

Use of Hupresin To Capture Red Blood Cell Acetylcholinesterase for Detection of Soman Exposure

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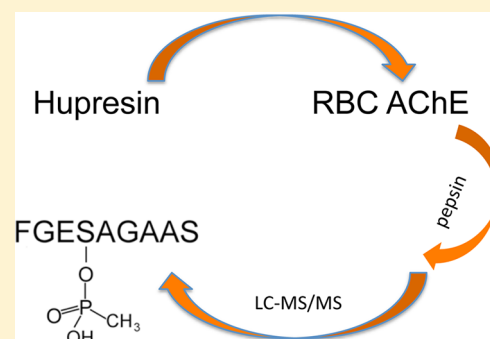
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ABSTRACT: Toxicity from acute exposure to nerve agents and organophosphorus toxicants is due to irreversible inhibition of acetylcholinesterase (AChE) in the nervous system. AChE in red blood cells is a surrogate for AChE in the nervous system. Previously we developed an immunopurification method to enrich red blood cell AChE (RBC AChE) as a biomarker of exposure. The goal of the present work was to provide an alternative RBC AChE enrichment strategy, by binding RBC AChE to Hupresin affinity gel. AChE was solubilized from frozen RBC by addition of 1% Triton X-100. Insoluble debris was removed by centrifugation. The red, but not viscous, RBC AChE solution was loaded on a Hupresin affinity column. Hemoglobin and other proteins were washed off with 3 M NaCl, while retaining AChE bound to Hupresin. Denatured AChE was eluted with 1% trifluoroacetic acid.

The same protocol was used for 20 mL of RBC AChE inhibited with a soman model compound. The acid denatured protein was digested with pepsin and analyzed by liquid chromatography tandem mass spectrometry on a 6600 Triple-TOF mass spectrometer. A targeted method identified the aged soman adduct on serine 203 in peptide FGESAGAAS. It was concluded that Hupresin can be used to enrich soman-inhibited AChE solubilized from 8 mL of frozen human erythrocytes, yielding a quantity sufficient for detecting soman exposure.



Chemical warfare nerve agents and organophosphorus pesticides are toxic to humans because they disrupt cholinergic nerve impulse transmission. The toxicants irreversibly inhibit acetylcholinesterase, resulting in accumulation of excess acetylcholine and loss of muscle function. Human acetylcholinesterase (AChE) in red blood cells (RBC) serves as a surrogate for AChE in the nervous system.¹ It should be possible to monitor toxicant exposure by measuring adducts on the active site serine of RBC AChE, using mass spectrometry assays similar to those for adducts on the active site serine of plasma butyrylcholinesterase.^{2–4} RBC AChE is not as easy to work with as plasma butyrylcholinesterase, because RBC AChE is membrane bound,⁵ and the concentration of RBC AChE is lower at 0.5 $\mu\text{g/mL}$ compared to plasma butyrylcholinesterase at 4–5 $\mu\text{g/mL}$.

We recently developed monoclonal antibodies that can be used to immunopurify RBC AChE after the RBC AChE has been solubilized by addition of 1% Triton X-100.⁶ The immunopurified RBC AChE was treated with a soman model compound and digested with pepsin. Liquid chromatography tandem mass spectrometry identified the FGESAGAAS peptide modified on the active site serine by aged soman. The method requires monoclonal antibodies and solid supports for immobilizing the antibodies.

The present report provides a simpler protocol for enriching RBC AChE to use for detecting nerve agent exposure. In place of antibodies, we used the commercially available affinity ligand Hupresin to capture RBC AChE. The AChE remained bound while contaminating proteins were washed off with 3 M NaCl and while the Hupresin was desalted by washing with water. Denatured, but not active AChE, was eluted with 1% trifluoroacetic acid. The denatured AChE was digested with pepsin and analyzed by liquid chromatography–tandem mass spectrometry. A targeted mass spectrometry method was used to detect the parent and daughter ions for the aged soman-labeled active site peptide of RBC-AChE.

