



HHS Public Access

Author manuscript

Chem Res Toxicol. Author manuscript; available in PMC 2016 April 19.

Published in final edited form as:

Chem Res Toxicol. 2015 February 16; 28(2): 256–261. doi:10.1021/tx500468h.

A Simplified Method for Quantifying Sulfur Mustard Adducts to Blood Proteins by Ultra-High Pressure Liquid Chromatography-Isotope Dilution Tandem Mass Spectrometry

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Abstract

Sulfur mustard binds to reactive cysteine residues, forming a stable sulfur-hydroxyethylthioethyl [S-HETE] adduct that can be used as a long-term biomarker of sulfur mustard exposure in humans. The digestion of sulfur mustard-exposed blood samples with proteinase K following total protein precipitation with acetone produces the tripeptide biomarker [S-HETE]-Cys-Pro-Phe. The adducted tripeptide is purified by solid phase extraction, separated by ultra-high pressure liquid chromatography, and detected by isotope dilution tandem mass spectrometry. This approach was thoroughly validated and characterized in our laboratory. The average interday relative standard deviation was 9.49%, and the range of accuracy was between 96.1-109% over a concentration range of 3.00 to 250. ng/mL with a calculated limit of detection of 1.74 ng/mL. A full 96-well plate can be processed and analyzed in 8 h which is five times faster than our previous 96-well plate method and only requires 50 μ L of serum, plasma, or whole blood. Extensive ruggedness and stability studies and matrix comparisons were conducted to create a robust, easily transferrable method. As a result, a simple and high-throughput method has been developed and validated for the quantitation of sulfur mustard blood protein adducts in low volume blood specimens which should be readily adaptable for quantifying human exposures to other alkylating agents.

Introduction

Sulfur mustard, bis(2-chloroethyl)sulfide, is a vesicant and alkylating agent that has been developed as a chemical weapon (CW).¹ The Chemical Weapons Convention classifies

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Supporting Information

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sulfur mustard as a Schedule 1 toxic chemical and prohibits its development, fabrication, stockpiling, and use.² The development of reliable methods for long-term detection of sulfur mustard exposure in clinical specimens is critical for monitoring accidental exposures during demilitarization activities and providing forensic evidence during a suspected chemical weapon release.

Sulfur mustard received the reputation “King of battle gases” during World War I as it was responsible for approximately 80% of all chemical casualties.³ Despite the Geneva Protocol of 1925 which banned its use, sulfur mustard was allegedly used as a CW in the mid-1930s in Ethiopia,⁴ the 1960s in Yemen,⁴ and the 1980s by Iraq during the Iran-Iraq conflict where it caused an estimated 40-50,000 casualties.⁵⁻⁷ Sulfur mustard continues to be a concern due to its history of implementation and the continued existence of legacy stockpiles and munitions disposed of at sea. Recently, unintentional exposures have occurred domestically during demilitarization of a sulfur mustard munition found in a clamshell driveway in Delaware,⁸⁻¹⁰ and by unsuspecting clam fishermen who accidentally dredged chemical ordnance.¹¹

Sulfur mustard is an alkylating agent which quickly reacts with nucleophiles such as DNA, RNA, water, lipids, peptides, and proteins via an episulfonium ion intermediate.^{12, 13} Sulfur mustard primarily targets the eyes, skin, and lungs.¹⁴ As a result, exposed individuals typically experience symptoms ranging from nausea, vomiting, and blistering of the skin to respiratory disorders or death in extreme cases.³ Exposures in the past have been diagnosed using DNA adducts and urinary metabolites as biomarkers.^{8, 9, 15, 16} Sulfur mustard can bind to DNA at several locations, including but not limited to the N⁷ and O⁶ position of Gua, and the N³ position of Ade, all of which can be measured via immunoassays or mass spectrometry.^{16, 17} DNA adducts are quickly repaired, or DNA synthesis is blocked to avoid further replication of the mutation.³ Urinary metabolites, such as thiodiglycol and 1,1'-sulfonylbis[2-(methylthio)ethane], have also been used to detect exposures in the past. Urine metabolites of sulfur mustard are useful biomarkers due to their abundance and non-invasive specimen collection methods.^{18, 19} However, these biomarkers are only present in the body up to two weeks post-exposure, limiting their usefulness for long-term examination of samples.²⁰

