Verification of Exposure to Organophosphates: Detection of an Unknown Cholinesterase Inhibitor

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ABSTRACT

Verification of exposure to nerve agents is relevant because the health risks of even low level exposures have not yet been fully explored. Within this context it is important to verify non-exposure in order to re-assure worried military personnel or citizens. Phosphylated butyrylcholinesterase is one of the most important biomarker to verify an exposure to nerve agents. Nerve agent adducts can be analyzed with LC-MS/MS by detection of a phosphylated nonapeptide that is derived after digestion of butyrylcholinesterase with pepsin. For a sensitive analysis (low degree of BuChE inhibition) the identity of the cholinesterase inhibitor has to be known in order to operate the LC-MS/MS instrument in the most sensitive SRM mode. In practice the identity of the cholinesterase inhibitor will not be known beforehand and the number of possible organophosphates is larger than thousand. However, the number of possible molecular masses of relevant organophosphates is approximately 170. By smart selection of these masses the number of MRM transitions that have to be acquired can even be reduced to 34 transitions, when all nerve agents from the OPCW schedule 1 list need to be screened.

Furthermore, a generic method will be presented that is based on the conversion of the phosphyl moiety conjugated to BuChE. After digestion of BuChE the phosphyl group is eliminated from the peptide under mild alkaline conditions. Subsequently, the formed dehydroalanine residue reacts with a specific nucleophile which results in the formation of a generic adduct that can be measured with LC-MS/MS using the most sensitive SRM mode. With the presented methodology a larger spectrum of BuChE inhibitors can be detected without paying too much compromise to the sensitivity.

1.0 INTRODUCTION

Recently we have developed several methods for the verification of exposure to nerve agents [1-3]. Besides the hydrolysis products of nerve agents, the most important biomarker is the adduct of the nerve agent to butyrylcholinesterase. One method utilizes the fluoride reactivation technique in which reactivatible phosphyl groups are released from the enzyme upon incubation with fluoride ions [4]. The other method focuses on the enzyme itself: after isolation from plasma and digestion with pepsin a phosphylated nonapeptide is analyzed with LC-MS/MS [5]. The mass of the phosphylated peptide is a reference to the identity of the nerve agent. In view of the low concentrations of the biomarkers, analyses are often acquired in the SIM (Single Ion Monitoring) or SRM (Single Reaction Monitoring) mode to adjust the mass spectrometer in the most sensitive mode. It means that the identity of the nerve agent must be known in advance in order to adjust the mass spectrometer for the adequate acquisition mass. In practice, the identity of the ChE inhibitor will not be known always, which means that the mass spectrometer must be operated in a scan mode to screen for possible ChE inhibitors. Three common

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daughter ions of the nonapeptide are 602, 673 and 778, which can serve as fixed product ions to perform a parent ion scan. It will be studied whether the identity of the ChE inhibitor can be noticed after a fixed product ion scan. Secondly, the consequences on the sensitivity of the assay will be studied as well.

An alternative approach is to convert the nerve agent adduct into a common adduct which can be measured with LC-MS/MS in the SRM mode. The identity of the nerve agent can not be deduced anymore, but the LC-MS analysis can be performed in the most sensitive (SRM) mode. The method is truly generic because all phosphylation groups that bind to the active site of BuChE will be detected. The conversion will be performed according to a method that is inspired from the detection of phosphylation sites as used in proteomic studies [6-8]. The phosphyl group will be eliminated by mild alkaline hydrolysis, which results in the formation of a dehydroalanine group. Next, the double bond reacts with a specific nucleophile that is added to the reaction mixture. The adduct can later be analyzed as a specific mass tag to the nonapeptide. The fixed product ion scan and conversion of phosphylated BuChE will be discussed in this report.

2.0 EXPERIMENTAL

2.1 Materials

Purified human butyrylcholinesterase (HuBuChE, E.C. 3.1.1.8) was obtained from Dr. B.P. Doctor of the Walter Reed Hospital, Washington DC. Centrifugal ultrafilters (Centricon YM-3, 3 kD or Amicon Ultra-15, 100 kD), were purchased from Millipore (Bedford, MA, USA). Pepsin (EC 3.4.23.1) was purchased from Roche Diagnostics (Almere, The Netherlands), Sarin, soman and VX were used from stocks within our laboratory. Human plasma was purchased from a blood bank (Sanquin, Leiden, The Netherlands). Bariumhydroxide was obtained from Riedel-de Haën. 2-(3-aminopropyl-amino)ethanolwas purchased from Fluka (Buchs, Switzerland).

