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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.

Keywords: Steroids; Diet; Isotope ratio mass spectrometry (IRMS); Doping control

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 δ^{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 δ^{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:

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

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 Delta^{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

etiocholanolone acetates, the oven temperature was increased from 80 °C (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. For the analysis of 5β-androstanediol, 5β-pregnanediol and androstenol acetates, the oven temperature was increased from 80 °C (1 min) to 270 °C (11.5 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. All urine samples of each athlete have been processed by batch to obtain a sufficient precision on the carbon isotope ratios (S.D. ∼ 0.3‰). Negative shifts of the δ^{13}C-value due to the formation of acetate were corrected [8].

2.4. Statistical analysis

Statistical analysis was performed using the SPSS 13.0 package for Windows (SPSS Inc., Chicago, USA). The normality of the distributions was assessed with the Kolmogorov–Smirnov test. Statistical significance for the carbon isotopic values of the first urine sample collected from each subject after residence periods in Kenya and South Africa with respect to the other samples was assessed with the Wilcoxon signed-ranks test. The significance level was set at $p < 0.05$.

3. Results and discussion

The 2-year longitudinal profile of urinary isotopic values (expressed as δ^{13}C-value) of measured steroids is depicted for each subject in Fig. 1, together with their residence periods in Africa. Using Wilcoxon signed-ranks test, significant changes ($p < 0.05$) are observed for the mean carbon isotopic values of the first urine sample collected from subject S1 after the residence periods in Kenya and South Africa. While mean carbon isotopic values of the urine sample of subject S2 directly collected after returning from the second period in South Africa are significantly different from the values of the other samples, the longitudinal profile of subject S3 reveals no significant variation throughout the 2-year study. After residence in African countries, the observed variations versus less negative delta values, i.e. versus enrichment in ^{13}C, may mainly be attributed to a change in diet [2]. Significant differences ($\Delta\delta^{13}$C-value > 3‰) are observed when the mean carbon isotopic values of urinary androsterone and etiocholanolone in Kenyan [3] and Swiss [4] populations are compared.

Our results tend to show a dynamic ^{13}C-enrichment upon diet change with significant inter-individual variations following time of residence and collection time interval of the urine sample after returning to Switzerland. Urine samples of subject S3 collected 2 and 1 months after the first and second 5-week residence in Kenya, respectively, do not show significant ^{13}C-enrichment, whereas a variation of about 1‰ is observed for the urine sample collected 2 weeks after returning to Switzerland (subject S2, Fig. 1). In contrast to subjects S1 and S2, the natural longitudinal variation in the carbon isotopic ratios of urinary steroids is normally distributed for subject S3, and the standard deviations for the steroids of interest are ranging from 0.21 to 0.38‰. The profile of subject S1 is characterized by a large variation ($\Delta\delta^{13}$C-values > 2.5‰) for the urine sample col-

