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Alkylation of Human Serum Albumin by Sulfur Mustard in Vitro and in Vivo: Mass Spectrometric Analysis of a Cysteine Adduct as a Sensitive Biomarker of Exposure

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To develop a mass spectrometric assay for the detection of sulfur mustard adducts with human serum albumin, the following steps were performed: quantitation of the binding of the agent to the protein by using [^{14}C]sulfur mustard and analysis of acidic and tryptic digests of albumin from blood after exposure to sulfur mustard for identification of alkylation sites in the protein. The T5 fragment containing an alkylated cysteine could be detected in the tryptic digest with micro-LC/tandem MS analysis. Attempts to decrease the detection limit for in vitro exposure of human blood by analysis of the alkylated T5 fragment were not successful. After Pronase treatment of albumin, *S*-[2-[(hydroxyethyl)thio]ethyl]Cys-Pro-Phe was analyzed by means of micro-LC/tandem MS, allowing a detection limit for in vitro exposure of human blood of 10 nM, which is 1 order of magnitude lower than that obtained by means of modified Edman degradation. The analytical procedure could be successfully applied to the analysis of albumin samples from Iranian victims of the Iran−Iraq war.

Introduction

We are engaged in the development of methods for retrospective detection of exposure to chemical warfare (CW)[1] agents, i.e., sulfur mustard (*1*−*7*), nerve agents (*8*, *9*), and arsenicals. Our methodology is based on analysis of long-lasting adducts that CW agents form with DNA and proteins. In this way, the CW agents to which casualties have been exposed can be definitively identified, whereas dosimetry of the exposure will also contribute to the proper treatment of the intoxication. The need for retrospective detection of exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome" (*10*). Recently, these attempts have led to a general interest in the effects of low-level exposure to CW agents (*11*) in which diagnosis and dosimetry of exposure are essential tools. In addition, our assays can be used in a variety of other applications, e.g., in biomonitoring of workers in destruction facilities of the CW agents and in forensic analyses in the case of suspected terrorist activities.

In previous reports, we described the interactions of the vesicant sulfur mustard with DNA (*1*, *2*) and hemoglobin (*3*, *6*). In addition, we developed a modified Edman procedure for the determination of the adduct of sulfur mustard with the N-terminal valine residue of hemoglobin (*4*). It was demonstrated that this procedure could be successfully applied to the analysis of blood samples of Iranian victims of the Iran−Iraq war (*7*). Attempts to lower the minimal exposure concentration of sulfur mustard in blood which can be detected with the modified Edman procedure have resulted in limited improvement. To enable detection of low-level exposure to sulfur mustard, we have explored the usefulness of adducts with other proteins. Adduct formation with human serum albumin (*12*) might be more efficient than with hemoglobin since albumin is not enclosed by a cell membrane. A drawback, however, is the faster elimination rate of albumin adducts (half-life of albumin of 20−25 days vs the life span of hemoglobin of 120 days), which restricts the use of albumin adducts to a few weeks after exposure has taken place. Covalent binding to albumin has been documented for ultimate carcinogens derived from various compounds, e.g., benzene (*13*, *14*), polycyclic aromatic hydrocarbons (*15*, *16*), food-borne compounds such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx;[1] *17*, *18*), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ;[1] *19*), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP;[1] *20*), and aflatoxin B_1 (*21*).

In this report, we demonstrate that albumin is effectively alkylated by sulfur mustard. Complete characterization of a cysteine−sulfur mustard adduct is presented, as well as a novel analytical procedure for assessing exposure to sulfur mustard based on this adduct, which is at least 1 order of magnitude more sensitive than the modified Edman degradation. The usefulness of the method is demonstrated by the analysis of albumin samples from Iranian victims of the Iran−Iraq war.

Materials and Methods

Chemicals. Caution: *Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.*

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[1] Abbreviations: CW, chemical warfare; HETE, [2-[(hydroxyethyl)-thio]ethyl]; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MRM, multiple-reaction monitoring; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TFA, trifluoroacetic acid.

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Technical-grade sulfur mustard was distilled before use to a gas chromatographic purity of >99.5%. [^{14}C]Sulfur mustard (specific activity of 15 mCi/mmol) was synthesized as described elsewhere (*22*). *S*-[2-[(Hydroxyethyl)thio]ethyl]-L-cysteine [(*S*-HETE)Cys][1] was synthesized as described by Grant and Kinsey (*23*). Constantly boiling 6 N HCl was obtained from Pierce (Rockford, IL). Iodoacetic acid, trypsin (TPCK-treated, type XIII from bovine pancreas, EC 3.4.21.4), and Pronase (protease type XIV from *Streptomyces griseus*, EC 3.4.24.31) were obtained from Sigma (St. Louis, MO). Human blood was obtained from healthy volunteers in our laboratory, with consent of the donor and approval of the TNO Medical Ethical Committee. Blood samples from Iranian victims were kindly provided by T. J. F. Savelkoul (Academic Hospital Utrecht, Utrecht, The Netherlands) and were stored at −70 °C.

