HHS Public Access

Author manuscript

Biomed Chromatogr. Author manuscript; available in PMC 2018 April 01.

Published in final edited form as:

Biomed Chromatogr. 2017 April ; 31(4): . doi:10.1002/bmc.3830.

A High-Throughput UHPLC-MS/MS Method for the Quantification of Five Aged Butyrylcholinesterase Biomarkers from Human Exposure to Organophosphorus Nerve Agents

Leigh Ann Graham[a], **Darryl Johnson**[b], **Melissa D. Carter**[c,*], **Emily G. Stout**[b], **Huseyin A. Erol**[b], **Samantha L. Isenberg**[a], **Thomas P. Mathews**[a], **Jerry D. Thomas**[c], and **Rudolph C. Johnson**[c]

[a]Battelle at the Centers for Disease Control and Prevention, Atlanta, GA

[b]Oak Ridge Institute for Science and Education Fellow at the Centers for Disease Control and Prevention, Atlanta, GA

[c]Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA

Abstract

Organophosphorus nerve agents (OPNAs) are toxic compounds that are classified as prohibited Schedule 1 chemical weapons. In the body, OPNAs bind to butyrylcholinesterase (BChE) to form nerve agent adducts (OPNA-BChE). OPNA-BChE adducts can provide a reliable, long-term protein biomarker for assessing human exposure. A major challenge facing OPNA-BChE detection is hydrolysis (aging), which can continue to occur after a clinical specimen has been collected. During aging, the *o*-alkyl phosphoester bond hydrolyzes, and the specific identity of the nerve agent is lost. To better identify OPNA exposure events, a high throughput method for the detection of five aged OPNA-BChE adducts was developed. This is the first diagnostic panel to allow for the simultaneous quantification of any Chemical Weapons Convention Schedule 1 OPNA by measuring the aged adducts methyl phosphonate (MeP-BChE), ethyl phosphonate (EtP-BChE), propyl phosphonate (PrP-BChE), ethyl phosphoryl (ExP-BChE), phosphoryl (P-BChE), and unadducted BChE. The calibration range for all analytes is 2.00 – 250. ng/mL, which is consistent with similar methodologies used to detect unaged OPNA-BChE adducts. Each analytical run is three minutes making the time to first unknown results, including calibration curve and quality controls, less than one hour. Analysis of commercially purchased individual serum samples demonstrated no potential interferences with detection of aged OPNA-BChE adducts, and quantitative measurements of endogenous levels of BChE were similar to those previously reported in other OPNA-BChE adduct assays.

[*]Corresponding author: M.D. Carter, Division of Laboratory Sciences, Centers for Disease Control and Prevention, Atlanta, GA 30341, USA. vsm8@cdc.gov Phone: 770-488-7263. Fax: 770-488-7581.

Disclaimer

The findings and conclusions in 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.

Conflict of Interest Disclosure

The authors declare no competing financial interest.

Keywords

butyrylcholinesterase; organophosphorus nerve agents; hydrolysis; aging; quantitative mass spectrometry

1. Introduction

Organophosphorus nerve agents (OPNAs) are compounds that are classified by the Chemical Weapons Convention (CWC) as prohibited Schedule 1 chemical weapons.(OPCW, 2005) There have been several documented OPNA uses, including the Iraq-Iran conflict in 1984, Japan in 1994 and 1995, and Syria in 2013.(Pita and Domingo, 2014, Suzuki et al., 1995, UN, 1984, Yanagisawa et al., 1995) OPNAs are toxic chemicals that induce nausea, vomiting, convulsions, seizures, and death by binding to the active site serine of the cholinesterase enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). (Grob and Harvey, 1953, Schecter, 2004) The toxicity of OPNAs, as well as their relative ease of synthesis, make them a concern for use as a weapon.(Evison et al., 2002) In addition to intentional exposure, there is also an increased risk for accidental exposure during the destruction of legacy stockpiles. This is especially of concern given the current efforts by the Organisation for the Prohibition of Chemical Weapons (OPCW) to eradicate OPNA stockpiles by 2023.(OPCW, 2015)

In the body, OPNAs can undergo hydrolysis, making direct measurement of the live agent of exposure difficult.(Black, 2010) Nerve agent metabolites can be detected in blood and urine; however, these metabolites are excreted from the body within two weeks following exposure.(Hamelin et al., 2014, Mawhinney et al., 2007, Riches et al., 2005) OPNAs also form protein adducts which can provide a previously reported two to three months for retrospective detection.(Black and Read, 2013) The Ellman activity assay has been used to determine OPNA exposure by measuring cholinesterase activity but lacks the specificity to distinguish OPNAs from organophosphorus pesticides or other compounds capable of inhibiting cholinesterases.(Ellman et al., 1961) Fluoride reactivation provides a sensitive method for detecting OPNA exposure, but agents that have undergone hydrolysis (aging) cannot be reactivated; therefore, rapidly aging OPNAs such as soman (GD) may not be detected.(Adams et al., 2004, Heilbronn, 1965, Heilbronn, 1965) Direct measurement of OPNA-adducted proteins has become an increasingly utilized method for retrospective detection.(Crow et al., 2014, Noort et al., 2006, Van der Schans et al., 2008, Williams et al., 2007) OPNAs readily react with BChE which provides a reliable biomarker for measuring OPNA exposure. OPNA-BChE adducts have relatively long half-lives (8–12 days), are abundant in serum and plasma, and can easily be extracted from blood since BChE is not membrane-bound.(Knaack et al., 2012, Pantazides et al., 2014, Sporty et al., 2010)

A major challenge facing direct measurement of OPNA-BChE adducts is an aging process caused by hydrolysis. The *o*-alkyl phosphoester bond of OPNA-BChE adducts ages over time in biological matrices, and the specific identity of the agent is lost (Figure 1).(Carter et al., 2013, Segall et al., 1993) For example, sarin (GB), cyclosarin (GF), GD, VX, VM, and VR all share a common methyl-phosphorus bond that is preserved after reaction with the

active site serine of BChE, giving rise to the aged methyl phosphonate biomarker, MeP-BChE.(Berry and Davies, 1966, Segall et al., 1993) Although the specific identity of the OPNA cannot be determined following aging, identification of MeP-BChE is indicative of organophosphorus exposure. Similarly, ethyl sarin (GE) and VE (O-ethyl-S-diethylaminoethylphosonothiolate) share a common ethyl-phosphorus bond, giving rise to the aged ethyl phosphonate biomarker, EtP-BChE. Only synthetic analogues of the methyl and ethyl OPNAs, such as propyl-sarin (Pr-GB), would be expected to form the propyl phosphonate biomarker, PrP-BChE. The methyl, ethyl, and propyl phosphonate adducts are unique to OPNAs and are not known to be formed by any organophosphorus pesticides.(Jiang et al., 2013) A phosphoryl adduct (P-BChE) can be formed by OPNAs such as tabun (GA) and GV (2-dialkylaminoalkyl N,N-dialkylphosphonamidofluoridate), but can also be generated from the industrial additive cresyl saligenin phosphate (CBDP).(Carletti et al., 2008, Liyasova et al., 2011) In the case of GA, aging through deamidation has also been reported, producing an ethyl phosphoryl adduct (ExP-BChE).(Barak et al., 2000, Elhanany et al., 2001) However, this additional adduct (+108 Da) is not specific to GA. Organophosphorus pesticides containing a diethoxyphosphate group can also undergo aging to produce ExP-BChE.(Jiang et al., 2013, Jiang et al., 2013)

The formation of aged OPNA-BChE adducts can continue to occur after a clinical specimen has been collected, highlighting the need for a method to quantify aged biomarkers.(Berry and Davies, 1966) Previous reports have described methods that could be used to detect aged biomarkers of OPNA exposure; however, these assays required additional sample analysis to identify or confirm the agent of exposure.(Noort et al., 2006, Van der Schans et al., 2008) To better identify OPNA exposure events, a high-throughput method for the detection and quantitation of aged OPNA-BChE adducts has been developed. The method described herein provides simultaneous quantification of P-BChE, MeP-BChE, ExP-BChE, EtP-BChE, PrP-BChE, and unadducted BChE biomarker peptides in human blood matrices. Monitoring this panel of aged OPNA-BChE biomarkers allows for the detection of all CWC Schedule 1 OPNAs.

2. Materials and methods

2.1 Materials

The following materials were purchased from Sigma Aldrich (St. Louis, MO, USA): phosphate buffered saline with Tween 20 (PBST) dry powder, dimethyl pimelimidate dihydrochloride, 0.2 M tris buffered saline (TBS) 10X concentrate, triethanolamine buffer solution, and pepsin from porcine gastric mucosa. The following items were purchased from Fisher Scientific (Hanover Park, IL, USA): Optima LC/MS grade formic acid, KingFisher shallow well plates (PN: 22-387-030), KingFisher 96 tip combs (PN: 22-387-029), KingFisher deep well plates (PN: 12-565-651), Fisherbrand deep well plates (PN: 12-566-121), Pierce protein precipitation plates, and Eppendorf PCR adhesive foil. Eppendorf heat-sealing foil was purchased from VWR (Radnor, PA, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile and deionized water were commercially available from Tedia (Fairfield, OH, USA). Ultrapure 18.2 MΩ-cm water was generated in-house with an Aqua Solutions Water Purification system (Jasper, GA, USA).

Dynabeads Protein G magnetic beads were obtained from Life Technologies (Carlsbad, CA, USA). BChE monoclonal antibodies from clone 3E8 were purchased from BioPorto (Hellerup, Denmark).

