HHS Public Access

Author manuscript

Anal Chem. Author manuscript; available in PMC 2018 February 07.

Published in final edited form as:

Anal Chem. 2017 February 07; 89(3): 1955–1964. doi:10.1021/acs.analchem.6b04441.

High-Confidence Qualitative Identification of Organophosphorus Nerve Agent Adducts to Human Butyrylcholinesterase

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

[a]Battelle at the Centers for Disease Control and Prevention, Atlanta, GA 30341

[b]Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA 30341

[c]Oak Ridge Institute for Science and Education Fellow at the Centers for Disease Control and Prevention, Atlanta, GA 30341

Abstract

In this study, a data-dependent, high-resolution tandem mass spectrometry (ddHRMS/MS) method capable of detecting all organophosphorus nerve agent (OPNA) adducts to human butyrylcholinesterase (BChE) was developed. After an exposure event, immunoprecipitation from blood with a BChE-specific antibody and digestion with pepsin produces a nine amino acid peptide containing the OPNA adduct. Signature product ions of this peptic BChE nonapeptide (FGES*AGAAS) offer a route to broadly screen for OPNA exposure. Taking this approach on an HRMS instrument identifies biomarkers, including unknowns, with high mass accuracy. Using a set of pooled human sera exposed to OPNAs as quality control (QC) materials, the developed method successfully identified precursor ions with <1 ppm and tied them to signature product ions with <5 ppm deviation from their chemical formulas. This high mass accuracy data from precursor and product ions, collected over 23 independent immunoprecipitation preparations, established method operating limits. QC data and experiments with 14 synthetic reference peptides indicated that reliable qualitative identification of biomarkers was possible for analytes >15 ng/mL. The developed method was applied to a convenience set of 96 unexposed serum samples and a blinded set of 80 samples treated with OPNAs. OPNA biomarkers were not observed in convenience set samples and no false positive or negative identifications were observed in blinded samples. All biomarkers in the blinded serum set >15 ng/mL were correctly identified. For the first time, this study reports a ddHRMS/MS method capable of complementing existing quantitative methodologies and suitable for identifying exposure to unknown organophosphorus agents.

*Correspondence to: M.D. Carter, Division of Laboratory Sciences, Centers for Disease Control and Prevention, Atlanta, GA 30341, USA. melissa.carter@cdc.hhs.gov.

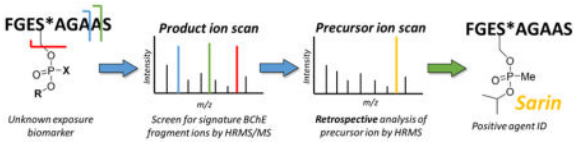
Disclaimer

The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services or the U.S. Centers for Disease Control and Prevention. The use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the U.S. Centers for Disease Control and Prevention.

Conflict of Interest Disclosure

The authors declare no competing financial interest

Graphical Abstract



Keywords

High Resolution Mass Spectrometry; Antibody Capture; Immunoprecipitation; Nerve Agents; Cholinesterase; Acetylcholinesterase; Butyrylcholinesterase

Introduction

Organophosphorus nerve agents (OPNAs)[1,2] are a class of internationally-banned chemical warfare agents that broadly target a family of enzymes known as serine hydrolases.[3] OPNAs disable serine hydrolases by phosphorylating their active site serine residues. Since OPNAs have such broad inhibitory activity, many different combinations of ligands retain potent activity. Therefore, over a thousand[4] distinct species of OPNA structures have been defined as schedule 1 agents by the Chemical Weapons Convention.[5]

Inhibition of acetylcholinesterase (AChE) in the nervous system by OPNAs is responsible for producing a number of characteristic toxic effects including seizures and associated motor convulsions. AChE inhibition leads to accumulation of acetylcholine in synapses and a loss of signal transmission.[2] Traditionally, inhibition of AChE was detected using the Ellman activity assay which measures choline production as a measure of enzyme activity.[6] This assay has been widely used for clinical assays, such as the detection of organophosphorus (OP) pesticide exposure.[7] While effective, activity-based assays cannot identify a specific agent in an exposure event. Hydrolyzed OPNA metabolites identify metabolites of specific agents from urine or blood;[8,9] however, these metabolites are short-lived, and the majority of excretion occurs days after an exposure has occurred.[10,11] Analysis of OPNA adducts to albumin offer another route to verify exposure.[12] However, albumin poorly reacts with V-series agents, limiting its application.

OPNAs also inhibit a related, more accessible enzyme from blood known as butyrylcholinesterase (BChE).[13] The relative abundance and longevity of BChE in blood makes it an ideal biomarker to assess OPNA exposure weeks after an exposure event.[14] BChE activity assays, similar to the Ellman assay, measure choline turnover but still cannot identify a specific causative agent.[15,16] Fluoride reactivation assays, developed after a sarin attack in a Tokyo subway in the late 1990s, were a major leap forward in post-exposure OPNA detection.[17] Typically, these assays utilize a nucleophilic fluoride anion to regenerate the specific nerve agent for GC-MS analysis from blood, identifying the specific agent used.[18]

Perhaps the most effective BChE-based detection methods center on UHPLC-MS/MS analysis of digest products. After immunoprecipitation of the enzyme from blood, BChE is

digested with pepsin to produce a nine amino acid peptide (FGES*AGAAS, nonapeptide) which contains the covalently-bound nerve agent for analysis.[19,20,21,22] However, in addition to their numerous structural combinations, many OPNAs undergo dealkylation after binding to BChE (Figure 1),[23] a process known as aging, which creates more potential biomarkers for analysis. Currently, all OPNA-BChE biomarkers must be targeted by quantitative assays to confirm exposure events. Supporting such a large number of targeted methods places a large burden on the public health laboratories responsible for assessing OPNA exposure.

While OPNAs have many structural combinations, product ions formed by the BChE nonapeptide under MS/MS conditions do not retain the specific OPNA adduct. Therefore, the same products ions are always observed regardless of which OPNA is bound.[24,25,26] These conserved product ions are signature b-ions (containing the nonapeptide amino terminus after loss of the OPNA adduct)[27] that can be broadly used by qualitative screening assays to determine OPNA exposure regardless of the initial precursor ion mass. For example, a blood sample taken from an exposed individual days after an exposure event may contain several distinct biomarkers of OPNA exposure, which requires complicated targeted analysis.[28,29] A BChE-based screening method could determine the presence of multiple biomarkers by identifying the signature b-ions that are uniformly generated. Given the large number of possible OPNAs, an exposure to an unknown OPNA may also occur. In this case, a targeted method would not detect exposure because the precursor ion mass-to-charge ratio (m/z) would be unknown. Previous studies used product ion scans in methods with conventional triple quadrupole instruments to screen for exposure.[30] However, their targeted design and low resolving power limited identification to only anticipated precursor ions.

