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Mission (im)possible? Gas chromatography/combustion/isotope ratio mass spectrometry of trace amounts of urinary norandrosterone

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Abstract

The origin of urinary 19-norandrosterone (NA) was investigated. In a first experiment, demethylating activity in some of NA-containing urine samples could be detected. Adding deuterated androgens such as $^{2}H_{5}$-androsterone (D_5-A) to suspicious urine samples resulted in the 19-nor analogue (D_5-NA). In a second study, an extensive cleanup-procedure was developed which allows the measurement of the $^{13}C/^{12}C$-ratios of urinary NA by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). It is based on two high performance liquid chromatography (HPLC) cleanup steps. With the first normal phase HPLC step, NA and androsterone (A) are separated from etiocholanolone (E). The second reversed phase HPLC cleanup of the NA-containing fraction separates NA from A. GC/C/IRMS of all three fractions delivers carbon ratios of the above mentioned analytes. Samples from excretion studies and doping control samples containing NA in low concentrations (< 10 ng/ml) were analysed. By comparing the $^{13}C/^{12}C$-ratios of NA, A and Etiocholanolone, the origin of NA in doping control samples could be elucidated as either endogenous or exogenous.

Introduction

Anabolic steroids have been prohibited for use in sport by the International Olympic Committee (IOC) since the olympic games in 1976. Nandrolone is known as an anabolic agent since the 1930s[1] and is mainly metabolized to conjugates of 19-norandrosterone (NA) and 19-noretiocholanolone (NE)[2]. The World Anti Doping Agency (WADA) Prohibited List 2005 sets a limit of 2 ng NA per ml urine for „adverse analytical findings“[3]. The question, whether urinary 19-norsteroids like NA might be of endogenous origin has been discussed

extensively. A review of this discussion was published by Bricout and Wright[4]. Recent findings show, that besides the possible sources discussed (exercise, contaminated nutritional supplements, intermediates during the biological synthesis of estrogens etc.), a demethylating activity in urine can be responsible for amounts of up to ca. 2 ng NA per ml urine: At the 22nd Cologne Workshop on Dope Analysis in March 2004, the doping control laboratories of Kreischa and Oslo reported, that some suspicious doping control samples (low amounts of NA, enhanced density, high concentrations of androsterone (A) and etiocholanolone (E)) were able to transform parts of A, E or their glucuronic acid conjugates into the corresponding 19-norsteroids[5]. To avoid false positive results in doping control, it is a pressing task to determine the origin of NA in urine, thus to decide, if it is a metabolite of exogenous administration of nandrolone or a related substance or the result of a transformation from A into NA.

As urine may lose the demethylation-activity due to e.g. storage conditions or an intake of nandrolone in addition to the active urine is possible, a single activity-test of suspicious samples can't be sufficient to demonstrate the origin of 19-norsteroids in urine. To differentiate between endogenous and synthesized naturally occuring steroids, the method of choice is gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS) which was presented for doping control purposes in 1994[6]: Synthesized steroids are normally made from *Dioscorea spp*. or soy[7], thus C_3-plants which are depleted in ^{13}C in contrast to C_4-plants. In consequence, synthesized steroids and their metabolites are also depleted in ^{13}C in contrast to endogenous steroids which derive from the diet. The challenge is to obtain $^{13}C/^{12}C$-ratios (expressed as $\delta^{13}C_{VPDB}$-values) from NA in urine at very low concentrations: $^{13}C/^{12}C$-ratios down to 2 ng/ml should be confirmed whereas the limit of detection for GC/C/IRMS-systems is about 10 ng NA per injection. Thus at least 10 ml of urine has to be cleaned up with almost no losses and due to the relatively high biological background, the cleanup has to be very thorough as baseline separation on the GC is required for reliable $\delta^{13}C_{VPDB}$-values. Hence, a cleanup-method for 10 ml urine was developed which allows reliable $^{13}C/^{12}C$-ratios of urinary NA down to 3 ng/ml urine or even lower to be obtained. The $\delta^{13}C_{VPDB}$-values can be compared with those from E and A which are cleaned up at the same time.

