Urinary Analysis of Four Testosterone Metabolites and Pregnanediol by Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry after Oral Administrations of Testosterone

Arnaud Maître, Christophe Saudan*, Patrice Mangin, and **Martial Saugy**

Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland

Abstract

The most frequently used method to demonstrate testosterone abuse is the determination of the testosterone and epitestosterone concentration ratio (T/E ratio) in urine. Nevertheless, it is known that factors other than testosterone administration may increase the T/E ratio. In the last years, the determination of the carbon isotope ratio has proven to be the most promising method to help discriminate between naturally elevated T/E ratios and those reflecting T use. In this paper, an excretion study following oral administration of 40 mg testosterone undecanoate initially and 13 h later is presented. Four testosterone metabolites (androsterone, etiocholanolone, 5α-androstanediol, and 5β-androstanediol) together with an endogenous reference (5β-pregnanediol) were extracted from the urines and the $\delta^{13}C/^{12}C$ ratio of each compound was analyzed by gas chromatography–combustion–isotope ratio mass spectrometry. The results show similar maximum $\delta^{13}C$-value variations (parts per thousand difference of $\delta^{13}C/^{12}C$ ratio from the isotope ratio standard) for the T metabolites and concomitant changes of the T/E ratios after administration of the first and the second dose of T. Whereas the T/E ratios as well as the androsterone, etiocholanolone and 5α-androstanediol $\delta^{13}C$-values returned to the baseline 15 h after the second T administration, a decrease of the 5β-androstanediol δ-values could be detected for over 40 h. This suggests that measurements of 5β-androstanediol δ-values allow the detection of a testosterone ingestion over a longer post-administration period than other T metabolites $\delta^{13}C$-values or than the usual T/E ratio approach.

Introduction

The detection of doping with testosterone is mainly based on the concentration measurement of the testosterone (T) to epitestosterone (E) ratio (T/E ratio). If this T/E ratio is greater than 6 in the urine of a competitor, it is mandatory that additional investigations should be conducted. Isotope ratio mass spectrometry (IRMS) may be used in this context as an additional analysis to help in deciding whether an elevated T/E ratio can be explained by a misuse of testosterone (1–4) or testosterone precursors (4,5). Indeed, it is known that other factors than testosterone administration may lead to T/E ratio exceeding 6 (6,7).

IRMS allows measurements of slight differences in the carbon isotope ratio ($^{13}C/^{12}C$) of the exogenous and endogenous testosterone. Synthetic testosterone is produced from precursors derived from plants with low ^{13}C content, whereas the ^{13}C and ^{12}C content in the natural endogenous form depends on the isotopic carbon composition of the food diet and is influenced by additional effects of human biological processing (8). Such differentiation between synthetic and endogenous origins has also been reported for other hormones such as epitestosterone (9), nandrolone (10), and corticosteroids (11).

Very few time profile IRMS studies with the chronological determination of T/E ratio after oral testosterone administration are reported in the literature (2,3,12). Chronological measurement of the carbon isotopic ratio of T metabolites was able to detect T administration in cases in which the T/E ratio remained below 6 (2). It must be noted that in this latter study the five volunteers were of Chinese ethnicity, and therefore, the cutoff limit of a T/E ratio of 6 that is suited for the caucasian race is questionable when used on a Chinese subject population. It is also worth mentioning that the excretion studies on two caucasian volunteers after one oral ingestion of T undecanoate (3). These excretion studies report the detection of exogenous T by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) after the T/E ratio had returned to below 6. The determination of exogenous T with GC–C–IRMS was possible for more than twice as long as with application of T/E ratio limits.

* Author to whom correspondence should be addressed: Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland. E-mail: csaudan@hospvd.ch.

This paper describes the time courses of isotopic ratio values in urine of androsterone (Andro), etiocholanolone (Etio), 5α-androstanediol (5αA), 5β-androstanediol (5βA), and the endogenous reference 5β-pregnanediol (5βP) in the frame of an excretion study following oral ingestion of testosterone initially and 13 h later by a healthy, male, caucasian volunteer (Figure 1). The profile of the T/E ratio is also compared with the results obtained by IRMS. These excretion studies are fundamental in understanding how the time profile of IRMS values is affected after multiple oral ingestions of testosterone. To our knowledge, there is no report of chronological application of the isotope ratio technology and the T/E ratio to monitor an excretion study with multiple oral testosterone ingestions.

