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Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols

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A gas chromatographic combustion isotope ratio mass spectrometric (GC/C/IRMS) method was used for studying the incorporation of exogenous testosterone enanthate into excreted urinary 5α- and 5β-androstane-3α,17β-diols. A multistep but straightforward work-up procedure produced a simple GC chromatogram of urinary steroid acetates composed principally of two androstanediols and pregnanediol. It is anticipated that such a method may form the basis of a doping control test for testosterone that could be used as a primary method during major sporting events or alternatively as a verification technique. Urine samples from five individuals were collected before and after administration of testosterone enanthate (250 mg). The $\delta^{13}C$ °/00 value of androstanediols was around −26 to −28 during the baseline period and decreased to about −29 to −30 in the days following synthetic testosterone administration. One of the other major steroids in the chromatogram, pregnanediol, was utilized as the "internal standard," because its $\delta^{13}C$°/00 values did not markedly change following testosterone administration, remaining at −25 to −27. In all subjects studied, the $\delta^{13}C$°/00 values for androstanediols were reduced sufficiently over 8 days to confirm administration of synthetic testosterone. Although steroids isolated from urine of normal individuals from 12 different countries gave values between −24 and −28, this seemed not to be related to nationality or region. The most likely variable is the proportion of plants with low and high carbon 13 content in the diet. This variable is likely to be more affected by individual food preferences than broad ethnic food divisions. In this paper, we propose a ratio of $\delta^{13}C$°/00 for androstanediols to pregnanediol as a useful discriminant of testosterone misuse, a value above 1.1:1.0 being indicative of such misuse. The work-up procedure was designed for batch analysis and to use only simple techniques, rather than employ further instrumentation, such as high-performance liquid chromatography (HPLC), in purifying steroids for GC/C/IRMS. (Steroids **62**:379–387, 1997) *© 1997 by Elsevier Science Inc.*

Keywords: isotope ratio mass spectrometry (IRMS); doping control; testosterone

Introduction

The increasing need in sport for proving testosterone misuse requires new methodologies. For many years, a testosterone/epitestosterone (T/E) excretion ratio determined by gas chromatography–mass spectroscopy (GC–MS) of greater than 6:1 has been used as the hallmark for confirmation of drug administration,[1] but this method is fallible. For one thing, occasional drug-free individuals give a ratio >6, and a high ratio can also be adjusted downward by simultaneous administration of epitestosterone. We have found that in eight Chinese subjects given 250 mg testosterone enanthate, only three gave T/E values >6 on more than 1 day, demonstrating a high rate of false negatives, at least in this racial group.

In 1990, Southan and co-workers[2] used isotope ratio mass spectrometry (IRMS) to show that synthetic testosterone had a different ^{13}C content than endogenous hormone. This is a reflection of the origin of the materials, because all testosterone, both endogenous and synthetic, is ultimately of plant origin. Gonadal testosterone is made from precursor molecules derived from a wide variety of vegetable mate-

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rials eaten by humans or by the animals humans eat. Individual plants discriminate to different degrees against $^{13}CO_2$, and particular species are known to have high or low levels of ^{13}C in their biomolecules.[3] In human bodies, the ^{13}C content, therefore, reflects an average of the ^{13}C content of all the plant material eaten by humans and our animal protein providers. Synthetic testosterone, in contrast, is generally made from a single plant species, mostly soy, so the ^{13}C content has a defined value reflecting the relatively low ^{13}C content of this plant. Thus, a significant difference in ^{13}C content between gonadal and soy testosterone could provide the basis for developing a definitive test for hormone misuse. Differences in carbon isotope ratios referred to by the symbol δ, defined as the difference in isotope ratio between the sample and an international carbonate standard "PDB." Although this is the accepted standard, for common usage a calibrated international standard of CO_2 is used. The values reported for δ carbon isotope ratios are

$$\delta^{13}C^{0}/_{00} = \left(\frac{\text{Ratio sample} - \text{Ratio PDB}}{\text{Ratio PDP}}\right) \times 1000$$

Becchi and coinvestigators have published pioneering studies on development of a method employing IRMS for determination of carbon isotope ratio of testosterone extracted from urine.[4,5] They demonstrated that, providing sufficient urine was available, the endogenous or exogenous origin of testosterone could be readily determined. A major remaining problem demonstrated by these studies relates to sensitivity of the analysis, because the quantity of urine collected from athletes is relatively small (about 75 mL), and this is divided into two, a primary (A) and a secondary sample (B). On each sample, nonsteroidal drug metabolites must be analyzed, as well as anabolic steroid screening and measurement of the T/E ratio.

We have attempted to improve the methodology to allow more sensitive analysis. It was decided to forgo any attempt to analyze testosterone itself and to concentrate on analysis of its metabolites 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD). Our overall objective was to easily produce a single sample for analysis containing a few defined steroids to include the androstanediols and steroids we call "endogenous reference compounds" (ERCs). An ERC, in this instance, is a steroid whose carbon isotope ratio could not be altered through administration of exogenous testosterone. Aguilera, Becchi, and co-workers in their most recent publication use cholesterol and 5-androstene-3β,17β-diol as ERC.[5] To achieve our overall objective, we developed simple methodology adaptable to batch analysis, which required no liquid chromatographic (HPLC) instrumentation. Using this methodology, we determined the $\delta^{13}C^{0}/00$ of the androstanediols present in urine following administration of testosterone enanthate to five volunteers. This communication presents the results of this study.

