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Elucidation of Original and Metabolic Sources of Ephedrines by Stable Isotope Analysis

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Introduction

Among a number of other stimulants, the effective WADA prohibited list mentions some *Ephedra* alkaloids. Ephedrine (E) is prohibited at a threshold level of 10 µg/mL, for cathine ("norpseudoephedrine", NP) a threshold level of 5 µg/mL has been established. In contrast phenylpropanolamine ("norephedrine", NE) and pseudoephedrine (P) are included into the Monitoring Program and are not considered as Prohibited Substances.

These compounds are metabolically related. E is degraded to NE and P is degraded to NP following administration. It should be of interest therefore, whether presence of NE (not prohibited) is possibly due to illicit administration of E on the one hand, and whether presence of NP (prohibited) is possibly due to unprohibited administration of P. E and P are ingredients of over-the-counter drugs active against coryza, allergic coryza and common cold. NE and NP have to be prescribed. They represent the active ingredients in some anorectics.

An efficient method to tackle questions of source assignment is analysis of stable isotope ratios. In the field of doping control this is routinely performed on $^{13}C/^{12}C$-ratios of steroids. During metabolism of steroids the carbon backbone is not significantly modified. Consequently the carbon isotope signature of synthetic steroid hormones will be more or less conserved. This in turn facilitates the detection of illicit steroid administration. In regard to ephedrines, it can be deduced in advance that the situation will be quite different. In large part ephedrines are metabolized via N-demethlyation. On the one hand this means that one out of ten carbon atoms is lost. It can't be expected, that ^{13}C is distributed statistically in organic molecules, and therefore significant changes of the $^{13}C/^{12}C$-ratios in the resulting nor-compound may occur. On the other hand, N-demethylation is likely to be associated by large ^{15}N-isotope effects, which will effect changes of the $^{15}N/^{14}N$-ratio in both, substrate and metabolite. These phenomena may be exploited in order to tell, whether occurrence of demethylated ephedrines is due to metabolism or administration.

Methods

Authentic samples of ephedrines were obtained from several over-the-counter drugs and from laboratory standard materials. Authentic NP, which in Germany is classified as an illegal drug, was a gift from the analytical laboratory of the German Federal Police Department (BKA, Karlsruhe). Urine samples containing ephedrines and corresponding metabolites were obtained from archived reaccreditation tests. The tests were conducted in 2003 and 2004, and the corresponding pooled specimens were stored at -40°C. Moreover, patients who had administered ephedrines against common cold were asked to contribute urine samples.

Carbon and nitrogen stable isotope analysis of the authentic materials were performed either by elemental analysis/isotope ratio mass spectrometry (EA/IRMS) or by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), depending on the purity of the material. By all means, hydrochlorides were converted to the free bases in order to guarantee GC amenability.

Urine samples were prepared according to the usual protocol for volatile stimulants. Briefly, 5 mL urine were adjusted to pH 14, and basic compounds were extracted with tert-butyl methyl ether (TBME). The extraction is supported by adding ca. 3 g of dried sodium sulfate. As a slight modification of the standard procedure, the extracts finally were dried and redissolved in 50 µL TBME.

Carbon and nitrogen stable isotope analysis of urinary ephedrines was performed by GC/C/IRMS. The analytical device is described in [2]. Two runs are required for either carbon or nitrogen analysis. $^{15}N/^{14}N$-Analysis presumes some modification of the setup. Water and carbon dioxide formed during the combustion process were removed cryogenically. Presence of CO_2 in the ion source interferes with m/z 28 and 29 (N_2) by formation of $CO^{\cdot+}$. Nitrous oxides were converted to N_2 over elemental copper at 600°C.

The column was an Agilent HP-5MS (Agilent Techn., Waldbronn, Germany). The length was 30 m, the ID was 0.32 mm, and the film thickness was 0.5 µm. The solvent was removed during 90 s at 10°C at a gas flow of 50 mL/min. Sample transfer was achieved at 250°C during another 90 s, where the GC temperature was set to 35°C. The temperature program encompassed three ramps (40°/min to 135°C, 8°/min to 275°C, 40°/min to 300°C). After the solvent vent interval the carrier gas flow was adjusted to 1.5 mL/min.

Results and Discussion

Figure 1 shows the carbon and nitrogen stable isotope ratios of authentic ephedrines from a variety of sources.

