Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts

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Abstract

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. Furthermore, in the case of a terrorist attack with CWA, rapid and reliable diagnosis of the exposure is essential. The present research aims at development of a mass spectrometric method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin.

The albumin assay 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 procedure for isolation of albumin from human blood could be substantially shortened by using affinity chromatography. The entire procedure, i.e., albumin isolation, pronase digestion and mass spectrometric analysis can be performed within 3 h.

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 – modified rat albumin was 2 days, which is in good correspondence with literature values for native rat albumin. 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 after the exposure.

A tentative SOP has been drafted, which has been demonstrated to U.S. Army Medical Research Institute of Chemical Defense in a satisfactory way.

The presented method has the potential to become a generic assay for diagnosis of exposure to a wide array of alkylating agents, which is highly relevant within the field of operational toxicology.

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Key words: adduct, diagnosis, low level exposure, mass spectrometry, sulfur mustard

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Introduction

Within the framework of previous cooperative agreements (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 (*1*). 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 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 (*2*) 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 cooperative agreement DAMD17-97-2-7002 we have drafted inter alia a standard operating procedure (SOP) for GC-NCI/MS determination of the sulfur mustard adduct to N-terminal valine in human hemoglobin (*3, 4*). With this method an in vitro exposure of 0.1 µM sulfur mustard could be detected. 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 (*5*), 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 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).

Furthermore, we found that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of the tripeptide (S-HETE)-Cys-Pro-Phe (see *Figure 1*), 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 (*6*). Using only 3 mg of albumin, we were able to detect *in vitro* exposure of human blood to 10 nM of sulfur mustard by applying this method. Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard. As part of Cooperative Agreement DAMD 17-02-2-0012, we here report on the further development of the albumin method into an SOP (*7*).

Experimental

Materials and methods

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). A number of LC/electrospray tandem MS analyses 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 V, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C.

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).

Tentative SOP for albumin – tripeptide assay

The plasma samples (1 mL) of interest were spiked with plasma (50 μL), isolated from blood exposed to 100 μM d_8-sulfur mustard. The sample (1 mL) was applied on a HiTrap™ Blue HP (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) affinity column that was incorporated into an FPLC system, after conditioning with buffer A (50 mM KH_2PO_4, pH 7; 10 mL). The column was eluted with buffer A (7 mL; 1 mL/min). A large peak was visible at 280 nm, corresponding with material having no affinity to the column material. Subsequently, the column was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 7 mL; 1 mL/min). UV positive (280 nm) material was collected (total volume 2.5 mL). The HiTrap column was regenerated by washing with buffer A (14 mL).

Subsequently, a PD-10 column (containing 10 mL 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 (2.5 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL). Next, the column was further eluted with aqueous NH_4HCO_3 (50 mM; 2.5 mL) and the eluate was collected.

Part of the purified albumin fraction (0.25 mL) was diluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL) 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.

Isolation of albumin can also be performed using a disposable syringe. In that case, plasma (0.5 mL) is diluted with 2 mL of buffer A and filtrated over a 0.45 micron acrodisc. This sample is applied to a pre-conditioned HiTrap™ Blue HP column. The applied sample is washed with 10 mL of A buffer and eluted with 3 mL of high salt buffer (buffer A containing 1.5 M KCl). This eluate is desalted (conditioned with 25 mL of NH_4HCO_3), by applying the entire sample on a PD 10 column followed by elution with 3 mL of a 50 mM NH_4HCO_3 solution. The digestion is carried out using 750 μL of the desalted solution with 100 μL of pronase (10 mg/mL), followed by a 10 kD filtration step after 2 h at 37ºC.

The filtrate was directly analyzed by means of LC/MS/MS or, in case of exposure levels < 0.1 μM, further processed by Sep-Pak C18 clean-up. In the latter case, a Sep-Pak C18 cartridge 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 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, redissolved in H_2O (50 µL) and analyzed.

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).

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.

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 HiTrap™ Blue HP 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).

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 for the LC-system were as described above for (S-HETE)Cys-Pro-Phe.
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 and MH^+ 494.2 → 113, respectively. Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

Results
In the original procedure, we isolated albumin by means of precipitation, which is rather time-consuming and precludes automation of the methodology. A literature search revealed that albumin can be removed from serum by affinity chromatography, which will facilitate the analysis of less abundant serum proteins (*8*). 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 HiTrap™ Blue HP columns. The affinity is based on specific interactions of the column material with amino acid residues in albumin. In a representative experiment, 1 mL of serum or plasma (containing a theoretical amount of 40 mg of albumin) was applied to a 1 mL HiTrap™ Blue HP column. Plasma constituents with no affinity to the column material rapidly eluted from the column, as evidenced by the UV pattern. Albumin is eluted from the column by applying a high salt buffer. This procedure takes 10 min. Subsequently, the fraction containing albumin (checked by UV) is desalted by means of gel filtration on a PD 10 column, which takes an additional 10 min. LC tandem MS analysis of pronase digests of the albumin fraction thus obtained gave similar results as found earlier by using the old procedure. The recovery of the isolation procedure was 50-60%. In case of exposure levels < 0.1 µM, a Sep-Pak C18 clean-up step of the digest is added, resulting in the detection of levels of approximately 10 nM. Furthermore, when larger batches of albumin (20 mg) are processed, levels of 1 nM can be determined.

The method could be set up within one day, and has been successfully demonstrated to a scientist of USA MRICD, in one of the laboratories of Centers for Disease Control & Prevention (CDC), Atlanta, GA, indicating the robustness of the assay.

In order to obtain information about the persistence of the albumin – sulfur mustard adduct, laboratory animals were exposed to the agent. Preliminary experiments, in which rat blood had been exposed *in vitro* to sulfur mustard, showed that the major product was (S-HETE)Cys-Pro-Tyr (see *Figure 2* 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. 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 HiTrap™ Blue HP columns, according to the tentative SOP. After treatment with Pronase, 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 3*. 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 *Figure 4*.

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 (*4*). 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, the samples were analyzed by means of GC-MS (negative ion chemical ionization) with single ion monitoring for the presence of d_0- and d_8-pentafluorophenylthiohydantoin. The results clearly demonstrate that the adduct level increases during the first days after the exposure (see *Figure 5*). 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.

Discussion

We succeeded in significantly shortening the procedure for albumin isolation and subsequent pronase digestion. We found that a commercially available affinity column for albumin could be advantageously used in the tentative standard operating procedure. 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. In future work, we plan to use columns with immobilized pronase. This 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.

The use of an internal standard, *i.e.*, a plasma sample isolated from blood that had been exposed to sulfur mustard-d_8, has also been worked out in combination with the affinity chromatography procedure. 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 (*9*) and to acrylamide (*10*). Apparently, the cysteine-34 residue in human serum albumin is highly prone towards alkylation by a wide array of electrophilic compounds. It is therefore envisaged that the presented method has the potential to become a generic assay for diagnosis of exposure to alkylating agents, which is highly relevant within the field of operational toxicology.

