Verification of Exposure to Cholinesterase Inhibitors: Generic Detection of OPCW Schedule 1 Nerve Agent Adducts to Human Butyrylcholinesterase

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Abstract

Phosphylated butyrylcholinesterase is one of the most important biomarkers to verify an exposure to nerve agents, and it can be analyzed with liquid chromatography–tandem mass spectrometry (LC–MS–MS) by detection of a phosphylated nonapeptide that results after digestion of butyrylcholinesterase (BuChE) with pepsin. For a sensitive analysis (low degree of BuChE inhibition), the identity of the cholinesterase inhibitor has to be known in order to use the LC–MS–MS instrument in the most sensitive selected reaction monitoring mode. In practice, the identity of the cholinesterase inhibitor will not be known beforehand, and the number of possible organophosphates is greater than 1000. However, the number of possible molecular masses of organophosphates is approximately 170. A method for which only 34 transitions in the multiple reaction monitoring mode have to be acquired in order to screen for an exposure to all Organization for the Prohibition of Chemical Weapons Schedule 1 nerve agents was developed.

Introduction

The development of analytical methodology for the verification of exposure to nerve agents is relevant because the results of the analysis of biomedical samples might be used as evidence for the confirmation of an alleged use of these agents. Recently, the OPCW (Organization for the Prohibition of Chemical Weapons) established a working group to discuss the possibility of analysis of biomedical samples as part of their verification program. Secondly, the health risks of even low-level exposures have not yet been fully explored and within this context it is important to verify non-exposure in order to reassure worried military personnel or citizens. 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 human butyrylcholinesterase (BuChE). One method utilizes the fluoride reactivation technique in which regenerable phosphoryl groups are released from the enzyme upon incubation with fluoride ions (4). Within the other method, the enzyme is isolated, digested with pepsin, and a phosphorylated nonapeptide, FGES(x)AGAAS, in which x represents the nerve agent adduct, is subsequently analyzed with liquid chromatography–tandem mass spectrometry (LC–MS–MS) (5). The mass of the phosphorylated nonapeptide 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 single ion monitoring (SIM) or single reaction monitoring (SRM) mode to adjust the MS in the most sensitive mode. This means that the identity of the nerve agent must be known in advance in order to adjust the in-
instrument for the adequate acquisition mass. In practice, the identity of the cholinesterase (ChE) inhibitor will not always be known, which means that the MS must be operated in a scan mode to screen for possible ChE inhibitors. The phosphorylated nonapeptide has three common daughter ions, m/z 778.4, 673.4, and 602.4, which represent the native peptide [MH]+ and the b8-x and b7-x fragments, respectively (see Figure 1). Earlier, it has been reported that the fragment ions of the MS–MS spectra of the phosphorylated peptides are the same independent of the identity of the nerve agent (5). Therefore, these daughter ions can serve as fixed product ions to perform a parent ion scan in order to discover the identity of the OPCW Schedule 1 nerve agent. We here present a strategy to verify an exposure to a previously unknown ChE inhibitor that can be detected using a fixed product ion scan, and the consequences for the sensitivity will be discussed as well.

Experimental

Materials

Citrate-preserved human plasma was purchased from a blood bank (Sanquin, Leiden, The Netherlands).

Centrifugal ultrafilters (Centricon YM-3, 3 kD, or Amicon Ultra-15, 100 kD), were purchased from Millipore (Bedford, MA). Pepsin (EC 3.4.23.1) was purchased from Roche Diagnostics (Almere, The Netherlands); nerve agents were used from stocks within our laboratory. All other chemicals were purchased from Merck (Darmstadt, Germany).

Instrumentation

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

Procedures

Inhibition of plasma samples with organophosphates. Human plasma was inhibited with the n-propyl analogue of VX [O-n-propyl S-(2-diisopropylaminoethyl) methylphosphonothioate]. The concentration of the nerve agent in plasma was 1.8 µM, which is a 36-fold excess compared to the approximate concentration of butyrylcholinesterase in plasma (50 nM). Inhibition of the sample was allowed for 2 h at room temperature. As a blank, non-inhibited plasma was used. The plasma samples were further processed as described.

