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Discriminative determination of alkyl methylphosphonates and methylphosphonate in blood plasma and urine by gas chromatography–mass spectrometry after *tert*.-butyldimethylsilylation

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Abstract

A method for determining two nerve gas hydrolysis products, alkyl (ethyl, isopropyl and pinacolyl) methylphosphonates (RMPAs) and methylphosphonate (MPA), separately, in human plasma and urine samples was developed, using two different deproteinization procedures. In the first method, the plasma sample was deproteinized by adding a fourfold volume of acetonitrile, followed by passing the supernatant through a Bond Elut strong anion-exchange (SAX) cartridge [fluoride (F^-) form]. After washing the cartridge with water and methanol, the RMPAs were eluted with a 3% (v/v) solution of methanolic ammonia, and analyzed by gas chromatography–mass spectrometry (GC–MS) after *tert*.-butyldimethylsilyl (TBDMS) derivatization. The detection yields of TBDMS derivatives of RMPAs were in the range of 69 to 99%, in contrast to the poor yields obtained when only acetonitrile deproteinization pretreatment was used (yield: 13–26%). The yield of the TBDMS derivative of MPA was very low (8%), however. In a the second method, a plasma sample was deproteinized by adding a half volume of 10% (w/v) trichloroacetic acid (TCA), and the resulting supernatant was extracted with diethyl ether to remove TCA, the aqueous fraction was then passed through a Bond Elut SAX cartridge. After washing the cartridge with 0.5% (v/v) methanolic ammonia, MPA was eluted with 3% (v/v) methanolic ammonia. The detection yield of the TBDMS derivative of MPA was nearly quantitative. A pretreatment method using SAX solid-phase extraction was also developed for the cleanup of a urine sample, in which the sample was directly applied to a Bond Elut SAX cartridge, followed by elution of the RMPAs and MPA with 3% (v/v) methanolic ammonia, which were then derivatized and analyzed by GC–MS. The detection yields of TBDMS derivatives of RMPAs and MPA were in the range of 61 to 97%.

Keywords: Derivatization, GC; Alkyl methylphosphonates; Methylphosphonates

1. Introduction

Sarin gas attacks by a Japanese Cult occurred in Matsumoto city and the Tokyo subway system in 1994 and 1995, causing numerous death and injuries [1]. This presents the first cases in which chemical warfare agents were indiscriminately released against

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the general population. Sarin, soman, tabun and VX are representative nerve gases that can be unleashed in the form of a vapor or aerosol [2]. When dispersed in public places, it is usually difficult to identify such substances directly using on-site detection because of their ready decomposition by hydrolysis to alkyl methylphosphonates (RMPAs): pinacolyl (PMPA), isopropyl (IMPA), and ethyl (EMPA) methylphosphonate. RMPAs can be further hydrolyzed to methylphosphonate (MPA) (Fig. 1) [3]. Tabun is hydrolyzed via a different pathway. RMPAs and MPA are all man-made compounds, and the determination of them provides indirect proof of the use or production of nerve gases. A number of analytical methods have been reported for the determination of RMPAs and MPA: gas chromatography (GC) [4,5], capillary electrophoresis (CE) [6], ion chromatography [7], high-performance liquid chromatography (LC) [8], CE–mass spectrometry (MS) [9], and LC–MS [10] and LC–MS–MS [11]. RMPAs and MPA cannot be directly analyzed by GC–MS because of their polar and water-soluble nature. They can, however, be analyzed after derivatization to volatile compounds [4,5,12,13]. Purdon et al. [12] reported that *tert*.-butyldimethylsilyl (TBDMS) derivatives are comparatively stable against hydrolysis and are also easily prepared. Japanese forensic laboratories have mainly utilized a GC–MS method for the identification of various types of causative substances in poisoning cases. Our laboratory has adopted this TBDMS derivatization method to identify polar compounds including the hydrolysis products of nerve gases in forensic investigation on sarin gas attack [14].

In cases of nerve gas attacks, various types of environmental samples (air, soil and water) and biological samples (blood, urine, mucosa, saliva and gastric contents) are analyzed and we found a low detectability of RMPAs and MPA in evidence samples. The detection of nerve gases and their related degradation products in soil samples is very difficult, and we consolidated a series of methods for the quantitative determination of RMPAs and MPA using strong anion-exchange (SAX) solid-phase extraction (SPE) followed by GC–MS after TBDMS derivatization [15,16]. This method can be also applied to various aqueous samples, including seawater and beverages [15].

