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Which factors control $^{13}\text{C}/^{12}\text{C}$ -ratios of urinary steroids?

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Abstract

There is considerable variation in the stable carbon isotope ratios ($\delta^{13}\text{C}$ values) of urinary steroids. The total variance necessarily is the sum of biological variability and analytical error. In a reference population of 56 students of physical education, both factors exhibited comparable orders of magnitude. In order to explain the biological variability, several possible factors were investigated by a questionnaire. Sex, physical activity, and travels to the Americas turned out to be significant. Different steroids exhibit different $\delta^{13}\text{C}$ values, where females exhibit different patterns than males. Additionally, in females oral contraception results in a significant depletion of $\delta^{13}\text{C}$ values. The latter phenomenon can possibly be explained by down-regulation of HMG-CoA reductase, the key enzyme in steroid biosynthesis. In fact, *in vitro* experiments showed that the reaction is associated by a significant ^{13}C isotope effect ($^{12}\text{k}/^{13}\text{k} = 1.0031 \pm 0.0004$). As this isotope effect finally propagates to androstanes and pregnanes at slightly different proportions, very small differences between the $\delta^{13}\text{C}$ values of these steroid classes theoretically may be induced.

Introduction

The ratio of the stable carbon isotopes ^{13}C and ^{12}C routinely is applied to confirm presence of synthetic steroid hormones or of their metabolites in urine. In particular, doping with synthetic testosterone or corresponding pro-hormones thus can be detected. For this purpose, the $\delta^{13}\text{C}$ values of relevant target compounds are compared to those of suited reference compounds. Both parameters however are subject to physiological variation. In order to substantiate the methodology, it is therefore of utmost importance that there be sound understanding of the physiological factors that possibly control the natural abundance of the stable carbon isotopes in urinary steroids. Unless this need is fulfilled, there will be always a significant chance that stable isotope data in doping control are challenged. It has happened for instance that - seconded by physiologists and biochemists - athletes who clearly tested

positive claimed that the results were due to special dietary habits or metabolic peculiarities.

Methods

A population of 56 students of physical education was investigated (36 males, 20 females). One female was pregnant and out of the other females 12 were using oral contraceptives.

In addition to delivery of a urine sample, the subjects were asked to complete a questionnaire. Dietary habits, exercise frequency, travels in the past 6 months, medication, health status and a number of other items thus could be investigated. Urinary steroids were analysed for $^{13}\text{C}/^{12}\text{C}$ as described in [1]. The analytes were androsterone (A), etiocholanolone (E), the corresponding 11-hydroxy congeners (OHA, OHE), and pregnanediol (PD).

Data were analysed by fitting linear mixed effects models. The software was “R” in the latest version, where the nlme-library was employed [2]. The $\delta^{13}\text{C}$ values of the steroids were considered the dependent variable. The identity of the respective compounds was considered a fixed effect (independent variable), just like any other possible physiological variable. Each subject was considered to be affected with a randomly distributed $\delta^{13}\text{C}$ baseline value, due to varying dietary input in the population. Factors were included and removed according to likelihood statistics.

As there was evidence for possible effects of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) on $\delta^{13}\text{C}$ values of endogenous steroids, the enzyme was investigated as described in [3]. Briefly, the catalytic portion of the enzyme was expressed and purified. The kinetic isotope effect associated with the catalysed reaction then was investigated by the competitive method [4].

Results & Discussion

The random variation between the subjects amounted to a standard deviation of $\pm 0.47\text{‰}$ on the δ -scale and the residual standard deviation was $\pm 0.45\text{‰}$. The latter typically is attributed to analytical error. As empirically the repeatability of stable isotope analysis of steroids is slightly better (ca. $\pm 0.3\text{‰}$), it can be concluded that there is still some— if not very much - unexplained variance in the data. Table 1 shows the significances of the fixed effects finally included into the model.