Experimental

Materials

Testosterone enanthate, Testoviron depot® was obtained from Schering, Japan. Reference steroids were obtained from Sigma (St. Louis, Missouri, USA), which was also the supplier of Girard reagent T (carboxymethyl, trimethyl ammonium chloride hydrazide) and sodium bismuthate. Sephadex LH 20 was a product of Pharmacia AB and Sep-pak® cartridges, a product of Waters Corp. (Milford, Massachusetts, USA). β-glucuronidase/aryl sulfatase was obtained from Sigma (Type H1) and Boehringer Mannheim (Mannheim, Germany). Solvents were of analytical grade.

Individuals studied

Eight Chinese male subjects aged 19–22 were studied, although GC/C/IRMS analysis was only conducted on five. Permission for undertaking these experiments was obtained from the Chinese National Research Institute of Sports Medicine, and consent was obtained from the participants. Spot morning urine samples were collected for 2 days prior to an intramuscular injection of 250 mg testosterone enanthate. Two urine samples (0–8 h and 8–24 h) were collected for the first 4 days after administration, although only aliquots of the early morning sample were subject to analysis. Morning spot urine samples were collected on the 5–9th days after administration and on days 11, 13, and 15.

Determination of testosterone/epitestosterone ratio (T/E)

Urinary testosterone and epitestosterone were quantified in all the samples using an adaptation of the method of Donike et al.[6] These measurements were carried out by one of us (YL) in China at the National Institute of Sports Medicine. T/E ratios were then determined.

Preparation of steroid extract for GC/C/IRMS

A flowsheet summarizing the methodology is shown in Figure 1. Urine (typically 25 mL) was extracted by Sep-pak® cartridge according to the method of Shackleton and Whitney.[7] Once dried, the extract was dissolved in 3 mL 0.1 M acetate buffer pH 5 and *Helix pomatia*-derived β-glucuronidase/sulfatase (12 mg Sigma

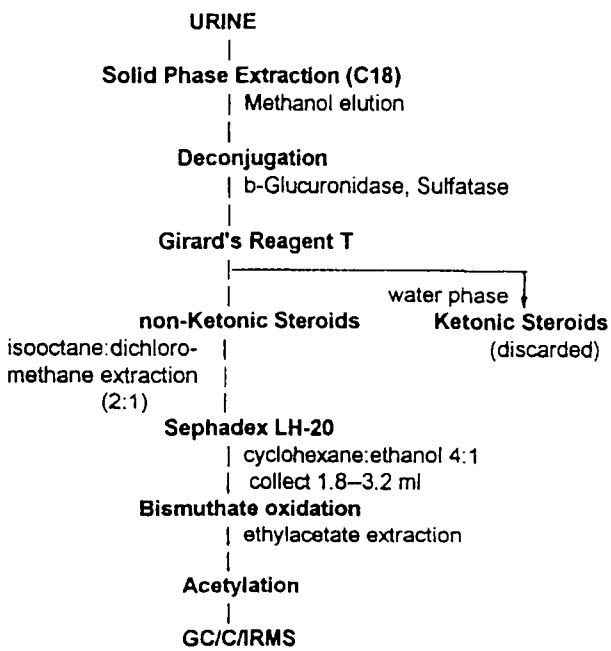

Figure 1 Flow sheet of the urinary extraction method.

type H1 powder, 100 μL Boehringer liquid enzyme) was added. Hydrolysis was allowed to proceed for 3 h at 55°C. A Girard separation was carried out to separate carbonyl-containing steroids (ketonic) from noncarbonyl-containing (nonketonic) steroids.[8,9] To the hydrolyzed urine mixture, 2 mL glacial acetic acid and 100 mg Girard reagent T was added. The solutions were placed in an oven at 100°C for 30 min. The nonketonic steroids were extracted by 2 × 5 ml isooctane: dichloromethane 2:1 (v/v), and the solvent was dried. Small columns of 0.5 Sephadex LH-20 were prepared in Pasteur pipettes, the Sephadex being allowed to swell in the cyclohexane: ethanol (4:1) solvent system before preparation.[10] The steroid extract dissolved in 100 μL of the same solvent mixture was added to these columns. Solvent eluting between 1.8 and 3.2 mL was collected and dried. Acetic acid (0.1 mL), water (0.1 mL), and 5 mg sodium bismuthate were added.[9] Oxidation was allowed to proceed for 2 h, and after neutralization (0.5 mL of 0.5 M acetate buffer), the mixture was extracted with 4 mL ethyl acetate. After drying, steroid acetates were prepared overnight with 50 μL acetic anhydride and 50 μL pyridine. The acetates were analyzed by GC/C/IRMS.

Gas chromatography combustion isotope mass spectrometry (GC/C/IRMS)

A schematic representation of the GC/C/IRMS instrumentation is illustrated in Figure 2. The acetylated steroid samples were kept refrigerated until analysis. Cyclohexane (20 μL; 99.9% pure from Sigma Chemicals 27-0625-8) was added to each vial, and one-tenth (2 μL) of each sample was injected splitless onto a J&W 30 m DB17 capillary column housed in a Fisons 8000 series GC. The injector was kept at 220°C. The temperature program was as follows: starting temperature 50°C (1 min), followed by rapid temperature increase (25° min) to 300°C, where it was held for 15 min.

The separated components were heart-split into the combustion furnace filled with copper oxide wires (Elemental Microanalysis Limited, UK) held at 850°C. The combustion gases were passed through a nafion membrane water removal trap, and the remaining CO_2 was analyzed on a Micromass isochrom isotope mass spectrometer. The mass spectrometer consisted of an electron impact source running at 400 μA trap current, the ionized CO_2 gas, was focused by a magnet onto three Faraday collectors. The ions collected were those at masses 44, 45, and 46. The Micromass data system calculated the areas of the beams and subtracted any background; whereupon, calculation of the ^{13}C δ values for the successive peaks were carried out.

