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Direct Quantitation of Methyl Phosphonate Adducts to Human Serum Butyrylcholinesterase by Immunomagnetic-UHPLC-MS/MS

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Abstract

Hydrolysis of G- and V-series organophosphorus nerve agents containing a phosphorus-methyl bond yields a methylphosphonic acid (MeP) product when adducted to human butyrylcholinesterase (BChE). The MeP adduct is considered a sign of “aging” and results in loss of the *o*-alkyl identifier specific to each nerve agent. After aging has occurred, common therapeutics like oximes cannot reactivate the cholinesterase enzyme and relieve cholinergic inhibition. Until now, a direct, quantitative method for determination of the MeP adduct to BChE was unavailable. Aged adducts in serum samples were processed by immunomagnetic separation of BChE by antibody conjugated bead, isotope-dilution, pepsin digestion, followed by UHPLC separation and detection by conventional electrospray ionization tandem mass spectrometry (ESI-MS/MS). Ions were detected in selected reaction monitoring (SRM) mode, and transition *m/z* 874.3 → 778.3 was used for quantitation. The analytical response ratio was linearly proportional to the serum concentration of MeP adducted peptide (MeP-P) over the nominal concentration range of 2.0–250 ng/mL, with a coefficient of determination R^2 ≥0.997. Intrarun accuracy expressed as %Relative Error (%RE) was ≤13.5, 16.3 and 3.20% at 2.0, 16 and 250 ng/mL, respectively; the corresponding precision expressed as %RSD was ≤11.9, 6.15 and 3.39%. Interday %RSD was ≤7.13, 5.69 and 1.91%. Recovery of MeP-P from serum was ≥68% across the validated concentration range, and contributions from matrix effects were minimal. The method provides a direct, quantitative measurement of MeP-P found in clinical samples suspected of nerve agent exposure and subjected to such post-sampling stresses as elevated temperature and extended shipping.

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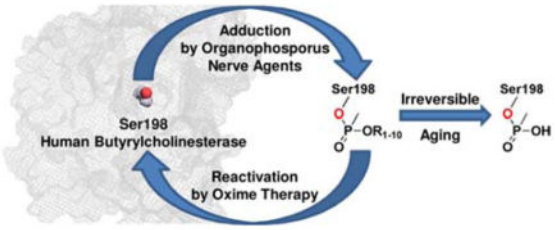
Conflict of Interest Disclosure

The authors declare no competing financial interest.

Graphical Abstract



Keywords

butyrylcholinesterase; organophosphorus nerve agent; cholinesterase inhibitors; aging; methyl phosphonic acid; methyl phosphonate

Introduction

A wide range of oxime compounds were introduced in 1955 as potent reactivators of sarin-inhibited cholinesterase (ChE) in both red blood cells and rat brain tissue.[1] The efficacy of oxime reactivators was subsequently tested in a kinetics study by Davies and colleagues who then reported a two-stage chemical process of ChE inhibition by organophosphorus compounds (OPs).[2] The process was described as an initial OP inhibition of ChE followed by a first-order rate conversion to a product that could no longer be reactivated by oximes.[2] These final products, formed by the hydrolysis of the *o*-alkyl phosphoester bond, are known now as the aged products of OPs.[3]

In G- and V-series organophosphorus nerve agents (OPNAs) containing a phosphorus-methyl bond, the aged product formed is a methylphosphonic acid (MeP) adduct.[4] As illustrated in Scheme 1, an OPNA binds to serine-198 of human butyrylcholinesterase (BChE) in blood by loss of its protonated leaving group. The structural identity of many OPNAs are preserved at this stage of BChE inhibition and can be identified by either fluoride reactivation[5] or protein digestion-HPLC-MS/MS.[6,7] Following the second stage of inhibition yielding the aged product MeP, the structural identity of the specific OPNA is lost. The MeP adduct, however, provides a means of retrospective detection of a prohibited Schedule 1 toxic chemical as identified by the Organization for the Prohibition of Chemical Weapons (OPCW)[8] and is specific to OPNAs containing the signature phosphorus-carbon bond, in this case the methyl bond. OP pesticides would not be expected to interfere with detection of the MeP adduct since pesticides do not contain the signature phosphorus carbon bond specified by the OPCW.

While storage conditions for clinical samples should not exceed 4 °C under ideal circumstances,[12] collection sites of potential OPNA exposures are often in uncontrolled environments and consequently, subject to greater stress. Likewise, OP-adduct stability is subject to the effects of delayed collection and long-distance shipping. Sampling conditions are of particular importance since previous indirect measurements of OP aging show that aging rates vary by *o*-alkyl branching on the phosphoester bond and by temperature.[4,13] By the criteria for *o*-alkyl branching of OPNAs, soman would be expected to age the fastest

followed in increasing order by cyclosarin, sarin, Russian VX and VX.[4] Hence, samples collected and stored at higher temperatures over longer periods of time are expected to have higher levels of the aged adducts, but without a method to measure them, the extent of the exposure may be underestimated or missed entirely. The likelihood of suboptimal sampling and storage conditions emphasizes the pressing need for a direct measurement of aged OPNA adducts in chemical emergencies.

