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DETECTION OF TRACE LEVELS OF THIODIGLYCOL IN BLOOD, PLASMA AND URINE USING GAS CHROMATOGRAPHY–ELECTRON-CAPTURE NEGATIVE-ION CHEMICAL IONISATION MASS SPECTROMETRY

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SUMMARY

A sensitive method has been developed for the detection and quantitative determination of thiodiglycol in blood, plasma and urine. Samples were extracted from Clin Elut columns and cleaned up on C_{18} Sep-Pak cartridges (blood, plasma) or Florisil Sep-Pak cartridges (urine). Tetradeuterothiodiglycol was added to the sample prior to extraction as internal standard. Thiodiglycol was converted to its bis-(pentafluorobenzoate) derivative and analysed by capillary gas chromatography–electron-capture negative-ion chemical ionisation mass spectrometry using selected ion monitoring. Levels of thiodiglycol down to 1 ng/ml (1 ppb) could be detected in 1-ml spiked blood and urine samples; calibration curves were linear over the range 5– or 10–100 ng/ml. Blood and urine samples from a number of control subjects were analysed for background levels of thiodiglycol. Concentrations up to 16 ng/ml were found in blood, but urine levels were below 1 ng/ml.

INTRODUCTION

The use of mustard gas, bis(2-chloroethyl) sulphide, in the Iran–Iraq conflict has stimulated renewed interest in the verification of mustard poisoning in victims of chemical warfare attacks. Although the isolation and detection of sulphur mustard immediately after spiking into blood or urine is relatively straightforward[1–3], the detection of free sulphur mustard in the body fluids of hospitalised casualties is unlikely, due to its chemical reactivity and extensive metabolism. A more fruitful compound for detection may be thiodiglycol, the hydrolysis product of sulphur mustard, which was shown by metabolism studies in rats to be excreted, either free or conjugated, as a minor metabolite in urine[4,5]. A method for the detection of thiodiglycol in urine was reported by Wils *et al.*[6], and a similar method has more recently been employed by Vycudilik[7] to analyse urine samples from casualties of chemical attacks. Since thiodiglycol is not easily isolated from aqueous media, both methods employed treatment of urine with concentrated hydrochloric acid to convert any thiodiglycol present to sulphur mustard. The latter was then recovered by adsorption from the headspace[6], or by steam distillation and extraction[7], and readily

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detected by gas chromatography–mass spectrometry (GC–MS). These methods enabled the detection of levels down to the equivalent of 10–20 ng of thiodiglycol in 10–20 ml aliquots of urine. They did not however discriminate between thiodiglycol, its conjugates, or indeed any other moiety which may convert to sulphur mustard on treatment with hydrochloric acid. An important finding reported by Wils *et al.*[6] was the detection of thiodiglycol, or other compound converting to mustard using the acid conversion procedure, in the urine of control subjects at levels up to 55 ng/ml. The identity of the endogenous compound present in urine was not determined, but the results indicated that the detection of thiodiglycol in urine using this method was ambiguous with regard to verification of exposure to sulphur mustard. In this present paper we report an alternative and very sensitive method of detecting thiodiglycol, which isolates the latter directly from biological fluids using a simple procedure. By appropriate pretreatment of the samples the method can discriminate between free thiodiglycol, conjugates sensitive to enzymatic hydrolysis, and other compounds hydrolysing to thiodiglycol under acidic conditions. The screening of ten control subjects for thiodiglycol is also reported.

EXPERIMENTAL

Materials

Thiodiglycol was purchased from Aldrich (Gillingham, U.K.) and redistilled before use. Standard solutions of thiodiglycol were made up in acetone at concentrations of 0.1–100 μg/ml. Fisons (Loughborough, U.K.) Distol-grade ethyl acetate and methanol, and Aldrich HPLC-grade toluene were used. For derivatisation pentafluorobenzoyl chloride (puriss) was purchased from Fluka Chemicals (Glossop, U.K.) and pyridine (Regis derivatisation grade) from Pierce and Warriner (Chester, U.K.). Florisil Sep-Pak and C_{18} Sep-Pak cartridges were purchased from Waters Assoc. (Northwich, U.K.) and were conditioned with methanol (5 ml) and ethyl acetate (5 ml) before use. Clin Elut columns (1003) were made by Analytichem International (Harbor City, CA, U.S.A.); they 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 and Warriner).