Proteomics offers an efficient route to screen for characteristic BChE nonapeptide product ions. Data-dependent high-resolution tandem mass spectrometry (ddHRMS/MS) methods, most often used to sequence peptides, collect data in two distinct layers. First, the instrument acquires a full mass spectrum with high resolving power and identifies the most intense ions in that scan. Next, the instrument selects the most intense ions for dissociation, collecting high-resolution spectra on the product ions generated and tying them to specific precursor ions.[31] Applying this analytical strategy to the OPNA-adducted nonapeptide would provide an effective route to screen for OPNA-BChE biomarkers by their signature b-ions and identify precursor ions with high mass accuracy. Ideally, this method would complement existing targeted methodologies using the same preparation, vastly increasing laboratory efficiency.

For the first time, this study describes the development, characterization, and validation of a BChE screening method capable of identifying unknown OPNA adducts to BChE through the analysis of signature product ions by UHPLC-ddHRMS/MS. High mass accuracy was observed across 23 independent quality control (QC) preparations of human plasma exposed to various OPNAs. Using these samples for characterization, precursor ions were identified with a mean mass accuracy of <1 ppm while signature BChE nonapeptide product ions were identified with a mean mass accuracy of <5 ppm. In addition to QC preparation, a set of 14 synthetic BChE biomarker peptides, representing both aged and unaged OPNA adducts, were analyzed and used to establish reliable qualitative identification of analytes above 15 ng/mL. Method validation focused on two sets of experiments. First, the method analyzed a

set of 96 individual presumably unexposed human plasma samples. All samples from this set yielded data consistent with a lack of OPNA exposure. The method also analyzed a blinded set of 80 serum samples treated with OPNAs. All biomarkers above 15 ng/mL were identified with a 100% success rate. Importantly, the method did not identify any false positives or false negatives in these samples. This method complements existing quantitative methods and increases confidence in exposure verification. More importantly, the work herein describes the first OPNA-BChE screening method capable of identifying exposure to an unknown OPNA.

Materials and Methods

Materials

HPLC-grade acetonitrile and water were obtained from Fisher Scientific (Waltham, MA). Tween-20, trimethylamine (TEA) buffer, phosphate buffered saline, dimethyl pimelimidate dihydrochloride and tris-buffered saline were obtained from Sigma-Aldrich (St. Louis, Mo). Dynabeads for immunomagnetic separation of BChE from blood products were obtained from Life Technologies (ThermoFisher Scientific, Grand Island, NY). King Fisher deep well plates, shallow well plates, and tip combs, protein precipitation plates, heat sealing foil, and 96-well autosampler plates were all obtained from Fisher Scientific (Waltham, MA). Synthetic peptides were used throughout this study for method optimization and isotopically-labeled peptides were used as internal standards. BChE, GB-BChE, P-BChE, VX-BChE, VR-BChE, and oCP-BChE synthetic peptides were supplied by The Netherlands Organisation for Applied Scientific Research (TNO; Rijswijk, Netherlands). Synthetic peptides for GE-BChE, GD-BChE, GF-BChE, PrP-BChE, PrGB-BChE, MeP-BChE, ExP-BChE, and EtP-BChE were obtained from Battelle Memorial Institute (Columbus, OH). As detailed in a previous publication, the following abbreviations were used for OPNA-BChE biomarkers: BChE – butyrylcholinesterase; P-BChE – phosphorylated BChE; MeP-BChE – methyl phosphonate-adducted BChE; EtP-BChE – Ethyl phosphonate-adducted BChE; ExP-BChE – Ethoxy-phosphoryl butyrylcholinesterase; Propyl-BChE – propyl phosphonate-adducted BChE; VX-BChE – VX- adducted BChE; GB-BChE – Sarin-adducted BChE; GE-BChE – Ethyl Sarin-adducted BChE; VR-BChE – Russian VX-adducted BChE; oCP-BChE – ortho-cresylphosphate-adducted BChE; GF-BChE – Cyclosarin-adducted BChE; GD-BChE – Soman-adducted BChE..[27] Isotopically-labeled and native peptides were matched from the same vendor. For example, native and isotopically-labeled BChE nonapeptide were both supplied by TNO.

Preparation of Quality Control (QC) Materials

Pooled human plasma (QC low, QCL) and plasma whose BChE was fully inhibited with GB (QC high, QCH) were obtained from Battelle Memorial Institute (Columbus, OH). Plasma was deemed fully inhibited by GB according to a loss of BChE enzyme activity. To prepare the methyl phosphonate adduct in plasma (QC mid, QCM), plasma fully inhibited by GB was incubated at 37°C for 17 days. BChE-free matrix blank was prepared by filtering pooled, untreated human plasma through a 10 kDa centrifugation filter (Millipore; Bellerica, MA). All QC materials were divided into 1 mL aliquots and stored at −70°C until needed for each assay.

Sample Preparation

Immunomagnetic isolation of BChE has been described previously.[27] The BChE monoclonal antibody (BioPorto, Hellerup, Denmark; clone ID 3E8) was conjugated to Protein G-coated DynaBeads (ThermoFisher Scientific, Grand Island, NY). Preparation of the beads began by aliquoting 2mL of beads and washing them with two-4 mL volumes of phosphate buffered saline containing Tween-20. Beads were next suspended in 8 mL of PBS Tween-20, and 400 μg of BChE antibody was added. Beads were rotated overnight (16 h) at room temperature. After incubation, the PBS Tween-20/antibody mixture was removed, and beads were washed with two volumes of triethanolamine (TEA) buffer (Sigma-Aldrich; St. Louis, IL). Beads were then incubated for 30 min in a 5.4 mg/mL solution of dimethyl pimelimidate in TEA buffer at room temperature. Supernatant was removed, and the beads were washed with 4 mL of PBS Tween-20. Beads were incubated again with 4 mL of TBS at room temperature for 15 min. Beads were washed two additional times in 2 mL volumes of PBS Tween-20 and re-suspended in 1.9 mL of PBS Tween-20. Beads were stored in this solution at 4°C until use.

