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The Application of a Single-Column GC–MS-MS Method for the Rapid Analysis of Chemical Warfare Agents and Breakdown Products

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Abstract

The development of one comprehensive gas chromatographic-triple quadrupole mass spectrometric (GC–MS-MS) method for the analysis of nerve agents and their breakdown products can pose a challenge due to significant differences in analyte volatility. Nerve agent breakdown products typically have a low volatility, requiring a derivatization step prior to analysis by gas chromatography (GC). However, nerve agent parent compounds are generally more volatile, which eliminates the need for derivatization and allows for direct analysis. Therefore, the analysis of these analytes is typically performed using separate analytical methods. This may require the use of multiple columns composed of different stationary phases to ensure the most efficient separation. With the wide selection of GC columns and derivatizing agents, it is potentially possible to develop a single-column/analytical method that is suitable for the detection of nerve agents and their breakdown products. We evaluated six nerve agents (tabun, sarin, soman, cyclosarin, VX and Russian VX) and the six corresponding breakdown products (EDPA, IMPA, PMPA, CMPA EMPA and MMPA). Chromatographic separation and multiple-reaction mode electron ionization detection of the nerve agents and silylated breakdown product derivatives were performed using an Agilent 7890 A gas chromatography (GC) equipped with a mid-polarity column, coupled to a 7000 triple quadrupole mass spectrometry system. A fast (12.5 min), highly sensitive (picogram) and selective method was achieved. The feasibility of this method for nerve agent and breakdown product detection in real samples was demonstrated using nerve agent-spiked human plasma at various exposure times (3 min, 1 h and 24 h). Five of the six nerve agents and all six breakdown products were successfully detected. This robust method has utility as a rapid screening tool to identify a specific nerve agent in a potential exposure event by simultaneous detection of the parent and or its corresponding breakdown product in plasma.

Introduction

Nerve agents are a class of organophosphorus chemicals that include the G agents tabun (GA), sarin (GB), soman (GD) and cyclosarin (GF) and the V agents VX and Russian VX (VR). First developed in 1936, they gained popularity during World War II as they were synthesized as weapons of war. Recent nerve agent use has been reported in the news (1, 2). In early 2017, the half-brother of North Korean leader Kim Jong Un was assassinated by VX poisoning. Several months later, there was a suspected sarin attack on Syrian civilians by the Syrian government.

Nerve agents are known inhibitors of both butyryl- (BChE) and acetylcholinesterase (AChE) enzymes. It is the inhibition of AChE that poses a serious threat to human health. AChE enzymes catalyze the breakdown of acetylcholine, which is necessary for nerve impulse transmission. Exposure to these extremely toxic chemicals can typically be characterized by miosis, salivation and convulsions

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and other neurological manifestations that can ultimately result in death. There is a short latency period, dependent on dose and route, so symptoms can present in minutes (3). The V series agents are the most toxic of the nerve agents, with low volatility, and are highly stable and persistent in the environment. Primary routes of exposure are skin, eye and inhalation (4). The G agents are ~10 times less toxic than the V agents (5). However, their high volatility makes them highly lethal when inhalation is the route of exposure. Once nerve agents are absorbed by the body they bind to the AChE and BChE. AChE-adducted and BChE-adducted nerve agents can persist for as short as minutes to days (typically G agents) before they undergo an irreversible aging process, spontaneous enzymatic reactivation or hydrolysis (6). AChE-adducted and BChE-adducted nerve agents can be found in the red blood cells and plasma of humans, respectively. The reactivation and hydrolysis processes form alkyl methylphosphonic acid (AMPA) breakdown products in the blood, which are rapidly excreted in the urine (7). AMPAs, ethyl hydrogen dimethylphosphoramidate (EDPA), isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA), cyclohexyl methylphosphonic acid (CMPA), ethyl methylphosphonic acid (EMPA) and 2-(methyl)propyl methylphosphonic acid (MMPA) are unique to each nerve agent precursor (as shown in Figure 1). The AMPAs can each serve as a marker of exposure, since they can be found in biological matrices such as urine, plasma and serum. They are less toxic and less volatile than their precursor; therefore they require derivatization for detection by GC.

The existing detection schemes for nerve agents or their breakdown products include chromatographic, electrochemical, colorimetric and spectrometric techniques (8–12). While a myriad of published methods have successfully detected nerve agents or AMPAs, currently no single method can simultaneously detect both the nerve agent and breakdown product. The nerve agents are mostly volatile, making GC the more suitable approach. On the other hand, liquid chromatography is ideal for the breakdown products. Each of these limitations likely contributes to the absence of a comprehensive method.

This present paper describes a method that can simultaneously detect GC-amenable nerve agents and non-volatile polar breakdown products in an individual sample using one column. The GC is suitable for chromatographic separation of both nerve agents and breakdown products with the use of a mid-polarity column and the application of a derivatizing reagent. The derivatizing reagent will convert the highly polar molecules to GC-amenable derivatives with the addition of a functional group that increases the compounds' volatility. The nerve agents remain intact and maintain their volatility. N-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA with 1% t-BDMCS) reagent converts the breakdown products to GC-amenable derivatives. The (14%-cyanopropyl-phenyl) polydimethylsiloxane stationary phase of the VF-1701 MS column improves the retention of the nerve agents and breakdown products generated for each standard calibration curve. This paper will present a rapid (12.5 min), highly sensitive (picogram [pg]) and selective method for the simultaneous analysis and detection of five nerve agents and their corresponding breakdown products.