The Netherlands Organisation for Applied Scientific Research (TNO; Rijswijk, Netherlands) provided synthetic native and isotopically-labeled BChE and P-BChE peptides. Battelle Memorial Institute (Columbus, OH, USA) provided synthetic native and isotopically-labeled MeP-BChE, ExP-BChE, EtP-BChE, and PrP-BChE peptides. For PrP-BChE, two isoforms are possible, *i*-PrP-BChE and *n*-PrP-BChE. These isoforms have the same mass, but could not be separated chromatographically. Therefore, this work only uses *i*-PrP-BChE synthetic standards, but the method would be able to detect either isoform. Commercial pooled serum was purchased from Bioreclamation, Inc. (Westbury, NY, USA) and TNO. Cresyl saligenin phosphate (CBDP) inhibited serum (98% BChE inhibition) was procured from TNO. GE (99.4% BChE inhibition), GV (99.3% BChE inhibition) and Propyl GB (98.8% BChE inhibition) inhibited serum were purchased from Battelle. Sarin-inhibited serum and plasma (98% inhibition) were obtained from Battelle Memorial Institute and the U.S. Army Edgewood Chemical Biological Center. A commercial convenience set consisting of serum from 96 healthy individuals with no expected OPNA exposure was purchased from Tennessee Blood Services (Memphis, TN, USA). The method used blood products acquired from commercial sources, and the work did not meet the definition of human subjects as specified in 45 CFR 46.102 (f). The commercial blood products were screened for the presence of pathogens prior to commercial sale.

2.2 Preparation of calibrators and quality controls

Amino acid analysis of synthetic peptides was carried out by New England Peptide (Gardner, MA, USA) prior to preparation of calibration and quality control solutions. Stock solutions were made for each native and isotopically-labeled standard at 1.00 mg/mL in ultrapure water. Native peptide stock solutions were combined and diluted in ultrapure water at concentrations of 2.00, 4.00, 8.00, 16.0, 32.0, 63.0, 125, and 250. ng/mL. A single internal standard (ISTD) solution was prepared that consisted of isotopically-labeled peptides P-[$^{13}C_9$]-BChE (450 ng/mL), MeP-[$^{13}C_9$,^{15}N]-BChE (250 ng/mL), ExP-[$^{13}C_9$]-BChE (250.ng/mL), EtP-[$^{13}C_9$,^{15}N]-BChE (250 ng/mL), [$^{13}C_9$]-BChE (250 ng/mL), and PrP-[$^{13}C_9$,^{15}N]-BChE (100 ng/mL) peptides. The structures of the isotopically-labeled peptides with sites of labeling can be found in Figure 2. 10 kDa MWCO filtered uninhibited human serum was used as matrix blank. The filtration was found to remove endogenous BChE from the serum, and therefore did not interfere with the detection of BChE calibrators.

Five quality control (QC) samples were prepared: high level QC (QH), mid-level BChE QC (QM-BChE), mid-level QC (QM), low level P-BChE QC (QL-P-BChE), and low level QC (QL). QL, QM, and QH were prepared by spiking synthetic peptides into 10 kDa filtered uninhibited serum at 10.0, 40.0, and 80.0 ng/mL. Pooled commercial uninhibited serum from TNO was used as QM-BChE. A 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (cresyl saligenin phosphate or CBDP) inhibited serum from TNO, which is known to hydrolyze to form the P-BChE adduct, was used as QL-P-BChE.(Schopfer et al., 2010) Uninhibited pooled human serum (QM-BChE) and pooled serum fully inhibited with oCP

(QL-P-BChE) were included in the QC characterization material to verify digestion by pepsin. Since these are serum-based QC materials, it ensures proper sample preparation for unknown samples. QC material characterization was completed over the course of 4 weeks during method validation ($n = 24$) and performed by five laboratory analysts following the CDC's multi-rule quality control system (MRQCS). (Caudill et al., 2008)

2.3 Sample preparation

Samples were prepared as previously described with additional optimization.(Johnson et al., 2015, Knaack et al., 2012, Pantazides et al., 2014) Briefly, a DynaMag-15 (Invitrogen, Carlsbad, CA) was applied to remove the solvent from 2 mL of Dynabeads Protein G magnetic beads. The beads were washed twice with 4 mL aliquots of PBST, and then 8 mL of PBST was added to the beads followed by 400 μg of anti-BChE monoclonal antibody. This mixture was allowed to rotate on a Dynal Sample mixer at speed setting 20 for one hour at room temperature; after which, the solvent was removed, and the beads were washed twice with 4 mL aliquots of triethanolamine buffer. A 4 mL aliquot of a 5.4 mg/mL solution of dimethyl pimelimidate dihydrochloride in triethanolamine buffer was added to the magnetic beads, and this was allowed to rotate for 30 minutes at speed setting 20 at room temperature. After the solvent was removed, 4 mL of TBS was added, and then the magnetic beads were allowed to rotate for 15 min at speed setting 20 at room temperature. The solvent was removed after rotation, and the magnetic beads were washed twice with 2 mL aliquots of PBST. Finally, 1.9 mL of PBST was added to the beads, and they were stored at 4 °C for use up to 3 months.

After antibody conjugated bead preparation, 125 μL aliquots of either serum or plasma were pipetted into a multiscreen HTS HV 0.45 μm opaque filter plate and then centrifuged for 5 minutes at 3000 × g and 20 °C. A 75 μL aliquot of the filtered serum or plasma was transferred into a KingFisher deep 96-well plate. A 50 μL aliquot of anti-BChE magnetic beads were added to the wells of a KingFisher shallow plate that corresponded to wells that contained serum or plasma. A KingFisher Flex magnetic particle processor (Thermo Scientific, Waltham, MA, USA), was used to transfer the anti-BChE beads to the sample plate containing the serum or plasma. The sample plate was then covered with adhesive foil, and allowed to shake for 45 min on an Eppendorf MixMate (Eppendorf, Hamburg, Germany) at 1400 rpm at room temperature. The KingFisher Flex was used to transfer the BChE bound magnetic beads to three deep well wash plates containing 500 μL PBST, and finally to a KingFisher shallow 96-well plate for digestion. The digestion plate contained 10 μL of 2 mg/mL pepsin in 0.6% formic acid in ultrapure water and 10 μL of the isotopically-labeled internal standard in each well. In wells that were used for calibration or that contained a synthetic peptide QC spike, 75 μL of the appropriate solution was added, and to the remaining wells, 75 μL of 0.6% formic acid in ultrapure water was added. The digestion plate was covered with adhesive foil and placed in an Eppendorf Thermomixer R (Hamburg, Germany) for 30 min at 37 °C with intermittent shaking at 1000 rpm for 10 sec every min. After digestion, the anti-BChE beads were removed from the digestion plate using the KingFisher Flex. A 285 μL aliquot of acetonitrile was added to each well of the protein precipitation plate used and followed by the addition of the digested samples. This plate was allowed to shake for 1 min at 1,000 rpm before being filtered through the protein

precipitation plate using a vacuum manifold, and a deep 96-well plate as a receiving plate. The samples were dried to completeness using a Porvair Ultravap (Porvair Sciences, Wrexham, UK) at 60 °C under a nitrogen stream. Samples were resuspended in 75 μL of 0.6% formic acid in ultrapure water for UHPLC-MS/MS analysis. A flowchart summarizing the sample preparation procedure has been included Scheme 1 for clarity.

2.4 UHPLC-MS/MS

The BChE biomarker peptides were evaluated using an Agilent 1290 Infinity series UHPLC system (Santa Clara, CA, USA) coupled with an Agilent 6490 triple quadrupole mass spectrometer with a jet stream ionization source, operating in positive ionization mode. The instrument was tuned and calibrated bimonthly using the Agilent ESI tuning mixture (P/N G1969) over a range of 50–1400 *m/z*. Chromatographic separation was carried out on a Restek Pinnacle DB Biphenyl 1.9 μm, 2.1 × 50 mm analytical column (Bellefonte, PA, USA). The column compartment and autosampler temperatures were set to 60 °C and 10 °C, respectively. Mobile phases consisted of 0.1% formic acid in (A) water and (B) acetonitrile, delivered at a flow rate of 300 μL/min. Gradient conditions increased mobile phase B concentration from 5% to 23% over 1.55 min, from 23% to 76% over 0.28 min, held 76% for 0.17 min followed by a re-equilibration step at 5% mobile phase B for 1.0 min. A total analysis time of 3 min per sample was achieved. The following parameters were used with the jet stream ionization source: drying gas temperature = 220 °C; drying gas flow = 11 L/min; nebulizer = 60 psi; sheath gas temperature = 350 °C; sheath gas flow = 11 L/min; capillary voltage = 3500 V; nozzle voltage = 1800 V; iFunnel high pressure RF = 190 V; iFunnel low pressure RF = 120 V; fragmenter voltage = 250 V. The mass spectrometer was operated in multiple reaction monitoring mode, with the two most selective transitions for the native BChE biomarker peptides being monitored for quantitation and confirmation. A single transition was monitored for each of the isotopically-labeled biomarker peptides. P-BChE and MeP-BChE transitions were acquired in time-segment 1 (0.00–1.54 min) with a 35 ms dwell time. ExP-BChE and EtP-BChE transitions were acquired in time-segment 2 (1.54–1.65 min) with a 20 ms dwell time. BChE and PrP-BChE transitions were acquired in time-segment 3 (1.65–1.90 min) with a 35 ms dwell time. See Table 1 for parameters for peptide detection.

2.5 Recovery and Matrix Effects

Since an isotopically-labeled BChE protein is not currently available, recovery of aged OPNA-BChE and unadducted BChE peptides was determined by adding calibrators at low (2.00 ng/mL), mid (32.0 ng/mL), and high (250 ng/mL) level spikes to the matrix blank serum prior to pepsin digestion (“processed”) or immediately following pepsin digestion (“unprocessed”). Recovery was assessed by comparing the average chromatographic peak area ($n = 4$) of the processed calibrators to the unprocessed calibrators. Matrix effects were evaluated by comparing the average peak area ($n = 4$) of unprocessed calibrators in matrix blank serum to calibrators spiked into 0.6% formic acid in water at low, mid, and high level spikes.

