Isotopic Fractionation of Endogenous Anabolic Androgenic Steroids and Its Relationship to Doping Control in Sports

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Abstract

The use of gas chromatography (GC)–combustion (C)–isotope ratio mass spectrometry (IRMS) demonstrates that a single oral administration of dehydroepiandrosterone (DHEA, 100 mg) to a male subject significantly lowers the ^{13}C content of etiocholanolone (Et) and androsterone (A) in the subject's urine. The difference in carbon isotope ratio ($\delta^{13}C$‰) values between Et and A increases from 1.6‰ at the time of administration to 5.1‰ at 26 h post-administration, indicating preferential metabolism of administered DHEA to form Et in relation to A. Multiple oral administrations of DHEA to a male subject reveals lower $\delta^{13}C$ values during the excretion period of Et (–31.7‰ to –34.6‰) and A (–31.4‰ to –33.0‰) to that of the $\delta^{13}C$ value of the administered DHEA (–31.3‰). Reference distributions of $\delta^{13}C$ Et and $\delta^{13}C$ A constructed from normal athlete populations within Australia and New Zealand show a small natural discrimination against ^{13}C in the formation of Et relative to A (mean = 0.3‰, n = 167, p = 0.007). Amplified differences between $\delta^{13}C$ Et and $\delta^{13}C$ A, and in vivo ^{13}C depletion measured by GC–C–IRMS are shown to be potentially useful for doping control.

Introduction

Abuse of endogenous (i.e., naturally occurring) steroids is one of the most important issues in sports. Doping control laboratories have been confronted with the challenge of finding criteria that allow endogenous steroids to be distinguished from their synthetic analogues in the urine of athletes. Dehydroepiandrosterone (DHEA) is an orally ingested weak endogenous androgen, sold in the United States as an over the counter "dietary supplement", which may be ingested by athletes with the aim of increasing levels of the more active androgens such as testosterone and dihydrotestosterone (1). The International Olympic Committee prohibits DHEA administration to an athlete (2), however the detection of administered DHEA by doping control laboratories has been difficult because of an incomplete understanding of DHEA metabolism as well as interindividual variations in urinary steroid excretion (3–9).

Attempts to detect DHEA abuse by gas chromatography (GC)–mass spectrometry (MS) alone have relied on the elevated excretion of the glucuronide conjugate of DHEA. Dehennin et al. (4) proposed that DHEA administration is indicated by a urinary concentration of DHEA–glucuronide exceeding the level of 300 ng/mL, based on reference studies showing the average normal concentration of DHEA–glucuronide to be less than 100 ng/mL. The usefulness of proposed limits such as this often depend on the metabolism of the individual athlete and the degree to which DHEA is converted to its sulfate conjugate (10). Obviously, more predominant conversion of DHEA to DHEA-sulfate will reduce the effectiveness of an administration marker based on DHEA–glucuronide. Previous studies by Kazlauskas (5) and Cawley (9) have found elevated DHEA–glucuronide excretions greater than 300 ng/mL for only 10 h after the administration of DHEA. Work by Ayotte et al. (6) has shown that DHEA administration resulted in increased excretion of C-7 hydroxylated metabolites: 7α- and 7β-hydroxy-DHEA. The detection of these compounds in the free/glucuronide steroid fraction routinely applied by doping control laboratories is difficult; however, because these two products are excreted as sulfate conjugates, which are not hydrolyzed by this process.

The use of GC–combustion (C)–isotope ratio MS (IRMS) analysis has been shown by various groups to be effective in detecting the administration of synthetic endogenous steroids (11–24). This is based on the principle of synthetic analogues of endogenous steroids, such as, DHEA, androstenedione, testosterone, and dihydrotestosterone having a considerably lower ^{13}C content compared with the naturally excreted compound (21). The GC–C–IRMS technique is capable of measuring these differences with a precision of 1‰ (25). A detailed description of the fundamental principles and nomenclature associated with the application of GC–C–IRMS analysis to doping control has been reported previously (26).