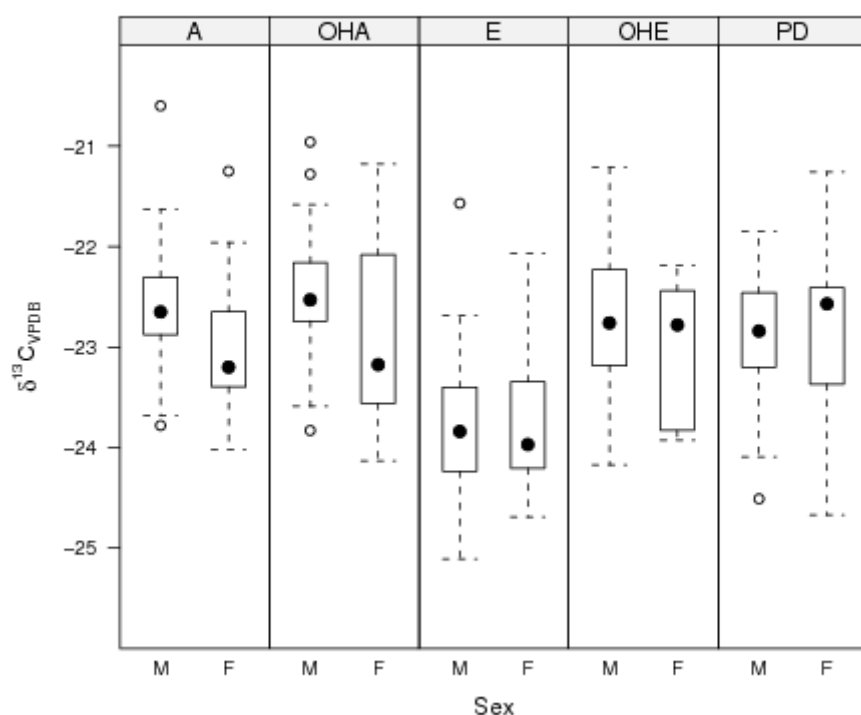
The factor “Steroid” is highly significant ($p < 0.001$). Therefore it has to be concluded that there is considerable isotope fractionation in the steroid metabolism. Physiologically, different $\delta^{13}\text{C}$ values have to be assumed for different compounds. Furthermore, there is a significant

interaction effect between the factors “Steroid” and “Sex” ($p=0.03$). Consequently different $\delta^{13}\text{C}$ patterns of the steroids have to be assumed for males and females. Both phenomena are illustrated in figure 1. The $\delta^{13}\text{C}$ values of the 5β -compounds (E, OHE, PD) are nearly identical for males and females. In contrast, the 5α -steroids A and OHA exhibit a ^{13}C depletion in females. Moreover, E generally is characterized by conspicuously small $\delta^{13}\text{C}$ values in both sexes.

Table 1. Significances of the factors included in the final mixed effects model

Factor	p-Value
Steroid	< 0.001
Steroid×Sex	0.03
Travels to the Americas	0.01
Exercise	0.02
Oral Contraception	0.04

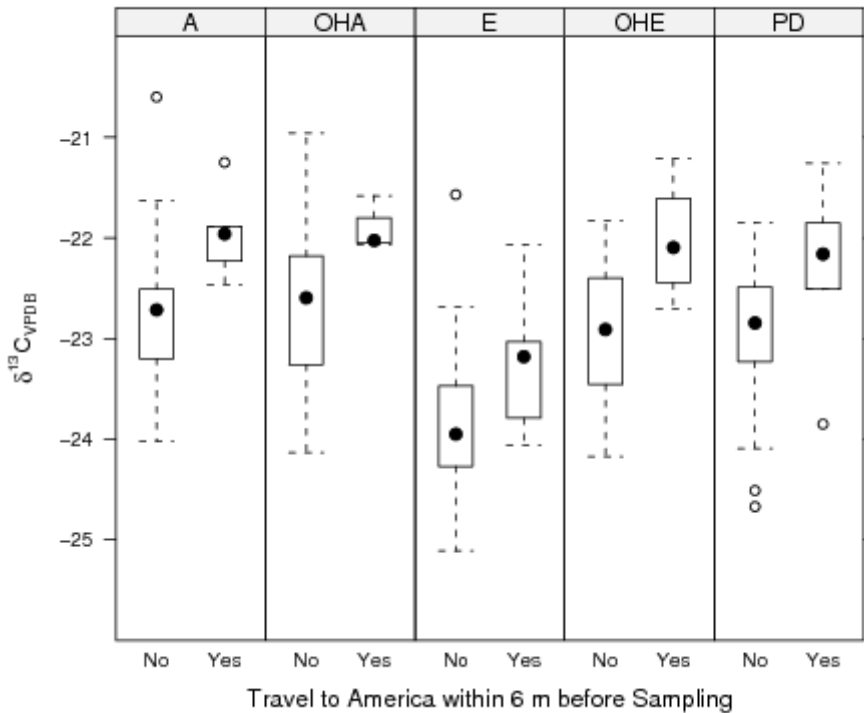
Figure 1. Boxplots of the $\delta^{13}\text{C}$ values of the investigated steroids grouped by factor “Sex”.



