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Specific and sensitive quantitation of 2,2′-dichlorodiethyl sulphide (sulphur mustard) in water, plasma and blood: application to toxicokinetic study in the rat after intravenous intoxication

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ABSTRACT

A sensitive and specific capillary gas chromatographic method has been developed to measure trace amounts of 2,2′-dichlorodiethyl sulphide (sulphur mustard) in environmental or biological samples. Sulphur mustard was isolated from water or plasma by a solid-phase extraction procedure and from blood by liquid–liquid extraction. The accuracy and precision of the methods were demonstrated using replicate analyses of spiked water, plasma or blood: within-run and between-run variabilities were less than 20%. These analytical methods were used to evaluate the rate of sulphur mustard degradation in water or plasma. Good linear calibration curves, with a detection limit of 45 ng/ml, were obtained for quantitation and determination of sulphur mustard in blood following its intravenous administration to rats. Initial toxicokinetic data were obtained.

INTRODUCTION

Although chemical weapons have been prohibited since the 1925 Geneva protocol, the old vesicant sulphur mustard (2,2′-dichlorodiethyl sulphide, mustard gas, SM) still represents a potential threat as a chemical warfare agent (*e.g.*, in the recent Gulf War). Thus, the use of SM has been revealed in the Iran–Iraq conflict by toxicological analysis and recognized by a United Nations team of experts [1,2].

Clinical effects due to the alkylating properties of SM have been described [3], but the mechanism of its biological actions is not yet well understood [4]. Toxicokinetic studies, such as blood SM kinetic investigations, could be correlated with physiological symptoms and lead to therapeutic procedures. In order to understand the effects of SM in the organism, or to prove an alleged SM attack, specific and sensitive methods for the detection, isolation, identification and quantitative analysis of trace amounts of SM in environmental and biological samples are required.

Since the first use of SM in World War I, numerous methods of detection have been reported. Classical non-sensitive colorimetric methods have been used [5,6]. More recently, other approaches have been described for the determination and identification of SM from air, soil and aqueous samples; they are mainly based on chro-

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matographic techniques, such as thin-layer chromatography (TLC) [7–10], gas chromatography (GC) [11–18], gas chromatography–mass spectrometry (GC–MS) [19–22], and high-performance liquid chromatography (HPLC) [23].

Very few papers describe measurements of SM in biological matrices. Traces were recovered in urine samples (1–30 ng/ml) of Iranian victims [24–26] or determined in spiked plasma samples (4.5 μg/ml) [27], using GC–MS or gas chromatography–electron-capture detection (GC–ECD), respectively. The vesicant was also qualitatively detected by GC–MS in abdominal fat and quantified in tissues and in body fluids by electrothermal atomic absorption spectrometry (ET-AAS) [28]. However, the qualitative and quantitative validation of these results were not investigated, *i.e.* extraction efficiencies, precisions, accuracies, etc.

This paper deals with clean-up procedures for handling environmental or biological samples. A rapid, simple solid-phase extraction procedure adapted to routine measurements was developed for aqueous and plasma samples. A liquid–liquid extraction technique improved the specificity of the method and was more suitable for blood toxicokinetic studies. The samples resulting from clean-up procedures were analysed by capillary GC with flame ionization detection (FID). The performances of these methods are presented with respect to recovery, limit of detection, linearity and reproducibility. The analytical methods have been optimized for the determination and quantitation of SM trace amounts in aqueous, plasma and blood samples. The *in vitro* SM degradation levels in spiked water or human plasma and the *in vivo* rate of SM disappearance in rat blood after intravenous SM intoxication (10 mg/kg) are reported.

EXPERIMENTAL

Chemicals and reagents

SM (2,2′-dichlorodiethyl sulphide) and the internal standard (2,2′-dichloropropyl sulphide) were obtained from CEB (Centre d'Etudes du Bouchet), Department of Chemical Synthesis. The purity of these compounds was more than 90%. 2-Propanol, methanol and sodium chloride were purchased from Merck (Nogent sur Marne, France). Ethyl acetate of analytical-reagent grade was supplied from SDS (Peypin, France).

Standard solutions

Stock solutions of SM and the internal standard were prepared in 2-propanol at a concentration of 20 mg/ml. Working solutions were diluted as required, usually to 1 mg/ml in 2-propanol. They were stored at 4°C and kept for 3 weeks without any degradation. The stability of SM stored in 2-propanol at 4°C was examined. It was stable over the entire test period (3 weeks): at a concentration of 1 mg/ml, 101.8, 99.5 and 94.9% of the initial concentration were recovered 7, 18 and 23 days, respectively, after preparation of the solutions.

The calibration graph was obtained by adding increasing known amounts of SM to water, plasma or blood. Samples were treated as described below.

