Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

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Sport und Buch Strauß, Köln, 2004

A. CAWLEY, R. KAZLAUSKAS, G. TROUT, A. GEORGE:
Pure and Applied Aspects of Carbon Isotope Ratio Analysis in Doping Control
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 231-240

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Pure and applied aspects of carbon isotope ratio analysis in doping control

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Introduction

Carbon isotope ratio (CIR; $\delta^{13}C$) analysis using Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) has demonstrated a high potential to detect the illegal administration of synthetically derived endogenous steroids [1-5]. There remains however, a clear need for WADA accredited laboratories to improve the use and understanding of this technique to meet the rigorous medico-legal demands of doping control. In the Sydney laboratory (ASDTL), the CIR is considered as an extension of the routine GC-MS steroid profile, not as a separate test. Using this methodology we aim to obtain as much information as possible from endogenous steroid concentrations, concentration ratios and $\delta^{13}C$ values that relate to the metabolism of the individual athlete.

Three key areas of endogenous steroid research have been identified:

- To improve the ability of the routine GC-MS steroid screen to identify suspicious samples from the majority of negative samples analysed by a laboratory. These samples would then undergo confirmation by GC-C-IRMS.
- To investigate the natural variation in $\delta^{13}C$ values within and between different ethnic populations with the aim of establishing reference intervals to support criteria relating to the detection of endogenous steroid abuse in athletes. Preliminary results of this study have been presented [6].

- To develop the ability to confirm the administration of specific endogenous steroids. While this poses a significant challenge, further investigation will provide valuable information on steroid metabolism and result in improved use of the GC-MS and GC-C-IRMS techniques.

To date, most CIR research has been concerned with improvements to the analytical process of the GC-C-IRMS technique. While this aspect is important and more work is required, in order to realise the full potential that this technique may bring to doping control it is also important to investigate the pure aspects of CIR analysis. The reporting of scientifically obscure numbers and symbols such as $\delta^{13}C$ = -20.0‰, -25.0‰ or -30.0‰ for example, will inevitably result in questions within the legal framework of doping control. It is therefore essential that analysts have a superior understanding of what these numbers mean and how they are derived in terms of steroid pharmacology and the analytical process.

A case study: Dehydroepiandrosterone (DHEA)

In recent years, the detection of dehydroepiandrosterone (DHEA) abuse has been discussed extensively at the Cologne workshop [7-10], not to mention the wider literature [4,11-14]. While conclusive methods of analysis have been difficult to report, it appears that evermore questions regarding DHEA metabolism are posed from the various studies conducted. This should be viewed in a positive manner as further investigations will provide more information relating to DHEA metabolism, which in turn will improve the detection of DHEA abuse in athletes using combined GC-MS/GC-C-IRMS methodology. Furthermore, consideration should also be given to the possibility that information relating to DHEA metabolism and improving the detection of its abuse could be used for the detection of other prohormone abuse such as androstenedione and the androstenediols.

Experimental

Two administration trials using DHEA from the same batch of capsules extracted were analysed in this study. Both had the informed consent of the subjects and approval of the Human Ethics

Committee of Southern Cross University for the administration of DHEA to male volunteers [15]. Baseline urine samples were collected at the time of initial DHEA administrations followed by regular urine collections.

Subject A (30 year-old male); was orally administered with a single dose of 100 mg DHEA. A total of thirteen urine samples were collected at regular intervals up to a 52-hour post-administration period.
Subject B (30 year-old male); was orally administered with 100 mg DHEA at morning and night for seven days. Twenty-three urine samples were collected at regular intervals over the seven day period from the first administration and then a further three collections of urine were made over 22-hours post-administration.
Urine samples were collected in plastic bottles and stored at –20 °C prior to analysis.

GC-C-IRMS analysis of etiocholanolone (Et), androsterone (A) and 11 ketoetiocholanolone (11-keto) requires minimal cleanup by solid phase extraction using BondElut Certify cartridges following enzyme hydrolysis (β-glucuronidase from *E.coli*) except that in this case we used a 3 mL urine sample. An underivatised steroid extract (3 μL) was then analysed by GC-C-IRMS (Thermo Delta Plus). 17α-methyltestosterone (120 μg/mL, 50μL) was added as an internal standard[6].

