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Bridging the Gap between Sample Collection and Laboratory Analysis: Using Dried Blood Spots to Identify Human Exposure to Chemical Agents

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Abstract

Public health response to large scale chemical emergencies presents logistical challenges for sample collection, transport, and analysis. Diagnostic methods used to identify and determine exposure to chemical warfare agents, toxins, and poisons traditionally involve blood collection by phlebotomists, cold transport of biomedical samples, and costly sample preparation techniques. Use of dried blood spots, which consist of dried blood on an FDA-approved substrate, can increase analyte stability, decrease infection hazard for those handling samples, greatly reduce the cost of shipping/storing samples by removing the need for refrigeration and cold chain transportation, and be self-prepared by potentially exposed individuals using a simple finger prick and blood spot compatible paper. Our laboratory has developed clinical assays to detect human exposures to nerve agents through the analysis of specific protein adducts and metabolites, for which a simple extraction from a dried blood spot is sufficient for removing matrix interferents and attaining sensitivities on par with traditional sampling methods. The use of dried blood spots can bridge the gap between the laboratory and the field allowing for large scale sample collection with minimal impact on hospital resources while maintaining sensitivity, specificity, traceability, and quality requirements for both clinical and forensic applications.

INTRODUCTION

Chemical emergencies have the potential to impact large numbers of persons, whether directly through illness and death, or indirectly through low dose exposures and the worried well. Public health response following a chemical release, including intentional, unintentional, and natural occurrences, consists of documenting the people injured and exposed as well as the geographical extent of the event. This information supports emergency response efforts by identifying the chemical agent which is crucial for mitigating additional exposures, including secondary exposures to medical personnel, and preparing for future events through materials distribution[1]. Although a significant amount of information

regarding individuals treated medically will be gathered post treatment, there is still a need to identify those with minimal symptoms and even no symptomology, who still may have been exposed. A clinical assay can be used to capture this information as long as the blood or urine is appropriately collected within a time window following exposure that is sufficient for specific biomarkers to be present.

Although fieldable assays are available for clinical sample analysis, there are advantages to rapidly collecting and transporting samples to established laboratories for analysis. The infrastructure within a clinical laboratory is designed to address sample chain of custody, ensure the safety and security of the personal information required with collection of clinical specimens, and maintain strict adherence to sample traceability ensuring the correct result is connected with the accompanying epidemiological data[2]. The controlled and regulated environment of the laboratory also ensures quality of the results while the isolation of the laboratory minimizes the impact of the chaos from the event on laboratory activities.

Traditional clinical sample collection of blood requires a trained phlebotomist to capture the sample and specific storage conditions to maintain the integrity of the blood sample (e.g. preventing clotting or hemolysis)[3]. Additionally, there are safety concerns of blood borne pathogens which have the potential to infect those handling contaminated samples, whether during collection, shipping, aliquoting, or analysis[4]. These issues further complicate transportation of samples requiring additional safe guards and cold transport to ensure safe delivery of viable samples. Dried blood spots (DBS) are an alternative matrix that addresses all of these issues and ultimately can reduce the requirements for trained personnel to collect samples[5], reduce the materials needed for sample collection, reduce transportation costs[6], and minimize potential blood borne pathogen exposures.

Clinical methods have relied upon dried blood spots for years, particularly in the field of newborn screening[7], as well as epidemiological[8] and environmental population studies[9]. Dried blood samples can be collected through a variety of mechanisms: samples can be directly spotted from a finger prick onto a DBS card, measured prior to spotting onto the DBS card using a pipette or capillary, or drawn from a finger into a microsampler device (Figure 1)[10]. The simplest means of sample collection, the lance and spot approach, presents challenges for quantitative analysis primarily due to the hematocrit of the blood. The hematocrit, or the measure of blood cells, impacts the spread of the blood prior to drying, subsequently resulting in different sample volumes from the same spot size. This issue can be addressed by analysis of a whole spot with a known volume[11]. Measured spots can be self-created using a capillary to deliver a set volume or in a laboratory setting using a pipette. This adds another level of complexity to the sample collection, which is undesirable for emergency response; however, a controlled volume spot does permit quantitation without any need for correction. In the event that a spot of undetermined volume is received, an analytical correction using a measure of protein content from the same spot can be used to attain accurate quantitative measurements[12]. However, this approach requires additional testing of each sample for both the analyte of interest and protein content and confirmation of a direct correlation between the protein content and the analyte concentration. Another alternative is a microsampler, which consistently draws 10 μL of blood regardless of

hematocrit[10]. This device enables self-sampling, with minimal equipment, while maintaining a constant volume for each sample necessary for quantitation.

