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## Enhanced Stability of Blood Matrices Using a Dried Sample Spot Assay to Measure Human Butyrylcholinesterase Activity and Nerve Agent Adducts

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### Abstract

Dried matrix spots are safer to handle and easier to store than wet blood products, but factors such as intra-spot variability and unknown sample volumes have limited their appeal as a sampling format for quantitative analyses. In this work, we introduce a dried spot activity assay for quantifying butyrylcholinesterase (BChE) specific activity which is BChE activity normalized to the total protein content in a sample spot. The method was demonstrated with blood, serum, and plasma spotted on specimen collection devices (cards) which were extracted to measure total protein and BChE activity using a modified Ellman assay. Activity recovered from dried spots was ~80% of the initial spotted activity for blood and >90% for plasma and serum. Measuring total protein in the sample and calculating specific activity substantially improved quantification and reduced intra-spot variability.

Analyte stability of nerve agent adducts was also evaluated, and the results obtained via BChE-specific activity measurements were confirmed by quantification of BChE adducts using a previously established LC-MS/MS method. The spotted samples were up to 10-times more resistant to degradation compared to unspotted control samples when measuring BChE inhibition by the nerve agents sarin and VX. Using this method, both BChE activity and adducts can be accurately measured from a dried sample spot. This use of a dried sample spot with normalization to total protein is robust, demonstrates decreased intra-spot variability without the need to control for initial sample volume, and enhances analyte stability.

### Keywords

Butyrylcholinesterase activity; Enzyme inhibition; Protein adducts; Dried blood spots; Organophosphorus nerve agents

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## Introduction

Butyrylcholinesterase (BChE, EC 3.1.1.8), a serine hydrolase closely related to acetylcholinesterase (AChE, EC 3.1.1.7), is a non-specific cholinesterase that hydrolyzes various choline esters.<sup>1</sup> BChE activity has been measured as an indicator of numerous conditions including organophosphate toxicity,<sup>2-3</sup> liver dysfunction,<sup>4</sup> and drug sensitivity.<sup>5</sup> Measuring BChE activity using the Ellman assay,<sup>6</sup> or a variation thereof,<sup>7</sup> is straightforward and can be performed using blood, plasma, or serum samples.

The use of filter paper to collect and dry blood samples for subsequent analysis has been widely recognized since the 1960s, when Guthrie and Susi demonstrated its use for neonatal screening of phenylketonuria.<sup>8</sup> Improvements in laboratory technologies have helped overcome the limitations of testing the small sample volumes obtained from dried blood spots (DBSs), leading to substantial growth in this area in recent years.<sup>9</sup> DBS and dried serum spots (DSSs) typically offer a number of advantages over methods such as venipuncture with vacuum tube collection. DBS sampling through a finger prick is less invasive and relatively painless compared to venipuncture.<sup>10</sup> Additional advantages include potentially increased analyte stability, reduced infection hazard, and compatibility with room temperature storage conditions, which removes the need for cold chain transportation of samples. However, it is important to verify that the analyte of interest is not lost or altered by the drying process when moving from a wet to a dried sample format. Confirmation of analyte stability is of particular concern for measuring enzymatic activity from a dried spot because the drying process can denature the enzyme or release proteases.<sup>10-11</sup>

The DBS sample format has been applied to a number of enzymatic assays including acid  $\alpha$ -glucosidase for the diagnosis of Pompe disease and lysosomal activity for the diagnosis of lysosomal storage disorders.<sup>12-13</sup> Researchers also have examined BChE activity from a DBS.<sup>14-15</sup> However, previous studies used a method that correlated a known punch size to the sample volume that was being analyzed. This method resulted in dried samples that only retained 60% of the original activity.<sup>14</sup> Although correlating punch size with sample volume is generally accepted, it overlooks the variability that can be introduced by punch location and hematocrit effect on the area of the DBS.<sup>16-19</sup>

In this work, we introduce a dried spot activity assay for determining BChE-specific activity, which is BChE activity normalized to the total protein content.<sup>20</sup> BChE activity and total protein eluted off of a dried blood, plasma, or serum spot was examined for intra- and inter-spot variability and compared to wet control samples. Analyte stability of the spotted samples was also examined and confirmed using a parallel mass spectrometry method.

