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LC–MS analysis of trimethoxyamphetamine designer drugs (TMA series)from urine samples

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ABSTRACT

A sensitive liquid chromatography–mass spectrometric (LC–MS) method for quantification of an active psychedelic hallucinogenic drugs (trimethoxyamphetamines) in human urine after solid-phase extraction (SPE) with C_{18} cartridge was developed and validated. Chromatographic separation was achieved on reversed-phase Phenomenex 3.0 μm Polar Plus column (150 mm × 2.1 mm) with acetonitrile −0.2% acetic acid as mobile-phase and the step gradient elution resulted in a total run time of about 20 min. The analytes were detected by using an electrospray positive ionization mass spectrometry in selected ion monitoring (SIM) mode. In the evaluated concentration range (10–200 ng/mL) ($R^2 \geq 0.998$) a good linear relationship was obtained. The lower limits of detection (LLODs) and quantification (LLOQs) ranged from 4.26 to 9.12 ng/mL and from 13.18 to 29.22 ng/mL, respectively. Average recoveries ranged from 68.52 to 97.90% in urine at the concentrations of 25, 50 and 100 ng/mL. Intra- and inter-day relative standard deviations were 3.70–10.77% and 7.63–12.94%, respectively. This LC–MS method proved to be robust and reliable, and suitable for the use as a confirmation method in clinical urine drug testing.

1. Introduction

TMAs, also known as trimethoxyamphetamines, are a family of isomeric psychedelic hallucinogenic drugs. For TMAs, six isomers of equal total formula, $C_{12}H_{19}NO_3$ and the same molecular mass, 225.28 g/mol exist, which present different mutual position of the three methoxy groups [1].

The TMAs are analogs of the phenethylamine cactus alkaloid mescaline. Several TMAs were firstly synthesized by the chemist Alexander Shulgin [2]. It is reported that some TMAs induce a range of psychedelic effects ranging from sadness to empathy and euphoria. They are substituted amphetamines, however, their behavior is different as compared to unsubstituted amphetamines, which are well known stimulant drugs.

TMA (3,4,5-trimethoxyamphetamine) was the first totally synthetic psychedelic phenethylamine found to be active in man [2,3]. TMA is already classified in Schedules I to the 1971 United Nations Convention on Psychotropic Substances.

The 2,4,5-trimethoxyamphethamine (TMA-2) was described as a hallucinogenic drug 10 times more effective than TMA. Due to the lack of specific scientific evidence, acute or chronic toxicity has not been confirmed in humans, but toxic effects cannot be excluded. There have been no reported cases of fatal or non-fatal intoxication [4]. However, TMA-2 carries potential risks common to already controlled substances. Therefore, the Council of European Union has adopted on 27 November 2003 a Decision, defining TMA-2 as a substance which is to be placed under control measures and criminal penalties in the EU Member States, in accordance with their national law and with their obligations under the 1971 United Nations Convention on Psychotropic Substances [5].

TMA-2 has been widely used as a recreational drug and sold on the black market and as a "chemical research"; in a minor extent, also TMA and TMA-6 (2,4,6-trimethoxyamphethamine) have been used in this way. These three isomers are significantly more active as hallucinogenic drugs, and have consequently been placed onto the illegal drug schedules in some countries such as Italy. The other three isomers TMA-3 (2,3,4-trimethoxyamphethamine), TMA-4 (2,3,5-trimethoxyamphethamine) and TMA-5 (2,3,6-trimethoxyamphethamine) are not known to have been used as recreational drugs [6].

We describe a method for identification and quantification of the three more active compounds of TMA series (TMA, TMA-2 and TMA-6) in human urine, using TMA-3 as the internal standard (IS) (Fig. 1). Urine analysis is generally employed to detect the

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abuse of drugs in clinical and forensic application and in surveillance of drug substitution. To the best of our knowledge, this is the first identification of compounds of TMA series performed by liquid chromatography–mass spectrometry with electrospray ionization (LC–ESI–MS). To date no multiresidual analytical methods for trimethoxyamphetamines in biological samples have been reported in literature. The determination of TMA-2 has been carried out in rat urine by gas chromatography–mass spectrometry (GC–MS) [7]. However, GC–MS usually requires derivatization prior to analysis in order to enhance the chromatographic performances. Besides, liquid chromatography coupled to mass spectrometry for amphetamines analysis does not require sample derivatization and has been successfully applied in biological samples and seized material [8–13]. In order to improve the sensitivity and selectivity of analysis of these stimulants, a sample pre-treatment procedure to remove protein and potential interfering endogenous is often required. Solid-phase extraction is the most widely used preconcentration procedure. As reported in our previous papers, SPE on C_{18} cartridge was successfully used for extraction/purification of methoxy and dimethoxyamphetamines from biological matrices [14–16]. The same extraction conditions can be extended to determine the molecular congeners as the trimethoxyamphetamines.

