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Determination of the main hydrolysis product of *O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothiolate, ethyl methylphosphonic acid, in human serum

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Abstract

For the unequivocal proof of the use of a nerve agent *O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothiolate (VX), a rapid, accurate and sensitive method which allows us to identify its main hydrolysis product ethyl methylphosphonic acid (EMPA) in human serum was explored by GC–MS. GC–MS analysis was performed after solvent extraction with acetonitrile in acidic conditions from the serum sample, which was previously deproteinized by micro-ultrafiltration, and subsequent *tert.*-butyldimethylsilyl derivatization with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) with 1% *tert.*-butyldimethylsilyl chloride (*t*-BDMSC). Linear calibration curves were obtained in the concentration range from 50 to 500 ng/ml for EMPA in the full-scan EI mode and from 5 to 50 ng/ml for EMPA in the SIM EI mode. The relative standard deviation obtained at a sample concentration of 50 ng/ml was 8.4% in the full-scan mode and 7.3% in the SIM mode. Upon applying the full-scan EI and CI mode, 40 ng/ml and 80 ng/ml were the detection limits. Using the SIM-EI mode, in which the ion at *m/z* 153 was chosen, the limit was 3 ng/ml.

Keywords: *O*-Ethyl *S*-2-Diisopropylaminoethyl methylphosphonothiolate; Ethyl methylphosphonic acid

1. Introduction

Owing to its strong acetylcholinesterase-inhibiting properties the nerve agent *O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothiolate (VX) is considered a chemical warfare agent in addition to isopropyl methylphosphonofluoridate (sarin, GB), 1,2,2-trimethylpropyl methylphosphonofluoridate (soman, GD), etc., by international conventions [1,2]. VX, the inhalation LD_{50} of which is 10 mg min/m^3 [2], is several hundred times stronger than HCN gas, and it can be said to be one of the strongest nerve agents. What is worse, in a murder case in 1994 in Osaka, VX was used and its strong killing and wounding properties have caused great shock and unrest in the world. This emphasizes the need for specific detection and identification methods for VX.

Owing to its chemical instability, VX is readily hydrolyzed [3–6] and it is also thought to be enzymatically and non-enzymatically (chemically)

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hydrolyzed in the human body to ethylmethylphosphonic acid (EMPA) and partly to methylphosphonic acid (MPA) [7] according to the scheme outlined in Fig. 1. For unequivocal proof of the use of VX, it will be, therefore, very important to have methods available for the identification and the determination of the hydrolysis products.

The determination of the products has often been studied by high-performance liquid chromatography (HPLC) [8], ion chromatography (IC) [9,10], capillary electrophoresis (CE) [11] and high-performance liquid chromatography–mass spectrometry (LC–MS) [12] with no derivatization, and by HPLC and gas chromatography–mass spectrometry (GC–MS) with derivatization [13–25]. Owing to its sensitivity and selectivity, GC–MS is at present one of the most suitable techniques for identification of these products.

EMPA is a polar, non-volatile compound. Therefore, prior derivatization to a volatile compound is necessary if GC–MS is to be employed. The common approach which has been used for derivatization of the methylphosphonic acids such as EMPA is methylation with diazomethane [14,15], on-column methylation with trimethylphenyl-ammonium hydroxide (TMPAH) [26], pentafluorobenzylation with pentafluorobenzylbromide (PFBBr) [23,24], trimethylsilylation with trimethylsilylating reagents [20–22] and *tert.*-butyldimethylsilylation with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [25].

However, almost all these procedures were developed for environmental samples such as soil and water rather than for biological samples such as human serum and urine. A simple extraction method from human serum and the following sensitive and selective analytical technique by GC–MS with prior derivatization for EMPA, which is thought to be a main hydrolysis product of VX in human body, were investigated.