Experimental

Chemicals and standards

Methanol (puriss., distilled before use) (MeOH) and tert-butyl methyl ether (distilled before use) (TBME) were purchased from KMF Laborchemie Handels GmbH (St. Augustin, Germany), *n*-hexane (gradient grade for liquid chromatography), isopropanol (p.a.) (IPA), cyclohexane (gradient grade for liquid chromatography) (CHX), potassium carbonate (p.a.), potassium hydrogencarbonate (p.a.), ammonium iodide and sodium phosphate (p.a.) from Merck (Darmstadt, Germany), β-Glucuronidase from E.coli from Roche Diagnostics (Mannheim, Germany), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Macherey-Nagel (Düren, Germany) (MN), ethanthiol (EtSH) from Fluka (Switzerland) and acetonitrile (gradient grade for liquid chromatography) (AcN) from JT Baker (Deventer, Holland). The steroids A and E were purchased from Sigma-Aldrich (Steinheim, Germany) and the reference standard 5α-androstane-3α,17β–diacetate from Steraloids (Newport, USA). NA, $^{2}H_4$-norandrosterone (D_4-NA), NE, $^{2}H_4$-etiocholanolone (D_4-E) and $^{2}H_5$-androsterone (D_5-A) were synthezised in our laboratory[8], [9].

Methods

Activity test

According to Thieme *et al.*[5], aliquots of 0,5 ml of urine samples were spiked with D_4-E and/or D_5-A, 5000 ng each. After incubation at 37°C for 15 hours in a water bath, a liquid-liquid extraction was carried out using 5 ml of TBME as extracting agent after adjusting the pH value to 9.6 by adding 250 µl of a 20% solution of K_2CO_3 and $KHCO_3$. After shaking for 5 min and centrifugation for 5 min at 1200 g, the ether layer was decanted and dried on a vacuum rotating evaporator. The derivatisation was performed for 1 hour at 50°C using 50 µl of a mixture of MSTFA:ammonium iodide:EtSH = 1000:2:3 (v/w/v).

All analyses were carried out on a high resolution mass spectrometer MAT 95 (Thermo Finnigan, Bremen, Germany) after chromatographic separation on an HP6890 gas-chromatograph (Hewlett & Packard, now Agilent Technologies, Palo Alto, USA). An HP-Ultra 1 column (17 m, 0.20 mm ID, 0.11 µm film thickness, crosslinked methyl silicone) was used at a constant pressure of 13 psi. The following temperature (T) program was applied: 185°C, 5°C/min to 240°C, 20°C/min to 310°C. Injection of 2 µl was performed in

split mode (split 1:20) using a CTC A 200 autosampler (CTC Analytics, Zwingen, Switzerland). A mass spectrometric resolution of 5000 was set for all HRMS experiments.The following ion traces were monitored: m/z 405.2645 (M^+ -15) and m/z 420.2879 (M^+) for NA-bis-trimethylsilylether (TMS) and NE-bis-TMS; m/z 409.2896 (M^+ -15) and m/z 424,3130 (M^+) for D_4-NA-bis-TMS and D_4-NE-bis-TMS; m/z 410.2958 (M^+ -15) and m/z 425.3192 (M^+) for D_5-NA-bis-TMS. The identification of D_5-NA and D_4-E was performed by comparison of the retention times and fragmentation pattern with the reference steroids NA, NE and D_4-NA (all synthesized in our laboratory).

