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Investigations on changes in $^{13}C/^{12}C$ ratios of endogenous urinary steroids after pregnenolone administration

Thomas Piper,* Christoph Schlug, Ute Mareck, and Wilhelm Schänzer

For the detection of possible misuse of naturally occurring anabolic androgenic steroids like testosterone (T), anti-doping laboratories use a combination of two techniques. One is molecular steroid profiling to evaluate urinary steroid concentrations and normal diagnostic ratios. The other is isotope ratio mass spectrometry (IRMS), in which the $^{13}C/^{12}C$ ratios of target analytes like T are compared to the $^{13}C/^{12}C$ ratios of endogenous reference compounds (ERCs). The $^{13}C/^{12}C$ of the most commonly used ERC, pregnanediol (5β-pregnane-3α,20α-diol, PD), can be influenced by administration of pregnenolone (3β-hydroxy-pregn-5-en-20-one, PREG). Therefore PREG administration bears the potential to circumvent IRMS testing for doping control samples.

In order to investigate the influence of PREG on PD and on other urinary excreted steroids, administration studies with oral and transdermal application of PREG were carried out. The influence of PREG administration on concentrations and $^{13}C/^{12}C$ ratios of all investigated target analytes was negligible. Only PD and 5β-pregnan-3α-ol-20-one (3aP) showed significant depletion in both their glucuronidated and sulfated steroids. The results suggest that appropriate alternative ERCs are: 11β-hydroxy-androsterone/etiocholanolone, 5β-pregnane-3α,17,20α-triol, pregn-5-ene-3β,17,20α-triol and cholesterol.

Due to its properties to disguise the misuse of anabolic steroids by influencing the $^{13}C/^{12}C$ ratio of PD, PREG should be considered to be added to the World Anti-Doping Agency (WADA) list of prohibited substances as a masking agent.

Keywords: carbon isotope ratio; pregnenolone; doping control; excretion study; steroid metabolism

Introduction

Pregnenolone (3β-hydroxy-pregn-5-en-20-one, PREG) is a key intermediate in the steroid biosynthesis from cholesterol to androgens and corticoids.[1,2] After PREG became commercially available in the 1940s, several administration studies were carried out to demonstrate the effectiveness of this steroid as a constitutional agent.[3–5] The results of these studies were ambiguous and the usefulness of PREG administration is still under discussion today.[6]

Regarding the enhancement of athletic performance, PREG is not considered to be a doping agent and is not listed on the World Anti-Doping Agency (WADA) list of prohibited substances.[7] However, several studies have demonstrated its potential to perform as a masking agent by influencing the carbon isotope ratio (CIR) of pregnanediol (5β-pregnane-3α,20α-diol, PD).[8–10]

In doping control analysis, samples showing suspicious steroid profile parameters such as an elevated testosterone (T) to epitestosterone (EpiT) ratio are forwarded to isotope ratio mass spectrometry (IRMS) determination in order to test for evidence of either the exogenous or endogenous origin of urinary steroids.[8–14] Therefore, the CIR of endogenous reference compounds (ERC) like PD are compared to the CIR of target compounds (TC) like T. CIR are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on the equation:

$$\delta^{13}C[‰] = \frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{std}}{\left(^{13}C/^{12}C\right)_{std}} \times 1000 \qquad (1)$$

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard.[15]

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

$$\Delta[‰] = \delta^{13}C_{ERC} - \delta^{13}C_{TC} \qquad (2)$$

For all Δ values of interest, reference ranges were determined and reference limits established.[13,14,16–19] By administration of PREG, the $\delta^{13}C$ value of PD is influenced which can result in a false negative IRMS test. This has already been demonstrated.[8–10] So one aim of this study was to investigate the influence of PREG administration on other possible ERC: 11β-hydroxy-androsterone (OHA_G), 11β-hydroxy-etiocholanolone (OHE_G), 5α-androst-16-en-3α-ol (16EN_G) and 5β-pregnane-3α,17,20α-triol (5bPT_G) excreted glucuronidated, cholest-5-en-3β-ol (CHOL_F) excreted unconjugated and pregn-5-ene-3β,17,20α-triol (PT_S) excreted as sulfo-conjugate.

