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Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols

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Abstract

We propose a new screening method for testosterone (T) doping in sport. The current method for detecting T administration is based on finding a T to epitestosterone ratio (T/E) in urine that exceeds six. The difficulties with T/E are that T administration does not always result in a T/E>6 and that a rare individual will have T/E>6 in the absence of T administration. Our previous studies reveal that carbon isotope ratio helps to determine the origin of the urinary T because the values for T and its metabolites decrease after the administration of exogenous T. In this study, we present a rapid and efficient screening sample preparation method based on three successive liquid–solid extractions, deconjugation with *E. coli* β-glucuronidase after the first extraction, acetylation after the second extraction, and a final extraction of the acetates. The $^{13}C/^{12}C$ of two T metabolites (5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol) and one pregnanediol as endogenous reference (5β-pregnane-3α,20α-diol) was measured by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) on 10 ml of urine collected from 10 healthy men before and after T administration. Following T administration, the $^{13}C/^{12}C$ of 5β-androstane-3α,17β-diol diacetate and 5α-androstane-3α,17β-diol diacetate declined significantly from −26.2‰ to −30.8‰ and from −25.2‰ to −29.9‰, respectively and the $^{13}C/^{12}C$ of 5β-pregnane-3α,20α-diol diacetate was unchanged. In addition, the ratio of androstanediols to pregnanediol increased in the post-T urines.

Keywords: Steroids; Testosterone

1. Introduction

After 1976, when the use of anabolic androgenic steroids (AAS) was prohibited by the International Olympic Committee (IOC), several techniques were

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developed to detect these compounds. At present, AAS are detected in urine samples by gas chromatography–mass spectrometry (GC–MS). However, pharmaceutical or exogenous testosterone presents special challenges as it is not possible to distinguish it from natural endogenous testosterone by GC–MS.

New possibilities for detecting exogenous T emerged following reports that the carbon isotope ratio ($^{13}C/^{12}C$) of exogenous T was different from that of endogenous T [1]. The $^{13}C/^{12}C$ of natural human testosterone is a function of the aggregate sum of the $^{13}C/^{12}C$ of the plants and animals we consume, and any additional effects of human biological processing. Synthetic testosterone is prepared from one plant source with an unusually low $^{13}C/^{12}C$. These slight differences can be detected through isotope ratio mass spectrometry [2–6]. In a T administration study that collected urines from subjects before and after T administration, we reported that the $^{13}C/^{12}C$ of urinary T and metabolites of T were significantly lower after T, and that the $^{13}C/^{12}C$ of metabolic precursors of T were unchanged [2]. Shackleton et al. [5] confirmed that T administration results in lower $^{13}C/^{12}C$ values for T metabolites, showed that the values remain low for 8 days following a dose of 250 mg of T enanthate, and demonstrated the potential applicability of the method for detecting administration of dehydroepiandrosterone (DHEA) and other steroids. These studies and others [7] are consistent with the view that carbon isotope ratio methods could be very useful in discovering the origin of T and other steroids in an athlete's urine.

The present method for detecting T administration is finding a ratio of T to epitestosterone (T/E) in urine by GC–MS that exceeds six [8]. While $T/E>6$ is an excellent indicator of T administration, it is not a definitive test because a few individuals who have not used T have a T/E greater than six [9,10], and, other factors may alter T/E [11]. Further, since the median T/E is ~1 [12], individuals with low or normal T/E may take T and not exceed the reporting threshold of six, thus a negative report ($T/E<6$) does not exclude T administration. Lowering the T/E ratio threshold would result in detecting more cases of naturally elevated T/E, but would still allow some use of synthetic T. Other indirect means of detecting T administration have been discussed [13], but unlike $^{13}C/^{12}C$, none have the potential to serve as a direct indicator of T administration.

If a $^{13}C/^{12}C$ method is to be useful for increasing the sensitivity of detecting T administration, it is necessary to develop a screening method that is rapid, efficient, reliable, and requires a small volume of urine. In our first reports [2–4], the method required ~30 ml and it was relatively slow due to the high-performance liquid chromatography (HPLC) step. Furthermore, in the interest of understanding the method, we determined the $^{13}C/^{12}C$ of several compounds: cholesterol, DHEA, androst-5-ene-3β,17β-diol (T precursors), T itself, and T metabolites 5α-androstane-3α,17β-diol (5α-adiol) and 5β-androstane-3α,17β-diol (5β-adiol).

In this paper, we present a new sample preparation method that only requires 10 ml of urine, does not require HPLC fractionation, and provides chromatograms with excellent separation of the compounds of interest and virtually flat baselines.

2. Experimental

2.1. Urine samples

Urines were obtained from two groups of healthy male subjects. Subjects A–E were 20–59 years of age and were participating in a 25-week study concerned with the effects of T on behavior. The protocol was approved by the Harvard Medical School institutional review board. The subjects received intramuscular T enanthate (300 mg/week) or placebo, and urines were collected weekly for 26 weeks. Subjects F–J were participating in an 8-day study to discover the effects of T infusions on the hypothalamic–pituitary–testicular axis. After collection of the baseline urine, the subjects received an infusion of aqueous testosterone at doses of 7 mg/1.7m^2/24-h for days 1–2, 14 mg/1.7m^2/24-h for days 3–4, and 28 mg/1.7m^2/24-h for days 5–6. Timed urine samples were collected on each day. The protocol was approved by the Harbor-UCLA Hospital Ethics Committee.