Extraction procedures

Water and plasma. The solid-phase C_{18} extraction column (3 ml) from Baker (Sochibo, Meudon-la-Forêt, France) was equilibrated with 3 ml of methanol, then 1 ml of sample in an 18% NaCl solution was loaded on the cartridge and passed through it by gentle vacuum aspiration (1 ml/min). The cartridge was dried under vacuum for 10 min. The adsorbed SM was eluted from the cartridge with 1 ml (0.5 ml twice) of ethyl acetate. An aliquot (1 μl) was injected into the GC system.

Blood. Liquid–liquid extraction was performed for the blood sample: 6 ml of whole blood was stabilized with 18% NaCl (1.08 g) and added to 50 μl (5 μg) of a 100 μg/ml internal standard solution. The solution was extracted twice with 3 ml of ethyl acetate after 30 s vigorous shaking. The mixture was centrifuged under 2987 *g* at 4°C for 3 min in a Rottanta RP (Hettich, Cera-labo, Aubervilliers, France). The organic phases were transferred into 10-ml venoject tubes (SNL, Bondoufle, France). All glass tubes were rinsed

with ethyl acetate. The combined organic layers (6 ml) were concentrated to 100 μl in a high-speed vacuum concentrator coupled to a refrigerated condensation trap (Savant, Bioblock Scientific, Vanves, France) for 20 min, then under a gentle stream of nitrogen. An aliquot (1 μl) was injected into the GC system. The extracts containing SM were kept at 4°C before injection.

GC analysis

Two gas chromatographs, a Delsi DI 700 (Argenteuil, France) and a Perking Elmer 8320 (Saint Quentin-en-Yvelines, France), equipped with a split–splitless injector and a flame ionization detector, were used.

Water and plasma samples were separated on the Delsi chromatograph, which was equipped with a 10 m × 0.22 mm I.D. CP Sil 19 CB silica capillary column (Chrompack, Les Ulis, France). Helium (l'Air Liquide, Bois-D'Arcy, France) was used as carrier gas. Experimental conditions were: inlet pressure, 0.45 bar; injector vent flow-rate, 2 ml/min; septum purge flow-rate, 2 ml/min. The initial column temperature of 70°C was held for 0.5 min, then raised at 30°C/min to 140°C, held for 2 min, then raised to 200°C at 39.9°C/min.

Blood samples were analysed on the Perkin Elmer 8320 chromatograph, which was equipped with the same capillary column as previously the Delsi. The inlet pressure of the carrier gas, helium, was 1.03 bar, the injector vent flow-rate was 20 ml/min, and the septum purge flow-rate was 2 ml/min. The initial column temperature of 60°C was held for 0.5 min, then raised at 15°C/min to 90°C, 1 min isothermal, thereafter increased at 30°C/min to 140°C, held for 4 min, then raised to 230°C at 30°C/min.

On both chromatographs, the detector was operated at 250°C and the injector at 200°C. The latter was maintained in splitless mode for 0.5 min.

Gas chromatography–mass spectrometry

A Nermag R10-10S mass spectrometer (Nermag, Argenteuil, France) coupled to a Delsi DI 700 gas chromatograph was used for the analysis of by-products related to SM. All glass was treated with a 1% aquasil solution in order to prevent the adsorption of metabolites. Separations were accomplished as described above. The mass spectrometer operating conditions were as follows: ion source temperature, 200°C; electron energy, 70 eV; ionization current, 200 μA; interface temperature, 220°C.

Stability of SM in water or human plasma

SM (300 μg/ml) was incubated at 20°C in aqueous samples and 37°C in biological media. Two aliquots of each matrix were removed at 0, 5, 10, 15, 30, 45, 60, 90, 120 and 240 min after *in vitro* SM contamination and placed in tubes containing 18% NaCl. The samples were extracted and analysed for SM.

Animal studies

A solution of SM in 2-propanol (10 mg/ml) was daily prepared and intravenously administered (10 mg/kg via the femoral vein) to fourteen groups of at least four male Wistar rats (Janvier, Le Genest Saint Isle, France) weighing 280–330 g. Blood samples were then collected from rats 2, 5, 10, 20, 35 and 45 min and 1, 1.5, 2, 3, 4, 5, 6 and 8 h after the intoxication, and placed into heparinized tubes placed in the ice. The samples were immediately treated as described above.

