Quantification of Nerve Agent VX-Butyrylcholinesterase Adduct from an Accidental Exposure

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Abstract

The lack of data in the open literature on human exposure to the nerve agent O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate (VX) gives a special relevance to the data presented in this study in which we report the quantification of VX-butyrylcholinesterase adduct from a relatively low-level accidental human exposure. The samples were analyzed by gas chromatography-high resolution mass spectrometry using the fluoride ion regeneration method for the quantification of multiple nerve agents including VX. Six human plasma samples from the same individual were collected after the patient had been treated once with oxime immediately after exhibiting signs of exposure. Detection limits of approximately 5.5 pg/mL plasma were achieved for the G-analogue of VX (G-VX). Levels of the G-VX ranged from 81.4 pg/mL on the first day after the exposure to 6.9 pg/mL in the sample taken 27 days after the exposure. Based on the reported concentration of human butyrylcholinesterase in plasma of approximately 80 nM, it can be calculated that inhibition levels of ≥ 0.05% of BuChE can be accurately quantified. These data further indicate that the fluoride ion regeneration method is a potentially powerful tool that can be used to assess low-level exposure to VX.

Introduction

Chemical warfare nerve agents are among the most toxic substances ever synthesized. O-Ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate (C11H19 NO3PS, VX) is an organophosphate chemical warfare agent and the most well-known of the V-series nerve agents. Nerve agents like VX work by rapidly binding to a serine residue in the active site of cholinesterase enzymes to form a phosphate or a phosphonate ester, which inhibits the action of these enzymes (1). Additionally, VX is odorless and tasteless, and can be distributed in liquid, aerosol, or vapor form (2). Because of its high viscosity, low vapor pressure (0.0007 mmHg), and environmental persistence, VX is primarily considered a percutaneous hazard (3). However, as a vapor or aerosol, VX can also pose a significant inhalation and ocular hazard. The current VX toxicity estimates for mild effects via inhalation/ocular exposure and threshold effects via percutaneous vapor exposure are 0.1 mg-min/m3 and 10 mg-min/m3, respectively. Because VX poses a significant threat even at low concentrations, the ability to quickly and easily verify exposure to VX using biological samples could be a valuable component in the diagnosis and treatment process.

The first and only documented non-military VX human exposure to date (4) reported results from a victim of the Osaka VX incident in December 1994. A human serum sample was collected from the victim and analyzed for the identification of ethyl methyphosphonic acid (EMPA), the primary hydrolysis product of VX, and 2-(diisopropylaminoethyl)methyl sulfide (DAEMS), the methylthioether of 2-(diisopropylamino)-ethanethiol (DAET, the second hydrolysis product of VX). Analysis of these two metabolites showed a high-level exposure to the agent, which was calculated to be 1.25 μg/mL EMPA and 143 ng/mL DAEMS.

Controlled human studies on the low-level response to nerve agents were carried out in 1965 (5). The purpose of these studies was to determine the dose of nerve agent that would decrease the cholinesterase enzyme activity to less than 30% after administration of the agents VX and sarin by intravenous or oral routes to normal human subjects. According to these studies, it was found that VX is 3–4 times more potent in red blood cell cholinesterase (RBC-ChE) depression than sarin, based on the amount of each one of the nerve agents necessary to inhibit the enzyme activity to 50% in humans. It was also reported that, in humans, VX-inhibited RBC-ChE enzyme undergoes spontaneous reactivation; VX-inhibited RBC-ChE ages very little and is responsive to oxime reactivation for as long as 48 h; and the dose of oxime required to reactivate VX-inhibited

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ChE is much smaller than the dose necessary for sarin-inhibited enzyme. No subject showed more discomfort than mild to moderate gastrointestinal symptoms, which a small dose of atropine easily controlled.

More recently, we analyzed clinical samples from an accidental, low-level human nerve agent exposure. In June 2006, a laboratory worker exhibiting signs of nerve agent exposure was treated with a Mark I antidote kit [pralidoxime chloride (600 mg) and atropine (2 mg)] and monitored for several hours at a local hospital. Post-exposure examination revealed that the worker was exposed to vaporized VX. In the present study, we demonstrate how the fluoride ion regeneration method (6–8) is a valuable and sensitive method for the retrospective quantitative analysis and for biomonitoring of exposure to nerve agents. The results indicate that the method can be used successfully to detect the agent even after the patient has been treated with oxime. Our results illustrate specific advantages in using the fluoride ion regeneration method and how it can serve as a complement to measuring nerve agent urinary metabolites.

