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Journal of Chromatography B, 701 (1997) 9–17

JOURNAL OF CHROMATOGRAPHY B

Detection of sarin hydrolysis products from sarin-like organophosphorus agent-exposed human erythrocytes

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Received 7 March 1997; received in revised form 23 June 1997; accepted 26 June 1997

Abstract

A sarin-like organophosphorus agent, [bis(isopropyl methyl)phosphonate; BIMP], was synthesized. This agent has the same phosphonate group as sarin and also has the same anti-acetylcholinesterase activity potency as sarin. The ID_{50} and LD_{50} values of BIMP in mice after intravenous injection were 3.9 nM and 0.8 mg/kg, respectively. The AChE activities of their red blood cells and brains were dose-dependently reduced by intravenous BIMP. After preparation of experimental BIMP-exposed human red blood cells, BIMP-bound acetylcholinesterase (AChE) was solubilized from erythrocyte membranes, purified by immunoaffinity chromatography, digested with trypsin, and the sarin hydrolysis products bound to AChE were released by alkaline phosphatase digestion. The digested sarin hydrolysis products were subjected to trimethylsilyl (TMS) derivatization and detected by gas chromatography–mass spectrometry. Isopropyl methylphosphonic- and methylphosphonic acids, which are the sarin hydrolysis products, were detected in experimental BIMP-exposed human red blood cells. This new method, which enables sarin's hydrolysis products to be detected in BIMP-exposed erythrocytes, is a useful tool for studying sarin-poisoning victims. © 1997 Elsevier Science B.V.

Keywords: Sarin; Bis(isopropylmethyl)phosphonate

1. Introduction

On March 20, 1995, the Tokyo subway system suffered from a horrifying terrorist attack with sarin gas (isopropyl methylphosphonofluoridate), the second attack experienced in Japan, that left twelve people dead and over 5000 injured [1–3]. We performed judicial autopsies on the four acute sarin-poisoning victims. A definitive method for the forensic diagnosis of sarin poisoning has not yet been established. Therefore, forensic toxicological studies of sarin poisoning are required. However, sarin is a highly toxic organophosphorus compound and is a potential chemical weapon as a nerve gas. The synthesis and uses of sarin in Japan, even for basic studies, are strictly controlled by law and under treaty and it is difficult to study experimental sarin toxicity in any laboratory except in the army. This provided a major barrier to progress in the pathophysiological study of sarin poisoning. The development of a substitute for sarin that has almost the same effect on acethylcholinesterase (AChE) would, therefore, be useful for forensic and clinical toxicological studies on sarin poisoning. In the study described here, we describe the synthesis of a sarin-like organophosphorus agent [bis(isopropyl

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0378-4347/97/$17.00
PII S0378-4347(97)00355-1

methyl)phosphonate, BIMP] and the development of a technique for diagnosing sarin poisoning.

2. Experimental

2.1. Chemicals and reagents

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), human recombinant AChE and trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated from bovine pancreas) were purchased from Sigma (St. Louis, MO, USA). Methylphosphonic acid was purchased from Aldrich (Milwaukee, WI, USA). Bovine intestinal alkaline phosphatase was purchased from Toyobo (Osaka, Japan). *N,O*-Bis(trimethylsilyl)trifluoroacetamide was obtained from GL Science (Tokyo, Japan). Centriplus 3 and Centriplus 30 were purchased from Amicon (Beverly, MA, USA). Prefilled chromatography column AG 50W cation-exchange resins were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Protein A Sepharose CL-4B and CNBr-activated Sepharose 4B were obtained from Pharmacia Biotech (Uppsala, Sweden). All other reagents were of the highest grade available commercially.

