Validation and Application of a GC–MS Method for Determining Soman Concentration in Rat Plasma Following Low-Level Vapor Exposure

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Abstract

A method for determining the chemical warfare agent soman (GD) in rat plasma has been validated and applied to low-level inhalation exposure studies currently being conducted. This method utilizes a fluoride ion-based regeneration assay with isotope dilution followed by large volume injection gas chromatography with ammonia chemical ionization mass spectrometric detection. Following sample preparation by solid phase extraction, chromatographic separation was achieved using a 14% cyanopropylphenyl/86% dimethyl polysiloxane capillary column with a total run time of 18.16 min. Soman and the deuterated isotope (2H4-soman) internal standard were detected using the selected ion monitoring mode and quantitated using the ammonia adduction ratio of m/z ions 200/204. A reproducible linear relationship was obtained for the quantitative concentration range of 10 pg on-column to 1000 pg on-column (r² = 0.9995) for standards in ethyl acetate with a detection limit of 5.65 pg on-column, and an average recovery of 93% in plasma. This sensitive method was successfully applied to the analysis of soman in rat plasma immediately post-exposure, resulting in the construction of dose-response plots.

Introduction

The chemical warfare nerve agent O-pinacolyl methylphosphonofluoridate (Figure 1), also known as soman (GD, CAS Registry Number: 96-64-0) is an extremely toxic organophosphorus compound that inhibits the enzymatic activity of acetylcholinesterase (AChE). In the reaction of soman with AChE, soman loses a fluoride ion (1) and phosphorylates the serine residue (Figure 2) in the active site of AChE, thereby inhibiting its ability to hydrolyze the neurotransmitter acetylcholine. This results in the accumulation of acetylcholine and overstimulation of cholinergic receptors (2). Signs of exposure

(R)-

Figure 1. Structure of soman.

Figure 2. Soman binding to the serine residue of AChE and “aging” of the soman-AChE complex: soman (A); serine residue of AChE (B); phosphorylated enzyme (c); carbocationic intermediate (d); dealkylation products (e); and aged enzyme (f). (Adapted from 20, 21).
to high levels of soman include muscle twitches, tremors, convulsions, seizures, salivation, miosis, and death attributable to respiratory failure (1). At lower doses, the majority of these toxic signs are not typically observed, necessitating the development of alternative exposure detection methods.

In today’s world, the use of chemical warfare agents such as soman poses a significant threat. Despite efforts to end the use of such agents (3), several incidents involving the terrorist use of nerve agents have occurred (4,5). In addition, nerve agent exposure is possible for military personnel on the battlefield. Therefore, the retrospective detection and filling of critical toxicological data gaps are of high importance in nerve agent research.

Currently, inhalation studies are being conducted to determine the toxic effects of low-level soman vapor exposure using animal models. To gain a better understanding of the dose-response relationship in these models, an estimate of the levels of soman present in the blood is needed. Methods have been previously developed to quantify the internal dose of various nerve agents after exposure. Such methods quantitate the activity of AChE in blood (6), unbound nerve agent in blood (“free agent”) (7–10), and hydrolysis products of the nerve agent in blood and urine (2,11–13). In addition, methods for the detection of soman specifically have been developed, including isotope dilution using liquid chromatography–tandem mass spectrometry (LC–MS–MS) for the detection of soman hydrolysis products in urine (13) and isotope dilution with the use of gas chromatography–tandem mass spectrometry (GC–MS–MS) and derivatization of urinary metabolites of soman (2). Some of these methods involve tedious sample preparation like derivatization of analytes prior to analysis, and others, such as the measurement of enzyme activity, do not achieve the sensitivity needed to detect low levels of soman (14). Although the LC–MS–MS methods reach the sensitivity levels needed and do not require derivatizing agents, they focus only on the hydrolysis products of soman, mainly pinacolyl methylphosphonic acid.