For QC and sample analysis, 125 μL of each sample was thawed and filtered through a 96-well 0.45 μm PVDF filter plate (Millipore; Bellerica, MA) and centrifuged at 3000 x*g* for 5 min. After this time, samples were transferred to a 96-deep-well plate, and magnetic beads were added using a Thermo Scientific KingFisher (Waltham, MA) liquid handling system. The bead-plasma suspension was shaken at 1400 RPM for 45 min. While shaking, a digestion plate was prepared by adding 75 μL of 0.6% formic acid to a 96-well plate followed by 10 μL of 2 mg/mL solution of pepsin also in 0.6% formic acid. A 10 μL volume of a 500 ppb solution of isotopically-labeled synthetic peptides (Battelle Memorial Institute and TNO) may also be added to this mixture (to verify injection and proper instrument function) and will not interfere with unknown identification. After incubation was complete, beads loaded with BChE were washed three times with PBS-tween and transferred to the digestion solution via the KingFisher. Beads were incubated at 37°C for 30 min and shaken for one minute intervals at 1000 RPM for 10 seconds. After incubation, the KingFisher was used to remove the beads from the digestion solution. The remaining digestion solution was mixed with 285 μL of acetonitrile to precipitate any remaining whole proteins and filtered through a protein precipitation plate (ThermoFisher Scientific, Waltham, MA) under vacuum. The eluent was then dried under a stream of nitrogen for 45 min. Samples were re-suspended in 0.6% formic acid and transferred to an Eppendorf 96-well PCR plate (Hauppauge, NY) for analysis.

UHPLC-ddHRMS/MS

For chromatographic resolution and separation of known analytes from matrix background, an Agilent 1290-series UHPLC system (Agilent Technologies, Santa Clara, CA) was equipped with a Phenomenex Kinetex Biphenyl 50 × 2.1 mm, 1.7μm, 100Å pore size column (Phenomenex, Torrance, CA). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The following gradient was used at a constant flow rate of 0.1 mL/min: 0 to 5 min, 2% B; from 5 to 35 min, B was linearly increased from 2% to 18%; from 35 to 50 min, B was linearly increased from 18% to 40%; from 50 to 50.1 min, B was increased to 90% and held until 55 min; after this time,

B was immediately taken back to 2% and the column was equilibrated for 5 min. For each analysis, the column temperature was kept at 60°C, and 10 μL of sample was injected. The autosampler compartment was set to 8°C, and the injection needle was washed for 20 seconds with a 10% solution of methanol in water.

Data-dependent acquisition was achieved with a Thermo Scientific QExactive mass spectrometer (ThermoFisher Scientific, Waltham, MA) set to ddMS2/SIM mode. Every 48 hours, the instrument performance was evaluated and calibrated using Thermo Scientific CalMix calibration solution. Parameters that fell out of control according to CalMix evaluation were also calibrated. Prior to analysis of QC samples, a purified mixture of native synthetic peptides in 0.6% formic acid (at 32 ppb) was injected to ensure mass accuracy and that data-dependent isolation and dissociation of ions was working optimally.

Data Acquisition and Processing

MS1 and MS2 spectra were stored on the instrument as data was collected. Instrument data sets were initially analyzed using Thermo Scientific QualBrowser version 3.1.66.10 using a mass range filter set to search for characteristic phosphate adducted BChE nonapeptide product ions (*m/z* 778.3366, *m/z* 673.2940 and *m/z* 602.2569) with a 10 ppm tolerance. Final analysis was conducted with the TraceFinder 3.2 software (ThermoFisher Scientific, Waltham, MA) set to screening mode. In this mode, the software can identify high mass accuracy product ions and precursor ions from both QC materials and unknowns. For data analysis of QC materials, a compound library was created based on precursor and product ion masses of those found in GB-inhibited pooled plasma, fully aged GB-inhibited pooled plasma, and uninhibited plasma. Data gathered on unknowns was first analyzed using QualBrowser as described above. Precursor ions identified were used to build custom libraries for unknown samples and analyzed in the blinded study. High resolution masses of unknowns and QC materials are exported from TraceFinder as a Microsoft Excel file for statistical analysis.

Convenience Set and Unknown Exposure Analysis

A convenience set of 96 individual human serum samples was obtained from Tennessee Blood Services (Tennessee Blood Services Corporation, Memphis, TN). 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). Samples were prepared as described above, and data analysis was completed using both QualBrowser and TraceFinder. Data were exported and analyzed to establish a mean *m/z* value and standard deviation.

A blinded set of 80 spiked samples were prepared and analyzed as described above. Unknown mass values were recorded and exported as high accuracy *m/z* values using TraceFinder. Data were then scored using the identities of each blinded sample in order to calculate a percent positive identification.

Safety Considerations

Appropriate safety control measures, including engineering, administrative, and personal protective equipment, were used for all procedures based on a site-specific risk assessment

that identified physical, health, and procedural hazards. OPNA-spiked serum or plasma was evaluated prior to receipt to ensure all live agent was quenched. Serum and plasma evaluated in this way present no additional risks outside of those present when working with human blood products. Additionally, all blood products are screened for potential blood borne pathogens prior to receipt. Universal precautions for working with these materials should be followed while using this method.

Results and Discussion

Method Development and Optimization

The proteomics literature[31] offers a route to reliably screen for signature product ions of adducted BChE nonapeptides. Known as data-dependent acquisition, this specific scan mode is regularly used to sequence and identify peptides present in a sample. Data-dependent acquisition first identifies the most intense ions in a precursor ion scan. Next, the instrument selects these ions for fragmentation in real time and collects product ion spectra on each before conducting another full scan to complete the duty cycle. Data-dependent acquisition constantly records and links together precursor and product ion spectra. Therefore, applying this instrument logic to BChE nonapeptide analysis on a high resolution instrument would provide high-confidence identification of characteristic product ions. This scan mode would also provide chemical formulation of unknown precursors that produce the characteristic fragment ions. Accurate mass data collected in the assay can serve as a reporting metric and provide limits of mass accuracy for both QC materials and product ion masses of unknowns. With these considerations in mind, a basic data collection and QC metric was envisioned for development on a QExactive mass spectrometer (Figure 2).