1,1,1′,1′-Tetradeuterothiodiglycol was prepared by reacting sodium sulphide with ethyl bromoacetate to give diethyl 2,2′-thiodiglycolate, followed by reduction with lithium aluminium deuteride. Purity was judged by MS to be >97%, containing < 0.1% of non-deuterated thiodiglycol. Samples of fresh urine and blood were collected from healthy male volunteers. Rat urine was collected over a 24-h period from two male Porton strain rats dosed intraperitoneally with sulphur mustard (1.58 mg/kg). Hydrolysis of conjugates was performed with β-glucuronidase type H-2 (Sigma, Poole, U.K.) (a crude solution of *Helix Pomatia*, possessing both β-glucuronidase and sulphatase activity), or with concentrated hydrochloric acid (BDH, Analar, Poole, U.K.).

Extraction and clean-up

Blood or plasma (1 ml), to which tetradeuterothiodiglycol (10 ng) in acetone (10 μl) was added, was absorbed onto a 3-ml Clin Elut tube, connected directly to a C_{18} Sep-Pak cartridge. The tube and cartridge were eluted with ethyl acetate (5 × 5 ml),

collecting the eluate in a 50-ml round bottomed flask. The extract was concentrated to *ca.* 1 ml on a rotary evaporator at 30°C, and then transferred to a 1-ml vial, rinsing the flask with methanol (0.5 ml). The combined extract and washings were concentrated to dryness under a stream of nitrogen at 40°C. If required this concentrate could be stored at −20°C.

Urine was treated similarly except that a normal-phase Florisil Sep-Pak cartridge was used in place of the C_{18} cartridge. For the enzymatic hydrolysis of conjugates, urine (1 ml) was buffered at pH 5 with 0.1 *M* sodium acetate–acetic acid (0.4 ml) and incubated with β-glucuronidase solution (0.1 ml) at 37°C for 24 h. Acid hydrolysis was performed by incubating urine (1 ml) with concentrated hydrochloric acid (0.1 ml) at 37°C for 48 h; the acid was then neutralised with 5 *M* sodium hydroxide. Extraction and clean-up were performed as above.

Derivatisation

To the dried residue from the clean-up was added pyridine (50 μl) and pentafluorobenzoyl chloride (10 μl). The mixture was vortexed and then stood at ambient temperature for 5 min. The solution was made up to 500 μl with toluene (440 μl), vortexed and centrifuged; aliquots (2 μl) of the supernatant were injected. This derivatised solution was stable for 4 weeks when stored at −20°C, but was usually analysed immediately.

GC–MS analysis

Analyses were performed using a Finnigan 4600 gas chromatograph–mass spectrometer. The gas chromatograph was fitted with a 25 m × 0.22 mm I.D. bonded-phase column coated with OV-1701, film thickness 0.25 μm (Thames Chromatography, Maidenhead, U.K.), inserted directly into the mass spectrometer source [additional analyses were performed using 12 m × 0.22 mm BP-5, BP-10 and BP-20 columns (SGE, U.K.)]. Helium at 15 p.s.i. was used as carrier gas. The oven was held at 90°C for 0.5 min, programmed from 90 to 230°C at 25°C/min, from 230 to 260°C at 4°C/min, and finally held at 260°C for 2 min. Splitless injections (2 μl) were made using a split delay of 0.5 min, septum purge 2 ml/min, split flow 50 ml/min; injector temperature, 265°C; transfer line temperature, 260°C.

