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Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography–tandem mass spectrometry

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Abstract

Gas chromatography–tandem mass spectrometry (GC–MS–MS) with selected-reaction monitoring was applied to the analysis of urinary metabolites of sulphur mustard, derived from the β-lyase pathway and from hydrolysis. In the case of β-lyase metabolites, a limit of detection of 0.1 ng/ml was obtained, compared to 2–5 ng/ml using single stage GC–MS with selected-ion monitoring. GC–MS–MS methodology was less useful when applied to the analysis of thiodiglycol bis(pentafluorobenzoate) using negative-ion chemical ionisation although selected-reaction chromatograms were cleaner than selected-ion chromatograms. The advantage of using GC–MS–MS was demonstrated by the detection of low levels of β-lyase metabolites in the urine of casualties who had been exposed to sulphur mustard.

1. Introduction

The recent use of chemical weapons in the Middle East [1,2] has stimulated the development of analytical methods for the confirmation of poisoning in casualties of chemical warfare. Recent studies in these laboratories have focussed on the development of analytical methods for the retrospective confirmation of poisoning by the vesicant sulphur mustard [$S(CH_2CH_2Cl)_2$]. Ten urinary metabolites of sulphur mustard were identified in the rat [3], seven of which were derived from an initial interaction of sulphur mustard with glutathione; two metabolites were derived from hydrolysis. Analytical methods, employing GC–MS with selected-ion monitoring (SIM), were developed for the detection of four of these metabolites in urine. Thiodiglycol [$S(CH_2CH_2OH)_2$] and its sulphoxide [$OS(CH_2CH_2OH)_2$], derived from the hydrolysis of sulphur mustard, were detected either separately [4,5] or as the single analyte thiodiglycol after reduction of the sulphoxide with titanium trichloride [5].

1, 1 - Sulphonylbis[2 - (methylsulphinyl)ethane] [$O_2S(CH_2CH_2SOCH_3)_2$] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane [$CH_3SOCH_2CH_2SO_2CH_2CH_2SCH_3$], two metabolites derived from the glutathione pathway after further metabolism involving the enzyme β-lyase, were reduced with titanium trichloride to the single analyte 1,1-sulphonylbis-[2-(methylthio)ethane] [$O_2S(CH_2CH_2SCH_3)_2$] [6].

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The occurrence of the above-mentioned metabolites in man has recently been demonstrated by the analysis of urine samples from two subjects accidentally exposed to sulphur mustard [7]. Limits of detection for these hydrolysis and β-lyase metabolites, using GC–MS-SIM, were in the range 1–5 ng/ml. However, at concentrations of analyte close to the limit of detection, the selected-ion current traces showed the presence of several other trace-level components with retention times within 1–2 min of those of the desired analytes, particularly for the β-lyase metabolites, thus limiting the specificity of the methods and the limits of detection obtainable. In real case samples this may impose serious limitations, since one of the problems commonly encountered with casualties of chemical warfare in remote conflicts is that the first urine sample may be collected several days after the exposure, when levels of urinary metabolites being excreted are low. An additional factor is that the casualties are likely to have been treated with various antibiotics and other medications [8] which produce additional excretion products in the urine, usually at much higher concentrations than the analytes of interest.

One of the few techniques which offers the possibility of improving both specificity and the limit of detection is tandem mass spectrometry (MS–MS) in combination with gas or liquid chromatography, as has been demonstrated in its application to the detection of drugs of abuse and many other trace analytes [9]. The specificity and sensitivity of GC–MS–MS has been demonstrated in the detection of chemical warfare agents spiked into air [10–12] and concrete [13], and in the analysis of environmental residues collected from the site of a chemical attack [14]. In this paper we report the application of GC–MS–MS methodology to the detection of metabolites of sulphur mustard in urine, and demonstrate useful improvements in comparison to the GC–MS methods previously reported from this laboratory. The application and advantage of GC–MS–MS methodology are demonstrated by the retrospective detection of β-lyase metabolites in urine samples collected in 1984 and 1988 from casualties of the conflicts in Iran and Iraq.

