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Stability studies of amphetamine and ephedrine derivatives in urine

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Abstract

Knowledge of the stability of drugs in biological specimens is a critical consideration for the interpretation of analytical results. Identification of proper storage conditions has been a matter of concern for most toxicology laboratories (both clinical and forensic), and the stability of drugs of abuse has been extensively studied. This concern should be extended to other areas of analytical chemistry like antidoping control. In this work, the stability of ephedrine derivatives (ephedrine, norephedrine, methylephedrine, pseudoephedrine, and norpseudoephedrine), and amphetamine derivatives (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA)) in urine has been studied. Spiked urine samples were prepared for stability testing. Urine samples were quantified by GC/NPD or GC/MS. The homogeneity of each batch of sample was verified before starting the stability study. The stability of analytes was evaluated in sterilized and non-sterilized urine samples at different storage conditions. For long-term stability testing, analyte concentration in urine stored at 4 °C and −20 °C was determined at different time intervals for 24 months for sterile urine samples, and for 6 months for non-sterile samples. For short-term stability testing, analyte concentration was evaluated in liquid urine stored at 37 °C for 7 days. The effect of repeated freezing (at −20 °C) and thawing (at room temperature) was also studied in sterile urine for up to three cycles. No significant loss of the analytes under study was observed at any of the investigated conditions. These results show the feasibility of preparing reference materials containing ephedrine and amphetamine derivatives to be used for quality control purposes.

Keywords: Ephedrine; Norephedrine; Methylephedrine; Pseudoephedrine; Norpseudoephedrine; Amphetamine; Methamphetamine; 3,4-Methylenedioxyamphetamine (MDA); 3,4-Methylenedioxymethamphetamine (MDMA); Stability; Urine; Doping control

1. Introduction

Knowledge of the stability of drugs in biological fluids is critical for proper interpretation of analytical results. Losses of analytes due to thermal or chemical degradation, enzymatic metabolism, hydrolysis, or the presence of interfering compounds due to severe matrix degradation, may take place due to improper transport, handling or sample storage conditions. As a consequence of analyte instability, reliability of analytical results is severely compromised. Stability testing can be used to explain discrepancies between reanalyses long after initial analyses and may help to determine time limits that must be imposed between the collection and analysis of samples for pharmacokinetic studies, or to identify the optimal storage conditions for specimens retained in drug-testing and forensic laboratories. For these reasons, stability assessment is considered a fundamental parameter for the validation of bioanalytical methods [1]. At the same time, the stability of drugs has to be also evaluated in order to prepare reference materials or samples to be distributed in intercomparison exercises to ensure that differences in results between laboratories are not related to drug instability or lack of sample homogeneity [2–5].

In the area of analytical toxicology, the stability of drugs of abuse in biological specimens has been extensively studied, mainly because their presence in these matrices may involve legal consequences for individuals. Furthermore, from 1988 DHHS Guidelines for Federal Workplace Drug Testing (USA) require that laboratories shall retain all confirmed drug-positive urine samples for at least 1 year in frozen storage [6]. This issue increased the interest of toxicological labora-

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tories to ensure that analyte instability will not affect analytical results during the retesting period. A large body of data is available on the stability in urine of phencyclidine [7–9], lysergic acid diethylamide [9–13], cannabinoids [8–20], morphine and codeine [8,9,17,21–26], and cocaine/benzoylecgonine [8,10,15,17,26–29].

The stability of amphetamine derivatives has been also studied by some authors [7–9,17,30]. In most of these works, real samples (clinical or forensic specimens) were used for stability testing, and time and temperature conditions were restricted to those most commonly used for this kind of samples, 1 year or less at −20 °C. Some factors that may contribute to the stability of drugs in stored urine samples, e.g. sample pH, type of storage container, and use of preservatives have been also evaluated. None of these studies addressed the stability of other psychostimulants like ephedrine derivatives.

The misuse of drugs in an attempt to enhance performance by athletes constitutes an offence in regulated sport practice and in many aspects the control of these substances (antidoping control) is similar to drugs of abuse testing. Nevertheless there are few studies addressing the stability of doping agents in urine [31–33].

Ephedrine and amphetamine derivatives are included in the list of prohibited substances in doping control for their stimulating effects. Unlike drug abuse testing, analytical findings of amphetamines in routine doping control are reported on a qualitative basis. However, ephedrines, are reported on a quantitative basis as threshold concentrations have been defined by the World Antidoping Agency (WADA) as a positive criterion for reporting results. The availability of reference materials for drug abuse and antidoping laboratories is especially important since they are often used to test the accuracy of quantitative methods. Few organizations provide Standard Reference Materials (i.e. the National Institute of Standards and Technology (NIST)) for psychostimulant drugs (mainly amphetamine and methamphetamine) but none for ephedrine derivatives. In this work, the stability in urine samples of several amphetamine derivatives (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA)) and ephedrine derivatives (ephedrine, cathine, methylephedrine, pseudoephedrine and norephedrine) has been studied. The systematic study of the stability of amphetamine and ephedrine derivatives in urine samples will be helpful to evaluate the feasibility of preparing reference materials to be used for internal quality control or to be distributed in intercomparison exercises.

2. Experimental

2.1. Chemical and reagents

Ephedrine, norephedrine hydrochloride, methylephedrine, pseudoephedrine and methamphetamine hydrochloride were supplied by Sigma Chemicals (St. Louis, MO, USA). Cathine hydrochloride was supplied by Mack (Illertissen, Germany). Amphetamine sulfate was provided by the Spanish Ministry of Health. MDMA, MDA, and the deuterated internal standards (I.S.) amphetamine-d_5 ((±)-1-phenyl-1,2,3,3,3-pentadeutero-2-aminopropane), methamphetamine-d_8 ((±)-1-phenyl-1,2,3,3,3-pentadeutero-2-trideuteromethylaminopropane) and MDA-d_5 ((±)-1-[3,4-(methylenedioxy)phenyl]-2-(1,2,3,3,3-pentadeuteroaminopropane)) were supplied by Cerilliant (Austin, TX, USA). MDMA-d_5 ((±)-1-[3,4-(methylenedioxy)phenyl]-2-(1,2-bideutero-2-trideuteromethylaminopropane)) was purchased from Lipomed (Arlesheim, Switzerland). Etaphedrine, used as I.S. for methylephedrine and norephedrine quantification was supplied by Merrel Dow Pharmaceuticals Ltd. Methylephedrine was used as I.S. for the quantification of ephedrine, pseudoephedrine and cathine.