Since aged adducts provide retrospective determination of prohibited Schedule 1 toxic chemicals, they should be measured. When measuring ChE activity by the Ellman assay,[9] the aged MeP adduct appears no different than the initial OPNA adduct because both adducts effectively inhibit ChE.[4] Likewise, the chemically stable MeP adduct is resistant to both oxime therapy and fluoride reactivation thereby preventing direct measurement.[10] The need for a method to measure the aged adduct is apparent due to the extensive detection capabilities of the MeP adduct and the absence of a quantitative, direct measurement. Therefore, we to present a quantitative analytical method that possesses the specificity and sensitivity of immunomagnetic separation-isotope dilution-UHPLC-MS/MS[11,14] for the direct measurement of MeP adduct to BChE in human serum.

Experimental Section

Materials

Synthetic unlabeled and stable isotopically-labeled MeP peptide standards (FGES[O-methylphosphonic acid]AGAAS (MeP-P) and $^{13}C_9$,^{15}N-FGES[O-methylphosphonic acid]AGAAS (MeP-P*), respectively) were obtained from Battelle Memorial Institute (Columbus, OH, USA). Peptide amino acid analysis (AAA) was performed at Midwest Bio-Tech, Inc. (Fishers, IN, USA) and determined to be 54% peptide content for MeP-P and 46% peptide content for MeP-P*. The percentages of peptide content are calculated by weight and the remainder indicates the presence of counter ions. Clone 3E8 BChE monoclonal antibodies were purchased from ThermoFisher Affinity Bioreagents (Rockford, IL, USA). Acetonitrile and deionized water HPLC grade solvents were available commercially from Tedia (Fairfield, OH, USA). Formic acid (98%), phosphate buffered saline with Tween 20 (PBST) dry powder, dimethyl pimelimidate dihydrochloride (DMP), 0.2 M Tris buffered saline (TBS) 10X concentrate, triethanolamine buffer solution and pepsin from porcine gastric mucosa were obtained from Sigma Aldrich (St. Louis, MO, USA). Dynabeads protein G were procured from Life Technologies (Carlsbad, CA, USA). KingFisher 96 Flex microplates (200 μL), KingFisher 96 tip combs for deep well magnets, KingFisher Flex microtiter deepwell 96 plates (v-bottom), protein precipitation plates, 2 mL 96-well Nunc plates, 96-well PCR plates (ABgene product), PCR foil and easy pierce 20 μm heat sealing foil were acquired from Fisher Scientific (Rockford, IL, USA). Non-sterile MultiScreen HTS HV 0.45 μm opaque filter plates were bought from EMD Millipore (Billerica, MA, USA).

Sample preparation

Samples were processed as described earlier[7,11] and recently optimized.[14] Briefly, immunomagnetic beads were prepared by applying a DynaMag-15 magnet to 2 mL of the

Protein G Magnetic Dynabeads. The supernatant was removed, and beads were resuspended in 4 mL of PBST. This washing step was repeated twice. Beads were again resuspended in PBST (8 mL) along with an additional aliquot of 400 μg of BChE monoclonal antibody. The mixture was allowed to rotate on a Dynal Sample Mixer (speed 20) overnight at room temperature. The following day, the supernatant was removed and 4 mL aliquots of triethanolamine buffer were used to wash the beads twice. Then, a 4 mL aliquot of a 27 mg DMP in 5 mL triethanolamine buffer was added to the beads and allowed to rotate on the Dynal Sample Mixer (speed 20) for 30 minutes at room temperature. The supernatant was removed and resuspended in 4 mL of TBS. This suspension was allowed to rotate as before for 15 minutes. The beads were washed twice in 2 mL of PBST prior to a final suspension in 1.9 mL of PBST. The bead suspension was stored at 4 °C until use or up to 3 months.

