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Short communication

Solid-phase extraction and reversed-phase high-performance liquid chromatographic determination of sulphur mustard in blood

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Abstract

A reversed-phase high-performance liquid chromatographic method is reported for the analysis of sulphur mustard in blood with the aid of solid-phase extraction sample preparation. Sulphur mustard is extracted from blood samples (both in vitro and in vivo) of rats with a solution of 0.05 *M* sodium dodecyl sulphate and pre-concentrated over Sep-Pak C_{18} cartridges pre-coated with Tween-20. A Polygosil C_{18} column is used with acetonitrile–water (52:48, v/v) as mobile phase for separation and sulphur mustard was detected at 200 nm.

1. Introduction

There is currently a great need for analytical techniques for identification of sulphur mustard [SM, 1,1′-thiobis-(2-chloroethane)] in biological fluids, such as blood, and to study the distribution and fate of SM in order to understand the mechanism of action and also for retrospective identification of poisoning due to SM. Presently, the methods to verify SM poisoning depend on the monitoring of some of the urinary metabolites of SM, i.e. thiodiglycol and its sulphoxides, by gas chromatography–mass spectrometry (GC–MS) [1–4]. Maisonneuve et al. [5] have reported a sensitive GC–MS method to determine SM in blood by liquid–liquid extraction or in plasma by a solid-phase extraction procedure. Recently, Hambrook et al. [6] demonstrated the presence of ^{35}S in blood and erythrocytes after cutaneous application of ^{35}S-labelled SM, and observed that the ^{35}S-labelled residue bound to erythrocyte was stable up to the life span of erythrocytes. Other than the methods employing GC–MS referred to above, there is no mentioning to our knowledge of any HPLC procedure relating to the detection of SM in blood or biological samples. We report here a reversed-phase HPLC method for the detection and quantitation of SM in blood using a solid-phase extraction (SPE) step specifically designed for recovering SM from blood.

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2. Experimental

2.1. Equipment

The HPLC equipment consisted of an LC-6A pump, a SIL-1A injector with a 200-μl loop, an SPD-6AV UV-Vis detector and a Chromatopac C-R3A integrator, all from Shimadzu (Tokyo, Japan).

2.2. Chemicals and solvents

SM used in this study was synthesized according to the standard procedure [7] and was found to be 99% pure through GC analysis. Acetonitrile used for the mobile phase was HPLC grade from Merck (Thane, India). Double-distilled water was prepared from the single distilled water previously refluxed for 24 h over acidified potassium permanganate in order to achieve very negligible interference with UV detection at 200 nm. Sodium dodecyl sulphate (SDS) and Tween-20 were obtained from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA), respectively. Sep-Pak C_{18} (SPC_{18}) cartridges used in the SPE were from Waters Associates (Milford, MA, USA).

2.3. Animal experiments

Wistar rats weighing between 175 and 225 g were used in this study. The LD50 of SM applied topically on the skin (after closely clipping the hair) of rats at the back near the tail region was 169 mg/kg as determined by Dixon's up and down method [8]. Blood was drawn from the retro-orbital plexus into heparinized tubes.

To obtain the detection limit and linearity of the response for SM quantitation, just prior to injection, various concentrations of SM (5–1000 ng per 10 μl) were prepared in acetonitrile–water (20:80, v/v) from a stock solution of SM in neat chilled acetonitrile and injected immediately onto the HPLC column. For in vitro studies, blood samples were spiked by vortex-mixing (30 s) with SM at 0.5, 1.0 and 2.0 μl/ml of blood in quadruplicate. These samples were processed either immediately (after 1 min) or after 30 min to study the precision and recovery of SM from blood samples. For in vivo studies, blood samples drawn after 30 min of exposure to SM (two concentrations of SM, 3.0 and 6.0 LD50 doses of SM, were employed for topical application on the skin of rats) were immediately processed for analysis.

2.4. Sample processing

Preparation of the SPC_{18} cartridges for SPE: Prior to use the cartridges were flushed with 2 ml of methanol followed by 2 ml of water. The cartridges were then coated in situ with the non-ionic surfactant Tween-20 by passing 10 ml of a 0.5% solution.

Blood (1 ml) from both in vitro and in vivo experiments was diluted with 40 ml of a solution of 0.05 *M* SDS and 1 *M* sodium chloride in 20% acetonitrile–water and was centrifuged at 10 000 *g* for 7 min in a MLW Janetzki K24 cold centrifuge (Leipzig, Germany) to remove cell debris. The supernatant was then passed through a SPC_{18} cartridge previously coated with Tween-20. After 2 washes with 1 *M* sodium chloride (20 ml each) the SPC_{18} was dried under vacuum. The SM thus trapped was eluted with 2 ml of acetonitrile. An aliquot of the extract was diluted with water (5-fold dilution to have the SM in acetonitrile–water, 20:80, v/v) and 10 μl of this mixture were injected immediately onto the column.