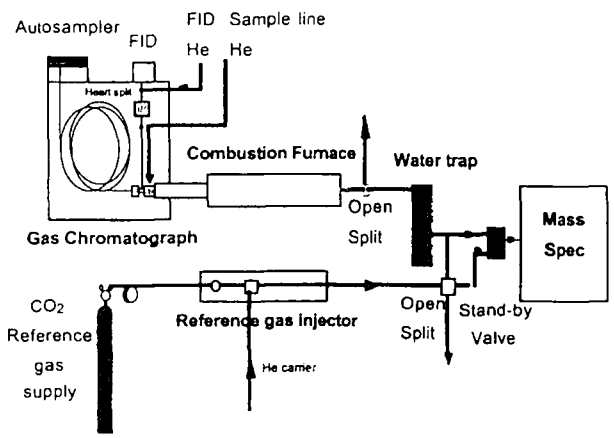

Figure 2 Schematic of the Micromass GC/C/IRMS instrument.

Results and discussion

Evaluation of gas chromatographic columns

The objective of the study was to measure $\delta^{13}C^0/00$ for urinary androstanediols formed as metabolites of testosterone. In our initial studies (Subject 1), we undertook chromatography on DB1 capillary columns but did not get resolution of the two diacetylated epimers (Figure 3A). Later employment of a DB17 column (Figure 3B) allowed separation of the epimers as well as the ERC, pregnanediol diacetate, and pregnanetriol diacetate (Figure 3B).

Confirmation of identity of steroids in extracts

Prior to sending the first samples for GC/C/IRMS analyses in England, the identities of the principal components of the chromatogram were confirmed by GC/MS. This was carried out on a Hewlett-Packard 5970 instrument housing a 15 meter DB1 capillary column. The peaks chosen for GC/C/IRMS analyses had retention times and electron impact mass spectra identical to those of 5α- and 5β-androstanediol diacetate and pregnanediol diacetate. Reference steroids for these compounds were also analyzed on the GC/C/IRMS instrument using both DB1 and DB17 columns, and these gave identical retention times to the urinary steroids. Pregnanetriol could also be analyzed by GC/C/IRMS.

Achievement of work-up procedure objective

The method developed and utilized had the following qualities. First, the Girard separation almost completely removed carbonyl containing steroids from the hydrolyzed extracts, which probably represent 75% of urinary steroids. Exceptions may be the 11-carbonyl containing steroids that probably do not react because of the hindered nature of that functional group. Second, a crude micro Sephadex LH-20 column separation effectively produced a fraction containing steroids with two and three functional groups. Third, sodium bismuthate oxidation was designed into the procedure as a means of removing remaining long-retention time pregnane metabolites, thus allowing shorter periods between injection. Many of the quantitatively more important metabolites are converted into 17-oxygenated C_{19} steroids by the procedure. Fourth, acetylation provided steroids with good gas chromatographic properties that were readily separated. Fifth, despite the complexity of the steroid fraction of urine, the final chromatogram was simple and composed of only a few peaks for which $\delta^{13}C^0/00$ could be determined with accuracy.

$\delta^{13}C^0/00$ value of the synthetic testosterone

Testosterone acetate prepared from the Japanese testosterone enanthate used for injection in these studies gave a $^{13}C^0/00$ value of −30.41, a value close to the lowest value obtained for androstanediol diacetate measurements obtained in the subjects studied following testosterone administration.

We also analyzed five other current products and one synthetic sample made more than 40 years ago. The following results were obtained: testosterone of Chinese manufacture −30.40; U.S. manufacture −30.38; two Czech products,