Experimental

The method used for this study was similar to that published by Holland et al. (8) for BuChE adducts. The method for urinary metabolites for VX was performed as published by Barr et al. (9).

Sample preparation

The six human plasma samples, obtained with informed consent from the patient, were collected in purple-top Vacutainers containing K$_2$EDTA. The blood samples were separated into red blood cells (RBC) and plasma fractions by centrifugation at 4500 rpm for 10 min at 10°C using an Allegra 25R centrifuge (Beckman Coulter, Palo Alto, CA). Following centrifugation, the plasma fraction was removed and immediately stored at -70°C, and the RBC fraction was refrigerated.

Serum samples [1-mL aliquots; standards, quality control (QC) materials, blanks, and unknowns] were thawed as necessary and placed in 15-mL glass tubes. Then 3 mL of 0.2M sodium acetate buffer at pH 3.5 and 200 µL of 5.25M aqueous KF were added to each tube. Samples were mixed thoroughly and incubated in a water bath at 40°C for 1 h. A solid-phase extraction manifold was prepared with Nexus® cartridges (Varian, Palo Alto, CA), disposable flow valve liners, and waste tubes. The cartridges were conditioned with 2-mL hexanes, 2 × 4 mL chloroform, and 2 × 5 mL water. After the samples were removed from the water bath, 10 µL of the 220-pg/mL internal standard solution containing labeled G-VX-CD$_3$ was spiked into each sample. Samples were mixed thoroughly and the calculated volumes of the native analytes working solutions (5.5, 55, or 550 ng/mL) were added to each one of the eight calibration standards spiked at 5.5, 55, or 550 ng/mL were added to each one of the eight calibration standards to achieve their final concentrations. After mixing, samples were poured onto the pre-conditioned cartridges, and the serum or plasma matrix was drawn through by vacuum into the waste tubes. The analytes were left trapped on the sorbent material while the columns were dried under vacuum for approximately 30 min. The analytes were eluted with 2.5-mL of chloroform, and 200 µL of ethyl acetate was added to the eluting volume. In the final step, the samples were concentrated to a final volume of approximately 50 µL in a TurboVap® LV evaporator (Zymark, Framingham, MA) set at 35°C and 10–15 psi nitrogen flow. Samples were transferred to 1.4-mL silanized glass autosampler vials [Chromacol (Sun-Sri, Duluth, GA) and analyzed by gas chromatography–high-resolution mass spectrometry (GC–HRMS).

**GC-HRMS analysis**

Sample analysis was performed on a Thermo Electron MAT 900 XL Trap double-focusing magnetic sector MS (Bremen, Germany) interfaced to an Agilent 6890 GC (Wilmington, DE). Injection was automated using an A200S autosampler (LEAP Technologies, Carrboro, NC). Ionization was accomplished in electron ionization mode and mass analysis by multiple ion detection in lock mode. Electron energy generally ranged from 40 to 68 eV, with a maximum accelerating voltage of 5 kV. The transfer-line temperature was kept at 210°C, and the source temperature was held at 230°C. FC-43 was used as the mass calibration compound during the analysis, and the instrument was tuned at approximately 7500 resolution (based on the 10% valley definition). The 2-pL injections were made in pulsed splitless mode, with a pulse pressure of 35 psi, a pulse time of 1 min, a purge flow of 50 mL/min, and a purge time of 0.9 min. A 4-mm internal diameter gooseneck liner with no glass wool was used, and the GC front inlet temperature was kept at 200°C. The GC column was a 60-m ZB-5 (0.25-mm i.d., 0.25-µm film thickness, Phenomenex, Torrance, CA). Research grade helium carrier gas was maintained at a constant flow of 1.0 mL/min. The GC program began at a temperature of 68°C for 2.5 min, ramped to 175°C at a rate of 6.7°C/min, held at this temperature for 16 min, and ended with a fast ramp to 310°C at a rate of 50°C/min holding for 2.5 min to clean the column of any possible sample residues.