RESULTS

Initial studies

Addition of NaCl to the samples before extraction was found to be helpful, because it prevented, by the common ion effect, the hydrolysis of SM in aqueous or biological media and the consequent formation of hemi-mustard (2-chloro-2'-hydroxydiethyl sulphide) and thiodiglycol (2,2'-dihydroxydiethyl sulphide). Several NaCl concentrations were tested in order to obtain a good reproducibility for extraction steps. The results are shown in Table I: the percentage of SM extracted increases with the NaCl concentration. A NaCl solution of 18% was chosen: at higher percentages, no yield improvement was noted and problems of SM solubility were also encountered.

TABLE I

RELATION BETWEEN SODIUM CHLORIDE CONTENT AND SM YIELD IN AQUEOUS SAMPLES

NaCl content content (%)	SM added (μg/ml)	Yield efficiency (mean ± S.D., $n = 6$) (%)	Yield (mean ± S.D.) (%)
0	10	71.1 ± 9.8	71.5 ± 5.1
	50	78.5 ± 16.8	
	100	66.3 ± 2.2	
	150	70.1 ± 12.1	
9	10	58.9 ± 9.4	73.9 ± 10.7
	50	82.3 ± 12.5	
	100	73.5 ± 10.9	
	150	80.9 ± 3.8	
18	10	81.2 ± 18.9	88.7 ± 6.5
	50	85.6 ± 17.7	
	100	92.4 ± 3.1	
	150	95.5 ± 4.7	

Hence, an 18% solution of NaCl was added immediately after sampling. This procedure, applied to SM extraction from environmental or biological samples, allowed mean yields of 88.7 ± 6, 80.7 ± 6.6 and 85.3 ± 10.8% from water, plasma and blood samples, respectively.

Method validation

Calibration curves and sensitivity. Standard curves were obtained with increasing amounts of SM without an internal standard for water or plasma, and in the presence of a constant amount of the internal standard for blood. SM was extracted as described in the extraction procedure. For all methods, the peak-area response of SM (aqueous and plasma samples) or the peak-area ratio between SM and the internal standard (blood samples) was plotted *versus* the concentration of SM (aqueous and plasma samples) or the concentration ratio between SM and the internal standard (blood samples). For each matrix, the detector response was linear for SM.

Two calibration curves were used for the calculation of the concentration of SM in water, plasma and blood samples, the first to quantify small amounts of SM and the second to titrate larger amounts. Calibration curves in the ranges 5–100 and 100–300 μg/ml SM were linear for water and plasma. Correlation coefficients ranged from 0.9818 to 0.9952. Calibration curves in the ranges 0.0416–0.166 and 0.166–1.66 μg/ml SM were linear for blood samples. Table II lists correlation coefficients, slopes and y-axis intercepts obtained for blood samples. These calibration curves were used to obtain the SM concentrations in spiked and unknown samples.

Assay precision and accuracy. The precision and the relative accuracy (recovery) of the method were examined by replicate analysis of freshly prepared water, plasma and blood samples: these samples were spiked with a known amount of SM and treated as unknown samples. The precision [expressed as coefficient of variation (C.V.)] is defined as the degree of agreement between replicate measurements of the same quantity. The accuracy, defined as the degree of agreement between the measured value and the true value, is expressed as relative accuracy, which is the measured value as a percentage of the true value [29]. Intra- and inter-variations were determined for each extraction procedure and matrix: low- and high-concentration controls were analysed six or ten times within a single assay, and in three or ten separate assays. Table III gives details of intra- and inter-assay C.V.s, mean concentrations found and relative accuracies for each method. The overall recovery ranged from 83.9 to 99.3%. The within-run and between-run precisions of

TABLE II

SLOPES, y-AXIS INTERCEPTS AND CORRELATION COEFFICIENTS DETERMINED FOR BLOOD SAMPLES

FID on PE 8320 with an internal standard (2,2'-dichloropropyl sulphide).

Linearity range	Slope	y-Axis intercept	Correlation coefficient
41.66 ng/ml to 0.166 μg/ml	1.32	−0.0044	0.9416
0.1666 μg/ml to 1.66 μg/ml	0.87	0.084	0.9967

methods ranged from 5.6 to 21.6%. These results show a good accuracy and precision for the tested concentrations, except in the case of 29.7% (found for spiked plasma samples with 10 μg/ml, which could be improved by using an internal standard).

Detection limits. Limits of detection, for a signal-to-noise ratio of 3, were determined according to Foley and Dorsey [30] for each matrix and extraction procedure. Values of 4.6 and 9.4 μg/ml SM were found for water and plasma samples, respectively, using the solid-phase extraction procedure; a value of 45 ng/ml was found for blood samples, using the liquid–liquid extraction procedure.

