Identification of Metabolites of Nerve Agent VX in Serum Collected from a Victim

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Abstract

A human serum sample collected from a victim of the Osaka VX incident was analyzed according to our developed technique for metabolites of VX. Gas chromatography–mass spectrometry (GC–MS) in full-scan electron impact and chemical ionization modes were used, and, for more reliable confirmation, GC–MS–MS was also employed. In the serum sample, both ethyl methylphosphonic acid and 2-(diisopropylamino-ethyl)methyl sulfide were detected. These results indicated that the techniques using GC–MS and GC–MS–MS were applicable to biological samples such as serum. These results also provide the first documented, unequivocal identification of the specific metabolites of VX in victim's serum and, furthermore, clarify a part of the metabolic pathway of VX in the human body.

Introduction

O-Ethyl *S*-2-diisopropylaminoethyl methylphosphonothioate (VX) strongly and readily binds to acetylcholinesterase and thereby inhibits this vital enzyme's normal biological activity in the cholinergic nervous system. It can be manufactured by relatively simple chemical techniques, and raw materials are inexpensive and readily available. Therefore, VX, along with isopropyl methylphosphonofluoridate (sarin, GB) and pinacolyl methylphosphonofluoridate (soman, GD), has been feared as a second "nuclear weapon" of poor countries. VX, whose inhalation LD_{50} is 10 mg • min/m^3, is several hundred times more poisonous than HCN gas via the respiratory system and approximately 100 times more poisonous than sarin through the skin (1–3) and is thus thought to be among the strongest nerve agents.

These nerve agents have been designated as chemical warfare agents (CW) and their use is forbidden by an international convention (1,2). However, the use of sarin in a chemical attack by Iraq against Kurdish communities in northern Iraq has been documented (4). More recently, CWs were used as tools for terrorism. 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. Thus, their strong killing and wounding properties have caused great shock and unrest in the world.

These three nerve agents contain the alkyl methylphosphono moiety in their chemical structures, and they are readily hydrolyzed to the corresponding alkyl methylphosphonic acids: VX to ethyl methylphosphonic acid (EMPA), sarin to isopropyl methylphosphonic acid (IPMPA), and soman to pinacolyl methylphosphonic acid (PMPA). The alkyl methylphosphonic acids are ultimately very slowly hydrolyzed to methylphosphonic acid (Figure 1) (5–10). Detection of methylphosphonic acids is usually performed for proof of the use of nerve agents.

The determination of methylphosphonic acids has been mainly studied using high-performance liquid chromatography (HPLC) (11), ion chromatography (IC) (7,9,12,13), capillary electrophoresis (CE) (10), HPLC–mass spectrometry (MS) (14), and CE–MS without derivatization and HPLC and gas chromatography–mass spectrometry (GC–MS) with derivatization (5,6, 16–23). Most of the determinations were from authenticated samples and environmental samples such as soil and water.

On the other hand, for unequivocal proof of exposure to these nerve agents, it is necessary to detect the unchanged nerve agents and their metabolites from biological samples such as blood, urine, and saliva collected from victims. In the human body, they are also thought to be enzymatically and/or spontaneously hydrolyzed to the corresponding methylphosphonic acids according to the pathways outlined in Figure 1. Until now, there were no published papers on their metabolism in the human body. Additionally, few available analytical methods that will allow us to determine the compounds in biological samples have been developed (13,22,23).

Quite recently, we analyzed the serum sample collected from a victim of the VX incident in December, 1994, in Osaka. In the analysis, several specific compounds that would be very important for the proof of exposure to VX could be detected. In this paper, the resulting detailed data will be shown, leading to clarification of a part of the metabolism of VX in the human body.

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Experimental

Materials

EMPA was purchased from Aldrich (Milwaukee, WI). The standard stock solution of EMPA was prepared in distilled water (1 mg/mL), and adjusted to the appropriate concentration with distilled water or human serum immediately before use. An internal standard (IS), diphenylmethane (DPM), was purchased from Wako (Osaka, Japan), and the IS solution was prepared in acetonitrile (the concentration being 100 µg/mL). The derivatization reagent used, *N*-methyl-*N*-(tertiary-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) with 1% tertiary-butyl-dimethylsilyl chloride (*t*-BDMSC), was purchased from GL Sciences (Tokyo, Japan).

