Automated Headspace Solid-Phase Microextraction and In-Matrix Derivatization for the Determination of Amphetamine-Related Drugs in Human Urine by Gas Chromatography–Mass Spectrometry

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Abstract

An automated extraction and determination method for the gas chromatography (GC)–mass spectrometry (MS) analysis of amphetamine-related drugs in human urine is developed using headspace solid-phase microextraction (SPME) and in-matrix derivatization. A urine sample (0.5 mL), potassium carbonate (5M, 1.0 mL), sodium chloride (0.5 g), and ethylchloroformate (20 µL) are put in a sample vial. Amphetamine-related drugs are converted to ethylformate derivatives (carbamates) in the vial because amphetamine-related drugs in urine are quickly reacted with ethylchloroformate. An SPME fiber is then exposed at 80°C for 15 min in the headspace of the vial. The extracted derivatives to the fiber are desorbed by exposing the fiber in the injection port of a GC–MS. The calibration curves show linearity in the range of 1.0 to 1000 ng/mL for methamphetamine, fenfluramine, and methylenedioxymethamphetamine; 2.0 to 1000 ng/mL for amphetamine and phentermine; 5.0 to 1000 ng/mL for methylenedioxyamphetamine; 10 to 1000 ng/mL for phenethylamine; and 50 to 1000 ng/mL for 4-bromo-2,5-dimethoxyphenethylamine in urine. No interferences are found, and the time for analysis is 30 min for one sample. Furthermore, this proposed method is applied to some clinical and medico-legal cases by taking methamphetamine. Methamphetamine and its metabolite amphetamine are detected in the urine samples collected from the patients involved in the clinical cases. Methamphetamine, amphetamine, and phenethylamine are detected in the urine sample collected from the victim of a medico-legal case.

Introduction

Methamphetamine and its metabolite amphetamine are powerful stimulants of the central nervous system and are used for commonly abused drugs in many countries. Recently, ring-substituted amphetamines such as methylenedioxyamphetamines (MDA), methylenedioxymethamphetamine (MDMA), and 4-bromo-2,5-dimethoxyphenethylamine (2C-B) have been used for abused drugs. Acute deaths have been reported after overdoses of amphetamine and its related drugs. A widespread and automated method is required for the routine analysis and screening of amphetamine-related drugs in biological materials for forensic, judicial, and clinical purposes. Many chromatographic methods have been published to determine amphetamine-related drugs in biological materials (1,2), in which the extraction of the target drugs from sample matrices prior to chromatography was necessary. However, free amines are difficult to extract from sample matrices and cannot be well-separated because of their high polarity and volatility. In the analysis of free amphetamines by gas chromatography (GC) or GC–mass spectrometry (MS), difficulties have also been encountered in sensitivity and reproducibility because of adsorption and interaction with the column. It resulted in poor peak resolution. Therefore, a derivatization is usually needed for the GC analysis of these compounds. Thus, sample preparation procedures become laborious and costly. In addition, the organic solvents used are toxic in both the human body and the environment.

Solid-phase microextraction (SPME), which was first reported by Pawliszyn's research group (3), is a unique technique for the extraction of organic compounds in aqueous samples. It is a simple and rapid sample preparation method that does not require an organic solvent. There are many applications in which volatiles and semivolatiles have been extracted from environmental, food, and biological materials (4). Some methods for the determination of amphetamines in biological materials using SPME have been reported that include both the direct immersion and headspace method (5–16). In our previous studies, two

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derivatization methods have been examined. One was injection-port derivatization with heptafluorobutyryl anhydride (HFBA) following headspace-SPME extraction (6,15), and the other was in-matrix derivatization with pentafluorobenzyl bromide (PFB-Br) (16). The former method resulted in damage to the stationary phase of the analytical column, thus the lifetime of the column was shortened. Furthermore, with this method it is important to quickly inject HFBA into the injection port just before inserting the fiber. However, the latter method needed to agitate the sample solution enough to avoid a formation of clods of proteins in the solution. Thus, it is difficult to apply these two methods to an automated procedure using commercial apparatuses.

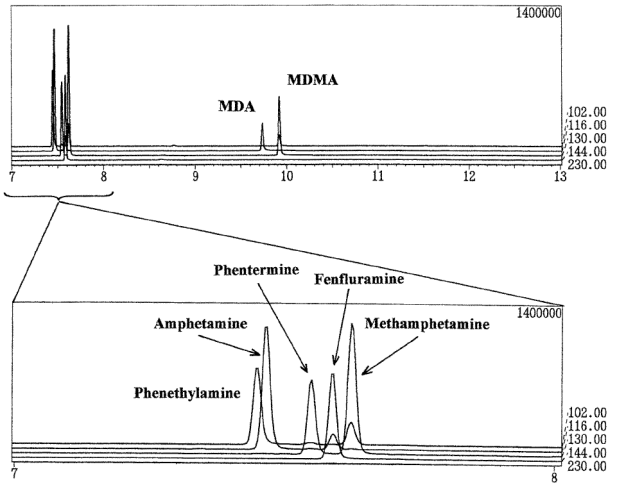

Figure 1. Typical SIM chromatogram of the ethylformate derivatives of amphetamine-related drugs.