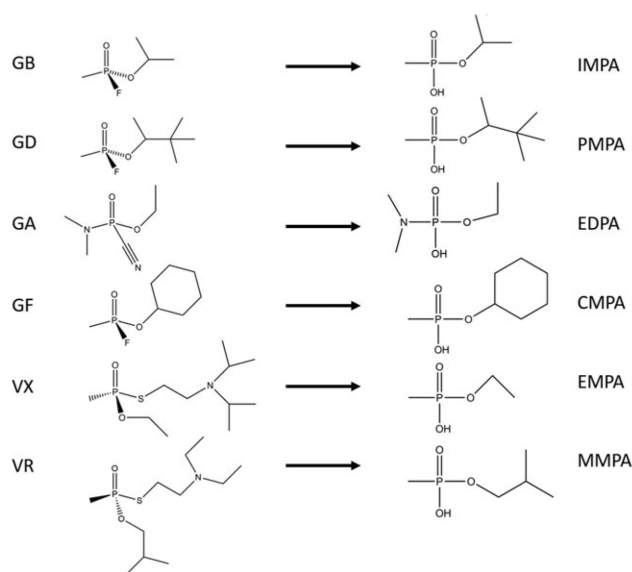

Figure 1. Structural depiction of nerve agents and their corresponding breakdown products.

Methods

Materials/reagents

Isopropanol (IPA) and dichloromethane (DCM) were purchased from Fisher Scientific (Waltham, MA, USA). Hydrochloric acid (HCl) (molecular biology grade, 36.5-38.0%) and MTBSTFA (with 1% t-BDMCS) were purchased from Sigma Aldrich (Allentown, PA, USA).

Dilute nerve agents GA, GB, GD, GF (less than 2 mg/mL) and VX and VR (less than 1 mg/mL) were obtained in IPA (with the exception of GA in multisol (48.5% water, 40% propylene glycol, 10% ethanol and 1.5% benzyl alcohol)) from the USAMRICD chemical agent vault. Stocks were stored at −80°C until used. Diisopropyl fluorophosphate (DFP), used as an internal standard for the nerve agents, was purchased from Sigma Aldrich (Allentown, PA, USA) and stored at −20°C until used. The nerve agent breakdown products (EDPA, IMPA, PMPA, CMPA, EMPA and MMPA) and the internal standard methylphosphonic acid-d$_3$ (MPA-d$_3$) were purchased from Cerilliant (Round Rock, TX, USA). The stocks were stored at −20°C until used. The structures of each compound are illustrated in Figure 1.

Pooled plasma (with sodium heparin) was purchased from Bioreclamation (Hicksville, NY). It was stored at −80°C until used.

Standard preparation

Standard mixtures were prepared in IPA at a stock concentration of 1000 ng/mL. A seven-point calibration curve was prepared in IPA at concentrations ranging from 1.95 to 125 ng/mL. The DFP and MPA-d$_3$ were spiked into the calibration standards at 100 ng/mL. The DFP was served as an internal standard for the intact nerve agents and MPA-d$_3$ for the breakdown products.

Two quality control standards (QCs), a low (LQC) and high (HQC), were prepared at 11.7 and 93.8 ng/mL concentrations by mixing two calibration standards (7.8 with 15.6 ng/mL and 62.5 with 125 ng/mL), respectively.

One milliliter aliquots of the calibration standards and QC standards were transferred to GC vials, and 20 µL of MTBSTFA (w/t-BDMCS) derivatizing reagent was added. The samples were derivatized for 1 h at 40°C, which resulted in the formation of tert-butyldimethylsilyl (TBDMS) breakdown product derivatives. The samples were immediately analyzed by GC–MS-MS. The derivatization step was completed each day for the inter-day study.

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Instrument analysis

Analysis was performed using an Agilent 7890 A GC system coupled to a 7000 triple quadrupole mass spectrometry system. Separation was achieved with a 30 m × 0.25 mm Agilent VF-1701 MS column with a film thickness of 0.25 µm. Helium carrier gas was set to a flow rate of 1.1 mL/min. Samples were injected at 1 µL volume into the single tapered liner containing no glass wool, enclosed in a splitless inlet heated at 230°C with a 1-min purge time. The oven was initially heated to 35°C, held for 1.5 min, and then ramped at 30°C/min to 275°C, then 5°C/min to 290°C. The transfer line, source and quadrupole were heated to 280°C, 230°C and 150°C, respectively. Multiple-reaction monitoring (MRM) mode was selected for the acquisition of data, to minimize interferences from the matrix, resulting in improved sensitivity and selectivity of the CWA. Positive electron impact ionization was employed using the parameters summarized in Table I.

To develop the MS–MS acquisition method, individual standards of each target analyte were first injected in full-scan mode. The retention times and precursor ions were determined. Product ion scan was performed to elucidate the most abundant product ions. After the precursor ions and product ions were selected, MRM scan was performed using the selected transitions to verify that they yielded the optimal peak shape and maximum peak area. The two most abundant transitions (quantitation and qualitative confirmation) for each analyte were monitored at different collision energies, and the collision energy with the greatest peak area was selected. One transition was selected for each internal standard. Multiple acquisition windows were established during the 12.5-min run time to optimize the mass spectral sensitivity. Therefore, the transition dwell times were dependent upon the number of transitions monitored per window and varied between analyte transitions. A total of 13 data acquisition points per chromatographic peak were collected. The acquisition parameters were optimized by analyzing the target analytes at various inlet, transfer line, source and initial oven temperatures and carrier gas flows to determine the conditions that yielded the optimal chromatographic separation, peak shape and abundance. The selected GC and MS–MS conditions are summarized in Table I.