The mass spectrometer was operated in the selected ion monitoring mode using negative ion chemical ionisation with methane as reagent gas; ion source pressure 0.8 Torr; source temperature 100°C; electron energy 150 eV; emission current 0.3 mA. M^- ions were monitored for thidiglycol bis(pentafluorobenzoate), *m/z* 510, and its tetradeuterated analogue, *m/z* 514; dwell time 0.157 s; total scan time 0.5 s. The retention time for thiodiglycol bis(pentafluorobenzoate) was *ca.* 10 min with the tetradeuterated analogue eluting 1.5 s earlier.

Quantitation was performed by comparing the computer integrated peak area for *m/z* 510 at the appropriate retention time with that for the internal standard monitoring *m/z* 514. Calibration curves were established in samples of blood and urine, previously shown to contain <1 ng/ml of thiodiglycol, spiked at concentrations of 1, 5, 10, 25, 50, 75 and 100 ng/ml. These calibration curves were shown to be superimposable, within the limits of experimental error, on calibration curves constructed from standard solutions of thiodiglycol and internal standard. A calibration was also established in a pooled plasma sample, found to contain 6 ng/ml of

endogenous thiodiglycol, and the calibration curve shown to be parallel to that constructed from standard solutions but with a positive offset corresponding to the background level. Because of the difficulty in obtaining truly blank samples, subsequent quantitation of unknowns was performed against a calibration curve constructed from standard solutions on the same day as the analysis. For similar reasons, recoveries were estimated using tetradeuterothiodiglycol by comparing the appropriate peak areas for 10 ng/ml spiked into blood and urine with those of standard solutions containing 10 ng. Glassware/reagent blanks were run as negative controls before the analysis of each unknown sample.

RESULTS AND DISCUSSION

The recovery and purification of low-molecular-weight water-soluble compounds from aqueous media presents a difficult problem. Generally a compromise between recovery and clean-up has to be accepted. In previous studies of water-soluble trichothecene mycotoxins we employed Clin Elut extraction columns to recover polar compounds from urine, followed by a chromatographic clean-up[9]. A similar though modified procedure was found suitable for thiodiglycol. Because of the moderate volatility of thiodiglycol, and the possibility of oxidation reactions or adsorption onto glassware, efforts were made to keep manipulations to a minimum. For blood and plasma, a sufficiently clean extract was obtained simply by passing the eluate from the extraction column directly through a reversed-phase silica C_{18} cartridge. For urine, which contains a high percentage of more polar materials, a normal-phase clean-up was preferred, a Florisil cartridge retaining the most polar components. Recoveries of tetradeuterothiodiglycol spiked at levels of 10 ng/ml were determined as 50–70% in

Fig. 1. NICI mass spectrum of thiodiglycol bis(pentafluorobenzoate).

blood, and 60–80% in urine (six determinations), the major losses being incurred at the extraction stage.

In our previous work[9] on the trace analysis of trichothecene mycotoxins, electron-capture negative-ion chemical ionisation (NICI) was found to give optimum sensitivity and selectivity for trace analysis in body fluids. We explored several possible derivatives of thiodiglycol which might be suitable for detection using NICI. The flophemesyl and heptafluorobutyryl derivatives gave very weak M^- ions. In contrast the bis(pentafluorobenzoyl) derivative was found to concentrate almost all of its ion current in the molecular ion, enabling great sensitivity to be obtained in the selected ion mode. Fig. 1 shows the NICI mass spectrum of thiodiglycol bis(pentafluorobenzoate). In contrast the electron-impact positive ion spectrum showed no molecular ion, the parent ion being *m/z* 239 ($C_6F_5COOCH_2CH_2^+$) with the non-informative ion *m/z* 195 ($C_6F_5CO^+$) also accounting for a large proportion of the ion current. Fig. 2 shows a selected ion current profile using NICI for 400 fg injected into the gas chromatograph. Although very weak additional ions are observable in the NICI spectrum, the concentration of the ion current into a single ion (plus isotopic ions) does present problems of confirmation of identification at trace levels. Raising the source temperature and pressure failed to induce fragmentation of the molecular ion. In this present work, to be confident that a compound we detected in control blood samples at the retention time of thiodiglycol was in reality thiodiglycol, we repeated the analysis using three additional GC columns of varying selectivity (BP-5, BP-10 and BP-20). In each case the compound eluted at the retention time for thiodiglycol bis(pentafluorobenzoate). Additional support for the identification was obtained from selected ion monitoring of *m/z* 510 at 5000 mass resolution using a VG 7070EQ magnetic sector instrument. The selected ion current profile at 5000 resolution, for a blood sample found to contain 13 ng/ml of thiodiglycol, is shown in Fig. 3.

