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Analysis of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, metabolites of sulphur mustard, in urine using gas chromatography–mass spectrometry

ROBIN M. BLACK*, RAYMOND J. CLARKE and ROBERT W. READ
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ (UK)
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ABSTRACT

A method has been developed for the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, which have been identified as urinary metabolites of sulphur mustard in the rat. The two metabolites were reduced to the single analyte 1,1′-sulphonylbis[2-(methylthio)ethane] by treatment of urine with acidic titanium trichloride. 1,1′-Sulphonylbis[2-(methylthio)ethane] was readily extracted from urine by passing through a C_8 reversed-phase extraction column, or by solvent extraction from a solid absorbent tube, and detected by gas chromatography–mass spectrometry using ammonia positive ion chemical ionisation. The limit of detection was 2 ng/ml for 1-ml samples of urine. There were no background levels of analyte in human or rat urine. If man metabolises sulphur mustard by a similar pathway, the detection of these metabolites should constitute firm evidence of an exposure to sulphur mustard.

INTRODUCTION

The recent proliferation and use of chemical weapons, particularly in the Middle East, plus the negotiations towards a verifiable chemical weapons treaty, have lead to increased attention being given to the retrospective confirmation of specific agent poisoning in victims of chemical weapons. Of particular concern is sulphur mustard, following its use in the Iraq–Iran conflict [1–3]. At present there is no unambiguous means of confirming a medical diagnosis of mustard poisoning in casualties who have been externally decontaminated from any traces of agent. Greatest attention has been given to the detection of excretion products derived from the hydrolysis of sulphur mustard as possible indicators of mustard poisoning. Methods for the detection of thiodiglycol, the simple hydrolysis product of sulphur mustard, in urine, have been reported by Wils and co-workers [4,5] and by Black and Read [6]. Black and Read [7] have also reported methods for the detection of thiodiglycol sulphoxide, which recent metabolism studies [8] have shown to be the major urinary excretion product derived from the hydrolysis of sulphur mustard. However, a com-

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plicating factor in the detection of these hydrolysis products is the presence of low background levels of analyte in normal urine which, although usually in the range 1–10 ng/ml, were as high as 36 ng/ml [7] and 55 ng/ml [4] in extreme cases. We therefore sought an alternative metabolite whose detection in urine could be accepted as unambiguous evidence of mustard poisoning, in addition to the detection of products derived from hydrolysis.

Nine urinary metabolites of sulphur mustard have been identified in recent studies in these laboratories [8]. Several of these are conjugates of mustard with N-acetylcysteine, most of which have poor mass spectrometric and/or gas chromatographic properties, mainly due to thermal instability. More promising from the viewpoint of analysis were two closely related products assumed to be derived from the further action of the enzyme β-lyase on cysteine conjugates. These metabolites are 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (**1**) and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane (**2**).

$O_2S(CH_2CH_2SOCH_3)_2$ $CH_3SCH_2CH_2SO_2CH_2CH_2SOCH_3$

1 **2**

We sought an analytical method which would detect both of these metabolites in urine down to levels of 5 ng/ml or lower. Compounds containing sulphoxide groups often possess poor gas chromatographic properties, giving rise to badly tailing peaks. An additional problem with these particular compounds was the possibility of thermal elimination of the CH_3SOH moiety to give an olefinic species $-SO_2CH=CH_2$, a process which is observed in the mass spectra of these compounds. The high polarity of the sulphoxide group also makes isolation from aqueous matrices more difficult. We therefore sought to reduce the sulphoxide groups to sulphide, which would increase both lipophilicity and thermal stability, and enable both metabolites to be detected as the common easily extractable analyte 1,1′-sulphonylbis[2-(methylthio)ethane] (**3**).

$O_2S(CH_2CH_2SCH_3)_2$

3

We had already adopted a similar approach for the simultaneous detection of thiodiglycol and its sulphoxide, by reducing thiodiglycol sulphoxide to thiodiglycol using acidic titanium trichloride [7]. This reagent had been previously employed by Nishimura *et al.* [9] to reduce dimethyl sulphoxide to dimethyl sulphide. In this present paper we report a method, employing gas chromatography–mass spectrometry (GC–MS), which enables both of the β-lyase metabolites (**1** and **2**) to be detected in urine as the single analyte 1,1′-sulphonylbis[2-(methylthio)ethane], after reduction with acidic titanium trichloride.

