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Urine stability, steroid profile and T/E ratio: towards an index of sample degradation.

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INTRODUCTION

The last revision of the WADA list reduced the threshold value for an elevated testosterone/epitestosterone (T/E) concentration ratio from 6 to 4. The WADA also released a technical document [1], fixing the criteria to assess sample degradation. A preliminary, retrospective evaluation of the samples received in the summer months of the last three years confirmed the empirical evidence that the degradation of the sample is often associated to an increase of T/E value, and that this pattern, especially now that the threshold of the T/E ratio has been reduced, can generate an additional workload for the laboratories.

We have tried to identify one or more suitable markers of sample degradation that, based on the data obtained by other antidoping laboratories [2-6], could be evaluated directly, possibly at the screening stage, reducing the need for additional confirmation and/or quantitation procedures on endogenous steroid hormones. Different potential markers of urine degradation (pH, metabolic by-products, deconjugated steroids and the variation of the concentration of testosterone, epitestosterone, DHT and DHEA in both total and free fraction) were considered; particularly, the effect of the storage temperature and of the urinary pH on the variation of the concentration of representative endogenous steroids, in both free and conjugated fraction, was considered, with the aim of verifying whether it would be possible to understand, directly from the screening procedures for the steroid hormones in the total and in the free fraction, whether a sample is to be considered degraded, thus avoiding unnecessary, time-consuming confirmation analysis. The significance of the proposed parameters was evaluated reconsidering all the data on the steroid fraction obtained on more than 2000 samples received by our laboratory in the period May-September 2004.

EXPERIMENTAL SECTION

In all experiments the urines underwent the screening procedures for both total and free anabolic steroids and analysed by GC/MS. The relative concentrations of the following steroids (glucuronate+free fraction) were measured: testosterone (Testo), epitestosterone (epiT), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), 5α-androstanedione and 5β-androstanedione.

Constant temperature study

Experiments have been carried out on 10 different pools of urines, collected for two days. One aliquot of 3 mL of each pool was immediately taken for endogenous steroids analysis (free and conjugated fraction). Each pool was divided in four groups, stored at different temperatures (–20 °C, 4 °C, 25 °C and 37 °C) for 20 days.

Fixed pH study

Experiments have been carried out on 6 different pools of urines, collected for two days. One aliquot of 3 mL of each pool was immediately taken for steroids analysis (free and conjugated fraction), then each pool of urine was divided in three groups (two pool each groups) and stored at 25 °C and 37 °C and at pH 5, 7 and 9. The pH values were checked daily, and, if necessary, adjusted. The characterization of the samples was carried out following the degradation of a non-buffered group of samples, analyzed concurrently.

Reference standards

The standards were obtained by NARL-Australia (testosterone, epitestosterone), and by Sigma Aldrich (dehydroepiandrosterone, dihydrotestosterone, 5α-androstanedione and 5β-androstanedione).

Analytical procedure

To 3 mL of urine, 50 µL of internal standard (17α-methyltestosterone), 1 mL of 0.2M phosphate buffer pH=7.4. and 30 µL of beta-glucuronidase from E. coli were added and hydrolysis was performed for 1 h at 50 °C. The buffered solution was then alkalinized with 1 mL of carbonate buffer and the steroids were extracted with 10mL of tert-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation, the etheral layer was transferred and evaporated to dryness under vacuum; the residue was derivatized by 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA):NH_4I:Dithioerythrytol (1000:2:4 v/w/w) and 1 µL of the derivatized extract was injected directly into the injection port. The sample preparation for the screening analysis of the free fraction consisted only in a liquid-liquid extraction a pH 9 and derivatization with the same reagent used for the total fraction.

