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A quantitative method to detect human exposure to sulfur and nitrogen mustards via protein adducts

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Abstract

Sulfur and nitrogen mustards are internationally banned vesicants listed as Schedule 1 chemical agents in the Chemical Weapons Convention. These compounds are highly reactive electrophiles that form stable adducts to a variety of available amino acid residues on proteins upon exposure. We present a quantitative exposure assay that simultaneously measures agent specific protein adducts to cysteine for sulfur mustard (HD) and three nitrogen mustards (HN1, HN2, and HN3). Proteinase K was added to a serum or plasma sample to digest protein adducts and form the target analyte, the blister agent bound to the tripeptide cysteine-proline-phenylalanine (CPF). The mustard adducted-tripeptide was purified by solid phase extraction and analyzed using isotope dilution LC-MS/MS. Product ion structures were identified using high-resolution product ion scan data for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF. Thorough matrix comparison, analyte recovery, ruggedness, and stability studies were incorporated during method validation to produce a robust method. The method demonstrated long term-stability, precision (RSD < 15%), and intra- and inter-day accuracies > 85% across the reportable range of 3.00–200 ng/mL for each analyte. Compared to previously published assays, this method quantitates both sulfur and nitrogen mustard exposure biomarkers, requires only 10 μL of sample volume, and can use either a liquid sample or dried sample spot.

Keywords

Sulfur mustard; Nitrogen mustard; Protein biomarker; Cysteine adducts; LC-MS/MS; Quantitative method

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1. Introduction

Sulfur and nitrogen mustards belong to a class of chemical warfare agents (vesicants) that produce painful blisters on exposed skin and can damage the eyes, lungs, and central nervous system [1]. Due to their high reactivity, historical use as chemical weapons, and ease of production, these vesicants are listed as Schedule 1 chemical agents in the Chemical Weapons Convention [2]. As a result, their production, synthesis, stockpiling, and use are strictly prohibited. Sulfur mustard (HD), or bis(2-chloroethyl)sulfide, was first used as a weapon during WWI and later in the Iraq-Iran conflict [3–5]. Additionally, several nitrogen mustards, bis(2-chloroethyl)ethylamine (HN1), bis(2-chloroethyl)methylamine (HN2), and tris(2-chlorethyl)amine (HN3), were stockpiled but never used as chemical weapons [6]. A detection method for HD, HN1, HN2, and HN3 exposures is necessary for preparedness during potential terrorist attacks as well as to monitor personnel involved with eliminating existing mustard agent stockpiles [7].

Urinary and plasma metabolites are used to monitor exposure to vesicants. Urinary metabolites are abundant and easy to collect; however, they are transient and are generally eliminated from the body within 1–2 weeks [8,9]. Key urinary metabolites of HD include the simple hydrolysis products thiodiglycol (TDG) and TDG sulfoxide as well as the β-lyase metabolites 1,1′-sulfonylbismethylthioethane (SMBTE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE) among others [10–14]. Both TDG and TDG sulfoxide are abundant in human urine post-exposure, but they are also present in unexposed individuals, making them biomarkers consistent with, but not specific for, exposure to HD [10]. The β-lyase metabolites are more specific biomarkers of HD exposure and have not been observed in unexposed individuals, unlike TDG. For the nitrogen mustards, N-ethyldiethanolamine (EDEA), *N*-methyldiethanolamine (MDEA), and triethanolamine (TEA) are common metabolites for HN1, HN2, and HN3, respectively [15]. However, previous studies by Lemire et al. showed high background concentrations for TEA in unexposed individuals making TEA an ineffective biomarker of exposure for HN3 [16].

Covalent adducts of sulfur and nitrogen mustards to macromolecules such as DNA and proteins are biomarkers of exposure that have a longer half-life compared to neat agents and urinary and plasma metabolites in the body. Vesicants can alkylate DNA via the aziridinium cation at N^7-guanine and N^3-adenosine or crosslink between guanines [17–19]. However, DNA adducts can be detected and repaired within days [10,17,20,21]. The reactive agents can bind to cysteine, lysine, histidine, aspartic acid, glutamic acid, and tyrosine residues as well as the N-terminus of proteins [18,22,23]. Sulfur and nitrogen mustard adducts to proteins such as albumin and hemoglobin are ideal since they are stable for several weeks to months in the blood [9,11].

Sulfur and nitrogen mustard adducts to reactive cysteine residues on proteins are well-established, long-term biomarkers of exposure. In 1999, Noort and colleagues first introduced a method which evaluated an HD adduct to highly reactive cysteine-34 position on human serum albumin [7]. Shortly after, Noort et al. [9] expanded this panel of mustard adducts that bind to reactive cysteines to include the chemotherapeutic drugs cyclophosphamide, melphalan, mechlorethamine, and nornitrogen mustard [15]. In 2008, Yeo et al. developed a method to simultaneously verify exposure to HD, HN1, HN2 and HN3 using the cysteine-34 adduct [25]. These initial methods involved complex sample clean-up techniques to extract the biomarkers from blood (e.g., affinity chromatography techniques) and required larger sample volumes and longer preparation times.

We built upon previously identified biomarkers of sulfur and nitrogen mustards. Here we report a high-throughput method which simultaneously quantitates sulfur and nitrogen mustard exposures via protein adducts to reactive cysteine in human serum or plasma matrices with increased selectivity and minimal sample volume. The reported method was developed using method parameters identified by Scientific Working Group for Forensic Toxicology (SWGTOX) [26] and FDA guidance on bioanalytical validation [27]. The Organisation for the Prohibition of Chemical Weapons (OPCW) accepts two different albumin-derived diagnostic markers containing the cysteine-34 adduct to confirm sulfur mustard exposure using biomedical sample analysis. The first, the tripeptide fragment cysteine-proline-phenylalanine (CPF) containing an agent-specific adduct on the cysteine, is the diagnostic marker which is produced when albumin containing samples are digested with proteinase K [24,25,28–30]. The second diagnostic bio-marker, the dipeptide fragment cysteine-proline (CP) also containing the agent-specific adduct on the cysteine, is produced when albumin containing samples are digested with pronase [31]. The method reported here quantitates the tripeptide adduct, which for simplicity will be referred to as the agent bound-CPF (i.e. HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF). In brief, 10 μL of serum or plasma is digested using proteinase K, purified using solid phase extraction (SPE), separated by UHPLC, and detected by isotope dilution MS/MS as depicted in Fig. 1.

