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14. ABSTRACT
The research described in this report is focused on the development of methods for diagnosis of exposure to chemical agents. In Volume I, the development of a Standard Operating Procedure (SOP) for diagnosis of exposure to sulfur mustard is described, based on mass spectrometric analysis of the sulfur mustard-adducted tripeptide (S-(2-hydroxylethylthioethyl)-Cys-Pro-Phe), derived from albumin. The SOP for the tripeptide adduct was successfully demonstrated to a scientist of USAMRICD. The method could be set up within one day and the scientist of USAMRICD was able to perform the entire assay on his own after 2 days. Furthermore, in Volume I the mass spectrometric analysis of histidine sulfur mustard adducts is extensively described, as well as an immunoslotblot assay for detection of sulfur mustard adducts to keratin.
In Volume II (addendum), it is described how several methods for diagnosis of exposure to chemical agents (sulfur mustard, Lewisite, phosgene, nerve agents) have been improved or developed and subsequently transferred to Centers for Disease Control & Prevention. Furthermore, within this context a generic method for detection of covalently modified human butyrylcholinesterase has been developed, in order to circumvent the shortcomings of the current (specific) assays for diagnosis of exposure to OP-anticholinesterases. Finally, various reference compounds have been synthesized and delivered to CDC.

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SUMMARY

The need for retrospective detection procedures for exposure to low levels of chemical warfare agents has been urgently illustrated by the conflicts in the Gulf Area and, especially, in the attempts to clarify the Gulf War Syndrome. In Volume I of this report, the research within the current cooperative agreement (DAMD17-02-2-0012) aims at:

1. development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin and of adducts to histidine in hemoglobin and albumin (**part A, B and C** of revised statement of work).
2. development of immunoslotblot assays for quantitation of protein – sulfur mustard adduct levels, using monoclonal antibodies already available from previous research in this field (**part D** of revised statement of work).

The albumin assay (**part A**) is based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin, a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties. The tripeptide assay could be substantially shortened after development of an affinity chromatography procedure which enables work-up, digestion and mass spectrometric analysis of a plasma sample within 3 hours.

With respect to animal experiments in order to obtain information about the persistence of the albumin-sulfur mustard adduct, the level of alkylation at the free cysteine residue in rat albumin was determined to be 5%. After pronase treatment of rat albumin, isolated from rat blood exposed to [^{14}C]-labelled sulfur mustard, the tripeptide (S-HETE)Cys-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe.

Subsequently, the persistence of the various sulfur mustard adducts has been studied, in particular the adduct to the Cys-34 residue in albumin. After exposure of rats (0.3 mg/kg, i.v.), the tripeptide adduct (S-HETE)Cys-Pro-Tyr could be determined until 7 days after the exposure; the observed half-life time of sulfur mustard - alkylated rat albumin was 2 days, which is in accordance with literature values. In the corresponding globin samples of these animals, the N-terminal valine adduct could still be determined after 28 days after the exposure. Remarkably, the maximum adduct level was reached after 2-3 days, implicating the presence of intact sulfur mustard in the animal during the first 2-3 days.

Plasma samples from marmosets which had been challenged (i.v.) with sulfur mustard within the context of our previous cooperative agreement, were processed. Albumin was isolated and digested with pronase. The tripeptide (S-HETE)Cys-Pro-Phe could still be detected in samples taken 28 days after 28 days. Taken into account that the mass spectrometer (Q-TOF) used for these experiments is not the most sensitive one for this kind of analyses, the retrospectivity for this particular adduct is high.

Derivatization of (S-HETE)Cys-Pro-Phe (e.g., with pentafluorobenzyl bromide) did not result in higher sensitivy during LC tandem MS. GC-MS derivatization was not successful either. The fluorescence derivatization of the tripeptide S-(2-hydroxylethylthioethyl)-Cys-Pro-Phe (S-HETE)Cys-Pro-Phe was investigated; the 5/6-carboxyfluoresceine (FAM) derivative of the tripeptide (S-HETE)Cys-Pro-Phe was prepared. When spiked pronase digests were subjected to derivatization with FAM succinimidyl ester, the FAM-tripeptide could be detected in the digest with capillary electrophoresis – laser-induced fluorescence, albeit after prior clean-up by reversed-phase HPLC. Unfortunately, the FAM-tripeptide could not be determined in a pronase digest of albumin isolated from a highly exposed blood sample.

With regard to analysis of the histidine-sulfur mustard adducts (N1- and N3-isomers, *i.e.*, the most abundant amino acid adducts formed after exposure to sulfur mustard; **part B**), a work-up procedure was developed for rapid isolation of these adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin. This procedure consists of an ion chromatography step by using Dowex 50WX8 (Na^+ form). Subsequent elution with diluted NH_4OH afforded highly purified histidine adducts, which could be further derivatized with Fmoc-Cl for electrospray LC tandem MS analysis. Derivatization of the histidine adducts with pentafluorobenzyl bromide in DMF, in the presence of triethylamine, afforded the corresponding tris-(pentafluorobenzyl) derivatives. The derivative exhibited favorable properties for LC-tandem MS analysis. Derivatization of the histidine adducts with trifluoroacetic acid anhydride for GC-MS analysis resulted in the formation of a tris(trifluoroacetyl) derivative with favorable mass spectrometric properties. For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared. It turned out that the 5/6-carboxyfluoresceine (FAM) group gives the best results with regard to sensitivity. The FAM derivatives could be determined at a level of approximately 25 pg/ml.
Despite all these efforts, the histidine adduct method proved to be much more laborious and far less sensitive than the method based on the LC tandem MS analysis of the tripeptide (S-HETE)Cys-Pro-Phe.Therefore, the latter was selected for developing into an SOP (**Part C**). An SOP for the tripeptide assay was drafted that included the use of a deuterated internal standard. The inter-individual and intra-individual variation of the in vitro sensitivity of human blood to sulfur mustard was determined, following the albumin – tripeptide (HETE-Cys-Pro-Phe) SOP. Furthermore, the day-to-day variability was determined. The SOP for the tripeptide adduct has been demonstrated to a scientist of USAMRICD, in an independent institute. The method could be set up within one day and the scientist of USAMRICD was able to perform the entire assay on his own after 2 days. It is envisaged that the scope of this method is not limited to sulfur mustard and that it can become a generic method for diagnosis of exposure to a wide array of alkylating agents.

With regard to the development of immunoslotblot assays (**Part D**) for detection of sulfur mustard adducts to proteins, several clones are available that produce antibodies which show specificity not only for hemoglobin alkylated with sulfur mustard (50 μM) but also for alkylated keratin. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 μg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μM.

Some problems arose with respect to reproducibility of the accessibility of the sulfur mustard adducts on keratin in the immunoassay and the stability of the antibody producing hybridomas. We selected new stable hybridomas producing antibodies directed against sulfur mustard adducts to keratin isolated both from previously exposed human callus and from freshly exposed human callus. It appeared that the accessibility of the sulfur mustard adducts

decreased after exposure at concentrations above 500 μM sulfur mustard, possibly due to the high degree of crosslinking by the bi-functional agent.
An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur mustard. Phage display is a relative new method that enables researchers to quickly evaluate a huge range of potentially useful antibodies, thereby bypassing the more costly and time-consuming hybridoma technique. The Tomlinson I and J phage libraries were used to select phage antibodies exhibiting affinity for sulfur mustard adducts on keratins, isolated from human callus. Two kinds of phage-antibodies were obtained: antibodies recognizing native keratin and antibodies recognizing keratin modified by sulfur mustard. These phage antibodies retained activity after repeated culturing and culturing in larger volumes.

In Volume II of this report, it is described how several methods for diagnosis of exposure to chemical warfare agents and other toxic compounds have been improved or developed and transferred to Centers for Disease Control & Prevention.

1. The modified Edman degradation of sulfur mustard-modified hemoglobin
2. The method for diagnosis of exposure to Lewisite
3. The method for diagnosis of exposure to phosgene
4. The albumin tripeptide assay for diagnosis of exposure to sulfur mustard
5. The nonapeptide assay for determination of exposure to nerve agents, based on pepsin digestion of human butyrylcholinesterase (HuBuChE)

In order to circumvent the shortcomings of the current (specific) assays for diagnosis of exposure to OP-anticholinesterases, a generic method for detection of covalently modified HuBuChE has been developed.
Finally, various reference compounds have been synthesized and delivered to CDC.

Volume I

Low level exposure to sulfur mustard: development of a SOP for analysis of albumin adducts and of a system for non-invasive diagnosis on skin

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IV RESULTS

LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionization
CBQCA	3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde
CDC	Centers for Disease Control & Prevention
CE	capillary electrophoresis
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
DMA	N,N'-dimethylacetamide
DMF	dimethylformamide
DTT	dithiothreitol
EC APCI	Electron Capture Atmospheric Pressure Chemical Ionization
EI	electron impact
FAM	5/6-carboxyfluorescein
FAM SE	5/6-carboxyfluorescein succinimidyl ester
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethyloxycarbonyl
FPLC	fast protein liquid chromatography
HETE	2-hydroxyethylthioethyl
(S-HETE)Cys-Pro-Phe	(S-2-hydroxyethylthioethyl)Cysteine-Proline-Phenylalanine
HFBI	heptafluorobutyryl imidazole
HOBT	N-hydroxybenzotriazole
HSA	human serum albumin
LIF	laser-induced fluorescence
MRM	multiple reaction monitoring
MUP	4-methylumbelliferyl phosphate
NICI	negative ion chemical ionization
NMP	N-methyl-2-pyrrolidone
PDAM	pyrenyl diazomethane
PFB	pentafluorobenzyl
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
SOP	standard operating procedure
TFA	trifluoroacetic acid
TIS	triisopropylsilane
THF	tetrahydrofuran
TYE	tryptone yeast extract
Z	benzyloxycarbonyl

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I INTRODUCTION

The use of chemical warfare agents in the Iran-Iraq war has learned that reliable methods for identification and verification of exposure to chemical warfare agents in alleged casualties were urgently needed, and not available at that time. Furthermore, experience with the casualties in the Iran-Iraq war and with other incidents learned that biopsies or autopsies of alleged victims often become available several days or even weeks after alleged exposure. Recently, the need for retrospective detection of exposure and, even more demanding, of low level exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome". Quantitation of low level exposure provides an indispensable basis to study the subtle toxic effects of such exposures. Moreover, the application of reliable procedures to exclude that even trace exposure to chemical agents has occurred will contribute to combat readiness of the soldier.

Within the framework of previous grants (DAMD17-88-Z-8022, DAMD17-92-V-2005 and DAMD17-97-2-7002) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (Benschop, 1991; Benschop and Van der Schans, 1995; Benschop *et al*., 2000). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays and upon the development of procedures for mass spectrometric analysis of the adducts. The immunochemical assays can be performed on small samples, are highly sensitive, and can be applied "on site" when properly developed. Results obtained from the GC-MS or LC-MS-MS analyses will confirm the immunochemical results and will provide information on the structure of the adducts. In this way, it can be established whether casualties have indeed been exposed to sulfur mustard, whereas dosimetry of the exposure will be a starting point for proper treatment of the intoxication.

The main advantage of detection of adducts of sulfur mustard in proteins over those to DNA is the expected much longer half-life of protein adducts (Skipper & Tannenbaum, 1990). Whereas in DNA of human skin most of N7-(2-hydroxyethylthioethyl)-guanine (N7-HETE-Gua)[1] has been removed two days after *in vivo* exposure (Van der Schans *et al*., 1994), it is expected that adducts to proteins have life-spans varying from several weeks to months. Consequently, the retrospectivity of the diagnosis in protein adducts is superior to that in DNA. Moreover, detection is supposedly also more sensitive in case of single, protracted, and intermittent exposure to sulfur mustard at low concentrations, since the protein adducts will accumulate.

Within the framework of our previous grant DAMD17-97-2-7002 we have drafted standard operating procedures (SOPs) for the immunoslotblot (ISB) assays of sulfur mustard adduct to DNA and for the GC-NCI/MS determination of the sulfur mustard adduct to N-terminal valine in human hemoglobin. The development of the SOPs exposed some problems. For instance, with regard to the assay for GC-NCI/MS determination of the sulfur mustard to N-terminal valine in human hemoglobin, we were not able to further decrease the lowest detectable exposure level, *i.e.*, 100 nM. Nevertheless, these SOPs represent the first practically useful methods that have been validated for diagnosis of exposure to sulfur mustard which can be applied in a sophisticated field laboratory. Although the lowest detectable exposure levels of these procedures appeared to be sufficient to prove mild exposure to sulfur mustard of Iranian soldiers in blood samples taken 3 weeks after exposure (Benschop *et al*., 1997), it is self-evident that further lowering of these detection limits is needed when even lower exposure levels should be firmly established. The need for research

[1] HETE: 2-(hydroxyethyl)thioethyl

on such detection of low level exposure and effects thereof has been formulated as a high priority research goal by the Department of Defense (1999).

Explorative research within the context of our previous grant DAMD17-97-2-7002 (Benschop *et al*., 2000) has yielded important clues for development of new SOPs that satisfy the abovementioned needs. Firstly, we have lowered the minimum detectable concentration of sulfur mustard in human blood by at least one order of magnitude by mass spectrometric analysis of adducted peptides from enzymatically digested albumin (Noort *et al*., 1999). Secondly, we have found that, as in the case of hemoglobin, adducts to histidine are by far the most abundant adducts in albumin (Noort *et al*., 1997). Thirdly, we have recently succeeded to raise monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratins. The latter opens the possibility to detect *in vivo* skin exposure to sulfur mustard in a non-invasive way under operational conditions, *e.g.*, by means of immunofluorescence techniques. If these qualitative immunoassays indicate that exposure to sulfur mustard has occurred, adduct levels have to be quantified in order to estimate the extent of sulfur mustard injury.

Consequently, we decided to continue our investigations on four topics, within the framework of the current Cooperative Agreement DAMD17-02-2-0012:

1. further development of the mass spectrometric analysis of the tripeptide (S-HETE)Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard.
2. perform further research on analysis of the most abundant adduct formed after exposure of proteins to sulfur mustard, *i.e.*, N1/N3-HETE-histidine.
3. develop the most suitable procedure into a SOP.
4. develop immunochemical assays in order to quantify levels of sulfur mustard adducts to proteins.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

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

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously: Nα-Fmoc-(N1/N3-HETE)histidine (Noort *et al*., 1997), [^{14}C]sulfur mustard (Fidder *et al*., 1999) and sulfur mustard-d_8 (Fidder *et al*., 1996a)

The following commercially available products were used:
Fluorescein-5-isothiocyanate (FITC 'isomer I'), 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), 5/6-carboxyfluorescein succinimidyl ester (FAM SE) and Oregon Green 488-X, succinimidyl ester 6-isomer (Molecular Probes Europe BV, Leiden, The Netherlands). Diethanolamine, acetonitrile (Baker Chemicals, Deventer, The Netherlands); human serum albumin (HSA), pentafluorobenzyl bromide, (Fluka, Buchs, Switzerland); dl-dithiothreitol (DTT), iodoacetic acid sodium salt, TPCK trypsin, bovine serum albumin, human hemoglobin, pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31), tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel) heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-fluorenylmethylchloroformate (Fmoc-Cl), β-mercaptoethanol, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP; Novabiochem); trifluoroacetic acid, thiodiglycol (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNAse T1, protease inhibitor cocktail mini tablets, (Boehringer, Mannheim, Germany); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); FITC-labeled 'goat-anti-mouse' (Southern Biotechnology Associates, Birmingham, AL), RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat, (Campina, Eindhoven, The Netherlands). Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands).
Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA). Centrex UF-2 (3 or 10 kDa molecular weight cut-off) centrifugal ultrafilters were procured from Schleicher & Schuell (Keene, NH). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden). Dowex 50WX8 was obtained from Fluka. The Tomlinson I & J phage libraries were generous gifts from Greg Winter (Centre for Protein Engineering, Cambridge, United Kingdom). Two *Escherichia coli* strains were used in this study as hosts for bacteriophages: TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* ΔM15] and HB2151 [K-12 *ara* Δ(*lac-pro*) *thi*/F' *proA*$^+$ *B*$^+$ *lacI*^{q} *Z* ΔM15].

Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing Na_2EDTA (15 mg).
Human callus was obtained from chiropodists. Human skin resulting from cosmetic surgery was obtained from a local hospital with consent of the patient and approval of the TNO Medical Ethical Committee.

II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).
Gel filtration on Sephadex G-75 (Pharmacia) was performed with a P-1 pump, GP-250 gradient programmer, Frac-100 fraction collector, a UV-1 optical unit (254 nm) and a UV-1 control unit (Pharmacia).
Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).
Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). Protein was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). An Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

Liquid chromatography experiments were run on an ÁKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).
HPLC with radiometric detection was performed using a Gilson (Villers-le-Bel, France) HPLC system consisting of a 305 master pump, a 306 slave pump, an 805 manometric module and an 811C dynamic mixer. The mobile phase consisted of a linear gradient (0'-20') of 0.1% (v/v) trifluoroacetic acid (TFA) in water to 48% (v/v) acetonitrile and 52% (v/v) water with 0.1% (v/v) TFA. The LC flow was 1 ml/min. The eluate was monitored at 214 nm with a Spectroflow 757 UV detector (Applied Biosystems, Ramsey, NJ) and with a radiometric detector (Radiomatic, model Flo-one Beta series A 500, Meriden, CT) with Ultima-Flo (Packard, Meriden, CT) as scintillation cocktail. Liquid scintillation countings were performed with a A2500 TR scintillation counter (Packard) with Hionic Fluor (Packard) as scintillation cocktail.
LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column 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 between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Other LC/electrospray tandem MS spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s, unless stated otherwise. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 eV, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C.
LC-tandem MS experiments during the method demonstration at CDC were recorded on an API4000 triple quadrupole instrument with a standard ionspray interface (Applied Biosystems, Toronto, Canada) and a Shimadzu (Kyoto, Japan) modular liquid chromatograph. 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 C18 (Phenomenex, Torrance, CA, USA) columns (both 150 mm x 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. A gradient of solvents C (H_2O with 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 minutes. 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 minutes. 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 d_8-tripeptide) 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.

APCI-MS was performed on a TSQ7000 (Finnigan) at TNO Food and Nutrition Institute (Zeist, The Netherlands).
Fourier Transform – Ion Cyclotron Resonance (FT-ICR) MS was performed on a 11.5-T instrument (Environmental Molecular Sciences Laboratory, Richland, Washington). Sector-ion trap mass spectrometry was performed on a BE ion trap MAT900 (Thermoquest, Bremen, Germany).
GC-NCI/MS analyses were carried out with a HP 5973 mass selective detector connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The column used was a Restek RTX-5SilMS capillary column (length 30 m, i.d. 0.25 mm, film thickness 1 µm). The oven of the chromatograph was kept at 100 °C for 1.5 min, the temperature was then progammed at 25 °C/min to 270 °C. Source temperature MS: 160 °C. Injection volume was 1 µl (containing about 1% of the total sample).
^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to tetramethyl silane. The solvent signals at 2.525 ppm (residual Me_2SO-d_5 in Me_2SO-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 (Me_2SO-d_6) or 77.1 ppm ($CDCl_3$) were used as a reference for ^{13}C NMR spectroscopy.
Radioactivity countings were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was performed on a Beckman P/ACE 5000 CE instrument equipped with a Laser Induced Fluorescence detector (Fullerton, CA, USA). Excitation wavelength was 488 nm, emission wavelength 520 nm. The fused silica capillary (i.d. 75 µm) was purchased from Composite Metal Services (The Chase, Hallow, Worces., UK). The length of the capillary was 47 cm (40 cm effective

length to the detector). Sample introduction was performed by pressure injection for 20 s. Separation voltage was 20 kV. The buffer used consisted of 20 mM Borax, pH 9.
Radioactivity countings were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.

III EXPERIMENTAL PROCEDURES

III.1 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric analysis of an alkylated tripeptide in albumin

III.1.1 Incubation of human blood with sulfur mustard, [^{14}C]sulfur mustard or sulfur mustard-d_8

A 1 M solution of sulfur mustard, [^{14}C]sulfur mustard (sp. act. 15 mCi/mmol) or sulfur mustard-d_8 in CH_3CN was prepared. Subsequently, the required dilution in CH_3CN was prepared and a well-defined amount was added to human blood (2-10 mL), resulting in a 1% end concentration of CH_3CN. After incubation for 2 h at 37 °C, plasma and erythrocytes were separated by centrifugation at 3,000 rpm for 5 min.

III.1.2 Isolation of albumin from plasma by precipitation (method used in initial procedure)

Albumin was isolated from human plasma according to a procedure described by Bechtold *et al.* (1992). Thus, shortly, whole blood was collected into an EDTA-containing vacutainer and separated into red blood cells and plasma. To the plasma an equal volume of 0.5 M $CaCl_2$ was added. The mixture was incubated at room temperature overnight, or for 1 h in the shortened version of the procedure and then centrifuged at 900*g* for 20 min. To the supernatant were added 4 volumes of 0.9% saline. Nine volumes of an acid/alcohol mixture (made by adding 1 ml 12 M HCl to 600 ml ethanol) were added dropwise to the supernatant. The mixture was incubated at 37 °C for 30 min and then centrifuged at 650*g* for 5 min. To the supernatant was added a volume of 0.2 M sodium acetate in 95% ethanol equal to 1/10 the total volume of the supernatant. After 15 min the mixture was centrifuged at 650*g* for 5 min, the supernatant discarded, and the albumin pellet washed with acetone. The mixture was centrifuged at 650*g* for 5 min and the supernatant discarded. The pellet was then washed in diethyl ether, centrifuged and allowed to dry overnight. Yields: 50-60 mg/ml plasma. Analysis with SDS PAGE showed coelution with commercially available human serum albumin.

III.1.3 Pronase digestion of large amounts of albumin and LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe

To a suspension of albumin (20 mg) in aqueous NH_4HCO_3 (50 mM; 5 ml) was added a solution of pronase (10 mg/ml; 660 µl) in aqueous NH_4HCO_3 (50 mM). After incubation for 2.5 h at 37 °C, the mixture was filtrated through a filter with a cut-off of 3 kDa with centrifugation at 4,000*g*, in order to remove the enzyme. Sep-Pak C18 clean-up of the sample was performed as follows. A Sep-pak C18 cartridge (model Classic) was rinsed 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 consecutively 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 with 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. The tripeptide (S-HETE)Cys-Pro-Phe is determined by multiple reaction monitoring (MRM) of MH^+ (*m/z* 470) → *m/z* 105 which corresponds with a fragment of thiodiglycol. Operation conditions were: cone voltage 30 - 35 V, collision energy 20 eV and argon pressure 3-4.10^{-3} mB. The injection volume was 40 µl. The LC-system comprised a microcolumn with Lichrosorb RP18 material (length 0.35 m, i.d.

0.32 mm). Gradient elution using H_2O/CH_3CN 95/5 with 0.2% HCOOH and H_2O/CH_3CN 2/8 with 0.2% HCOOH as eluent A and B, respectively, was performed as follows.
0-5 min: flow 0.1 ml/min, 100% eluent A; 5-25 min: flow 0.6 ml/min, 100% A to 70% A; 25-45 min: flow 0.6 ml/min, 70% A to 0 % A. Flow rates were reduced by means of an LC Packings splitter (Amsterdam, The Netherlands) which was placed before the injection valve: 0-5 min, 2 $\rightarrow$ 10 µl/min and subsequently 10 µl/min.

III.1.4 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 100 µM sulfur mustard-d_8. Next, these samples were applied to a HiTrap Blue Sepharose column and desalted on a PD-10 column, as described in subsection III.3.1.
Pronase digestion of a sample (0.25 mL) of the purified albumin fraction (diluted with aqueous NH_4HCO_3; 50 mM; 0.5 mL), followed by LC/MS/MS analysis, showed a linear relationship between exposure level and peak ratio.

III.1.5 Derivatization of (S-HETE)Cys-Pro-Phe with pyrenyl diazomethane (PDAM)

To a solution of (S-HETE)Cys-Pro-Phe (0.7 µmol; 0.33 mg) in CH_3CN/H_2O, 1/1, v/v, was added pyrenyl diazomethane (PDAM; 0.4 mg; 1.6 umol), dissolved in CH_3CN (0.1 ml). The mixture was mixed with a Vortex and subsequently stirred for 2 h at room temperature, under the exclusion of light. After 2 h, the reaction mixture was analyzed by means of HPLC, and the chromatograms were compared with the chromatograms obtained after analysis of separate solutions of PDAM and tripeptide. The newly formed compound was isolated from the reaction mixture and analyzed by LC-MS. Electrospray MS: *m/z* MH^+ 684.2. Yield: 0.4 mg of a yellowish solid (0.6 µmol).

III.1.6 Derivatization of (S-HETE)Cys-Pro-Phe with FAM-SE, followed by CE-LIF detection

Derivatization was performed by dissolving synthetic (S-HETE)Cys-Pro-Phe (0.25 mg) in N-methylpyrollidon (100 µl). Next, DIPEA (5 µl) and 5,6-FAM-SE (10 µl of a stock solution of 200 mg/mL in DMSO) was added. After incubation for 2 h, aqueous hydroxylamine solution (1.5 M, adjusted to pH 8.5 with 5 M NaOH, diluted 1/1 with water; 200 µl) was added and the mixture was incubated for another hour.
The fluorescent derivative was purified using a Seppak C18 cartridge. First, the cartridge was equilibrated by rinsing with methanol (2 mL), followed by water (2 mL). The reaction mixture was applied, and the cartridge washed with water (4 mL). Finally the product was eluted using methanol (2 mL). HPLC showed two closely eluting peaks (5- and 6 isomer of FAM-(S-HETE)Cys-Pro-Phe); the correct mass was confirmed by ES-MS; *m/z* 828.3 (MH^+).
Quantification was performed using absorbance measuring at 494 nm at pH 8, according to the protocol of the manufacturer. Yield: 56%, uncorrected for losses for analyses.
Capillary electrophoresis experiments were performed on a Beckman P/ACE 5000 (Beckman, Fullerton, CA, USA) capillary electrophoresis instrument equipped with laser induced fluorescence detection. A fused silica capillary (i.d. 75 µm, Polymicro, Phoenix AZ, USA) was mounted in the capillary cartridge and maintained at 25 °C. The total length of the capillary was 47 cm and the effective length from inlet to detection window was 40 cm. Samples were introduced by pressure injection for 3 s at 0.5 psi. Separation voltage was 20

kV. The running buffer consisted of 100 mM Boric acid adjusted to pH 9.3 with sodium hydroxide. A concentration of 3 nM was easily detected using CE-LIF.

III.1.7 Derivatization of (S-HETE)Cys-Pro-Phe with FAM-SE in pronase digests

First, pronase digests from albumin (from 0.5 ml of plasma, isolated from blood exposed to 0 or 1 mM sulfur mustard) were purified by using the Sep-pak C18 method (vide infra, SOP). The appropriate fractions were concentrated and the residue dissolved in NMP (0.1 mL) or 0.1 M $NaHCO_3$ (0.1 ml). DIPEA (5 µL; in case of NMP as solvent) and 6-FAM SE (25 µL of a 10 mg/ml solution in DMF) was added to 10 ul of the abovementioned solution. After 2 h, the reaction was quenched with hydroxylamine solution. Half of the reaction mixture was applied to HPLC analysis; the region where FAM (S-HETE)Cys-Pro-Phe elutes was collected and analyzed by means of CE-LIF. Prior to CE-LIF analysis, samples were diluted 10 times.
In spiking experiments, pronase digests were spiked with synthetic (S-HETE)Cys-Pro-Phe (1 mg/ml) and derivatization was carried out in 0.1 M $NaHCO_3$.

III.1.8 Synthesis of (S-d_8-HETE)-Cys-Pro-Phe

To a solution of Cys-Pro-Phe (1.8 mg; 5 µmol) in CH_3CN/H_2O, 1/1, v/v; 1 ml) was added 5% $NaHCO_3$ (1.25 mL). Subsequently, a solution of d_8-sulfur mustard in CH_3CN (0.5 M; 25 µL) was added. The solution was stirred for 2 h at room temperature. HPLC analysis showed conversion into one major product. Subsequently, the solution was neutralized with 1 M aqueous HCl and the product was purified by means of reversed phase HPLC. Mass spectrometric analysis showed the expected molecular mass; no d_0-derivative could be detected. Yield: 1.8 mg (3.7 µmol; 75%) of a colourless oil.

III.1.9 Derivatization of (S-HETE)Cys-Pro-Phe for LC tandem MS analysis

Derivatization was accomplished according to Singh *et al.* (2000). Shortly, to a solution of the adduct (5 µg) in acetonitrile (50 µl) was added acetonitrile/pentafluorobenzyl bromide (19/1, v/v; 50 µl) and a solution of KOH in EtOH (8/1000, w/v; 50 µl). The mixture was heated at 60 ºC for 30 min. Subsequently, the mixture was concentrated and analyzed by LC/electrospray tandem MS on a TSQ7000 instrument.

III.1.10 Synthesis of (S-HETE)Cys-Pro-Tyr

This compound was synthesized on solid phase, in an analogous way as (S-HETE)Cys-Pro-Phe, using N-Fmoc-(S-HETE)Cys (Noort *et al*., 1995). Tandem mass spectrometric data: *m/z* 486.2 (MH^+), 469.2 (MH^+ - NH_3), 279.2 (Pro-Tyr), 137.0 ($^+S\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}OH$), 105.0 ($^+CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}OH$).

III.1.11 Determination of persistence of sulfur mustard adducts in rats exposed to sulfur mustard (i.v.)

Male Wistar WU rats (approximately 300 g) were purchased from Harlan, The Netherlands. The animals were allowed to eat and drink ad libitum. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocols for animal experiments were approved by the TNO Committee on Animal Care and Use. Rats (three animals per time point) were exposed to a dose of 0.3 mg sulfur mustard/kg

(i.v.). Sulfur mustard was diluted to a concentration of 6 mg/mL in 2-propanol. Just before injecting the rats, the sulfur mustard solution was diluted with saline to give a concentration of 0.3 mg/mL. After anesthesia with Dormicum/Hypnorm, two rats at a time were injected with this freshly prepared solution (1 mL/kg, i.v.) in the penis vein.
At the requisite time points (10 min, 1 h, 6 h, 1, 2, 3 ,7, and 28 days) after exposure, animals were killed by decapitation, blood (ca. 7 ml/rat) was collected in heparinized tubes and centrifuged (2500 rpm, 5 min) to separate plasma from erythrocytes. The plasma samples were stored at -20°C until further work-up. The erythrocytes were washed three times (2500 rpm, 5 min) with PBS and were subsequently stored at -20°C.

III.1.12 Preparation of "internal standards" for analysis of albumin and hemoglobin adducts

Rat blood (10 mL, heparinized) was incubated with sulfur mustard-d_8 in acetonitril (100 µM; final acetonitril concentration 1%) for 2 h at 37 °C. Next, plasma was separated from erythrocytes by centrifugation (2500 rpm, 5 min). The plasma was stored at -20 °C and was used as such as internal standard for analysis of the plasma samples generated throughout the animal experiments. The erythrocytes were washed three times (2500 rpm, 5 min) with PBS, and hemolyzed in water. Subsequently globin was isolated according to the procedure described by Bailey *et al*. (1987) and used as internal standard for analysis of the globin samples generated throughout the animal experiments.

III.1.13 Work-up of rat plasma samples

To rat plasma (0.5 mL) was added plasma (25 µL), isolated from rat blood, exposed to 100 µM sulfur mustard-d_8. This mixture was diluted with buffer A (2 mL, 50 mM KH_2PO_4, pH 7.0) and filtrated using a 0.45 µm filter disc in order to remove solid particles. Next, the sample was applied on a Hi Trap Blue Sepharose column (Amersham Biosciences, 1 mL, capacity 20 mg HSA/mL gel) and washed with buffer A (12 mL). Next, albumin was eluted using buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7.0). The entire wash and elution steps were monitored with a UV lamp at 280 nm. UV positive material was collected, resulting in a total volume of 3 mL. These 3 mL samples were desalted using a PD-10 desalting column (Amersham Biosciences). The PD-10 column was equilibrated using a solution of NH_4HCO_3 (50 mM, 25 mL). Next, the sample consisting of buffer B (3 mL) and albumin was applied. The albumin was eluted using 3 mL of NH_4HCO_3 solution (50 mM).

III.1.14 Pronase digestion of rat albumin, followed by LC/MS/MS analysis

Part of the above solution (0.75 mL, containing maximal 4.8 mg albumin) was digested using Pronase (100 µL, 10 mg/mL in 50 mM NH_4HCO_3) for 2 h at 37 °C. After 2 h the mixture was filtrated using a 10 kD ultrafilter. The filtrate was analyzed using Q-TOF LC-MS and LC/MS/MS for the presence of (S-HETE)Cys-Pro-Tyr and its deuterated analogue.
Conditions LC-system
Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

Time (min)	% eluent A	% eluent B	Flow (ml/min)
0	100	0	0.1
5	100	0	0.6
50	30	70	0.6

The flow of 0.6 ml/min was split before the column to 35 µl/min. Column: PepMap C18, 3 µm, 15 cm x 1 mm. Loop: 50 µl.

Conditions triple quad MS

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Tyr and (S-d_8-HETE)Cys-Pro-Tyr to the most intense fragment (HETE):

MH^+ 486.2 → 105

MH^+ 494.2 → 113

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

III.1.15 Analysis of rat globin samples according to the Standard Operating Procedure for modified Edman degradation of globin

Globin (20 mg), isolated from rat blood, was dissolved in formamide (2 ml). Subsequently, a solution of globin isolated from blood exposed to sulfur mustard-d_8 (100 µM) in formamide (20 mg/ml; 50 µl) was added. Next, pyridine (8 µl) and pentafluorophenyl isothiocyanate (8 µl) were added and the mixture was incubated at 60 °C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 × 1 ml) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jouan RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with water (2 × 0.5 ml), aqueous Na_2CO_3 (0.1 M, 0.5 ml) and water (0.5 ml). The organic layer was dried ($MgSO_4$), evaporated to dryness using the centrifugal evaporator and dissolved in toluene (100 µl).

Next, a Florisil cartridge was conditioned with methanol/dichloromethane (1/9, v/v; 2 ml) and dichloromethane (2 ml), respectively. The toluene solution was applied on the cartridge, which was subsequently washed with dichloromethane (2 ml) and methanol/dichloromethane (1/9, v/v; 1 ml). The thiohydantoin was eluted with methanol/dichloromethane (1/9, v/v; 1.5 ml). The latter eluate was evaporated to dryness and dissolved in toluene (100 µl). To this solution heptafluorobutyryl imidazole (10 µl) was added and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was washed with water (2 × 100 µl), aqueous Na_2CO_3 (0.1 M, 100 µl) and finally with water (100 µl). The toluene layer was dried ($MgSO_4$), concentrated to 30 µl and analyzed with GC-MS.

Conditions of the GC-MS were as follows:

Column: Restek 451548 Rtx-5Sil-MS, length 30 m, i.d. 0.25 mm, film thickness 1 µm. Carrier gas: helium. Oven temp.: 140 ºC (1 min) – 20 ºC/min → 260 (18 min). Inj. volume: 1 µl via autosampler. Inj. temp. 270 ºC. Inj. Mode: pulsed splitless. Pulse pressure/time 200 kPa/0.2 min. Splitless time : 1 min.

MSD transferline: 240 ºC. MS quad: 130 ºC. MS source: 160 ºC. EMV: 1576 V. MSD mode: SIM, *m/z* 564, *m/z* 572. Dwell time : 80 msec.

III.2 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric and fluorescence-based analysis of histidine adducts

III.2.1 Isolation of globin from human blood

Globin was isolated from human blood samples as described by Bailey *et al*. (1987). The red blood cells were washed four times with saline and lysed with water. After 30 min in ice/water, they were centrifuged for 30 min at 25,000*g* (4 °C). The supernatant was poured

into a stirred mixture of concentrated HCl/acetone (1/100, v/v) at -20 °C. After decanting the supernatant, the precipitate was washed with concentrated HCl/acetone (1/100, v/v), acetone and ether, and dried. For some experiments, the crude globin was purified by chromatography on a G-25 Sephadex column, using 0.1 M formic acid, 6 M urea and 50 mM dithiothreithol as an eluent. UV-positive fractions were pooled and dialyzed three times against a 1 mM phosphate buffer, pH 7. Finally, the globin was dialyzed against water for 2 h and lyophilized to give a white fluffy compound.

III.2.2 Acidic hydrolysis of globin

Globin (20 mg), isolated from human blood, was hydrolyzed in constant boiling HCl (6 M, 110 ºC, 16 h) in hydrolysis tubes. Next, the clear solution was evaporated to dryness and coevaporated several times with water.

III.2.3 Hydrazinolysis of globin

Globin (26 mg), isolated from human blood which had been exposed to 10 µM sulfur mustard, was dissolved in hydrazine monohydrate (4.5 mL) and heated at 100 ºC overnight. The resulting pink solution was concentrated under reduced pressure. Analysis with LC-MS showed the presence of both HETE-His-N_2H_4 (MH^+ 274) and HETE-His-OH (MH^+ 260).

III.2.4 Procedure for isolation of histidine adducts by means of cation-exchange chromatography, derivatization with Fmoc-Cl, and Sep-Pak C18 clean-up

A disposable syringe (10 mL size) was filled with Dowex WX8 resin (Na^+ form; 3 mL) and the resin was washed with aqueous acetic acid (15 mL; pH 5.5). A solution of globin acidic hydrolysate (from 3 mg of globin; coevaporated several times with water, to evaporate excess HCl) in aqueous acetic acid (1 mL; pH 5.5) was applied to the column. The column was washed with 15 mL aqueous acetic acid (pH 5.5) in order to remove uncharged and weakly basic amino acids. Subsequently, the column was washed with water (10 mL) to remove acetic acid. Finally, N1/N3-HETE-histidine and other basic amino acids were removed from the column using aqueous NH_4OH (15 mL, pH 10.5). The latter eluate was evaporated to dryness. Fmoc derivatization and Seppak C18 purification were performed as described earlier (Noort *et al*., 1997).

III.2.5 Synthesis of N1- and N3-HETE-L-histidine

These compounds were synthesized according to the procedure described in the final report of Cooperative Agreement DAMD17-92-V-2005.
The analytical data were in full agreement with earlier obtained data.

III.2.6 Derivatization procedures for GC-MS analysis of histidine adducts

Derivatization of N1/N3-HETE-histidine with trifluoroacetic anhydride and GC-MS analysis of the derivative
N1/N3 HETE-histidine (1.0-10 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure, ethyl acetate (1 ml) and trifluoroacetic anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15

min-90 min and evaporated under reduced pressure. GC-MS analysis showed that a derivative with 3 trifluoroacetyl groups had been formed.
GC-NICI-MS: *m/z* 561 (HETE-His-OMe.3 TFA). GC-EI-MS: 141 (CF_3-C(O)-O-$C_2H_4^+$), 201 (CF_3-C(O)-O-C_2H_4-S-$C_2H_4^+$)
detection limit: 3 pg

Derivatization of HETE-histidine with heptafluorobutyric anhydride and GC-MS analysis of the derivative
N3-HETE-histidine (1.0 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure ethyl acetate (1 ml) and heptafluorobutyric anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15 min and evaporated under reduced pressure.
GC-NICI-MS: *m/z* 665 (HETE-His-OMe.2 HFB)
note: N1 HETE-histidine was also derivatisized with HFBA, but the desired adduct could not be detected.

Derivatization of N3-HETE-histidine with PFPA and GC-MS analysis of the derivative
N3-HETE-histidine (1.0 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure ethyl acetate (1 ml) and pentafluoropropionic anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15 min and evaporated under reduced pressure.
GC-NICI-MS: *m/z* 565 (HETE-His-OMe.2 PFP).

Derivatization of N1-HETE-histidine methyl ester with hydrazine
Boc-N1-HETE (OtBu)-His-OMe (18.3 mg) was dissolved in TFA/H_2O (95/5, v/v; 2 ml) and left at room temperature for 2 h. The solution was concentrated under reduced pressure and the residue was coevaporated with ethanol (2x 2 ml). The residue was dissolved in isopropanol (210 µl) and hydrazine monohydrate (36 µl) was added. After standing at room temperature overnight the solution was concentrated under reduced pressure and the residue was coevaporated with methanol (2x 2 ml). LC-MS: *m/z* (MH^+) 274.1.

Derivatization of N1-HETE-histidine hydrazide
N1-HETE-histidine hydrazide was dissolved in THF (1 mg/ml). To 200 µl of this solution trifluoroacetic anhydride, pentafluoropropionic anhydride or N-methyl-bis-trifluoroacetamide (60 ul) was added and the mixture was heated at 110 °C for 15 min. The mixtures were concentrated under reduced pressure. MS analysis showed that HETE-histidine-hydrazide was very reactive towards THF; only THF adducts could be detected.

III.2.7 Fluorescence derivatization of histidine adducts

FITC derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To an aqueous solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.5 ml; 3.6 mM) was added aqueous Na_2CO_3 (0.5 ml; 0.2 M) and a solution of fluorescein-5-isothiocyanate (FITC 'isomer I') in ethanol (1 ml; 2.5 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 4 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC,

affording yellow-orange solids. Electrospray MS: *m/z* 649 (MH^+), 325 (MH_2^{2+}) for both derivatives.
The N1- and N3-(2-hydroxyethylthioethyl)histidine FITC derivatives had slightly different retention times.
In aqueous solution, the compounds slowly (16 h at room temperature) rearranged into compounds with *m/z* 631 (MH^+; probably the corresponding thiohydantoins), which had a slightly different retention time.

CBQCA derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To an aqueous solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.65 ml; 2.9 mM) was added a solution of 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) in methanol/0.2 M aqueous KOH, 80/1, v/v (0.65 ml; 10 mM) and an aqueous KCN solution (1.3 ml; 20 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 3 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC, affording dark pink solids. Yield for the N3 adduct: 0.4 mg; 0.7 µmol; 38% based on histidine derivative. Electrospray MS: *m/z* 556 (MH^+), 452 (MH^+ - $HOCH_2CH_2SCH_2CH_2^+$).

Oregon Green 488-X derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To a solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.9 µmol) in aqueous $NaHCO_3$ (2 ml; 0.2 M) was added a solution of Oregon Green 488-X, succinimidyl ester 6-isomer in ethanol (1 ml; 2 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 4 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC, affording orange solids. Electrospray MS: *m/z* 789 (MNa^+), 767 (MH^+), 384 (MH_2^{2+}).

FAM derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To a solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (1 mg) in aqueous $NaHCO_3$ (1 ml, 0.1 M, pH 8.3) was added a solution of 5- and 6-carboxyfluorescein succinimidyl ester in DMF (150 µl, 42 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 5 h, HPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were purified by elution on Seppak C18.
Yield: 0.23 mg N3 isomer and 0.39 mg N1 isomer (based on ^{1}H-NMR spectroscopy).
Electrospray MS: *m/z* 309.6 (MH_2^{2+}), 618.1 (MH^+) for both isomers.

III.2.8 Isolation of N1- and N3-HETE-L-histidine by reversed-phase HPLC

In order to isolate the N1/N3-HETE-histidine adduct, solutions of globin (isolated from blood exposed to 0 and 10 mM sulfur mustard) were prepared in 6 M HCl (10 mg/mL). The globin solutions were subjected to acidic hydrolysis (16 h, 110 ºC) and the resulting amino acid mixtures were used to set up isolation procedures based on reversed-phase HPLC.

An acidic hydrolysate (20 mg of blank globin in 6 M HCl (2 mL), spiked with N1/N3-HETE-histidine, both 100 microgram) was neutralized with NH_4OH (concentrated, 1.5 mL). Part (1 mL) was purified using a preparative C18 column (250 mm x 10 mm; 5 u; Alltima).
A low gradient profile was applied in order to separate the N1/N3-HETE-His from the other amino acids in the acidic hydrolysate. Flow rate: 3.3 mL/min. Buffers used: A = 0.1% TFA in H_2O, B = 0.1% TFA in 80% CH_3CN. First, the column was eluted with 0% B for 5 min; next a gradient was applied to 50% B in 20 min and finally the column was washed with 100 % B and equilibrated again to 0 % A with 10 mL of A buffer. N1- and N3-HETE-HIS eluted at about 15 min using this system.
Fractions were collected and analyzed by LC/MS with and without derivatization with Fmoc-chloride. MS confirmed the presence of the adducts in the collected fractions.
Next, globin, isolated from blood exposed to 50 mM sulfur mustard was used to test this isolation procedure. Again, the hydrolysate was fractionated on a preparative Alltima C18 column and the collected fraction was lyophilized to dryness and taken into water (100 µl). Part (10 µl) was used for LC-MS analysis, part (45 µl) was used for FMOC derivatization and part (45 µl) was used for derivatization with TFAA (GC-MS analysis).

III.2.9 Derivatization of acidic hydrolysates with Fmoc-Cl for LC tandem MS analysis

Derivatization with Fmoc-Cl was performed as described earlier (Noort *et al*., 1997).

III.2.10 Derivatization of acidic hydrolysates with TFAA for GC-MS analysis

Lyophilized hydrolysate (45 µl) was taken up in HCl/methanol (2 mL, 2 M) and heated at 80 ºC. The sample was dried and coevaporated with ethyl acetate (0.5 mL). Next, ethyl acetate (1 mL) and trifluoroacetic anhydride (350 µl) were added and the mixture heated at 110 ºC for 30 min. The samples were dried and dissolved in toluene (100 µl) and analyzed with GC/MS.

III.3 Development of Standard Operating Procedures for analysis of sulfur mustard adducts to proteins

III.3.1 Standard Operating Procedure for albumin – tripeptide assay

The plasma sample (0.5 mL) of interest was spiked with plasma (25-50 µL), isolated from blood exposed to 1 - 100 µM d_8-sulfur mustard. The sample was diluted with buffer A (50 mM KH_2PO_4, pH 7; 2 ml), filtrated over a filter disk (0.45 µm), applied on a HiTrap™ Blue HP cartridge (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) that had been equilibrated with buffer A (10 mL). The cartridge was eluted with buffer A (10 mL) by means of a syringe, within one minute. Subsequently, the cartridge was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 3 mL). The latter effluent was collected. The HiTrap column can be regenerated by washing with buffer B (10 ml) and subsequently with buffer A (10 mL). Next, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (3 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 3 mL). The effluent was collected.

Part of the purified albumin fraction (0.75 ml) was used and subsequently Pronase was added (100 µl of a freshly prepared solution (10 mg/ml) in 50 mM NH_4HCO_3), followed by incubation for 2 h at 37 ºC. The digests were filtrated through molecular weight cut-off filters

(10 kD) under centrifugation at 2772 g in order to remove the enzyme. The filtrate was analyzed by means of LC/MS/MS.

For low exposure levels, the filtrate (after 10 kD filtration) was processed Seppak C18 cartridge. To this end, the cartridge was conditioned with methanol (5 mL), followed by 0.1% TFA/water (5 mL). The pronase digest was applied, and washed with water (2 mL), containing 0.1 % TFA, 10 % CH_3CN/water (2 mL,0.1 % TFA), 20 % CH_3CN/water (2 mL,0.1 % TFA), and finally eluted with 40% CH_3CN/water (2 mL,0.1 % TFA). The eluate was evaporated to dryness, redissolved in water (0.1 % TFA, 50 µl and analyzed with LC/MS/MS.

Conditions LC-system

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

Time (min)	% eluent A	% eluent B	Flow (ml/min)
0	100	0	0.1
5	100	0	0.6
50	30	70	0.6

The flow of 0.6 ml/min was split before the column to 35 µl/min. Column: PepMap C18, 3 µm, 15 cm x 1 mm. Loop: 50 µl.

Conditions triple quad MS

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Phe and (S-d_8-HETE)Cys-Pro-Phe to the most intense fragment (HETE):

MH^+ 470.2 → 105

MH^+ 478.2 → 113

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

III.3.2 Determination of interindividual variation

Blood was taken from volunteers (with informed consent; 10 persons) and exposed to sulfur mustard (0.1 µM), as described above. After incubation for two hours at 37ºC, the plasma layer was separated from the erythrocytes. From each of the abovementioned plasma samples, 0.5 mL was mixed with 25 µl of "internal standard" plasma (25 µl; isolated from blood that had been exposed to sulfur mustard-d_8, resulting in a virtual exposure level of approximately 1 µM sulfur mustard-d_8. Each plasma sample was further processed according to the SOP.

III.3.3 Determination of intraindividual variation

Blood was taken from volunteers (3), three times, at three different time points (3 months between). Blood samples were exposed to sulfur mustard (0.1 µM) and further processed as desribed in the SOP.

III.3.4 Determination of day-to-day variability of SOP

A single blood sample was exposed to sulfur mustard (0.1 µM); plasma was isolated. At four different time points (within a time period of 6 months), aliquots of the plasma sample were processed according to the SOP.

III.3.5 Demonstration of SOP to USAMRICD scientist

The demonstration was carried out in April 2004. For details, see Appendix 1.

III.4 Development of immunoslotblot assays for quantitative analysis of sulfur mustard adducts to proteins

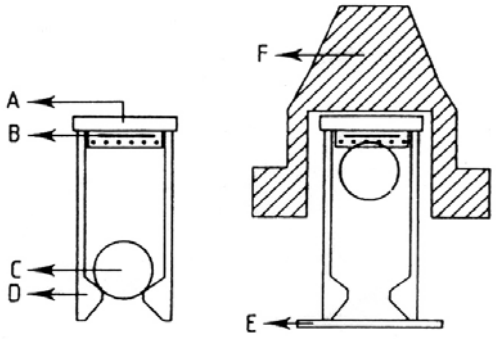


Figure 1. Schematic diagram of the device for skin exposure to air saturated with sulfur mustard vapor. At the inner side of the plastic cap (A) a piece of filter paper (B) was placed, onto which 3 µl of liquid sulfur mustard was applied. The bottom side of the glass cylinder was closed by a stainless steel ball (C). When the air in the cylinder (a volume of ca. 2 ml) had become saturated with sulfur mustard vapor (at 28 °C: ca. 1100 mg/m^3), the cylinder was placed onto the skin (E) and the ball was lifted with a magnet (F) for the desired period of time.

III.4.1 Exposure of human skin to saturated sulfur mustard vapor and extraction of epidermal keratins

Human skin samples (9 × 0.25 cm^2) were exposed to saturated sulfur mustard vapor (0, 2, 4 or 8 min) by using the device represented in Figure 1. The estimated sulfur mustard concentration at 28 °C close to the skin was 1100 mg.m^{-3}. After the exposure, the epidermis was separated from the dermis by heat shock (2 min at 60 ºC, followed by 5 min at 0 ºC). The epidermis was cut into 3 or 4 pieces, and transferred into a 4 ml vial. Next, low salt buffer (10 mM Tris/150 mM NaCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml), containing a protease inhibitors cocktail (1 tablet/12 ml buffer), was added. The mixture was shaken for 1 h at 0 ºC. The epidermis was sedimented, the upper layer was discarded and replaced by high salt buffer (10 mM Tris/150 mM NaCl/1.5 M KCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). The mixture was shaken for 1 h at 0 ºC. Next, the epidermis was sedimented; the upper layer was discarded and replaced by washing buffer (10 mM Tris/150 mM NaCl/3 mM EDTA; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). After shaking for 30 min at 4 ºC, the epidermis was sedimented. The liquid layer was discarded and the epidermis was extracted with lysis buffer (20 mM Tris/1 mM EDTA/2% SDS/1 mM DTT; pH 7.4, 2 ml), containing protease inhibitors, under gentle shaking overnight at room temperature. The mixture was

centrifuged at 5,000*g* for 5 min and the supernatant containing the keratins (0.87 mg/ml) was stored at –70 ºC. Before treatment with 0.5 M NaOH, the keratin solutions were dialyzed against H_2O in a Slyde-A-Lyzer cassette (size: 0.1-0.5 ml).

III.4.2 Preparation of skin cryostat sections

After exposure, a piece of the skin was cut from the central part of the treated area and fixed in methanol/acetic acid (3/1 v/v, 1.5 h at 4 °C), rehydrated by incubation overnight in 70% ethanol at 4 °C, followed by incubation in 5% sucrose at 4 °C for 1.5 h. Next, the pieces were stretched between microscope slides and stored at –20 °C.
Alternatively, the piece of skin was immediately stretched between microscope slides, without fixation, and stored at -20 °C.
For the preparation of cryostat sections, a small piece of skin was embedded in Tissue Tek (O.C.T. compound, Miles Inc., Elkhart, USA). Subsequently, cryostat sections (5 mm thickness) were prepared at -35 °C with a cryostat microtome (2800 Frigocut, Rechert-Jung, Leica, Rijswijk, The Netherlands) on slides precoated with a solution of 3-aminopropyl tri-ethoxysilane (2% in acetone). The slides were stored at room temperature. In the case of non-fixated skin, the cross-sections were fixated with 70% ethanol, washed with TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.4) and stored at room remperature.

III.4.3 Immunofluorescence microscopy of skin cryostat sections

The preparation of skin cryostat sections has been described in Subsection III.4.2. Immunofluorescence microscopy of sulfur mustard-keratin adducts in skin sections was performed analogously to the procedure described for detection of N7-HETE-Gua (Benschop *et al*., 2000). Briefly, the following procedure was applied after fixation of the skin section with 70% ethanol on aminoalkylsilane-precoated slides and washing with TBS:

- precoating with TBS + 5% milkpowder (30 min at room temperature);
- treatment with antibody specific for sulfur mustard-exposed keratin; supernatants of up to 32 selected monoclonal antibodies in a 1:1 dilution in TBS containing 0.05% Tween 20 and 0.5% gelatin (overnight at 4 °C);
- treatment with a second antibody, FITC-labeled ‘goat-anti-mouse’, 75-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a LSM-41 laser scanning microscope. The fluorescence of the FITC group above the stratum corneum and of the propidium iodide were measured consecutively to visualize the presence of sulfur mustard-keratin adducts in the stratum corneum and the DNA in the nuclei. The fluorescein staining was used to determine the presence of sulfur mustard-keratin. Adduct levels were estimated from the brightness of the fluorescence above the stratum corneum. The second image, from the propidium iodide staining, served to localize nuclei on the image.

III.4.4 Isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residue was stirred in a buffer (5 ml; pH 7.4)

containing Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residue was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M).
The crude keratin was purified on a G 75 column (100 × 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized, affording 20 mg crude keratin/100 mg callus. In spite of the dialysis step it appeared that the protein content was only 7-22%.
Because the latter procedure resulted in a precipitate upon dialysis, particularly in the case of keratin containing sulfur mustard adducts, several other keratin-purification procedures were applied (see Results section). It appeared that precipitation could be avoided when, before dialysis, the keratin fractions were incubated at 65 °C for 10 min. However, this procedure did not improve the accessibility of the sulfur mustard adducts on keratin.
Positive results were obtained when the crude keratin, in 0.5-ml aliquots, was purified through a PD10 column (Sephadex G-25, Amersham), followed by desalting and concentrating of the pooled appropriate fractions over a Centrex UF-2 filter (molecular cut-off 10 kDa, KD10, 1-ml aliquots, each followed by two 1-ml washings with water). Representative concentrations: 20-30 mg keratin/ml. Effects of still remaining salt and SDS could be eliminated by the strong dilution in PBS before application in the immunoassay.

III.4.5 Bio-Rad assays for protein content

For the assessment of the protein content originally the Bio-Rad assay as described previously (Benschop and Van der Schans, 1995) was applied. However, it appeared that the keratin solutions obtained as described in the previous section still contain components which interfere with this assay. Therefore, in those cases the so-called RC DC protein assay (Bio-Rad) was applied according to the manufacturer. Briefly, 125 µl of RC reagent I was added to 25 µl of sample (or standard protein solution) and incubated for 1 min at room temperature. Then 125 µl of RC reagent II was added, followed by centrifugation for 3-5 min at 15,000g. Supernatant was discarded and 127 µl of reagent A' was added and incubated at room temperature for 5 min until the precipitate was completely dissolved. Next, 1 ml of DC reagent B was added and the mixture was incubated for 15 min at room temperature. Then, absorbance was read at 750 nm.

III.4.6 Exposure of human callus to sulfur mustard

To a suspension of human callus (70-100 mg) in 0.9% NaCl (100 µl) was added a solution of an appropriate concentration of sulfur mustard in isopropanol (100 µl). The mixture was incubated for 6 h at 37°C. Isolation of keratin was performed as described in Subsection III.4.3.

III.4.7 Immunoassays (ELISA) with hybridoma-supernatants

As described under 'Results' the screening of hybridoma supernatants was subject to detailed investigation. So far the following procedures yielded optimal results:
The hybridoma-supernatants were tested in a direct ELISA against globin isolated from human blood treated with sulfur mustard (0, 50, 100, 500 µM). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted globin dissolved in PBS to a final concentration of 10-12.5 µg/ml. Of these

dilutions 50 µl was added per well and incubated 1 h at room temperature. The plates were washed three times with PBS. Next, the plates were incubated with PBS containing 2% FCS for 30 min at room temperature. After emptying the plates, the hybridoma supernatants were added, diluted 100-500 times in PBS with 0.05% Tween 20 and 1% FCS. Of these dilutions 50 µl was added per well and incubated for 30 min at room temperature. After washing with PBS containing 0.05% Tween 20 (three times), the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 500-2000 times in PBS containing 0.05% Tween 20, and 1% FCS, was added (50 µl/well) and the plates were incubated for 60 min at room temperature. After four washings with PBS containing 0.05% Tween 20, a solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 30 min.
For the screening of supernatants in a direct ELISA against sulfur mustard adducts to keratin the same procedure was applied, with the following modifications. The plates were coated with keratin isolated from human callus treated with sulfur mustard (0 or 50 µM), dissolved in PBS to a final concentration of 5 µg/ml. After washing with PBS, the plates were incubated with PBS containing 2% FCS, followed by washing with PBS containing 0.05% Tween 20 (three times). Hybridoma supernatants were screened in a 1:5 dilution. Further processing was the same as described above for plates coated with globin.

III.4.8 Immunoslotblot procedure for the detection of sulfur mustard adducts to keratin

Essentially, the procedure developed for the detection of sulfur mustard adducts to DNA (Benschop *et al*., 2000) was applied. Briefly, a. solution of keratin (200 µl, 2.5 µg/ml PBS) isolated from a human callus sample exposed to sulfur mustard was spotted on a nitrocellulose filter. All samples were blotted in triplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the keratin was immobilized by UV-crosslinking (50 mJ/cm^2). Next, the filter was incubated with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min and washed three times with PBS + 0.1% Tween 20. Next, the filter was incubated with 1st antibody (1H10, directed against sulfur mustard adducts to human keratin) diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each. Next, the filter was incubated with 2nd antibody (directed against the 1st antibody) diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each.
Finally, a 1:100 mixture of solution B with solution A (of the Enhanced Chemiluminescence Blotting Detection System) was preincubated in a water bath for at least 30 min at 25 °C. Then, free (wash) solution was removed from the filter with filter paper and position A12 and H1 marked with ball point (not a felt pen!). The filter was placed in a closely fitting box. After addition of substrate solution (10 ml) incubation was performed for 1 min.
Next, the filter was wrapped in plastic (straight from the substrate solution) without air bubbles. Liquid was pressed out, the filter was transferred in plastic into a luminometer cassette and placed in the luminometer. Luminescence was measured according to the required program. For each sample the mean luminescence of triplicate samples was calculated.

III.5. Production of new monoclonal antibodies directed against sulfur mustard adducts to keratin

Because earlier antibodies directed against sulfur mustard adducts to keratin, including the 1H10, seemed to have lost their specificity, we decided to produce new antibodies. Because some modifications were applied the updated procedures have been described below. In addition, a new procedure, the phage display assay, was applied.

III.5.1. Solid phase synthesis of peptides containing an Nω-HETE-glutamine or Nω-HETE-asparagine residue

The following peptides containing modified asparagine or glutamine residues were synthesized as described earlier (Benschop *et al* 2000):

1. G-V-V-S-T-H-(Nω-HETE)Q-Q-V-L-R-T-K-N-K
2. G-I-Q-(Nω-HETE)Q-V-T-V-N-Q-S-L-L-T-P-L-N-K
3. G-V-M-(Nω-HETE)N-V-H-D-G-K-V-V-S-T-H-E-K

III.5.2 Immunization of mice for generation of antibodies against synthesized haptens

Four mice were immunized (*s.c.*) with 100 µl of a mix of the three antigens (about 1 mg/ml) and stimune in a ratio of 1:1.25. Blood samples of all mice were taken after 7 days to test the serum for antibody response against keratin treated with 0.05 or 1 mM sulfur mustard, with a direct ELISA. A positive response was not observed against sulfur (*s.c.*) mustard treated proteins after 21 days. Therefore, the mice received a second immunization with the same mix of haptens at 7 weeks after the first immunization. After the second immunization still no positive response was observed against sulfur mustard treated proteins. However, there was a positive response against each of the three adducted peptides used for immunization in comparison to the corresponding non-adducted peptides. A booster with antigen (volume up to 0.2 ml) was administered (*i.p.*, according to the manufacturer of stimune) 4 weeks later to two mice, one without and one with stimune. After 3 days the animals were killed with CO_2 anesthesia and the blood was collected by heart puncture. A cell suspension of the spleen was prepared for the production of hybrid cell strains.
The other two mice received a booster, without stimune, after another 2 months and were used for spleen cell collection for the production of hybrid cell strains.
The whole procedure was repeated with another four mice but immunization was carried out with keratin isolated from human callus exposed to 1 mM sulfur mustard.

III.5.3 Production of hybrid cell strains

The spleen cells of the mouse were isolated for fusion with SP2/0 plasmacytoma cells. The SP2/0 plasmacytoma cells were grown in RPMI1640-medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 mM ß-mercaptoethanol. Spleen cells and SP2/0 cells were washed twice in RPMI-medium without serum. Next, 10^8 spleen cells were added to 10^7 SP2/0 cells and centrifuged (20 min at 50*g*). The supernatant was removed and the cells were exposed to fusion conditions by brief consecutive incubations of a mixture of these cells in 41% and 25% PEG 4000 as follows. The cell pellets were resuspended for 1 min in 41% PEG-solution (0.5 ml). Next, 25% PEG-solution (0.5 ml) was added and the mixture was shaken slowly for 1 min. RPMI-medium without serum

(4 ml) was added twice and the cell suspension was shaken slowly for 2 min. The cell suspension was incubated for 15-30 min at room temperature and then centrifuged (20 min at 50*g*). The supernatant was removed and the pellet resuspended in RPMI-medium with 10% FCS. The cells were seeded in a 75-cm^2 culture flask and incubated overnight. After 24 h of incubation, the cells were centrifuged (20 min at 10*g*) and were resuspended in complete RPMI-medium (38 ml; the same medium as used for growing of SP2/0 cells) supplemented with HAT-medium, *i.e.*, 0.1 mM hypoxanthine, 16 mM thymidine, and 0.4 mM aminopterine. Hybridomas were selected in HAT-medium because they can grow in this medium whereas SP2/0 cells do not survive; spleen cells cannot be cultured. The cells were seeded in 96-well polystyrene culture plates in HAT-medium. Hybrid cells were cultured and refreshed in this selective HAT-medium and their supernatants were screened for specific antibody production in a direct ELISA. Cells producing specific antibodies against sulfur mustard treated proteins were recloned twice by limiting dilution as will be described in the next subsection.

III.5.4 Cloning of hybridomas by limiting dilution

Cells of the fusion mixture producing specific antibodies against sulfur mustard treated protein (keratin) were counted by light-microscopy and diluted in HAT-medium to a concentration of 50, 10 and 5 cells/ml. Per well of 96-well culture plates, 0.1 ml of one of these solutions was added resulting in 5, 1 and 0.5 cell/well. The plates were incubated for eight days without refreshing the medium. Subsequently, the amount of clones per well was counted. The supernatants of wells with only one clone were tested for specific antibody activity against sulfur mustard treated hemoglobin. Clones showing a positive result were recloned once again by limiting dilution to make sure that monoclonal antibodies would be obtained.

