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U. GÜNTNER, U. FLENKER, C. AYOTTE, M. UEKI, W. SCHÄNZER:
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D/H Isotope Analysis of Endogenous Steroids - First Results

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Abstract

$^{13}C/^{12}C$-analysis of endogenous steroids has proven a valuable tool to elucidate the origin of these compounds. Whether measurement of the D/H isotope ratio has a similar potential is widely unknown.

We analyzed reference samples and samples from excretion studies. Additionally we reanalyzed some samples showing presence of exogenous steroids as judged by $^{13}C/^{12}C$. The urines had different geographic origin. Due to the low abundance of deuterium the measurements were restricted to androsterone and etiocholanolone. These compounds show sufficient concentrations in urine.

D/H ratios in human androgens are similar to the values for sterols of other organisms. Deuterium is depleted by 200–370‰ *vs.* VSMOW.

For the time being unequivocal evidence for the origin of androgens by D/H analysis is missing. This might be due to unsolved analytical problems. Exchange of hydrogens during sample handling seems to be an important issue. Exchange with ambient material easily might blur isotopic signatures because the D/H values of androgens are extremely depleted. Nonetheless some trends can be identified.

1 Introduction

The analysis of $^{13}C/^{12}C$ in endogenous steroids has become a standard tool in doping analysis to provide evidence for the application of synthetic steroids. The effectivity of this method relies on the fact that synthetic testosterone (T) and its synthetic prohormones are depleted in ^{13}C compared to the corresponding biogenous compounds [1]. However problems might arise when baseline levels of $^{13}C/^{12}C$ are low. This will be the case in regions with diet predominantly derived from C-3 plants. Moreover recently gels for transdermal application of T have been developed. This effects a slow and smooth release of this hormone [2, 3]. Because under these circumstances the endogenous synthesis of androgens will not be suppressed completely it is likely that the isotope signature of T metabolites excreted in urine under treatment with T gel is not controlled exclusively by the synthetic T. Another element which possibly exhibits characteristic isotopic signatures is hydrogen. As the relative mass difference between deuterium (D, ^{2}H) and protium (H, ^{1}H) is far bigger than between ^{13}C and ^{12}C, it can be expected that those signatures will be even more pronounced [4, 5].

All hydrogen in lipids ultimately is derived from water. Because meteoric water shows systematic variation of D/H depending on geographic factors, it is to be expected that this isotopic signature is reflected in biosynthesized lipids. Significant D/H fractionation occurs during biosynthesis of lipids [4, 6]. But the biochemical pathway for the production of urinary steroids is identical for different individuals. It is likely therefore that the D/H of the ambient water represents an offset which will propagate through the biotransformation.

2 Material and Methods

2.1 Urine Samples

Reference samples were analyzed from 15 urines collected in Cologne, from 9 urines collected in Montreal and from 12 urines collected in Tokyo. Urines were also analyzed from one hypogonadal patient treated with testosterone gel TestoGel®50 (Jenapharm, Jena, Germany) for several weeks.

2.2 Sample Preparation

Urine samples were prepared according to Geyer *et al.* [7].

An HPLC cleanup step was added. It was performed on an Agilent LC 1100. The column used was a LiChrospher®100 RP-18, 5µm, 250 x 4 mm (Merck, Darmstadt, Germany). The mobile phase was constituted by acetonitrile/water starting at 30% acetonitrile increased linearly to 100% within 20 min. The flow was set to 1 mL/min. The UV detector was set to a wavelength of 192 nm.

2.3 Isotope Analysis

D/H analysis requires much more material than isotope analysis of the stable carbon isotopes. This is due to the low abundance of deuterium in nature. Its relative abundance is only 0.015% [8]. Therefore only the most abundant T metabolites - etiocholanolone (E) and androsterone (A) - were subject of the present study. The HPLC fraction containing E and A was evaporated to dryness and dissolved in cyclohexane. The concentration was adjusted to 250 µg/mL for D/H measurement and to 100 µg/mL for $^{13}C/^{12}C$ analysis. The solvent was spiked with 5α-androstane-3β-ol as reference standard.

