DOCUMENTATIE
TNO Prins Maurits Laboratorium
Dq6-1014

 Chem. Res. Toxicol. **1996,** *9,* 788−792

Monitoring of in Vitro and in Vivo Exposure to Sulfur Mustard by GC/MS Determination of the N-Terminal Valine Adduct in Hemoglobin after a Modified Edman Degradation

Alex Fidder,[†] Daan Noort,[†] Ad L. de Jong,[‡] Hendrik C. Trap,[†] Leo P. A. de Jong,[†] and Hendrik P. Benschop*,[†]

Department of Chemical Toxicology and Department of Analysis of Toxic and Explosive Substances, TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands

Received December 29, 1995[⊗]

We report that exposure to the chemical warfare agent sulfur mustard can be monitored by means of a modified Edman degradation involving selective release of the N-terminal valine adduct of hemoglobin with the agent. The degree of alkylation of the N-terminal valine in human hemoglobin is approximately 1−2% of the total alkylation induced in hemoglobin upon treatment of human blood with sulfur mustard. After modified Edman degradation, followed by derivatization with heptafluorobutyric anhydride, the obtained pentafluorophenyl thiohydantion derivative of the valine adduct could be analyzed at a ≥0.5 fmol level by means of GC/MS under negative ion chemical ionization conditions. Applying this procedure, in vitro exposure of human blood to ≥0.1 μM of sulfur mustard could be determined. In vivo exposure of guinea pigs could also be established at 48 h after intoxication intravenously with 0.5 mg/kg (0.06 LD_{50}) of the agent.

Introduction

The use of chemical warfare agents in the Iran−Iraq war (1980−1988) has shown that reliable methods are urgently needed for detection of the nature and extent of poisoning with these agents in order to assure optimal treatment of the casualties. Recently, the need for retrospective detection of exposure to such agents has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome" (*1*). Furthermore, these methods can also be used for verification of alleged noncompliance to the recently signed treaty to ban chemical weapons (*2*), to biomonitor workers in plants for the destruction of chemical warfare agents, and to establish or confirm terroristic use of chemical warfare agents (*3*).

Experiences with casualties of chemical warfare agents in the Iran−Iraq war and in other incidents (*4*) made clear that biopsies of victims often become available several days or even weeks after alleged exposure. In this context, we have explored the feasibility of immunochemical assays of DNA adducts with the vesicant and strongly alkylating agent sulfur mustard [bis(2-chloroethyl) sulfide] (*5*), based on detection of *N*7-alkylated guanine residues (*6*, *7*). This method allows us to detect in vitro exposure of human blood to ≥0.07 μM sulfur mustard[1] and of human skin to sulfur mustard vapor at Ct values ≥300 mg·min·m^{-3}, i.e., at a minimum Ct value that does not cause blisters (*8*).

We now focus our attention toward mass spectrometric analysis of sulfur mustard adducts to hemoglobin. Measurement of hemoglobin adducts is a well-established method for biomonitoring of environmental or occupational exposure to alkylating carcinogens (*9*). As these adducts are generally stable and in many cases have the same lifetime (ca. 4 months) in vivo as the native protein (*10*), they may be detectable even if a substantial period of time has elapsed since the exposure took place. In addition, globin is readily accessible in relatively large amounts (ca. 140 mg/mL of blood). This approach stands in contrast with detection of DNA damage, which may be repaired within days.

A versatile method for analyzing N-alkylated valine residues in hemoglobin has been developed by Törnqvist et al. (*11*). This method is based on selective cleavage of the adduct from hemoglobin by using the modified Edman reagent pentafluorophenyl isothiocyanate (PFPITC).[2] In the preceding paper (*12*), we used LC−tandem mass spectrometry to elucidate structural modifications in hemoglobin due to exposure to sulfur mustard and showed, e.g., that the N-terminal valine residues of both α- and β-globin are prone to alkylation by sulfur mustard. The second chloroethyl moiety of sulfur mustard is expected to be hydrolyzed to a hydroxyethyl group (see Scheme 1). In the present study, we investigate the feasibility of the modified Edman procedure for trace analysis of the N-alkylated valine formed after in vitro exposure of human blood to sulfur mustard and after in vivo exposure of guinea pigs to the agent. In our case, the obtained pentafluorophenyl thiohydantoin (PFPTH)

* To whom correspondence should be adressed, at the TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands. Telephone: +31 15 284 35 29; Telefax: +31 15 284 39 63.