Most research to date has demonstrated the use of GC–C–IRMS

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analysis to show a decrease in the ^{13}C content of steroid diol metabolites following administrations of synthetic endogenous steroid analogues, particularly testosterone, towards the value of the isotope ratio in the synthetic material. The isolation of steroid diol metabolites from the urine matrix is complex because of the low concentrations that are excreted and the need to remove all the ketosteroids that are present in high levels, since the ketosteroids interfere with the diol analysis. To improve the use of GC–C–IRMS analysis, Rogerson et al. (27) developed a method to determine $\delta^{13}C$ values using the more abundant ketosteroids metabolites: androsterone (A) and etiocholanolone (Et). Because the GC–C–IRMS analysis involves combustion of the substances to form carbon dioxide, the chromatograms produced are for ions *m/z* 44, 45, and 46 and do not contain any structural information. It is therefore important to reinject the sample into a GC–MS system, preferably utilizing the same run conditions to ensure that the peaks seen on the GC–C–IRMS are the correct compounds (Figure 1). Under the chromatographic conditions described herein, baseline separation is achieved for A and Et that eliminates any $\delta^{13}C$ bias that would affect the results.

The method using ketosteroid metabolites reported in this paper was used to investigate the effect that the administration of single and multiple doses of DHEA to healthy male volunteers had on δ^{13} values of A, Et, and 11-ketoetiocholanolone (11-ketoEt), which was not affected by the administered drug. The $\delta^{13}C$ of 11-ketoEt was measured as an endogenous reference compound that reflected the natural value*, and that was solely dependent on the diet of the subject and was not affected by metabolites of androgens because of its metabolic formation via the cortisone/cortisol pathway (28). Changes in $\delta^{13}C$ values of Et and A relative to 11-ketoEt following administration of DHEA and subsequent criteria that may be applied by doping control laboratories have been presented previously (26). The studies in this article aim to increase the understanding of DHEA metabolism following its administration and to present some reference $\delta^{13}C$ values obtained from a normal population to enable more effective use of GC–C–IRMS analysis to detect DHEA administration in doping control.

Experimental

Steroids and reagents

Steroid standards of A, Et, 11-ketoEt, and 17α-methyltestosterone (17-MeT) were obtained from Sigma Chemical Company (St. Louis, MO). DHEA capsules (100 mg, batch No. 37033) were obtained from KAIZEN (Los Angeles, CA). β-Glucuronidase from *Escherichia coli* (*E. Coli*) K12 (EC 3.2.1.31) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). BondElut solid-phase extraction (SPE) cartridges were obtained from Varian (Harbor City, CA). Ultrahigh purity helium, high-purity oxygen, and carbon dioxide gases were obtained from BOC gases (Sydney, Australia). The water used was obtained using a Milli-Q water purification system purchased from Millipore (Bedford, MA). All other reagents and solvents were of analytical grade.

Isolation of DHEA from capsules

The contents of two capsules of DHEA (100 mg), taken from the same batch administered to the two subjects in this study, were ground to a fine powder. Duplicate amounts of approximately 10 mg from each capsule were shaken using a rotary shaker with methanol (5 mL) in a clean screw top test tube for 1 h. An aliquot (100 µL) of the resulting supernatant was added to water (5 mL) and shaken for 10 min. SPE was used to obtain the DHEA fraction. BondElut Certify (C8/SCX) columns were placed onto a vacuum manifold (IST, Hengoed, U.K.) before they were conditioned with methanol (2 mL) and water (2 mL). The samples in aqueous solution were loaded onto the columns. The columns were then washed with water (2 mL) and methanol–water (10:90, 2 mL) before being dried under vacuum for 30 min. This was followed by a wash with hexane (2 mL) and further drying under vacuum for 60 min. The steroid fraction was eluted from the columns with ethyl acetate–methanol (95:5, 2 mL). The 17-MeT (120 µg/mL, 200 µL) was added as an internal standard before the fraction was evaporated to dryness under nitrogen. The dry residue was dissolved in dichloromethane (200 µL) to obtain a concentrate. An aliquot (20 µL) of this concentrate was diluted to a volume of 200 µL with dichloromethane to obtain a sample for GC–C–IRMS analysis.

The administration trials

Two administration trials using DHEA from the same batch of capsules extracted were analyzed 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 (29). Baseline urine samples were collected at the time of initial DHEA administrations followed by regular urine collections.

Subject A

A single dose of 100 mg DHEA was orally administered to a 30-year old male. A total of thirteen urine samples were collected at regular intervals up to a 52-h post-administration period.

Subject B

At morning and night for 7 days, 100 mg DHEA was orally administered to a 30-year old male. Twenty-three urine samples were collected at regular intervals over the 7 day period from the first administration, and 3 further collections of urine were made over the 22 h post-administration.