2.5. Chromatography

The column (25 cm × 4 mm I.D.) was packed with Polygosil C_{18} (5 μm) (Macherly-Nagel, Düren, Germany) according to the procedure prescribed by the manufacturer. The mobile phase was acetonitrile–water (52:48, v/v), at a flow-rate of 1.4 ml/min. The UV detector was set at 200 nm with a detection attenuation of 4.

2.6. Statistical analysis

Linear least-squares regression analysis for the standard curve calibration and two-way analysis

of variance for the between-day and within-day precision were performed [9].

3. Results

Fig. 1 shows a chromatogram with SM eluting at 5.8 min. The calibration plot for SM was linear in the 10–1000 ng range with acetonitrile–water (20:80, v/v) as the injection solvent. Regression analysis of the data (12 points) give the linear equation as log(area of the curve) = 2.63 + 1.01 log(concentration), the standard deviation of the slope and intercept being 0.016 and 0.035 respectively, with a correlation coefficient of 0.998. The within-day and between-day coefficients of variation for a single concentration (100 ng/10 μl) were found to be 6.2% and 3.1%, respectively. The limit of detection of the method at a signal-to-noise ratio of 3:1 was 2.5 ng/10 μl using SM standard at a detection attenuation of 2.

The peak for SM in the SPE extracts from SM spiked blood was identified with respect to the standard of SM, 100 ng/10 μl (Fig. 2). The results of the validation of the method by the in vitro experiments are listed in Table 1. The method was shown to be accurate and precise (between-day and within-day variation <8%). The recovery of SM was estimated to be 29 ± 2.2 (after 1 min) and 24 ± 1.7% (after 30 min). The lowest detection limit of SM in blood was 30 μg/ml with an injection volume of 10 μl. SM was also clearly detected in the rat blood at 30 min after the dermal application of SM (Fig. 3). The concentrations of SM detected in these samples were 210–280 ng/10 μl (3 LD50) and 650–760 ng/10 μl (6 LD50) and the recoveries of SM from blood of these rats were 4.6 ± 0.32% (3 LD50) and 7.0 ± 0.27% (6 LD50) considering the volume of blood as 10% of the bodyweight in grams.

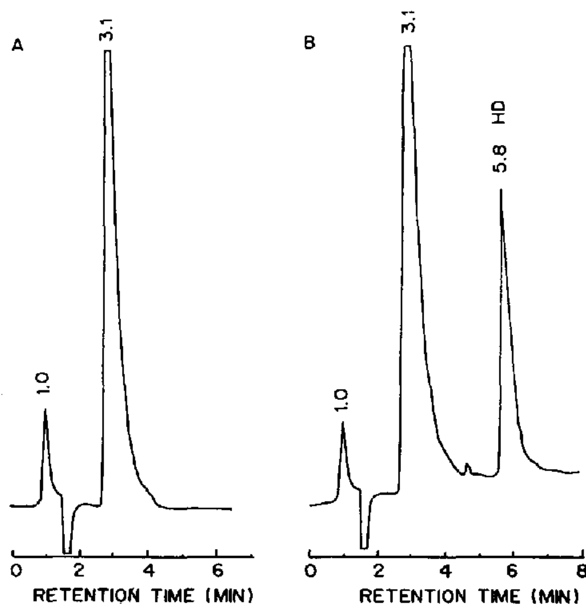

Fig. 1. Chromatograms of blank solvent (A) (20% acetonitrile) and a SM standard (100 ng/10 μl) (B). Conditions as given in Experimental.

