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Short communication

Longitudinal profiling of urinary steroids by gas chromatography/combustion/isotope ratio mass spectrometry: Diet change may result in carbon isotopic variations

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Abstract

Longitudinal profiling of urinary steroids was investigated by using a gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) method. The carbon isotope ratio of three urinary testosterone (T) metabolites: androsterone, etiocholanolone, 5β-androstane-3α,17β-diol (5β-androstanediol) together with 16(5α)-androsten-3α-ol (androstenol) and 5β-pregnane-3α,20α-diol (5β-pregnanediol) were measured in urine samples collected from three top-level athletes over 2 years. Throughout the study, the subjects were living in Switzerland and were residing every year for a month or two in an African country. ^{13}C-enrichment larger than 2.5‰ was observed for one subject after a 2-month stay in Africa. Our findings reveal that ^{13}C-enrichment caused by a diet change might be reduced if the stay in Africa was shorter or if the urine sample was not collected within the days after return to Switzerland. The steroids of interest in each sample did not show significant isotopic fractionation that could lead to false positive results in anti-doping testing. In contrast to the results obtained with the carbon isotopic ratio, profiling of urinary testosterone/epitestosterone (T/E) ratios was found to be unaffected by a diet change.
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1. Introduction

Endogenous steroids are produced from cholesterol in the body. Cholesterol is derived from an average of a wide variety of feed vegetal and animal precursors or synthesized from precursors of feed origin. In plant tissue, the main source of variation in $^{13}C/^{12}C$ isotopic ratio (expressed in $\delta^{13}C$-value) is derived from the different photosynthetic pathways for carbon dioxide fixation. Plants incorporate carbon dioxide photosynthetically by three different mechanisms: the Calvin cycle (C_3) pathway, the Hatch-Slack (C_4) pathway and the Crassulacean acid metabolism (CAM) pathway. The C_3 pathway results in a large change in the carbon isotope proportions relative to atmospheric carbon dioxide and hence discriminates more strongly against the heavier isotope ^{13}C compared to the C_4 pathway. Main representatives of C_3 group are wheat, rice, potato, barley, grape, oats and sugar beet, whereas maize, sugar cane, millet and pineapple are the important species of the C_4 group. The $\delta^{13}C$-values of C_3 and C_4 plants are ranging from −35 to −22‰ and from −20 to −8‰, respectively [1].

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 naturally ^{13}C-enriched constituents. Because common food ingredients are maize, millet and sugar cane (C_4 plants) in certain areas of Africa, it is expected that the basic ^{13}C enrichment of the body store will be high for local populations [2]. It is known that urine samples collected from a country like Kenya have a higher content of ^{13}C in steroids than western or oceanian countries [3,4].

Although carbon isotope dynamic studies of urinary steroids in relation with diet changes have been shown to produce significant variations in cattle [5,6], little is known on exchange

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rates of ^{12}C and ^{13}C during steroid biosynthesis in humans. A GC/C/IRMS method is used to investigate longitudinal variations over a 2-year period of carbon isotopic ratio ($^{13}C/^{12}C$) of urinary steroids in selected top-level Caucasian male athletes and also assess on their profiles, the possible influence of environmental/diet changes induced by intercontinental traveling with variable residence time periods. These data should demonstrate the use of IRMS analysis for longitudinal profiling and provide insights into physiological variations of steroid carbon isotopes, also with the aim of avoiding misinterpretation in isotope ratio mass spectrometry (IRMS) anti-doping testing.

2. Experimental

2.1. Subjects and experimental protocol

Three top-level Caucasian male athletes (runners) aged between 28 and 30 years participated in a 2-year longitudinal study. This study is a part of the project "Top Level Sport Without Doping" of the Swiss Federal Office for Sport (FOSPO) with the aim of promoting doping-free sport and protecting the right of athletes to compete in a fair and ethical sport environment. The participants gave their written informed consent and accepted to produce urine samples for both announced and unannounced testing. Throughout this 2-year study, the subjects were residing from 1 to 2 months a year in either Kenya or South Africa, and were living in Switzerland for the rest of the time or were in competition in a European country for 2-week maximum period. Although no detailed diet report was requested, the subjects declared that they had fully adapted their diet to local food, with the two main changes being an increase in maize consumption and substituting cane sugar for beet sugar. In addition, none of the athletes consumed food supplement or prohormones during the entire study. Urine samples were divided into 20 ml flasks and stored without additives at −20 °C until analysis.