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 (5), which was washed with 20 mL of 20 mM phosphate buffer (15 mM KH2PO4 and 5 mM Na2HPO4, 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 20 mM phosphate buffer and 7 mL 350 mM sodium chloride (350 mM NaCl in 20 mM phosphate buffer). Finally, butyrylcholinesterase was eluted with 10 mL of 1000 mM NaCl (1000 mM in 20 mM phosphate buffer).

Digestion of BuChE with pepsin. The BuChE solution obtained after procainamide affinity extraction was concentrated using a 100 kD cut-off filter. The residue was washed with 5% formic acid (2 × 2 mL). The residue (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 residue. 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 prewashed (0.5 mL water) 3 kD cut-off filter. The filter was washed with 150 µL 5% formic acid solution and the fluid was filtered and pooled with the first filtrate. This solution was used for LC–MS–MS experiments.

LC–MS–MS of pepsin digests. Stationary phase was a PepMap C18 column (15 cm × 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. The pump flow was 50 µL/min. The gradient program was 0–5 min: 100% A, flow 10 → 50 µL/min; 5–60 min: 100% A → 70% B. Injection volume was 20 µL. Subsequently, ion chromatograms of m/z 778.4, the most selective fragment originating from the loss of the phosphoryl moiety from the protonated molecular ion, were generated. Triple quad: MRM transitions of daughter ion 778.4 were recorded. Source CID was 12 V and collision energy was 31 V, with argon as the collision gas at an indicated pressure of 1.5 mTorr. Scan time was 0.1 s.

Results and Discussion

Human plasma was incubated with the n-propyl analogue of VX to inhibit BuChE completely. BuChE was isolated using the procainamide affinity extraction procedure, 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 nonapeptides have three daughter ions in common (602.4, 673.4, and 778.4) independent of the nerve agent that is bonded to the enzyme. The daughter ions can serve as fixed product ion in a parent ion scan. Normally, the ion chromatogram of a transition to daughter ion 778.4 is the "cleanest" chromatogram (5). Therefore, the following parent ion scan [874–1014] → 778.4 was acquired (Figure 2A). A significant background level is present and several peaks that have daughter ion 778.4 in common are visible. The peak at 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 916.4 (mass of n-propyl-VX adduct to the nonapeptide) is shown (Figure 2B). The signal of the peak compared to the background is better compared to the chromatogram in Figure 2A, but the signal-to-noise ratio of the indicated peak in relation to the background is still not striking.

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 the OPCW Schedule 1 components reveals that only side chains of O-alkyl (< C10) and O-cycloalkyl (< C10) and alkyl (< C3) are considered (see also Figure 3) (6). The number of different combinations of organophosphorus compounds (including all iso-alkylforms) are more than thousand, but the number of mass combinations for linear O-alkyl alkylphosphonates is reduced to only 13 discrete combinations with steps of 14 mass units corresponding with one methylene group (i.e., 874, 888, 902, 916, 930, 944, 958, 972, 986, 1000, 1014, 1028, and 1042) (see Table I, first column). Some masses are more likely to occur than others. Mass 874 can occur only in one form and is represented by the nonapeptide with the methylphosphonic acid 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 of less importance that the propyl group can exist as an n-propyl or i-propyl group because these groups show the same mass. V-agents generate the same kind of adduct and are also in this category (Table I, first column). The occurrence of unusual combinations such as methyl sarin, m/z 888, O-decyl propyl phosphonofluoridate, m/z 1042, and analogue 1028 and 1014 is also unlikely. We choose to perform the analysis again while measuring only nine transitions in MRM mode, 874 → 778, 902 → 778, 916 → 778, 930 → 778, 944 → 778, 958 → 778, 972 → 778, and 986 → 778. Figure 4 shows the total ion current (TIC) chromatogram of nine transitions of an n-propyl-VX exposed sample and a blank sample. Although the selectivity of the analysis is significantly increased, an additional peak in the chromatogram of the exposed sample cannot be observed. This means that the individual extracted ion chromatograms have to be evaluated.