Quantitation of excreted steroids (free and conjugated fraction) was performed by GC-MS on an Agilent 5890/ 5973A, in electron impact (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone (HP1), ID 0.20 mm, film thickness 0.11 µm. The carrier gas was helium (flow rate: 1 mL/min, split ratio 1:10), and the temperature program was as follows: 180 °C (hold 4.5 min), 3 °C/min to 230 °C, 20 °C/min to 290 °C, 30 °C/min to 320 °C; transfer line temperature: 280 °C. Acquisition was carried out in selected ion monitoring (SIM) of the following fragments: m/z 432 for testosterone, epitestosterone and DHEA; m/z 275 for 5α-androstanedione and m/z 417 for 5β-androstanedione. All values of urine concentration were calculated by the peak areas of the detected signals relative to the internal standard methyltestosterone (m/z 301). For calibration of the GC/MS instrument, the following reference mixtures were used (table 1 and table 2). All experimental data are reported in the figures 1-11.

Table1: Methanolic solutions

Compound	**Internal code**	**CalG1 50uL**	**CalG2 50uL**
		Conc. ng/mL (3 mL urine)	**Conc. ng/mL (3 mL urine)**
Testosterone	Test2-001	10	40
Epitestosterone	Epi-002	10	40
Androsterone	H047-002	500	1000
Etiocholanolone	H080-001	500	1000
DHEA	DHEA-001	10	40
DHT	DHT-002	10	20
5αAndrostanedione	5ADIONE-002	10	40
5βAndrostanedione	5BDIONE-001	10	40
Androstenedione	ASTE-001	10	20

Table2: Blank urine spiked with the target compounds

Compound	**Internal code**	**USP 1**	**USP 2**
		Conc. ng/mL (3 mL urine)	**Conc. ng/mL (3 mL urine)**
Testosterone	Test2-001	10	40
Epitestosterone	Epi-002	10	40
Androsterone	H047-002	500	1000
Etiocolanolone	H080-001	500	1000
DHEA	DHEA-001	10	40
DHT	DHT-002	10	20
5aAndrostanedione	5ADIONE-002	10	40
5bAndrostanedione	5BDIONE-001	10	40
Androstenedione	ASTE-001	10	20

RESULTS AND DISCUSSION

Influence of the temperature

From the data reported in Figures 1-3 it can be concluded that the most relevant parameters involved - and detectable - in the urine degradation process are the followings:

- formation (first in the conjugated fraction and then in the free fraction) of 5α-androstanedione and 5β-androstanedione: the urinary concentrations of these substances increase quickly during storage at 37 °C, more slowly at 25 °C. No difference (as far as the steroid profile is concerned) was detected, over a 20-day period, between 4 °C and - 20 °C;
- increased concentration of testosterone, epitestosterone, DHEA and DHT in the free fraction, recorded when the urine was stored at 37 °C.
- rapid increase and than decrease of DHT and DHEA in the conjugated fraction;
- increase of the pH value, recorded when the urine was stored either at 37 °C or at 25 °C.

Influence of pH

Figures 4-7 show that the effect of pH can be summarized as follows:

- the degradation is very rapid at 37 °C, slower but still pronounced at room temperature, while no difference (as far as the steroid profile is concerned) was detected between 4 °C and - 20 °C on a 30-day period;
- the pH value appears to be critical for the degradation process, i.e. at 37 °C: the process is very slow at pH 5.0. At pH 7.0 the process is very fast;
- the degradation is still quick at pH 9.0, but it goes on for a short time.

Parameters validation

In this phase we have studied ten pools of urine, with the aim of fixing a tentative cut-off value for the two most reliable markers of urine degradation, i.e. 5α-androstanedione and 5β-androstanedione. Data from Figures 8-11 show that when the percentage of epitestosterone and testosterone in the free fraction is higher than the 5% of the conjugated fraction, the value of 5α-androstanedione and 5β-androstanedione in the total fraction is around 7 and 20 ng/mL respectively. To validate this hypothesis we have reconsidered all the "A" samples (total: 2965) analyzed in our laboratory during the summer period (from May to September 2004).

The results of this retrospective study were the followings:

- 247 of these samples were found to match our degradation criteria;
- all those samples (27) with a T/E > 6, and subsequently found to be degraded, matched our degradation criteria;

- reconsidering the new limit (T/E >4), 138 had a T/E ratio (from the screening) imposing a confirmation analysis, and 43 (31%) could be considered degraded on the basis of the limits proposed for the screening analysis (TMS-derivatives, total fraction), without running any confirmation.