Adducts to blood proteins are a potential tool for assessing exposure once urinary metabolites are no longer detectable. Sulfur mustard forms adducts to hemoglobin at Val, Glu, and His residues which can be present for up to 120 days.²¹ However, hemoglobin is not commonly used as a biomarker due to the intrinsic sensitivity of these adducts to acids/bases and instability during mass spectrometry analysis.²² Sulfur mustard can also form stable adducts to human serum albumin (HSA) at its reactive Cys-34 residue which has made HSA a useful tool for long-term detection of exposure despite the shorter half-life of 20-25 days.¹³

A method to quantify sulfur mustard-HSA adducts was originally developed by The Netherlands Organisation for Applied Scientific Research (TNO; Rijswijk, The Netherlands)²³ and further modified by Andacht *et al.*²⁴ Sulfur mustard binds to the single reactive Cys residue (Cys-34) of HSA producing the stable sulfur-hydroxyethylthioethyl [S-

HETE] adduct. When HSA is digested with pronase, a tripeptide is observed ([S-HETE]-Cys-Pro-Phe) and can be quantified via LC-MS/MS. The previously reported method was a labor intensive process which precipitated blood proteins using trichloroacetic acid and then enriched HSA using Cibacron blue affinity. The HSA enriched samples were then digested with pronase, filtered, and concentrated using solid phase extraction (SPE) prior to LC-MS/MS analysis.²⁴ The concentration of [S-HETE]-Cys-Pro-Phe in unknown samples was then calculated from a calibration curve comprised of known sulfur mustard spikes into blank plasma.

Here we report a straightforward and simplified method for measuring sulfur mustard adducts to HSA in human blood specimens. A 96-well plate can be prepared in 3.5 h and analyzed via isotope dilution UHPLC-MS/MS in 4.5 h, totaling 8 h from start to finish, which is five times faster than previous methods.²⁴ Required sample volume has also decreased four-fold while maintaining sensitivity using proteinase K to generate the [S-HETE]-Cys-Pro-Phe biomarker. The presented method quantifies exposure using a synthetic peptide calibration curve as opposed to a calibration curve based on sulfur mustard spiked into matrix at different concentrations. Ruggedness, stability, and matrix effect experiments were performed, and as a result, a sensitive, high-throughput analytical method for assessing sulfur mustard exposure via measurement of protein adducts in human blood specimens has been developed and validated. Since the method does not selectively enrich albumin, it should be readily adaptable for measuring exposure to other alkylating agents.

Experimental Procedures

Reagents and Materials

HPLC-grade methanol, ammonium bicarbonate, Optima™ acetone, heat sealing foil, 96-well deep well plates (Fisher Scientific; Hanover, IL); Optima™ LC/MS-grade formic acid (Sigma-Aldrich; St. Louis, MO); proteinase K isolated from *Tritirachium album* (Millipore; Billerica, MA); HPLC-grade water and acetonitrile (Tedia; Fairfield, OH); 96-well PCR plates and adhesive PCR foil (Eppendorf; Hauppauge, NY); Kinetex C18 column (2.1 x 50 mm, 1.3 μm) (Phenomenex; Torrance, CA); Oasis Hydrophilic/Lipophilic Balanced (HLB) 96-well (30 mg) SPE plates (Waters; Milford, MA); Environmental Protection Agency ultra-dilute sulfur mustard (10 μg/mL) reference standard in isopropanol (Lawrence Livermore National Laboratory; Livermore, CA).