Urine samples were collected in plastic bottles and stored at –20°C prior to analysis.

Isolation of steroid metabolites from urine

Urine samples (2 mL) were adjusted to pH 7.0 by the addition of phosphate buffer (0.2M, 1.5 mL) before enzyme hydrolysis with β-glucuronidase from *E. coli* K12 (50 µL) for 1.5 h at 50°C. The hydrolyzed steroid metabolites were then separated from the urine matrix using the SPE method described for the isolation of DHEA from the capsule preparation. The 17-MeT (120 µg/mL, 200 µL) was added as the GC–C–IRMS volumetric internal standard. The fraction obtained was evaporated to dryness under

* Pregnanediol, also an endogenous reference compound, coelutes with the 11-ketoEt but its concentration is usually lower. Assuming a different isotopic composition, the effect of the small amount of pregnanediol would be to increase the standard deviation of the isotope ratio for 11-ketoetiocholanolone for a normal population of samples.

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nitrogen before the dry residue was dissolved in dichloromethane (100 µL) and transferred to a vial for GC–C–IRMS analysis.

GC–C–IRMS conditions

The system used was a Finnigan-MAT Delta Plus with a GC Combustion III from Thermo Finnigan (Bremen, Germany) for separation and online combustion of the steroid metabolites. The carrier gas was helium with a constant flow of 1.8 mL/min and initial pressure of 17.3 psi. The injection volume was 2 µL in splitless mode at 280°C with an interval of 0.5 min. The column (30–m × 25 –mm i.d.) was a Hewlett-Packard HP-50+ cross-linked 50% phenyl-methyl siloxane (0.25 µm film thickness). The column temperature was programmed from 180°C for 1 min to 250°C at 12°C/min, to 280°C at 3°C/min, then finally to 300°C at 15°C/min and held for 4 min. The combustion interface was used with an oxidation reactor temperature of 940°C. High purity oxygen gas was flushed through the furnace for 3600 s prior to analysis of a sequence. The reduction reactor temperature was 620°C. The software operating the IRMS system was ISODAT 7.4.

The $\delta^{13}C$ of Et, A, 11-ketoEt, and 17-MeT were determined in each of the urine samples from both administration studies. This required careful definition of each of the peaks relative to the background to ensure there was no systematic bias caused by erroneous integration. The $\delta^{13}C$ calculated by the software was relative to the calibrated value of $\delta^{13}C$ = –30.49‰ that was determined by comparison with the primary standard, Vienna Pee Dee Belemnite (CSIRO, 1999). This value was set for the reference CO_2 peak defined by the same procedure as the steroid peaks. Stability and reproducability of GC–C–IRMS measurements was monitored by determining $\delta^{13}C$ 17-MeT in each sample that was always close to its value of –32.8‰ (CSIRO, 1999), measured by combustion ^{13}C analysis within a standard deviation of 0.7‰.

GC–MS analysis

Each sample extract analyzed by GC–C–IRMS was also analyzed by full-scan GC–MS under the same GC conditions previously outlined using a Hewlett-Packard HP 5890 GC coupled to a HP 5970 mass selective detector (MSD). This was performed in order to ensure spectral identification of A, Et, 11-ketoEt, and 17-MeT and verify the purity of these peaks. The MSD acquired data in scan mode from 40 to 450 amu and electron impact spectral comparison was made to standards run at the same time.

Table I. GC–C–IRMS Results From the Single Administration of DHEA to Subject A in Relation to Post-Administration Time

		$\delta^{13}C$ (‰)			
Sample	Time (h)	Et	A	11-ketoEt	17-MeT
A0	0	–24.2	–22.6	–21.0	–32.5
A1	2	–25.7	–24.0	–20.9	–32.9
A2	5	–31.8	–30.0	–20.0	–33.3
A3	9	–32.1	–30.0	–19.3	–32.6
A4	11	–31.1	–28.6	–20.8	–32.8
A5	22	–31.4	–26.9	–21.1	–33.1
A6	26	–31.4	–26.3	–21.5	–33.5
A7	28	–30.8	–25.7	–20.4	–32.9
A8	30	–30.6	–25.7	–20.9	–33.7
A9	34	–30.1	–25.2	–20.6	–32.7
A10	41	–29.8	–25.1	–21.4	–32.9
A11	46	–29.3	–24.8	–21.9	–32.4
A12	48	–28.0	–24.1	–21.6	–32.3
A13	52	–26.3	–23.9	–21.6	–32.9

Reference population study

GC–C–IRMS analysis was conducted on a total of 167 urine samples collected from Australian (n = 59) and New Zealand (n = 108) athletes following approval of the Australian Institute of Sport Ethics Committee and the informed consent of each participant (30). Each of the athletes signed a guarantee that they had not ingested any substance prohibited by the Olympic Movement Anti-Doping Code (2), to ensure, as best as possible, that their urine samples could be used to represent a normal, drug-free population.

