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Improved sample preparation and subsequent investigations on population based thresholds for carbon isotope ratios of urinary steroids

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Abstract

Within the existing methods for carbon isotope ratio (CIR) determinations one of the main challenges was the separation of steroids with 5α- and 5β-conformation like androsterone (AND) and etiocholanolone (ETIO) or 5α-androstanediol (5a-Diol) and 5β-androstanediol (5b-Diol). Switching to a different HPLC column allowed for complete separation of ETIO and AND and 5a-Diol and 5b-Diol. This change in sample preparation necessitated the determination of a new reference population to establish reliable thresholds for the improved method.
Therefore n = 67 males and females were investigated enclosing the glucuronidated steroids ETIO, AND, 5a-Diol, 5b-Diol, pregnanediol, 16-androstenol, 11-ketoetiocholanolone, testosterone, epitestosterone and dehydroepiandrosterone (DHEA). Additionally, to corroborate earlier findings on steroids excreted sulfated, the following steroids from the sulfate fraction were included: ETIO, AND, DHEA, epiandrosterone and 17α- and 17β-androstenediol.
Regarding the glucuronidated steroids, over all the new limits are lower than the existing ones. For ETIO and AND the differences are small suggesting that the co-elution of both compounds had no significant influence on the measured CIR. A significant difference was found for 5a-Diol and 5b-Diol, suggesting that the old method introduced a systematic offset on these values. For sulfated steroids, the values were comparable but showed smaller standard deviations, now equal to the ones for glucuronidated steroids.
This investigation demonstrates the necessity to re-evaluate reference based thresholds after changing the method for CIR determinations even though most changes are mainly due to improved measurements resulting in smaller standard deviations.

Introduction

The existing method for CIR (carbon isotope ratio) determinations enabled the valid measurement of ten different urinary steroids out of one urine specimen using a twofold HPLC (high performance liquid chromatography) clean up [1]. One of the main challenges was the clean up and separation of steroids with 5α- and 5β-conformation like androsterone (AND) and etiocholanolone (ETIO) or 5α-androstanediol (5a-Diol) and 5β-androstanediol (5b-Diol). Switching to a different HPLC column solved these problems and enabled complete separation. This change in sample preparation necessitated the determination of a new reference population (RefPop) to establish reliable thresholds for the new method.

Experimental

HPLC clean up
While the sample preparation was only slightly adjusted compared to the existing methods for glucuronidated and sulfated steroids [1-3], the change in HPLC purification was the crucial step. As depicted in Figure 1, baseline separation of ETIO and AND and 5a-Diol and 5b-Diol was achieved maintaining the possibility to collect all other steroids of interest.
All HPLC fractionation steps were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) with a XBridge™ Shield RP18 5 mm (4.6 x 250 mm) column protected with a XBridge™ Shield RP18 5 mm (4.6 x 20 mm) guard column purchased from Waters (Baden-Dättwil, Switzerland). The injection volume was 50 µL and the flow rate 1 mL/min. A linear gradient was used increasing from 40/60 ACN/H_2O to 60% ACN in 18 min and then within 1 min to 98 % ACN. After 11 min at 98 %, the

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column was re-equilibrated for 10 min. The resulting fraction collection times are listed in Table 1. For purification of acetylated steroids the gradient was changed to start with 60/40 ACN/ H_2O, increased to 98 % ACN in 16 min, was hold for 9 min and subsequent the column was re-equilibrated as above. Separation of fraction I_S_Ac started with 70/30 ACN/H_2O, increased to 85 % ACN in 20 min, then to 98 % in 2 min, hold for 8 min and then re-equilibration for 10 min.