In preliminary *in vitro* experiments with [^{14}C]-labelled sulfur mustard we had found that the free cysteine-34 residue in rat albumin is also prone to alkylation by sulfur mustard. LC-tandem MS analysis showed 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 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 in the rat (1-3 days; see for instance Troester *et al.* (*11*). The albumin adduct was no longer detectable after 7 days after the exposure. It should be pointed out that the half-life time of albumin in humans is much longer (approximately 16 days).

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. This phenomenon was also observed in the previous Cooperative Agreement (DAMD17-97-2-7002), in which a marmoset was exposed to sulfur mustard (*1*). However, this pilot experiment was performed with only one animal. This finding justifies the research for novel scavengers. Interestingly, Langenberg *et al*. (*12*) 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 has also been observed by Maisonneuve *et al*. (*13*), 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. 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 (*14*)). The adduct to the N-terminal valine could still be detected after 28 days (end of experiment).

Conclusions

1. The alkylated cysteine-34 residue in human serum albumin is a highly sensitive biomarker for exposure to sulfur mustard and to related alkylating compounds.
2. A rapid isolation procedure for albumin, based on affinity chromatography, has been implemented in the method for albumin adduct determination.
3. The use of an internal standard has been optimized.
4. The standard operating procedure for determination the sulfur mustard alkylated cysteine-34 residue in albumin could be successfully demonstrated to USAMRICD.
5. 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.
6. 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.
7. The level of N-terminal valine adduct increases during the first 2-3 days after the exposure, indicating the presence of intact sulfur mustard during that period.
8. The presented method has the potential to become a generic assay for diagnosis of exposure to a wide array of alkylating agents, which is highly relevant within the field of operational toxicology.

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Figures

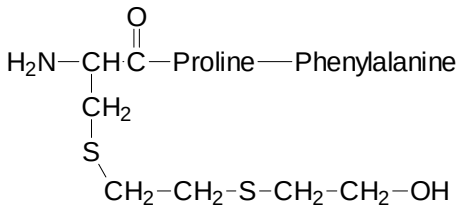

Figure 1. Chemical structure of tripeptide – sulfur mustard adduct, (S-HETE)Cys-Pro-Phe, obtained after pronase digestion of albumin alkylated by sulfur mustard.

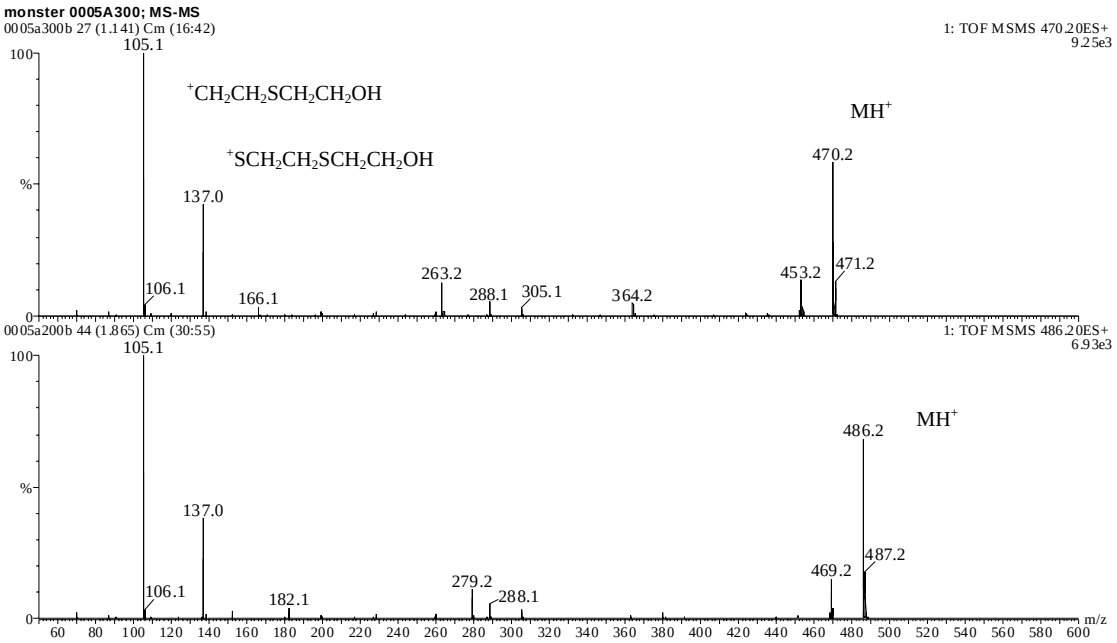

Figure 2. Comparison of tandem MS spectra (recorded on Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE)Cys-Pro-Tyr (lower panel), demonstrating the intense fragment ion with *m/z* 105 in both cases.

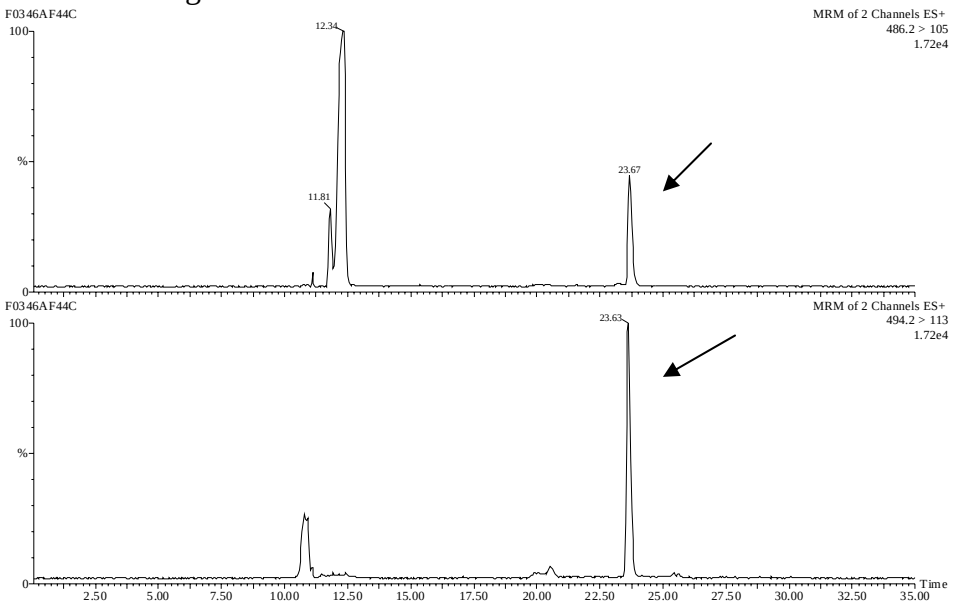

Figure 3. LC-tandem MS analysis of (S-HETE)Cys-Pro-Tyr (arrow) in a pronase digest of albumin, using the multiple reaction monitoring scanning mode for the transition m/z 486 (MH^+) → 105. Albumin was isolated from plasma taken from a rat, 1 h after exposure to sulfur mustard (0.3 mg/kg). The sample 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).