2-(Diisopropylaminoethyl)methyl sulfide (DAEMS) and an IS, 2-(diisopropyl-aminoethyl)methoxide (DAEMO), were synthesized in our laboratory according to the procedures described here later. The standard stock solution of DAEMS was prepared in distilled water (100 µg/mL) and adjusted to the appropriate concentration with human serum immediately before use. The IS solution was prepared in dichloromethane (concentration, 30 µg/mL).

Sodium thiomethoxide and sodium methoxide were purchased from Wako, and 2-(diisopropylamino)ethyl chloride hydrochloride (DAEC • HCl) were purchased from Aldrich. They were used for synthesis of DAEMS and DAEMO. Other chemicals used were of analytical grade.

Synthesis of DAEMS

DAEMS was synthesized according to the following procedure. A 50-mL round bottom recovery flask containing 0.5 g of sodium thiomethoxide dissolved in 5 mL of absolute methanol was fitted with an efficient water-cooled condenser and a dropping funnel that was charged with 1 g of free DAEC prepared from DAEC • HCl. DAEC was added dropwise over a period of 10 min to the stirred methanol solution maintained at 10–15°C. After the addition was complete, the mixture was boiled under reflux for 2 h. The reaction mixture was cooled, and 5 mL of distilled water was added. The solution was extracted with 10 mL of dichloromethane. The extract was dried with anhydrous sodium sulfate and evaporated. The resultant mixture was placed in a distillation flask and distilled under reduced pressure, and the fraction distilling at 108–111°C (27-mm Hg) was collected (compound X). For the assignment of compound X, ^{1}H-NMR and GC–MS were employed.

Samples for the ^{1}H-NMR spectra were prepared in standard 5-mm sample tubes at a concentration of approximately 10 mg/mL in $CDCl_3$. The ^{1}H-NMR spectra were obtained using a Varian (Palo Alto, CA) GEMINI 2000 Fourier-transform NMR spectrometer system operating in the frequency sweep mode. A sweep width of 4500 Hz was used to record the spectra.

In the 1H-NMR spectrum of compound X, a singlet at δ_H 2.12 and a doublet at δ_H 0.994, the result of one S-methyl and two *N*-isopropyl groups, respectively, were observed. The signal from the two methin protons appeared at δ_H 2.99 as a septet. Four methylene protons between the S and N atoms appeared at δ_H 2.45 ~ 2.65 as a characteristic AA'BB' pattern, representing the structure of DAEMS.

Based on this examination, compound X was assigned to DAEMS, and the purified DAEMS was used as the authentic standard.

The IS, DAEMO, was synthesized according to the same procedure as DAEMS except that sodium methoxide was used instead of sodium thiomethoxide.

GC–MS and GC–MS–MS

GC–MS was carried out on a JEOL JMS-SX102AQQ hybrid MS (JEOL, Tokyo, Japan) interfaced to a Shimadzu GC-17A GC (Shimadzu, Kyoto, Japan). A fused-silica capillary column Hewlett Packard Ultra 2 (25 m × 0.32-mm i.d., 0.52-µm film thickness) was used for separation. Injections were manually done in the splitless mode at 270°C. The column oven temperature was raised from 70 to 300°C at 10°C/min for the analysis of EMPA. For the analyses of volatile metabolites, the oven temperature was maintained at 70°C for 2 min and then raised at 10°C/min to 300°C. The temperature of the transfer line between the GC and the MS was set at 250°C. High-purity helium, at a linear velocity of 45 cm/s, was used as the carrier gas. The EI operating parameters were as follows: resolution, 1000; source temperature, 200°C; electron energy, 70eV; ionizing current, 300 µA; ion multiplier, 1.0kV; and accelerating voltage, 10kV. The iso-butane CI operating conditions were as follows: resolution, 1000; source pressure, 3×10^{-4} Pa; source temperature, 200°C; electron energy, 200eV; ionizing current, 300 µA; ion multiplier, 1.2kV; and accelerating voltage, 10kV. Data were collected from *m/z* 50 to 800 at a scan rate of 0.5 s/scan.

