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Detection of cresyl phosphate-modified butyrylcholinesterase in human plasma for chemical exposure associated with aerotoxic syndrome

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Abstract

Aircrew complain of illness following a fume event in aircraft. A chemical in jet engine oil, the neurotoxicant, tri-o-cresyl phosphate, after metabolic activation to cresyl saligenin phosphate, makes a covalent adduct on butyrylcholinesterase (BChE). We developed a mass spectrometry method for detection of the cresyl phosphate adduct on human BChE, as an indicator of exposure. Monoclonal mAb2, whose amino acid sequence is provided, was crosslinked to cyanogen bromide-activated Sepharose 4B and used to immunopurify plasma BChE treated with cresyl saligenin phosphate. BChE was released with acetic acid, digested with pepsin, and analyzed by LC-MSMS on the 5600 Triple TOF mass spectrometer. Peptide FGES₁₉₈AGAAS with an added mass of 170 Da from cresyl phosphate on serine 198 was detected as parent ion 966.4 Da. When characteristic daughter ions were monitored in the MSMS spectrum the limit of detection was 0.1% cresyl saligenin phosphate inhibited plasma BChE. This corresponds to 2×10^{-9} g in 0.5 ml, or 23×10^{-15} moles of inhibited BChE in 0.5 ml plasma. In conclusion, a sensitive assay for exposure to tri-o-cresyl phosphate was developed. Laboratories that plan to use this method are cautioned that a positive result gives no proof that tri-o-cresyl phosphate is toxic at low levels.

Keywords

aerotoxic syndrome; mass spectrometry; butyrylcholinesterase; monoclonal antibody mAb2

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Introduction

Flight crews on commercial and military aircraft have complained of illness associated with exposure to chemicals in the cabin and cockpit air [1–6]. During a fume event, chemicals from jet engine oil and hydraulic fluid leak into the bleed air through faulty seals. Over an eighteen month period between January 2006 and June 2007 470 fume events were reported in the U.S. commercial fleet, or an average of 0.86 events per day [7]. A review of incident reports between 1998 and 2003 from the Australian Defense Force aircraft found that 0.08 to 2.5 fume events occurred per 1000 hours of flying [8]. In 1999 it was estimated that there were over 300 fume events world-wide [9]. Inflight neurotoxic symptoms include cognitive deficits, headache, eye, skin and upper airway irritation, muscle pain, and diarrhea [3, 4]. The illness associated with fume events has been named aerotoxic syndrome [2]. Exposure to chemicals is suspected to be the cause of aerotoxic syndrome, but this has not been proven. A laboratory test proving exposure is needed.

The chemicals in jet engine lubricating oil and hydraulic fluid include the organophosphorus esters tributyl phosphate, triphenyl phosphate, dibutylphenyl phosphate, diphenylbutylphosphate, isopropylphenyl-phenyl phosphate, di-isopropylphenyl phenyl phosphate, bis isopropylphenyl-diphenyl phosphate, and tricresyl phosphate [10, 11]. They are added to the oil to serve as anti-wear agents and flame retardants. Only one of these, tricresyl phosphate, is a known neurotoxicant. The ortho isomers of tricresyl phosphate cause degeneration of the peripheral nerves and spinal tract, progressing to paralysis of the extremities in man [12]. Tricresyl phosphate is a mixture of ten isomers. Tri-o-cresyl phosphate (TOCP) is a minor component in jet engine oil, constituting no more than 0.01% of the added tricresyl phosphate.

Schindler et al. developed a gas chromatography-mass spectrometry assay for the metabolites of organophosphorus esters in jet engine oil [13]. They analyzed urine from 332 pilots and cabin crew who reported exposure to fumes during their last flight. The 55 control urines were from unexposed persons from the general population. Compared to the control samples, the flight crew had significantly higher levels of dibutyl phosphate (a metabolite of tributyl phosphate and dibutylphenyl phosphate) and diphenyl phosphate (a metabolite of triphenyl phosphate, diphenylbutylphosphate, isopropylphenyl diphenyl phosphate, and bis isopropylphenyl diphenyl phosphate). However, they did not find the di-o-cresyl phosphate metabolite of TOCP. Only one sample contained metabolites of m- and p-tricresyl phosphates. Metabolite levels were very low, indicating a slight occupational exposure to organophosphorus chemicals. The study of metabolites in urine provided no evidence of exposure to TOCP. This finding can be re-interpreted to mean that all of the TOCP formed covalent adducts with protein targets and that a more definitive assay would analyze protein adducts.

In the present work we developed a method to measure exposure to TOCP by analyzing protein adducts. TOCP is metabolically converted to cresyl saligenin phosphate [14], as indicated in Figure 1. Cresyl saligenin phosphate (CBDP) is highly reactive with human butyrylcholinesterase (BChE), an enzyme in blood that captures cresyl saligenin phosphate and makes a permanent bond with it. The reaction rate of CBDP with BChE is among the

fastest known, similar to that with nerve agents [15]. Figure 2 shows that cresyl saligenin phosphate reacts with BChE to make a covalent bond on the active site serine 198. The adduct immediately ages to o-cresyl phosphoserine-BChE and releases saligenin. A second aging step yields phosphoserine-BChE and releases o-cresol. The aged BChE adducts indicated in Figure 2 have been observed by mass spectrometry as well as by crystal structure analysis of pure BChE treated with CBDP in vitro [15, 16].