Furthermore, the impact of PREG administration on other urinary steroids, which are usually used as TCs, was of interest as here the reported results were equivocal.[8–10] The following steroids excreted as glucuronides were determined: androsterone (A_G),

* *Correspondence to: Thomas Piper, German Sport University Cologne, Institute of Biochemistry, Am Sportpark Müngersdorf 6, 50933 Köln, Germany. E-mail: Thomas.Piper@chuv.ch*

German Sport University Cologne, Institute of Biochemistry, Am Sportpark Müngersdorf 6 50933 Köln, Germany

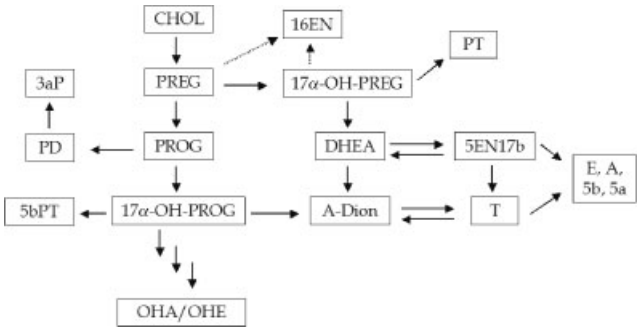

Figure 1. Simplified steroid metabolism. PROG – progesterone; A-Dion – androst-4-ene-3,17-dion. Further information in the text.

etiocholanolone (E_G), T_G, EpiT_G, dehydroepiandrosterone (DHEA_G) and 5α- and 5β-androstane-3α,17β-diol (5a_G and 5b_G). Special attention was paid on steroids excreted as sulfo-conjugates as some cases in the literature on a direct metabolism of PREG sulfate (PREG_S) to DHEA_S were reported.[20,21] Investigated sulfo-conjugated steroids were: A_S, E_S, epiandrosterone (EpiA_S), DHEA_S, androst-5-ene-3β,17β-diol (5EN17b_S) and androst-5-ene-3β,17α-diol (5EN17a_S).

In addition, the metabolic fate of administered PREG was of interest; therefore the urinary concentrations and CIR of PD_G and PD_S and of 5β-pregnan-3α-ol-20-one (3aP_G) and 3aP_S were investigated together with 5bPT_G, PT_S and 5bPT_S. These steroids were chosen as they are all early metabolites of PREG (Figure 1) and should reflect the CIR of administered PREG without large dilution by endogenously produced steroids.[1,2]

Experimental

Chemicals and steroids

Chromabond® C18 cartridges were obtained from Macherey & Nagel (Düren, Germany). Acetone (for gas chromatography), pyridine, acetic anhydride (distilled before use), glacial acetic acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). *Tert.*-butyl methyl ether (TBME, distilled before use) was from KMF Laborchemie (St Augustin, Germany), β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany), and steroid reference material (A, E, OHA, OHE, PD, PREG, DHEA, T, 16EN, 5bPT, EpiA, CHOL and 5α-androstane-3β-ol (RSTD)) was supplied by Sigma (Steinheim, Germany). 5a, 5b, 3aP, PT, 5EN17b and 5EN17a were purchased from Steraloids (Newport, USA). β-Estradiol-3,17-diacetate (EST) was from Riedel-de Haen (Seelze, Germany). All solvents and reagents were of analytical grade.

Excretion studies

Oral administration

One healthy male volunteer (35 years, 77 kg, 171 cm) administered two capsules of BIOVEA® Pregnenolone containing 50 mg PREG each. Prior the administration study, three capsules were homogenized and prepared according to routine procedures for nutritional supplements to identify all steroidal ingredients and to determine the δ^{13}C value of PREG.[22] The only steroid found was PREG with a δ^{13}C value of -31.1 ± 0.05‰ (n = 3).

Three blank urine samples were collected one day before and directly before the administration and then all urine samples were collected for the following five days resulting in a total number of 28 urine samples. All urine samples were stored frozen until preparation. Four samples were discarded after steroid profile determination due to low urinary steroid concentrations.

The study was approved by the ethical committee of the German Sport University, and written consent was given by the participant.

Transdermal administration

After a wash-out period of three weeks, the same healthy male volunteer started with application of BIOVEA® Pregnenolone cream. This preparation was tested prior administration, too, and only contained PREG with a δ^{13}C value of -31.1 ± 0.10‰ (n = 3). Three blank urine samples were collected one day before and directly before the administration. The volunteer applied cream containing approx. 30 mg of PREG every evening on arms and chest for six consecutive days. During the administration period, the morning urine sample and one evening urine sample prior the next application was collected. After cessation, the following seven morning urines were collected, resulting in a total of 21 urine samples. All specimens were stored frozen until preparation. The study was approved by the ethical committee of the German Sport University, and written consent was given by the participant.

Sample preparation

Analytes have to be efficiently isolated and purified before GC/C/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 high performance liquid chromatography (HPLC) clean-up was employed.