Additional advantages for DBS may be the reduction in sample preparation steps and costs. For some analyses, the extraction of the analytes from the DBS can replace a previous solid phase extraction or liquid-liquid extraction step. Furthermore, extraction from the DBS is simple, straightforward, and requires minimal solvents, reducing time to results and overall costs of analysis.

To test the efficacy of dried samples for exposures assessment, three assays used to identify human exposure to nerve agents were evaluated. As cholinesterase inhibitors, a measure of cholinesterase activity serves as a non-specific test of exposure and can be measured from butyrylcholinesterase (BChE) in plasma, serum, or whole blood[13]. More specific methods include the identification of BChE bound nerve agents[14] and hydrolyzed nerve agents, often referred to as organophosphorus nerve agent (OPNA) metabolites[15]. Typical sample preparation involved protein precipitation followed by specific chemical extraction (solid phase extraction) or biological recognition (antibody capture) of the analytes of interest to isolate the analyte from the matrix. Subsequent chromatographic separation and tandem mass spectrometry were used for identification and quantitation.

This study evaluated multiple dried sampling approaches, including dried blood spots and microsamplers for the determination of human exposure to nerve agents. Specific biomarkers evaluated were OPNA metabolites and OPNA adducts to BChE. BChE activity, a determination of unadducted BChE, was also evaluated for efficacy in DBS, including a correction of concentration to the total protein content. Precision and accuracy, including comparability to traditional methods, were determined along with an assessment of stability for select analytes with known degradation issues.

METHODOLOGY

Preparation of calibrators and controls

Whole blood and serum, purchased from Tennessee Blood Services (Memphis, TN) were used as a control sample for both BChE activity and BChE. This pooled blood was also fortified to prepare controls and calibrators for OPNA metabolites. Metabolites of GB (sarin), GD (soman), GF (cyclosarin), VX, and VR (Russian VX) purchased from Cerilliant (Round Rock, TX) were diluted in water and spiked directly into pooled blood. Calibrators were volumetrically prepared at concentrations ranging from 5 – 500 ng/mL with control samples prepared at 30 and 250 ng/mL. Control samples for GB-BChE, and VX-BChE were prepared by spiking live agent (GB and VX) into serum as described previously[14].

Calibrators for BChE activity were prepared from recombinant human BChE (Protexia) in filtered 4% nonfat dried milk at concentrations ranging from 0.05 – 6.4 U/mL[14]. Calibrators for GB-BChE, and VX-BChE were prepared from the synthetic peptides (TNO, Rijswik, the Netherlands) at concentrations ranging from 1–250 ng/mL[14, 16].

Spots were prepared by pipetting 5, 40, or 300 μL onto preprinted Whatman 903 protein saver cards (Fisher Scientific, Waltham, MA) and allowed to dry at room temperature for a minimum of 2 hours prior to storage with desiccant or humidity sponge (VWR International, Radnor, PA) in a zipper close bag at a minimum temperature of −70 C. Mitra™ microsamplers (Neoteryx, Torrance, CA) were prepared by following the manufacturer's instructions, allowing for a minimum 2 hour drying time before storage with desiccant or humidity sponge in a zipper close bag at a minimum temperature of −70 C.

Sample preparation of dried samples

Dried blood spots containing OPNA metabolites (including calibrators and controls) were prepared for analysis by punching (using a standard 0.25 in single-hole punch) the entire 5 μL spot from the card or removing the microsampler tip from the holder and placing the punch/tip into a 96-well plate along with 25 μL of 23.8 ng/mL isotopically labeled internal standard (Cerilliant, Round Rock, TX) and 250 μL of HPLC-grade methanol (Tedia, Fairfield, OH). The plate was sealed with adhesive foil and mixed for 10 minutes using a ThermoLab Systems Wellmix (Hudson, NH). The supernatant was transferred into a clean 96-well plate and concentrated to dryness in an UltraVap (Porvair Sciences, Leatherhead, U.K) at 70 °C using a flowrate of 70 L/min. The residue was then reconstituted and mixed thoroughly with 100 μL of 5% water in HPLC-Grade acetonitrile (Tedia, Fairfield, OH).