Experimental

Chemicals

All solvents and reagents of analytical grade purity were purchased from Fluka (Buchs, Switzerland). 5α-Androstan-3β-ol acetate, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5β-pregnane-3α,20α-diol, etiocholanolone, and androsterone with a purity of at least 99.5% were obtained either from Steraloids Inc. (Newport, RI) or Sigma Chemical Co. (St. Louis, MO). Bakerbond spe™ 500-mg or 1000-mg octadecyl C_{18} disposable extraction columns were obtained from J.T. Baker (Phillipsburg, NJ). β-Glucuronidase from Escherichia coli in a 50% glycerol solution (pH 6.5, 140 U/mL at 37°C) was supplied by Roche Diagnostics GmbH (Mannheim, Germany). The reference carbon dioxide gas was purchased from Carbagaz (Domdidier, Switzerland). The mixture of three alkanes, C_{15} (n-pentadecane), C_{20} (n-eicosane), and C_{25} (n-pentacosane), was supplied by Chiron AS (Trondheim, Norway).

Figure 1. Metabolic pathways of testosterone.

Dosage and collection of urine samples

A 40-mg testosterone undecanoate capsule (Panteston®, Organon SA, France) were self-administered orally initially and 13 h later by a healthy, male, caucasian volunteer. Baseline urine samples were obtained before initial administration, and 39 subsequent spot urine samples were collected over a period of 100 h after the first capsule administration. Reference urine samples were collected from 40 caucasian male subjects. In this excretion study, the spot urine with the T/E ratio of 36 at $t = 2.3$ h was selected to be the positive quality control urine (QC-pos). The negative quality control urine (QC-neg) was obtained from a healthy subject. Each urine was aliquoted into 15-mL cryogenic tubes and stored without additives at –20°C until analysis.

Sample preparation

The urine sample (10 mL) was centrifuged in glass tubes at 2500 rpm for 5 min and was applied onto a 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 residue was dissolved in 1 mL of 0.2M phosphate buffer (pH 7.0) and 50 µL of β-glucuronidase was added as supplied. After incubation at 50°C during 1 h (or at 37°C overnight) in a thermostated water bath, the deconjugated steroids were extracted on C_{18} column (500 mg) by washing with 6 mL acetonitrile/H_2O (20:80, v/v) and subsequently by 6 mL acetonitrile/H_2O (35:65, v/v) and finally eluted with 12 mL acetonitrile. The eluate was evaporated to dryness under a nitrogen stream (Turbo Vap LV evaporator, Zymark, Hopkinton, MA), and then the residue was dried over phosphorus pentoxide for 20 min. Acetylation of the extract was carried out in 50 µL of pyridine and 50 µL of acetic anhydride at 60°C for 1 h incubation. The reaction medium was evaporated to dryness and subsequently the residue was redissolved in 3 mL of acetonitrile/H_2O (50:50, v/v). The solution was applied onto a C_{18} column (1000 mg). The fraction F1 containing androsterone and etiocholanolone was obtained by elution with 15 mL acetonitrile/H_2O (75:25, v/v) after washing with 12 mL of acetonitrile/H_2O (50:50, v/v). Finally, the fraction F2 with the androstandiols and the pregnanediol was obtained by eluting with 12 mL of acetonitrile. The derivatized steroids of fractions F1 and F2 were spiked with 40 µL and 10 µL of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/mL in methanol), respectively. Then both fractions F1 and F2 were evaporated to dryness and finally dissolved in 500 µL and 40 µL of cyclohexane, respectively.