2. Experimental

2.1. Materials

1,1-Sulphonylbis[2-(methylsulphinyl)ethane], 1-methylsulphinyl - 2 - [2-(methylthio)ethylsulphonyl]ethane, 1,1-sulphonylbis[2-(methylthio)-ethane], thiodiglycol sulphoxide and the appropriate deuterated standards were synthesised as reported previously [15,16]. Thiodiglycol was purchased from Aldrich Chemical Co. (Gillingham, UK) and was redistilled before use. Fisons (Loughborough, UK) Distol grade solvents were used.

2.2. Samples

Urine samples were collected from five Iranian casualties of vesicant poisoning, who were undergoing treatment in the University of Ghent hospital in 1984 [8]. Details of the history, symptomatology and treatment of these patients (code-numbered C1–C5) have been published by Willems [8]. Two urine samples were collected on 30 March 1988 from two patients undergoing treatment in a London hospital, following a chemical attack on the Kurdish town of Halabja on 17 March 1988. The samples were stored at −20°C on receipt at CBDE, except for a single period of thawing and refreezing for thiodiglycol analysis.

2.3. Extraction and clean up

Urine samples (1 ml) were treated with acidic titanium trichloride to reduce thiodiglycol sulphoxide to thiodiglycol and the two β-lyase metabolites to the single analyte 1,1-sulphonylbis[2-(2-methylthio)ethane] [5,6]. Additional sample preparation was as reported previously [4–6] except that the final residue was dissolved in acetonitrile (5 μl) and toluene (45 μl) (instead of neat toluene) to improve solubility. Thio-

diglycol was converted to its bis(pentafluorobenzoate) derivative [4] for GC–MS–MS analysis.

2.4. GC–MS–MS analysis

Analyses were performed using a Finnigan-MAT TSQ 700 triple stage quadrupole mass spectrometer operated in the multiple-reaction monitoring mode, interfaced to a Varian 3400 gas chromatograph. All analyses of urine samples from casualties were preceded by a glassware/system blank.

1,1-Sulphonylbis[2-(2-methylthio)ethane]

Minor modifications were made to the conditions reported previously [6]. The gas chromatograph was fitted with a 25 m × 0.22 mm I.D. BP-10 column (SGE, Milton Keynes, UK), film thickness 0.25 μm, plus 50 cm × 0.22 mm I.D. BP-1 retention gap, film thickness 0.25 μm. The GC oven was held at 120°C for 1.5 min, heated from 120 to 260°C at 12°/min and held at 260°C for 15 min. Helium was used as carrier gas at 137 kPa. Injections (1 μl) were made using a septum programmable injector fitted with a buffered liner (Restek, supplied by Thames Chromatography, Maidenhead, UK), previously deactivated with dimethyldichlorosilane; the injector was held at 90°C for 0.5 min, heated from 90 to 260°C at 170°/min, and held at 260°C for 15 min.

MS–MS conditions were optimised to give maximum peak size. Ammonia was used as chemical ionisation (CI) gas, source pressure 800 Pa (gauge reading), source temperature 150°C, electron energy 120 eV, emission current 400 μA, electron multiplier 1500 V, sensitivity 10^{-8} A/V. Argon was used as collision gas at a pressure of 0.053–0.066 Pa, collision cell offset −10 eV. MS1 was tuned to transmit the quasimolecular ($M + NH_4^+$) ions of the analyte and internal standard and MS2 was tuned to detect selected product ions; reactions monitored were m/z 232→75 and m/z 238→78 (internal standard); dwell times were 0.25 s, with a 3-min delay before the filament and multiplier were turned on.

Quantitation was performed by comparing the integrated peak area for the product ion m/z 75 from 1,1-sulphonylbis[2-(methylthio)ethane] with the peak area for m/z 78 from the internal standard. A calibration curve was established using samples of normal human urine (1 ml) spiked with 1,1-sulphonylbis[2-(methylsulphinyl)ethane] at concentrations of 0, 0.1, 0,2, 0.5, 1, 2, 5 and 10 ng/ml, plus 1,1′-sulphonylbis-(trideuteromethylsulphinyl)ethane] as internal standard at a concentration of 5 ng/ml. Recoveries of β-lyase metabolites were determined in four replicate samples spiked with 1,1-sulphonylbis[2-(methylsulphinyl)ethane] at 1 ng/ml, by comparison of the peak area for m/z 75 with that from a blank urine extract to which an equivalent amount of 1,1-sulphonylbis[2-(methylthio)ethane] (0.87 ng) had been added.

