Quantitative Analysis of the Sulfur Mustard Hydrolysis Product Thiodiglycol (2,2'-Sulfobisethanol) in In Vivo Microdialysates Using Gas Chromatography Coupled With Pulsed Flame Photometric Detection

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Abstract

An analytical method employing gas chromatography is presented for assessing the concentrations of the sulfur mustard hydrolysis product thiodiglycol (TDG) in cutaneous in vivo microdialysates. The use of a pulsed flame photometric detector allows for selective detection of the analyte following solvent exchange and derivatization with heptafluorobutyric anhydride. Quantitative assessment is performed using thiodipropanol (TDP) as a surrogate internal standard. A linear relationship and a very significant correlation (r^2 = 0.9982) between the ratio of TDG and TDP concentrations and the ratio of the square root of peak heights is demonstrated. The suitability of the analytical method is verified by the evaluation of blank in vivo microdialysates spiked with known amounts of TDG. The limit of detection in microdialysates is 0.200 nmol/mL (24.4 ng/mL) and the limit of quantitation was 0.364 nmol/mL (44.4 ng/mL). The presented method provides selective, sensitive, rapid, and high-throughput analysis of microdialysates containing TDG, providing an efficient alternative for high-performance liquid chromatography and capillary electrophoresis techniques.

Introduction

Sulfur mustard (2,2'-dichloro-diethyl sulfide) is a potent alkylating substance and well-known chemical warfare agent employed several times in military operations during the 20th century (1). Even though it causes lethal intoxication less frequently than other warfare agents, injured victims develop local and systemic symptoms that are painful, incapacitating, and tend to require a prolonged healing period (2).

In an effort to provide a more thorough understanding of the fate of sulfur mustard in the skin, in vivo dermal microdialysis experiments are performed in our laboratory. In these investigations, the agent is delivered onto the surface of the skin of rats, and a small probe (placed intradermally) is used to sample interstitial fluid for solutes. The collected specimens are analyzed for thiodiglycol (TDG), a hydrolysis product of sulfur mustard (3).

A number of methods aimed at the analysis of TDG have been previously described. Although some of these methods employ liquid chromatography (4–7) [frequently considered the instrument of choice for analyzing microdialysates (8)] and the related technique micellar electrokinetic chromatography (9), the application of gas chromatography (GC) has been much more common. Various detection techniques have been used, GC with mass selective detection (MSD) being the most popular among them (10). TDG has been measured by all of these methods in environmental matrices including soil (11,12), metals (13), and concrete (14), as well as in biological samples such as blood (15), urine (16,17), and skin specimens (18,19).

A pulsed flame photometric detector (PFPD) was employed in this study for the detection of the sulfur-containing compound TDG. The PFPD is an improved flame photometric detector, allowing a time-programmable detection of the emitted and filtered light. As the time profile of light emission is characteristic for each element of the periodic table, the detection can be focused on signals originating from a specific element with high sensitivity and a very low background (20). An important feature of the PFPD is that when it is empoyed to detect sulfur, the PFPD response varies in proportion to the square of the change in the amount of sulfur reaching the detector.

Previously, PFPD has been employed successfully in the quantitative analysis of inorganic and organic sulfur-containing compounds in various matrices, including petrochemical samples (21), beer (22), food products (23), and water (24).

Experimental

Chemicals and gases

All substances used were of the highest available grade. TDG [bis-(2-hydroxyethyl)-sulfide], thiodipropanol [bis-(3-hydrox-

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ypropyl)-sulfide] (TDP), triethylamine, and heptafluorobutyric anhydride (HFBA) were procured from Sigma-Aldrich (Budapest, Hungary). SupraSolv-grade benzene*, SupraSolv-grade methanol, and 25% ammonia solution were obtained from Merck (Budapest, Hungary). Ethanol absolute was obtained from Reanal Finomvegyszergyár (Budapest, Hungary). All gases employed were at least 4.5 grade and were acquired from Messer Hungarogáz (Budapest, Hungary).