2.6 Stability and Ruggedness

In order to determine the stability of assay materials, the calibrators and QCs were subjected to cycles of freeze/thaw and various storage temperature conditions. The effects of calibrator and QC storage temperatures were examined after 8 freeze-thaw cycles, each from −70 °C to room temperature. The stability of the calibrators and QM-BChE material was evaluated by storing the materials at 4 °C, room temperature, or 37 °C, and evaluating them after 4, 8, 24, 48, and 72 hours. Analysis of QM-BChE material was extended, also collecting data after 5 and 7 days. Evaluation was based on calculated concentrations, and storage conditions were determined acceptable if calculated concentrations were within ±2 standard deviations of their characterized means (Tables 2 and 3, *n=24*). The ruggedness of the method was evaluated on five parameters that represent steps in the UHPLC-MS/MS analysis that might be impacted by changes to the validated assay. The resuspension buffer (0.1% formic acid and 0.6% formic acid), UHPLC organic mobile phase (acetonitrile and methanol), LC column lot (8 different products lots), LC gradient slope (linear and increased slope), and LC flow rate (± 100 μL/min) were varied individually, and the effect of each was evaluated using QC samples. Evaluation was based on the calculated QC concentrations, and altered parameters were determined to have an impact on the assay if the QC concentrations were outside of ±2 standard deviations of their characterized means (Table 3).

2.7 Matrix Blank and Convenience Set Analysis

Previous methods developed for the quantification of OPNA adducts to BChE have used GB- or GA-inhibited serum as the matrix blank. These inhibited materials would interfere with the measurement of aged biomarkers because GB- and GA-BChE undergo aging to MeP-BChE, P-BChE, or ExP-BChE. Each of these materials, unaged GB-inhibited serum, unaged GA-inhibited serum, and filtered serum were used to prepare calibration curves following the sample preparation protocol described in section 2.3. A comparison of the slopes of the calibration curves in GB-inhibited, GA-inhibited and 10 kDa filtered serum was carried out to determine if the 10 kDa filtered serum would be within 5% and therefore an acceptable matrix blank. The validated method was also applied to analyze a convenience set of 96 commercially-obtained individual human serum samples to assess background and possible interferences in an unexposed population.

2.8 Evaluation of Nerve Agent Spiked Materials

To demonstrate the utility of this assay, human serum spiked with GE, GV, and propyl GB (Pr-GB) were incubated at 37 °C for at least one week and analyzed ($n = 5$) using the validated method. Each of the agents in Figure 3 are expected to undergo hydrolysis to form aged products. GE and VE (Figures 5A and 5D) are expected to form EtP-BChE, while GV (Figure 3B) would form P-BChE, and Pr-GB (Figure 3C) would form PrP-BChE. For thermal stability studies, sarin (GB) inhibited serum and plasma were analyzed prior to and during aging of GB-BChE. Individual aliquots of inhibited serum and plasma were incubated ex vivo at 4 °C, 22 °C (benchtop) and 37 °C over the course of one month. Approximately 15 time points (n=1) were collected for each temperature and stored at −70 °C until analysis in the panel. Upon analysis, the concentration of each biomarker was determined and plotted against time ($\ln[C] = -k_{obs}t + \ln[C]_0$). First order conditions were

assumed from previous literature and applied to the GB-BChE decay in order to calculate the resulting rate constant (k) and half-life ($t_{1/2}$) using $t_{1/2}=\frac{\ln 2}{k_{obs}}$. First order conditions were applied again when calculating the rate constant of MeP-BChE and unadducted BChE formation. Likewise, first order conditions were used to calculate the rate constant of the regeneration of BChE activity. The activation energy of decay was calculated by $\ln\left(\frac{k_{obs_1}}{k_{obs_2}}\right)=\frac{-E_a}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)$, where T1 = 310 K and T2 = 295 K, and R is the ideal gas constant (1.987 cal·K-1·mol-1). The pre-exponential factor, A, was calculated using $k=Ae^{-E_a/(RT)}$.

Finally, the untargeted analysis used to look for potential new BChE biomarkers from the observed GB-BChE decay was processed the same as the diagnostic BChE biomarkers and analyzed by UHPLC-HRMS/MS. Discrimination of potential decay product biomarkers was completed using ddMS2 scan filtering for the signature $[M+H]^+$ BChE peptide fragments *m/z* 673.2940, 602.2569 and 778.3366 within 10 ppm error at 70,000 resolution. HRMS/MS experiments were carried out on a Thermo Q-Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer.

2.9 Data Acquisition and Processing

MassHunter Workstation Software, LC/MS Data Acquisition for 6400 Series Triple Quadrupole v. B.06.00, Build 6.0.6025.3 SP3 was used for data acquisition. MassHunter Workstation Software Quantitative Analysis v. B.06.00 SP01, build 6.0.388.1 was used for spectral analysis and quantitation. Accuracy was reported in percent relative error, % RE = $[C_e-C_t]/C_t \times 100$, where C_e and C_t are the experimental concentration determined from the calibration curve and the theoretical concentration, respectively. The percent relative standard deviation, % RSD = $SD/C_{avg} \times 100$, was calculated as a measure of assay precision, where C_{avg} and SD are the average calculated concentration and the standard deviation, respectively.

Calibration curves were created for each analyte in matrix blank serum by plotting the area of the analyte divided by the area of the internal standard versus the expected calibrator concentration, using linear regression with a 1/x weighting over the range of 2.00–250. ng/mL (n = 24). Analyte detection was confirmed using a confirmation ion ratio, which is the ratio of the quantitation ion area to the confirmation ion area.

2.10 Safety considerations

The analysis of BChE and OPNA adducts to BChE posed no greater risk to analysts than general peptide analyses. Universal safety precautions were followed when handling biological specimens such as blood products.

3. Results and discussion

3.1 UHPLC-MS/MS optimization

The MS/MS fragmentation spectra for BChE and MeP-BChE have been previously reported. (Carter et al., 2013, Fidder et al., 2002, Knaack et al., 2012, Sporty et al., 2010) When

adducted BChE peptides (FGESAGAAS) are fragmented using collision-induced dissociation, β-elimination of the OPNA adduct is observed, converting the adducted serine into a dehydroalanine (*m/z* 778.3) (Figure 4). For the MeP-BChE, EtP-BChE, PrP-BChE, and P-BChE peptides, quantitation was based on the transition of each respective $[M+H]^+$ precursor ion to the product ion *m/z* 778.3. Confirmation of the MeP-BChE, EtP-BChE, PrP-BChE, and P-BChE peptides was based on the transition of each respective $[M+H]^+$ precursor ion to the product ion *m/z* 673.3, which results from the loss of the OPNA adduct and further fragmentation to the b8 ion. Due to ion intensities, ExP-BChE quantitation was based on the transition from the precursor ion $[M+H]^+$ *m/z* 904.3 to the product ion *m/z* 673.3 and confirmed by the transition from the precursor ion $[M+H]^+$ *m/z* 904.3 to the product ion *m/z* 778.3. In-source fragmentation was not observed for the OPNA-BChE or unadducted BChE peptides. The MS/MS transitions used for the native and labeled peptides can be found in Table 1.

The Restek Pinnacle DB Biphenyl (1.9 μm, 2.1 × 50 mm) analytical column was chosen for chromatographic separation, using a gradient of increasing mobile phase B as follows: 5–23% (1.55 min), 23–76% B (0.28 min), 76% B (0.17 min), 5% B (1.00 min). The retention time for calibrators and QCs are provided in Table 2. Time-segmented multiple reaction monitoring mode gave at least 15 data points across each peak at the lowest reportable limit (LRL) and also allowed for optimization of dwell time for each segment. An extracted ion chromatogram displaying all biomarker peptides is presented in Figure 5. P-BChE and MeP-BChE transitions were acquired in time-segment 1 (0.00–1.54 min) with a 35 ms dwell time. ExP-BChE and EtP-BChE transitions were acquired in time-segment 2 (1.54–1.65 min) with a 20 ms dwell time. BChE and PrP-BChE transitions were acquired in time-segment 3 (1.65–1.90 min) with a 35 ms dwell time. See Table 1 for parameters for peptide detection.

3.2 Accuracy and Precision

To assess accuracy, precision, linearity, and limits of detection, data from 24 processed analytical runs (calibration curve and QCs) were evaluated. LC-MS/MS data was collected over four weeks, processing no more than two analytical runs per day. Calibration curves were linear for all analytes with a coefficient of determination value $R^2 \geq 0.980$ over the range of 2.00–250. ng/mL. The slope and y-intercept of the calibration curve were determined by linear least squares regression with 1/x weighting using the MassHunter Quantitative Analysis software package. Table 2 lists the interday accuracy and precision for each analyte at the lowest and highest calibrator levels, as well as the limit of detection (LOD) values that were calculated using the Taylor method.(Taylor, 1987) The lowest calibrator for each analyte was used as the lowest reportable limit. Determination of the method's accuracy and precision followed the guidelines in the FDA's guidance for bioanalytical method validation and thus show applicability for the analysis of clinical samples.(FDA, 2001) All analytes at all calibration levels demonstrated precision within 15 %RSD, with the exception of the 2.00 ng/mL calibrator for EtP-BChE which had a calculated %RSD of 24. All analytes were observed within 15 %RE, with the exception of the 2.00 ng/mL calibrator for BChE and PrP-BChE which had calculated %RE values of 18 and 25, respectively.