2.2. Preparation of bis(isopropyl methyl)-phosphonate

BIMP is one of the dialkyl dialkylpyrophosphonates [4] and has the same phosphonate group as sarin. Dialkyl dialkylpyrophosphonates are analogues of the organic pyrophosphates, which are potential insecticides. It is thought that dialkyl dialkylpyrophosphonates might have the potential to be anti-cholinergic agents. In order to prepare isopropyl methylphosphonic acid (IMPA), one of the hydrolysis products of sarin, 500 mg (5.2 mmol) of methylphosphonic acid and 450 mg (7.8 mmol) of isopropyl alcohol were placed in 10 ml of dry pyridine and reacted with 1.61 g (7.8 mmol) of *N,N'*-dicyclohexylcarbodiimide (DCC) for 6 h at room temperature. The resulting white precipitate was removed by filtration and the solvent was removed by evaporation in vacuo in a water bath. IMPA was purified by thin-layer chromatography (TLC) on cellulose gel-coated plates using a solvent system of 25% ammonia water–isopropyl alcohol (75:25, v/v) and was eluted from the gel using methanol. This purification procedure was performed twice more, after which, the purified IMPA was reacted with DCC to produce the IMPA dimer, bis(isopropyl methyl)phosphonate (BIMP, M.W. 258). BIMP was purified by TLC, as described above, and stored in an ampule at −80°C until required for use. BIMP was analyzed qualitatively by gas chromatography–mass spectrometry (GC–MS) using a 1-μl aliquot of a solution of BIMP in 1 ml of ethyl acetate. The GC–MS conditions used are described in Section 2.8. Gloves and masks were worn at all times, while BIMP was being used. After finishing the experiments, the remaining BIMP was placed in water to hydrolyse it.

2.3. Inhibition of acetylcholinesterase by BIMP

BIMP and diisopropyl fluorophosphate (DFP), which was used as a positive control, were dissolved in 0.1 *M* sodium phosphate buffer (pH 8.0) containing 10% (v/v) methanol. Bovine erythrocyte AChE, (0.02 U) was preincubated with several concentrations (final concentration, 0.1 n*M*–0.1 m*M*) of BIMP and DFP at 25°C for 30 min, after which, the remaining AChE activities were measured by the method of Ellman et al. [5].

2.4. Preparation of AChE immunoaffinity column

A 0.5-ml volume of human recombinant AChE solution in saline, containing 0.1 mg of protein, was emulsified with an equal volume of complete Freund's adjuvant. Male albino rabbits, weighing 2.5–3.0 kg, were injected with this emulsion subcutaneously. They received 1.0 ml of the emulsion once every two weeks for six weeks, and then once a month for two months. Blood was collected from a carotid artery ten days after the final injection and was allowed to clot at 4°C. The serum was separated by centrifugation and served as the source of antiserum. The rabbit IgG fractions were separated using a Protein A Sepharose CL-4B column [6]. Then, in order to prepare the immunoadsorbent, the separated rabbit IgG fractions were bound to the cyanogen bromide-activated Sepharose 4B gel [7]. The immunoadsorbents were packed in a 15×1.0 cm

column (packed bed volumes, 70×10 mm) and equilibrated in 10 m*M* Tris-buffered saline (TBS, pH 7.4).

2.5. Animal experiments

Various doses of BIMP (0.6, 0.9, 1.2 and 2.4 mg/kg) were injected into the tail veins of Balb/c mice (weighing 20–25 g) and the control group received an injection of vehicle into the tail vein. After death or 30 min after injection, the brains and samples of blood were taken and their AChE activities were measured using the method of Ellman et al. [5].

2.6. Preparation of experimental sarin-exposed human red cells

BIMP (100 μg) was dissolved in 0.1 ml of methanol, added to 50 ml of human heparinized