Convenience Sample Set

A convenience set from 150 healthy individuals with no known exposure to sulfur mustard was purchased from Tennessee Blood Services (Memphis, TN). This convenience set contained 50 individual serum, plasma, and blood specimens to evaluate potential interferences. As this study used de-identified specimens acquired from commercial sources, the work did not meet the definition of human subjects research as specified in 45 CFR 46.102 (f).

Calibrators and Quality Controls

Native and d_8 -isotopically labeled [S-HETE]-Cys-Pro-Phe synthetic peptides were synthesized by TNO (Figure 1). The peptides were purified using HPLC and verified to be > 90% pure using HPLC-UV. The eight-point calibration curve (3.00, 6.00, 12.5, 25.0, 50.0, 100., 200. and 250. ng/mL) was prepared in 0.1% formic acid in HPLC-grade water, aliquoted, and stored at -70°C until use. A 35.0 ng/mL stock solution of d_8 -isotopically labeled peptide in 0.1% formic acid in HPLC-grade water was aliquoted and stored at -70°C .

Quality control (QC) materials were made from a stock solution of plasma spiked with sulfur mustard by TNO. In summary, pooled plasma was spiked to a final concentration of 100 μM sulfur mustard (> 98% pure) in acetonitrile and incubated at 37°C for 6 h. The 100 μM sulfur mustard spiked plasma stock was analyzed by TNO for residual sulfur mustard using GC/MS before shipment to the Centers for Disease Control and Prevention. Three QC materials, QC Low (QCL), QC Mid (QCM) and QC high (QCH), were prepared by diluting the 100 μM sulfur mustard plasma stock with pooled plasma from Tennessee Blood Services which were then aliquoted and stored at -70°C . The matrix blank sample was pooled serum from Bioreclamation (Westbury, NY). Each QC level was characterized as part of the method validation using 22 analytical runs.

Safety Considerations

The analysis of sulfur mustard adducts to Cys-Pro-Phe are not expected to pose a risk greater than general peptide analyses. Universal precautions, including the use of proper personal protection equipment and biological safety cabinets, were used by trained personnel when handling clinical specimens. Plasma and serum pools were screened by the vendors, in accordance with FDA regulations, to be free of Hepatitis B, Hepatitis C, *Treponema Pallidum* (Syphilis), and HIV.

Sample Preparation

Matrix blank samples for the calibration curve, QC materials, and any possible clinical samples were thawed and centrifuged at 3,000 $x g$ for 5 min to remove particulates. Fifty microliters of each QC, matrix blank, and unknown sample were transferred without disturbing the pellet to a 96-well deep well plate. Acetone (300 μL) was added to each well containing sample to precipitate all proteins present. The sample plate was covered with adhesive foil, shaken at 500 rpm for 30 s using a MixMate[®] (Eppendorf; Hauppauge, NY), and centrifuged at 3,000 $x g$ for 5 min at 20°C . The supernatant was removed without disturbing the protein pellet, and the pellet was then air dried at room temperature.

Internal standard (20 μL) was added to each well containing sample. Each calibrator (50 μL) was added to the wells containing the pellet formed by the matrix blank samples to construct an eight-point calibration curve in matrix. Fifty microliters of 0.1% formic acid in HPLC-grade water was added to the remaining wells including QCs and unknowns to ensure homogeneity. An aliquot of 400 μL 50 mM ammonium bicarbonate (pH 7.8) and 100 μL of 10 mg/mL proteinase K in 50 mM ammonium bicarbonate was added to all wells. The plate

was covered with adhesive foil and shaken at 1,000 rpm for 90 min at 50°C on a Thermomixer R® (Eppendorf; Hauppauge, NY) for sample digestion.