2. Experimental

2.1. Materials

EMPA was purchased from Aldrich (Milwaukee, WI, USA). 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 prior to use. An internal standard (I.S.), diphenylmethane (DPM), was purchased from Wako (Osaka, Japan), and the I.S. solution was prepared in acetonitrile (the concentration being 100 μg/ml). Other chemicals used were of analytical grade. The derivatization reagent used for analysis, *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) with 1% *tert.*-butyldimethylsilyl chloride (*t*-BDMSC) was purchased from GL Sciences (Tokyo, Japan). Micro-ultrafiltration units were purchased from Millipore (Dradford, MA, USA).

2.2. Gas chromatography–mass spectrometry

GC–MS was carried out on a JEOL JMS-SX102AQQ double focusing mass spectrometer (JEOL, Tokyo, Japan) interfaced to a Shimadzu GC-17A gas chromatograph. A fused-silica capillary column DB-1 (column dimension and film thickness, being 30 m×0.32 mm I.D. and 0.25 μm) (J&W Scientific, Rancho Cordova, CA, USA) was used for separation. Injections were effected manually in the splitless mode at 270°C. The column oven temperature was maintained at 80°C for 2 min and then raised at 15°C/min to 300°C. The temperature of the transferline between gas chromatograph and the mass spectrometer was set at 250°C. High purity helium, at a linear velocity of 45 cm/s, was used as carrier gas. The EI operating parameters were as follows: resolution, 1000; source temperature, 200°C; electron energy, 70 eV; ionizing current, 300 μA; ion multi-

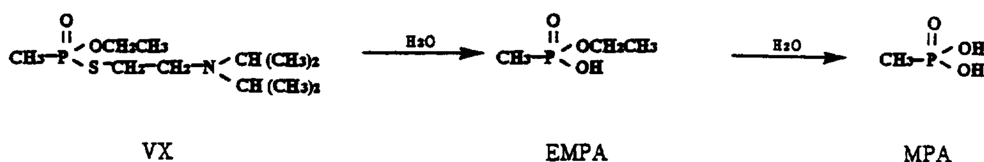

Fig. 1. Generalized hydrolysis pathway for VX.

plier, 1.0 kV; and accelerating voltage, 10 kV. Isobutane CI operating conditions were as follows: resolution, 1000; source pressure, $3 \cdot 10^{-4}$ Pa; source temperature, 200°C; electron energy, 200 eV; ion multiplier, 1.2 kV; and accelerating voltage, 10 kV. Data were collected from *m/z* 50–800 at a scan rate of 0.5 s/scan.

2.3. Sample preparation

Serum samples were prepared as follows (Fig. 2): Serum samples (1 ml) were deproteinized with micro-ultrafiltration units, and subsequently the filtrate was extracted with 1 ml of dichloromethane (centrifugation facilitates the separation of layers), and the aqueous layer was separated (organic layer was analyzed for VX and their volatile degradation products present). The aqueous layer was acidified with 1 ml of oxalate buffer (pH 1.68) and additional 0.6 g of sodium chloride was added for salting-out (the addition of sodium chloride enables us to separate 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 screw-capped pyrex tube and evaporated 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 volume of the reaction mixture was injected into the GC–MS system for determination of *t*-BDMS derivative of EMPA (Fig. 2).

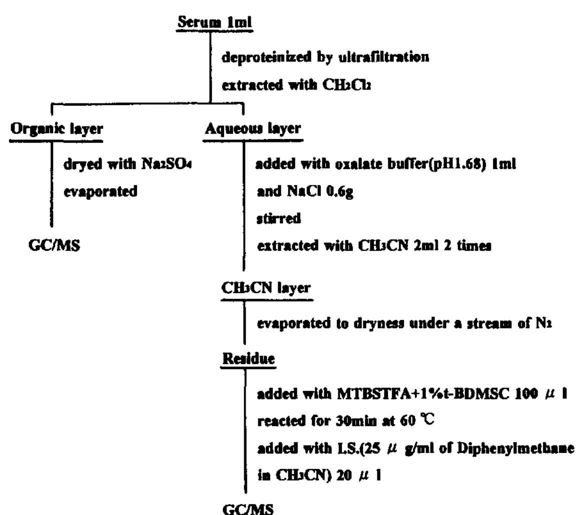

Fig. 2. Procedure for the preparation of serum samples.