The linearity of the method for quantitation was good over the range 5–100 ng/ml in plasma and urine, and 10–100 ng/ml in blood (linearity at higher levels was not assessed). The non-linear lower parts of the curve were consistent and quantitation at very low levels was performed using calibration points at 0, 1, 5 and 10 ng/ml. The calibration curve obtained for thiodiglycol spiked into urine is shown in Fig. 4. Regression analysis of the linear portion of the curve gave the equation y =

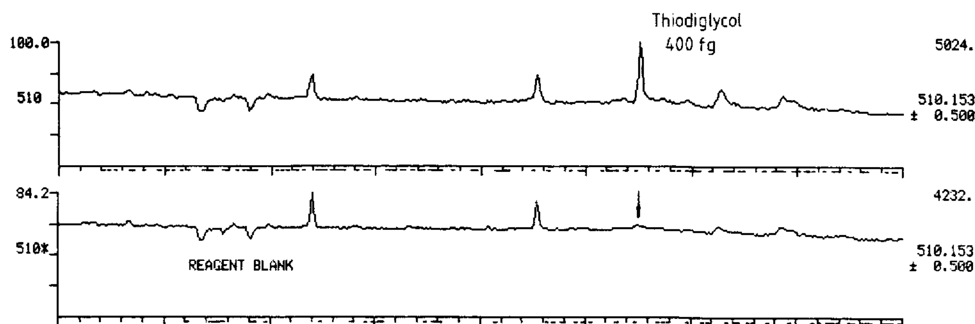

Fig. 2. Selected ion current profile monitoring *m/z* 510 for thiodiglycol bis(pentafluorobenzoate), equivalent to 400 fg thiodiglycol injected.

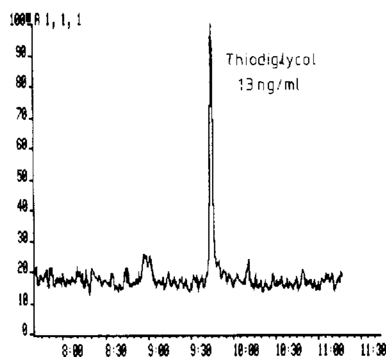

Fig. 3. Selected ion current profile monitoring *m/z* 510 at 5000 resolution, showing the detection of thiodiglycol (13 ng/ml) in a control blood sample.

$0.101x - 0.142$ ($r = 0.999$), compared to a calibration from standards, $y = 0.100x - 0.247$ ($r = 0.999$). The calibration curve for blood (obtained several weeks earlier) gave the equation $y = 0.149x - 0.694$ ($r = 0.999$), compared to a calibration from standards, $y = 0.150x - 0.555$ ($r = 0.998$). Moderate to good precision was obtained. Six replicate determinations on a sample of blood, found to contain a natural background of thiodiglycol determined as 12 ng/ml, gave a coefficient of variation of 5.3%. Four replicate determinations for samples of blood, previously shown to contain natural levels of thiodiglycol determined as 6 ng/ml and spiked with additional thiodiglycol at 50 ng/ml, gave a coefficient of variation of 4.6% (mean 53 ng/ml). Six replicate determinations for urine spiked at 20 ng/ml gave a coefficient of variation of