Method validation

Linearity

This method was validated for linearity, limit of detection (LOD), lower limit of quantitation (LLOQ), intra- and inter-day precision, and intra- and inter-day accuracy. Calibration curves were prepared for GA, GB, GD, GF, VX, VR, EDPA, IMPA, PMPA, CMPA, EMPA and MMPA with calibrator concentrations at 1.95, 3.91, 7.81, 15.6, 31.3, 62.5 and 125 ng/mL. Two internal standards were assigned, DFP for the nerve agents and MPA-d_3 for the breakdown products. Regression coefficients were calculated from the weighted equations ($1/x$ for all agents and breakdown products, except $1/x^2$ for VX and VR) that were generated for each standard calibration curve.

Inter-day precision and accuracy

Five calibration curves and five sets of QC standards were analyzed for five consecutive days, one curve and QC set each day, to examine the inter-day precision and accuracy. Precision was expressed as % coefficient of variation (%CV) and was calculated as the standard deviation (SD)/mean of calculated concentration. Accuracy was calculated as % difference between the actual and theoretical concentration.

Intra-day precision and accuracy

Five calibration curves and five sets of QC standards were analyzed in one day to assess the intra-day precision and accuracy.

Table I. GC–MS-MS acquisition parameters

Target analyte	RT (min)	Transition ions (*m/z*)	Dwell time (ms)	CE (eV)
GB	5.58	Quant 98.9 -> 80.9	38	15
		Qual 124.9 -> 98.9	38	15
GD	6.67	Quant 126 -> 82	38	0
		Qual 126 -> 99	38	10
GA	7.43	Quant 132.9 -> 105.9	38	5
		Qual 70.1 -> 43	38	5
GF	7.65	Quant 82 -> 66.9	38	40
		Qual 98.9 -> 66.9	38	5
VX	9.68	Quant 114.1 -> 72	12	5
		Qual 114.1 -> 43	12	20
VR	9.76	Quant 99 -> 56	12	15
		Qual 71 -> 56	12	5
EMPA TBDMS derivative	7.85	Quant 153.1 -> 74.9	24	20
		Qual 181.0 -> 153.1	24	10
IMPA TBDMS derivative	7.92	Quant 153.1 -> 74.9	24	20
		Qual 194.9 -> 153.1	24	10
EDPA TBDMS derivative	8.18	Quant 209.8 -> 181.8	38	10
		Qual 181.8 -> 138.8	38	10
MMPA TBDMS derivative	8.45	Quant 153.1 -> 74.9	24	20
		Qual 194.9 -> 153.1	24	10
PMPA TBDMS derivative	8.86	Quant 153.1 -> 74.9	24	20
		Qual 194.9 -> 153.1	24	10
CMPA TBDMS derivative	9.64	Quant 153.1 -> 74.9	12	20
		Qual 194.9 -> 153.1	12	10
DFP	6.17	Quant 127 -> 101	75	10
MPA-d_3 TBDMS derivative	8.95	Quant 156 -> 74.9	24	10

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Limit of detection and lower limit of quantification

The analyte's relative response was plotted against the theoretical concentrations of the standard solutions to generate calibration curves. The linear regression equation was calculated by the method of least squares. The LOD was determined by extrapolation using the signal-to-noise value of the lowest calibration standard to calculate the concentration that would yield a signal-to-noise value of 3. The LLOQ was defined as the concentration at the lowest calibrator that met the acceptance criteria of ≤20% accuracy and precision. Intra-day and inter-day accuracy and precision were evaluated using five replicates of a low- and high-QC standard at concentrations of 11.72 and 93.75 ng/mL, respectively. An acceptable accuracy (% error) was determined based on a QC value of ≤15% of the true value. A precision (% CV) of ≤15% between replicates was considered acceptable. All calibrators and QCs had a minimum S/N ratio of greater than 3:1.

Spiked plasma sample preparation

The efficiency of the GC–MS-MS method to accurately detect and identify the nerve agents and hydrolysis breakdown products in plasma was assessed. This analysis will show the effects of time on nerve agent hydrolysis and breakdown product formation. Triplicate plasma samples (500 µL) were fortified with a 10 ng/mL mixture of GA, GB, GD, GF, VX and VR in IPA. Three exposure times (3 min, 1 h and 24 h) at room temperature were assessed to allow for the detection of varying degrees of nerve agent and breakdown products in the samples. The reactions were quenched to prevent further conversion of the nerve agents to breakdown products, and the analytes were extracted with the addition of 500 µL of DCM. Concentrated HCl (50 µL) was added to each sample to decrease the pH and for protein precipitation. DFP and MPA-d_3 were spiked at 100 ng/mL to serve as internal standards for the nerve agents and breakdown products, respectively. The samples were vortexed for 30 s, then centrifuged with an Eppendorf Centrifuge 5418 R (Hamburg, Germany) at 13,000 rpm and 20°C for 10 min. The organic layer was transferred to a GC vial. The 3-min and 1-h time point samples were frozen overnight at −80°C until the 24-h time point sample was processed. Aliquots of 100 µL of each sample were transferred to a GC vial insert and derivatized with 2 µL of MTBSTFA (with 1% t-BMDCS) for 1 h at 40°C. Samples were analyzed by GC–MS-MS. Triplicate negative control samples containing internal standard (100 ng/mL) spiked in plasma containing the extraction solvent to minimize hydrolysis. Positive control samples containing a mixture of nerve agents (10 ng/mL), breakdown products (10 ng/mL) and internal standards (100 ng/mL) spiked in plasma that contained extraction solvent were also extracted. The negative and positive control samples were extracted immediately after spiking. The normalized percent recoveries were estimated by calculating the adjusted peak area of the sample analyte relative to the adjusted peak area of the positive control, multiplied by 100. The positive control represents the 100% concentration of 10 ng/mL mixture of nerve agents and breakdown products in plasma.

