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Development of an automated on-line pepsin digestion–liquid chromatography–tandem mass spectrometry configuration for the rapid analysis of protein adducts of chemical warfare agents

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ABSTRACT

Rapid monitoring and retrospective verification are key issues in protection against and non-proliferation of chemical warfare agents (CWA). Such monitoring and verification are adequately accomplished by the analysis of persistent protein adducts of these agents. Liquid chromatography–mass spectrometry (LC–MS) is the tool of choice in the analysis of such protein adducts, but the overall experimental procedure is quite elaborate. Therefore, an automated on-line pepsin digestion–LC–MS configuration has been developed for the rapid determination of CWA protein adducts. The utility of this configuration is demonstrated by the analysis of specific adducts of sarin and sulfur mustard to human butyryl cholinesterase and human serum albumin, respectively.

1. Introduction

The ability of rapid monitoring of chemical exposures after a terrorist or military attack with chemical (warfare) agents is considered of utmost importance, both to provide reliable diagnosis of exposure as a starting point for proper medical treatment [1] and to confirm non-exposure of the so-called "worried well" (see, e.g., [2,3]). In this respect, biomedical samples such as urine and blood can provide valuable qualitative or quantitative information on an individual's exposure to chemical agents (see, e.g., [4,5]). Exposure to chemical agents often results in the formation of stable and persistent covalent protein adducts in the body. For example, a nerve agent exposure will result in the formation of adducts to human butyryl cholinesterase (HuBuChE) [5], whereas the vesicant sulfur mustard will give rise to rather random adducts, e.g., to human serum albumin (HSA) [6–8]. Similar covalent protein adducts are often observed in drug toxicology (see, e.g., [9–11]), in metabolism (e.g., [12–16]) and from post-translational modification (e.g., [17,18]). The analysis of covalent protein adducts is usually performed by enzymatic digestion of the modified protein and subsequent mass spectrometric analysis of the resultant peptide or amino acid adducts. Due to the long manual sample handling steps, these methods are time-consuming and rather laborious. Automated on-line systems, as reported earlier by Shen et al. [19] and more recently by Hoos et al. [20], will afford increased sample throughput after terrorist or military incidents and will also be advantageous for use in field laboratories. We here report the development of an automated configuration to verify and monitor CWA (chemical warfare agent) exposures, using on-line enzymatic digestion–LC–tandem MS.

2. Experimental

2.1. Reagents

Caution: sulfur mustard is a primary carcinogenic, vesicant and cytotoxic agent. Sarin is a potent cholinesterase inhibitor and it is extremely toxic. These compounds should be handled only in fume hoods and by experienced personnel. Sulfur mustard and sarin (purity >95% according to ^{1}H-NMR analysis), were taken from our own laboratory stock. Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). Pepsin was purchased from Roche (Almere, The Netherlands). Milli-Q water was obtained from a local Milli-Q Academic system (Millipore, Bedford, MA). Formic acid was purchased from Merck (Darmstadt, Germany). Purified HuBuChE, as a 350 nM solution in water, was kindly donated by Dr. Doctor (WRAIR, WA, USA).

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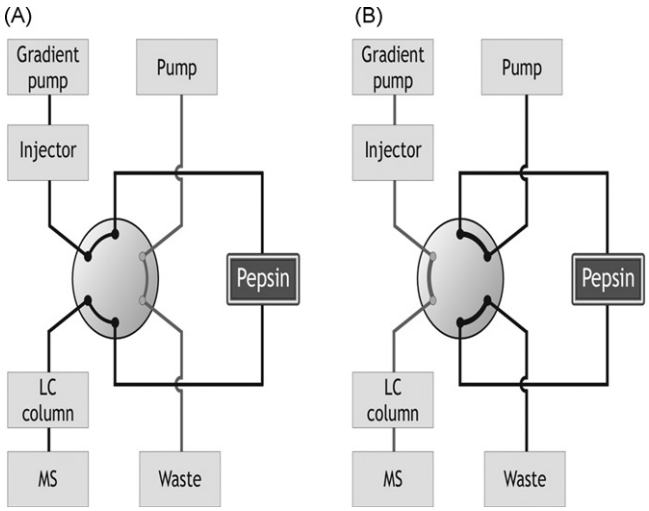

Fig. 1. Schematic representation of the analysis system. The configuration consists of a switching valve connected to a gradient LC pump, a sampler (inject), a pepsin cartridge, an isocratic LC pump and an LC column directly coupled to the mass spectrometer. (A) Shows the valve connections during the injection of the sample, digestion, and trapping on the LC column. (B) Shows the valve after switching for gradient elution of the peptides from the LC column and concomitant reconditioning of the pepsin cartridge.

2.2. Sample preparation

Purified HuBuChE (solution in water; 350 nM) was fully inhibited with sarin at a final concentration of 1 μg/ml, at room temperature for 30 min. Human blood, obtained from healthy volunteers in the laboratory, was incubated with various concentrations of sulfur mustard. Subsequently, plasma was separated from erythrocytes by centrifugation, and albumin was isolated from the plasma by affinity chromatography over a HiTrap Blue cartridge (0.7 cm × 2.5 cm, 1 ml; GE Healthcare, Hoevelaken, The Netherlands). After isolation, the protein sample was split for either off-line pepsin digestion or on-line digestion with immobilized pepsin.