Each sample set was analyzed in the following order and consisted of eight calibration standards spiked at 5.5, 16.5, 27.5, 82.5, 220, 1100, 3300, and 11,000 pg/mL of native standards; the low and high QC pool and a serum blank, then the unknowns. To ensure no analyte carryover if more than one unknown was included in a single batch, additional serum blanks were prepared and analyzed before analysis of the unknowns.

**Data analysis**

Data analysis and quantification using internal standards were performed using the Xcalibur™ software (Thermo Electron). Each of the individual data files was reviewed and baselines were drawn manually, as needed, to ensure proper identification and quantification. The relative response ratios of the standards were plotted versus concentration to create an eight-point calibration plot, weighted by the reciprocal of the standard concentration. This plot was used for quantification of QC samples, blanks, and unknowns. The ratio of the confirmation ion to the quantification ion was evaluated as a requirement for a positive identification and confirmation of a
nerve agent exposure. After quantification in Xcalibur, data were further analyzed by SAS (SAS Institute, Cary, NC) and in-house CDC software to achieve automated statistical QC analysis.

An exponential decay curve was fitted to all measured values for G-VX over the sample collection period using Microsoft Excel, using the assumption that each sample was collected at the same time of day. The half-life of VX-BuChE adduct was estimated from this decay curve.

Results and Discussion

Post-exposure plasma samples were drawn from the exposed individual on days 1, 6, 8, 13, 20, and 27 after a suspected accidental inhalation of VX. Urine samples were collected on days 1 and 6 after the exposure. We successfully quantified G-VX in all of the patient plasma samples, from day 1 through day 27 following the VX exposure and following treatment with oxime. Figure 1 shows days' 1 and 27 reconstructed ion chromatograms for the patient plasma samples and a blank serum. It is important to note that the patient sample yielded peaks with good signal-to-noise ratios for both the native fragment ion at m/z 99.0011 and the confirmation ion at m/z 111.0011. The calculated signal-to-noise ratio for the m/z 99.0011 analyte ion was 98:1 for day 1 and 25:1 for day 27. The method limit of detection is 5.5 pg/mL for G-VX.

The level of G-VX regenerated in the sample collected 1 day after exposure was 81.4 pg/mL which corresponds to 650 femtoles/mL VX. Individual calibration curves were acquired for each of the six patient samples; their quality is similar to that shown in Figure 2, and all QC measurements were in control (data not shown). The calculated amount of G-VX in the patient samples can be seen in Table I. Concentrations in the patient samples ranged from 81.4 pg/mL G-VX collected on day 1 to 6.9 pg/mL G-VX collected on day 27 following exposure. Levels of the other three nerve agents monitored by our method, sarin, soman, and cyclosarin, were below the method limits of detection for these agents.

The urine samples collected on days 1 and 6 were also analyzed for the detection of ethyl methylphosphonic acid by GC–MS–MS. The limit of detection for this analyte is 0.9 ng/mL. Results for these two samples were below the limit of detection. In that regard, an interesting observation is that the VX-BuChE adduct method was sensitive enough to confirm this low-level exposure while the urinary metabolite levels were below the limit of detection. Additionally, the timing of urine collection after exposure is critical for the detection of specific urinary metabolites to a single acute event. If urine is collected too soon, the nerve agent may not have had a chance to be metabolized and be excreted. If a urine sample is collected too late, then most of the nerve agent hydrolysis products may have already been excreted. Because the lifetime of the adduct is the same as the average lifetime of the BuChE.

Figure 1. Reconstructed ion chromatograms for the G-analogue of VX (G-VX); m/z 99.0011 for native G-VX fragment ion in patient plasma sample collected 1 day after exposure (A); m/z 99.0011 for native G-VX fragment ion in patient plasma sample collected 27 days after exposure (B); and the same ion, monitored for a blank serum sample (C). This figure is a composite of three separate gas chromatography–high resolution mass spectrometric analyses. Traces B and C in the analysis of sample 6 and the blank were normalized to match the trace from the analysis of the patient sample in day 1 after exposure.

Figure 2. Eight-level standard curve, covering the range 5.5 pg/mL to 11,000 pg/mL of G-VX, used to quantify the exposed patient sample collected 1 day after exposure. Inset: detail of the lowest five standards.