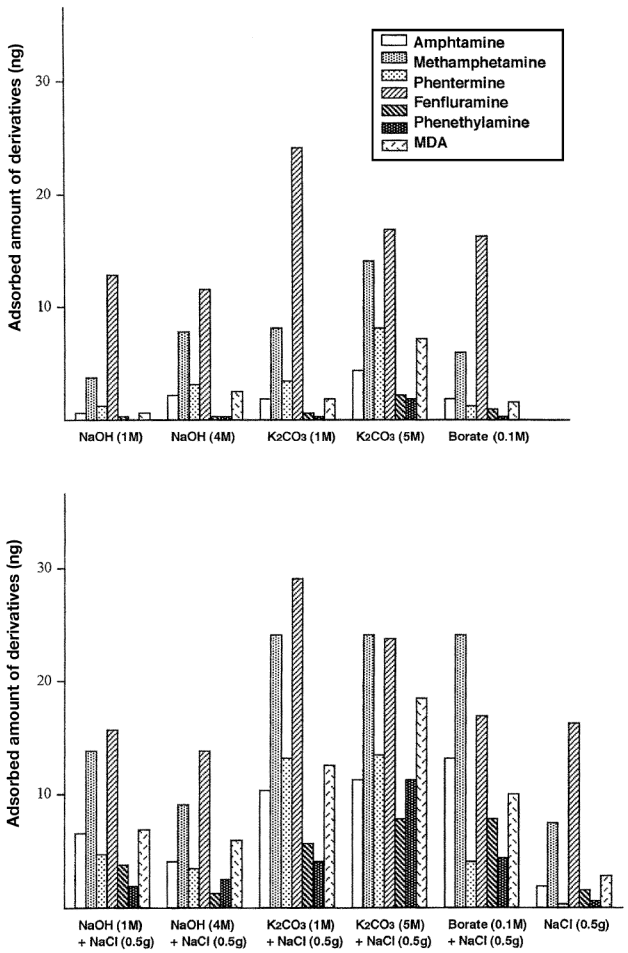

Figure 2. The effect of additives on adsorbed amounts of the ethylformate derivatives extracted from a spiked urine sample (500 ng/mL).

The aim of this study was to develop an automated method for the simultaneous determination of amphetamines and ring-substituted amphetamines in urine samples. Recently, alkylchloroformates have been used for the derivatization of amines in biological matrices (17). The alkylchloroformates quickly reacted with primary and secondary amines in an aqueous sample that kept only an alkali condition. Therefore, these reagents were suitable for our aim to derivatize amphetamine-related drugs. Although automated SPME methods for the determination of amphetamines in urine have been reported (11,14), the fiber was immersed into a urine sample and these methods were not optimized. In these immersed methods, nonvolatile and high molecular compounds were extracted simultaneously. Thus, sensitivity or resolution was decreased, and the fiber and the analytical apparatus were damaged by introducing the endogeneous compounds and excess reagent.

In this study, the headspace method was used to minimize endogenous interferences and damage to the apparatus and extend the half-life of the fiber. We performed an investigation to select a suitable derivatizing reagent for the separation of the derivatives and optimize the extraction condition of the derivatives in human urine. Finally, this proposed method was applied to clinical and medico-legal cases of methamphetamine intoxication.

Experimental

Materials

Amphetamine hydrosulfate and pentadeuterated methamphetamine (methamphetamine-d_5) hydrochloride were supplied by Dr. Hara of Fukuoka University (Fukuoka, Japan). Fenfluramine hydrochloride, MDA, MDMA, and phenethylamine were purchased from Sigma (St. Louis, MO). Methamphetamine hydrochloride was purchased from Dainihon Pharmaceutical Co. (Osaka, Japan). Phentermine hydrochloride was supplied by Dr. Kimoto of Kinki Regional Narcotic Control Office (Osaka, Japan). 2C-B was synthesized in our laboratory. Other reagents and solvents used were purchased at the highest commercial quality from Wako Pure Chemical Inc. (Osaka, Japan). Stock standard solutions (1.0 mg/mL) were dissolved in water or ethanol and stored at 4°C in a refrigerator.

A drug-free urine collected from a healthy adult male was used to make blank or spiked urine samples containing amphetamine-related drugs. Drug-free urine and urine samples collected from clinical and medico-legal cases were kept frozen at –20°C until analyzed.

A manual assembly of SPME with a replaceable extraction fiber coated with polydimethylsiloxane (PDMS, 100 µm) was purchased from Supelco (Tokyo, Japan). The fibers were conditioned in a GC injection port at 250°C for 1 h prior to use.

