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Isotope dilution LC/MS/MS for the detection of nerve agent exposure in urine

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Abstract

Organophosphorus nerve agents (OPNA), chemically related to and derived from organophosphate insecticides, constitute a clear and present threat to both military and civilian targets. Military regimes and terrorist organizations have demonstrated the will and ability to produce mass casualties by dispersing organophosphorus nerve agents, which, in turn could terrorize populations and overwhelm healthcare systems. A high throughput, robust and sensitive analytical protocol has been developed for the quantitation of the urinary metabolites of sarin (GB), soman (GD), VX, Russian VX (RVX) and cyclohexylsarin (GF) utilizing solid phase extraction (SPE) followed by High Performance Liquid Chromatography (HPLC)–isotope dilution tandem mass spectrometry (LC/MS/MS). The method has demonstrated linearity and reproducibility (1–200 ng/mL) for all analytes and has a Limit of Quantitation (LOQ) $\leq$ 0.5 ng/mL for all analytes (S/N $\geq$ 10/1). The method was validated by performing 20 individual analyses over 10 days by five scientists with all values falling within two standard deviations of the mean.
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Keywords: Nerve agents; LC/MS/MS; Method development

1. Introduction

Nerve agents are among the most toxic chemical substances known at this time and pose a two-fold threat making them an ideal weapon for rogue states and terrorist organizations. The first effect is physiological due to their acute toxicity. The second effect is psychological due to their ability to cause panic in affected or perceived affected populations which could overwhelm health care systems.

Following exposure OPNAs irreversibly inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the central and peripheral nervous systems resulting in toxic accumulation of acetylcholine (ACh) [1–3]. During normal function, AChE hydrolyzes ACh to both choline and acetic acid. Choline is then regenerated to ACh [1]. When AChE is inhibited by a nerve agent, or other organophosphorus compound, it becomes incapable of degrading ACh causing the peripheral and central nervous system synapses to continuously signal. The clinical effects of nerve agents are the result of fatigue at the receptor site due to continuous and persistent stimulation of ACh. The broad spectrum of symptoms associated with exposure to nerve agents (e.g. rhinorrhea, ocular effects, respiratory difficulties and chest pain), are due to the aforementioned stimulation by ACh at the muscarinic, nicotinic and central nervous system synapses [1]. Military grade nerve agents generally display a greater toxicity to humans and laboratory mammals [2,3]. Alternately, in the case of the sarin attacks in the Toyko subway, the sarin used was from a crude synthesis and of relatively poor purity, yet the toxicological effects were pronounced [4].

In animal studies exposure to nerve agents resulted in the parent compounds hydrolyzing to their corresponding phosphonic acids and covalently binding to enzymes and tissue proteins [1–3]. Urinary excretion of the phosphonic acid metabolite is the primary elimination route for nerve agents following exposure [4]. In the body the primary leaving group is fluorine for GB, GD and GF. For VX the leaving group is the thiomoiety. The phosphonic acid resulting from the leaving group acts as an agent specific marker for quantitating each nerve

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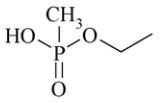
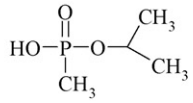


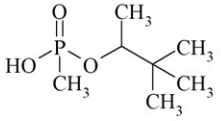
VX Acid

Molecular Weight =124.08
Molecular Formula =$C_3H_9O_3P$



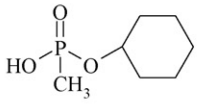
Sarin (GB) Acid

Molecular Weight =138.10
Molecular Formula =$C_4H_{11}O_3P$



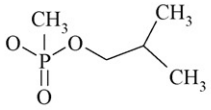
Soman (GD) Acid

Molecular Weight =180.19
Molecular Formula =$C_7H_{17}O_3P$



Cyclohexyl methylphosphonic (GF) Acid

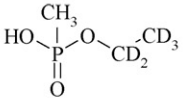
Molecular Weight =178.17
Molecular Formula =$C_7H_{15}O_3P$



RVX Acid

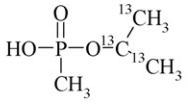
Molecular Weight = 152
Molecular Formula = $C_5H_{13}O_3P$

(a)



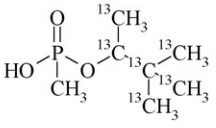
VX Acid

Molecular Weight =129
Molecular Formula =$C_3H_4D_5PO_3$



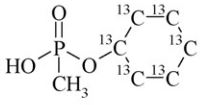
Sarin (GB) Acid

Molecular Weight =141.07
Molecular Formula =$C_1{}^{13}C_3H_{11}O_3P$



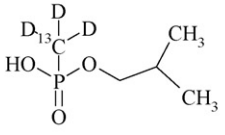
Soman (GD) Acid

Molecular Weight =186.12
Molecular Formula =$C_4{}^{13}C_6H_{14}O_3P$



Cyclohexyl methylphosphonic (GF) Acid

Molecular Weight =184.10
Molecular Formula =$C_1{}^{13}C_6H_{17}O_3P$



RVX Acid

Molecular Weight = 156
Molecular Formula = $^{13}CC_4H_{13}O_3P$

(b)

Fig. 1. (a) Structures of the analytes chosen for this study. (b) Structures of the isotopically labeled internal standards.

