Vol. 15, No. 2, 2024
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Establishment and Evaluation of GC/MS Methods for Urinalysis of Multiple Phenethylamines

Po-Han Shih, Tsung-Hsien Lin, Shih-Ting Zeng, Shu-Yu Fan, Chi-Zong Zang*, Ya-Chun Ko, Ya-Hui Hsu, Shou-Chieh Huang, Mei-Chih Lin, Su-Hsiang Tseng

Taiwan Food and Drug Administration, Ministry of Health and Welfare, Taipei, Taiwan

Received April 19, 2024, Revised May 24, 2024, Accepted May 28, 2024
First published on the web June 30, 2024; DOI: 10.5478/MSL.2024.15.2.79

Abstract : Over the past few decades, new psychoactive substances (NPS) have become prevailing. With the widespread emergence of NPS, phenethylamines (PEAs) have become one of the groups abused most which PEAs, along with other stimulants, make up the majority of stimulants. When determining the NPS, the methods for screening and confirmation are crucial which assesses the reliability of testimony. In this study, a set of GC/MS methods employing two derivatizing agents for determining 76 target PEAs in urine was established and further applied for authentic sample analysis. Five PEAs (*N,N*-DMA, PMMA, 4-CA, amphetamine, and methamphetamine) with contents over their LLOQs were detected in thirteen of the twenty tested samples. In order to compare the result from the GC/MS methods with the previously established LC-MS/MS method, Cohen's kappa coefficient and McNemar's test were applied for statistical analysis. Perfect agreement between GC/MS and LC-MS/MS techniques for determining target PEAs is demonstrated by the Kappa coefficient for each of the five detected targets.

Keywords : GC/MS, new psychoactive substance, phenethylamines, statistical analysis, urine

Introduction

The analytical methods for screening and confirmatory test are essential for identifying substances of abuse which conducts the credibility of testimony. The preliminary test used to identify the targets from urine samples over their threshold values is known as screening test.[1] The confirmatory test recognized as secondary test is performed by contrasting the positive result from screening test.[1–4]

In forensic analysis, immunoassays are used for drug screening because of their high sensitivity and ease of use. However, limitations such as constantly changing new psychoactive substances (NPS) and the inability to distinguish between false negative/false positive results limit their advantages over NPS analysis.[5–7] In contrast, chromatographic techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are efficient tools for screening or confirmatory tests and are widely used in forensic science and clinical toxicology applications for their excellent sensitivity and specificity.

The adoption of analytical methods that are sensitive, specific, and conclusive is essential for the testing of substances of abuse, while the methods applied meet strict criteria for sample integrity, legal documentation, validation of targets identified and confirmation of the results.[1] It should demonstrate equivalent effectiveness in target identification no matter what method is used in confirmatory test. In this context, statistical measures are an efficient tool to assess the compatibility from analytical methods. When assessing the degree of agreement/disagreement (independence/difference) between assessors of binary variables (positive/negative or yes/no), Cohen's Kappa Coefficient and McNemar's test are two metrics used most often.[8–12]

With the widespread emergence of NPS in recent decades, phenethylamines (or PEAs for short) have become one of the groups abused most which PEAs, along with other stimulants, make up the majority of stimulants. By 2023, PEA and other stimulants account for the majority of the reported NPS, with 398 items in total.[17] Physical and psychological effects associated with PEAs include high blood pressure, hyperthermia, hallucinations, agitation, aggression, dissociation, attention deficit hyperactivity disorder, liver and kidney failure, and overdose can lead to severe poisoning and even death.[18]

*Reprint requests to Chi-Zong Zang
https://orcid.org/0000-0001-6015-0391
E-mail: heavenincry@fda.gov.tw

Materials and methods

Reagents

Seventy-six standards and four internal standards (IS) were obtained from six vendors. The detailed information is shown in Table 1. Among all, the standards obtained from GreenChem were commission-synthesized products associated with Taiwan Food and Drug Administration (TFDA) and have been identically assessed by NMR, HRMS, and FT-IR, whereas the purity for all items are above 98%. Ammonium hydroxide and methanol were analytical grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethyl acetate and isopropanol were analytical grade purchased from Avantor (Phillipsburg, NJ, USA). Sodium hydroxide, dipotassium phosphate, monopotassium phosphate, and glacial acetic acid were analytical grade purchased from E. Merck (Darmstadt, Germany). The derivatization agents heptafluorobutyric acid (HFBA) and pentafluoropropionic anhydride (PFPA) were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Normal human urine, certified drug negative containing 0.01% sodium azide, was purchased from UTAK Laboratories, Inc. (Santa Clarita, CA, USA). The solid-phase extraction (SPE) cartridge Bond Elut SPEC DAU cartridge (15 mg, 3 mL) was purchased from Agilent Technologies (Santa Clara, CA, USA). A total of 20 authentic urine samples were provided by 6 different analytical laboratories authorized by local law enforcement agencies of Taiwan. The authentic urine samples were stored at -20°C and acclimated to room temperature before analysis. The sampling of urine specimens in this study followed the regulations of the Ministry of Health and Welfare, Taiwan.

Instrumentation

The GC/MS analysis was performed on an Agilent 8890 gas chromatograph (GC) system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 7693A autosampler and connected to an Agilent 5977C mass selective detector (MSD). The chromatographic analysis was performed using an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm). Helium was used as carrier gas at a flow rate of 0.9 mL/min. The target PEAs were divided into two groups (see Table 1) and the parameters of GC/MS analysis are as follows. Group I analytes: temperature program of GC started at the initial temperature of 120°C and held for 0.5 min, increased to 180°C at 15°C/min, increased to 200°C at 3°C/min, and then increased to 285°C at 30°C/min and hold for 3 min, whereas the total run time was within 17 min; the injector was set at 260°C as the inlet temperature under splitless mode with injection volume of 2 μL; the ion source was set at 230°C and interface temperature of 280°C under electron ionization (EI) mode at 70 eV. Group II analytes: temperature program of GC started at the initial temperature of 100°C and held for 1 min, increased to 265°C at 10°C/min, and then increased to 300°C at 20°C/min and hold for 5 min, whereas the total run time was within 24.3 min; the injector was set at 285°C as the inlet temperature under splitless mode with injection volume of 1.5 μL; the ion source was set at 230°C and interface temperature of 300°C under EI mode at 70 eV. The data acquisition was carried out under total ion monitoring mode in the scanning range of 30 to 550 *m/z* to determine the retention time and characteristic mass fragments for each analyte so that the selected ion monitoring (SIM) mode could be applied for detecting the targets in the sample. The instrumentation as well the preparation of standard solutions and urine samples for LC-MS/MS analysis were in accordance with those published in a previous study and are presented as Supplementary Material 1 (SM 1).[19]

Preparation of standard solutions

The 76 PEA standards and 4 IS for GC/MS analysis were separately dissolved in methanol to the concentration of 1.0 mg/mL as stock solutions, whereas the working solutions were prepared by diluting the stock solutions with methanol to the concentration of 10 μg/mL. Before use, all stock and working solutions were stored at -20°C and allowed to acclimate to room temperature before use.

