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GC–MS and LC–MS analysis of nerve agents in body fluids: Intra-laboratory verification test using spiked plasma and urine samples☆,☆☆

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ABSTRACT

The purpose of this study was to check the applicability of different analytical methods for the identification of unknown nerve agents in human body fluids. Plasma and urine samples were spiked with nerve agents (plasma) or with their metabolites (urine) or were left blank. Seven random samples (35% of all samples) were selected for the verification test. Plasma was worked up for unchanged nerve agents and for regenerated nerve agents after fluoride-induced reactivation of nerve agent-inhibited butyrylcholinesterase. Both extracts were analysed by GC–MS. Metabolites were extracted from plasma and urine, respectively, and were analysed by LC–MS. The urinary metabolites and two blank samples could be identified without further measurements, plasma metabolites and blanks were identified in six of seven samples. The analysis of unchanged nerve agent provided five agents/blanks and the sixth agent after further investigation. The determination of the regenerated agents also provided only five clear findings during the first screening because of a rather noisy baseline. Therefore, the sample preparation was extended by a size exclusion step performed before addition of fluoride which visibly reduced baseline noise and thus improved identification of the two missing agents. The test clearly showed that verification should be performed by analysing more than one biomarker to ensure identification of the agent(s).

1. Introduction

Nerve agents (Scheme 1) are highly toxic organophosphorus compounds which may cause severe poisoning in mammals by inhibiting the acetylcholinesterase (AChE) activity [1–4]. Therefore, their production, stockpiling, and use has been proscribed by the Chemical Weapons Convention (CWC) [5]. However, the past two decades have shown that stockpiling and use of nerve agents cannot be completely prohibited: sarin (GB) and tabun (GA) have been used by Iraq [6,7] and cyclosarin (GF) was produced in Iraq [8,9].

Besides the use of chemical weapons in military conflicts, an asymmetric threat started to cause concern about the use of chemical warfare agents by terrorists: during the nineties of the last century Japan has been the target of the Aum Shinrikyo sect: VX was used in an assassination of a young Japanese in 1994 in Osaka [10] and sarin was used in Matsumoto in 1994 [11,12] as well as in the Tokyo subway incident in 1995 [12,13]. In case of the homicidal use of VX, the victim died without giving a hint on the cause of his death. According to signs and symptoms, poisoning by an organophosphorus pesticide was assumed but could not be proven. Only when a suspected person was caught 6 months later, he testified that the murder was committed with VX. After that, the metabolites of VX could be found in the stored samples from the victim [10]. In the Matsumoto case, it took 7 days until sarin could be identified in pond water as agent used for the attack [11].

These examples show that analytical methods for the identification of nerve agents in body fluids are important components of verification of alleged use.

The choice of suitable analytical methods for the analysis of incorporated nerve agents first depends on the reaction of nerve agents with body constituents (see Scheme 2): Nerve agents are

Abbreviations: AChE, Acetylcholinesterase; BChE, Butyrylcholinesterase; CHMPA, O-cyclohexyl methylphosphonic acid; CVX, Chinese VX, O-n-butyl S-2-diethylaminoethyl methylphosphonothiolate; DFP, Diisopropyl fluorophosphate; EHMPA, O-(2-ethyl)hexyl methylphosphonic acid; EMPA, O-ethyl methylphosphonic acid; GA, Tabun, O-ethyl N,N-dimethyl phosphoramidocyanidate; GB, Sarin, O-isopropyl methylphosphonofluoridate; GD, Soman, O-pinacolyl methylphosphonofluoridate; GF, Cyclosarin, O-cyclohexyl methylphosphonofluoridate; iBMPA, O-iso-butyl methylphosphonic acid; IMPA, O-isopropyl methylphosphonic acid; nBMPA, O-n-butyl methylphosphonic acid; PhPA, Phenylphosphonic acid; PMPA, O-pinacolyl methylphosphonic acid; VR, Russian VX, O-iso-butyl S-2-diethylaminoethyl methylphosphonothiolate; VX, O-ethyl S-2-diisopropylaminoethyl methylphosphonothiolate.

