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Development and validation of a qualitative GC-MS method for methamphetamine and amphetamine in human urine using aqueous-phase ethyl chloroformate derivatization

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Abstract: Methamphetamine (MA) is the most common and available drug of abuse in Korea and its primary metabolite is amphetamine (AP). Detection of AP derivatives, such as MA, AP, phentermine (PT), MDA, MDMA, and MDEA by the use of immunoassay screening is not reliable and accurate due to cross-reactivity and insufficient specificity/sensitivity. Therefore, the analytical process accepted by most urine drug-testing programs employs the two-step method with an initial screening test followed by a more specific confirmatory test if the specimen screens positive. In this study, a gas chromatography-mass spectrometric (GC-MS) method was developed and validated for confirmation of MA and AP in human urine. Urine sample (500 μL) was added with N-isopropylbenzylamine as internal standard and ethyl chloroformate as a derivatization reagent, and then extracted with 200 μL of ethyl acetate. Extracted samples were analysed with GC-MS in the SIM/Scan mode, which were screened by Cobas c311 analyzer (Roche/Hitachi) to evaluate the efficiency as well as the compatibility of the GC-MS method. Qualitative method validation requirements for selectivity, limit of detection (LOD), precision, accuracy, and specificity/sensitivity were examined. These parameters were estimated on the basis of the most intense and characteristic ions in mass spectra of target compounds. Precision and accuracy were less than 5.2 % (RSD) and ±14.0 % (bias), respectively. The LODs were 3 ng/mL for MA and 1.5 ng/mL for AP. At the screening immunoassay had a sensitivity of 100% and a specificity of 95.1 % versus GC-MS for confirmatory testing. The applicability of the method was tested by the analysis of spiked urine and abusers' urine samples.

Key words: validation, qualitative analysis, aqueous-phase derivatization, urine, methamphetamine, GC-MS

1. Introduction

The administration of addictive drugs, such as narcotic substances (e.g., cocaine, heroin, morphine, amphetamine (AP)), leads to the loss of the regulatory functions involved in glutamate secretion due to the excessive secretion of dopamine. When the administration is constantly repeated, a loss of control owing to drug intake followed by drug addiction will ensue.[1] Such drugs not only exhibit potent effects but

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also increase the difficulty of predicting side effects. As a result, the level of damage caused by addiction to a drug is substantially high.[2,3]

With the recent spread of drug trafficking via the internet and social network service (SNS), the scale of drug smuggling has considerably increased with a steady increase in the number of drug offenders.[4,5] According to the White Paper on Drug-Related Crimes of the Supreme Prosecutors' Office, the annual number of drug offenders in Korea was 11,916 in 2015; 14,214 in 2016; 14,123 in 2017; and 12,613 in 2018.[6] Despite the deviations caused by differences in the level and frequency of narcotic control each year, the number has constantly been >10,000 for four consecutive years, thereby indicating the continuous increase in drug offenders.

Among narcotic substances, methamphetamine (MA; Philopon) is a highly addictive CNS stimulant and the most abused drug in Korea.[7] MA undergoes the metabolic processes in the body to produce AP as its main metabolite.[8] The pharmacological effect of MA is similar to that of AP; however, because it is more easily transported through the blood-brain barrier, it exhibits a stronger stimulant effect on the CNS with reinforced drug effects.[9] The most well-known AP derivatives include 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxymetamphetamine (MDMA), 3,4-Methylenedioxy-*N*-ethylamphetamine (MDEA), and phentermine (PT).[10]