Pretreatment of urine samples

The pretreatment of the urine samples was carried out using a Bond Elut SPEC DAU SPE cartridge on a Biotage Rapid Trace$^+$ SPE Workstation system (Vimpelgatan, Switzerland). The sample solution was prepared by mixing 2 mL of the homogenized urine sample, 20 μL of the IS solution (equivalent 100 ng/mL), and 1 mL of 0.1 M phosphate buffer solution (pH 6). The setting of automatic SPE is as follows: Condition, methanol, 1 mL, 12 mL/min, to waste; condition, water, 1 mL, 12 mL/min, to waste; load, sample solution, 2 mL, 1.8 mL/min, to waste; purge-cannula, water, 5 mL, 42 mL/min, to cannula; rinse, 0.1 M acetic acid, 1 mL, 3 mL/min, to waste; purge-cannula, water, 5 mL, 42 mL/min, to cannula; rinse, methanol, 1 mL, 3 mL/min, to waste; dry, nitrogen gas, 2 bar, 4 min; collect, elution buffer (dichloromethane/isopropanol/25% ammonia, 80:20:2, v/v/v), 2 mL, 4.8 mL/min, to fraction; purge-cannula, methanol, 5 mL, 42 mL/min, to cannula. After the extraction, collect the eluent and transfer to a micro-reaction vial. Evaporate the eluent to dryness by gently flushing with a steam of nitrogen gas at 40°C and keep the residue. The residue was then derivatized by the following procedure: dissolve the residue with 50 μL of HFBA (or PFPA) and 50 μL of ethyl acetate. Cap the micro-reaction vial, vortex the residue solution to homogeneity, and react at 90°C for 15 min. Cool the solution to room temperature and evaporate with nitrogen gas at 40°C until dry. Dissolve the derivatized residue with 100 μL of ethyl acetate and then apply to GC/MS analysis. Drug-free urine (DFU) consisted of urine but without spiking any target analyte was used as the blank matrix and negative control.

The carryover was assessed by injecting DFU (n = 3) immediately after analysis of the urine samples spiked with the concentration of the highest calibrator (800/1000/2000 ng/mL) and the response ratio of residual to LLOQ was then calculated. The limit of carryover is 20% which is considered ignorable on target analysis. To evaluate the selectivity, different DFU samples (n = 20) were analyzed and no interfering peaks appeared during the target analysis i.e. the absence of evident interfering signals from the matrix at retention times nearby the characteristic ions.

Validation of GC/MS method

In the practice of forensic analysis, the validation is assessed to ensure the reliability and feasibility of the proposed method. In this study, the validation was performed in accordance with the guidelines "Working Group for Forensic Toxicology Standard Practices (SWGTOX) for Method Validation in Forensic Toxicology" and "Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens".[20,21] The methods were validated in terms of carryover, selectivity, linearity, sensitivity, extraction recovery, accuracy, and precision. To assess the linearity, the standard solutions of 76 PEAs (n = 3) were analyzed at six concentration levels ranging from 50 to 2000 ng/mL (IS of 100 ng/mL included). The calibration curve was plotted from the peak area ratio of standard/IS versus the concentration of standard using the least-square method, and the acceptable correlation coefficient (r) value was greater than 0.995. Quantification of target analytes was determined using a calibration curve based on the IS method within an established linear range. The acceptable ranges for qualitative and quantitative determination were as follows: relative ion ratio > 50%, RSD ± 20%; relative ion ratio 20–50%, RSD ± 25%; relative ion ratio 10–20%, RSD ± 30%; relative ion ratio ≤ 10%, RSD ± 50%. The acceptable retention time deviation of target was ± 0.2 min. Sensitivity was evaluated in terms of the LOD and LLOQ (lower limit of qualification) defined by the estimated signal-to-noise ratios (S/N) of 3 and 10, respectively. Six replicates (n = 6) were used for each analyte. The quality control (QC) was performed on analyte spiked urine samples. The SPE recovery, intra-day and inter-day precision (% CV) and accuracy (%) were evaluated in triplicate over five different runs at three concentration levels from low to high (100–1000 ng/mL). The acceptable range of recovery, precision and accuracy are 80%–120% and ± 20%, respectively. To extract the detailed explanation above, a flowchart was drawn to express it as shown in Figure 1.

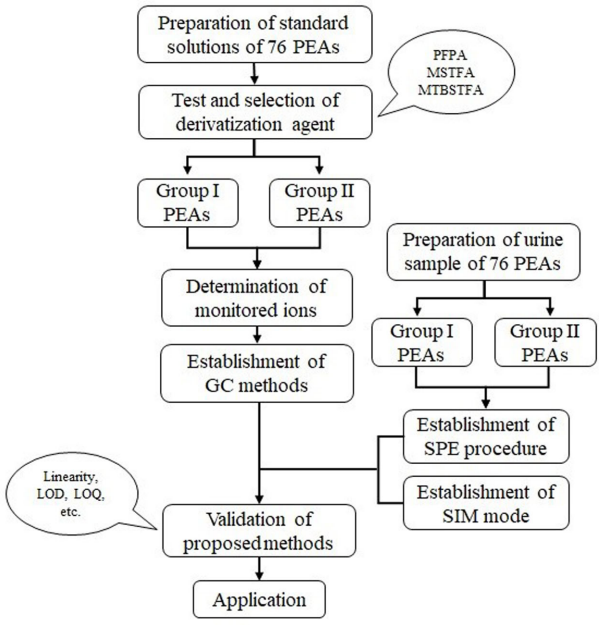

Figure 1. Flowchart for the development of the proposed methods.

Experimental

Determination of target analyte

Twenty authentic urine samples were analyzed applying the presented GC/MS methods. The established LC-MS/MS is also applied as a comparison. The cutoffs for positivity of target analyte were based on the legal threshold or lower limit of quantification (LLOQ) of each item. Specifically, the legal thresholds for five items in Taiwan are as follows: 500 ng/mL for amphetamine; 500 ng/mL for methamphetamine while amphetamine is detected of above 100 ng/mL; 500 ng/mL for MDMA. Meanwhile, it is identified as MDMA positive when MDMA and MDA are detected simultaneously with individual amount less than 500 ng/mL while the total amount is above 500 ng/mL; 500 ng/mL for MDA; 500 ng/mL for MDEA. The overall identification of a sample is defined as positive if one or more target analytes are detected above their cutoff concentration. Conversely, a negative is defined as long as the target is not detected or is below the cutoff concentration. The above identification must meet the acceptance criteria for relative ion intensity (see Table S1 in supplemental material). Furthermore, Cohen's kappa coefficient and McNemar's test were evaluated on the analysis results obtained by both methods to examine the consistency and difference between the results.

Statistical analysis

The consistency and difference of diagnostic tests (binary outcome, positive/negative or +/–) for urinalysis of PEAs in authentic samples applying GC/MS and LC-MS/MS was evaluated by the Cohen's kappa coefficient (κ) and McNemar's test (chi-square x^2) with 2 × 2 contingency tables. The data a and d in the matrix represent the number of agreements

(positive samples of target analyte), whereas b and c represent the number of disagreements (negative samples of target analyte). The data n represents the total number of agreements and disagreements (i.e. a + b + c + d). The definition of κ is as follows:

$$\kappa = \frac{p_o - p_e}{1 - p_e}$$

where p_o is the relative observed agreement among raters and p_e is the hypothetical probability of chance agreement among raters, described as follows:

$$p_o = \frac{a+d}{n}$$

$$p_e = \frac{(a+b)(a+c)}{n} \times \frac{(c+d)(b+d)}{n} = \frac{(a+b)(a+c)+(c+d)(b+d)}{n^2}$$

The x^2 of McNemar's test is conducted as follows:

$$\chi^2 = \frac{(b-c)^2}{b+c}$$

Meanwhile, if the total number n is less than 40, the Yates continuity correction is applied for compensating the deviations from the theoretical probability distribution.[22] The corrected x^2 is defined as follows:

$$\chi^2 = \frac{(|b-c|-1)^2}{b+c}$$

The McNemar's test was assessed by the online calculator.[23]