In an attempt to overcome the limitations of previous techniques, a different method was developed for the quantification of soman in plasma and was applied to a toxicokinetic investigation in guinea pigs and rhesus monkeys (11). The method measures bound nerve agent using an excess of fluoride ions to shift the equilibrium of the soman–serine binding reaction that occurs in the active site of acetylcholinesterase to regenerate intact soman. Similar methods have been reported previously for the nerve agent sarin (15). Even though these methods have proven to be reliable and sensitive detection methods for the identification of nerve agent exposures (15,17), they do not overcome soman-specific difficulties attributed to its rapid rate of dealkylation (“aging”) (Figure 2). Once the irreversible “aging” reaction of the soman–acetylcholinesterase complex has occurred, the regeneration method is no longer able to regenerate the parent compound. Adams et al. (11) report large measurement variations in levels of regenerated soman attributed to rapid “aging” and variability in the post-exposure analysis time, demonstrating the need for immediate sample processing post-exposure.

In the present study, a modified version of the fluoride regeneration assay and instrumental analysis method described previously (15) is validated and used immediately after soman vapor exposure to quantitate the concentration in rat plasma. This method uses solid-phase extraction (SPE) of regenerated soman and a deuterated isotope internal standard (2H4-soman, Figure 3) followed by analysis using large volume injection GC with ammonia chemical ionization MS detection. This sensitive method was successfully applied to the analysis of soman in rat plasma, with the amount of soman regenerated from the blood correlating with the dose of soman to which the animals were exposed.

Methods

Chemicals and reagents
Soman and 2H4-soman (soman-d4) were procured through the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Analytical grade ethyl acetate (99.5+% purity), 2-propanol (> 99.5% purity), sodium sulfate anhydrous granular (≥ 99% purity), and potassium fluoride (99+% purity) were obtained from Sigma-Aldrich Chemical (St. Louis, MO). Sodium hydroxide (99.4% purity) was obtained from Fisher Chemicals (Fair Lawn, NJ), and the glacial acetic acid (99.7+% purity) also used to make the buffer solution was obtained from Sigma-Aldrich Chemical. Ammonium hydroxide (99.99+% purity) and methanol (99.0+% purity) were obtained from Sigma-Aldrich Chemical, and nitrogen compressed gas (ultra high purity) and helium compressed gas (ultra high purity) were from CT&S (Albentown, PA).

Animal use
Groups consisting of five adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 225–300 g at the time of testing were exposed to soman vapor in a whole body dynamic airflow chamber for 60 min. A total of 25 male rats were exposed to concentrations ranging from 0.139 to 0.401 mg/m3, and doses, expressed as concentration x time (Ct), ranging from 8.3 to 24.1 mg-min/m3. Following exposure, 1000 µL of whole blood was collected from a tail snip for use in the regeneration assay described in the following sections. All experiments and procedures were approved by the U.S. Army Edgewood Chemical Biological Center Institutional Animal Care and Use Committee, and conducted in accordance with the requirements of Army Regulation 70-18 and the National Research Council’s “Guide for the Care and Use of Laboratory Animals” (18).
Soman vapor generation

Whole body vapor exposures were conducted using a Rochester-style 750-L dynamic airflow inhalation chamber. Soman vapor generation was accomplished using a glass saturator cell as described previously (19). Briefly, saturated soman vapor streams were generated by placing neat liquid soman into a glass multi-pass saturator cell containing a hollow ceramic cylinder and allowing the nitrogen carrier gas to flow through the saturator cell and over the soman-wetted ceramic cylinder. The temperature of the saturator cell and the flow rate of nitrogen determined the amount of soman vapor generated. Soman concentrations were then determined using GC with flame photometric detection (Agilent Technologies, Wilmington, DE).

Stock solutions and calibration standards preparation

Stock solutions of soman and soman-d₄ were prepared in hexane at concentrations of 1.100 mg/mL and 1.698 mg/mL, respectively. Intermediate levels used as working standards were prepared by diluting stock solutions in ethyl acetate at concentrations of 110, 11, and 0.1 pg/mL for soman and 3.396 µg/mL for soman-d₄. Calibration standards were prepared by spiking a known amount of soman and soman-d₄ stock or working standards directly into ethyl acetate resulting in nine concentration points spanning the range of 10–1000 pg injected on-column of soman and a constant 998.424 pg on-column of soman-d₄. All stock and standard solutions were stored at -20°C.