GC–CI–MS–MS was carried out on a JMS-SX102AQQ hybrid mass spectrometer (JEOL) interfaced to a Shimadzu GC-17A GC (Shimadzu). The chromatographic conditions were the same as those for the GC–MS. Argon, at a pressure of 2.2×10^{-6} Torr, was used as the collision gas. The other MS–MS operating parameters were as follows: resolution, 1000; source pressure, 3×10^{-4} Pa; source temperature, 200°C; electron energy, 200eV; accelerating voltage, 10 kV; ionizing current, 300 µA; collision energy, 50 eV; and

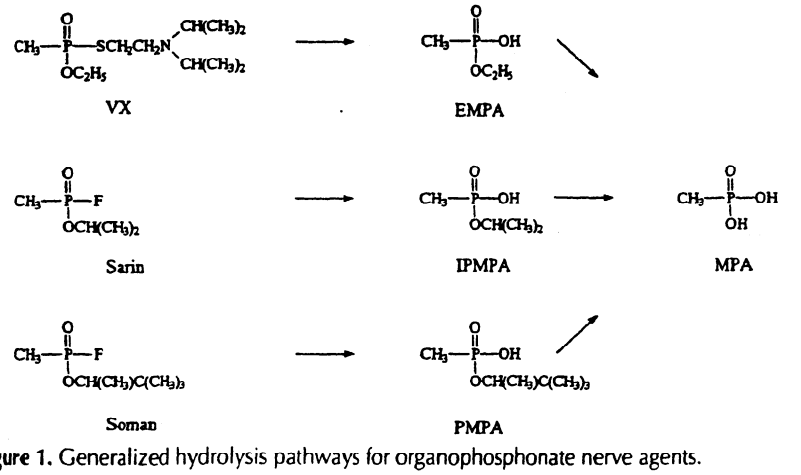

Figure 1. Generalized hydrolysis pathways for organophosphonate nerve agents.

ion multiplier, 1.2 kV. The product ion spectra were recorded from *m/z* 50 to 300 at a scan rate of 1 scan is using the pseudo-molecular ions as the precursor ions (*m/z* 176 and 239 in the analysis of DAEMS and the *t*-BDMS derivative of EMPA, respectively).

Sample preparation

Serum samples were prepared as follows (Figure 2): A 1-mL volume of serum sample was extracted twice with 1 mL of dichloromethane (centrifugation facilitates the separation of layers). Both the organic and aqueous layers were separated. The organic layer was used for the determination of VX and its volatile metabolites, and the aqueous layer was used for the determination of water-soluble compounds such as EMPA.

The organic layer was dried with anhydrous sodium sulfate, transferred to a 13 × 100-mm screw-capped Pyrex tube, and evaporated carefully under a gentle stream of nitrogen at ambient temperature. The residue was dissolved into 100 µL of dichloromethane, and 5 µL of DAEMO solution (IS) was added to it. A 1-µL aliquot of the extract was injected into the GC–MS and GC–MS–MS.

The aqueous layer was deproteinized by adding 1 mL of acetonitrile, and the supernatant was separated by centrifugation. The supernatant was acidified with 1 mL of oxalate buffer (pH 1.68), and an additional 0.6 g of sodium chloride was added for salting out. The addition of sodium chloride enabled us to separate the aqueous layer and acetonitrile layer. The solution was extracted twice with 2 mL of acetonitrile (centrifugation facilitates the separation of layers). The organic layer was transferred to a 13 × 100-mm screw-capped pyrex tube and evaporated just to dryness under a stream of nitrogen at 60°C. The residue was derivatized by adding 100 µL of MTBSTFA with 1% *t*-BDMSC to the tube and then heating the tube at 60°C for 30 min. At the end, 20 µL of DPM solution (I.S.) was added to the tube. A 1-µL aliquot of the reaction mixture was injected into the GC–MS and GC–MS–MS.