III.5.5 Immunoassays (ELISA) with the polyclonal antisera and hybridoma-supernatants

The polyclonal antisera and hybridoma-supernatants were tested in a direct ELISA against keratin isolated from human callus treated with sulfur mustard (0, 50, 1000 µM) and against the immunogen itself (if available in sufficient amounts). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted keratin or peptides dissolved in PBS to a final concentration of 5 µg/ml. Of these dilutions 50 µl was added per well and incubated for 45 min at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20. (The plates were not incubated with PBS containing 1% FCS). The polyclonal antisera and the hybridoma supernatants were diluted 10-1,000 times and 5-100 times, respectively, in PBS with 0.05% Tween 20 and 1% FCS. Of these dilutions 50 µl was added per well and incubated for 60 min at 37 °C. After washing, the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 1:1,000 in PBS containing 0.05% Tween 20, 0.5% gelatin, and 5% FCS, was added (50 µl/well) and the plates were incubated for 60 min at 37 °C. After three washings with PBS containing 0.05% Tween 20, the plates were washed once with 0.1 M diethanolamine, pH 9.8 (100 µl). A solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 1 h.

III.5.6. Production of new monoclonal antibodies directed against sulfur mustard adducts to keratin by phage display

As alternative for the selection of sulfur mustard-keratin-adduct specific antibodies we applied the so-called phage display technique instead of the classical hybridoma technique.

Bacterial strains and growth conditions.
Two *Escherichia coli* strains were used in this study as hosts for bacteriophages: TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5/*F' *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* ΔM15] and HB2151 [K-12 *ara* Δ(*lac-pro*) *thi*/F' *proA*$^+$ *B*$^+$ *lacI*^{q} *Z* ΔM15]. Both strains were grown on tryptone yeast extract (TYE) plates (containing 1% glucose and antibiotics when required. Cultures were grown in 2× TYE broth (2× TY).

Preparation of antigens.
Keratin was isolated from human callus exposed to various concentrations of sulfur mustard up to 1 mM as described above.

Phage display library.
The Tomlinson I & J libraries (a generous gift from Greg Winter, Centre for Protein Engineering, Cambridge, United Kingdom) were used to select phage antibodies. The Tomlinson I & J libraries each comprise over 100 million different single chain antibody variable region fragments scFv. For general information and references on phage display of antibodies see Winter *et al.* (1994).

Rescue of the phage library.
Rescue of the libraries was performed by inoculating 10^{10} PFU of both I and J libraries in strain TG1 in 2× TY broth (TY, 200 ml) containing glucose (Glu, 1%) and ampicillin (Amp, 100 µg/ml) (2× TY+G+Amp) and incubated at 37 °C with shaking (at 250 rpm) until the optical density at 600 nm (OD_{600}) was approximately 0.4. Fifty milliliters of this culture was infected with KM13 helper phage ($2x10^{11}$) and incubated at 37 °C for 30 min. The infected cells were collected by centrifugation (3000 × *g* for 10 min) and resuspended in 2× TY (100 ml) containing glucose (0.1%), Amp (100 µg/ml) and kanamycin (Kan, 50 µg/ml) (2× TY-0.1% Glu+Amp+Kan). Subsequently, the cells were incubated overnight at 30 °C and with shaking at 250 rpm. Bacterial cells were removed by centrifugation at 3,300 × *g* for 30 min., and phages present in the supernatant were precipitated with 1/5 v/v 20% polyethylene glycol 6000 (1/5 v/v 20%), NaCl (2.5 M) (PEG/NaCl) at icewater for at least 1 hr. After centrifugation at 3,300 *g* for 30 min the pellet was resuspended in PBS (1/50 v/v) and centrifuged again at 11,600 *g* for 10 min to remove bacterial debris. The final concentration of the phage was in the range of 10^{12} -10^{-13}/ml.

Selection procedure.
Maxisorp immunotubes (5.0 ml; Nunc, Roskilde, Denmark) were coated with 4 ml of antigen *i.e.* unexposed keratin and keratin exposed to 1 mM sulfur mustard in PBS, at a concentration of 5 µg/ml, at 4°C or 16 h. Subsequently, the immunotubes were blocked with PBS containing 2% skimmed milk (MPBS) for 2 h at room temperature (RT) and washed three times with PBS. The phage library, about $10^{12\text{-}13}$ phages in 4 ml MPBS, was added, and the tubes were

incubated on a turntable for 60 min at RT, followed by a standing incubation for 60 min at RT. For subtractive selection of phages showing affinity for keratin exposed to 250µM sulfur mustard, the phage library was first added to immunotubes coated with 5 µg/ml untreated keratin at 4°C for 16 h. After incubation, the library was transferred to an immunotube, which was coated with keratin exposed to 250µM sulfur mustard, 5µg/ml, at 4°C for 16 h. After incubation, the tubes were washed 20 times with PBS containing 0.05% Tween 20 (PBST). Subsequently, while being rotated on a under-and over turntable bound phages were eluted in 500 µl 1 mg/ml trypsin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) at RT for 10 min. To determine the number of eluted phages, *E. coli*, grown to an OD_{600} of 0,4 was infected for 30 min at 37°C and cells were plated in a serial dilution on TYE+Amp+G plates and grown at 16 h at 37°C. This selection procedure was repeated for another two rounds. In the second and third round 2% Bovine Serum Albumin (BSA; Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) and 2% MPBS were used as blocking agent, respectively.

Production of monoclonal phage antibodies.

After the third and final round of selection, individual colonies were inoculated into 100 µl 2xTY+G+Amp and grown at 37 °C for 16 hr. Subsequently an inoculum of 5µl was transferred to 200 µl fresh 2xTY+G+Amp and bacteria were grown to an early log-phase. Then, 25 µl 2xTY+G+Amp containing 10^9 trypsin-sensitive helper phage KM13 was added to each well. After an incubation at 37 °C for 1 h, with shaking at 250 rpm, cells were collected by centrifugation at 1,800 *g* for 10 min and resuspended in 200 µl 2xTY+Amp+Kan, and grown overnight at 30 °C at 250 rpm. These culture supernatants were contained with monoclonal phage antibodies (MoPhAbs).

For the production of larger quantities of MoPhAbs the pellet was resuspended in 50 ml 2xTY+Amp+Kan and cells were grown at 30 °C for 16 h. Then, the cells were pelleted by centrifugation at 3,300 *g* for 15 min. The supernatant was mixed with 1/5 v/v PEG/NaCl and by an incubation on ice water for 2 h. The precipitated MoPhAbs were pelleted by centrifugation at 3,300 *g* for 30 min. The pellet was resuspended in 2 ml PBS and stored at 4 °C. For long term storage at -70 °C glycerol (15% end concentration) was added.

Expression of soluble antibody fragments.

For expression of soluble antibody fragments (single chain variable fragments, scFvs) the *E. coli* nonsuppressor strain, HB2151, was infected with phages as described for TG1. Individual HB2151 colonies were transferred into 150 µl of 2×TY+Amp-G. Plates were incubated at 37 °C, with shaking at 250 rpm, until the OD_{600} of the cells was about 0.9. For induction of the scFvs Isopropyl-β-D-thiogalactopyranoside (IPTG) (Eurogentec, Seraing, Belgium) was added to a final concentration of 1 mM. Plates were incubated for 16 to 24 h at 30 °C (with shaking at 250 rpm). Bacteria were centrifuged at 1,800 × *g* for 10 min and cell supernatants were collected for further use.

Screening MoPhAbs and scFvs by ELISA.

High affinity microtiter plates (Costar, Corning, USA) were coated with 100 µl antigen (5µg/ml) as described above for the coating of the immunotubes. Before incubation with the MoPhAbs or scFvs the plates were washed three times with PBS and blocked with 2% MPBS for 2 h at RT. Culture supernatants of superinfected TG1 cells containing MoPhAbs and supernatants containing scFvs were used in 2% MPBS and incubated for 60 min at RT. Plates were washed three times with PBS containing 0.05% Tween 20. MoPhAbs were detected by using the Detection Module Recombinant Phage Antibody system (Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations, using an HRP-anti-M13 as

second antibody, which is specific for the viral major coat protein VIII. Binding of soluble antibody fragments was detected with HRP-anti-Protein A (ICN Biomedicals, Inc., Aurora, U.S.A) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as substrate. Emission was measured at 415nm. These experiments were repeated at least once.

Immunoslotblot.

The immunoslotblot assay was essentially the same as described earlier (see section III.4.9) with some modifications. Keratin samples (isolated from human callus unexposed or exposed to 250 μM, 500 μM and 1mM sulfur mustard) were diluted to a concentration of 1-5 μg/ml in PBS. The samples were immobilized on nitrocellulose using a Minifold Dot blot manifold (Schleicher and Schuell). For this 3 pieces of blotting paper were pre-wetted in PBS and 1 piece of nitrocellulose (pore size 0.1 μm) was pre-wetted in aquadest and PBS. Then, 50-200 μl of the samples was pipetted in triplicate on the filter. The dots were then rinsed with 400 μl PBS. The nitrocellulose was removed from the filter support and allowed to dry on air for 15 min. The keratin was crosslinked to the nitrocellulose with UV using a GS Gene Linker (Biorad) at 50 mJ.

To prevent non-specific binding of the MoPhAb, the nitrocellulose filters were incubated with 2% milkpowder in PBS + 0.1% Tween (PT) for 1 h at RT. The nitrocellulose was washed 4 times with PT. Then, 10 ml PBS containing, 2% milkpowder and approximately $1x10^{13}$ MoPhabs was added to the filter and incubated overnight at 4 °C under continuous shaking. Then, the filter was extensive washed four times with PT and incubated with 10 ml PBS contained with 2% milkpowder and 5000-fold diluted horseradish peroxidase (HRP) conjugated to mouse anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), during 2 h at RT under continuous shaking. The filter was then extensively washed with PT as stated above. In the mean time substrate (BM Chemiluminescence blotting substrate, POD (Roche)) and solution A (10 ml/filter) were prewarmed to 25 °C. After 30 min 1/100 volume of substrate solution B was added and incubated for another 30 min at 25°C. Subsequently, the filter was incubated with the substrate solution for 1 min. The filter was placed in a plastic bag. The chemiluminescence was measured with a luminometer (MicroBeta Trilux; Wallac, EG & G Bertold, Breda, The Netherlands)).

PCR screening for full length insert.

Clones producing the desired MoPhabs were checked for the presence of full length VH and Vκ insert. DNA was isolated by using the High Pure PCR Preparation Template Kit (Roche, Almere, the Netherlands) according to the manufacturers' protocol. PCR was performed using the primers LMB3 (CAGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) under conditions described elsewhere (Winter *et al*., 1994). Annealing temperature was set at 55 °C and extension time was 2 min. Primers were purchased at OPERON Biotechnologies (Cologne, Germany). The size of the amplified DNA was estimated on a 2% agarose gel at a current of 100 mA, using the Superladder-low 100bp as reference (Eurogentec, Seraing, Belgium). DNA was visualized using ultraviolet light. Full length VH and Vκ insert should result in a DNA fragment of 935bp. A PCR product without insert would result in a DNA fragment of 329bp.

IV RESULTS

IV.1 Further development of the mass spectrometric analysis of the tripeptide (S-HETE)-Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard

IV.1.1 Introduction

Results obtained within the framework of our previous grant DAMD17-97-2-7002 showed that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of the tripeptide (S-HETE)Cys-Pro-Phe, which could be conveniently isolated and determined in a rather sensitive way by micro-LC/electrospray tandem MS with multiple reaction monitoring at an absolute detection limit of 4 pg (Noort *et al.*, 1999). 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 nitrogen-mustard derivatives (Noort *et al*., 2002a). We here elaborate this method in more detail, in order to investigate whether it can be transformed in a Standard Operating Procedure. Various detection methods have been pursued, in order to make the method as fast, as sensitive and as reproducible as possible.

IV.1.2 Shortening of the procedure for albumin isolation by precipitation

The procedure for isolation of albumin from human blood samples, as routinely used in our laboratory, consists of overnight incubation of the plasma sample with $CaCl_2$ in order to precipitate the immunoglobulins, followed by a number of other washings and precipitation steps (Bechtold *et al*., 1992). This procedure could be substantially shortened by performing the incubation with $CaCl_2$ for only 1 h, instead of overnight. Identical levels of (S-HETE)Cys-Pro-Phe were found after pronase digestion, in comparison to albumin samples which had been isolated according to the original procedure.

IV.1.3 Upscaling of pronase digestion of albumin

In previous experiments only 3 mg of albumin was digested with pronase, which enabled the detection of an exposure level of 10 nM. We reasoned that the use of larger amounts of albumin, in combination with purification of the pronase digest by means of Sep-Pak C18 cartridges, might lead to significantly lower observable exposure levels. Indeed, when 20 mg samples of albumin were digested with pronase, followed by Sep-Pak clean-up of the digests, the lowest detectable exposure level could be improved by one order of magnitude (*i.e.*, 1 nM) (see Figure 2). However, the reproducibility of this procedure is not yet at the same level, compared with the 3 mg procedure. The micro-LC columns severely suffered from clogging, due to the relatively large amounts of material introduced. More attention should be paid to further clean-up of the digests prior to the actual LC/electrospray tandem MS analysis on the triple-quad instrument.

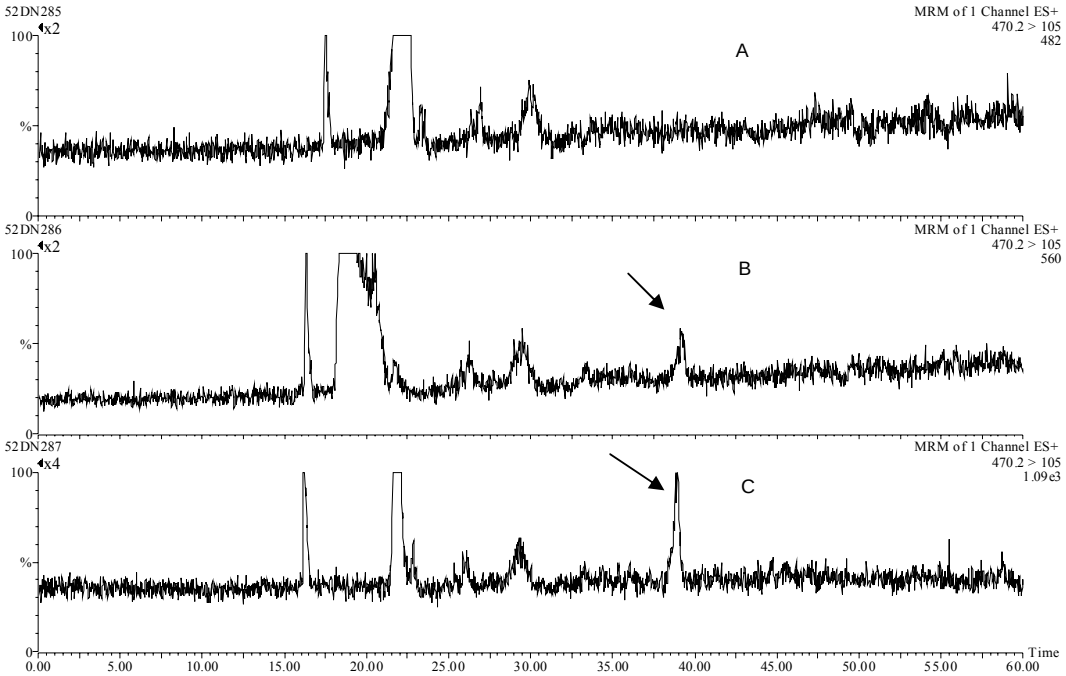


Figure 2. Trace level LC/electrospray tandem MS analysis of (S-HETE)Cys-Pro-Phe in pronase digest of albumin (20 mg) after purification on Sep-Pak C18, measuring the transition *m/z* 470 (MH^+) ---> 105. Albumin was isolated from non-exposed blood (A) or from human blood that was exposed to 1 nM (B). Panel C represents the 1 nM digest after spiking with synthetic (S-HETE)Cys-Pro-Phe. The arrow indicates the peak for (S-HETE)Cys-Pro-Phe.

IV.1.4 Isolation of albumin from plasma by affinity chromatography

It is clear that the isolation of albumin by means of precipitation is rather time-consuming, which limits the utility of the method. Moreover, the current isolation method precludes automation of the methodology. A literature research revealed that albumin can be removed from serum by affinity chromatography, which will facilitate the analysis of less abundant serum proteins (Sato *et al*., 2002). 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.

IV.1.5 Use of albumin alkylated by d_8-sulfur mustard as an internal standard

The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to d_8-sulfur mustard, has been worked out. This will enable quantitative analyses of unknown samples. With this procedure a stock solution in 4 M urea of powdered albumin, isolated from blood which had been exposed to 0.1 mM d_8-sulfur mustard is added to an "unknown" albumin sample, prior to pronase digestion. In this way, the internal standard can be added to the actual sample more accurately than by weighing. The use of d_8-sulfur mustard-alkylated albumin rather than the synthetically accessible d_8-tripeptide has the advantage that it provides evidence whether pronase digestion has indeed occurred. The tripeptide obtained after digestion of albumin, isolated from human blood that had been exposed to d_8-sulfur

mustard, had similar mass spectrometric properties. Satisfactory calibration curves were obtained.

We also found that plasma, isolated from blood exposed to a well-defined amount of d_8-sulfur mustard, could be used as an internal standard in the assay which uses the affinity chromatography procedure for isolation of albumin. This is more convenient than using a solution of powdered albumin (see Figure 3 for dose-response curve).

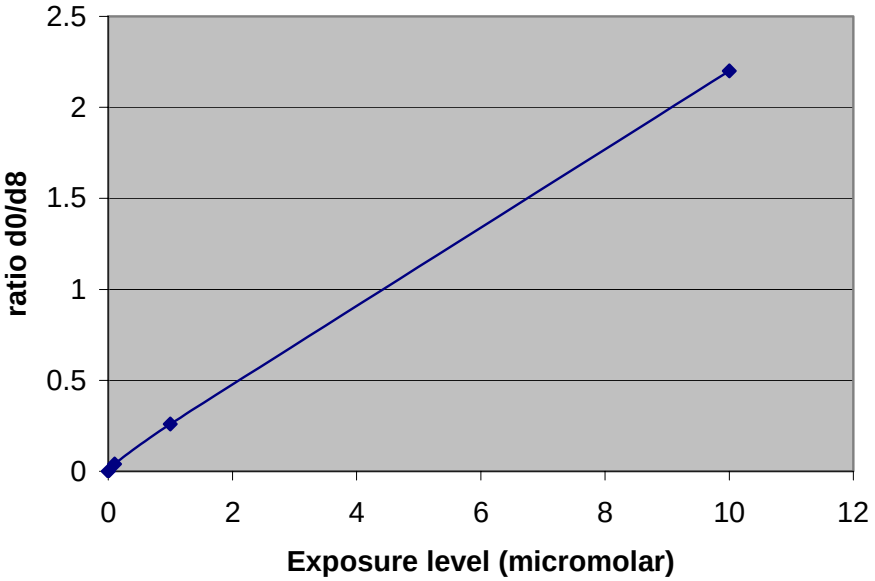


Figure 3. Dose – response curve for LC – tandem MS analysis after pronase digestion of albumin isolated by affinity chromatography from human blood that was exposed to various concentrations of sulfur mustard, in the presence of a fixed amount of internal standard which had been isolated from blood which had been exposed to d_8-sulfur mustard.

See Figure 4 for representative ion chromatograms for analysis of (S-HETE)Cys-Pro-Phe and (S-d_8-HETE)Cys-Pro-Phe in a Pronase digest of albumin, and Figure 5 for the corresponding MS/MS spectra. The procedure was used in the animal experiments.

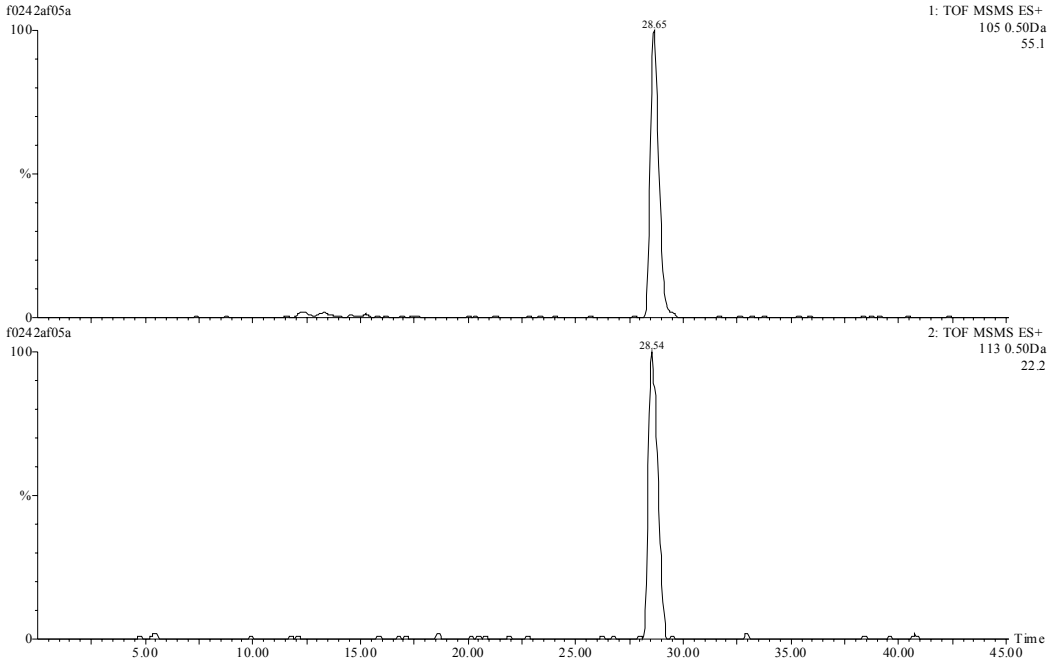


Figure 4. LC-tandem MS analysis (Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-*d8*-HETE)Cys-Pro-Phe (lower panel) in a pronase digest of albumin. Albumin was isolated from plasma (1 ml) by affinity chromotagraphy, followed by desalting on a PD-10 column. The plasma was isolated from human blood that had been exposed to sulfur mustard (10 µM). Prior to isolation of albumin, 50 µl of a plasma sample was added, isolated from blood that had been exposed to sulfur mustard-d_8 (100 µM).

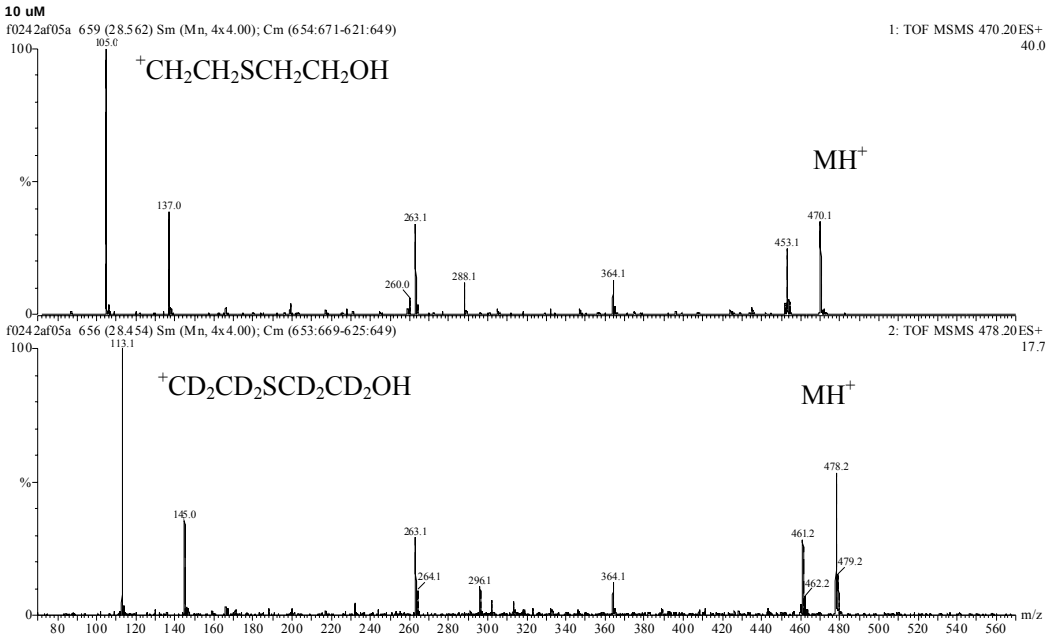


Figure 5. Tandem MS spectra (Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-d_8-HETE)Cys-Pro-Phe (lower panel).

IV.1.6 Sector ion trap MS and Fourier transform – ion cyclotron resonance MS of (S-HETE)Cys-Pro-Phe

A number of analyses of pronase digests were run a sector ion trap instrument (BE ion Trap MAT900) at Thermoquest (Bremen, Germany). Unfortunately, the instrument used proved to be less sensitive than our Q-TOF instrument. The main reason for this was the sample matrix and the deviating fragmentation pattern (less intense fragment *m/z* 105). An exposure level of human blood of 100 μM could still be detected.
Further analyses were run on an FT-ICR instrument at the Environmental Molecular Sciences Laboratory (EMSL; Richland, Washington). Pronase digests of human serum albumin were prepared. Since we were not allowed to store the samples on dry ice during the flight to USA, we tested the stability of the digests. Digests were stored in 4 ml glass vials at room temperature and were analyzed each day. The amount of alkylated tripeptide did not decrease during storage. However, when the same samples were stored in eppendorf tubes, the presence of alkylated tripeptide could not longer be demonstrated after three days! After optimization of the instrument (11.5 Tesla FT instrument), the alkylated tripeptide could be determined with a detection level of approximately 200 ng/ml (2 ng absolute), which is a factor of 10 less sensitive than our Q-TOF instrument. In a pronase digest the adduct could not be determined. Altogether, it seems that the FT-ICR technique is not a good technique for measurement of trace amounts of low-molecular analytes in complex biological matrices.

IV.1.7 Derivatization of (S-HETE)Cys-Pro-Phe for LC tandem MS analysis

It has recently been reported that mass spectrometric detection of fluorinated amino acid derivatives under Electron Capture Atmospheric Pressure Chemical Ionization (EC APCI) conditions results in far better detection limits, when compared to normal electrospray MS (Singh *et al*., 2000). We were anxious to find out whether this technique could also be applied to analysis of (S-HETE)Cys-Pro-Phe. Thus, derivatization of (S-HETE)Cys-Pro-Phe with pentafluorobenzyl bromide (PFB-Br) in the presence of KOH in acetonitril afforded predominantly the di-PFB derivative of the tripeptide, with the PFB groups on the amino group and on the carboxylic acid group (Figure 6). Mass spectrometric analyses indicated that at least for the synthetic reference compound, the fluorinated derivative could be determined slightly more sensitive than the underivatized compound. Care has to be taken however: only standard solutions of the purified derivative have been analyzed in this stage. Derivatization yields and the effect of a matrix, *i.e*., a mixture of other derivatized amino acids, have not been taken in consideration yet.

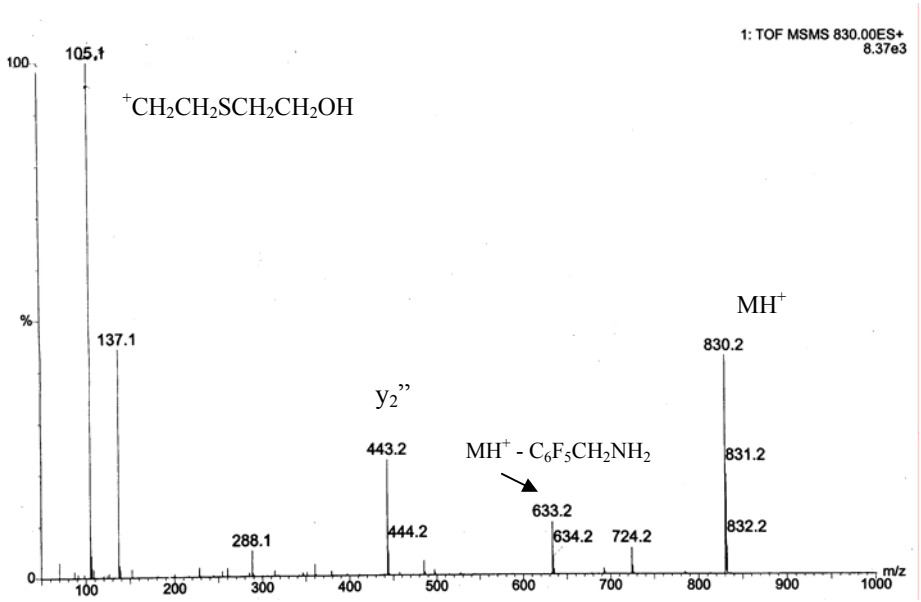


Figure 6. Tandem MS spectrum of bis(pentafluorobenzyl) derivative of (S-HETE)Cys-Pro-Phe.

IV.1.8 Derivatization of (S-HETE)Cys-Pro-Phe for fluorescence detection

Several reagents were explored for fluorescence derivatization of (S-HETE)Cys-Pro-Phe. Initially,we did not succeed in modification of the NH_2 function of (S-HETE)Cys-Pro-Phe for reasons unknown. We therefore directed our attention towards derivatization of the carboxylic acid moiety of the tripeptide. Reaction with commercially available pyrenyl diazomethane gave rise to a fluorescently labeled compound that was stable enough to allow purification by reversed-phase HPLC. However, the detection limit for analysis of this compound was only in the low µg/ml range, i.e., far less sensitive than mass spec analysis. Based on the positive results obtained for derivatization of the histidine adduct with 5/6-carboxyfluorescein succinimidyl ester (FAM-SE), we re-examined the derivatization of the tripeptide with FAM-SE. It appeared that after derivatization and removing excess reagent with hydroxylamine, the peak for the reagent or byproduct was exactly on the same spot as the derivatized tripeptide. CE-LIF analysis of this peak showed two closely eluting peaks, with equal intensity (see Figure 7). The limit of detection was estimated to be 0.3 nM. Mass spectrometric analysis showed the correct mass (see Figure 8).

Subsequently, a more diluted solution of (S-HETE)Cys-Pro-Phe (7 µg/ml) was derivatized with FAM-SE and the mixture was analyzed by means of CE-LIF (see Figure 9). Comparison with the blank (no (S-HETE)Cys-Pro-Phe added) showed the presence of an extra peak at 5.2 min.

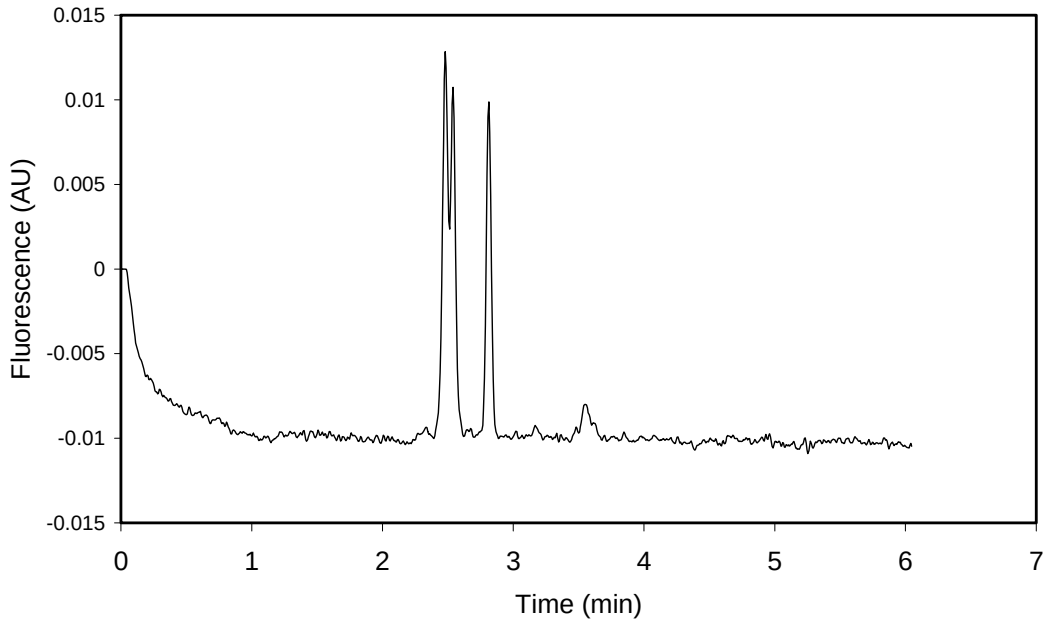


Figure 7. Capillary electrophoresis – laser-induced fluorescence detection of 5/6-carboxyfluoresceine (FAM) derivative of (S-HETE)Cys-Pro-Phe, after derivatization of reference standard with FAM-succinimidyl ester (FAM-SE, 5/6-isomer), followed by Sep-Pak C18 clean-up. The double peak left represents the desired derivative (mixture of 5- and 6-isomer); the peak on the right is fluoresceine, at a concentration of 345 pg/ml.

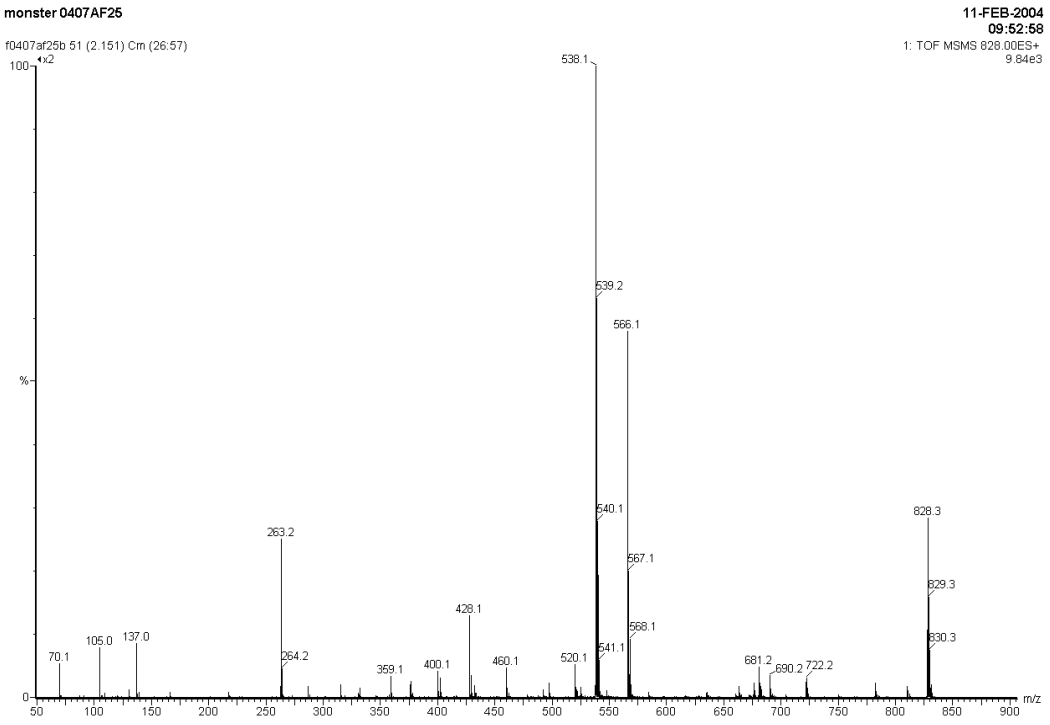


Figure 8. Electrospray MS spectrum of N-5/6-carboxyfluoresceine derivative of (S-HETE) Cys-Pro-Phe.

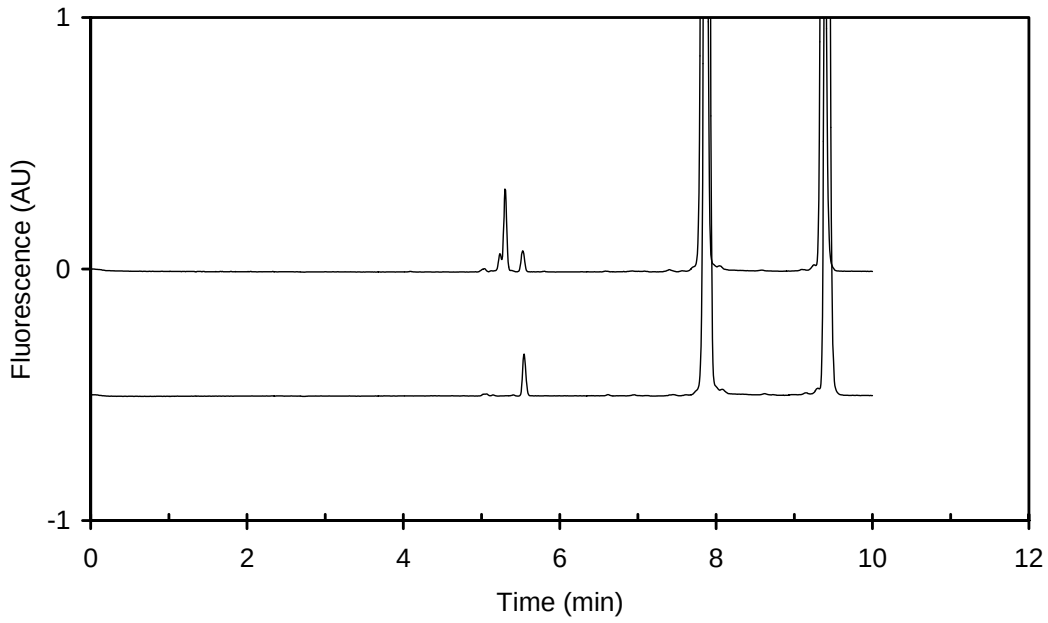


Figure 9. Capillary electrophoresis of 6-FAM derivative of (S-HETE)Cys-Pro-Phe after derivatization at low concentration (7 µg/ml). Synthetic (S-HETE)Cys-Pro-Phe was derivatized with 6-carboxyfluoresceine succinimidyl ester and purified by means of Sep-Pak C18. Upper trace: 6-FAM labeled (S-HETE)Cys-Pro-Phe. Lower trace: blank (no tripeptide added during derivatization). Migration time of 6-FAM(S-HETE)Cys-Pro-Phe 5.2 min

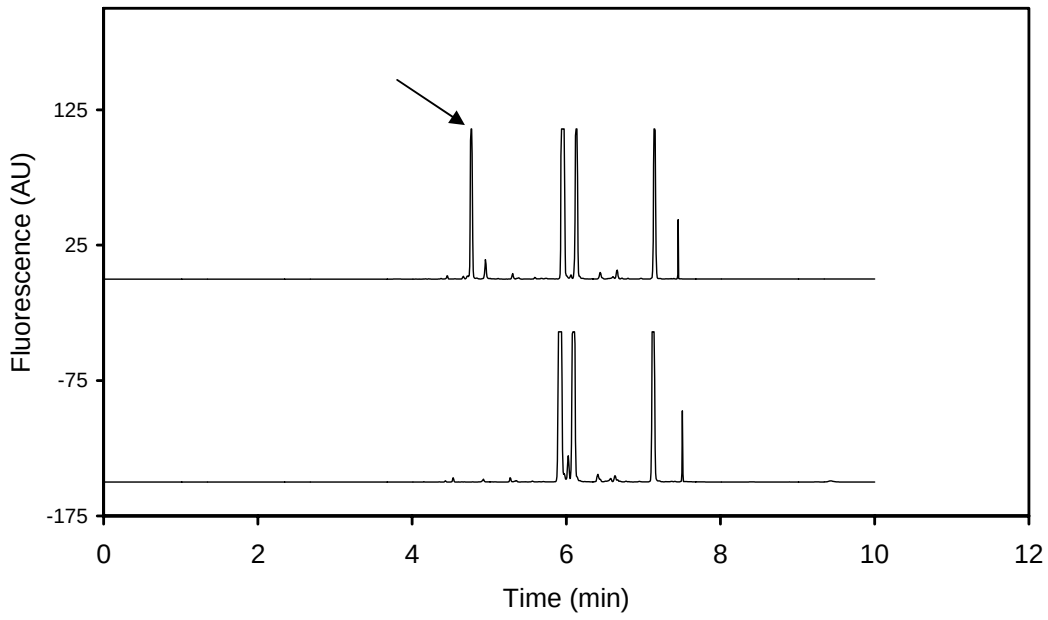


Figure 10. Analysis of (S-HETE)Cys-Pro-Phe in a pronase albumin digest, after derivatization with 6-carboxyfluoresceine succinimidyl ester. Upper trace: albumin pronase digest spiked with synthetic (S-HETE)Cys-Pro-Phe. Lower trace: blank albumin pronase digest.

Subsequently, a pronase digest of albumin was spiked (at 1 mg/ml) with (S-HETE)Cys-Pro-Phe and subsequently derivatized with FAM-SE 6-isomer in 0.1 M $NaHCO_3$. The digest was also derivatized without the addition of (S-HETE)Cys-Pro-Phe. The result is shown in Figure 10. Although the sample was spiked at a very high level, the result was encouraging since it was demonstrated that the tripeptide could be derivatized in a relevant matrix.
Next, pre-purified (Sep-pak C18) pronase digests of albumin that had been isolated from blood that had been exposed to sulfur mustard (1 mM) were derivatized with FAM-SE 6-isomer. The derivatized digest was pre-purified by means of reversed-phase HPLC, and finally analyzed by means of CE-LIF. A similar digest from a non-exposed blood sample was also analyzed. Both NMP and 0.1 M $NaHCO_3$ were used as solvents. A representative result is shown in Figure 11. The trace for the reference compound is also shown. It can be seen that in the area in the electropherogram where the FAM-(S-HETE-Cys)Pro-Phe derivative elutes an interfering peak is present, which precludes unambiguous analysis.

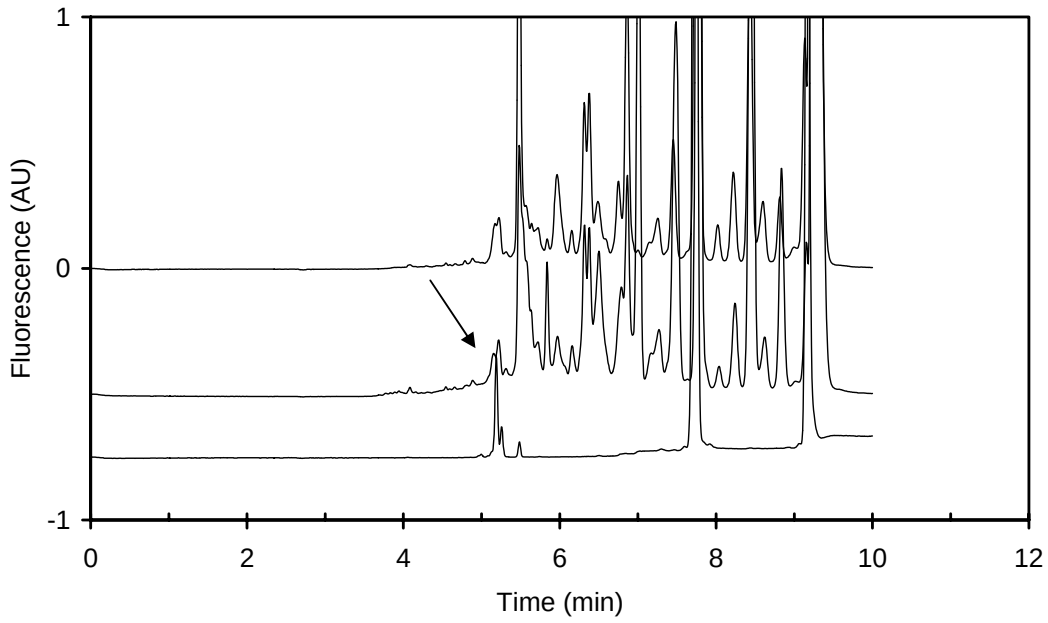


Figure 11. Capillary electrophoresis of albumin pronase digest, after derivatization with 6-FAM-SE. Before and after derivatization the mixture was pre-purified with HPLC.
Upper trace: pronase digest of blank albumin
Middle trace: pronase digest of albumin from blood exposed to 1 mM sulfur mustard
Lower trace: FAM(S-HETE)Cys-Pro-Phe; migration time FAM(S-HETE)Cys-Pro-Phe 5.2 min

IV.1.9 Persistence of albumin adducts

In order to obtain information about the persistence of the albumin – sulfur mustard adduct, laboratory animals were exposed to the agent. We prefer to use the rat for these studies because the amino acid sequence of rat albumin has been published. Furthermore, it has been reported that the modified tripeptide Cys-Pro-Tyr results from rat albumin modified at Cys-34

by the food-borne carcinogens IQ (Turesky *et al*., 1987) and PhIP (Alexander *et al*, 1997), after pronase treatment. Experiments, in which rat blood was exposed *in vitro* to sulfur mustard, showed that the major product was (S-HETE)Cys-Pro-Tyr (see Figure 12 for tandem MS spectrum), and that the level of alkylation of the free cysteine residue in rat albumin was approximately 5%. Electrospray tandem mass spectrometric analyses could be performed in an analogous way, *i.e.*, by selecting the charged molecular ion in the first MS and measuring the highly selective 105 fragment in the second mass spectrometer. Consequently, the rat was chosen as laboratory animal for studying the persistence of the albumin adduct.

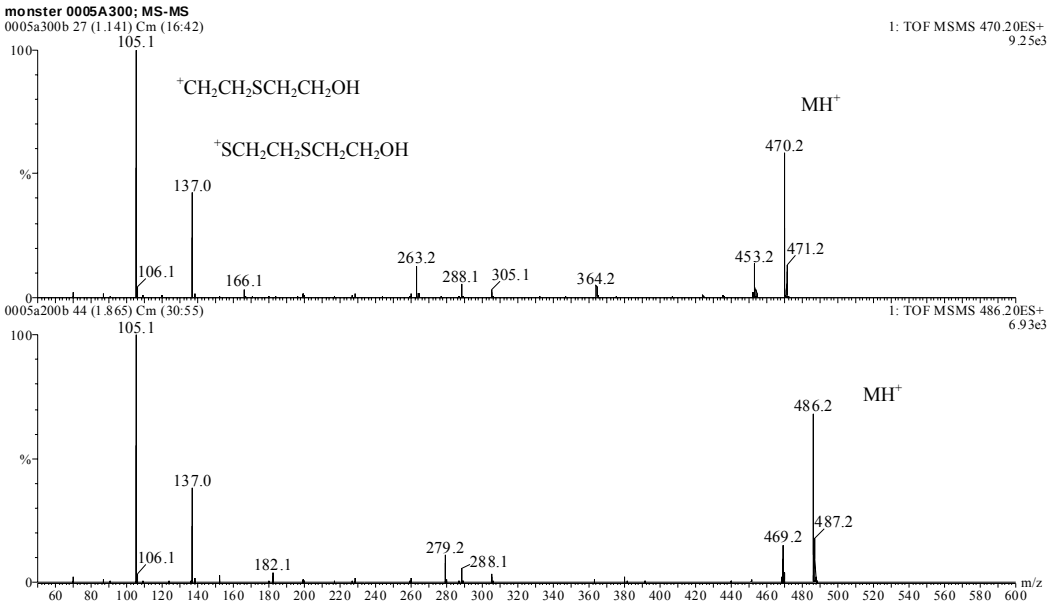


Figure 12. Comparison of tandem MS spectra (recorded on Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE)Cys-Pro-Tyr (lower panel), demonstrating the intense fragment ion with *m/z* 105 in both cases.

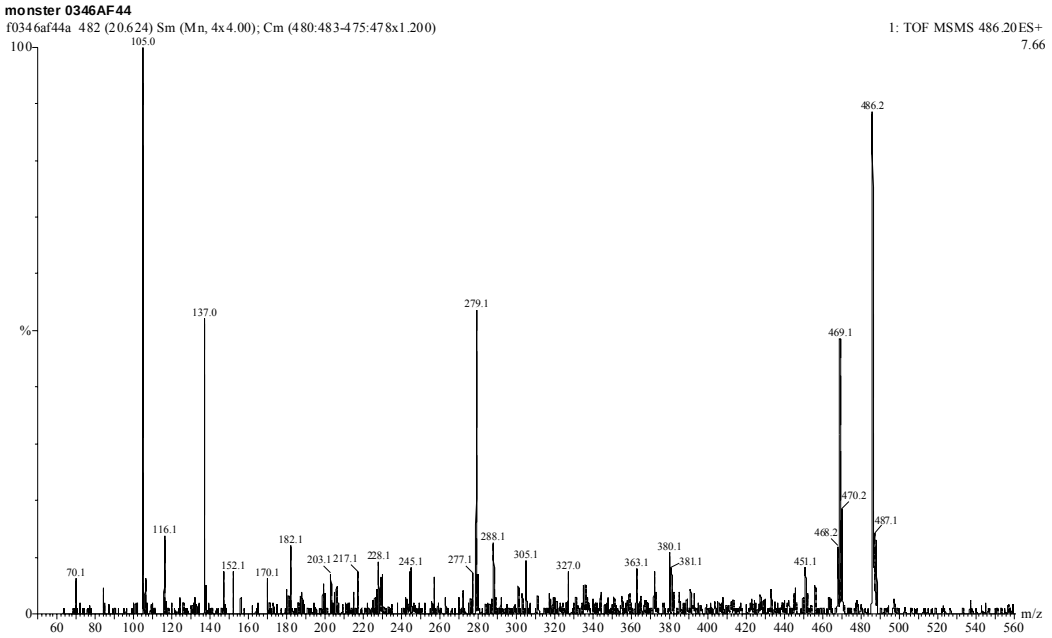


Figure 13. Full scan electrospray tandem MS spectrum (recorded on Q-TOF instrument) of molecular ion (MH^+ 486.2) of sulfur mustard tripeptide adduct (S-HETE)Cys-Pro-Tyr, obtained after pronase digestion of albumin from rat blood, isolated 1 h after exposure *in vivo* to sulfur mustard (0.3 mg/kg, i.v.).

The animal study was performed as follows. For each time point to be studied 3 rats were taken. A dose of 0.3 mg/kg sulfur mustard was administrated (i.v.) to the animals, and blood was collected after 10 min, 1 h, 6 h, 1, 2, 3, 7 and 28 days after exposure. At the indicated time points, blood was collected (approximately 7 mL per rat). Plasma was separated from the erythrocytes by centrifugation and after addition of well-defined amount of internal standard (i.e., plasma from rat blood that had been exposed to 100 µM sulfur mustard-d_8) albumin was isolated by affinity chromatography on Blue Sepharose cartridges, according to the tentative SOP. After treatment with Pronase, the resulting digests were analyzed qualitatively by means of LC tandem MS-MS on a Q-TOF instrument, with recording of full MS-MS spectra, in order to verify that indeed the tripeptide (S-HETE)Cys-Pro-Tyr was formed *in vivo*; see Figure 13 for a full MS-MS spectrum.

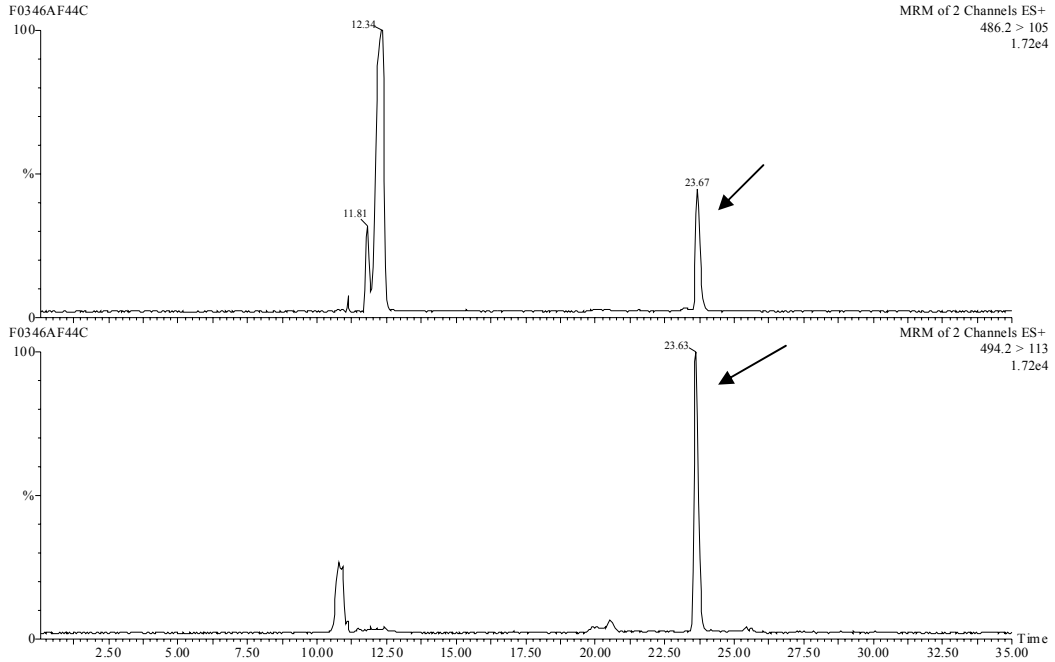


Figure 14. LC-tandem MS analysis of (S-HETE)Cys-Pro-Tyr (arrow) in a pronase digest of albumin, using the multiple reaction monitoring scanning mode for the transition *m/z* 486 (MH^+) → 105. Albumin was isolated from plasma taken from a rat, 1 h after exposure to sulfur mustard (0.3 mg/kg). The sample was analyzed in the presence of albumin, isolated from rat blood exposed to sulfur mustard-d_8 *in vitro* (corresponding to 5 µM sulfur mustard-d_8). Upper panel: analysis of (S-HETE)Cys-Pro-Tyr (multiple reaction monitoring of the transition *m/z* 486 (MH^+) → 105). Lower panel: analysis of (S-d_8-HETE)Cys-Pro-Tyr (multiple reaction monitoring of the transition *m/z* 494 (MH^+) → 113)

Subsequently, the samples were analyzed by means of LC tandem MS on a triple-quad instrument for more sensitive analysis by using the multiple reaction monitoring mode. A representative analytical run is given in Figure 14. Significant amounts of the tripeptide could be observed, which rapidly decreased in time (half-life of sulfur mustard - modified albumin: 2 days). For the time-course of the albumin adduct level see Table 1 and Figure 15.

Table 1. Peak ratios of d_0/d_8 (S-HETE)Cys-Pro-Tyr levels after pronase digestion of albumin samples of rats after exposure to sulfur mustard (0.3 mg/kg, i.v.)

sample	d_0/d_8 (1st series)	d_0/d_8 (2nd series)	d_0/d_8 (3rd series)	d_0/d_8 (mean)
10 min	0.29	0.22	*	0.25
1 h	0.46	0.42	0.32	0.40
6 h	0.37	0.34	0.34	0.35
1 d	0.20	0.16	0.20	0.18
2 d	0.16	0.17	*	0.16
3 d	0.16	0.10	0.16	0.14
7 d	0.033	0.032	0.039	0.035
28 d	0**	0**	0**	0**

* animal died just after administration of sulfur mustard

** the level of d_0-(S-HETE)Cys-Pro-Tyr was below the detection limit

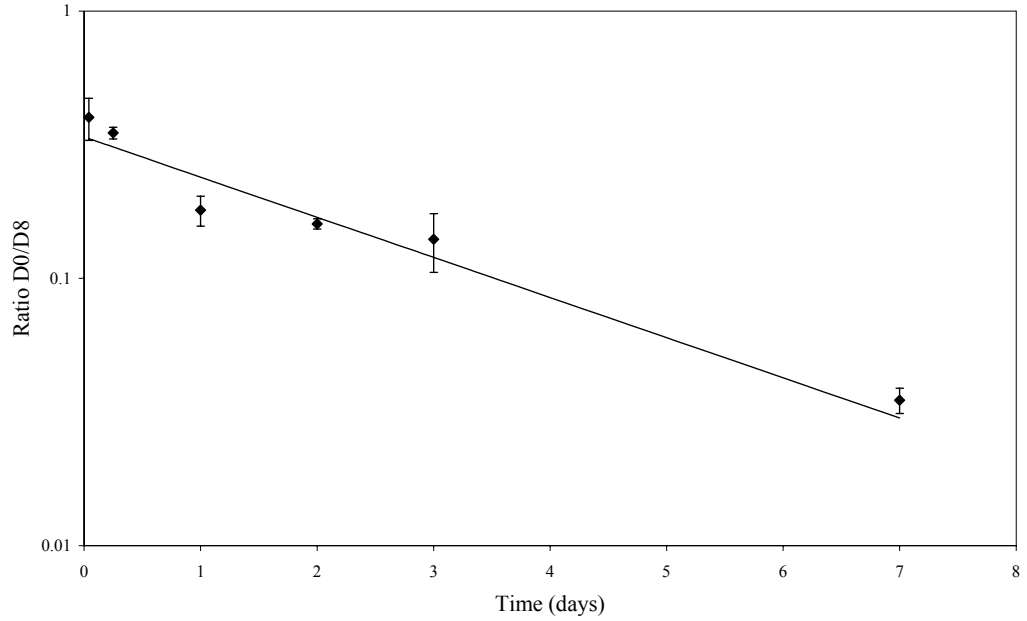


Figure 15. Persistence of alkylated cysteine in albumin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, albumin was isolated by affinity chromatography and analyzed by using the tentative SOP for determination of (S-HETE)Cys-Pro-Tyr. Plasma from rat blood exposed to sulfur mustard-d_8 (100 μM) was used as an internal standard.

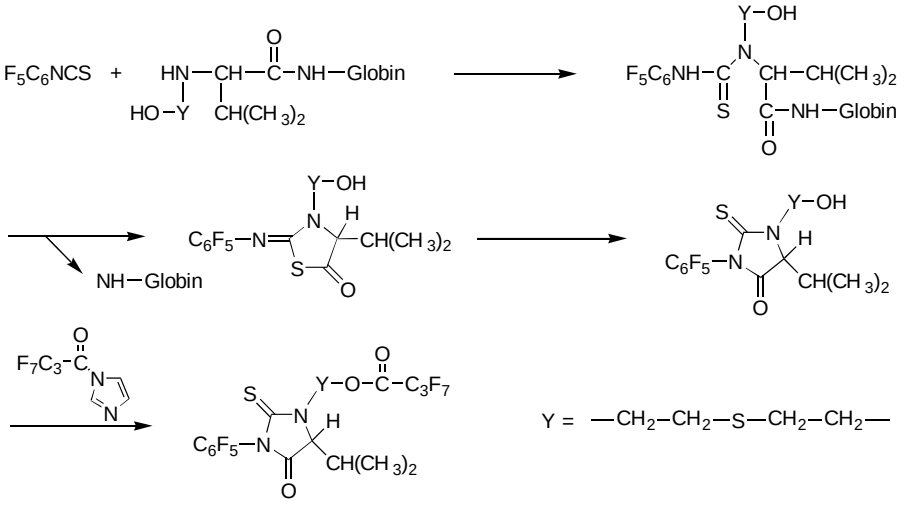


Figure 16. Modified Edman degradation of globin alkylated by sulfur mustard.

Subsequently, the N-terminal valine adduct levels were determined in the corresponding erythrocyte samples according to the Standard Operating Procedure (SOP) reported during the previous Cooperative Agreement DAMD17-97-2-7002. Globin was isolated and subsequently a well-defined amount of globin isolated from blood that had been exposed sulfur mustard-d_8 was added, and the mixture was processed according to the SOP. Thus, shortly, the globin

samples were dissolved in formamide and pentafluorophenylisothiocyanate was added. After incubation for 2 h at 60 ºC, the pentafluorophenylthiohydantoin was isolated by extraction and was further derivatized by treatment with heptafluorobutyrylimidazole (see Figure 16). The samples were analyzed by means of GC-NICI-MS with single ion monitoring for the presence of d_0- and d_8-pentafluorophenylthiohydantoin. A representative analytical run is shown in Figure 17. The results of the various analyses are given in Table 2 and Figure 18.

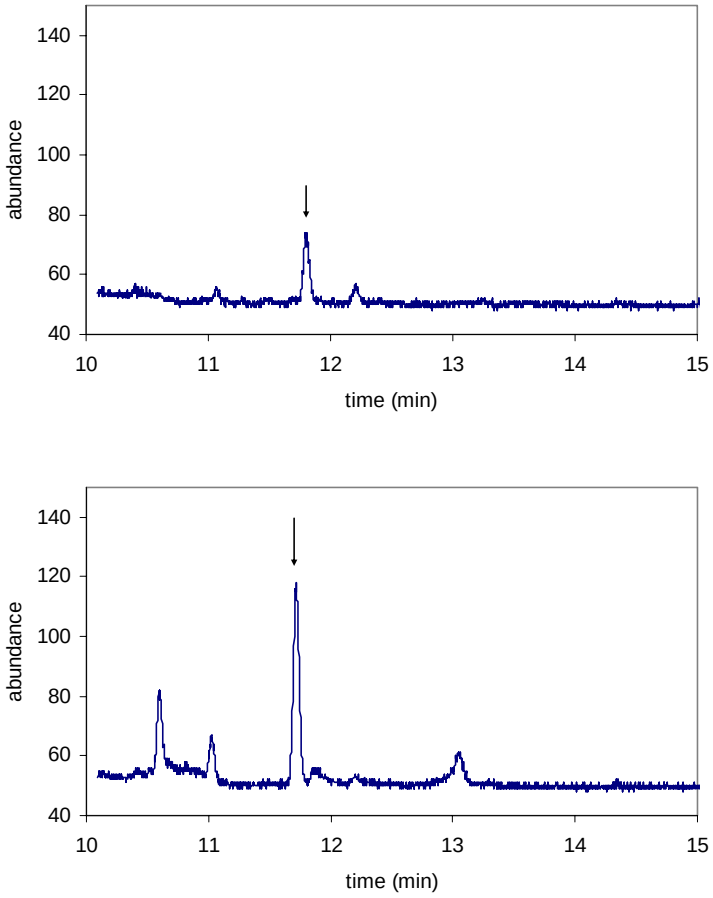


Figure 17. Representative GC-NCI/MS analysis of globin (20 mg) isolated from blood taken from a rat, 7 days after administration of sulfur mustard (0.3 mg/kg, i.v.), after modified Edman degradation. Globin isolated from rat blood treated with 100 µM sulfur mustard-d_8 served as an internal standard. Ion chromatograms after monitoring for *m/z* 564 (analyte; upper panel) and *m/z* 572 (internal standard; lower panel).

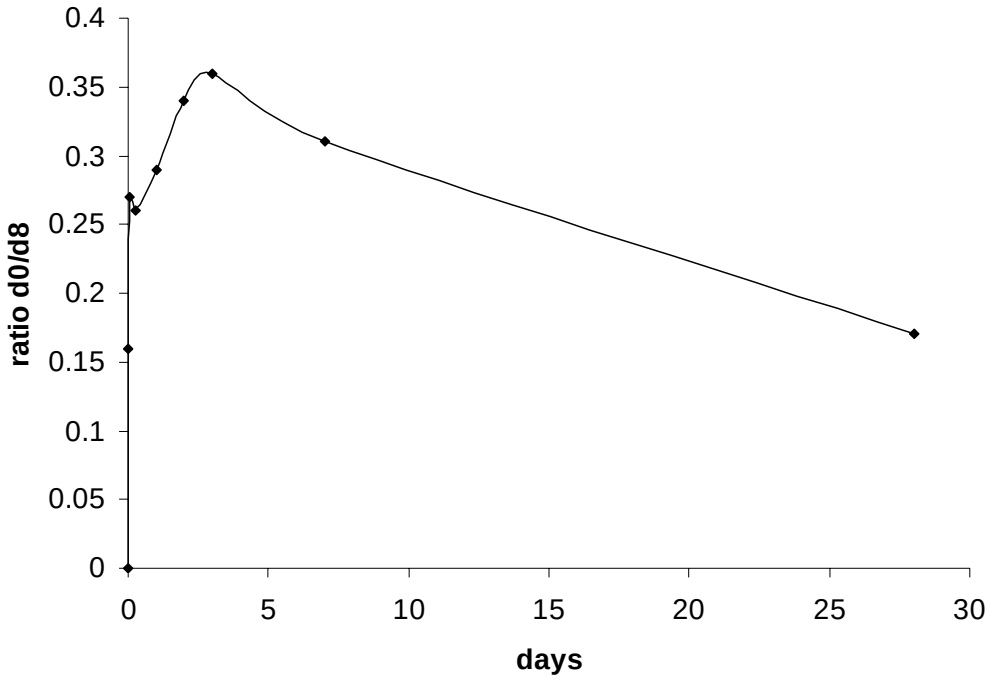


Figure 18. Persistence of alkylated N-terminal valine in hemoglobin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, globin was isolated and analyzed by using the SOP for determination of alkylated N-terminal valine. Globin from rat blood exposed to sulfur mustard-d_8 (100 µM) was used as an internal standard.