EXPERIMENTAL

Materials

1,1′-Sulphonylbis[2-(methylthio)ethane] (**3**) was synthesised from 1,1′-sulphonylbis(2-chloroethane) and methanethiol. 1,1′-Sulphonylbis[2-(methylsulphinyl)ethane] (**1**) and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane (**2**) were prepared by oxidation of **3** with sodium periodate. 1,1′-Sulphonylbis[2-(trideuteromethylsulphinyl)ethane] was synthesised by an analogous procedure using [$^2H_3$]methanethiol. Full experimental details will be reported elsewhere [10]. Standard solutions were made up in methanol at concentrations of 0.1–10 μg/ml.

Titanium trichloride (15% solution in 20–30% hydrochloric acid) was purchased from Aldrich (Gillingham, UK). Fisons (Loughborough, UK) Distol-grade solvents were used. Chem Elut tubes and Bond Elut columns (Analytichem International, Harbor City, CA, USA) were purchased from Jones Chromatography (Hengoed, Mid Glamorgan, UK). Chem Elut tubes were washed with methanol (3 × 5 ml) and dried in a vacuum oven at 60°C prior to use. All glassware was pretreated with Aquasil siliconising fluid (Pierce & Warriner, Chester, UK).

Samples of human urine were collected from volunteer laboratory workers. Samples of rat urine were collected and supplied by Biology Division, CDE. They were stored at −20°C prior to analysis.

Preliminary experiments

Solutions of metabolites **1** or **2** (10 μg) in water (1 ml) were treated with titanium trichloride solution (0.5 ml) at 75°C for 15 min or at 40°C overnight [7]. The cooled solution was absorbed onto a 5-ml Chem Elut tube, extracted with dichloromethane (2 × 8 ml), and analysed by full scanning GC–MS. Recoveries were estimated by comparison with a standard solution of 1.1′-sulphonylbis[2-(methylthio)ethane].

Extraction and clean-up

Method A. To urine (1 ml) was added 1,1′-sulphonylbis[2-(trideuteromethylsulphinyl)ethane] (50 ng) as internal standard. Titanium trichloride solution (0.4 ml) was then added and the sample incubated at 40°C overnight (16 h). The sample was transferred to a 3-ml (500 mg) C_8 Bond Elut column (previously conditioned with 2 × 2 ml methanol and 2 × 2 ml water) mounted on a Vac Elut vacuum manifold (Jones Chromatography), and the solution allowed to seep through the sorbent bed under gravity; a light vacuum (120–250 mmHg) was then applied to remove unretained material. Partial clean-up was performed by washing the column with water (2 × 0.5 ml) followed by methanol–water (40:60) (2 × 0.5 ml) under gentle suction (*ca.* 50 mmHg), and the column then sucked dry under maximum vacuum (water pump) for 5 min. The analyte was eluted with acetone (2 × 0.5 ml) using gentle suction (*ca.* 50 mmHg), the eluate was transferred to a 1-ml vial and concentrated to dryness under a stream of nitrogen or using a centrifugal evaporator.

Method B. To urine (1 ml) was added 1,1′-sulphonylbis[2-(trideuteromethylsulphinyl)ethane] (50 ng) and titanium trichloride solution (0.4 ml) and the sample incubated at 40°C overnight (16 h). The sample was then transferred to a 3-ml Chem Elut tube connected in series to a 3 ml (500 mg) silica Bond Elut column (previously

conditioned with 2 × 2 ml dichloromethane–*n*-hexane, 40:60) mounted on a Vac Elut vacuum manifold. The analyte was extracted from the Chem Elut tube with dichloromethane–*n*-hexane (40:60) (2 × 5 ml) and the eluate passed directly through the silica column using gentle suction (*ca.* 50 mmHg). The Chem Elut tube was removed and the retained analyte eluted from the silica column with acetone (3 × 0.5 ml) using gentle suction (*ca.* 50 mmHg). The eluate was transferred to a 1-ml vial and concentrated to dryness under a stream of nitrogen or using a centrifugal evaporator.