Results and discussion

The administration studies

The single dose administration illustrated in this study provided information relating to the metabolism kinetics of DHEA. The multiple administration represented a more realistic study, as it is expected that an athlete would most likely administer supraphysiological amounts (greater than 200 mg per day) for extended periods of time in order to gain any of the perceived advantages that DHEA may provide. Saturation effects may also be observed that reveal pharmacological differences between endogenous and synthetic DHEA metabolism. Chromatographic separation of Et and A is achieved using the HP 50+ (50%

phenylmethylsilicone) capillary GC column (Figure 1). 11-keto is measured as an endogenous reference compound (ERC) that is produced in a separate biosynthetic pathway to that of the androgens such that its $\delta^{13}C$ value accounts for dietary and other natural variations of the individual. The $\delta^{13}C$ value of 17α-methyltestosterone (17-MeT) is measured as an internal standard for quality control. Variation in this measurement is allowed within 0.3‰ of the certified value, based on the uncertainty estimate discussed herein. The results of the single dose study have been presented previously [16]. Unexpectantly, a time-dependent difference between $\delta^{13}C$ Et and $\delta^{13}C$ A (**$\delta^{13}C$ Et - $\delta^{13}C$ A**) was observed during the excretion of the administered DHEA (Figure 2). This provided evidence of isotopic fractionation whereby DHEA administration results in metabolic discrimination of the isotopes of Et in preference to A resulting in a 3.5 ‰ increase of **$\delta^{13}C$ Et - $\delta^{13}C$ A**. Similar results presented by Flenker and Schänzer [17] have been described as kinetic isotope effects arising from reduction of the double bond between C-4 and C-5 in Δ^4-steroids. There is the possibility, with greater understanding of the pharmacokinetics involved, that fractionation patterns may provide useful post-administration analysis criteria to confirm and distinguish the abuse of particular endogenous steroids. The multiple administration results are shown by Figure 3. Unexpectedly, minimum $\delta^{13}C$ Et and $\delta^{13}C$ A values were ^{13}C depleted (up to 3.3‰ and 1.7‰ respectively) compared to the DHEA used for the administration ($\delta^{13}C$ = -31.3‰). This study raises the important question of how the apparent *in vivo* ^{13}C depletion of steroid metabolites in relation to their precursor is possible. In relation to doping control this phenomenon, if shown to in fact exist, represents an advantageous outcome that would most likely result in longer detection periods for endogenous steroid abuse than would otherwise be the case. These results will be presented in more detail in the near future [18].

An estimation of measurement uncertainty (MU) for GC-C-IRMS analysis

Estimations of MU have the potential to increase understanding of analytical techniques that would serve to improve the legal defensibility of methods in a sensitive political and forensic field such as doping control. Compliance with the International Organization for Standardization (ISO) standard 17025 does not necessitate large volumes of tedious work. Under normal circumstances, a laboratory would have access to estimations of method ruggedness from appropriate method validation procedures. It should also be considered that the guideline for MU

(clause 5.4.6.2) stated by ISO is a "reasonable estimate" that can be interpreted being "fit for purpose" of the laboratory and the method. A comprehensive study of MU applied to doping control analysis has been provided recently by Ventura *et al.* [20]. There are unique considerations in relation to MU estimates for GC-C-IRMS. In essence, GC-C-IRMS is not a quantitative technique *per se*. Instead of measuring the quantity of a particular substance, GC-C-IRMS is concerned with measuring the relative stable isotopic composition of a substance. The ISO-Guidelines on Uncertainty in Measurement (GUM) proposes the use of a "bottom-up" approach to developing estimates of MU [21]. To reduce complexity, a "cause and effect" analysis can be used in conjunction with such an approach (Figure 4). This begins with a definition of the measurand (i.e. what is being measured), which in the case of GC-C-IRMS analysis of steroids is the $\delta^{13}C$ measurement on units of per mille (‰). The individual components of MU then need to be identified and listed in order of decreasing contribution. In theory, the major contribution to GC-C-IRMS MU is the precision of the technique, from extraction through to instrumental analysis. This may be viewed as the combination of *intra*-assay variation or repeatability, and *inter*-assay variation or reproducibility. To quantify the contribution made by precision to the overall MU a relatively simple, but statistically significant repeat extraction and analysis of the same QC negative urine was undertaken. The standard uncertainties of *intra*- and *inter*-assay standard deviations were calculated as standard errors of the mean to give a value of 0.24‰.