Reliable data-dependent selection of the BChE nonapeptide between samples was the first critical consideration for the method's success. Since data-dependent acquisition relies on the identification of abundant peaks in a precursor ion scan, enrichment of the BChE nonapeptide was necessary. Current OPNA-BChE detection methods developed within our laboratory rely heavily on the immunomagnetic separation of BChE from human blood products.[27] This approach decreases sample complexity and ensures high fidelity data-dependent identification of analytes between samples. The conservation of a single sample preparation between multiple methods has the added benefit of increasing laboratory efficiency and decreasing costs since a single preparation may be used for several different analytical tests.

Instrument method development began by optimizing source parameters and collision energies on synthetic BChE nonapeptides to produce the characteristic product ions for identification. Using these peptides, a normalized collision energy (NCE) of 22% induced regular dissociation patterns among potential analytes. Figure 3 shows the dissociation patterns optimized and observed for this method. These spectra represent product ions of the nonapeptides generated from untreated plasma (BChE nonapeptide), plasma treated with GB (GB-BChE), and aged GB-treated plasma, which hydrolyzes to the methyl phosphonate adduct to BChE (MeP-BChE). After optimization, the method reliably generated all characteristic BChE nonapeptide product ions with mass accuracies of 5 ppm or less.

Data-dependent acquisition on the QExactive mass spectrometer requires careful optimization of resolving power, trap fill times, and dynamic exclusion to balance sensitivity, accuracy, and reproducibility. While these parameters affect spectral quality and sensitivity, they also affect duty cycle times. Longer duty cycles are potentially problematic since they may result in the loss of analyte selection for fragmentation in a data-dependent assay. Duty cycle times were therefore carefully considered throughout instrument method optimization.

Prior to introduction into the orbitrap for mass analysis, ions are collected in a transient, curved ion trap, termed the c-trap. Maximum injection times (maximum IT) into the c-trap can be extended to boost sensitivity within a method. While longer trap fill times increase sensitivity, they also lengthen instrument duty cycles times. Maximum IT works with the automatic gain control (AGC) target to set a maximum threshold of ions collected in the c-trap. Thus, c-trap fill times vary throughout a method based on ion abundance. Finally, the underfill ratio was set to 1%, bringing the intensity threshold to a value of 1.3×10^3 cps. Working with the AGC target value, the underfill ratio dictates a minimum ion intensity for ddHRMS/MS selection. The set value for this method is quite low. However, the high sample purity obtained after BChE immunomagnetic precipitation significantly decreases potential interference from the sample matrix. Thus, having a low intensity threshold increases the likelihood of BChE nonapeptide selection without the potential of matrix interference.

High resolution mass analysis takes place in the QExactive's orbitrap mass analyzer. In the orbitrap, resolving power directly correlates with scan times and is defined as the separation of ions at a given m/z. Since peak width is essential for resolution in a mass spectrum, resolving power is defined as full width at half maximum (FWHM). To properly balance duty cycle times and analyte separation from matrix background, full scan resolution was set to 70,000 FWHM and product ion scan to 17,500 FWHM. These values provided optimal peak resolution from matrix background while keeping duty cycles as low as possible. A high fidelity data-dependent acquisition method was developed by carefully balancing these specific parameters and others. A more detailed list of optimized method parameters can be found in the supplemental information.

Despite the high sample purity after immunomagenetic separation of BChE from blood products, method chromatography was also optimized to further separate analytes from matrix background and ensure high fidelity data-dependent analysis. A 60 minute binary gradient of water and acetonitrile with 0.1% formic acid provided ideal peak shape and separation of known nonapeptides from matrix background. As a measure of chromatographic performance, a mixture of synthetic BChE nonapeptides were analyzed (Figure 4). The mixture evaluated in this experiment consisted of both unaged adducts and aged adducts to BChE, representative of potential findings following an OPNA exposure. The synthetic peptides used for this experiment were present at 32 ng/mL. In general, more polar unadducted and aged BChE species eluted earlier in the chromatographic gradient. The more hydrophobic unaged biomarkers required a higher percentage of organic solvent for elution. Despite the shallow gradient, some peaks still co-eluted, such as EtP-BChE and ExP-BChE. No challenges were encountered in distinguishing their data-dependent

identification given the significant mass differences of these co-eluting species. Some nonapeptide biomarkers shared identical chemical compositions, such as PrP-BChE and VX-BChE, but were resolved chromatographically. These results suggested that ddHRMS/MS and UHPLC parameters were sufficiently optimized to identify all potential OPNA adducts.

As a final measure of method performance prior to characterization, synthetic BChE nonapeptides were analyzed at specific concentrations of 2, 4, 8, 16, 32, 64, and 125 ng/mL. These peptide mixtures included G- and V- series nonapeptides. This set also included nonapeptides with poor ionization corresponding to unadducted BChE nonapeptide, P-BChE, and oCP-BChE. Therefore, this set of analytes is highly representative of the range of biomarkers likely to be observed after an OPNA exposure event. These peptide pools were examined in a matrix background at each concentration to establish a range of method performance. Method performance was considered good when reproducible data-dependent selection and fragmentation of peptides was observed. At concentrations below 15 ng/mL, the characteristic BChE fragment ions of OPNA-BChE biomarkers were not reproducibly observed for all analytes. Therefore, the method was considered to have good performance for biomarker identification at concentrations above 15 ng/mL. Analysis of convenience set samples in a previous study found that the human BChE nonapeptide has an average concentration of 40 ng/mL after immunoprecipitation.[24] Therefore, biomarker values at or above the threshold of 15 ng/mL are reasonable to expect after an exposure incident.

Method Validation

Qualitative method validation focused on three specific areas: data-dependent dissociation of analytes, product ion mass accuracy, and precursor ion mass accuracy. With these parameters in mind, human plasma treated with OPNAs served as the best possible QC material to assess method performance since it would provide all possible matrix effects from clinical samples during characterization. Orbitrap *m/z* resolving power varies with ion mass,[32,33] so OPNA-treated plasma samples were chosen based on a range of potential biomarker masses. QCL for this method was pooled human plasma which only yielded the unadducted BChE nonapeptide after work up (*m/z* 796.3472) while QCH is pooled human plasma fully inhibited with GB yielding the GB-BChE nonapeptide after preparation (*m/z* 916.3812). Finally, QCM was pooled human plasma fully inhibited with GB and aged for three weeks at 37 °C. This QC yields the aged product, MeP-BChE, after work up (*m/z* 874.3342). Human BChE exists as a glycosylated homotetramer of four 100 kDa subunits in blood,[34] so a matrix blank of human plasma prepared by 10 kDa MWCO filtration was chosen and shown to produce no detectable BChE nonapeptide product ions. Using these QC materials and matrix blank, four separate analysts prepared and collected data on 23 independent sets of QC materials over the course of four weeks. No more than two QC sets were acquired each day,[35] and the high mass accuracy data was exported into an Excel spreadsheet for upload into a data management (STARLIMS) system equipped with statistical analysis software (SAS, Carey, NC).

