II.8.2 VX and its decomposition products

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Introduction

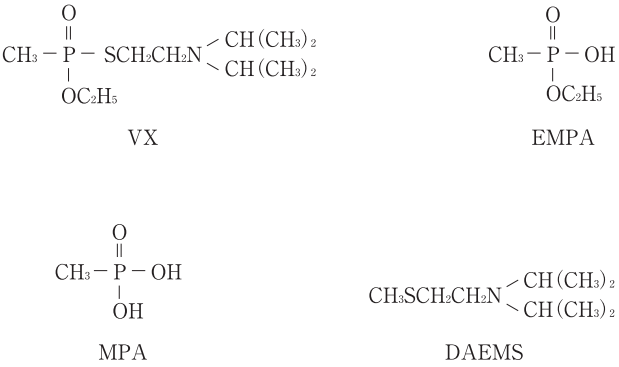
An organophosphorus nerve agent VX (*O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothiolate, *Figure 2.1*) shows potent inhibitory action on acetylcholinesterase; its development, production, stockpiling and use are being prohibited by the CWC international treaty as a chemical weapon together with those of sarin and soman. In addition, even material compounds for VX synthesis are being also controlled strictly.

In the world history, there had been no records on the use of VX in any international dispute. However, in December 1994, a murder terrorism incident using VX committed by a cult group took place in Osaka, Japan. The very high poisoning potency of VX proven in the incident surprised the whole world with a shock and anxiety.

VX is easily hydrolyzed under alkaline conditions, and also in the environmental water and soil to produce ethylmethylphosphonic acid (EMPA) and further methylphosphonic acid (MPA) [1]. VX is rapidly hydrolyzed by both chemical and enzymatic reactions in mammalian bodies to produce EMPA and 2-(diisopropylaminoethyl)methyl sulfide (DAEMS)[a]. These metabolites or decomposition products are detected for verification of the use of VX [2].

Many methods for EMPA and MPA mainly in environmental water and soil were reported using ion chromatography with indirect photometric detection [3], capillary electrophoresis [4], GC/MS after methylation [5], silylation [6–8] and pentafluorobenzyl (PFB) derivatization [9, 10], LC/MS [11] and CE/MS [12, 13] both without any derivatization, LC/MS after derivatization and LC/MS/MS [14]. In actual terrorism cases using VX, the detection of its metabolite products from urine and blood is essential. In this chapter, the details for GC/MS analysis of VX metabolites in human serum[b] are described.

Figure 2.1



Structures of VX and its hydrolyzed products/metabolites.

Reagent and their preparation

- A 10-mg aliquot of EMPA (Aldrich, Milwaukee, WI, USA) is dissolved in 10 mL distilled water (1 mg/mL) to prepare aqueous stock solution. Just before use, the solution is appropriately diluted with blank human serum to prepare the standard specimens.
- A 10-mg aliquot of DAEMS is dissolved in 100 mL distilled water to prepare aqueous stock solution (100 μg/mL). Just before use, the solution is appropriately diluted with blank human serum to prepare the standard specimens. DAEMS can be synthesized by reacting 2-(diisopropylamino)ethyl chloride hydrochloride[c] (Aldrich) with sodium thiomethoxide (Aldrich) [2].
- A 10-mg aliquot of diphenylmethane (DPM, internal standard = IS, Aldrich and other manufacturers) is dissolved in 100 mL acetonitrile (100 μg/mL).
- *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide + 1 % *tert*-butyldimethylchlorosilane (Pierce, Rockford, IL, USA) is directly used for *tert*-butyldimethylsilyl (*t*-BDMS) derivatization.
- A 1-mg aliquot of 2-(diisopropylaminoethyl)methoxide (DAEMO, IS) is dissolved in 100 mL dichloromethane (10 μg/mL). DAEMO can be synthesized by reacting 2-(diisopropylamino)ethyl chloride hydrochloride with sodium methoxide (Aldrich and other manufacturers) [2].
- Other reagents are of the highest purity commercially available.

