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Journal of Chromatography B, 695 (1997) 237–244

JOURNAL OF CHROMATOGRAPHY B

Method for the analysis of the methylphosphonic acid metabolites of sarin and its ethanol-substituted analogue in urine as applied to the victims of the Tokyo sarin disaster

Masayasu Minami[a,*], Da-Mei Hui[a], Masao Katsumata[a], Hirofumi Inagaki[a], Camille A. Boulet[b]

[a]*Department of Hygiene and Public Health, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113, Japan*
[b]*Detection and Identification Section, Defense Research Establishment, Suffield, PO Box 4000, Medicine Hat, Alb. T1A 8K6, Canada*

Received 10 June 1996; revised 13 March 1997; accepted 24 March 1997

Abstract

An analysis method for the methylphosphonic acid metabolites of sarin in urine using trimethylsilyl derivatization and flame photometric detection is described in this report. Authentic reference standards of isopropyl methylphosphonic acid (IMPA) and ethyl methylphosphonic acid (EMPA) as well as methylphosphonic acid were employed to estimate the concentration in human urine. A sample pretreatment procedure was developed for urine using a column of cation-loaded ion-exchange resins (Ag^{+}-, Ba^{2+}- or H^{+}-Dowex) and adjusting the pH of the eluate from the column to 3.75–3.85 improved recovery of the target compounds. The eluate was evaporated to dryness under vacuum prior to trimethylsilylation, to remove water and any hydroxy- or amino-carrying volatile substances. The sarin metabolites, because of their low volatility, were concentrated and could be derivatized for analysis. The use of synthesized authentic sarin and ethylsarin metabolites, i.e., IMPA and EMPA, made it possible to establish the necessary sample pretreatment procedures for derivatization and gas chromatography–flame photometric detection (GC–FPD) analysis. The detection limits were 0.025 ppm both for EMPA and IMPA, and 0.625 μM for MPA, respectively. This method can be useful for estimating the exposure level to sarin by assaying the metabolites in urine and it is applicable to a large numbers of samples. © 1997 Elsevier Science B.V.

Keywords: Sarin; Methylphosphonic acid

1. Introduction

On the 20th of March, 1995, our medical school hospital accepted 30 acutely ill patients. These patients were exposed to the chemical warfare agent sarin, as a result of a terrorist attack on the Tokyo subway system. Four of these patients were critically ill from the exposure. To estimate the possible sarin exposure levels of these patients for better treatment and for evaluating the risk of delayed neurotoxic symptoms [1], we required a simple analysis method for the sarin metabolites in biological fluids. The method needed to be relatively rapid, sensitive and simple, in order to be able to deal with a large numbers of urine samples obtained during the clinical monitoring of the patients. A gas chromatograph equipped with flame photometric detector was selected for the detection of trimethylsilyl derivatized

*Corresponding author.

PII S0378-4347(97)00203-X

phosphorous compounds. To develop this method, we synthesized the likely metabolites of sarin and ethylsarin, isopropyl and ethyl methylphosphonic acids (IMPA and EMPA, respectively), as standards. In this method, we took advantage of the low volatility of the sarin and ethylsarin phosphonic acid metabolites and of the selectivity of the gas chromatography–flame photometric detection (GC–FPD) system for phosphorus-containing substances [2–4].

Gas chromatography, or thermospray liquid chromatography with mass spectrometric detection (GC–MS or LC–MS) is often used for the analysis of organophosphorus chemical warfare agents and their degradation products in soil and environmental water [5–9] and in food [10,11]. Shih et al. [12,13] applied a GC–MS method to the monitoring of organophosphorus warfare agents in biological samples from experimental animals. The recovery efficiency and selectivity for the target compounds can be affected by other substances in the sample [14] and these contaminants also can affect the chromatographic resolution and performance, especially in the case of GC–MS equipment, resulting, for example, in difficulties in stabilizing the baseline of the chromatogram after long-term use. The sensitivity of the GC–MS system is excellent, but the selectivity for organophosphorus compounds is not always sufficient to detect those chemicals in biological materials, which contain various kinds of interferents. The effect of these interferents and contaminants can be a difficult problem when attempting to analyse exceptionally large numbers of biological samples (as collected from the patients of the Tokyo sarin disaster), because of the specificity of and selectivity to the organophosphorus compounds [2–4].

