DETERMINATION OF LOW MOLECULAR WEIGHT THIOLS USING MONOBROMOBIMANE FLUORESCENT LABELING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

By Robert C. Fahey and Gerald L. Newton

The bromobimanes constitute a class of reagents introduced by Kosower and coworkers\(^1\) as useful reagents for labeling of the thiol group. Monobromobimane (mBBr) has proven to be especially useful as a reagent for the analysis of low molecular weight thiols. The reagent itself is only weakly fluorescent but selectively reacts with thiols to yield highly fluorescent and stable thioethers (mBSR) which can be easily detected at the picomole level (Eq. 1). The bimane

\[
\text{mBBr} + \text{RSH} \rightarrow \text{mBSR} + \text{HBr} \quad (1)
\]

derivatives can be separated by electrophoresis\(^3\), paper chromatography\(^3\), and ion exchange chromatography\(^4\) but high-performance liquid chromatography (HPLC) has proven to be the most useful separation method\(^5\). This chapter describes methods for the preparation and HPLC analysis of monobromobimane derivatives of low molecular weight thiols in extracts of biological samples and discusses typical problems encountered in the development and application of these methods.

Materials

Materials and sources were as follows: HPLC grade sodium perchlorate and 50% sodium hydroxide - Fisher Scientific; methanol and acetonitrile - Burdick and Jackson; 5-sulfosalicylic acid - MCB; tetrabutylammonium phosphate - Kodak;
Puriss grade methanesulfonic acid - Fluka; glacial acetic acid, sodium sulfide, sodium sulfate, sodium thiosulfate, potassium chloride, dibasic potassium phosphate, sodium acetate, and boric acid - Mallinckrodt; diethylenetriaminepentaaetic acid (DTPA), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), Trizma base (Tris), 2-pyridyl disulfide, reduced glutathione, L-homocysteine, ergothioneine, pantetheine, 2-mercaptoethanesulfonic acid, N-acetylcycteine, penicillamine, thioglycollic acid and N-ethylmorpholine - Sigma; methyl disulfide - Aldrich; monobromobimane - mBBr, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid (HEPPS), cysteine hydrochloride, high purity dithiothreitol (DTT), and 2-mercaptoethanol - Calbiochem; L-cystinyl-bis-glycine - Vega; WR-1065 - Dr. Leonard Kedda, National Cancer Institute. A sodium methanesulfonate stock solution was prepared from methanesulfonic acid by addition of one equivalent of sodium hydroxide and dilution to 4 M. γ-Glutamylcysteine was prepared by the method of Strumeyer and Bloch and 4'-phosphopantetheine by the method of Wang, Shuster, and Kaplan.

**Preparation of Samples**

Several different types of samples are typically needed in the course of a given application and these include the thiol standard sample, the reagent blank, the unknown sample, and the unknown control. The thiol standard solutions were prepared from authentic stock solutions of the individual thiols of interest. Thiol stock solutions were prepared at high concentration (10-20 mM) and low pH (10 mM methanesulfonic acid) to minimize losses due to oxidation, except for sulfide which was prepared in 50 mM HEPPS at pH 8.0. Varying level of purity and degree of hydration often made it difficult to prepare stocks of precisely known concentration and it was therefore convenient to check the final concentration by titration of the thiol with Ellman's reagent. Standard solu-
tions of methanethiol, 2-mercaptopyridine, and cysteinylglycine were prepared from the corresponding disulfides by reaction with one equivalent of DTT in 50 mM HEPPS, pH 8.0, containing 5 mM DTPA for 10 min at 50°C under argon. The derivatization reaction was carried out at high concentration to maximize the reaction rate with mBBr and minimize competing side reactions; a 1-2 mM stoichiometric excess of mBBr was used to insure rapid and complete reaction. In a typical reaction the thiol (final concentration 1 mM) was added to a solution containing 50 mM HEPPS, 5 mM DTPA, and 2-3 mM mBBr at pH 8.0 and reaction allowed to proceed for 10 min under dim lighting at room temperature. Methanesulfonic acid was added to 25 mM prior to storage. Acetic acid has also been used to lower the pH for storage but acetate reacts with mBBr to yield a fluorescent product which may interfere in some analyses. Under these conditions the half life for reaction of GSH and other cysteine derivatives was ~20 sec whereas sulfite (t$_{1/2}$ ~2 min), penicillamine (t$_{1/2}$ ~1 min) and CoA (t$_{1/2}$ ~1.5 min) reacted more slowly but were >95% reacted at 10 min. Since heavy metal ion levels were low and the thiol concentration was high, losses due to oxidation were negligible and exclusion of air during preparation of standard samples was not required. When thiol standard solutions (10 µM) of the mBBr derivatives of cysteine, GSH and WR-1065 in 1% acetic acid were stored as single aliquots in Eppendorf centrifuge tubes, no significant loss was observed at 4, -20, or -70°C for 20 months. However, standard solutions repeatedly frozen at -20°C and thawed showed significant losses over short periods. The bimane derivatives are photosensitive and should be protected from light during preparation and storage.

Preparation of cell extracts for analysis is more complicated and the optimal procedure varies with cell type. Loss of thiol due to oxidation is an important problem which is minimized by using heavy metal chelators and limiting
the exposure of the thiol to oxygen while at high pH. Trace levels of peroxide in buffers can also oxidize thiols so that high purity, slowly autoxidized buffers, such as Tris and HEPPS, have been found preferable to easily autoxidized buffers, e.g. N-ethylmorpholine, when low thiol levels are to be analyzed. Loss of thiol or thiol derivative due to enzymic degradation represents another major problem; thus, attempts to extract kidney, which is rich in $\gamma$-glutamyltranspeptidase and dipeptidase activities, without enzyme inactivation results in extensive conversion of GSH to cysteine. Enzymes must, therefore, be inactivated during the extraction process and this can be accomplished by extracting either in acid or in organic solvent. The reagents selected for such extractions and for subsequent labeling of cell extracts must not react significantly with mBBr. Although mBBr reacts selectively with thiols, it also reacts slowly with amines, phosphate, carboxylates, and other nucleophiles when these are present at high millimolar or molar concentrations to yield fluorescent products at micromolar levels which can interfere with the analysis.

