Quantitation of Fluoride Ion Released Sarin in Red Blood Cell Samples by Gas Chromatography-Chemical Ionization Mass Spectrometry Using Isotope Dilution and Large-Volume Injection

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Abstract

A new method for measuring fluoride ion released isopropyl methylphosphonofluoridate (sarin, GB) in the red blood cell fraction was developed that utilizes an autoinjector, a large-volume injector port (LVI), positive ion ammonia chemical ionization detection in the SIM mode, and a deuterated stable isotope internal standard. This method was applied to red blood cell (RBC) and plasma ethyl acetate extracts from spiked human and animal whole blood samples and from whole blood of minipigs, guinea pigs, and rats exposed by whole-body sarin inhalation. Evidence of nerve agent exposure was detected in plasma and red blood cells at low levels of exposure. The linear method range of quantitation was 10-1000 pg on-column with a detection limit of approximately 2-pg on-column. In the course of method development, several conditions were optimized for the LVI, including type of injector insert, injection volume, initial temperature, pressure, and flow rate. RBC fractions had advantages over the plasma with respect to assessing nerve agent exposure using the fluoride ion method especially in samples with low serum butyrylcholinesterase activity.

Introduction

Chemical warfare nerve agents such as sarin (GB, isopropyl methylphosphonofluoridate, CAS 107-44-8) represent some of the most toxic substances available for misuse by nations and terrorist groups. The acute toxicity of nerve agents and organophosphorus pesticides is caused by inhibition of acetylcholinesterase, which produces an excess of the neurotransmitter acetylcholine in central, peripheral, and neuromuscular synapses. The results of excess acetylcholine include miosis, sweating, excessive salivation, muscle fasciculation, seizure, and respiratory failure. Typically, organophosphorus inhibitors of acetylcholinesterase and similar esterases react covalently with the serine residue at the active site to produce the O-alkyl methylphosphonylated enzyme and a free leaving group (1). In the case of sarin, the leaving group is fluoride ion. The inhibition is frequently considered irreversible because the enzyme is incapable of removing the O-alkyl methylphosphonate in a timely manner without the addition of exogenous nucleophilic chemicals such as oximes (2-pralidoxime, 2-PAM). Over time, the O-alkyl methylphosphononylated enzyme undergoes a process known as “aging” in which the O-alkyl group is removed by hydrolysis to produce the highly stable methylphosphonylated enzyme. After aging, the enzyme cannot be reactivated even in the presence of oximes, fluoride ion, or other chemicals.

Exposure assessment is critical with respect to justifying the course of medical treatment of exposed individuals and preventing further contamination from secondary exposures. Historically, exposure assessment was limited to criteria based on medical observations of the signs and symptoms of cholinergic crisis and blood cholinesterase inhibition. However, cholinesterase inhibition is not specific, and many different chemicals will cause a decrease in enzyme activity. Furthermore, variations in cholinesterase activity within individuals and populations have made minor exposures difficult to detect.

In addition to reacting with cholinesterase, nerve agents such as sarin hydrolyze under physiological conditions to produce alkyl methylphosphonates. Exposure verification methods using either gas chromatography (GC) or liquid chromatography (LC) were developed for sarin, cyclohexylsarin, VX, and related compounds that could analyze for their alkyl methylphosphonate metabolites in blood, tissue, and/or urine (2-4). The hydrolysis product of sarin, isopropyl methylphosphonic acid (IMPA), was found in the serum, brain tissue, and urine of the Tokyo subway victims (5-8). Evidence of sarin exposure in the Tokyo subway victims was also provided by the electrospray tandem mass spectrometric (MS) analysis of phos-
Phosphorylated nonapeptides from serum butyrylcholinesterase after pepsin digestion (9). This method has the ability to detect nerve agent inhibited butyrylcholinesterase, even after aging has occurred. Another recent analytical development to improve the specificity and sensitivity of exposure assessment includes a GC–MS method for measuring fluoride ion regenerated alkyl methylphosphonofluoridates from plasma butyrylcholinesterase (both human and animal). This method was able to verify sarin exposure in the plasma samples of certain victims of the Aum Shinrikyo cult terrorist attacks in a Matsamoto neighborhood in 1994 and the following year in the Tokyo subway (10).