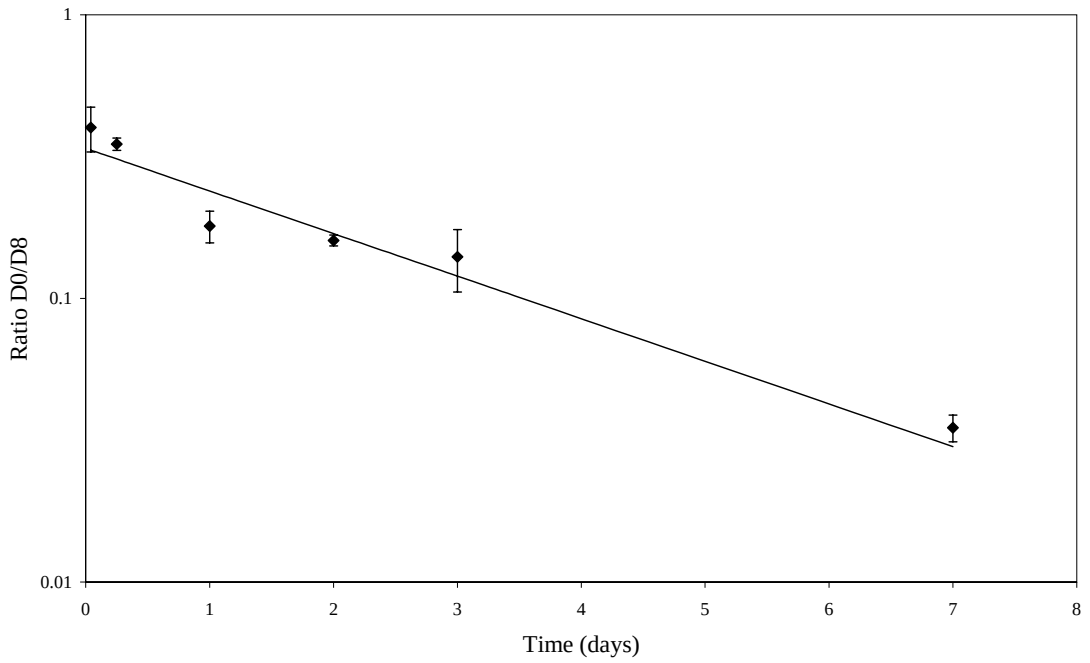

Figure 4. Persistence of alkylated cysteine in albumin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, albumin was isolated by affinity chromatography and analyzed by using the tentative SOP for determination of (S-HETE)Cys-Pro-Tyr. Plasma from rat blood exposed to sulfur mustard-d_8 (100 µM) was used as an internal standard.

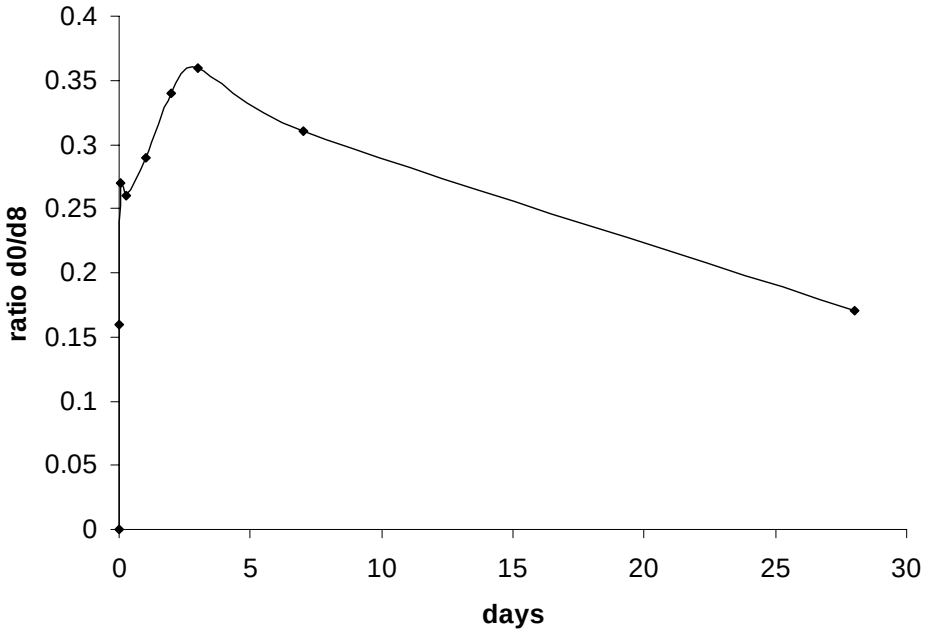

Figure 5. Persistence of alkylated N-terminal valine in hemoglobin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, globin was isolated and analyzed by using the SOP for determination of alkylated N-terminal valine. Globin from rat blood exposed to sulfur mustard-d_8 (100 µM) was used as an internal standard.

Low level exposure to sulfur mustard: Development of an SOP for analysis of albumin adducts

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Why analysis of bio-medical samples

- **Diagnosis in case of casualties**
- **Low level exposure (Gulf War Syndrome)**
- **Biomonitoring (health surveillance)**
- **Verification (forensic)**
- ***Also: confirmation of non-exposure!***

Highly relevant as a "Medical Chemical Defense Product for the Warfighter and Homeland Defense"!

(Noort et al., Toxicol. Appl. Pharmacol. 184, 116-126 (2002))