2. Materials and methods

2.1. Chemicals and materials

Lyophilized native and $^{13}C_9$,^{15} N-isotopically labeled HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF custom peptides (chemical purity > 98%) were synthesized by Battelle Memorial Institute (Columbus, OH). The phenylalanine residue of the isotopically labeled peptide was fully enriched at each nitrogen and carbon with ^{15}N and ^{13}C. Stock solutions of plasma spiked to a final concentration of 16 μg/mL HD (99.7% pure in acetone), 100 μg/mL HN1 (99% pure in acetone), 100 μg/mL HN2 (79.7% pure in acetone), and 100 μg/mL HN3 (99.7% pure in acetone) were also prepared by Battelle. All spiked plasma stocks were analyzed for residual agents (< 47 ng/mL free agent) using GC/MS before shipment to the Centers for Disease Control and Prevention (CDC). Pooled human plasma collected from whole blood donations was purchased from Bioreclamation (Westbury, NY) for use as controls and agent-enriched plasma stocks. Plasma pools were screened by the vendor, in accordance with Food and Drug Administration regulations, to be free of hepatitis B, hepatitis C, syphilis, and HIV. HPLC-grade water, acetonitrile, and methanol were purchased from MG Scientific (Pleasant Prairie, WI). Ammonium bicarbonate, ammonium formate, 96-well PCR plates, and heat sealing foil were purchased from Fisher Scientific (Hanover, IL). Optima LC/MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO). Proteinase K isolated from *Tritirachium album* (cat #70663) was purchased from EMD Millipore (Billerica, MA). Kinetex C18 columns (2.1 × 50 mm, 1.3 μm particle size) were purchased from Phenomenex (Torrance, CA). Oasis hydrophilic/lipophilic balanced (HLB) 96-well (30 mg) SPE plates were purchased from Waters (Milford, MA). Mitra™ microsampling devices were purchased from Neoteryx (Torrance, CA). Additional lots of proteinase K enzyme were purchased from different vendors to compare digestion efficiency (Section 3.10): lots 76230Y and 17916 from Thermo Fisher (Hanover, IL); lot J62051 from Alfa Aeser (Tewksbury, MA); and lots 193504 and 193981 from MP Biomedical (Santa Ana, CA).

2.2. Preparation of the calibration curve, internal standard, and quality control materials

A stock solution (1 μg/mL each) of HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF in HPLC-grade water was made from the lyophilized peptides. The stock solution was diluted with HPLC-grade water to construct a seven-point calibration curve: 3.00, 6.00, 12.5, 25.0, 50.0, 100, and 200 ng/mL. The 15 ng/mL internal standard (ISTD) solution was prepared from the lyophilized $^{13}C_9$,^{15}N-labeled HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF peptides in HPLC-grade water. The calibration curve and ISTD solution were aliquoted and stored at −70 °C until use. There were no isotopic contributions between the native and labeled compounds.

Quality control (QC) materials were made by diluting the agent-enriched plasma stocks to two levels (QC low [QCL] and QC high [QCH]) using control plasma. The agent-enriched plasma stocks were diluted in control plasma to a final concentration of ~7.5 ng/mL and ~75 ng/mL of HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF for QCL and QCH, respectively. This same control plasma sample served as the matrix blank (MB). The MB sample was processed with the QCs to monitor for contamination and to serve as a function check for the instrument since the MB sample contains ISTD. The QC and MB materials were aliquoted and stored at −70 °C until use. Each QC concentration was statistically determined during materials characterization.

2.3. Convenience sample set

A commercial convenience sample set from Tennessee Blood Services (Memphis, TN) was used to evaluate baseline levels, analyte specificity, and any chromatographic interferences. The convenience set was comprised of 50 serum, 50 plasma, and 50 whole blood samples from unexposed individuals (150 unmatched samples). The samples were purchased from a commercial source and were de-identified; therefore, the work did not meet the definition of human subjects research as specified in 45 CFR 46.102 (f).

2.4. Sample preparation

Samples were prepared in a similar fashion as reported in Pantazides et al. [24]. Key features of the new method included the addition of nitrogen mustard analytes, a reduction in sample volume and digestion time, and a change in SPE conditions. Reported here, 10 μL of each calibrator in water, QCs, MB, and sample were added to a 96-well PCR plate. Calibration slope comparison studies showed equivalency between matrices; therefore, calibrators were stored in water to improve long-term stability of the calibrators. ISTD (10 μL) and 10 mg/mL proteinase K in 50 mM ammonium bicarbonate (100 μL) were added to each well to be analyzed. The PCR plate was heat sealed and the samples were digested for 60 min at 50

°C on a PCR heating block. After incubation, the PCR plate was briefly centrifuged to reduce the potential for cross-well contamination that could occur when removing the heat-sealed foil.

Sample cleanup was performed using an Oasis 30 mg HLB SPE plate. The plate was conditioned with HPLC-grade methanol (1 mL) followed by HPLC-grade water (1 mL) using a vacuum manifold. The entire digested sample (~120 μL) was loaded onto the SPE plate and pulled through by vacuum. The samples were washed with 5% methanol in 50 mM ammonium bicarbonate (1 mL) and then eluted into a deep 96-well plate with HPLC-grade acetonitrile (0.6 mL). The eluent was dried under a nitrogen stream at 60 °C. The samples were reconstituted in 0.1% formic acid in HPLC-grade water (50 μL) and stored at −70 °C until LC-MS/MS analysis.

2.5. Dried sample analysis

The potential of analyzing dried plasma and serum samples was assessed with Mitra™ microsampling devices. In brief, the hydrophilic polymer-based Mitra™ microsampling devices were used to collect QCL and QCH samples following the manufacturer's instructions and dried at room temperature for a minimum of 2 h. Each microsampling device wicks up 10 μL of sample with an RSD of 3.05%, according to the manufacture's certificate of conformance. Once dry, the tip of the microsampling device was transferred to the 96-well PCR plate and processed through the method as an unknown. The tip of the microsampling device was completely submerged in buffer to ensure uniform digestion.

