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Stability Studies of Selected Doping Agents in Urine Samples (I): Caffeine and Ephedrine Derivatives

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Stability studies of doping agents in urine samples.

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Introduction

According to different international quality standards, homogeneity and stability must be verified from production to end-use when producing control samples for intercomparison exercises or reference materials (1-3). Stability is the ability of a material, when stored under specified conditions, to maintain a stated property (e.g. concentration value) within the pre-selected limits within a delimited period of time. Knowledge of the stability of drugs in biological samples is critical for proper interpretation of analytical results. Furthermore, stability data is used to establish recommended storage conditions for a given reference material. Stability assessment is considered a fundamental parameter for the validation of bioanalytical methods (4).

Not surprisingly, most data available in the literature refers to the stability of some drugs of abuse (e.g. 11nor-9-carboxy-Δ^9-tetrahydrocannabinol, morphine/codeine, cocaine/benzoylecgonine, amphetamine derivatives etc...) in biological specimens (5-16). This in part is due to the fact that their presence in these matrices may involve legal consequences for individuals. Additionally these determinations are strictly regulated in some settings. Stability studies of drugs of abuse are commonly addressed to determine optimal storage conditions for clinical samples as well as control materials. The misuse of doping agents constitutes an offence in regulated sport practise and conceptually the control of these substances in biological fluids is quite similar to drugs of abuse testing.

In this work, the protocol for assessing the stability of compounds of the IOC list of Prohibited Substances and Methods in sport is described. The objective of stability testing in doping control is to identify and evaluate any significant modification or degradation of the analytes in urine (or for some substances in whole blood) when subjected over time to different storage conditions (particularly temperature). To conduct complete stability studies is rather complex and out of the scope of the present work. The protocol developed is mainly focused in the evaluation of the suitability of urine samples after being exposed to different temperature conditions. These are defined as the most commonly encountered for their intended use as test items in external quality assurance schemes, or as reference materials.

Protocol for stability studies

Sample preparation

Urine samples spiked with known concentrations of the analytes of interest were used for stability testing. A single concentration was evaluated for each substance. Except for those substances where there is a pre-defined cut-off concentration (that was used as a reference), for the rest of analytes concentrations selected tried to mimic those normally encountered in routine doping control analysis (17). Reference compounds of the highest quality commercially available were used. A pooled urine blank was obtained from healthy volunteers, selected according to inclusion and exclusion criteria described in the clinical protocol for excretion studies approved by the corresponding Ethical Committee (*Protocol for the performance of excretion studies of banned substances in healthy volunteers*) or purchased (Drug Negative Urine, Bio-Rad Laboratories, Hercules, California, USA).

In some cases, when reference materials (parent compounds or metabolites present in urine) are not commercially available, the urine for stability testing could be obtained from excretion studies after the administration of the drug to healthy volunteers according to the clinical protocol approved by the corresponding Ethical Committee.

Blank urine verification

Regardless of its origin, the blank urine sample was subjected to verification before the addition of the analytes. The verification consisted of the measurement of physico-chemical

parameters (e.g. pH, specific gravity,..), and the analysis to check the absence of interfering compounds and dope agents.

If the blank urine was of commercial origin and was distributed in different shipping containers, but all the containers belonged to the same production batch, only one of them was subjected to verification. Otherwise, each container was checked. Each collection container of urine samples from volunteers was checked separately before pooling.
To verify the absence of interfering compounds and dope agents the urine sample was analysed following standard screening methods routinely used in doping control. In addition, the confirmation method for the specific analyte that would be added to the urine was applied to check its absence. Current criteria (quantitative or qualitative) in antidoping regulations for reporting samples as positive or negative were applied.

Specific gravity and sample pH were also measured. The specific gravity had to be higher than 1.01 g/mL. The sample pH values must be within 5 and 7. In some urine samples with pH values higher than 6, the pH was adjusted within 5 and 6 with diluted hydrochloric acid.

In urine samples obtained from healthy volunteers, when pooling was necessary to obtain an adequate volume, the physico-chemical parameters were measured in the resulting final blank urine pool.

