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Journal of Chromatography B, 795 (2003) 167–177
JOURNAL OF CHROMATOGRAPHY B
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Stability studies of selected doping agents in urine: caffeine☆

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Received 26 March 2003; received in revised form 2 July 2003; accepted 9 July 2003

Abstract

The stability of caffeine in urine samples has been studied. A high-performance liquid chromatography (HPLC) method for the quantification of caffeine in urine samples was validated for that purpose. The method consists of a liquid–liquid extraction at alkaline pH with chloroform-2-propanol (9:1, v/v) with a salting out effect. 7-Ethyltheophylline was used as internal standard (ISTD). Analyses were performed with an Ultrasphere ODS C18 column using water/acetonitrile (90:10, v/v) as a mobile phase at a flow rate of 1 ml/min. Ultraviolet absorption at 280 nm was monitored. Extraction recoveries for caffeine and 7-ethyltheophylline were 81.4 ± 6.0 and 87.3 ± 5.7%, respectively. The calibration curves were demonstrated to be linear in the working range of 6–30 μg/ml ($r^2 > 0.990$). The limit of detection and the limit of quantitation were estimated as 0.7 and 2.0 μg/ml, respectively. Precisions in the range of 1.5–9.2 and 4.1–5.8% were obtained in intra- and inter-assay studies, respectively, using control samples containing 10, 14 and 26 μg/ml of caffeine. Accuracies ranging from 2.9 to 7.4% for intra-assay experiments, and from 3.9 to 5.4% in inter-assay studies were obtained. Stability of caffeine in urine samples was evaluated after long- and short-term storage at different temperature conditions. The batches of spiked urine were submitted to sterilization by filtration. No adsorption of the analyte on filters was observed. Before starting stability studies, batches of reference materials were tested for homogeneity. For long-term stability testing, caffeine concentration in freeze-dried urine stored at 4 °C and in liquid urine samples stored at 4, −20, −40 and −80 °C was determined at several time intervals for 18 months. For short-term stability testing, caffeine concentration was evaluated in liquid urine stored at 37 °C for 7 days. The effect of repeated freezing (at −20 °C) and thawing was also studied for up to three cycles. The stability of caffeine was also evaluated in non-sterile samples stored at −20 °C for 18 months. No significant loss of the compound was observed at any of the investigated conditions.

Keyword: Caffeine

1. Introduction

Stability may be defined as the ability of a material, when stored under specified conditions to maintain a stated property (e.g. concentration) within the specified limits for a specified period of time. The objective of stability testing is to identify and evaluate any significant degradation of the analytes

☆ Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

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doi:10.1016/S1570-0232(03)00557-9

when subjected to storage over time at different conditions.

Knowledge of the stability of drugs in biological fluids is critical for proper interpretation of analytical results. For this reason, stability assessment is considered a fundamental parameter for the validation of bioanalytical methods [1]. Since the analysis of biological samples for drug testing is not usually performed immediately after sample collection, it is very important to use optimal storage conditions for which the drug has been demonstrated to be stable during the storage time. Most data available in the literature in the area of analytical toxicology, refer to the stability of drugs of abuse in biological specimens (e.g. 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC–COOH), morphine/codeine, cocaine/benzoylecgonine or amphetamine derivatives) [2–13], mainly because their presence in these matrices may involve legal consequences for individuals.

The stability of drugs has to be also evaluated in order to prepare samples for intercomparison exercises or reference materials. According to different international quality standards, homogeneity and drug stability must be verified from production to end-use when producing this type of samples [14–16] to ensure that differences in results between laboratories are unrelated to drug instability or sample inhomogeneity. In these cases, stability data may be also useful in planning transport and to establish recommended storage conditions for a given reference material.

The misuse of drugs as ergogenic aids by athletes constitutes an offence in regulated sport practise and conceptually the control of these substances in biological fluids, named antidoping control, is similar to drugs of abuse testing. In antidoping control, it is also important to ensure that analyte instability will not affect the analytical results during the retesting period. This is especially significant for those analytes that have threshold concentrations as positivity criteria. Nevertheless, this kind of information is seldom addressed. One of the few exceptions is the stability of unconjugated testosterone in urine [17].