2.6. Instrumentation and data acquisition/processing

Product ion scans were collected using high-resolution mass spectrometry (HRMS) which aided in the identification of new fragmentation ions for nitrogen mustard adducts to cysteine-34. A Thermo QExactive HF quadrupole Orbitrap mass spectrometer (Bremen, Germany) was equipped with a heated electrospray ionization (HESI) source and operated in positive ion mode. The following parameters were applied: spray voltage 3.00 kV, capillary temperature 300 °C, sheath gas 12.0 L/min, auxiliary gas 1.00 L/min, and S-lens RF levels 40.0 V. Nitrogen was used for the spray stabilization, higher-energy collision dissociation cell, and damping gas in the C-trap. The full scan and all-ion-fragmentation (AIF) were acquired at a resolving power of 120,000 FWHM with an AIF scan range of 107.3–907.1 m/z. The automatic gain control target was 1e6, with a maximum injection time of 100 ms. Samples were infused using a built-in syringe pump and were fragmented at collision energies (CE) of 10, 25, and 50 eV. Data acquisition and analysis were performed using QExactive software (version 2.3) and XCalibur (version 3.0).

Once specific fragmentation ions were identified using HRMS, a quantitative, triple quadrupole (QQQ) MS method was developed. Chromatographic separation was carried out on an Agilent 1290 Infinity UHPLC system (Santa Clara, CA) equipped with an auto-sampler, binary pump, and heated column compartment. Analytical separation was performed using a Phenomenex Kinetex C18 column (50 mm × 2.1 mm, 1.3 μm particle size). Mobile phase A (MPA) consisted of 5 mM ammonium formate in HPLC-grade water and mobile phase B (MPB) consisted of 0.1% formic acid in HPLC-grade acetonitrile.

Following a needle wash with MPB (6 s), sample (2.5 μL) was injected onto the column with initial conditions of 2% MPB. A linear increase of the solvent to 10% MPB occurred from 0.00 to 2.0 min, followed by a second linear increase to 25% MPB from 2.01 to 3.5 min, an immediate ramp to 80% MPB at 3.51 min (held until 4.0 min), and an immediate return back to initial conditions at 4.01 (held until 4.5 min). The flow rate was initially 350 μL/min until 2.01 min at which point the flow rate increased to 450 μL/min for the remainder of the run. The column temperature was maintained at 70 °C, and the sample storage compartment was set to 4 °C.

The UHPLC system was coupled to a 6500 Sciex QQQ mass spectrometer (Framingham, MA) equipped with a TurboIonSpray source operating in positive ion mode. Data was collected in multiple-reaction monitoring (MRM) mode where the two most sensitive transitions were monitored to serve as the quantitation (quant) and confirmation (confirm) transitions for the native analytes. A single MRM transition was recorded for each of the isotopically labeled compounds corresponding to the quant transition of their native analogues. The following optimized parameters were applied for the detection of all analytes: curtain gas (12 psi), collision gas (10 psi), IonSpray Voltage (4000 V), temperature (600 °C), Ion Source Gas 1 (85), Ion Source Gas 2 (85), and dwell time (15 ms).

Data acquisition and quantitative analysis were performed utilizing Analyst (version 1.6) and MultiQuant software (version 3.0.2). The 7-point calibration curve was established by plotting the response ratio (analyte area divided by the ISTD area) against the expected calibrator concentration and performing a 1 / x weighted linear regression. The response was linear over the entire range of 3.00–200 ng/mL with a coefficient of determination (R^2) of 0.990 or greater for each analyte. The confirmation ion ratio (CIR) was calculated by dividing the confirmation ion peak area by the quantitation ion peak area. The mean CIR from the analytical standards was established and evaluated on a batch-by-batch basis.

2.7. Method validation and QC material characterization

The method validation included inter- and intra-day precision, accuracy by recovery of a known addition of each analyte, linearity, limit of detection (LOD), ruggedness, stability, specificity, and an evaluation of matrix effects. The validated method was used to characterize concentration limits for the low and high QC materials and to establish the acceptable limits for calibrator concentration, calibration slope, peak retention time, and minimum ISTD area. QC characterization was completed over two months with 24 analytical batches performed by three analysts with a maximum of two analytical batches per day. Each analytical batch included a calibration curve, MB, and two QCs (QCL and QCH). After limits were established, the QC materials were evaluated against modified Westgard rules [32,33].

3. Results and discussion

3.1. Mass spectrometry development

HRMS was used to confirm previously established fragment ions and identify new fragment ions for nitrogen mustards. Full-scan and MS^2 spectra have previously been reported for

HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF [7,25]. We report additional unique transitions, with no observed endogenous interferences, that proved to be both reliable and sensitive. Briefly, HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF solutions (500 ng/mL) for both the native and isotopically-labeled peptides were directly infused into the HRMS instrument to collect full scan and product ion scan (MS^2) spectra. The MS^2 spectra were used to elucidate the structures of new product transitions with a mass error range < 0.50 ppm. The exact mass, mass error, and proposed chemical formulas and structures of monitored product fragments collected are shown in Table 1. After initial HRMS studies a final QQQ MRM method was developed and optimized. Overlays of product ion scans from the QQQ instrument collected at CE of 10, 25, and 50 eV for each analyte are shown in Fig. 2. Less sensitive HN3-CPF product ions, 497.2 → 301.1 m/z (quant) and 497.2 → 152.1 m/z (confirm), were chosen over 497.2 → 364.1 m/z due to a coeluting interfering peak that could not be resolved. All transition specific source settings for the QQQ instrument are shown in Table 2.