A detailed description of sample preparation was published elsewhere [14,17,19] and will herein only be described in brief: 10–20 ml of urine was applied on a conditioned C18 solid-phase extraction cartridge, washed with 2 ml of water and eluted triply with 1 ml of methanol (MeOH). After adding 10 µl of a solution containing 100 µg/ml RSTD in acetone, the dried residue was dissolved in 1 ml of sodium phosphate buffer and extracted with 5 ml of TBME to separate CHOL. The aqueous residue was hydrolysed with β-glucuronidase, adjusted to pH 9.6 with 0.5 ml of potassium carbonate buffer and again extracted with 5 ml TBME. The organic layer (containing formerly glucuronidated steroids) was transferred into a conical test tube. The pH of the aqueous residue was adjusted to 5 with 100 to 150 µl of glacial acetic acid followed by another solid phase extraction. The sulfo-conjugated steroids were eluted with MeOH/ethyl acetate and hydrolyzed with ethyl acetate/sulfuric acid. Then 10 µl of a solution containing 100 µg/ml RSTD in acetone were added. 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 5 ml of TBME (sulfo-conjugated steroids). After centrifugation, the organic layer was transferred into a conical test tube.

Urinary steroid concentrations

A 0.5 ml aliquot of each of the abovementioned organic layers was prepared to determine the amount of different steroids. The glucuronidated steroids were determined according to routine sample preparation procedures.[23] Both dried aliquots of the unconjugated and sulfo-conjugated fraction were acetylated by

adding 50 µl of pyridine and 50 µl of acetic anhydride and incubation for 45 min at 70 °C. After evaporation to dryness the samples were transferred into auto-sampler vials, evaporated, redissolved in 10 µL of TBME and forwarded to gas chromatography/mass spectrometry (GC/MS) determination.

For this purpose, a GC Agilent 6890 coupled to a mass selective detector MSD Agilent 5973 was used. The GC system was equipped with a Macherey & Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). The injections were performed splitless at 300 °C. The initial oven temperature of 60 °C was held for 1.5 min, increased at 40 °C/min to 240 °C, followed by a ramp at 2 °C/min to 260 °C, and 40 °C/min to the final temperature of 300 °C. A constant flow of 1.2 ml/min with helium as carrier gas was used. The MSD acquired data in single ion monitoring mode. The following ions were used for quantification and identification, respectively: RSTD (243/258), E and A (272/244), PD (284/269), DHEA (270/255), 5EN17b and 5EN17a (314/286) and CHOL (368/353)[*m/z*].

HPLC clean-up

The 4.5 ml TBME residue of each fraction was evaporated to dryness, re-dissolved in 200 µl of acetone, transferred into HPLC auto-sampler vials and evaporated. Most of the clean-up methods for steroids excreted glucuronidated and sulfo-conjugated have already been published elsewhere.[14,17,19] For the unconjugated CHOL, no HPLC clean-up was necessary and for 5bPT and 3aP the existing methods could be used, only the fraction collection times had to be adjusted. 5bPT was collected from 13.3 to 14.5 min and 3aP from 19.3 to 20.3 min.

GC/C/IRMS measurements

All samples were measured on an Agilent 6890 Gas Chromatograph (Waldbronn, Germany) coupled to a Delta plus XP gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via a custom made GC combustion interface (Patent application number: DE 10 2006 015 258 B3 2007.10.11).[24] The GC system was equipped with the same column as mentioned above. Injection was performed with a Gerstel (Mühlheim an der Ruhr, Germany) KAS unit at 50 °C in solvent vent mode with a helium flow of 100 ml/min. Injection volumes ranged from 2 to 5 µl of TBME. The initial temperature was maintained for 3 min and increased at 40 °C/min up to 260 °C, then at 2 °C/min to 280 °C and then at 40 °C/min up to 295 °C and kept for 3 min. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.4 ml/min. The combustion furnace was operated at 950 °C.

Correction for the acetate moiety

All determined values were corrected for the influence of the acetate moiety as described in literature.[14,25]

Method validation

The developed methods for CHOL, 5bPT and 3aP were validated by means of linear mixing models using Eqn 3:[14,26,27]

$$\delta^{13}C_m = (\delta^{13}C_e - \delta^{13}C_a)\frac{c_e}{c_m} + \delta^{13}C_a \qquad (3)$$

with c_x = corresponding concentration and $\delta^{13}C_x$ = corresponding $\delta^{13}C$ value; subscript m stands for mixture, e for endogenous and a for added standard.