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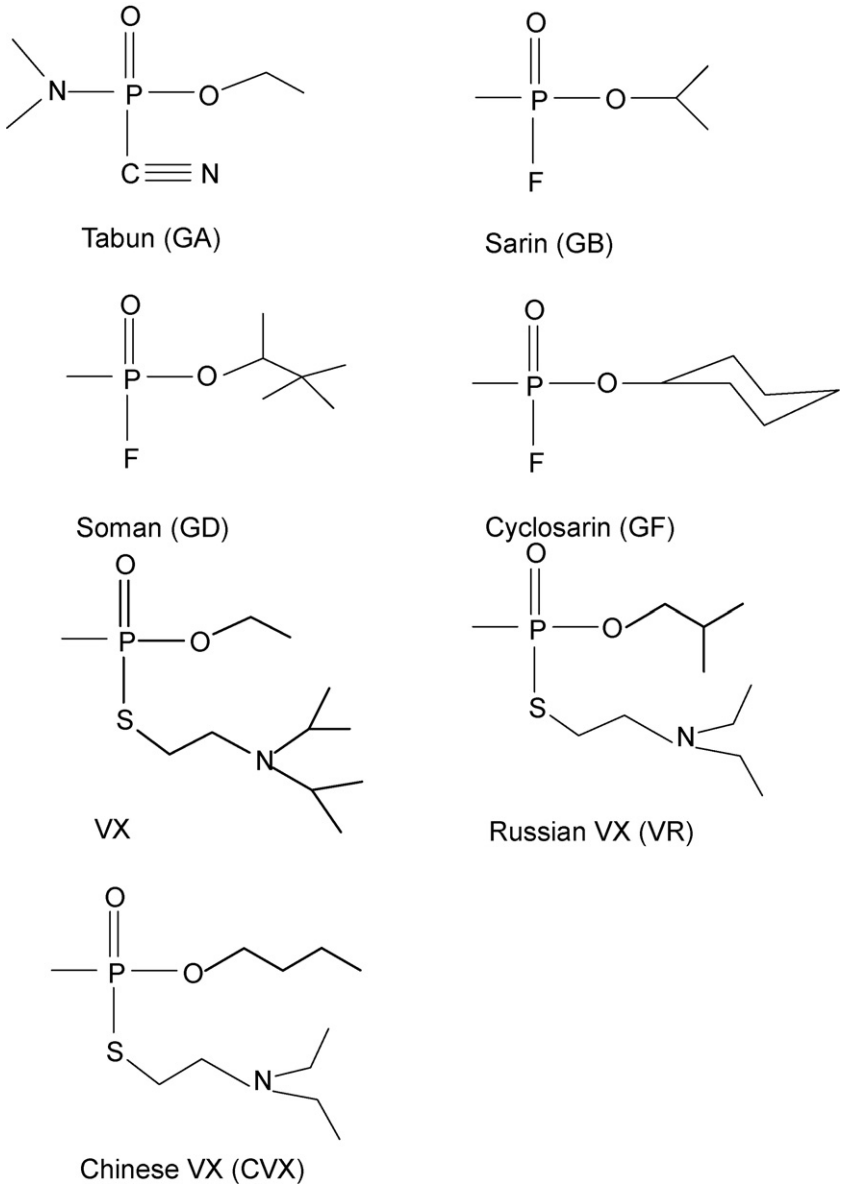
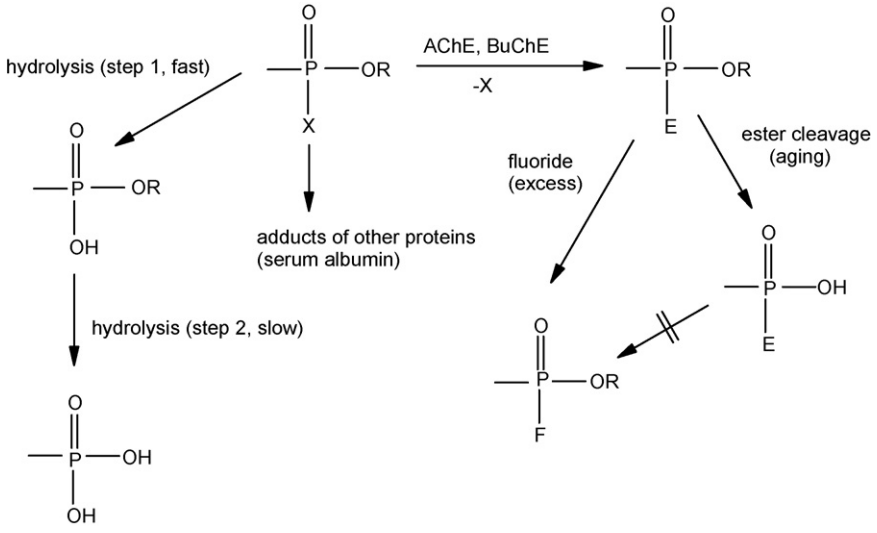
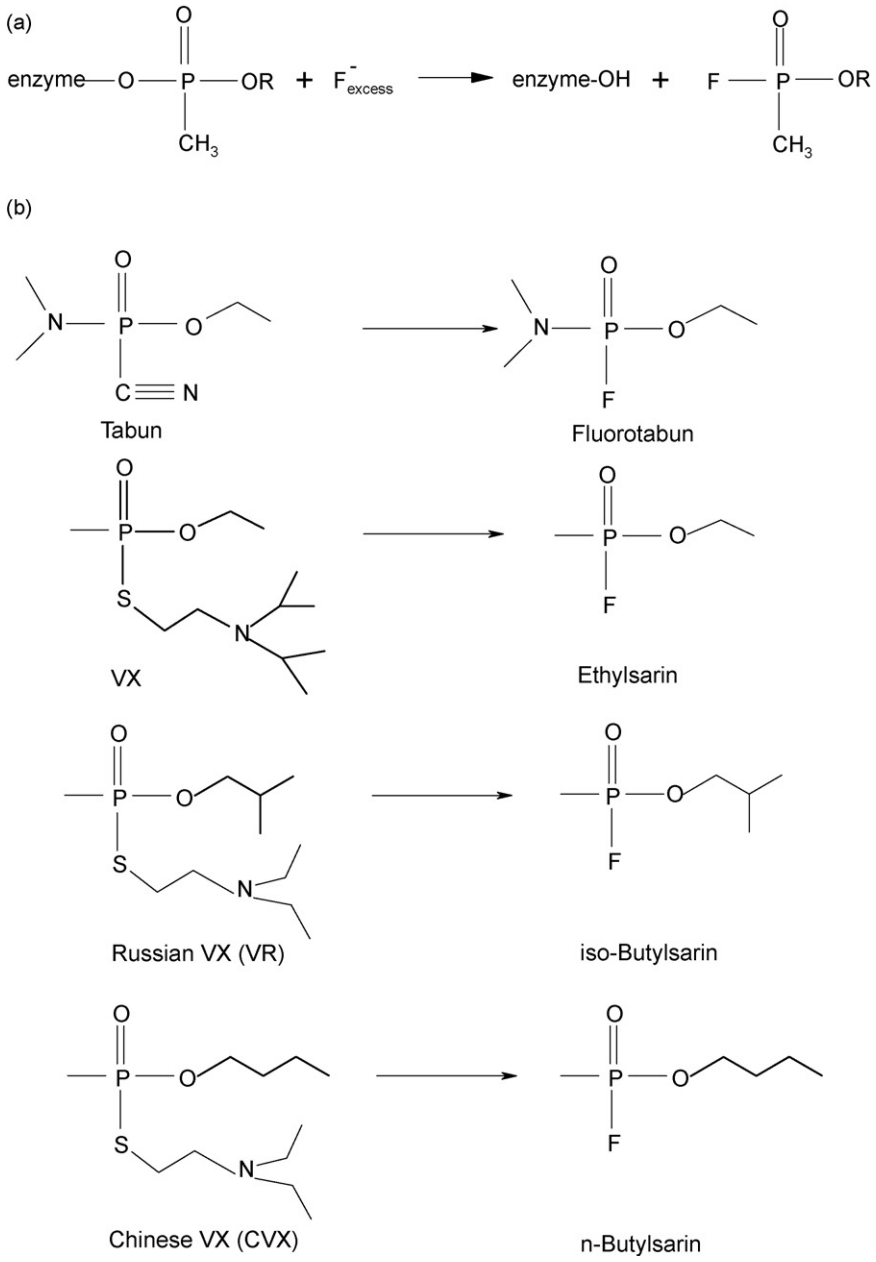