Results

The $\delta^{13}C$ of DHEA in the capsules administered to subjects A and B was determined to be –31.3‰ with a standard deviation of 0.5‰ obtained from triplicate IRMS analysis of duplicate extractions from two capsules (12 injections). A full-scan mass spectrum was collected of each DHEA sample to confirm the presence of DHEA in the administered capsules. The purity of the fraction

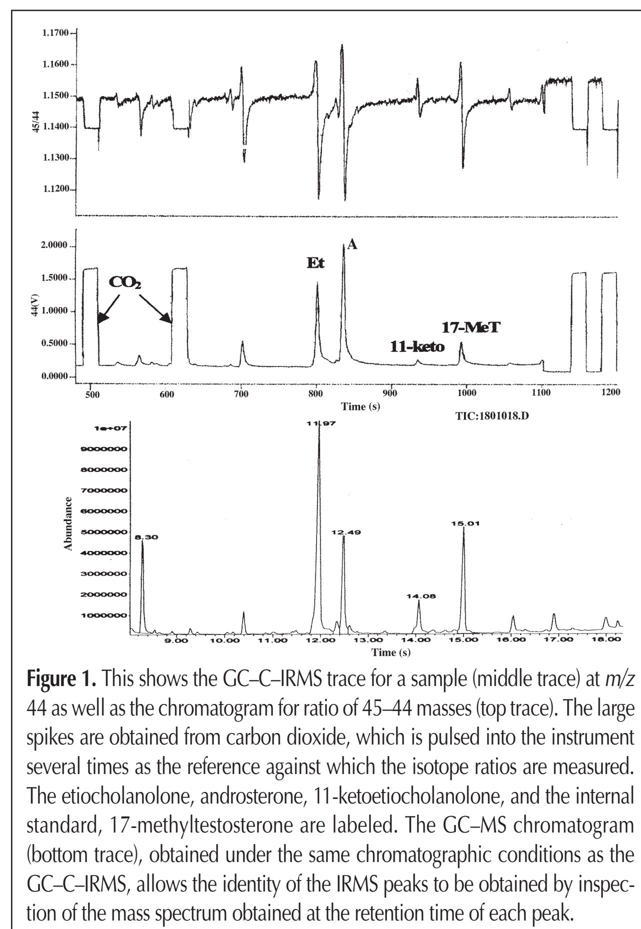

Figure 1. This shows the GC–C–IRMS trace for a sample (middle trace) at m/z 44 as well as the chromatogram for ratio of 45–44 masses (top trace). The large spikes are obtained from carbon dioxide, which is pulsed into the instrument several times as the reference against which the isotope ratios are measured. The etiocholanolone, androsterone, 11-ketoetiocholanolone, and the internal standard, 17-methyltestosterone are labeled. The GC–MS chromatogram (bottom trace), obtained under the same chromatographic conditions as the GC–C–IRMS, allows the identity of the IRMS peaks to be obtained by inspection of the mass spectrum obtained at the retention time of each peak.

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collected from the capsules was evaluated by searching for any other peaks in the chromatogram. No other peaks were observed, only DHEA and 17-MeT (the internal standard), indicating the fraction was pure.

The GC–C–IRMS results obtained from urinary steroid extracts of each of the time point collections from the single administration of DHEA to subject A are given in Table I and have been discussed previously (26).

In support of previous studies (11–24), Et and A were both observed to have lowered δ^{13}C values following administration of DHEA. Unexpectedly, however, a time-dependent difference between δ^{13}C Et and δ^{13}C A (**δ^{13}C Et–δ^{13}C A**) was observed during the excretion of the administered DHEA (Figure 2). At the beginning of administration, **δ^{13}C Et–δ^{13}C A** was 1.6‰ in this individual before increasing to a maximum value of 5.1‰ at 26 h post-administration, then decreasing to 2.4‰ at 52 h post-administration.

