Determination of the Origin of Urinary Noranadrosterone-Traces by Gas Chromatography Combustion Isotope Ratio Mass Spectrometry

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Introduction

The steroid 19-norandrosterone (19NA) is an ongoing problem in doping control: On the one hand, it is, besides 19-noretiocholanolone (19NE), the most abundant metabolite of the potent synthetic anabolic steroid 19-nortestosterone (19NT)[1]. On the other hand, small amounts are biosynthesized by pregnant women and further evidence exists for physiological origin of this compound. Therefore, the World Anti Doping Agency (WADA) introduced a threshold concentration of 2 ng 19NA per ml urine to discriminate 19NT abuse from biosynthetic origin[2].

Recent findings show however, that formation of 19NA resulting in concentrations above the threshold level might be due to demethylation of androsterone (A) in urine. This steroid is excreted by healthy persons in milligram amounts per day[3].

To avoid false positive results, the origin of urinary 19NA in doping control samples could be elucidated by $^{13}C/^{12}C$-analysis. Synthetic 19NT is derived from C_3-plants by partial synthesis and shows $\delta^{13}C_{VPDB}$-values of around -28‰. Endogenous steroids are less depleted in ^{13}C due to a dietary mixture of C_3- and C_4-plants.

Comparing $\delta^{13}C_{VPDB}$-values of A as endogenous reference compound (ERC) with19NA should reveal unambiguously the origin of urinary NA.

Samples

11 urine samples containing 19NA from pregnant women and from excretaion studies with different prohormones were analyzed as control samples as well as blank urines spiked with 19NA-glucuronide in different concentrations. 25 19NA-containing samples from routine analysis were then analyzed.

Measurement

$^{13}C/^{12}C$-analysis

Injection	Splitless mode; manually up to 3 µl out of at least 5 µl methanol
GC	HP 5890 II Optima δ3 (Macherey/Nagel); 20 m x 0.25 mm ID; film thickness 0.25 µm
C	GC Combustion Interface II (Finnigan MAT) oxidation reactor (CuO) at 940°C
IRMS	Delta C (Finnigan MAT)

GC/MS

A similarly equipped GC/MS was used to check each sample for coelutions

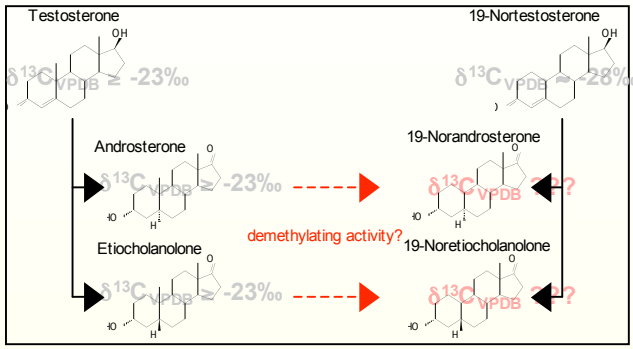

Fig. 1: metabolism of selected steroids and illustration of expected $\delta^{13}C_{VPDB}$-values

Sample Preparation

After solid phase extraction of 10 ml urine, steroid glucuronides were hydrolyzed enzymatically and separated at alkaline pH with tert-butyl methyl ether. A semipreparative normal phase HPLC on a $(CH_3)_2$N-propyl column was applied for purification to separate 19NA and A from E. 19NA and A were further purified and separated by HPLC on C_{18}-material. GC/C/IRMS-measurements were performed without derivatization

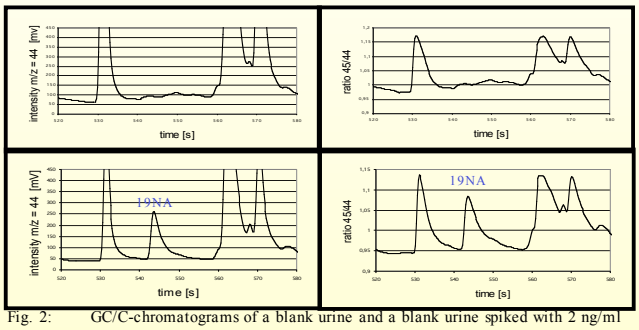

Fig. 2: GC/C-chromatograms of a blank urine and a blank urine spiked with 2 ng/ml 19NA

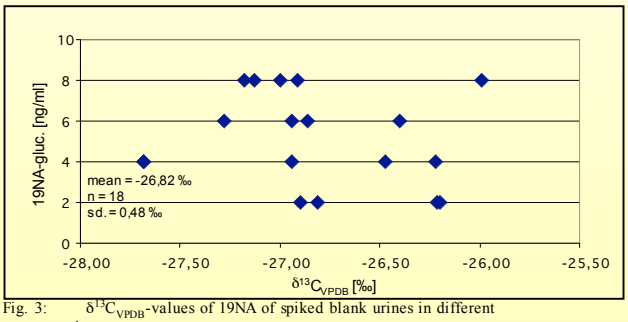

Fig. 3: $\delta^{13}C_{VPDB}$-values of 19NA of spiked blank urines in different concentrations