Applications to water, plasma and blood samples

The described methods were applied to analyses of *in vitro* contamination by SM in both aqueous and plasma media. The chemical degradation of recovered SM was followed *versus* time, and the resulting profiles are shown in Fig. 1. About 90 min after the contamination, the SM levels were below the detection limit. A rapid monoexponential decrease was observed, with a half-life of 4.03, 4.5, 6.6, 7.85, and 8.12 min in distilled water, plasma, mineral water, pH 4 buffer (acetic acid, sodium acetate) and pH 10 buffer (sodium tetraborate, sodium hydroxide), respectively. About 20 min after contamination (Fig. 2), these different media were analysed for the by-prod-

TABLE III

INTRA- AND INTER-ASSAY PRECISION AND RELATIVE ACCURACY OF ANALYTICAL METHODS FOR DETERMINATION OF SM IN DIFFERENT MATRICES

Matrix	Quality control concentration added (μg/ml)	Within-run precision			Between-run precision		
		Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Recovery (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Recovery (%)
Aqueous	10	11.61 ± 1.50 (n = 10)	12.9	83.9	9.39 ± 1.2 (n = 10)	13.1	93.9
	100	96.92 ± 5.48 (n = 10)	5.70	97.0	103.6 ± 8.6 (n = 10)	8.3	96.5
	200	201.5 ± 17.5 (n = 10)	8.70	99.3	193.2 ± 10.9 (n = 10)	5.6	96.6
Plasma	10	8.45 ± 2.51 (n = 10)	29.7	84.5			
	100	102.9 ± 9.76 (n = 10)	9.5	97.1			
	200	195.6 ± 32.6 (n = 10)	16.4	99.3			
Blood	0.166	0.149 ± 0.025 (n = 5)	16.75	89.00	0.15 ± 0.024 (n = 15)	16.34	89.78
	0.166	0.156 ± 0.034 (n = 4)	21.61	93.30			
	0.166	0.146 ± 0.021 (n = 6)	14.60	87.70			
	1.25	1.115 ± 0.054 (n = 5)	4.83	89.24	1.16 ± 0.08 (n = 17)	7.54	93.99
	1.25	1.237 ± 0.082 (n = 6)	6.64	99.02			
	1.25	1.162 ± 0.087 (n = 6)	7.45	92.92			

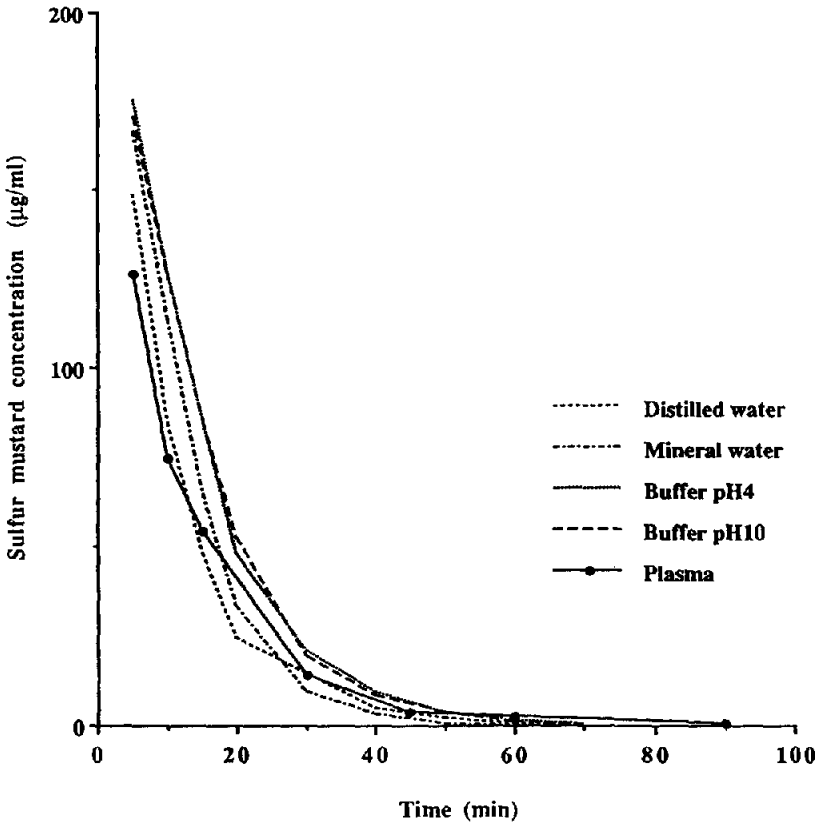

Fig. 1. Concentration–time profiles of SM in several different aqueous media and in human plasma.

ucts related to SM by GC–MS. Hemi-mustard (HM), thiodiglycol (TDG) and 2,2′-diisopropoxydiethyl sulphide were detected and identified. These products do not appear in a standard solution of SM, which demonstrates that they are degradation products obtained from SM and not GC artefacts.