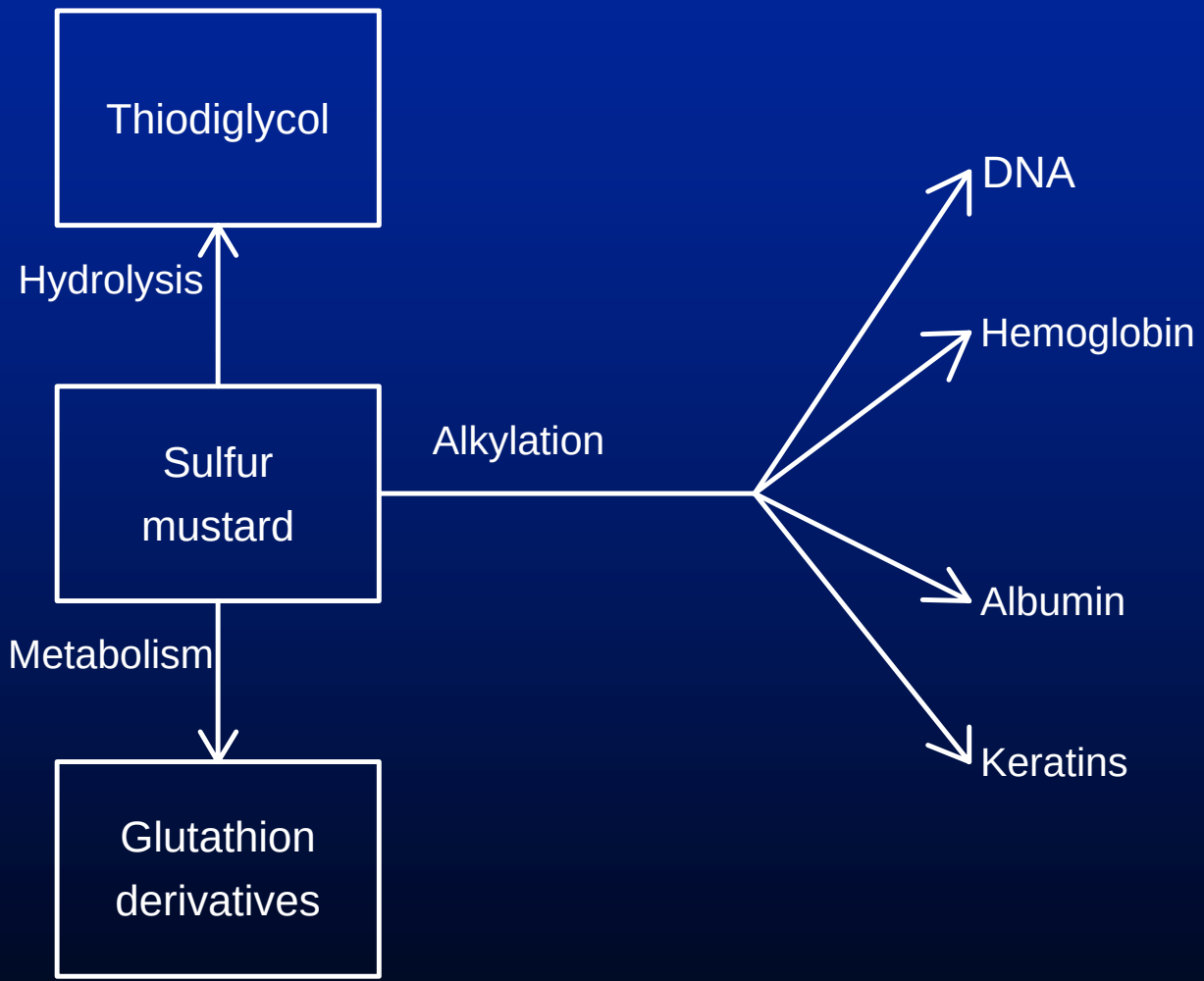
Approach:

Retrospectivity

- Intact agent
- Metabolites
- Adducts with DNA
- Adducts with proteins

- Mass spectrometry
- Immunoassay

What happens after sulfur mustard exposure?



Albumin

- abundant protein in plasma (40 mg/ml)
- reactive amino acids, e.g., Cysteine-34 residue
- not enclosed by cell membrane
- long half-life time (approx. 16 days in humans)

Earlier work

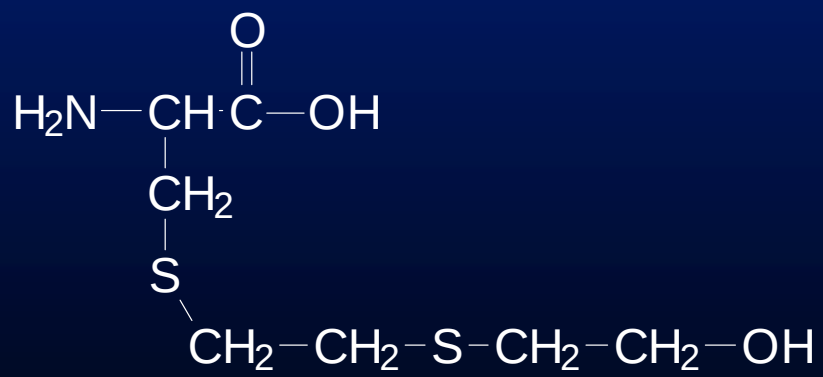
using ^{14}C-labelled sulfur mustard:

(*Fidder et al. Chem. Res. Toxicol. 12, 715-721, 1999)*

Albumin extensively alkylated by sulfur mustard

- Trypsin digestion: random alkylation
- 5% of bound radioactivity bound to Cys-34 residue

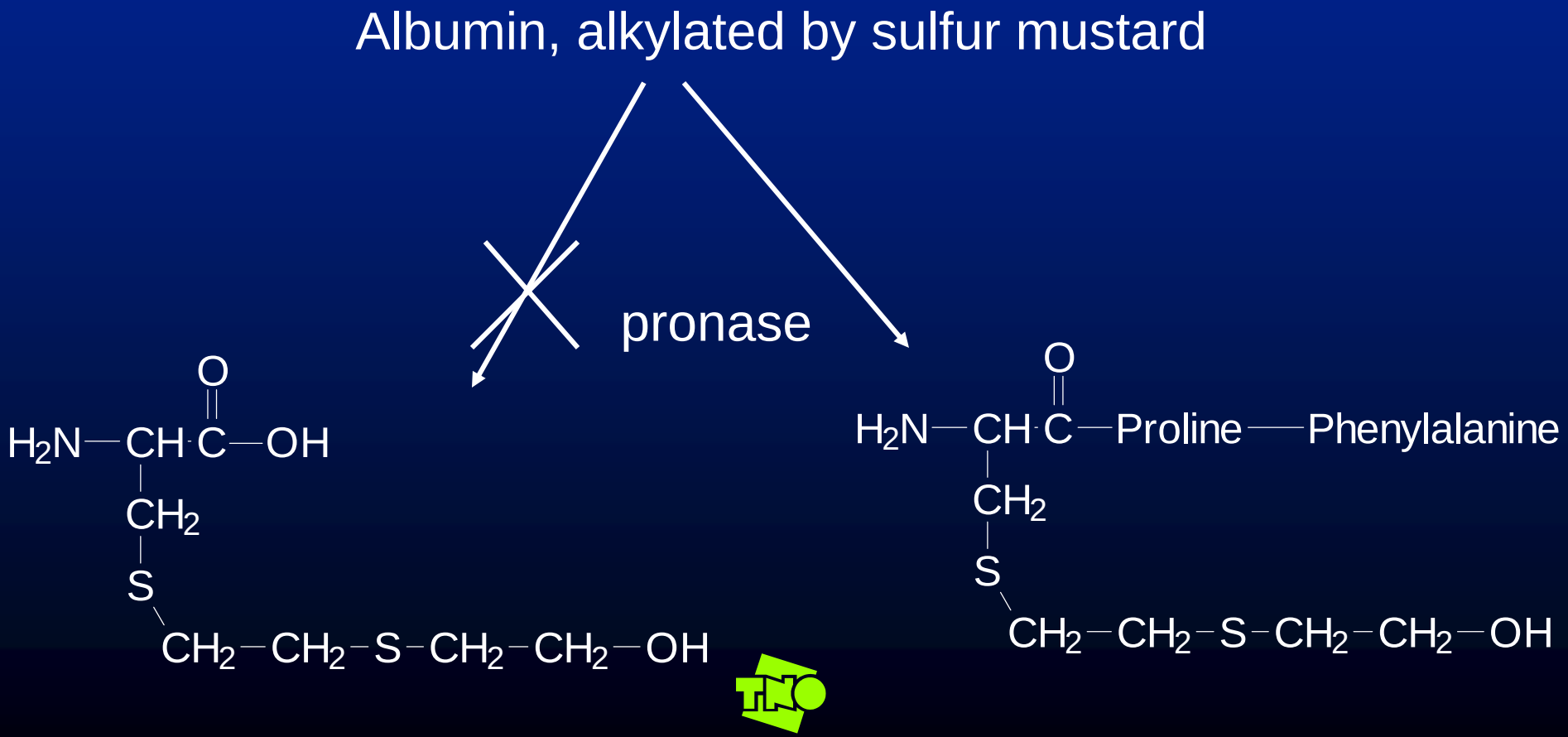
Chemical structure of cysteine adduct:



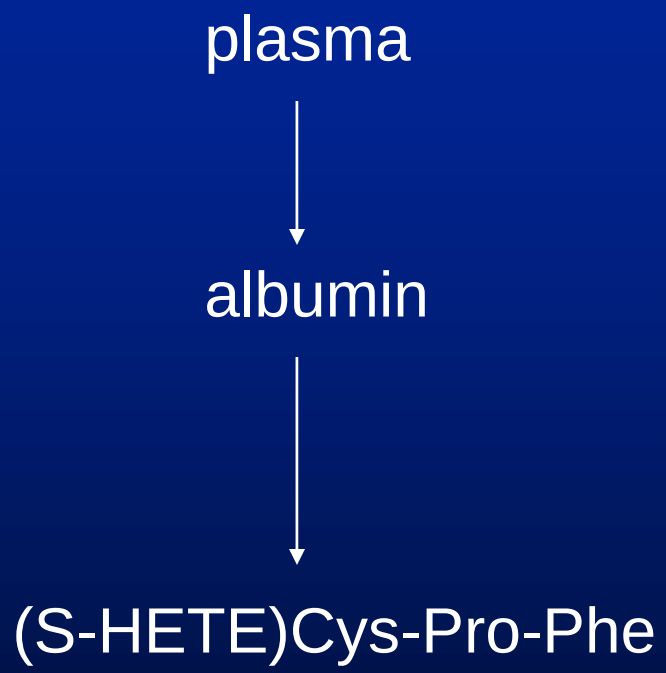
(S-2-hydroxyethylthioethyl)-cysteine
(S-HETE)cysteine

Serendipity

…..while searching for a method for mild hydrolysis of albumin in order to obtain the cysteine-sulfur mustard adduct for GC-MS analysis



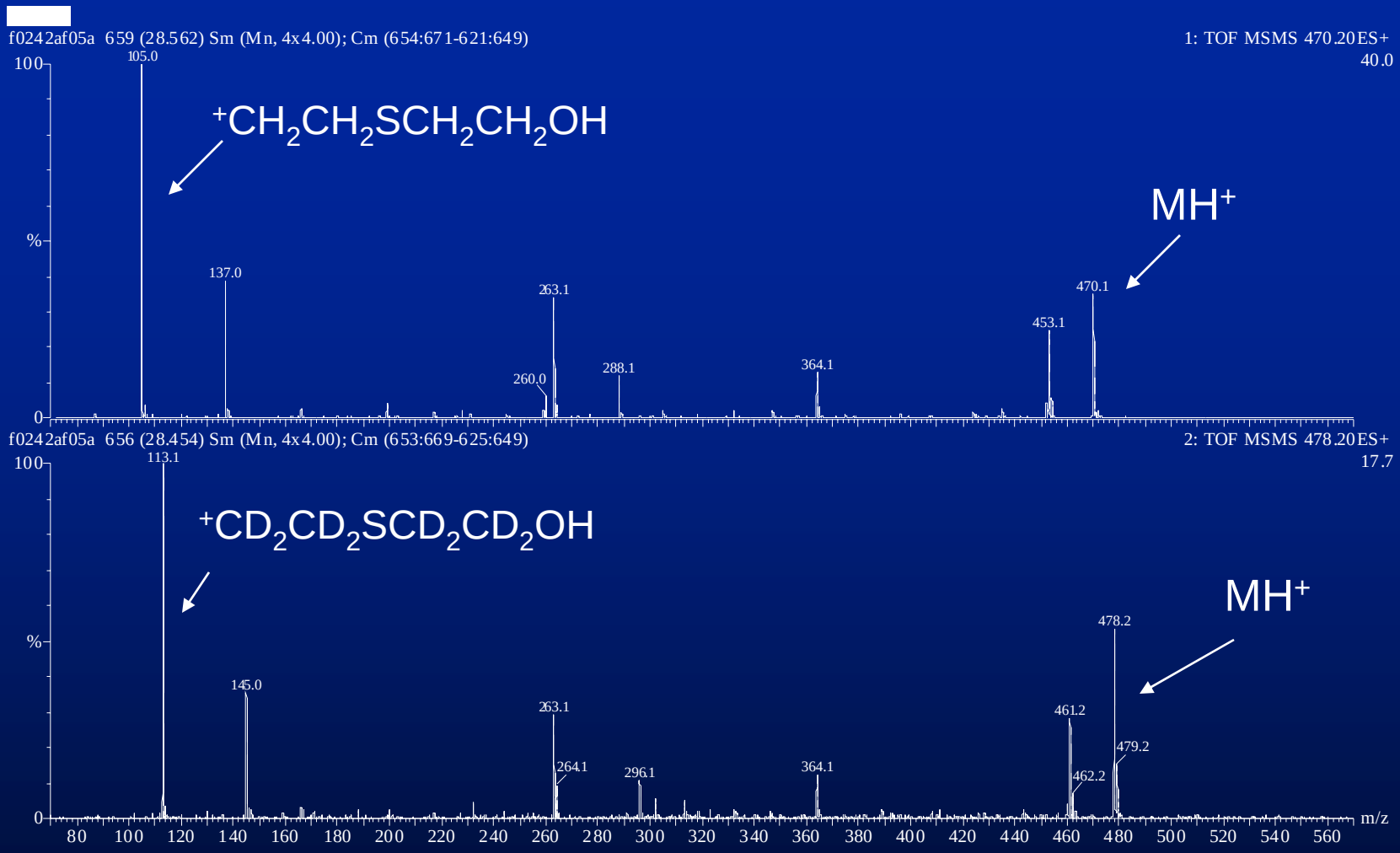
Standard Operating Procedure



Steps

- Addition of internal standard (plasma from blood exposed to *d8*-sulfur mustard)
- Affinity chromatography (0.25 h), desalting (0.25 h)
- Pronase digestion (2 h)
- Filtration (0.5 h)
- Purification by Sep-Pak C18 (1 h)
- LC-tandem MS analysis (1 h)