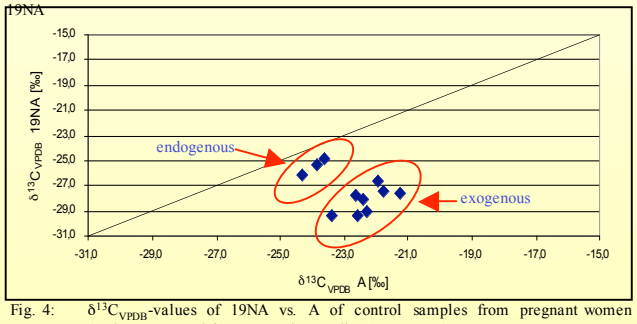

Fig. 4: $\delta^{13}C_{VPDB}$-values of 19NA vs. A of control samples from pregnant women (endogenous) and from excretion studies (exogenous)

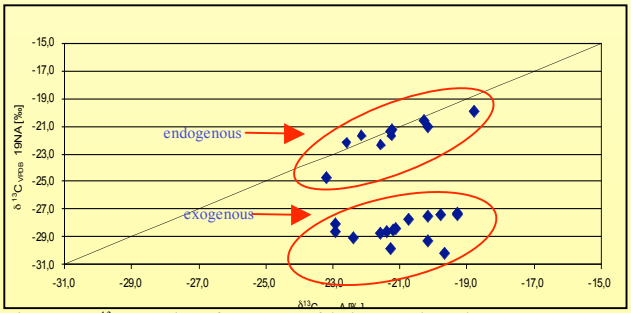

Fig. 5: $\delta^{13}C_{VPDB}$-values of 19NA vs. A of doping control samples

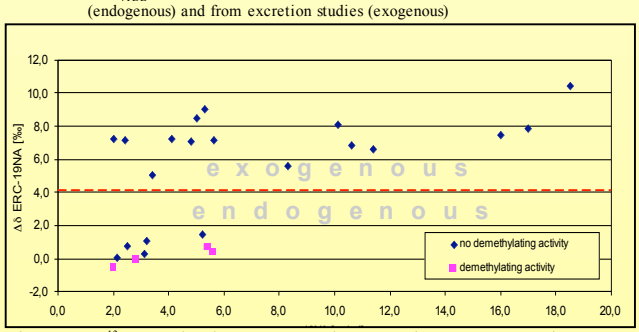

Fig. 6: $\Delta\delta^{13}C_{VPDB}$-values between ERC and 19NA vs. the 19NA-concentration

Results

Fig. 2 shows GC/C-chromatograms of a blank urine (top) and a 19NA-spiked urine (bottom). Especially the ratio 45/44 indicates, that no confounding coelution is present. All samples were also checked for coelutions by GC/MS with the same result. **Fig. 3** indicates the precision of the $^{13}C/^{12}C$-analysis which is acceptable for concentrations down to 2 ng 19NA per ml urine. **Fig. 4** shows $\delta^{13}C_{VPDB}$-values of 19NA vs. A of control samples. Endogenous values derive from urine of pregnant women whereas exogenous values are from urine of excretion studies with prohormones. The data obtained allow to discriminate steroid abuse from biosynthetic origin. **Fig. 5** is similar to fig. 4, but 19NA-containing doping control samples are presented. Normally, A was the ERC; in three cases, 11OH-A was used as reference compound because the δ-value of A was influenced by the abuse of other hormones. Obtained data reveals steroid abuse unambiguously. **Fig. 6** shows the Δδ-values of 19NA and ERC of the same samples vs. the concentration of NA. Demethylating activity is marked, if present as well as a heuristic threshold value.

Conclusions

Data of urine samples are presented with a 19NA concentration of more than 5 ng/ml which don't show (any more) demethylating activity but endogenous $\delta^{13}C_{VPDB}$-values.

To avoid false positive results in doping control, IRMS is obligate to distinguish between doping or not for 19NA-traces.

References:

[1] Schänzer, W., et al., *Metabolism of Nortestosterone, Norandrostenedione and Norandrostenediol. Identification of 3a-hydrosyestr-4-en-17-one glucuronide and 3a,16a-dihydroxy-5a-estran-17-one glucuronide and sulphate*, in *Recent Advances in Doping Analysis. Proceedings of the 12th Cologne Workshop on Dope Analysis, 10th to 15th April 1994*, W. Schänzer, et al., Editors. 2001, Sportund Buch Strauß: Klön. p. 155-174.

[2] WADA Laboratory Committee. *Reporting Norandrosterone Findings. Technical Document TD2004NA, World Anti Doping Agency*: 2004.

[3] Thieme, D., et al., *Kinetics of in-situ demethylation of endogenous steroids in urine samples.*, in *Recent Advances in Doping Analysis. Proceedings of the Manfred Donike Workshop. 22nd Cologne Workshop on Dope Analysis, 7th to 12th March 2004*, W. Schänzer, et al., Editors. 2005, Sportund Buch Strauß: Köln. p. 177-188.