## Experimental Section

### Materials

Pooled serum was purchased from Bioreclamation (Westbury, NY), and a portion was spiked with either the organophosphorus nerve agent sarin (GB) or VX at Battelle Memorial Institute (Columbus, OH) to ensure >98% BChE inhibition. Pooled plasma and pooled blood were purchased from Tennessee Blood Services (Memphis, TN). Blood, serum, and plasma

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pools were screened by the vendors, in accordance with Food and Drug Administration regulations, to be free of hepatitis B, hepatitis C, syphilis, and HIV. Nonfat dry milk was purchased from LabScientific (Livingston, NJ). Anti-BChE monoclonal antibody (clone 3E8), EMD Millipore Amicon centrifugal filter units, EMD Millipore MultiScreen filter plates, microtiter plates, and Whatman 903 protein saver cards were purchased from Fisher Scientific (Waltham, MA). Whatman 903 protein saver cards were used in this work as they meet Clinical and Laboratory Standards Institute guidelines for specimen collection devices.<sup>21</sup> Phosphate buffer (PB), dithiobis-nitrobenzoic acid (DTNB), and butyrylthiocholine were purchased from EQM Research (Cincinnati, OH). Avioq HIV-1 DBS elution medium, zipper close bags, humidity sponges, and Pierce micro BCA protein assay kits were purchased from VWR International (Radnor, PA). Pepsin from porcine gastric mucosa, phosphate buffered saline with Tween (PBST), triton X-100, and formic acid (~98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). DynaBeads Protein G superparamagnetic beads were purchased from Life Technologies (Carlsbad, CA). Aquasil C18 HPLC columns (1.0 × 50 mm, 3 μm) were purchased from Thermo Scientific (Waltham, MA). Recombinant human BChE (Protexia) from PharmAthene was received as a gift from Dr. Douglas Cerasoli at the United States Army Medical Research Institute of Chemical Defense (Aberdeen Proving Grounds, MD). Ultra-pure water was generated in-house using an Aqua Solutions (Jasper, GA) Type I Ultra-Pure 18.2 MΩ RO+DI system.

### Preparation of Dried Spots

Dried blood, serum, and plasma spots were prepared in a class 2 biosafety cabinet. Depending on the experiment, 25-300 μL of sample was spotted in the middle of the preprinted circle on a protein saver card. The cards were allowed to dry for 2 h in the biosafety cabinet. The cards were then folded and placed in a zipper close bag containing a humidity sponge.<sup>22</sup> At the desired time, a card was removed from the zipper bag, and a standard 0.25 inch single-hole punch was used to create 1-10 discrete discs from the dried spots. Unless it was noted that whole spot analysis was performed, samples analyzed for only BChE activity and total protein consisted of three discrete punches from a single spot. Samples analyzed for BChE activity, total protein, and BChE peptide biomarkers required additional sample and utilized 10 discrete punches from four spots on a single card. The discs from each sample were placed in the well of a 1.2 μm polyvinylidene difluoride MultiScreen filter plate. Depending on the experiment, 40 or 100 μL of elution buffer (ultra-pure water, 0.1% Triton X-100, or DBS elution medium) was pipetted on top of the discs. Samples consisting of three discs used 40 μL of elution buffer while samples of 10 discs used 100 μL of elution buffer. The discs were incubated at room temperature (22°C) for 10 min. The filter plate was then placed on a new 96-well PCR plate and centrifuged at 3000 × g for 5 min. The plate in which the filtrate was collected was heat sealed and stored at -70°C until analysis.

### Preparation of BChE Calibrators and QC Samples

BChE activity calibrators were prepared from a stock solution of recombinant human BChE (Protexia) of a known activity (54,608 U/mL). A blocking solution was prepared by filtering 4% nonfat dried milk with an Amicon Ultra-15 100 kDa centrifuge filter unit. The concentrated BChE enzyme stock was diluted in blocking solution to final concentrations of

0.05, 0.10, 0.20, 0.40, 0.80, 1.6, 3.2, and 6.4 U/mL and stored in 100  $\mu$ L aliquots at  $-70^{\circ}\text{C}$ . Use of the blocking solution prevented loss of enzyme through nonspecific adsorption to tube and well plate surfaces while processing samples.

Three different quality control (QC) samples were prepared and used to monitor each activity experiment. For use in preparing the QC samples, we created heat-inactivated plasma to reduce the BChE activity of the plasma sample. This inactivated plasma was created by heating pooled plasma to  $60^{\circ}\text{C}$  for 15 min to flocculate fibrinogen. After initial heating, the plasma was centrifuged for 10 min at  $10,000g$ . The supernatant was transferred to a new tube without disturbing the pellet. The supernatant plasma was then heated for a second time to  $68^{\circ}\text{C}$  for 5 min to inactivate BChE. Subsequent analysis of the heat-inactivated plasma revealed  $>99\%$  inactivation. QC samples consisted of: QC high (QCH; pooled plasma), QC mid (QCM; 1:2 dilution of QCH in heat-inactivated plasma), and QC low (QCL; 1:20 dilution of QCH in heat-inactivated plasma). All QC samples were stored in 100  $\mu$ L aliquots at  $-70^{\circ}\text{C}$ . Two analysts characterized the QC samples through 20 analytical runs, with a maximum of 2 runs per day. The characterized samples were used to establish quality metrics for subsequent analytical runs. Quality metrics included evaluation of QCs against amended Westgard rules<sup>23-24</sup> and verification that no QC was outside of 3 SD or that 2 consecutive QCs were outside of 2 SD.