Instrumentation. HPLC was performed using two Waters (Bedford, MA) 510 pumps, a gradient programmer (Waters, model 660), and a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden). The eluent (flow rate of 1 mL/min) was 0.1% trifluoroacetic acid (TFA)[1] in H_2O with a linear gradient to 0.1% TFA in CH_3CN/H_2O (various ratios). The eluate was monitored at 214 or 254 nm with a Spectroflow 757 UV detector (Applied Biosystems, Ramsey, NJ) and with a radiochromatography detector (Radiomatic, model Flo-one Beta series A 500, Meriden, CT), using Ultima-Flo (Packard, Meriden, CT) as the scintillation cocktail. Liquid scintillation countings were performed using a Minaxi Tri-Carb 4000 series scintillation counter (Packard) using Hionic Picofluor (Packard) as the scintillation cocktail. LC/tandem MS analyses were performed on a VG Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.) which was coupled to an HPLC system consisting of two Waters solvent delivery systems (models 590 and 510) under the control of a solvent programmer (Waters, model M680). For gradient LC, the milliliters per minute flow rate from the delivery system was converted to a microliters per minute flow rate using an Accurate microflow processor (model IC-70-CAP, LC Packings, Zürich, Switzerland). A microcolumn with PRP-1 material (0.32 mm × 250 mm) or with Lichrosorb RP18 material (0.32 mm × 350 mm) was used. Samples were applied through a Valco injection valve (VICI, Schenkon, Switzerland), equipped with a 10 or 40 μL sample loop. Conditions for mass spectrometric analyses are given in the relevant entries. Peptides were synthesized on an Abimed (Langenfeld, Germany) AMS 422 peptide synthesizer using commercially available building blocks (Novachem, Läufelfingen, Switzerland). Slide-a-Lyzer cassettes (0.1−0.5 mL) were obtained from Pierce. Sep-Pak C18 cartridges (model Classic, short body) were obtained from Waters (Milford, MA). Centrex UF-2 (10 kDa molecular mass cutoff) centrifugal ultrafilters were obtained from Schleicher & Schuell (Keene, NH).

Incubation of Human Blood with Sulfur Mustard or [^{14}C]Sulfur Mustard and Isolation of Albumin. To human blood (5 mL) was added a solution of sulfur mustard or [^{14}C]sulfur mustard in CH_3CN (50 μL). After incubation (under gentle shaking) for 2 h at 37 °C, plasma and erythrocytes were separated by centrifugation at 3000*g*. Subsequently, albumin was isolated from the plasma according to a procedure described by Bechtold et al. (*13*). Representative yields were 50−60 mg/mL plasma.

Acidic Hydrolysis of Albumin and Derivatization with Fmoc-Cl for Subsequent HPLC Analysis with Radiometric Detection. Albumin from blood which had been exposed to [^{14}C]sulfur mustard (1 mM, specific activity of 15 mCi/mmol) was hydrolyzed with acid. To this end, the albumin sample (14 mg) was dissolved overnight in constantly boiling 6 N HCl (1 mL). The solution was transferred to a vacuum hydrolysis tube, and the vial was carefully rinsed with 6 N HCl (1 mL). The tube was cooled in liquid nitrogen and evacuated, after the contents solidified. Subsequently, the tube was heated at 110 °C. After 24 h, the solution was concentrated under vacuum and the residue was coevaporated with H_2O (3 × 1 mL) to remove traces of HCl. A small part of the hydrolysate ($^1/_{20}$) was dissolved in borate buffer (0.2 M, pH 7.8, 1.5 mL). Subsequently, a solution of Fmoc-Cl in acetone (15 mM, 1.5 mL) was added, and the sample was shaken vigorously for 1 min. The sample was then washed with hexane (5 × 1 mL), and the aqueous layer was used for HPLC analysis with radiometric detection.

Tryptic Digestion of Albumin. To a solution of albumin (3 mg, isolated from blood which had been exposed to [^{14}C]sulfur mustard or sulfur mustard) in a buffer (300 μL) containing 6 M guanidine hydrochloride, 100 mM Tris-HCl, and 1 mM EDTA (pH 8.3) (with 2 M NaOH) was added dithiothreitol (5 mg), and the solution was incubated at 55 °C for 40 min. Subsequently, iodoacetic acid (sodium salt, 10 mg) was added and the mixture incubated at 40 °C for 30 min. The clear solution was transferred into a Slide-a-Lyzer cassette (0.1−0.5 mL), and the solution was dialyzed against aqueous 50 mM NH_4HCO_3 (3 L) for 16 h. Trypsin (2% w/w) was added, and the mixture was incubated at 37 °C for 4 h.

Synthesis of the Sulfur Mustard Adduct of T5 of Albumin. To a solution of (*S*-HETE)Cys (1 mmol, 225 mg) in dioxane/H_2O (5 mL, 1/1, v/v) were added Fmoc-Cl (1 mmol, 260 mg) and Na_2CO_3 (270 mg) under stirring at 0 °C. After 4 h at 0 °C, stirring was continued at room temperature for 16 h. The solution was washed with petroleum ether 60−80, and the aqueous layer was acidified (pH 3) with 1 M $KHSO_4$ (20 mL). The aqueous layer was extracted with ethyl acetate (2 × 20 mL). The organic layers were collected, dried ($MgSO_4$), and concentrated, giving a colorless oil (400 mg, 89%). FPLC analysis showed the presence of one main compound. This compound was used without further purification for the solid-phase synthesis of the sulfur mustard adduct of the T5 tryptic fragment of albumin, i.e., A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-(*S*-HETE)C-P-F-E-D-H-V-K. After splitting from the resin, one main compound resulted according to FPLC analysis. Electrospray MS: *m/z* 1269.8 (MH_2^{2+}), 847.0 (MH_3^{3+}), 635.5 (MH_4^{4+}).