BChE nonapeptides from QCL, QCM, and QCH lots were reproducibly selected for dissociation across all 23 preparations and produced the three signature product ions

consistently. After selection and dissociation, product ions were reliably identified with high mass accuracy (< 5 ppm standard deviation in mass from the mean) (Table 1). Acquiring QC precursor ion masses at a higher resolving power (70,000 FWHM) provided a mass measurement with < 1 ppm deviation across 23 QC sets (Table 2). Thus, when analytes were present at a concentration of > 15 ng/mL, the method reliably identified biomarkers with high confidence. The high mass accuracy and precision observed in the validation data highlights the strength of this method when analyzing clinical samples exposed to a potentially unknown OPNA. Additionally, the mass accuracy serves as an ideal complement to existing quantitative assays of OPNA exposure – providing more confidence in reporting an exposure event. The mass accuracy limits established from method characterization data by the SAS calculation provided limits of mass variance for OPNA-BChE nonapeptide in QC materials. These limits serve as a guide for method performance between samples sets and establish criteria for reporting data in the case of an exposure event.

Method Stability and Ruggedness

Previous characterization and validation studies performed on related BChE adduct methods detailed material stability and digestion conditions.[24,36] For this study, the parameters most critical to method performance, specifically data-dependent selection of BChE nonapeptides were identified and tested to establish method limitations and ruggedness. Separation of OPNA analytes from matrix background was a major factor for method performance in characterization, so initial solvent composition, UHPLC gradient slopes, and flow rates were varied. Changes in these parameters only had a slight impact on performance which varied between analytes (see supplemental information for details).

Since the method relies on the accurate identification of fragment ion masses for assessment of exposure, fragmentation energies and HRMS mass calibration were varied to establish their effect on method performance. Collision energies were varied by 10% and exhibited only modest impacts on analyte identification. Finally, high mass accuracy was left uncalibrated for 12 days and showed no significant impact on method performance. Taken together, these results suggest this method is stable and amenable to use in clinical laboratories using defined limits for data reporting. Additional details of ruggedness evaluation can be found in the supplemental information.

96 Individual Matrix Sample Convenience Set

Pooled human plasma was used throughout method characterization; however, the method is expected to test for exposure in individuals. Therefore, a commercial convenience set of 96 individual, presumably unexposed samples was used to establish how reproducibly signature product ions were observed in individuals. Importantly, mutations to the BChE nonapeptide detected in this method occur at extremely low rates, < 0.01%,[37,38] and should have a minimal impact on this method. The acquired precursor and product ion spectra of the BChE nonapeptide from all 96 patient samples showed no significant (< 5 ppm) mass deviation between individuals (Figure 5). Furthermore, no spectra yielded a precursor ion indicative of OPNA exposure, and all 96 individual samples yielded the precursor ion expected for BChE nonapeptide with < 1 ppm deviation in mass accuracy.

Theoretically, methods broadly screening for OPNA adducts to BChE have the capability to detect exposure to organophosphorus (OP) pesticides. Indeed, previously developed methods using low resolution instruments projected the presence of low levels of OP pesticide exposure.[16,39] Of the 96 individual serum samples evaluated in our convenience set, none possessed biomarkers consistent with OP pesticide exposure, possibly due to either the size of the convenience set or the method's current lowest reportable limit.

80 unknown exposure samples

As a final validation of method performance, a set of 80 blinded samples was analyzed to simulate those that may be received in a suspected exposure event. To ensure optimal method performance and sample preparation, the 80 blinded samples were split into four batches of 20 samples, each batch analyzed alongside a QC characterization set. The analyst prepared and analyzed the blinded samples and QC sets according to the established method protocol. In a suspected exposure event, a variety of OPNA-BChE biomarkers may be observed at different concentrations. Unknown, agent-spiked plasma sample specimens analyzed in this experiment also contained a variety of different biomarkers at the concentrations anticipated in a suspected exposure event. This set also included samples where only a fraction of BChE was inhibited. Unexposed samples were also included in the set to further assess the rate of false positive identifications. The comparison of the theoretical value versus experimentally-determined value was used to score the method's performance. The results of these blinded sets and the total number biomarkers present versus those identified can be found in Table 3.

TraceFinder identifies mass features using a custom reference library. Libraries in TraceFinder are easily editable to add new or unknown analytes. For the purposes of this blinded study, the analyst updated a reference library based on the positive identification of key product ion masses (m/z 602.2569, 673.2940 and 778.3366) from ddHRMS/MS spectra.

The 80 unknown specimens were a combination of individual unexposed serum, serum exposed to one agent, serum exposed to multiple agents, aged serum, and other human matrices such as urine. Analytes present above the established performance limit of 15 ng/mL were correctly identified in every sample. Some unknown samples were mixtures of multiple pooled human serum samples exposed to different individual agents. These mixtures resulted in a 6 to 1 dilution of the anticipated analytes, below the method's performance limit. In addition to the method's 100% success rate, no false positive or negative identifications were observed. The results collected in this final evaluation of method performance demonstrate how this method significantly increases the number of observable analytes within a single method while maintaining a high level of accuracy and precision.

Conclusions

This study presents a new tool for public health investigators to screen for more potential biomarkers after an OPNA exposure event. The developed method centers on the identification of signature product ions of the BChE nonapeptide which are produced after exposure to OPNAs. Since specific OPNA-BChE analytes are not targeted by this method,

every possible OPNA-BChE nonapeptide biomarker can be screened in a single analytical run. This method reliably identified biomarkers with extremely high mass accuracy by their signature product ions throughout characterization. When this method was applied to a set of 80 potentially exposed samples, it identified 100% of the biomarkers within its reportable range. A commercial convenience set determined less than 1% false positive identification rate of OPNA-BChE biomarkers for this test. This method is currently employed within our laboratory alongside a targeted assay for high-priority OPNA-BChE nonapeptide biomarkers. The data generated by this qualitative method complements our targeted assay to increase confidence in biomarker identification and catch any potential unknown OPNA-BChE biomarker. Currently, this method is not suitable for high-throughput screening. However, we plan to continue improving future versions of this method by increasing analyte sensitivity for detection below 15 ng/mL and decreasing analytical run times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Defense Threat Reduction Agency, Office of Public Health Preparedness and Response at the Centers for Disease Control, and the Oak Ridge Institute for Science Education. The authors would like to thank Ms. Chariety Sapp for dispensing individual serum for convenience set analysis and distribution of blinded samples.