Travels to the Americas significantly increase the $\delta^{13}\text{C}$ values of endogenous steroids ($p=0.01$). This can be attributed to the relatively large contribution of C4 plants (corn) to human diet in this region. The effect was to be expected on principle. It is interesting, that the

changed stable isotope signature of the diet still is visible after periods of up to six months. However, it may be that subjects partly maintain American diet after return to Europe. The effect clearly can be seen in figure 2. All steroids are affected to the same degree.

Figure 2. Effects of travels to the Americas on $\delta^{13}\text{C}$ values of the investigated steroids.



Interestingly, increasing exercise frequency clearly results in an increase of the $\delta^{13}\text{C}$ values of endogenous steroids ($p=0.02$, figure 3). The effect however is identical for all the compounds. No significant corresponding interaction effect was found and figure 3 confirms the parallel nature of the change. The result possibly can be explained by larger proportions of carbohydrates in the diet following higher exercise frequencies. Carbohydrates generally exhibit higher $\delta^{13}\text{C}$ values than lipids [5]. It is also possible that the *de novo* biosynthesis of steroids preferably starts from carbohydrates at higher workload because a larger proportion of lipids is utilized in order to yield energy.

Regarding the females only, oral contraception results in a general depletion of the $\delta^{13}\text{C}$ values ($p=0.04$, figure 4). The relative statistical weakness of the result is certainly due to the small subpopulation ($n=20$). However the effect is quite pronounced (ca -0.7‰) for each of

the compounds.

Figure 3. Effects of different exercise frequencies on $\delta^{13}\text{C}$ values of the investigated steroids.