Sample preparation

The sample preparation method followed the regeneration assay previously developed by Jakubowski et al. (15). Whole blood (1000 µL) from both control and exposed rats was collected from a tail snip in Microtainer K₂EDTA-containing tubes (BD Vacutainer®, Franklin Lake, NJ) and then centrifuged at 15,000 rpm for 5 min (Thermal International Equipment Company, Micromax model, radius = 7.5 cm) to separate the plasma and red blood cell (RBC) fractions immediately post-exposure. For both control and exposed samples, a 200-µL aliquot of plasma was weighed. To each plasma sample, 1 mL of acetate buffer (pH 3.5), 200 µL of 6M potassium fluoride, and a soman-d₄ spike (yielding a concentration of 20 ng/mL in the final extract) were then added. Plasma samples from unexposed rats were spiked with a known amount of soman and soman-d₄ and used as positive controls. Negative controls were also employed by using unexposed rat plasma and spiking only with the soman-d₄. Samples were vortex mixed, centrifuged at 15,000 rpm for 5 min, and the supernatant was then transferred to C₁₈ SPE cartridges (Sep-Pak, Waters, Milford, MA) pre-conditioned with 1 mL ethyl acetate, 1 mL 2-propanol, followed by 1 mL of acetate buffer. Samples were filtered through the SPE cartridge and the filtrates discarded. The original sample pellet was re-suspended in 1 mL acetate buffer and 200 µL 6M potassium fluoride, vortex mixed, and centrifuged. The supernatant was once again transferred to SPE cartridges and filtered. The cartridges were then washed with 500 µL acetate buffer, swabbed to remove residue, and dried under a vacuum. Soman and soman-d₄ were eluted by adding 1 mL of ethyl acetate to the cartridge. The eluent containing soman/soman-d₄ was collected in glass tubes containing sodium sulfate to dry the extract. The extracts were then filtered through a 0.2-µm syringe microfilter (PN 4436, Pall Life Sciences, Ann Arbor, MI), followed by an additional sodium sulfate rinse with 500 µL of ethyl acetate. Final sample volume was dependent on exposure concentration, with higher concentrations requiring dilution with ethyl acetate, and lower concentrations requiring concentration with nitrogen compressed gas.

Analytical method

Analytical separation and detection was achieved using an Agilent Technologies GC model 6890 coupled with an MS Detector (model 5973 MSD, Agilent Technologies). Injections of 50 µL of extract were made by autoinjector (model 7673, Agilent Technologies) into a large volume injector port (PTV Inlet, Agilent Technologies). The inlet was cooled to ~30°C by liquid nitrogen and held for 5.1 min. The temperature then ramped at a rate of 720°C/min for a final inlet temperature of 225°C. Solvent vent time was 5.0 min and the vent flow was 300 mL/min. Purge flow was 500 mL/min and purge time 8.7 min. An intermediate polarity deactivated guard column (5 m x 0.32 mm i.d., Restek, Bellefonte, PA) and an Rtx-1701 analytical column (30 m x 0.32 mm x 1.0-µm film thickness, Restek) were used with helium as the carrier gas with a constant flow rate of 3 mL/min. The GC had an initial oven temperature of 35°C for 5 min which was then ramped to 164°C at a rate of 25°C/min. The temperature was then increased to 170°C at a rate of 2°C/min. Finally, the temperature was ramped to 270°C at a rate of 50°C/min and held at this temperature for 3 min for a total runtime of 18.16 min. Elution times for the diastereomeric mixtures of soman and soman-d₄ were typically ~11.4 min as shown in Figure 4.