N-Methyl-bis-trifluoroacetamide (MBTFA) of gas chromatography grade was purchased from Macherey-Nagel (Düren, Germany). β-Glucuronidase from *Helix Pomatia* (HP-2) was purchased from Sigma. Ultra pure water was obtained using Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Bond Elut Certify® solid-phase extraction columns were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). Methanol was of high-performance liquid chromatographic (HPLC) grade. Other reagents were of analytical-reagent grade. A 1.1 M acetate buffer, pH 5.2, was prepared by adjusting the pH of a 1.1 M sodium acetate solution with acetic acid (glacial) 100% anhydrous. A 0.1 M phosphate buffer, pH 6, was prepared by adjusting the pH of a 0.1 M potassium dihydrogen phosphate solution with a 1 M potassium hydroxide solution.

Drug-free urine used for preparing spiked samples was purchased from Bio-Rad Laboratories (Irvine, CA, USA), and analyzed to verify the absence of any of the analytes of interest.

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 (100 μm of minimum thickness and 75% of porosity), and two glass fiber filters (380 μm and 200 μm of minimum thickness respectively, both with a 90% of porosity) were used. For the sterilizing filtration, a membrane filter of modified polyvinylidene fluoride and 0.22 μm of pore size was used.

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

2.2. Standard solutions

Separate stock standard solutions (1 mg/mL expressed as free-bases) of all the compounds studied and the internal standards were prepared using methanol as a solvent. The working standard solutions of 100 μg/mL and 10 μg/mL were prepared by a 1:10 and a 1:100 dilution, respectively, of the stock standard solutions with methanol. Solutions were stored at −20 °C.

2.3. Analytical methods

2.3.1. Analysis of ephedrine derivatives

To 2 mL of urine samples, 20 μL of the corresponding I.S. solution (methylephedrine or etafedrine, 1 mg/mL) was added. The urine samples were made alkaline by adding 0.2 mL of

0.5 M potassium hydroxide solution and extracted with 2 mL of *tert*-butyl methyl ether with salting-out effect (1 g of anhydrous sodium sulfate). After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 3000 × *g*), the organic phase was transferred into injection vials.

A gas chromatograph (HP 5890 series II) equipped with a nitrogen-phosphorus detector and an autosampler (HP 7673A) was used. Separation was carried using a cross-linked 5% phenyl-methylsilicone capillary column (12 m × 0.2 mm i.d., and 0.33 μm film thickness) (HP, Ultra A). Helium was used as carrier gas at a flow rate of 0.5 mL/min measured at 180 °C and as make up gas at a flow-rate of 35 mL/min. Air and hydrogen detector flows were set at 85 and 4 mL/min, respectively. Initial temperature was set at 90 °C and programmed to rise at 20 °C/min to 300 °C (held for 4 min). Total run time was 14.5 min. The sample injection volume was 3 μL. Samples were injected in the split mode with a split ratio 1:10. Injector and detector temperatures were set at 280 °C.

For quantification, calibration samples containing 1 μg/mL, 5 μg/mL, 10 μg/mL, 15 μg/mL and 25 μg/mL were used for ephedrine, methylephedrine and cathine, and 5 μg/mL, 10 μg/mL, 15 μg/mL, 25 μg/mL and 50 μg/mL for norephedrine and pseudoephedrine. Control samples containing 6 μg/mL of cathine, 12 μg/mL of ephedrine or methylephedrine, and 30 μg/mL of norephedrine or pseudoephedrine were used.

2.3.2. Analysis of amphetamine derivatives

To 1 mL of urine samples, 50 μL of the corresponding I.S. solutions (amphetamine-d_5, methamphetamine-d_8, MDMA-d_5 or MDA-d_5) was added. The pH of the urine samples was adjusted to 5.2 by adding 1 mL of 1.1 M acetate buffer. Enzymatic hydrolysis was performed by adding about 5000 Fishman units of β-glucuronidase (50 μL) to each sample and incubating in a water bath for 3 h at 55 °C. This method has been developed for its application to real antidoping control samples. For this reason, since metabolites of amphetamine derivatives are excreted in urine as their glucuronide conjugates, an enzymatic hydrolysis is needed to detect amphetamines abuse. After hydrolysis, the pH of the samples was adjusted to 6 by adding 1 mL of 0.1 M phosphate buffer. Bond Elut Certify® columns were conditioned by washing with 2 mL of methanol and 2 mL of 0.1 M phosphate buffer. The columns were prevented from drying. After applying the urine samples, columns were washed with 1 mL of 1 M acetic acid and 6 mL of methanol. Analytes were eluted with 2 mL of ethyl acetate containing 2% ammonium hydroxide. Eluates were reduced to dryness under a nitrogen stream in a water bath at 40 °C with the previous addition of 20 μL of MBTFA to prevent losses, and kept in a vacuum oven with di-phosphorus pentoxide during at least 60 min. Trifluoroacetyl derivatives (TFA) were formed by redissolving the dry extracts with 50 μL of MBTFA and incubating them at 70 °C for 30 min.

