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Measurement uncertainty for the determination of amphetamines in urine by liquid-phase microextraction and gas chromatography-mass spectrometry

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ABSTRACT

A gas chromatography-mass spectrometry method for the determination of amphetamines in urine samples by means of liquid-phase microextraction was validated, including calculation of measurement uncertainty. After extraction in the three-phase mode, acceptor phase was withdrawn from the fiber and the residue was derivatized with trifluoroacetic anhydride. The method showed to be very simple, rapid and it required a significantly low amount of organic solvent for extraction. The limits of detection were 10 and 20 μg/L for amphetamine and methamphetamine, respectively. The calibration curves were linear over the specified range (20 μg/L to 1400 μg/L; $r^2 > 0.99$). The method showed to be both precise and accurate and a relative combined uncertainty of 2% was calculated. In order of importance, the factors which were more determinant for the calculation of method uncertainty were: analyte concentration, sample volume, trueness and method precision.

1. Introduction

According to the United Nations Office on Drugs and Crime, amphetamines are the second class of most used illicit drugs in the world. Their consumption for recreational purposes has increased significantly over the past years due to its easy availability and low cost. Amphetamine (AM) and methamphetamine (MA) are two important central nervous system stimulants that are often abused both by drug addicts and recreational users [1–4].

Urine drug testing has been widely applied in forensic and clinical toxicology and the most commonly techniques used for the extraction or pretreatment of compounds present in biological fluids for further chromatographic analysis are liquid- liquid extraction (LLE) [5,6] and solid phase extraction (SPE) [7–11]. However, current trends in analytical chemistry suggest to use smaller quantities of samples and to develop methods which use minimum volumes of organic solvents. Recently, two solvent-free sample pretreatment techniques, the liquid-phase microextraction (LPME) and the hollow fiber LPME (HF-LPME) techniques, firstly introduced by Pedersen-Bjergaard and Rasmussen [12], attracted increasing attention for the analysis of illegal drugs [13–15]. They can be considered as evolutions of solvent microextraction. In particular, HF-LPME is based on the use of a cylindrical porous membrane made of polypropylene, which presents low cost and it is disposable [16–21].

For many reasons, forensic toxicologists are being asked to determine and report measurement uncertainty (MU) in their methods for drug analysis [22]. MU has been increasingly required in analytical toxicology by quality management standards in order to demonstrate that a laboratory is producing data that are fit for purpose and is necessary as a validation parameter in international standards such as ISO 17025 [23]. The uncertainty associated with the result of a measurement, characterizes the dispersion of the values that can be fundamentally attributed to that result. It is a measure of confidence that helps in decision with respect to decisional values. The assessment of the MU was originated from the area of analytical chemistry, and its importance has been lately spread to forensic analytical chemistry, to support effective decision making [24,25].

Two approaches for the estimation of measurement uncertainty are generally proposed: top-down, based on inter-laboratory studies or validation studies results, and bottom-up, where all conceivable sources of uncertainty are systematically evaluated. The latter has been recommended by the Guide to the Expression of Uncertainty in Measurement (GUM). In general, ISO/IEC 17025 which defines the requirements for accreditation of laboratories refers to both approaches [26]. The process of estimation of measurement uncertainty using a bottom-up

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approach involves four steps: specification of the measurand, identification of sources of uncertainty based on the elaboration of a "cause and effect" diagram, quantification of uncertainty components and calculation of the combined standard uncertainty [27–30].

Therefore, the aim of the present work was to validate the determination of amphetamines in urine samples by using HF-LPME and gas chromatography mass spectrometry, and to calculate the uncertainty of the method.

2. Experimental

2.1. Chemicals and reagents

Methanolic solutions of amphetamines at a concentration of 1000 mg/L and the internal standards AM-*d5* (deuterium label on ring) and MA-*d5* (deuterium labeled on side chain) at a concentration of 100 mg/L were obtained from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Sodium hydroxide, hydrochloric acid and sodium chloride were purchased from Merck (Darmstadt, Germany). Dihexyl ether, trifluoroacetic anhydride (TFAA) and ethyl acetate was purchased from Sigma–Aldrich (MO, USA).

Hollow-fiber Q3/2 Accurel KM polypropylene (600 μm, 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). Gel-loading pippeter tips Round CC 4853 (0.5 mm; 1–200 μL) were purchased from Costar (Corning, NY, USA). Extractions were conducted using a multi-tube vortexer model VWR VX-2500 (Thorofare, NJ, USA).

2.2. Preparation of standards solutions

Working solutions of AM and MA, the internal standards AM-*d5* and MA-*d5*, at concentrations of 10 mg/L, were prepared from the stock solutions by pipetting 100 μL of the stock solutions and diluting with 900 μL of methanol. Stock solutions obtained from Cerilliant were stored at 2° to 8 °C when not in use.