Figure 4. GC-MS extracted ion chromatograms of plasma blank at m/z 200 (A); plasma blank at m/z 204 (B); recovered soman at m/z 200 from rat plasma at 401.5 pg on-column (C); recovered soman-d₄ at m/z 204 from rat plasma at 1000 pg on-column (D); soman standard in ethyl acetate at m/z 200 at 401.5 pg on-column (E); and internal standard soman-d₄ in ethyl acetate at m/z 204 at 1000 pg on-column (F). The double peaks were manually integrated together for all quantitation.
MS detection was accomplished using positive ion ammonia chemical ionization (CI) in the selected ion monitoring (SIM) mode with a deuterated stable isotope as an internal standard. CI source conditions were optimized using Fluoroether E3 (CAS Registry Number: 3330-16-3, also known as PFDTD; Agilent Technologies) tuning compound and methane as a reagent gas. An ammonia tune was conducted before each sequence to optimize ionization parameters. The ammonia adduct ion ratio of m/z 200/204 (soman/soman-d4) was used for quantitation. Mass spectra were obtained at a dwell time of 50 ms for each ion (3.77 cycles/s). After manually integrating the characteristic double peaks of soman together, quantitation of unknown samples was determined by using the slope and intercept calculated by linear regression analysis of calibration curves (Enhanced Chemstation Data Analysis, MSD Chemstation D.01.02.16, Agilent Technologies).

Results and Discussion

Method validation

Method validity was assessed by determining the linearity, sensitivity, selectivity, accuracy, precision, recovery, and stability of soman spiked into control rat plasma samples. Initial testing showed that a larger amount of soman was recovered from plasma than RBCs (data not shown), suggesting that there may be more binding sites or more unbound agent in the plasma (14). Alternatively, it may be an indication that “aging” occurs to a lesser extent or at a slower rate in plasma than RBCs. RBCs dry out when stored for long periods of time, making it difficult to recover the desired amount of sample. Given the greater amount of soman present in the plasma, and the poor recovery from older RBC samples, the regeneration method utilized in the present study was validated in plasma only.

Linearity. Figure 5 shows the response (peak area) ratio of soman to the internal standard (soman-d4). Instrument linearity and reproducibility are demonstrated using no less than seven calibration concentration standards and comparing the slope, intercept, and correlation of five calibration curves over four consecutive weeks. The data show good correlation (r^2 = 0.9995) over two orders of magnitude with 0.08% variance and a slope variance of 3.76%. The intercept did not change significantly over the course of the study.

Sensitivity and selectivity. The sensitivity of this method was established by determining the limit of quantitation (LOQ) and the limit of detection (LOD). The LOQ is the minimum concentration of the analyte that can be accurately and pre-

Table 1. Accuracy and Precision of the Regenerated Soman Method*

<table>
<thead>
<tr>
<th></th>
<th>Mean (pg on-column)</th>
<th>% Relative Error</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC (9.9 pg on-column)</td>
<td>9.04</td>
<td>8.69</td>
<td>0.76</td>
</tr>
<tr>
<td>MQC (401.3 pg on-column)</td>
<td>395.48</td>
<td>1.50</td>
<td>16.29</td>
</tr>
<tr>
<td>HQC (990 pg on-column)</td>
<td>895.69</td>
<td>9.53</td>
<td>29.43</td>
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</table>

* Five rat plasma sample replicates were spiked with soman and soman-d4 at the LQC, MQC, and HQC; soman recovered was reported as pg on-column from which the mean was calculated.

Figure 5. Peak-area ratio of soman to soman-d4 versus pg soman injected onto the column. Each point represents the peak-area ratio of soman to soman-d4 at each standard calibration concentration (10-1000 pg on-column). These points were obtained from five calibration curves over four consecutive weeks using seven or more standard concentrations for each curve.