2.2. Reagents for GC–C–IRMS sample preparation

The following steroids were obtained from

Steraloids (Wilton, NH, USA): androst-5-ene-3β,17β-diol diacetate, 5β-androstane-3α,17β-diol diacetate, 5α-androstane-3α,17β-diol diacetate, 5α-androstane-3β,17β-diol diacetate, 5β-androstane-3β,17β-diol diacetate, 5β-pregnane-3α,20α-diol diacetate, 5β-pregnane-3α,17,20α-triol-3,20-diacetate, 5α-androstane-3α, 17α-diol, 5α-androstane-3β,17α-diol, 5α-pregnane-3β,20β-diol diacetate, 5α-pregnane-3α,20β-diol diacetate, androst-5-ene-3β,17α-diol diacetate, pregn-4-ene-3β,20α-diol, pregn-4-ene-3β,20β-diol, 5β-pregnane-3α,20β-diol diacetate, 5β-pregnane-3β,20α-diol diacetate, 5β-pregnane-3β, 20β-diol diacetate, 5α-pregnane-3α,20α-diol diacetate, pregn-5-ene-3β,20α-diol diacetate, 5α-androstane-3α,17β-diol and 5β-androstane-3β,17α-diol diacetate.

Sigma (St. Louis, MO, USA) provided 5α-pregnane-3β,20α-diol, and Boehringer Mannheim (Indianapolis, IN, USA) provided β-glucuronidase enzyme from *E. coli*. Solid phase extractions were carried out on Bakerbond solid-phase extraction columns filled with reverse phase octadecyl silica (J.T. Baker 7020-07 and 7020-13, Gallade Chemical, Fontana, CA, USA). All solvents and reagents were of analytical grade purity.

2.3. Overview of the analyses

Each urine underwent two different extraction and cleanup procedures and three different MS analyses. Firstly, our routine extraction procedure for anabolic steroids was performed followed by GC–MS [14]. This procedure estimated the T/E and the concentrations of T, E, 5β-adiol and 5α-adiol. Secondly, the new extraction and derivatization procedure was performed, followed by GC–MS to determine the identity and purity of peaks. Thirdly, the same extract was analyzed by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) to measure the carbon isotope ratio.

2.4. Routine steroid screen

The routine steroid screen includes addition of [1,2-$^{2}H_{2}$] testosterone to 2.5 ml of urine, liquid–solid extraction on a Bond-Elut C_{18} cartridge, enzymatic deconjugation with β-glucuronidase from *E. coli*, formation of trimethylsilyl derivatives, and GC–MS analysis in the selected ion monitoring mode as previously described [14]. Concentrations of T, 5β-adiol and 5α-adiol were estimated. All urine samples were found negative for exogenous anabolic androgenic steroids. As expected, the T/E of the post-T administration urines were greater than six (Table 1).

2.5. New steroid extraction for screening by GC–C–IRMS analysis

The steroids were extracted, purified, and acetylated as shown in Fig. 1. Urine (10 ml) was extracted by solid-phase extraction (SPE) on C_{18} column (500 mg), the conjugated steroids were eluted with methanol (8 ml) and the eluate evaporated to dryness. To hydrolyze the conjugated steroids, the methanolic residue was redissolved in 320 μl of 0.2 *M* phosphate buffer (pH 7.0), and 80 μl of β-glucuronidase was added as supplied. After incubation at 60°C for 1 h, the deconjugated steroids were extracted by SPE on C_{18} column (500 mg), by washing with 12 ml of methanol–water (20:80, v/v) and eluting with acetonitrile (12 ml). Acetylation of the extracts was carried out using pyridine (50 μl) and acetic anhydride (50 μl) for 1 h at 60°C incubation. The reaction medium was evaporated to dryness. The dried residue from acetylation was redissolved in 3 ml of acetonitrile–water (50:50, v/v). The steroid extract was purified by SPE on C_{18} column (1 g and 6 ml capacity). The fraction with the androstanediols and pregnanediols was obtained by elution with acetonitrile after washing with 12 ml of acetonitrile–water (50:50, v/v) and 15 ml of acetonitrile–water (75:25, v/v). The derivatized steroids were dried and dissolved in 25 μl of cyclohexane. This solution was analyzed by GC–MS and by GC–C–IRMS.