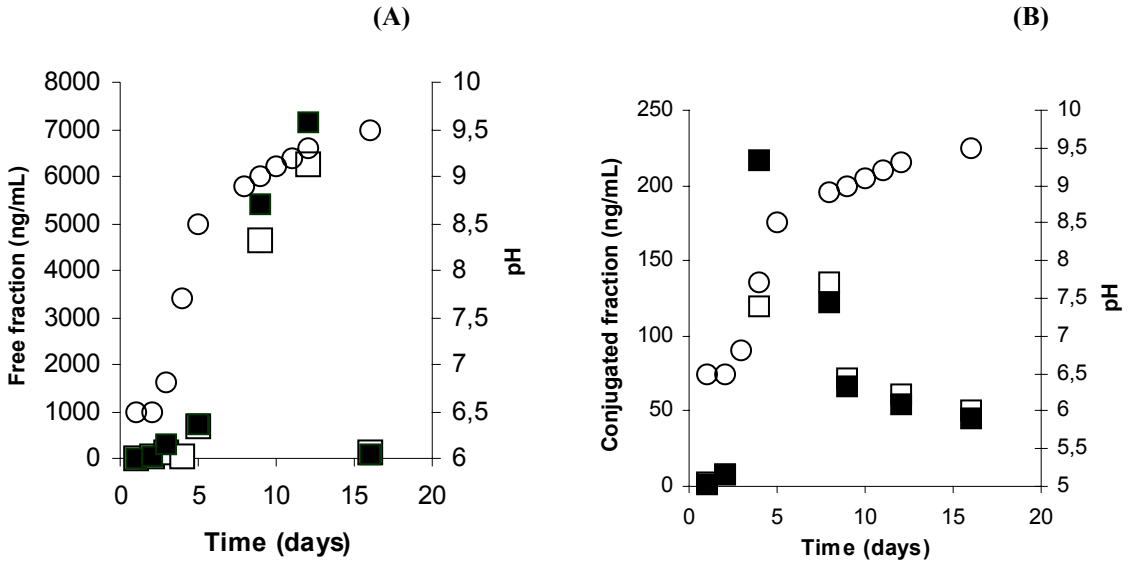

Figure 1. Variation as a function of time of the urinary concentration of: 5α-androstanedione (□), 5β-androstanedione (■) and of the urinary pH (○), at 37 °C, both in the free **(A)** and in the conjugated **(B)** fraction.

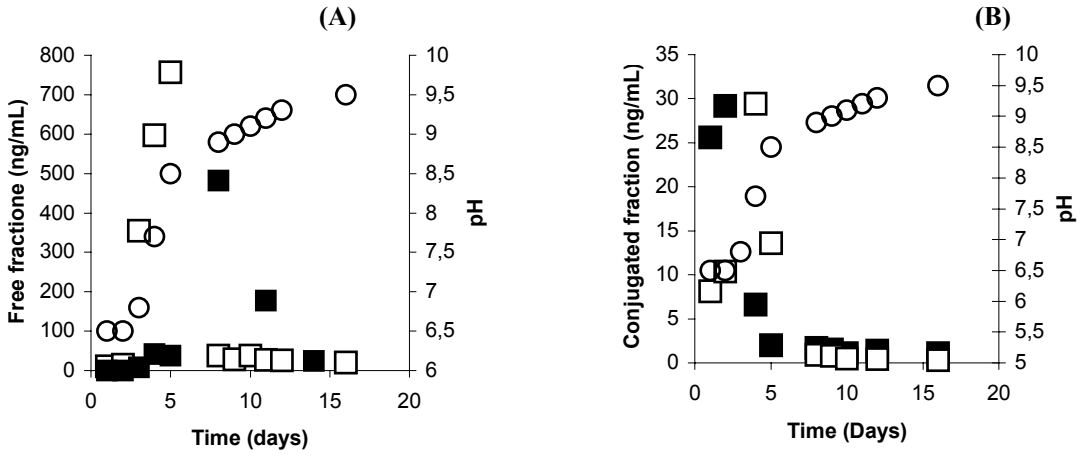

Figure 2. Variation as a function of time of the urinary concentration of: DHT (□), DHEA (■) and of the urinary pH (○), at 37 °C, both in the free **(A)** and in the conjugated **(B)** fraction.