$^{13}C/^{12}C$ measurement was performed by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The analytical instrument was a GC 5890 II (Agilent Technologies) coupled to a Delta C gas isotope ratio mass spectrometer by combustion interface II (ThermoFinnigan, Bremen, Germany). The GC column was a Optima δ3 (Macherey & Nagel, Düren, Germany). Dimensions were 17 m x 0.25 mm. The film thickness was 0.25 µm. Helium was used as the carrier gas at a constant pressure of 30 psi.

1 µl was injected in splitless mode (splitless time 1.5 min) at 300°C. An A200S autosampler (CTC Analytics, Zwingen, Switzerland) was employed at an injection speed of 5.5 µl/s.

The initial column temperature was set to 60°C for 1.5 min followed by an increase of 30°C/min up to 265°C. A second ramp of 3°C/min followed. The final temperature of 295°C was held for 2 min. $^{13}C/^{12}C$ is expressed as $\delta^{13}C_{PDB}$, where the working standard (CO_2, $\delta^{13}C_{PDB}$=-3‰) was calibrated *vs.* an *n*-alkane mixture [9].

Measurement of D/H was performed by gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS). The system was a GC 6890 II (Agilent Technologies) coupled to a Delta Plus XP gas isotope ratio mass spectrometer by a combustion interface III (ThermoFinnigan, Bremen, Germany). The GC column was a J&W HP-5ms (Agilent Technologies). Dimensions: 30 m x 0.32 mm. The film thickness was 0.25 µm. Helium was used as the carrier gas at a constant flow of 3 mL/min. Injection parameters were identical to $^{13}C/^{12}C$-measurements. The working standard (H_2, δD_{VSMOW}=-690‰) was calibrated *vs.* the same calibration mixture as the CO_2 gas.

The initial temperature was set to 60°C for 1.5 min followed by an increase of 30°C/min up to 250°C. This temperature was kept constant for 5 min. The temperature then was increased at 10°C/min up to the final temperature of 295°C which was held for 5 min. D/H is expressed either as δD_{VSMOW} or as δD *vs.* reference standard.

3 Results and Discussion

Figure 1 depicts the dependence of D/H of the reference standard (RSTD) from the order of injection. 5 repeated measurements of RSTD were performed before and 3 repeated measurements were performed after injection of 13 biological samples. It can be seen that a significant trend is present in the data from the RSTD. When measured in common with biological material on the other hand, the variability of the RSTD increases and shows significant covariation with the values of the urinary steroids. Several explanations are possible for these phenomena:

1. Hydrogen might be exchanged with the environment.
2. Hydrogen might be exchanged with the solvent.
3. Hydrogen might be exchanged between E and A on the one hand and RSTD on the other, which might be mediated by any acidic compound present in the sample.