GC–MS

The GC–MS used was a Shimadzu (Kyoto, Japan) GC-17A and

QP-5000 equipped with a 30-m × 0.25-mm-i.d. fused-silica capillary column (Supelco, PTE-5, 0.25-µm film thickness). The oven temperature was set at 80°C for 3 min and then programmed from 80°C to 220°C at 40°C/min, 220°C to 280°C at 8°C/min, and held at 280°C for 3 min. The temperatures of the injection port and the interface were set at 250°C and 230°C, respectively. The splitless injection mode was used. Helium with a flow rate of 0.8 mL/min was used as a carrier gas. The ions used for quality were *m/z* 91, 116, and 207 for amphetamine; *m/z* 91, 102, 130, and 221 for methamphetamine; *m/z* 91 and 130 for phentermine; *m/z* 72, 116, 144, and 159 for fenfluramine; *m/z* 91, 102, and 193 for phenethylamine; *m/z* 102, 128, 230, 242, and 332 for 2C-B; *m/z* 116, 135, and 251 for MDA; and *m/z* 102, 130, 135, and 265 for MDMA at selected ion monitoring (SIM). Ions used for quantitation were *m/z* 102 for phenethylamine; *m/z* 116 for amphetamine and MDA; *m/z* 130 for methamphetamine, MDMA, and phentermine; *m/z* 144 for fenfluramine; *m/z* 230 for 2C-B; and *m/z* 134 for methamphetamine-d_5 (the internal standard).

Combi PAL

Combi PAL (CTC Analytics, Zwingen, Switzerland) is a multifunctional autosampler for the headspace and liquid GC injection system. As an option, an SPME fiber can be attached to this system and an automated SPME method can be applied for the analysis of many compounds. This apparatus can heat the sample during SPME extraction by the difference from a Varian autosampler. This fully automated analytical system consists of two components: a Combi PAL (a sample preparation device) and GC–MS. The control of the instrumentation was effected through preinstalled Combi PAL software (Cycle Composer). This software was used to create and edit the sample preparation and control the operation of Combi PAL. Using this software, extraction conditions (e.g., extraction temperature, time, and agitation rate) can be changed flexibly.

SPME procedure

A urine sample (0.5 mL), K_2CO_3 (5M, 1.0 mL), NaCl (0.5 g), ethylchloroformate (20 µL), and the internal standard (0.01 mg/mL, 30 µL) were placed into a 10-mL vial and sealed rapidly with a silicon septum and a vial cap. The vials were put in the sample tray. The following procedures (extraction and analysis) were worked automatically. The SPME needle was inserted into the vial, and the extraction fiber was exposed in the headspace. The vial was then heated at 80°C for 15 min. The vial was rotated at 250 rpm during the SPME extraction. After extraction, the fiber was pulled back into the needle and the needle was then inserted into the injection port of a GC–MS. The fiber was exposed for 3 min in the injector.

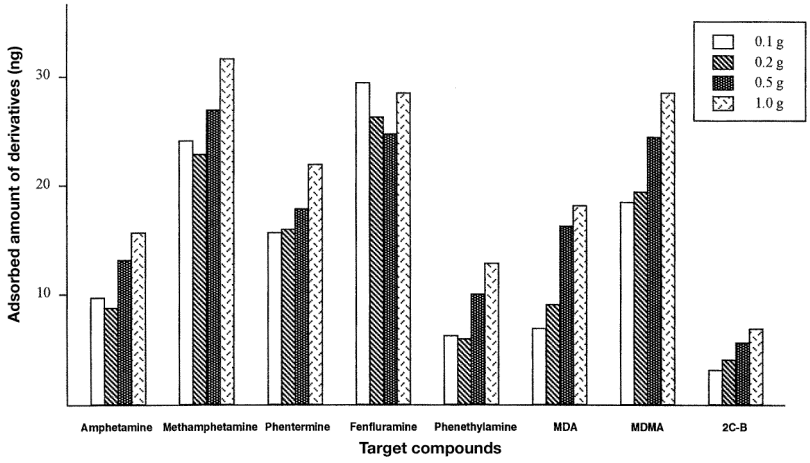

Figure 3. The effect of sodium chloride on adsorbed amounts of the ethylformate derivatives extracted from a spiked urine sample (500 ng/mL).