Methods based on detection of alkyl methylphosphonates have been useful because they indicate the individual was exposed to a chemical that yields this product. Thus, sarin readily hydrolyzes chemically and enzymatically to isopropyl methylphosphonic acid (IMPA) which has been measured in the blood, tissue, and urine. However, IMPA can also be produced by O,O'-diisopropyl methylphosphonate (11) and bis(O-isopropyl methyl) pyrophosphate (12). Analysis of IMPA by GC has required derivatization, an added complex and time-consuming step to the sample preparation.

Of the methods available to assess nerve agent exposure, the fluoride ion regeneration method has demonstrated some unique advantages over cholinesterase inhibition assays and the alkyl methylphosphonate methods. The first advantage is greater specificity, especially with respect to methods based on cholinesterase activity. These methods can only indicate that some factor has decreased enzyme activity. The specificity advantage over the alkyl methylphosphonate methods results from the ability of the fluoride ion method to discriminate between toxic cholinesterase inhibitors and relatively non-toxic precursors of the alkyl methylphosphonates. In order to yield detectable results with the fluoride ion method, the toxic agent must have first reacted with the cholinesterase. Therefore, it is required to have a suitable leaving group that permits the nucleophilic reaction at the serine residue. However, with respect to the alkyl methylphosphonate methods, the source of an alkyl methylphosphonate in a physiological sample is not specific to a nerve agent exposure. Alkyl methylphosphonates will be present in blood and urine after exposure to nerve agents, the alkyl methylphosphonates themselves, or their related dialkyl methylphosphonates. The second advantage of the fluoride ion method is the generation of volatile nerve agents as analytes, which negates the need for derivatization as is necessary with the GC-based alkyl methylphosphonate methods.

This paper presents an extension of the fluoride ion regeneration method that incorporates solid-phase extraction (SPE), ammonia chemical ionization (CI) in the positive ion mode, a large-volume injector (LVI) with an autosampler, and a stable isotope internal standard for measuring sarin in red blood cell (RBC) samples. In the course of developing modifications to this method, the following performance characteristics were established, including optimizing LVI parameters, identifying the useful reportable range, estimating the accuracy and precision, and determining detection limits. In addition, advantages of analyzing the RBC fraction of the blood in cases of suspected exposure to sarin have been presented.

The method validation studies presented here utilized blood samples collected from Gottingen minipigs, which are being used as a model of nerve agent inhalation exposure. They share many important anatomical and physiological characteristics with humans (13), including the near absence of circulating carboxylesterase in the blood. This is important because carboxylesterase, which is present in rat blood, has been shown to react with nerve agents. This, in turn, can have an impact on the animal model’s sensitivity to nerve agent-induced toxic effects (14).

Experimental

Chemicals and materials

Analytical grade solvents were obtained from Aldrich Chemical Company (Milwaukee, WI). Sarin and 2H6-sarin (2H6-isopropyl methylphosphonofluoridate) of purity greater than 97% were procured from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Gases were obtained from Messer, Inc. (Chattanooga, TN) and had a minimum purity of 99.999%. All chemicals, solvents, and gases were used as obtained with no further purification.

Standard preparation and characterization

Sarin was weighed and diluted in hexane to give a concentrated stock solution of 1.829 µg/mL. The concentrated stock solution of 2H6-sarin was 1.415 µg/mL in hexane. Hexane was used for the concentrated stock because refrigerated solutions of sarin in hexane are stable for extended periods. The sarin concentrated stock was then diluted serially in ethyl acetate to produce two working standards at 1.829 µg/mL and 0.1829 µg/mL. These solutions were used to spike red blood cell samples of approximately 0.2000 g to generate concentrations in the final extract of 0.2195, 0.4938, 1.006, 1.994, 4.024, 8.048, 12.07, 15.91, and 19.94 ng/mL. 2H6-Sarin was diluted in ethyl acetate to a working standard of 2.829 µg/mL that was then used to spike the samples in order to yield a final concentration of 20.00 ng/mL of final extract.

Control samples

Whole blood from pre-exposed Gottingen minipigs (9–12 kg, Marshall Farms, North Rose, NY) and Sprague-Dawley rats (314–344 g, Charles Rivers Laboratories, Wilmington, MA) were used as control samples. Human whole blood was collected from laboratory volunteers.