Following bead preparation, serum samples of 125 μL each were manually pipetted into a non-sterile, multiscreen HTS HV 0.45 μm opaque filter plate, sealed with adhesive PCR foil, and centrifuged at 3,000 x g for 5 minutes at 20 °C to remove any fibrous tissue. An aliquot of 75 μL for each filtered serum sample was pipetted into a 96-well KingFisher deep well plate. To the wells receiving calibrators, a 75 μL aliquot of matrix “blank” was added to address potential matrix effects. The matrix blank added to calibrator wells was a tabun adducted human serum QC material. This matrix blank was chosen because it served as a serum sample depleted of unadducted BChE and would not chemically age to form the MeP adduct. An aliquot of 50 μL of prepared BChE antibody-coated magnetic beads was pipetted into all corresponding serum-containing wells of a 96-well KingFisher shallow well plate. A ThermoScientific KingFisher Flex magnetic particle processor was used to relocate the BChE antibody-coated magnetic beads to serum-containing wells. BChE protein was bound to the antibody beads when shaken with an Eppendorf MixMate at 1,400 rpm for 2 hours at room temperature. The BChE protein bound beads were transferred by the KingFisher to three deep-well wash plates containing 500 μL PBST and finally, to a plate containing 10 μL of 2 mg/mL pepsin. The pepsin digestion plate also contained a 10 μL aliquot of 850 ng/mL internal calibrator MeP-P* and a 75 μL aliquot of 0.6% formic acid. Calibrator wells also received a 10 μL aliquot of MeP-P calibrator (2, 4, 6, 16, 32, 63, 125 or 250 ng/mL). The digestion plate was maintained at 37 °C and mixed at 1000 rpm for 10 seconds per minute for 30 minutes on an Eppendorf Thermoshaker. The KingFisher was used to remove beads from the sample. An acetonitrile aliquot of 285 μL was made to a 0.2 μm filter, Pierce 2 mL protein precipitation plate. The digested samples were then added by manual pipetting to the precipitation plate. A vacuum manifold was used to filter samples through the protein precipitation plate and eluted into a 2 mL 96-well Nunc plate. The eluted samples were dried to completeness under nitrogen pressure using a TurboVap maintained at 70 °C. The dried samples were resuspended in 75 μL of 0.6% formic acid and transferred to a 96-well v-bottom PCR plate. The plate was heat sealed with foil for UHPLC-MS/MS analysis.

Preparation of stock solutions

Pooled human serum adducted with sarin or tabun obtained from Battelle Memorial Institute (Columbus, OH, USA) was used to prepare samples for quality control (QC). Tabun QC material served as a matrix blank because it was depleted of unadducted BChE and would not chemically age to form the MeP adduct. Therefore, the matrix blank represents the

complexity of sample matrices but does not contribute to the analyte measured. Final MeP QC material was prepared by *ex vivo* aging of the sarin adducted materials at 40 °C for 96 hours. The final aged material was split, half to be used as QC high material and the other half to be diluted 1:1 with control (unadducted) pooled human serum and used as QC low material. The MeP QC materials were characterized during method validation.

UHPLC-MS/MS

MeP-P levels were determined in human serum using an Agilent Technologies 6460 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) with a jet stream ionization interface operated in positive ionization mode. The instrument was tuned and calibrated bi-monthly over a mass range of *m/z* 118–2720 using the Agilent ESI tuning mixture (P/N G1969) and autotune procedure. UHPLC elution was performed using an Agilent 1290 Infinity series UHPLC system. Samples (3 μL) were injected onto a Waters Acquity BEH C18 Column (2.1 x 50 mm, 1.7 μm) (Waters, Milford, MA, USA). Column compartment and autosampler temperatures were 60 °C and 10 °C, respectively. Mobile phases were 0.1% formic acid in (A) water and (B) acetonitrile. The following gradient conditions were used at a constant 300 μL/min flow rate and average back pressure of 600 bar: 0–0.01 min 2%B; 0.01–1.33 min linear gradient 60%B; 1.33–1.34 min linear gradient 2%B; 1.34–2.0 min 2%B. The following optimized instrument parameters were applied for the detection of the unlabeled analyte and the isotopically-labeled internal calibrator: drying gas temperature = 350 °C; drying gas flow = 5 L/min; nebulizer = 35 psi; sheath gas temperature = 400 °C; sheath gas flow 12 L/min; capillary = 5000 V; nozzle voltage = 1000 V; cell accelerator voltage = 30 V; fragmenter = 200 V; dwell time = 25 ms. Detection of MeP-P was by selected reaction monitoring (SRM) (MeP-P quantitation ion *m/z* 874.3 → 778.3, collision energy = 30 V; confirmation ion *m/z* 874.3 → 673.3, collision energy = 30 V and MeP-P* internal calibrator *m/z* 884.3 → 788.3, collision energy = 30 V) at ‘unit’ resolution of 0.7 amu full width at half maximum height.

Convenience sample set

A set of 94 individual human sera samples was commercially obtained from Tennessee Blood Services (Memphis, TN, USA) to evaluate baseline level responses in a population where exposure was not expected.

