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Journal of Chromatography B, 855 (2007) 115–120
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of 13 amphetamine related drugs in human whole blood using an enhanced polymer column and gas chromatography–mass spectrometry☆

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Received 5 October 2006; accepted 1 March 2007
Available online 19 March 2007

Abstract

Metamphetamine (MA) is one of the most frequently encountered abused drugs in Japan and the Triage™ immunoassay kit is often used to screen for this drug. However, immunoassay screening also gives positive results with other structurally related compounds, such as 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), *p*-methoxyamphetamine (PMA), an ephedrine metabolite and *β*-phenethylamine (PEA). Therefore, it is important to develop a simple and reliable method which can determine these drugs simultaneously. This paper describes a simple method for simultaneous identification and quantification of 13 amphetamine related drugs in human whole blood. The method consists of a solid phase extraction using a new polar-enhanced Focus™ column followed by acetylation and gas chromatography–mass spectrometry in the scan mode. Tetradeuterated MA and trideuterated methylephedrine (ME) were used as internal standards. As the Focus™ column required only simple extraction steps and provided a clean extract, identification of each drug was feasible even at low concentrations. The calibration curves were linear over the concentration range from 50 to 5000 ng/ml for all drugs with correlation coefficients that exceeded 0.99. The lower limits of detection of the drugs were 5–50 ng/ml. The absolute recoveries for the drugs were 65–95% and 64–89% at concentrations of 100 and 1000 ng/ml, respectively. Accuracy and precision data were satisfactory when using 2 internal standards. The applicability of the assay was proven by the analysis of blood samples in forensic cases. This method should be most useful for confirmation of positive immunoassay results for amphetamines and related drugs.

Keywords: Amphetamines; Ephedrine; Enhanced polymer column; Gas chromatography-mass spectrometry

1. Introduction

In addition to the classical stimulant methamphetamine (MA), abuse of amphetamine-derived designer drugs, such as 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), N-methyl-1-(3,4-methylene dioxyphenyl)-2-butanamine (MBDB) and *p*-methoxyamphetamine (PMA), has significantly increased in Japan among juveniles, and is now a serious social problem [1]. The immunoassay screening kit, Triage™, has been used for preliminary screening of MA using urine samples, but this kit also gives positive results for the above amphetamine-derived designer drugs, an ephedrine (EP) metabolite (norephedrine) and some prescription drugs, such as tetracaine and ranitidine [2]. In forensic toxicological cases, another problem is that samples obtained from putrefied bodies often give positive results in immunoassay analysis because of putrefactive bases, such as *β*-phenethylamine (PEA), produced during the process of putrefaction of the body [3]. Therefore, positive results must be confirmed by a second independent method that is at least as sensitive as the screening test and that provides the highest level of confidence in the result.

Multi-analyte procedures that enable screening and quantification of the above-mentioned drugs simultaneously are an ideal tool because they can confirm the existence of several important compounds following injection of a single sample extract, and

☆ This paper was presented at the 31st Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Nagoya, Japan, 28–29 September 2006.

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1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.03.002

estimation of the level of poisoning can be made at the same time. This is particularly useful for the analysis of blood samples.

Numerous methods have been developed for the analysis of amphetamine (AP), MA and amphetamine-derived designer drugs using gas chromatography (GC), liquid chromatography (LC), gas chromatography–mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS) [4–6]. However, there are few multi-analyte procedures for these drugs in blood samples. Peters et al. developed a reliable GC–MS method which can simultaneously quantify 16 amphetamines, amphetamine- and piperazine-derived designer drugs and 2 metabolites, *p*-hydroxyamphetamine and *p*-hydroxymethamphetamine in human plasma [7]. Kankaanpää et al. developed a rapid GC–MS assay for 15 amphetamine-type stimulant drugs in human blood using a simple extraction-derivatization technique [8]. In both methods, the drugs were derivatized with heptafluorobutyric anhydride (HFBA) and analyzed in the selected-ion monitoring (SIM) mode. These methods have been thoroughly validated and are accepted as a reliable quantification procedure for these drugs; however, it was critical to set 12 time windows in order to analyze many drugs in SIM mode [7] and some ephedrine-type drugs gave 2 peaks with HFBA [8].

