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Analysis of the sulphur mustard metabolites thiodiglycol and thiodiglycol sulphoxide in urine using isotope-dilution gas chromatography–ion trap tandem mass spectrometry

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Abstract

A sensitive method has been developed for the trace analysis of the sulphur mustard metabolite thiodiglycol (TDG) in urine, and its oxidation product thiodiglycol sulphoxide (TDGO) after reduction to thiodiglycol. Thiodiglycol was extracted from urine by solid phase extraction onto a polymeric cartridge and, after isolation, converted to its bis-heptafluorobutyryl derivative with heptafluorobutyryl imidazole. An ion trap mass spectrometer in selected reaction monitoring mode detected spiked concentrations down to 0.2 ng/ml with a signal to noise ratio > 3:1. Urine, from human volunteers with no known exposure to sulphur mustard, contained detectable but very low concentrations (<0.2 ng/ml) of thiodiglycol, consistent with previous observations using different methodologies. Combined concentrations of thiodiglycol and thiodiglycol sulphoxide were determined after reduction of the latter with titanium trichloride. In this case higher background levels (up to 3 ng/ml) were observed, consistent with the sulphoxide being the major excretion product of the two metabolites. The method was applied to urine samples, stored frozen for 13 years, from two casualties of accidental mustard poisoning. Levels of thiodiglycol were 1 and 3 ng/ml, which increased to 78 and 104 ng/ml after treatment of the urine with titanium trichloride.

Keywords: Sulphur mustard; Thiodiglycol; Thiodiglycol sulphoxide; Urine; Isotope-dilution; Gas chromatography; Ion trap tandem mass spectrometry

1. Introduction

1.1. Background

The development, production, stockpiling and use of chemical weapons is prohibited under the Chemical Weapons Convention [1]. In cases of alleged use of chemical warfare (CW) agents, environmental samples may be collected and analysed for agents and their degradation products as supporting evidence of a CW attack [2]. Biomedical samples, e.g. blood and urine, may be analysed for biological markers of poisoning as evidence that individuals have been exposed to a CW agent [3–5]. Such samples would also be collected for forensic purposes in the event of a terrorist release of a CW agent, e.g. following the 1995 Tokyo subway release of the nerve agent sarin [6]. Biomedical sample analysis also has applications in exposure monitoring, e.g. in individuals engaged in demilitarization activities, and for diagnosis of poisoning prior to the administration of medical countermeasures.

The blister agent sulphur mustard remains one of the CW agents of greatest concern, because of its advantageous physical properties and ease of production. A number of biological markers of exposure to sulphur mustard have been identified, including urinary metabolites and adducts with blood proteins and DNA [3–5]. The major urinary metabolites of sulphur mustard are derived from competing reactions with water and glutathione [7]. Both types of metabolite have been detected in urine collected from casualties of CW attacks and from individuals accidentally exposed to sulphur mustard [8–13]. Thiodiglycol (TDG), the simple hydrolysis product of sulphur mustard, and its oxidation product thiodiglycol sulphoxide (TDGO), are excretion products in rat and man [14,8–11], the sulphoxide appearing to be the major elimination product. Their disadvantage as urinary biomarkers is that trace levels, particularly of the sulphoxide, are present in the urine of non-exposed individuals [8,9,15–17], presumably from environmental or dietary

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exposure to TDG, or to a chemical that is metabolised to TDG and TDGO. Abnormal levels of these metabolites do, however, provide supportive evidence of exposure to sulphur mustard, as recently affirmed by a temporary working group of experts at the Organisation for the Prohibition of Chemical Weapons. Confirmation of exposure can be obtained by the parallel detection of β-lyase metabolites [10,18–20], derived from an initial reaction of sulphur mustard with glutathione. In this paper we report a sensitive method for the detection of TDG, and TDGO after reduction to TDG, using gas chromatography interfaced to a bench-top ion trap mass spectrometer.