The detection antibody of the immunoassay may cross-react with structurally related compounds that possess a similar antigenic determinant to the target analyte or their limited assay specificity/sensitivity may lead to reduced accuracy and reliability. Due to such issues, urine drug testing often adopts a 2-step process, where the positive result from the primary screening is evaluated via a secondary confirmation. The immunoassay that is often employed in the screening step is not only easy to use and automated, but also produces relatively rapid analysis results. [11,12] As a result, most screening steps utilize immunoassay. The forensic laboratories in Korea also utilize immunoassay during primary screening. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are the methods that are mainly used for secondary confirmation.[13,14] Although GC-MS is one of the most widely used methods in narcotic analysis, an efficient analysis with this method requires an additional process of derivatization that increases pretreatment time according to the substance that will be analyzed. Thus, in recent studies, there has been a notable trend to apply LC-MS/MS in the analyses as it does not require derivatization. Compared to GC-MS, the utility of LC-MS/MS is higher for analyzing substances that exhibit relatively high polarity among biological samples, with an additional benefit of reduced pretreatment time as the analysis can proceed without the process of derivatization.[15] GC-MS however allows the simultaneous use of selected ion mode (SIM) and scan mode, with the following benefits: i) the mass spectral library can be used in scan mode to detect unknown substances; and ii) the influence of the matrix is comparatively less than that in LC-MS/MS.[16,17]

For the confirmatory tests used in urine drug testing, the requirements for the validation of qualitative analytical methods vary according to the industry committees and regulatory agencies.[18,19] Similarly, when the effectiveness of qualitative analyses is being evaluated, different requirements are suggested by different studies; however, limit of detection (LOD) and specificity/sensitivity are the commonly recommended parameters. This finding may be attributed to the adjustment of parameters to suit the purpose of the qualitative analyses, which may differ from the requirements needed to validate quantitative analytical methods. Other parameters include selectivity, accuracy and precision, and ruggedness.[20-22]

Despite many advantages of GC-MS, time-consuming derivatization step has long been demonstrated as a major drawback. To reduce the time required for derivatization, aqueous-phase ethyl chloroformate derivatization, a process involving the simultaneous performance of extraction and derivatization to successfully reduce pretreatment time, was applied in the present study. The aqueous-phase alkyl chloroformate derivatization is known to be applicable

to the analyses of amino acids, fatty acids, and amines. Further, methyl, ethyl, and isobutyl chloroformates are known as the most commonly used derivatization reagents.[23-26] To obtain a more highly purified extract of the supernatant prior to instrumental analysis, high-speed centrifugation at 30,000 g was used to minimize the interference in this study. Further, to reduce the time required to concentrate the extract, an aliquot from the centrifuged supernatant was directly injected into the GC-MS system for analysis. To validate the qualitative GC-MS analysis developed in this study, the selectivity, accuracy and precision, and the utility of the analysis were determined using urine samples spiked with working standard solution and urine samples obtained from authentic drug users, while the specificity/sensitivity was determined by comparing the GC-MS results to those from the immunoassay.

2. Materials and Methods

2.1. Reagents and apparatus

d-AP (1000 μg/mL in methanol) and *d*-MA (1000 μg/mL in methanol) were purchased from Cerilliant (Austin, TX, USA). Amine Mixture-6 (250 μg/mL in methanol; the standard solution containing a mixture of AP, MA, PT, MDA, MDMA, and MDEA) was also purchased from Cerilliant. The internal standard (IS), N-isopropylbenzylamine (IPBA), was purchased from Toronto Research Chemicals (Ontario, Canada). To carry out the immunoassay, ONLINE DAT Amphetamines II reagent was purchased from Roche Diagnostics (Manheim, Germany). Ethyl chloroformate (ECF), the reagent for aqueous-phase alkyl chloroformate derivatization, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The organic solvents, ethyl acetate (HPLC grade) and methanol (HPLC grade), were purchased from J.T. Baker/Avantor (Center Valley, PA, USA). Potassium hydrogen carbonate, which was employed during pretreatment, was purchased from Wako (Osaka, Japan). All other reagents used in this study were above the ACS grade.

d-MA and *d*-AP were prepared by serial dilutions based on the purpose of use. The standard solution containing the drug mixture and IS solution were prepared through dilution with methanol in final concentrations of 100 μg/mL and 3 μg/mL, respectively. These standard solutions were stored at -20 °C until use.

