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Urine Storage Conditions and Steroid Profile Analysis

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Introduction

The International Olympic Committee [1] regards an elevated urinary concentration ratio testosterone glucuronide/ epitestosterone glucuronide (T/E ratio) above 6 as analytical proof for the administration of testosterone, provided that an endocrinological investigation including previous and/or subsequent controls could exclude a natural elevated T/E ratio. For the interpretation of the concentrations of endogenous steroids (steroid profile) [2, 3] in urine it is of outmost importance that the sample taking and storage conditions are adequate to prevent changes in the concentrations of steroids caused by bacterial contamination or thermal degradation [4, 5]. Questions have been raised whether the results of urinary steroid analysis may reflect microbial transformations rather than true baseline concentrations unless the samples are analysed immediately or snap-frozen in sterile containers [6]. In order to investigate these possible changes in urine due to storage conditions and to try to reproduce unpublished results where production of testosterone in urine was postulated, the following experiment was undertaken.

Materials and methods

Study design

Spot urines from 12 volunteers (6 females and 6 males) were collected and one aliquot was immediately taken for steroid analysis (free and conjugated endogenous steroids, see below). Four other aliquots were stored at -20, 4, 20 and 37 °C for 5 days. A fifth aliquot was stored at 4 °C for 3 days and subsequently at 20 °C for 4 days. The later conditions were applied in order to simulate the storing conditions in an actual doping case.

Bacterial growth analysis

Parts of the fresh urine samples and the samples stored at 37 °C were cultivated according to standard procedures in our hospital and standard procedures for identification of bacteria were applied.

Steroid analysis

The following endogenous steroids were quantified according to routine doping analysis procedures [7, 4]: androsterone, etiocholanolone, testosterone, epitestosterone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstane-3,17-dione. The quantitative measurements were made for both the free and combined (free + glucuronides) fraction. If otherwise not stated the results from the combined fractions are given. Additionally the pH values of the urines were measured with a Metrohm 744 ph meter and the densities determined with an ATAGO UR1 refractometer.

Results

When analysing the spot urine samples for bacterial growth immediately after sample delivering and after urine storage at "elevated" temperatures for several days the results in table 1 were obtained.

	Analysed immediately	4 °C for 3 days + 20 °C for 4 days	37 °C for 5 days
Number of samples with more than 1000 bacteria per ml	1	6	8
Sample %	8.3	50	67

Table 1: *Bacterial growth in urine samples stored at different conditions*

The following bacteria were identified: Enterococcus sp., Klebsiella pneumoniae, Klebsiella ozaenae, E. coli, Streptococcus agalactiae, Staphylococcus aureus and coagulase negative Staphylococci.

The relative changes of steroid concentrations and pH values due to different storage conditions are shown in figures 1 and 2. Storage at 20 °C compared to -20 °C for 5 days did

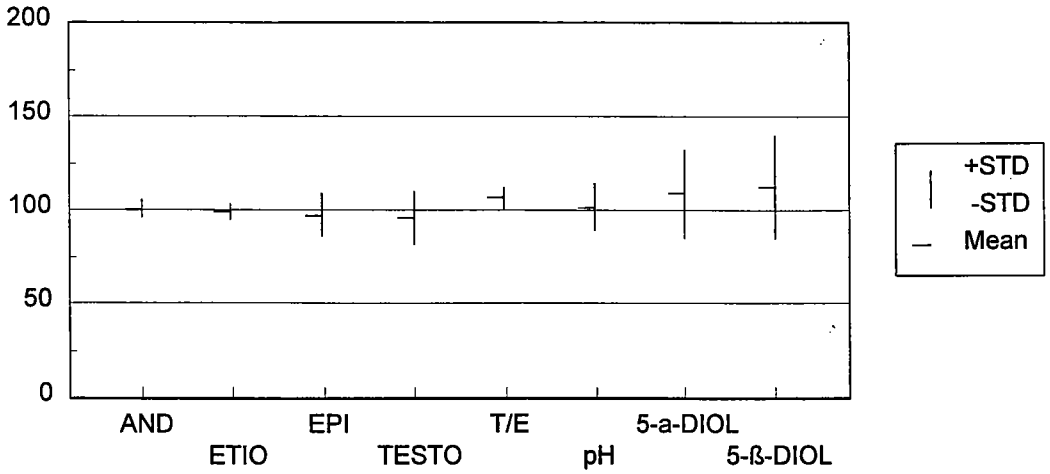

***Figure 1**: Relative changes (%) of steroid concentrations and pH values for urine samples stored for 5 days at 20 °C compared with -20 °C (100%). (Androsterone (AND), etiocholanolone (ETIO), epitestosterone (EPI), testosterone (TESTO), testosterone/epitestosterone ratio (T/E), 5α-androstane-3α,17β-diol (5-α-DIOL), 5β-androstane-3α,17β-diol (5-ß-DIOL))*