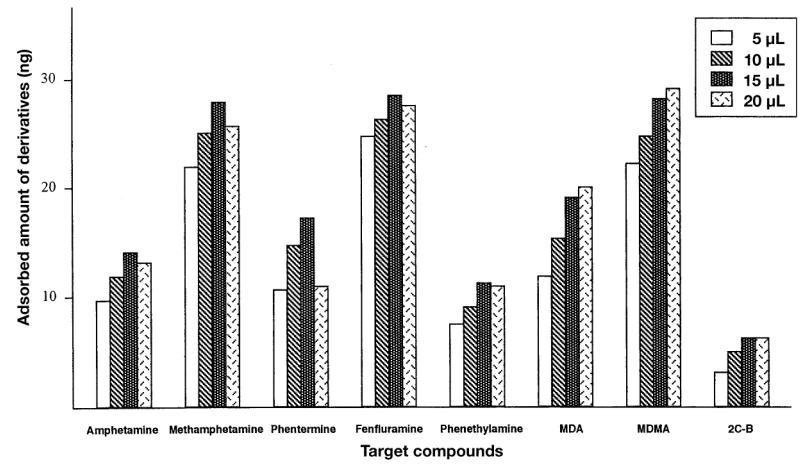

Figure 4. The effect of ethylchloroformate on adsorbed amounts of the ethylformate derivatives extracted from a spiked urine sample (500 ng/mL).

Analytical data

In order to examine the effect of additives on the target analyte extracted from urine, eight different conditions were tested for additives. When the optimal condition was examined, a urine sample spiked with 500 ng/mL of amphetamine-related drugs was prepared. The fiber was exposed for 20 min at 80°C and then analyzed. In order to determine the effect of the sodium chloride amounts on the adsorbed amount of amphetamine-related drugs, four different amounts (0.1, 0.2, 0.5, and 1.0 g) of sodium chloride were added in the vial. In order to determine the effect of the amount of the derivatizing reagent on the adsorbed amount of the amphetamine-related drugs, four different amounts (5, 10, 15, and 20 µL) of ethylchloroformate was added in the vial.

In order to determine the effect of the extraction temperature and time on the adsorbed amount of amphetamine-related drugs, the fiber was exposed to four different temperatures (70°C, 80°C, 90°C, and 100°C) for seven different times (3, 5, 10, 15, 20, 30, and 40 min).

Method validation

In order to determine calibration curves, urine samples spiked simultaneously with eight amphetamine-related drugs at concentrations ranging from 0.1 to 2000 ng/mL (14 points) were prepared and analyzed using the previously mentioned procedure. The analysis was performed in triplicates at each point. The calibration curves

were obtained by plotting the concentration and the peak-area ratio between target compounds and methamphetamine-d_5. Reproducibility was evaluated by analyzing urine samples containing three different concentrations (5.0, 50, and 500 ng/mL) of amphetamine-related drugs on the same day in six replicates (intraday reproducibility) and over six consecutive days in duplicates (interday reproducibility).

Results and Discussion

Selection of alkylchloroformates

Three alkylchloroformates (ethylchloroformate, propylchloroformate, and butylchloroformate) were examined for the derivatization of amphetamines in order to achieve a good chromatographic separation. Each derivatized amphetamine was prepared according to the method found in reference 17. In brief, amphetamines (1 µg) and a buffer (1 mL) were placed into a test tube. Ethyl acetate (1 mL) was added to the test tube and shaken for 5 min. The organic layer was transferred to a new tube and dehydrated by anhydrous sodium sulfate. The organic solution (1 µL) was injected to a GC–MS. The derivatives of methamphetamine and phentermine have the same base peak ions at any tested aklylchloroformate. The retention time of these two peaks was very closed, and the peaks were overlapped in the chromatogram when using butylchloroformate for the reagent. Thus, the limit of detection and quantitation of these derivatives decreased, and the problem of a misidentification had happened. Using propylchloroformate, an amphetamine derivative was always coeluted with a phenethylamine derivative at any tested condition, though these two derivatives have different base peak ions (whereas when using ethylchloroformate as the derivatizing reagent the better chromatographic separation was obtained). In Figure 1, the simultaneous separation of eight amphetamine-related drugs was shown. The order of elution of the analytes was phenethylamine, followed successively by amphetamine, phentermine, fenfluramine, methamphetamine, MDA, MDMA, and 2C-B.

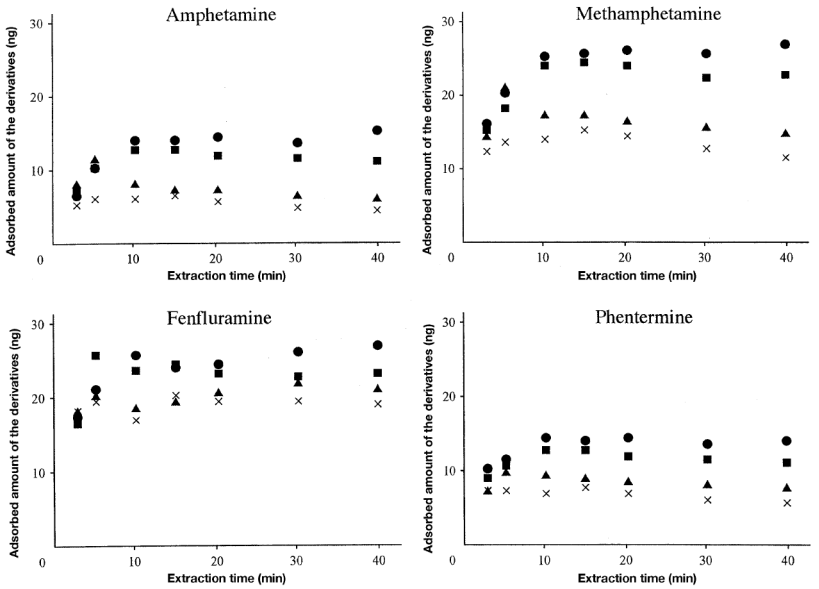

Figure 5. The extraction temperature–time profile on adsorbed amounts of the ethylformate derivatives extracted from a spiked urine sample (500 ng/mL): (●) 70°C, (■) 80°C, (▲) 90°C, and (×) 100°C.