Table 2. Peak ratios of d_0/d_8 pentafluorophenylthiohydantoin derivatives after modified Edman degradation of globin samples of rats after exposure to sulfur mustard (0.3 mg/kg, i.v.)

sample	d_0/d_8 (1st series)	d_0/d_8 (2nd series)	d_0/d_8 (3rd series)	d_0/d_8 (mean)
10 min	0.15	0.17	*	0.16
1 h	0.37	0.24	0.19	0.27
6 h	0.29	0.25	0.24	0.26
1 d	0.24	0.29	0.34	0.29
2 d	0.33	0.36	*	0.34
3 d	0.40	0.31	0.38	0.36
7 d	0.32	0.32	0.30	0.31
28 d	0.18	0.16	0.17	0.17

* animal died just after administration of sulfur mustard

The results clearly demonstrate that the adduct level increases during the first days after the exposure. This implicates that there is still intact sulfur mustard present in the animal. Comparison of the two time-courses, i.e, from hemoglobin adduct and albumin adduct levels, learns that in the rat the hemoglobin adduct is far more persistent than the albumin adduct, as should be expected in view of the life time of the rat erythrocyte and the half life of rat albumin.

We have also performed some experiments with plasma of marmosets that had been exposed to sulfur mustard. These plasma samples had been isolated from blood samples taken during experiments for determination of the persistence of hemoglobin adducts, within the context of

our previous cooperative agreement DAMD17-97-2-7002, and had been stored for 2 years at –70 ºC. Albumin was isolated from these samples using the precipitation procedure. Unfortunately, the amino acid sequence of marmoset albumin is not known. Nevertheless, we digested small amounts (3 mg) of the albumin samples and analyzed the digests for the presence of the (S-HETE)Cys-Pro-Phe. The first three samples (1 h, 1 day, and 7 days after administration of 4.1 mg/kg sulfur mustard, iv) were digested and analyzed without the use of an internal standard. The presence of (S-HETE)Cys-Pro-Phe could be demonstrated, which indicates that marmoset albumin contains a free cysteine function near Pro-Phe, which is prone to alkylation by sulfur mustard. The levels after 1 h and 1 day were comparable, while the sample taken after 7 days was approximately 1.6 less intense. The other samples (*i.e.*, taken after 14, 21, 28 and 56 days) were digested and analyzed in the presence of an internal standard (albumin from human blood that was exposed to d_8-sulfur mustard). The estimated exposure levels were 1.49, 0.76 and 0.37 µM (compared to the internal standard) for the 14, 21, and 28 days samples, respectively (see Figure 19). In the sample which had been taken after 56 days, the observed peak was below the detection limit. It must be stressed, however, that the digests had not been pre-purified by means of Sep-Pak C18 and that the analyses were run on a Q-TOF instrument instead of triple-quad instrument, which has higher sensitivity. When we assume that alkylation does not influence the life time of the protein, we can derive that the half-life time of marmoset albumin is 7 days (for humans: 20-25 days).

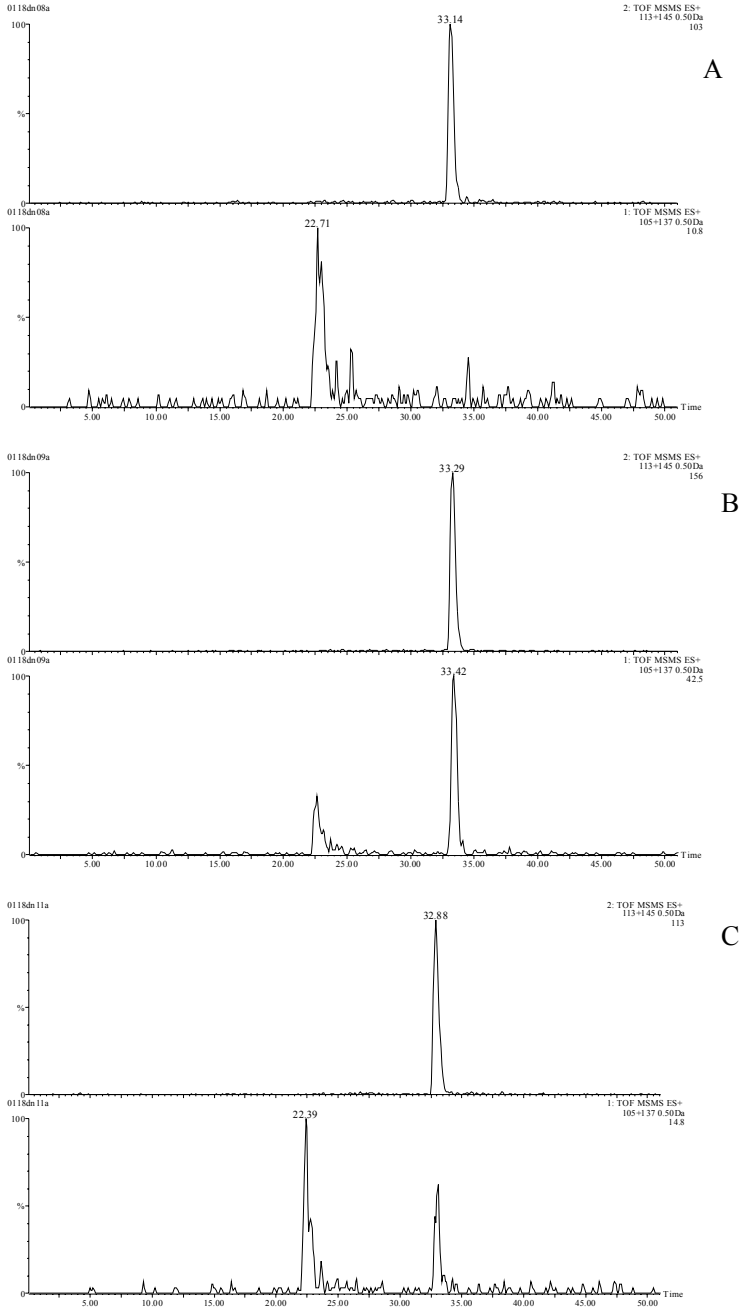


Figure 19. LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe in pronase digests of albumin isolated from plasma taken from a marmoset which had been exposed (i.v.) to sulfur mustard (4.1 mg/kg). The sample was analyzed in the presence of albumin, isolated from human blood which had been exposed to d_8-sulfur mustard (corresponding with a level of 5 μM d_8-sulfur mustard). Panel A: internal standard + blank marmoset albumin sample. Panel B: internal standard + marmoset albumin from blood sample taken 14 days after intoxication. Panel C: internal standard + marmoset albumin from blood sample taken 28 days after intoxication. In each panel (A-C), the upper trace represents (S-d_8-HETE)Cys-Pro-Phe (resulting from digestion of d_8-sulfur mustard-alkylated albumin), while the lower trace represents (S-HETE)Cys-Pro-Phe (resulting from digestion of albumin from the marmoset).

IV.2 Further development of methods for sensitive analysis of N1/N3-(2-hydroxyethylthioethyl)histidine

IV.2.1 Introduction

In hemoglobin and albumin from human blood exposed to sulfur mustard, 34% and 28% of total adduct level could be ascribed to N1/N3-HETE-histidine, respectively (Noort *et al*., 1997). In view of this abundance and the stability of the adduct under acidic conditions, which enables its quantitative release from proteins, we investigated the mass spectrometric analysis of this adduct for which no sensitive method is currently available. The only published method is based on LC/electrospray tandem MS analysis of the 9-fluorenyl-methoxycarbonyl (Fmoc) derivative of N1/N3-HETE-histidine, which allowed the determination of an exposure level of human blood *in vitro* of 10 μM sulfur mustard from the adducts formed in hemoglobin (Noort *et al*., 1997). We have elaborated whether analysis of the histidine adduct can form the basis of a Standard Operating Procedure.

IV.2.2 Development of a work-up procedure for isolation of N1/N3-HETE-histidine based on ion exchange chromatography

We developed a work-up procedure for isolation of the histidine-sulfur mustard adducts from amino acid mixtures resulting from acidic hydrolysis of hemoglobin or albumin. This procedure consists of an ion exchange chromatography step by using Dowex 50 WX8 (Na^+ form). In this way, uncharged amino acids were eluted with aqueous acetic acid. After elution with water, elution with diluted NH_4OH afforded purified histidine adducts, which were further derivatized with Fmoc-Cl. Further clean-up could be achieved by elution on a Seppak C18 cartridge (see Figure 20).

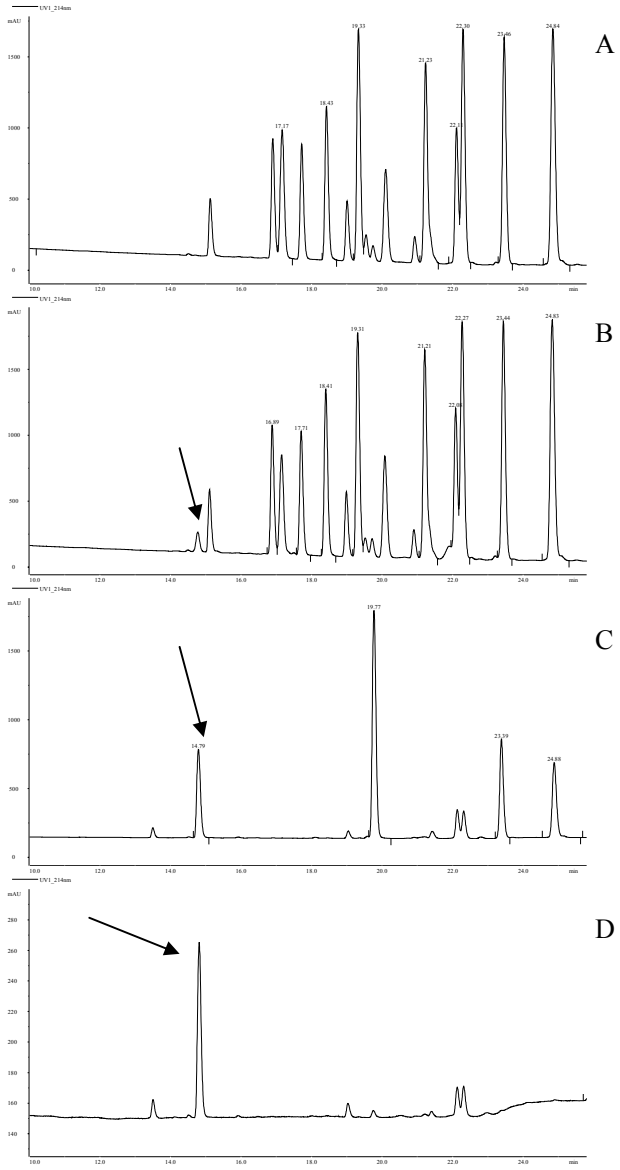


Figure 20. HPLC analysis of acidic digests of human globin, spiked with synthetic N1/N3-HETE-histidine, after derivatization with Fmoc-Cl. (A) non-spiked acidic globin hydrolysate, (B) globin acidic hydrolysate spiked with N1/N3-HETE-histidine, (C) globin acidic hydrolysate spiked with N1/N3-HETE-histidine after pre-purification by cation-exchange chromatography, and (D) after purification of the digest of panel C on Sep-Pak C18. The arrow indicates the peak for the Fmoc-N1/N3-HETE-histidine derivatives.

IV.2.3 Direct isolation of histidine adduct by means of reversed-phase HPLC

It was attempted to directly isolate N1/N3-HETE-histidine from acidic digests by means of semi-preparative reversed-phase HPLC, i.e., without derivatization. Subsequent direct LC-tandem MS would circumvent the laborious derivatization of the amino acid mixtures. Figure 21 shows a semi-preparative HPLC run from spiked digests. Appropriate fractions were

collected, concentrated and analyzed by LC/MS with and without derivatization with Fmoc-Cl. The adduct could indeed be detected by applying this method, when highly spiked hydrolysates or hydrolysates from highly exposed globin samples were used. Unfortunately, during micro-LC tandem MS analysis the retention time of the underivatized adduct was highly variable, which precluded routine analyses (see Figure 22 and 23 for a successful analysis). Basically, one can argue that this method can be used advantageously for preparative purification of the adduct from acidic or enzymatic digests. Subsequent derivatization of the isolated fraction with either Fmoc-Cl for LC-tandem MS analysis or with TFAA for GC-MS analysis can then be used as an appropriate analytical method. However, this will render the final method very laborious.

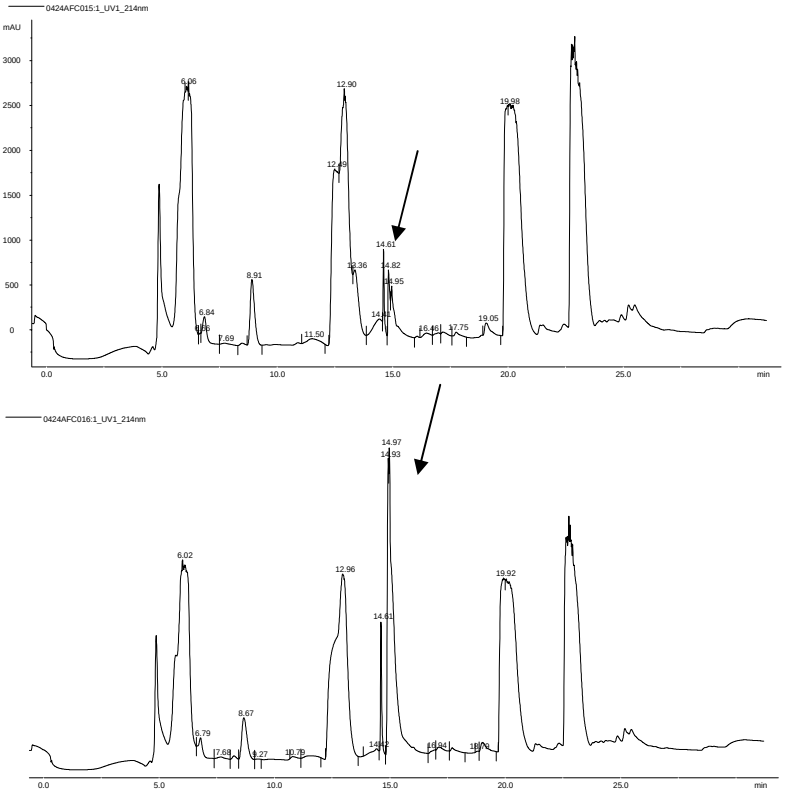


Figure 21. Preparative reversed-phase HPLC purification of acidic hydrolysates of globin (7 mg) spiked with N1/N3-HETE-His, after neutralization with conc. NH_4OH solution.
Trace A: acidic hydrolysate spiked with 30 microgram of each isomer. Trace B: acidic hydrolysate spiked with 90 microgram of each isomer. Fractions of 1 min each were collected and analyzed for N1/N3-HETE-His by means of LC tandem MS.

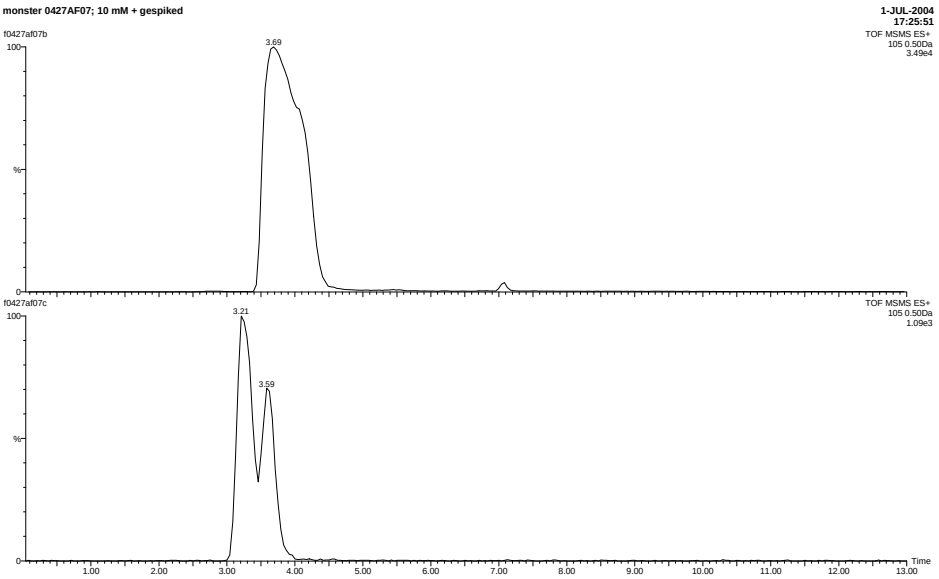


Figure 22. Direct LC-tandem MS analysis of histidine adduct in pre-purified acidic digest of globin, spiked with N1- and N3-(HETE)-histidine. Upper trace: without dilution. Lower trace: diluted sample, showing the individual isomers. Column used: PepMap C18 column.

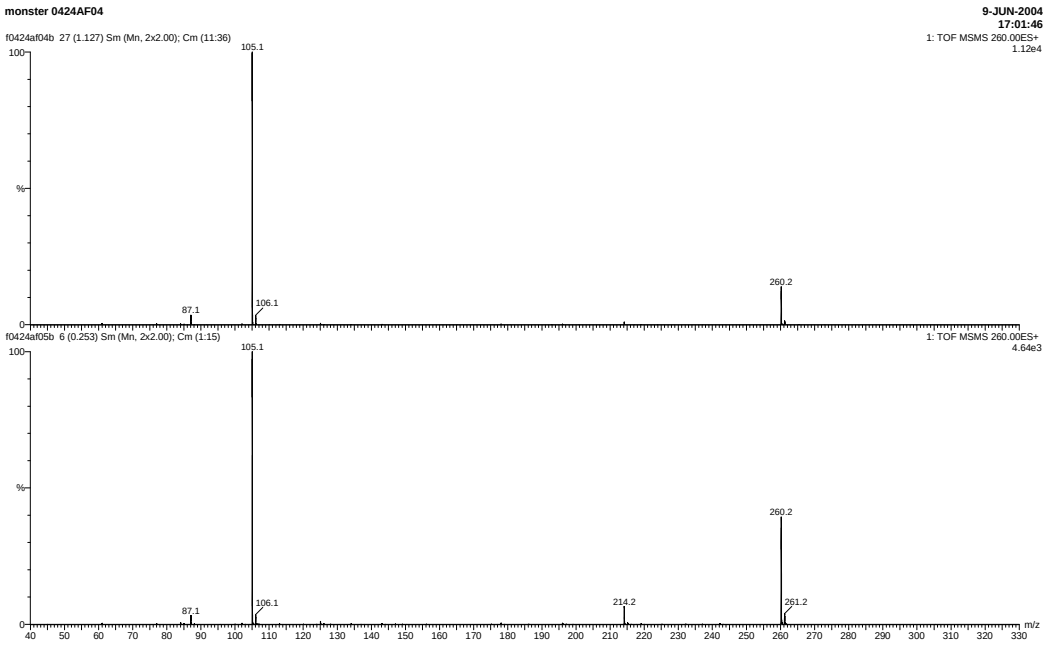


Figure 23. Direct LC-tandem MS analysis of histidine adduct in pre-purified acidic digest of globin, spiked with N1- and N3-(HETE)-histidine; tandem MS spectra of individual isomers. (*m/z* 260, MH^+).

IV.2.4 Hydrazinolysis of globin and analysis of HETE-histidine hydrazine derivatives

As an alternative to acidic hydrolysis of globin, it was attempted to degrade globin by means of hydrazinolysis, ***i.e.***, by treatment with hydrazine, as was reported by Helleberg ***et al***. (2000) We were curious to find out whether the resulting HETE-histidine hydrazine derivative has more favorable properties for LC-MS or GC-MS analysis than the native adduct. Treatment of N1-HETE-histidine methyl ester with hydrazine monohydrate in isopropanol resulted in the desired HETE-histidine-N_2H_4 derivative. Treatment of globin with hydrazine monohydrate resulted in the formation of the same HETE-histidine-N_2H_4 derivative, as well as HETE-histidine-OH derivative, as evidenced by LC-tandem MS analysis. Pure hydrazine should probably give only the hydrazide derivative. The hydrazide derivative proved to be highly unstable, even towards solvents as THF, and could not be further derivatized for GC-MS analysis. We decided to skip further work on hydrazinolysis and analysis of HETE-histidine hydrazide derivatives.

IV.2.5 Derivatization of N1/N3-HETE-histidine for GC-MS analysis

From previous research (Noort *et al*., 1997) it appeared that GC-MS based procedures for analysis of the histidine adducts were troublesome, mainly due to the high polarity or thermal instability of the derivatives. It was found that reaction of the methyl ester of the histidine adducts with trifluoroacetic anhydride afforded a derivative which could be analyzed in a rather sensitive

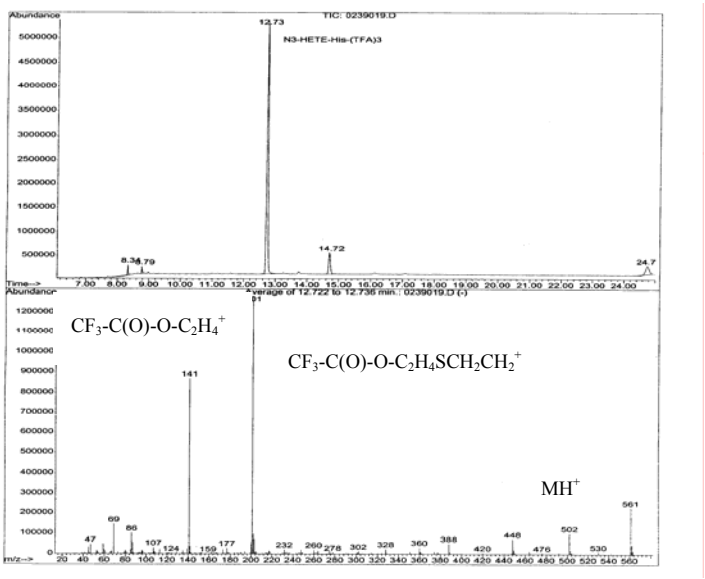


Figure 24. GC-EI-MS analysis of tris(trifluoroacetyl)-N3-(HETE)-histidine.

way by GC-MS (detection limit: 3 ng/ml; 3 pg absolute); see Figure 24. Reaction of N3-HETE-histidine methyl ester with heptafluorobutyric anhydride gave rise to the formation of a bis(heptafluorobutyryl)derivative. However, reaction of N1-HETE-histidine methyl ester with heptafluorobutyric anhydride did not proceed. Reaction with pentafluoropropionic anhydride gave the corresponding bis(pentafluoropropionyl)derivative. Derivatization with trifluoroacetic acid afforded the best results. Unfortunately, it was found that GC-MS analysis was not reproducible.

IV.2.6 Derivatization of N1/N3-HETE-histidine for LC-tandem MS analysis

In our previous cooperative agreements we found that derivatization of N1/N3-histidine with Fmoc-Cl afforded the corresponding Fmoc derivative that could be analyzed rather sensitively by means of LC-tandem MS (Noort *et al*., 1997). Recently, it has been reported (Singh *et al*., 2000) that mass spectrometric detection of fluorinated amino acid derivatives under EC APCI conditions (vide supra) results in far better detection limits, when compared to normal electrospray MS. Derivatization of N1/N3-HETE-histidine with pentafluorobenzyl bromide in DMF, under the agency of triethylamine, afforded the corresponding tris-pentafluorobenzyl (PFB) derivatives. Unfortunately, we were not able to analyze these derivatives with GC-MS. ^{1}H-NMR analysis of the derivative showed that the imidazole function had been modified with a PFB group. This might explain the failure of GC-MS analysis: such a derivative has a permanent positive charge. Fortunately, this derivative exhibited favorable properties for LC/electrospray tandem MS analysis. It has a long retention time, compared to other derivatives, which might enable Sep-Pak C18 clean-up prior to the actual MS analysis.

IV.2.7 Derivatization of N1/N3-HETE-histidine for HPLC with laser-induced fluorescence detection

Laser-induced fluorescence has been used for detection of yoctomole amounts of amino acid derivatives, whereas detection at single molecule level has been shown, albeit under extremely controlled conditions (Chen and Dovichy, 1994). This technique, however, is less selective than mass spectrometry. Various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared (see Figure 25).
Fluorescein-5-isothiocyanate (FITC 'isomer I') was selected because it is one of the most widely used fluorescent dyes. Isothiocyanates form reasonably stable thioureas upon reaction with amines. A drawback is its relatively low reactivity. FITC derivatized amine groups have an excitation maximum at 492 nm and an emission maximum at 520 nm. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) was selected because of the advantage that the unreacted dye is non-fluorescent. The reaction with primary amines involves an intramolecular ring closure, in the presence of cyanide ions. CBQCA derivatized amine groups have an excitation maximum at 468 nm and an emission maximum at 560 nm.
Oregon Green 488-X, succinimidyl ester, 6-isomer is a fluorescein derivative. Conjugates of Oregon Green 488 dyes have several advantages compared to conjugates of fluorescein. These include greater photostability, higher fluorescence and less quenching. In this particular case the succinimidyl ester was used because it is well-known that succinimidyl esters are more reactive than isothiocyanates. For all abovementioned fluorescent dyes the desired conjugates with N1- and N3-(2-hydroxyethylthioethyl)histidine could be prepared in good yields. Surprisingly, the derivatives of FITC slowly rearranged into compounds which are probably the corresponding thiohydantoins (based on mass spectrometry). The derivatives were isolated by semi-preparative FPLC, using a reversed-phase column, and characterized with electrospray MS. The derivatives were stored as dry solids and proved stable for several months.In our hands the most promising fluorescein-based reagent was 5/6-carboxyfluorescein (FAM) succinimidyl ester. The derivatization reaction proceeded smoothly and the stability of the carboxamide derivatives was excellent. The individual derivatives of both N1-HETE-His and N3-HETE-His were isolated by semi-preparative FPLC, using a reversed-phase column, and characterized with electrospray MS. The UV spectrum of one of the derivatives is given in Figure 26. With the fluorescent derivatives in hand, the detection limits were determined for analysis with capillary electrophoresis with laser induced fluorescence (CE-LIF) detection. See Figure 27 for a representative analysis of the reference compound. The detection limits of the FAM N1-HETE- and FAM N3-HETE-histidine derivatives were 24 and 21 pg/ml, respectively.

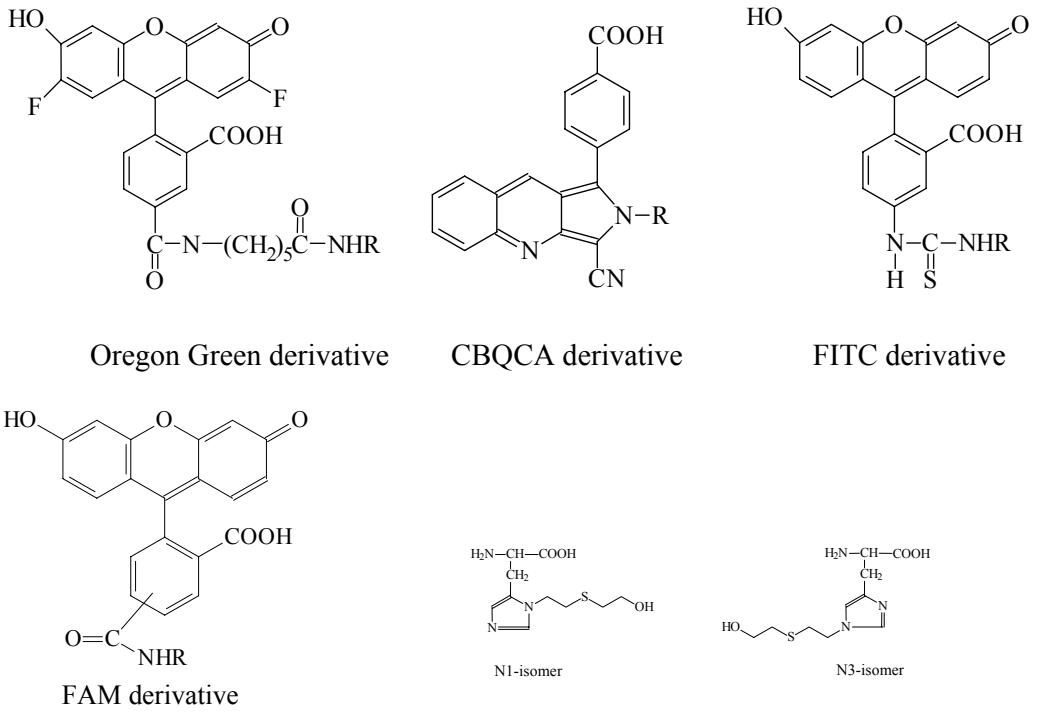


Figure 25. Fluorescent derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine.

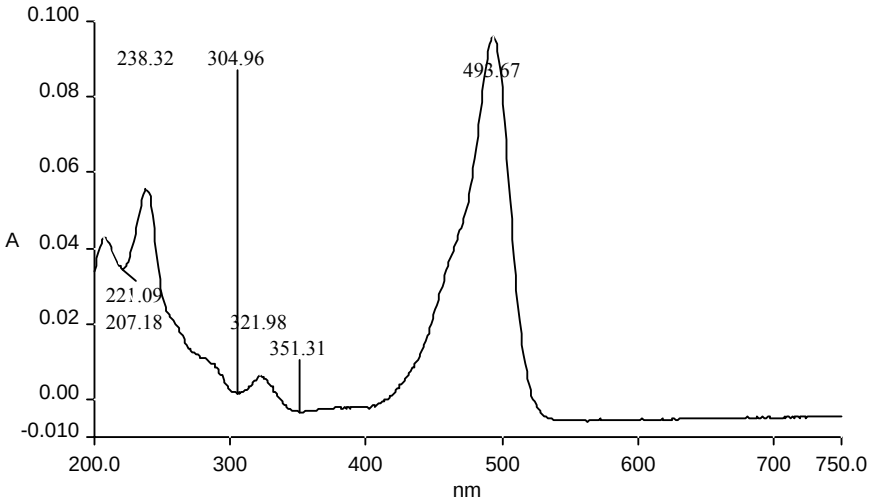


Figure 26. UV spectrum of FAM derivative of N1-HETE-histidine.

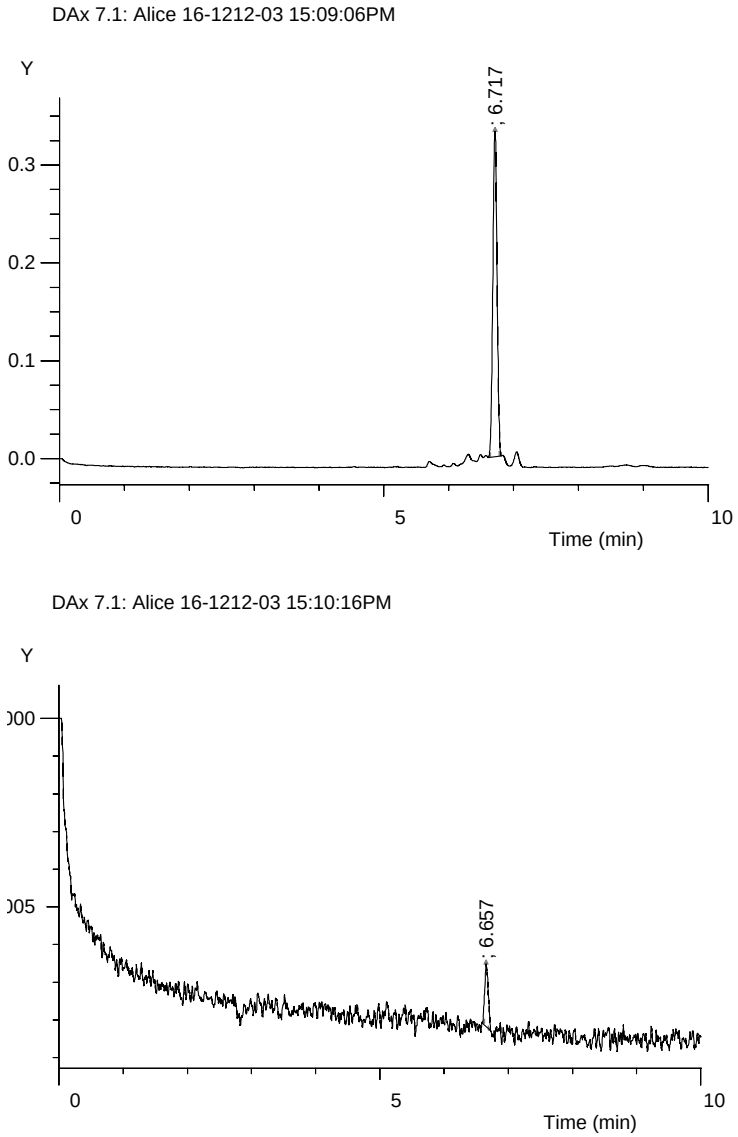


Figure 27. CE-LIF analysis of FAM derivative of N1-HETE-histidine at two different concentrations: 4.9 ng/ml (upper panel) and 24 pg/ml (lower panel).

The derivatization of amino acid mixtures (containing the histidine adducts) with FAM succinimidyl ester has also been studied. Unfortunately, even in highly spiked samples (microgram/ml level), we could not identify the FAM N1/N3-HETE-histidine derivatives, when the derivatization was carried out as performed for (S-HETE)Cys-Pro-Phe (see above).

IV.3 Development of Standard Operating procedure for albumin - tripeptide assay

During the course of the project it turned out the albumin tripeptide method was the method of choice for development into an SOP. A tentative SOP was drafted converted into a definitive version after a number of slight modifications. Also, the interindividual and intraindividual variation of adduct formation to Cys-34 in human serum albumin, and the day to day variability of the assay was determined. Finally, the SOP has been demonstrated to a scientist of USAMRICD.

IV.3.1 Inter-individual and intra-individual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin – tripeptide assay

Blood samples were drawn from 10 volunteers with informed consent, and subsequently exposed to 0.1 µM sulfur mustard. Work-up of the samples was performed simultaneously. After incubation for 2 h at 37 ºC, plasma was isolated. Internal standard plasma (isolated from human blood that had been exposed to d_8-sulfur mustard) was added to a virtual exposure level of d_8-sulfur mustard of 1 µM. The samples were worked up according to the SOP. The results are given in Table 3.

Table 3. Inter-individual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin tripeptide assay

volunteer	d_0/ d_8 – tripeptide ratio
1	0.11
2	0.10
3	0.12
4	0.13
5	0.13
6	0.13
7	0.12
8	0.12
9	0.12
10	0.11
Mean ± s.d.	0.12 ± 0.01

Subsequently, the intraindividual variation was determined. From 3 persons blood samples were taken at three different time points (3 months in between) and exposed to sulfur mustard (0.1 µM). Plasma was isolated and the samples were processed according to the SOP and analyzed by LC/tandem MS for the ratio d_0/d_8 (S-HETE)Cys-Pro-Phe. The series at the third time point was lost during processing. The results are shown in Table 4.

Table 4. Intra-individual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin tripeptide assay

volunteer	d_0/d_8 – tripeptide ratio (time point 1)	d_0/d_8 – tripeptide ratio (time point 2)
1	0.13	0.13
2	0.12	0.13
3	0.11	0.13

IV.3.2 Day-to-day variability of SOP for tripeptide assay

The day-to-day variability was determined in the following way. A blood sample from a single volunteer was exposed to sulfur mustard (0.1 μM) and subsequently plasma was prepared. At four different time points (within a time period of 6 months), aliquots of the plasma sample were processed according to the SOP and analyzed by LC/tandem MS for the ratio d_0/d_8 (S-HETE)Cys-Pro-Phe. At one of the time points, the sample was lost during processing. The results are shown in Table 5.

Table 5. Day-to-day variability of SOP for albumin tripeptide assay

Time point	Ratio d_0/d_8
1	0.13
2	0.12
3	0.13

IV.3.3 Demonstration of the SOP for the tripeptide assay to a USAMRICD scientist

As was also the case for SOP's developed in the previous cooperative agreement DAMD17-97-2-7002, i.e., the SOP for modified Edman degradation and the SOP for immunochemical analysis of DNA adducts, the current SOP for the tripeptide assay was demonstrated to a scientist of USAMRICD. The aim was to demonstrate that (1) the assay could be set up in an independent laboratory within a short period of time and that (2) the assay was easy to perform by a person that had no experience with the procedure. In this particular case the demonstration was performed within the laboratories of the Centers for Disease Control and Prevention (CDC), because the LC-tandem MS instrument at ICD was not fully operational (yet) at that particular time.

The LC tandem MS system represented in Figure 28 was used for the analyses. Initially, both columns are equilibrating with 100% C at 50 uL/min. Solvent C from pump #3 flows through the autosampler and through column #1. A 50uL injection is carried out, and the sample is loaded onto column #1 for 5 minutes. At this time, the valves V1 & V2 are switched, so that column #1 is connected to the gradient pumps (#1 & #2) and to the MS. A solvent gradient from 0% C to 100% C is delivered over the next 25 mins (i.e., between 5 and 30 mins after the injection), and the sample elutes to the MS. Meanwhile, column #2 is connected to pump #3 and to waste, and is equilibrated at 75 uL/min with 100% solvent C. Between 30 and 32 min after injection, both columns are equilibrated with 100% C at 50 uL/min. At 32 min after injection, the valves V1 & V2 are switched again so that pump #3 is now connected to column #2. At ~33 min, the next sample injection is made and the process is repeated using the alternate column for sample separation.

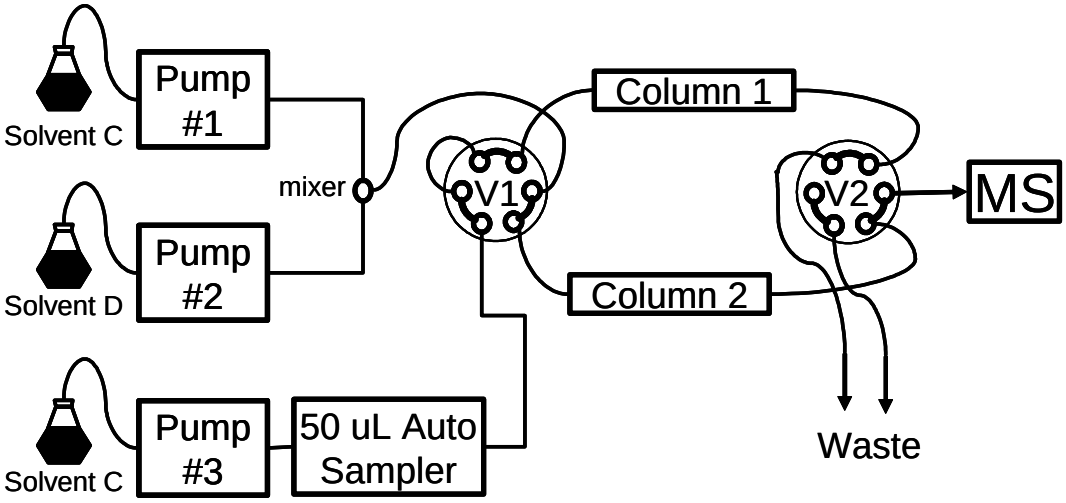


Figure 28. LC tandem MS set-up used during the method demonstration

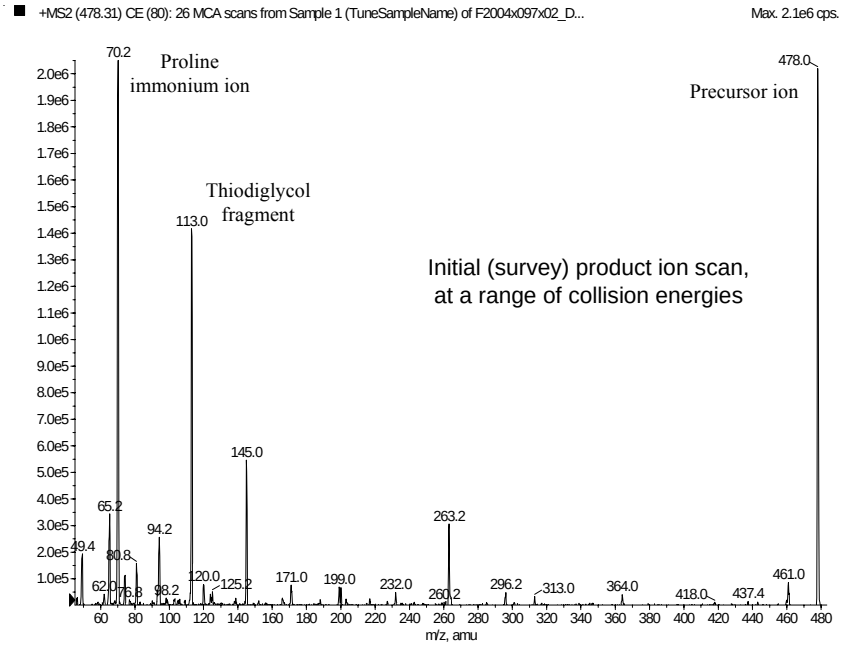


Figure 29. Initial (survey) product ion scan of (S-d_8-HETE)Cys-Pro-Phe (*m/z* 478).

The following experiments were performed:

1. MS/MS optimization on the synthetic (S-d_8-HETE)Cys-Pro-Phe, including a composite initial MS/MS fragment ion scan of *m/z* 478 and optimization of collision energy for the eight best transitions. The deuterated peptide adduct was taken, in order to not contaminate the instrument.
2. Processing (i.e., albumin isolation, pronase digestion) and mass spectrometric analysis of several plasma samples, that had been isolated from human blood that had been exposed to various concentratrions of sulfur mustard.

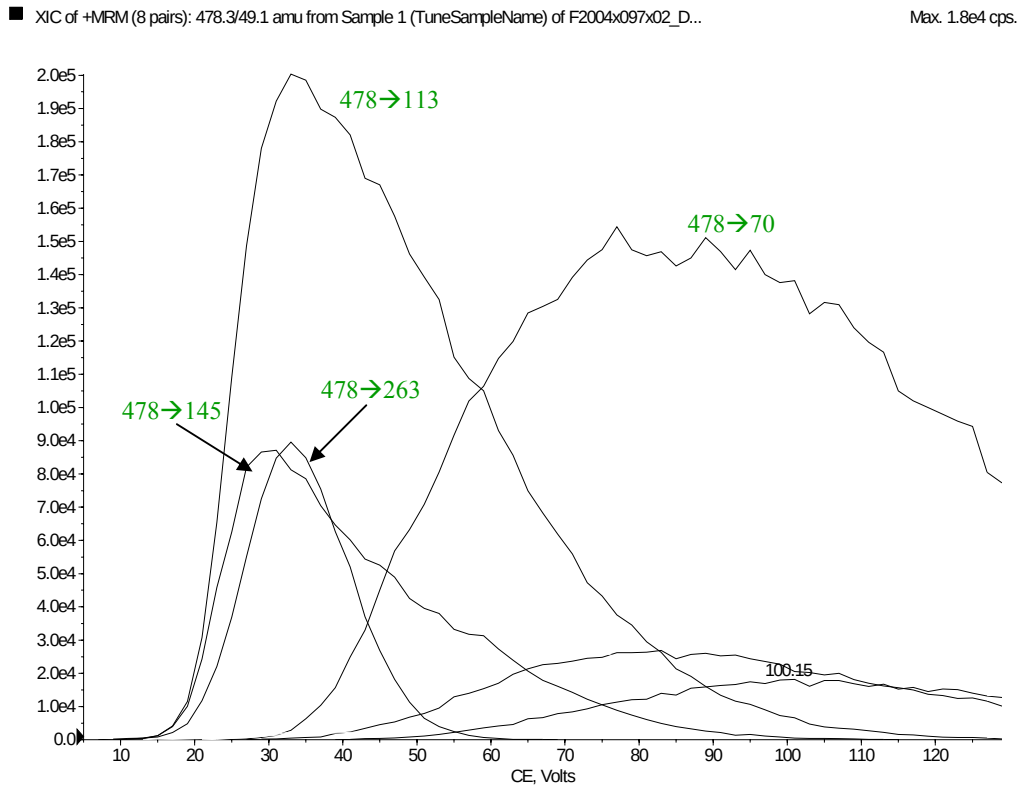


Figure 30. Optimization of collision energies for the best eight MRM transitions of (S-d_8-HETE)Cys-Pro-Phe.

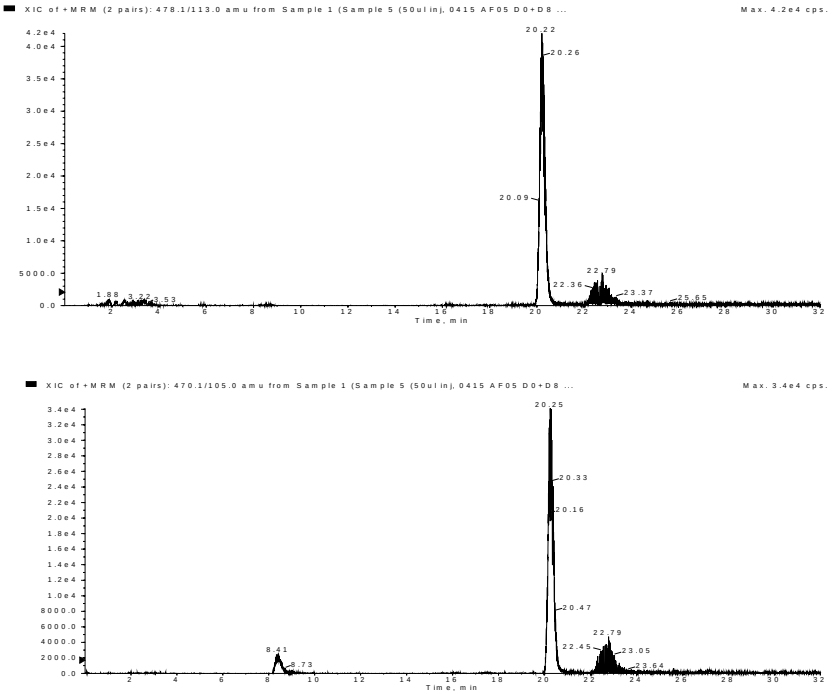


Figure 31. LC tandem MS analysis of pronase digest of albumin isolated from blood that had been exposed to sulfur mustard (10 µM; upper trace); internal standard at 10 µM (lower trace).

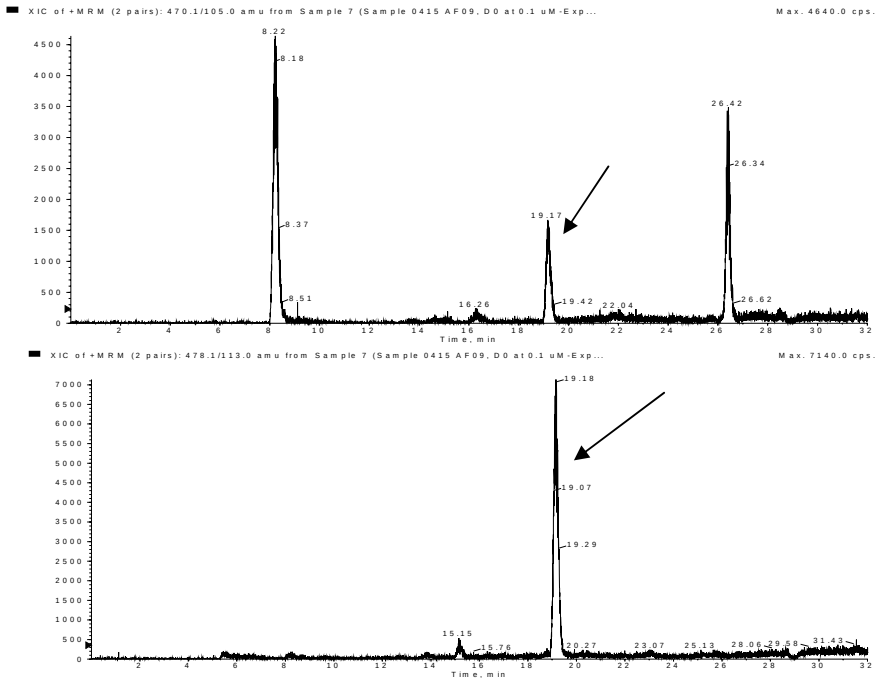


Figure 32. LC tandem MS analysis of pronase digest of albumin isolated from human blood after exposure to sulfur mustard (0.1 µM) (upper trace). The lower trace represents the d_8-internal standard (virtual exposure level: 0.5 µM).

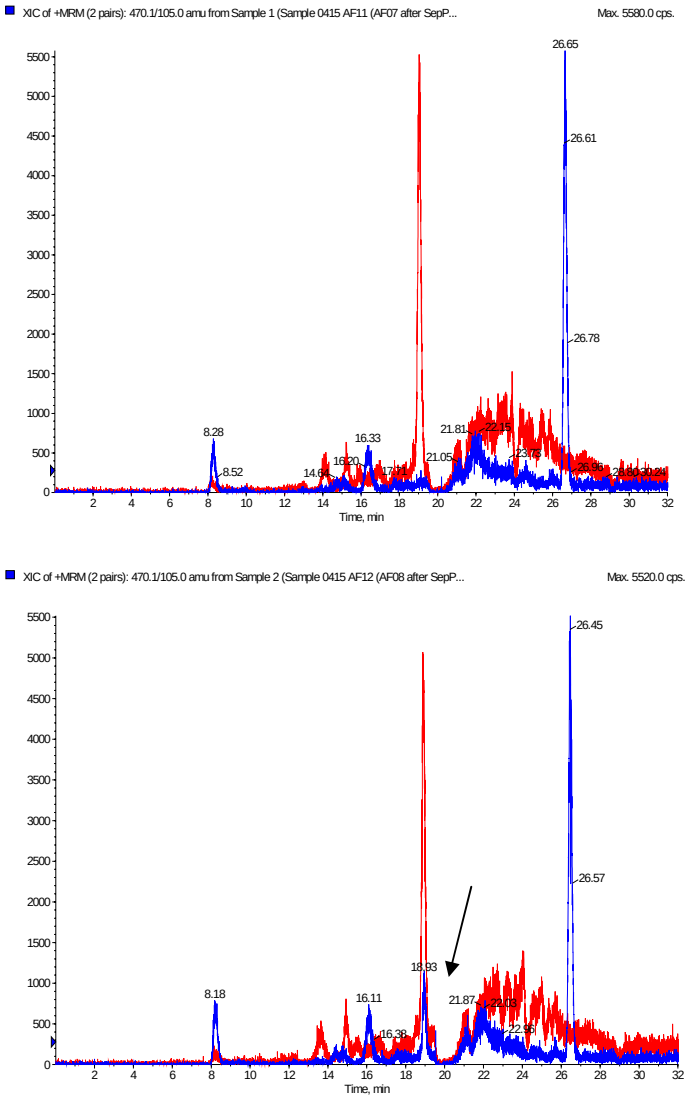


Figure 33. LC tandem MS analysis of pronase digest of albumin isolated from unexposed human blood (upper panel) and exposed human blood (10 nM; lower panel), after Sep Pak cleanup of the digest. The blue trace represents the (S-d_0-HETE)Cys-Pro-Phe, and the red trace represents the d_8-internal standard, spiked at a virtual level of 0.05 µM.

For establishing the right conditions for the instrument, the d_8-tripeptide reference standard was used (see Figures 29 and 30). The use of the corresponding d_0-reference standard was circumvented, because of possible contamination of the instrument. The method could be readily set up within one working day. First, a plasma sample with a relatively high exposure level (10 µM) was used (see Figure 31). The signal for d_0-sulfur mustard and d_8-internal standard were of comparable intensity, as was to be expected. Similar results were obtained for lower exposure levels (0.1 µM); see Figure 32. A 10 nM exposure level could still be detected when a Sep-Pak C18 step was incorporated in the procedure (see Figure 33).
The sensitivity of the instrument used at the CDC laboratory was comparable to that obtained at TNO.

IV.4 Optimization of immunoassay for screening of available antibodies against sulfur mustard adducts to proteins

IV.4.1 Introduction

The following steps were taken in our general approach to the development of an immunochemical assay for the detection of sulfur mustard adducts with proteins. It has been attempted to further improve the sensitivity and reproducibility of the ELISA for the screening of monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratin. Next, some of the most promising clones were further characterized and applied in the development of the generally more sensitive immunoslotblot assay.

IV.4.2 Screening of monoclonal antibodies directed against sulfur mustard adducts to hemoglobin and globin in a direct ELISA and optimization of the assay

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to hemoglobin. The antibodies were supplied as supernatants of clones obtained from fusions after immunization of mice with peptide haptens containing a histidine-sulfur mustard adduct derived from human globin or which have been adducted with sulfur mustard to a cysteine residue (Noort *et al*., 1995). The following procedure was applied:

1. Coating of plates with hemoglobin (50 µl of 5 µg/ml, isolated from human blood exposed to 0 and 50 µM sulfur mustard) in PBS for 1 h at room temperature,
2. washing with PBS (3 times),
3. blocking of free places with 2% FCS in PBS (30 min at room temperature),
4. washing with PBS + 0.05% Tween (3 times),
5. adsorption of 1st ab (in duplicates): supernatants of clones obtained from sulfur mustard-his-peptides fusions, and of 3H6, obtained from a mouse immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin, were applied in a 1:5 dilution, 60 min at room temperature,
6. washing with PBS + 0.05% Tween (3 times),
7. adsorption of 2nd ab: 1/1000 diluted GAM-alkaline phosphatase in PBS+0.05% ween+1% FCS, 55 min at room temperature,
8. washing with PBS + 0.05% Tween (3 times), and with DEA (1 time),
9. incubation with MUP in reaction buffer, pH 9.8, 45 min at 37 °C,
10. measurement of fluorescence with microtiter plate reader.

Supernatant of clone 3H6 did not show any specificity for alkylated hemoglobin. Also other supernatants from clones from immunizations with an adducted his-peptide derived from hemoglobin, did not show any specificity for alkylated hemoglobin. Because there was some doubt about the alkylated hemoglobin used for binding to the wall of the well on the microtiter plate we isolated new hemoglobin and globin from blood exposed to sulfur mustard. Also with these antigens no positive result was obtained.

A single clone, 183 3D5-2E11, showed some specificity for alkylated hemoglobin and alkylated globin.
Another clone, Val-1H2 (directed against the sulfur mustard adduct to the N-terminal valine of the α-chain of globin) showed some specificity against alkylated globin on plates pre-coated with glutaraldehyde.

These puzzling data stressed the need to study the different steps in the immuno assay in more detail. Parameters varied were the following:

A Sub-culturing of clone Val-1H2, to select monoclonals with freshly prepared supernatants.
B Concentration of hemoglobin or globin used for coating: 5, 10 or 20 µg/ml.
C Coating with hemoglobin in water or PBS.
D Coating with hemoglobin, old and fresh.
E Coating with globin, old and fresh.
F Coating with hemoglobin and globin over a larger exposure range (0-1 mM sulfur mustard).
G Coating with trypsinized albumin and globin.
H Pre-coating with glutaraldehyde (5%) or poly-l-lysine (100 µg/ml) (60 min at room temperature).
I Pre-coating with glutaraldehyde (0.1, 0.2 and 1%) or poly-l-lysine (10 or 100 µg/ml) (o/n at 4 °C), and coating with globin (1, 5, 10 or 20 µg/ml, 1 h at room temperature).
J Application of BioStab immunoassay stabilizer (after pre-coating with 0.2% glutaraldehyde and coating with globin, 10 µg/ml); no wash after addition of BioStab.
K Coating overnight at 4°C, at 37 °C dry, at 37 °C wet or for 1 h at room temperature with continuous vibration.
L Coating in bicarbonate, pH 9.6, instead of PBS.
M Blocking of free places with water + 5% FCS or PBS + 2% FCS.
N Variation of time and temperature of treatment with 1st ab and 2nd ab.

The following results were obtained:

Sub-cloning of clone Val-1H2

Several monoclonals were selected after subcloning of clone Val-1H2. Two of these, IIIE8 and IIIG10, were selected and their supernatants used for further optimization of the immuno assay.

Variation of coating conditions

- Although originally positive results were obtained when applying pre-coating with glutaraldehyde, in later experiments this result could not be reproduced. Even, pre-coating with glutaraldehyde, followed by coating with globin, resulted in enhancement of a-specific binding of antibody.
- Coating with hemoglobin (5 or 10 µg/ml) instead of globin resulted in extreme high (a-specific) fluorescence, even with 1:500 diluted supernatant (to the same extent as with the combination glutaraldehyde and globin).
- Pre-coating with poly-l-lysine resulted in high background fluorescence.
- Application of BioStab resulted only in a marginal improvement.
- Coating in bicarbonate instead of PBS appeared to be not an improvement.
- The concentration of globin during coating appeared to be important: 10 µg/ml gave good results both with coating for 1 h or overnight (wet), whereas with 5 µg/ml in combination with coating for 1 h at room temperature a reverse effect was observed and with coating overnight (wet) strong non-specific adsorption of antibody occurred.
- The blocking of free places with FCS appeared to be efficient as could be concluded from the low background fluorescence when only 2nd ab was added to the coated plates (without pre-coating and with coating in PBS).

Variation of time and temperature of treatment with 1st ab and 2nd ab

The best results were obtained when adsorption of both 1st ab and 2nd ab occurred for a short period (30 min) at room temperature. Upon application of the conditions which facilitate adsorption (optimal binding temperature, 37 °C, and longer incubation periods, 1h), then a-specific binding of 1st ab to globin) was preferentially increased. Enhancement of globin

concentration (to 12.5 µg/ml) resulted in a lower specific fluorescence, but a-specific binding was still more decreased. Under these conditions, even at a 1:2000 dilution of 2nd ab a distinct difference in fluorescence was observed between non-alkylated globin and globin alkylated with 100 µM sulfur mustard.

These data suggest that the immuno assay for screening of monoclonals producing antibodies against sulfur mustard adducts to globin can still further be improved by suppressing the a-specific binding of 1st ab to globin, for example by carrying out the 1st antibody adsorption at a higher concentration of Tween 20. When this results in a more sensitive and more reproducible assay than after preparing fresh supernatant of each clone, all other available monoclonal clones can be screened for efficiency of ab- binding.

IV.4.3 Screening of monoclonal antibodies directed against sulfur mustard adducts to keratin in a direct ELISA

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to keratin. More or less the same procedure was applied as originally applied for the screening of anti-sulfur mustard-globin clones, described in section IV.2.1., except that coating of plates was carried out with keratin (50 µl of 5 µg/ml, isolated from human callus exposed to 0 and 50 µM sulfur mustard) in PBS for 1 h at room temperature.

Several clones could be selected with a high specificity for keratin exposed to sulfur mustard. A number of these are presented in Table 6.

Other supernatants, including 1H10, appeared to have lost their activity. For that reason 28 clones, all originating from the same clone 1.3-C2 from the mouse immunized with peptide 1 + 2, were cultured again to produce fresh supernatant. Although there was some differentiation in growing rate of the cell cultures, all supernatants appeared to be active, indicating that all clones were still able to produce antibodies specific for sulfur mustard adducts to keratin.

In an attempt to reproduce the screening results with antibodies specific for alkylated keratin, some problems arose. On keratin samples newly prepared from callus (obtained from other local chiropodists) exposed to sulfur mustard no specificity was observed. On the originally prepared keratin samples, all clones tested were still positive. Because this suggested that something was wrong with the isolation of the keratin samples several isolation procedures, described in literature were applied, but all without the wanted specificity for adducts of sulfur mustard to the keratin.
Briefly, we applied a modified version of the original method (Sun and Green, 1978) by homogenizing the suspension (10 min), followed by sonification during the extraction phase. Also the method of Steinert (1975, 1978) was applied in which extraction occurred in Tris (20 mM), urea (8 M) and β-mercaptoethanol (0.1 M). Upon extraction all samples were centrifuged (30 min 3500 rpm) and the supernatants dialysed against water. Because dialysis resulted in the formation of a precipitate, this step was substituted by filtration through Centrex UF-2 filters (molecular cut-off 10 kDa) followed by lyophilization.
Further studies on the protein samples indicated that assessment of the keratin concentrations in the samples, pipetted from the filters after three washings with water, resulted in erroneous results when the BioRad method was applied. This suggests that in spite of the repeated washings the keratin samples still contained compounds which interfere with the protein measurements. In addition, when applying the more reliable protein precipitation method a large variation in protein concentration (from 0.2 up to 3 mg/ml) was obtained. Both phenomena may have a confounding effect on the responses obtained in the direct ELISA.

In the current studies variations are applied in both the exposure conditions of human callus to sulfur mustard and the isolation procedure of keratin to improve the reproducibility of the response of sulfur mustard exposed keratin in the direct ELISA. In addition, the originally obtained primary clones which produced antibodies specific for sulfur mustard adducts to keratin will be subcloned, followed by selection in a direct ELISA with a coating of keratin isolated from newly sulfur mustard exposed human callus or keratin treated in solution with sulfur mustard.

A complicating factor appeared to be the fact that contamination of the isolated keratins with small amounts of SDS (essential for extraction of keratin) interfered with the binding of these keratins in the immunoassays which might result in erroneous outcomes of the immunoassays.

IV.4.4 Variations in isolation of keratin from human callus

In an attempt to reproduce the screening results with antibodies specific for alkylated keratin, some problems arose. On keratin samples newly prepared from callus (obtained from other local chiropodists) exposed to sulfur mustard no specificity was observed. On the originally prepared keratin samples, all clones tested were still positive. Because this suggested that something was wrong with the isolation of the keratin samples, several isolation procedures described in literature were applied, but all without the desired specificity for adducts of sulfur mustard to the keratin.
Briefly, we applied besides the originally applied method (Sun and Green, 1978) a modification of this method by homogenizing the suspension (10 min), followed by sonication during the extraction phase. Also, the method of Steinert (1975) was applied in which extraction occurred in Tris (20 mM), urea (8 M) and β-mercaptoethanol (0.1 M). Upon extraction all preparations were centrifuged (30 min 3500 rpm) and the supernatants dialysed against water. Because dialysis resulted in the formation of a precipitate this step was substituted by filtration through a KD10 filter followed by lyophilization.
Precipitation of keratin during dialysis could also be prevented by denaturing the crude protein by heating at 65 °C for 10 min, but this did not result in an improved accessibility of the sulfur mustard adducts.

Further studies on the protein samples indicated that assessment of the keratin concentrations in the samples, pipetted from the filters after two washings with water, resulted in erroneous results when the BioRad method was applied. This suggests that in spite of the repeated washings the keratin samples still contained compounds which interfere with the protein measurements. In addition, when applying the more reliable RC DC method a large variation in protein concentration (from 0.2 up to 3 mg/ml) was obtained. Both phenomena may have a confounding effect on the responses obtained in the direct ELISA.

A significant improvement in the isolation procedure appeared to be the application of a brief homogenization step of the human callus suspension in 0.9% NaCl, immediately after sulfur mustard exposure, followed by extraction overnight in Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M), as described by Steinert (1975) and purification over a PD10 column and finally desalting by filtration through a KD10 filter.