Micro-LC/Tandem MS Analyses of Tryptic Albumin Digests. A PRP-1 column (0.3 mm × 250 mm) was used in the LC system. Eluent A consisted of 95/5 H_2O/CH_3CN containing 0.5% HCOOH, and eluent B consisted of 2/8 H_2O/CH_3CN containing 0.5% HCOOH. The following flow scheme was applied: 100% eluent A at a flow rate of 0.1 mL/min from 0 to 5 min and, subsequently, 100% eluent A to 100% eluent B at a flow rate of 0.3 mL/min from 5 to 90 min. Flow rates were reduced by a preinjector split: at a rate of approximately 3−10 μL/min from 0 to 5 min and, subsequently, constant at a rate of 10 μL/min. The LC column was directly connected to the electrospray probe. The injection volume was 10−40 μL. Analyses were performed in the multiple-reaction monitoring (MRM) mode (transition $MH_3^{3+} \rightarrow$ *m/z* 1071.0, 1014.5, and 978.5). Operating conditions were as follows: cone voltage of 35 V, collision energy of 12 eV, argon pressure of 5 × 10^{-3} mbar, and dwell of 1.5 s/channel.

Pronase Hydrolysis of Albumin and Micro-LC/Tandem MS Analysis of (*S*-HETE)Cys-Pro-Phe. To a suspension of albumin (3 mg) in aqueous NH_4HCO_3 (50 mM, 750 μL) was added a solution of Pronase (10 mg/mL) in aqueous NH_4HCO_3 (50 mM, 100 μL). After incubation for 2.5 h at 37 °C, the mixture was filtered through a centrifugal ultrafilter (10 kDa cutoff) with centrifugation at 4000*g*. The filtrate was analyzed with micro-LC/tandem MS. In this case, the tripeptide (*S*-HETE)Cys-Pro-Phe is determined by MRM of MH^+ (*m/z* 470) → *m/z* 105 ($HOCH_2CH_2SCH_2CH_2^+$) which corresponds with a fragment of thiodiglycol. Operation conditions were as follows: cone voltage of 30−35 V, collision energy of 20 eV, and argon pressure of 3−4 × 10^{-3} mbar. The injection volume was 40 μL. The LC system comprised a PRP-1 column (0.32 mm × 350 mm). Gradient elution using 95/5 H_2O/CH_3CN with 0.5% HCOOH and 2/8 H_2O/CH_3CN with 0.5% HCOOH as eluents A and B, respectively, was performed as follows. The flow scheme was as follows: 100% eluent A at a flow rate of 0.1 mL/min from 0 to 5 min and, subsequently, 100% eluent A to 100% eluent B at

Table 1. Binding of [^{14}C]Sulfur Mustard to Human Serum Albumin upon Treatment of Human Blood with Various Concentrations of the Agent

[^{14}C]sulfur mustard concentration (μM)	% total radioactivity bound to albumin	μmol of [^{14}C]sulfur mustard bound per 1000 μmol of albumin
1.3	21	0.43
13	20	4.1
130	20	41
1300	18	370

a flow rate of 0.5 mL/min from 5 to 90 min. Flow rates were reduced by a splitter: from 0 to 5 min, 2 → 10 μL/min, and subsequently, 10 μL/min. This procedure was used for analysis of the Iranian blood samples.

Improved Procedure for Determination of (*S*-HETE)-Cys-Pro-Phe with Micro-LC/Tandem MS. The procedure for analysis of the adducted tripeptide (*S*-HETE)Cys-Pro-Phe was improved by means of the following methods.

(1) Sep-Pak C18 Cleanup of the Sample. A Sep-Pak C18 cartridge was conditioned with MeOH (5 mL) followed by 0.1% TFA/H_2O (5 mL). The filtered Pronase digest was applied to the cartridge. The cartridge was rinsed with 0.1% TFA/H_2O (2 mL), 0.1% TFA/10% CH_3CN (2 mL), 0.1% TFA/20% CH_3CN (2 mL), and finally 0.1% TFA/40% CH_3CN (2 mL). The 40% CH_3CN eluate was collected, concentrated, and redissolved in H_2O (50 μL). The sample was now ready for LC/MS analysis.

(2) Modified Liquid Chromatography Procedure. It was found that application of a microcolumn with Lichrosorb RP18 material (0.32 mm × 350 mm), in combination with a slightly modified gradient, improved the detection limit of the micro-LC/tandem MS analysis.

Gradient elution using 95/5 H_2O/CH_3CN with 0.2% HCOOH and 2/8 H_2O/CH_3CN with 0.2% HCOOH as eluents A and B, respectively, was performed as follows. The flow scheme was as follows: 100% eluent A at a flow rate of 0.1 mL/min from 0 to 5 min and, subsequently, 100% eluent A to 30% eluent B at a flow rate of 0.6 mL/min from 5 to 25 min and, finally, 70% eluent A to 100% B at a flow rate of 0.6 mL/min from 25 to 45 min. Flow rates were reduced by means of a splitter: from 0 to 5 min, 2 → 10 μL/min, and subsequently, 10 μL/min.

