Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

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Sport und Buch Strauß, Köln, 2004

R.K.R. TAYLOR, A.T. CAWLEY, R. KAZLAUSKAS, G.J. TROUT, A.V. GEORGE:
Validity of Carbon Isotope Ratio Measurements for Decomposed Urine Samples
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 491-495

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Validity of carbon isotope ratio measurements for decomposed urine samples

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Introduction

Unintentional deconjugation of steroids in urine samples sent to WADA accredited laboratories for analysis is an indication of microbiological action. Criteria used to subsequently detect this decomposition in urine samples include changes in pH, a percentage of free testosterone greater than 5 as measured by Gas Chromatography-Mass Spectrometry (GC-MS) and the presence of 5a and 5b-androstane-3,17-diones, [1,2]. These decomposed samples no longer have steroid profile values that can be representative of the originally collected urine due to steroid interconversions and metabolism by microorganisms. It has been proposed that carbon isotope ratio ($\delta^{13}C$ (‰)) measurements made by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) on the urinary residual androsterone (A) and etiocholanolone (Et) may alleviate issues associated with the accurate measurement of the testosterone to epitestosterone (T/E) ratio conducted on samples that would be deemed to be decomposed by doping control laboratories [2].

This problem will arise when samples that present GC-MS screening T/E values of greater than 6 but GC-MS confirmation of these samples reveal that a significant amount of testosterone (T) is detected in the unconjugated (free) form. This presents an interesting scenario when GC-C-IRMS analysis of these samples reveals ^{13}C depleted T metabolite values that would indicate administration of an endogenous steroid (Table 1 shows results for 2 such cases).

Table 1: Cases of decomposed urine samples with high T/E values

	CASE 1	CASE 2
T/E	8.2	4.0
Free T (%)	24.6	11.2
δ^{13}C Et (‰)	-28.5	-28.3
δ^{13}C A (‰)	-27.2	-27.3
δ^{13}C ERC (‰)	-23.1	-21.4
Difference (‰)	4.8	6.4

Experimental

Urine was collected, with informed consent, from 15 volunteers (5 female, 10 male) aged between 20 and 45 years of age. Separate aliquots (3 mL) of these urine samples were stored for 1, 2 and 3 months at -10 and 22.4 °C. Measurements of pH and specific gravity were made prior to the initial analysis and each monthly analysis.

T/E determinations were made following the routine T confirmation procedure: addition of d_3T (4 µg/mL, 50 µL) as surrogate, isolation of urinary steroid conjugates using Serva PADII resin, reconstitution of the dried conjugate fraction with phosphate buffer pH 7 (0.2 M, 1.5 mL), extraction with ether to give the free fraction, enzyme hydrolysis of the aqueous layer using β-glucuronidase from *E.coli* K12 (EC 3.2.1.31, 50 µL) at 50 °C for 1.5 hours, hexane extraction of the hydrolysate at pH 10, addition of 17α-methylT (4 µg/mL, 50 µL) as internal standard, then derivatisation of the free and conjugated fractions to form TMS enol-ethers (MSTFA/TMSI, 55 µL). Measurements were made using an Agilent 6890/5973 GCMS using a HP1 column (17m, 0.11um film, 0.2mm diameter) using routine screening conditions.

GC-C-IRMS analysis was carried out on the residual androsterone, etiocholanolone and 11-ketoetiocholanolone (internal marker) using the conditions described previously [4] on steroid extracts obtained following the addition of 5α-androstan-3β-ol (100 µg/mL, 50 µL) as surrogate and the addition of phosphate buffer pH 7 (0.2 M, 1.5 mL) before enzyme hydrolysis as described above. Solid phase extraction of the hydrolysed urinary steroid metabolites was

performed using Varian BondElut ® Certify cartridges that were conditioned with methanol (3 mL), water (3 mL), loaded with the sample hydrolysate, washed with water (3 mL), water/methanol (90:10, 3 mL), hexane (1 mL), then eluted with ethyl acetate/methanol (95:5, 3 mL). 17α-methylT (100 μg/mL, 50 μL) was added as internal standard.