Table I. Summary of Post-Exposure Samples, Collection Dates, and Results after Analysis and Quantification for a Subject Exposed to Low Levels of VX in a Laboratory Setting

<table>
<thead>
<tr>
<th>Collection Date</th>
<th>Days Post-Exposure</th>
<th>Results (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/2/2006</td>
<td>1</td>
<td>81.2</td>
</tr>
<tr>
<td>6/7/2006</td>
<td>6</td>
<td>47.1</td>
</tr>
<tr>
<td>6/9/2006</td>
<td>8</td>
<td>24.2</td>
</tr>
<tr>
<td>6/14/2006</td>
<td>13</td>
<td>18.1</td>
</tr>
<tr>
<td>6/21/2006</td>
<td>20</td>
<td>11.0</td>
</tr>
<tr>
<td>6/28/2006</td>
<td>27</td>
<td>6.9</td>
</tr>
</tbody>
</table>

* Accidental exposure occurred on June 1, 2006.
† The method LOD for G-VX is 5.5 pg/mL.
adducts can be analyzed relatively soon after the exposure and over a much longer period of time.

Figure 3 plots the calculated amounts of G-VX versus days after exposure. Reference range studies carried out in our laboratory with 100 individual serum samples from people with no known or suspected exposure to VX (8) indicated that no level of G-VX is above the method limit of detection in individuals who are not exposed to VX and we found no interferences. With the assumption that the background pre-exposure level for the individual who was exposed in this study is also below the method limit of detection, and that free VX is absent, we estimated the half-life of VX-BuChE adduct to be 7.5 days. This is consistent with previously published measurements of the half-life for butyrylcholinesterase of between 5 and 16 days (10).

Immediately after showing symptoms of organophosphate toxicity, the individual examined in this study was administered one Mark I nerve agent antidote kit [pralidoxime chloride (600 mg) and atropine (2 mg)]. Initially, the individual reported blurred vision and miosis. Delayed symptoms included mild rhinorhea and eyelid muscle fasciculation. Miosis and other symptoms disappeared within 24 h of the exposure. Later review concluded that the individual was exposed to low levels of vaporized VX for approximately 60 min. Air monitoring results indicated that, nearest the suspected exposure area, 26 times the worker population limit (WPL) for VX vapor exposure, which is 10⁻⁶ mg/m³, was measured for VX (using a G-VX analytical method for VX). However, because the monitoring was done hours after the exposure, the actual vapor concentration during the exposure incident may have been higher. Although, in this case, it is not possible to calculate the “actual” exposure concentration, estimates of possible concentrations would easily fall within the range recently predicted for miosis in humans, which is at the EC₀ (miosis) for a 120-min exposure at 0.000087 mg/m³ and at the EC₅₀ (miosis) for a 120-min exposure at 0.000333 mg/m³ (11).

No other human VX-exposure measurements have been documented in the open literature. However, measurements of other nerve agent BuChE adducts have been previously reported (6), specifically in serum samples from the victims of the sarin terrorist attack in Tokyo, Japan in 1995 and from sarin incident at Matsumoto a few months earlier. It was found that, after reactivation with fluoride ions and analysis by high-resolution single ion monitoring GC–MS, the amount of regenerated sarin in the samples from the Tokyo incident was ≤ 4.1 ng sarin/mL serum. Because of the excellent sensitivity in our current method for measuring nerve agent adducts, we were able to detect levels much lower than those reported for the Tokyo subway incident in a subject who showed only mild symptoms. The ability to detect exposure at such low levels could prove critical in distinguishing victims of a low-level exposure from a chemical terrorism attack from the “worried well”.

Conclusions

There is little data in the peer-reviewed literature on levels of nerve agents in clinical samples following human exposure. The use of the appropriate deuterium isotopic labeled standards coupled with GC–HRMS provided a highly selective analysis and facilitated precise and accurate quantification of the nerve agent VX in the plasma-3 of an accidentally exposed individual. In this case, the level of VX exposure was relatively low, and the symptoms appear to have been minor. Nevertheless, by using our method we were able to confirm the presence of VX (in its fluoridated form) in samples at a level of 6.9 pg/mL collected as late as 27 days following the single exposure event. To put the amount of the detected adduct into perspective, it corresponds to around 0.05% BuChE inhibition in an individual with normal BuChE levels of 80nM (12), thus demonstrating this method’s outstanding sensitivity.

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