Thiodiglycol and thiodiglycol sulphoxide

GC conditions for the analysis of thiodiglycol bis(pentafluorobenzoate) were as reported previously [4,5]. For GC–MS–MS, methane was used as CI gas, source pressure 930 Pa (gauge reading); other source conditions were as above. Argon was used as collision gas at a pressure of 0.13 Pa, collision cell offset +10 eV. MS2 was tuned to transmit the product ion m/z 167 ($[C_6F_5]^-$) and MS1 was tuned to detect the parent M^- ions m/z 510 and 514 (internal standard). Quantitation was performed by comparison of integrated peak areas as described above.

3. Results and discussion

3.1. β-Lyase metabolites

For the analysis of β-lyase metabolites, minor modifications were made to the GC conditions reported previously [6]. The use of a septum programmable injector minimised thermal degradation in the injection port, which was occasionally observed at the higher injection temperature used previously. The collision activated dissociation (CAD) spectrum of the quasimolecular ion m/z 232 ($[M + NH_4]^+$) of 1,1-sulphonylbis[2-

(methylthio)ethane] is shown in Fig. 1, together with a selected-reaction chromatogram, monitoring the fragmentation m/z 232→75, which demonstrates the sensitivity for a standard (100 pg injected). Under the conditions used, which gave optimum sensitivity, m/z 75 is the only characteristic product ion, formed by scission of the C–SO_2 bond; a protonated molecular ion at m/z 215 is also observed in the CAD spectrum resulting from loss of ammonia. The greater specificity of GC–MS–MS compared to GC–MS is shown in Fig. 2 which compares the selected-ion chromatograms for m/z 232, obtained using single-stage GC–MS-SIM from urine spiked at 1 and 10 ng/ml, with the selected-reaction chromatograms obtained using GC–MS–MS-SRM from urine spiked at 1 and 0.1 ng/ml. The chromatograms for analyte concentrations of 1 ng/ml demonstrate the dramatic improvement in the signal-to-noise ratio obtained using GC–MS–MS. No peak was observed in the GC–MS-SIM chromatogram at the appropriate retention time above the base-line noise, and a number of extraneous components appear in the chromatogram. In contrast, the GC–MS–MS-SRM chromatogram shows a signal-to-noise ratio of ca. 50:1 for the analyte peak with no other observable peaks over a retention window of 13 min. The chromatogram in Fig. 2 for urine spiked at 0.1 ng/ml illustrates the limit of detection using GC–MS–MS (based on a signal-to-noise ratio of 5:1), and even at this level no additional peaks were observed across the chromatogram.

The method gave a linear calibration over the range 0.1–10 ng/ml for 1,1-sulphonylbis[2-(methylsulphinyl)ethane] spiked into urine, with a calibration coefficient 0.9999 and slope 0.207. Good precision was obtained for urine spiked at