Preparation of standard solutions

Calibration solutions and samples for testing the suitability of the calibration curve (Figure 1) were prepared by dissolving TDG in benzene containing 0.05 mol/L triethylamine. Calibration solutions contained TDG at concentrations of 0.41, 0.82, 1.23, 1.64, 2.05, 2.87, and 3.69 nmol/mL (50.0, 100.0, 150.1, 200.1, 250.1, 350.1, and 450.2 ng/mL, respectively). For recovery studies from aqueous samples, 10.0-µL microdialysates collected in vivo were spiked with methanol solutions containing known amounts of TDG. Benzene solutions containing 1.0 nmol/mL (150 ng/mL) TDP and 0.05 mol/L triethylamine were used as the internal standard in all cases.

Preparation of nonaqueous samples

Aliquots (100.0 µL) of the TDG and TDP solutions were transferred to 2-mL silanized vials, and 10.0 µL of HFBA was added. Vials were shaken using a Velp Scientifica ZX3 instrument (Spektrum 3D, Debrecen, Hungary) at 10 Hz for 1 min and were kept at room temperature for 1 min. Aqueous ammonia solution (500 µL, 5%) was added to the mixtures, which were then vortexed at 40 Hz for 5 min. The benzene layers were used for the injections.

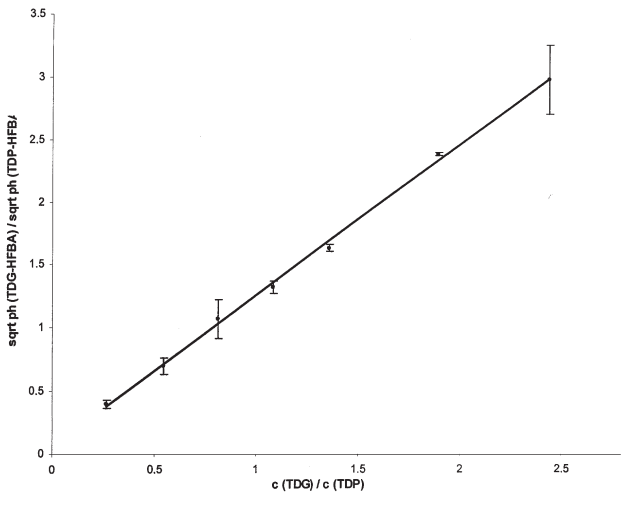

Figure 1. Calibration plot used for the quantitation of TDG in nonaqueous samples and microdialysates. The ratio of the concentrations of TDG and the internal standard TDP is plotted against the ratio of the square root of peak heights of TDG-HFBA and TDP-HFBA. For each calibration point, the mean of the values obtained from running three calibration solution series (indicated in Table I as A, B, and C) is plotted. Error bars represent standard deviation of these values. See text for further details. (Square root of peak height = sqrt ph.)

* Benzene can cause acute hepatotoxicity as well as cancer. Proper safety requirements must be considered when working with this chemical.

Preparation of in vivo microdialysates

A 10.0-µL aliquot of each microdialysate was spiked with 100.0 µL TDP solution. Ethanol absolute (350 µL) was added to the mixture, which was then evaporated to complete dryness at 40°C under a gentle stream of nitrogen using a Pierce Reacti-Therm Heating Module No. 18790 and a Reacti-Vap Evaporator No. 18780 (Pierce Co., Rockford, IL). Benzene (100.0 µL) containing 0.05 mol/L triethylamine was added to the residue and the rest of the sample preparation procedure was performed as described for nonaqueous samples.

GC conditions and data

A Varian CP-3800 GC and PFPD with an R647 photomultiplier were used (Varian Co., Middelburg, the Netherlands). Injections were made by a Varian CP-8400 autosampler. Separation was performed on a CP-Sil 8 CB low bleed/mass spectrometry column (30-m × 0.25-mm i.d., 0.25-µm film thickness). The carrier gas was hydrogen, and column flow was 1.0 mL/min. One-microliter splitless injections were made with the injector temperature set at 250°C and a split time of 2.0 min. The temperature program was 60°C, held 2.0 min, then ramp at 10°C/min to 175°C, then at 40°C/min to 250°C and held 1.0 min (total run time of 16.38 min). The detector temperature was 200°C, photomultiplier voltage was 490 V, gate delay was 6.0 ms, gate width was 20.0 ms, trigger level was 200 mV, and gain factor was 20. Air1 flow (combustor chamber) was set at 17.0 mL/min, Air2 flow (ignitor chamber) at 10.0 mL/min, and hydrogen flow at 11.0 mL/min. A sulfur filter (394 nm) was installed in the detector. A Varian Star GC workstation (version 6.20) software was employed for system control and data acquisition. Solvent runs were performed after every 10 sample runs in order to verify that no interference was produced by any substances contaminating the chromatographic system.

