U. Flenker, C. von Kuk, U. Güntner, F. Hülsemann, V. Gougoulidis, W. Schänzer

Influence of Changes in Diet on the Dynamics of $^{13}C/^{12}C$ in Selected Urinary Steroids: Diet free from Cholesterol

Institute of Biochemistry, German Sport University Cologne

1 Introduction

The abuse of synthetic testosterone and its pro-hormones can be detected by measurement of the $^{13}C/^{12}C$-ratio of testosterone or its metabolites [1]. The method makes use of the fact, that synthetic steroids usually are derived from C-3 plants [7], which exhibit a significant depletion of ^{13}C during photosynthesis [10]. On the other hand most humans feed on a mixture of C-3 and C-4 plants. C-4 Plants perform photosynthesis by a different mechanism than C-3 plants and do discriminate far less against heavy isotopomers [3]. Consequently a comparison of the $^{13}C/^{12}C$ ratios of testosterone metabolites and steroids from an independent pathway can indicate the administration of synthetic steroids. Typically synthetic compounds are isotopically lighter than their human derived counterparts. A difference of three per mil on the delta scale has been written down as a threshold level in sports legislation [11]. Androgens, corresponding prohormones or metabolites thereby represent the targets. Any endogenous steroid from an independent metabolic pathway may serve as a reference in contrast.

It is assumed that the carbon flux through the biosynthetical pathway of steroids is isotopically equilibrated, although formally this is not mentioned. Disturbances of this equilibrium theoretically could effect a false positive finding. This is due to the possibility that different urinary steroids might exhibit different adaptation times. If for instance the reference compounds approximate faster towards a new isotope ratio than the target compounds, this will result in significant differences of the isotope ratios until both groups will have reached the new equilibrium.

Such a disturbance occurs each time the isotope ratio of the food sources is changed. Consequently the time constants for the (isotopic) equilibration of relevant steroid hormones with dietary intake are of great interest here. In order to elucidate the corresponding processes, we performed a switch from usual European feeding habits to a C-4 plant

dominated diet, which is relatively enriched in . C-4 plants constitute a considerable proportion of human diet in arid areas. At the same time the diet in North and Latin America is rich in C-4 plant derived material due to the relative importance of beef. Cattle-breeding heavily relies on corn nourishment where corn exhibits C-4 metabolism and is strongly enriched in ^{13}C.

We present preliminary results from two out of the six subjects who volunteered for this study. The study was approved by the ethics committee of the Sports University.

2 Methods

Six subjects (4 males, 2 females) took part in the study. Physical activity ranged from moderate fitness training in most subjects to elite endurance sport in two male subjects.

The experimental diet was dominated by corn and sorghum products. It was mostly free from cholesterol, as no animal products were consumed. Suitable (*i. e.* ^{13}C enriched) products were purchased from local markets and stores. All products were homogenized, dried and carbon isotope analysis was performed by elemental analysis/isotope ratio mass spectrometry (EA/IRMS). For this purpose a Euro EA 3000 elemental analyzer (Hekatech, Wegberg, Germany) was coupled to a delta C isotope ratio mass spectrometer (Thermo, Bremen, Germany) by a custom made interface. Foodstuff with delta values larger than -20 per mil on the VPDB scale was included in the study.

During the experimental period lunch was prepared by the catering facilities of the German Sport University. Every day one meal was sampled, homogenized and submitted to isotope analysis. Lunch was taken in common at the Sport University, whereas the subjects were provided with sufficient amounts of raw products to prepare breakfast and diner at home.Home made meals therefore were not strictly standardized. However the number and properties of the ingredients were restricted and the participants therefore consumed meals of very similar isotopic composition. The subjects were asked to keep a journal of all consumed products. The ^{13}C enriched diet was maintained for 28 days.

Urines were collected five times a day. Urine samples also were collected seven days before and 20 days following the experimental period. The sampling was not carried out at fixed times. However the subjects were asked to approximate regular intervals. The urines were stored at 4°C until further analysis. Urine samples were prepared and steroids were analyzed by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) as described elsewhere [4]. The procedure was identical to our routine procedure for confirmation of T/E cases [5]. The compounds analyzed were androsterone (A),

etiocholanolone (E), 11-hydroxyandrosterone (OHA), 11-hydroxyetiocholanolone (OHE) and pregnanediol (PD).