Synthesis of (*S*-HETE)Cys-Pro-Phe. *N*-Fmoc-*S*-HETE-Cys was used for coupling to immobilized Pro-Phe by manual addition of the Fmoc derivative and coupling reagents to the resin (10 μmol scale). After the peptide was split from the resin with TFA, one main compound resulted, according to FPLC analysis. ^{1}H NMR and mass spectrometric data were in accordance with the proposed structure. The compound was used as a reference for tandem MS experiments. Electrospray MS: *m/z* 470 (MH^+), 105 ($HOCH_2CH_2SCH_2CH_2^+$).

Results

Quantitation of Binding. For quantitation of sulfur mustard binding to albumin, human blood was incubated with ^{14}C-labeled agent (1300, 130, 13, and 1.3 μM; specific activity of 15 mCi/mmol) for 2 h at 37 °C. After isolation of albumin, the protein (2 mg) was dissolved in a solution of 1 M urea in 0.9% NaCl and the radioactivity was determined with liquid scintillation counting. A survey of the results is given in Table 1.

Identification of Alkylation Sites for Sulfur Mustard in Albumin. In a first attempt to identify amino acids which are alkylated in albumin by sulfur mustard, the protein isolated from blood which had been exposed to [^{14}C]sulfur mustard (1 mM) was treated with 6 N HCl. After subsequent derivatization of the amino acid mixture with Fmoc-Cl, two main peaks were present in the HPLC chromatogram (Figure 1). Peak 1 is probably [^{14}C]-thiodiglycol, resulting from hydrolysis of adducts with

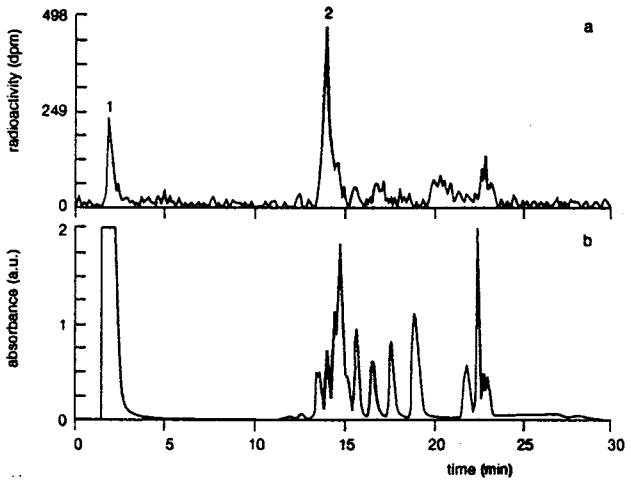

Figure 1. HPLC analysis (Pep-RPC 5/5 column) of an acidic hydrolysate of albumin isolated from human blood that was exposed in vitro to 1 mM [^{14}C]sulfur mustard, after derivatization with Fmoc-Cl: (a) detection of radioactivity and (b) UV detection (254 nm). The eluent (flow rate of 1 mL/min) was 0.1% TFA in H_2O with a linear gradient to 0.1% TFA in CH_3CN/H_2O (48/52, v/v) over the course of 20 min; peak 1 is probably thiodiglycol and peak 2 *N*α-Fmoc-*N*1/*N*3-HETE-histidine.

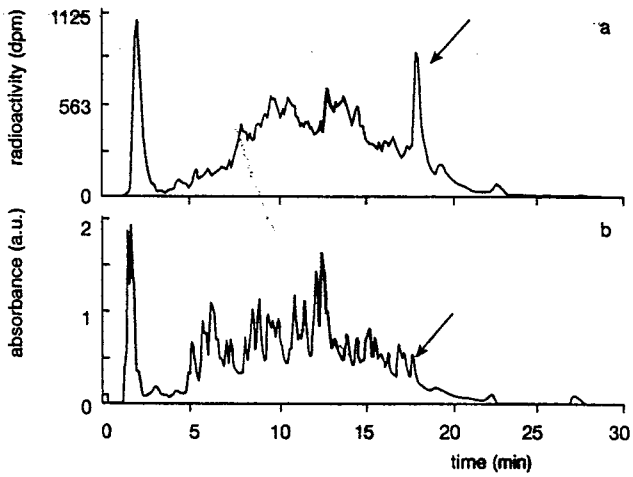

Figure 2. HPLC chromatogram (Pep-RPC 5/5 column) of a tryptic digest of albumin isolated from human blood that was treated in vitro with [^{14}C]sulfur mustard (10 mM): (a) detection of radioactivity and (b) UV detection (214 nm). The eluent was 0.1% TFA in H_2O with a linear gradient to 0.1% TFA in CH_3CN/H_2O (48/52, v/v) over the course of 20 min. The arrows denote the peak for the alkylated T5 fragment.

glutamic or aspartic acid. Peak 2, which contains 28% of the total radioactivity bound to albumin, coelutes with the Fmoc derivative of the synthetic histidine–sulfur mustard adduct, i.e., *N*α-Fmoc-*N*1/*N*3-[2-[(hydroxyethyl)-thio]ethyl]histidine.

