Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (4)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1997

S. HORNING, H. GEYER, M. MACHNIK, W. SCHÄNZER, A. HILKERT, J. OEßELMANN:
Detection of Exogenous Testosterone by 13C/12C Analysis
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (4). Sport und Buch Strauß, Köln, (1997) 275-283

S. Horning, H. Geyer, M. Machnik, W. Schänzer
A. Hilkert* and J. Oeßelmann*

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

Institute of Biochemistry, German Sports University,
Carl-Diem-Weg 6, D-50933 Köln, Germany
*Finnigan MAT GmbH,
Barkhausenstraße 2, D-28197 Bremen, Germany

Introduction

Testosterone misuse in sport continues to be a problem for the athlete as well as the analyst. The testosterone/epitestosterone (T/E) ratio, measured by gas chromatography/mass spectrometry (GC/MS), is a useful indicator for testosterone administration, but there are instances where the urinary T/E ratio (T/E > 6) can lead to false negatives. In other cases, elevated T/E ratios obtained from individuals claiming to be drug free need to be evaluated, e.g., in a controlled study, to avoid false positives. Recently, there have been investigations on the use of carbon isotope ratio measurements to detect testosterone administration [1,2]. In 1994 Beechi *et al.* [1] presented a novel, effective method for confirming suspected testosterone doping using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Their method employed HPLC isolation of urinary (50 mL) steroids (testosterone, 5β-androstane-3α,17β-diol, dehydroepiandrosterone and cholesterol), followed by derivatization (acetylation with acetic anhydride in pyridine) and GC/C/IRMS analysis (mass spectral analysis of $^{12}CO_2$ and $^{13}CO_2$ formed via combustion of testosterone eluting from the gas chromatograph [3]). As synthetic testosterone has a ^{13}C abundance different from that of endogenous testosterone, determination of the $^{13}C/^{12}C$ ratio can be effectively used to detect testosterone misuse.

Modern, commercially available combustion isotope ratio GC/MS (irm-GC/MS) instruments easily reach detection thresholds below 10 ng (10 ng testosterone (0.035 nmole), or 0.66 nmole CO_2); however, a large number of ions of the minor isotope are necessary to obtain precision at the 0.1‰ level [4]. According to specifications, the Delta C irm-GC/MS instrument (Finnigan MAT, Bremen, Germany) can precisely determine an atomic percent excess (APE) of 0.0002 (0.2‰) with 1 nmole CO_2 (corresponding to 0.053 nmole (15.16 ng) testosterone). Hence, we reasoned that with efficient sample preparation techniques it should be possible to obtain reliable data carbon ratio on as little as 300 ng testosterone dissolved in 30 µl solvent (0.035 nmole/µL).

We have undertaken two excretion studies (oral application of 40 mg testosterone undecanoate to male volunteers) to determine the effectiveness of GC/C/IRMS for detecting testosterone administration. In parallel, excretion rates of the testosterone metabolites [5] were determined and testosterone/epitestosterone (T/E) ratios were measured, as it was of interest to see if synthetic testosterone could be detected in cases where the T/E ratio returned to its normal value. Carbon isotope ratios were determined for testosterone and, in a few cases, for testosterone metabolites, 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD), as their excretion rates increased significantly following testosterone administration.

Experimental

Urine samples were collected from two men (ages 33 and 39) following oral application of 40 mg testosterone undecanoate (Organon, Oberschleißheim, 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 normal procedure [6]. Based on the testosterone concentration, an appropriate volume of urine was aliquoted (between 2 and 20 mL) to obtain at least 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 dissolved 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. After hydrolysis, 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.

Testosterone and the two diol metabolites, 5αAD and 5βAD, were separated and purified using immunoaffinity chromatography (IAC) and high-performance liquid chromatography (HPLC) according to the described method [7]. The immunoaffinity antibody was raised in rabbits and directed towards testosterone-3-carboxy-methyloxime-bovine serum albumin. As the steroid is A-ring coupled, the antibody is selective towards the 17β-hydroxy group in the D-ring and is cross reactive to other 17β-hydroxy steroids, i.e., 5αAD and 5βAD. HPLC fractionation (Hewlett Packard (HP) 1090) of the IAC eluate was performed with a HP analytical column (LiChrospher RP 18, 5µ, 125x4 mm i.d.) with a water+acetonitrile mobile phase gradient increasing linearly from 30% acetonitrile to 100% in 17.5 min at a flow of 1 mL/min. Testosterone, eluting at 7.9-8.6 min, 5βAD (8.9-9.4 min) and 5αAD (9.5-10.0 min) were collected in glass tubes and the residue dried under vacuum.