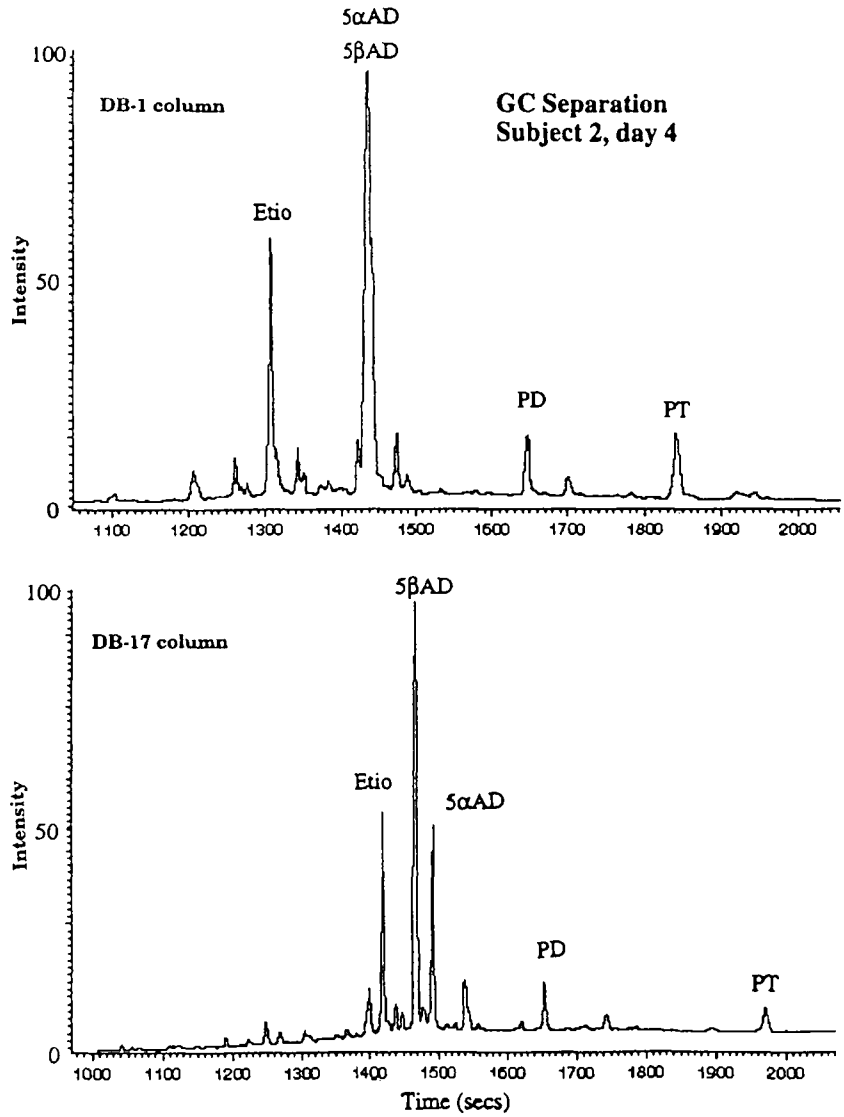

Figure 3 Gas chromatographic separation (on GC/C/IRMS instrument) of acetylated urinary steroid fraction. This sample was initially separated on a DB1 column, which failed to resolve the 5α- and 5β-androstanediols (upper panel). Later employment of a DB17 column permitted individual measurement of the epimers (lower panel). Both columns also resolved etiocholanolone, produced by bismuthate oxidation of pregnanetriol, pregnanediol, and nonoxidized pregnanetriol.

−29.28 and −29.15, respectively; and one Russian −30.22. The U.S. sample of more than 40 years old gave a value of −33.18, probably reflecting the different plant origin of synthetic steroids made long ago. We believe that testosterone is currently made mostly from soy by-products, although originally sterol constituents of the Mexican yam were the primary precursors.

Results for individual subjects

With the exception of Subject 1, a DB17 capillary column was utilized that allowed separate analysis of 5α- and 5-β androstanediols. Graphs illustrating the $\delta^{13}C^{0}/00$ results are shown in Figure 4A–F. In all cases, the $\delta^{13}C^{0}/00$ values for the androstanediols fell significantly following testosterone administration.

Figure 4 illustrates two other features. One is the adequacy of making single measurements of $\delta^{13}C^{0}/00$ for each sample. Panel B and C show results obtained by plotting the average of duplicate measurements (B) and of the *first* measurement of the duplicate pair (C). Essentially these graphs are identical, suggesting duplicate GC/C/IRMS analyses of the same preparative extract are unnecessary. The second feature relates to the trend of slightly increasing δ values for pregnanediol during the study period of Subject 3 (Figure 4D). Because this is not caused by random irreproducibility we proposed that the individual changed his diet substantially to foodstuffs with higher $\delta^{13}C^{0}/00$ values.