MATERIALS AND METHODS

Hupresin was synthesized by Emilie David at the CHEMFORASE company, Mont-Saint-Aignan, France, emilie.david@chemforase.com. The soman model compound *O*-pinacolyl methylphosphonothiomethyl (soman *Sp*-thiomethyl) was synthesized in the laboratory of John Cashman⁷ and stored in

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dimethyl sulfoxide at $-80\text{ }^{\circ}\text{C}$. Packed red blood cells were a gift from the Nebraska Medical Center blood bank. Recombinant human AChE (rHuAChE) (P22303) was expressed in Chinese Hamster Ovary cells and purified by procainamide affinity chromatography.⁶ Porcine pepsin (from stomach mucosa) was from Sigma-Aldrich P-6887. YM-10 regenerated cellulose centrifugal filter 10000 NMWL, MRCPR010, was from Merck Millipore Ltd.

No-Ghost Method for Solubilizing RBC AChE. Frozen RBC (80 mL) were thawed and diluted with 120 mL of 1% Triton X-100 in PBS, 0.1% azide. This solubilized the membrane-bound AChE but left a small amount of insoluble debris. The debris was removed by centrifugation in microfuge tubes for 30 min at 14000 rpm (12000g). The solubilized no-ghost RBC AChE solution was red, but not viscous. No-ghost RBC AChE activity was 1.8 u/mL.

The classical method for preparing RBC AChE washes away hemoglobin before solubilizing the membrane-bound AChE with Triton X-100.^{8,9} The hemoglobin-free cells are called ghosts. To simplify the process, we did not prepare red cell ghosts and therefore named our preparation no-ghost RBC AChE.

Enzyme Activity Assays. AChE activity was measured in 0.1 M potassium phosphate pH 7.0 at $25\text{ }^{\circ}\text{C}$ with 1 mM acetylthiocholine iodide in the presence of 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) on a Gilford spectrophotometer interfaced to a MacLab data recorder (ADInstruments, Inc.). No-ghost RBC AChE solutions (5 μL) were preincubated with 1.98 mL of 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) in buffer for 10 min or more to deplete free sulfhydryl groups before the AChE reaction with acetylthiocholine was started by addition of 0.02 mL of 0.1 M acetylthiocholine. The increase in absorbance at 412 nm was converted to micromoles acetylthiocholine hydrolyzed using the extinction coefficient $13600\text{ M}^{-1}\text{ cm}^{-1}$.¹⁰ Units of activity are expressed as micromoles per min. AChE u/mL (pH 7) were converted to mg/mL using the conversion factor of 5000 u/mg.¹¹

Mass Spectrometry Standard for a Soman Model Compound-Inhibited Human AChE. rHuAChE in 0.5 mL of 20 mM TrisCl pH 7.5, 0.19 M NaCl had an activity of 40000 u/mL, which calculates to 8 $\mu\text{g}/\mu\text{L}$. After overnight incubation with 4 μL of soman *Sp* thiomethyl dissolved in dimethyl sulfoxide, AChE activity was reduced 99.9% to 84 u/mL. Aliquots of the soman-inhibited rHuAChE were digested with pepsin for 2 h at $37\text{ }^{\circ}\text{C}$ at a constant ratio of 40 μg rHuAChE and 80 μg pepsin in various volumes of 1% formic acid ranging from 800 μL to 25 μL . A 1 μL aliquot from each digest was examined by MALDI-TOF mass spectrometry with dihydroxybenzoic acid matrix in negative mode to identify the conditions that yielded a good signal for the aged soman adduct on the active site peptide FGESAGAAS. All digests ranging from 0.05 to 1.6 μg AChE per μL yielded the expected mass for the aged soman adduct. The best signal was for the highest AChE concentration. Digests were filtered through a YM-3 spin filter to remove intact pepsin and stored at $-20\text{ }^{\circ}\text{C}$ until used as standards in the 6600 Triple-TOF mass spectrometer. We have confidence that the quantity of rHuAChE digest applied to the mass spectrometer in Figure 4A was 8 μg because the rHuAChE sample was not subjected to the sample preparation steps used for RBC AChE and therefore had minimal losses.