Electron-impact (EI) mass spectra of SM derivatives are shown in Fig. 3. The mass/charge (m/z) ratios and relative intensities (in parentheses) of the typical ion fragments of HM were 140 (22%), 109 (100%)), 104 (50%), 91 (51%), 73 (32%), 61 (96%), and 45 (79%), probably due to molecular ion $[OH–CH_2–CH_2–S–CH_2–CH_2–Cl]^{+\cdot}$, $[CH_2–S–CH_2–CH_2–Cl]^{+}$, $[HO–CH_2–CH_2–S–CH=CH_2]^{+\cdot}$, $[HO–CH_2CH_2–S–CH_2]^{+}$, $[CH_2–S–CH=CH_2]^{+}$, $[CH_2–CH_2–S–H]^{+}$, [HC–S] or $[C_2H_5–O]^{+}$, respectively. The m/z values and relative intensities of the typical ion fragments of TDG were 104 (36%), 91 (38%), 61 (100%), and 45 (67%), probably due to $[HO–CH_2–CH_2–S–CH=CH_2]^{+}$, $[HO–CH_2–CH_2–S–CH_2]^{+}$, $[CH_2–CH_2–S–H]^{+}$, [HC–S] or $[C_2H_5–O]^{+}$, respectively, with the molecular ion at m/z 122 $[S(CH_2–CH_2–OH)_2]^{+\cdot}$. The m/z values and relative intensities of the typical ion fragments of 2,2′-diisopropoxydiethyl sulphide were 118 (19%), 105 (13%), 91 (28%), 86 (61%), 73 (34%), 61 (100%), and 43 (94%), probably due to $[S=CH–CH_2–O–iC_3H_7]^{+\cdot}$. $[S–CH_2–O–iC_3H_7]^{+}$, $[HO–CH_2–CH_2–S–CH_2]^{+}$, $[S(CH=CH_2)_2]$, $[CH_2–O–iC_3H_7]$, $[CH_2–CH_2–S–H]^{+}$, and $[iC_3H_7]$, respectively. About 30 min after contamination, these degradation products were still detectable.

For pH 4 buffer, four other degradation products were found. The interpretation of their mass spectra is in progress in our laboratory. For hu-