This method is simple, clean and should be easily applied to epidemiological and clinical studies.

2. Experimental

2.1. Materials

Trimethoxyamphetamines (Fig. 1) were prepared in our laboratory at their maximum level of purity, as reported in literature [2]. Following the synthesis, the final products were identified by ^{1}H NMR. Melting points (m.p.) were determined with a Kofler hot stage microscope. IR spectra were acquired using a PerkinElmer 1760-X IFT. The product characterization by ^{1}H NMR spectrometry was carried out using a Varian VXR 200.

TMA: m.p. 218 °C; ^{1}H NMR ($CDCl_3$) δ: 6.41 (s, 2H, Arom); 3.87 (s, 6H, 2xOCH_3); 3.83 (s, 3H OCH_3) 3.25–3.1 (m, 1H, CH); 2.87–2.27 (m, 2H, CH_2); 1.50 (s, 2H, NH_2); 1.45 (d, 3H, CH_3).

TMA-2: m.p. 130 °C; ^{1}H NMR ($CDCl_3$) δ: 6.69 (s, 1H, Arom); 6.52 (s, 1H, Arom); 3.83 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 3.79 (s, 3H, OCH_3); 3.24–3.05 (m, 1H, CH); 2.68–2.37 (m, 2H, CH_2); 1.49 (s, 2H, NH_2); 1.10 (d, 3H, CH_3).

TMA-3 (IS): m.p. 148 °C; ^{1}H NMR ($CDCl_3$) δ: 6.83 (s, 1H, Arom); 6.61 (s, 1H, Arom); 3.88 (s, 6H, 2xOCH_3); 3.63 (s, 3H, OCH_3); 3.30–3.01 (m, 1H, CH); 2.18–2.38 (m, 2H, CH_2); 1.82 (s, 2H, NH_2); 1.11 (d, 3H, CH_3).

TMA-6: m.p. 200 °C; ^{1}H NMR ($CDCl_3$) δ: 6.13 (s, 2H, Arom); 3.61 (s, 3H, OCH_3); 3.78 (s, 6H, 2xOCH_3); 3.20–2.93 (m, 1H, CH); 2.78–2.42 (m, 2H, CH_2); 1.80 (s, 2H, NH_2); 1.07 (d, 3H, CH_3).

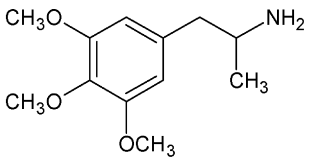
Deionized and distilled water was purified through a Milli Q water system (Millipore, Bedford, MA, USA). All reagents and solvents were purchased at the highest commercial quality. LC-grade acetonitrile and acetic acid (HOAc) were procured from Mallinckrodt J.T. Baker (Deventer, Holland). Bond Elut C_{18} solid-phase extraction columns (100 mg/mL) were purchased from Alltech (Milano, Italy) and mounted on a VacElut vacuum manifold (Supelco, Milano, Italy). Solvent filters (cellulose acetate and nylon 0.45 μm) were obtained from Nalgene Company (Rochester, NY, USA).

Aqueous stock solutions (1.0 mg/mL) of trimethoxyamphetamines were prepared, stored at +4 °C, and diluted with Milli Q water to appropriate concentrations before use. Drug-free specimens collected from a healthy adult male were used to make blank and spiked samples containing TMAs. The urine samples were kept frozen at −20 °C.

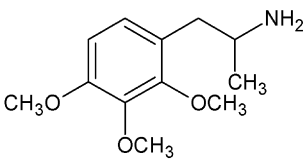
2.2. LC–ESI–MS set up

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC/MSD equipped with a diode-array detector and an autosampler (G1313A) was used for LC separation. Chromatographic separation was achieved using a Polar Plus column (150 mm × 2.1 mm, 3 μm) (Phenomenex, Torrance, CA, USA) fitted with a 3 μm Polar Plus security guard cartridge (4 mm × 2.1 mm) (Phenomenex, Torrance, CA, USA). The column temperature was maintained at 35 °C. The mobile-phase consisted of Eluent A water with 0.2% HOAc and Eluent B acetonitrile. The solvents were filtered through a 0.45 μm membrane prior to use. The separation was performed in a run time of 25 min under gradient conditions with a flow rate of 0.3 mL/min and was followed by clean-up and equilibration stage. The gradient elution ranged from 5% (t = 0 min) to 70% acetonitrile (t = 22 min) (Table 1). The injection volume was 10 μL.

