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Improvements in the Methodology of Monitoring Sulfur Mustard Exposure by Gas Chromatography–Mass Spectrometry Analysis of Cleaved and Derivatized Blood Protein Adducts

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Abstract

An analytical method for determining exposure to 2,2'-dichlorodiethyl sulfide (sulfur mustard, HD) has been enhanced. The method is based on the cleavage of adducted HD (protein-hydroxyethylthioethyl esters) to produce thiodiglycol. Following cleavage, a deuterated internal standard is added, and the analytes are extracted, derivatized, and analyzed by gas chromatography–negative ion chemical ionization-mass spectrometry. Inclusion of a concentration step, addition of solid sodium bicarbonate to neutralize excess derivatization reagent, and optimization of method and instrument conditions provided dramatic increases in signal-to-noise ratio. A five-day precision and accuracy study was conducted, including interday and intraday unknown analysis. Linearity was verified by a $R^2 > 0.9995$ for all five curves evaluated. The precision and accuracy of the assay were demonstrated to be excellent by evaluation of the interday and intraday unknown samples (< 10% relative standard deviation and relative error in most cases). Statistical treatment of the method blanks and calibration results demonstrated a reduction in the limit of quantitation from 25nM (HD, human plasma, in vitro) to 1.56nM. Sample and calibration stability through the analytical sequence was established by the inclusion of continuing calibration verification standards (< 5% error). Short-term sample stability was verified by reinjection of a calibration set after 18 days ($R^2 = 0.9997$). Quantitative agreement with the previous method was supported by the analysis of a 50nM standard protein sample (HD, rat plasma) with both methodologies (< 1% error).

Introduction

Human exposure to chemical warfare agents (CWA) such as 2,2'-dichlorodiethyl sulfide (sulfur mustard; HD) continues to be a significant concern for both military and civilian populations. Sulfur mustard has been used in WWI and in more recent conflicts in the Middle East. The worldwide stockpile of HD poses a threat to humanity because of its potential use as part of a terrorist action. In accordance with the Chemical Weapons Convention, chemical demilitarization efforts are ongoing, and risks to the worker population are also of concern. The current potential for human exposure to HD necessitates further development of sensitive and selective analytical methodologies.

Definitive identification of HD as the agent involved in a particular incident is critical to the assessment of extent of exposure, medical follow-up, and political/legal ramifications involved with intentional use. More sensitive bioanalytical methods not only allow detection of lower levels of exposure, but they also lengthen the time window in which to observe retrospective detection.

Several reviews identifying methods designed to monitor exposure to chemical warfare agents including HD in biological matrices have been published (1–3). A variety of methods have been developed specifically for HD exposure. These include the immunoassay of HD bound to DNA (4), the gas chromatography–mass spectrometry (GC–MS) method for the N-terminal valine adduct of hemoglobin (5–8), a GC–MS method of HD adducts from skin keratin (7), the liquid chromatography–MS–MS method of the albumin/sulfur mustard adducts (9), and an immunological assay for the detection of mustard adducts to skin tissue (10). The DNA, hemoglobin, and albumin methods have each been used to confirm human exposure to HD. Most recently, definitive methods were used to confirm HD as the agent involved following an accidental human exposure in the summer of 2004 (11–13).

This report describes sensitivity improvements made to a previously published method for detecting HD exposure (14). The technique involves the formation and monitoring of HD adducts cleaved from blood proteins such as albumin and

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globin. Both albumin and globin contain numerous free carboxylic acid groups from aspartic acid and glutamic acid that are alkylated by the electrophilic HD, in vivo, to give hydroxyethylthioethyl (HETE) esters. These esters are cleaved or hydrolyzed with dilute base to yield thiodiglycol (TDG). Following the addition of a deuterated form of TDG (TDG-d_8), the analytes are extracted into ethyl acetate and then derivatized with pentafluorobenzoyl chloride. The resultant bis(pentafluorobenzoyl) ester of thiodiglycol (2,2'-thiobisethanol dipentafluorobenzoate, TDGPFB) and its isotopic analogue (TDG-d_8PFB) are then analyzed by GC–negative ion chemical ionization (NCI)-MS with methane reagent gas in the selected ion monitoring mode (7,13–16).

The present paper discusses modifications to the original method that have resulted in lowering detection levels from the previously reported limit of quantitation (LOQ) of 25nM (HD to plasma, in vitro) to 1.56nM. The purpose of this report is to provide evidence demonstrating the increased sensitivity and specificity afforded by these modifications. Excellent linearity, precision, accuracy, and stability were demonstrated through a precision and accuracy study. In addition, statistics were applied to define the limit of detection (LOD) and LOQ achieved. We herein report the details of the analytical procedure, its sensitivity, reproducibility, and short-term stability.

