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Investigations on the deconjugation processes of conjugated urinary steroids during degradation with ^{18}O labelled water.

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Introduction

Recent investigations on the influence of urinary degradation on carbon isotope ratios (CIR) of endogenous steroids showed a strong shift in CIR of unconjugated steroids especially at the beginning of deconjugation.[1] The difference between conjugated steroids (gluco- and sulfo-conjugates) and free steroids was found to be more than 1‰. This finding was surprising as it is generally assumed that deconjugation occurs without involvement of the steroidal backbone. Both, glucuronides and sulfates should be cleaved under retention of the oxygen atom at the steroid (Figure 1). In contrast, a cleavage occurring between the steroid and the oxygen could help explaining the found isotopic fractionation accompanied by degradation.

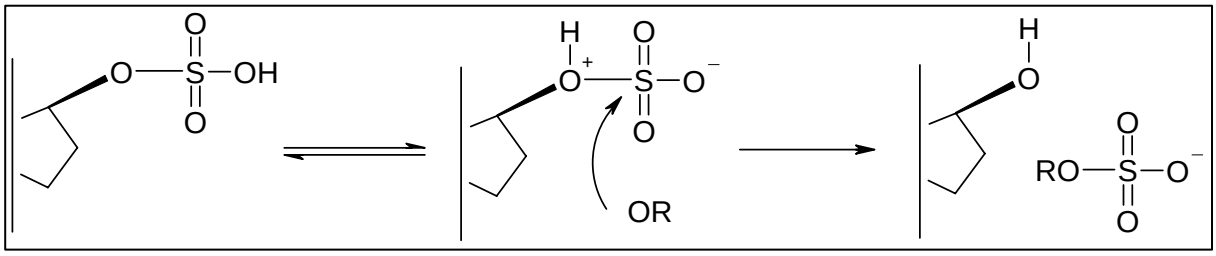

Figure 1: Proposed cleavage of a sulfo-conjugated steroid.[2] Glucuronidated steroids should behave the same.[3]

Experimental

To investigate a possible exchange of oxygen during degradation of urine samples, 4 blank urines were divided and one part was fortified with 0.25 mL of ^{18}O labelled water prior to degradation at 37°C. The following steroids have been investigated over the course of 3 months:

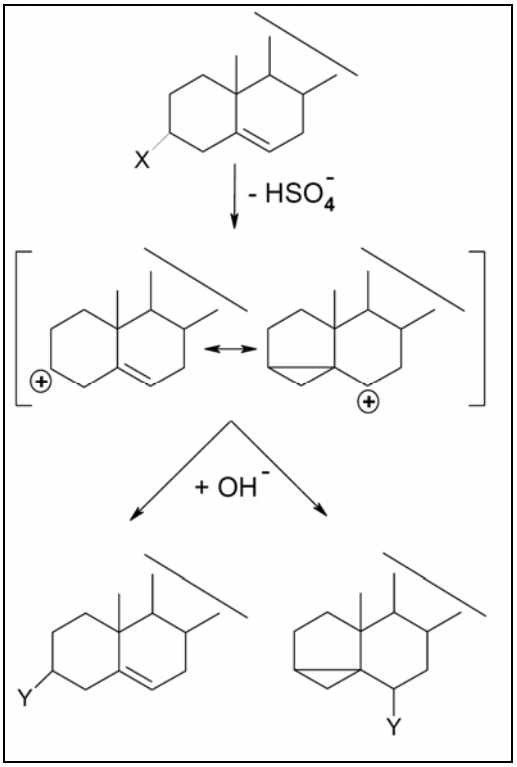

Figure 2: Proposed reaction mechanism for the formation of **DCM** and unconjugated **DHEA**.[1]

- 3α-hydroxy-5α-androstan-17-one (**A)**;
- 3α-hydroxy-5β-androstan-17-one (**E**);
- 3β-hydroxy-androst-5-en-17-one (**DHEA**)
- 3α,5-cyclo-5α-androstan-6β-ol-17-one (**DCM**)

These steroids have been chosen as in accordance with a prior ascertained reaction mechanism both **DHEA** and **DCM** should incorporate oxygen atoms from the surrounding water during degradation of sulfo-conjugated DHEA (Figure 2).[1]

E and A conjugates in contrast should be cleaved according to the above mentioned reaction mechanism (Figure 1) without incorporation of oxygen atoms.