For this reason, as part of a larger project (ALADIN 2002) this work aims to study the stability of selected doping agents to prepare samples to be used in interlaboratory comparison exercises or as reference materials. For this purpose, a protocol for stability testing and the evaluation criteria of the stability data were defined [18]. This protocol is focused in the systematic evaluation of the suitability of urine samples after being exposed to the different temperature conditions most commonly encountered for their intended use as test items in external quality assurance schemes, or as reference materials.

In human doping control a restriction exists on the urinary concentration of caffeine for its stimulant activities. In this work, the stability protocol and the evaluation criteria proposed have been applied to determine the stability of caffeine in urine. The adequacy of different criteria used to evaluate stability will be discussed.

2. Experimental

2.1. Chemicals and reagents

Caffeine (USP quality) was supplied by Sigma. 7-Ethyltheophylline, used as internal standard (ISTD) was synthesized from theophylline, supplied by Sigma. 2-Propanol, acetonitrile, chloroform and methanol were of high-performance liquid chromatography (HPLC) grade. Other reagents were of analytical-reagent grade. Deionisated water used in the HPLC mobile phase was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Filters for clarification of urine and for sterilizing filtration were supplied by Millipore (Millipore Ibérica, Barcelona, Spain). For clarification of the urine one cellulose reinforced disc membrane filter of 90 mm of diameter, 100 μm of minimum thickness and 75% of pore size, and two glass fiber filters, one with 90 mm of diameter and 380 μm of minimum thickness and another with 75 mm of diameter and 1200 μm of minimum thickness, both with a 90% of pore size were used. For the sterilizing filtration of the urine a membrane filter of modified polyvinylidene fluoride and 0.22 μm of pore size was used.

Cryotubes of 3.6 and 4.5 ml for storage at −40 and −80 °C were supplied by Labclinics (Barcelona, Spain). Polipropylene tubes of 5 ml for storage at −20 and 4 °C were supplied by Vidra Foc (Barcelona, Spain).

Sodium carbonate buffer was prepared by mixing 100 g of sodium carbonate anhydrous and 50 g of sodium hydrogen carbonate. For the systhesis of

7-ethyltheophylline, 2 g of anhydrous theophylline were dissolved in 50 ml of 0.1 M NaOH aqueous solution and mixed with 5 ml of ethyl iodide in a three-neck round bottom flask. The reaction mixture was heated at reflux for 6 h. After a second addition of 5 ml of ethyl iodide the resulting mixture was stirred overnight. The organic phase was extracted with benzene (2 ml × 60 ml) and washed with deionized water (2 ml × 75 ml). Then, the organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum yielding 7-ethyltheophylline as a pale yellow powder. The purity of the solid obtained was verified by thin layer chromatography (TLC), and HPLC. TLC was carried out on precoated silica gel merck 60 F_{254} (0.25 mm) sheets and using a mixture of chloroform–methanol (9:1, v/v) as a mobile phase. HPLC was carried out using the method described in this paper.

Stock standard solutions of 4 mg/ml of caffeine and 1 mg/ml of 7-ethyltheophylline (internal standard) were prepared using methanol as a solvent. The working standard solution of 400 μg/ml of caffeine was prepared by 1:10 dilution of the stock standard solutions with methanol. All solutions were stored at −20 °C.

2.2. Instrumental analysis

Chromatographic analysis was performed in a Series II 1090L liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array UV detector. The instrument was linked to a Hewlett-Packard chemstation for liquid chromatography. Separation was carried out using an Ultrasphere ODS column with 3 μm particle size (7.5 cm × 0.46 cm i.d.) (Beckman, San Ramon, CA, USA).

The mobile phase was a mixture of water and acetonitrile with gradient elution. The initial acetonitrile content (20%) was maintained for 4.5 min and then increased to 30% in 1 min, maintained there for 2.5 min, decreased to 10% in 0.5 min and stabilized for 0.5 min before the next injection. The flow rate of the mobile phase was maintained at 1 ml/min. The detector was set to monitor the signal at 280 nm. In addition, the full ultraviolet spectrum between 190 and 400 nm of the detected peaks was stored in the data system.

Peak areas were measured and the ratio with the peak area of the internal standard was used for all calculations.

2.3. Sample preparation procedure

To 1 ml of urine samples, 10 μl of ISTD solution (7-ethyltheophylline, 1 mg/ml) were added. The urine samples were made alkaline by the addition of 100 mg of sodium carbonate buffer (pH 9.0) and extracted with 7 ml of a mixture of chloroform-2-propanol (90:10, v/v). After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 3000 × g), the organic layer was separated and evaporated to dryness under a nitrogen stream in a water bath at 40 °C. Dried extracts were reconstituted in 100 μl of a mixture of deionized water–acetonitrile (90:10, v/v) by vigorous vortex mixing. Volumes of 10 μl were injected into the chromatographic system.

