Shila Jain, Tejinder Kaur, Tripta Garg, Abhinav Srivastava, Arpit Soni, Alka Beotra

Effect of storage condition on $^{13}C/^{12}C$ ratios and steroid profile

National Dope Testing Laboratory, New Delhi, India

Abstract

The storage of urine samples prior or during transportation to laboratory is critical as high temperature may lead to chemical, enzymatic and bacterial degradation thereby affecting sample constitution [1,2]. The present study was aimed to identify any changes in steroid profile and delta values of $^{13}C/^{12}C$ ratios of endogenous steroids in the urine after storage of samples at 37°C up to 5 days. The urine samples of six male volunteers were collected and each sample was spilt into six 10 ml aliquots. One aliquot was processed for steroid profile analysis and Carbon Isotope Ratio (CIR) measurements. The remaining five aliquots were stored at 37°C up to five days and one aliquot of each volunteer was processed daily & injected on Gas Chromatograph Mass Spectrometer Detector and Gas Chromatograph Isotope Ratio Mass Spectrometer (GC-C-IRMS) for steroid profiling and CIR measurements. There was no significant decrease in androsterone (A) and etiocholanolone (E) concentrations in any of the samples stored upto 5 days at 37°C except one sample with decreased A and E concentration on 5th day. This sample also showed traces of 5**α-** and 5**β**-androstenedione indicating early bacterial growth. There was no change in $\delta^{13}C$ values for A, E, 5**α**-diol and 5**β**-diol in any of the sample stored until 5th day even in the sample which showed early sign of bacterial growth. Further work is in progress with more number of negative and positive samples.

Introduction

Urine samples transferred to WADA accredited laboratories may be exposed to high temperatures and improper storage conditions in hot climate countries. Transport and storage of urine samples at high temperature may lead to bacterial degradation [1]. The effect of degradation processes on steroid concentrations and diagnostic ratios are well investigated but effect of degradation on CIR has been only investigated by Piper et al. [1,2]. Therefore, aim of the present study was to identify any changes in steroid profile and delta values of $^{13}C/^{12}C$ ratios of endogenous steroids in the urine samples after storage of samples at 37°C (the most prevalent ambient temperature) up to 5 days.

Experimental

Reagents and Chemicals

Reference standards of endogenous steroids and deuterated internal standards were procured from Sigma-Aldrich (USA) and National Measurement Institute (Australia). C-18 sample preparation cartridges were procured from RFCL Ltd, β-glucuronidase enzyme (E. coli) was from Roche Diagnostics (USA). All other solvents and chemicals were either of high performance liquid chromatography (HPLC) grade or analytical grade.

Collection of urine samples

The urine samples of six male volunteers who declared not to use any steroids, prohormones or nutritional supplements, were collected and each sample was spilt into six aliquots. One aliquot of each sample was processed for steroid profile analysis and Carbon Isotope Ratio (CIR) measurements. The remaining five aliquots were stored at 37°C up to five days. One aliquot of each volunteer was processed every day for steroid profiling and CIR measurement.

Urine steroid profile determination

Routine sample preparation procedure consisting of solid phase clean up, enzymatic hydrolysis, solvent extraction and derivatization followed by Gas Chromatography-Mass Spectrometry (GC-MSD) analysis for steroid profiling of androgenic anabolic steroids (AAS) was employed [3].

 ISBN 978-3-86884-038-4

Determination of CIR

The samples were prepared using enzymatic hydrolysis, solid phase extraction (C18 cartridges), HPLC cleanup and derivatization before injecting on Gas Chromatography Combustion Isotope ratio Mass Spectrometry (GC-C-IRMS). Sample preparation prior to HPLC purification and analytical parameters for GC-C-IRMS is described elsewhere [4].

HPLC Purification

The clean-up of all analytes was achieved by a two-fold HPLC fractionation. The details of fraction collection are depicted in Table-1. Analytical parameters for 1st and 2nd HPLC cleanup are given in Table-2.

	Fraction No.	Collection Time (Min) From	To	Compound Collected*
First Fractionation	1	9.4	10.4	11-keto
	2	11.0	12.0	T
	3	12.2	14.4	EpiT, DHEA,5a, 5b
	4	14.4	16.1	A,E
	5	17.3	18.4	PD
Second Fractionation	2-1	8.3	9.4	T-Ac
	2-2	18.2	19.6	RSTD-Ac
	3-1	7.2	8.3	EpiT-Ac
	3-2	9.5	10.5	DHEA-Ac
	3-3	20.1	22.5	5a-Ac, 5b-Ac

*Abbreviations
11-keto: 11-ketoetiocholanone; T:Testosterone; EpiT: Epitestosterone; A: Androsterone
E: Etiocholanone; 5a: 5α-androstane-3α, 17β-diol;5b: 5β-androstane-3α, 17β-diol
RSTD: 5a-androstane-3b-ol ; DHEA: Dehydroiepiandrosterone; PD: Pregnanediol;
Ac:Acetate