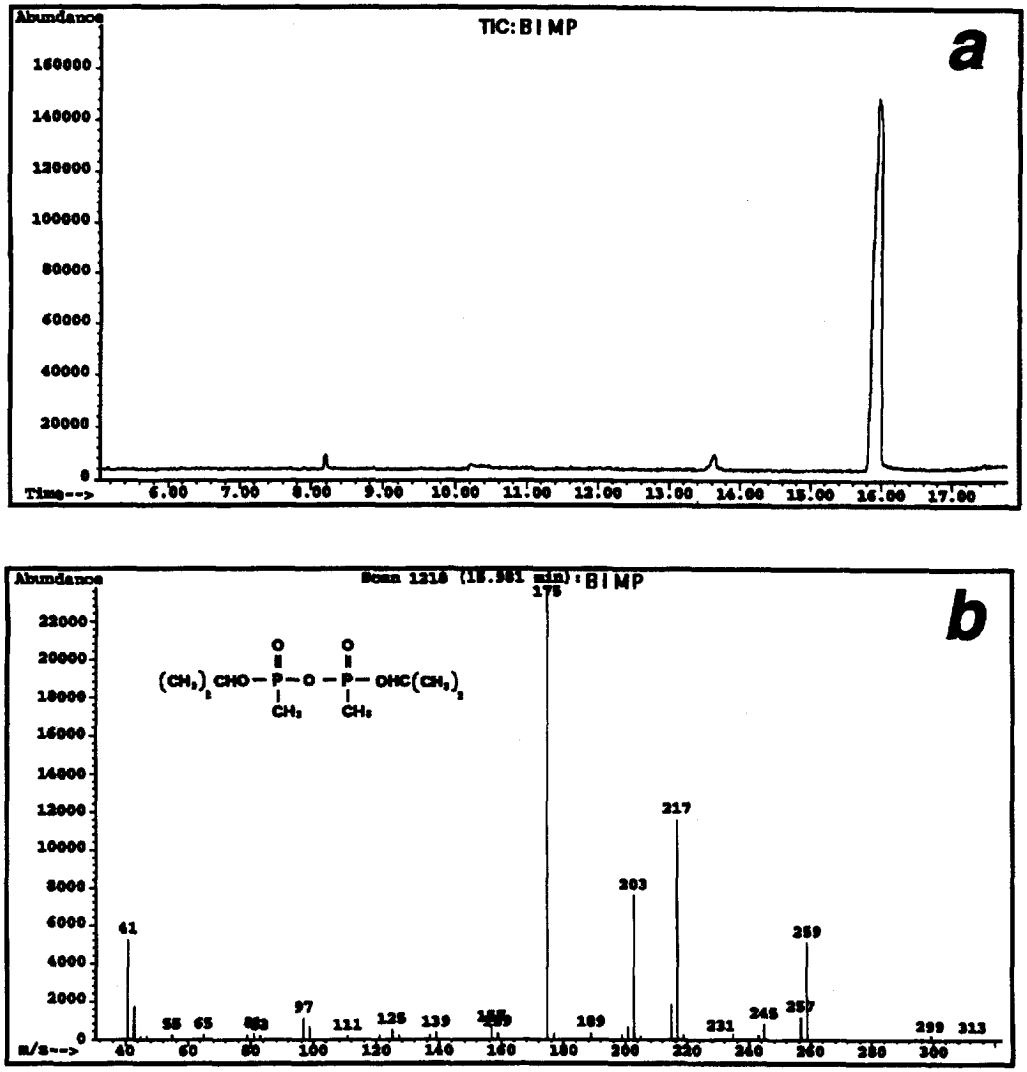

Fig. 1. Chromatograms of BIMP synthesized without TMS derivatization. (a) The total ion chromatogram (TIC). (b) The CI mass spectrum of the peak observed at about 16 min on the TIC.

blood, incubated at 25°C for 30 min and centrifuged at 1000 *g* for 30 min at 4°C. The plasma and buffy coat were removed and the red blood cells were suspended in TBS and centrifuged at 1000 *g* for 30 min at 4°C. This red blood cell washing procedure was repeated twice more. The AChE activity of washed red blood cells was inhibited almost completely by BIMP.