Results and discussion

Method development

When analyzing drugs and substances of abuse, the selectivity of GC/MS method influences the identification of target analytes. Therefore, various measures have been taken to improve the performance of chromatographic analysis, including derivatization, complexation, and ion-pair formation. Of the reactions listed above, the derivatization is the most commonly used for GC/MS analysis.[24] The derivatization process was introduced in this study to improve the detection and resolution of target PEA analysis. In the pretest, various derivatization reagents, including *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), HFBA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), PFPA, and trifluoroacetic anhydride (TFAA) were tested. The scheme chart of derivatization of PEAs was expressed as Figure 2. Among the derivatization agents tested, HFBA and PFPA were observed to have the main effect on improving GC/MS detectability in the analysis of target PEA (data not shown). The target PEAs were divided into two groups according to the adapted derivatization agents. The derivatized monitoring ions were collected as shown in Table 1, and the total ion chromatogram (TIC) of PEAs in urine spiked at 500 ng/mL under SIM mode is shown in Figure 3. The chromatographic separation was achieved within 17 and 23 minutes in separate runs. From Figure 3, it is observed that the SIM mode not only eliminates interference from the urine matrix, but also demonstrate processing efficiency in multi-target analysis by monitoring designated ions. Based on the above advantages, SIM mode has emerged as an efficient technique to analyze multiple PEA

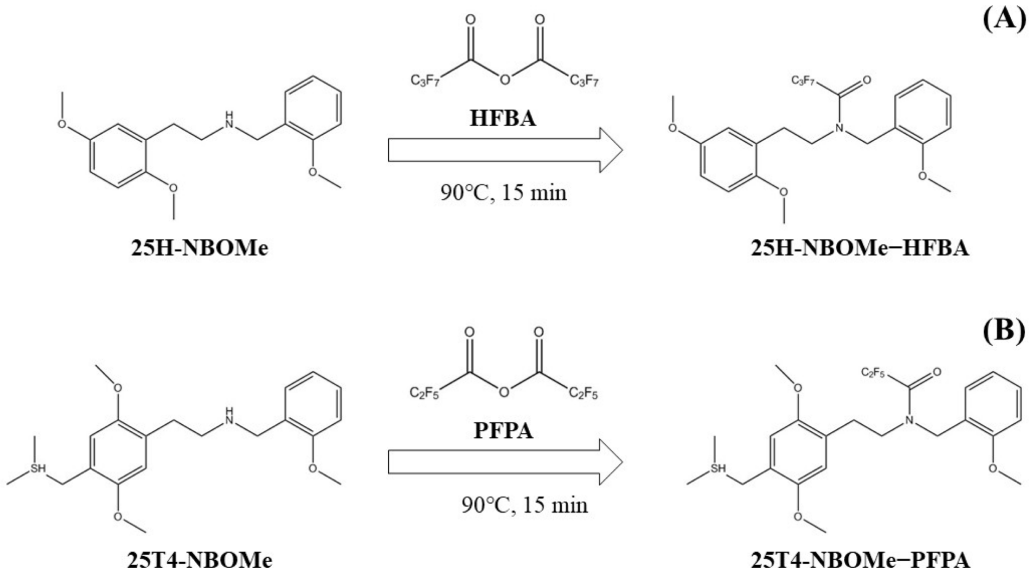

Figure 2. Scheme chart of derivatization applying HFBA and PFPA for two groups of PEAs. (A) Group I, take 25H-NBOMe as example; (B) Group II, take 25T4-NBOMe as example.

Table 1. MRM parameters for 76 targets and 4 IS of PEAs by GC/MS

Group	Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Distributor	Monitored ions (*m/z*)	Corresponding Internal standard	Derivatization agent
I	1	*N,N*-DMA (*N,N*-dimethylamphetamine)	33286-27-0	$C_{11}H_{17}N$	163.2	a	72*, 91, 56	MDA-d_5	HFBA
	2	4-MA (4-methylamphetamine)	41632-56-8	$C_{10}H_{15}N$	149.2	a	132*, 105, 240	MDA-d_5	
	3	4-CA (4-chloroamphetamine)	3706-38-5	$C_9H_{12}ClN$	169.7	a	240*, 152, 125	MDA-d_5	
	4	4-BA (4-bromoamphetamine)	58400-88-7	$C_9H_{12}BrN$	214.1	b	240*, 196, 198	MDA-d_5	
	5	4-CMA (4-chloromethamphetamine)	30572-91-9	$C_{10}H_{14}ClN$	183.7	a	254*, 152, 210	MDA-d_5	
	6	PMMA (*para*-methoxymethamphetamine)	3398-68-3	$C_{11}H_{17}NO$	179.3	a	148*, 254, 210	MDA-d_5	
	7	PMEA (*para*-methoxyethylamphetamine)	93963-24-7	$C_{12}H_{19}NO$	193.3	a	148*, 268, 121	MDA-d_5	
	8	5-APDB (5-(2-aminopropyl)-2,3-dihydrobenzofuran)	152623-94-4	$C_{11}H_{15}NO$	177.2	b	133*, 160, 134	MDA-d_5	
	9	Fenproporex (3-(1-Phenylpropan-2-ylamino)propanenitrile)	16397-28-7	$C_{12}H_{16}N_2$	188.3	a	293*, 118, 56	MDA-d_5	
	10	2C-E (2,5-dimethoxy-4-ethylphenethylamine)	923013-67-6	$C_{12}H_{19}NO_2$	209.2	a	179*, 192, 405	MDA-d_5	
	11	5-MAPDB (5-(2-methylaminopropyl)-2,3-dihydrobenzofuran)	-	$C_{12}H_{17}NO$	191.2	b	133*, 160, 254	MDA-d_5	
	12	MMDA (5-methoxy-3,4-methylenedioxyamphetamine)	60676-84-8	$C_{11}H_{16}NO_3$	209.2	a	165*, 192, 405	MDA-d_5	
	13	DOC (2,5-dimethoxy-4-chloroamphetamine)	42203-77-0	$C_{11}H_{16}ClNO_2$	229.7	b	185*, 212, 155	MDMA-d_5	
	14	Proscaline (2-(3,5-Dimethoxy-4-propoxyphenyl)ethanamine)	61367-69-9	$C_{13}H_{21}NO_3$	239.3	a	167*, 180, 435	MDMA-d_5	
	15	Clobenzorex (*N*-[(2-Chlorophenyl)methyl]-1-phenylpropan-2-amine)	5843-53-8	$C_{16}H_{18}ClN$	259.8	c	125*, 127, 118	MDMA-d_5	
	16	25C-NBF (4-chloro-*N*-[(2-fluorophenyl)methyl]-2,5-dimethoxy-benzeneethanamine)	1539266-21-1	$C_{17}H_{19}ClFNO_2$	323.8	b	109*, 198, 185	MDMA-d_5	
	17	3,4-DMA-NBOMe (*N*-(o-methoxybenzyl)-3,4-dimethoxyamphetamine)	-	$C_{19}H_{25}NO_3$	315.4	b	121*, 178, 151	MDMA-d_5	

Table 1. Continued.

