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Reversed-phase chromatographic method for specific determination of glutathione in cultured malignant cells

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ABSTRACT

A chromatographic method for the specific determination of glutathione in malignant cell lines is described. The method is based on the ability of glutathione-S-transferase to specifically and quantitatively conjugate glutathione to 1-chloro-2,4-dinitrobenzene and chromatographic quantitation of the resultant conjugate, dinitrophenyl-S-glutathione, by reversed-phase liquid chromatography. The assay can be performed on 20 000 *g* supernatants of cell homogenates without acid extraction. 2-Mercaptoethanol, a sulfhydryl compound often used as a thiol-protective agent to preserve enzymatic activities of a number of enzymes, did not interfere with glutathione determination by this method. The dinitrophenyl-S-glutathione isolated from either standard glutathione samples or from cell homogenates was shown to be identical to authentic dinitrophenyl-S-glutathione using mass spectrometry. Recovery of glutathione in standard samples by the current method was identical to that determined using 5,5′-dithiobis(2-nitrobenzoic acid). Exogenous glutathione added to supernatants of cell homogenate in the presence or absence of 2-mercaptoethanol was also completely recovered.

INTRODUCTION

Glutathione (GSH)-related detoxification mechanisms have been implicated as mediators of alkylating agent resistance in a number of cell lines [1–5]. Specific quantitation of cellular GSH content is often necessary during studies of drug resistance in cultured malignant cells. For specific measurement of GSH, a number of chemically or enzymatically synthesized derivates of GSH [6] can be separated and quantified by high-performance liquid chromatography (HPLC) [7,8]. Because of the reactive nature of GSH, other cellular thiols, and the formation of derivatives of other cellular thiols, the specificity of these assays for GSH can be ensured only under rigorously standardized conditions [6]. These constraints can render GSH measurements less reproducible. Flow cytometric methods for quantifying GSH in tumor cells using monochlorobimane have also been developed [9–11]. However, since these methods require elaborate instrumentation which are not widely available, their use is limited. Interference by an exogenous sulfhydryl-protecting agent such as 2-mercaptoethanol (2-ME) and the need to remove protein sulfhydryls are common additional constraints of most methods for specific determination of GSH. These con-

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straints have hindered the understanding of interactions of cytosolic GSH with protein sulfhydryls, mixed disulfides of GSH and protein (PSSG), and GSH-related enzymes and their relationship with chemotherapeutic drug resistance.

In order to facilitate studying the relationship of GSH with protein sulfhydryls and GSH–protein mixed disulfides, we have devised a simple GSH-specific chromatographic assay which can be performed directly on cell homogenates eliminating the need for acid extraction. The specificity of this method stems from the ability of glutathione-S-transferase (GST) to conjugate electrophiles only to GSH under the present assay conditions. The method is based on the observation that in the presence of several-fold excess of 1-chloro-2,4-dinitrobenzene (CDNB) concentration, GST can quantitatively convert GSH in a reaction mixture into the GSH-CDNB conjugate, dinitrophenyl-S-glutathione (Dnp-SG). In this paper, we describe a reversed-phase HPLC method to isolate and quantify Dnp-SG as a measure of GSH from the reaction mixtures of both standard solutions and cell homogenates. In addition, we have shown that the fast atom bombardment (FAB) mass spectra of the HPLC peak corresponding to Dnp-SG formed in cell homogenates was indeed identical to that of authentic Dnp-SG. Comparison of GSH levels obtained by this method and that by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method [12] in several malignant cell lines in culture indicates general agreement.

EXPERIMENTAL

Reagents and solutions

CDNB, DTNB, GSH, purified equine liver GST, and 2-ME were purchased from Sigma (St. Louis, MO, USA). HPLC-grade reagents including acetonitrile and trifluoroacetic acid (TFA) were purchased from Pierce (Rockford, IL, USA). RPMI-1640 medium, penicillin–streptomycin solution, phosphate-buffered saline (PBS), fetal calf serum (FCS), and other tissue culture supplies were purchased from Grand Island Biological (Gaithersburg, MD, USA).