Carbon isotope ratio measurements were performed using a Delta C irm-GC/MS instrument. The GC (HP 5890) was equipped with a SGE BPX5 column (17 m x 0.22 mm i.d., 0.25 µm film thickness) using helium as the carrier gas and injections were made either in the splitless mode or on-column (using a HP 0.53 mm i.d. precolumn). Standard solutions (10 µg/µL) of testosterone from Schering (Berlin, Germany) Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany) were dissolved in methanol and 2 µl aliquots were injected in the splitless mode. Extracts obtained from urine were dissolved in acetone and analyzed using on-column injection.

Carbon isotope ratios were expressed as the relative difference between the sample and an international standard (Pee Dee Belemnite). Calibrated CO_2 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), while for the urine samples the standard deviation was slightly larger.

Results and Discussion

This study was two-fold: excretion rates were determined for testosterone and metabolites after oral ingestion of testosterone-undecanoate and carbon isotope ratio measurements were performed on testosterone and, in a few cases, 5αAD and 5βAD, extracted from urine. Excretion rates for testosterone, androsterone, etiocholanolone, 5αAD and 5βAD following ingestion of testosterone undecanoate are listed in Table 1 together with the T/E ratio. Note that the excretion rates for 5αAD and 5βAD remained elevated even after testosterone returned to its normal value.

Table 1. Urinary excretion rates of testosterone and metabolites after ingestion of 40 mg testosterone undecanoate.

Time h	T/E	r T µg/h	r A µg/h	r E µg/h	r 5αAD µg/h	r 5βAD µg/h
- 7.4						
0	1.1	2.8	238	120	4.5	10.5
2	22.2	112	2135	742	5.4	16.2
4	6.9	30.9	1080	326	15.3	47.4
6	2.7	9.8	558	183	11.1	19.7
8	3.1	11.7	453	233	11.0	22.0
10	1.0	4.6	444	309	13.4	26.9
11.8	0.9	3.7	426	298	12.6	22.8
13.8	1.0	3.6	322	181	9.9	20.4
16.8	1.1	2.6	213	145	7.9	17.3
24	0.9	2.9	240	163	6.7	15.6

Testosterone (T), androsterone (A), etiocholanolone (E), 5α-androstane-3α,17β-diol (5αAD), 5β-androstane-3α,17β-diol (5βAD) determined by GC/MS analysis using deuterated internal standards (D3-testosterone and D4-etiocholanolone) for quantification [8]. T/E area ratio of testosterone to epitestosterone.

Carbon isotope ratio measurements were performed for standard solutions (10 ng/mL) of commercially available testosterone, 5αAD and 5βAD (see Table 2). A 2 µL aliquot of the testosterone sample (1.32 nmole CO_2) was analyzed by GC/C/IRMS in the splitless mode and yielded a narrow GC signal at m/z 44 ($^{12}CO_2$) of more than 0.3 V. The steroids were not

derivatized as this adds carbon atoms and modifies the isotopic ratio of the molecule. Also, derivatization can lead to isotopic fractionation, e.g., reaction with acetic anhydride, $(CH_3CO)_2O$, can result in fractionation since the anhydride has two reactive centers, and when one possesses a ^{13}C atom it reacts at a slightly different rate.

Table 2. $\delta^{13}C‰$ values for commercially available testosterone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol.

Substance	Source	$\delta^{13}C‰$
Testosterone	Schering	-28.14
Testosterone	Serva	-28.75
Testosterone	Sigma	-28.82
5α-androstane-3α,17β-diol	Sigma	-32.83
5β-androstane-3α,17β-diol	Sigma	-30.10

Commercially available testosterone showed little variation in $\delta^{13}C‰$ values (Table 2). Results of isotopic measurements for testosterone in urine of several males (non-testosterone users) and one male who had ingested 40 mg testosterone undecanoate are shown in Table 3. For all of the samples in Table 3, less than 10 mL of urine was used to obtain a sufficient amount of testosterone for GC/C/IRMS analysis.