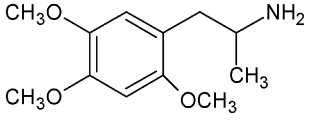
Mass spectrometric detection was performed using an Agilent G1946 (MSD 1100) single stage quadrupole instrument equipped with an electrospray atmospheric pressure ionization source. The system was calibrated with the procedures provided by Agilent; the mass spectrometer was optimized with an infusion of 0.25 μg/mL TMAs mixture at a flow rate of 100 μL/min. The LC–MS system was programmed to divert column flow to waste for 4.5 min after



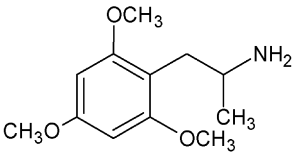
TMA (3,4,5-trimethoxyamphetamine)



IS = TMA-3 (2,3,4-trimethoxyamphetamine)



TMA-2 (2,4,5-trimethoxyamphetamine)



TMA-6 (2,4,6-trimethoxyamphetamine)

Fig. 1. Chemical structures of trimethoxyamphetamines.

Table 1
Chromatographic gradient program

Time (min)	% B	Flow (mL/min)
0	5	0.3
15	28	0.3
18	28	0.3
20	50	0.3
22	70	0.3
25	5	0.3

injection, after which time flow was directed into the mass spectrometer that operated in positive ion mode. For quantitative measurement of analytes, selected ion monitoring (SIM) was employed. In the ESI ion source, trimethoxyamphetamines isomers formed predominantly the deaminated ion at *m*/*z* 209.1. For each compound, two mass fragments were monitored with one fragment (*m*/*z* 209.1) used for quantification and the other fragment (*m*/*z* 226.1, $[M+H]^+$) used for the additional confirmation of identity. The following ESI conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 9.5 L/min; nebulizer gas (nitrogen) at a pressure of 42 psi; capillary voltage in positive mode at 3400 V; fragmentor voltage at 83 V.

2.3. Sample preparation

Amphetamines were extracted using our previously described procedure for other amphetamine analogous [14–16]. Briefly, urine samples (1 mL) were spiked with 50 ng of IS (TMA-3) and mixed with hydrogen carbonate buffer (100 mM, pH 10, 1 mL). The mixture was applied to a Bond Elut C_{18} extraction column, previously activated and conditioned with 1 mL of methanol and 1 mL of 100 mM hydrogen carbonate buffer (pH 10). After application of the sample, the column was washed with 2 mL of Milli Q water and dried by passing a stream of air for 5 min. The analytes were then eluted with 2 mL of methanol and the eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 1 mL of the mobile-phase.

2.4. Method validation

The analytical validation was performed according to the accepted guidelines [17,18]. The selectivity of the method was evaluated by analyzing urine from 9 healthy non-drug-consuming subjects. The method was also assessed to determine matrix effect on the ion suppression or enhancement according to Annesley [19]. Nine drug-free urine samples from different sources were extracted and subsequently fortified with analytes at three different concentrations (25, 50 and 100 ng/mL). Peak areas obtained from the extracts were compared with the corresponding peak areas produced by the reference solutions at the same concentration. The ion suppression percentage was calculate according to the following equation: Matrix effect (%) = (mean peak area "reconstituted extract in urine" – mean peak area "reference solution") × 100/mean peak area "reference solution".

Blank urine samples, extracted as described above, were fortified with 50 ng/mL of IS (TMA-3) and appropriate amounts of trimethoxyamphetamines at concentrations ranging from 10 to 200 ng/mL. The linearity of the compound-to-IS peak ratio versus the theoretical concentration was verified in urine by using a $1/x$ weighted linear regression. The regression coefficients (R^2) and the curvature were tested on a set of five calibration curves. The lower limit of detection (LLOD) and the limit of quantification (LLOQ) were calculated as $y_{LOD} = b + 3S.D._b$ and $y_{LOQ} = b + 10S.D._b$, where b = intercept and $S.D._b$ = standard deviation of the intercept [20].

Intra- and inter-day precision and accuracy were determined at three concentration levels (25, 50 and 100 ng/mL) by preparing and analyzing, on the same day and on five different days, respectively, five replicates for each level. The precision was expressed as relative standard deviation (R.S.D. (%)) and the accuracy was evaluated as [mean found concentration/nominal concentration] × 100. These criteria are compliant with the FDA guidelines for bioanalytical method validation [21].