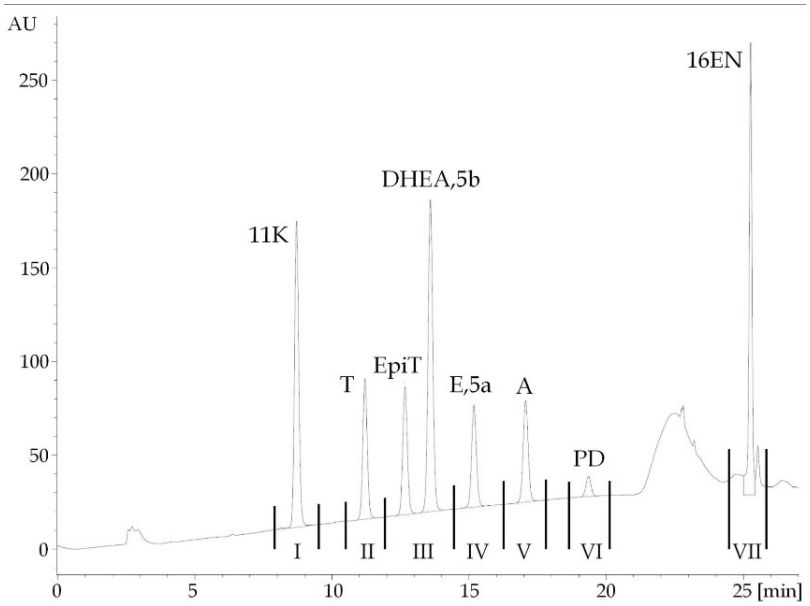

Figure 1: UV-chromatogram (wavelength 195 nm) of a standard containing all steroids of interest from the glucuronidated fraction.

Gluc	Fraction	Steroid	Time[min]	Sulf	Fraction	Steroid	Time [min]
Free	I	11K	8.8-10.0	Free	I_S	DHEA, 17a, 17b	12.7-15.1
	II	T	11.3-12.5		II_S	ETIO, EpiA	15.1-17.4
	III	EpiT, DHEA, 5b-Diol	13.9-15.4		III_S	AND	17.4-19.0
	IV	ETIO, 5a-Diol	15.4-17.4				
	V	AND	17.4-19.0				
	VI	PD	19.9-21.2				
	VII	16EN	25.1-26.1				
Ac	II_Ac	T_Ac	9.0-10.0	Ac	I_S_Ac	DHEA_Ac	8.7-10.1
	III_Ac	EpiT_Ac	8.4-9.6			17a_Ac	14.2-15.6
		DHEA_Ac	10.1-11.6			17b_Ac	15.6-17.0
		5b-Diol_Ac	15.1-16.4				
	IV_Ac	ETIO_Ac	10.5-11.8				
		5a-Diol_Ac	15.3-16.6				

Table 1: List of different fractions collected for steroid clean up and separation together with collection times.

GC/C/IRMS measurements

All samples were measured on an Agilent 7890 gas chromatograph (Waldbronn, Germany) coupled to a Delta V gas isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) via the GC combustion interface (GCC III, Thermo Electron). A detailed description of the method has already been published [3].

Reference Population

The RefPop consisted of 38 males and 29 females, aged from 20 to 54 years. All volunteers were healthy, performed regular exercising up to 20 h/week and were recruited from students and employees of the German Sports University and the Swiss Laboratory for Doping Analysis.

Results and Discussion

Population based thresholds

All $\delta^{13}C_{VPDB}$ and all Δ-values were found Gaussian distributed (Shapiro-Wilk test, a = 0.05) and therefore enabled parametrical calculation of threshold values by adding the threefold SD resulting in the 99.7% reference limit. Thresholds for Δ-values could be calculated with all possible ERC and exemplarily the results for PD are listed in Table 2.

Over all these results are comparable to the already published ones [1,2]. For glucuronidated steroids the limits were found slightly lower due to smaller SD. Larger differences between the populations or methods were visible for epitestosterone (EpiT), 5b-Diol and 5a-Diol. While the difference for EpiT can solely be attributed to improved clean up and resulting smaller SD during measurement (as already found in another investigation [4]), the difference for 5a-Diol and 5b-Diol seemed to be due to the changes in the HPLC method. The complete separation of both steroids during the clean up prevented any mutual influence. Interestingly, the impact of this novel separation on the higher concentrated steroids ETIO and AND was comparatively low, maybe because they are often found in urine at approximately equal concentrations. The SD for sulfated steroids were smaller than in the preceding study, now comparable to those of glucuronidated steroids.