BChE adducts were eluted from microsamplers using 100 μL of HPLC-grade water (Tedia, Fairfield, OH). The supernatant was then mixed with magnetic beads coated with BChE specific antibodies. The beads were removed from the supernatant, washed, and incubated into buffer and enzyme to digest the protein. The digested samples were filtered, concentrated to dryness, and reconstituted in 75 μL of 0.6% formic acid.

DBS and microsamplers were reconstituted in 100 μL of HPLC-grade water for evaluation of BChE activity and total protein content.

LC-MS/MS instrumental analysis

For the OPNA metabolites, a volume of 5 μL of each sample was injected onto an Agilent 1290 LC System (Santa Clara, CA) and separated isocratically using a Waters (Milford, MA) HILIC HPLC column (3 μm, 2.1 × 50 mm) with 14% 20 mM ammonium acetate (Fluka, St. Louis, MO) and 86% acetonitrile as the mobile phase at a flow rate of 500 μL/min. Analytes were detected using turbo ion electrospray tandem mass spectrometry on a Sciex 6500 triple quadrupole mass spectrometer (Foster City, CA) in negative ion mode as described previously[11].

The prepared BChE adduct samples and peptide calibrators were similarly separated and detected using reverse phase C18 chromatography coupled to tandem mass spectrometry as described previously[14, 16].

Calibration curves for BChE adducts and OPNA metabolites were prepared relative to internal standard response with 1/x weighting. All calibration curves met the r^2 criteria of 0.990 for acceptance. Methods were characterized for precision and accuracy by an evaluation of quality control samples analyzed with respect to a single calibration curve, not

exceeding two runs per day. Between 7 and 20 replicates were used to characterize each method or sample type. Recoveries were determined by comparing fortified matrix samples extracted to extracted matrix samples post-spiked at the same concentration. Matrix effects were determined by comparing solvent samples to extracted matrix samples post-spiked at the same concentration.

Activity and protein measurements

BChE activity measurements were performed using a modified Ellman assay, detailed previously[14]. Calibrators and controls were diluted 1:5 with blocking solution in a protein LoBind plate and then 10 μL were mixed with phosphate buffer and dithiobis-nitrobenzoic acid (EQM Research, Cincinnati, OH). The plate was placed on a preheated BioTek Synergy H4Microplate Reader (Winooski, VT) and allowed to mix. After 10 min, 20 μL of 20.0 mM butyrylthiocholine was added to each well, mixed for 10 s, and measured at 412 nm for plasm/serum samples and 430 nm for blood samples. This cycle of shaking and measurements was repeated for 20 min. The change in absorbance was plotted over time and used to determine a slope for each well. A linear least-squares regression with 1/y weighting was used to quantify activity via a calibration curve of standards.

Total protein content was determined using a Pierce micro BCA (bicinchoninic acid) protein assay kit (VWR International, Radnor, PA) performed in triplicate according to the manufacturer's instructions and documented previously[14]. Simply, a calibration curve was created using the provided standard from 5 – 200 μg/mL. Calibrators and samples were diluted 1:1000. BCA working reagent (100 μL) was added to 100 μL of sample, mixed, and incubated for 2 h at 37 °C. The absorbance at 562 nm was measured and a second degree polynomial regression with 1/y weighting was used to quantify total protein via a calibration curve of standards.

DATA AND RESULTS

Previously, we developed an analytical panel of biomarkers to identify human exposures to organophosphorus nerve agents using plasma or serum[16–18]. This broad sweeping approach accommodated identification of low dose exposures, but required the appropriate handling and storage of whole blood, plasma, or serum to obtain a viable sample for analysis. To address the challenges of whole blood sample collection, shipping, and storage, dried blood samples were evaluated using each of the methods referenced to determine if this sample source was amenable for these analyses.