GC–C–IRMS analysis

The carbon isotope measurements were performed on a Delta^{Plus} IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A GC (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separations were achieved on an HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (J&W Scientific, 30 m × 0.25-mm i.d., 0.15-µm film thickness). The injector temperature was set to 280°C.
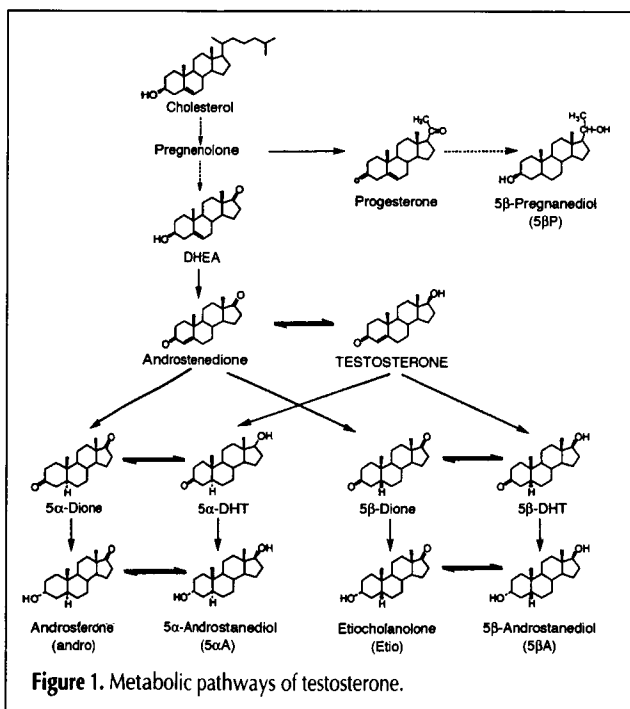
The combustion and reduction oven temperatures were set to 940°C and 600°C, respectively. Reference carbon dioxide gas pulses (20-s durations) were introduced at four different times during the course of the chromatographic separation. For the calibration of the reference gas with the alkane mixture of known $\delta^{13}C$-values, the oven temperature was increased from 80°C (1 min) to 270°C (7.0 min) at 15°C/min. For the analysis of the androsterone and etiocholanolone acetates (fraction F1), the oven temperature was increased from 80°C (1 min) to 270°C (8.3 min) at 15°C/min, then to 300°C at 35°C/min, and maintained at the final temperature for 3 min. For the analysis of the fraction F2 containing the androstandiols and the pregnanediol acetates, the oven temperature was increased from 80°C (1 min) to 270°C (11.5 min) at 15°C/min, then to 300°C at 35°C/min, and maintained at the final temperature for 3 min. The volume of injection was 1–2 μL, depending on the estimated concentrations of the compounds of interest as evaluated in the frame of the T/E ratio determination. The extracts were injected twice in the splitless mode, and the replicate measurements were averaged.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}C/\text{‰} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}}$$

Negative shifts of the $\delta^{13}C$-value due to the formation of an acetate were corrected as follows:

$$D_{OH} = D_{OAC} + 2m \frac{(D_{OAC} - D_{AC})}{n}$$

Where D_{OH} is $\delta^{13}C$-value for the underivatized steroids, D_{OAC} is the $\delta^{13}C$-value for the acetylated steroids, D_{AC} is the $\delta^{13}C$-value for the acetylating reagent, n is the number of carbon atom in a molecule, and m is the number of hydroxyl groups to be acetylated.

All subsequent $\delta^{13}C$-values have been corrected for this negative shift. The D_{AC} value of our acetylating reagent was determined to be –48.8‰ according to a published procedure (13).

Determination of steroid concentration and T/E ratio

Urinary testosterone and epitestosterone were quantified in all the samples and the concentration of the steroids of interest were quantified according to the method of Nolteernsting et al (14).

Results and Discussion

Isotope measurements, accuracy, and reproducibility of the method

The reference carbon dioxide gas, which is pulsed several times during the course of the chromatographic separation, is calibrated by a mixture of three alkanes, C_{15} (n-pentadecane),

C_{20} (n-eicosane), and C_{25} (n-pentacosane), from Chiron AS with $\delta^{13}C$-values of –30.22, –33.06, and –28.21‰, respectively. The internal standard (5α-androstan-3β-ol acetate) was measured together with this alkane mixture and the $\delta^{13}C$ value was –31.9‰ (n = 50, SD = 0.2‰).