Group	Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Distributor	Monitored ions (*m/z*)	Corresponding Internal standard	Derivatization agent
I	18	25B-NBF (4-bromo-*N*-[(2-fluorophenyl)methyl]-2,5-dimethoxy-benzeneethanamine)	1539266-17-5	$C_{17}H_{19}BrFNO_2$	368.2	b	109*, 242, 244	MDMA-d_5	HFBA
	19	25G-NBOMe (2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl] -3,4-dimethylbenzeneethanamine)	1797132-54-7	$C_{20}H_{27}NO_3$	329.4	b	192*, 121, 525	MDMA-d_5	
	20	25I-NBF (*N*-(2-fluorobenzyl)-4-iodo-2,5-dimethoxyphenethylamine)	1539266-13-1	$C_{17}H_{19}FINO_2$	415.2	b	109*, 290, 277	MDMA-d_5	
	21	Amphetamine	2706-50-5	$C_9H_{13}N$	135.2	d	240*, 118, 117	MA-d_8	
	22	FPBA (1-(4-fluorophenyl)butan-2-amine)	23292-09-3	$C_{10}H_{14}FN$	167.2	a	109*, 254, 135	MDA-d_5	
	23	Methamphetamine	300-42-5	$C_{10}H_{15}N$	149.2	d	254*, 210, 118	MA-d_8	
	24	4-FEA (4-fluoroethamphetamine)	3823-31-2	$C_{11}H_{16}FN$	181.2	a	268*, 136, 269	MA-d_8	
	25	5-F-2-MOA (5-fluoro-2-methoxyamphetamine)	-	$C_{10}H_{14}FNO$	183.2	a	166*, 167, 379	MDA-d_5	
	26	3-F-4-MOA (3-fluoro-4-methoxyamphetamine)	-	$C_{10}H_{14}FNO$	183.2	a	166*, 167, 379	MDA-d_5	
	27	MDDMA (*N,N*-dimethyl-3,4-methylenedioxyamphetamine)	74341-79-0	$C_{12}H_{17}NO_2$	207.2	b	72*, 73, 135	MDA-d_5	
	28	MDA (3,4-methylenedioxyamphetamine)	6292-91-7	$C_{10}H_{13}NO_2$	179.2	a	162*, 240, 375	MDA-d_5	
	29	DMA (2,5-dimethoxyamphetamine)	2801-68-5	$C_{11}H_{17}NO_2$	195.3	a	151*, 178, 391	MDA-d_5	
	30	Lefetamine	24301-90-4	$C_{16}H_{19}N$	225.3	a	134*, 135, 118	MDA-d_5	
	31	DOM (4-methyl-2,5-dimethoxyamphetamine)	15588-95-1	$C_{12}H_{19}NO_2$	209.3	a	165*, 405, 135	MDA-d_5	
	32	MDMA (3,4-methylenedioxymethamphetamine)	64057-70-1	$C_{11}H_{15}NO_2$	193.2	e	254*, 162, 210	MDMA-d_5	
	33	MDEA (3,4-methylenedioxy-*N*-ethylamphetamine)	82801-81-8	$C_{12}H_{17}NO_2$	207.3	a	268*, 240, 403	MDMA-d_5	
	34	Mescaline	832-92-8	$C_{11}H_{17}NO_3$	211.3	a	194*, 407, 226	MDA-d_5	

Table 1. Continued.

Group	Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Distributor	Monitored ions (*m/z*)	Corresponding Internal standard	Derivatization agent
I	35	Benzphetamine	5411-22-3	$C_{17}H_{21}N$	239.4	d	91*, 148, 92	MDMA-d5	HFBA
	36	2C-C (4-chloro-2,5-dimethoxyphenethylamine)	88441-15-0	$C_{10}H_{14}ClNO_2$	215.7	a	198*, 185, 411	MDMA-d_5	
	37	2C-I (2,5-dimethoxy-4-iodophenethylamine)	64584-32-3	$C_{10}H_{14}INO_2$	307.1	a	290*, 277, 503	MDMA-d_5	
	38	4-EA-NBOMe (4-ethyl-*N*-(o-methoxybenzyl)amphetamine)	-	$C_{19}H_{25}NO$	283.4	b	121*, 146, 91	MDMA-d_5	
	39	25H-NBOMe (2-(2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine)	1566571-52-5	$C_{18}H_{23}NO_3$	301.4	b	164*, 497, 151	MDMA-d_5	
	40	25P-NBOMe (2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl]-4-propyl-benzeneethanamine)	1539266-43-7	$C_{21}H_{29}NO_3$	343.5	b	206*, 121, 539	MDMA-d_5	
	41	MA-d_8 (Methamphetamine-d_8) (I.S.)	136765-40-7	$C_{10}H_7D_8N$	157.3	e	261*, 231	-	
	42	MDA-d_5 (3,4-methylenedioxyamphetamine-d_5) (I.S.)	136765-42-9	$C_{10}H_8D_5NO_2$	184.3	e	167*, 244	-	
	43	MDMA-d_5 (3,4-methylenedioxymethamphetamine-d_5) (I.S.)	136765-43-0	$C_{11}H_{10}D_5NO_2$	198.3	e	258*, 213	-	
II	44	Phentermine	1197-21-3	$C_{10}H_{15}N$	149.2	d	204*, 132, 164	AM-d_8	PFPA
	45	4-FA (4-fluoroamphetamine)	64609-06-9	$C_9H_{12}FN$	153.2	a	190*, 136, 109	AM-d_8	
	46	4-FMA (4-fluoromethamphetamine)	52063-62-4	$C_{10}H_{14}FN$	167.2	a	204*, 160, 136	AM-d_8	
	47	EMA (*N*-Ethylamphetamine)	16105-78-5	$C_{11}H_{17}N$	163.2	a	218*, 190, 118	AM-d_8	
	48	PMA (4-methoxyamphetamine)	3706-26-1	$C_{10}H_{15}NO$	165.2	a	121*, 148, 122	AM-d_8	
	49	5-APB (5-(2-aminopropyl)benzofuran)	286834-80-8	$C_{11}H_{13}NO$	175.2	b	131*, 158, 190	AM-d_8	
	50	2C-H (2,5-Dimethoxyphenethylamine)	3166-74-3	$C_{10}H_{15}NO_2$	181.2	f	164*, 121, 151	MDA-d_5	
	51	5-AEDB (5-(2-aminoethyl)-2,3-dihydrobenzofuran)	796869-33-5	$C_{10}H_{13}NO$	163.2	b	133*, 146, 309	MDA-d_5	
	52	4-MTA (4-methylthioamphetamine)	14116-06-4	$C_{10}H_{15}NS$	181.3	a	164*, 137, 327	MDA-d_5	

Table 1. Continued.

Group	Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Distributor	Monitored ions (*m/z*)	Corresponding Internal standard	Derivatization agent
	53	5-MAPB (5-(2-methylaminopropyl)benzofuran)	1823925-53-6	$C_{12}H_{15}NO$	189.2	b	204*, 158, 131	MDA-d_5	
	54	Mefenorex	17243-57-1	$C_{12}H_{18}ClN$	211.7	a	266*, 268, 190	MDA-d_5	
	55	2C-D (2,5-dimethoxy-4-methylphenethylamine)	25505-65-1	$C_{11}H_{17}NO_2$	195.3	a	178*, 341, 179	MDA-d_5	
	56	5-EAPB (5-(2-ethylaminopropyl)benzofuran)	1823776-22-2	$C_{13}H_{17}NO$	203.3	b	218*, 158, 159	MDMA-d_5	
	57	DOET (2,5-dimethoxy-4-ethylamphetamine)	22004-32-6	$C_{13}H_{21}NO_2$	223.3	a	179*, 206, 149	MDMA-d_5	
	58	MBDB (*N*-methyl-α-ethyl-3,4-methylenedioxyphenethylamine)	128767-12-4	$C_{12}H_{17}NO$	207.3	b	218*, 176, 135	MDMA-d_5	
	59	TMA (3,4,5-trimethoxyamphetamine)	5688-80-2	$C_{12}H_{19}NO_3$	225.3	c	181*, 208, 182	MDMA-d_5	
	60	TMA-2 (2,4,5-trimethoxyamphetamine)	1083-09-6	$C_{12}H_{19}NO_3$	225.3	a	181*, 151, 208	MDMA-d_5	
II	61	TMA-6 (2,4,6-trimethoxyamphetamine)	23815-74-9	$C_{12}H_{19}NO_3$	225.3	a	181*, 151, 208	MDMA-d_5	PFPA
	62	6-Cl-MDMA (1-(6-chloro-1,3-benzodioxol-5-yl)-*N*-methylpropan-2-amine)	-	$C_{11}H_{14}ClNO_2$	227.7	b	204*, 196, 160	MDMA-d_5	
	63	Escaline	3166-82-3	$C_{12}H_{19}NO_3$	225.3	a	167*, 180, 371	MDMA-d_5	
	64	DOB (4-bromo-2,5-dimethoxyamphetamine)	29705-96-2	$C_{11}H_{16}BrNO_2$	274.1	a	229*, 231, 256	MDMA-d_5	
	65	3C-P (4-propoxy-3,5-dimethoxyamphetamine)	-	$C_{14}H_{23}NO_3$	253.3	a	167*, 194, 399	MDMA-d_5	
	66	6-Br-MDMA (1-(6-bromo-1,3-benzodioxol-5-yl)-*N*-methylpropan-2-amine)	-	$C_{11}H_{14}BrNO_2$	272.1	b	204*, 160, 240	MDMA-d_5	
	67	2C-B (4-bromo-2,5-dimethoxyphenethylamine)	56281-37-9	$C_{10}H_{14}BrNO_2$	260.1	a	242*, 229, 244	MDMA-d_5	
	68	DOI (2,5-dimethoxy-4-iodoamphetamine)	42203-78-1	$C_{11}H_{16}INO_2$	321.2	a	277*, 304, 467	MDMA-d_5	
	69	Lisdexamfetamine	608137-33-3	$C_{15}H_{25}N_3O$	236.4	f	121*, 91, 230	MDMA-d_5	
	70	2C-T-2 (2,5-dimethoxy-4-ethylthiophenethylamine)	681160-71-4	$C_{12}H_{19}NO_2S$	241.4	a	256*, 419, 257	MDMA-d_5	