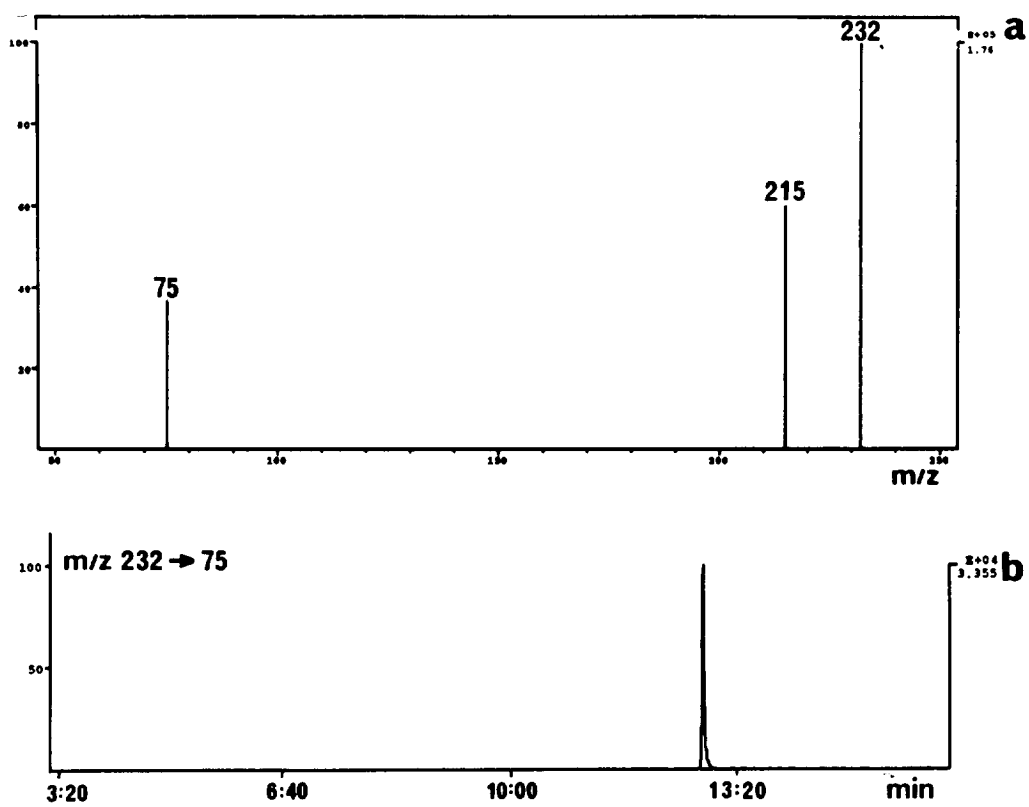

Fig. 1. (a) CAD spectrum of 1,1'sulphonylbis[2-(methylthio)ethane], m/z 232 (NH_3 positive CI); (b) selected-reaction chromatogram (m/z 232→75, monitoring the product ion) of a standard (100 pg injected).

