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The determination of organophosphonate nerve agent metabolites in human urine by hydrophilic interaction liquid chromatography tandem mass spectrometry

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Abstract

A sensitive, robust isotope dilution LC/MS/MS method is presented for the quantitative analysis of human urine for the alkyl methylphosphonic acid metabolites of five organophosphorus nerve agents (VX, rVX or VR, GB or Sarin, GD or Soman, and GF or Cyclosarin). The selective sample preparation method employs non-bonded silica solid-phase extraction and is partially automated. While working with a mobile phase composition that enhances the electrospray ionization process, the hydrophilic interaction chromatography method results in a 5-min injection-to-injection cycle time, excellent peak shapes and adequate retention ($k' = 3.1$). These factors lead to limits of detection for these metabolites as low as 30 pg/mL in a 1-mL sample of human urine. The quality control data (15 and 75 ng/mL) demonstrate accurate (−0.5 to +3.4%) and precise (coefficients of variation of 2.1–3.6%) quantitative results over the clinically relevant urine concentration range of 1–200 ng/mL for a validation set of 20 standard and quality control sets prepared by five analysts over 54 days. The selectivity of the method is demonstrated for a 100-individual reference range study, as well as the analysis of relevant biological samples. The combined sample preparation and analysis portions of this method have a throughput of 288 samples per day.

Keywords: Hydrophilic interaction chromatography; HILIC chromatography; LC/MS/MS; Organophosphorus nerve agents; Nerve agent metabolites; Chemical warfare agent; Chemical terrorism

1. Introduction

Chemical warfare agents are possible terrorist weapons. Consequently, the assessment of human exposure to these agents has recently gained attention [1]. Quantification of phases I and II metabolites of chemical warfare agents by gas chromatography or liquid chromatography, coupled with mass spectrometric detection, is the historic and most common approach for this assessment [2–9], although other detectors have been employed [10]. Researchers are also investigating the detection of chemical warfare agents and their degradation products in environmental samples [11–15]. Some researchers also employ various detector strategies in the absence of a chromatographic separation [16–18].

The organophosphorus nerve agents were employed in terrorism incidents in Japan in 1994 and 1995 [1]. Germany first developed this class of chemical agents (G-agents). Britain, the United States (VX) and the former Soviet Union (rVX) further developed them as chemical warfare agents. These compounds react with the serine residue in the active site of the enzyme acetylcholinesterase, inhibiting hydrolysis of the neurotransmitter acetylcholine within nerve synapses. Excessive acetylcholine results in over stimulation and eventual paralysis of various

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muscles, with death usually resulting from paralysis of respiration. Because organophosphorus nerve agents are toxic (μg/kg), are simple to synthesize and are possibly available from chemical stockpiles, they remain a great concern for those who must respond to acts of terrorism.

The peer-reviewed literature contains numerous sample preparation methods for the quantitative analysis of urine for organophosphonate nerve agent metabolites. Sample preparation methods have been reported employing reverse phase solid-phase extraction (SPE) [2], strong anion exchange SPE [3,6], liquid–liquid extraction [7], azeotropic distillation/concentration [5] and multi-step SPE with derivatization [9]. Unless very acidic conditions are used, reverse-phase methods typically result in poor recoveries for small polar acidic compounds. Because of non-specific elution of acidic components of urine along with the phosphonic acid nerve agent metabolites, strong anion exchange methods can result in highly contaminated samples. We report a more selective, partially automated sample preparation procedure employing solid phase extraction with non-bonded silica.

The specificity afforded by chromatography, coupled with mass spectrometric detection with isotope dilution, produces methods generally preferred for clinical and forensic analysis. Shih et al. reported a gas chromatography–mass spectrometry (GC/MS) method [2]. Fredriksson et al. [3] published a GC/MS/MS method employing a hybrid sector quadrupole mass spectrometer, while Driskell et al. reported two GC/MS/MS methods employing triple quadrupole mass spectrometers [5,7]. Riches et al. reported a method employing GC/MS/MS on an ion trap instrument [9]. The specificity of tandem mass spectrometry and the ability to detect the native analytes without derivatization makes liquid chromatography tandem mass spectrometry (LC/MS/MS) an appealing analysis technique. We report a novel chromatographic method based on hydrophilic interaction chromatography that offers several advantages over existing methods, including reduced limits of detection from biological matrices.

