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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.

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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.

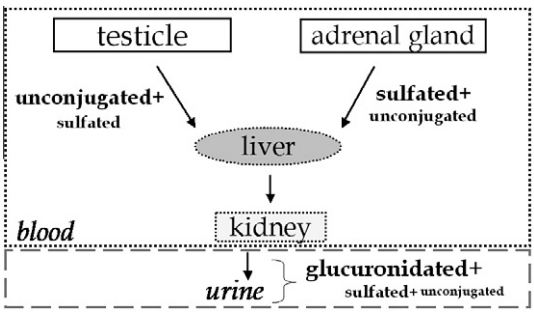

Fig. 1. Schematic of endogenous steroid production and excretion. Glucuronidation takes place at the liver, further information in the text.

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

their steroid profile were chosen for further investigations on IRMS.

2.4. Sample preparation CIR

Analytes have to be efficiently isolated and purified before GC/C/IRMS (gas chromatography/combustion/IRMS) analysis in order to avoid co-elution of compounds and to keep in readiness the ability to measure differently concentrated urinary steroids in comparable amounts. Both aspects are necessary for valid $^{13}C/^{12}C$ determinations. Therefore, extensive sample preparation followed by twofold HPLC clean up was employed.

A detailed description of sample preparation was published elsewhere [7,31] and will herein only be described briefly: 5–20 mL of urine were applied on a conditioned C18 solid-phase extraction cartridge, washed with 2 mL of water and eluted triply with 1 mL of MeOH; the dried residue was dissolved in 1.5 mL of sodium phosphate buffer and extracted with 4 mL of TBME to separate unconjugated steroids; the aqueous residue was hydrolysed with β-glucuronidase for 60 min at 50 °C, and again extracted with 4 mL TBME twice; the organic layer (containing formerly glucuronidated steroids) was transferred into a test tube and evaporated to dryness, re-dissolved in two times 150 μL of MeOH, transferred into a HPLC auto-sampler vials and evaporated.

The aqueous residue was further prepared to liberate the sulfated steroids [14]. The pH was adjusted to 5 with 100–150 μL of glacial acetic acid followed by another solid phase extraction. The sulfo-conjugated steroids were eluted with MeOH/ethyl acetate and hydrolysed with ethyl acetate/sulfuric acid. After adding 0.5 mL of methanolic sodium hydroxide and evaporation to dryness, the residue was reconstituted in 5 mL of water and extracted with 4 mL of TBME twice. After centrifugation, the organic layer (containing formerly sulfo-conjugated steroids) was transferred into a conical test tube and evaporated to dryness, re-dissolved in two times 150 μL of MeOH, transferred into an auto-sampler vial and evaporated.

2.5. HPLC-cleanup of glucuronidated steroids

In order to remove all interfering or co-eluting compounds prior to GC/C/IRMS measurements, two consecutive high performance liquid chromatography (HPLC) fractionation steps were employed. Both were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) with a Waters (Baden-Dättwil, Switzerland) XBridge™ Shield RP18 3.5 μm (4.6 × 250 mm) column protected with a XBridge™ Shield RP18 3.5 μm (4.6 × 20 mm) guard column.

The dried residue was dissolved in 50 μL of a mixture containing acetonitrile/water (50/50, v/v), the injection volume was 50 μL and the flow rate 1 mL/min. A linear gradient was used increasing from 40/60 acetonitrile/water to 60% acetonitrile in 16 min and then within 6 min to 98% acetonitrile. After 8 min at 98%, the column was re-equilibrated for 10 min. Before each batch of samples, a standard solution containing approximately 40 μg/mL of TESTO, DHEA and 16EN, 200 μg/mL of PD and 100 μg/mL of ETIO and AND each was injected twice to determine the retention times for fraction collection. The automatic fraction collector Agilent 1200 was programmed to prepare 5 fractions: The first containing TESTO (collection time from 10.7 to 12.0 min), the second comprising DHEA and 5b-AD (12.8–14.5 min), the third including ETIO and 5a-AD (14.6–16.4 min), the fourth with AND (16.5–17.8 min) and the fifth encompass the ERC PD (19.0–20.5 min). The different fractions were evaporated to dryness under a stream of air. All fractions were acetylated due to considerably improved separation and peak shape of steroids on both the HPLC and the GC column.

Therefore, 50 μL of pyridine and 50 μL of acetic anhydride were added. The mixture was incubated for 60 min at 70 °C and evaporated to dryness under a stream of air and the dried residue was transferred with two times 150 μL of MeOH into auto-sampler vials.

Fractions 1, 2 and 3 were further purified by an additional HPLC fractionation using a different gradient starting with 60/40 acetonitrile/water, increasing to 80% acetonitrile in 16 min and then within 6 min to 98% acetonitrile. After 8 min at 98%, the column was re-equilibrated for 10 min. Again; the mixture containing acetonitrile/water was used as solvent for injection with 50 μL and the flow rate was 1 mL/min. A standard solution containing approximately 40 μg/mL of TESTO_Ac (testosterone acetate), DHEA_Ac and 16EN_Ac as internal standard plus 100 μg/mL of ETIO_Ac, 5b-AD_Ac and 5a-AD_Ac each was injected twice to determine the retention times for fraction collection. Fractions were collected (TESTO_Ac from 8.7 to 9.8 min, DHEA_Ac from 10.0 to 11.5 min, 5b-AD_Ac from 14.8 to 16.0 min, ETIO_Ac from 10.5 to 11.8 min and 5a-AD_Ac from 15.3 to 16.5 min) and evaporated to dryness. The dry residues were re-dissolved in two times 100 μL of MeOH, transferred into auto-sampler vials and evaporated.

