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Investigation of dried blood sampling with liquid chromatography tandem mass spectrometry to confirm human exposure to nerve agents

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Abstract

A method was developed to detect and quantify organophosphate nerve agent (OPNA) metabolites in dried blood samples. Dried blood spots (DBS) and microsampling devices are alternatives to traditional blood draws, allowing for safe handling, extended stability, reduced shipping costs, and potential self-sampling. DBS and microsamplers were evaluated for precision, accuracy, sensitivity, matrix effects, and extraction recovery following collection of whole blood containing five OPNA metabolites. The metabolites of VX, Sarin (GB), Soman (GD), Cyclosarin (GF), and Russian VX (VR) were quantitated from 5.0 – 500 ng mL^{-1} with precision of ≤16% and accuracy between 93 – 108% for QC samples with controlled volumes. For unknown spot volumes, OPNA metabolite concentrations were normalized to total blood protein to improve interpretation of nerve agent exposures. This study provides data to support the use of DBS and microsamplers to collect critical exposure samples quickly, safely, and efficiently following large-scale chemical exposure events.

Keywords

Dried blood spots; nerve agent metabolites; microsamplers; emergency response; mass spectrometry; liquid chromatography

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5. Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the US Department of Health and Human Services.

1. Introduction

Organophosphate nerve agents (OPNA) remain a public health threat due to their continued use despite international prohibitions [1, 2]. Dissemination of these compounds through warfare and terrorist activities has resulted in human exposures causing injury or death [3]. These exposures demonstrate the need to identify the exposure agent as well as differentiated from the worried well from persons with low-dose exposures to monitor for delayed health effects. Exposure to OPNAs can be confirmed by the detection of specific biomarkers, including protein adducts and metabolites [4–8]. The alkyl phosphonic acid metabolites, which readily form by hydrolysis in the body, can be used to diagnose OPNA poisonings; these metabolites have been measured in blood products following human exposures to VX and sarin (GB) as well as in animal studies with GB and cyclosarin (GF) [9–12]. Concentrations of the OPNA metabolites are rarely reported; however serum levels of isopropyl methylphosphonic acid (IMPA, metabolite of GB) in victims following chemical weapons attacks in Tokyo and Matsumoto ranged from 2 to 136 ng/mL [10].

Methods to detect and quantify these metabolites in clinical matrices have been established using LC-MS/MS [6, 10, 12–16] and GC-MS [17–19]. Sample preparation continues to be critical for analysis to reduce potential interferences and ionization effects and increase sensitivity for MS detection. Previous studies have focused on isolation of OPNA metabolites from blood and urine matrixes using ion exchange, reverse phase, and normal phase SPE [14, 20, 21]. While these protocols have proven effective, they can be time consuming, costly, and may require a larger sample volume (50–1000 μL) [14, 20, 21].

Rapid sample collection is crucial to identify affected persons, due to decreasing concentrations of metabolites. Additionally, blood concentrations of OPNA metabolites may vary greatly depending on the length of time from exposure to sample collection, the level of exposure to the agents, and individual biological factors [9, 12]. Alternatives to invasive, labor-intensive blood draws are needed to accelerate and simplify the sample collection process. The use of blood spotting cards [22, 23] and microsamplers [24, 25] may be implemented outside of the clinical setting, thus providing suitable alternatives to traditional blood tubes.

Dried blood samples have been used for epidemiological disease surveillance to rapidly collect samples from large numbers of people [26, 27]. There are multiple advantages to using dried blood spots (DBS) over traditional blood collection techniques. Dried blood samples are collected by spotting or sorbing venous blood or blood from a finger prick onto filter cards or microsamplers. Collecting dried blood samples is generally faster and less invasive than a blood draw, uses a smaller volume of blood, and requires minimal training, meaning that self-sampling may be possible. Furthermore, blood spot cards can be maintained at ambient temperature with desiccant and do not require cold storage. Finally, because blood is dried on the collection cards, samples are considered stable and non-hazardous during storage and transport [28, 29].

Impacts resulting from variations in the blood must be considered when performing analytical quantitation from dried blood samples. The most critical factor to address is

hematocrit (HCT), a measure of red blood cell (RBC) volume, which can cause spot size variability and heterogeneous distribution of blood components [30]. These two issues may be mitigated by analysis of the entire spot [31]. However, extraction differences due to chemical binding to blood have also been identified in samples with different HCT and must be investigated [28, 30].

For accurate quantitation of OPNA metabolites in a suspected exposure sample, the volume of blood sample must be known [30, 31]. Pipetting can be used to control the spot volume; however, in an emergency, more user-friendly devices such as microsamplers and controlled-volume capillaries may be beneficial. Microsamplers are devices used to obtain dried blood samples for quantitative bioanalysis and have been documented to collect the same volume of blood across HCT variations without the need for additional volume measurement [24]. Because microsamplers are polymeric instead of cellulose, differences in extraction from each sorbent could be possible. For these reason, microsamplers were evaluated along with traditional DBS in this study.