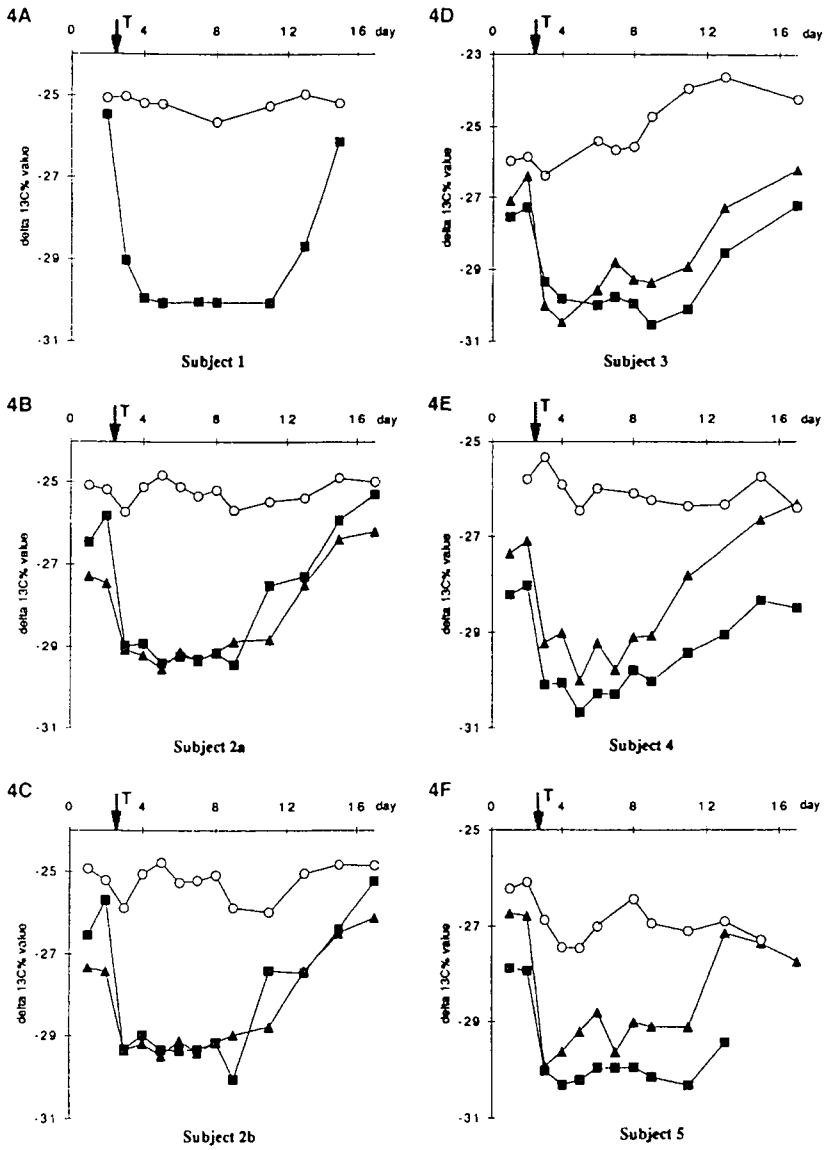

Figure 4 Values of $\delta^{13}C^{0}/00$ for all five subjects throughout the study period. The open circles represent pregnanediol, the closed rectangles 5βAD and closed triangles 5αAD. 4A shows the values obtained for Subject 1 in which only one baseline sample was analyzed, the testosterone being administered on the 2nd day of study. Both androstanediols were measured together on a DB1 column, 4B shows plots for Subject 2 obtained by averaging values for the duplicate runs, and 4C shows equivalent plots through only using the first analysis. 4C, 4D, and 4E are plots of Subjects 3, 4, and 5. For Subjects 2–5 the testosterone was administered after the second baseline sample (day 3 of study).

Designing a method for universal use demands final agreement of numerative values above and below which individuals are considered to have or to have not illicitly used a drug. In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstanediol $\delta^{13}C^{0}/00$ values less than −28.3 during the control period, and establishing a conservative cut-off value of −29.0 would clearly pick up all drug users for about 7 days after administration. Additionally, we considered that measurement of analyte/ECR ratios could allow numerative values to be established that are to some degree independent of variables introduced through using the methodology in different laboratories with different instrumentation. In Figure 5A, we show the $\delta^{13}C^{0}/00$ ratio of combined androstanediols (average of 5α- and 5β-) to pregnanediol (PD) for the five individuals studied so far. All baseline ratios fell below 1.08, and from these data, we would suggest that a ratio of greater than 1.1 could be used to confirm testosterone administration. When we averaged the data for the five individuals, we found that the AD/PD ratio value of 1.1 was exceeded for 11 days (Figure 5B). Because a combined androstanediol measurement is carried out in the Aguilera et al. studies,[5] comparable data could be prepared from their measurements, although in their case, cholesterol would be the ERC used for ratio determination.

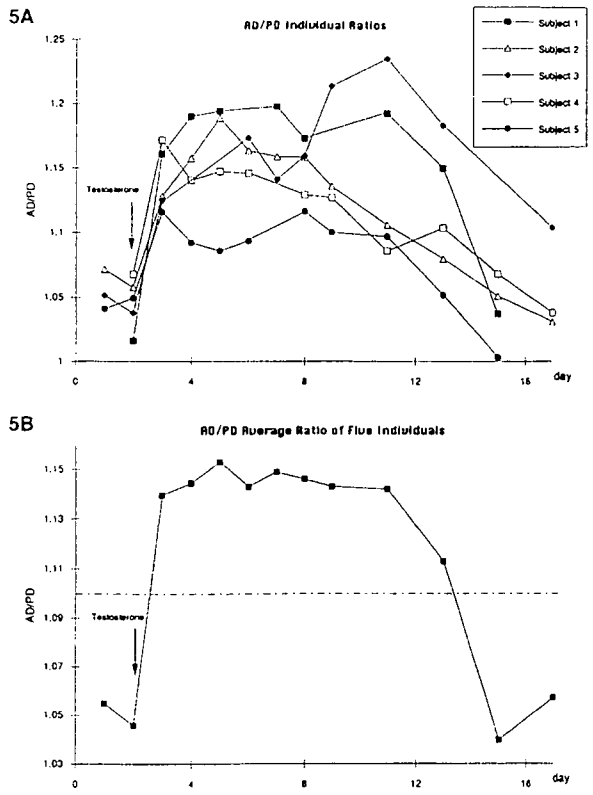

Figure 5 Androstanediol/pregnanediol ratio. A) ratios of $\delta^{13}C^0/00$ for the androstanediols (mean of 5α and β) to pregnanediol for the five subjects; B) average of the ratio for the five subjects for each day; the dotted line represents the proposed discriminant value (1.10) for confirmation of testosterone administration.

Reproducibility of isotope measurements and accuracy

For the five subjects studied, ideally 26 or more isotope measurements were made for each steroid; i.e., 13 duplicate analyses. A few samples were lost or contaminated, and occasionally only single measurements were made on a sample. As a representative analysis, Table 1 gives all of the measurements for Subject 2. Using these data, there are two features of accuracy and reproducibility that can be quantified. One is the variation in δ measurement between duplicate analyses of the same sample and the other the overall variation occurring during analyses of a complete series from one individual. For duplicates, the reproducibility was excellent and did not differ significantly between the five subjects studied. Considering the data shown in Table 1, averages and standard deviations were measured for each duplicate for the two androstanediol and pregnanediol diacetates that give values in the $\delta^{13}C^0/00$ −23 to −30 range. Means were then determined of the standard deviations. For pregnanediol, the mean standard deviation between duplicates was ± 0.21 with a range of ± 0.02 to ± 0.71. Comparable results were obtained for the androstanediols (± 0.11 for 5α-androstanediol and ±0.25 for 5β-androstanediol). These results were considered to represent excellent reproducibility and (as previously noted) suggested that the methodology could be used with *single* determinations by GC/C/IRMS of $\delta^{13}C^0/00$ for each steroid. This was illustrated graphically in Figure 4B and C, which showed both the $\delta^{13}C^0/00$ values for the average of duplicates and for the first analysis. The graphs were essentially identical.

