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On-line solid-phase extraction liquid chromatography-continuous flow frit fast atom bombardment mass spectrometric and tandem mass spectrometric determination of hydrolysis products of nerve agents alkyl methylphosphonic acids by *p*-bromophenacyl derivatization

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Abstract

For proof of the presence of chemical warfare agents sarin, soman and VX, a rapid, accurate and sensitive method which allows us to determine their hydrolysis products ethyl methylphosphonic acid, isopropyl methylphosphonic acid and pinacolyl methyl phosphonic acid was explored by using continuous flow frit fast atom bombardment (FAB) LC–MS and LC–MS–MS. After derivatization of analytes with *p*-bromophenacyl bromide, LC–MS–MS analyses for screening were performed by a flow injection method. The three alkyl methylphosphonic acids (AMPAs) were eluted within 5 min, and the detection limits for the three AMPAs ranged from 1 to 5 ng/ml. For confirmation of the screening results, LC–MS–MS analysis with chromatographic separation was conducted by using a narrow bore column. The three AMPAs were all eluted with excellent separation within 25 min, and the detection limits ranged from 1 to 20 ng/ml. Quantitative measurement was performed by LC–MS in selected ion monitoring (SIM) mode with chromatographic separation. Linear calibration curves were obtained for the three AMPAs and the detection limits ranged from 0.5 to 3 ng/ml. The relative standard deviation for peak area ranged from 3.4 to 6.0% at 50 ng/ml for the three AMPAs.

Keywords: Mass spectrometry; Derivatization, LC; Chemical warfare agents; Sarin; Soman; VX; Alkyl methylphosphonic acids

1. Introduction

In 1915, in the Great War, chemical warfare agents (CWAs) appeared on the battle field for the first time. Because of the inhumanity of CWAs, their use was forbidden with the conclusion of the Geneva Protocol in 1925. Organophosphorus nerve agents such as sarin, soman and VX could be mass-produced by means of fairly simple chemical techniques and equipment. The precursor compounds were inexpensive and readily available. Since then, they have, therefore, been still developed, manufactured and stockpiled, and have often been used in international conflicts [1–4]. In 1993, 130 countries signed the Chemical Weapons Convention (CWC) to ban not only their use but also their production and

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stockpiling and additionally to control the precursor products. The CWC is now under implementation by the Organization for the Prohibition of Chemical Weapons (OPCW).

More recently in Japan, CWAs were used as tools for terrorism by a cult group; Sarin was used to commit indiscriminate murders in Matsumoto City in 1994 and the Tokyo subway in 1995, and VX was used to commit murder in Osaka in 1994. With these incidents as a turning point, measures of help for victims of terrorist attacks with biological or chemical weapons have been developed, mainly in Europe and the USA. Particularly, in the USA, the Chemical/Biological Incident Response Force (CBIRF) has been organized to save victims by rapid identification of the agents used. Until now, there are no such countermeasures in Japan. However, it is of urgent necessity to develop the reliable analytical methods which will allow us to provide evidence of the prior nerve agent presence.

Sarin, soman and VX are readily hydrolyzed to the corresponding alkyl methylphosphonic acids (AMPAs), and ultimately very slowly hydrolyzed to methylphosphonic acid (MPA) (Fig. 1) [4–10]. Up to now, there are few published papers on the metabolism of these nerve agents in the human. However, in the human, they are also thought to be enzymatically or/and spontaneously hydrolyzed to these methyphosphonic acids according to the pathways outlined in Fig. 1 [4,11,12], and some analytical methods which allow us to promote or to facilitate human metabolism studies have been applied to biological samples [12–20]. Thus, for proof of the use of the nerve agents, it will be very important to have methods available for the determination and identification of their hydrolysis products.

The analysis of methylphosphonic acids has been primarily studied using high-performance liquid chromatography (HPLC) with flame photometric detection [21–23], ion chromatography (IC) [20,24–26], capillary electrophoresis [27,28], high-performance liquid chromatography–mass spectrometry (LC–MS) and/or tandem mass spectrometry (LC–MS–MS) [13,29–35] and capillary electrophoresis–mass spectrometry [36], direct inlet secondary ion mass spectrometry [37] and fast atom bombardment (FAB) MS [38], with no derivatization, and HPLC with ultraviolet or fluorescence detection [39,40],