Stabilization treatments

The blank urine was submitted to different stabilization treatments: filtration for clarification and stabilization by adding sodium azide (NaN_3). The clarification of the urine was performed using a peristaltic pump and a steel filter holder containing three filters: one cellulose reinforced disc membrane filter of 90 mm of diameter, 100 μm of minimum thickness and 75% of porosity 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 porosity. NaN_3 was added to samples as preservative to a final concentration of 0.1% (w/v), except when samples were subjected to freeze-drying.
After stabilization, the blank urine was spiked with the adequate volume of solution of the analyte.

Sterilizing filtration

Once prepared, the urine sample was filtered through a sterilizing filter and aliquoted under sterile conditions. The sterilizing filtration was performed using a membrane filter of modified polyvinylidene fluoride, 0.22 μm of pore size. The process of filtration and aliquoting was performed in sterile conditions in a laminar flow cabinet using equipment and materials previously sterilized. Sterile aliquots were placed in appropriate containers for its storage at the different temperatures intended for stability testing.

Verification procedure of the potential adsorption of analytes on sterilizing filters

The potential adsorption of the analytes onto the sterilizing filter was investigated. When such kind of phenomenon appears, the analyte present in the initial volumes of the filtered sample tends to saturate the filter. Once the filter is saturated, adsorption is no longer observed. The verification procedure consists in assessing the minimum volume of sample that had to be wasted at the beginning of the sterilizing filtration process in order to minimise the adsorption phenomenon that may alter the homogeneity of aliquots. For this purpose, a minimum volume of 500 ml of urine spiked with the analyte at the same concentration as the stability sample was prepared. One aliquot of the sample of 50 mL was separated and stored at –20°C until analysis without going through the filtration step. The sterilizing filtration process was applied to the remaining urine sample, collecting aliquots of 50 ml. Aliquots obtained after the sterilizing filtration were taken at regular intervals (aliquots number 2, 4, 7 and 10) and analysed (5 replicates), together with the samples not subjected to sterilizing filtration, using the corresponding quantitative method. The Dixon's test (α=5%) was applied to detect outliers in the replicates of each sample aliquot. The adsorption of the analyte in the sterilizing filter was estimated by the analysis of variance (ANOVA) or the student's t-test. The statistical test was applied to the 5 replicate determinations per aliquot, once outliers (if any) excluded. The objective of this analysis is to determine if there is an adsorption phenomenon. If that is the case, concentrations should differ depending on the aliquot considered. In addition this analysis should help in defining the initial volume of sample to be discarded. The results obtained for each aliquot subjected to sterilizing filtration were in addition compared with those obtained for the aliquot of sample not subjected to it.

Sample storage

After the sterile filtration and aliquoting process, and the verification of the sample homogeneity, the samples subjected to stability testing were stored at the different temperature conditions according to the proposed protocol.

Homogeneity testing

Homogeneity of urine samples was checked to ensure that differences between aliquots (e.g. in concentration values) would not significantly affect the evaluation of the stability results. For this purpose a 5% of the aliquots of sample prepared for stability testing, and a minimum of 5, were taken at random at the end of the production step, after the material was packaged in its final form. Each sample aliquot (replicates, n=5) was analysed applying the analytical method used for stability testing.

Homogeneity of the sample batch was estimated by the analysis of variance (ANOVA) method. The Dixon's test (α=5%) was also applied to detect outliers in the replicates of each aliquot. A one-way ANOVA was applied to the 5 replicate determinations per unit, once outliers excluded. The between-aliquots variance had to be statistically lower than the within-aliquots variance (α=0.05) to conclude that the batch was homogeneous.

Experimental design for stability testing

The proposed experimental strategy for stability testing includes the evaluation of the stability of the analytes in urine after long and short-term storage, and after several freeze and thaw cycles of the samples.

Long-term stability testing includes, for a period of 24 months, the analysis of samples stored at different temperatures at prefixed time intervals. The stability of the analyte in liquid urine was evaluated at 4°C and -20°C. For some compounds, -40°C was also evaluated. Additionally, the stability of the analyte in freeze dried aliquots, stored at 4°C, was evaluated. A stock of samples was also stored at -80°C for comparison purposes. Samples stored at each condition were analysed (n=5 replicates) using the quantitative methods applied in routine doping control in our laboratory. Following the initial quantitative analysis, samples are planned to be reanalysed at 1, 2, 3, 6, 9, 12 and 24 months after sample preparation. The

sample pH was measured at each time interval to ensure that no microbiological degradation of the urine sample occurred during storage, as it may affect the evaluation of the analytical results.