The residues were dissolved in toluene (100 μl) before GC–MS analysis.

GC–MS analysis

Preliminary experiments were performed using a VG7070EQ double focussing magnetic sector mass spectrometer, coupled to a HP 5890 gas chromatograph. The analytical method was developed using a Finnigan 4600 GC–MS system. The gas chromatograph was fitted with a 25 m × 0.22 mm I.D. BP10 silicone bonded phase column (SGE, Milton Keynes, UK), film thickness 0.25 μm, inserted directly into the mass spectrometer ion source. Helium at 20 p.s.i. was used as carrier gas. Splitless injections (1 μl) were made, with a 4 mm injector insert, split delay 0.5 min, septum purge 2 ml/min; injector temperature 260°C. The GC oven temperature was held at 90°C for 0.5 min, programmed from 90 to 180°C at 20°C/min, from 180 to 240°C at 10°C/min and finally held at 240°C for 2 min. The GC–MS interface oven and transfer line were held at 260°C. The mass spectrometer was operated in the selected ion monitoring mode using positive ion chemical ionisation with ammonia as reagent gas; source pressure 0.6 Torr; source temperature 120°C. The ion source was operated at 120 eV with an emission current of 0.3 mA.

The retention time of 1,1′-sulphonylbis[2-(methylthio)ethane] was *ca.* 10 min 20 s and that for the internal standard 10 min 17 s. For detection and quantitation, the ion at *m/z* 232 $[M + NH_4]^+$ was monitored for 1,1′-sulphonylbis[2-(methylthio)ethane], and *m/z* 238 for the reduced hexadeuterated internal standard; the ion at *m/z* 184 was monitored for additional confirmation at levels above *ca.* 20 ng ml. Dwell time was 0.08 s, total scan time 0.3 s.

Quantitation was performed by comparing the computer integrated peak area for the ion *m/z* 232 at the appropriate retention time with that for the reduced hexadeuterated internal standard monitoring *m/z* 238. Calibration curves were established for samples of human and rat urine spiked with 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (**1**) at levels of 0, 2, 5, 50, 100, 250 and 500 ng/ml.

RESULTS AND DISCUSSION

Preliminary experiments

Preliminary experiments indicated that both of the metabolites **1** and **2** were unstable to GC; neither compound eluted from non-polar silicone bonded phase columns. In contrast, the bis-methylthio reduction product **3** possessed good GC properties, giving a narrow well shaped peak under the conditions employed. The reduction of the metabolites **1** and **2** to the bis-methylthio product **3**, using titanium trichloride in hydrochloric acid, occurred in >95% yield at a temperature of 75°C for 15 min, or alternatively at 40°C overnight (16 h). At the higher temperature some

leakage of the acidic reagent around the caps of the plastic micro-centrifuge tubes tended to occur and for convenience the lower temperature was adopted, as was used for the reduction of thiodiglycol sulphoxide to thiodiglycol [7].

Isolation and clean-up

Two methods were developed for the isolation of **3**. The first method is intended to be used for the analysis of urine specifically for these β-lyase products. The second method was designed so that it could be integrated into our procedure for determining the hydrolysis products thiodiglycol sulphoxide and thiodiglycol [7] using a single sample of urine. The procedure employed for the analysis of the combined hydrolysis products also involves an initial reduction with acidic titanium trichloride.

Preliminary extraction experiments indicated that liquid–liquid extraction of **3** from aqeuous solution with dichloromethane was inefficient (*ca.* 30% recovery from 1 ml of water using 3 × 2 ml portions of dichloromethane). Superior recoveries were achieved either by retention of the analyte on reversed-phase silica cartridges, or by extraction with dichloromethane after absorption of the urine onto a solid absorbent tube.