GC/MS conditions

GC column: a DB-1 fused silica capillary column (30 m × 0.32 mm i.d., film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA).

GC/MS conditions[d]; instrument: a Shimadzu QP5050 gas chromatograph connected with a mass spectrometer (Shimadzu Corp., Kyoto, Japan); column (oven) temperature for EMPA: 80 °C (2 min) → 15 °C/min → 300 °C; column (oven) temperature for DAEMS: 50 °C (2 min) → 10 °C/min → 300 °C; injection temperature: 270 °C; injection mode: splitless; interface temperature: 250 °C; EI electron energy: 70 eV; CI reagent gas: isobutane.

Procedures

i. Analysis of VX and its volatile metabolite [2]

i. A 1-mL volume of serum is mixed with 1 mL dichloromethane, shaken and centrifuged; the dichloromethane layer is transferred to another test tube. To the above aqueous phase, 1 mL dichloromethane is again added, shaken and centrifuged.
ii. The resulting dichloromethane layers are combined and dehydrated by adding anhydrous Na_2SO_4. The clear dichloromethane solution is transferred to a glass vial and carefully evaporated to dryness under a stream of nitrogen gas at room temperature[e].
iii. The residue is dissolved in 100 μL of the dichloromethane solution of DAEMO (IS solution); a 1-μL of it is injected into GC/MS for analysis[f].

ii. Analysis of EMPA [2, 8]

i. The remaining aqueous layer in the above procedure 1) is mixed with 1 mL acetonitrile and centrifuged for deproteinization[g].
ii. The resulting supernatant solution is mixed with 1 mL of 0.05 M oxalate buffer solution (pH 1.68)[h], 0.6 g NaCl and 2 mL acetonitrile[i], shaken and centrifuged. The resulting acetonitrile layer is transferred to another test tube. To the remaining aqueous phase, 2 mL acetonitrile is again added, shaken and centrifuged.
iii. The acetonitrile layers obtained are combined, dehydrated with anhydrous Na_2SO_4; the clear acetonitrile layer is transferred to a Pyrex test tube[j], and evaporated to dryness under a stream of nitrogen with heating at 60 °C.
iv. The residue is mixed with 100 µL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide + 1 % *tert*-butyldimethylchlorosilane and heated at 60 °C for 30 min for *t*-BDMS derivatization[k].
v. To the above reaction mixture, 20 µL of the diphenylmethane (IS) acetonitrile solution is added and mixed; a 1-µL aliquot of it is subjected to GC/MS analysis[l].

Assessment of the method

Figure 2.2 shows a total ion chromatogram (TIC), a mass chromatogram measured at *m/z* 114 and EI and CI mass spectra[m] for DAEMS (500 ng/mL) extracted from human serum. If VX remains, it is extracted into the dichloromethane layer; however in most cases except for massive VX exposure, VX cannot be detected, because of its rapid metabolism and decomposition in human bodies.