IRMS-method

The method comprises the following steps:

1. Reversed phase solid phase extraction (RP SPE)
2. Enzymatic hydrolysis of glucuronides
3. Liquid/liquid extraction (LLE)
4. 1st normal phase high performance liquid chromatography (NP-HPLC) purification on a dimethylaminopropyl column: Separation in two fractions
5. 2nd RP-HPLC purification on a C_{18} column: Separation of one fraction in two subfractions
6. GC/C/IRMS
7. Interpretation of results

sample preparation

1. Reversed Phase SPE:

The RP SPE cartridges from MN (Chromabond C_{18}, 500 mg, 6 ml) were conditioned with first 2 ml MeOH, then 2 ml H_2O. 10 ml of urine was placed on the column and washed with 2 ml H_2O. Elution was performed with 2 ml MeOH.

2. Enzymatic hydrolysis of glucuronides:

The dried eluate was dissolved in 1 ml sodium phosphate buffer (0,2 m, pH 7). 50 µl β-glucuronidase was added and incubated 1h at 50°C.

3. Liquid Liquid Extraction:

250 µl carbonate buffer (200 g/l, K_2CO_3/$KHCO_3$ = 1/1) was added and extracted with 5 ml TBME. The organic layer was transferred into a conical test tube and evaporated to dryness. This extract was transferred with two washings of 100 µl MeOH to an HPLC-Vial with micro-insert and dried in a desiccator.

4. *1st NP-HPLC on a dimethylaminopropyl column from MN: EC 250/4 Nucleosil 100-5 $N(CH_3)_2$ + referring pre-column*

The aim of the first HPLC is to separate NA and A together from E and necessary cleanup for GC/C/IRMS. To estimate the retention times and the collection pattern for NA, A and E, a mixed standard of each 5 µg in 50 µl *n*-hexane/IPA = 90/10 (v/v) was injected into an Agilent 1100 HPLC-system, equipped with degasser, quaternary pump, autosampler, column-oven and UV-detector. The mobile phase was 96% *n*-hexane and 4% IPA for the first 15 min. followed by 9 min. column-washing with 80% IPA and 20% *n*-hexane. Reconditioning of the HPLC column was achieved by washing 9 min isocratically with the initial mixture. The temperature was set to 50°C, the flow rate was 1 ml/min and detection took place at 200 nm. After dissolving the extracts in 50 µl *n*-hexane/IPA = 90/10 and injection of 50 µl into HPLC, fractions were collected with an automatic fraction collector (Foxy 200) from Isco (Lincoln, Nebraska, USA). After drying the sample-fractions, the NA + A-fraction was transferred with twice 100 µl MeOH into another HPLC-vial with micro-insert and dried again. The E-fraction was clean enough for GC/C/IRMS (conditions see below).

5. *2nd RP-HPLC on a C_{18} column: LiChroCART 250-4 LiChrospher 100 RP_{18} EC (5 µm) + pre-column: LiChroCart 25-4 LiChrospher 100 RP_{18} (5 µm), both from Merck*

The aims of the second HPLC are to separate NA from A and to eliminate further matrix-compounds with regard to the low amounts of NA in urine. To estimate the retention times and the collection pattern for A and NA, a mixed standard of each 5 µg in 50 µl MeOH was injected into the above mentioned Agilent 1100 HPLC-system. The mobile phase was a mixture of H_2O and AcN starting with 30% AcN increasing to 100% within 20 min. Reconditioning of the HPLC column was achieved by washing 5 min isocratically with the initial mixture. The temperature was set to 24°C, the flow rate was 1 ml/min, detection took place at 200 nm. After dissolving the NA + A-fraction in 50 µl MeOH and injection into HPLC, NA and A were seperated from each other according to the above estimated collection pattern. After drying both fractions, cleanup is sufficient for GC/C/IRMS.