Scheme 1. Structural formulae of nerve agents.



Scheme 2. Fate of nerve agents in blood.



Scheme 3. (a) General reaction of the fluoride-induced reactivation of nerve agent-inhibited BChE. (b) Formation of fluoridated nerve agents during fluoride-induced reactivation of tabun- or V-agent-inhibited BChE.

quickly bound to the cholinesterases which are then inhibited [14,15], and other proteins like serum albumin are also involved in adduct formation [16–21]. In addition, G-type nerve agents are rapidly hydrolysed by phosphotriesterases like human serum paraoxonase (PON 1) [22–26] to form O-alkyl methylphosphonic acids which are eliminated *via* the renal system [27–29]. Second, the time interval between exposure and sampling is essential for the choice of biomarkers for the analysis of nerve agents. Unchanged G-type nerve agents can be found in plasma only for a few hours whereas for V-type agents, e.g. VX, a persistency of about 40 h has been reported by van der Schans et al. [30]. The hydrolytic products of all nerve agents are eliminated from plasma within 1–2 days [31,32] but can be determined in urine up to 2 weeks [33]. Finally, the fluoride-induced reactivation of nerve agent-inhibited BChE (see Scheme 3) can be performed for at least up to 3 weeks after exposure [31,34], because the life-time of this protein adduct is only limited by the *de novo*-synthesis of the BChE.

In our laboratory, a test battery covering (i) GC–MS analysis of unchanged nerve agents after extraction from plasma, (ii) GC–MS analysis of regenerated nerve agents after fluoride-induced reactivation of inhibited BChE, (iii) LC–MS analysis of O-alkyl methylphosphonic acids after extraction from plasma, and (iv) LC–MS analysis of O-alkyl methylphosphonic acid after extraction from urine was established [35]. To test the applicability of this battery, we performed an intra-laboratory study simulating a rather short time interval between “exposure” (spiking) and sampling. Therefore, 20 samples were spiked with nerve agents (plasma) or with the corresponding O-alkyl methylphosphonic acid (urine). Seven samples were selected randomly which underwent the whole test battery consisting of the above-mentioned methods.

Table 1
Master list of sample spiking.

Sample number #	Agent (9 mL plasma)	Metabolite (13 mL urine)	Volume added (μL)	Concentration in plasma (ng/mL)	Concentration in urine (ng/mL)
1	CVX	nBMPA	10	111	76.9
2	GF	CHMPA	12.8	142	98.5
3	GF	CHMPA	8.2	91.1	63.1
4	GA	–[a]	6.4	71.1	–
5	Blank	Blank	0.0	0.0	0.0
6	GB	IMPA	10	111	76.9
7	Blank	Blank	0.0	0.0	0.0
8	VX	EMPA	7.3	81.1	56.2
9	GD	PMPA	7.4	82.2	56.9
10	GB	IMPA	20	222	154
11	VR	iBMPA	10	111	76.9
12	Blank	Blank	0.0	0.0	0.0
13	VR	iBMPA	8.3	92.2	63.8
14	GA	–[a]	10	111	–
15	GB	IMPA	7.0	77.8	53.8
16	VX	EMPA	14.6	162	112
17	GF	CHMPA	5.9	65.5	45.4
18	CVX	nBMPA	11	12.1	84.6
19	GA	–[a]	17.4	193	–
20	GD	PMPA	14.8	164	114

[a] In case of tabun, no stable metabolite is available as reference for spiking of the urine samples.

