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Determination of amphetamine and methamphetamine in urine by solid phase extraction and ion-pair liquid chromatography–electrospray–tandem mass spectrometry

Ming-Ren Fuh*, Ti-Yu Wu, Tzuen-Yeuan Lin

Department of Chemistry, Soochow University, P.O. Box 86-72, Taipei, Taiwan

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Abstract

A method using a solid phase extraction (SPE) and ion-pair liquid chromatography–electrospray–tandem mass spectrometry (LC–ES–MS/MS) was developed for determination of amphetamine (Amp) and methamphetamine (mAmp) in urine samples. A reversed phase C_{18} column was utilized for LC separation and MS/MS was used for detection. Trifluoroacetic acid was added to the mobile phase as an ion-pairing reagent. MS^2 was employed for quantitative determination. In addition, d_8-amphetamine and d_8-methamphetamine were used as internal standards. An Oasis HLB SPE cartridge, which has hydrophilic and lipophilic functions, was utilized for sample pre-treatment. Recoveries ranging from 97.3 to 102.1% were measured. Good linear ranges, 5–500 ng/ml, for Amp and mAmp were determined. The detection limit of each analytical compound, based on a signal-to-noise ratio of 3, was approximately 1 ng/ml. The applicability of this newly developed method was examined by analyzing several urine samples from drug users.

Keywords: Amphetamine; Methamphetamine; Ion-pair LC; LC–ES–MS

1. Introduction

As the number of illegal users of amphetamine (Amp) and methamphetamine (mAmp) increases dramatically, the determination of these drugs has become an important task [1,2]. Urine sample analysis is generally used to examine the abuse of these stimulants. Various analytical methods for the measurement of these compounds in biological samples have been reported, including gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) with fluorescence detection, capillary electrophoresis and LC–electrospray (ES)–MS [3–9]. GC–MS is the most widely used method because of good sensitivity and unambiguous identification of analytes [3]. However, derivatization is often needed for the GC–MS analysis of these compounds.

LC–MS has emerged as a sensitive and selective analytical method in drug analysis [10–13]. Due to the complex nature of urine, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS analysis. Solid phase extraction (SPE) has been demonstrated as an effective sample pre-treatment procedure to remove protein and potential interfering endogenous components in urine and to pre-concentrate the analytical compounds [14–16].

In this study, we explored the utilization of SPE and ion pair LC–ES–MS/MS for quantitative determination of Amp and mAmp in urine. A mix-mode SPE was utilized for urine sample pre-treatment. Ion pair LC–ES–MS/MS was used for the quantitative determination of amphetamine and methamphetamine. The application of this newly developed assay was demonstrated by examining several urine samples from drug addict suspects.

* Corresponding author. Tel.: +886 2 28819471x6821; fax: +886 2 28812685.
E-mail address: msfuh@mail.scu.edu.tw (M.-R. Fuh).

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2. Experimental

2.1. Chemicals

Purified water from a Milli-Q system from Millipore Corp. (Bedford, MA, USA), and HPLC grade acetonitrile and methanol (Milinckrodt Baker, Paris, KY, USA) were used. Trifluoroacetic acid (TFA) was purchased from Riedel-de Haen AG, Germany. Amp, d_8-Amp, mAmp and d_8-mAmp were from Cerilliant Corp. (Austin, TX, USA). Ammonium hydroxide was obtained form J.T. Baker (Phillipsburg, NJ, USA).

2.2. Equipment

An HP 1100 LC system (Hewlett-Packard Co., Palo Alto, CA, USA) consisting of a quaternary pump, an online degaser and an autosampler was used. Mass spectrometric detection was performed using an Agilent series LC/MSD trap SL instrument equipped with an electrospray ionization source that was operated in the positive mode with the spray voltage set at −3.5 kV. The capillary exit voltage was 102 V. Agilent 1100 series LC/MSD Trap software (version 4.0) was utilized for system control, data acquisition and data analysis. Heated N_2 gas (350 °C, 8 l/min) was used to evaporate solvent from the electrospray chamber, and compressed N_2 gas (40 psi) was used for nebulization. MS/MS mode and multiple reaction monitoring (MRM) were employed for quantitative measurement. The isolation width for precursor ions was 2. The settings for the MRM were: Amp, *m/z* 136 → 119 and 91 mAmp, *m/z* 150 → 119 and 91; d_8-Amp, *m/z* 144 → 127 and 96; d_8-mAmp, *m/z* 158 → 124 and 92. MS/MS data acquisition was performed under the following conditions: normal scan speed, *m/z* range 50–230, ion charge control (ICC) target 30,000 and maximum accumulation time 300 ms.