2.4. Assay validation

The following parameters were evaluated in the method validation: selectivity/specificity, heteroscedasticity, linearity, limits of detection and quantitation, recovery, stability, and intra- and inter-assay precision and accuracy, according to a protocol previously described [19].

2.5. Experimental design for stability testing

2.5.1. Preparation of samples for stability testing

Caffeine-free urine (blank urine) was obtained from healthy volunteers, selected according to the inclusion and exclusion criteria described in the clinical protocol for excretion studies approved by the local Ethical Committee. The blank urine was analysed to verify the absence of caffeine and any interfering compounds.

2.5.1.1. Sterile samples. The blank urine was clarified by filtration using three different filters (one cellulose reinforced disc membrane filter and two glass fiber filters of different pore size). After addition of sodium azide (0.1% w/v), 1 l of blank urine was spiked with caffeine to a target concentration of 14 μg/ml. Then, the urine sample was filtered through a sterilizing filter and distributed in aliquots under sterile conditions in a laminar flow cabinet. Sterile aliquots were placed in appropriate containers for its storage at the different temperatures intended for stability testing. A few aliquots were subjected to a freeze-dried process (Bioanalytical Services Ltd., Cardiff, UK).

The potential adsorption of caffeine onto the sterilizing filter was investigated by comparing caffeine concentrations obtained before and after the filtration process. About 500 ml of urine sample were filtered, and aliquots of 50 ml collected. One aliquot taken before the sterilizing filtration (aliquot 0) and four aliquots taken at regular intervals after the sterilizing filtration process (aliquots 2, 4, 7 and 10) were analysed.

Five aliquots of the sample prepared were taken at random at the end of the production step and analysed for homogeneity testing.

2.5.1.2. Non-sterile samples. A non-sterile sample was prepared by spiking blank urine with caffeine to a target concentration of 14 μg/ml. No stabilization treatments were applied.

2.5.2. Storage conditions

2.5.2.1. Sterile samples. The proposed experimental strategy includes the evaluation of the stability after long- and short-term storage, and after going through repeated freeze and thaw cycles.

- Long-term stability testing of caffreine was evaluated in liquid urine stored at 4, −20 and −40 °C, and in freeze-dried urine stored at 4 °C for 1, 2, 3, 6, 9, 12 and 18 months. An additional stock of samples was stored at −80 °C as a reference condition for comparison purposes.
- Short-term stability testing was evaluated in urine samples stored at 37 °C for 3 and 7 days. A stock of samples was stored at −20 °C for comparison purposes.
- The stability of caffeine after going through three freeze (at −20 °C) and thaw (at room temperature) cycles was also evaluated.

2.5.2.2. Non-sterile samples. The stability of the caffeine in non-sterile urine stored at −20 °C was evaluated at 1, 2, 3, 6, 9, 12 and 18 months.

2.5.3. Sample analysis

Aliquots of the sample from each storage condition (five replicates) were analysed at each storage time using the quantitative method described above. Calibration samples containing 6, 12, 16, 22 and 30 μg/ml of caffeine were prepared in duplicate. A control sample of 14 μg/ml of caffeine was analysed in triplicate in each analytical batch for internal quality control purposes. The replicates of each aliquot of sample were analysed at random in the analytical batch.

The pH of the urine sample was also measured before subjected to the extraction method.

2.5.4. Calculations

The Dixon's test ($\alpha = 5\%$) was applied to detect outliers in the replicates ($n = 5$) of each aliquot ($n = 5$) of sample. Homogeneity, adsorption of caffeine on the sterilizing filter and stability were evaluated by applying an ANOVA test ($\alpha = 5\%$) to aliquots, once outliers of replicates (if any) excluded (SPSS for Windows, version 9.0).

For long-term stability testing the ANOVA test was used to compare results of each storage condition at each prefixed time interval with the reference value. In addition, differences with respect to the reference value (percentages of change) were determined for each storage condition. To further investigate any deviation from reference values suggesting sample degradation, a linear trend analysis of concentrations at different storage times was also determined for each storage condition.