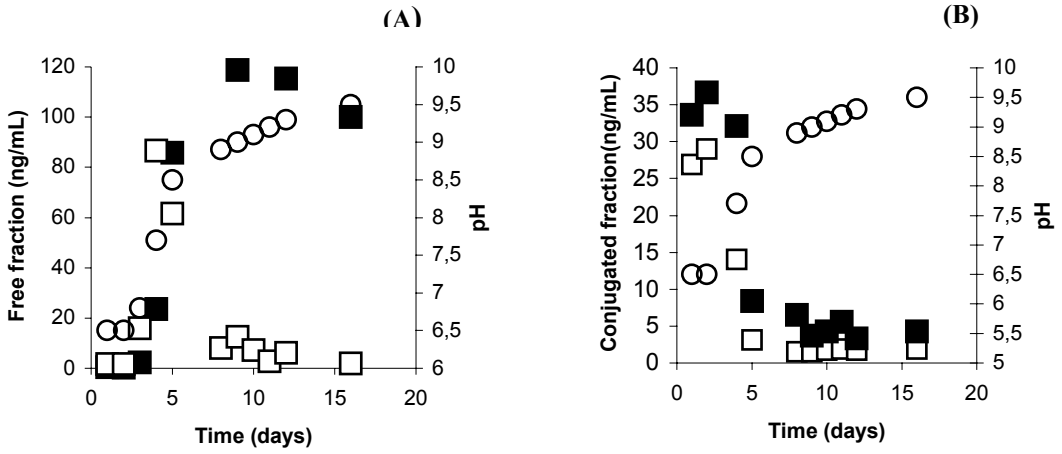

Figure 3. Variation as a function of time of the urinary concentration of: Testosterone (□), Epitestosterone (■) and of the urinary pH (○), at 37 °C, both in the free **(A)** and in the conjugated **(B)** fraction.

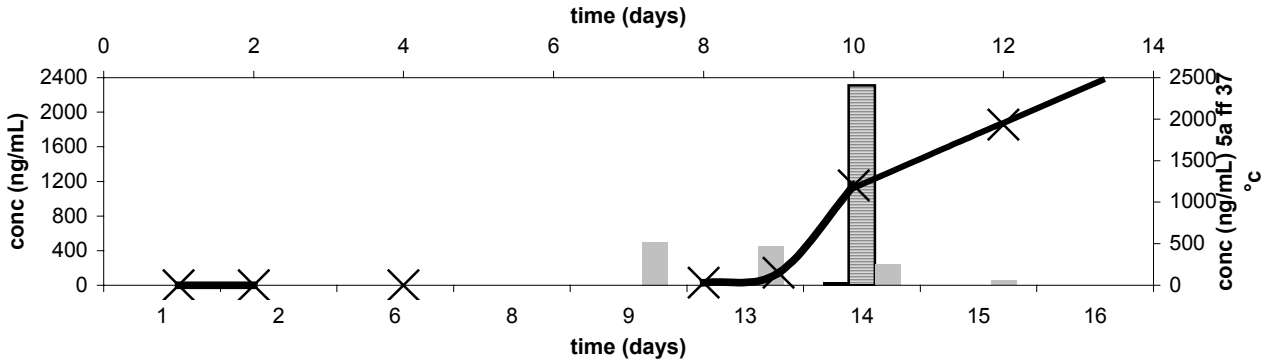

Figure 4. Variation as a function of time of the urinary concentration of 5α-androstanedione (free fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (×).