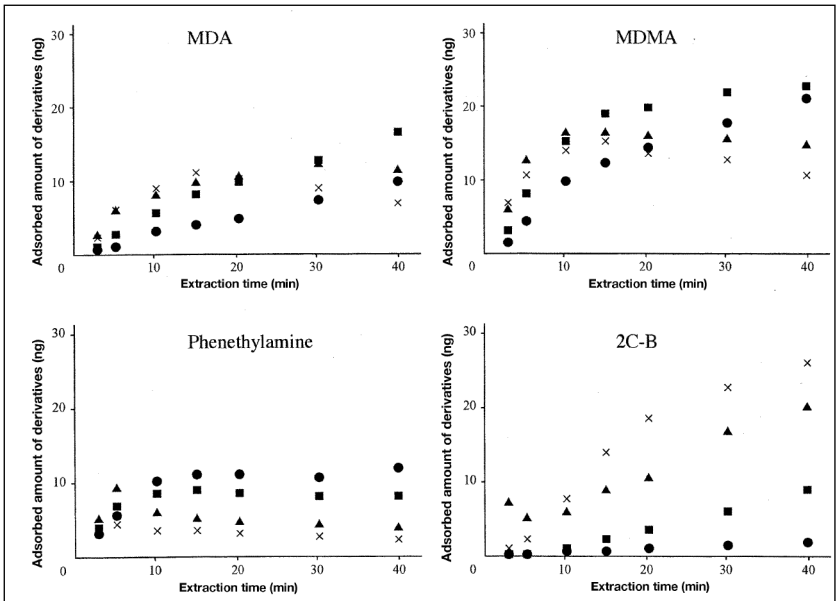

Figure 6. The extraction temperature–time profile on adsorbed amounts of the ethylformate derivatives extracted from a spiked urine sample (500 ng/mL) (continued): (●) 70°C, (■) 80°C, (▲) 90°C, and (×) 100°C.

Effect of SPME parameters

Headspace extraction has several advantages. First, the SPME fiber is protected from adverse effects caused by nonvolatile substances present in the sample matrices. Moreover, sample matrix modifications can be done without affecting the SPME fiber. Therefore, the headspace extraction mode was used for extracting analytes from biological materials in our studies. In general, the concentration of semivolatile compounds in the gaseous phase at room temperature is small, and headspace extraction rates for these compounds are substantially lower. Extraction rates of these compounds from headspace can be improved by changing the extraction parameters (i.e., increasing the extraction temperature or using the agitation) (18). The dramatic change with the equilibration time that is associated with an increase in temperature causes increases in both the Henry's constant of the analyte and the diffusion coefficient. Consequently, a less amount of analytes can be extracted at equilibrium. Additionally, this is also because the distribution constant decreases as the extraction temperature increases. Therefore, it is important to carefully optimize the extraction parameters for acceptable sensitivity.

In order to obtain acceptable precision and sensitivity, the extraction parameters (e.g., temperature, time, and pH) were examined. Before starting the SPME extraction, the preheating was not carried out to equilibrate the analytes between the headspace and the sample matrix in this study because the preheating procedure was not effective when methamphetamines in blood were extracted using SPME in our previous study.

In order to determine the effect of using alkaline for adjusting pH and sodium chloride on the adsorbed amount of the derivatives, the fiber was exposed in the headspace at 80°C for 20 min. The adsorbed amounts of the derivatives onto the fiber were dependent on the concentration of alkaline with and without sodium chloride (Figure 2). The recoveries of the derivatives from urine in the presence of both potassium carbonate (5M) and sodium chloride (0.5 g) were higher than other additives, and the coefficients of variation were smaller. The added amount of sodium chloride was changed to determine the effect on the adsorbed amount of the derivatives. The adsorbed amount of the derivatives was increased with that of sodium chloride and maximized at 1.0 g (Figure 3). However, sodium chloride was attached to the fiber and the repeatability was worthwhile in the addition of 1.0 g. Therefore, the added amount of sodium chloride was 0.5 g in the vial in order to extend the half-life of the fiber.