3. Results and discussion

3.1. Derivatization reagent

To compare the availability of derivatization reagents for the GC–MS analysis of EMPA, an on-column methylating reagent TMPAH, an acylating reagent PFBBr, a trimethylsilylating reagent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and *tert.*-butyldimethylsilylating reagent MTBSTFA with 1% *t*-BDMSC, which were described in previous papers, were used as derivatization reagents.

After treatment of EMPA (5 μg) with each derivatization reagent described above, 20 μl of I.S. solution were added, and a 1-μl volume of each reaction mixture was analyzed by GC–MS. According to the method described by J.A. Tornes et al. [26], on-column methylation was performed by dissolving EMPA in methanol solution of TMPAH and then injecting the mixture into the GC–MS instrument. PFB derivatization was carried out by incubating EMPA, PFBBr and potassium carbonate in acetonitrile for 3 h at 90°C according to the method described by V. Bardarov et al. [27]. In accordance with the method described by J.G. Purdon [25], TMS derivatization and *t*-BDMS derivatization were conducted by treating EMPA with BSTFA and MTBSTFA with 1% *t*-BDMSC for 30 min at 60°C, respectively.

As is shown in Fig. 3, the highest sensitivity was obtained when MTBSTFA with 1% *t*-BDMSC was used, resulting in rapid and simple derivatization without a special catalyst. Thus, we chose MTBSTFA with 1% *t*-BDMSC as a derivatization reagent for EMPA.

3.2. Derivatization reaction

In order to determine the proper conditions for derivatization, several derivatization procedures were

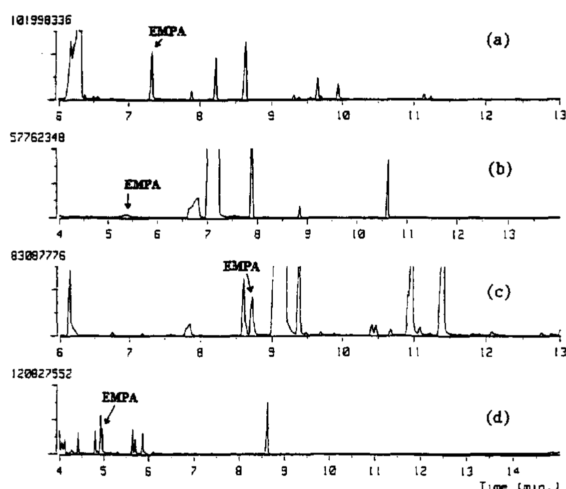

Fig. 3. Total ion chromatograms obtained for 5 μg of EMPA after derivatization with (a) MTBSTFA+1% *t*-BDMSC, (b) TMPAH, (c) PFBBr and (d) BSTFA. The chromatographic conditions for (a), (c) and (d) are given in Section 3.1. The condition for (b) is as follows: the column temperature was maintained at 50°C for 1 min and then raised at 15°C/min to 250°C.

examined. Deproteinized serum fortified with EMPA (500 ng/ml) was used as sample.

Extraction was performed according to the method described in Section 2.3 in this paper, and the extracts were reacted with MTBSTFA with 1% *t*-BDMSC at room temperature, 40°C and 60°C for 10 min, 30 min and 1 h to compare the yields of *t*-BDMS derivative of EMPA by GC–MS. As Purdon et al. described [25], the yields were constant independently of the reaction time and temperature, and derivatization of EMPA at all three temperatures and all three reaction times appears to be complete. Based on the above comparison, we chose 60°C and 30 min in the present study.