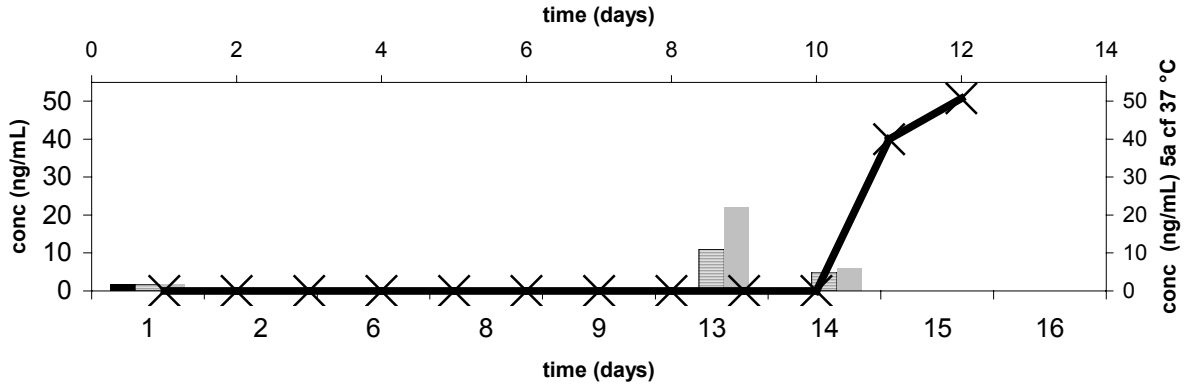

Figure 5. Variation as a function of time of the urinary concentration of 5α-androstanedione (conjugated fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (×).

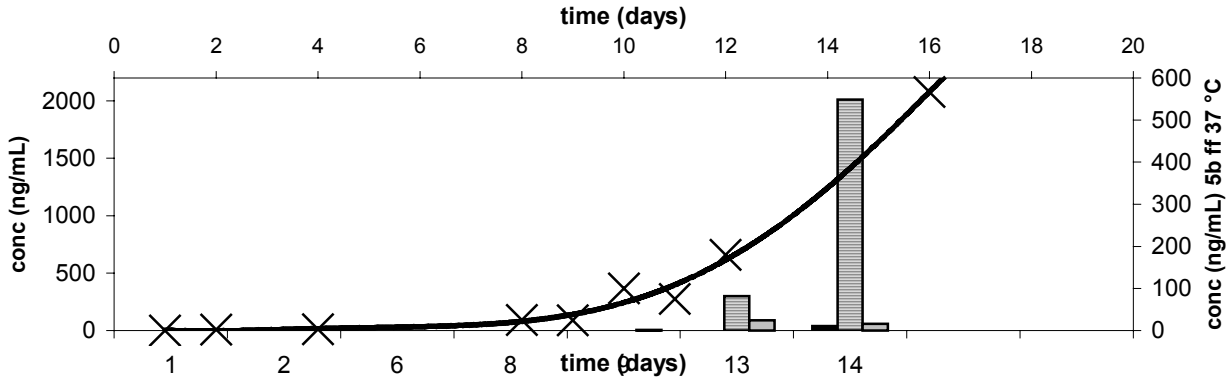

Figure 6. Variation as a function of time of the urinary concentration of 5β-androstanedione (free fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (×).

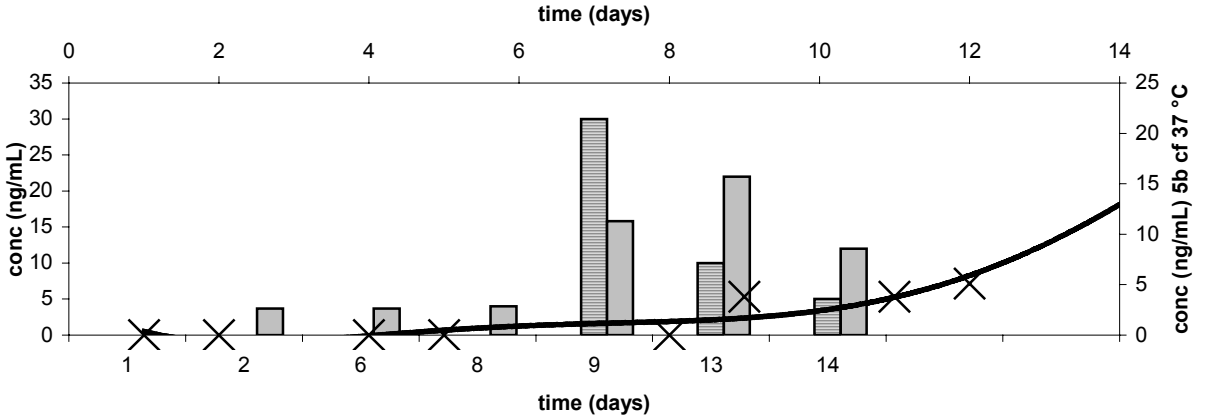

Figure 7. Variation as a function of time of the urinary concentration of 5β-androstanedione (conjugated fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (×).