2.6. HPLC-cleanup of sulfated steroids

The same equipment was used to clean up the formerly sulfated steroids. For the first run the same gradient was employed as above. The standard mixture contained DHEA, ETIO and AND and three fractions were collected and acetylated. Afterwards only fraction 1 containing DHEA was forwarded to the second HPLC purification using the same method as above. The fraction containing ETIO also comprised EPIA (3β-hydroxy-5α-androstan-17-one). All aliquots were transferred to auto-sampler vials and forwarded to IRMS analysis.

2.7. GC/C/IRMS measurements

All samples were measured on an Agilent 7890 Gas Chromatograph (Waldbronn, Germany) coupled to a Delta V gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via the GC combustion interface (GCC III, ThermoElectron). Injections were performed in the splitless mode at 280 °C with injection volumes ranging from 1 to 3 μL cyclohexane. The GC column was a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm) from Agilent. The initial oven temperature of 70 °C was maintained for 2 min and increased at 30 °C/min up to 270 °C, then at 2 °C/min to 290 °C and then at 30 °C/min up to 300 °C and kept for 3 min. Carrier gas was purified He (purity grade 4.9) with a constant flow of 1.4 mL/min. The combustion furnace was operated at 940 °C. Data was acquired using ISODAT® 3.0 software (ThermoElectron).

2.8. Correction for the acetate moiety

All determined values were corrected for the influence of the acetate moiety as described in literature [7,32]. All $\delta^{13}C$ values reported within this article are for the underivatized steroids.

2.9. Measurement protocol IRMS

For IRMS analysis, 2 consecutive sample preparations were conducted. First, 3 blank urines and 3 post-administration urines from each of the chosen subjects was prepared according to the described method. During this first step PD, ETIO, AND, TESTO, 5a-AD and 5b-AD excreted glucuronidated were under investigation at which PD served as ERC and the other steroids as TC. Each steroid from each specimen was injected once, so for each subject a mean value for the blank samples and for the post-administration samples could be calculated together with the according standard deviation (SD).

For the second preparation the remaining portions of the respective blank and post-administration samples were pooled due to low urine volume. Then the pooled samples were prepared as described and now ETIO, AND (to confirm the first measurements) and DHEA excreted glucuronidated as well as ETIO, AND, EPIA and DHEA sulfated were under investigation to allow for comparison between both excretion forms and to identify possible differences. Each steroid from each pooled sample was injected three times in order to enable calculation of mean value and SD and to facilitate statistical treatment in a similar way to the first investigations.

To each single steroid injection RSTD was added to monitor possible instrument drifts during the measurements and each batch of RSTD values obtained for a single steroid before and after the administration was tested for statistical difference.

2.10. Statistical analyses

The CIR values were tested for statistical significant differences with Student's *t*-test as CIR data can be assumed to be Gaussian-distributed [7,13,14]. For urinary concentration data, the same assumption was made. Additionally, tests were performed with the Wilcoxon signed-rank test as a non-parametrical method. As these calculations yield the same results as for the *t*-test, the latter was chosen.

3. Results and discussion

3.1. Steroid profile data

3.1.1. Concentration ratios

In Fig. 2 the impact of HCG administration on urinary excreted steroids is exemplarily depicted for subject A. Usually, in doping control analysis the TESTO/EPIT ratio (both steroids excreted glucuronidated) is the preferred marker to identify TESTO or TESTO-prohormone administration. This protocol is based on the fact that an elevated serum TESTO level leads to a decrease in endogenous TESTO and EPIT production by down-regulation of the testicular production via the hypothalamus-pituitary-gonad-axis, which strongly increases the TESTO/EPIT ratio. In the case of a HCG administration this marker fails as both steroids excreted by the testis are up-regulated and the ratio maintains nearly unchanged. This phenomenon was observed in all volunteers and has already been described in literature [24,33,34]. The response on other possible concentration ratios constructed with TESTO like TESTO/DHEA or TESTO/ETIO showed a higher impact after administration (Fig. 2). The same holds true for ratios constructed with other direct TESTO-metabolites like 5a-AD or 5b-AD and DHEA or ETIO. Over all, these ratios are affected by large inter-individual variability and were not found elevated beyond a reference population based threshold throughout the complete study for all individuals. (Reference based thresholds were estimated using a recently established reference population investigated for their urinary concentrations of endogenous formestane consisting of 91 male and female volunteers [31]).

To estimate the power of subject-based thresholds in this circumstance, the long-term stability of these markers within one subject would have to be investigated first. Usually, concentration ratios are more stable than corresponding urinary steroid concentrations, but for the ratios used here, no data is at hand.

3.1.2. Absolute concentrations

For absolute urinary concentrations, reference based thresholds are well investigated and established and explicitly fixed by the World Anti-Doping Agency (WADA) in the relevant technical document [35–38]. The majority of all samples were found with no suspiciously elevated values after HCG administration. Within the ten subjects under investigation only 3 out of 177 post-administration urine specimens showed elevated urinary concentrations beyond reference based thresholds after correction for specific gravity: 2 for TESTO and 1 for 5a-AD.

