NOTES & TIPS

An Improved Monobromobimane Assay for Glutathione Utilizing Tris-(2-carboxyethyl)phosphine as the Reductant

M. T. Anderson,* J. R. Trudell,† D. W. Voehringer,* I. M. Tjioe,* L. A. Herzenberg,* and L. A. Herzenberg*,[1]

Department of Genetics and †Department of Anesthesia, Stanford University School of Medicine, Stanford, California 94305

Received November 11, 1998

Increased levels of oxidative stress are associated with many disease states including HIV infection, malignancy, renal insufficiency, and chronic alcohol abuse. Consequences of oxidative stress include a decrease in the concentrations of glutathione found within an organism's fluids and tissues. The monobromobimane (mBrB)[2] assay is a commonly used measure of glutathione. In this assay, glutathione (GSH) is reacted with mBrB to form a fluorescent adduct that is readily detectable by HPLC using fluorescence detection. Within biological samples, the majority of free glutathione is present as GSH. However, a less abundant fraction is composed of glutathione dissulfide (GSSG). The levels of oxidized glutathione levels are derived by measuring both total and reduced glutathione levels and then subtracting the reduced glutathione levels from the total glutathione levels. Before the GSSG component can be measured with the mBrB assay, it must first be converted into the GSH form. This conversion has generally entailed the addition of a large excess of a reducing agent such as dithiothreitol (DTT) or sodium borohydride ($NaBH_4$). However, these reductants are also reactive with mBrB and in the course of this side reaction, the labeling reagent is consumed and a background fluorescent peak is generated (1). This background peak can obscure the detection of the glutathione–bimane product. To circumvent these difficulties, several different modifications of the mBrB assay have been introduced. These include addition of greater amounts of mBrB, use of separate assay conditions for the analysis of purified glutathione and for the analysis of blood-derived samples, and insertion of additional step in the assay in which the excess reductant is removed before it can interfere with the glutathione to bimane conjugation (1, 2).

Over the past 15 years, many mBrB assay protocols have been published; however, a need for one that is simpler and more generally useful remains. We anticipated that creating such a protocol would require finding a reductant that could be used in lower amounts. Consequently, we explored the use of trialkylphosphines because they are powerful reductants that need to be present in only slight molar excess for complete reduction of dissulfides. One member of this family of compounds is tributylphosphine (TBP). However, TBP possesses the negative characteristics of being poorly soluble in water and of possessing a bad odor. Because of these negative characteristics, we did not experimentally pursue the use of TBP. Another member of this family is Tris-(2-carboxyethyl)phosphine (TCEP) which has only recently become commercially available as the result of the identification of an efficient means of its synthesis (3). TCEP is highly soluble in water and it is nonvolatile.

To incorporate TCEP as the reductant in the mBrB assay, we modified an established protocol (4) in several ways. We replaced the DTT reductant with TCEP. We set the amount of TCEP added as that amount which would reproducibly allow the measurement of at least 1 mM glutathione within the acidified extracts of whole blood. We adjusted the concentration of mBrB to be approximately twice (1.75) the concentration of TCEP. Also, because TCEP is a good reductant even at acidic pHs, we buffered the pH of the reduction step to 8.0, the optimal pH for selective labeling of GSH by mBrB.

The modified mBrB assay protocol

Reagents. Monobromobimane and TCEP were obtained from Molecular Probes (Eugene, OR) and stored dessicated until use. Glutathione and glutathione disulfide, sulfosalicylic acid, and DTT were from Sigma Chemical Co. (St. Louis, MO). *N*-Ethylmorpholine, acetic acid, and methanol were purchased from Fisher Scientific (Pittsburgh, PA).

Sample preparation. Whole blood was obtained from volunteers and prepared for analysis essentially as de-

[1] To whom correspondence should be addressed. Fax: (650) 725-8564. E-mail: lenherz@darwin.stanford.edu.

[2] Abbreviations used: mBrB, monobromobimane; GSH, glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol; $NaBH_4$, sodium borohydride; TBP, tributylphosphine; TCEP, Tris-(2-carboxyethyl)phosphine; FACS, fluorescence-activated cell sorting.

Analytical Biochemistry **272,** 107–109 (1999)
Article ID abio.1999.4153
0003-2697/99 $30.00

scribed by Anderson *et al.* (4). In brief, immediately after the blood was drawn, it was chilled, diluted 1:1 with 10% sulfosalicylic acid, and then stored at −80°C. To remove the precipitated protein, the acidified blood was thawed on ice and then twice clarified by centrifugation at 1200*g* for 10 min at 4°C. Samples consisting of only purified glutathione were prepared by resuspending either the GSH or GSSG powder with a solution consisting of 5% sulfosalicylic acid plus 50 mM DTT (5).