Inhibition of RBC AChE with Soman *Sp*-Thiomethyl. No-ghost RBC AChE in 0.6% Triton X-100 was treated with soman *Sp*-thiomethyl. This soman model compound has a thiomethyl group in place of the fluoride ion in authentic

soman¹² and has the *Sp*-configuration. The soman model compound had been diluted into dimethyl sulfoxide so that a 4 μL aliquot added to 50 mL of RBC AChE reduced its AChE activity 93% from 1.8 to 0.12 u/mL after overnight incubation at $4\text{ }^{\circ}\text{C}$.

Hupresin for Partial Purification of No-Ghost RBC AChE. No-ghost RBC AChE was prepared by adding 12 mL of 1% Triton X-100 in PBS, 0.1% azide to 8 mL of human RBC. The RBC had been stored frozen at $-20\text{ }^{\circ}\text{C}$, but were thawed before use. Cell debris was removed by centrifugation. AChE activity measured with acetylthiocholine was 1.8 u/mL. The 20 mL solution was loaded on 2 mL of Hupresin packed in a Pharmacia C10/10 column. Hemoglobin and other proteins were washed off with 20 mL of 0.1 M potassium phosphate pH 7, 20 mL of 1 M NaCl in buffer, and 20 mL of 3 M NaCl. Hupresin was desalted with 20 mL of water before bound AChE was eluted with 1 mL aliquots of 1% trifluoroacetic acid. About 15% of the AChE activity was lost in the flow through during loading and washing.

The Hupresin column was cleaned with 20 mL of 0.1 M NaOH to remove a brown color from heme and equilibrated with 0.1 M potassium phosphate pH 7. We previously reported that the binding capacity of Hupresin was unchanged by sanitation with 0.1 M NaOH.¹³ The same 2 mL Hupresin column was used to partially purify soman-inhibited no-ghost RBC AChE using the protocol described above.

SDS Gel Electrophoresis and Digestion with Trypsin. The 1 mL fractions of RBC AChE in 1% trifluoroacetic acid were dried, dissolved in 50 μL of SDS gel loading buffer and heated in a boiling water bath for 3 min. A 20 μL aliquot of each fraction was run on a precast 4–20% gradient gel to identify the protein-containing fractions. To determine whether RBC AChE had eluted with 1% TFA, gel bands were excised, digested with trypsin, and analyzed by LC-MS/MS as described.¹³

Digestion with Pepsin. A 20 mL solution of no-ghost RBC AChE was inhibited with the soman model compound to 7% of its original activity, from 1.8 to 0.12 u/mL. During the 16 h treatment with the soman model compound, the adduct lost the pinacolyl group leaving only methylphosphonate on the active site serine. Soman-inhibited AChE in 20 mL was partially purified by affinity chromatography on 2 mL of Hupresin as described for control, uninhibited RBC AChE. The trifluoroacetic acid-extracts containing soman-inhibited RBC AChE were dried in a vacuum centrifuge. The dry pellet was brown. The pellet was dissolved in 50 μL of 0.1% formic acid. The brown solution was digested with 300 μL of a freshly prepared 2 mg/mL pepsin solution in 0.6% formic acid for 2 h at $37\text{ }^{\circ}\text{C}$. The brown material was removed by filtering the digest through a YM-10 spin filter. The colorless filtrate was dried, dissolved in 40 μL of 0.1% formic acid and centrifuged for 30 min at maximum speed in a microfuge (12000g). The top 10 μL were transferred to an autosampler vial. A 5 μL aliquot was injected into the liquid chromatography tandem mass spectrometer instrument. We had expected a yield of 0.7–7 μg of RBC AChE if losses during sample preparation were minimal. However, the yield appeared to be 0.007 μg based on signal intensity in the MSMS spectrum in Figure 4B compared to that for the control sample in Figure 4A.