Data processing

Data were processed using Mass Hunter Workstation Quantitative Analysis B.05.00. The criteria used to characterize each analyte were retention time, relative response, peak algorithm, quantitation and confirmation transitions. Quantification was performed by the integration of the area under the target peak of the MRM chromatogram relative to the integrated area under the internal standard peak. A weight of $1/x$ was applied to the chromatographic data for the target analytes ($1/x^2$ for the V agents) to generate the linear regression fit. Graphical representations were prepared using Graphpad Prism 7 software.

Results and Discussion

The six nerve agents and six metabolites were successfully separated and identified using the developed method. The extracted ion chromatograms for a representative quantitative and qualitative ion transition for each target nerve agent and breakdown product at 15.6 ng/mL are shown in Figures 2 and 3. Figure 4 depicts the representative chromatograms for the internal standards (DFP and MPA-d_3) at 100 ng/mL.

The validation results are within the acceptance criteria set forth in the previous method validation section. Tables II and III detail the figures of merit that were obtained during the validation study.

Selectivity

The rapid GC oven program allowed for the separation of all six nerve agents and six breakdown products within 12.5 min. The peaks in the extracted ion chromatogram were each baseline resolved, with superior peak symmetry. Method selectivity was achieved with the combination of chromatographic retention and mass spectrometric MRM transitions. Additionally, a second transition was used for confirmation.

Linearity

The calibration curves were linear from 1.95 to 125 ng/mL for all analytes except the V agents (linearity from 7.80 to 125 ng/mL). The mean correlation coefficient (r^2) values were all >0.9957 except for the V agents which were >0.9571.

Inter-day accuracy and precision

For all agents and breakdown products, inter-day accuracy and precision (expressed as %CV) ranged from 0% to 11% and 0% to 10%, respectively. All nerve agent and breakdown products fell within the validation acceptance criteria for method accuracy (85–115%) and method precision (≤15%).

Intra-day accuracy and precision

The intra-day accuracy and precision for all agents and breakdown products ranged from 1% to 10% and 1% to 11% (expressed as % CV), respectively. All nerve agent and breakdown products fell within the validation acceptance criteria for method accuracy (85–115%) and method precision (≤15%).

LOD and LLOQ

The LOQs for all analytes were 1.95 ng/mL (except 7.80 ng/mL for VX and VR). The LODs across all analytes were in the pg/mL range, between 3.18 and 780 pg/mL. The calculated values are presented in Table III.

Method development

Due to the range of polarities of the target analytes, the mid-polarity VF-1701 MS column was implemented to achieve better separation of the more polar analytes such as the V agents. The analytes

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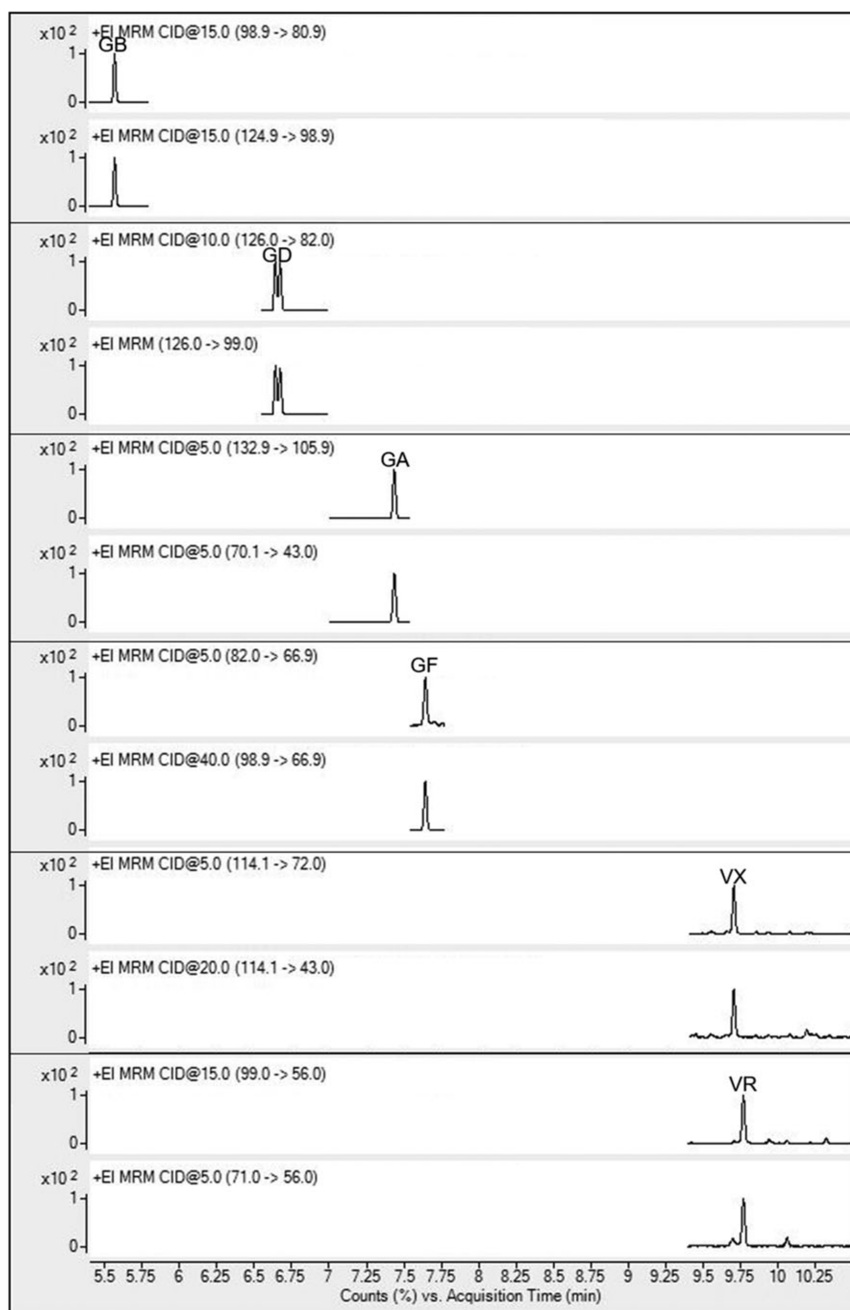