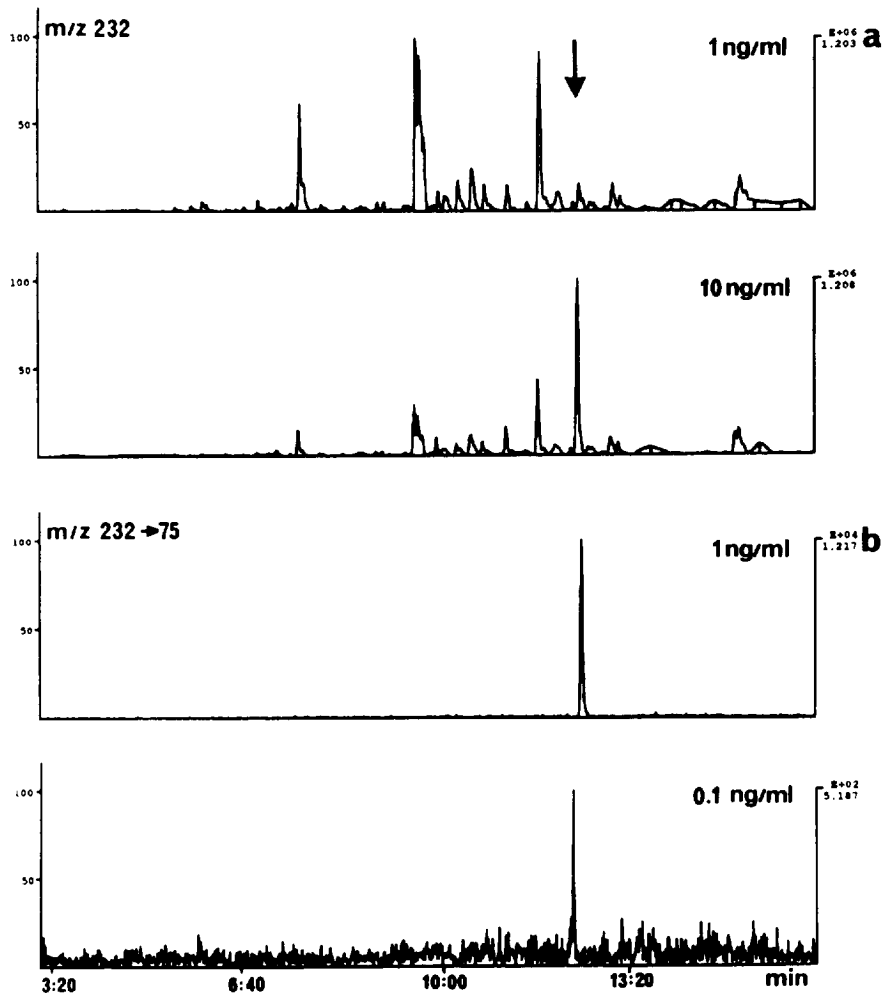

Fig. 2. (a) Selected-ion chromatograms, monitoring m/z 232 ($M + NH_4^+$), showing the detection of 1,1′-sulphonylbis[2-methylsulphinyl)ethane], spiked into human urine at 1 and 10 ng/ml; (b) selected-reaction chromatograms, m/z 232→75 monitoring the product ion, showing the detection of 1 and 0.1 ng/ml spiked into human urine.

1 ng/ml. Determined values ranged from 0.96 to 1.17 ng/ml, mean 1.09 ng/ml, coefficient of variation (C.V.) 6.4%; this compared with a C.V. of 2.95% obtained at 50 ng/ml using single-stage GC–MS-SIM [6]. Recoveries, determined in quadruplicate in normal urine spiked with 1,1-sulphonylbis[2-(methylsulphinyl)ethane] at a concentration of 1 ng/ml, were 48, 49, 51 and 56% compared with a mean recovery of 88% at levels of 50 ng/ml using GC–MS [6]. A possible limitation of the method is the absence of a suitable additional product ion for confirmation of identification. Monitoring the reaction m/z 232→215 (loss of NH_3) gave traces that were similar to SIM chromatograms. However, additional support for identification was obtained by substituting a BPX5 GC column, which gave a significantly reduced retention time for the analyte.

3.2. Thiodiglycol and thiodiglycol sulphoxide

The most sensitive GC–MS assay reported for thiodiglycol and its sulphoxide employs negative-ion chemical ionisation (NICI) of the bis(pentafluorobenzoate) derivative of thiodiglycol [4,5].

The ion current is concentrated almost entirely in the molecular anion, which provides good sensitivity and selectivity, but does not provide additional structurally characteristic ions for confirmation of identity. The CAD spectrum of the molecular anion of thiodiglycol bis(pentafluorobenzoate) is shown in Fig. 3. Not surprisingly, the product ion current is concentrated in the two ions $[C_6F_5]^-$ (m/z 167) and $[C_6F_5CO_2]^-$ (m/z 211) derived from the pentafluorobenzoyl moiety; the use of MS–MS therefore does not significantly increase the specificity of detection using NICI, and reaction monitoring of these product ions gave chromatograms that were not significantly cleaner than GC–MS-SIM chromatograms. However, monitoring the parent ion of m/z 167 provided excellent sensitivity as illustrated in Fig. 3 for 0.2 pg injected, and reduced the chemical background observed in the selected-ion current chromatograms as illustrated in Fig. 4 for Ghent urine sample C1. The true limit of detection for thiodiglycol and its sulphoxide was not determinable in urine, since normal urine contains very low background levels (usually in the range 1–12 ng/ml), mostly of the sulphoxide [5]. The use of GC–MS–MS in this case does not therefore dramatically improve the analysis although selected-reaction chromatograms are significantly cleaner than selected-ion chromatograms.