2. Experimental

2.1. Reagents

The metabolites of sarin (isopropyl methylphosphonofluoridate) and of ethylsarin (ethyl methylphosphonofluoridate), i.e., IMPA and EMPA, both of which are viscous fluids at room temperature, were synthesized at the Defence Research Establishment Suffield (Sawyer et al. [14]). IMPA and EMPA were synthesized by treating the corresponding alkyl methylphosphonochloridates with water. The products were distilled and analyzed by proton [15] and phosphorus NMR [16] for purity. Purity was greater than 95%. Methylphosphonic acid (MPA) and ethylphosphonic acid (EPA) were purchased from Aldrich (Milwaukee, WI, USA). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from GL Sciences (Tokyo, Japan). Dowex 50WX2 resins were from Dow Chemicals (Midland, MI, USA). Other reagents used were of analytical grade, unless otherwise indicated.

Stock solutions of EMPA and IMPA were prepared by adding 10 μl of the pure substance to 10 ml of water, giving a concentration of $1 \cdot 10^3$ ppm (v/v). MPA (10 m*M*) and 17.7 m*M* EPA were dissolved in water and used as the stock solutions. The stock solutions of EMPA, IMPA or MPA were diluted 200–1000 fold in water for use as the authentic standard, 1–5 μl of which was spiked into the sample for testing the recovery and for other purposes.

2.2. Apparatus and chromatographic conditions

The GC apparatus (GC9A, Shimadzu, Kyoto, Japan) was equipped with a split–splitless inlet, a flame photometric detector (FPD-9, Shimadzu) with a filter with an absorption peak at 526 nm, a data integrator (CR5A, Shimadzu) and a fused-silica column (CBPI-M50-0.25, Shimadzu; 50 m×0.25 mm I.D.).

The carrier gas (helium) had a flow-rate of 2.0 ml/min. The split mode was set at a ratio of 1:20. The operating temperature conditions were: injection port temperature, 150°C; detector temperature, 250°C. The oven temperature was programmed as follows: time 0, 100°C, to 200°C in 5 min, then at 200°C for the next 5 min and to 290°C in 5 min, with the temperature being maintained for 5 min.

A Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA) was used for removing water and volatile substances from the sample.

Pooled urine samples were obtained from ten laboratory staff (aged 22 to 57 years, mean 28.4 years) who had no abnormal physical or clinical laboratory signs (urine analysis, liver function tests,

blood cell counts) at the annually conducted health examination and who were not involved in the sarin disaster. Urine samples were pooled to obtain a representative sample for spiking experiments. The same pooled urine was also used for recovery experiments by spiking it with appropriate amounts of the standards of the organophosphorus compounds. Samples from two of the sarin-exposed patients were assayed serially from the day of exposure until seven days after the accident, for time course analyses of EMPA, IMPA and MPA levels in urine after exposure. The sampling time was as indicated in Section 3 (Fig. 5). The samples were stored at −20°C until use.

2.3. Column pretreatment and trimethylsilylation conditions

The sample treatment procedures after the column processing step were as shown in Fig. 1. After the Speed Vac evaporation of the column-treated sample, the methylphosphonic acids remained in the vial, because of their high boiling temperature. The reagent for trimethylsilylation, comprising ten volumes of BSTFA and one volume of TMCS, was mixed immediately before use. A 200-μl volume of the reagent for trimethylsilylation was added to the column-treated and dried sample in a new capped vial and the mixture was heated at 100°C for 1 h. A 1-μl volume of the trimethylsilylated sample was injected into the GC–FPD apparatus. The recovery efficiency of the spiked standards in urine was determined using EPA as an internal standard.