Table 1
Binary gradient parameters

Time (min)	Rate (μL/min)	%B: MeOH
0.00	200	20
1.50	200	20
2.00	200	90
2.25	200	100
8.5	200	100
8.6	200	20
10.00	200	20

Table 2
Mass spectrometer parameters

Ion spray voltage	−4500
Temperature	350 °C
Collision gas	10 psi
Curtain gas	35 psi
Nebulizer gas (gas 1)	45 psi
Heating gas (gas 2)	50 psi

agent following exposure [5]. The polar nature of the resulting phosphonic acid metabolite readily lends itself to LC/MS/MS analysis without the need of derivatization. In addition, the animal and human acid metabolites detected following nerve agent exposure are the same hydrolysis products found when the parent compounds are exposed to the environment [5]; indicating the potential application of this method to environmental samples.

Numerous manuscripts have detailed analytical approaches for the quantitation of hydrolysis products from nerve agents

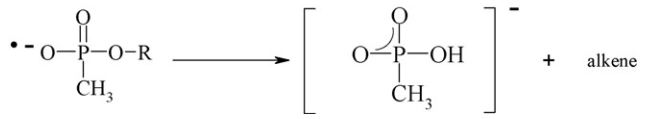


Fig. 3. Fragmentation scheme for the phosphonic acids. The analytes undergo cleavage at the C—O bond resulting in transfer of a H atom and loss of an alkene.

[6–11] or nerve agent metabolites [12,13] utilizing mass spectrometry. Black et al. presented a Gas Chromatography/Mass Spectrometry (GC/MS) with Selected Ion Monitoring (SIM) and GC coupled to tandem mass spectrometry (MS/MS) [6] and a Liquid Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometric (LC/APCI-MS) method [7–9]. Creasy reported a LC/MS method using post-column derivatization using trimethylphenyl ammonium hydroxide [10]. Hill and co-workers described a rapid screening method utilizing ambient pressure ion mobility mass spectrometry [11]. Another approach is to quickly measure the nerve agent metabolites in urine utilizing isotope dilution GC/MS/MS post-event [13]. Barr et al. developed a sensitive and robust method with simple sample preparation utilizing isotope dilution GC/MS/MS, for the detection of the nerve agent metabolites VX, GB, GD, GF and Russian VX in urine that is an improvement over existing GC/MS/MS methods [14].

This research focused on the development of a rapid analytical protocol for the quantitation of nerve agent phosphonic acid metabolites in urine [15]. The protocol utilized anion exchange solid phase extraction (SPE) followed by isotope dilution LC/MS/MS analysis. SPE was utilized because it has proven to be robust and rapid. Additionally SPE can be automated, reducing human error and further expediting the

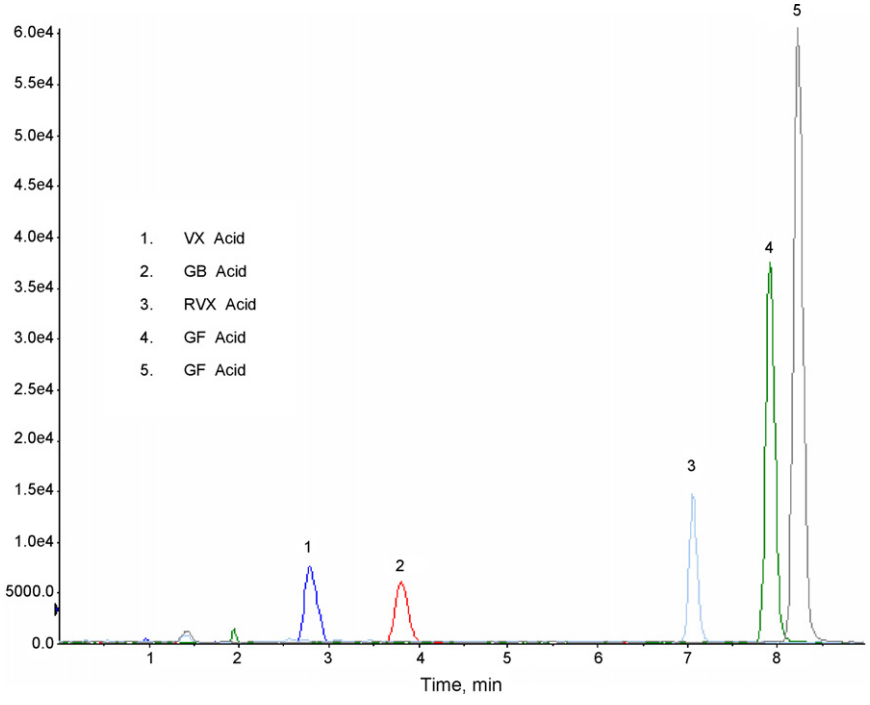


Fig. 2. LC/MS/MS chromatogram for a urinary extract of the target analytes at 1 ng/mL.

extraction process. Extraction efficiencies were found to range from 62% to 87% for the analytes in the protocol and distinct baseline separation was achieved for all analytes. By targeting the analysis to the phosphonic acids, derivatization associated with traditional GC/MS(/MS) methods is not necessary, saving sample preparation time. Tandem mass spectrometry was chosen due to the inherent specificity and sensitivity of the technique.