Within a 17-day series of datapoints for duplicate assays for a single individual, the results show considerably less reproducibility—notice the peaks and valleys of the pregnanediol plots in Figure 4. In any physicochemical analyses, it is, of course, possible to have excellent reproducibility but compromised values. For accuracy and reproducibility assessment between a series of different samples, we use the δ measurement of the ERC pregnanediol. The administration of testosterone should in no way change the $\delta^{13}C^0/00$ value for pregnanediol that has to be produced from cholesterol via several intermediates. In a perfect analysis, the measured ^{13}C content of pregnanediol would not be expected to change during the 17-day study period unless, of course, the individual alters his or her diet markedly during the study period. Even with dietary modification, the ^{13}C content of body molecular constituents would be expected to change only slowly.

The highest and lowest duplicate average $\delta^{13}C^0/00$ values for pregnanediol for the five subjects differed by −0.92 to −2.96. As previously mentioned, with the exception of Subject 2, the average duplicate values seemed to rise and fall randomly during the 17-day study period. What

Table 1 $\delta^{13}C^0/_{00}$ values obtained from duplicate GC/C/IRMS analyses for Subject 2 before and following testosterone administration

	Subject 2		
	5βAD	5αAD	PD
Day 1	−26.55	−27.33	−24.93
	−26.15	−27.20	−25.34
	−26.66	−27.28	−25.15
Day 2	−25.68	−27.42	−25.20
	−25.95	−27.49	−25.17
Day 3[a]	−29.33	−29.36	−25.88
	−28.63	−28.81	−25.61
Day 4	−28.99	−29.19	−25.06
	−28.88	−29.24	−25.21
Day 5	−29.34	−29.49	−24.78
	−29.48	−29.61	−24.87
Day 6	−29.37	−29.11	−25.26
	−29.12	−29.16	−24.97
Day 7	−29.33	−29.42	−25.22
	−29.31	−29.28	−25.46
Day 8	−29.16	−29.16	−25.09
	−29.18	−29.14	−25.30
Day 9	−30.06	−28.98	−25.88
	−28.83	−28.78	−25.52
Day 11	−27.41	−28.78	−25.99
	−27.63	−28.88	−24.99
Day 13	−27.47	−27.42	−25.05
	−27.12	−27.59	−25.72
Day 15	−26.39	−26.50	−24.84
	−25.50	−26.28	−24.96
Day 17	−25.24	−26.12	−24.86
	−25.36	−26.32	−25.13

Abbreviations: 5βAD, 5β-androstane-3α,17β-diol; 5αAD, 5α-androstane-3α, 17β-diol and PD, pregnanediol
[a]The first collection following testosterone administration.

is the source of this irreproducibility? Because we know that the reproducibility of $\delta^{13}C^0/00$ measurements of individual steroids in duplicate GC/C/IRMS runs is excellent, we must assume that the *measured* $\delta^{13}C^0/00$ values for individual chromatographic peaks are accurate. If these are accurate, then any difference in the value from the "true" value for the steroid determined must represent some minor contamination of the steroid peak by components with greater or less $\delta^{13}C^0/00$ values. We have proposed that the trend to increasing values for Subject 2 may be because of dietary changes toward foodstuffs with greater ^{13}C content.

These minor irreproducibilities, which typically give rise to a range of about −1.5 in the δ value for all datapoints during the study period, will not affect the outcome of a drug test, because in no cases do the lowest values breach the discriminant value for the positives.

Testosterone/epitestosterone ratios

Table 2 gives the T/E values for five individuals studied here (Subjects 1–5) and three other subjects whose steroids were not analyzed by the IRMS technique (Subjects 6–8). The data show that the maximal increase in the ratio for each of the individuals was between 12 and 68 times baseline level, although it was noticeable that only 4 of the 8 subjects gave T/E values greater than 6 and 1 of these only modestly exceeded it on one occasion (T/E 6.69). The T/E ratio of the five subjects studied here have been included in Figure 5C as compared to the $\delta^{13}C^0/00$ AD/PD ratio for the same individuals.

$\delta^{13}C^0/00$ steroid values for different nationalities

Figure 6 shows the $\delta^{13}C^0/00$ values for duplicate analyses of the androstanediols and PD from 15 individuals of 11 different nationalities in addition to the Chinese. In some cases, it was possible only to obtain values for the 5β androstanediols because of the low amounts of 5αAD present. The results demonstrate that there are differences among racially and regionally varied individuals with undetermined diet, but we must remember that part of this variation may be attributable to the analytical inaccuracies previously discussed. However, all values were greater than −28.2, which should be compared to the values of less than −29.2