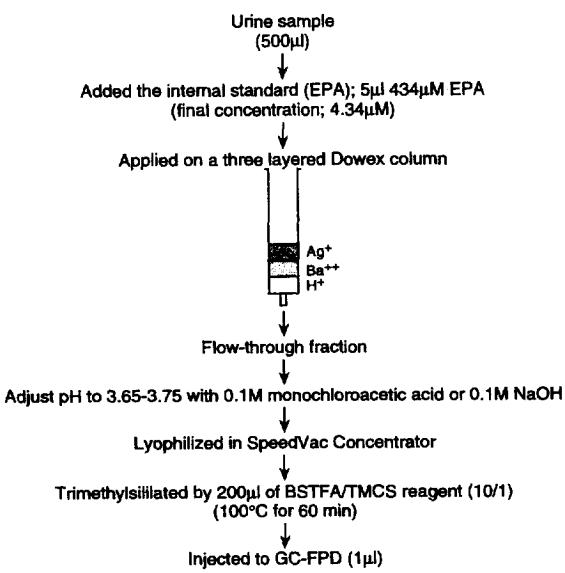

Fig. 1. Schematic representation of the pretreatment procedure for biological samples.

3. Results and discussion

3.1. Pretreatment of urine samples

The following ion-exchange column treatment procedure was devised for the sample to reduce the quantity of precipitate in solution prior to derivatization and GC analysis. The recovery test was used to test the efficiency of the pretreatment process. Dowex 50WX2 resins were washed consecutively with alkaline (0.1 *M* NaOH), acid (0.1 *M* HCl) and water and loaded with silver (Ag^+; using 0.1 *M* $AgNO_3$) or barium (Ba^{2+}; using 0.1 *M* $BaCl_2$) ions for the removal of Cl^-, or SO_4^{2-} ions, respectively, in the pretreatment procedure for urine samples. The resin pile were to adsorb Cl^- and SO_4^{2-} ions and urine pigments to Ag^+-, Ba^{2+}- and H^+-loaded layers, respectively. The procedures after the column treatment step were the same as shown in Fig. 1.

The biological samples contain large quantities of OH- and NH_2-carrying substances, such as water, volatile alcohols and volatile amines, which consume the trimethylsilylation reagent and hinder the efficiency of the reaction. These substances could be removed under reduced pressure by the Speed Vac system. However, another problem originated from a large precipitating mass in the reaction vial appearing after the Speed Vac desiccation procedure. This precipitate appeared to inhibit the trimethylsilylation reaction and reduce the derivatization efficiency of methylphosphonic acid esters trapped in the precipitated mass. We, therefore, used a three-layered Dowex column to remove as much precipitate as possible from the sample. Black et al. [10] used Dowex resins for the separation of MPA, but they did not utilize the resins for removing these reaction-disturbing substances. A solid-phase extraction cartridge for the pretreatment of biological samples was reported by Shih et al. [12], but in our hands, MPA

and EPA could not be adsorbed onto the cartridge. We, therefore, abandoned the solid-phase extraction procedure in favour of the ion-exchange method.

3.2. Optimization of trimethylsilyl derivatization

A 1-μl volume of 5 m*M* chloroacetate–NaOH buffer, pH 3.85, was added to the authentic standards, as shown in Table 1, and the vial content was evaporated to dryness under vacuum before trimethylsilylation. If one uses only BSTFA for trimethylsilylation [5] of biological samples, the recovery of trimethylsilylated compounds from the samples is not always considered to be satisfactory. Thus, we added TMCS to BSTFA to increase the efficiency of the derivatization reaction, as indicated by the chromatographic peak height, and the ratio of TMCS to BSTFA was optimized. It was found that a ratio of 10:1 BSTFA–TMCS was suitable for these derivatization reactions (Table 1).

3.3. Effect of pH on trimethylsilylation

First, we have to take the reactivity of water to TMS reagent [17,18] into consideration. This problem could be overcome using the desiccation procedure with a Speed Vac. The methylphosphonic acid ester was thought to be easily trimethylsilylated, when it was kept in a barely dissociable, or non-ionized, state before the pretreatment [14,18].

Table 1
Experiment to find the optimal composition of reagents for trimethylsilylation

BSTFA–TMCS ratio	Relative peak height			
	EMPA	IMPA	MPA	EPA
1:0	1.000	1.000	1.000	1.000
4:1	0.687	0.594	0.682	0.366
5:1	0.750	0.595	1.611	0.695
1:1	0.370	0.300	0.655	0.252
9:1	1.032	0.867	1.330	0.798
10:1	1.023	0.961	0.941	1.179

The mixture of alkyl phosphonic acids, containing EMPA (FC in SwS; 0.25 ppm; v/v), IMPA (FC in SwS; 0.25 ppm; v/v), MPA (FC in SwS; 3.13 m*M*) and EPA (FC in SwS; 4.35 m*M*) was used for testing the efficacy of trimethylsilylation. The peak height was the indicator of the efficacy and a 1:10 ratio of the TMS reagents (TMCS–BSTFA) seemed satisfactory. If the proportion of TMCS is larger compared to BSTFA, the relative peak height decreases. The effect seems more conspicuous for EPA than for the other phosphonates.