The nine residue active site peptide FGESAGAAS is the result of cleavage at serine, a nonclassical cleavage site for pepsin. Unusually high quantities of pepsin are required to produce the nine-residue peptide, as pointed out by Fidler et al.¹⁴ and confirmed by others.^{2–4,6,15–17} Digestion with lower quantities of pepsin yields a mixture of active site peptides, containing 12 residues or more. The 9-residue peptide is ideal for mass

spectrometry analysis because the short peptide ionizes readily and yields a fragmentation spectrum that contains at least three characteristic fragment ions.

Liquid Chromatography Tandem Mass Spectrometry for Pepsin-Digested Protein. Data were acquired on a 6600 Triple-TOF mass spectrometer (AB Sciex) fitted with a nanospray source. Peptides were separated by ultra high pressure liquid chromatography (Eksigent, Dublin, CA) before they were introduced into the mass spectrometer. Two data acquisition schemes were employed. The first was a traditional data dependent acquisition scheme to which we added an organophosphorylation category to the Data Dictionary and Parameter Translation files to facilitate detection of organophosphate-modified peptides. This scheme was used for analyzing the rHuAChE control sample. Data were searched against the Uniprot/Swissprot Jan2015.fasta database with Protein Pilot v 5.0 software (AB Sciex). Details of this mass spectrometry method are reported in Dafferner et al.⁶

In the second scheme, a targeted Product Ion method designed for the aged soman adduct on peptide FGESAGAAS was used. The targeted method was used to increase the sensitivity of detection. Details of the method were based on the results from the rHuAChE control. Product Ion data acquisition was performed on the singly charged mass for the active site peptide labeled with aged soman in positive mode (FGESAGAAS + 78 Da + H⁺ = 874.35 Da, where 78 Da is the added mass for aged soman). A data acquisition cycle consisted of two steps. An MS survey scan was made over a mass range of 400–900 Da with an accumulation time of 1000 ms. This was followed by a Product Ion MSMS scan whenever the 874.35 Da parent ion appeared in the survey scan. Data were analyzed with Peak View v 2.1 (AB SCIEX). An Extracted Ion Chromatogram for the 874.35 Da mass was constructed from both the mass spectral and MSMS fragmentation data.

RESULT

Hupresin. The Hupresin affinity ligand is based on a derivative of the chemical huprine, which is a condensation product of tacrine and huperzine-A. Huprine derivatives were synthesized by French scientists who were searching for a new acetylcholinesterase (AChE) inhibitor to use for treating Alzheimer's disease.¹⁸ They synthesized dozens of derivatives and found one, Hup-19, with characteristics suggesting it might work as an affinity ligand. They cross-linked Hup-19 to Sepharose and purified recombinant human butyrylcholinesterase expressed from insect cells to 90% purity in a single step.¹⁹ Emilie David at the CHEMFORASE company, Mont-Saint-Aignan, has made the Hupresin affinity gel available for purchase in small and large quantities.

Binding and Elution of No-Ghost RBC AChE from Hupresin. A 2 mL column of Hupresin bound 94% of the AChE activity in 20 mL of no-ghost RBC AChE in 0.6% Triton X-100, PBS. Hemoglobin and other contaminating proteins easily passed through. Washing with 3 M NaCl eluted an additional 10% of the original AChE activity. No reagent was identified that released nondenatured, active AChE from Hupresin in good yield. This is not a drawback for projects that aim to identify adducts on the active site serine of AChE by mass spectrometry. The denatured AChE was released from Hupresin with 1% trifluoroacetic acid. Fractions that contained protein were identified by SDS gel electrophoresis. Figure 1 shows that all five fractions contained the same set of proteins. The most intense Coomassie blue stained bands are in lane 2, representing