2.6. GC–MS analysis of acetylated derivatives

GC–MS analyses of a standard solution of acetylated derivatives of 5β-pdiol, 5β-adiol and 5α-adiol, and the acetylated derivatives from the extracted urines were performed on a Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with an HP-50+ (50% phenyl–methylpolysiloxane) fused-silica capillary column (30 m×0.25-mm I.D., 0.15 μm film thickness) linked to an HP 5971 mass

Table 1
Summary of T/E and carbon isotope ratio ($\delta^{13}C$‰) data in subjects receiving weekly doses of testosterone enanthate for 7 weeks (subjects A–E) and data on urine samples collected from subjects receiving by infusion for 7 days (subjects F–J)

Subject	Urine	Collection Day	T/E	5β-pdiol		5β-adiol		5α-adiol		Difference	
				($\delta^{13}C$)‰	Mean	($\delta^{13}C$)‰	Mean	($\delta^{13}C$)‰	Mean	(5β-adiol)-(5β-pdiol)	(5α-adiol)-(5α-pdiol)
A	1	0	2.3	−25.91	−26.11	−26.77	−27.04	−25.12	−25.29	0.9	0.8
				−26.31		−27.31		−25.46			
	2	49	61.2	−25.27	−25.23	−33.07	−33.35	−29.00	−28.99	8.1	3.8
				−25.19		−33.63		−28.99			
B	3	0	0.4	−25.41	−25.33	−26.10	−26.23	−24.63	−24.64	0.9	0.7
				−25.25		−26.36		−24.65			
	4	28	19.7	−25.64	−25.72	−32.23	−32.20	−30.31	−30.27	6.5	4.5
				−25.81		−32.17		−30.23			
C	5	0	0.8	−24.96	−24.99	−24.85	−24.78	−24.34	−24.36	0.2	0.6
				−25.02		−24.70		−24.37			
	6	28	53.2	−24.65	−24.62	−30.86	−30.95	−30.87	−30.74	6.3	6.1
				−24.59		−31.04		−30.60			
D	7	0	1.1	−25.10	−25.13	−25.76	−25.64	−23.77	−23.68	0.5	1.4
				−25.16		−25.52		−23.59			
	8	28	22.3	−24.72	−24.60	−29.92	−29.97	−28.63	−28.53	5.4	3.9
				−24.48		−30.01		−28.42			
E	9	0	0.2	−24.16	−23.91	−25.80	−25.71	−25.65	−25.66	1.8	1.7
				−23.66		−25.62		−25.66			
	10	49	10.2	−23.32	−23.30	−30.11	−30.20	−30.96	−30.96	6.9	7.7
				−23.28		−30.28		−30.95			
F	11	0	1.5	−25.10	−25.14	−26.12	−26.07	−25.66	−25.67	0.9	0.5
				−25.17		−26.02		−25.67			
	12	6	11.7	−26.07	−26.11	−31.05	−31.06	−30.31	−30.32	5.0	4.2
				−26.14		−31.07		−30.32			
G	13	0	1.2	−25.61	−25.72	−25.81	−25.98	−24.59	−24.62	0.3	1.1
				−25.83		−26.15		−24.64			
	14	7	10.8	−26.10	−26.04	−31.00	−31.11	−29.69	−29.68	5.1	3.6
				−25.97		−31.22		−29.67			
H	15	0	1.6	−25.64	−25.65	−26.33	−25.46	−24.72	−24.79	0.2	0.9
				−25.65		−26.59		−24.85			
	16	5	15.4	−26.34	−26.17	−31.43	−31.27	−31.27	−31.24	5.1	5.1
				−25.99		−31.11		−31.21			
I	17	0	0.97	−25.42	−25.41	−26.71	−26.77	−27.25	−27.31	1.4	1.9
				−25.39		−26.83		−27.36			
	18	7	14.2	−24.30	−24.48	−28.37	−28.40	−28.99	−29.06	3.9	4.6
				−24.66		−28.43		−29.13			
J	19	0	0.91	−25.84	−25.79	−26.84	−26.83	−25.72	−25.61	1.0	0.2
				−25.74		−26.82		−25.50			
	20	5	11.4	−24.73	−24.78	−29.30	−29.35	−29.43	−29.47	4.6	4.7
				−24.82		−29.40		−29.51			

selective detector. The injections (2 μl) were made in the splitless mode using helium as carrier gas (76 kPa). The oven temperature was increased from 80°C (1 min) to 270°C (6 min) by 15°C/min, then to 300°C at 25°C/min, and maintained at the final temperature for 1 min.

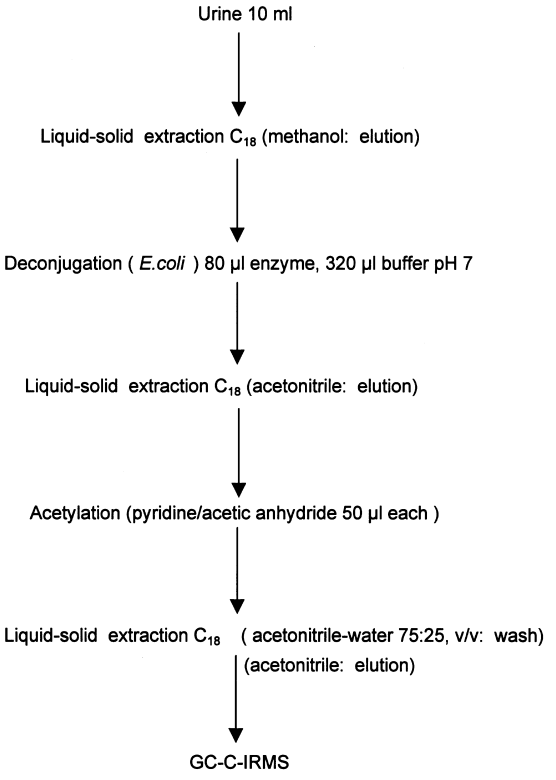

Fig. 1. Flow chart summarizing the sample preparation steps for screening urine samples by GC–C–IRMS.