After digestion, an Oasis HLB SPE plate was conditioned with 1 mL methanol followed by 1 mL HPLC-grade water on a vacuum manifold. The entire digested sample was loaded onto the SPE plate and drawn through the bed using the vacuum manifold. The wells were washed with 1 mL 20% methanol in HPLC-grade water and eluted into a new 96-well deep well plate with 500 µL acetonitrile. The samples were dried under nitrogen pressure (50 psi) at 70°C, reconstituted in 50 µL 0.1% formic acid in HPLC-grade water, transferred to a 96-well PCR plate, and heat sealed with pierceable foil.

UHPLC-LC/MS

The [S-HETE]-Cys-Pro-Phe analyte was analyzed on an AB Sciex 6500 triple quadruple mass spectrometer (Framingham, MA) interfaced with an Agilent 1290 Infinity series UHPLC (Santa Clara, CA). Data was collected with Analyst 1.6.2 (AB Sciex) using selective reaction monitoring (SRM) with a dwell time of 25 ms for each of the three transitions (Table 1). The TurboIonSpray source was operated in positive ion mode and under the following parameters: curtain gas= 12 psi; collision gas= 10 psi; ionspray voltage= 4000 V; temperature= 600°C; gas source 1= 85 psi; and gas source 2= 90 psi. The collision energy and declustering, entrance, and collision cell exit potentials were independently optimized for quantitation ion, confirmation ion and internal standard and are reported in Table 1. The fragmentation patterns of these peptides have been previously reported.²⁴ The peptide extract (5 µL) was separated by reversed-phase chromatography using a Kinetex C18 analytical column heated to 70°C. Mobile phases were 0.1% formic acid in HPLC-grade water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). A linear gradient with a flow rate of 350 µL/min was run up to 21.5% mobile phase B over 1.8 min, followed by a step to 80% mobile phase B held for 0.2 min, then an immediate return to 2% mobile phase B for column equilibration. The total run time was 3.0 min per sample. The segmented gradient yielded elution times of 2.0 min (\pm 0.2 min) for the [S-HETE]-Cys-Pro-Phe analyte. Extracted ion chromatograms were assessed using Analyst 1.6.2 for correct analyte retention time and peak shape.

Method Validation

Data from 22 analytical runs was evaluated to determine intra- and interday accuracy and precision (relative standard deviation, %RSD), and linearity for all calibrators and QCs and ruggedness, stability, recovery, and matrix effects experiments were performed as part of the validation process. Each analytical run contained an eight-point calibration curve in matrix blank, three QCs (QCL, QCM, and QCH), and a matrix blank sample. A maximum of two runs were prepared and analyzed per day over the span of four weeks by four analysts.

Results and Discussion

A new approach to monitor sulfur mustard adducts to blood proteins without specifically enriching for the protein such as HSA was developed to increase analyte recovery while increasing throughput. Previous sulfur mustard adduct methods isolated HSA using

Cibacron blue prior to enzymatic digestion.²⁴ The disadvantages of enriching for HSA are that the process is laborious, time-consuming, costly, and not completely specific for the target protein. The currently reported method non-specifically captures proteins in a sample and theoretically could be expanded to measure adducts on any Cys that is reactive.

In previous sulfur mustard protein adduct methods, pronase (synonyms: pronase E, actinase E, or protease type XIV from *Streptomyces griseus*) was used to generate the [S-HETE]-Cys-Pro-Phe peptide.^{23, 24} However, commercially available pronase is comprised of a mixture of proteases resulting in significant variability between different lots and vendors of enzyme. During our own digestion studies, pronase cleaved proteins to form the tripeptide adduct but would also further digest to form the dipeptide [S-HETE]-Cys-Pro over time. The ratio between the dipeptide and tripeptide was found to be highly variable; therefore, a more reproducible digestion enzyme was desired to improve method robustness and transferability. Alternate enzymes including chymotrypsin, trypsin, and proteinase K were investigated. Chymotrypsin yielded a specific six amino acid peptide, Leu-Gln-Gln-Cys-Pro-Phe, containing the HETE adduct, but was not pursued due to the high cost of chymotrypsin and labor intensive process which required reducing and alkylating the sample prior to digestion. Trypsin yielded a 21 amino acid peptide containing the HETE adduct but also required reducing and alkylating the sample. Despite not typically being considered a proteomics grade enzyme, proteinase K was selected since it reliably produced the [S-HETE]-Cys-Pro-Phe tripeptide, was affordable, and could be used with a simple digestion protocol.