3.2. Liquid chromatography development

A variety of chromatographic conditions were tested to achieve optimal peak symmetry, analyte retention, and chromatographic resolution from interferences. Multiple reversed phase and mixed-mode HPLC columns were tested including silica-based C18, biphenyl, and porous graphitic carbon columns and mixed-mode weak anion/cation exchange and strong anion/cation exchange columns. In addition, several mobile phase solvents containing different organics (various compositions of water, methanol, and acetonitrile), concentrations of salts (0 to 10 mM ammonium formate), and pH (ranging from 2 to 9) were tested. Ultimately, the silica-based C18 column (50 × 2.1 mm, 1.3-μm particle size) and 5 mM ammonium formate in HPLC-grade water (MPA) and 0.1% formic acid in acetonitrile (MPB) were selected. This combination of column and solvents was selected since it resolved issues regarding multiple peaks and was the most successful at separating the peptides from interferences while maintaining good retention and peak shape which were common issues with the nitrogen and sulfur mustard peptides, respectively. The total delay time of the system was ~0.543 min based on a column void volume of 120 μL, gradient delay of 70 μL, and flow rate of 350 μL/min. With these conditions, nitrogen mustard peptides eluted in ~7–8% MPB while the sulfur mustard peptide eluted in ~17% MPB.

The gradient profile and flow program varied between the nitrogen and sulfur mustard peptides to attain optimal chromatographic resolution for each analyte and improve the method throughput. A gradient slope of $y = 4x$ (where y is %B and x is time) at a flow of 350 μL/min was required to separate the nitrogen mustard analogs and eliminate transition specific interfering endogenous peaks, particularly for HN3-CPF. The gradient slope was increased to 10 and the flow rate to 450 μL/min to elute HD-CPF with improved S/N and throughput. Under these conditions HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF eluted at 3.27 ± 0.02 min, 2.19 ± 0.04 min, 1.90 ± 0.04 min, and 1.95 ± 0.04 min, respectively. HN3-CPF and HN2-CPF were not chromatographically resolved but were resolved by mass spectrometry. Chromatograms for the MB, lowest calibrator (3.00 ng/mL), and QCL for each analyte are shown in Fig. 3. Note that in Fig. 3 there is a resolved peak in HN2-CPF and HN3-CPF that is only present in plasma or serum-based samples (i.e. MB and QC samples). This peak is not present in the chromatograms of the solvent-based peptide calibrators.

3.3. SPE development

Multiple SPE plates and sample loading, washing and elution conditions were compared to assess analyte extraction recovery and performance. Protein precipitation plates plus several reverse-phase, mixed-mode, and hydrophilic SPE plates were compared. Ultimately, the 30 mg HLB SPE plate was selected since it achieved the highest analyte recovery. Sample loading was optimized by comparing analyte recovery from samples (120 μL) directly loaded onto the SPE plate to samples diluted with 50 mM ammonium bicarbonate or water (1 to 2 mL) prior to SPE extraction. There were no significant changes in analyte recoveries between undiluted and diluted samples; therefore, the undiluted procedure was selected for simplicity. Multiple wash solvents (water, 1 to 15% methanol in water, and 0 to 0.5% formic acid in water), volumes (0.5 to 2.0 mL wash) and number of washes (1 to 3 washes) were investigated. As a result, a wash condition of 1 mL 5% methanol in 50 mM ammonium bicarbonate was selected since the 5% organic solvent eluted polar compounds without prematurely eluting our analytes of interest. Finally, several elution conditions were evaluated including different organic solvent strengths (75 to 100% methanol or acetonitrile), volumes (0.2 to 2.0 mL elution), and number of elutions (1 to 4 elutions). A single elution with 0.60 mL acetonitrile was selected since it effectively eluted the analytes of interest in a relatively low volume which led to a faster solvent evaporation time. Methanol gave similar analyte recoveries but required higher elution volume and longer solvent evaporation times.

3.4. Analyte extraction recovery, relative ionization efficiency, and matrix compatibility

SPE optimization included the evaluation of analyte recovery and relative ionization efficiency. In brief, a MB sample was processed in triplicate through the digestion step and split into two parts prior to SPE. One part was enriched with a low-level calibrator (12.5 ng/mL) before SPE while the second part was enriched with the same calibrator after SPE. Both samples were then dried after SPE and reconstituted in 40 μL 0.1% formic acid in water and 10 μL ISTD and analyzed via HPLC-MS/MS. Extraction recovery was calculated by comparing the response ratios of a calibrator added pre-SPE versus post-SPE. The relative ionization efficiency was calculated by comparing the peak area of the analytes added to MB post-SPE versus the peak areas of the same concentration of analytes added to solvent. Based on these results, a 30 mg HLB SPE plate using a 1 mL 5% methanol in 50 mM ammonium bicarbonate wash with a single 600 μL acetonitrile elution yielded a SPE recovery of 86.3 ± 5.8%, 94.5 ± 7.6%, 85.8 ± 3.2%, and 84.0 ± 4.6% for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively. Under the same SPE conditions the relative ionization efficiency was 85.9 ± 2.6%, 114.1 ± 2.0%, 92.0 ± 6.4%, and 117.6 ± 1.0% for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively.

Matrix comparisons were performed to determine whether serum, plasma, and whole blood could be used interchangeably. Calibration curves were prepared in triplicate in solvent, plasma, serum, and whole blood and processed using the method. Slopes within 5% of each other were considered interchangeable. Serum, plasma, and solvent were all within ± 5%;

therefore, they can be used interchangeably. Whole blood was over the 5% threshold. As a result, whole blood needs to be separated prior to analysis or analyzed using calibrators prepared in whole blood.

3.5. Limit of detection

The LOD for each analyte was established using the method outlined by Taylor in *Quality Assurance in Chemical Measurements* [34]. The standard deviations (SDs) from 20 analytical batches of the three lowest calibrators (3.00, 6.00, and 12.5 ng/mL) were plotted against their respective concentration. The LOD was calculated as 3-times the y-intercept of this plot (S_0). The calculated LOD for each analyte was 1.06 ng/mL, 2.20 ng/mL, 1.76 ng/mL, and 1.32 ng/mL for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively. The lowest calibrator, 3.00 ng/mL, was used as the method's lower reportable limit.