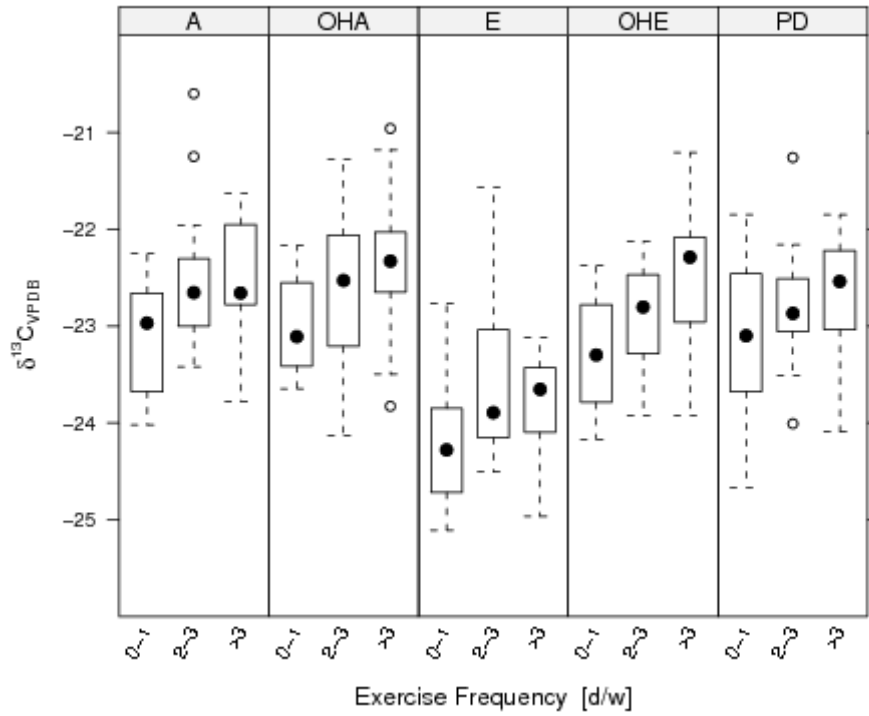
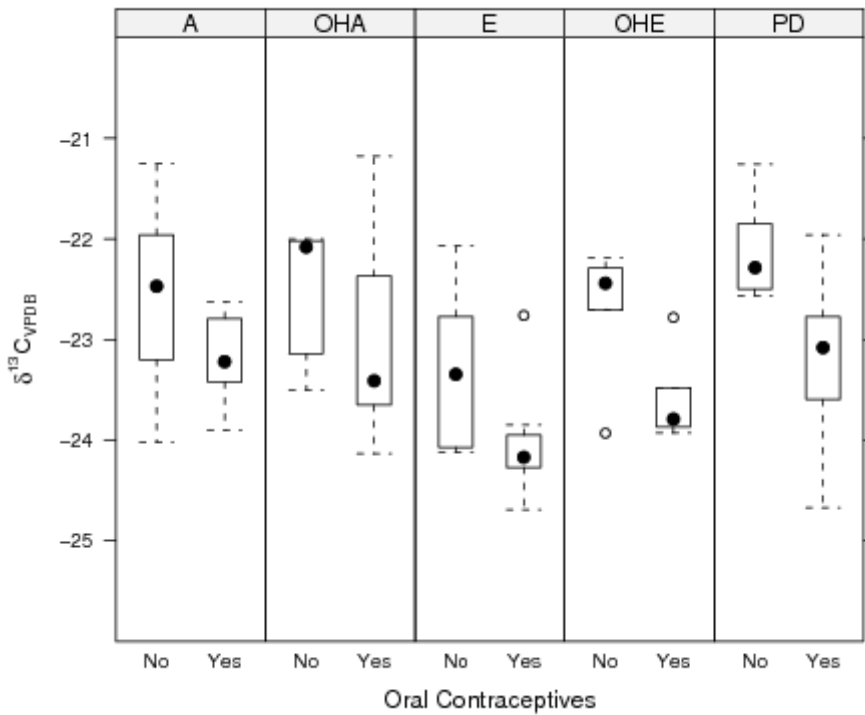
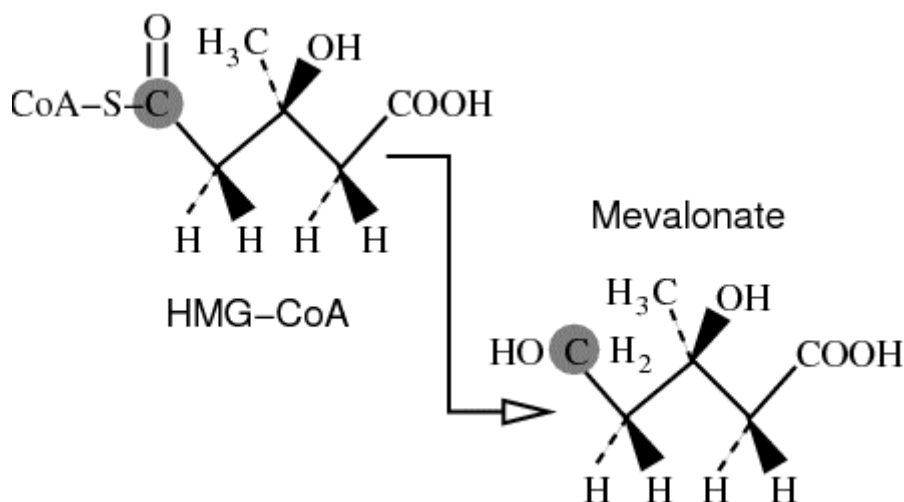


Figure 4. Effects of oral contraception on $\delta^{13}\text{C}$ values of the investigated steroids.