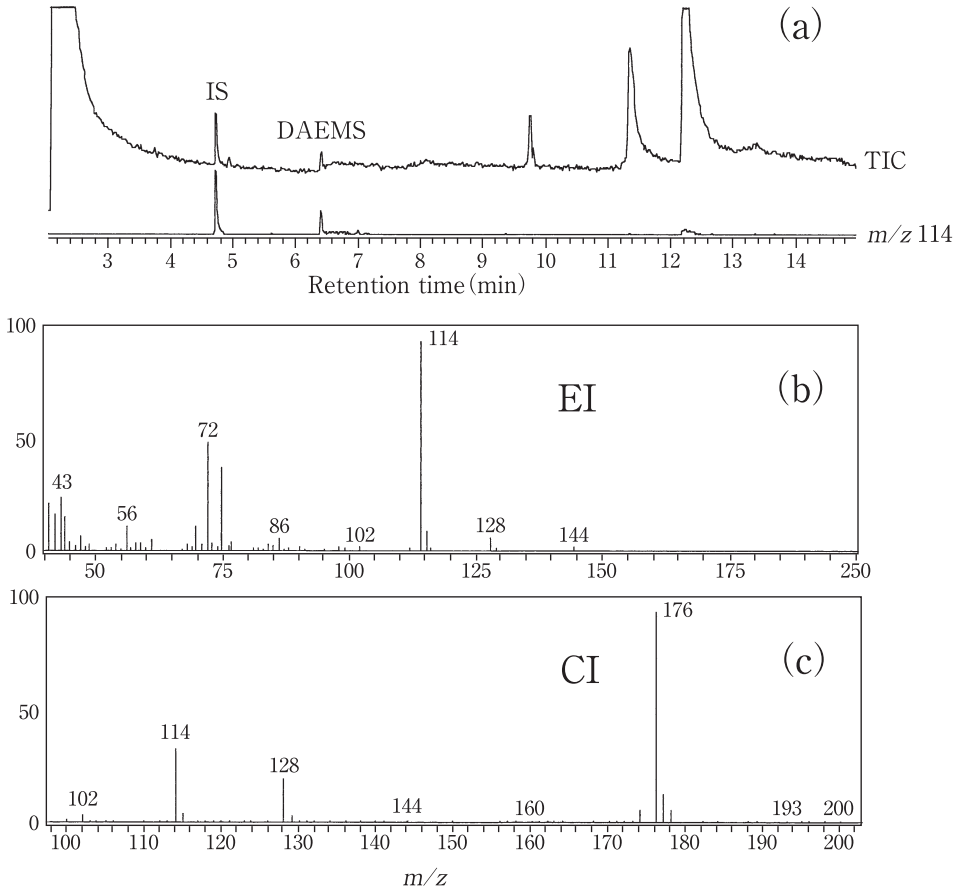
The detection limits of DAEMS in serum are about 50 ng/mL in the scan mode and about 5 ng/mL in the SIM mode.

Figure 2.3 shows a TIC, mass chromatograms and mass spectra[n] for EMPA (1 µg/mL) extracted from human serum. In humans, who have been exposed to VX, MPA also appears together with EMPA in serum. However, in the present method, the extraction efficiency of MPA is as low [o] as several %; only a trace level of MPA can be detected or it is not detectable in most cases. It should be pointed out that MPA can be equally produced from some organophosphorus nerve agents, such as sarin and soman; the detection of only MPA does not enable specification of a chemical weapon used. The identification of EMPA is most important to verify the exposure to VX.

The detection limit of EMPA in human serum is about 10 ng/mL in the scan mode and about 1 ng/mL in the SIM mode.

Usually, for qualitative analysis of organophosphorus nerve agents, the detection and identification of their metabolite alkyl methylphosphonic acid are carried out. In the VX poisoning cases, DAEMS due to the leaving group can be detected together with EMPA; the detection of both compounds highly enhances the reliability for verification of VX exposure.

Figure 2.2



GC/MS analysis for DAEMS in serum. (a) a total ion chromatogram (TIC) and a mass chromatogram; (b) an EI mass spectrum of DAEMS; (c) a CI mass spectrum of DAEMS.

