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Kinetic Isotope Effects during Metabolism of Δ^4-Steroids

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Abstract

On principle kinetic isotope effects (KIEs) may occur under two conditions: At a rate limiting step and at a branchpoint of substance flux. The reduction of the double bond of Δ^4-steroids is the rate limiting step during metabolism of testosterone and related hormones. At the same time it represents a branchpoint, because two types of isomers occur. Isotopic fractionation therefore may be expected here. We could show that different δ^{13}C-values in fact can be measured for androsterone and eticholanolone, the most abundant representatives of each of the two branches. Different δ^{13}C-values as well could be observed between the 19-nor analogues of these compounds after administration of 19-norandrostendione to one volunteer.

1 Introduction

Kinetic isotope effects (KIEs), *i.e.* the fractionation of isotopes during reactions that are under kinetic control, fundamentally can be observed when

1. there is non-quantitative conversion of the educt(s) and
2. when different reaction constants exist for the different isotopes of the elements involved.

Lack of quantitative conversion occurs either at rate limiting steps or at branchpoints of the reaction. Due to the differing zero point energies, different reaction rates of isotopically substituted chemical bonds have to be assumed virtually all the time a bond is broken or formed [11, 12]. Whether this becomes manifest consequently depends on the fulfillment of the condition mentioned first.

The initial and rate limiting step during phase-1 metabolism of androgenous anabolic steroids is the reduction of the double bond between C-4 and C-5 [10]. This process constitutes a major branchpoint in steroid metabolism, because two classes of metabolites are formed (5α- and 5β-androstanes) and because the reaction appears to be irreversible under biological conditions. Two different enzymes are responsible for this, sited in separated compartments of the cell. As a bond between two carbons is affected, this step of steroid metabolism is a perfect candidate for fractionation of carbon isotopes to an detectable extent.

The most abundant metabolites resulting from this part of the metabolism of androgens (testosterone, androstendione, DHEA *etc.*) are androsterone (A) and etiocholanolone (E). A represents the 5α-, E the 5β-branch. We were interested in possible differences of the $^{13}C/^{12}C$-ratio between these compounds under normal biological conditions, *i.e.* in steady-state. At the same time we hypothesized that after administration of a synthetic Δ^4-steroid time dependent differences of the $^{13}C/^{12}C$-ratio could be observed between the two metabolic branches. We monitored the development of the $^{13}C/^{12}C$-ratio of 19-noretiocholanolone (NE) and 19-norandrosterone (NA) after oral application of a single bolus of 19-norandrostendione to one volunteer.

2 Methods

2.1 Analysis of E and A

Nine urine samples (U-1 – U-9) were analyzed after routine analysis had shown no evidence for presence of exogenous steroids. One quality control urine (QC) collected and pooled from eight males known to be free from synthetic steroids was analyzed as well. 5ml of urine were prepared.

Sample preparation on principle followed the method described in [7]. To achieve isolation of E and A before isotopic analysis a new HPLC cleanup procedure was developed:

- Solvents: H_2O/CH_3CN, starting at a ratio of 70:30 linearly changed to 45:55 within 17min.
- Flow: 1.8 ml/min.
- Column: Hewlett & Packard, ODS Hypersil, 250×4 mm, 5μm.

Several columns of similar specifications were tested. Only the model mentioned above yielded sufficient seperation of E and A.

After evaporation of the solvent the HPLC-fractions were taken up in 2-propanol. As the concentrations of E and A were known from routine steroid profiling, concentrations could be adjusted to $\approx$100μg/ml uniformly in all resulting samples.

Isotopic measurements were performed by gas chromatography / combustion / isotope ratio mass spectrometry (GC/C/IRMS). General setup of the whole device was as described in [7]. The chromatographic column was a M&N Optima-δ3 (Macherey & Nagel, Düren, Germany), 17m length, 0.25mm inner diameter, 0.25μm film thickness. Injections (1μl) were performed in splitless mode at a headpressure of 30psi. Temperature was isothermal at 60 °C for 90s, raised to 246 °C at 30K/min and then raised to 278 °C at 3K/min. Finally the temperature was raised to 290 °C at 30K/min and was kept constant for 5min.

Five replicate measurements were performed on each HPLC-fraction. Data were analyzed by means of ANOVA.

Table 1: Two factor ANOVA for five replicate measurements of the δ^{13}C-values of androsterone and etiocholanolone (factor 'Ster.') nested within ten urine samples (factor 'Spl.'). Factor 'Spl.' as well as its interaction with factor 'Ster.' shows highly significant influence.

