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# Simultaneous determination of methamphetamine and amphetamine in human urine using pipette tip solid-phase extraction and gas chromatography–mass spectrometry

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**Abstract**

Methamphetamine and amphetamine were extracted from human urine samples using pipette tip solid-phase extraction (SPE) with MonoTip $C_{18}$ tips (pipette tip volume, 200 μl), in which $C_{18}$-bonded monolithic silica gel was fixed. A sample of human urine (0.5 ml) containing methamphetamine, amphetamine, and *N*-methylbenzylamine as internal standard (IS), was mixed with 25 μl of 1 M sodium hydroxide solution. The mixture was extracted into the $C_{18}$ phase of the SPE tip by 25 repeated aspirating/dispensing cycles using a manual micropipettor. Analytes retained in the $C_{18}$ phase were then eluted with methanol by five repeated aspirating/dispensing cycles. After derivatization with trifluoroacetic anhydride, analytes were measured by gas chromatography/mass spectrometry with selected ion monitoring in the positive-ion electron impact mode. Recoveries of methamphetamine, amphetamine, and IS spiked into urine were more than 82.9, 82.2, and 78.2%, respectively. Regression equations for methamphetamine and amphetamine showed excellent linearity in the range of 0.25–200 ng/0.5 ml. Limit of detection was 0.04 ng/0.5 ml for methamphetamine and 0.05 ng/0.5 ml for amphetamine. Intra- and inter-day coefficients of variations for both stimulants were not greater than 10.8%. The data obtained from actual determination of methamphetamine and amphetamine in autopsy urine samples are also presented for validation of the method.


*Keywords:* Pipette tip solid-phase extraction; SPE tip; Methamphetamine; Amphetamine; Gas chromatography; Mass spectrometry

## 1. Introduction

Methamphetamine is a central nervous system stimulant that produces euphoria, hallucinations, increased alertness, and wakefulness [1]. The drug's strong reinforcing and addictive potentials contribute to abuse, and tolerance to its psychotropic effects leads to use of toxic doses. In Japan, methamphetamine is the most commonly abused drug. Due to widespread abuse of methamphetamine, drug testing for methamphetamine and its metabolites is routinely performed in clinical and forensic laboratories. Methamphetamine and its metabolites are generally assessed by urine analysis. Within a 24-h period, about 43% of an administered methamphetamine dose is excreted as such in urine in unchanged form, and approximately 5% of it is discharged as amphetamine [2].

Many methods have been reported to assess methamphetamine and amphetamine in human urine samples using gas chromatography (GC) [3], high-performance liquid chromatography (HPLC) [4,5], GC/mass spectrometry (MS) [6–12], HPLC–MS [13,14], capillary electrophoresis (CE) [15], and CE–MS [16]. Most of these methodologies employ extraction methods, such as liquid–liquid extraction (LLE) [3,4,9,13,15,16], and solid-phase extraction (SPE) [3,5–8,10–14], in order to remove impurities contained in urine samples. Although LLE and SPE methods may successfully extract methamphetamine and amphetamine from human urine samples, the large amount of

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organic solvent used in extraction procedures can cause health and environmental problems. In addition, off-line LLE and SPE methods are laborious, and time-consuming.

The pipette tip SPE (PT-SPE) method is now an essential tool for purification, concentration, and selective isolation (by affinity and metal chelator) of proteins and peptides in genomics, proteomics, and metabolomics [17–20]. To minimize required volumes of solvents and samples, SPE is available in a miniaturized format, such as SPE tips [21,22]. Recently, a new SPE tip, the MonoTip $C_{18}$ tip, was developed in Japan for purification of proteins, and peptides from aqueous samples [23]. In this device, monolithic silica, consisting of continuous mesoporous silica skeletons and through-pores, is fixed in the end of 10- and 200-μl pipette tips and the monolithic silica surface is chemically modified with the $C_{18}$ phase. The sample solution is aspirated and dispensed through the MonoTip $C_{18}$ tip for extraction of analytes using a micropipettor before HPLC or HPLC–MS analysis. An advantage of MonoTip $C_{18}$ tips for sample preparation is that extraction can be carried out more easily and rapidly than with conventional SPE cartridges. The small bed volume and sorbent mass within the MonoTip $C_{18}$ tip allow for the use of a reduced solvent volume, smaller elution volume, reduced time for the evaporation step, and higher throughput.