2.7. Sample treatment

Ghosts of the human experimental sarin-exposed red blood cells were prepared according to the method of Hanahan and Ekholm [8]. The isolated ghosts were resuspended in 40 ml Tris–HCl buffer containing 1% (w/v) CHAPS [9,10], incubated for 30 min at 4°C and then centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant, in which over 90% of the AChE in the red blood cells was solubilized, was collected. The supernatant was concentrated by ultrafiltration using Centriplus 30. The concentrated supernatant was applied to the AChE immunoaffinity column, and allowed to make contact with the immunoadsorbent matrix at room temperature for 60 min. After incubation, the column was washed with 40 ml of 0.1 *M* Tris (pH 8.8, containing 0.1% CHAPS and 0.5 *M* NaCl) and the bound AChE was then eluted with 40 ml of 0.1 *M* acetate (pH 3.0, containing 0.1% CHAPS and 0.5 *M* NaCl). The eluted solution was neutralized with 1 *M* Tris (pH 8.8) and then the eluate was concentrated again by ultrafiltration using Centriplus 30. The purified AChE was digested with trypsin (20 μg/mg protein) at 37°C for 24 h. The digested peptide solutions were alkalinized by adding 1 *M* Tris–HCl buffer (pH 10.0) and then incubated with bovine intestinal alkaline phosphatase (3 U/mg protein) at 37°C for 48 h. The high-molecular-mass peptide digestion products were removed by ultrafiltration using Centriplus 3; positive ions were removed by passing the solution through the prefilled chromatography column (AG 50W cation-exchange resins), the water was evaporated off and the residue was dissolved in a small volume of pyridine. An aliquot of this pyridine solution was mixed with an equal volume of the trimethylsilylation agent, bis(trimethylsilyl)trifluoroacetamide [containing 1% (v/v) trimethylchlorosilane], and left to stand for 60 min at room temperature prior to analysis [11].

2.8. GC–MS analyses

GC was performed using a Hewlett Packard gas chromatograph 5890B equipped with a capillary column (30 m×0.25 mm I.D.; film thickness, 0.25 μm) HP-5MS. Helium, at 10.9 p.s.i., was used as a carrier gas. The oven was heated to 40°C for 2 min, from 40 to 150°C at 8°C/min, from 150 to 280°C at 15°C/min, and then maintained at 280°C for 10 min. GC–MS analyses were carried out on a Hewlett Packard mass spectrometer 5889, into which 1 μl of each sample was injected in the splitless mode. The electron impact (EI)-MS operating conditions were as follows: Electron energy, 70 eV and ion source temperature, 200°C. The methane chemical impact (CI)-MS operating conditions were as follows: Electron energy, 230 eV and ion source temperature, 200°C.

3. Results and discussion

Fig. 1 shows the chromatograms of BIMP synthesized without trimethylsilyl (TMS) derivatization. Fig. 1a shows the total ion chromatogram of BIMP, which is a sharp peak at about 16 min and Fig. 1b is the CI mass spectrum of this peak. In the CI mass spectrum, peaks of *m/z* 259 and 299, which are due to $[M+H]^+$ and $[M+C_3H_5]^+$, respectively, were observed. It was confirmed that the product synthesized was BIMP.

The ID_{50} values of BIMP and DFP were 3.9 and 150 n*M*, respectively (Fig. 2), and the LD_{50} of BIMP in mice after intravenous injection was 0.8 mg/kg. The AChE activities of the brain and blood samples were reduced dose-dependently by intravenous injection of BIMP (Fig. 3). In light of these results, we consider that BIMP has enough anti-AChE activity to be used in experiments on sarin poisoning.

Sarin is a highly toxic organophosphorus agent that is easily hydrolyzed, especially under alkaline conditions. Usually, the detection of sarin itself and/or isopropyl methylphosphonic acid in the blood of sarin victims is very difficult. Sarin binds to a

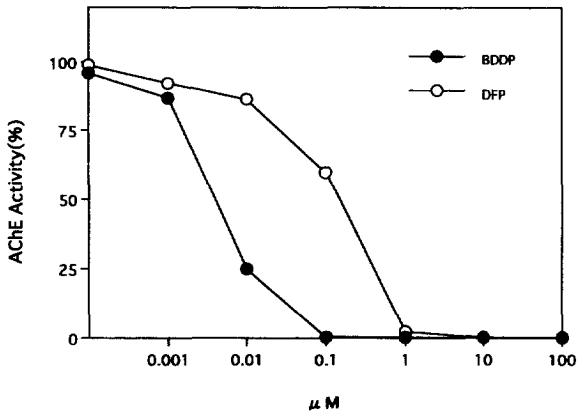

Fig. 2. Inhibition activity of bovine erythrocyte AChE by BIMP (solid circles) and DFP (open circles).

hydroxyl group of serine in the active site of the AChE molecule and strongly inhibits its enzyme activity [12]. Finally, its isopropyl ester is hydrolyzed, known as a phenomenon called aging, to methylphosphonic acid, which is conjugated to the serine residue in the enzyme molecule. Each AChE molecule that is bound to sarin contains one molecule of isopropyl methylphosphonoserine or methylphosphonoserine [12]. If the isopropyl methylphosphonic- and/or methylphosphonic acids that are conjugated to AChE in the blood or tissues of sarin-poisoned victims are detected, it would provide strong evidence of sarin poisoning.