At first, we thought that we could ignore the ionic dissociation occurring in sample solution before the desiccation process [17,19–21], because the liquid phase of the specimen was evaporated to dryness before trimethylsilylation. However, we found in the preliminary pilot study that the pH of the sample solution before the evaporation procedure has a considerable effect on trimethylsilylation, even after desiccation using a Speed Vac. Thus, we have to take the ionic dissociation of the substance of interest in the hydrophilic sample solution into account, even when the liquid phase of the sample has been evaporated to dryness before trimethylsilylation. Because the pH-dependent ionic dissociation constant of the methylphosphonic acids lose dissociating characteristic into ions in more acidic, or alkaline solution than the pK_a of the substance according to the principle of acid–base equilibrium [22], the degree of trimethylsilylation was investigated under different pH conditions of the sample before evaporation of the mixture of authentic standard solutions, which were diluted from the respective stock solutions as described in Section 2; 5 μl EMPA [the final concentration in the solution spiked with the substance (FC in SwS); 0.5 ppm, v/v], 5 μl IMPA (FC in SwS; 0.5 ppm, v/v), 5 μl MPA (FC in SwS; 3.13 μ*M*) and 5 μl EPA (FC in SwS; 4.34 μ*M*). These were dissolved in 1 ml of solutions with different pH values. The relation between the peak height of the chromatogram and the pH value of the solution is shown in Fig. 2. The efficiency of trimethylsilylation corresponds to the peak height, which is highest at about pH 3.8 and lowest at about pH 3.1 and 5.1 (Fig. 2).

3.4. Effect of urea and glucose on trimethylsilylation

Almost all biological fluids, such as urine and blood, contain a small quantity of urea and glucose. These substances hinder analysis in trimethylsilylation and could not be totally removed from the

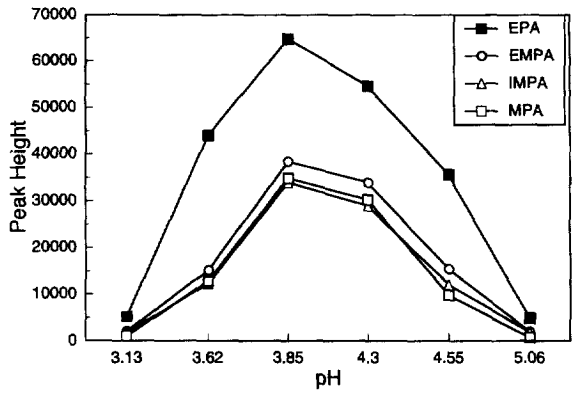

Fig. 2. Effect of pH on trimethylsilylation. The dissociating characteristics of phosphonic acid esters and phosphonic acids into ions in hydrophilic solution disturbs the trimethylsilylation reaction, even in a degassed and desiccated state. Each test solution consists of 5 μl of the buffer solution and 1 ml of degassed water. A 5-μl volume of the respective standard solutions, the mixed external standard solution consisting of 5 μl of EMPA [final concentration in the solution spiked with the substance (FC in SwS); 0.5 ppm, v/v], 5 μl of IMPA (Fc in SwS; 0.5 ppm, v/v), 5 μl of MPA (FC in SwS; 3.13 μ*M*) and 5 μl of the internal standard, EPA (FC in SwS; 4.35 μ*M*) were spiked in 1 ml of the buffered solution.