Sample preparation

Two mL of urine were adjusted to pH 7 and extracted with 5 mL of *tert.*-butyl methyl ether. The organic layer was transferred and evaporated under a stream of nitrogen. The analytes were derivatized by means of trimethylsilylation and methyltestosterone was used as internal standard.[4]

GC-MS/MS method

All measurements were performed on an Agilent GC 7890 A coupled to an Agilent 7000B Triple Quad mass spectrometer. The GC column was a J&W Scientific Ultra I (OV-1) with 17 m length, 0.2 mm inner diameter and 0.11 µm film thickness. Injections (2.5 µl) were performed in split mode (1:10) at 300°C with a constant pressure of 14.6 psi. The oven temperature started at 185°C, raised to 234°C with 4°C/min, than with 40°C/min to 310°C and held for 2 min.

	^{18}O	^{16}O
DCM	434 → 419	432 → 417
DHEA	434 → 419	432 → 417
E	436 → 421	434 → 419
A	436 → 421	434 → 419

Table 1: Used ion transitions.

The mass spectrometer was operated in MRM mode with nitrogen as collision gas. The ion transitions are listed in Table 1 and the chromatographic resolution is exemplarily depicted in Figure 3:

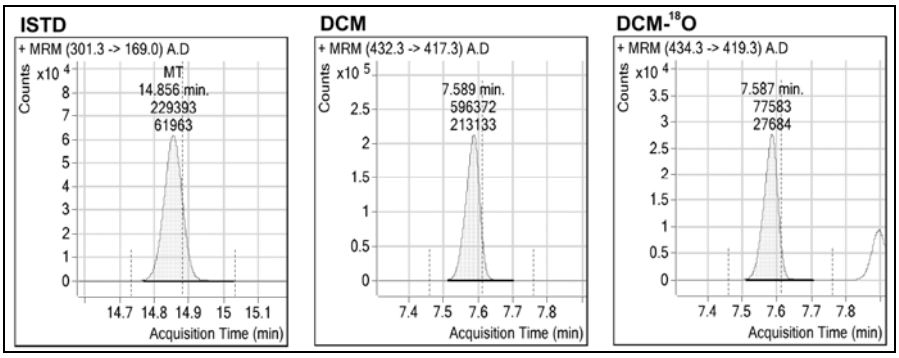

Figure 3: GC-MS/MS chromatograms of methyltestosterone (ISTD) and both ion transitions used for DCM.

Results and discussion

As in the former study,[1] a large inter-individual variability between the different urines was found. Two results are presented exemplarily in Figures 4 and 5. Depicted are the changes in the ratio of ^{18}O divided by ^{16}O over the course of time. The initial ratio was arbitrarily set to zero. Urine A (Figure 4) shows the expected trends for the different steroids. **E** and **A** are not influenced in their oxygen isotope ratio (OIR) and these scatter around the starting value. The large variation found in this sample is caused by the low amounts of unconjugated **E** an **A** liberated by degradation.