Buffer A (10 m*M* potassium phosphate, pH 7.0) and buffer B (100 m*M* potassium phosphate, pH 6.5) were prepared with and without 1.4 m*M* 2-ME. 2-ME was also added to PBS to a final concentration of 1.4 m*M*. Buffer A was used to prepare fresh stock solutions of GST (75 U/ml) and GSH (10 m*M*) and to make serial dilutions. CDNB (20 m*M*) was prepared by dissolving 40.5 mg of CDNB in 10 ml of absolute ethanol. Dnp-SG was enzymatically prepared, purified, and quantified as previously described [13]. After assessing purity by HPLC and thin-layer chromatography, dilutions of this preparation of Dnp-SG were made in buffer B. DTNB solution was prepared by dissolving 20 mg of DTNB in 100 ml of 1% sodium citrate. Other reagents for the GSH assay by the DTNB method were prepared as previously described [14].

Cell lines and homogenates

NCI-H69, NCI-H82, and NCI-H1436 human small cell lung cancer cell (SCLC) lines were obtained from Dr. Adi F. Gazdar (National Cancer Institute, Bethesda, MD, USA). The P3HR1 and EB2 Human Burkitt's lymphoma cell lines were obtained through the American Tissue Culture Collection (Rockville, MD, USA). The Molt-16 human T-cell lymphoblastic leukemia cell line was obtained from Dr. R. Graham Smith (University of Texas Southwestern Medical School, Dallas, TX, USA). All cell lines were grown in suspension cultures and maintained and passed every three days in RPMI medium containing 10% FCS and 1% penicillin–streptomycin at 37°C and 5% CO_2 in a water-jacketed humidified Forma Scientific incubator. Cells growing in log phase were harvested and washed twice with PBS (with or without 2-ME) to remove medium. Next, the cells were subjected to hypotonic lysis in buffer A (with or without 2-ME) and homogenized for 1 min by sonication in a Sonifier at 100 W with the homogenate in an ice bath. Following this, the homogenate was immediately centrifuged at 20 000 *g* for 10 min at 4°C to minimize interference by γ-glutamyltranspeptidase (a membrane-associated enzyme which degrades GSH). The supernatant fractions were used to

determine GSH by the HPLC and the DTNB methods.

GSH determination by quantifying Dnp-SG using HPLC

For quantifying Dnp-SG, HPLC was performed on a Pharmacia-LKB FPLC system using a Pharmacia PepRPC-C_{18} (100 mm × 10 mm I.D.) reversed-phase column. The mobile phase consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 100% acetonitrile (solvent B). The flow-rate was maintained at 1.5 ml/min. The column effluent was monitored at 230 and 340 nm using an LKB 2141 variable-wavelength monitor. A double-gradient program was developed using the Pharmacia-LKB LCC 500 Plus controller to optimize the separation of Dnp-SG from CDNB and GSH. For the first 17 ml, the gradient was increased from 0 to 30% B, and from 17 to 27 ml the gradient was increased from 30 to 70% B and maintained at 70% B from 27 to 35 ml. The column was then washed with 10 ml of 100% B and 10 ml of 0% B sequentially before the next assay. During the development of the assay, extracted samples of reaction mixtures containing supernatant of cell homogenate were injected sequentially up to three times to determine whether fouling of the reversed-phase column by contaminants during repetitive use affected the retention time or the size of the Dnp-SG peak. Up to ten assays were performed each day with blank samples injected at the beginning and end of each day to monitor elution of contaminants. The column was washed overnight at 0.1 ml/min with 100% B with monitoring at 230 and 340 nm to ensure that the column was adequately washed.

The reaction mixture was prepared by successive addition of 20 μl of GST stock solution (75 U/ml), 830 μl of buffer B, 50 μl of 20 m*M* CDNB solution, and 100 μl of either standard solution of GSH or cell homogenate (prepared in buffer with or without 2-ME). The reaction was terminated after 5 min by freezing the reaction mixture. The reaction mixture (1 ml) was lyophilized, and the Dnp-SG was extracted with 50% aqueous ethanol (0.2 ml). A 25-μl aliquot of the extract was injected onto the column. The area of chart paper under the 340-nm absorption peak corresponding to Dnp-SG was cut out using a razor blade and weighed on an analytical Mettler balance. A standard curve was generated by plotting GSH concentration in standard solutions obtained by the DTNB method *versus* weight (mg) of the chart paper corresponding to the Dnp-SG peak.