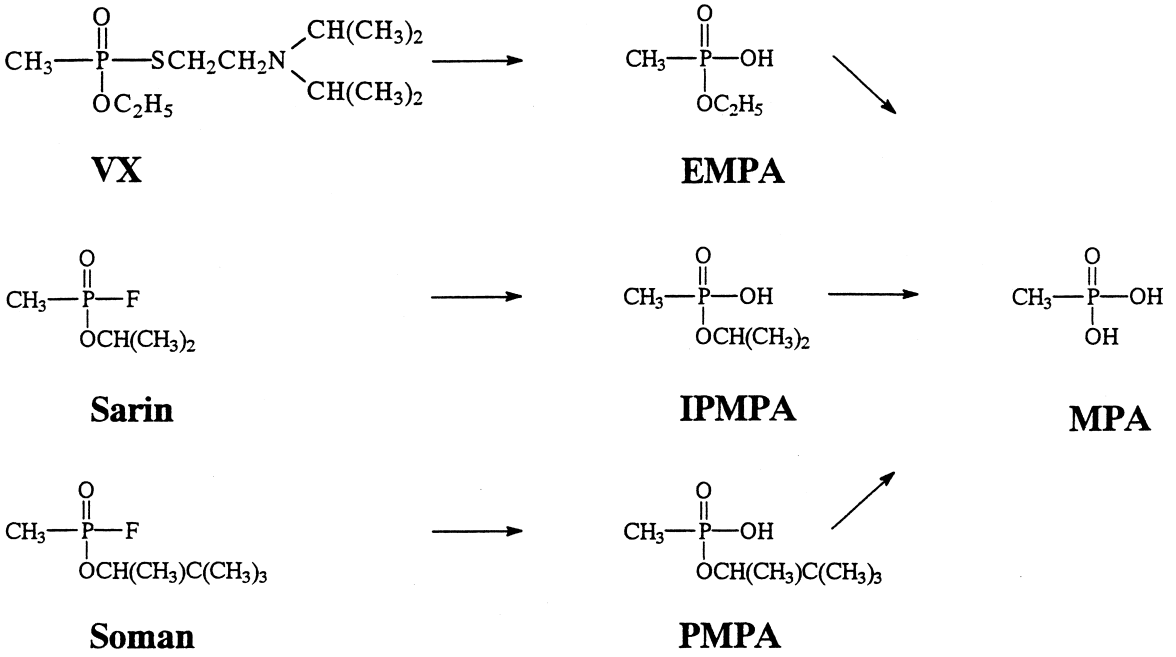
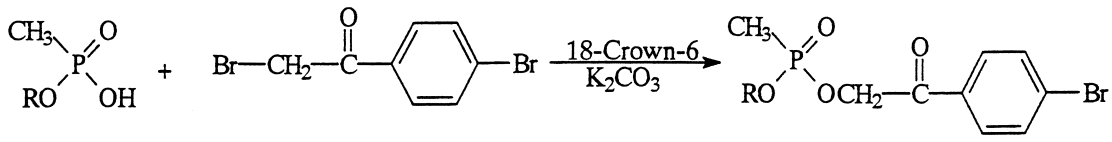


Fig. 1. Generalized hydrolysis pathways for organophosphonate nerve agents.



Alkyl methylphosphonic acid (AMPA) **p-Bromo phenacyl bromide (PBPB)** **AMPA PBP ester**

Where R = CH_3CH_2- : EMPA
R = $(CH_3)_2CH$- : IPMPA
R = $(CH_3)_3CCH(CH_3)$- : PMPA

Fig. 2. PBP derivatization of AMPAs.

GC, GC–MS and/or GC–MS–MS with derivatization such as methylation, pentafluorobenzylation, trimethylsilylation and *tert.*-butyldimethylsilylation [7,9,10,12,14–19,41–47]. We have also reported analytical methods for the detection of methylphosphonic acids in human serum using GC–MS [19] and IC [20]. In the present study, an attempt was made to develop sensitive, rapid and simple screening and determination methods by combining on-line solid-phase extraction (SPE) and continuous flow-frit FAB LC–MS or LC–MS–MS following derivatization with labeling reagent for HPLC, *p*-bromophenacyl bromide (Fig. 2).

2. Experimental

2.1. Materials

Ethyl methylphosphonic acid (EMPA) and pinacolyl methylphosphonic acid (PMPA) were purchased from Aldrich (Milwaukee, WI, USA) and isopropyl methylphosphonic acid was synthesized in our laboratory. The standard solutions of the three AMPAs were prepared in distilled water (1 mg/ml), and adjusted to the appropriate concentration with distilled water, river water or human serum immediately prior to use. *p*-Bromophenacyl bromide (PBPB) was purchased from GL Science (Osaka, Japan).