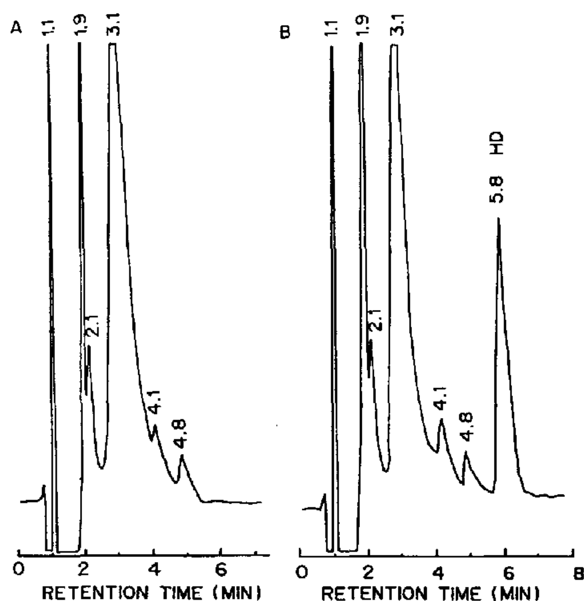

Fig. 2. Chromatograms of blank blood (A) and a spiked blood sample (B) of 0.6 mg/ml SM (absolute amount injected onto the column: 102 ng/10 μl). Conditions as given in Experimental.

4. Discussion

The purpose of this study was to explore the possibility of detecting SM in blood by a suitable

Table 1
Accuracy, precision, and recovery for the analysis of SM in rat blood

Concentration (mg/ml)	Accuracy (%)	C.V. (%)	n^a	Precision (%)	
				Between-day	Within-day
0.6	99	5.4	12	5.0	7.1
1.2	108	10.5	12	5.4	7.6
2.4	112	11.3	12	5.4	7.6
Recovery (mean ± S.D.) (%) 24 ± 1.7%					

[a] n = Number of replicates, divided over three runs.

sample handling technique taking into consideration the peculiarities of SM, viz., the easy hydrolysis to thiodiglycol in aqueous solution and its loss due to co-volatilization in the more common volatile organic solvents like chloroform and dichloromethane used in conventional solvent extraction methods (our unpublished data). Another important point was to obtain a

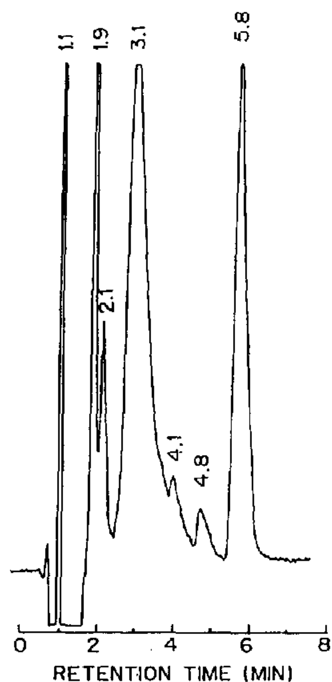

Fig. 3. Chromatogram showing the detection of SM in rat blood at 30 min after dermal application of 3 LD50 SM (the concentration of SM detected being 230 ng/10 μl as calculated from the peak area). Conditions as given in Experimental.

protein-free extract in order to prevent any damage to the column (C_{18} silica). Moreover, we could not employ the method of Desilets et al. [10], designed for direct injection of biological samples onto the semi-permeable reversed-phase (SRP) C_{18} column (prepared in situ by passing a 0.5% solution of non-ionic surfactant, Tween-20). We found that the in situ coated Tween-20 was not stable as seen by the large baseline drifts at 200 nm.

The rationale behind the use of an SDS-based solvent for the initial extraction of SM from blood is that SDS has been successfully used in some HPLC assays for both diluting the sample (plasma) and simultaneously releasing the drug bound to the proteins [11]. In our experience, in many in vivo experiments SM could not be detected when blood or plasma from animals exposed to SM was diluted in 1 *M* sodium chloride alone; however, SM could be detected clearly using a plain micellar solution of SDS (0.05 *M*). Washes with 1 *M* sodium chloride helped to prevent hydrolysis of SM and also to remove the polar impurities from the extract. The detection of SM employing SDS solvent extraction suggests that SM is loosely associated with erythrocytes and that a 1 *M* sodium chloride solution is not sufficiently strong to liberate SM from the erythrocytes. The low recovery of SM obtained in the SPE procedure can be attributed to the rapid hydrolysis of SM in aqueous solution especially when vortex-mixed, in spite of its very low solubility [12]. The limit of detection of the method can further be enhanced, if desired, simply by increasing the sample size (volume of

injection) in a weak solvent to 100–1000 μl compared with the 10 μl used presently. We believe that the SPE procedure described here could be useful for both HPLC and GC–MS determination of SM.

5. Conclusions

SM can be detected and quantitated by reversed-phase HPLC at 200 nm. SM from blood samples of albino rats exposed to either 3 or 6 LD50 percutaneous dose (in vivo) can be clearly detected by employing sample extraction with micellar SDS solution with 1 *M* sodium chloride in acetonitrile–water (20:80, v/v) followed by SPE on a Tween-20 coated SPC_{18} cartridge.

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