Table 1. Calculated values for the linear mixing models referring to the equation y = a* x + b. a represents the Δ value (endogenous steroid minus added standard) and b the $\delta^{13}C$ value of the standard (mean and SD). All values in $\delta^{13}C_{VPDB}$[‰]

Steroid	a[‰]	SD[‰]	b[‰]	SD[‰]	Std[‰]	SD[‰]
CHOL	2.3	0.30	−25.4	0.17	−25.3	0.30
5bPT_G	12.0	0.36	−29.5	0.19	−29.8	0.12
3aP_G	11.8	0.18	−31.3	0.10	−31.3	0.10
5bPT_S	10.5	0.39	−30.2	0.20	−29.8	0.12
3aP_S	10.0	0.45	−31.9	0.23	−31.3	0.10

A comparison with the linear equation (y = a*x + b) shows that the corresponding equation of the resulting line of best fit represents the difference in $\delta^{13}C$ values between the endogenous steroid and the added standard ($\delta^{13}C_e - \delta^{13}C_a$) as its slope. The absolute $\delta^{13}C$ value of the standard is represented by the intercept on the y-axis (b = $\delta^{13}C_a$). Usually *Keeling plots* are used if one of the mentioned values is not directly measurable. In our case, all values were measurable and so it was possible to divert the mixing model from its intended use. If isotopic fractionation occurred during sample preparation, it would become obvious as the line of best fit would not intercept the y-axis by a numerical value representing the $\delta^{13}C$ value of the added standard. By calculating the least squares fit it is possible to evaluate standard deviations (SDs) for the described method, if assumed that errors for the concentrations are negligible.

Results and Discussion

Method validation

The approach of linear mixing models was chosen to study the method's validity for CHOL, 5bPT and 3aP. The results are listed in Table 1. The validation results are comparable to those found for other investigated steroids and prove the validity of the method.[14,17,19] According to these findings, no isotopic fractionation was taking place during sample preparation.

Urinary steroid concentrations

Oral administration

In Figure 2, the results of PD_G, 3aP_G and 5bPT_G excreted as glucuronides are depicted. For standardization the concentration ratios of the respective steroid divided by EpiT were calculated. Both PD_G/EpiT_G and 3aP_G/EpiT_G showed a strong increase directly after administration of PREG reaching a first maximum ca. 12 h after application and a second one after approx. 26 h. In contrast, 5bPT_G/EpiT_G only showed a diurnal cycle and no response to PREG administration.

The results obtained for sulfo-conjugated steroids standardized by 5EN17a_S are depicted in Figure 3. Again the ratios of PD_S/5EN17a_S and 3aP_S/5EN17a_S increased after administration while the 17α-hydroxylated compound PT_S stayed at the same level as before. The concentrations of the sulfo-conjugates scatter more than the ones of glucuronidated steroids, a phenomenon namely attributed to sample preparation and already reported in literature.[19,28–31] The relative increase after ingestion of PREG was found higher for sulfates than for glucuronides (>100-fold *vs* ~30-fold) which is explicable by very low urinary