Data acquisition and processing

Data acquisition was carried out on MassHunter Workstation Software, LC/MS Data Acquisition for 6400 Series Triple Quadrupole v. B.05.00, build 5.0.5027.0. Spectral analysis and quantitation were carried out utilizing MassHunter Workstation Software Quantitative Analysis v. B.05.00, build 5.0.291.0. Accuracy was reported in terms of percent relative error $\%RE=[(C_e-C_t)/C_t] \times 100$ where C_e is the experimental concentration determined from the calibration curve, and C_t is the theoretical concentration. Percent relative standard deviation, $\%RSD=(SD/C_{avg})\times 100$, was calculated as a measure of assay precision, where C_{avg} is the average calculated concentration, and SD is the standard deviation of C_{avg}. Peak area ratios of MeP-P/MeP-P* were plotted against expected concentration to construct calibration curves from a series of eight MeP-P calibrators in matrix blank serum. Each calibrator was injected (n=21) and validated over the range of 2.0–

250 ng/mL. QC material characterization was completed over the course of two weeks during method validation (n=21) and performed by three laboratory analysts.

Safety considerations

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

Results and discussion

Indirect measurements of OPNA aging have been made using oxime therapy in conjunction with ChE activity assays.[4] Oxime therapeutics such as PAM-2, TMB-4, LüH-6, HI-6 and HLö-7 are considered antidotes to OPNA poisoning since they break the phosphoserine bond by nucleophilic substitution, reactivating ChE.[15] The principle behind pairing oxime therapy with cholinesterase activity assays is that reactivation by oxime, and consequently the recovery of ChE activity, will decrease as aging occurs. The indirect assay model lacks specificity for the ChE being reactivated and is unable to identify the aged adduct formed. The method we describe overcomes the current lack of specificity and has an added benefit of adequate sensitivity to monitor expected levels of the aged OPNA adduct.

Separation and detection

Fragmentation of synthetic MeP-P and MeP-P* resulted in the predominant product ions *m/z* 778.3 and 788.3, respectively (Figure 1). Quantitation was based on the MeP-P transition *m/z* 874.3 → 778.3 resulting from the β-elimination of methylphosphonic acid from Ser-198 and yielding a dehydroalanine. Likewise, quantitation of MeP-P* was based on the transition *m/z* 884.3 → 788.3. The MeP-P confirmation transition *m/z* 874.3 → 673.3 was a result of β-elimination and collision induced fragmentation to the b8 ion. This fragmentation was reported previously for OPNA adducts to BChE.[7] In-source fragmentation was not observed for MeP-P or MeP-P*. UHPLC separation of MeP-P was achieved using a 1.3 minute linear gradient from 2% to 60% mobile phase B, yielding a MeP-P elution time of 0.6 minutes (Figure 2). A 90% accuracy was observed for the peak signal intensity increase between low (2 ng/mL) and high (250 ng/mL) calibrators as compared to the theoretical increase anticipated (Figures 2A–B). The peak signal intensity of the lowest calibrator at 2 ng/mL was 2-fold higher than matrix blank serum containing no analyte (Figure 2C).

Linearity

The peak area ratios of MeP-P/MeP-P* were linearly proportional to the concentration of MeP-P over the nominal concentration range of 2–250 ng/mL with a coefficient of determination $R^2 \geq 0.9974$ and a line equation of $y = 0.0144 \pm 0.00021x - 0.00078 \pm 0.0024$. The validated concentration range is consistent with earlier measurements for OPNA adducts to BChE in clinical samples and the general BChE abundance of 40–80 nM expected in plasma.[7,11,16] The Taylor[17] calculation for the lower limit of detection was 0.96 ng/mL and was only slightly higher than that reported for the detection of the free acid in

human urine.[18] The reportable range was from the lowest calibrator 2 ng/mL to the highest calibrator 250 ng/mL. The need for an isotope-dilution calibration curve was determined by comparing MeP-P peak areas in matrix blank serum and aqueous matrices. The slopes of the calibration curves differed by 13%. When the isotopically labeled MeP-P* was used, and the peak area ratios of MeP-P/MeP-P* were reported, the difference in serum and aqueous line slopes decreased to 5%, a minimal contribution from matrix effects. In order to address the measured difference between matrices, calibrators were added to matrix blank serum for consistency with clinical sample matrices analyzed.