2.2 Instrumentation

Fixed product ion scan analyses were performed on a TSQ Quantum Ultra triple quad mass spectrometry instrument from Thermo Scientific (Breda, The Netherlands). HPLC system was an Acquity system from Waters (Milford, MA, USA).

Derivatized BuChE was analyzed on a Q-TOF mass spectrometer from Micromass (Altrincham, UK) equipped with a standard Z-spray electrospray interface. The LC system consisted of an Alliance 2690 HPLC gradient system (Waters, Milford, MA, USA).

2.3 Procedures

2.3.1 Inhibition of plasma samples with organophosphates

Human plasma was inhibited with sarin, soman, VX or dichlorvos. The concentration of the OP in plasma was 3.7 – 7 µM, which is a 75-140 fold excess compared to the approximate concentration of HuBuChE in plasma (50 nM). Inhibition of the sample was allowed for 2 hrs at room temperature. As a blank, non-inhibited plasma (0.5 ml) was used. The plasma samples were further processed as described below.

2.3.2 Isolation of HuBuChE from human plasma

A disposable 10 mL mini-extraction column (tube ABIMED AMS 422 peptide synthesizer, Gilson, Villiers le Bel, France) was filled with 2 mL procainamide-gel, which was washed with 20 mL of phosphate buffer (15 mM KH_2PO_4 and 5 mM Na_2HPO_4, pH 6.9) Then, 1 mL of plasma sample was gently

mixed with the procainamide-gel. After 30 min at room temperature, the gel was washed with 5 mL phosphate buffer and 7 mL 350 mM sodium chloride (350 mM NaCl in phosphate buffer). Finally, HuBuChE was eluted with 10 ml 1000 mM NaCl in phosphate buffer.

2.3.3 Digestion of HuBuChE with pepsin

The HuBuChE solution obtained after procainamide affinity extraction was concentrated using a 100 kD cut-off filter. The retentate was washed with 5% formic acid (2x 2 mL). The retentate (approximately 200 µL) was transferred to a 4 ml glass vial; the filter was rinsed with 250 µl 5% formic acid. The rinse fluid was combined with the retentate. Pepsin solution (50 µl of a 0.2% (i.e., 2 mg/ml) solution in 5% formic acid) was added. After incubation for 2 h at 37 °C, the incubation mixture was filtrated through a pre-washed (0.5 ml water) 3 kD cut-off filter. The filter was washed with 150 µl 5% formic acid solution and the fluid was filtrated and pooled with the first filtrate. This solution was used for LC-tandem MS experiments.

2.3.4 LC-tandem MS of pepsin digests

Stationary phase was a PepMap C18 column (15 cm x 1 mm, 3 µm particles) from LC-Packings (Amsterdam, The Netherlands). The mobile phase consisted of a gradient of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile. Gradient program was 0'-5': 100%A, flow 0.1→0.6 mL/min; 5'-60': 100% A->70% B, flow 0.6 mL/min. The pump flow (0.6 mL/min) was reduced to a column flow of approx. 50 µL/min by a splitter (LC-packings). Injection volume was 10-20 µl. QTOF: Electrospray MS-MS spectra of the protonated molecular ion were recorded using a cone voltage of approximately 35 V and a collision energy of approximately 30 eV. Subsequently, ion chromatograms of m/z 778.4, the most selective fragment originating from the loss of the phosphyl moiety from the protonated molecular ion, were generated. Triple quad: MRM transitions of daughter ion 778 were recorded. Source CID was 12 V and collision energy was 31 V.

2.3.5 Conversion of pepsin digests of HuBuChE with $Ba(OH)_2$ and 2-(3-aminopropylamino)ethanol and subsequent LC-tandem MS analysis

Isolation of HuBuChE from plasma and subsequent pepsin digestion was carried out as described above. The filtrate was concentrated, coevaporated with 50 mM NH_4HCO_3 (2x 0.5 ml), and dissolved in an aqueous solution of $Ba(OH)_2$ and 2-(3-aminopropylamino)ethanol (100 and 50 mM, respectively; 0.2 mL). After incubation for 1 h at 37 ºC, the reaction was quenched by the addition of acetic acid (10 microliter). The resulting solution was analyzed with LC/MS/MS (QTOF configuration) as described above, but with a collision energy of 18 eV.