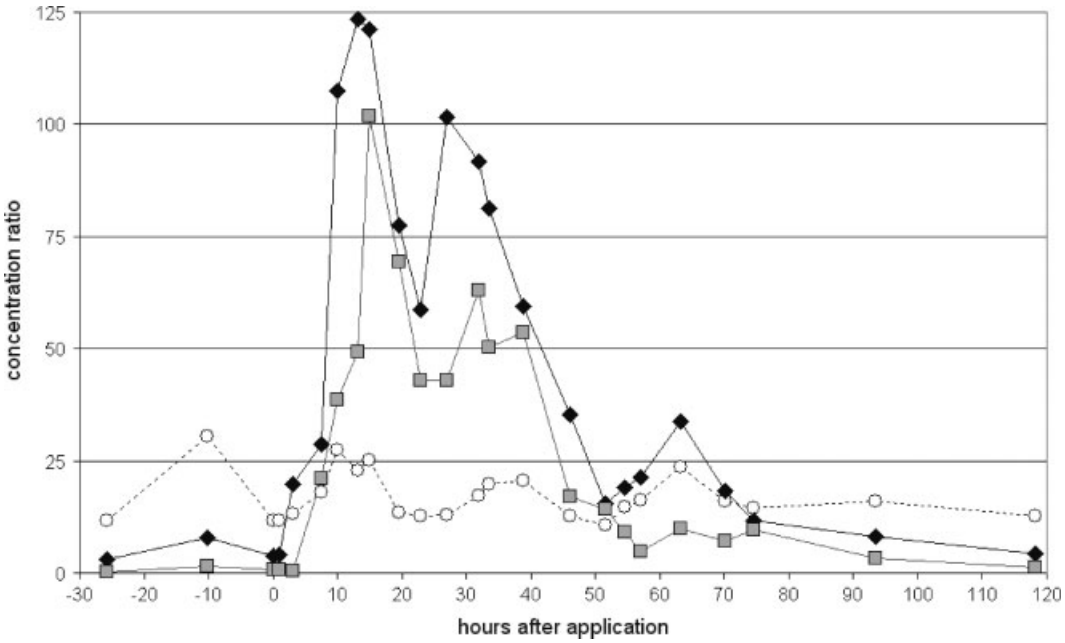

Figure 2. Urinary concentration ratios of steroids excreted as glucuronides. Depicted are PD_G/EpiT_G (black diamonds), 3aP_G/EpiT_G (grey squares) and 5bPT_G/EpiT_G (open circles) after oral administration of 100 mg PREG at 0 h.

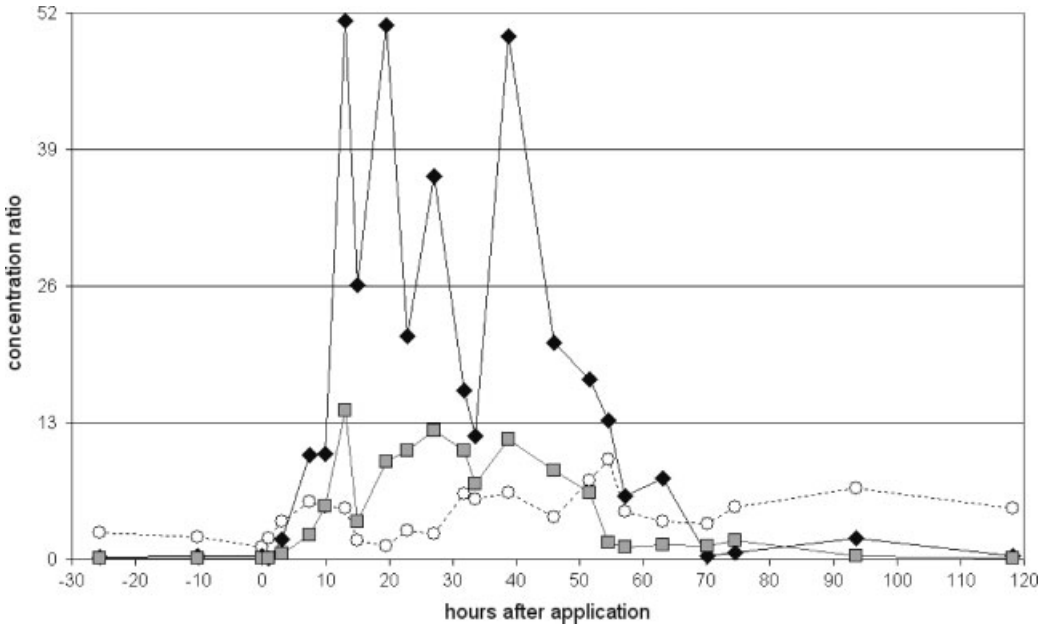

Figure 3. Urinary concentration ratios of steroids excreted as sulfo-conjugates. Depicted are PD_S/5EN17a_S (black diamonds), 3aP_S/5EN17a_S (grey squares) and PT_S/5EN17a_S (open circles) after oral administration of 100 mg PREG at 0 h.

concentrations of PD_S and 3aP_S ($\leq$10 ng/ml) in the blank urines. Both steroids seem to be preferentially excreted glucuronidated.

Absolute urinary concentrations found for PD_G and 3aP_G after administration were ca. 2100 ng/ml and 1200 ng/ml and for PD_S and 3aP_S ca. 1400 ng/ml and 440 ng/ml, respectively. All other investigated steroids showed diurnal variations but did not increase after the oral intake of PREG.

Transdermal administration

Over the whole time period of PREG cream administration, none of the investigated steroids were influenced. Not even PD_G, which scattered around a mean value 3.6 $\pm$ 1.16 for its PD_G/EpiT_G concentration ratio.