The standard aqueous solution of PBP derivative of every AMPA was prepared by derivatizing AMPA (1 mg each) with PBPB and purifying by preparative thin-layer chromatography, and was employed for examination of mass spectra and product ion spectra.

Acetonitrile was of HPLC grade, and other chemicals used were of analytical grade. Mobile phases were prepared by dissolving the appropriate reagents in distilled water.

2.2. On-line SPE system

The dual-column system used in the present study consisted of two 10AD pumps, a SIL-10A auto injector equipped with a 50-μl sample loop, a six-port valve, a degassing unit, a column oven (Shimadzu, Kyoto, Japan), and mass spectrometer (Fig. 3).

The analytical process was as follows: A 50-μl aliquot of derivatized sample was injected by the auto injector into the sample loop, and was transferred to the trapping column at a flow-rate of 200 μl/min (status 1); 1.5 min after the injection, the switching valve was turned to the alternate position, so that the trapping column and main separation column were in-line (status 2).

By backflushing mode, the mobile phase used in the main separation carried the enriched analytes from the trapping column to the main separation column at a flow-rate of 100 μl/min. After a 3-min

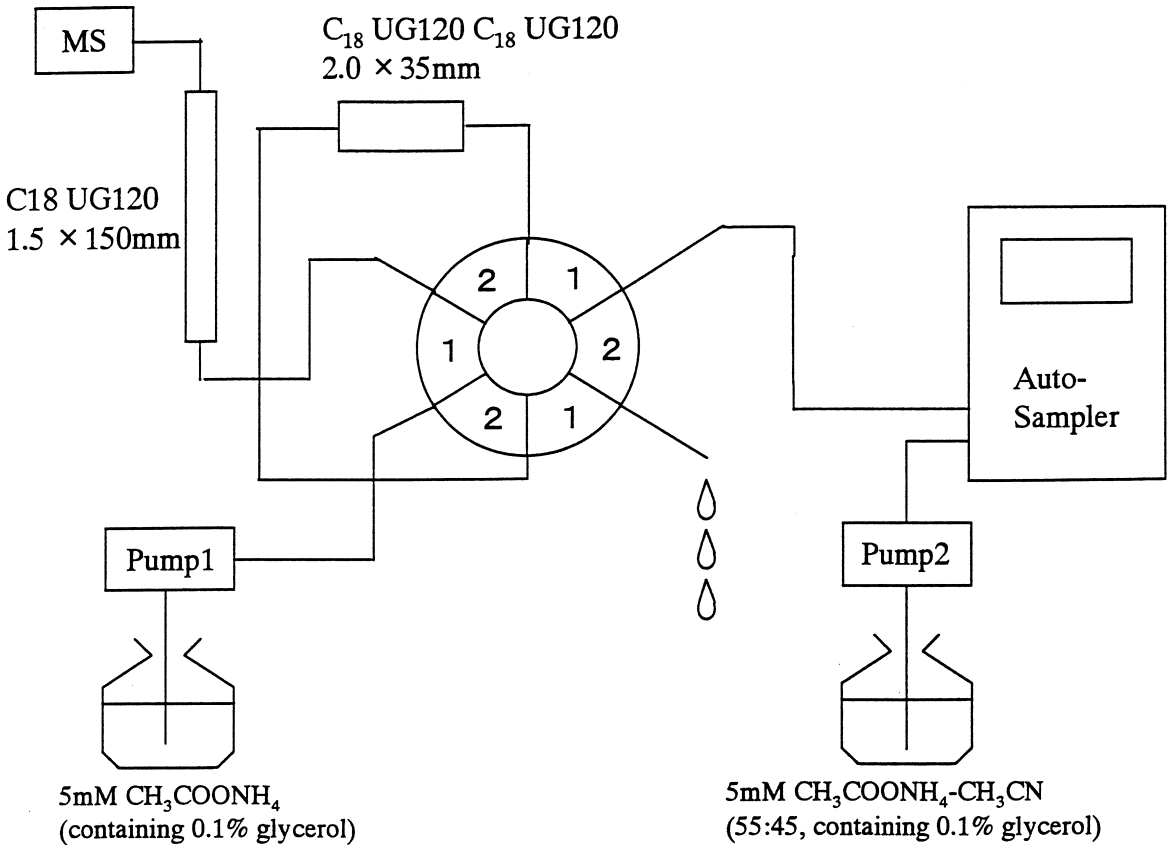


Fig. 3. Schematic diagram of column switching system.

transfer time, the switching valve was turned back to the initial position to disconnect the trapping column and the main separation column, so as to recondition the former. The analytes were eluted from the main column with the mobile phase used for the main separation. The eluent was passed to the FAB interface by splitting 40:1.