To evaluate DBS for detection and quantitation of OPNA metabolites, spots of 5 μL were prepared. This small DBS permitted a single punch to capture the entire spot, ensuring the analysis of a known volume, while minimizing the impact of hematocrit. OPNA metabolites were extracted from the dried sample using methanol coupled with a small volume of aqueous internal standard; this combination achieved ~100% recovery of all analytes from the DBS with a short incubation period at room temperature. The direct extraction of OPNA metabolites from DBS resulted in minimal matrix effects (−2.27–2.24%), eliminating the need for additional sample preparation such as solid phase extraction as was necessary for the wet whole blood, plasma, and serum samples[11, 17]. Precision ranged from 6.17–14.2%

and accuracy ranged from 93.6–111% for two quality control samples at 30 and 250 ng/mL assessed over a period of 66 days, with a maximum of two analyses per day. Furthermore, the analyte peak areas from the DBS were consistent with equivalent concentrations injected on column from the traditional whole blood sample preparation [Figure 2]. This further supports the DBS extraction as an efficient and effective means of sample preparation for this analysis. The reportable range for the detection of OPNA metabolites from DBS was sufficient to identify exposures; however, the reduced sample volume (5 μL) resulted in an order of magnitude less sensitivity than previously achieved with wet samples[11]. Given the excellent reduction in matrix effects noted for these compounds, increasing the initial dried sample volume may alleviate these sensitivity issues.

Larger volume dried blood spots (40 μL) were prepared, eluted, and analyzed for BChE activity. First, it was confirmed that the activity of the BChE protein was retained through the drying and elution process. However, initial experiments identified a disparity between the dried spot results from whole blood, plasma, and serum when compared to the unspotted matrix matched control sample. When the dilution differences were accounted for, the final BChE activity recovered was ~80% for whole blood and >90% for plasma and serum. To address this bias, the measured BChE activity was correlated to the total protein content for each sample. This normalization reduced the disparity between spotted and unspotted samples of different dilutions when water was used as the elution solution.

Multiple individual punches from a 300 μL DBS were measured for BChE activity. It was noted that there was some variance depending on the location of the punch from the DBS; this effect was particularly significant for plasma and serum spots. When the normalization to the total protein content for each spot was applied, the variance between the corrected results diminished substantially. This further confirms reporting relative to protein content provides an accurate result for BChE activity in spots of unknown volume as well as any location within a spot, which may impact the percent of protein content.

The preparation of a DBS without a known volume is simple for collection, but still presents challenges from a laboratory perspective by requiring additional testing to assure accurate results. Another possibility that addresses a known volume with a simple sample collection is a microsampler. This device uses volumetric absorptive microsampling (VAMS™) technology that has consistently demonstrated a draw of 10 μL of whole blood, regardless of hematocrit. These samplers wick the blood without the need for additional volume measurement and can be directly collected from a finger prick. Microsamplers were evaluated for use in the analysis of OPNA metabolites, BChE activity, and BChE adducts, to provide a suite of analytical methods applicable to identification of nerve agent exposures.

OPNA metabolites were extracted from the microsamplers using methanol as previously completed with DBS. Since BChE activity and BChE-adducts are measurements of the same protein, elution with water, established with DBS for BChE activity, was applied to the microsamplers. The BChE adducts were further prepared for analysis through BChE antibody capture followed by an enzyme digestion. The resulting nonapeptide was compared to synthetic nonapeptide calibrators for quantitation. All three methods resulted in precision <14% and accuracy from 80.0–108% as shown in Figure 4. These analytical results indicate

that the use microsamplers are a viable alternative to DBS with high ease of sample collection needed for use during an exposure event.

Dried samples offer many advantages, including simplified sample collection and analysis, while maintaining the needed precision, accuracy, sensitivity, and ease of use. Furthermore, the shipping and storage of dried samples only requires minimal space and few regulated conditions as long as the DBS or microsamplers are maintained in a cool, dry place. With proper storage, dried samples may also provide increased analyte stability. BChE adducts, documented to have degradation issues[16], were assessed for stabilization within dried spots.