Table 1. Continued.

Group	Item	Analyte	CAS No.	Formula	Molecular weight (g/mol)	Distributor	Monitored ions (*m/z*)	Corresponding Internal standard	Derivatization agent
II	71	2C-T-4 (2,5-Dimethoxy-4-isopropylthiophenethylamine)	868738-44-7	$C_{13}H_{21}NO_2S$	255.4	a	270*, 271, 433	MDMA-d_5	PFPA
	72	2C-T-7 (2,5-dimethoxy-4-n-propylthiophenethylamine)	850140-15-7	$C_{13}H_{21}NO_2S$	255.4	a	270*, 163, 433	MDMA-d_5	
	73	25D-NBOMe (2-(2,5-dimethoxy-4-methylphenyl)-*N*-(2-methoxybenzyl)ethanamine)	1539266-35-7	$C_{19}H_{25}NO_3$	315.4	b	178*, 165, 121	MDMA-d_5	
	74	25C-NB3OMe (4-chloro-2,5-dimethoxy-*N*-[(3-methoxyphenyl) methyl]benzeneethanamine)	1566571-57-0	$C_{18}H_{22}ClNO_3$	335.8	b	198*, 121, 185	MDMA-d_5	
	75	25B-NBOMe (*N*-(2-methoxybenzyl)-4-bromo-2,5-dimethoxy-phenethylamine)	1539266-15-3	$C_{18}H_{22}BrNO_3$	380.2	a	121*, 244, 242	MDMA-d_5	
	76	25I-NBOMe (*N*-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine)	1043868-97-8	$C_{18}H_{22}INO_3$	427.3	a	121*, 290, 277	MDMA-d_5	
	77	25T2-NBOMe (4-(ethylthio)-2,5-dimethoxy-*N*-[(2-methoxy-phenyl)methyl]benzeneethanamine)	1539266-51-7	$C_{20}H_{27}NO_3S$	361.5	b	256*, 539, 257	MDMA-d_5	
	78	25T4-NBOMe (2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl]-4-[(1-methylethyl)thio]benzeneethanamine)	1566571-73-0	$C_{21}H_{29}NO_3S$	375.5	b	270*, 553, 271	MDMA-d_5	
	79	25T7-NBOMe (2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl]-4-(prop-ylthio)benzeneethanamine)	1539266-55-1	$C_{21}H_{29}NO_3S$	375.5	b	270*, 553, 271	MDMA-d_5	
	80	AM-d_8 (Amphetamine-d_8) (IS)	145225-00-9	$C_9D_8H_5N$	143.3	e	126*, 193	-	
	81	MDA-d_5 (3,4-methylenedioxyamphetamine-d_5)	136765-42-9	$C_{10}H_8D_5NO_2$	184.3	e	136*, 167	-	
	82	MDMA-d_5 (3,4-methylenedioxymethamphetamine-d_5)	136765-43-0	$C_{11}H_{10}D_5NO_2$	198.3	e	208*, 344	-	

a. GreenChem Corporation (Taichung, Taiwan)
b. Cayman Chemical (Ann Arbor, MI, USA)
c. LGC Limited (Teddington, UK)
d. Sigma-Aldrich Corporation (St. Louis, MO, USA)
e. Cerilliant Corporation (Austin, TX, USA), methanolic solutions (1 mg/mL)
*Quantifier ion

analogues especially NPS with the same frame structure, by monitoring fragment ions in a specific detection window with fixed retention times. This technique allows the identification and quantification of PEA-type substances with high accuracy and specificity. Good separation of the various analogues was achieved by applying appropriate SIM program settings determined by retention time. The cross contribution was further investigated through subsequent method validation to ensure limited impact on the analysis and sufficient specificity for quantification of analytes with the same mass pattern.

Method validation

In forensic analysis, the reliability and credibility of an analytical process influence court decisions; therefore, the method validation is indispensable. The results showed that