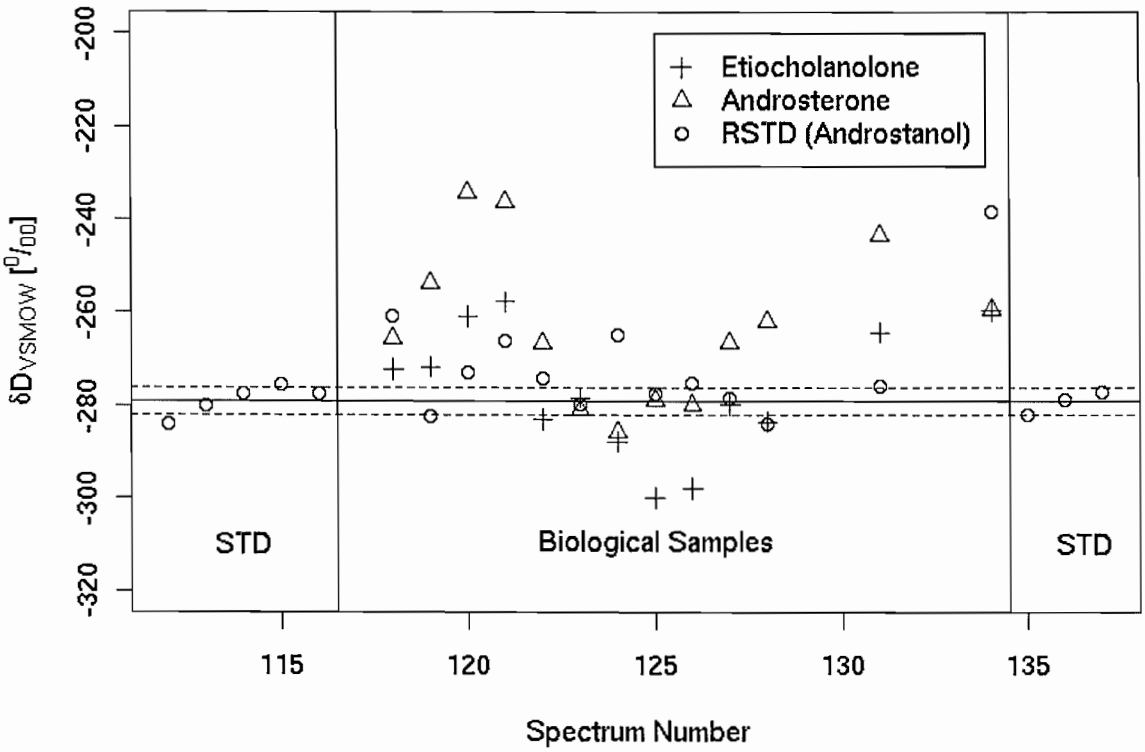

Figure 1: D/H of RSTD *vs.* sequence line. Vertical lines indicate beginning and end of measurement of biological samples. Horizontal lines indicate mean (solid) ± standard deviation (dashed) of RSTD calculated without measurement of biological samples.

The measurements of RSTD before and after the measurements of E and A were done repeatedly from 2 vials containing a standard mixture. Once punctured it is likely that isotopic equilibration with ambient water proceeds. In addition the standard mixture contained a small amount of 2-propanol. The D/H depleted material preferably will evaporate while the enriched residue equilibrates with the analytes. Both hypotheses can explain the positive trend in D/H of RSTD.

The hypothesis of hydrogen exchange between biological compounds and RSTD is supported by analysis of the correlation of D/H between those compounds. Figure 2 shows the outcome: D/H of both biological steroids exhibit significant correlations to the D/H of the RSTD. This indicates a common source of hydrogen for the three compounds. Necessarily exchange of hydrogen must have occurred, regardless of the primary source, which to a certain degree might be atmospheric water.