Analyte stability of GB-BChE and VX-BChE was evaluated using fortified serum samples as well as dried versions of the same fortified samples at 4, 22, and 37 °C. The control for this experiment was maintained at −80 °C for the duration of the experiment, which was conducted isochronously. Over the period of 14 days, the dried serum samples were more resilient to loss of the GB or VX inhibitor from the BChE protein when compared to the samples in solution (Figure 5). At the highest temperature (37 °C) the unspotted serum samples exhibited a decrease in GB-BChE and VX-BChE proteins with approximate 85% losses determined. However, the dried serum samples exhibited no trend that would indicate the loss GB-BChE or VX-BChE. These results support the use of dried samples as a mechanism to minimize stability issues for these analytes.

CONCLUSIONS

Clinical methods have been developed and evaluated that perform comparably in plasma or serum and dried whole blood spots as well as microsamplers. With these results, dried samples can be used as an effective tool for capturing epidemiological samples in the event of a chemical exposure. They resolve many issues that may impede the collection of samples from potentially exposed persons, including cumbersome sample measurement and collection as well as biological hazards and cold shipment and storage concerns. Furthermore, the simplification of sample preparation with sufficient sensitivity for exposure identification supports the use of dried blood samples. As additional response assays are developed which measure biomarkers of exposure, DBS and microsamplers can further be incorporated into public health response, taking advantage of the many benefits.

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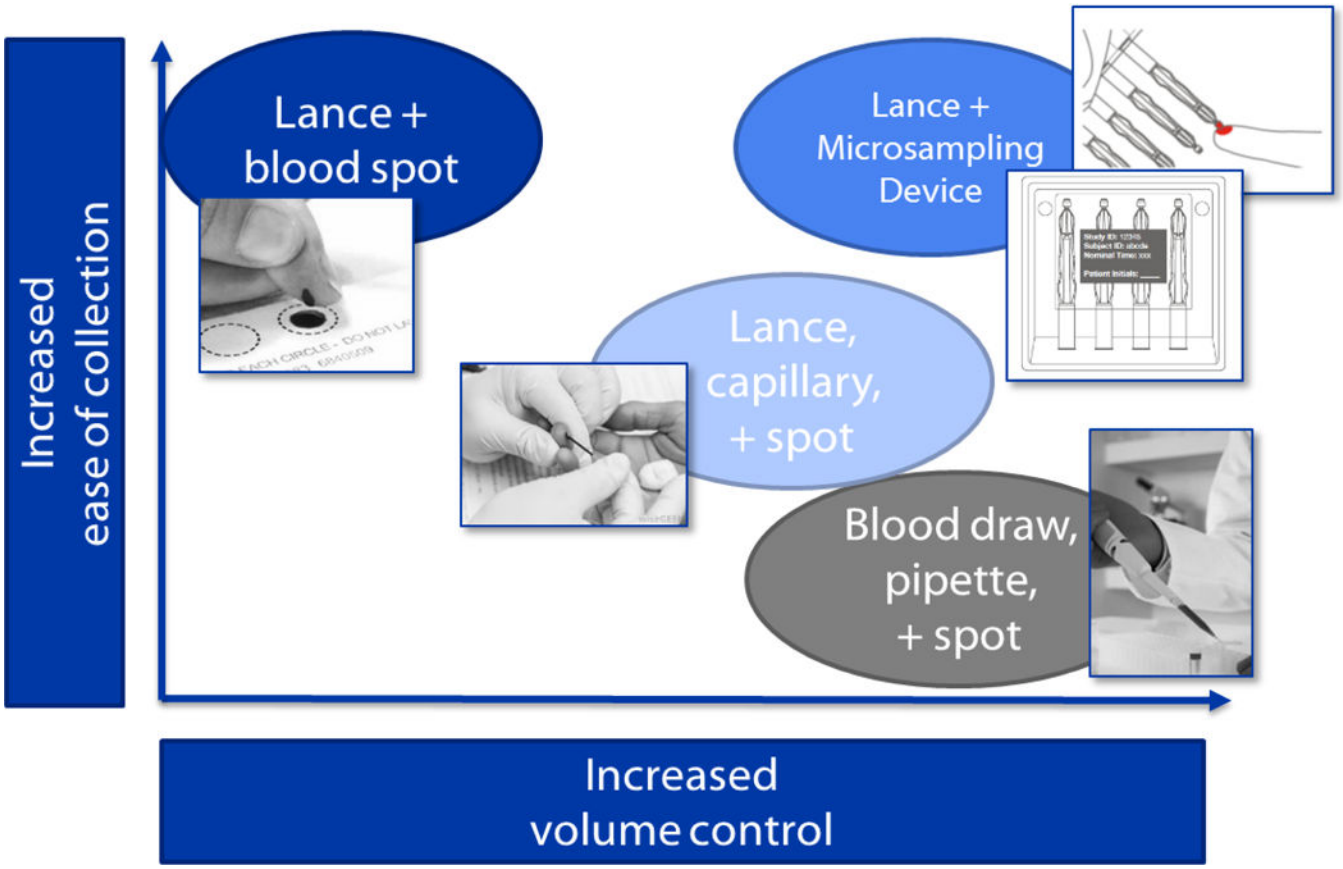