Nevertheless, significant individual changes in urinary steroid concentrations were found as listed in Table 1. TESTO, DHT and 5a-AD were all significantly increased in 3 out of the 4 subjects chosen for CIR investigations, EPIT and 5b-AD in 2 and AND in 1. The special case of subject F, not showing any significant changes and partly even decreasing concentrations will be discussed in particular later on.

The 3 blank and 3 post-administration samples were chosen according to the above mentioned concentrations ratios. For subject A for example the specimens collected after 3, 4 and 4.7 days (Fig. 2) were selected.

To sum up these findings, it will hardly be possible to detect the administration of HCG by neither concentrations ratios nor absolute urinary concentrations. As the few samples with elevated concentrations would have been forwarded to IRMS analysis according

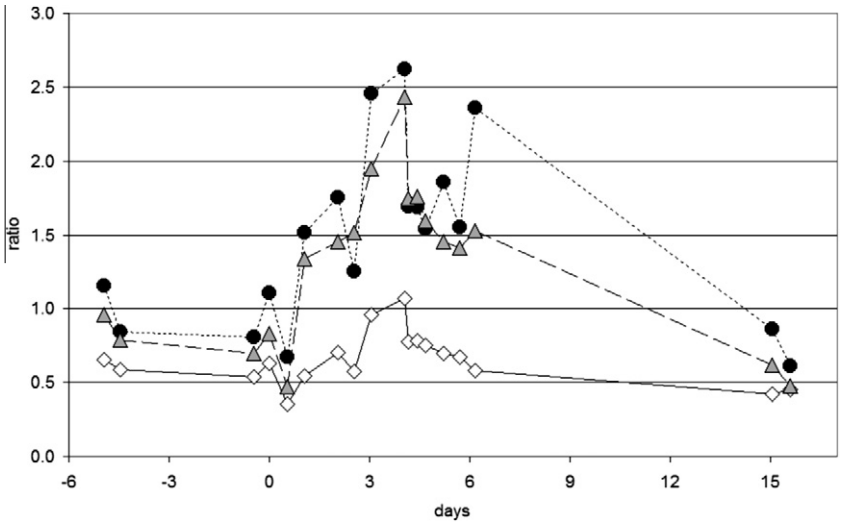

Fig. 2. Urinary concentration ratios of subject A. The first HCG administration took place on day 0. Black circles represent the ratio of TESTO divided by ETIO multiplied by 100, grey triangle TESTO/DHEA and open diamonds TESTO/EPIT.

Table 1
Mean urinary concentrations in ng/mL with standard deviations of the selected specimens for CIR analysis.

Subject	TESTO	EPIT	AND	ETIO	DHT	5a-AD	5b-AD	DHEA
A-before	33 ± 2	57 ± 7	5123 ± 1160	3696 ± 757	5 ± 2	70 ± 11	122 ± 19	43 ± 5
A-after	123 ± 33	131 ± 27	7851 ± 1111	5438 ± 847	14 ± 2	193 ± 55	377 ± 79	61 ± 17
p	**	**	*	ns	**	*	**	ns
C-before	54 ± 9	11 ± 2	3552 ± 1029	2642 ± 722	8 ± 3	85 ± 24	214 ± 136	53 ± 5
C-after	162 ± 30	32 ± 2	4872 ± 476	3648 ± 467	23 ± 5	285 ± 80	474 ± 152	52 ± 8
p	**	**	ns	ns	*	*	ns	ns
F-before	45 ± 19	27 ± 12	3446 ± 1887	4195 ± 2452	8 ± 5	95 ± 56	359 ± 121	46 ± 23
F-after	117 ± 124	34 ± 29	1815 ± 1348	1746 ± 1389	17 ± 18	219 ± 190	462 ± 386	49 ± 35
p	ns	ns	ns	ns	ns	ns	ns	ns
J-before	46 ± 12	34 ± 11	5707 ± 614	2920 ± 537	9 ± 4	89 ± 21	234 ± 99	76 ± 4
J-after	159 ± 45	64 ± 33	3680 ± 2621	2752 ± 1781	28 ± 6	191 ± 25	728 ± 166	65 ± 23
p	**	ns	ns	ns	*	**	*	ns

Samples were collected before administration ($n = 3$, before) and after administration ($n = 3$, after). Values found in highly concentrated urines were corrected to a specific gravity of 1.02. Level of significance: $^{*}p < 0.05$; $^{**}p < 0.01$; ns – not significant (Student *t*-test).

to WADA regulations [37], the impact of HCG on CIR was carefully investigated.

3.2. Instrument drift

The possible influence of instrument drift was monitored and could be excluded during these measurements as no trend or significant change of RSTD values was observed within this study. The mean value for all RSTD determinations ($n = 240$) was $\delta^{13}C_{VPDB} = -30.4 \pm 0.46‰$ and within the direct comparison between before and after administration values the mean difference was 0.03‰ and never found to exceed 0.36‰.