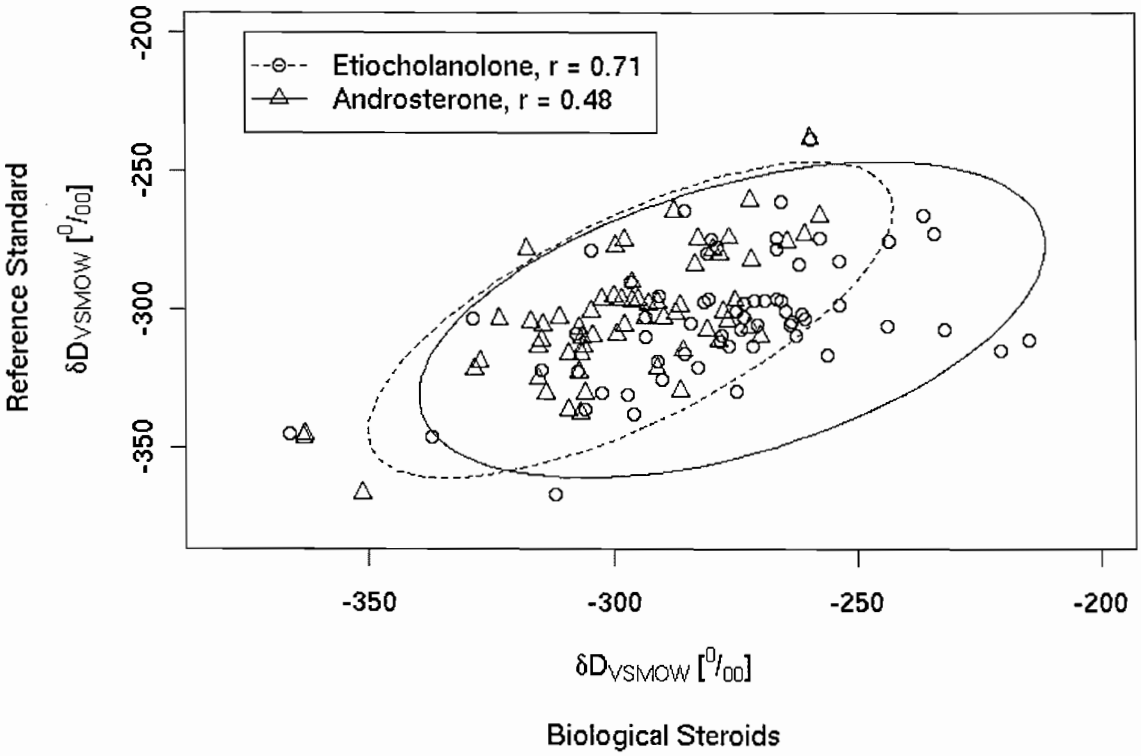

Figure 2: Correlation of δD in biological steroids (etiocholanolone, androsterone) and δD in reference standard.

The data in figures 1 and 2 are calculated *vs.* VSMOW *via* calibrated H_2. Due to the findings so far we decided to report any further D/H measurements *vs.* RSTD. This at least partly should eliminate systematic errors due to hydrogen exchange.

Another fact speaking in favor of employment of a steroid as standard is the extremely low δD_{VSMOW} of the reference H_2. It was determined as –690‰. This corresponds to a D/H of $\approx 4.65e^{-05}$ and certainly does not allow accurate measurements. In spite of all objections concerning the accuracy of the data it has to be stated that the magnitude of δD_{VSMOW} measured for biological steroids is well comparable to the range of the values measured by Sessions [10], who reported values from –325‰ to –205‰ for sterols from different species whereas our data range from –366‰ to –215‰.

Figure 3 shows D/H *vs.* RSTD in E and A from reference urines from different regions. Whereas the values from Tokyo and Cologne are comparable the samples from Montreal exhibit a composition which is isotopically lighter. This is consistent with the fact that D as well as ^{18}O become depleted in atmospheric water with increasing distance from the sea [5, 11].