2. Experimental

2.1. Chemicals and materials

The organophosphorus nerve agent metabolites chosen for investigation were methyl isopropyl methylphosphonic acid (GB acid), pinacolyl methylphosphonic acid (GD acid), cyclohexyl methylphosphonic acid (GF acid), ethyl methylphospho-

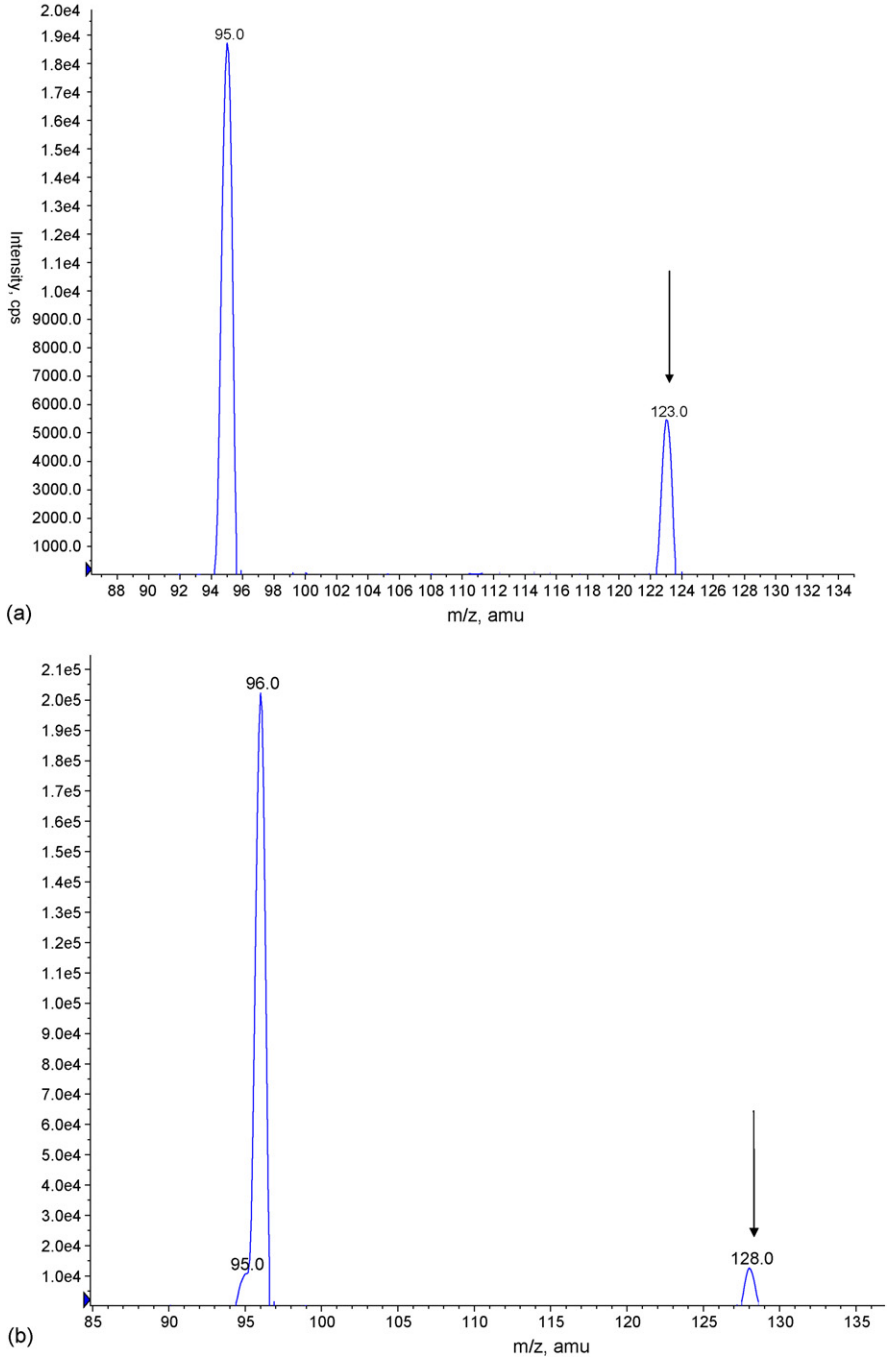


Fig. 4. (a) Tandem mass spectrum of VX Acid and the isotopically labeled internal standard (b). The product ion at *m/z* 96 for the internal standard points to transfer of a H atom after loss of the alkene during fragmentation. (c) Tandem mass spectrum of GB Acid and the labeled internal standard (d). (e) Tandem mass spectrum of GD Acid and the labeled internal standard (f) (g) Tandem mass spectrum of GF Acid and labeled GF Acid (h). (i) Tandem mass spectrum of RVX Acid. The product ion at *m/z* 99 for the internal standard (j) is a result of the ^{13}C and deuterium labels on the methylphosphonic acid.