1.2. Analytical methods for thiodiglycol

1.2.1. Sample preparation

Small, neutral, non-ionisable polar molecules are the most difficult to selectively isolate from aqueous solution. In our original procedure we used liquid extraction of TDG from urine absorbed onto diatomaceous earth (Chem Elut), followed by clean-up by elution through a C_{18} or Florisil cartridge [15]. This provided an effective method of isolation for TDG when combined with derivatisation with pentafluorobenzoyl chloride and negative ion chemical ionization mass spectrometry (NICI–MS), but the extract contained relatively large amounts of extraneous material. For the much more polar TDGO, the requirement for a very polar solvent for extraction from diatomaceous earth resulted in even larger amounts of extraneous material [16]. Because of the difficulty of selective isolation from urine, our preferred method for TDGO was to reduce it to TDG in situ using titanium trichloride [19]. A number of solid phase extraction (SPE) procedures have since been reported for the isolation of TDG, using C_{18}, carbon, and polymeric cartridges [21,17]. In this paper we report the effective use of a polymeric cartridge.

TDG and TDGO may be partially excreted as glucuronide conjugates, and treatment of urine with the enzyme glucuronidase may liberate significant additional amounts of the free metabolites [15,17].

1.2.2. Choice of derivative

TDG (for most applications) and TDGO require derivatisation prior to gas chromatographic analysis [22]. Trimethylsilyl or *tert*-butyldimethylsilyl derivatives are commonly used for environmental analysis, but generally do not provide sufficiently low limits of detection for biomedical sample analysis [23]. In our previous method for the analysis of TDG in urine [15], we converted TDG to its bis-pentafluorobenzoyl derivative using pentafluorobenzoyl chloride. This derivatising agent converts TDGO to the same derivative, reduction occurring during the derivatisation reaction [16]. The advantage of this method is that it is inherently the most sensitive for the analysis of TDG when combined with NICI–MS. Limits of detection in urine were ∼1 ng/ml using single stage GC–MS [15] and ∼0.1 ng/ml using GC–MS–MS [19]. A disadvantage of the method is that derivatised residues contain a high chemical background and can only be analysed for trace levels using NICI–MS. Jakubowski et al. [24] converted TDG to its bis-heptafluorobutyryl derivative using heptafluorobutyric anhydride (HFBA), which provided detection limits down to 1 ng/ml using electron ionisation and selected ion monitoring. HFBA and other acylating reagents, including heptafluorobutyryl imidazole (HFBI), were evaluated in our laboratory [25] and found to give much reduced chemical background in trace analysis compared to pentafluorobenzoyl chloride, particularly when combined with positive chemical ionisation (PCI) MS. We report the use of HFBI as derivatising agent below. More recently, HFBA has been used for combined TDG/TDGO measurements [17] in urine, after reduction of TDGO to TDG with titanium trichloride [16].

1.2.3. Instrumentation

Our original method for TDG [15] was based on GC–NICI–MS using a quadrupole mass spectrometer in selected ion monitoring (SIM) mode. Some enhancement in terms of reduced chemical background was obtained using GC–MS–MS and selected reaction monitoring on a triple sector quadrupole instrument [19]. For broader application, it is desirable that methods should be transferable to cheaper instrumentation rather than requiring expensive research grade mass spectrometers. For the present work we have utilized a bench-top ion trap mass spectrometer in MS–MS mode to improve selectivity. Ion trap instruments are particularly attractive for trace analysis because they offer the high selectivity of tandem mass spectrometry at moderate cost [26].

2. Experimental

2.1. Materials

TDG and titanium trichloride (15% solution in 20–30% hydrochloric acid) were obtained from Sigma–Aldrich (Gillingham, UK). TDGO and the deuterated internal standard TDG-d_4 [$S(C^2H_2CH_2OH)_2$] were synthesized by the Organic Chemistry Group, Dstl, Porton Down. Distol grade acetonitrile from Fisher Scientific Ltd. (Loughborough, UK), water from a Millipore Milli-Q water system, and heptane (HPLC grade) from Fisher Scientific Ltd. (Loughborough, UK) were used as solvents. HFBA and HFBI were obtained from Sigma–Aldrich (Poole, UK). Stock solutions of the analyte standards and internal standards were prepared in acetonitrile and stored in a refrigerator. For spiking urine samples the deuterated standards were prepared at a concentration of 1.0 μg/ml in acetonitrile. TDG and TDGO were also prepared at a concentration of 1.0 μg/ml and further diluted to 0.1 and 0.01 μg/ml for spiking at lower concentrations.

For samples treated with glucuronidase, glucuronidase H-2 crude solution from *Helix pomatia* (500 μl) from Sigma–Aldrich (Poole, UK) was dissolved in 0.1 M sodium acetate buffer solution, pH 5.0 (10 ml).