In a second attempt, the protein was digested with trypsin after reduction of the disulfide bridges with dithiothreitol and carboxymethylation of the free cysteine residues with iodoacetic acid (*24*). HPLC analysis of tryptic digests gave reproducible chromatograms. A large number of radioactive peaks were observed, demonstrating random alkylation of albumin by sulfur mustard (Figure 2). The large peak in the early region of the chromatogram probably represents [^{14}C]thiodiglycol (cleavage of ester adducts) and small alkylated peptides. One peak in the late-eluting region of the chromatogram, containing 4–5% of the total radioactivity, was fully separated from other peptide material. With higher

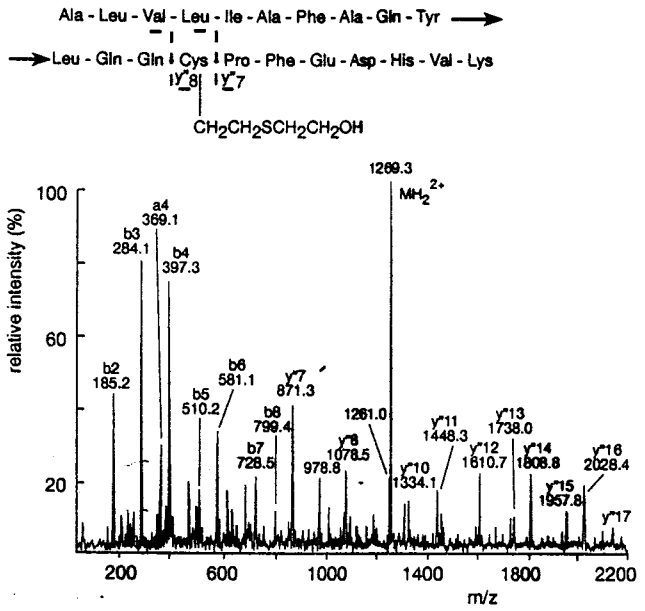

Figure 3. Product ion spectrum of molecular ion MH_2^{2+} (*m/z* 1269) of the alkylated T5 peptide in a tryptic digest of albumin isolated from human blood that was exposed to sulfur mustard (10 mM).

exposure levels (>5 mM), an additional peak in the UV region could be observed which coincided with the radioactive peak. Mass spectrometric analysis of a tryptic digest of albumin from blood exposed to 10 mM sulfur mustard identified the late-eluting peak as a compound with an ion at *m/z* 1269.3, which corresponds with MH_2^{2+} of the alkylated T5 fragment, i.e., [[2-(hydroxyethyl)thio]-ethyl]-(A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C-P-F-E-D-H-V-K) ($MW_{monoisotopic}$ of 2536.3 Da, $MW_{average}$ of 2538.0 Da). Tandem MS experiments showed that alkylation had indeed occurred at Cys^{34}, which is clearly demonstrated in the *m/z* values of fragments y''_7 and y''_8 (for the nomenclature, see ref *25*), corresponding to values for a nonalkylated and an alkylated fragment, respectively (Figure 3). Moreover, the radioactive peak of the peptide in the tryptic digest coeluted with synthetic S-alkylated T5, which could be readily attained by solid-phase synthesis.[2]

Assay for Detection of the Alkylated T5 Fragment. Since the alkylated T5 fragment represented a relatively high percentage of the total radioactivity bound to albumin and was fully separated from other peptides, it seemed worthwhile to investigate whether LC/tandem MS analysis of this peptide in a tryptic digest of albumin is suitable for retrospective detection of exposure to sulfur mustard. The detection limit of selective ion recording (SIR) for the ion at *m/z* 1269.5 (MH_2^{2+}) was 10 pg for the synthetic compound. In the MRM mode, the following transitions were recorded: *m/z* 846.3 (MH_3^{3+}) → *m/z* 1071.0, 1014.5, and 978.5 (doubly charged y_{17}, y_{16}, and y_{15}, respectively); *m/z* 846.3 (MH_3^{3+}) → *m/z* 185.0, 284.2, and 397.3 (b_2, b_3, and b_4, respectively); and *m/z* 1269.5 (MH_2^{2+}) → *m/z* 185.0, 284.2, and 397.3.

[2] Previously, peptides containing a cysteine–sulfur mustard adduct were synthesized by employing a building block in which the HETE group was protected with a *tert*-butyl group (*31*). We now found that the hydroxyl function can be left unprotected (at least for these particular sequences), i.e., employing *N*-Fmoc-*S*-HETE-cysteine as a building block for solid-phase peptide synthesis. The resulting crude product consisted mainly of the desired S-alkylated T5 and was used without further purification.

The lowest detection limit was obtained from combined recording of the first three transitions, i.e., *m/z* 846.3 → *m/z* 1071.0, 1014.5, and 978.5, allowing a detection of ≥15 pg of the alkylated peptide.

For analysis of trypsinized albumin samples, MRM was the method of choice since SIR was not specific enough. The detection limit for the adduct was now increased to 45 pg (from standard addition, 3/1 S/N). The lowest detectable concentration for in vitro exposure of human blood appeared to be approximately 1 μM. Unfortunately, serious problems were encountered with blank samples, since small signals were observed at the same retention time as the alkylated T5 fragment at exposure levels of ≤0.5 μM. Since impurities in the iodoacetic acid might be responsible for the interferences in the blank, the reduction and alkylation of the isolated albumin were omitted. This had an adverse effect on the lowest detectable concentration, since large fragments were formed which coeluted with the alkylated T5 fragment.