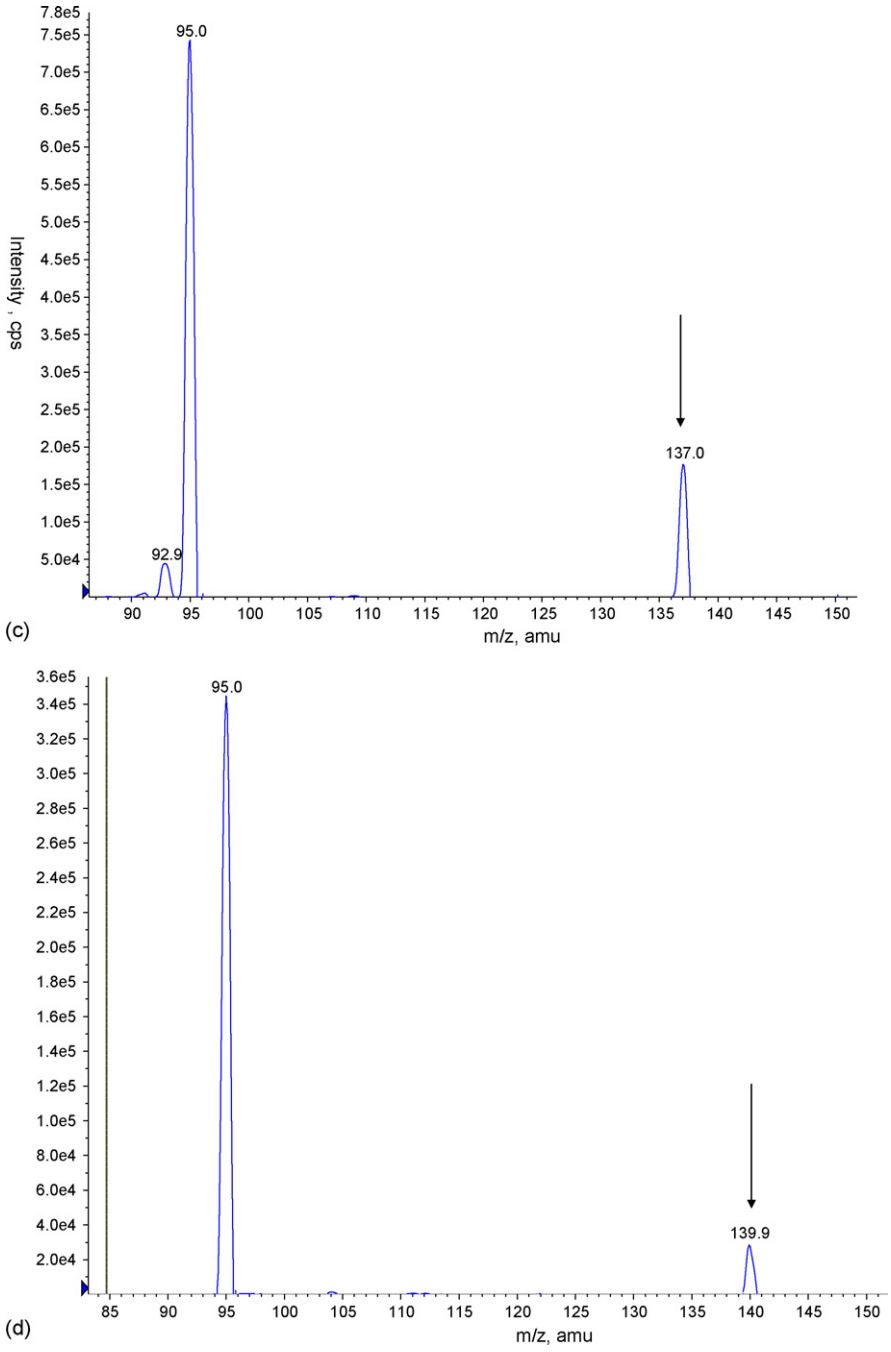


Fig. 4. (*Continued*)

nic acid (VX acid) and isobutyl methylphosphonic acid (RVX acid) (Fig. 1a). Materials were purchased as both neat standards and in prescreened urine from Cerilliant (Round Rock, TX). Stock solutions from neat materials were made from each standard and diluted in methanol for individual analysis and MS optimization. From this stock solution, a mixture of the metabolites was created and diluted for a representative calibration curve (1–200 ng/μL).