The GC–C–IRMS results obtained from the multiple administration of DHEA to subject B twice daily are shown in Figure 3. Daytime administrations of DHEA were at approximately 7:00 am, and those at night were at approximately 7:00 pm. The usefulness of 11-ketoEt as an endogenous reference compound was confirmed by the observation that its δ^{13}C value remained essentially unchanged throughout the study. At the initial administration, δ^{13}C Et and δ^{13}C A were –24.5‰ and –23.2‰, respectively. Following multiple DHEA administrations, values of δ^{13}C Et and δ^{13}C A were observed between –27.8‰ and –34.6‰, with the minimum δ^{13}C Et value of –34.6‰ at 144 h and the minimum δ^{13}C A value of –33.0‰ at 129 h. These results displayed δ^{13}C values of Et and A that were ^{13}C depleted (up to 2.5‰ and 3.0‰, respectively) in relation to those observed after the single DHEA administration to subject A. Unexpectedly, however, minimum δ^{13}C Et and δ^{13}C A values were also ^{13}C depleted (up to 3.3‰ and 1.7‰, respectively) compared with the DHEA used for the administration (δ^{13}C = –31.3‰).

The effect of multiple DHEA administration on **δ^{13}C Et–δ^{13}C A** is shown in Figure 4. Between the second administration at t =

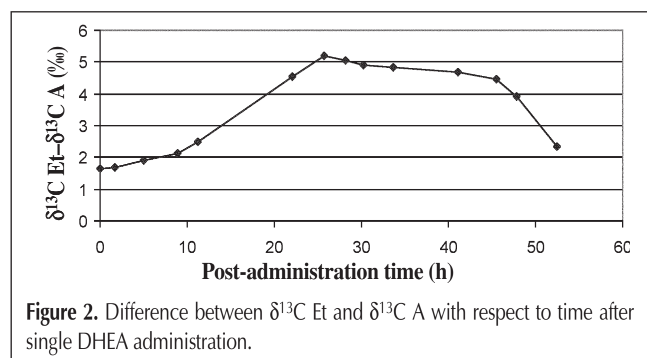

Figure 2. Difference between δ^{13}C Et and δ^{13}C A with respect to time after single DHEA administration.

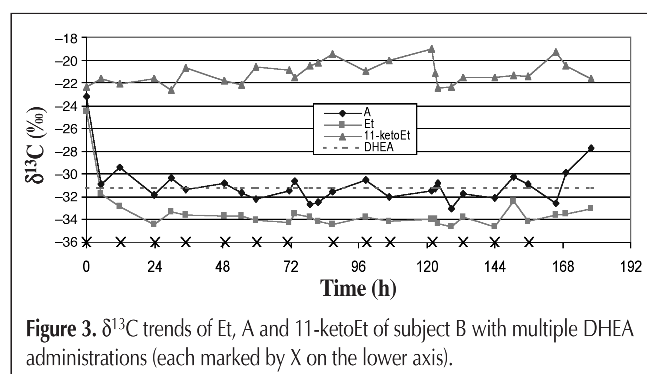

Figure 3. δ^{13}C trends of Et, A and 11-ketoEt of subject B with multiple DHEA administrations (each marked by X on the lower axis).

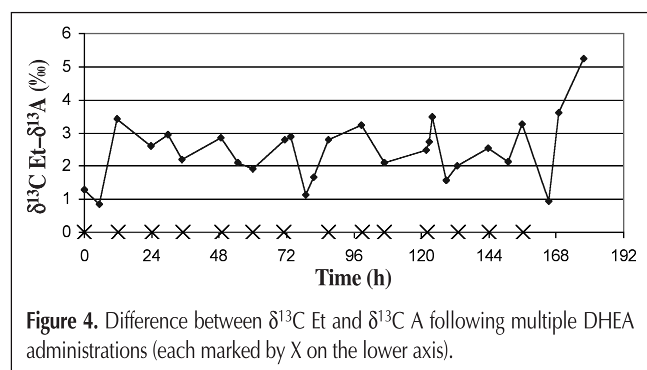

Figure 4. Difference between δ^{13}C Et and δ^{13}C A following multiple DHEA administrations (each marked by X on the lower axis).