In the human body nerve gases are readily hydrolyzed to RMPAs, and become partially bound to proteins such as cholinesterases (ChEs), resulting in the formation of nerve gas adducts [17,18]. Therefore, to verify victims' exposure to a nerve gas, it is necessary to detect either hydrolysis products, RMPAs and MPA, or nerve gas protein adducts [17–19]. The level of hydrolysis products and ad-

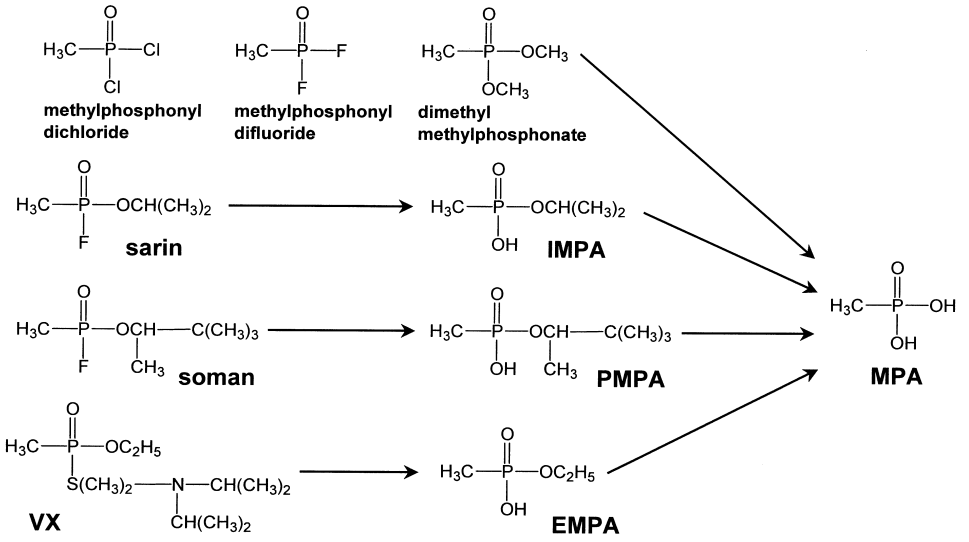

Fig. 1. Hydrolysis pathway of nerve gases and their synthetic intermediates.

ducts in biological fluids can reflect the extent of the exposure of nerve gases. Prior to the GC or GC–MS analysis, a cleanup is needed in order to separate RMPAs and MPA from complex-matrix biological samples. Several reports have appeared relative to GC–MS analysis from serum [13,20–22] and urine samples [13,20,22–24]. In this respect, only RMPAs, but not MPA, have been analyzed. The reason for this may be due to the technical difficulty in dealing with polar MPA and also that the detection of this secondary hydrolysis product was not necessary, in these cases. However, the determination of MPA is important because it is not only an RMPAs hydrolysis product, but is also a degradation product of methylphosphonyl dichloride, methylphosphonyl difluoride and dimethyl methylphosphonate, all of which are intermediates used in the synthesis of nerve gases [14].

We wish to report here on the quantitative determination of RMPAs and MPA in human plasma and urine samples. We examined the efficiency of deproteinization methods using organic solvents and acids on the recoveries of RMPAs and MPA from plasma samples. In combination with SAX-SPE, two different deproteinization methods were developed for use in GC–MS analysis after TBDMS derivatization. A simple SPE pretreatment method for urine samples is also described.

2. Experimental

2.1. Reagents

Bond Elut SAX cartridges, regular type, 500 mg/3 ml, strong anion-exchange SPE cartridges, were obtained from Varian (Harbor City, CA, USA). *N*-Methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). MPA, EMPA and PMPA were obtained from Aldrich (Milwaukee, WI, USA). IMPA was prepared as previously reported [25]. All other chemicals used were of analytical grade. All aqueous solutions were prepared with distilled, deionized water.

Stock solutions, MPA, EMPA, IMPA and PMPA were prepared in acetonitrile (1.5 mg/ml for MPA, 3.0 mg/ml for EMPA, IMPA and PMPA) and stored in tightly capped glass vials at −20 °C. Working solutions (50 ng–5 μg each, for GC–MS analysis) were prepared by diluting the acetonitrile stock solution with additional acetonitrile. Stock solutions, MPA, EMPA, IMPA and PMPA in water (3.0 mg/ml) were also prepared and stored at 4 °C. Working solutions (150 μg each, for CE analysis) were prepared by diluting the aqueous stock solution with distilled water.

Human plasma samples were prepared from outdated transfusion blood obtained from Tokyo Metropolitan Police Hospital (Iidabashi, Tokyo, Japan). Human urine samples were collected from volunteers at our institute.