Minipig, guinea pig, and rat sarin exposure

In conducting this study, investigators adhered to the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication No. 86-23, 1985, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, Washington, D.C.). These investigations were also performed in accordance with the requirements of AR 70-18, “Laboratory Animals, Procurement, Transportation, Use, Care,
and Public Affairs", and the U.S. Army Edgewood Chemical and Biological Center Institutional Animal Care and Use Committee (IACUC), which oversee the use of laboratory animals. Pigs were surgically prepared under anesthesia with jugular canulas for serial blood sampling during exposure. Whole-body sarin exposure of minipigs was conducted in a 1000-L chamber using concentrations ranging from 0.01 to 12.8 mg/m³. No anesthetic, pretreatment, or post-treatment drugs were given to test animals in order to simulate realistic exposure conditions. Whole-body sarin exposure of rats and guinea pigs was conducted in a 750-L chamber using concentrations ranging from 2 to 54 mg/m³. Details of the inhalation exposure apparatus and methods are published elsewhere (15). Cannulated minipigs were also injected subcutaneously behind the ear with sub-lethal doses of GB in saline. Blood samples (7–10 mL) were collected in ethylenediaminetetraacetic acid (EDTA) tubes before, during, and after the agent exposures.

Sample preparation

Whole blood spiking studies used human, minipig, guinea pig, or rat blood stabilized with EDTA that was spiked to levels from 1 to 15 ng/mL blood. The whole blood was mixed gently by inversion for 10 min at room temperature before separation of the plasma and RBC by centrifugation at 15000 rpm for 5 min (Micromax, Thermo IEC, Needham Heights, MA). After separation, the plasma samples were frozen until analysis and RBC samples were refrigerated at 5°C.

Blood samples were collected from minipigs via a jugular catheter before, during, and after whole-body exposure to sarin vapor. Blood samples from rats were collected from the tail vein before and after whole-body exposure to sarin. In the case of agent-induced lethality, blood from dead rats was collected via cardiac puncture. Blood was taken from guinea pigs by toenail clip. All blood samples were collected in standard EDTA-containing tubes and then centrifuged at 15000 rpm for 5 min to separate the plasma from the red blood cells. Plasma was frozen until analysis and RBCs were refrigerated at 5°C.

RBC and plasma samples were extracted using C₁₈ SPE columns (200 mg Sep-Pak, Waters Associates, Millipore Corp., Milford, MA) which were first conditioned with 1 mL each of ethyl acetate, followed by isopropanol, and then pH 3.5 acetate buffer. The SPE columns were not allowed to dry after the acetate buffer and were covered until they were used. The type of sample determined the exact treatment that was used to generate the final ethyl acetate extract for GC–MS analysis. For RBC samples, amounts were weighed in a tared centrifuge microvial with sample sizes ranging from 0.2 to 0.5 g. To RBC samples, 1 mL of acetate buffer and 200 µL of KF solution (6M) were added, and the mixture was vortex mixed for 10–20 s and then centrifuged at 15000 rpm for 5 min. The sediment at the bottom of the microvial was resuspended with 750 µL of acetate buffer and 200 µL of KF solution. This mixture was vortex mixed, followed by centrifugation at 15000 rpm for 5 min, and the resulting liquid was then added to the original solution. Fifteen minutes after the original addition of buffer and KF, the combined reaction mixture was allowed to drain through the conditioned SPE column under a gentle vacuum. After complete draining, the SPE column was washed with 500 µL of acetate buffer and allowed to dry using a light vacuum to pull air through the column for 3 min. The analytes were eluted with 1 mL of ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was removed from the collection tube and filtered through a 0.2-µm nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial for analysis.

For plasma samples, 200-µL aliquots were weighed to the nearest 0.1 mg in a tared 2.0-mL microvial. Acetate buffer (1 mL) and 100 µL of KF solution (6M) were added and the mixture was vortex mixed for 10–20 s. This initial reaction mixture was transferred onto the conditioned C₁₈ SPE cartridge. The sample microvial was washed with a mixture of 750 µL acetate buffer and 100 µL KF solution which was added to original reaction mixture on the SPE column. The reaction mixture was the filtered by the conditioned C₁₈ column. The analytes were eluted with 1 mL of ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was removed from the collection tube and filtered through a 0.2-µm nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial for analysis.