Urine samples were collected from six healthy male volunteers, with no history of exposure to sulphur mustard, and were stored at −20 °C prior to analysis. Pooled urine was created by mixing equal volumes of these six samples. Urine samples previously collected from casualties accidentally exposed to sulphur mustard from a 1914–1918 War munition [10] were also stored

at −20 °C and had previously been thawed and re-frozen several times.

2.2. Sample preparation

2.2.1. Preliminary experiments

In some preliminary experiments, a number of solid phase extraction cartridges were investigated for the isolation of TDG and TDGO from water. Direct LC–MS analysis of the extracts was used to determine recoveries. This work showed that considerable oxidation of TDG to TDGO can take place on commonly used SPE cartridges, particularly carbon-based cartridges.

2.2.2. Optimized procedure

Pooled urine samples (1 ml) were spiked with TDG at the appropriate level, and internal standard (10 ng), and left to stand for 2 h. The samples were absorbed onto 3 ml/60 mg Oasis HLB cartridges (Waters Ltd., Elstree, UK) conditioned with acetonitrile (1 ml) and water (1 ml). The SPE cartridges were then washed with water (500 μl) and dried under slight vacuum for 5 min. The cartridges were eluted with acetonitrile (2 × 0.75 ml). The extracts were carefully evaporated to dryness using a Thermo-Electron SpeedVac centrifugal vacuum evaporator (40 °C), and the residues re-dissolved in acetonitrile (200 μl). The derivatising agent HFBI (30 μl) was added and the samples heated at 50 °C for 30 min. After cooling, water (200 μl) was added to react with excess derivatising agent. The acetonitrile/water mixture was extracted with heptane (2 × 300 μl), the two fractions were combined, and concentrated to approximately 50 μl under a stream of nitrogen prior to analysis. The TDG-$(HFB)_2$ derivative (see Fig. 1) is relatively volatile and is subject to significant losses if the extract is concentrated to dryness. The derivatives were found to be stable when stored in the refrigerator at 4 °C for several days.

For combined analysis of TDG and TDGO, the internal standard TDG-d_4 (10 ng) was added to the urine samples (1 ml). Titanium trichloride solution (400 μl) was added and the samples were heated for 1 h at 75 °C [16]. The solutions were neutralized with 10 M sodium hydroxide solution (400 μl) and centrifuged (2000 × *g*) for 5 min. The supernatant was withdrawn and processed as described above.

In some experiments, urine samples (1 ml) were treated with a solution of the enzyme glucuronidase (250 μl) and incubated at 37 °C overnight.

2.2.3. Recoveries

To assess the recovery of TDG from the SPE cartridge, urine samples (1 ml), to which internal standard TDG-d_4 (10 ng) had been added, were spiked with 10 μl of a 1 μg/ml solution of TDG in acetonitrile (=10 ng/ml TDG) either before or after the SPE extraction process. The recovery, expressed as a percentage, was calculated as the peak area ratio for the sample spiked before SPE extraction with that after extraction. Derivatisation efficiency was determined by comparing peak areas for a spiked extract from a urine sample with that from the same quantity of analyte spiked directly into acetonitrile, derivatised, and processed through the remainder of the method.

2.2.4. Calibration

Calibration was carried out by spiking urine pooled from six volunteers with no known exposure to sulphur mustard with known quantities of thiodiglycol and the internal standard. The spiking concentration range was 0.2–20 ng/ml with internal standard concentration 10 ng/ml. A calibration graph was constructed by plotting the ratio of the peak areas for TDG and TDG-d_4 against the concentration of spiked TDG.