2.7. GC–C–IRMS analysis

GC–C–IRMS analyses were performed on an HP 6890 gas chromatograph connected to a Micromass Isoprime isotope ratio mass spectrometer. The GC was equipped with an DB-17 column (J&W Scientific, 50%phenyl–methylpolysiloxane, 30 m×0.25-mm I.D., 0.25 µm film thickness). Helium was the carrier gas (76 kPa). Samples were injected in the splitless mode at 285°C. The GC separation began at 80°C for 1 min, then the oven temperature increased at 15°C/min to 270°C (6 min), then at 25°C/min to 300°C, and maintained at the final temperature for 1 min. The temperature of the combustion oven was 850°C and the oxidative catalyst was copper oxide pellets (0.5-mm thickness in a 6-mm quartz tube, 60 cm×65-mm I.D.)

The symbol δ is the standard notation for expressing carbon isotope ratios and it is defined as the relative difference in isotope ratio between the sample and an international standard (calcium carbonate from a fossil, *Belemnitella americana*) obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite or 'PDB') [15] and relatively rich in ^{13}C, calculated as:

$$\delta^{13}C‰ = \frac{R_{sample} - R_{PDB}}{R_{PDB}} \times 1000$$

where R_x refers to the $^{13}C/^{12}C$ of the sample or international standard and is represented by $^{13}C‰$ ('per mil'). Each extract was injected twice into the GC–C–IRMS and the replicate measurements were averaged.

3. Results and discussion

A principal objective of the new sample preparation scheme presented here was to develop a rapid method for screening urine samples by GC–C–IRMS. To accomplish this, it was necessary to eliminate the HPLC purification step from our previous methods [2–4]. Secondly, we aimed to reduce the sample volume requirements from ~30 to 10 ml or less. Thirdly, a screening method must be rapid and efficient, therefore we wished to adapt the method to batch processing in order to improve sample analysis time and sample throughput. A flowchart summarizing the procedure is shown in Fig. 1. The principal features are three successive liquid–solid extractions, deconjugation with *E. coli* β-glucuronidase after the first extraction, acetylation after the second extraction, and a final extraction of the acetates.

3.1. Chromatography

Carbon isotope ratio measurements require rigorous attention to detail during sample preparation because impurities can result in spurious $\delta^{13}C‰$ values. Chromatographic resolution is the most critical parameter because the isotopic measurement of the peaks of interest are altered by coeluting or poorly separated peaks. Figs. 2 and 3 show the characteristics of the GC–MS and GC–C–IRMS chromatograms of our procedure. Fig. 2 shows a flat baseline and enough resolution of the three peaks of