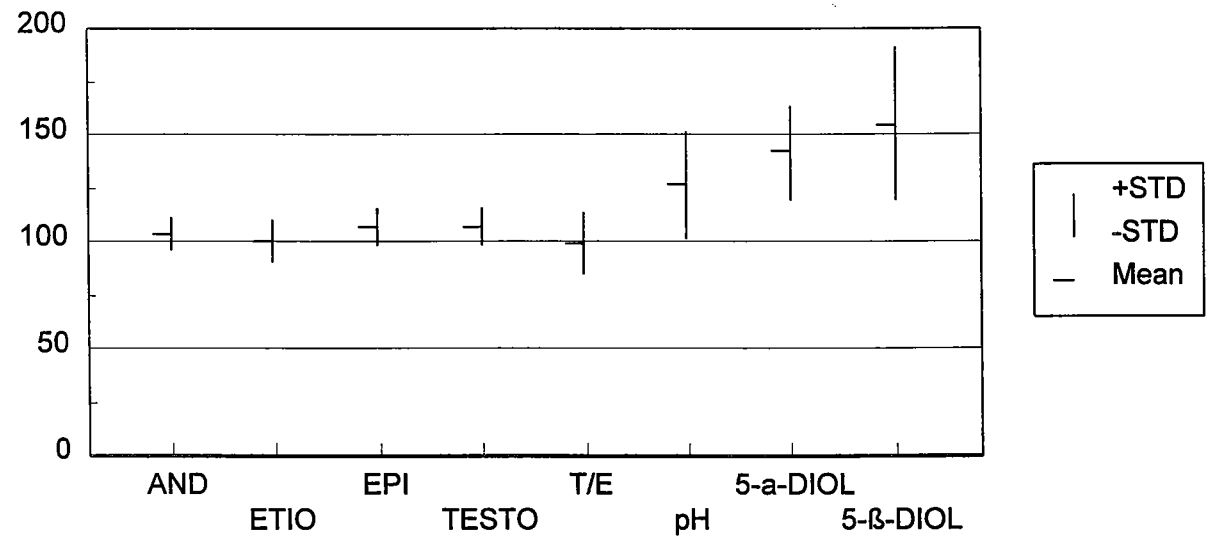

***Figure 2**: Relative changes (%) of steroid concentrations and pH values for urine samples stored for 5 days at 37 °C compared with -20 °C (100%).*

not change the concentrations of androsterone, etiocholanolone, testosterone, epitestosterone, the T/E ratio and pH-values and only slightly increased the concentrations of 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol. When storing the samples at 37 °C for 5 days, significantly increased values, compared to the storage at -20 °C, for the pH values and the concentrations of 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol were observed.According to these changes in the urines stored at 37 °C it is of interest to monitor the production of 5α-androstane-3,17-dione and to correlate it to the identifications of bacterial growth as well as the pH values(Table 2).

Sample	pH	Bacterial growth > 1000/ml urine	c (Androstanedione) [ng/ml]
1	9.4	--	--
2	5.7	Staphylococcus	--
3	5.8	--	60
4	9.0	Staphylococcus	390
5	5.1	Enterococcus	--
6	6.5	Enterococcus, E. coli, Klebsiellae	10
7	6.1	--	--
8	7.3	Enterococcus, Staphylococcus.	100
9	9.0	Enterococcus, Staphylococcus.	1200
10	7.6	Staphylococcus	430
11	9.1	--	430
12	9.2	Staphylococcus	340

Table 2: *5-α-Androstane-3,17-dione production , bacterial growth and pH values in urine samples stored at 37 °C for 5 days.*

The synthesis of 5-α-androstane-3,17-dione from the oxidation of androsterone and etiocholanolone mainly, indicates enzymatic and microbial activities in urines stored at 37 °C. In most of the samples with 5-α-androstane-3,17-dione production the pH value was significantly increased. Addtionally the elevated temperature might thermally contribute to

the deconjugation of steroid conjugates[8]. Therefore we determined the percentage of free endogenous steroids in relation to the bacterial growth (Table 3). In several urines, especially those with strong bacterial growth, steroids appeared up to 90 % in free form, while under all other storing conditions (≤ 20 °C) no free steroids (< 2%) were detected. When we added sodium azide as a preservative (1 g/l) to four fresh urine ssamples and incubated them at 37 °C for 5 days, only minor amounts of free steroids were detected.