For the screening, the eluent was introduced in the FAB interface without analytical separation, by back flush from the trapping column.

2.3. LC conditions

The column used for the main separation was a CAPCELL PAK UG C_{18} (1.5 mm I.D.×150 mm, Shiseido, Tokyo, Japan). The column for trapping (preconcentration of analytes) was a CAPCELL PAK UG C_{18} (2.0 mm I.D.×35 mm, Shiseido, Tokyo, Japan).

The mobile phase employed in the main separation and trapping were 5 m*M* ammonium acetate–acetonitrile (55:45, v/v, containing 0.1% glycerol) and 5 m*M* ammonium acetate (containing 0.1% glycerol), respectively. flow-rates of the mobile phases were 100 μl/min and 200 μl/min, respectively. All columns were operated at 30°C.

2.4. FAB MS and MS–MS

FAB ionization mass spectrometry (FAB MS) and FAB ionization tandem mass spectrometry (FAB MS–MS) were performed with a JMS-SX102AQQ hybrid mass spectrometer (BEQQ type) (JEOL, Tokyo, Japan) equipped with a continuous flow frit–FAB interface. Xenon was used for FAB ionization,

and argon, at a pressure of 2.2×10^{-6} Torr, was used as the collision gas for collision-induced dissociation (CID) in the MS–MS analysis. The other MS and MS–MS operating parameters were as follows: accelerating voltage, 8 kV; resolution, 1000; and collision energy, 50 eV. Data were collected from m/z 50 to 500 at a scan rate of 3 s/scan, and the product ion spectra were recorded using every protonated molecule as the precursor ion (m/z 323 for EMPA, 337 for IPMPA and 379 for PMPA). In the MS–MS analysis for confirmation with the separation column, the precursor ion was programmed as follows: m/z 323 at the retention of 0–8.5 min, m/z 337 at 8.5–18.2 min and m/z 379 at 18.2–30.0 min.

2.5. Sample preparation

Serum samples were prepared as follows: A 1-ml volume of serum sample was deproteinized by adding 2 ml of acetonitrile. The supernatant was separated by centrifugation, transferred to a 13×100 mm screw-capped pyrex tube and evaporated just to dryness under a gentle stream of nitrogen at 80°C. The residue was dissolved into 200 μl of acetonitrile, and was derivatized by mixing with 5 mg of PBPB, 1 mg of 18-crown-6 and 1 mg of K_2CO_3 and then heating at 80°C for 30 min. After cooling, the reaction mixture was evaporated just to dryness under a gentle stream and dissolved into 1 ml of dichloromethane. The dichloromethane solution was applied to an prewashed Bond Elut SI cartridge (Varian, CA, USA). Subsequently, the cartridge was washed with 2 ml of dichloromethane and then 1 ml of dichloromethane–methanol (9:1, v/v). The retained analytes were eluted using 1 ml of dichloromethane–methanol (9:1, v/v). The eluate was evaporated just to dryness under a gentle stream and dissolved into 300 μl of distilled water. The aliquot (50 μl) was injected into the on-line SPE LC–MS system.

For the analyses of environmental water samples such as river water and pond water, the sample was centrifuged and the 1-ml aliquot of supernatant was directly evaporated just to dryness under a gentle stream of nitrogen at 80°C. Subsequently, the residue was derivatized, by the procedure described above, and analyzed.

3. Results and discussion

3.1. PBP derivatization of methylphosphonic acids

To optimize the *p*-bromophenacyl (PBP) derivatization, several procedures were examined. At first, the derivatization of AMPAs in acetonitrile–water was applied to the serum sample according to the PBP derivatization previously described by Bossle et al. [39]. However, we were not successful in the application of this method and, therefore, we examined PBP derivatization of serum samples in pure acetonitrile.

The serum sample spiked with three AMPAs (each concentration being 100 ng/ml) was used to examine the extent of derivatization and to define optimum reaction conditions. After deproteinization of the serum samples, reactions were carried out using 18-crown-6 and K_2CO_3 as the catalyst at room temperature, and at 80°C for 30, 60 and 120 min to compare the yields of each PBP derivative of AMPAs by FAB LC–MS. The derivatization yields showed that the reaction was already complete after 30 min. Therefore, 30 min at 80°C was used as reaction conditions throughout further work.