6. *GC/C/IRMS*

Measurement of $^{13}C/^{12}C$-ratios was performed by GC/C/IRMS. A GC 5890 II (Hewlett & Packard, now Agilent Technologies) was coupled to a Delta C gas isotope ratio mass spectrometer (ThermoFinnigan) by a combustion interface II (ThermoFinnigan). The GC column was a Optima δ3 (MN). Dimensions of the column were 17 m length and 0.25 mm inner diameter. The film thickness was 0.25 µm. A retention gap (deactivated fused

silica, 2 m length, 0.32 mm inner diameter) was connected to the analytical column to improve splitless conditions. Helium (purity 5.0 ~ 99.999%) was the carrier gas at a constant pressure of 30 psi.

Up to 3 µl from at least 5 µl of the sample dissolved in MeOH were injected in splitless mode (splitless time 1:30 min) at 300°C. The initial oven temperature was set to 60°C for 1:30 min followed by an increase of 30°C/min up to 265°C. A second ramp of 3°C/min followed during which separation of the relevant compounds was achieved. The final temperature of 295°C was held for 2 min.

Samples usually were injected manually. In some cases an autosampler (A200S, CTC Analytics) could be employed. It was operated at an injection speed of 5.5 µl/s.

An isotopically characterized reference standard (5α-androstane-3α,17β-diol-diacetate) was co-injected (0.5 µl, 100 µg/ml) to control chromatographic conditions and validity of the calculated isotope ratios. $^{13}C/^{12}C$-ratios are expressed as $\delta^{13}C_{VPDB}$, where the working standard (carbon dioxide, $\delta^{13}C_{VPDB}$ = -3.0‰) was calibrated *vs.* an *n*-alkane mixture[10].

Results and discussion

Validity of the cleanup

Fig. 1 shows a GC/C-chromatogram of the relevant time period of the NA-fraction of a purified blank urine. The left panel shows the intensitiy of m/z = 44: Almost no biological components coelute in the relevant time period for NA between the first two peaks. The right panel of fig. 1 shows the ratio of the masses 45/44 (with an offset of each 100 mV for 45 and 44): The ratio is quite constant within the relevant time period which is also promising for NA-containing samples.

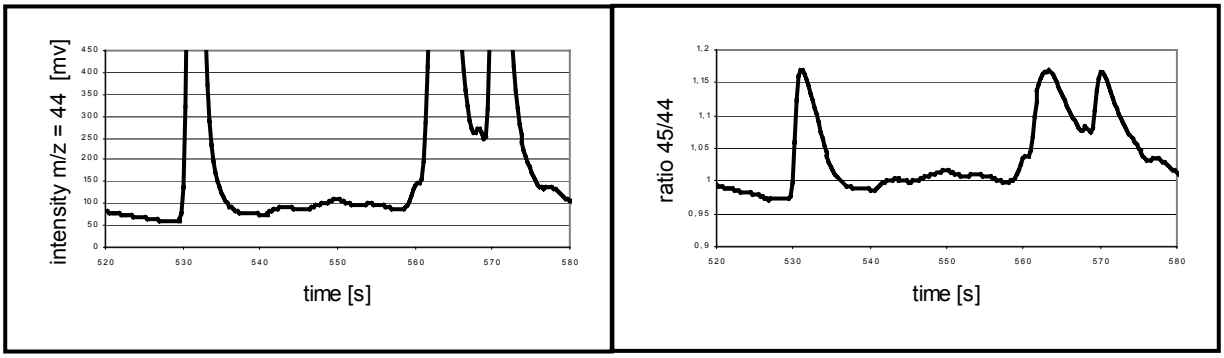

Fig. 1: GC/C-chromatogram of the relevant time period of the NA-fraction of a blank urine; intensity m/z = 44 on the left and the corresponding ratio 45/44 on the right

Fig. 2 is similar to fig. 1, but a urine was analysed which was spiked with 4 ng NA per ml as glucuronic acid conjugate. The response (left panel) is quite qood and the ratio 45/44 (right panel) indicates, that no relevant coelutions are present which might be covered by NA.