† Department of Chemical Toxicology, TNO Prins Maurits Laboratory, Rijswijk.

‡ Department of Analysis of Toxic and Explosive Substances, TNO Prins Maurits Laboratory, Rijswijk.

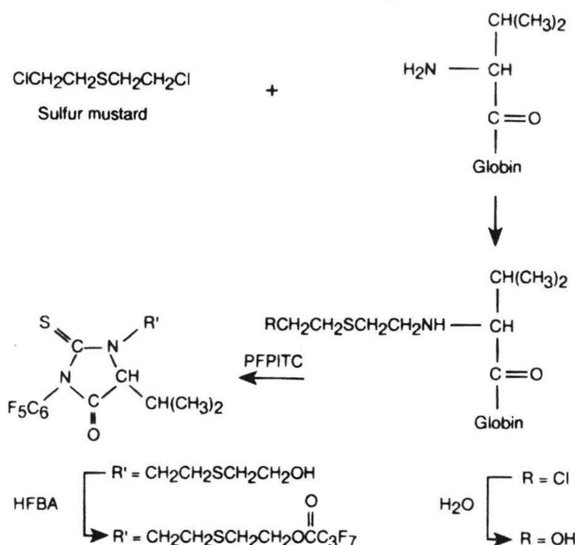
⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] In ref 7, N7-alkylated guanine residues in white blood cells could be detected after in vitro exposure of human blood to 2 μM sulfur mustard. We now are able to detect exposure as low as 0.07 μM in vitro.

[2] Abbreviations: DMSO, dimethyl sulfoxide; HFBA, heptafluorobutyric anhydride; NICI, negative ion chemical ionization; *N*-HETE-DL-Val, *N*-[2-[(hydroxyethyl)thio]ethyl]-DL-valine; PFPITC, pentafluorophenyl isothiocyanate; PFPTH, pentafluorophenyl thiohydantoin; SIM, selective ion monitoring.

S0893-228x(95)00215-3 CCC: $12.00 © 1996 American Chemical Society

Scheme 1. Reaction of Sulfur Mustard with N-Terminal Valine of Globin and Subsequent Modified Edman Degradation Followed by Reaction with Heptafluorobutyric Anhydride



derivative can be analyzed at the femtomole level by GC/MS [operating in negative ion chemical ionization (NICI) mode combined with selective ion monitoring (SIM)] most effectively after acylation of the hydroxyethyl moiety with heptafluorobutyric anhydride (HFBA, see Scheme 1).

Experimental Procedures

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

HFBA, PFPITC, diethyl ether, methanol, toluene, pyridine, and formamide (all analytical grade) were purchased from Fluka (Buchs, Switzerland). (±)-2-Bromo-3-methylbutyric acid, 2-mercaptoethanol, and 2-bromoethylamine hydrobromide were purchased from Acros (Beerse, Belgium). Technical-grade sulfur mustard was distilled before use to a gas chromatographic purity exceeding 99.5%. 1,1,2,2-Tetradeuterio-2-chloroethanol was purchased from Isotec Inc. (Miamisburg, OH). Sodium [^{35}S]sulfate (42−52 TBq/mmol, carrier free) was purchased from Amersham International (Houten, The Netherlands). Human blood was obtained from healthy volunteers in our laboratory, with consent of the donor and approval of the TNO Medical Ethical Committee.

Animals. Male guinea pigs (Dunkin-Hartley, type strain Crl; Charles River, Sulzfeld, Germany), weighing 400−500 g, were used. They were housed in metabolism cages during the experiment and maintained with food and water *ad libitum*. The protocol for the animal experiment was reviewed and approved by the TNO Committee for Animal Care and Use.