CIR of urinary steroids

Oral administration

The δ^{13}C values for all investigated steroids showed parallel trends as for the urinary concentrations. Most of the steroids were not influenced by the oral administration of PREG. All of these steroids are listed in Table 2. With standard deviations (SD) ranging from

Table 2. CIR mean values of all steroids apparently not influenced after administration of 100 mg PREG orally. Listed are the mean values for n = 24 specimens together with the belonging standard deviation (SD). F – steroids excreted unconjugates, G – steroids excreted glucuronidated and S – steroids excreted sulfo-conjugated

Steroid	mean	SD
CHOL_F	−22.9	0.31
E_G	−23.6	0.64
E_S	−23.9	0.18
A_G	−21.8	0.26
A_S	−22.5	0.18
DHEA_G	−21.0	0.44
DHEA_S	−20.7	0.12
5bPT_G	−21.9	0.28
5bPT_S	−22.3	0.24
T_G	−23.0	0.70
EpiT_G	−23.8	0.36
5a_G	−23.4	0.46
5b_G	−22.8	0.44
OHA_G	−22.4	0.37
OHE_G	−22.8	0.60
16EN_G	−22.4	0.45
EpiA_S	−22.2	0.39
5EN17b_S	−21.6	0.83
5EN17a_S	−21.7	0.75
PT_S	−22.2	0.53

0.1 to 0.8 ‰ for n = 24 determinations this demonstrates the excellent repeatability of the used methods.

Only PD and 3aP showed significant depletion after administration in both the glucuronidated (Figure 4) and the sulfo-conjugated steroids (Figure 5).

The glucuronidated analytes showed pre-administration values of −21.9‰ and −23.2‰ for PD_G and 3aP_G, respectively. After administration values were depleted to −29.9‰ and −30.5‰ for both steroids and therefore did not reach the value of the administered PREG with −31.1‰. This can be explained by an ongoing endogenous production of both steroids despite the administration of large amounts of PREG. Both analytes showed a strong depletion for approx. 60 h and then started to return to their pre-administration values without reaching them within the examined period of 120 h. 5bPT_G remained constant over the whole investigated time (Table 2).

The sulfated steroids showed a similar pattern (Figure 5). After administration of PREG, CIR were depleted from their starting values of ca. −23‰ down to values around −30‰ for both steroids. Again, after approx. 60 h the CIR started to return to basal values without reaching them in the investigated time period of 120 h. The sulfo-conjugated steroids showed more depleted values at the end of the study in contrast to the glucuronidated ones. A possible explanation for this might be the faster turnover of the pool of glucuronidated pregnane-steroids in contrast to the sulfated pool. This is supported by the fact that in the undisturbed metabolism pregnane-steroids are preferentially excreted as glucuronides. So these pools should equilibrate faster after an exogenous disturbance.

Metabolic fate of PREG

Interestingly, both 3aP_G and 3aP_S were influenced slower than PD_G and PD_S directly after administration of PREG. While PD showed strongly depleted values 55 min after administration, the belonging 3aP values were not (3aP_G) or only slightly (3aP_S) depleted. The concentration ratios in Figures 2 and 3 show the same delayed response of 3aP. This is in contrast to published metabolic pathways describing the transformation of PROG to PD via 5β-pregnane-3,20-dion, then 3aP and PD as end product.[32] However, in our study the 20α-hydroxysteroid-dehydrogenase seemed to act before or simultaneously with the 3α-hydroxysteroid-dehydrogenase. So the metabolic pathway should be as depicted in Figure 1.

Furthermore, neither 5bPT nor PT showed any depletion after oral administration of PREG in this study (Figures 4 and 5 and Table 2). As depicted in Figure 1, both steroids should be direct metabolites of PREG or PROG with 17α-hydroxylated steroids as intermediates. The 17α-hydroxylase is one of the key enzymes in steroid metabolism as this hydroxylation is prerequisite for the following 17,20-lyase transforming C21-steroids to C19-steroids.[1,2] Obviously, no 17α-hydroxylation of the administered PREG took place as none of the both investigated 17α-hydroxylated steroids showed any significant depletion after administration. This lack of 17α-hydroxylation explains the results obtained for all C19-steroids. Without hydroxylation the side chain cleavage is impossible.

The enzyme 17α-hydroxylase/17,20-lyase (CYP17A1) is only found in leydig cells, thecal cells and the adrenal cortex bound to the smooth endoplasmic reticulum.[2,33] The orally administered PREG is namely metabolized in the liver, where the CYP17A1 is not found.[2] The small amount of PREG absorbed into the blood circulation seems not, or only to an insignificant amount, to reach the abovementioned steroid production sites in our investigated individual. So no C19-steroids were produced from the administered PREG.