Calculating signal-to-noise ratio

The signal-to-noise ratio (s/n) was calculated by dividing the height of the peak of TDG by the root-mean-square noise acquired in a 30-s interval immediately preceding the retention window (defined as the retention time ± 6 s) of the analyte. Calculation of the noise was performed by the data acquisition software.

Limits of detection and quantitation

The limit of detection (LOD) was defined as s/n of 3.3, and s/n of 10 was considered to be the limit of quantitation (LOQ). The LOQ was 1.82 times (square root of 3.3) the LOD because of the quadratic response of the PFPD.

Quantitation

The ratio of the concentrations of TDG and TDP was plotted against the response (the response was calculated as the ratio of the square root of peak heights of TDG and TDP). The calibration curves were obtained by performing linear regression on the calibration points. The linearity of the calibration curves was tested by preparing three series of calibration solutions independently. Each calibration solution was analyzed three times, and the average of the three results was considered for further calculations. Each point of the final curve put to the linearity test was

obtained by taking the mean of the responses acquired for a given *x* value. Statistical evaluation was carried out using Microsoft Excel 2000 (Budapest, Hungary).

Results

Figure 2A shows a typical chromatogram of a processed cutaneous microdialysate containing bis[(2-*O*-perfluorobutyryl) ethyl]sulfide (TDG heptafluorobutyric ester) and the internal standard bis[(2-*O*-perfluorobutyryl)propyl]sulfide (TDP heptafluorobutyric ester). The retention times, as well as the shape and width of the chromatographic peaks of the derivatives, were found to be acceptable and reproducible with the employed instrument parameters. In solvent and method blank runs, peaks were absent in the retention window (retention time of peak ± 6 s) of the analyte and that of the internal standard (Figure 2B).

All detector parameters were set as recommended by the manufacturer. With these settings, the LOD for TDG was 0.20 nmol/mL and the LOQ was 0.36 nmol/mL.

The linearity of the relationship between the ratio of the concentrations of TDG and TDP and the response factors were tested as proposed in the literature (25). Figure 1 shows the tested calibration curve (see the Experimental section for details of obtaining the curve). Responses obtained for each calibration point in three parallel studies are displayed in Table I. A linear regression performed on the calibration points using the method of least-squares yielded a straight line with a slope of 1.1992 ± 0.0225 and an intercept of 0.0545 ± 0.0313. The determination coefficient of the curve was 0.9982, indicating a very good linearity of the relationship between the dependent and independent variables. The standard deviation of residuals was 0.0421. Residuals were distributed randomly above and below the fitted line, supporting that the linear fit was reasonable. The *y*-intercept was statistically indistinguishable from zero ($p = 0.05$).

In order to verify the suitability of the calibration process, three solutions containing 0.615, 2.131, and 3.443 nmol/mL TDG each (75.0, 259.9, and 420.1 ng/mL, respectively) were processed and analyzed. The concentration of TDG in these solutions was calculated using the calibration curve shown in Figure 1. The deviation