The extraction recoveries were determined at low, medium and high concentrations. Recoveries were calculated by comparing the analyte/IS peak area ratios (R_1) obtained from extracted plasma samples with those (R_2) from the standard solutions at the same concentration.

3. Results and discussion

This paper reported an analytical method that employs a solid-phase extraction using a Bond Elut C_{18} cartridge, followed by liquid chromatography–mass spectrometry.

Different gradients of acetonitrile and 0.2% HOAc were assayed at a constant flow rate of 0.3 mL/min in order to get all analytes separated. Acetonitrile was chosen as the organic solvent because it provided a higher sensitivity and lower background noise than methanol. The three analytes and the IS were well separated from any endogenous components in urine matrix using the chromatographic conditions reported in Table 1.

Mass spectra were acquired using direct infusion of each standard in 0.2% HOAc applying ESI ionization sources in both negative and positive mode. These preliminary experiments showed effective results only in positive ESI mode.

Fig. 2 shows the chromatograms of urine extracts, blank (A) and spiked at 50 ng/mL (B). No significant interfering peaks were observed at the retention times of TMAs and IS in blank urine extraction which are shown in Fig. 2A.

Full scan mass spectra were monitored from *m*/*z* 100 to 350 (Fig. 3). Trimethoxyamphetamine compounds contain an amine function which can be easily deaminated. Thus highly abundant fragment at *m*/*z* 209.1 was observed for all analytes and selected as the quantifier ion. For additional confirmation of identity was monitored the proton adduct $[M+H]^+$ at *m*/*z* 226.1.

With regard to the validation method, regression was linear over the tested concentration range (10–200 ng/mL), with regression coefficients (R^2) calculated from five calibration curves for each compound. The average slope and intercept were reported in Table 2. Although the range of linearity is low, it includes the critical value for regulatory cut-off levels in routine analysis of drugs of abuse in urine [22].

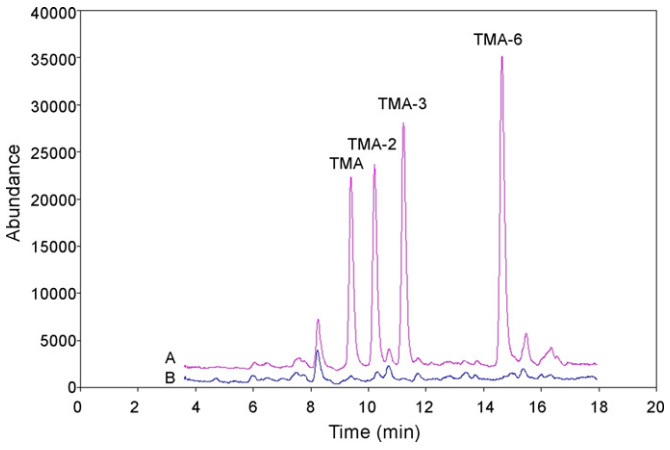


Fig. 2. Extract of blank urine (A) and urine sample spiked with 50 ng/mL of trimethoxyamphetamines (B).