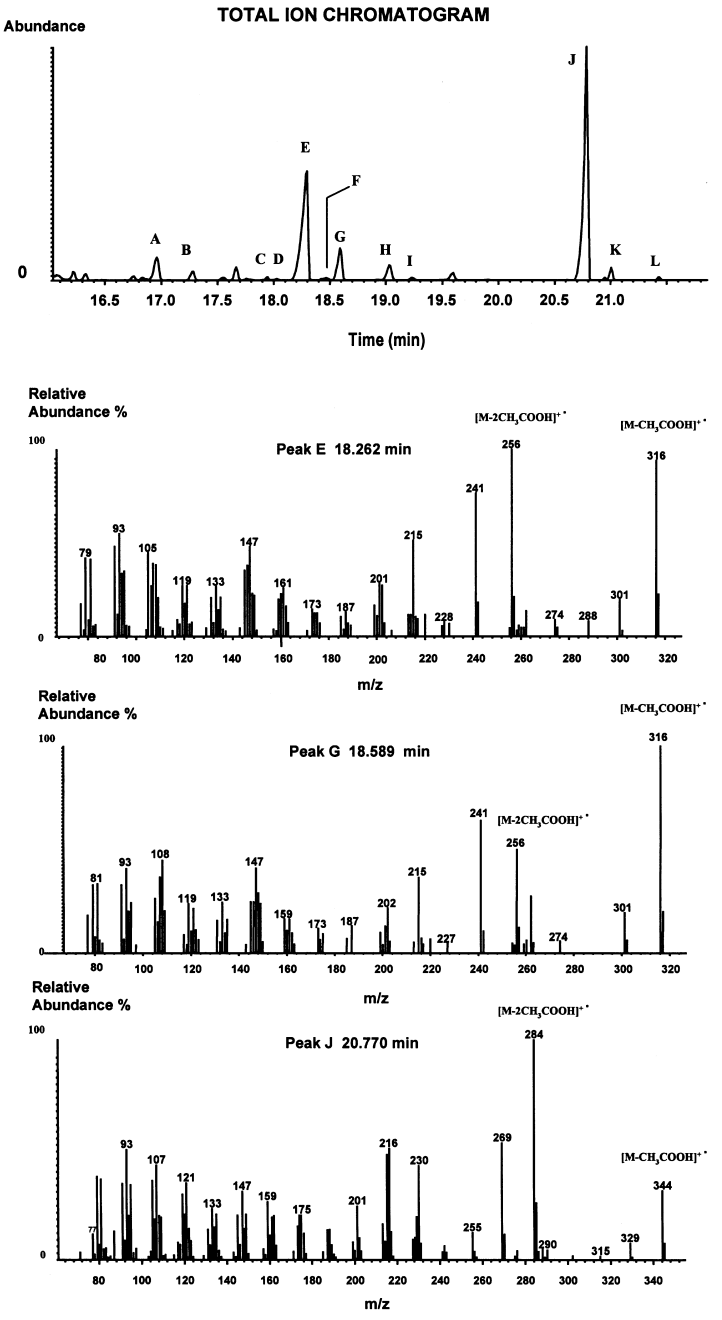

Fig. 2. Total ion chromatogram, mass spectra of compounds used in GC–MS analysis. A and B = unidentified androstene. C = 5β-androstane-3α,17α-diol diacetate. D = unidentified androstene. E = 5β-androstane-3α,17β-diol diacetate (5β-adiol-diacetate). F = 5α-androstane-3α,17α-diol diacetate and androst-5-ene-3α,17β-diol diacetate. G = 5α-androstane-3α,17β-diol diacetate (5α-adiol-diacetate). H = androst-5-ene-3β,17α-diol diacetate. I = androst-5-ene-3β,17β-diol diacetate. J = 5β-pregnane-3α,20α-diol diacetate (5β-pdiol-diacetate). K = 5α-pregnane-3α,20α-diol diacetate. L = pregn-5-ene-3β,20α-diol diacetate.

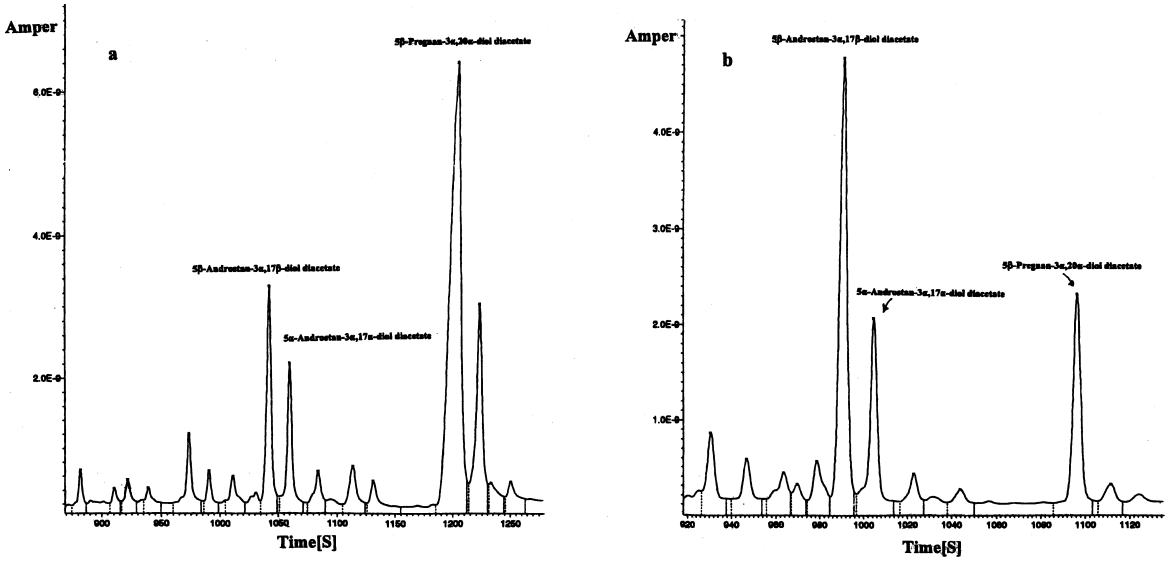

Fig. 3. GC–C–IRMS chromatograms from a negative (a) and a positive (b) urine.

interest. Fig. 3 also has a flat baseline and background characteristic of GC–C–IRMS. Examples of the spectra of the peaks of interest and their retention times are shown in Fig. 2.

The characteristics of the chromatograms were optimized by three successive SPE extractions. In the first step, the conjugated steroids were extracted using C_{18}-SPE [16]. Following enzymatic hydrolysis, we found that a second SPE step gave higher recoveries compared to liquid extraction. In addition, the second SPE step substantially improved the quality of the chromatograms, and it was accomplished with a minimum volume of elution solvent (12 ml).

