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Detection of Exogenous Steroids by $^{13}C/^{12}C$ Analysis

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Abstract

Urinary steroid profiles (steroid ratios) are used to control the misuse of endogenous steroids such as testosterone and dihydrotestosterone. For example, the testosterone/epitestosterone (T/E) ratio, measured by gas chromatography/mass spectrometry (GC/MS), is used to control testosterone administration. When the ratio lies outside the normal range (T/E > 6), the sample is positive (or a recommendation is made for a follow-up study). Similarly, dihydrotestosterone (DHT) misuse is controlled by comparing the ratios of the urinary excreted diols, 5α-androst-ane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD).

Recent investigations have illustrated the effectiveness of carbon isotope ratio mass spectrometry to detect and confirm testosterone administration. The method is based on comparison of the carbon isotope ratio ($^{13}C/^{12}C$) of testosterone to other endogenous steroids (testosterone precursors and metabolites). As exogenous (synthetic) testosterone has a lower ^{13}C abundance, measurement of the urinary testosterone carbon isotope ratio can be used to prove testosterone misuse. In fact, the carbon isotope ratios can be used to determine testosterone administration even when the T/E ratio is at its normal value (T/E=1).

With support from the International Cycling Federation we have been able to continue our preliminary investigations with testosterone and have developed a method for confirming testosterone misuse based on carbon isotope ratio mass spectrometry. The method is based on carbon isotope ratio measurements of testosterone, testosterone metabolites, androsterone, etiocholanolone, 5αAD and 5βAD, and testosterone precursors, e.g., pregnanediol, pregnanetriol and cholesterol. In addition, we have expanded the existing procedure to detect misuse of other endogenous steroids, including dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA).

Introduction

The misuse of endogenous steroids such as testosterone (T), dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA) is an acute problem in sports and presents a challenge for sports drug testing laboratories. Currently, IOC laboratories monitor exogenously administered steroids using steroid profiles and ratios, e.g., the testosterone/epitestosterone (T/E) ratio. However, in some instances testosterone administration can go undetected using the urinary T/E ratio (T/E > 6) [1]. The T/E>6 ratio for determination of testosterone administration rapidly falls from values higher than 20 to below the cut-off level of 6 within a short time after ingestion (or injection) of testosterone undecanoate. Conceivably, an athlete could administer small doses of testosterone on a regular (daily) basis, even during competition, and this would not be detected in the drug testing laboratory using the T/E ratio.

The drug testing laboratory is also required to distinguish between positive and false positive cases of endogenous steroid use. An elevated T/E ratio from an individual claiming to be drug free and with no previous history of steroid misuse must be evaluated with care. Often times an elevated T/E ratio is due to a low level of epitestosterone (E), and in such cases it is useful to determine urinary concentrations of testosterone and other steroids (e.g., testosterone precursors and metabolites) and normalize the concentrations based on the urine density to determine whether or not the testosterone concentration is excessively high. Some federations also ask that an athlete participate in an endocrinological study under controlled conditions to assure that a false positive is avoided [2].

At the 1996 Manfred Donike Workshop we presented a method for determining and confirming testosterone misuse based on carbon isotope ratio monitoring (irm-GC/MS) [1]. The method was designed to utilize a small volume of urine (>10 mL), normally the maximum amount available in samples submitted to the drug testing laboratory, and was modeled after the method presented by Beechi *et al.* [3]. We chose a combination of immunoaffinity chromatography (IAC) and HPLC fractionation for purification of testosterone from urine and determined carbon isotope ratios for underivatized testosterone and two testosterone metabolites, 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD). With this sample preparation technique, it was possible to obtain reliable carbon ratio data on as little as 300 ng testosterone (0.035 nmole/μL). For males (non-testosterone users), the testosterone

carbon isotope content ($\delta^{13}C‰$) was found to range between -21 and -24 (expressed as the relative difference between the sample and an international standard, Pee Dee Belemnite). Following ingestion of testosterone undecanoate, the $\delta^{13}C‰$ value decreased to below -29. The lower $\delta^{13}C‰$ value results from the fact that synthetic testosterone has a lower ^{13}C content than naturally occurring testosterone. The difference in the $^{13}C/^{12}C$ isotope ratio allows one to confirm testosterone administration.