Assay for Detection of Sulfur Mustard Adduct with Cys^{34} in Albumin after Pronase Digestion. Subsequently, we attempted to hydrolyze the alkylated T5 fragment with Pronase to enable GC/MS analysis of the adducted cysteine residue after derivatization. After removal of the enzyme by filtration through a centrifugal ultrafilter (10 kDa cutoff), analysis of the incubation mixture with LC/MS did not show the presence of the single adducted amino acid. Instead, several small peptides were present, containing the alkylated cysteine residue, i.e., (*S*-HETE)Cys-Pro ($MW_{monoisotopic}$ of 322.1 Da), (*S*-HETE)Cys-Pro-Phe ($MW_{monoisotopic}$ of 469.2 Da), and Gln(*S*-HETE)Cys-Pro-Phe ($MW_{monoisotopic}$ of 597.2 Da). These peptides could also be detected after Pronase hydrolysis of a tryptic digest from sulfur mustard-exposed albumin.

We investigated whether these peptides could be detected after direct digestion of albumin. Micro-LC/MS analyses with selective ion recording were performed with the Pronase digest of albumin exposed to a relatively high concentration of sulfur mustard (5 mM). The peak area of the dipeptide was ca. 3/4 of that for the tripeptide, whereas the alkylated tetrapeptide was not present in a significant amount. The dipeptide coeluted as a broad peak with other peptides and amino acids. The tripeptide eluted as a sharp peak, despite the large injection volume (40 μL) relative to the column dimensions. For quantitation of the amount of tripeptide formed after Pronase digestion, experiments were carried out with albumin isolated from blood which had been exposed to [^{14}C]sulfur mustard (1 mM). Upon HPLC analysis of a Pronase digest, a small peak was observed (Figure 4) which coeluted with the synthetic tripeptide and which contained ca. 6% of the total radioactivity, which is slightly higher than the percentage determined to be present as alkylated T5 after tryptic cleavage (4–5%). It was established that elution of the Pronase digest on a Sep-Pak C18 cartridge led to considerable cleanup of the sample. Elution was performed with 0.1% TFA/H_2O, 0.1% TFA/10% CH_3CN, 0.1% TFA/20% CH_3CN, and finally 0.1% TFA/40% CH_3CN. The rapidly eluting compounds (amino acids and dipeptides) were present in the 0.1% TFA/H_2O and the 0.1% TFA/10% CH_3CN fractions. The tripeptide was exclusively present in the 40% CH_3CN layer. The extent of recovery of the tripeptide after filtration was 100%, whereas the extent of recovery of the Sep-Pak procedure was 87%. Use of ultrafilters with

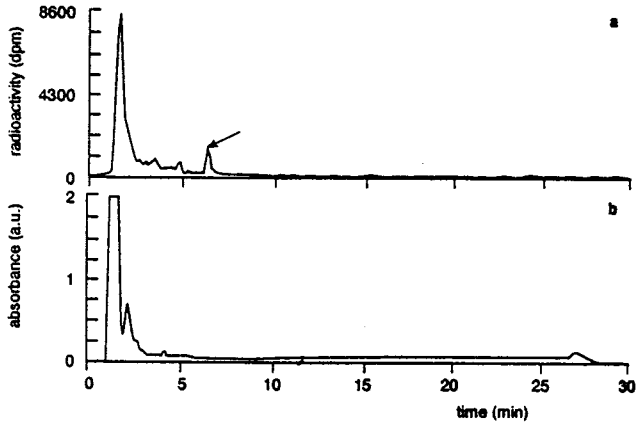

Figure 4. HPLC analysis (Pep-RPC 5/5 column) of (*S*-HETE)-Cys-Pro-Phe (arrow) in a Pronase digest of albumin isolated from human blood that was exposed to [^{14}C]sulfur mustard (1 mM): (a) detection of radioactivity and (b) UV detection (214 nm). The eluent (flow rate of 1 mL/min) was 0.1% TFA in H_2O with a linear gradient to 0.1% TFA in CH_3CN/H_2O (80/20, v/v) over the course of 20 min. The arrow denotes the peak for the alkylated tripeptide.

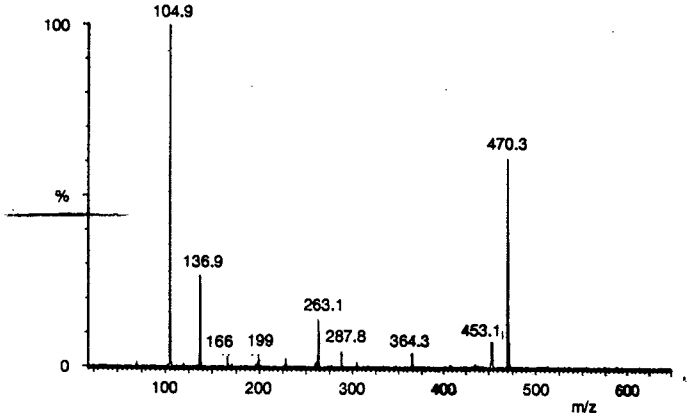

Figure 5. Product ion spectrum of molecular ion MH^+ (*m/z* 470) of synthetic (*S*-HETE)Cys-Pro-Phe: *m/z* 453 ($MH^+ - NH_3$), 137.1 ($HOCH_2CH_2SCH_2CH_2S^+$), and 104.9 ($HOCH_2CH_2SCH_2$-CH_2^+).

a cutoff of 3 kDa instead of ultrafilters with a cutoff of 10 kDa for removal of the enzyme after digestion did not improve the analysis of the tripeptide. We also found that it was important to use relatively large amounts of Pronase, i.e., a Pronase/albumin ratio of 1/3 (w/w). When the Pronase/albumin ratio was lowered, the level of formation of the tetrapeptide Gln-(*S*-HETE)Cys-Pro-Phe was increased, whereas prolonged digestion (>2.5 h) gave increased amounts of dipeptide. We also found that less tripeptide was formed when 10 mg of albumin was digested, instead of 3 mg, in the same volume and with the same enzyme/albumine ratio of 1/3, applying the same incubation conditions.

