Retrospective Detection of Exposure to Sulfur Mustard: Improvements on an Assay for Liquid Chromatography–Tandem Mass Spectrometry Analysis of Albumin/Sulfur Mustard Adducts

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Abstract

We here report on the further development of the method comprising the pronase digestion of albumin alkylated by sulfur mustard and the subsequent mass spectrometric analysis of an adducted tripeptide. This includes significant improvements in both the albumin isolation procedure and the automation of the microliquid chromatography–electrospray-tandem mass spectrometric analysis. We also report on the results of a small reference range study, in which we have established that there are no detectable interferences in sera from unexposed individuals.

Introduction

Within the framework of our program directed towards the development of methods for diagnosis of exposure to chemical warfare agents, we recently reported (1) that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of (*S*-2-hydroxyethylthioethyl)-Cys-Pro-Phe [(S-HETE) Cys-Pro-Phe] (1) (Figure 1). This tripeptide could be isolated and analyzed in an extremely sensitive way by microliquid chromatography (LC)–electrospray-tandem mass spectrometry (MS) with multiple reaction monitoring (MRM) at an absolute detection limit of 4 pg. Using only 3 mg albumin, we were able to detect in vitro exposure of human blood to 10nM sulfur mustard by applying this method. Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard. Interestingly, we recently showed that this method can also be applied to demonstrate exposure (in vitro as well as in vivo) to a wide range of other alkylating agents (2,3). In the present work, we report on the simplification and reduction in the time required for the procedure, for eventual use in a field laboratory. This includes significant improvements in both the albumin extraction procedure and the automation of the micro-LC–electrospray-tandem MS analysis. We also report the results of a small reference range study, in which we have established that there are no detectable interferences in sera from unexposed individuals.

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Experimental

Materials and instrumentation

Pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31) and bovine serum albumin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Centrex UF-2 (10 kDa molecular mass cutoff) centrifugal ultrafilters were

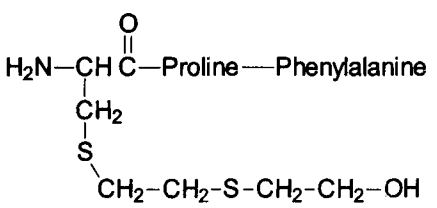

Figure 1. Chemical structure of tripeptide/sulfur mustard adduct *S*-(2-hydroxyethylthioethyl)-Cys-Pro-Phe[(S-HETE)Cys-Pro-Phe] obtained after pronase digestion of albumin alkylated by sulfur mustard.

procured from Schleicher & Schuell (Keene, NH). HiTrap Blue HP columns (1 mL) and PD-10 columns were purchased from Amersham Biosciences (Uppsala, Sweden). Incubation of whole blood samples was performed as described earlier (1). Formic acid, acetonitrile, KH_2PO_4, KCl, and NH_4HCO_3 were purchased from Fluka (Buchs, Switzerland). Sulfur mustard and sulfur mustard-d_8 were used from stocks within our laboratory. Human blood was obtained from a blood bank.

LC–electrospray-tandem MS experiments were conducted on two systems. The first system was used for optimization of the procedure and was comprised of a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, U.K.) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA). The chromatographic hardware consisted of a precolumn splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10- or 50-µL injection loop mounted, and a PepMap C_{18} (LC Packings) or Vydac C_{18} column (both 150 mm × 300-µm i.d., 3-µm particles; Grace Vydac, Hesperia, CA). A gradient of eluents A [H_2O/0.2% (v/v) formic acid] and B [acetonitrile/0.2% (v/v) formic acid] was used to achieve separation. The flow delivered by the LC was split precolumn to allow a flow of approximately 6 µL/min through the column and into the electrospray-MS interface. MS–MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy of 20 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