In some cases, microsamplers may not be available and pre-measurement of the blood volume prior to spotting may not be feasible. These samples can still be used to identify exposure to OPNAs; however, accurate quantitation may be challenging. Comparison of metabolite concentrations to previous exposures is desired to better interpret the exposure event. A previous study normalized OPNA protein adduct concentration with total protein content in DBS to achieve this goal. Through this correction, a more comparative value for diagnostic interpretation was achieved [5].

For the first time a novel method has been developed to quantitate metabolites of VX, GB, soman (GD), GF, and VR in dried blood using analysis by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry (MS/MS). The method was evaluated for precision, accuracy, sensitivity, matrix effects, and extraction recovery. Effects of hematocrit on quantitation were investigated for DBS and microsampling devices. Additionally, the normalization of metabolite to protein content was studied to support diagnostic interpretation of results from DBS without controlled volume collection. This application of DBS can support the rapid collection of exposure samples following an emergency event.

2. Materials & Methods

2.1 Chemicals and Reagents

Metabolites of the nerve agents GB, isopropyl methylphosphonic acid (IMPA); GD, pinacolyl methylphosphonic acid (PMPA); GF, cyclohexyl methylphosphonic acid (CMPA); VX, ethyl methylphosphonic acid (EMPA); and VR, 2-(methyl)propyl methylphosphonic acid (MMPA), were purchased from Cerilliant (Round Rock, TX). Isotopically labeled internal standards (ISTD) were also purchased from Cerilliant as a mixture (500 ng mL^{-1} in water for each compound; limited distribution). The ISTD was comprised of the following: D_5 EMPA, $^{13}C_3$ IMPA, $^{13}CD_3$ MMPA, $^{13}C_6$ PMPA, and $^{13}C_6$ CMPA. HPLC-grade acetonitrile and HPLC-grade methanol were purchased from Tedia Company, Inc. (Fairfield, OH). Pelletized ≥99.0% ammonium acetate was purchased from Fluka (St. Louis, MO).

Deionized (DI) water (>18mΩ·cm) was prepared on-site using an installed water purification system (Aqua Solutions, Inc., Jasper, GA). Pooled whole blood and a convenience set of individual whole blood samples were purchased from Tennessee Blood Services (Memphis, TN). Use of deidentified spiked blood from a commercial source was not deemed to constitute human subject research.

2.2 Solutions

A stock solution of each analyte was prepared in methanol to yield a final concentration of 30 μg mL^{-1}. From these stock solutions, three working solutions containing all analytes were prepared at concentrations of 0.25, 1.0, and 10 μg mL^{-1} in methanol. A working ISTD solution was prepared by diluting the 500 ng mL^{-1} solution containing all isotopically-labeled analytes to 23.8 ng mL^{-1} in DI water. All solutions were stored at −20 °C.

Six calibrators were prepared volumetrically in pooled human whole blood by diluting the working solutions to achieve final concentrations of 5.0, 10, 50, 100, 200, and 500 ng mL^{-1}. Two quality control samples (QCs) were prepared in the same manner as the calibrators at low (QL) and high (QH) concentrations of 30 and 250 ng mL^{-1}, respectively. Five individual whole blood samples were fortified volumetrically with the working solutions to final concentrations of 30 ng mL^{-1} and 300 ng mL^{-1}. All calibrators, QC samples, and individual blood samples (blank and fortified) were prepared as 5 μL spots on preprinted Whatman 903 Protein Saver Cards (Fisher Scientific, Waltham, MA). QC samples were also spotted at 100 μL on the same preprinted cards for additional studies.

All calibrator and control samples were also prepared using 10 μL Mitra™ microsamplers (Neoteryx, Torrance, CA) following the manufacturer's instructions. Prepared cards and microsamplers were dried at ambient temperature for a minimum of two hours before analysis or storage in plastic bags with desiccant at −20 °C.

2.3 Hematocrit study samples and measurement

Blood samples with varied HCT levels were prepared by centrifugation (15 min, 3500 RPM) of whole blood treated with K2-EDTA to divide the RBC from the plasma. The RBC were then washed three times with phosphate buffered saline, and fresh plasma was added to create hematocrit levels of 15, 24, 60, and 70%. These samples were fortified volumetrically with the working solutions for final concentrations of 30 ng mL^{-1} and 300 ng mL^{-1}, and prepared as DBS and with microsamplers as described above.