Figure 1.
Potential sampling mechanisms for dried spots relative to volume control and sample collection.

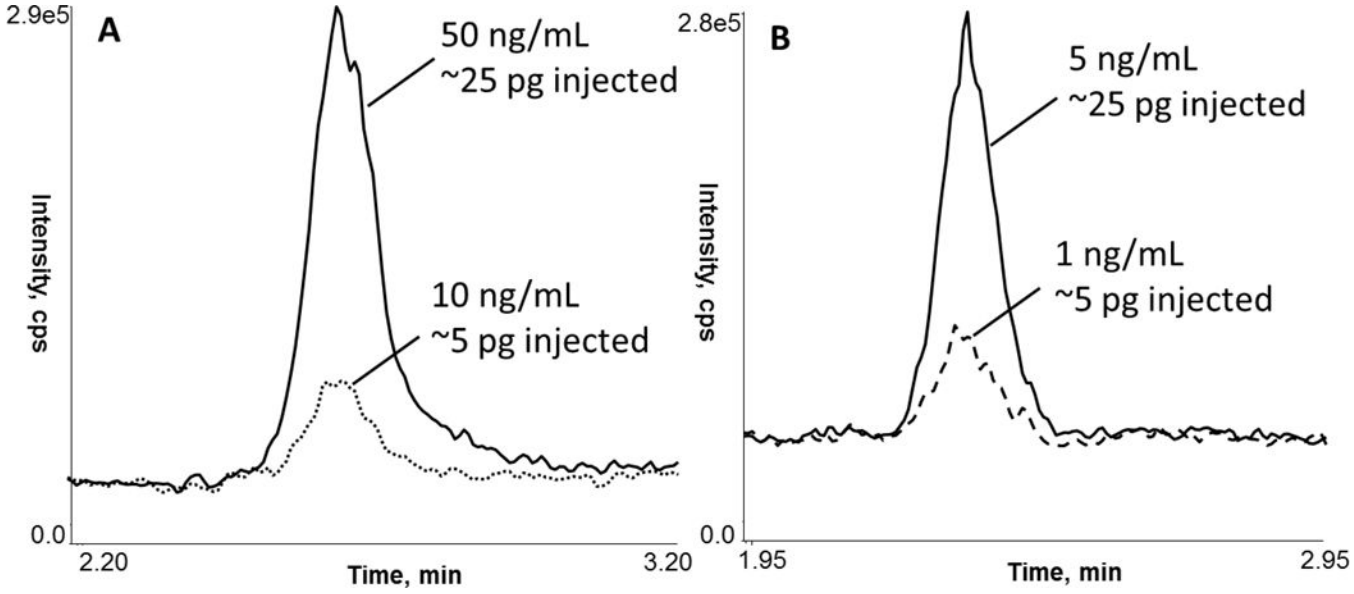

Figure 2.

Comparison of GB (sarin) metabolite chromatograms from (A) dried blood spot (5 μL sample) and (B) unspotted serum (50 μL sample) with commensurate injection amounts.