Instrumental analysis

Injections of 50 µL of extract were made by autoinjector into the LVI (model PTV, Agilent Technologies, Wilmington, DE) using the following parameters: initial temp −20°C; initial time 5.1 min; final temp 225°C; rate 720°C/min (maximum ballistic heating as listed in the Agilent manual); vent time 5.00 min; vent flow 300 mL/min; purge flow 50 mL/min; and purge time 8.7 min.

Either an HP-5MS (Agilent Technologies) or a Rtx-1701 (Restek, Inc., Bellefonte, PA) was used with a flow rate of 3 mL/min (63 cm/s) and using the following GC (Agilent Technologies model 6890) oven program: −20°C (9.3 min) to 50°C at 40°C/min, to 64°C at 2°C/min, and to 275°C (2 min) at 50°C/min.

MS detection (Agilent Technologies model 5793 MSD) was by chemical ionization with ammonia reagent gas in the positive ion mode using the m/z 158/164 ammonia adduct ion ratio (GB/²H₆-GB) for quantitation and the m/z 175 (GB) and 181(²H₆-GB) ions as qualifiers. Linear internal standard calibration curves for GB were generated from 10 to 1000 pg using standards in ethyl acetate.

Data processing and analysis

The Agilent software (Agilent Technologies model 6890) provided with the MS was used to process and analyze the data. The software allowed automatic analysis of the internal standard method based on the analyte area ratios of the peaks at their respective retention times. A trained analyst checked the data for proper peak selection, peak shape, evaluation of baseline, and interferences.

Quantitation

Calibration standards were prepared from ethyl acetate enriched with concentrations of 0.20, 0.50, 1.00, 2.00, 4.00, 8.00, 12.0, 16.0, and 20.0 ng/mL of native sarin and 20.0 ng/mL deuterated sarin. Because 50 µL of each standard was injected
were evaluated. The lower limit of the range could be extended by a factor of 10 by concentrating the sample volume to 0.1 mL. The 14%-cyanopropylphenyl 86%-dimethylpolysiloxane (RTX-1701) column yielded excellent resolution and retention time consistency. Precision and accuracy was demonstrated at the 10, 200, and 1000 pg spike levels (on column), which yielded 12.89 ± 0.578 pg (4.48% RSD, relative standard deviation), 237.4 ± 13.11 pg (5.52% RSD), and 1100 ± 42.65 pg (3.88% RSD), respectively. The result of the MDL calculation was 1.8 pg on column. Furthermore, the root-mean-square signal-to-noise ratio for a 10-pg GB spike was 150, which indicated there was an abundance of signal at the lowest standard, which corresponded to the limit of quantitation. Recoveries were determined by comparing the instrument response to sarin directly injected into the GC–MS versus the same amount in spiked RBC samples processed through the entire method. Percent recoveries were determined by comparing the instrument response to sarin directly injected into the GC–MS versus the same amount in spiked RBC samples processed through the entire method. Percent recovery.

**Recovery.** Recoveries were determined by comparing the instrument response to sarin standards in ethyl acetate directly injected into the GC–MS versus the same amount in spiked RBC samples processed through the entire method. Percent recovery was studied by means of recovery assays, which represented control minipig RBC samples of approximately 0.200 g spiked at three concentrations to generate 10, 200, and 1000 pg on column (n = 5 each). Before spiking, the samples were treated with the pH 3.5 acetate buffer and 200 μL of potassium fluoride solution (6M) to simulate the biomarker regeneration conditions. After reagent addition and agent spiking the samples were fortified with 2H6-GB as the internal standard.

**Precision.** The precision of the method was determined as the repeatability of the recoveries of five replicates at each of three spiking levels within a day (10, 200, and 1000 pg on column). Long-term precision was evaluated over the course of eight months by analysis of control minipig RBC samples (0.2–0.4 g) spiked to generate 200 pg on column. Sample matrix was spiked after addition of acetate buffer and KF solution.

**Method detection limit (MDL).** The MDL was determined by the analysis of seven replicate minipig RBC samples spiked near the detection limit. The standard deviation of the replicates is multiplied by the one sided Student t value of 3.143 (for six degrees of freedom at the 99% confidence level) to generate the MDL (16).

