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Case study: GC/C/IRMS data of an unsusual urine sample

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Abstract

A doping control urine sample showing a testosterone/epitestosterone (T/E) ratio higher than 4 (9.1) in the initial testing procedure was repeated and additionally analyzed with the gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). The GC/C/IRMS results indicated an exogenous origin of the testosterone (T) and T metabolites. The confirmation analysis showed a T/E ratio of 7.9, but the amount of free T was determined as 24% of total excreted T. The sample shows neither an elevated pH value, nor an altered steroid profile due to the formation of 5α -androstane-3,17-dione or 5 β -androstane-3,17-dione. The GC/C/IRMS analysis was repeated to confirm the exogenous origin of the T and T metabolites in the combined and conjugated fraction in parallel.

The GC/C/IRMS results of the conjugated and combined fraction show neglectable differences between the δ^{13} C values in the different sample preparations. The Δ^{13} C-values met the criteria to report an Adverse Analytical Finding.

The reason for the presence of high amounts of unconjugated steroids could not be clarified.

Introduction

Based on TD2004EAAS (valid until December 2013) to report an Adverse Analytical Finding of an elevated T/E value, the concentration of free T and/or E in the specimen was not allowed to exceed 5% of the respective glucuroconjugates [1]. A doping control urine sample (collected in late 2013) with a T/E ratio greater than 4, showed an exogenous origin of T and T metabolites as well as significant amounts of free steroids with no evidence of bacterial degradation. To verify if the separation of the free fraction affects the δ^{13} C-values, both fractions (conjugate- as well as combined fraction) of this sample were analyzed. The analysis of the free fraction itself was not possible due to the lack of A sample volume.

Experimental

Aliquots of the urine sample were prepared according to the common (modified) procedures for the detection of anabolic androgenic steroids by gas chromatography / (tandem)-mass spectrometry (GC/MS/MS) and GC/C/IRMS [2,3].

Results and Discussion

As depicted in Table 1, the sample contains significant amounts of free steroids (e.g. T 24%), but shows neither an elevated pH value, nor an altered steroid profile due to the formation of 5α -androstane-3,17-dione or 5β -androstane-3,17-dione. Based on the current technical document TD2004EAAS (valid until December 2013), the concentration of free T and/or E in the specimen was not allowed to exceed 5% of the respective glucuroconjugates to report an Adverse Analytical Finding of an elevated T/E value [1].

Poster



	A [ng/mL]	Etio [ng/mL]	5α [ng/mL]	5β [ng/mL]	T [ng/mL]	E [ng/mL]
Conjugated fraction	9409	4544	268	235	50	5.9
Free fraction	833	1690	50	51	11.8	0.4
Free (%)	9	37	19	22	24	7
A: androsterone Etio: etiocholanolone		5α: 5α- 5β: 5β-	androstane-3α,17(androstane-3α,17(T: testosterone E: epitestosterone		

Table 1: Steroid profile results in conjugated and free fraction

In the currently valid technical document TD2014EAAS the confirmation procedure of a sample is necessary on request of the Testing Authority, when the steroid profile of the sample cannot be processed by the Adaptive Model in ADAMS. The presence of free T is interpreted as sign of microbial degradation and has to be reported as well, but is no longer a decision criterion for an Adverse Analytical Finding. [4]

A separation of the free fraction for the GC/C/IRMS confirmation procedure is not recommended or required in any of the respective Technical Documents [3,4]. However, the Cologne (inhouse) standard operating procedure includes the separation of the free fraction as additional cleaning step to remove coeluting and disturbing substances [3].

	ΟΗΑ	ΡD	Etio	Α	Τ	5α	5β
	[δ ¹³ C]	[δ ¹³ C]	[δ ¹³ C]	[δ ¹³ C]	[δ ¹³ C]	[δ ¹³ C]	[δ ¹³ C]
Conjugated fraction	- 21.83	- 21.36	- 29.07	- 27.67	- 28.95	- 29.18	- 29.20
Combined fraction	- 21.85	- 21.53	- 29.07	- 27.26	- 28.91	- 29.20	- 29.09
OHA: 11β-hydroxyandrosterone		A: androsterone		5α: 5α-androstane-3α,17β-diol		T: testosterone	
PD: pregnanediol		Etio: etiocholanolone		5β: 5β-androstane-3α,17β-diol			

Table 2: GC/C/IRMS (δ^{13} C-values) results in conjugated and combined fraction



The GC/C/IRMS results of the conjugated and combined fraction show neglectable differences between the δ^{13} C values in the different sample preparations; no fractionation could be detected (Table 2).

These results are in accordance with Piper et al [5], who found during the degradation of urine samples within a couple of weeks no significant change in carbon isotope ratios.

The Δ^{13} C-values met the criteria to report an Adverse Analytical Finding (Tables 3a,3b) [6]. The reason for the presence of high amounts of unconjugated steroids could not be clarified.

	OHA-Etio	ΟΗΑ-Α	ΟΗΑ-Τ	ΟΗΑ-5α	ΟΗΑ-5β
	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]
Conjugated fraction	7.24	5.84	7.12	7.35	7.37
Combined fraction	7.22	5.41	7.06	7.35	7.24
OHA: 11β-hydroxyandrosterone		A: androsterone		5α: 5α-androstane-3α,17β-diol	
T: testosterone		Etio: etiocholanolone		5β: 5β-androstane-3α,17β-diol	

Table 3a: GC/C/IRMS (Δ^{13} C-values) results in conjugated and combined fraction

	PD-Etio	ΡD-Α	ΡD-Τ	ΡD-5α	ΡD-5β
	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]	[Δ ¹³ C]
Conjugated fraction	7.71	6.31	7.59	7.82	7.84
Combined fraction	7.54	5.73	7.38	7.67	7.56
T: testosterone A: androsteron		A: androsterone	5α: 5α-androstane-3α,17β-diol		
PD: pregnanediol Etio: etiocholano		Etio: etiocholanolor	ne 5β: 5β-androstane-3α,17β-diol		

Table 3b: GC/C/IRMS (Δ^{13} C-values) results in conjugated and combined fraction



Conclusions

In this urine sample the "free" excreted steroids cannot be considered as a sign for bacterial activity.

The presence of unconjugated steroids in high amount compared to the conjugated steroids (Table 1) show no influence on the δ^{13} C-values of the analyzed steroids (Table 2). The carbon isotope measurements show no fractionation when the conjugated steroids were analyzed following the separation of the unconjugated ("free") fraction and compared to the combined steroid fraction. It is possible to have a "degraded" sample due to high free T and for it still to be a doping case.

References

[1] WADA Technical Document TD2004EAAS, v 1.0, 13 August 2004 (valid until 31 December 2013)

[2] Mareck U, Geyer H, Flenker U, Piper T, Thevis M, Schänzer W (2007): Detection of dehydroepiandrosterone misuse by means of gas chromatography-combustion-isotope ratio mass spectrometry. *Eur. J. Mass Spectrom.* 13, 419-426
[3] Piper T, Mareck U, Geyer H, Flenker U, Thevis M Platen P, Schänzer W (2008): Determination of ¹³C/¹²C ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *RCM* 22: 2161-2175.

[4] WADA Technical Document TD2014EAAS, v 1.0, January 2014

[5] Piper T, Geyer H, Schänzer W (2010): Degradation of urine samples and its influence on the ¹³C/¹²C ratios of excreted steroids. Drug Test. Analysis **2**: 620-629.

[6] WADA Guidelines for Detection of EAAS by GC-C-IRMS Analysis, v 1.0, January 2014

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