Calculated concentrations of all QC materials from interday and intraday analysis were found to be within 15 %RSD with the exception of QL-P-BChE, which had an interday %RSD of 22 (Table 3). The serum used for QL-P-BChE was inhibited with CBDP, which undergoes hydrolysis to form P-BChE.(Schopfer et al., 2010) Previously reported methods were used to confirm that the CBDP-inhibited serum was not fully aged.(Johnson et al., 2015) Therefore, the level of P-BChE in this material was expected to increase with time resulting in a greater %RSD. The imprecision observed in this QC is a reflection of adduct stability in the material and not the assay. Future editions of this assay will require more stable P-BChE QC materials.

3.3 Recovery and Matrix Effects

Since an isotopically-labeled BChE protein is not commercially available, extraction efficiency of aged OPNA-BChE and unadducted BChE peptides was assessed by adding calibrators (2.00, 32.0, and 250. ng/mL) to matrix blank serum either before ("processed") or after ("unprocessed") pepsin digestion. The processed samples were representative of the sample preparation used for synthetic peptide calibrators in this method. The average peak areas ($n = 4$) from these samples were used to evaluate recovery. The mean percentage recoveries for all analytes were ≥ 76% at all concentrations evaluated (Table 4). Potential losses in recovery may be attributed to nonspecific pepsin digestion and peptide adsorption to plastic surfaces.

Matrix effects were assessed by comparing the average peak area of unprocessed calibrators that were added to matrix blank serum after pepsin digestion and calibrators that were spiked into 0.6% formic acid in water. This was carried out with low, mid, and high calibrators. Matrix effects ranged from 5–17% for aged adducts and accounted for an approximate 35% reduction in mean peak area for unadducted BChE.

3.4 Stability and ruggedness

All calibrators and QC materials were found to have concentrations within the acceptable characterized values that were determined by the MRQCS for up to 8 freeze-thaw cycles. All calibrators were found to be stable up to 72 hours at 4 °C, room temperature, and 37 °C. QM-BChE was found to be stable up to 7 days at 4 °C, room temperature, and 37 °C, with the BChE concentration at each temperature/time point within the acceptable characterized QC range.

Method ruggedness was evaluated for all analytes using five parameters that represent steps in the UHPLC-MS/MS analysis that might be impacted by changes to the validated assay. The resuspension buffer of peptides prior to UHPLC-MS/MS analysis was evaluated with 0.6% and 0.1% formic acid in water and no differences were observed. The LC organic mobile phase was evaluated by using either acetonitrile or methanol, each containing 0.1% formic acid. While both organic mobile phases produced QC results within ± 2 standard deviations of their characterized means, signal intensity was significantly higher (i.e. > 50%) with acetonitrile. The use of a linear gradient did not alter method performance, but increasing the gradient slope did impact calculated QC concentrations. When gradient slope was increased (5–35% B, 35–85% B), 13.3% (2 of 15) of QCs were outside of ± 2 standard

deviations of their characterized means. Changes to the LC flow rate were evaluated at 200 μL/min and 400 μL/min. The decreased flow rate impacted the assay with 20% of QCs falling outside of their characterized values, most likely due to insufficient equilibration of the chromatograph. The increased flow rate had some impact on assay accuracy with 6.7% of QCs falling outside of their characterized concentration. The use of LC columns from multiple product lots showed potential to significantly impact the assay due to shifts in retention time. Of the eight product lots evaluated, three produced results in which at least one analyte's retention time was shifted by more than 10%. This method uses segmented MRM to maximize dwell time, and significant shifts in retention time can prevent MS/MS analysis if the peak elutes outside of its analysis segment. When time segments were removed and dwell times were reduced to account for increased cycle time, the assay produced accurate quantitation, but chromatographic peak areas were reduced which could lead to decreased sensitivity.

3.5 Matrix blank serum and convenience set analysis

The matrix blank material used in this method consisted of unadducted pooled human serum filtered through a 10 kDa molecular weight cutoff filter. This is different from other methods developed in this laboratory which have used GB-inhibited serum or GA-inhibited serum as a matrix blank.(Carter et al., 2013, Johnson et al., 2015, Pantazides et al., 2014) These inhibited matrices could not be selected for this method since GB inhibited BChE ages to MeP-BChE and GA inhibited BChE can age to form P-BChE or ExP-BChE. All three of these aged products are measured in this assay so use of GA or GB inhibited serum would interfere with the detection of aged biomarkers as the materials aged. A comparison of matrix blank sera was conducted, and it was determined that slopes of calibration curves in unaged GB-inhibited, unaged GA-inhibited, and 10 kDa filtered serum differed by less than 5%; therefore, 10 kDa filtered serum was determined to be an acceptable alternative matrix blank. Calibrators were also processed in 0.6% formic acid water, but calibration curve slopes differed by more than 5% when compared to GA and GB inhibited serum so 0.6% formic acid in water was not used as a matrix blank.

To assess background and possible interferences, a commercially-purchased convenience set of 96 individual human serum samples with no expected nerve agent exposure was analyzed. The average BChE nonapeptide concentration in human serum was found to be 40.0 ± 9.8 ng/mL with a range of 18.4 – 61.7 ng/mL, which was in agreement with results previously reported.(Pantazides et al., 2014) A BChE nonapeptide concentration of 40.0 ng/mL in serum is representative of a BChE enzyme concentration of 50 nM which is consistent with previously reported concentrations of BChE in human serum.(Lockridge, 2015, Noort et al., 2002, Tacal and Lockridge, 2010) Only the unadducted BChE biomarker was found to be present in the convenience set; no aged OPNA-BChE adducts were detected.

3.6 Evaluation of nerve agent spiked materials

Following an OPNA exposure event, blood samples undergo aging whether *in vivo* or *ex vivo*. This clinical assay provides an expansion of laboratory capabilities, allowing for the detection of all prohibited Schedule 1 OPNAs. This assay can also play an important role in the detection of OPNA exposure for patients who have received oxime therapy. The aged

adduct would be the only BChE biomarker expected to remain following oxime therapy since unaged adducts would be lost during BChE reactivation.(Berry and Davies, 1966)

OPNAs are known to age at different rates depending on the composition of the *o*-alkyl group; therefore, the ability to measure aged adducts becomes increasingly important.(Berry and Davies, 1966) Previously developed methods were used to study the thermal stability of sarin in serum and plasma.(Carter et al., 2013, Pantazides et al., 2014) When GB inhibited serum and plasma matrices were stored at 22 °C and 37 °C, the decay half-life of GB-BChE (-•-) was monitored respective to the storage temperature and matrix (Figure 6). The plot of GB-BChE decay in serum at 37 °C is shown in Figure 6, where the linear fit is $y = 0.0226x + 3.5972$, $R^2=0.9578$. The calculated half-lives (Table 5) of decay at 22 °C are similar (<1 % difference) intra-matrix, but in serum, the biomarker GB-BChE had a 15 % longer half-life than in plasma. At 37 °C, an expected decrease in biomarker half-life was observed for GB-BChE. The measurable change in biomarker growth or decay did not exceed one half-life for sarin inhibited serum and plasma matrices stored at 4 °C.

According to previous reports on biomarker formation, the authors assumed first order conditions in the calculation of this work's rate constants.(Aurbek et al., 2009, Davies and Green, 1956, Ellman et al., 1961, Evans et al., 2008) In doing so, the *ex vivo* change in biomarker stability at 37 °C in GB inhibited serum and plasma was calculated (Table 6). For each analyte, an assumed first order rate constant (k) was reported. The decay for GB-BChE was 0.02 h^{-1} in both serum and plasma. The formation of MeP-BChE from GB-BChE was 0.05 h^{-1} in serum and 0.03 h^{-1} in plasma. The formation of unadducted BChE from GB-BChE was 0.03 h^{-1} in serum and 0.01 h^{-1} in plasma. Activation parameters for GB-BChE were calculated from the temperature dependence of the rate constants in GB inhibited serum and plasma assuming first order conditions (Table 6). The activation energy of GB-BChE was calculated to be 14 kcal/mol in serum and 9.7 kcal/mol in plasma. The pre-exponential factor for GB-BChE was found to be 7.1×10^7 h^{-1} in serum and 1.3×10^5 h^{-1} in plasma.

Since hydrolysis of GB-BChE leads to the formation of MeP-BChE and unadducted BChE, each biomarker was monitored in an example *ex vivo* study of sarin inhibited pooled serum stored at 37 °C (Scheme 2). At t=0 of the study, 100% of the BChE target monitored was observed as the GB-BChE biomarker at 41 ng/mL. After 861 hours (36 days), the MeP-BChE remained the most abundant biomarker (22 ng/mL, 54%), and unadducted BChE was still present (8.3 ng/mL, 20%). The GB-BChE biomarker, however, was no longer detected. Since the MeP-BChE and unadducted BChE biomarkers only accounted for 74% of the initial GB-BChE concentration, an untargeted analysis was used to search for a singular unknown decay product. A number of masses were identified, but they neither accounted for only one decay product of GB-BChE nor possessed the signature MS/MS fragmentation expected of the adducted BChE biomarkers.

Additional agents were evaluated for their respective aged adducts to BChE in spiked serum. As anticipated, GE aged to form EtP-BChE, which was measured to be 40.6 ± 5.3 ng/mL in the GE-spiked serum. GV aged to form P-BChE and was determined to be 29.8 ± 3.4 ng/mL in the GV-spiked serum. Serum inhibited with Pr-GB aged to form PrP-BChE with a

measured concentration of 15.1 ± 0.7 ng/mL. All nerve agent-spiked serum samples had a BChE inhibition of at least 98% so changes in aged biomarker concentrations are likely the result of differences in the rates of aging. Of note, synthetic peptide standards for GE-, GV-, and Pr-GB-BChE were not available at the time of this work to measure their unaged BChE adduct concentrations.