Experimental

Materials

Ethyl acetate, sodium bicarbonate, anhydrous sodium sulfate, pyridine, methanol, and pentafluorobenzoyl chloride were obtained from Sigma-Aldrich (St. Louis, MO) and used without purification. Sodium hydroxide (1N) and hydrochloric acid (3N) were purchased from Fisher Scientific (Waltham, MA). Sulfur mustard was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD); purity as determined by nuclear magnetic resonance spectroscopy was 97.5%. The internal standard (IS) octadeuterothiodiglycol (TDG-d_8) was obtained from Ash Stevens (Detroit, MI). Previous analysis of TDG-d_8 by GC–MS demonstrated no evidence of undeuterated material that could potentially interfere with the assay of the native compound. Bond Elut 500-mg silica (Si) solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA) were conditioned with 3 mL of ethyl acetate before use. Pooled human plasma (with sodium heparin) was obtained from Bioreclamation (Hicksville, NY).

Sample preparation

For this study, a standard curve (consisting of seven concentration levels of HD) was generated on five different days from protein stocks. In addition, each day also incorporated the processing of a blank sample (no internal standard or analyte added), a method blank (no analyte added), and three unknown concentrations (see Intraday and interday precision and accuracy studies) from protein stocks.

Calibration curve protein stocks. The calibration curve protein stocks were prepared as follows. Sulfur mustard (100 µL, 5.00µM) in ethanol was added to 9.90 mL of human plasma. This spiked solution was then serially diluted with human plasma; the final HD concentrations obtained were 50.0, 25.0, 12.5, 6.25, 3.13, 1.56, and 0.78nM. Blank protein stock was also prepared from the same lot of human plasma. The samples were incubated at 40°C with gentle shaking for 2 h. Protein was precipitated and twice washed with acetone, then air dried at ambient temperature in a desiccator.

Interday and intraday precision and accuracy unknown protein stocks. The protein stocks for the unknown samples were prepared as follows: Human plasma was spiked with a solution of HD in ethanol to a level corresponding to 50.0nM. The 50.0nM spiked solution was then serially diluted with plasma to provide additional concentrations corresponding to 25.0, 12.5, 6.25, 3.13, and 1.56nM. Three unknown concentrations (37.5, 9.38, and 2.34nM HD in plasma) were prepared from these dilutions. These unknown solutions were incubated at 40°C with gentle shaking for 2 h. The protein was precipitated and twice washed with acetone and air dried at ambient temperature in a desiccator.

Protein preparation for analysis. All data points originated at the protein stage (i.e., each day, protein was weighed from the stocks prepared and processed through the sample preparation procedure to provide a unique data point). Precipitated protein was weighed to approximately 25 mg and then treated with 1N NaOH (200 µL) and heated at 70°C for 120 min. The digested protein mixture was spiked with TDG-d_8 (20 µL, 30 ng/mL) and neutralized using 3N HCl (70 µL). The sample was extracted with ethyl acetate (1.0 mL), and the extract was dried with sodium sulfate (400 mg). A portion of the organic layer was removed (800–900 µL) and treated with sodium sulfate to promote further drying. Derivatization of TDG and TDG-d_8 to their respective pentafluorobenzoyl entities was accomplished with the addition of pyridine (15 µL) and the derivatizing compound pentafluorobenzoyl chloride (20 µL). The solution was shaken for 10 min, followed by the addition of methanol (10 µL) and sodium bicarbonate (40 mg) to neutralize any residual derivatizing reagent. The ethyl acetate extract was dried by adding sodium sulfate (400 mg). Then a portion (600–700 µL) of the extract was applied to an SPE cartridge, and the eluent was collected. An additional 1.0 mL of ethyl acetate was passed through the SPE cartridge, and the combined fractions were concentrated to 50–100 µL under nitrogen at 60°C (Evaporator, Organomation Associates, Berlin, MA). The resulting solution was analyzed by GC–NCI-MS with methane as the reagent gas.

Precision and accuracy studies involved preparation from protein stock and subsequent analysis of the unknowns over the course of five days. Intraday and interday preparation and analysis were performed.