2.2. Urine samples

To determine the LOD and specificity/sensitivity of the method, a mixture of urine samples obtained from subjects who had not taken any drugs was used as the blank sample. The specificity/sensitivity was determined by adding the reference material containing the analytes *d*-MA and *d*-AP, mixed in a 9:1 (*v/v*) ratio, to the blank sample. Thereafter, a set of diluted solutions of 1000, 500, 300, 200, 100, 50, and 25 ng/mL concentrations was prepared. To measure the ratio of the quantifier ion peak against the qualifier ion peak for the analytes, the Amine Mixture-6 reference material was added to the blank sample to prepare a set of diluted solutions of 1000, 500, 300, 200, 100, and 50 ng/mL concentrations.

Herein, urine samples from 166 subjects suspected of drug intake were collected and subjected to testing following a request from the district prosecutor's office and police stations in the Yeongnam area. The samples were stored in a 4 °C refrigerator for 20 days after receipt; if a sample required subsequent analyses, it was separately marked and stored in a -20 °C freezer until use.

2.3. GC-MS analysis

The immunoassay instrument used for primary screening was the Cobas c311 analyzer (Roche/Hitachi). The GC-MS used for secondary confirmation was the 7890 Gas Chromatograph/5975 Mass Selective Detector (Agilent Technologies, Avondale, PA, USA). DB-5MS UI (30 m × 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) was employed as the separation column. The temperature of the column was maintained at 100 °C for 1 min, and steadily raised to 220 °C at a rate of 15 °C/min. After maintaining the column at 220 °C for 2 min, the temperature was raised to 300 °C at a

rate of change of 40 °C/min; the column was maintained at 300 °C for 2 min. The injector and the GC interface temperatures were set to 250 °C and 280 °C, respectively. The flow rate of helium as the carrier gas was 1.0 mL/min, and the split mode was used as the injection method with a 10:1 ratio. A 2-μL volume of the sample was injected into the 7693 automatic liquid sampler (Agilent Technologies) for analysis.

The temperature of the ionization source in GC-MS was set to 230 °C; the ionization method was set to the electron ionization (EI) mode; and the analysis was carried out in SIM/scan mode. Based on the mass spectra of the analytes obtained in full scan mode, a quantifier ion and two qualifier ions were selected. Additionally, one characteristic ion was selected from the mass spectrum of IS for subsequent analyses. The ratio between quantifier ion and qualifier ion was calculated using the SIM chromatogram. To carry out the qualitative analyses using GC-MS, the generally applied permissible deviation of ±20 % was set for the ratio between the quantifier ion and qualifier ion according to the Laboratory Guidelines published jointly by the Society of Forensic Toxicologists and the American Academy of Forensic Sciences.[27]

2.4. Sample preparation

A 500-μL volume of the urine sample was mixed with 50 μL of IS (3 μg/mL), 50 μL of saturated $KHCO_3$ solution, 200 μL of ethyl acetate, and 50 μL of ECF. Thereafter, the mixture was transferred to a polypropylene tube for centrifugation (1.5 mL, Eppendorf). By performing a 30-s vortex at a rate of 3,200 rpm, extraction and derivatization (extractive derivatization) were simultaneously carried out. Following high-speed centrifugation of the sample at 30,000 g for 10 min at room temperature, 50 μL of the supernatant was transferred to a vial. Thereafter, a 2-μL aliquot was injected into the GC-MS system for analyses.

2.5. Method validation in qualitative analysis

To validate the methods based on qualitative analysis, the following were evaluated: selectivity, limit of detection (LOD), accuracy and precision, and specificity/sensitivity.