### BChE Activity Measurements

Activity measurements were performed using a modified version of the Ellman assay.<sup>6</sup> Calibrators, QCs, and samples were diluted 1:5 by combining 10  $\mu$ L of calibrators, QCs, or samples with 40  $\mu$ L of blocking solution in individual wells of a protein LoBind plate. The diluted samples were then mixed on an Eppendorf (Hamburg, Germany) MixMate plate shaker for 5 min at 1200 rpm. Activity analysis for each diluted sample was performed in triplicate in a clear 96-well microtiter plate. The plate was prepared by adding 150  $\mu$ L of 69 mM PB (pH 7.4), 20  $\mu$ L of 3.0 mM DTNB, and 10  $\mu$ L of diluted calibrators, QCs, or samples to individual wells. The microtiter plate was then placed in a BioTek Synergy H4 Microplate Reader (Winooski, VT) preheated to  $37^{\circ}\text{C}$ . The plate was mixed in the plate reader by shaking for 10 min to allow thorough mixing of all reagents and quenching of any free sulfhydryl groups before activity measurements. After 10 min, the plate reader added 20  $\mu$ L of 20.0 mM butyrylthiocholine to each well to serve as substrate for active BChE. The plate was then mixed for 10 s by shaking, followed by measurement of absorbance at 412 nm for plasma or serum samples and 430 nm for blood samples. The shaking-absorbance measurement cycle was repeated for 20 min. The change in absorbance over time was plotted and used to determine an accurate slope for each reaction well. BioTek Gen5 software was used to perform a linear least squares regression with 1/y weighting and quantify activity via a calibration curve of standards. The QCs and coefficient of determination of the calibration curve were examined to verify that the run was within quality specifications stated above.

### Determination of Total Protein

Total protein in each sample was quantified using the Pierce micro BCA (bicinchoninic acid) protein assay kit and measured in triplicate. The assay was performed per the

manufacturer's instructions. Briefly, a 7-point calibration curve was created by diluting the BSA standard provided by the manufacturer to concentrations of 5, 10, 25, 50, 100, 150, and 200  $\mu\text{g/mL}$ . Calibrators and samples were diluted 1:100 with water in a protein LoBind plate, followed by a 1:10 dilution, to yield a final 1:1000 dilution in water. BCA working reagent was prepared by mixing micro BCA reagents A, B, and C at a ratio of 25:24:1. In a clear 96-well microtiter plate, 100  $\mu\text{L}$  of the diluted calibrators or samples were combined with 100  $\mu\text{L}$  of BCA working reagent. The plate was sealed with an adhesive foil, mixed on a plate shaker for 1 min at 1000 rpm, and then incubated at 37°C for 2 h. After incubation, absorbance at 562 nm was measured on the microplate reader. Gen5 software was used to perform a 2nd degree polynomial regression with 1/y weighting to quantify total protein via a calibration curve of standards.

### Quantification of BChE Peptide Biomarkers

Quantification of BChE and GB/VX-adducted BChE was performed using isotope dilution LC-MS/MS as described in previous work from this laboratory,<sup>25</sup> with an adjustment to use 50  $\mu\text{L}$  of sample instead of 75  $\mu\text{L}$  because of limited sample volume. Samples were filtered through a 0.45  $\mu\text{m}$  PVDF filter, and 50  $\mu\text{L}$  of filtered sample was incubated with anti-BChE antibody functionalized magnetic beads to immuno-capture BChE from the samples. All bead transfer steps were performed on a Thermo Scientific KingFisher Flex magnetic particle processor. After shaking 2 h at 1400 rpm, the magnetic beads, along with any immuno-captured BChE, were washed with PBST and transferred to a digestion plate which was then sealed with an adhesive foil. The digestion plate contained pepsin, an internal standard mix (<sup>13</sup>C<sub>9</sub>-isotopically labeled 9 amino acid peptides representative of digested unadducted BChE, GB adducted BChE, and VX adducted BChE), and 0.6% formic acid. The digestion plate was heated to 37°C for 30 min with 10 s of shaking at 1000 rpm every minute. After digestion, the beads were removed from the solution. Undigested proteins were removed through addition of acetonitrile to the samples in a protein precipitation plate. The eluent from the precipitation plate was dried with nitrogen at 70°C in a Biotage (Uppsala, Sweden) Turbo Vap-96, resuspended in 0.6% formic acid, and heat sealed until analysis. Biomarker peptides were analyzed on an Agilent 6460 triple quadrupole mass spectrometer (Santa Clara, CA) interfaced with an Agilent 1290 Infinity series HPLC. Peptides were separated via reversed-phase chromatography on an Aquasil C18 column. Data were collected with Agilent MassHunter 6.0 using multiple reaction monitoring. Quantification was performed using 8 peptide calibrators processed at the same time as samples. QCs, processed concurrently with samples, and the coefficient of determination of each standard curve were examined to verify the run was within quality specifications established in previous work.<sup>25</sup>