3. Results and discussion

Caffeine is a performance enhancing agent in sport and therefore is one of the compounds included in the list of classes of prohibited substances and methods of doping according to the International Olympic Committee and the World Anti-doping Agency. Due to extensive social consumption caffeine is prohibited at urinary concentrations above 12 μg/ml [20]. Accordingly, it is a candidate to be used for preparing reference materials or samples to be distributed in intercomparison exercises, with special interest for antidoping control laboratories.

3.1. Validation results

The analytical method used in this study to quantify caffeine in urine has been demonstrated to comply with the criteria for the validation of quantitative methods established according to the requirements of

different international organizations and regulatory authorities [1,21–27].

No interferences were detected at the retention times of caffeine and 7-ethyltheophylline, after the analysis of five different blank urines. In Fig. 1, the chromatogram obtained after analysis of a blank urine is compared with those obtained after analysis of a urine spiked with 14 μg/ml of caffeine.

The behaviour of the variance over the calibration range (homoscedasticity/heteroscedasticity of the procedure) was evaluated by applying the Levene test ($\alpha = 5\%$) to the results obtained after analysis of the calibration curve in quadruplicate. The procedure was found to be heteroscedastic, so peak area ratios between caffeine and the ISTD were subjected to a proportional weighted least-square regression analysis. Determination coefficients (r^2) up to 0.990 in all calibrations were observed. The *F*-test for comparison of variances was not significant ($\alpha = 5\%$), indicating adequate adjustment of the data to the proposed linear model over the range of 6–30 μg/ml.

The standard deviation of the estimated concentration values of the lowest calibration point (6 μg/ml) was used as a measure of the noise of the analytical system. The limits of detection and quantitation, defined as 3.3 and 10 times the value of noise, respectively, were estimated at 0.7 and 2.0 μg/ml.

Extraction recoveries for caffeine and 7-ethyltheophylline were 81.4±6.0 and 87.3±5.7%, respectively. No influence of time on the responses of caffeine and 7-ethyltheophylline was found.

Precision and accuracy were determined by the analysis of three replicates of control urine samples with concentrations of 10, 14 and 26 μg/ml of caffeine. Precision was expressed as the relative standard deviation (R.S.D.) of the control sample concentrations, and accuracy was expressed as the relative error (ERR) of these concentrations. Results obtained for intra-assay precision and accuracy, and inter-assay precision and accuracy are presented in Tables 1 and 2, respectively. Intra-assay precision and accuracy ranged from 1.5 to 9.2 and 2.9 to 7.4%, respectively. Inter-assay values were in the range of 4.1–5.8% for precision, and 3.9 to 5.4% for accuracy.

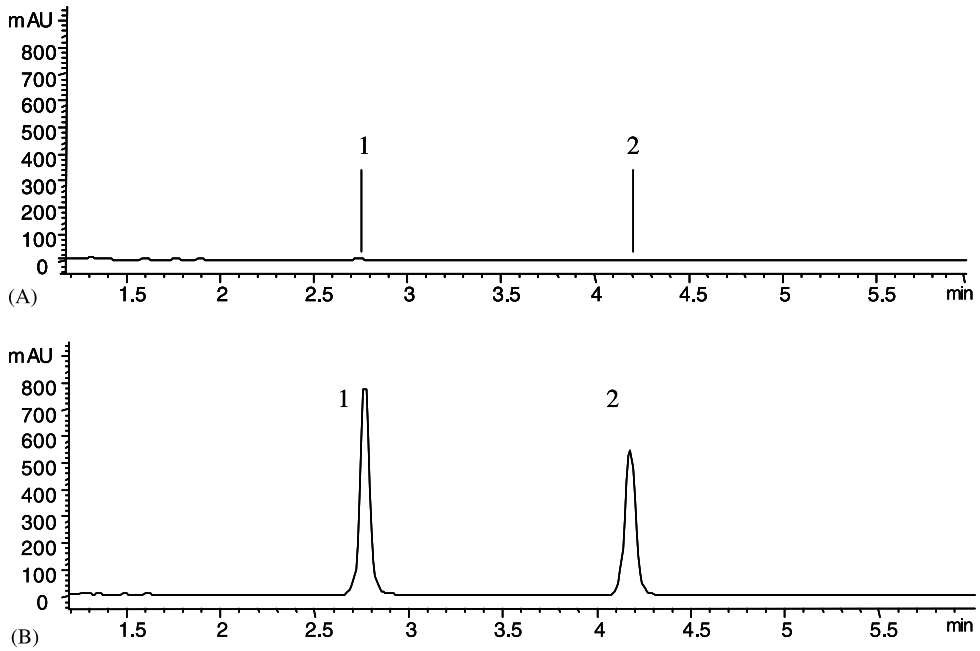

Fig. 1. HPLC chromatograms obtained after analysis of a blank urine (A) and a control urine spiked with 14 μg/ml of caffeine (B). Peak 1 corresponds to caffeine and peak 2 corresponds to 7-ethyltheophylline (ISTD).