To evaluate the selectivity, 10 different urine samples were analyzed and evaluated by monitoring the interference on the retention times (RT) of analytes. Further, whether the RT of the analyte and IS were affected by interference was determined and compared by using the peaks on the chromatogram. The presence of interference caused by cross reactive compounds was determined by adding the analyte that is most likely to be detected in the authentic urine samples to the blank sample. The compounds used in the selectivity evaluation were MDMA, MDA, and MDEA, which exhibit high cross-reactivity, and PT, which has a high frequency of detection.[28]

The LOD was determined by using the standard deviation between the signal (S) from the results of 10 urine samples with the addition of the reference material of identical concentrations (10 ng/mL of MA and 5 ng/mL of AP) and the noise (N) obtained from 10 blank samples; the result with an S/N ≥ 3 was selected as the LOD.

The precision and accuracy were estimated to determine the reproducibility of the results. Precision indicates the proximity of the measured values obtained from repeated analyses when several aliquots from a homogenized sample are employed. In contrast, accuracy indicates the difference between nominal and measured values. To estimate the precision and accuracy, homogenized QC samples of three concentrations (50 ng/mL, 200 ng/mL, 1000 ng/mL)) were prepared, and six aliquots were used for repeated measurements. In the present study, the measured mean values were within 15 % (bias) of the nominal value for accuracy while the relative standard deviation (RSD) should not exceed 15 % for precision.

The specificity/sensitivity was evaluated by comparing the results of immunoassay to those from GC-MS.[29] The Cobas c311 analyzer was used as the immunoassay instrument used in the analysis while ONLINE DAT Amphetamines II with a cut-off of 300 ng/mL was used as the reagent. The screening result from the immunoassay was deemed positive if

equal to or above the cut-off value. The result was deemed true positive (TP) when the screening result was positive and the GC-MS had detected both MA and AP. However, it was deemed true negative (TN) when both instruments reported a negative result. If the screening result was positive but the GC-MS result was negative (i.e., neither MA nor AP was detected), the result was deemed false positive (FP). If the immunoassay led to a negative result but the GC-MS led to a positive result (i.e., at least one (MA or AP) was detected), the result was deemed false negative (FN). Herein, accuracy was calculated using (TP+TN)/(TP+TN+FN+FP) while specificity and sensitivity were calculated using TN/(TN+FP) and TP/(TP+FN), respectively.

3. Results and Discussion

3.1. Sample preparation

To reduce the time taken for derivatization, which accompanies the GC-MS method, the aqueous-phase alkyl chloroformate derivatization was carried out to simultaneously achieve extraction and derivatization. The derivatization was carried out using ECF as the derivatization reagent, which exhibits a high reactivity to amine compounds.[30] Additionally, ethyl acetate was used as the extraction solvent. By performing a 30-s vortex of the urine sample with the added derivatization reagent and extraction solvent, extractive derivatization was carried out. High-speed centrifugation was performed at 30,000 g for 10 min at room temperature to efficiently remove the interference. Thereafter, the pure extract from the supernatant could be obtained. The conventional method of analysis involves solid-phase extraction and derivatization with trifluoroacetic anhydride as an acylating agent. In addition, it requires approximately 70 min for single sample preparation and instrumental analysis.[31] Conversely, the analytical method used in this study required approximately 15 min for single sample