Figure 2. Representative extracted ion chromatograms of quantitative (top) and qualitative (bottom) ion transitions for 15.6 ng/mL target nerve agent standards.

exhibited improved chromatographic resolution and peak symmetry with the application of a single tapered inlet liner free of glass wool. The low GC initial oven and inlet temperatures increased the peak abundance for the early eluting compounds, for instance, GB and GD. However, higher peak abundance was observed at higher inlet temperatures for the late eluting compounds. Therefore, the selected inlet temperature was in the mid-range of those optimal temperatures. A flow rate of 1.1 mL/min ensured the elution of all 12 analytes and internal standards within 10 min. The additional 2.5-min runtime allows for high-temperature column conditioning to eliminate contaminants in dirty matrices. The collision energies selected for each analyte transition had the highest peak abundance while retaining superior peak shape.

Method validation

The GC–MS-MS method was validated for linearity, LLOQ, accuracy and precision. The reduction of column and source contamination was critical to the successful validation. A solvent delay was employed at the beginning of the run to send the solvent and derivatizing reagent to the split vent. The detector was also turned off during times that the analytes were not eluting.

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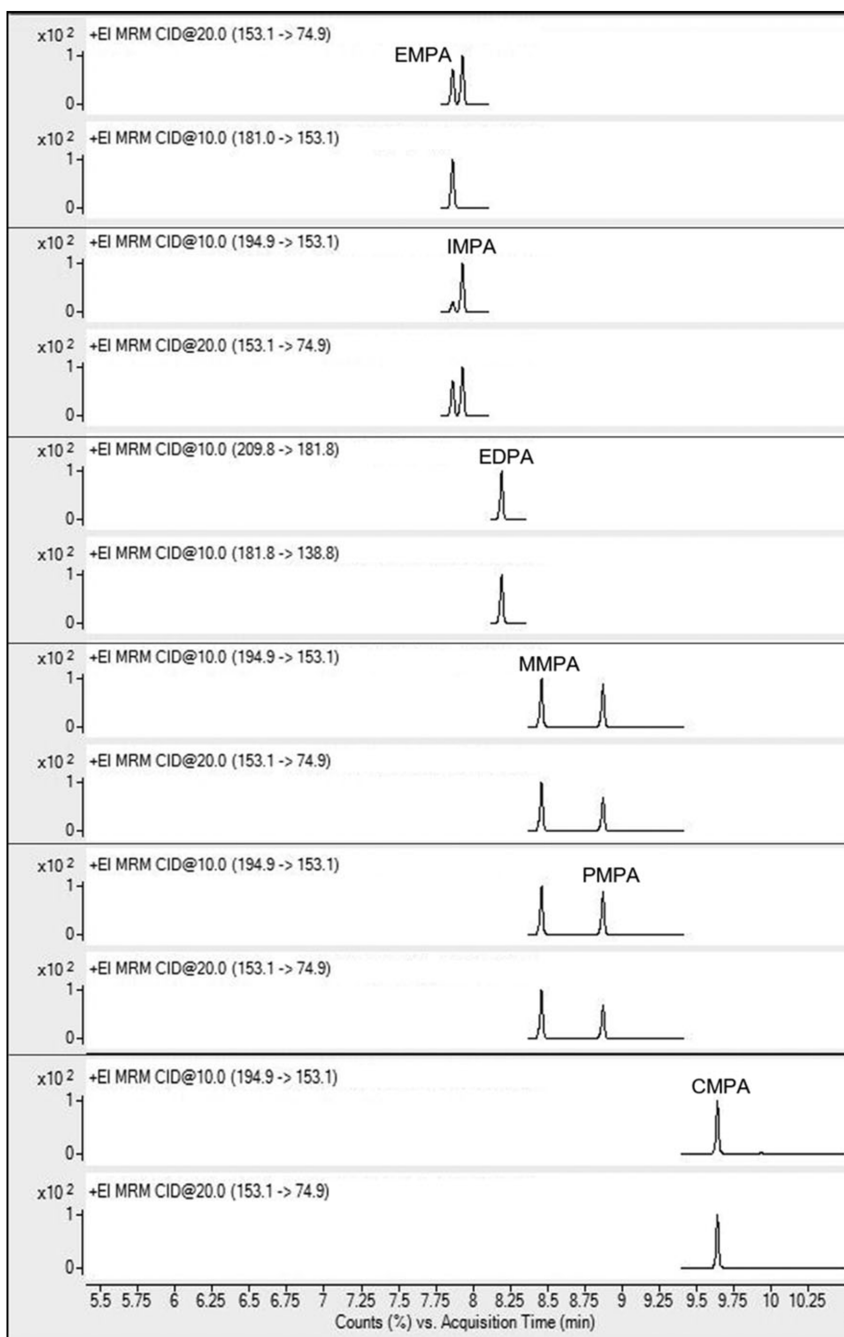

Figure 3. Representative extracted ion chromatograms of quantitative (top) and qualitative (bottom) ion transitions for 15.6 ng/mL target nerve agent breakdown product standards.