References

1. Soltaninejad K, Shadnia S. Basic and Clinical Toxicology of Organosphosphorus Compounds. 2014:25–43.
2. Sidell, FR. Medical Aspects of Chemical and Biological Warfare. United States of America; 1997. p. 129-179.
3. Bachovchin DA, Cravatt BF. Nat Rev Drug Discovery. 2012; 11:52–68. [PubMed: 22212679]
4. van der Schans MJ, Fidder A, van Oeveren D, Hulst AG, Noort D. J Anal Toxicol. 2008; 32:125–130. [PubMed: 18269804]
5. Annual Report. Organisation for the Prevention of Chemical Weapons; 2014.
6. Ellman GL, Courtney KD, Andres V, Featherstone RM. Biochem Pharmacol. 1961; 7:88–95. [PubMed: 13726518]
7. Ciesielski S, Loomis DP, Mims SR, Auer A. Am J Public Health. 1994; 84:446–451. [PubMed: 8129063]
8. Black RM, Clarke RJ, Read RW, Reid MTJ. J Chromatogr A. 1994; 662:301–321. [PubMed: 8143028]
9. Read RW, Black RM. J Chromatogr A. 1999; 862:169–177. [PubMed: 10596974]
10. Riches J, Morton I, Read RW, Black RM. J Chromatogr B: Anal Technol Biomed Life Sci. 2005; 816:251–258.
11. Mawhinney DB, Hamelin EI, Fraser R, Silva SS, Pavlopoulos AJ, Kobelski RJ. J J Chromatogr B: Anal Technol Biomed Life Sci. 2007; 852:235–243.
12. Black RM, Harrison JM, Read RW. Arch Toxicol. 1999; 73:123–126. [PubMed: 10350193]
13. De Bisschop HCJV, De Meerleer WAP, Willemsf JL. Biochem Pharmacol. 1987; 36:3587–3591. [PubMed: 3675615]
14. Black RM. J Anal Toxicol. 2008; 32:2–9. [PubMed: 18269786]
15. Namba T, Nolte CT, Jackrel J, Grob D. Am J Med. 1971; 50:475–492. [PubMed: 4324629]
16. Grob D, Harvey AM. Am J Med. 1953; 14:52–63. [PubMed: 13016586]

17. Polhuijs M, Langenberg JP, Benschop HP. Toxicol Appl Pharmacol. 1997; 146:156–161. [PubMed: 9299607]
18. Adams TK, Capacio BR, Smith JR, Whalley CE, Korte WD. Drug Chem Toxicol. 2015; 27:77–91.
19. Sporty JL, Lemire SW, Jakubowski EM, Renner JA, Evans RA, Williams RF, Schmidt JG, van der Schans MJ, Noort D, Johnson RC. Anal Chem. 2010; 82:6593–6600. [PubMed: 20617824]
20. Fidder A, Hulst AG, Noort D, de Ruiter R, van der Schans MJ, Benschop HP, Langenberg JP. Chem Res Toxicol. 2002; 15:582–590. [PubMed: 11952345]
21. Knaack JS, Zhou Y, Abney CW, Jacob JT, Prezioso SM, Hardy K, Lemire SW, Thomas J, Johnson RC. Anal Chem. 2012; 84:9470–9477. [PubMed: 23083472]
22. Noort D, Fidder A, van der Schans MJ, Hulst AG. Anal Chem. 2006; 78:6640–6644. [PubMed: 16970345]
23. Carletti E, Aurbek N, Gillon E, Loiodice M, Nicolet Y, Fontecilla-Camps JC, Masson P, Thiermann H, Nachon F, Worek F. Biochem J. 2009; 421:97–106. [PubMed: 19368529]
24. Pantazides BG, Watson CM, Carter MD, Crow BS, Perez JW, Blake TA, Thomas JD, Johnson RC. Anal Bioanal Chem. 2014; 406:5187–5194. [PubMed: 24604326]
25. Johnson DC, MD, Crow BS, Isenberg SL, Graham LA, Erol HA, Watson CM, Pantazides BG, van der Schans MJ, Langenberg JP, Noort D, Blake TA, Thomas JD, Johnson RC. J Mass Spectrom. 2015; 50:683–692. [PubMed: 26149113]
26. Carter MD, Crow BS, Pantazides BG, Watson CM, Thomas JD, Blake TA, Johnson RC. Anal Chem. 2013; 85:11106–11111. [PubMed: 24205842]
27. Graham LA, Johnson D, Carter MD, Stout EG, Erol HA, Isenberg SL, Mathews TP, Thomas JD, Johnson RC. Biomed Chromatogr. 2016
28. Segall Y, Waysbort D, Barak D, Ariel N, Doctor BP, Grunwald J, Ashani Y. Biochemistry. 1993; 32:13441–13450. [PubMed: 8257680]
29. Fidder A, Hulst AG, Noort D, de Ruiter R, van der Schans MJ, Benschop HP, Langenberg JP. Chem Res Toxicol. 2002; 15:582–590. [PubMed: 11952345]
30. van der Schans MJ, AF, van Overen D, Hulst AG, Noort D. J Anal Toxicol. 2008; 32:125–129. [PubMed: 18269804]
31. Yates JR, Ruse CI, Nakorchevsky A. Annu Rev Biomed Eng. 2009; 11:49–79. [PubMed: 19400705]
32. Hardman M, Marakov AA. Anal Chem. 2003; 75:1699–1705. [PubMed: 12705605]
33. Marakov AA, Denisov E, Kholomeev A, Balschun W, Lange O, Strupat K, Horning S. Anal Chem. 2006; 78:2113–2120. [PubMed: 16579588]
34. Lockridge O, Adkins S, La Du BN. J Biol Chem. 1987; 262:12945–12952. [PubMed: 3115973]
35. Caudill SP, Schleicher RL, Pirkle JL. Stat Med. 2008; 27:4094–4106. [PubMed: 18344178]
36. Johnson D, Carter MD, Crow BS, Isenberg SL, Graham LA, Erol HA, Watson CM, Pantazides BG, van der Schans MJ, Langenberg JP, Noort D, Blake TA, Thomas JD, Johnson RC. J Mass Spectrom. 2015; 50:683–692. [PubMed: 26149113]
37. Bartels CF, Jensen FS, Lockridge O, van der Spek AFL, Rubinstein HM, Lubrano T, La Du BN. Am J Hum Genet. 1992; 50:1086–1103. [PubMed: 1570838]
38. Lockridge O, Norgren RB Jr, Johnson RC, Blake TA. Chem Res Toxicol. 2016
39. van der Schans MJ, Hulst AG, van der Riet-van Oeveren D, Noort D, Benschop HP, Dishovsky C. Chem-Biol Interact. 2013; 203:96–102. [PubMed: 23123253]