3.3. Application to the analysis of urine samples

The clear advantage of using GC–MS–MS was demonstrated by the analysis of urine samples which had been collected in 1984 and 1988 from

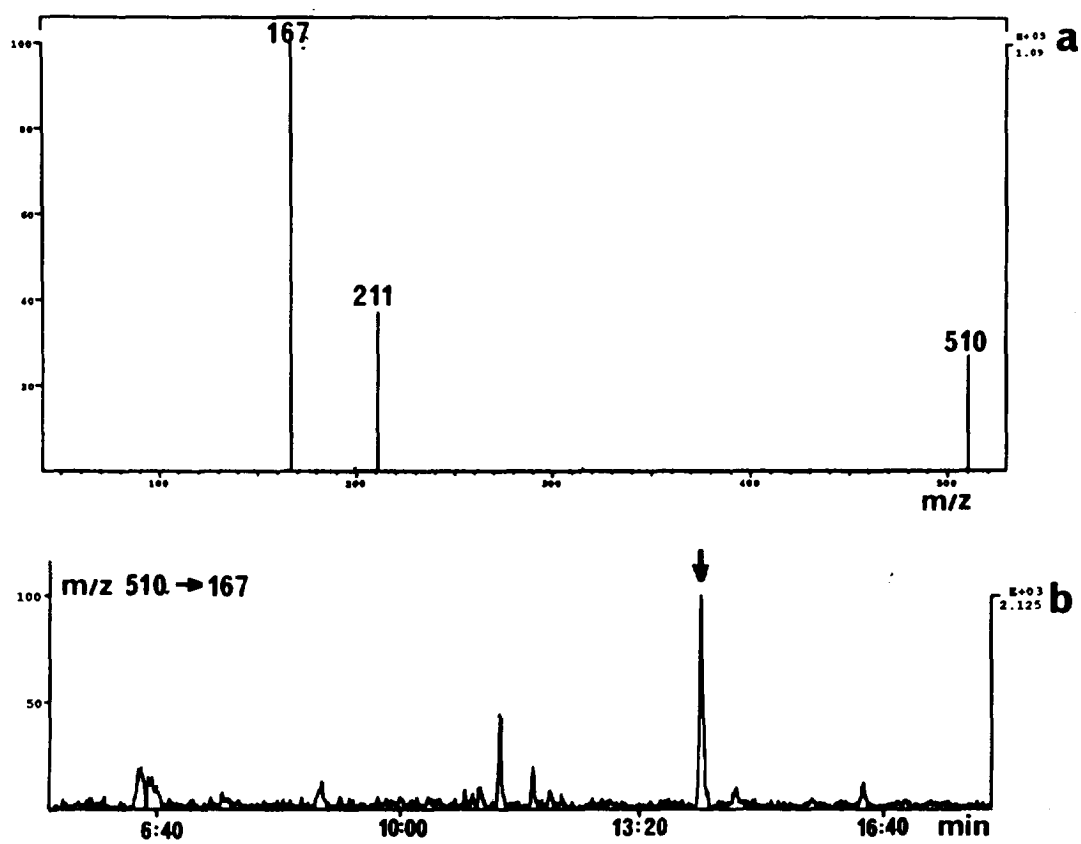

Fig. 3. (a) CAD spectrum of thiodiglycol bis(pentafluorobenzoate), m/z 510 (CH_4 NICI); (b) selected-reaction chromatogram, m/z 510→167 monitoring the parent ion, of a standard (0.2 pg injected).