3.0 RESULTS

3.1 Generic assay: Fixed product ion scan

Human plasma was incubated with the n-propyl analogue of VX to inhibit BuChE completely. BuChE was isolated using the procainamide affinity extraction, digested with pepsin and analyzed with LC-MS/MS. It was supposed that the identity of the inhibitor was not known beforehand, which means that a specific acquisition mass could not be established. The nonapetides have three daughter ions in common (602, 673 and 778) which can serve as fixed product ion in a parent ion scan. Normally the ionchromatogram of a transition to daughter ion 778 is the cleanest chromatogram, therefore a parent ion scan of daughter ion 778 was acquired and Figure 1 shows the result.

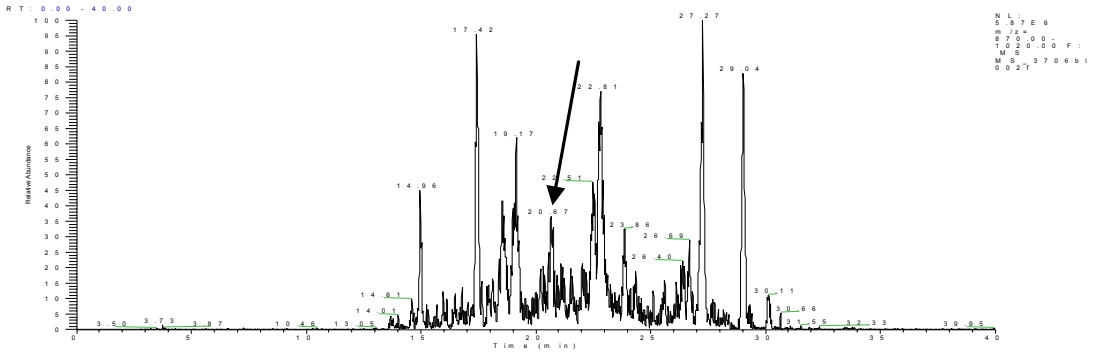

Figure 1: TIC (Total Ion Current) chromatogram of fixed product ion scan (m/z 874-1014) of daughter ion 778 of pepsin digest of inhibited HuBuChE isolated from human plasma. Peak indicated by arrow is peak of interest.

Figure 1 shows a significant background level and several peaks are visible which have daughter ion 778 in common. In this acquisition mode a blank sample was not analyzed. The peak at retention time 20.67 min is the peak of interest. The signal is not high in relation to the background but signal strength is improved when the extracted ion chromatogram of mass 916 (=mass of nonapeptide with n-propyl-VX adduct) is shown (Figure 2).

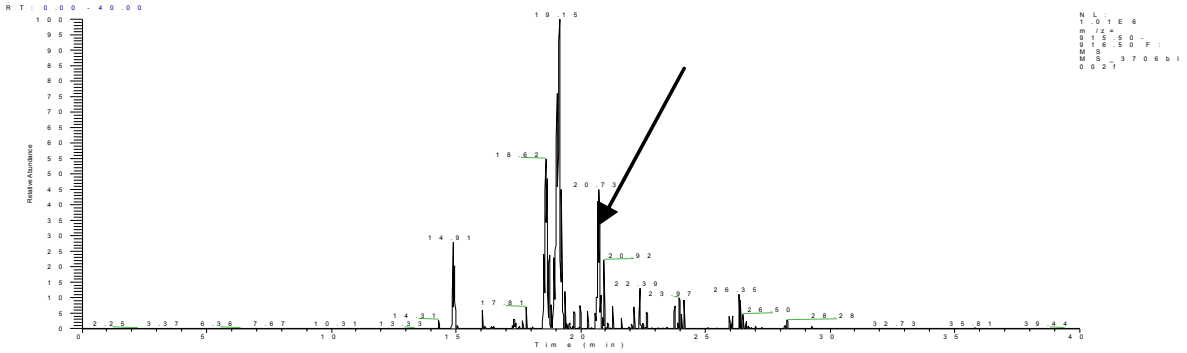

Figure 2: Extracted ion chromatogram m/z 916 acquired after a fixed product ion scan (m/z 874-1014) of daughter ion 778 of pepsin digest of inhibited BuChE isolated from human plasma. Peak indicated by arrow is peak of interest.

The signal of the peak compared to the background is improved compared to the chromatogram in Figure 1 but the signal to noise ratio of the indicated peak in relation to the background is still not eye-catching.