2.3. Instrumental analysis and data acquisition

The analyses were performed using an Agilent 6850 Network GC System gas chromatograph coupled with an Agilent 5975 Series quadrupole mass selective detector (MSD) (Wilmington, DE, USA). Samples were injected into the GC–MS by means of an autosampler (Agilent 7693). Chromatographic separation was achieved on a HP-5MS fused-silica capillary column (30 m × 0.25 μm × 0.1 μm film thickness) (Agilent Technologies, Wilmington, DE, USA) using helium as the carrier gas at 0.6 mL/min at a constant flow rate mode. The MSD was operated by electron ionization (EI) (70 eV) in selected ion monitoring (SIM) mode. The injector was operated in the splitless mode and injection volume was 1.0 μL. The injector and transfer line temperature were 220 °C and 250 °C, respectively. The oven temperature was maintained at 80 °C for 2 min, programmed at 20 °C/min with a hold at 200 °C for 5 min. Programmed again at 25 °C/min with a hold at 275 °C for 1 min (run time 12 min). The following ions were chosen for identification and quantification: AM, *m/z* 91 (45%), 118 (50%), 140 (base peak); AM-*d5* 96 (90%), 123 (base peak); MA *m/z* 110 (35%), 118 (30%), 154 (base peak); MA- *d5*, *m/z* 122 (30%), 158 (base peak). The underlined ions were used for quantification.

The acceptance criteria for qualification were: retention time within 2% compared with standards analyzed in the same batch and relative abundance of qualifier ions with relative intensities which match those of standards analyzed in the same batch with an allowable error of ±20% [31].

The software used for data acquisition was Agilent ChemStation Enhanced Data Analysis Software. Data were further processed with Microsoft Excel® 2010.

2.4. Urine samples

Authentic human urine samples ($n = 10$) were obtained from the Laboratory of Toxicological Analysis of University of São Paulo, as part of workplace drug testing programs. These samples were tested by immunoassay methods using Syva EMIT immunoassay II Plus on a Dimension analyzer (Siemens Erlangen, Germany) and samples with positive results for the amphetamines group (AM/MA) (cut off > 1000 μg/L) in the screening test were selected for confirmation by the technique developed in our present work. The protocol of this study was previously approved by the Faculty of Pharmaceutical Sciences Ethics Committee, University of São Paulo, Brazil (Ethics Protocol Approval no. 3001).

2.5. Sample preparation

In a 5-ml glass tube, 50 μL of IS working solutions (AM-*d5* and MA-*d5* at 10 mg/L) were added to 1.0 mL of urine. The pH was adjusted using 200 μL of NaOH solution at a concentration of 1 mol/L (pH > 12) and the content was then transferred to an Eppendorf tube containing 10 mg of NaCl. A hollow fiber was cut into 9-cm segments and its pores were filled with dihexyl ether (organic phase). The lumen of the hollow fiber was filled up with 30 μl of HCl solution at a concentration of 0.01 mol/L (acceptor phase) by means of a micropipette. The fiber was submerged into the urine sample solution in a U-shape configuration and the system was submitted to agitation at 1200 rpm for 60 min in an orbital shaker. The extraction process was carried out at room temperature (20–24 °C), avoiding formation of bubbles and evaporation of the organic solvent impregnated into the fiber. The acceptor phase was then withdrawn from the fiber and dried under nitrogen stream (N_2) and the residue was derivatizated with 50 μL of TFAA and 50 μL of ethyl acetate at 70 °C for 15 min. After cooling down, the samples were dried at 40 °C under N_2 stream) and re-suspended in 50 μL of ethyl acetate. An aliquot of 1.0 μL was then injected into the GC-MS system.

2.6. Optimization of the method

A Box-Behnken experimental design was applied for three factors that should be relevant for HF-LPME efficiency: acceptor phase (HCl at 0.01 and 0.1 mol/L), time of extraction (30 and 60 min) and molarity of the donor phase (NaOH at 0.1 and 2.0 mol/L). A total of 17 samples (with 5 center points) were evaluated by the absolute area produced by each analyte in all tested conditions. The statistical software Origin® (version 9) was used in the design of the experiment and to perform the result analysis through surface response.

2.7. Validation of the method

The method was validated according to international guidelines and recommendations for validation of analytical methods in Forensic Toxicology [31–34], establishing selectivity, limit of detection (LOD), lowest limit of quantification (LLOQ), linearity and linearity range, recovery, precision, accuracy, dilution integrity.

2.7.1. Selectivity

To evaluate selectivity, ten different drug-free urine samples were extracted and analyzed according to the previously described method. Additionally, ten blank urine samples fortified with