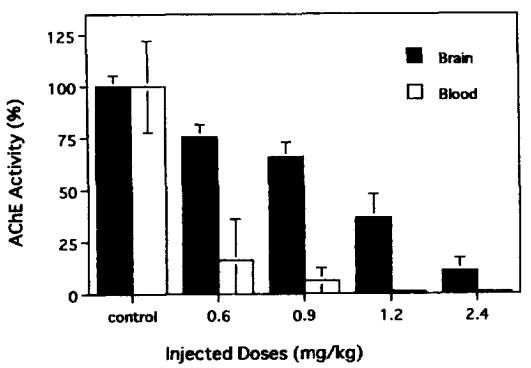

Fig. 3. Inhibitory effects of BIMP on mouse blood and brain AChE activity.

BIMP possesses anti-AChE activity and the same phosphonate group as sarin. Therefore, after binding to the AChE molecule, it would behave like sarin and we can prepare something resembling sarin-exposed human samples by using BIMP. Our preliminary experiments on several human organs and human blood have shown that AChE activity is greatest in the erythrocytes (data not shown). Hence, we prepared human experimental sarin-exposed blood samples by treating them with BIMP in vitro and these were used to detect sarin hydrolysis products.

In blood, amphipathic AChE dimers of a globular form (G_2) [13,14] are anchored in the plasma membrane by a covalently attached glycoinositol phospholipid [15–17]. Therefore, the first step was to prepare ghosts of the human experimental sarin-exposed red blood cells. AChE was then solubilized from the ghosts using the non-denaturing detergent, CHAPS. In order to cleave isopropyl-methylphosphonic- and/or methylphosphonic acids from the solubilized AChE, enzymatic digestion with alkaline phosphatase, which can digest both phosphoric and methyl phosphonic esters, was performed. However, the active site of the AChE molecule lies at the base of a narrow gorge that is 20 Å in depth [18], which prevents alkaline phosphatase from coming into contact with isopropyl-methylphosphonoserine and/or methylphosphonoserine at the active site, necessitating some conformational modification of the molecular structure of AChE to make alkaline phosphatase active. Taking the amino acid sequence of human AChE [19] into consideration, the active site of AChE could be degraded by trypsin to a 41-residue peptide (about 4.1 kDa). Alkaline phosphatase digestion of solubilized AChE after trypsin digestion was very useful for releasing the sarin hydrolysis products from AChE. Fig. 4 shows the total ion chromatogram of the TMS derivatives of the substances digested from normal (*a*) and BIMP-exposed (*b*) red blood cells. No prominent peak is observed in Fig. 4a. Figs. 5 and 6 show the EI and CI mass spectra of the peaks observed at 10.553 and 11.273 min, respectively. In the chromatograms of a TMS derivative of the authentic isopropyl methylphosphonic acid, the total ion chromatogram has one sharp peak at

