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Influences of β-HCG administration on carbon isotope ratios of endogenous urinary steroids

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ABSTRACT

Several factors influencing the carbon isotope ratios (CIR) of endogenous urinary steroids have been identified in recent years. One of these should be the metabolism of steroids inside the body involving numerous different enzymes. A detailed look at this metabolism taking into account differences found between steroids excreted as glucuronides or as sulphates and hydrogen isotope ratios of different steroids pointed out possibility of unequal CIR at the main production sites inside the male body – the testes and the adrenal glands.

By administration of β-HCG it is possible to strongly stimulate the steroid production within the testes without influencing the production at the adrenal glands. Therefore, this treatment should result in changed CIR of urinary androgens in contrast to the undisturbed pre-treatment values.

Four male volunteers received three injections of β-HCG over a time course of 5 days and collected their urine samples at defined intervals after the last administration. Those samples showing the largest response in contrast to the pre-administration urines were identified by steroid profile measurements and subsequent analysed by GC/C/IRMS. CIR of androsterone, etiocholanolone, testosterone, 5α- and 5β-androstanediol and pregnanediol were compared. While pregnanediol was not influenced, most of the investigated androgens showed depleted values after treatment. The majority of differences were found to be statistically significant and nearly all showed the expected trend towards more depleted δ^{13}C-values.

These results support the hypothesis of different CIR at different production sites inside the human body. The impact of these findings on doping control analysis will be discussed.

1. Introduction

The application of carbon isotope ratio (CIR) measurements to sports drug testing is nowadays a well-established method to provide evidence for the intake of testosterone (17β-hydroxy-androst-4-en-3-one, TESTO) or testosterone prohormone like dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one, DHEA) by athletes [1–9]. In order to prove either the exogenous or the endogenous origin, CIR of endogenous reference compounds (ERC) like pregnanediol (5β-pregnane-3α,20α-diol, PD) are compared to the CIR of target compounds (TC). CIR are expressed as δ^{13}C values against the international standard Vienna Pee Dee Belemnite (VPDB) based on the equation:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{std}} - 1, \quad (1)$$

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard [10,11].

Differences between ERC and TC are expressed as Δ values based on the equation:

$$\Delta = \delta^{13}C_{ERC} - \delta^{13}C_{TC} \quad (2)$$

According to the current WADA document in effect for isotope ratio mass spectrometry (IRMS) determinations, a Δ value larger than 3‰ indicates the exogenous source of the TC [12]. Several recent publications pointed out that this threshold is inapplicable to

Abbreviations: CIR, carbon isotope ratios; TESTO, testosterone; DHEA, dehydroepiandrosterone; ERC, endogenous reference compound(s); TC, target compound(s); VPDB, Vienna Pee Dee Belemnite; IRMS, isotope ratio mass spectrometry; HCG, human chorionic gonadotropin; TBME, *Tert.*-butyl methyl ether; MeOH, methanol; AND, androsterone; ETIO, etiocholanolone; PD, pregnanediol; RSTD, androstanol; 5a-AD, 5α-androstanediol; 5b-AD, 5β-androstanediol; 16EN, 3α-hydroxy-5α-androst-16-ene; EPIT, epitestosterone; DHT, dihydrotestosterone; GC/C/IRMS, gaschromatography/combustion IRMS; HPLC, high performance liquid chromatography; Ac, acetate; EPIA, epiandrosterone; SD, standard deviation; WADA, World Anti-Doping Agency; PREG, pregnenolone; CHOL, cholesterol; CYP17, cytochrome P450 17α-hydroxylase-17,20-lyase.

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some pairs of ERC and TC, as investigations on reference populations showed Δ values to be influenced by endogenous isotopic fractionation factors, which results in a different threshold value for those steroids [5–9,13,14].

Only little is known about these influencing factors. While the absolute $\delta^{13}C$ values of endogenous steroids are strictly correlated to the individuals diet resulting in basal values between −16‰ and −25‰, no difference in Δ values was found to be correlated to this [7,8,15,16]. It has been reported that some enzymatic reactions within the human body can result in enrichment or depletion of selected steroids, especially after administration of these steroids or their precursors [5,7,13,17]. But urinary steroid concentrations seem not to be correlated to CIR in general, which would be expected if enzymatic reactions solely influence $\delta^{13}C$ values [7,14].

In addition, differences in CIR of steroids excreted glucuronidated and sulphated have been found to exceed 1‰, which could hardly be attributed only to differences in phase II metabolism or steroid secretion by the kidney [9,18–20]. Those findings are supported by isotopic ratios measured on the other abundant element in the steroid backbone, hydrogen. These results suggest that different steroid production sites within the body might reflect different CIR [21]. This hypothesis is depicted in Fig. 1 and will be explained in the following:

In men the two main production sites for steroids are the testes and the adrenal glands [22–26]. Steroids excreted from the testes into the blood circulation are mainly unconjugated and only a small amount is liberated in its sulfated form. In the adrenal gland it was found to be the other way around and more sulfated than unconjugated steroids are excreted [18,25,27]. During the passage through the liver unconjugated steroids are glucuronidated to a high extent and sulfated only to a small amount. Sulfated steroids remain mostly unchanged and constitute a separated pool of steroids within the plasma, especially DHEA-sulfated which is found with high plasma concentrations. All conjugated steroids are excreted into urine via the kidney. In urine, approximately 70% of all steroids are found glucuronidated and 30% sulfated besides a very small amount of unconjugated steroids (<2%). These values are largely generalized and subjected to a large inter-individual variability [14,28].