Hematocrit levels were measured by centrifuging the blood in a capillary tube at 12000 RPM using a M24 hematocrit centrifuge (LW Scientific, Lawrenceville, GA) and measuring HCT using a micro-capillary reader (Damon/IEC Division, Needham Heights, MA).

2.4 Methanol extraction of full spot DBS and microsamplers

Dried blood samples were prepared for analysis by removing the entire 5 μL DBS using a standard 0.25 in single-hole punch or removing the 10 μL microsampler tip from the holder, and placing the punch/tip into a 2 mL 96-well NUNC plate (Thermo Scientific, Hudson, NH). A 25 μL aliquot of ISTD (23.8 ng mL^{-1}) was added to each well, followed by 250 μL

of methanol. The plate was then sealed with adhesive PCR Sealing Foil (Thermo Scientific, Hudson, NH) and vortexed on high for 10 min using a ThermoLab Systems Wellmix (Thermo Scientific, Hudson, NH). The supernatant was transferred to a second NUNC plate and dried using a TurboVap (Caliper, Hopkinton, MA) at 70 °C under 75 L min^{-1} of nitrogen until the solvent was evaporated. Samples were reconstituted using 100 μL of 5% DI water in acetonitrile, mixed using the Wellmix on high for 1 min, and the entire 100 μL transferred to a 150 μL 96-well PCR plate (Thermo Scientific, Hudson, NH). The PCR plate was sealed using an Easy Pierce 20 μm Foil Sheet (Thermo Scientific, Hudson, NH) and ABgene plate sealer (Thermo Scientific, Hudson, NH) and placed in the instrument autosampler for analysis.

2.5 Water extraction of full spot and partial DBS

DBS were prepared for analysis by using a standard 0.25 in single-hole punch to remove the entire 5 μL DBS from the card, or a single punch from a 100 μL DBS, and placing the punch into a 2 mL 96-well NUNC plate. A 25 μL aliquot of ISTD (23.8 ng mL^{-1}) was added to the well, followed by 250 μL of DI water. The plate was then sealed with an adhesive PCR Sealing Foil and vortexed on high for 10 min using a Wellmix. A 25 μL portion of each sample was transferred to a separate NUNC plate and set aside for total protein analysis. The remaining supernatant was transferred to another NUNC plate, and 1 mL of acetonitrile was added to each well to facilitate drying. The solvent was evaporated in a Porvair TurboVap (Ashland, VA) at 70 °C using the following procedure: evaporate for 15 min at a flow rate of 30 L min^{-1}, then mix for 20 sec on the Wellmix; evaporate for 15 min at a flow rate of 50 L min^{-1}, then mix for 20 sec; and finally evaporate at a flow rate of 70 L min^{-1} until dry. Samples were reconstituted with 100 μL of 5% DI water in acetonitrile, mixed using a Wellmix on high for 1 min, and the entire 100 μL was transferred to a 150 μL 96-well PCR plate. The PCR plate was sealed using Easy Pierce 20 μm Foil Sheet and AB gene plate sealer, and placed in the instrument autosampler for analysis.

2.6 Instrumental analysis

The HPLC separation was performed using an Agilent 1290 HPLC (Santa Clara, CA) with a Waters Atlantis® HILIC 2.1 mm × 50 mm (3-μm particles) HPLC column (Milford, MA) maintained at 35°C by a column thermostat. A 10 μL volume of sample was injected onto the column followed by a 10 sec needle wash using methanol. Chromatographic separation of the OPNA metabolites was achieved using an isocratic mobile phase consisting of 88:12 acetonitrile:20 mM ammonium acetate in DI water with an initial flow rate of 500 μL min^{-1}. The flow rate was increased to 1000 μL min^{-1} at 3.01 min to remove late-eluting impurities and returned to 500 μL min^{-1} at 5.01 min, ending the run and allowing the system to equilibrate prior to the subsequent injection.

The mass analysis was performed using an API 5500 triple quadrupole MS from Sciex (Foster City, CA). Two negative ion transitions per analyte were monitored using multiple reaction monitoring (MRM), and one transition was monitored for each ISTD as described by Schulze [2016]. Analyte-specific MS parameters were optimized for the highest signal intensity. The MS settings were as follows: curtain gas (CUR), 35 psi; nebulizer gas (GS1), 40 psi; turbo gas (GS2), 40 psi; GS2 temperature (TEM), 550 °C; collision gas (CAD),

medium setting (producing a pressure reading of nitrogen of 2.8 X 10^{-5} Torr); ionspray potential (IS), −4000 V; entrance potential (EP), −10; interface heater (IHE), set to “on.”

The data were analyzed using Analyst® software (Sciex, version 1.6.2). Linear regression analysis of the calibrator concentration versus the ratio of the quantification ion area to the ISTD ion area with 1/x weighting was used for quantitation. All calibration curves met or exceeded a minimum correlation coefficient requirement of 0.990.