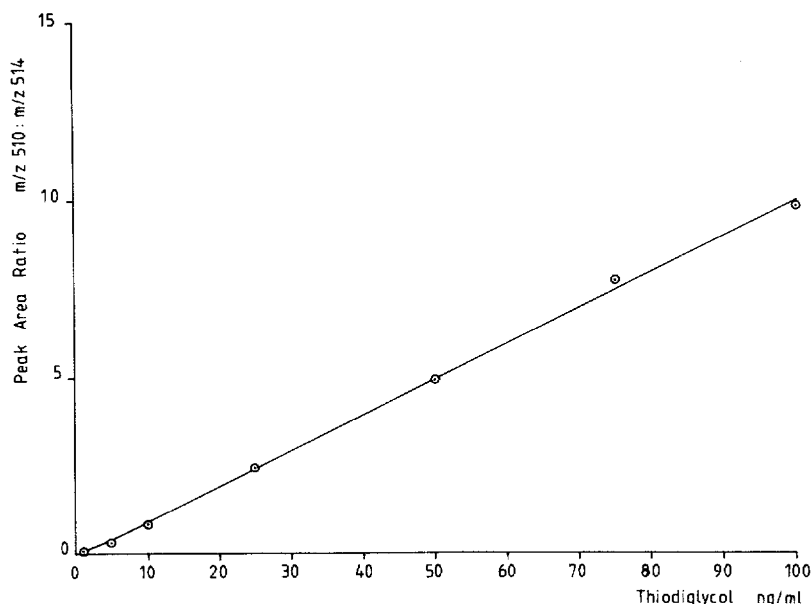

Fig. 4. Calibration curve for thiodiglycol in urine (slope = 0.101, correlation = 0.999).

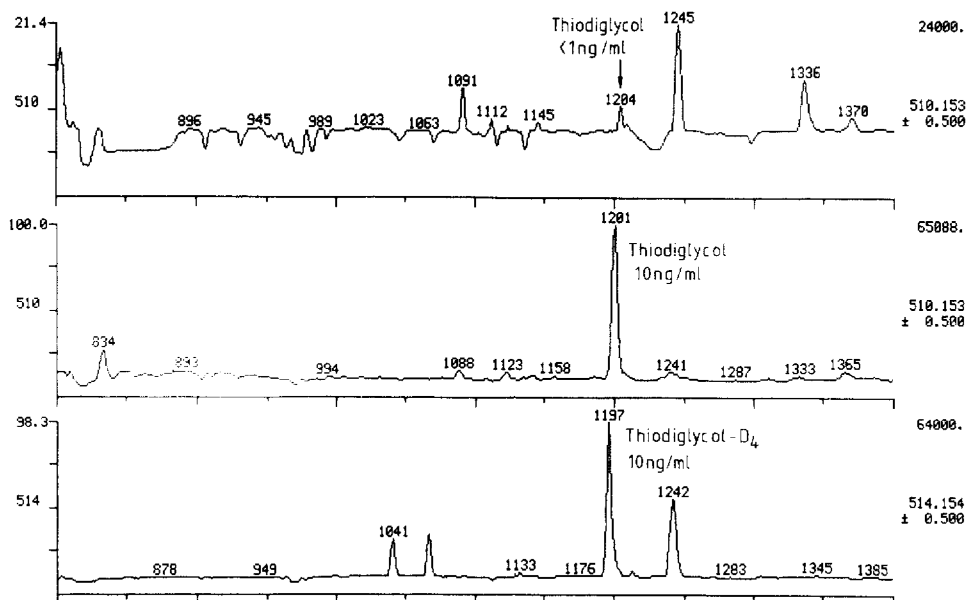

Fig. 5. Selected ion current profiles showing the detection of trace levels (< 1 ng/ml) of thiodiglycol in a control blood sample (upper), the same sample spiked with 10 ng/ml (middle), and the trace for the internal standard (10 ng/ml) (lower).