	Df	SSQ	MSQ	F	p
Spl.	9	20.71	2.30	18.60	< 0.0001
Spl.×Ster.	10	24.61	2.46	19.89	< 0.0001
Resid.	80	9.90	0.12		

Table 2: Linear model for the δ^{13}C-values of 19-noretiocholanolone (NE) and 19-norandroterone (NA) depending on time after application of 100mg 19-norandrostendione. Intercepts show significant difference. Slopes are siginificantly different, where the slope for NA is not significantly different from zero.

	δ^{13}C	SE	p
NE	-30.25	0.24	< 0.0001
NA	-28.83	0.24	< 0.0001

	$\frac{d\,\delta^{13}C}{dt}$	SE	p
NE	0.045	0.015	< 0.01
NA	-0.024	0.015	≈ 0.12

2.1.1 Analysis of 19-norsteroids

One volunteer (male, 49y, 76kg) orally administered 100mg of 19-norandrostendione. Urine samples were collected 10, 16.2 and 20 hours after application. Samples were treated as described above. NA and NE were isolated by HPLC and underwent isotopic analysis in different runs. Again five replicate measurements were performed.

Data were analyzed by iterative fitting of linear models with 'time' being the independent variable.

3 Results

Table 1 shows the results of a two factor ANOVA for the δ^{13}C-values of A and E (factor 'Ster.') nested within the ten analyzed urine samples (factor 'Spl.'). Factor 'Spl.' and the interaction of 'Spl.' and 'Ster.' are highly significant. A graphical representation of the corresponding data is given in figure 1. Data are presented as $\bar{x} \pm s$ of δ^{13}C-values of E and A for each urine sample *vs.* the grand mean. Figure 1 also contains asterisks indicating significant differences ($p \leq 0.05$) in δ^{13}C-values between E and A within sample after BONFERRONI-correction. In nine out of ten samples the average δ^{13}C-values of E is lower than that of A. Out of these nine cases seven are significant.

Table 2 shows the parameters of the linear model describing the dependence of the ^{13}C-enrichment of NE and NA on time after application of 19-norandrostendione. Model selection techniques (iterative analysis of variance) showed that different intercepts and different slopes are indeed necessary to describe the data. No evidence for curvature was found in the data. A graphical representation of the model is given in figure 2.

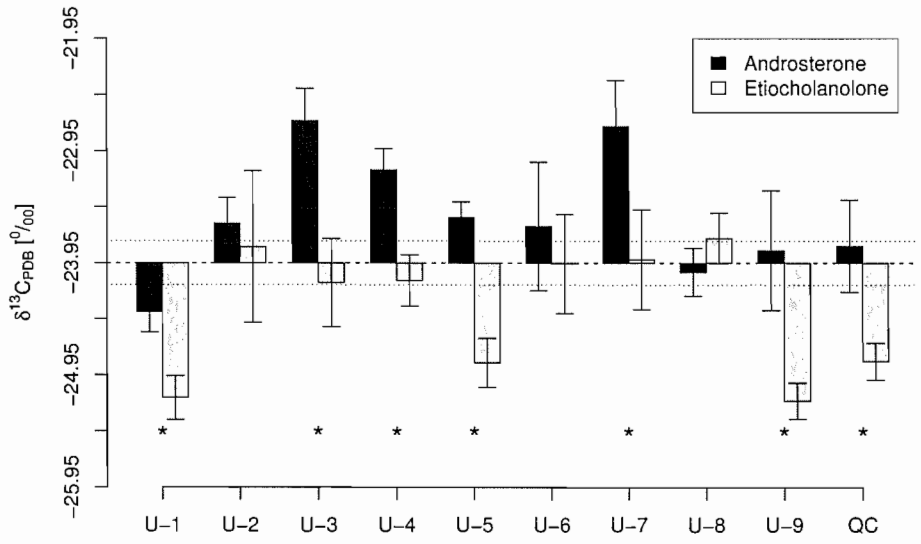

Figure 1: δ^{13}C-values of androsterone (A) and etiocholanolone (E) from nine routine urine samples (U-1 – U-9) and from one urine collected and pooled from eight males (QC). Values are represented as $\bar{x} \pm s$ relative to grand mean. Dotted lines indicate 99%-confidence limits of the grand mean. Asterisks indicate significant difference between E and A within sample after BONFERRONI-correction.