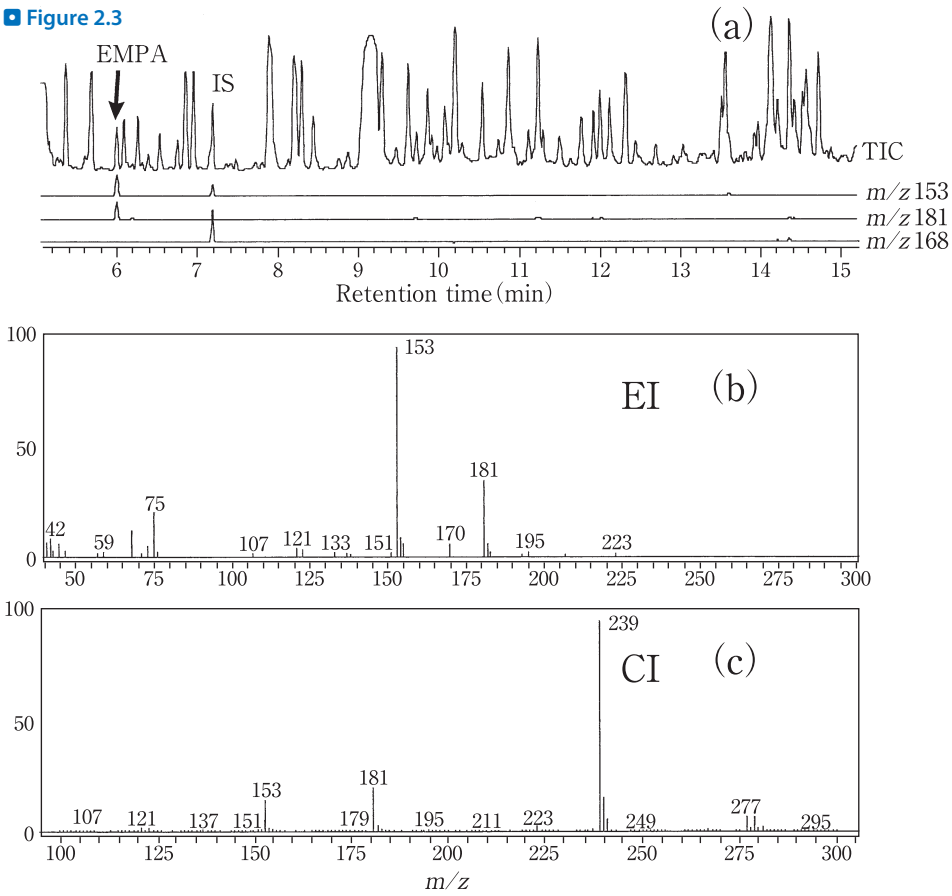
Poisoning case, and toxic and fatal concentrations

VX is much less volatile than sarin, but is highly permeable through the skin; usually victims are exposed to an aerosol or a liquid form of VX. The absorption of VX through the eye mucosa and the skin results in its poisoning.

In the murder terrorism with VX taking place in Osaka, Japan, 1994, VX was sprayed on the back of the neck of the victim using a syringe (the exposure amount not known); he died 10 days later. As VX poisoning symptoms, marked miosis and lowered levels of cholinesterase activity characteristic for organophosphorus compound poisoning appear first; in severer poisoning, dyspnea, enhanced sweating, convulsion attack, respiratory arrest and finally cardiopulmonary arrest leading to death can be observed.

VX is said to be the most potent poison among the nerve agents. There are no precise data on the toxicity of VX in humans; there are only estimated values based on the experimental