A HP 6890 series gas chromatograph system equipped with a quadrupole mass spectrometer (HP 5973 mass selective detector) and autosampler (HP 7683 series injector) was used. Separation was performed using a cross-linked 5% phenyl-methylsiloxane capillary column (12 m × 0.2 mm i.d., 0.3 μm film thickness) (HP, Ultra-2) connected to 1 m of retention gap (HP deactivated column, 0.32 mm i.d.). Helium was used as carrier gas at a flow rate of 1.0 mL/min (measured at 180 °C). The oven was maintained at 70 °C for 2 min, and then the following rates were programmed: from 70 °C to 160 °C at 30 °C/min; from 160 °C to 170 °C at 5 °C/min; from 170 °C to 200 °C at 15 °C/min; and from 200 °C to 280 °C at 30 °C/min with a total run time of 11.67 min. Samples were injected in the splitless mode. The injector and the interface temperatures were set at 280 °C. The MS was operated using electron impact ionization (70 eV) and selected ion monitoring acquisition mode. Three ions were monitored for each substance and used as qualifying ions for their identification (deviations in ion ratios higher than 20% were not accepted). The ions used for quantification were *m/z* 118 for amphetamine-N-TFA, *m/z* 154 for methamphetamine-N-TFA, *m/z* 154 for MDMA-N-TFA, *m/z* 162 for MDA-N-TFA, *m/z* 123 for amphetamine-d_5-N-TFA, *m/z* 161 for methamphetamine-d_8-N-TFA, *m/z* 158 for MDMA-d_5-N-TFA, and *m/z* 167 for MDA-d_5-N-TFA.

For quantification, calibration samples were prepared to final concentrations of 10, 500, 1000, 1500 and 2000 ng/mL for all analytes. Control samples used contained 1000 ng/mL of MDMA and MDA (in-house preparation), and 616 ng/mL of amphetamine and 602 ng/mL of methamphetamine (Urine control Level C3 Low Opiate, Bio-Rad Laboratories).

2.4. Assay validation

The analytical methods were validated according to a validation protocol previously described [34]. The following parameters were evaluated: selectivity/specificity, heteroscedasticity, linearity, limits of detection and quantification, recovery, stability, and intra- and inter-assay precision and accuracy.

2.5. Experimental design for stability testing

2.5.1. Sterile samples

2.5.1.1. Preparation of samples. Spiked urine samples listed in Table 1 were prepared as follows. Blank specimens were stabilized with sodium azide (0.1%, w/v) and clarified by filtration using three different filters (one cellulose reinforced disc membrane filter and two glass fiber filters of different pore size) before spiked with the adequate volumes of the standard solution of the drug to result in the target concentrations listed in Table 1. Then, samples were filtered through a sterilizing filter and, after discarding the initial 100 mL (dead volume of the equipment), they were distributed in aliquots under sterile conditions in a laminar flow cabinet. Adsorption of the analytes in the sterile filter was evaluated by comparison of the samples obtained before (aliquot 0) and after (aliquots 1–4) the filtration process. The homogeneity of each urine sample was checked by analyzing five aliquots taken at random at the end of the production step.

2.5.1.2. Storage conditions. According to a protocol previously described [35], the following stability conditions were studied:

- Long-term stability was evaluated by storing samples at 4 °C and −20 °C for 1, 2, 3, 6, 9, 12, 18 and 24 months. An addi-

Table 1
Composition of the samples, target and threshold concentrations (defined by the World Anti-Doping Agency) of the analytes

Sample	Compound	Target concentration (ng/mL)	Threshold concentration
Sterile sample			
Sample 1	Ephedrine	12000	10000 ng/mL
Sample 2	Norephedrine	30000	None[a]
	Methylephedrine	12000	10000 ng/mL
Sample 3	Cathine	6000	5000 ng/mL
	Pseudoephedrine	30000	None[a]
Sample 4	Amphetamine	1000	None
	MDA	1000	None
Sample 5	Methamphetamine	1000	None
	MDMA	1000	None
Non-sterile sample			
Sample 6	MDMA	620	None
Sample 7	MDMA	1250	None
Sample 8	Methamphetamine	700	None

[a] Threshold concentration before 2005: 25000 ng/mL.

tional stock of samples was stored at −80 °C as reference condition for comparison purposes.
- Short-term stability was evaluated in 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 the analytes after going through three freeze (at −20 °C) and thaw (at room temperature) cycles was also evaluated.

2.5.2. Non-sterile samples

2.5.2.1. Preparation of samples. Non-sterile urine samples were prepared as follows. Blank specimens stabilized with sodium azide (0.1%, w/v) were spiked with the adequate volumes of the standard solution of the drug to result in the target concentrations listed in Table 1. They were distributed in aliquots and stored in the conditions described in the next paragraph. The homogeneity of each urine sample was checked by analyzing five aliquots taken at random at the end of the production step.

2.5.2.2. Storage conditions. The following stability conditions were studied in non-sterile samples:

- Long-term stability was evaluated by storing samples at 4 °C and −20 °C for 6 months. The initial concentration obtained after sample preparation was taken as reference value for comparison purposes.
- Short-term stability was evaluated in samples stored at 37 °C for 7 days. A stock of samples was stored at −20 °C for comparison purposes.

2.5.3. Samples analyzed

Aliquots (five replicates) from each storage condition of the samples prepared for stability testing (Table 1) were analyzed at different time intervals according to the stability testing protocol described above. Calibration samples were prepared in duplicate. A control sample (three replicates) was analyzed in each analytical batch. The replicates of each aliquot of sample were analyzed at random in the analytical batch.

2.5.4. Calculations

The Dixon's test ($\alpha = 5\%$) was applied to detect outliers in the replicates ($n = 5$) of each aliquot of sample. Homogeneity, adsorption of the analytes on the sterilizing filter and stability were evaluated by applying an ANOVA test ($\alpha = 5\%$) (SPSS for Windows, version 11.0), once outliers of replicates (if any) were excluded. For stability testing, the ANOVA test was used to compare concentrations obtained at each storage condition (C_x) with a reference value (concentration of aliquots of sample stored at the reference condition).