Intraday unknowns. Five sets (N = 5) of unknown samples corresponding to 37.5, 9.38, and 2.34nM HD in plasma were generated along with a calibration curve and five method blanks, in a single day. Precision was assessed by calculating the relative standard deviation (RSD) expressed as a percentage for each group of samples. Accuracy was reported as the percent error from the difference between the calculated and expected concentrations.

Interday unknowns. Five sets ($N = 5$) of unknown samples corresponding to 37.5, 9.38, and 2.34nM HD in plasma, plus method blanks, were generated along with a calibration curve over the course of 5 days ($N = 5$). The unknown concentrations were calculated from the calibration curve generated on the day the samples were analyzed. Precision and accuracy were calculated as described.

Method blank study. A method blank study was performed to assess the limits of detection (LOD) and quantification (LOQ) of the assay. Seven ($N = 7$) samples were prepared. All method blanks contained IS with no analyte.

Instrumentation

GC–NCI-MS analyses were performed on an Agilent Technologies (Santa Clara, CA) 6890 GC interfaced to an Agilent 5973 mass-selective detector. The GC was fitted with a DB-5ms bonded phase capillary column (30 m × 0.25-mm i.d., 0.25-µm film thickness). Helium was used as the carrier gas in the ramped flow mode. The initial flow rate was set to 1.2 mL/min and held for 13.8 min; after elution of the peaks of interest, the flow rate was increased to 2 mL/min. The oven temperature was held initially at 80°C for 1 min, programmed from 80 to 225°C at 30°C/min, from 225 to 300°C at 50°C/min, and held at 300°C for 3 min. Pulsed splitless injections of 1 µL were made using an Agilent 7683 autosampler. The injection port temperature was set to 250°C, split vent delay to 2 min, and the transfer-line temperature to 280°C. Typical retention times were 11.3 min for TDG-d_8PFB and 11.4 min for TDGPFB.

The MS detection was conducted using NCI with methane as the reagent gas. The source and quadrupole temperatures were set at 150 and 120°C, respectively. The monoisotopic molecular ion and the M+1 ion were monitored for the TDGPFB (*m/z* 510 and 511) and TDG-d_8PFB internal standard (*m/z* 518 and 519). The dwell time for each ion in the selected ion monitoring (SIM) mode was 100 ms. The instrument was initially tuned in accordance with the manufacturer's autotune protocol. The emission current was then set to approximately 100 µA, and the electron energy was set to approximately 100 eV. These conditions provided optimum analyte and IS counts.

Data analysis

Graphic representations and linear regression analyses were conducted with GraphPad Prism software, version 4.03, 2005 (GraphPad Software, San Diego, CA). Calculations for TDG concentrations and other statistical treatments were performed with Microsoft Excel 2003, SP2 (Microsoft, Redmond, WA).

Results and Discussion

The purpose of this report is to illustrate the increased sensitivity resulting from alterations to the previously published method (14). A comprehensive study was conducted to optimize the method with the goal of minimizing detection levels and enhancing overall signal-to-noise. The previously reported lower limit of detection was at the 25nM exposure level (HD to plasma, in vitro).

Concentration of the extract prior to GC–NCI-MS analysis was investigated as a means to lower the LOD for the assay. HD exposure levels ranging from 1.6 to 400nM were prepared in accordance with the published assay and analyzed after an approximately 10-fold concentration of the final extract. The resultant chromatograms exhibited retention time migration, baseline elevation, and poor peak shape reproducibility, which degraded as the analytical sequence progressed. When performing instrument maintenance after analysis of the concentrated extracts, the inlet gold seal was found to be severely discolored.

Evaluation of the concentrated extract with GC–(positive ion) electron ionization (EI)-mass spectrometry indicated a large peak consistent with the mass spectrum of pentafluorobenzoic acid, suggesting that unreacted derivatization reagent was present. After evaluating several methods of neutralizing the excess reagent, the addition of solid sodium bicarbonate was found to remove virtually the entire pentafluorobenzoic acid peak from the GC–EI-MS chromatogram. The previously published method included a neutralization step using an aqueous solution of sodium bicarbonate; however, this step was subsequently removed from the method because it was deemed unnecessary. The addition of a concentration step to the sample preparation protocol reestablished the necessity for neutralization. Applying this adjustment resulted in cleaner baselines with lower background counts (optimized method baseline dropped from 2000 counts down to approximately 200 counts).