Another possible explanation would be a direct 20α-hydroxylation of PREG by 20α-hydroxylase. The formed 20α-hydroxy-PREG acts as a competitive inhibitor for 17α-hydroxylase and might prevent the formation of 17α-hydroxy-PREG by this way.[34]

Placement in the context of former studies

Whether the reported influence of PREG administration on the CIR of 16EN and 5b[10] is due to inter-individual variance in the absorption and following metabolism of PREG or might be due to analytical differences has to be clarified in further studies. As for the formation of 16EN the 17α-hydroxylation might not be necessary [35,36] its sole formation might be probable. An influence of 5b without E being involved in contrast is quite strange. Unfortunately, no 5EN17a was determined in the study carried out by Saudan *et al*. as this steroid should be produced in parallel to 16EN by the metabolism suggested by Weusten *et al*. In our study, neither showed an influence after PREG administration.

The reported direct transformation of PREG_S to DHEA_S[21] could also not be affirmed in our study. As it only took place in a homogenate of a human adrenal, where the compartmentalization of the different enzymes is abrogated and in an individual suffering from adrenal cancer, this finding is not too surprising.

Transdermal application

In the specimens collected during the transdermal application of PREG cream, only the CIR of PD were determined. As not even PD showed any influence in its δ^{13}C values (mean value for n

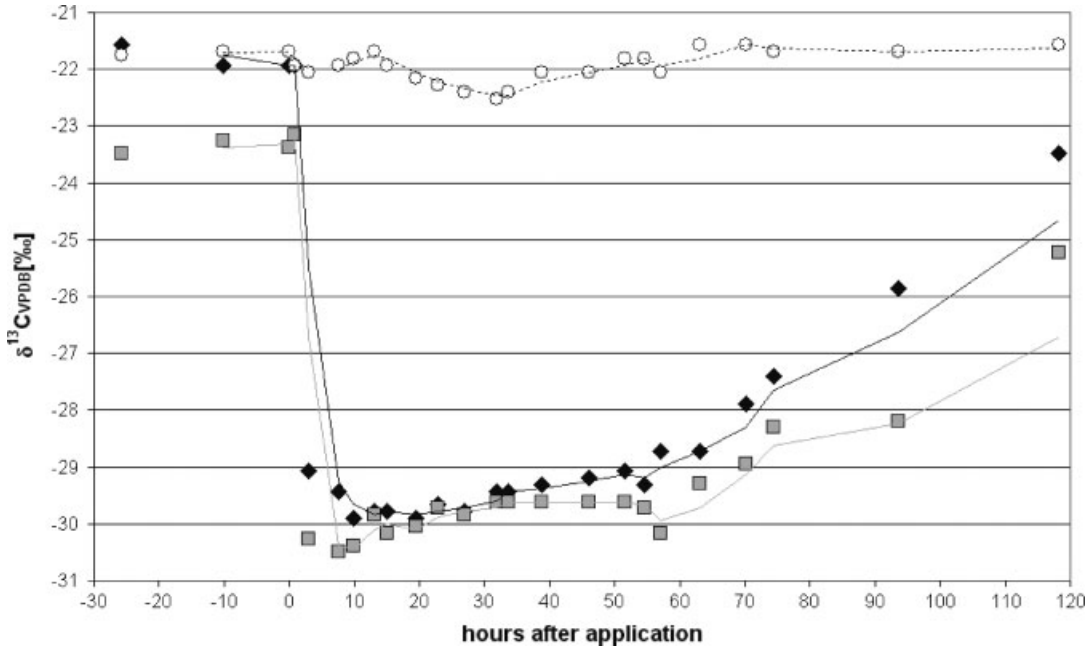

Figure 4. Changes in CIR of three urinary steroids excreted glucuronidated after administration of 100 mg PREG orally. Open circles represent 5bPT_G, black diamonds PD_G and grey squares 3aP_G. All values in δ^{13}CVPDB [‰]. The trend lines demonstrate the moving average ($k = 2$).