In previous studies, we demonstrated that the PT-SPE method was effective for extraction of antihistamine drugs from human plasma with good recovery, linearity, and reproducibility [24,25]. In this paper, establishment of the PT-SPE procedure for assessing methamphetamine and amphetamine from human urine samples using MonoTip $C_{18}$ tip and GC–MS analysis, is reported.

## 2. Experimental

### 2.1. Materials

Methamphetamine hydrochloride and *N*-methylbenzylamine as internal standard (IS) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and Wako Pure Chemicals Industries (Osaka), respectively. Amphetamine sulfate was synthesized according to the method of Lindeke and Cho [26]. Trifluoroacetic (TFA) anhydride was obtained from Pierce (Rockford, IL, USA). MonoTip $C_{18}$ tips (pipette tip volume, 200 μl; $C_{18}$-bonded monolithic silica gel with diameter of 2.8 mm, thickness of 1 mm, mesopore size of 15 nm, and through-pore size of 15–25 μm) were purchased from GL Sciences Inc. (Tokyo, Japan). Other common chemicals used were of the highest purity, and commercially available. Drug-free urine samples were obtained from healthy volunteers, and stored at −80 °C until use.

### 2.2. Preparation of standard solutions and quality control (QC) samples

Stock standard solutions of methamphetamine, amphetamine, and IS were prepared separately by dissolving appropriate amounts of each compound in methanol to achieve a concentration of 1 mg/ml. All stock solutions were stored at 4 °C. Working standard solutions of the compounds were prepared by serial dilution of stock standard solutions with methanol. A series of 0.05-ml standard solutions were evaporated to dryness under a gentle stream of nitrogen. Residues were reconstituted in 0.5 ml drug-free urine to prepare calibration standards containing 0.25–200 ng/0.5 ml for methamphetamine and amphetamine, and 50 ng/0.5 ml for IS. QC samples were prepared by the same procedure as used for calibration standards, and concentrations were 0.25–200 ng/0.5 ml for both methamphetamine and amphetamine, and 0.5–200 ng/0.5 ml for IS.

### 2.3. PT-SPE procedure

Extraction of methamphetamine, amphetamine, and IS from human urine was achieved using a MonoTip $C_{18}$ tip. After attaching the tip onto a Pipetman P200 pipette (Gilson SAS, Villiers-le-Bel, France), preconditioning of the tip was done by aspirating and dispensing (to waste) 200 μl methanol, and then 200 μl distilled water through the tip. For new tips, this procedure was repeated twice to reduce background noise. Twenty-five microliters of 1 M sodium hydroxide solution were added to 0.5 ml of a urine sample containing methamphetamine, amphetamine, and IS in a clean sample tube (1.5 ml). A 200-μl aliquot of the sample was aspirated into the conditioned MonoTip $C_{18}$ tip, and dispensed back into the same sample tube. These two steps are referred to as one aspirating/dispensing cycle. In this work, extraction of methamphetamine, amphetamine, and IS onto the $C_{18}$ phase of the tip was performed by 25 repeated aspirating/dispensing cycles. The tip was then washed by aspirating 200 μl distilled water, and dispensing the eluate as waste. After washing, the tip was placed on a vacuum manifold, and dried under vacuum for 3 min to remove any traces of water. Finally, analytes and IS were eluted from the tip with 100 μl methanol into a vial (4 ml) by five repeated aspirating/dispensing cycles. After addition of one drop of acetic acid, eluates were evaporated to dryness under a stream of nitrogen. The dried residue was used for derivatization.

### 2.4. Derivatization

Methamphetamine, amphetamine, and IS were derivatized with TFA anhydride. A 100-μl aliquot of TFA anhydride–ethyl acetate (5:1, v/v) was added to each residue, and samples were capped, mixed, and heated at 80 °C for 10 min with an aluminum block heater (Reacti-Therm™ Heating/Stirring Model; Pierce). After cooling to room temperature, the solvent was then evaporated to dryness under a stream of nitrogen, and residues were reconstituted in 100 μl ethyl acetate. A 2-μl aliquot of sample solution was subjected to GC–MS analysis. Chemical structures of the derivatives are shown in Fig. 1.

### 2.5. GC–MS conditions

All analyses were performed using a Shimadzu GC-2010 gas chromatograph interfaced with a Shimadzu QP-2010 quadrupole mass spectrometer (Shimadzu Corp., Kyoto, Japan).