3.3. CIR of major compounds

As major compounds ETIO, AND and PD were considered as these are usually under investigation during routine IRMS analysis and always found in urine specimens in sufficient concentrations for IRMS analysis. PD was chosen as ERC as this compound is not or nearly not excreted by the testis. Despite the fact that high amounts of 3β-hydroxy-pregn-5-en-20-one (PREG), the presumed precursor of PD, are found within testicular tissue, no PD is found in venous blood of testis [22,39,40]. An increase in testicular steroid neo-genesis should therefore not influence the CIR or concentrations of PD. As depicted in Fig. 3, no significant change or trend in CIR could be observed for PD.

In contrast, for both TC, ETIO and AND, a clear trend to more depleted CIR values after HCG administration is obvious. For the 5β-steroid ETIO this difference is found significant in subjects A ($p = 0.013$) and F (0.015) and for the 5α-steroid AND in subjects A (0.012), C (0.011) and F (0.016). The differences in mean values account for 0.6–1.0‰ and could be verified during the second sample preparation (vide infra).

These results support the hypothesis of different CIR at different production sites inside the body.

3.4. CIR of minor compounds

Within this study TESTO, 5a-AD and 5b-AD are considered as minor compound due to their lower urinary concentrations, which are often under investigation to confirm conspicuous samples in doping control analysis. In 3 out of the 4 investigated subjects the trend to more depleted values after investigation is found again (Fig. 4). For TESTO the difference was found to be as large as 1.8‰ in subject J ($p = 0.004$) and resulted in significant difference of 1.1‰ in subject A (0.024). Interestingly, in subject F the difference was −0.9‰ (0.049). The androstanediols 5a-AD and 5b-AD gave the same picture. A strong trend to more depleted values was given in subjects A, C and J with significant differences for 5a-AD (A – 0.018; C – 0.048) and for 5b-AD only with subject C ($p = 0.017$). And for subject F there was nearly no change found in both androstanediols with a slightly trend again to negative differences.

To sum up these findings, 3 subjects strongly support the hypothesis of different CIR at the testes while subject F seems to behave contradictory. While the main urinary metabolites of TESTO, ETIO and AND showed depleted values after HCG administration, the precursor itself and the intermediates 5a-AD and 5b-AD did not. A possible explanation for these findings might be the unique stimulus of the testes caused by HCG. Due to its long half-life of elimination of about 24 h, HCG remains in the blood circulation for quite a long time. This causes a biphasic stimulus of the steroid production in the testis with a time period between both maxima of approximately 24 h as depicted in Fig. 5. Interestingly, after this long and extensive stimulus, the steroid production in the testis is completely down-regulated due to exhaustion of receptors and production capacities. For a particular time period of 10 to 20 h the testes hardly produce any steroids [41,42].

As shown in Fig. 6 and mentioned above, for subject F the ratios of the steroid concentration did behave different from the other subjects and the strongest impact of HCG administration was found between 5.7 and 6.3 days. Despite the elevated ratios and in contrast to all other subjects no significant increase in absolute concentrations was observable for these urine specimens (Table 1).

The samples under investigation for CIR of subject F originate from this late phase of suppressed steroid production in the testes and this might offer an explanation for the enriched values of TESTO. At that point in time, the contribution to urinary TESTO from the testes should be negligible, so the more depleted part of urinary TESTO was missing which could result in more enriched values for this subject as TESTO then mainly originated from peripheral metabolism. This would also explain the balanced values for 5a-AD and 5b-AD, hardly showing any influence, as they are metabolites of TESTO but do not have their solely source here. In contrast, ETIO and AND as the main and final metabolites still show the influence of former input of depleted TESTO and did not return to starting values or show the inverse trend of TESTO as a high percentage of these metabolites originate from other sources than the testes under normal physiological conditions.

With the data at hand it will not be possible to reveal the source of this outliers unambiguously and while the presented explanation is sound, it does not claim to be the only possible scientific interpretation of these results.

3.5. CIR of sulfated steroids

With the second sample preparation of now pooled urine samples the findings of the first investigations could be verified for ETIO and AND as depicted in Fig. 7. For the glucuronidated steroids