4. Conclusions

A quantitative method was developed for the confirmation of exposure to OPNAs in clinical samples through detection of aged OPNA-BChE adducts. The method allows for the detection of all CWC Schedule 1 OPNAs by monitoring their resulting aged OPNA-BChE biomarkers P-BChE, MeP-BChE, ExP-BChE, EtP-BChE, and PrP-BChE, as well as unadducted BChE. In the analysis of a commercial convenience set of 96 individual serum samples, no aged OPNA-BChE adducts were observed and quantitative measurements of unadducted BChE were in agreement with previous studies using similar methodologies. This method allows for the quantitation of unadducted BChE and the aged OPNA-BChE biomarkers over a concentration range of 2.00 to 250. ng/mL and has demonstrated high intraday and interday accuracy and precision of calibration standards and spiked QC materials. The analysis of pooled uninhibited serum QC materials demonstrated high assay precision and is in agreement with FDA guidance for bioanalytical methods. The various aged OPNA-BChE biomarkers provide valuable information by confirming OPNA exposure over a prolonged period of time.

Acknowledgments

The authors would like to extend a special thank you to Professor Emeritus James K. Hurst of Washington State University and Dr. B. Rey de Castro of the CDC's Division of Laboratory Sciences, Professor and Director Gary Suizdak of the Scripps Center for Metabolomics, and Dr. Daan Noort of The Netherlands Organization for Applied Scientific Research for providing subject matter expertise toward the work's merit in biomarker kinetics evaluation, untargeted mass spectrometric method design, and OPNA biomarker stability applications to the field, respectively. The authors would like to thank Ms. Chariety Sapp of the CDC's Incident Response Laboratory for reference materials storage management, Dr. Bobby N. Brewer of Battelle Memorial Institute for support in reference material preparation, Dr. Ronald A. Evans of the U.S. Army Edgewood Chemical Biological Center for support in reference material preparation, Dr. Kristie L. Rose of Vanderbilt University School of Medicine for support in discussions toward untargeted analyses, and the Defense Threat Reduction Agency (IAA 11-005-12430), Oak Ridge Institute for Science and Education, and Centers for Disease Control and Prevention for project financial support.

References

Adams T, Capacio B, Smith J, Whalley C, Korte W. The Application of the Fluoride Reactivation Process to the Detection of Sarin and Soman Nerve Agent Exposures in Biological Samples. Drug and chemical toxicology. 2004; 27(1):77–91. [PubMed: 15038250]

Aurbek N, Thiermann H, Eyer F, Eyer P, Worek F. Suitability of human butyrylcholinesterase as therapeutic marker and pseudo catalytic scavenger in organophosphate poisoning: A kinetic analysis. Toxicology. 2009; 259(3):133–9. [PubMed: 19428953]

Barak D, Ordentlich A, Kaplan D, Barak R, Mizrahi D, Kronman C, et al. Evidence for PN bond scission in phosphoroamidate nerve agent adducts of human acetylcholinesterase. Biochemistry. 2000; 39(5):1156–61. [PubMed: 10653663]

Berry WK, Davies DR. Factors influencing the rate of "aging" of a series of alkyl methylphosphonyl-acetylcholinesterases. Biochem J. 1966; 100(2):572–6. [PubMed: 5968554]

Black RM. History and perspectives of bioanalytical methods for chemical warfare agent detection. Journal of Chromatography B. 2010; 878(17):1207–15.

Black RM, Read RW. Biological markers of exposure to organophosphorus nerve agents. Archives of toxicology. 2013; 87(3):421–37. [PubMed: 23371414]

Carletti E, Li H, Li B, Ekström F, Nicolet Y, Loiodice M, et al. Aging of cholinesterases phosphylated by tabun proceeds through O-dealkylation. Journal of the American Chemical Society. 2008; 130(47):16011–20. [PubMed: 18975951]

Carter MD, Crow BS, Pantazides BG, Watson CM, Thomas JD, Blake TA, et al. Direct quantitation of methyl phosphonate adducts to human serum butyrylcholinesterase by immunomagnetic-UHPLC-MS/MS. Anal Chem. 2013; 85(22):11106–11. [PubMed: 24205842]

Carter MD, Crow BS, Pantazides BG, Watson CM, Thomas JD, Blake TA, et al. Direct Quantitation of Methyl Phosphonate Adducts to Human Serum Butyrylcholinesterase by Immunomagnetic-UHPLC-MS/MS. Analytical chemistry. 2013; 85(22):11106–11. [PubMed: 24205842]

Caudill SP, Schleicher RL, Pirkle JL. Multi-rule quality control for the age-related eye disease study. Stat Med. 2008; 27(20):4094–106. [PubMed: 18344178]

Crow BS, Pantazides BG, Quinones-Gonzalez J, Garton JW, Carter MD, Perez JW, et al. Simultaneous measurement of tabun, sarin, soman, cyclosarin, VR, VX, and VM adducts to tyrosine in blood products by isotope dilution UHPLC-MS/MS. Anal Chem. 2014; 86(20):10397–405. [PubMed: 25286390]

Davies DR, Green AL. The kinetics of reactivation, by oximes, of cholinesterase inhibited by organophosphorus compounds. The Biochemical journal. 1956; 63(4):529–35. [PubMed: 13355845]

Elhanany E, Ordentlich A, Dgany O, Kaplan D, Segall Y, Barak R, et al. Resolving pathways of interaction of covalent inhibitors with the active site of acetylcholinesterases: MALDI-TOF/MS analysis of various nerve agent phosphyl adducts. Chemical research in toxicology. 2001; 14(7): 912–8. [PubMed: 11453739]

Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7:88–95. [PubMed: 13726518]

Evans RA, Jakubowski EM, Muse WT, Matson K, Hulet SW, Mioduszewski RJ, et al. Quantification of sarin and cyclosarin metabolites isopropyl methylphosphonic acid and cyclohexyl methylphosphonic acid in minipig plasma using isotope-dilution and liquid chromatography-time-of-flight mass spectrometry. Journal of Analytical Toxicology. 2008; 32(1):78–85. [PubMed: 18269798]

Evison D, Hinsley D, Rice P. Chemical weapons. BMJ. 2002; 324(7333):332–5. [PubMed: 11834561]

FDA. US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV); 2001. Guidance for industry: bioanalytical method validation.

Fidder A, Hulst AG, Noort D, de Ruiter R, van der Schans MJ, Benschop HP, et al. Retrospective detection of exposure to organophosphorus anti-cholinesterases: mass spectrometric analysis of phosphylated human butyrylcholinesterase. Chem Res Toxicol. 2002; 15(4):582–90. [PubMed: 11952345]

Grob D, Harvey AM. The effects and treatment of nerve gas poisoning. Am J Med. 1953; 14(1):52–63. [PubMed: 13016586]

Hamelin EI, Schulze ND, Shaner RL, Coleman RM, Lawrence RJ, Crow BS, et al. Quantitation of five organophosphorus nerve agent metabolites in serum using hydrophilic interaction liquid chromatography and tandem mass spectrometry. Anal Bioanal Chem. 2014; 406(21):5195–202. [PubMed: 24633507]

Heilbronn E. Action of fluoride on cholinesterase. Acta chem scand. 1965; 19:1333. [PubMed: 5850143]

Heilbronn E. Action of fluoride on cholinesterase—II: In vitro reactivation of cholinesterases inhibited by organophosphorous compounds. Biochemical pharmacology. 1965; 14(9):1363–73. [PubMed: 5857535]

Jiang W, Cashman JR, Nachon F, Masson P, Schopfer LM, Lockridge O. Mass spectrometry method to identify aging pathways of Sp-and Rp-tabun adducts on human butyrylcholinesterase based on the acid labile PN bond. toxicological sciences. 2013; 132(2):390–8. [PubMed: 23345579]

Jiang W, Murashko EA, Dubrovskii YA, Podolskaya EP, Babakov VN, Mikler J, et al. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of titanium oxide-enriched peptides for detection of aged organophosphorus adducts on human butyrylcholinesterase. Analytical biochemistry. 2013; 439(2):132–41. [PubMed: 23624322]

Johnson D, Carter MD, Crow BS, Isenberg SL, Graham LA, Erol HA, et al. Quantitation of ortho-cresyl phosphate adducts to butyrylcholinesterase in human serum by immunomagnetic-UHPLC-MS/MS. J Mass Spectrom. 2015; 50(4):683–92. [PubMed: 26149113]

Knaack JS, Zhou Y, Abney CW, Jacob JT, Prezioso SM, Hardy K, et al. A high-throughput diagnostic method for measuring human exposure to organophosphorus nerve agents. Analytical chemistry. 2012; 84(21):9470–7. [PubMed: 23083472]

Knaack JS, Zhou Y, Abney CW, Jacob JT, Prezioso SM, Hardy K, et al. A high-throughput diagnostic method for measuring human exposure to organophosphorus nerve agents. Anal Chem. 2012; 84(21):9470–7. [PubMed: 23083472]

Liyasova M, Li B, Schopfer LM, Nachon F, Masson P, Furlong CE, et al. Exposure to tri-o-cresyl phosphate detected in jet airplane passengers. Toxicology and applied pharmacology. 2011; 256(3):337–47. [PubMed: 21723309]

Lockridge O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinc, and potential therapeutic uses. Pharmacology & Therapeutics. 2015; 148:34–46. [PubMed: 25448037]

Mawhinney DB, Hamelin EI, Fraser R, Silva SS, Pavlopoulos AJ, Kobelski RJ. The determination of organophosphonate nerve agent metabolites in human urine by hydrophilic interaction liquid chromatography tandem mass spectrometry. Journal of Chromatography B. 2007; 852(1):235–43.