In previous reports, we described mass spectrometry methods for detecting the aged phosphoserine adduct of BChE [17, 18]. Those methods required multi-step purification and enrichment of the target peptide starting from 25–50 ml of blood and employed a MALDI TOFTOF 4800 mass spectrometer to obtain a limit of detection of about 0.05% labeling on BChE (with a signal-to-noise of 5). In the present report we describe purification of BChE from 0.5 ml of human plasma in a single step, using a highly specific antibody, followed by a simple peptic digestion and mass spectral analysis by electrospray LC-MSMS mass spectrometry on a Triple TOF 5600 mass spectrometer. This method can detect the cresyl phosphate adduct on BChE with a limit of detection of 0.1% of the BChE (signal-to-noise of about 100). Even at the limit of detection both MS and MSMS spectra can be acquired to firmly establish the identity of the adduct. The Triple TOF 5600 offers both higher resolution and better mass accuracy than the MALDI TOFTOF 4800. In addition, use of electrospray mass spectrometry makes the present method more quantitatively reproducible than our previous method that used MALDI mass spectrometry, a process that is well known to be only semi-quantitative.

Materials

Outdated human plasma from the University of Nebraska Hospital Blood Bank was volunteer blood collected by the Red Cross. 2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide (CBDP, also called cresyl saligenin phosphate), the toxic metabolite of tri-ortho cresyl phosphate, was synthesized by the Canadian National Single Small Scale Facility (DRDC Suffield). CBDP was dissolved in acetonitrile and stored at –80°C. The following were from Sigma-Aldrich: pepsin from porcine stomach mucosa cat no. P-6887 and Protein G Sepharose cat no. P-3296. The following were from EMD Millipore Amicon (Billerica, MA): Amicon Ultra-15 centrifugal filter with an Ultracel-10K regenerated cellulose membrane, 10 kDa cutoff, cat no. UFC901024; Ultrafree-MC centrifugal filter unit with a 0.45 µm Durapore membrane, 0.45 µm cutoff, cat no. UFC3 OHV 00; Microcon-10 centrifugal filter Ultracel YM-10 regenerated cellulose 10,000 NMWL, MRCPT010. CNBr-activated Sepharose 4B was from Amersham Pharmacia Biotech AB, cat no. 17-0430-01. Mouse ascites fluid containing a monoclonal antibody to human BChE, mAb2, was developed in the laboratory of Jacques Grassi [19] and stored frozen for over 20 years. The monoclonal had been made against pure human BChE provided by Patrick Masson. Frozen hybridoma cells were from Stéphanie Simon. Pure human BChE used as a positive control in gels, was purified from human plasma by ion exchange and procainamide affinity chromatography [20]. The accession number for human BChE is P06276 in the Swiss Protein databank.

Purification of mAb2

Ascites fluid (6 mL) was clarified by centrifugation. Fat was removed by filtration through 0.45 μ m Durapore centrifugal filters. Protein G Sepharose (5 mL) packed in a Pharmacia C10/20 column was equilibrated with 20 mM sodium phosphate pH 7.4 at room temperature in preparation for loading the defatted, filtered ascites fluid. The flow-thru was saved for a second passage through Protein G. The column was washed with 150 mL of 20 mM sodium phosphate pH 7.4 until the eluant had an absorbance at 280 nm of 0.015. Antibody was eluted with four 3 mL aliquots of 0.1 M citric acid pH 2.6 into tubes preloaded with 1.2 mL of 1 M dibasic sodium phosphate, 0.5 M sodium hydroxide, thus adjusting the monoclonal to pH 7.3 as it came off the column. The procedure was repeated 9 times, the starting material for each cycle being the flow-thru from the previous application. Yield of monoclonal was calculated from absorbance at 280 nm, using 1.4 as the absorbance of a 1 mg/mL solution. The yield from the first cycle was 3.8 mg. The combined yield of mAb2 from 9 cycles was 17 mg from 6 mL of ascites fluid.

The monoclonal was intended for crosslinking to Sepharose via amine groups. Therefore the buffers for purification of mAb2 contained no Tris and no azide. The purified mAb2 was concentrated in a 10,000 MW cutoff ultracentrifugal filter to 5 mg/mL. The final buffer was 0.125 M sodium phosphate, 0.04 M citrate pH 7.3.

mAb2 crosslinked to Sepharose

Preservatives were removed from 1 g of CNBr-activated Sepharose by adding the dry beads to 50 mL of cold 1 mM HCl. The beads were washed with 0.2 M sodium phosphate pH 7.4 to raise the pH. Swollen beads (4 mL) were pelleted by centrifugation and the liquid discarded. Crosslinking was initiated by addition of 1 mL of 5 mg/mL mAb2 in 0.125 M sodium phosphate, 0.04 M citrate pH 7.3, for a total volume of 5 mL. The 50 mL tube was rotated at room temperature for 30 hours. Measurement of absorbance at 280 nm indicated that 99% of the monoclonal had bound to the beads. Beads were washed 2X with phosphate buffered saline (PBS, 20 mM sodium/potassium phosphate, 150 mM sodium chloride pH 7.4), 1X with 0.2 M NaCl, 20 mM TrisCl, 1 mM EDTA pH 7.5, 0.05% NaN₃, and 1X with Tris buffered saline (20 mM TrisCl, 150 mM sodium chloride pH 7.4) containing 0.2% Tween-20. The 4 mL of swollen mAb2-Sepharose beads were stored in a 10 mL suspension of PBS containing 0.05% azide at 4°C.