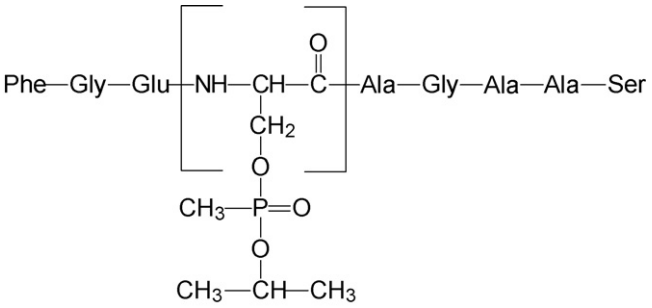

Fig. 2. Chemical structure of the nonapeptide adduct FGES*AGAAS resulting from pepsin digestion of human butyryl cholinesterase, after exposure to sarin.

2.3. On-line digestion

The instrument configuration, depicted in Fig. 1, consisted of (1) a binary pump, Waters 600 (Waters, Manchester, U.K.), and a poroszyme immobilized pepsin cartridge, 30 mm × 2.1 mm (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), (2) an Alliance type 2690 liquid chromatograph (Waters), combined with a six-port switching valve (VICI, Bester, Amstelveen, The Netherlands) and an analytical LC column (PepMap C_{18}, 15 cm × 1 mm ID; LC Packings, Amstelveen, NL), and (3) a Q-TOF hybrid tandem mass spectrometer (Micromass, Manchester, U.K.)

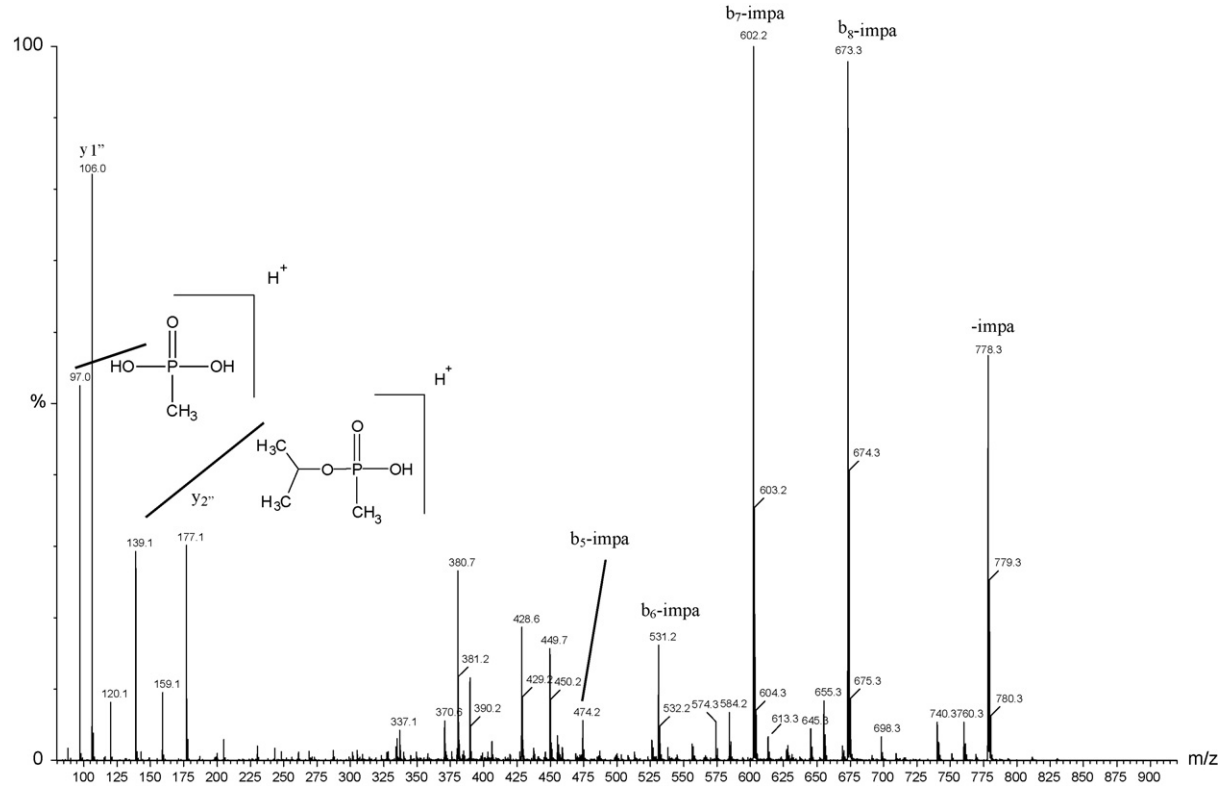

Fig. 3. Product ion spectrum of $[M + 2H]^{2+}$, m/z 458.7, of the modified nonapeptide FGES*AGAAS obtained from pepsin digestion of sarin-exposed HuBuChE (impa = isopropyl methylphosphonic acid); cone voltage 17 V and collision energy 16 eV.