The labeling reaction. For analysis by this procedure, 20 μl of sample was transferred with a Hamilton Gas-tight syringe 1702 configured with a Chaney adapter (Hamilton, Reno, NV) to a 12 × 100 mm borosilicate glass tube. The aliquot was then neutralized and buffered by the addition of 240 μl of a 1:1.7:5.3 mixture of 1.0 M *n*-ethylmorpholine, pH 8.0, 0.05 M NaOH and H_2O. The GSSG present in the sample was then reduced by the addition of 30 μl of 7.5 mM TCEP and the reduction was allowed to proceed for 20 min at 22°C. The borosilicate tube was then wrapped in aluminum foil to protect the reaction mixtures of the subsequent steps from light. The GSH within the sample was derivatized by the addition of 40 μl of 10 mM mBrB dissolved in 50% acetonitrile and this reaction was allowed to proceed for 20 min at 22°C. The reaction was stopped by the addition of 25 μl of 5.8 M perchloric acid and then stored on ice until HPLC.

Chromatography. Reaction products were analyzed on a Hewlett Packard Model 1050 HPLC (Hewlett Packard, Palo Alto CA) equipped with an autosampler accessory that was programmed to inject 25 μl into the sample line. Separations were achieved with a Vydac 5-μm C18 HPLC column of 25 cm by 0.46 cm (Vydac Model 201TP54; Hesperia, CA). The fluorescent products eluting from this column were detected with a Kratos Spectroflow 980 fluorescence detector (Applied Biosystems, Ramsey, NM) with excitation at 360 nm and emission at 470 nm. The Spectoflow 980 detector was connected to a Hewlett Packard Vectra Computer that was configured with a Hewlett Packard 359ODD A/D interface board.

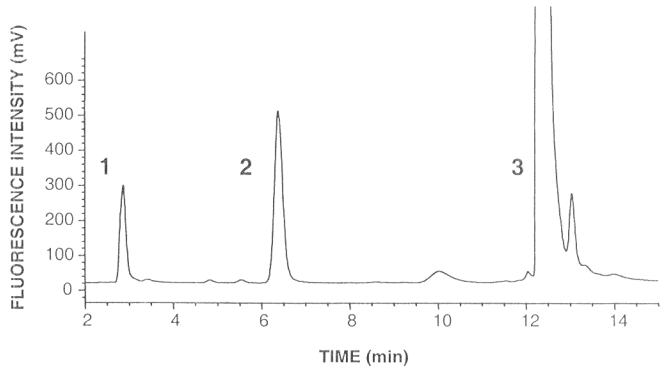

FIG. 1. Direct relationship between the GSSG in samples and the peak area of the bimane–glutathione product. A series of samples with increasing concentrations of GSSG were analyzed with the modified mBrB assay. The peak areas of the glutathione–bimane products were determined with the Hewlett Packard Chemstation. The amount of GSSG in the sample and the peak area of the glutathione–bimane product were directly related over at least a range of 6.125 mM (39 pmol) to 800 mM (20 nmol).

The integrated areas of the fluorescent peaks were calculated with the Hewlett Packard Chemstation software program. Elution buffers were based upon an acetate buffer consisting of 0.21% acetic acid (w/v) titrated to pH 3.9 with 1 M NaOH. Elution solution A was 86% acetate buffer and 14% methanol. Elution solution B was 10% acetate buffer and 90% methanol. The column was pre-equilibrated by passing 100% A:0% B at 1.0 ml/min at 30°C through it. After injection of sample, the applied material was eluted in three steps, 100% A from 0 min to 5 min, followed by a first linear gradient of 100% A to 75% A:25% B from 5 to 10 min and then a second linear gradient of 75% A:25% B to 100% B from 10 to 20 min. With these chromatographic conditions, the sulfosalicylic acid/void volume peak eluted at 2.8 min, the glutathione–bimane peak at 6.5 min, and the bimane–reductant peak eluted at 12.6 min. Samples were analyzed in triplicate and bars representing the standard deviation of the de-

TABLE 1

	Peak area of glutathione ($\times 10^6$)	
	Observed	Expected
10 μl (150 μM GSSG standard)	13.2	
20 μl of WB extract	97.1	
10 μl (150 μM GSSG standard) + 20 μl of WB extract	111	110

Note. Glutathione peak areas detected in mixtures consisting of whole blood and purified GSSG were identical to the glutathione peak areas detected when these two components were analyzed separately. Samples were analyzed in at least duplicate with the described TCEP monobromobimane assay.