Method A. Various reversed-phase silica extraction columns retained **3** from aqueous solution. Recovery experiments from urine spiked with **1**, after titanium trichloride treatment and using acetone as eluent, indicated recovery to decrease in the order $C_8 > C_{18} >$ Ph > CH > CN, the more polar phases CH and CN giving poor recoveries. C_8 Bond Elut columns were selected for further method development. Elution experiments with 2 × 0.5-ml volumes containing increasing amounts (10, 20, 30% etc.) of methanol in water indicated 40% methanol to be the maximum concentration which did not elute the analyte, and this solvent was therefore used for partial removal of more polar extraneous materials from the column. Elution of the analyte could be achieved with methanol, acetone, acetonitrile or dichloromethane; acetone gave the most consistent recoveries. Six replicate recoveries from urine, spiked with 50 ng/ml of **1**, ranged from 82 to 94%, mean value 87.7%, with a coefficient of variation of 4.5%.

Method B. For the simultaneous determination of thiodiglycol and its sulphoxide, urine is treated with acidic titanium trichloride under conditions similar to those described above and is then absorbed onto a Chem Elut solid absorbent tube prior to extraction. Since the reduced β-lyase product **3** is considerably more lipophilic than thiodiglycol, selective extraction using a relatively non-polar solvent should be possible prior to the extraction of thiodiglycol with ethyl acetate. Dichloromethane extraction of the Chem Elut tube gave virtually quantitative recoveries of **3**. The polarity of the solvent was then gradually decreased by substituting increasing amounts of *n*-hexane; the optimum concentration which efficiently recovered **3** (*ca.* 90%), with minimal (*ca.* 10%) extraction of thiodiglycol, was hexane–dichloromethane (60:40). The eluate was partially cleaned up by retention of the analyte on a silica Bond Elut column which was then eluted with acetone. Six replicate recoveries from urine, spiked with 50 ng/ml of **1**, were in the range 83–103%, mean value 95.3%, with a coefficient of variation of 7.6%.

GC–MS analysis

Electron impact (EI) ionisation, methane chemical ionisation (CI) and ammo-

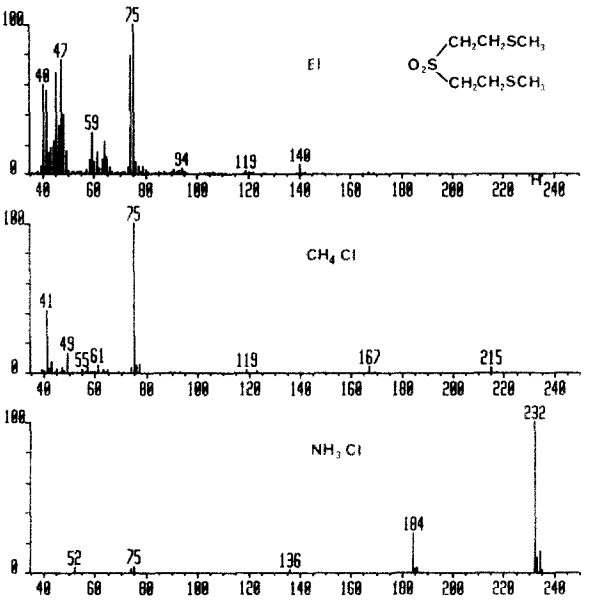

Fig. 1. EI, methane CI and ammonia CI mass spectra of 1,1′-sulphonylbis-[2-(methylthio)ethane].

nia CI positive ion mass spectra of **3** are shown in Fig. 1. Only ammonia CI gave a high intensity (100%) quasimolecular ion and was therefore adopted as the method of choice. Some variation in the ammonia CI mass spectra was observed when using the magnetic sector instrument, particularly as the source became dirty; ions at *m/z* 136, 138 and 140 became more intense and the quasimolecular ion weaker, especially with small amounts injected. Our experience with several metabolites of sulphur mustard has been that their mass spectra are sensitive to temperature and matrix effects, presumably due to thermal decomposition reactions. Such variability was minimal when using GC quadrupole MS, which can operate at much lower source temperatures. The $[M+NH_4]^+$ ion at *m/z* 232 was monitored for quantitation and the fragment ion at *m/z* 184, formed by loss of CH_3SH, for confirmation at levels above *ca.* 20 ng/ml. Signal-to-noise ratios for this ion were poor at levels of analyte below 20 ng/ml. Additional sulphur isotope containing ions at *m/z* 234 and 186 could also be monitored if required for additional confirmation. The ion at *m/z* 136 was unsuitable for detection due to the heavy background present in urine at this mass.