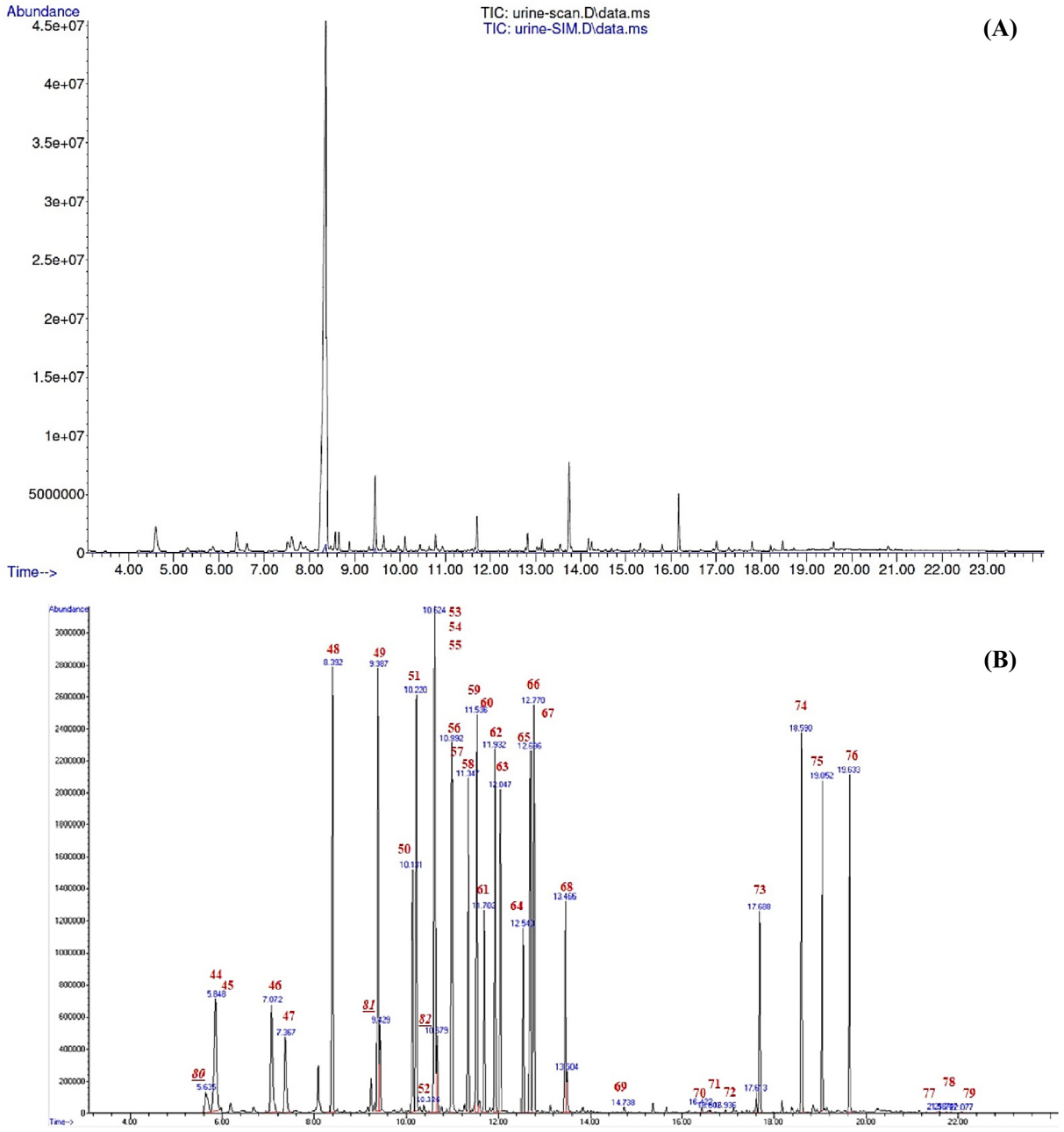

Figure 3. Total ion chromatograms (TIC) of blank urine, 76 targets, and 4 IS at 500 ng/mL in urine, ordered by item number of analytes from Table 1: (A) HFBA-derivatized blank urine under scanning and SIM modes; (B) and (C), HFBA-derivatized Group I PEAs under SIM mode; (D) PFPA-derivatized blank urine under scanning and SIM modes; (E) PFPA-derivatized Group II PEAs under SIM mode.

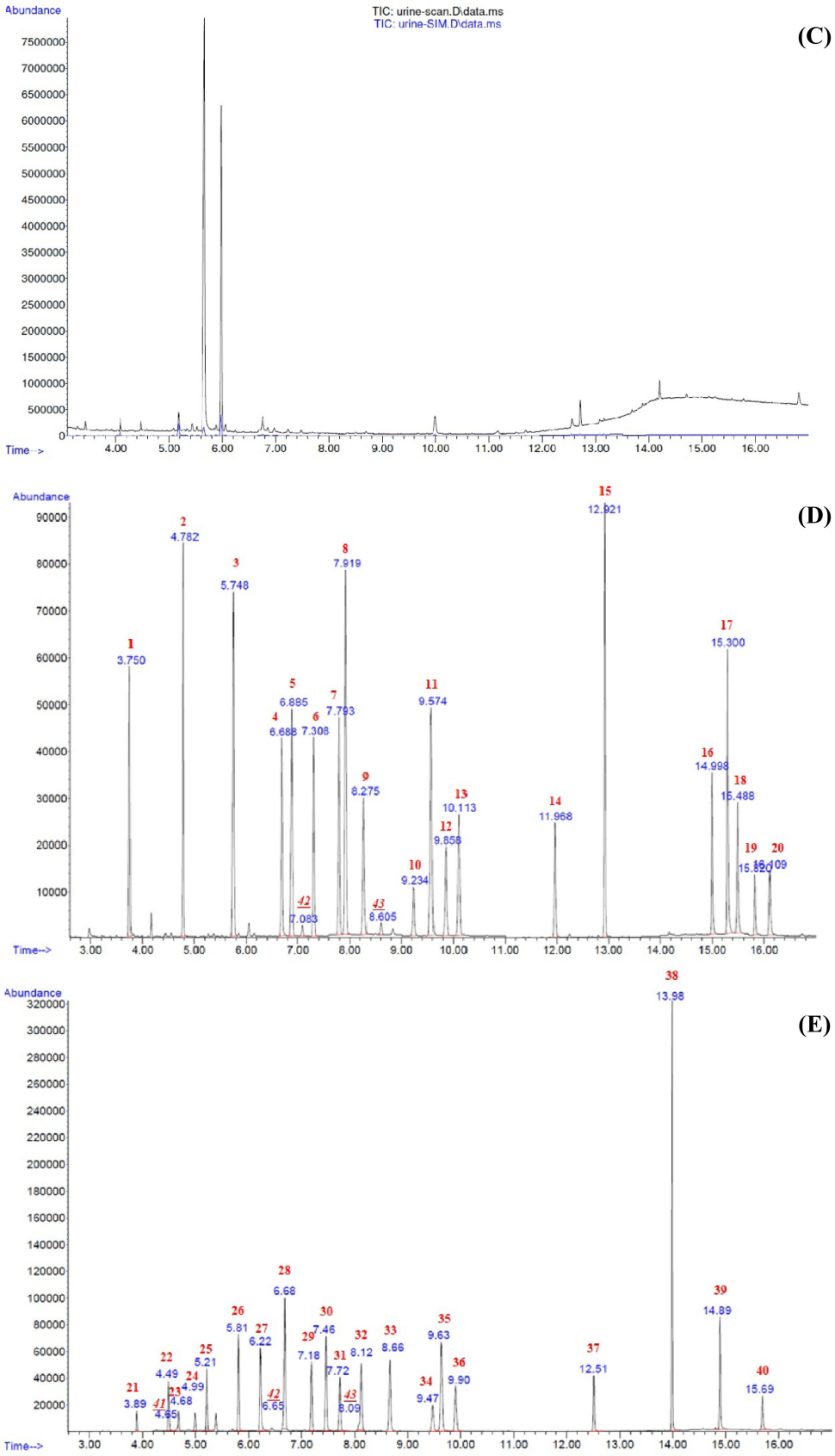

Fig. 3. Continued.

Table 2. Linearity, LOD, and LLOQ of 76 targets and 4 IS of PEAs for GC/MS[a]

Group	Item	Analyte	Concentration range (ng/mL)	Linearity (R)	LOD (ng/mL)	LLOQ (ng/mL)
	1	*N,N*-DMA	50-1000	0.9985	20	50
	2	4-MA	100-1000	0.9995	50	100
	3	4-CA	100-1000	0.999	50	100
	4	4-BA	50-1000	0.998	20	50
	5	4-CMA	50-1000	0.998	10	50
	6	PMMA	50-1000	0.9985	20	50
	7	PMEA	50-1000	0.9985	10	50
	8	5-APDB	50-1000	0.999	20	50
	9	Fenproporex	50-1000	0.999	20	50
	10	2C-E	50-1000	0.9985	20	50
	11	5-MAPDB	50-1000	0.9965	10	50
	12	MMDA	50-1000	0.9955	20	50
	13	DOC	100-1000	0.9995	50	100
	14	Proscaline	50-1000	0.9985	20	50
	15	Clobenzorex	50-1000	0.999	10	50
	16	25C-NBF	100-1000	0.9995	50	100
	17	3,4-DMA-NBOMe	50-1000	0.999	20	50
	18	25B-NBF	50-1000	0.9985	20	50
	19	25G-NBOMe	50-1000	0.9965	20	50
	20	25I-NBF	50-1000	0.997	20	50
	21	Amphetamine	50-2000	0.9959	20	50
I	22	FPBA	50-2000	0.9994	20	50
	23	Methamphetamine	50-2000	0.9993	20	50
	24	4-FEA	100-1000	0.9998	50	100
	25	5-F-2-MOA	50-2000	0.9999	20	50
	26	3-F-4-MOA	50-2000	0.9999	20	50
	27	MDDMA	100-2000	0.9994	50	100
	28	MDA	50-2000	1	20	50
	29	DMA	50-2000	0.9997	20	50
	30	Lefetamine	100-2000	0.9981	50	100
	31	DOM	50-2000	0.9982	20	50
	32	MDMA	50-2000	0.9996	20	50
	33	MDEA	100-2000	0.9996	50	100
	34	Mescaline	100-1000	0.9997	50	100
	35	Benzphetamine	50-2000	0.9998	20	50
	36	2C-C	50-1000	0.9989	20	50
	37	2C-I	50-1000	0.9981	20	50
	38	4-EA-NBOMe	50-1000	0.999	20	50
	39	25H-NBOMe	50-1000	0.9986	20	50
	40	25P-NBOMe	50-1000	0.9961	50	50
	41	MA-d_8	-	-	-	-
	42	MDA-d_5	-	-	-	-
	43	MDMA-d_5	-	-	-	-

Table 2. Continued.

