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SHORT COMMUNICATION

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Quantitative analysis of O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage

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Abstract A convenient and rapid micro-anion exchange liquid chromatography (LC) tandem electrospray mass spectrometry (MS) procedure was developed for quantitative analysis in serum of O-isopropyl methylphosphonic acid (IMPA), the hydrolysis product of the nerve agent sarin. The mass spectrometric procedure involves negative or positive ion electrospray ionization and multiple reaction monitoring (MRM) detection. The method could be successfully applied to the analysis of serum samples from victims of the Tokyo subway attack and of an earlier incident at Matsumoto, Japan. IMPA levels ranging from 2 to 135 ng/ml were found. High levels of IMPA appear to correlate with low levels of residual butyrylcholinesterase activity in the samples and vice versa. Based on our analyses, the internal and exposure doses of the victims were estimated. In several cases, the doses appeared to be substantially higher than the assumed lethal doses in man.

Key words Chemical warfare agents · LC tandem MS · Multiple reaction monitoring · O-isopropyl methylphosphonic acid · Sarin

Introduction

Recently, a new type of proliferation of chemical warfare agents has emerged with the use of nerve agents by the Aum Shinrikyo sect in terrorist attacks, e.g., with sarin in Matsumoto and in the Tokyo subway (Brackett 1996) and with VX in Osaka, Japan (Nozaki et al. 1995). Sensitive and reliable methods are required to establish the nature and extent of exposure to such agents. Methods developed to date take advantage of rapid binding of nerve agents to acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) and of hydrolysis of the agents to the corresponding alkyl methylphosphonic acids.

With respect to the first approach, we developed a procedure for analysis of phosphylated cholinesterases, which is based on Reactivation of the phosphylated enzymes with fluoride ions: this converts the organophosphate moiety quantitatively into the corresponding phosphofluoridate, which is subsequently isolated and quantitated (Polhuijs et al. 1997). Application of this method to serum samples from the Japanese victims clearly showed that these people had been exposed to an organophosphate with the structure $iPrO(CH_3)P(O)X$, presumably with X = F (sarin; see Fig. 1). A much more laborious and qualitative method, reported by Nagao et al. (1997) and by Matsuda et al. (1998), is based on isolation and trypsinization of inhibited cholinesterases, subsequent treatment with alkaline phosphatase, followed by isolation, derivatization and gas chromatography-mass spectrometry (GC-MS) analysis of the released phosphyl moiety.

With respect to the second approach, Minami et al. (1997) demonstrated the presence of the second fingerprint of exposure to sarin, i.e., O-isopropyl methylphosphonic acid (IMPA; Fig. 1), in urine of Japanese victims, by employing GC with flame photometric detection after trimethylsilylation. Several other laboratories have reported the use of GC-MS for analysis of IMPA in biological samples (see, for example, Shih et al. 1991; Black et al. 1994; Fredriksson et al. 1995). GC-MS procedures, although highly sensitive, are quite laborious while requiring extensive sample preparation including ion-exchange chromatography as well as derivatization steps. In some laboratories liquid chromatography-mass spectrometry (LC-MS) has been applied to the analysis of IMPA (Wils and Hulst 1988; Tørnes 1996; Borrett et al. 1996; Black and Read 1997, 1998), which provides a rapid screening of samples with

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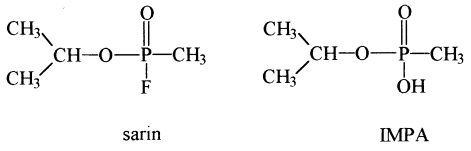

Fig. 1 Chemical structures of sarin and O-isopropyl methylphosphonic acid (IMPA)

minimal sample pretreatment. However, biological samples were not involved in these particular cases. We report here on the development of a new and rapid LC tandem MS method for quantitative determination of IMPA in blood and its application to the analysis of serum samples from victims of the terrorist attacks in Matsumoto and Tokyo. Based on these analyses, we estimated the internal and exposure doses of the victims.