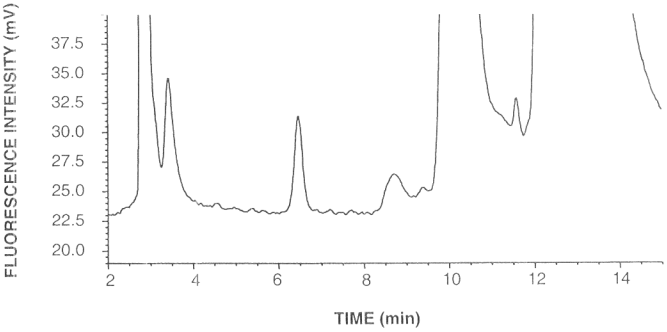

FIG. 2. Representative chromatogram of 800 μM glutathione. The sample was prepared and analyzed as described by the method described. Sulfosalcylic acid, glutathione–bimane adduct, and bimane–reductant eluted at 2.8, 6.5, and 12.6 min., respectively.

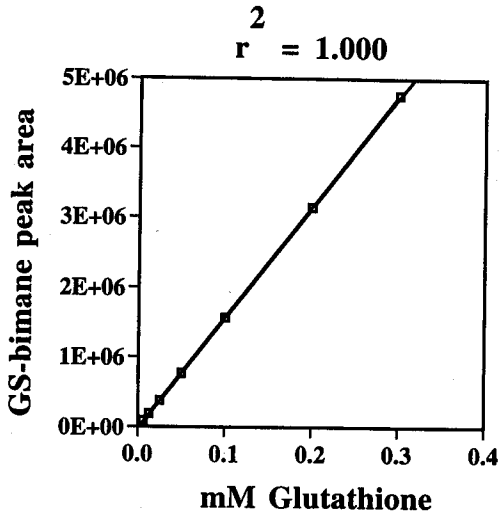

FIG. 3. Representative chromatogram obtained from the analysis of the glutathione present within FACS sorted cells. Approximately 1×10^6 purified naïve CD4 human T cells ($CD4^+$, $CD45^+$, $CD62L^+$) were FACS sorted, washed free of serum, and resuspended in 100 μl of 5% sulfosalicylic acid plus 50 μM DTT. The samples were then analyzed with our modified mBrB assay. The peak at 6.5 min. is the glutathione–bimane peak.

termination were smaller than the symbol designating the mean value of the determination.

Results and Discussion

We found that with our assay conditions, TCEP completely reduced the GSSG that was present within the samples to GSH. Furthermore, the presence of TCEP did not interfere with the labeling of GSH by mBrB nor with the detection of the glutathione–bimane product. This was true for samples consisting of either purified glutathione or acidified extracts of whole blood. As evidence for this, when samples possessing the same molar equivalents of glutathione in either the GSH or GSSG forms were assayed, the resultant fluorescent glutathione–bimane peaks were the same size (Table 1). Also, when GSSG standards with concentrations ranging between 6.125 and 800 μM in glutathione were assayed, a linear relationship was obtained between the concentration of glutathione in the sample and the size of glutathione–bimane peak measured (Fig. 1). This linear response was obtained regardless of the sample type, since the addition of defined amounts of GSSG to the acidified extracts of whole blood led to increases in the glutathione–bimane peak that were of the same area as that obtained when that amount of GSSG was assayed by itself (Table 1). Finally, we observed that the glutathione–bimane peaks could be unambiguously attributed and that they were well separated from other fluorescent entities that were generated (Fig. 2).

To date, we have used our modified assay to monitor changes in glutathione resulting from pharmacologic intervention of the HIV disease with *N*-acetylcysteine (6). We have also used this assay to investigate the redox changes in T cell subsets that occur in the course of HIV infection (manuscript in preparation). In these studies, T cells from uninfected individuals were sorted using fluorescence-activated cell sorting (FACS) to obtain pure populations of the various T cell subsets. The amount of glutathione present in the various purified populations of cells was then determined with our modified assay. A representative chromatogram from these analyses is shown in Fig. 3. We anticipate that the simplicity and general utility of this assay will lead to its wide use.

Acknowledgment. These studies were supported by NIH Grant CA42509 to Leonard A. Herzenberg.

REFERENCES

1. Baeyens, W., VanderWeken, G., and DeMoerloose, P. (1987) *Chromatographia* **23,** 717–721.
2. Fenton, S. S., and Fahey, R. C. (1986) *Anal. Biochem.* **154,** 34–42.
3. Han, J. C., and Han, G. Y. (1994) *Anal. Biochem.* **220,** 5–10.
4. Anderson, A., Isaksson, A., Brattstrom, L., and Hultberg, B. (1993) *Clin. Chem.* **39,** 1590–7.
5. Svardal, A. M., Mansoor, M. A., and Ueland, P. M. (1990) *Anal. Biochem.* **184,** 338–46.
6. Herzenberg, L. A., De Rosa, S. C., Dubs, J. G., Roederer, M., Anderson, M. T., Ela, S. W., and Deresinski, S. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1967–1972.