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High-throughput toxicological analysis of Methamphetamine, MDA and MDMA from human plasma by LC-MS/MS

Laurian Vlase[1], Daniela-Saveta Popa[2], Felicia Loghin[2], Sorin E. Leucuta[1]*

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Abstract**:** A new high-throughput liquid chromatography coupled with mass spectrometry (LC-MS/MS) method for the quantification of methamphetamine (MA), methylendioximeth-amphetamine (MDMA) and methylendioxiamphetamine (MDA) in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a 15:85 (v/v) mixture of methanol and 0.1% (v/v) formic acid in water at 45ºC with a flow rate of 1 mL/min. The detection was performed in MRM mode using an ion trap MS equipped with an APCI ion source, positive mode. The ion transitions monitored were m/z 150→ m/z (91+119) for MA, m/z 180→ m/z 163 for MDA and m/z 194→ m/z 163 for MDMA, respectively. The human plasma samples (0.2 mL) were precipitated using 7% perchloric acid in water (0.1 mL) and aliquots from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity ($r > 0.996$), precision and accuracy (< 13%) over the concentration ranges of 2.03-169.04 ng/mL for MA, 2.08-173.44 ng/mL for MDA, and 2.48-206.92 ng/mL for MDMA, respectively. The recoveries were between 88.3-110.4%. The method is not expensive, it needs a minimum time for plasma sample preparation and has a run-time of 2.5 min for instrument analysis (retention times of MA, MDA and MDMA were 1.8, 1.9 and 2.0 min, respectively). The developed and validated LC-MS/MS method is very simple and more rapid than other similar methods. It can be useful for the rapid quantification of MA, MDMA and MDA in human plasma in forensic toxicology analyses.

Key words: Methamphetamine, MDA, MDMA, LC-MS/MS, forensic analyses, high-throughput assay

Methamphetamine (MA) and methylendioxymethamphetamine (MDMA), commonly named Ecstasy (Fig.1), are popular designer drugs currently used worldwide. They are dangerous drugs, having an increased toxic potential. MA is a psychotropic drug acting as "dynamite" for the nervous system. It excites indirectly the dopaminergic and adrenergic receptors in the brain, stimulates the release of cathecolamines in the synaptic cleft and inhibits their neuronal reuptake.

MDMA is an entactogen acting mainly on serotoninergic neurons. It produces massive serotonin release, blocks its neuronal reuptake and inhibits its synthesis. The feeling of interior void installed after psychotropic effect is induced by the depletion of serotonin at cortical level, and can last for 24 hours. After repeated exposure to MDMA, the serotoninergic activity diminishes in a dose-depending mode consecutively to destruction of

**) Corresponding author: PhD, associated professor, University of Medicine and Pharmacy "Iuliu Hatieganu", Department of Toxicology; Address: 8, Victor Babes, Cluj-Napoca 400012, Romania, Tel./fax 0040264450555; E-mail: dpopa@umfcluj.ro or dspopa@yahoo.fr*
University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, 8, Victor Babes 400012, Romania: 1) Department of Pharmacutical Technology and Biopharmaceutics: 2) Department of Toxicology

serotoninergic neurons and severe reduction of triptophan-hydrolase activity, the enzyme involved in neuronal serotonin synthesis.

The principal active metabolite of MDMA is methylendioxyamphetamine (MDA) (Fig.1), formed by N-demetylation. It has been found that it also produces the destruction of serotonergic neurons [1,2].

Analysis of amphetamines is important in forensic toxicology for workplace drug testing, criminal justice, drug abuse treatment and sport doping control programs. Gas chromatography / mass spectrometry (GC-MS) is the most common instrumental technique used for simultaneous analysis of MA, MDA and MDMA in different matrices such as serum [3], urine [4,5], hair [6,7], saliva [8,9] and sweat [10], but an isolation-step performed by liquid–liquid extraction (LLE) [3,5,7] or solid-phase extraction (SPE) [6-10] and derivatisation is required. This pretreatment of samples makes the analysis longer and more expensive. Several high-performance liquid-chromatography (HPLC) methods with fluorescence detection have been reported for the analysis of MDA and MDMA in serum and whole blood [11], oral fluid [12] or urine [11,13], as well as for the analysis of MA and amphetamine in plasma and hair samples [14]. Prior to HPLC analysis an isolation of analytes is also required, performed generally by LLE [11-14], a time-consuming step that increases the cost of the assay and can affect the recovery.