It is questionable whether it is necessary to perform a full fixed product ion scan of the daughter ions. Close examination of the guidelines of OPCW Schedule 1 components reveals that only side chains of O-alkyl (<C10) and O-cycloalkyl (< C10) and alkyl (<C3) are considered. The number of different combinations of organophosphate are more than thousand, but the number of mass combinations for linear O-alkyl alkylphosphonates is reduced to only 13 combinations, i.e. 874, 888, 902, 916, 930, 944, 958, 972, 986, 1000, 1014, 1028 and 1042. Some masses are more likely to occur than others. Mass 874 can occur only in one form and is represented by the MPA adduct. Mass 916 can occur three times as O-propyl methyl phosphyl (sarin), O-ethyl ethyl phosphyl and O-methyl propyl phosphyl adduct. Mass 1042 can occur only as an O-decyl propyl phosphyl adduct. It is left out of consideration that the propyl group can exits as n-propyl or i-propyl group because these groups show the same mass. V-agents generate the same kind of adduct and are in this category. The occurrence of weird combinations such as methyl sarin, m/z 888, O-decyl propyl phosphonfluoridate, m/z 1042, and analogue 1028 and 1014 is also unlikely. We choose to perform the analysis again while measuring only nine transitions, 874 → 778, 902 → 778, 916 → 778, 930 → 778, 944 → 778, 958 → 778, 972 → 778, and 986 → 778. Figure 3 shows the TIC-chromatogram of nine transitions of an exposed sample and a blank sample. Both chromatograms look almost identical, which means that the individual extracted ion chromatograms had to be studied.

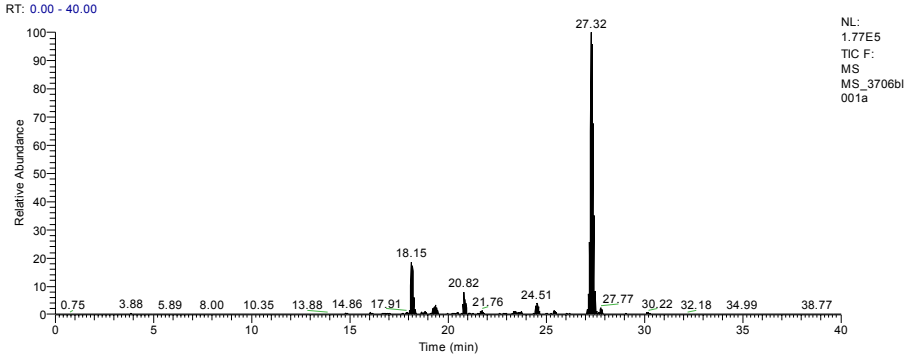

Figure 3 TIC-chromatogram of 9 transitions with daughter ions 778. Acquistion parent ions were 874, 902, 916, 930, 944, 958, 972, 986 and 1000. Upper: exposed sample (n-propyl-VX) Lower: non exposed sample

Figure 4 shows the extracted ion chromatograms of mass 874. The two chromatograms look different from each other but a distinct peak that represents the phosphylated nonapeptide cannot be found. Figure 5 shows the extracted ion chromatograms of mass 916. The figure shows clearly the presence of an additional peak compared to the non exposed sample. Indeed mass 916 represents the nonapeptide conjugated with the n-propyl analogue of VX and is the same mass of the O-i-propyl methylphosphyl adduct which is generated by sarin. The peak was detected while measuring 9 transitions and it is remarkable that the sensitivity decreased only to approx. 80% of the original signal strength measured in the SRM mode (chromatograms not shown).