2.2. Plasma sample pretreatment for solid-phase extraction

A 1-ml volume of human plasma sample was spiked with 50 μl of an aqueous solution of RMPAs and MPA (150 μg each, for CE analysis) or 25 μl of an acetonitrile solution of RMPAs and MPA (75 ng–2.6 μg each, for GC–MS analysis), vortex-stirred, and allowed to stand at room temperature for 2 h. A 4-ml volume of acetonitrile was added to the sample, and the suspension was vigorously mixed for 1 min. The mixture was centrifuged at 1500 *g* for 5 min, and the resulting supernatant was concentrated to less than 1 ml under reduced pressure at 50 °C on a Model EC-57CS centrifugal evaporator (Sakuma Seisakusho, Tokyo, Japan), and adjusted to 1 ml with water. The resulting solution was subjected to SPE treatment.

Alternatively, a 1-ml volume of human plasma sample was spiked with 50 μl of an aqueous solution of MPA (150 μg, for CE analysis) or 25–30 μl of an acetonitrile solution containing MPA (50 ng–5 μg for GC–MS analysis) and allowed to stand at room temperature for 2 h. A 0.5-ml volume of 10% (w/v) trichloroacetic acid (TCA) was added to the sample, the mixture was vortex-mixed for 1 min, and then centrifuged at 1500 *g* for 5 min. The resulting supernatant was transferred to another 10-ml screw-capped glass tube and 4 ml of diethyl ether was added. The mixture was shaken for 5 min, centrifuged at 1500 *g* for 1 min, and the upper organic layer was discarded. A 4-ml volume of diethyl ether was added to the remaining lower layer, and this

washing procedure was repeated a second time. The residual aqueous layer was heated at 45 °C for 10 min to evaporate the remaining diethyl ether. After cooling, the resultant solution was subjected to SPE treatment.

2.3. Urine sample pretreatment for solid-phase extraction

A 0.5-ml volume of human urine was spiked with a solution containing RMPAs and MPA and was vortex-stirred, and allowed to stand at room temperature for 2 h. Then the mixture was subjected to SPE treatment. For CE and GC–MS analysis, the amount of spiked solution of RMPAs and MPA were 50 μl of aqueous solution (150 μg each) and 25–30 μl of acetonitrile solution (60 ng–4 μg), respectively.

2.4. Solid-phase extraction procedure

SPE was performed according to a previously described method [15] with minor modifications. The SPE cartridge was attached to a Waters Extraction manifold (Waters, Milford, MA, USA), and conditioned with 2 ml of methanol followed by 2 ml of water, activated with 12 ml of a 1 *M* sodium fluoride solution and washed with 5 ml of water. The pretreated human plasma or urine sample (0.5–1 ml) described in Sections 2.2 and 2.3 were applied to the cartridge. For the urine sample or the plasma sample pretreated by acetonitrile deproteinization, the cartridge was washed with 3 ml of water followed by 2 ml of methanol, MPA and RMPAs were eluted with 5 ml of a 3% (v/v) methanolic ammonia solution. For the plasma sample pretreated by TCA deproteinization, the cartridge was washed with 3 ml of water, 2 ml of methanol and an additional 2 ml of 0.5% (v/v) methanolic ammonia, and MPA was eluted with 4 ml of 3% (v/v) methanolic ammonia. The eluted fraction was concentrated and dried under reduced pressure at 60 °C on a centrifugal evaporator, and then subjected to CE and TBDMS derivatization followed by GC–MS.

2.5. Capillary electrophoresis

For the quantitative determination of RMPAs, MPA and TCA, CE was performed according to a previously described method [15], using a Quanta 4000E system (Waters). CE was fitted with a fused-silica capillary (30 cm×75 μm I.D.). The voltage was set at 30 kV with a positive power supply, and the temperature was maintained at 25 °C. The electrophoresis buffer was composed of 100 m*M* boric acid containing 10 m*M* benzoate (pH 6.0). Samples were injected hydrostatically for 30 s, and indirectly detected at 254 nm.

