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Effect of microbial degradation on steroid profile and IRMS analysis: A case study

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Abstract

Effect of microbial degradation on the steroid profile in urine samples has been well investigated but not much is known about its effects on carbon isotope ratios (CIR)[1-3]. Transportation of urine samples, collected for doping control analysis does not always meet ideal conditions of storage which may stimulate bacterial contamination affecting urinary steroids concentration. According to WADA technical document TD2004 EAAS (valid till Dec. 2013), the presence of bacterial degradation and free steroids invalidate the urine sample for reporting Adverse Analytical Findings (AAF)[4]. This paper presents result of one sample received from an international client in 2013, which showed signs of bacterial degradation (presence of high concentration of 4-androstendione, 5α- and 5β-androstanedione and a T/E ratio > 10). Further investigation showed that most of the endogenous steroids excreted were in free form (more than 50%) implying a strong indication of degradation. The influence of bacterial degradation on CIR of different urinary steroids was investigated in the combined, unconjugated and conjugated fractions. The peak heights and $\delta^{13}C$ values of urinary steroids in the combined fraction were comparable with those observed in the free fraction. The GC/C/IRMS analysis was consistent with exogenous origin of endogenous steroids, meeting the criteria to report an AAF. This sample could not be reported as AAF as all the target compounds were present in un-conjugated form, thereby not meeting WADA criteria of reporting AAF[4]. Further work is in progress with more number of samples to ascertain that CIR is independent of influence of bacterial growth affecting steroid profile.

Introduction

Urine samples exposed to high temperatures and/or improper storage conditions in hot climate countries may be subjected to bacterial degradation [1]. The effect of degradation processes on steroid concentrations and diagnostic ratios are well investigated but limited work is carried out to know its effect on CIR[1-3]. WADA technical document TD2004 EAAS for reporting and evaluation of T/E-ratios invalidate specimens containing more than 5% of free testosterone (T) and/or epitestosterone (E) for reporting Adverse Analytical Findings (AAF)[4]. A doping control urine sample showing T/E ratio greater than 4 (12.2) in the initial testing procedure showed strong bacterial degradation. Inspite of steroid profile being invalid, the present study was carried out to understand the impact of strong bacterial degradation on GC/C/IRMS analysis.

Experimental

Chemicals and Reference Standards

Reference standards of endogenous steroids were procured from Sigma-Aldrich, USA and National Measurement Institute (NMI), Australia. C-18 sample preparation cartridges were procured from Ranbaxy Fine Chemicals Ltd, India, β- glucuronidase enzyme (E. Coli) was from Roche Diagnostics, USA. All other reagents/solvents were of High Performance Liquid Chromatography Grade (HPLC-grade) or analytical grade.

Urine Samples

A batch of 26 samples, received from an international client in 2013, was processed according to routine screening procedure for anabolic steroids. Out of 26 samples, one sample showed strong signs of bacterial degradation and a T/E ratio

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> 10.0. The pH and the specific gravity of this sample was 7.21 and 1.014 respectively.

GCMSD/ and GC/C/IRMS Analysis

A fresh aliquot of the sample was prepared for the quantification of T/E by gas chromatography mass spectrometry (GCMSD)[6]. Additionally, this sample was also analysed by gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) to confirm the exogenous origin of the T metabolites. GC/C/IRMS analysis of sample was performed both with ether washing (conjugated fraction) and without ether washing (combined fraction) prior to enzymatic hydrolysis [6].

High Performance Liquid Chromatography (HPLC) purification/cleanup was performed in accordance with the routine sample preparation procedures and thereafter injected onto GC/C/IRMS after acetylation. The free fraction separated from the conjugated sample was also processed by routine screening procedure for GC/C/IRMS analysis including HPLC clean-up (free fraction). Delta$^{13}C/^{12}C$ ($\delta^{13}C/^{12}C$) values for androsterone (Andro), etiocholanolone (Etio), and 5α- and 5β-androstane-3α,17β-diol (5α-diol & 5β-diol), testosterone (T), epitestosterone (E) and 11-keto-etiocholanolone (11keto) pregnanediol (PD) as endogenous reference compound (ERC) were measured.

