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Degradation kinetics of selected threshold compounds in biological samples: effect of different external conditions

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Abstract

In this study we are presenting short-term and long-term protocols to investigate the stability of triamcinolone, triamcinolone acetonide, prednisone, betamethasone, deflazacort and formoterol in male and female urines at different temperatures (25, 37, 55°C for short-term; -20 and 4°C for long-term) and pH values (5.0, 7.0, 9.0).
To assess the degradation rate and nature, the concentration of the target analytes, pH, specific gravity and nitrites levels were measured every day for 7 days in short-term stability protocol, and every three days for three months in long-term stability protocol. Results were compared with those obtained in aliquots stored at -80°C, since at this temperature thermal/microbial degradation is totally inhibited.
Concerning short-term stability, data showed that, in normal conditions, although all studied substances underwent extensive degradation after 7 days at 37°C and moderate degradation at 25 and 55°C, their levels did not decrease remarkably for at least 4 days; besides, degradation kinetics appeared to be pH-dependent, being slower at pH 5.0 and 9.0 than at pH 7.0; additionally, since degradation at 37°C was more pronounced than 55°C, it can be assessed that both microbiological and thermal factors affect substances stability and the increase of pH values and nitrites concentration at 37 and 25°C confirms this evidence. Long-term stability results showed that investigated substances were stable at -20°C for 91 days and at 4°C for at least 30 days. Finally, no marked differences were observed in degradation rate between female and male urine.

Introduction

Since the analysis of biological samples for antidoping controls is not performed immediately after collection, knowledge about stability of prohibited drugs in urines is crucial for proper interpretation of the analytical results. Recently studies were conducted on stability of several classes of substances included in the WADA prohibited list [1] such as stimulants [2-4], diuretics [2,5], anabolic agents [6-8] and narcotics [9,10]. Nevertheless, limited information is presently available about degradation of glucocorticoids and β2-agonists.
The stability of some glucocorticoids and β2-agonists was investigated in different storage conditions to ensure that the procedures established by the WADA and actually in use to collect, transport and store urine samples for doping control tests do not affect the reliability of the analytical results.

Experimental

Stability study protocol

Starting from previous investigators experience [2,4], a protocol for long- and short-term stability testing was set up. 6 pools of urines (3 from female and 3 from male subjects) were obtained mixing different blank urines (5 for each pool). Each pool, spiked with triamcinolone, triamcinolone acetonide, prednisone, betamethasone, deflazacort (at the MRPL concentration, 30%nbspng/mL) and formoterol (at the threshold concentration, 40 ng/mL), was divided in four stocks, three adjusted to pH 5.0, 7.0, 9.0 and one without pH adjustment; each stock was further divided in aliquots stored as it follows:
short-term stability study: aliquots were stored at 25, 37, 55°C for 7 days, simulating transportation conditions of collected samples towards the WADA assigned laboratory;

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long-term stability study: aliquots were stored at -20 and 4°C for 91 days, simulating the storage conditions of urine samples; reference aliquots: aliquots stored at -80°C were considered as reference samples for comparison purposes, being any degradation process highly unlikely at that temperature.

Sample analysis

The concentration of the target analytes were determined, in triplicate, daily and every three days for short- and long-term studies respectively, after sample dilution 1:20 with aqueous solution of internal standards (exemestane, final concentration 30 ng/mL, for glucocorticoids and formoterol deuterated, final concentration 50 ng/mL, for formoterol) and direct injection into LC-MS/MS system (analytical parameters in Tab.1). For all analytes, the ratio (A_T/A_{-80}) between the mean (n=3) of the area ratios analyte/internal standard obtained at each storage condition tested (A_T) and the mean (n=3) of the area ratios analyte/internal standard obtained at the reference condition (A_{-80}) was determined in function of the storage time. Nitrites levels (bacterial contamination index) were measured using urine test strips Meditrol 10+Leuko; additionally, pH values were monitored and if necessary corrected in samples stored at fixed pH (5.0, 7.0, 9.0).