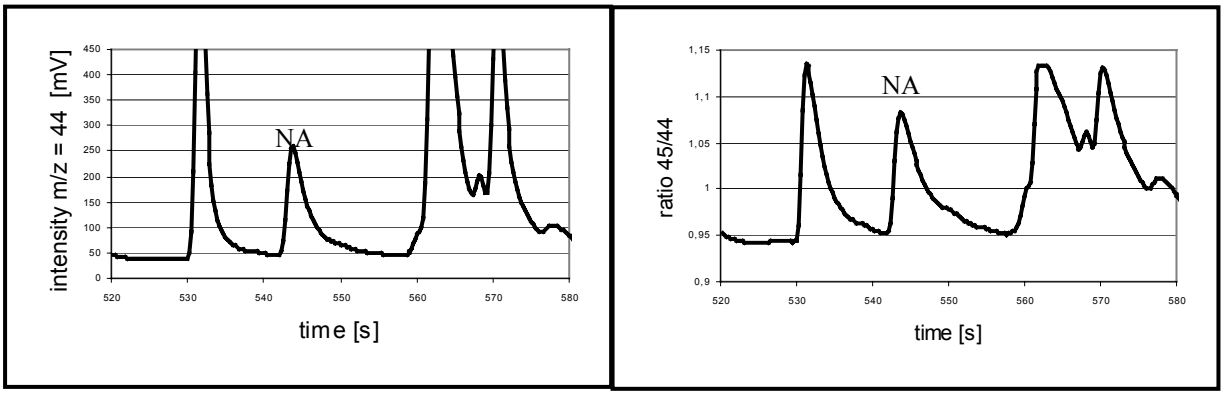

Fig. 2: GC/C-chromatogram of the relevant time period of the NA-fraction of a urine sample spiked with 4ng NA/ml; intensitiy m/z = 44 on the left and the corresponding ratio 45/44 on the right

To further check for coelutions, the samples were also injected into a GC/MS which was equipped similar to the GC/C/IRMS. Until now, no urine showed disturbing coelutions in the relevant time period. Fig. 3 shows, that the obtained δ-values of NA in NA-glucuronide spiked urine samples are quite constant for different concentrations with a total standard deviation of 0,48 ‰ (n=18) although the response of the 2 ng/ml urine was sometimes close to the limit of the dynamic linear range of the IRMS.

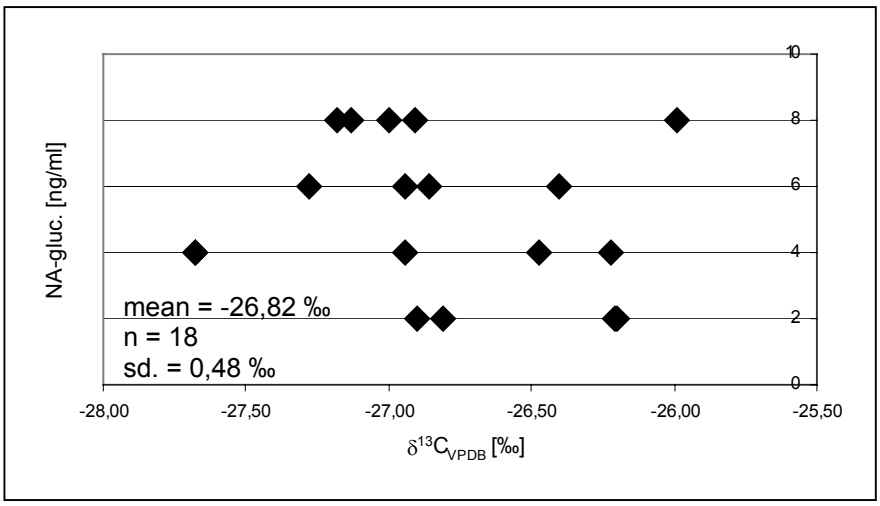

Fig. 3: $\delta^{13}C_{VPDB}$-values of NA of NA-glucuronide spiked urine specimens

Fig. 4 shows $\delta^{13}C_{VPDB}$-values of control samples. Theoretical identity of both parameters is indicated. The exogenous values derive from urine samples from excretion studies with different prohormones whereas the endogenous values close to the identity line are from urine samples of pregnant women. The two groups are clearly separated from each other as NA of

exogenous origin is much more depleted in the ^{13}C-amount than NA of endogenous origin. So the difference in the carbon isotope ratios between NA and A is much larger, if the source of NA is of exogenous origin and the method was promising for doping control samples.

