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Kinetic of in-situ demethylation of deuterated endogenous steroids in urine samples

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Abstract

The appearance of traces of 19-norsteroids in routine doping control samples is a well established phenomenon. In a selection of suspicious urine samples, the relevance of 19-demethylation could be confirmed by incubation of the samples with corresponding deuterated steroids. The identity of the formed steroids (e.g. 19 noretiocholanolone-d5) was confirmed according to required criteria.

Kinetic data (i.e. time, concentration and temperature dependence as well as stereo specificity) of demethylation were examined. The time course of steroid conversion, which is naturally subjected to inter-individual variations, was found to obey a first order kinetics.

In-situ demethylation was observed in free steroids as well as for conjugates (the latter resulting in intact 19-norsteroid-glucuronides). Urine samples may be partially protected by the addition of stabilizing agents (EDTA, sodium azide).

1. Introduction

The appearance of suspicious signals indicating the presence of low amounts of norandrosterone (NA) and noretiocholanolone (NE) in doping control urine samples (fig. 1) is well described. Various potential origins of this phenomenon are documented e.g. the endogenous production in women during pregnancy or in the ovulation period of the menstrual cycle (1, 2), endogenous production in men (3) and contamination of meat or nutrition supplement (4, 5). The examination of correlations between low concentrations of norandrosterone and physical exercise led to contradictory results (6, 7).

Norandrosterone glucuronide was described to be stable under various storage conditions (8), and screening results can usually be confirmed in retained aliquots or B-samples regardless their different storage conditions.

To discriminate between low endogenous levels from exogenous application, cut-off values of 2 ng/mL NA for males and 5 ng/mL (modified to 2 ng/mL in 2004) for females were defined (9). Recommendations to correlate NA concentrations with of endogenous steroid levels, e.g. (androsterone (A)+etiocholanolone (E)) / NA of 1000 (male) or 500 (female) were proposed (10).

This study deals with the in-situ formation of NA and NE in stored urine samples, which was suggested according to an apparent accumulation of NA and NE in certain batches after longer transportation times. The objective of this study was to identify suspicious samples, to simulate and study the eventual conversion of endogenous steroids by incubation of these samples with corresponding deuterated standards (A-d4, E-d5).

2. Experimental

2.1. Sample material

Suspicious urine samples with low screening concentrations of NA or NE (>0.5 ng/mL) were included in this incubation study. Sample characteristics are listed in Table 1, samples were typically characterised by an elevated specific gravity and high endogenous steroid concentrations (androsterone and etiocholanolone > 1µg/mL). Transportation times range from 2-7 days, pH values did not show the typical deviations of steroid concentrations indicating bacterial contamination. The statistical significance of the kinetic studies is limited by the restricted amount of sample material.

2.2. Incubation

The urine samples were spiked with either a mixture of A-d4 and E-d5 or A-d4 glucuronide (substrate concentrations ranged from 1 to 4 µg/mL) and stored up to 120 hours at room temperature (23 ± 2 °C) to simulate the transportation period or 12 h at 37°C (accumulation of urine in the bladder).

2.3. Material and reagents

All urine samples were pre-cleaned by a solid phase extraction procedure using amberlite XAD-2 resin (100 - 200 micron, Serva). After loading 2.5 mL of urine, the columns were

washed twice with water and eluted with methanol. After evaporation the extracts were reconstituted with phosphate buffer (KH_2PO_4 (0.07M) + Na_2HPO_4 (0.07M) (170 : 70 v/v) pH 6.5) and hydrolysed using 20 µL of β-glucuronidase from Escherichia coli (Roche, Germany) at 50 °C (3 h). Subsequently, liquid-liquid extraction of the samples was carried out using 2*2 mL of n-pentane + methanol (Merck, reagent grade, 24:1 v/v) as extracting agent after adjusting the pH value to 9 by adding 250 mg of a solid buffer ($NaHCO_3$ (KMF, Germany) + K_2CO_3 (Merck, Germany, both reagent grade 84:138 w/w)). The residues were dried in a nitrogen stream and derivatised using 40 µL of a mixture of N-methyl-trimethylsilyl-trifluoro-acetamide (MSTFA GC-grade, Macherey & Nagel, Germany) + ammonium iodide (analytical grade, Merck, Germany) + n-propanethiol (reagent grade, Merck) (5 mL + 25 mg + 10 µL) at 55 °C for 30 min. Certified steroid reference standards (NA, NE, epitestosterone-d3 (eT-d3), NA-d4, NE-d5, NT-d3, A-d4, E-d5 and A-d4 glucuronide were purchased from NARL, Australia. NE-d3 was obtained from Radian, USA.