Quantifying GSH by the DTNB method

A DTNB method slightly modified from that previously described by Beutler *et al.* [14] was used to determine GSH concentrations in the stock solutions. Briefly, 300 μl of *m*-phosphoric acid precipitating solution were added to 200 μl of either GSH solution or buffer A (blank) incubated for 5 min, then centrifuged in an Eppendorf microfuge at 16 000 *g* for 10 min. To 800 μl of 0.3 *M* sodium phosphate in a 1.5-ml glass cuvette, 200 μl of the resulting supernatant were added. The absorbance at 412 nm was measured against the blank in a Gilford Response spectrophotometer and designated "Abs1". Subsequently, 100 μl of DTNB solution were added to both blank and reaction cuvettes and the absorbance at 412 nm was measured and designated "Abs2". The concentration of GSH (m*M*) in the standard solution was calculated using the previously reported E_{412} of the reaction product [12], 2-nitro-5-mercaptobenzoic acid (13.6 mM^{-1} cm^{-1}) and a dilution factor of 13.75 in the following equation:

$$\text{GSH concentration (m}M\text{)} = \frac{(\text{Abs2} - \text{Abs1}) \times 13.75}{13.6\ mM^{-1}\ cm^{-1}}$$

Recovery of exogenously added GSH

Known amounts of GSH were added to the supernatant of cell homogenate prepared with and without 1.4 m*M* 2-ME. The resulting solutions were assayed for GSH by the method as described above. The recovery (%) of GSH was determined from a plot of nanomoles of GSH recovered in the reaction mixture *versus* the number of nanomoles of exogenous GSH added to the reaction mixture.

RESULTS

The minimum GST activity and optimal CDNB concentration necessary to completely conjugate 100 nmol of GSH (contained in 1 ml of 0.1 m*M* GSH) in the reaction mixture were determined by monitoring the absorbance of the reaction mixture at 340 nm in a Gilford Response spectrophotometer. At conditions stated above, up to 100 nmol of GSH in 1 ml of reaction mixture were completely conjugated as indicated by no further increase in absorbance at 340 nm after approximately 3 min (data not presented).

Quantifying authentic Dnp-SG

Fig. 1 shows the separation of Dnp-SG (peak 1) from CDNB as a sharp peak during HPLC performed using the PepRPC 100 mm × 10 mm I.D. reversed-phase C_{18} column in the Pharmacia-LKB FPLC system. The plot of Dnp-SG quantified by HPLC *versus* concentration of Dnp-SG obtained spectrophotometrically showed excellent linearity and passed through the origin (data not presented), indicating the ability of our chromatographic method to accurately quantify Dnp-SG. Concentrations of Dnp-SG in a number of samples of authentic Dnp-SG prepared in a blinded fashion were measured spectrophotometrically and by HPLC and found in close to perfect agreement (data not presented).

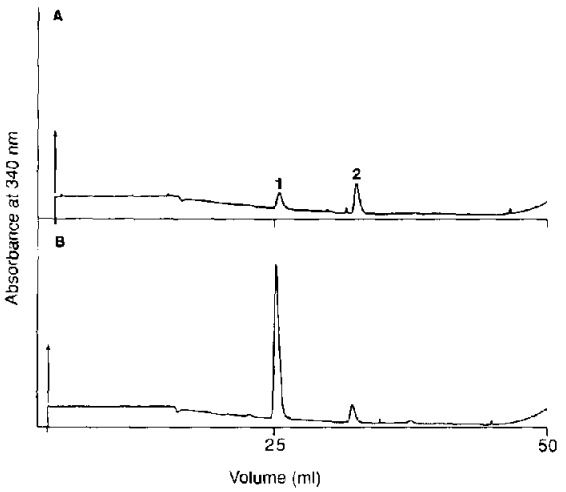

Fig. 1. Separation of Dnp-SG and CDNB by HPLC. In (A) 5 nmol and in (B) 50 nmol of GSH were incubated with 1 m*M* CDNB, 1.5 U of GST, and buffer A in 1 ml of reaction mixture for 5 min, and 25 μl of reaction mixture were injected on a reversed-phase column. Peaks 1 and 2 correspond to Dnp-SG and CDNB, respectively. Details are given in the text.