2. Experimental

2.1. Chemicals and SPE cartridges

Original nerve agents (sarin, soman, tabun, cyclosarin, VX, Russian VX (VR) and Chinese VX (CVX)) as well as reference chemicals for regenerated nerve agents (fluorotabun, ethylsarin, n-butylsarin, and iso-butylsarin; see Scheme 2b) were made available by the German Ministry of Defence. Diisopropyl fluorophosphate (DFP) was obtained from Fluka (Taufkirchen, Germany). Ethyl methylphosphonic acid (EMPA), pinacolyl methylphosphonic acid (PMPA), and phenylphosphonic acid (PhPA) were purchased from Aldrich (Taufkirchen, Germany). Isopropyl methylphosphonic acid (IMPA), iso-butyl methylphosphonic acid (iBMPA), n-butyl methylphosphonic acid (nBMPA), cyclohexyl methylphosphonic acid (CHMPA), and (2-ethylhexyl) methylphosphonic acid (EHMPA) were purchased from TNO Defence, Security and Safety (Rijswijk, The Netherlands). Purity of the reference compounds (>95%) was provided by the supplier and further surveyed by in-house NMR (Bruker 400 MHz microbay with BBO probe, Bruker Biospin, Rheinstetten, Germany).

The solvents (methanol, 2-propanol, hexane and acetonitrile) were purchased from Merck (Darmstadt, Germany) of the highest grade available and were used without further purification. Ammonia, potassium acetate, sodium dihydrogen phosphate monohydrate, solid sodium hydroxide, hydrochloric acid, perchloric acid 70%, acetic acid, and formic acid, were all of "p.a".-grade and obtained from Merck (Darmstadt, Germany) while potassium fluoride ("ultra") was from Fluka (Taufkirchen, Germany).

Isolute-ENV+ (25 mg, 1 or 10 mL) and isolute octyl SPE cartridges (25 mg, 1 mL) were from Biotage (Uppsala, Sweden), HyperSep amino cartridges (100 mg, 1 mL) were from ThermoHypersil-Keystone (Dreieich, Germany), and NAP-5 columns (size exclusion gel) were obtained from Amersham Biosciences (Uppsala, Sweden).

Purified water was prepared by a PureLab system from USF (Ransbach-Baumbach, Germany) which was fed with deionised water from an Elect 80 system, also from USF.

EDTA-blood was collected from volunteers, and plasma was separated by centrifugation. It was pooled and frozen at −20 °C until use.

Urine was collected from volunteers, pooled and frozen at −20 °C until use.

2.2. Standard solutions

The stock solutions were prepared as 0.01 vol.% solutions in hexane (nerve agents, regenerated agents, and DFP) or in methanol (metabolites) which were stable for 6 months at room temperature. To prepare calibration standards the requested stock solutions were diluted with 2-propanol to give final concentrations of 100 ng/mL (nerve agents and DFP) or with 5 vol.% formic acid (metabolites and PhPA or EHMPA, the internal standards for the plasma and urine method, respectively). The calibration standards were always freshly prepared before starting an experiment.

2.3. Plasma deproteinisation

Due to rapid hydrolysis of nerve agents, primarily G-agents, in native plasma it was indispensable to precipitate the plasma proteins immediately after spiking. Therefore, 75 μL 1 M $HClO_4$ were added to 1 mL plasma followed by 15 μL 1 M potassium acetate solution after shaking the mixture briefly. Then, the mixture was centrifuged at 16,750 × *g* for 10 min in a Hettich Rotina 35 R (Tuttlingen, Germany). The supernatant was separated and frozen at −60 °C until sample processing.

2.4. Sample spiking

20 samples, each of 9 mL plasma and 13 mL urine, respectively, were spiked or left blank in an arbitrary order with different volumes (see Table 1) of the respective single stock solutions, i.e. 100 μg/mL nerve agent in hexane and 100 μg/mL O-alkyl methylphosphonic acid in methanol. To simplify the spiking procedure the volumes of the metabolite solutions were the same as for the neat nerve agent solutions although this did not provide the equimolar concentration.

The master list of the spiking procedure is given in Table 1. The operators for sample processing did not get any information on this master list until all procedures were finished.

When the spiking was done three 1 mL portions of each spiked plasma sample were separated, deproteinized as described before and frozen at −60 °C for the analysis of original agents. The remaining 6 mL plasma and the urine samples were frozen directly after spiking at −60 °C.

2.5. Selection of the test samples

Before sample processing, an independent scientist of the institute who was not involved in spiking, sample preparation or blinding selected 7 numbers out of 20: #3, #7, #8, #11, #12, #15 and #20. All sample tubes with these numbers were thawed and divided for analysis: 2× 0.5 mL plasma for the fluoride-induced reactivation, 2× 1 mL plasma and 2× 4 mL urine for the determination of the metabolites. The remaining plasma and urine samples were frozen again as reference samples. For the determination of the unchanged nerve agents only two of the three deproteinised plasma samples which had been frozen separately were thawed, too. The third plasma sample remained frozen as reference sample.