	PD-ETIO	PD-AND	PD-T	PD-EpiT	PD-DHEA	PD-5a-Diol	PD-5b-Diol	PD-ETIOS	PD-ANDS	PD-EpiA	PD-DS	PD-17a	PD-17b
Mean	1.36	0.23	1.41	1.86	-0.29	1.12	1.08	0.23	-0.61	0.73	-1.68	0.20	0.09
SD	0.53	0.42	0.78	0.74	0.61	0.77	0.81	0.7	0.58	0.73	0.74	0.76	0.7
RefLim	3.0	1.5	3.8	4.1	1.6	3.5	3.6	2.4	1.2	3.0	0.6	2.1	2.2

Table 2: Reference limits calculated against PD for all possible target compounds under investigation. All values are in $\delta^{13}C_{VPDB}$ [‰].

Differences in $\delta^{13}C_{VPDB}$ values

In contrast to earlier studies, no significant difference could be detected between male and female samples, perhaps as the majority of women did not use any contraceptives. The only significant difference was found between steroids excreted glucuronidated and sulfated (Figure 2). For dehydroepiandrosterone (DHEA) the difference was 1.4 ‰, for ETIO 1.1 ‰ and for AND 0.9 ‰.

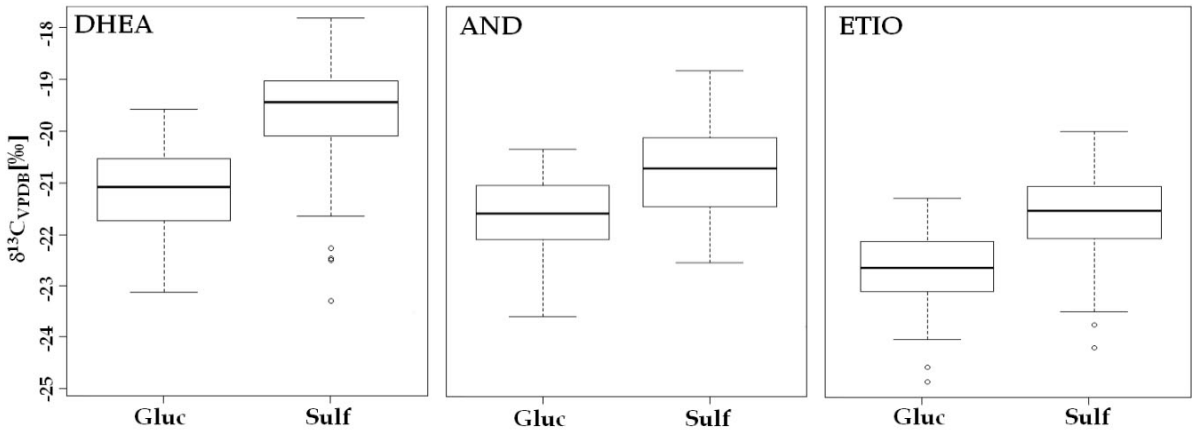

Figure 2: $\delta^{13}C$-values of DHEA, AND and ETIO excreted glucuronidated and sulfated. All differences are highly significant (Student´s t-test, $p < 0.001$)

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Conclusions

Reference based thresholds still constitute the most reliable way in CIR determinations to distinguish between endogenous or exogenous origin or urinary steroids, which has been demonstrated several times within the last years [1-6]. Major changes in sample preparation always necessitate new determination of limits. The results obtained within this study basically confirm the results of earlier investigations. Furthermore, using the example of 5a-Diol and 5b-Diol, the imperative to reevaluate reference-based thresholds is clearly demonstrated.

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