Recovery of GSH in standard solution by the HPLC method

After complete conjugation, Dnp-SG and residual CDNB in reaction mixtures containing standard GSH were separated by HPLC. Tracings of absorbance at 340 nm (Fig. 1A and B) confirm that in the absence of 2-ME, Dnp-SG was the only compound in the reaction mixture other than CDNB with significant absorption at 340 nm with a signal-to-noise ratio of 10:1. FAB mass spectrometry of the Dnp-SG isolated by HPLC from standard samples as well as from cell homogenates showed the expected $[M + H]^+$ ion at *m/z* 474.2 (data not presented) suggesting that Dnp-SG peak isolated and quantified is indeed authentic Dnp-SG. In the presence of 2-ME the tracing of absorption at 340 nm revealed evidence of other products, possibly due to the reaction of CDNB with 2-ME. However, these products were clearly separable from Dnp-SG. Fig. 2A shows that GSH added to buffer A is completely recovered and within the error of measurement, the absence or presence of 2-ME does not significantly affect the recovery (98 *versus* 102%, respectively).

Quantifying GSH in supernatant of cell homogenates in six malignant cell lines by the HPLC method

Each HPLC assay required approximately 1 h. We performed up to ten assays per day. Blank samples containing only buffer injected on the column at the beginning and end of each day revealed minimal contaminants with absorption at 230 or 340 nm. Repeated injections of samples of reaction mixtures containing the same amount of supernatant of cell homogenate up to three times revealed variation in the area under the curve of the Dnp-SG peaks of less than 10%, and the retention times of the peaks varied by less than 1%.

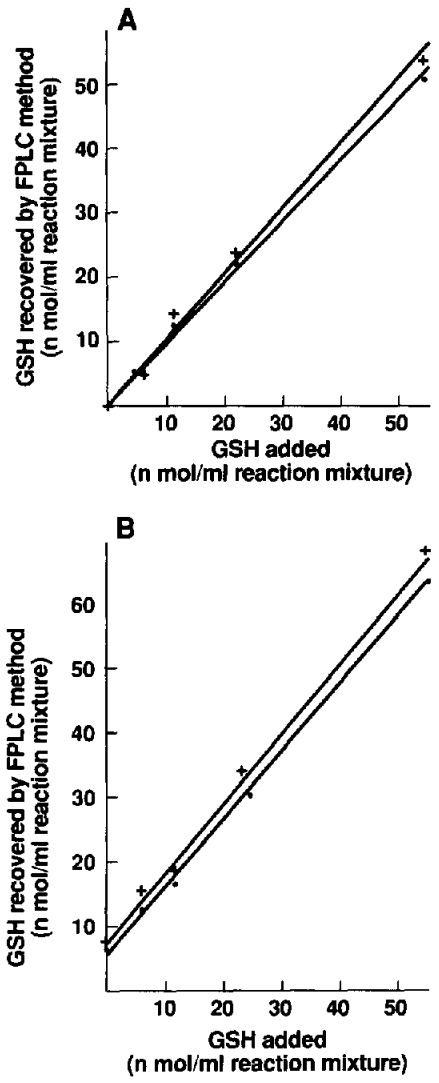

Fig. 2. Recovery of exogenously added GSH by the HPLC method. Known concentrations of standard GSH were added to (A) phosphate buffer prepared with (+) and without (●) 2-ME and (B) supernatant of H1436 cell homogenate prepared in buffer with (+) and without (●) 2-ME. Details are given in the text.

Reproducible size of the Dnp-SG peak and retention times indicated that the reversed-phase sorbent was not being significantly fouled by contaminants in the reaction mixture. We attributed this to adequate column washing and to the removal of most contaminants by ethanol extraction prior to sample injection. Fig. 2B demonstrates the quantitative recovery of exogenous

TABLE I

COMPARISON OF GSH CONCENTRATIONS IN SIX HUMAN MALIGNANT CELL LINES AS DETERMINED BY THE HPLC AND DTNB METHODS

Determinations of GSH by the two methods were carried out using the procedures described in the text.

Cell line	GSH (mean ± S.D.) (nmol/mg of protein)	
	HPLC method	DTNB method
P3HR1	57.0 ± 5.1 (n = 5)	43.6 ± 1.5 (n = 3)
Molt-16	17.4 ± 0.9 (n = 3)	20.1 ± 0.8 (n = 3)
EB2	68.4 ± 6.1 (n = 3)	49.8 ± 3.7 (n = 3)
NCI-H69	76.2 ± 9.7 (n = 9)	78.6 ± 2.0 (n = 3)
NCI-H82	91.5 ± 13.1 (n = 8)	77.0 ± 1.0 (n = 3)
NCI-H1436	57.6 ± 7.9 (n = 6)	52.1 ± 1.1 (n = 3)