2.6. General procedures of sample processing

For solid-phase extraction (SPE) a VacMaster system for 20 samples from Separtis (Grenzach-Whylen, Germany) was used in connection with a vacuum station CVC 200 from Vacuubrand (Wertheim, Germany).

Work-up of all samples was performed in duplicate and aliquots of all eluates obtained during sample preparation were injected twice into the respective chromatographic system. Thus, four individual chromatograms were recorded for each of the test procedures providing sixteen analyses (obtained from four test procedures) for interpreting the results of the complete sample processing.

For calibration, standard mixtures containing 100 ng/mL of the respective analytes were injected six-fold in the beginning, three-fold in the middle and again three-fold at the end of each series to assess the stability of the chromatographic system.

All methods described here and published recently [35] have been validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), reproducibility (within day and from day to day), accuracy, and robustness which were passed within a deviation range of 10% maximum.

2.7. GC–MS analysis of original nerve agents after extraction from plasma

The GC–MS system consisted of a 5890 gas chromatograph and a 5972 mass selective detector, both from Agilent Technologies (Waldbronn, Germany). It was equipped with a cold injection system (CIS 3) from Gerstel (Mülheim a.d. Ruhr, Germany) connected to a VF 5 MS capillary column of 30 m × 0.25 mm I.D. × 0.25 μm film thickness from Varian (Darmstadt, Germany). Helium (5.0) was used as carrier gas at a constant flow rate of 1.3 mL/min which was passed through an oxygen absorber and a molecular sieve from Varian (Darmstadt, Germany) before reaching the gas chromatograph. For the injection of a 5 μL sample, the CIS was programmed in the solvent venting mode starting from 40 °C which was held for 1.1 min while the split exit was open. Then, the split exit was closed and the system was heated to 190 °C at a rate of 12 °C/s (hold time: 0.10 min) and further to 260 °C (also at a rate of 12 °C/s) which was held for 1 min. During this period the analytes were evaporated and passed to the column which was kept at 50 °C for 1.8 min. After the injection was done, the oven was heated to 80 °C (no hold) at a rate of 10 °C/min, further to 180 °C/min at a rate of 20 °C/min (hold time: 0.5 min) and finally to 300 °C at a rate of 30 °C/min. The final temperature was held for 1 min. The mass spectrometer (transfer line: 280 °C, source temperature: 230 °C, quadrupole: 150 °C) was used in the SIM mode applying the following mass ions for detection: *m*/*z* 86, 99, 114, and 127. A chromatogram from the analysis of a standard mixture is shown in Fig. 1A.

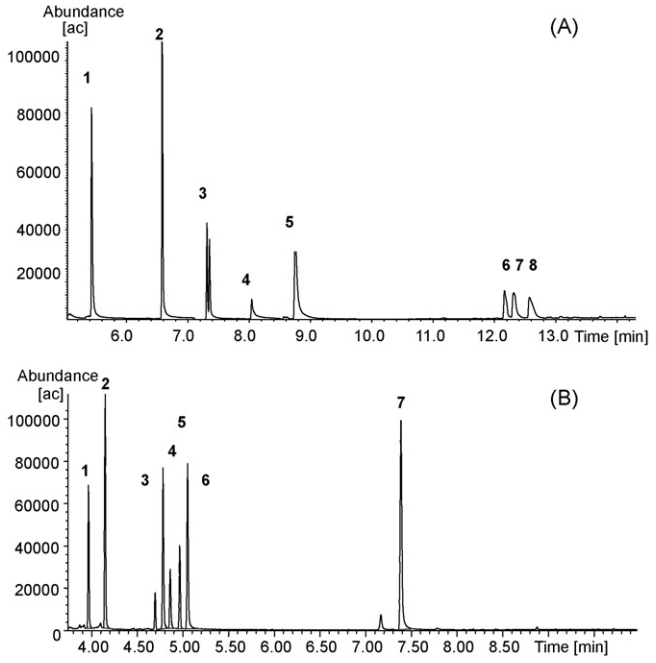


Fig. 1. Standard chromatograms by GC–MS (*c* = 100 ng/mL in 2-propanol). (A) Separation of the neat nerve agents with 1 GB, 2 DFP (IS), 3 GD, 4 GA, 5 GF, 6 VX, 7 VR and 8 CVX. (B) Separation of the regenerated nerve agents with 1 ethylsarin, 2 GB, 3 iso-butylsarin, 4 fluorotabun, 5 DFP (IS), 6 n-butylsarin and 7 GF, unknowns from reference compounds.

2.8. Sample preparation for the analysis of original nerve agents in plasma

1 mL deproteinised plasma was spiked with 20 μL of the internal standard solution (500 ng/mL DFP in 2-propanol), mixed and then passed over a preconditioned (1 mL methanol, 1 mL purified water) cartridge (C8, 25 mg, 1 mL). After adsorption, the cartridge was sucked dry by vacuum. For elution, the cartridge was hung into a 15 mL Falcon tube and 100 μL 2-propanol were added to the cartridge. Then, the Falcon tube was placed in a centrifuge and the 2-propanol was sucked through the cartridge bed by centrifuging at 212 × *g* for 1 min and then at 5300 × *g* for 3 min. After centrifugation the cartridge was removed and placed again on the SPE station while the collected eluate was thoroughly mixed before it was transferred from the Falcon tube to a GC vial. 5 μL of the eluate which contained the G-agents were injected into the GC–MS system.

