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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SULFUR MUSTARD REACTION WITH AMINO ACIDS AND PROTEINS

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ABSTRACT

The interaction of sulfur mustard with aminoacids and proteins has been investigated in this study. Rats were injected with sublethal doses of sulfur mustard subcutaneously and intraperitoneally. At different time intervals, plasma and urine samples were collected. The binding affinity of sulfur mustard with urinary and plasma proteins and enzymes was studied for the first time using non-ideal size exclusion high-performance liquid chromatography (HPLC). We observed that sulfur mustard reacts with proteins and enzymes containing cystein, arginine, and lysine residues.

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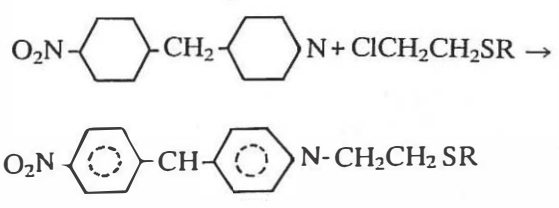
INTRODUCTION

Sulfur mustard or 2, 2-dichlorodiethyl sulfide is a well known alkylating agent and has been used as a vesicant chemical warfare agent.[1,2] Under physiological conditions, sulfur mustard can react with the amino (-NH2), thio (-SH) and carboxyl (-COOH) groups of aminoacids and proteins.[3,5]

Several methods have been developed for the detection of sulfur mustard and its metabolites. For example, in the presence of 4-P-nitrobenzyl-pyridine and under alkaline condition, a blue complex is formed as shown in reaction I.[6]



(I)

Another method for the detection of sulfur mustard is based on the *in vivo* incorporation of 35S in aminoacids and proteins.[7] Thin-layer chromatography (TLC) has also been used by Samuel and Appler for the detection of sulfur mustard and its metabolites.[8,9] Recently, Wils and his co-workers have developed a

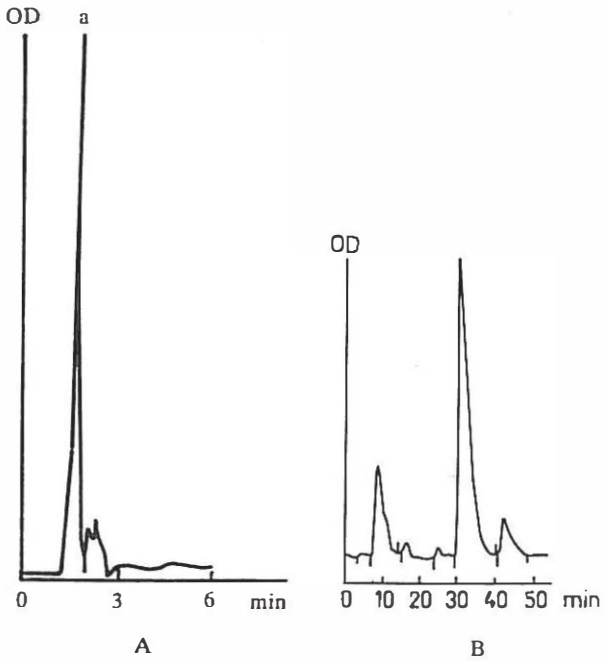


Fig. 1. Typical chromatograms of a rat urine control sample (A) and of rat urine samples obtained at 9 h intervals after injection of the rat with sulfur mustard. Column: ODS, Eluent: acetonitrile and water (30:70 V/V) flow-rate: 0.5 ml/min. Peaks: a=chloramine-T, b=thiodiglycol and c=sulfur mustard.

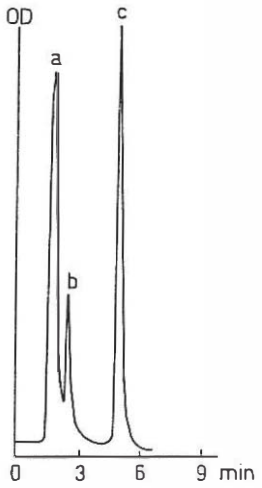


Fig. 2. Typical chromatogram of rat plasma samples obtained at 24 h intervals after injection of the rat with sulfur mustard. Column: TSK G 2000 SW, Eluent: acetonitrile and water (30:70 V/V) flow-rate = 0.5 ml/min.