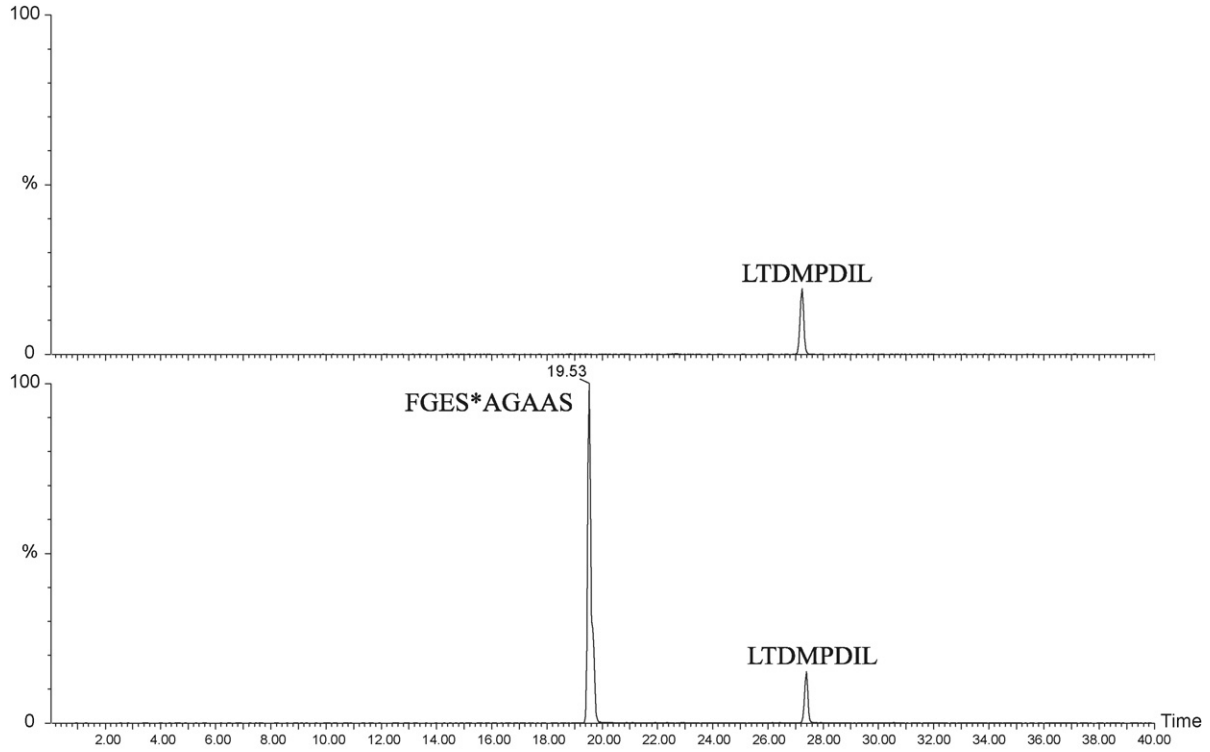

Fig. 4. Summed ion chromatogram of product ions *m*/*z* 602.3, 673.3, and 778.3 of nonapeptide adduct FGES*AGAAS $[M+H]^+$, *m*/*z* 916.3, in a pepsin digest of sarin-inhibited HuBuChE; upper trace: digest of native HuBuChE; lower trace, digest of sarin-inhibited HuBuChE; cone voltage 17 V and collision energy 16 eV. The peak at 27.4 min is the peptide LTDMPDIL, which has a $[M+H]^+$ of 917.4 and a b_6-fragment of 673.3.