The method has been expanded to include several endogenous steroids to obtain a profile of steroid $\delta^{13}C‰$ values. The profiling technique allows one to detect not only testosterone administration, but also the misuse of other endogenous steroids such as epitestosterone, DHT, and DHEA. Steroids in the profile include, cholesterol, pregnanediol, pregnanetriol, androsterone, etiocholanolone, DHEA, testosterone, DHT, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol and 5-androstene-3β,17β-diol. The steroids are extracted from urine after enzymatic hydrolysis (β-glucuronidase from *Escherichia coli*) and purified using HPLC fractionation, prior to analysis with gas chromatography, combustion, isotope ratio mass spectrometry (GC/C/IRMS). With this methodology actual testosterone and DHT positive cases have been confirmed. At the 1997 Manfred Donike Workshop and in recent publications, Shackleton *et al.* [4,5] have presented a similar method for the detection of exogenously administered steroids using the metabolites androstanediol and 5-androstenediol for profiling.

Experimental

In an excretion study, urine samples were collected from two men (ages 34 and 40) following oral application of testosterone undecanoate (Organon, Oberschleißheim, Germany) and DHEA (Sigma, Deisenhofen, Germany). The urine samples were stabilized with NaN_3 and stored at 4°C. Steroids were isolated from urine and quantified by GC/MS, according to the standard procedure [6]. Based on the testosterone concentration, an appropriate volume of urine was aliquoted (between 2 and 20 mL, adjusted to obtain 500 ng testosterone) for GC/C/IRMS analysis. Urine was passed through an Amberlite XAD-2 column (Pasteur pipette, closed with a glass bead, bed height 2 cm), the column washed with 2 mL bidistilled water and the conjugated and unconjugated steroids eluted with 2 mL methanol. After drying, the residue was redissolved in 1 mL 0.2 M sodium phosphate buffer and the unconjugated

steroids were separated by extraction with 5 mL *tert.*-butylmethyl ether. The steroid conjugates were enzymatically hydrolyzed (50 µL β-glucuronidase from *Escherichia coli*, Boehringer, Mannheim, Germany) for 1 h at 50°C. The extract was alkalized with 250 µl of an aqueous 20% buffer solution of K_2CO_3:$KHCO_3$ (1:1, w/w) and the steroids extracted in 5 mL *n*-pentane. After drying, the residue was dissolved in 75 µL MeOH and 50 µL was fractionated via HPLC.

Table 1. HPLC and GC/C/IRMS retention times.

Substance	**HPLC (min)**	**GC/C/IRMS (s)**
Testosterone	7.7	713
5-Androstene-3β,17β-diol	7.7	639
5β-Androstane-3α,17β-diol	8.6	619
DHEA	8.6	629
Pregnanetriol	8.7	852
5α-Androstane-3α,17β-diol	9.1	638
DHT	9.5	666
Etiocholanolone	9.6	612
Androsterone	10.1	631
Pregnanediol	11.4	753

HPLC fractionation using water+acetonitrile mobile phase with gradient increasing from 30 to 100% acetonitrile in 17.5 min at 1 mL/min flow. GC/C/IRMS retention time for m/z 44 (CO_2) resulting from combustion of the steroid. DB5-MS column (17 m x 0.25 mm i.d., 0.25 µm film thickness). Splitless injection. He flow 2 mL/min at 120°C. Temperature program: Initial 120°C, 20°C/min to 220°C, 5°C/min to 310°C.