Also to be considered is the contribution of traceability. In theory, this would be relatively small but may vary significantly between laboratories due to different methods of CO_2 reference gas calibration. A dual-inlet IRMS allows internal determination of the $\delta^{13}C$ value of CO_2 reference gas, however without such a system laboratories such as ASDTL require external calibration. Thus the CO2 gas in a large cylinder (BOC Gases, Sydney Australia, Laser Grade) was first calibrated by an external reference laboratory for use on our system. This was provided by the Commonwealth Science and Industry Research Organisation (CSIRO) with respect to the International Atomic Energy Agency (IAEA) NBS-19 standard distributed by the U.S. National Institute of Standards and Technology (NIST). The CSIRO instrument (Thermo MAT 252) was calibrated in relation to the NBS-19 limestone standard using elemental analysis (EA) flash combustion. The accuracy of these measurements in relation to the agreed value served as a

rectangular distribution with which to calculate a standard uncertainty of 0.0081‰. The standard deviation (0.0087‰) of eight (8) NBS-19 measurements represented the uncertainty associated with the precision of this determination. These standard uncertainties were combined with the standard deviation of eight (8) CO_2 reference gas measurements made relative to NBS-19 to give an overall traceability value of 0.01‰. The CSIRO devision also measured the isotope ratio of the 17α-methyltestosterone used as an internal standard and our values agreed within the Uncertainty of the measurements.

Due to the lack of a suitable certified reference material (or CRM) a bias component is not included in this estimate, however it may represent the most important of all contributions to GC-C-IRMS MU. Consensus values from proficiency trials could be used to estimate the bias in a laboratory's measurement, resulting in more rigorous method development or the application of correction factors. This emphasises the importance of well designed and regular inter-laboratory proficiency trials as a measure of GC-C-IRMS reproducibility. In the absence of an estimate for the bias component of MU, an internal laboratory MU estimate is reasonable to satisfy ISO 17025 regulations. Combining the precision and traceability components reveals the insignificance of the latter. Of importance however, is the fact that small contributions such as traceability are investigated using the "bottom-up" approach, itself a demonstration of ISO 17025 compliance. Hence the combined standard uncertainty (u) was 0.24‰. At a confidence level of 95% commonly used in doping control analysis, a coverage factor (k) of 2 can be applied to produce an estimate of expanded uncertainty (U) to be 0.48‰ for the measurement at –20.6‰. Finally, verification of MU estimates is required to demonstrate compliance to ISO 17025. For this purpose a positive QC sample, which is a composite of urine samples with T/E values greater than 6, was analysed with 49 sets of analysis. The standard deviation of $\delta^{13}C$ ERC for the 49 measurements was determined to be 0.3‰, which is less than the estimated U. Hence the proposed estimate is supported.

Acknowledgements

This work was possible with the financial contribution of the Department of Industry, Tourism and Resources of the Australian Commonwealth Government. The School of Chemistry of the

University of Sydney provided a scholarly stipend for this work and financial assistance for its presentation at the 2004 Manfred Donike Workshop on Dope Analysis. Special mention is made for Dr Robert Weatherby at Southern Cross University for organising the administration studies.