Table 1
Intra-assay precision and accuracy obtained in the quantification of caffeine added to urine samples in three different days

Concentration (μg/ml)	Assay	*n*	Estimated concentration (mean ± S.D.) (μg/ml)	Precision (R.S.D., %)	Accuracy (relative error, %)
10	1	3	9.6 ± 0.4	4.1	4.2
	2	3	9.3 ± 0.5	5.9	7.0
	3	3	9.4 ± 0.3	2.9	5.9
14	1	3	13.6 ± 0.2	1.5	2.9
	2	3	14.5 ± 1.3	9.2	7.4
	3	3	13.6 ± 0.3	2.1	3.1
26	1	3	26.3 ± 1.5	5.6	4.6
	2	3	25.5 ± 1.2	4.5	4.0
	3	3	24.1 ± 0.6	2.5	7.3

3.2. Adsorption on the sterilizing filter

According to intramural data obtained for different drugs of abuse (e.g. THC–COOH) in the context of external quality control activities in drugs of abuse testing [28,29], some analytes are adsorbed on the filter used for the sterilizing filtration of the sample. This phenomenon may alter the homogeneity of the different aliquots of a filtrated sample, and therefore, it has to be investigated for each analyte. The verification procedure consists of assessing the minimum volume of sample that has to be wasted at the beginning of the sterilizing filtration process in order to minimise the adsorption phenomenon.

Results obtained for five aliquots of the sample analysed to check the adsorption of caffeine on the sterilizing filter are shown in Fig. 2. Differences between caffeine concentrations obtained before and after the filtration process were not statistically significant ($P < 0.05$). According to these results, no retention of caffeine on the filtration system was observed. Thus, a waste of 100 ml of sample (dead volume of the equipment) was considered to be appropriate.

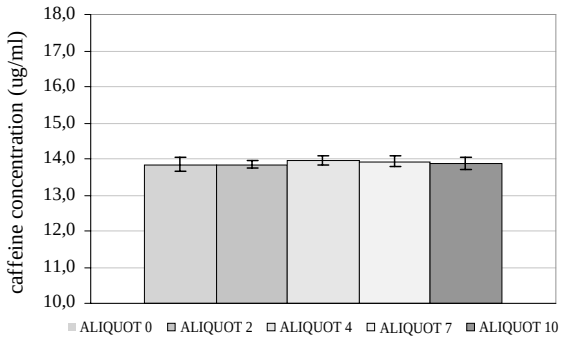

Fig. 2. Study of the adsorption of caffeine onto the sterilizing filter. Mean values of caffeine concentrations ($n = 5$) obtained for different aliquots of non-sterile (aliquot 0) and sterile (aliquots 2, 4, 7 and 10) urine.

3.3. Homogeneity testing

Differences between concentration values of the five aliquots analysed for homogeneity testing were not statistically significant ($P < 0.05$). According to these data the sample batch was considered homogeneous, indicating the adequacy of the procedure used to prepare samples containing caffeine.

Table 2
Inter-assay precision and accuracy obtained in the quantification of caffeine added to urine samples

Concentration (μg/ml)	*n*	Estimated concentration (mean ± S.D.) (μg/ml)	Precision (R.S.D., %)	Accuracy (relative error, %)
10	9	9.5 ± 0.4	4.1	5.4
14	9	14.0 ± 0.8	5.8	3.9
26	9	25.5 ± 1.2	4.7	4.6

3.4. Stability data

Stability of caffeine in urine was evaluated at different storage times and under different storage conditions using the validated method. The protocol used for stability testing was mainly focused on the evaluation of the suitability of urine samples after being exposed to those different temperature conditions most commonly encountered for their intended use as test samples in external quality assurance schemes, or as reference materials.