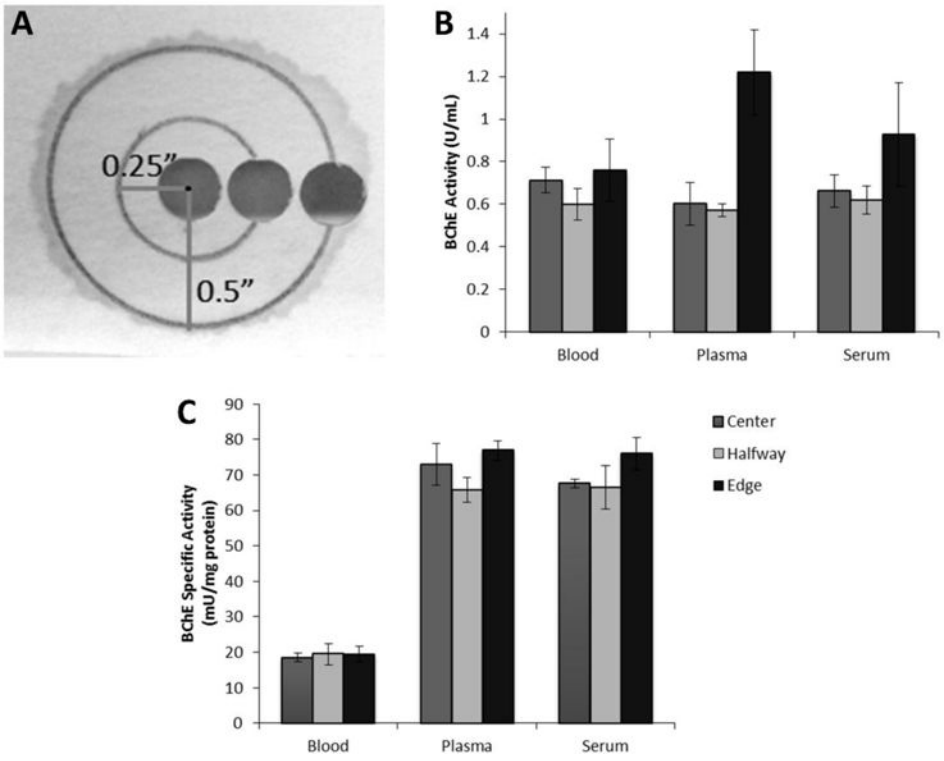

Figure 3.

Sampling location within dried spots (A) to determine variance of butyrylcholinesterase (BChE) activity across individual dried blood, plasma, and serum spots (B) and the reduction of variance between individual spot samples through normalization of the BChE activity to total protein content (C)[14].