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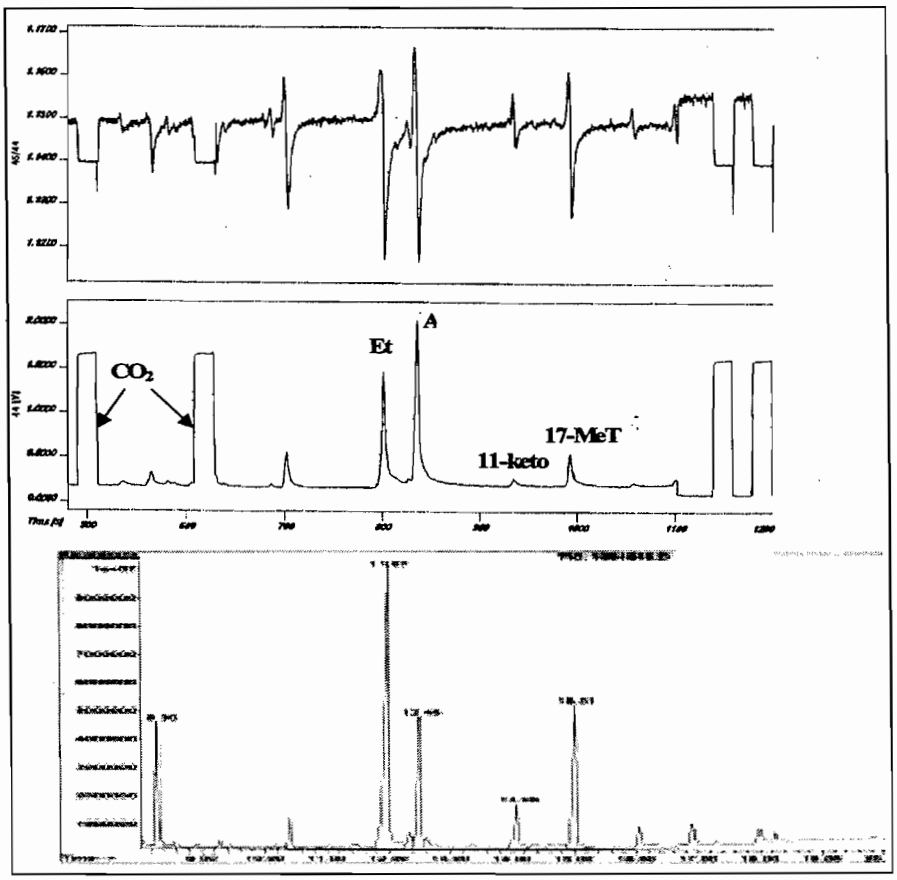

Figure 1: This shows the GC-C-IRMS trace for a sample (middle trace) at m/z 44 as well as the chromatogram for ratio of 45/44 masses (top trace). The GCMS chromatogram (bottom trace) allows the identity of the IRMS peaks to be obtained by inspection of the mass spectrum under each peak.

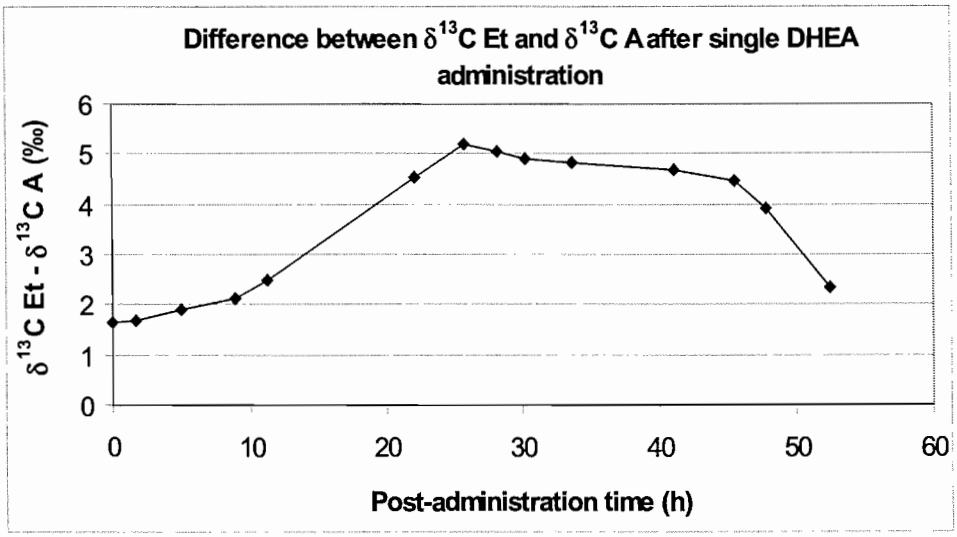

Figure 2: Difference between δ^{13}C Et and δ^{13}C A with respect to time after single DHEA administration