Although analyte stability is recommended to be evaluated at different concentration levels, in this work only one concentration of the compound has been studied. A concentration approximately 20% above the positivity criterion (14 μg/ml) has been used to assess caffeine stability. The working range of the calibration also tries to bracket the threshold concentration, independently that the analytical method has a LOD and a LOQ which allow to analyse urine samples with concentrations below the lowest calibrator.

3.4.1. Long-term stability

No significant changes with respect to the initial value were observed for the pH values measured at the different storage times for all the conditions tested. Therefore, it can be assumed that the sterilisation by filtration was effective and no microbiological degradation of the urine, which may affect the evaluation of the stability results, occurred during sample storage [30].

The evaluation of caffeine long-term stability was performed by comparison with a reference value: either the initial concentration (C_0) or the concentration obtained at a condition that may be considered as reference like −80 °C ($C_{-80\,^\circ C}$), a temperature at which any thermal or microbiological degradation is assumed to be unlikely. The evaluation criteria may differ according to the reference value used. When comparing with the initial concentration differences should be higher than the inter-assay precision of the method at the studied concentration to consider the analyte unstable at a given storage condition, while when comparing with aliquots of the sample stored at −80 °C and analysed the same day, differences should be higher than the intra-assay precision. Taking into account the validation results (Tables 1 and 2), a limit of 10% appears to be reasonable for caffeine changes.

Since intra- and inter-assay precisions of the analytical method for caffeine quantification are very similar, evaluation of the results obtained for stability testing by comparison with either the initial concentration (C_0) or the concentration obtained at −80 °C provide similar conclusions. Although comparison with the initial concentration is the criteria most commonly used to evaluate stability data published in the literature [7,8,10,11], high differences with respect to the initial concentration could be related to a lower inter-assay precision of the analytical method and not to an actual degradation of the analyte, which may lead to misleading conclusions. For this reason, for analytes with lower inter-assay precision, comparison with a reference condition like −80 °C and analysed the same day is recommended.

Concentrations and changes in caffeine concentration (expressed as percentage) obtained at the different storage conditions are shown in Table 3 and graphical data are presented in Fig. 3. Percentage changes with respect to the initial concentration were lower than 10% in all storage conditions at the studied time intervals. In Fig. 4, ratios between the concentrations obtained for the aliquots of the sample stored at the different conditions tested and the concentration in aliquots of the sample stored at −80 °C are shown. Ratios ranging from 0.96 to 1.03 were obtained.

Changes in caffeine concentration with respect to the reference value (either the initial concentration or the concentration of the aliquot stored at −80 °C) were found to be statistically significant ($P < 0.05$) at some of the conditions tested. However, in the worst case these changes were within ±6% of the initial concentration (Table 3, Fig. 3) and lower than the inter-assay precision of the method. Thus, according to the evaluation criteria described above this changes were considered irrelevant. In agreement with the inter-assay precision obtained in the validation, it is reasonable to conclude that the greatest contributor to the observed changes was the variability of the analytical method. Percentage changes in caffeine concentration with respect to aliquots of the sample stored at −80 °C were also lower than 5% (Fig. 4). According to these results, no relevant change in concentration of the caffeine was observed at any of the investigated conditions.

The slope of the linear regression of concentration versus storage time was used to evaluate trends at each

Table 3
Concentration values and differences (% of change) between the initial concentrations and the concentrations obtained after 1, 2, 3, 6, 9, 12 and 18 months in sterile liquid urine stored at 4, −20 and −40 °C, in freeze-dried urine stored at 4 °C, and in non-sterile liquid urine stored at −20 °C. The initial concentrations were: 14.33 μg/ml for non-sterile samples and 13.84 μg/ml for the rest of the conditions