2.6. tert.-Butyldimethylsilylation and gas chromatography–mass spectrometry

The SPE eluent was subjected to TBDMS derivatization followed by GC–MS, according to a previously described method [15]. The SPE eluent was concentrated under reduced pressure at 60 °C on a centrifugal evaporator, transferred to a 1-ml screw-capped glass vial (Nichiden Rika Garasu, type MV-7, Tokyo, Japan), and completely dried by centrifugal evaporation. A 50-μl volume of MTBSTFA and 50 μl of acetonitrile, which contained 25 μg/ml of anthracene [internal standard (I.S.) for quantitative analysis], were added, homogenized by sonication for 5 min and incubated at 60 °C for 1 h. A 1-μl volume of the mixture was applied to the GC system described below. The GC–MS system consisted of an HP 6890 series gas chromatograph combined with a HP 5973 quadrupole mass selective detector (Yokogawa Analytical Systems, Tokyo, Japan). The stationary phase was a capillary column HP-5 MS (30 m×0.25 mm I.D., 0.25 μm in thickness, Yokogawa Analytical Systems). The carrier-gas (helium) flow-rate and the splitter ratio were adjusted at 0.8 ml/min and 50, respectively. The injection port, transfer line and ion source were maintained at 250, 280 and 230 °C, respectively. Electron ionization (ionization energy 69.9 eV, ionization current 34.6 μA) was used as the ionization mode. The oven temperature was controlled by a program [starting at 90 °C (1 min hold), increasing to 290 °C at 20 °C/min (5 min hold)]. The acquisition mass range was 50–550, and the scan rate was 0.8 scans/s. Acquisition was started 4 min after sample injection. Total ion chromatogram of standard TBDMS derivatives of RMPAs and MPA is shown in Fig. 2A. The extracted ion chromatograms were obtained at *m*/*z* 153 ($[M-57\ (tert.\text{-butyl})-(\text{alkyl})+H]^+$) for the