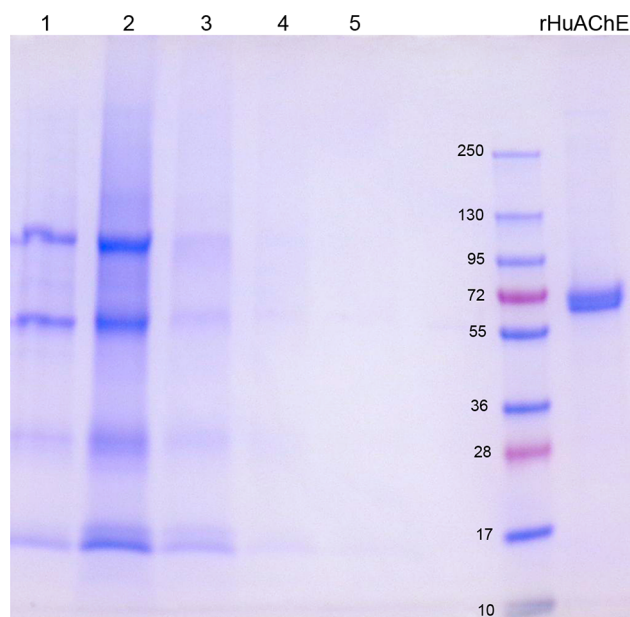


Figure 1. RBC AChE eluted off 2 mL Hupresin, visualized on an SDS gel stained with Coomassie blue. Lanes 1–5 show proteins in fractions 1–5. The fractions were dried, dissolved in 50 μ L of SDS/dithiothreitol loading buffer, and 20 μ L was loaded per lane. Truncated recombinant human AChE Q552stop MW 65 kDa is in the last lane.

the proteins in fraction 2. Bands at about 95 and 55 kDa were digested with trypsin and analyzed by LC-MS/MS.¹³ Human AChE (P22303) was the most abundant protein in both bands. Other proteins in these bands were plasminogen (P00747), retinal dehydrogenase (P00352), actin (P63261), spectrin (P02549) and hemoglobin beta (P68871). The control rHuAChE in Figure 1 has a molecular weight of 65 kDa. Reduced RBC AChE has a molecular weight of 75 kDa.¹¹ It was concluded that RBC AChE was recovered from Hupresin as a partially purified, denatured protein that comigrated with other proteins on an SDS gel.

Detection of Soman Model Compound-Labeled AChE. The structure of the soman model compound that inhibited no-ghost RBC AChE is shown in Figure 2. This model

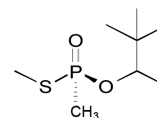


Figure 2. Soman model compound (*O*-pinacolyl methylphosphonothiomethyl) has a thiomethyl group in place of fluoride in authentic soman.

compound produced the same adduct on AChE as authentic soman^{7,12} and aged to yield an added mass of 78 Da. The structure of the aged soman adduct on the active site serine of human AChE is in Figure 3.

The fragmentation and elution properties of the soman-labeled AChE active site peptide (FGESAGAAS + 78 Da) were determined in preliminary experiments with rHuAChE. Recombinant human AChE was inhibited with soman *Sp*-thiomethyl and digested with pepsin. Peptic peptides were introduced into the Triple TOF 6600 mass spectrometer using a data dependent acquisition method and the results analyzed by comparison with the Uniprot/Swissprot database. The singly charged, active-site peptide containing the aged soman adduct with a mass of

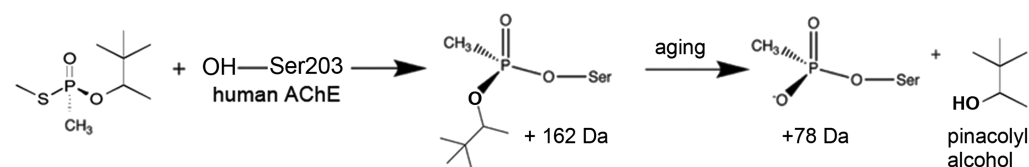


Figure 3. Soman model compound makes a covalent bond with the active site serine 203 of human AChE (P22303). The initial adduct has an added mass of +162 Da that rapidly ages to a stable adduct with an added mass of +78 Da and release of pinacolyl alcohol.