In order to minimize the damage to the fiber and GC–MS, the added amount of ethylchloroformate to the sample was examined. The adsorbed amounts of ring-substituted amphetamine (MDA, MDMA, and 2C-B) derivatives to the fiber were dependent on the amount of ethylchloroformate and maximized at 20 µL (Figure 4). However, the adsorbed amounts of amphetamine (amphetamine and the other compounds) derivatives to the fiber were maximized at 15 µL ethylchloroformate and decreased over the amount. Therefore, the added amount of ethylchloroformate was selected to be 20 µL.

Automated SPME methods for the determination of amphetamines in urine have been reported (11,14). In these reports, however, the extraction temperature was not optimized because the autosamplers could not heat the sample during the extraction period. In order to optimize the extraction temperature and time on the adsorbed amount of amphetamine-related drugs, the vial was heated at four different temperatures (70°C, 80°C, 90°C, and 100°C) for seven different periods (3, 5, 10, 15, 20, 30, and 40 min). The result is shown in Figures 5 and 6. The equilibrium of the amphetamines was reached at approximately 70°C or 80°C for 10 min, except for MDA, MDMA, and 2C-B. The equilibrium of MDA and MDMA was reached at approximately 90°C or 100°C for 15 min. The equilibrium of 2C-B could not be reached at up to 100°C for the tested periods. The adsorbed amounts of the amphetamine derivatives dramatically decreased as the extraction temperature increased from 80°C to over 90°C. However, the adsorbed amounts of the derivatives of the ring-substituted amphetamines slightly increased with the extraction temperature from 80°C to 90°C. Therefore, the fiber was exposed in the headspace of the vial at 80°C for 15 min. The recoveries of amphetamine-related drugs from the spiked urine (500 ng/mL) in this method were 3.2% to 10.0%, and the coefficients of variation were 1.17% to 8.07%. Typical SIM chromatograms extracted from the spiked urine are shown in Figure 7.

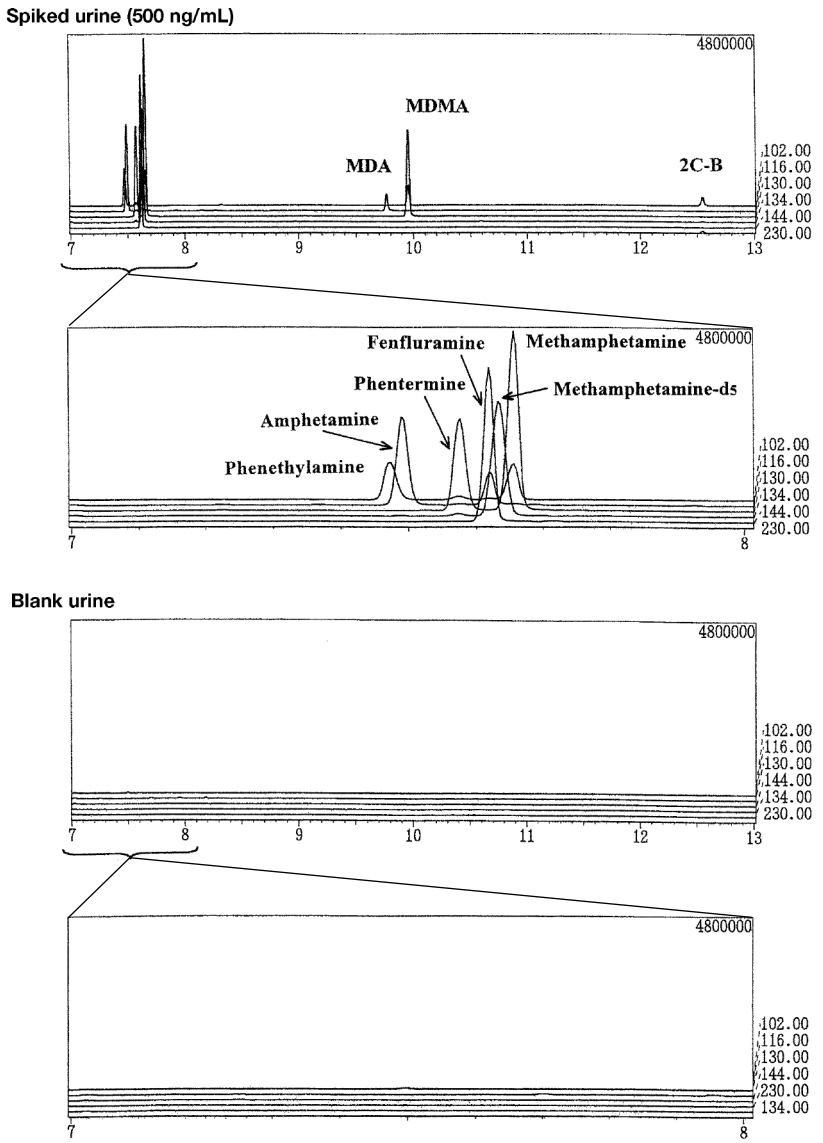

Figure 7. Typical SIM chromatograms of the ethylformate derivatives extracted from the spiked and blank urine samples.