2.4. Instrumental analyses

All analyses were carried out on a high resolution mass spectrometer AutoSpec M (MicroMass, Great Britain) after chromatographic separation on an HP5980 gas chromatograph (Hewlett Packard, USA). An HP-5MS column (12.5 m, 0.20 mm ID, 0.33 µm film thickness, crosslinked 5% phenylmethyl silicone) was used at a helium flow of approximately 1.0 mL/min. The following temperature gradient was applied: 150°C (0.5 min), then at 12.5°C/min to 315°C. Injection (1µL) was in splitless mode using a CTC 200 autosampler (CTC Analytics, Switzerland). A mass spectrometric resolution of 10 000 was applied for all HRMS experiments, low resolution full scan data were recorded at a resolution of 1.000.

3. Results and discussion

3.1. Formation of 19-norandrosterone and 19-noretiocholanolone

The first incubation experiment (after 12 hours, 37°C) using the suspicious urine sample #4 showed clearly the formation of NA-d4 and NE-d5 (fig. 2). The identity of the deuterated steroids was confirmed by comparison of retention times and ion ratios with corresponding reference standard materials (e.g. NA-d4, NE-d3). Moreover, a full scan mass spectrum of NE-d5, in good accordance with the NE mass spectrum, could be obtained. The

identification of additional amounts of NE-d4 is due to the presence of E-d4 in the deuterated standard.

3.2. Conversion kinetics

The influence of concentration and temperature dependence on the formation of NA-d4 and NE-d5 is shown in fig. 4 and 5. A predominant formation of the 5β isomer was observed in each experiment. The ratio NE-d5/NA-d4 ranged from 2 to 10 at identical substrate concentrations. The final norsteroid concentrations were approximately proportional to the amount of incubated deuterated steroids (examined in a range between 1 and 4µg/mL, fig. 4). The time course of the reactions corresponded to a exponential kinetics. Half of the maximal saturation concentrations were typically produced in the time frame of 20-45 hours.

3.3. Conjugation

The formation of NA appeared to be independent of the conjugation of the substrate (fig. 6). After incubation of A-d4 glucuronide, the formation of intact NA-d4 glucuronide was observed. The relative amount of free NA-d4 in the sample was about 1.3% of the glucuronide, the kinetic parameters of free and conjugated steroids do not show a significant difference. The conversion rates of the A-d4 glucuronide appeared to be about 50% higher, compared to the free A-d4. However the experiments were not fully compatible, because the incubation of free steroids included a mixture of A-d4 and E-d5, 4µg/mL each, while the glucuronide experiment was carried out with 4µg/mL A-d4 glucuronide.

3.4. Stabilization

Attempts to suppress the formation of 19-norsteroids by addition of either EDTA or sodium azide were partially successful. The conversion rate of steroids dropped to 22% (NA-d4) and 25% (NE-d5) after treatment with EDTA and to 59% (NA-d4) and 79% (NE-d5) after application of NaN_3 , each compared to the untreated urine samples. The incubation was carried out at default conditions (12h at 37 °C).

3.5. Microbial characterisation

There was no evidence of unusual bacterial activity in the respective samples. Steroid profiles did not show significant deviations which are typical for bacterial contaminations, attempts of a microbacterial characterisation of one of the most potent urine samples (table 1, sample 4) did not show any positive result. A general PCR screening (technical details are published

elsewhere (11)) for bacteria in 50 routine doping control samples indicated a positive response in 16 samples. Two of the active urines were included in this study and did not contain any bacterial DNA.

3.6. Estimation of uncertainty

The identity of deuterated NA and NE could be confirmed according to conventional identification criteria. These norsteroids were produced in selected incubated urine samples, while no formation was observed in corresponding blank (i.e. not incubated with deuterated androsterone) or in incubated but inactive urine samples. The conversion reactions show a reasonable time courses and are temperature and concentration dependent.

However, a remaining qualitative uncertainty is due to the excess amounts of substrate compared to the target analyte. The observed formation of NA, NE and NA-glucuronide could be theoretically due to any appropriate reaction of a contaminant of the deuterated reference standard. The presence of a suitable group of impurities 5β-estrane-3α,17ξ-diol-d5, which could easily form NE-d5 by oxidation of the 17-hydroxy group, could however be excluded.