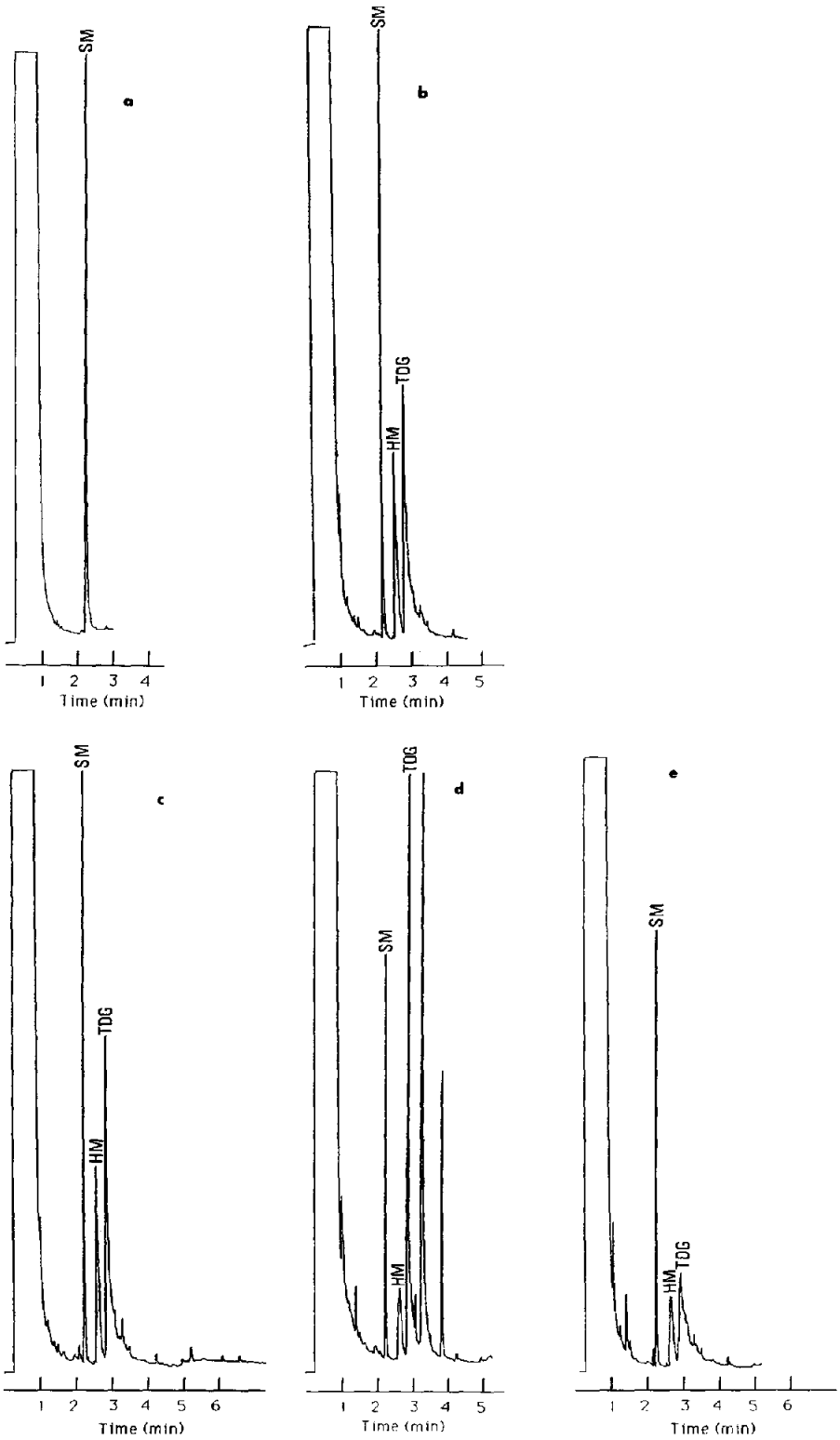

Fig. 2. Chromatograms of (a) standard solution of SM containing 100 μg/ml (amount of SM injected was 100 ng), (b) mineral water, (c) distilled water, (d) pH 4 buffer and (e) pH 10 buffer, 20 min after contamination with SM (300 μg/ml).

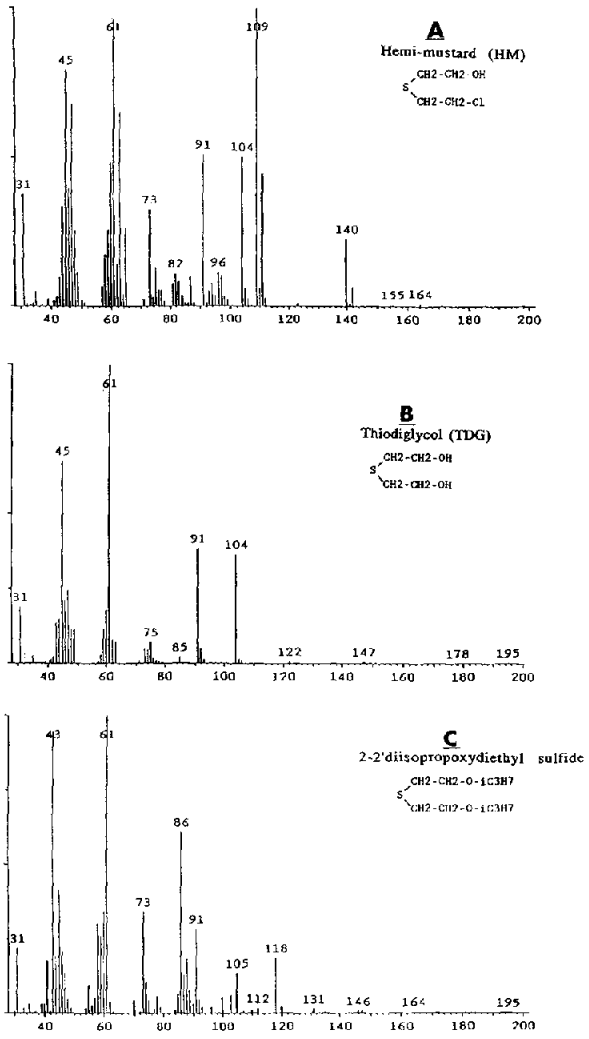

Fig. 3. Electron-impact mass spectra of SM metabolites in mineral water, distilled water or pH 10 buffer, 20 min after contamination. The scale on the x-axis is in mass-to-charge (m/z) units, and on the y-axis, relative abundance (%). (A) Hemi-mustard; (B) thiodiglycol; (C) 2,2'-diisopropoxydiethyl sulphide.

man plasma, no metabolite was observed 20 min or 45 min after contamination. These results are in good agreement with the high affinity of SM towards red blood cells and proteins.

The liquid–liquid extraction procedure and the GC–FID analysis described above were applied to the determination of SM in rat blood, after intravenous administration of 10 mg/kg SM (Fig. 4). As shown in Fig. 5, blood concentrations of SM decreased biexponentially, with a half-life of 5.56 min for the first elimination phase and 3.59 h for the second. More details of the toxicokinetic results will be published elsewhere [31]. SM was detected in the rat blood samples 8 h after its intravenous administration.