Table 3. Characteristics of urine samples analyzed by GC/C/IRMS

Urine	Type	[T] (ng/mL)	T/E	Volume (mL)	$\delta^{13}C‰$ T
1	Blank	99.7	4.5	6	-21.3
2	Blank	65.2	3.6	8	-23.2
3	Blank	73.9	1.8	10	-24.3
4	Blank	50.5	1.1	10	-24.4
5	Blank	85.8	1.0	6	-23.2
6	6 h after T admin.	964	18.0	2	-29.5
7	8 h after T admin.	664	8.0	2	-28.6

All urine samples obtained from males. Samples 5-7 are from an individual before and after ingestion of 40 mg testosterone undecanoate. Other urine samples are from non-testosterone users. GC/MS analysis used to determine testosterone concentration and T/E ratio [8]. Volume refers to the urine volume used for extraction of testosterone for GC/C/IRMS analysis.

Four of the non testosterone users (urine samples 2-5) had similar δ‰ testosterone values, reflecting the fact that they had a common (European) origin (the origin of sample 1 is unknown). Interestingly, urine samples 1, 2 and 3 have elevated T/E ratios, but their δ‰ values are within the normal range. For samples 6 and 7, obtained 6 and 8 h after testosterone ingestion, there is a large decrease in the $\delta^{13}C‰$ testosterone value due to the different (lower) ^{13}C abundance in synthetic testosterone.

Results of isotopic measurements for testosterone in urine collected at 2 h intervals after ingestion of 40 mg testosterone undecanoate are shown in Figure 1. Included in the plot are the T/E ratios (see Table 1). As has been shown in other studies [9], the T/E ratio increased rapidly following testosterone administration but returned to lower values within a short time (after 6 h the T/E ratio was 2.7).