The extraction method allows the separation of androsterone (Andro) and etiocholanolone (Etio) acetates from the 5α-androstanediol (5αA), 5β-androstanediol (5βA), and pregnanediol (5βP) acetates on the C_{18} column by eluting with different ratio mixture of acetonitrile/H_2O (15). Before the GC–C–IRMS analysis, an internal standard (5α-androstan-3β-ol acetate) with a known $\delta^{13}C$-value is spiked in both fractions. Figure 2 shows an example of GC–C–IRMS chromatograms of m/z 44 for fractions F1 and F2. The chromatograms show symmetrical peaks for the compounds of interest, no tailing, and no evidence of co-eluting compounds.

Each batch includes positive (QC-Pos) and negative (QC-Neg) quality controls and the measurements are performed for all metabolites in the linear range of the IRMS. The purpose of the quality controls is to verify the reproducibility of the extraction procedure and the GC–C–IRMS measurements. The two incubation procedures given in the experimental part have been verified with the positive and negative quality controls to
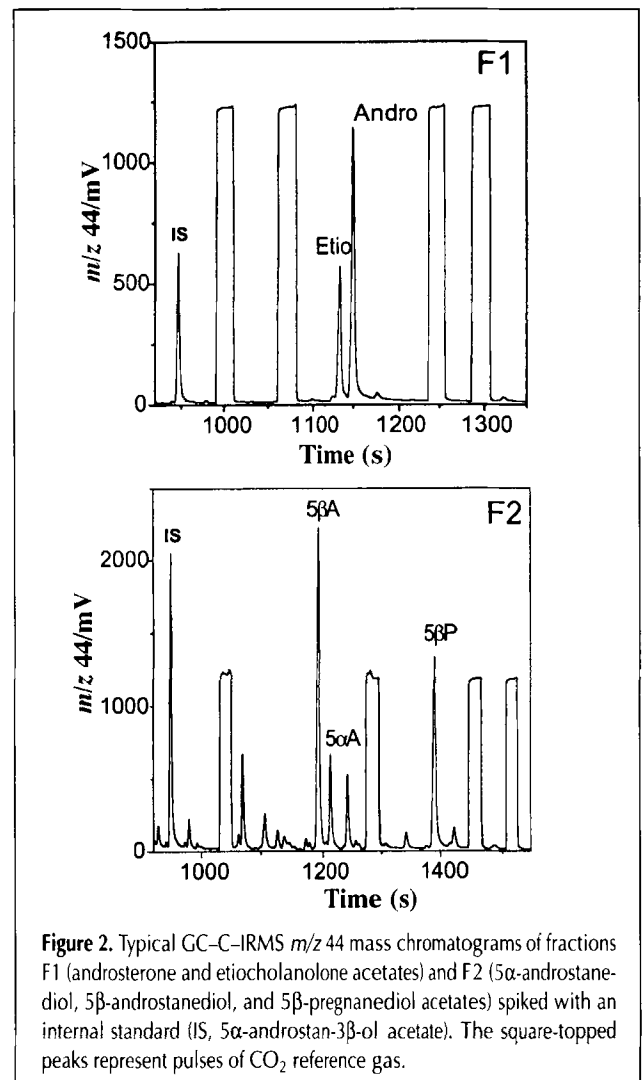

Figure 2. Typical GC–C–IRMS m/z 44 mass chromatograms of fractions F1 (androsterone and etiocholanolone acetates) and F2 (5α-androstanediol, 5β-androstanediol, and 5β-pregnanediol acetates) spiked with an internal standard (IS, 5α-androstan-3β-ol acetate). The square-topped peaks represent pulses of CO_2 reference gas.

