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Preliminary results on $\delta^{13}C$ values of endogenous steroids in Cuban population.

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Introduction

The detection of endogenous steroids produced naturally for the human body has always been a major challenge for anti-doping scientists as the administered steroids are chemically identical to that produced in the body, but $^{13}C/\ ^{12}C$ ratio in the synthetic product is slightly different. GC/C/IRMS is a powerful tool able to detect and confirm the abuse of anabolic steroid which occur naturally in the body such as testosterone.[1,2] However, the number of $\delta^{13}C$ profiling studies of urinary steroid has been limited [3] even though this topic represents a breakthrough in doping control. The aim of the present work was to investigate the delta values ($\delta^{13}C$) of the endogenous steroids to establish reference ranges in Cuban population.

Materials and methods

Reference population: Urine samples were collected from Cuban athletes (20 males, 10 females) and healthy volunteers (12 males, 8 females) n=60.

Urine sample preparation: Extraction of 10 mL of urine was carried out by the standard operating procedure to detect the endogenous steroids profile. The dry residue was reconstituted with methanol and sample purification was carried out by HPLC following previous works.[4,5] Four fractions (F1-4) were collected and analyzed by GC/MS and GC/C/IRMS.

Instrument: GC/C/IRMS Finnigan Delta Plus IRMS coupled with a HP 6890 Gas Chromatograph. Analytes separation was achieved on a capillary column SPB-5 (30 m x 0.25 mm I.D x 0.25 film thickness). Helium at 2 mL/min was used as carried gas. The oven temperature programme started at 150°C for 1 min and then increased to 260°C at 30 °C/min rate. Later, it was increased to 290 °C at 1.5 °C/min rate, then up to 300 °C at 6 °C/min and finally it was kept at 300°C for 2 min. Splitless injection mode at 280°C was used. Injection volume was 2-4 µL. The oxidation and reduction reactors were operated at 940 °C and 650 °C, respectively.

Results and discussion

In order to find out the naturally occurring $\delta^{13}C$ values of the selected steroids and their associated Δ values, the reference population (n=60) was investigated. The $\delta^{13}C$ values of the following endogenous steroids were measured: 11-ketoetiocolanolone (11-keto) and 11β-hydroxyandrosterone (OHA) (F1), androsterone (A) and etiocholanolone (E) (F2), pregnanediol (PD) (F3) and androstenol (16-EN) (F4) (figure 1). PD, OHA, 11-keto and 16-EN were taken as endogenous reference compounds (ERCs). All steroids were injected without derivatization. Table I summarizes the results from reference population evidencing natural discrimination against ^{13}C in the formation of E relative to A.

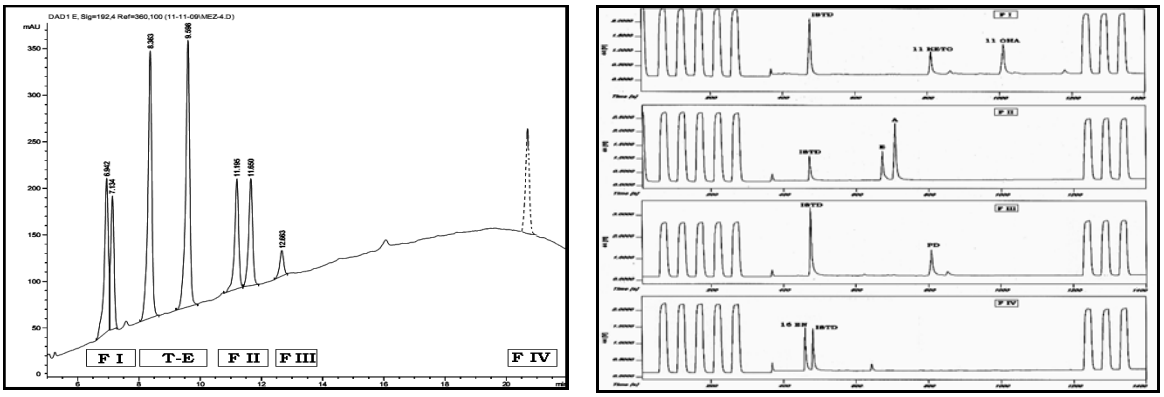

Figure 1. HPLC and GC/C/IRMS chromatograms of each fraction.