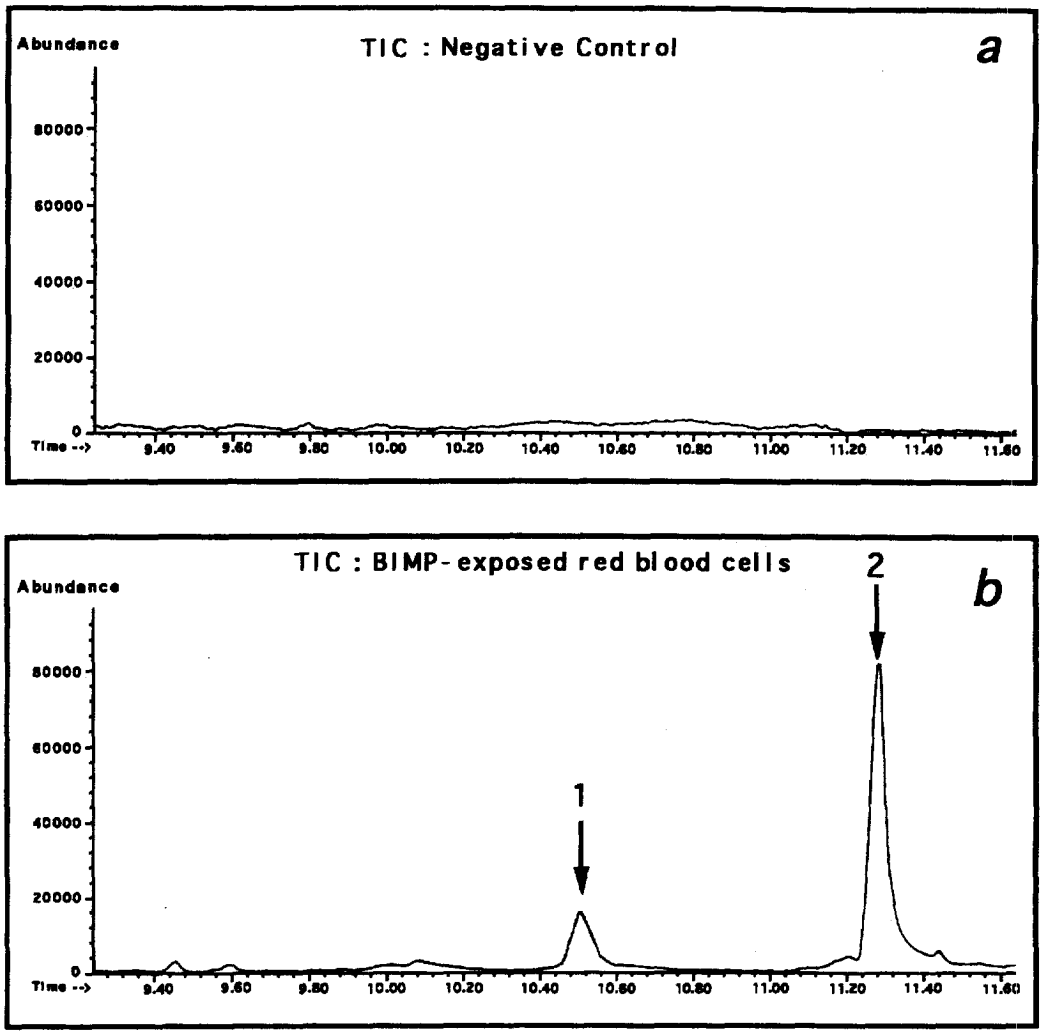

Fig. 4. Chromatograms of the TMS derivatives of digested substances from normal red blood cells (a) and BIMP-exposed red blood cells (b). 1=isopropyl methylphosphonic acid and 2=methylphosphonic acid.

10.5267±0.1266 min (n=6) and the EI mass spectrum of this sharp peak shows peaks at m/z 209, which is due to $[M-H]^+$, m/z 195, which is due to $[M-CH_3]^+$ and m/z 153 [20]. In the CI mass spectrum of it, peaks of m/z 211, 239 and 251, which are due to $[M+H]^+$, $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$, respectively, and a peak of m/z 153 were observed. In the chromatograms of the TMS derivative of an authentic methylphosphonic acid sample, the total ion chromatogram has one sharp peak at 11.2628±0.1346 min (n=6) and the EI mass spectrum of this sharp peak shows peaks at m/z 240, the parent peak, and m/z 225. Compared with the fragment patterns of the EI- and CI-MS of authentic isopropyl methylphosphonic and methylphosphonic acids, the parent peak and other prominent peaks were also present in the chromatograms of the TMS derivatives obtained from the BIMP-exposed red blood cells shown in Figs. 5 and 6, respectively. The retention times of these substances, shown in Figs. 5