Figure 2.3

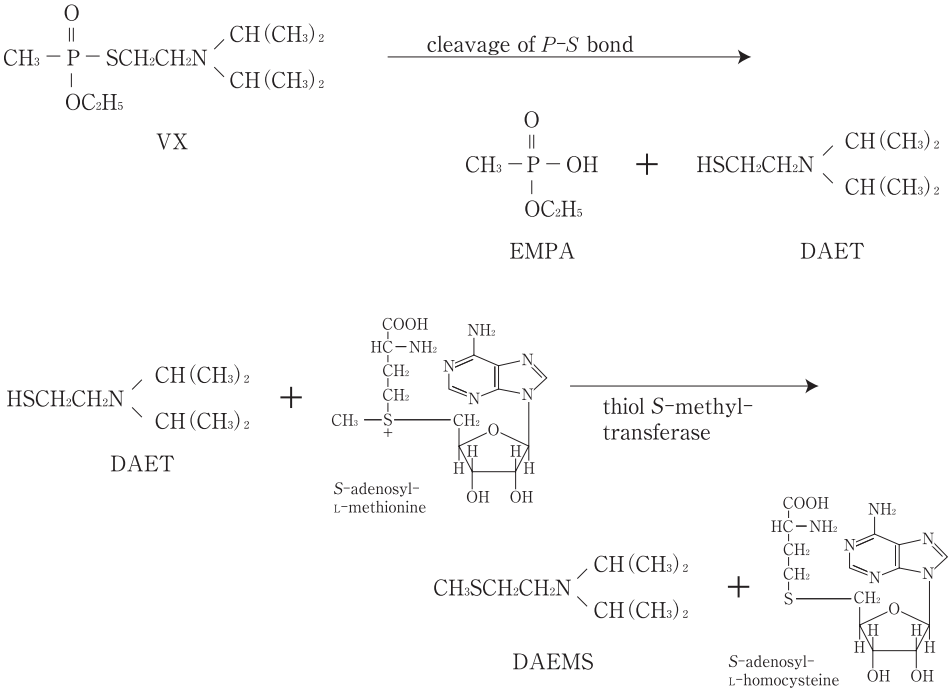


GC/MS analysis for EMPA in serum. (a) a TIC and mass chromatograms; (b) an EI mass spectrum of the *t*-BDMS derivative of EMPA; (c) a CI mass spectrum of the same derivative of EMPA.

data of animals. According to the reports by the US army [15,16], the minimal toxic and lethal concentrations of VX *via* the airway are said to be 1.1×10^{-5} mg · min/m^3 and 0.1 mg · min/m^3, respectively. According to a report by WHO [17], the percutaneous lethal doses of VX are estimated to be 2–10 mg.

In the above VX- poisoned victim, VX could not be detected from his serum, which had been sampled 1 h after the exposure; however EMPA and DAEMS, the metabolites of VX, could be detected [18].

Figure 2.4



Main metabolic pathways for VX in human bodies.

Notes

a) When VX is absorbed into human bodies, it is rapidly hydrolyzed by chemical and enzymatic reactions with allylesterase to yield EMPA and 2-(diisopropylamino)ethanethiol (DAET). The DAET formed is immediately subjected to methyl-conjugation by the action of thiol *S*-methyltransferase being contained in the endoplasmic reticulum to produce DAEMS [18]. This reaction requires *S*-adenosyl-L-methionine (activated methionine) as coenzyme (*Figure 2.4*).
In addition, the disappearance of the resulting DAEMS from blood is very rapid. According to rat experiments made by Tsuchihashi et al. [18], DAEMS could be detected from 10 min after intraperitoneal administration of a large dose of DEAT (20 mg/kg), but was at detection limit levels only 3 h after the administration. Therefore, in humans, DAEMS may become undetectable only several hours after exposure to VX; thus the blood specimens should be sampled as soon as possible.

b) Urine specimens can be also analyzed with the same procedure.

c) 2-(Diisopropylamino)ethyl chloride hydrochloride is designated as one of the Schedule 1 chemicals listed by CWC. It is also being strictly controlled by the domestic laws. To purchase the compound, proper legal procedures including various documents clarifying the purpose of its use are required.