2. Experimental

2.1. Materials

The analytical standard samples in a range of clinical relevance (1, 2, 5, 10, 25, 50, 100 and 200 μg/L of each analyte in synthetic urine), quality control samples (15 and 75 μg/L of each analyte in synthetic urine) and their isotopically labeled analogues (500 μg/L in water) were purchased from Cerilliant Corporation (Round Rock, TX). The analytes were comprised of: EMPA (ethyl methylphosphonic acid, CAS 1832-53-7), the metabolite of VX (*O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothioate, CAS 50782-69-9); IMPA (isopropyl methylphosphonic acid, CAS 1832-54-8), the metabolite of GB (isopropyl methylphosphonofluoridate CAS 107-44-8); PMPA (pinacolyl methylphosphonic acid, CAS 616-52-4), the metabolite of GD (pinacolyl methylphosphonofluoridate, CAS 96-64-0); CMPA (cyclohexyl methylphosphonic acid, CAS 1932-60-1), the metabolite of GF (cyclohexyl methylphosphonofluoridate, CAS 329-99-7); MMPA (2-(methyl)propyl methylphosphonic acid, CAS 1604-38-2), the metabolite of rVX (*O*-2-(methyl)propyl *S*-2-(diethylaminoethyl) methylphosphonothioate CAS 159939-87-4).

The internal standards were isotopically labeled as follows: EMPA, ethyl-D_5; IMPA, isopropyl-$^{13}C_3$; PMPA, trimethylpropyl-$^{13}C_6$; CMPA, cyclohexyl-$^{13}C_6$; IMPA, methylphosphonyl-^{13}C, D_3. Fig. 1 shows the structures of the organophosphorus metabolites and internal standards.

Organic-free 18.2 MΩ Type I water from a purifier purchased from Aqua Solutions, Inc. (Jasper, GA) was used in these studies. HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ). Omnipur ammonium acetate (97%) was purchased from EMD Chemical, Inc. (Gibbstown, NJ). Individual human urine samples for reference range studies were obtained from Tennessee Blood Services Corporation (Memphis, TN).

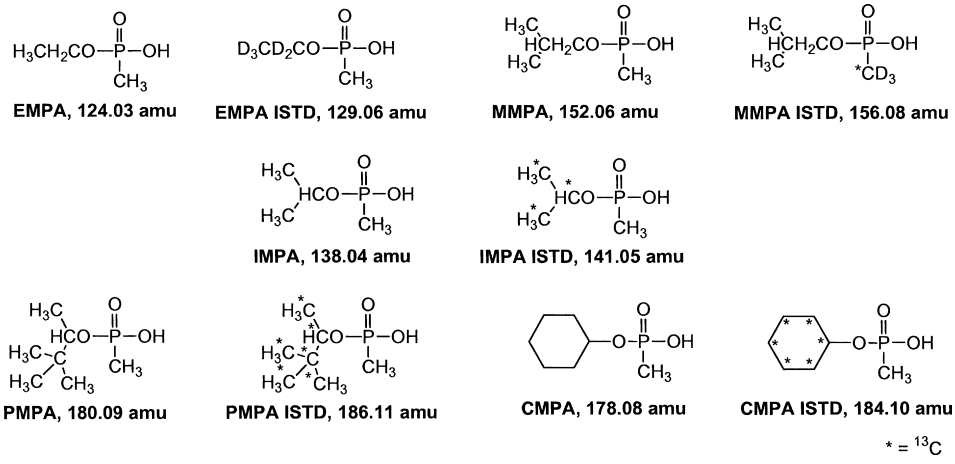


Fig. 1. Structures and monoisotopic masses of the analytes and internal standards employed in this method. EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

2.2. Sample preparation

One hundred microliters of internal standard solution was transferred to a 15-mL conical centrifuge tube. Six milliliters of acetonitrile was added to the tube, followed by 2 s of vortexing. One milliliter of unknown, standard, or QC sample was then added to the tube, vortexed for 2 s and subsequently taken to dryness at 70 °C under 15 psi of nitrogen flow in a TurboVap sample concentrator (Caliper Corporation, Hopkinton, MA). The residue was then extracted twice with 1.5 mL of a solution of 5% water in acetonitrile. The extracts were combined in a 12 mm × 75 mm test tube and transferred to a Gilson 215 SPE (Gilson, Inc., Middleton, WI) for the automated solid-phase extraction procedure.

The Gilson 215 SPE was programmed to perform the following procedure. The Phenomenex Strata Si-1 SPE cartridges (3 mL, 500 mg) were pretreated with 4.8 mL of 25% water in acetonitrile, followed by 3 mL acetonitrile. A 2.75 mL of sample extract was then loaded onto the cartridge at a rate of ∼3 mL/min. This was followed by a two-step rinse of 2 mL acetonitrile, followed by 4 mL of 10% water/90% acetonitrile. The analytes were then eluted with 2.5 mL of 25% water/75% acetonitrile into a fresh 12 mm × 75 mm test tube.

The eluant was evaporated to dryness at 70 °C with 15 psi of nitrogen in a TurboVap with occasional vortexing to assure that the analytes were concentrated near the bottom of the test tube. The sample was reconstituted in 200 μL 5% water in acetonitrile, heated in the TurboVap at 70 °C for 1 min, vortexed for 20 s and transferred to a 350-μL polypropylene screw-top autosampler vial.