2.7 Total Protein Measurements

Total protein content was determined using a Pierce Micro BCA (bicinchoninic acid) Protein Assay Kit (VWR International, Radnor, PA) and performed in triplicate according to the manufacturer’s instructions, using the Biotek PowerWave HT microplate spectrometer (Winooski, VT) as described previously [5].

2.8 Method Validation and Characterization

Data from 20 replicate calibration curves and quality control (QC) samples for DBS and microsamplers were evaluated to assess accuracy and precision. Accuracy was determined by calculating the relative percent difference between the experimentally determined and theoretical concentrations for the QC samples. Precision was determined by calculating the RSD of twenty replicates of the two QC samples. Whole blood from 80 deidentified patient samples from individuals with no anticipated exposure to OPNAs were screened for potential matrix interferences. Five individual whole blood samples were also fortified at 30 and 300 ng mL^{-1}, spotted or sorbed, and analyzed to assess method performance.

Extraction recovery and matrix effects were evaluated in pooled blood at 30 and 250 ng mL^{-1} for both DBS (n=4) and microsamplers (n=4). Dried blood samples spiked before and after extraction were compared to determine extraction recovery. Dried blood samples fortified after extraction were then compared to standards prepared in 5% DI water in acetonitrile to quantify matrix effects.

$$Extraction\ Recovery\ (\%) = (Response\ from\ post\ extraction\ spike - Response\ from\ pre\ exraction\ spike)/Response\ from\ post\ spike\ x100\%$$

$$Matrix\ Effects\ (\%) = (Response\ from\ solvent\ spike - Response\ from\ post\ exraction\ spike)/Response\ from\ solvent\ spike\ x100\%$$

LODs were estimated by multiplying the SD (S_0) of the lowest calibrator (5 ng mL^{-1}) by three as described by Taylor [32].

3. Results and Discussion

Various dried blood samples were evaluated for the determination of OPNA metabolites in whole blood. An extraction of 5 L DBS and 10 μL dried blood microsamplers was developed and validated for the quantitation of these five metabolites using LC-MS/MS.

Since samples received following an emergency or large-scale exposure event may vary in spot volume, the normalization of OPNA metabolite concentration to total protein concentration was also investigated to correlate results from DBS with uncontrolled volumes for diagnostic interpretation.

In initial experiments, 5 μL DBS were extracted with 5% DI water in acetonitrile to align the extraction solvent with the established HILIC chromatographic separation and avoid the need for additional sample preparation steps [14, 33]. Using this extraction, OPNA metabolites could not be detected at 50 ng mL^{-1}; therefore, a more polar solvent and an additional concentration step were investigated to increase extraction efficiency and improve sensitivity. DI water and methanol were selected for extraction evaluation. The extraction with DI water successfully removed the OPNA metabolites from the blood card, but additional time was required to concentrate the extract. The use of methanol was ultimately selected as it successfully extracted the metabolites with the least concentration time needed. The volume of solvent used for extraction was also investigated. Minimal differences in response and reproducibility were noted between 1000 μL, 500 μL, and 250 μL of extraction solvent; therefore, a volume of 250 μL of methanol was selected. These extraction parameters were applied to a 30 ng mL^{-1} DBS sample and analyzed by HILIC LC-MS/MS (Figure 1). Contamination of the single-hole punch was also investigated. For the blank paper samples and blank DBS samples punched immediately following the highest calibrator, no responses were detected.

DBS were initially extracted in glass vials using sonication; however, this process was labor and material intensive. Incorporation of 96-well plates permitted the use of multichannel pipettes for solvent addition and transfer steps, reducing sample preparation time. Use of this container was anticipated to impact DBS extraction; therefore, mixing time was optimized for the 96-well plate format to ensure the optimum recovery of the OPNA metabolites. Mixing times of 5, 10, and 15 min were evaluated, with a 10 min mixing selected since the response and reproducibility increased from 5 to 10 min, but no further increases were observed at 15 min. The final extraction recoveries from DBS in 96-well plates were comparable to those obtained in individual glass vials. Following the success of this process for DBS, microsamplers were prepared in the same manner.

With this protocol, extraction recoveries were determined at 30 and 250 ng mL^{-1} for both DBS and microsamplers using pooled whole blood (Table 1). For microsamplers, the extraction recoveries were slightly lower for the more polar metabolites, IMPA (87.9%) and EMPA (85.0%); this difference was not observed for DBS. The remaining metabolites, PMPA, CMPA, and MMPA, were extracted from the spots and microsamplers with recoveries all above 95.2%, indicating a near complete recovery of analyte.