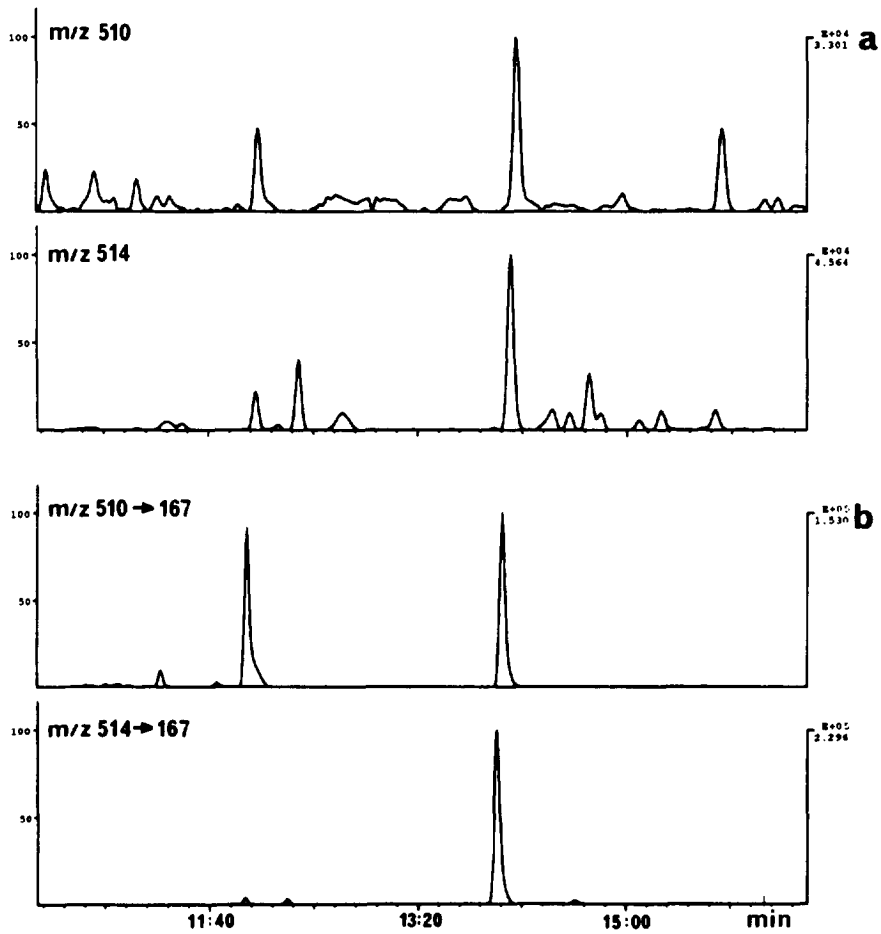

Fig. 4. (a) Selected-ion chromatograms monitoring m/z 510 (M^-) and 514 (M^-, internal standard), and (b) selected-reaction chromatograms monitoring m/z 510 and 514 as parent ions of m/z 167, showing the detection of thiodiglycol plus thiodiglycol sulphoxide (66 ng/ml) in Ghent sample C1.

casualties of vesicant poisoning; these samples had been stored for up to 10 years at −20°C. The urine had been collected approximately 10 and 13 days following an exposure to sulphur mustard, so levels of urinary metabolites were expected to be very low (in rats the major portion of a cutaneous dose was excreted within 48 h [17]). The results of the analyses are shown in Table 1. β-Lyase metabolites were detected in all seven of the urine samples; the preceding glassware blanks were all negative. In four of the five samples collected in 1984, levels of β-lyase metabolites (0.5–5 ng/ml) were close to or below the limit of detection of GC–MS-SIM. In the exceptional case of urine from Ghent casualty C1, very high levels were detected (220 ppb) and this subject died 24 h after the sample was collected [8]. Concentrations of β-lyase metabolites (0.1 and 0.3 ng/ml) close to the limit of detection were found in the urine from the two London patients. Even at these low levels the GC–MS–MS-SRM traces, such as that shown in Fig. 5, were surprisingly clean across a 13-min window although a single additional component was observed in the Ghent urine samples with a retention time ca. 90 s longer than that for the reduced β-lyase metabolites. With the exception of Ghent patient C1, the combined levels of

Table 1
Detection of hydrolysis and β-lyase metabolites of sulphur mustard in the urine of human subjects exposed to sulphur mustard

Subject	Thiodiglycol + sulphoxide (ng/ml)	β-Lyase metabolites (ng/ml)
Ghent C1	66, 72	220
Ghent C2	28, 27	0.5
Ghent C3	nd	1
Ghent C4	nd	5
Ghent C5	32, 34	1
London 1	11[a]	0.1
London 2	11[a]	0.3
Control	11	<0.1

[a] Thiodiglycol < 1 ng/ml.
nd = not determined due to insufficient sample.