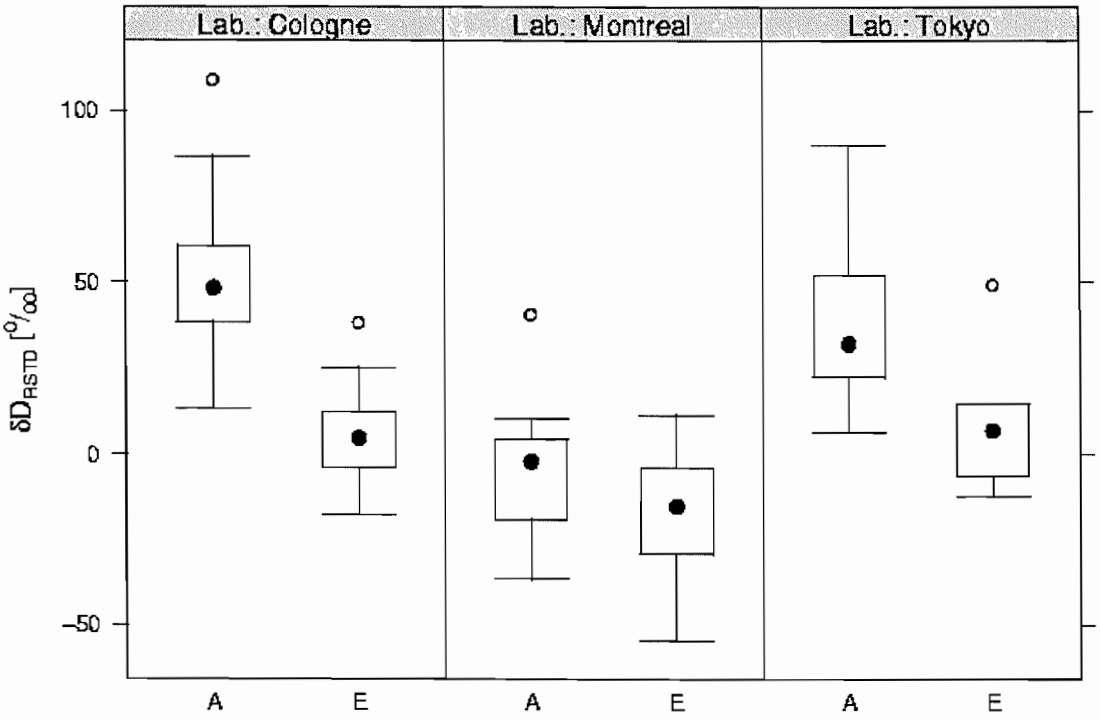

Figure 3: D/H in androsterone (A) and etiocholanolone (E) extracted from reference samples of different regional provenance.

A similar effect exists for increasing altitude and increasing geographical latitude. Figure 4 illustrates the distribution of D/H in precipitation over the world. It can be recognized that the values in Western Europe and Japan are similar. The eastern part of Canada shows D/H depletion.