3.6. Accuracy and precision

Accuracy and precision were evaluated during peptide recovery experiments and by analyzing intraday and interday QC results. To evaluate accuracy, a concentrated stock peptide solution was added into two different matrix material lots to a final concentration of 6.00, 25.0, and 100 ng/mL in triplicate on two different days, and each sample was analyzed as an unknown. The recovery percentage (average final concentration divided by the added concentration) was 90.0%, 91.2%, 93.8%, and 90.4% for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively. All recovery values were within the allowable limit of 85–115% [27]. Precision was assessed by analyzing intraday and interday results of two QC levels (QCL and QCH) over the course of 20 analytical batches from 10 days. The relative standard deviation (RSD) was calculated by dividing the SD of 20 analytical batches by the mean concentration for each analyte. The RSD of the intraday and interday QC results were within the 15% threshold limit for all analytes as shown in Table 3.

3.7. Analytical specificity

A convenience set comprised of 50 blood, 50 serum, and 50 plasma specimens from 150 unexposed individuals was screened to evaluate for analytical specificity and chromatographic interferences. No endogenous peaks were present in any of the 150 samples. In addition, CIR was monitored to add another level of selectivity for this method and to provide further confidence in the quantitative results. The CIR was approximately 0.44 ± 0.08 for HD-CPF, 0.19 ± 0.03 for HN1-CPF, 0.18 ± 0.02 for HN2-CPF, and 0.72 ± 0.11 for HN3-CPF for the QC characterization. There was a bias high for CIR at the lower limit of the method due to the low signal-to-noise.

3.8. Dried sample analysis

Dried plasma and serum was investigated as a sample matrix to simplify shipping and transportation of samples. The advantages of dried samples are that they reduce infection hazards and can be transported at room temperature. In 2016, John et al. first introduced a method which evaluated the dipeptide adduct HD-CP, using dried sample spots on two microsampling devices, Mitra$^{\text{TM}}$ microsamplers and Noviplex$^{\text{TM}}$ DUO cards [31]. We expanded upon their research and evaluated mustard adducts to the CPF biomarker using dried sample spots. To evaluate dried samples, the QCL and QCH samples (n = 8) were spotted onto a Mitra™ microsampling device and run through the method as previously described (Section 2.5). The concentration results from the dried QCL samples were 0.1%, 0.4%, 11.6%, and 1.9% of the mean QC value characterized from plasma for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively. Furthermore, the concentration results from the dried QCH samples were 8.3%, 13.1%, 10.4%, and 9.9% of the mean QC value characterized from plasma for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF, respectively. Since all concentration values were within 15% of the characterized mean value of liquid plasma, the 10 μL microsampling dried sample spot devices can be used as an alternate to liquid sample. Microsampling devices did not work with whole blood samples which was expected due to previous matrix comparison studies between serum, plasma, and whole blood (Section 3.4). Since this was a preliminary study of dried samples, future dried sample analyses need to be conducted to evaluate stability and ruggedness.

3.9. Stability

Stability of each analyte was assessed through a series of freeze-thaw cycles, storage of unprocessed and extracted samples at room temperature, and long-term storage at −70 °C. Previous stability studies were performed on HD-CPF [24], but this is the first time nitrogen mustards have undergone rigorous stability studies for extracted peptides and inhibited materials. In each of these experiments, two QC levels (QCL and QCH) were evaluated in triplicate. Furthermore, low and high-level synthetic peptides (12.5 and 100 ng/mL) were evaluated through each stability experiment to simulate prepared samples. Freeze-thaw experiments were performed by cycling the peptides and QCs three times between room temperature and −70 °C and allowing them to equilibrate to each temperature. Bench-top stability of unprocessed samples was conducted by storing peptides and QC samples at room temperature for 24 h prior to sample processing. Bench-top stability of processed samples was conducted by processing the samples through all steps of the reported method and storing the prepared samples at room temperature for 24 h before LC-MS/MS analysis. Finally, long-term stability was performed by storing the peptides and QC samples at temperatures ranging from 4 °C to −70 °C for 1 month. These rigorous stability studies showed that all analytes were stable at each condition and had values within ± 15% of the nominal concentration.

3.10. Ruggedness testing

Ruggedness was evaluated by varying sample preparation parameters that were expected to have the greatest variation. Since the calibration curve and ISTD are present as the final digested products, all of the ruggedness parameters focused on the digestion parameters. The following five digestion parameters were assessed: enzyme concentration, heating block type, and digestion volume, time, and temperature. Heating blocks were compared since some devices uniformly heat the entire sample (i.e. PCR heating blocks), while other devices heat one surface of the sample (i.e. Thermomixers). To evaluate ruggedness, QCL and QCH samples were measured in triplicate at ± 20% of the final conditions for all five parameters and analyzed as unknowns. For example, when evaluating digestion time the QCs would be digested for 45 min (−20% of control) and 75 min (+20% of control) and analyzed as unknowns. All values were within the expected limit of 85–115% of the characterized concentration with a few exceptions that are shown in Table 4. In addition, six proteinase K lots from different vendors were evaluated (vendor details are listed in Section 2.1). These different vendor lots of proteinase K were selected due to their variation in purity, cost, and quality. Proteinase K from Millipore was selected due to its low cost and consistency between lots.

4. Conclusions

In the event of a chemical exposure, it is vital to have multiple orthogonal methods to measure biomarkers of exposure. By having a panel of confirmatory biomarkers, researchers have the ability to detect and confirm chemical exposures in a variety of sample types (urine, blood, etc.) under different collection timelines (immediate collection to several weeks post-exposure). In addition, having multiple biomarkers allows scientists to meet minimum requirements set by the OPCW of having two or more biomarkers to confirm exposure. Furthermore, the use of a panel of biomarkers improves the degree of confidence in a clinical result.