Single-step immunopurification of butyrylcholinesterase (BChE) from human plasma

For mass spectrometry analysis of adducts on BChE, a 0.5 mL aliquot of human plasma yielded enough BChE (1.9 μ g) to see as little as 0.1% adduct. However, in initial trials we tested the purification protocol with 5 mL of human plasma. A 1 mL suspension of mAb2-Sepharose was briefly centrifuged to allow removal of liquid before the 0.4 mL beads were added to 5 mL of human plasma. The mixture was rotated at room temperature overnight. Based on BChE activity remaining in the unbound liquid, it was estimated that 95–98% of the BChE had bound to the beads. The same high efficiency of BChE binding to mAb2-Sepharose was obtained when the protocol used 0.5 mL human plasma to bind to 0.04 mL beads.

BChE activity

Plasma BChE activity was measured at 25°C in 0.1 M potassium phosphate buffer pH 7.0 containing 0.5 mM 5,5'-dithiobis-nitrobenzoic acid and 1 mM butyrylthiocholine. The increase in absorbance at 412 nm due to the reaction of thiocholine with the 5,5'-dithiobis-nitrobenzoic acid was converted to μ moles of butyrylthiocholine hydrolyzed per min using the extinction coefficient $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ for the yellow, 2-thio-p-nitro-benzoic acid product [21].

Calculation of BChE protein

One unit of BChE activity is the amount that hydrolyzes 1 μ mole butyrylthiocholine in 1 minute in an assay that contains 1 mM butyrylthiocholine in pH 7.0 potassium phosphate buffer at 25°C. Units of BChE activity were converted to μ g BChE protein using the fact that 1 mg pure BChE has 720 units of activity. Thus, a human plasma sample with a BChE activity of 2.9 units per mL contains 2 μ g BChE in 0.5 mL (47 nM).

Release of BChE from mAb2-Sepharose

Beads containing BChE captured from plasma were washed 1X with PBS, 2X with Tris buffered saline containing 0.2% Tween-20, and 3X with water. BChE from 5 mL plasma was released from 0.4 mL beads with 0.4 mL of 0.4 M acetic acid pH 2.6. The acid treated beads were regenerated by addition of 1 M TrisCl pH 7.5. BChE from 0.5 mL plasma was released from 0.04 mL beads with 50 μ L acid after the beads were transferred to a 0.45 μ M Durapore ultracentrifugal filter. Centrifugation separated the beads from the BChE. The eluted BChE intended for mass spectrometry analysis was digested with pepsin. Alternatively, acid released BChE intended for SDS gel electrophoresis was dried in a vacuum centrifuge. BChE activity was detectable when the acid-released BChE was neutralized with an equal volume 1 M TrisCl pH 8.5.

Gel electrophoresis

Polyacrylamide 4–30% gradient gels were poured in a Hoefer gel apparatus. Gels were stained for protein with Coomassie blue, or for BChE activity by the method of Karnovsky and Roots [22]. Each lane of the BChE activity stained gel was loaded with 10 μ L containing 0.02 units of BChE activity.

Amino acid sequence of monoclonal mAb2

A vial of frozen hybridoma cells was sent to SydLabs, Inc. (Malden, MA) for PCR amplification of the mRNA of the light and heavy chains from mAb2. The nucleotide and deduced amino acid sequences were derived from the PCR products.

Standards for limit of detection of the cresyl phosphate adduct on BChE

Human plasma was titrated with CBDP until 98% of the BChE activity was inhibited. A 100-fold molar excess of CBDP over BChE was added to plasma to achieve 98% inhibition. The 100-fold molar excess was required because of the way the experiment was performed. CBDP was titrated by adding aliquots of plasma over the course of 8 hours until detectable BChE activity remained. During the 8 hour titration a significant amount of the CBDP was

likely to have hydrolyzed. To determine whether the CBDP-treated plasma was free of unreacted CBDP, an aliquot of the treated plasma was incubated with pure human BChE. Activity measurements showed no inhibition of pure BChE, thus demonstrating the absence of unreacted CBDP. The limit of detection samples were prepared by mixing CBDP-treated plasma with untreated plasma to make samples containing 0, 0.1, 0.5, 1.0, 2.0 and 50% CBDP-treated plasma.

The BChE from 0.5 mL of each sample was captured on mAb2-Sepharose (0.04 mL beads) and released with 50 μ L of 0.4 M acetic acid pH 2.6 to yield a maximum of 1.9 μ g of BChE. The acid-released BChE was digested at 37°C for 2 hours with 2 μ L of a fresh 1 μ g/ μ L pepsin solution prepared in 5% formic acid. The digests were centrifuged through Microcon Ultracel-10 filters with a 10,000 MW cutoff to clear the digest of particulates that could plug the liquid chromatography system, and to stop the digestion by separating pepsin from the peptides. The filter was washed prior to applying the sample by centrifuging 0.4 mL of water through the membrane. Centrifugation of the peptides through the membrane took 15 minutes at 14,000 $\times$ g. The membrane was washed with 50 μ L of 5% formic acid to release residual peptides. The 100 μ L filtrate was taken to dryness in a vacuum centrifuge, redissolved in 15 μ L of 0.1% formic acid, and centrifuged at 14,000 $\times$ g in a microcentrifuge for 5 min to pellet any particulate matter. The upper 10 microliters were transferred to autosampler tubes.