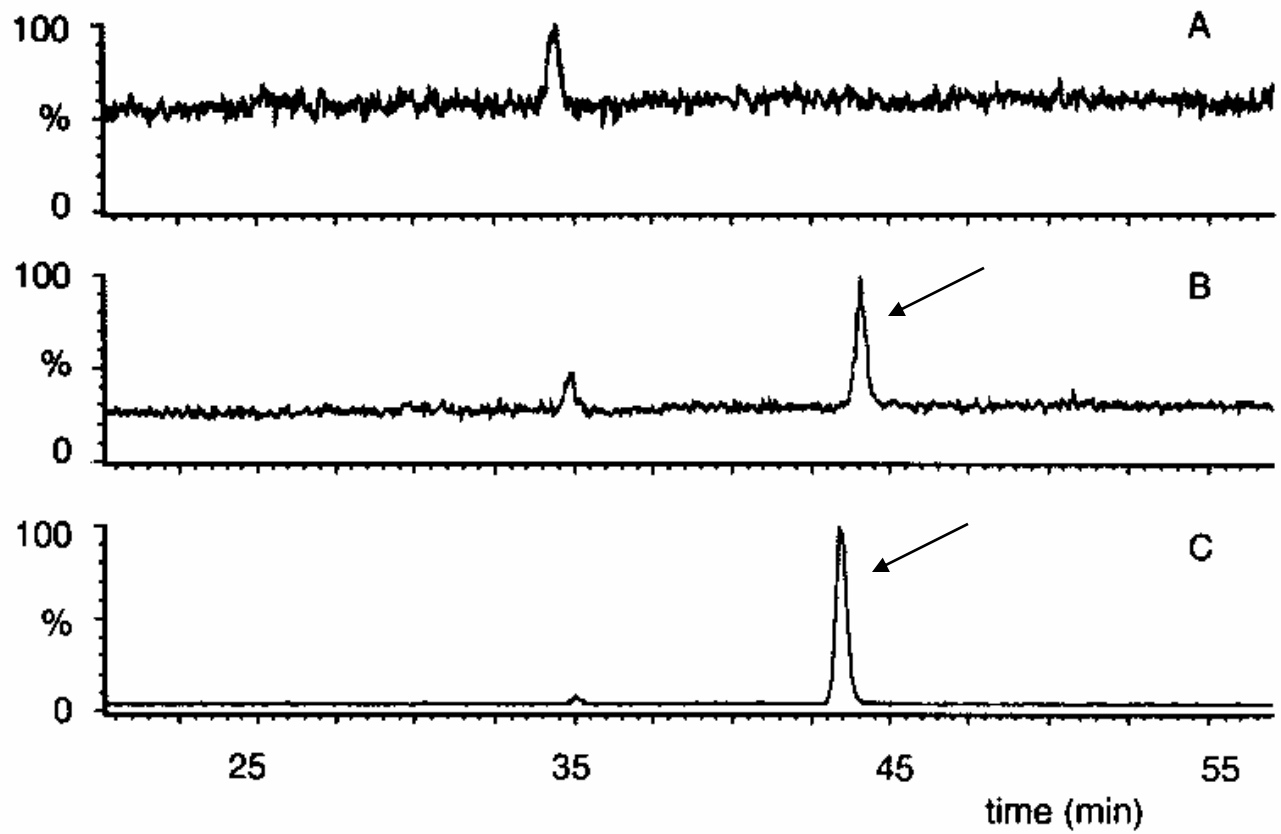
(See also: Noort et al., J. Anal. Toxicol., in press)



Tandem MS spectra of (S-HETE)Cys-Pro-Phe (upper panel) and (S-*d8*-HETE)Cys-Pro-Phe

Typical LC-MS/MS runs of (S-HETE)-Cys-Pro-Phe in a pronase digest of albumin

m/z 470 (MH^+) -> 105



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10 nM HD

100 nM HD

Lowest detectable levels, in human blood *in vitro*

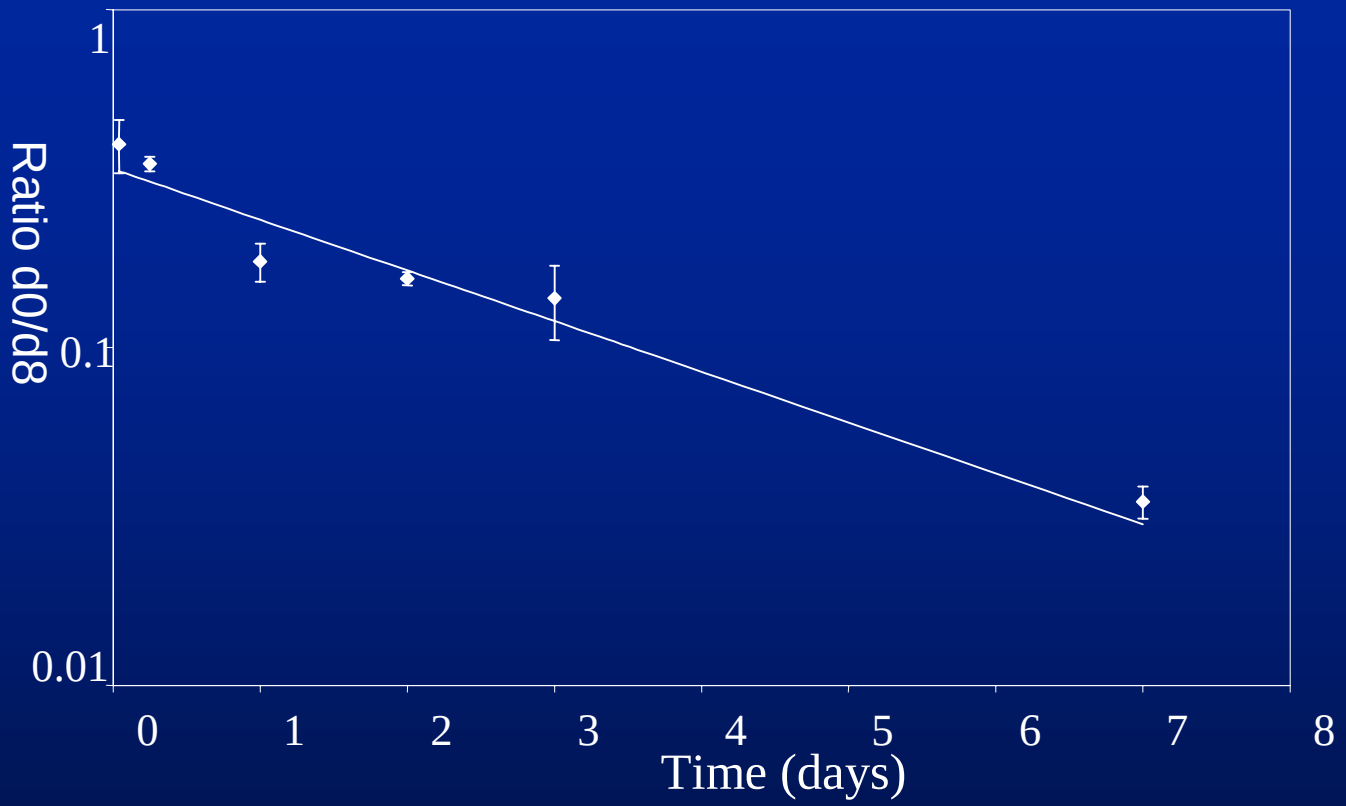
- **Without Sep-pak clean-up:** **100 nM**
- **With Sep-pak clean-up:** **10 nM**
- **Using 20 mg albumin, with Sep-pak clean-up** **1 nM**

