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APPLICATION NOTE

Direct Analysis of Amphetamine Stimulants in a Whole Urine Sample by Atmospheric Solids Analysis Probe Tandem Mass Spectrometry

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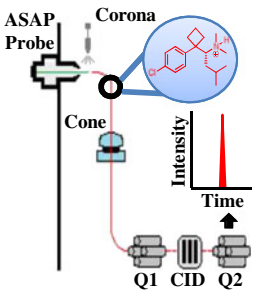

Abstract. Amphetamine-type stimulants (ATS) are among illicit stimulant drugs that are most often used worldwide. A major challenge is to develop a fast and efficient methodology involving minimal sample preparation to analyze ATS in biological fluids. In this study, a urine pool solution containing amphetamine, methamphetamine, ephedrine, sibutramine, and fenfluramine at concentrations ranging from 0.5 pg/mL to 100 ng/mL was prepared and analyzed by atmospheric solids analysis probe tandem mass spectrometry (ASAP-MS/MS) and multiple reaction monitoring (MRM). A urine sample and saliva collected from a volunteer contributor (V1) were also analyzed. The limit of detection of the tested compounds ranged between 0.002 and 0.4 ng/mL in urine samples; the signal-to-noise ratio was 5. These results demonstrated that the ASAP-MS/MS methodology is applicable for the fast detection of ATS in urine samples with great sensitivity and specificity, without the need for cleanup, preconcentration, or chromatographic separation. Thus ASAP-MS/MS could potentially be used in clinical and forensic toxicology applications.

Keywords: ASAP-MS/MS, Ambient mass spectrometry, Biological fluid, Amphetamine stimulants, Forensic toxicology

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Introduction

Amphetamine-type stimulants (ATS) are among the illicit stimulant drugs that are most often used worldwide [1]. ATS act by inducing the release of monamines from nerve terminals in the brain. These drugs markedly affect the mental function and elicit behavior such as insomnia, anxiety, aggressive behavior, and addiction potential. Moreover, in some countries, ATS have been the first choice for the treatment of obesity in women [2].

In recent years, an increasing number of analytical methodologies based on high-performance liquid chromatography mass spectrometry (HPLC-MS) [3, 4] and gas chromatography mass spectrometry (GC-MS) [1, 5] have been developed to detect ATS. However, most of these methods involve time-consuming procedures, especially tedious and elaborate sample pretreatment that includes liquid-liquid extraction, solid phase extraction, and sometimes derivatization to enhance sensitivity [5]. In this context, a major challenge nowadays is to develop a fast and efficient methodology that requires minimal sample preparation to analyze trace levels of ATS in biological fluids.

In the last decade, one of the most important advances in the field of mass spectrometry was the development of ambient mass spectrometry (AMS) techniques, such as desorption electrospray ionization (DESI), direct analysis in real time (DART), easy ambient sonic spray ionization (EASI), and atmospheric solids analysis probe mass spectrometry (ASAP) [6]. In ASAP-MS, the operator simply dips the end of the sealed glass capillary into the solid sample or solution, to coat the outside of the capillary with the sample. The operator then removes any excess sample, inserts the capillary into an ASAP probe, and introduces the probe into the atmospheric pressure

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ionization (API) source. Sample vaporization occurs under the hot nitrogen stream of the electrospray (ESI) or atmospheric pressure chemical ionization (APCI) desolvation gas. An APCI-like process promotes sample ionization in the gas phase, where the corona discharge needle produces a plasma that ionizes the analytes and generates protonated molecules in the presence of water or radical ions in dry conditions [7]. A significant advantage of ASAP-MS is that introduction of the sample into the mass spectrometer ion source dismisses the need for a vacuum lock or an interface with HPLC [6]. Moreover, the analytes can be ionized under open-air and then directly and rapidly analyzed with high throughput and minimal simple pretreatment [8]. ASAP-MS has been successfully applied to analyze biological fluids [8], steroids [9], some drugs [10, 11], and metabolites in urine [8, 12].

Here we examine whether atmospheric solids analysis probe coupled to tandem mass spectrometry (ASAP-MS/MS) provides adequate sensitivity to detect ATS in urine samples.