In our previous method [3,4] the acetates were purified by HPLC, a procedure which inevitably carries some risk of isotopic discrimination. In the new procedure it is important to show that discrimination does not occur during the final acetonitrile–water (75:25, v/v) wash. To accomplish this we determined the $\delta^{13}C$‰ values on the diacetates of 5β-pregnane-3α,20α-diol, 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol after washing with 15 and 21 ml of acetonitrile–water (75:25, v/v). In order to ensure a range of $\delta^{13}C$‰ values and heterogeniety, we studied eight urines obtained from four subjects, before and after T administration. As shown in Table 2, the wash volume did not significantly influence the $\delta^{13}C$‰ values of the diacetates ($p=0.2$, before and $p=0.06$, after). In addition, we noted that the 15 ml wash eliminated the underivatized and monoacetylated steroids, while the 21 ml wash resulted in some loss of androstanediol diacetates. The underivatized keto steroids are also

Table 2
Effect of the volume wash of acetonitrile–water (75:25, v/v) on the carbon isotope ratio of diacetylated steroids 5β-pdiol, 5α-adiol, and 5β-adiol in four pre-T and four post-T urine at the third SPE clean-up

Urine	Compound	Volume of Wash (ml)			
		15	21	15	21
		Pre-T urine		Post-T urine	
1	5β-pdiol	−25.82	−25.70	−25.36	−25.64
2		−26.11	−25.12	−24.78	−24.69
3		−25.14	−24.65	−24.48	−24.07
4		−25.07	−23.09	−24.62	−24.62
1	5β-adiol	−25.90	−25.56	−31.60	−30.95
2		−27.04	−26.09	−30.95	−32.58
3		−24.36	−23.52	−28.40	−30.94
4		−25.08	−26.88	−31.11	−33.10
1	5α-adiol	−25.48	−25.34	−28.91	−29.24
2		−26.46	−23.52	−30.74	−31.99
3		−25.29	−26.38	−29.67	−29.02
4		−25.62	−26.07	−29.06	−31.53

eliminated during the first 12 ml wash with acetonitrile–water (50:50, v/v).

3.2. Analysis time and urine volume

In our previous publications, the HPLC step was rate-limiting with the result that a batch of 10 samples required nearly 2 days to process [2–4]. The new method enables a batch of 10 samples to be extracted and analyzed by GC–C–IRMS analysis in 8 h. In our previous publications [2–4], it was necessary to use 30 ml of urine in order to obtain satisfactory estimates of the ^{13}C‰ of T. The typical male urine received in our doping control laboratory contains ~40 ng/ml of T, whereas the median concentrations of 5β-adiol and 5α-adiol are 121 and 68 ng/ml, respectively. These concentrations were determined by summarizing our database of ~10 000 male urines. The higher concentration of the diols enables the new analysis to be carried out on 10 ml of male urine. We determined the concentration of the diols from the GC–MS screening analysis, then selected a sample volume that would provide approximately 700 ng of each diol.

3.3. Choice of enzyme for deconjugation

The three diols of interest are excreted as both glucuronide and sulfate conjugates [17], therefore the specific advantages and disadvantages of deconjugating with β-glucuronidase from *E. coli* or *H. pomatia* are considered. The *H. pomatia* enzyme gives a greater yield of sulfates; however, the 3 h hydrolysis time is a distinct disadvantage for a rapid screening method. *E. coli* enzymatic hydrolysis takes only 1 h due to a high specific activity, which provides chromatograms with excellent separation of 5β-adiol, 5α-adiol, and 5β-pdiol from surrounding peaks. In addition, when *H. pomatia* was used to deconjugate, the chromatograms were more complex because several isomers of the diols of interest were detected.

3.4. Selection of T metabolites and of andogenous reference

Following a dose of T, only 1–2% is excreted as T conjugates and another 4–5% is excreted as 5β-adiol and 5α-adiol [17–19]. Some dihydrotestosterone and other minor metabolites of T are detected in human urine but the concentrations are too low for carbon isotope ratio measurements. The majority of administered T is excreted as androsterone and etiocholanolone. These two steroids are present in large amounts (1–3 μg/ml); however, they are the final elimination path for many steroids, therefore the effect of T administration on their ^{13}C‰ values is diluted. Although we are investigating the ^{13}C‰ of androsterone and etiocholanolone, at the present time the optimal metabolites of T for ^{13}C‰ analysis are 5β-adiol and 5α-adiol.

The endogenous reference concept is important to the analysis because the selected compound serves as an internal control. The ^{13}C‰ of the endogenous reference reflects the diet of the individual and will vary among individuals [2–5]. By using an endogenous reference, one can better estimate the degree of incorporation of exogenous T into T and its metabolites. Furthermore, one can calculate derived variables such as the ratio of ^{13}C‰ of endogenous reference/metabolite or the difference between the ^{13}C‰ of the endogenous reference and metabolite.