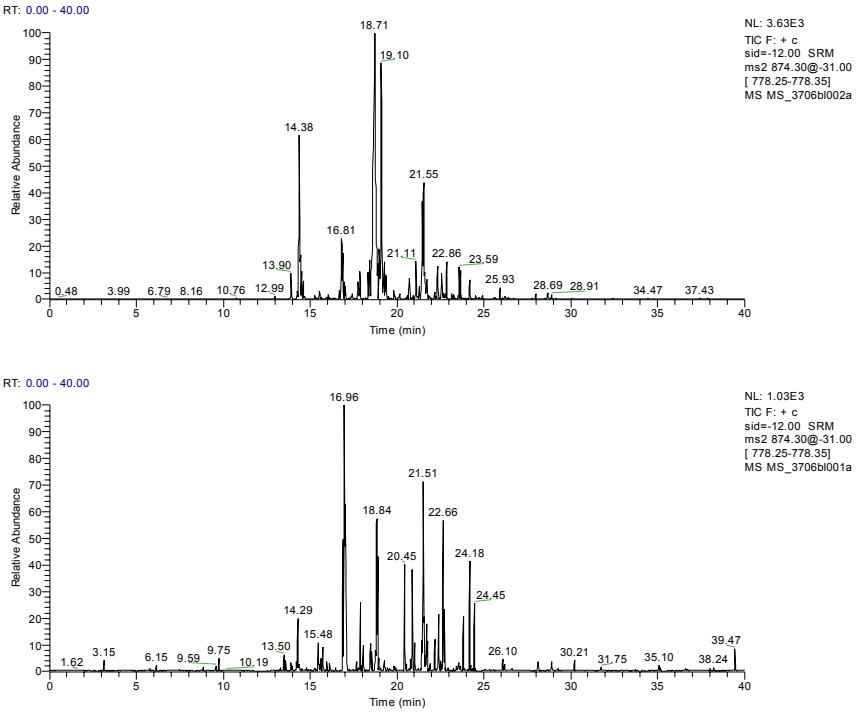

Figure 4 Extracted ion chromatogram 874 → 778 measured in MRM mode (nine transitions were recorded) Upper exposed sample (n-propyl-VX), Lower exposed sample

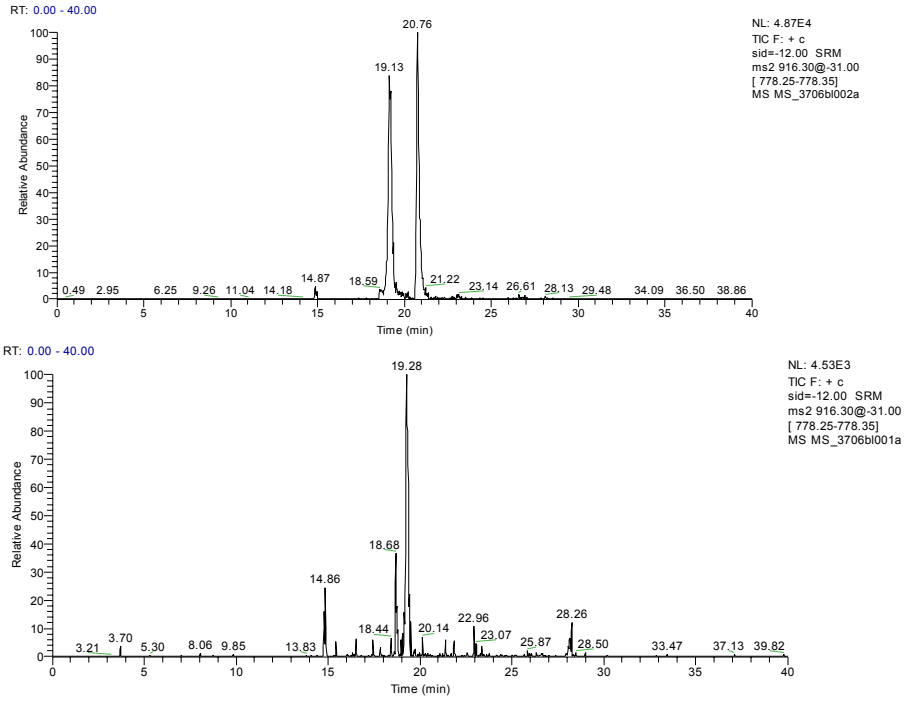

Figure 5 SRM chromatogram 916 → 778 measured in MRM mode (nine transitions were recorded) Upper exposed sample (n-propyl-VX), Lower non exposed sample

The linear alkyl phosphonates are not the only group that exist as a nerve agent, for example adducts caused by tabun derivatives or O-cycloalkyl alkyl phosphonates will result in mass tags that are not in the collection of masses that were mentioned earlier. For these categories of inhibitors it is required to set up a new collection of masses. Table 1 shows the masses of the phosphylated nonapeptides that are derived after inhibition by these different types of nerve agents. It should be mentioned that the dialkylamido group of tabun is replaced by a hydroxyl group during the pepsin digestion which takes place under acidic conditions (pH<2). The number of mass possibilities is therefore determined by the number of C-atoms in the O-alkyl group increased with one in case of aging (m=876). Interestingly, the mass of the peptides with a cyclotabun adduct are the same as the masses of the masses of the peptides with linear sarin adducts, which means that the number of masses that need to be screened can be further reduced. By the way, masses like 890, 904 and 932 show up in the array of masses that are no longer exclusively related to nerve agents, but also to adducts that can be formed by pesticides such as (aged) dichlorvos (m/z 890 and 904) and (aged) paraoxon (m/z 904 and 932).

