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Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids excreted as sulphates: method validation and reference population.

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Introduction

More than 1/3 of all steroids are excreted as conjugates. The main excretion forms in urine are glucuronides and sulphates. While the $^{13}C/^{12}C$ ratio of glucuronidated steroids has been under investigation in the recent years,[1-4] nearly no data was published for sulfated steroids.[5] So a method was developed to investigate the $\delta^{13}C$ values of dehydroepiandrosterone (DHEA), etiocholanolone (E), androsterone (A), epiandrosterone (EpiA), 5-androsten-3β,17β-diol (5EN17b), 5-androsten-3β,17α-diol (5EN17a) and 5-pregnen-3β,17,20α-triol (PT) excreted as sulphates.

Experimental

The sample preparation for sulphated steroids was carried out subsequent to the preparation for glucuronides.[4] In Figure 1 a flow scheme is presented.
Sufficient clean up of all analytes was achieved by a twofold HPLC fractionation of foremost underivatized and then acetylated steroids. HPLC conditions were similar to the ones published for glucuronided steroids.[4] In Figure 2 UV-chromatograms of the standards used to fix retention times of analytes are shown.

aqueous residue (after routine sample preparation) → adjust pH = 5 with glacial acetic acid → SPE conditioning, loading, washing → dry SPE in an desiccator over P_2O_5 → elute SPE with methanol /ethyl acetate (30/70) → solvolysis with ethyl acetate/H_2SO_4 (100 mL/200 mg) → 1h at 55°C, than add methanolic NaOH → dry , redisolve in 2 mL H_2O and extract with TBME → HPLC clean up

Figure 1: Flow scheme of sample preparation

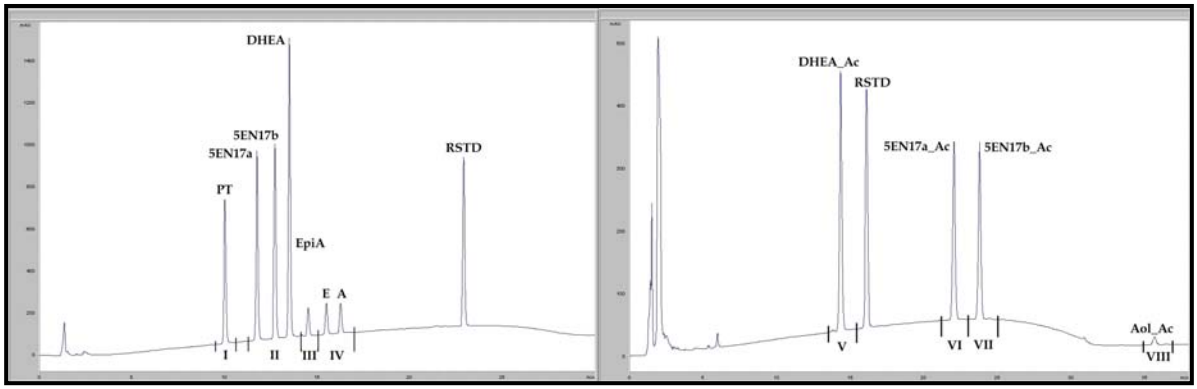

Figure 2: HPLC chromatograms of the first clean up (left) and the second clean up (right). Fraction II from the first run was acetylated and fractionated again to achieve sufficient clean up. Fraction I was acetylated prior analyses and Fractions III and IV were determined underivatized. 17β-estradiol-diacetate (RSTD) was used as reference standard for HPLC and androstanol-acetate (Aol_Ac) to control the acetylation step.

GC/C/IRMS measurements

All samples were measured on a Hewlett-Packard HP5890 Series II Gas Chromatograph (Böblingen, Germany) coupled to a Delta C gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via the GC Combustion Interface II (ThermoElectron). The GC system was equipped with a Macherey & Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). Injection was performed cool-on-column at 50°C. After keeping the initial temperature for 0.5 min it was increased with 30°C/min up to 250°C, then with 2°C/min to 270°C, finally with 15°C/min to 295°C and maintained for 2min. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.2 mL/min.