Experimental

The amphetamine (**1**), methamphetamine (**2**), ephedrine (**3**), sibutramine (**4**), and fenfluramine (**5**) standard compounds (Figure 1) were purchased from Cerilliant (Round Rock, TX, USA). Formic acid (FA; Sigma-Aldrich, St. Louis, MO, USA), acetonitrile (ACN, HPLC grade), and methanol (CH_3OH, HPLC grade) were acquired from Merck (Darmstadt, Germany). The standard solutions were prepared by diluting the stock solutions of the stimulants (1.0 mg/mL) in CH_3OH. The resulting solutions were stored at –20 °C throughout the development of the methodology.

The samples were analyzed on a Xevo TQ-S tandem quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Waters ASAP probe. The instrument was operated by using the same source conditions optimized in the positive APCI mode, as follows: API source temperature = 150 °C, desolvation gas flow = 200 L/h, desolvation gas temperature = 200 °C, cone gas flow = 150 L/h, and nebulizer gas flow = 7 bar. The corona current and the cone voltage were optimized to 8 μA and 40 V, respectively, to improve detection sensitivity.

All the samples were analyzed in the multiple reaction monitoring (MRM) scan mode. For the collision-induced dissociation (CID) technique, collision energy (CE) values ranging from 5 to 50 eV (with stepup to 5 eV each time) were optimized for each compound. The samples were injected into the ASAP source via direct infusion. Argon at 0.15 mL/min was used as collision gas. The dwell time established for each transition was 0.105 s, and the interscan delay was set to the automatic mode. The resulting MS data were processed with the MassLynx v4.1 software.

First, stock solutions of compounds **1**–**5** alone were prepared at a concentration of 1.0 mg/mL by dilution of the standard compounds in CH_3OH. Next, a urine pool solution containing compounds **1**–**5** at concentrations ranging from 0.5 pg/mL to 100 ng/mL was prepared by dilution of the stock solutions in 2 mL of whole urine without previous cleanup, preconcentration, or purification. The whole urine used in this experiment was obtained from a volunteer who did not use amphetamine stimulants. Another experiment was performed by using a urine sample and saliva collected from a volunteer contributor (V1) who was using sibutramine for weight loss. Ethical approval for this study was granted by the ethical committee of our University (Ethics Committee of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, Ribeirão Preto, Brazil - letter no. 32/2012). The volunteers who participated in the study gave their consent prior to inclusion. The ASAP probe containing a sealed glass capillary was dipped five times into the urine pool solution to a minimum depth of 5 mm, and the excess liquid was removed. Then, the capillary was attached to the MS probe, and the urine pool solution (urine spiked with compounds **1**–**5**) or the urine sample and saliva collected from V1 was directly introduced into the ASAP source used in the MS/MS experiments. In this setup, the minimum sample volume required for analysis was 300 μL. Prior to use, the glass capillary was rinsed with a mixture of H_2O/CH_3OH (50:50, v/v) and subsequently inserted

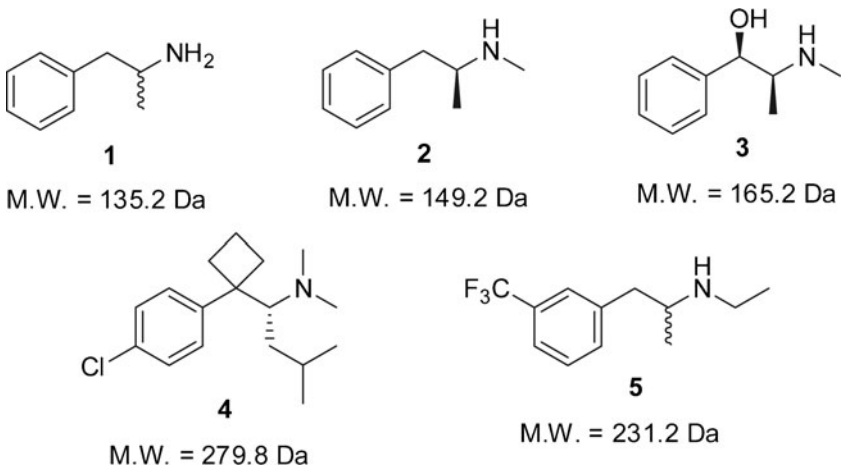

Figure 1. Chemical structures of amphetamine (**1**), methamphetamine (**2**), ephedrine (**3**), sibutramine (**4**), and fenfluramine (**5**)