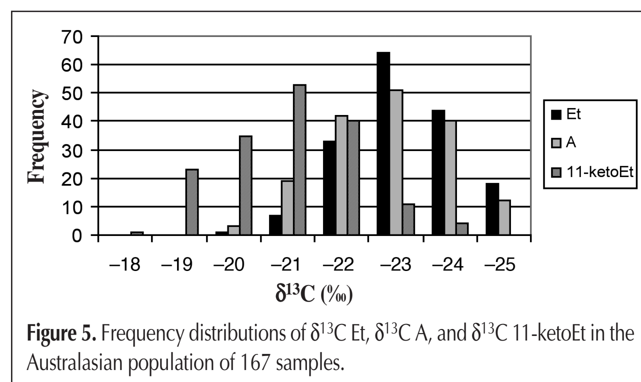

Figure 5. Frequency distributions of δ^{13}C Et, δ^{13}C A, and δ^{13}C 11-ketoEt in the Australasian population of 167 samples.

Table II. Statistics Extracted From the Frequency Distributions of δ^{13}C Et, δ^{13}C A, and δ^{13}C 11-ketoEt in the Australasian Reference Population of 167 Samples

	δ^{13}C (‰)				
	Et	A	11-ketoEt	Et – A	Avg. (Et,A) – 11-ketoEt
Mean	–23.2	–22.9	–20.9	0.3	2.0
Median	–23.2	–22.9	–20.9	0.3	2.1
SD	1.04	1.12	1.24	1.14	0.93
Maximum	–20.0	–19.6	–18.1	2.9	3.9
Minimum	–25.8	–25.1	–24.5	–2.8	–0.9

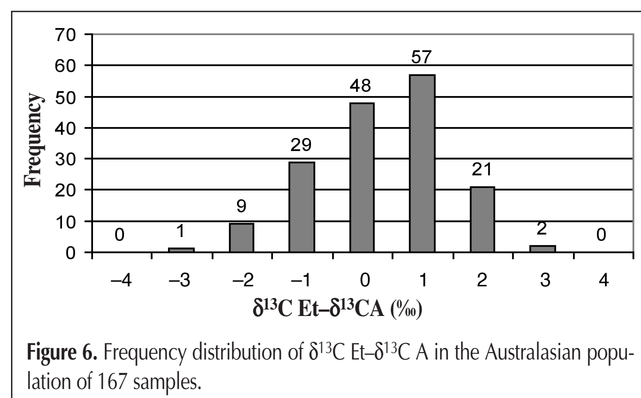

Figure 6. Frequency distribution of δ^{13}C Et–δ^{13}C A in the Australasian population of 167 samples.

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12 h and the final administration at t = 156 h, **δ^{13}C Et–δ^{13}C A** had an average value of 2.5‰ compared with the initial value of 1.3‰. After the final DHEA administration, **δ^{13}C Et–δ^{13}C A** increased to a value of 5.3‰ at t = 178 h, a 4.0‰ increase from the initial value. This occurred at 22 h post-administration, therefore, showing a similar time-dependent increase of **δ^{13}C Et–δ^{13}C A** as observed in the single administration study to a separate subject. Unfortunately, the post-administration urine collection period was too short in the multiple administration study to properly verify the trend observed in Figure 2.

Frequency distributions of δ^{13}C Et and δ^{13}C A representing the reference data set collected from 167 samples analyzed by GC–C–IRMS are shown in Figure 5. δ^{13}C Et values displayed a slightly right-skewed distribution around the –23.2‰ mean, and δ^{13}C A values were observed to have a relatively normal distribution around a mean value of –22.9‰ (Table II). There were no samples in the δ^{13}C distributions of Et and A with values more ^{13}C depleted than –25.8‰ and –25.1‰, respectively. Variation in δ^{13}C Et, δ^{13}C A, and δ^{13}C 11-ketoEt values with standard deviations of 1.04‰, 1.12‰, and 1.24‰, respectively, is presumed to be the result of experimental variation and dietary effects.

The frequency distribution of **δ^{13}C Et–δ^{13}C A** in the reference population demonstrates a small natural preference for ^{13}C depletion in the formation of Et relative to A (Figure 6). This does not appear to be evident when considering only the mean and median **δ^{13}C Et–δ^{13}C A** value of 0.3‰ and the equidistant range of approximately zero. Detailed analysis of the **δ^{13}C Et–δ^{13}C A** distribution however, reveals that more than double the number of samples (48%) were found to contain Et that was ^{13}C depleted in relation to A (δ^{13}C Et < δ^{13}C A), when compared to the 23% of samples that contained A that was ^{13}C depleted relative to Et (δ^{13}C A < δ^{13}C Et). There was no preferential fractionation (i.e., δ^{13}C Et = δ^{13}C A) in 29% of samples.