2.3. Instrumental analysis

The HPLC separation was performed on an Agilent 1100 HPLC with a well-plate autosampler (Santa Clara, CA). The HPLC column employed was a Waters Atlantis HILIC 2.1-mm × 50-mm with 3-μm particles. This hydrophilic interaction column consists of high purity, non-bonded silica particles. The injector was programmed to draw 5 μL of sample, wash the injector needle for 5 s in the wash port in 100% methanol and inject the sample onto the column. The mobile phase consisted of 86% acetonitrile and 14% 20 mM ammonium acetate at an initial flow rate of 500 μL/min. Following elution of the analytes, the flow rate was increased to 1000 μL/min at 2.51 min to remove any late eluting impurities. The flow rate was returned to 500 μL/min at 3.91 min to provide a stable pressure for the subsequent injection. This program allows a 5-min injection-to-injection cycle time.

The mass spectral analysis was performed on an API 4000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, CA) controlled by Analyst software. The mass spectrometer was operated in negative-ion, selected-reaction-monitoring (SRM) mode. The specific operating conditions are listed in Table 1 with the proposed fragment ions. The specific settings used were curtain gas (CUR), 35 psi; nebulizer gas (GS1), 40 psi; turbo gas (GS2), 40 psi; GS2 temperature (TEM), 550 °C; collision gas, nitrogen; collision gas (CAD), 7, producing a gas pressure reading of 3.5×10^{-5} Torr; ionspray potential (IS), −4000 V; entrance potential (EP), −10; interface heater (IHE), on.

2.4. Data analysis

The data were analyzed using Analyst 1.4, which was provided with the instrument. This software allows review of the chromatograms for retention times, baselines and possible interferences. Quantitative analysis of the data by automated and manual integrations, linear regression and calculation of accuracies and correlation coefficients was also performed with this software package. The chromatographic data were smoothed three times prior to integration with a bunching factor between 1 and 3, and fitted by linear regression using $1/x$ weighting.

2.5. Safety precautions

The techniques and materials in this method do not pose any special hazards. General considerations include exercising universal precautions, including wearing appropriate personal protective equipment, when handling chemicals and urine samples. The high voltage employed in electrospray ionization should also be considered a hazard, and the safety interlocks provided by the instrument manufacturer should not be defeated. For safety considerations specific to the instrument employed, please consult the manufacturer.

3. Results and discussion

3.1. Sample preparation

Initial samples were prepared by a strong anion exchange (SAX) procedure and analyzed by hydrophilic interaction chromatography on a 2.1 mm × 150 mm Alltima HILIC column obtained from Alltech Associates. Unfortunately, as can be seen in Fig. 2A, this sample preparation resulted in coeluting interferences with EMPA. Because the SAX procedure extracts the analytes based on their acidity, this technique was most likely extracting a high number of other acidic species present in urine, leading to the coeluting interferences.

Polar SPE media were investigated to take advantage of the hydrophilic nature of these analytes. For polar media extraction, the analytes had to be transferred from urine into an organic solvent. This was accomplished by azeotropic distillation of the water by adding acetonitrile at a 6:1 ratio to urine and evaporating to dryness in a TurboVap at 70 °C. The reconstitution was optimized with 5% water in acetonitrile, as higher concentrations of water tended to dissolve more matrix and affected the chromatography, while lower water content displayed variable recovery behavior between samples. Extraction on silica (Phenomenex Strata Si-1) gave very consistent recovery and elution behavior for these analytes when compared to C2, C1 and CN solid phase extraction media. Interestingly, the matrix components displayed chromatographic behavior, appearing as bands after drying during fraction collection studies. Once the bands

Table 1
Selected reaction monitoring (SRM) settings with proposed fragment ions

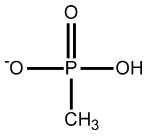
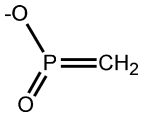
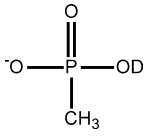
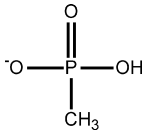
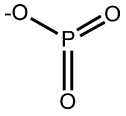
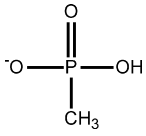
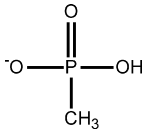
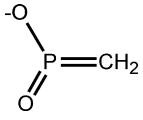
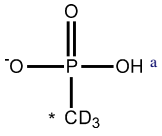
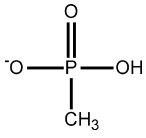
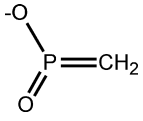
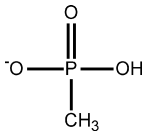
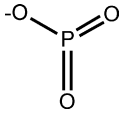
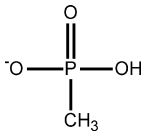
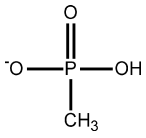
Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Proposed product ion	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
EMPA-quantitation	−123	−95		−40	−20	−8
EMPA-confirmation	−123	−77		−55	−40	−8
EMPA-internal std	−128	−96		−40	−17	−8
IMPA-quantitation	−137	−95		−60	−23	−10
IMPA-confirmation	−137	−79		−75	−40	−10
IMPA-internal std	−140	−95		−60	−21	−8
MMPA-quantitation	−151	−95		−90	−18	−8
MMPA-confirmation	−151	−77		−90	−30	−8
MMPA-internal std	−155	−99	 [a]	−90	−18	−8
PMPA-quantitation	−179.1	−95		−90	−15	−8
PMPA-confirmation	−179.1	−77		−90	−27	−5
PMPA-internal std	−185.1	−95		−90	−15	−5