3.2. Derivative stabilities

The authentic AMPAs (1 μg each) were derivatized with PBPB as described in this paper, and the resultant reaction mixture was dissolved in 1 ml of distilled water following evaporation of acetonitrile. The aqueous solution was stored at ambient temperature. LC–MS analyses were accomplished over a period of 3 days. Neither of the PBP derivatives of the AMPAs displayed significant degradation for 3 days, which indicates that relatively long term stability of the PBP derivatives can be expected even in aqueous solution.

3.3. HPLC conditions

It is known that ion intensity obtained in FAB MS frequently depends largely on the concentration of the buffering salt in the mobile phase. In an optimization study, the highest intensity for every PBP derivative was obtained at the concentration of ammonium acetate below 5 mM. A concentration of

5 m*M* ammonium acetate in the mobile phase was chosen on consideration of peak shape in the chromatogram.

Additionally, to optimize the separation of PBP derivatives of the three AMPAs, suitable mobile phase composition was explored using the acetonitrile–ammonium acetate buffer (containing 0.1% glycerol) system. The acetonitrile concentrations were varied between 40 and 60% and *k* values of the derivatives were measured. As shown in Fig. 4, a decrease in acetonitrile concentration led to larger *k* values and, hence, to better separation. At an acetonitrile concentration below 50%, adequate separation of even PMPA diastereomers was achieved. In view of these results, we chose 45% acetonitrile for further work, because all compounds were eluted with adequate separation and in minimum time (within 20 min).

3.4. Concentration of analytes with trapping column

In the trace analyses of environmental or biological samples using LC–MS, the detection limits of the analytical systems are sometimes not sufficient, and an improvement of the detection limits is required. In addition, direct injection of biological fluids or surface water often compromises sensitivity, mainly due to the presence of protein, salt, etc. For a solution to these problems, on-line SPE, which allows us to clean-up samples, enrich analytes and directly introduce column effluent into the MS interfaces, has been used, especially in environmental applications [48]. In the present study, we explored the elution of the enriched PBP derivatives of AMPAs by backflushing, subsequent to their concentration on the head of the trapping column.

When focusing the analytes, it is very important to minimize the width of their focused band. Thus, we employed 5 m*M* ammonium acetate (containing 0.1% glycerol) as the mobile phase for trapping. In addition, too large a precolumn load often causes breakthrough of analytes in on-line SPE. However, the breakthrough of the analytes was not observed even when loading as much volume as 50 μl on to the trapping column.

When eluting the enriched analytes from the trapping column by backflushing, an acetonitrile concentration above 20% in the eluent gave excellent results. We chose 45% as the acetonitrile concentration, which was the same composition as the mobile phase used in the main separation.

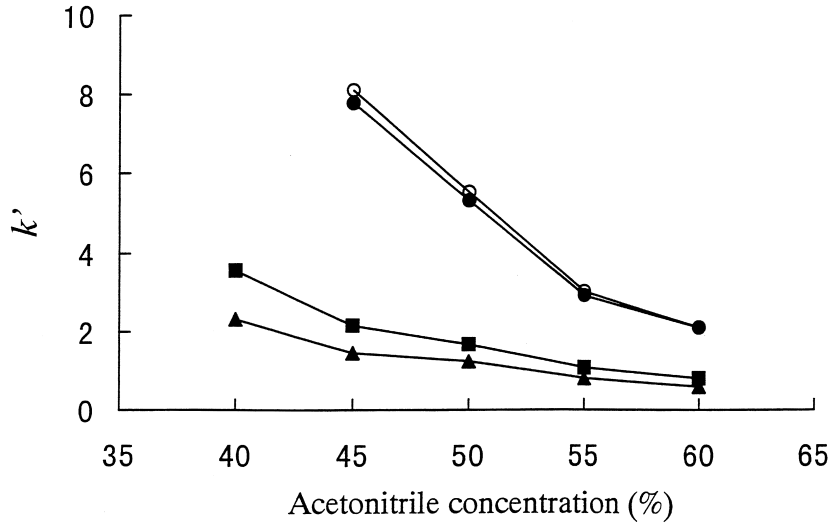


Fig. 4. Effect of acetonitrile concentration on *k′* values of PBP derivatives of AMPAs. Acetonitrile was mixed with 5 m*M* ammonium acetate buffer containing 0.1% glycerol. Symbols: ▲, EMPA; ■, IPMPA; ○ and ●; PMPA (diastereomers).

3.5. FAB MS and FAB MS–MS analyses of PBP derivatives of AMPAs

Before employing FAB MS–MS, FAB mass spectra of PBP derivatives of three AMPAs were measured in both positive and negative modes by a flow injection technique, to compare sensitivity and spectral profile. As samples, the standard aqueous solution of every PBP derivative of AMPAs was used.