Materials and methods

To a serum sample (50 µl) was added an aqueous solution of IMPA-d_3 (10–150 ng/ml; 50 µl), water (350 µl) and concentrated sulphuric acid (12.0 N; 27 µl). The mixture was extracted twice with isobutanol/toluene (1:1, v/v; 1.1 ml). After thorough mixing, the phases were separated by centrifugation. The organic phase was collected, concentrated to a small volume (20–50 µl) by heating under a gentle stream of nitrogen, and analysed with LC tandem MS on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). Operating conditions were: capillary voltage 3 kV (negative ions) or 4 kV (positive ions), cone voltage 30 V, collision energy 12 eV, gas (Ar) cell pressure $3 \cdot 10^{-3}$ mBar, dwell time 1.5 s. For negative ion electrospray MS with MRM the following transitions were monitored: m/z 137 ($[M\text{-}H]^-$) $\rightarrow$ m/z 95 ($[M\text{-}H - C_3H_6]^-$) for IMPA and m/z 140 $\rightarrow$ m/z 98 for IMPA-d_3. For positive ion electrospray MS with MRM the following transitions were monitored: m/z 139 ($[M+H]^+$) $\rightarrow$ m/z 97 ($[M+H - C_3H_6]^+$) for IMPA and m/z 142 $\rightarrow$ m/z 100 for IMPA-d_3. The LC system consisted of a microbore column (20 cm $\times$ 320 µm) containing PRP-X100 10 µm particles, with H_2O/CH_3CN (1:1, v/v), containing 0.5% HCOOH, as the eluent. The flow rate was 20 µl/min (by means of LC-packings splitter; flow of Waters 590 high performance liquid chromatography (HPLC) pump, 0.6 ml/min). The injection volume was 2 µl.

Results and Discussion

We adapted the method described by Little et al. (1986) for isolation of IMPA from serum, which implies acidification of the sample and subsequent extraction with isobutanol/toluene. With this procedure the recovery was established to be 70–100%, as determined by using [^{14}C]IMPA. Our method is less laborious than that used by Fredriksson et al. (1995) which involves anion-exchange chromatography. We recently reported methods to detect exposure to sulphur mustard, based on tandem electrospray MS analysis with multiple reaction monitoring (MRM) of sulphur mustard adducts with DNA and haemoglobin (Fidder et al. 1996; Noort et al. 1997). The same MRM technique was applied to the mass spectrometric detection of IMPA, while the microbore anion-exchange LC-system described by Kientz et al. (1992), after slight modification, was selected for analyte separation.

For both negative and positive ion electrospray MS with MRM the loss of propene (C_3H_6) from the (de)protonated molecular ion was monitored. The absolute detection limits for aqueous standards of IMPA were 10 pg and 2 pg, for analysis in the negative and positive ion mode, respectively. In comparison with other LC-MS methods for analysis of IMPA (Wils and Hulst 1988; Tørnes 1996; Borrett et al. 1996; Black and Read 1997, 1998), this method is more sensitive. Only one peak was observed when serum samples, spiked with IMPA, were analysed in the negative ion mode, demonstrating the high selectivity of this method. In this case, the detection limit for analysis of IMPA in serum was 4 ng/ml, when the processed sample volume was 20 µl. For analysis in the positive ion mode the sensitivity was slightly better (absolute detection limit 2 pg; detection limit in serum 1 ng/ml) but the selectivity was impaired, when compared with analysis in the negative ion mode. O-isopropyl trideuteromethylphosphonic acid (IMPA-d_3^1)[1] was used as an internal standard, having a response equal to that of IMPA. The recovery of the entire method was determined to be 70–110%, by comparison of a blank serum sample spiked with IMPA and a standard solution of IMPA. This is in accordance with the earlier obtained recovery by using radioactively labelled IMPA (vide supra). The complete procedure, including work-up, takes approximately 2 h, and is described in the Materials and methods section.

The serum samples of Japanese victims of the Matsumoto incident and the Tokyo subway incident were processed and analysed as described in the Materials and methods section. In the first series of analyses the amounts of IMPA were estimated using an external standard. On the basis of these results a proportional amount of IMPA-d_3 was added prior to work-up of the samples in the final series of experiments. The results are summarized in Table 1 together with the BuChE activities measured within 1.5–2.5 h after the incident (Polhuijs et al. 1997). The mass chromatograms of a typical LC tandem MS analysis of a Japanese serum sample are shown in Fig. 2.

A number of samples was analysed with both positive ion and negative ion electrospray MS, giving virtually identical results. Samples containing low amounts of IMPA (≤4 ng/ml) were analysed in the positive ion mode only. Obviously, substantial amounts of IMPA are present in most of the serum samples, which provides additional evidence for exposure to nerve agents with the structure iPrO(Me)P(O)X. Significantly lower IMPA levels were found in blood samples taken approximately 2 h later (victims 1, 2 and 3), which is in accordance with a ready excretion of the highly polar IMPA, at least partly into urine (Minami et al. 1997).