Table 1 (*Continued*)

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Proposed product ion	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
CMPA-quantitation	−177.1	−79		−90	−35	−5
CMPA-confirmation	−177.1	−95		−90	−18	−5
CMPA-internal std	−183.1	−95		−90	−18	−5

EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.
[a] * denotes ^{13}C.

that contained the analytes were identified, the extraction procedure was optimized to recover the analytes. An LC/MS/MS chromatogram collected on a 2.1 mm × 50 mm Atlantis HILIC column is shown in Fig. 2B for the extract of internal standards spiked into an individual urine sample.

3.2. LC/MS/MS analysis

One of the initial concerns with this method was optimization of the electrospray ionization conditions. This was studied by infusing the isotopically labeled internal standards through a three-way connection into the HPLC eluant and varying the solvent composition via the HPLC pumps. Initial testing of analyte response versus solution composition revealed increasing signal intensity and signal-to-noise ratio with increasing acetonitrile content, with the largest increase in signal and S/N ratio measured above 80% acetonitrile (Fig. 3). At nearly 100% acetonitrile content, this trend reversed itself due to unstable electrospray ionization conditions and a low concentration of suitable proton acceptors needed to form the anionic analyte species [19].

To take advantage of the S/N benefits afforded by high acetonitrile content, hydrophilic interaction chromatography [20] was chosen as the separation technique. Fig. 4A shows the SRM chromatogram of the quantitative transitions of the native analytes of the lowest calibration solution (1 ng/mL). Fig. 4B displays the lack of interferences from blank human urine, while Fig. 4C demonstrates comparable S/N for a 2 ng/mL spike of native analytes into a relatively concentrated human urine sample. The column capacity factor, k', was calculated to be 3.1 for PMPA on the Atlantis column, insuring adequate retention on the stationary phase. Due to the lack of strong interactions between the acidic analytes and the silica surface, the peak shapes displayed very little tailing. We propose that the primary interaction

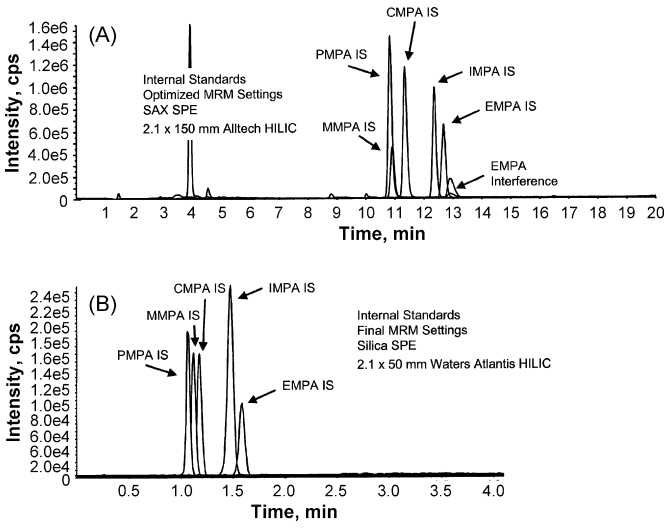


Fig. 2. (A) SRM chromatogram from the 2.1 mm × 150 mm column of the internal standards extracted from pooled human urine by SAX SPE. (B) The SRM chromatogram from the 2.1 mm × 50 mm column of the internal standards extracted from a sample of the same urine pool by Silica SPE. EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

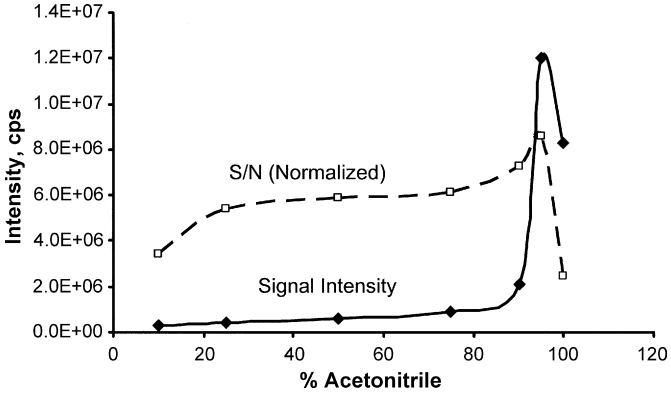


Fig. 3. The enhancement of the response and S/N of the infused analytes as the concentration of acetonitrile is increased. EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