The tripeptide was conveniently detected with micro-LC/tandem MS under MRM conditions: transition *m/z* 470 (MH^+) → *m/z* 105 ($HOCH_2CH_2SCH_2CH_2^+$) (see Figure 5 for the mass spectrum). A clean blank was obtained when albumin isolated from nonexposed blood was used. Replacement of the PRP-1 column with a Lichrosorb C18 (5 μm) microcolumn and slight modification of the gradient led to a detection limit of 4 pg for the tripeptide. With this improved procedure, including the Sep-Pak C18 cleanup step, we achieved a detection level of 10 nM sulfur mustard in human blood. For such an analysis, 3 mg of albumin is needed, which is present in 120 μL of

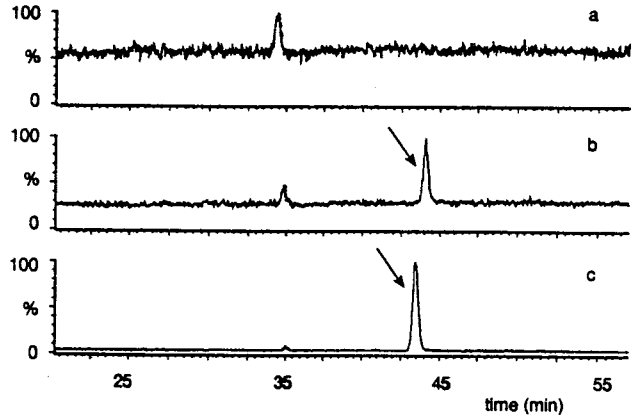

Figure 6. Ion chromatogram of (*S*-HETE)Cys-Pro-Phe (arrow) in a Pronase digest of albumin (3 mg) after purification on Sep-Pak C18, using the MRM scanning mode for the transition from *m/z* 470 (MH^+) to *m/z* 105. Albumin was isolated from nonexposed human blood (a) and from human blood that was exposed to 10 (b) or 100 nM sulfur mustard (c).

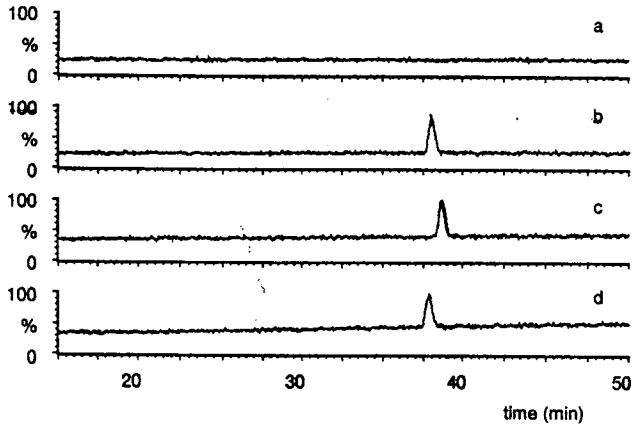

Figure 7. Ion chromatogram of (*S*-HETE)Cys-Pro-Phe in a Pronase digest of albumin (3 mg), using the MRM mode for the transition from *m/z* 470 (MH^+) to *m/z* 105. Albumin was isolated from nonexposed human blood (a), from human blood that was exposed to 1 μM sulfur mustard (b), and from blood taken from two Iranian victims 8–9 days after alleged exposure to sulfur mustard (c and d).

human blood (see Figure 6). It was estimated from the peak areas for the tripeptide in the micro-LC/tandem MS spectra that the relationship between exposure level (10 nM to 10 μM) and adduct level is linear over the entire range.

Analysis of Iranian Blood Samples. We applied the procedure to analysis of blood samples taken from nine Iranian casualties of the Iran–Iraq war (1986), which were treated at the Academic Hospital Utrecht. The blood samples had been kept at −70 °C since that time. These victims had been exposed to sulfur mustard 8–9 days earlier, while some of them had donned gas masks during the whole exposure period or part thereof. All patients suffered from skin injuries compatible with sulfur mustard intoxication; some of them had respiratory difficulties. Albumin was isolated from the blood sample without prior separation of erythrocytes and plasma, since the red blood cells had been hemolyzed during storage. In all cases, except for the blank samples, the tripeptide could be detected (see Figure 7 for an example). The adduct levels correspond to estimated levels after in vitro exposure of human blood to agent concentrations ranging from 0.4 to 1.8 μM.

Discussion

In our continuous search for sensitive, retrospective methods for detecting exposure to CW agents, we initiated a study of sulfur mustard binding to albumin.