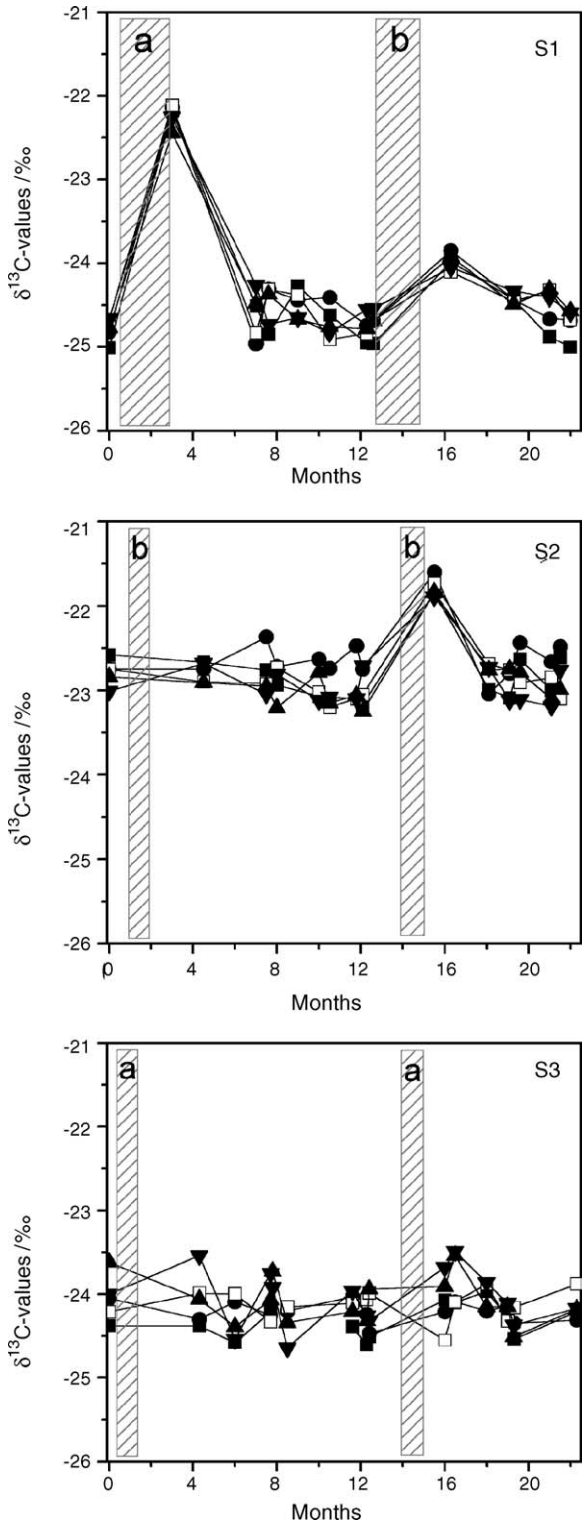

Fig. 1. Two-year longitudinal profiles of urinary δ^{13}C-values of androsterone (▲), etiocholanolone (▼), 5β-pregnanediol (●), 5β-androstanediol (■) and androstenol (□) for athletes S1, S2 and S3. The striped rectangles denote the residence periods in either Kenya (a) or South Africa (b). Residence time of subject S1 in Africa was of 10 and 8 weeks for the first and second year, respectively. Subjects S2 and S3 resided for 5 weeks per year in South Africa and Kenya, respectively. Collection time interval of the first urine after the first residence in Africa was of 1, 8 and 9 weeks for subjects S1, S2 and S3, respectively. After the second residence in Africa, the urine samples were collected at the earliest 6, 2 and 4 weeks for subjects S1, S2 and S3, respectively.

lected 1 week after returning from Kenya and a much smaller variation for the urine sample collected 6 weeks after the stay in South Africa. Nevertheless, it is not possible from our data to anticipate a larger ^{13}C-enrichment in subject S1 if (1) he would have resided for a longer period of time in Kenya and (2) the urine sample would have been collected the day after returning to Switzerland.

The selected urinary steroids are all derived from cholesterol, but are produced along different metabolic pathways. Therefore, significant differences in ^{13}C-enrichment of these steroids induced by different carbon exchanges could take place within a short period of time after diet changes with ^{13}C-enriched ingredients. For the first urine samples collected after intercontinental stays of subjects S1 and S2, differences less than 0.4‰ are found between the steroids of interest. Considering the within-assay precision on IRMS measurements, these results show that similar carbon redistribution along the different metabolic pathways was reached, either with enrichment in ^{13}C or with a return to basal $^{13}C/^{12}C$. For subject S3, we obtain larger differences ($\Delta\delta^{13}C$-values up to 0.9‰) for the first urine sample collected after both 1-month stays in Kenya.

The International Olympic Committee (IOC) Medical Commission banned the use of testosterone in 1982 and stated that a urinary T/E ratio above 6 was a sufficient proof of testosterone abuse. After while, it became apparent that certain individuals had T/E ratios naturally elevated above 6 [9], the IOC stated that a follow-up was needed for T/E ratios above this value to prove physiological or pathological circumstances. In August 2004, a technical document from the World Anti-Doping Agency (WADA) was adopted with the need of submitting the sample to IRMS analysis for determination of the $^{13}C/^{12}C$ ratio of selected steroids, if the urinary T/E ratio value is equal or greater than 4 as well as for altered steroid profiles with testosterone metabolites and DHEA concentration greater than fixed cut-off concentrations [10]. If IRMS analysis does not readily indicate exogenous administration of steroids for urinary samples with T/E ratio $\geq$4, the result is reported as inconclusive and further longitudinal studies have to be performed.

As exogenous testosterone or precursors contain less ^{13}C than their endogenous homologues, it is expected that urinary steroids with a more negative $^{13}C/^{12}C$ ratio originate from pharmaceutical source [11]. According to the technical document of the WADA Laboratory Committee, a result would be reported as consistent with the administration of a steroid when the $^{13}C/^{12}C$ value measured for the metabolites differs significantly, i.e. by 3.0‰ or more from that of the urinary reference steroid chosen [10]. We observe that carbon isotopic differences between testosterone metabolites (androsterone, etiocholanolone and 5β-androstanediol) and endogenous references (androstenol and 5β-pregnanediol) are less than 0.8, 0.7 and 0.9‰ for subjects S1, S2 and S3, respectively. Therefore, these results do not indicate a doping with testosterone or a precursor, according to August 2004 WADA criteria.

For the urinary T/E–time profiles of each subject, it was found that the T/E ratios are normally distributed. All subjects have a remarkably stable T/E ratio throughout the study with coefficients of variation less than 15%. The mean T/E ratio was 1.37 (range: 1.09–1.56, $n = 9$), 0.96 (range: 0.84–1.14, $n = 10$) and 1.18 (range: 0.80–1.36, $n = 10$) for subjects S1, S2 and S3, respectively. These results are in accordance with longitudinal T/E ratio profiles of drug-free males [12]. Nevertheless, it should be mentioned that these T/E–time profiles together with longitudinal carbon isotopic variations may point out a doping with endogenous steroids, but are likely not sensitive enough to detect a doping with anabolic steroids of synthetic origin [13].

In conclusion, our findings have shown that the stay, the length of stay in an intercontinental country and time collection of urine samples might be factors affecting the ^{13}C content of steroids, but do not induce significant isotopic fractionations that could lead to false positive results in anti-doping analysis. Nevertheless, this study is a starting point and further investigations should be performed with a higher number of male and female subjects, together with different residence times and urine sample collections to describe a general kinetic model of carbon enrichment or depletion in steroids. IRMS has been claimed to be the definitive solution for interpretation of abnormal endogenous profiles in drug testing. However, when drug-testing authorities interpret IRMS results, they should have detailed information about residencies and travels of the athlete (whereabouts) as diet might influence the $^{13}C/^{12}C$ values of urinary steroids.

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