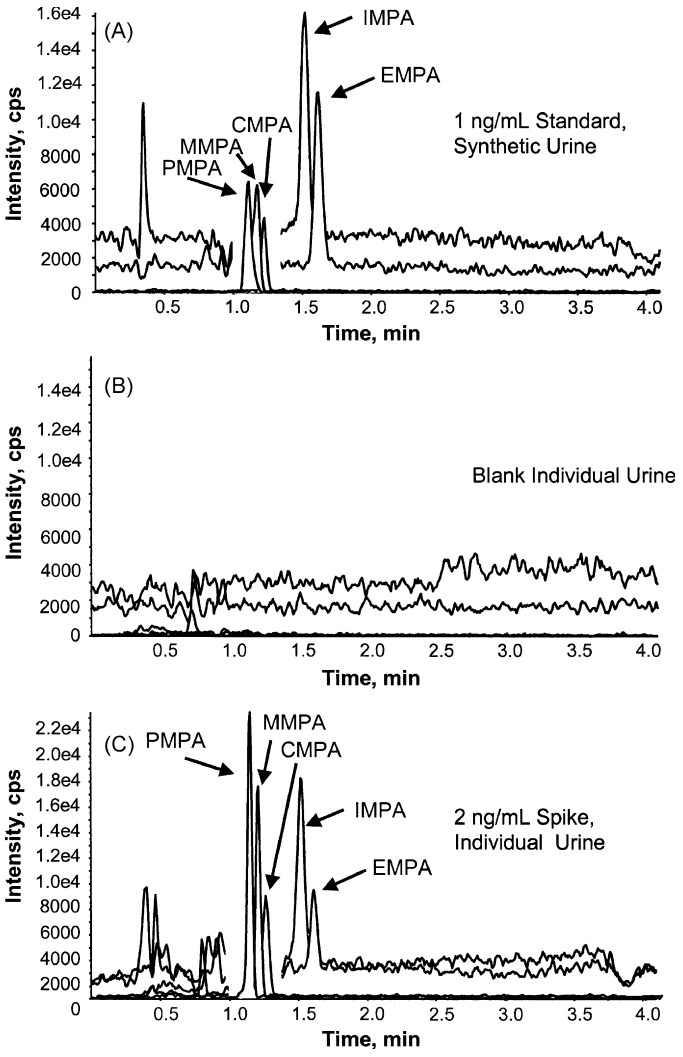


Fig. 4. (A) SRM chromatogram of the 1 ng/mL standard extracted from synthetic urine. (B) SRM chromatogram of an extracted blank individual urine. (C) SRM chromatogram of an individual urine spiked with 2 ng/mL of the analytes that showed a lower than normal recovery. Note that the confirmation ions and internal standard transitions are not shown for clarity. EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

for retention is partitioning into the aqueous layer formed around the silica particles, consistent with the hydrophilic interaction chromatography mechanism [20].

3.3. Linearity

When the overall signal intensity was higher than $\sim 5 \times 10^6$ counts/s, the detector began to display non-linear response. This caused non-linear calibration curves for most of the transitions within the clinically important 1–200 ng/mL range. To obtain linear calibration curves, we made several changes in the mass spectrometer parameters. A high baseline signal from the mobile phase of ~5000 counts/s, presumably due to a solvent cluster, was one factor causing non-linearity for IMPA and EMPA. The declustering potential was raised 10 V for these two transitions to help lower this background, while still retaining adequate sensitivity for the analytes. For the quantitation and confirmation ion transitions of MMPA, PMPA and CMPA, the non-linearity was found to be due to very high response. The collision energy was lowered to bring the signal of the highest calibrators to $\sim 3 \times 10^6$ counts/s. Additionally, the electron multiplier voltage was lowered from 2100 to 1700 V. These modifications resulted in linear calibration curves over the range from 1 to 200 ng/mL with R values exceeding 0.999. While these modifications lowered the S/N of the measurements, the lowest calibrators still demonstrated sufficient S/N to ensure adequate sensitivity.

3.4. LOD and recovery

Analytical methods employed to assess human exposure to toxic chemicals from terrorism activities must be able to accurately quantify the resultant metabolites (or other biomarkers) over a clinically significant range. The analysis must be sensitive enough to quantify the metabolites from a reasonably sized sample at a concentration corresponding to the lowest level of exposure that results in a human health effect. Because of a lack of human exposure data regarding organophosphorus nerve agents – and many other chemical warfare agents – this level is not well understood. Following the terrorism event in the Tokyo Subway, hospitalized patient samples analyzed by other methods displayed urine concentrations of IMPA greater than 10 ng/mL [10]. To account for differences in exposure route and sensitivity, and to allow for longer sampling times post-exposure, extending this range to at least 1 ng/mL – if not lower, given the uncertainty of exposure data – would be useful.