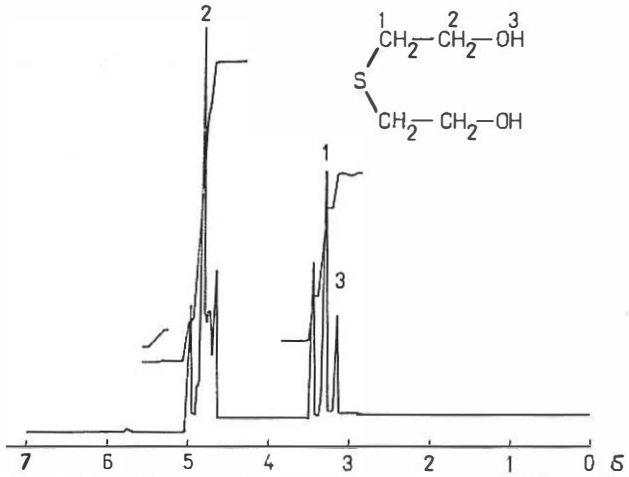


Fig. 3. Typical NMR spectra of plasma sample obtained 24 h intervals after injection of the rat with sulfur mustard. Solvent: CDC13, SA =1 × 1/6, SW = 500.

new method for analysis of sulfur mustard and thiodiglycol or 2,2-dihydroxydiethyl sulfide.[10] Analysis of sulfur mustard and similar compounds wih HPLC was first developed *in vitro* by Bosse.[11] In the present study, we have used for the first time HPLC method to investigate the interaction of sulfur mustard with proteins in biological systems *in vivo*.

MATERIALS AND METHODS

Chemicals

Methanol, glycerol, chloramine-T and acetonitrile (LC grade) were purchased from Merck (Darmstadt, F.R.G).

Apparatus

All the analyses were performed on Pharmacia LKB HPLC Model 2151 (A), and repeated on a Shimadzu HPLC model LC-4A (B). A mass-spectrometer model 311-A, a nuclear magnetic resonance (NMR) model T-60A, and an infrared (IR) model 267 were also used.

Chromatography

The apparatus A. consisted of two pumps, model 2150; a UV detector model 2151; a Sample injector, model 7125; and two G 2000SW (10 μm particle size, 60 cm×7.5 mm I.D.) and one ODS (30μm particle size, 25 cm×4 mm I.D.) columns which were immersed in a constant temperature water bath.

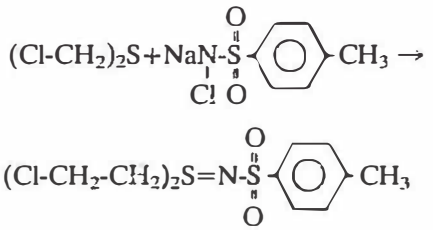
The apparatus B consisted of a UV detector, model SPD-2AS; an automatic sample injector and an ODS (30 μm particle size, 25 cm×4 mm I.D.) column which was immersed in a constant temperature water bath. The mobile phase consisted of acetonitrile and water (30:70), the flow-rate was 0.5 ml/min. The column effluent was monitored a 254 nm.

Injection

Different concentrations of sulfur mustard were used in 0.01% glycerol and injected to the MUSK rats (body weight 140-220 g) at sublethal doses subcutaneously (800 mg/kg) and intraperitoneally (100 mg/kg). Control groups received 0.01% glycerol only. At different time intervals (0.5, 1, 2, 3, 5, 9, 16, 24, 28, 72, 96, 168 h) plasma and urine samples were collected.

Sample Derivatization

Due to poor UV absorption of sulfur mustard and its metabolites, their derivatizations with arenesulfonyl sulfilimines (chloramine-T) were obtained before injection to HPLC. The product (reaction II) showed that it has maximum absorption at 254 nm.