Urinary standards from Cerilliant were received in sealed ampules containing 1.8 mL of matrix with the following analyte concentrations: 1 ng/mL; 2 ng/mL; 5 ng/mL; 10 ng/mL; 25 ng/mL; 50 ng/mL; 100 ng/mL; and 200 ng/mL. Cerilliant also provided: blank urine; two quality control samples at concentrations of 15.0 ng/mL (QCL) and 75.0 ng/mL (QCH) for each compound; and isotopically labeled nerve agent metabolites [GB acid ($^{13}C_3$), GD acid ($^{13}C_6$-ester), GF acid ($^{13}C_6$), VX acid (ethyl-d_5) ester, and Russian VX acid (methylphosphonyl ($^{13}C_1$), d_3 at a concentration of 500 ng/mL (Fig. 1b). Urine used in preparation of working materials was collected and pooled from laboratory volunteers and prescreened for nerve agent metabolites. Standard materials and urine were stored at – 20 °C. HPLC grade formic acid, acetic acid, acetonitrile, and methanol were purchased through Fisher Scientific (Fairlawn, NJ). In-house deionized water was treated with an E-Pure water

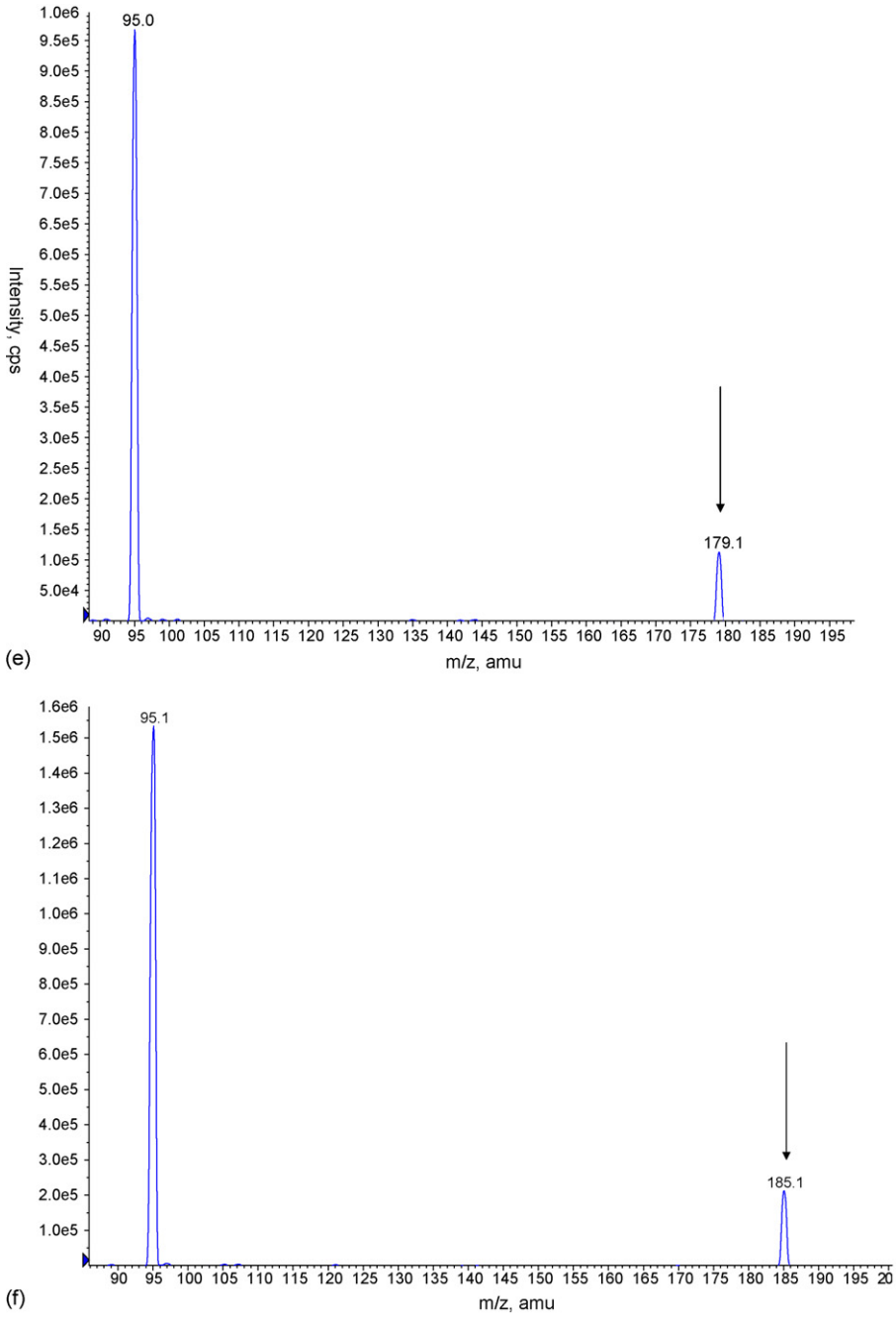


Fig. 4. (*Continued*)

purification system (Barnstead International, Dubuque, IA) to yield organic-free 18.3 MΩ water.