Matrix effects, evaluated at 30 and 250 ng mL^{-1}, ranged from 26.1–33.3% for DBS and from 60.3–83.3% for microsamplers for all five analytes. The absence of matrix effects was defined as zero; therefore, the microsamplers had almost double the matrix effects as compared to the DBS. An assessment of matrix effects for comparable volumes, using two DBS spots equaling 10 μL of sample, did not indicate that volume was a significant contributing factor for matrix effects. Therefore, this variation in matrix effects between the

sample collection types was likely a difference in retention of blood components to the blood spot cards (composed of cellulose) as compared to the microsampler (composed of polymeric sorbent) [24].

The estimated LOD values (Table 1) were below the lowest calibrator of 5 ng mL^{-1} for all analytes. Variability at 5 ng mL^{-1} was 9.8–14.5% and 11.1–16.3% for DBS and microsamplers respectively; mean accuracies at the same level were within 89.6–106% for all five analytes for both sample collection types. This data in combination with the estimated LOD supports the selection of 5 ng mL^{-1} as the lowest reportable limit for these samples.

Twenty curves for both DBS and microsamplers were prepared and analyzed to determine method accuracy and precision. Multiple analysts participated in curve preparation and analysis, and no more than two curves were analyzed per day. Accuracy of the QC means for all analytes fell between 93.0 – 108% for microsamplers and 93.7–108% for DBS, which were within FDA recommended guidelines of 85 – 115% (Table 2) [34]. Coefficient of variation for the QCs for both DBS and microsamplers ranged from 4.79–16.0%. QC variability was lower overall for DBS (average 8.15%) as compared to the microsamplers (average 13.5%). Challenges preparing the microsampling devices from pre-collected blood occasionally resulted in an excess of sample on the shoulder of the tip, which may have contributed to this increase in bias [24].

Unfortified individual whole blood samples were evaluated for the presence of potentially interfering components. No peaks were detected at corresponding retention times for any of the five OPNA metabolites in 80 individual unexposed blood samples prepared and extracted from DBS. This observation was confirmed for microsamplers using a subset of these individual unexposed samples. Therefore, the potential for false positives from the matrix was determined to be minimal.

OPNA exposure samples are difficult to obtain; therefore, blank individual blood samples were fortified with OPNA metabolites for method assessment. As described before, hematocrit impacts the extraction of analytes from the matrix; therefore, potential biases from variations in HCT were evaluated for DBS and microsamplers. The typical reported ranges of HCT in healthy adults is from 35–50% [35]; higher or lower values may occur when health of an individual is compromised. To evaluate the normal HCT range, individual blood samples (HCT values 25.8–47.0%) were fortified at 30 and 300 ng mL^{-1} and evaluated as unknowns for both DBS (n=5) and microsamplers (n=4). The mean accuracy for all five metabolites at 300 ng mL^{-1} ranged from 91.0–114% with precision not exceeding 12.6% for both sample types. At 30 ng mL^{-1} the mean accuracy ranged from 76.4–100%, with precision not exceeding 7.19% (with the exception of EMPA extracted from microsamplers which had a variability of 22.6%). Overall, these results confirm the effectiveness of this method to extract OPNA metabolites from DBS and microsamplers from individual blood samples within the typical reported HCT range in healthy adults without significant biases or variance.

To investigate the impact of HCT outside the normal range, pooled blood with adjusted HCT ranging from 15–70% was fortified with OPNA metabolites at 30.0 and 300 ng mL^{-1} and used to prepare DBS and microsamplers. All hematocrit levels were measured prior to fortification with OPNA metabolites and preparation of DBS and microsamplers. No correlation between HCT and recovery was observed for DBS both spike levels (Figure 2). Microsamplers, however, showed a slight bias at lower HCT for EMPA and were strongly correlated with a low bias at high HCT for all analytes. Thus the use of mictosamplers exceeds the method accuracy expectation at hematocrit levels below 25.8 for EMPA and above 47.0 for all analytes tested [34].

In the event of an emergency, samples may be collected in a variety of ways, including DBS of uncontrolled volume. To obtain quantitative data from an unknown volume of blood, normalization to a consistent component of whole blood is needed. A previous study has referenced total protein for normalization of other OPNA biomarkers from DBS [5]. To evaluate this process for OPNA metabolites, 100 μL DBS were prepared using fortified whole blood containing the five nerve agent metabolites. A single punch constituting approximately 40% of the blood volume was extracted with 100% methanol, using the 5 μL DBS protocol described above. Although the metabolites were successfully extracted, the blood proteins fixed irreversibly on the card, and total protein could not be measured. A solution of 100% DI water successfully extracted both the metabolites and proteins from the DBS. An aliquot of the aqueous extract was then used to measure total protein. The remaining aqueous extract was concentrated by evaporation, reconstituted in 5% DI water in acetonitrile, and analyzed by HILIC LC-MS/MS.