thiodiglycol and its sulphoxide detected were higher than the levels of β-lyase metabolites detected. This observation is consistent with the excretion pattern observed in rats [17] where levels of hydrolysis products excreted were significantly higher than those of β-lyase metabolites from ca. 3 days after exposure, possibly due to slow release of covalently bound thiodiglycol from acidic sites on proteins and nucleotides. Unfortunately, the detection of thiodiglycol and its sulphoxide does not provide an unequivocal indication of exposure to sulphur mustard because of the low levels present in normal urine [4,5,18,19]. Separate aliquots of the urine samples from the Ghent patients were previously reported by Wils et al. [18] to contain thio-

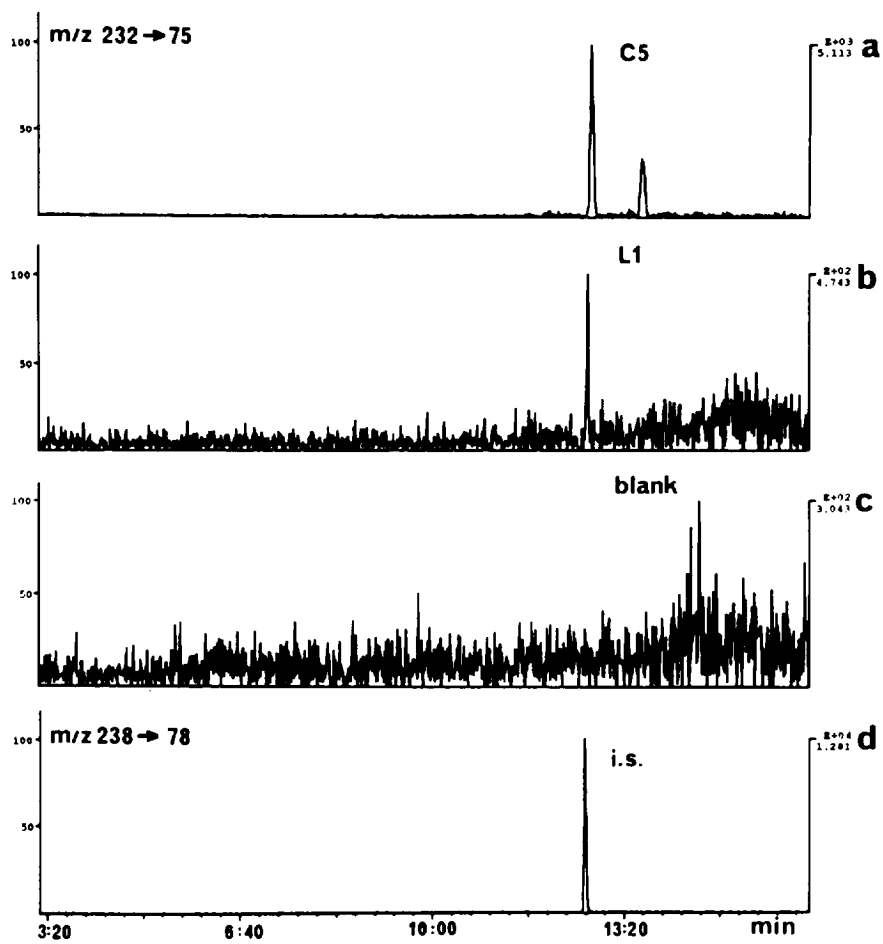

Fig. 5. Selected-reaction chromatograms showing the detection of β-lyase metabolites: (a) Ghent patient C5 (1 ng/ml); (b) London patient L1 (0.1 ng/ml); (c) glassware blank for L1; (d) internal standard (5 ng/ml).

diglycol at concentrations ranging from 15 to 90 ng/ml (samples were coded G1–G5 [18]), using a method which converts thiodiglycol back to sulphur mustard; levels of thiodiglycol up to 55 ng/ml were reported for control samples. In contrast, β-lyase metabolites of sulphur mustard have not been observed in normal urine and no interferences have been observed at the appropriate retention time using GC–MS–MS-SRM. The detection of β-lyase metabolites using GC–MS–MS methodology therefore provides a sensitive and unequivocal biological marker of exposure to sulphur mustard, for a period up to at least two weeks following exposure depending on the dose absorbed.

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