In addition, to evaluate long-term stability, ratios of the mean values of concentrations obtained at each test condition (C_x) to the mean value of concentrations obtained at the reference condition ($C_{-80°C}$) were monitored over time. Differences with respect to the reference value (percentages of change) were determined for each storage condition. A linear trend analysis of concentrations and ratios of concentrations to the reference value ($C_x/C_{-80°C}$) at different storage times was also determined for each storage condition.

3. Results

3.1. Validation results

Results of the validation of the procedures for the quantification of ephedrines and amphetamines are summarized in Tables 2 and 3, respectively. Both procedures were found to be heteroscedastic, so peak area ratios between the analytes and the corresponding internal standard were subjected to a proportional weighted least-square regression analysis. Determination coefficients (r^2) up to 0.990 in all calibrations were obtained. The F tests for comparison of variances were not significant ($p > 0.05$), indicating adequate adjustment of the data to the proposed linear model over the corresponding measure range (Tables 2 and 3).

No interferences were detected at the retention times of the analytes and the internal standards after the analysis of five different blank urine samples. Limits of quantification (LOQ) ranged from 0.4 μg/mL to 2.7 μg/mL for ephedrine derivatives and from 71.0 ng/mL to 83.4 ng/mL for amphetamine derivatives (Tables 2 and 3).

No influence of injection time on the responses of the analytes and the internal standards were found. Extraction recoveries between 50% and 70% were obtained for the ephedrine derivatives. Extraction recoveries were >60% for amphetamine and MDA, and >90% for methamphetamine and MDMA.

Precision and accuracy were determined by the analysis of three replicates of control urine samples at three different concentration levels. Precision was expressed as the relative standard deviation (R.S.D.%) of the concentrations obtained for each replicate of the control samples (QC sample) and accuracy was expressed as the relative standard error (R.E.%) of these concentrations. Results of intra and inter-assay preci-

Table 2
Results of the validation of the analytical method for the quantification of ephedrines

Precision and accuracy					LOQ (μg/mL)	Recovery % (mean ± S.D.)	*U*% (*k* = 2)
QC sample (μg/mL)	Intra-assay		Inter-assay				
	R.S.D.%	R.E.%	R.S.D. %	R.E.%			
Ephedrine							
4	0.8–12.2	11.8–17.6	8.2	14.5			
8	2.1–7.0	5.7–13.2	6.3	9.2	1.2	59.6 ± 6.7	13.2
20	1.3–5.2	7.8–11.4	3.3	9.5			
Methylephedrine							
4	0.3–7.7	5.5–10.2	6.0	7.4			
6	1.1–2.6	2.2–5.3	2.7	4.2	1.3	67.5 ± 7.7	12.2
20	0.8–9.6	7.8–11.5	5.4	8.9			
Norephedrine							
8	0.9–13.3	5.3–10.8	9.1	7.7			
12	1.3–7.9	1.4–5.1	4.3	2.7	2.7	56.5 ± 2.8	8.0
40	1.4–5.6	1.8–3.9	3.6	2.9			
Cathine							
4	7.1–8.6	9.6–15.7	10.1	12.3			
6	2.4–6.6	6.5–15.3	6.1	11.5	2.0	68.8 ± 7.4	12.4
20	2.0–2.5	2.2–4.7	3.3	3.6			
Pseudoephedrine							
8	5.1–11.3	6.8–9.5	6.9	7.8			
12	1.3–3.5	3.8–6.7	2.5	5.2	0.4	69.6 ± 6.1	6.0
40	1.5–2.2	9.7–10.5	1.7	10.1			

R.S.D.: relative standard deviation; R.E.: relative error; LOQ: limit of quantification; *U*: uncertainty.

sion and accuracy obtained for ephedrines and amphetamines are presented in Tables 2 and 3, respectively. Both methods showed good precision and accuracy for all the analytes, <20% for the low-concentration control urine samples and <15% for the medium and high-concentration control urine samples.

3.2. Stability study

The potential adsorption of the analytes on the sterilizing filter was first evaluated. Differences between concentrations obtained for the 5 aliquots of sample analyzed were not statistically significant ($p > 0.05$) (Table 4). A slight decrease in con-

Table 3
Results of the validation of the analytical method for the quantification of amphetamines

Precision and accuracy					LOQ (ng/mL)	Recovery % (mean ± S.D.)	*U*% (*k* = 2)
QC sample (ng/mL)	Intra-assay		Inter-assay				
	R.S.D.%	R.E.%	R.S.D.%	R.E.%			
Amphetamine							
200	1.5–19.5	2.4–18.6	11.8	8.2			
750	4.4–8.7	5.9–7.4	6.9	6.5	83.4	59.5 ± 19.3	16.6
1750	0.9–3.0	2.3–3.7	3.5	2.8			
Methamphetamine							
200	2.3–18.0	1.7–12.8	14.7	10.2			
750	3.9–8.8	6.5–8.6	6.2	7.1	76.6	90.0 ± 4.7	10.0
1750	1.0–3.3	3.2–4.1	3.8	3.8			
MDMA							
200	1.8–18.1	2.1–12.3	7.1	9.4			
750	4.1–7.7	4.2–6.4	5.8	5.3	71.0	95.9 ± 6.1	12.0
1750	1.4–2.6	1.6–3.4	3.0	2.5			
MDA							
200	1.5–14.4	2.8–10.4	7.5	6.8			
750	4.9–11.7	3.6–11.8	8.7	8.2	78.4	65.6 ± 17.6	11.6
1750	3.1–10.5	2.2–17.2	9.7	8.9			

R.S.D.: relative standard deviation; R.E.: relative error; LOQ: limit of quantification; *U*: uncertainty.

