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Note

Quantification of thiodiglycol in urine by electron ionization gas chromatography–mass spectrometry

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Thiodiglycol (2,2′-thiodiethanol) has been classified as a major in vivo degradation product of bis(2-chloroethyl) sulfide (sulfur mustard gas, HD) [1,2]. Therefore, the presence of thiodiglycol in biological fluids should be evidence of mustard exposure. The analysis of urine samples from Iranian Chemical warfare casualties for thiodiglycol after alleged mustard exposure has been accomplished on two occasions [3,4]. Thiodiglycol levels were elevated in most of the exposed soldiers. However, in one study some of the control urine samples indicated levels of thiodiglycol higher than that of the exposed Iranians [3]. The method of analysis used in both verification studies was a headspace sampling gas chromatographic–mass spectrometric (GC–MS) technique that relied on conversion of the thiodiglycol back to the more volatile mustard. A more recent assay by Black and Read [5] demonstrated the existence of control urines with less than 1 ng/ml thiodiglycol. Analysis was by GC–MS using electron-capture negative-ion chemical ionization of the bis(pentafluorobenzoate) derivative.

Our objectives were to develop a simpler assay capable of verifying mustard exposure. The use of electron ionization in the positive-ion mode would increase the number of instruments capable of performing the assay. Another advantage to using electron ionization was the generation of structurally characteristic fragments of thiodiglycol that aided in identification of the analyte in the complex biological matrix. To validate the assay and sample handling

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procedures both rat and guinea pig models were used. The rats were exposed subcutaneously to mustard while guinea pigs received a vapor exposure. Also, the assay was used to gather control data on volunteer human urine levels of thiodiglycol. High control levels of thiodiglycol would render the assay useless in all but gross mustard contamination situations.

EXPERIMENTAL

Materials

The deuterated thiodiglycol was purchased from Ash Stevens (Detroit, MI, U.S.A.). Thiodipropanol, undeuterated thiodiglycol, nordihydroguaiaretic acid (NDGA), heptafluorobutyric anhydride, trifluoroacetic anhydride, and ethylene diaminotetraacetic acid were procured from Aldrich (Milwaukee, WI, U.S.A.). The glucuronidase with sulfatase activity was from a partially purified preparation of Helix pomatia purchased through Sigma (St. Louis, MO, U.S.A.). Molecular sieves (Davison, Baltimore, MD, U.S.A.) were 5-Å beads. Thiodiglycol sulfone and thiodiglycol sulfoxide were gifts from Dr. William Korte.

Sample preparation

To 1 ml of urine in a polypropylene vial add 1.0 μg of d_8-thiodiglycol and 1.0 μg of thiodipropanol (0.1 ml of a 10 μg/ml solution each). Note, if thiodipropanol has already been added as a preservative then more need not be added to the sample. Add 1000 U or more of β-glucuronidase with sulfatase activity and seal the vial. After sitting for 1 h at room temperature adjust the pH of the urine to 3–4 by using small amounts of 50% hydrochloric acid and test the resulting urine with pH paper. Note, if acid is added too quickly the sample may violently effervesce causing possible loss of sample. If the sample was over-acidified then 1 *M* sodium hydroxide was used to make the adjustment back to the proper range. Samples were evaporated at 90°C over a stream of nitrogen to near dryness (30–45 min). Several molecular sieves (two to five beads of grade 522, 5 Å size) were added to each vial along with 0.3 ml of ethyl acetate (stored over molecular sieves). Heptafluorobutyric anhydride (HFBA) (0.3 ml) was added to each sample and the entire mixture was sealed and allowed to sit for 60 min at 60°C. Derivatized samples were then centrifuged and the liquid phase was separated by pipet into a clean vial. The precipitate was washed with a mixture of 0.3 ml dry ethyl acetate and 0.1 ml HFBA, then centrifuged for 15 min. The combined liquid phases were then evaporated at 60°C over nitrogen to near dryness. The samples were then redissolved in 0.2 ml of dry ethyl acetate and subjected to GC–MS analysis.

Instrumental

An HP 5970B gas chromatograph–mass-selective detector (Hewlett-Packard, Avondale, PA, U.S.A.) was used in the selected-ion mode for detection of the following fragment ions: derivatized thiodiglycol at m/z 300 and 301, derivatized d_8-thiodiglycol at m/z 309 and 522, and derivatized thiodipropanol at m/z 328 and 542. Splitless injections of 1–2 μl were made using the following conditions: injector temperature, 220°C; column (DB5, 30 m$\times$0.25 mm I.D., 0.25-μm film) temperature, 100°C (0.5 min), then at 3.5°C/min to 140°C, then at 50°C/min to 250°C and hold (2 min); carrier gas, helium at 30 cm/s; ionization voltage, 70 eV. A standard curve of concentration versus peak-area ratio was prepared for 0, 1, 5, 10, 50, 100, and 500 ng of thiodiglycol per ml of urine using either 1.00 μg of d_8-thiodiglycol or thiodipropanol per ml of urine as the internal standard. Control urine was checked for background thiodiglycol before it was used to prepare the standard curve. The control urine was kept in the freezer to be used when necessary. In general, the control contained less than 1 ng/ml thiodiglycol to be usable.