Correction for the acetate (Ac) moiety

All the $\delta^{13}C/^{12}C$ values obtained by GC/C/IRMS analysis were corrected for the influence of the acetate moiety as described in literature [7].

Results and Discussion

GCMSD Analysis

The screening data of this sample on GCMSD showed a T/E-ratio greater than 4 (12.2) and signs of degradation i.e. presence of high concentration of 4-androstendione, 5α- and 5β-androstanedione.

The confirmation analysis showed a T/E ratio of 7.0 but the amount of free T and /or E and other free steroids were found to be more than 50% (Table-1). As per TD2004EAAS (valid till December 2013) which was applicable at the time of analysis of this sample, to report an AAF of an elevated T/E value, the concentration of free steroids should not be more than 5% [4]. As per TD2014EAAS, a sample showing signs of microbial degradation that may cause an alteration of the steroid profile may not be suitable for inclusion in longitudinal study profiles. The presence of free T is interpreted as sign of bacterial degradation and has to be reported as well, but is no longer a decision criterion for an AAF [5].

	Fraction	A (ng/ml)	Etio (ng/ml)	5α-diol (ng/ml)	5β-diol (ng/ml)	E (ng/ml)	T (ng/ml)	T/E
Screening	Combined fraction	279.6	1910.6	25.2	489.4	11.0	134.1	12.2
Confirmation	Conjugated fraction	4.2	17.4	0.6	2.6	0.6	4.4	7.0
	Free Fraction	238.1	1278.3	18.9	287.0	9.3	68.6	7.4
	% in Free Fraction	98	99	97	99	94	94	

Table 1. Steroid profile results in the combined, conjugated and free fraction.

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GC/C/IRMS Analysis

The GC/C/IRMS analysis of the conjugated fraction revealed that the peak intensity of all the target compounds was below 0.10 nA which implies a non-reliable delta value measurement and also failed the identification criteria on GCMSD. But when the GC/C/IRMS analysis was performed with the combined fraction, interestingly the peak heights and $\delta^{13}C$ values of urinary metabolites or target compounds (TC) in the combined fraction were comparable with the free fraction (Fig. 1 and 2).

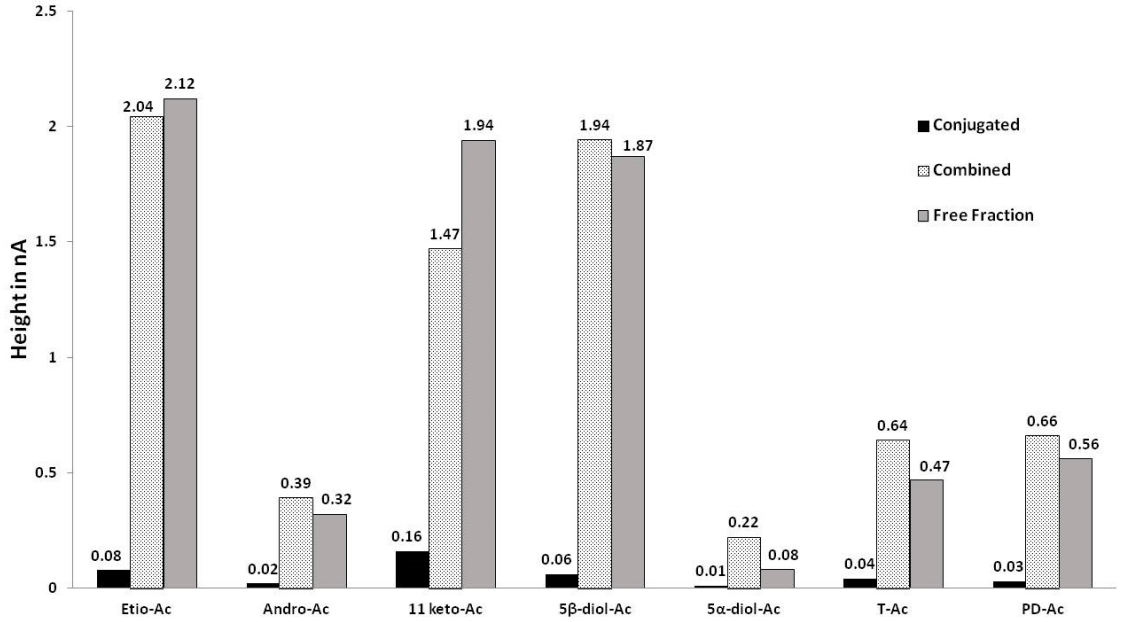

Figure 1. Comparison of peak heights of target compounds and endogenous reference compounds (ERC) in conjugated, combined and free fractions.