DBS of 5 and 100 μL volumes were prepared from a whole blood sample fortified at 200 ng mL^{-1}, extracted with DI water, and analyzed as above. The OPNA metabolites were quantitated relative to a calibration curve prepared from 5 μL DBS extracted with DI water. As expected, the volume difference resulted in a significant bias between the samples (Figure 3A); thus, the analyte concentrations from both 5 μL and 100 μL sub-punch DBS were normalized to total protein content to better compare the results. A relative ratio of metabolite concentration to protein content significantly improved the comparison between the whole and partial spots (Figure 3B). This correlation provides needed information to improve interpretation of OPNA exposures from DBS with unknown blood volumes.

The methods presented here have the ability to detect human exposure to five nerve agents from dried blood samples. Although other methods have reported lower detection limits [6, 13, 36], these unique methods will identify the presence of OPNA metabolites as low as 5 ng/mL. Furthermore, the analysis of a single dried blood sample can be completed in approximately 30 min, providing a rapid response, similar to previous publications [36]. However, the greatest advantage of this analytical approach is the ability to detect and quantitate neve agent exposure from dried blood samples, which will be benifical in the event of a large scale exposure

4. Conclusions

Dried blood samples may be an effective tool of collecting human exposure samples from a large chemical release. Five nerve agent metabolites were detected from 5 μL DBS and 10 μL microsamplers from 5–500 ng mL^{-1} with precision <16% and accuracy between 93–108% for QC samples. Evaluation of individual samples confirmed lack of interferences and minimal biases resulting from matrix effects or HCT variations, with the exception of microsamplers for HCT outside of the normal healthy range. Bias observed when evaluating DBS of uncontrolled volumes was substantially reduced by normalizing measured OPNA metabolite concentrations to total protein. This normalized measurement will improve interpretation of nerve agent exposures when using alternative collection techniques such as DBS when volume of blood is not known. The use of microsamplers could facilitate self-sampling supporting the need for rapid collection of clinical samples; however, controlled volume DBS is preferred as filter paper for preparation of spots is readily available and there are minimal quantitation issues related HCT.

Abbreviations:

OPNA	Organophospate nerve agent
DBS	dried blood spot
GB	Sarin
GD	Soman
GF	Cyclosarin
VR	Russian VX

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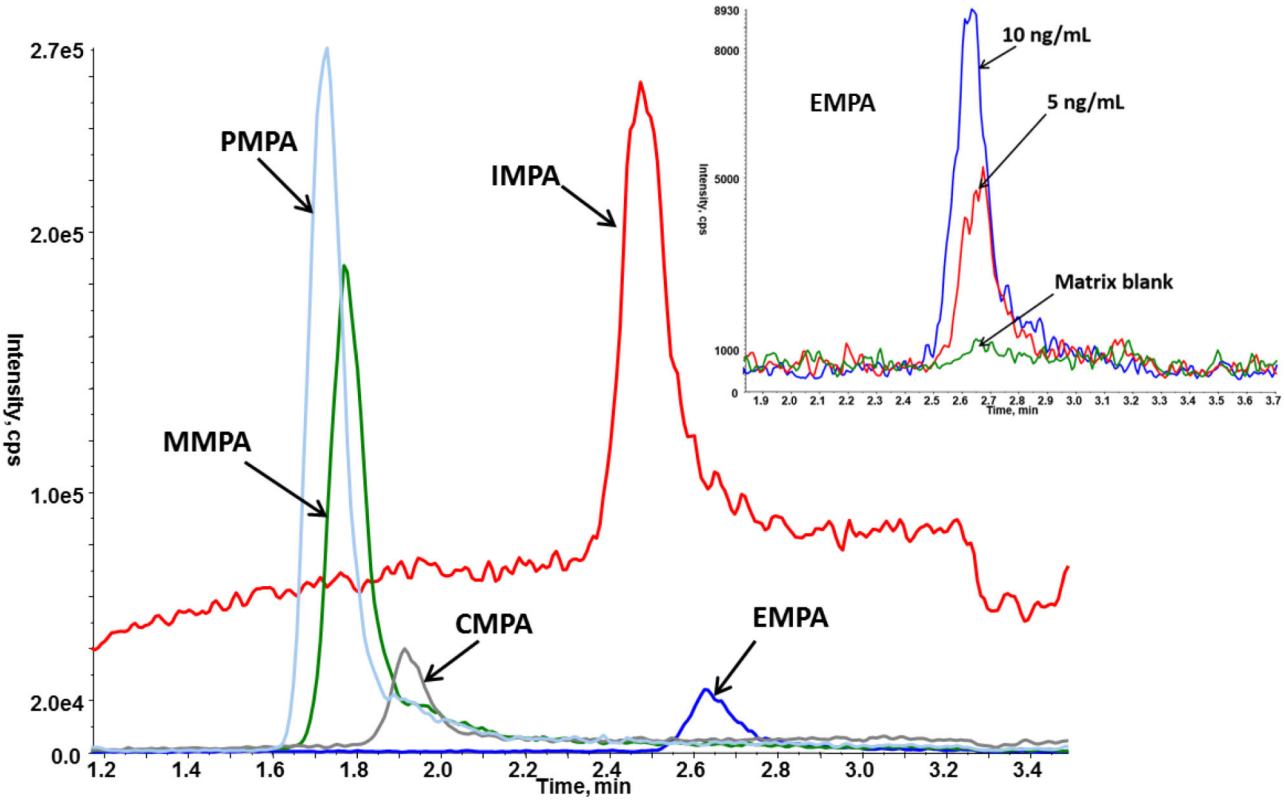