4.1% (mean 19.3 ng/ml). Based on a signal-to-noise ratio of at least 3:1 the method readily detected levels of 1 ng/ml spiked into 1-ml samples of blood and urine previously shown to contain < 1 ng/ml. Fig. 5 shows the selected ion current profiles for a sample of blood found to contain < 1 ng/ml of thiodiglycol and after spiking with an additional 10 ng/ml. The ion current for thiodiglycol is seen against a clean background (the data system has normalised the background for the unspiked sample to the highest peak). Plasma behaved similarly to whole blood. Fig. 6 shows selected ion current profiles for a sample of urine which contained no detectable thiodiglycol above the noise level (*i.e.* < 1 ng/ml), and the same sample spiked with 10 ng/ml of thiodiglycol. The background monitoring of the molecular ion of thiodiglycol was again relatively clean, but some background was observed in human urine monitoring the molecular ion for the deuterated internal standard; it did not however interfere with quantitation.

During subsequent work we attempted to extend the method to the pentafluorobenzoates of the sulphoxide and sulphone analogues of thiodiglycol. Under similar derivatising conditions the sulphoxide was found to convert in high yield to thiodiglycol bis(pentafluorobenzoate), a reaction which presumably involves a variation of the Pummerer rearrangement of sulphoxides; this reaction is being investigated. The method therefore does not distinguish between thiodiglycol and its sulphoxide, a possible metabolite. It can be extended to the sulphone whose bis(pentafluorobenzoyl) derivative also concentrates nearly all of its ion current in the M^- ion, m/z 542 using NICI. However peak shape was poor using similar GC conditions and sensitivity and detection limits were about an order worse.

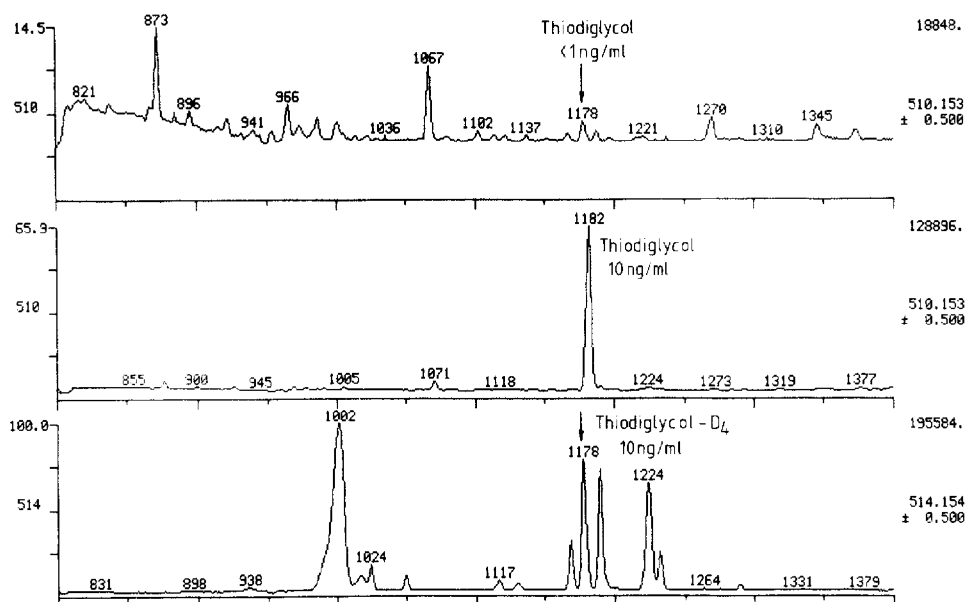

Fig. 6. Selected ion current profiles from a control urine sample (upper), the same sample spiked with thiodiglycol (10 ng/ml) (middle), and the trace for the internal standard (lower).

