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## **Structural investigation of candidate proteins in urine samples with demethylation activity**

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### **Introduction**

It is a matter of fact that an in-situ formation of low levels of NA and NE from androsterone and etiocholanolone, respectively, exists. The transformation of deuterated androgens to the corresponding norsteroids after incubation was observed in selected samples which fulfil the criteria  $NA/NE < A/E$  as postulated in previous studies [1]. Recent studies have shown that microbial activity (investigated by bacteria PCR) is not correlated to artificial produced norsteroids [2]. The process of demethylation seems to be initiated by the presence of certain proteins with enzymatic activity. The aim of this study was to separate and to identify eligible proteins by investigation of urines with and without degradation characteristics.

### **Experimental**

Three blank urines without demethylation characteristic and four instable urines exhibiting values of NE higher than 0.5 ng/mL and fulfilling the criteria  $NA/NE < A/E$  were selected from the routine doping control. The content of proteins in urine of healthy humans is known to be very low. For this reason, urine proteins were initially separated by ultrafiltration (cut-off 10kDa). Subsequently, the filtrate (1mL) containing peptides with molecular weight about 5-10 kDa was concentrated by lyophilisation until the volume was reduced to 60-100 $\mu$ L. The enzymatic activity of demethylation proved to be preserved during this sample preparation. Sodium dodecyl sulfat-polyacrylamide gel electrophoresis analysis (SDS-PAGE) of the lyophilised urine extract (15 $\mu$ L) was performed on a 16% separating gel according to the protocol developed for the separation of small proteins in the molecular weight range from 1kDa to 10kDa [3]. The bands were visualized directly in the gel by silver staining [4]. The procedure is compatible with subsequent mass spectrometric analysis. For this purpose, the bands of interest were manually excised from the gel, destained and in-gel tryptic digestion was carried out. All samples were analysed by LC-MS-MS to obtain protein sequence information for identification by database searches (Mascot).

## Results

► Urinary proteins with a relevant weight from 5-10kDa were separated from all investigated samples. In comparison to the utilized protein molecular weight standard, the staining of separated urine peptides occurred with strong delay indicating a poor amount of them. Therefore, the prolonged impact time of the developing solution leads to unfavourably broad and deeply stained bands in case of the molecular weight control standards. The pattern of proteins in the mentioned molecular weight range seems to be very similar both in stable and instable urines. (Figure 1)

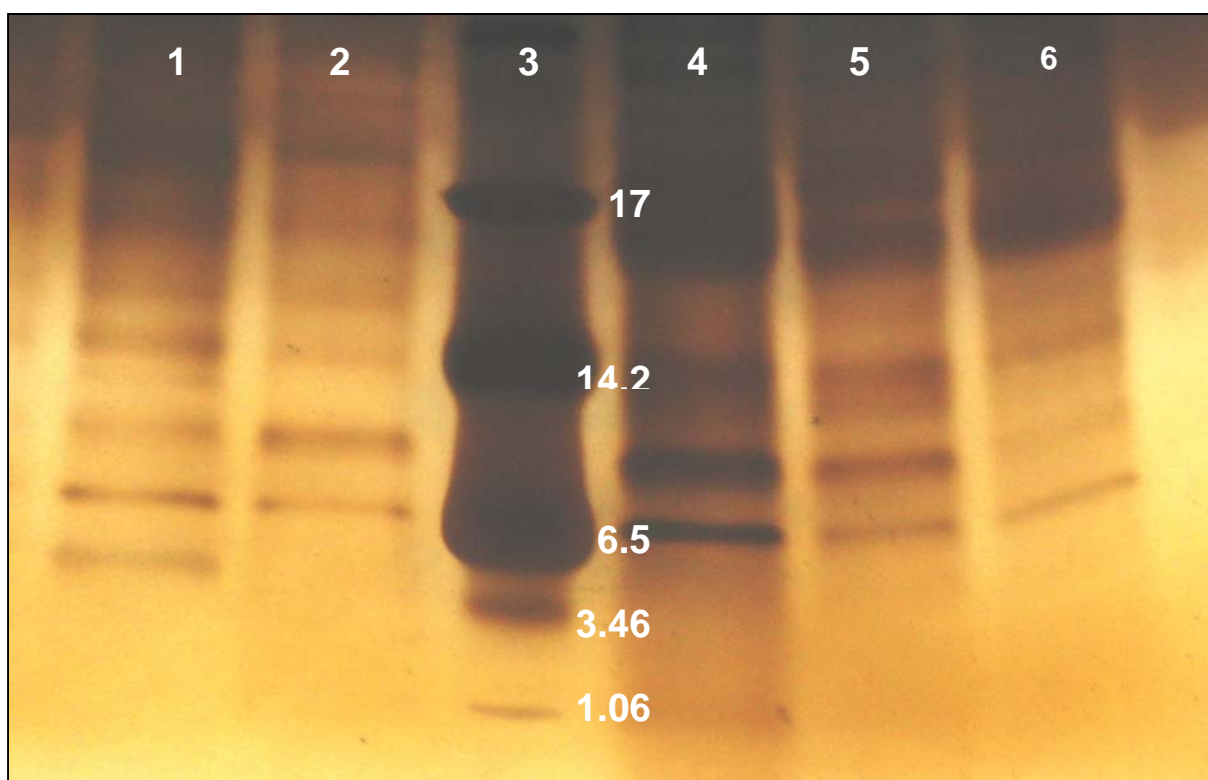


Figure 1. SDS-Page map of instable urines (lane 1, 2) and stable urines (lane 4,5,6); lane 3 ultra low molecular weight marker

► As a result from LC-MS-MS analyses and subsequent database searches six different proteins could be identified (see table below). Ubiquitin and Keratin are commonly excreted in urine of humans. Both compounds were observed in stable as well as instable samples indicating their insignificance for the demethylation activity. The presence of cystatin, psoriasin and sarcosin only in active urine samples does not explain the in-situ formation of norsteroids. Only sarcosin (N-methylglycine) is involved as methyl group acceptor in the cholin-betain metabolism. It is assumed that the formation of sarcosin is a sign for a higher methyl group transfer in the individual.

<i>protein</i>	<i>stable urine</i>	<i>instable urin</i>
ubiquitin	+	+
keratin	+	+
dermicidin	+	+
cystatin-A	-	+
psoriasin	-	+
sarcosin	-	+

Table 1. Identified proteins in stable versus instable urines (Mascot results)

### Conclusion

Several suitable proteins were detected in instable urines but none of them seems to be clearly associated with demethylation activity. Therefore, other co-factors potentially influencing or causing this reaction have to be taken into consideration.

### Reference

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