Urinary steroids (see Table 1) were separated using HPLC according to the described method [1]. HPLC fractionation (Hewlett Packard (HP) 1090) was performed with an analytical column (HP LiChrospher RP 18, 5µ, 125x4 mm i.d.) using a water+acetonitrile mobile phase gradient increasing linearly from 30 to 100% acetonitrile in 17.5 min at 1 mL/min. The eluting steroids were collected in glass tubes by hand or with a fraction collector (Gilson FC 205, Middleton, Wisconsin, USA) and the elute dried under vacuum. The fractions were collected in continuous groups so that it could be confirmed that part of the substance did not elute in the previous- or later-fraction. This can lead to isotopic fractionation, as the ^{13}C component elutes earlier than the ^{12}C component in the HPLC analysis.

Etiocholanolone and androsterone were usually collected in a single fraction in a separate HPLC run using a small aliquot (15 μL) of the original 80 μL sample. A large time window was used for collection to avoid any isotopic fractionation in the HPLC analysis. The remainder (ca. 10 μL) of the sample was analyzed by GC/MS after enol-TMS-derivatization (MSTFA/TMIS). Cholesterol was extracted (*tert.*-butylmethyl ether) from alkalized (KOH) urine (5 mL) and reconstituted in methanol prior to GC/C/IRMS analysis.

For GC/C/IRMS analysis the samples were redissolved in freshly distilled methanol, with the amount of methanol adjusted to obtain a final concentration of ca. 10 ng/μL. Carbon isotope ratio measurements were performed using a Finnigan MAT Delta C instrument (Finnigan MAT GmbH, Bremen, Germany). The GC (HP 5890) was equipped with a 17 m J&W DB5-MS column (0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, California, USA) with helium as the carrier gas and injections (1 μL) were made in the splitless mode using a closed (single-sided) insert. Helium flow through the GC column was 2 mL/min at 120° and 2.5 mL/min was added with the backflush valve opened. The GC was temperature programmed from 120°C (0 min) to 220°C (20°C/min), and then ramped at 5°C/min. GC retention times are listed in Table 1. A metal four-way connector (Valco, Schenkon, Switzerland) was used to join the GC column to the capillary columns leading to the combustion oven and backflush valve. No difference could be noted (in chromatography and isotopic ratios) using a glass connector. A FID detector was installed and could be used in place of the combustion oven to verify GC performance. To determine $\delta^{13}C‰$ values, calibrated CO_2 ($\delta^{13}C‰$ -3.66) gas was pulsed into the ion source during the backflush (solvent elution) period and the relative difference between the $^{13}C/^{12}C$ ratio of the sample and the CO_2 calibrant was calculated and displayed as $\delta^{13}C‰$. The standard deviation for the standard solutions was smaller than 0.2‰ (n>5).

Standard mixtures (10 ng/μL) of testosterone, androsterone, etiocholanolone, 5αAD, 5βAD, pregnanediol and pregnanetriol (Table 2), were used to test the performance and stability of the instrument. Under certain GC conditions (e.g., dirty insert or bleeding column) $\delta^{13}C‰$ values for testosterone were too negative (as much as 10%). Interestingly, the $\delta^{13}C‰$ value for testosterone was more sensitive to GC conditions than for any other standard steroids. The $\delta^{13}C‰$ value for siloxan (column bleeding component) is near that of the analyte and with some columns the bleeding content can be very high and can vary over the width of

the GC peak and influence the $\delta^{13}C‰$ value. The combustion reactor (where the sample eluting from the GC is pyrolzed and the carbon formed is oxidized to yield $CO_2/^{13}CO_2$) was stable for more that one year and seldom needed to be reoxidized (normally less than once per week).

Results and Discussion

Standard mixtures of steroids at 10 ppm concentration [10 ng/μL] were used to confirm proper operation of the GC/C/IRMS instrument. These steroids and their $\delta^{13}C‰$ values are listed in Table 2.

Table 2. $\delta^{13}C‰$ values of commercially available steroids used as standards.

Substance	$\delta^{13}C‰$
Testosterone	-29.4
Androsterone	-32.1
Etiocholanolone	-24.5
5α-Androstane-3α,17β-diol	-34.7
5β-Androstane-3α,17β-diol	-30.7
Pregnanediol	-30.4
Pregnanetriol	-30.7

$\delta^{13}C‰$ values for underivatized steroids. GC conditions given in Table 1.