3.3. Sample preparation

3.3.1. Deproteinization of serum samples

To explore a suitable deproteinization method for serum samples, two procedures were examined; serum samples were deproteinized by adding perchloric acid, which is commonly used for deproteinization of biological samples, and deproteinization by ultrafiltration. In the former case, large amounts of alkali were needed for neutralization of excess acid and large volumes of salts were precipitated on evaporating to dryness which disturbed the following derivatization. On the other hand, in the latter case no treatment was needed after deproteinization and only a very little volume of salt was precipitated. Ultrafiltration was chosen for deproteinization.

3.3.2. Extraction procedure

To avoid disturbance in the detection of small peaks, sample preparation is one of the most essential processes. Particularly when samples are serum containing a number of unknown compounds, suitable sample preparation can lead to good results. In the present study, the following three procedures were tested and the results obtained from GC–MS of *t*-BDMS derivatives were compared: the three procedures were liquid–liquid extraction (referred to as procedure A), solid-phase extraction (referred to as procedure B), and evaporation to dryness under a stream of nitrogen at 60°C without extraction (referred to as procedure C). Deproteinized serum to which EMPA was added (500 ng/ml) was used as sample.

For procedure A, a suitable extraction solvent was first explored using diethyl ether, dichloromethane, chloroform–isopropanol (3:1), and acetonitrile. Serum samples were extracted in advance with dichloromethane, and the aqueous solution was extracted with the solvent described above after acidifying with 1 ml of oxalate buffer and subsequent salting out with a saturating amount of sodium chloride. The extracts were treated according to the method described in Section 2.3 and resultant recoveries were compared. As shown in Table 1, the best recovery was obtained by using acetonitrile, and we, therefore, chose acetonitrile as extraction solvent.

For procedure B, serum samples were extracted in

Table 1 guttermin=0.1
Recoveries of EMPA with various extracting solvents

Extracting solvent	Recovery (mean±S.D., n=3) (%)
Dichloromethane	<1
Diethyl ether	2.2±0.1
Chloroform–isopropanol (3:1)	44.0±2.9
Acetonitrile	90.7±6.4

advance with dichlorometane and the aqueous solution was prepared as follows: Sep-Pak QMA (anion-exchanger) cartridges were prewashed successively with 10 ml of methanol and 10 ml of distilled water. The aqueous solutions were diluted with 4 ml of distilled water and loaded on the prewashed cartridge. Subsequently, the cartridge was washed with 2 ml of distilled water and then 2 ml of methanol. The retained compounds were eluted using 2 ml of 0.1 *M* hydrochloric acid–methanol. Then, the eluates were evaporated to dryness under a stream of nitrogen at 60°C.

The total ion chromatograms shown in Fig. 4a–c were obtained for serum samples which were prepared according to procedure A, B and C, respectively. One can see large impurity peaks which disturbed detection of *t*-BDMS derivatives of EMPA in the chromatogram in Fig. 4c, and a long time would be needed for treatment of a large volume of serum sample. Therefore, procedure C was ruled out. In Fig. 4a and b, the *t*-BDMS derivative of EMPA was eluted without any disturbance by impurities in the serum. The smallest number of impurity peaks appeared in the chromatogram in Fig. 4b, suggesting that procedure B is preferred to procedure A. However, the recoveries of EMPA obtained were 90.7±6.4% (n=3) and 59.7±5.6% (n=3) for procedure A and B, respectively. Based on the above comparison, we finally chose procedure A for sample preparation in the present study.

3.3.3. Effect of pH on the extraction

In the extraction of EMPA, it was found that the recovery of EMPA changes depending largely on the pH of the sample. Therefore, we explored the optimization of the pH of the serum sample on the extraction. Deproteinized serum to which EMPA was added (500 ng/ml) was used as sample. Serum samples were extracted in advance with dichlorometane and the aqueous solution was extracted with acetonitrile after adjusting the pH with 1 *M* hydrochloric acid or buffer solution and subsequently salted out with a saturating amount of sodium chloride. The pH of the aqueous solution was varied between 1 and 6 and recoveries of EMPA were measured. As shown in Fig. 5, lower pHs gave the better recoveries. Below pH 2, the recoveries were constant and higher than 90%. Therefore, pH was adjusted to 2 with oxalate buffer in the present study.