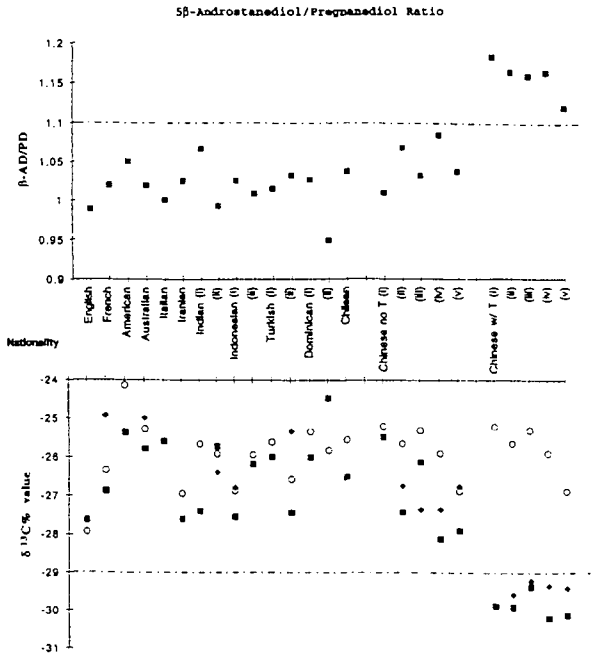

Figure 6 The lower panel shows the $\delta^{13}C^0/00$ values for the androstanediols and pregnanediols for individuals of different nationality, while the upper panel shows the 5βAD/PD ratio. The open circles represent PD, the closed diamonds 5αAD and closed rectangles 5βAD. The baseline values for the Chinese represent the mean of the two measurements, while the Chinese "testosterone" values represent the average of the 7 days after drug administration. Pregnanediol was below detection level in the Chilean urine, so pregnanetriol values were reported instead.

found in the subjects studied following testosterone administration. The mean value for the 5βAD/PD ratio was 1.02 ± 0.03 with a range of 0.95 to 1.07, which compares to the average value for the Chinese baseline samples of 1.05 ± 0.03 (range 1.01 to 1.08). The ratio values for the testosterone administered subjects were all >1.15 in the first days after injection. These data support the previous cut-off ratio value of 1.1:1.0 suggested on the basis of studying the Chinese subjects before and after testosterone administration.

There are notable features with regard to the ^{13}C content

Table 2 Urinary testosterone/epitestosterone ratios for the eight subjects

Study day	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subjec 7	Subject 8
1	0.26	1.28	4.69	0.10	0.12	0.17	0.08	0.11
2	0.33	1.48	5.26	0.11	0.11	0.22	0.06	0.11
3[a]	0.65	8.77	29.3	0.26	0.7	1	1.07	0.42
4	1.95	24.2	36.9	1.77	6.69	4.13	1.36	2.98
5	2.81	45.8	41.8	2.01	5.91	3.53	1.53	7.23
6	2.79	33.2	61.8	1.96	3.71	3.10	1.45	7.48
7	3.03	15.4	54.9	1.91	4.24	3.65	1.68	7.16
8	4.13	36.4	57.7	1.48	5.95	3.08	0.97	4.89
9	5.3	20.3	39.7	1.51	5.78	1.43	1.04	3.81
11	3.2	26.5	33.0	0.58	3.69	0.97	0.40	3.69
13	0.77	2.63	3.42	0.68	3.41	0.20	0.07	1.93
15	0.22	0.99	4.37	0.19	2.27	0.15	0.06	0.21
17	0.26	2.02	4.80	0.13	0.21	0.20	0.08	0.08

[a]Day of testosterone administration.

of steroids in urine from individuals of different countries. One is that there is a considerable spread in values but one that is unrelated to race and "typical regional diets," at least based on the small sample studied. The lowest and highest $\delta^{13}C^0/00$ among this group differed by 4 units (−24 to −28); however, both these sets of values came from Caucasians. The remaining samples from different nationalities and ethnic groups fell within this range, a particularly important finding with respect to the current study, because it suggests that the results obtained following testosterone administration are universally applicable.

Diet of individuals must have the greatest part to play in the establishment of an individual's $\delta^{13}C$ value. Because all food originates as plant life (including animal protein), it was interesting to research what is known of $\delta^{13}C^0/00$ values for dietary plants. Smith and Epstein[3] published the ^{13}C content of 104 plants, but relatively few were commonly used foodstuffs. A few were high $^{13}C/^{12}C$ plants (corn, sugar cane, grain, and sorghum) averaging around $\delta^{13}C^0/00$ −14, but most were categorized as a separate group of low $\delta^{13}C^0/00$ species having values between −23 and −30. These include dietary oil precursors (olive, sunflower, and castor) and various vegetables (wheat, grass, bamboo, peas, squash, radish, beets, and citrus fruit). Because typical worldwide diets of human and domestic animals use mostly low $\delta^{13}C^0/00$ plants, we would anticipate that $\delta^{13}C$ values for excreted steroids would fall in the −23 to −28 range, as found. The $\delta^{13}C^0/00$ value of an individual plant species does not vary significantly dependent on geographic location,[3] and variation only manifests itself in plants grown in urban areas where CO_2 from fossil fuels increases the ^{12}C content slightly. This clearly does not markedly affect agricultural foodstuffs. For the purposes of this assay technique, it is fortuitous that synthetic testosterone is obviously derived from a very low $\delta^{13}C^0/00$ plant species, otherwise no distinction would be possible between endogenous and exogenous compound.

Practicality of methodology for use in doping control

We compared our $\delta^{13}C^0/00$ values for androstanediol with those of Aguilera et al., who used similar methodologies but different instrumentation.[5] They studied individuals of different nationality and ethnicity than ours. For the purposes of this comparison, we averaged our values for 5α- and 5β-androstanediol, because their values were based on measurement of the combination. For baseline samples, we obtained an averaged $\delta^{13}C^0/00$ of −26.87, which agreed excellently with Aguilera's[5] value of −26.52. For androstanediols measured during testosterone administration, they obtained an average value of −32.44; whereas, our value (the mean of duplicates of the lowest values for each individual) was −30.21. Although our minimum value is not as low as that of Aguilera, it was still well below the baseline level, so it could be used for proving testosterone abuse. The difference in results between the laboratories could be attributed to their use of a testosterone with lower $\delta^{13}C^0/00$ (its origin was not reported) or unequal calibration of the different instruments used.