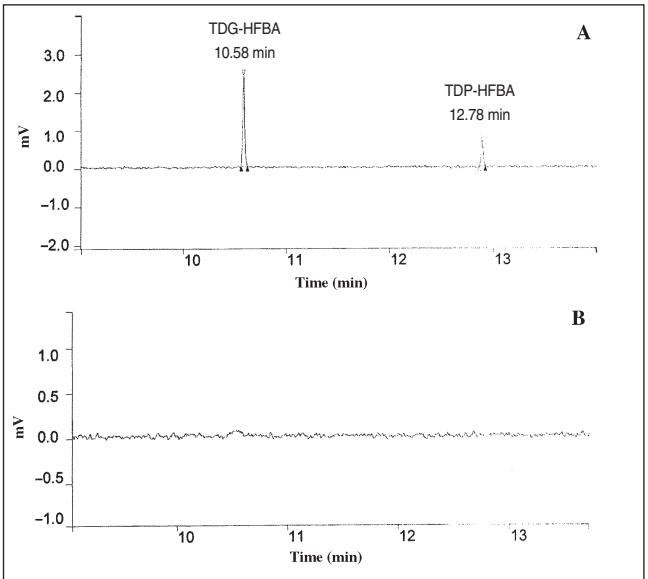

Figure 2. A typical chromatogram of a microdialysate containing 1.64 nmol/mL TDG and spiked with 1.0 nmol/mL TDP (internal standard) (A), and chromatogram of a blank microdialysate (B). No interference was ever observed in the retention window of either the analyte or the internal standard.

Table I. Statistical Evaluation of Detector Responses For the HFBA Adducts of TDG and TDP, Obtained in Three Calibration Solution Series*

c (TDG)/c (TDP)	sqrt ph† (TDG-HFBA)/sqrt ph† (TDP-HFBA)				
	Series A	Series B	Series C	Mean ± SD‡	RSD§ %
0.27	0.37	0.43	0.38	0.39 ± 0.03	8.17
0.54	0.68	0.77	0.64	0.70 ± 0.07	9.56
0.81	1.21	1.09	0.9	1.07 ± 0.16	14.65
1.08	1.37	1.32	1.27	1.32 ± 0.05	3.79
1.36	1.66	1.61	1.61	1.63 ± 0.03	1.77
1.89	2.39	2.37	2.38	2.38 ± 0.01	0.42
2.44	3.24	2.99	2.69	2.97 ± 0.28	9.26

* The mean values and standard deviations were employed to produce the plot in Figure 2. Each calibration value was provided by taking the average of the responses obtained in three replicate measurements.
† SQRT PH = square root of peak height.
‡ SD = standard deviation.
§ RSD = relative standard deviation.

Table II. Calculated Concentrations of TDG in Nonaqueous Samples Evaluated to Verify the Suitability of the calibration Plot Displayed in Figure 1*

	Sample 1	Sample 2	Sample 3
Calculated concentration (nmol/mL)	0.628	2.096	3.193
Nominal concentration (nmol/mL)	0.615	2.131	3.443
Error (%)†	2.11	1.64	7.26

* Analysis was performed on HFBA adducts.
† Error represents the deviation of calculated from theoretical concentrations.

Table III. Repeatibility of the Quantitation of TDG Spiked in Blank In Vivo Microdialysates

Sample number	Concentration found (nmol/mL)	Error %*
1	0.818	1.09
2	0.783	5.32
3	0.817	1.21
4	0.813	1.69
5	0.769	7.01
Mean (nmol/µL)	0.800	
Standard deviation (nmol/µL)	0.022	
RSD (%)	2.75	

* Error values represent the deviation of calculated from theoretical concentrations. Analysis was performed on HFBA adducts.

of the measured concentrations from the nominal values was 2.11–7.26% (Table II). The reproducibility of the entire sample preparation process was tested by preparing and analyzing five microdialysates acquired in vivo and spiked with TDG at a concentration of 0.827 nmol/mL (100.9 ng/mL). The dialysates were collected in an experiment in which no sulfur mustard was applied. Again, the results were obtained using the calibration curve displayed in Figure 1 (see Table III for numerical data). The calculated concentrations deviated from the nominal value by 1.1–7.1%, with the mean concentration being 2.4% lower than expected.

Discussion

One of the advantages of the microdialysis technique is that it allows the local and continuous monitoring of the interstitial concentrations of low-molecular-weight substances (26). This, on the other hand, posed a number of challenges in analytical method development. First, approximately 40 samples were to be processed in each microdialysis experiment. In order to be able to handle these quantities, it was decided that the preparation of samples include as few steps as possible. Because of the large number of samples, the duration of GC runs also had to be kept low, and cost-effectiveness was also a consideration. Nevertheless, high sensitivity of detection was still a requirement because the presence of TDG in microdialysates is an unambiguous indicator of penetrating fractions of sulfur mustard, however small its concentration may be.