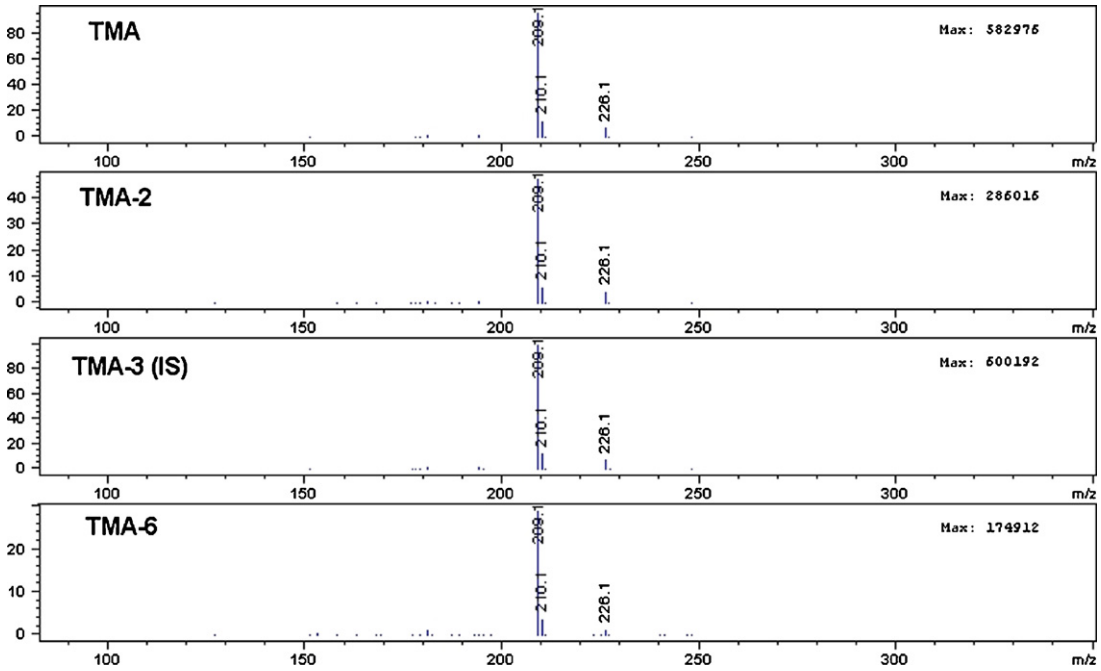


Fig. 3. Mass spectra of TMA, TMA-2, TMA-3 and TMA-6.

Table 2
Validation parameters

Compound	R^2 ± S.D.	Slope ± S.D.	Intercept ± S.D.	LOD (ng/mL)	LOQ (ng/mL)
TMA	0.997 ± 0.002	0.633 ± 0.060	−0.071 ± 0.025	4.26	13.18
TMA-2	0.996 ± 0.004	0.663 ± 0.057	0.105 ± 0.026	9.12	18.18
TMA-6	0.997 ± 0.003	1.400 ± 0.121	−0.061 ± 0.064	6.64	29.22

S.D. = Standard deviation.

Table 3
Intra- and inter-day precision and accuracy

Compound	Conc. (ng/mL)	Repeatability (R.S.D.), $n = 5$		Accuracy (%), $n = 5$	
		Intra-day	Inter-day	Intra-day	Inter-day
TMA	25	3.70	9.74	106.57	105.66
	50	6.99	7.63	101.90	101.79
	100	7.03	9.54	97.28	96.35
TMA-2	25	4.81	12.35	107.06	111.37
	50	10.77	12.32	98.13	105.54
	100	11.40	12.42	95.89	103.08
TMA-6	25	6.28	12.94	98.40	97.88
	50	9.40	10.50	97.48	99.09
	100	10.02	12.10	97.70	101.08

R.S.D. = Relative standard deviation.

Table 4
Recovery and matrix effect

Compound	Spiked conc. (ng/mL)	Recovery (%) ($n = 5$)	Matrix effect (%) ($n = 9$)
TMA	25	87.60	11.61
	50	87.44	14.23
	100	89.90	2.24
TMA-2	25	69.90	14.8
	50	68.52	13.5
	100	79.04	10.8
TMA-6	25	92.40	2.92
	50	95.86	0.41
	100	97.70	8.01

The lower limits of detection (LLODs) and quantification (LLOQs) were from 4.26 to 9.12 ng/mL and from 13.18 to 29.22 ng/mL, respectively (Table 2). This level of sensitivity was comparable to that reported for similar analytes by capillary electrophoresis–mass spectrometry (CE–MS) [14–16] and was suitable for confirmation of the presence of the analytes in urine samples.

To determine the intra-day precision and accuracy, five replicate analyses were performed with spiked urine samples at 25, 50 and 100 ng/mL for all trimethoxyamphetamines. IS was added, and the samples were extracted and analyzed within 1 day. Inter-day precision and accuracy were determined by repeating this procedure at the same concentrations on five different days. Data thus obtained were evaluated in reference to the IS (Table 3). Acceptable precisions with relative standard deviation (R.S.D. (%)) below 15% at every concentration except for the lower limit of quantitation (LLOQ) where 20% R.S.D. was acceptable [17]. The method was found to be precise with a R.S.D. < 15% for all substances. Recoveries were evaluated by analyzing urine samples with three different concentrations (25, 50 and 100 ng/mL); these results and the matrix effect data are summarized in Table 4. Very good recoveries were achieved at pH 10 for all analytes. No significant relative matrix effect was observed, because it was <15% in all cases (Table 4). Therefore, it was concluded that the matrix does not affect the accuracy and precision of TMAs determination.

4. Conclusion

The first LC–MS method for simultaneous identification and quantification of the three active compounds of TMA series (TMA, TMA-2 and TMA-6) was validated for human urine. In view of the simplicity, sensitivity and selectivity, the method is recommendable for the determination of these amphetamine derivatives in clinical pharmacology, bioavailability studies and forensic toxicology. LC–MS analysis of trimethoxyamphetamines does not require derivatization and is comparable in sensitivity, accuracy and precision to GC–MS and CE–MS for analogous compounds [7,14–16,23].

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