A modified open one-compartment model was tried to fit to the data [6]. The corresponding equation is (C0 + dC) - dC · exp(-k · t), where C0 is the initial concentration, dC is the increase, k is the system constant and t is the time. k Expresses the turnover of the system for a given unit of time. The modification simply comprised substituition of the final concentration by (C0 + dC). This was done because dC necessarily must vary between subjects due to a differing C0. But it should be identical for all compounds within one individual. In contrast the final concentration will be identical for all individuals but may vary between compounds due to metabolic isotope fractionation. The software used was R version 2.0.0 [9] where the nlme-library was required [8].

3 Results and Discussion

Figure 1 shows the results obtained from a male subject (M-4) with moderate physical activity. The zero-point on the abscissa corresponds to the beginning of the experimental diet. Starting at different intercepts all steroids show a parallel increase in . No evidence for different half-lives could be found. The common half-live is 386 h, which corresponds to a system constant of 0.0018/h. That means that per hour roughly 2 per mil of the steroidogenic carbon pool is exchanged. The increase in ^{13}C likewise could be assumed to be equal for all compounds. It was estimated to be 2.7 per mil.
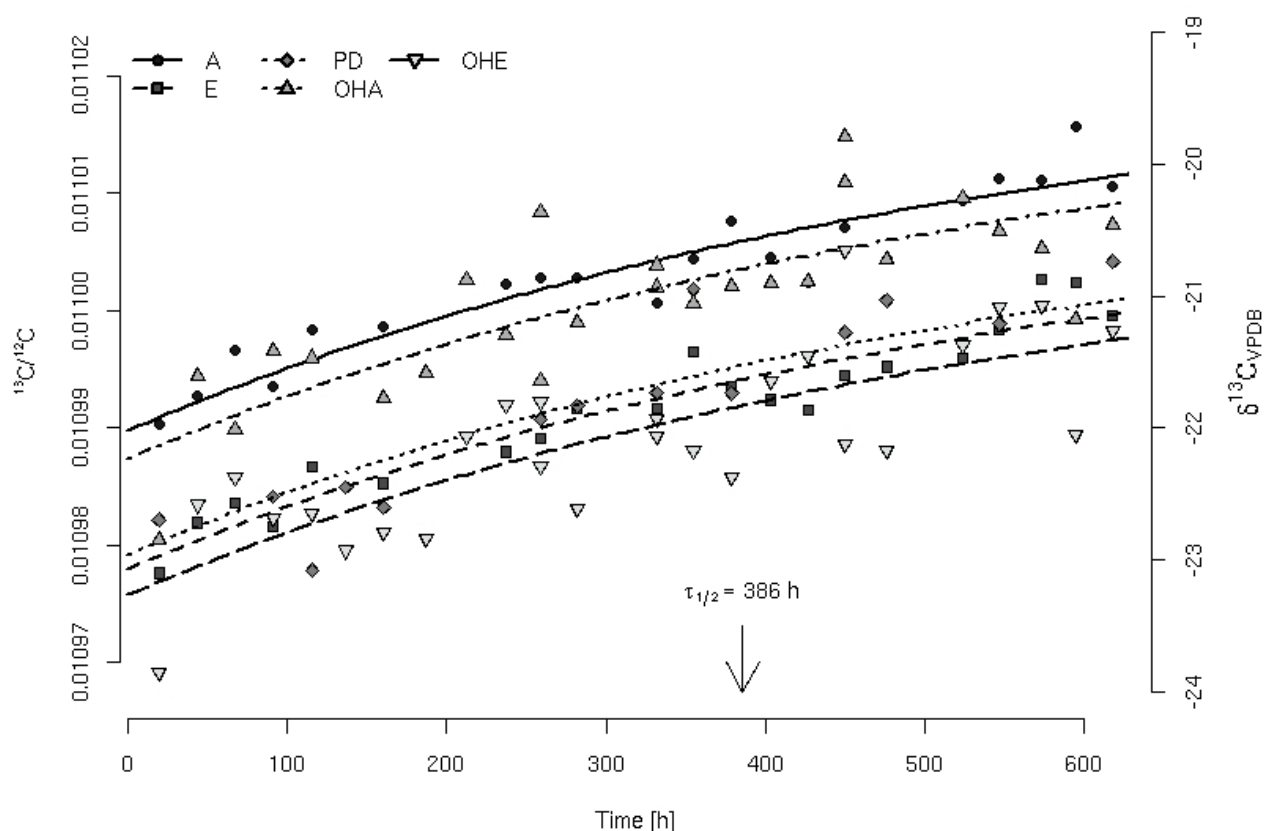
Figure 1: Dynamics of in five urinary steroids during experimental diet. Male subject (M-4), moderate physical activity, height 1.70 m, weight 70 kg.