Moreover, there are additional analytical restrictions for these hypothetical impurities. The respective amounts of the impurity should be comparable in all standards but the structure must be fully specific with respect to

- degree of deuteration (d4 in A-d4, d5 in E-d5)
- conformation (5α in A-d4, 5β in E-d5) and
- conjugation (glucuronide in A-gluc-d4, free in A-d4)

which appears to be an unlikely (but not impossible) option.

The accuracy of quantitative results may be impeded by disturbation of the internal standards by other conversion products of the substrate. Epitestosterone-d3 had to be replaced as internal standard due to its co-elution with 5α-androstanedione-d3, formed as side product in the incubation studies. However, similar effects may lead to a certain systematic suppression of NA and NE concentrations.

4. Conclusions

There is clear evidence, that deuterated 19-NA and (or) 19-NE is formed in stored urine samples after incubation of the samples with A-d4 and (or) E-d5. The identity of the respective 19-norsteroids was confirmed by retention times and 3 diagnostic fragment ions

(mostly monitored as selected ion recording in high resolution MS, full scan mass spectrum in one case). Time, substrate concentration and temperature dependence of the norsteroid formation in incubation experiments provide evidence of an in-situ synthesis of the norsteroids in active urine samples.

Even under extreme conditions (6 days storage at 37 °C) the conversion of A-d4 to NA-d4 did not exceed a relative amount of 0.1 %, formation rates of NE-d5 were always significantly (2-10 times) higher than NA-d4.

The possibility of a formation of NA in stored samples should be considered,

- if the relative amount is lower than 1 per 1000 of androsterone (mandatory regulations should be adopted),
- quantitative discrepancy between individual analyses occur (in particular an elevation of NE with increasing time of storage) or
- the relative amount of NE/E is significantly higher than NA/A.

Duration and conditions (storage temperature) of urine samples transportation should be optimised, a partial stabilization could be achieved by addition of metabolic inhibitors (e.g. EDTA).

The incubation of such specimens with A-d4 or E-d5 and an identification of the formation of the corresponding deuterated 19-norsteroids could be helpful in those cases.