## Results

### Recovery of BChE Activity from Dried Spot and Normalization to Total Protein

Initial experiments confirmed that BChE activity was retained despite the drying and elution processes. The approximate volumes required to fill the preprinted circle on the protein saver card were 40  $\mu\text{L}$  for blood, 25  $\mu\text{L}$  for plasma, and 25  $\mu\text{L}$  for serum. Cards were spotted with those volumes, dried, and stored overnight at room temperature. The entire spot was

then cut out and eluted using 50  $\mu\text{L}$  of water, 0.1% triton X-100, or DBS elution medium. Previous work has demonstrated that spotting a known volume and excising the entire spot can yield efficient recovery of activity.<sup>26</sup> Our results confirm that activity is efficiently recovered when following that approach (Fig. 1A). Accounting for the dilution introduced by spotting 25 or 40  $\mu\text{L}$  of sample and eluting with 50  $\mu\text{L}$  of buffer, activity recovered from the entire DBS was  $\sim 80\%$  while activities recovered from the entire dried plasma and DSS were  $>90\%$  of the initial activity. Quantification of total protein in each sample enables a specific activity to be calculated, which does not require information about initial sample volume and any dilution factors that were introduced during processing of the spots. Water and 0.1% triton X-100 both yielded specific activities that were equivalent to the unspotted control (Fig. 1B); therefore, water was chosen as the optimal elution medium for subsequent work.

### **BChE Activity Measured at Various Locations on a Dried Spot**

Samples collected during a field study might be of an unknown or inconsistent volume spotted onto a card. Often this potential issue is addressed by associating a sample volume to a punch size and eluting samples from a discrete number of punched discs instead of eluting the entire spot.<sup>14</sup> However, associating an initial sample volume with a particular punch size does not account for variances among samples, and the concentration of analyte can vary throughout the spot. Using pooled blood, plasma, or serum, we determined that a saturated 0.25-inch punch contained 15  $\mu\text{L}$ , 7.5  $\mu\text{L}$ , and 7.5  $\mu\text{L}$ , respectively, of initial sample. To determine the possibility of analyte variability across a dried spot despite a consistent punch size, we assessed large dried spots. Either 300  $\mu\text{L}$  of blood or 200  $\mu\text{L}$  of serum or plasma was spotted, dried, and stored overnight. The sample volumes were chosen such that the dried spot would be approximately 1.25 inches in diameter. The following day, 3 locations were processed separately from the same spot. The 3 locations analyzed were the middle of the spot, where the sample was initially spotted (center); 0.25 inch from the center (halfway); and 0.5 inch from the center (edge). Despite a consistent punch size, activity measured at several locations on the dried spot (Fig. 2) varied greater than 2-fold between punch locations.

### **BChE Activity Recovered Following Extended Storage**

Analyte stability over time is also an important aspect in using dried spots for sample collection. To evaluate the stability of active BChE when stored on a dried spot for an extended period, we analyzed samples of blood, plasma, and serum for specific activity. Each sample was then spotted, dried, and stored in a zipper close bag with a humidity sponge for 40 days at 22°C. Samples were then processed and analyzed for specific activity (Fig. 3). Samples stored as dried spots for 40 days at room temperature largely retained activity compared to controls.

### **Adduct Stability Over 14 Days at Various Temperatures**

When assessing samples for cholinesterase inhibition, proper storage and handling is essential. Even irreversible inhibitors, such as organophosphorus nerve agents and pesticides, continue to undergo chemical reactions after sample collection.<sup>27-29</sup> Reactions can include aging of the inhibiting adduct and spontaneous reactivation of the enzyme

through loss of the adduct, and either reaction will skew quantification. To evaluate the impact of DSS processing and storage on BChE adducts, we performed a 2-week experiment. Pooled serum partially inhibited with GB and VX was spotted and stored under various conditions and compared to unspotted controls stored under the same conditions. Storage conditions evaluated were 4°C, 22°C, and 37°C, and for 1, 3, 7, and 14 days. The specific activity of DSS samples was significantly more stable over time and much closer to the unspotted control serum at time zero (Fig. 4). Over time and with increasing temperature, unspotted samples increased in activity, indicating spontaneous reactivation of BChE and loss of the inhibiting adduct. The degradation of the sample was confirmed by assessing the same samples for adducted and unadducted BChE peptides via LC-MS/MS. In unspotted controls, the BChE biomarker peptide concentration increased (Fig. 5A), whereas the adducted peptide biomarker concentrations decreased (Fig. 5B & 5C).

