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Population study on $\Delta\delta^{13}C$ values of endogenous steroids in Romania

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Abstract

As part of the method validation for establishing the exogenous source of endogenous steroids in human urine by estimating the $^{13}C/^{12}C$ ratio by GC/C/IRMS technique, a population study on the $\Delta\delta^{13}C$ values of a Romanian reference population has been conducted. The results of the study are presented. For each pair of target compound (TC)-endogenous reference compound (ERC) an individual reference interval was calculated as average ± 3 standard deviations. The reference intervals calculated on the basis of a population study are including both the natural variation within the reference population of these values and the uncertainty of the method of measurement. The reference interval, outside which the measured values are no longer compatible with a natural occurrence, presented variations from one TC-ERC pair to another, supporting the recommendation to calculate individual reference intervals for each TC-ERC pair.

Introduction

Although chemically identical, the $^{13}C/^{12}C$ isotopic ratio in the synthetic product is different from the naturally produced steroid. The $^{13}C/^{12}C$ isotopic ratios for the endogenous steroids can be accurately measured by the GC/C/IRMS technique [1-4]. The biochemical processes are producing some kinetic isotopic effects [5,6] and measurement precision of $\delta^{13}C$ values is not the same for each compound [2]. Therefore, is expected that the $\Delta\delta^{13}C_{ERC-TC}$ values have different acceptance ranges and is strongly recommended that each laboratory conducts a population study on a statistically significant reference population, to calculate the reference intervals for each ERC-TC pair.

Experimental

Reference population:
Urine sample collected from Romanian healthy volunteers (39 male, 11 female), not medicated with steroids, corticosteroids or other medication that might interfere with the target or reference compounds analyzed.
Sample preparation: The sample preparation is comprehensively described in reference [2]. A 5-20 mL urine sample, depending on the previously measured steroid profile, was prepared by a standard operating procedure for steroids. The dry residue was reconstituted in acetonitrile/water 1/1 and a first HPLC clean-up carried-out. Six fractions were collected, internal standard 5α-androstan-3β-ol (RSTD) added, dried and acetylated (with 50 µL acetic anhydride and 50 µL piridine, at 70°C, 45 min). Four fractions, reconstituted in cyclohexane, were injected in GC/C/IRMS; the fraction with testosterone and the fraction with DHEA were reconstituted in acetonitrile/water 1/1 and submitted to a second HPLC clean-up before injection.

HPLC clean-up:
The collected fractions and collection periods are shown in Table 1. The clean-up was performed on an Agilent 1100 series HPLC system with an Agilent 1364A automatic fraction collector and a Merck analytical column LiCrospher 100RP-18 (5 µm) 250x4 mm with precolumn LiCrospher 100RP-18 (5µm) 4x4 mm. The column thermostat was set at 30°C. The injection volume was 50 µL, the flow rate 1 mL/min and the DAD wavelength 192 nm. For HPLC clean-up 1, a linear gradient was used increasing from 30:70 acetonitrile/water to 100% acetonitrile in 25 min; after 5 min at 100% acetonitrile, the column was reequilibrated for 6 min. For HPLC clean-up 2, a linear gradient was used increasing from 60:40 acetonitrile/water to 100% acetonitrile in 30 min; after 10 min at 100% acetonitrile, the column was reequilibrated for 6 min.

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GC/C/IRMS analysis:
All samples were measured on an Agilent 7890A gas chromatograph coupled through GCIsolink and CONFLO IV to a DELTA V PLUS isotope ratio mass spectrometer (Thermo Scientific). The GC system was equipped with a HP-50+ column (30 m, 0.25 mm ID, 0.25 µm film thickness). The injection volume was 2 µL; the injections were performed splitless at 280 °C. A constant flow of 2.4 mL/min of helium carrier gas was used. The initial oven temperature of 60 °C was held for 2 min, increased at 40 °C/min to 260 °C, followed by a ramp at 3 °C/min to 290 °C and 40 °C/min to the final temperature of 295 °C and held for 3 min. The oxidation reactor was operated at 1030 °C and oxidized for 60 min after each sequence of 60-70 injections. The water removal was done by a Nafion membrane. The $\delta^{13}C$ value of the CO_2 reference gas was calibrated towards the CU/USADA-33-1 standard.

Results and Discussion

The acetylated Etiocholanolone (E), Androsterone (A), Testosterone (T), Dehydroepiandrosterone (DHEA), 5α-androstanediol (5a) and 5β-androstanediol (5b) were measured as target compounds (TC). The acetylated 11-ketoetiocholanolone (11K), Pregnanediol (PD) and 5α-androst-16-en-3α-ol (16-androstenol, 16EN) were measured as endogenous reference compounds (ERC). All measured $\delta^{13}C$ values were corrected for the contribution of the acetate groups.