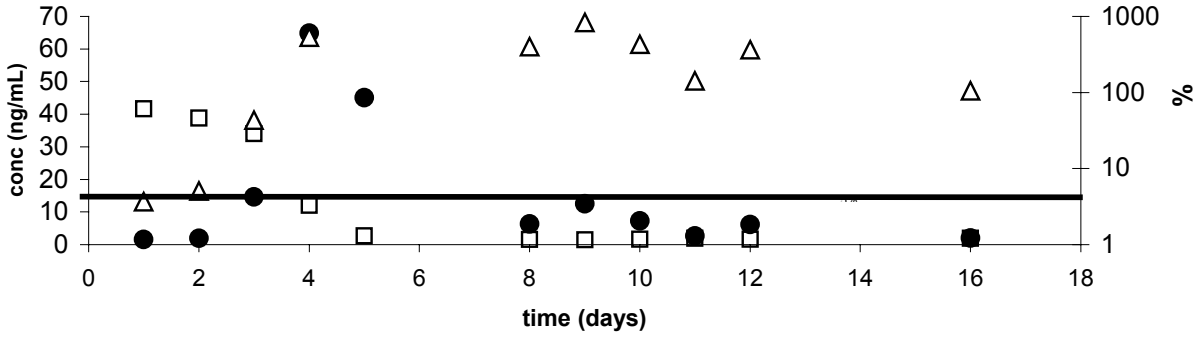

Figure 8 . Variation as a function of time of the urinary concentration of testosterone in the ●= free fraction, □=conjugated fraction and △=% free fraction/conjugated fraction

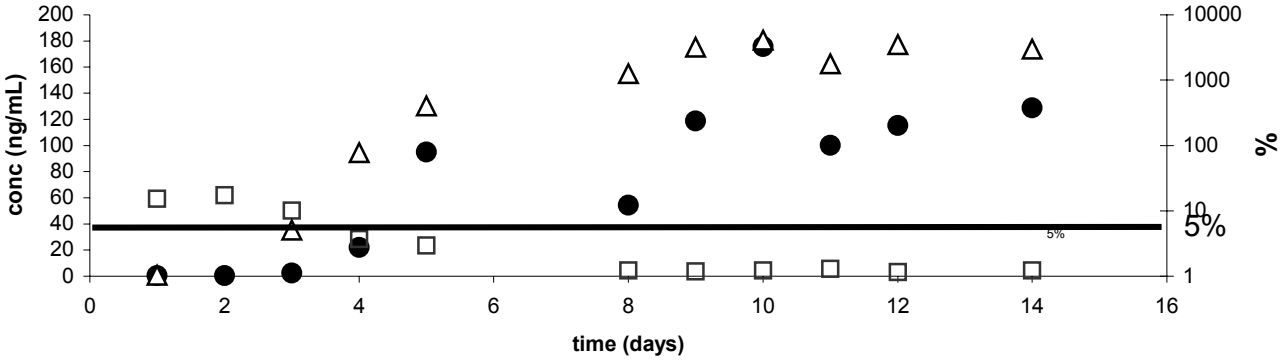

Figure 9 . Variation as a function of time of the urinary concentration of epitestosterone in the ●= free fraction, □=conjugated fraction and △=% free fraction/conjugated fraction