## Discussion

Dried sample spots offer a number of advantages over unspotted blood products for testing. However, intra-spot variability and unknown sample volumes must be addressed before dried spots can be used as a sample format for BChE activity and adduct analysis. When a known volume is spotted and the entire spot is analyzed, efficient recoveries can be achieved. However, without knowing the volume spotted, it is not possible to determine if discrepancies are the result of low activity, dilution of the sample, or inefficient elution of the spot. We demonstrated that measuring both activity and total protein content provide a basis to overcome these issues. After the total protein of the sample was assessed and specific activity was calculated, samples eluted with water or 0.1% triton X-100 (Fig. 1) can be directly compared to unspotted control samples.

Use of the DBS elution medium resulted in low specific activities for plasma and serum. These samples had artificially high (~130%) recoveries of total protein. The medium contains sodium chloride and triton X-100, which at high levels can, according to the manufacturer, interfere with accurate protein quantification.

Measuring specific activity, as opposed to activity alone, also addressed issues related to punch location. The activity at the edge of plasma and serum spots was significantly higher and more variable than activity at the center or halfway locations of the same spot (Fig. 2B). Plasma demonstrated the greatest edge effects compared to the other two locations (2.1-fold increase in activity), followed by serum (1.5-fold increase), and blood (1.2-fold increase). Total protein from the same discs exhibited a correlated trend (plasma: center = 8.2 mg/mL, halfway = 8.7 mg/mL, edge = 15.9 mg/mL; serum: center = 9.8 mg/mL, halfway = 9.4 mg/mL, edge = 12.3 mg/mL), and visually, the edges of the plasma and serum spots were darker (Fig 2A). Specific activity normalizes this effect, indicating that differences in activity around the edge of the spot result from an increase in total protein concentration and not an increase in BChE concentration alone. In all three matrices, normalization to total protein greatly reduced the intra-spot variability: from 12.0% to 2.6% relative standard deviation (RSD) for blood, 45.7% to 7.9% RSD for plasma, and 22.9% to 7.4% RSD for serum.

Storing samples as a dried spot helped to maintain sample integrity. Samples with normal levels of activity, stored as a dried spot, retained >90% of their initial activity, even after 40 days of storage at room temperature (Fig. 3). This is important because it indicates that dried spots could be transported under ambient temperatures and still remain viable for analysis. Additionally, samples with inhibited levels of BChE activity were more resilient to loss of the inhibitor and spontaneous reactivation when stored as a DSS (Table 1). Under the least ideal storage conditions tested (37°C, 14 days), samples stored in solution exhibited at least 10-fold more reactivation than those stored at the same conditions on a dried card. The enhanced stability of the DSS samples was confirmed by LC-MS/MS data quantifying unadducted BChE, GB-adducted BChE (GB-BChE), and VX-adducted BChE (VX-BChE) via biomarker peptides (Fig 5). Under the conditions tested, biomarker peptides from DSS samples exhibited no detectable trend indicative of an increase in unadducted BChE peptide or a decrease in inhibitor-adducted BChE peptides. However, unspotted samples stored at analogous conditions exhibited an increase in BChE peptide and a decrease in GB-BChE and VX-BChE peptides. After 14 days at 37°C, BChE peptide increased 0.28 ng/mg protein; this 437% increase indicates the extent of spontaneous reactivation. GB-BChE peptide decreased 0.18 ng/mg protein (85% decrease) and VX-BChE decreased 0.29 ng/mg protein (87% decrease), for a total decrease in adducted peptide of 0.47 ng/mg protein. The residual loss of adducted peptide not accounted for by spontaneous reactivation (0.19 ng/mg protein) might be indicative of aging of the nerve agent adducts. GB and VX adducts age to a methylphosphonate adduct, which was not measured in this work.

## Conclusions

This new method, which utilizes total protein normalization for determining BChE activity from a dried sample spot, merges the benefits of dried samples spots with the quantitative robustness of wet blood product methods. The normalization step allows the assay to overcome the variability of punch location without knowing the initial sample volume. Additionally, the dried sample format eliminates the need for cold storage and transportation of samples before analysis, which may be of great importance in the event of a large scale emergency involving cholinesterase inhibitors. Using the reported method, the specific activity of the spotted samples was found to be in agreement with unspotted controls, and DSS samples exhibited increased analyte stability when compared to unspotted controls. This observed increase in stability was confirmed by quantifying organophosphorus nerve agent adducts to BChE via a previously established LC-MS/MS method.