To further improve robustness, proteinase K digestion was rigorously tested and optimized. Conditions such as digestion time and temperature, enzyme concentration, enzyme buffer (i.e. ammonium bicarbonate) concentration, and pH were separately assessed against the validated conditions using the QCs at values $\pm 20\%$ of the final conditions to determine ruggedness. The three QC materials were run in triplicate under each test condition. All parameters had insignificant influence on the adducted tripeptide when varied $\pm 20\%$ except for a digestion temperature of $+20\%$ of the characterized value which yielded greater analyte levels.

To further explore the effects of temperature on proteinase K and to ensure reliable production of the [S-HETE]-Cys-Pro-Phe peptide, several lots of proteinase K were investigated at various temperatures. In brief, five different lots of proteinase K that had been used during method characterization were used to digest QCM material at 40°C, 50°C, 60°C, and 70°C, each run in triplicate and analyzed in one day (Table 2). The digestion temperature of 60°C yielded higher signal but also increased %RSD above the characterized limits. In addition, proteinase K has been previously reported to become unstable at temperatures $> 60^\circ\text{C}$.²⁵ This method is intended to be transferred to other laboratories; therefore, it is essential to operate under stable conditions to ensure interoperability.

SPE optimization was conducted to improve method performance. SPE recovery was determined by spiking a low level [S-HETE]-Cys-Pro-Phe peptide (12.5 ng/mL) into matrix blank samples pre- and post-SPE. Internal standard was added prior to transferring the sample to a 96-well PCR plate at the end of the method to normalize for any instrumental

variations. The recovery from the HLB SPE was 53 (\pm 4.1%) with no significant loss from the dry down process. Several experiments were conducted to improve recovery including exchanging SPE plates and wash volumes and conditions; however, HLB yielded the highest recovery.

The eight-point calibration curve was spiked into water, serum, plasma, and whole blood and run in triplicate to evaluate matrix effects. The response, which was calculated using the area of the analyte over the area of internal standard, was plotted against the peptide concentration. All calibration curves were linear and had slopes within 5% of each other.

Statistical analysis of the 22 analytical runs was conducted to validate the method. The linear range for [S-HETE]-Cys-Pro-Phe was 3.00-250. ng/mL with an average coefficient of determination value of >0.99 and an average line equation of $y = 0.054x - 0.0052$ with %RSD $<10\%$ for slope and y-intercept. The limit of detection (LOD) was determined using the Taylor method²⁶ in which the four lowest calibrators (3.00, 6.00, 12.5 and 25.0 ng/mL) were analyzed for SD and plotted against their respective concentration. The LOD was calculated as three times the y-intercept and was determined to be 1.74 ng/mL. The lowest calibrator (3.00 ng/mL) is the lowest reportable limit (LRL); results are not reported below the LRL. As shown in Figure 2, a sulfur mustard spiked plasma sample (100 nM) was compared to this reported method's lowest calibrator (3.00 ng/mL or 6.3 nM [S-HETE]-Cys-Pro-Phe) and matrix blank. As expected, the lowest calibrator peak is clearly distinguishable from the matrix blank sample. The average ion ratio for the calibration curve (peak area of confirmation ion divided by the peak area of the quantitation ion) of 0.47 (\pm 0.083) was used to confirm detection. Accuracy from the 22 analytical runs was determined by dividing the average of the calculated concentration for each calibrator by the expected concentration. Relative standard deviation was established by dividing the SD of each calibrator by the average calculated concentration of the same calibrator. The average calculated concentration, SD, accuracy, and %RSD from the validation is shown in Table 3.