Retention time migration was addressed by adding a column conditioning procedure to the instrument method. Increasing the final oven temperature to 300°C for 3 min with an increased carrier gas flow rate of 2 mL/min proved to condition the column and elute high boiling components between runs. Also, the MS parameters were adjusted to optimize the ion source conditions for resonance electron capture ionization (see Instrumentation section) to take advantage of the electronegative nature of the di-pentafluoro derivatives. This re-

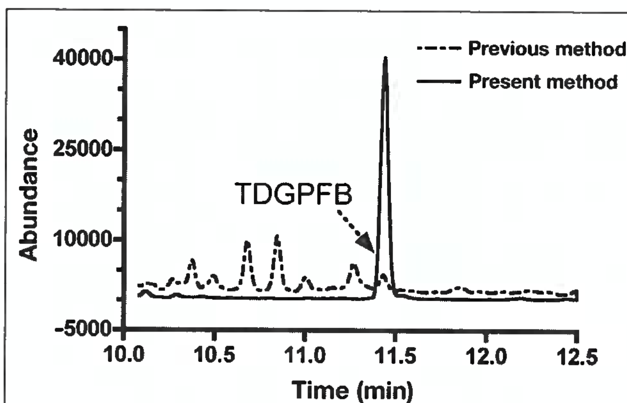

Figure 1. Overlaid 50nM standard *m/z* 510 extracted ion chromatograms: The dashed chromatogram originates from protein obtained from a 50nM standard spike (HD, rat plasma) prepared and analyzed using the previous method; the solid chromatogram represents the same 50nM standard protein sample prepared (from the protein stage) and analyzed using the modified method. The TDGPFB analyte peak is indicated at 11.4 min. Note the dramatic improvement in signal-to-noise ratio.

sulted in enhanced signal-to-noise ratios, reproducible retention times, and more accurate peak integrations.

Continued refinement of the method resulted in adjustments to the extract volumes used during the derivatization and SPE procedures to maximize the final concentration of analyte. In addition, the HD solvent used for the calibration curve generation was switched from saline to ethanol. This change improved the miscibility of the agent and solvent combination, alleviated agent degradation concerns (hydrolysis in saline), and increased the precision of the exposure procedure. Together, these improvements resulted in a dramatic increase in sensitivity and selectivity (Figure 1).

These improvements also allowed a lower calibration range to be evaluated through the accompanying precision and accuracy study. From the cumulative results of previous experiments a calibration range of 0.78 to 50.0nM HD exposure level was chosen. The internal standard spiking solution concentration was also lowered 10-fold in accordance with the lower levels being investigated.

From the interday studies, five calibration curves were generated by plotting the ratio of the analyte peak area to internal standard peak area, divided by the protein weight, as a function of concentration. Linearity was demonstrated by the R^2 values obtained for the curves (> 0.9995; Table I). It is generally accepted that the precision determined at each level in the calibration curve should not exceed 15% RSD except for at the lower limit of quantitation (LLOQ), where it should not exceed 20% RSD (17). The precision of the present calibration curve data is in agreement with these guidelines with the exception of the 1.56nM RSD, which is marginally over 15% (15.3%, see Table I). The five-day calibration curve illustrates the interday reproducibility obtained (Figure 2).

The precision and accuracy of the method is demonstrated through the interday and intraday results for three concentrations (2.34, 9.38, and 37.5nM), which were analyzed as unknowns (Table II). The lower recoveries observed (negative % error) suggest a slight negative bias in the accuracy of the method; however, the calculated percent error for all concentrations, intraday and interday, was less than 10%.

The LOD of the method was estimated by equation 1 according to Skoog et al. (18):

Table I. Mean Standard Values Calculated from Daily Calibration Curves*

	Standard Concentration (nM)							
	0.78	1.56	3.13	6.25	12.5	25.0	50.0	R^2
Day 1	0.89	1.95	2.93	6.44	12.2	24.6	50.3	0.9997
Day 2	0.91	1.76	3.22	6.48	12.0	24.5	50.3	0.9996
Day 3	0.97	1.31	3.14	6.22	12.1	25.8	49.7	0.9995
Day 4	0.94	1.58	3.01	6.22	12.8	24.5	50.2	0.9998
Day 5	0.82	1.48	3.12	6.15	13.0	24.5	50.1	0.9997
Average	0.91	1.62	3.08	6.30	12.4	24.8	50.1	
% Deviation from expected	15.9	3.4	–1.3	0.8	–0.7	–0.9	0.3	
STD Deviation	0.06	0.25	0.11	0.15	0.45	0.6	0.2	
RSD	6.3	15.3	3.7	2.4	3.6	2.3	0.5	

* The concentration of each standard data point was calculated from the equation of the linear regression line for the day it was analyzed. The R^2 value for each day is indicated.