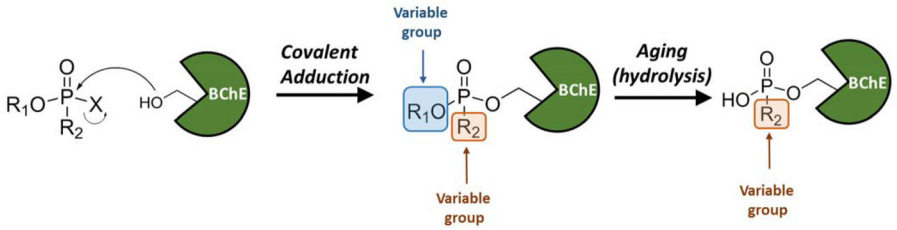


Figure 1. Adduction mechanism of OPNAs to BChE

OPNAs inhibit their target serine hydrolases (SHs), such as BChE, by covalently binding to the active site serine. Aging of OPNA adducts occurs in blood whereby alkyl-phosphate variable groups (blue) on the OPNA adduct are non-specifically hydrolyzed to the corresponding phosphonic or phosphoric acid while the alkyl-phosphonate variable groups (red) remain unchanged. OPCW-CWC nomenclature defines R_1 as a variable alkyl group (i.e. ethyl, methyl, propyl, or cyclohexyl) or a hydroxyl group; R_2 can be either a variable alkyl group or an amino alkyl group.

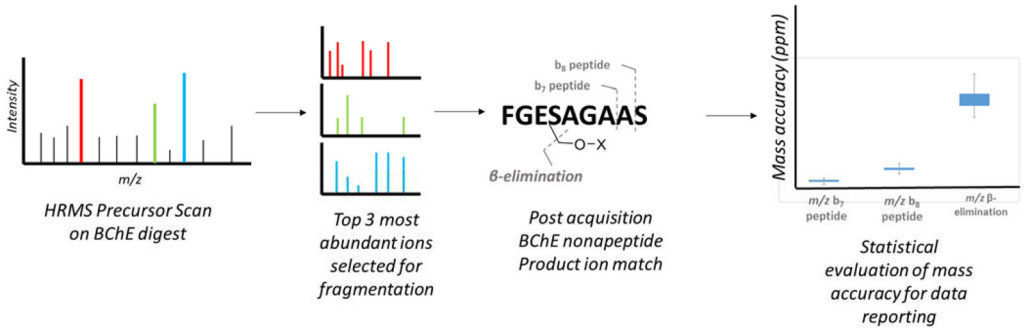


Figure 2. Data dependent BChE method workflow

Data-dependent analysis of peptides begins with a survey of the most abundant ions in a precursor ion scan. These ions are then selected for fragmentation in real time by the instrument. After data-dependent acquisition of a BChE peptic digest, product ion scans are searched for characteristic product ions of the BChE nonapeptide. These product ions are linked to the precursor scans where an abundant analyte was first identified. After acquisition and recording, these high mass accuracy values are statistically analyzed for external reporting.

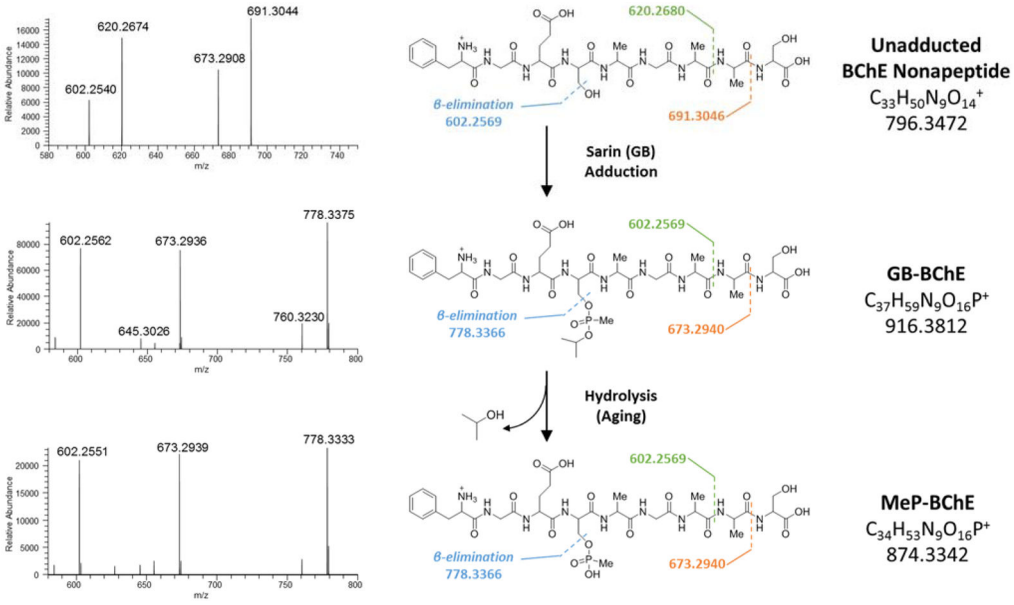


Figure 3. Principles and verification of ddHRMS/MS method development for unadducted BChE and adducted BChE biomarkers

After the immunoprecipitation of the BChE protein from blood, serum or plasma and digestion with pepsin, the resulting BChE nonapeptide produces signature product ions upon collision-induced dissociation. These product ion masses correspond to cleavages along the peptide backbone (b_7 and b_8) and β-elimination ions from serine 198. These losses happen at different rates depending on phosphorylation. Product ions identified from representative ddHRMS/MS spectra are shown alongside their theoretical masses and cleavages from the specific nonapeptide structures (highlighted in blue, green, and orange).