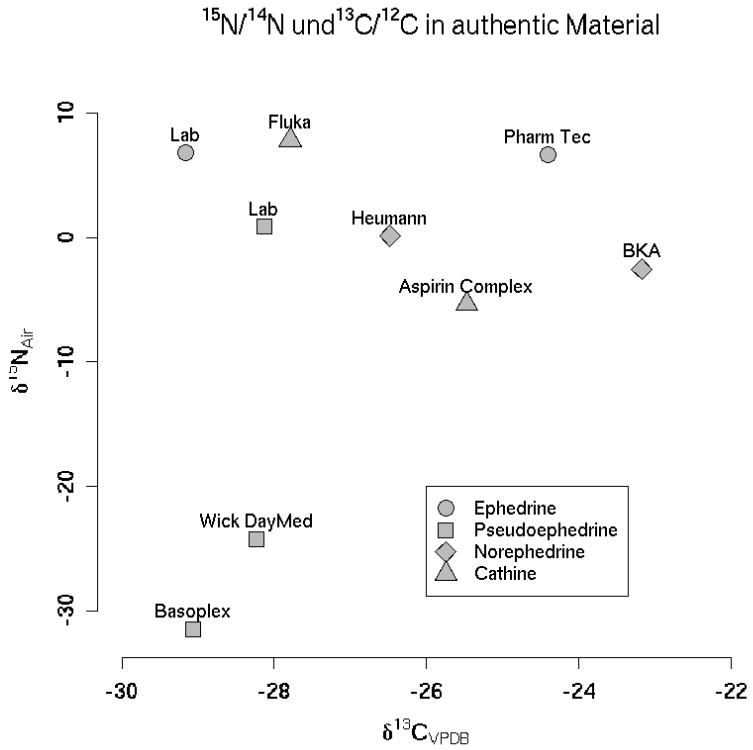

Figure 1: Carbon and nitrogen stable isotope ratios of authentic ephedrines

Both parameters exhibit large variation, which can be attributed to different sources of the raw materials and to the charcteristics of the repspective production processes. The distribution of the $^{15}N/^{14}N$-ratios exhibits an interesting pattern. $\delta^{15}N$-values lower than -5 per mil are unlikely to occur naturally [3]. It can be hypothesized therefore, that the observed distribution of the $\delta^{15}N$-values reflects either natural or synthetic origin of the respective compound.

The metabolism of E and P is dominated by N-demethylation [1]. However, the parent compound is much more abundant in urine tnan the metabolite. This allows some interesting conclusions concerning the change of the stable isotope ratios in these molecules. It is to be assumed, that due to kinetic isotope effects (KIE), the metabolite becomes ^{15}N depleted *vs.* the substrate. At the same time a similar effect is to be expected for ^{13}C in the methyl group. The latter however is lost, and therefore the effect can't be observed in the metabolite. In contrast

^{13}C preferably will stay in the substrate. Moreover, methyl groups tend to be ^{13}C -depleted [5], and consequently the loss of this moiety will result in even stronger ^{13}C -enrichment of the parent compound.

Because of the C/N-ratio of *Ephedra*-alkaloids (10:1), the observed isotope effect will be most pronounced for nitrogen. Changes in $^{13}C/^{12}C$ simply will be "diluted", as isotope ratios for whole molecules are measured.

Figure 2 shows stable isotope data (repeated measurements, mean ± std dev) from pooled urine samples collected during a P execretion study.

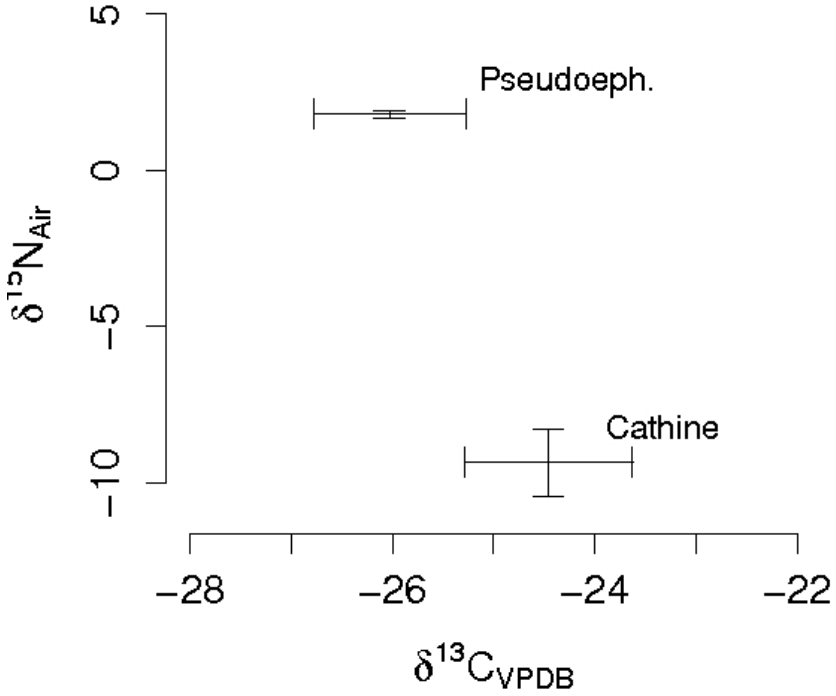

Figure 2: $\delta^{13}C$- and Figure 2: $\delta^{15}N$-values of pseudoephedrine and cathine in pooled urine samples after administration of pseudoephedrine

These data are in full accordance with theory. The metabolite (NP) is depleted in ^{15}N by ca. 10 per mil. At the same time the parent compound is enriched in ^{13}C by ca. 1.5 per mil. Although both compounds will change their isotope ratios over time, the difference is expected to remain more or less unchanged.