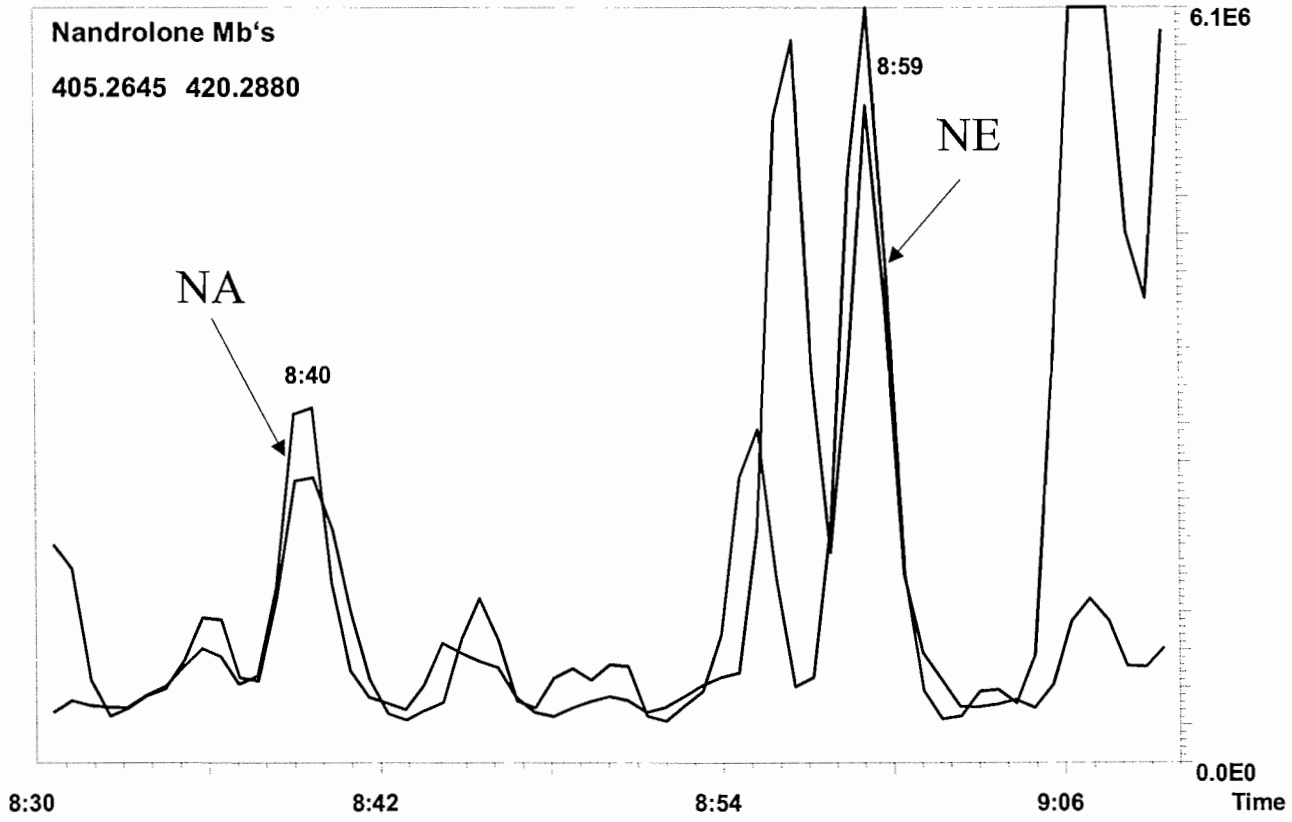

Figure 1: Trace amounts of norandrosterone and noretiocholanolone are occationally detected in doping control urine samples (eg. Sample #4 according to table 1). NE (1.2 ng/mL) exceeded the concentration of NA (0.5 ng/mL) significantly, which was a typical observation in the majority of cases.

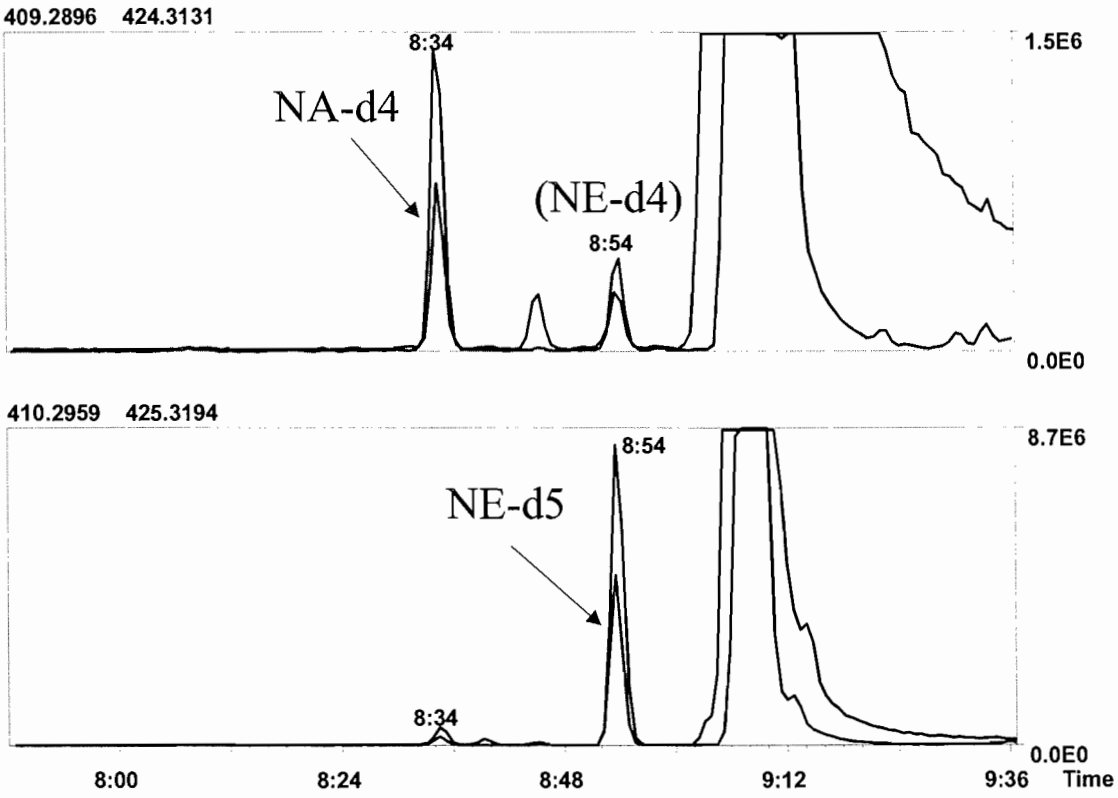

Figure 2: Detection of NA-d4 and NE-d5 in urine sample #4 (table1, fig.1) after incubation with androsterone-d4 and etiocholanolone-d5.