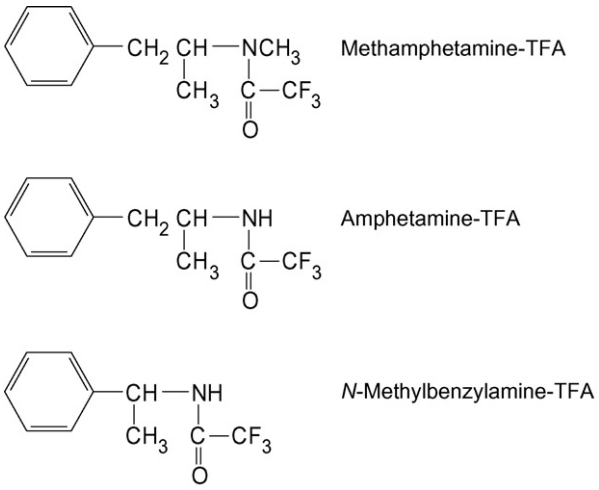


Fig. 1. Chemical structures of TFA derivatives of methamphetamine, amphetamine, and *N*-methylbenzylamine (IS) used in this study.

The GC–MS was operated with an interface temperature of 300 °C, and an ionization source temperature of 250 °C. The mass spectrometer was tuned daily using perfluorotributylamine. A solvent delay of 3.5 min was set to protect the filament from oxidation. Chromatographic separation was achieved using a DB-5MS fused silica capillary column (30 m × 0.32 mm i.d., film thickness, 0.25 μm; J&W Scientific, Folsom, CA, USA). Helium with a minimum purity of 99.99995% was used as carrier gas at a flow rate of 2 ml/min. The gas chromatograph was equipped with a split/splitless injection port operated at 250 °C. Samples were injected in the splitless mode at a column temperature of 60 °C, then the splitter was opened after 1 min. The gas chromatograph oven temperature was programmed as follows: initial temperature, 60 °C for 1 min; from 60 to 200 °C at a rate of 20 °C/min and finally from 200 to 300 °C at a rate of 40 °C/min. Final temperature was held for 4.5 min. The mass spectrometer was operated in the positive-ion electron impact (EI) mode. EI mass spectra were obtained at an ionizing energy of 70 eV, and at an emission current of 60 μA. Quantification was carried out in the selected ion monitoring (SIM) mode. In order to select the monitoring ion for methamphetamine, amphetamine, and IS, mass spectra were obtained from injections of TFA derivatives of the analyte standards into the GC–MS. Table 1 summarizes major fragment ions for derivatives of methamphetamine, amphetamine, and IS. The monitored ions for quantitative analysis were *m/z* 154 for methamphetamine, *m/z* 118 for amphetamine, and *m/z* 217 for IS.

### 2.6. Evaluation of recovery, quantification, and linearity

Recoveries were calculated by comparing chromatographic peak areas obtained from extracts of QC samples with those obtained by direct GC injection of TFA derivatives of standard compounds dissolved in ethyl acetate. Recoveries were determined at four different concentrations of methamphetamine, amphetamine, and IS. Regression equations for methamphetamine and amphetamine extracted from human urine were obtained by fitting the ratio of the peak area of the analyte to that of the IS (50 ng) versus concentration of analytes. Concentrations of calibrators ranged from 0.25 to 200 ng/0.5 ml for both methamphetamine and amphetamine (10 calibrators: 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng/0.5 ml). Equations were then used to calculate concentrations of QC samples or autopsy samples. Intra-day coefficient of variation (CV) and accuracy were determined by replicate analysis of QC samples spiked with four different concentrations of methamphetamine and amphetamine. The same procedure was repeated for 5 days in order to determine inter-day CV and accuracy.

The limit of detection (LOD) was defined as the lowest concentration of analyte spiked in urine that could be detected with a signal-to-noise ratio of at least 3. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve that could be measured with a signal-to-noise ratio of at least 10, a intra-day CV ≤20%, and an accuracy of 80–120%. The upper limit of quantification (ULOQ) was obtained on the calibration curve with an acceptable intra-day CV ≤20%, and an accuracy of 80–120%. In the present study, however, ULOQ was set at ≤200 ng/0.5 ml, to protect the MS detector from excessive ions. The acceptance criterion for the correlation coefficient was >0.999.