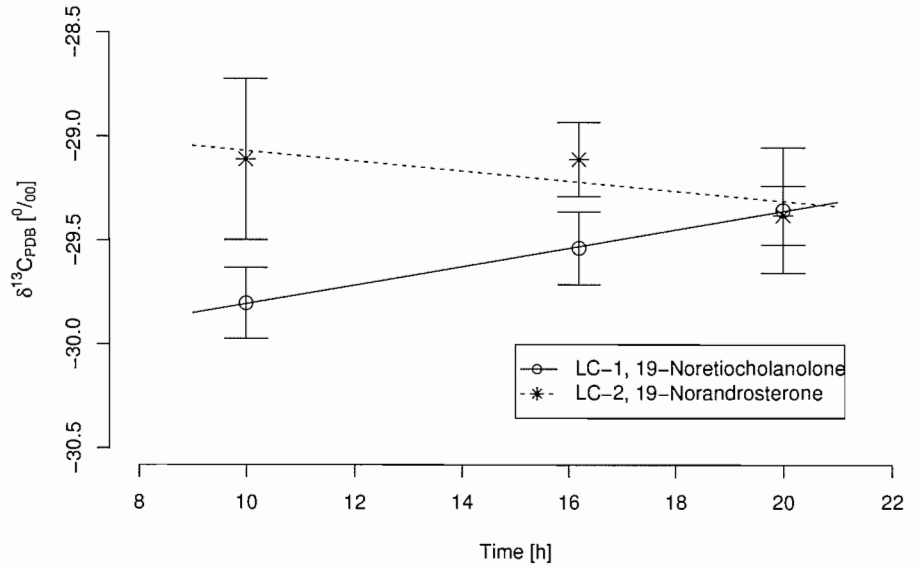

Figure 2: δ^{13}C-values of 19-norandosterone and 19-noretiocholanolone after administration of 100mg 19-norandrostendione. Symbols and error bars show $\bar{x} \pm s$. Fitted lines represent the linear model described in table 2.

4 Discussion

The isotopic data for E and A clearly indicate a fractionation of the stable carbon isotopes ^{13}C and ^{12}C during the reduction of the double bond between C-4 and C-5. E is depleted in ^{13}C relative to A. The degree of fractionation appears to differ from individual to individual. Isotopic fractionation theoretically depends on

1. isotopic fractionation during the reaction itself (probably different in 5α- and 5β-reduction),
2. the substrate concentration and
3. the magnitude of the fluxes of metabolites through both pathways.

Hence from the present data a definite model cannot be formulated. Nevertheless our observations are in good accordance with theory: Given that a depletion of ^{13}C occurs during 5β-reduction the residual and therefore the 5α-metabolites must be isotopically enriched. Biological variation of the activities of any of the two relevant enzymes will cause different fluxes through the metabolic pathways, which easily could explain the significant interindividual variation observed in differences between $\delta^{13}C$-values of E and A.

5α- and 5β-androstanes are frequently analyzed with respect to their $^{13}C/^{12}C$-ratios in doping control to detect abuse of synthetic steroids in sport [1–7, 9]. Published results are somewhat contradictory concerning possible isotope fractionation however. In a recent study Aguilera *et al.* found that acetylated 5β-androstane-3α, 17β-diol was isotopically slightly enriched *vs.* acetylated 5α-androstane-3α, 17β-diol [5]. In an earlier investigation Aguilera *et al.* found no significant difference in isotopic composition between A and E [4]. Again acetylated compounds were used for GC/C/IRMS analysis. Flenker *et al.* reported ^{13}C-depleted E relative to A using underivatized material [7]. Aguilera *et al.* presented similar data based on acetylated androstanediols once more [3].

One possible reason for the inconsistent results might be that GC/C/IRMS is sensitive towards lack of chromatographic baseline seperation. Depending on the chromatographic conditions 5α- and 5β-isomers of androstanes are difficult to separate. Goodman and Brenna [8] report an apparent enrichment of around 2 $^{0}/_{00}$ for the first of two coeluting compounds (methyltridecanoate and butylated hydroxytoluene) at an overlap of 10 % valley. Increasing overlap worsens the situation dramatically. Former work of our group [7] likewise showed that in contrast to isolated measurements of the same laboratory standards E apparently is ^{13}C-depleted *vs.* A when the two compounds are measured together. Chromatographic seperation was better than 10 % valley.

Unfortunately the reported isotopic fractionations are in an order of magnitude that allows for explanation by true KIEs as well as by systematic analytical error. In the present study we circumwent possible problems caused by lack of chromatographic separation by introduction of HPLC isolation of both compounds of interest.

The data obtained from our execretion study with 19-norandrostendione generally confirm the results. Obviously 5β-metabolites of androgens are depleted in ^{13}C *vs.* their 5α-counterparts. This supports our hypothesis of a KIE present during 5β-reduction. As the

substrate concentration decreases the isotopic fractionation decreases as well. No evidence was found for a KIE during 5α-reduction.

However theoretically two approximately parallel curves should be obtained for the two branches with or without isotope effect present in 5α-metabolism. Our results suggest different slopes for ^{13}C in NE and NA over time. It has to be emphasized that the slope for NA is not significantly different from zero. A possible explanation might be a distribution of the substrate into different compartments **before** the Δ^4-reduction. It is known that 5α- and 5β-reductase indeed are sited in the endoplasmatic reticulum and in the cytoplasm of hepatocytes respectively [10].

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