Table 1. ASAP-MS/MS Ion Transitions, Instrument Settings for the Standard Compounds 1-5, LOD (S/N = 5), and repeatability at 1.0 ng/L (n = 3) for the compounds analyzed in whole urine

Compound	Precursor ion $[M + H]^+$ (*m/z*)	Specific product ion (*m/z*)	Target ion transition	DP[a] (V)	CE[b] (eV)	LOD (ng/mL)	Repeatability (RSD, %)
Amphetamine (**1**)	136	91 ($C_7H_7^+$)	136 → 91	20	15	0.40	10.3
Methamphetamine (**2**)	150	91 ($C_7H_7^+$)	150 → 91	30	15	0.10	14.4
Ephedrine (**3**)	166	148 ($[\mathbf{3}+H-H_2O]^+$)	166 → 148	30	10	0.19	10.4
Sibutramine (**4**)	280	125 ($C_7H_6Cl^+$)	280 → 125	10	25	0.003	13.8
Fenfluramine (**5**)	232	159 ($CF_3C_7H_7^+$)	232 → 159	20	20	0.002	12.2

[a] DP = declustering potential
[b] CE = collision energy

into the ASAP probe at 500 °C for at least 20 s to clean the surface of the capillary from any residual contamination. In the case of urine samples spiked with compounds **1**–**5** and of the urine sample collected from V1, the same procedure was carried out under the aforementioned conditions. A new glass capillary was used for each sample to minimize the chances of carryover and cross-contamination [8]. All the analyses were conducted in triplicate.

Results and Discussion

Prior to the ASAP-MS/MS experiments, a full scan (ASAP-MS) spectrum of the urine pool solution was obtained, and the peaks of the protonated molecules of the target compounds were identified (Supplementary Figure S1, see Supplementary Material). Then, specific product ions for each target compound were used during MRM analysis. Table 1 shows the precursor ions (protonated molecules) and the specific product ions of compounds **1**–**5**, as well as the optimum declustering potential (DP) and collision energy (CE) values of each compound. At optimum CE values, the specific product ion of each compound was the most intense in the MS/MS spectrum of the corresponding protonated molecules.

Doue and co-workers demonstrated that the desolvation gas temperature affects desorption of the analytes from the probe because complete vaporization of compounds without thermal degradation is the main condition to reach a signal with higher intensity [9]. In this context, the effects of the desolvation gas temperature (from 150 °C to 250 °C) on the signal intensity were also examined in this work.

Our findings revealed that the response signals of the analytes were at a maximum for a desolvation gas temperature of 200 °C. Whole urine and the urine pool solution (urine spiked with compounds **1**–**5** at concentrations from 0.5 pg/mL to 100 ng/mL) were analyzed under the optimized ASAP-MS/MS conditions. Figure 2 shows the MRM traces of the whole urine and urine spiked with analytes **1**–**5**. Analysis of MRM traces from Figure 1 attested to the specificity of the MRM method for the fast detection of the selected ATS in urine samples via ASAP-MS/MS. Sensitivity (LOD) and repeatability at 1.0 ng/mL were determined for the five tested compounds (Table 1). LOD values were determined from urine samples (n = 3) as the lowest concentration of each analyte yielding signal-to-noise (S/N) ratios of at least 5:1. The RSD values regarding the repeatability of each analysis remained between 10% and 14% for whole urine. These values were measured by sequential repetitive injection of the same sample (n = 3), followed by averaging of the peak area values of