### 2.7. Forensic autopsy samples

Urine samples were obtained from four forensic autopsy cases from the Department of Legal Medicine, Aichi Medical University School of Medicine. Urine toxicology screening was previously performed by immunoassay (Triage™ Drugs of Abuse Panel plus Tricyclic Antidepressants, Biosite Diagnostic Inc., San Diego, CA), and results were 'positive' for amphetamines and 'negative' for phencyclidine, benzodiazepines, cocaine, cannabinoids, opiates, barbiturates, and tricyclic antidepressants, for all urine samples. Following screening, all urine samples were stored at –80 °C until determination of methamphetamine and amphetamine. This study was

Table 1
Mass spectral characteristics of TFA derivatives of methamphetamine, amphetamine, and *N*-methylbenzylamine (IS) obtained in the positive-ion EI mode

| Compound (TFA derivative) | MW[a] | Monitored ions[b] |
|---|---|---|
| Methamphetamine | 245 | **154**[c] (100), 118 (47), 110 (34), 91 (17) |
| Amphetamine | 231 | 140 (94), **118** (100), 91 (42) |
| *N*-Methylbenzylamine (IS) | 217 | **217** (57), 148 (23), 91 (100) |

[a] MW, molecular weight.
[b] *m/z* and relative intensity (%).
[c] Quantification ion of each compound is printed in bold.

approved by the Ethics Committees of both Showa University School of Medicine and Aichi Medical University School of Medicine.

## 3. Results and discussion

### 3.1. Optimization of extraction conditions for MonoTip $C_{18}$ tips

The number of aspirating/dispensing cycles is a critical parameter for extraction recovery for the PT-SPE method using MonoTip $C_{18}$ tips. Extraction profiles of various amounts of methamphetamine, amphetamine, and IS were examined by plotting analyte recovery from urine samples versus aspirating/dispensing cycles (Fig. 2). Extraction of the compounds reached equilibrium after 25 aspirating/dispensing cycles at both low (20 ng/0.5 ml) and high (200 ng/0.5 ml) concentrations. Therefore, we decided that the number of aspirating/dispensing cycles of extraction should be 25 cycles (roughly 1.5 min). In the elution process, analytes were eluted from the MonoTip $C_{18}$ tip into a vial (4 ml) by aspirating/dispensing 100 μl methanol through the tip several times. Results showed that the number of aspirating/dispensing cycles for desorption was not significant for the compounds tested. However, to achieve sufficient recovery within a short period of time, five repeated aspirating/dispensing cycles (roughly 10 s) with 100 μl methanol were used in the elution step.

In the present study, all extraction procedures including conditioning, sampling (extraction), washing, drying, and elution by the MonoTip $C_{18}$ tips required approximately 8 min. However, the time required to manually perform conventional cartridge SPE was reported to be >20 min [11,27,28]. Therefore, the use of MonoTip $C_{18}$ tips is recommended for rapid extraction of methamphetamine and amphetamine from human urine.

### 3.2. Method validation

Fig. 3 shows SIM chromatograms obtained for extracts from 0.5 ml human urine in the presence (20 ng of each compound) or absence of test compounds. Distinct peaks appeared for the three analytes and retention times for IS, amphetamine, and methamphetamine were 5.68, 5.91, and 6.68 min, respectively (Fig. 3, lower panel). While small impurity peaks were observed for blank urine, no interfering peaks were found around peaks of the test compounds (Fig. 3, upper panel).

Recoveries of methamphetamine, amphetamine, and IS from urine samples using the present method are presented in Table 2. Recoveries of methamphetamine, amphetamine, and IS were 82.9–88.3%, 82.2–88.2%, and 78.2–82.7%, respectively, and were considered satisfactory. Regression equations for both methamphetamine and amphetamine extracted from human urine exhibited good linearity in the range of 0.25–200 ng/0.5 ml. The equations and correlation coefficients were: $y = 0.12795x - 0.01347$ and $r = 0.99999$ for methamphetamine, and $y = 0.07431x - 0.00334$ and $r = 0.99994$ for amphetamine. LODs of methamphetamine and amphetamine under optimal conditions were 0.04 and 0.05 ng/0.5 ml, respectively. LLOQ and ULOQ corresponding to lowest and highest