In the positive mode, the protonated molecular ion $[M+H]^+$ was observed in every mass spectrum of PBP derivatives, and especially in those with derivatives of EMPA and IPMPA as the base ions. FAB MS often suffers notoriously from alkali ion adduct such as $[M+Na]^+$ and $[M+K]^+$. However, in the present experiment, such ion adduct was not observed, because all salt was washed away after on-line SPE trapping of the analytes. In the negative mode every mass spectrum was dominated by the corresponding AMPA anion $[M-PBP]^-$, and no other significant fragmentation was observed.

The absolute ion intensity of $[M+H]^+$ for the EMPA and IPMPA PBP derivatives was approximately three times higher than that of $[M-PBP]^-$, while the former was slightly lower than the latter for the PMPA PBP derivative. This shows that the $[M+H]^+$ ions are preferable to $[M-PBP]^-$ as a precursor in product ion MS–MS experiments. Additionally, the higher molecular mass precursor ion would often be more favorable for MS-MS analysis. Based on the above comparison, we selected the $[M+H]^+$ ions of AMPAs PBP derivatives in positive mode for MS–MS analysis.

Fig. 5 shows the product ion spectra after CID of the $[M+H]^+$ ion produced by positive FAB ionization for three AMPAs PBP derivatives. In the present study, we selected ^{81}Br $[M+H]^+$ as a precursor ion from two isotopic species of $[M+H]^+$, because the background chemical noise from the serum sample is less for ^{81}Br $[M+H]^+$ than for ^{79}Br $[M+H]^+$ in a single MS measurement of PBP derivatives of EMPA and PMPA. In every product ion spectrum, some characteristic product ions, such as $[CH_3PO(O-PBP)OH+H]^+$ at m/z 295 due to alkene loss, $[PBP]^+$ at m/z 199 and $[BrC_6H_4CH_2]^+$ at m/z 171, which were isotopically pure species, were observed with relatively high intensity. Additionally, the relative intensity of the alkene loss signal increases with the size of the alkyl group of the AMPAs.

3.6. Screening by flow injection LC–MS–MS

For rapid, sensitive and accurate screening, flow injection LC–MS–MS analysis without chromatographic separation was used, but concentration of the analytes by on-line SPE was performed, as described above, and product ion spectra of selected $[M+H]^+$ ions were monitored. As samples, spiked serum and river water were employed.

The PBP derivatives were all eluted within 5 min (Fig. 6), and excellent product ion spectra for every derivative were obtained, with scarcely any interference except that some fairly weak ions due to impurities in sample matrices were observed in the spectra. The full scan detection limits were 1 ng/ml, 1 ng/ml and 5 ng/ml for EMPA, IPMPA and PMPA in river water, and 3 ng/ml, 1 ng/ml and 5 ng/ml for EMPA, IPMPA and PMPA in human serum, respectively.

3.7. Confirmation by LC–MS–MS

In addition to the accurate screening of AMPAs in environmental and biological samples, we also explored a reliable method for confirmatory analysis. For this purpose, MS–MS with chromatographic separation was conducted following the concentration of analytes by column switching as described above, and product ion spectra were measured for three AMPAs PBP derivatives by selecting their $[M+H]^+$ ions as precursor ions. As samples, spiked serum and river water were employed.

The total ion chromatograms obtained for spiked serum and river water are shown in Fig. 7(a)and (b). The PBP derivatives were all eluted within 25 min in well-separated peaks and the diastereomers of the PMPA PBP derivative were also sufficiently separated. Excellent product ion spectra for every derivative were obtained with less interference than in screening. The detection limits were 2 ng/ml, 1 ng/ml and 20 ng/ml for EMPA, IPMPA and PMPA in river water, and 5 ng/ml, 1 ng/ml and 20 ng/ml for EMPA, IPMPA and PMPA in human serum, respectively. Thus, the combination of the screening and the following confirmation would give more