Group	Item	Analyte	Concentration range (ng/mL)	Linearity (R)	LOD (ng/mL)	LLOQ (ng/mL)
	44	Phentermine	50-1000	0.9965	25	50
	45	4-FA	50-1000	0.9973	25	50
	46	4-FMA	50-1000	0.9996	25	50
	47	EMA	50-1000	0.9997	25	50
	48	PMA	50-1000	0.9987	25	50
	49	5-APB	50-1000	0.9962	25	50
	50	2C-H	25-1000	0.9976	10	25
	51	5-AEDB	25-1000	0.9965	10	25
	52	4-MTA	100-800	0.9964	50	100
	53	5-MAPB	50-1000	0.9976	25	50
	54	Mefenorex	50-1000	0.9981	25	50
	55	2C-D	100-1000	0.9951	50	100
	56	5-EAPB	50-1000	0.9978	25	50
	57	DOET	25-1000	0.9981	10	25
	58	MBDB	50-1000	0.9992	25	50
	59	TMA	50-1000	0.9983	25	50
	60	TMA-2	100-1000	0.9957	50	100
	61	TMA-6	100-1000	0.9954	50	100
	62	6-Cl-MDMA	50-1000	0.9964	25	50
II	63	Escaline	25-1000	0.9986	10	25
	64	DOB	25-1000	0.999	10	25
	65	3C-P	50-1000	0.997	25	50
	66	6-Br-MDMA	50-1000	0.9956	25	50
	67	2C-B	25-1000	0.9956	10	25
	68	DOI	100-1000	0.9966	50	100
	69	Lisdexamfetamine	200-1000	0.9971	100	200
	70	2C-T-2	200-800	0.9961	100	200
	71	2C-T-4	200-800	0.9984	100	200
	72	2C-T-7	200-800	0.9965	100	200
	73	25D-NBOMe	100-1000	0.9986	50	100
	74	25C-NB3OMe	50-1000	0.9977	25	50
	75	25B-NBOMe	50-1000	0.9978	25	50
	76	25I-NBOMe	100-1000	0.9975	50	100
	77	25T2-NBOMe	200-800	0.9985	100	200
	78	25T4-NBOMe	300-800	0.9986	200	300
	79	25T7-NBOMe	300-800	0.9997	200	300
	80	AM-d_8	-	-	-	-
	81	MDA-d_5	-	-	-	-
	82	MDMA-d_5	-	-	-	-

the carryovers for 76 PEAs were all less than 0.01% (data not shown), and no interference was seen in the subsequent analysis of the samples. In the evaluation of selectivity, no obvious impact from the above elements have been found and indicated the proposed methods as a selective technique for analyzing PEAs. Subsequently, the linearity was assessed through setting up calibration curves with the concentration range of 25 to 2000 ng/mL, based on the correla-

tion coefficient, r. The r values of the calibration curve for all analytes was above 0.995, and the determined LOD and LLOQ were 10–200 ng/mL and 25–200 ng/mL, respectively (see Table 2, alone with LC-MS/MS result of Table S2 in supplemental material). In particular, the targets lisdexamfetamine, 2C-T-2, 2C-T-4, 2C-T-7, 25T2-NBOMe, 25T4-NBOMe, and 25T7-NBOMe showed higher LOD and LLOQ which can be attributed to their low volatility. The obtained SPE extraction recovery was 80.2%–111.6% for all analytes, corresponding to the criteria of 80%–120%. To assess accuracy and precision, intra-day and inter-day experiments were performed using triplicate samples at three concentration levels. The ion ratio was over 50%, and the accuracy ranged from -12.5% to 18.9%. These values are within ±20% according to the respective criteria and are satisfactory. The results of extraction recovery, precision, and accuracy are shown as Table S3 in supplemental material.

Application

The goal of this study was to establish a set of derivatized GC/MS methods that could be used to analyze authentic urine samples and identify 76 PEAs. The current methods were applied to the analysis of authentic urine samples that were supplied by the accredited local analytical laboratories. A total of 20 samples were examined, and the results of urinalysis for targets above the LLOQs are shown in Table 3. Thirteen out of 20 samples tested positive for five target PEAs including *N,N*-DMA, PMMA, 4-CA, amphetamine, and methamphetamine. The detected concentration levels ranged from 94.5 ng/mL to 124.0 μg/mL.

Meanwhile, the authentic samples were also analyzed using the previously established LC-MS/MS method, to ensure whether there was equivalence between the two techniques in PEA monitoring.[19] The findings of the statistical assessments are shown as Table 4 (comparative result as Table S4 in supplementary material). In the LC-MS/MS analysis, a perfect agreement (kappa coefficient value = 1) was observed when the dilution rates of urine samples were 50× for *N,N*-DMA, PMMA, and 4-CA, and 500× for

Table 3. Targets detected above LLOQ from authentic urine samples applying GC/MS

Sample No.	Target detected	Content (μg/mL)	RSD (%)
1	*N,N*-DMA	94.5 ng/mL	4.94
	PMMA	196.9 ng/mL	0.85
2	*N,N*-DMA	239.2 ng/mL	3.26
	4-CA	1.5	3.55
5	*N,N*-DMA	655.1 ng/mL	0.84
6	*N,N*-DMA	317.6 ng/mL	0.78
11	Amphetamine	15.9	8.73
	Methamphetamine	82	11.7
13	Amphetamine	163.9 ng/mL	0.74
	Methamphetamine	1.2	0.36
14	Amphetamine	3	2.95
	Methamphetamine	30.3	0.81
15	Amphetamine	20	5.2
	Methamphetamine	95.6	0.81
16	Amphetamine	448.4 ng/mL	0.74
	Methamphetamine	43.8	2.33
17	Amphetamine	10.6	4.58
	Methamphetamine	68.3	4.27
18	Amphetamine	1.8	0.35
	Methamphetamine	21.3	1.11
19	Amphetamine	4	0.72
	Methamphetamine	22.1	0.47
20	Amphetamine	10.5	0.74
	Methamphetamine	124	1.19

Table 4. Statistical analysis for target determination applying LC-MS/MS and GC/MS methods

N,N-DMA		LC-MS/MS (50× dilution fold)		
		Positive	Negative	Total
GC/MS	Positive	4	0	4
	Negative	0	16	16
	Total	4	16	20
Kappa coefficient		1		
P value		NA		
x^2		NA		

PMMA		LC-MS/MS (50× dilution fold)		
		Positive	Negative	Total
GC/MS	Positive	1	0	1
	Negative	0	19	19
	Total	1	19	20
Kappa coefficient		1		
P value		NA		
x^2		NA		

4-CA		LC-MS/MS (50× dilution fold)		
		Positive	Negative	Total
GC/MS	Positive	1	0	1
	Negative	0	19	19
	Total	1	19	20
Kappa coefficient		1		
P value		NA		
x^2		NA		

Table 4. Coninued.