HPLC clean-up 1		
Fraction	**Analyte**	**Collection period (min)**
I	11K	11.2-12.4
II	T	13.5-14.7
III	5a, 5b, EpiT, DHEA	14.7-16.8
IV	E, A	16.8-19.2
V	PD	19.2-20.6
VI	16EN	28.4-29.8
HPLC clean-up 2		
Fraction	**Acetylated analyte**	**Collection period (min)**
II-1	T_ac	12.8-14.2
II-2	RSTD_ac	37.1-38.5
III-1	EpiT_ac	11.2-12.6
III-2	DHEA_ac	14.9-16.3
III-3	5b_ac	24.3-25.8
III-4	5a_ac	25.8-27.2
III-5	RSTD_ac	37.1-38.5

Table 1. HPLC clean-ups collected fractions and collection periods

Table 2 shows a summary of $\delta^{13}C$ values of endogenous steroids. The mean $\delta^{13}C$ values showed that, among ERCs, 16EN is the most enriched in ^{13}C and the 11K the most depleted. E is more depleted in ^{13}C than A; this might be the result of E peak tailing under A peak; although the chromatographic separation was satisfying, the additional peak tailing introduced by the oxidation reactor and the existing post GC column dead volumes still affects the measurement of $\delta^{13}C$ values for E and A. Part of that difference could be explained by a kinetic isotopic effects arising from reduction of the double bond between C-4 and C-5 [6], but this effect should have result also in a 5b more depleted than 5a [2], which was not noticed. T and 5a are also relatively depleted; this might be due to background interference, more visible on the endogenous steroids with lower concentration in the urine sample. A few $\delta^{13}C$ values for T and 5a were even bellow -28 ‰, although all urines used in the study were negative. Therefore, an adverse analytical decision should not be based on a single abnormal $\delta^{13}C_{TC}$ or $\Delta\delta^{13}C_{ERC\text{-}TC}$ value; also, instead of using a unique threshold for all TCs, reference intervals should be calculated for each TC. The minimum $\delta^{13}C$ for all selected TCs and ERCs were close to 2-fold and above 3-fold SD, providing the opportunity to propose criteria based on 3 SD confidence limits.

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	E	A	T	DHEA	5b	5a	11K	PD	16EN
Mean	-25.74	-22.14	-26.19	-23.32	-24.98	-26.77	-23.83	-23.07	-22.91
SD	0.94	0.80	1.38	0.97	1.10	1.21	0.95	0.84	1.13
Max	-23.85	-20.62	-23.75	-21.29	-22.71	-24.38	-21.62	-21.33	-20.88
Min	-27.59	-23.90	-28.94	-25.41	-27.05	-29.45	-25.90	-24.93	-25.65
m-2s	-27.63	-23.74	-28.96	-25.26	-27.19	-29.19	-25.72	-24.76	-25.18
m-3s	-28.57	-24.54	-30.34	-26.23	-28.29	-30.40	-26.67	-25.60	-26.31

Table 2. Summary of $\delta^{13}C$ values of endogenous steroids from Romanian population

Table 3 shows a summary of $\Delta\delta^{13}C_{ERC\text{-}TC}$ values. The reference interval, calculated as 3-fold SD around the average, accounts for both natural variation and method uncertainty. Since the synthetic steroids are more depleted in ^{13}C than the endogenously produced steroids, a significant exogenous contribution is expected to increase the $\Delta\delta^{13}C_{ERC\text{-}TC}$ above the upper limit of the reference interval (m+3s). Some of the upper limits are close to the 3 ‰ criterion, but most are either much lower or higher, due mainly to averages significantly biased from 0 ‰; all SDs were close to 1 ‰.

	11K-E	11K-A	11K-T	11K-DHEA	11K-5β	11K-5α
Mean	+2.05‰	-1.73‰	+2.46‰	-0.61‰	+1.10‰	+2.75‰
SD	0.97‰	0.99‰	1.10‰	1.30‰	0.92‰	1.20‰
m-3s	*-0.9‰*	*-4.7‰*	*-0.8‰*	*-4.5‰*	*-1.7‰*	*-0.9‰*
m+3s	**+5.0‰**	**+1.2‰**	**+5.7‰**	**+3.3‰**	**+3.9‰**	**+6.4‰**

	PD-E	PD-A	PD-T	PD-DHEA	PD-5β	PD-5α
Mean	+2.68‰	-0.79‰	+3.44‰	-0.35‰	+1.80‰	+3.86‰
SD	0.77‰	0.62‰	1.28‰	0.65‰	0.88‰	0.74‰
m-3s	*-0.4‰*	*-2.6‰*	*-0.4‰*	*-1.6‰*	*-0.9‰*	*-1.6‰*
m+3s	**+5.0‰**	**+1.1‰**	**+7.3‰**	**+2.3‰**	**+4.4‰**	**+6.1‰**

	16EN-E	16EN-A	16EN-T	16EN-DHEA	16EN-5β	16EN-5α
Mean	+2.77‰	-0.79‰	+3.41‰	+0.46‰	+1.89‰	+4.06‰
SD	1.11‰	0.90‰	1.16‰	1.27‰	1.01‰	1.14‰
m-3s	*-0.6‰*	*-3.5‰*	*-0.1‰*	*-3.3‰*	*-1.1‰*	*-0.6‰*
m+3s	**+6.1‰**	**+1.9‰**	**+6.9‰**	**+4.3‰**	**+4.9‰**	**+7.5‰**

Table 3. Summary of $\Delta\delta^{13}C_{ERC\text{-}TC}$ values of endogenous steroids from Romanian population

Conclusions

A statistical population study on the $\delta^{13}C$ and $\Delta\delta^{13}C_{ERC\text{-}TC}$ values of a Romanian reference population (50 volunteers) was conducted. The $\Delta\delta^{13}C_{ERC\text{-}TC}$ reference intervals varied significantly among the endogenous reference compound-target compound (ERC-TC) pairs supporting the WADA recommendation of calculating the reference intervals in each laboratory for each ERC-TC pair. The reference intervals calculated on the basis of a population study are including both the natural variation of the $\Delta\delta^{13}C_{ERC\text{-}TC}$ within the reference population and the uncertainty of the method.

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