Figure 6. Average soman recovery versus time (days) demonstrating stability when stored in plasma matrix alone (A) and plasma with the addition of assay reagents (B). The average recovery of soman spiked (99 ng/g) into control rat plasma (n = 5) decreased significantly over the course of four weeks when stored with and without acetate buffer and potassium fluoride in 4°C, indicating that soman is not stable at either step of the regeneration assay.
cisely quantified with a peak-to-peak signal-to-noise (S/N) ratio of 10:1. The LOD is the smallest amount that can be detected with a peak-to-peak S/N ratio of 3:1. The average LOQ in plasma was calculated to be 18.83 pg on-column and the average LOD in plasma was 5.65 pg on-column. Selectivity was demonstrated by comparing six blank plasma samples and six spiked plasma samples, corresponding with the lowest point on the calibration curve (9.9 pg on-column). Each blank sample was analyzed for potential interferences and no interfering signals of consequence at the retention times of soman or soman-d₄ were found as shown in Figure 4.

**Accuracy, precision, and recovery.** The accuracy and precision of the method was determined by injecting five replicate plasma samples at three known concentrations, low quality control (LQC), medium quality control (MQC), and high quality control (HQC) over the expected range of the method (Table I). The relative errors reported for the replicates were 8.69%, 1.50%, and 9.53% for the LQC, MQC, and HQC, respectively, indicating that the method is both accurate and precise. Precision of the instrument, or instrument repeatability, was measured by injecting the same three levels of known concentrations (n = 1 at each level) five times each and determining the relative standard deviation (RSD) for each series. The RSDs for the five repeated injections at each level were 3.0% or less. Method recovery was determined by comparing soman and soman-d₄ extracted from plasma samples to those spiked directly into ethyl acetate (unextracted). The percent recovery for both soman and soman-d₄ were based on the area count responses reported by the data analysis software. The recoveries for the LQC, MQC, and HQC were 101%, 93%, and 87%, respectively.

**Stability.** A series of stability tests was conducted to assess the stability of soman at different points throughout the regeneration assay in order to determine how rapidly the steps of the assay must be completed following receipt of soman containing samples. The stability of soman in plasma alone stored at 4°C and in plasma with the addition of 1 mL acetate buffer (pH 3.5) and 200 µL potassium fluoride solution stored at 4°C, both intermediate steps in the regeneration assay, was determined. In addition, soman stability in ethyl acetate, the final matrix of the regeneration assay, was assessed.

**Agent stability in plasma matrix.** To evaluate how stable soman is in the plasma matrix alone, five control rat plasma samples were spiked with soman at a concentration of 99 ng/g, which is equivalent to the HQC validation samples (990 pg on-column) in six sets (initial, 24 h, week 1, week 2, week 3, week 4) for a total of 30 samples. The initial set was prepared immediately after spiking by adding the internal standard soman-d₄ and then completing the regeneration assay, and the other samples were stored at 4°C until ready for preparation, with soman-d₄ added at the time of extraction. The results show a rapid decrease in the average percent recovery of soman as time in storage increased (Figure 6A).

**Agent stability in plasma matrix with assay reagents.** To determine how stable soman is in the plasma matrix with the addition of assay reagents, the experimental setup previously discussed was followed with the addition of 1 mL acetate buffer (pH 3.5) and 200 µL potassium fluoride before spiking. Again, the initial set was prepared immediately after spiking while the others were stored in acetate buffer and potassium fluoride at 4°C until the appropriate time, with soman-d₄ added at the time of extraction. The results show a rapid decrease in the average percent recovery of soman as the time in storage increased (Figure 6B).

Both the soman-spiked plasma samples stored with and without the addition of buffer and potassium fluoride show a rapid decrease in recoveries over the course of four weeks. These results indicate that soman is not stable at either point during the regeneration assay when stored at 4°C, demonstrating the need for rapid sample preparation immediately after soman exposure in order to obtain recoveries that accurately reflect the internal dose. Future studies will involve a stability test in plasma with the addition of buffer but without potassium fluoride as well as conducting the same series of tests in RBCs. In addition, analyzing the hydrolysate product pinacolyl methylphosphonic acid levels and the soman-AChE complex to compare the rate of hydrolysis and aging will better our understanding of the relationship of time and soman stability in biological matrices.