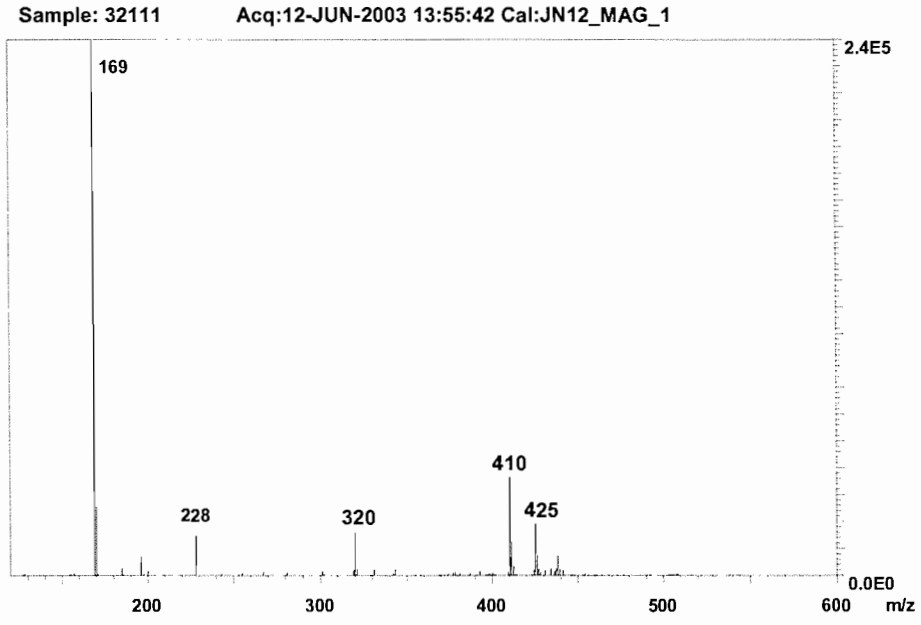

Figure 3: Full scan mass spectrum of NE-d5 in the urine sample #4, formed after incubation with etiocholanolone-d5.