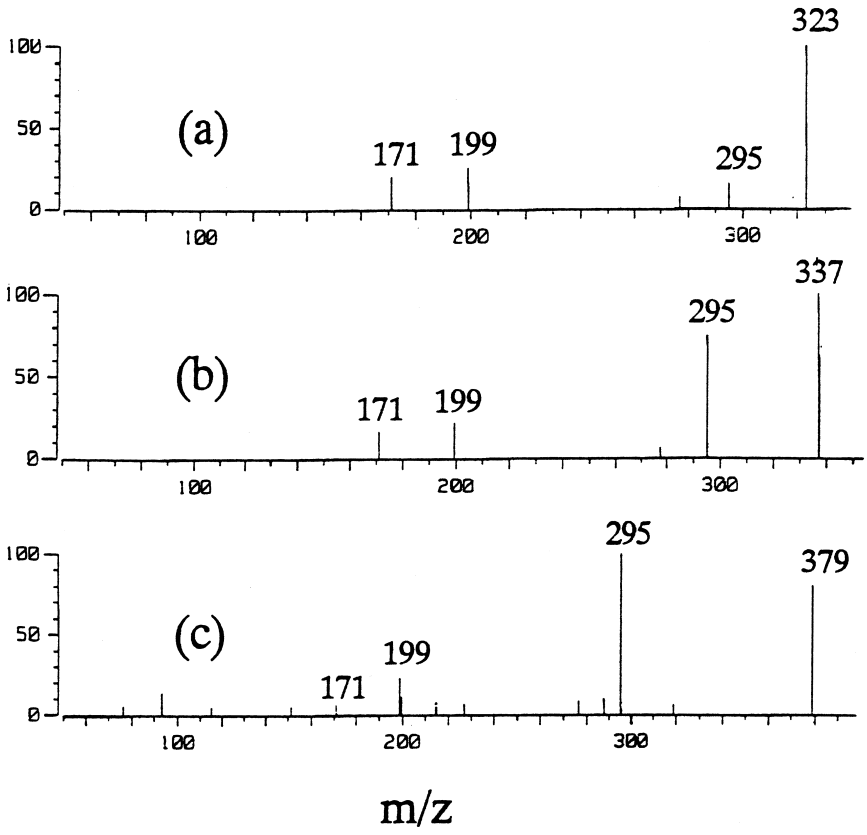


Fig. 5. Product ion spectra of PBP derivatives of (a) EMPA, (b) IPMPA and (c) PMPA produced by CID of the $[M+H]^+$ ions.

reliable identification, leading to proof of the presence of nerve agents.

3.8. Quantitative analysis using LC–SIM–MS

To evaluate linearity and reproducibility, FAB LC-MS analyses under selected ion monitoring (SIM) mode were carried out for spiked serum samples without an internal standard, and calibration curves were constructed for the $[M+H]^+$ ion (m/z= 323 for EMPA, 337 for IPMPA and 379 for PMPA).

The FAB SIM-MS analysis showed a good linearity throughout the concentration range from 5 to 100 ng/ml for EMPA and IPMPA (EMPA: $y=2.12x+0.225$, $r^2=0.998$; IPMPA: $y=4.81x+2.35$, $r^2=0.999$) and from 10 to 100 ng/ml for PMPA ($y=0.427x+0.346$, $r^2=0.999$). Relative standard deviation values obtained at the sample concentration of 50 ng/ml were 4.9% for EMPA, 3.4% for IPMPA, and 6.0% for PMPA ($n=3$). The detection limits in SIM mode were estimated to be 1 ng/ml for EMPA, 0.5 ng/ml for IPMPA, and 5 ng/ml for PMPA at a signal-to-noise ratio of 3:1.

To evaluate its accuracy in the serum and environmental water sample analyses, spiked serum and river water and standard aqueous solution were run in the SIM mode. Resultant peak areas ($[M+H]^+$ ions) were compared for the three AMPAs. The recovery values of serum and river water samples to corresponding standard aqueous solutions were calculated (Table 1). The recovery for EMPA from the serum sample was a little lower because of