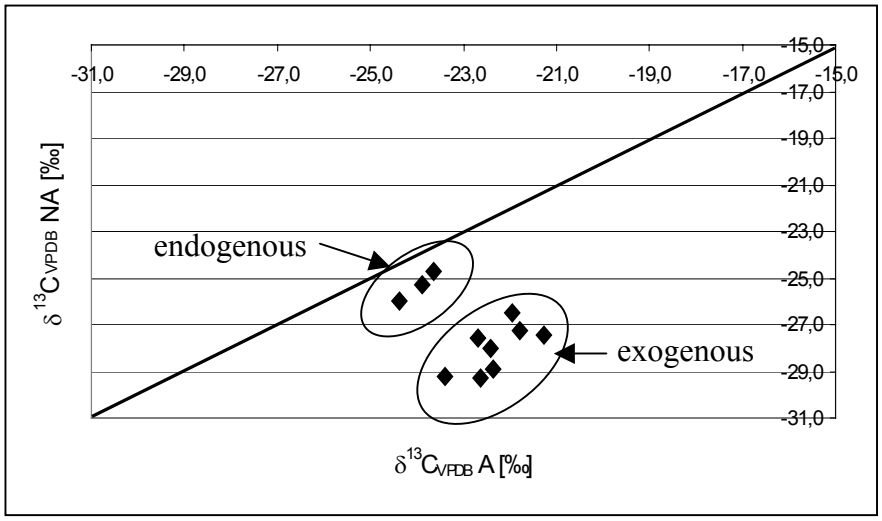

Fig. 4: $\delta^{13}C_{VPDB}$-values of NA vs. A of quality control samples

Analysis of doping control samples

This method was used to analyse 25 NA-containing doping control samples. Fig. 5 shows the $\delta^{13}C_{VPDB}$-values of NA vs. the selected endogenous reference compound (ERC) and a bisector. With exception of two samples where the δ-values of A were influenced by the use of prohormones of Testosterone, A was used as ERC. For the other two samples, $\delta^{13}C_{VPDB}$-values of 11-OH-A as ERC were used, obtained by routine analysis. Again, two groups can be identified. In the first group, δ-values scatter randomly around the bisector and the differences between the δ-values of NA and ERC are smaller than 2 ‰. In these samples, NA is obviously of endogenous origin. The other group shows differences in the δ-values between NA and ERC larger than 4 ‰ and up to more than 10 ‰. δ-values of this group clearly show, that NA is of exogenous origin.