Results and Discussion

Analyses of a serum sample obtained from the victim of VX incident

Case history of VX incident. The victim (28-year-old male) was attacked by two members of a cult group. They sprinkled VX on his neck with a disposable syringe. He suddenly cried out and fell down on the road. He was carried to a hospital and received therapy, but he died 10 days later. Because of his symptoms, which included miosis and a low level of cholinesterase activity, poisoning with organophosphorus pesticides was suspected at first, but the cause of the death was not clarified by postmortem examination.

About 6 months later, his death was discovered to be due to the attack with VX according to the testimony of suspected persons. Other details such as the concentration, purity, and amount of VX used were not clear.

Thus, we were asked to analyze his serum, which was collected approximately 1 h after his exposure to VX and kept frozen at –20°C until analysis. The analyses were performed according to the method described in the experimental section.

GC–MS and GC–MS–MS analyses of separated aqueous layer. The separated aqueous layer was extracted with acetonitrile. After *t*-BDMS derivatization of the extract, the GC–MS analysis was conducted in both the full-scan EI and isobutane CI modes.

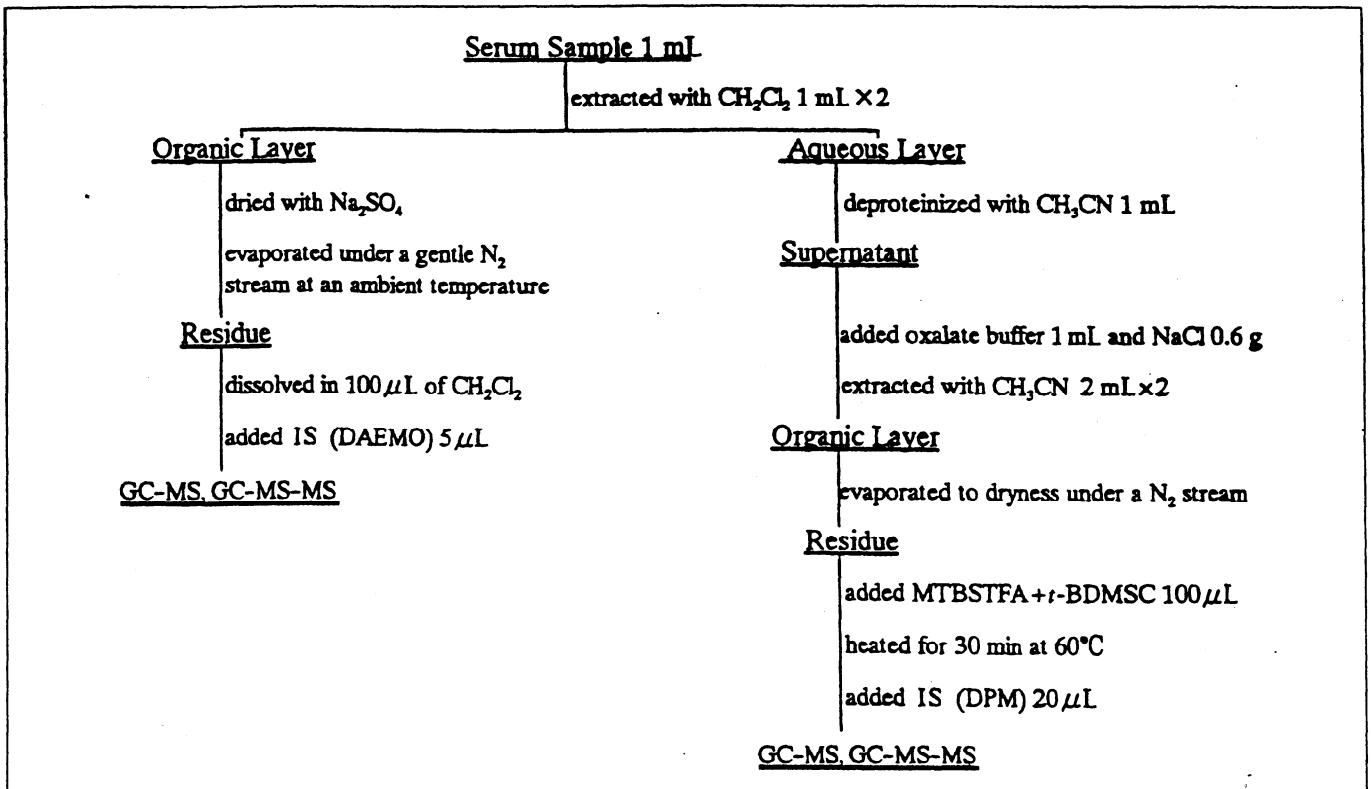

Figure 2. Procedure for the determination of VX and its metabolites from serum sample.