Since IMPA must have entered systemically as intact nerve agent and in view of its rapid in vivo hydrolysis to IMPA (almost instantaneously in the case of the

[1] IMPA-d_3 was synthesized by hydrolysis of sarin-d_3 and was purified by distillation under reduced pressure

Table 1 Butyrylcholinesterase activity and O-isopropyl methylphosphonic acid (IMPA) levels in serum samples from victims of terrorist attacks in Tokyo and Matsumoto

Incident	Victim number[a]	BuChE activity (arbitrary units)	IMPA (ng/ml serum)[b]
Tokyo	1	21	100 ± 2
	1[c]	28	86 ± 6 (87)[d]
	2	126	24 ± 3
	2[c]	134	9 ± 1
	3	126	26 ± 1 (26)[d]
	3[c]	116	15 ± 2 (13)[d]
	4	126	16 ± 1 (14)[d]
	5	583	4 ± 0[d]
	6	818	2 ± 0[d]
	7[e]	1100	n.d.[f]
	8	1131	6 ± 0[d]
	9[e]	804	24 ± 1
	10	66	43 ± 1
	11[e]	172	66 ± 3 (61)[d]
Matsumoto	12	166	78 ± 1
	13	52	136 ± 1 (135)[d]
	14[e]	224	2 ± 0[d]
	15[e]	1460	n.d.[f]
	16[e]	761	11 ± 0
	17[e]	1172	n.d.[f]
	18[e]	1186	3 ± 0[d]

[a] Victims were male, unless noted otherwise
[b] Analysed (in duplicate) with negative ion electrospray mass chromatography (MS) with multiple reaction monitoring (MRM), unless noted otherwise
[c] Second sample taken from a victim at 2–2.5 h after arrival in hospital, whereas all other samples were taken within 1.5 h after hospitalization
[d] Analysed (in duplicate, except for the determinations mentioned *in parentheses*) with positive ion electrospray MS with MRM
[e] Female victim
[f] n.d., Not detectable, which is below the detection limit of 1 ng/ml serum

(+)-enantiomer), it is not unreasonable to assume that the degree of initial inhibition of BuChE correlates with the amount of IMPA in the serum samples, which were taken within 1.5 h after exposure. As presented in Fig. 3, a high content of IMPA in serum does appear to correlate with a low residual BuChE activity and vice versa. In this respect, we would like to stress that although the purity of the sarin used by the terrorists is not known precisely, it is unlikely that the high IMPA levels in the serum samples of the victims are due to other organophosphates present in the terrorists' sarin. For example chlorosarin, which should also give IMPA after hydrolysis in serum, is highly moisture sensitive and hydrolyses almost instantaneously upon exposure to air. In addition, in vivo hydrolysis of di-O-isopropyl methylphosphonate, which could also have been an impurity in the terrorists' sarin, is too slow to give significant IMPA levels in serum.

Based on the same arguments explained above, the determined levels of IMPA in serum can be used to calculate the internal dose of sarin in the victims. As indicated by the results obtained by Little et al. (1986) after exposure of mice to [^{3}H]sarin and similar results