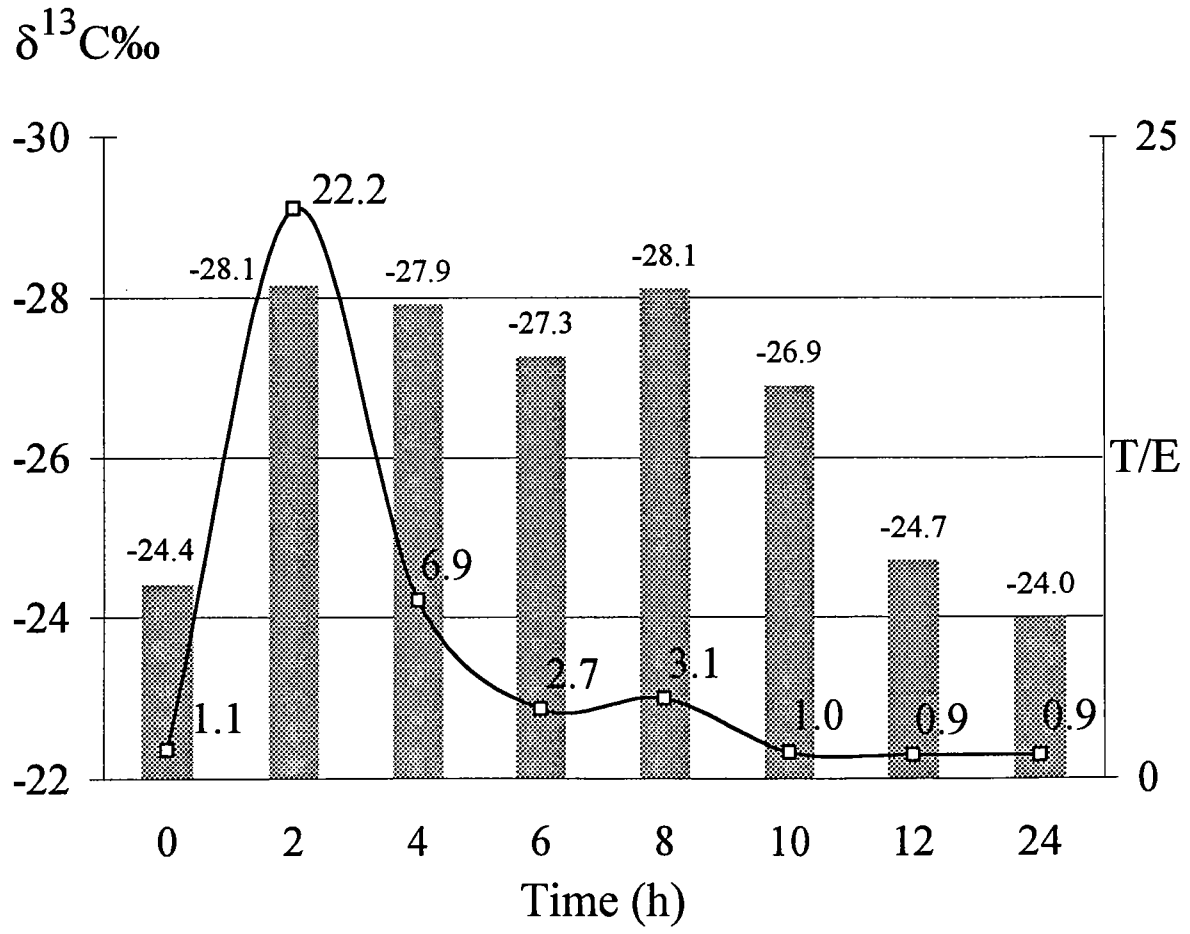

Figure 1. $\delta^{13}C‰$ testosterone values (vertical bars) and T/E ratios (□, solid line is used as a guide) determined at 2 h intervals following ingestion of 40 mg testosterone undecanoate.

Using the T/E > 6 ratio to determine testosterone doping, only the urine samples collected at 2 and 4 h after administration would be considered positive. Other factors, such as the increased 5βAD rate of excretion (Table 1), or the testosterone concentration and A/T ratio (androsterone to testosterone), which are determined in GC/MS screening, also point to testosterone misuse, but these are secondary indicators [10]. The $\delta^{13}C‰$ values provide direct evidence for application of synthetic testosterone and these values remain changed long after the T/E ratio has decreased below 6.

The excretion rates for 5αAD and 5βAD were elevated for nearly 24 h after testosterone administration (Table 1). Therefore, it was of interest to determine the $\delta^{13}C‰$ values for these two metabolites, as they might also show decreased values and lead to prolonged detection of testosterone misuse. As these species also bind to the testosterone immunoaffinity antibody, they were present in all of the urine immunoaffinity extracts and no extra sample workup (or increased urine volume) was needed to obtain sufficient amounts for GC/C/IRMS analysis. In fact, due to the large differences in GC retention times between testosterone and 5βAD, it is feasible during HPLC fractionation to collect these two species in a single glass tube and to analyze both together in one GC/C/IRMS analysis.

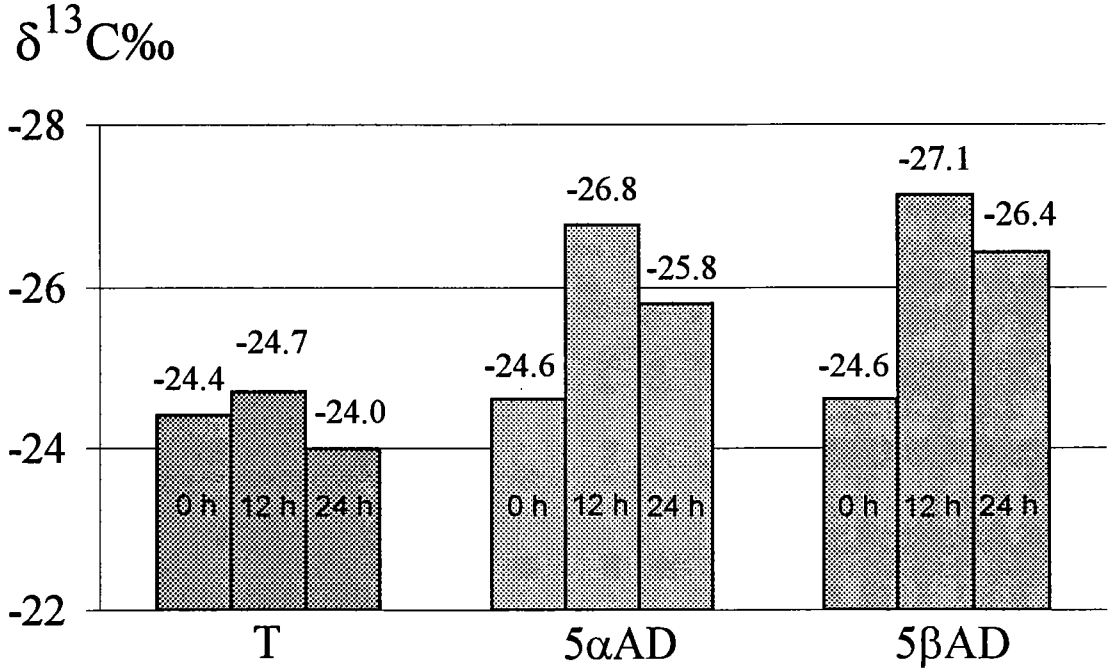

Figure 2. $\delta^{13}C‰$ values for testosterone (T), 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD) determined prior to ingestion of 40 mg testosterone undecanoate and 12 and 24 h thereafter. Pertaining information is given in Table 1.