Figures 3 and 4 reveal that this is not strictly the case. These data were obtained from a

patient, who had applied E. As expected, NE is ^{13}C -enriched *vs.* E, where this parameter doesn't change significantly with time. The $\delta^{13}C$-values of the E however unexpectedly exhibit variation. A possible explanation can be given by consideration of metabolic pathways other than demethylation. In fact Sever *et al.* [4] observed degradation to benzoic acid, which is likely to be also associated by ^{13}C isotope effects. This hypothesis is supported by the $\delta^{13}C$ value of the pure drug, which contains less ^{13}C than urinary E.

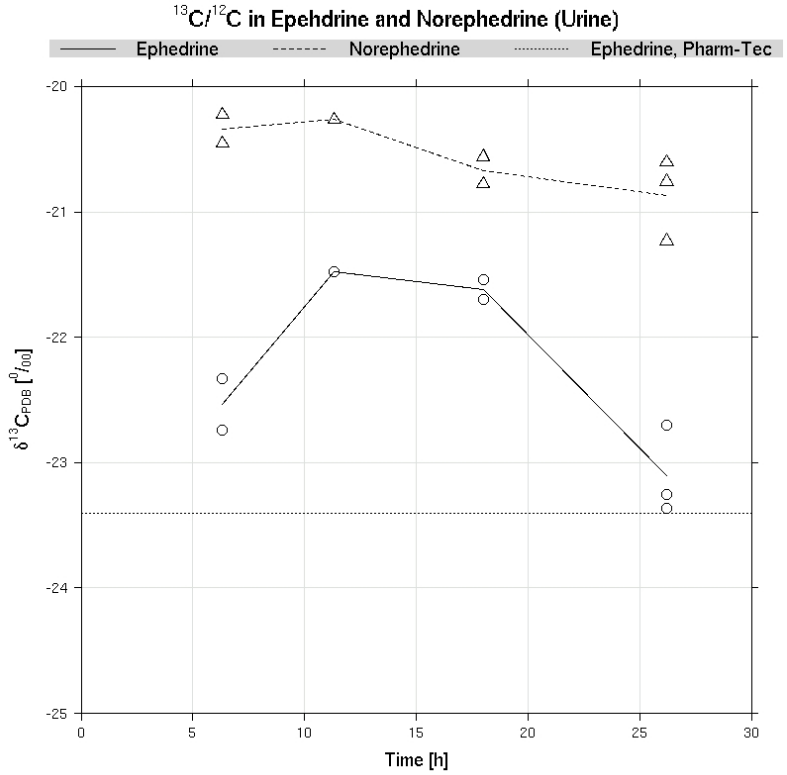

Figure 3: $\delta^{13}C$ -values of urinary ephedrine and norephedrine after application of ephedrine. The horizontal line indicates the $\delta^{13}C$ -value of the administered drug.

Figure 4 shows the corresponding $\delta^{15}N$-values. As is to be expected, there is a constant difference between the compounds, where the metabolite is enriched in the heavier isotope. However, theory predicts an increasing enrichment in ^{15}N for both compounds. The opposite is the case. This finding currently can not be explained, and requires further investigation. Nonetheless, as already suggested by the results from the pooled samples, if urinary NP or NE are supposed to be products of P or E respectively, it is to be postulated that there be a difference in $\delta^{15}N$ of ca. 10 per mil between the two compounds.

