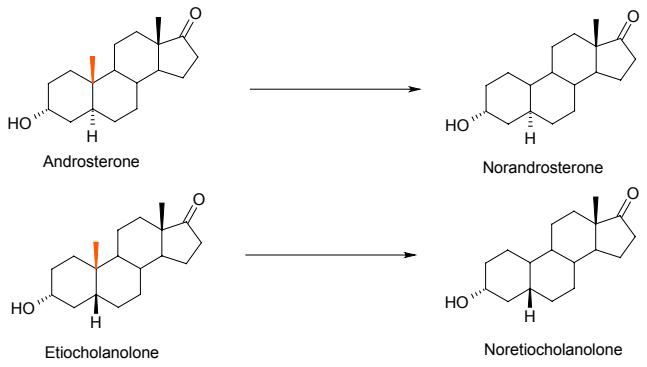
C. Rautenberg[1)], J. Grosse[1)], P. Anielski[1)], D. Thieme[2)], P. Hemmersbach[3)],
H.S. Lund[3)] and R.K. Mueller[1)]

Aspects of the in situ formation of 19-norsteroids

[1)] Institute of Doping Analysis and Sports Biochemistry, D-01731 Kreischa, Germany
[2)] Institute for Legal Medicine, D-80337 Munich, Germany
[3)] Hormone Laboratory, Section for Doping Analyses, Aker University Hospital Oslo, Norway

Introduction

Norandrosterone (NA) and noretiocholanolone (NE) are the two main urinary indicators to detect the misuse of nandrolone and/or related prohormones (e.g. norandrostenedione, norandrostenediol). Recent studies have shown the possibility of an in situ formation of low levels of NA and NE originating from androsterone and etiocholanolone, respectively. [1].



The goal of this study was to investigate some aspects of this in-situ formation. For this purpose a larger number of samples was investigated to obtain more information about factors potentially influencing or causing this process, e.g. storage conditions.

Experimental

In total 30 A samples and the corresponding B samples exhibiting screening values of NE higher than 0.5 ng/mL and fulfilling the criterion $NA/NE < A/E$ (A=androsterone, E=etiocholanonolone) were selected based on the routine doping control. Two aliquots of each sample were prepared, one control without addition, and one with addition of deuterated substrates. For this purpose the urine samples were spiked with androsterone-d4 and etiocholanone-d5 at concentrations comparable to the endogenous level of their non-

deuterated analogues (4µg/mL, solution in methanol was evaporated before 1 mL urine was added) and incubated at 37 °C for 20 hours. Sample preparation was carried out both with and without enzymatic hydrolysis (β-glucuronidase from *E. coli*) after purification by solid phase extraction (XAD-2; 100-200 µm) to differentiate between unconjugated and conjugated fraction. The solution was adjusted to pH 9 ($NaHCO_3$ / KCO_3) and extracted with n-pentane/ methanol (24:1). After evaporation to dryness the samples were derivatised with a mixture of MSTFA/ ammonium iodide/ n-propanethiole.

Epitestosterone-d3 and nortestosterone-d3 were utilised as internal standards.

All samples were analysed by GC-HRMS to detect the norsteroids NA, NE as well as NA-d4 and NE-d5 formed as result of the demethylation from androsterone-d4 and etiocholanolone-d5, respectively. The measuremenst were carried out in SIM modus and focused on following ions: 405.2645 (NA and NE); 409.2896 (NA-d4); 410.2959 (NE-d5); 435.3068 (epitestosterone-d3); 421.2912 (nortestosterone-d3)

Results

► The formation of the norsteroids NA-d4 and NE-d5 after incubation was observed in 27 from a total of 30 selected samples. As a consequence of this activity the concentrations of NA and NE increased during the incubation, accordingly. Differences between A and B samples were determined in several cases.

The demethylation of androgens occurred both in A and B sample in about 43 % of the suspicious specimens. In 47% of the samples exhibiting the feature of active urines the transformation could only be proved in the B samples. The corresponding A samples were completely inactive. No degradation effect at all was observed in 10% of the samples although the selection criteria were fulfilled (*see Figure 1*).