Animal exposure studies

Thirty-six male rats were divided into six experimental groups of six rats each. Groups 1 and 2 were given a moderate subcutaneous dosage (750 μg/kg or one third of an LD_{50}) of mustard. Groups 3 and 4 were given a lower subcutaneous dose (450 μg/kg) of mustard. Groups 5 and 6 served as control groups and were not dosed with mustard. Urines were collected from the six groups at various time intervals by using metabolic cages. Collection continued until chemical analysis of the urine shows that the mustard breakdown product thiodiglycol is not significantly higher than that of the control groups or until a steady background has been achieved. During and after collection of urine from groups 1, 3, and 5 the urine was held on ice or refrigerated until the assays was performed. Half of the cold protected samples contained the chemical stabilizers as follows: a combination of 0.001% NDGA and 0.01% EDTA or NDGA alone. All urines contained 1.00 μg/ml thiodipropanol. Urine from groups 2, 4, and 6 were collected, also split in half, and left at room temperature until the assays were performed. Half of the collected samples that were not kept on ice contained the chemical stabilizers listed above. The collected urines were assayed to determine the concentration of thiodiglycol at various time intervals after dosing the rats with mustard.

Hairless guinea pigs were exposed to mustard vapor over 6 cm^2 of their back or periods of up to 8 min. Vapor exposure was by passive diffusion from mustard-coated pieces of filter paper placed into 1-cm^2 plastic caps. Up to six caps were then fixed onto the backs of the nude guinea pigs for a specified time period. Urine was collected 24 h after exposure by draining the bladder with a syringe. Urine sample size varied from 0.2 to 0.8 ml.

RESULTS AND DISCUSSION

The sample preparation scheme developed in this study was a significant departure from past thiodiglycol assays. Because of its polar and very hydrophilic nature thiodiglycol has been very difficult to extract from biological matrices. Our strategy was to derivatize the sample with excess HFBA after initial acidification and drying steps. Trifluoroacetic anhydride (TFAA) was also evaluated but its derivatives in this case were found to be less stable than the HFBA derivatives in solution. After derivatization, the thiodiglycol was more lipophilic and would extract into ethyl acetate. Amines and amino acids would form salts that would be insoluble in organic solvents. Moderately acidic solutions of thiodiglycol are stable to many reactions even at temperatures in excess of 100°C [6]. The extent of derivatization was measured to be 95% disubstituted, 4% monosubstituted, and 1% unreacted. Using this sample preparation scheme it was possible to quantify thiodiglycol from 5–500 ng in spiked rat or human urine in 3 h or less. Previously reported assays required 24–48 h or more to quantify samples. We have detected thiodiglycol down to 1 ng/ml in urine. However, the signal-to-noise ratio at 1 ng/ml was only 2–3. Furthermore, the regression curve representing data between 1 and 10 ng/ml was non-linear but reproducible. Therefore, a range from 5 to 500 ng/ml was considered to be the working range of the assay. The loss of linearity between 5 and 10 ng only slightly affected the standard curves that were generated. Since all exposed Iranian soldiers had thiodiglycol levels higher than 10 ng/ml a working range of 5–500 ng would be more than adequate.

The ability to assay the underivatized analyte was used to determine the extent of derivatization with HFBA. The relative abundance of the molecular ion of thiodiglycol ranged from 1 to 5% of the base ion at *m/z* 61. The $[M-18]^+$ ion was prominent in the thiodiglycol spectrum. In general, loss of HOR (R = H, HFB, TFA) produced prominent fragments in the mass spectra of all forms of thiodiglycol and thiodipropanol whether derivatized or not. Additionally, the loss of –OR produced significantly abundant ions for only the HFBA- and TFA-derivatized forms of thiodiglycol and thiodipropanol. The HFBA derivative produced analytically useful fragments at high molecular weights ranging from *m/z* 241 to 301. Fragments at *m/z* 300 and 301 represented the loss of one $-OCOC_3F_7$ group leaving most of the analyte molecule intact. Therefore the identity of the HFBA-derivatized analyte was confirmed by the characteristic fragments. However, the molecular ion of both the HFBA and TFA derivatives had low relative abundances (i.e. for the HFBA derivative the M^+ was only 10–20% of the base at *m/z* 241) as was also noted by Black and Read [5]. HFBA was still the derivative of choice in the analysis of thiodiglycol by electron ionization positive-ion detection despite the low molecular ion abundance. Fragments *m/z* 300 and 301 were structurally informative and showed high relative abundances of 70 and 50%, respectively. Analogous fragmenta-

tions of the deuterated internal standard produced ions at m/z 307 and 309. Molecular ions of HFBA derivatives could be used for quantification of 50–500 ng/ml thiodiglycol in urine. Spectra of the HFBA derivatives and the assay as a whole were reproduced on two other Hewlett-Packard MSD systems as a means of ensuring portability.