Results and discussion

Changes in pH values.

The pH of the urines changed during the storage at room temperature to basic pH values indicating formation of ammonia. The range of change was 0.3 to 3.4 pH units.

Correlation of $\delta^{13}C$ values and free T

No urine sample displayed significant variation (>1‰) between $\delta^{13}C$ values of Et and A measured initially and those obtained following storage. No sample was found to have $\delta^{13}C$ values of Et or A that were ^{13}C depleted (i.e. more negative) than the proposed threshold of -27.0‰ [3]. The % free T for all samples varied significantly (up to 68%) as time progressed. Consequently, no correlation was observed between changes in $\delta^{13}C$ values and % free T (Figure 1).

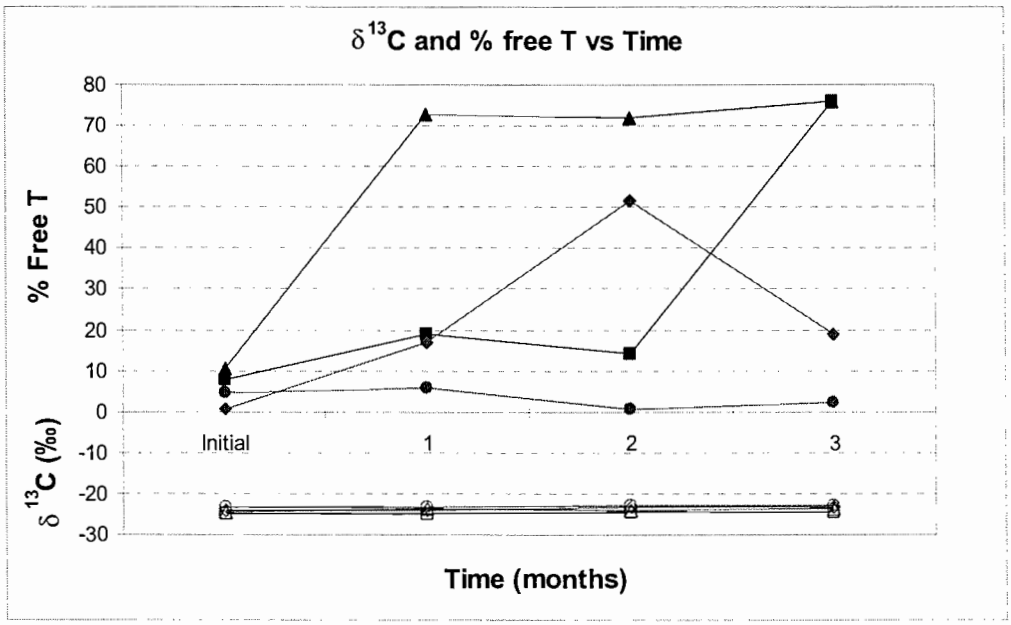

Figure 1: Selected samples showing no correlated change between $\delta^{13}C$ and % free T values

Analysis of decomposition products

The $\delta^{13}C$ values of 5β-androstanedione and 5α-androstanedione were determined for samples that contained sufficient amounts of each. No difference could be established between the $\delta^{13}C$ values of Et and A with their respective C-3 keto analogues. The results from samples 10 and 12 are shown in Table 2 as an example.

Table 2: $\delta^{13}C$ values of Et and A and their respective decomposition products

	SAMPLE 10	**SAMPLE 12**
$\delta^{13}C$ Et (‰)	-23.6	-22.9
$\delta^{13}C$ 5β-dione (‰)	-24.0	-22.3
$\delta^{13}C$ A (‰)	-23.3	-21.9
$\delta^{13}C$ 5α-dione (‰)	-23.8	-20.3

Conclusion

Fractionation (i.e. discrimination of ^{13}C) of the isotopic distribution due to enzymatic processes was not observed from microbiological degradation of urinary steroid metabolites. These results demonstrate the possibility of $\delta^{13}C$ measurements being valid for urine samples deemed to be decomposed based current criteria of % free T or androstanediones.

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