The key enzyme during steroid biosynthesis is HMGR. It catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate. Figure 5 shows the reaction scheme. HMGR is extensively regulated and it is well known that e. g. steroids may effect reduced activity of this enzyme. As a consequence, the reaction might become more limiting during oral contraception and possible isotope effects present in the reaction will then show up in the products. In the meantime we precisely could measure the ^{13}C isotope effect associated with the HMGR reaction [3]. The value is $^{12}\text{k}/^{13}\text{k} = 1.0031 \pm 0.0004$. Full rate limitation by HMGR and complete propagation of the effect to cholesterol, the biochemical precursor of steroids, would thus result in a maximum ^{13}C depletion of ca. 3‰. So, the observed value of 0.7‰ is reasonable and suggests partial limitation. Mechanistically, is to be assumed that the isotope effect is mostly restricted to the position C-1 of mevalonate, the immediate product of HMGR. Different classes of steroids (androstanes, pregnanes) however incorporate this carbon atom at different proportions. Figure 6 shows the positions of mevalonate C-1 in the cholesterol backbone. Theoretically it is therefore possible that differences in $\delta^{13}\text{C}$ between pregnanes and androstanes are induced by oral contraception.

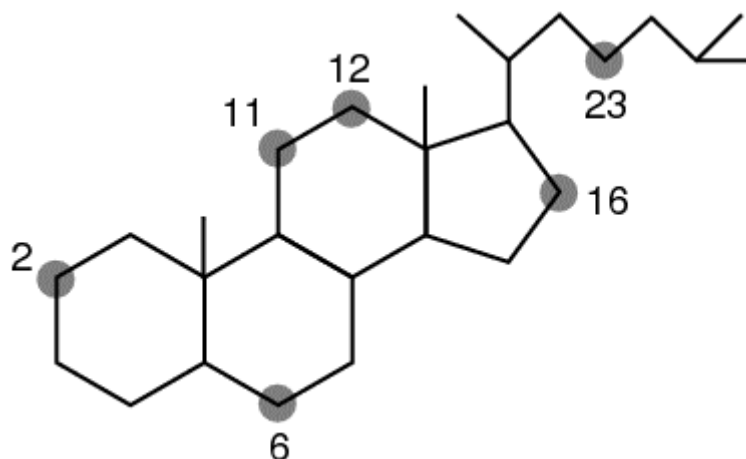
Figure 5. Scheme of the reaction catalysed by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). C-1 of mevalonate is marked.



If the isotope effect was fully restricted to C-1 of mevalonate, a local depletion of 18.6‰ would result (6 x 3.1). The effect would propagate into pregnanes by a weight of 5/21 (5 times C-1 from mevalonate, 21 carbon atoms in total). At full limitation a depletion of 4.4‰ would result. A corresponding calculation for androstanes yields a value of $18.6 \times 5/19 =$

4.9‰. So, a relative difference of ca. 0.5‰ between pregnanes and androstanes theoretically could be induced. As this represents a “worst case scenario” it is not to be expected that oral contraception represents a considerable problem when stable isotope analyses of steroids will have to be evaluated for purposes of doping control. It has to be mentioned that the calculations are based on considerable simplifications, however.

Figure 6. Positions of C1 of mevalonate after incorporation into cholesterol.



Conclusions

$\delta^{13}\text{C}$ values of endogenous steroids are not fixed values. There are numerous factors that physiologically may influence these parameters. Significant factors are sex, dietary input, travels, and exercise frequency. The latter two factors probably exert the corresponding effects also by affecting dietary input. There is also significant variation between different steroids, which reflects isotope fractionation in the metabolism. Moreover, oral contraception bears relevance. As could be demonstrated *in vitro*, the phenomenon is possibly mediated by isotope effects present in the reaction catalysed by HMGR, the key enzyme in steroid biosynthesis. A considerable proportion of the variance present in the $\delta^{13}\text{C}$ values of endogenous steroids can be explained by the abovementioned factors. This improved understanding of the observable variation of the isotope signatures of urinary steroids helps to substantiate stable isotope methodology in doping control. The factors found to be significant seem to exert their effects on the different compounds in parallel. Due to the comparably small magnitude of the effect present in the final products, the exception represented by the HMGR reaction is possibly negligible.

References

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