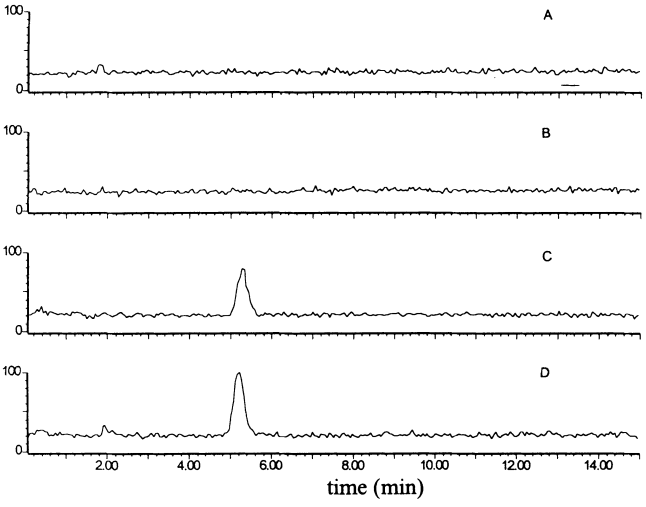

Fig. 2 Negative ion liquid chromatography (LC) tandem electrospray mass spectrometry (MS) using the multiple reaction monitoring scanning mode for transition m/z 137 → m/z 95 (IMPA) or m/z 140 → 98 (O-isopropyl trideuteromethylphosphonic acid; IMPA-d_3). Traces A and B (IMPA and IMPA-d_3, respectively) represent a blank serum sample; traces C and D (IMPA and IMPA-d_3, respectively) represent a Japanese serum sample (50 µl; second sample of victim no. 3) to which a solution of IMPA-d_3 (21 ng/ml; 50 µl) had been added. The LC system consisted of a microbore column (20 cm × 320 µm) containing PRP-X100 (10 µm particles), with H_2O/CH_3CN (1:1, v/v), containing 0.5% HCOOH, as the eluent. Flow rate, 20 µl/min, injection volume, 2 µl

obtained with soman in non-human primates, i.e., marmosets (De Jong et al. unpublished results), it may be assumed that free IMPA is distributed almost uniformly in the organism, while an amount of sarin equivalent to two-thirds of free IMPA is bound covalently. It thus follows that the internal dose ranges from $5/3 \times 2$ to $5/3 \times 135$ ng/ml, which corresponds with 3.3–230 µg/kg. Assuming an average body weight

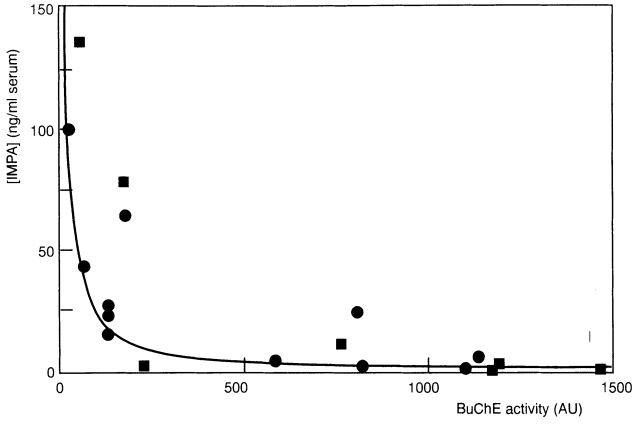

Fig. 3 Inhibition of butyrylcholinesterase (BuChE) activity in serum samples of victims of terrorist attacks with sarin in Tokyo (●) and Matsumoto (■), measured within 1.5 h after exposure, and concentration of hydrolysed sarin (IMPA) in these samples. The drawn curve follows the equation: [IMPA] = −0.70 + 25/BuChE activity, with [IMPA] in ng/ml serum and BuChE activity in arbitrary units (AU)

of 62.5 kg, this corresponds with an overall internal dose of 0.21–15.0 mg/individual. Evidently, these are minimum values since the blood samples were taken at least 1.5 h after the exposure while it has been observed that the blood levels of IMPA decrease rapidly with time (see Table 1). Our estimated internal doses of sarin are approximately an order of magnitude higher than the values reported by Minami et al. (1997), which were derived from time-integrated urinary values of IMPA.

It is tempting, albeit somewhat speculative, to compare the calculated internal doses with the estimated value of 50% lethal concentration time (LCt_{50}) of sarin in man, i.e., 100 mg min m^{-3} (Ivarsson et al. 1992; Black and Harrison 1996). Assuming an average respiratory minute volume of 20 l/min and an averaged retention for inhaled sarin in man of 60% (Oberst 1961) the internal lethal dose would be 1.2 mg/person, which corresponds in our calculations with a serum level of IMPA of *ca*. 11.5 ng/ml. In spite of the uncertainties in our assumptions, e.g., with regard to respiratory minute volume and exposure time which affects the acute toxicity of sarin, it may be concluded that several of the victims have been exposed to highly toxic concentrations of sarin. In some cases the C*t*-values are substantially higher than the assumed LCt_{50}. Presently, we cannot explain this discrepancy.

In conclusion, a rapid method for analysis of IMPA in serum samples has been described, which could be applied to the analysis of serum samples of victims of the two terrorist attacks with sarin. Probably, this method can be extended to the analysis of other O-alkyl phosphonic acids. Since the low molecular weight metabolite IMPA is readily excreted into the urine, this method will be applicable to analysis of blood samples taken in a rather limited time span after exposure. Based on reasonable assumptions, the measured serum levels of IMPA can be used to determine the overall internal dose of sarin entered systemically into the victims.

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