The second LC–electrospray system consisted of an API4000 triple quadrupole instrument with a standard ionspray interface (Applied Biosystems, Toronto, ON, Canada) and a Shimadzu (Kyoto, Japan) modular LC. In this case, the chromatographic hardware incorporated three high-pressure pumps, two six-port switching valves, an autosampler with a 50-µL injection loop, and two identical Luna C_{18} (Phenomenex) columns (both 150 × 1-mm i.d., 5-µm particles) in parallel. This LC system was configured such that no flow splitting was necessary and that when the first column was being eluted the second column was being equilibrated in preparation for analysis of the next sample, as shown in Figure 2. A gradient of solvents C [H_2O/1% (v/v) formic acid] and D [80:20 acetonitrile/H_2O plus 1% (v/v) formic acid] was delivered at 50 µL/min. Injections of 50 µL were typically made. The samples were loaded onto the selected analytical column during a 5-min period using Solvent C at 50 µL/min and then both of the six-port valves were switched. Elution was by means of a linear gradient, ramped from 0% D commencing immediately after the valve switching, to 100% D after a further 25 min. During elution of the selected analytical column, Solvent C was used for regeneration of the selected off-line column at a flow rate of 75 µL/min. The cycle time was 33 min. MS–MS (MRM) transitions from *m/z* 470.1 to 105.0 (for the tripeptide) and from *m/z* 478.1 to 113.0 (for the tripeptide-d_8) were recorded at unit resolution on both Q1 and Q3 (i.e., with ion peaks between 0.60- and 0.80-*m/z* units wide at half-maximum height) at a declustering potential of 65 V and a collision energy of 35 eV, with nitrogen as the collision gas.

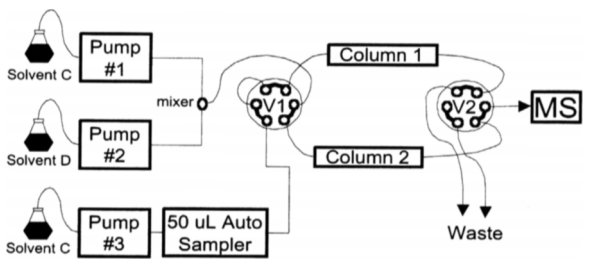

Figure 2. LC setup for automated microbore parallel-column analysis using identical 1-mm i.d. columns. The Shimadzu LC system used here was capable of delivering high-quality gradients at 50 µL/min, and the system controller was capable of managing three high-pressure pumps and two switching valves. Taking the dead volumes in the autosampler components and flow lines into account, a minimum loading time of 4.0 min was required to ensure that > 99% of each injected sample (50 µL in every case) reached the head of the column before the two six-port valves were switched and the autosampler flow was diverted to waste. V1, V2: six-port switching valves, shown with flow channels set for elution of Column 2 to the MS while Column 1 is equilibrated.

Isolation of albumin from human plasma using affinity chromatography

Plasma (1 mL) was applied on a HiTrap™ Blue HP (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) affinity column that was incorporated into a fast protein liquid chromatography (FPLC) system after conditioning with buffer A (50mM KH_2PO_4, pH 7; 10 mL). The column was eluted with buffer A (7 mL; 1 mL/min). A large peak was visible at 280 nm, corresponding with material having no affinity to the column material. Subsequently, the column was eluted with buffer B (50mM KH_2PO_4, 1.5M KCl, pH 7; 7 mL; 1 mL/min). UV positive (280 nm) material was collected (total volume 2.5 mL). The HiTrap column was regenerated by washing with buffer A (14 mL).

Subsequently, a PD-10 column (containing 10 mL Sephadex G 25 material) was equilibrated with 50mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (2.5 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50mM, 0.5 mL). Next, the column was further eluted with aqueous NH_4HCO_3 (50mM, 2.5 mL), and the eluate was collected.

Digestion of albumin obtained after affinity chromatography, with addition of internal standard

Plasma samples (1 mL), isolated from blood exposed to different concentrations of sulfur mustard, were spiked with plasma (50 µL) isolated from blood exposed to 100µM sulfur mustard-d_8. Next, these samples were applied to a HiTrap Blue Sepharose column and desalted on a PD-10 column as described.

Part of the purified albumin fraction (0.25 mL) was diluted with aqueous NH_4HCO_3 (50mM; 0.5 mL) and, subsequently, Pronase was added [100 µL of a freshly prepared solution (10 mg/mL) in 50mM NH_4HCO_3], followed by incubation for 2 h at 37°C. The digests were filtered through molecular mass cutoff filters (10 kDa) under centrifugation at 2772 × *g* in order to re-

move the enzyme. The filtrate was analyzed by means of LC–MS–MS.