Table 1 Masses of phospylated nonapeptides derived after pepsin digestion of HuBuChE inhibited with different classes of nerve agents

Linear Sarin mass	Cyclo Sarins mass	Linear Tabun mass	Cyclo Tabuns mass
874	914	876	916
888	928	890	930
902	942	904	944
916	956	918	958
930	970	932	972
944	984	946	986
958	998	960	1000
972	1012	974	1014
986	1026	988	
1000	1040	1002	
1014		1016	
1028			
1042			

Conclusive, after pepsin digestion of inhibited BuChE the total of masses that need to be scanned for is 34. Interestingly the sensitivity of the assay reduces to only 80% of the original signal strength when ten transitions are measured in the MRM mode. In case that the identity of the inhibitor is not known it can be chosen to perform 4 analyses in which nine transitions are measured each time. With that strategy a main part of the spectrum of nerve agents can be screened for.

3.2 Derivatization of phosphylated site in BuChE

The selection of acquisition masses that were mentioned in the previous section does not cover the possible inhibition by all organophosphates. Therefore, a novel and generic assay for OP biomonitoring was developed that is based on pepsin digestion of HuBuChE, followed by base-catalyzed beta-elimination of the phosphyl moiety and subsequent Michael addition of a suitable nucleophile. It results in one common modified nonapeptide, that can be analyzed by means of LC tandem MS. This methodology was inspired from the proteomics field where the method is used to detect phosphylated proteins or phosphylated sites in proteins.

Plasma was incubated with various nerve agents. After complete inhibition of BuChE was achieved, BuChE was extracted and digested with pepsin as described above. Next the sample was hydrolyzed with

barium hydroxide and derivatized with 2-(3-aminopropylamino)-ethanol [9]. In Figure 7 the sample preparation procedure is shown. The methodology proved to be viable with plasma samples that had been exposed to various OP's (see Figure 8). It can also be seen that the non-exposed sample shows no peaks, which indicates that the result is true negative. This outcome was not obvious because the unmodified serine can undergo an elimination reaction upon alkaline hydrolysis resulting in the same dehydroalanine [10]. Apparently, the elimination reaction of the serine itself proceeds not so fast as the elimination of the phosphonyl group. Two peaks were observed, which can be explained by the fact that due to the formation of a dehydroalanine residue and subsequent attack of the nucleophile on the double bond, the resulting amino acid loses its chiral integrity. Since the chirality of the other amino acids in the peptide does not change, a diastereomeric mixture of two peptides results. Performing the modification reaction on the peptide level has the additional advantage that a two-step approach can be followed. First, the generic method can be used for an initial screening of samples and after finding a positive sample, the original pepsin digest can be analyzed in a more specific way as described in the previous section in order to unravel the identity of the OP inhibitor.

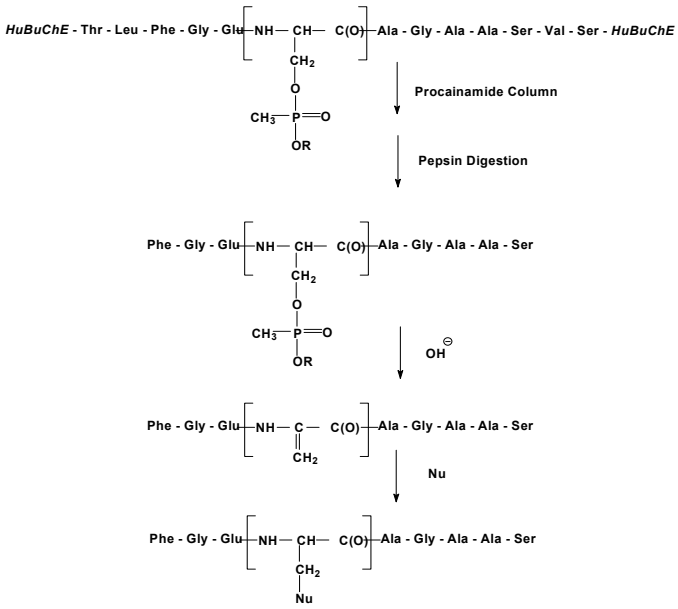

Figure 7 Methodology for conversion of phosphylated HuBuChE into a common nonapeptide.