The residuals of the model have a standard deviation of ±0.3 per mil. This is in perfect agreement with the empirical precision of a GC/C/IRMS system. Therefore the model and the fit appear to be quite reasonable. Diagnostic plots (not shown) as well gave support to the assumption of an appropriate model.

Because virtually no cholesterol was consumed all systematic changes in $^{13}C/^{12}C$ of steroids must be mostly – if not completely – due to *de novo* synthesized compounds. The steroidal increase in can be observed immediately after change to the C-4 based diet. It can be concluded therefore that a considerable fraction of urinary steroids is rapidly and directly synthesized from dietary acetate sources. Obviously this applies to all compounds to the same degree. From the evidence given so far no problems arise to doping control. No change of differences in $^{13}C/^{12}C$ between different steroids is caused by the experimental diet. This can be concluded because no evidence for different half-lives of different steroids could be found. The model employed here is the simplest possible. Whereas it could be shown to be quite

230

appropriate for subject M-4, a look at figure 2 immediately reveals that this is not necessarily true for different persons.
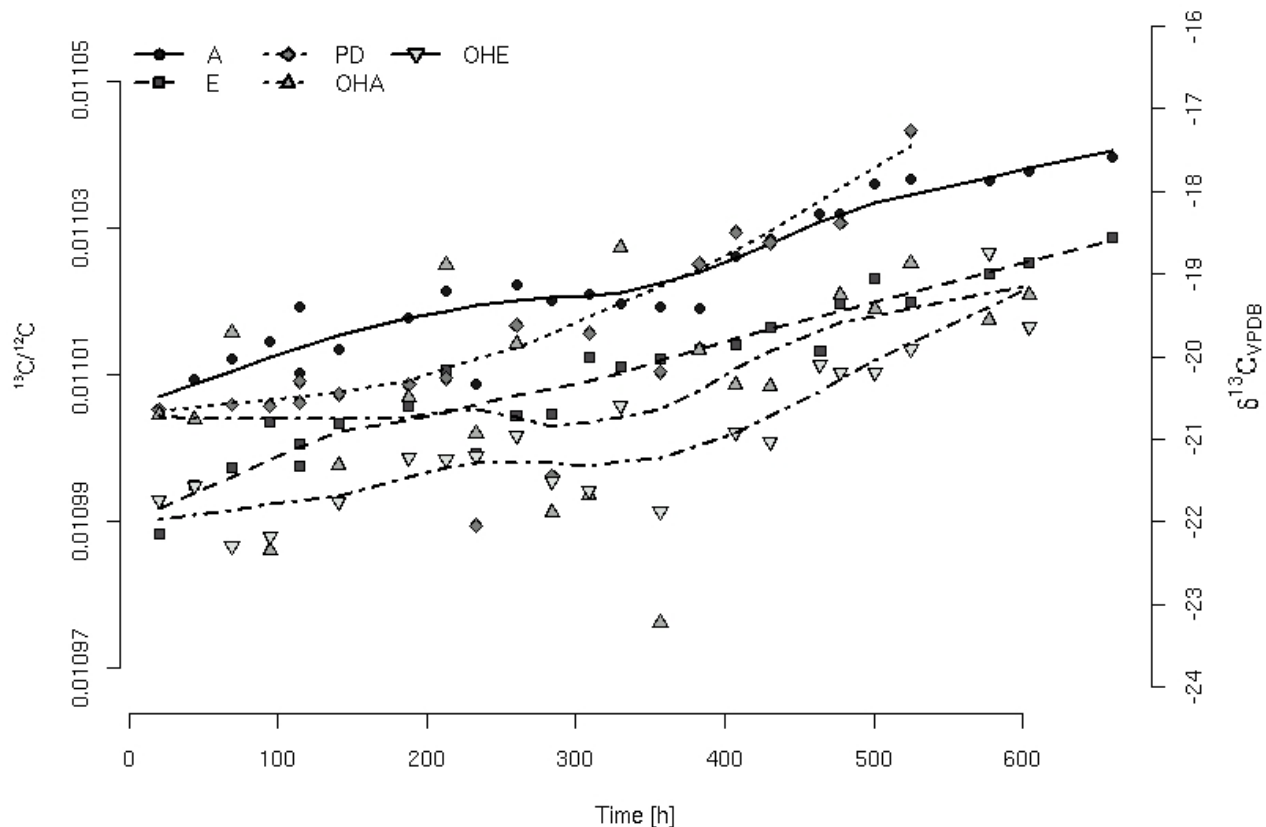