Table 4
Concentration values (ephedrines, μg/mL; amphetamines, ng/mL) and differences (percentages of change) between the mean values of concentration ($n = 5$) obtained for the aliquots of sample not subjected to sterilizing filtration (aliquot 0) and taken after the filtration process (aliquots 1–4)

	Aliquot 0	Aliquot 1		Aliquot 2		Aliquot 3		Aliquot 4	
	Mean ± S.D.	Mean ± S.D.	% change	Mean ± S.D.	% change	Mean ± S.D.	% change	Mean ± S.D.	% change
Ephedrine	11.4 ± 0.7	10.8 ± 0.5	−4.8	10.7 ± 0.6	−5.5	10.5 ± 0.8	−7.7	10.5 ± 0.3	−7.2
Norephedrine	28.7 ± 1.5	26.8 ± 1.0	−6.6	27.2 ± 1.5	−5.3	26.6 ± 1.4	−7.4	27.0 ± 0.9	−5.9
Methylephedrine	12.4 ± 0.9	12.0 ± 0.2	−3.0	11.9 ± 0.1	−3.9	12.0 ± 0.2	−3.1	12.0 ± 0.2	−3.3
Pseudoephedrine	27.5 ± 1.0	27.9 ± 0.6	1.7	29.1 ± 1.9	5.9	26.8 ± 1.2	−2.4	28.1 ± 0.7	2.2
Cathine	4.7 ± 0.2	4.8 ± 0.1	1.7	5.0 ± 0.3	6.2	4.7 ± 0.2	−0.8	4.9 ± 0.1	4.2
Amphetamine	955.7 ± 15.3	954.2 ± 14.5	−0.2	961.4 ± 38.1	0.6	952.9 ± 7.7	−0.3	946.4 ± 21.8	−1.0
MDA	952.2 ± 14.1	918.7 ± 38.2	−3.5	899.4 ± 32.9	−5.5	930.1 ± 11.7	−2.3	867.0 ± 35.5	−8.9
Methamphetamine	953.7 ± 12.4	933.1 ± 12.8	−2.2	936.3 ± 2.7	−1.8	932.4 ± 13.6	−2.2	911.5 ± 20.5	−4.4
MDMA	1012.1 ± 13.6	985.5 ± 5.2	−2.6	988.7 ± 13.0	−2.3	979.3 ± 4.4	−3.2	970.4 ± 2.3	−4.1

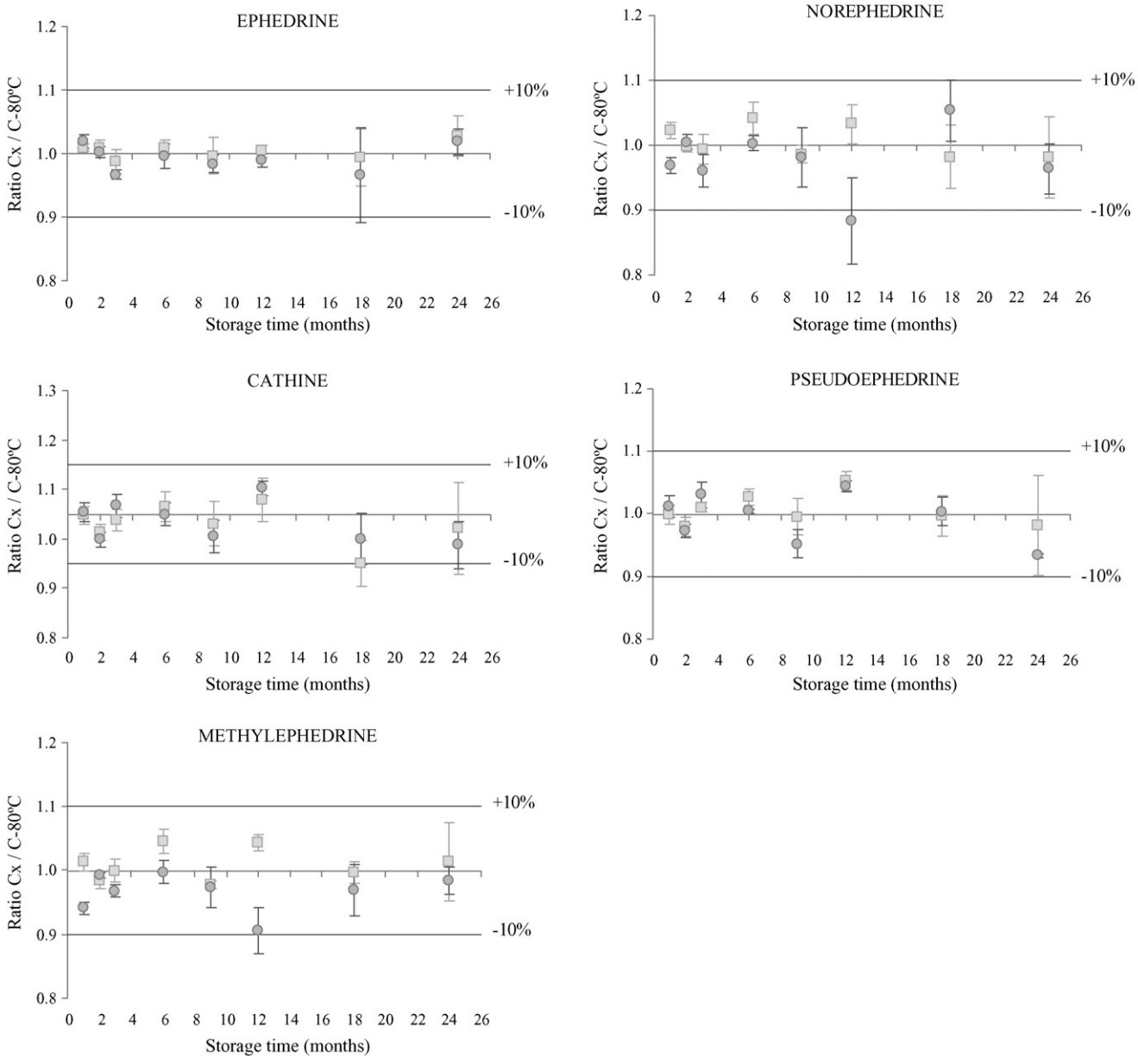

Fig. 1. Long-term stability of ephedrine, norephedrine, methylephedrine, cathine and pseudoephedrine. Ratios between the mean values of concentration ($n = 5$) obtained at the different storage conditions (C_x) and the mean value of concentration of the sample stored at −80 °C ($C_{-80°C}$). (◯), −20 °C; (□), 4 °C.

centration is observed in aliquots taken after filtration (aliquots 1–4) with respect to the aliquot taken before the filtration process (aliquot 0) for some of the compounds.

