NOTES & TIPS

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

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) 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 disulfide (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 (NaBH₄). 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 triallylphosphines because they are powerful reductants that need to be present in only slight molar excess for complete reduction of disulfides. 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 5.0, 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 desiccated 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-
scribed by Anderson et al. (4). In brief, immediately after
the blood was drawn, it was chilled, diluted 1:1 with 10% 
Sulfoalicylic 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 1200g
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%
Sulfoalicylic acid plus 50 mM DTT (5).

The labeling reaction. For analysis by this proce-
dure, 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 boros-
licate 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 H2O. 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 alu-
minum foil to protect the reaction mixtures of the sub-
sequent 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 Pack-
ard, Palo Alto CA) equipped with an autosampler acces-
sory 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 Spec-
troflow 980 fluorescence detector (Applied Biosystems,
Ramsey, NM) with excitation at 360 nm and emission at
470 nm. The Spectroflow 980 detector was connected to a
Hewlett Packard Vectra Computer that was configured
with a Hewlett Packard 3590DD A/D interface board.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak area of glutathione (×10⁴)</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Observed</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>10 µl (100 µM GSSG standard)</td>
</tr>
<tr>
<td>20 µl of WB extract</td>
</tr>
<tr>
<td>10 µl (100 µM GSSG standard) +</td>
</tr>
<tr>
<td>20 µl of WB extract</td>
</tr>
</tbody>
</table>

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.

The integrated areas of the fluorescent peaks were cal-
culated 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-
FIG. 3. Representative chromatogram obtained from the analysis of the glutathione present within FACS sorted cells. Approximately $1 \times 10^6$ purified naïve CD4 human T cells (CD4+, CD45+, CD62L+) were FACS sorted, washed free of serum, and resuspended in 100 μl of 5% salicylic acid plus 50 μM DTT. The samples were then analyzed with our modified mBrB assay. The peak at 0.5 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