Determination of Testosterone

Using the method described, an actual T/E positive case was confirmed by GC/C/IRMS analysis. The sample was obtained from a male athlete in competition. The T/E ratio was 14.7, the testosterone concentration 358 mg/mL and the A/T ratio 12.3 (androsterone/testosterone). The athlete participated in an endocrinological study [2] in order that baseline T/E ratios could be established. The baseline T/E ratio was found to be 1.0. Carbon isotope ratio mass spectrometry analysis was performed on several steroids isolated from the positive urine and the baseline urine obtained during the endocrinological study. Cholesterol and pregnanediol were chosen as reference markers, as their $\delta^{13}C‰$ values are not influenced by exogenously administered testosterone. Androsterone and etiocholanolone were selected since they are major metabolites of testosterone and their $\delta^{13}C‰$ values should differ in the two samples. The results are shown in Figure 1.

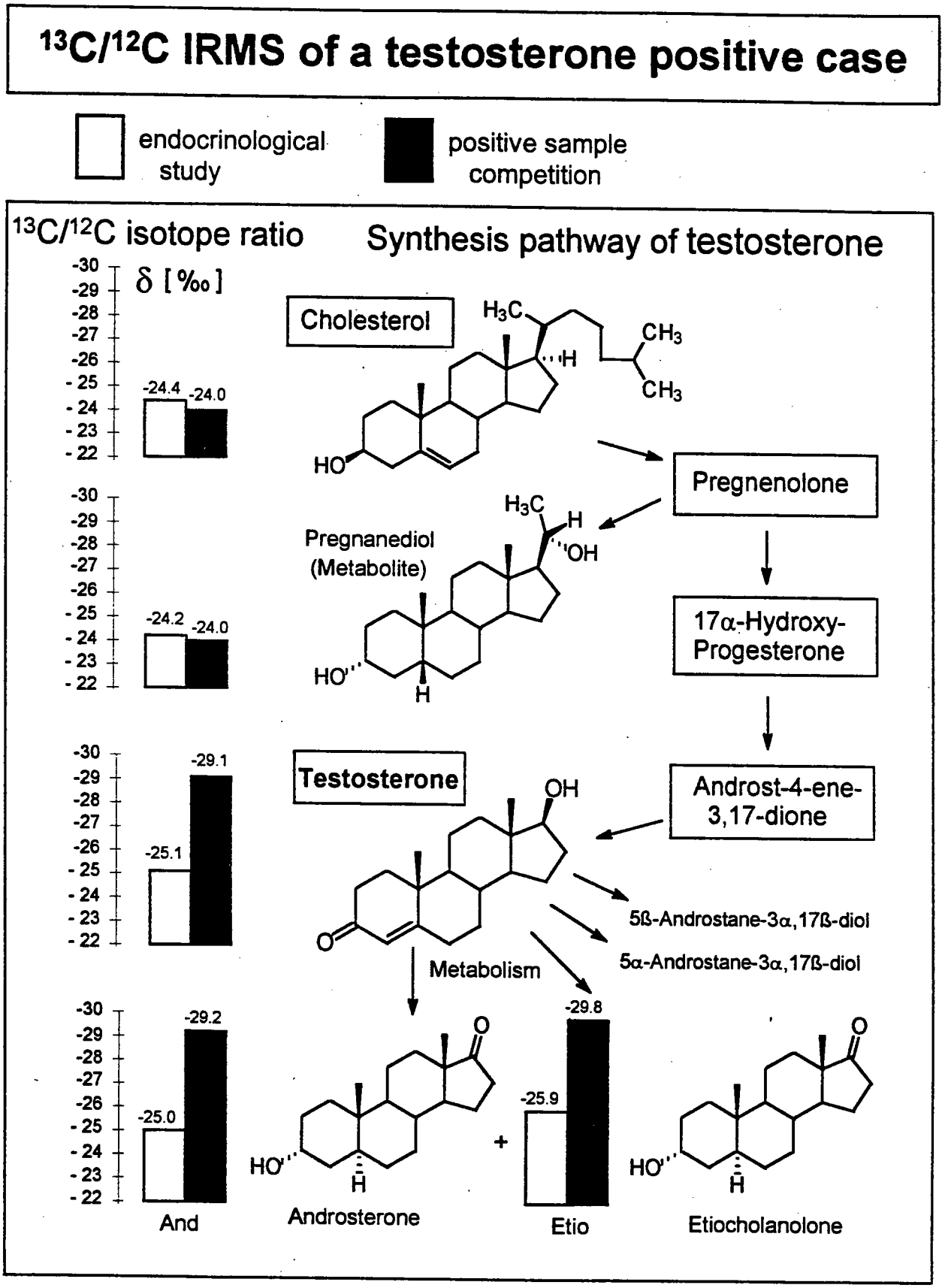

Figure 1. $\delta^{13}C‰$ values for steroids isolated from a urine sample obtained during competition (solid bar, T/E 14.7) and during an endocrinological study (open bar, T/E 1.0).

The $\delta^{13}C‰$ values for cholesterol and pregnanediol in both samples are around -24, which is normal for males in Europe. The $\delta^{13}C‰$ values for testosterone and its metabolites androsterone and etiocholanolone are much lower in the sample obtained during competition, as a result of testosterone administration. Of interest is the range of values (-24.0 to -25.9) obtained for the different steroids in the profile during the endocrinological study. Studies are in progress to determine how $\delta^{13}C‰$ values range in an individual steroid profile.

Determination of 5α-Dihydrotestosterone