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The LLOQ for the analytes is within the range detected in clinical samples (13). The higher LLOQ for the V agents is attributed to their tendency to stick to the inlet liner. The characteristic sticky nature of the V agents causes a decreased peak abundance, resulting in a shorter linear range.

DFP proved to be a good selection as an internal standard for the nerve agents. Similar in structure to the nerve agents, DFP was expected to exhibit behavior similar to that of the nerve agents. Therefore, the method was highly sensitive for DFP. MPA-d_3 served as the internal standard for the breakdown products. Although MPA-d_3 is a deuterated analog of the secondary breakdown product of the nerve agents, the peak abundance was lower than that of the primary breakdown products at the same concentration. This was likely due to increased polarity of MPA-d_3, as compared to the breakdown products. Therefore, a high concentration of MPA-d_3 was necessary to ensure consistent recovery of the internal standard.

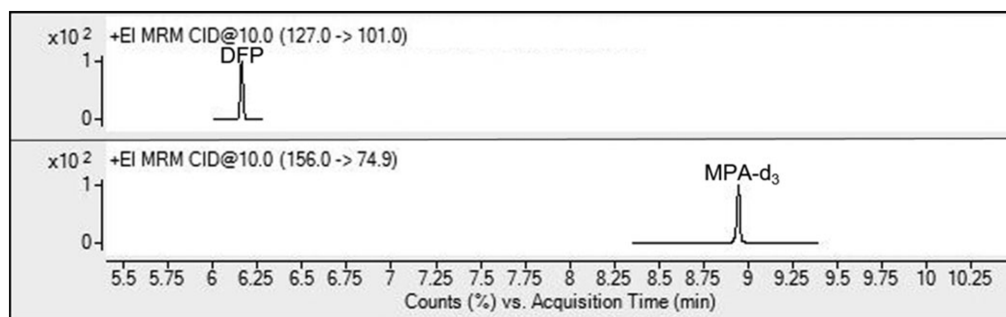

Figure 4. Representative extracted ion chromatograms of quantitative ion transitions for 100 ng/mL internal standards.

Table II. Figures of merit for the nerve agents and breakdown products

Analyte	Intra-day				Inter-day			
	% CV		% Error		% CV		% Error	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
GB	3	2	3	2	2	0	1	1
GD	3	2	2	2	1	0	2	4
GA	2	1	4	3	0	1	3	3
GF	11	2	2	5	10	1	4	2
VX	3	2	5	9	5	4	3	10
VR	9	8	9	10	8	4	3	11
EMPA TBDMS derivative	2	2	3	1	4	1	5	0
IMPA TBDMS derivative	3	2	4	1	6	2	1	1
EDPA TBDMS derivative	3	3	5	3	7	2	2	0
MMPA TBDMS derivative	2	1	4	2	4	1	2	1
PMPA TBDMS derivative	2	2	4	1	4	2	2	2
CMPA TBDMS derivative	1	1	7	2	4	2	4	1

%CV—coefficient of variation (%), LQC—low-quality control standard, HQC—high-quality control standard.

Table III. Summary of validation result

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Mean (min–max) R^2		Regression Fit	Weight	Linear Range (ng/mL)
			Intra-day	Inter-day			
GB	0.003	1.95	0.9997 (0.9991–1.000)	0.9996 (0.9993–0.9999)	Linear	$1/x$	1.95–125
GD	0.013	1.95	0.9996 (0.9992–0.9999)	0.9994 (0.9991–0.9996)	Linear	$1/x$	1.95–125
GA	0.128	1.95	0.9982 (0.9976–0.9988)	0.9978 (0.9969–0.9988)	Linear	$1/x$	1.95–125
GF	0.781	1.95	0.9987 (0.9986–0.9989)	0.9989 (0.9975–0.9995)	Linear	$1/x$	1.95–125
VX	0.279	7.81	0.9807 (0.9770–0.9850)	0.9781 (0.9700–0.9864)	Linear	$1/x^2$	7.81–125
VR	0.287	7.81	0.9747 (0.9667–0.9867)	0.9698 (0.9571–0.9825)	Linear	$1/x^2$	7.81–125
EMPA TBDMS derivative	0.046	1.95	0.9996 (0.9993–0.9999)	0.9981 (0.9957–0.9992)	Linear	$1/x$	1.95–125
IMPA TBDMS derivative	0.031	1.95	0.9996 (0.9992–0.9999)	0.9992 (0.9985–0.9999)	Linear	$1/x$	1.95–125
EDPA TBDMS derivative	0.011	1.95	0.9990 (0.9985–0.9996)	0.9979 (0.9972–0.9984)	Linear	$1/x$	1.95–125
MMPA TBDMS derivative	0.034	1.95	0.9986 (0.9973–0.9996)	0.9992 (0.9979–0.9998)	Linear	$1/x$	1.95–125
PMPA TBDMS derivative	0.033	1.95	0.9994 (0.9987–0.9999)	0.9992 (0.9985–0.9997)	Linear	$1/x$	1.95–125
CMPA TBDMS derivative	0.027	1.95	0.9988 (0.9974–0.9996)	0.9995 (0.9987–0.9999)	Linear	$1/x$	1.95–125