Exposure of purified keratin to sulfur mustard resulted in precipitation of the keratin, even after exposure to only 100 μM sulfur mustard. This suggested that crosslinking by sulfur mustard made the protein insoluble and which also made the sulfur mustard adducts less accessible for the antibodies at higher degrees of crosslinking.
The homogenization of the pieces of human callus treated with sulfur mustard made the sulfur mustard adducts more accessible for the antibodies. However, at high exposure levels (more than 500 μM sulfur mustard) the accessibility of sulfur mustard adducts was still decreased.

Exposure to 250 µM sulfur mustard resulted in a higher response than exposure to 1 mM sulfur mustard. A typical experiment is presented in Figure 34.

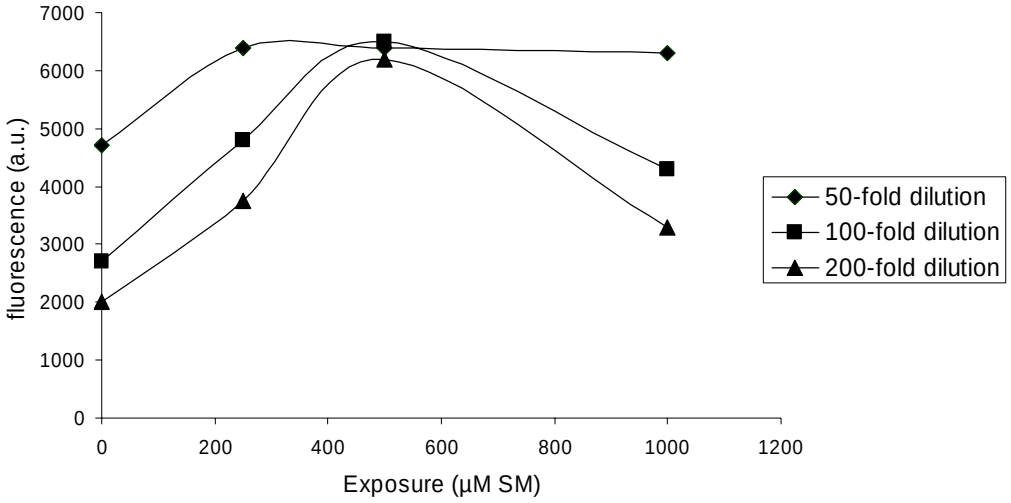


Figure 34. Direct ELISA with keratin isolated from human callus exposed to sulfur mustard (0, 250, 500 and 1000 µM sulfur mustard) with supernatants of clone 5C7 at 50-, 100- and 200-fold dilution. Data points are averages of two independent measurements of the same sample. The range between these points was about 10%.

These data clearly indicate that after high exposure the accessibility of sulfur mustard adducts has been decreased.

IV.4.5 Selection of newly prepared monoclonal antibodies directed against sulfur mustard adducts to keratin in a direct ELISA

The originally obtained primary clones which produced antibodies specific for sulfur mustard adducts to keratin were tested in a direct ELISA. Unfortunately, the 9 primary clones tested did not show any significant specific activity on newly prepared keratin (isolated from human callus treated with 50 µM sulfur mustard). Also, subcloning of 2 of the primary clones did not result in significant specific monoclonals.
For that reason, we decided to produce new antibodies. In total 8 fusions were carried out with spleen cells of mice immunized with a mix of the 3 sulfur mustard-adducted peptides mentioned before or with keratin isolated from human callus exposed to 1 mM sulfur mustard.
The mice received two immunizations followed by a booster without stimune. One mouse received a booster with stimune. Fusion of spleen cells from all mice resulted in clones producing specific antibodies on keratin isolated from human callus treated with 1 mM sulfur mustard (except mouse RM03). The mouse boostered without stimune did not behave different from the other mice. In total about 4000 clones were tested. An overview of the selection is presented in Table 6.

Table 6. Overview of the selection of hybridomas producing antibodies directed against keratin isolated from human callus treated with 1 mM sulfur mustard

Mouse	immunization	96-well	24-well	6-well	25 cm2	75 cm2	frozen
RM01	mix of 3 SM-peptides	63	25	15	9	4	**3H4**,2D10,**1C6**, 1B8
RM02	mix of 3 SM-peptides, with stimune	72	21	6	4	1	3F9,5A7
RM03	mix of 3 SM-peptides	1					
RM04	mix of 3 SM-peptides	27	8	7			1D10,2D4,**2E4**,3F6,**4F7**,5E8
RM05	SM keratin	22	6	7	6	2	5A8,**5C7**
RM06	SM keratin	12	8	5	7	1	3F8,3F9,4F11
RM07	SM keratin	20	12	8	1		3D5,2H1
RM08	SM keratin	37	15	7	2	1	**1D6**,**1E8**,2F12,3F12,4D3,4E10,5A6

Promising hybridoma-cultures in the 6-well state, or further cultured, were frozen. The most promising frozen clones have been printed in bold (more then a factor of 2 difference between exposed and unexposed keratin at 25-fold or more dilution of culture supernatant).

From mouse RM03 no specific hybridomas could be selected. From all other mice two or more promising hybridoma-cultures have been selected.

IV.4.6. Characterization of the newly prepared antibodies directed against keratin isolated from human callus treated with sulfur mustard.

Antibodies of clone 5C7 were applied in an immunoslotblot assay (Figure 35).

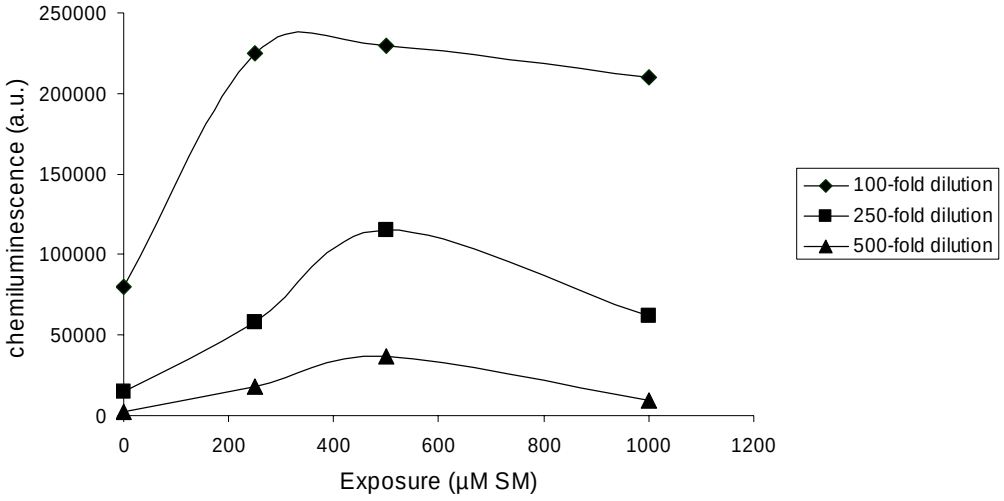


Figure 35. Immunoslotblot assay with keratin isolated from human callus exposed to sulfur mustard (0, 250, 500 and 1000 µM sulfur mustard) with supernatants of clone 5C7 (see Table 6) at 100-, 250- and 500-fold dilution. Keratin was applied in 0.5 µg aliquots in the assay. Data points are averages of two independent measurements of the same sample. The range between these points was about 10%. Chemiluminescence values above 200000 a.u. are less reliable because of the upper limits of the luminometer.

These data showed more or less the same pattern as with the direct ELISA and phage display antibodies. Again, after 1 mM sulfur mustard the accessibility of the adducts seemed to be decreased, resulting in a lower chemiluminescence.
Also supernatants of the hybridoma cultures, 3H4, 1C6 and 4F7, applied in an immunoslotblot assay showed promising specificities (data not shown).

These data indicate that, albeit not as efficient as found before with 1H10 antibodies, new antibodies are available which recognize sulfur mustard-adducts to keratin isolated from human callus exposed to sulfur mustard. In future experiments it should be attempted to further improve the accessibility of the sulfur mustard adducts on keratin.

IV.5 Cross reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin.

Clone 3H6, which had been derived from a fusion with an adducted cysteine of a peptide derived from hemoglobin, showed some specificity for both alkylated keratin (Table 7).

The same clone, and some clones derived from fusions with an adducted his-peptide derived from hemoglobin did not show any specificity for alkylated human serum albumin. However, when applied on microtiter plates coated with alkylated keratin some clones derived from fusions with an adducted his-peptide derived from hemoglobin, showed some binding (Table 8). This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Table 7. Antibody specificities of clones obtained from a fusion after immunization with haptens of partial sequences of keratin containing glutamine (1,2) or asparagin (3)-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 μM or 50 μM sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

clone	peptides used for immunization	Antibody response against keratin exposed to sulfur mustard solution of .	
		0 μM	50 μM
2.3D7	3	124	537
3.2G8	1+2+3	294	1165
1.1A8		2969	4013
1.2B6	1+2	275	1445
2.3D9	3	337	2709
2.2B5	3	494	3380
1.3C2-1D9	1+2	810	3423
1.3C2-1B4	1+2	641	3766
1H10	1+2	975	3791
1H10(2)	1+2	618	3745
3H6		586	1268

Table 8. Antibody specificities of clones obtained from a fusion after immunization with haptens of partial sequences of hemoglobin, containing histidine-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 µM or 50 µM sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

clone	Peptide* used for immunization	Antibody response against keratin exposed to sulfur mustard solution of .	
		0 µM	50 µM
D5-2E9	1	228	2628
4A3-1B3	3	238	2891
4A3-1C2	3	246	2668
4A3-2A10	3	299	2943
4A3-2B7	3	293	2996
4A3-2F4	3	327	2969
4A3-2F6	3	240	2498
4A3-1A7	3	160	1623
4A3-2G7	3	135	1578
4A3-2E8	3	397	3274

*These peptides (1: AFSDGLA(N1/N3-HETE)HLDNLK, corresponding with residues 70-82 of human β-globin; 3: L(N1/N3-HETE)HVDPENFRLLGNVK, corresponding with residues 96-109 of human β-globin) have been described in detail previously (Benschop *et al*., 2000).

IV.6 Characterization of monoclonal antibodies directed against sulfur mustard adducts to proteins

IV.6.1 Antibodies against sulfur mustard adducts to keratin

Nine clones producing antibodies against sulfur mustard adducts to keratin were selected for further characterization and subcloning. As described previously, 32 monoclonal clones, all originating from clone 1.3C2, have been selected of which antibodies showed specificity against keratins isolated from human callus treated with 50 µM sulfur mustard (Benschop *et al*., 2000).
Antibodies of one clone, 1H10, were characterized further. Cross-reactivity toward the 3 hapten peptides (GVVSTH(N-ω-HETE)QQVLRTKNK (1); GIQ(N-ω-HETE)QVTVNQSLLTPLNK (2) and GVM(Nω-HETE)NVHDGKVVSTHEK (3)) and the corresponding nascent peptides was assessed in a competitive ELISA (Table 9).

Table 9. Antibody specificity of clone 1H10 obtained from a mouse immunized with peptides 1+2 derived from adducted keratin

50% inhibition in competitive ELISA with		
	sulfur mustard-peptide (nmol/well)	Nascent-peptide (nmol/well)
Peptide 1	>20	>20
Peptide 2	1.4	6.5
Peptide 3	7.6	>20

With 1.4 nmol/well of peptide 2 50% inhibition was observed, whereas with the corresponding nascent peptide 6.5 nmol/well was required to achieve the same extent of inhibition. Peptide 3, which was not used for the immunization resulting in clone 1H10, also showed some cross-reactivity (50% inhibition with 7.6 nmol/well). All other peptides tested, including peptide 1 used for the immunization, did not show any cross-reactivity in the concentration range tested, *i.e.*, 50% inhibition is not achieved at amounts less than 20 nmol/well.

IV.7 Development of the immunoslotblot assay for the detection of sulfur mustard adducts to proteins

IV.7.1 Sulfur mustard adducts to hemoglobin

As described previously, several clones of which the antibodies recognized alkylated hemoglobin were obtained from mice immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys$_{93}$ through leu$_{106}$-lys of the β-chain of hemoglobin (Noort *et al.*, 1995). One of these, 3H6, was further characterized. These antibodies recognized sulfur mustard-modified hemoglobin in a dose-dependent way. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA. However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have attempted to apply these antibodies to an immunoslotblot assay for alkylated hemoglobin, but so far without lowering the minimum detectable concentration.

IV.7.2 Sulfur mustard adducts to human keratin

We applied the immunoslotblot assay, developed for the detection of N7-HETE-Gua in DNA, on sulfur mustard adducts with keratin using the antibodies 1H10 directed against these adducts. Keratin was isolated from human callus exposed to sulfur mustard. In Figure 36 the data are summarized.

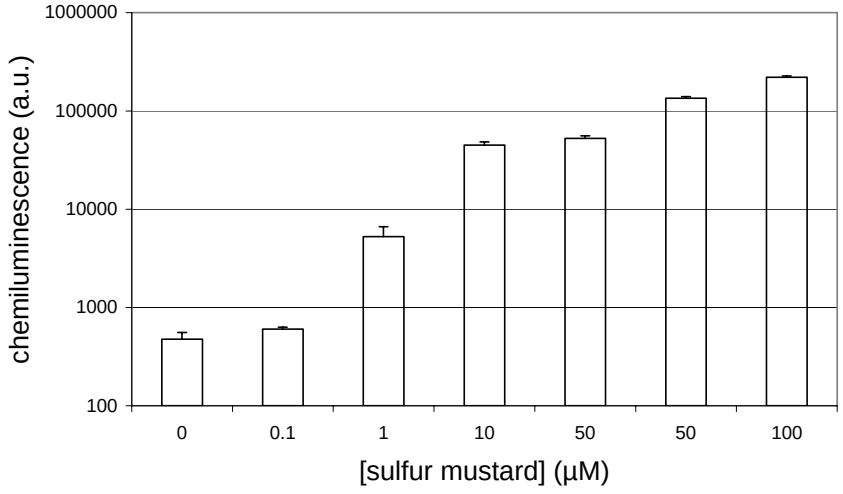


Figure 36. Immunoslotblot assay on keratin of sulfur mustard exposed human callus. Human callus was exposed to sulfur mustard at concentrations ranging from 0 to 100 μM sulfur mustard. Keratin was isolated and applied in 0.5 μg aliquots in the assay. Data are the mean of triplicates. The error bars represent the standard error of the mean.

IV.8 Selection of recombinant antibodies by antibody phage display

Antibody phage display is a very powerful technique for selecting recombinant antibodies from a large library (Winter *et al*., 1994). An antibody phage library consists of the variable regions of heavy (VH) and light (Vκ) chains of human antibodies, which are randomly combined and linked together by a polypeptide linker to form a single-chain fragment (scFv). These scFvs are fused to a minor coat protein of bacteriophage M13, pIII, resulting in phages displaying antibody fragments; phage antibodies (PhAbs). The display of scFvs on a filamentous phage offers the possibility to select PhAbs without using hybridoma technology. Phage antibodies are selected by panning the library for several rounds on an immobilized antigen. At present, large synthetic libraries are available, which are created from unrearranged V gene segments from nonimmunized healthy human donors. These libraries can be used to select antibodies against any given antigen, including foreign antigens, self antigens, nonimmunogenic antigens, and toxic antigens. The goal of this study was to select antibodies, PhAbs or scFvs, to detect adducts of sulfur mustard to keratin in human skin (see also Bikker *et al*., 2007).

IV.8.1 Selection of phage antibodies.

The Tomlinson I and J phage libraries were used to select MoPhAbs and scFvs exhibiting affinity for keratin sulfur mustard adducts. Three rounds of (subtractive)selection were performed on human keratin isolated from the callus, which was exposed to 250 µM, 1mM sulfur mustard as well as unexposed keratin (Figure 37). MoPhAbs derived from the successive selection rounds were screened for their capacity to distinguish keratin that was exposed to sulfur mustard from unexposed keratin. After the final selection, 768 individual, randomly chosen colonies from both libraries (I and J); in total 1536 colonies were used to prepare culture supernatants contained with MoPhAbs. In first instance these MoPhAbs were screened in an ELISA based assay for antigen recognition. Thirty four clones produced MoPhAbs which were able to distinguish untreated keratin from keratin that was exposed to 1mM sulfur mustard (Figure 38); nineteen showing affinity for keratin exposed to 250µM sulfur mustard and fourteen clones showing affinity for untreated keratin. All thirty four clones were selected for the production of larger phage quantities as described in the experimental procedures. Two clones retained activity, even after repeated culturing, and culturing in larger volumes. These two clones: clone ID2E1 showing affinity for exposed keratin over unexposed keratin, and clone IAD7 showing affinity for unexposed keratin over exposed keratin, were selected for more detailed characterization.

IV.8.2 PCR Screening.

Clones ID2E1 and IAD7 were checked for the presence of full length VH and Vκ insert. The DNA fragment that was obtained by PCR of DNA isolated from clone IAD7 had an estimate size of approximately 930bp, indicating the presence of full length VH and Vκ inserts (Figure 39). Molecular characterization revealed two unique amino acid sequences, showing sequence variation in the CRD regions of both light and heavy chains (see Table 10). Sequences are available from GenBank under accession nos. DQ184510 (VH ID2E1), DQ184511 (Vκ ID2E1), DQ184512 (VH IAD7), and DQ184513 (Vκ IAD7).

IV.8.3 Characterization of MoPhabs

The MoPhAbs of clone IAD7 and ID2E1 were characterized by the ISB assay and ELISA. Clone ID2E1 was selected for binding to keratin, which was exposed to 250 µM sulfur mustard. The MoPhAbs expressed by clone ID2E1 showed most distinctive recognition patterns in the ELISA assay. Antibody recognition to keratin exposed to increased concentrations sulfur mustard shows a maximal signal at 250µM sulfur mustard (Figure 40A). The results point towards specific recognition of alkylated amino acid sequence(s). At higher exposure levels, 500µM and 1mM, antibody recognition is somewhat reduced, but remains higher than the signal with unexposed keratin. The decreased interaction at higher exposure level could possibly be a result of the formation of additional inter-, or intra molecular crosslinks, decreasing the availability of the recognition site, which principally could be a monoadducts or an inter-or intramolecular crosslink.

MoPhAb IAD7 was directed against unexposed keratin. The binding characteristics of the MoPhAbs expressed by clone IAD7 were best distinctive in the ISB assay (Figure 40B). Optimal resolution was obtained when spotting 200 µl at a concentration of 2.5 µg/ml on the nitrocellulose filter. Increased exposure levels resulted in a decreased emission. This means that increased adduct levels results in a decreased stability of the antibody-keratin interaction. These results point towards the idea that the antibody-binding site is situated at an amino acid sequence, which is alkylated upon exposure; the formation of mono adducts, or crosslinks cause such structural alterations that effective antibody binding is disturbed. Both types of MoPhAbs tested did not show any binding activity to the blocking reagents milk powder and BSA (data not shown).

IV.8.4 Expression of soluble antibody fragments.

Clones ID2E1 and IAD7 were selected for expression of scFvs. The *E. coli* non suppressor strain, HB2151, was infected with phages from both clones, and induced with IPTG. Culture supernatants, putative contained with single chain antibody variable region fragments (scFvs), were collected to be detected by ISB or ELISA further use. In both cases no signal was obtained, even after repeated attempts under different conditions. This suggested that HB2151 did not express any detectable amount of scFvs, or scFvs with the destined affinity.
After DNA sequencing we found that ID2E1 contains a stop codon (see Table 10). Strain TG1 suppresses termination of translation by a stop codon and introduces a glutamate residue at these positions. In contrast HB2151 is a non-suppressor strain: it terminates translation in case of a stop-codon, explaining the absence of a signal. In line with the previous, we feel it tempting to envisage that in case of IAD7, were no signal could be detected too, the presence of a putative stop-codon between the scFv gene and g*III*, is the cause of the termination of translation of the geneproduct, the scFv-pIII fusion gene. An other explanation might be that the affinity of the scFv in a soluble form is too inefficient to be detected in ISB or ELISA, possibly due to avidity effects.

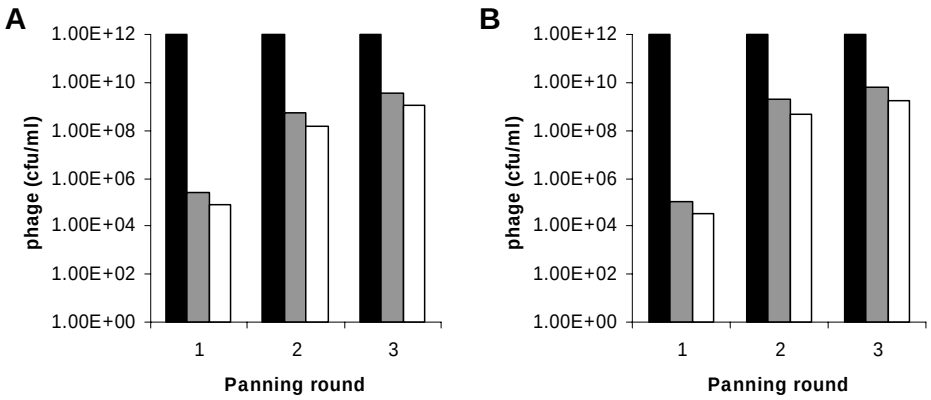


Figure 37. Number of eluted and amplified phages after each of the three rounds of selection. (A) subtractive selection of phages with affinity for keratin exposed to 250µM sulfur mustard. (B) subtractive selection of phages with affinity for unexposed keratin. The back columns indicate the number of (amplified) phages after each round, which were used for the next round of selection. The gray columns represent the numbers of phages representing the unbound fraction of phages after exposure to keratin (Fig 37A) and keratin exposed to 1mM sulfur mustard (Fig 37B) after each round. This way the bound phages were 'subtracted' from the phage population that was used for exposure to the desired antigen. The white bars represent the number of phages which were obtained after elution of the desired antigen: (A), keratin exposed to 250µM; (B), unexposed keratin, and were used for amplification. The titer was determined as pfu/ml (pfu= plaque forming unit).

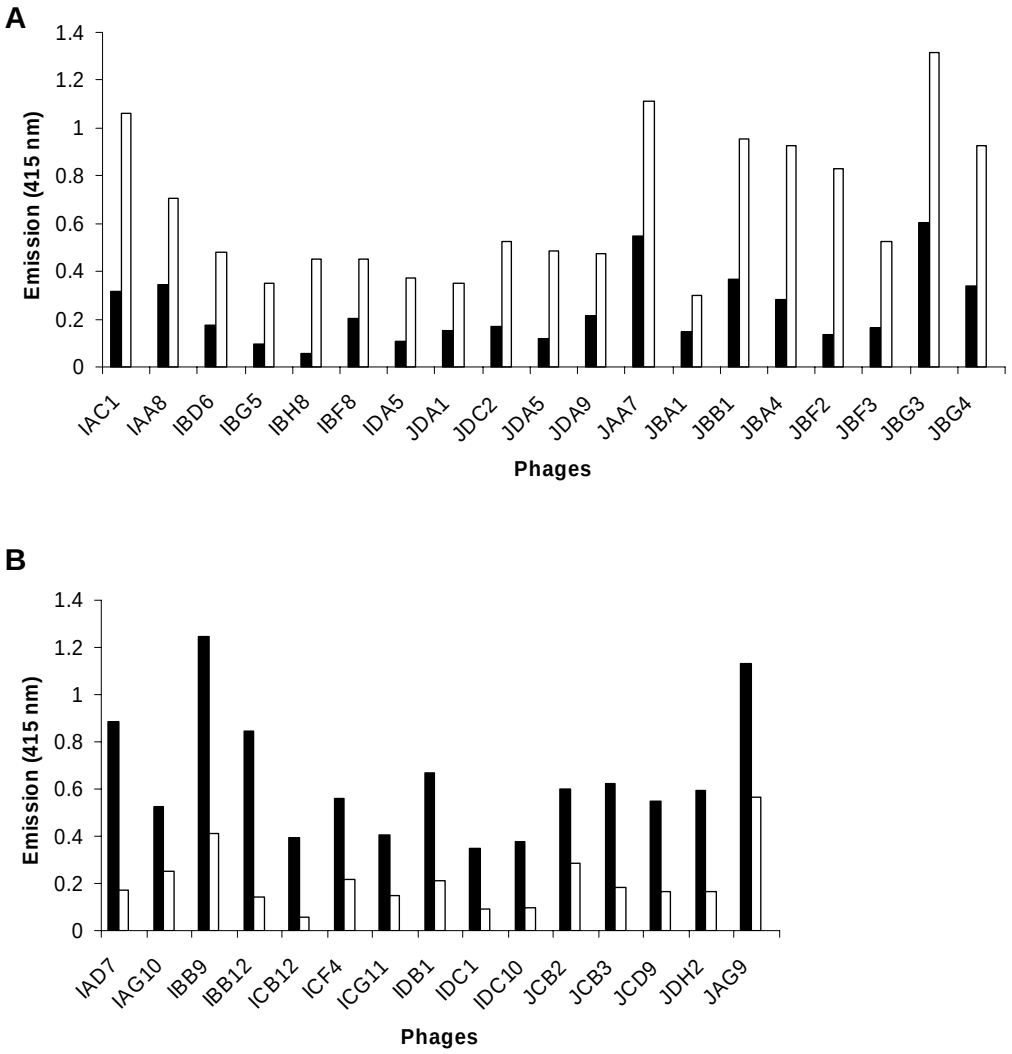


Figure 38. Screened culture supernatants of clones containing MoPhAbs which were able to distinguish keratin from keratin, which was exposed to sulfur mustard. (A) MoPhAbs showing affinity for keratin exposed to 250µM; (B) MoPhAbs showing affinity for unexposed keratin. White bars, affinity to unexposed keratin; grey bars, affinity to keratin exposed to 250µM sulfur mustard; black bars, affinity to keratin exposed to 1mM sulfur mustard. The affinity is quantified in emission of ABTS at 405nm.

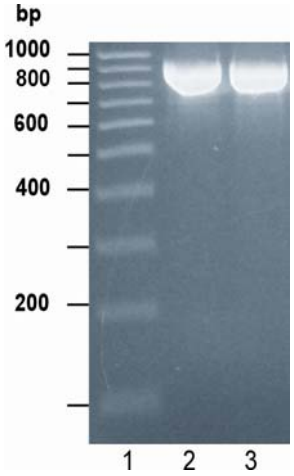
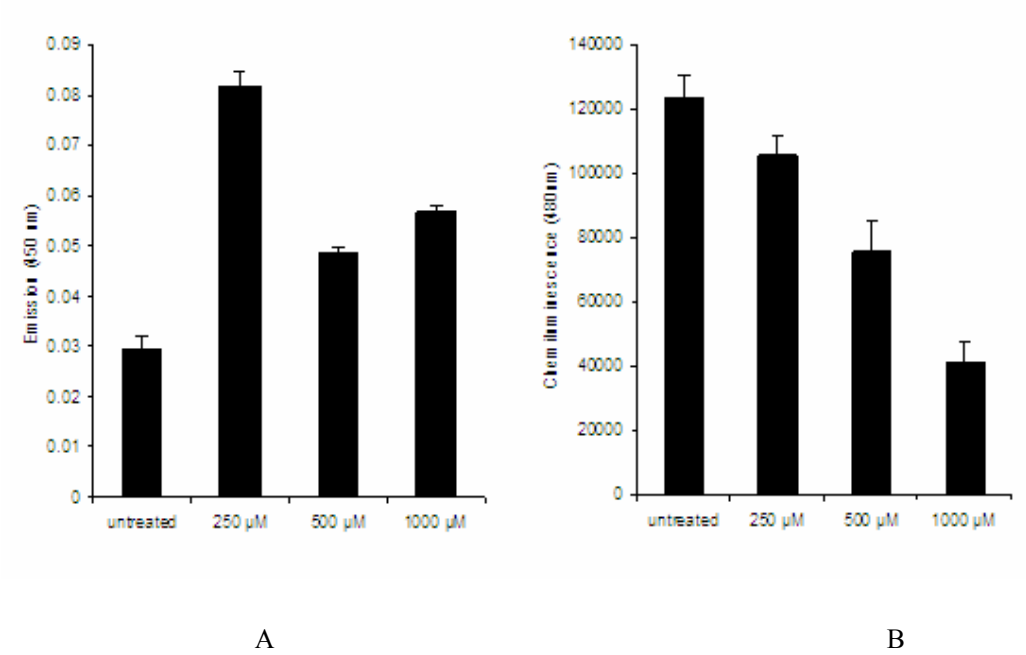


Figure 39. The clones ID2E1 and IAD7 were checked by PCR for the presence of full length Vκ and VH insert. 2% agarose gel loaded with lane 1; reference DNA Superladder-low, lane 2; PCR product of clone ID2E1, lane 3; PCR product of clone IAD7. The size of this DNA fragment corresponded to DNA contained with a complete Vκ and VH insert.



A B

Figure 40. Typical examples of binding activity of (A; left diagram) MoPhAb ID2E1 in ELISA, (B; right diagram) MoPhAb IAD7 in the ISB assay. Values are mean values + SEM (standard error of the mean), $P \leq 0.05$.

Table 10. Human germline V_H and Vκ fragments from clone ID2E1 and IAD7 FR, framework region; CDR, complementarity-determining region. Sequences are available from GenBank under accession numbers DQ184510 (VH ID2E1), DQ184511 (VL ID2E1), DQ184512 (VH IAD7), and DQ184513 (VL IAD7). E represents translation of an amber stopcodon to glutamine in *E. coli* TG1 *supE* strain. Highlighted amino acids indicate differences between ID2E1 and IAD7.

Heavy chains	FR1	CDR1	FR2	CDR2	FR2	CDR3	FR4
ID2E1	QLLESGGGLVQPGGSLELSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	SITSAGYGTCYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	ASSTFDY	WGQGTLVTVSS
IAD7	QLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	SIYTNGNNTTYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	NTAYFDY	WGQGTLVTVSS

Light chains	FR1	CDR1	FR2	CDR2	FR2	CDR3	FR4
ID2E1	DTQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	YASYLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQTYTAPDT	FGQGTKVEIKR
IAD7	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	SASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQAGNAPTT	FGQGTKVEIKR

Appendix 1. Demonstration of tripeptide SOP to USAMRICD scientist

The work plan and protocols had been sent to people involved at USMRICD and CDC, in advance of the actual visit. Also, a list of required items/chemicals had been sent to CDC in advance of our visit. All items/chemicals were present upon arrival.
Scientists/technicians involved:
Dr. J.R. (John) Barr (CDC, Emergency Response & Air Toxicants Branch), analytical chemist, mass spectrometrist.
A. (Adrian) Woolfitt (CDC, Emergency Response & Air Toxicants Branch), analytical chemist, mass spectrometrist.
J.R.(Rick) Smith (USMRICD), analytical chemist, mass spectrometrist.
A. (Alex) Fidder (TNO-PML), bio-organic chemist.
Dr. D. (Daan) Noort (TNO-PML), bio-organic chemist.

Reference samples and plasma samples to be used had been sent, stored on dry ice, to CDC in March, 2004. Since then the samples had been stored at –20 ºC
0410AF04: (S-d_0-HETE)Cys-Pro-Phe
0410AF05: (S-d_8-HETE)Cys-Pro-Phe
0410AF06: plasma (5 ml), isolated from blood, exposed to 0 uM HD
0410AF07: plasma (5 ml), isolated from blood, exposed to 100 uM d_0-HD
0410AF08: plasma (5 ml), isolated from blood, exposed to 100 uM d_8-HD

Monday, April 5, 2004.
Buffers were prepared for the affinity isolation procedure for albumin. Instead of KH_2PO_4, NaH_2PO_4 was used.
Blank plasma was obtained from CDC (as replacement for 0410AF06), just to be sure it was truly blank.
Plasma sample 0410AF07 was diluted with blank plasma to a (virtual) 10 uM exposure level.
Adrian had already prepared mass spec instrument (Sciex, API 4000), as described in "Retrospective detection of exposure to sulfur mustard: improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin – sulfur mustard adducts". (Noort *et al*., 2004).
In contrast to the work plan, we did not start with analyzing the reference standards, because of contamination risks: the instrument had already been used for analysis of the tripeptide numerous times and analytical conditions were clear.
Samples prepared, in duplicate, according to protocols:
500 ul 0 uM d_0 + 0 ul 100 uM d_8 ("double blank")
450 ul 0 uM d_0 + 50 ul 100 uM d_8 ("blank")
450 ul 10 uM d_0 + 50 ul 100 uM d_8 ("10 uM exposed")
Codes of samples obtained after pronase digestion, ready for LC tandem MS analysis:
0415AF01: double blank
0415AF02: double blank
0415AF03: blank
0415AF04: blank
0415AF05: 10 uM exposed
0415AF06: 10 uM exposed
Sample 0415AF05 gave satisfactory analytical data; the rest of the samples were analyzed overnight. Erroneously, some samples were indicated with a "0414" code instead of "0415".

Tuesday, April 6, 2004.

1. Plasma dilutions were prepared, ranging from 0, 0.01, 0.1 and 1 uM by dilution of the 0410AF07. To 500 ul of the 0 and 0.01 d_0 uM samples was added 25 ul 1 uM d_8 plasma, resulting in an end concentration of 0.05 uM. To 500 ul of the 0.1 and 1 uM d_0 samples, 25 ul of 10 uM d_8 plasma was added, resulting in an end concentration of 0.5 uM.
2. Processing of the samples was performed according to the protocols (single experiments, no duplicate). Since only a ¼ part of the worked-up, desalted albumin solution is taken for pronase digestion, the other ¾ part was stored at –20 ºC for experiments on Wednesday.
Codes of samples obtained after pronase digestion, ready for LC tandem MS analysis:

0415AF07: 0 uM d_0/0.05 uM d_8
0415AF08: 0.01 uM d_0/0.05 uM d_8
0415AF09: 0.1 uM d_0/0.5 uM d_8
0415AF10: 1 uM d_0/0.5 uM d_8
The samples were analyzed the same day. However, severe problems occurred with instrument (leakage within the electrospray probe; needle was clogged). Problems were partially fixed and the samples were run overnight again.

Wednesday, April 7, 2004.

1. The analyses of 0415AF07-10 looked satisfactorily. Sensitivity was good Instrument was fixed.
2. New pronase digests were prepared, by taking double amounts (1.5 ml) of desalted albumin samples. In this case, the double amount of pronase (200 ul of 10 mg/ml) was added.
Codes of samples obtained after pronase digestion:
0415AF11: 0 uM d_0/0.05 uM d_8
0415AF12: 0.01 uM d_0/0.05 uM d_8
0415AF13: 0.1 uM d_0/0.5 uM d_8
0415AF14: 1 uM d_0/0.5 uM d_8
These samples were not analyzed, but half of each sample was further processed on Seppak. The remaining half was stored in the freezer.

3. During the course of the pronase digestion, Rick and Adrian practiced the isolation of albumin from plasma, under supervision of Alex.
4. Eluents for the Sep-Pak cartridge procedure were prepared: 0.1% TFA/water, 0.1% TFA/10% acetonitrile in water, 0.1% TFA/20% acetonitrile in water and 0.1% TFA/40% acetonitrile in water.
5. 850 ul (half of sample) of the filtrated pronase digests were further processed on Sep-Pak C18 classic cartridges. The 40 % acetonitrile fractions were collected and evaporated.
Codes of samples after Sep-Pak clean-up:
0415AF15: 0 uM d_0/0.05 uM d_8
0415AF16: 0.01 uM d_0/0.05 uM d_8
0415AF17: 0.1 uM d_0/0.5 uM d_8
0415AF18: 1 uM d_0/0.5 uM d_8
(N.B. By mistake, these codes were not used during the analyses!; on the analytical runs they appear as “0415AF07-10 after Seppak”; these samples were analyzed on Thursday, April 8)

6. Concentration of the samples by using the Savant Speedvac apparatus proceeded too slowly. Therefore, the samples were lyophilized overnight in a Virtis “Genesis” 25 EL lyophilization unit.

Thursday, April 8, 2004.

1. Samples 0415AF15-18 (i.e. with codes "0415AF07-10 after Seppak") had been nicely concentrated. The residues were dissolved in 0.1% TFA in water (55 ul) and analyzed according to the protocol.
2. Analytical data were satisfactory; peak ratios d_0/d_8 were as expected.
3. All LC tandem MS runs were compiled into a Powerpoint file by Adrian.
4. It was decided that it was not necessary to run samples at Friday, April 9.

V DISCUSSION

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; albumin adducts (Part A)

We succeeded in significantly shortening the procedure for albumin isolation and subsequent pronase digestion. First, we found that the precipitation step with $CaCl_2$ can also be performed for only one hour, instead of overnight. Eventually, *i.e.*, after pronase digestion and LC-tandem MS analysis, the same results were obtained, irrespective of the time period of the $CaCl_2$ step. Furthermore, we found that a commercially available affinity column for albumin could be advantageously used. After the affinity chromatography procedure and rapid desalting of the albumin on a PD10 column, the purified albumin sample is recovered as a solution in aqueous NH_4HCO_3. The thus obtained solution can be used immediately for pronase digestion.

The use of an internal standard, *i.e.*, a plasma sample isolated from blood that had been exposed to d_8-sulfur mustard, has also been worked out in combination with the affinity chromatography procedure. In previous experiments we used a 4 M urea solution of powdered d_8-sulfur mustard alkylated albumin. However, we found that the precipitated protein had poor solubility in 4 M urea. Moreover, the presence of large amounts of urea has negative effects on the LC-tandem MS analyses of the pronase digest.

The minimal observable exposure level (in vitro) could be lowered a factor of 10, *i.e.*, 1 nM, by using larger amounts of albumin (20 mg instead of 3 mg). However, due to the large amounts of peptide material injected the micro-HPLC column was often overloaded and showed poor separation characteristics. Therefore, the 1 nM level could not reproducibly be analyzed.

Attention has been paid to the fluorescence derivatization of the sulfur mustard peptide adduct (S-HETE)Cys-Pro-Phe, derived from human serum albumin. We could derivatize the reference standard (S-HETE)Cys-Pro-Phe with 5/6 carboxyfluorescein succinimidyl ester (FAM-SE) and succeeded in subsequent analysis by means of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). Furthermore, we succeeded in detecting the tripeptide adduct in a pronase digest spiked at a high level with the synthetic reference standard, albeit after pre-purification with reversed-phase HPLC. Pre-purification of the samples was required because otherwise proper analysis by means of capillary electrophoresis was not possible (overloading of the capillary). Also, it is important to perform the derivatization reaction on a sample that is as clean as possible. Unfortunately, the adduct could not be detected in a derivatized pronase digest from albumin that had been isolated from blood after exposure to sulfur mustard (1 mM). In the area in the electropherogram where the FAM-(S-HETE)Cys-Pro-Phe derivative elutes an interfering peak is present, which precludes unambiguous analysis. Furthermore, we believe that at low adduct concentrations and in the presence of highly abundant competing amino acids, the derivatization reaction proceeds too sluggishly. However, even when the latter problems can be solved, we believe that the fluorescence-based method can never surpass the MS-based method because of the quite laborious sample prep, e.g., pre-purification of samples with HPLC before CE-LIF analysis, required for proper CE-LIF analysis, and the lower selectivity of CE-LIF compared to LC-MS/MS.

Columns with immobilized pronase, or other digestive enzymes might enable the construction of an automated system of (reactor) columns, coupled to a tandem mass spectrometer, in which an unprocessed plasma sample can be introduced. This would be highly convenient for use under field laboratory conditions, and more importantly, would speed up the actual diagnosis (see Volume II of this report for several exploratory experiments).

In *in vitro* experiments with [^{14}C]-labelled sulfur mustard we found that the free cysteine-34 residue in rat albumin is also prone to alkylation by sulfur mustard. It was found by LC-tandem MS analysis that a tripeptide (S-HETE)Cys-Pro-Tyr was formed after pronase digestion. The level of alkylation at the particular cysteine residue was approximately 5%. On the basis of these results we believe that the rat is a good animal model for studying the persistence of the adduct. Rats were exposed (i.v.; 0.3 mg/kg) to sulfur mustard and at certain time points the animals were killed and their blood was collected. After addition of internal standard, albumin was isolated from the plasma by affinity chromatography. Globin was isolated from the erythrocytes by precipitation in acetone/HCl. Subsequently, the albumin samples were treated with pronase and the digests were analyzed by means of LC-tandem MS. Initially, an increase in adduct level could be observed. Subsequently, the adduct level decreased rapidly. The observed half-life time of sulfur mustard alkylated albumin was 2 days, which is in accordance with literature values for albumin adducts (1-3 days; see for instance Troester *et al*. (2002). The albumin adduct was no longer detectable after 7 days after the exposure. We have to keep in mind, however, that for humans the half-life of albumin is much higher (20-25 days), implicating that the adduct might be detected for a longer period of time after exposure.
The corresponding globin samples were analyzed for the presence of adducts to the N-terminal valine residues. Remarkably, the adduct level clearly increased during the first two days, which implicates that there is still free sulfur mustard present during that time, which causes accumulating damage. Interestingly, Langenberg *et al*. (1998ab) already observed a long terminal half-life of sulfur mustard in blood after intravenous exposure of hairless guinea pigs to sulfur mustard. The slow elimination of unchanged sulfur mustard was already observed by Maisonneuve *et al*. (1993), after i.v. administration of the agent to rats. Obviously, the long terminal half-life of sulfur mustard in blood is the result of redistribution from the tissues into the blood. Probably, sulfur mustard accumulates in the adipose tissue after which it can slowly re-enter the blood stream. Also, it might accumulate in the cell membranes, *e.g*., of the red blood cell, from which it is slowly released. This phenomenon was also observed in a previous Cooperative Agreement (DAMD17-97-2-7002), in which a marmoset was exposed to sulfur mustard. However, this experiment was performed with only one animal (see below for further information on this past experiment). The N-terminal valine adduct level decreased more or less linearly, in accordance with the life-time of the erythrocyte of the rat (reported life-time of rat erythrocyte 65 days (Walker *et al*., 1992). The adduct to the N-terminal valine could still be detected after 28 days. Since the aim of these experiments was to study the persistence of the albumin adducts in vivo, we have used i.v. administration (as was also stated in the Statement of Work) of sulfur mustard for these initial experiments in order to be sure that well-defined amounts of sulfur mustard were available for adduct formation. Nonetheless, there are various clues that sulfur mustard will also enter the blood stream during more 'realistic' battlefield exposure scenarios, e.g., by inhalation or by percutaneous exposure. For instance, Langenberg *et al* (1998ab) reported measurable concentrations of DNA-sulfur mustard adducts in blood of hairless guinea pigs after nose-only exposure to sulfur mustard (dose corresponding to 1 LCt50). It is evident that in such case also albumin adducts will be formed since, in contrast to DNA, albumin is not enclosed within a cell membrane. In the same study, DNA adducts could be analyzed after percutaneous exposure (1 Lct50) of hairless guinea pigs to sulfur mustard vapor; even intact sulfur mustard could be analyzed in this case up to concentrations of 12 ng/ml (75 nM) blood. Such concentrations, and also lower concentrations, will definitely give rise to the formation of measurable concentrations of albumin adducts.

Within the context of our previous cooperative agreement marmosets had been exposed to sulfur mustard and blood samples had been taken at regular time points for determination of the N-terminal valine adduct to sulfur mustard. It appeared that the N-terminal valine adduct was still detectable 94 days after the exposure had occurred. We had stored the plasma samples (we had only used the erythrocytes in our previous studies) in the freezer for use at a later stage. The amino acid sequence of marmoset albumin was not known, so actually we did

not really know whether there is any free cysteine residue present in this protein. After pronase digestion of a small amount of albumin from the exposed marmoset, the tripeptide (S-HETE)Cys-Pro-Phe could be analyzed. As should be expected, the particular albumin adduct was quite stable in the marmoset in vivo, probably as stable as the protein itself. The adduct could still be determined after at least 28 days after the exposure. It has to be remarked, however, that the analyses were run on a Q-TOF instrument, and not on a triple-quad instrument, which is generally better equipped to measure transitions resulting in a better sensitivity. When it is assumed that the life-time of albumin is not affected by alkylation with sulfur mustard, the half-life of marmoset albumin is approximately 7 days.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; histidine adducts (Part B)

Although histidine adduct are the most abundant adducts resulting after exposure of proteins to sulfur mustard, there is no sensitive method for their analysis available. For the histidine adducts a convenient isolation procedure from acidic hydrolysates was developed based on cation exchange chromatography. Unfortunately, this method proved to be rather irreproducible until now. Furthermore, a derivatization method for GC-MS analysis was developed, which allows the analysis of the histidine adducts as tris(trifluoroacetyl)derivatives. The detection limit for GC-MS analysis of this compound is 3 ng/ml; 3 pg absolute.
Attempts to analyze the histidine adduct as a hydrazide, resulting from treatment of globin with hydrazine, failed mainly due to the instability of the hydrazide functionality.

Derivatization of the histidine adduct with pentafluorobenzyl bromide resulted in the formation of the (tris)pentafluorobenzyl derivative. This derivative could not be analyzed by means of GC-MS, probably because of the charged imidazole ring. However, it turned out that it has favorable properties for LC tandem MS analysis. It has a long retention time, compared to other derivatives, which might enable Sep-pak clean-up of real samples containing the particular adduct.

We also studied the feasibility of fluorescence detection of sulfur mustard-histidine adducts. Although HPLC with fluorescence detection is much less specific than mass spectrometric detection, it has the advantage that yoctomole amounts of amino acid derivatives can be analyzed. A number of fluorescent derivatives of the histidine-adduct were synthesized, which were used as reference compounds. The carboxyfluoresceine (FAM) derivative of N1/N3-histidine was chosen for further analyses, because it can be prepared quite easily using a commercially available reagent and it proved to be a stable derivative, in contrast to the FITC derivative that slowly rearranged. Capillary electrophoresis with LIF detection gave the best results. Although the detection limit of the FAM derivatives of N1/N3-HETE-histidine was determined to be 20-25 pg/ml, this method could not be applied to processed acidic digests of globin or albumin. As was the case with the tripeptide adduct, we believe that the derivatization reaction of the adduct does not proceed at low concentration levels, probably due to high abundant components like native amino acids. Furthermore, one of the disadvantages of not following an MS-based approach is that it is hard to distinguish between all the fluorescent derivatives, especially when the compound of interest is only present in low amounts compared to other compounds as is definitely the case when analyzing adducts.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adduct; comparison of the albumin – tripeptide method with the histidine adduct method (part C)

Comparison of the two methods, i.e., tripeptide adduct and histidine adduct method, reveals the following. The disadvantages of the histidine-adduct method are obvious. In order to release the adduct from the protein, an acidic hydrolysis step (with a duration of 16 h) is required that results in a mixture of amino acids that have basically identical properties. Subsequently, laborious ion-exchange and derivatization steps are required in order to differentiate between the various components. Furthermore, the ion-exchange work-up step was not readily reproducible. The procedure for analysis of the tripeptide adduct (S-HETE)Cys-Pro-Phe originating from albumin is a very rapid, straightforward method for diagnosis of exposure to sulfur mustard, and is in our view at this moment superior to the method for determination of histidine adducts. The work-up of the plasma sample is minimal, no derivatization is required, and the method can probably be automated. A Standard Operating Procedure (SOP) was drafted on the basis of a tentative version. The SOP for analysis of the tripeptide adduct was demonstrated to a scientist of USAMRICD. In this particular case the demonstration was performed within the laboratories of the Centers for Disease Control and Prevention (CDC), because the LC-tandem MS instrument at ICD was not fully operational (yet) at that particular time. The demonstration was well-prepared: reference compounds, a list with required materials, chemicals etc., protocols and a work-plan were sent in advance to both parties (CDC and USAMRICD). All required items arrived on time and, if required, were stored at -20 ºC. We did not start with the analysis of reference standards, because we did not want to contaminate the instrument with the analyte. The analysis started with a 10 µM exposure level, including isotope dilution by using plasma isolated from blood that had been exposed to d_8-sulfur mustard. Peak ratios d_0/d_8 were as to be expected. There were no problems with contaminated blanks. Next, lower-exposed samples were studied; results were satisfactory. Finally, the Sep-Pak C18 procedure was demonstrated in order to enhance the sensitivity of the procedure. Overall outcome of the method demonstration was that it was possible to set up the SOP at a different, well-equipped laboratory within a relatively short period of time (one working day), and that it was possible to fully transfer the method to an analytical scientist, within two working days.
The interindividual variation of the in vitro sensitivity of human blood to sulfur mustard was determined, following the SOP for the tripeptide assay. The deviation found was within the range of 10%. Furthermore, the intra-individual variation and the day-to-day variability of the assay was also shown to be acceptable.
Currently, the tripeptide method is routinely used at CDC in case of of alleged exposures to sulfur mustard. It is envisaged that the scope of the tripeptide method is not limited to sulfur mustard and that it can become a generic method for diagnosis of exposure to a wide array of alkylating agents.

Immunochemical protein adduct analysis (Part D)

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Therefore, antibodies were raised against S-HETE-cysteine in partial sequences of human hemoglobin in our previous studies (Benschop *et al*., 1995). However, the minimum detectable concentration obtained for *in vitro* exposure of human blood with these antibodies was only 50 µM of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts was a major topic of the current study.
Investigations were performed on three proteins, *i.e.*, hemoglobin, albumin, and keratin. The accessibility of the adducts for immunochemical analysis is supposed to increase in this order, *i.e.*, hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma,

whereas keratins in the skin are directly accessible from the environment for sulfur mustard and for reagents.

Immunochemical analysis of hemoglobin adducts; characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin
Antibodies (clone 3H6) raised against an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin, were further characterized. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA. However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have attempted to apply these antibodies in an immunoslotblot assay to alkylated hemoglobin. Several other clones were obtained from the above-mentioned immunization which produced antibodies that recognize alkylated hemoglobin. These clones are now available for screening against sulfur mustard adducts to globin.

Immunochemical analysis of hemoglobin adducts: antibodies against peptide haptens containing a histidine-sulfur mustard adduct
N1/N3-HETE-Histidine is the most abundant amino acid adduct formed in hemoglobin (and albumin, *vide infra*) after exposure of human blood to sulfur mustard (Noort *et al*., 1997). In addition, three out of the five sites of alkylation within the tertiary structure of hemoglobin are histidine residues, *i.e.*, α-his_{20}, β-his_{77} and β-his_{97} (Noort *et al*., 1997). Therefore, partial sequences of hemoglobin containing these adducted amino acids were synthesized as haptens and used for raising antibodies (Benschop *et al*., 2000).
From all haptens, clones were obtained producing antibodies with specificity for hemoglobin treated with 50 μM sulfur mustard. For the screening of these clones in order to select the most effective antibodies the test procedure should be optimized first. It appeared that coating of the plates with 10-12.5 μg/ml globin and applying a rather high dilution of 2^{nd} ab (1:1000 or 1:2000) resulted in the highest specificity for the detection of sulfur mustard adducts to globin. These data suggest that the immuno assay for screening of monoclonals producing ab's against sulfur mustard adducts to globin can still further be improved by suppressing the a-specific binding of 1^{st} ab to globin, for example by carrying out the 1^{st} ab adsorption at a higher concentration of Tween 2.

Immunochemical analysis of albumin adducts
An electrophilic compound has to cross the cell membrane of the erythrocyte in order to react with hemoglobin. Therefore, adduct formation with plasma proteins might be more efficient. The most abundant plasma protein is albumin, which has a relatively slow turn-over in human beings (half-life 20-25 days). It was found that a proportional amount of sulfur mustard (approximately 20%) was bound to albumin isolated from human blood treated with sulfur mustard (Benschop *et al*., 2000).
We attempted to raise antibodies against the synthesized T5 fragment (*vide supra*) of human albumin containing an alkylated cysteine and against the alkylated human serum albumin itself. Unfortunately, all originally positive clones lost their specificity for alkylated human albumin during the selection process. These data suggest that the immunogenicity of the HETE-moiety as present in this peptide is not very high and that possibly the alkylated T5-sequence in the sulfur mustard-exposed albumin is poorly accessible.
Antibodies raised against the other alkylated proteins (hemoglobin and keratin) did not show any cross-reactivity with sulfur mustard adducts to albumin.

Immunochemical analysis of keratin adducts
In addition to the respiratory tract, the skin is a major target for vesicants such as sulfur mustard. Proteins in the skin, particularly those in the stratum corneum, are readily accessible to agents. Since keratin is the most abundant protein in stratum corneum and epidermis, methods for retrospective detection of skin exposure to sulfur mustard were developed in the present study.

Keratins (MW 40-70 kDa) form the backbone of the intermediate filaments (IFs) in epithelial tissues (Sun and Green, 1978; Steinert, 1978). In basal epidermal cells almost 30% of all synthesized proteins are keratins. Their structures are closely related and can be represented by a central α-helix rich domain (length 300-350 residues) flanked on either side by non-helical domains of variable size and chemical character. The helical segments contain heptad repeats of hydrophobic residues. In addition, a conserved periodic distribution of acidic (aspartic acid, glutamic acid) and basic (arginine, histidine and lysine) amino acids is found in IFs. The termini contain *inter alia* inexact repeats of glycine and (phospho)serine residues. Amino acid sequences of a number of human keratins have been documented (Steinert, 1978). Most data indicate that the end domains are predominantly located on the surface of the IFs.

For development of an immunochemical assay, two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, as earlier described (Benschop *et al*., 2000). This approach appeared to be very successful for raising antibodies (Van der Schans *et al*., 2002). Fusions from mice immunized with only one peptide or with a mixture of two or three peptides resulted in specific antibodies to sulfur mustard adducts in keratin isolated from human callus. Antibody 1H10 was raised against adducts present in end domains of keratins K5, K14 and K1, of which K5 and K14 are two of the most important keratins in basal cells and K1 is the main keratin in the stratum corneum. The competitive ELISA turned out that antibody 1H10 only recognised sulfur mustard adducts to peptide 2 which contained amino acid sequences present both in end domains of K1 and K5. Furthermore, it also appeared important to apply freshly prepared supernatants of cultures of clones producing specific antibodies.
The data obtained with the immunoslotblot assay suggest that a lower detection limit of 0.2 μM sulfur mustard exposure should be feasible (or 0.3 s exposure to saturated sulfur mustard vapor). As can be derived from the binding data of [^{14}C]sulfur mustard to keratin, presented previously (Benschop *et al*., 2000), this corresponds to 25 fmol of adducted sulfur mustard at 0.5 μg of keratin blotted to the nitrocellulose filter, and to 1 sulfur mustard adduct among $5x10^7$ unadducted amino acids in keratin, or 1 sulfur mustard adduct per 10^5 keratin molecules.
Some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. Unfortunately, the experiments with 1H10 antibodies could not be reproduced with newly prepared keratin from sulfur mustard-exposed human callus. Nevertheless, newly prepared antibodies could be successfully applied in the immunoslotblot assay, albeit with a lower sensitivity.

Cross-reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin

Cross-reactivity was observed for antibodies raised against partial sequences of hemoglobin containing a histidine-sulfur mustard adduct. These antibodies show specificity not only for alkylated hemoglobin but also for alkylated keratin. This suggests that the specificity depends in some cases mainly on the presence of the 2-hydroxyethylthioethyl moiety and not on the amino acid to which this moiety is bound. If so, these antibodies can be valuable for immunochemical staining applications, *e.g.*, in histochemical identification of alkylated cell proteins in human skin.
These antibodies did not show specificity to albumin or trypsinized albumin alkylated by sulfur mustard. Similar results were obtained for one of the clones raised against a partial sequence of keratin containing a glutamine-sulfur mustard adduct, which showed a positive response on sulfur mustard-exposed human skin. We were not successful in raising antibodies against sulfur mustard treated albumin when using a partial sequence of albumin containing the major adduct formed in this protein with sulfur mustard (cysteine-34, *vide supra*). These results may suggest that sulfur mustard adducts in albumin are poorly accessible.

Selection of recombinant antibodies by phage display

The purpose of this part of the study was to generate antibodies to detect sulfur mustard adducts on human keratin. In addition to the classical pathway of antibody generation as performed by the hybridoma technique, we explored the suitability of phage display technologies. In comparison to phage display, the hybridoma technique is significantly more expensive and time consuming. Moreover, phage display does not require immunizations and the sacrifice of laboratory animals.

By using the Tomlinson I & J libraries (Winter *et al*., 1994) we have been able to select a single MoPhAb, denoted IAD7, which was able to distinguish unexposed keratin from keratin that was exposed to various concentrations sulfur mustard (Bikker *et al*., 2007). MoPhAb IAD7 is directed against the native, unexposed keratin. Exposure to increased concentrations of sulfur mustard results in a decreased affinity to keratin, as shown in ELISA and ISB. It is plausible that structural changes in the protein, caused by adduct formation and crosslinking (Byrne *et al*., 1996; Dillman *et al*., 2003) may hinder effective antigen recognition. Based on these characteristics MoPhAb IAD7 would be a suitable antibody that may be used in the detection and diagnose of sulfur mustard exposure to the human skin. We argue that MoPhAb IAD7 may be used in parallel with antibodies which recognize the actual sulfur mustard adduct as complementary antibody or negative control.

We have also been able to generate an antibody (ID2E1) that recognizes keratin that had been modified by sulfur mustard, by applying phage display (Bikker *et al*., 2007). Antibody recognition to keratin exposed to increased concentrations sulfur mustard shows a maximal signal at 250µM sulfur mustard. The results point towards specific recognition of alkylated amino acid sequence(s). At higher exposure levels antibody recognition is somewhat reduced, but remains higher than the signal with unexposed keratin. The decreased interaction at higher exposure level could possibly be a result of the formation of additional inter-, or intra molecular crosslinks, thereby decreasing the availability of the recognition site. It is noteworthy to mention that these results are very much in line with those obtained with the antibodies that were obtained with the hybridoma technique. These antibodies show an optimal signal at 500 µM sulfur mustard, also decreasing at higher exposure levels.

VI KEY RESEARCH ACCOMPLISHMENTS

1. The alkylated cysteine-34 residue in albumin is a highly sensitive biomarker for exposure to sulfur mustard.
2. A novel, rapid isolation procedure for albumin, based on affinity chromatography, has been applied for diagnostic purposes.
3. The lowest detectable exposure level in human blood for determination of the alkylated cysteine-34 residue in albumin with the tripeptide method has been improved with a factor of 10 (from 10 nM to 1 nM), by increasing the amount of albumin. However, in this form the procedure proved to be poorly reproducible.
4. The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to sulfur mustard-d_8, has been worked out completely.
5. A derivatization procedure was developed for analysis of the tripeptide (S-HETE)Cys-Pro-Phe with CE-LIF.
6. It was determined that the tripeptide can be determined in derivatized pronase digests by means of CE-LIF, but only when spiked at high levels.
7. The particular albumin sulfur mustard adduct can be detected in the rat *in vivo* at least 7 days after the exposure, with a half-life of approximately 2 days for the albumin adduct, which is in accordance with literature values for rat albumin.
8. The adduct to the N-terminal valine residue in hemoglobin can be detected in the rat *in vivo* at least 28 days after the exposure.
9. The level of N-terminal valine sulfur mustard adduct increases during the first 2-3 days after the exposure, indicating the presence of intact sulfur mustard during that period.
10. The particular albumin - sulfur mustard adduct is stable in the marmoset *in vivo*, at least 28 days after the exposure.
11. A convenient isolation procedure for the histidine adducts has been developed, based on cation-exchange chromatography.
12. A GC-MS derivatization reaction for the histidine adducts of sulfur mustard has been developed, which enables relatively sensitive detection of the adduct. However, no satisfactory results were obtained when the adduct was in a complex matrix.
13. Several fluorescent derivatives of the histidine adducts have been prepared; the 5/6-carboxyfluorescein (FAM) derivatives could be detected at a level of 20-25 pg/ml with capillary electrophoresis – laser induced fluorescence detection.
14. Upon comparison of the tripeptide method and the histidine method, the tripeptide method is by far the best candidate to be developed into a Standard Operating Procedure.
15. The interindividual -, intraindividual - , and day-to-day variability of the tripeptide assay were determined to be acceptable.
16. An SOP for the tripeptide assay was drafted.
17. The SOP was successfully demonstrated and transferred to a scientist of USAMRICD.
18. Several clones are available for producing antibodies which show specificity not only for hemoglobin alkylated with 50 µM sulfur mustard but also for alkylated keratin.
19. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
20. An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin.
21. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 µg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 µM.
22. Although not as efficient as found before with 1H10 antibodies, new antibodies have been prepared which recognize sulfur mustard-adducts to keratin isolated from human callus exposed to sulfur mustard.
23. An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur

mustard. To our knowledge, this is the first report describing the use of phage display for the detection of post-translational modifications.

24. Thirty four culture supernatants, contained with MoPhabs, were able to distinguish untreated keratin from keratin that was exposed to sulfur mustard.
25. Two clones, IAD7 and ID2E1, recognizing unmodified keratin and keratin modified by sulfur mustard, respectively, retained their activity after repeated culturing and culturing in larger volumes.
26. The kinetics of both hybidoma acquired antibodies as well as the phage antibodies directed agains sulfur mustard adducts on keratins are comparable. At low exposure levels (up to 250 μM sulfur mustard) there is an increase of antibody binding, whereas at higher exposure levels (> 500 μM sulfur mustard) the signal is decreasing. These results strongly suggest that the decreased interaction at higher exposure level could be a result of the formation of additional inter-, or intra molecular crosslinks decreasing the availability of the recognition site.

VII REPORTED OUTCOMES (from beginning of cooperative agreement to end, including modification)

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NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

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Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins". Presented by D. Noort at "8th European ISSX Meeting", Dijon, France. April 27 – May 1, 2003.

Methods for biomonitoring of individuals (involved by destruction of CW's) who may be contaminated with low concentrations of sulfur mustard, lewisite, phosgene and OP's. Presented by G.P. Van der Schans at meeting of CEPA IEX 13.11, Destruction of old chemical munitions, April 2004, Civitavecchia, Italy.

Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Presented by D. Noort at Bioscience Review , Hunt Valley MD, May 16-21, 2004.

Biomonitoring of exposure to chemical warfare agents. Presented by D. Noort at 2004 Joint SOFT/TIAFT Meeting, August 30 – September 3, 2004, Washington DC.

VIII CONCLUSIONS

1. The albumin-tripeptide assay, based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties, is a very fast and sensitive assay for biomonitoring of exposure to sulfur mustard.
2. For detection with laser-induced fluorescence, various derivatization methods have been explored for the tripeptide. The most convenient derivative turned out to be the 6-carboxyfluoresceine (FAM) derivative. However, the tripeptide could not be detected in a pronase digest of sulfur mustard alkylated albumin, after derivatization with FAM succinimidyl ester and subsequent CE-LIF detection. Mass spectrometry is still the method of choice for this procedure.
3. After pronase treatment of rat albumin, isolated from rats exposed to [^{14}C]-labelled sulfur mustard *in vivo*, the tripeptide (S-HETE)Cys-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe.
4. The albumin adduct (S-HETE)Cys-Pro-Phe) was not longer detectable in the rat 7 days after the exposure.
5. The half-life time of sulfur mustard – alkylated albumin was 2 days, which is in accordance with literature values for other albumin adducts formed in rats.
6. The corresponding globin samples were analyzed for the presence of adducts to the N-terminal valine residues. The adduct to the N-terminal valine could still be detected after 28 days.
7. The globin adduct level clearly increased during the first 2-3 days, which implicates that there is still free sulfur mustard present during that time, which causes accumulating damage. Subsequently, the adduct level decreased more or less linearly, in accordance with the life-time of the erythrocyte of the rat.
8. Direct isolation of the histidine adduct from pronase or acidic digests by means of preparative reversed-phase HPLC could be effected, but was not yet reproducible due to variation in retention time of the adduct
9. For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared. The most convenient derivative turned out to be the 5/6-carboxyfluoresceine derivative, which could be analyzed at a level down to 20-25 pg/ml with capillary electrophoresis – laser induced fluorescence (CE-LIF) detection.
10. Upon comparison of the tripeptide method and the histidine method, the tripeptide is by far the best candidate to be developed into a Standard Operating Procedure.
11. The interindividual and intraindividual variation, and the day-to-day variability of the tripeptide assay are within an acceptable range.
12. The SOP drafted for the tripeptide assay could be successfully demonstrated to a scientist of USAMRICD in a different laboratory indicating the high utility of the procedure.
13. The screening procedure for the selection of monoclonal antibodies against adducts of sulfur mustard with proteins (globin, albumin and keratin) has been improved. The most suitable antibodies are used for the development of an immunoslotblot assay for retrospective detection of exposure to sulfur mustard.
14. So far the best results were obtained with the detection of sulfur mustard adducts to keratin isolated from human callus exposed to sulfur mustard. Unfortunately, some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. It appeared that the accessibility of the sulfur mustard adducts decreased at sulfur mustard exposures above 0.5 mM. We selected new clones producing antibodies specific for exposed keratin, of which several retained their activity upon continued culturing.