Discussion

The single administration study provided evidence of isotopic fractionation with DHEA giving metabolic discrimination of the isotopes of Et in preference to A resulting in a 3.5 ‰ increase of **δ^{13}C Et–δ^{13}C A**. Fractionation resulting in Et being ^{13}C depleted in relation to A has been described by Flenker et al. (31) as being the result of kinetic isotope effects arising from reduction of the double bond between C-4 and C-5 in Δ^4-steroids. In theory, this step would present an ideal opportunity for fractionation of carbon atoms because it is a rate-limiting branchpoint of substrate flux where two sets of isomers (5α- and 5β-) are formed that involve non-quantitative conversion (31,32). Isotopic fractionation is thought to be different in hepatic 5α- and 5β- reduction where steroid precursors are distributed into different cell compartments: the endoplasmic reticulum and cytoplasm, containing 5α- and 5β-reductase, respectively (31). This assumes that most of the Et and A comes directly from DHEA via reduction of the $C_{5,6}$ double bond, and reactions at C-3 and C-17 are minor pathways.

This study involving Δ^5-steroids supports that of Flenker et al. involving Δ^4-steroids. It remains unclear, whether fractionation occurred during $C_{4,5}$ reduction of androstenedione and testosterone produced from DHEA by biochemical transformations at C-3 and C-17, or during $C_{5,6}$ reduction of DHEA itself (or both). The concentration of substrate may also have a significant effect on flux in metabolic pathways, thereby contributing to isotope fractionation. This would influence the pharmacokinetics of DHEA as demonstrated by previous studies showing metabolic overload giving rise to abnormally high production of Et and A (4–6,8,9). The prolonged ^{13}C depletion of Et indicated less dilution from endogenous metabolic sources than was expected, thereby providing evidence of a feedback mechanism reducing natural Et production. Direct reduction appears to be the most likely contribution to isotopic changes because it can be noted that the difference between δ^{13}C Et and δ^{13}C A was not observed in studies involving administration of androstenedione (27) and testosterone (22). The ^{13}C depletion of carbon isotopes in Et relative to A may provide a specific marker of DHEA administration in athletes post-administration.

The multiple oral administration of DHEA (100 mg) to subject B provided δ^{13}C values that were more likely to be representative of the dosing regime of athletes using DHEA. In order to gain the perceived benefits from DHEA administration (1), an athlete would most likely take large doses over a prolonged period of time. The fluctuating trend of **δ^{13}C Et–δ^{13}C A** following repeated administrations of DHEA was further evidence of changing ^{13}C fractionation patterns resulting from the metabolism of excess DHEA to form Et and A. Analysis of the data from this study also raises the important question of how the apparent in vivo ^{13}C depletion of steroid metabolites in relation to their precursor is possible. No reported biochemical mechanism describing induced ^{13}C depletion was found in the literature, thus, further work is required to establish its origin. One possible mode of investigation would be the adoption of site specific natural isotopic fractionation– ^{13}C NMR that has been successfully used in fatty acid metabolism research (33). This methodology may enable quantitative determination of ^{13}C depletion that occurs at particular carbon sites (C-5 for instance) within the steroid molecule following administration of synthetic analogues of endogenous steroids.

The δ^{13}C value of 11-ketoEt may be incorporated into analysis criteria by comparison with the δ^{13}C values of Et and A to determine whether administration of DHEA has occurred. It has been proposed that a calculated difference of δ^{13}C 11-ketoEt from the averaged value of δ^{13}C Et and δ^{13}C A greater than 4.0‰ is indicative of illegal administration (26). The results from the multiple administration of DHEA to subject B show this averaged difference to be greater than the proposed value of 4.0‰ throughout the study. The significant effect of DHEA on the value of this averaged difference was noted with the change from 1.5‰ at the time of the initial administration to a maximum of 13.8‰ at 166 h.