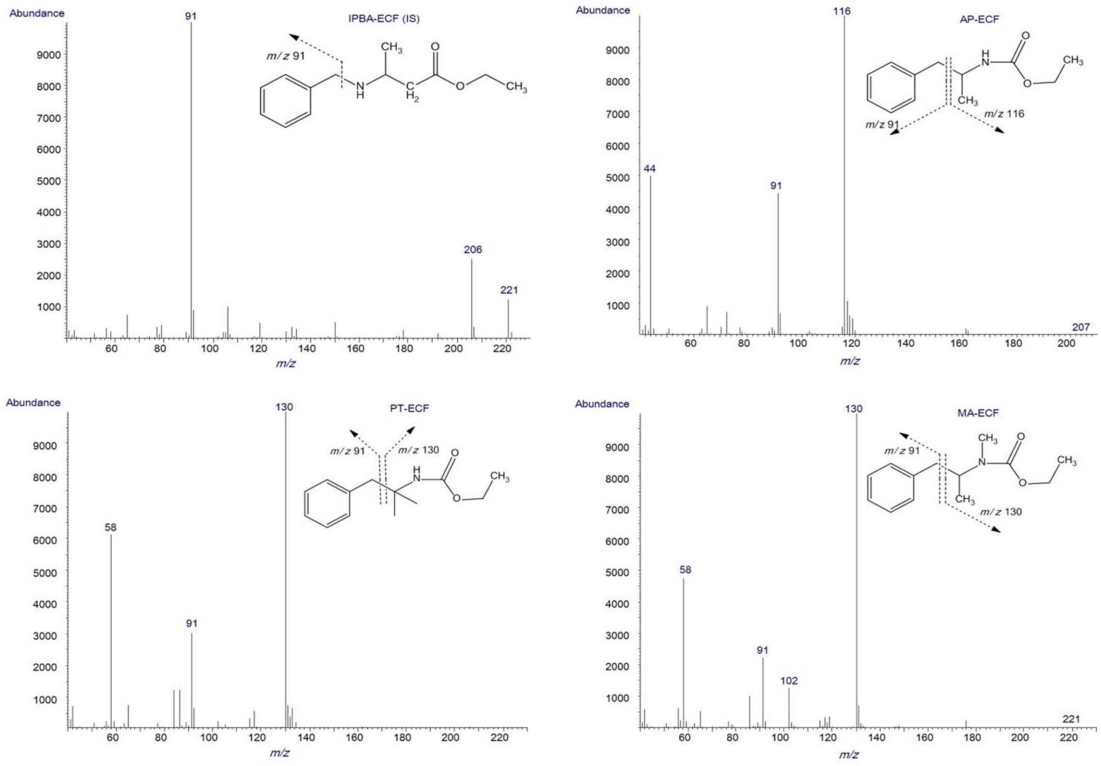

Fig. 1. Chemical structures of the analytes and mass spectra for ECF-derivatives.