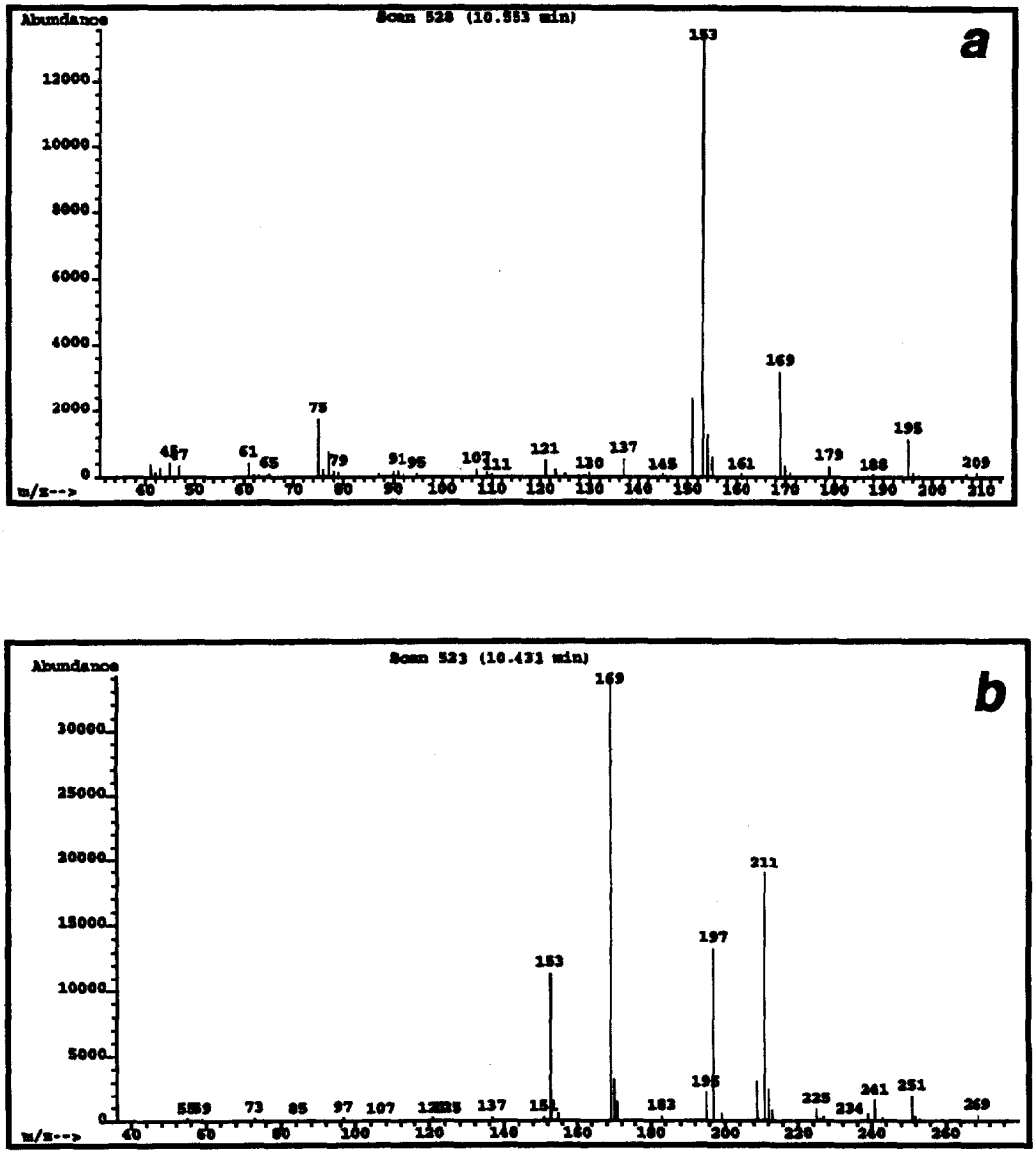

Fig. 5. EI-MS (a) and CI-MS (b) of the peak observed at about 10.550 min in Fig. 4.

and 6, were within the ranges of those of the TMS derivatives of authentic isopropyl methylphosphonic and methylphosphonic acids, respectively. Thus, we were able to confirm that the former fragments were from the TMS derivative of isopropyl methylphosphonic acid and the latter ones were from that of methylphosphonic acid.