Instrumentation. HPLC was performed using two Waters 510 pumps (Bedford, MA), a gradient programmer (Waters, Model 660), and a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden). The eluent (flow rate 1 mL/min) was 0.1% trifluoroacetic acid in water with a linear gradient to 0.1% trifluoroacetic acid in acetonitrile/water (4/1 v/v) in 25 min. The eluate was monitored at 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 scintillation cocktail. Liquid scintillation countings were performed using a Minaxi Tri-Carb 4000 series scintillation counter (Packard) using Hionic Picofluor (Packard) as scintillation cocktail. Thermospray LC/MS spectra were recorded on a Nermag (Paris, France) R10-10C quadrupole instrument equipped with a thermospray ion source (Nermag), which was coupled to the LC system via a Vestec (Vestec Co., Houston, TX) thermospray interface. The LC system comprised a Waters 510 HPLC pump and an RP-18 column (Lichrosorb 5 μm particles) using aqueous methanol in varying ratios as eluent. The mass spectrometer was operated in the positive ion mode. The temperature of the thermospray vaporizer ranged from 250 to 260 °C during elution, while the temperature of the ion block was maintained at 230 °C. The scan time was 1 s for m/z 100−450. GC/MS was performed using a VG 70-250S (Fisons Instruments, Altrincham, U.K.) mass spectrometer operated in the NICI mode (methane) with an ion source temperature of 200 °C and an ionization energy of 70 eV. The ion source pressure was 2 mPa. The gas chromatograph (Hewlett Packard 5890A) was equipped with an on-column injector (Carlo Erba, Milan, Italy) and a CP-SIL 5 CB fused silica capillary column (Chrompack, Middelburg, The Netherlands, length 50 m, i.d. 0.32 mm, film thickness 0.25 μm). The oven of the chromatograph was kept at 120 °C for 5 min; the temperature was then programmed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min. ^{1}H and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA) VXR 400S spectrometer operating at 400.0 and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to TMS. The solvent signals at 2.525 ppm (residual DMSO-d_5 in DMSO-d_6) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference for ^{1}H NMR spectroscopy, whereas the solvent signals at 39.6 ppm (DMSO-d_6) or 77.1 ppm ($CDCl_3$) were used as a reference for ^{13}C NMR spectroscopy.

Synthesis. Sulfur Mustard-d_8. This compound was synthesized from sodium sulfide and 1,1,2,2-tetradeuterio-2-chloroethanol according to a published method (*13*). The obtained octadeuterated thiodiglycol was treated with thionyl chloride in dichloromethane under reflux. Evaporation followed by vacuum distillation at 10 Pa afforded sulfur mustard-d_8. The purity, as checked by GC/MS, analysis was 95%. The main impurity (4%) was identified as 1,4-dithiane-d_8.

^{13}C NMR ($CDCl_3$) δ 42.4 (CD_2Cl), 33.7 (CD_2S); GC/MS (EI$^+$) m/z 166 [M$^+$], 115 (M$^+$ − CD_2Cl], 67 [$C_2D_4Cl^+$], 30 [$C_2D_3^+$].

[^{35}S]Sulfur Mustard. This compound was synthesized as described in ref 12. Radiochemical purity (TLC) 97%, chemical purity (GC) 95%; sp act. 314 MBq/mmol.

2-[(2-Aminoethyl)thio]ethanol was synthesized by reaction of 2-bromoethylamine hydrobromide with 2-mercaptoethanol, according to a method described by Peck et al. (*14*). After workup, the residue was purified by distillation under reduced pressure (125−126 °C/40 Pa). Yield 11.9 g (98 mmol, 66%) of a colorless oil. ^{1}H NMR (DMSO-d_6) δ 3.55 (t, 2H, HOCH_2−), 2.70 (t, 2H, −CH_2NH$_2$), 2.58 (t, 2H, HOCH$_2$CH_2S−), 2.55 (t, 2H, −SCH_2CH$_2$OH).

***N*-[2-[(Hydroxyethyl)thio]ethyl]-DL-valine (*N*-HETE-DL-Val)** was synthesized by reaction of 2-[(2-aminoethyl)thio]ethanol with (±)-2-bromo-3-methylbutyric acid according to a method described by Murthy et al. (*15*). Yield: 2.2 g (10 mmol, 23%).

^{1}H NMR (D_2O) δ 0.96 (d, 3H, CH$_3$), 1.00 (d, 3H, CH$_3$), 2.17 (m, 1H, CH(CH$_3$)$_2$), 2.71 (t, 2H, SCH_2CH$_2$OH), 2.85 (m, 2H, NCH$_2$CH_2S), 3.21 (m, 2H, NCH$_2$), 3.45 (d, 1H, NCH), 3.70 (t, 2H, CH_2OH); ^{13}C NMR (D_2O) δ 18.21, 18.92 (2 × CH$_3$), 28.06 (SCH$_2$CH$_2$N), 30.12 (CH(CH$_3$)$_2$), 34.05 (HOCH$_2$CH$_2$S), 47.43 (NCH$_2$), 61.04 (CH$_2$OH), 69.14 (CHN); LC/MS m/z 222 [MH$^+$].