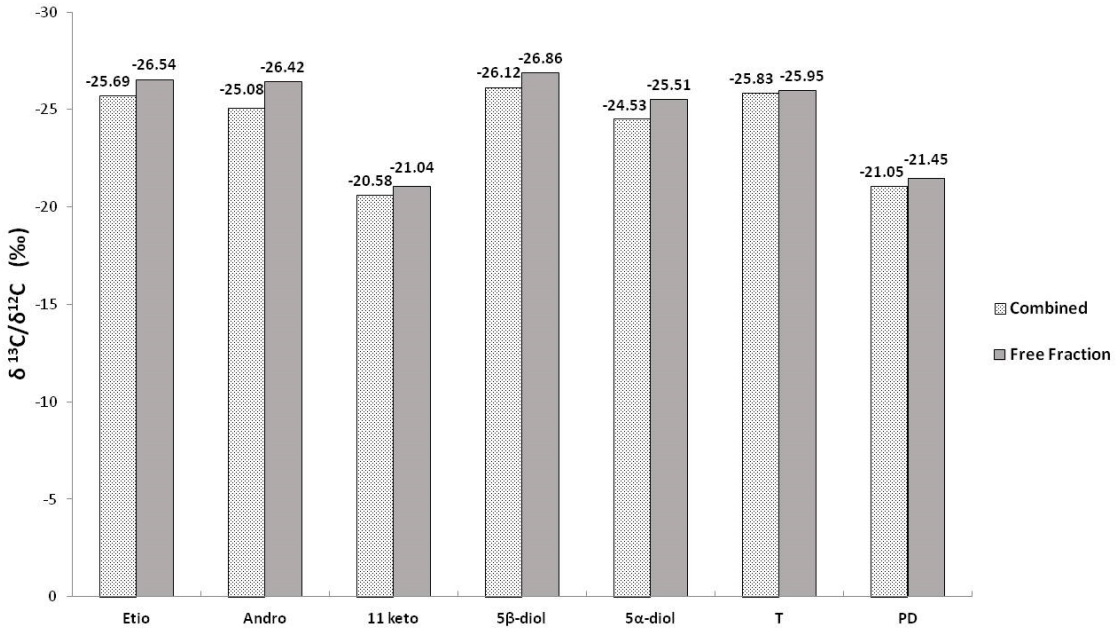

Figure 2. Comparison of $\delta^{13}C$ values (‰) of target compounds and endogenous reference compounds in combined and free fractions.

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The delta delta difference of $^{13}C/^{12}C$ ($\Delta\delta^{13}C/^{12}C$) values of Andro, Etio and T from ERC (11keto-etio) and $\Delta\delta^{13}C/^{12}C$ values of 5α-diol and 5β-diol from ERC (PD) was greater than 3 per mil (‰) indicating an exogenous origin of endogenous steroids or meeting the criteria to report an AAF (Fig. 3).
The GC/C/IRMS findings were consistent with an exogenous origin of the target compounds but the sample could not be reported for AAF as all the target compounds were mainly present in the free fraction (unconjugated form) which invalidated the sample [4]. The standard deviation (SD) for the target compounds and ERCs of the method varied from 0.5 to 1‰.