Month	Freeze-dried urine 4 °C		Sterile liquid urine								Non-sterile liquid urine	
	Mean ± S.D. ($n = 5$)	% Change	4 °C		−20 °C		−40 °C		−80 °C		−20 °C	
			Mean ± S.D. ($n = 5$)	% Change	Mean ± S.D. ($n = 5$)	% Change	Mean ± S.D. ($n = 5$)	% Change	Mean ± S.D. ($n = 5$)	% Change	Mean ± S.D. ($n = 3$)	% Change
1	13.64 ± 0.19	−1.4	12.84 ± 0.30	−7.2	13.59 ± 0.25	−1.8	13.61 ± 0.16	−1.7	13.41 ± 0.20	−3.1	13.73 ± 0.09	−4.2
2	13.01 ± 0.15	−6.0	13.74 ± 0.22	−0.7	13.52 ± 0.21	−2.3	13.19 ± 0.14	−4.7	13.39 ± 0.21	−3.2	13.94 ± 0.17	−2.7
3	13.67 ± 0.21	−1.2	13.53 ± 0.51	−2.2	13.98 ± 0.14	1.0	13.84 ± 0.21	0.0	13.95 ± 0.12	0.8	14.17 ± 0.80	−4.3
6	12.83 ± 0.18	−7.3	13.01 ± 0.10	−6.0	13.21 ± 0.17	−4.5	13.33 ± 0.13	−3.7	13.30 ± 0.11	−3.9	13.94 ± 0.15	−2.7
9	13.65 ± 0.13	−1.4	13.91 ± 0.13	0.5	13.80 ± 0.11	−0.3	13.89 ± 0.10	0.4	14.03 ± 0.08	1.4	13.84 ± 0.29	−3.4
12	13.05 ± 0.07	−5.7	13.32 ± 0.30	−3.8	13.22 ± 0.20	−4.5	13.35 ± 0.13	−3.5	13.00 ± 0.05	−6.1	13.23 ± 0.14	−7.7
18	13.56 ± 0.63	−2.0	14.00 ± 0.03	1.2	13.49 ± 0.21	−2.5	13.44 ± 0.69	−2.9	13.81 ± 0.15	−0.2	13.78 ± 0.39	−3.8

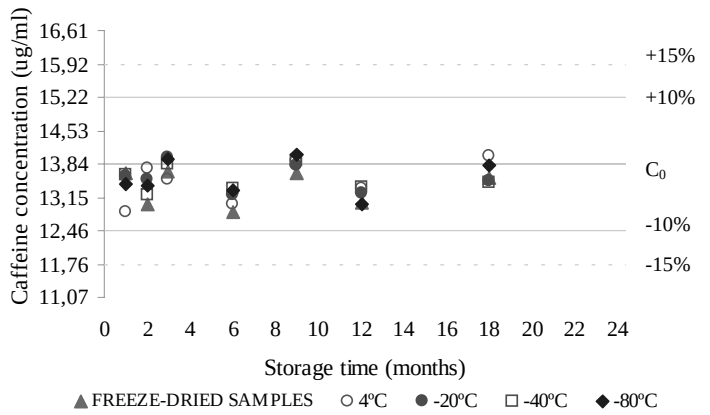

Fig. 3. Mean values of concentration ($n = 5$) obtained at the different storage conditions studied for long-term stability testing (C_0, initial concentration).

storage condition. Trend analysis may help in an early detection of sample degradation. The slopes did not deviate significantly from zero at any of the storage conditions tested, excluding trends of drug degradation.

Changes lower than 10% in caffeine concentration were also observed after the long-term storage of the non-sterile urine sample at −20 °C. Concentration values of caffeine in the non-sterile urine measured over 18 months at different time intervals are presented in Table 3 and Fig. 5. These results are important due to the fact that storage at −20 °C of non-sterile samples (e.g. B positive samples awaiting for a final decision from international sport federations) is a common current practice in most antidoping control laboratories.

3.4.2. Short-term stability

Changes in caffeine concentration (expressed as percentage of deviation over the reference value) are shown in Table 4. Differences lower than 10% and non-statistically significant ($P < 0.05$) were observed at the storage times evaluated.

Evaluation of short-term stability of caffeine was also performed by comparison with a reference value, in this case caffeine concentration in aliquots of the sample stored at −20 °C. Differences were lower than the intra-assay precision of the method and

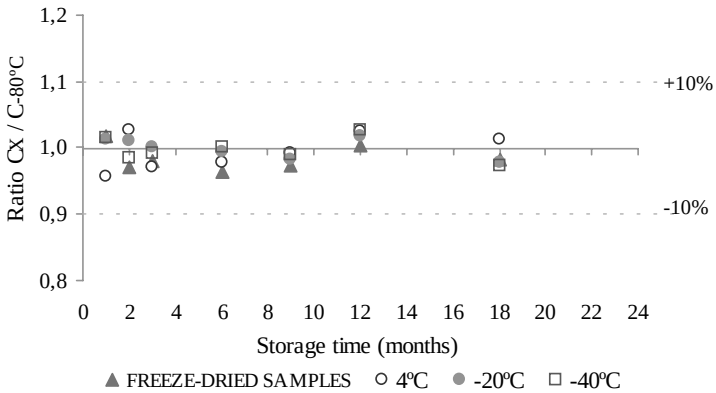

Fig. 4. Ratios between the mean values of concentration ($n = 5$) obtained at the different storage conditions studied (C_x) for long-term stability testing and the mean value of concentration ($n = 5$) of the sample aliquots stored at −80 °C ($C_{-80\,^\circ C}$).