1-[2-[(Hydroxyethyl)thio]ethyl]-*dl*-5-isopropyl-3-(pentafluorophenyl)-2-thiohydantoin. *N*-HETE-DL-Val (10 mg) was dissolved in a mixture of aqueous $KHCO_3$ (5 mL, 0.5 M) and 1-propanol (2.5 mL). PFPITC (25 μL) was added, and the mixture was heated for 2 h at 45 °C. The PFPTH derivative was extracted with *n*-heptane (2 × 10 mL) and concentrated to dryness. The conversion of *N*-HETE-DL-Val to the pentafluorophenyl thiohydantoin (PFPTH) derivative was 94% (determined by comparison with an exact amount of $CHCl_3$ added to the ^{1}H NMR sample).

^{1}H NMR ($CDCl_3$) δ 4.40 (ddd, 1H, NCH_AH$_B$), 4.34 (d, 1H, NCH), 3.81 (t, 2H, CH_2OH), 3.65 (ddd, 1H, NCH$_A$$H_B$), 3.09 (ddd, 1H, NCH$_2CH_AH_B$), 2.85 (ddd, 1H, NCH$_2CH_A$$H_B$), 2.83 (m, 2H, SC$H_2CH_2$OH), 2.43 (m, 1H, NCHC$H$), 1.27 (d, 3H, CHC$H_3$), 0.99

(d, 3H, $CHCH_3$); GC/MS (EI+) *m/z* 428 [M+], 410 [M+ − H_2O], 383 [M+ − CH_2CH_2OH], 351 [M+ − SCH_2CH_2OH].

***N*-HETE-DL-Val-Leu-Ser-Pro-Ala-Asp-Lys-OH** was synthesized on a solid support using (fluorenylmethoxy)carbonyl-protected amino acids according to standard procedures. The modified valine residue was incorporated as *N*-[(*tert*-butyloxy)-carbonyl]-*N*-HETE-DL-Val. LC/MS *m/z* 834 [MH+].

Exposure of Human Blood to Sulfur Mustard and Isolation of Globin. A solution of sulfur mustard, sulfur mustard-d_8, or [35S]sulfur mustard in acetonitrile was added to human blood (20 mL; final acetonitrile concentration 1%), and the mixture was incubated overnight at 37 °C in a shaking water bath. Globin was isolated from blood samples according to Bailey et al. (*16*).

Modified Edman Degradation of Globin (*17*). Alkylated globin (20 mg, unless otherwise stated) was dissolved in formamide (2 mL), after which pyridine (6 μL) and PFPITC (6 μL) were added. The mixture was incubated overnight at room temperature followed by 2 h at 45 °C. After extraction of the formamide layer with diethyl ether (3 × 1.5 mL), the combined diethyl ether fractions were dried under a stream of nitrogen. The residue was dissolved in toluene (2 mL). Subsequently, the toluene layer was extracted with water (2 × 1 mL), 0.1 M Na_2CO_3 (1 × 1 mL), and water (1 × 1 mL). After subsequent drying with $MgSO_4$ and evaporation to dryness, the residue was dissolved in toluene (0.5 mL), HFBA (10 μL) was added, and the solution was heated at 45 °C for 10 min. After cooling, the solution was extracted with phosphate buffer (0.5 mL, pH 6.0; 1 M). The toluene layer was dried ($MgSO_4$) and evaporated to near dryness. Finally, the residue was redissolved in toluene (100 μL) and analyzed by GC/MS (NICI); *m/z* 624 [M•−], 604 [M•− − HF], 584 [M•− − 2 HF], 564 [M•− − 3 HF], 544 [M•− − 4 HF].

Exposure of Guinea Pigs to Sulfur Mustard and Isolation of Globin. Three guinea pigs were used for in vivo experiments. Blood taken from one of the animals was used for the isolation of blank globin. The others were anaesthetized with 2.5% halothane in N_2O/O_2 (62/38), and sulfur mustard was injected intravenously into the dorsal penis vein (1 mL of a solution of 0.5 or 5 mg/mL saline, respectively). After 48 h, blood was drawn from the carotid artery and collected in a heparinized tube. Globin was isolated as described before.

Results and Discussion

Development of a Modified Edman Procedure for Determination of N-Terminal Valine Alkylated by Sulfur Mustard. In view of the strong alkylating properties of sulfur mustard, we assumed that alkylation of N-terminal valine in hemoglobin would occur as observed with other alkylating compounds (*9*). In order to study the feasibility of the modified Edman degradation procedure for determination of the sulfur mustard adduct with N-terminal valine, we first synthesized the thiohydantoin of *N*-HETE-DL-Val. Reaction of the latter with PFPITC gave a single product (yield 94%), the structure of which was in accordance with the desired PFPTH, as corroborated by 1H NMR, 13C NMR, and GC/MS (EI+).