Mass spectral data acquisition on the Triple TOF 5600 mass spectrometer

For a detailed description of the liquid chromatography-mass spectral methods see Supplementary Data.

Analysis of the mass spectral data

Data were analyzed with the aid of PeakView v1.2.0.3 (AB Sciex). Quantitation of the MS data was initiated by creating an Extracted Ion Chromatogram (XIC) for the parent ion, using the “Extracted Ions Dialog” (see Figure 3 for an illustration using 2%-labeled FGESAGAAS cresyl phosphate). The MS total ion data (Figure 3A) were interrogated with the exact mass for the peptide, using a mass window width of 0.05 Da. The exact singly-charged masses were determined by combining the exact monoisotopic mass for FGESAGAAS, 796.3472 Da (calculated from the Protein Prospector algorithm MS Product, <http://prospector.ucsf.edu>) with the exact monoisotopic added masses for cresyl phosphate (170.0127 Da) or phosphate (79.9663 Da) (calculated with Protein Prospector algorithm MS Isotope). The exact doubly-charged masses were calculated from the exact singly-charged masses by adding the mass of a proton (1.00782 Da) and dividing by two. A peak corresponding to the elution of the targeted peptide was identified in the XIC (for example at 31.32 minutes for FGESAGAAS cresyl phosphate, Figure 3B). Fragmentation data were used to establish the identity of each peptide and to define its elution time. The extracted peak was selected using a 0.4 minute window (Figure 3C). For accurate quantitation it was critical to use the same size window when selecting data from each sample. An MS spectrum for the selected data from the extracted ion chromatogram was created by clicking the “Displays a Spectrum for the Selection” toolbar icon (Figure 3D). The monoisotopic peak from the appropriate isotopic family was selected using a 0.2 Da window (Figure 3E).

A second 0.2 Da background reference window was selected from a portion of the MS spectrum that contained no peaks. The background-corrected peak height for this mass (Subtracted Height) was determined with the aid of the “Graph Selection Info” algorithm (Signal/Noise option). The Subtracted Height is defined as the maximum intensity of the signal in the selected area minus the average intensity for the background selection. Subtracted Height is directly related to the quantity of the peptide in the sample. MS analyses were performed for FGESAGAAS cresyl phosphate, FGESAGAAS-phosphate and FGESAGAAS unlabeled.

Quantitation of the MSMS data employed a similar strategy. XICs were created by interrogating the MSMS data sets from both FGESAGAAS-phosphate and FGESAGAAS cresyl phosphate using the 778.3366 Da fragment ion, with a 0.05 Da mass window width. The same fragment ion could be used for both adducts because it represents loss of the adduct plus a water molecule from the parent ion in each case. A peak corresponding to the elution of the targeted peptide was selected using a 0.4 minute window and the complete MSMS spectrum displayed. The fragment ions at 673.2940 and 602.2569 Da were selected from the MSMS spectrum using a 0.1 Da window and used for background-corrected quantitation. Again, the same fragments could be used for both adducts because after loss of the adduct mass plus water the resulting peptide is the same in both cases. This type of targeted analysis on the fragment ions is tantamount to the more familiar multiple reaction monitoring analysis except that more fragment ion information is obtained.

Results

Immunopurification of BChE from human plasma

The average human plasma sample contains about 2 µg BChE in 0.5 mL plasma (47 nM). This corresponds to 23 pmoles of BChE (molecular weight 85 kDa). In this report we show that the yield of purified BChE from 0.5 ml of plasma is sufficient to detect as little as 0.1% cresyl phosphate-modified BChE. The BChE must, however, be purified from plasma. We developed a one-step purification protocol using monoclonal mAb2 [19] crosslinked to Sepharose. Overnight incubation of 0.04 ml of mAb2-Sepharose beads with 0.5 ml plasma selectively captured the BChE. The SDS gel in Figure 4 (lane 4) shows that proteins released from beads with acid included BChE monomer (85 kDa) and BChE dimer (170 kDa). The band at 85 kDa was confirmed to be BChE by mass spectrometry analysis of the trypsin-digested gel slice. The major contaminating proteins in the pepsin digested acid extract were immunoglobulins.

Activity assays showed that 95–98% of the BChE activity was gone from plasma, and that the missing BChE activity was bound to mAb2 beads. The residual BChE activity in plasma after incubation with mAb2-Sepharose is shown in Figure 5A lane 5. The low activity in lane 5 contrasts with the high BChE activity in the same plasma (lane 4) before incubation with mAb2 beads. BChE bound to mAb2 beads has catalytic activity as shown in Figure 5A lane 2. The acid extracted, neutralized BChE consists predominantly of tetramers, as shown in Figure 5A lane 3. The gel was counterstained with Coomassie blue in Figure 5B to demonstrate that the protein loading for the plasma samples in lanes 4 and 5 was similar,

thus confirming that the low BChE activity in Figure 5A lane 5 is explained by selective extraction of BChE from plasma.

The yield of active BChE released from mAb2 beads with acetic acid was low, even when the pH of the BChE was immediately adjusted to pH 7. This is consistent with the poor stability of BChE activity at low pH. For mass spectrometry analysis of pepsin-digested BChE it was unnecessary to have active BChE enzyme. A tight-binding immunoprecipitation protocol was ideal.