Quantitation

Method A gave a linear calibration over the range 10–500 ng ml for metabolite **1** spiked into human urine, with a correlation coefficient 0.999, slope 0.0246, slight curvature was observed below 10 ng/ml; quantitation in this lower range was performed against calibration points at 0, 2, 5 and 10 ng/ml. Normal blank human urine gave a clean background at the retention time of interest as shown in Fig. 2. Ten samples of urine, from seven subjects, were analysed and all found to be clear at the retention time for the analyte **3**. The limit of detection and quantitation, monitoring

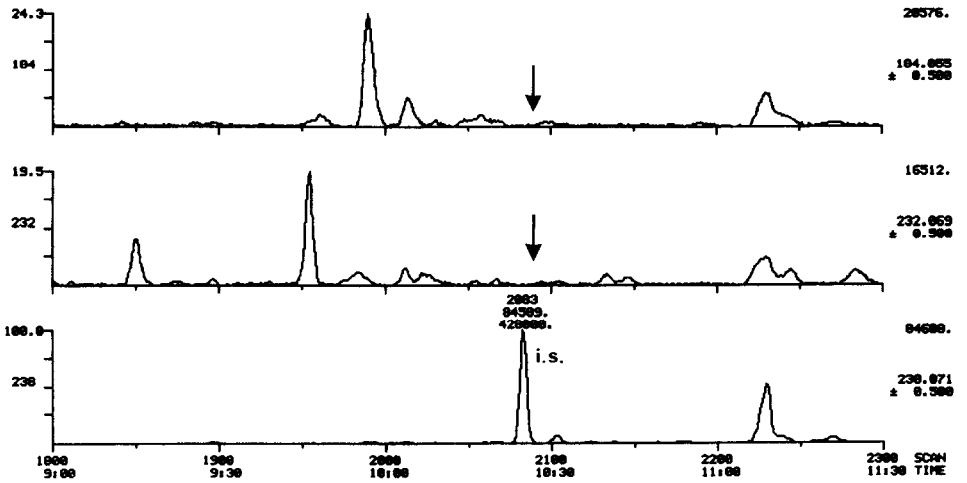

Fig. 2. Selected ion current chromatograms (method A) showing the absence of analyte in human urine monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

the quasimolecular ion and based on a signal-to-noise ratio of 3:1, was 2 ng/ml. The signal-to-noise ratio was lower for the ion at *m/z* 184 and monitoring this ion was useful only at levels above *ca.* 20 ng/ml. Fig. 3 shows selected ion current chromatograms from a sample of human urine spiked with **1** at a concentration of 50 ng/ml. The precision of the method was good; six replicate analyses of urine spiked at 50 ng/ml gave values of 48–52 ng/ml (Table I), mean value 49.8 ng/ml, with a coefficient