The efficacy of the modified Edman procedure was studied in more detail using the synthetic peptide *N*-HETE-DL-Val-Leu-Ser-Pro-Ala-Asp-Lys-OH, i.e., the adducted N-terminal heptapeptide from the α-chain of hemoglobin. After modified Edman degradation, no further thiohydantion could be removed from the reaction mixture after three extractions with diethyl ether as determined with GC/MS (NICI). The adduct level detected after a second treatment with the modified Edman reagent added less than 1% to the total adduct detected. Assuming that PFPITC reacts in a straightforward way with protein-bound *N*-HETE-Val (cf. Scheme 1), these results indicate an almost quantitative release of *N*-HETE-DL-Val.

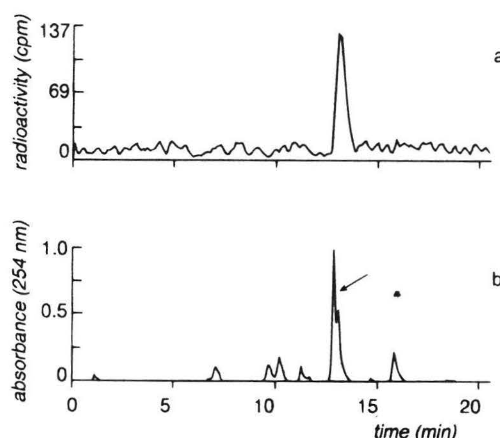


Figure 1. Reversed-phase HPLC analysis of a reaction mixture obtained after modified Edman degradation of globin isolated from human blood after exposure to 5 mM [35S]sulfur mustard. The synthetic PFPTH derivative of *N*-HETE-DL-Val was coeluted (arrow). (a) Radiometric detection; (b) UV detection.

Quantitation of the *N*-HETE-Val Levels in Hemoglobin Exposed to Sulfur Mustard. Analysis of globin isolated from [35S]sulfur mustard-treated human blood provided quantitative information on the extent of hemoglobin alkylation. It was found that an approximately proportional part of the alkylating agent was bound to globin, almost independent of the [35S]sulfur mustard concentration used, i.e., 25%, 19%, 27%, 24%, and 29% of the total radioactivity was incorporated into the protein after treatment of human blood with 5000, 1000, 10, 1, and 0.1 μM of the agent, respectively.

The levels of adducted valine were determined in a similar way as reported by Christakopoulos et al. (*17*). After modified Edman degradation of globin isolated from blood that had been exposed to 1 and 5 mM [35S]sulfur mustard, analysis of the organic extract by reversed-phase HPLC with radiometric detection showed a single radioactive peak which coeluted with the synthetic PF-PTH of the valine adduct (Figure 1). When we assume that the yield of the Edman degradation reaction is almost quantitative (*vide supra*), the level of valine adduct is approximately 1% of the total radioactivity bound to globin. It follows from the results obtained in these experiments that an average of one alkylated valine residue is formed per 4500 or 900 globin chains after exposure of human blood to 1 or 5 mM [35S]sulfur mustard, respectively. On the other hand, when the amount of radioactivity in the organic extract was directly measured, i.e., without HPLC separation, the valine adduct level was determined to be ca. 2%. A similar degree of alkylation of N-terminal valine was reported by Hambrook et al. (*18*) after exposure of human blood to 0.05, 0.1, and 0.5 mM [35S]sulfur mustard, i.e., an average value of 3.5 ± 1.5% as found from the overall radioactivity released from globin upon modified Edman degradation.

In the preceding paper (*12*), we demonstrated that the N-terminal valine residue of both α- and β-globin chain became adducted upon exposure of blood to sulfur mustard. This result was verified using the modified Edman degradation. Thus, globin isolated from blood after exposure to 5 mM [35S]sulfur mustard was separated into its α- and β-chains by chromatography on a CM-Sepharose CL-6B column according to Bergmark et

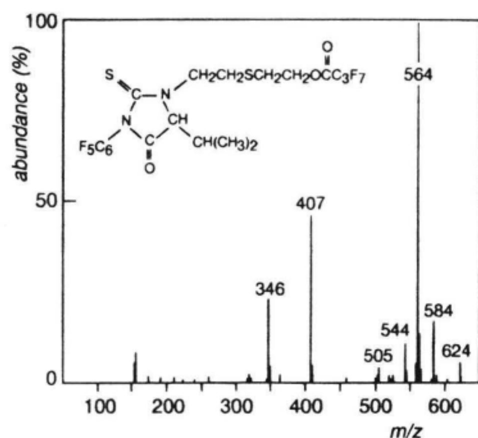


Figure 2. GC/MS (NICI) spectrum of the PFPTH of N-(2-heptafluorobutyroethylthioethyl)-DL-valine (synthetic reference); m/z for $M^{\bullet-} = 624$.