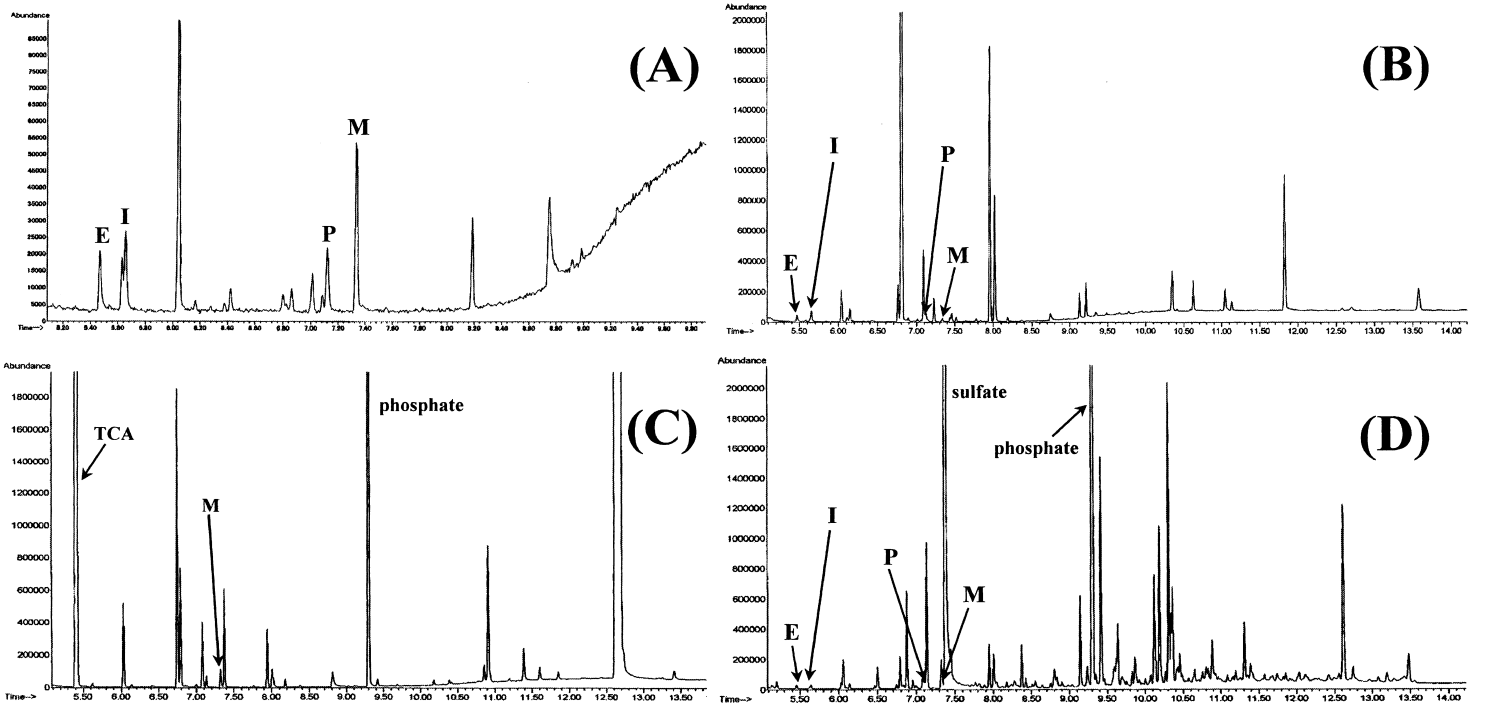

Fig. 2. Total ion chromatogram of standard TBDMS derivatives of RMPAs and MPA (A), serum (B and C) and urine (D) samples. (A) A 50 μl acetonitrile solution containing 25 μg/ml of RMPAs and MPA, 25 μg/ml of anthracene (I.S.) and 50 μl of MTBSTFA was reacted at 60 °C for 1 h, and the resultant reaction mixture was subjected to GC–MS as described in the Experimental section. (B) Serum (1 ml) spiked with 2.5 μg of RMPAs and MPA was pretreated by deproteinization with acetonitrile and SPE. (C) Serum (1 ml) spiked with 3.0 μg of MPA was pretreated by deproteinization with TCA and SPE. (D) Urine (0.5 ml) spiked with 2.5 μg of RMPAs and MPA was pretreated by SPE. Detailed conditions are described in the Experimental section. Peaks of TBDMS derivatives of EMPA (E), IMPA (I), PMPA (P), MPA (M), TCA, sulfate, and phosphate are shown on the chromatograms.

TBDMS derivatives of EMPA, IMPA and PMPA, m/z 267 ($[M-57$ (*tert*.-butyl)$+H]^+$) for the TBDMS derivative of MPA, m/z 178 for the I.S., respectively. The analyte concentrations were obtained from the peak area ratios of the analytes to I.S., using a standard calibration curve, prepared by plotting the peak area ratios against the designated concentration of the analytes (0.2–30 μg/ml in TBDMS reaction mixture, triplicate). The standard calibration curve was prepared in every series of analyzing samples by GC–MS [25].

3. Results and discussion

3.1. Deproteinization of human plasma samples for the extraction of RMPAs and MPA

Blood plasma contains high concentration of proteins which interfere with a GC–MS analysis. Moreover, the adsorption of RMPAs and MPA to proteins prevents their quantitative recovery. Therefore, a deproteinization step is essential. Deproteinization using an organic solvent was initially examined. Human plasma spiked with RMPAs and MPA were mixed with acetonitrile, a polar organic solvent, and the supernatant was analyzed by CE. CE was employed to quantify the true amount of RMPAs and MPA in the aqueous biological samples. As shown in Table 1B, the recovery of MPA was low (31%) compared to the RMPAs (77–90%). MPA is more polar than RMPAs. RMPAs have acidic pK_a values, and MPA has two pK_a values of 2.2 and 7.7 [26]. It is assumed that part of the precipitate contained protein-bound MPA, since protein denaturation using an organic solvent failed to completely disrupt the binding interaction between MPA and proteins. The detection yields of both RMPAs and MPA by GC–MS were low (<26%, Table 1B). This can be attributed to the suppressive effect of divalent cations and neutral compounds in the plasma on TBDMS derivatization, as was observed in the case of soil samples [25,27,28]. Further purification using SAX-SPE is necessary to achieve satisfactory yields in the TBDMS derivatization of RMPAs.

Acetone and methanol were also examined as

Table 1
Recoveries and detection yields of alkyl methylphosphonates and methylphosphonate in human plasma

	(A) Non-pretreatment,	(B) Deproteinization with acetonitrile		(C) Deproteinization and SPE	
	GC–MS[a]	CE[b]	GC–MS[c]	CE[d]	GC–MS[e]
PMPA	0.15±0.27	77.4±11.2	25.6±4.1	79.9±7.1	98.8±5.4
IMPA	0.27±0.16	89.9±9.1	13.8±0.53	74.5±1.2	71.3±3.9
EMPA	0.32±0.16	79.3±7.3	13.1±0.95	69.4±2.8	69.3±5.3
MPA	0.18±0.13	30.9±5.0	1.58±0.25	20.4±3.7	7.5±2.1

[a] Human plasma containing MPA and RMPAs (each 6 μg), was directly treated by *tert*.-butyldimethylsilylation, and analyzed by GC–MS. The detection yield is defined as the percentage value of the peak area ratio of TBDMS derivatives to the internal standard, compared to the value for acetonitrile solution containing the same concentrations of MPA and RMPAs. Values represent an average of three determinations±standard deviation.

[b] Human plasma containing MPA and RMPAs (each 150 μg) was pretreated by deproteinization with acetonitrile, and analyzed by CE. The recovery is defined as the percentage value of the peak area of MPA and RMPAs, compared to the value for an aqueous solution containing the same concentrations of MPA and RMPAs. Values represent an average of three determinations±standard deviation.