Noort D, Benschop HP, Black RM. Biomonitoring of Exposure to Chemical Warfare Agents: A Review. Toxicology and Applied Pharmacology. 2002; 184(2):116–26. [PubMed: 12408956]

Noort D, Fidder A, Van der Schans M, Hulst A. Verification of exposure to organophosphates: generic mass spectrometric method for detection of human butyrylcholinesterase adducts. Analytical chemistry. 2006; 78(18):6640–4. [PubMed: 16970345]

OPCW. [Accessed July 22, 2016] Convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction. 2005. Available from: https://www.opcw.org/fileadmin/OPCW/CWC/CWC_en.pdf

OPCW. Major Milestone Reached as 90% of Global Chemical Weapon Stocks Destroyed. Organisation for the Prohibition of Chemical Weapons; 2015. Available from: https://www.opcw.org/news/article/major-milestone-reached-as-90-of-global-chemical-weapon-stocks-destroyed/ [Accessed July 22, 2016]

Pantazides BG, Watson CM, Carter MD, Crow BS, Perez JW, Blake TA, et al. An enhanced butyrylcholinesterase method to measure organophosphorus nerve agent exposure in humans. Analytical and bioanalytical chemistry. 2014:1–8. [PubMed: 24220758]

Pantazides BG, Watson CM, Carter MD, Crow BS, Perez JW, Blake TA, et al. An enhanced butyrylcholinesterase method to measure organophosphorus nerve agent exposure in humans. Anal Bioanal Chem. 2014; 406(21):5187–94. [PubMed: 24604326]

Pita R, Domingo J. The Use of Chemical Weapons in the Syrian Conflict. Toxics. 2014; 2(3):391.

Riches J, Morton I, Read RW, Black RM. The trace analysis of alkyl alkylphosphonic acids in urine using gas chromatography–ion trap negative ion tandem mass spectrometry. Journal of Chromatography B. 2005; 816(1):251–8.

Schecter WP. Cholinergic symptoms due to nerve agent attack: a strategy for management. Anesthesiol Clin North America. 2004; 22(3):579–90. viii. [PubMed: 15325720]

Schopfer LM, Furlong CE, Lockridge O. Development of diagnostics in the search for an explanation of aerotoxic syndrome. Anal Biochem. 2010; 404(1):64–74. [PubMed: 20447373]

Segall Y, Waysbort D, Barak D, Ariel N, Doctor BP, Grunwald J, et al. Direct observation and elucidation of the structures of aged and nonaged phosphorylated cholinesterases by 31P NMR spectroscopy. Biochemistry. 1993; 32(49):13441–50. [PubMed: 8257680]

Sporty JL, Lemire SW, Jakubowski EM, Renner JA, Evans RA, Williams RF, et al. Immunomagnetic separation and quantification of butyrylcholinesterase nerve agent adducts in human serum. Analytical chemistry. 2010; 82(15):6593–600. [PubMed: 20617824]

Sporty JL, Lemire SW, Jakubowski EM, Renner JA, Evans RA, Williams RF, et al. Immunomagnetic separation and quantification of butyrylcholinesterase nerve agent adducts in human serum. Anal Chem. 2010; 82(15):6593–600. [PubMed: 20617824]

Suzuki T, Morita H, Ono K, Maekawa K, Nagai R, Yazaki Y, et al. Sarin poisoning in Tokyo subway. The Lancet. 1995; 345(8955):980–1.

Tacal O, Lockridge O. Methamidophos, dichlorvos, O-methoate and diazinon pesticides used in Turkey make a covalent bond with butyrylcholinesterase detected by mass spectrometry. Journal of Applied Toxicology. 2010; 30(5):469–75. [PubMed: 20229498]

Taylor, JK. Quality assurance of chemical measurements. CRC Press; Boca Raton, FL: 1987.

UN. United Nations Report of the Specialist Appointed by the Secretary-General to Investigate Allegations by the Islamic Republic of Iran Concerning the Use of Chemical Weapons. 1984.

Van der Schans M, Fidder A, Van Oeveren D, Hulst A, Noort D. Verification of exposure to cholinesterase inhibitors: generic detection of OPCW Schedule 1 nerve agent adducts to human butyrylcholinesterase. Journal of analytical toxicology. 2008; 32(1):125–30. [PubMed: 18269804]

Williams NH, Harrison JM, Read RW, Black RM. Phosphylated tyrosine in albumin as a biomarker of exposure to organophosphorus nerve agents. Archives of toxicology. 2007; 81(9):627–39. [PubMed: 17345062]

Yanagisawa N, Morita H, Nakajima T, Okudera H, Shimizu M, Hirabayashi H, et al. Sarin poisoning in Matsumoto, Japan. The lancet. 1995; 346(8970):290–3.

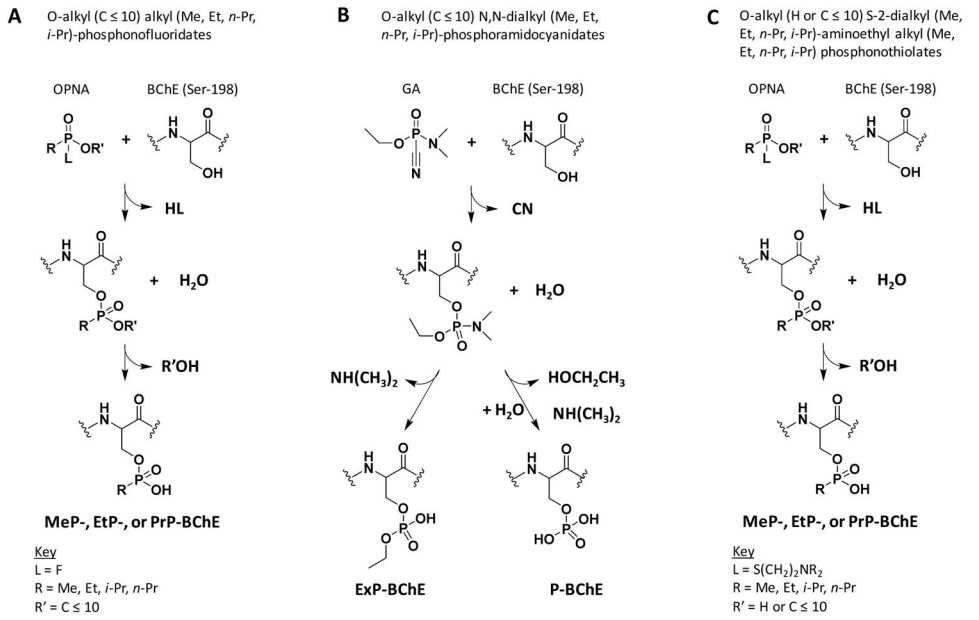


Figure 1.

Schedule 1 OPNAs inhibit BChE at Ser-198 and age to form MeP-BChE, EtP-BChE, PrP-BChE, ExP-BChE, or P-BChE. The CWC defines Schedule 1 OPNAs as (**A**) *o*-alkyl (C ≤ 10) alkyl (methyl, ethyl, *n*-propyl, or *i*-propyl)-phosphonofluoridates (ex. GB, GD, GE, GF, Pr-GB); (**B**) *o*-alkyl (C ≤ 10) N,N-dialkyl (methyl, ethyl, *n*-propyl, or *i*-propyl)-phosphoramidocyanidates (ex. GA); (**C**) *o*-alkyl (H or C ≤ 10) S-2-dialkyl (methyl, ethyl, *n*-propyl, or *i*-propyl)-aminoethyl alkyl (methyl, ethyl, *n*-propyl, or *i*-propyl) phosphonothiolates (ex. VE, VM, VR, VX).(OPCW, 2005)

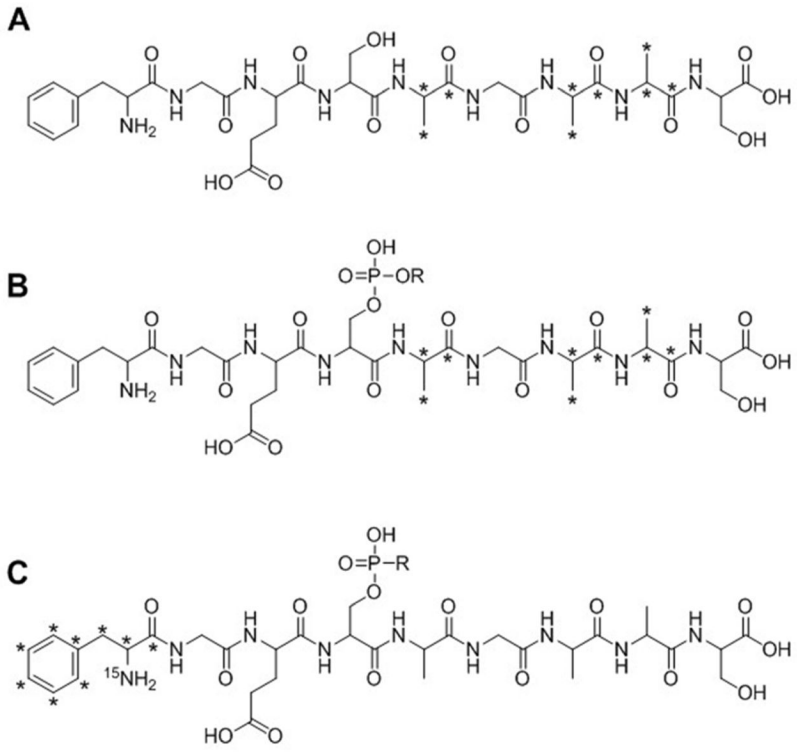


Figure 2.

Chemical structures of the isotopically-labeled (**A**) unadducted BChE peptide (FGESAGAAS), (**B**) P-BChE (R = H) and ExP-BChE (R = C_2H_5), and (**C**) MeP-BChE (R = CH_3), EtP-BChE (R = C_2H_5), and PrP-BChE (R = C_3H_7). An asterisk (*) indicates sites of ^{13}C-isotope labeling.