Before deuterated thiodiglycol was available, thiodipropanol was used as the internal standard. Table I presents an example of the precision of the spiked rat urine method when using thiodipropanol as the internal standard. Precision at 1 ng/ml was a very acceptable: relative standard deviation (R.S.D.) = 8.2%. At 500 ng/ml the precision (R.S.D.) was 1.5% Regression analysis of the curve using the mean values in Table I gave the equation $y = 0.00150x - 0.0115$ ($r = 0.999$). One disadvantage to the use of thiodipropanol was its late retention time (approximately 13 min) which limits assay output. Also, thiodipropanol reacted slower with the HFBA reagent than thiodiglycol which can cause major deviations in linearity if not taken into consideration. In general, stable-isotope internal standards have demonstrated superior attributes for MS assays. Precision improved slightly with the deuterated internal standard. Regression analysis of the data from the deuterated internal standard assay gave $y = 0.00423x + 0.00154$ ($r = 0.999$). Once the d_8-thiodiglycol was available, thiodipropanol was used as a stabilizer and to decrease binding effects of the analyte.

The best test of the assay was seen in the analysis of urine samples collected from rats after a subcutaneous injection or guinea pigs after vapor exposure of neat distilled mustard. Mustard at 750 and 450 μg/kg was injected into rats and produced no overt symptoms of exposure. Blood hematology appeared normal and the exposed rats gained weight at the same rate as the control group. Pathology performed on random rats indicated only a small region of redness at the site of injection which was the same for dosed versus control rats. Urine was collected and analyzed from both exposure groups and from a control group. All urines were collected over thiodipropanol. In addition, further stabilization

TABLE I

PRECISION OF PEAK-AREA RATIOS FOR THIODIGLYCOL IN SPIKED RAT URINE ($n = 5$)

Concentration (ng/ml)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0	0.006333 ± 0.00330	52.1
1	0.03096 ± 0.00255	8.24
10	0.04926 ± 0.00440	8.92
100	0.3022 ± 0.0175	5.80
500	1.874 ± 0 0275	1.46

was attempted by using one of the following treatments: (1) keeping the samples on ice as they were collected; (2) addition of the antioxidant NDGA; (3) a combination of NDGA and EDTA. One group of urines was collected and held at room temperature containing only thiodipropanol. Results for the 24-, 48-, and 116-h samples have been shown in Table II. These results were gathered before the deuterated internal standard was available; therefore thiodipropanol was the internal standard. The control groups did not contain measurable amounts of thiodiglycol. In general, the additional efforts to stabilize thiodiglycol beyond the inclusion of thiodipropanol provided no clear-cut increase in stability. Trace levels of thiodiglycol (1–15 ng/ml) could be measured for up to a week post-exposure in the rat.

Guinea pig vapor exposure produced thiodiglycol levels of 34.4–297 ng/ml (Table III). Validation of the thiodiglycol assay by using a vapor exposure model has not been previously reported. This greatly increases the confidence of verifying an actual exposure situation which would probably involve vapor exposure.

Thirty volunteer human urine samples were collected and assayed. Results indicated that levels were below the detection limits of the assay. These results agreed with those of Black and Read [5]. In our opinion, despite the limited survey of human control levels, the verification of mustard exposure is possible if samples can be stabilized and procured fast enough to ensure integrity. How-

TABLE II

THIODIGLYCOL LEVELS IN RAT URINE AFTER MUSTARD EXPOSURE

HD dose (μ/kg)	Thiodiglycol concentration (ng/ml)		
	20 h	48 h	116 h
750	196 ± 159	116 ± 76.6	17.2 ± 13.2
450	70.5 ± 19.5	60.6 ± 38.0	6.1 ± 1.4
0	<1	<1	<1

TABLE III

THIODIGLYCOL LEVELS IN GUINEA PIG URINE AFTER VAPOR MUSTARD EXPOSURE

Sample	Sample volume (ml)	Thiodiglycol concentration (ng/ml)
GP1	0.1	75
GP2	0.1	34
GP4	0.5	185
GP5	0.5	297
GP6	0.5	176

ever, a diversified set of control urines from many different cultures would aid in the validation of the assay.

Attempts to quantify the oxidation products of thiodiglycol by extending the HFBA assay were not fully successful. The thiodiglycol sulfoxide rearranged to produce the bis(HFB) derivative of thiodiglycol as was seen by Black and Read [5]. However, the yield appeared to be less than 30% as judged by the presence of three other peaks in the ion chromatogram that share common major fragments. We have been pursuing the structure of the three extraneous peaks. This was an advantage to using electron ionization with the HFBA derivatives. At least markers of the oxidized thiodiglycol products were visible and structural information could be gathered. Thiodiglycol sulfone did not produce any useful chromatographic peaks before or after derivatization under the conditions of our assay.

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