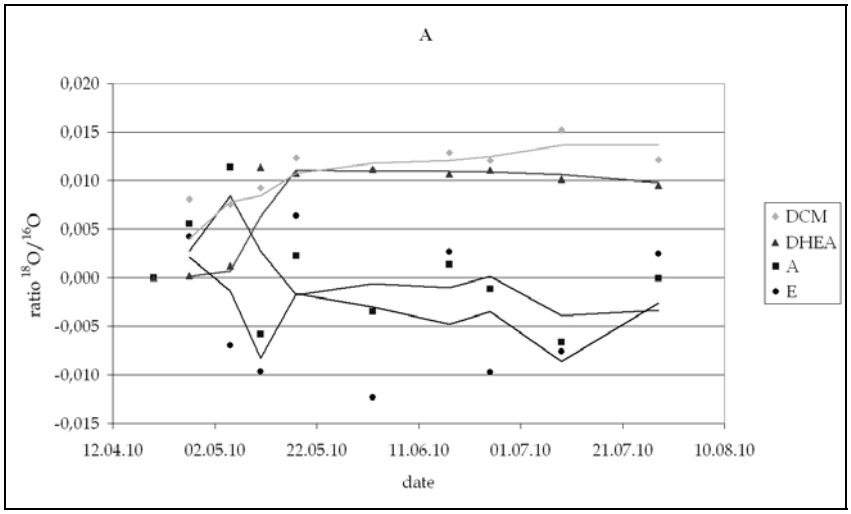

Figure 4: Changes in the ratios of $^{18}O/^{16}O$ over time in one male urine.

The OIR of **DHEA** and **DCM** increase during the study as ^{18}O from the urinary matrix is incorporated according to the reaction mechanism depicted in Figure 2.

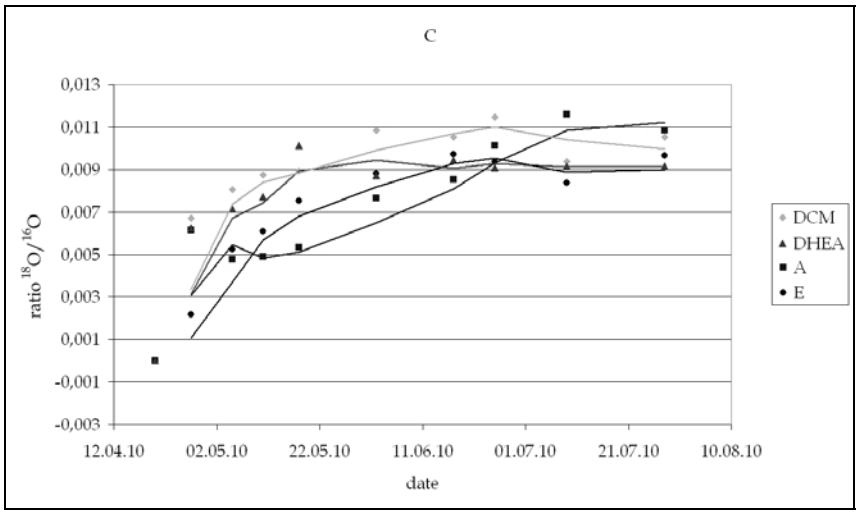

Figure 5: Changes in the ratios of $^{18}O/^{16}O$ over time in one female urine.

A different and unexpected behaviour is shown in Figure 5. During the degradation of urine C, all investigated steroids show a similar trend in their OIR. Especially in the beginning it is more pronounced for **DHEA** and **DCM**, but **E** and **A** are obviously influenced, too.

Three out of the 4 investigated urines showed this influence on **E** and **A**. The relative content of ^{18}O in the urines was elevated by approx. 20% due to the addition of labelled water. The largest relative change in OIR of **E** and **A** accounted for 13%, in one sample **DCM** changed for 22%.

Conclusion

During the processes of degradation it seems possible that the oxygen-carbon-bond is involved in the chemical or enzymatic cleaving reactions which might allow for an explanation of the found changes in CIR during degradation. Further investigations will be necessary to support these findings and enable a sound explanation of the isotopic fractionation taking place during deconjugation of steroids.

Literature

1) Piper T, Geyer H, Schänzer W. (2010) Degradation of urine samples and its influence on the $^{13}C/^{12}C$ ratios of excreted steroids. *Drug Test. Analysis* **11-12**, 620-629.
2) Burstein S, Lieberman S. (1958) Kinetics and Mechanism of Solvolysis of Steroid Hydrogen Sulfates. *J. Am. Chem. Soc.* **80**, 5235-5239.
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4) Donike M, Zimmermann J. (1980) Preparation of trimethylsilyl, triethylsilyl and tert-butyldimethylsilyl enol ethers from ketosteroids for investigations by gas-chromatography and mass-spectrometry *J. Chrom.* **202**, 483-486.