3.4. Derivative stability

Serum samples to which EMPA was added (500 ng/ml) were extracted according to the method described in this paper and the extracts were derivatized using MTBSTFA with 1% *t*-BDMSC. The reacting mixtures were stored both at room temperature (about 25°C) and in a freezer, and GC–MS was carried out for a period of 7 days. The *t*-BDMS derivative of EMPA displayed no significant degradation at either temperature, and the results obtained indicate that the derivatized EMPA may be safely stored without significant degradation even in the derivatized mixture of serum extracts for up to 7 days at room temperature.

3.5. GC–MS of t-BDMS derivative of EMPA

Serum samples containing EMPA (500 ng/ml) were prepared according to the method established here, and for the resultant derivatization mixture a GC–MS study was conducted in both full-scan EI and CI modes.

The resultant TIC is depicted in Fig. 6a, where the *t*-BDMS derivative of EMPA is eluted in about 7.2 min without any disturbance by impurities in the serum.

As shown in Fig. 6b, a fragmentation ion at m/z 153, due to $[M-C_4H_9-C_2H_5]^+$ formation, dominated the EI mass spectrum of the *t*-BDMS derivative of EMPA. Less intense ions at m/z 181, due to $[M-C_4H_9]$, m/z 223, due to $[M-CH_3]$, and m/z 75 were also observed. The isobutane CI mass spectrum, illustrated in Fig. 6c, was dominated by the proton adduct ion $[M+H]^+$ (pseudo-molecular ion) at m/z 239.

3.6. Quantitative analysis and resultant detection limits

To see how reliably the methods established here work, quantitative measurements by GC–MS in both

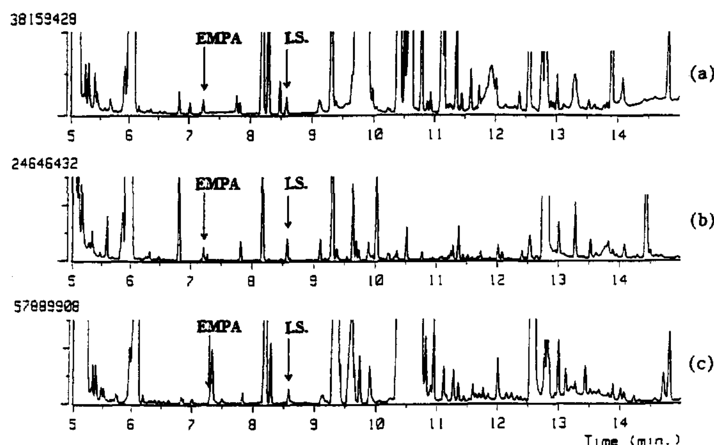

Fig. 4. Total ion chromatograms obtained for a fortified serum after sample preparation by (a) solvent extraction with acetonitrile, (b) solid-phase extraction with Sep-Pak QMA and (c) non-extraction.

full-scan and selected ion monitoring (SIM) EI modes were carried out in the presence of DPM as the I.S. for fortified serum sample in which the concentration of the added EMPA was varied. In both modes, the predominant ions (m/z 153 for the t-BDMS derivative of EMPA and 168 for DPM) were chosen for the quantitation.