Isolation and digestion of albumin from patient samples without the use of affinity chromatography

Albumin was purified from individual plasma samples (0.2 mL) collected from 80 patients, none of whom had been exposed to sulfur mustard, following our previous method (1), which is based on a lengthy series of selective precipitation steps originally described by Bechtold et al. (4). These plasma samples were obtained from a blood bank in Tennessee but were not linked with any personal or demographic information. To 3 mg of each purified albumin sample was added an amount of albumin that had been purified from blood exposed to 100µM sulfur mustard-d_8, such that the level of internal standard was equivalent to 330nM exposure. Pronase digestion was carried out for 2.5 h at 37°C at an enzyme/substrate ratio of 1:3. Digestion was stopped by centrifugation using 10 kDa molecular mass cutoff filters as described, and LC–MS–MS analyses were carried out using the triple-quadrupole mass spectrometer.

A set of quantitation standards were prepared at the same time as the patient samples by mixing albumin prepared from whole blood that had been exposed to 1µM sulfur mustard with bovine serum albumin (BSA). The BSA, which has the same amino acid sequence as human serum albumin (HSA) in the region of Cys-34 and that had previously been shown to be free of any interfering materials, was also used as a blank material. These standards ranged from approximately 3 up to 200nM exposure and were intended to cover a range near the method limit of detection because high levels of (S-HETE)Cys-Pro-Phe were not expected to be present in the patient samples. The sulfur mustard-d_8 internal standard was present at a level equivalent to 330nM exposure throughout.

Results and Discussion

Isolation of albumin from plasma by affinity chromatography

The isolation of albumin by means of precipitation is rather time consuming, which limits the utility of the tripeptide method. For example, a minimum of 48 h typically elapsed between receipt of 10 serum samples and the availability of quantitative LC–MS–MS data on those samples, in our hands. Moreover, the laborious isolation methods precluded automation of the methodology. This would seriously limit the usefulness of the original method in the event of a large-scale contamination of a population with sulfur mustard. A literature search revealed that albumin can be removed from serum by affinity chromatography, which will facilitate the analysis of less abundant serum proteins (5). We reasoned that this procedure might be suitable for isolation of albumin from serum. The affinity material, having Cibacron Blue F3G-A as ligand, is commercially available in small columns. The affinity is based on specific interactions of the column material with amino acid residues in albumin.

In a representative experiment, 1 mL serum or plasma (containing approximately 40 mg albumin) was applied to a 1-mL HiTrap Blue column. Plasma constituents with no affinity to the column material rapidly eluted from the column, as evidenced by the UV pattern. Albumin is eluted from the column by applying a high salt buffer. This procedure took 10 min. Subsequently, the fraction containing albumin (checked by UV) was desalted by means of gel filtration on a PD 10 column, which took an additional 10 min. The purified albumin sample was recovered as a solution in aqueous NH_4HCO_3 that can be used immediately for pronase digestion. LC–MS–MS analysis of pronase digests of the albumin fraction thus obtained gave similar results as those found earlier by using the old procedure. The entire procedure (i.e., from blood sample to a digest that is ready for analysis) took only 3 h.

Use of albumin alkylated by sulfur mustard-d_8 as an internal standard

We found that plasma, isolated from blood exposed to a well-defined amount of sulfur mustard-d_8, could be used as an internal standard in the assay that uses the affinity chromatography procedure for isolation of albumin (see Figure 3 for a dose-response curve). This will enable quantitative analyses of unknown samples. A representative LC–tandem MS chromatogram is given in Figure 4; see Figure 5 for the corresponding tandem MS spectra.

Triple-quadrupole LC–MS–MS analysis of unexposed patient samples

Using our original method for albumin purification (1,4), we have investigated the method detection limits using a relatively robust and rugged high-throughput liquid chromatographic system based on 1-mm internal diameter column hardware and having an optimum flow rate of 50 µL/min. We were able to obtain a limit of detection (LOD) equivalent to the exposure of whole blood to approximately 1.5nM sulfur mustard and a limit of quantitation of approximately 4.5nM using the