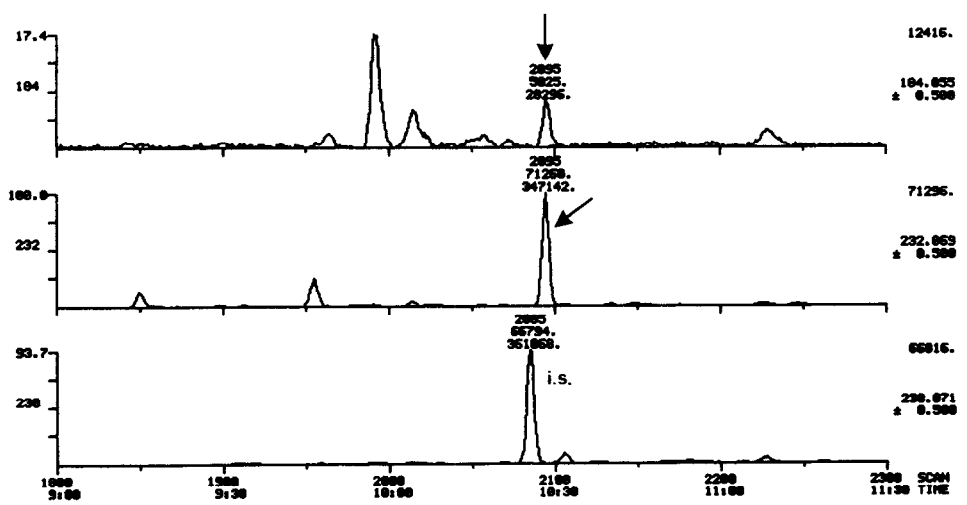

Fig. 3. Selected ion current chromatogram (method A) showing the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (50 ng/ml), spiked into human urine, monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

TABLE I

PRECISION DATA FOR HUMAN URINE SPIKED WITH 50 ng/ml OF I

	Recovery (ng/ml)	
	Method A	Method B
	48	51
	51	49
	52	50
	50	49
	49	51
	49	52
Mean	49.8	50.3
Coefficient of variation (%)	2.95	2.4
σ_{n-1}[a]	1.47	1.21

[a] σ_{n-1} = Standard deviation.

of variation of 2.95%. Rat urine gave a linear calibration over the range 5–500 ng/ml. Six replicate analyses of a sample of rat urine, collected 5 days after cutaneous dosing with 2 μmol of sulphur mustard, gave values of 28–32 ng/ml, mean value 30 ng/ml, with a coefficient of variation of 0.9%. No analyte was detected in control samples of urine from 4 rats. There were however many more extraneous peaks in the selected ion current chromatograms derived from rat urine. Fig. 4 shows selected ion current chromatograms from a sample of rat urine, collected 5 days after a cutaneous exposure to mustard (2 μmol), found to contain β-lyase metabolites (≡ 32 ng/ml of I.

Method B gave a linear calibration for human urine spiked with I over the range 10–500 ng/ml, with slight curvature below 10 ng/ml. The limit of detection and

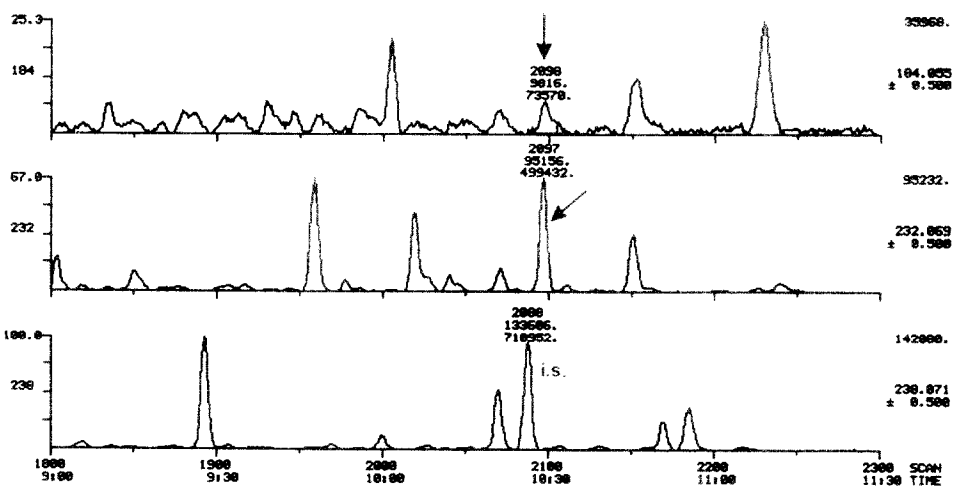

Fig. 4. Selected ion current chromatograms (method A) showing the detection of β-lyase metabolites (≡ 32 ng/ml of I) in rat urine, 4 days after a cutaneous application of sulphur mustard (318 μg), monitoring m/z 184 (upper) and m/z 232 (middle), and the response to the internal standard (50 ng/ml) monitoring m/z 238 (lower). Time in mins.