15. The screening procedure for the selection of monoclonal antibodies against adducts of sulfur mustard with proteins (globin, albumin and keratin) has been improved. The most suitable antibodies are used for the development of an immunoslotblot assay for retrospective detection of exposure to sulfur mustard.
16. An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies. Two clones, IAD7 and ID2E1, produced antibodies that recognize unmodified keratin and keratin modified by sulfur mustard, respectively.

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XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. D. Noort
Dr. G.P. van der Schans
Dr. B.L.M. van Baar
Mr. A. Fidder
Mrs. R.H. Mars-Groenendijk
Mr. A.G. Hulst
Mrs. S. de Kant
Mrs. C.E.A.M. Degenhardt-Langelaan
Mr. E. Wils
Mr. S.H. van Krimpen

Volume II

Development of methods for diagnosis of exposure to chemical warfare agents

LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionization
BAL	British Anti Lewisite
CDC	Centers for Disease Control & Prevention
CVAA	2-chlorovinylarsonous acid
DMA	N,N'-dimethylacetamide
DMF	dimethylformamide
DTT	dithiothreitol
EMPA	O-ethyl methylphosphonic acid
EC APCI	Electron Capture Atmospheric Pressure Chemical Ionization
EI	electron impact
Fmoc	fluorenylmethyloxycarbonyl
HETE	2-hydroxyethylthioethyl
(S-HETE)Cys-Pro-Phe	(S-2-hydroxyethylthioethyl)Cysteine-Proline-Phenylalanine
HFBI	heptafluorobutyryl imidazole
HOBT	N-hydroxybenzotriazole
HSA	human serum albumin
IMPA	O-isopropyl methylphosphonic acid
L1	lewisite
MPA	methylphosphonic acid
MRM	multiple reaction monitoring
NICI	negative ion chemical ionization
NMP	N-methyl-2-pyrrolidone
OP	organophosphate
PAB	phenylarsine-British Anti Lewisite complex
PDT	1,3-propanedithiol
PFB	pentafluorobenzyl
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
SOP	standard operating procedure
TFA	trifluoroacetic acid
TIS	triisopropylsilane
THF	tetrahydrofuran
Z	benzyloxycarbonyl

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I INTRODUCTION

The work described in this report has been performed by the direction of Centers for Disease Control and Prevention (CDC), as an extension of cooperative agreement DAMD 17-02-2-0012.
The work is focused on

1. Transfer of methods for diagnosis of exposure to chemical warfare agents and toxic scheduled chemicals
2. Improvement of existing assays
3. Development of a general method for detection of covalent cholinesterase inhibitors
4. Development of assays to detect exposure to other toxic chemicals, likely to be used for chemical terrorism

Ad 1. Transfer of methods
We have proposed that two methods will be transferred to CDC: the method for diagnosis of lewisite exposure (Fidder *et al*., 2000) and an alternative method for diagnosis of sulfur mustard exposure, i.e., the modified Edman degradation of sulfur mustard-modified hemoglobin (Fidder *et al*., 1996; Noort *et al*., 2004a). In more detail:

a. Protocols for the modified Edman degradation and the method for diagnosis of Lewisite exposure will be delivered to CDC. The procedures will be demonstrated at CDC by TNO employees.
b. The following reference materials will be delivered to CDC:
    - frozen blood samples (erythrocytes; 5 ml) which have been exposed to various (three) concentrations of Lewisite
    - the reference compound lewisite – British Antilewisite complex (5x 1 mg)
    - internal standard phenylarsineoxide – British Antilewisite complex (5x 1 mg)
    - pentafluorophenyl thiohydantoin of the N-terminal D/L-valine sulfur mustard adduct (1 mg)
    - globin samples (100 mg), isolated from blood which has been exposed to three different concentrations of sulfur mustard
    - globin, isolated from human blood which had been exposed to d_8-sulfur mustard (10 μM; 100 mg).

Ad 2. Improvement of existing assays
a. Sulfur mustard adducts
We had proposed to attempt to automate the albumin-tripeptide assay. The bottleneck of the albumin – tripeptide assay has been the laborious and lengthy (overnight!) isolation procedure for albumin from human plasma. Recently, TNO-PML developed a rapid isolation procedure for albumin from human plasma based on affinity chromatography, which takes only 30 min. In combination with a column of immobilized pronase, this might enable the development of a fully automated analytical system. Examples of such systems with other digestion enzymes have been reported in the literature (Hsieh *et a.l*, 1996; Nadler *et al*., 1996) and could also be applied for protein adduct determination (Shen *et al*, 2000). The advantages of such an automated system is that it reduces the reaction time, minimizes sample loss, improves cleavage efficiency and affords more reproducible digests. It is envisaged that the developed methodology will enable processing of large numbers of human blood samples. First, it will be investigated whether immobilized pronase gives rise to the formation of the adducted tripeptide as well.

Expected deliverables
- a methodology for rapid isolation of albumin from human plasma
- a methodology for albumin digestion on a column of immobilized pronase
- implementation of the two abovementioned methodologies into an automated system

b. Assays for nerve agent adducts
In addition to the fluoride reactivation method, we recently developed an alternative mass spectrometric assay for diagnosis of exposure to nerve agents, which is based on LC-tandem MS analysis of a phosphylated nonapeptide in a pepsin digest of human butyrylcholinesterase (HuBuChE; Fidder *et al*., 2002), *i.e*., one of the major targets of nerve agents. The advantages of this method are that it bypasses the use of highly toxic analytical standards and that it can be used for a wide variety of agents, including those that suffer from aging, a process which precludes the use of the fluoride reactivation method.
In case of screening of large numbers of human blood samples for assessment of exposure to nerve agents, a procedure for automated isolation and digestion of phosphylated HuBuChE, *i.e*., the biomarker of interest, is highly desirable. In the current nonapeptide assay, isolation of HuBuChE is performed by affinity chromatography on a column filled with procainamide gel; the gel is prepared in our laboratory. However, this isolation procedure is quite laborious. Although we made some advances in developing a more rapid procedure based on procainamide gel, it still lacks reproducibility. Therefore, we had proposed
a. to further elaborate the rapid version of the isolation procedure for HuBuChE.
b. to further work-out the use of an internal standard within this procedure.
c. to explore the use of immobilized pepsin (commercially available) for automation of the procedure.
d. to synthesize a batch of procainamide gel and deliver it to CDC
e. to synthesize a number of phosphylated nonapeptides, to be used as reference standards.

Expected deliverables
- a rapid procedure for isolation and digestion of HuBuChE
- an LC-tandem MS assay for the phosphylated nonapeptide, including the use of an internal standard
- a batch of procainamide gel for isolation of HuBuChE by affinity chromatography.

Ad 3. Generic method for detection of covalent cholinesterase inhibitors
One of the problems with the current assays for assessment of organophosphate exposure is that one has to know in advance for which type of nerve agent to screen for during mass spectrometric analysis. However, it is impossible to screen for adducts of all kinds of inhibitors. Therefore, a generic method for detection of phosphylated HuBuChE is highly needed. In this respect, our attention was raised by the generic method which is used for the detection of phosphorylation sites in proteins in the field of proteomics (Oda *et al*., 2001). According to this method, proteins are treated with mild base in order to eliminate the phosphate function, resulting in the formation of a dehydroalanine residue in the protein. Subsequently, the thus formed dehydroalanine residue is reacted with a thiol, containing a tag, *e.g*., a biotin group; the protein is then enzymatically digested and the digest is purified by means of streptavidin affinity chromatography. Recently we have applied this methodology to phosphylated HuBuChE (i.e. after inhibition with a nerve agent) in preliminary experiments. Elimination readily occurred and introduction of various thiol-containing groups proceeded smoothly. Subsequent pepsin digestion gives rise to the formation of the desired nonapeptide. Until yet, we have not found the right “mass tag”, in order to obtain a nonapeptide with an

enhanced response in the mass spectrometer. When this methodology indeed works, "positive" samples can be subjected to further analyses in order to identify the used nerve agent. It is expected that the methodology is suitable for a vast array of organophosphates. In analogy to the proteomics work, we can also apply a biotin-containing tag and isolated the modified nonapeptide by streptavidin affinity chromatography.
We had proposed to further develop this methodology into an assay that can be applied as a first screening method for large numbers of samples.

Expected deliverable
- a general assay for biomonitoring of exposure to organosphosphates based on the abovementioned technologies.

Ad 4. Development of assays to detect exposure to other toxic chemicals, likely to be used for chemical terrorism

a. Phosgene
Previous research within TNO-PML has shown that upon *in vitro* exposure of human blood to phosgene an intramolecular adduct to Lys-195 and Lys-199 residues in albumin (Noort *et al*., 2000) is formed. A tandem mass spectrometric assay was developed for analysis of the tryptic fragment containing this intramolecular lysine adduct, which enabled the detection of exposure of human blood to ≥ 1 μM phosgene *in vitro*. This tryptic fragment is a dimer of two peptides, bridged by the carbonyl moiety derived from phosgene. Due to the particular urea bridge in the peptide its fragmentation is restricted to only a few fragments, rendering it very suitable for sensitive LC-tandem MS analysis.
One of the drawbacks of the method is the rather lengthy procedure for albumin isolation and reduction/carboxymethylation of cysteine bridges. In this respect, it is envisaged that the rapid isolation procedure for albumin (see above) can also be applied to this methodology. Furthermore, there have been reports in the literature that describe the on-line reduction/carboxymethylation of proteins (Hsieh *et al*., 1996; Nadler *et al.*, 1996; Shen *et al*., 2000), which would render the procedure much less laborious.
In summary, it was proposed to
- further develop this method for routine use, *e.g*., by performing the reduction/carboxymethylation of albumin on-line.
- transfer the resulting method to CDC
- synthesize a well-defined amount (0.050 mg) of adducted peptide and transport it to CDC.
  Expected deliverables
- a rapid method for biomonitoring exposure to phosgene, based on adducts to albumin
- tranfer of this method to CDC
- a well-defined amount of reference peptide

The following subjects focus on toxic, readily accessible compounds for which no methods for retrospective detection of exposure are available yet.

b. Cyanide
Cyanide (as HCN or salt) is a readily available toxicant for which no method is available for retrospective detection of exposure. Little is known of any possible adduct formation of cyanide; reaction of cyanide with cystine has been reported to proceed through an intermediate thiocyanate, which subsequently reacts intramolecularly with an amino function

of cystine, giving a five-membered ring. It can be envisaged that a similar reaction might occur with a disulfide function within a protein, followed by further reaction with a nearby lysine residue. We had proposed to examine this in more detail by exposure of human blood to radioactively labelled cyanide. Covalent attachment of radioactivity to proteins can easily be detected and quantitated. The identity of potential adducts will be elucidated by LC tandem MS and by comparison with synthetic standards. In summary, the feasibility of an assay for monitoring exposure to cyanide, based on adducts to proteins, will be investigated.
Deliverable
- a report describing the performed research, including full experimental details.

Phosphine
Phosphine is a highly toxic gas which is inter alia used by farmers as a grain fumigant (Newton *et al.*, 1999). It is also an important by-product during the (illegal) manufacturing of metamphetamine (Burgess, 2001). The derivatives aluminum phosphide and zinc phosphide, which generate phosphine upon exposure to moisture, are used as rodenticidal agents (Guale *et al*., 1994). Especially the phosphide derivatives are regarded by the FBI as potential candidates for use in chemical terrorism because they are readily accessible at low cost.
Whether phosphine can form (persistent) adducts to bio-macromolecules such as proteins is not known. The conversion of oxyhaemoglobin to methaemoglobin has been described in the literature (Chin *et al*, 1992), but further data have not been reported. We had proposed whether adduct formation occurs with proteins by using tritiated phosphine, in a similar way as described for cyanide. In case stable adducts with proteins are formed, an assay will be developed for mass spectrometric analysis of the most suitable adduct. In summary, the feasibility of an assay for monitoring exposure to phosphine, based on adducts to proteins, will be investigated.
Deliverable
- a report which includes full experimental details.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

Tripeptide assay

- Human plasma exposed to d0 and/or d8 sulfur mustard (e.g. 1, 5, 10, 50, 100 µM)
- S-HETE-Cys-Pro-Phe for calibration/tuning of instrument
- Pronase, (protease type XIV, Bacterial from Streptomyces Griseus, E.C. 3.4.24.31), Sigma, P 5147
- KH_2PO_4, Fluka,
- KCl, Fluka,
- NH_4HCO_3, Fluka,

Modified Edman degradation

- Human globin from blood that had been exposed to d0 and/or d8 sulfur mustard (e.g. 1, 5, 10, 50, 100 µM)
- Formamide, Fluka, 47671
- Pyridin, 99.9+ %, Sigma Aldrich 270407
- Pentafluorophenyl isothiocyanate PFPITC, Fluka, 76755
- Heptafluorobutyrylimidazole (HFBI), Pierce, 44211
- Sodium chloride, p.a.
- Concentrated hydrochloric acid, p.a.
- Acetonitrile, p.a.
- acetone, p.a.
- diethyl ether, p.a.
- toluene, p.a.
- Dichloromethane, p.a.
- Na_2CO_3, p.a.
- $MgSO_4$, p.a.
- Liquid nitrogen
- Methanol, p.a.
- $KHCO_3$, p.a.
- 2-propanol, p.a.
- heptane, p.a.

Phosgene method

- Reference peptide-phosgene: [ASSAK*QR][LK*ZASLQK], C=O bridge between K* residues, Z= S-carboxymethylcysteine
- Human plasma exposed to various concentrations phosgene
- Trypsin (TPCK treated), Type XIII, E.C. 3.4.21.4, Sigma, T-8642 ca 10.000 units mg/ solid
- Sodium iodoacetate, Fluka, 57858
- Dithiothreitol, Fluka, 43817
- NH_4HCO_3
- Guanidine.HCl
- Tris.HCl
- EDTA

Lewisite method

- Hemolyzed red blood cells isolated from blood exposed to various concentrations of L1
- Heptafluorobutyryl imidazole (HFBI), Pierce, 44211
- 2,3-dimercaptopropanol (BAL), Riedel-de Haen, 64046
- CVAA-BAL
- Phenylarsenic-BAL
- CVAA-BAL-HFB; must be prepared freshly by derivatization of L1-BAL with heptafluorobutyryl imidazole
- Nitrogen or preferably argon.
- Acetonitril, Biosolve
- Toluene, Merck
- Methanol, Merck
- Dichloromethane, Biosolve
- $MgSO_4$, Fluka
- KH_2PO_4, Fluka
- KCl, Fluka
- Bleach

Nonapeptide assay for OP biomonitoring

- Butyrylthiocholine iodide, Sigma (Bornem, Belgium).
- Pepsin (EC3.4.23.1), Roche Applied Science 10108057001 (Almere, the Netherlands)
- 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Fluka
- Sepharose 4B, Fluka
- Procainamide.HCl, Fluka
- Cyanogen bromide, Fluka
- ε-Aminohexanoic acid and formic acid, Fluka
- NH_4HCO_3
- Na_2CO_3
- NaH_2PO_4
- Na_2HPO_4
- NaCl

Generic assay for OP biomonitoring

- Pepsin (EC 3.4.23.1), Roche Applied Science 10108057001 (Almere, The Netherlands)
- Sepharose 4B, Fluka
- Procainamide.HCl, Fluka
- $Ba(OH)_2$, Riedel-de Haen
- 1H,1H,2H,2H perfluorodecane-1-thiol, Fluorous Technologies Inc. Pittsburgh, PA,
- Ethanethiol, Fluka
- N-Phenylethylenediamin, Fluka
- 4-(2-Aminoethyl)aniline, Fluka
- 2-Phenylethanethiol, Fluka
- 3-(2-Aminoethylamino)propylamine, Fluka
- N-Biotinylated cysteamin, Fluka
- 1,4 Bis-(3-aminopropylamino)butane,
- 2(3-Aminopropyl-amino)ethanol benzylamin (I), Fluka
- 1-(2-Aminoethyl)piperidine, Fluka
- 2-Mercaptoethylamine, Fluka
- 1,3 Bis(3-aminopropyl)1,1,3,3, Fluka

- Tetramethyldisiloxane, Fluka

II.2 Instrumentation/devices

Tripeptide assay

- Plastic tubes, 13 mL, Sarstedt, 60541685
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- pH meter
- Acrodisc filters, 0.45 µm, PVDF, Waters, WAT200510
- Amicon Ultra-4, centrifugal Filter devices, 10000 MWCO, UFC801096, Millipore
- Centrifuge tubes
- Pasteur pipettes
- disposable syringes, 3.0 cc, slip tip, Aldrich, Z19211-2
- HiTrap Blue HP, Amersham Biosciences, 17-0412-01, 5 x 1 mL columns
- PD-10 columns, Amersham Biosciences, 17-0851-01, 30 pre-packed columns
- 4 mL screw neck vials, 45 x 14.7 mm, Alltech associates, 98110
- 13-425 solid cap. Black propylene with TFE, Alltech, 98430
- LC-MS-MS (triple-quadrupole or Q-TOF instrument)
- Gilson pipettes, 20, 100 and 1000 µL and tips

Modified Edman degradation

- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, Aldrich, order no. Z10642-9
- Vortex Vibrofix, VF1 electronic, Janke & Kunkel
- Vacuum concentrator, Jouan RC10-10
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- Ultracentrifuge (25000 g), Centrikon T-234
- Gilson pipettes, 20, 100 and 1000 µL and tips
- Measuring cylinders, 100 and 500 mL
- Tubes for ultracentrifuge, resistant to 25000 g or more
- Heat gun
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Glass wool,
- Disposable syringes, 2 mL
- Injection needles
- Tubing, to be used between syringes and pasteur pipettes
- Vials 300 µL, or 200 µl inserts for larger vials
- Caps for 300 µL vials
- Capper
- Florisil cartridges, classic, Waters, WAT051960
- Pasteur pipettes, long and small
- GC-NICI-MS
- Seppak C18 cartridges, classic, Waters, WAT 051910

Phosgene method

- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, ordered from Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, ordered from Aldrich, order no. Z10642-9
- Vortex Vibrofix, VF1 electronic, Janke & Kunkel
- Vacuum concentrator, Jouan RC10-10
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- Waterbath
- Slide-a-lyzer dialysis cassettes (10 kD, 0.1-0.5 mL, Pierce Prod nr 66415)
- HiTrap Blue Sepharose columns (Amersham Biotech)
- PD-10 columns (Amersham Biotech)
- Pasteur pipettes, long and short
- Gilson pipettes, 20, 100 and 1000 µL and tips
- 10 kD molecular weight cut-off filters (2 mL)
- Glass wool,
- LC/MS/MS (triple-quadrupole or Q-TOF)
- Pepmap C18 HPLC column (LC Packings) or comparable
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Disposable syringes, 2 mL
- Injection needles
- 0.45 micron syringe filters (Waters, 25 mm PVDF, Prod Nr, WAT200510)

Lewisite method

- Seppak C18 cartridges (classic, Millipore)
- Pasteur pipettes, long and small
- Glass wool,
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, ordered from Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, ordered from Aldrich, order no. Z10642-9
- GC/MS, with possibility for EI conditions and a CP-Sil 5 CB column or comparable
- Vacuum concentrator, Jouan RC10-10
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials

Nonapeptide assay for nerve agents

- Amicon (100 kD, 15 mL) and Microcon (3 kD, 0.5 mL) ultra centrifugal filter devices, Millipore (Bedford, MA, USA).
- Peptide synthesis tubes, with filter (2 ml), MultiSynTech, GMBH, Germany
- Heraeus Centrifuge equipped for centrifugation of 100 kD centrifugal devices (Heraeus,Hanau, Germany)
- Eppendorf centrifuge equipped for centrifugation of 3 kD centrifugal devices (Eppendorf. Hamburg, Germany)

- 96 well absorbance reader Asys UVM340 (Eugendorf, Austria)

Generic assay for nerve agents

- Centrifugal ultrafilters (Centricon YM-3, 3 kD or Amicon Ultra-4, 10 kD), were purchased from Millipore, Bedford, MA.
- Further as described for "Nonapeptide assay for nerve agents"

Peptide synthesis

Solid phase peptide synthesis was carried out on a Syro 2000 (Multisyntech, Germany) peptide synthesizer on a 10 µmol scale, using commercially available amino acids and customized Fmoc-based protocols. After synthesis, the peptides were split off from the resin and purified to homogeneity with semi-preparative HPLC.

HPLC

Analytical and semi-preparative HPLC was performed on an AKTA Explorer chromatography system (Amersham Pharmacia Biotech). Analytical column used: Alltech Alltima C18 250 x 4.6 mm; 5 µm particle size. Semi-preparative column used: Alltech Altima C18, 250 x 10 mm; 5 µm particle size

III EXPERIMENTAL PROCEDURES

III.1 Improvement and demonstration of existing methods

III.1.1 Albumin – tripeptide assay

Standard Operating Procedure

The plasma sample (0.5 mL) of interest was spiked with plasma (25-50 µL), isolated from blood exposed to 1 - 100 µM d_8-sulfur mustard. The sample was diluted with buffer A (50 mM KH_2PO_4, pH 7; 2 ml), filtrated over a filter disk (0.45 µm), applied on a HiTrap™ Blue HP cartridge (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) that had been equilibrated with buffer A (10 mL). The cartridge was eluted with buffer A (10 mL) by means of a syringe, within one minute. Subsequently, the cartridge was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 3 mL). The latter effluent was collected. The HiTrap column can be regenerated by washing with buffer B (10 ml) and subsequently with buffer A (10 mL). Next, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (3 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 3 mL). The effluent was collected.

Part of the purified albumin fraction (0.75 ml) was used and subsequently Pronase was added (100 µl of a freshly prepared solution (10 mg/ml) in 50 mM NH_4HCO_3), followed by incubation for 2 h at 37 ºC. The digests were filtrated through molecular weight cut-off filters (10 kD) under centrifugation at 2772 g in order to remove the enzyme. The filtrate was analyzed by means of LC/MS/MS.

For low exposure levels, the filtrate (after 10 kD filtration) was processed on a Seppak C18 cartridge. To this end, the cartridge was conditioned with methanol (5 mL), followed by 0.1% TFA/water (5 mL). The pronase digest was applied, and washed with water (2 mL), containing 0.1 % TFA, 10 % CH_3CN/water (2 mL,0.1 % TFA), 20 % CH_3CN/water (2 mL,0.1 % TFA), and finally eluted with 40% CH_3CN/water (2 mL,0.1 % TFA). The eluate was evaporated to dryness, redissolved in water (0.1 % TFA, 50 µ and analyzed with LC/MS/MS.

<u>Conditions LC-system</u>

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

Time (min)	% eluent A	% eluent B	Flow (ml/min)
0	100	0	0.1
5	100	0	0.6
50	30	70	0.6

The flow of 0.6 ml/min was split before the column to 35 µl/min. Column: PepMap C18, 3 µm, 15 cm x 1 mm. Loop: 50 µl.

<u>Conditions triple quad MS</u>

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Phe and (S-d_8-HETE)Cys-Pro-Phe to the most intense fragment (HETE):

MH^+ 470.2 → 105

MH^+ 478.2 → 113

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

Analyses at TNO (Q-TOF instrument)

LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (water with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column 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 between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Analyses at CDC (triple-quad instrument)

LC-tandem MS experiments during the method demonstration at CDC were recorded on an API4000 triple quadrupole instrument with a standard ionspray interface (Applied Biosystems, Toronto, Canada) and a Shimadzu (Kyoto, Japan) modular liquid chromatograph. 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 C18 (Phenomenex, Torrance, CA, USA) columns (both 150 mm x 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. A gradient of solvents C (H_2O with 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 minutes. 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 minutes. 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 d_8-tripeptide) 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.

Preparation of a column containing immobilized pronase

Poros AL (Applied Biosystems, Foster City, CA, 2.7 g) material was suspended in a buffer (5 mL, 100 mM Na_2HPO_4, pH 7.2, 19 mM benzamidine. To this suspension, a solution of pronase (125 mg) dissolved in phosphate buffer (pH 7.2, 100 mM Na_2HPO_4) was added. Next, $NaCNBH_3$ (10 mg) was added and the emulsion was incubated for 16 h at RT. The Poros material was filtered and washed with phosphate buffer and finally incubated with ethanolamine (10 mL 1 M in phosphate buffer) and $NaCNBH_3$ (10 mg) for two h. The material was washed with water, taken up in water (12 mL) and a part (0.9 g) was packed into a column (4.6 mm x 100 mm) according to the instructions given by the manufacturer. The column was tested with albumin isolated from blood exposed to sulfur mustard (1 mM) and with a set of randomly chosen peptides. To test the column, a thermostat was used to keep the

column at 37 ºC. Several conditions were attempted, e.g., stationary digestion and digestion by pumping around, and digestion times were varied. Unfortunately, evidence for enzymatic digestion could not be obtained.

Digestion of human serum albumin with pepsin and mass spectrometric analysis of digest
Human plasma (2 mL) was exposed to a solution of sulfur mustard in acetonitril (20 µl of a 1 M solution, resulting in a 10 mM exposure level) for 2 hours at 37 ºC. A blank sample was included by incubating plasma (2 mL) with acetonitril (20 µl). Next, part of the plasma samples (0.5 mL) were diluted with buffer A (2 mL, 50 mM KH_2PO_4, pH 7.0).
This solution was filtered over a 0.45 micron Acrodisc filter and applied to a preconditioned (10 mL buffer A) HiTrap Blue Sepharose column. The column was washed with buffer A (2 x 10 mL) and eluted with buffer B (3 mL, 50 mM KH_2PO_4, 1.5M KCl, pH 7.0).

Desalting was performed by applying the sample to a PD-10 column (conditioned with 5 % formic acid, 25 mL). Part (0.5 mL) of the the eluate (3 mL) was digested using pepsin (50 µls of a 2% solution in formic acid (5% in water). After two hours the samples were filtrated over a 10 kD MWCO filter. The filtrate was analyzed using LC-MS. The main adducts found were AEVSKL (MH_2^{2+}, 375.7), KPLVEEPQNL (MH_2^{2+}, 635.8) and LGMFLYE (MH_2^{2+}, 488.0). The position of the (2-hydroxyethyl)thioethylgroup could not be determined, probably due to the instable character of the adducts. In view of the peptides' structures, probably glutamic acid residues are involved.

III.1.2 Standard Operating Procedure for modified Edman degradation of globin, modified by sulfur mustard

Procedure for modified Edman degradation of globin alkylated by sulfur mustard
Globin (20 mg), from blood exposed to sulfur mustard was mixed with globin (20 mg) isolated from blood exposed to sulfur mustard-d_8 (10 µM) and dissolved in formamide (2 ml). Next, pyridine (8 µl) and pentafluorophenyl isothiocyanate (8 µl) were added and the mixture was incubated at 60 °C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 × 1 ml) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jouan RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with water (2 × 0.5 ml), aqueous Na_2CO_3 (0.1 M, 0.5 ml) and water (0.5 ml). The organic layer was dried ($MgSO_4$), evaporated to dryness using the centrifugal evaporator and dissolved in toluene (100 µl).
Next, a Florisil cartridge was conditioned with methanol/dichloromethane (1/9, v/v; 2 ml) and dichloromethane (2 ml), respectively. The toluene solution was applied on the cartridge, which was subsequently washed with dichloromethane (2 ml) and methanol/dichloromethane (1/9, v/v; 1 ml). The thiohydantoin was eluted with methanol/dichloromethane (1/9, v/v; 1.5 ml). The latter eluate was evaporated to dryness and dissolved in toluene (100 µl). To this solution heptafluorobutyryl imidazole (10 µl) was added and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was washed with water (2 × 100 µl), aqueous Na_2CO_3 (0.1 M, 100 µl) and finally with water (100 µl). The toluene layer was dried ($MgSO_4$), concentrated to 30 µl and analyzed with GC-MS.

For GC-MS analysis, an HP 5973 mass selective detector was connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The system was operated in the NICI mode (methane) with a source temperature of 150 °C and an ionization energy of 70 eV. The column used was a CPSil 5 CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands) or a Hewlett Packard HP-5 column (5% phenyl methyl siloxane; length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). The oven of the chromatograph was kept at 120 °C for 5 min, the temperature was then progammed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min. Injection volume was 1 µl (containing about 1% of the total sample).
Ion chromatograms were recorded after monitoring for *m/z* 564 (M^{-} - 3 HF, analyte) and 572 (M^{-} - 3 HF, internal standard).

III.1.3 Standard Operating Procedure for verification of phosgene exposure

Reduction/carboxymethylation of albumin
Guanidine.HCl (M = 95.53, 57.3 g), Tris (hydroxymethyl)aminomethane (M= 121.14, 1.21 g) and EDTA (M= 292.24, 29 mg) were dissolved in water to give a volume close to 98 mL; the pH was adjusted to pH 8.3 with a few drops HCl (6 M) and finally the volume was adjusted to 100 mL with water.
Dissolve DTT (50 mg) in guanidine buffer (3 mL, 6 M guanidine.HCl, 100 mM TRIS, 1 mM EDTA, pH 8.3). Part (0.3 mL) of this solution is added to lyophilized albumin samples that were obtained by means of affinity chromatography, as described for the tripeptide assay. These solutions are incubated at 55 ºC for 40 min. Next, iodoacetic acid (10 mg) is added and the mixture is incubated for 30 min at 40 C , followed by dialysis against 50 mM NH_4HCO_3 (3x 1L) at RT in a Slide-A-Lyzer dialysis cassette (10000 MWCO, 0.1-0.5 mL). The last dialysis step is performed overnight at RT.

Trypsin digestion
Dialyzed (reduced and carboxymethylated) albumin was transferred from the slide-a-lyzer cassettes into vials (4 mL). Next, trypsin was added (2 % w/w). For this purpose, trypsin (6 mg) was dissolved in buffer (5 mL, 50 mM NH_4HCO_3). Part (50 µl) was added to the dialyzed solution of reduced and carboxymethylated albumin in buffer. Incubation was performed at 37°C for 4 h. Finally, the sample was filtrated through a pre-rinsed (50 mM NH_4HCO_3, 3x 1 mL) 10 kDa MW cut-off filter with centrifugation at 3200g, to remove the enzyme. The resulting trypsin digests were analyzed by means of LC tandem mass spectrometry.

ESI-LC-tandem MS conditions:
Eluent A: 0.2 % formic acid/water
Eluent B: 0.2 % formic acid acetonitril
A gradient was applied from:
100 % A (5 min, 0.1 mL/min)
100 to 30 %A in 60 min (0.6 mL/min)
The flow of 0.6 mL/min was reduced by a LC-packings splitter to ca. 40 µl/min.
Column: PepMap C18, 3 micron, 15 cmx 1 mm, loop: 10 µl
MS conditions (Q-TOF):
Full scan product ion spectra (MS/MS) were recorded of the doubly charged native adduct peptide (MH_2^{2+} 861.2) and of the [^{13}C] analogon (MH_2^{2+} 861.7) at a cone voltage of 35 V and a collision energy of 35 eV. Argon gas pressure was 10^{-4} mBar.

For more sensitive detection of (ASSAK*QR)(LK*CcmASLQK), analyses were performed in the multiple-reaction monitoring (MRM) mode (transitions $[M + 2H]^{2+}$ *m/z* 861.0 >747.5 and $[M + 2H]^{2+}$ *m/z* 861>773.5 on a Quattro II triple-quadrupole mass spectrometer. Operating conditions were as follows: cone voltage 40 V; collision energy 30 eV, argon pressure, 5 x 10^{-3} mBar; and dwell time, 1 s. LC conditions were as described above.

III.1.4 Standard Operating Procedure for verification of Lewisite exposure

Reagent preparation:
BAL (M = 124.23, d = 1.239 g/mL, 1 mL = 10 mmol) solution: dilute 10 µl BAL (100 micromole) with 990 µl acetonitril to achieve an end concentration of 100 mM.

Internal standard preparation:
PAB (Phenylarsine – BAL complex; M=274) solution: dissolve 1 mg to an end concentration of 100 micromolar in acetonitril; 1 mg=3.64 micromole. In 3.64 mL: 1 mM. Dilute this solution 1:10 to give 100 micromolar.

Sample work-up (improved procedure)
Incubate washed and lysed erythrocytes (1 mL) with BAL solution (100 mM solution in CH_3CN; 10 µl) and PAB solution (100 micromolar , 10 µl) for 1 h at RT.
Add water (9 mL) to the lysed erythrocytes, apply to a conditioned Seppak C18 classic cartridge (conditioned by washing with methanol (10 mL), water (10 mL) consecutively), and wash with water (20 mL). Elute L1-BAL and PAB with dichloromethane/acetonitril (4/1, v/v, 3 mL), concentrate with a vacuum concentrator after a first volume reduction using a stream of air , coevaporate (2 x 0.5 mL toluene), dissolve in toluene (100 µl) and add HFBI (10 µl). Heat (50 ºC) for 1 h. After cooling the samples were diluted with toluene (200 µl). Next, wash with water (4 x 100 µl). Dry over $MgSO_4$ and analyze with GC/MS. In case of exposure levels < 1 micromolar it is recommended to concentrate to a smaller end volume.

GC-MS conditions
Mass spectrometer (Agilent 5973N MSD)
Vacuum: 10^{-6} Torr
Source temperature: 200 ºC
Multiplier: 2200 V
Emission: 34.6 microampere
ionisation energy: 70 eV
Ionisation mode: EI
Acquisition mode:SIM
Dwell time: 100 ms
Mass: *m/z* 454 and 470
Type recording: GC-MSD
Injection volume: 1 µl, splitless

Gas chromatograph (HP 6890)
Column: factorfour VF-5ms, 50 m x 0.25 mm, df 0.25 micrometer
Carrier gas: helium, constant flow, 1 mL/min
Temperature: 120 (1), 8 ºC/min, 280 (5)
Interface: 280 ºC

Alternative method for diagnosis of phosgene exposure

To washed and lysed erythrocytes (1 mL) isolated from blood exposed to Lewisite, a solution of 1,3-propanedithiol (PDT) in acetonitril (10 µl, 0.1 mM) was added and incubated for 2 h at 37 ºC. Next, the erythrocyte solution was diluted with water (2 mL) and extracted with toluene (2 x 0.5 mL). The toluene layers were combined and evaporated to dryness in a vacuum concentrator. Next, the concentrated sample was reconstituted in toluene (20 µl) and analyzed by GC/MS. The GC inlet was held at 245°C, helium flow rate 1.0 ml/min, The following oven temperature program was used: initial temperature 120°C, held for 1 min, next ramped at 55°C /min to 207 °C, followed by final ramp at 8°C /min to 280°C held for 5 min, (16.71 min total run time). The GC–MS transfer line was held at 300°C and the MS quadrupole and source heaters were maintained at 150°C and 230°C, respectively.

III.1.5 Nonapeptide assay for OP biomonitoring

Preparation of procainamide gel

Approximately 125 mL of Sepharose 4B gel suspension in ethanol/water was placed in a funnel with a glass filter and washed with 1 liter of water, re-suspended in 0.2 M phosphate buffer (pH 11.5; 150 ml) and cooled at 4 °C. Cyanogen bromide (7 g in 15 mL acetonitrile/water 1:1) was added gently and the suspension was stirred for 10 min. Next the suspension was washed with water (1 L) and immediately transferred into ε-aminohexanoic acid solution (1.6 g ε-aminohexanoic acid in 150 mL 0.2 M sodium carbonate, 0.2 M sodium bicarbonate and 0.4 M sodium chloride, pH 9). The mixture was stirred for 48 h at 4 °C. Next the gel was washed with water, re-suspended in water (150 mL) and after addition of 4.2 g procainamide.HCl adjusted to pH 4.5. EDC (N-3-dimethylaminopropyl)-N-ethylcarbodiimide; 7 g) was added and the pH adjusted again to pH 4.5. The pH slowly rose, so the pH had to be adjusted a few more times. When the pH remained relatively stable, the mixture was stirred in the cold room for 48 - 60 h. Next, the gel was washed with water (1.5 L). The wash fluid was collected and extinction was measured at 278 nm ε^{270} =16150 $M^{-1}cm^{-1}$). Calculation revealed that 29 µmol of procainamide was bound to 1 mL of gel.
The gel is stored in water (150 ml), containing 0.02% NaN_3.

Inhibition of plasma samples with nerve agent

Human plasma was inhibited with sarin, d7-sarin, soman, VX or tabun. The concentration of the nerve agent in plasma was 1 µg/ml which corresponds with 3.7 – 7 µM which is a 75- 140 fold excess compared to the concentration of BuChE (50 nM). Inhibition of the sample was allowed for 2 hrs at room temperature. Next 0.5 ml of nerve agent inhibited plasma was mixed with 0.5 ml d7-sarin inhibited plasma (internal standard). For one sample (blank) 0.5 ml non-inhibited plasma was mixed with 0.5 ml d7-sarin inhibited plasma. The plasma samples were further processed as described below.

Isolation of HuBuChE from human plasma

A disposable 10 mL mini-extraction column (tube ABIMED AMS 422 peptide synthesizer, Gilson, Villiers le Bel, France) was filled with 2 mL procainamide-gel, which was washed with 20 mL of phosphate buffer (15mM NaH_2PO_4 and 5 mM Na_2HPO_4, pH 6.9) Then, 1 mL of plasma sample was gently mixed with the procainamide-gel. After 30 min at room temperature, the gel was washed with 5 mL phosphate buffer and 5 mL 150 mM sodium chloride (150 mM NaCl in phosphate buffer). Finally, HuBuChE was eluted with 7 ml 600 mM NaCl in phosphate buffer.

Digestion of HuBuChE with pepsin

BuChE solution obtained after procainamide affinity extraction was concentrated using a 100 kD cut-off filter. The retentate was washed with 5% formic acid (2 x 2 mL). The retentate (approximately 200 µL) was transferred to a 4 ml glass vial; the filter was rinsed with 250 µl 5% formic acid. The rinse fluid was combined with the retentate. Pepsin solution (50 µl of a 0.2% (i.e., 2 mg/ml) solution in 5% formic acid) was added. After incubation for 2 h at 37 °C, the incubation mixture was filtrated through a pre-washed (0.5 ml water) 3 kD cut-off filter. The filter was washed with 150 µl 5% formic acid solution and the fluid was filtrated and pooled with the first filtrate. In case of 100% inhibition, this solution was used for LC-tandem MS experiments. In case a low observable detection level was desired, the combined filtrates were concentrated and the residue was dissolved in water with 5% formic acid (100 µl) and analyzed with LC-tandem MS.

LC-tandem MS of pepsin digests

The LC system consisted of an Alliance 2690 HPLC gradient system (Waters, Milford, MA, USA). The mass spectrometer was a Q-TOF instrument (MicroMass, Altrincham, UK) equipped with a standard Z-spray electrospray interface. Stationary phase was a PepMap C18 column (15 cm x 1000 µm, 3 µm particles) from LC-Packings (Amsterdam, The Netherlands). The mobile phase consisted of a gradient of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile. Gradient program was 0'-5': 100%A, flow 0.1-0.6 mL/min; 5'-60': 100% A->70% A, flow 0.6 mL/min. The pump flow (0.6 mL/min) was reduced to a column flow of 40 µL/min by a splitter (LC-packings). Injection volume was 10 µl. Electrospray MS-MS spectra of the protonated molecular ion were recorded using a cone voltage of approximately 35 V and collision energy of approximately 30 eV. Subsequently, ion chromatograms of *m/z* 778.4 that were obtained after selection and fragmentation of the protonated molecular ion, were recorded.

Ellman assay

The wells of a 96 well plate were filled with 100 µl DTNB solution (0.8 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM phosphate buffer, pH 8.0). Next 10 µl of the sample was added wherafter 100 µl BuSChI solution (0.8 mM Butyrylthiocholine iodide in water) was added. The plate was stirred for 10 s and the absorbance at 412 nm was read in the well plate reader. After a certain period the absorbance was measured again. The net increase of the absorbance in time is a measure of the HuBuChE activity in the sample.

On-line pepsin digestion of HuBuChE

The instrumental set-up consisted of a CapLC from Waters (Micromass, Wythenshawe, Manchester, UK) combined with a six-port switching valve (VICI, Bester Amstelveen, The Netherlands) for the direction of the flows. An LCQ Deca XP Max ion-trap mass spectrometer (Thermo Electron, San Jose, CA, USA) was used for the MS and MS/MS analysis. The first step in the procedure was the injection of a protein sample; a small aliquot is injected (2 µL) using the autosampler, situated on the CapLC. In case of purified HuBuChE, a 380 nM solution was diluted 100 times with water containing 0.2% formic acid. The sample was directed to the immobilized pepsin cartridge (Applied Biosystems, Nieuwkerk a/d Ijssel, NL), which is placed over an automated six-port switching valve, and the flow-through is directly transferred to the LC column (300 µm ID, 150mm C18 Pepmap LCpackings, Amstelveen, NL). The conditions in the pepsin cartridge equalled the begin conditions of the LC gradient (95% H_2O: 5% ACN 0.2%FA, 6 µL/min). Thus, the digestion

cartridge and LC column were switched in-line, resulting in trapping of the formed peptides in the on-line digestion on top of the analytical column. After a 20 min. digestion time, the valve was switched and a linear gradient elution (in 30 min to 20% H_2O: 80% ACN 0.2 % TFA 6 µL/min) of the trapped peptides took place. During the LC-analysis the digestion cartridge was reconditioned.

III.2 Generic assay for OP biomonitoring

Alkaline hydrolysis of inhibited HuBuChE followed by Michael addition of ethanethiol and pepsin digestion

Purified HuBuChE (250 µl of a stock solution; 5 nmol) was 100 % inhibited with either VX, sarin or soman (10 equivalents). The solution was concentrated over a 10 kD ultrafilter (4000 rpm, 30 min) to remove excess inhibitor and the retentate was dissolved in an aqueous solution of $Ba(OH)_2$ and ethanethiol (100 and 50 mM, respectively; 0.5 mL). The mixture was incubated for 2 hours at 37 ºC and neutralized with TFA/CH_3CN (1 %). The samples were concentrated over a 10 kD filter and washed with a solution of NH_4HCO_3 (50 mM, 3 x 1 mL). After the third wash step, the concentrate was dissolved in aqueous formic acid (5 %, 0.55 mL) containing pepsin (0.10 mg). The mixture was incubated for 2 h at 37 ºC, after which the mixture was concentrated over a 3 kD ultrafilter. The filtrate was analyzed with LC/MS/MS.

Michael addition of ethanethiol after pepsin digestion of (inhibited) HuBuChE

Purified HuBuChE (blank or inhibited; 1 nmol) was digested with pepsin as described above. After filtration of the peptic digest over a 3 kD filter, the filtrate was now lyophilized to dryness and redissolved in 200 µl of $Ba(OH)_2$ (100 mM) and ethanethiol (50 mM). After 1 h at 37 ºC, the reaction was quenched with acetic acid (5 µl) and the mixture was used for analysis with LC/MS/MS.

Generic procedure for diagnosis of exposure to nerve agents

Isolation of HuBuChE from plasma and subsequent pepsin digestion was carried out as described previously. The filtrate was concentrated, coevaporated with 50 mM NH_4HCO_3 (2x 0.5 ml), and dissolved in an aqueous solution of $Ba(OH)_2$ and 2-(3-aminopropylamino)ethanol (100 and 50 mM, respectively; 0.2 mL). After incubation for 1 h at 37 ºC, the reaction was quenched by the addition of acetic acid (10 µl). The resulting solution was analyzed with LC/MS/MS.

MS conditions used:

Eluent A: H_2O(0.2 % formic acid)

Eluent B: CH_3CN (0.2 % formic acid)

Gradient:	Time	%A	%B	Flow (mL/min)
	0	100	0	0.1
	5	100	0	0.6
	60	30	70	0.6

The flow of 0.6 mL/min was reduced by a LC-packings splitter to a column flow of ca 40 µl/min.

Column: PepMap C18, 3 µm, 15 cm*1 mm, loopsize 10 µl

Optimization MS/MS (parent ion, collision energy).

Parent ion	Fragment ion (peakintensity eV)	Fragment ion (peakintensity eV)
MH_2^{2+} 448.7	720.3 (429 at 18 eV)	791.4 (583 at 18 eV)

III.3 Feasibility studies for development of assays for diagnosis of exposure to toxic chemicals

III.3.1 Exploratory work on cyanide

Incubation of model peptide oxytocine with sodium cyanide

Oxytocine (CYIQNCPLG, with a disulfide bridge between both cysteine residues, 1 mg, 1 micromole) was dissolved in PBS (1 mL) giving a 1 millimolar solution. To this solution, NaCN was added (4.9 mg) resulting in an end concentration of 100 mM CN^-.
The mixture was incubated at 37 °C for 2 days. At regular points in time, samples were taken for HPLC and LC-MS analyses. After 2 h, HPLC analysis showed the appearance of an extra peak at 8.9 min. A blank sample, containing only oxytocine, only showed a peak at 11 min. After 2 days, no oxytocin was visible in the HPLC chromatogram, but two other peaks appeared. MS analysis demonstrated the presence of NCS-CPLG(NH2), NCS-CYIQ and of NCS-CYIQN.

Incubations of human blood with ^{14}C labeled KCN

Human blood (5 x 4 mL) was exposed to several concentrations (0, 0.01, 0.1, 1 and 10 mM) of ^{14}C labeled KCN (53 mCi/mmol) for 24 h at 37 °C. Subsequently, the blood samples were divided into erythrocytes and plasma by centrifugation. The erythrocytes were washed with 3 x 5 mL PBS. The remaining washed erythrocytes were lysed with water (2 mL). Globin was isolated from the above erythrocytes using acid acetone precipitation. Radioactivity counting was performed after each step on each fraction.

III.3.2 Exploratory work on phosphine

Incubation of model peptide oxytocine with phosphine

Phosphine gas was generated by adding a pellet of Magtoxin WM (60% Mg_3P_2; gives PH_3 after reaction with water; gift from Degesch Benelux) to a flask, fitted with a dropfunnel filled with water and a gas inlet and outlet system. The flask was first gently flushed with a stream of nitrogen to prevent ignition of the formed phosphine (spontaneous in air above 1.8%). A pellet of Magtoxin was added to the flask. A drop of water was allowed to fall on the pellet in order to start the immediate generation of phosphine gas. The outlet of the flask was connected via a plastic tubing to a Pasteur pipette in order to bubble the phosphine through a solution of oxytocin in PBS (1 mg/mL, 5 mL total). A total of four pellets were used to generate phosphine gas; each pellet generates approximately 200 mg of phosphine gas according to the manufacturer. After the last pellet had disappeared, the tube containing oxytocin and phosphine gas was closed and left at room temperature for 5 days. The normal solubility of phosphine gas in water is 31 mg/100 mL (9 mM) at 17 ºC. In our case, we generated 800 mg of gas and had this slowly bubbled through a PBS solution (5 mL); therefore, the maximum end concentration of about 9 mM is reasonable to assume.
After 5 days, the tube containing the reaction mixture was opened and nitrogen was bubbled through for 30 min. A sample (100 µls) of the reaction mixture was analyzed with LC/MS. No differences could be observed between the exposed sample and a control sample (non-exposed oxytocine).

III.4 Synthesis of various reference materials

III.4.1 (S-HETE)Cys-Pro-Phe for albumin tripeptide assay

Coupling

First, immobilized Pro-Phe (120 µmol) was prepared on a Syro peptide synthesizer according to standard protocols. Subsequently, coupling with Fmoc-Cys-S-HETE-OtBu was effected on a 90 µmol scale to give (S-HETE)-CPF.

Briefly, the resin was swollen in dichloromethane (5 ml) for 30 minutes and the peptide coupling reagents mixture was prepared. PyBop (281 mg), HOBt (73 mg) and Fmoc-Cys-HETE-OtBu (232 mg) were dissolved in N-methyl-2-pyrrolidone (NMP; 700 µl).

The coupling reaction was performed in two separate columns A (50 µmol) and B (40 µmol), due to the relatively high volumes. To column A the reagents mixture (388 µl), diisopropylethylamine (105 µl) and extra NMP (100 µl) were added. Column B was also treated with the mixture (312 µl), diisopropylethylamine (84 µl) and NMP (100 µl). The reaction mixtures were kept under argon for two hours.

Fmoc removal

The resin was washed with NMP (5 x 3 ml), treated with 20% piperidine/N,N-dimethylacetamide (DMA) (3 x 1 ml) for 10 minutes and washed with DMA (3 x 3 ml). Finally the resin was washed with NMP (5 x 3 min).

Cleavage

The synthesized S-HETE-CPF was cleaved from the resin with trifluoracetic acid/ triisopropylsilane (95/5) according to a standard protocol, precipitated with diethyl ether/pentane (1/1), washed and dried. The peptide was dissolved in water/acetonitrile and analysed with HPLC.

Purification and quantification

The product ((S-HETE)Cys-Pro-Phe) was purified with HPLC, analysed with LC-MS and concentrated under vacuo. The peptide (weighed amount: 11 mg) was dissolved in a mixture of water and acetonitrile (1/1, 2 ml) and quantified with HPLC by comparison with an already quantified standard (total yield: 10.05 mg). Finally, the peptide solution was divided into small portions (50 µg, 250 µg and 1 mg) and concentrated.

ES-MS data: *m/z* 470 (MH^+); 453 ($MH^+ - NH_3$); 364 $MH^+ - H_3CCH_2SCH_2CH_2OH$); 263, y_2''; 166, y_1''; 137, $^+SCH_2CH_2SCH_2CH_2OH$; 105, $^+CH_2CH_2SCH_2CH_2OH$.

III.4.2 Synthesis of pentafluorophenylthiohydantoin derivative of N-terminal valine for modified Edman degradation of sulfur mustard modified hemoglobin

First, a $KHCO_3$/2-propanol solution was prepared by dissolving 0.5 M $KHCO_3$ (100 ml) in 2-propanol (50 ml). N-(2-Hydroxyethylthioethyl)valine (50 mg ; 0.21 mmol) was dissolved in $KHCO_3$/2-propanol (20 ml ; pH = 8.84) and pentafluorophenylisothiocyanate (25 µl ; 0.8 eq.) was added. The mixture was stirred and heated in a water bath (40^o C) for 2 hours. Subsequently, the mixture was cooled to room temperature and extracted with hexane (2 x 25 ml). The hexane layer was concentrated under reduced pressure until a colorless syrup remained. The residue was taken up into dichloromethane (200 µl) and purified using a short column of silica gel (1% methanol/dichloromethane as eluent). The fractions were analysed

by TLC (1% methanol/dichloromethane) and GC-MS: in each case only one spot/peak could be observed. The purified product was dissolved in $CDCl_3$ and quantified: 56 mg. The solution was concentrated under vacuo and the residue was taken up in dichloromethane (1 ml), divided into small portions (1 and 10 mg), concentrated and stored at -70 ºC.
GC-MS (EI) data: *m/z*, 60 $[C_2H_4]^{+\cdot}$, 104 $[CH_2CHSCH_2CH_2OH]^{+\cdot}$, 130 $[C_6H_{12}NS]^+$, 351, loss of $^{\cdot}SCH_2CH_2OH$, 383, loss of $^{\cdot}CH_2CH_2OH$, 410, loss of H_2O, 428, $M^{+\cdot}$.

III.4.3 Synthesis of phosgene albumin adduct O=C-(T25-28)

N-Fmoc-cysteine-(S-carboxymethyl tert-butyl ester)
Cysteine.HCl (10.3 mmol; 1.63 g) was dissolved in 50 mM NH_4HCO_3 solution (100 ml). The pH of the reaction mixture was adjusted to 8.2 with NH_4HCO_3. Acetonitrile (50 ml) and t-butyl bromoacetate **(**2.4 g; 1.8 ml; 12.2 mmol) were added; the pH of the reaction mixture slowly decreased to 8.0. After stirring for 2 h at room temperature, TLC analysis (eluent: ethylacetate/n-butanol/acetic acid/water, 2/1/1/1, v/v/v/v; colorization with I_2) showed complete conversion into a higher running product. The reaction mixture was concentrated and subsequently the concentrate dissolved in water/dioxane (2/1, v/v, ca. 150 ml). The pH of the solution was adjusted to 8 with Na_2CO_3. Fmoc-Cl (13.5 mmol; 3.5 g), dissolved in 20 ml acetone, was added in portions. After stirring for 1 h, TLC analysis (eluent 8% $MeOH/CH_2Cl_2$) showed complete conversion. After adjusting the pH to 8 with aqueous NaOH, the mixture was extracted with petroleum ether. Subsequently, the mixture was acidified to pH 3.5 and extracted with ethyl acetate. The ethyl acetate fraction was dried ($MgSO_4$) and concentrated. Yield: 1.5 g (33% for two steps). Electrospray LC-MS: *m/z* 480 (MNa^+), 458 (MH^+), 402 ($MH^+ - C_4H_8$).

O=C-(T25-T28)
Peptide synthesis was performed according to standard protocols for solid phase peptide synthesis.
The peptides (Z-Ala)-Ser-Ser-Ala-Lys-Gln-Arg(NO_2) (4.9 mg; 5.3 µmol) and (Z-Leu)-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys(Z) (6.2 mg; 5.1 µmol) were dissolved in water/acetonitrile (v/v, 1/1; 5 ml). The pH of the solution was adjusted to 9 with diluted NaOH solution. A 20% solution of phosgene in toluene (0.1 ml) was diluted with acetonitrile (0.9 ml). Small aliquots (25 µl) of this solution were added to the peptides solution, after which the pH of the solution was brought to pH 9 with aqueous NaOH (40 mM). It was verified with LC tandem MS that, in addition to the symmetrical products, the desired product had been formed. The desired product was isolated by means of semi-preparative reversed phase HPLC. Yield: 1.2 mg.
A part (0.6 mg) of the protected product was deblocked by treatment with HF in anisole in a Teflon container. To the peptide anisole (50 µl) and liquid HF (1 ml) was added. The reaction mixture was stored for 2 h at 0 ºC. HF was removed under a stream of N_2 gas; the residue was dissolved in 0.2 M acetic acid (2 mL). The solution was extracted with diethylether (2 ml). Purification of the crude product was effected by means of reversed phase HPLC (Source column). Yield: 175 µg.

III.4.4 Synthesis of standards for Lewisite method

Synthesis of CVAA-BAL

To a solution of L1 (415 mg; 2 mmol) in acetone (30 mL), BAL (286 mg; 2.3 mmol) was added in small amounts within 30 min. Subsequently, the mixture was concentrated, and CVAA-BAL was isolated and purified using silica gel column chromatography, with toluene/ethylacetate (100/0 to 95/5, v/v) as the eluent. Appropriate fractions (analyzed using TLC; eluent: ethyl acetate/toluene, 1/4 ,v/v) were collected and concentrated. Yield: 76 mg (0.3 mmol, 15%) of a slightly colored oil. The product was a mixture of two isomers (ratio: 7/3) as determined by NMR spectroscopy. CVAA-BAL (code: 0439AF01; 5 vials containing 1 mg each). The samples were stored at CDC at -70 ºC.

^{1}H-NMR ($CDCl_3$):

δ (ppm): 6.65 (d, 1H,J= 14.3 Hz, = C(Cl)H, isomer II), 6.55 (d, 1H, J=14.3 Hz, =C(As)H, isomer II), 6.52 (m, 2H, J= 14.2 Hz, CH =CH, isomer I), 4.14-3.10 (m, 5H, 2 x CH_2, 1 x CH; both isomers), 1.96 (b, 1H,OH, isomer I), 1.88 (b, 1H, OH, isomer II).

^{13}C-NMR ($CDCl_3$):

δ (ppm): 136.5 (=C, isomer II), 134.7 (=C, isomer I), 128.0 (=C, isomer II), 127.7 (=C, isomer I), 64.5 (CH_2OH, isomer I), 63.7 (CH_2OH, isomer II), 62.5 (CH, isomer II), 58.7 (CH, isomer I), 44.0 (CH_2, isomer II), 43.2 (CH_2, isomer I).

Synthesis of internal standard phenylarsine-BAL

Phenylarsine-BAL (PAB), which was used as an internal standard, was prepared starting from phenylarsine oxide and purified as described for CVAA-BAL. Yield: 630 mg (70%). The product was a mixture of two isomers (ratio 7/3), according to ^{1}H-NMR. Phenylarsine-BAL (code 0441AF02; 5 vials containing 1 mg each).

The samples were stored at CDC at -70 ºC.

^{1}H-NMR ($CDCl_3$):

δ (ppm): 7.4-8.0 (m, 5H, H-aromatic), 5.2 (m, 1H, OH), 4.0-2.8 (m, 5H, 2 x CH_2, 1 x CH)

^{13}C-NMR ($CDCl_3$):

δ (ppm): 129-128 (C-aromatic), 63.3 (CH_2OH; isomer I), 63.0 (CH_2OH, isomer II), 62.4 (CH, isomer I), 58.4 (CH, isomer II), 43.1 (CH_2, isomer II), 42.3 (CH_2, isomer I).

III.4.5 Synthesis of reference peptides for nonapeptide assay for nerve agents

Synthesis of bis(di-isopropylamino)chlorophosphine

To a solution of phosphotrichloride (14ml, 0.158 mol) in diethyl ether (240 ml) di-isopropylamine (95.8g, 0.947 mol) was slowly added under argon atmosphere. The mixture was stirred for 6.5 hours at reflux temperature and 16 hours at room temperature. The di-isopropylamine•HCl salt was removed by filtration and the solvent by evaporation. The residue was distilled under atmospheric pressure in order to remove the remaining di-isopropylamine, followed by distillation under reduced pressure (4 mmHg, 140ºC). The product distilled at a temperature of 115 – 118 ºC.

Yield 34.4 g (82%); ^{31}P NMR (85% H_3PO_4) δ (ppm): 133.5.

Synthesis of bis(di-isopropylamino)methylphosphine

To a cold (acetone/CO_2 bath) solution of bis(di-isopropylamino)chlorophosphine (13.29g, 49.8mmol) in diethyl ether was added dropwise a solution of methylmagnesiumbromide (17ml, 51.0mmol, 3.0M in diethyl ether) under an argon atmosphere. After 30 minutes at low temperature the reaction mixture was stirred for 1 hour at room temperature. The precipitate

was removed by filtration and the filtrate was distilled under normal pressure in order to remove the solvent. This was followed by distillation under reduced pressure (1mbar, 110ºC). The product distilled at a temperature of 92-93 ºC. Yield: 9.43 g (77%). ^{31}P NMR (85% H_3PO_4) δ (ppm): 39.9.

Synthesis of O-isopropyl-bis(di-isopropyl)phosphoamidite (A)
Isopropyl alcohol (536µl, 421mg, 7.0 mmol) and sym-collidine•HCl (80 mg, 0.5mmol), that had been dried by coevaporation with acetonitrile (3 x 5ml), were dissolved in dichloromethane (5ml). Bis(diisopropylamino)methylphosphine (1.24 g, 5 mmol) was added and the solution was stirred for 24 hours at room temperature. TEA (0.5ml) was added and the reaction mixture was concentrated. The product was purified by silica gel column chromatography with hexane/TEA (19/1, v/v) as eluent. Yield: 0.90 g (88%).

Synthesis of O-ethyl bis(di-isopropyl)methyl phosphoamidite (B)
This reagent was prepared as described for the synthesis of reagent A; ethanol (410 µl, 323mg, 7.0mmol) was used instead of isopropyl alcohol. Yield: 0.78 g (82%).

Synthesis of O-p-methoxybenzyl bis(di-isopropyl)-methylphosphoamidite (C)
This reagent was prepared as described for the synthesis of reagent A; methoxybenzyl alcohol (870 µl, 962mg, 7.0mmol) was used instead of isopropyl alcohol. Yield 1.15 g (82%).
^{31}P NMR (85% H_3PO_4) δ (ppm): 122.2.

Synthesis of bis(dimethylamino)ethoxyphosphine
To a solution of ethyldichlorophosphite (5.5 g; 37.4 mmol) in diethylether (20 ml) was added a solution of dimethylamine (6.11 g, 135.8 mmol) in diethylether (20 ml), while the mixture was kept under an Argon overpressure at 10 ºC. The reaction mixture was stirred for 3 h. The formed dimethylamine.HCl salt was removed by filtration and the filtrate was concentrated. The residue was distilled under reduced pressure (12-14 mm Hg) at 60 ºC. The product distilled at a temperature of 45 ºC.
^{31}P NMR (85% H_3PO_4) δ (ppm): 137.7.

Synthesis of O-p-methoxybenzyl(N,N-dimethyl)ethoxyphosphoroamidite (D)
This reagent was prepared as described for the synthesis of reagent A. In this case bis(dimethylamino)ethoxyphosphine (825 mg, 5 mmol) was used instead of bis(diisopropylamino)methylphosphine, and methoxybenzyl alcohol (962 mg, 7.0 mmol) was used instead of isopropanol. Yield: 0.464 g (30%). ^{31}P-NMR (85% H_3PO_4) δ (ppm): 146.4.

Phosphorylation of the immobilized nonapeptide
First, the protected and immobilized nonapeptide Boc-FGE(tBu)SAGAAS(tBu) (10µmol) was washed consecutively with dimethylacetamide, t-amyl alcohol, 20% acetic acid in t-amyl alcohol, t-amyl alcohol, diethyl ether and under vacuum dried on P_2O_5. Phosphitylating reagent (0.15mmol, 15eq in 800µl acetonitril) and ^{1}H-tetrazole (167µl, 0.075mmol, 0.45M in acetonitril) were added to the immobilized peptide and the mixture was stirred for 16 hours. *tert*-Butyl hydroperoxide solution (80% in water; 100 µl) was added and the mixture was stirred for one hour. The peptide (still immobilized) was once more washed according to the previous described method.
In case of isopropyl methylphosphonyl nonapeptide, reagent A was used, in case of ethyl methylphosphonyl nonapeptide reagent B, in case of methylphosphonyl nonapeptide reagent C and in case of O-ethylphosphoryl nonapeptide reagent D.

Cleavage from the resin and deprotection
The immobilized phosphonylated peptide was cleaved from the resin and deprotected by treatment with a TFA/TIS solution (95/5, v/v); 200 µl of this solution was added six times every 5 minutes. After the last addition, the resin was filtered off and left in the TFA/TIS solution for 2 hours after which the solution was removed. After dissolving the residue in water followed by freeze drying the desired peptide adduct was obtained. They were purified by semi-preparative HPLC using an Altima C18 column.

Analytical data (TOF MS ES^+):
"nonapeptide" denotes FGES*AGAAS, with S* phosphylated.
Isopropyl methylphosphonyl nonapeptide: *m/z* 916.4 (MH^+), 458.7 (MH_2^{2+})
Ethyl methylphosphonyl nonapeptide: *m/z* 902.3 (MH^+), 451.7 (MH_2^{2+})
Methylphosphonyl nonapeptide: *m/z* 874.3 (MH^+), 437.7 (MH_2^{2+})
Ethyl phosphoryl nonapeptide: *m/z* 904.3 (MH^+), 452.7 (MH_2^{2+})

Analytical data (TOF MS/MS ES^+):
Characteristic, unique fragments:
Isopropyl methylphosphonyl nonapeptide: no MS/MS available of synthetic peptide adduct.
Ethyl methylphosphonyl nonapeptide: *m/z* 442.7(MH_2^{2+} - H_2O).
Methylphosphonyl nonapeptide: *m/z* 428.7 (MH_2^{2+} - H_2O).
Ethyl phosphoryl nonapeptide: *m/z* 443.7 (MH_2^{2+} - H_2O).