Intraday accuracy and %RSD of the calibrators were established as part of the validation. Four calibrator levels (6.00, 25.0, 100., and 250. ng/mL) were spiked into matrix blank and analyzed independently (n=5) to determine %RSD and accuracy (Table 3). The intraday relative standard deviation was 5.08%, and the range of accuracy was between 92.7-102% with a coefficient of determination value of > 0.998 .

The QC materials were characterized using the modified Westgard rules.^{27, 28} Using these parameters, a QC was excluded if it fell outside three SDs of the confidence interval or if two consecutive QC results fell outside the two SD confidence interval. Interday %RSD was established over 22 analytical runs (Table 4). The intraday %RSD of the QC material was 8.6, 8.1 and 10.1% for QCL, QCM and QCH, respectively. To calculate %RSD, five individual preparations of each QC material were simultaneously prepared. The average standard deviation from the five individual preparations was divided by the average calculated concentration from the same five QCs to calculate %RSD.

Peptide calibrator and QC sample storage temperatures and freeze/thaw stability were evaluated. Peptides and QC materials were stored at -70°C , -20°C , 4°C , room temperature,

and 37°C and analyzed weekly for four weeks. Peptide solutions were not stable at 4°C for one week and at -20°C for three weeks, but were stable at -70°C for at least 4 weeks. As a result, all peptides were stored at -70°C and monitored for change in concentration via QC values (Figure 3). Both native and isotopically labeled peptides were subjected to freeze/thaw cycles ranging from -70°C to room temperature. The peptides were stable for two freeze/thaw cycles. The stability of the QC materials was analyzed in a similar fashion and was stable for at least four weeks when stored at room temperature or colder. QC materials were subjected to five freeze/thaw cycles ranging from -70°C to room temperature and found to be stable.

To measure background levels of sulfur mustard and to monitor for interferences, a convenience set of 150 individual samples was analyzed. The convenience set from Tennessee Blood Services (Memphis, TN) was comprised of 50 blood, serum, and plasma specimens from 150 presumably unexposed individuals. Results showed that all 150 samples were negative for [S-HETE]-Cys-Pro-Phe and no interferences were present. QCs served as positive controls in lieu of clinical specimens from sulfur mustard exposed individuals which were not readily available for analysis.

Binding recovery of sulfur mustard-spiked materials was determined by spiking agent into serum, plasma, and blood. A solution of sulfur mustard (> 98% pure) in isopropanol (10 µg/mL) was spiked into serum, plasma, and blood at three levels (0, 35 and 70 ng/mL), incubated at 37°C for 6 h and immediately analyzed using the reported method. As a result, the percent recovery between expected and actual calculated concentration for the analyte [S-HETE]-Cys-Pro-Phe was 41 (± 1.1%) for serum and plasma and 8.4 (± 0.6%) for whole blood. The discrepancy between serum/plasma and blood can be caused by sulfur mustard binding to DNA or alternate blood proteins such as hemoglobin. Overall, low yield of cysteine adducts can be contributed to hydrolysis of sulfur mustard²⁹ or binding to alternate proteins.²¹

Conclusion

We developed a simplified high-throughput method to quantify sulfur mustard adducts to HSA in clinical blood specimens via isotope dilution UHPLC-MS/MS. Sample preparation time was significantly reduced by measuring cysteine adducts to hydroxyethylthioethyl in total protein rather than isolating adducts following HSA enrichment. Thorough digestion studies ensured that [S-HETE]-Cys-Pro-Phe was reliably produced by proteinase K, significantly improving the robustness of this approach over previously reported methods. In addition, SPE recovery was investigated to improve method performance. Upon optimization, the method was validation using 22 analytical runs to evaluate accuracy, %RSD, linearity, stability, and matrix effects. Using serum, plasma, and blood from 150 presumably unexposed individuals, we verified that no background levels of sulfur mustard or interfering chromatographic peaks were present. Binding recovery of sulfur mustard was investigated by spiking sulfur mustard into serum, plasma, and whole blood and measuring the tripeptide biomarker, [S-HETE]-Cys-Pro-Phe. Recovery of spiked agent was 41% for serum and plasma and 8.4% for whole blood. As a result of these efforts, the reported

method can measure sulfur mustard exposure via the hydroxyethylthioethyl adduct to HSA in low volume clinical blood matrix specimens in 8 hours.