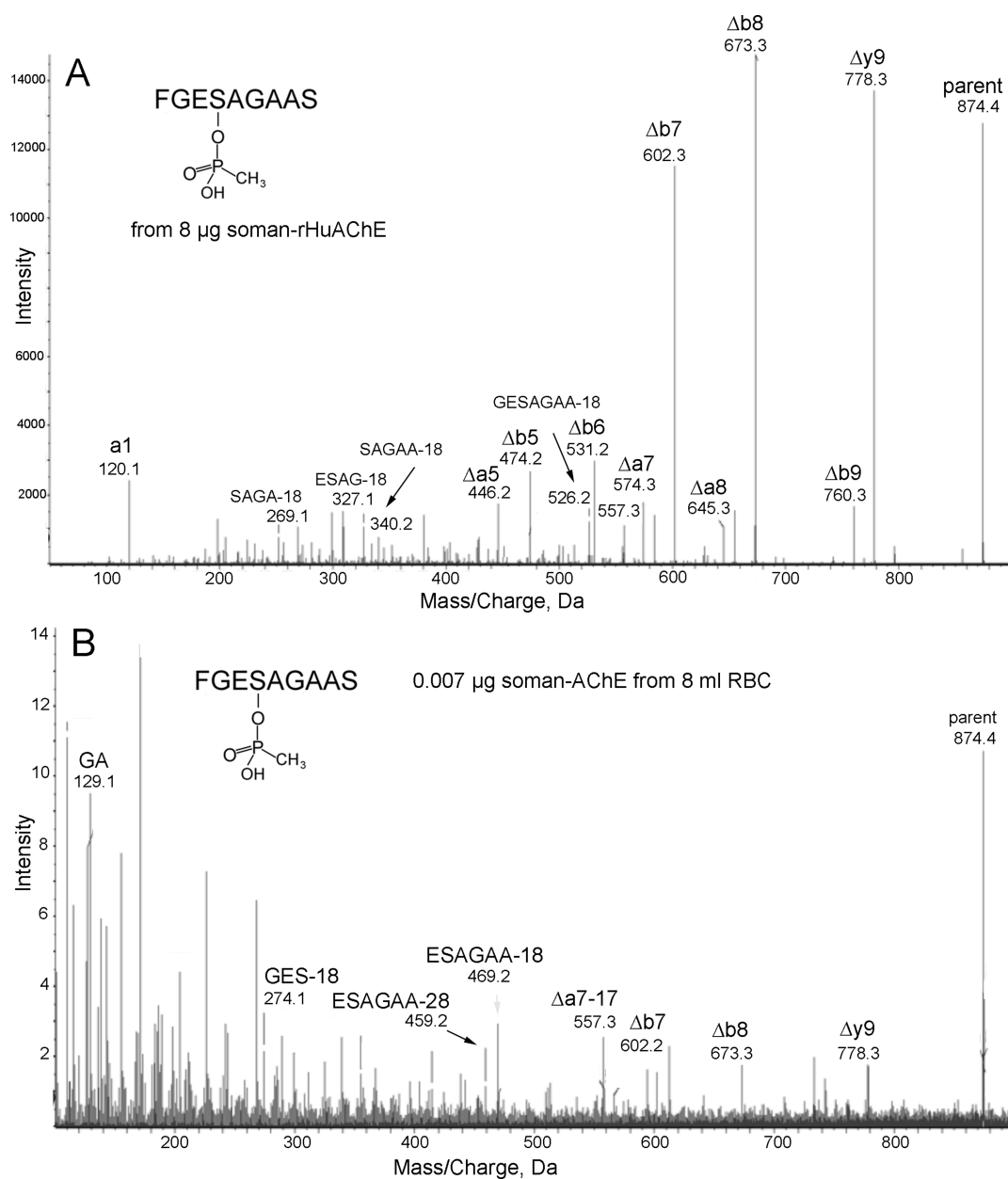


Figure 4. Fragmentation spectra of parent ions 874.4 from 8 μg of soman-inhibited, pepsin-digested rHuAChE in (A) and 0.007 μg from RBC AChE in (B). The Δ symbol indicates ions that have lost methylphosphonate and a molecule of water during collision-induced fragmentation. The masses of internal fragment ions are consistent with β -elimination of the aged soman adduct and a molecule of water.

874.4 Da eluted at 15.3 min. The fragmentation spectrum in Figure 4A confirms that the methylphosphonate adduct from aged soman is attached to Ser4; fragment ions at 778.36, 673.31, and 602.27 Da are the most intense.

Pepsin digestion of the soman-labeled no-ghost RBC AChE also yielded the nine residue active site peptide FGESAGAAS

modified on serine 4 with an added mass of +78 Da for the aged soman adduct. Targeted analysis in the 6600 Triple TOF mass spectrometer showed that the aged soman-labeled peptide eluted at 14.91 min, similar to the standard peptide. As shown in Figure 4B the parent ion had a mass of 874.4 Da and fragment ions at 778.3, 673.3, and 602.2 Da. The 874.4 Da mass is

consistent with the peptide sequence FGESAGAAS with an added mass of 78. The fragment ions Δy_9 (778.3), Δb_8 (673.3), and Δb_7 (602.2) have lost the soman adduct plus a molecule of water, thus converting the modified serine to dehydroalanine. It was concluded that soman exposure was detected in RBC AChE after the soman-inhibited no-ghost RBC AChE was partially purified on Hupresin.

DISCUSSION

Rationale for Analyzing RBC AChE for Nerve Agent Exposure. The physiologically important target for nerve agent toxicity is AChE in the nervous system. AChE in red blood cells does not function in nerve impulse transmission, but it has the same reactivity with nerve agents as AChE in the nervous system. When AChE in red blood cells is inhibited more than 50% by a single dose of nerve agent, humans show signs of toxicity.