In our previous studies, we determined the ^{13}C‰ of T precursors cholesterol acetate, androst-5-ene-3β,17β-diol diacetate, and dehydroepiandrosterone acetate. We have abandoned cholesterol because the origin of urinary cholesterol is not well understood. Dehydroepiandrosterone acetate and androst-5-ene-3β,17β-diol diacetate provide satisfactory ^{13}C‰ measurements [2,3]; however, they are not present in sufficient quantities after deconjugation with *E. coli*. In the present assay, the recovery of 5β-pdiol is high and its chromatographic properties are excellent. In addition, Shackleton et al. report that the ^{13}C‰ of 5β-pdiol is not affected by T administration and they find it to be a good internal reference standard [5].

3.5. Detection of testosterone administration

A principal objective of this work was to determine if our new method further confirms the utility of ^{13}C‰ measurements in detecting T administration. The T/E of the pre-T urines were all less than 2.3 (mean = 1.1, range = 0.2–2.3). The mean post-T urine T/E was 23 (range = 10.2–61). These data confirm that T administration increases

T/E and that the samples are suited for determining the effect of T administration on the $\delta^{13}C‰$ of andogenous reference and metabolites.

To strengthen our confidence in the method, the analyst conducting the GC–C–IRMS and data reduction was blind to the content of the samples and had no knowledge of the clinical protocol. Table 1 shows the T/E and $\delta^{13}C‰$ values for 10 subjects who submitted pre-T and post-T urines. Subjects A–E received T enanthate (300 mg/week) and the post-T urines were collected in the 4th or 7th week of weekly T administration, and subjects F–J received T by intravenous infusion and the post-T urine was collected on days 5–7. Following T administration, the $\delta^{13}C‰$ values for both diols decreased. The mean pre-T and post-T $\delta^{13}C‰$ values for 5β-adiol were −26.2 (C.V.=2.6%) and −30.8 (C.V.=5.6%), respectively. This difference was significant at $p<0.001$. Likewise the mean pre-T and post-T $\delta^{13}C‰$ values for 5α-adiol were −25.2 (C.V.=3.9%) and −29.9 (C.V.=3.1%), respectively ($p<0.001$). The magnitude of the mean decrease was 4.6 for 5β-adiol and 4.7 for 5α-adiol. These findings confirm that the

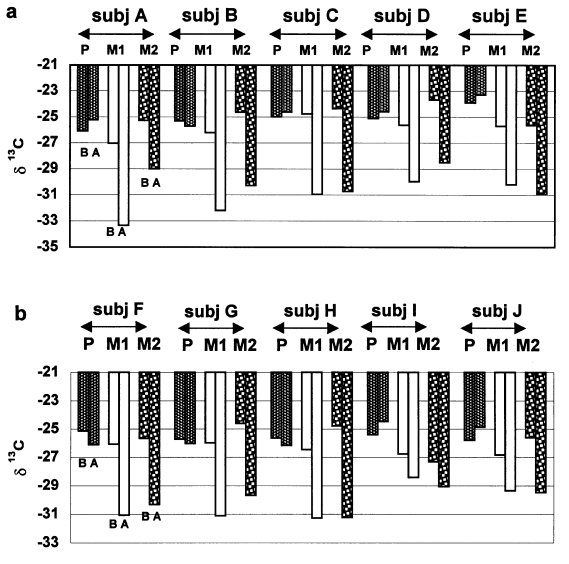

Fig. 4. Mean carbon isotope ratio values ($\delta^{13}C‰$) of pregnanediol (P=5β-pdiol), and T metabolites (M1=5β-adiol), (M2=5α-adiol) in five subjects who received T enanthate (3a) and five subjects who received T by infusion (3b). Pre-T $\delta^{13}C‰$ values are labeled before (B) and post-T $\delta^{13}C‰$ values are labeled after (A).

$\delta^{13}C‰$ of T metabolites decreases following T administration.

As expected, the carbon isotope ratio for 5β-pdiol was not affected by exogenous T administration. The mean pre-T and post-T $\delta^{13}C‰$ values were −25.3 (C.V.=2.4%) and −25.1 (C.V.=3.7%), and these values were not significantly different ($p=0.34$). These results concur with those obtained by Shackleton et al. [5], and confirm that the $\delta^{13}C‰$ of T precursors are not affected by T administration. In addition, the $\delta^{13}C‰$ values for 5β-pdiol were similar to the values observed in our previous work for other T precursors: cholesterol (−24.6‰), DHEA (−24.3‰), and androst-5-ene-3β,17β-diol (−26.2‰) [2–4].

Fig. 4a and b summarize the data such that the consistency of the pre-T $\delta^{13}C‰$ measurements and the magnitude of the change in the post-T samples are apparent. The pre-T and post-T samples are shown as pairs with the pre-T $\delta^{13}C‰$ on the left, and the post-T values on the right (indicated with arrows for subj A). It is noteworthy that the magnitude of the post-T change in the $\delta^{13}C‰$ of 5β-adiol and 5α-adiol in subjects I and J (Fig. 4b) is less than that of the other subjects. These two subjects were the only Asian–Americans in the study and there is some evidence that Asians may differ from non-Asians in the magnitude of the effect of exogenous T on T/E [5].