Methamphetamine		LC-MS/MS (50× dilution fold)			LC-MS/MS (500× dilution fold)		
		Positive	Negative	Total	Positive	Negative	Total
GC/MS	Positive	5	4	9	9	0	9
	Negative	0	11	11	0	11	11
	Total	5	15	20	9	11	20
Kappa coefficient		0.579			1		
P value		0.046			NA		
x^2		4			NA		

Amphetamine		LC-MS/MS (50× dilution fold)			LC-MS/MS (500× dilution fold)		
	Positive		Negative	Total	Positive	Negative	Total
GC/MS	Positive	1	7	8	8	0	8
	Negative	0	12	12	0	12	12
	Total	1	19	20	8	12	20
Kappa coefficient		0.146			1		
P value		0.014			NA		
x^2		6.036			NA		

amphetamine and methamphetamine, indicating a consistent result. Insufficient dilution rate (50×) of urine samples (in terms of amphetamine and methamphetamine) could result in invalid LC-MS/MS identification, making it impossible to reduce the matrix effect to meet the criterion for ion ratio of qualifiers, and hence a dilution rate of 500x was introduced to ensure consistency of the results.

The bulk of the analytical methods for identifying multiple PEAs in urine was established using LC-MS/MS.[25–27] In contrast, the GC/MS is generally considered to be less advantageous than the LC-MS/MS in terms of sensitivity, selectivity, and adaptability. Nonetheless, this study established the effective GC/MS methods with different derivatization process in identifying multiple PEAs. The current methods were suggested as an effective tool in screening/confirmatory testing as LC-MS/MS for identifying multiple PEAs, according to the result from statistical assess.

Conclusions

This study presents a set of derivatized GC/MS methods for detection of 76 target PEAs in urine. The proposed methods were applied to the analysis of authentic urine samples, of which 13 samples were detected positive for six identified targets in total out of 20 samples. The results of the urinalysis were statistically analyzed and compared with those obtained using the LC-MS/MS method that was previously established. The statistical results suggested that GC/MS was as efficient as LC-MS/MS for PEA urinalysis. Furthermore, this study provided a systematic technique for preliminary or confirmatory testing of PEAs, and the methods can be used to identify drug components in urine in the future.

Acknowledgments

This work was supported by the Taiwan Food and Drug Administration, Ministry of Health and Welfare, R.O.C.

References

1. Lum, G.; Mushlin, B. *Lab. Med.,* **2004**, 35, 368, DOI: https://doi.org/10.1309/QHJCKA4235EGPEGF
2. Akhgari, M.; Bahmanabadi, L.; Iravani, F. S. S.; Jokar, F. *Toxicol. Anal. et Clin.* **2021**, 33, 109, DOI: https://doi.org/10.1016/j.toxac.2020.10.089
3. Graziano, S.; Anzillotti, L.; Mannocchi, G.; Pichini, S.; Busardò, F. P. *J. Pharm. Biomed. Anal.* **2019**, 163, 170, DOI: https://doi.org/10.1016/j.jpba.2018.10.011
4. Favretto, D.; Pascali, J. P.; Tagliaro, F. *J. Chromatogr. A* **2013**, 1287, 84, DOI: https://doi.org/10.1016/j.chroma.2012.12.049
5. Staack, R. F.; Fritschi, G.; Maurer, H. H. *J. Chromatogr. B* **2002**, 773, 35, DOI: https://doi.org/10.1016/S1570-0232(01)00619-5
6. Kraemer, T.; Wennig, R.; Maurer, H. H. *J. Anal. Toxicol.* **2001**, 25, 333, DOI: https://doi.org/10.1093/jat/25.5.333.
7. Hawks, R. L.; Chiang, C. N. *Urine testing for drugs of abuse. Research monograph series 73. National Institute on Drug Abuse.* U.S. Department of Health and Human Services **1986**. https://archives.nida.nih.gov/sites/default/

files/monograph73.pdf
8. Casati, S.; Angeli, I.; Ravelli, A.; Del Fabbro, M.; Minoli, M.; Orioli, M. *Forensic Sci. Int.* **2019**, 304, 109951, DOI: https://doi.org/10.1016/j.forsciint.2019.109951
9. Warrens, M. J. *J. Psychol. Psychother.* **2015**, 5, 1000197, DOI: https://doi.org/10.4172/2161-0487.1000197
10. Feuerman, M.; Miller, A. R.; *J. Eval. Clin. Pract.* **2008**, 14, 930, DOI: https://doi.org/10.1111/j.1365-2753.2008.00984.x
11. Pembury Smith, M. Q. R.; Ruxton, G. D. *Behav. Ecol. Sociobiol.* **2020**, 74, 133, DOI: https://doi.org/10.1007/s00265-020-02916-y
12. ElMehy, A.; Wood, D.; El Madah, E.; Hassan, N.; Saad, K.; Dargan, P. *Asia Pac. J. Med. Toxicol.* **2022**, 11, 4, DOI: https://doi.org/10.22038/apjmt.2022.19912
13. Landis, J. R.; Koch, G. G. *Biometrics* **1977,** 33, 159, DOI: https://doi.org/10.2307/2529310
14. Ti, L.; Tobias, S.; Lysyshyn, M.;, Laing, R.; Nosova, E.; Choi, J. C.; Arredondo, J.; McCrae, K.; Tupper, K.; Wood, E. *Drug Alcohol Depend.* **2020**, 212, 108006, DOI: https://doi.org/10.1016/j.drugalcdep.2020.108006
15. Nwala, G. C.; Ibeneme, C. A.; Ojinnaka, N. C. *Int. j. sci. res.* **2021**, 10, 930, DOI: https://www.ijsr.net/getabstract.php?paperid=SR21311103801
16. Gjerde, H.; Gjersing, L.; Furuhaugen, H.; Bretteville-Jensen, A. L. *Subst. Use Misuse* **2019**, 1337, DOI: https://doi.org/10.1080/10826084.2019.1580295
17. *UNODC Early Warning Advisory on NPS - Summary Dashboard*, assessed May 2024. Available at:? https://www.unodc.org/LSS/Page/NPS/DataVisualisations
18. Mercolini, L. Chapter 20 - New Psychoactive Substances: An Overview, In A. Dasgupta (2nd Ed.), *Critical Issues in Alcohol and Drugs of Abuse Testing*, Academic Press, **2019**, 247. DOI: https://doi.org/10.1016/B978-0-12-815607-0.00020-4
19. Fan, S. Y.; Zang, C. Z.; Shih, P. H.; Ko, Y. C.; Hsu, Y. H.; Lin, M. C. Tseng, S. H.; Wang, D. Y. *Forensic Sci. Int.* **2021**, 325, 110884, DOI: https://doi.org/10.1016/j.forsciint.2021.110884
20. Scientific Working Group for Forensic Toxicology. *J. Anal. Toxicol.* **2013**, 37, 452, DOI: https://doi.org/10.1093/jat/bkt054
21. Laboratory and Scientific Section of the United Nations Office on Drugs and Crime, *Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens,* United Nations Office on Drugs and Crime **2009**. Available at: https://www.unodc.org/documents/scientific/validation_E.pdf
22. Giannini, E. H.; Design, measurement, and analysis of clinical investigations. *In Textbook of Pediatric Rheumatology* **2005**, 142, DOI: https://doi.org/10.1016/B978-1-4160-0246-8.50012-7
23. Omnicalculator online https://www.omnicalculator.com/statistics/mcnemars-test
24. He, Y. Liquid-based microextraction techniques for environmental analysis. In *Comprehensive Sampling and Sample Preparation* **2012,** 835, DOI: https://doi.org/10.1016/B978-0-12-381373-2.00116-2
25. Wagmann, L.; Maurer, H. H. *Exp. Pharmacol.* **2018**, 252, DOI: https://doi.org/10.1007/164_2017_83
26. Simão, A. Y.; Antunes, M.; Marques, H.; Rosado, T.; Soares, S.; Gonçalves, J.; Mário, B.; Maristela, A.; Gallardo, E. *Bioanalysis* **2020**, 12, DOI: https://doi.org/10.4155/bio-2020-0148
27. Di Trana, A.; Mannocchi, G.; Pirani, F.; La Maida, N.; Gottardi, M.; Pichini, S.; Busardò, F. P. *J. Anal. Toxicol.* **2020**, 44, DOI: https://doi.org/10.1093/jat/bkaa103