Ultimately, rapid collection and testing of clinical samples is most desirable after an exposure event. This work highlights the fact that test results related to cholinesterase inhibition or adducts can skew from the time of collection to the time of analysis depending on how samples are collected and stored. For that reason, utilizing the reported approach may be critical for accurate analysis of large numbers of samples when refrigerated shipping and storage capabilities are limited.

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this article 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 U.S. Department of Health and Human Services.

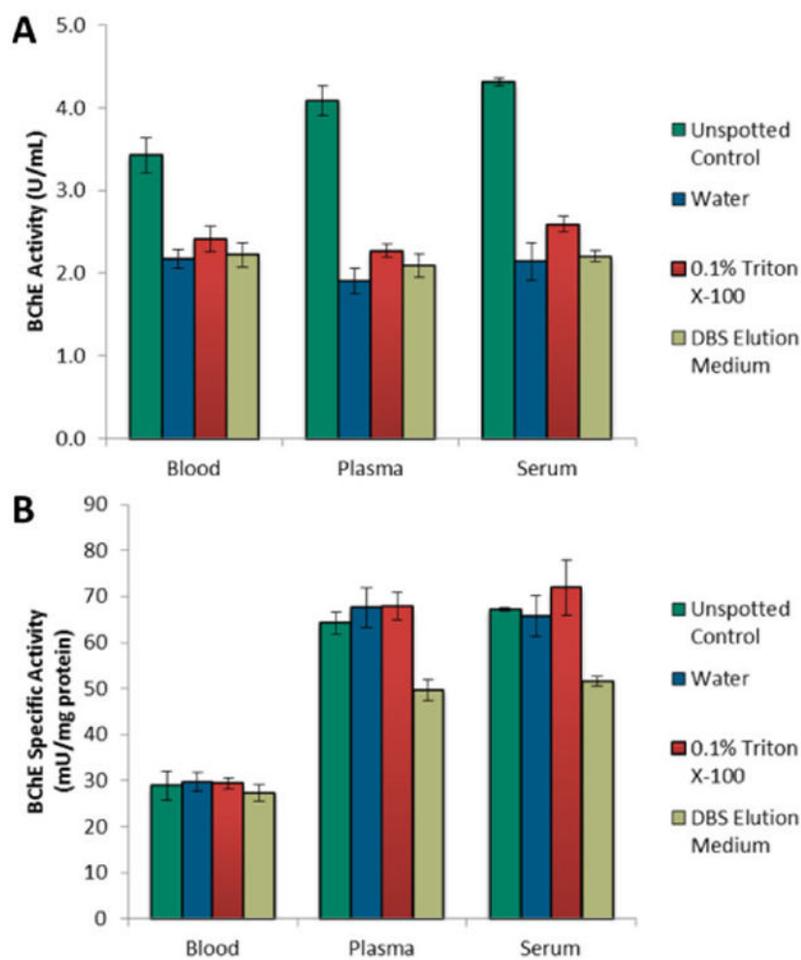
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## Abbreviations

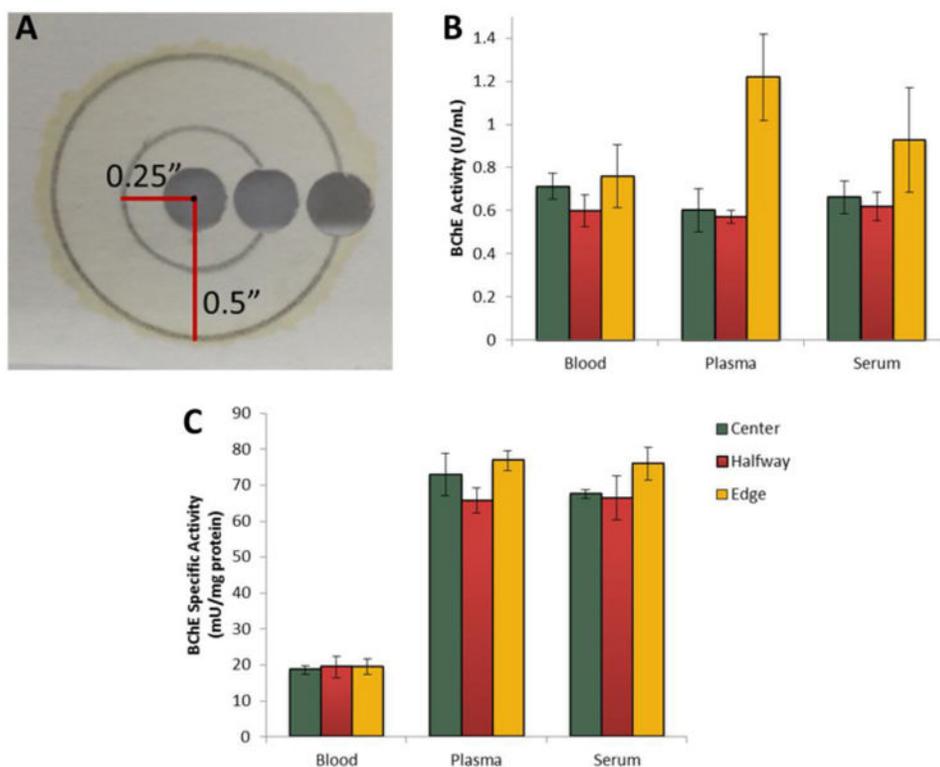
<b>BChE</b>	Butyrylcholinesterase
<b>AChE</b>	Acetylcholinesterase
<b>DBS</b>	Dried blood spot
<b>DSS</b>	Dried serum spot