$$\Delta x_{\min} = \bar{x}_1 - \bar{x}_b > ts_b \sqrt{\frac{N_1 + N_b}{N_1 N_b}} \quad \text{Eq.1}$$

where $\Delta x_{\min}$, the minimum detectable quantity (i.e., the LOD), is equal to a value greater than the difference between the average response (x_1) and the average blank response (x_b). The subscript b refers to the blank determination, t is the probability statistic (dependent upon degrees of freedom and desired confidence interval), s_b is the standard deviation of the blank set, and N is the number of determinations. The ratio of the noise area observed at the retention time of the analyte in the m/z 510 amu ion chromatogram to the internal standard peak area (m/z 518 amu ion chromatogram) per milligram protein of the method blanks was evaluated. The LOD at 99% confidence level (t = 3.71) was estimated as > 0.45nM from the Method blank study (N = 7). The same statistical treatment applied to a larger data set, that is, all method blanks analyzed over the entire study [N = 16; Method blank study (N = 7), Interday study (N = 5), Intraday study (N = 4)], with concentrations calculated by the five-day average calibration curve, resulted in a LOD of > 0.52nM at the 99% confidence level (t = 3.11).

To further support the above calculations, the method LOD at the 99% confidence level was also estimated according to the definition of the American Chemical Society (ACS) (19) as $3s_0$, where s_0 is the value of the standard deviation as the concentration approaches zero. The s_0 was estimated from the y-intercept of the linear regression of the plot of the standard deviation of the seven standard levels versus the concentration (20). Because at least seven replicates at each level are necessary to provide useful statistics, the values obtained from two additional calibration curves (R^2 = 0.9999 for both) generated after the precision and accuracy study were included in the cal-

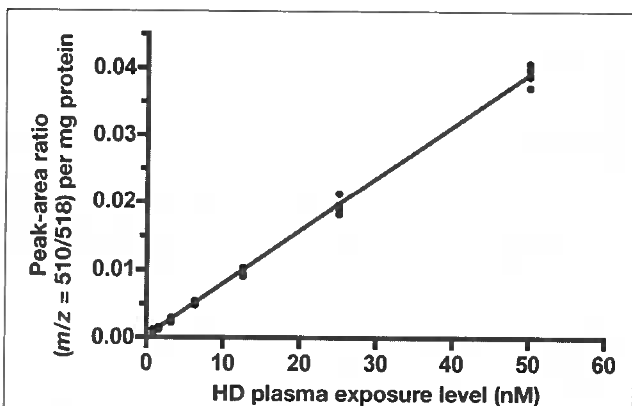

Figure 2. Five-day calibration curve (n = 5): The curve derived from the linear regression analysis of all calibration points generated over the course of the five-day precision and accuracy study illustrates the interday reproducibility and linearity of the method (R^2 = 0.9974).

culation. This treatment resulted in an LOD of 0.55nM that is in agreement with the previous calculations. Taking into account the range of LODs (> 0.45–0.55nM) calculated here, the estimated LOQ of the method lies between 1.51 and 1.83nM, as calculated by applying the ACS definition (LOQ = $10s_0$, i.e., [LOD/3] × 10) (19). These calculated values agree with empirical observation (Figure 3).

This LOD is based upon the primary ions observed for the bis(pentafluorobenzoyl) ester of thiodiglycol (TDGPFB) and the internal standard, its octadeutero isotopic analogue (TDG-d_8PFB). Because we monitor both the monoisotopic and M+1 ions, we can perform a similar calculation with the M+1 data. Statistical treatment of the method blank data was applied as described (18) for the M+1 ion peaks of *m/z* 511 (analyte) and *m/z* 519 (IS). Because no calibration curve was constructed from the M+1 ion data, the LOD was expressed as the ratio of peak areas per milligram protein. The LOD, calculated using equation 1, corresponds to > 0.00043 per mg protein at the 99% confidence level, which computes [(LOD/3) × 10] to an LOQ of > 0.0014 per mg protein. The 0.78 and 1.56nM calibration data were evaluated against these calculated LOD and LOQ values. All standard data provided ratio per milligram protein values above the LOD; all of the 0.78nM standards provided ratios per mg protein values below the LOQ (average = 0.00076 per mg protein) and all of the 1.56nM standards provided ratios per milligram protein values marginally below the LOQ (average = 0.0012 per mg protein); however, the 1.56nM data lies above the LOQ when it is calculated at the 95% confidence level (LOQ_{95} > 0.0010 per mg protein).