2.3. Gas chromatography–mass spectrometry

A Thermo-Electron GCQ ion trap GC–MS system was used for analysis. The GC was fitted with a 0.5 m fused silica IP deactivated guard column and a 30 m, 0.25 mm i.d., 0.25 μm film thickness Rtx-5MS capillary column (Thames-Restek UK Ltd., Buckinghamshire). The injector was fitted with a 4 mm internal diameter single gooseneck injector liner deactivated with dichlorodimethylsilane. The injector was operated in splitless

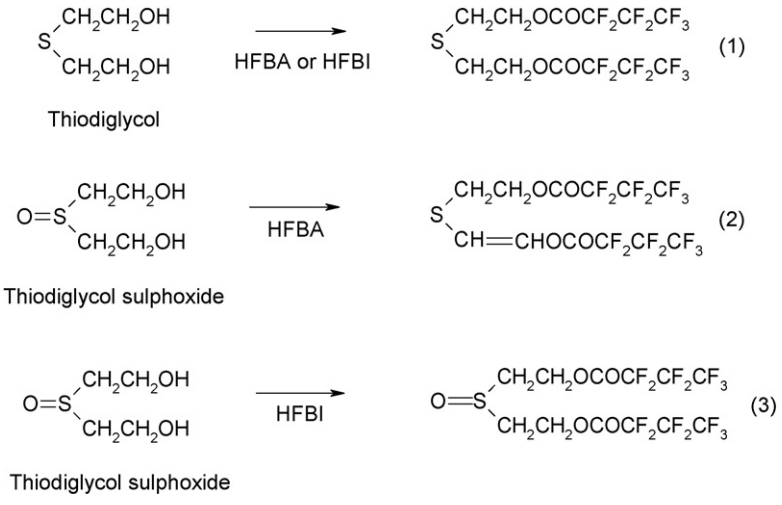

Fig. 1. Products from the reaction of TDG and TDGO with HFBA and HFBI.

injection mode at a temperature of 250 °C (purge delay 1.0 min). Auto-Sep T (SGE Ltd.) low bleed septa were used in the injector. The carrier gas was helium (constant velocity 40 cm/s). The oven temperature was held at 80 °C for 1 min, programmed from 80 to 280 °C at 20 °C/min and held at 280 °C for 2 min. An injection volume of 1 μl was used.

The ion trap mass spectrometer was operated using positive ion chemical ionization with methane as reagent gas. The ion trap was configured to operate in selected reaction monitoring (SRM) mode. In this mode a precursor ion is isolated in the trap and fragmented; only ions from a characteristic fragmentation are recorded. The precursor ions chosen for selected reaction monitoring for TDG and TDG-d_4 $(HFB)_2$ derivatives were *m/z* 301 and 305, respectively (isolation width = 3.0) and the respective product ions were *m/z* 241 and 243. The parameters excitation voltage and *q* value were optimised manually as follows. First scans were performed with excitation voltages of 1, 5 and 10 V. The excitation voltage giving the largest peak area was selected (in this case 1 V). A further experiment was then performed at excitation voltages of 0.5, 1.0 and 1.5 V. An excitation voltage of 1.0 V gave the largest peak area and was selected as the optimum value. Scans were then performed at a low, medium and high *q* value, with excitation voltage 1 V, from which low *q* value (0.225) was selected as optimal. The maximum ion time for each scan was 25 ms, excitation time was 16 ms, and the ion source temperature 180 °C.

3. Results and discussion

No satisfactory method is currently available that separately determines TDG and TDGO in urine samples. There are three obstacles to achieving this objective. Firstly, it is difficult to isolate TDGO, which is much more polar than TDG, from aqueous solution, other than by concentration to dryness or by use of a very polar solvent after absorption onto diatomaceous earth. Secondly, a small amount of oxidation of TDG to TDGO may occur either during SPE or derivatisation. Thirdly, TDGO may produce three different types of derivative according to the particular reagent used [21,25].

3.1. Sample preparation

A number of solid phase extraction procedures were investigated for TDG and TDGO, using direct LC–MS analysis for the determination of recoveries [27]. Carbon, which has been used previously as an absorbent for TDG [21], retained both analytes, but all of the carbon phases investigated resulted in some oxidation of TDG to TDGO (in extreme cases up to 60%). Similar behaviour was observed with polymeric phases based on styrene divinylbenzene copolymers. The Oasis HLB cartridge, which is a hydrophilic-lipophilic water wettable copolymer based on *N*-vinylpyrrolidone and divinylbenzene, showed no such oxidation and gave a good recovery for TDG as observed by LC–MS. Boyer et al. [17] have recently reported the successful use of this cartridge. It did not retain TDGO, but TDGO could be recovered from the void and wash from the cartridge if required; it is less susceptible to evaporative losses than TDG. More hydrophobic cartridges such as C_{18} showed variable recoveries of TDG and almost no retention of TDGO. Because of the lack of a satisfactory extraction procedure for TDGO, reduction to TDG with titanium trichloride remains our preferred method. Additional clean-up of the extracts was achieved subsequent to derivatisation, by liquid–liquid extraction into heptane. The combination of polymeric SPE recovery from urine, derivatisation with HFBI, and liquid–liquid extraction of the derivatised extract into heptane, produced a much cleaner extract than our previous method, which used extraction from diatomaceous earth and derivatisation with pentafluorobenzoyl chloride [15,16].