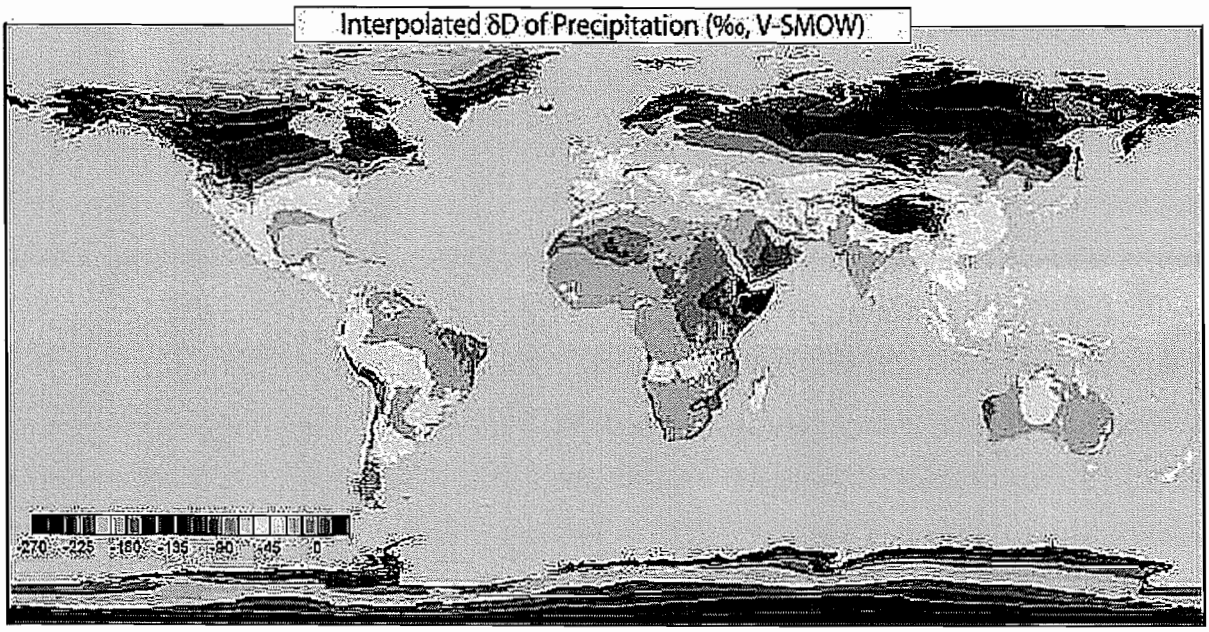

Figure 4: Distribution of D/H in precipitation over the world. Map taken from [11].

Figure 5 shows the distribution of D/H in the reference samples from Cologne and the distribution of routine samples judged as "positive" for presence of exogenous androgens by $^{13}C/^{12}C$ of E and A.

Generally E shows lower D/H-values than A. It is not easy to obtain complete baseline separation for these compounds and it is known that systematic errors in the calculation of isotope ratios can result from lack of chromatographic resolution [12]. On the other hand it could be shown that kinetic isotope effects are likely to be the reason for the relative depletion of $^{13}C/^{12}C$ in E compared to A [13].

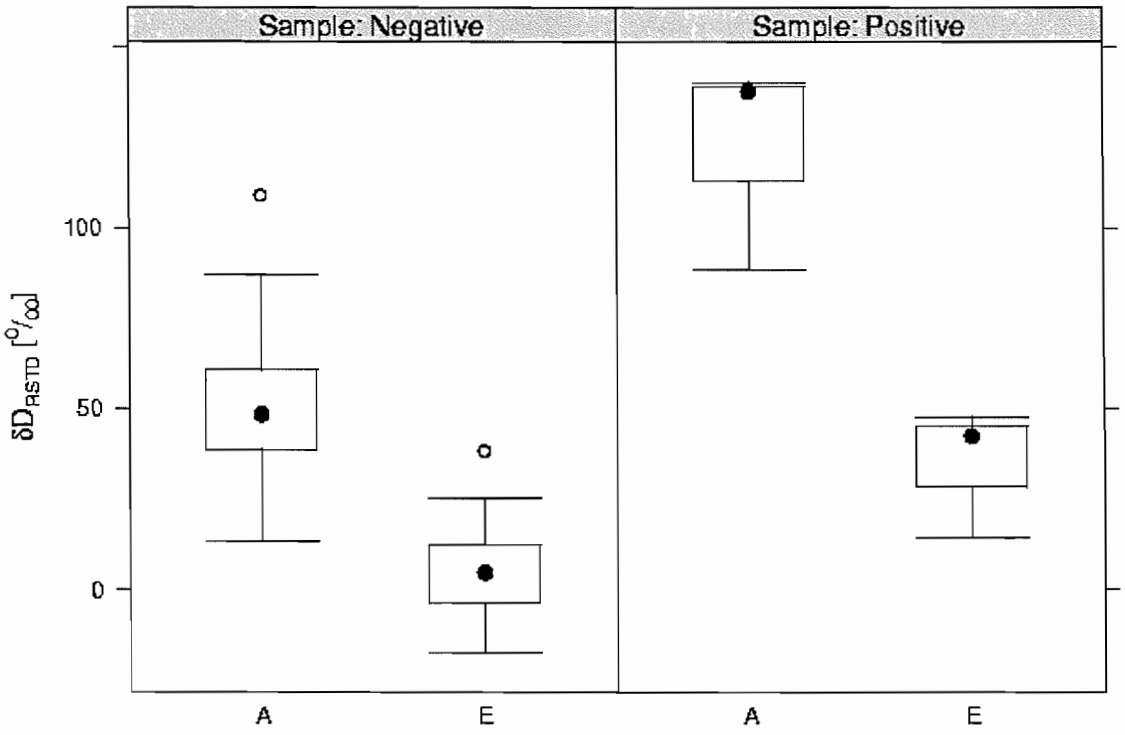

Figure 5: D/H in androsterone (A) and etiocholanolone (E) from reference samples collected in Cologne (Negative) and from routine samples showing evidence for presence of synthetic steroids (Positive) according to $^{13}C/^{12}C$.