Past researchers have thoroughly investigated HD-adducts to proteins, including adducts to cysteine-proline-phenylalanine (HD-CPF) and to a lesser extent, nitrogen mustards (HN1, HN2, and HN3) bound to the same tripeptide (HN1-CPF, HN2-CPF, and HN3-CPF). The cysteine-34 adduct is an effective biomarker of exposure since 41% and 8.4% of HD-agent added in vitro bound to the cysteine-34 residue in serum/plasma and whole blood, respectively [24]. The reported method is unique since it is the first quantitative method to simultaneously measure HD, HN1, HN2, and HN3 biomarkers of exposure in a high-throughput fashion while maintaining sensitivity and robustness. In addition, this method uses unique quantitation and confirmation transitions for HN1-CPF and HN3-CPF that are free of endogenous interferences and yield reproducible chromatography. Previous concerns regarding the lack of selective HN3 biomarkers of exposure were addressed by finding new HN3-CPF fragmentations products (497.2 → 301.1 *m/z* and 497.2 → 152.2 *m/z*). In the reported method, all transitions were monitored and thoroughly evaluated using a convenience set from 150 different individuals to examine for any endogenous chromatography interferences. By combining these analytes into one method and by updating the transitions, we increased the coverage for confirming exposures to Schedule 1 vesicants.

During an investigation of potential chemical agent exposure, there never seems to be enough sample. The reported method requires only 10 µL of sample. Reducing the required sample volume allows for each specimen to be analyzed by other diagnostic methods to look for additional confirmatory biomarkers or other potential exposure chemicals or to be archived for future examination. In addition, this method is compatible with either dried or liquid samples which allows for the potential expansion into field-ready applications and simplifies the shipping process.

Ruggedness was evaluated to design a robust, transferrable method and to understand which method parameters have the greatest impact on analyte variation. Stability was evaluated to ensure that method materials and samples were stable at temperature ranging from room temperature to −70 °C for extended times as either the unprocessed or processed samples. Overall, this method demonstrated high-level precision, accuracy, and linearity, and improved LODs for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF. As a result of these efforts, we have designed a robust high-throughput method that can allow an analyst to process and analyze 96 samples in 9.25 h. Furthermore, this method may be expanded to include alternate matrices and additional analytes with similar chemistries.

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Abbreviations list

HD	sulfur mustard (bis(2-chloroethyl)sulfide)
HN1	bis(2-chloroethyl)ethylamine
HN2	bis(2-chloroethyl)methylamine
HN3	tris(2-chlorethyl)amine
TDG	thiodiglycol
SMBTE	1,1′-sulfonylbismethylthioethane
SBMSE	1,1′-sulfonylbis[2-(methylsulfinyl)ethane]
EDEA	N-ethyldiethanolamine
MDEA	*N*-methyldiethanolamine
TEA	triethanolamine
CPF	cysteine-proline-phenylalanine
SWGTOX	Scientific Working Group for Forensic Toxicology
OPCW	Organisation for the Prohibition of Chemical Weapons
SPE	solid phase extraction
MRM	multiple-reaction monitoring
UHPLC-MS/MS	ultra-high pressure liquid chromatography tandem mass spectrometry
CDC	Centers for Disease Control and Prevention

HLB	hydrophilic/lipophilic balanced
ISTD	internal standard
QC	quality control
QCL	QC low
QCH	QC high
MB	matrix blank
HRMS	high-resolution mass spectrometry
HESI	heated-electrospray-ionization
AIF	all-ion-fragmentation
CE	collision energy
MPA	mobile phase A
MPB	mobile phase B
Quant	quantitation
Confirm	confirmation
R^2	coefficient of determination
CIR	confirmation ion ratio
LOD	limit of detection
SD	standard deviation
RSD	relative standard deviation

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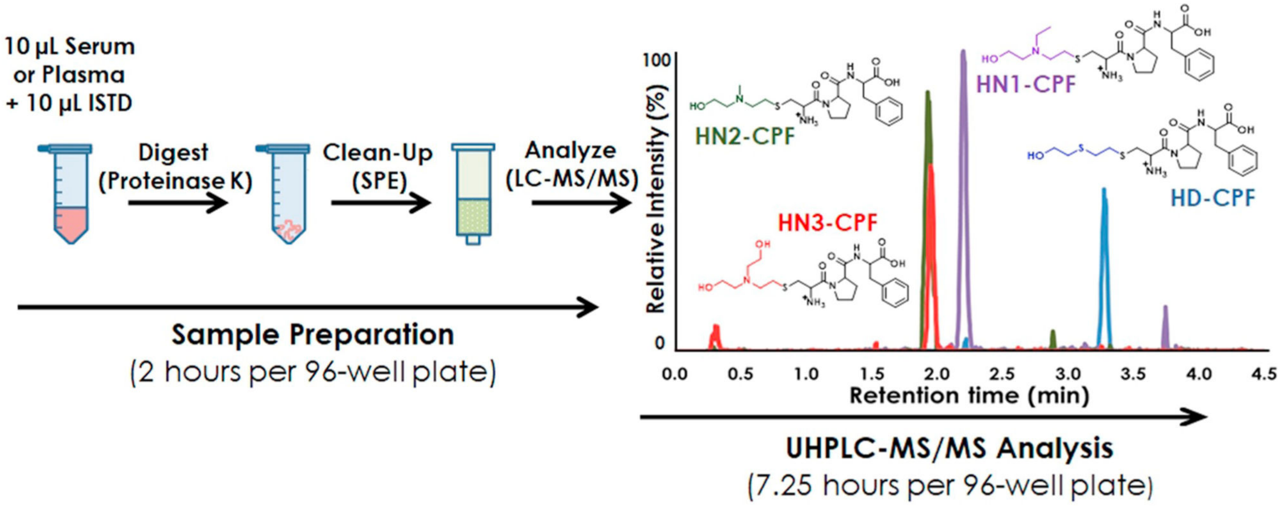


Fig. 1.

Method schematic for the analysis of sulfur mustard (HD) and nitrogen mustards (HN1, HN2, and HN3) bound to the tripeptide, cysteine-proline-phenylalanine (CPF).