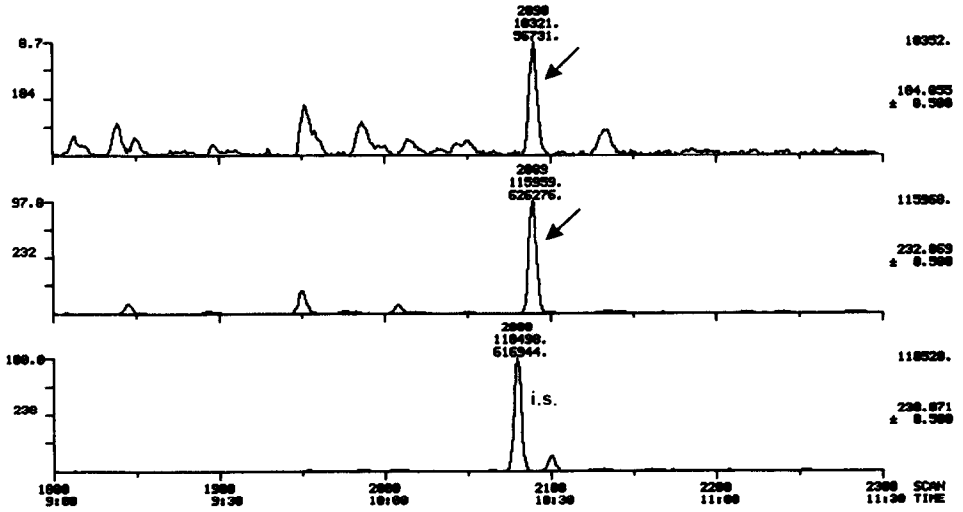

Fig. 5. Selected ion current chromatograms (method B) showing the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (50 ng/ml), spiked into human urine, monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

quantitation monitoring *m/z* 234 was 2 ng/ml. Again precision was good; six replicate analyses of urine spiked with **1** at 50 ng/ml gave values of 49–52 ng/ml (Table I), mean value 50.3 ng/ml, with a coefficient of variation of 2.4%. A selected ion current chromatogram showing the detection of 50 ng/ml of **1** in spiked human urine is shown in Fig. 5. As with method A, control samples of urine from seven subjects were all negative for the analyte. Rat urine gave a linear calibration over the range 2–500 ng/ml, and again control samples from 5 rats were negative.

Both methods provide sensitive analytical procedures for the detection of β-lyase-derived metabolites of sulphur mustard, down to levels of 2 ng/ml in urine. Method A gave marginally cleaner traces from blank urine samples although there was little difference around the retention time of interest. In contrast to the detection of the hydrolysis products, thiodiglycol and its sulphoxide, there was no indication of any background levels of analyte in normal human or rat urine. Method A is preferred for the analysis of samples from casualties, provided sufficient urine is available to allow several different assays to be performed. Method B has been employed for the quantitative measurement of the urinary excretion of **1** and **2** plus the combined hydrolysis products, thiodiglycol and its sulphoxide, following cutaneous administration of sulphur mustard to rats. The results of these studies will be reported in a separate paper [11].

The applicability of the general method to the retrospective identification of mustard poisoning will, of course, depend on whether man also metabolises sulphur mustard by the same pathway, and on the elimination profile. The latter is currently being determined in rats but the question of metabolism in humans will only be answered if suitable samples become available in the event of a chemical attack.

CONCLUSIONS

A sensitive and specific method has been developed for the detection of two urinary metabolites of sulphur mustard, which are assumed to be derived from the action of β-lyase on cysteine conjugates. The metabolites were detected as the single analyte 1,1'-sulphonylbis-[2-(methylthio)ethane] after reduction with titanium trichloride. In contrast to the detection of hydrolysis products of sulphur mustard, there was no indication of any background levels of analyte in human or rat urine. If man metabolises sulphur mustard by the same pathway, then the detection of these metabolites should provide firm evidence of exposure to mustard.

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