give comparable results. The within-assay precision was determined by extracting six aliquots of QC-Pos and QC-Neg and injecting each once within the same day. For the determination of the between-assay precision, 20 aliquots of QC-Pos and QC-Neg were extracted by 4 different technicians over a period of 18 months, and injected once each. For the within-assay precision the overall means and SD for etiocholanolone, androsterone, 5β-androstanediol, 5α-androstanediol, and 5β-pregnanediol in

the QC-neg were −24.6 (± 0.1), −24.2 (± 0.2), −23.9 (± 0.1), −23.6 (± 0.3), and −23.2 (± 0.2) ‰, respectively, whereas in the QC-pos, −27.9 (± 0.3), −27.7 (± 0.2), −28.2 (± 0.2), −28.0 (± 0.2), and −23.2 (± 0.2) ‰ were obtained. For the between-assay precision, the overall means and SD for etiocholanolone, androsterone, 5β-androstanediol, 5α-androstanediol, and 5β-pregnanediol in the QC-neg were −24.4 (± 0.4), −24.0 (± 0.4), −24.3 (± 0.3), −24.3 (± 0.4), and −23.7 (± 0.4) ‰, respectively. For the QC-pos, means and SDs of −27.8 (± 0.4), −27.9 (± 0.4), −27.8 (± 0.4), −28.4 (± 0.5), and −23.2 (± 0.3) ‰ were obtained. These results indicate that the SDs for each compound measured with QC-neg and QC-pos are slightly higher in the case of the between-assay precision compared to the within-assay precision. Although this method allows for the $\delta^{13}C$-value determination of five steroids, the SDs are comparable to existing IRMS methods used for two T metabolites (12,16).

$\delta^{13}C$ Values following oral testosterone administration

The GC–C–IRMS and the T/E ratio results from the excretion study following oral administration of 40 mg testosterone undecanoate initially and 13 h later is shown in Figure 3. Figure 3 also shows the mean and the mean-3SD $\delta^{13}C$-values for androsterone, etiocholanolone, 5αA, 5βA, and 5βP obtained after analysis of a reference population of healthy caucasian males (n = 40). Table I lists also the mean $\delta^{13}C$-values, the median $\delta^{13}C$-values, the SDs, the $\delta^{13}C$ range values of androsterone, etiocholanolone, 5αA, 5βA, and 5βP, together with the results obtained by Ueki and Okano (5) for 5αA, 5βA, and 5βP from a population of 450 athletes who had participated to the XVII Olympic Winter Games in Nagano (Japan). This population with a majority of Caucasian subjects may be compared to our data because a correction factor was used to convert all measured $\delta^{13}C$-values of the acetylated compounds to give values for underivatized steroids. A correction factor was not applied for the population studies obtained by Shakelton et al. (2) and Aguilera et al. (16). As listed in Table I, only 5αA, 5βA, and 5βP may be compared with the data of Ueki and Okano (5). There are striking differences between the ranges found in the two laboratories with mean differences of 6.1, 4.2, and 2.3‰ for 5αA, 5βA, and 5βP, respectively. Moreover, our SDs are much lower to those obtained by Ueki and Okano (5). These results may be explained by a difference of the analytical method or from the diet heterogeneity of the population. Indeed, the diet of subjects from Europe is different from that of America and Japan and before the XVII
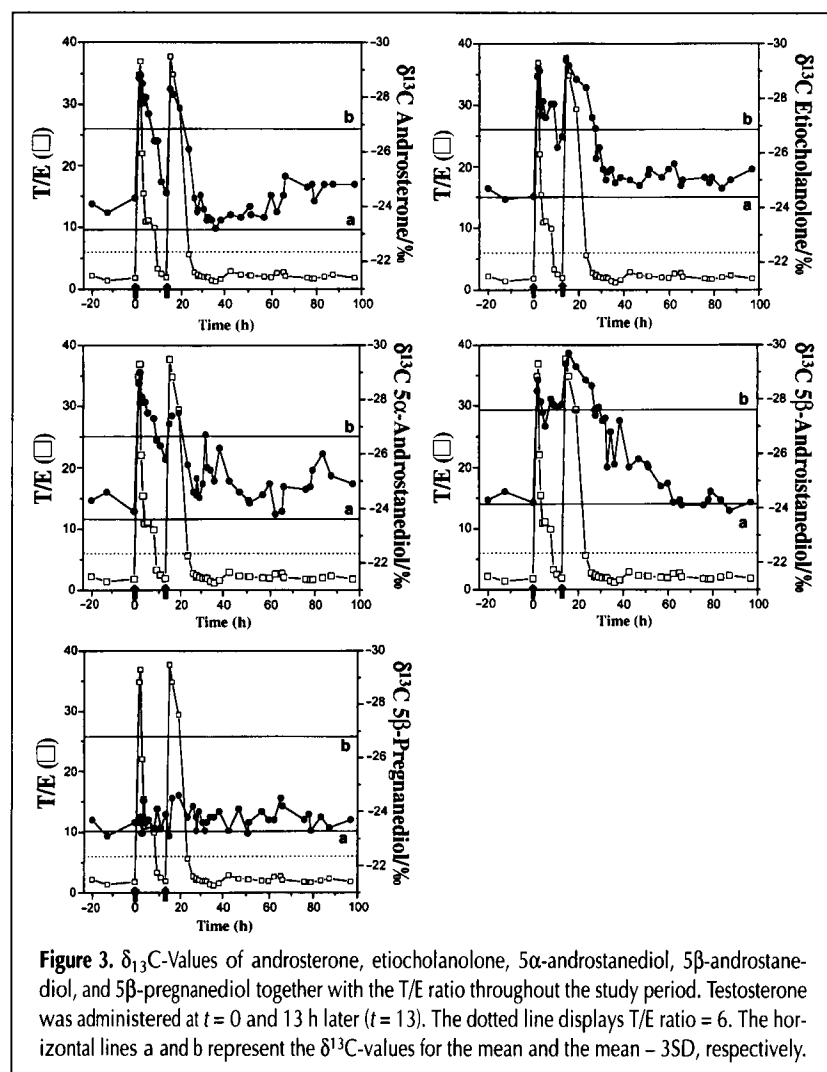
Figure 3. $\delta_{13}C$-Values of androsterone, etiocholanolone, 5α-androstanediol, 5β-androstanediol, and 5β-pregnanediol together with the T/E ratio throughout the study period. Testosterone was administered at t = 0 and 13 h later (t = 13). The dotted line displays T/E ratio = 6. The horizontal lines a and b represent the $\delta^{13}C$-values for the mean and the mean − 3SD, respectively.