As depicted in Fig. 7, the analysis showed good linearity throughout the concentration range from 50 to 500 ng/ml for EMPA in the full-scan EI mode ($y=3.06\cdot10^{-3}x-0.08$) and from 5 to 50 ng/ml for EMPA in the SIM EI mode ($y=9.76\cdot10^{-4}x+1.05\cdot10^{-3}$). The relative standard deviation obtained at the sample concentration of 50 ng/ml was 8.4% in the full-scan mode and 7.3% in the SIM mode. For the full-scan EI mode, the detection limit was 40 ng/ml, at which the three most intense ions (m/z 153, 181, 75) could be observed and the intensity of noises were about the same as that of m/z 75 in a background-subtracted mass spectrum. When the SIM EI technique, in which the ion at m/z 153 was chosen, was applied, the limit was estimated to be 3 ng/ml based on a S/N ratio of ca. 3:1. Furthermore, the limit for the full-scan CI mode was 80 ng/ml at a S/N ratio of 3:1.

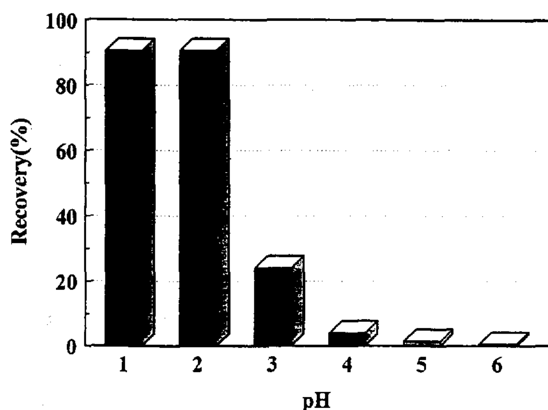

Fig. 5. Effect of sample pH on extraction.

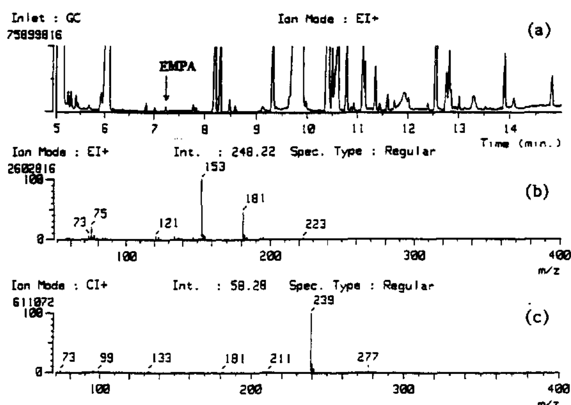

Fig. 6. (a) Total ion chromatogram (TIC) in EI mode, and (b) EI and (c) CI mass spectra obtained for a fortified serum sample.

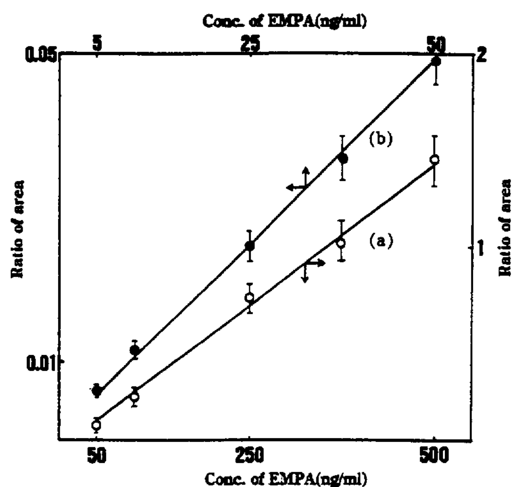

Fig. 7. Calibration curves of EMPA by GC–MS in (a) scan mode and (b) SIM mode obtained for a fortified serum sample.

4. Conclusion

In the present study, a rapid, accurate and sensitive detection method for EMPA in serum was developed using GC–MS analysis after extraction with acetonitrile and successive derivatization with MTBSTFA+1% *t*-BDMSC. In the method, the sample preparation procedure offers good recovery of EMPA, high sensitivity and reliable reproducibility of the results. Using GC–MS, EMPA in serum can be identified conveniently and accurately, leading to the unequivocal proof of the use of VX. Furthermore, the method described in this paper can also be applied to a great range of samples such as soil and environmental water, and it could become a powerful method for the reliable detection in biological and environmental samples of the use of VX.

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