**Agent stability in solvent.** Using the samples from the previously discussed matrix stability analysis, a test was conducted to evaluate how stable soman is in ethyl acetate, the final matrix after extraction, in a freezer at −20°C (normal storage conditions for our samples awaiting analysis). The five extracted samples at each of the six time points were injected four times under the same GC–MS conditions over the course of 29 days.

**Table II. Recovery of Soman Stored in Ethyl Acetate at −20°C Over 29 Days**

<table>
<thead>
<tr>
<th>Time Stored in Ethyl Acetate Post Sample Preparation</th>
<th>Time Stored in Acetate Buffer and KF Prior to Sample Preparation</th>
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<tbody>
<tr>
<td>Initial 24 h 1 week 2 weeks 3 weeks 4 weeks</td>
<td>Initial 24 h 1 week 2 weeks 3 weeks 4 weeks</td>
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<td></td>
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<tr>
<td>Initial</td>
<td>964 ± 9 673 ± 12 197 ± 20 57 ± 12 13 ± 3 2 ± 2</td>
</tr>
<tr>
<td>24 h</td>
<td>673 ± 12 197 ± 20 57 ± 12 13 ± 3 2 ± 2</td>
</tr>
<tr>
<td>7 days</td>
<td>200 ± 22</td>
</tr>
<tr>
<td>8 days</td>
<td>58 ± 10 12 ± 1</td>
</tr>
<tr>
<td>13 days</td>
<td>675 ± 15</td>
</tr>
<tr>
<td>14 days</td>
<td>986 ± 5</td>
</tr>
<tr>
<td>15 days</td>
<td>672 ± 12 197 ± 21 60 ± 9</td>
</tr>
<tr>
<td>21 days</td>
<td>195 ± 21</td>
</tr>
<tr>
<td>22 days</td>
<td>982 ± 7</td>
</tr>
<tr>
<td>28 days</td>
<td>650 ± 59 59 ± 10</td>
</tr>
<tr>
<td>29 days</td>
<td>960 ± 77</td>
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</table>

* Control rat plasma samples were spiked with soman (99 ng/g), stored in acetate buffer and potassium fluoride for the specified period of time (0–4 weeks), and then analyzed at four different times over the course of 29 days, and recoveries recorded as pg on-column ± standard deviation. Storing samples under these conditions post sample preparation is typical for samples awaiting analysis. Results show that the ratio of soman to soman-d₄ remains stable for up to 29 days in ethyl acetate; n = 5 for each time point.
Analysis of the four aliquots at each time point showed no significant decrease in the amount of soman recovered (Table II), indicating that the ratio of soman to soman-d₄ remained stable in ethyl acetate at -20°C.

**Application of regenerated soman detection in rat plasma**

In applying this method, it should be noted that there are limitations. One limitation is that the bound and free agent fractions cannot be quantified separately without the use of an additional assay because the regenerated compound is identical to the parent compound. In addition, this method is not able to quantitate other biotransformation pathway products such as hydrolysis or “aging” products. Despite these limitations, this method has been successfully applied previously to the detection of regenerated soman in rat plasma and red blood cells following 240 min whole-body inhalation exposures (14). In this study, levels of regenerated soman were measured in plasma samples immediately post-exposure and were correlated with the soman exposure concentration. Obtaining a closely correlated dose-response is typically difficult for soman because of its rapid “aging” (11). Additionally, the same study reported that regenerated soman was detected in the plasma at every concentration tested (0.033 to 0.280 mg/m³). This is a significant finding because soman was detected at vapor levels that did not produce significant inhibition of AChE activity, a typical biomarker of nerve agent exposure, demonstrating that this method is approximately threefold more sensitive than the AChE assay. In the present study, exposure of groups of rats to various concentrations of soman vapor for 60 min resulted in dose-dependent detection of soman in plasma (Figure 7). Linear regression analysis of regenerated soman data yielded an r² value of 0.754, demonstrating a solid relationship between soman exposure concentration and levels of regenerated soman. Using this method in conjunction with both free agent and hydrolysis product assays will provide a more complete picture of the pharmacokinetics and biotransformation of soman in vivo.

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