SOP was demonstrated to U.S. Army

- Method was demonstrated to J.R. Smith of USAMRICD
- Demonstration took place at CDC (laboratory of Dr. J. Barr), using an identical mass spectrometer as present at USAMRICD
- Method could be set up within one working day, giving similar results as at TNO-PML

Animal experiments

- In case of rat albumin: (S-HETE)Cys-Pro-Tyr results
- Rats were exposed (i.v.) to 0.3 mg/kg
- Blood samples drawn at various time points
- Plasma samples were processed according to the SOP
- Digests analyzed by means of LC-tandem MS
- Adduct levels were quantified by means of internal standard

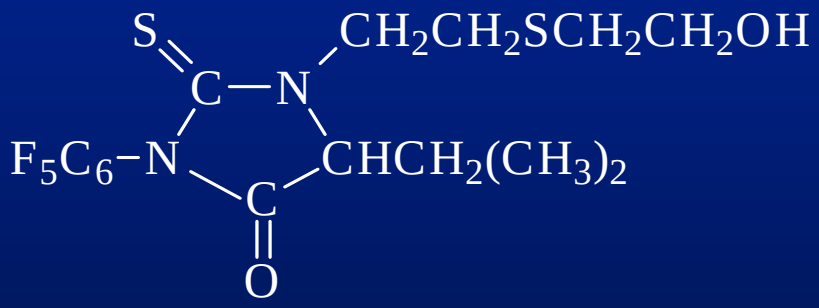


Persistence of alkylated cysteine in albumin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.), by analysis of (S-HETE)Cys-Pro-Tyr.

Plasma from rat blood exposed to sulfur mustard-*d8* (100 µM) was used as an internal standard.

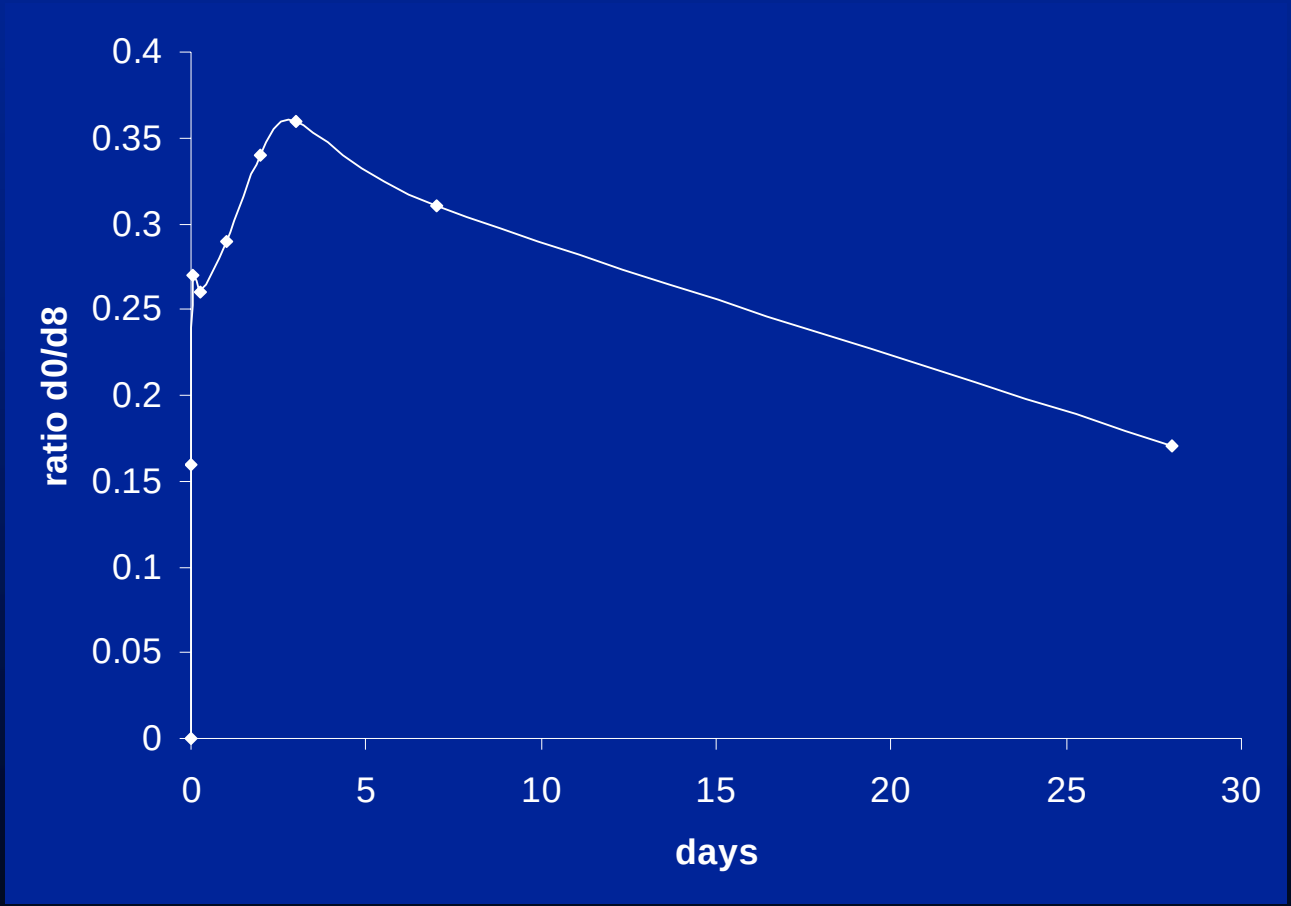
Hemoglobin adducts were also determined:

- **Modified Edman degradation of globin**
  - Pentafluorophenylisothiocyanate
- **Derivatization with HFBA**
  - Heptafluorobutyrylimidazole
- **Analysis with GC-MS**
- **Detection limit 100 nM SM in blood**