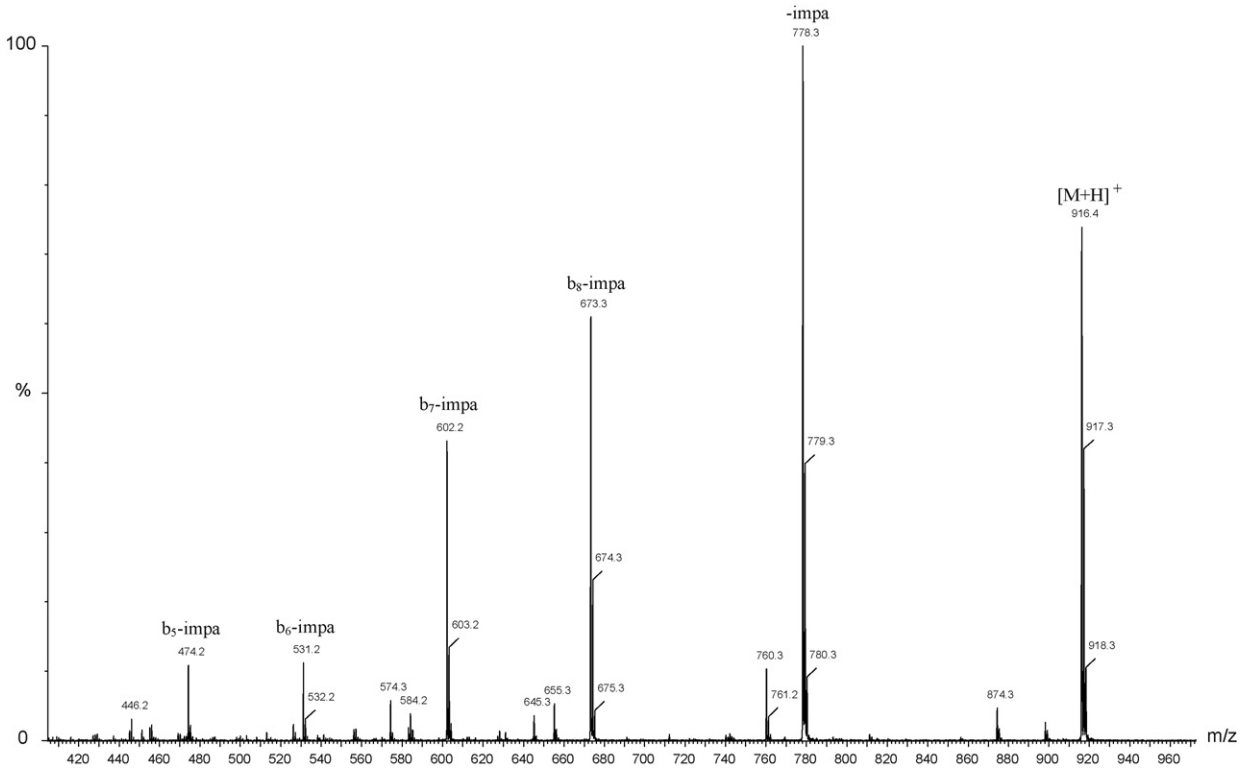

Fig. 5. Product ion spectrum of $[M+H]^+$, *m*/*z* 916.3, of the modified nonapeptide FGES*AGAAS, obtained from pepsin digestion of sarin-exposed HuBuChE; cone voltage 35 V and collision energy 30 eV.

Table 1
Sulfur mustard adduct peptides observed after pepsin digestion of HSA from sulfur mustard-exposed blood

Adduct peptide[a]	MH_2^{2+}
YLQQC*PFED	623.7
LQQC*PFED	542.2
LQQC*PFEDHVKL	780.9
LGMFLYE*	488.7
KPLVE*EPQNL	635.8
AE*VSKL	375.7

[a] (*) After the modified residue marks the site of the 2-hydroxyethylthioethyl (HETE) moiety in the peptide.

equipped with a standard Z-spray type electrospray ionization (ESI) interface. After injection of 10 μl of an aqueous sample, the protein analytes were led through the immobilized pepsin cartridge, while the effluent flow was directly transferred to the LC column. Because the conditions in the pepsin cartridge were set as the starting conditions of the LC gradient (eluent A: H_2O with 0.2% formic acid), this resulted in trapping of the digest peptides at the front end of the analytical column. After a 5 min digestion time, the valve was switched and the trapped peptides were then eluted from the column by a linear gradient. The gradient of eluents A and B (acetonitrile with 0.2% formic acid) was programmed as follows: 100% A → 70% B in 60 min. The pump flow of 0.6 ml/min was split pre-column with a pre-column splitter (type Acurate 400; LC Packings) to allow a flow of approx. 40 μl/min through the pepsin cartridge, LC column and ESI interface. During the LC-analysis cycle, the digestion cartridge was flushed with eluent A (100 μl/min) to prepare for the next injection. The individual LC separated digest peptides were analyzed using a Q-TOF mass spectrometer that was operated at a cone voltage between 15 and 35 eV, employing nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 l/h, respectively). MS/MS product ion spectra were scanned over the full mass range

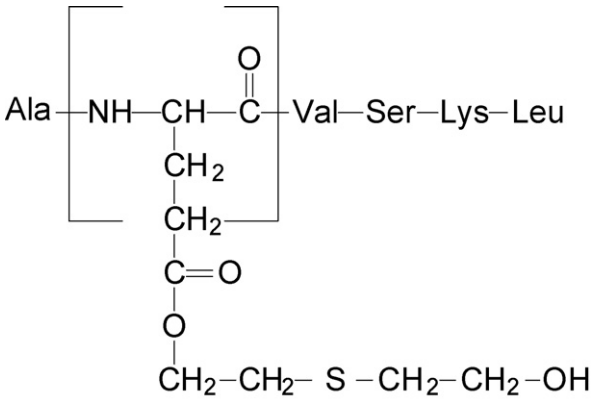

Fig. 7. Chemical structure of the sulfur mustard adduct peptide AE*VSKL obtained after pepsin digestion of serum albumin isolated from sulfur mustard-exposed human blood.

of 50–900 Da, with a scan cycle time of 2.5 s and using a collision energy as indicated in the various figure legends, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