[c] Human plasma containing MPA and RMPAs (each 2 μg) was pretreated by deproteinization with acetonitrile, subjected to *tert*.-butyldimethylsilylation and analyzed by GC–MS. The detection yield is defined as the percentage value of the peak area ratio of TBDMS derivatives to the internal standard, compared to the value for acetonitrile solution containing the same concentrations of MPA and RMPAs. Values represent an average of three determinations±standard deviation.

[d] Human plasma containing MPA and RMPAs (each 150 μg), was pretreated by deproteinization with acetonitrile and SPE, and analyzed by CE. The recovery is defined as the percentage value of the peak area of MPA and RMPAs, compared to the value for an aqueous solution containing the same concentrations of MPA and RMPAs. Values represent an average of three determinations±standard deviation.

[e] Human plasma containing MPA and RMPAs (each 3 μg), was pretreated by deproteinization with acetonitrile and SPE subjected to *tert*.-butyldimethylsilylation and analyzed by GC–MS. The detection yield is defined as the percentage value of the peak area ratio of TBDMS derivatives to the internal standard, compared to the value for acetonitrile solution containing the same concentrations of MPA and RMPAs. Values represent an average of eight determinations±standard deviation.

deproteinization solvents. The recovery values of RMPAs and MPA were similar to those in acetonitrile pretreatment (data not shown). However, the supernatant was slightly turbid. Therefore, acetonitrile was chosen as a deproteinization solvent for pretreating plasma for extracting RMPAs.

Deproteinization with strong acid was also examined. Our laboratory had adopted a deproteinization procedure using perchloric acid ($HClO_4$) for the determination of RMPAs and MPA in blood samples by TBDMS GC–MS [14], and we experienced low reproducibility in this procedure. Instead of blood samples, a plasma sample was examined for $HClO_4$ deproteinization. A 0.5-ml volume of an aqueous solution of 3.5% (v/v) $HClO_4$ was added to 1 ml of a human plasma sample spiked with RMPAs and MPA, and the solution was vortex-mixed, followed by neutralizing the supernatant with 0.5 *M* potassium hydroxide to remove excess perchlorate ion by the precipitation as potassium perchlorate. The recoveries of RMPAs and MPA in the supernatant were quantitative (as evidenced by CE), but the detection yields by GC–MS were not reproducible (<30% for RMPA, 50–150% for MPA). Further pretreatment using SAX-SPE was examined (Section 3.2).

We also examined the efficiency of TCA deproteinization on the quantitative extraction of RMPAs and MPA from a plasma sample. A 0.5-ml volume of 10% (w/v) TCA was added to 1 ml of plasma, and the resulting mixture was vortex-mixed and centrifuged. RMPAs and MPA were quantitatively recovered in the supernatant fraction (verified by CE, data not shown). However, neither the TBDMS derivatives of RMPAs nor MPA were detected, because the large quantity of TCA interfered with the TBDMS derivatization. In order to remove the TCA included in the deproteinized fraction, diethyl ether extraction was examined and the quantity of the remaining TCA in deproteinized fraction was determined by CE analysis. About 33 mg of TCA was estimated to be present in 1 ml of the deproteinized supernatant, and nearly 70% of the TCA (23 mg) was removed by extraction with diethyl ether. The pK_a value of TCA is 0.7 at 25 °C [29], and the, pH values of the TCA deproteinized supernatant before and after ether extraction were less than 1 and about 4, respectively. In spite of the very low pH conditions, MPA was not extracted by ether during the TCA extraction (Table 2A). In contrast, RMPAs, especially PMPA, were transferred to the ether layer, and the recoveries of RMPAs in the extracted aqueous fraction were low.

Table 2
Recoveries and detection yields of methylphosphonate in human plasma

	(A) Deproteinization with TCA,	(B) Deproteinization and SPE	
	CE[a]	CE[b]	GC–MS[c]
MPA	100.9±5.2	79.4±4.1	114.7±9.0

[a] Human plasma containing MPA (150 μg), was pretreated by deproteinization with TCA, and analyzed by CE. Value represents an average of three determinations±standard deviation.

[b] Human plasma containing MPA (150 μg), was pretreated by deproteinization with TCA and SPE, and analyzed by CE. Value represents an average of eight determinations±standard deviation.

[c] Human plasma containing MPA (4 μg) was pretreated by deproteinization with TCA and SPE, subjected to *tert*.-butyldimethylsilylation and analyzed by GC–MS. Value represents an average of eight determinations±standard deviation.