Figure 6 depicts the relation of D/H and $^{13}C/^{12}C$ in E and A from reference samples collected in Cologne. The isotope ratios show a positive correlation which is more pronounced for A. The values for a hypogonadal patient treated with testosterone gel (TestoGel®50) are added. The evaluation of $^{13}C/^{12}C$ alone would not allow for a clear discrimination from the reference samples. Inclusion of D/H clearly represents a diagnostic improvement. This holds true although D/H alone just like $^{13}C/^{12}C$ does not clearly fall outside the range observed for the reference samples. Instead the combination of both parameters provides greater significance.

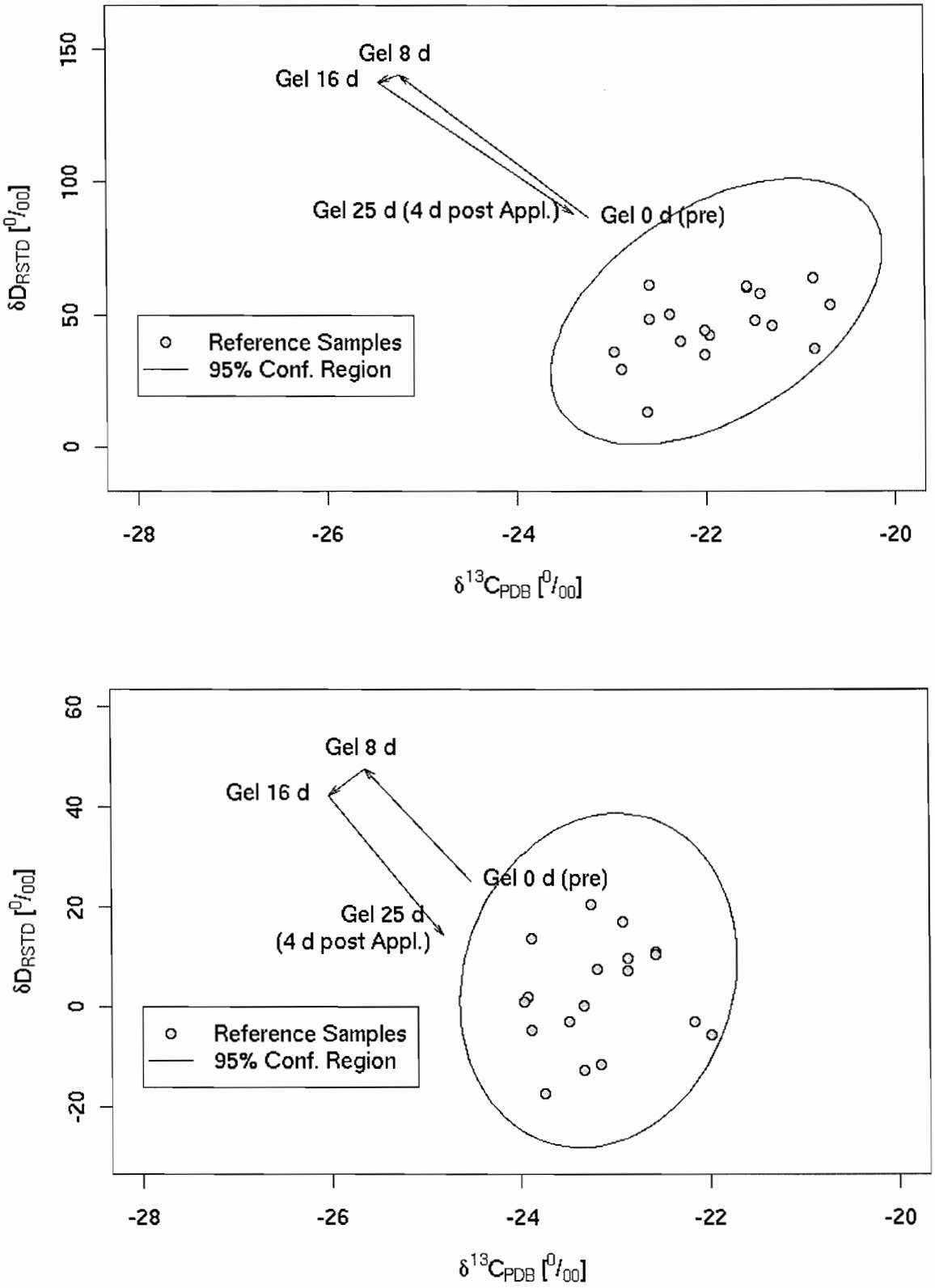

Figure 6: D/H *vs.* $^{13}C/^{12}C$ in androsterone (upper panel) and etiocholanolone (lower panel) from reference samples collected in Cologne and from samples collected from a hypogonadal patient treated with TestoGel®50 for several weeks.

References

[1] Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun. Mass Spectrom.* 1994; **8**: 304.

[2] Jenapharm. Product information TestoGel®. 2003.

[3] Swerdloff RS, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto AM, Snyder PJ, Weber T, Longstreth J, Berman N, the Testosterone Gel Study Group. Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. *J. Clin. Endocrinol. Metab.* 2000; **85**(12): 4500.

[4] Hayes JM. Fractionation of the isotopes of carbon and hydrogen in biosynthetic processes. *Reviews in Mineralogy and Geochemistry*. 2001; **43**: 225.
URL: http://www.nosams.whoi.edu/general/jmh/index.html (February 2004).

[5] Hoefs J. *Stable Isotope Geochemistry*. Springer, Berlin. 1997.