To desorb the V-agents, the same cartridge was rinsed with 1 mL 0.5 vol.% ammonia solution followed by 1 mL hexane. Without drying the solid phase after the hexane wash it was overlaid again with 100 μL 2-propanol. Elution by centrifugation and handling of the eluate was performed in the same manner as above. Again, 5 μL of the second eluate were injected into the GC–MS system.

2.9. GC–MS analysis of the regenerated nerve agents after fluoride-induced reactivation of nerve agent-inhibited butyrylcholinesterase

For this method the same configuration as described above was used. Only the injection of the 5 μL sample and the separation of the analytes differed slightly: The solvent venting mode was performed at 40 °C (1.01 min hold time) and the final temperature was set at 190 °C reached by a rate of 12 °C/s and held for 2 min. The oven program started at 60 °C without hold time, proceeded first to 120 °C at a rate of 20 °C/min (no hold time), then to 125 °C at a rate of 3 °C/min and ended at 200 °C, which was held for 1 min. For

detection, the following mass ions were used in the SIM mode: *m*/*z* 99, 101, and 126. A chromatogram from the analysis of a standard mixture is shown in Fig. 1B.

2.10. Sample preparation for the analysis of regenerated nerve agents after fluoride-induced reactivation of inhibited butyrylcholinesterase

First, a 0.5 mL plasma sample was passed over a NAP 5 size exclusion column to remove small molecules. Then, the protein containing eluate was mixed with 100 μL 10 M potassium fluoride solution and incubated at room temperature for 30 min. Afterwards, the mixture was passed over an amino cartridge (preconditioned with 1 mL methanol and 1 mL 1 M hydrochloric acid) to remove excess fluoride (which is extremely hazardous to the capillary column). The sample was eluted completely from the ion exchange cartridge by adding 100 μL purified water. Then, the resulting eluate was mixed with 20 μL of the internal standard solution (500 ng/mL DFP in 2-propanol) and was transferred onto a preconditioned (1 mL methanol, 1 mL purified water) ENV+ cartridge (25 mg, 1 mL) for final SPE. The elution of the regenerated nerve agent was performed by 200 μL 2-propanol using the vacuum system, and 5 μL of this eluate were finally injected into the GC–MS system.

2.11. LC–MS analysis of the nerve agent metabolites after extraction from plasma or urine

The LC–MS system consisted of a Sciex API 150 single quadrupole mass spectrometer from Applied Biosystems (Darmstadt, Germany) and a System 200 LC (two micropumps, autosampler, column oven, and degasser) from Perkin-Elmer (Rodgau-Jügesheim, Germany), which was connected to a Hypercarb column 100 mm × 2.1 mm from ThermoHypersil-Keystone (Dreieich, Germany) kept at an oven temperature of 30 °C. Separation was performed by gradient elution applying the following gradient program: 100% A (2 vol.% formic acid) at a flow rate of 150 μL/min was held for 1 min. Between 1 and 2 min the mobile phase changed to 80% A/20% B (pure acetonitrile) at a flow rate of 175 μL/min which was held for 5 min. After that, the mobile phase changed further to 80% B within 1 min which was kept constant for 6 min. At last, the gradient turned back to 100% A and a flow rate of 150 μL/min which was held for 4 min for equilibration.

Ionisation was provided by an ESI source used in the negative mode at −4200 V. Spray gas and curtain gas were set at 9 and 11 a.u., respectively, while the mass spectrometer parameters were set automatically during the MS tune procedure for the following mass ions: *m*/*z* 123 (EMPA), 137 (IMPA), 151 (nBMPA and iBMPA), 157 (PhPA), 177 (CHMPA), 179 (PMPA), and 207 (EHMPA).

Fig. 2 shows the chromatograms obtained from analysing the standard mixtures by LC–MS.

2.12. Sample preparation for the analysis of O-alkyl methylphosphonic acids in plasma

1 mL plasma was mixed with 300 μL 1 M perchloric acid, rigidly mixed and centrifuged at 16,752 × *g* for 10 min. The supernatant was collected, mixed with 10 μL internal standard solution (10 μg/mL PhPA in methanol), rigidly mixed again and transferred to an ENV+ cartridge (25 mg, 1 mL) preconditioned with 1 mL methanol and 1 mL purified water. The bed was rinsed with 1 mL purified water and overlaid by 250 μL of a 1:1-mixture of methanol and 0.5 vol.% ammonia. The eluate was collected and reduced to 125 μL in a Jota RVC (vacuum concentrator from Christ, Osterode, Germany) by evaporating methanol and ammonia. The residue was then reconstituted to solution with 250 μL of 10 vol.% formic acid, and 20 μL of this mixture were injected into the LC–MS system.