Chromatographic conditions		**Mass spectrometric conditions**		
Instrument: Agilent 1200 Rapid Resolution Series HPLC		**Instrument: API 4000 QTrap**		
Column: Supelco Discovery C18 (150 x 2.1, 5 µm)		**Ion source: positive electrospray ionization (ESI)**		
Injection volume: 20 µL		**Temperature source: 550°C**		
Flow rate: 250 µL/min		**Voltage: 5000 V**		
Mobile phases: **water 0.1 % formic acid (mobile phase A),** **acetonitrile 0.1% formic acid (mobile phase B)**		**Aquisition mode: single reaction monitoring (SRM)**		
Gradient		***Analyte***	***Monitored transition for analyte determination (m/z)***	***Collision energy (eV)***
Time (min)	**%B**	**Triamcinolone**	**395.3/357.2**	**25**
0.0	**30**	**Triamcinolone acetonide**	**435.2/415.3**	**17**
5.0	**60**			
8.0	**80**	**Prednisone**	**359.0/323.0**	**30**
10.0	**100**	**Betamethasone**	**393.3/355.2**	**25**
		Deflazacort	**442.0/121.0**	**30**
		Formoterol	**345.0/149.0**	**30**
		Exemestane (ISTD)	**297.2/149.0**	**30**
		Formoterol deuterated (ISTD)	**349.0/125.0**	**30**

Table 1: Instrumental conditions

Results and Discussion

Concerning short-term stability, all substances under investigation showed marked signs of degradation after 7 days at all storage conditions tested, although their concentration remained stable for the first 4 days; in particular prednisone, deflazacort and triamcinolone seemed to be more affected by degradation processes than formoterol, triamcinolone acetonide and betamethasone (Fig.1). For all substances, degradation rates were closely related to storage temperature and pH; in fact, the urinary levels of the analytes decreased faster in aliquots stored at 37°C than in aliquots stored at 25°C and 55°C; additionally, degradation resulted extensive at pH 7.0 and moderate at pH 5.0 and 9.0.

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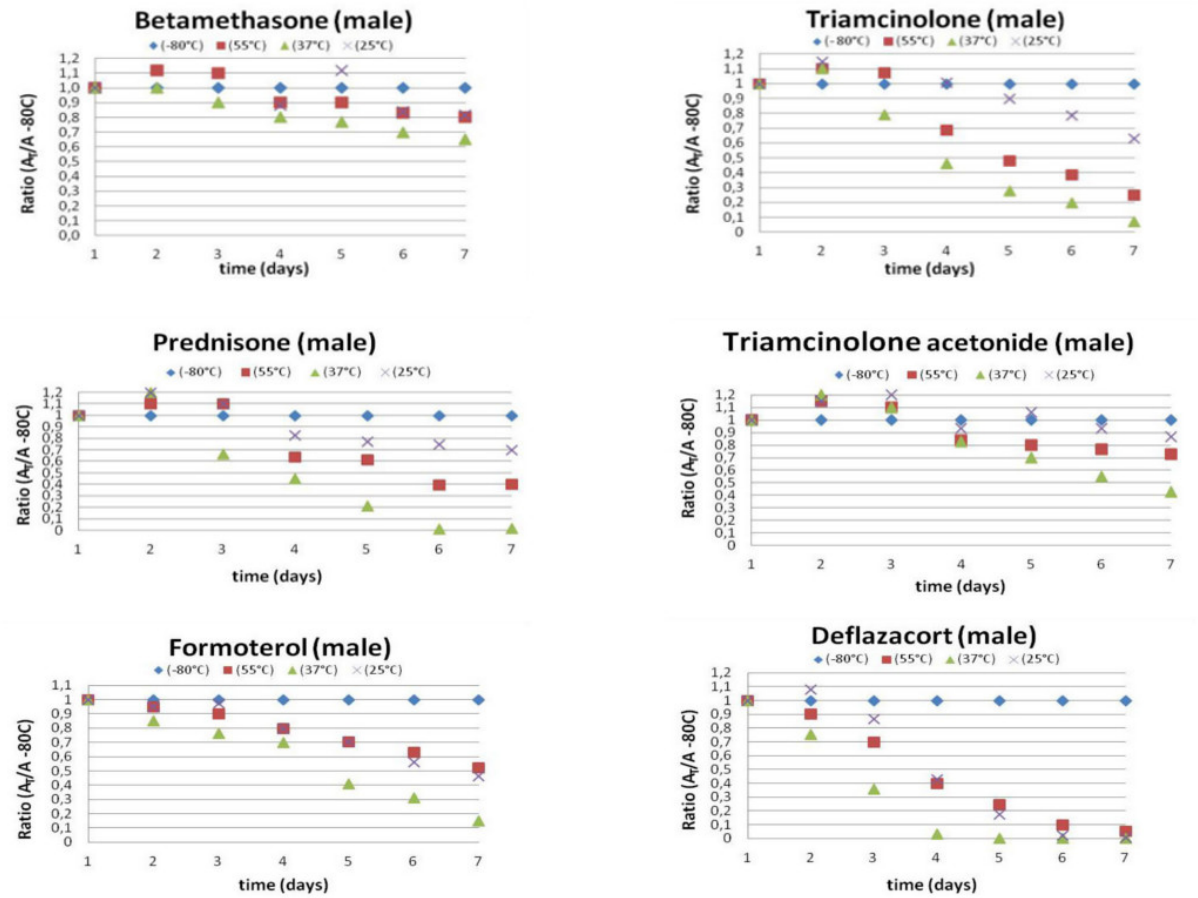