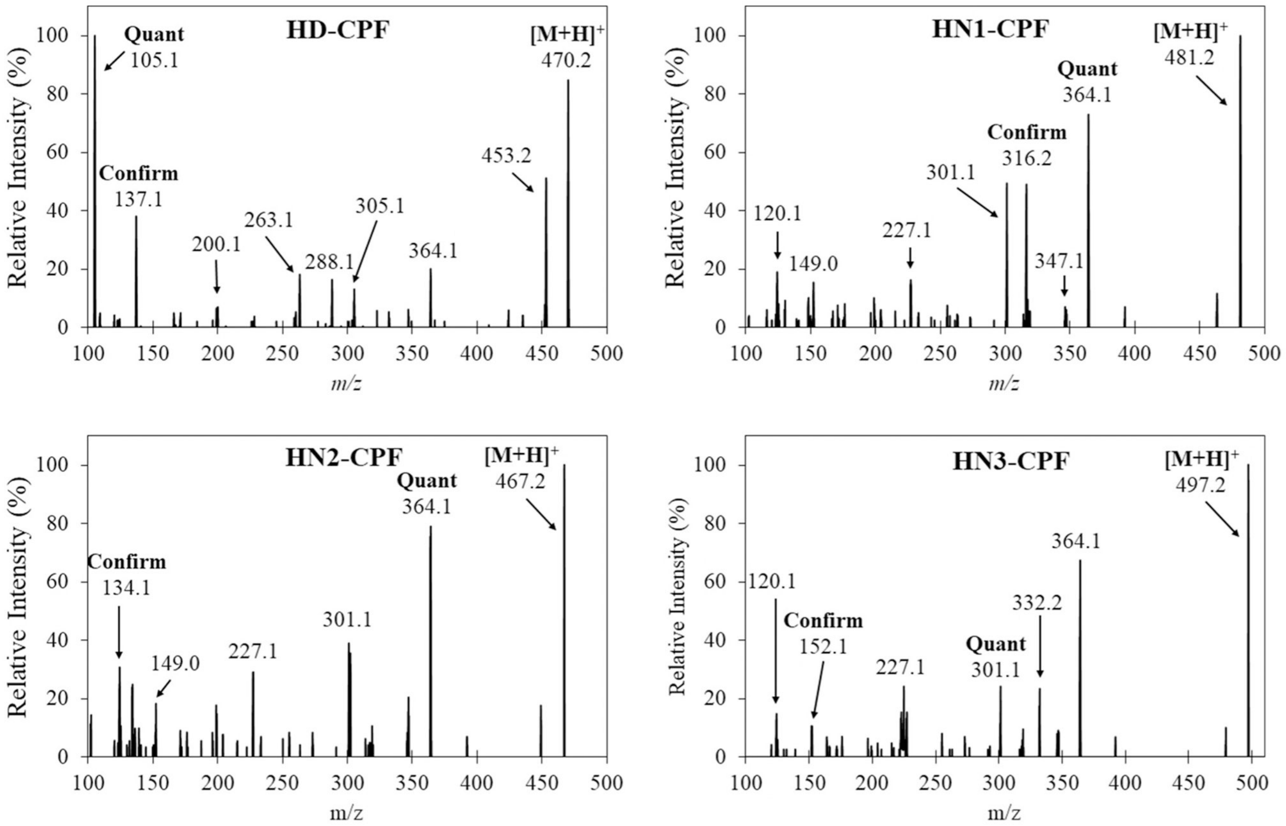


Fig. 2.

Product ion scans (MS^2) for sulfur mustard (HD) and nitrogen mustards (HN1, HN2, and HN3) adducted to the tripeptide cysteine-proline-phenylalanine (CPF). Each scan shows an overlay of collision energies at 10, 25, and 50 eV. The precursor $[M+H]^+$, quantitation (quant), and confirmation (confirm) ions are labeled.

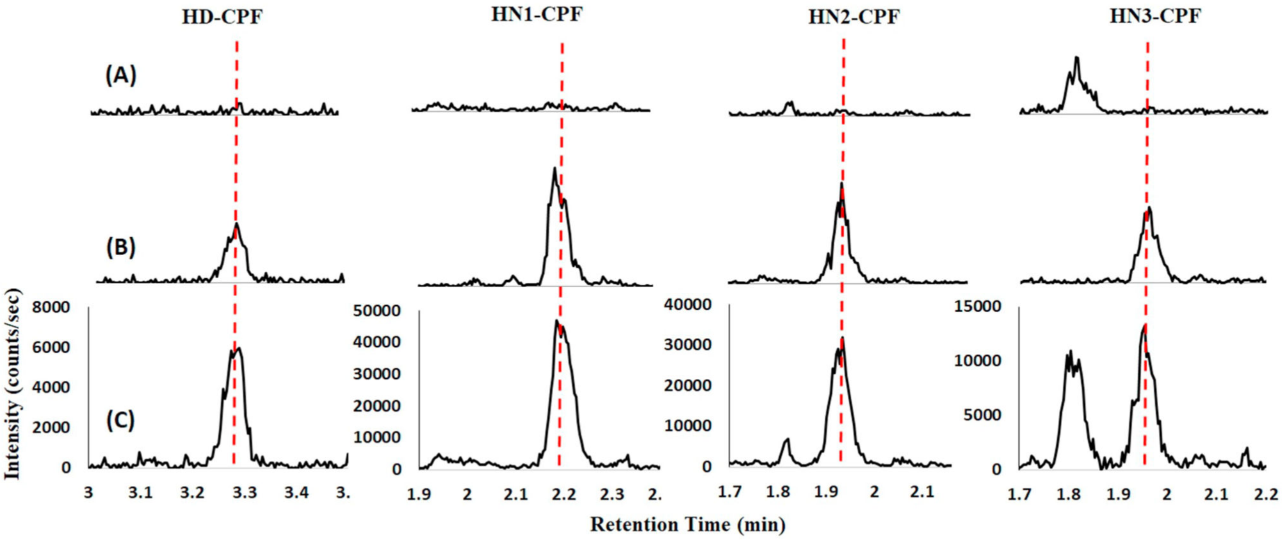


Fig. 3.

Representative chromatograms of A) matrix blank (MB), B) lowest calibrator (3.00 ng/mL) and C) quality control low (QCL) samples for sulfur (HD) and nitrogen mustards (HN1, HN2, and HN3) bound to the tripeptide cysteine-proline-phenylalanine (HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF). The peak intensity scale is fixed for all chromatograms within each analyte. The dotted line represents the expected retention time.