Application to spiked plasma samples

Five of the six nerve agents (GA, GD, GF, VX and VR) and all six of the breakdown products (EDPA, IMPA, PMPA, CMPA, EMPA and MMPA) in fortified plasma were successfully detected and identified with the validated GC–MS-MS method. There was a positive relationship between the nerve agent exposure time in plasma and the simultaneous degradation of nerve agent and formation of the breakdown product. Degradation of the nerve agent was observed by the decrease in peak area and the formation of breakdown product by the increase in peak area. GB was not detected in the plasma. The detected analyte recoveries were normalized against the mean positive control recoveries to determine the final detected concentrations. Respectively, Table IV and Figure 5 summarize the normalized mean peak responses and normalized percent recoveries for

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Table IV. Mean concentration (ng/mL) of analytes present in spiked plasma extracts

Analyte	Exposure time					
	3 min		1 h		24 h	
	Mean conc.	Std dev	Mean conc.	Std dev	Mean	Std dev
GB	ND	ND	ND	ND	ND	ND
GD	3.18	0.120	1.52	0.00800	0.868	0.0545
GA	8.21	0.483	2.12	0.0835	1.523	0.0136
GF	1.62	1.06	1.09	0.295	0.820	0.137
VX	8.23	3.26	5.51	1.36	3.98	0.240
VR	9.44	2.12	7.06	0.396	2.17	0.310
EMPA	1.86	0.386	2.31	0.752	3.65	0.421
IMPA	2.86	0.534	5.33	1.10	9.59	1.53
EDPA	3.98	1.01	3.93	0.933	4.25	1.67
MMPA	0.788	0.0977	1.09	0.127	4.65	0.729
PMPA	2.24	0.376	4.83	0.922	8.48	1.44
CMPA	2.41	0.370	4.09	0.775	7.37	1.32

ND, not detected.

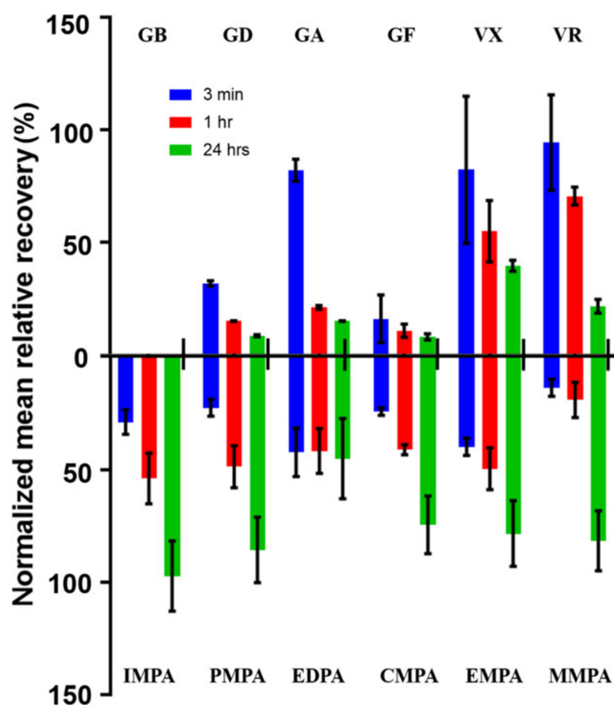

Figure 5. Graphical representation of nerve agents and breakdown products detected in spiked plasma standards at 3 min, 1 h and 24 h time points. The error bars display the standard deviation of the mean (n = 3) relative recovery.

nerve agent and breakdown products detected in the spiked plasma samples.

The spiked nerve agents and breakdown products were extracted from the plasma samples with DCM. The DCM was effective at preventing further hydrolysis of the nerve agent from occurring as well as extraction of the less polar nerve agents from the matrix. DCM was added to the plasma of the positive control plasma samples directly before spiking with the nerve agent/breakdown product standard mixture. Nerve agent/breakdown products partition immediately into the DCM thus minimizing any breakdown processes that may occur, which allowed the positive control to serve as a 100% response plasma standard. The application of the HCl to the plasma samples assisted in crashing the proteins out of solution as well as reducing the pH to protonate the breakdown products to help partition them to the organic phase. The extraction solvent was effective at the isolation of both the non-polar nerve agents and the polar breakdown products from the plasma.

GB was the only nerve agent that was not detected. It is well known that the parent agents, especially G-type, are relatively short-lived due to hydrolysis and rapid binding to plasma/tissue proteins (14). Animal toxicokinetic studies following intravenous agent administration demonstrate the short residence time is especially related to G-agents relative to more persistent V-type agents (15, 16). It is highly likely that the inability to detect GB was attributable to both rapid protein binding and conversion to IMPA after it was spiked into plasma.

The goal of this study was to develop a simple process for extracting and analyzing free agents and related AMPA products from plasma. The procedure has utility as a rapid screening tool to facilitate verifying the identity of a specific nerve agent in a potential exposure event by detecting the parent and or its corresponding breakdown product. Results from method validation in plasma indicate excellent precision and accuracy. Benefits include a simple, rapid and robust means to simultaneously identify and quantify the analytes in plasma using a single-sample preparation process and GC–MS-MS analysis. Since the method focuses on the intact agent or the AMPA analytes, it does not address analysis of agent bound to proteins such as cholinesterases or human serum albumin. As such it does not account for all the processes involved in the total disposition of the agent. Also not assessed was the complete hydrolysis of any of the AMPA's to the common product MPA. Since the source of MPA cannot be related back to a specific agent identity, its presence in a plasma sample provided no information on the parent agent from which it was derived and was not considered for the method.