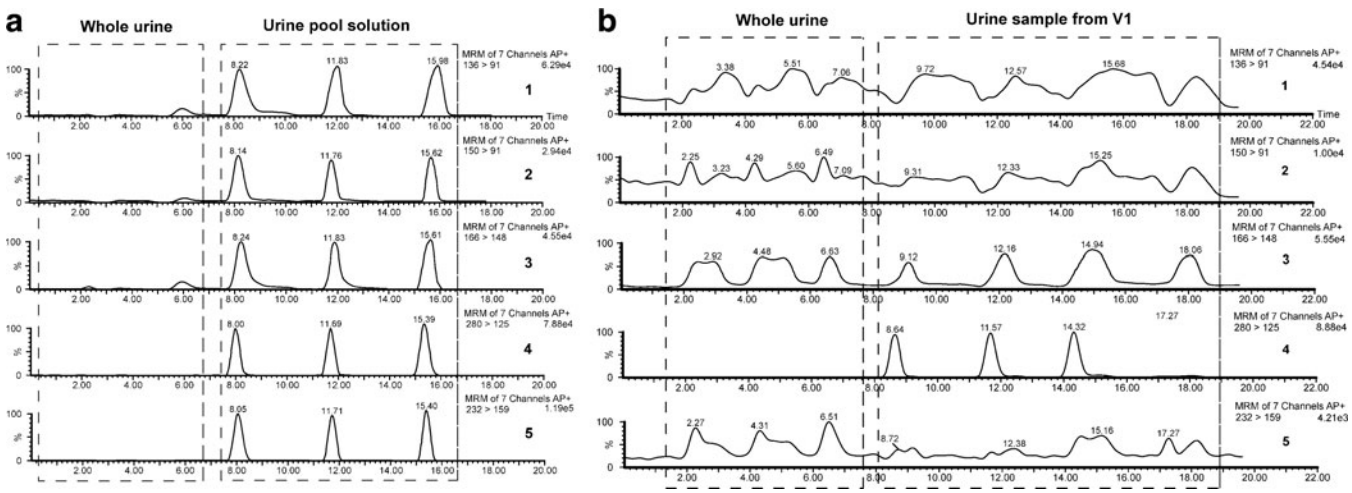

Figure 2. MRM traces obtained from **(a)** whole urine and the urine pool solution (urine spiked with compounds **1–5** at concentrations of 1.0 ng/mL). Analyses of the urine pool solution and whole urine were performed in triplicate **(b)** urine sample from V1 with the respective channels for compounds **1–5**. Analyses of the urine sample from V1 and whole urine were performed in triplicate

compounds from MRM traces. The experiments indicated that the LOD values obtained for direct analysis of compounds **1**–**5** ranged between 0.002 and 0.4 ng/mL for whole urine (S/N = 5, Table 1).

The selectivity of the MRM method used herein was able to differentiate ATS in the presence of other urine sample components. Interfering peaks did not emerge in the ion chromatograms obtained during individual analysis of each analyte (data not shown). The LOD values for amphetamine (**1**) and methamphetamine (**2**) were 0.1 ng/mL and 0.4 ng/mL, respectively. Finally, a urine sample (Figure 2) and saliva (Supplementary Figure S2, see Supplementary Material) from V1 were analyzed by ASAP-MS/MS. The methodology proposed in this work provided unambiguous identification of sibutramine in both samples, which demonstrated the potential of this method to detect, not to quantify, ATS in urine samples and saliva with no preconcentration and/or cleanup steps. The stability of ATS in these fluids is higher compared with other biological matrixes, such as plasma and blood. In addition, the concentration of most of these compounds in urine exceeds the values found in other biological matrixes [12]. Although LC-MS/MS is the analytical technique of choice to quantify ATS [4] because it offers better precision and linearity range compared with other techniques, these results suggest that further studies to evaluate the potential of ASAP-MS/MS to quantify ATS are necessary.

In this study, there was no carryover (Supplementary Figure S3A, see Supplementary Material) or sample matrix suppression (Supplementary Figure S3B, see Supplementary Material) during direct analysis of the complex urine components, as evidenced by the absence of analyte signals in whole urine. In addition, the complex mixture of compounds in the urine matrix did not significantly interfere in the direct detection of the selected ATS. On the other hand, severe matrix effects emerge during the LC-MS/MS analysis of drugs, mainly because the electrospray source (ESI-MS) is highly susceptible to other components present in the matrix, which may result in signal suppression or enhancement [13].

Conclusions

The proposed ASAP-MS/MS method allowed identification of ATS drugs (amphetamine, methamphetamine, ephedrine, sibutramine, and fenfluramine) in whole urine with excellent sensitivity and specificity. Direct detection of the drug sibutramine in the urine sample and saliva of a volunteer user was also possible. Because the developed method dismisses the need for chromatographic separation, consumption of organic solvents and the analysis time are smaller. The results of this study indicate that ASAP-MS/MS is a sensitive, specific, and high-throughput method for analysis of ATS in urine and saliva samples—it requires no sample preparation and can potentially detect ATS in these biological fluids for clinical and forensic toxicology purposes. Further studies aiming to quantify ATS and detect their metabolites using ASAP-MS/MS are underway.

Acknowledgments

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