3.6. Additional markers of T administration

To further elucidate the relationship between the $\delta^{13}C‰$ of pregnanediol and androstanediols we calculated two derived variables, namely the differences in $\delta^{13}C‰$ between 5β-adiol and 5β-pdiol and between 5α-adiol and 5β-pdiol. As shown in Table 1, the maximum difference in (5β-adiol)–(5β-pdiol) and (5α-adiol)–(5β-pdiol) in urines obtained pre-T were 1.8 and 1.9, respectively; these pre-T values should be compared to the minimum post-T differences which are 3.9 and 3.6, respectively. The maximum post T differences were 8.2 and 7.7, respectively. A t-test (paired) on the pre-T and post-T difference scores for (5β-adiol)–(5β-pdiol) was significant ($p<0.001$). Similarly the (5α-adiol)–(5β-pdiol) difference scores were significant ($p<0.001$). This confirms that the difference between the $\delta^{13}C‰$

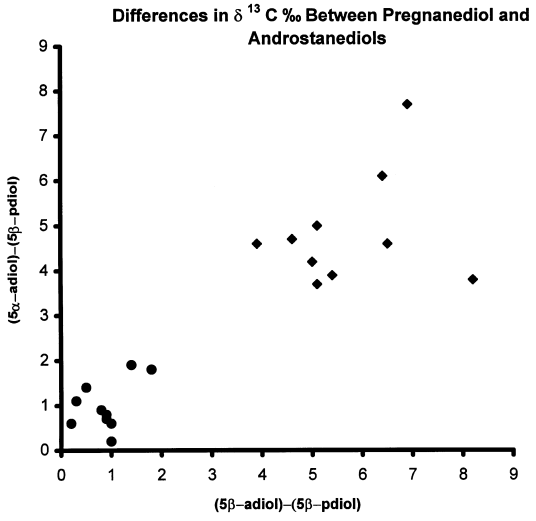

Fig. 5. Testosterone metabolite-pregnanediol δ^{13}C‰ values of pre-T (●) and post-T (♦) urines collected from 10 subjects. The *X*-axis is the difference between 5β-adiol and 5β-pdiol (5β-adiol)–(5β-pdiol) and the *Y*-axis is the difference between 5α-adiol and 5β-pdiol (5α-adiol)–(5β-pdiol).

values for the andogenous reference and metabolites accurately discriminate between urines collected before and after T administration. Fig. 5 plots these difference scores for the pre-T and post-T urines. The values on the pre-T urines cluster in the lower left and all values are less than 1.9. The post-T urines all have difference scores that exceed 3.6.

The ratio of $^{13}C/^{12}C$ values of the androstanediols (AD) to pregnanediol (PD) has been reported to be a useful indicator of T administration [5]. Accordingly, we calculated the mean $^{13}C/^{12}C$ of the two androstanediols (5α-adiol + 5β-adiol)/2 and the ratio (5α-adiol + 5β-adiol)/2*5β-pdiol (AD/PD). The range of AD/PD $^{13}C/^{12}C$ values for the pre-T urines is 0.981–1.074, which is in excellent agreement with the maximum baseline value of 1.08 reported by Shackleton et al. [5]. Further, the AD/PD ratios in the post-T urines (range 1.167–1.312) were all below 1.1, the value suggested by Shackleton et al. [5] to distinguish between T users and non-users.

As far as the acetate group's contribution is concerned the δ^{13}C‰ values that we obtained are very close to those obtained by Shackleton et al. [5] and Aguilera [20] even though different source batches of acetic anhydride were used. Finally, the conclusions concerning a negative or positive case are not affected by the discrepancies between derivatized and free compounds as they remain consistent within a normal range variation. In addition, the contribution of the acetate group is the same for the endogenous references and the testosterone metabolites.

4. Conclusions

We propose a new screening method for the detection of exogenous testosterone administration using GC–C–IRMS. The method meets the requirements for use in routine screening. Firstly, only 10 ml of male urine was required to obtain satisfactory estimates of δ^{13}C‰. Secondly, by replacing the HPLC step in our previous procedure with three successive solid-phase extractions, greater speed and efficiency was achieved by processing samples in batches. Thirdly, the choice of deconjugating with β-glucuronidase from *E. coli* rather than from *H. pomatia* resulted in high quality chromatograms with excellent resolution of the peaks of interest. The method gives consistent and reproducible δ^{13}C‰ values with a mean C.V. for replicates of 1.6%. We confirmed that T administration results in a decrease in the δ^{13}C‰ values of T metabolites, and we found significant differences between the δ^{13}C‰ values of (5β-pdiol)–(5β-adiol) obtained on the pre-T and post-T urines, and between the (5β-pdiol)-(5α-adiol) of the pre-T and post-T urines.

We propose that the method described herein be used to supplement the T/E results obtained GC–MS. In the case of an elevated T/E and normal δ^{13}C‰ values there would be no need to notify the athlete that additional testing is necessary. The sample would be reported negative. With further experience, we anticipate that samples with low δ^{13}C‰ values and T/E>6 could be reported positive for T. Finally, the method may detect cases of T administration characterized by low δ^{13}C‰ values and a T/E that is less than six.

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