DISCUSSION

The aim of this study was to produce rapid, simple and reliable analytical methods for the quantitative measurement of SM in environmental and biological samples. This requires reproducible extraction procedures, because most of the variations and imprecisions of a method arise during pretreatment. Optimization of the extraction procedures for SM in water, plasma and blood is discussed here. Maximum and reproducible detector responses (specificity, sensitivity and reproducibility of retention times) for SM and its internal standard were sought, and a capillary column coated with CP Sil 19 CB was successfully used for GC–FID analysis. TLC [7–10] and GC [11–15] suffered from a poor reproducibility and sensitivity, and HPLC [23] required a long derivatization step (60 min).

Owing to the low concentrations of SM encountered in this study, investigations were based on solid-phase extraction (using an ODS stationary phase and ethyl acetate as eluent). The influences of the sorbent and the solvent on extraction efficiencies were discussed by Callebat *et al.* [32]. Under these conditions it was possible to obtain linear calibration curves, and to analyse SM samples ranging from 5 to 300 μg/ml extracted from water or plasma matrices. The described method offers several advantages: a rapid one-step extraction for both matrices, good accuracy and precision and adequate sensitivity without laborious analytical steps. The reliability of this procedure makes the method suitable for routine analysis for the evaluation of SM in water or plasma, and an important analytical tool to check an alleged SM use by the measurement of SM persistence in the contaminated media and by the semi-quantitative determination of the by-products related to SM.