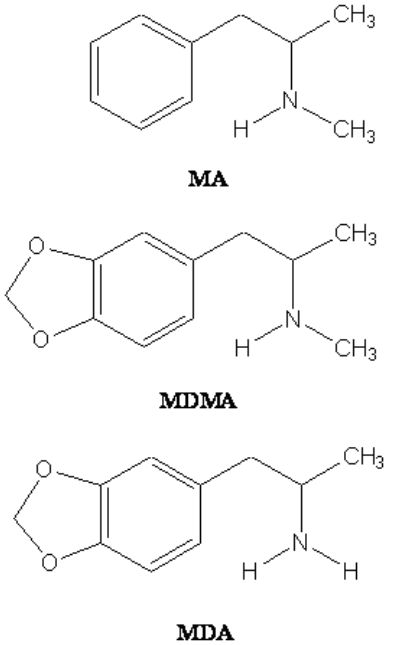

Fig.1. Chemical structures of methamphetamine (MA), 3,4-methylen-dioxymethamphetamine (MDMA) and 3,4-methylen-dioxyamphetamine (MDA)

The liquid chromatography coupled with mass spectrometry tandem (LC-MS/MS) offers considerable advantages by its powerful performances: speed, selectivity, sensitivity and robustness. Sample preparation is more simple and rapid and often includes precipitation of proteins (PP) and/or extraction before chromatographic analysis. The aim of this work was to develop and validate a new simple and efficient high throughput LC-MS/MS assay for the simultaneous quantification of MA, MDMA, and its main active metabolite, MDA, in human plasma.

Experimental

Reagents

Methamphetamine (MA), methylendioximethamphetamine (MDMA) and methylen-dioxiamphetamine (MDA) were reference standards from Lipomed (Switzerland). Methanol of HPLC-grade, formic acid and 70% perchloric acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water pro injections was purchased from the Infusion Solution Laboratory of the University of Medicine and Pharmacy Cluj-Napoca (Romania). The human blank plasma was supplied by the Transfusion Centre Cluj-Napoca (Romania) from healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of a G1312A binary pump, an in-line

G1379A degasser, an G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 15:85 (v/v) mixture of methanol and 0.1% (v/v) formic acid in water at 45 ºC with a flow rate of 1 mL/min. The detection was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionisation (APCI) ion source, in positive mode: dry gas nitrogen at 5 L/min, dry gas temperature 350ºC, vaporizer 400°C, nebulizer 60 psi (nitrogen), capillary 2200 V. The ion transitions monitored were m/z 150→ m/z (91+119) for MA, m/z 180→ m/z 163 for MDA and m/z 194→ m/z 163 for MDMA, respectively.

Standard solutions

The stock solutions of MA (845 μg/mL), MDA (867 μg/mL) and MDMA (1035 μg/mL) were prepared by dissolving appropriate quantities in methanol. Two working solutions (8.45 μg/mL and 169.04 ng/mL for MA, 8.67 μg/mL and 173.44 ng/mL for MDA, and 10.35 μg/mL and 206.92 ng/mL for MDMA, respectively) were prepared by appropriate dilutions in drug-free human plasma. These solutions were used to prepare plasma standards with the concentrations of 2.03, 6.76, 13.52, 27.05, 54.09, 108.19, and 169.04 ng/mL for MA, 2.08, 6.94, 13.88, 27.75, 55.50, 111.00, and 173.44 ng/mL for MDA, and 2.48, 8.28, 16.55, 33.11, 66.21, 132.43, and 206.92 ng/mL for MDMA, respectively. The resultant plasma standards were pipetted into 15 mL polypropylene tubes and stored at -20ºC until analysis. As quality control (QC) samples were used three concentrations from calibration range for each substance: second level (lower), fortieth level ng/mL (medium) and sixtieth level (higher).

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinized with 7% perchloric acid in water (0.1 mL). After vortex-mixture (10 s) and centrifugation (6 min at 5000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 30 μL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing the three analytes with those obtained from different plasma blank samples (n = 6).

The concentrations of MA, MDA and MDMA were determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined for five calibration series (n = 5) by the least squares analysis: y = b + ax, weighted (1/y) linear regression, where y - peak area and x - analyte concentration.

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The absolute recoveries were measured by comparing the response of each analyte from spiked plasma samples with the response from a standard solution with the same analyte

concentration, prepared in mobile phase and processed in the same manner with plasma sample.