We found that a proportional amount of sulfur mustard (ca. 20%) was bound to albumin isolated from human blood treated with various concentrations (1.3 μM to 1.3 mM) of ^{14}C-labeled agent, indicating a linear relationship between exposure concentration and adduct level. This linear relationship could be further extended to a concentration as low as 10 nM. Similar results were previously obtained for binding of sulfur mustard (0.1 μM to 5 mM) to hemoglobin (*4*). Although the latter protein is enclosed in erythrocytes, it binds an even somewhat greater fraction of the agent (ca. 25%).

To identify adducted amino acids, both an acidic hydrolysate and tryptic digests of albumin isolated from blood that had been exposed to [^{14}C]sulfur mustard were analyzed by means of HPLC. (*N1*/*N*3-HETE)Histidine was identified as its Nα-Fmoc derivative in the acidic hydrolysate from coelution with the synthetic product as the major adducted amino acid, as was also the case for human hemoglobin. The peak of this adducted amino acid accounted for 28% of the total radioactivity bound to the protein. Although the histidine adduct is rather abundantly formed in albumin and is stable during acidic hydrolysis, its future use as a biomarker for exposure to sulfur mustard will depend on the development of a satisfactory method of analysis (*6*).

The analyses of the tryptic digests showed a radioactive fragment containing 4–5% of the radioactivity, which was separated from other peptide fragments. The albumin fragment was identified by micro-LC/tandem MS analysis as the T5 tryptic fragment alkylated at Cys^{34}, as we did previously for hemoglobin (*3*). Further evidence for its identity was obtained by coelution with the synthetic alkylated T5 upon HPLC analysis. Cys^{34}, which is the only free cysteine residue of the protein, has previously been identified as a nucleophilic site capable of reacting with electrophiles (see, for example, refs *13*, *14*, and *19*). In the crystal structure of albumin (*26*), Cys^{34} is protected in a crevice where it probably interacts with His^{39} and Glu^{82}, allowing the stabilization of the deprotonated thiol and causing its pK_a to be relatively low, between 5 and 7 (*27*). This might explain the enhanced reactivity of the Cys^{34} residue, although the histidine residues mentioned earlier might even be more reactive.

The lowest detectable concentration for in vitro exposure of human blood to sulfur mustard was found to be 1 μM using micro-LC/tandem MS analysis under MRM conditions of the alkylated T5 fragment in the albumin tryptic digest. Enhancement of the minimum detectable concentration was hampered by small signals that were observed in the blank samples at the same retention time as the analyte. In a second approach toward a quantitative analysis of the alkylated Cys^{34} residue, the modified peptide fragment was further digested with Pronase, which led to the formation of di-, tri-, and tetrapeptides containing the alkylated cysteine residue. The most abundant fragment, i.e., the tripeptide (*S*-HETE)Cys-Pro-Phe, is also formed after direct digestion of adducted albumin by Pronase. The tetrapeptide was formed in trace amounts, whereas the dipeptide (*S*-HETE)Cys-Pro was formed to a slightly lesser extent than the tripeptide, which totals the amount of Cys^{34} alkylation up to 10%. Compared to that at hemoglobin, alkylation at cysteine is more pronounced for albumin (*6*). The incomplete hydrolysis of (alkylated) albumin by Pronase has been observed by others. For instance, after Pronase treatment of rat albumin adducted with the food-borne carcinogen IQ (*19*) or PhIP (*28*), a similar tripeptide Cys*-Pro-Tyr resulted, with Cys* being the adducted Cys^{34} residue. Analogously, the modified tripeptide His*-Pro-Tyr has been reported, with His* being the adducted His^{146} residue, which resulted from Pronase digestion of albumin exposed to benzo[*a*]pyrene antidiol epoxide (*29*). Black et al. (*30*) also observed incomplete hydrolysis with Pronase of globin which had been alkylated by sulfur mustard.

We optimized the enzymatic degradation of adducted albumin, the workup and the micro-LC/tandem MS analysis, resulting in a simple, fast, reliable, and sensitive method. Using only 3 mg of albumin, we were able to detect exposure of human blood to a sulfur mustard concentration of 10 nM. Moreover, the method could successfully be applied to the analysis of blood samples of Iranian victims of the Iran–Iraq war. In this respect, the results were in accordance with those obtained after modified Edman degradation of globin from the same blood samples (estimated levels corresponding to concentrations ranging from 1 to 2 μM; unpublished results). Presently, our method enables detection of exposure of human blood to a sulfur mustard concentration of 10 nM, which is 10 times lower than after modified Edman degradation of globin, as reported earlier (*4*). This opens the way for detection and dosimetry of low-level exposure to sulfur mustard.

In conclusion, we have shown that albumin is effectively alkylated by sulfur mustard and that the Cys^{34} residue is an important alkylation site. In addition, we have developed a new method based on the combination of micro-LC and tandem MS for establishing exposure to sulfur mustard by analysis of an adducted tripeptide resulting from Pronase hydrolysis of albumin. This method is less laborious and more sensitive than the modified Edman degradation and does not require derivatization steps. We believe that this approach can generally be applied for dosimetry of exposure to other compounds which bind to Cys^{34}. The synthetic alkylated T5 fragment will be used for raising monoclonal antibodies, which might be useful for immunochemical detection of exposure to sulfur mustard.

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