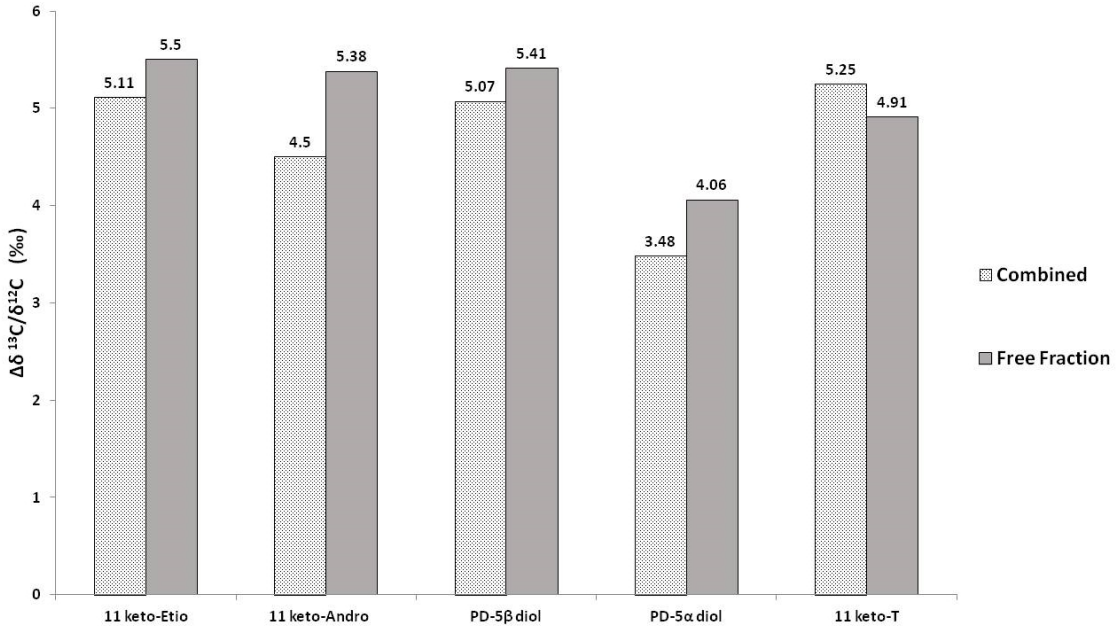

Figure 3. Comparison of $\Delta\delta^{13}C/^{12}C$ of target compounds and endogenous reference compounds in combined and free fractions.

Conclusions

In the present case study, the presence of free excreted steroids is attributed to a microbial degradation of the sample as evident from the GC-MSD analysis. The GC/C/IRMS findings were consistent with an exogenous origin of the target compounds but the sample could not be reported as AAF, as all the target compounds were mainly present in the free fraction (unconjugated form) which invalidated the sample as per the applicable TD2004EAAS. It is rather preliminary to conclude that bacterial degradation in a urine sample has no effect on $\delta^{13}C/^{12}C$ values of steroids. Studies with a larger number of samples including exogenous and endogenous, showing bacterial growth are in progress to ascertain the hypothesis that the CIR is independent of the availability of drugs in the free form in the samples due to bacterial growth.

References

1. Piper T,Geyer H,Schänzer W, (2010), Degradation of urine samples and its influence on the $^{13}C/^{12}C$ ratios of excreted steroids,*Drug Test Anal2(*11-12*),*620-29.

2. Mareck U, Geyer H, Opfermann G, Thevis M, Schanzer W. (2008) Factors influencing the steroid profile in doping control analysis, *J. Mass Spectrom,* 43, 877-91.

3. Tsivou M, Livadara D, Georgakopoulos DG, Koupparis MA, Atta-Politou J, Georgakopoulos CG (2009) Stabilization of human urine doping control samples: microbial degradation of steroids. *Anal Biochem*, 388(1), 146-54.

4. WADA TD2004 EAAS World Antidoping Agency, Montréal, Canada.(2004): http://www.wada-ama.org/Documents/World_AntiDoping_Program /WADP-IS- Laboratories/Technical_Documents (access date 24.03.2014)

5. WADA TD2014 EAAS World Antidoping Agency, Montréal, Canada.(2014): http://www.wada-ama.org/Documents/World_AntiDoping_Program /WADP-IS- Laboratories/Technical_Documents (access date 24.03.2014)

6. Jain S, Kaur T, Garg T, Shrivastava A, Soni A, Beotra A (2012) Effect of storage conditions on carbon isotope ratios of endogenous steroids. In: Schanzer W, Geyer H, Gotzmann A, Mareck U, (eds.) *Recent Advances in Doping Analysis (20)*, Koln, pp 217-220.

7. Piper T, Mareck U, Geyer H, Flenker U, Thevis M, Platen P, Schanzer W. (2008) Determination of 13C/12C ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes, *Rapid Comm Mass Spectrom,*22, 2161-2175.

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