As with testosterone, the naturally occurring steroid 5α-dihydrotestosterone (DHT) has potential for misuse since it has anabolic effects and it is difficult to determine its presence in urine. DHT has been removed from the market in most countries, but ample supplies are rumored to exist. Statistical methods based on ratios of steroid concentrations have been developed to monitor DHT misuse. In the Cologne laboratory the ratio 5αAD/5βAD > 1.5 is used as an indicator for DHT administration in males [7]. Other parameters, such as the DHT concentration (> 21 ng/mL), the ratio A/E > 1.5 (androsterone/etiocholanolone) and the ratio DHT/E > 8.2 have also been found to be useful, but none of these parameters provide unequivocal evidence for DHT misuse.

Urine samples collected after sublingual DHT administration to a male volunteer [7] were examined by GC/C/IRMS to determine the effectiveness of the isotope technique. As indicated at the 1996 Workshop [1], the diol (5αAD and 5βAD) metabolites are long-term indicators for testosterone misuse, and it is expected that they should be equally informative for controlling DHT misuse. DHT metabolism is different from that of testosterone as only 5α products are formed (see Figure 2). Measuring $\delta^{13}C‰$ values for the 5α (androsterone and 5αAD) and 5β (etiocholanolone and 5βAD) metabolites should allow one to determine the presence of exogenously administered DHT and testosterone and distinguish between the two.

Table 3 lists $\delta^{13}C‰$ values for several steroids isolated from urine samples collected up to 24 h after sublingual application of DHT. $\delta^{13}C‰$ values for DHT could be determined only at 6 and 16 h after administration due to its low concentration in urine. The $\delta^{13}C‰$ value for DHT was largely negative compared to testosterone, pregnanediol and pregnanetriol. As expected, the $\delta^{13}C‰$ values for the 5α metabolites (5αAD and androsterone) are negative compared to their 5β isomers (5βAD and etiocholanolone).