LC–MS is becoming a powerful tool to supplement GC–MS because it permits the confirmatory analysis of polar or non-volatile compounds without derivatization. In the analyses of amphetamines, several screening procedures have been reported [9,10]. However, LC–MS techniques still have some drawbacks because the fragmentation can vary considerably between different instruments and reduction of the ionization of an analyte by co-eluting compounds, the so-called ion suppression effect, is observed [5]. Wood et al. developed a rapid quantification method for 6 amphetamine-related drugs, MA, AP, MDA, MDMA, 3,4-methylenedioxyethylamphetamine (MDEA) and EP, in human plasma and oral fluid by LC–MS–MS [11]. They selected a deuterated internal standard (IS) for each drug in order to compensate for the ion suppression effect in different matrices.

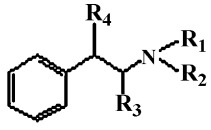
Recently, we devised a rapid screening method for 30 abused drugs including amphetamines, in human urine using a new polar-enhanced Focus^{TM} column followed by acetylation and GC–MS in the scan mode [12].

As this procedure uses the scan mode for the GC–MS analysis and acetylation as derivatization, it potentially offers the possibility of quantifying a wide variety of drugs in a single-step procedure. Therefore, we attempted to quantify 13 amphetamine related drugs, AP, MA, dimethylamphetamine (DMA), phenylpropanolamine (PPA), EP, methylephedrine (ME), MDA, MDMA, MBDB, PMA, *p*-methoxymethamphetamine (PMMA), 4-methylthioamphetamine (4MTA) and PEA, in human whole blood using this technique (Fig. 1).

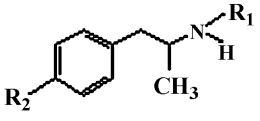
2. Materials and methods

2.1. Reagents

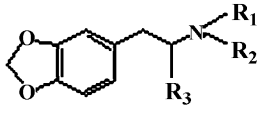
MA hydrochloride was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). PPA hydrochloride was purchased from Sigma-Aldrich Co. (St Louis. MO, USA). EP hydrochloride was purchased from Junsei Pharmaceutical Co. Ltd. (Tokyo, Japan). ME was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). AP sulfate, MDA hydrochloride, MDMA hydrochloride, DMA hydrochloride, PMA hydrochloride, PMMA hydrochloride, 4MTA hydrochloride, tetradeuterated MA hydrochloride (MA-d_4) and trideuterated ME (ME-d_3) were synthesized in our laboratories using previously published methods [13–15]. The isotopic purities of MA-d_4 and ME-d_3 in relation to the quantification of respective MA and ME were more than 99% for both compounds. MBDB hydrochloride, as 1 mg/ml free base (w/v in methanol), was purchased from Cerilliant (Austin, TX, USA). PEA was purchased from Wako Pure Chemical Industries



Dimethylamphetamine
DMA; R_1=CH_3, R_2=CH_3, R_3=CH_3, R_4=H
Methylephedrine
ME; R_1=CH_3, R_2=CH_3, R_3=CH_3, R_4=OH
β-Phenethylamine
PEA; R_1=H, R_2=H, R_3=H, R_4=H
Amphetamine
AP; R_1=H, R_2=H, R_3=CH_3, R_4=H
Methamphetamine
MA; R_1=CH_3, R_2=H, R_3=CH_3, R_4=H
Phenylpropanolamine
PPA; R_1=H, R_2=H, R_3=CH_3, R_4=OH
Ephedrine
EP; R_1=CH_3, R_2=H, R_3=CH_3, R_4=OH



p-Methoxyamphetamine
PMA; R_1=H, R_2=OCH_3
p-Methoxymethamphetamine
PMMA; R_1=CH_3, R_2=OCH_3
4-Methoxythioamphetamine
4MTA; R_1=H, R_2=SCH_3



3,4-Methylenedioxyamphetamine
MDA; R_1=H, R_2=H, R_3=CH_3
3,4-Methylenedioxymethamphetamine
MDMA; R_1=CH_3, R_2=H, R_3=CH_3
N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MBDB; R_1=CH_3, R_2=H, R_3=C_2H_5

Fig. 1. Structures, names and abbreviations of the investigated compounds.