Results

The chromatographic conditions, especially the composition of the mobile phase, were optimized in several trials to achieve the good MS signals, the short retention times of analytes and consequently a high-throughput analysis.

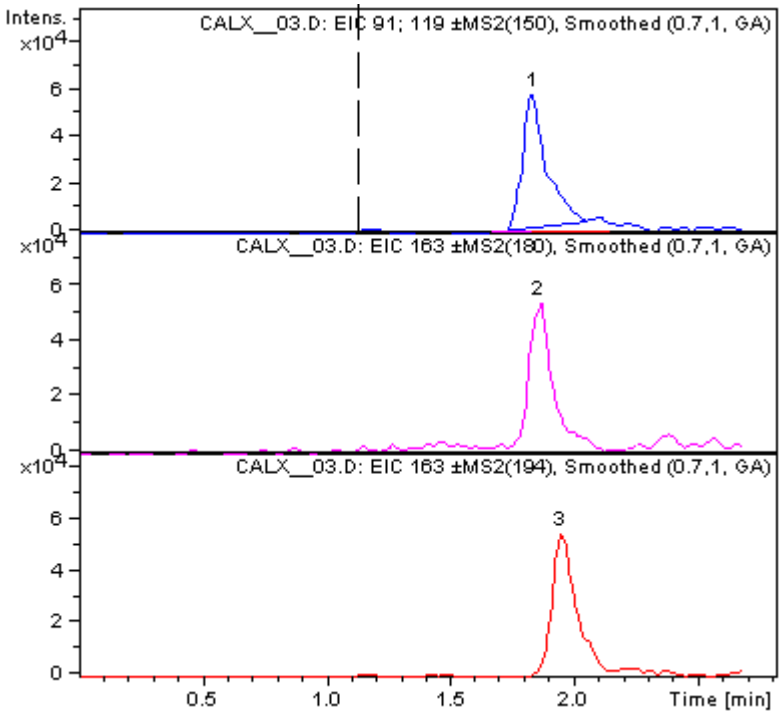

Fig. 2 Representative chromatograms of (up) blank plasma and plasma spiked with (1) MA, (2) MDA and (3) MDMA at lower limits of quantification (2 ng/ml for MA and MDA, and 2.5 ng/ml for MDMA, respectively) (retention times: MA - 1.8 min, MDA – 1.9 min, MDMA – 2.0 min)

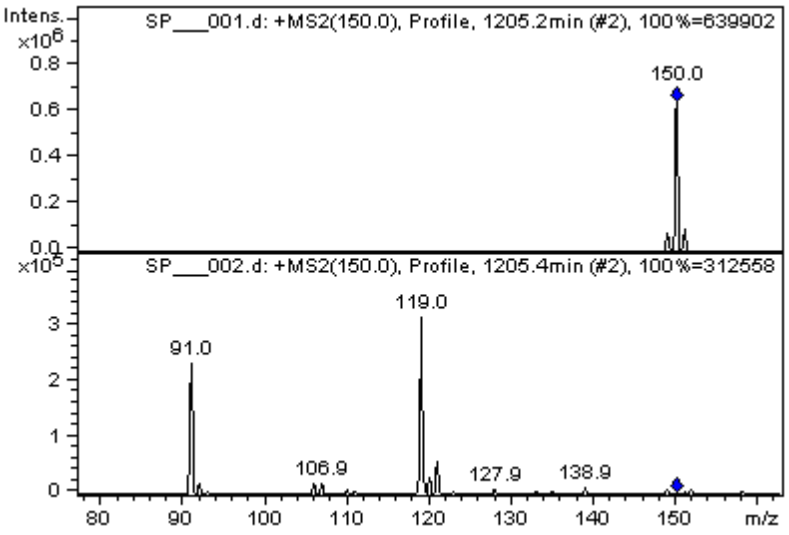

Fig. 3 Full-scan spectra (up) and MS/MS spectra (down) of MA

The best results were obtained with the mixture of methanol and 0.1% (v/v) formic acid in water (15:85, v/v) under isocratic conditions. In the selected chromatographic conditions, the retention times of MA, MDA and MDMA were 1.8, 1.9 and 2.0 min, respectively, and the analytical run-time was 2.5 min for instrument analysis. No interfering peaks from the endogenous plasma components were observed at the retention times of the three analytes. Representative chromatograms of plasma spiked with MA, MDA and MDMA at LLOQ are shown in Fig.2.