The method LOD was determined statistically [21] from data obtained from human urine samples spiked with native analyte at four different concentrations approaching the LOD. Six replicate samples at each concentration were spiked with diluted internal standard solution at 1 ng/mL and taken through the extraction procedure. The standard deviation of the six replicate measurements was plotted as a function of concentration. This data was fitted using linear regression, and the y-intercept calculated to determine the standard deviation at a concentration of zero. The method LOD for the quantitation ion transition of each analyte is defined as three times this standard deviation value and was determined as IMPA, 240 pg/mL; EMPA, 160 pg/mL; MMPA, 75 pg/mL; PMPA, 30 pg/mL; CMPA, 50 pg/mL. This corresponds to 6, 4, 2, 0.8 and 1.3 pg on-column, respectively. These results indicate that our low calibrator is both above the generally accepted lower limit of quantitation (LOQ) [21] and placed to bracket the clinically relevant range. Further, if future research discloses the need for certain analytes, a lower calibration range could be developed.

We calculated the method recovery from data obtained from spiking pooled human urine. The pool was divided into two groups of 12 samples. One group was enriched with the native analytes to give three samples at 5.7, 10.9, 57.1 and 109.1 ng/mL. Both groups were taken through the sample preparation procedure without the addition of stable-isotope, labeled internal standard solution. Following the final reconstitution in a solution containing internal standards, the second group of samples

Table 2
Accuracy and precision of QC measurements over 20 runs

Analyte	Mean (ng/mL)	S.D.	%R.S.D.	Mean accuracy (%)
15 ng/mL				
IMPA	15.39	0.43	2.81	102.58
PMPA	15.40	0.39	2.54	102.69
CMPA	15.32	0.32	2.10	102.16
MMPA	15.33	0.51	3.35	102.17
EMPA	15.51	0.51	3.30	103.37
75 ng/mL				
IMPA	75.55	2.33	3.08	100.73
PMPA	75.18	2.25	3.00	100.24
CMPA	74.63	2.54	3.41	99.51
MMPA	75.85	2.76	3.64	101.13
EMPA	74.92	2.13	2.85	99.89

EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

was enriched with the native analytes while the corresponding samples in the first group received the same volume of solvent. A calibration line was built from the group of samples enriched post-extraction, and those enriched pre-extraction were quantified against it. The average percent accuracy of the calculated values was taken as percent recovery at each concentration, which fell within $100 \pm 3\%$ across the concentration range for each analyte. The average recovery for each analyte was 35% EMPA, 59% IMPA, 75% MMPA, 80% CMPA and 89% for PMPA. The low recoveries for EMPA and IMPA do not affect the method accuracy and precision; they are accounted for by the stable isotope-labeled internal standards, and the low standard is well above the method LOD.

3.5. Method validation

Methods employed to quantitatively determine human exposure to chemical warfare agents must consistently produce data with a high degree of confidence. These results may determine a course of medical treatment, predict long-term health effects and become forensic evidence in a court of law. Consequently, the selected method must demonstrate a high degree of accuracy and precision over an extended period of time while it is performed by multiple analysts. We found our method was very robust and displayed accuracy and precision well within accepted limits [22].

The accuracy was established by analyzing enriched synthetic urine QC materials multiple times and comparing the analysis mean to the known concentration. Over a period of 54 days, five analysts performed 20 analyses for each of the two concentration levels. The means, standard deviations and mean accuracies for the QC samples (enriched to 15 and 75 ng/mL) are shown in Table 2. The means are less than one standard deviation (S.D.) from the prepared concentration. The mean accuracy for each analyte, expressed as a percentage of the expected value, is shown in Table 2—it ranged from −0.5 to +3.4%. These data define the accuracy of the method.

The method intra-day precision shown in Table 2 was determined by calculating the average coefficient of variation (CV) of repeated measurements on synthetic urine samples. The method precision, determined by calculating the average CV of 20 repeated measurements on the QC materials over a 7-week period, ranges from 2.1% to 3.6%. These values reflect both intra- and inter-day variations and demonstrate the excellent reproducibility of the method.

3.6. Throughput

Method throughput is important for response to terrorism events. During such events, thousands, if not tens of thousands of samples, could be received, and the analytical results must be produced quickly. The throughput of the analytical method is 288 samples per day. The 5-min injection-to-injection cycle time is afforded by the low back-pressure and excellent peak shapes obtained on this column at higher flow-rates, and the lack of any late-eluting peaks that could interfere with subsequent injections. Throughput could be easily increased by various means, including increasing flow rates, the addition of equipment to allow column switching, or both. The throughput of the entire sample preparation procedure is about 300 samples per day. But the procedure shows inter-analyst variation due to the speed in which the manual steps involved are completed, such as pipetting and solution transfers.