Amino acid sequence of mAb2

The amino acid sequences of the heavy and light chains of mAb2 were deduced from the mRNA sequences. Figure 6 confirms that the monoclonal is an IgG1 bearing a kappa light chain [19]. This is the first report of the amino acid sequence of a monoclonal to human BChE. The nucleotide and amino acid sequences for the heavy and light chains are deposited under GenBank accession numbers BankIt1690185 Seq1 KJ141199 and BankIt1690188 Seq2 KJ141200.

LC-MSMS analysis of the cresyl phosphate adduct on BChE

The 5600 Triple TOF mass spectrometer has high mass accuracy, improved sensitivity and improved resolution, capabilities not available in previous mass spectrometers we have used for analyzing BChE adducts. Using a targeted approach on the Triple TOF, we selected four parent ion masses for fragmentation. MSMS data were collected for the singly-charged FGESAGAAS phosphate adduct at 876.3135 Da, and the singly-charged FGESAGAAS cresyl phosphate adduct at 966.3599 Da, as well as for their doubly-charged counterparts. Fragmentation of only four parent ions allowed more data acquisition time than normal to be devoted to the MS scan (300 ms) and to each MSMS scan (150 ms) thereby increasing the sensitivity for detection of the ions of interest. Enrichment of the modified BChE peptides was not required. Using this strategy it was possible to detect the cresylphosphate adduct on 0.1% of the total human BChE in 0.5 mL plasma, which represents very low dose TOCP exposure.

Samples for mass spectrometry analysis were prepared from immunopurified BChE digested with pepsin. Peptides in the filtered digest were separated by liquid chromatography before mass spectral data acquisition. Background-corrected peak height data (Subtracted Height) were obtained for parent ions FGESAGAAS, FGESAGAAS cresylphosphate, and FGESAGAAS-phosphate. Signals were observed for singly-charged ions but not for doubly-charged ions.

Subtracted Height MS values for each of the observed peptides are given in Supplementary Table I along with the elution times. The quantity of unlabeled FGESAGAAS was essentially the same in each of the five samples listed. The average Subtracted Height for unlabeled FGESAGAAS was 3636 ± 1180 (i.e. $\pm 32\%$). The peak position was 796.3529 ± 0.0025 Da which is only 0.0057 Da larger than the exact, theoretical mass of unmodified FGESAGAAS.

For FGESAGAAS phosphate, only the 50% labeled sample gave reasonable MS spectra. The observed peak position was 876.3289 Da which was only 0.0151 Da larger than the exact, theoretical mass of 876.3135 Da. At 2% labeling the observed peak position was already 0.1216 Da too large, making it unreliable.

MS data for FGESAGAAS cresyl phosphate yielded reasonable values for Subtracted Height down to 1% labeling with an average peak position of 966.3797 ± 0.0018 Da. However, the peak position at 0.5% labeling was 0.0866 Da too large making it unreliable. Figure S1, in Supplementary Data, illustrates the difference between extracted ion chromatograms for unlabeled FGESAGAAS with 2%-labeled FGESAGAAS cresyl phosphate. In conclusion the sensitivity from the MS data for FGESAGAAS cresyl phosphate was reliable to only 1% labeling.

Similar analyses were performed on the MSMS data. An extracted ion chromatogram was created using the MSMS data and the appropriate parent ion mass. The peak corresponding to the elution of the parent ion was then interrogated to reveal the corresponding fragment ions. Subtracted Heights were determined for the extracted ion chromatography peak and for the associated fragment ions. Subtracted Height values for all levels of CBDP labeling are reported in Supplementary Table II. Reliable signals were obtained down to 0.1% labeling for fragments from FGESAGAAS cresyl phosphate.

0.1% is the lowest level of labeling tested, making it the limit of detection for this study. Figure 7A shows the full range extracted ion chromatogram for the 966.3599 Da parent ion taken from the 0.1% labeled sample. The peak at 31.30 minutes corresponds to the expected elution time for cresyl phosphate FGESAGAAS. This peak is well resolved from the noise (Figure 7B). Analysis yielded a Subtracted Height of 243 and a signal-to-noise of 13. Figure 7C shows a full range MSMS spectrum of the peptide eluting between 31.0 and 31.70 minutes. Three characteristic fragments from cresyl phosphate FGESAGAAS are present in this spectrum: 602.26, 673.29 and 778.34 Da. Refer to the annotated MSMS spectrum of cresyl phosphate FGESAGAAS in Figure 10 for the expected fragment masses. These fragments are more clearly evident in the expanded MSMS spectrum shown in Figure 7D. Analysis yielded Subtracted Height and signal-to-noise values for the 602.26 Da mass of 5.5 and 70; for the 673.29 Da mass of 9.4 and 124, and for the 778.34 Da mass of 5.3 and 73.

Plasma used in these studies contained 3.8 μ g BChE/ml. At 0.1% labeling, the concentration of labeled BChE is 3.8 ng/ml or 46 fmoles per ml. The upper limit for the amount of cresyl phosphate FGESAGAAS peptide in a 0.1% labeled plasma preparation is therefore 44 pg/ml. These values do not take into account losses due to sample preparation, to missed cleavages during peptic digestion, and to formation of the aged phospho FGESAGAAS adduct.