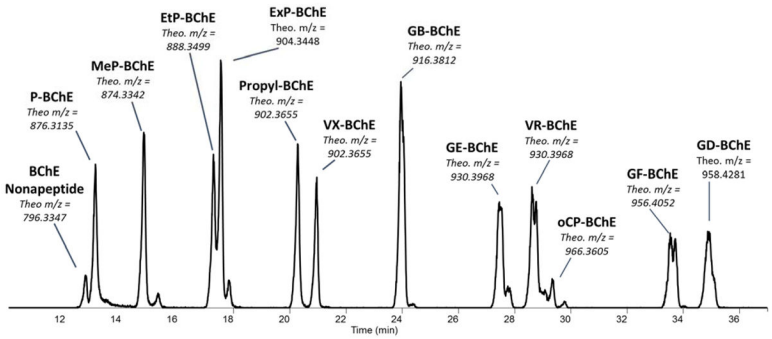


Figure 4. Extracted ion chromatogram of possible analytes following OPNA exposure

A mixture of synthetic peptides used in targeted methods following OPNA exposure were examined using the UHPLC-ddHRMS/MS method. Mass deviation of precursor ion species from theoretical was less than 10 ppm. Abbreviations: BChE – butyrylcholinesterase; P-BChE – phosphorylated BChE; MeP-BChE – methyl phosphonate-adducted BChE; EtP-BChE – Ethyl phosphonate-adducted BChE; ExP-BChE – Ethoxy-phosphoryl butyrylcholinesterase; Propyl-BChE – propyl phosphonate-adducted BChE; VX-BChE – VX- adducted BChE; GB-BChE – Sarin-adducted BChE; GE-BChE – Ethyl Sarin-adducted BChE; VR-BChE – Russian VX-adducted BChE; oCP-BChE – *ortho*-cresylphosphate-adducted BChE; GF-BChE – Cyclosarin-adducted BChE; GD-BChE – Soman-adducted BChE.

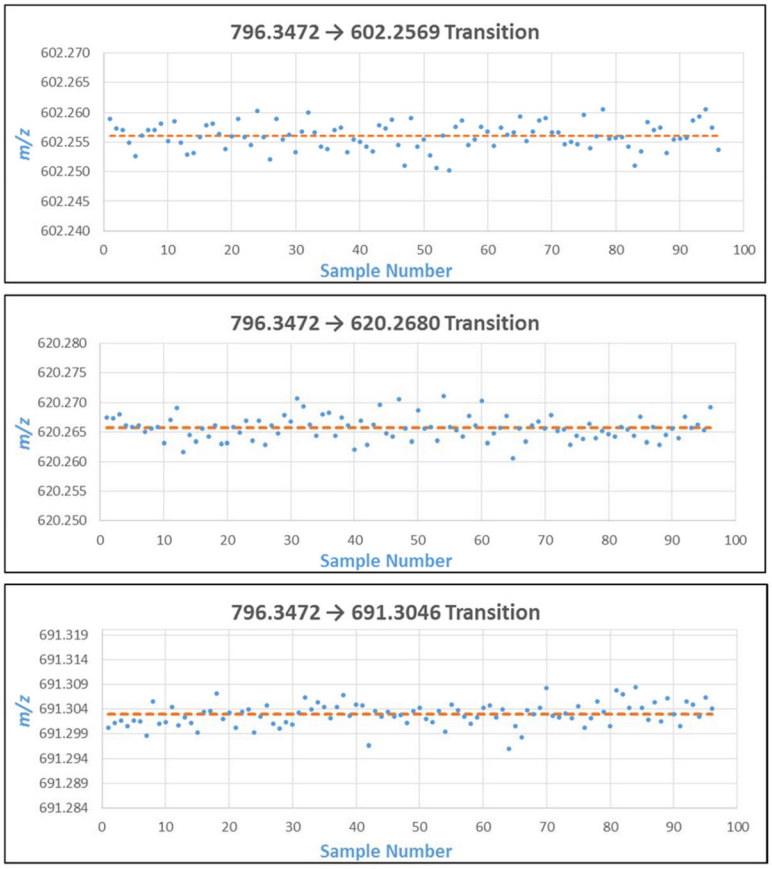


Figure 5. Identification of unadducted BChE nonapeptide ions from a convenience set of 96 individual matrix samples

Analysis of 96 individual, presumably unexposed serum samples was performed. All 96 samples produced signature product ions consistent with the unadducted BChE nonapeptide with less than 10 ppm mass deviation. These product ions were traced successfully to identification of the precursor ion of the unadducted BChE nonapeptide with less than 1 ppm deviation from theoretical mass.

Table 1

High mass accuracy evaluation of product ions from method validation (n=23).

	AnalyteCode	−2σ Window	Mean Product Ion *m/z* (±σ ppm)	+2σ Window
QCL: BChE	796 → 602	602.2519	602.2551 (±2.7 ppm)	602.2583
	796 → 620	620.2508	620.2652 (±4.3 ppm)	620.2706
	796 → 691	691.2982	691.3018 (±2.7 ppm)	691.3054
QCM: MeP-BChE	874 → 602	602.2536	602.2568 (±2.6 ppm)	602.2600
	874 → 673	673.2903	673.2929 (±2.0 ppm)	673.2955
	874 → 778	778.3328	778.3362 (±2.2 ppm)	778.3396
QCH: GB-BChE	916 → 602	602.2544	602.2564 (±1.6 ppm)	602.2584
	916 → 673	673.2907	673.2935 (±2.1 ppm)	673.2963
	916 → 778	778.3330	778.3360 (±2.0 ppm)	778.3390

Table 2

High mass accuracy evaluation of precursor ions from method validation (n=23).

	Precursor Ion Theoretical	−2σ Window	Experimental (±σ ppm)	+2σ Window	RT (min)	RT (%CV)
QCL: BChE	796.3472	796.3465	796.3471 (±0.4 ppm)	796.3478	12.6	2.5
QCM: MeP-BChE	874.3342	874.3335	874.3344 (±0.5 ppm)	874.3353	14.8	1.8
QCH: GB-BChE	916.3812	916.3795	916.3809 (±0.75 ppm)	916.3822	23.7	1.1

Table 3

Results of 80 blinded clinical specimens prepared to simulate an OPNA exposure.

Batch	Total biomarkers	Total identified/reportable range	Score %
1	32	29/29	100
2	29	26/26	100
3	20	17/17	100
4	19	19/19	100
Total	**100**	**91/91**	**100**

* Biomarkers below the threshold of 15 ng/mL were not included in this total.