Table 1

Tentative identification of A) product ions and B) precursor and product ion structures for HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF based on collected high resolution MS^2 data.

Analyte	Molecular Formula	Calculated $[M+H]^+$ *(m/z)*	Observed $[M+H]^+$ *(m/z)*	Mass Error (ppm)	Collision Energy (eV)	Predicted Product Ion Mass *(m/z)*	Formula
HD-CPF	$C_{21}H_{31}N_3O_5S_2$	470.17779	470.17790	0.01	25	105.03739	$[M-C_4H_9OS+H]^+$
					25	137.00902	$[M-C_4H_9OS_2+H]^+$
HN1-CPF	$C_{23}H_{36}N_4O_5S$	481.24792	481.24770	0.21	25	364.13223	$[M-C_6H_{14}NO+H]^+$
					25	316.16879	$[M-C_{14}H_{26}N_3O_3S+H]^+$
HN2-CPF	$C_{22}H_{34}N_4O_5S$	467.23227	467.23229	0.02	25	364.13223	$[M-C_5H_{12}NO+H]^+$
					25	134.06340	$[M-C_{22}H_{34}N_4O_5S+H]^+$
HN3-CPF	$C_{23}H_{36}N_4O_6S$	497.24283	497.24237	0.46	50	301.11813	$[M-C_{16}H_{16}N_2O_4+H]^+$
					50	152.07056	$[M-C_8H_9NO_2+H]^+$

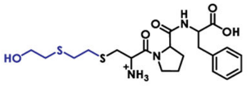
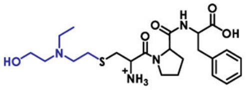
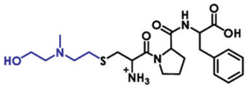
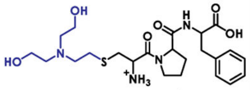
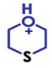
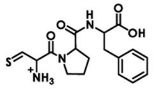
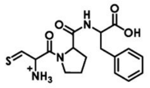
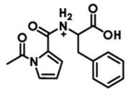
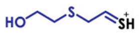
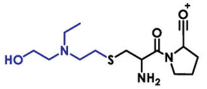
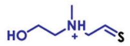
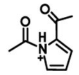
	HD-CPF	HN1-CPF	HN2-CPF	HN3-CPF
Precursor	 470.17790 m/z	 481.24770 m/z	 467.23229 m/z	 497.24237 m/z
Product (Quant transition)	 105.03739 m/z	 364.13223 m/z	 364.13223 m/z	 301.11813 m/z
Product (Confirm transition)	 137.00902 m/z	 316.16879 m/z	 134.06340 m/z	 152.07056 m/z

Table 2

Multiple-reaction monitoring (MRM) parameters using a Sciex 6500 triple quadrupole mass spectrometer. Quantitation transition (quant), confirmation transition (confirm), internal standard transition (ISTD), declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CxP).

Parameter	Transition (m/z)	DP (V)	EP (V)	CE (V)	CxP (V)
HD-CPF_Quant	470.2→105.1	60	8	27	20
HD-CPF_Confirm	470.2→137.1	60	8	27	20
HD-CPF_ISTD	480.1→105.1	60	8	27	10
HN1-CPF_Quant	481.2→364.1	80	8	30	20
HN1-CPF_Confirm	481.2→316.2	80	8	25	20
HN1-CPF_ISTD	491.3→374.1	80	8	30	20
HN2-CPF_Quant	467.2→364.1	80	10	28	20
HN2-CPF_Confirm	467.2→134.1	80	10	30	20
HN2-CPF_ISTD	477.1→374.1	80	10	30	20
HN3-CPF_Quant	497.2→301.1	80	10	40	20
HN3-CPF_Confirm	497.2→152.1	80	4	40	20
HN3-CPF_ISTD	507.2→374.1	80	8	30	20

Table 3

Intraday and interday precision. Values are the relative standard deviation (RSD) for each analyte at the quality control low (QCL) and quality control high (QCH) from 20 analytical batches over 10 days.

	HD-CPF		**HN1-CPF**		**HN2-CPF**		**HN3-CPF**	
	QCL	**QCH**	**QCL**	**QCH**	**QCL**	**QCH**	**QCL**	**QCH**
Intraday (RSD)	10.1%	10.7%	10.8%	7.6%	9.0%	9.6%	9.1%	7.0%
Interday (RSD)	9.3%	7.8%	11.6%	7.8%	13.5%	6.6%	9.2%	9.0%

Table 4

Ruggedness from quality control low (QCL) samples for sulfur mustard (HD) and nitrogen mustards (HN1, HN2, and HN3) adducted to the tripeptide cysteineproline-phenylalanine (CPF), abbreviated as HD-CPF, HN1-CPF, HN2-CPF, and HN3-CPF when A) digestion parameters were varied ± 20% of the final method parameters and B) six lots of proteinase K from different vendors were compared.

A) Accuracy from initial measurement

Parameters	Final method parameter	HD-CPF		HN1-CPF		HN2-CPF		HN3-CPF	
		−20% of final parameter	+20% of final parameter	−20% of final parameter	+20% of final parameter	−20% of final parameter	+20% of final parameter	−20% of final parameter	+20% of final parameter
Enzyme conc	10 mg/mL	89%	103%	87%	90%	86%	105%	85%	106%
Digestion time	60 min	89%	102%	89%	103%	86%	102%	86%	104%
Digestion temp	50 °C	88%	97%	91%	102%	84%	94%	80%	97%
Digestion volume	120 μL	114%	95%	123%	98%	112%	91%	121%	99%
Heating block	ThermoCycler	107%	93%	108%	102%	95%	94%	106%	103%

B) Accuracy from initial measurement

Company	Cat #	HD-CPF	HN1-CPF	HN2-CPF	HN3-CPF
Millipore	70663–5	Control			
Affymetrix	76230Y	115%	87%	92%	95%
Alfa Aeser	J622051	103%	96%	92%	95%
MP Biomed	193504	96%	120%	95%	92%
MP Biomed	193981	98%	115%	93%	96%
Thermo	17916	100%	125%	96%	102%