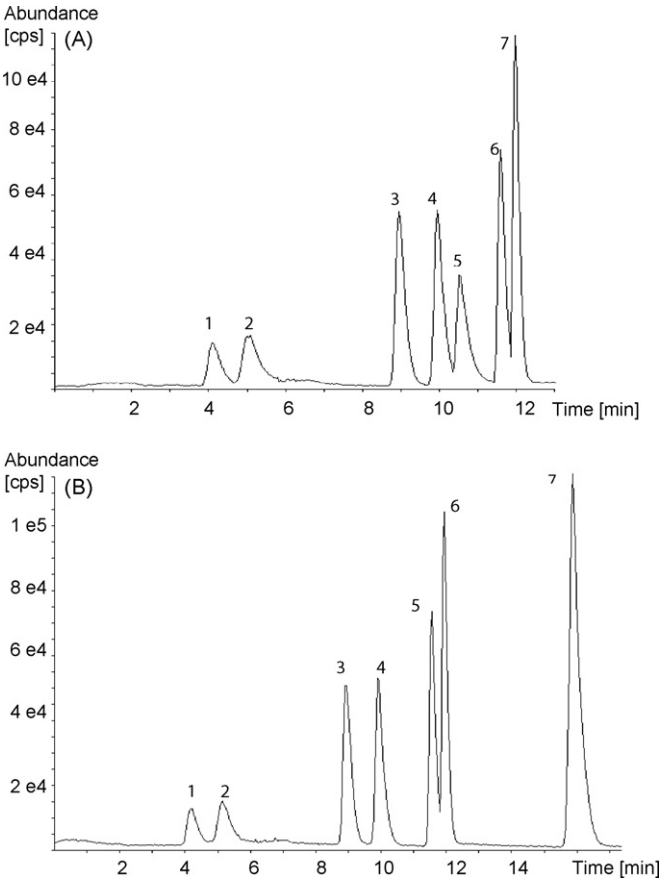


Fig. 2. Standard chromatograms by LC–MS (*c* = 100 ng/mL in 5 vol.% formic acid). (A) Metabolites in plasma with 1 EMPA, 2 IMPA, 3 iBMPA, 4 nBMPA, 5 PhPA (IS), 6 CHMPA and 7 PMPA. (B) Metabolites in urine with 1 EMPA, 2 IMPA, 3 iBMPA, 4 nBMPA, 5 CHMPA, 6 PMPA and 7 EHMPA (IS).

2.13. Sample preparation for the analysis of O-alkyl methylphosphonic acids in urine

Aliquots of 4 mL urine samples were centrifuged at 5300 × *g* to remove the urinary sediment. The supernatant was then mixed with 300 μL concentrated hydrochloric acid and a 40 μL aliquot of the internal standard solution (10 μg/mL EHMPA [36] in methanol). After mixing rigidly, 12 mL purified water were added for dilution before the sample was transferred to a preconditioned (1 mL methanol, 1 mL purified water) ENV+ cartridge (25 mg, 10 mL). The bed was sucked dry and eluted with 1 mL of the same mixture used for plasma processing. Ammonia and methanol were removed in the vacuum concentrator and the residue (500 μL) was made up to 1 mL by 10 vol.% formic acid. 20 μL of this solution were injected into the LC–MS system.

3. Results and discussion

3.1. Analysis of the unchanged nerve agents

The samples #3, #7, #12, #15, and #20 could be unequivocally identified: either the agent spiked into the plasma was determined or there was no doubt that the plasma had been left blank. In samples #8 and #11 small peaks were seen within the retention window of the V-agents, but signal to noise was too low for identification. After analysing the second eluates (see Section 2.8) by

Table 2
Results of the analysis of unchanged nerve agents after extraction from plasma.

Sample number #	Nerve agent	Remarks	Additional measurements	Results of the additional measurements
3	GF	u.i.		
7	No agent	n.c.s.		
8	Small peak within the retention window of V-agents	S:N < 3:1	GC–MS analysis of the second eluate (see Section 2.8)	V-agent
11	Small peak within the retention window of V-agents	S:N < 3:1	GC–MS analysis of the second eluate (see Section 2.8)	VR
12	No agent	n.c.s.		
15	GB	u.i.		
20	GD	u.i.		

u.i.: agent unequivocally identified by retention time and ratio of mass ions.
n.c.s.: negative control sample.

GC–MS, too, the agent in sample #11 could be unequivocally identified as VR. Nevertheless, it still was not possible to identify the agent in sample #8. Thus, the second eluates were additionally injected into the LC–MS system using the flow injection mode (FIA). After that, it was clear that indeed a V-agent was present in sample #8. But, as the analysis was performed in the FIA mode (without a column) it was not possible to decide which of the isomeric V-agents exactly had been spiked into the plasma.

Table 2 summarizes the results for the seven selected samples processed as described in Section 2.8.

3.2. Analysis of the regenerated nerve agents after fluoride-induced BChE reactivation

The search for regenerated nerve agents after fluoride-induced reactivation of BChE provided definitive results in five of seven samples (Table 3). The chromatograms of samples #3 and #8 were very noisy preventing unequivocal identification.

NAP 5 size exclusion columns were used during method development in order to remove any free agent from the mixture before starting fluoride-induced BChE reactivation. Hence, we used NAP 5 columns after thawing the reference plasma samples. By applying this additional sample processing step all small molecules were removed from plasma resulting in a substantial noise reduction facilitating interpretation in all cases.

A different problem was presented by sample #20: to our surprise, we found a well defined double peak at a retention time where no other fluoridated nerve agent eluted, which looked similar to the typical double peak of soman obtained by the method for neat agents. According to a TNO report on fluoride-induced reactivation [37], it was not possible to find a soman peak after reactivation of soman-inhibited human BChE due to rapid aging of the BChE-soman complex. Therefore, we omitted soman from method development for the fluoride-induced reactivation. However, Renner et al. [3] presented a method to recover soman by excess fluoride from the plasma of rats exposed by soman.