Table I. Absolute $\delta^{13}C$-Values of Androsterone, Etiocholanolone, 5αA, 5βA, and 5βP of a Healthy, Caucasian, Male Population*

$\delta^{13}C$-value /‰	Mean		SD		Range		Median this study
	This study	ref 5	This study	Ref 5	This study	Ref 5	
Androsterone	−23.2	–	1.23	–	−21.2 to −25.3	–	−23.2
Etiocholanolone	−24.4	–	0.83	–	−22.1 to −25.8	–	−24.6
5αA	−23.6	−17.5	1.00	3.53	−21.8 to −25.9	−10.5 to −24.6	−23.8
5αA	−24.2	−20.0	1.15	2.75	−22.0 to −26.3	−15.2 to −24.6	−24.3
5αP	−23.3	−21.0	1.17	1.65	−20.7 to −25.6	−17.2 to −23.8	−23.2

* (n = 40). Data obtained by Ueki and Okano (5) for an athlete population of males and females of various ethnicities (n > 350).

Olympic Winter Games in Nagano (Japan), the athletes did not eat the local diet for a sufficient time to have a measurable change of the steroids $\delta^{13}C$-values in urine. Differences to ethnicity may not explain the ranges of the $\delta^{13}C$-values for 5αA, 5βA, and 5βP in a population (2,16).