Before stability testing, the homogeneity between aliquots was verified for all samples. Differences between concentrations of the five aliquots analyzed for homogeneity testing were not statistically significant ($p > 0.05$) (data not shown), indicating that all the sample batches prepared for stability testing were homogeneous.

3.2.1. Long-term stability

The evaluation of long-term stability for sterile samples was performed by comparison with the concentration of aliquots of the sample stored at −80 °C and analyzed together with the test samples. Ratios between the concentrations of the sample stored at the different conditions and the concentration of the sample stored at −80 °C are shown in Figs. 1 and 2, for ephedrine and amphetamine derivatives, respectively. For the ephedrine derivatives, the maximum decrease in analyte concentration for samples stored at 4 °C was −10%, observed for cathine. Maximum variations in concentration obtained for the sample stored at −20 °C were observed for norephedrine, ranging from −12% to 5%. Amphetamine derivatives showed lower differences throughout the study. Changes obtained for the sample stored at 4 °C ranged from a maximum decrease of −2%, observed with amphetamine, to a maximum increase of 4% observed with MDA. Maximum changes in concentration observed for the samples stored at −20 °C were obtained for MDA, with maximum variations within ±5%.

To further investigate any deviation from the reference value suggesting loss of stability over time, trend analysis of ratios ($C_x/C_{-80°C}$) was evaluated. The slopes of the linear regressions did not deviate significantly from zero at any of the storage conditions tested for any of the analytes investigated.

In non-sterile samples, changes of concentration after 6 months of storage at 4 °C and −20 °C were not relevant for MDMA and methamphetamine. For MDMA (sample 6), changes in concentration of 5.8% and −1.7% were observed after storage of the sample at 4 °C and −20 °C, respectively (684.9 ± 21.4 ng/mL and 658.2 ± 8.2 ng/mL) compared to the initial concentration (647.3 ± 10.4 ng/mL). For methamphetamine (sample 8), changes in concentration of 2.0% and 8.5% were observed after storage of the sample at 4 °C and −20 °C, respectively (645.7 ± 4.4 ng/mL and 687.3 ± 24.3 ng/mL) compared to the initial concentration (633.2 ± 15.3 ng/mL).

3.2.2. Short-term stability

Short-term stability was studied for some of the ephedrine derivatives (ephedrine, norephedrine, methylepehedrine), and for all the amphetamine derivatives. Evaluation of short-term stability data was also performed by comparison with a reference value, in this case concentration of the analyte in aliquots