Samples of blood and urine from ten male volunteers were analysed for thiodiglycol. The results are summarised in Table I. Only two of the blood samples contained levels below 1 ng/ml, though both of these showed detectable peaks at the retention time for thiodiglycol (*e.g.* Fig. 5). Levels up to 16 ng/ml were detected, most being around the 10 ng/ml level. In contrast none of the urine samples, taken from the

TABLE I

ANALYSIS OF BLOOD AND URINE SAMPLES FROM CONTROL SUBJECTS

– = No sample.

Subject No.	*Thiodiglycol (ng/ml)*	
	Blood	*Urine*
1	13	<1*
2	11	<1
7	12	<1*
8	9	<1
9	16	<1*
10	10	<1
11	10	<1
12	6	<1
59	<1	–
60	<1	–

* Also <1 ng/ml after treatment with β-glucuronidase and conc. hydrochloric acid.

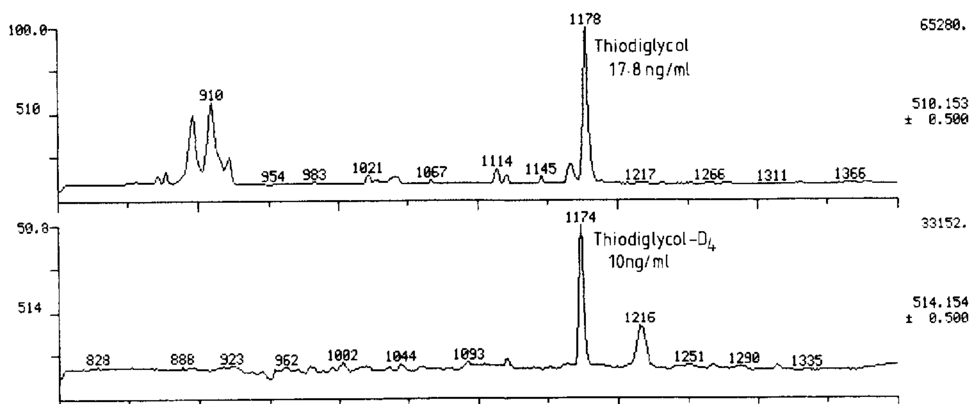

Fig. 7. Selected ion current profile showing the detection of thiodiglycol (18 ng/ml) in rat urine.

same volunteers at the same time as the blood samples, contained free thiodiglycol at levels above 1 ng/ml. Urine from subjects with the highest levels in blood was also analysed after treatment with β-glucuronidase (which also possesses some sulphatase activity), and after treatment with hydrochloric acid. Again levels of thiodiglycol present remained below 1 ng/ml. These differences between blood and urine levels may simply reflect dilution in urine though metabolism is an additional possibility. The analyses of urine samples from control subjects contrast with those reported by Wils *et al.*[6], who found levels up to the equivalent of 55 ng/ml. These differences may reflect variations in diet, or possibly result from the differing hydrolysis conditions used although treatment of our samples with hydrochloric acid at 95–100°C for 1 h gave results no different from those obtained when incubated at 37°C. Clearly a much larger number of control subjects will need to be analysed for thiodiglycol before any firm conclusions can be drawn about endogenous levels.

To demonstrate that the method could detect thiodiglycol in biological fluids after exposure to sulphur mustard, analyses were performed on samples of urine from two rats dosed intraperitoneally with sulphur mustard (1.59 mg/kg). No thiodiglycol was detected in the rat urine prior to dosing. Relatively small amounts of free thiodiglycol (18 ng/ml, both rats) were observed in the 24-h urine (Fig. 7). Much less background was observed in rat urine monitoring m/z 514 of the internal standard. Treatment of the rat urine with β-glucuronidase increased levels of thiodiglycol more than five fold (91 and 148 ng/ml), and treatment with concentrated hydrochloric acid more than 10 fold (256 and 388 ng/ml). Elimination profiles for thiodiglycol and its conjugates, and the identification of these conjugates and other metabolites present, are currently under investigation.

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