Recovery

To determine the extraction efficiency of MeP-P during sample processing, matrix blank serum samples containing low-, mid- and high level calibrator spikes along with internal calibrator were added either prior to pepsin digestion (Processed) or immediately before UHPLC-MS/MS analysis (Unprocessed) (Table 1). The processed and unprocessed additions of calibrator to matrix samples differ by the step in the protocol in which the calibrator was added to the sample. For the unprocessed samples, calibrators were added immediately before analysis by UHPLC-MS/MS. The processed samples denote the addition of calibrators at the pepsin digestion step of the sample work-up. Recovery was calculated based on the ratio of peak areas of processed MeP-P versus unprocessed MeP-P. The mean (n=4) percent recovery for MeP-P calibrators was 68±0.5%, 75±0.1% and 70±0.1% at 2.00, 16.0 and 250 ng/mL, respectively. Plate adhesion and nonspecific pepsin digestion are reasonable sources of loss in protein and peptide based methods.[19]

Precision and accuracy

The intrarun and interrun accuracy and precision for the quantitation of MeP-P were recorded over the course of two weeks (Table 2). Multiple calibrators were analyzed within single batch runs. The MeP-P intrarun %RE was ≤13.5%, ≤16.3% and ≤3.20% at the low-, mid- and high-level calibration concentrations, respectively. Corresponding %RSD values were ≤11.9%, ≤6.15% and ≤3.39%. Interrun accuracy and precision were calculated from the mean MeP-P concentration from each of the three interday batch runs. The %RE for interday runs was ≤10.8%, ≤12.5% and ≤2.00%, and the corresponding %RSD was ≤7.13%, ≤5.69% and ≤1.91%. Low and high QC materials were run in conjunction with each calibration curve and calculated to be 10.0±1.4 ng/mL and 19.8±1.9 ng/mL, respectively (n=21). The %RSD of these QCs over a two week period was calculated at 14% for the QC low material and 10% for the QC high material. The method's accuracy and precision follow the guidelines in the FDA's guidance for bioanalytical method validation and thus show applicability for the analysis of clinical samples.[20]

Stability

To assess the temperature effects on the analytical response ratio of MeP-P calibrators, peptides were allowed to stand for 0, 4, 8 and 24 hours at 4, 22 and 37 °C prior to the addition of internal calibrator (Figure 3). The average response ratios (n=3) obtained at successive time points were within ±10% of initial values for up to 24 hours at all temperatures. The effects of storage were also evaluated by measuring peak area ratios after four freeze-thaw cycles from −70°C to 25°C and found to be stable ±10% of the theoretical.

Application to Baseline Samples and *Ex Vivo* Aging

A convenience set of 94 individual serum samples was purchased to assess baseline levels of MeP adducted to human BChE. All samples were found to be <2.0 ng/mL with no peaks detected. Common OPs used in commercially available pesticides such as dichlorvos and malathion (the most widely used in the United States in 2007)[21] would not be expected to interfere with MeP adduct detection since they do not contain the signature phosphorus-carbon bond crucial to MeP identification. Therefore, no MeP adducts were expected in these samples since detection would suggest exposure to a prohibited OPCW Schedule 1 toxic chemical containing the signature phosphorus-methyl bond, extremely unlikely outside the chemical warfare context.

Serum materials known to contain OPNAs with the phosphorus-methyl bond, however, would be likely to exhibit aging to the MeP adduct. In fact, when various archived (stored at −70 °C for ≥2 years) OPNA QC materials (soman, cyclosarin, sarin, Russian VX and VX) adducted at >99.9% BChE inhibition were evaluated using this method, MeP adducts were determined to be present at increasing levels over time. For instance, archived materials from 2004 had higher levels of MeP-P present than more recent 2013 material lots. As reported by Berry and Davies,[4] the OPNAs were observed to age at different rates dependent on the branching of the carbon chain on the *o*-alkyl phosphoester bond.

Our method for MeP-P is an important addition to the detection of OPNA exposure. Following oxime therapy, an aged adduct would be the only BChE biomarker expected to remain because the BChE would be primarily reactivated.[21] Our method also provides a means of evaluating the change in OPNA QC materials over time. With the occurrence of *ex vivo* aging, MeP-P levels in sarin adducted human serum were observed to triple in less than four hours at 37 °C while the same effect at 22 °C was not observed until 32+ hours. Sarin adducted materials stored at 4 °C were monitored for 72 hours and no increase in MeP-P levels was observed. While the initial sarin *ex vivo* aging experiments do not reflect the intricacies of *in vivo* aging, they do emphasize the importance of post-collection sample handling and storage. Clinical samples shipped or stored at temperatures greater than 4 °C would be expected to have higher levels of MeP-P present upon laboratory receipt and analysis. In samples of soman exposure, MeP-P would likely be the only detected BChE biomarker since soman is the fastest of the OPNAs to age.[4] The addition of a method to measure aging in OPNA analysis is therefore one of significant value for retrospective detection.

Conclusions

A method for the direct quantitation of MeP adducts to human BChE in serum was validated over the concentration range of 2.0–250 ng/mL. With a high intra- and inter-day accuracy and precision, this method can be used reliably for human serum samples. The MeP-P analyte was stable and afforded the necessary specificity for retrospective detection of prohibited OPCW Schedule 1 toxic chemicals. The method provides a sizeable improvement in specificity over indirect measurements that relied on the loss or reactivation of cholinesterase activity to determine whether aging had occurred. Furthermore, the presence

of OPNA exposure can be evaluated more accurately by detecting the MeP adduct and improve responses to OPNA chemical emergencies.