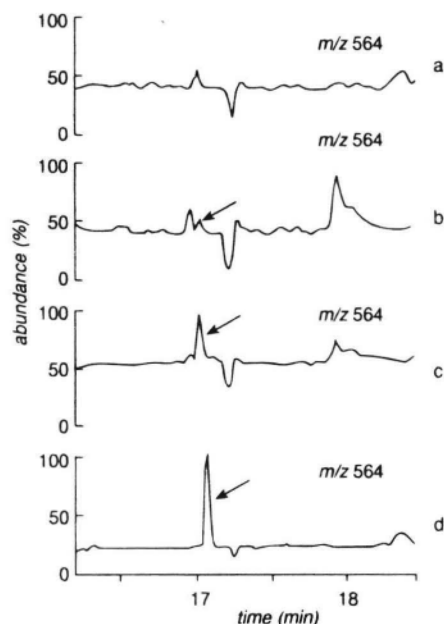


Figure 3. GC/MS (NICI) analysis of the PFPTH of N-(2-heptafluorobutyroethylthioethyl)valine obtained from modified Edman degradation (and subsequent derivatization with HFBA) of globin (20 mg) isolated from human blood after exposure to various concentrations of sulfur mustard. Ion chromatograms after monitoring for m/z 564 ($M^{\bullet-}$ − 3 HF). (a) 0 μM; (b) 0.1 μM; (c) 1 μM; and (d) 10 μM exposure to sulfur mustard.

al. (*19*). The [^{35}S]PFPTH derivative of N-HETE-Val was detected upon HPLC analysis of the reaction mixtures obtained after treatment of both α- and β-globin with the modified Edman reagent, indicating that each chain is prone to N-terminal alkylation by sulfur mustard. This was also found for alkylation of hemoglobin with methyl bromide after in vitro exposure of blood to the agent (*20*).

Detection of in Vitro Exposure of Human Blood to Sulfur Mustard. GC/MS (NICI) analysis of a blank globin sample treated with the modified Edman reagent showed two peaks interfering with the peak for the target compound. This interference was circumvented by further derivatization of the HETE side chain in the thiohydantoin with HFBA, which also improved the chromatographic performance of the analyte. The product N-(2-heptafluorobutyroethylthioethyl)valine PFPTH could be detected by GC/MS (NICI) down to a level of 25 fmol at full scan (m/z 200−800) and of 0.5 fmol (S/N = 3) under SIM conditions (m/z 564 [$M^{\bullet-}$ − 3 HF]; see

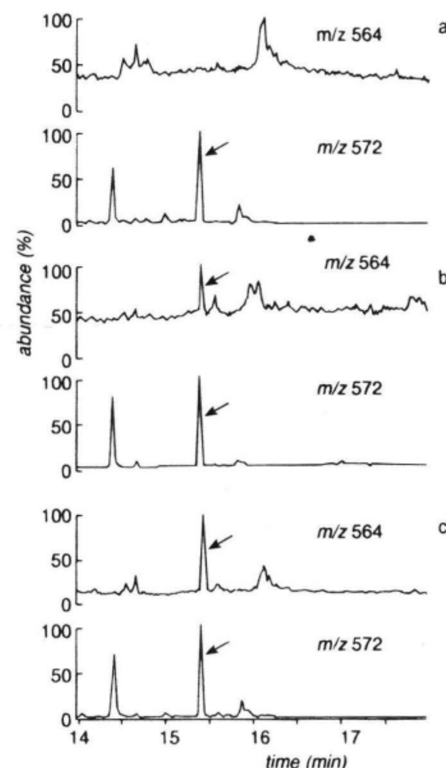


Figure 4. GC/MS (NICI) analysis of PFPTHs obtained after modified Edman degradation of globin samples (20 mg) from male guina pigs treated with an intravenous dose of (a) 0 mg/kg, (b) 0.5 mg/kg (0.06 LD_{50}), and (c) 5 mg/kg sulfur mustard (0.6 LD_{50}). Globin (350 μg) isolated from human blood treated with 1 mM sulfur mustard-d_8 served as an internal standard. Mass chromatograms (SIM) illustrating measured amounts of PFPTH of N-(2-heptafluorobutyroethylthioethyl)valine (m/z 564 [$M^{\bullet-}$ − 3 HF], upper panels) and PFPTH of N-(2-heptafluorobutyroethylthioethyl-d_8)valine (m/z 572 [$M^{\bullet-}$ − 3 HF], lower panels).