**Results**

The isotope dilution method demonstrated the ability to detect nanomolar amounts of GB despite the complexity of the red blood cell matrix. The reportable range was 10–1000 pg of sarin on column which, for a 50-μL injection, equals a GB concentration range of 1.428–142.8 nanomoles in a final ethyl acetate volume of 1 mL. The lower limit of the range could be extended by a factor of 10 by concentrating the sample volume to 0.1 mL. The 14%-cyanopropylphenyl 86%-dimethylpolysiloxane (RTX-1701) column yielded excellent resolution and retention time consistency. Precision and accuracy was demonstrated at the 10, 200, and 1000 pg spike levels (on column), which yielded 12.89 ± 0.578 pg (4.48% RSD, relative standard deviation), 237.4 ± 13.11 pg (5.52% RSD), and 1100 ± 42.65 pg (3.88% RSD), respectively. The result of the MDL calculation was 1.8 pg on column. Furthermore, the root-mean-square signal-to-noise ratio for a 10-pg GB spike was 150, which indicated there was an abundance of signal at the lowest standard, which corresponded to the limit of quantitation. Recoveries were determined by comparing the instrument response to sarin directly injected into the GC–MS versus the same amount in spiked RBC samples processed through the entire method. Percent recoveries were determined by comparing the instrument response to sarin standards in ethyl acetate directly injected into the GC–MS versus the same amount in spiked RBC samples processed through the entire method. Percent recovery was studied by means of recovery assays, which represented control minipig RBC samples of approximately 0.200 g spiked at three concentrations to generate 10, 200, and 1000 pg on column (n = 5 each). Before spiking, the samples were treated with the pH 3.5 acetate buffer and 200 μL of potassium fluoride solution (6M) to simulate the biomarker regeneration conditions. After reagent addition and agent spiking the samples were fortified with 2H6-GB as the internal standard.

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ers at 10-, 200-, and 1000-pg on-column spike levels were 55 ± 3, 48 ± 10, and 39 ± 4%, respectively. Adjusting the results for recovery was unnecessary because of the use of a stable isotope internal standard. Representative data demonstrating the linear regressions generated by both GB-spiked minipig RBC and GB-ethyl acetate standards are shown in Figure 1. On a daily basis, the calibration was checked by analyzing GB standards in ethyl acetate and analyzing matrix spike samples (0.2-0.4 g RBC) spiked to yield 200 pg of GB on column (Figure 2). The 95% and 99% confidence limits of the matrix spike recoveries were calculated based on the mean and standard deviation of the spiking results, which was calculated to be 97.8 ± 9.22. The 95% confidence limit was equal to 97.8 ± (9.22), and the 99% confidence limit was equal to 97.8 ± 2.7(9.22). The analytes were stable for at least four weeks in separated blood samples stored either at 5°C for RBC or below -10°C for plasma.

For native GB, the ions at m/z 158 and 175 represented the [M+NH₄]⁺ and [M+NH₃+NH₄]⁺ adduct ions, respectively. Analogous ions were seen for the deuterated internal standard. Figure 3 is an example of the extracted ion chromatograms of a minipig red blood cell sample extract after whole-body GB vapor exposure at approximately 0.04 mg/m³ for 60 min using the developed method. The positive ammonia CI conditions also generated higher ammonia-GB adducts which were used as qualifying and tuning ions.

Control samples were analyzed to determine if the analyte or an interference was present in blood from laboratory animals or the general population. For minipigs, guinea pigs, and rats, the blood samples were either from animals before exposure or from control animals. For the human blood, samples from several volunteers were collected. There was no measurable amount of GB in any of the control blood samples tested.

Results for whole blood from control animals and human volunteers that were spiked with various amounts of GB to determine the distribution of agent between the plasma and the red blood cells are presented in Table I. The utility of the method for describing the absorption of GB during an inhalation exposure and a subcutaneous injection in the minipig is shown in Figure 4. The upper panel (A) presents the fluoride ion regenerated GB (R-GB) results of the 10-min whole-body exposure of GB at a concentration of 5.1 mg/m³ in a 10.5-kg animal. The lower panel (B) presents the 12.5 μg/kg subcutaneous injection of GB, diluted in saline, in a 10.0-kg minipig. The blood samples were separated into RBC and plasma fractions and then analyzed. In all minipig GB exposure studies, levels of regenerated GB found in the RBC fraction were 10 to 40 times greater than levels found in the plasma.