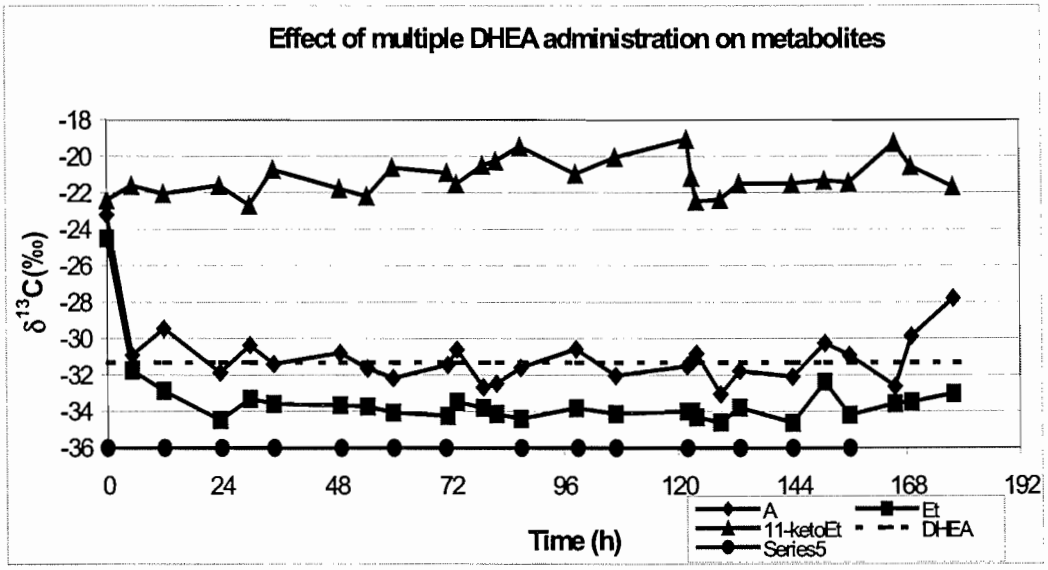

Figure 3: $\delta^{13}C$ trends of Et, A and 11-ketoEt of subject B with multiple DHEA administrations (each marked by • on the lower axis)

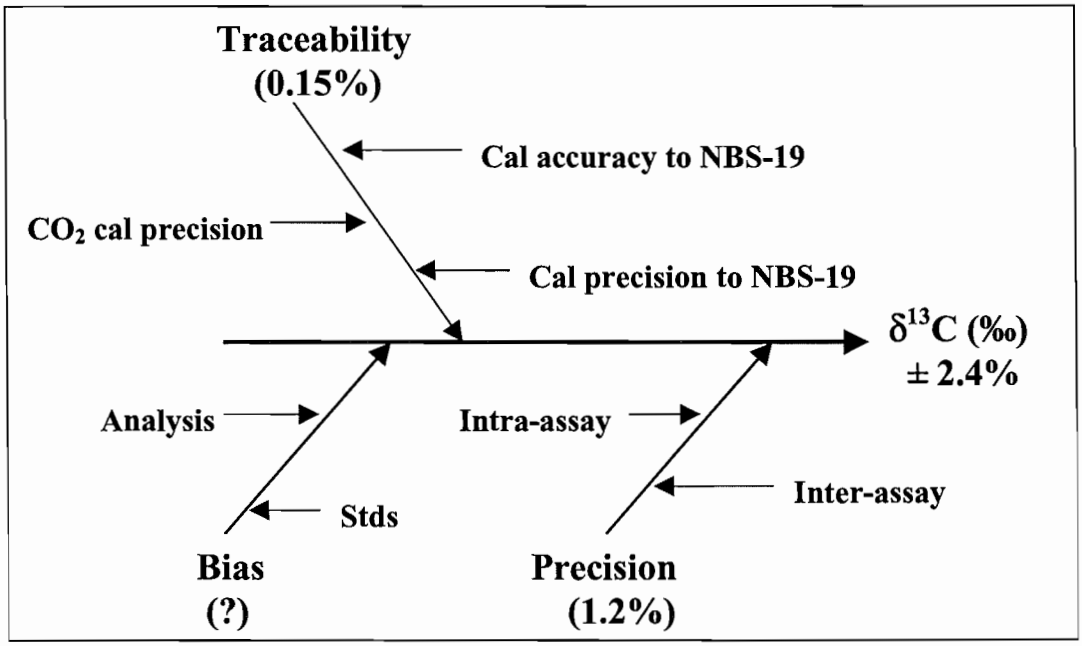

Figure 4: Cause and effect diagram showing components of GC-C-IRMS MU contributing to U = ± 0.48‰ for a measurement at –20.6‰.