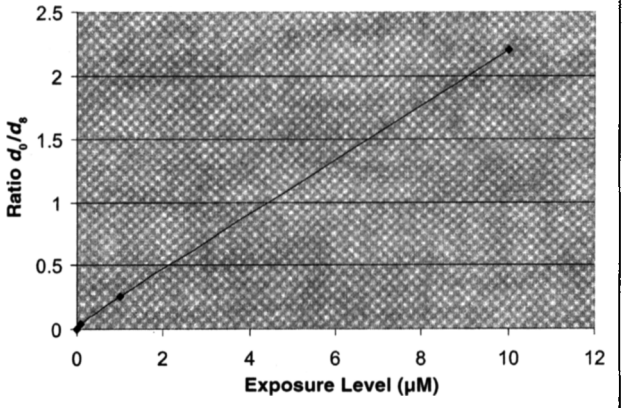

Figure 3. Dose-response curve for LC–MS–MS analysis after pronase digestion of albumin isolated by affinity chromatography from human blood that was exposed to various concentrations of sulfur mustard in the presence of a fixed amount of internal standard that had been isolated from blood, which had been exposed to sulfur mustard-d_8 (concentrations used 0.1, 1, and 10µM, with ratios d_0/d_8 found 0.04, 0.26, and 2.2, respectively).

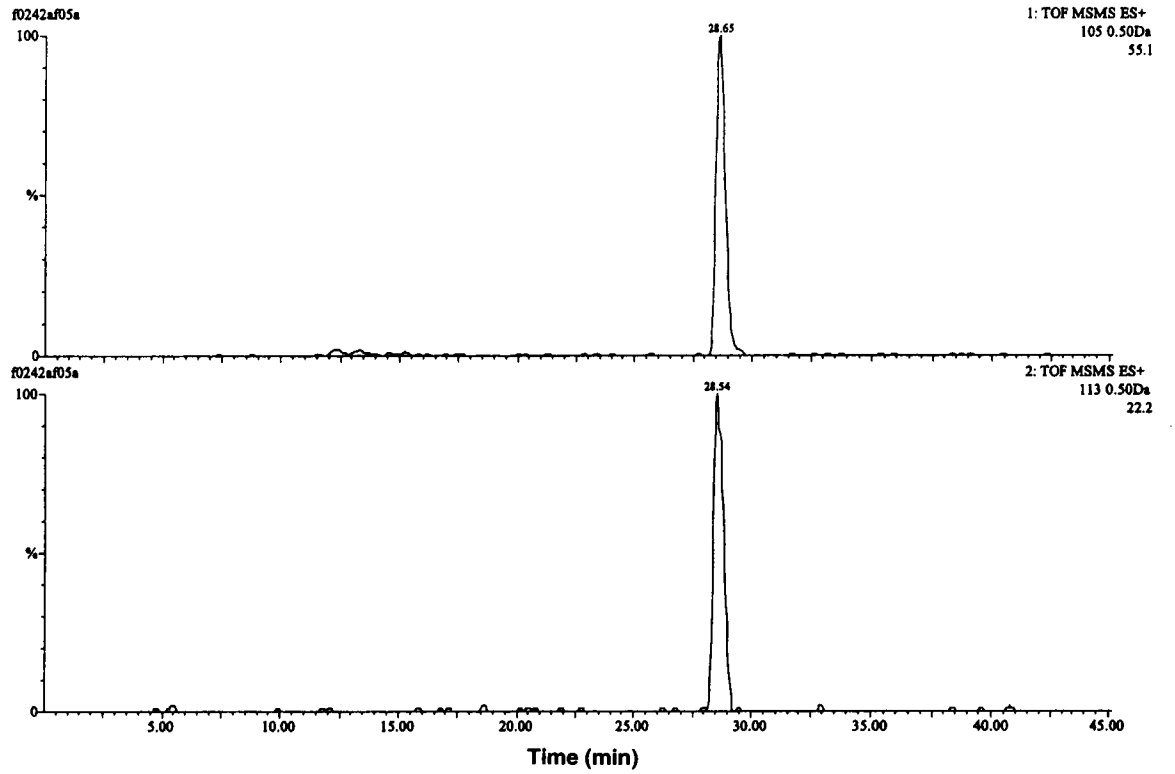

Figure 4. LC–tandem-MS analysis (Q-TOF system) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE-d_8)Cys-Pro-Phe (lower panel) in a pronase digest of albumin. Albumin was isolated from plasma (1 mL) by affinity chromotagraphy, followed by desalting on a PD-10 column. The plasma was isolated from human blood that had been exposed to sulfur mustard (10μM). Prior to isolation of albumin, 50-μL plasma sample was added, isolated from blood that had been exposed to sulfur mustard-d_8 (100μM). The charged molecular ion (*m/z* 470 for upper panel and 478 for lower panel) was selected in the first MS, and the highly selective thiodiglycol derived fragment (*m/z* 105 for upper panel, and 113 for lower panel) was selected in the second MS of the Q-TOF system.