Recently, the residues of chemical warfare agents and their degradation products were reported to have been detected in environmental samples from a Kurdish village [20], but there are no reports of the detection of sarin degradation products in human specimens. We think that our procedure will be useful for analyses of the blood and tissues of sarin

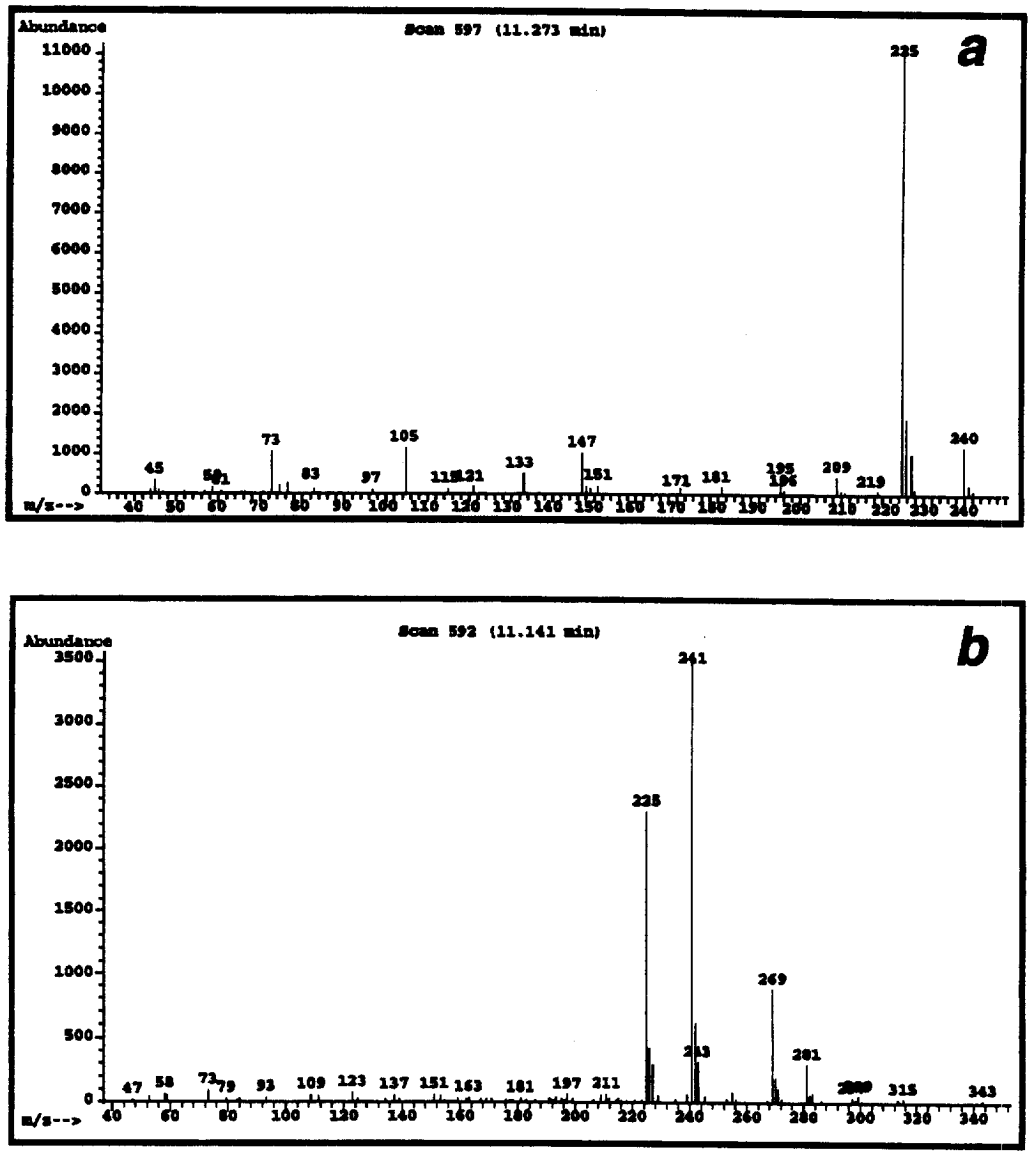

Fig. 6. EI-MS (a) and CI-MS (b) of the peak observed at about 11.270 min in Fig. 4.

victims. We applied this method to the examination of the four blood samples from victims of the Tokyo sarin attack, and the hydrolysis products of sarin were detected in all samples (data not shown).

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