A comparison of the relative changes that can occur in R-GB levels postmortem in plasma and RBC samples is shown in Figure 5. Each group of 10 animals was exposed to GB vapor for 10 min at concentrations ranging from 23 to 39 mg/m³. Samples were taken 60 min after the end of the exposure. The average results of the surviving animals were compared to the dead animals within each group.

### Table I. Whole Blood Analysis for GB After Either Spiking or Exposure

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spike Level (ng)</th>
<th>Plasma (ng/g)</th>
<th>RBC (ng/g)</th>
<th>Total Spike % Recovery</th>
<th>% in Plasma</th>
<th>% in RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minipig spike</td>
<td>1.39</td>
<td>0.068</td>
<td>0.777</td>
<td>60.8</td>
<td>8.1</td>
<td>92.0</td>
</tr>
<tr>
<td>Minipig spike</td>
<td>5.49</td>
<td>0.521</td>
<td>2.828</td>
<td>61.0</td>
<td>15.6</td>
<td>84.4</td>
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<tr>
<td>Minipig spike</td>
<td>12.80</td>
<td>3.260</td>
<td>5.250</td>
<td>66.5</td>
<td>38.3</td>
<td>61.7</td>
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<tr>
<td>Minipig spike</td>
<td>16.46</td>
<td>5.242</td>
<td>5.647</td>
<td>66.2</td>
<td>48.1</td>
<td>51.9</td>
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<tr>
<td>Human spike</td>
<td>2.10</td>
<td>0.509</td>
<td>0.516</td>
<td>40.0</td>
<td>49.7</td>
<td>50.4</td>
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<tr>
<td>Human spike</td>
<td>4.08</td>
<td>1.210</td>
<td>0.848</td>
<td>40.2</td>
<td>58.8</td>
<td>41.2</td>
</tr>
<tr>
<td>Human spike</td>
<td>8.07</td>
<td>3.063</td>
<td>1.420</td>
<td>43.8</td>
<td>68.3</td>
<td>31.7</td>
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<tr>
<td>Guinea Pig spike</td>
<td>10.1</td>
<td>6.44</td>
<td>2.75</td>
<td>75.9</td>
<td>70.1</td>
<td>29.9</td>
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<tr>
<td>Rats exposed</td>
<td>na</td>
<td>379.0</td>
<td>16.40</td>
<td>na</td>
<td>95.8</td>
<td>4.20</td>
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<tr>
<td>Minipig exposed</td>
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<td>0.4813</td>
<td>15.47</td>
<td>na</td>
<td>3.02</td>
<td>96.98</td>
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<tr>
<td>Guinea Pigs exposed</td>
<td>na</td>
<td>41.02</td>
<td>4.70</td>
<td>na</td>
<td>88.69</td>
<td>11.31</td>
</tr>
<tr>
<td>Guinea Pigs exposed*</td>
<td>na</td>
<td>0.84</td>
<td>0.84</td>
<td>na</td>
<td>0.0</td>
<td>100.00</td>
</tr>
</tbody>
</table>

* Average n = 3.

Discussion

Optimal LVI conditions were dependent on the physical properties of the analyte because the liquid nitrogen cryotrap must be set to a temperature that will effectively stop the analyte but not the solvent. GB analysis conditions that needed to be optimized for the LVI included injection insert, injection volume, initial temperature, initial time, pressure, purge flow rate, and purge time. An injection insert packed with Tenax was critical for operation of the method. Although solvents such as methyl acetate, hexane, and methanol were evaluated, they were found to be problematic to the sample preparation or to the LVI. An earlier study found that methanol was optimal for the analysis of sarin by capillary GC using LVI (17). Ethyl acetate could not be used because it was incompatible with the thermionic detector that was being employed. The LVI in this earlier study could not be automated because of deactivated fused-silica tubing that needed to be connected and removed with each injection.