[6] Sessions AL, Burgoyne TW, Schimmelmann A, Hayes JM. Fractionation of hydrogen isotopes in lipid biosynthesis. *Organic Geochem.* 1999; **30**: 1193.

[7] Geyer H, Schänzer W, Mareck-Engelke U, Nolteernsting E, Opfermann G. Screening procedure for anabolic steroids - the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds.), *Proceedings of the 15th Cologne Workshop on Dope Analysis, 23rd to 28th February 1997*, number 5 in Recent advances in doping analysis. Sport & Buch Strauß, Köln. 1998; 99–101.

[8] Platzner I. Appendix 1, table of naturally occuring isotopes. In Platzner I (ed.), *Modern Isotope Ratio Mass Spectrometry*, Chemical Analysis - A Series of Monographs on Analytical Chemistry and its Applications. John Wiley & Sons, Chichester, 2nd edition. 1999; 487–497.

[9] Schimmelmann A. Hydrogen and carbon stable isotope (D/H, $^{13}C/^{12}C$) standards for organic compound specific investigations. Technical report, Indiana University. 2002.
URL: http://php.indiana.edu/~aschimme/hc.html (August 2002).

[10] Sessions AL. *Hydrogen isotope ratios of individual organic compounds*. Ph.D. thesis, Indiana University. 2001.

[11] Bowen G. Maps of the stable isotope composition of precipitation. 2004.
URL: http://www.es.ucsc.edu/~gbowen/Isomaps.html (February 2004).

[12] Goodman KJ, Brenna JT. Curve fitting for restoration of accuracy for overlapping peaks in gas chromatography/combustion isotope ratio mass spectrometry. *Anal. Chem.* 1994; **66**: 1294.

[13] Flenker U, Schänzer W. Kinetic isotope effects during metabolism of Δ4-steroids. In Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds.), *Proceedings of the 19th Cologne Workshop on Dope Analysis, 18th to 23rd March 2001*, number 9 in Recent Advances in Doping Analysis. Sport & Buch Strauß, Köln. 2001; 179–185.