The reference population gives an indication of the expected variation of δ^{13}C Et, δ^{13}C A, and δ^{13}C 11-ketoEt within a drug-free sample population of athletes from Australia and New Zealand. Approximations at a 99.7% confidence level (i.e., mean plus three standard deviations) imply that the minimum δ^{13}C Et and δ^{13}C A values from both groups were –26.3‰. These results are in support of the proposed δ^{13}C = –27.0‰ (26) to provide an effective value for doping control laboratories to confirm DHEA administration. Comparison of this absolute δ^{13}C value with the multiple

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administrations of DHEA to subject B shows that all samples after administration for the collection period would be found to be positive when applied to both δ^{13}C Et and δ^{13}C A values. The single DHEA administration to subject A shows that a post-administration detection period of 48 hours would result from a –27.0‰ criterion applied to δ^{13}C Et, although it would be much shorter (11 h) if applied δ^{13}C A. These changes are considerably longer than effects observed on metabolite concentrations as a means of detection of the parent substance (5,9).

Metabolic fractionation is illustrated (Figure 5) showing the δ^{13}C reference distribution of 11-ketoEt, the endogenous marker that is produced in a separate biosynthetic pathway to the androgens, to be ^{13}C-enriched relative to the δ^{13}C reference distributions of Et and A in the Australasian population. This is further highlighted in Figure 7, showing 99% of the population as having positive averaged differences. A consequence of this work finding larger-than-expected natural differences between the δ^{13}C values of Et and A relative to 11-ketoEt is that the proposed criteria (26) may need to be revised. Even though there were no samples in the Australasian reference population that had averaged differences greater than 4.0‰ (Figure 7), a 99.7% confidence level applied to this distribution revealed that the maximum expected value from athletes not doping may be as large as 4.8‰. The criteria would however, be supported by a 95% confidence level (i.e., mean plus two standard deviations) applied to this distribution. In relation to detecting DHEA abuse, averaged difference values remained far greater than 4.8‰ throughout the multiple administration study. In fact, the smallest difference observed was 8.7‰, which was recorded 22 h after the final administration.

The differences observed between δ^{13}C Et and δ^{13}C A produced from completely endogenous sources and those observed following DHEA administration can also be compared to propose analysis criteria. Statistical interpretation of the reference population suggests that a **δ^{13}C Et–δ^{13}C A** value greater than 3.7‰ could be used to indicate DHEA administration. This would also represent a 48-h post-administration detection period based on the single administration of DHEA to subject A.

Conclusion

This study demonstrated the use of GC–C–IRMS analysis of urinary steroid metabolites to provide more information on metabolic pathways following illegal steroid administration. Alteration in δ^{13}C values of A and Et in relation to each other showed a preference for prolonged formation of ^{13}C depleted Et from administered DHEA. Metabolism of orally administered DHEA, with respect to the proportions forming Et and A, appears to be related to pharmacokinetic mechanisms resulting from excess DHEA in the body. Furthermore, it displays significant differences to that of endogenous DHEA. The complex nature of steroid metabolism in the body was also highlighted following multiple administrations of DHEA, showing in vivo ^{13}C depletion of androgen metabolites. Comparison of reference values obtained from a normal population with those obtained from administration studies allowed several criteria to be proposed for GC–C–IRMS analysis in doping control. This includes the absolute values of Et and A, use of an endogenous marker representing reference values that is a necessary component of any doping control process, and changes in the values of metabolites reflecting kinetic isotopic fractionation (**δ^{13}C Et–δ^{13}C A**). It is envisaged that, in the future, such measurements as a combinatorial factor will provide protocols that are valuable for confirming the administration of synthetic analogues of particular endogenous steroids.

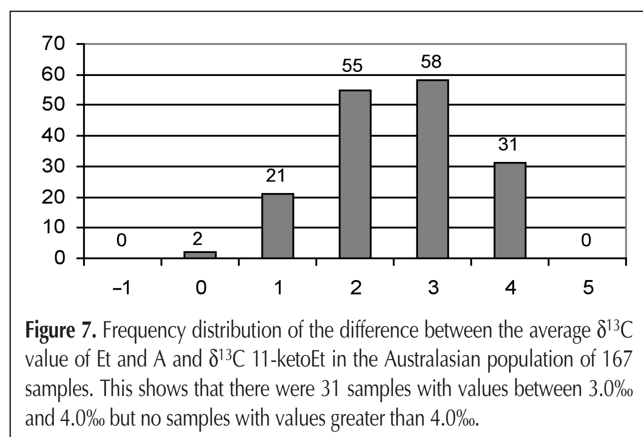

Figure 7. Frequency distribution of the difference between the average δ^{13}C value of Et and A and δ^{13}C 11-ketoEt in the Australasian population of 167 samples. This shows that there were 31 samples with values between 3.0‰ and 4.0‰ but no samples with values greater than 4.0‰.

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