The GC peak of TDG tails on most types of chromatographic columns; therefore, after analysis derivatization is preferable. In the majority of previous studies, nonpolar GC columns were used following derivatization with silylating agents such as *N*-methyl-(*N*-tert-butyldimethylsilyl)-trifluoroacetamide (13,14) and bis(trimethylsilyl)trifluoroacetamide (12), but acylation with pentafluorobenzoyl chloride (16) and HFBA (17) has also been reported. In one case, the separation of underivatized TDG took place on a polar column (27).

HFBA was selected for this purpose because TDG-HFBA and TDP-HFBA adducts were retained well by the column used, and the acylation reaction was found to reach equilibrium within 2 min at ambient temperature. Triethylamine was added to accelerate the reaction. Unreacted HFBA was decomposed by adding water containing 5% ammonia in order to prevent it from attacking the surface of the inner wall of the liner or the stationary phase of the column. A short vortex allowed heptafluorobutyric acid that was formed in the reaction to be transferred to the aqueous phase.

The small volume (10–40 µL) of the microdialysates obtained in these experiments was a limiting factor for the manipulation of samples, especially because TDG was usually present in parts-per-trillion amounts. Therefore conventional extraction techniques were not employed for sample preparation. Because dialysates contain a large number of polar solutes and TDG has a hydrophilic character, extraction of the dialysates with a nonpolar organic solvent was not expected to either recover TDG or remove interferences selectively and with high efficiency. Solid-phase extraction, on the other hand, would have been time-consuming and would have required an additional solvent exchange step (from the eluent to benzene) prior to derivatization. Instead, a single solvent exchange was performed, which allowed milder conditions (40°C) during evaporation along with a 5–10-min evaporation time.

PFPD allowed for fast chromatographic separation. Although only the peaks of the analyte and the internal standard were present in the chromatograms, aliquots prepared from in vivo dialysates were expected to contain a large number of matrix components that remained undetected but could contaminate various parts of the GC. In order to reduce this contamination, an additional ramp in temperature (40°C/min to 250°C) was included in the oven program. The absence of peaks in solvent blanks as well as in blank in vivo dialysates confirmed that the HFBA adducts of TDG and TDP were detected without any interference.

It is common to transform the quadratic relationship between the dependent and independent variables into a linear one. In most reports published previously on quantitation using PFPD, linear relationships have been obtained by plotting the square root of peak areas (28) or peak heights (22). The advantage of this approach is that the evaluation of linear relationships is more established than that of secondary polynomial relationships. Because of the options of data acquisition software, evaluation was based on the square root of peak heights in the present work. Although a square root circuit was included in the instrument that was used, the s/n obtained in this way was approximately 10 to 15 times lower than that obtained when the square root circuit was not used, yielding a considerable drop in detection and quantitation limits. In contrast to this, the post-measurement taking of the square roots of the heights of only the two peaks of interest allowed a lower noise amplitude and, at the same time, higher s/n and lower LOD and LOQ.

Calculated concentrations of TDG were good estimates of the nominal concentrations in both benzene solutions and in spiked in vivo microdialysates with their deviation from the nominal values being acceptably small and in the same range. These results indicate that no matrix effect selective for TDG or TDP was present in microdialysates. This allows for faster preparation of the calibration solutions and thus contributes to shorter sample preparation. The LOD and LOQ were adequately low because in the in vivo experiments, microdialysis samples were found to contain TDG at levels between 0 and approximately 30 nmol/mL. As TDP is significantly cheaper than labelled TDG, its use as an internal standard during GC–MSD analyses of TDG might also be worthy of consideration.

Conclusion

The use of GC with PFPD allowed an efficient and high-throughput analytical approach for the assessment of TDG in microdialysates obtained in vivo from the subcutaneous tissues of rats without extensive sample cleanup efforts. With the presented method, fast, selective, and sensitive analysis is feasible because no sulfur-containing compounds can be detected in the intersti-

tial fluid of rats unexposed to sulfur mustard. It has been demonstrated that TDP is an appropriate internal standard for these analyses.

As the presented procedure allows for the routine analysis of in vivo microdialysates, it can be employed in the development of an animal model for the evaluation of decontaminants and candidate antidotes against sulfur mustard injury.

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