2.3. Preparation of standard solutions

Stock solutions (100 ng/ml) of all drugs and internal standards were prepared in methanol. The working solutions were diluted with methanol/water (20/80, v/v) to appropriate concentration weekly. All solutions were kept in a refrigerator (4 °C) when not in use. The standard curve range was 5–500 ng/ml for both Amp and mAmp. Drug-free urine collected from five healthy volunteers were used for method development.

2.4. SPE sample preparation and LC separation

Prior to SPE extraction, urine sample (10 ml) was deproteinated with TFA (100 μl), vortexed for 3 min, and centrifuged for 5 min at 1500 × *g*. An aliquot of supernatant fluid (1 ml) was used for SPE extraction. This is for deproteination and to pre-form [analyte–TFA] molecules. A mixed mode Oasis HLB SPE cartridge (1 ml, 30 mg, Waters Corp., Milford, MA, USA) was utilized for extraction. It is packed with a macro-porous copolymer that has hydrophilic as well as lipophilic functions. The SPE cartridge was first conditioned with methanol and water prior to urine sample loading. The acidified urine sample was introduced to SPE cartridge. Afterward, the SPE cartridge was further washed with 1 ml of 5/95 (v/v) methanol/2% ammonium hydroxide mixtures and 1 ml of 20/80 (v/v) methanol/2% ammonium hydroxide mixtures to remove the endogenous components in urine sample. Alkaline solution was used as washing solution to convert the analytes to neutral molecules that were retained in the SPE cartridge during washing procedure. Acidic solution (20/80 methanol/2% acetic acid, v/v) was utilized for eluting the analytical components. This eluted solution was evaporated to dryness under nitrogen gas and then reconstituted with mobile phase.

A Symmetry Shield RP18 column (2.1 mm × 150 mm, 5 μm, Waters Corp.) was used for LC separation. A mixture of acetonitirle–water (10/90, v/v) with 0.05% trifluoroacetic acid was used as a mobile phase. The flow rate was set at 0.2 ml/min. Injection volume was 10 μl.

3. Results and discussion

3.1. LC/MS/MS analysis

The interaction between the possible free silanol groups in the packing material of LC column and the amino groups in protonated amphetamine and methamphetamine would cause severe tailing effect in LC separation. The LC separation of this study was a modification of an ion-pair liquid chromatography method published by this laboratory [17]. Three major ions ($[M+H]^+$, $[C_9H_{11}]^+$ and $[C_7H_7]^+$) were detected while $[M+H]^+$ was selected as precursor ion for MS/MS. The sum of intensities of the two fragment ions ($[C_9H_{11}]^+$ and $[C_7H_7]^+$ at *m/z* 119 and 91) was used for LC/MS/MS quantitative measurement of amphetamine and methamphetamine. Analogous MS/MS fragmentations were observed for the deuterium labeled internal standards (d_8-Amp and d_8-mAmp). The characteristic MS^2 fragment ions and quantitative ions of each analyte and internal standard are listed in Table 1. The typical chromatograms of standard solution and spiked urine samples are shown in Fig. 1. The retention times of Amp and mAmp are 5.7 and 6.7 min, respectively.