To test for background signals that might confound the proposal that 0.1% labeling represents a reasonable low limit of detection unlabeled BChE was examined. MSMS data for the unlabeled BChE sample were extracted for the 966.3599 Da parent ion of cresyl phosphate FGESAGAAS. Though no peak was detected in the extracted ion chromatogram, in the time frame expected for elution of cresyl phosphate FGESAGAAS (31.0–31.7

minutes, see Figure 1E in the Supplemental Data), fragments eluting in this region were examined. No signals were detected at 602.26, 673.29 or 778.4 Da (see Figure 8). This is especially clear in the expanded spectrum (Figure 8B). Unlabeled BChE shows no trace of a signal at the positions characteristic for cresyl phosphate FGESAGAAS. Thus the proposition that 0.1% cresyl phosphate-labeled BChE can be detected is confirmed.

The fragments that are present in Figure 8 tentatively correspond to the sequence (GP)SFL/I(AP)FL/I that is associated with pepsin. This peptide is from the tail of a peak that elutes maximally at 30.60 minutes.

A standard curve of Subtracted Height for the 673.3 Da mass versus the percent labeling of BChE by CDBP is shown in Figure 9. There is a linear correlation between 0.1 and 2 percent labeling with an R^2 coefficient of 0.9627. Inclusion of the 50% labeled point improves the R^2 value to 0.9999.

The mass at 966.38 Da was identified as the cresyl phosphate modified FGES₁₉₈AGAAS peptide by the fragmentation spectrum in Figure 10 which was taken from the 50% labeled sample. The parent ion includes the mass of cresyl phosphate (170 Da). The 188 Da interval between 778.35 and 966.38 Da is consistent with the loss of cresyl phosphate (170 Da) and water (18 Da) from the parent ion. The 69 Da interval between b3 and b4 indicates that the b4 ion has dehydroalanine in place of serine. The ion at 778.35 Da and the b4-b8 ions have all lost cresyl phosphate plus a molecule of water. The cresyl phosphate modified residue is identified as serine 198 by the fact that the b4-b8 ions have lost water (18 Da) whereas b2 and b3 have not. Loss of water occurs in the beta-elimination reaction that converts cresyl phospho serine to dehydroalanine. Conversion of the organophosphate-labeled serine to dehydroalanine is typical behavior for mass spectral fragmentation of organophosphorylated serine. For FGESAGAAS phosphate, reliable signals from the MSMS data were obtained down to only 1% labeling. Signal-to-noise values at 1% were 80-fold above background. At 0.5% and below, the Subtracted Height values were equivalent to those seen in the 0% labeled sample.

Discussion

Improved method for analysis CDBP-labeled human BChE

Previously we employed an analysis scheme for CDBP-labeled BChE that required 25 ml of plasma; a 2-step, partial purification of the BChE from plasma involving column chromatograph both on Q-Sepharose and on Procainamide Sepharose; concentration of the relatively large volume of purified protein with an Amicon stirred cell; peptic digestion; enrichment of the FGESAGAAS phospho peptide with titanium oxide; and mass spectral analysis on the MALDI TOF 4800 mass spectrometer (Liyasova et al., 2011). With this method we were able to detect as little as 0.05% labeled BChE, with a signal-to-noise ratio of about five. However, the elaborate procedure provided many opportunities for loss of sample or introduction of contamination. The Amicon stirred cell was a particular worry with regard to introduction of trace contamination because thorough cleaning of this device is difficult. Furthermore, the MALDI-TOF mass spectrometer is notorious for difficulties in reliably obtaining signals from samples present in low concentration. In the current report,

we describe a simpler and cleaner method for detection of CBDP-labeled BChE that requires only 0.5 ml of plasma. It involves a single-step immunopurification of the BChE to near homogeneity; use of small-volume, disposable filters; peptic digestion; omission of the peptide enrichment step due to the high level of initial purification; and mass spectral analysis on the Triple TOF 5600 mass spectrometer using electrospray ionization. With this method we are able to detect as little as 0.1% labeled BChE with a signal-to-noise ratio of about 100. The simplified procedure improves confidence in the results, especially for samples that have low levels of labeling.

Exposure to TOCP is not ruled out

Schindler et al. reported a failure to detect metabolites of TOCP in urine from 332 pilots (Schindler et al., 2013). The TCP metabolites measured by Schindler et al. were di-o-cresyl phosphate, di-m-cresyl phosphate, and di-p-cresyl phosphate [13]. These were selected based on metabolite studies in cats and rats [23, 24]. However, the mechanism described in Figure 2 shows that none of these metabolites are produced in the reaction of CBDP with BChE, where the expected metabolites are saligenin and o-cresol. Nomeir and Abou-Donia [23] found that the major TOCP metabolite in the urine of cats was o-cresol, a compound that was not investigated by Schindler et al. Therefore, the work of Schindler et al. does not rule out the possibility that a fume event exposes personnel to low levels of TOCP, that could be detected as adducts on BChE.

Cresyl phosphate adduct of BChE

Previously we focused on the phosphate adduct of BChE [17, 18] because we had a method to enrich the phosphorylated BChE peptide on titanium oxide. In the present report, peptide enrichment was not required. It was still important to purify BChE from plasma, but it was not necessary to enrich the adducted peptide. The 5600 Triple-TOF mass spectrometer performed better than the 4800 MALDI-TOF mass spectrometer we had used in the past. In positive mode the cresyl phosphate peptide ionized with a higher intensity than the phosphorylated peptide. Therefore, we focused on the cresyl phosphate adduct of BChE. As little as 0.02 pmoles of cresyl phosphate-modified FGESAGAAS peptide could be detected on the 5600 Triple TOF mass spectrometer with confidence. For comparison, a 0.5 ml sample of human plasma contains 23 pmoles of BChE.