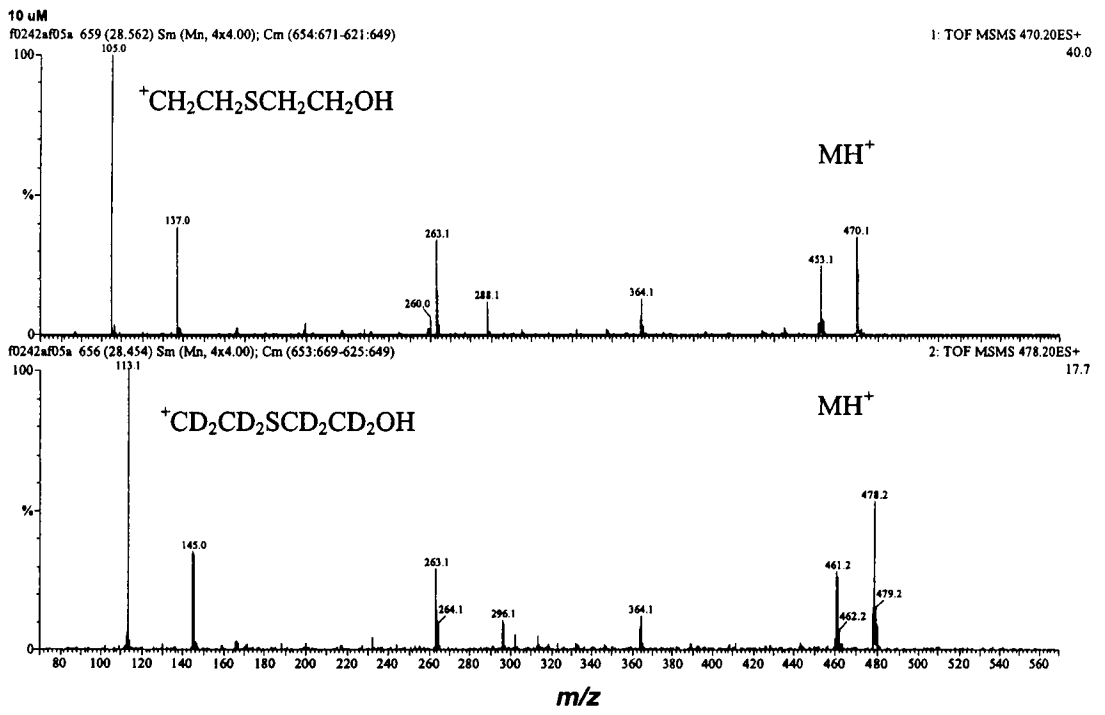

Figure 5. Tandem-MS spectra of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE-d_8)Cys-Pro-Phe (lower panel) recorded on the Q-TOF system.

triple-quadrupole MS. Dose-response curves closely approximated to linear in the range of exposures between 3 and 200nM, with R^2 values typically around 0.998 (data not shown). Although the 300-µm i.d. LC columns, which we have usually employed, give exquisite LC–electrospray-MS–MS sensitivity, the analytical system was not very rugged and could require significantly more user intervention and troubleshooting than might be expected to be available in a field laboratory. Furthermore, it is generally accepted that it is much easier to automate the LC injections with 1-mm i.d. columns than it is to automate smaller-bore systems (even if a fully integrated micro-LC system is available). Although there will be some loss of sensitivity, in our opinion the improvements in robustness and ruggedness and the ease of automation can provide significant advantages for high-throughput analyses.

Using the high-throughput LC system (Figure 2) in conjunction with the original albumin purification method, we have been able to evaluate a set of 80 patient serum samples for the presence of background levels of sulfur mustard adduct (or possible low-level interferences) in an unexposed population. All 80 patient samples were determined to contain undetectable levels of the tripeptide adduct (i.e., approximately 1.5nM or less), as shown in Figure 6. This is of particular importance because significant background levels of thiodiglycol (TDG), a urinary metabolite of sulfur mustard, have been reported in the literature regarding unexposed populations (6,7). Of the 80 individual patient serum samples analyzed for (S-HETE)Cys-Pro-Phe, 78 corresponding urine samples had also been analyzed for TDG in our laboratories (6). The TDG measurements for these 78 samples ranged between nondetectable (for an LOD of approximately 0.17 ng/mL) and 19.2 ng/mL of urine (after normalization against the levels of creatinine, to correct for differing urine concentrations), with an arithmetic mean of 4.3 ng/mL and a geometric mean of 2.8 ng/mL. The geometric mean, calculated as the nth root of the product of all n values, is useful because it is much less sensitive to outlying values than the arithmetic mean. Although this reference range study was carried out on a relatively small scale, it would appear that the albumin tripeptide method is not subject to the limitations imposed on TDG measurements because of an inherent background level. We chose to use the original albumin purification method for this study because it routinely yielded high purity albumin samples and facilitated comparison with the results of alternate LC–MS–MS strategies (for example, the use of 0.32-mm i.d. columns). However, in future work we intend to reanalyze the same patient samples using the affinity-based cleanup procedure.