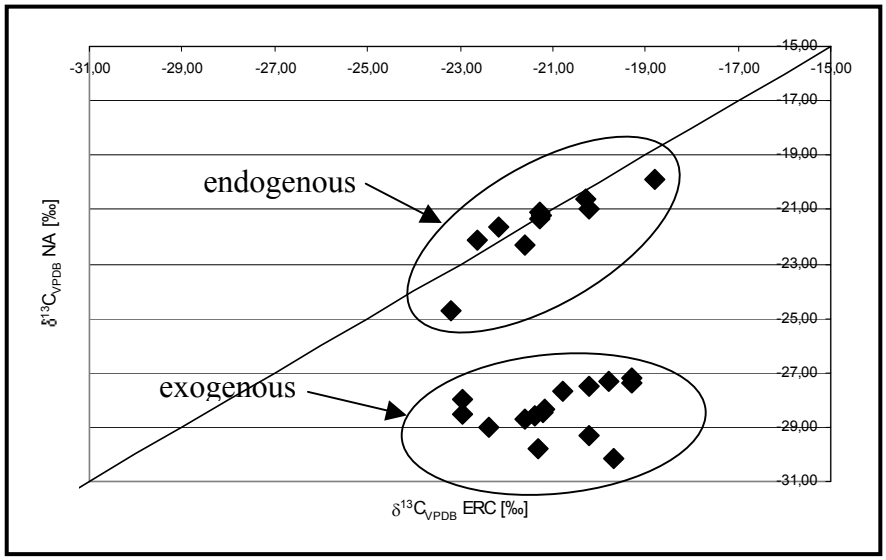

Fig. 5: $\delta^{13}C_{VPDB}$-values of NA vs. ERC of doping control samples

Fig. 6 shows the Δδ-values between NA and ERC vs. the concentration of NA in urine, corrected for specific gravity if necessary of the same samples as in fig. 5. The specimens indicated by circles did not show demethylating activity whereas the 5 samples indicated by crosses (2 samples at 2 ng/ml on top of each other) were active. The ones above the dotted line at about 3.8 ‰ can be considered as „positive“ whereas the samples below this line showed endogenous δ-values for NA. Only 5 of the 10 „negative“ samples showed demethylating activity. The other 5 urine specimens may have lost its activity due to storage conditions. The highest endogenous concentration was 5.6 ng/ml urine for an active urine and 5.2 ng/ml urine for a not active urine. Exogenous $\delta^{13}C_{VPDB}$-values of NA were identified over the whole concentration range.

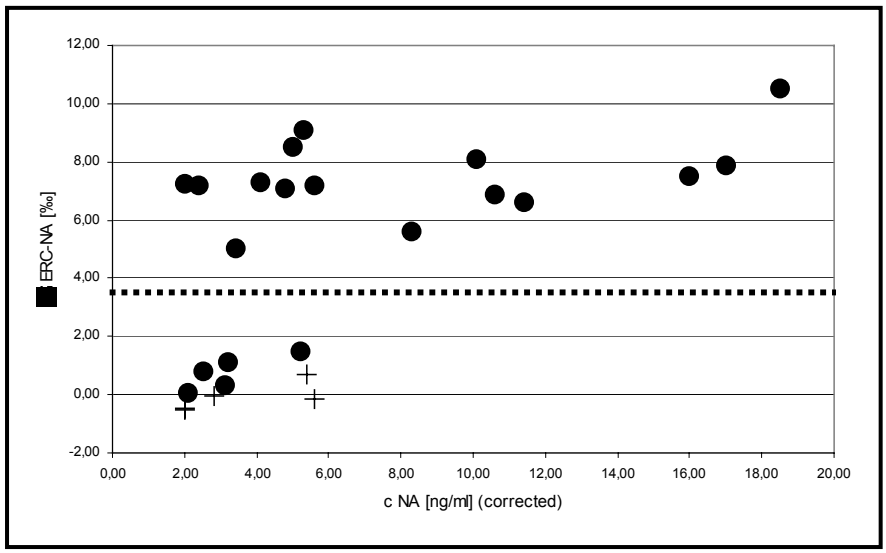

Fig. 6: Δδ-values between NA and ERC vs. the concentration of NA in urine

Summary and conclusion

As known before, urinary NA-traces can be of endogenous origin. A method based on two HPLC cleanup steps was developed to prove an abuse with nandrolone or other 19-norsteroids by GC/C/IRMS. The method can obtain $\delta^{13}C_{VPDB}$-values of NA down to a concentration of 3 ng/ml urine or less. Data of doping control samples are presented with a NA-concentration of up to 5.2 ng/ml urine which did not show demethylating activity, but the carbon isotope ratio of NA showed endogenous values. So quantification combined with a check for a demethylating activity can not prove a doping offence unequivocally. IRMS is necessary to distinguish between doping or not for NA.

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