3.7. Unknown sample analysis

The method monitors two SRM transitions per analyte. The integrated area of the first transition is ratioed against that of the internal standard for quantitation purposes, and is referred to as the quantitative transition. The integrated area of the second transition, or qualifier transition, is ratioed to that of the quantitative transition and is used for qualitative purposes. The average value of this ratio for each analyte is calculated on a daily basis from the values obtained from the standard and quality control samples. In the case of an unknown sample, this average value ±20% is used to confirm the presence of the analyte. Table 3 shows the average error in this ratio for five standard

Table 3
Average percent error in response ratio for each analyte in the standard and quality control samples

Concentration (ng/mL)	Average error (%) ($N = 5$)				
	EMPA	IMPA	MMPA	PMPA	CMPA
1	6.3	4.4	5.3	4	2.7
2	6.3	1.9	5.9	8	5
5	5.6	8.7	2.9	2.1	3.2
10	3.6	2.8	2.6	2.2	1.5
15	3.9	4	1.7	2.7	3.9
25	2.4	4	2	2.5	2.8
50	2.8	2.9	1.3	3	2
75	2.2	2.5	1.7	1.9	2
100	3	1.9	1.4	2.6	1.7
200	1.5	3.6	1.7	2	2.1

EMPA is the metabolite of VX, IMPA is the metabolite of GB, MMPA is the metabolite of rVX, PMPA is the metabolite of GD and CMPA is the metabolite of GF.

and quality control sets for each analyte randomly selected from the validation data. The average confirmation ratio for this data set was: EMPA, 0.15; IMPA, 0.34; MMPA, 0.54; PMPA, 0.23; CMPA, 2.3. Typical S/N for the confirmation ion transition for the 1 ng/mL standards were: EMPA, 20; IMPA, 220; MMPA, 265; PMPA, 50; CMPA, 215. It should be noted that the response of the confirmation ion transition of EMPA and IMPA are optimized for maximum intensity, while the remaining analytes are optimized for linear response over the calibration range.

A set of 20 individual human urine samples were obtained from Tennessee Blood Services. These samples were spiked with various combinations of the native analytes (at 7, 13, 38, 80 and 150 ng/mL), including several intentionally left blank and were processed by a second analyst who was unaware of the spiking levels. The total time for sample preparation, including the standard and QC sets, was 3 h. The total time for analysis was 2.75 h. With the two processes overlapped, the total actual analysis time was 4.5 h. There were no false positives or negatives. The percent accuracy of the results from the spiked samples ranged from EMPA, 85–100%; IMPA, 87–101%; MMPA, 97–104%; PMPA, 97–103%; CMPA, 96–99%. These results demonstrate the effectiveness of this method to distinguish between those samples containing, and those samples not containing, these nerve agent metabolites.

A separate study was undertaken to compare this method with the established GC/MS/MS method of Barr et al. [7]. Six separate laboratories were issued blind unknown samples with five different concentrations of each analyte. Three of the laboratories tested the samples using both the GC/MS/MS and LC/MS/MS methods, while three tested using only the LC/MS/MS method. The accuracy to the consensus mean for all analytes fell within ±2.5% for both methods, except for GF, where it was +10% for the GC/MS/MS method. The %RSD ranged from 3.8% to 5.7% for the LC/MS/MS method and 9.6–14.8% for the GC/MS/MS method, depending on the analyte. The results were analyzed with Proc GLM using a two-factor ANOVA setup, where one factor was the method employed and the second was the consensus mean. Results of the analysis showed that the two methods had comparable accuracy for IMPA, PMPA and MMPA, while the LC/MS/MS method was more accurate for CMPA and EMPA.

3.8. Limitations of method; interfering substances and conditions

No natural environmental exposure is known to produce these nerve agent metabolites in urine. Accordingly, for non-exposed persons the reference range is expected to approach zero. A reference range study for the nerve agent metabolites was conducted on urine samples from 100 individuals obtained from Tennessee Blood Services. Analysis of these samples showed that background levels were below our LOD.

During the process of automating the solid phase extraction, we discovered an interference in the CMPA SRM transition of 177–95. This interference was due to an impurity in the 12 mm × 75 mm test tubes used with the Gilson 215, probably a residue of the manufacturing process. Because this interference is separated chromatographically, it does not affect the accuracy or precision of the method. It does, however, affect the throughput of the method, as it elutes near 4.5 min. To remove this impurity, we will investigate different manufacturers, as well as precleaning the tubes.