From the cumulative results of the statistical treatment applied herein and those observed, we set the LOQ at 1.56nM for this method and instrument combination (Figure 3). Often, the most pertinent applicable question is whether or not HD exposure has occurred. The described statistical treatment of the precision and accuracy data provides method criteria for evaluating responses near the LOD. A positive response detected below the LOQ (1.56nM) must meet the LOD criteria for both the main analyte response (> 0.55nM) and the isotopic molecular ion response (a ratio per mg protein value > 0.00043 per mg protein) to be considered significant at the 99% confidence level.

Table II. The Precision and Accuracy of the Method*

	Intraday			Interday		
Expected Concentration (nM)	Mean calculated concentration (nM)	Precision RSD (%)	Accuracy error (%)	Mean calculated concentration (nM)	Precision RSD (%)	Accuracy Error (%)
2.34	2.20	4.0	–6.3	2.24	12.1	–4.4
9.38	8.85	2.5	–5.6	8.85	4.8	–5.6
37.5	35.9	3.2	–4.3	37.2	1.8	–0.9

* For the interday studies, each concentration was prepared from precipitated protein and analyzed one time by applying the calibration curve generated on the same day. Five days were studied. For the intraday study, each concentration was prepared from precipitated protein and analyzed five times by applying the calibration curve generated on the same day. Precision was measured by calculating the relative standard deviation (RSD) expressed as a percentage for each group of samples. Accuracy was reported as percent error from the difference between the calculated and expected concentrations.

To investigate the calibration curve and sample stability throughout the analytical sequence, continuing calibration verification standards (CCV, 6.25nM) were employed at the end of each sequence or set of 10 injections. A total of six CCVs were analyzed against the calibration curve generated on the same day. The same vial of midpoint calibration standard was reinjected. All recoveries were within ± 5% of the expected value (range –1.8 to 2.9% error). To evaluate the short-term sample stability, the calibration GC vials analyzed on day 4 of the precision and accuracy study were recapped and refrigerated (4°C). The samples were reinjected 18 days later. The linear regression analysis of the curve provided an R^2 = 0.9997 (previously obtained R^2 = 0.9998).

To illustrate the increased sensitivity of the assay, a sample of protein previously prepared from rat plasma spiked with HD (saline) at the 50nM level was utilized. The protein was weighed and processed using the improved assay. It was analyzed against the HD (ethanol) curve discussed in the previous paragraph. Quantitation resulted in a value of 49.6nM (accuracy = –0.74% error). An overlay of the resultant chromatogram with one obtained from the analysis of the same protein sample analyzed eight days earlier under the previous assay conditions demonstrated a dramatic increase in the signal-to-noise ratio (Figure 1).

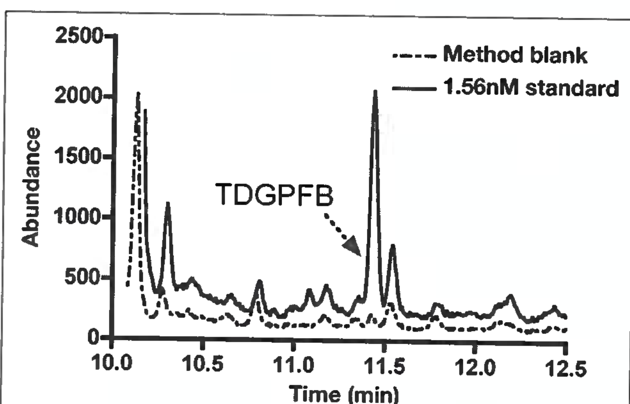

Figure 3. Overlaid method blank and 1.56nM standard *m/z* 510 extracted ion chromatograms: The dashed chromatogram represents a method blank protein sample and the solid chromatogram represents a 1.56nM standard protein sample, both analyzed in the same analytical sequence using the modified method. The TDGPFB analyte peak is indicated at 11.4 min.

Conclusions

Improvements have been instituted in the sample preparation and analysis protocols for the monitoring of sulfur mustard exposure by GC–MS analysis of blood protein adducts. Dramatic reduction in baseline counts and overall noise was

obtained. A five-day precision and accuracy study was conducted. Linearity was demonstrated by $R^2 > 0.9995$ for interday calibration curves. Precision and accuracy was demonstrated to be excellent by analysis of unknown samples. A statistical treatment for the handling of a response near the LOD was proposed at the 99% confidence level. These improvements have lowered the reported LOQ of the method from 25 to 1.56nM in vitro HD exposure to human plasma.

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