(II)

Afterclean up steps, urine and plasma samples were derivatized with chloramine-T. Briefly, to these samples 1 ml methanol and water (30:70 V/V) and a appropriate amount of chloramine-T was added in 10 ml plastic tubes containing the appropriate volume of the sample. The mixture was then heated at 60°C for 1 hr. with the help of a magnetic mixer.

Assay method

The samples were eluted with an acetonitrile and water (30:70 V/V) mix. Standards were prepared and assayed in parallel plasma and urine samples. Each sample was run in duplicate. Fractions obtained from

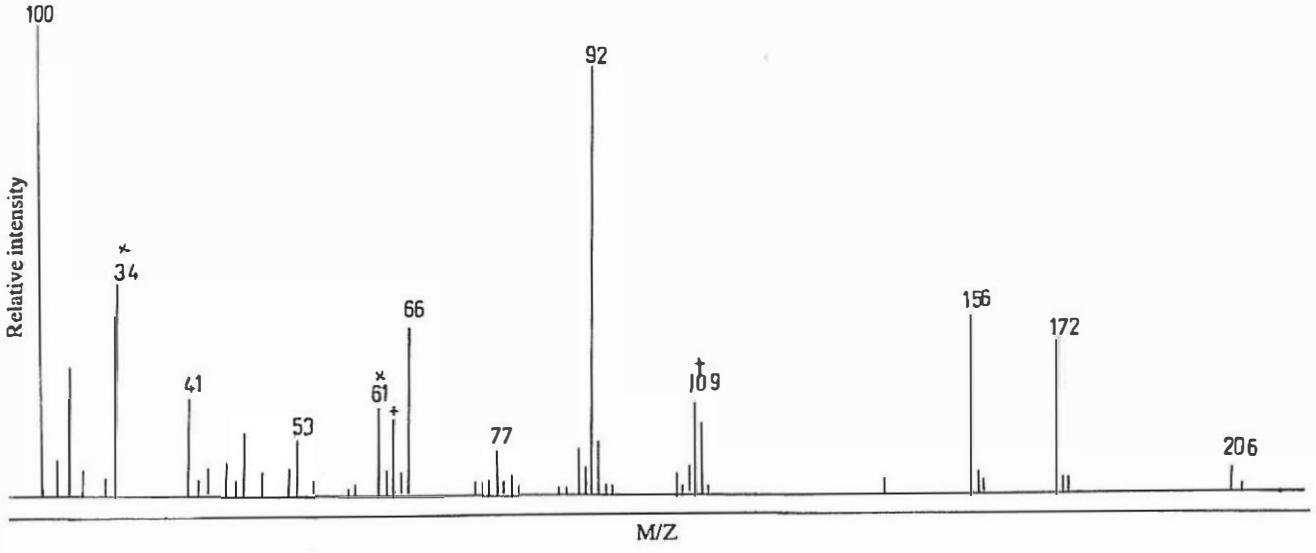


Fig. 4. Electron-impact mass spectra of plasma sample obtained at 24 h intervals after injection of the rat with sulfur mustard. pressure: 10/14 torr, scan= 60 sec, temperature: 120-140°C.

the urine and plasma samples by HPLC were also detected by NMR and MS and compared with the standard solution.

Recovery, Precision, and Detection Limits

The recovery of sulfur mustard and its metabolites was measured with 20μg and without sulfur mustard. Within each assay coefficients of variation were calculated from twenty consecutive injections of the same standard solution. The detection limits of sulfur mustard on signal-to-noise ratio of 5, were determined by injections of diluted standard solutions and diluted sample solutions. The reproducibility and sensitivity of the method was excellent. The advantage was that in a single assay it was possible to separate compounds with different polarity without time consuming preparations and it was possible to assay various plasma and urine samples for each determination. The values represent mean±SD% of the control. $P<0.01$ vs control. N=6-9 for each determination.