2.2. Sample preparation

One milliliter of urinary standard, 1 mL of 0.05 M acetic acid, and 50 μL of internal standard were transferred to a 15 mL conical centrifuge tube. The centrifuge tube was vortex mixed for 30 s prior to solid-phase extraction. Pooled urinary spikes were prepared by combining 1 mL of urine, 1 mL of 0.05 M acetic acid, 10 μL of stock solution, and 50 μL of internal standard in a 15 mL conical centrifuge tube and vortex mixed. SPE was performed on an Isolute PE-AX (3 mL/200 mg) anion exchange cartridge (Argonaut, Foster City, CA). Prior to loading the sample, the extraction cartridge was conditioned with 1 mL of methanol followed by 1 mL of deionized water under gravity elution. The acidified, diluted sample was loaded onto the cartridge; the centrifuge tube was rinsed with 300 μL of deionized water, vortex mixed, and added to the SPE cartridge. The cartridge was then washed with 1 mL of deionized water before elution. Analytes were eluted two times with 1 mL of methanol (5% formic acid) under gravity, at a flow rate of approximately 1 mL/min. The eluant was concentrated to 300 μL using a Turbovap LV (Caliper Life Sciences, Hopkinton, MA) under N_2 (15 psi) at 70 °C. The concentrated eluant was transferred to a 300 μL insert in a 1.8 mL autosampler vial for LC/MS/MS analysis.

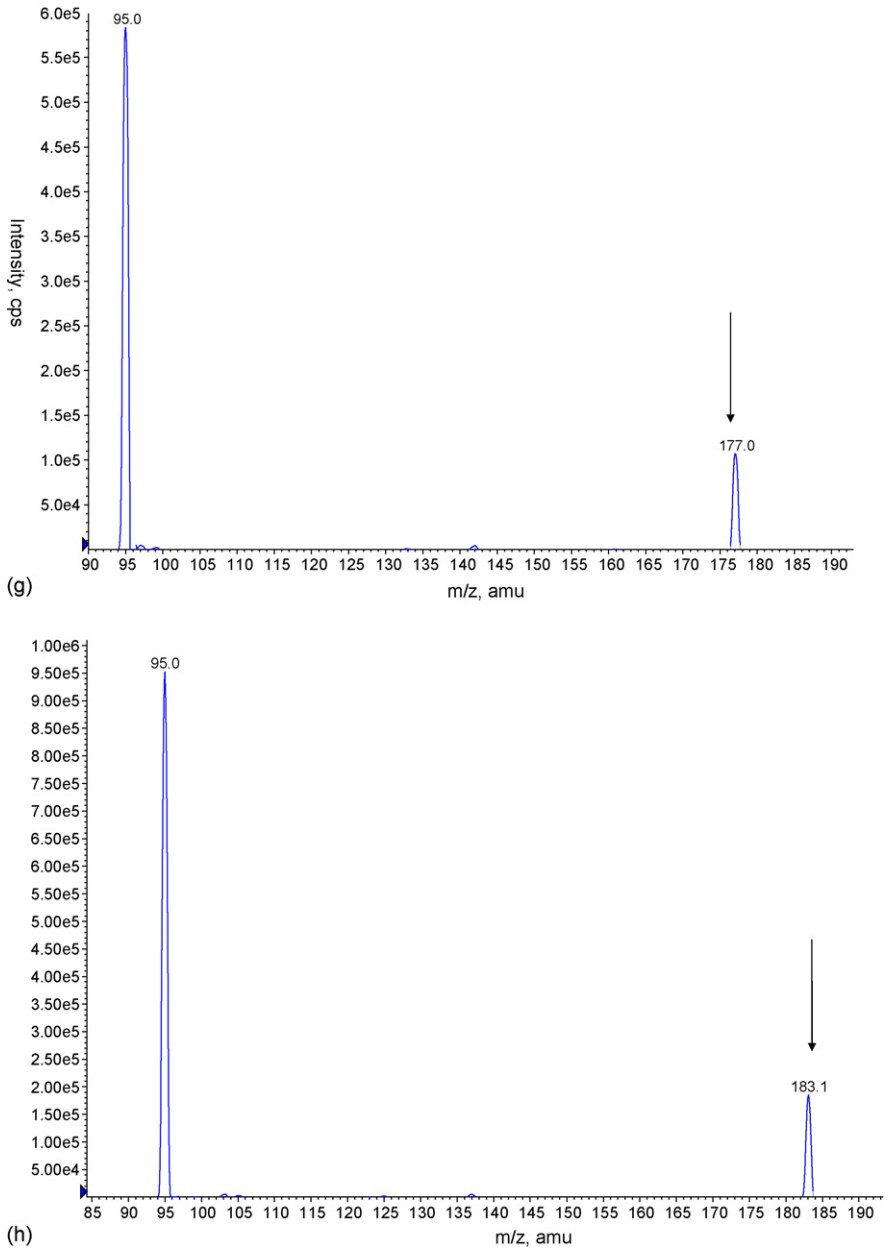


Fig. 4. (*Continued*)

Automated extractions were performed using tabless SPE cartridges (Argonaut, Foster City, CA) coupled to a Gilson 735 liquid handler (Middleton WI.) operating in the four-needle configuration. The automated extraction method requires 20 min for 96 samples versus 40 min for 12 samples using manual extraction.