3.2. Solid-phase extraction of the deproteinized fraction of human plasma samples

It was impossible to quantitatively determine RMPAs and MPA by TBDMS GC–MS, for the deproteinized plasma fraction examined in Section 3.1, even though quantitative recoveries were achieved (verified by CE). Therefore, we investigated the usefulness of purifying the RMPAs and MPA by SAX-SPE. We adopted a cleanup pretreatment method using a Bond Elut SAX cartridge, which has been successfully applied to soil and other forensic samples [15].

Acetonitrile deproteinization enabled the quantitative extraction of RMPAs but not MPA from a human plasma sample (Table 1B). In contrast, the detection yields by TBDMS GC–MS were low for RMPAs. We applied the above-mentioned SAX-SPE procedure for the cleanup of the acetonitrile-deproteinized fraction. As a result, many unknown peaks were observed on TBDMS GC–MS which interfered with the detection of the TBDMS derivatives (data not shown). The reason for this severe interference is as follows. In our original SAX procedure [15], the cartridge was washed with only 5 ml of water after loading the samples. This amount

is inadequate for completely removing interferents during the washing step. Therefore, instead of washing 5 ml of water, the cartridge was washed with a solution of 3 ml of water and 2 ml of methanol. Additional methanol washing could efficiently remove hydrophobic interferents remaining in the deproteinized fraction, and the GC–MS detection was significantly improved. Both the recoveries and the detection yields of RMPAs were in excess of 69% (Table 1C). Human plasma contains large quantities of inorganic anions: 10–19 μg of sulfate (SO_4^{2-} and HSO_4^-) and 32–51 μg of phosphate ($H_2PO_4^-$ and HPO_4^{2-}) per ml [30]. TBDMS-derivatives of phosphate and sulfate were observed as peaks in GC–MS of the acetonitrile deproteinization-SAX-SPE fraction, but they did not interfere with the detection of TBDMS derivatives of the RMPAs (Fig. 2B). Accordingly, a pretreatment method involving acetonitrile deproteinization combined with SAX-SPE appears to be suitable for the quantitative cleanup of RMPAs from plasma samples. Using this method, the detection limits of RMPAs ($S/N=3$ on the extracted ion chromatogram of m/z 153) were 30 ng per 1 ml of plasma, and the calibration curves were linear in the concentrations range of 75 ng to 2.6 μg per ml of plasma. The above mentioned determination level corresponds to the toxic level (43–136 ng/ml) of IMPA in the case of sarin gas attack [31]. If splitless injection or selected ion monitoring mode is adopted in GC–MS analysis, the exposure level can be determined.

We examined the efficiency of the SAX-SPE method on the removal of $HClO_4$ from the deproteinized plasma fraction. The supernatant was passed through the Bond Elut SAX cartridge, and RMPAs and MPA were eluted with 5 ml of 3% (v/v) methanolic ammonia. Although RMPAs and MPA were quantitatively recovered from the plasma samples, as evidenced by the CE method, the TBDMS derivatives of RMPAs and MPA were not detected by GC–MS. This is attributed to interference by a huge peak derived from unknown compounds. The same interference was observed for the plasma blank solution, and it is assumed that the surface of the SPE cartridge was partially decomposed by $HClO_4$ and the unknown compounds were eluted with methanolic ammonia, thus interfering with the detection of the TBDMS derivatives. Accordingly, $HClO_4$ deproteinization is unsuitable for the cleanup of RMPAs and MPA.

TCA deproteinization enabled the quantitative extraction of MPA but not RMPAs from human plasma samples. In contrast, the detection yield for MPA by TBDMS GC–MS was low. As a result, the SAX-SPE procedure was adopted for the cleanup of the TCA deproteinization fraction. TCA has a weaker ionic strength than MPA, and MPA is, therefore, retained more strongly by the SAX-SPE cartridge. To remove residual TCA from the ether-washed fraction, the concentration of ammonia and the volumes of the washing and eluting solution were modified. TCA was removed by elution with 2 ml of 0.5% (v/v) methanolic ammonia, and MPA was nearly quantitatively eluted with 4 ml of 3% (v/v) methanolic ammonia (Table 2B). On GC–MS of a TBDMS derivatized solution of the SAX-SPE fraction from TCA deproteinized plasma, three main peaks appeared, which were derived from TBDMS-derivatized TCA, TBDMS-derivatized phosphate and TBDMS-derivatized unknown compounds (Fig. 2C), but these peaks did not overlap with the peak corresponding to the TBDMS derivative of MPA. The detection limit of MPA ($S/N=3$ on the extracted ion chromatogram of m/z 267) was 33 ng per ml of plasma. The calibration curve was linear in the concentration range of 50 ng to 5 μg per ml of plasma. The detection yield of MPA was quantitative (Table 2B). Somewhat higher yield value (115%) may be ascribed to the interference from background contaminants. Accordingly, a pretreatment method including TCA deproteinization, ether extraction combined with SAX-SPE is suitable for the cleanup of MPA from plasma samples.