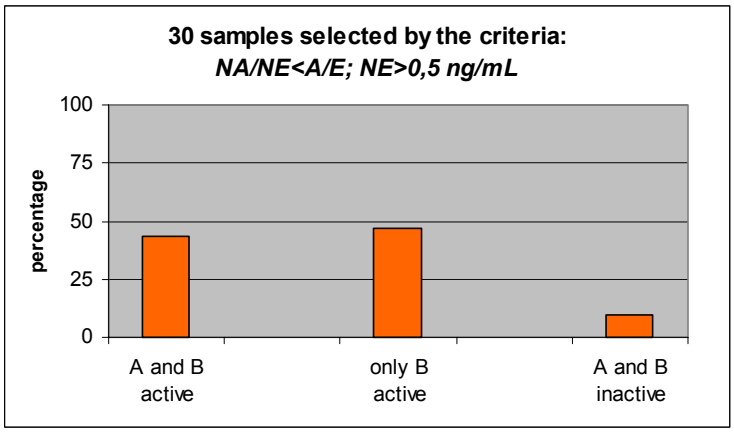


Fig. 1: Occurrence of demethylation activity in selected samples

In some cases (*Table 1*; No. 2 and 3) the inactivity may be due to the fact, that the ratio A/E was not significantly higher than NA/NE. However, the conversion did not always occur even though the selection criteria were fulfilled. In this case (*Table 1*; No. 1) other reasons have to be taken into consideration.

Table 1: Relative amounts of the5α- and 5β–isomers of inactive samples

No.	lab code	sample	NA	NE	NA/NE	A/E	NA-d4	NE-d5
1	41347	A	0,2	0,5	0,4	1,4	0	0
		B	0,2	0,3	0,6		0	0
2	42141	A	0	1,3	0	0,15	0	0
		B	0	0,9	0		0	0
3	42956	A	2,5	4	0,6	0,6	0	0
		B	2	4,9	0,4		0	0

► Incubation of several suspicious samples was repeated within a time interval of about 6 to 9 weeks after arrival in the lab (*Table 2*). The in-situ formation of deuterated norsteroids could only be confirmed in the first incubation of A samples carried out close to the arrival. A subsequent incubation with the corresponding B samples reconfirmed the conversion reaction.

Table 2: Results of incubation depending on storage conditions (A samples stored at5°C; B samples frozen at -20°C)

No.	lab code	sample	NA	NE	A/E	NA-d4	NE-d5	date of incubation after arrival in the lab
1	42488	A	2,2	6,2	0,7	0,6	0,8	3 weeks
		A	2,3	8,2		0	0	8 weeks
		B	0,6	2,3		0,6	0,9	5 months
2	42939	A	1,7	1,9	0,6	0,2	0,2	3 weeks
		A	2	1,9		0	0	9 weeks
		B	1,7	1,8		0,4	0,6	5 months
3	43347	A	1,2	1,6	1,8	0,8	1,4	2 weeks
		A	1,8	2,2		0	0	6 weeks
		B	0,5	0,7		0,7	1,4	5 months

Conclusions

► The suspicion of an existing demethylation activity was confirmed in about 90% of all samples characterised by the condition *NA/NE<A/E*. It has to be pointed out that in the cases of inactivity of the A samples the transformation reaction reoccured in the corresponding B samples, indicating that the degradation activity might change, diminish, or even disappear depending on the storage conditions and presumably associated factors.

► A re-incubation of three A samples supported this fact. In these repeated experiments no formation of 19-norsteroids was observed at all and the urine specimens seem to have lost

their activity within 4 weeks after the first incubation. During this time the samples were stored at 5 °C. In contrast to this, the activity of all B samples (frozen at - 20 °C after arrival in the lab) was preserved over a period of several months.

Reference

[1] J. Grosse, P. Anielski, P. Hemmersbach, H. Lund, R.K. Mueller, C. Rautenberg, D. Thieme: Formation of 19-norsteroids by in situ demethylation of endogenous steroids in stored urine samples. Steroids 2005; 70: 499-506

Acknowledgement

This project was supported by WADA and the Federal Institute of Sports Science (Bonn, Germany, VF 0414/042/2004-2006).