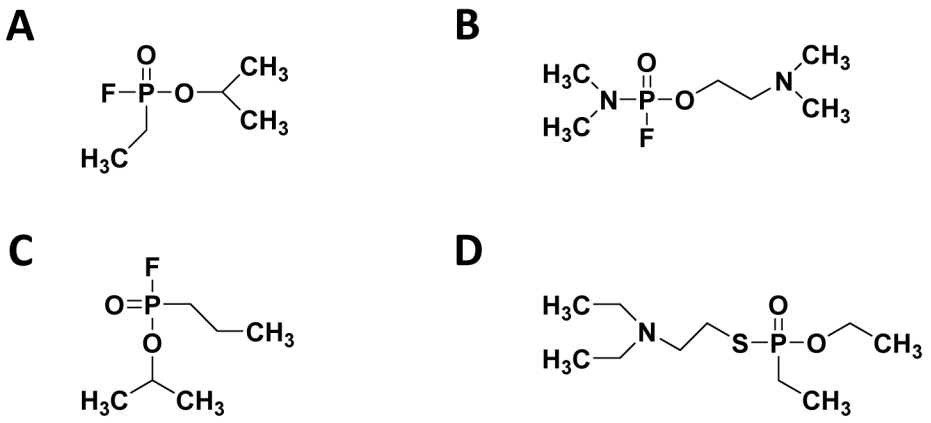


Figure 3.

Chemical structures of the organophosphorus nerve agents (**A**) GE (ethyl sarin), (**B**) GV (2-dialkylaminoalkyl N,N-dialkylphosphonamidofluoridate), (**C**) Pr-GB (propyl-sarin), (**D**) VE (O-ethyl-S-diethylaminoethylphosonothiolate).

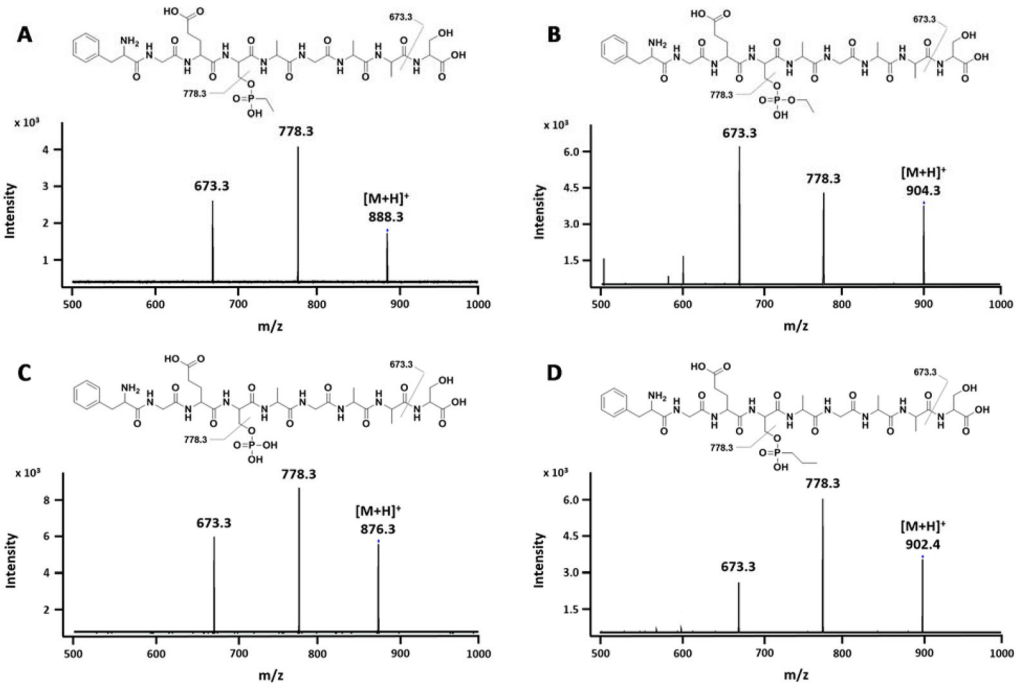


Figure 4.

Chemical structure and product ion mass spectrum of the precursor ion for the synthetic peptide (**A**) EtP-BChE ($[M+H]^+$ *m/z* 888.3), (**B**) ExP-BChE ($[M+H]^+$ *m/z* 904.3), (**C**) P-BChE ($[M+H]^+$ *m/z* 876.3), and (**D**) PrP-BChE ($[M+H]^+$ *m/z* 902.4). Fragmentation spectra were collected by scanning the mass range *m/z* 500–1000, using a collision energy of (**A**) 35V, (**B**) 34V, (**C**) 32V, (**D**) 35V. The dashed lines indicate the proposed sites of fragmentation. The peptide sequence shown is FGE**S**AGAAS.

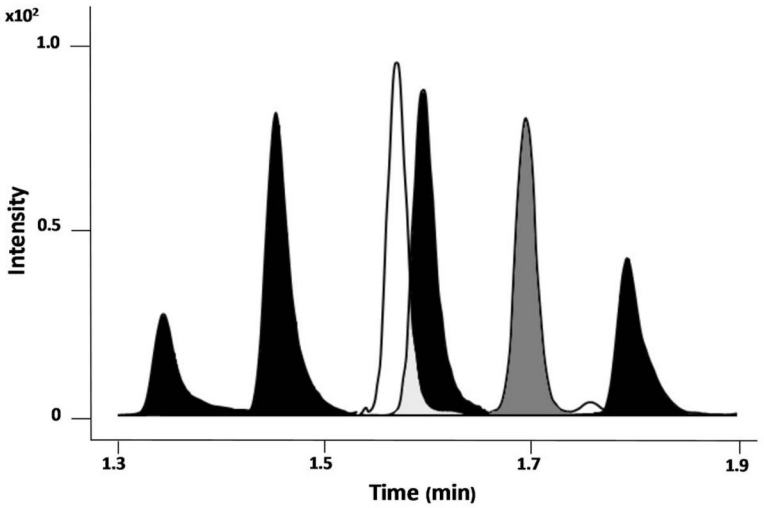


Figure 5.

Overlay of extracted ion chromatograms of synthetic peptides for unadducted BChE and aged OPNA-BChE biomarkers. The black-filled peaks are aged OPNA-BChE peptides (P-BChE, MeP-BChE, EtP-BChE, and PrP-BChE), the black outline with no fill is the aged ExP-BChE peptide, and the black outline with gray fill is the unadducted BChE peptide.

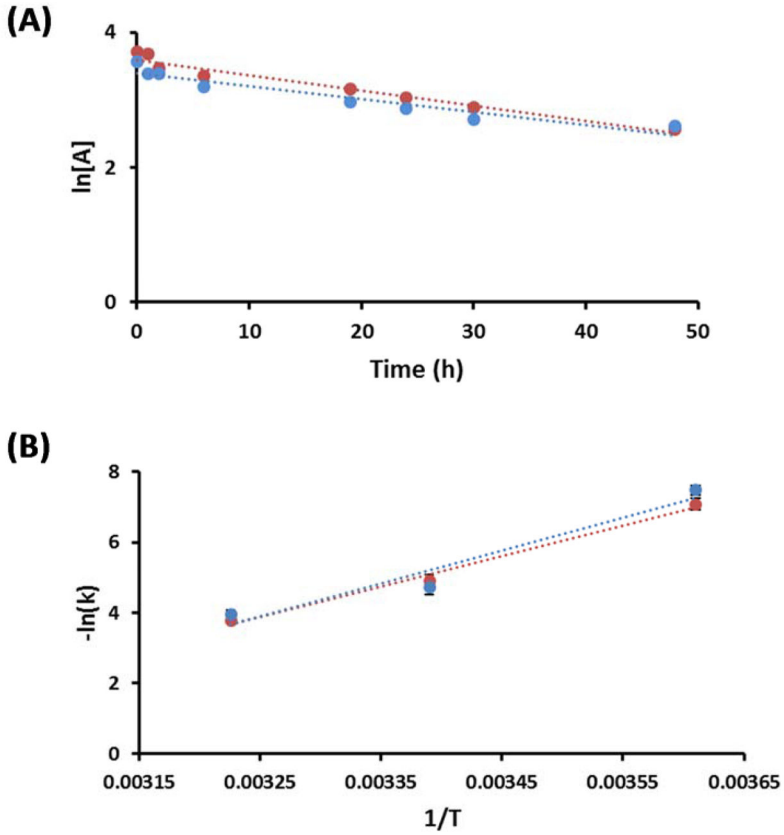
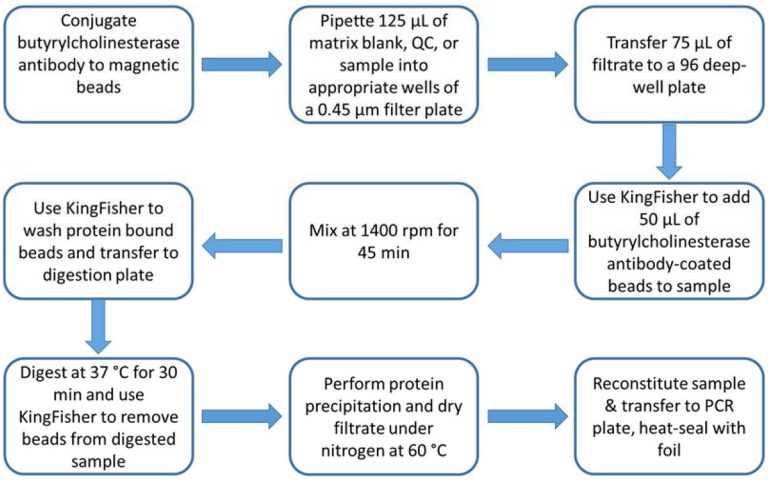
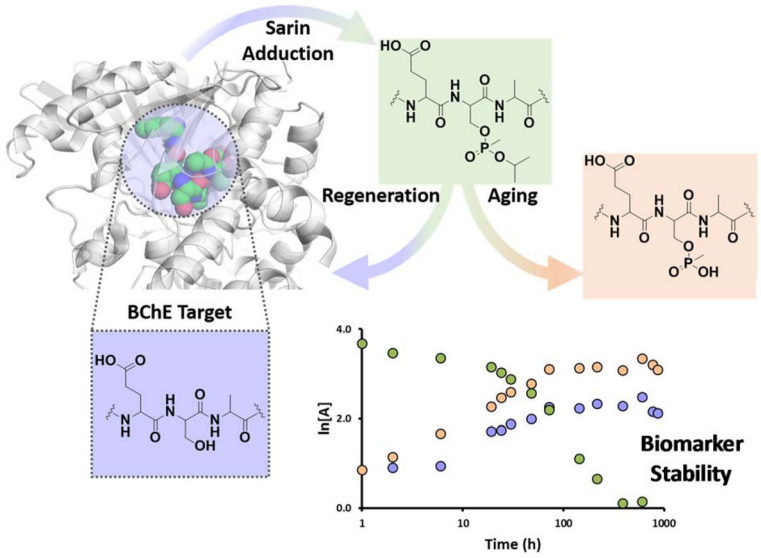


Figure 6.