3.3. Pretreatment of urine samples

We attempted to detect TBDMS derivatives of RMPAs and MPA directly from the urine samples, but under the dry-up conditions used for drying, the urine sample could not be dissolved in the TBDMS reagent. Therefore, a cleanup method using SAX-SPE prior to GC–MS analysis was examined. For urine samples, a deproteinization procedure was not necessary prior to SPE cleanup. The Bond Elut SAX cartridge used in this investigation has an anion-exchange capacity of 350 μequiv. [30], and all the

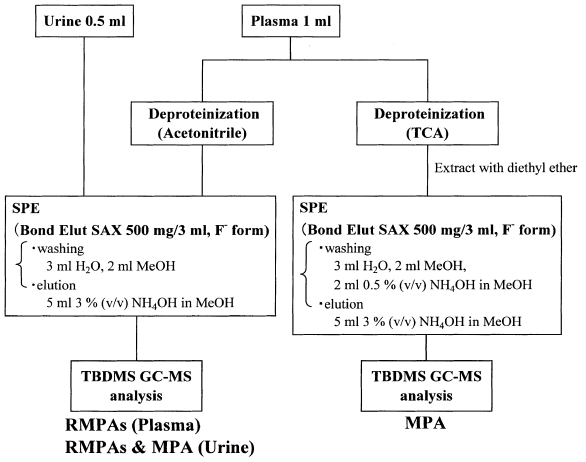

Fig. 3. The schemes showing the methodology used in sample preparation.

anion compounds present in 0.5 ml of the urine sample were retained on the SPE cartridge. The urine sample was directly applied to SPE, under the same conditions as were used in the acetonitrile deproteinization procedure. As shown in Table 3A, RMPAs and MPA were nearly quantitatively recovered (73–88%), as verified by CE, and the TBDMS derivatives of RMPAs and MPA in urine were quantitatively detected. Various types of organic and inorganic compounds in urine samples were detected by GC–MS as TBDMS-derivatives. In particular, two main peaks corresponding to TBDMS-derivatized sulfate and phosphate were observed (Fig. 2D). In humans, the volume of urine excreted is in the range of 1000 to 1600 ml/day, and the quantities of inorganic sulfate and phosphate in human urine are 1.4–3.3 and 1.9–2.3 g/day, respectively [30]. TBDMS-derivatized sulfate was eluted close to the elution position of TBDMS-derivatized MPA, and the base peaks of TBDMS-derivatized sulfate and MPA are m/z 269 and 267, respectively. Accordingly, the detection yield of MPA was influenced by the presence of sulfate, and, as a result, so the detection limit of MPA ($S/N=3$ on the extracted ion chromatogram of m/z 267) was 700 ng per ml of urine. The detection limits of RMPAs were 60 ng per ml of urine. The calibration curve was linear in the concentration range of 140 ng–5.6 μg (for RMPAs) and 700 ng–5.5 μg (for MPA) per ml of urine.

Table 3
Recoveries and detection yields of alkyl methylphosphonates and methylphosphonate in human urine

	(A) SPE and CE[a]	(B) SPE and GC–MS[b]
PMPA	–[c]	75.6±10.4
IMPA	75.9±2.6	60.6±8.2
EMPA	87.5±1.7	70.3±8.5
MPA	73.2±1.4	96.8±15.9

[a] Human urine containing MPA and RMPAs (each 150 μg), was pretreated by SPE and analyzed by CE. Values represent an average of three determinations±standard deviation.

[b] Human urine containing MPA and RMPAs (each 1.5 μg) was pretreated by SPE, treated by *tert*.-butyldimethylsilylation, and analyzed by GC–MS. Values represent an average of eight determinations±standard deviation.

[c] Not determined.

4. Conclusion

A quantitative determination of both RMPAs and MPA in plasma and urine by GC–MS after TBDMS derivatization was established, and is shown schematically in Fig. 3. RMPAs in plasma samples were determined after acetonitrile deproteinization followed by SAX-SPE. MPA in the plasma sample was determined after TCA deproteinization, ether extraction followed by SAX-SPE. RMPAs and MPA in urine sample were determined after SAX-SPE. This is the first report describing the quantitative determination of MPA in plasma samples by TBDMS GC–MS.

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