Figure 1: Data referred to one of the male urine pools stored at not fixed pH value for short-term stabilty study (7 days). Graphics report the ratio (A_T/A_{-80}) between the mean (n=3) of the area ratios analyte/internal standard obtained at each storage condition tested (A_T) and the mean (n=3) of the area ratios analyte/internal standard obtained in reference samples (A_{-80}), in function of storage time.

It is established that thermal and microbiological degradation processes can affect analyte stability in biological samples [5,8] and that at pH 7.0 and T=37°C microorganism proliferation is more likely to occur. From the present study, being the analyte degradation at 37°C higher than 55°C, it can be assessed that both microbial and thermal processes occurred. Supplementary parameter measurements strengthened this assumption: in samples stored at 37°C and pH 7.0 the highest amount of nitrites (index of bacterial contamination) was observed; additionally, in samples stored at 37°C without pH adjustment, a marked increase of pH value (Fig.2) was observed over the 7 days.

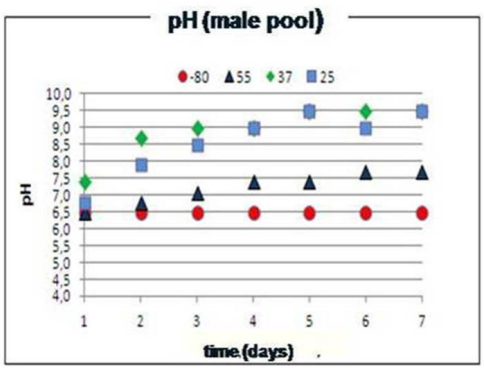

Figure 2: pH values increase in one of the male urine pools stored at not fixed pH for short-term stability study.

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Long-term stability results (Fig.3) showed that all investigated substances were stable at -20 °C for the whole period (91 days) at all pHs considered; weak degradation occurred for triamcinolone and formoterol and a slithly higher degradation occurred for prednisone and deflazacort, in samples stored at 4°C and pH 7 and 9, but in any case degradation started after the 30th day of the study. Finally no marked differences in degradation rates were observed between female and male pools.