2.3. Instrumentation

Analytical separations were performed with an Agilent 1100 HPLC (Wilmington, DE) equipped with a 3.5 μm 2.1 mm × 150 mm Xterra MS column (Waters, Milford, MA) at 40 °C. Five microliter injections of the extract were made on the HPLC using a mobile phase consisting of H_2O (0.5% formic acid) (Solvent A) and MeOH (Solvent B). The mobile phase gradient is given in Table 1.

The analytes were analyzed by negative ion electrospray/tandem mass spectrometry operating in multiple reaction monitoring (MRM) mode on an API 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA). The mass spectrometer settings are listed in Table 2. Individual compound specific parameters (i.e. declustering potentials, collision energies, entrance potentials, and collision cell exit potentials) were optimized for each analyte.

3. Results and discussion

Utilization of LC/MS/MS for the quantitation of organophosphorus nerve agent metabolites demonstrated protocol sensitivity, selectivity and robustness. The phosphonic acids ionized readily under negative electrospray conditions, chromatograph-

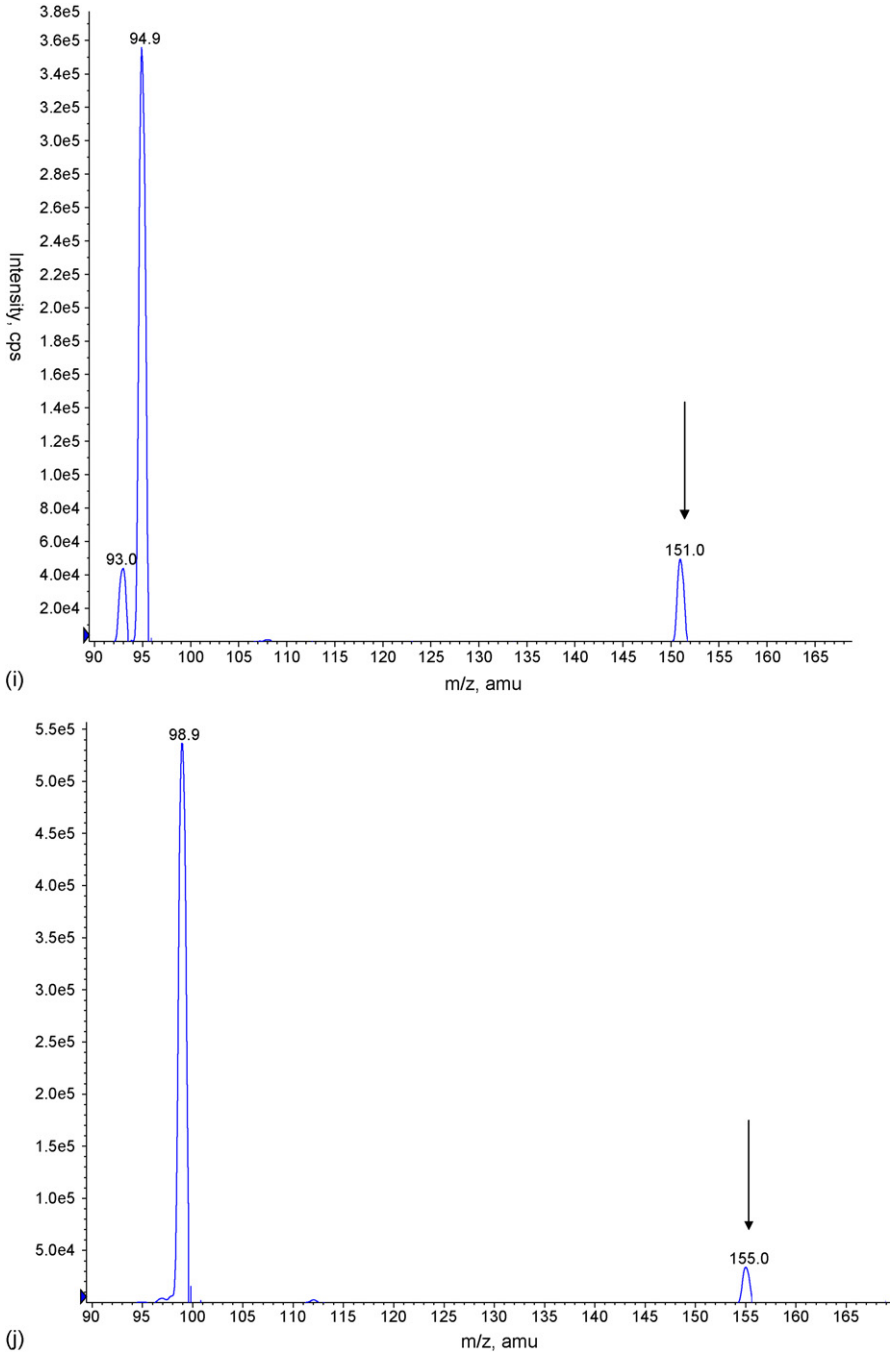


Fig. 4. (*Continued*).

ically all peaks were well resolved, and the product ions from collision-induced dissociation are clean and distinct (Fig. 2). The analytes are believed to fragment through cleavage of the carbon–oxygen bond through a four-centered transition resulting in loss of an alkene to yield a methylphosphonic acid at *m*/*z* 95 (Fig. 3).