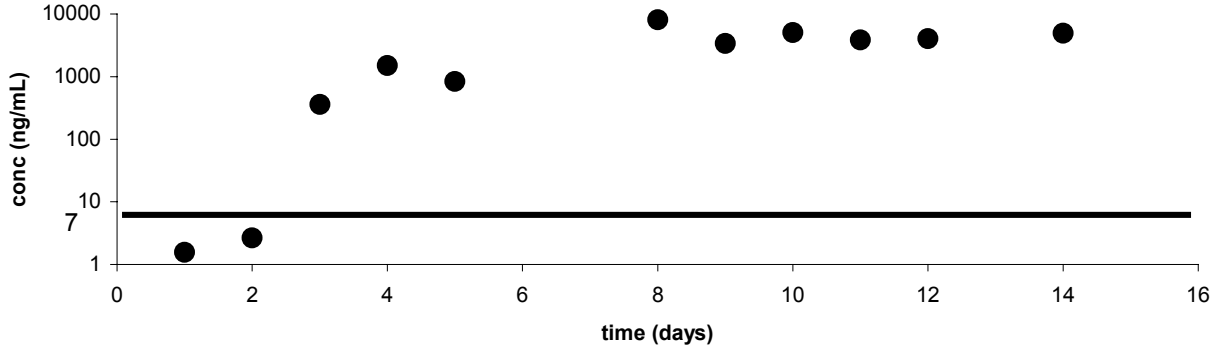

Figure 10 . Variation as a function of time of the urinary concentration of 5α-androstanedione in the total fraction.

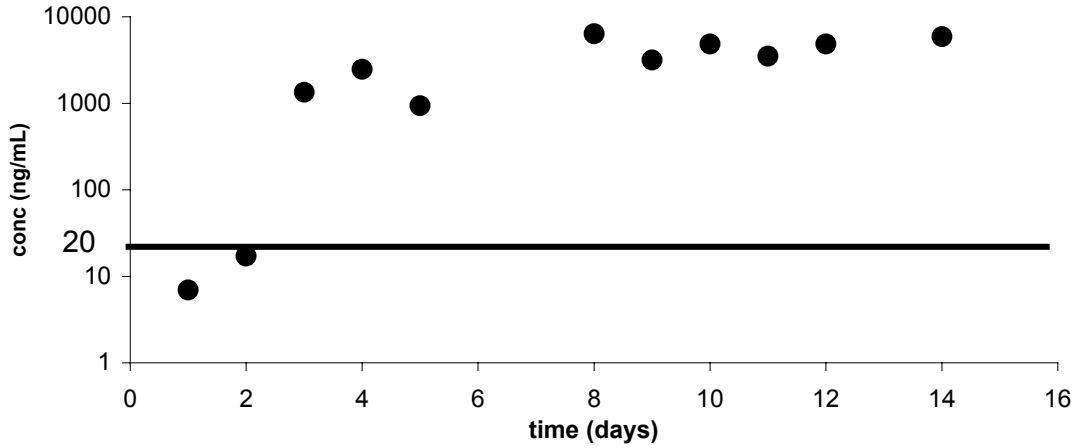

Figure11 . Variation as a function of time of the urinary concentration of 5β-androstanedione in the total fraction.

CONCLUSIONS

- The lowering of the T/E threshold value from 6 to 4 imposes a careful examination of potential markers of urine degradation (corresponding, according to ref. [1] to a testosterone and/or epitestosterone concentration ratio free/conjugated > 5%): the full quantitative confirmation in the free and total fraction of the testosterone and epitestosterone concentration may exceed the overall capacity of the laboratory (especially in the summer months).
- If the screening "IVa" (TMS-derivatives, free fraction) is performed, the simple presence of 5α-androstanedione and 5β-androstanedione is a reliable index of sample degradation: a window in the screening macro is easily added and monitored (presence/absence).
- If only the screening "IVb" (TMS-derivatives, total fraction) is performed, the concentration of 5α-androstanedione and 5β-androstanedione in the total fraction above a confidence threshold value (tentatively 7 ng/mL and 20 ng/mL respectively) is a reliable index of sample degradation.
- Alternatively, the concentration of 5α-androstanedione and 5β-androstanedione can be estimated by the height ratio 5α/ISTD and/or 5β/ISTD (in our case deuterated epitestosterone). The instrumental stability and repeatability of these parameters suggest that the laboratory internal threshold can represent a valid index for the preliminary assessment of sample degradation.

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