Table I. Summary of $\delta^{13}C$ of endogenous steroids from Cuban population (n= 60).

	$\delta^{13}C$ (‰)					
	E	A	PD	OHA	11-KETO	16-EN
MEAN	-21.89	-20.66	-21.14	-21.07	-20.86	-21.37
SD	0.99	0.78	1.14	1.11	1.2	1.08
MAX	-20.27	-19.22	-18.79	-18.26	-18.23	-19.67
MIN	-23.77	-22.17	-23.16	-22.42	-22.83	-23.65
MEAN - 3SD	-24.86	-23	-24.56	-24.4	-24.46	-24.61

Viewing data set without need to account for statistical variable, minimum $\delta^{13}C$ values of A, E, PD, OHA, 11-keto and 16-EN were determined to be -23.77 ‰, -22.17 ‰, -23.16 ‰, -22.42 ‰, -22.83 ‰ and -23.65 ‰, respectively. The mean of $\delta^{13}C$ values obtained from the selected steroids showed that OHA is more enriched in ^{13}C than PD, and 16-EN is the most depleted. E was more depleted in ^{13}C than A. It has been described as being the result of kinetic isotope effects arising from reduction of the double bond between C-4 and C-5 in Δ^4-steroids.[6]

Reference limits for doping control can be calculated using a simple statistic test on the basis of confidence intervals derived from the population mean and population standard deviation (SD).[7] Many authors state that 3-fold SD show appropriate reference limits.[1,8,9] The minimum $\delta^{13}C$ for all selected TCs and ERCs were between the 2-fold and 3-fold SD providing the opportunity to propose criteria based on 3 SD confidence limits of -24.86 ‰ and -23 ‰ for E and A, respectively. No sample in data set had $\delta^{13}C$ values lees than -28 ‰, the limit established by WADA to consider a metabolite as a product of endogenous steroids abuse.[10] Because of the absolute $\delta^{13}C$ values of endogenous steroids can vary in a large range from -16 ‰ to -26 ‰,[11,12] Δ values have demonstrated to be more reliable to detect the misuse of synthetic steroids, considering that both ERCs and target compounds (TCs) are influenced by the diet. Not all pairs of endogenous steroids yield the same Δ values within one individual due to a natural occurring offset, which is attributed to isotopic fractionation occurring with a consistent isotopic fractionation factor of each enzymatic reaction or diffusion process during steroids metabolism.[1] Hence, for doping control purposes, decisive criteria should be based on laboratories' reference populations.[4] To detect doping, the mean values of $\delta^{13}C$ E and A or its absolutes values can be subtracted from the $\delta^{13}C$ value of the ERCs to provide a $\Delta\ \delta^{13}C$ measurement capable of distinguishing between endogenous or exogenous values of E and A. In this work the maximum $\Delta\ \delta^{13}C$ observed was -2.02 ‰ to the pair 11-keto-TC, a value that can be proposed as an absolute limit. All $\Delta\ \delta^{13}C$ values were less than 3 ‰.

It is known that diet may change the carbon isotopic composition. The difference in the ^{13}C enrichment of food products in the diet and even in the food chain is caused by different contribution of natural ^{13}C-enriched constituents. The variation in ^{13}C abundance foodstuffs is mainly the result of isotopic fractionation in plants. Preliminary results have reveals two primary clusters of E and A values derived from C_3 diets ($\delta^{13}C$= -23.0 ± 1.0 ‰) and C_4 diets ($\delta^{13}C$= -20.1 ± 1.3 ‰).[13,14] Our data set includes our population in the C_4 group, with $\delta^{13}C$ values comparables with Center America and North America countries like Mexico and United Stated, respectively.[7]

Conclusions

The $\delta^{13}C$ values of endogenous steroids from Cuban population are presented. The $\Delta\delta^{13}C$ values between endogenous steroids and several ERCs were less than 3 ‰. It is a proposed criteria based on 3 SD confidence limits of -24.86 ‰ and -23 ‰ for E and A, respectively. Our data set includes our population in the C_4 group, with $\delta^{13}C$ values comparables with Center and North America countries like Mexico and United Stated. This reference

population will grow over time with the benefit of increasing statistical information for the already implemented use of steroids.

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