3. Results and discussion

3.1. Sarin-exposed HuBuChE

One of the methods of choice to determine nerve agent-inhibited HuBuChE is based on HuBuChE extraction from plasma by means of procainamide affinity chromatography, followed by pepsin digestion and mass spectrometric analysis of the formed nonapeptide FGES*AGAAS with LC–MS (Liquid chromatography–mass spectrometry)/MS, with S* being the nerve agent modified serine residue [5]. In case of sarin, the nonapeptide

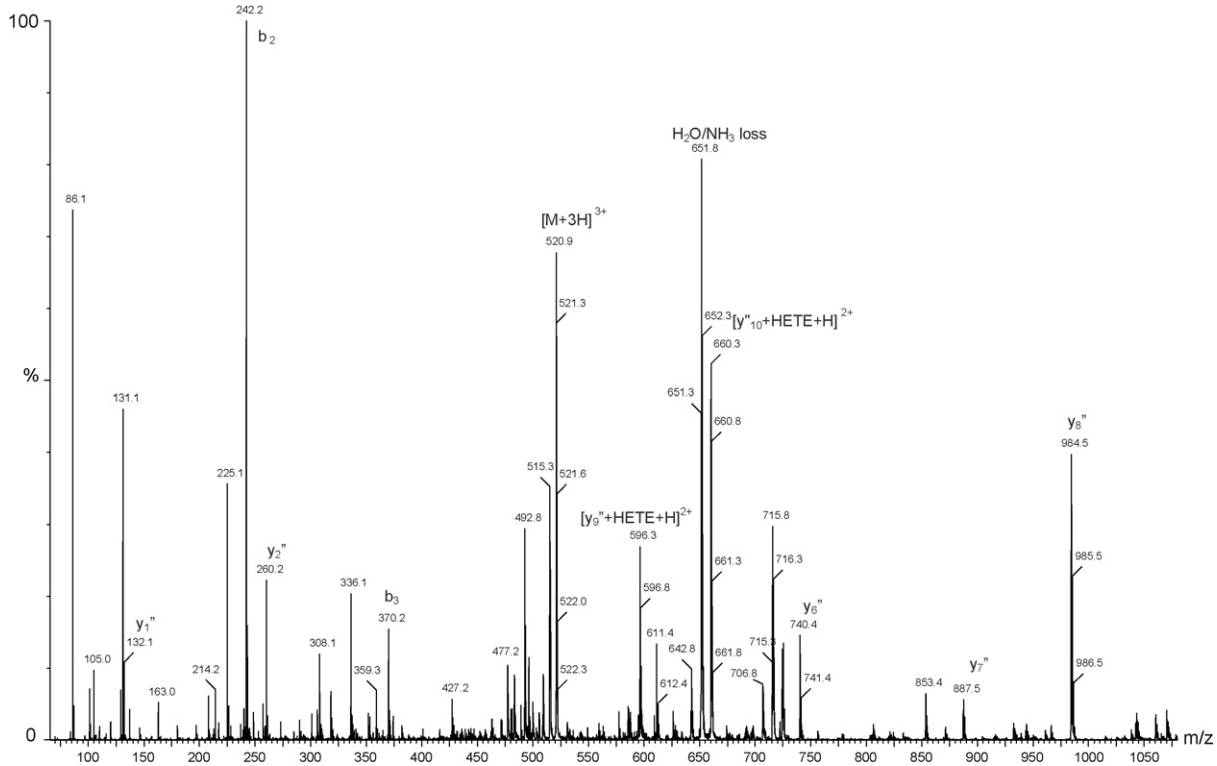

Fig. 6. Product ion mass spectrum of $[M+3H]^{3+}$, *m/z* 520.9, of LQQC*PFEDHVKL from a pepsin digest of sulfur mustard-exposed human serum albumin (HETE = 2-hydroxyethylthioethyl); cone voltage 18 V and collision energy 16 eV. The peak at *m/z* 651.8 is from the loss of H_2O/NH_3 from the parent ion.