<b>GB</b>	Sarin
<b>PB</b>	Phosphate buffer
<b>DTNB</b>	5,5'-dithiobis-(2-nitrobenzoic acid)
<b>PBST</b>	Phosphate-buffered saline with tween
<b>QC</b>	Quality control
<b>BCA</b>	Bicinchoninic acid
<b>RSD</b>	Relative standard deviation



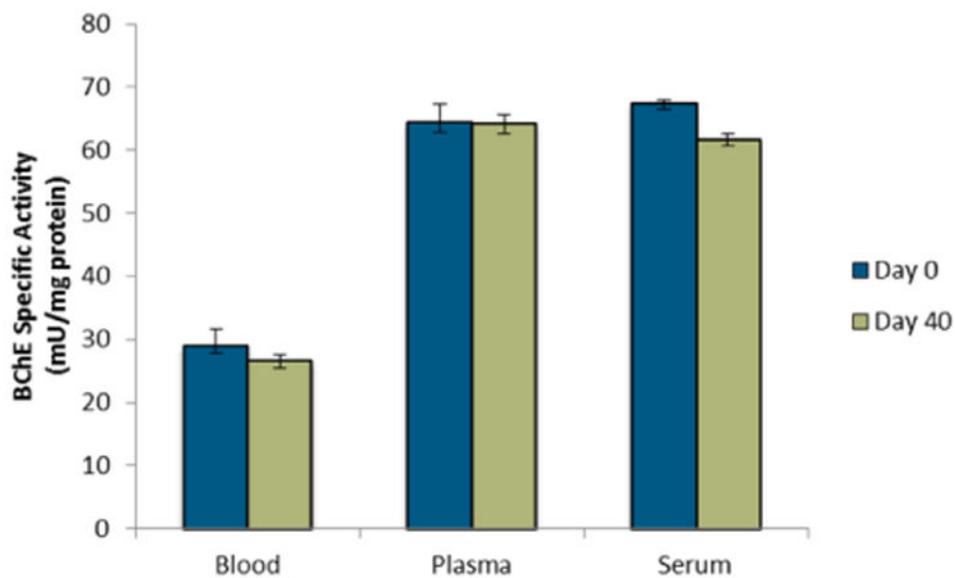
**Fig. 1. Recovery of BChE activity from dried spots**

(A), BChE activity recovered from dried samples using various eluents compared to an unspotted control aliquot (white). (B), BChE specific activity determined by normalizing activity to total protein in each sample. Data shown as the mean  $\pm$  SD (n = 5).



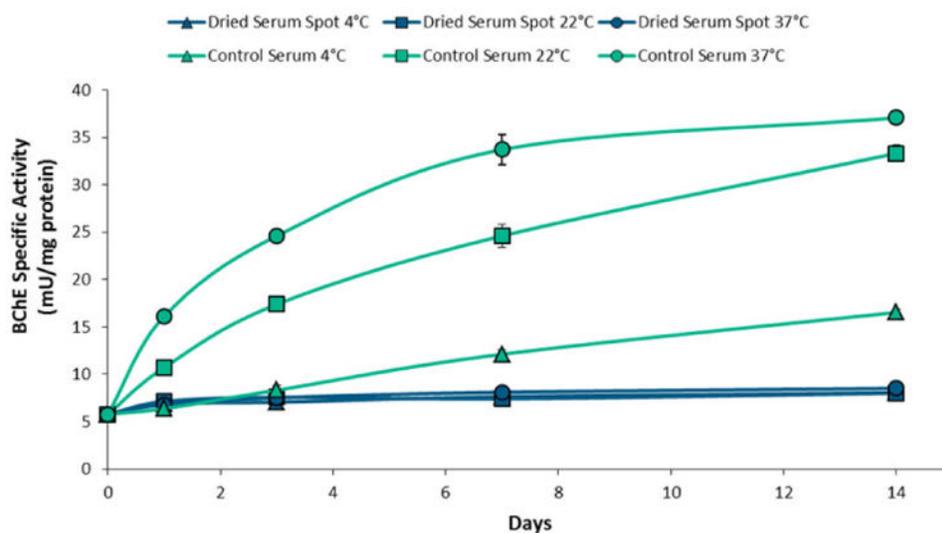
**Fig. 2. Effect of punch location on BChE activity**