As reported in previous studies (2,8), the $\delta^{13}C$-value for 5bP should not be affected by the administration of testosterone since it has to be produced from cholesterol via several intermediates (Figure 1). A change of the $\delta^{13}C$-value for 5βP and for T metabolites could be expected if the individual modify drastically the diet during the study period. In the 41 urines collected during the excretion study, we determined a mean $\delta^{13}C$-value of –23.7‰ with SD = ± 0.4‰, range: –22.6 to –24.6‰ and median = –23.7‰ for 5βP. The SD value is similar to what obtained with the between-assay of QC-neg and QC-pos and shows that the $\delta^{13}C$-values for 5βP remain constant during the time course of the excretion. These findings reveal no dietary modification during the excretion study and are in agreement with previous investigations (2,8).

Interestingly, T/E ratio reach a maximum of 36 after both administrations and this value is higher than what has been reported (3) after an oral dose of 40 mg testosterone undecanoate for two caucasian individuals (T/E ratio = 22). These differences may be rationalized as an interindividual variability in metabolism (17), such as genetic differences in the cytochrome P450 system (7) and preferential biotransformation of the steroids. Differences in volume distribution and body weights may also explain these results.

It is noteworthy that the T/E ratio increased rapidly following testosterone administration but returned below 6 after 10 and 15 h for the first and second T administrations, respectively (Figure 3). Therefore only the urines collected during this period would be considered as suspect and subject to further procedures (6). Carbon isotope ratio measurements are also used for that purpose in detecting testosterone abuse. Similarly to the T/E ratio, the $\delta^{13}C$-values of the four T metabolites decrease rapidly after T administration with a difference of about 5‰ with respect to the endogenous reference 5βP. These findings together with the fact that the values are out of the range as defined by the population mean – 3SD for all T metabolites would be strong indicators of testosterone abuse. Indeed, a difference superior to 4‰ with respect to the endogenous reference 5βP is indicative of an exogenous T ingestion (8,16). This difference of 4‰ is slightly higher to 3SD for all T metabolites of the reference population (Table I).

The results show similar maximum $\delta^{13}C$-value variations for the T metabolites and concomitant changes of the T/E ratios after administration of the first and the second dose of T. Whereas androsterone, etiocholanolone, and 5α-androstanediol δ-values returned to the baseline 15 h after the second T administration, a decline in the 5α-androstanediol δ-values could be detected over 40 h. This finding may be related to different metabolic pathways with different kinetics from testosterone to T metabolites. For instance, it is known that urinary 5α-androstanediol arise from both hepatic and peripheral metabolism whereas urinary 5β-androstanediol is considered to arise only from hepatic metabolism (16). One should also consider the wash out period between the administration of the first and the

second dose. About 10.3 h after the second T dose, it may be observed on the T/E ratio profile that there is likely no residual effect on this parameter from the first dose, whereas it may not be excluded from the 5β-androstanediol and ethiocholanolone $\delta^{13}C$-values. Other excretion studies on different subjects with multiple T ingestions are variable dose schedules would be needed to test this effect.

It may be also emphasized that after ingestion of the second dose of T, there is a spot urine with a T/E ratio below 6 (T/E = 5.6 at t = 23.3 h) that shows for androsterone, etiocholanolone, 5α-androstanediol, and 5β-androstanediol a difference of 2.3, 4.6, 1.8, and 4.9‰, respectively, with the $\delta^{13}C$-value of the endogenous reference 5βP. For the subsequent spot urine at t = 26 h, a T/E ratio of 2.7 was obtained with a $\delta^{13}C$-value superior to 4‰ for 5β-androstanediol. From these results and other studies (3,12), it may be asked in routine procedure: 1. If the T/E ratio at 6 is still a valid cutoff and 2. What are the IRMS criteria to declare an exogenous T ingestion, that is, does all the measured T metabolite $\delta^{13}C$-values or does only one have to be superior to 4‰. Further excretion studies with multiple T ingestions should help in the future in deciding which criteria should be unilaterally adopted.

In conclusion, our results suggest that measurements of 5β-androstanediol δ-values allow the detection of a testosterone ingestion over a longer postadministration period than other T metabolites $\delta^{13}C$-values or than the usual T/E ratio approach. A difference of 4‰ with respect to the endogenous reference 5βP should be used as indicative of an exogenous T ingestion. As discussed previously, the use of a reference population to report positive samples is questionable.

Acknowledgment

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