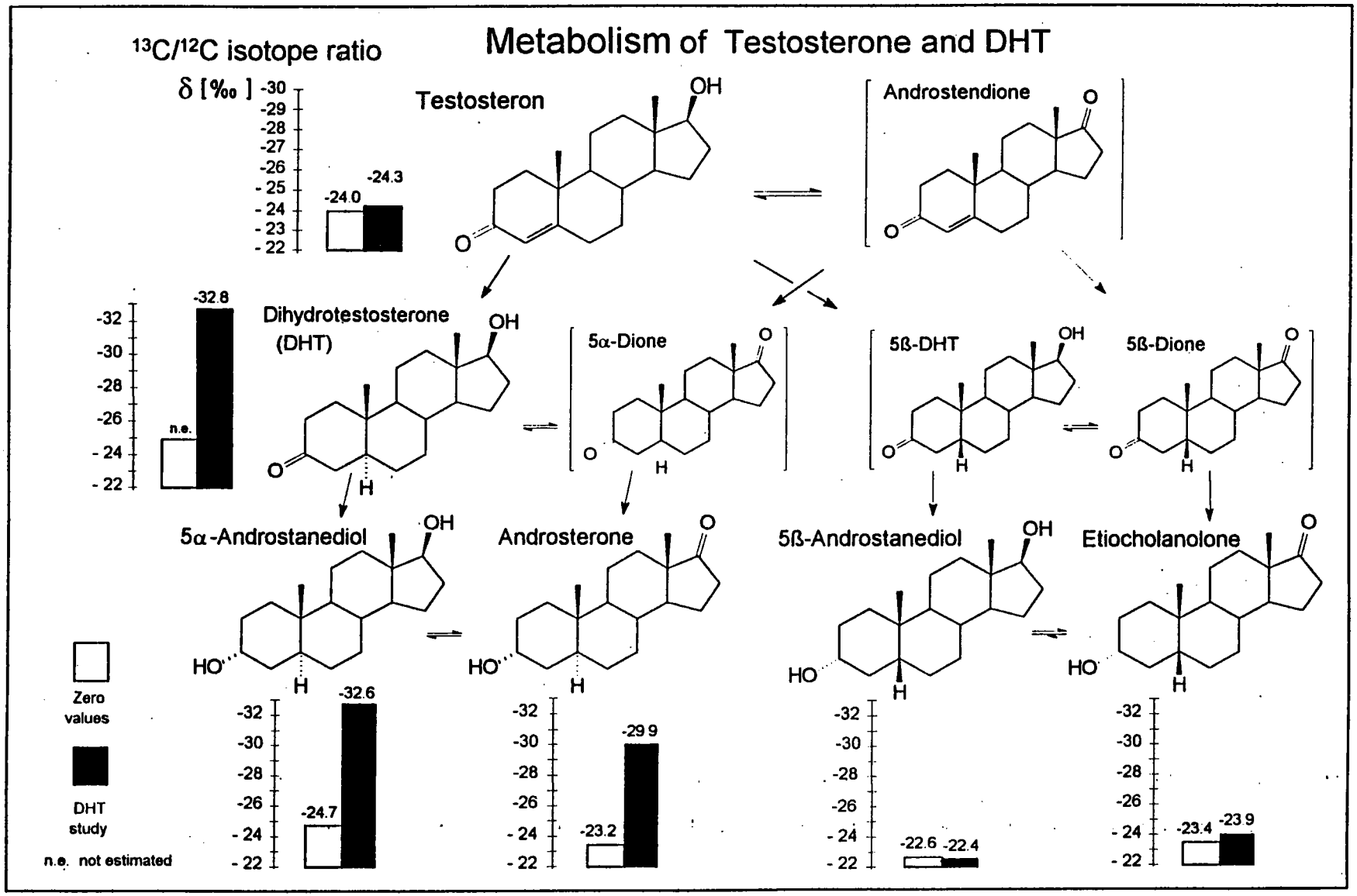

Figure 2. $\delta^{13}C‰$ values for steroids isolated from urine samples obtained prior (open bar) and 6 h after 25 mg sublingual DHT administration (solid bar) to a male volunteer.

Table 3. $\delta^{13}C‰$ values of steroids isolated from urine following DHT administration.