While deconjugation occured, we could not detect in our experiments a synthesis of testosterone. Urinary testosterone concentrations (combined fraction) remained within the analytical precision constant under all examined storage conditions (Figure 3). The T/E ratio (combined fraction) did not raise to elevated values in our experiments (Figure 4).

Sample	Bacterial growth	Density [g/ml]	pH	AND [%]	5α-DIOL [%]	5β-DIOL [%]	TESTO [%]
1	--	1,004	9.4	0	0	0	0
2	+	1,015	5.7	5	0	21	0
3	--	1,027	5.8	1	21	47	61
4	+	1,008	9.0	4	0	29	0
5	+	1,010	5.1	4	0	0	0
6	++	1,014	6.5	23	67	30	44
7	--	1,007	6.1	0	0	0	0
8	++	1,018	7.3	70	82	55	94
9	++	1,013	9.0	1	8	18	54
10	+	1,023	7.6	9	18	44	61
11	--	1,028	9.1	1	0	1	17
12	+	1,007	9.2	5	17	17	35

***Table 3**: Percentage of free steroid concentrations in urine after storage at 37 °C for 5 days.*

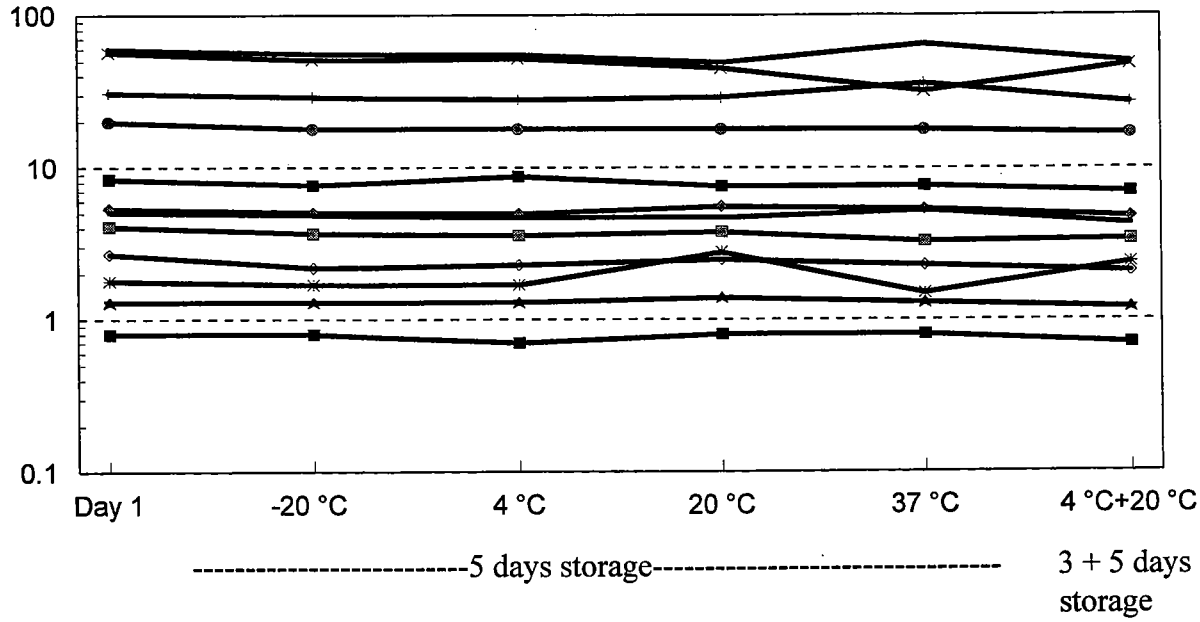

Figure 3: *Urinary testosterone concentrations in ng/ml (combined fraction) in 12 spot urines after different storage conditions.*