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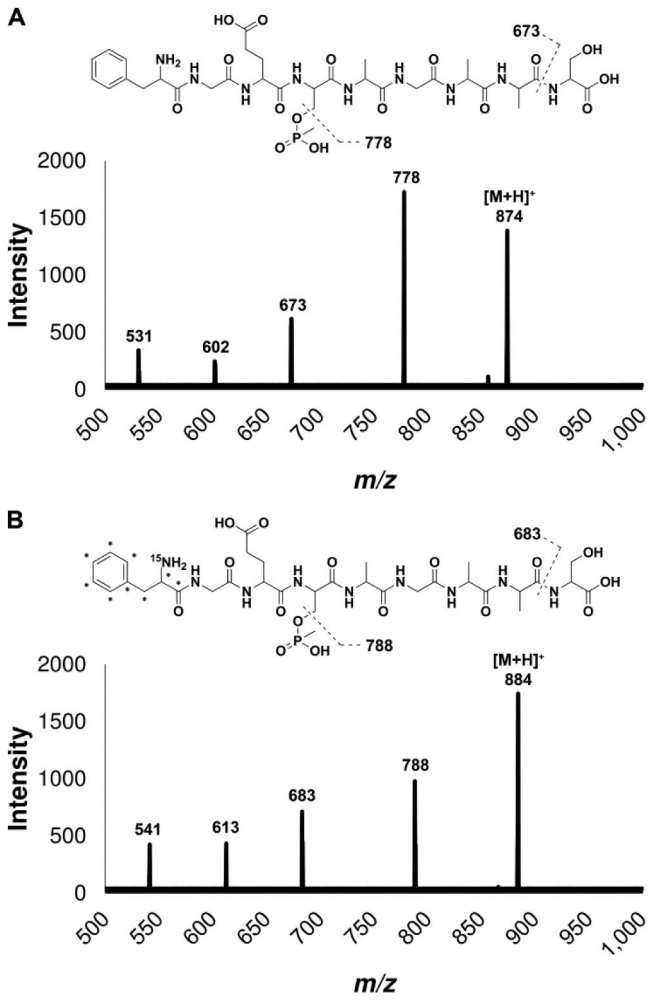


Figure 1.

Chemical structures and product ion mass spectra of the precursor ions of **A**) MeP-P ([M +H]$^+$ *m/z* 874.3) and **B**) MeP-P* ([M+H]$^+$ *m/z* 884.3). Asterisks indicate a carbon-13 label, and the peptide shown is FGESAGAAS. Spectra were collected at a collision energy of 30 V over the mass range *m/z* 500–1000. The dotted line in each chemical structure indicates the proposed site of fragmentation. This fragmentation was reported previously for OPNA adducts to BChE.[7]

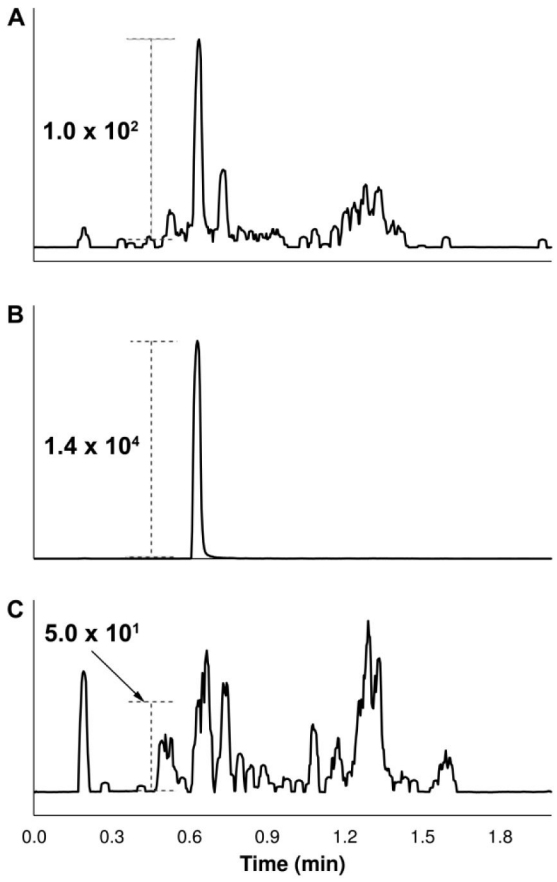


Figure 2.