2.2. Quantitative analysis of urinary steroids

Quantitative analyses of urinary testosterone (4-androsten-17β-ol-3-one) and epitestosterone (4-androsten-17α-ol-3-one) glucuronides were performed using similar procedures of a previously reported method [7]. The urine sample (2.5 ml) was spiked with 20 μl of an internal standard (17α-methyltestosterone, 0.020 mg/ml in methanol) and was then applied onto a 500 mg C_{18} solid phase column (JT Baker, Phillipsburg, NJ, USA) previously conditioned with 5 ml of methanol and 5 ml of water. Elution was carried out three times with 1 ml of methanol and the solvent was evaporated to dryness under a stream of nitrogen at 40 °C (Turbo Vap LV evaporator, Zymark, Hopkinton, MA, USA). To hydrolyze the glucoconjugated steroids, the residue was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/ml at 37 °C) was added as supplied (Roche Diagnostics GmbH, Manheim, Germany). The hydrolysis was completed in 1 h at 50 °C or at 37 °C overnight in a thermostated water bath. After addition of about 200 mg solid carbonate buffer (Na_2CO_3–$NaHCO_3$, 1:10 by weight), the sample was extracted with 5 ml of *tert*-butylmethylether (TBME) with shaking for 10 min. After centrifugation (2500 × *g* for 5 min), the organic phase was collected and dried with solid Na_2SO_4. The derivatization was carried out by addition of 50 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)/NH_4I/ethanethiol (1000/2/3; v/w/v). The reaction mixture was heated in a stopped vial at 60 °C for 20 min. The samples (2 μl) were injected at 300 °C with a 10:1 split on a Hewlett-Packard 6890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7683 auto-sampler and coupled with a HP 5973 mass selective detector (MSD). GC separation was achieved on HP-1 column (17 m × 0.2 mm i.d., 0.11 μm film thickness) from J&W Scientific (Folsom, CA, USA) operated with a helium inlet pressure of 20 psi. The oven temperature was increased from 181 to 230 °C at 3 °C/min, and then to 310 °C at 40 °C/min and held for 4 min. The electronic beam energy was set at 70 eV in the electronic impact (EI) mode.

The analyses were performed in single ion monitoring mode (SIM) with $m/z = 432$ for both testosterone and epitestosterone. For each substance, a six-point calibration curve was established ($R^2 > 0.996$) using available reference material (Steraloids Inc., Newport, RI, USA) in the 5–250 ng/ml concentration range for testosterone and epitestosterone.

2.3. GC/C/IRMS analysis of urine samples

The symbol δ is the standard notation for expressing carbon isotope ratios $^{13}C/^{12}C$. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}\text{C}\,(‰) = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \times 1000$$

Urinary androstenol (16(5α)-androsten-3α-ol), 5β-pregnanediol (5β-pregnane-3α,20α-diol), androsterone (5α-androstan-3α-ol-17-one), etiocholanolone (5β-androstan-3α-ol-17-one) and 5β-androstanediol (5β-androstane-3α,17β-diol) were extracted in two different fractions following a previously described method for IRMS analysis [8]. A $\text{Delta}^{\text{Plus}}$ XL IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A Gas Chromatograph (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used for separation and online combustion of the steroid metabolites. Chromatographic separations were achieved on a HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness) from J&W Scientific (Folsom, CA, USA). The carrier gas was helium with a constant flow of 1.0 ml/min and initial pressure of 13.6 psi. The injection volume was 2 μl in splitless mode at 280 °C. The combustion and reduction oven temperatures were set to 940 and 600 °C, respectively. For the analysis of the androsterone and

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