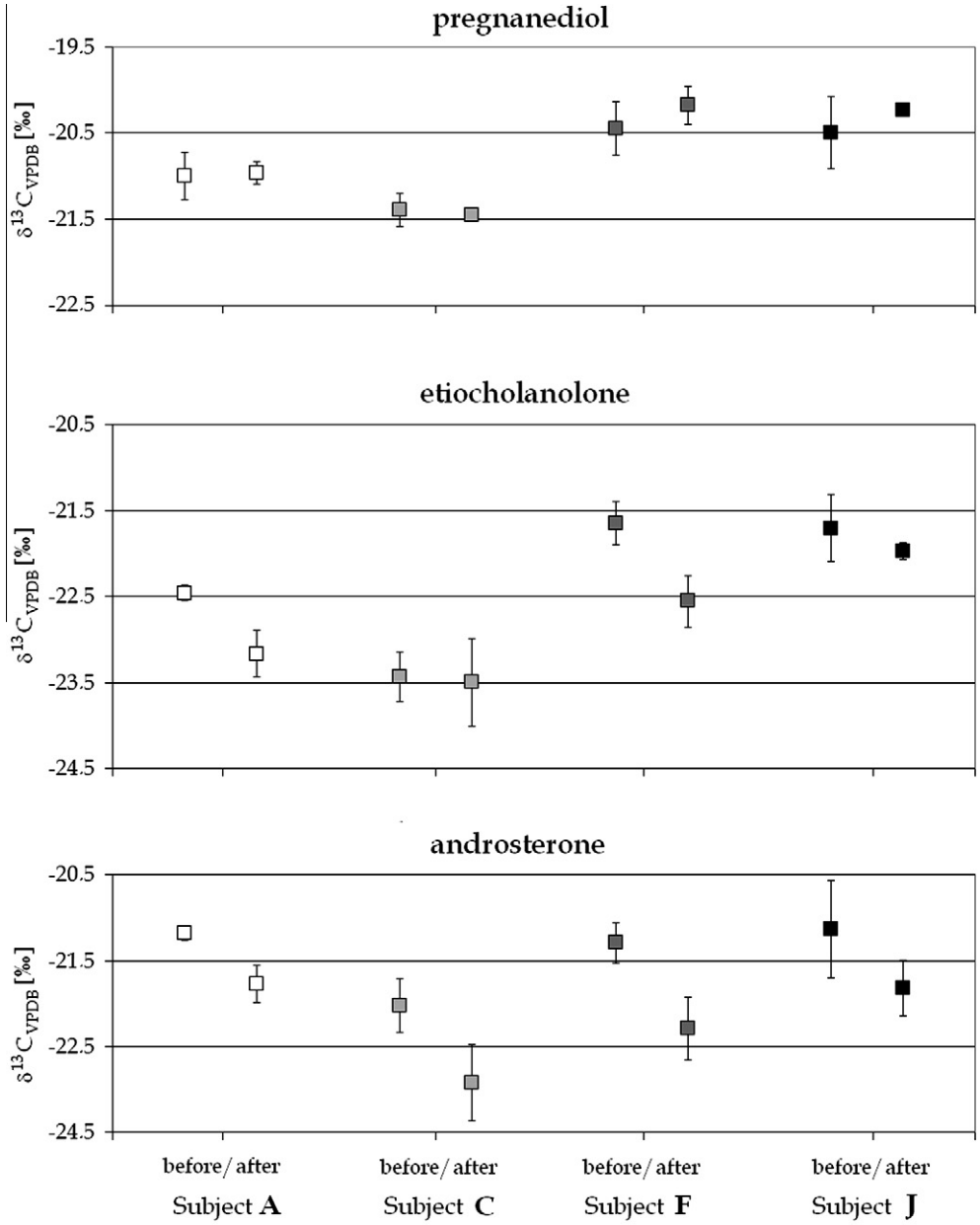

Fig. 3. Mean CIR values of major compounds before ($n = 3$) and after HCG administration ($n = 3$), the error bars depict 1 SD.

differences in mean CIR values between both preparations were in average smaller than 0.26‰.

The level of significance of differences between values before and after administration increased for all subjects. This is due to the fact that for the pooled samples with threefold injection only the measurement uncertainty and not the combination of sample preparation and measurement uncertainty accounted. Nevertheless, for the sulfated steroids most of the differences were not found to be significant on a level of $p < 0.05$ but all show the expected trend towards more depleted values after administrations of HCG. The main reason for the missing significance is the larger SD CIR measurements of sulfated steroids are afflicted with [14].

As already reported in literature [9,20], a stable offset can be found between steroids excreted glucuronidated and sulfated with a mean value around 0.7‰. For our subjects, the values differ for about 1.2‰ with an extraordinary large value found for subject J between AND- glucuronidated and AND-sulfated of 2.6‰.

While the influence of HCG administration on ETIO-glucuronidated and ETIO-sulfated seems to be quite similar, a significant difference for AND can be found. AND- glucuronidated showed values depleted by approx. 0.8‰ whereas AND-sulfated only was depleted by 0.4‰. A possible explanation for this finding might be the larger pool of AND-sulfated in contrast to ETIO-sulfated [14,43]. The overall contribution from testicular AND to the pool of AND-sulfated should therefore be smaller as the one of testicular ETIO to ETIO-sulfated resulting in a smaller difference in CIR for AND-sulfated.

Due to low urinary concentrations, DHEA-glucuronidated could only be measured in 2 individuals. In both a highly significant depletion was found (subject A: 1.1‰, $p = 0.006$ and subject J: 1.6‰, $p = 0.009$). In DHEA excreted sulfated, no change of isotopic values was detected in any of the subjects, which is not surprising taking into account the large pool size of DHEA-sulfated.

The generally similar behaviour of steroids excreted glucuronidated and sulfated after HCG application supported our hypothesis