Peak signal intensity of extracted ion chromatograms of MeP-P in human serum matrix. Chromatograms were acquired from matrix blank serum spiked with **A**) 2 ng/mL MeP-P (6 pg injected), **B**) 250 ng/mL MeP-P (750 pg injected) and **C**) no analyte. Detection was based on MeP-P $[M+H]^+$ *m/z* 874.3 → 778.3; each sample was prepared for a 75 μL serum volume. Analyte quantitation was calculated using the adduct peak area response ratio MeP-P/MeP-P*. Signal intensity is indicated by dotted vertical brackets.

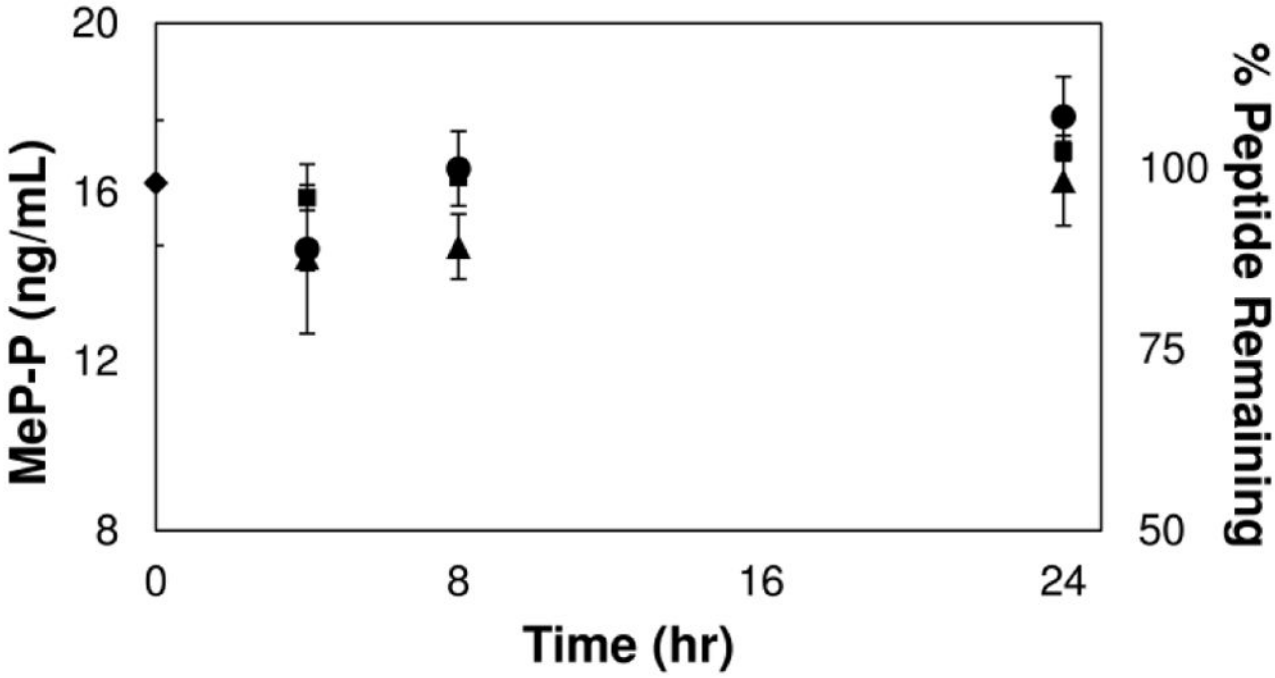
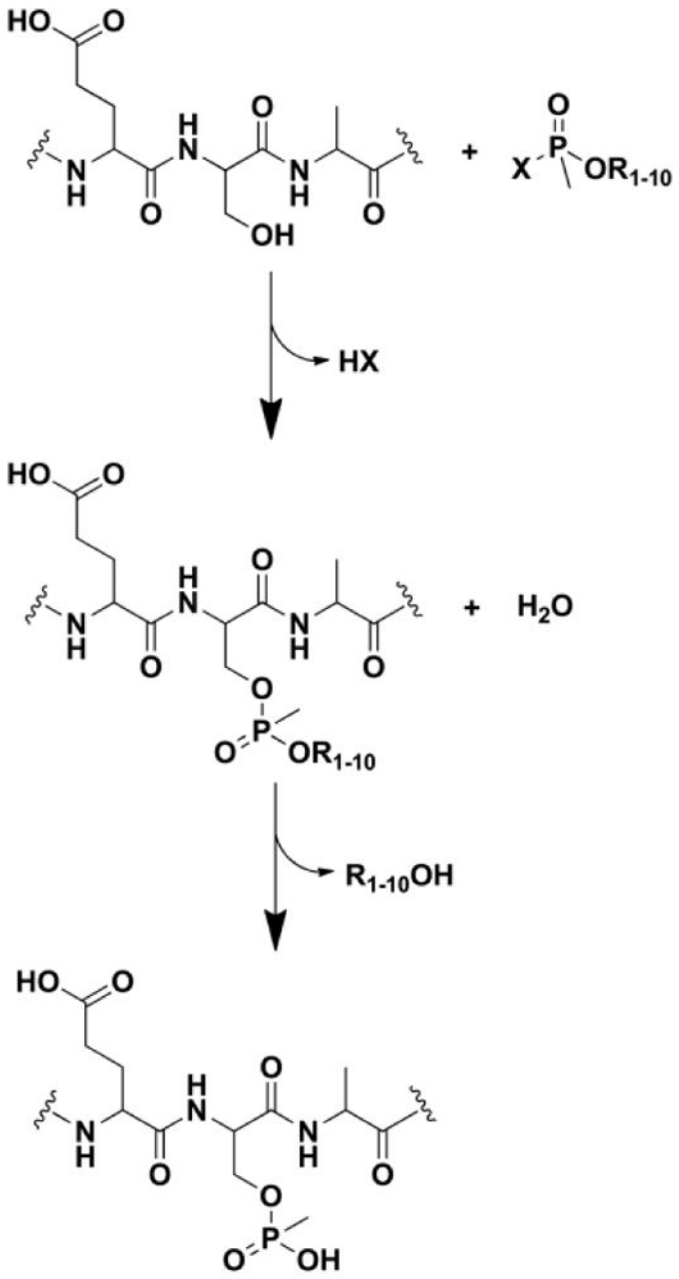