(Osaka, Japan). Medazepam hydrochloride was provided by Shionogi & Co. Ltd. (Osaka, Japan).

Trifluoroacetic acid (TFA) and ethyl acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetic anhydride was purchased from Sigma-Aldrich Co. (St Louis. MO, USA). Pyridine (silylation grade) was purchased from Pierce (Milwaukee, WI, USA). The Focus™ column was purchased from Varian, Inc. (Lake Forest, CA, USA). The other chemicals were of analytical reagent grade.

2.2. Standard solutions

Most drugs (5 mg as free base) were dissolved in methanol and the volume was adjusted to 5 ml, to obtain a concentration of 1000 ng/μl. PPA and EP were dissolved in 0.01 M hydrochloric acid. ME was dissolved in a mixture of methanol and 0.01 M hydrochloric acid (9:1). These solutions were further diluted in methanol to 100, 10 and 1 ng/μl.

2.3. Biological samples

Human whole blood samples obtained at the time of autopsy were stored at −20 °C until analysis and drug-free human whole blood samples were obtained from healthy volunteers and used as control samples.

2.4. Extraction and derivatization procedure

One milliliter of whole blood was mixed with 10 μl IS solution containing 1000 ng/ml each of MA-d_4 and ME-d_3 in a centrifuge tube (10 ml), and 3 ml acetone was added. The mixture was vortex-mixed for 30 s and centrifuged at 850 × *g* for 5 min. The supernatant was transferred to another 10 ml centrifuge tube and as much acetone as possible was evaporated under a stream of nitrogen (<0.5 ml). Then, 1 ml distilled water was added, and the mixture was shaken and centrifuged. The supernatant was applied to a Focus™ column previously conditioned sequentially with 1 ml methanol and 1 ml distilled water. The column was rinsed sequentially with 1 ml distilled water and 1 ml 30% acetonitrile (ACN). The analytes were eluted with 1 ml ACN/distilled water/TFA (90:10:0.1, v/v). Then the eluate was evaporated to dryness under a stream of nitrogen at 60 °C. The residue was dissolved in 50 μl pyridine, and 50 μl acetic anhydride was added to the solution for acetylation. The mixture was kept at 60 °C for 30 min, then the solvent was evaporated to dryness at room temperature. The residue was dissolved in 200 μl ethyl acetate, and a 2 μl aliquot of the solution was subjected to analysis by GC–MS.

2.5. GC–MS conditions

The apparatus used was an Agilent 6890 GC combined with an Agilent 5973 MS. A HP-5 ms fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with 5% phenylmethylsilicone stationary phase was used. The splitless injection mode was selected with a valve off time of 2 min. The GC–MS conditions were as follows: the initial temperature 60 °C was held for 2 min, the temperature was programmed to 300 °C at a rate of 20 °C/min; this temperature was maintained for 10 min. The injection port and transfer line temperatures were 250 and 280 °C, respectively. The carrier gas was helium and the constant pressure mode was used. The retention times were fixed using the retention time locking (RTL) technique with medazepam as the locking compound. We set the retention time of medazepam to 13.00 min and the pressure 19.117 psi was set as the RTL condition. The full-scan mode (scanning range 50–550 amu) was used and one quantifier and one qualifier ion were selected for each drug in the mass chromatogram as shown in Table 1. The presence of drugs was confirmed by the relative intensities of the qualifier ion against the quantifier ion which were to be within ±20% of those obtained from the respective reference substance and retention time of the respective drug, with the difference being within ±2.0% of the mean value of 3 measurements of spiked blood samples. As the RTL method

Table 1
Retention time, target and qualifier ions and relative response of 13 amphetamine-related drugs