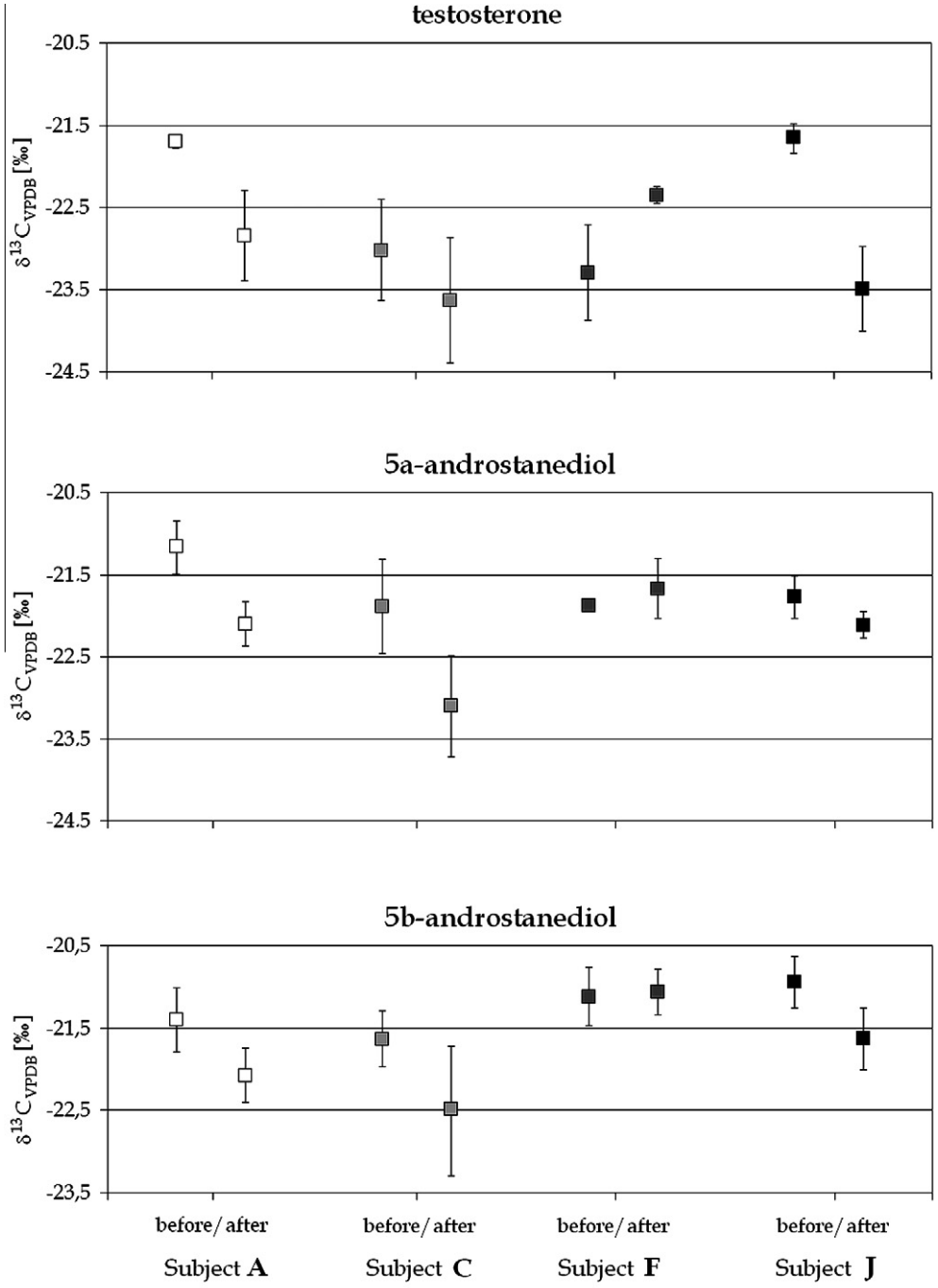

Fig. 4. Mean CIR values of minor compounds before (n = 3) and after HCG administration (n = 3), the error bars depict 1 SD.

and gave another hint that the influence of Phase II metabolism might not be solely responsible for the found differences between both excretion forms. Of course, these finding cannot exclude an influence of Phase II metabolism and different experiments will have to be conducted for clarification.

3.6. Impact on doping control analysis

The results obtained during this study lead to two main conclusions regarding sports drug testing. First, with the help of CIR it will not be possible to detect the misuse of HCG by athletes. The found significant changes in CIR can only be detected in the direct comparison of blank and administration samples. For a single spot urine which will be provided for doping control analysis by an athlete, the depletion caused by the additional stimulation of the testes was too low to elevate Δ-values beyond the defined WADA threshold of 3‰. Only for the Δ-value of PD-TESTO the threshold was exceeded in two samples (subject J, 3.4‰ and 3.6‰). But reference population based thresholds taking into account the isotopic fractionation during steroid metabolism were never exceeded within this study [7].

Second, and as a direct conclusion of the first finding, the influence of different CIR at different production sites inside the body on the CIR of urinary excreted steroids cannot, in any normal physiological state, result in a false positive doping control test. The CIR values found within this study were a result of a strong stimulus

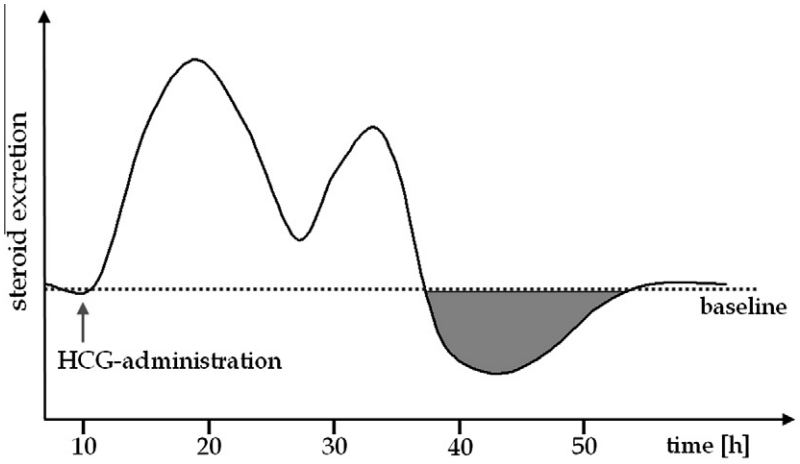

Fig. 5. Steroid excretion of the testis after stimulus by HCG administration.