Substance	$\delta^{13}C‰$ 0 h	6 h	16 h	24 h
Testosterone	-24.0	-24.3	-24.5	-24.6
Pregnanediol	-22.6	-22.8	-23.2	-24.0
Pregnanetriol	-22.7	-21.8	-23.0	-24.3
DHT	n.d.	**-32.8**	**-30.4**	n.d.
Androsterone	-23.2	**-29.9**	**-28.7**	-24.1
5α-Androstane-3α,17β-diol	-24.7	**-32.6**	**-33.6**	**-30.9**
Etiocholanolone	-23.2	-23.6	-23.9	-23.8
5β-Androstane-3α,17β-diol	-22.3	-22.6	-22.4	-23.3

Urine samples collected from a male volunteer after sublingual application of 25 mg DHT.
n.d. not determined.

$^{13}C‰$ values for 5αAD and 5βAD up to 70 h after DHT administration are given in Table 4. Included are concentration ratios of 5αAD/5βAD, where 5αAD/5βAD > 1.5 is used as the cutoff for DHT administration. At 24 h the 5αAD/5βAD ratio is below the cutoff level and this sample would not be considered positive (a false negative, based on further information obtained from GC/C/IRMS analysis). The 5αAD $\delta^{13}C‰$ value indicates DHT administration after 24 h, as well as at 32 h and 44 h. In this case, GC/C/IRMS provides a three fold increase in retrospectivity, as compared to the 5αAD/5βAD > 1.5 cutoff based on GC/MS analysis.

Table 4. Urinary 5αAD/5βAD ratios and $\delta^{13}C‰$ values for 5αAD and 5βAD following DHT administration.

Time (h)	5αAD/5βAD	$\delta^{13}C‰$ 5βAD	5αAD
0	0.32	-23.8	-24.7
16	**2.64**	-22.4	**-33.6**
24	1.00	-23.3	**-30.9**
32	0.97	-23.8	**-27.6**
44	0.64	-23.5	**-25.9**
69	0.31	-23.8	-25.0

Urine samples collected from a male volunteer after sublingual application of 25 mg DHT.
5αAD/5βAD determined by GC/MS analysis with cutoff for DHT administration at 1.5 [7].

In 1996 the IOC issued a DHT proficiency test to all accredited IOC laboratories. After GC/MS analysis and confirmation of the results, remaining aliquots (6 mL) of the three samples were subjected to GC/C/IRMS analysis. The results are summarized in Table 5. $\delta^{13}C‰$ values for 5αAD are negative in two of the samples (-29.8 and -25.3), as compared to 5βAD and the reference steroids ($\delta^{13}C‰$ values are on the order of -20), which can be taken as proof of DHT administration. Remarkable are the rather positive $\delta^{13}C‰$ values for the reference steroids (pregnanediol, pregnanetriol, etiocholanolone and 5βAD) in these samples.

Table 5. $\delta^{13}C‰$ values of steroids in DHT samples issued at the 1996 IOC proficiency test.

	$\delta^{13}C‰$		
Substance	**Urine 1**	**Urine 2**	**Urine 3**
Androsterone	-20.3	**-24.1**	-19.6
5α-Androstane-3α,17β-diol	-19.9	**-29.8**	**-25.3**
Etiocholanolone	-20.3	-20.5	-19.6
5β-Androstane-3α,17β-diol	-19.9	-20.4	-19.9
Pregnanediol	-20.5	-21.4	-20.0
Pregnanetriol	-21.2	-20.9	-20.8

Urine samples were obtained from a diabetic patient undergoing DHT therapy [8].

Determination of Dehydroepiandrosterone

Recently there have been reports of misuse of dehydroepiandrosterone (DHEA). It is banned by the IOC, however, it is available in the United States as a nutritional supplement. There is some doubt as to the effectiveness of DHEA as an androgenic (anabolic) agent, but as it is a banned substance, measures must be taken to check for its presence. Thus far, little has been done by the IOC laboratories to address this problem. GC/MS data and steroid profiles from a DHEA excretion study were presented at the 1997 Workshop [9].

In a preliminary study, urine samples collected after ingestion of 40 mg DHEA (free form, rather than the sulfate conjugate) to a male volunteer were examined by GC/C/IRMS. Urine samples collected prior and 4 h after DHEA administration were subjected to enzymatic hydrolysis using sulfatase and β-glucuronidase from *Helix pomatia* and purified by HPLC fractionation. Two additional steroids, DHEA and 5-androstene-3β,17β-diol, were recovered by HPLC. Table 6 lists $\delta^{13}C‰$ values for steroids isolated from the urine samples. The $\delta^{13}C‰$ value of the exogenously administered DHEA was determined as -35.5.