Nevertheless, the main part of steroids found glucuronidated in urine should originate from the testes while a larger quantity of steroids produced in the adrenal glands should be found in the fraction of sulfated steroids. If we now assume that steroids produced in the testes have depleted CIR in contrast to steroids emerging from the adrenal glands, most of the above mentioned findings could be explained, especially the differences in CIR between both main excretion forms – glucuronidated and sulfated.

To test for this hypothesis, urines collected after administration of β-human chorionic gonadotropin (HCG) were investigated regarding both their steroid concentrations and CIR of selected steroids excreted both glucuronidated and sulfated. Administration of HCG to males result in a strong stimulation of the steroid production within the testes while no stimulating effects on the adrenal gland come about [23,29]. This strong stimulus of the TESTO-production in the Leydig cells should have an influence on the CIR of urinary steroids that are metabolites of testicular TESTO. Other steroids not synthesized in the testes should not be affected in their CIR.

The verification of this hypothesis will provide a deeper insight into the nature of CIR of endogenous steroids and will help explaining the variability of CIR found in athlete's urine specimens. This improved knowledge will support the decision-making on doping control samples and so further validate the existing methods used for TESTO-misuse detection.

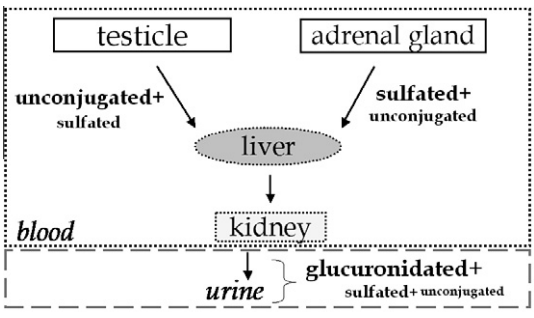

Fig. 1. Schematic of endogenous steroid production and excretion. Glucuronidation takes place at the liver, further information in the text.

2. Experimental

2.1. Chemicals and steroids

Bakerbond™ SPE Octadecyl columns were purchased from J.T. Baker (Deventer, Netherlands). Pyridine, acetic anhydride and sulphuric acid were from Sigma–Aldrich (Buchs, Switzerland) and β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany). *Tert.*-butyl methyl ether (TBME) was obtained from Acros (Geel, Belgium), methanol (MeOH), ethyl acetate and sodium hydroxide from Merck (Darmstadt, Germany) and acetonitrile from Biosolve (Valkensward, Netherlands). All solvents and reagents were of analytical grade. Steroid reference material (3α-hydroxy-5α-androstan-17-one (AND); 3α-hydroxy-5β-androstan-17-one (ETIO); 5β-pregnane-3α,20α-diol (PD); DHEA and TESTO) was supplied by Sigma–Aldrich (Steinheim, Germany). 5α-Androstane-3α,17β-diol (5a-AD), 5β-androstane-3α,17β-diol (5b-AD), 3α-hydroxy-5α-androst-16-ene (16EN) and 3β-hydroxy-5α-androstane (RSTD) were purchased from Steraloids (Newport, RI, USA).

2.2. HCG administration study

A detailed description of the complete excretion study can be found elsewhere and will herein only be described in brief [30]. Ten healthy men aged between 21 and 29 years received 3 HCG injections (2000 I.U. Choriomon®) on days 0, 2 and 4 in the morning. Four blank urine specimens were collected before the first administration and two blank urines were collected 11 days after the last administration. After the third injection, volunteers collected every spot urine for 48 h. All samples were divided into several vials and stored until analysis at −20 °C. The Ethical Commission for Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Lausanne, Switzerland) approved this protocol and all volunteers gave written consent.

Out of this population of 10 subjects, a subset of four individuals (named A, C, F and J) was selected for further IRMS investigations due to their steroid profile.

2.3. Steroid profile

An aliquot of each specimen was prepared according to the already published method suitable to determine the amount of different endogenous steroids for routine doping control samples [30]. The following steroids of interest were determined: TESTO, EPIT (17α-hydroxy-androst-4-en-3-one), DHEA, 5a-AD, 5b-AD, AND, ETIO and DHT (17β-hydroxy-5α-androstan-3-one). The so compiled steroid profile was investigated for influences of HCG administration and was used to ascertain the urine volume requisite for IRMS analysis. Four volunteers showing strong variations in