Calibration curves and reproducibility

The calibration curves showed linearity in the range of 1.0 to 1000 ng/mL for methamphetamine, fenfluramine, and MDMA; 2.0 to 1000 ng/mL for amphetamine and phentermine; 5.0 to 1000 ng/mL for MDA; 10 to 1000 ng/mL for phenethylamine; and 50 to 1000 ng/mL for 2C-B in urine (Table I). The correlation coefficients of the calibration curves were 0.995 to 0.999. The limit of detection in urine was 0.5 to 50 ng/mL. This proposed method gave a substantial amount

for the detection of therapeutic levels and had a smaller coefficient of variation. The intraday and interday coefficients of variation for three different concentrations in urine were 1.17% to 8.07% and 2.98% to 12.2%, respectively (except for 2C-B) (Table II). Considering the intraday and interday coefficients of variation with the liquid–liquid or solid-phase extraction methods, the proposed method was found to be more reproducible.

Table I. Quantitation Limit and Linearity of the Method

Compounds	Limit of detection (ng/mL)	Range of linearity (ng/mL)	Linearity*	Correlation coefficient
Amphetamine	0.5	2.0–1000	$y = 1.47x + 0.0039$	0.999
Methamphetamine	0.5	1.0–1000	$y = 2.86x + 0.0061$	0.999
Phentermine	0.5	2.0–1000	$y = 1.57x - 0.0021$	0.999
Fenfluramine	0.5	2.0–1000	$y = 2.23x - 0.0027$	0.999
Phenethylamine	5.0	10–1000	$y = 0.57x + 0.0015$	0.999
MDA	2.0	5.0–1000	$y = 0.33x - 0.004$	0.996
MDMA	0.5	1.0–1000	$y = 1.80x - 0.0036$	0.998
2C-B	10	50–1000	$y = 0.05x - 0.001$	0.995

* x = amounts of analytes (µg/mL) and y = peak-area ratio.

Table II. Accuracy, Intraday, and Interday Precision for the Analysis of Amphetamine Analogues

	Intraday* ($n = 6$)		Interday† ($n = 12$)	
	mean ± SD‡	%CV§	mean ± SD	%CV
Amphetamine				
5	5.18 ± 0.31	6.04	5.18 ± 0.51	9.79
50	54.6 ± 2.57	4.17	47.9 ± 2.82	5.89
500	502 ± 5.9	1.17	504 ± 16.2	3.22
Methamphetamine				
5	4.90 ± 0.20	4.13	4.99 ± 42.5	8.51
50	50.1 ± 1.92	3.82	51.9 ± 1.96	3.77
500	492 ± 7.3	1.41	502 ± 14.5	2.89
Phentermine				
5	5.21 ± 0.36	6.92	4.48 ± 0.33	7.46
50	51.6 ± 2.17	4.22	50.0 ± 3.80	7.59
500	520 ± 19.7	3.79	479 ± 22.5	4.70
Fenfluramine				
5	6.01 ± 0.44	7.24	5.51 ± 0.43	7.87
50	50.3 ± 2.16	4.31	50.9 ± 3.09	6.08
500	529 ± 22.9	4.33	496 ± 30.0	6.05
Phenethylamine				
5	5.30 ± 0.34	6.51	5.23 ± 0.50	9.63
50	51.1 ± 3.52	6.90	47.9 ± 4.12	8.61
500	512 ± 13.1	2.55	482 ± 30.6	6.35
MDA				
5	4.96 ± 0.40	8.07	5.92 ± 0.72	12.2
50	50.7 ± 3.55	7.01	47.1 ± 4.80	10.2
500	485 ± 23.4	4.81	418 ± 31.9	7.63
MDMA				
5	5.38 ± 0.33	6.11	5.18 ± 0.49	9.52
50	50.2 ± 2.94	5.85	49.1 ± 2.44	4.96
500	495 ± 18.9	3.83	477 ± 30.1	6.32
2C-B				
5	–	–	–	–
50	44.8 ± 3.86	8.63	45.6 ± 12.1	26.5
500	476 ± 45.2	9.49	461 ± 100	21.7

* Performed on a single day of analysis.
† Performed over six consecutive days in duplicates.
‡ SD, standard deviation.
§ CV, coefficient of variation.

Application of medico-legal and clinical cases

This proposed method was applied to human urine samples obtained from eight patients having methamphetamine poisoning. Typical SIM chromatograms of some medico-legal and clinical cases are shown in Figure 8. A sharp and symmetrical peak of amphetamines was obtained without the disturbance of endogenous interferences. Methamphetamine, amphetamine, and phenethylamine were detected in the urine sample collected from the victim of a medico-legal case. It is well-known that phenethylamine occurs by decomposition. Phenethylamine could be detected from the urine of the victim, because some months passed posthumously in this case. Methamphetamine and its metabolite amphetamine were detected in the urine samples collected from the patients of the clinical cases.