Mechanism of toxicity in aerotoxic syndrome is unknown

This section addresses the important issue of cause-and-effect. That some people get sick on jet aircraft after exposure to fumes from the engines is well documented. The specific compound in the fumes that is responsible for making those people sick and the mechanism by which they become sick is still unclear. We have focused on detection of exposure to TOCP because it is currently the most popular candidate for the cause of this problem. Our assay provides a convenient and sensitive test for exposure to TOCP. However it does not prove that TOCP is the cause of the illness. Moreover, a negative test for TOCP from a sick passenger does not exclude the possibility that other compounds in the fumes caused their symptoms. The doses of TOCP that have been found to cause paralysis are high [25] compared to the low doses that might possibly be present in the cabin air of aircraft. Air sampling has detected tricresyl phosphate [26–28], but not TOCP or the more toxic mono-o-

cresyl phosphate [29] in cabin air. If future studies associate cresyl phosphate adducts on BChE with aerotoxic syndrome, the result will only prove exposure, but will not explain illness. Inhibition of BChE does not cause illness. Adduct levels on BChE are expected to be low, in the range of 3% or less [18], suggesting that toxicity may be due to chemicals other than TOCP. It has been argued that other chemicals in jet oil may contribute to toxic symptoms [2, 30]. Proof of exposure to other organophosphorus chemicals in jet engine oil is already provided by the GC-MSMS results of Schindler et al. who found elevated levels of dibutyl phosphate and diphenyl phosphate in the urine of aircrew and aircraft maintenance technicians [13, 31]. Abou-Donia reported increased levels of autoantibodies against nervous system proteins in flight crew [32]. In conclusion, a positive result for the cresyl phosphate adduct on BChE in persons exposed to fumes in the cabin air, would confirm that the fume event occurred, and that some of the chemicals from the jet engines were absorbed. However, a positive result for the cresyl phosphate adduct on BChE will provide no proof that toxicity is caused by low dose exposure to TOCP.

Immunopurification of BChE from plasma in a single step

Immunopurification of BChE from human plasma for the purpose of analyzing adducts on BChE has been reported previously. Sporty et al. and Carter et al. used the commercially available monoclonal 3E8 coupled to Dynabeads Protein G to immunopurify BChE from 0.5 ml and 0.075ml of human plasma, respectively [33, 34]. Marsillach et al. coupled monoclonal 3E8 to Dynabeads epoxy to purify BChE from 0.2 ml of plasma [35]. Monoclonal 3E8 (also called HAH 002-01 and ab17246), was created in the laboratory of Norgaard-Pedersen [36] and is presently sold by Thermo Scientific, catalog # HAH0020102. The high cost of monoclonal 3E8 and the absence of information regarding the 3E8 amino acid sequence make it prohibitive to crosslink 5 mg/ml to Sepharose.

Though mAb2 is not commercially available, the amino acid and nucleotide sequences provided in the present report make it possible to make a stable CHO cell line that secretes the monoclonal. In our hands, mAb2 extracted the same percentage (65%) of BChE from 0.5 ml of plasma compared to 3E8 when these antibodies were bound to Dynabeads Protein G according to the Sporty protocol [33].

Not accepting samples for analysis

We have provided this improved method for analyzing exposure to TOCP with the expectation that the information will be useful to laboratories interested in testing human blood samples. However, the University of Nebraska Medical Center will no longer accept samples from individuals interested in having their blood tested for exposure to TOCP because we have not been able to obtain permission from the University's Institutional Review Board for this test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BChE	butyrylcholinesterase
XIC	extracted ion chromatogram
MSMS	tandem mass spectrometry
MS	mass spectrometry
MALDI-TOF	matrix assisted laser desorption ionization time of flight
LC-MSMS	liquid chromatography-tandem mass spectrometry
TOCP	tri-ortho cresyl phosphate
CBDP	cresyl saligenin phosphate alternatively 2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide
PBS	phosphate buffer saline 20 mM sodium/potassium p 50 mM sodium chlor 7.4

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Figure 1.
Metabolic conversion of TOCP to CDBP. Cytochrome P450 enzymes metabolically activate tri-ortho-cresyl phosphate to the toxic CDBP.

Figure 2.

Reaction of CBDP with BChE to make two types of aged adducts. BChE makes a covalent bond with CBDP on serine 198. The ring-opened adduct has not been observed. However both types of aging products have been identified by mass spectrometry and x-ray crystallography. The o-cresyl phospho adduct adds 170 Da to the mass of the active site serine 198, while the phosphate adduct adds 80 Da.

Figure 3.