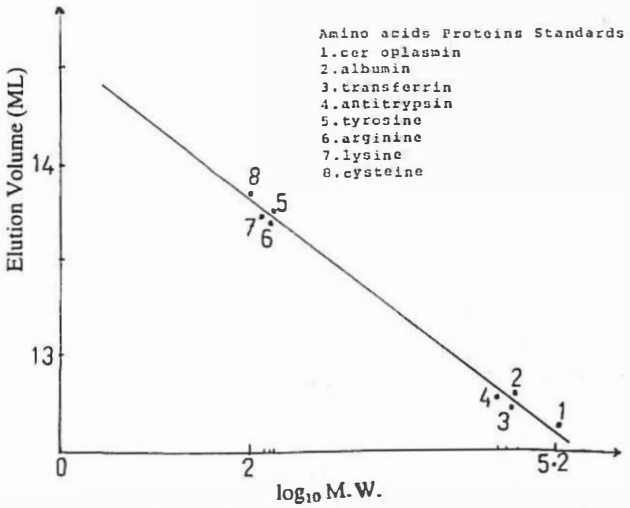


Fig. 5. The relationship between elution volume and the logarithm of the molecular mass of protein standards.
Column: TSK G 2000 SW, Eluent: acetonitrile and water (30:70 V/V), Flow rate: 0.5 ml/min.

RESULTS

From different time interval samples only a typical number of them at 9 and 24 h intervals after injecting the rat with sulfur mustard were selected.

Fig. 1 shows typical chromatograms of rat urine control samples (A) and rat urine samples obtained at 9 h intervals after injection of the rat with sulfur mustard (B). The chromatograms showed satisfactory resolution and symmetrical peak shapes, both with the standards (A) and samples (B). The retention time were 1.39 min for chloramine-T (Fig. 1A), 3.27 min for thiodiglycol and 6.27 min for sulfur mustard (Fig.1B). The results were reproducible throughout all analyses.

Fig.2 shows typical chromatograms of rat plasma samples obtained to 24 h intervals
rat with sulfur mustard. The retention times (Fig. 2) were 5.77, 19.90, 24.33, 31.18 and 38.76 min for plasma proteins. Again the results obtained were reproducable throughout all analyses. Under the experimental conditions used, sulfur mustard and its metabolites were strongly retained on the column during the program.

Fig.3 shows typical NMR spectra of a single fraction sample. Fig.4 shows electron-impact mass spectra of plasma sample obtained at 24 h intervals after injectic
of the rat. The peaks at m/z 158 $(CH_2CH_2Cl)_2S$, m/z 109 ($-CH_2SCH_2CH_2OH$), m/z 104($-CH_2SCH_2CH_2Cl$)

and peaks at m/z 34, 41, 53, 61, 66, 77, 92 and 206 indicated that sulfur mustard reacted with glutathione.

Fig.5 shows the relationship between the elution volume and the logarithm of the molecular mass of protein standards.

DISCUSSION

Previously, the interaction of sulfur mustard with amino (-NH2), thio (-SH) and carboxyl (-COOH) groups of aminoacids have been studied by TLC and GC techinqes.[3,5] It is obvious that these toxic compounds cause damage to brain, liver, kidney and heart.[12,13] Sulfur mustard interacts with tissue macromolecules such as DNA and results in growth inhibition, chromosomal aberration of sulfur mustard and its metabolites with aminoacids. A precise technique to insight these kinds of interactions is very important. Previously developed techniques are limited to the identification of sulfur mustard in the biological samples. However, with HPLC technique we could analyze the fractions and the possible interaction of these toxic compounds to proteins.The sensitivity of this separation is comparable with that obtained by previous investigators.[3,4,7] This techinque is applied to determine the interaction of sulfur mustard and its metabolites with different proteins from a variety of biological sources. The site of this kind of interaction is the cysteine, arginine and lysine residues (Fig. 5).

Use of urine and plasma in this study facilitates to minitoring the toxicity of human subjects to sulfur mustard.

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