²⁰ Repeated exposure to sarin over a period of days can depress RBC AChE to near zero without symptoms ensuing.²¹ RBC AChE is a reliable surrogate for AChE in muscle.²²

Red blood cells persist in the circulation with a half-life of 33 days and a lifetime of 120 days.²³ This suggests that exposure to nerve agents or other organophosphorus toxicants may be detectable on RBC AChE in blood drawn weeks after the exposure incident.

Antihuman AChE monoclonal antibodies immobilized on Sepharose beads can be used to enrich nerve-agent inhibited RBC AChE.⁶ Hupresin is less expensive than antibodies, works as well as antibodies, and can be cleaned with 0.1 M NaOH and reused at least 7× without reducing binding capacity for no-ghost RBC AChE.¹³ This makes the commercially available Hupresin a cost-effective reagent for enriching RBC AChE to be used for detection of organophosphorus toxicant exposure including nerve agents and organophosphorus pesticides.

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Notes

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. The authors declare no competing financial interest.

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ABBREVIATIONS

AChE, acetylcholinesterase
FGESAGAAS, PheGlyGluSerAlaGlyAlaAlaSer active site peptide in AChE
MALDI-TOF, matrix assisted laser desorption ionization–time-of-flight mass spectrometry
MSMS, fragmentation spectrum
PBS, phosphate buffered saline
RBC, red blood cells
RBC AChE, red blood cell acetylcholinesterase
rHuAChE, recombinant human AChE
Soman *Sp*-thiomethyl, *O*-pinacolyl methylphosphonothio-methyl
TFA, trifluoroacetic acid
u/mL, units/mL

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