Figure 1.

Chromatogram of all analytes (30 ng mL^{-1}) extracted from a 5 μL dried blood spot (DBS); Inset: EMPA extracted from a 5 μL DBS fortified at 10, 5, and 0 ng mL^{-1} (matrix blank).

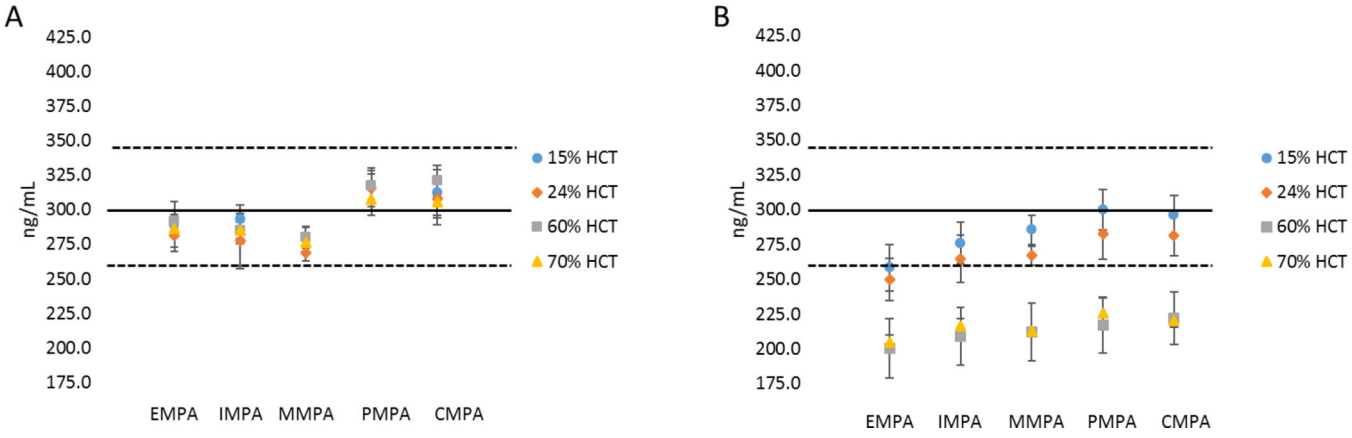

Figure 2.

Accuracy with precision error bars of (A) dried blood spots (DBS) and (B) microsamplers with n=4 replicates at each HCT level for the quantitation of five nerve agent metabolites from individual samples at 300 ng mL^{-1} in varied HCT. The dotted lines represent 15% accuracy.