3.2. Derivatisation

The major reaction products identified from the reaction of TDG and TDGO with HFBA and HFBI are shown in Fig. 1. The reaction product of TDG with HFBA and HFBI under the conditions described is the bis-heptafluorobutyryl derivative TDG-$(HFB)_2$. For TDGO, the major reaction product with HFBA was derived from a Pummerer rearrangement, in which the sulphoxide oxygen reacts with the derivatizing agent, followed by rearrangement and elimination to give the olefinic derivative **2**. Small amounts of **2** were also observed in the derivatisation of TDG with HFBA, arising from oxidation; this became more significant at the low concentrations likely to be encountered in urine. In contrast, HFBI showed insignificant oxidation of TDG and afforded simple derivatisation of TDGO with retention of the sulphoxide function to give the bis-heptafluorobutyryl derivative **3**. Total ion current (TIC) chromatograms showing the products of the reaction of TDG and TDGO with HFBI are shown in Fig. 2. A very small amount of the oxidised product can be seen in the chromatogram for TDG.

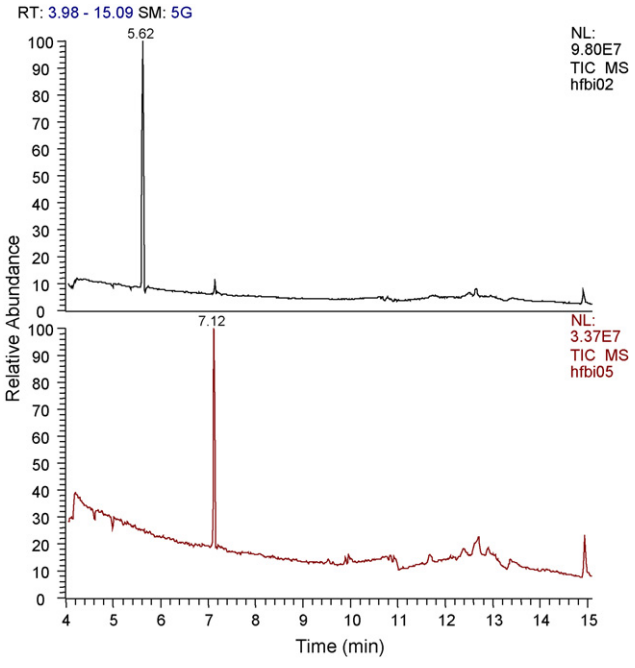

Fig. 2. TIC chromatograms resulting from the GC–MS analysis of the reaction products of TDG (top) and TDGO (bottom) with HFBI.

Table 1
Details of EI and CI (methane) ion trap mass spectra of heptafluorobutyryl esters of TDG and TDGO

Compound	EI *m/z* (relative abundance in parentheses)	CI *m/z* (relative abundance in parentheses)
TDG (HFBI)	241 (100), 85 (54), 86 (38), 301 (22), 69 (18), 300 (15), 242 (9)	301 (100), 241 (9)
TDGO (HFBA)	453 (100), 299 (88), 239 (41), 169 (35), 69 (27), 197 (25), 512 (24), 241 (17), 147 (17), 119 (13)	299 (100), 513 (24), 453 (23), 300 (10)
TDGO (HFBI)	241 (100), 169 (25), 69 (25), 213 (10), 119 (8)	317 (100), 531 (35), 241 (60), 301 (12), 345 (10)

HFBI has the further advantage that it does not produce acidic by-products, and the unreacted reagent is potentially less damaging to GC columns than HFBA. HFBI was therefore selected as the derivatising agent of choice. Similar products to **2** have been observed from TDGO derivatised with TFAA, and Pummerer derived products are also observed with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [22,25]. Simple derivatisation to the bis-trimethylsilyl derivative is observed with bis-(trimethylsilyl)trifluoroacetamide (BSTFA).