The result of this proposed method was compared with the conventional method (6) using eight urine samples from individual patients who were taking methamphetamine. The diagrams are shown in Figure 9. The correlation values were 0.899 and 0.856 for amphetamine and methamphetamine, respectively. The results of this method were in good agreement with that of the conventional method.

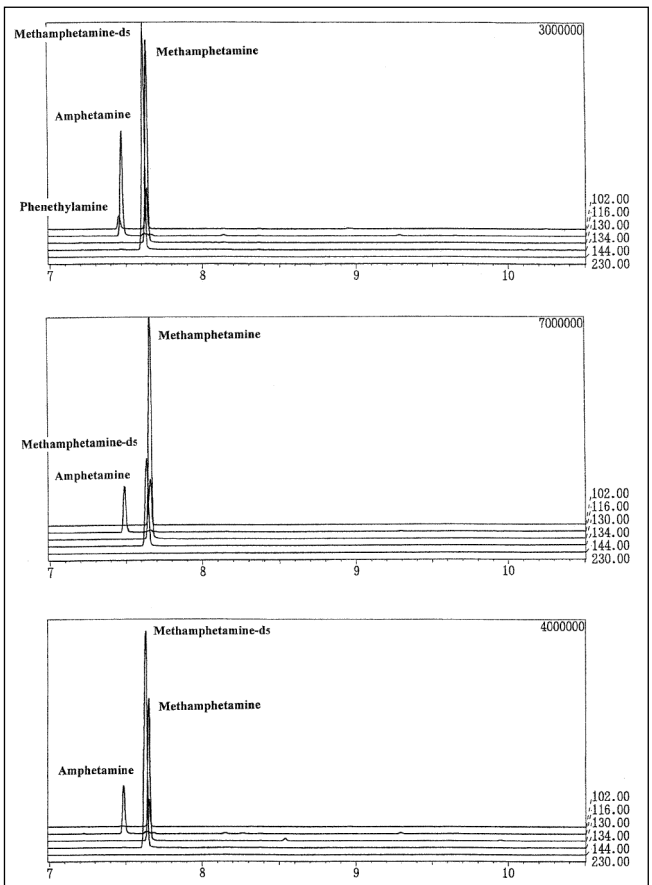

Figure 8. SIM chromatograms of the ethylformate derivatives from medico-legal and clinical cases.

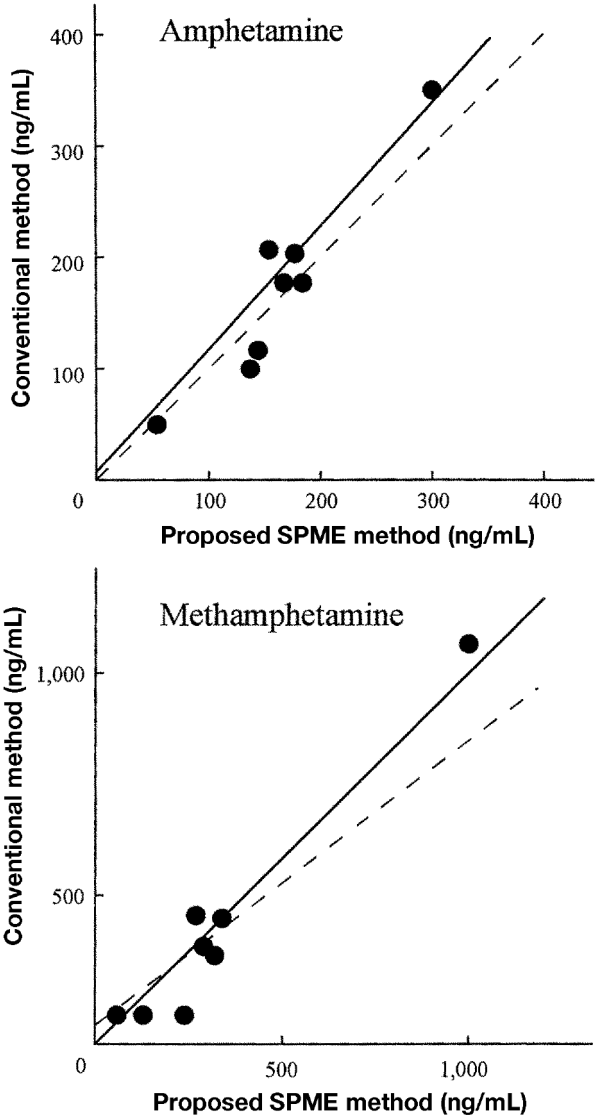

Figure 9. The regression for the amphetamine and methamphetamine concentration in urine samples determined with the proposed method versus the conventional method. The line of quality is indicated by a solid line, and the linear regression of the urine samples is indicated by a segmented line: amphetamine ($y = 1.13x - 20.69$, $r^2 = 0.899$) and methamphetamine ($y = 0.792x + 48.41$, $r^2 = 0.856$).

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