Validation

The approach of linear mixing models was chosen to study the method's validity.[4] With every batch of samples a blank urine was prepared in order to monitor the method's repeatability over time (here 6 months). In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in all fractions, it was necessary to scan all samples on a GC/MS system using settings comparable to the IRMS setup.

Reference population

The reference population included 67 subjects (students and employees of the German Sport University, Cologne), 25 females and 42 males, aged between 18 to 46 years. Body weights ranged from 50-100 kg, body heights from 160-203 cm, and all participants exercised up to 6

times per week. All subjects provided single spot urine samples, and the specimens were stored at –20°C until analysis.

Results and discussion

Method validation

The results of the linear mixing models are listed in Table 1. The test failed for none of the implemented steroids. The standard deviations (SD) for all steroids were found slightly larger than for the comparable glucuronidated steroids[4] (mean SD of ± 0.39 ‰ instead of ± 0.28 ‰). In Table 2 the results of the blank urine prepared with each batch of samples of the reference population are shown. Again the SDs are slightly larger than for glucuronides but still satisfying for $\delta^{13}C$ determinations. The larger SD might originate from the solvolysis reaction, which did not yield the same amount of free steroid for every sample preparation.

	a [‰]	SD [‰]	b [‰]	SD [‰]
PT	10.8	0.47	-30.5	0.22
E	11.1	0.38	-34.2	0.18
A	9.4	0.43	-30.1	0.20
DHEA	12.2	0.32	-32.2	0.15
EpiA	2.3	0.35	-18.6	0.18
5EN17b	9.0	0.38	-31.5	0.19
5EN17a	12.6	0.40	-33.1	0.22

Table 1: Results of the linear mixing models; referring to the linear equation y = ax + b

	PT [‰]	E [‰]	A [‰]	EpiA	5EN17a	5EN17b	DHEA [‰]
mean	-20.8	-22.9	-21.7	-21.8	-21.9	-22.2	-20.5
SD	0.30	0.81	0.76	0.49	0.50	0.76	0.39

Table 2: Results of the blank urine prepared over a period of six month (n = 6)

Reference population

In order to determine the $\delta^{13}C$ values of all steroids at natural abundance and their associated Δ values, the reference population comprised n = 67 subjects was investigated. From a total of 469 determinations only 11 were not accepted due to steroid concentration below the limit of detection (n = 5) or HPLC failure (n = 6). The absolute $\delta^{13}C$ values of all steroids cover a range from -19.2 ‰ for one DHEA to -26.6 ‰ for one 5EN17b determination and are

therefore in the expected range for urinary steroids.[6] In Table 3 the calculated Δ values and the resulting reference limits are listed. Each Δ value was tested for Gaussian distribution (Shapiro Wilk test) and no significant discrepancies were detected which allowed for parametrical calculation of reference limits. Over all it can be stated that the limits are higher than for glucuronides because of larger SDs and because of the more enriched endogenous reference compound PT used here.

	ΔPT-E	ΔPT-A	ΔPT-EpiA	Δ PT-5EN17a	ΔPT-5EN17b	ΔPT-DHEA
mean	2.3	1.0	1.3	1.6	2.3	0.2
SD	0.97	1.05	0.89	1.10	1.29	1.03
ref lim	5.2	4.2	4.0	4.8	6.2	3.3

Table 3: Δ values of the reference population. The reference limits (ref lim) are calculated by adding the threefold SD to the mean value which results in a 99.7 % reference interval.

Conclusion

The developed method offers the potential to investigate steroids excreted as sulphates in addition to glucuronidated steroids. The calculated reference limits are effectual and the method can be applied for doping control, but first results showed no substantial improvement in detection times in contrast to existing methods. Nevertheless will this method be a valuable tool for investigations on steroid metabolism and related influences on $\delta^{13}C$ values.

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