Relative abundances for the major ions in the mass spectra recorded for TDG and TDGO with HFBA and HFBI are shown in Table 1. The intense ion at $m/z = 301$ $[(CH_2)_2S^+CH_2CH_2OCOC_3F_7]$ for the TDG-$(HFB)_2$ derivative observed in CI mode allows for a highly sensitive analysis. In contrast, TDGO derivatives **2** and **3** gave spectra in which the ion current was distributed over a range of ions (even in CI mode), and this suggests that a less sensitive assay would be achievable.

3.3. Recoveries after SPE and derivatisation

The recovery, expressed as a percentage, was calculated as the peak area ratio for the sample spiked before SPE extraction with that after extraction. Derivatisation efficiency relates to a comparison of a spiked urine sample extract and a control sample in which the same quantity of analyte was derivatised directly in acetonitrile. SPE recovery for TDG was $52 \pm 4\%$ ($n = 3$) and the derivatisation efficiency $50 \pm 5\%$ ($n = 3$). The recovery was observed to be somewhat lower for the combined TDG/TDGO method (based on signal from internal standard) but was not calculated. A recovery of 28% has been reported by Boyer et al. [17].

3.4. Measurement of free TDG

The method showed good linearity over the range 0.2–10 ng/ml ($y = 0.1207x + 0.0009$ [$n = 9$]). TDG was just detectable above the background in the unspiked pooled urine sample and some of the individual samples, but was significantly lower than the lowest spiked calibration standard analysed (0.2 ng/ml). Selected reaction monitoring chromatograms for pooled urine spiked with 0.5 ng/ml TDG, and a sample from the volunteer giving the highest background reading of the six samples, are shown in Fig. 3. Further studies including the analysis of samples from the same person on several occasions have demonstrated similarly low levels of TDG. Although we have analysed relatively few samples we have not observed any samples with TDG levels in excess of 0.5 ng/ml. There is, however, a need for more data on background levels of free TDG in the European population. The background level of TDG was too low in our samples to measure any significant effect from pretreatment with the enzyme glucuronidase. The precision of the method was evaluated at a concentration of 10 ng/ml ($n = 3$) on two occasions. The coefficient of variation for each set of measurements was 6.2 and 5.0%.

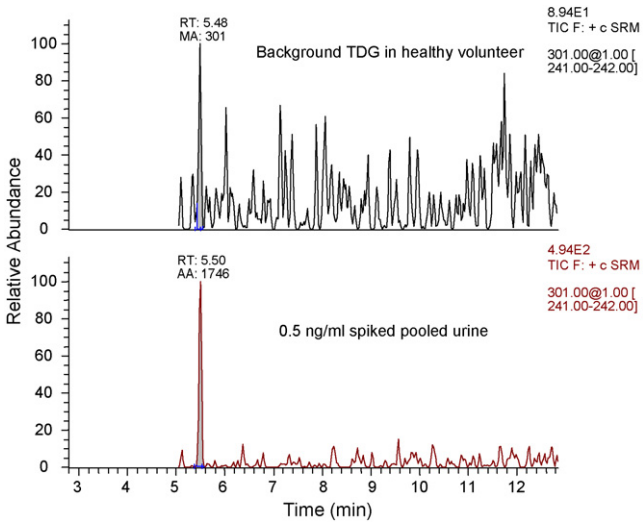

Fig. 3. SRM chromatograms derived from urine from a healthy volunteer and pooled human urine spiked with TDG (0.5 ng/ml).

Samples of urine from two surviving casualties of accidental sulphur mustard poisoning were analysed. The SRM chromatograms from one of these analyses are shown in Fig. 4. Levels of TDG in these samples were estimated to be 2 ng/ml (Subject 2) and 3 ng/ml (Subject 1) and are similar to those measured using a previous method [10]. Only a slight increase in the peak area ratios, from 0.24 to 0.27 and 0.41 to 0.43, respectively, was observed following treatment of the samples with glucuronidase.