Our method relies on the ability of small concentrations of water in acetonitrile to solvate ionic species. Therefore, the technician must take care to measure separately the water and acetonitrile portions of the sample preparation reagents and mobile phase, followed by thorough mixing. Further, we found that because of the design of the instrument, during aspiration aged transfer tubing in the Gilson 215 SPE unit can change the water content of the sample preparation reagents, resulting in lowered recoveries. The tubing should be inspected regularly and replaced when necessary.

4. Conclusions

A validated isotope dilution LC/MS/MS method was developed for the analysis of the metabolites of five organophosphorus nerve agents from human urine: ethyl methylphosphonic acid, isopropyl methylphosphonic acid, pinacolyl methylphosphonic acid, cyclohexyl methylphosphonic acid and 2-(methyl)propyl methylphosphonic acid. The selective sample preparation, the hydrophilic interaction chromatography and the selected reaction monitoring analysis produce low limits of detection and data with excellent precision and accuracy. The robustness of this method is demonstrated by the excellent quality control data produced by five different analysts over a period of 54 days, as well as the excellent accuracy and precision of the multi-laboratory method comparison study.

Given the possibility of interference from organophosphate pesticides method selectivity is a major concern for trace-level analysis methods—especially for methods employed to determine exposure. The 100-individual reference range study displayed no interferences with the analytes in this study. Further, the reproducibility of the confirmation ion ratio adds additional specificity to this analysis. Possibly, when assessing exposure to some organophosphate pesticides, technicians could also use this method for other analytes.

This method is clearly suitable for use in the response to a chemical terrorism incident to assess nerve agent exposure across multiple laboratories. We obtained accurate results from the analysis of 20 unknown individual urine samples, with no false positives or negatives. Future investigations will include increased throughput of this method by looking at faster analysis and sample preparation techniques. Another interesting pursuit would be reduced limits of detection and development of a new calibration range that will allow detection of these metabolites at extended times post-exposure. The true LOD of which this system is capable is estimated as at least two times lower for EMPA and IMPA, and 10 times lower for MMPA, PMPA and CMPA, if the instrument is optimized for sensitivity rather than linear response over the current concentration range. Additionally, researchers could investigate the applicability of this method for environmental and food samples, given that the analytes also represent the initial hydrolysis products of the nerve agents.

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References

[1] J. Newmark, Neurol. Clin. (2005) 623.
[2] M.L. Shih, J.R. Smith, J.D. McMonagle, T.W. Dolzine, V.C. Gresham, Biol. Mass Spectrom. 20 (1991) 717.
[3] S.A. Fredriksson, L.G. Hammarstrom, L. Henriksson, H.A. Lakso, J. Mass Spectrom. 30 (1995) 1133.
[4] T. Nakajima, K. Sasaki, H. Ozawa, Y. Sekijima, H. Mortia, Y. Fukushima, N. Yanagisawa, Arch. Toxicol. 72 (1998) 601.
[5] W.J. Driskell, M. Shih, L.L. Needham, D.B. Barr, J. Anal. Toxicol. 26 (2002) 6.
[6] M. Kataoka, Y. Seto, J. Chromatogr. B 795 (2003) 123.
[7] J.R. Barr, W.J. Driskell, L.S. Aston, R.A. Martinez, J. Anal. Toxicol. 28 (2004) 372.
[8] J.R. Barr, J. Anal. Toxicol. 28 (2004) 305.
[9] J. Riches, I. Morton, R.W. Read, R.M. Black, J. Chromatogr. B 816 (2005) 251.
[10] M. Minami, D.-M. Hui, M. Katsumata, H. Inagaki, C.A. Boulet, J. Chromatogr. B 695 (1997) 237.
[11] R.M. Black, R.W. Read, J. Chromatogr. A 794 (1998) 233.
[12] R.W. Read, R.M. Black, J. Chromatogr. A 862 (1999) 169.
[13] M. Noami, M. Kataoka, Y. Seto, Anal. Chem. 74 (2002) 4709.
[14] Q. Liu, X. Hu, J. Xie, Anal. Chim. Acta 512 (2004) 93.
[15] P.A. D'Agostino, J.R. Hancock, C.L. Chenier, C.R. Jackson Lepage, J. Chromatogr. A 1110 (2006) 86.
[16] K.A. Joshi, M. Prouza, M. Kum, J. Wang, J. Tang, R. Haddon, W. Chen, A. Mulchandani, Anal. Chem. 78 (2006) 331.
[17] P. Mulchandani, W. Chen, A. Mulchandani, Anal. Chim. Acta 568 (2006) 217.
[18] Y.R. Shu, A. Su, J. Liu, C. Lin, Anal. Chem. 78 (2006) 4697.
[19] N.B. Cech, C.G. Enke, Anal. Chem. 72 (2000) 2717.
[20] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[21] J.K. Taylor, Quality Assurance of Chemical Measurements, Lewis Publishers, Chelsea, MI, 1987.
[22] American Chemical Society Committee Report, Anal. Chem. 55 (1983) 2210.