Conclusion

A robust GC–MS-MS method for the simultaneous detection and identification of nerve agents and breakdown products was validated. Results in this report reveal a sensitive and selective method that offers high accuracy and precision. The LLOQs were 1.95 ng/mL (7.80 ng/mL for V agents) for all agents and breakdown products. The LODs ranged from 0.003 to 0.780 ng/mL. All reported least squares regression values were >0.99 (except for the V agents, >0.95). The feasibility of this method for the detection of nerve agents and metabolites in biological samples was demonstrated with their successful detection in spiked plasma samples.

Disclaimer

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References

1. Gladstone, R. (2017) Report confirms that chemical arms were used on Syrian villagers. *New York Times*. https://www.nytimes.com/2017/06/29/world/middleeast/syria-sarin-chemical-weapons-united-nations.html (accessed Oct 17, 2017).
2. Paddock, R.C., Sang-Hun, C. (2017) Kim Jong-nam was killed by VX nerve agent, Malaysians say. *New York Times*. https://www.nytimes.com/2017/02/23/world/asia/kim-jong-nam-vx-nerve-agent-.html (accessed Oct 17, 2017).
3. Geoghegan, J., Tong, J.L. (2006) Chemical warfare agents. *Continuing Education in Anaesthesia Critical Care & Pain*, **6**, 230–234.
4. Centers for Disease Control and Prevention. (2015) "Facts about VX," Emergency Preparedness and Response. *CDC*. https://emergency.cdc.gov/agent/vx/basics/facts.asp (accessed Oct 17, 2017).
5. Spradling, K.D., Dillman, J.F. (2011) The molecular toxicology of chemical warfare agents. *Advances in Molecular Toxicology*, **5**, 113.
6. Delfino, R.T., Ribeiro, T.S., Figueroa-Villar, J.D. (2009) Organophosphorus compounds as chemical warfare agents: a review. *Journal of the Brazilian Chemical Society*, **20**, 407–428.
7. Smith, J.R., Capacio, B.R. (2009) Mass spectrometry applications for the identification and quantification of biomarkers resulting from human exposure to chemical warfare agents. *NATO Science and Security Series A: Chemistry and Biology, Detection of biological agents for the prevention of bioterrorism*, 191.
8. Barr, J.R., Driskell, W.J., Aston, L.S., Martinez, R.A. (2004) Quantitation of metabolites of the nerve agents sarin, soman, cyclohexylsarin, VX, and Russian VX in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology*, **28**, 372–378.
9. Hamelin, E.I., Schulze, N.D., Shaner, R.L., Coleman, R.M., Lawrence, R.J., Crow, B.S., *et al.* (2014) Quantitation of five organophosphorus nerve agent metabolites in serum using hydrophilic interaction liquid chromatography and tandem mass spectrometry. *Journal of Analytical and Bioanalytical Chemistry*, **406**, 5195–5202.
10. Stan'kov, I.N., Sergeeva, A.A., Derevyagina, I.D., Konovalov, K.V. (2003) Gas-chromatographic determination of sarin, soman, and o-isobutyl-s-2-(n,n-diethylamino)ethyl methylphosphonothioate (a VX-like compound) traces in water. *Journal of Analytical Chemistry*, **1058**, 160–164.
11. Liu, G., Lin, Y. (2005) Electrochemical sensor for organophosphate pesticide and nerve agents using zirconia nanoparticles as selective sorbents. *Journal of Analytical Chemistry*, **77**, 5894–5901.
12. Kanga, M.J., Burks, R.M., Atwater, J., Lukowicz, R.M., Williams, P., Holmes, A.E. (2017) Colorimetric sensor arrays for the detection and identification of chemical weapons and explosives. *Critical Reviews in Analytical Chemistry*, **47**, 138–153.
13. Nakajima, T., Sasaki, K., Ozawa, H., Sekjima, Y., Morita, H., Fukushima, Y., *et al.* (1998) Urinary metabolites of sarin in a patient of the Matsumoto sarin incident. *Archives of Toxicology*, **72**, 601–603.
14. Capacio, B.R., Smith, J.R., Gordon, R., Haigh, J.R., Barr, J.R., Lukey, B.J. Clinical detection of exposure to chemical warfare agents. In: Romano, J.A., Jr., Lukey, B.J., Salem, H. (eds). Chemical Warfare Agent: Chemistry, Pharmacology, *Toxicology, and Therapeutics*, 2nd edition. CRC Press, Taylor and Francis Group, LLC: Boca Raton, Florida, 2008; pp 501–548.
15. Benschop, H.P., De Jong, L.P.A. (1991) Toxicokinetics of soman: species variation and stereospecificity in elimination pathways. *Neuroscience and Biobehavioral Reviews*, **15**, 73–77.
16. van der Schans, M.J., Lander, B.J., van der Wiel, H., Langenberg, J.P., Benschop, H.P. (2003) Toxicokinetics of the nerve agent (±)VX in anesthetized and atropinized hairless guinea pigs and marmosets after intravenous and percutaneous administration. *Tox Appl Pharmacol*, **191**, 48–62.

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