The biomarker GB-BChE was evaluated *ex vivo* at 22 °C and 37 °C from GB inhibited serum ( ) and plasma ( ). (**A**) The decay of GB-BChE in serum and plasma is shown at 37 °C, where $y = -0.0226x + 3.60$, $R^2 = 0.958$ and $y = -0.0195x + 3.40$, $R^2 = 0.909$, respectively and (**B**) the temperature dependence of k_{obs} is plotted where $y = 9391x - 26.6$, $R^2 = 0.951$ and $y = 8652x - 24.2$, $R^2 = 0.990$.



Scheme 1.
Flowchart summarizing sample preparation.



Scheme 2.

Upon hydrolysis of the GB-BChE ( ) biomarker, MeP-BChE ( ) and BChE ( ) form via aging and regeneration.

Table 1

Optimized electrospray ionization-MS/MS parameters.

Analyte	Ion Type	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cell Accelerator (V)	Collision Energy (V)
P-BChE	Quantitation	876.3	778.3	4.0	32
	Confirmation	876.3	673.3	5.0	33
	ISTD	885.3	787.3	5.0	33
MeP-BChE	Quantitation	874.3	778.3	5.0	30
	Confirmation	874.3	673.3	5.0	33
	ISTD	884.3	788.3	5.0	30
ExP-BChE	Quantitation	904.4	673.3	5.0	34
	Confirmation	904.4	778.3	5.0	36
	ISTD	913.4	787.4	4.0	36
EtP-BChE	Quantitation	888.3	778.3	5.0	35
	Confirmation	888.3	673.3	5.0	35
	ISTD	898.4	788.3	5.0	35
BChE	Quantitation	796.3	691.3	2.5	25
	Confirmation	796.3	620.3	2.5	27
	ISTD	805.3	700.3	2.5	25
PrP-BChE	Quantitation	902.4	778.3	5.0	35
	Confirmation	902.4	673.3	5.0	33
	ISTD	912.4	788.3	5.0	35

Table 2

The retention time, expected concentration, mean calculated concentration from characterization, accuracy, precision, limit of detection (LOD), and coefficient of determination (R^2) values of BChE biomarker peptides ($n = 24$).

Analyte	Retention Time (min)	Expected Conc. (ng/mL)	Mean Calculated Conc. (ng/mL)	% RE	% RSD	LOD[a] (ng/mL)	$R^2 \pm SD$
P-BChE	1.36	2.00	2.26	13	13	1.90	0.994 ± 0.005
		250.	256	2.4	3.7		
MeP-BChE	1.46	2.00	2.19	9.5	8.2	1.34	0.997 ± 0.003
		250.	254	1.5	3.2		
ExP-BChE	1.58	2.00	2.25	12	13	2.16	0.995 ± 0.003
		250.	254	1.7	3.8		
EtP-BChE	1.60	2.00	2.06	2.8	24	1.66	0.989 ± 0.004
		250.	248	−1.0	6.1		
BChE	1.70	2.00	2.36	18	8.1	0.663	0.995 ± 0.003
		250.	255	2.1	3.3		
PrP-BChE	1.81	2.00	2.50	25	7.0	2.04	0.995 ± 0.004
		250.	254	1.5	2.9		

[a]Taylor method. (Taylor, 1987)

Table 3

Interday (n = 24 from QC characterization) and intraday (n = 5) precision of quality control materials.

Analyte	QC Level	Mean Interday Conc. (ng/mL)	Interday % RSD	Mean Intraday Conc. (ng/mL)	Intraday % RSD
P-BChE	QL-P-BChE	18.5	22	15.0	5.8
	QM	40.1	6.3	42.5	6.5
	QH	82.9	7.5	84.4	9.3
MeP-BChE	QL	9.18	7.5	9.43	5.2
	QM	37.5	11	39.0	6.5
	QH	79.8	7.7	81.1	4.6
ExP-BChE	QL	9.41	5.5	9.17	3.7
	QM	35.4	11	37.5	5.6
	QH	85.5	10	90.4	3.2
EtP-BChE	QL	10.7	14	10.2	10
	QM	46.3	15	48.7	10
	QH	107	14	106	2.3
BChE	QL	8.45	8.3	8.66	9.8
	QM-BChE	40.8	14	36.8	11
	QH	81.0	10	79.4	4.7
PrP-BChE	QL	9.39	8.0	9.17	2.2
	QM	40.2	11	39.9	4.4
	QH	84.9	11	83.0	4.4

Table 4

Average recovery of calibrators in matrix blank serum added before (“processed”) or after (“unprocessed”) pepsin digestion. (n = 4)

Analyte	Calibrator(ng/mL)	Processed(mean area ± SD)	Unprocessed(mean area ± SD)	% Recovery[a] ± SD[b]
P-BChE	2.00	$(4.12 \pm 0.75) \times 10^2$	$(4.81 \pm 0.72) \times 10^2$	86 ± 2
	32.0	$(5.71 \pm 0.32) \times 10^3$	$(7.16 \pm 0.52) \times 10^3$	76 ± 1
	250.	$(4.89 \pm 0.31) \times 10^4$	$(6.41 \pm 0.35) \times 10^4$	76 ± 1
MeP-BChE	2.00	$(9.85 \pm 1.23) \times 10^2$	$(1.10 \pm 0.07) \times 10^3$	89 ± 1
	32.0	$(1.36 \pm 0.05) \times 10^4$	$(1.78 \pm 0.11) \times 10^4$	76 ± 1
	250.	$(1.23 \pm 0.10) \times 10^5$	$(1.60 \pm 0.59) \times 10^5$	77 ± 1
ExP-BChE	2.00	$(8.67 \pm 1.30) \times 10^2$	$(1.02 \pm 0.10) \times 10^3$	85 ± 1
	32.0	$(1.40 \pm 0.05) \times 10^4$	$(1.77 \pm 0.08) \times 10^4$	79 ± 1
	250.	$(1.24 \pm 0.10) \times 10^5$	$(1.63 \pm 0.06) \times 10^5$	76 ± 1
EtP-BChE	2.00	$(1.30 \pm 0.24) \times 10^3$	$(1.55 \pm 0.25) \times 10^3$	84 ± 3
	32.0	$(1.03 \pm 0.13) \times 10^4$	$(1.40 \pm 0.20) \times 10^4$	73 ± 1
	250.	$(9.90 \pm 1.08) \times 10^4$	$(1.28 \pm 0.14) \times 10^5$	77 ± 1
BChE	2.00	$(4.99 \pm 0.64) \times 10^2$	$(5.75 \pm 0.60) \times 10^2$	87 ± 1
	32.0	$(8.78 \pm 0.51) \times 10^3$	$(1.11 \pm 0.10) \times 10^4$	79 ± 1
	250.	$(7.66 \pm 0.78) \times 10^4$	$(1.01 \pm 0.07) \times 10^5$	76 ± 1
PrP-BChE	2.00	$(1.53 \pm 0.18) \times 10^3$	$(1.72 \pm 0.21) \times 10^3$	89 ± 1
	32.0	$(3.87 \pm 0.18) \times 10^4$	$(4.92 \pm 0.28) \times 10^4$	79 ± 1
	250.	$(3.57 \pm 0.39) \times 10^5$	$(4.59 \pm 0.36) \times 10^5$	78 ± 1

[a] $\%\ \text{Recovery} = [(\text{mean}_{\text{proc}})/(\text{mean}_{\text{un}}) \times 100$

[b] $\text{SD}(\%\ \text{Recovery}) = \%\ \text{Recovery} \times [(\sigma_{\text{un}}/\text{mean}_{\text{un}})^2 + (\sigma_{\text{proc}}/\text{mean}_{\text{proc}})^2]^{1/2}$

Table 5

GB-BChE half-lives were calculated in serum and plasma at 22 °C and 37 °C.

$t_{1/2}$ (h)*	22 °C	37 °C
Serum	93 (20%)	31 (10%)
Plasma	79 (20%)	36 (10%)

*RSD

Table 6

Observed *ex vivo* thermal stability of GB-BChE.

	BChE	MeP-BChE	GB-BChE		
	$k_{(obs)}$ *formation* (h^{-1})*	$k_{(obs)}$ *formation* (h^{-1})*	$k_{(obs)}$ *decay* (h^{-1})*	E_a *(kcal/mol)**	*A* (h^{-1})*
Serum	0.03 (20%)	0.05 (20%)	0.02 (9%)	14 (20%)	7.1×10^7 (8%)
Plasma	0.01 (20%)	0.03 (20%)	0.02 (20%)	9.7 (30%)	1.3×10^5 (10%)

* RSD