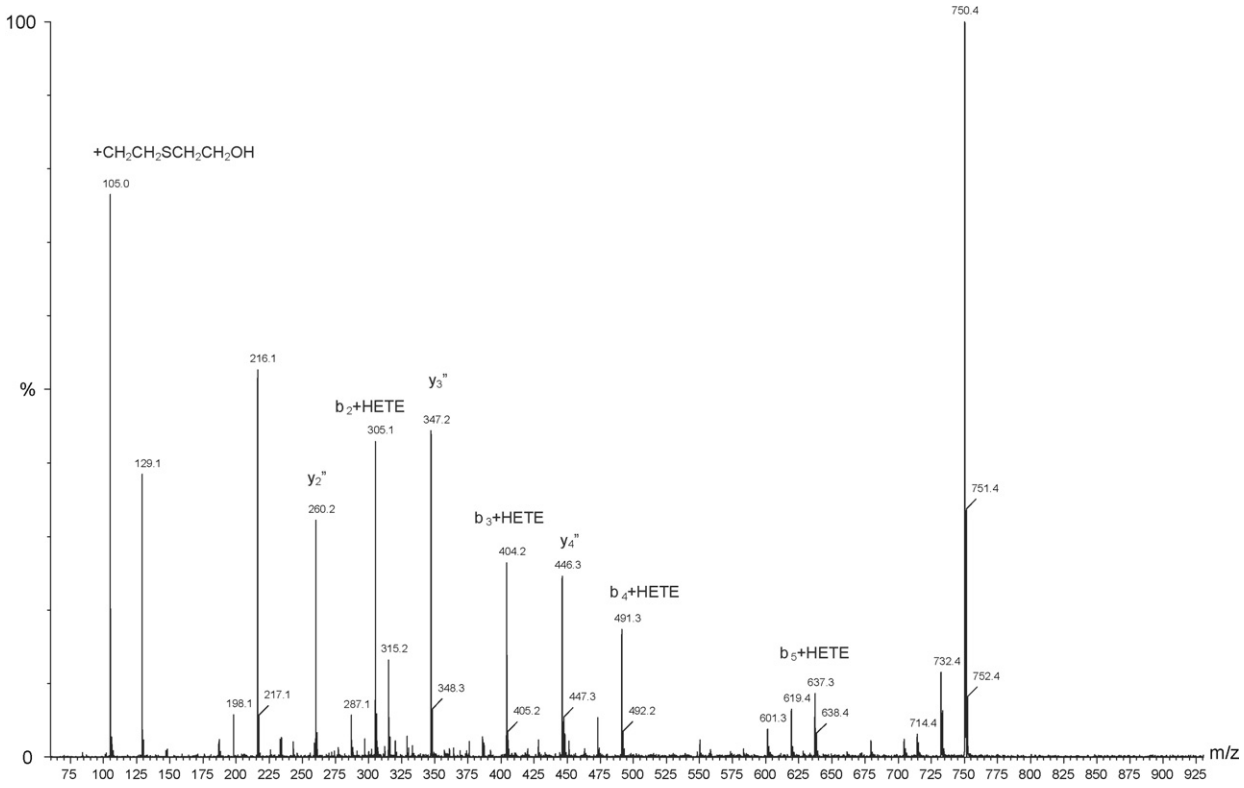

Fig. 8. Product ion mass spectrum of $[M+H]^+$, m/z 750.4, of AE*VSKL in a pepsin digest of sulfur mustard-exposed albumin (HETE = 2-hydroxyethylthioethyl); cone voltage 30 V and collision energy 33 eV.

depicted in Fig. 2 is formed. It is well-known from the literature [5] that only the Ser-198 residue within the active site becomes modified after exposure of human butyrylcholinesterase to an organophosphate. We have recently shown that this strategy can be used for monitoring exposure to a wide range of organophosphates [21].

Purified HuBuChE that had been inhibited by sarin was used for initial experiments with the on-line configuration. Indeed, the

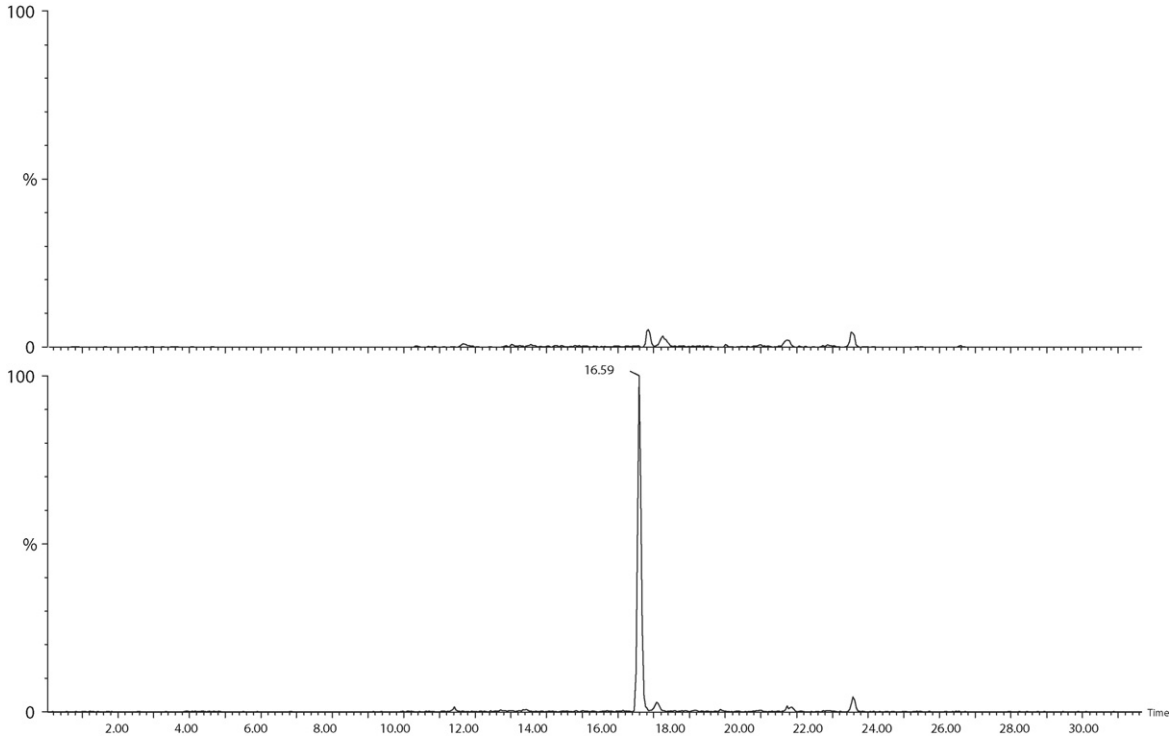

Fig. 9. Summed ion chromatogram of m/z 105.0 and 646.4 product ions from $[M+H]^+$, m/z 750.4, of AE*VSKL in a pepsin digest of serum albumin isolated from human blood that had been incubated with sulfur mustard; upper trace: 0 mM exposure; lower trace, 100 μM exposure.