Previously, the fluoride ion reactivation method was used on serum samples to retrospectively detect exposure (10). Serum or plasma was considered the preferred sample because man and most animals have circulating esterases such as butyrylcholinesterase that provides a pool of suitable binding sites. Red blood cells contain acetylcholinesterase that also reacts with organophosphorus nerve agents. In humans, a 27-fold excess of binding sites in the serum/plasma butyrylcholinesterase has been reported as compared to the red blood cell acetylcholinesterase (10). These studies did not actually spike whole blood to demonstrate the 27-fold excess in the serum/plasma over the red blood cells. However, there are examples of the preferential
binding of nerve agents to acetylcholinesterase over butyrylcholinesterase (18). Despite the preferential binding to acetylcholinesterase, the majority of the accessible GB should be located in the serum/plasma because of the large excess of binding sites according to previous studies.

Spiking whole blood (human) did not necessarily result in levels of GB that were much greater in plasma than in red blood cells (Table I). At the 2.10-ng GB spiking level, the percent recovered from both sources was almost identical. As the spiking level increased the percentage in the human plasma increased but never achieved a 27-fold excess as had previously been reported (10). This may indicate that there are more binding sites in the red blood cells than just those related to acetylcholinesterase. A similar trend was seen with the minipig spiking results. However, as the percentage of R-GB in the plasma increased with increasing spiking level the results deviated from what would have been seen during an actual inhalation or subcutaneous exposure in the minipig. During all exposure experiments in the minipig, from miosis to lethal levels, the red blood cell samples yielded 10–50 times the amount of R-GB found in the plasma. Figure 4 illustrates R-GB data for inhalation and subcutaneous injection in the minipig that supports the differences seen between RBC and plasma levels. Pigs have low levels of butyrylcholinesterase and almost no carboxylesterase, which could explain the predominance of the nerve agent in the red blood cells (19). As spiking levels increased above a certain concentration in the blood, which is not usually achieved in vivo, binding of lower affinity sites in the plasma became competitive. A tyrosine residue of serum albumin has been shown to bind nerve agents and may represent one of the lower affinity sites (20).

Table I also has listed results from rats and guinea pigs for comparison. Rats produced the highest recoveries of regenerated GB from the plasma with over 95% of the recovered agent. Rats are known to have plasma carboxylesterase (21) as well as plasma butyrylcholinesterase and acetylcholinesterase that produce a large sink for organophosphorus nerve agents (21,22). Similar to those collected from the rats, the samples collected 1 h after exposure of guinea pigs to GB vapor showed R-GB recoveries in the plasma that were a factor of eight greater than the levels in the RBC. If the same guinea pigs are sampled (minus one that died) on day 7, then the levels in the plasma are not detectable while small amounts can still be quantitated in the red blood cells. Published studies of regenerated nerve agents in guinea pig plasma showed that the regenerated agent levels dropped rapidly in the living animals but decreased much slower postmortem (23).

The fluoride ion regeneration method in RBC samples is a potential dosemetric tool that may allow comparisons of different routes of exposure. To be an effective dosemetric, levels of R-GB need to reflect the absorption of agent in an animal or human. The ability of the fluoride ion regeneration method in RBC samples to follow the course of an exposure in the minipig is demonstrated in Figure 4. Inhalation exposure of GB produced a steady rise after an initial delay in the levels of R-GB that are seen. In contrast, the subcutaneous exposure produced a more rapid rise in R-GB levels followed by a plateau. The plasma R-GB levels responded in a similar fashion but at much-reduced levels.

The relative change in R-GB levels in rat plasma and RBC samples before and after death (Figure 5) demonstrates the stability of bound GB in the red blood cells. Plasma levels drop quickly after death in rats although overall levels are still greater than those found in the RBC samples. However, because of its stability, bound GB in RBC samples would appear to be a better source to estimate the exposure dose.

Conclusions

An ammonia chemical ionization GC–MS method has been developed for the analysis of the nerve agent GB in RBC samples.
after fluoride ion reactivation involving a simple and rapid sample preparation with a GC run time of about 25 min per sample. The fluoride ion regeneration method in RBC samples has some distinct advantages over other methods that evaluate GB exposure. These include increased specificity and the ability to assess exposures in humans and animals despite large variations that exist in the levels of plasma cholinesterase and other plasma binding sites. The fluoride ion regeneration assay using RBC samples is the most sensitive method to describe and monitor exposure in animals that lack GB plasma binding sites in rats. Overall, evaluating both plasma and RBC samples for R-GB develops a more complete picture of nerve agent exposure.

References