First, we injected a soman standard solution (100 ng/mL in 2-propanol) applying the conditions of the fluoride-induced reactivation method to prove whether this double peak would really be soman. Indeed, the double peak of the soman standard eluted exactly at the same time as the peak from sample #20. Then, we spiked two new plasma samples with soman and reactivated them according to the procedure described in Section 2.10 including the size exclusion step to remove free soman. Again, we received the characteristic double peak of soman. Thus, it is to assume that soman was bound to serum albumin as shown by Black et al. [17] and Williams et al. [21] and that it was regenerated during processing from its serum albumin adduct – a procedure presented by Adams et al. [38].

3.3. Analysis of the O-alkyl methylphosphonic acids extracted from plasma

As shown in Table 4, only sample #8 could not be identified. Two reasons for this finding are conceivable: first, the metabolites were not directly spiked into the plasma but were formed by hydrolysis during the spiking procedure at room temperature. The process was stopped by freezing the samples. Hydrolysis of VX is considered to be substantially slower than that of the G-agents due to the higher stability of the P–S bond compared to the P–F bond in G-agents [39]. Hence, the spiking time might have been too short to form enough VX metabolite for analysis. Second, the recovery of O-ethyl methylphosphonic acid (EMPA) was found to be only about 20% when the samples are processed as described in Section 2.12 while the other metabolites were recovered completely from plasma. This seems to be due to the moderate acidic conditions during extraction

Table 3
Results of the analysis of regenerated nerve agents after fluoride-induced reactivation of nerve agent-inhibited BChE.

Sample number #	Regenerated agent found	Remarks	Additional measurements	Results of the additional measurements
3		Very noisy baseline	Size exclusion step (see Section 2.10)	GF
7	No agent	n.c.s.		
8		Very noisy baseline	Size exclusion step (see Section 2.10)	Ethylsarin
11	Iso-butylsarin	Rather noisy baseline		
12	No agent	n.c.s.		
15	GB	u.i.		
20	Double peak similar to soman seen in the method for unchanged nerve agents	No reactivation of soman-inhibited BChE because of aging [37]	Soman standard and soman plasma control	GD

u.i.: agent unequivocally identified.
n.c.s.: negative control sample.

Table 4
Results of the analysis of the O-alkyl methylphosphonic acids after extraction from plasma.

Sample number #	Metabolite found	Corresponding agent	Remarks
3	CHMPA	GF	u.i.
7	No metabolite	–	n.c.s.
8			No hint found
11	iBMPA	VR	u.i.
12	No metabolite	–	n.c.s.
15	IMPA	GB	u.i.
20	PMPA	GD	u.i.

u.i.: agent unequivocally identified.
n.c.s.: negative control sample.

Table 5
Results of the analysis of the O-alkyl methylphosphonic acids after extraction from urine.

Sample number #	Metabolite found	Corresponding agent	Remarks
3	CHMPA	GF	u.i.
7	No metabolite	–	n.c.s.
8	EMPA	VX	u.i.
11	iBMPA	VR	u.i.
12	No metabolite	–	n.c.s.
15	IMPA	GB	u.i.
20	PMPA	GD	u.i.

u.i.: agent unequivocally identified by retention time and $[M-1]^-$.
n.c.s.: negative control sample.

from plasma which are not sufficient to suppress the dissociation of this phosphonic acid completely. In subsequent pilot experiments the acidic character of the extraction mixture was fortified resulting in elevated recoveries for EMPA, which was then found with 60%. Unfortunately, the modified procedure was not yet available during this intra-laboratory test.

3.4. Analysis of the O-alkyl methylphosphonic acids extracted from urine

In this case, the metabolites were directly spiked into the urine which simplified the detection of all agents. Therefore, we succeeded in identifying EMPA in sample 8 without any further measurements.

Although this was an artificial verification situation, it can be assumed that it would be easier to identify the agent(s) in a patient's urine than in a patient's plasma sample because the metabolites are eliminated rather quickly from plasma *via* the renal system leading to higher agent concentrations in urine compared to plasma.

Table 5 summarizes the results of the extraction procedure from urine.

4. Conclusion

All seven samples examined for the intra-laboratory verification test were correctly identified. No differences between the analytical results and the master list (Table 1) which was finally consulted for comparison could be detected. But, this intra-laboratory verification test clearly proved the need for the analysis of more than one biomarker analysed for the unequivocal verification. Best, a test battery for the analysis of different biomarkers is applied, but different biomarkers are only available when sampling is done within the first week after exposure. In case of delayed sampling fluoride-induced reactivation of inhibited BChE in plasma samples and analysis of O-alkyl methylphosphonic acids in urine are mandatory.

Original nerve agents in plasma are only available as a biomarker when sampling is performed within the first hours after exposure, and the collected plasma is stabilised for transportation by protein precipitation immediately after blood drawing and plasma separation. Furthermore, the plasma samples have to be frozen at −78 °C (solid carbon dioxide) until they arrive at the laboratory. Therefore, the test battery for unequivocal identification of nerve agents cannot be extended to the original agents extracted from plasma.

The presented intra-laboratory study emphasized the necessity to improve sample processing during fluoride-induced reactivation by size exclusion chromatography for removal of small molecules as a general processing step. Additionally, it could be shown that soman can be detected in plasma treated with excess fluoride.

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