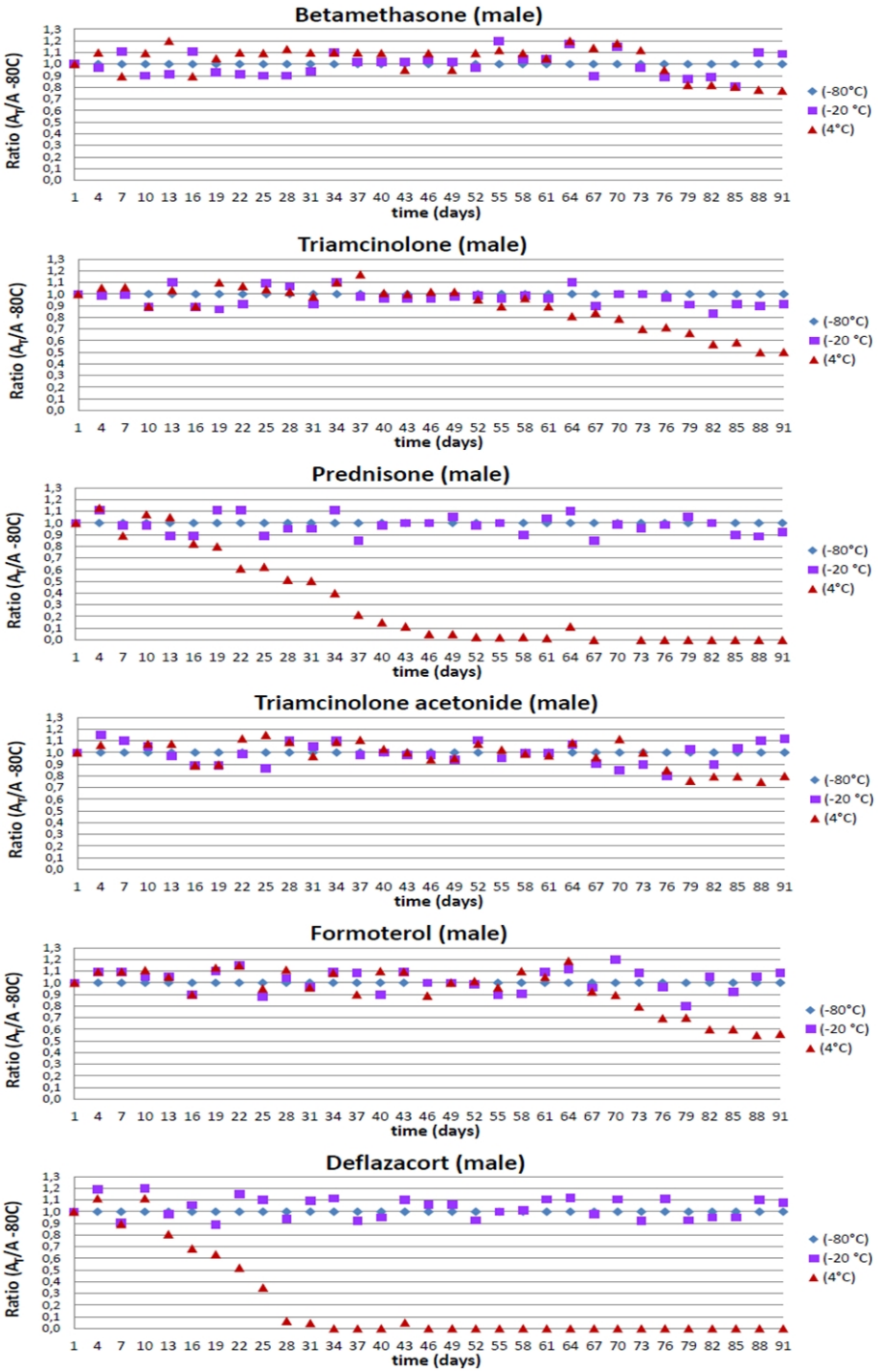

Figure 3: Data referred to one of the male urine pools stored at not fixed pH value for long-term stabilty study (91 days). Graphics report the ratio (A_T/A_{-80}) between the mean (n=3) of the area ratios analyte/internal standard obtained at each storage condition tested (A_T) and the mean (n=3) of the area ratios analyte/internal standard obtained in reference samples (A_{-80}), in function of storage time.

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Conclusions

According to the experimental data collected in this study, triamcinolone, triamcinolone acetonide, prednisone, betamethasone, deflazacort and formoterol are subject to both thermal and microbiological degradation processes, these being more or less pronounced depending on both the storage time and the environmental conditions (temperature and pH). Nonetheless, the procedures established by WADA and presently in use to collect, store and transport the urine samples for doping control tests, if correctly followed, are adequate to ensure the stability of the investigated substances and the reliability of the analytical results reported by the accredited laboratories.

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