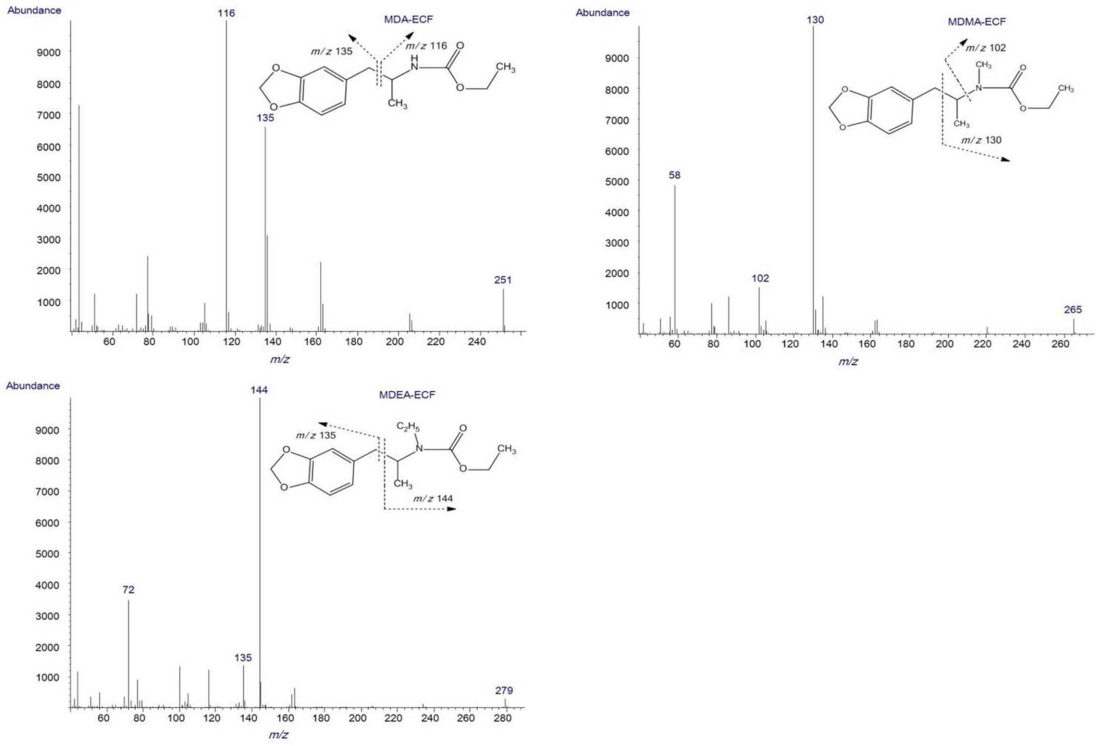

Fig. 1. Continued

preparation and 25 min when instrumental analysis was included. Such findings indicate that the use of the aqueous-phase ethyl chloroformate derivatization is effective at reducing workup required to perform an analysis. Additionally, a substantial cost reduction effect was observed regarding pretreatment owing to the use of solid-phase extraction cartridge, organic solvent, and disposable test tubes.

3.2. GC-MS analysis

To optimize the RT and peak shape of the analytes on the chromatogram, the GC temperature program was varied to enhance the separation of the analytes. Because the adherence of amine groups to the inner wall of a nonpolar capillary tube may hinder separation, prior to instrumental analysis, derivatization was carried out to convert the amine groups (-NH, -NH_2) in the analytes to nonpolar groups to enhance separation and sensitivity. The RT of each component was specified according to full-scan mass spectra of derivatized analytes obtained from GC-MS analysis. One characteristic quantifier ion and two qualifier

Table 1. Retention times, molecular weight, ions selected for GC-MS analysis of ECF-derivatives of the analytes

Compound	Retention time (min)	Molecular weight	Quantifier ion (*m/z*)	Qualifier ions (*m/z*)
IPBA (IS)	7.37	221	206	91, 221
AP	7.47	207	116	91, 65
PT	7.57	221	130	58, 91
MA	7.67	221	130	91, 102
MDA	10.24	251	116	135, 251
MDMA	10.41	265	130	135, 102
MDEA	10.74	279	144	116, 135