sample by treatment of the column. We investigated the tolerable level of these contaminants in the sample for trimethylsilylation. A 1-ml volume of buffer (5 m*M* chloroacetic acid–NaOH, pH 3.85), the pH of which gave the maximum peak height, as mentioned in Section 3.3, was used for testing the effect of glucose, or urea, on trimethylsilylation. The test solution consisted of (1) 500 μl of a mixture composed of 5 μl EMPA (FC in SwS; 0.5 ppm,v/v), 5 μl IMPA (FC in SwS; 0.5 ppm, v/v), 5 μl MPA (FC in SwS; 3.13 μ*M*) and 5 μl EPA (FC in SwS; 4.34 μ*M*) in the buffer and (2) 500 μl of the respective substance in a final concentration of 1–5 g/l glucose, or 5–50 g/l urea, dissolved in the buffer. There were no detectable differences among the peak heights, which was thought to be an indicator of the sensitivity, if we adjusted the pH 3.82–3.85 in the sample with the possible trimethylsilylation interferents. The problem associated with urea and glucose could also be overcome using EPA as the internal standard and diluting the urine to give urea or glucose concentrations in the 0–100 or 0–5 g/l region, respectively.

Table 2
Recovery of spiked authentic standards in urine

	Concentration	Urine		
		mean	S.D.	C.V.
EMPA	0.025 ppm	92.4	4.47	4.84
	0.05 ppm	92.3	3.64	3.94
	0.075 ppm	91.2	5.50	6.03
IMPA	0.025 ppm	97.1	0.89	0.92
	0.05 ppm	96.1	4.27	4.44
	0.075 ppm	90.8	4.61	5.80
MPA	0.025 μ*M*	98.3	2.28	2.32
	0.05 μ*M*	93.9	4.34	4.65
	0.075 μ*M*	95.6	5.42	5.67

The mean and S.D. in each cell of the table were calculated from 10–12 values. S.D. indicates standard deviation.

3.5. Recovery efficiency from control urine samples after sample pretreatment

The recovery efficiency of authentic samples in control urine was examined. Final concentrations (FC) of (1) 0.025, 0.05 or 0.075 ppm (v/v, μl/ml) of EMPA; (2) 0.025, 0.05 or 0.075 ppm (v/v or μl/ml) of IMPA; (3) 0.625, 1.25 or 1.88 μ*M* MPA, as the external standard; and (4) 5 μl of 434 μ*M* EPA, as the internal standard, were spiked into 500 μl of the pooled control urine (the FC of EPA; 4.34 μ*M*). The spiked control urine was passed through the column after adding 50 μl of 1 *M* chloroacetic acid to it and the pH of the eluate from the column was adjusted to 3.6–3.85. The same spiking procedure was also carried out with authentic substances into 500 μl of 5 m*M* chloroacetic acid–NaOH buffer, pH 3.85, as a control. The GC peak height of the spiked standard was compared with that of the standards in the control urine after column treatment by calculating the ratio (%) of the chromatographic peak height [(the height relevant to the biological sample/that of the buffer)×100=the recovery rate (%)] for the respective standard. The recovery rate was 90.9–98.3% for urine samples. The coefficient of variance of the recovery rate was 0.92–6.03% (Table 2).

3.6. Calibration curves for control urine and spiked samples

The same procedures as cited in Fig. 1 were employed for comparing the calibration curves derived from the GC peak height value of the spiked standard in the pooled urine which was passed through the column with those of the spiked standard in the control buffer (5 m*M* chloroacetic acid, pH 3.85), where the column treatment step was omitted and the subsequent sample processing steps were employed. There is little difference between the calibration curves derived from the biological material spiked with the standards and the curve from the control buffer (pH 3.85) spiked with the standard substances (Fig. 3), indicating that the salts, amines and alcohols in the biological material did not appear to reduce the regression coefficient of the curve. This demonstrated that the problem associated with contaminating substances was overcome by the pretreatment procedure and by the use of the internal standard, EPA.