Peak no.	Drug name	IS used	Retention time (min)	Target and qualifier ions (*m/z*) (relative response in parentheses)	
1	DMA	2	6.74	72 (100)	91 (7.4)
2	ME-AC	1	8.57	72 (100)	162 (1.3)
3	PEA-AC	1	8.64	104 (100)	163 (18.2)
4	AP-AC	2	8.67	86 (100)	118 (68.8)
5	MA-AC	2	9.24	58 (100)	100 (46.3)
6	PPA-2AC	1	10.16	86 (100)	107 (15.5)
7	PMA-AC	2	10.26	148 (100)	121 (34.3)
8	EP-2AC	1	10.58	58 (100)	100 (56.3)
9	PMMA-AC	2	10.77	58 (100)	148 (41.9)
10	MDA-AC	2	10.87	162 (100)	135 (28.6)
11	4MTA-AC	2	11.34	164 (100)	137 (22.9)
12	MDMA-AC	2	11.36	58 (100)	162 (42.9)
13	MBDB-AC	2	11.69	72 (100)	176 (36.4)
IS-1	ME-*d*3-AC		8.55	75 (100)	165 (1.2)
IS-2	MA-*d*4-AC		9.22	62 (100)	104 (46.1)

was used in our screening method, the retention time once fixed with reference substance (medazepam in this study) remained unchanged.

2.6. Preparation of calibration curves

Blood samples were prepared containing AP, MA, DMA, PPA, ME, EP, PMA, PMMA, MDA, MDMA, MBDB, 4MTA, and PEA at concentrations of 50, 100, 500, 1000, 2500 and 5000 ng/ml, each containing 1000 ng/ml IS. These samples were extracted in the same manner as described above. The calibration curve was obtained by plotting the peak area ratio of the respective drug to the IS versus the amount of respective drug.

2.7. Estimation of recovery and precision

The absolute recoveries of each drug in whole blood samples at two different concentrations, 100 and 1000 ng/ml, were determined by comparing the average peak area of the derivative of each drug in the samples ($n = 3$) with that in the standard solutions ($n = 3$). QC samples were prepared at concentrations of 100 and 1000 ng/ml and analyzed as described above. Within-day precision ($n = 5$, as relative standard deviation, RSD (%)) and accuracy were calculated based on the prepared calibration curves.

3. Results and discussion

3.1. Deproteinization procedure

In forensic toxicological cases, putrefied and coagulated whole blood samples have to be analyzed, thus deproteinization and centrifugation steps before loading onto the SPE column are often necessary. We have evaluated two deproteinization procedures using ACN and acetone in comparison with simple dilution of whole blood with 1 ml distilled water. Although the highest recovery of drugs was obtained by the simple dilution procedure, colored materials were not completely removed at the purification step with a Focus™ column and this may damage the column quickly. As the deproteinization by acetone gave a higher recovery of drugs than that by ACN and produced colorless extracts, acetone was chosen as the deproteinization solvent. Removal of acetone after the deproteinization step was essential in order to obtain a high recovery of drugs at the purification step using a Focus™ column. In the case of plasma samples, simple dilution with 1 ml distilled water followed by centrifugation is used before loading onto the column.

3.2. Selection of the SPE column

Thirteen drugs were extracted from whole blood samples using several types of SPE columns: a silica-based mixed-mode column (non-polar C_8 and a strong cation exchanger), a polymer-based column (polymerized hydrophilic and hydrophobic monomer) and a recently developed multifunctional polymer column (Focus™). According to the manual provided by Varian Inc. [16], this column can retain various drugs with a wide range of polarity (from polar to non-polar drugs) by polar-enhanced sorbent with hydrogen-bond donor and acceptor sites, dipole–dipole and hydrophobic interactions.

Among the 3 different SPE columns examined, the Focus™ column gave the highest recovery of drugs and colorless clean extracts were obtained. Therefore, quantification of each drug at low concentrations was feasible even although the scan mode was used instead of the SIM mode. In case quantification of the drugs at concentrations near to the detection limit was required, the method can be easily switched to SIM mode using the same extract.