Short-term stability was evaluated on liquid urine stored at 37°C for 3 and 7 days. A stock of aliquots was also stored at -20°C for comparison purposes. Stability samples used to determine the stability of the analytes after 3 or 7 days of storage at 37°C were removed from the incubator and stored at –20°C until analysis. Sample aliquots, corresponding to days 0, 3 and 7, were analysed (n=5 replicates) in the same analytical batch.

The effect of repeated freezing (at –20°C) and thawing was also studied for up to 3 cycles (F/T 1, F/T 2 and F/T 3). After the repeated freeze and thaw cycles, 5 replicates of each sample aliquot were analysed using the corresponding quantitative method. A summary of time and temperature conditions is presented in Table 1.

Table 1.- Stability testing: summary protocol

Assay	**Time period**	**Storage temperature**								
		-80°C	**-40°C**	**4°C**	**-20°C**			**4°C**		**37°C**
				F/T 0	F/T 1	F/T 2	F/T 3	Freeze dried	Liquid	
Assay 1	Month 0	-	-	1	1	1	1	-	-	-
Assay 2	Month 0, Day 3	-	-	-	1	-	-	-	-	1
Assay 3	Month 0, Day 7	-	-	-	1	-	-	-	-	1
Assay 4	Month 1	1	1	-	1	-	-	1	1	-
Assay 5	Month 2	1	1	-	1	-	-	1	1	-
Assay 6	Month 3	1	1	-	1	-	-	1	1	-
Assay 7	Month 6	1	1	-	1	-	-	1	1	-
Assay 8	Month 9	1	1	-	1	-	-	1	1	-
Assay 9	Month 12	1	1	-	1	-	-	1	1	-
Assay 10	Month 18	1	1	-	1	-	-	1	1	-
Assay 11	Month 24	1	1	-	1	-	-	1	1	-
Total assays*		**8**	**8**	**1**	**11**	**1**	**1**	**8**	**8**	**2**

* Total assays refer to the number of aliquots to be tested. Several replicates of each aliquot (n=5) were subjected to analysis.

Results evaluation for stability testing

The evaluation of analyte stability is performed by comparison with a reference value. For long-term stability the reference value was considered either the initial concentration or the concentration obtained at a reference condition, i.e. concentration of the analyte in urine sample stored at -80°C, a temperature at which any thermal or microbiological degradation is unlikely. For short-term stability the reference value was the concentration of the analyte of urine sample aliquots stored at -20°C.

The Dixon's test (α=5%) was applied to detect outliers in the replicates of each sample aliquot. The ANOVA or the Student's t-test was applied to the 5 replicate determinations per aliquot, once outliers excluded, to compare the results of each storage condition with the reference value. In addition, differences between the mean values of concentration at a given storage condition and the reference value were calculated. Differences with respect to the reference value had to be higher than 15% (criterium commonly used for the acceptance of the intra- and inter-assay precision in the validation of analytical methods) and statistically significant ($p<0.05$) to consider the analyte unstable at a given storage condition.

For long-term stability, the slope of the linear regression of concentrations at different storage times was also used to evaluate trends at each storage condition that may help in an early detection of sample degradation. In this case, the slope had to be not significantly different from zero.

A one-way ANOVA was also applied to determine the analyte stability after repeated freeze and thaw cycles. Results obtained for each freeze and thaw cycle (F/T 1, 2 and 3) were compared with those obtained for sample aliquots not subjected to freeze and thaw (F/T 0).

Conclusions

Protocols for homogeneity and stability testing for dope agents in urine samples to be used in external quality assurance schemes or as reference materials have been developed. The feasibility and adequacy of the protocols for the objectives proposed have been studied with

samples containing caffeine and ephedrine derivatives (ephedrine, norephedrine, methylephedrine, pseudoephedrine, and norpseudoephedrine).

According to the evaluation criteria proposed, most of the compounds were found to be stable in all storage conditions during the periods of time evaluated up to now (caffeine and ephedrine 9 months, rest of compounds 6 months).

Stability studies with other dope agents are planned to be performed within the framework of the project *ALADIN 2002* dealing with the set-up of an external quality assurance scheme for antidoping control laboratories.

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