All peptides seem to loose the phosphonyl moiety easily during fragmentation. Characteristic common fragments:
778.3 (MH^+ - phosphonyl moiety).
673.3 (b_8 – phosphonyl moiety).
602.3 (b_7 – phosphonyl moiety).
531.2 (b_6 – phosphonyl moiety).
177.1 (a_2/y_2'').
106.1 (y_1").

IV RESULTS

IV.1 Demonstration and further development of SOP for the albumin-tripeptide assay for detection of sulfur mustard exposure

Background

Noort *et al*. (1999, 2004b) published a sensitive method for detecting exposure to sulfur mustard based on pronase digestion of albumin, and subsequent mass spectrometric analysis of the resulting tripeptide Cys*-Pro-Phe, with Cys* the sulfur mustard-modified Cys-34 residue of albumin.

This assay was demonstrated to a scientist of USAMRICD in 2004, as was described in Volume I of this report. The actual demonstration was performed at CDC; consequently, a CDC scientist took also part in the demonstration.

Method demonstration

Especially the use of affinity chromatography for isolation of albumin was further explored. The demonstration has been described in detail in Volume I of this report and has also been published as a joint publication (Noort *et al*., 2004b).

Attempts for automation of the albumin assay

The automation of the μliquid chromatography – electrospray – tandem mass spectrometric analysis was initiated, by attempting to perform the albumin digestion on-line. Especially in case of large numbers of samples, the availability of an automated system is highly convenient. In the first instance, the immobilization of pronase on Poros column material was attempted (see Riggs *et al*., 2001). Unfortunately, after immobilization of pronase we were not able to detect the tripeptide anymore in the eluate of the Poros column. We therefore turned our attention to pepsin; columns containing immobilized pepsin are commercially available (*vide infra* for the nonapeptide assay). First, the in-solution pepsin digestion of albumin from plasma that had been exposed to sulfur mustard was studied. The main adducts found were AEVSKL (MH_2^{2+}, 375.7), KPLVEEPQNL (MH_2^{2+}, 635.8) and LGMFLYE (MH_2^{2+}, 488.0); see Figure 1. The position of the (2-hydroxyethyl)thioethylgroup is not sure in all cases, due to the instable character of the adducts, although for these particular fragments glutamic acid residues are involved. Unfortunately, we did not yet succeed in the digestion of sulfur-mustard alkylated albumin with immobilized pepsin.

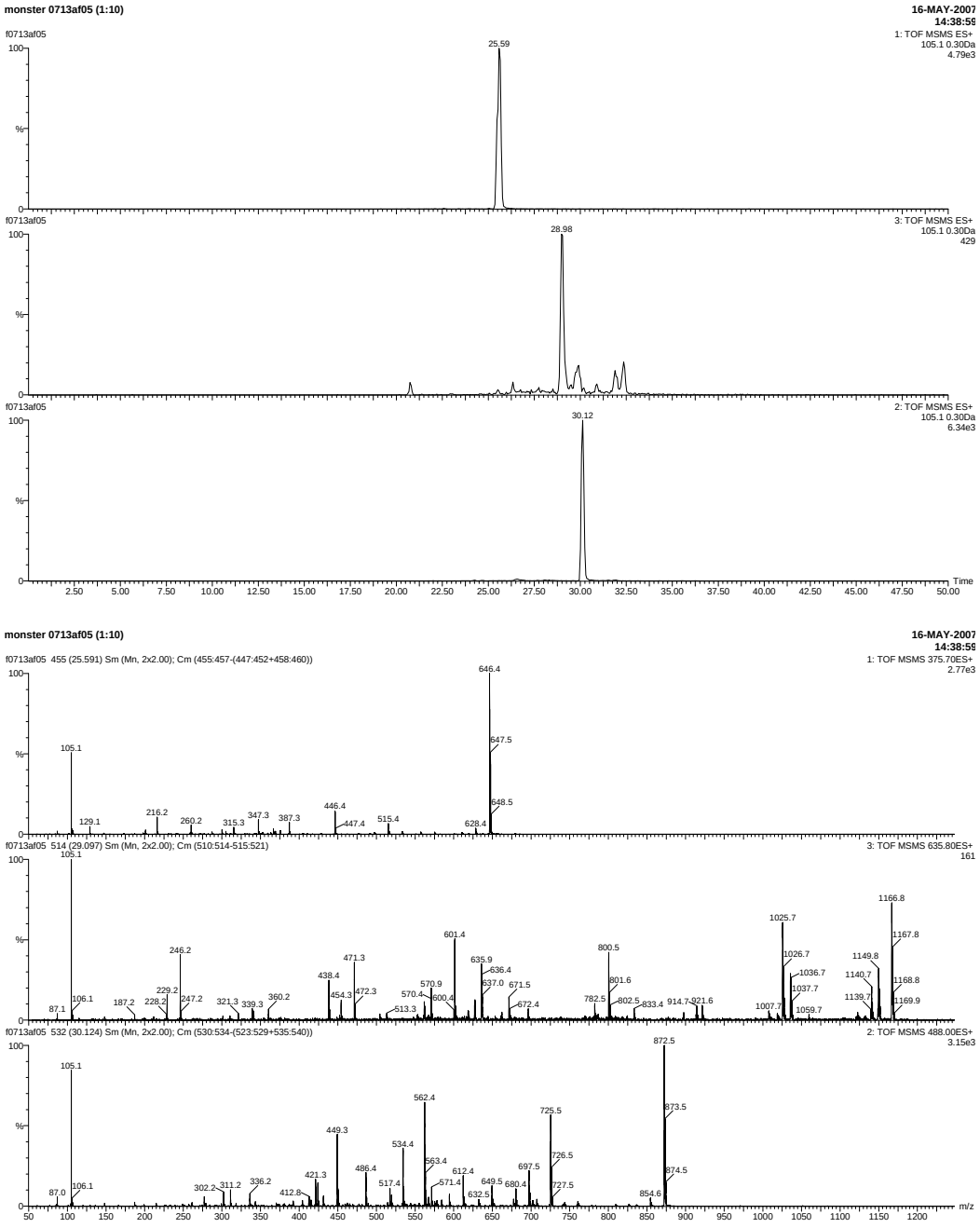


Figure 1. LC-tandem MS analysis of pepsin digests of albumin isolated from human blood that had been exposed to sulfur mustard. Upper three traces, 2-hydroxyethylthioethyl adducts of AEVSKL (MH_2^{2+}, 375.7), KPLVEEPQNL (MH_2^{2+}, 635.8) and LGMFLYE (MH_2^{2+}, 488.0), respectively. Lower three traces, corresponding tandem mass spectra

IV.2 Demonstration of the modified Edman degradation of sulfur mustard modified hemoglobin

Background

Sensitive methods have been reported for sulfur mustard adducts with proteins. In case of the N-terminal valine globine sulfur mustard adduct, the alkylated amino acid can be selectively

cleaved using a method developed for other alkylating agents (Törnqvist *et al*., 1996). Reaction of globin with pentafluorophenyl isothiocyanate releases the alkylated amino acid as the hydantoin, which is further derivatized to its heptafluorobutyryl derivative and analyzed by GC-MS or GC-MS/MS (Fidder *et al*, 1996; Noort *et al*., 2004a). The actual chemistry on which the modified Edman degradation is based, is depicted in Figure 2.

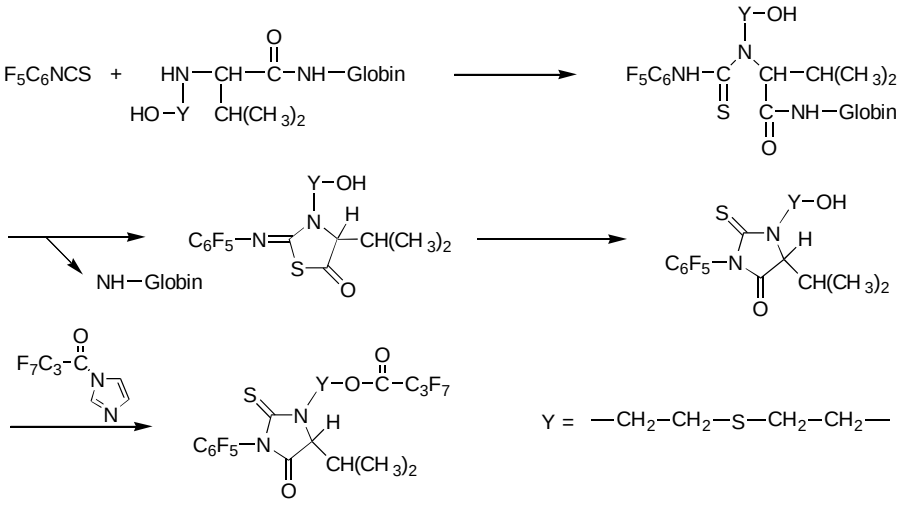


Figure 2. Modified Edman degradation of sulfur mustard-modified globin

Method demonstration

The method demonstration was focused on two objectives. The first objective was to see whether the method for modified Edman degradation of sulfur mustard-modified globin and subsequent GC-MS analysis could be installed within a relatively short period (4 days) of time. The second objective was to evaluate the use of LC tandem MS analysis for analysis of the underivatized pentafluorophenylthiohydantoin derivative obtained after modified Edman degradation.

A batch of underivatized pentafluorophenylthiohydantoin was synthesized in our laboratory, starting from synthetic N-terminal valine - sulfur mustard adduct (see Figure 3 for GC-MS analysis).

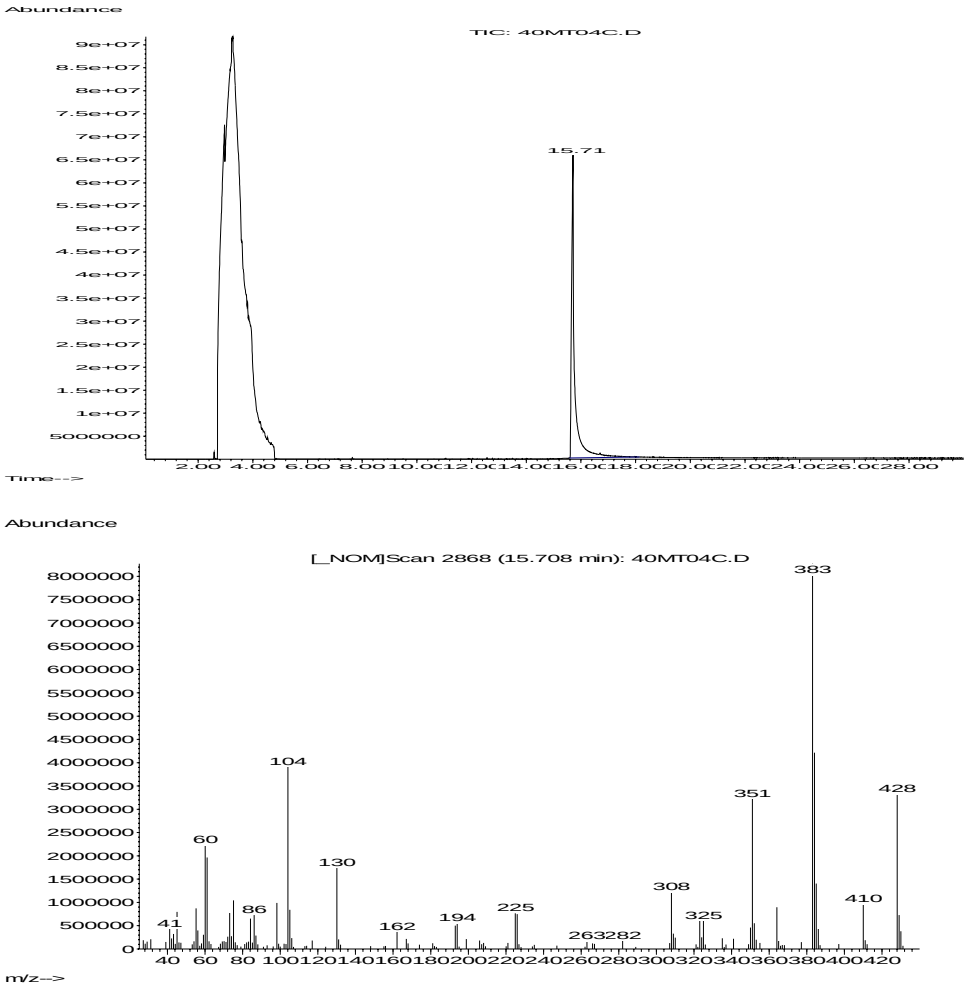


Figure 3. GC-MS analysis of pentafluorophenylthiohydantoin derivative of sulfur mustard adduct of N-terminal valine; upper trace GC-MS chromatogram; lower trace, EI-MS spectrum.

With regard to the first objective, it turned out that GC-EI-MS analysis of the pentafluorophenylthiohydantoin gave too many fragments. Since NICI analysis on the MAT900 is not sensitive enough, it was decided to use a triple quad-GC-MS (TSQ7000). The synthetic reference standard, after derivatization with HFBI, gave the expected mass spectrum after GC-NICI-MS; comparable spectra were obtained. For the already processed samples, a 10 nM level could still be detected. This is an improvement of the analyses performed at TNO, since in the latter case only a 100 nM exposure level could be detected. With regard to newly prepared samples, i.e. samples processed at CDC by a CDC-worker and a TNO-worker, rather variable results were obtained presumably due to concentration of processed samples was performed by heating under a stream of dry nitrogen and that relatively high amounts of reference materials were used for instrument optimization, during work-up of relatively low-level exposed globin samples.
With regard to the second objective, it turned out that LC tandem MS analysis can be applied for analysis of the specific pentafluorophenylthiohydantoin derivative to globin samples, processed according to the modified Edman degradation. The transition *m/z* 429 → 130 was selected for MRM (see Figures 4 and 5).

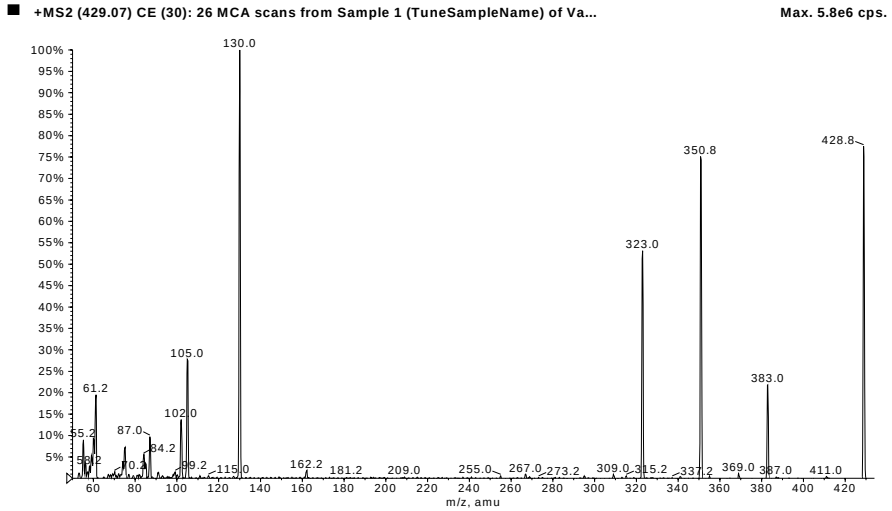


Figure 4. ESI/MS/MS product ion spectrum of pentafluorophenylthiohydantoin derivative of N-terminal valine adduct of sulfur mustard. This is a composite spectrum, created during automatic optimization, using a range of collision energies.

It turned out that the native standard had a rather long retention time (30 min) when a gradient of 99% water/1 % formic acid to 80%/20% CH_3CN, 1% formic acid was applied. Also, we suspected that the solubility of the pentafluorophenylthiohydantoin in aqueous solutions would be a problem. Best results were obtained with a gradient from CH_3CN/water, 1/1, 1% formic acid to 98% CH_3CN, 1% water, 1% formic acid, when the compound was dissolved in CH_3CN/water, 1/1, 1% formic acid.

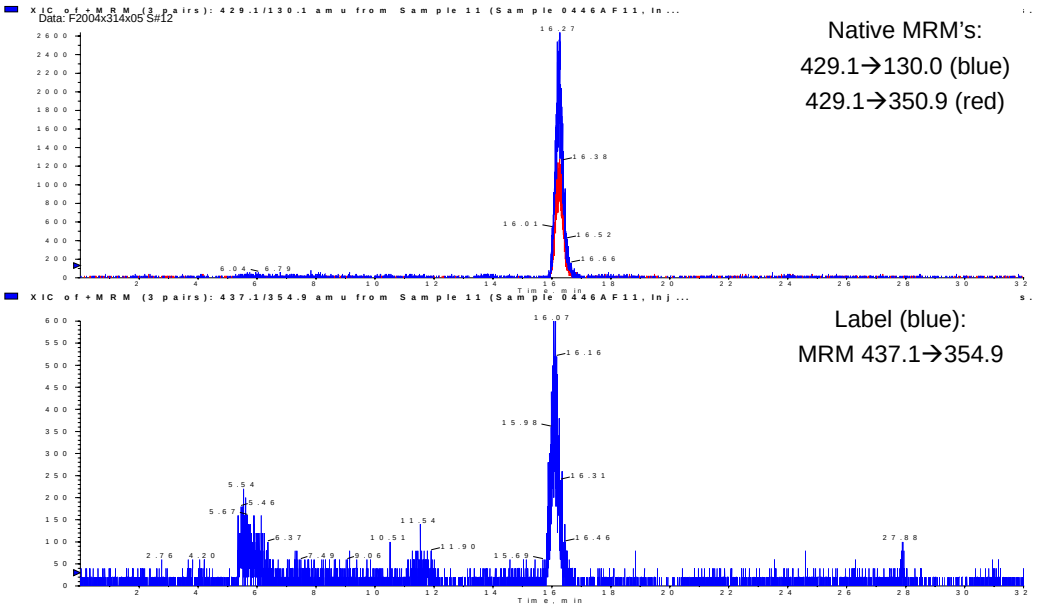


Figure 5. LC tandem MS analysis of a processed globin sample from blood that had been exposed to sulfur mustard. The upper trace represents the analysis of the native adduct (1 µM exposure level), and the lower trace represents the analysis of the d_8-deuterated adduct (5 µM exposure level).

The compounds appear to give relatively poor ionization by positive ion electrospray. The signal strength is much weaker than we routinely observe with albumin sulfur mustard tripeptide adducts. MS/MS fragmentation is good, with 3 or 4 strong ions to choose from. The estimated detection limit for the valine adducts is > 0.1 µM exposure level.

IV.3 Demonstration of the method for diagnosis of exposure to phosgene

Background

Phosgene is a highly reactive nucleophile with a half-life < 1 s in water. With regard to biological fate, it has been reported that phosgene reacts with glutathione to form a bis-conjugate (Fabrizi *et al*., 2000), and with cysteine to form 2-oxothiazolidine-4-carboxylic acid (Kubic and Anders, 1980). Also, it can react with the polar heads of phospholipids (Di Consiglio *et al*., 2001). Phosgene has also been reported to bind to haemoglobin and albumin (Noort *et al*., 2000). During exposure of human serum albumin to phosgene the lysine 195 and 199 residues are bridged, under formation of a urea-type bridge. Trypsin digestion of the phosgene-modified albumin results in the formation of a bis-peptide containing the normal tryptic fragments T25-T26 and T27-T28, with T25 and T27 linked by a carbonyl group (see Figure 6; Noort *et al*., 2000).

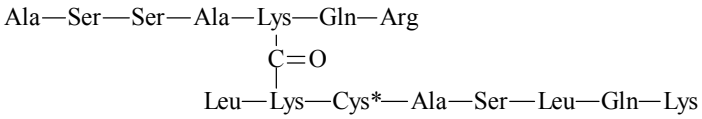


Figure 6. Structure of the phosgene adduct (ASSAK*QR)(LK*ZASLQK), with a C=O bridge between K* residues and with Z as carboxymethylcysteine [O=C-(T25)(T28)]

Method demonstration

The method was demonstrated to CDC workers as described in Appendix 2 of the experimental part. In advance of the actual demonstration, a number of reference compounds, already processed samples, and (exposed) samples to be processed at CDC had been sent to CDC. It appeared that the method could be relatively easy transferred, having in mind that the instrument (API 4000 Qtrap) had been installed only one week prior to the demonstration (see Figure 7 for a representative run).

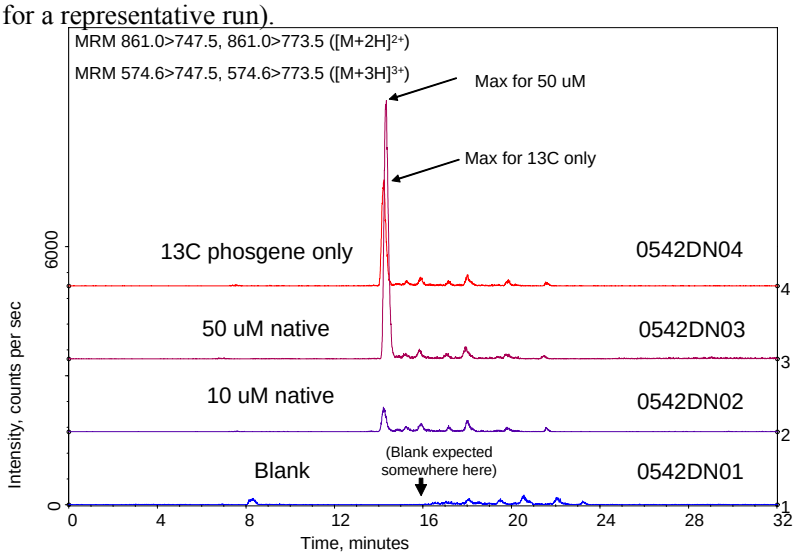


Figure 7. Tandem MS analysis of tryptic digests of albumin from ^{13}C-phosgene and normal phosgene-exposed plasma samples; MRM traces were summed.

From Figure 7 it became obvious that the ^{13}C-labelled internal standard cannot be used due to crossover of signals. Analogously, when the transition MRM 861.7 → 774.5 (i.e. the preferred MRM for the internal standard derived peptide adduct) was monitored, crossover was also observed for the native peptide adduct. As was also the case during earlier analyses (Noort *et al*., 2000), no real clean blank could be obtained (see Figure 8). The reason for this is still unknown, but probably the presence of large amounts of carbon dioxide (i.e.,"inactivated phosgene") may play a role in it.

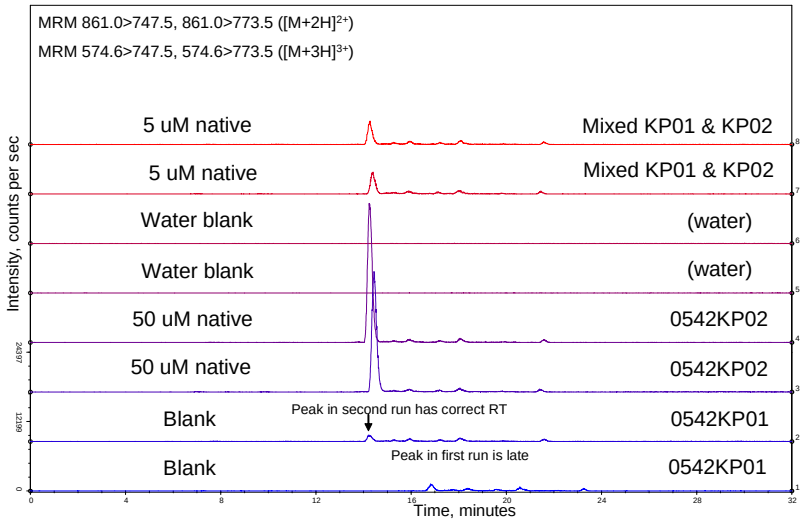


Figure 8. Tandem MS analysis of tryptic digests of albumin from phosgene-exposed plasma samples; MRM traces were summed. These particular samples were processed independently by a CDC worker.

Synthesis of reference peptide O=C-(T25-T28)

The most pragmatic way for the synthesis of the reference peptide is by reaction of phosgene with the two individual peptides Ala-Ser-Ser-Ala-Lys-Gln-Arg and Leu-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys. In that case, we should deal with the following. First, certain functional groups (N-terminal amino-groups, the NH_2 of the C-terminal lysine residue and the arginine group) have to be protected, since otherwise too many side-products will be formed, giving a complex, probably inseparable mixture of products. Second, in any case, symmetrical products will be formed, such as (Ala-Ser-Ser-Ala-Lys*-Gln-Arg)$_2$-C=O and (Leu-Lys*-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys)$_2$=O, with Lys* the site of carbonylation.

Having this in mind, we envisaged that the following peptide building blocks had to be synthesized:

1. (Z-Ala)-Ser-Ser-Ala-Lys-Gln-Arg(NO_2)
2. (Z-Leu)-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-(Lys-Z)

Schematically, the synthesis proceeds as depicted in Figure 9.

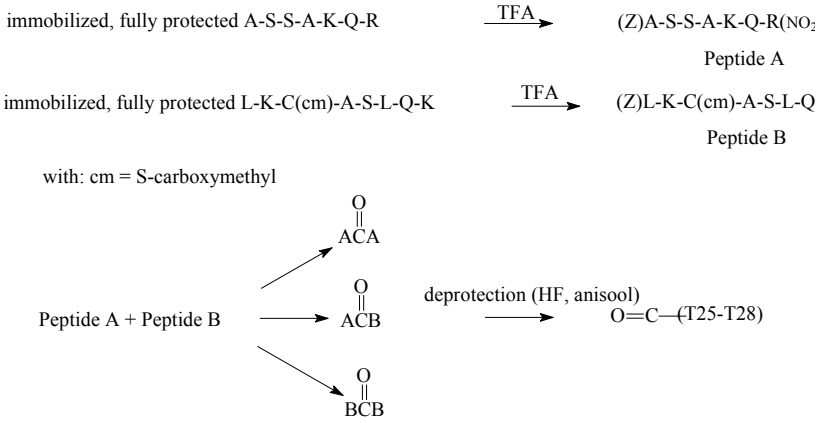


Figure 9. Synthetic methodology towards phosgene – albumin biomarker peptide.

The benzyloxycarbonyl (Z)-group was selected as protective group for the N-terminal amino functions, for the ε-NH_2 group of lysine and for the nitro group of the guanidine moiety in arginine. These protective groups are resistent against trifluoroacetic acid required for splitting the peptide from the resin. The peptides were further synthesized by means of standard Fmoc solid phase peptide chemistry, i.e., with acid-labile protective groups for the other side chains.
The particular cysteine building block, which was not commercially available at that time, was prepared by reaction of cysteine with tert-butyl bromoacetate, followed by reaction with Fmoc-Cl. Fmoc-Lys(Z) and Fmoc-Arg(NO_2) were coupled to SAC-Tentagel. The desired resins were obtained in good yield. The capacity of the resins was 18 µmol/100 mg for NH_2-Arg(NO_2) and 24 µmol/100 mg for NH_2-Lys(Z)-resin. The peptides were synthesized according to standard protocols on a solid-phase peptide synthesizer. Cleavage from the resin was effected by treatment with trifluoroacetic acid/water, 95/5, v/v.

The partially protected peptides were purified by means of semi-preparative reversed phase HPLC. Subsequently, they were linked with a carbonyl bridge through reaction with phosgene at pH 9. Except for the desired compound, also two symmetrical products were formed, according to LC tandem MS. The desired, partially protected bis-peptide was isolated and purified by means of semi-preparative reversed phase HPLC; see Figure 10 for MS spectrum.

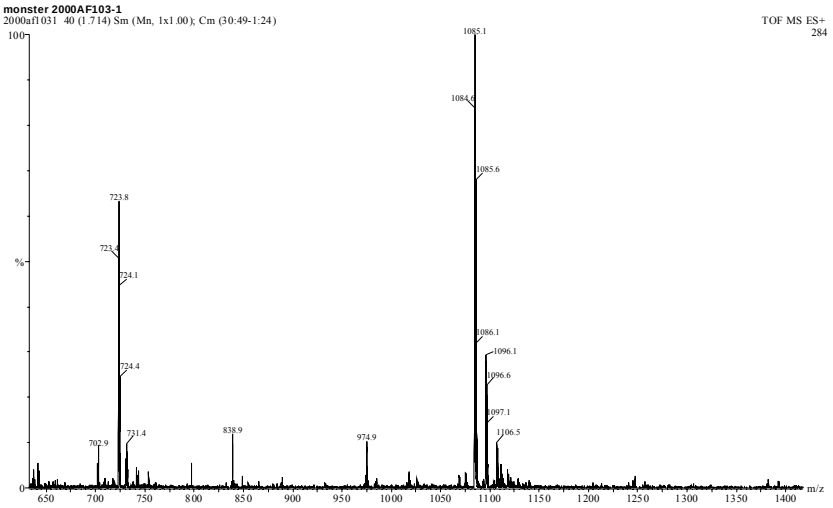


Figure 10. Electrospray MS spectrum of partially protected, synthetic O=C-(T25-T28): ((Z-Ala)-Ser-Ser-Ala-Lys*-Gln-Arg(NO_2))---(C=O)---((Z-Leu)-Lys*-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-(Lys-Z), with Lys* as Lys-195 and Lys-199 of human serum albumin.

Subsequently, the product was deprotected. Deprotection with BBr_3/TFA resulted in a non-defined product. Treatment with HF.pyridine resulted only in removal of the Z-groups; the NO_2 group remained on the Arg residue. Deprotection with HF/anisole yielded the desired deprotected product. The product was analyzed by means of LC tandem MS and had identical retention time and MS spectrum as the bis-peptide adduct obtained after trypsin digestion of phosgene-modified albumin (see Figure 11).

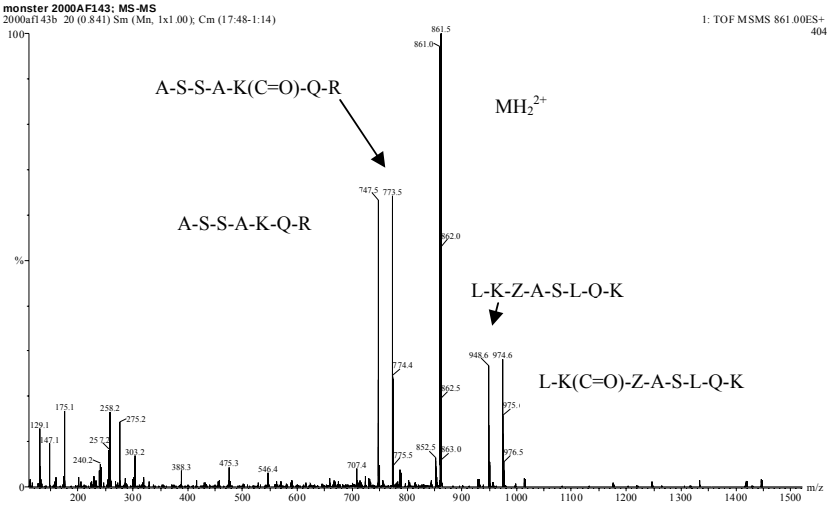


Figure 11. Electrospray tandem MS spectrum of synthetic, fully deprotected O=C-(T25-T28).

IV.4 Approach for automation of the assay for diagnosis of phosgene exposure

In collaboration with the group of Prof. Dr. Irth of the Free University of Amsterdam (The Netherlands), we have explored the possibilities to automate certain steps in the assay for

diagnosis of phosgene exposure. To this end, the approach depicted in Figure 12 will be followed:

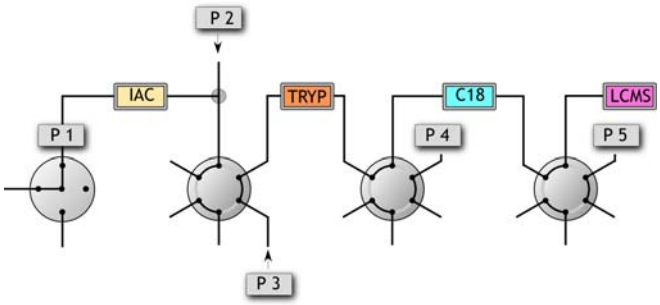


Figure 12. Analytical approach towards automation of phosgene assay; IAC, immunoaffinity chromatography for albumin; TRYP, immobilized trypsin; C18, C18 SPE cleanup.

In the first step, albumin will be isolated from plasma by immunoaffinity chromatography. It is believed that this will give a more pure sample than isolation by normal affinity chromatography. In the second step, the albumin will be enzymatically cleaved on a column containing immobilized trypsin. Subsequently, the digest will be reconcentrated on a short C18 SPE column, after which the sample will be analyzed by means of LC-tandem MS. From the above scheme it is obvious that the carboxymethylation step has been omitted. Until now, it is not yet possible to perform a carboxymethylation step on-line. So, we should either focus on a slightly different biomarker that does not contain the carboxymethylated cysteine residue, or the carboxymethylation should be performed off-line.
Until now, attention has been focused on automation of the C18 SPE desalination of the synthetic adduct. The peptide could readily be analyzed, giving identical mass spectral data.
We continued with the immunoaffinity isolation of modified HSA from plasma, followed by the off-line sample handling, i.e., sample lyophilization, carboxymethylation, and trypsin digestion. Finally, the sample was analyzed with SPE-LC-MS analysis, as described above. The following results were obtained (see Figure 13); in this case, 30 µl of the plasma sample was processed.

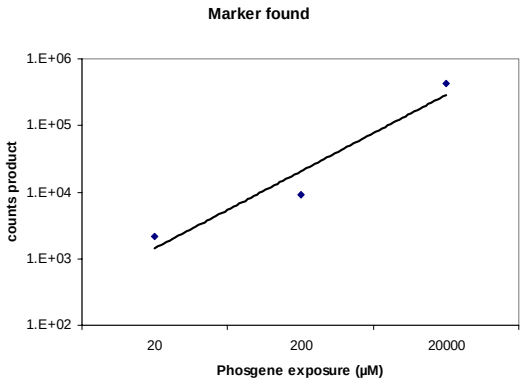


Figure 13. Semi-quantitative dose-effect relationship of formed O=C-(T25-T28) in processed albumin, isolated from plasma that had been exposed to various concentrations of phosgene. Sample work-up consisted of albumin isolation by immunoaffinity chromatography, and off-line sample handling, i.e., sample lyophilization, carboxymethylation and trypsin digestion.

IV.5 Demonstration of the method for diagnosis of exposure to Lewisite

Background

Trivalent arsenic has a high affinity for thiol groups. Upon incubation of human blood with ^{14}C-lewisite, 93% of the total radioactivity was found in the erythrocytes, with 25-50% associated with globin (Fidder *et al*., 2000). LC tandem MS of tryptic digests indicated the presence of several binding sites, and specifically identified a crosslink between the cysteine-93 and cysteine-112 residues of β-globin. The affinity for thiols has also been exploited in analytical methods. We use an analytical method that employs 2,3-dimercapto-1-propanol (BAL) for displacement of bound lewisite residues or hydrolyzed lewisite (CVAA) residues (see Figure 14). The resulting derivative is further derivatized with a heptafluorobutyryl group, which was initially meant to enable GC-NICI analysis. Unfortunately, the HFB group was easily split off under NICI conditions. However, the HFB derivative enabled sensitive analysis under EI conditions, allowing the detection of exposure levels of > 1 nM (see Fidder *et al*, 2000).

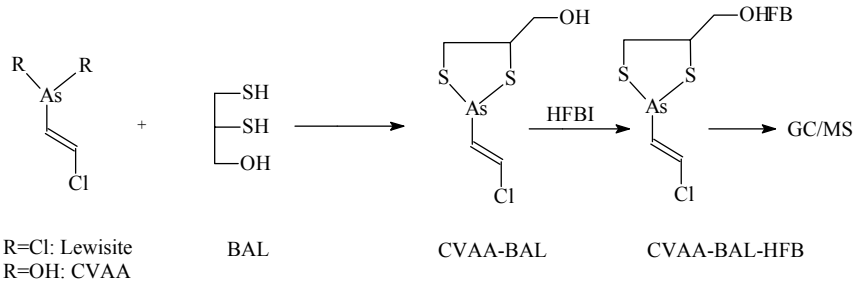


Figure 14. Chemical structures of dichloro(2-chlorovinyl)arsine (Lewisite, L1), 2-chlorovinylarsonzuur (CVAA), British Anti-Lewisite (BAL) and conversion into CVAA-BAL-HFB derivative.

Demonstration

The already processed samples that were sent to CDC gave almost identical analytical results, as obtained at TNO. Also, the reference standards that had been prepared could be analyzed satisfactorily. However, work-up of samples at CDC gave rather variable results, which could mainly be attributed to slightly different conditions, materials and chemicals that were used. Also, the instrument we used had not been calibrated with the synthetic standard in advance of the actual method demonstration. In a later stage, the method has been slightly changed with regard to the concentration of the samples; after introduction of the heptafluorobutyryl group, the sample was diluted with a small amount of toluene (0.2 ml) in order to facilitate further work-up. This procedure was demonstrated to the CDC technician in a later stage of the project (May 2007). The results obtained at CDC were now entirely comparable with the results obtained at TNO. Also, during this visit work was carried out on an alternative assay (see below), which proved to be more rapid and straightforward to perform than the BAL assay.

New assay for diagnosis of exposure to lewisite

During a later visit to CDC, additional work has been performed on diagnosis of exposure to Lewisite. In a collaborative effort with CDC, we elaborated a method that was originally used for analysis of urine samples, which is based on derivatization of CVAA with 1,3-propanedithiol (see Figure 15), followed by GC-MS analysis (Wooten *et al*., 2002). Applying

this method to blood samples resulted in a very rapid and straightforward method, allowing the analysis of an exposure level of 0.1 μM lewisite. This has not yet been optimized, however. A detailed description of the demonstration, including figures, can be found in Appendix 2.

Compared to the older method using BAL/HFBI, this method is much faster and easier to perform. An advantage is that the CVAA-PDT is not very volatile, which simplifies sample handling. It is clear that some adjustments to the method still have to be made with regards to determination of the efficacy of the derivatization procedure, as well as of the extraction procedure.

Recommendations/suggestions for improvement/further research:

1. investigate the effect of shortening incubation time with PDT (use the highly exposed blood sample for this)
2. investigate the effect of increasing the temperature for incubation with PDT (use the highly exposed blood sample for this).
3. increase the size of the blood sample.
4. decrease the amount of toluene used for reconstitution of the concentrated sample.
5. instead of reconstitution of the concentrates extracts, try the SPME method with elevated desorbing temperatures.

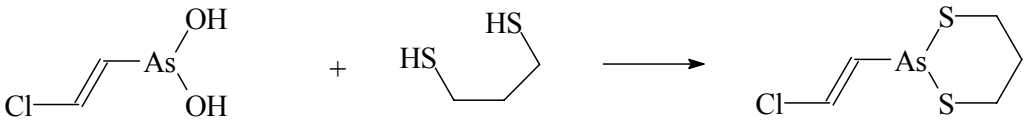


Figure 15. Extraction/derivatization of CVAA with 1,3-propanedithiol.

IV.6 Development of the nonapeptide assay for diagnosis of exposure to cholinesterase inhibitors

Background

The biochemical target for nerve agents is the enzyme acetylcholinesterase (AChE). Nerve agents inhibit this enzyme by phosphylation of a serine hydroxyl group within the active site. A similar reaction will occur with the related enzyme human butyrylcholinesterase (HuBuChE). AChE and HuBuChE, which have half-lives of 5-16 days, provide excellent biomarkers. HuBuChE is usually preferred to AChE because it is much more abundant in blood plasma than AChE in erythrocytes and more easy to isolate.

A rather straightforward method for diagnosis of exposure to nerve agents comprises the displacement of the bound organophosphorus moiety by incubation with a large excess of fluoride ion, under formation of an alkyl methylphosphonyl fluoridate (e.g. Degenhardt *et al*., 2004). However, this method is not suitable for soman and for poorly reactivatable compounds.

A more versatile, but also more laborious method of detection involves isolation of HuBuChE from plasma using affinity SPE, digestion with pepsin, and LC-tandem MS detection of a phosphylated nonapeptide (Fidder *et al*., 2002). This method surpasses the limitations of the fluoride reactivation assay.

Improvement of nonapeptide assay

A number of experiments have been performed in order to study the recovery of HuBuChE during procainamide affinity extraction. Human plasma (1 mL) was loaded on a cartridge that

was filled with 2 mL procainamide gel. After equilibration for 30 min, the gel was washed with 5 or 10 ml 20 mM phosphate buffer and 5 or 10 ml 150 mM NaCl in phosphate buffer. Finally, HuBuChE was eluted with 7 ml 600mM NaCl in phosphate buffer. The HuBuChE activity in each fraction was measured with the Ellman assay. The HuBuChE activity in the plasma sample was also measured using the Ellman assay and the amount of HuBuChE (activity x volume) was set at 100%.

Table 1. Recovery of HuBuChE isolation from human plasma using procainamide affinity extraction (n=2)

Fraction	Volume (mL)	Recovery	Volume (mL)	Recovery
1	5	0 %	10	0 %
2	10	7.8 % ± 0.6	5	1.8 % ± 1.3
3	7	76 % ± 4.2	7	81.0 % ± 4.2
4	5	3 % (n=1)	5	1.4 % (n=1)
Total		87 % ± 4.8		84.2 % ±5.5

Fraction 1: 20 mM phosphate buffer
Fraction 2: 20 mM phosphate buffer + 150 mM NaCl
Fraction 3: 20 mM phosphate buffer + 600 mM NaCl
Fraction 4: 20 mM phosphate buffer + 600 mM NaCl

The data in Table 1 show that the recovery of HuBuChE in fraction 3 ranges between 76-81%. The amount of HuBuChE in fraction 2 is significantly higher when the amount of 150 mM NaCl wash solution was doubled. The higher elution strength of this buffer will probably ensure a better sample cleanup but the recovery of HuBuChE will be lower as well.

Table 2. Recovery of HuBuChE isolation from human plasma using procainamide affinity extraction (n=4)

# ml gel	1	2
Fraction		
1	0.6 % ± 0.3	0.8 % ± 0.2
2	6.3 % ± 1.8	3.0 % ± 0.5
3	68.5 % ± 3.5	83.2 % ± 3.5
Total	75.4% ± 5.6	87 % ± 4.2

Fraction 1: 5 mL, 20 mM phosphate buffer
Fraction 2: 5 mL, 20 mM phosphate buffer + 150 mM NaCl
Fraction 3: 7 mL, 20 mM phosphate buffer + 600 mM NaCl

Table 2 shows the effect of the amount of procainamide gel in the cartridge. The recovery of HuBuChE in fraction 3 is higher using the 2-mL procainamide gel cartridge than using the 1-mL gel filled cartridge. Table 2 shows also that the amount of HuBuChE in fraction 2 is higher in the cartridge that was filled with 1 mL procainamide gel, which means that 1 ml of procainamide gel is the minimum amount to ensure sufficient retention of HuBuChE.

It is remarkable that the total recovery of all fractions is lower than 90%. It must be emphasized that the recovered HuBuChE was present in a high salt buffer. The high salt concentration might interfere with the reaction of the Ellman assay. Experiments revealed that the measured HuBuChE activity was 10% lower when HuBuChE was dissolved in high salt

buffer, i.e. 600 mM NaCl. The recoveries of HuBuChE in fraction 3 shown in the tables are therefore underestimated.

All experiments were performed using the same batch of procainamide gel. It is advised to perform these recovery studies every three months and also once a fresh prepared gel is going to be used, because various or aged batches may show different retention characteristics.
Depending on the characteristics of the gel it might be necessary to adjust the volume and ionic strength of the elution buffer. It appeared that the wash solution (fraction 2) sometimes needed a concentration of 350 mM NaCl to ensure sufficient sample clean-up and the elution buffer required a concentration of 1000 mM NaCl to elute BuChE from the procainamid gel.

Although the recovery of HuBuChE is rather optimized, the other purpose of this sample preparation step, i.e., sample cleanup, remains underexposed. The extent of sample cleanup can be evaluated by the study of the LC-MS/MS chromatograms, in which the signal to noise ratio needs to be optimized and the chromatograms must be true negative for non-exposed HuBuChE. It is advised to maximize the extent of sample cleanup and to accept that it will be at the cost of recovery. A lower recovery implies a loss of sensitivity in that sense that lower levels of inhibition cannot be detected. However, the lowest concentration of detection can easily be improved by preconcentration of the sample, by evaporation of the solvent, prior to analysis with LC-MS. A higher recovery of BuChE at the cost of lower sample clean up will be disadvantageous reflected by dirtier chromatograms and eventually lower sensitivity caused matrix effects during the LC-MS analysis.

It was investigated whether the extent of sample cleanup could be further improved by additional sample preparation steps such as ethanol precipitation. Results showed that an ethanol precipitation step did not have a significant effect on the analytical results.

Figure 16 shows the LC-MS/MS ion chromatogram of a pepsin digest of HuBuChE that was isolated from 100% sarin-inhibited human plasma, following the fast method of HuBuChE isolation, using only 2 ml of procainamide gel. Figure 15 shows the corresponding MS/MS spectrum. It is estimated that approximately 10% inhibition can still be detected according to this methodology, depending on the structure of the inhibitor.

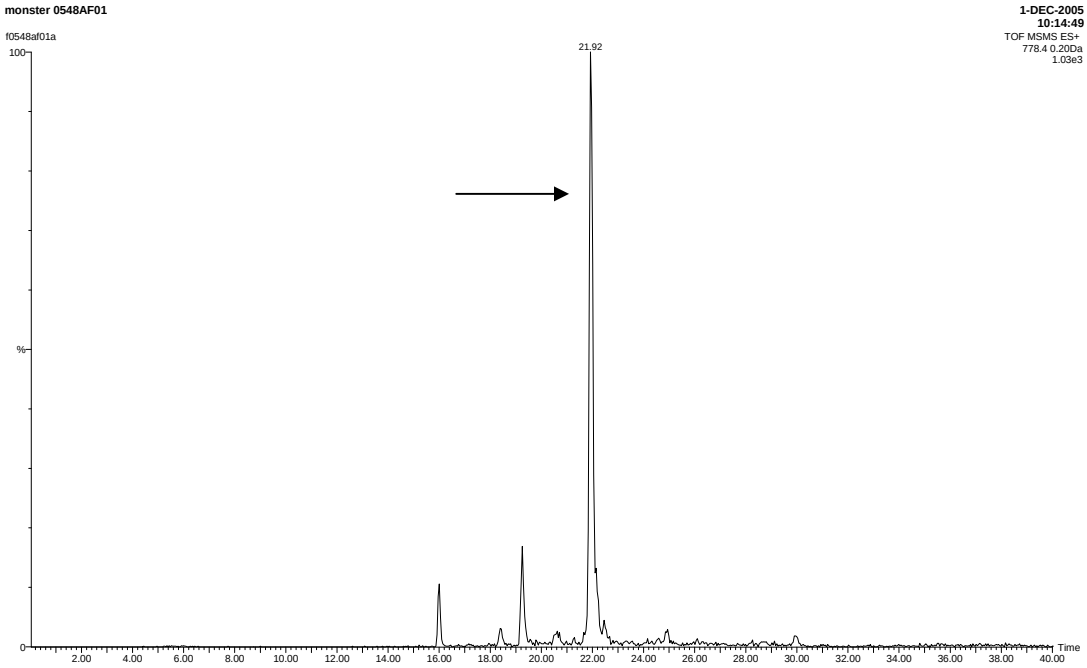


Figure 16. Ion chromatogram of pepsin digest of 100% sarin-inhibited HuBuChE isolated from human plasma. MS/MS conditions: *m/z* 916 → 778.4. The arrow indicates the peak of the phosphylated nonapeptide.

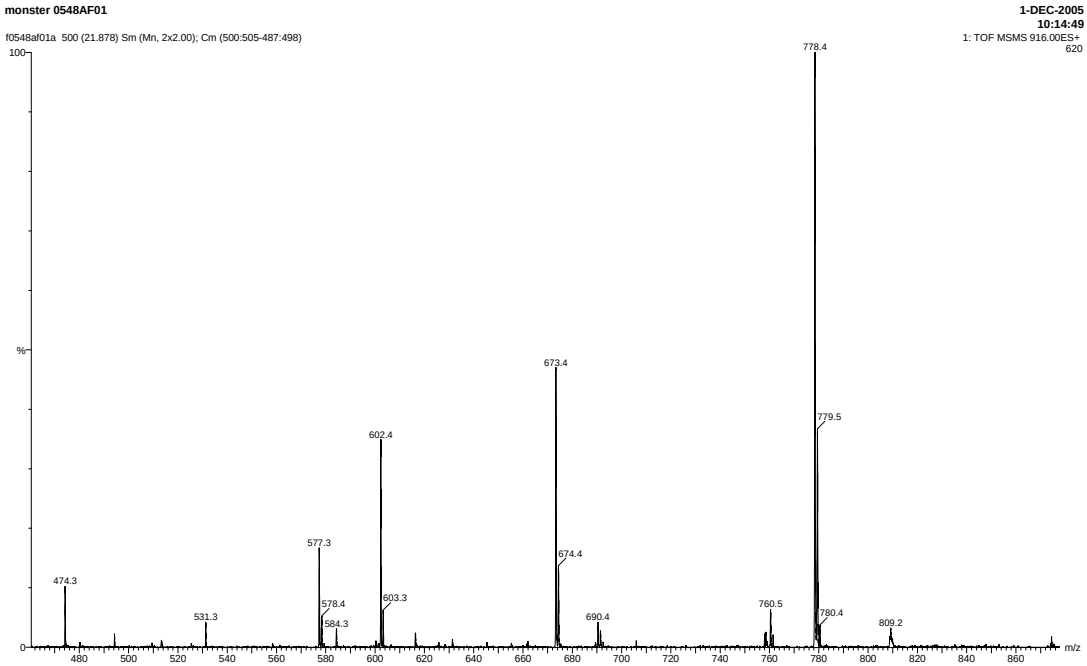


Figure 17. Product ion spectrum of molecular ion MH^+ (*m/z* 916) of nonapeptide FGES*AGAAS derived after pepsin digestion of sarin inhibited HuBuChE isolated from human plasma.

Use of internal standard

For internal standard purposes, it was proposed to use d_7-sarin, i.e. d_7-isopropyl methylphosphonofluoridate. The advantage of using sarin is that it does not age very rapidly after inhibition of HuBuChE. Also, the mass difference is large enough to circumvent cross-over with non-labelled sarin. We prefer to use plasma fully inhibited with d_7-sarin as the actual internal standard to be added to the "unknown" sample; a similar strategy was followed in the albumin tripeptide assay for diagnosis of exposure to sulfur mustard. In Figures 18 - 20 the use of d_7-sarin inhibited plasma is exemplified.

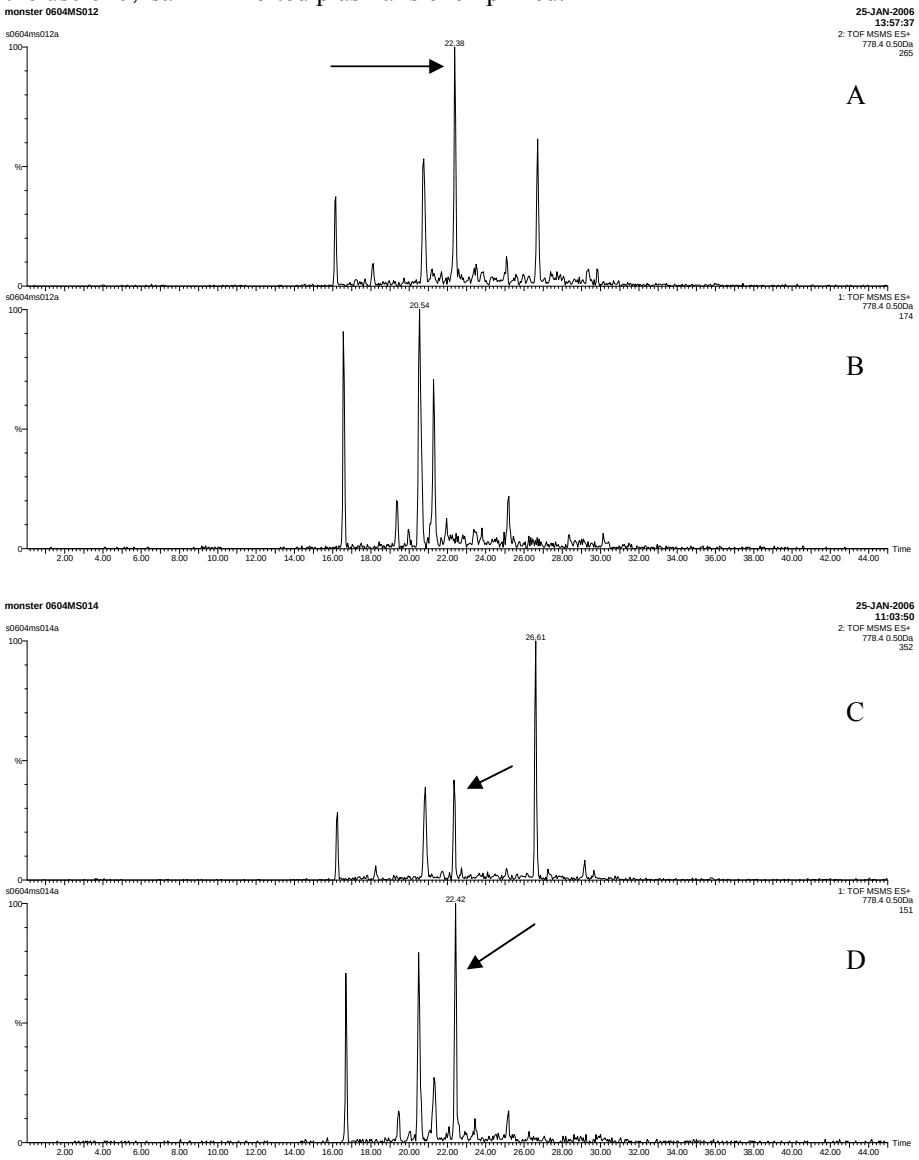


Figure 18. LC tandem MS analysis of FGES(IMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and sarin-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 916.4 (MH^+); d_0-sarin (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 916.4 (MH^+); d_0-sarin (exposed)

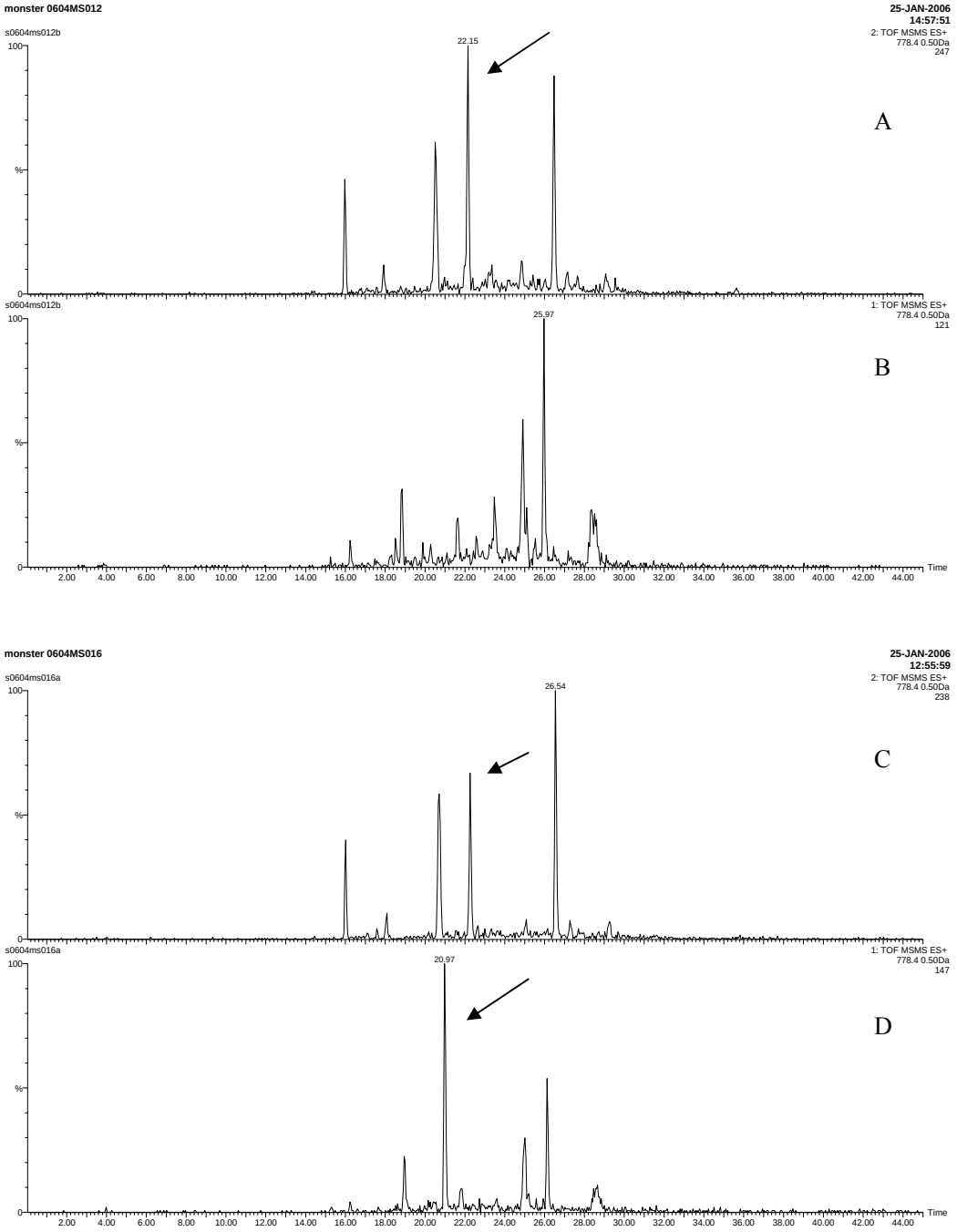


Figure 19. LC tandem MS analysis of FGES(EMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and VX-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 902.4 (MH^+); VX (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 902.4 (MH^+); VX (exposed)

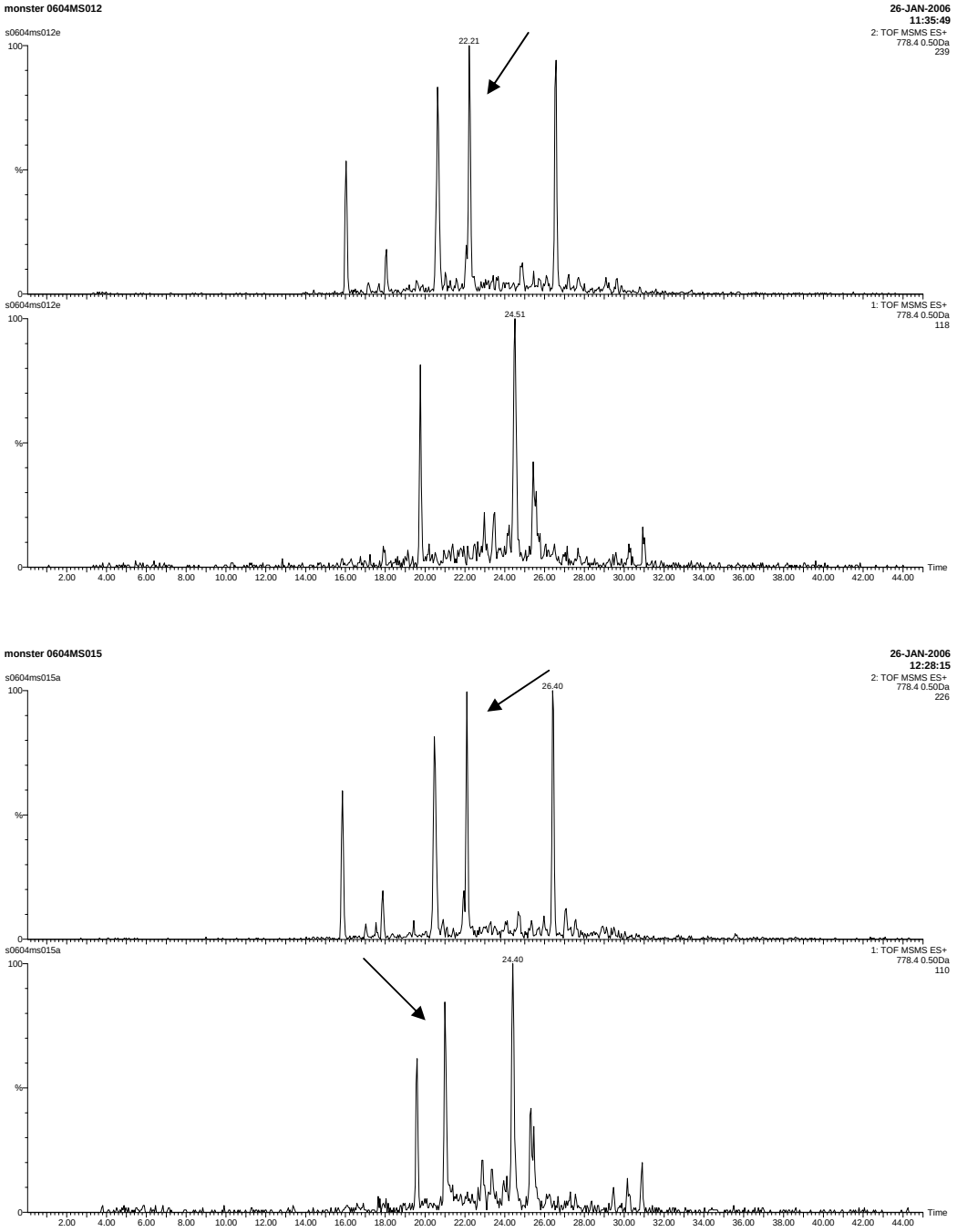


Figure 20. LC tandem MS analysis of FGES(MPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and soman-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 874.4 (MH^+); soman (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 874.4 (MH^+); soman (exposed)

Currently, multiple reaction monitoring (MRM) experiments of FGESAGAAS peptides are performed on a triple-quad MS instrument (TSQ Quantum Ultra, ThermoFinnigan). A triple quad instrument is better equipped to perform these analyses which is reflected in a better signal to noise ratio. Figure 21 shows the analysis of the FGES(EMPA)AGAAS that was derived after pepsin digestion of BuChE isolated from plasma that was exposed to VX. The same sample was analyzed on the QTOF instrument, shown in Figure 19. It is clear that a better signal to noise ratio is obtained using the triple quad instrument. .