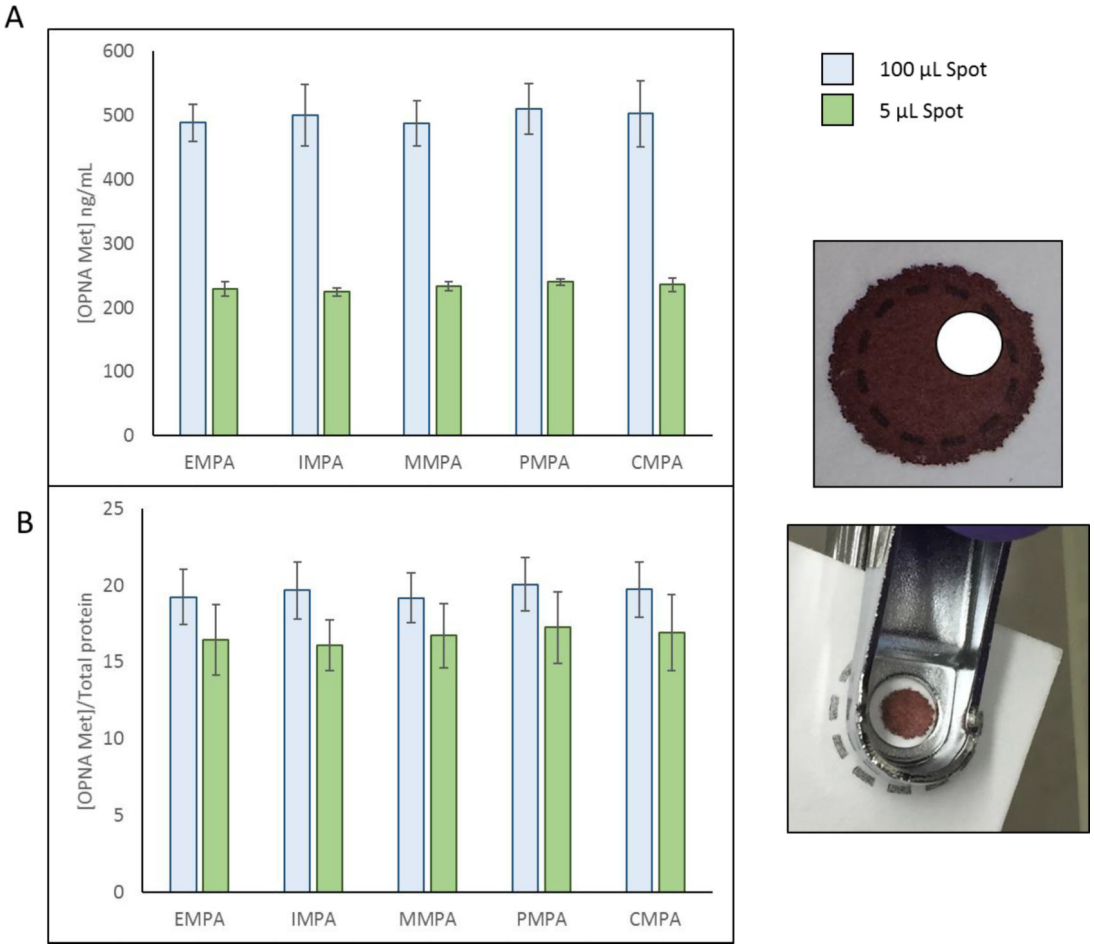

Figure 3.

Quantitation of five nerve agent metabolites, uncorrected for total protein (A) and corrected for total protein (B) at 200 ng mL^{-1} from 5 µL DBS and a sub-punch from 100 µL DBS (n=3)

Table 1.

Extraction recovery and matrix effects in % and LOD for five nerve agent metabolites using dried blood spots and microsamplers. Matrix effects and extraction efficiency were calculated at a concentration of 250 ng mL^{-1} (n=4) for both sample collection types the RSD is included in parentheses.

	Dried Blood Spots (DBS)			Microsamplers		
	Extraction Recovery (%)	**Matrix Effects (%)**	**Calculated LOD (ng mL^{-1})**	**Extraction Recovery (%)**	**Matrix Effects (%)**	**Calculated LOD (ng mL^{-1})**
EMPA	85.0 (4.27)	33.3 (9.36)	1.88	109 (14.2)	60.2 (11.3)	1.95
IMPA	87.9 (3.54)	26.3 (16.7)	2.09	103 (15.2)	78.8 (13.0)	1.95
PMPA	103 (10.1)	26.1 (16.3)	1.93	105 (17.7)	83.3 (14.5)	1.55
CMPA	102 (10.6)	28.1 (13.1)	2.73	102 (16.5)	79.1 (13.9)	1.64
MMPA	95.2 (10.0)	32.6 (11.9)	1.78	107 (18.9)	74.3 (16.7)	1.30

Table 2.

Accuracy and precision (%CV) for quality control samples at 30 and 250 ng mL^{-1} for five nerve agents metabolites from dried blood spots and microsamplers n=20

	Dried Blood Spots (DBS)				Microsamplers			
	QC Low(30 ng mL^{-1})		QC High(250 ng mL^{-1})		QC Low(30 ng mL^{-1})		QC High(250 ng mL^{-1})	
	Accuracy (%)	%CV	Accuracy (%)	%CV	Accuracy (%)	%CV	Accuracy (%)	%CV
EMPA	103	9.70	94.1	5.11	100	12.6	106	14.5
IMPA	103	15.4	94.0	6.53	93.0	15.3	106	13.8
PMPA	108	8.32	94.7	7.10	100	12.3	108	11.9
CMPA	103	10.3	93.7	6.36	101	16.0	105	13.5
MMPA	108	7.91	95.5	4.79	97.7	11.7	105	13.1