The resultant total ion chromatogram (TIC) is depicted in Figure 3A. A fairly small peak appeared at a retention time of 8.4 min in the TIC, and its EI mass spectrum was characterized by a predominant fragment ion at *m/z* 153 and less intense fragment ions at *m/z* 181, 75, and 223 (Figure 3B). Careful examination of the spectrum revealed that the ions at *m/z* 75, 153, 181, and 223 originated from $[HOSi(CH_3)_2]^+$, $[CH_3PO(OH)OSi(CH_3)_2]^+$, $[CH_3PO(OC_2H_5)OSi(CH_3)_2]^+$, and $[CH_3PO(OC_2H_5)OSi(CH_3)(CH_3)_3]^+$, respectively. Also, a CI mass spectrum obtained from the peak was dominated by the pseudo-molecular ion at *m/z* 239, suggesting that the molecular weight of the compound causing the peak was 238 (Figure 3C). The spectra and the retention time obtained from the peak agreed well with those from the *t*-BDMS derivative of the standard EMPA.

For the confirmation of the result from the above mentioned GC-MS, the same sample was further analyzed by GC–CI– MS–MS, in which *m/z* 238 was selected as the precursor ion. Figure 3D depicts the positive full-scan product ion spectrum produced from the peak described here previously. The spectrum that gave some specific product ions at *m/z* 73, 75, 153, and 195 corresponding to $[Si(CH_3)_3]^+$, $[HOSi(CH_3)_2]^+$, $[CH_3PO(OH)OSi(CH_3)_2]^+$, and $[CH_3PO(OC_2H_5)OSi(CH_3)_2CH_2]^+$, respectively, was identical to that of the *t*-BDMS derivative of the standard EMPA. According to these data, EMPA was proven to be contained in the serum sample.