Figure 2: Dynamics of in five urinary steroids during experimental diet. Female subject (F-4), moderate physical activity, height 1.65 m, weight 55 kg.

The $^{13}C/^{12}C$ in urinary steroids exhibit much more complicated kinetics in this individual. The one-compartment model is obviously quite inadequate and also technically it did not converge. For illustrative purposes the non-parametric lowess scatterplot smoother [2] was applied instead. On the one hand the introduction of more than one compartment is needed. But on the other hand the assumption that mixing within compartments occurs rapidly compared to changes from metabolism, is possibly not correct. In compartment models these spatial factors are neglected [6] which is potentially inadequate. Moreover the shapes of the curves are different for different compounds. This is particularly noticeable for PD and it shows an increase similar to an exponential curve and no asymptotic behavior during the experimental period. E and A conversely possess two plateaus and an asymptote is suggested. OHA and OHA behave comparable to A and E, but the initial slope is much smaller. Instead

the increase shows a retarded begin but appears to approximate to the same asymptote. The difference between E or A and any of the other compounds did not exceed 3 per mil. However from the data of F-2 it can be deduced that isotopic changes in diet might represent a problem for doping control.

Acknowledgments

References

[1] Michel Becchi, Rodrigue Aguilera, Yves Farizon, Marie-Magdeleine Flament, Hervé Casabianca, and Patrick James. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun. Mass Spectrom.*, 8:304–308, 1994.

[2] W. S. Cleveland. LOWESS: a program for smoothing scatterplots by robust locally weighted regression. *Am. Stat.*, 35:54, 1981.

[3] G. D. Farquhar. On the nature of carbon isotope discrimination in species. *Australian J. Plant Physiol.*, 10:205 − 226, 1983.

[4] Ulrich Flenker, Stevan Horning, Eckhart Nolteernsting, Hans Geyer, and Wilhelm Schänzer. Measurement of -ratios to confirm misuse of endogenous steroids. In W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, editors, *Proceedings of the 16th Cologne Workshop on Dope Analysis, 15th to 20th March 1998*, number 6 in Recent advances in doping analysis, pages 243–256, Köln, 1999. Sport & Buch Strauß.

[5] Ute Güntner and Ulrich Flenker. *Nachweis exogener Androgene im Humanurin anhand der Bestimmung der -Verhältnisse mittels Gaschromatographie/Isotopenver-hältnismassenspektrometrie*. Deutsche Sporthochschule Köln – Institut für Biochemie, Köln, 2003. Internal document SOP_HS1002.DOC, Version 01, Effective Date 2003/03/31.

[6] Dieter M. Imboden and Sabine Koch. *Systemanalyse*. Springer-Verlag, Berlin, Heidelberg, 2003.

[7] Axel Kleemann and Hermann J. Roth. *Arzneistoffgewinnung: Naturstoffe und Derivate*. Thieme, Stuttgart, 1983.

[8] José C. Pinheiro and Douglas M. Bates. *Mixed-Effects Models in S and S-PLUS*. Statistics and Computing. Springer, New York, Berlin, Heidelberg, 2000.

[9] R Development Core Team. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria, 2004. ISBN 3-900051-00-3.

[10] J. C. Vogel. *Fractionation of the Carbon Isotopes during Photosynthesis*. Springer-Verlag, Berlin, Heidelberg, New York, 1980.

[11] WADA Laboratory Committee. *Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio and Other Endogenous Steroids*. World Anti Doping Agency, Montreal, 2004. WADA document TD2004EAAS.