The detection was performed in MRM mode by monitoring the ion transitions: m/z 150→ m/z (91+119) for MA (Fig.3), m/z 180→ m/z 163 for MDA (Fig.4) and m/z 194→ m/z 163 for MDMA (Fig.5), respectively.

The calibration curves were linear over all the studied concentration ranges in human plasma, with the correlation coefficients greater than 0.996. The LLOQ were 2.03 ng/mL for MA, 2.08 ng/mL for MDA, and 2.48 ng/mL for MDMA, respectively. The values obtained for intra-day and

inter-day precision and accuracy during the validation for plasma are shown in Table 1 and Table 2, respectively.

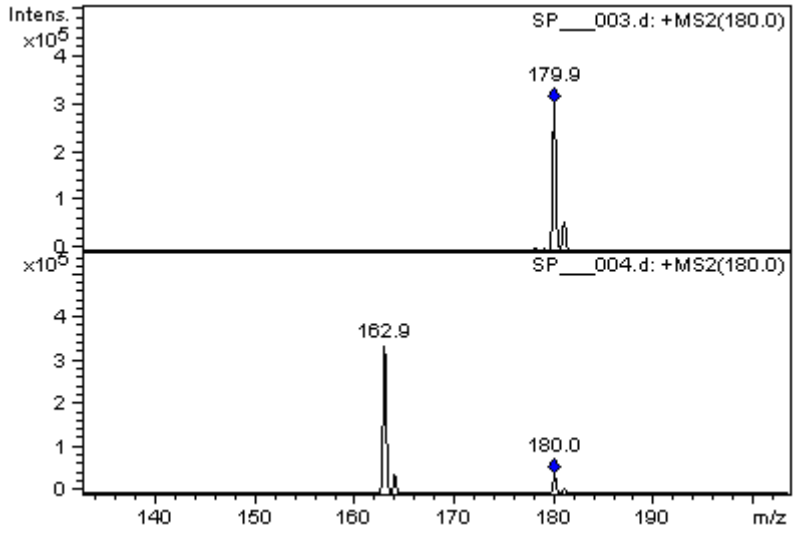

Fig. 4 Full-scan spectra (up) and MS/MS spectra (down) of MDA

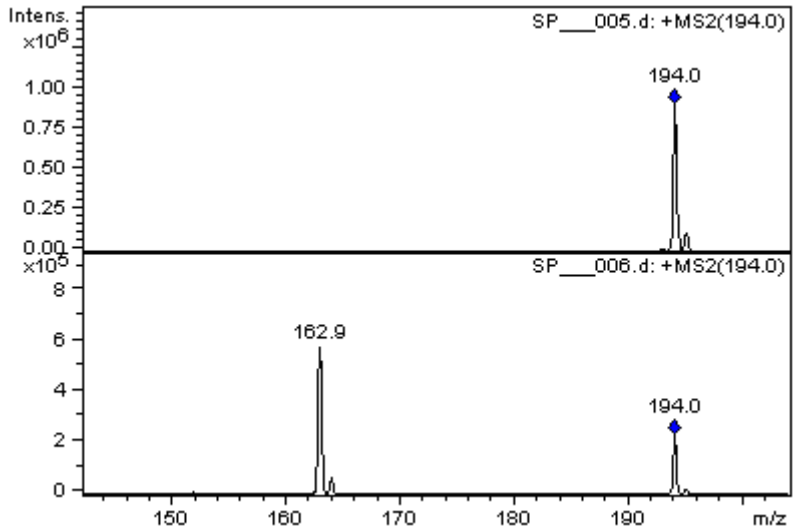

Fig. 5 Full-scan spectra (up) and MS/MS spectra (down) of MDMA

Discussion

In LC-MS/MS assays the sensitivity depends on MS detection mode, but the method used for sample preparation may influence the chromatographic background level and can generate matrix suppression effect. We propose a very simple and rapid pretreatment of plasma samples including only PP with 7% perchloric acid in water and direct injection into the chromatographic system from the supernatant obtained after centrifugation, with good recoveries (between 88.3-110.4%). Shima et al [15], Sergi et al [16], and Wood et al [17] have also analyzed amphetamines from human plasma or blood by LC-MS/MS after PP with methanol. The other methods reported in the literature include an isolation step by SPE to eliminate the impurities and to increase the sensitivity. But this operation increases the time of analysis, increases the costs and can affect the recovery. Therefore, Concheiro et al obtained a LOQ of 2 ng/mL after SPE with OASIS HLB cartridges and LC-MS analysis [18], but the recoveries were >70%. Cheze et al obtained a better LOQ of 0.1 ng/mL after extraction with Toxitube A cartridges and LC-MS/MS analysis [19], but the recoveries were also >66%.