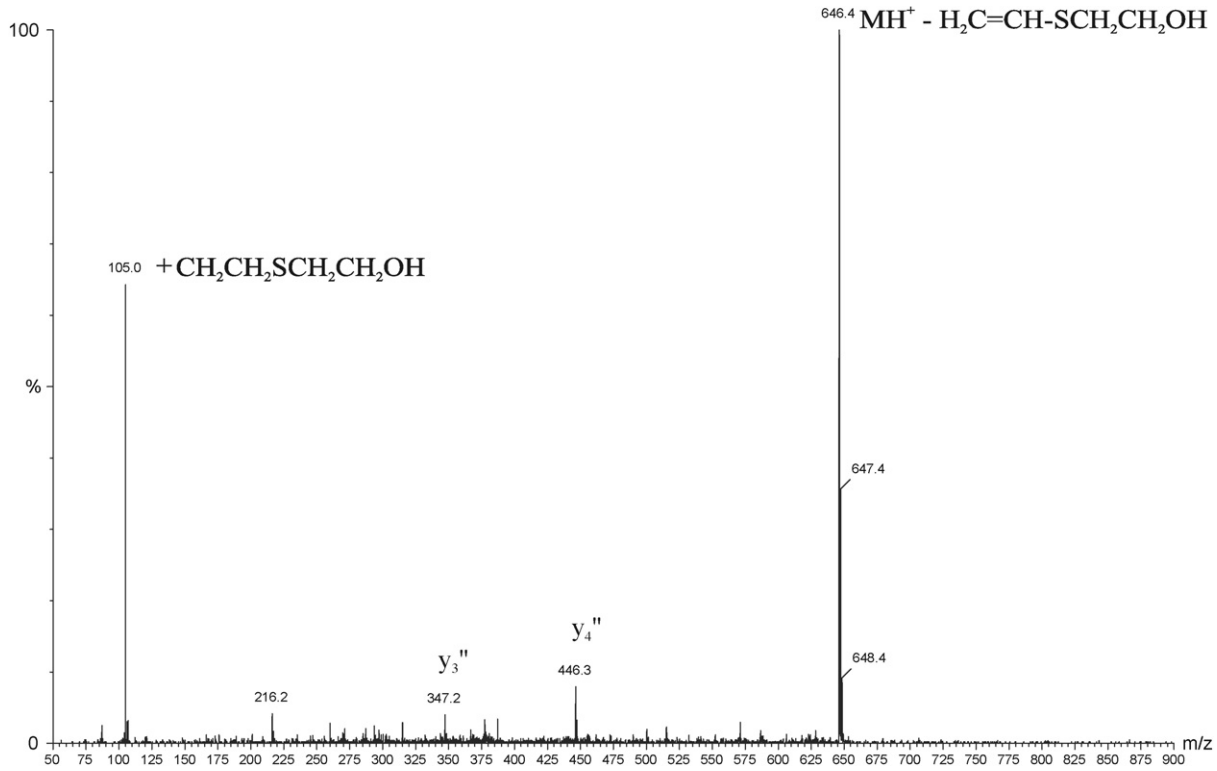

Fig. 10. Product ion mass spectrum of $[M+2H]^{2+}$, *m/z* 375.7, of AE*VSKL from on-line pepsin digestion LC–MS/MS analysis of sulfur mustard-exposed albumin; cone voltage 16 V; collision energy 14 eV.

expected nonapeptide was observed and the structure of the peptide adduct can be derived from the product ion mass spectrum of the doubly protonated molecules, *m/z* 458.7 (see Fig. 3). The relatively low abundant b_2 (*m/z* 205.1), b_3 (*m/z* 334.2), and y''_5 (*m/z* 376.2) fragment ions exclusively carry unmodified amino acid residues, confirming that the serine residue is the adduct site. The peptide was analyzed more sensitively and selectively when the singly protonated molecule at *m/z* 916.3 was selected as the precursor. Fig. 4 shows the ion chromatograms for the nonapeptide adduct in both an exposed and non-exposed HuBuChE sample. Fig. 5 shows the corresponding product ion spectrum, with the $[M+H]^+$ *m/z* 916.3 precursor selected.