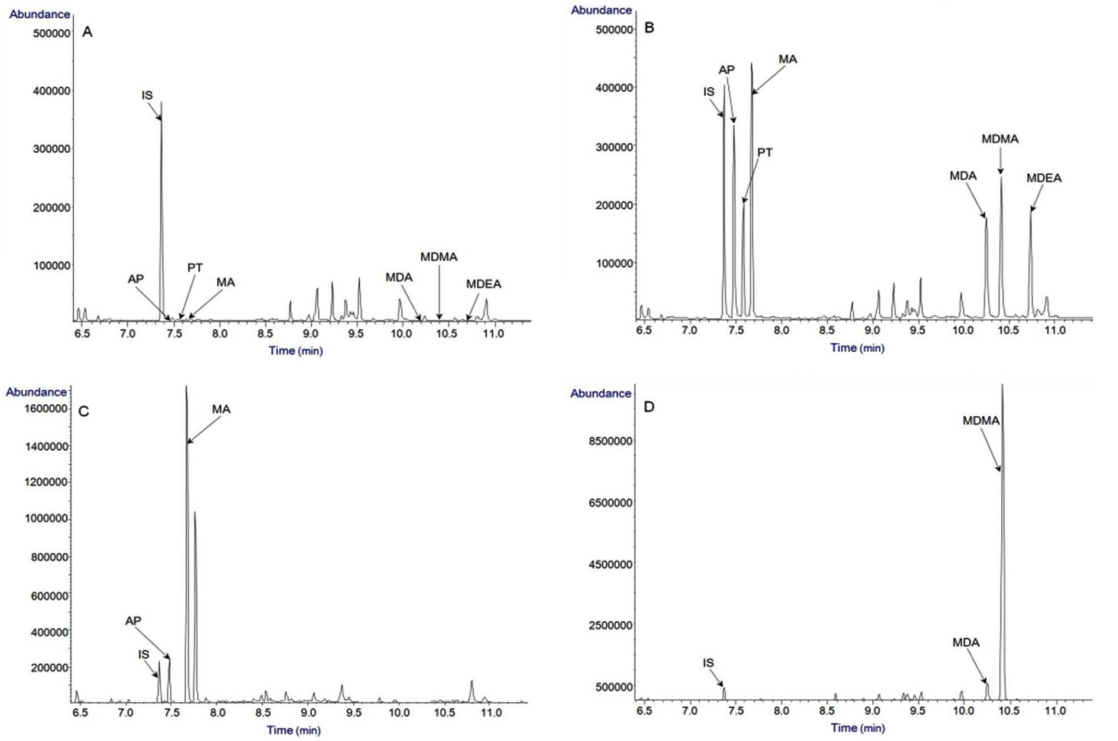

Fig. 2. Representative GC-MS extracted ion chromatograms of (a) blank urine with IS, (b) spiked urine containing 300 ng/mL of AP, PT, MA, MDA, MDMA and MDEA (c) MA positive urine and (d) MDMA positive urine samples.

ions were selected for qualitative analysis. *Fig.* 1 contains the mass spectrum used to examine the analytes and the chemical structure of the compounds after derivatization. *Table* 1 contains the data of RT, quantifier ions, and qualifier ions.

The GC-MS chromatogram obtained with the optimized GC-MS conditions is presented in *Fig.* 2. As demonstrated by the chromatograms of blank sample in *Fig.* 2A and sample spiked with the analytes in *Fig.* 2B, no interference was observed.

For the qualitative analyses performed with GC-MS, the permissible range of the ion ratio was within ± 20 % of the mean values for the ratio of the quantifier ion peak against the qualifier ion peak obtained with the aliquots (n = 5) prepared by the addition of MA and AP to the blank sample at concentrations of 50, 100, 200, 300, 500, and 1000 ng/mL. Based on our findings, the peak height of the quantifier ion (n = 1) and qualifier ion (n = 2) of MA was 4.2 and 7.7, respectively, while the permissible range was 3.3-5.0 and 6.1-9.2. For AP, the peak heights were 1.4 and 6.9, while the permissible range was 1.1-1.7 and 5.5-8.3.

3.3. Validation of qualitative analytical method

To validate the qualitative GC-MS analysis performed with aqueous-phase ethyl chloroformate derivatization, the selectivity, LOD, accuracy and precision, and specificity/sensitivity were estimated. To determine selectivity, the drug-free urine samples (n = 10) were analyzed. Based on our findings, no interference occurred in the analysis of MA or AP. Moreover, when interference was examined after the blank sample was mixed with MDMA, MDA, MDEA, and PT, which are the compounds most likely to be found in authentic urine samples, no interference was observed. Additionally, the separation was clear during the analysis of MA or AP.

When the quantification range for MA and AP was set to 25-1000 ng/mL, the determination coefficient (r^2)

Table 2. Accuracy and precision in measurements (n=6)

Compound	Nominal concentration (ng/mL)	Accuracy (% bias)	Precision (% RSD)
d-AP	5	-5.8	5.2
	20	-0.8	2.5
	100	-0.3	1.8
d-MA	45	14.0	1.6
	180	-9.6	0.5
	900	0.6	1.7

Accuracy calculated as [(mean calculated concentration - nominal concentration)/ nominal concentration] × 100
Precision expressed as RSD (relative standard deviation) of the peak area ratios of analyte/internal standard

of the calibration curve was > 0.9992, with linearity within the range. Moreover, the calculated LOD was 3.0 ng/mL for MA and 1.5 ng/mL for AP.