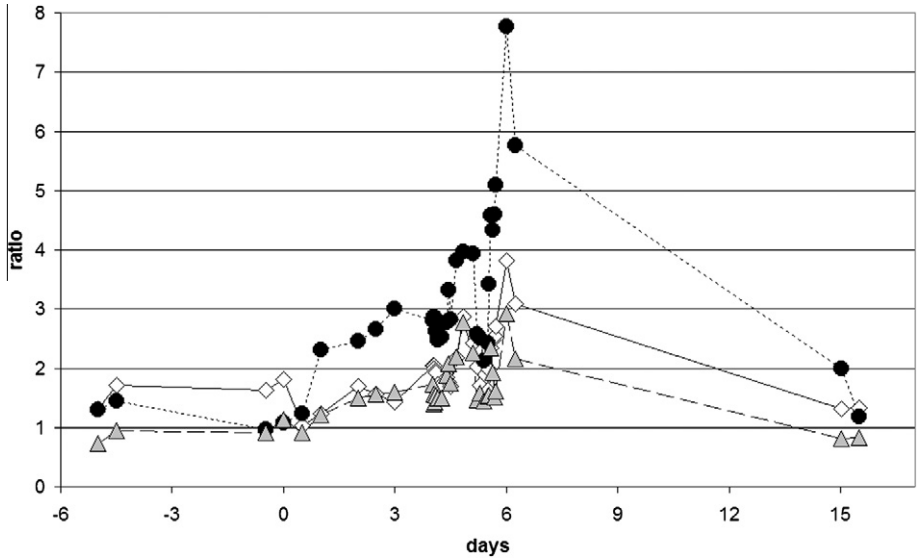

Fig. 6. Urinary concentration ratios of subject F. The first HCG administration took place on day 0. Black circles represent the ratio of TESTO divided by ETIO multiplied by 100, grey triangle TESTO/DHEA and open diamonds TESTO/EPIT.

and un-physiological high contribution of the testes to urinary excreted steroids and our reference population based thresholds covered even these values.

3.7. Possible explanations for the found differences in CIR

Three different approaches would be conceivable, namely different CIR of cholesterol (CHOL) at the different production sites, isotopic fractionation caused by 17α-hydroxylase,17,20-lyase (CYP17) or isotopic fractionation caused by similar enzymes resulting in different CIR due to varying fluxes in the network.

3.7.1. Cholesterol

All endogenous steroids are derived by enzymatic conversion of CHOL [25,26,44]. CHOL itself is to a large extend synthesized *de novo* from the body and only partly taken up from the diet [45]. The main production site for endogenous CHOL is the liver and the CHOL excreted here rapidly equilibrates with the plasma pool of CHOL. From this pool the adrenal gland takes up the complete amount of necessary CHOL while in the testes both circulating CHOL uptake and synthesis of CHOL take place [46]. A difference in CIR of the starting material CHOL would be detectable in all metabolites and could explain differences found in excreted steroids. Additionally, both production sites differ in the transport mechanism for CHOL from the outside to the inner mitochondrial membrane [39]. An isotopic discrimination associated with this transport would result in deviating values, too.

3.7.2. CYP17

17a-Hydroxylation and subsequent 17,20-cleavage of PREG or progesterone is the essential branching point in steroids synthesis [22,26,47]. Despite the same enzyme being found at both production sites, the proportion of enzymatic activity differs dramatically [48]. If the enzyme is hardly active like in the adrenal glumerolosa, mineral corticoids are synthesized. In the adrenal glands, where the 17α-hydroxylase activity is dominant, corticosteroids are produced and in the testes, where the 17,20-lyase activity outperforms the hydroxylation, androgens originate [48–50]. Isotopic fractionation which comes along with these enzymatic activities would be reflected by all particular steroids and would fit perfectly with our observations within this study. Here, further investiga-