Loss of the most volatile analytes, AP and MA, often seen during the evaporation step of the eluting solvent was not observed with this column, since the drugs were eluted with an acidic solvent.

3.3. Focus™ column protocols

After deproteinization of the sample, pH adjustment was carried out in order to recover all drugs from the Focus™ column efficiently. When the different buffer solutions of pH 9.0, 10.0, 11.0 and 12.28 were examined, the extraction efficiency of EP, PPA and ME significantly decreased at a higher pH probably because of the hydroxy group in these drugs, although no significant difference was observed for AP and MA. We therefore repeated the examination using buffer solutions with a lower pH, 7.5, 8.0, 8.5 and 9.0, and distilled water, and found that adding distilled water gave the highest recovery for all drugs.

The extraction protocol for the Focus™ column provided by Varian Inc, was slightly modified in order to improve the drug recovery. Thirty % ACN as rinse solvent led to the highest recoveries. The eluting solvent recommended was a mixture of methanol, ACN, distilled water and acid. According to the manual, distilled water, ACN and methanol disrupt hydrogen bond acceptor, dipole–dipole and hydrophobic interactions, respectively. Adding acid, the bonding between the hydrogen donor and analytes having a hydrogen acceptor is dissociated. Among the acids examined, TFA, formic acid and acetic acid, TFA gave only clean extracts and the highest recovery.

3.4. Selection of internal standard

Deuterated standards are known to be the best IS for drug quantification in GC–MS analysis. In our study, ephedrine-type drugs and other amphetamine related drugs showed slightly different extraction efficiencies at low concentrations. Therefore, we selected 2 deuterated analogues as the IS, ME-d_3 (IS-1 for ME, PEA, PPA and EP) and MA-d_4 (IS-2 for AP, MA, PMA, PMMA, MDA, MDMA, MBDB and 4MTA), and the reproducibility of our method was significantly improved and reliable qualification data for all 13 drugs were obtained.

3.5. Derivatization procedure

Many derivatization procedures, including trifluoroacetylation (TFA) [17], heptafluorobutyrylation (HFB) [7,8,18,19], acetylation (AC) [20], pentafluorobenzenesulfonylation (PFBS)

[21], and alkylchloroformation (PCF) [22,23], have been reported for the analysis of amphetamines. Among them, HFB is probably the most widely used in many laboratories because of the high sensitively of the derivatives. However, when HFBA is used for derivatization this can lead to high background levels and degradation of the stationary phase of the GC column, if excess reagent is not completely removed before injection into the GC–MS system [24]. Since excess reagent could not be removed by evaporation owing to its low volatility, alkaline washing was needed [7]. In our preliminary study, we examined several derivatization procedures including HFB, trimethylsilylation (TMS), *tert*-butyldimethylsilylation (TBDMS) and acetylation. Among them, acetylated extracts gave the least interfering peaks on the chromatogram and a single derivatization product was obtained. On the other hand, derivatives were incompletely formed in the case of many drugs due to steric hindrance of the bulky TMS, HFB and *tert*-butyldimethylsilyl (*t*BDS) groups. We therefore chose simple acetylation as the derivatization procedure. After acetylation, the reagent was evaporated under a stream of nitrogen at room temperature in order to avoid the evaporation of highly volatile compounds, such as AP and MA. DMA was not acetylated.

Acetylation of the drugs has another advantage in that we can analyze drugs having multi-functional groups or a higher molecular weight without increasing the molecular weight significantly (+42) compared with TFA (+96), HFB (+196) or TMS (+72). Therefore, we believe that acetylation is applicable to a variety of drugs with a wide molecular weight range and will examine this in future studies.