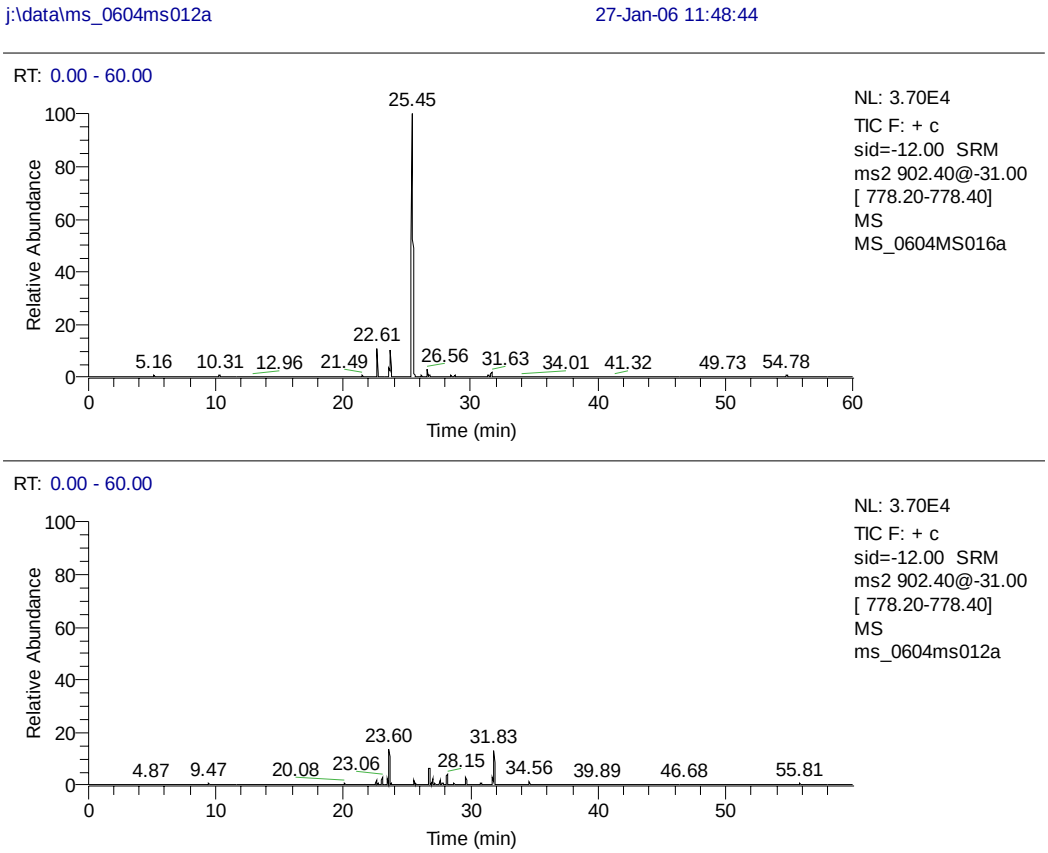


Figure 21. LC tandem MS analysis of FGES(EMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; VX-exposed sample (upper trace) and blank sample (lower trace). Multiple reaction monitoring of *m/z* 902.4 (MH^+) → 778.4

IV.7 Synthesis of reference peptides for nonapeptide assay

The synthesis of four different nonapeptide reference peptides has been performed, following a synthetic route as described in Fidder *et al*. (2002). The peptides will serve as reference compounds for analysis of pepsin digests that are derived from HuBuChE that had been exposed to sarin, soman, VX or tabun. Basically, the synthetic route we followed is outlined in Figure 22. Four different phosphylating reagents A-D were prepared (see Figure 23) and used for reaction with immobilized FGES*AGAAS, with S* unprotected to allow modification, i.e., reaction with the phosphylating agent. After oxidation of the phosphite function, followed by cleavage from the resin and deprotection (Figure 24), the peptides were purified to homogeneity by means of reverse phase HPLC. According to this synthesis methodology, milligram amounts of the required nonapeptides could be obtained, which gave

satisfactory mass spectrometric data. The peptides could be used for infusion experiments in order to optimize settings of the MS.

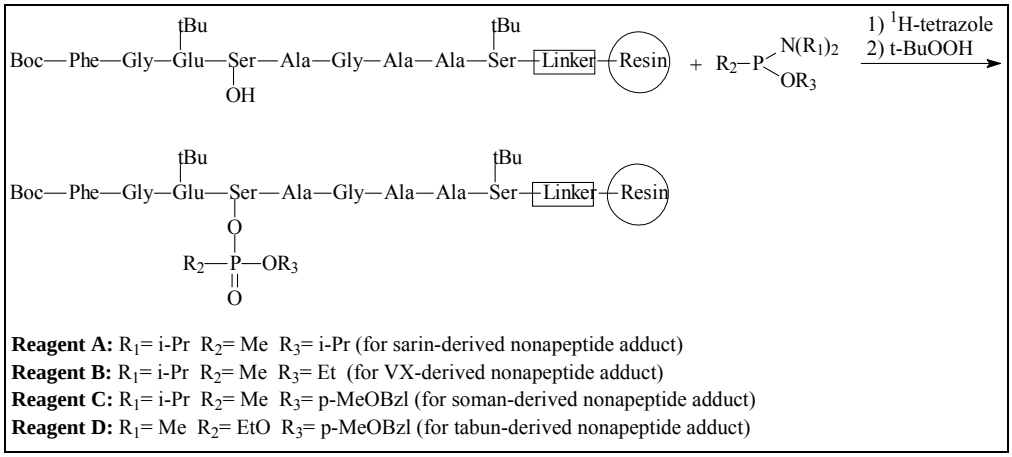


Figure 22. Solid phase synthesis of phosphylated nonapeptide reference compounds

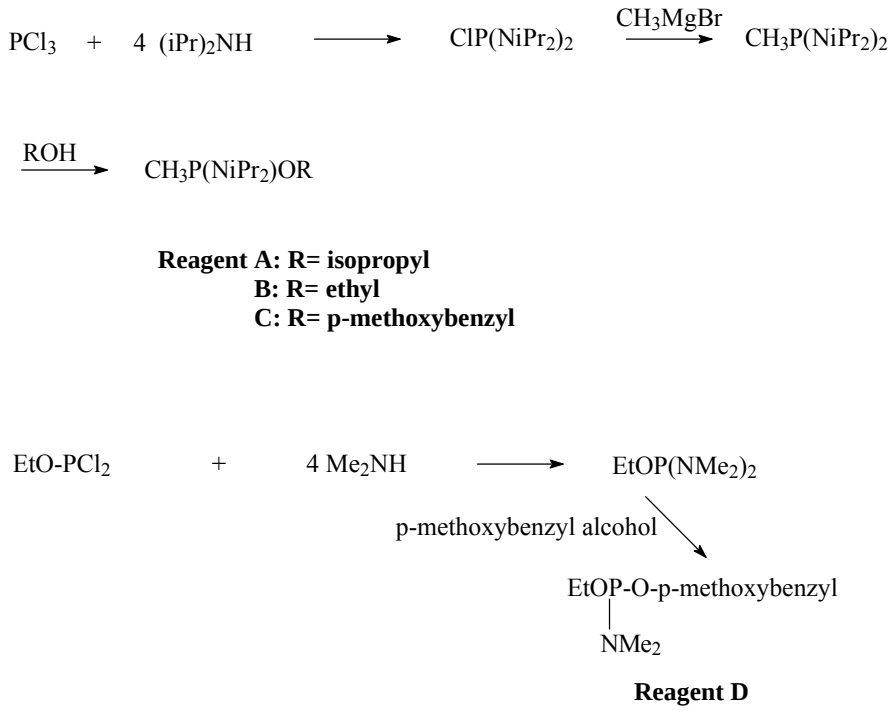


Figure 23. Synthesis of phosphylating reagents required for on resin phosphylation of HuBuChE nonapeptide derivatives.

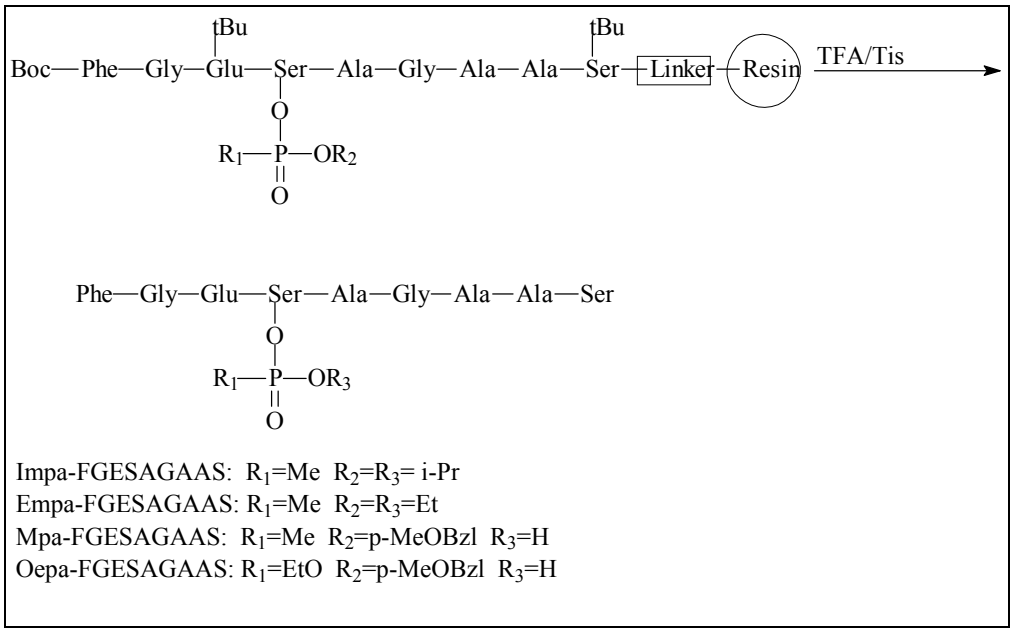


Figure 24. Cleavage from resin and concomitant deprotection of phosphylated nonapeptides

Various MS spectra of the four reference peptides are given in Figures A3.1-A3.4 of Appendix 3.

IV.8 Development of an on-line digestion method for HuBuChE adducts

In addition to the abovementioned off-line method for detection of adducts to butyrylcholinesterase, we have explored the development of an on-line digestion – LC tandem MS method. The off-line method is time-consuming due to the long manual sample handling steps. On-line methods can be automated and are useful for a rapid screening of large numbers of samples. We here describe the first steps towards such a method.
The total set-up for the on-line digestion of HuBuChE (see Figure 25) consists of a gradient pump, an auto sampler, a six-port switching valve, an immobilized pepsin cartridge and an LC column, coupled to an ion trap mass spectrometer.

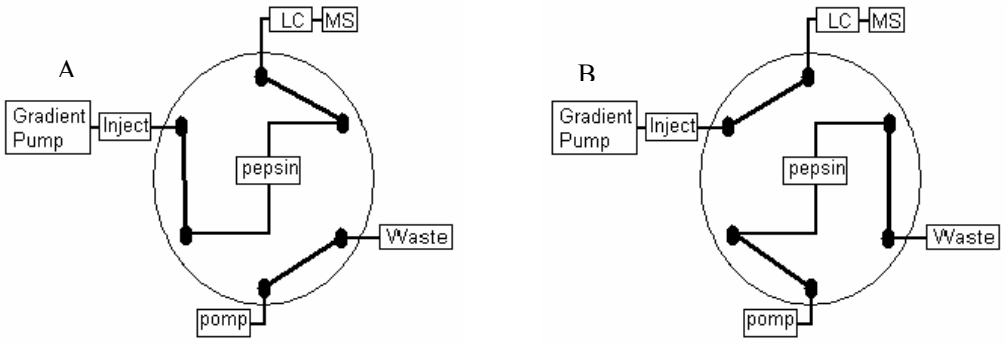


Figure 25. Schematic set-up of the automated analysis system; The set-up consists of a switching valve, containing a gradient pump, an autosampler (inject), a pepsin cartridge, a second LC pump and an LC-column which is directly coupled to the MS. Figure 25A shows the valve during the injection of the sample, digestion and trapping on the LC column. Figure 25B shows the valve after switching for gradient elution of the peptides from the LC column.

For the determination of the applicability and timing of the on-line digestion set-up a synthetic peptide, FGES*AGAAS, with S* is O-(dimethylphospho-serine) was used, which is relatively inert to pepsin digestion. The testing included the optimization of the switching time and of the LC gradient. Comparison between injections of the marker peptide on the LC-MS alone or on the total configuration, including pepsin column, showed no decrease of peak area in the MS analysis. In Figure 26 three chromatograms are shown. The top chromatogram shows full scan range (*m/z* 350-1200) MS. The middle chromatogram shows the same injection as A, with mass 904.27 (MH^+; this ion proved to be more intense than MH_2^{2+}**)** of FGES*AGAAS. Chromatogram C shows an LC-MS/MS run on mass 904.27 (different run). These chromatograms show that the system is capable of detecting the marker peptide, and therefore the switching times of the set-up are suited for the detection of the model peptide.

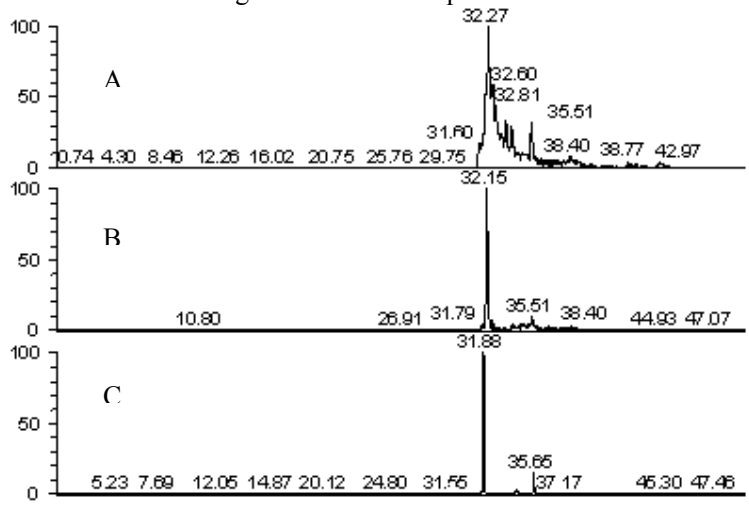


Figure 26. Analysis of synthetic FGES*AGAAS, with S* dimethylphosphoserine, on the on-line digestion LC-MS system. A) Total ion chromatogram (TIC) obtained from injection of the synthetic peptide, B) Extracted chromatogram of *m/z* 904.27, from the same run, C) MS/MS trace of the marker (*m/z* 904.27), obtained from a second run.

In Figure 27 the corresponding mass spectra are shown.

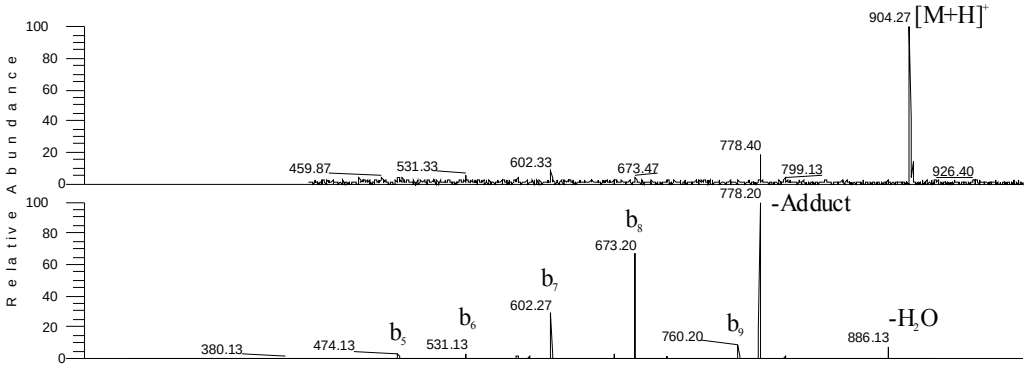


Figure 27. Mass spectrometric analysis of FGES*AGAAS, with S* dimethylphosphoserine. Upper trace: MS spectrum. Lower trace: MS/MS spectrum.

Next, purified native HuBuChE was injected (3.8 nM; 2 µl) to explore the on-line pepsin digestion. Indeed, the nonapeptide FGESAGAAS could be analyzed in the on-line digest (see Figure 28); the MS/MS spectra obtained showed the expected b_5 to b_9-ions of the marker peptide. The digestion time (the time the injection sample plug was on the pepsin cartridge) was optimised; digestion for 20 minutes gave optimal results; no difference could be observed with a digestion time of 30 or 40 minutes.

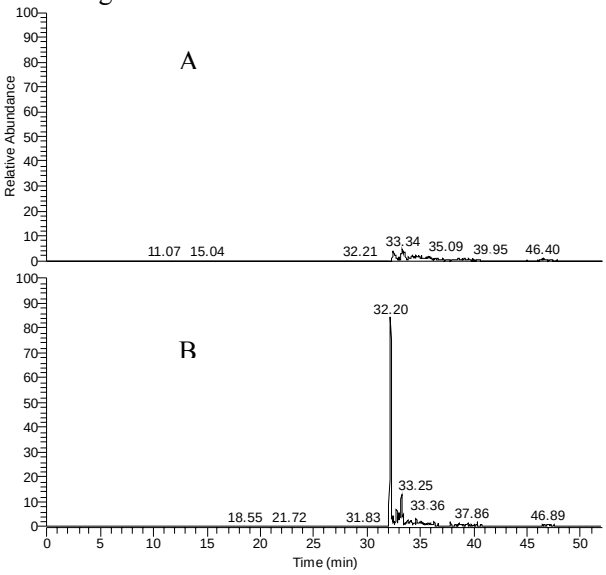


Figure 28. LC tandem MS analysis of an on-line digest of native HuBuChE. Upper trace: solvent injection. Lower trace: native HuBuChE. The MS/MS was set for the marker peptide FGESAGAAS (without adduct) *m/z* 796.3. The chromatograms are shown at normalized relative abundance.

Further proof of the applicability of the on-line set-up was obtained by injection of HuBuChE that had been exposed to sarin. The expected nonapeptide adduct could indeed be detected, giving identical mass spectrometric data (see Figures 29 and 30).

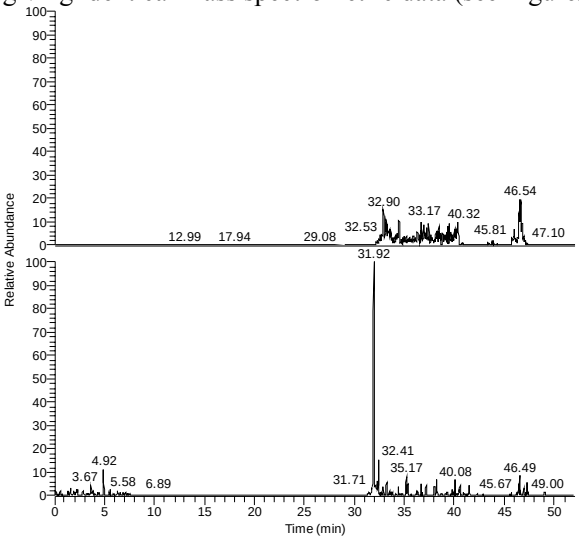


Figure 29. LC tandem MS analysis of an on-line pepsin digest of HuBuChE that had been exposed to sarin. Upper trace: native HuBuChE. Lower trace: sarin-exposed HuBuChE. The MS was tuned for FGESAGAAS, with S* the O-isopropylmethylphosponyl moiety, with *m/z* 916.2. The chromatograms are shown at normalized (to highest peak) relative abundance.

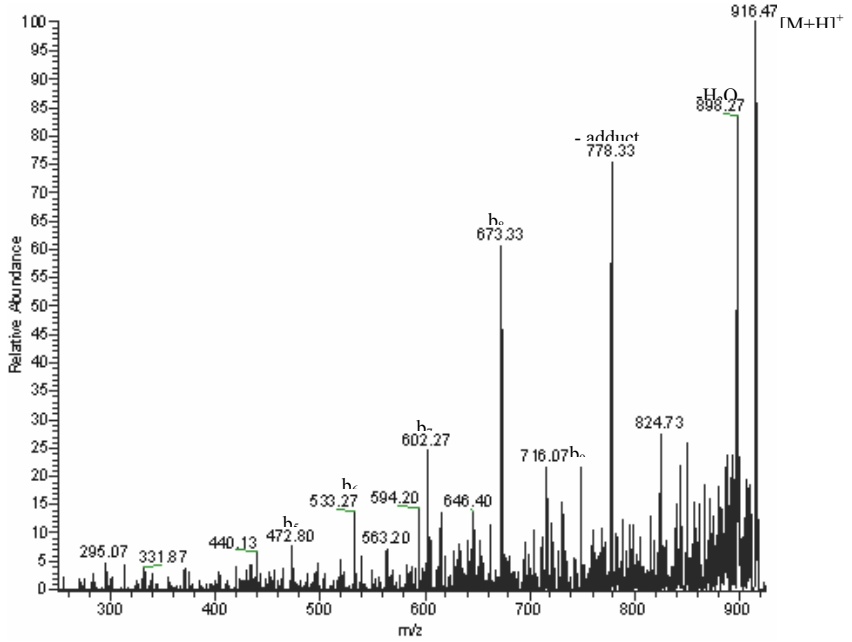


Figure 30. MS/MS spectrum of nonapeptide FGES*AGAAS, with S* = O-isopropylmethylphosphonylserine, after on-line pepsin digestion of sarin exposed HuBuChE.

At yet, we have not been able to detect the nonapeptide adduct in a plasma sample that had been exposed to sarin.
Next, further assessment of the analytical parameters of the system was performed. The limits of detection (LOD; with regard to amount of HuBuChE injected) were determined by serial (n=6) injections (2 μL) of different concentrations of intact native HuBuChE. This resulted in a LOD of 10.6 fmol (0.85 ng) protein with S/N ratio of 3.
The repeatability of the marker in LC-MS mode is 0.245% relative standard deviation (RSD) in retention time and 10.26 % RSD in peak area. This relatively high relative standard deviation is probably due to the fact that digestion rate in on-line digestion differs from injection to injection.

IV.9 Development of a generic assay for OP biomonitoring

Background
In recent years (Noort *et al*, 2002b) we have developed a number of assays in order to detect exposure to nerve agents. The fluoride reactivation method is based on the principle that upon incubation of phosphylated binding sites (for example HuBuChE in plasma) with a large excess of fluoride ions, the phosphyl moiety is quantitatively converted into the corresponding phosphono-or phosphorofluoridate (Degenhardt *et al*, 2004). The latter can be isolated by solid phase extraction on a C18 cartridge and quantitated by GC/NPD or GC/MS. The other method is based on mass spectrometric determination of specific peptide adducts that result after pepsin digestion of adducted HuBuChE (Fidder *et al*, 2002). This method surpasses the limitations of the fluoride-reactivation method since it can deal with HuBuChE inhibited with organophosphates that cannot be reactivated or that rapidly age (e.g. soman).

One of the problems with both assays for assessment of organophosphate exposure is that one has to know in advance for which type of nerve agent to screen for during mass spectrometric analysis. It is possible to perform parent ion scan, but this will be at the cost of sensitivity, which means that lower degrees of inhibition cannot be detected anymore. Therefore a generic mass spectrometry-based method for detection of phosphylated HuBuChE is highly needed. In this respect, our attention was raised by a general method which is used for the detection of phosphorylation sites in the field of proteomics (e.g., *Oda et al*, 2001). According to this method, proteins are treated with mild base in order to eliminate the phosphate function, resulting in the formation of a dehydroalanine residue in the protein. Subsequently, the thus formed dehydroalanine residue is subjected to a Michael addition with a simple or functionalized thiol or amine. We here report that this methodology (see also Noort *et al*., 2006), albeit in a slightly modified form, can be applied to nerve agent-inhibited HuBuChE and that the result is irrespective of the agent used (see Figure 31).

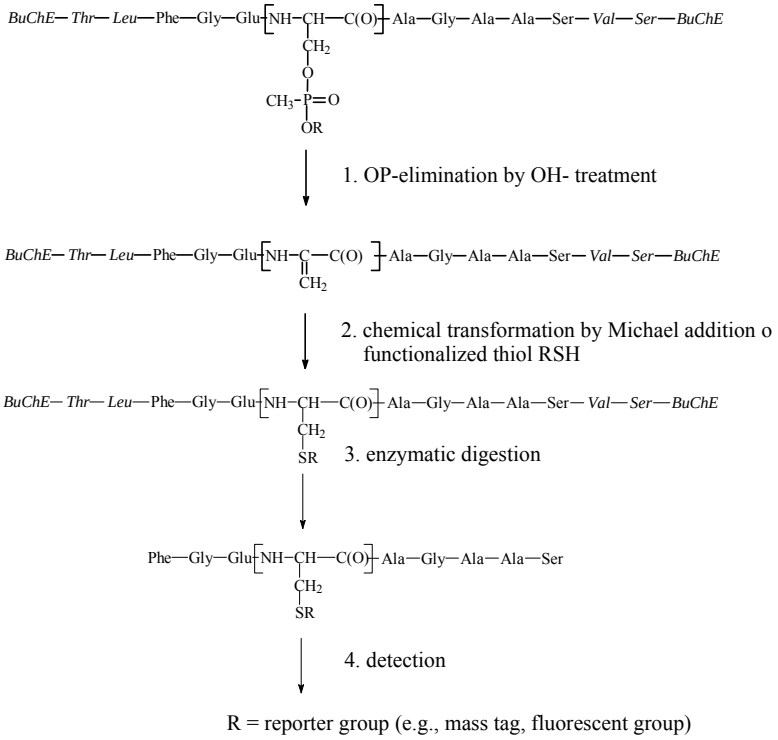


Figure 31. Initial strategy towards generic assay for diagnosis of exposure to OP compounds.

Synthetic FGE(p)SAGAAS was used to discover the most optimal conditions for introduction of a nucleophilic group like a thiol or amine. We first used ethanethiol as nucleophile since it has been used in the field of proteomics. It appeared that performing the reaction in $Ba(OH)_2$ (100 mM) and a nucleophile (50 mM) for 1 h at 37 ºC was most suitable. Longer reaction times would result in hydrolysis of non-phosphorylated serine residues to dehydroalanine, resulting in false positives.

Next, purified HuBuChE was used in order to detect the possibility of a generic assay. Three different nerve agents (VX, sarin, soman) were used to inhibit a portion of purified HuBuChE. The inhibited enzymes were subjected to alkaline hydrolysis like described before, followed by Michael addition of ethanethiol, resulting in all cases in the same S-ethyl FGECAGAAS (see Figures 32 and 33).

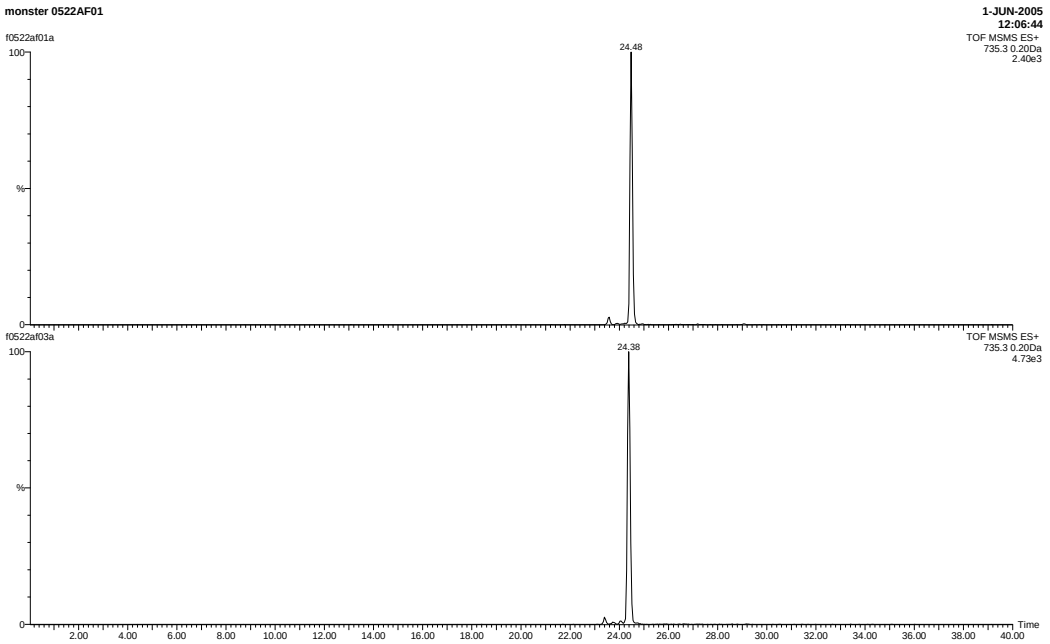


Figure 32. Ion chromatogram of *m/z* 735.3 of FGE(S-ethyl)CAGAAS obtained after processing pepsin digest of purified HuBuChE with $Ba(OH)_2$ and EtSH. Upper panel: sample inhibited with VX. Lower panel: sample inhibited with soman. In the first MS, the ion with *m/z* 840.3 (MH^+) had been selected.

The drawback of using ethanethiol, unfortunately, was that the derived S-ethyl nonapeptide lies in a rather "crowded" region of the chromatogram. Furthermore, the degree of fragmentation of the particular S-ethyl nonapeptide is very high (see Figure 33).

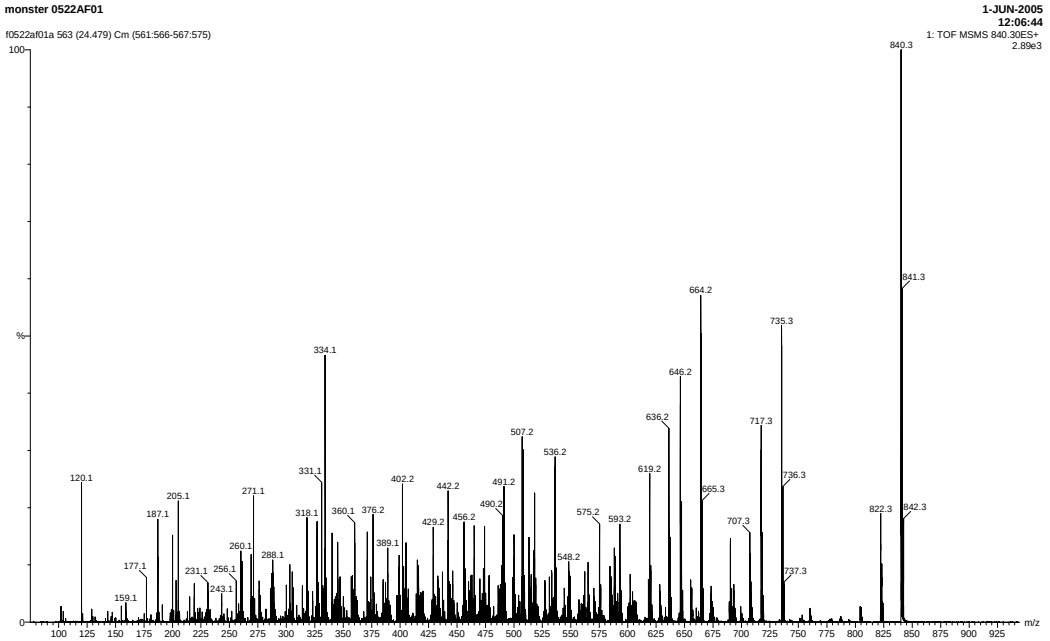


Figure 33. Product ion spectrum of molecular ion MH+ (*m/z* 840.3) of FGE(S-ethyl)CAGAAS obtained after processing pepsin digest of purified HuBuChE with $Ba(OH)_2$ and EtSH.

Again, synthetic FGE(p)SAGAAS was used to evaluate the use of nucleophiles other than ethanethiol. See Figure 34 for a list of nucleophiles that were used. The cysteamine derivatives were prepared in our laboratory. It appeared that the nonapeptide that results by using 2(3-aminopropylamino)ethanol (H) (see Figure 35 for chemical structure of adduct) has both favorable chromatographic properties and mass spectrometric properties; a nice MH_2^{2+} fragment could be observed (see Figures 36).

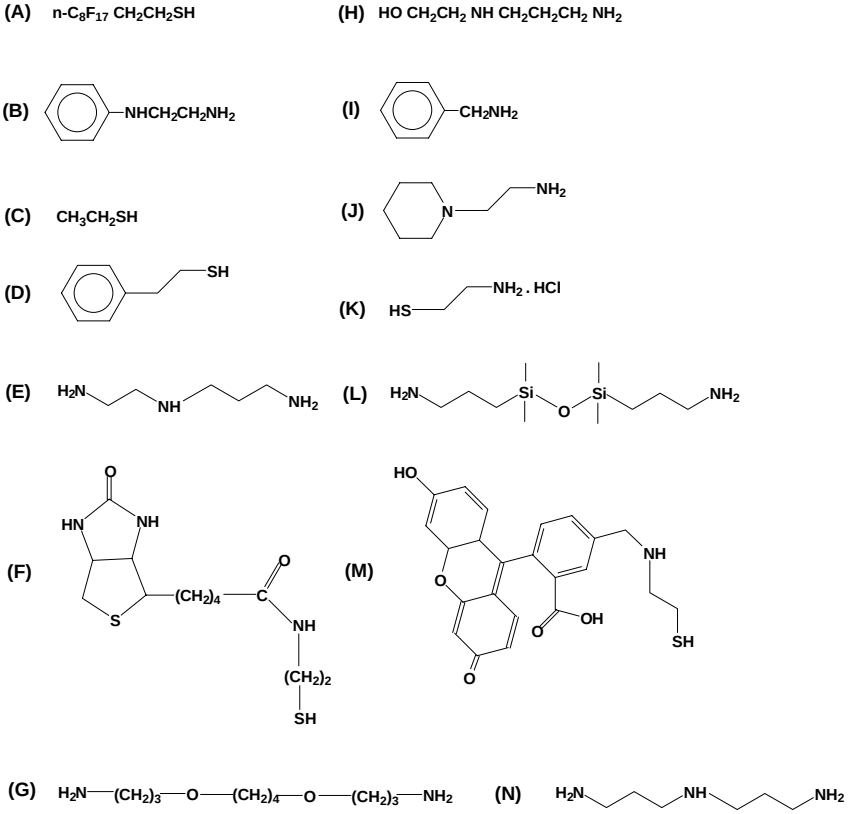


Figure 34. Structures of nucleophiles used for modification of FGE(p)SAGAAS residues after alkaline hydrolysis.

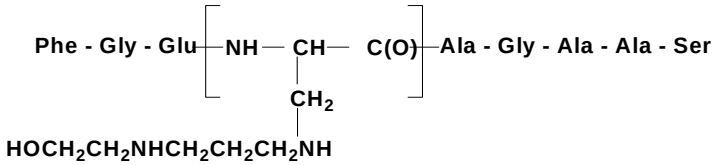


Figure 35. Structure of the modified nonapeptide FGEXAGAAS, with X the converted phosphoserine residue, as analyzed after conversion of the phosphylated serine residue with 2(3-aminopropylamino)ethanol by means of alkaline hydrolysis/Michael addition.

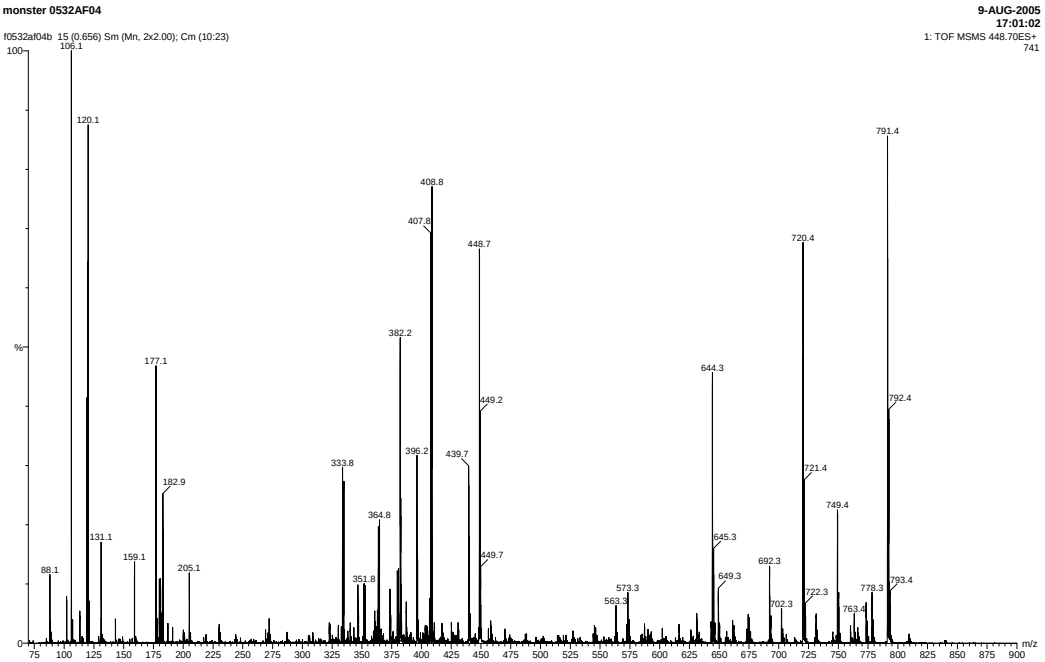


Figure 36. Product ion spectrum of *m/z* 448.7 (MH_2^{2+}) resulting from conversion of synthetic FGE(p)SAGAAS by alkaline hydrolysis and concomitant reaction with $HOCH_2CH_2NHCH_2CH_2CH_2NH_2$

It appeared that the direct alkaline hydrolysis and concomitant introduction of the selected nucleophile of HuBuChE was troublesome, probably due to precipitation of various proteins in the alkaline matrix. We decided to perform the pepsin digestion of isolated HuBuChE prior to the combined alkaline treatment/Michael addition.

This approach proved to be viable with plasma samples that had been exposed to various OP's (see Figures 37 and 38). Two peaks were observed, which can be explained by the fact that due to the formation of a dehydroalanine residue and subsequent attack of the nucleophile on the double bond, the resulting amino acid loses its chiral integrity. Since the chirality of the other amino acids in the peptide does not change, a diastereomeric mixture of two peptides results. The eventual method is depicted in Figure 39. Performing the modification reaction on the peptide level has the additional advantage that a two-step approach can be followed. First, the generic method can be used for an initial screening of samples and after finding a positive sample, the original pepsin digest can be analyzed in a more specific way as described by Fidder *et al* (2002), in order to unravel the identity of the OP inhibitor.

It can be envisaged that the present method can be used as a rough screening method in case of large numbers of samples, as is to be expected after a terrorist incident. Subsequently, after finding a positive sample, the digest can be analyzed in a more specific way, by reanalyzing the original digest for phosphylated nonapeptides in a more thorough and laborious way. Furthermore, the method might be valuable for epidemiological studies for determination of total OP pesticide exposure. In conclusion, with the presented method HuBuChE inhibition can now accurately be monitored by mass spectrometry, without advance knowledge of the structure of the inhibitor.

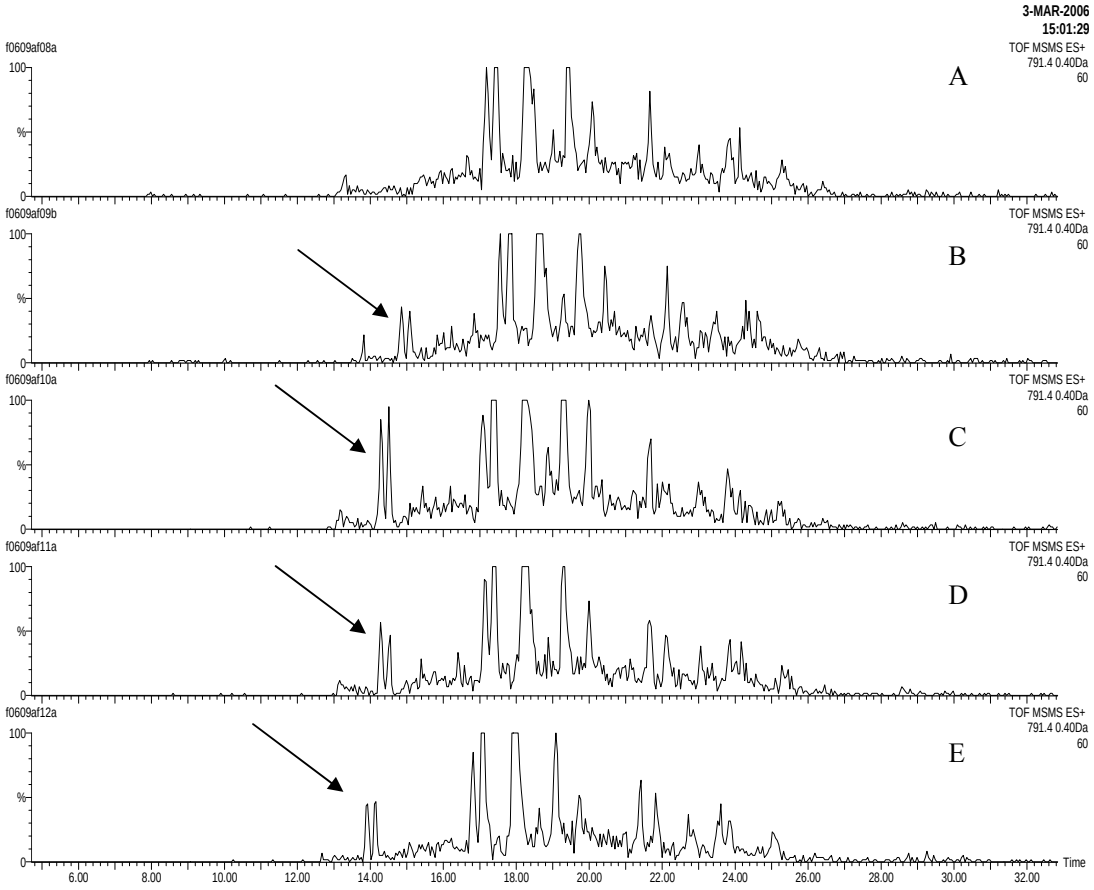


Figure 37. Ion chromatograms of *m/z* 791.4 in processed pepsin digests of isolated HuBuChE from plasma samples. Panel A represents the ion chromatogram of a processed digest from blank plasma. Panels B-E represent the ion chromatograms of digests from plasma, that had been exposed to sarin, soman, VX and dichlorvos, respectively. The digests were subjected to modification with 2(3-aminopropylamino)ethanol, under the agency of $Ba(OH)_2$. The arrows indicate the peak of the modified FGEXAGAAS. In the first MS, $[M+2H]^{2+}$, *m/z* 448.7 was selected.

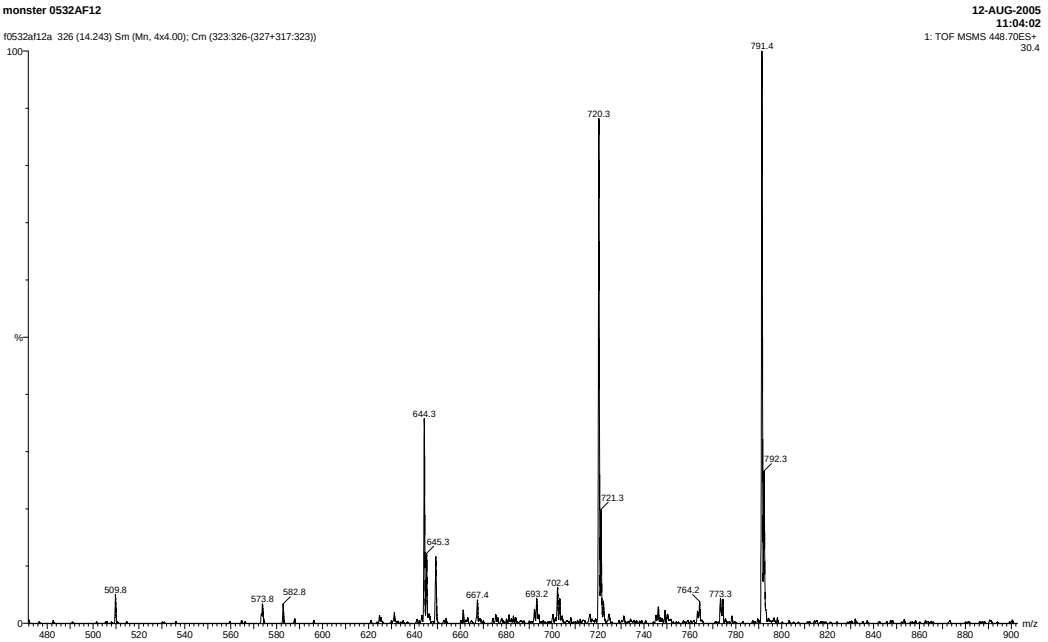


Figure 38. Part of the product ion spectrum of molecular ion MH_2^{2+} (*m/z* 448.7) resulting from conversion of FGE(p)SAGAAS by alkaline hydrolysis and concomitant reaction with $HOCH_2CH_2NHCH_2CH_2CH_2NH_2$, after processing of a pepsin digest of HuBuChE isolated from a plasma sample that had been exposed to sarin.

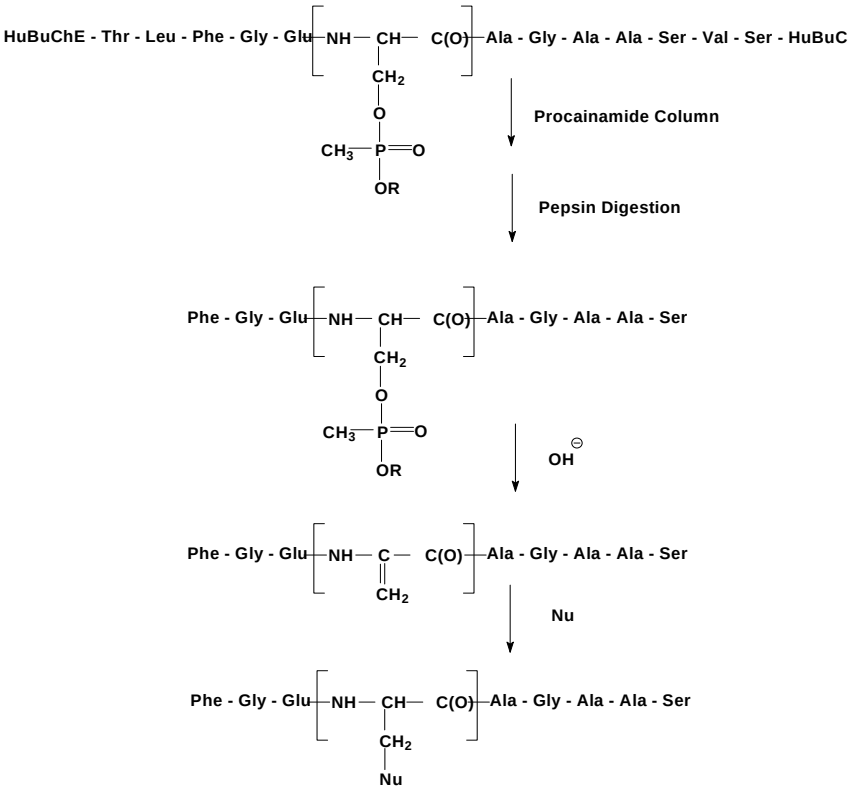


Figure 39. Reaction sequence to convert phosphylated HuBuChE into a modified nonapeptide

IV.10 Exploratory work on protein adduct formation with cyanide

Since we had a clue that cyanide can interact with disulfide bridges in proteins, we decided to first study the interactions of cyanide with a model peptide containing a disulfide bridge. For this purpose, we selected the commercially available oxytocine (CYIQNCPLG), which contains a disulfide bridge between the two cysteine residues. Upon incubation with NaCN at 37 ˚C, several products resulted. After 2 h, a peak appeared in the HPLC chromatogram; according to MS analysis ist identity was (NCS)-CYIQNCPLG. After 2 days, no oxytocin peak was visible in the HPLC chromatogram, but two other peaks appeared. MS analysis showed the presence of NCS-CPLG(NH_2), NCS-CYIQ and of NCS-CYIQN. These findings are in correspondence with the results found by Wood and Catsimpoolas (1963). Subsequently, human blood samples were exposed to ^{14}C-labelled KCN. After isolation of globin and albumin, covalently bound radioactivity to these proteins was determined (see Table 3 for results). Part of the globin samples (3 mg) and albumin solutions (containing 3 mg) originating from 1 and 10 mM exposure to ^{14}C labeled KCN were subjected to enzymatic digestion, using pronase or trypsin. The digested samples were analyzed using HPLC with UV and radiometric detection.

Table 3. Covalently bound radioactivity after incubation of human blood with ^{14}C-KCN

Exposure level	nmol CN-/mg albumin	% of total CN- bound to albumin	nmol CN-/mg globin	% of total CN- bound to globin
0	0	0	0	0
0.01	0.001	0.2	0.01	14
0.1	0.01	0.2	0.02	2.8
1	0.1	0.3	0.2	2.8
10	3	0.6	4.3	6

In case of trypsin digestion of globin (10 mM exposure), distinct radioactive peaks could be observed in the HPLC chromatogram, indicating covalently bound radioactivity (see Figure 40). The pronase digest only showed peaks at the beginning of the chromatogram, indicating the presence of smaller (more polar) amino acid and/or peptide adducts. In case of albumin, similar results were obtained.

Attempts to identify distinct adduct peptides with LC/MS were not successful, despite the information that was obtained during the preliminary experiments with model compound oxytocin.

IV.11 Exploratory work on protein adduct formation with phosphine

In case of phosphine, we followed a similar approach as for cyanide. Thus, the model peptide oxytocine was exposed to a saturated solution of phosphine in an aqueous buffer and modifications were determined by means of LC-tandem MS. No modifications could be detected, and therefore we stopped our activities (after consultation with CDC).

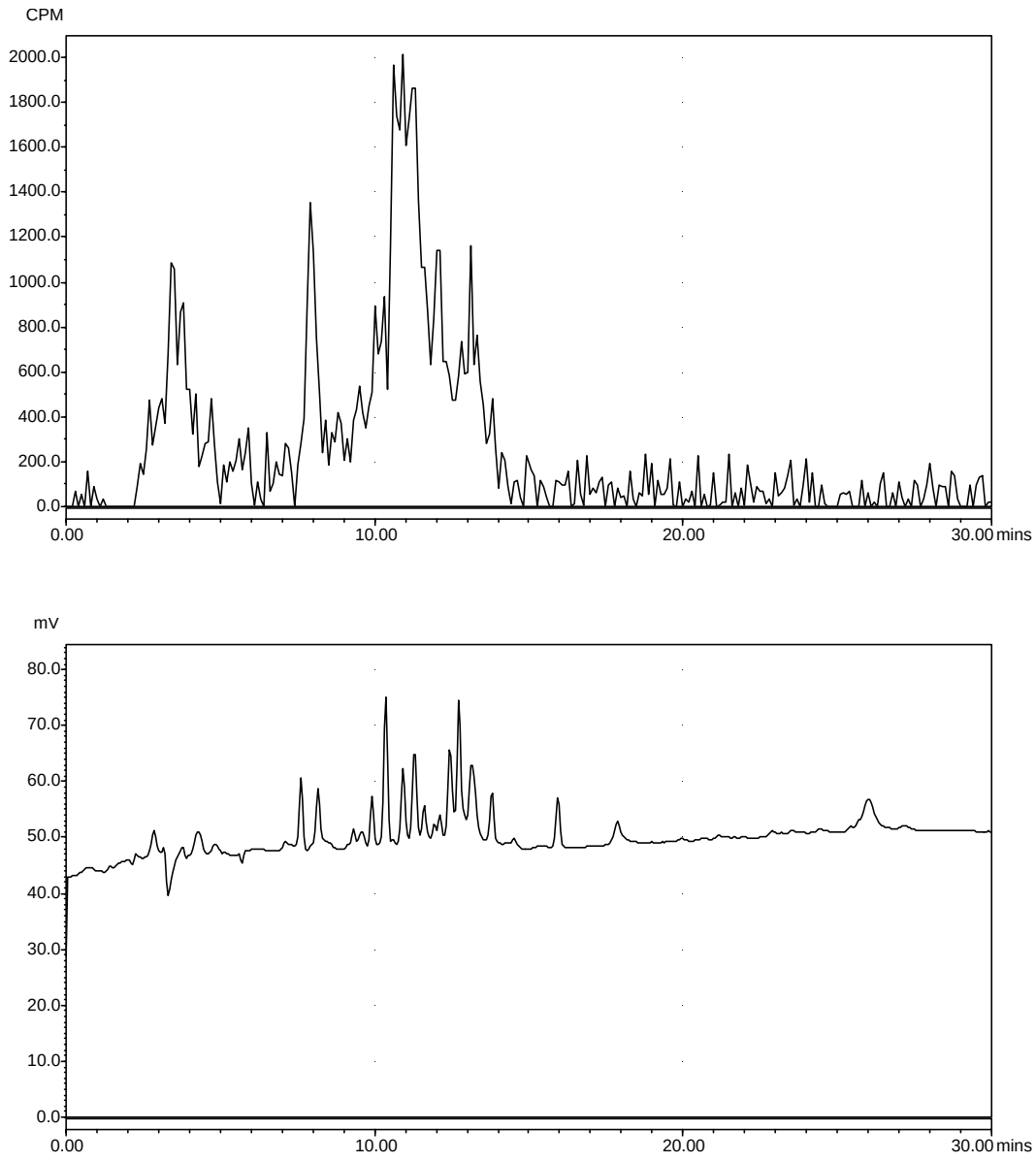


Figure 40. HPLC analysis of a trypsin digest of globin, isolated from blood exposed to 10 mM [^{14}C]KCN. Upper chromatogram: radioactivity detection. Lower chromatogram: UV detection.

Appendix 2. Detailed reports of methods transfers

1. Demonstration of albumin tripeptide assay

This assay was demonstrated simultaneously to US Army and to CDC; see Volume I of this report.

2. Modified Edman degradation

(Monday, November 8, 2004)

Standards

Reference standard 9705AF03 (pentafluorophenylthiohydantoin of valine-sulfur mustard adduct; 1.8 mg according to quantitative ^{1}H-NMR analysis), was dissolved in CH_3CN (180 µl), to give a 10 mg/ml stock solution. 3 further dilutions were prepared: 0.1 mg/ml, 1 µg/ml, 10 ng/ml (no codes were given).

The 1 µg/ml solution was used for setting-up the LC tandem MS method. Various ion combinations for MRM were explored; strongest intensity was obtained for *m/z* 429 → *m/z* 130 (identity of this fragment is still unknown).

The HR-GCMS Mat 900 was further optimized with already processed samples 0440AF01-05 for analysis under EI conditions (EI is the most sensitive mode on this instrument).

Processing of globin samples

Globin samples were weighed: see Table A2.1.

Table A2.1. Globin samples processed by modified Edman degradation

Globin sample	Description	Amount used (mg)	Internal standard	Sample code after modified Edman degradation
0442AF01	blank	24.4	0442AF07; 50 µl	0446AF01
0442AF03	10 nM	24.1	0442AF07; 50 µl	0446AF02
0442AF04	100 nM	20.0	0442AF07; 50 µl	0446AF03
0442AF05	1 µM	21.2	0442AF08; 50 µl	0446AF04
0442AF06	10 µM	22.2	0442AF08; 50 µl	0446AF05
0442AF07	d_8, 2 µM	25.1 in 1.25 ml formamide	n.a.	n.a.
0442AF08	d_8, 100 µM	28.7 in 1.43 ml formamide	n.a.	n.a.
0442AF09	d_0/d_8, 5/5 µM	23.9	n.a.	0446AF06

The weighed globin samples were dissolved in the appropriate amounts of formamide according to the work-plan. Incubation with pentafluorophenyl isothiocyanate was effected before 12 pm (2 h reaction time, during lunch).

Work-up of the modified Edman degradation reactions was performed according to the procedure described in the work-plan. After 3x extraction with toluene, the combined toluene layers were washed consecutively with water (2x 0.5 ml), aqueous 0.1 M Na_2CO_3 (0.5 ml), water (0.5 ml), and dried on $MgSO_4$. The samples were evaporated to dryness with a Zymark TurboVap Evaporator while heating at 30 ºC by applying a N_2 flow, dissolved in toluene (200 µl; normally 100 µl when samples are not split) and stored as such at –20 ºC. The toluene-layers of the first run of modified Edman degradations were split in two equal portions: one portion for the LC-MS method and 1 portion for the GC-MS method (after derivatization with

heptafluorobutyryl imidazole). The samples for the LC-tandem MS method were evaporated to dryness and subsequently dissolved in CH_3CN/H_20, 20/80, v/v (100 µl).
Codes of the various samples (underivatized!!): see Table A2.1.
Codes for the LC-tandem MS samples (after splitting): see Table A2.2.

Table A2.2 Samples for LC-tandem MS analysis

Sample code	description
0446AF07	Half of 0446AF01; blank/d_8
0446AF08	Half of 0446AF02; 10 nM/d_8
0446AF09	Half of 0446AF03; 100 nM/d_8
0446AF10	Half of 0446AF04; 1 µM/d_8
0446AF11	Half of 0446AF05; 10 µM/d_8
0446AF12	Half of 0446AF06; 5 µM/5 µM d_8

LC tandem MS analysis of samples 0446AF07 and 0446AF12 was performed overnight.

(Tuesday, November 9, 2004)
LC tandem MS analysis
Initial results of LC-tandem MS analyses of samples 0446AF07 and 0446AF12 were slightly disappointing: relatively small signals were obtained for the analyte. It was decided to focus first on the 0446AF12 sample, and then to continue with the other samples 0446AF08-0446AF11 (see Table A2.2). The 0446AF08-11 samples were run overnight (Tuesday → Wednesday).

Analysis of blood samples of accidentally exposed person
From blood sample labelled 04-905-0035-SPRB2 (under top label the sample was labelled 04-905-0035-P1; under the latter label was the original label "Mould 081704"; hemolyzed blood) and blood sample labelled 04-905-0031-SPRB2 (under top label the samples were labelled 04-905-0031-B1; under the latter label was the original label "Mould 072204"; whole blood), 0.5 ml (half of total sample) was pipetted into Eppendorf tubes. Codes for the two portions of samples that will be processed: 0446DN01 (Mould 081704) and 0446DN02 (Mould 072204).
Centrifuged (in Eppendorf tubes) in order to see whether there was still any layer separation. No layer separation could be observed. To each sample water (0.5 ml) was added in order to allow for (further) hemolysis (30 min). Subsequently, globin was isolated according to the commonly used procedure by precipitation in HCl/acetone, followed by washing with acetone and diethyl ether. The entire procedure was performed in the presence of a CDC scientist. The obtained globin material appeared similar to globin samples obtained after isolation of regular blood samples. The globin samples were air-dried, after which they will be subjected to modified Edman degradation.
Codes: 0446DN03 (120 mg) is derived from 0446DN01. 0446DN04 (93 mg) is derived from 0446DN02.

GC-EI-MS analysis of already processed samples 0440AF01-05; HFB derivatization of standard
Analysis did not proceed quite satisfactory yet. It was decided to derivatize a small amount of 9705AF03 with heptafluorobutyrylimidazole, for use as reference for the GC-MS analysis. Thus, 10 µl of the 10 mg/ml stock solution of 9705AF03 in CH_3CN was diluted to 1 ml with

toluene. Subsequently, heptafluorobutyric imidazole (100 µl) was added and the reaction mixture was incubated at 60 ºC for 30 min. After washing, drying etc. a clear solution resulted (0446AF13; approximately 0.1 mg/ml). Prior to analysis, this solution was further diluted to approximately 1 µg/ml (0446AF14).

Samples 0446AF01-06 were derivatized with heptafluorobutyryl imidazole according to the described procedure (ratio toluene/HFBI = 9/1); derivatization at 60 ºC for 30 min. Subsequently, the samples were washed with water (2 x 0.1 ml), aqueous 0.1 M Na_2CO_3 (0.1 ml), water (0.1 ml) and dried on $MgSO_4$. Sample codes, see Table A2.3.

Table A2.3. Sample codes after modified Edman degradation and subsequent HFBI derivatization

Globin sample	Description	Internal standard*	Sample code after modified Edman degradation	Sample code after subsequent derivatization with HFBI
0442AF01	blank	0442AF07; 50 µl	0446AF01	0446AF15
0442AF03	10 nM	0442AF07; 50 µl	0446AF02	0446AF16
0442AF04	100 nM	0442AF07; 50 µl	0446AF03	0446AF17
0442AF05	1 µM	0442AF08; 50 µl	0446AF04	0446AF18
0442AF06	10 µM	0442AF08; 50 µl	0446AF05	0446AF19
0442AF09	d_0/d_8, 5/5 µM	n.a.	0446AF06	0446AF20

* solutions of internal standard globins were used as described in Table A2.1

(Wednesday, November 10, 2004)
GC-EI-MS analysis
GC-EI-MS analysis on the MAT900 gave too many fragments. Since NICI on the MAT900 is not sensitive enough, it was decided to use a triple quad-GC-MS (TSQ7000), operated by Dr. J. Driskell. First, the 1 µg/ml standard was evaluated; a rather large peak resulted. The fragment with *m/z* 564 was the most abundant fragment; comparable results were obtained at TNO-PML.

Globin sample work-up
Globin samples of an accidentally exposed person, 0446DN03 (21.4 mg) and 0446DN04 (20.4 mg), were subjected to modified Edman degradation. Both samples readily dissolved in formamide. Globin 0442AF07 (50 µl of a solution of 25.1 mg in 1.25 ml formamide) was used as an internal standard.
Two globin samples were used for exercising the method: 0442AF01 (blank) and 0442AF05 (1 µM exposed). The procedure as described above was followed. At the end of the day, the samples were concentrated with a Zymark TurboVap Evaporator while heating at 60 ºC by applying a N_2 flow and stored as such at –20 ºC.

Table A2.4. Globin samples (exercise and "real" samples)

Globin sample	Description	Amount used (mg)	Internal standard*	Sample code after modified Edman degradation and subsequent derivatization with HFBI
0446DN03	"real" sample	21.4	0442AF07; 50 µl	0446AF21
0446DN04	"real" sample	20.4	0442AF07; 50 µl	0446AF22
0442AF01	blank	23.6	0442AF07; 50 µl	0446DA01
0442AF05	1 µM	20.3	0442AF08; 50 µl	0446DA02

*solutions of internal standard globin were used as described in Table A2.1

LC-tandem MS analyses

The LC tandem MS analyses of samples 0446AF07-12 showed that an exposure level of 1 µM could still be determined; gradient elution of 50/50% CH_3CN/H_2O to 100% CH_3CN was now applied and samples had also been dissolved in CH_3CN/H_2O (50/50, 100µl). The peak of the internal standard was present at a level that could be expected on the basis of concentrations: the internal standard was present at a level equivalent to 5 µM. For a similar transition, the peak for the native adduct in the 10 µM sample was twice as large as for the deuterated compound. Again, the transition of *m/z* 429 (MH^+) → *m/z* 130 showed the highest intensity. The identity of the *m/z* 130 fragment is yet unknown. Exploratory research using relatively high standards was also performed on the TSQ Quantum LC tandem MS.

(Thursday, November 11, 2004)

GC-NICI-MS analyses

GC-NICI-MS analyses on the TSQ7000 were continued. There were some problems with regard to the peak ratio d0/d8: the d8 level could be observed at the level that could be expected. However, the level of the d0 was much too high, and was also present in the blank. The reason for this was yet unclear. It was decided to use a less steep temperature program for the analyses; also, first the already processed samples 0440AF01-05 were to be analyzed. It seemed that by using the temperature program used at TNO-PML the chromatography improved; it seemed as if a 10 nM exposure level could still be detected. Peak ratios d0/d8 were as should be expected.

However, some of the newly prepared samples did not look good, e.g., in 0446AF16 the level of d8-pentafluorophenylthiohydantoin was satisfactory, but the level of the native pentafluorophenylthiohydantoin was much too high (see GC-NICI-MS results in Appendix 2). Sample 0446AF18 looked satisfactory.

HFB derivatization

Introduction of HFB on exercise samples was performed by CDC scientist according to the described procedure under supervision of TNO scientist.

Introduction of HFB on "real" samples was performed according to the described procedure; resulting sample codes 0446AF21 and 0446AF22 (see Table A2.4).

Exercising isolation of globin

From blood samples labelled 04-905-0032-SPRB2 (under top label the sample was labelled 04-905-0032-B1; under the latter label was the original label Mould072704) and 04-905-0036-SPRB2 (under top label the sample was labelled 04-905-0036-P1; under the latter label

was the original label Mould082304), was taken 0.5 ml for isolation of globin. Codes of samples to be processed: 0446DN05 (Mould072704) and 0446DN06 (Mould082304); codes of resulting globin samples: 0446DN07 (87 mg; derived from Mould072704) and 0446DN08 (116 mg; derived from Mould082304).