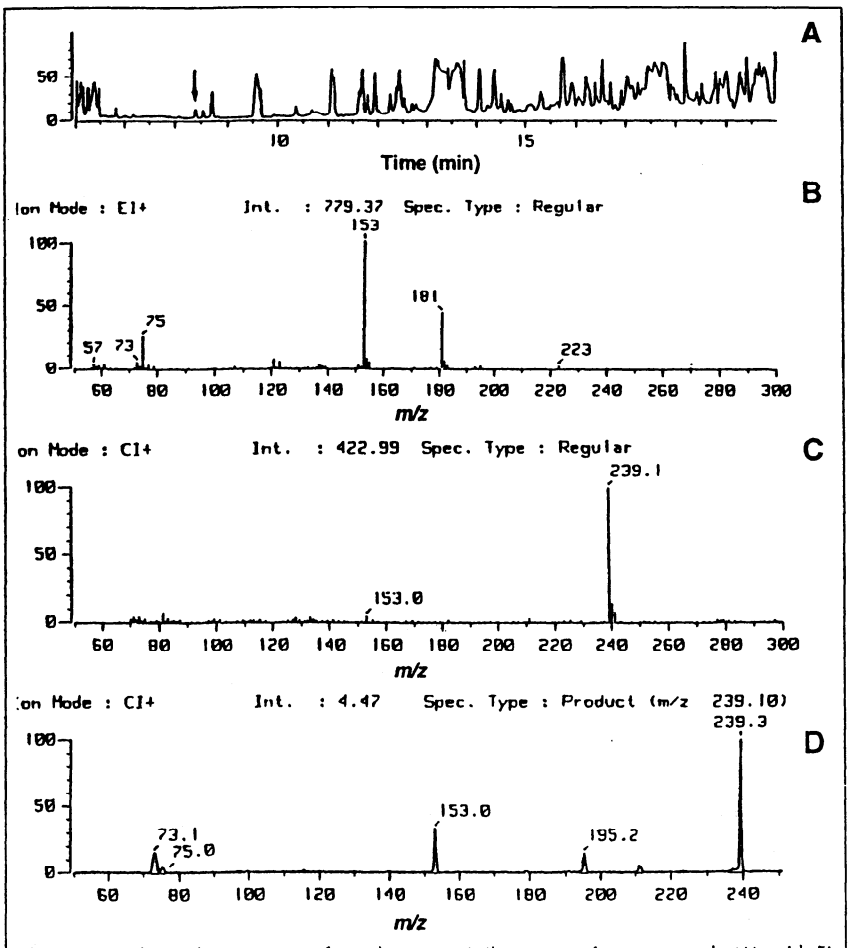

Figure 3. Total ion chromatogram from the acetonitrile extract of serum sample (A), with EI (B), isobutane CI full scan spectra (C), and product ion spectrum of $[M+H]^+$ confirming the identification of the *t*-BDMS derivative of EMPA (D). The arrow indicates the peak assigned to the *t*-BDMS derivative of EMPA.

In addition, to estimate the concentration of EMPA in the serum sample, a quantitative measurement by GC–MS in the selected ion monitoring (SIM) EI mode was carried out in the presence of DPM as the IS. The calibration curve was constructed with fortified serum samples in which the concentration of the added EMPA was varied, and the predominant ions (*m/z* 153 for the *t*-BDMS derivative of EMPA and 168 for DPM) were chosen for the quantitation.

The analysis showed good linearity throughout the concentration range from 0.1 to 5 µg/mL for EMPA, and the concentration of EMPA in the serum sample estimated using the calibration curve was 1.25 µg/mL.

GC–MS and GC–MS–MS analyses of separated dichloromethane layer. The dichloromethane extract was directly analyzed by GC– MS in both the full-scan EI and isobuthane CI modes. The resultant TIC is depicted in Figure 4A. At the retention time of 7.1 min in the TIC, a fairly small peak was observed, and its EI mass spectrum was characterized by a predominant fragment ion at *m/z* 114 and less intense fragment ions at *m/z* 72, 75, and 128 (Figure 4B). Considering the data on the related compounds of VX previously described by D'Agostino et al. (24), careful examination of the spectrum disclosed that the ions at *m/z* 114, 72, 75, and 128 were due to $[(iPr)_2N{=}CH_2]^+$, $[(iPr)N{=}CH_2]^+$, $[CH_3SCH_2CH_2]^+$, and $[(iPr)\text{-}(CH_3C{=}CH_2)NHC_2\text{-}H_5]^+$, respectively. The CI mass spectrum was dominated by a pseudo-molecular ion at *m/z* 176, suggesting that the molecular weight of the desired compound was 175 (Figure 4C). Based on the these data, we assigned DAEMS to this compound.

The sample was further analyzed by GC–CI–MS–MS, in which *m/z* 176 was selected as the precursor ion. The positive full-scan product ion spectrum produced from the desired compound is depicted in Figure 4D. It shows a specific product ions at *m/z* 75 corresponding to $[CH_3SCH_2CH_2]^+$, representing the structure of DAEMS.