3.6. GC–MS analysis

Fig. 2 shows the total ion chromatogram of derivatized extracts of whole blood spiked with 1000 ng/ml each of 13 drugs and IS, and Table 1 shows the retention times, quantifier and qualifier ions and relative responses of these 13 drugs. Sharp peaks attributed to each drug were obtained and no interfering peaks deriving from the sample matrix were observed. There was some peak overlapping: PEA-AC and AP-AC, and 4MTA-AC and MDMA-AC had nearly the same retention times (8.64 and 8.67 min, 11.34 and 11.36 min). These drugs were easily differentiated by selecting specific ions for each drug. Since the isotopic purities of MA-d_4 and ME-d_3 were more than 99%, the quantification of respective MA and ME at levels around their detection limit was feasible. By using the retention time locking technique, the retention time of each drug was fixed and, thus, identification of each drug was easy even after cutting the column edge as a maintenance procedure in order to obtain sharp peaks. Another advantage was that GC–MS analysis using the full scan mode makes it possible to screen other drugs not included in this study at the same time.

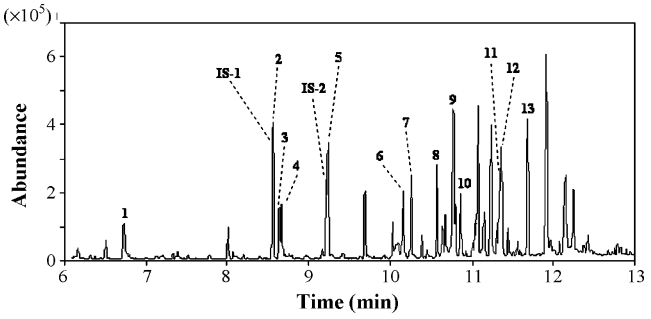


Fig. 2. Total ion chromatogram of the derivatized extracts from whole blood containing 1000 ng/ml each of the 13 amphetamine-related drugs (1: DMA, 2: ME-AC, 3: PEA-AC, 4: AP-AC, 5: MA-AC, 6: PPA-2AC, 7: PMA-AC, 8: EP-2AC, 9: PMMA-AC, 10: MDA-AC, 11: 4MTA-AC, 12: MDMA-AC, 13: MBDB-AC, IS-1: ME-d_3-AC, IS-2: MA-d_4-AC).

Table 2 shows the extraction recovery (%), lower limit of detection, intra-day precision and accuracy for each drug obtained by this method. The calibration curves were linear over the concentration range from 50 to 5000 ng/ml for all drugs with correlation coefficients over 0.99. The lower limit of detection for each drug, at a signal-to-noise ratio of 3, ranged from 5 to 50 ng/ml. The intra-day precision (RSD, %) of this method at concentrations of 100 and 1000 ng/ml was 2.0–7.6% and 1.6–4.7%, respectively. The calculated recoveries for the 13 drugs were 65–95% at a concentration of 100 ng/ml, and 64–89% at a concentration of 1000 ng/ml.

Table 2
Extraction recovery, accuracy, intra-day precision and lower limit of detection

Analyte	Recovery (%)		Intra-day precision (mean ± SD RSD (%))				LOD
	100	1000	100		1000		
DMA	69	75	111.4 ± 2.2	2.0	1016.5 ± 46.4	4.6	7
ME	76	73	107.5 ± 2.9	2.7	1022.5 ± 16.7	1.6	50
PEA	95	72	102.1 ± 7.7	7.6	1030.7 ± 31.8	3.1	50
AP	72	70	106.6 ± 3.9	3.7	950.2 ± 33.2	3.5	7
MA	88	89	103.6 ± 7.5	7.3	963.5 ± 37.5	3.9	7
PPA	88	64	98.4 ± 2.2	2.2	1015.1 ± 48.1	4.7	50
PMA	65	71	113.3 ± 3.4	3.0	976.6 ± 24.1	2.5	7
EP	78	67	100.4 ± 6.4	6.4	1007.3 ± 21.4	2.1	50
PMMA	66	77	104.0 ± 7.6	7.3	1018.5 ± 16.2	1.6	50
MDA	68	70	102.7 ± 4.5	4.4	990.0 ± 28.4	2.9	7
4MTA	70	71	114.6 ± 4.3	3.7	949.5 ± 28.5	3.0	10
MDMA	71	75	102.1 ± 6.9	6.8	1050.4 ± 16.0	1.5	7
MBDB	76	76	102.8 ± 2.5	2.5	1014.1 ± 18.4	1.8	5

Unit of concentration: ng/ml.