(Friday, November 12, 2004)
GC-NICI-MS analysis of samples 0446AF21 and 0446AF22 did not show the presence of the pentafluorophenylthiohydantoin-HFB derivative. However, no peaks for the internal standard could either be observed.
Analysis of the samples 0446DA01 and 0446DA02 showed only peaks for sample 0446DA02; however the ratio d0/d8 analyte was 1:2, and should have been 1:5.

3. Demonstration of phosgene method
(Monday, October 17, 2005)
Method will be performed together with the CDC scientists Kerry Preston and Adrian Woolfitt.
Already processed samples 0535AF10 (blank), 0535AF12 (10 microM), 0535AF15 (50 microM) and 0535AF17 (100 microM ^{13}C-phosgene), stored at CDC at –70 °C, were thawed. 0.5 ml of each sample was transferred to 6 ml tubes (without caps; other tubes did not fit in Eppendorf rotor). The samples were lyophilized, after freezing in solid CO_2, using an Eppendorf Vacufuge. Two runs: blank + ^{13}C, then 10 + 50 microM exposed samples. Samples were fully lyophilized after 2 h, giving a white residue. The samples were stored in the freezer (-20 °C) overnight.
In the meantime the LC tandem MS instrument (type: Applied Biosystems MDS Sciex 4000 Q Trap; newly installed instrument) was prepared for analysis of samples, standards etc.
The guanidine buffer was prepared according to the work plan (sent by e-mail to Maria Solano, Tuesday October 11, 2005).

(Tuesday, October 18, 2005)
The lyophilized samples of Monday were treated with DTT and iodoacetic acid (Na+ salt), together with a CDC scientist, according to the work-plan. Deviation from work plan: 50 mg iodoacetic acid, Na+ salt was dissolved in 180 µl guanidine buffer; 40 µl of this solution added to the DTT-reduced sample solutions.
Dialysis started around 13.00 (1 liter 50 mM NH_4HCO_3) per sample. Dialysis buffers refreshed at 15.30 and at 17.00. Continued overnight.
LC tandem MS analysis of already processed sample 0535AF25 (100 microM exposure) was started. No real conclusive results after one injection. Therefore, the reference standard (0511AF01; 10 microgram) was chosen for optimization of the instrument. The sample was dissolved in water containing 0.2% formic acid to an appropriate concentration.

(Wednesday, October 19, 2005)
Samples were removed from dialysis cassettes and digested with trypsin, as described below (*method was not incorporated in work plan*):
Dialyzed (reduced and carboxymethylated) albumin was transferred from the slide-a-lyzer cassettes into vials (4 mL). Next, trypsin was added (2 % w/w). For this purpose, trypsin (6 mg) was dissolved in buffer (5 mL, 50 mM NH_4HCO_3). Part (50 µl) was added to the dialyzed solution of reduced and carboxymethylated albumin in buffer. Incubation was performed at 37°C for 4 h. Finally, the sample was filtrated through a pre-rinsed (50 mM NH_4HCO_3, 3x 1 mL) filter with a 10 kDa cutoff with centrifugation at 3200g, to remove the enzyme.

The resulting trypsin digests were coded as follows:

Blank:	0542DN01
10 microM exposure:	0542DN02
50 microM exposure:	0542DN03
100 microM ^{13}C-labelled exposure:	0542DN04

Two samples (0535AF10 (blank), and 0535AF15 (50 microM exposure)) were selected for practicing the method. The procedure as described above was performed, starting with lyophilization.

(Thursday, October 20, 2005)

Samples 0542DN01-04 had been analyzed overnight. The 10 microM (0542DN02) level could not be distinguished from the blank; in the 50 microM (0542DN03) level the adduct could be detected. The sensitivity of instrument was further improved.
The CDC scientist trypsinized the two dialyzed samples, according to the work plan. Injection volume was changed to 50 µl. The results looked much better then.
Codes of trypsin digests:

Blank:	0542KP01
50 microM:	0542KP02

These samples will be analyzed by means of LC tandem MS.

(Friday, October 21, 2005)
Analyses of the phosgene bis-peptide adduct looked nice. The recorded spectra were compiled and the project closed.

4. Demonstration of lewisite method

(Monday, October 17, 2005)
Method will be performed together with CDC scientists Maria Solano and Joe Wooten.
Instrument to be used: MAT 900 XL. The GC-column was installed (type: Varian CP-Sil 5CB, 50 m length, 0.25 mm i.d., 0.25 mm film thickness)
Start with already processed samples 0536AF13-17. These samples had already been analyzed at TNO; it seemed as if the vial-inserts had been leaking their constituents into the vial. For this reason, 20 µl of toluene were added to each sample. Although the absolute intensity of the signals will now definitely decrease, the ratios between analyte and internal standard should not be different. The first sample that was analyzed was 0536AF17 (10 microM exposure). Satisfactory result: see file C:\Xcalibur\..\Oct,05\mo529001. The other samples were run overnight.
Since not all reagents had arrived at CDC, the work up of samples 0536AF08-0536AF12 was to performed at Tuesday (deviation from work plan).

(Tuesday, October 18, 2005)
Analytical results obtained from overnight GC-MS runs: looked satisfactory at first sight; chromatograms need still to be integrated. After a second look, the peak ratio of the analysis of 0536AF16 gave the same result as 0536AF17 (both ca 7). Probable course might have been a switch of samples. All samples will be reanalyzed (see Thursday).

Stock solutions were prepared for BAL (100 mM and PAB (100 microM), according to work plan.
From the exposed, washed and lysed erythrocyte samples 0536AF08-12, 1 ml was taken for processing. The rest was transferred into new vials and stored at –70 °C.
New codes for samples to be processed:
0542AF01 (1 ml from 0536AF08)
0542AF02 (1 ml from 0536AF09)
0542AF03 (1 ml from 0536AF10)
0542AF04 (1 ml from 0536AF11)
0542AF05 (1 ml from 0536AF12)

To these samples (0542AF01-05), 10 µl of BAL solution and 10 µl of PAB solution were added and the samples were incubated for 4 h (deviation from work plan; not relevant). The samples were diluted with water (9 mL). Sep-Pak C18 cartridges were conditioned according to the work plan. The samples were applied and the cartridges were eluted according to the work plan. For each sample, the appropriate fraction was isolated and concentrated; due to the unavailability of an appropriate device for concentrating samples in the way it is performed at TNO, the samples were concentrated under nitrogen in a special device (TurboVap evaporator). The remaining water was removed by concentration in the Vacufuge. The blank was concentrated in two steps; step 1: volume reduction using a stream of air; step 2: concentration in Vacufuge.
For future work this week, it was decided to split the samples (organic layer from aqueous layer) and to concentrate them separately.

(Wednesday , October 19, 2005)
Samples 0542AF01-05 were concentrated to dryness and coevaporated twice with toluene (0.5 mL). Next, the samples were dissolved in toluene (100 µl) and transferred to a 0.5 mL GC vial with a conically shaped bottom. Heptafluorobutyryl imidazole (10 µl) was added and the reaction mixtures were incubated for 1 h at 50° C. It has to be mentioned that the HFBI used was a red troubled liquid (had been stored in refrigerator), while it should be a white to light yellow solid. Next, the reaction mixtures were washed with water (100 µl), dried over $MgSO_4$ in a pasteur pipette, and analyzed with GC/MS.
The remaining, washed, exposed erythrocytes (samples 0536AF08-12) were divided in two portions of 1 mL. To one set of these samples (coded as 0542JW01-05), 10 µl of BAL solution and 10 µl of PAB solution were added and the samples were incubated for 1h according to the work plan. Next, work-up was performed according to the work plan. The eluate, consisting of two layers, was now divided into two separate fractions in order to speed up the concentration step. The upper (aqueous) layer was concentrated in the Vacufuge, while the lower (organic) layer was concentrated to a small volume using a stream of nitrogen. Finally both fractions were combined again, evaporated to dryness, and coevaporated twice with toluene (0.5 mL). It was decided to postpone the derivatization with HFBI to Thursday, using newly ordered HFBI.
The set of samples (0536AF13-17) prepared in The Netherlands was reanalyzed; unfortunately, sample 0536AF15 did not contain enough material to be analyzed again. The set of samples (0542AF01-05), prepared at CDC, was also analyzed.
In case of 0536AF13-17, the peak ratios now corresponded with the peak ratios found at TNO. Since the samples had been diluted with toluene on Monday (there was not enough sample anymore, probably due to leakage of the insert in the vial), the lowest exposure level

that could be analyzed was approximately 20 nM. The sample (0536AF16) that showed a deviating peak ratio on Tuesday, was now satisfactory.
The other set (0542AF01-05) gave low responses, probably due to the derivatization that did not proceed satisfactory (bad quality of HFBI; see Wednesday). Even the 100 nM exposure level (0542AF03) could not be detected anymore. The 1 microM exposure (0542AF04) gave a peak ratio analyte/internal standard that corresponded with earlier analyses. The 10 microM exposure level, however, showed a deviating peak ratio (19 instead of 6 – 7).

(Thursday, October 20, 2005)
A CP-Sil 5b column was installed in GC/MS (exactly the same type as was used for the analysis at TNO) of J. Wooten in order to get a mirror view of the results we obtained at TNO.
The MAT 900 XL was further optimized in order to improve the sensitivity.
Fresh HFBI (Pierce) had arrived; colourless and clear liquid (at room temp). Processed samples 0542JW01-05 were derivatized by CDC scientist with fresh HFBI, according to the work-plan, and were worked up further.
Codes after derivatization:
0542JW01 → 0542JW06 (blank)
0542JW02 → 0542JW07 (10 nM)
0542JW03 → 0542JW08 (100 nM)
0542JW04 → 0542JW09 (1 microM)
0542JW05 → 0542JW10 (10 microM)

Unfortunately, layer separation appeared to be difficult this time, using the conically shaped GC vials. When the toluene layer was extracted with water (4 x 0.1 mL) each time the water layer was collected in a tube. When performing the drying step, it appeared that most of the liquid consisted of water. So it was decided to add 100 µl of toluene to the collected water extracts, and dry that again over $MgSO_4$.
Analysis showed the expected peaks only for sample 0542JW10; the intensity of the peaks was, however, much lower than for the corresponding sample 0542AF05. The reason for this is still unknown.

5. Determination of CVAA in blood by using 1,3-propanedithiol, as an improved method for diagnosis of Lewisite exposure

In the following report, the development of a method is described for determination of bound CVAA residues in human blood, as an alternative to the method using British Anti-Lewisite as described by Fidder *et al*. (2000). This method is based on the method published by Wooten *et al* (2002), in which 1,3-propanedithiol is used for complexation of CVAA, followed by GC-MS analysis. The detailed report describes the experimental work, as performed by Joe Wooten (CDC), Alex Fidder and Daan Noort (both TNO Defense, Security and Safety) in the period May 1 – May 9, 2007.

Tuesday, May 1
Alex Fidder started working with Joe Wooten on the lewisite assay. This method has been transferred to CDC in 2006, but after that demonstration the method had been slightly improved, i.e., by using more toluene during the final extraction steps. After discussions with Joe it followed that the slightly improved procedure for sample work-up (as e-mailed by Alex

to Joe) was indeed successful and that comparable results were obtained for samples that had been both analyzed at CDC and at TNO.
In the afternoon, the procedure that Joe uses for CVAA in urine samples was applied to blood samples. This procedure is based on the following principle:
1,3-Propanedithiol (PDT) is used for complexation of CVAA. $^{13}C_2$, D_2-labelled CVAA is used as an internal standard. In case of urine, 10 µl (PDT) is added to 1 ml of urine; then the sample is heated for 10 min (70 C). Subsequently, an SPME (solid phase micro extraction) device is placed in the headspace of the sample. After a certain period of time, the headspace is removed and thermodesorbed.
This procedure was now applied to whole blood samples. Results were variable, although in most instances the analytes could be detected.

Preparation of samples:
Five concentrations of washed and lysed L1 exposed blood samples were prepared (see Table A.2.5) for analysis by adding 1-ml blood to a 10-ml SPME headspace vial. The sample vials were crimp-sealed and placed on the sample tray of the twin-tier autosampler fitted on the GC/MS system. Upon initiation, the autosampler added 10 µl of 1,3-propane dithiol (PDT) to each vial. The vial was then moved to the heater agitator set at 70°C with agitation. The SPME fiber was inserted into the headspace for 10 min; derivatization and SPME extraction occurred simultaneously.
Analytical Procedures:
SPME–GC–MS analyses were performed on a HP 6890 Gas Chromatograph coupled to a HP5973 Mass-Selective Detector. The GC inlet port, held at 245°C, was fitted with a narrow-bore 0.75 mm I.D. SPME injection liner. The gas chromatograph was fitted with a Varian CP-Sil 8 CB low bleed capillary column (30 m, 0.25mm, 0.25 µm film thickness) and maintained with helium flow rate of 1.0 ml/min. The following oven temperature program was used: initial temperature 180°C, held for 1 min, next ramped at 15°C /min to 218°C, followed by final ramp at 40°C /min to 250°C (2.64 min total run time). The GC–MS transfer line was held at 300°C and the MS quadrupole and source heaters were maintained at 150°C and 230°C, respectively.

Wednesday, May 2
Alex continues working on the lewisite assay. Two types of experiments were carried out:

1. an experiment with 1,3-propanedithiol in an analogous way as the experiments with British Anti Lewisite, i.e., after incubation with 1,3-propanedithiol, perform the extraction of CVAA-1,3-propanedithiol by using a SepPak C18 cartridge and elution with acetonitrile/dichloromethane
2. as 1, but perform a liquid-liquid extraction with toluene on the washed and lysed erythrocytes

More specifically: see table A2.5

Table A2.5 Incubation of washed/lysed erythrocyte samples with 1.3-propanedithiol (PDT), part I

Sample code	Amount (ml)	Exposure level (micromolar)	Internal standard (µl)	PDT solution (µl)
0646AF01	2	0 (Blank)	100	20
0646AF03	2	0.1	100	20
0646AF04	2	1	100	20
0646AF05	2	10	100	20
0646AF07	2	100	100	20

The above mentioned samples (lysed and washed erythrocytes, 2 mL) were incubated for two hours at 37 ˚C with internal standard and PDT.

The PDT solution was made by dissolving 20 µls of 1,3-propanedithiol in 2 mL of acetonitril, resulting in a 100 mM solution. The internal standard was already diluted as a solution in water of 1.04 microgram/mL (6 micromolar).
The internal standard (1,2 ^{13}C, 1,2 ^{2}H –chlorovinylarsonous) acid was obtained from Los Alamos Laboratories.
After the incubation the samples were split into two equal portions, and were worked up according to two methods, giving two series of samples:

Series 1: traditional SepPak C18 isolation

0718AF01 (blank), 0718AF02 (0.1 µM), 0718AF03 (1 µM), 0718AF04 (10 µM), 0718AF05 (100 µM)

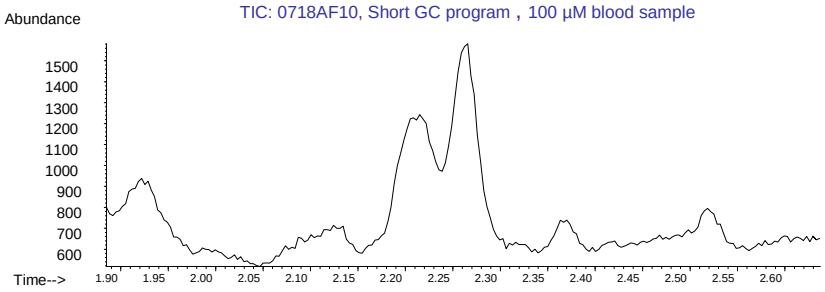
Series 2: extraction with toluene

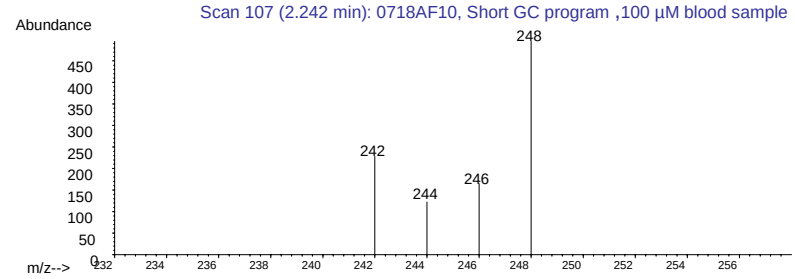
0718AF06 (blank), 0718AF07 (0.1 µM), 0718AF08 (1 µM), 0718AF09 (10 µM), 0718AF10 (100 µM)

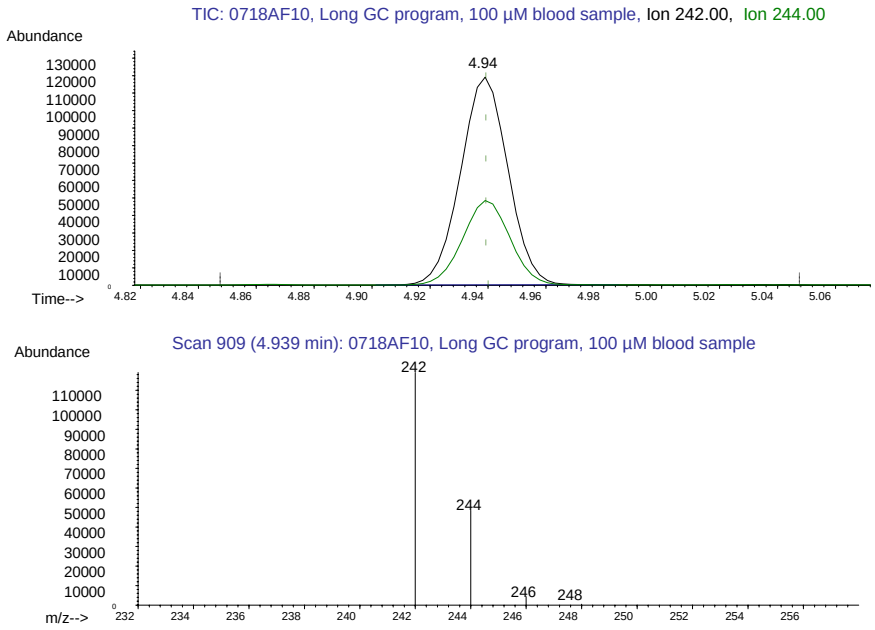
Thursday, May 3

Analysis of the samples did not yet give very satisfactory results; it was argued that the 1,3-propanedithiol-derivatized CVAA might be lost during concentration of the samples.
For the second series (direct extraction with toluene) results were also a little bit variable.

These samples were initially run using the short GC program in SIM mode, the next day the same samples were rerun using the long GC program in SIM mode. After comparing the results from the two GC programs, it was clear that the long GC program produced better analytical results (see graphics below).







It was decided to focus first on a highly exposed sample (100 µM) and incubate that sample with 1,3-propanedithiol; work-up by extraction with toluene (vials labeled as 0718AF11 and 12, blank and exposed respectively) and with ethyl acetate (vials labeled 0718AF13 and 14, blank and exposed respectively) (1.5 ml solvent added each). The toluene was nicely separated from the lysed erythrocytes.
Extraction with ethyl acetate resulted in coagulation of the hemoglobin and/or other proteins; the samples could not be used anymore. From the toluene layer, 0.2 ml was used for direct GC-MS analysis (0718AF15 and 16, blank and exposed respectively) and another 0.2 ml was concentrated using a vacuum concentrator. The concentrated samples were reconstituted in the same amount of toluene (0718AF17 and 18, blank and exposed respectively). The samples were analyzed overnight.

Monday, May 7
Preparing standard for GC/MS analysis:
Take 100 µls of the stock solution of deuterated/^{13}C-labelled CVAA (1.04 microgram/mL). This solution was lyophilized and subsequently PDT solution was added (100 mM; 10 µl) and 990 µl acetonitril. After 10 min this solution is diluted 10x, giving an end concentration of 10 ng/mL. This standard can be analyzed by GC/MS, confirming the retention time and fragmentation pattern of the analytes prepared earlier this week. A fresh solution of 100 mM PDT was prepared (MW = 108; d = 1.08). Samples were incubated for 10 min.
Sample codes:
0719AF01: blank (empty, only CH_3CN + PDT)
0719AF02: standard ($^{13}C_2/D_2$- CVAA + CH_3CN + PDT).
A dilution was prepared by taking 20 µL 0719AF01 or 02, and 180 µL toluene, before analysis with GC-MS. Sample codes: 0719AF07 and 0719AF08 (dilution of 0719AF01 and 02 respectively).

After analyzing the standard that had been prepared in this way, no CVAA-PDT derivative could be analyzed! Therefore, it was decided that derivatization of the standard was carried out under aqueous conditions.

From the internal standard solution $^{13}C_2/D_2$ CVAA (1.04 microg/ml), 100 µl was taken. To this solution, 10 µl of a PDT solution (0.1 mM in acetonitril) was added. After 10 min incubation at 37 ºC (not at 70 ºC, as was performed by Joe Wooten for urine samples) a sample with code 0719AF09 resulted.
After extraction with toluene (1 ml), the organic layer was transferred to a new vial. Code: 0719AF10. Part of this solution (20 µl) was diluted with 180 µl toluene. This solution, coded 0719AF11, was used for GC-MS analysis. Remarkably, the derivative could also not be detected.

Because this experiment did not give satisfactory results, we switched to the method Joe Wooten used for derivatizing urine samples.
100 µl $^{13}C_2/D_2$ CVAA or CVAA solution (1.04 microg/ml or 1.23 microg/ml) was incubated with PDT (10 µl; neat) at 70 C for 10 min.
Codes: 0719AF12 and AF13 respectively. Both samples were extracted with toluene (1 ml), and centrifuged. Next, the organic layers were transferred to new vials, encoded 0719AF14 and AF15, respectively. Part (20 µl) of 0719AF14 and 15 were diluted with 180 µl of toluene; codes 0719AF16 and 17. These samples were analyzed by GC-MS overnight.

In the mean time, blood samples were processed (0646AF01, blank, and 0646AF07, 100 µM). Samples were both treated with neat (10 µl) PDT as well as diluted PDT (0.1 mM solution in CH_3CN).
Sample codes:
0719AF03: blank, 1 ml + 0.1 mM PDT (10 µl)
0719AF04: blank, 1 ml + neat PDT (10 µl)
0719AF05: 100 µM, 1 ml + 0.1 mM PDT (10 µl)
0719AF06; 100 µM, 1 ml + neat PDT (10 µl)
The samples were diluted with 2 ml of water, extracted with 1 ml of toluene; in case of samples 04 and 06, the water layers were much more turbid than compared with the other samples. The samples were centrifuged (10 min; 1000 rpm) and 200 µl of the toluene layer was used for GC-MS/MS analysis.
Samplecodes:
0719AF18, 19, 20, 21 (extracts of 0719AF03, 0719AF04, 0719AF05 and 0719AF06, respectively, and analyzed overnight.

Tuesday, May 8
In samples 0719AF16 and 17 (derivatized standards) no analyte could be analyzed; the reason for this remains unclear.
In samples 0719AF20 and 21 the PDT-CVAA adduct could be analyzed under full scan conditions.

The GC inlet was held at 245°C, helium flow rate 1.0 ml/min, The following oven temperature program was used: initial temperature 120°C, held for 1 min, next ramped at 55°C /min to 207 °**C** , followed by final ramp at 8°C /min to 280°C held for 5 min, (16.71 min total run time). The GC–MS transfer line was held at 300°C and the MS quadrupole and source heaters were maintained at 150°C and 230°C, respectively.
The analyte's level in the sample from the diluted PDT looked even a bit better than the sample that was obtained with neat PDT. The blanks 0719AF18 and 19 were clean. The spectrum of the compound was comparable with the spectrum obtained in earlier work (see Joe Wooten's earlier laboratory notebook)

It was decided to analyze a blank sample (0719AF19) and an exposed sample (0719AF20) under single ion monitoring conditions, using 3 ions (181, 242, 244). A nice clean chromatogram was obtained, showing a peak with the right retention time. When an older sample was analyzed (100 µM exposure), which had been concentrated after extraction, a peak with a similar retention time was observed, but much higher. This provisionally indicates that no analyte is lost during concentration of the toluene extract. In order to elaborate this further, it was decided to work up some of the lower exposed blood samples under identical conditions, and analyze them subsequently under the SIM conditions that were set up for the selected samples (see above), and analyze them both directly after extraction as well as after concentration and reconstitution. Alex processed the following samples, see table A2.6. Incubation with PDT solution for 2 h.

Table A2.6. Incubation of washed/lysed erythrocyte samples with 1.3-propanedithiol (PDT), part II

Sample code	Amount (ml)	Exposure level (micromolar)	Ml PDT solution
0646AF01	1	0 (Blank)	10
0646AF03	1	0.1	10
0646AF04	1	1	10
0646AF05	1	10	10
0646AF07	1	100	10

After work-up (extraction with 1 ml of toluene) the extracts were divided into two parts. The first parts (100 µl), for direct GC-MS analysis, had the following codes (see Table A2.7). The second parts (500 µl) were concentrated to dryness in a vacuum concentrator and reconstituted in 50 µl toluene.

Table A2.7. Incubation of washed/lysed erythrocyte samples with 1.3-propanedithiol (PDT), part III

Original sample code	Exposure level (micromolar)	Toluene extract (first part)	Toluene extract after reconstitution (second part)
0646AF01	0 (Blank)	0719AF27	0719AF32
0646AF03	0.1	0719AF28	0719AF33
0646AF04	1	0719AF29	0719AF34
0646AF05	10	0719AF30	0719AF35
0646AF07	100	0719AF31	0719AF36

In case of series 0719AF27-31 a nice relationship between level of the CVAA-PDT derivative and the exposure level was found. The 1 microM sample could still be detected (see Figure A2.1).
After concentration and reconstitution in toluene (series 0719AF32-36), signals were increased (see Figure A2.2).
After comparison with series 0719AF27-31 it seems that an increase in peak area of approximately a factor of 8-9 was obtained after concentrating the sample a factor of 10. Thus, apparently the CVAA-PDT derivative is not very volatile, and concentration of the toluene extracts does not give severe problems.

Wednesday, May 9

Based on the results of May 8, the following experiments were carried out:

Three samples were prepared

1. 1 ml 0646AF03 (0.1 micromolar L1 exposure) + 10 µl PDT solution (0.1 mM in acetonitrile); incubation at 37 ºC for 30 min
2. 1 ml 0646AF03 (0.1 micromolar L1 exposure) + 10 µl PDT solution (0.1 mM in acetonitrile) + $^{13}C_2/D_2$-CVAA (50 µl, 6 microM); incubation for 30 min
3. as 1, but incubation for 60 min (further processed by Joe, due to time constraints).

Sample codes after extraction:

1 becomes 0719AF37

2 becomes 0719AF38

3 becomes 0719AF39

Extraction of the samples was carried out with 2x 0.5 ml toluene; the extracts were combined, concentrated and reconstituted in 20 µl of toluene. Analysis of 0719AF37 with GC/MS showed a significance increase in signal, i.e., roughly 6 times higher than in case of sample 0719AF33 (see Figure A2.3). The entire toluene extract had been used for concentration. Analysis of 0719AF38 showed a comparable signal for the native CVAA-PDT derivative and a much larger coeluting signal for the $^{13}C_2$, D_2-CVAA-PDT derivative (see ion *m/z* 181; ions 246 and 248 not shown in chromatogram). See Figure A2.4 for characteristics of $^{13}C_2$, D_2-CVAA-PDT derivative. Analysis of sample 0719AF39 failed.

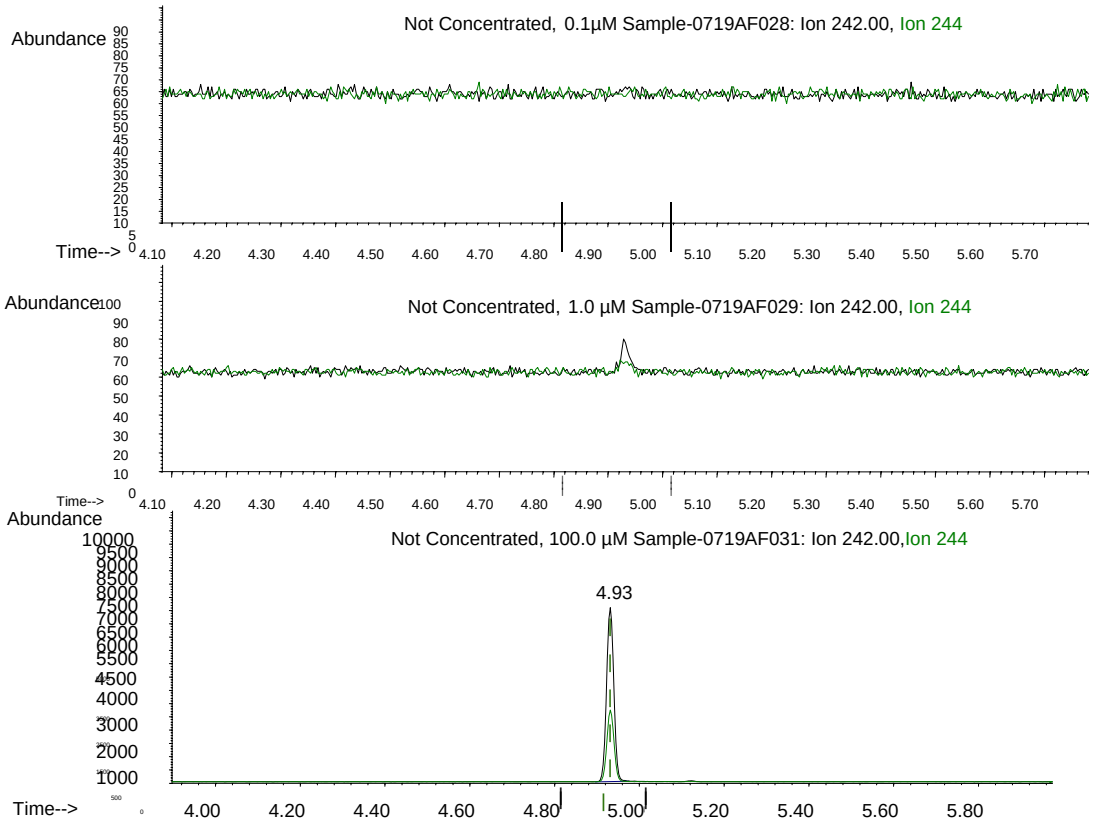


Figure A2.1. GC-MS analysis of of CVAA-PDT derivative in toluene extracts, obtained after PDT derivatization of human blood samples, without concentration/reconstitution.

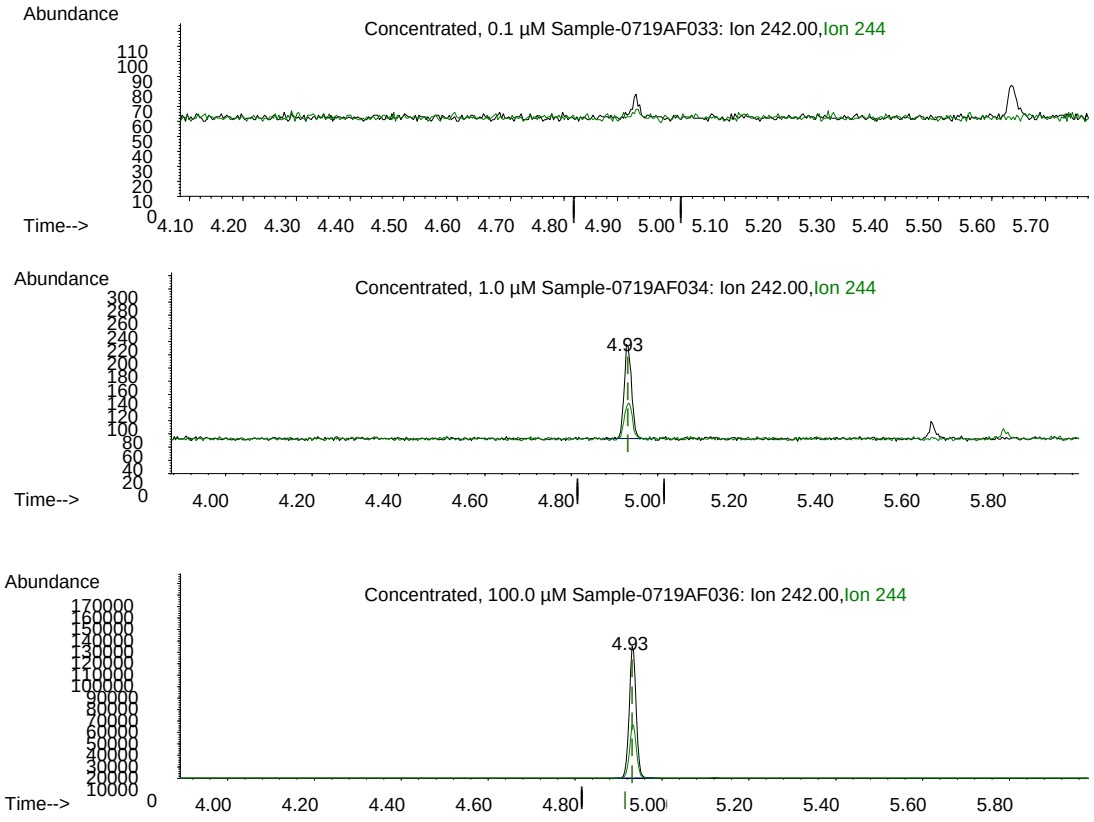


Figure A2.2. GC-MS analysis of CVAA-PDT derivative in reconstituted toluene extracts, obtained after PDT derivatization of human blood samples.

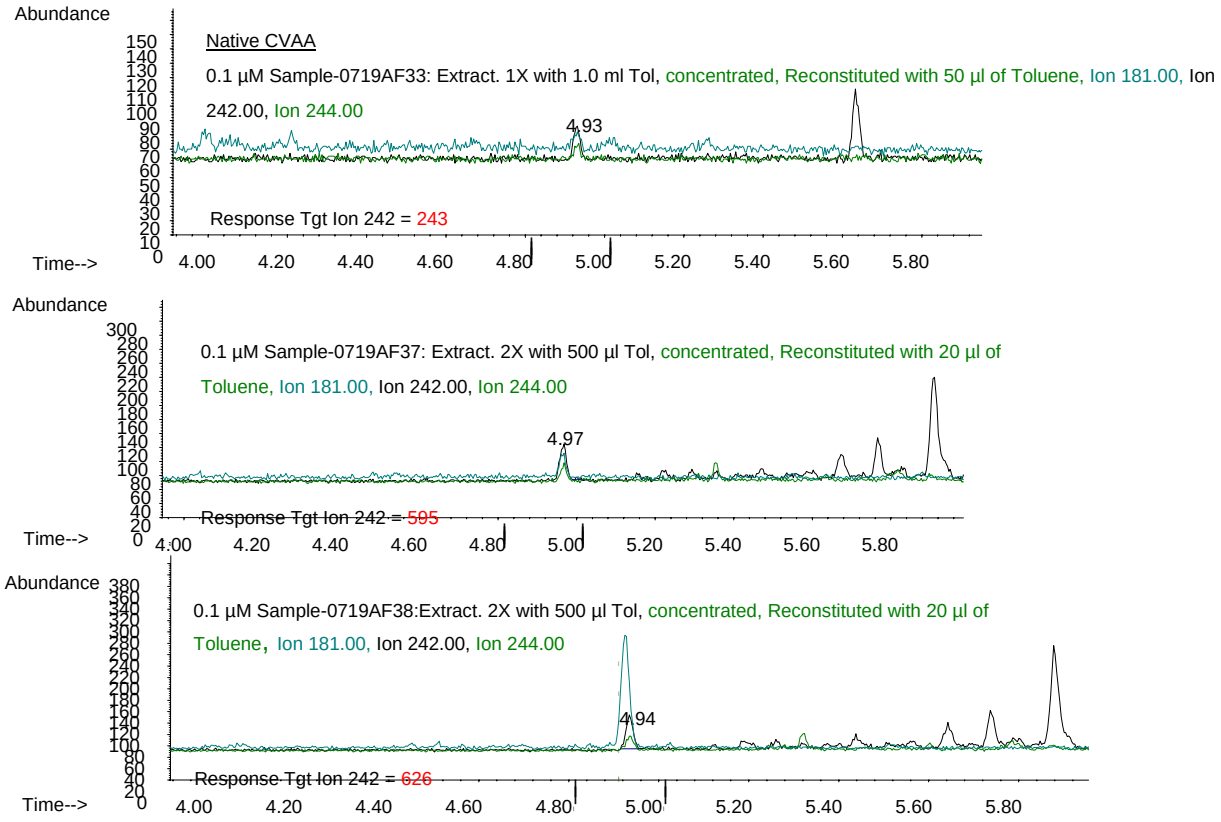


Figure A2.3. GC-MS analysis of CVAA-PDT derivative in reconstituted toluene extracts; samples 0719AF37 and 38 (containing internal standard); comparison with sample 0719AF33.

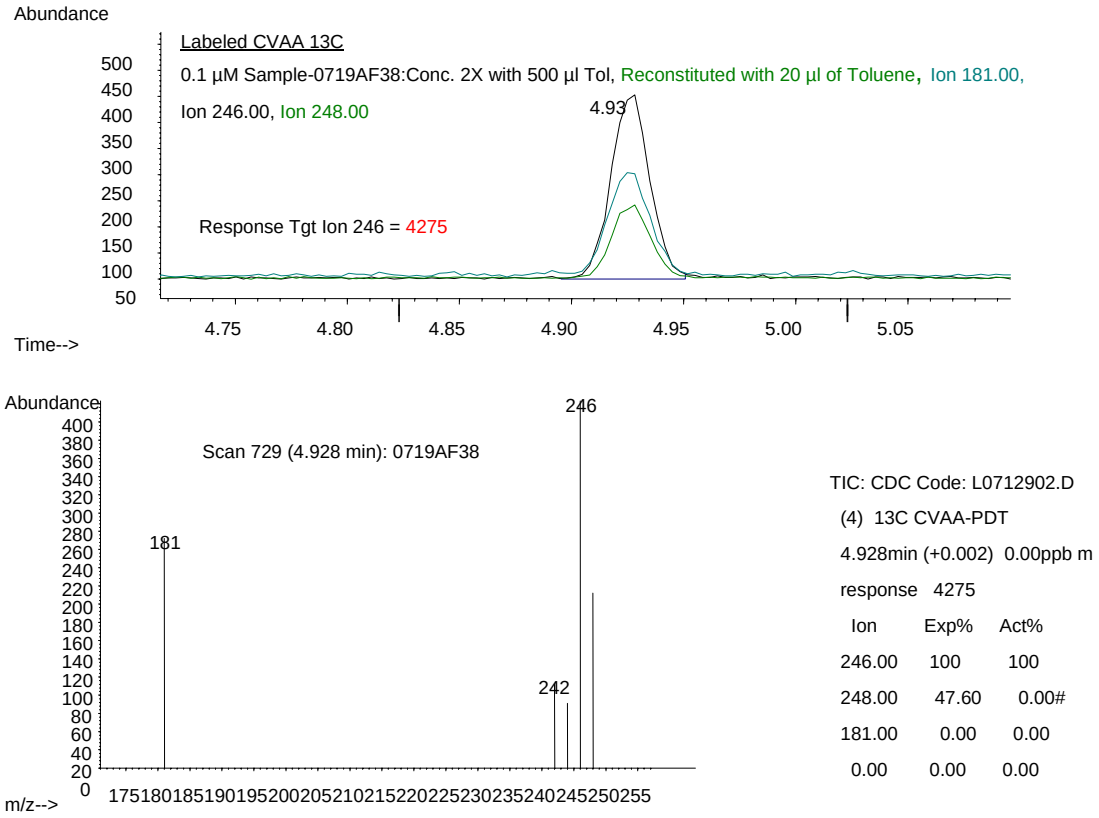


Figure A2.4. Characteristics of internal standard $^{13}C_2$, D_2-CVAA-PDT in sample 0719AF38. Upper figure, ion chromatogram; lower figure, part of mass spectrum.

Appendix 3. Mass spectrometric data of synthetic phospho-nonapeptides

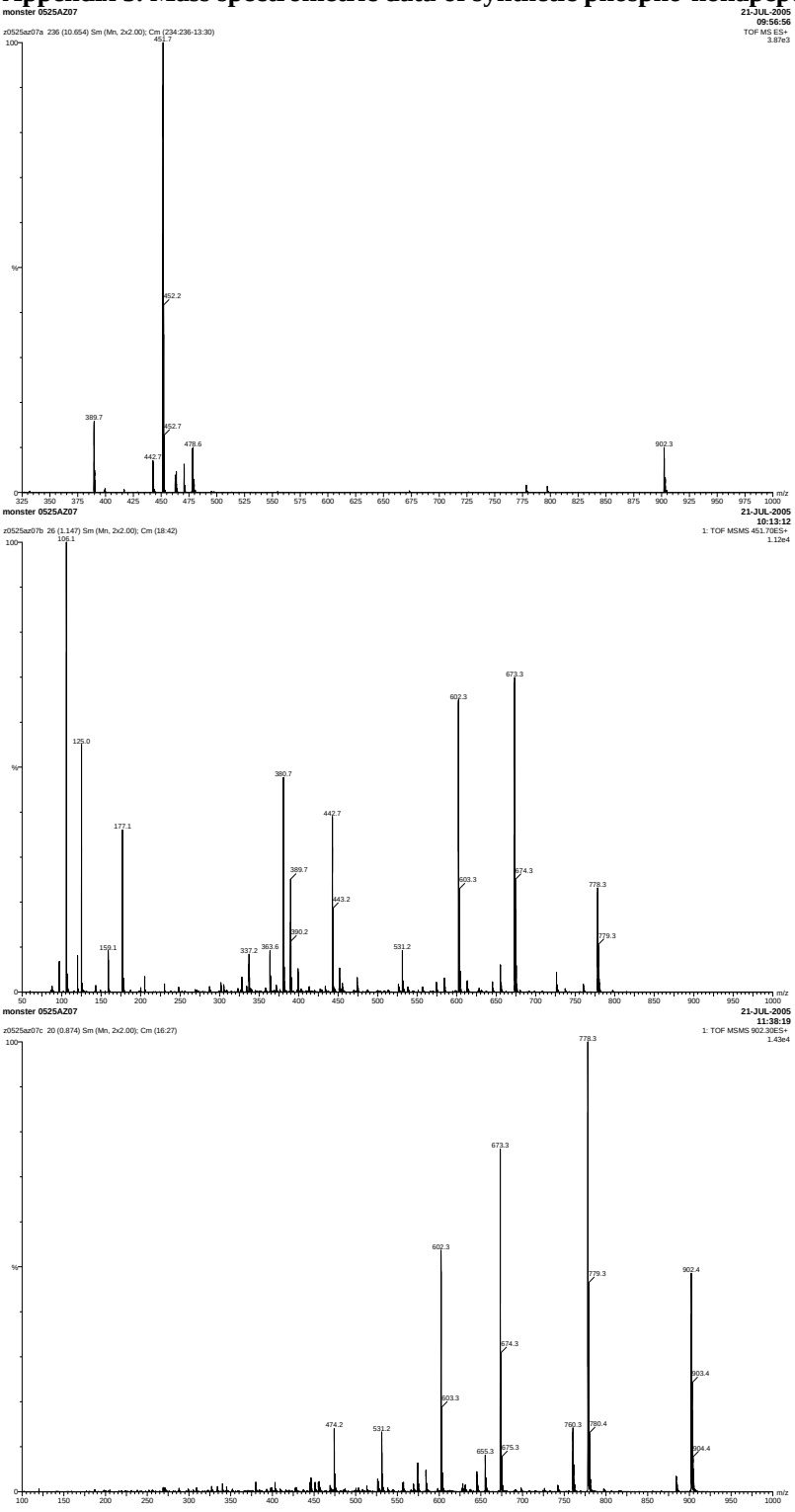


Figure A3.1. Mass spectra of synthetic FGES(EMPA)AGAAS, i.e., the nonapeptide derived from VX-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Middle trace: product ion spectrum of *m/z* 451.7 (MH_2^{2+}). Lower trace: product ion spectrum of *m/z* 902.3 (MH_2^{2+})

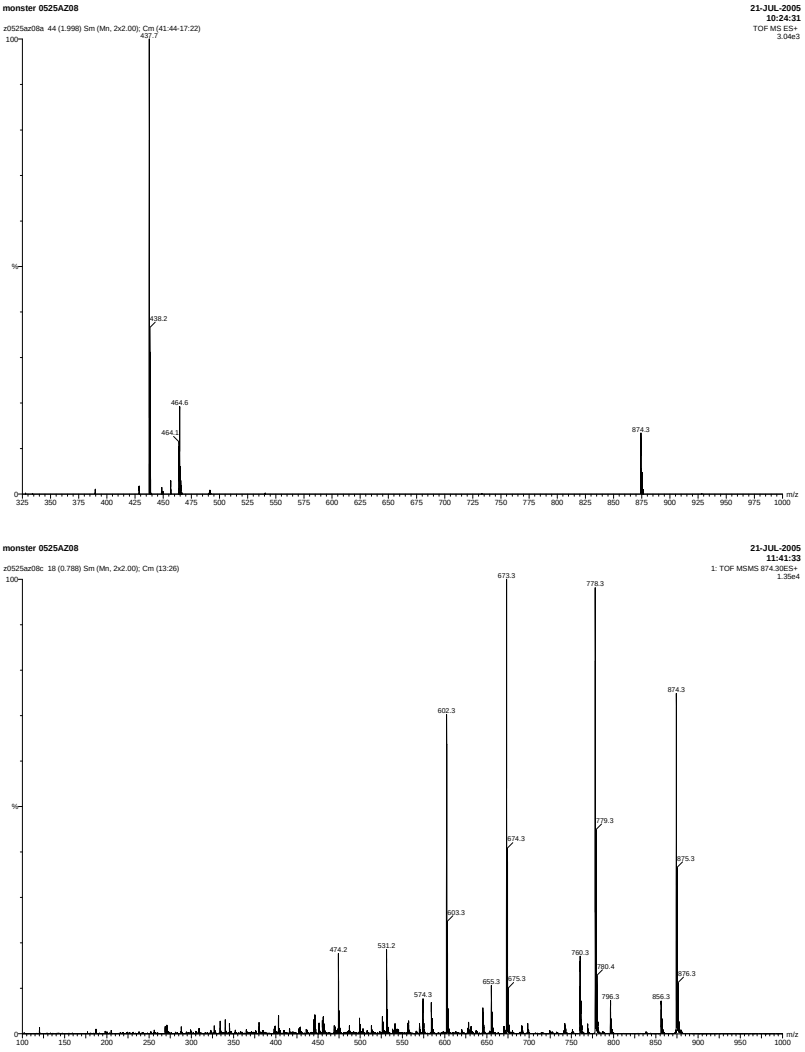


Figure A3.2. Mass spectra of synthetic FGES(MPA)AGAAS, i.e., the nonapeptide derived from aged soman-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Lower trace: Product ion spectrum of *m/z* 874.3 (MH^+).

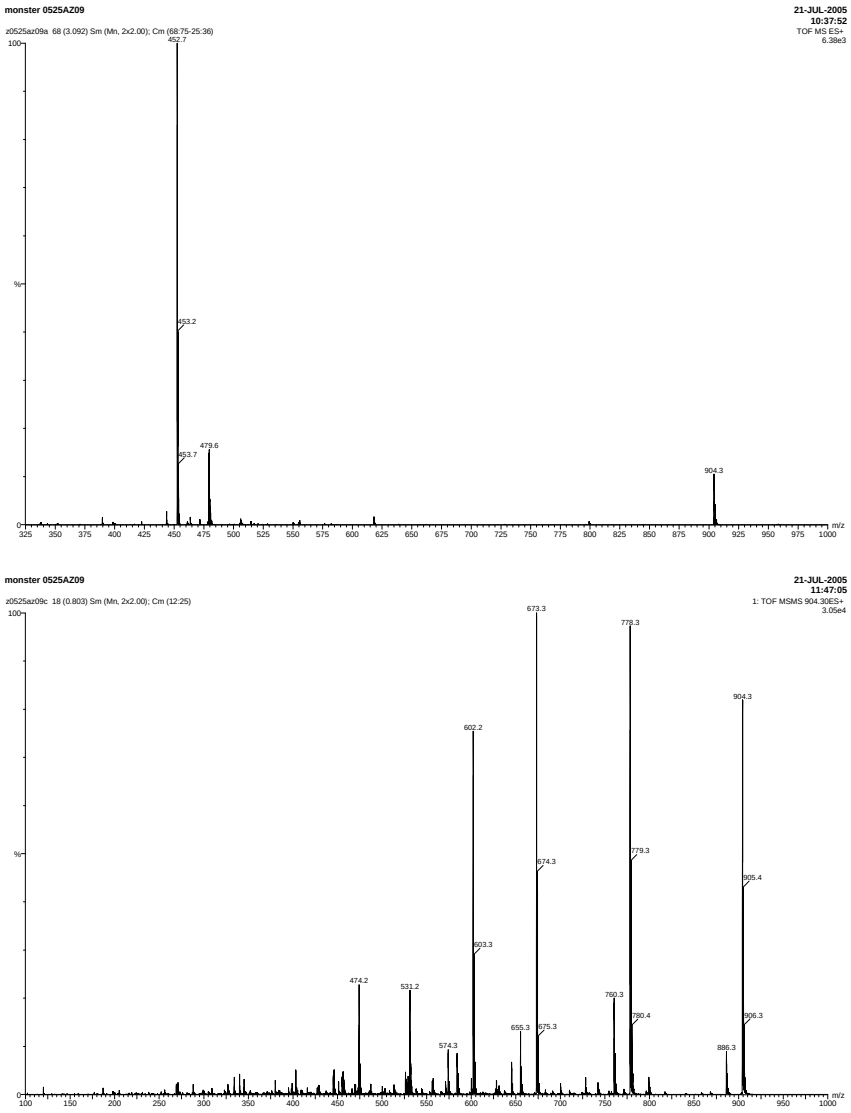


Figure A3.3. Mass spectra of synthetic FGES(O-ethylphospho)AGAAS, i.e., the presumed nonapeptide derived from tabun-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Lower trace: product ion spectrum of *m/z* 904.3 (MH^+).

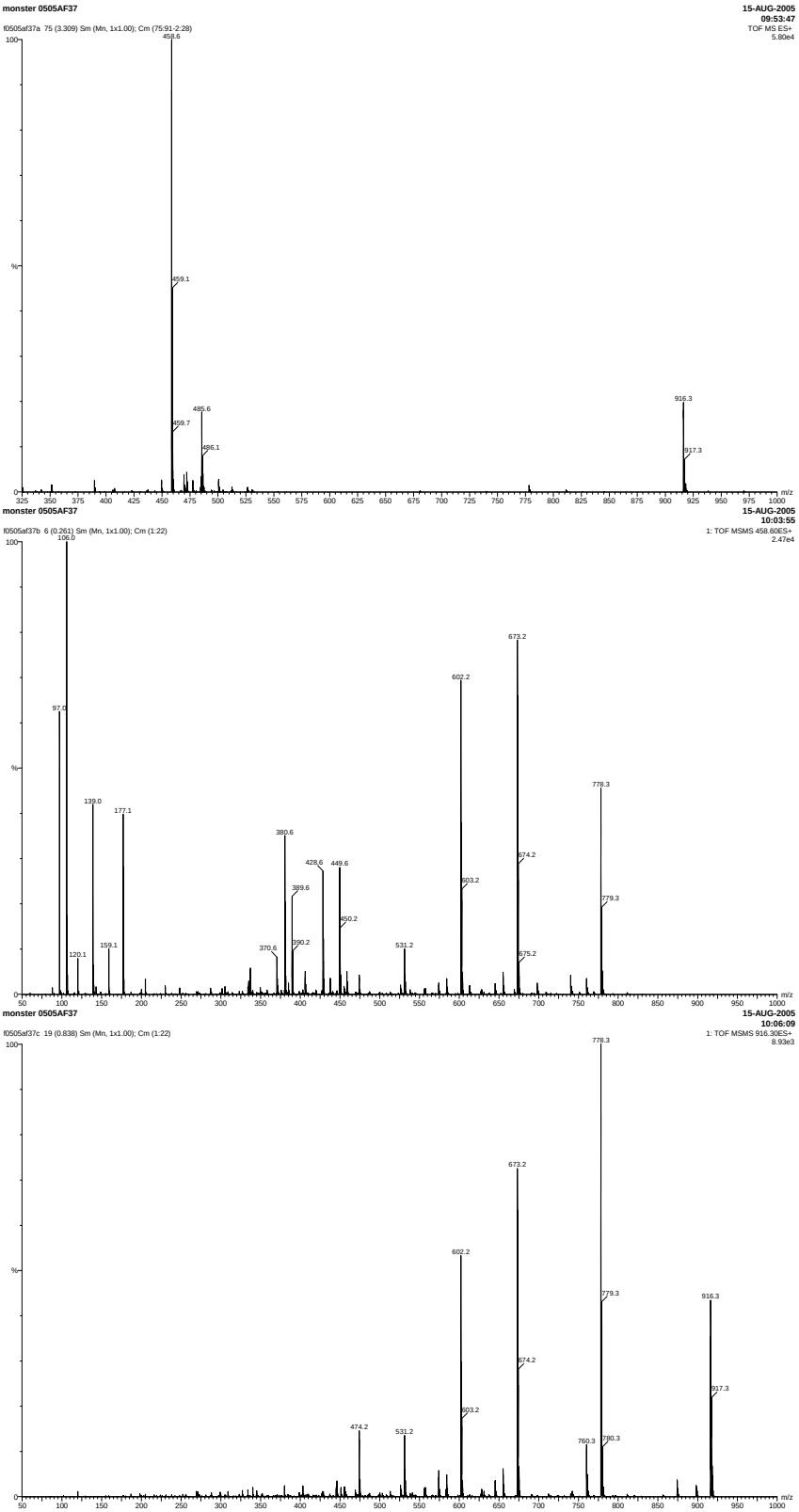


Figure A3.4. Mass spectra of synthetic FGES(IMPA)AGAAS, i.e., the nonapeptide derived from sarin-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Middle trace: product ion spectrum of *m/z* 458.6 (MH_2^{2+}). Lower trace: product ion spectrum of *m/z* 916.3 (MH^+).

V DISCUSSION

Method transfer

Several methods have been transferred to CDC during this project. In general, due to the availability of the reference compounds, the methods could relatively easily be set up. In general, it is recommended that the mass spectrometers should be fine-tuned a few weeks in advance with the synthetic reference standards.

During performance of the work, several relevant observations/recommendations were made:

Modified Edman degradation

- It seems that LC tandem MS analysis of the pentafluorophenylthiohydantoin obtained after modified Edman degradation of sulfur mustard-alkylated globin is not very sensitive. However, it should be recognized that only 10 mg of globin was used (samples were split after modified Edman degradation). Since the traces for the various MRM transitions are quite clean, it might be advantageous to use more globin material (e.g. 50 mg) in order to detect lower exposure levels.
- The samples prepared by modified Edman degradation at TNO could be analyzed satisfactorily; it turns out that the sensitivity of the TSQ7000 GC-MS instrument at CDC is higher than the sensitivity of the HP5973 MSD at TNO.
- Some of the samples prepared during the method transfer gave deviating results. A number of reasons can be given for this:
    - Instead of concentrating the samples by centrifugal evaporation, as described in the original procedure, the samples were concentrated by heating under a stream of nitrogen. This might result in a significant loss of analyte. It is recommended not to deviate from the standard procedure.
    - Working with relatively high level reference standards during sample preparation of low level exposed globin samples can cause severe problems.
- GC-NICI tandem MS might result in cleaner ion chromatograms and increased sensitivity.

Phosgene method

- It is recommended to use a similar column (PepMap C18, 15 cm x 1 mm; 3 mm) as used at TNO for future analyses.
- It is recommended to dissolve synthetic reference standard in a mixture of water/acetonitrile, containing HCOOH or TFA, and dilute further with water containing HCOOH or TFA
- In order to speed up the method: try to replace the dialysis step using dialyis cassettes by a washing step on molecular weight cut-off filters. According to the manufacturers instructions, the filters are compatible with 6 M guanidine buffers. In addition, it might be worthwhile to try to perform the tryptic digestion on the molecular weight cut-off filter itself.

Lewisite method

- Use 4 mL vials for collecting the Seppak C18 eluate; these vials should fit in a rotor for the Eppendorf Vacufuge

- Use an adjustable heat gun to concentrate the sample volume of 3 mL until an amount of approx 1 mL has been obtained, in order to avoid "bumping" during the concentration step in a vacuum concentrator.
- In order to facilitate the manipulations during extraction after introduction of the HFB group, it is recommended to add some extra toluene (0.5 ml) after performance of the derivatization reaction. After extraction and drying, the toluene layer may be concentrated by evaporation of the solvent.

Improvement of existing assays

Albumin – tripeptide method for analysis of sulfur mustard adducts

Reference compounds (synthetic tripeptide adduct, plasma samples) have been prepared and transferred to CDC. Furthermore, attention has been paid to the use of columns with immobilized enzymes. In case of immobilized pronase, no tripeptide could be obtained. We therefore explored the use of pepsin, which is commercially available in an immobilized form and which also afforded promising results in case of HuBuChE adducts. Pepsin digestion of sulfur mustard-modified albumin afforded some distinct peptide adducts; as was also the case with HuBuChE, the protein did not have to be pretreated by means of reduction/carboxymethylation. Unfortunately, we did not see the expected adducts upon use of immobilized pepsin, which might indicate that albumin is less susceptible to digestion of immobilized pepsin.

Assay for OP biomonitoring based on LC-tandem MS analysis of pepsin digests of HuBuChE

The pepsin digestion of HuBuChE is a powerful method for diagnosis of an exposure to an organophosphate. The isolation of HuBuChE from plasma has been further examined. We succeeded in a high-yield isolation of HuBuChE by using only 2 ml of procainamide gel; subsequent pepsin digestion of the crude protein material afforded a digest that could readily be analyzed for the presence of the phosphylated nonapeptide. Furthermore, a methodology for the preparation of various phosphylated nonapeptides, derived from HuBuChE inhibited with VX, sarin, soman and tabun, has been worked out; the peptides are now available as reference compound in milligram quantities. Both procainamide gel and reference peptides have been transferred to CDC. Furthermore, the possibility of an on-line system with a column containing immobilized pepsin in combination with a semi-automated LC-MS system, has been explored. The purpose of this on-line set-up is that it provides faster analysis and that it is less laborious, and it should result in better reproducibility and robustness. This allows higher throughput of samples, which could be favourable in case of large-scale incidents concerning nerve agent exposure. In case a purified OP-modified HuBuChE sample was run through this system, the desired phosphylated nonapeptide could be analyzed. At yet, the on-line system was not successful with HuBuChE samples that were isolated from plasma. Furthermore, in the current setting, the serum sample still needs an off-line isolation of HuBuChE, which is a low abundant protein in serum. However, the presented data are promising.

Development of novel assays

Novel assay for diagnosis of lewisite exposure

In close collaboration with CDC the basis for a straightforward, rapid method for diagnosis of exposure to lewisite was established; this method is based on the derivatization with 1,3-propanedithiol (PDT) of bound and unbound CVAA residues within human blood. Advantage

of this method is that further derivatization of the resulting CVAA-PDT complex is not necessary, and that the sample can easily be concentrated without sample loss. Furthermore, run times are short (< 10 min).

Generic assay for OP biomonitoring

We succeeded in developing a novel and generic assay for OP biomonitoring, that is based on pepsin digestion of HuBuChE, followed by base-catalyzed elimination of the phosphyl moiety and subsequent Michael addition of a suitable nucleophile. This results in one common modified nonapeptide, that can be analyzed by means of LC tandem MS. The availability of a phosphylated nonapeptide as model compound significantly helped in finding the most suitable nucleophile, *i.e.,* 2-(3-aminopropylamino)ethanol. It turned out that the phosphyl elimination/Michael addition reaction sequence gave the most reproducible results on the peptide level, which means that the pepsin digestion has to be carried out first. This strategy has the additional advantage that after splitting the sample after pepsin digestion, a two-step approach can be followed. First, the generic method can be used for an intitial screening of samples and after finding a positive sample, the pepsin digest can be analyzed in a more specific way as described in paragraph IV.6, in order to unravel the specific nature of the OP inhibitor.

Exploratory work on cyanide and phosphine

In case of cyanide, we obtained evidence for the formation of covalent adducts with albumin and hemoglobin. Distinct radioactive peaks resulted after trypsin digestion of albumin or globin, isolated from human blood that had been exposed to radioactively labeled (^{14}C) KCN. Unfortunately, we were unable to unravel the identity of peptide adducts, although we had obtained a number of promising clues on the mechanism of adduct formation by using a peptide model compound (oxytocine).

In case of phosphine, no evidence could be obtained of any chemical reaction with a model peptide (oxytocine), although it is envisaged that phosphine is a reactive compound.

VI KEY RESEARCH ACCOMPLISHMENTS

1. Reference materials for the modified Edman degradation of sulfur mustard-modified hemoglobin have been prepared and shipped to CDC.
2. The method for modified Edman degradation of sulfur mustard-modified hemoglobin has been transferred to CDC.
3. Reference materials for the method for diagnosis of exposure to Lewisite have been prepared and shipped to CDC.
4. The method for diagnosis of exposure to Lewisite has been transferred to CDC.
5. An alternative method for diagnosis of exposure to Lewisite, based on CVAA derivatization with 1,3-propanedithiol, has been developed in close collaboration with CDC.
6. The method for diagnosis of exposure to phosgene has been optimized and transferred to CDC.
7. Reference materials for the method for diagnosis of exposure to phosgene have been prepared and shipped to CDC.
8. The albumin tripeptide assay has been further improved.
9. Reference materials for the albumin tripeptide assay have been synthesized and shipped to CDC.
10. The nonapeptide assay for determination of exposure to nerve agents, based on pepsin digestion of HuBuChE, has been optimized.
11. On-line pepsin digestion of phosphylated HuBuChE, resulting in the phosphylated nonapeptide, has been accomplished; this can form the basis for an automated analytical system.
12. A methodology for the solid phase synthesis of phosphylated nonapeptides has been developed.
13. Various reference standards for the nonapeptide assay for determination of exposure to nerve agents have been prepared and transferred to CDC.
14. A generic method for detection of covalently modified HuBuChE has been developed.

VII REPORTED OUTCOMES (from beginning of cooperative agreement to end, including modification)

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Presentations

Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins". Presented by D. Noort at "8th European ISSX Meeting", Dijon, France. April 27 – May 1, 2003.

Methods for biomonitoring of individuals (involved by destruction of CW's) who may be contaminated with low concentrations of sulfur mustard, lewisite, phosgene and OP's. Presented by G.P. Van der Schans at meeting of CEPA IEX 13.11, Destruction of old chemical munitions, April 2004, Civitavecchia, Italy.

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VIII CONCLUSIONS

1. Several methods for diagnosis of exposure to scheduled compounds could be successfully transferred to CDC.
2. The method for modified Edman degradation of sulfur mustard modified hemoglobin can also be performed by using LC tandem MS; however, in that case the method is less sensitive.
3. The first attempts for semi-automation, i.e., by on-line digestion of modified proteins, of assays for diagnosis of exposure to various chemical agents proved to be successful.
4. The nonapeptide assay for diagnosis of exposure to cholinesterase inhibitors has been converted into a less laborious method.
5. An alternative method for diagnosis of exposure to Lewisite, based on CVAA extraction/derivatization with 1,3-propanedithiol, has been developed. This method is less laborious than the method based on extraction/derivatization with British Anti-Lewisite (BAL).
6. The developed generic assay for diagnosis of exposure to cholinesterase inhibitors is a powerful method that enables the detection of covalently modified HuBuChE.
7. The developed methodology for synthesis of phosphylated nonapeptides derived from HuBuChE enables the milligram-scale production of specific reference standards.

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XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

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Dr. M.J. Van der Schans
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Dr. F.J. Bikker
Mr. A. Fidder
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