Table 1: Details of fraction collection during first and second HPLC clean-up

Instrument Condition				
Instrument	:	Waters Alliance 2695 separation module with automated fraction collector		
Column	:	LiChroCART® 250 X 4 mm i.d., 5 µm particle size		
Guard Colum	:	LiChroCART® 25 X 4 mm i.d., 5 µm particle size		
Flow rate	:	1 ml/min		
Gradient	:		Acetonitrile	Water
(for 1st clean up)		Initial	30	70
		25 min	100	0
		30 min	100	0
		35 min	30	70
		40 min	30	70
Gradient	:		Acetonitrile	Water
(for 2nd clean up)		Initial	70	30
		33 min	100	0
		38 min	100	0
		38.01 min	70	30
		43 min	70	30
Temperature : 35°C		**Injection Vol.**: 50 µl		**Wavelength**: 192 nm

Table 2: Instrument parameters for first and second HPLC clean-up

ISBN 978-3-86884-038-4

GC/MS Identification

In order to identify steroids from co-eluting peaks all the fractions were injected in scan mode (m/z 40 to 400) into GC (Agilent 6890) coupled to a mass selective detector.

Results and Discussion

There was no significant decrease in androsterone (A) and etiocholanolone (E) concentrations in any of the samples after storage upto 5 days at 37°C except in one sample (volunteer-6) which showed decrease in A and E concentration on 5th day (Figure 1). This sample also showed traces of 5α- and 5β-androstanedione in routine screening procedure of anabolic steroids which indicated early bacterial growth. There was no significant change in $\delta^{13}C$ values for A, E, 5α-diol (5α) and 5b-diol (5β) in any of the sample of six volunteers stored until 5th day. However, the 5th day sample of 6th volunteer which showed early signs of bacterial growth did not show any change in the $\delta^{13}C$ values (Figure 2).

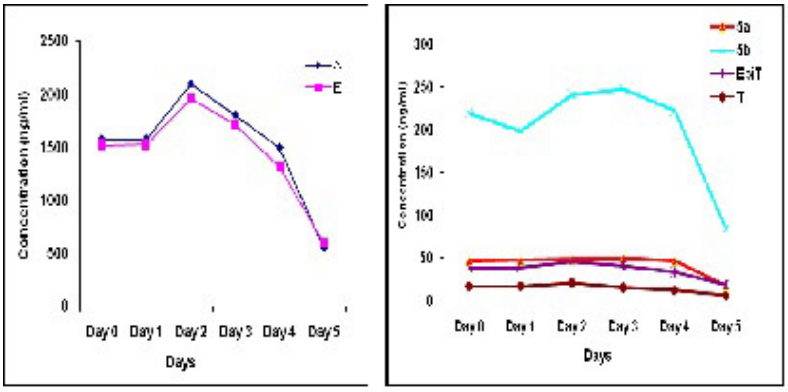

Figure 1: Changes in urinary concentrations of endogenous steroids in urine samples of volunteer 6: 1a) A and E; 1b)5a, 5b, EpiT and T

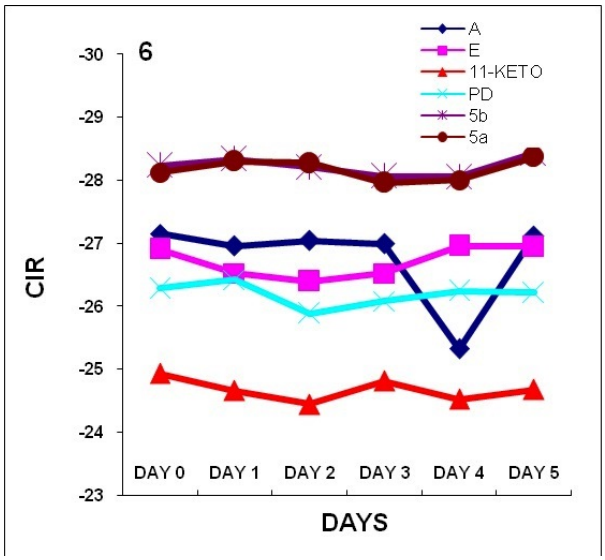

Figure 2: Changes in CIR of endogenous steroids in 6th volunteers over the five day time period

ISBN 978-3-86884-038-4

The relevance of present work in the context of temperature conditions prevalent in various parts of India is important and requires more work to conform preliminary findings. Though it is premature to say but the preliminary findings of the present work are in agreement to the extensive work done by Piper et al. [1] Further work is in progress with more number of samples and with extended time of storage at various temperature conditions.

Conclusions

The findings of our preliminary study show that storage of samples at 37°C for 5 days have no significant influence on $^{13}C/^{12}C$ ratios and concentration of endogenous steroids in urine samples. Further work is in progress with more number of negative and positive samples.

References

[1] Tsivou M, Livadra D, Georgakopoulos D G, Koupparis M A, Atta-Politou J, Georgakopoulos C G. (2009) Preservation of urine doping control samples-Preliminary results. Anal. Biochem 388, 146-155
[2] 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 Anal.* **2**, 11-12, 620-629.
[3] Jain S, Lal R, Garg T, Haseen J, Goswami M, Vandana Soni A, Beotra A, Kaur T. (2011) Comparative study of endogenous steroid profile of Indian sportspersons with other Commonwealth Games 2010 sportspersons. In Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (19)*, Köln, pp190-194
[4] Kaur T, Jain S, Bhula B, Lal R, Beotra A, Prasad GBKS. (2010). Delta values of endogenous steroids in Indian population. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (16)*, Köln, pp175-178

 ISBN 978-3-86884-038-4