3.2. Quantitative analysis and detection limit

Quantitative measurement was performed based on the ratio of peak area of each analyte to peak area of individual internal standard. The linearity of this newly developed assay was examined using a series of standard solutions and each standard was analyzed in triplicate. The evaluations of linearity and detection limit are summarized in Table 2. For each analyte, two linear ranges were determined from 5 to

Table 1
ES–MS^2 results for analytes and internal standards

	Fragmentation energy (V)	Precursor ion	MS^2 ion[a,b]
Amp	0.90	$[M+H]^+$	$[M+H]^+$ (136), **$[C_9H_{11}]^+$** (119), **$[C_7H_7]^+$** (91)
mAmp	0.87	$[M+H]^+$	$[M+H]^+$ (150), **$[C_9H_{11}]^+$** (119), **$[C_7H_7]^+$** (91)
d_8-Amp	0.75	$[M+H]^+$	$[M+H]^+$ (144), **$[C_9H_3D_8]^+$** (127), **$[C_7H_2D_5]^+$** (96)
d_8-mAmp	0.90	$[M+H]^+$	$[M+H]^+$ (158), **$[C_9H_6D_5]^+$** (124), **$[C_7H_6D]^+$** (92)

[a] *m*/*z* of each ion in parenthesis.
[b] Quantitation ions are in bold.

Table 2
Retention time, linearity and detection limit

	Retention time (min)	Calibration curve[a,b]	Range (ng/ml)	r^2	D.L.[c] (ng/ml)
Amp	5.7	$Y = 0.072X - 0.026$	5–100	0.999	1.0
		$Y = 0.034X + 0.685$	100–500	0.999	
mAmp	6.7	$Y = 0.089X - 0.051$	5–100	0.999	1.0
		$Y = 0.052X + 0.865$	100–500	0.999	

[a] *Y*: peak area ratio of standard and internal standard; *X*: concentration (ng/ml).
[b] Concentrations of standard: 5–100: 5, 10, 30, 50, 75, 100 ng/ml; 100–500: 100, 200, 300, 400, 500 ng/ml.
[c] D.L.: detection limit.

100 and 100 to 500 ng/ml, respectively. The detection limit for each analyte based on a signal-to-noise ratio of 3 was 1.0 ng/ml approximately and the limit of quantification is 5 ng/ml, respectively. The sensitivity of the present method is superior or equal to those previously reported results [3,4,18–20]. However, this method requires no derivatization procedure.

Table 3
Recovery of spiked urine sample[a]

	50 ng/ml	200 ng/ml	200 ng/ml
Amp	102.1 ± 1.5	100.7 ± 2.8	101.2 ± 4.8
mAmp	100.2 ± 1.9	99.3 ± 2	97.3 ± 3.7

[a] Recovery (%). Average ± standard deviation ($n = 5$).

3.3. Extraction recovery

In order to evaluate the extraction recoveries of SPE procedure, various spiked urine samples and standard solutions were analyzed. The recovery was determined by the response of a spiked urine sample as a fraction of that of a corresponding standard solution. The results of recovery study are summarized in Table 3. Good recoveries ranging from 97.3 to 102.1% were obtained. In addition, the chromatographic performance and MS/MS spectral quality for urine samples were not significantly less than those for standard solutions. These results indicated that no matrix effect or ion suppression was observed and this assay was suitable for the analysis of these compounds in urine samples.