Figure 3.

Stability of MeP-P calibrator in 0.1% formic acid buffer at (-■-) 4 °C, (-▲-) 22 °C and (-●-) 37 °C. The peptides were analyzed following incubation at the desired temperature for (-◆-) 0, 4, 8 and 24 hours. The MeP-P* internal calibrator was added to each sample prior to analysis. The calculated MeP-P concentration and % peptide remaining are plotted (n=4); error bars represent ±SD.



Scheme 1.

Inhibition of human BChE and the hydrolysis of the OPNA adduct phosphoester bond at serine-198. This process is also known as aging of the OPNA adduct. OPNAs with the above chemical structure, such as sarin and VX, are known to age and produce a MeP adduct. *Key: X= F or* $SCH_2CH_2N(R_{1–3})_2$*;* $R_{1–3}$*= methyl, ethyl, n-propyl or iso-propyl; and* $R_{1–10}$*= 1–10 carbon chain.*

Table 1

Recovery of MeP-P from serum at low-, mid- and high level spiking concentrations added prior to pepsin digestion (Processed) or immediately before UHPLC-MS/MS analysis (Unprocessed).

	Processed (n=4)		Unprocessed (n=4)			
Calibrator .conc. (ng/mL)	**Mean area**	**SD**	**Mean area**	**SD**	**% Recovery[a]**	**(SD)[b]**
2.00	255	23	377	179	68	0.5
16.0	1867	183	2501	152	75	0.1
250.	30691	1544	44135	1691	70	0.1

[a] %Recovery = [(mean processed area/mean unprocessed area) × 100)]

[b] SD %Recovery = %Recovery × $[(SD_{un}/Mean_{un})^2+(SD_{proc}/Mean_{proc})^2]^{1/2}$.

$Mean_{(un\ or\ proc)}$ and $SD_{(un\ or\ proc)}$ are the means or standard deviations calculated for the peak areas of the un/processed serum.

Table 2

Intrarun and interrun accuracy and precision for MeP-P in human serum over two weeks.

Calibrator conc. (ng/mL)	Day	Calc. conc. (ng/mL)	% Error	Mean calc. conc. (ng/mL)	Intraday %RSD	[a] Mean % Error	[b] Interday %RSD
2.00	1	1.97	−1.50	2.07	6.83	3.50	7.13
		2.17	8.50				
	7	1.90	−5.00	2.08	11.9	3.75	
		2.25	12.5				
	14	2.16	8.00	2.22	3.51	10.8	
		2.27	13.5				
16.0	1	15.5	−3.13	15.4	1.38	−4.06	5.69
		15.2	−5.00				
	7	14.6	−8.75	14.0	6.06	−12.5	
		13.4	−16.3				
	14	15.6	−2.50	15.0	6.15	−6.56	
		14.3	−10.6				
250.	1	244	−2.40	250	3.39	0.00	1.91
		256	2.40				
	7	252	0.800	255	1.66	2.00	
		258	3.20				
	14	254	1.60	254	0.279	1.40	
		253	1.20				

[a]Defined as the [(mean calc, conc.-theoretical conc.)/theoretical conc.] × 100.

[b]Defined as the %RSD of the calculated concentrations on days 1, 7 and 14.