LC-MS assay

The selected chromatographic conditions assure a high-throughput analysis, with an analytical run-time of 2.5 min. Other LC-MS/MS or LC-MS published methods for simultaneous quantification of MA, MDA and MDMA in human blood have long enough run-times, with retention times more than 3.5 min [15,16,18,19]. The method developed by Wood et al has a total analysis time of less than 20 min (including sample preparation), while our method needs a total analysis time of ~ 10 min.

Initially an ion suppression (poor analytical signal for analytes produced by competition for ionization between analytes and the others endogenous compounds) was observed with an ESI source. To avoid ion suppression an APCI source was selected for the analysis because it is less influenced by this phenomenon. The detection was performed in MRM mode by selecting for each analyte the specific ion transitions to obtain the best signal-to-noise ratios.

Table 1. The intra-day accuracy, precision and recovery data for the measurements of MA, MDA and MDMA in human plasma (n = 5)

Analyte	Nominal concentration (ng/ml)	Measured concentration		Precision (%)	Accuracy (%)	Recovery	
		ng/ml	± SD			(%)	± SD
MA	2.03	2.23	0.17	7.6	9.9	103.3	7.5
	6.76	6.71	0.51	7.7	-0.8	92.0	14.1
	27.05	26.10	0.80	3.1	-3.5	92.2	20.4
	108.19	113.55	3.62	3.2	5.0	104.5	11.5
MDA	2.08	2.18	0.12	5.6	5.0	91.4	14.1
	6.94	6.87	0.50	7.3	-1.0	99.4	9.6
	27.75	30.18	1.92	6.4	8.7	97.0	11.0
	111.00	119.92	4.36	3.6	8.0	88.3	8.0
MDMA	2.48	2.66	0.18	6.7	7.2	103.7	5.2
	8.28	8.87	0.64	7.2	7.2	96.0	7.0
	33.11	34.98	1.56	4.5	5.7	98.3	12.3
	132.43	139.06	4.78	3.4	5.0	98.8	10.6

Table 2. The inter-day accuracy, precision and recovery data for the measurements of MA, MDA and MDMA in human plasma (n = 5)

Analyte	Nominal concentration (ng/ml)	Measured concentration		Precision (%)	Accuracy (%)	Recovery	
		ng/ml	± SD			(%)	± SD
MA	2.03	2.21	0.02	0.9	8.8	101.1	13.2
	6.76	6.58	0.67	10.1	-2.7	91.8	16.9
	27.05	25.16	1.16	4.6	-7.0	110.4	9.3
	108.19	110.41	5.69	5.2	2.1	97.6	11.5
MDA	2.08	2.00	0.16	7.9	-4.0	102.6	11.2
	6.94	6.78	0.67	9.9	-2.3	103.0	7.9
	27.75	26.99	0.59	2.2	-2.8	107.9	7.5
	111.00	122.26	7.26	5.9	10.1	90.7	10.2
MDMA	2.48	2.31	0.30	13.0	-7.1	109.5	4.9
	8.28	8.43	0.83	9.9	1.8	99.7	6.2
	33.11	35.36	3.63	10.3	6.8	93.1	15.2
	132.43	137.54	9.23	6.7	3.9	97.5	3.7

Assay validation

The method was validated in accordance with international regulations [20,21,22]. The obtained results proved a good linearity ($r > 0.996$), sensitivity (LLOQ - 2.03 ng/mL for MA, 2.08 ng/mL for MDA, and 2.48 ng/mL for MDMA, respectively), accuracy and precision over the studied concentration ranges for all analytes. All values for accuracy and precision were within recommended limits (Table 1 and Table 2). The recovery values were

between 88.3-110.4%, which means no analyte loss during sample preparation due to adsorption on precipitated proteins.

Conclusion

The developed LC-MS/MS assay is simple, rapid, accurate and not expensive. In comparison with other published LC-MS/MS assays for simultaneous quantification of MA, MDA and MDMA in human plasma our method performs better in terms of speed (sample preparation and chromatographic run-time up 10 min) and costs, which are essential attributes for methods used in routine analysis. The method was validated and successfully applied for MA and MDMA abuse testing. It is a high-throughput method and can have wide applications in forensic toxicology and sport doping control programs.

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