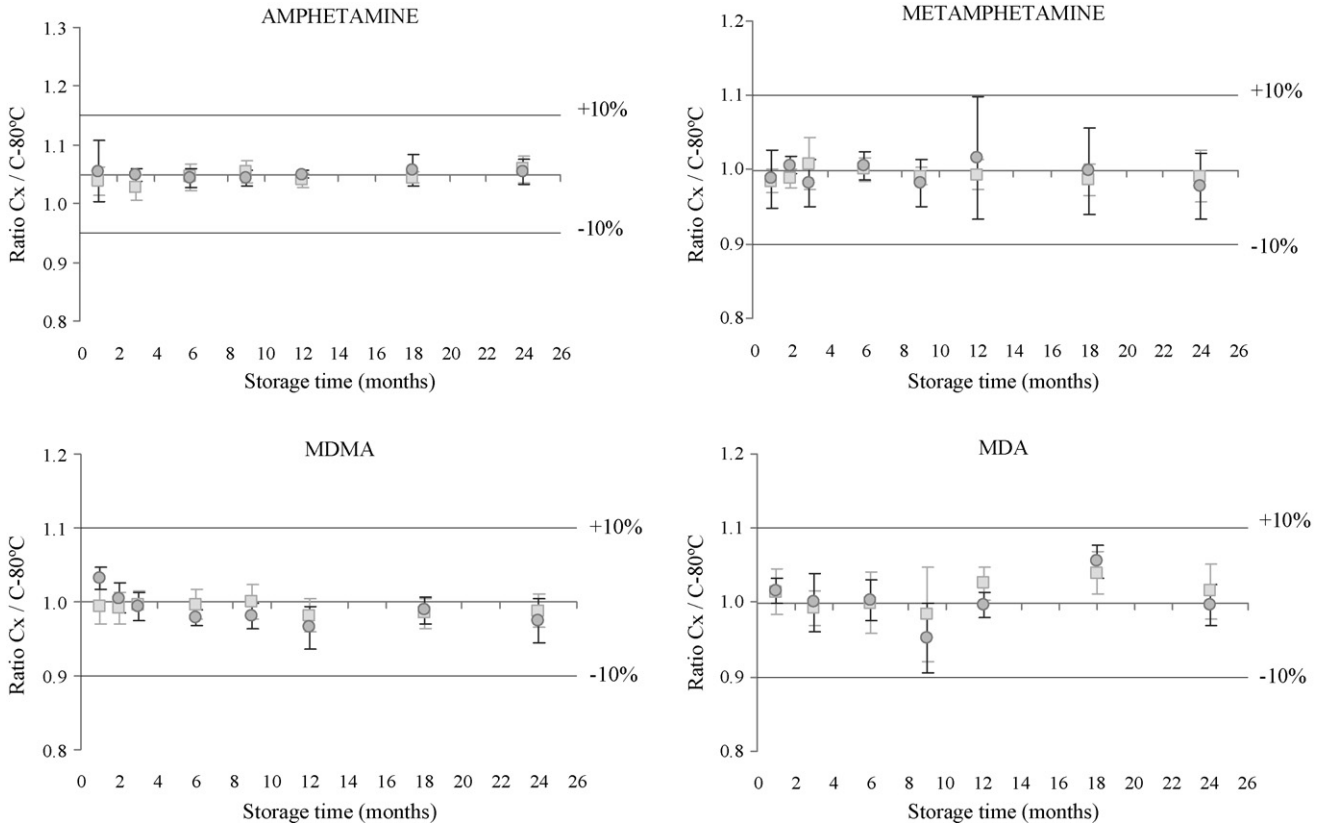

Fig. 2. Long-term stability of amphetamine, methamphetamine, MDMA and MDA. Ratios between the mean values of concentration ($n = 5$) obtained at the different storage conditions (C_x) and the mean value of concentration of the sample stored at −80 °C ($C_{-80°C}$). (◯), −20 °C; (□), 4 °C.

Table 5
Concentration values (ephedrines, μg/mL; amphetamines, ng/mL) and 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).

	−20 °C	37 °C–Day 3		37 °C–Day 7	
	Mean ± S.D.	Mean ± S.D.	% change	Mean ± S.D.	% change
Sterile samples					
Ephedrine	11.4 ± 0.3	11.5 ± 0.4	0.9	11.3 ± 0.4	−0.6
Norephedrine	28.9 ± 1.0	28.8 ± 1.1	−0.5	28.5 ± 1.1	−1.5
Methylephedrine	10.5 ± 0.1	10.4 ± 0.2	−0.3	10.3 ± 0.4	−1.4
Amphetamine	884.8 ± 6.6	895.9 ± 7.3	1.3	886.0 ± 15.5	0.1
MDA	906.0 ± 50.3	905.0 ± 18.4	−0.1	885.9 ± 29.2	−2.2
Methamphetamine	911.0 ± 11.4	917.9 ± 7.0	0.8	906.0 ± 22.0	−0.5
MDMA	998.3 ± 2.5	968.8 ± 26.9	−3.0	979.9 ± 4.6	−1.8
Non-sterile samples					
Methamphetamine	671.0 ± 19.9	–	–	687.3 ± 22.8	2.4
MDMA	1236.0 ± 20.8	–	–	1216.6 ± 48.1	−1.6

of the sample stored at −20 °C. Concentrations and changes in analyte concentration (expressed as percentage of deviation over the reference value) are shown in Table 5. Differences <1.5% and not statistically significant ($p > 0.05$) were observed for ephedrine, norephedrine, and methylepehedrine. Slight variations in concentration (<3%) were also obtained for the amphetamine derivatives. These differences were only statistically significant ($p < 0.05$) for MDMA after 7 days of storage at 37 °C, although the low percentage of variation (−1.8%) indicates that this difference can be considered irrelevant from a practical point of view.

For non-sterile samples, changes observed in concentrations of MDMA and methamphetamine after 7 days of storage at 37 °C were <2.5% and not statistically significant (Table 5).

3.2.3. *Freeze–thaw stability*

Concentrations of the ephedrine and amphetamine derivatives studied in the aliquots subjected to repeated freeze and thaw cycles are shown in Table 6. Concentrations with respect to the aliquot not subjected to freeze and thaw (F/T 0) did not show a significant decrease ($p < 0.05$) for any of the analytes investigated.

4. Discussion

According to the World Anti-Doping Code [36], amphetamines and some ephedrine derivatives are included in the list of classes of prohibited substances and methods of doping for their stimulating activities [37]. Currently, only ephedrine, methylephedrine, and cathine are prohibited, while norephedrine and pseudoephedrine were removed from the list in 2004 and placed on the WADA Monitoring Program [38]. The monitoring program allows WADA to detect patterns of misuse of these substances, in order to decide whether or not they should be added to the prohibited list. Furthermore, the use of some herbal dietary supplements containing ephedrine derivatives as "legal" alternatives to illicit drugs of abuse has raised in the last years [39]. Because they are considered natural and are available without prescription, the misconception is that these supplements are all healthy and safe [40]. This may result in an increase in the number of positive cases for ephedrine abuse not only in doping control but in the general population. Thus, amphetamine and ephedrines should be closely scrutinized in antidoping control laboratories and are candidates to be used for preparing reference materials for quality control purposes, or samples to be distributed in intercomparison exercises.

Table 6
Concentration values (ephedrines, μg/mL; amphetamines, ng/mL) and differences (percentages of change) between the mean values of concentration ($n = 5$) obtained for the aliquots of sample not subjected (F/T 0) and subjected to freeze and thaw cycles (F/T 1, F/T 2, F/T 3)

	F/T 0	F/T 1		F/T 2		F/T 3	
	Mean ± S.D.	Mean ± S.D.	% change	Mean ± S.D.	% change	Mean ± S.D.	% change
Ephedrine	13.0 ± 0.13	13.1 ± 0.1	0.7	13.1 ± 0.1	1.1	13.1 ± 0.1	0.8
Norephedrine	30.4 ± 1.0	30.4 ± 0.5	−0.03	30.4 ± 0.5	−0.1	30.3 ± 0.8	−0.4
Methylephedrine	11.1 ± 0.3	11.2 ± 0.2	0.8	11.1 ± 0.1	−0.1	11.0 ± 0.3	−1.3
Pseudoephedrine	30.4 ± 0.5	31.0 ± 0.3	1.9	31.1 ± 0.7	2.4	31.5 ± 0.3	3.8
Cathine	5.5 ± 0.2	5.6 ± 0.1	1.4	5.7 ± 0.2	3.4	5.9 ± 0.1	6.1
Amphetamine	1101.6 ± 22.9	1105.0 ± 28.2	0.3	1098.0 ± 21.6	−0.3	1122.5 ± 30.2	1.9
MDA	1056.8 ± 13.5	1033.0 ± 52.2	−2.3	1014.8 ± 72.5	−4.0	1058.1 ± 32.0	0.1
Methamphetamine	999.6 ± 13.4	1014.1 ± 6.5	1.5	1000.8 ± 23.6	0.1	1013.3 ± 11.0	1.4
MDMA	984.2 ± 7.4	979.6 ± 16.0	−0.5	983.3 ± 7.2	−0.1	986.8 ± 18.0	0.3