Herein, the product ion at *m*/*z* 95 points to a delocalized charge between the phosphorus and oxygen bonds with a hydrogen atom dispersed among the oxygen molecules. Evidence supporting this scheme is based on the fragmentation of VX acid versus the deuterium labeled internal standard (Fig. 4a). As can be seen in Fig. 4a, when the carbon–oxygen bond is broken the resulting product ion at *m*/*z* 96 points to transfer of a deuterium atom most likely from the terminal methyl group. This fragmentation held true for GB acid, GD acid, GF acid and their respective internal standards (Fig. 4c–h). The RVX acid and the corresponding internal standard produced product ions at *m*/*z* 95 and *m*/*z* 99, respectively (Fig. 4i). The difference in *m*/*z* values stems from the ^{13}C and deuterium labels in the methylphosphonic acid of the internal standard.

The calibration curves for each compound were linear through the dynamic range (1–200 ng/μL) with an extracted urinary limit of quantitation, found to be (S/N ≥ 10/1) of 0.5 ng/μL or 8.33 pg on column for all analytes. The limiting factor in over-

Table 3
Validation results from 20 independent analyses

	VX			GB			GF			GD			RVX		
	R	QCL	QCH	R	QCL	QCH	R	QCL	QCH	R	QCL	QCH	R	QCL	QCH
Mean	0.9992	15.20	74.27	0.9990	15.235	75.05	0.9991	15.84	75.830	0.9984	16.250	75.010	0.99937	15.495	74.994
S.D.	0.0006	0.897	3.036	0.0004	0.525	3.447	0.0006	0.936	3.230	0.0011	0.906	3.651	0.00043	0.814	3.865
% R.S.D.	0.0613	5.90	4.09	0.0450	3.34	4.59	0.0610	5.90	2.26	0.1129	5.58	4.87	0.1129	5.25	5.16
Target value		15.0	75.0		15.0	75.0		15.0	75.0		15.0	75.0		15.0	75.0
95% CI		1.76	5.95		1.029	6.76		1.83	6.33		1.78	7.16		1.60	7.58
95% CI min		13.44	68.32		14.02	68.29		14.01	69.5		14.47	71.36		13.895	82.57
95% CI max		16.96	80.22		16.26	81.81		17.67	82.16		18.03	82.12		17.095	67.41

The samples were analyzed over a 10-day period by five scientists.

all sensitivity was found to be the decreased signal intensity of VX acid in relation to the other analytes in the protocol. However, 1.66 pg on column was observed for each analyte and the limit of detection (LOD) for the other analytes (i.e. GB acid, GD acid, GF acid and RVX acid) would be much lower than 1.66 pg on column based on a 3 to 1 signal to noise ratio. Lastly, when capillary HPLC was performed, concentrations of 20 fg on column were observed; however, capillary HPLC was found to increase the need for instrument maintenance and cleaning which took away from the overall ruggedness of the method.

The robustness of the analytical method was determined by performing 20 individual analyses by five different scientists over a period of 10 days using spiked urinary standard curves and QCs provided by Cerilliant (Round Rock, TX). Reproducibility of the method was statistically examined using process control charts for both the quality control low (true value 15.00 pg/μL) and quality control high (75.00 pg/μL) values. These values are represented in Table 3 as 95% confidence intervals. In both cases, the QCL and QCH ranges were within three standard deviations with the majority of results falling within two standard deviations of the true value. The method displayed reproducibility for all five scientists with the calibration curves yielding an $r \geq 0.9984$ for all analytes (Table 3). As can be seen in Table 3, the percent relative standard deviations (% R.S.D.) of the 20 analyses were all ≤5.90% further emphasizing the reproducibility of the method.

4. Conclusion

A robust, high throughput and sensitive method has been developed for the quantitation of the human urinary metabolites of VX, RVX, GB, GD, and GF. The protocol is straightforward and rapid, capable of providing 120 analyses/24 h period with minimal labor input from the analyst. Techniques such as column switching may be employed to increase this number; however, those studies were not performed. The method was shown to be highly sensitive with a LOD ≤ 200 fg on column for all analytes which would allow for accurate quantitation of exposure measurement as well as providing reliable analytical results for long-term biomonitoring. The analytical protocol will provide public health laboratories the ability to make informed responses based on accurate analytical data.

Acknowledgements

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