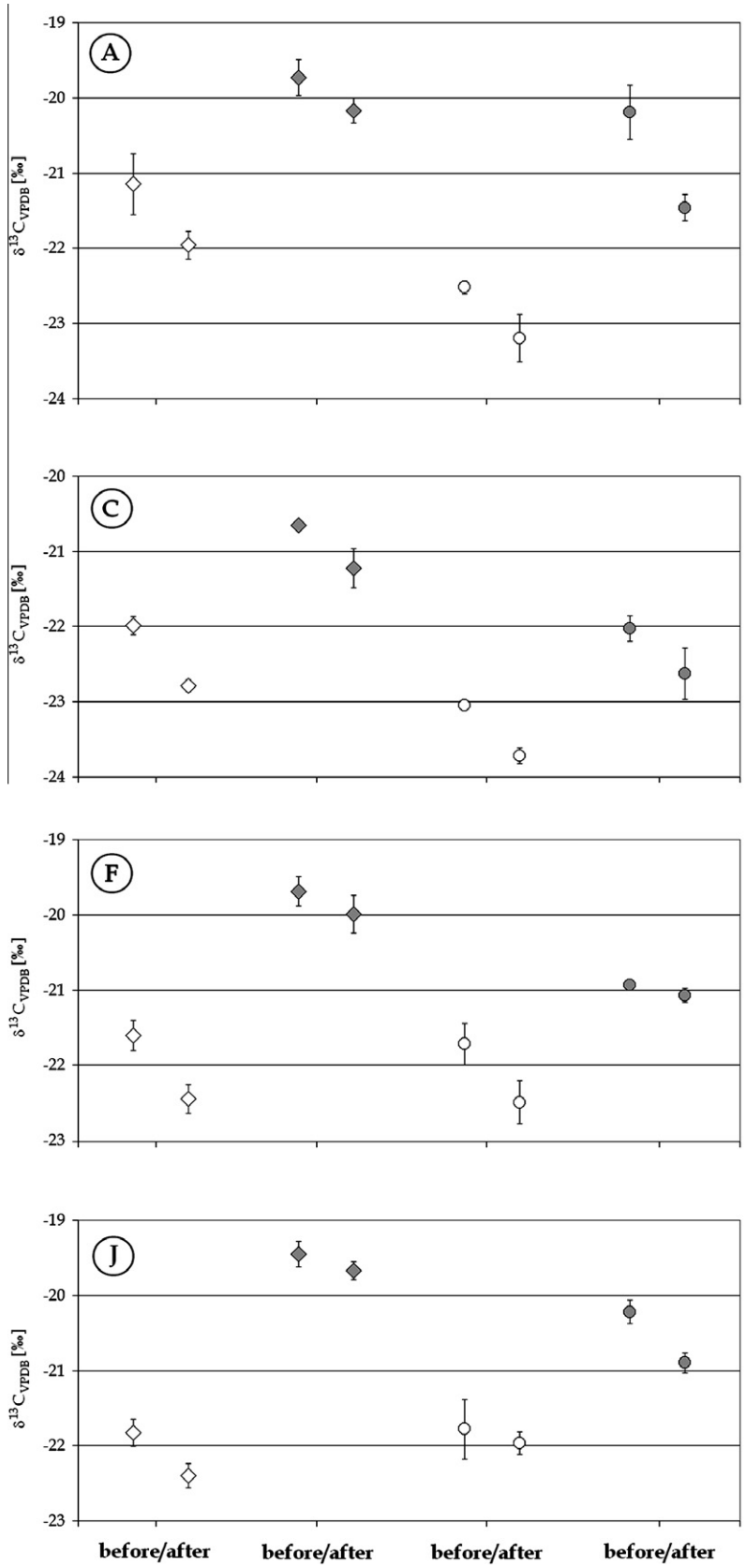

Fig. 7. Comparison of mean CIR values of glucuronidated and sulphated compounds before and after HCG administration, each sample was injected three times and the error bars depict 1 SD. Open diamonds – androsterone excreted glucuronidated, grey diamonds – androsterone excreted sulfated, open circles – etiocholanolone excreted glucuronidated, grey circles – etiocholanolone excreted sulfated.

tions by isolating the enzyme and testing for isotopic fractionation seems easily possible and will be conducted in the future.

3.7.3. Network-flux

If both the starting material CHOL has similar CIR at both sites and no isotopic fractionation is taking place with CYP17, a third possibility would be different fluxes of steroids through the same underlying network of steroid synthesis. Overall, as stated for CYP17, the enzymes converting steroids into each other are the same throughout the whole body. This enzyme identity should also be reflected by similar isotopic fractionation for each step of conversion. The main difference is the amount of steroids flowing through the network. As already mentioned the adrenal gland and the testes produce different amount of steroids using the same synthesis routes. Under steady state conditions the isotopic fractionation should be equal but as soon as the equilibrium is disturbed, isotopic composition of precursors and products will change according to the amount of transformed steroids [51,52]. And as a steady state cannot be assumed in both production sites (circadian excretion in the adrenal glands and pulsatile excretion at the testes) this could result in different CIR. Especially as in the adrenal glands one intermediate (DHEA) is nearly completely separated from the flux by sulfatation, which does not take place at the testes.

One drawback of this hypothesis is the fact that until now no correlation between urinary concentrations and CIR of excreted steroids has been found. If the amount of steroid production is correlated with a distinctive CIR, the urinary steroids should at least partly reflect this.

4. Conclusion

Within this study the hypothesis of different CIR at different steroid production sites was investigated by measuring CIR of selected urinary steroids after HCG administration. The results clearly demonstrate the influence of HCG application of the CIR of excreted steroids. As HCG only stimulates the steroid production at the testes, this finding can be taken as proof for more depleted values for steroids produced at the testes as for steroids from other sources like the adrenal glands.

The found difference will be helpful in explaining inter-individual variance in δ- and Δ-values and might even explain intra-individual variation as the contribution of the adrenal glands to urinary excreted steroids shows a diurnal variation while the testes excrete steroids pulsatile.

Of course, these findings do not exclude numerous other factors, which might influence the CIR of urinary steroids like the Phase II metabolism or particular enzymes from the UGT family responsible for inter-conversion or formation of steroids inside the different compartments of the body. Further research will be necessary to elucidate the impact of such factors on CIR of excreted steroids and might reveal the enzymatic influence.

As the detected influence was relatively small it will not be possible to use CIR to detect the misuse of HCG in doping control samples. And again, it could be shown that the influence of HCG on the urinary steroid profile is too small for this purpose, too.

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