The accuracy (bias) and precision (RSD) of the analysis results are presented in *Table* 2. Based on our findings, the estimated accuracy was - 9.6 – 14.0 % while precision was approximately 5.2 %. The accuracy and precision were acceptable as they fell between ±15 % and within 15 %, respectively.

The specificity and sensitivity of the assays were calculated by comparing the results from the immunoassay to those from GC-MS, as depicted in *Table* 3. Among the urine samples, the immunoassay using the Cobas c311 analyzer and GC-MS had specificity and sensitivity of 95.1 % and 100 %, respectively, for MA. Notably, forensic laboratories are most interested in the false negative rate (FN rate, %); however, this was 0 %. Such finding indicates the complete absence of a false negative case (i.e., 0 false negative among the 166 authentic urine samples analyzed). However, a false positive rate (FP rate, %) of 4.2 % obtained because of cross-reactivity. By conducting a secondary confirmation using GC-MS, the compounds eliciting cross-reactivity were identified. The representative FP case is reported in *Fig.* 2D. The accuracy of the primary screening and secondary confirmation model was 95.8 % in this study.

3.4. Forensic applications

The usefulness of GC-MS method developed for secondary confirmation was investigated using the positive (n = 31) and negative (n = 135) urine samples retrieved at the primary screening with the immunoassay instrument. The GC-MS result for an MA subject and an MDMA subject is presented in *Fig.* 2C and *Fig.* 2D, respectively. The subject whose results are presented in *Fig.* 2C tested positive at the primary screening. Further, when the secondary GC-MS analysis was carried out, the case was finally confirmed to be MA positive owing to the detection of AP and MA. Similarly, the subject whose results are presented in *Fig.* 2D tested positive at the primary screening. The secondary GC-MS analysis also detected MDMA and MDA, confirming positive MDMA; the primary screening result was caused by cross-reactivity. Thereafter, the subject was finally confirmed as an FP case for MA. The analysis of 166 authentic urine samples verified the utility of the method developed in this study (i.e., primary screening by immunoassay coupled with secondary confirmation by GC-MS). The analysis results could be used to clearly determine whether the subject had taken MA.

Table 3. Test results and performance of Cobas c311 analyzer vs GC-MS

Test results					Performance		
		GC-MS			Sensitivity (%)	100	TP/(TP+FN)×100
		Positive	Negative	Sum	Specificity (%)	95.1	TN/(TN+FP)×100
Cobas	Positive	24 (TP)	7 (FP)	31	FP rate (%)	4.2	FP/(TP+TN+FN+FP)×100
	Negative	0 (FN)	135 (TN)	135	FN rate (%)	0.0	FN/(TP+TN+FN+FP)×100
	Sum	24	142	166	Accuracy (%)	95.8	(TP+TN)/(TP+TN+FN+FP)×100

TP: true positives; TN: true negatives; FP: false positives; FN: false negatives

4. Conclusions

In this study, we sought to address a persistent drawback regarding the derivatization process of GC-MS. By applying the method of aqueous-phase ethyl chloroformate derivatization for extractive derivatization, we could significantly reduce the pretreatment time. Further, by applying high-speed centrifugation at 30,000 g for sample purification, we could minimize the interference from the urine sample matrix. By using a small amount of extraction solvent and directly injecting a portion of the centrifuged supernatant into the GC-MS system, the additional concentration step could be eliminated. The qualitative GC-MS method developed in the present study was tested according to validation requirements to verify its effectiveness. When the novel method was applied to 166 authentic urine samples submitted for testing, the qualitative analysis results for MA and AP could be rapidly and accurately obtained without any interference and with excellent resolution.

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