(A), A representative dried spot used to determine the effect of punch location on recovered activity. “Center” is a disc punched from the center point of the spot, “halfway” is a disc punched 0.25 inch from the center point, and “edge” is a disc punched 0.5 inch from the center point. (B), BChE Activity recovered from various punch locations across a dried spot. (C), BChE specific activity of the same samples. Data shown as the mean  $\pm$  SD ( $n = 3$ ).



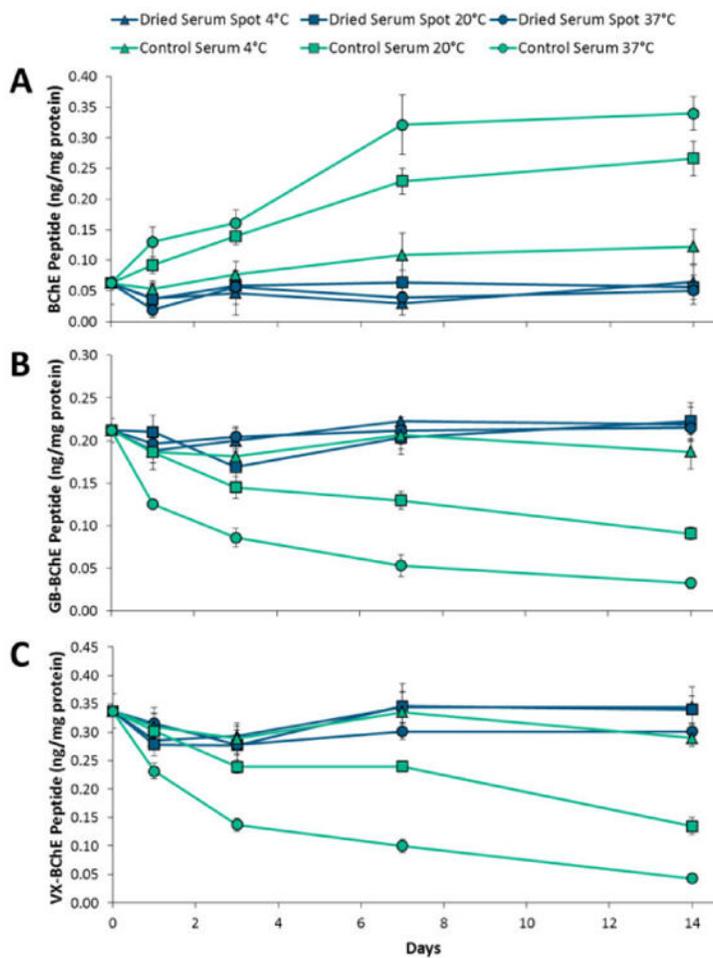
**Fig. 3. Stability of BChE activity after extended storage**

BChE specific activity of an unspotted control sample on day zero, compared to an aliquot that was spotted and stored at room temperature for 40 days before analysis. Data shown as the mean  $\pm$  SD (n = 3).



**Fig. 4. Enhanced resistance to spontaneous reactivation of inhibited samples stored as dried spots**

BChE specific activity of dried serum spots compared to unspotted controls at 4°C, 22°C, or 37°C, and for 1, 3, 7, or 14 days. Increasing BChE specific activity is indicative of the loss of inhibiting adduct from the enzyme and the reformation of the native active enzyme. Data shown as the mean  $\pm$  SD (n = 4).



**Fig. 5. Increased adduct stability of inhibited samples stored as dried spots**  
 LC-MS/MS analysis of biomarker peptides indicative of BChE (A), GB adducted BChE (B), and VX adducted BChE (C). Adduct stability is detected by the change in stored samples compared to an unspotted control on day zero. Data shown as the mean  $\pm$  SD (n = 4).

**Table 1**

Increase in BChE specific activity of dried serum spots (DSS) and control serum relative to initial activity.

	<b>1 day</b>	<b>3 days</b>	<b>7 days</b>	<b>14 days</b>
DSS at 4°C	19%	22%	32%	39%
Serum at 4°C	10%	44%	110%	186%
DSS at 22°C	23%	31%	28%	39%
Serum at 22°C	85%	201%	326%	477%
DSS at 37°C	18%	30%	40%	47%
Serum at 37°C	179%	326%	483%	541%