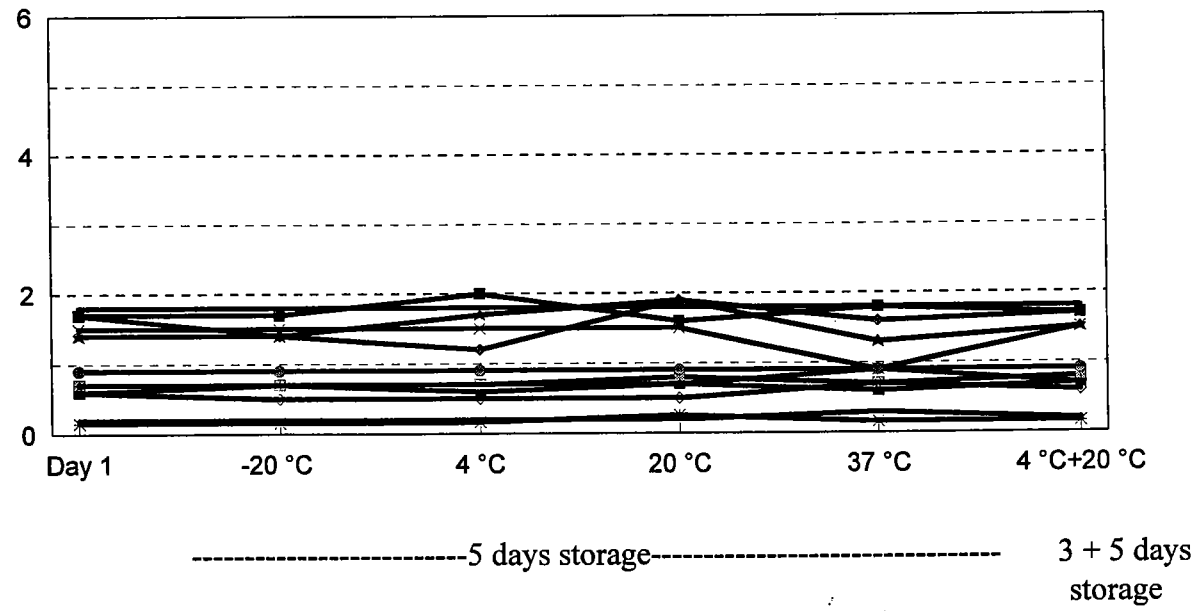

Figure 4: *Urinary testosterone/epitestosterone ratios (combined fraction) in 12 spot urines after different storage conditions.*

Discussion and conclusions

Spot urine samples taken from athletes for the purpose of doping control do normally not contain significant concentrations of bacteria (> 1000 / ml urine). Nevertheless one can never exclude the possibility of bacterial contamination, and the storage conditions from the time of sample taking to the start of the analysis may be in favour for bacterial growth. We have to take into account that neither are sterilised sampling devices used nor snap-freezing of the urine samples. Additionally, bacterial contamination may arise from an infection the athlete is suffering.

The experiment performed in this study only covers some of the possibilities of what bacterial grotwth may result in, because of the limited number of urine samples and volunteers. Nevertheless, we get some interesting results which might be clarified or better understood through follow-up studies with the incubation of sterilised urine samples with defined types of bacteria.

In our experiments, the storage of spot urine samples at 20 °C for 5 days lead to a considerable growth of bacteria in 50% of the samples. In these samples we did neither observe a synthesis of 5-α-androstane-3,17-dione nor the deconjugation of testosterone. The concentration of testosterone and the T/E ratio was constant. A considerable degradation of different steroids at this temperature, as earlier reported [9], could not be reproduced.

When increasing the temperature to 37 °C a considerable bacterial growth was observed in 2/3 of the samples, and we found the synthesis of 5-α-androstane-3,17-dione in several urines together with an increase of the concentrations of 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol. Additionally, in several urine samples the percentage of free steroid concentrations were markedly increased, while the overall concentration of testosterone was not changed.

We conclude, that possible effects on the concentration of endogenous steroids through unfavourable storing conditions and bacterial growth have to be taken into account when interpreting urinary steroid profiles. Situations where changes in endogenous steroid concentrations might have occurred, can be revealed through monitoring the pH value, the concentrations of androstanediones and the presence of considerable amounts of free steroid

metabolites. In such a situation the results concerning endogenous steroids should be interpreted with care[5].

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