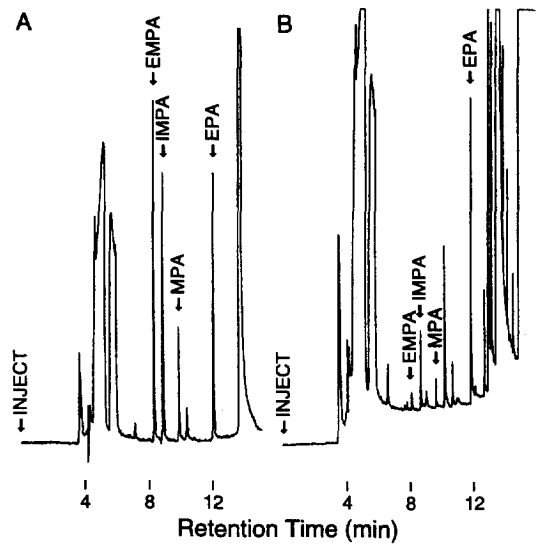

Fig. 4. Examples of gas chromatograms of (A) pooled normal urine spiked with authentic standards of methyl- and ethylphosphonic acids and methylphosphonic acid alkyl esters and (B) the urine sample from the most seriously intoxicated patient which was spiked with ethylphosphonic acid, as the internal standard.

3.7. GC analysis of the patients' urine samples

Fig. 4A shows the chromatogram of authentic standards spiked in normal urine which had been passed through the pretreatment process (Fig. 1). Fig. 4B shows the gas chromatogram of the specimen obtained from one of the most seriously intoxicated patients, as described below.

A 5-μl volume of the solution containing 434 μ*M* EPA (FC; 4.34 μ*M*) was added to urine, as the internal standard, before the pretreatment process was undertaken. The linearity of the calibration line

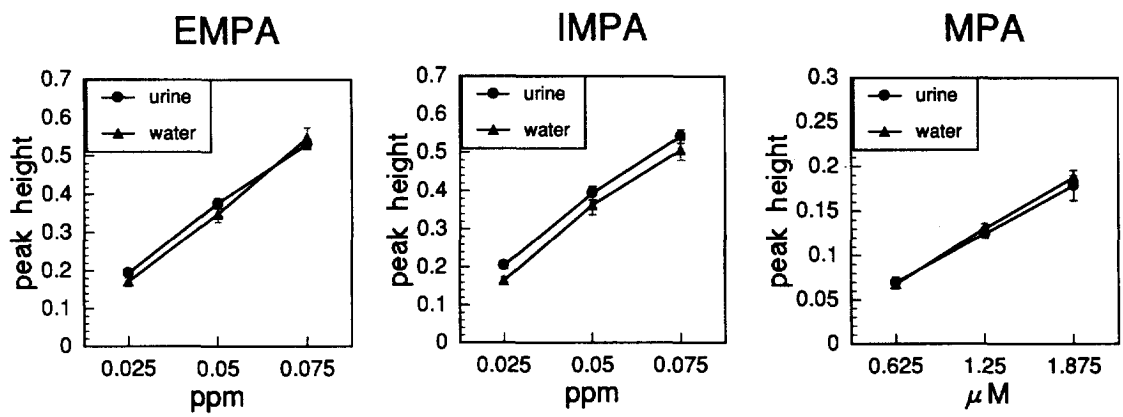

Fig. 3. Calibration curves for methylphosphonic acid esters (EMPA and IMPA) and methyl phosphonic acid (MPA) in urine samples using ethylphosphonic acid (EPA) as the internal standard. EPA (4.35 μ*M*; I.S.) was spiked into the pooled urine. The curves for urine samples following Dowex treatment are as shown in Fig. 1; the ▲-line indicates the curve derived from the treatment with buffer (0.005 *M* chloroacetic acid–NaOH, pH 3.85), which was passed through the same spiking procedure as for the pooled urine. The vertical line indicates the standard error.

was satisfactory and the detection limits were 0.025 ppm both for EMPA and IMPA, and 0.625 μ*M* for MPA. Shih et al. [12] reported that in spiked urine samples the peak area ratios were linear over concentration ranges from 10–200 ng/ml for IMPA. They obtained a quantification limit of 10 ng/ml with their method using GC–MS. Our detection limits for IMPA and EMPA were a little larger than those of Shih et al. [12], but were not much larger than the limits obtained by them using GC–MS.