Figure 5 shows the extracted ion chromatograms of mass 874, 916, 930, and 944 of an exposed and non-exposed sample. It is clear that only the chromatogram of the exposed sample with mass 916 shows 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 nonapeptide with the O-i-propyl methylphosphyl adduct, which is generated by sarin. The retention times of nonapeptides with other nerve agent adducts are slightly different with a window of approximately 3 min. It implicates that one cannot focus on only one retention time. The ion chromatogram of an exposed sample must be screened for the presence of an additional peak compared with the ion chromatogram of a non-exposed sample. Figure 5 shows that the transition to daughter ion 778.4 is rather unique, as shown by the rather "clean" chromatograms of the unexposed samples.

In this case it is not difficult to point out the peak that represents the nonapeptide with the nerve agent adduct.

The peak was detected while measuring nine transitions, and it is remarkable that the peak height decreased only to approximately 80% of the original signal strength measured in the SRM mode using the same sample (chromatograms not shown).
Table I. Masses of Phosphorylated Nonapeptides (FGES\(x\)AGAAS)\(^*\) Derived After Pepsin Digestion of Human Butyrylcholinesterase Inhibited with Different Classes of Nerve Agents

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<tr>
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<th>Linear Tabun Mass</th>
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* \(x\) stands for nerve agent adduct.

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Figure 4. TIC-chromatogram of nine transitions in MRM mode with daughter ion 778.4. Acquisition ions were 874, 902, 916, 930, 944, 958, 972, 986, and 1000: exposed sample (n-propyl-VX) (A) and non-exposed sample (B).

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Adducts derived from cyclic sarin or tabun derivatives will result in a series of masses with steps of 14 mass units, but different from those of the linear sarin derivatives (see Table I, second and third column). It should be mentioned that the dialkylamido group of tabun is replaced by a hydroxyl group during 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. Interestingly, the mass of the peptides with a cyclotabun adduct are the same as 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 (see first and fourth columns in Table I). 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 resulting in a methylphosphate or dimethylphosphate adduct (mass of the nonapeptide m/z 890 and 904) and (aged) paraoxon resulting in an ethylphosphate or diethylphosphate adduct (m/z 904 and 932). In fact, positive results obtained might be false positives because pesticides are not OPCW Schedule 1 compounds. If a nonapeptide with these masses is found, repeating the digestion with pepsin at higher pH might be considered. At pH 4.7, pepsin remains active, but the alkylamido group remains attached to the phosphorus atom, thus resulting in the mass of a nonapeptide with an intact tabun adduct that is higher than that generated by the organophosphate pesticides. The collection of masses mentioned in the first column of Table I is rather unique. After a survey of 900 pesticides, no compound was found that would result in the formation of a nonapeptide with the same mass as displayed in the first two columns of Table I (7). Other potential ChE inhibitors such as carbamates bear a nitrogen atom, which means that the mass of the nonapeptide will always be different from the masses mentioned in Table I.

Thus, from Table I, it can be derived that...
after pepsin digestion of inhibited BuChE the total of masses that need to be scanned for is 34. In a case where the identity of the inhibitor is not known and one is searching for a possible exposure to an OPCW Schedule 1 compound, it can be chosen to perform four analyses in which nine transitions are measured each time. In that case, exposure to all Schedule 1 nerve agents can be monitored while keeping the loss of sensitivity within acceptable limits.

Conclusions

The adduct of a nerve agent to butyrylcholinesterase is one of the most important and persistent biomarkers for nerve agent exposure (half-life 8–12 days). A method was developed for the verification of exposure to nerve agents, without having pre-knowledge of the identity of the inhibitor. This 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 34 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. The method can be used as a first screening to look for a possible exposure to a nerve agent. Subsequently after finding a positive sample, the digest can be analyzed while acquiring more daughter ions (778.4, 673.4, and 602.4) to obtain a true identification of the nonapeptide with nerve agent adduct.

Acknowledgment

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References