3.5. Measurement of combined TDG/TDGO

Levels of combined TDG/TDGO, after treatment of urine with titanium trichloride, were measurable in all samples from healthy volunteers and ranged from 1 to 4 ng/ml. The average level was 2 ng/ml and agreed closely with the level recorded in the pooled sample (2 ng/ml). A linear calibration was obtained for the pooled urine spiked with TDG in the range 0.5–100 ng/ml ($y = 0.0779x + 0.343$ [$n = 8$]). The significant y intercept is consistent with there being low background levels, particularly of the sulphoxide, in human urine. The precision of the method was evaluated at a concentration of 10 ng/ml ($n = 3$) on two occasions. The coefficient of variation for each set of measurements was 9.2 and 9.4%.

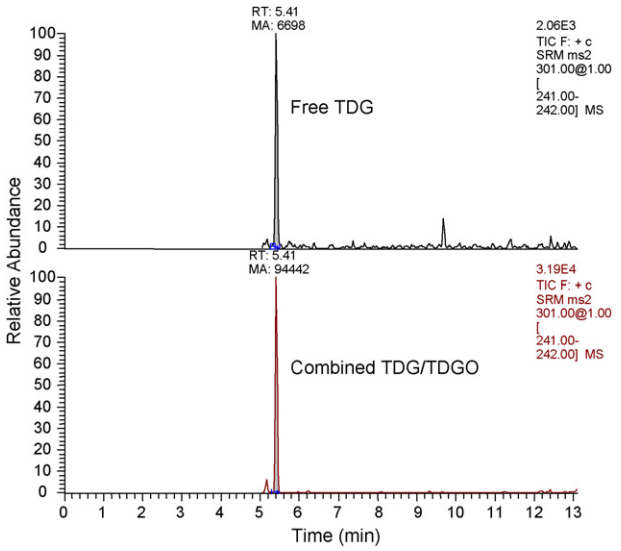

Fig. 4. SRM chromatograms derived from the urine of a surviving casualty of sulphur mustard poisoning (Subject 1) before (top) and after treatment with acidified titanium trichloride.

The samples from casualties of sulphur mustard poisoning [10], measured on two occasions against a calibration using spiked pooled urine, gave levels of 78 and 76 ng/ml (Subject 2) and 101 and 104 ng/ml (Subject 1). These values are higher than measured by a previous method in which the levels were 45 and 69 ng/ml, respectively [10]. In our previous method we isolated TDG by absorption onto diatomaceaous earth (Chem Elut) and elution with ethyl acetate/methanol [18]. The samples had been stored frozen for 10 years, with periodic thawing, between the analyses. In the absence of control samples stored over similar periods, it is not possible to explain the difference in these results. One possibility is that additional TDG/TDGO has been released from macromolecules over the storage period. The measurements support the findings made previously in our laboratory [10] and by Boyer et al. [17] that TDGO levels are higher than those for TDG in the general population and in casualties of sulphur mustard poisoning.

Within experimental error these values were unaffected by pre-treatment of the samples with glucuronidase. Peak area ratios were 7.1 and 6.6 (Subject 2) and 9.1 and 8.8 (Subject 1) treated with and without the enzyme, respectively. Fresh urine samples from a healthy volunteer treated with and without the addition of the enzyme glucuronidase, prior to analysis by the combined TDG/TDGO method, showed a small increase in signal in comparison to untreated samples. However, the background level in the samples was generally too low to provide accurate measurements.

3.6. Measurement of free TDGO

Derivatisation with HFBA and HFBI was investigated for the measurement of free TDGO in urine samples. The void and wash volume from the Oasis HLB cartridge was evaporated to dryness and derivatised. Neither derivative (**2** or **3**) transferred to the heptane phase in the liquid–liquid extraction part of the clean up and so samples were evaporated to dryness, dissolved in toluene and extracted with water to remove excess derivatising agent. Although satisfactory results were obtained in the recovery of TDGO from water samples, poor results were obtained from urine. This was also the case when a carbon cartridge was used to remove additional extraneous material from the urine extract.

4. Conclusions

The method described provides a rapid and sensitive procedure for the determination of thiodiglycol and its sulphoxide in urine. An alternative sample preparation procedure to that used previously in our laboratory provides good recovery and much cleaner extracts for introduction into the GC–MS. The use of an ion trap mass spectrometer provides the high specificity and resulting signal to noise ratios obtainable with tandem mass spectrometry at moderate cost in comparison to most other tandem MS instrumentation. The method was successfully applied to urine samples, previously found to contain thidiglycol and its sulphoxide, from two casualties of accidental sulphur mustard poisoning.

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