Our methodology is multistep, albeit built around low-technology procedures. Within the technique, there is only one chromatographic separation, and that was designed to produce a broad fraction rather than the isolation of individual steroids. The key to the method is separate isolation of nonketonic steroids, a procedure that in one extraction step removes 75% of unwanted steroids from urine. A second classical procedure, the oxidative removal of side chains from 17-hydroxypregnane steroids removes many of the remaining complex steroids from the mixture, resulting in a cleaner chromatogram, although in the future, we may simplify the method by discontinuing this procedure if satisfactory results can be obtained. Typically, without using any automation in wet chemistry procedures, a batch of samples requires 1 day of technician time to extract and derivatize. The mass spectrometry was automated, and latterly 20 minutes were required for each run. The maximum number of samples that could be analyzed per 24 h was, therefore, 72.

The length of time that the 250-mg testosterone enanthate dose (equivalent to 180-mg free testosterone) clearly manifested itself in decreased $\delta^{13}C^0/00$ value was 8–10 days. This compares well with other parameters studied on this cohort. For example, plasma testosterone was measured, and from being grossly elevated in the first few days after administration, it returned to normal by the 8th day, although the steroid itself may well be largely of exogenous origin on this occasion.[11] In addition, we report the T/E ratios that are particularly interesting, because essentially only three out of the eight individuals achieve values >6 on more than 1 day, showing a high rate of false negatives. This may not be a universal finding and may well be related to the race (Asian) of the studied individuals. However, it once again emphasizes the importance of developing alternate methodology for proving testosterone misuse. The elevated T/E ratios (although most are <6) fall significantly by the 11th day of the study in all subjects.

We believe that our method based on androstanediol measurement has the needed sensitivity for use in doping control. Although we analyzed all samples in duplicate and plotted the graphs from their averages, individual analyses were equally informative (Figure 4B, C). We would have no hesitation in confirming testosterone administration based on a single measurement. We have been able to get up to five analyses of a single sample using splitless gas chromatographic injection. This represents an equivalent of 5 mL urine for each analysis, which meets sample availability in doping control. Use of a solid injection device for GC, such as glass minivials in a carousel,[12] could improve this sensitivity greatly, because the total sample could be volatized and passed into the GC/IRMS instrument. Testing of a urine volume of 2 mL by our procedure and the Micromass IRMS instrument would be realistic. Our method has two obvious advantages over that of Aguilera et al.[5] One is that the two androstanediols are determined separately, so even if one peak was too small or showed evidence of contamination, the second peak could give the required value. A second advantage is that all analytes are measured in the same chromatogram; whereas, in Aguilera et al.'s study, at least two separate GC/C/IRMS analyses are required to measure the isotope ratio in the androstanediols and their

ERC cholesterol acetate. However, in fairness, they did have a more difficult assay through the inclusion of testosterone itself among the analytes.

Manipulating the result of a drug test to give a "negative" result following testosterone administration would be difficult. Although the AD/PD ratio could be normalized by taking commercial pregnanediol (Sigma, $\delta^{13}C^0/00$ value −32.12) the low "absolute" value for pregnanediol would give the situation away. Similarly, commercial androstanediols also have very low $\delta^{13}C^0/00$ values (Sigma −34.00) so self-administration of these compounds would not increase the δ values of testosterone derived metabolites. Probably the only way to fool such a test would be to alter the diet drastically over a long period of time through exclusively ingesting foodstuffs with $\delta^{13}C^0/00$ values below −29. False positives could be obtained by ingestion of nonproscribed steroids such as the now commonly used dehydroepiandrosterone (DHEA), which also partially metabolizes to androstanediols. However, whether testosterone or DHEA was being administered could easily be determined from marked differences in the total urinary steroid profile.

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References

1. Catlin DH, Cowan DA, De la Torre R, Donike M, Fraisse D, Oftegro H, Hatton CK, Starcevic B, Becchi M, de la Torre X, Norli H, Geyer H, Walker CJ (1996). Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J Mass Spectrom* **31**:397–402.
2. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, Mitchell D, Wheeler M, Brooks RV (1990). Programme and Abstracts of The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, p. 306.
3. Smith BN, Epstein S (1971). Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol* **47**:380–384.
4. Becchi M, Aguilera R, Farizon Y, Flament IM-M, Casabianca H, James P (1994). Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun Mass Spectrom* **8**:301–308.
5. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope JHG (1996). Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom* **31**:169–176.
6. Donike M, Bärwald KR, Klosterman K, Schänzer W, Zimmerman J (1983). *Sport*. Leistung und Gesundheit, Cologne, Germany, p. 293.
7. Shackleton CHL, Whitney JO (1980). Use of Sep-pak® cartridges for urinary steroids extraction: Evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* **107**:231–243.
8. Girard A, Sandulesco G (1936). Sur une nouvelle série de réactif du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la characterisation microchinique des aldehydes et cétones. *Helv Chim Acta* **19**:1095–1107.
9. Bush IE (1961). *The Chromatography of Steroids*. Pergamon Press Ltd., Oxford, UK, pp. 358–371.
10. Setchell KDR, Shackleton CHL (1973). The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. *Clin Chim Acta* **47**:381–388.
11. de la Torre X, Segura J, Farré M, Yang Z, Li Y, Wu M (1995). Steroid profile in different ethnic groups after testosterone ethanate administration. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis*, Sport und Buch Strauß, Köln, Germany, pp. 59–80.
12. Shackleton CHL, Honour JW (1976). Simultaneous estimation of urinary steroids by semiautomated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin Chim Acta* **69**:267–283.