Quantitative analysis of the cresyl phosphate-modified FGESAGAAS peptide of human BChE. A) Total ion chromatogram for a pepsin digest of 2% CDBP-labeled BChE immunopurified from human plasma by binding to mAb2 beads. B) Extracted ion chromatogram for 966.3599 Da, i.e. the cresyl phosphate labeled FGESAGAAS peptide. The FGESAGAAS cresyl phosphate peptide elutes at 31.32 minutes. Identification of the mass eluting at 31.32 minutes as FGESAGAAS cresyl phosphate was made by analysis of the fragments from the corresponding MSMS spectrum. The mass eluting at 15.36 minutes in panel B was evaluated by MSMS and found not to be FGESAGAAS cresyl phosphate. C) An expansion of the XIC with the shaded box indicating the selected time range used for quantitation (31.1–31.5 minutes). D) MS spectrum for peptides eluting between 31.1–31.5 minutes. The minor signal marked 968.5188 indicates the region of the spectrum containing the FGESAGAAS cresyl phosphate peptide. E) Expanded MS spectrum showing the peaks at 966.3774 and 967.3858 Da that are consistent with the cresyl phosphate labeled FGESAGAAS peptide. The shaded box at 966.329–966.429 indicates the area of the spectrum used for quantitation. The shaded box at 966.8–966.9 Da indicates the region used for background correction.

Figure 4.

SDS gel of BChE released from mAb2-Sepharose with 0.4 M acetic acid pH 2.6. Control mAb2 alone in lanes 1, 2, 3. BChE was extracted from 0.5 ml human plasma by binding to 0.04 ml mAb2-Sepharose, and released with acetic acid (lane 4). Pure BChE controls in lanes 5, 6, 7, 8. Albumin in lane 9. MW markers in lane 10.

Figure 5.

Nondenaturing gradient polyacrylamide gel stained for BChE activity and counterstained with Coomassie blue shows that plasma is almost completely depleted of BChE after incubation with mAb2-Sepharose and that the BChE bound to mAb2 beads is catalytically active. Lane 1, monoclonal mAb2; lane 2, plasma BChE bound to mAb2-Sepharose beads; lane 3, 0.02 units of acid extracted, neutralized BChE released from mAb2-Sepharose; lane 4, 0.02 units of control plasma; lane 5, the same plasma showing residual BChE after incubation with mAb2-Sepharose; lane 6, 0.02 units of pure BChE control; lane 7, human albumin.

IgG1 heavy chain of mAb2

QVQLQQSGAE LVRPGSSVKI SCKASGYAFS SYWMNWVKQR PGQGLEWIGQ
 IYPGDSDTNY NGKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCARDY
 GKDHFDYWGQ GTTLTVSSak ttpsvvypla pgsaaqtnsm vtlgclvkgy
 fpepvtvtwn sgsllsgvht fpavlqsdly tlsssvtvps sprpsetvtc
 nvahpasstk vdkkivprdc gckpcictvp evssvfifpp kpkdvltitl
 tpkvtcvvvd iskddpevqf swfvddvevh taqtqpreeq fnstfrsvse
 lpimhqdwln gkefkcrvns aafpapiekt isktkgrpka pqvytipppk
 eqmakdkvsl tcmitdffpe ditvewqwnq qpaenykntq pimntngsyf
 vysklmvqks nweagntftc svlheglhnh htekslshsp gk

Kappa light chain of mAb2

DIVMTQSQKF MSTSVGDRVS VTCKASQSVG TNVAWFQQTP GQSLKVLIYS
 ASNRYSGVPD RFTGSGSGTD FTLTISNVQS EDLAEYFCQQ FNSYPYTFGG
 GTKLAIKRad aaptvsifpp sseqltsgga svvcflnnfy pkdinvkwki
 dgserqngvl nswtdqdsd stysmsstlt ltkdeyerhn sytceathkt
 stspivksfn r nec

Figure 6.

Amino acid sequences of the heavy and light chains of mouse monoclonal mAb2. The variable regions are in upper case letters. The constant regions are in lower case letters. The heavy chain contains 442 amino acids. The light chain contains 214 amino acids.

Figure 7.

Illustration of the signal-to-noise in the MSMS data from the 0.1%-labeled cresyl phosphate FGES₁₉₈AGAAS peptide. Panel A is a full range extracted ion chromatogram for the parent ion at 966.3599 Da. Panel B is an expanded view of Panel A. Panel C shows the MSMS fragments from the parent ion eluting in the 31.30 minute peak. Panel D is an expanded view of Panel C.

Figure 8.

Negative control for the presence of cresyl phosphate FGESAGAAS. MSMS data for the unlabeled BChE sample were extracted for the 966.3599 Da parent. Panel A, shows MSMS fragments for the peptide eluting in the 31.0 to 31.7 minute window. Panel B is an expanded view of Panel A. No ions characteristic of cresyl phosphate FGESAGAAS are present.

Figure 9.

Limit of detection. The Subtracted Height value of the MSMS signal for the 673.3 Da fragment ion versus the % CBDP inhibited plasma BChE. The subtracted height was generated by the “Graph Selection Info” algorithm in the “Peak View” software (AB Sciex, Framingham, MA) and is defined as the maximum peak height in a selected area minus the average background noise. The line is a linear fit for the data out to 2% inhibition, with the y-axis intercept set to zero ($y = 73.099x$; $R^2 = 0.9627$). If the 50% inhibited point is included the fit improves to $R^2 = 0.9999$. The limit of detection is taken to be 0.1% CBDP-inhibited plasma BChE recovered from 0.5 ml plasma.

Figure 10.

MS/MS fragmentation spectra confirming the identity of the 966.4 Da parent ion as the cresyl phosphate modified FGES₁₉₈AGAAS peptide. The MS/MS spectrum is taken from 50% labeled BChE. A complete bion series is present.