Acknowledgements

The authors would like to thank the members of the Chemical Laboratory Response Network and Analytical Response laboratories for technical assistance during the preparation of this manuscript.

Funding Sources

This work was supported by the Centers for Disease Control and Prevention, Office of Public Health Preparedness and Response, the Oak Ridge Institute for Science, and Education, and the Defense Threat Reduction Agency (11-005-12430).

Abbreviations

S-HETE	sulfur-hydroxyethylthioethyl
CW	chemical weapon
TNO	The Netherlands Organisation for Applied Scientific Research
SPE	solid phase extraction
HLB	Hydrophilic/Lipophilic Balanced
SRM	selective reaction monitoring
QC	Quality control
QCL	QC Low
QCM	QC mid
QCH	QC high
LOD	limit of detection
LRL	lowest reportable limit
%RSD	relative standard deviation

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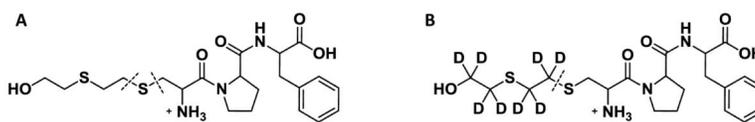


Figure 1. The chemical structures of (A) native and (B) d₈-isotopically labeled [S-HETE]-Cys-Pro-Phe peptide. The fragmentation sites for the quantitation and confirmation transitions are shown with dashed lines.

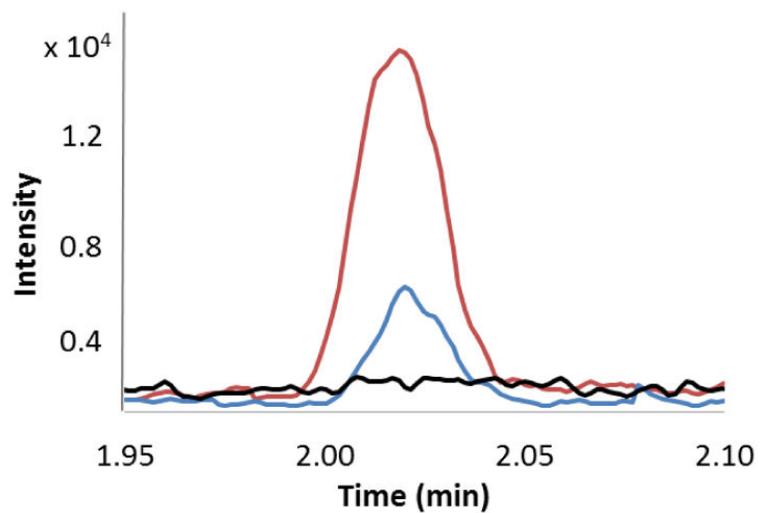


Figure 2. Chromatograph comparing sulfur mustard spiked into plasma (100 nM) (red line), lowest [S-HETE]-Cys Pro-Phe peptide calibrator of 6.30 nM (3.00 ng/mL) (blue line), and matrix blank sample (black line).