Figure 2 for a mass spectrum), assuming that derivatization with HFBA proceeds quantitatively.

The modified Edman degradation procedure combined with GC/MS (NICI) analysis with SIM allowed us to detect an exposure level as low as 0.1 μM sulfur mustard in human blood (Figure 3). This result is comparable to the detection limit for exposure of blood to the agent on the basis of immunochemical detection of alkylated N7-guanine residues (*7*)[1] in white blood cells. Evidently, immunochemical results can be confirmed by the method described in this paper.

Various amounts (20−700 μg) of globin isolated from human blood after exposure to 1 mM sulfur mustard and a fixed amount (350 μg) of globin isolated from human blood that was exposed to 1 mM of sulfur mustard-d_8 (internal standard) were treated with PFPITC according to the modified Edman procedure. GC/MS (NICI) analyses were performed under SIM conditions (m/z 564 and 572 for analyte and internal standard, respectively). A linear relationship ($y = 0.000911x + 0.0178$ with correlation factor 0.9987) was obtained when plotting the ratio (y) found for the peak areas of analyte and internal standard against the amount of globin used (x; in μg).

In addition, GC/MS (NICI) analyses were performed after modified Edman degradation of a fixed amount (350 μg) of the internal standard (globin isolated from blood exposed to 1 mM of sulfur mustard-d_8) and a fixed amount (20 mg) of globin isolated from human blood after exposure to various concentrations of sulfur mustard (0.1, 1, 10, and 100 μM). The plot obtained for the ratio of

the peak areas of analyte and internal standard vs the exposure concentration of sulfur mustard also showed a linear relationship. In combination with the linear relationship found between total adduct level in globin and exposure level, these results confirm that the percentage of valine adduct formed in globin is independent of the exposure concentration. A similar conclusion was already drawn for exposure of human blood to 1 and 5 mM of [^{35}S]sulfur mustard (*vide supra*).

Detection of in Vivo Exposure of Guinea Pigs to Sulfur Mustard. In order to assess the feasibility of the modified Edman procedure for detection of in vivo exposure to sulfur mustard, a preliminary experiment was performed with guinea pigs. Blood samples were taken 48 h after intravenous administration of 5 mg/kg (0.6 LD_{50})[3] or 0.5 mg/kg (0.06 LD_{50}) of sulfur mustard to the animals, and globin was isolated. After applying the modified Edman procedure and subsequent reaction with HFBA, GC/MS (NICI) showed the presence of the PFPTH of *N*-HETE-Val (see Figure 4b,c) in each case, whereas no adduct could be detected in a sample from a non-treated animal (Figure 4a).

In conclusion, a method has been developed for monitoring in vitro and in vivo exposure to sulfur mustard based on a modified Edman degradation. The results indicate that this method will be useful for sensitive detection of in vivo exposure to sulfur mustard as well as for confirmation of immunochemical detection (7) of exposure to the agent.

Acknowledgment. This work was supported in part by Grant DAMD17-92-V-2005 from the U.S. Army Medical Research and Materiel Command, Fort Detrick, Frederick, MD, and in part by the Directorate of Military Medical Services of the Ministry of Defence, The Netherlands. The authors are grateful to Gerrit W. H. Moes and Saskia de Kant for preparing [^{35}S]sulfur mustard and sulfur mustard-d_8, to Herma J. van der Wiel for performing the animal experiments, to Albert G. Hulst for performing LC/MS analyses, and to Simon H. van Krimpen for performing NMR analyses. We express our gratitude to Dr. P. B. Farmer and co-workers (MRC Toxicology Unit, Leicester, U.K.) for their help and hospitality during the start-up of the project.

References

(1) [illegible]ble, D. (1994) Back into the storm. Reanalyzing health effects of the Gulf War. *Anal. Chem.* **66**, 805A–808A.

(2) Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction. Opened for Signature, Paris, France, Jan 13, 1993.

(3) Van Biema, D. (1995) Prophet of poison. *Time Magazine* **145** (*13*), 14–21.