3.8. Time course analysis of the metabolites in urine

Fig. 5 shows the time course analysis of the metabolite, IMPA, in urine samples from the most seriously intoxicated patient who remains comatose and in the intensive care unit of our hospital and from another patient who was hospitalized and discharged after two days and currently has no particular symptoms. The maximum IMPA concentration was obtained in urine within 12 h, suggesting that the biological half life of sarin and ethylsarin is short, as pointed out by Shih et al. [12]. All of the patients hospitalized in our ICU showed no abnormal signs concerning renal function, and their heights and weights were within the normal Japanese range. The only exceptions were in their levels of acetylcholinesterase (AChE) in erythrocyte membranes, serum cholinesterase (ChE) and serum creatine phosphokinase. We can roughly estimate the total exposure quantities of sarin and ethylsarin from the time-integrated urinary values of IMPA and EMPA, respectively, by assuming that the daily creatinine output of the patients into urine was the same as the Japanese reference value for adults, i.e., 0.7–1.5 g/day [23]. The estimated sarin exposure values are 0.13–0.25 and 0.016–0.032 mg/person for the comatose and the less severely intoxicated patients, respectively.

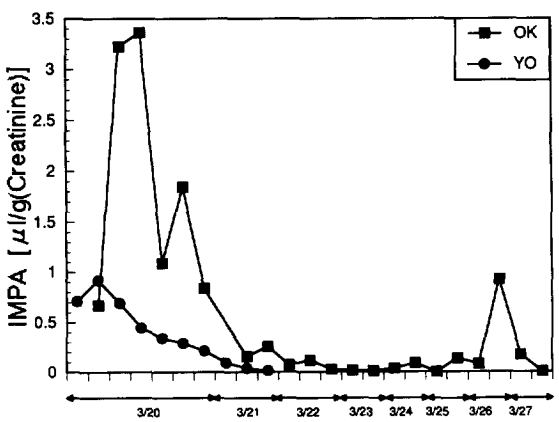

Fig. 5. Time course of the sarin metabolite (IMPA) in urine from patients taken immediately after exposure and for up to seven days. The examples shown are from two patients; the most seriously intoxicated one, who is still in the Intensive Care Unit of our medical school hospital and the other one, who was hospitalized for three days and showed no subsequent ill-effects.

Considering the estimated sarin exposure value, the value obtained from the comatous person slightly exceeds the published lethal dose [24,25] and explains the low lethality in the victims who received treatment in hospitals [26]; 12 (dead)/5500 (visited hospitals), and the earlier appearance (1 to 3 h after sarin exposure) of isopropyl alcohol [27], which, according to the metabolic pathway of sarin, should he excreted at the same time in urine as MPA, whose peak excretion level was 10–18 h after the exposure (detailed data not shown). Possible synthetic by-products, which may have been present in the sarin employed in the terrorist attack, for example, diisopropyl methylphosphonate, ethylisopropyl methylphosphonate and diethyl methylphosphonate, have also contributed to the observed metabolic products, since these dialkyl methylphosphonic acids would also be biotransformed rapidly into metabolites such as EMPA, IMPA, MPA and isopropanol by hydrolysis or by esterases, such as acetylcholine esterase (our unpublished data).

4. Conclusion

We have developed a method for the analysis of alkyl methylphosphonic acids, possible metabolites of sarin, in urine samples. This method was used for the analysis of urine samples obtained from victims who were exposed to sarin in a terrorist attack on the Tokyo subway system and who were admitted to our hospital. A GC–FPD method was chosen because of its reported sensitivity and selectivity for organophosphorus compounds [1–3]. A column pretreatment procedure was needed to remove interferents from the biological matrix, such as salts and proteins,

which affected the efficiency of the trimethylsilylation reaction. This procedure, involving Dowex-column pretreatment, adjustment of the eluate pH and evaporation to dryness prior to derivatization, was used on spiked samples of buffer, control urine and patients' samples to demonstrate the utility of the method. The use of authentic sarin and ethylsarin metabolites, IMPA and EMPA, made it possible to establish the necessary pretreatment procedures for the GC–FPD assay. This method can be used for the analysis of large numbers of biological samples in a clinical laboratory and may be useful for evaluating the level of exposure to sarin by estimation of the alkyl methylphosphonic acid metabolites in urine.

Acknowledgments

We are much indebted to Dr. John Clement of Canadian DRES for encouraging us to prepare this manuscript, to Drs. Louis Smith, Norman Ray, David Read and Martin K. Johnson of British MRC Toxicology unit for valuable discussion through e-mails and facsimiles as to the relevance of biological monitoring methods for risk evaluation of the exposed victims.

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