In order to determine the optimal digestion time, the nonapeptide adduct signal intensity was established after 5, 10, 20, 30, and 60 min residence time of inhibited HuBuChE inside the immobilized pepsin cartridge. It turned out that a residence time of 5 min afforded the same level of adduct peptides as longer residence times. Considering that off-line in solution pepsin digestion usually takes 2 h [5], the on-line digestion with immobilized pepsin is much faster. The yield of adduct nonapeptide from on-line pepsin digestion was approximately 70% of the yield after off-line in solution digestion. Similar digestion yields in on-line treatment have been observed earlier for various other proteins, for example albumin, as reported previously [22]. In conclusion, the on-line digestion performed satisfactory with a digestion time of 5 min.

The performance of the system was further evaluated in terms of limit of detection (LOD) and repeatability. The limit of detection was determined from six 10 μl injections of decreasing concentration of intact sarin-inhibited HuBuChE, down to the level that produced an S/N ratio of 3. The observed method LOD was 1.06 nM, corresponding to a total amount of 10.6 fmol (0.711 ng) of HuBuChE protein. The repeatability was determined with a solution of exposed HuBuChE (21.1 nM), from six repeated 10 μl injections. The repeatability of the marker analysis in LC–MS mode and determined from the TIC chromatogram is 0.25% relative standard deviation (R.S.D.) in LC retention time and 10% R.S.D. in LC peak area. The repeatability at 1.06 nM was comparable to that at 21.1 nM. Much of the peak area R.S.D. is probably due to the fact that the digestion rate in on-line digestion slightly differs from one injection to another. With the observed LOD and repeatability, the overall system performance was deemed acceptable for high throughput application. In case of a 10% inhibition level, which is actually a sign-free dose, the modified BuChE level would be approximately 6 pmol/ml (normal BuChE levels in human plasma are 60 nM). When matrix effects are not taken into consideration, this level would still be detectable in case 1 ml of blood is processed.

3.2. Sulfur mustard-exposed HSA

Enzymatic digestion of sulfur mustard-exposed HSA has previously been performed in our laboratory with pronase, to give the tripeptide C*PF, with C* the modified cysteine (C34) residue containing the 2-hydroxyethylthioethyl (HETE) moiety [6,7]. Unfortunately, we did not succeed in producing immobilized pronase that still displayed the required activity. We therefore explored the possibility of pepsin digestion of sulfur mustard-exposed HSA. First, pepsin digestion was performed in solution with albumin samples from highly exposed blood, to track the most prominent adduct peptides. The detected adduct peptides are summarised in Table 1. Subsequent analyses with the on-line pepsin digest configuration confirmed the qualitative off-line results from highly exposed blood. A number of adducts containing the modified C34 residue were observed, corresponding to the adduct peptide we normally analyze as C*PF after pronase treatment. In Fig. 6 the spectrum of the adduct peptide LQQC*PFEDHVKL is shown as an example. The site of modification was deduced from the product ion mass spectrum, as the C-terminal fragments up to y''_8 and N-terminal fragments up to b_3 correspond to unmodified amino acid

residues. Although these observations corresponded to the findings of our earlier work, the C34 modified digest peptides were not the most abundant adduct peptides. We observed a number of peptides with the HETE moiety attached to a glutamic acid (E) residue, where AE*VSKL (see, Fig. 7 for chemical structure) turned out to be the most abundant adduct peptide after pepsin digestion. The product ion mass spectrum of this peptide, given in Fig. 8, shows characteristic fragments from which the site of modification was deduced. The relatively weak ion y''_5 at m/z 679.4 is the first y-fragment carrying the HETE modification. Fig. 9 presents the summed ion chromatogram m/z 105.0 and 646.4 for this adduct peptide from the on-line pepsin digestion LC–MS/MS analysis of albumin isolated from sulfur mustard-exposed blood. The corresponding product ion spectrum of $[M+2H]^{2+}$ of the adduct peptide, m/z 375.7, is shown in Fig. 10. Again, a 5 min residence time on the pepsin cartridge was used, based on abovementioned results with modified HuBuChE. This shows that the sulfur mustard adducts of HSA can be analyzed by on-line pepsin digestion – LC–MS/MS.

In the current setting, it is estimated that an in vitro exposure to sulfur mustard in the low μM range can still readily be detected. From earlier studies we know that these levels are realistic [4]. For concentration levels below 1 μM it can be envisaged that the modified peptide AE*VSKL is a good candidate for analysis on triple-quad instruments through multiple reaction monitoring of the collision induced decay of $[M+2H]^{2+}$, m/z 375.7 to the fragment ions m/z 105.0 and 646.4, which would greatly enhance the sensitivity.

4. Conclusion

We have described the development of an automated on-line pepsin digestion LC–MS/MS method for rapid determination of exposure to CWA, and we have shown its utility for detection of sulfur mustard and sarin exposure. The on-line configuration provides fast analysis times and less laborious sample processing than analysis with off-line digestion in solution. The on-line approach will allow a much higher throughput of samples, which will be advantageous for monitoring in case of incidents with chemical agents. Inclusion of on-line protein isolation in the current configuration, for example by means of a cartridge containing the requisite affinity gel, would render the approach even more powerful and would enable the use of crude plasma samples without further additional work-up.

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