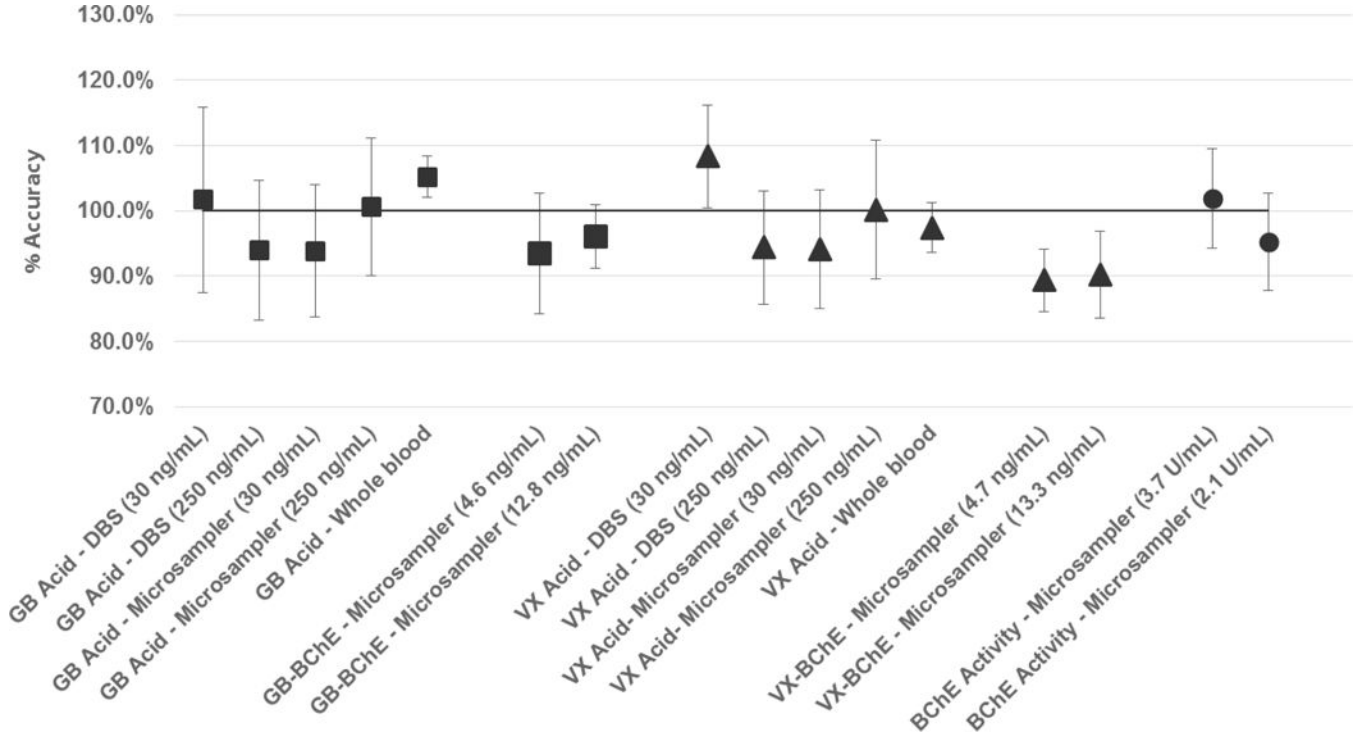

Figure 4.

Measured accuracy and precision (represented as error bars) of GB and VX nerve agent biomarkers in unspotted whole blood, dried blood spots, and microsamplers.

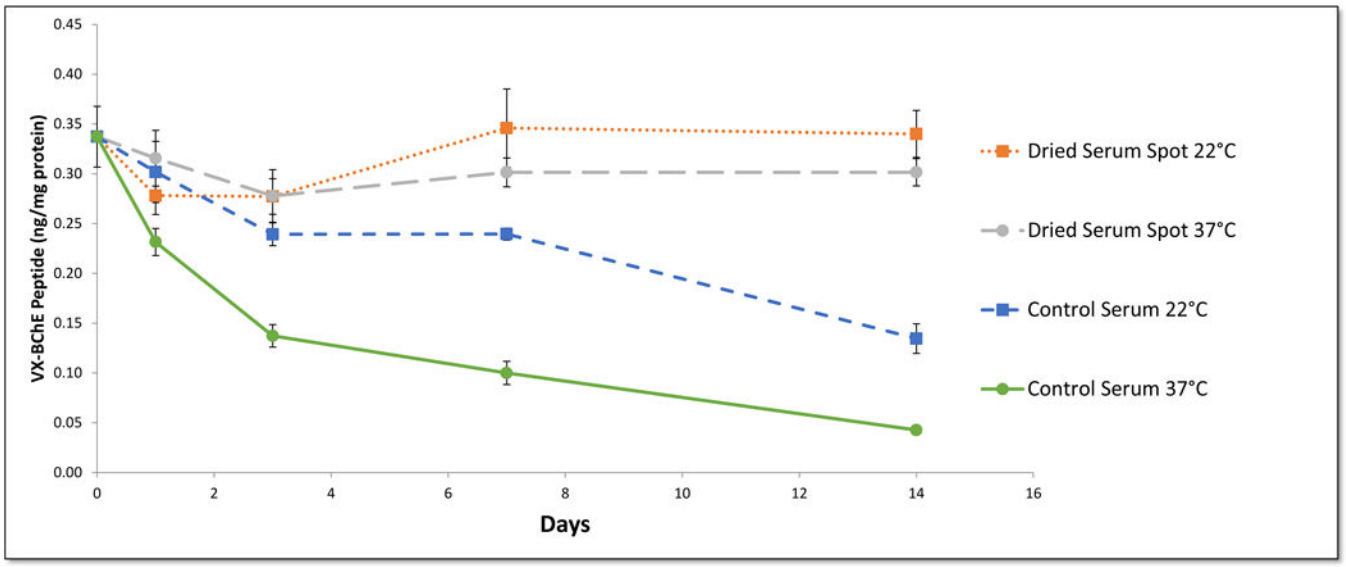

Figure 5.

Stability of butyrylcholinesterase bound VX (VX-BChE) relative to total protein content in dried serum spots and unspotted serum stored at 22 and 37 °C for 14 days[14].