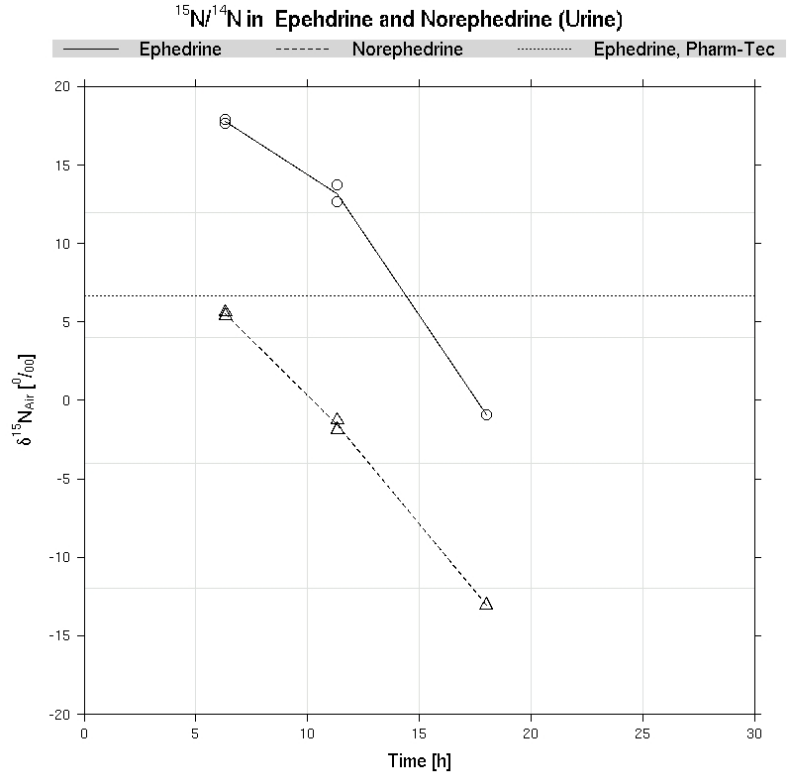

Figure 4: $\delta^{15}N$ -values of urinary ephedrine and norephedrine after application of ephedrine. The horizontal line indicates the $\delta^{15}N$-value of the administered drug.

Figure 5 shows $\delta^{13}C$-values from a patient who administered P. There is a more or less parallel trend in the values for P and NP, as is to be expected. However, there is considerable variation in the $\delta^{13}C$-values of NP, which is surprising. Moreover, the NP value temporarily falls below the value of the pure drug. Urinary P even stays lower in $^{13}C/^{12}C$ all the time. This can't be explained physiologically. Sooner it is doubtfull, whether the medicamentation is isotopically homogenous.

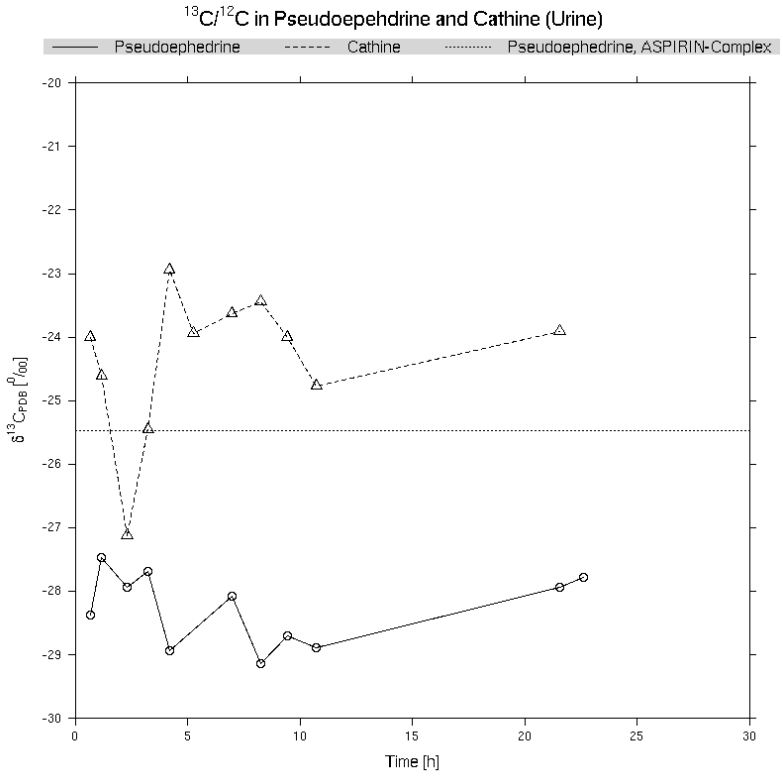

Figure 5: δ^{13}C-values of urinary pseudoephedrine and cathine after application of ephedrine. The horizontal line indicates the δ^{13}C-value of the administered drug.

Conclusions

The trends in the stable isotopic data of urinary ephedrines can be explained only with difficulties. Nonetheless there are general patterns, that are in accordance with theoretical considerations and which may help to elucidate the relationships between the methylated and demethylated compounds. If urinary NE or NP are present as metabolites, a strong ^{15}N-depletion and a small ^{13}C-enrichment will be observed compared to the parent compound. Due to the large variability of the stable isotope ratios of the pure drugs, it is very unlikely that this pattern will be observed, when both compounds have been administered separately. These findings however require confirmation by extended studies.

References

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