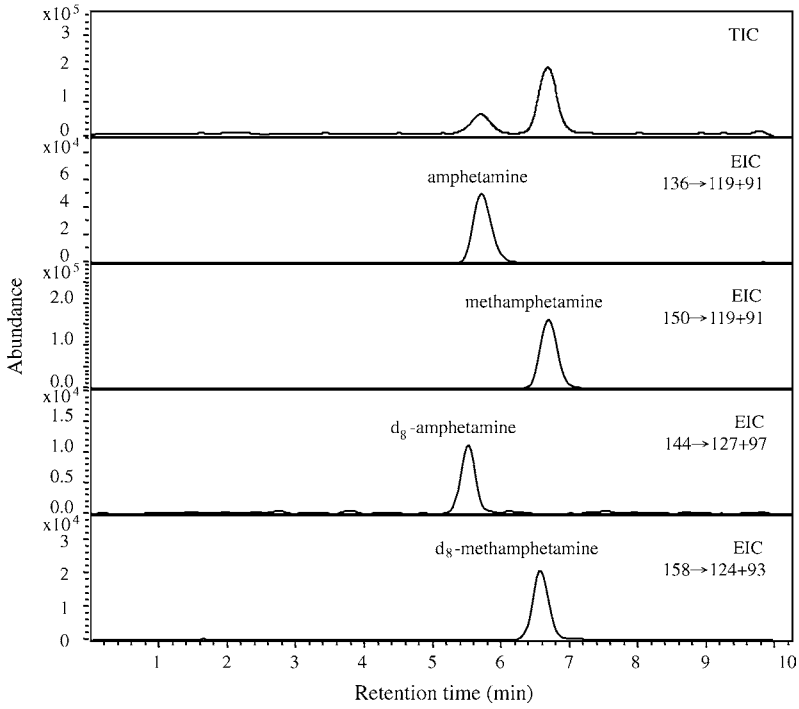

Fig. 1. Reconstructed ion chromatograms of a spiked urine sample (100 ng/ml for each analytical compound and 10 ng/ml internal standard).

Table 4
Intra-day and inter-day precision and accuracy

	Intra-day ($n = 3$)			Inter-day ($n = 3$)		
	50 ng/ml	200 ng/ml	500 ng/ml	50 ng/ml	200 ng/ml	500 ng/ml
Amp						
Mean	47.8	201.5	505.4	48.2	206.7	497.8
Accuracy (%)	95.6	100.8	101.1	96.4	103.4	99.6
CV	6.6	4.9	3.7	6.9	4.9	5.7
mAmp						
Mean	52.7	199.7	503.8	50.8	202.3	503.4
Accuracy (%)	105.4	99.9	100.8	101.6	101.1	100.7
CV	4.2	3.6	2.9	6.3	6.2	4.9

3.4. Precision and accuracy

Precision and accuracy were examined by the replicate analyses of amphetamine and methamphetamine spiked urine samples and the results are summarized in Table 4. The intra-day and inter-day precision showed coefficients of variance (CV) ranging from 2.9 to 6.6% and 4.9 to 6.9%, respectively. The accuracy of the method was expressed by [mean measured concentration/theoretical value] × 100%; accuracies ranging from 95.6 to 105.4% were determined.

3.5. Examination of urine samples of drug users

This newly developed analytical assay was applied to urine samples collected from five drug addicts and the results are summarized in Table 5. These samples were analyzed by a GC–MS method at Institute of Forensic Medicine, Ministry of Justice [3]. Four urine samples from methamphetamine users were examined. Amphetamine and methamphetamine were found in all the samples, with concentrations ranging from 44 to 3022 and 652 to 14,988 ng/ml, respectively. The data were evaluated by comparing the means with Student's *t*-test [21]. We found that the results from GC–MS and on-line SPE LC/MS/MS methods are not significantly different at the 95% confidence level.

Table 5
Results of real sample analysis

Sample	Drug found	GC–MS (ng/ml)	LC/MS/MS[e] (ng/ml)
A[a]	Amp mAmp	N.D.	44
		723	652
B[b]	Amp	1247	1338
	mAmp	8339	7847
C[c]	Amp	780	850
	mAmp	3615	3840
D[d]	Amp	2928	3022
	mAmp	12595	14988

[a] Sample was diluted 5-fold prior to on-line LC/MS/MS analysis.
[b] Sample was diluted 20-fold prior to on-line LC/MS/MS analysis.
[c] Sample was diluted 10-fold prior to on-line LC/MS/MS analysis.
[d] Sample was diluted 50-fold prior to on-line LC/MS/MS analysis.
[e] $n = 3$.

4. Conclusion

In this paper, an analytical method to determine amphetamine and methamphetamine in urine was developed utilizing a SPE pre-treatment and ion pair LC–ES–MS/MS. Ion pair LC effectively minimizes the tailing effect caused by the interaction between the packing material of LC column and the protonated analytical molecules [22]. SPE sample pre-treatment procedure effectively removed the endogenous components in urine sample. No matrix effect or ion suppression was observed. Good extraction recoveries ranging from 97.3 to 102.1% were obtained. This newly developed method has been successfully applied to analyze several urine samples from drug users. Good agreement between the results from this method and a standard GC/MS method was obtained.

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