Confirmation and quantitative analysis of DAEMS. For the confirmation of the assignment of DAEMS to the compound, DAEMS was synthesized in our laboratory according to the procedure described in the Experimental section and subjected to GC–MS and GC–MS–MS analyses. The data were collected under the same operating conditions as in the analyses of the serum sample, and the resultant retention time and mass spectra were compared with those from the assigned peak. The spectra and the retention time of the assigned peak were almost identical with those of authentic DAEMS, thus confirming the assignment.

In order to estimate the concentration of EMPA in the serum sample, quantitative measurements by GC–MS in the SIM-EI mode were performed using DAEMO as the IS. The calibration curve was constructed with spiked serum samples in which the concentration of the added DAEMS was varied. As the fragment

ion at *m/z* 114 was present as the predominant ion in both the mass spectra of DAEMS and DAEMO, the ion at *m/z* 114 was selected for the quantitation.

The analysis gave good linearity throughout the concentration range from 50 to 1000 ng/mL for DAEMS, and the concentration of DAEMS in the serum sample estimated using the calibration curve was 143 ng/mL.

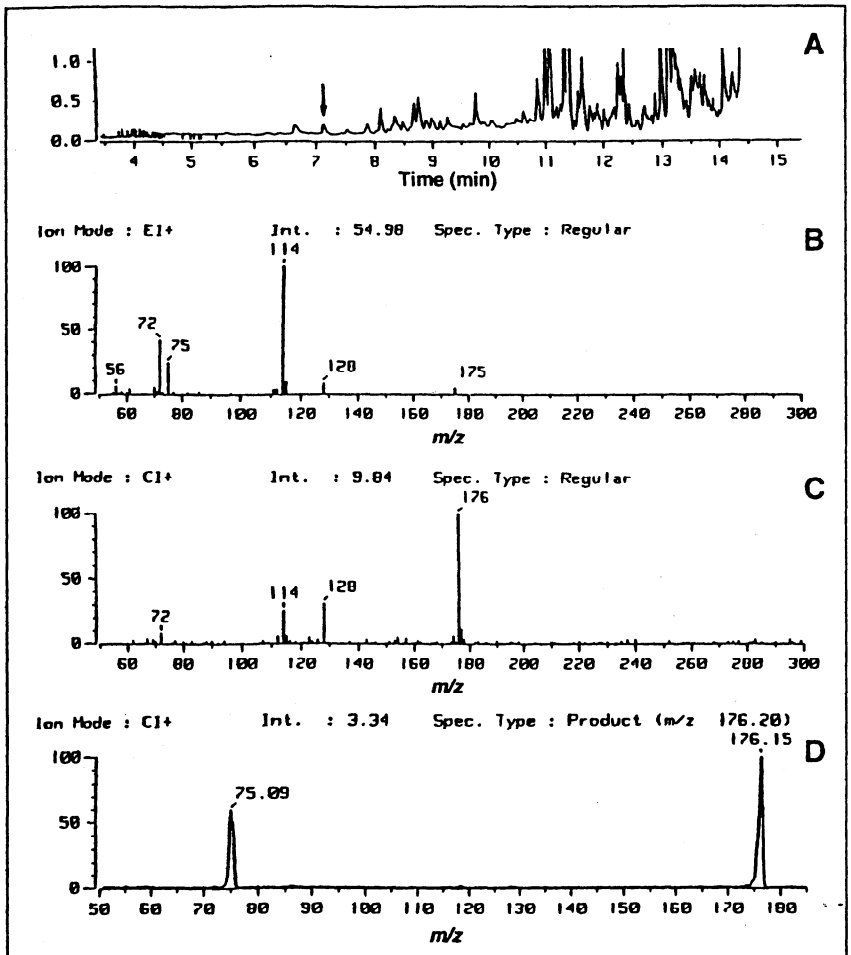

Figure 4. Total ion chromatogram from the dichloromethane extract of serum sample (A), with EI (B), isobutane CI full scan spectra (C), and product ion spectrum of $[M+H]^+$ confirming the identification of DAEMS (D). The arrow indicates the peak assigned to DAEMS.