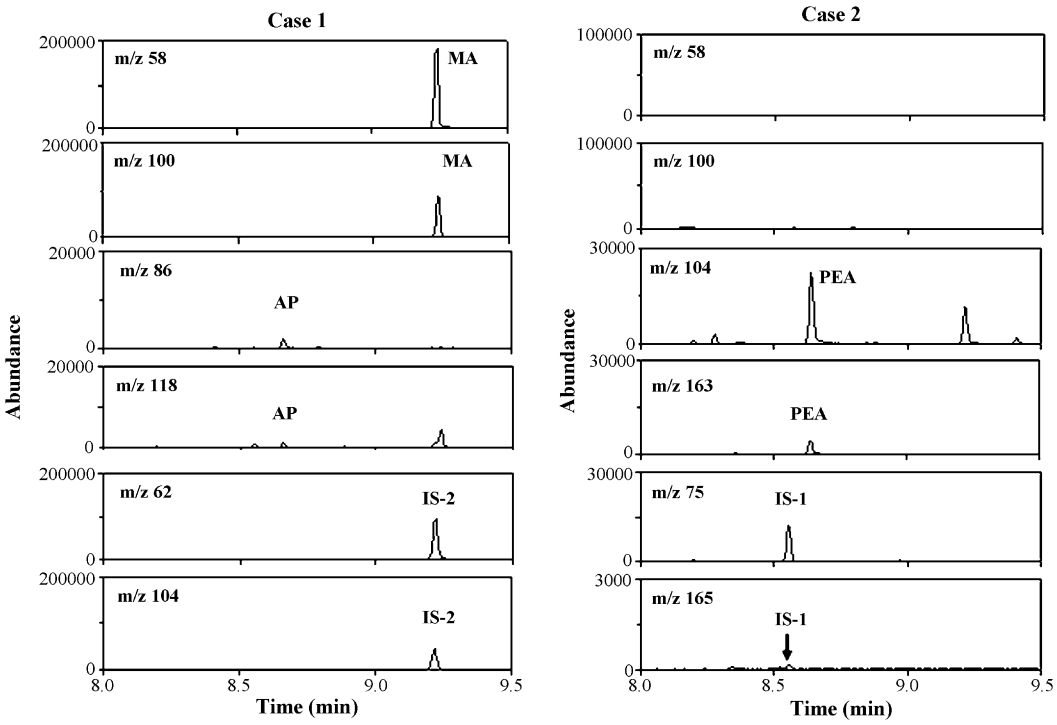


Fig. 3. Typical mass chromatograms of the derivatized extracts from whole blood samples in autopsy cases.

3.7. Practical application

The developed method was successfully applied to whole blood samples in 2 autopsy cases. In case 1, a man released from jail 2 days earlier had a cardiopulmonary arrest while taking a sauna and died soon after being delivered to an emergency hospital. In case 2, a man was found dead floating in the sea. In both cases, Triage™ urine screening showed positive results for amphetamines. Fig. 3 shows the typical mass chromatograms of the whole blood samples obtained by our method. In case 1, MA and AP were clearly detected at concentrations of 1453 and 92 ng/ml, respectively. Thus, drug abuse of MA was confirmed.

In case 2, PEA was only detected at a concentration of 5375 ng/ml and, thus, the positive result in the Triage™ screening was found to be due to putrefaction of the body.

4. Conclusions

We have developed a reliable GC–MS method to determine 13 amphetamine related drugs in human whole blood. This method should be most useful for confirmation of positive immunoassay results for amphetamines and related drugs in forensic as well as in clinical toxicological cases.

Acknowledgments

This work was supported by Grant-in-Aid for Scientific Research (No. 16390194) from Ministry of Education, Science, Technology, Sports and Culture.

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