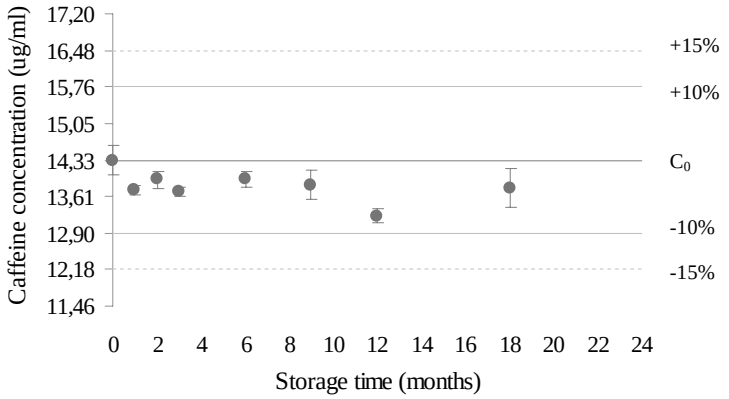

Fig. 5. Concentrations (mean ± S.D., $n = 3$) of caffeine in non-sterile urine stored at −20 °C over 18 months.

non-statistically significant ($P < 0.05$). According to these results, caffeine was found to be stable at 37 °C in sterile urine samples for at least 1 week.

3.4.3. Freeze–thaw stability

Caffeine stability was also demonstrated in urine samples subjected to three freeze and thaw cycles. Concentrations of caffeine in the aliquots subjected to repeated freeze and thaw cycles are shown in Fig. 6. Differences in concentration with respect to the aliquot not subjected to freezing and thawing were found to be non-statistically significant ($P < 0.05$).

In summary, data obtained in the different stability testing studies carried out in this work demonstrate that caffeine is stable in sterile and non-sterile urine samples for all the conditions tested. According to these results, sterile urine samples can be stored at the less demanding conditions (liquid urine at 4 °C) for at least 18 months. Furthermore, our studies have shown the adequacy of storing non-sterile samples containing caffeine at −20 °C, the most usual conditions of storage of biological samples.

Table 4
Concentration values and the differences (percentages of change) between the mean values of concentration ($n = 5$) obtained after 3 and 7 days of storage at 37 °C, and the reference value (sample stored at −20 °C) (12.86 ± 0.22 μg/ml, mean ± S.D., $n = 5$)

Day	Mean ± S.D. ($n = 5$)	% Change
3	13.20 ± 0.64	2.6
7	12.89 ± 0.52	0.2

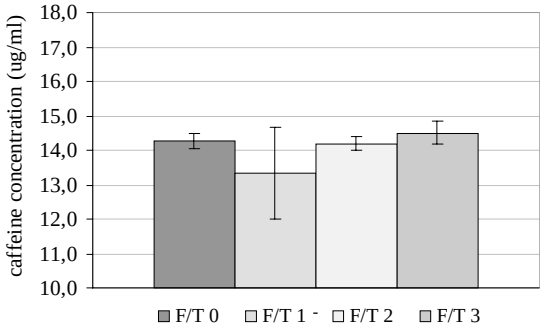

Fig. 6. Study of the stability of caffeine in urine samples after three freeze and thaw (F/T) cycles. Mean values of caffeine concentrations ($n = 5$) obtained for the aliquots of sample not subjected (F/T0) and subjected to repeated freeze and thaw cycles (F/T1, F/T2 and F/T3).

In this work, the feasibility and adequacy of the stability testing protocol and the recommended criteria for evaluating stability data have been demonstrated. In cases when the inter-assay precision of the analytical procedure is good, as in the case of caffeine, comparison with the initial concentration may be feasible. However, for analytes with lower inter-assay precision comparison with a reference condition analysed the same day is strongly recommended.

Acknowledgements

The present work has been supported by European Commission grant *ALADIN 2002*: *Analytical*

Laboratories for Antidoping control: International Network for External Quality Assessment (contract number G7RT-CT-2000-05022).

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