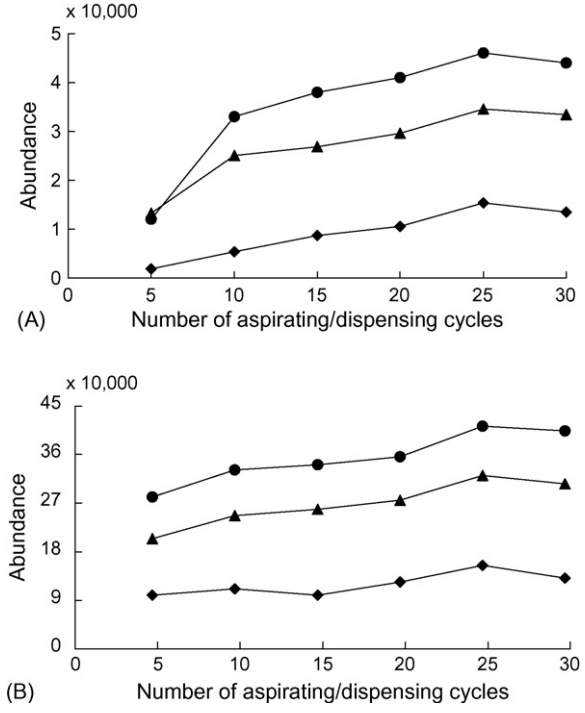


Fig. 2. Effects of numbers of aspirating/dispensing cycles on extractions of methamphetamine (●), amphetamine (▲), and IS (■) in human urine using MonoTip $C_{18}$ tips. Amount of each compound spiked into 0.5 ml urine was 20 ng and 200 ng in graphs A and B, respectively. Each data point represents mean of duplicate determinations.

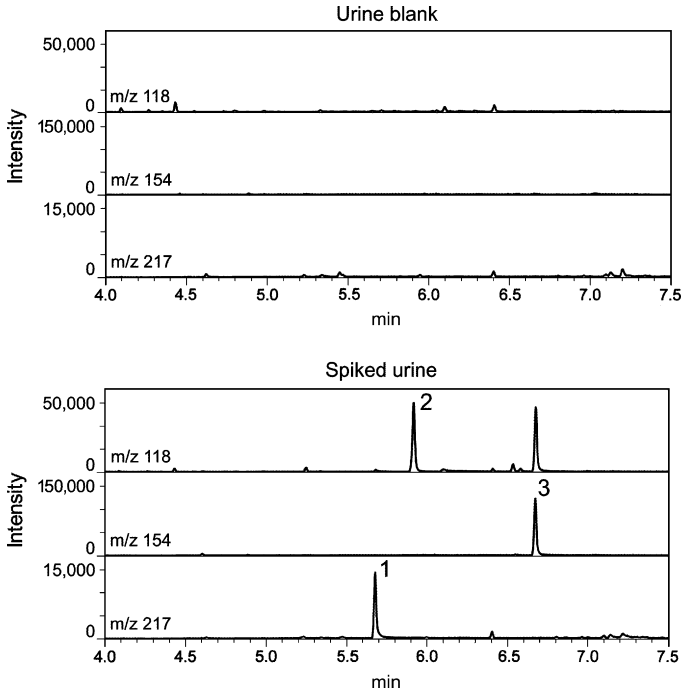


Fig. 3. SIM chromatograms for methamphetamine, amphetamine, and IS extracted from human urine using MonoTip $C_{18}$ tips. Amounts of the three compounds spiked into 0.5 ml urine were 20 ng. Peaks: 1, IS; 2, amphetamine; 3, methamphetamine.

Table 2
Recovery data for methamphetamine, amphetamine, and *N*-methylbenzylamine (IS) from QC samples using the present method

| Compound | Amount added (ng/0.5 ml) | Amount extracted[a] (ng/0.5 ml) | Recovery (%) |
|---|---|---|---|
| Methamphetamine | 200 | 176.5 ± 13.1 | 88.3 |
| | 50 | 41.4 ± 3.64 | 82.9 |
| | 5 | 4.21 ± 0.21 | 84.1 |
| | 0.5 | 0.42 ± 0.04 | 82.9 |
| Amphetamine | 200 | 173.6 ± 16.0 | 86.8 |
| | 50 | 41.1 ± 1.95 | 82.2 |
| | 5 | 4.41 ± 0.22 | 88.2 |
| | 0.5 | 0.41 ± 0.03 | 82.9 |
| *N*-Methybenzylamine (IS) | 200 | 159.4 ± 9.49 | 79.7 |
| | 50 | 39.1 ± 2.60 | 78.2 |
| | 5 | 4.07 ± 0.34 | 81.4 |
| | 0.5 | 0.41 ± 0.02 | 82.7 |

[a] Values represent means ± S.D. of four to five experiments.