Remarkably all of the endogenous steroids showed decreased $\delta^{13}C‰$ values, except pregnanetriol. There appears to be some conversion of DHEA (free) to testosterone, although this result must be confirmed. This study indicates that the diols, 5αAD and 5βAD, can be used as indicators for DHEA administration, and that it is not necessary to perform hydrolysis using *Helix pomatia*, making the method amenable to the standard procedure. Of course, analysis of the sulfate conjugate, 5-androstene-3β,17β-diol, provides evidence for DHEA administration and excludes testosterone. Studies are ongoing with DHEA to determine the long-term retrospectivity of the diols, 5αAD and 5βAD, as compared to the sulfate conjugate, 5-androstene-3β,17β-diol. In addition, studies are being performed with DHEA-sulfate.

Table 6. $\delta^{13}C‰$ values of steroids isolated from urine following DHEA administration.

	$\delta^{13}C‰$	
Substance	**0 h**	**4 h**
DHEA	-24.0	-33.2
5-androstene-3β,17β-diol	-23.6	-34.2
Testosterone	-24.4	-30.3
Androsterone	-23.8	-29.9
Etiocholanolone	-23.9	-30.3
5α-Androstane-3α,17β-diol	-23.6	-32.8
5β-Androstane-3α,17β-diol	-23.2	-31.2
Pregnanediol	-24.2	n.d.
Pregnanetriol	-23.9	-24.2

Urine samples collected from a male volunteer after ingestion 40 mg DHEA (free form). $\delta^{13}C‰$ of administered DHEA -35.5. Urine samples subjected to enzymatic hydrolysis using sulfatase and β-glucuronidase from *Helix pomatia*. n.d. Not determined.

Conclusion

Carbon isotope ratio mass spectrometry is an effective means of detecting exogenously administered testosterone, DHT and DHEA. This assumes, of course, that synthetic sources will continue to have a lower ^{13}C content than naturally occurring steroids. There is a significant increase in the retrospectivity of administration using metabolic end-products (diols) such as 5αAD, 5βAD and, presumably, 5-androstene-3β,17β-diol, as compared to the parameters obtained by GC/MS, T/E > 6 and 5αAD/5βAD > 1.5. The method described can be applied to other steroids with potential for misuse such as boldenone, nandrolone and

epitestosterone. Investigations are also ongoing with steroids isolated from animals (equine and bovine).

High performance GC/C/IRMS instruments have detection thresholds in the low ng range, thus it is possible to perform carbon isotope ratio measurements using a small amount of sample (less than 10 mL of urine). This is especially important in sport drug testing, as there is a limited volume of urine available. It is conceivable that all samples obtained in the drug testing laboratory could be subjected to GC/C/IRMS analysis since the analysis times are short and the instrument is fully automated. The major drawback at this time is the time required for sample preparation and the cost of the instrument.

In this study HPLC was utilized for sample isolation, rather than immunoaffinity chromatography (IAC) [1], as it was of interest to obtain an isotope ratio profile for a wide range of steroids. Pregnanediol, pregnanetriol, androsterone, etiocholanolone and DHEA do not interact with the antibody raised against testosterone-3-carboxy-methyloxime-bovine serum albumin. A screening method utilizing cholesterol (obtained via liquid-liquid extraction) and 17β-OH steroids (obtained via IAC) might be efficient (in terms of time) for a screening analysis, although the method presented by Shackleton *et al.* [5], based on Girard T reagent separation and Sephadex LH-20 chromatography, needs to be examined more closely. In addition, the influence of derivatization (enol-TMS with MSTFA/TMIS and acetylation with pyridine/acetic anhydride) must be investigated, as some preliminary experiments have shown that following acetylation there was a substantial decrease in $\delta^{13}C‰$ values.

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