GSH added to supernatant of cell homogenate of the H-1436 cell line. This indicates the accuracy of the method and provides evidence that under the specified assay conditions in this cell line, GSH is not degraded by γ-glutamyltranspeptidase or other enzymatic or chemical reaction. Preparation of acid extract which is required in most other methods is not necessary in the current HPLC method. The recovery of added GSH in the presence of 2-ME was somewhat higher (110%) compared to that in the absence of 2-ME (103%). This 7% difference is presumably due to the ability of 2-ME to prevent formation of oxidized GSH (GSSG) which is not measured by our assay. Similar results were observed with the other cell lines (data not presented). Results of the GSH content determined in six malignant cells lines by our HPLC and the DTNB method (Table I) show general agreement in GSH values obtained by the two methods. Lower values of total non-protein sulfhydryl (NPSH) by the DTNB method may have been due to oxidation of GSH or other non-protein thiols during homogenization or acid precipitation.

DISCUSSION

A variety of assays for measurement of GSH

are available but the enzymatic and HPLC assays are felt to be the most sensitive and specific [6]. Our assays combines the specificity of enzymatic assays and HPLC methods for the measurement of GSH in homogenate with little interference from other cellular thiols or even 2-ME. Our assay is based on chromatographically quantifying Dnp-SG, the enzymatic product from the conjugation of CDNB and GSH. GSTs conjugate CDNB to GSH in preference to other sulfhydryl compounds [15]. The only naturally occurring sulfhydryl other than GSH to show activity with GST are homoglutathione, reported only in plants [16], and γ-glutamylcysteine [17]. Although GSTs present in cell homogenates can catalyze formation of Dnp-SG, the incubation time is quite variable depending on the GST content of the particular cell line (data not presented). To make the assay convenient, we use a relatively large quantity of GST to quickly complete the reaction. If longer incubation time (10–15 min) is not of concern, then as little as 0.2 U of commercial GST can be used to catalyze complete conjugation of up to 100 nmol of GSH with CDNB, thus reducing the cost of the assay significantly. We have also used GSTs purified from several other sources in this assay and did not observe any differences in assay efficiency and recovery compared with the commercially available equine liver GST (data not presented). Commercial preparations of GST may contain variable amounts of GSH and, therefore, should be extensively dialyzed before use.

Under standardized conditions, the present HPLC method was sensitive enough to give reproducible GSH measurements in samples containing as little as 5 μM (5 nmol/ml) GSH. The specificity for GSH and the ability to accurately determine the GSH content by this method was supported by results of experiments measuring GSH recovery from standard solutions and that of exogenously added GSH to supernatant of cell homogenates. The FAB mass spectra (data not presented) confirm that the compound formed in standard GSH and in cell homogenates which has the same retention time during HPLC as pure Dnp-SG is indeed indentical to authentic Dnp-SG, decreasing the possibility that a non-GSH-containing metabolite of CDNB caused the increase in Dnp-SG peak area.

GSH levels obtained by our method in the six cell lines (Table I) are within the range of GSH content reported by other methods in various malignant cell lines [18–20]. Although GSH measurements by the present HPLC method and the DTNB method were comparable for all standard solutions, the agreement between GSH measurement by our method and the total NPSH measurement by the DTNB method was not as good for some of the cell lines. Because determination of Dnp-SG by our method appears to be quite accurate as indicated by the near perfect recovery of exogenously added GSH in cell homogenates, we believe that this discrepancy was due to an artifact due to the low value of NPSH by the DTNB method rather than an overestimation by the present method. This belief is supported by previous studies which indicate that during the acid precipitation of cell homogenates, peroxide-mediated oxidation of GSH and other NPSHs occurs to a significant extent [21]. It has previously been shown that the concentration of GSH in cell homogenates as measured by a specific chromatographic method [22] remains unaltered for up to 1 h. On the other hand, DTNB-sensitive NPSH content of homogenates has been shown to decrease considerably within 1 h [23]. It is therefore possible that lower values obtained for DTNB-sensitive NPSH for some of the cell lines may be due to the unstable nature of DTNB-sensitive NPSH.

Because our assay does not require acid precipitation for measurement of GSH we are attempting to develop a modification of this assay for measuring PSSG using enzymatic methods in order to examine the relationship of alkylating agent resistance with PSSG status in cultured malignant cells.

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