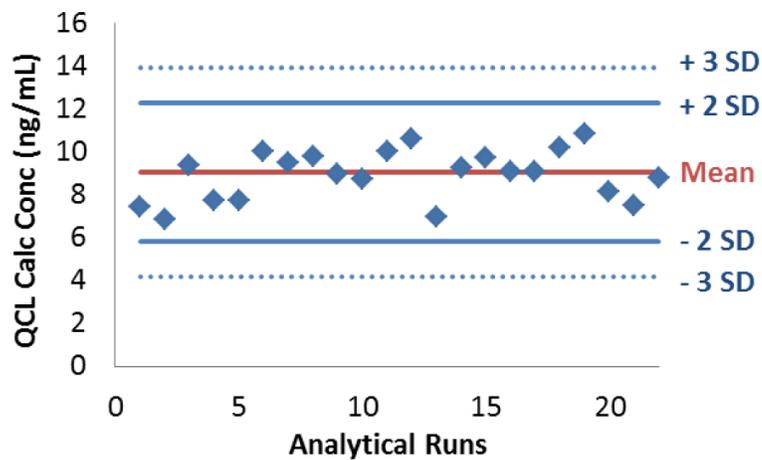


Figure 3. 4-week stability of QCL when stored at -70°C . *SD*, standard deviation; *QCL*, quality control low.

Table 1

Selected Reaction Monitoring (SRM) transitions for the UHPLC-MS/MS analysis of sulfur mustard adducts to Cys-Pro-Phe.

Transition	Peptide Sequence	Precursor Ion Q1 (<i>m/z</i>)	Product Ion Q3 (<i>m/z</i>)	Decustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)
Quantitation Ion	[S-HETE]-Cys-Pro-Phe	470.2	105.1	60	2.0	29	7.0
Confirmation Ion	[S-HETE]-Cys-Pro-Phe	470.2	137.1	60	7.0	28	11
Internal Standard	<i>d</i> ₈ -[S-HETE]-Cys-Pro-Phe	478.2	113.1	60	11	27	7.0

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Table 2

Effects of digestion temperature on the formation of [S-HETE]-Cys-Pro-Phe from QCM material using five different lots of proteinase K each run in triplicate.

Digestion Temperature	Calculated Concentration (\pm SD) (ng/mL)	%RSD
40°C	36.8 (\pm 5.40)	14.7%
50°C	40.5 (\pm 5.05)	12.5%
60°C	64.5 (\pm 17.4)	27.0%
70°C	44.1 (\pm 24.5)	55.6%

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Table 3

Calculated interday and intraday concentration, accuracy, and relative standard deviation for the [S-HETE]-Cys-Pro-Phe peptide calibrators.

Calibrator (ng/mL)	Interday (n = 22)			Intraday (n = 5)		
	Calculated Concentration (\pm SD) (ng/mL)	Accuracy (%)	Relative Standard Deviation (%)	Calculated Concentration (\pm SD) (ng/mL)	Accuracy (%)	Relative Standard Deviation (%)
3.00	3.28 (\pm 0.310)	109	9.49	--	--	--
6.00	5.77 (\pm 0.380)	96.1	6.66	5.56 (\pm 0.280)	92.7	5.08
12.5	12.3 (\pm 0.840)	98.7	6.78	--	--	--
25.0	24.5 (\pm 1.34)	98.1	5.46	24.4 (\pm 0.590)	97.4	2.40
50.0	48.3 (\pm 2.40)	96.6	4.96	--	--	--
100.	100. (\pm 4.03)	100.	4.02	99.8 (\pm 4.41)	102.	4.68
200.	202. (\pm 6.89)	101	3.41	--	--	--
250.	250. (\pm 9.82)	100.	3.93	242. (\pm 10.4)	97.7	4.18

Table 4

Interday relative standard deviation of the sulfur mustard protein adduct QC materials (n=22) over four weeks. *QCL* quality control low, *QCM* quality control mid, *QCH* quality control high.

Quality Control	Calculated Concentration (\pm SD) (ng/mL)	Relative Standard Deviation (%)
QCL	9.05 (\pm 1.62)	17.9
QCM	36.7 (\pm 5.00)	13.6
QCH	76.9 (\pm 9.20)	12.0

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