(4) Davey, B. L. (1992) Investigations into alleged use of chemical warfare agents in Angola (1986–1990). Presented at: the Workshop on Alleged Use of Chemical Weapons, Geneva, 16–17 Dec, 1992.

(5) Lawley, P. D. (1984) Carcinogenesis by alkylating agents. In *Chemical Carcinogenesis* (Searle, C. E., Ed.) 2nd ed., Vol. 1 pp 325–484, American Chemical Society, Washington, DC.

(6) Fidder, A., Moes, G. W. H., Scheffer, A. G., van der Schans, G. P., Baan, R. A., de Jong, L. P. A., and Benschop, H. P. (1994) Synthesis, characterization, and quantitation of the major adducts formed between sulfur mustard and DNA of calf thymus and human blood. *Chem. Res. Toxicol.* **7**, 199–204.

(7) Van der Schans, G. P., Scheffer, A. G., Mars-Groenendijk, R. H., Fidder, A., Benschop, H. P., and Baan, R. A. (1994) Immunochemical detection of adducts of sulfur mustard to DNA of calf thymus and human white blood cells. *Chem. Res. Toxicol.* **7**, 408–413.

(8) Papirmeister, B., Feister, A. J., Robinson, S. I., and Ford, R. D. (1991) *Medical Defense against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*, CRC Press, Boca Raton, FL.

(9) Törnqvist, M. (1994) Epoxide adducts to N-terminal valine of hemoglobin. *Methods Enzymol.* **231 B**, 650–657 and references therein.

(10) Osterman-Golkar, S., Ehrenberg, L., Segerbäck, D., and Hällström, I. (1976) Evaluation of genetic risks of alkylating agents. II. Haemoglobin as a dose monitor. *Mutat. Res.* **34**, 1–10.

(11) Törnqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. *Anal. Biochem.* **154**, 255–266.

(12) Noort, D., Verheij, E. R., Hulst, A. G., de Jong, L. P. A., and Benschop, H. P. (1996) Characterization of sulfur mustard induced structural modifications in human hemoglobin by liquid chromatography–tandem mass spectrometry. *Chem. Res. Toxicol.* **9**, 781–787.

(13) Blatt, A. H. (1946) *Organic Syntheses*, Collect. Vol. II, 3rd printing, pp 576–578, Wiley, New York.

(14) Peck, R. M., O'Connell, A. P., and Creech, H. J. (1966) Heterocyclic derivatives of 2-chloroethyl sulfide with antitumor activity. *J. Med. Chem.* **9**, 217–221.

(15) Murthy, M. S. S., Calleman, C. J., Osterman-Golkar, S., Segerbäck, D., and Svensson, K. (1984) Relationship between ethylation of hemoglobin, ethylation of DNA and administered amount of ethyl methanesulfonate in the mouse. *Mutat. Res.* **127**, 1–8.

(16) Bailey, E., Brooks, A. G. F., Dollery, C. T., Farmer, P. B., Passingham, B. J., Sleightholm, M. A., and Yates, D. W. (1988) Hydroxyethylvaline adduct formation in haemoglobin as a biological monitor of cigarette smoke intake. *Arch. Toxicol.* **62**, 247–253.

(17) Christakopoulos, A., Bergmark, E., Zorec, V., Norppa, H., Mäki-Paakkanen, J., and Osterman-Golkar, S. (1993) Monitoring occupational exposure to styrene from hemoglobin adducts and metabolites in blood. *Scand. J. Work Environ. Health* **19**, 255–263.

(18) Hambrook, J. L., Howells, D. J., and Schock, C. (1993) Biological fate of sulphur mustard (1,1′-thiobis(2-chloroethane)): uptake, distribution and retention of ^{35}S in skin and in blood after cutaneous application of ^{35}S-sulphur mustard in rat and comparison with human blood in vitro. *Xenobiotica* **23**, 537–561.

(19) Bergmark, E., Belew, M., and Osterman-Golkar, S. (1990) Separation and enrichment of alkylated globin chains as a means of improving the sensitivity of hemoglobin adduct measurement. *Acta Chem. Scand.* **44**, 630–635.

(20) Sannolo, N., Carbone, V., Ferranti, P., Fiume, I., Mamone, G., and Malorni, A. (1995) Mass spectrometric analysis of haemoglobin adducts formed by methyl bromide in vitro. *J. Chromatogr. B* **670**, 349–353.

TX9502150

[3] Unpublished results, J. P. Langenberg and H. J. van der Wiel, TNO Prins Maurits Laboratory, Rijswijk, The Netherlands.