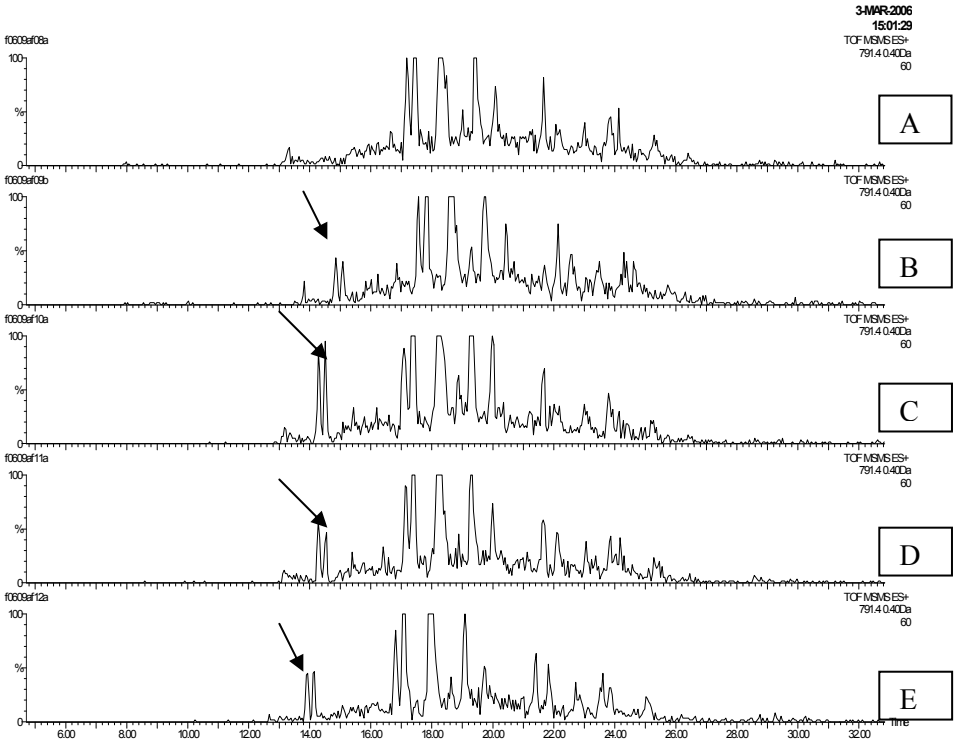

Figure 8 Ion chromatograms of *m/z* 791.4 in processed pepsin digests of isolated HuBuChE from plasma samples. Panel A represents the ion chromatogram of a processed digest from blank plasma. Panels B-E represent the ion chromatograms of digests from plasma, that had been exposed to sarin, soman, VX and dichlorvos, respectively. The digests were subjected to modification with 2-(3-aminopropylamino)ethanol, under the agency of $Ba(OH)_2$. The arrows indicate the peak of the modified FGEXAGAAS. In the first MS, $[M+2H]^{2+}$, 448.7 was selected.

4.0 CONCLUSION

The adduct of nerve agent to butyrylcholinesterase is an important and persistent biomarker for nerve agent exposure (half live 8-12 days). Two different methodologies were developed for the verification of exposure to nerve agent without having pre-knowledge of the identity of the inhibitor. The first method is based on the smart selection of acquisition masses for the measurements of MRM transitions of the modified nonapeptide that results after digestion of BuChE with pepsin. It appeared that the measurement of 42 transitions is sufficient to screen for all OPCW schedule 1 nerve agents. The sensitivity of the assay decreased to only 80 % compared to the most sensitive SRM mode, which means that inhibition levels down to 10% of control values can be detected.

In addition, a novel method was developed which is based on the conversion of the phosphylated serine in BuChE to a common adduct. The phosphyl group is eliminated through mild alkaline hydrolysis and the resulting dehydroalanine residue reacted with 2-(3-aminopropylamino)ethanol. The formed common adduct could be measured with LC-MS using the most sensitive SRM mode, independent of the nerve agent that was used. The method needs further improvement with regard to sensitivity to be suitable for screening for exposure to organophosphates at relevant levels.

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