Ephedrine derivatives are commonly included in cold and allergy medicines, as well as in over-the-counter food and weight-loss supplements. As their ergogenic effects are reached at doses higher than therapeutic ones, high cut-off concentrations have been established by WADA in an attempt to distinguish between its therapeutic use and its misuse. Accordingly, the analyte stability was studied at one concentration level for each compound: for threshold substances (cathine, ephedrine and methylephedrine), concentrations slightly above (approximately 20%) the positivity criterion proposed by WADA [37] were used, and for the rest of analytes the selected concentrations tried to be close to those normally encountered after drug intake (see Table 1).

The use of analytical methods properly validated is necessary to obtain reliable results when performing stability studies The analytical methods used in this work to quantify ephedrine and amphetamine derivatives in urine have 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 [1,41–47].

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 interlaboratory comparisons, or as reference materials [35]. Apart from the storage conditions studied in this work (liquid urine samples stored at 4 °C and −20 °C), a similar protocol for long-term stability testing of lyophilized aliquots stored at 4 °C has been applied and the results obtained for samples containing the ephedrine and amphetamine derivatives have recently been published [48]. The stability of some of this substances in non-sterile samples was also evaluated in usual storage conditions of samples analyzed in routine antidoping control (4 °C and −20 °C).

According previous in-house data obtained for other drugs (e.g. cannabis metabolite THC–COOH) in the context of external quality control activities on drugs of abuse testing [49,50], some analytes may be adsorbed on the filter used for the sterilizing filtration of the sample. This phenomenon may alter the homogeneity of the different aliquots of a filtered sample, and therefore, it is important to investigate it for each analyte. The verification procedure consists in assessing the minimum volume of sample that has to be wasted at the beginning of the sterilizing filtration process in order to minimize the adsorption phenomenon. In our hands, no significant changes in concentration between the different aliquots of sample analyzed was observed for any of the analytes investigated. The decrease in concentration observed for some analytes in aliquots taken after the filtration process with respect to the aliquot taken before, indicates a slight retention of the analytes in the sterilizing filter that however, does not affect the homogeneity of the sample. Thus, a waste of 100 mL of sample (dead volume of the equipment) was considered to be appropriate. The sterility of the urine sample was monitored by using previously described methods [49–51].

For long-term stability, statistically significant changes ($p < 0.05$) with respect to the reference condition (−80 °C) were only observed for the ephedrine derivatives at some of the storage conditions tested. However, for both ephedrines and amphetamines, changes in concentration did not exceed the intra-assay precision of the corresponding analytical methods. Thus, these differences can be attributed to the variability of the analytical method rather than to analyte degradation, and were considered irrelevant. Accordingly, no significant trends in analyte degradation were observed at any of the storage conditions tested. No relevant changes in concentrations were also observed for MDMA and methamphetamine after storage at 4 °C and −20 °C for 6 months comparing with initial concentration in non-sterile samples.

The results obtained for amphetamine and methamphetamine urine samples stored at −20 °C agree with those of Moody et al. [17], who reported no significant change in analytes concentration for up to 17 months. Other studies have also demonstrated the stability of these drugs in non-preserved urine at different time and temperature conditions. Hughes et al. [7] reported the stability of amphetamine and methamphetamine in spiked urine samples stored at 4 °C for up to 6 months. Dugan et al. [9] studied the stability in clinical samples tested before and after 1 year of storage at −20 °C, and Paul et al. [8] investigated the effect of freezing (at −16 °C to −18 °C) on the concentration of amphetamine and methamphetamine in spiked urine samples stored for 45 days. In the same way, our observations are also in accordance with those obtained by Clauwaert et al. [30], who demonstrated the stability of MDMA and MDA in non-preserved urine samples stored at −20 °C, 4 °C and 20 °C for 21 weeks.

The same criteria as for long-term stability was used to evaluate short-term stability data. Changes in analyte concentration with respect to the reference condition (−20 °C) were lower than the intra-assay precision of the analytical methods (Table 5). Results show the stability of all the analytes investigated in sterile and non-sterile urine samples after being subjected to 37 °C for 7 days. Analyte stability was also demonstrated in sterile urine after going through 3 freeze and thaw cycles.

In summary, data obtained in the different stability studies carried out in this work demonstrates the stability of ephedrine and amphetamine derivatives in preserved sterile and non-sterile (only MDMA and methamphetamine were studied) urine samples in all the conditions of time and temperature evaluated. According to our observations, urine samples containing ephedrine and amphetamine derivatives can be stored at the least demanding conditions studied, i.e. 4 °C, for up to 24 months for sterile samples, and for up to 6 months for non-sterile samples. The methodology presented when applied to other analytes may help to determine optimal storage conditions for urine samples to be used as reference materials and for positive urine samples that should be retained in drug-testing and antidoping control laboratories. The study demonstrates the feasibility of preparing certificate reference materials of successfully studied analytes. This is of special interest for those analytes for which a cut-off concentration has been established as positivity criterion for reporting adverse analytical findings, such as amphetamine derivatives in drugs of abuse testing, and ephedrine, methylephedrine and cathine in antidoping control.

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