Although solid-phase extraction on a C_{18} col-

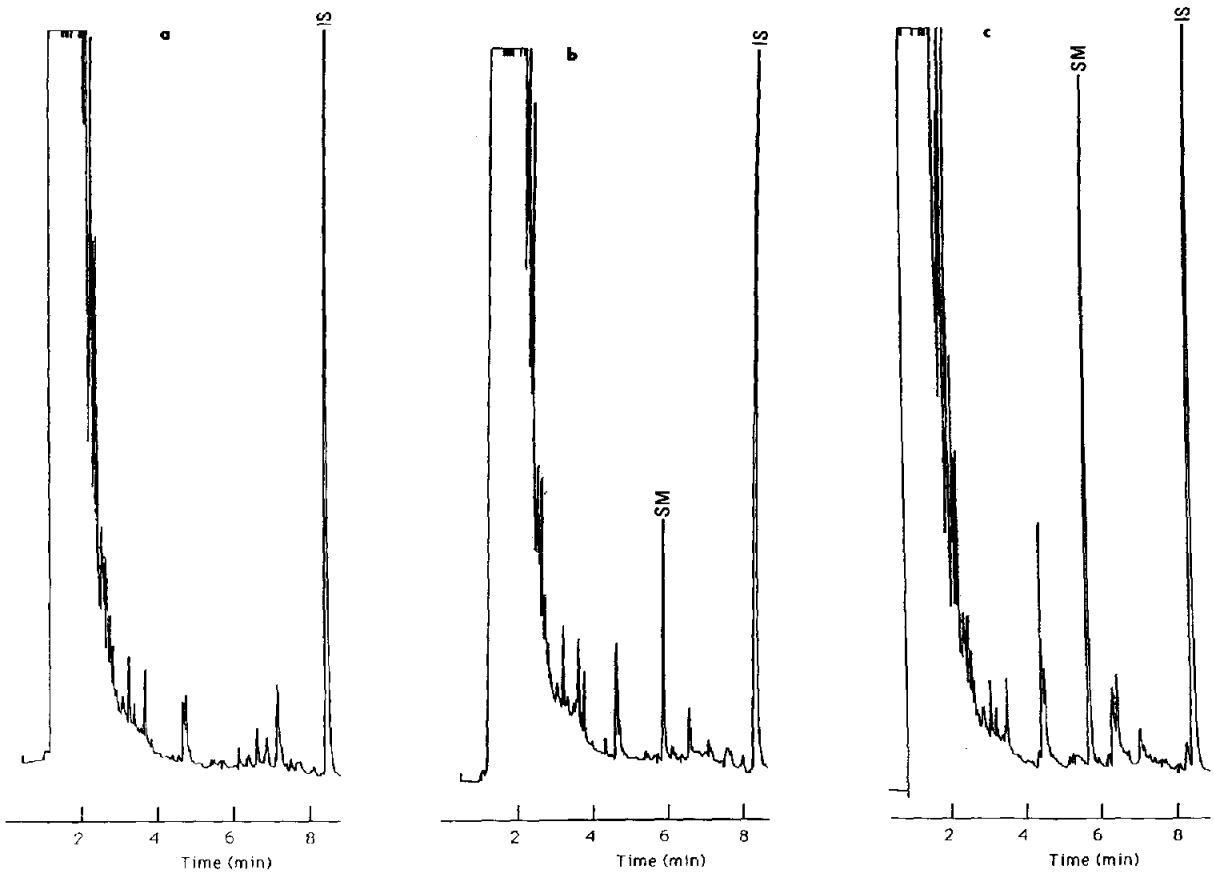

Fig. 4. Chromatograms of extracted blood samples. (a) Blank blood containing 833.3 ng/ml internal standard (IS); (b) spiked blood containing 166.6 ng/ml SM and 833.3 ng/ml IS; (c) blood 10 min after an intravenous dose of SM (10 mg/kg) containing 833.3 ng/ml IS.

umn was optimum for water or plasma matrices, it was unadapted for *in vivo* SM identifications in blood. This difference reflects the natural fluctuations between *in vitro* contaminations and *in vivo* intoxications. The separation and quantitation of SM in biological fluids present many difficulties. On the one hand, the concentrations of such a compound in physiological systems are at trace levels (low ng/ml), owing to its rapid degradation and/or metabolism; on the other hand, endogenous compounds interfere with the determination of SM in blood extracts. Matrix problems were reduced by the use of the liquid–liquid clean-up procedure, which proved to be better for the recovery of SM from extracts of whole blood. The use of an internal standard is recommended to overcome the losses occurring during the sample clean-up and to obtain a reproducible recovery. The precisions, within or between analytical series, were acceptable at the low concentrations (*e.g.* 166 ng/ml) that occur under these physiological conditions. An analytical method for SM, using liquid–liquid extraction, was found to be reliable for biological fluid analysis (limit of detection of 45 ng/ml). It was sensitive enough to measure the blood kinetics of SM in rats intravenously dosed with 10 mg/kg SM. The data on SM elimination in rat blood are, to our knowledge, the first report on *in vivo* SM blood kinetics. The clinical interpretations of SM, which have been well described, remain to be correlated with the low detected value of unchanged SM in the systemic circulation.

Higher extraction efficiencies were obtained

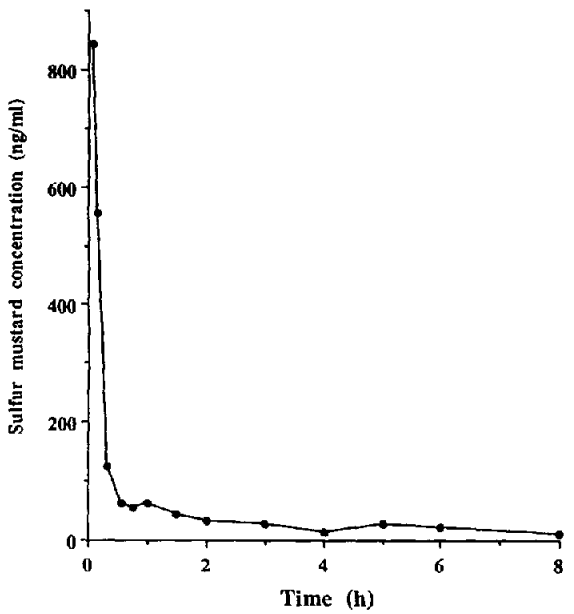

Fig. 5. Concentration–time profile of SM in blood obtained from intravenously dosed rats (10 mg/kg SM).

with NaCl. The decomposition of SM in aqueous or biological solutions may be attributed to its rapid hydrolysis to hemi-mustard and thiodiglycol. Sodium chloride has been reported to be effective in reducing SM hydrolysis in aqueous media [33]. Sass and Steger [16] have reported that chloride ions prevented ethylenesulphonium ion formation (the rate-determining step of SM hydrolysis), thus giving a higher degree of stability to SM. Vycudilik and Machata [24–26] and Heyndrickx *et al.* [27] stabilized SM, in biological media, before extraction, by the addition of a saturated NaCl solution. Here, the best NaCl concentration has been experimentally determined [32]. A NaCl solution of 18% was shown to prevent the hydrolysis of SM to its by-products and to recover maximal amounts of SM in different matrices without losses, for 15 min. Above 18%, the yield efficiency did not improve, but problems of SM solubility occurred.

CONCLUSION

To our knowledge, this is the first report of a systematic method for the quantitative analysis of SM in either environmental (water) or biological (plasma, blood) samples. In order to obtain reliable results from sample handling to quantitative analysis, the precision, accuracy, linearity, detection limit, specificity, sensitivity and retention times have all been studied. The procedures described in this paper are well suited for a large number of samples, enabling routine analysis to be performed.

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