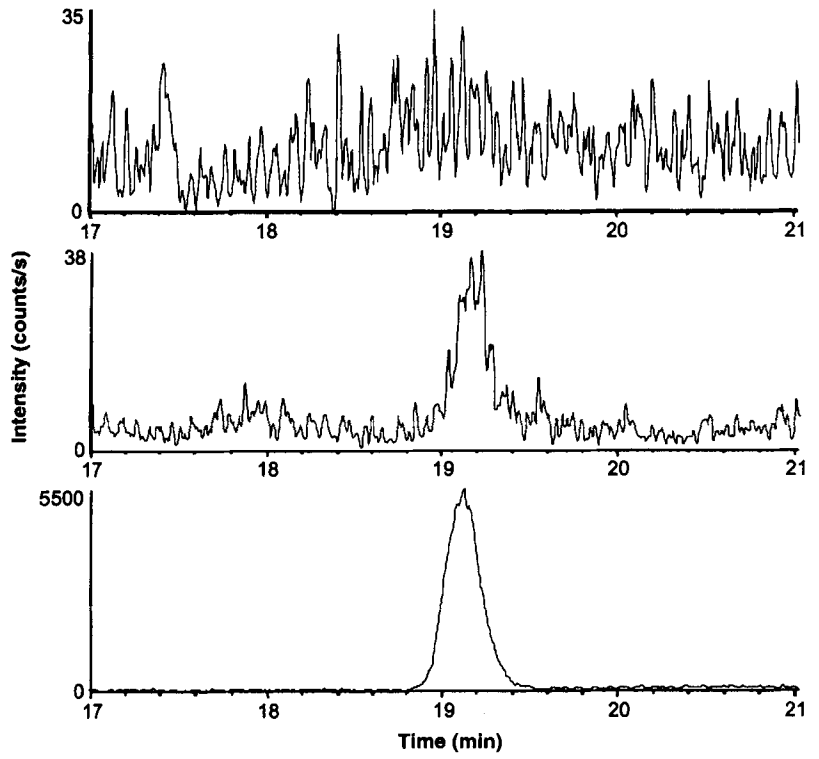

Figure 6. Detail of a representative LC–tandem MS analysis (API4000 triple-quadrupole instrument) of (S-HETE)Cys-Pro-Phe in a pronase digest of albumin from an unexposed individual (upper panel) and in an albumin standard corresponding to an exposure at the 3nM level (center panel). The lower panel shows the chromatogram for the (S-HETE-d_8)Cys-Pro-Phe internal standard at a level of 330nM exposure. Albumin was isolated from plasma (0.2 mL) by the precipitation method and, after pronase digestion, was analyzed using parallel 150 × 1-mm i.d. LC columns coupled to a triple-quadrupole MS. The traces, which are smoothed to reduce noise, show the MRM transitions from m/z 470.1 to 105.0 (top and center panels) for the native tripeptide and m/z 478.1–113.0 (lower panel) for the tripeptide-d_8.

Conclusions

In conclusion, we have presented a rapid and significantly improved method for diagnosis of human exposure to sulfur mustard. In future experiments, we plan to use columns with immobilized pronase. This might enable the construction of an automated system of (reactor) columns, coupled to a tandem MS, to which an unprocessed plasma sample can be introduced. This would be highly convenient for use under field laboratory conditions and would speed up the actual diagnosis. The results of a small reference range study suggest that unexposed populations should not have detectable levels of sulfur mustard/albumin adducts, further increasing the usefulness of this method for detection and quantitation of retrospective exposure. We plan to extend the scope of the reference range study, using the new methods for sample preparation along with the automated LC–MS–MS analysis.

Acknowledgments

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