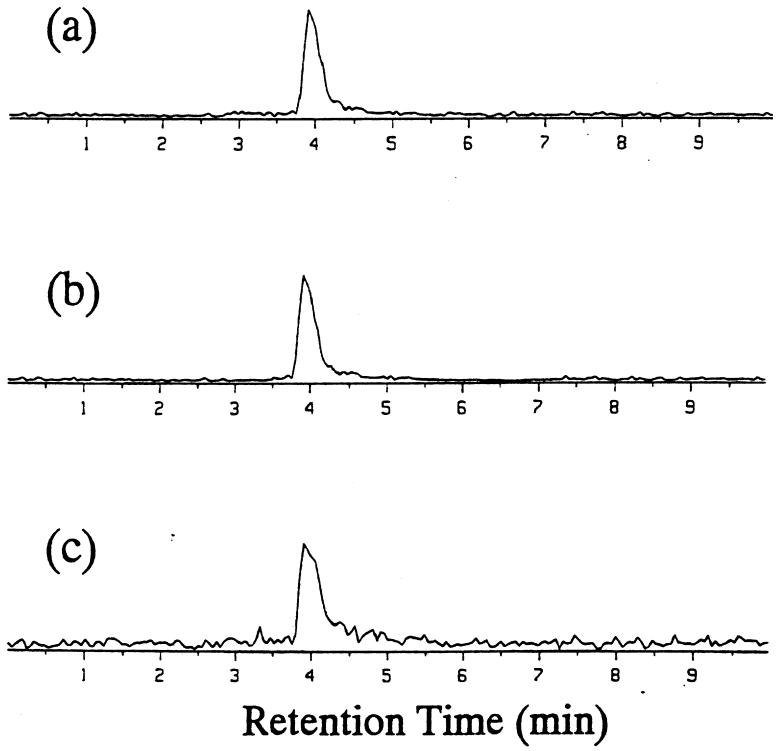


Fig. 6. Total ion chromatograms by flow injection LC–MS–MS screening analyses for (a) EMPA, (b) IPMPA and (c) PMPA in a spiked serum sample. The $[M+H]^+$ at m/z 323 (EMPA), 337 (IPMPA) and 379 (PMPA) were used as the precursor ions. Every chromatogram was obtained from one run. The concentrations of all the analytes added to the serum were 20 ng/ml.

disturbance by impurities in serum, while those for the other compounds were sufficiently high.

4. Conclusion

A rapid, accurate and sensitive analytical method for screening and determination of AMPAs in human serum and environmental water was developed by coupling HPLC with on-line SPE and continuous flow frit–FAB MS and MS–MS following PBP derivatization. In the simple and rapid screening by flow-injection LC–MS–MS, high reliability and sensitivity were obtained. Using a narrow bore column LC–MS–MS after the screening, AMPAs can be identified more accurately, leading to the reliable identification of AMPAs. In the quantitative analysis, LC–SIM-MS offered good linearity, sensitivity and reproducibility.

It is generally thought that LC–FAB-MS and MS–MS would be obsolete and inferior to LC–APCI/ESI–MS. However, we have achieved the low ppb level of detection limits and good reproducibility by combining continuous flow frit FAB with preconcentration by on-line SPE and PBP derivatization. This shows that the present method would compare favorably with the methods using APCI [30,31]. For large quantities of experimental samples, the sample preparation in the present method may become too elaborate because the procedure of aqueous sample evaporation is relatively time-consuming. However, in such cases, the combination of the method described in this paper and the previously reported extraction technique with acetonitrile [19] would

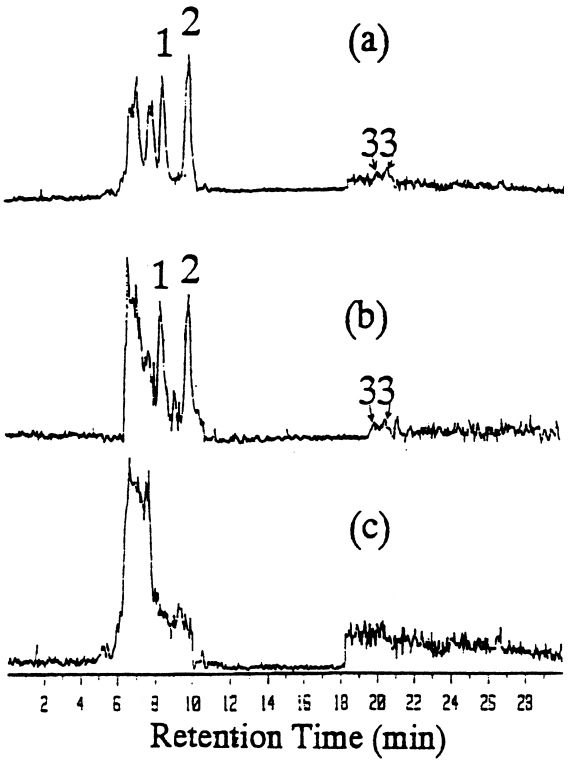


Fig. 7. Total ion chromatograms obtained for (a) spiked river water, (b) spiked serum and (c) blank serum. Every chromatogram was obtained from one run. The concentrations of all the analytes added to the serum and the river water were 20 ng/ml. Peaks in (a) and (b): 1=EMPA; 2=IPMPA; 3=PMPA (diastereomers).

Table 1
Recoveries of AMPAs from serum and river water

Sample	Recoveries (mean±S.D., n=3) (%)		
	EMPA	IPMPA	PMPA
Serum	68.9±3.38	88.3±3.00	88.0±5.28
River water	87.7±4.05	93.4±3.27	94.1±5.52

lower the detection limits, and would become a powerful method.

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