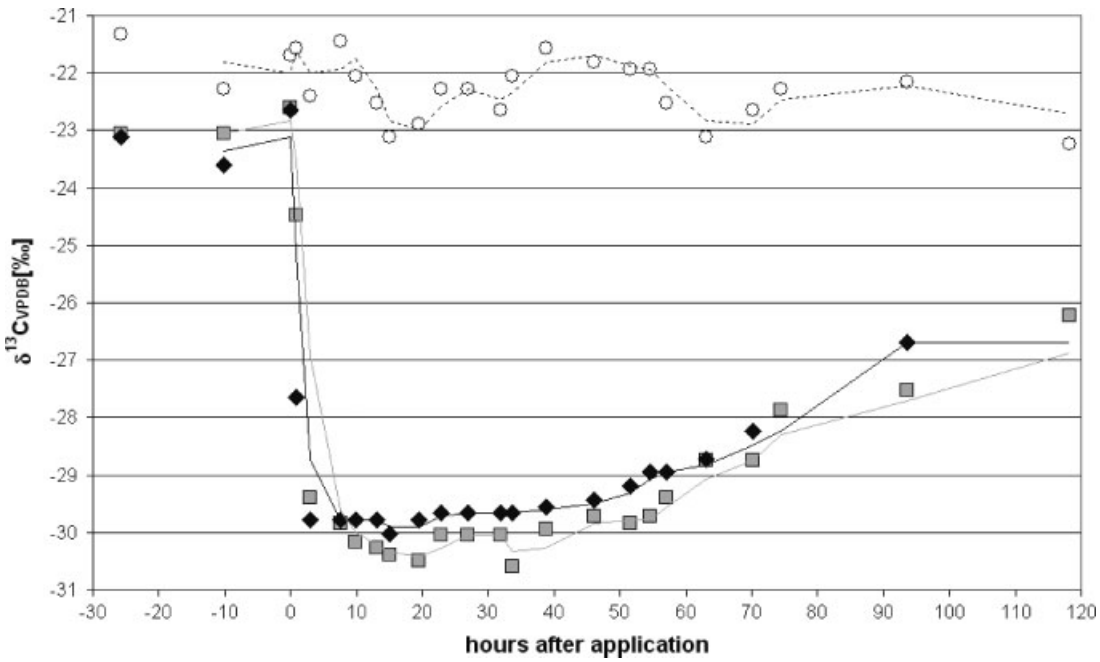

Figure 5. Changes in CIR of three urinary steroids excreted sulfo-conjugated after administration of 100 mg PREG orally. Open circles represent PT_S, black diamonds PD_S and grey squares 3aP_S. All values in δ^{13}CVPDB [‰]. The trend lines demonstrate the moving average ($k = 2$).

= 21 determinations -22.1 ± 0.28‰), all other steroids were not forwarded to IRMS analysis as obviously the PREG was not absorbed through the stratum corneum barrier of the skin at all.

CIR of possible ERCs

In the case of PREG administration, all investigated ERCs showed no influence either in their urinary concentrations or their CIR (Table 2) with the exception of PD and 3aP of course. From the fraction of unconjugated steroids, CHOL could be used, but as urinary CHOL is not necessarily derived from steroid metabolism but rather from endogenous cells of the urogenital system, this might be a limitation for the use of it.[37] From the glucuronidated fraction, OHA, OHE and 5bPT are potential ERCs and excreted as sulfo-conjugates namely PT can be used. The use of 5bPT_S might be limited by the relative small amounts of this steroid excreted as sulfate. As an influence of 16EN has already been shown and therefore can not be excluded, this steroid should not be used as an ERC in the investigation of a specimen suspicious for PREG administration.

Table 3. Δ values obtained from two different reference populations (both with n = 66 subjects).[14,17,19] Listed are the mean values, the standard deviations and the calculated reference limits. All values in $\delta^{13}C_{VPDB}$ [‰]

	OHA-PD	DHEA-PD	PT-PD
mean	0.46	0.42	1.41
SD	0.60	0.85	0.80
ref-lim	2.3	3.0	3.8

The uninfluenced ERC offer an alternative to reveal PREG administration by establishing reference limits between PD and other ERC. According to unpublished results of previously investigated reference populations these limits can be calculated by adding the 3-fold SD to the mean value.[14,17,19] The results are listed in Table 3; unfortunately no reference population based values of 5bPT was available. 16EN has been excluded because of the abovementioned reasons.

The use of usually employed TC seems not advisable as PREG is supposed to be used as a masking agent in the case of T or T-prohormone administration.

Conclusion

Orally administered PREG has a strong impact on the CIR of PD and 3aP. Therefore, its potential as a masking agent to circumvent IRMS testing by lowering the $\delta^{13}C$ value of the ERC PD in the context of doping control analysis could be demonstrated once more. All other investigated ERCs were not influenced by PREG administration and allow for a clear discrimination between endogenous PD and PD influenced by an administration of PREG. 3aP can also be used to prove PREG misuse. As the results for 16EN are contradictory at the moment, this steroid should not be used as an ERC in cases suspicious for PREG administration.

The metabolism of PROG to PD seems not to follow the in literature described way but to encompass at least a simultaneously hydroxylation of positions C3 and C20. Here further studies might help to clarify the metabolic fate of PREG and to elucidate the reasons for the lack of 17α-hydroxylation.

Due to its potential to act as a masking agent, PREG should be considered for addition to the WADA list of prohibited substances.

Acknowledgment

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