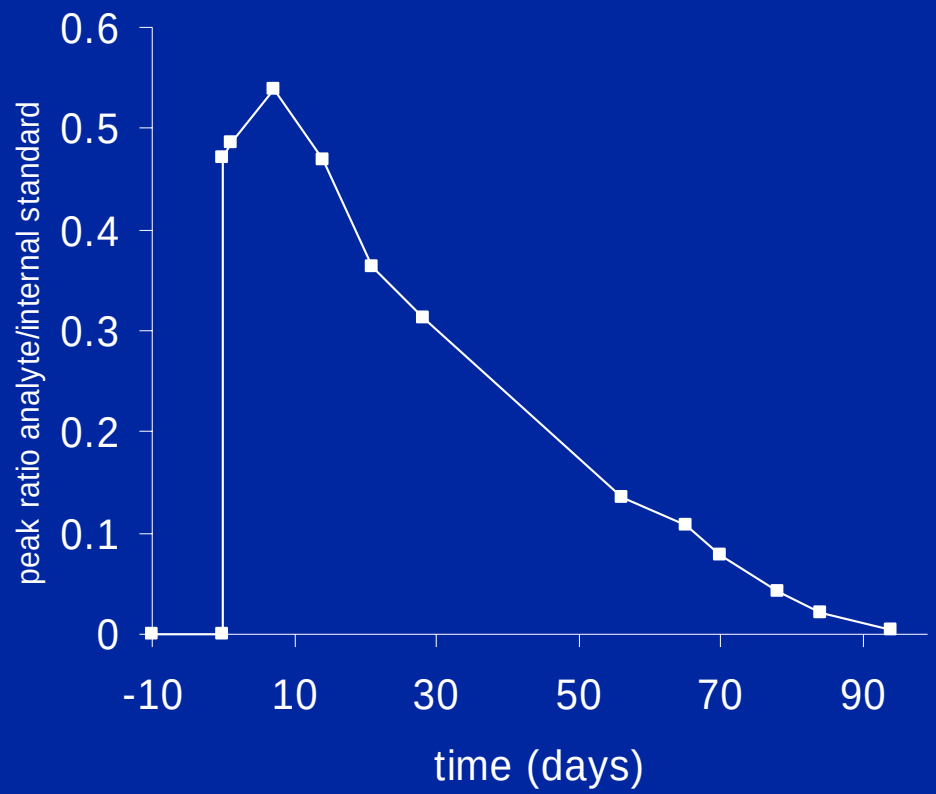


(see: Noort et al., J. Anal. Toxicol., in press)

Corresponding N-terminal valine adducts in rat hemoglobin



For comparison:
Persistence of alkylated N-terminal valine in hemoglobin of a marmoset after SM administration (4.1 mg/kg, i.v.)

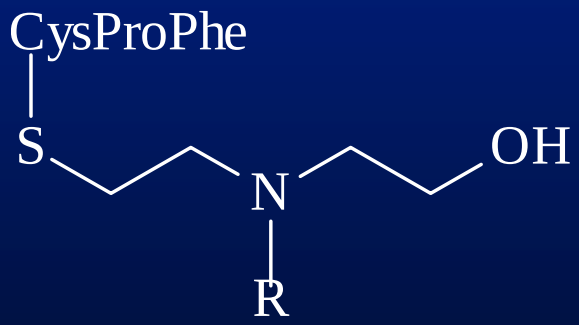


Spin-off: nitrogen mustards, e.g., melphalan *in vivo*

Melphalan:

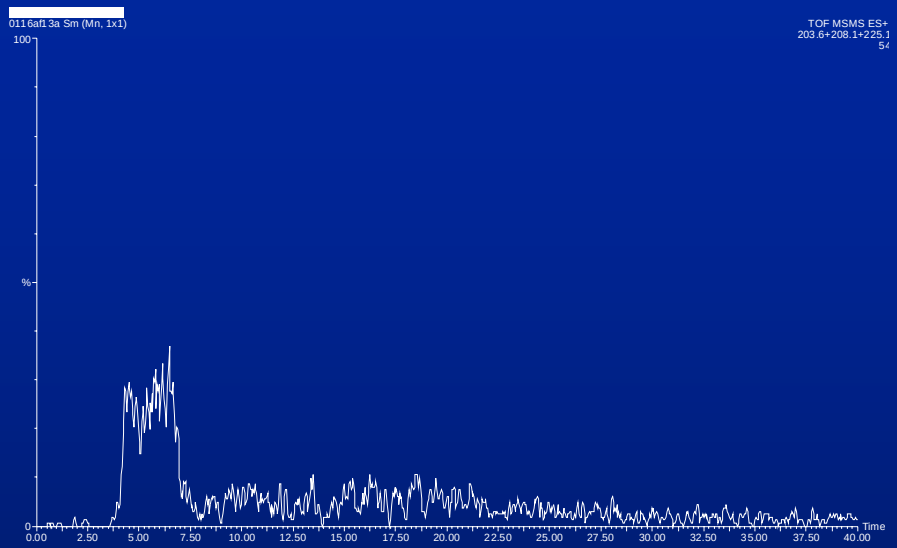
with R = 4-phenyl-alanine

Expected adduct



(See: Noort et al., Arch. Toxicol. 76, 83-88, 2002)

In vivo:



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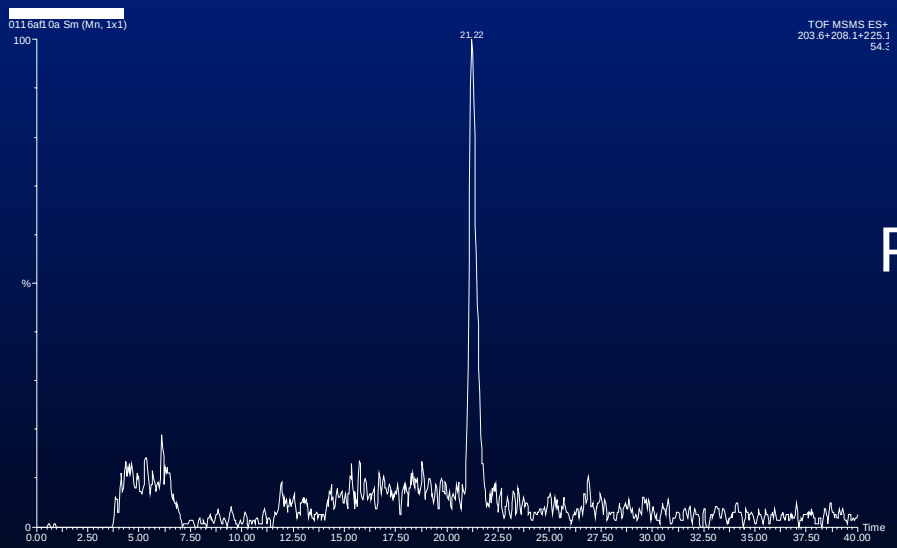

Patient 1; 135 mg melphalan I.v.

Generic assay for alkylating agents

Biomonitoring of exposure to a wide array of carcinogens and alkylating agents covered in one assay!

e.g., for application within operational toxicology

- **Acrylamide (see Noort et al. Arch. Toxicol. 77, 543-545, 2003)**
- **diesel exhaust (benzene, polycyclic aromatic hydrocarbons), to be developed**

Conclusions

- The alkylated cysteine-34 residue in human serum albumin is a highly sensitive biomarker for exposure to sulfur mustard.
- A rapid isolation procedure for albumin, based on affinity chromatography, has been implemented in the method for albumin adduct determination.
- The standard operating procedure for determination the sulfur mustard alkylated cysteine-34 residue in albumin could be successfully demonstrated to USAMRICD.

Conclusions (*continued*)

- 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.
- 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.
- The level of N-terminal valine adduct increases during the first 2-3 days after the exposure, indicating the presence of intact sulfur mustard during that period.
- It is envisaged that the presented method has the potential to become a generic assay for biomonitoring exposure to a wide array of alkylating compounds, which is highly relevant within the field of operational toxicology.

Acknowledgements

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