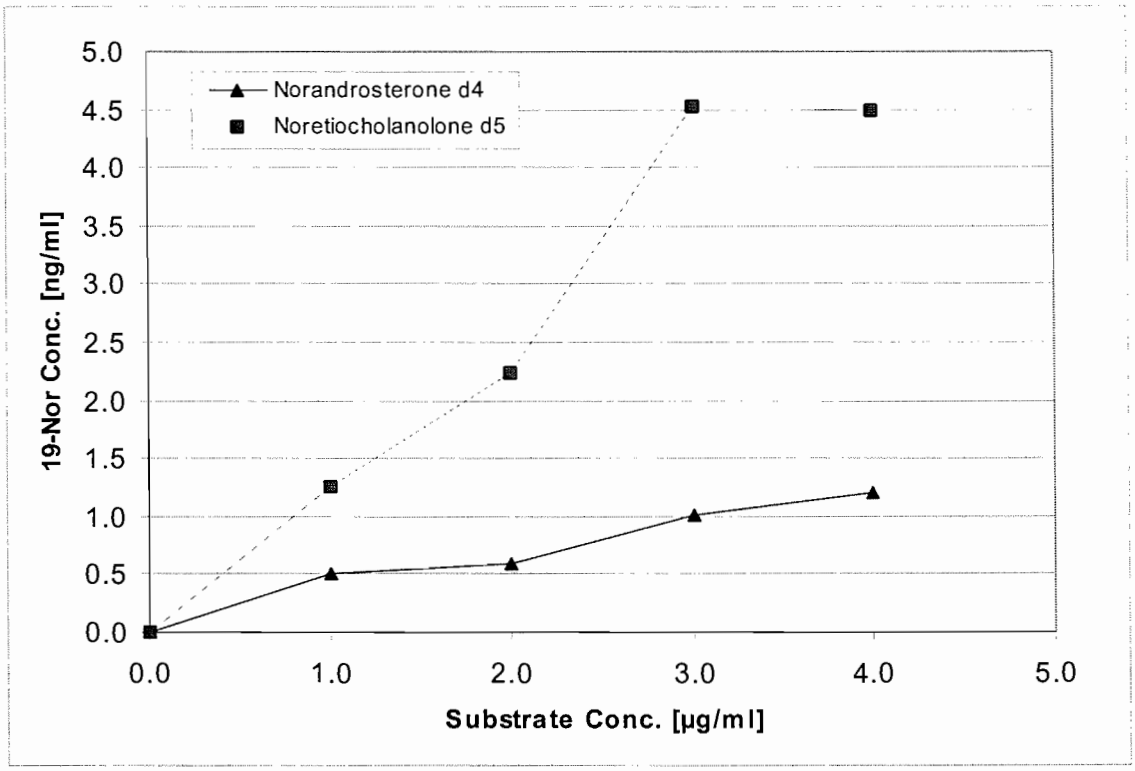

Figure 4: Substrate concentration (A-d4 and E-d5) and formation of norsteroids.

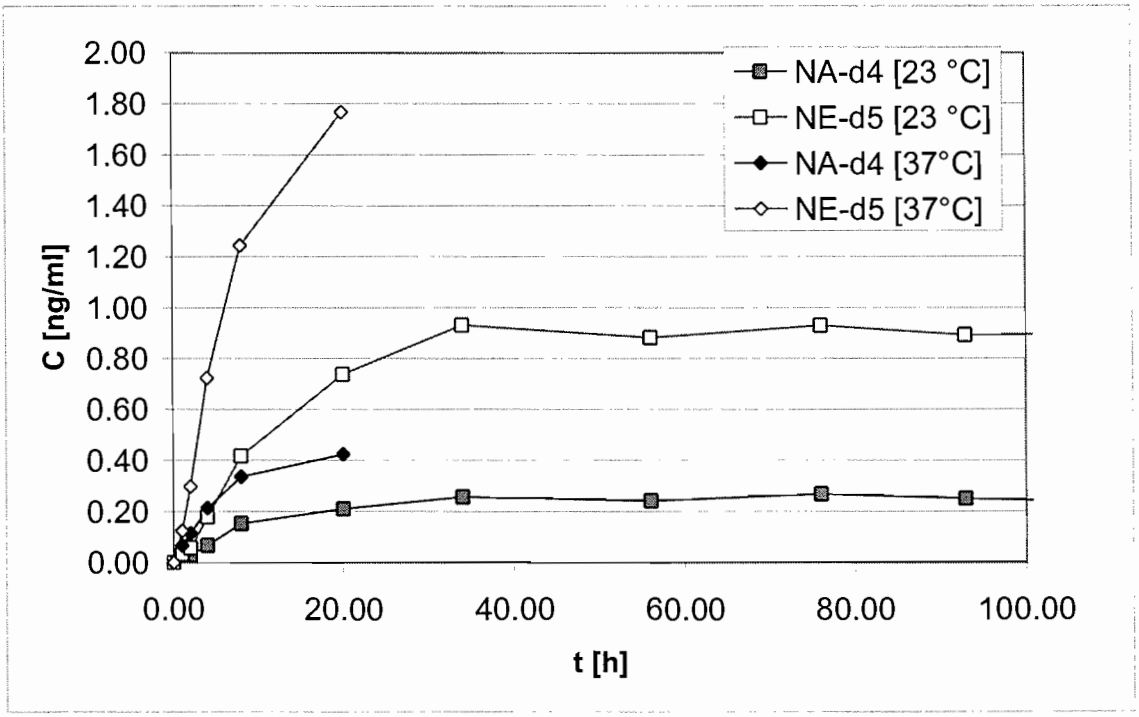

Figure 5: Influence of time and temperature on the formation of norsteroids in sample #4. (substrate concentrations A-d4 and E-d5 were 4µg/ml)