$\delta^{13}C‰$ values for testosterone, 5αAD and 5βAD determined prior to testosterone administration and at 12 and 24 h thereafter are shown in Figure 2. There is a significant decrease in the δ‰ value for 5αAD and 5βAD even after 24 h, despite the fact that δ‰ for testosterone has returned to its baseline value. It is not clear as to why the 5αAD and 5βAD δ‰ values remain decreased long after testosterone has returned to its normal value unless there is a difference in the clearance rates for the metabolites. Further studies must be performed to confirm these observations.

Conclusion

In this study we have shown that oral testosterone administration does not lead to a prolonged increase in the urinary T/E ratio. An alternative, and improved, method for detecting exogenous testosterone is GC/C/IRMS. Irm-GC/MS instruments have detection thresholds in the low ng range, hence, it is possible to perform carbon isotope ratio measurements using a small amount of substance (urine). This is especially important in drug testing, as there is a limited volume of urine available, and some urine must always be set aside to confirm a suspected positive screening result. Immunoaffinity chromatography, using an antibody raised against testosterone-3-carboxy-methyloxime-bovine serum albumin, combined with HPLC fractionation is one means of isolating testosterone and other steroids from urine. The urine volume needed for isolating testosterone for GC/C/IRMS analysis is based on the testosterone concentration. In this study the urine volume was adjusted to obtain a minimum of 500 ng testosterone, which for normal samples from males corresponds to less than 10 mL urine. As a guideline, samples with concentrations on the order of 10 ng/μL are well suited for GC/C/IRMS analysis, provided the sample is reasonably pure and that good GC conditions (low background and low column bleeding) prevail.

Synthetic and endogenous testosterone have significantly different ^{13}C contents. After administration of testosterone the $\delta^{13}C‰$ testosterone value decreased and remained so, even after the T/E ratio returned to its normal value. Normally GC/C/IRMS would be employed to confirm testosterone misuse detected by GC/MS screening (T/E ratio); however, the fact that the $\delta^{13}C‰$ testosterone value remains decreased for samples where the T/E ratio has returned to 1, illustrates the need to investigate the use of GC/C/IRMS in routine screening.

References

1. Becchi M., Aguilera R., Farizon Y., Flament M.M., Casabianca H. and James J., *Rapid Commun. Mass Spectrom.* **8**, 304 (1994).

2. Aguilera R., Beechi M., Casabianca H., Hatton C.K., Catlin D.H., Starcevic B. and Pope J.H.G., *J. Mass Spectrom.* **31**, 169 (1996).

3. Brand W., *J. Mass Spectrom.* **31**, 225 (1996).

4. Merritt D.A., Freeman K.H., Ricci M.P., Studley S.A. and Hayes J.M., *Anal. Chem.* **67**, 2461 (1995).

5. Schänzer W., Metabolism of anabolic androgenic steroids: 5α- and 5β-reduction of 3-keto-4-ene steroids, in W. Schänzer (Editor), *Recent Advances in Doping Analysis (4) - Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, Sport und Buch Strauß, Köln 1997.

6. Donike M., Ueki M., Kuroda Y., Geyer M., Nolteernsting E., Rauth S., Schänzer W., Schindler U., Völker E., Fujisaki M., *J Sports Med. Phys. Fitness* **35**, 235 (1995).

7. Schänzer W., Delahaut P., Geyer H., Machnik M. and Horning S., *J. Chromatogr. B.* **687**, 93 (1996).

8. Schänzer W., Donike M., Synthesis of deuterated steroids for GC/MS quantificationf of endogenous steroids, in M. Donike (Editor), *Recent Advances in Doping Analysis (2) - Proceedings of the 12th Cologne Workshop on Dope Analysis 1994*, Sport und Buch Strauß, Köln 1995, p. 93.

9. Zimmermann, J., Untersuchung zum Nachweis von exogenen Gaben von Testosteron, Thesis Deutsche Sporthochschule Köln, Germany 1986.

10. Geyer H., Schänzer W., Mareck-Engelke U., Donike M., Factors influencing the steroid profile, in M. Donike (Editor) , *Recent Advances in Doping Analysis (3) - Proceedings of the 13th Cologne Workshop on Dope Analysis 1995*, Sport und Buch Strauß, Köln 1996, p. 95.