal as suggested by Ricci *et al*.[54] is incorporated into modern software.

Reliance on software-automated peak and background definition should be viewed with caution. These are most commonly performed by a 'summation' method.[55] The start and end of a peak are detected, and the background is

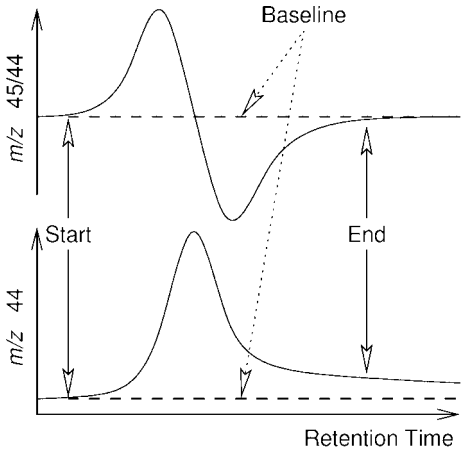

Figure 4. An example of correct peak and baseline definition as required from GC–C–IRMS analysts.

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described as a square or trapezoidal area beneath the peak. Raw data are summed over the length of the peak, and the background area is subtracted. Improvements in the GC–C–IRMS precision, particularly at low signal levels, have been made with the use of curve-fitting software in conjunction with digitizers that operate at 24-bit resolution.[55] This followed a research that demonstrated the potential for curve fitting to increase the accuracy of δ^{13}C measurements.[52,56] Continued development of software algorithms that remove subjectivity in assessing the background relative to GC–C–IRMS peaks will see greater improvements in precision, particularly analyst-to-analyst variation.[57]

Exploiting the information of isotope signals

Together with a greater understanding of, and improvements in the GC–C–IRMS system design, doping control laboratories continue to contribute important advances in physiological steroid research with the use of GC–C–IRMS. One recent example is the aim for compound-specific detection of prohormone abuse. It was previously accepted that IRMS capabilities can provide an answer simply as to whether 'an' endogenous steroid has been administered. Beyond analyzing known steroid metabolites, a novel approach has been reported[43] that takes advantage of urinary transformations specific to endogenous steroid precursors. These transformations may be metabolic or chemical in nature but the marker compounds produced from such mechanisms, if present at appropriate concentrations, allow complementary GC–MS and GC–C–IRMS analysis for screening and confirmation, respectively. The methodology of 'looking outside the metabolic box' exposed the limitations that research confined to the analysis of known metabolites places on doping control. Furthermore, the measurement of urinary originating from ingested synthetic material, expands the scope of steroid analysis from a 'metabolite-only' domain to a true urinalysis procedure.[58]

ISSUES FOR DOPING CONTROL

Sample preparation

Valid δ^{13}C measurements require isotopic fractionation during sample preparation to be excluded.[59] Semipreparative HPLC steroid purification methods combined with optimum GC–C–IRMS conditions can produce excellent chromatographic resolution required for the accurate reporting of δ^{13}C values. Consideration is needed, however, of the ability of reversed-phase HPLC to effectively separate isotopologs[60] and how this extends intuitively to the purification of steroids as a potential source of error. Indeed, as important as careful peak definition in the GC–C–IRMS analysis is a prerequisite for accurate results, chromatographic separation by HPLC has been shown to significantly alter the ^{13}C content of a steroid when the entire peak was not collected.[61]

Conversion of steroid metabolites to their acetate derivatives is another issue confronted by doping control laboratories. Acetylation, while an effective means of improving chromatographic resolution of steroids by increasing their volatility, represents a fractionation process. For each acetate group, two carbon atoms have been added to the steroid molecule, thereby altering its native δ^{13}C value. Additionally, kinetic isotope effects predominate when the reaction does not achieve completion. To compensate for these problems, an internal standard can be added pre- and postderivatization to correct steroid δ^{13}C values for the presence of acetate moieties and monitor the effectiveness of the procedure.[29] Of importance to the interpretation of $\Delta\delta^{13}$C values is the equivalence of hydroxyl moieties for the androgen metabolites and the ERC used for such measurements. For instance, the derivatization of A and Et should be accompanied with an ERC such as 11keto-Et containing one hydroxyl group. Similarly, $\Delta\delta^{13}$C measurements derived from derivatized $\alpha\alpha\beta$-diol and $\beta\alpha\beta$-diol should be made in relation to an ERC such as PD containing two hydroxyl groups. Considering all these potential errors, analysts should be aware that derivatization and the subsequent use of correction factors may limit the precision advantage of the GC–C–IRMS technique to doping control. In addition, excellent results from underivatized compounds can be achieved with conventional interfaces and midpolar columns so long as the prerequisites mentioned previously are fulfilled.

δ^{13}C reference intervals

A particularly important issue for doping control has been the limited δ^{13}C profiling studies of urinary steroids. There is an assumption that laboratories should conduct GC–C–IRMS analysis on at least 50 urine samples from each sex as a validation exercise to establish laboratory reference intervals. A practical introduction to the use of reference values and respective computations has been provided by Solberg and Gräsbeck.[62] The possibility then remains that a comprehensive δ^{13}C profiling database can be established for urine samples collected from elite athletes. In the meantime, preliminary results have revealed two primary clusters of Et and A values derived from C-3 diets ($\delta^{13}C = -23.0 \pm 1.0$‰) and C-4 diets ($\delta^{13}C = -20.1 \pm 1.3$‰).[63,64] It remains, however, based on global distributions to determine what significance diet, together with other factors such as ethnicity, age and sex, have on δ^{13}C variations. This is a particular need with the testing of athletes that compete at various locations throughout the world. The task of compiling δ^{13}C data from a large number of urine samples is a major challenge considering the complicated nature of GC–C–IRMS instrumentation. Added to this is the requirement for a sample population representing a diverse group of reference individuals to establish legally defensible δ^{13}C sanction criteria derived from reference intervals.

CONCLUSION

The introduction of IRMS to doping control represents a major breakthrough. The unequivocal detection of the abuse of synthetic steroid copies is now often possible. However, high performance in GC–C–IRMS is not problem-free. Neither issues of sample preparation nor 'ordinary' GC–MS methodology can be transferred offhand. In contrast, the demands of analytical stable isotope technologies by far exceed those of more conventional methods. It must be

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considered that this inevitably yields higher workloads. In particular, this applies to the demands of sample purity and GC resolution to instrument maintenance. Stable isotope-based inference, likewise, proved to be anything but simple. Whereas it had been hoped that synthetic and endogenous steroids in urine could easily be discriminated by their respective $\delta^{13}C$ values, it has turned out that varying baseline values will require careful study and evaluation of reference populations. In conclusion, GC–C–IRMS is a powerful and fascinating methodology, but it must be considered that handling maintenance and inference can be delicate and may represent major challenges to the fraternity in general and not to the unwary alone.

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