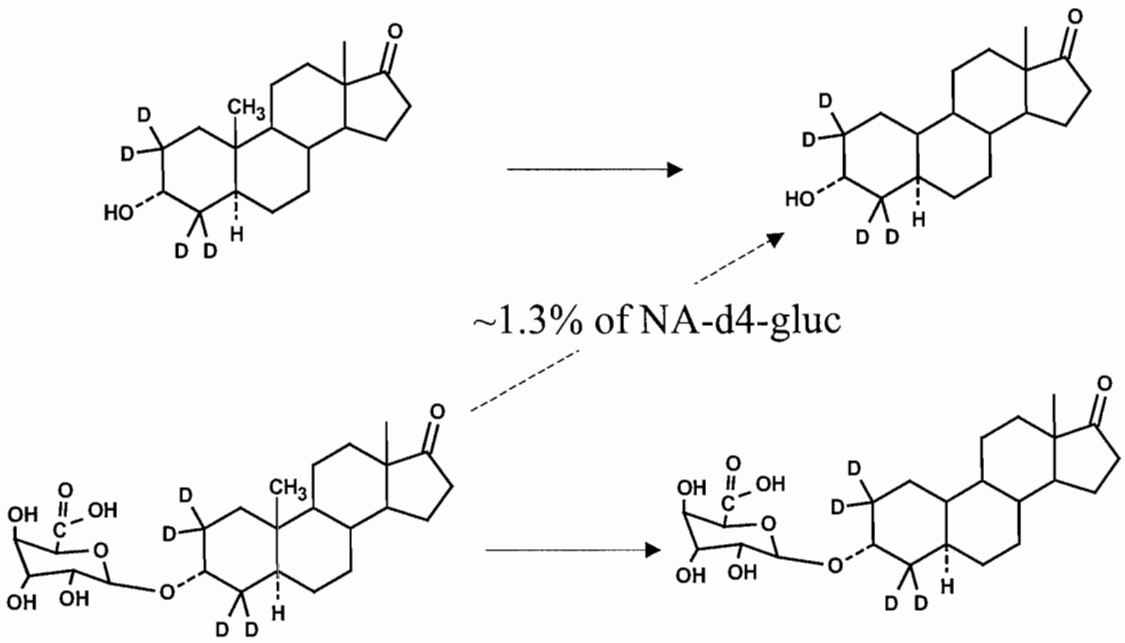

Figure 6: Free norandrosterone-d4 was produced after incubation experiments with unconjugated A-d4, while incubation with A-gluc-d4 resulted predominantly in the formation of NA-d4-glucuronide.

Table 1: Characteristics of the urine samples included into the incubation studies. In the majority of the suspicious cases (except 5, 8 and 13) the formation of deuterated 19 steroids was observed.

No	Discipline	Gender	Spec. Grav.	pH	Transp. [days]	A [µg/ml]	E [µg/ml]	NA [ng/ml]	NE / NA	NA-d4 [ng/ml]	NE-d5 / NA-d4
1	fencing	?	1.018	5.6	2	1.7	2.6	1.3	2.6	pos.	10
2	cycling	m	1.029	5.6	3	1.2	3.2	0.6	5.0	pos.	4.5
3	fencing	f	1.029	5.6	4	3.0	3.2	0.8	1.9	pos.	3.7
4	fencing	f	1.026	5.3	4	4.8	5.8	0.5	2.4	pos.	2.6
5	**cycling**	**m**	**1.027**	**5.1**	**5**	**3.1**	**1.0**	**0.6**	**0.3**	**neg**	**neg**
6	swimming	f	1.022	5.3	3	3.4	1.7	2.1*)	1.3*)	0.1*)	2*)
7	tennis	f	1.010	5.2	7	1.1	1.2	1.0*)	2.1*)	0.4*)	3.5*)
8	**tennis**	**f**	**1.028**	**5.2**	**6**	**8.2**	**10**	**0.6*)**	**2,3*)**	**neg**	**neg**
9	tennis	f	1.028	5.3	6	4.9	4.4	1.0*)	1.7*)	pos.	4.8
10	tennis	f	1.032	5.2	7	6.6	5.6	2.2*)	1.5*)	0.6*)	2.8*)
11	tennis	f	1.019	5.0	7	2.3	3.9	0.3*)	2.0*)	pos.	4.9
12	tennis	f	1.015	5.3	7	2.7	2.0	0.8*)	1.8*)	neg	trace
13	**tennis**	**f**	**1.016**	**5.2**	**7**	**4.2**	**4.7**	**0.5*)**	**2.0*)**	**neg**	**neg**
14	tennis	f	1.031	5.4	6	1.8	2.1	0.2	1.5	pos	5.5
15	tennis	f	1.018	5.4	7	2.8	2.7	0.4	1.7	pos	4.4

*) confirmed quantity

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