Proof of the use of VX

In the human body, VX is thought to be enzymatically and/or spontaneously hydrolyzed to the EMPA and 2-(diisopropylamino)ethanethiol (DAET). In our GC–MS analyses, EMPA was detected, and DAEMS, the methylthioether of DAET, was also detected instead of DAET. In general, thiols are readily methylated and metabolized to the corresponding methylthioethers in the human body according to the biosynthetic reaction, which is catalyzed by *S*-adenosyl-l-methionine-mediated thiol *S*-methyltransferase (25). Thus, we consider that VX was hydrolyzed to DAET, and the DAET was further metabolized to DAEMS by methylation following the previously mentioned enzyme route (Figure 5).

To date, EMPA has been considered as a target for the proof of the use of VX. However, EMPA and DAEMS were detected in the serum sample in our analyses, and it led to more reliable proof of the use of VX.

Conclusion

We successfully applied our newly developed method, which allows us to determine the hydrolysis products of nerve agents, to the analyses of the serum sample collected from a victim of the VX incident. In the analyses, DAEMS (concentration 143 ng/mL) and EMPA (concentration 1.25 µg/mL) were detected. Especially, DAEMS is considered to be the methyl-conjugated compound of DAET, the

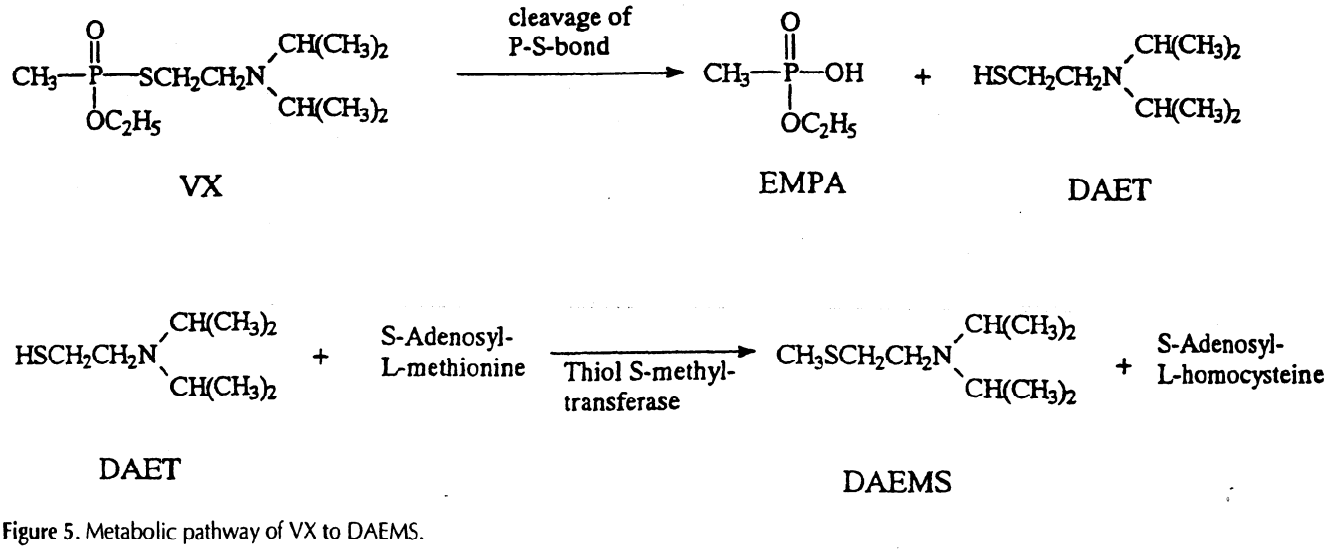

Figure 5. Metabolic pathway of VX to DAEMS.

hydrolysis product of VX. The detection of DAEMS in the biological sample was first reported in this paper. The detection of both EMPA and DAEMS provides more reliable proof of the use of VX than that previously reported, and clarifies a part of the metabolism of VX in human body.

However, the production rate of DAEMS, the detection period of DAET, etc. are not clear, and future studies on these subjects will be required to clarify the metabolism of VX.

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