d) Upon the use of *t*-BDMS derivatization of EMPA, the final solution containing a large amount of the derivatization reagent has to be injected into GC/MS, resulting in the marked contamination of the ion source of an MS instrument. The lighting-up time for the filament should be delayed as much as possible to protect the ion source and the analytical part of the instrument.
e) DAEMS is highly volatile, and thus its solvent should be evaporated at a lower temperature gradually and carefully. If VX itself remains, there is a danger of the secondary exposure for an analyst; the manipulations including the above evaporation should be done inside a draft chamber.
f) For quantitative analysis of DAEMS, various concentrations of DAEMS are spiked into blank serum specimens containing a fixed amount of DAEMO (IS) each and extracted as described before. Since the base peaks of DAEMS and DAEMO (IS) equally appear at *m/z* 114, the SIM measurements should be made using this single ion to construct a calibration curve for DAEMS consisting of peak area ratio of DAEMS to IS on the vertical axis and DAEMS concentration on the horizontal axis. A peak area ratio obtained from a test specimen is applied to the calibration curve to calculate a DAEMS concentration.
g) For deproteinization, the ultrafiltration or perchloric acid can be also used. When the perchloric acid is used, the supernatant solution should be neutralized with sodium bicarbonate before the extraction procedure.
h) The pH adjustment can be made using 1 M hydrochloric acid solution. Since the p*K*a value of EMPA is 2.75, the pH of the aqueous layer should be not higher than 2.0 to extract EMPA into an organic layer efficiently; at higher than pH 3.0, the efficiency becomes much lower.
i) Usually, acetonitrile and water are well miscible and difficult to be separated. However, the addition of a saturable amount of NaCl, the acetonitrile layer is distinctly separated from the aqueous layer by the salting-out effect.
j) The adsorption of EMPA to the Pyrex glass test tube is much less than that to a usual glass test tube.
k) For derivatization of EMPA, trimethylsilylation, PFB derivatization and methyl esterification can be also used. However, in the analysis by EI-MS, the *t*-BDMS derivatization gives the highest sensitivity.
   The GC/MS analysis in the negative ion chemical ionization (NICI) mode, the PFB derivatization with pentafluorobenzyl bromide (PFBBr) is most useful. By this method, the sensitivity ten to several ten times higher than that by the positive ion EI method can be obtained. In the mass spectrum, only a single peak at *m/z* 123 due to $[M–PFB]^-$ appears. However, in the NICI mode, the optimization of conditions is relatively complicated; it does not seem recommendable for wide use. Therefore, a simple positive EI method, which is highly reproducible, has been presented here.
l) For quantitative analysis of EMPA, a calibration curve is constructed with a similar procedure to that described in the above commentary[f]. However, ions at *m/z* 153 and 168 for *t*-BDMS derivative of EMPA and diphenylmethane (IS), respectively, are used for SIM measurements.
m) The base peak for DAEMS appearing at *m/z* 114 in the EI mass spectrum (*Figure 2.2)* is a fragment ion due to $[(iPr)_2\ N{=}CH_2]^+$. Since the same fragment ion at *m/z* 114 can be observed also in the spectrum of VX as the base peak [9], it is easy to examine the coexistence of VX and DAEMS by SIM measurements at *m/z* 114.

In the mass spectrum of DAEMS, other fragment ions at *m/z* 72,128 and 75, due to $[(iPr)N = CH_2]^+$, $[(iPr)(CH_3C = CH_2)NHC_2H_5]^+$ and $[CH_3SCH_2CH_2]^+$, respectively, also appear. Very small molecular peak (M^+) can be observed at *m/z* 175.

n) In EI mass spectra for *t*-BDMS derivatives, intense peaks due to $[M–57]^+$ are usually observed. However, in the case of the *t*-BDMS derivative of EMPA, a fragment ion, having a structure of $[CH_3PO(OH)OSi(CH_3)_2]^+$, is produced by desethylation, and appears at *m/z* 153 as the base peak; the $[M–57]^+$ peak due to $[CH_3PO(OC_2H_5)OSi(CH_3)_2]^+$ also appears at *m/z* 181 with intensity of about 50 %.
Except for EMPA, the *t*-BDMS derivatives of isopropylmethylphosphonic acid (IPMPA) and pinacolylmethylphosphonic acid (PMPA), the decomposition products of sarin and soman, respectively, also show their base peaks at *m/z* 153. Therefore close attention should be payed to the discrimination of EMPA from IPMPA or PMPA. However, for all compounds, the relatively intense $[M–57]^+$ peaks also appear; they can be indicators of their identities. The differences in retention times can be also used for their identification. In addition, upon analysis of EMPA in a specimen, it is essential to analyze its authentic compound simultaneously for comparison.

o) To detect MPA with high efficiency, the aqueous phase can be directly evaporated to dryness without acetonitrile extraction. When the volume of the aqueous phase is small, it can be realized; but when the volume is large, it requires a long time for evaporation to dryness. In addition, in the aqueous phase, many impurity compounds derived from a specimen matrix are included; in such cases, the extraction with acetonitrile may give better results, though its recovery rate is low.

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