Table 3
Intra- and inter-day coefficients of variation (CV) and accuracy for methamphetamine and amphetamine concentrations in QC samples measured by the present method

| Compound | Amount added (ng/0.5 ml) | Intra-day[a] | | Inter-day[b] | |
|---|---|---|---|---|---|
| | | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) |
| Methamphetamine | 200 | 7.30 | 87.4 | – | – |
| | 50 | 9.58 | 93.2 | 8.69 | 96.2 |
| | 5 | 9.61 | 88.9 | 7.29 | 90.0 |
| | 0.25 | 9.57 | 88.4 | 8.27 | 96.3 |
| Amphetamine | 200 | 10.8 | 98.2 | – | – |
| | 50 | 9.27 | 98.4 | 5.68 | 92.0 |
| | 5 | 8.12 | 91.6 | 9.22 | 89.4 |
| | 0.25 | 7.42 | 90.2 | 10.5 | 86.4 |

[a] Intra-day CVs were calculated from measurements of six to seven spiked samples on the same day.
[b] Spiked urine kept at 4 °C and analyzed on 5 separate days, with one sample each day.

concentration levels of the concentration range, were 0.25 and 200 ng/0.5 ml, respectively, for both methamphetamine and amphetamine. Intra- and inter-day CVs and accuracy were evaluated by assessing QC samples prepared in human urine, and are summarized in Table 3. Intra-day CVs at all concentrations examined were less than 9.61 and 10.8% for methamphetamine and amphetamine, respectively, whereas inter-day CVs at all concentrations examined were less than 8.69 and 10.5% for methamphetamine and amphetamine, respectively. Accuracy was in the range of 86.4–98.4% for all concentrations. Thus, the data indicated that the method was quite suitable for quantification of methamphetamine and amphetamine levels in human urine samples from 0.25 to 200 ng/0.5 ml.

Table 4
Methamphetamine and amphetamine concentrations in urine of four autopsy cases

| Case number | Methamphetamine (μg/ml)[a] | Amphetamine (μg/ml)[a] |
|---|---|---|
| 1 | 51.7 | 3.32 |
| 2 | 30.5 | 2.84 |
| 3 | 116 | 4.97 |
| 4 | 45.7 | 1.66 |

[a] Values are the means of duplicate determinations.

### 3.3. Application of the method to forensic autopsy cases

To demonstrate the forensic applicability of the present method, concentrations of methamphetamine and amphetamine in urine were determined in four forensic autopsy cases. Amount of *N*-methylbenzylamine added as IS was 50 ng to 0.5 ml of urine. However, due to high concentrations of methamphetamine and amphetamine present in the urine samples, urine samples were diluted with blank urine obtained from healthy subjects. Levels of methamphetamine and amphetamine in autopsy urine

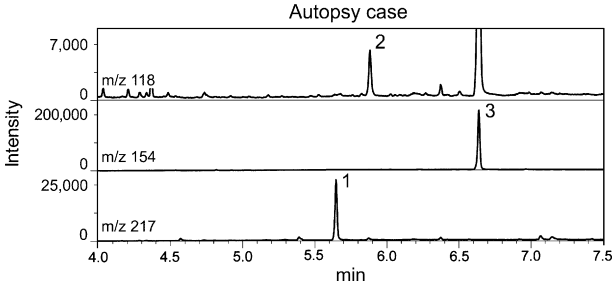


Fig. 4. SIM chromatograms obtained from an autopsy urine sample (case 1). Amount of *N*-methylbenzylamine used as IS was 50 ng for 0.5 ml urine. Peak numbers are the same as specified in Fig. 3.

samples are summarized in Table 4. Typical SIM chromatograms obtained from autopsy case 1 are shown in Fig. 4.

## 4. Conclusion

To the best of our knowledge, this is the first report dealing with PT-SPE and GC–MS for simultaneous determination of methamphetamine and amphetamine from human body fluids. Compared to LLE and conventional SPE, the present PT-SPE technique reduced sample extraction time, and organic solvent consumption. Under optimized conditions, good recovery, linearity, and reproducibility were obtained. The present method was successful in simultaneously quantifying methamphetamine and amphetamine in urine of forensic autopsy cases with the possibility of methamphetamine abuse.

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