As a guide to selection of appropriate reagents we include in Table 1 the retention times for reagent derived products obtained with various components. Each analysis protocol should include a reagent blank, a sample treated identically to the unknown sample but with the cells or tissue omitted, which can be used to identify peaks arising from the reagents. A second blank, the unknown control, is prepared by reacting the thiols present in the cell extract with NEM or DTNB prior to derivatization with mBBr. Since most thiols react with NEM and DTNB to form derivatives that are unreactive with mBBr, this sample serves as a check on the assignment of thiols in unknown samples and allows the identification of fluorescent nonthiol components contributed by cells. 2-Pyridyl disulfide can be used in place of DTNB but produces greater background due to the more intense fluorescence of the 2-mercaptopyridine bimane derivative.
Extraction of animal tissues using methanesulfonic acid to denature enzymes is illustrated by the following protocol: ~200 mg of fresh or frozen tissue was homogenized for 1 min in 1 ml ice cold 200 mM methanesulfonic acid using a Tekmar or Brinkman polytron homogenizer. An equal volume of 4M sodium methanesulfonate was added and the sample centrifuged 5 min in an Eppendorf microcentrifuge. The clear supernatant was diluted 1:3 into 200 mM HEPPS-methanesulfonate (pH 8.0) containing 5 mM DTPA and 3mM mBBr, and reaction allowed to proceed 10 min under dim light. After addition of methanesulfonic acid to 100 mM, the sample was centrifuged again and stored at -70°C until analyzed. For preparation of unknown control samples, 5 mM NEM (or 2 mM DTNB) was substituted for mBBr and the reaction allowed to proceed for 5 min after which mBBr was added to 2 mM and the reaction continued for 10 min before acidification. A procedure for extraction of animal tissue with sulfosalicylic acid, labeling with mBBr, and HPLC analysis for glutathione has been described by Anderson.

Use of acetonitrile as an organic protein denaturant is illustrated in the following protocol for analysis of mung bean: dried mung beans were ground to a fine powder in a mortar and pestle. The powder (200 mg) was placed in a septum-capped 3 ml vial and the vial flushed with argon. The extraction buffer (50% aqueous acetonitrile containing 50 mM HEPPS - pH 8.0, 5 mM DTPA and 2 mM mBBr) was preheated to 60°C and flushed with argon. A syringe was used to transfer 1 ml of extraction buffer to the vial containing the mung bean powder and the vial was then intermitantly vortexed while heating at 60°C for 10 min. After cooling the vial, methanesulfonic acid was added to 50 mM. The sample was transferred to a 1.5 ml Eppendorf centrifuge tube and centrifuged 5 min in an Eppendorf microcentrifuge. Samples were stored at -20°C and diluted at least 1:1 in 10 mM methanesulfonic acid to lower the acetonitrile content prior to HPLC analysis. In the control sample, 5 mM NEM (or 2 mM DTNB) was used in place
of mBBr and reaction allowed to proceed 10 min, after which mBBr was added to 2 mM and the reaction continued an additional 10 min, all at 60°C. The elevated temperature was required because organic solvents decrease the rate of reaction of mBBr with most thiols. Under these conditions the t\textsubscript{1/2} for GSH is <15 sec but the t\textsubscript{1/2} for CoA is ~1.5 min. Even at this elevated temperature it was considered desirable to test the use of a higher pH to insure complete derivatization. This was complicated by the fact that amine buffers react more extensively with mBBr at higher pH. However, substitution of HEPPS in the above procedure with 50 mM borate, pH 9.0, resulted in minor reagent peaks (Fig. 1A) and gave quantitative results which were only slightly higher than achieved using HEPPS, pH 8.0.

Acidified cell extracts have generally been found to be stable for weeks when stored at -70°C and showed no change when allowed to stand at room temperature in an autoinjector for up to 24 hours. An exception is the CoA derivative which is converted to the dephospho-CoA derivative with a half-life of about 10 hours in 1% acetic acid at room temperature.

**Chromatography**

All analyses were carried out on a Varian Model 5060 high performance liquid chromatograph equipped with a Waters WISP Model 710B autoinjector, an LDC Fluoromonitor III (Model 1311) fitted with a standard flow cell and 360 nm excitation/410-700 nm emission filters, and a Nelson Model 444 data system.

Three HPLC protocols have proven useful for different types of sample analysis. These are illustrated with various standard mixtures in Fig. 1 and retention times for standard thiol derivatives are tabulated in Table I. Separation of the greatest number of thiol derivatives was achieved with Method 1 which utilized a 4.6 x 250 mm Altex Ultrasphere ODS 5 μ analytical column.
equipped with a Brownley MPLC guard column containing an OD-GU 5μ C-18 cartridge. Solvent A was 0.25% aqueous acetic acid titrated to pH 3.5 with concentrated sodium hydroxide; solvent B was methanol. Solvents were filtered through 0.2 μ nylon filters (Rainin Scientific) in a Millipore solvent filter apparatus. The elution protocol (24°C, 1.2 ml per min) employed linear gradients as follows: 0 min, 15% B; 5 min, 15% B; 15 min, 23% B; 45 min, 42% B; 65 min, 75% B; 67 min, 100% B; 70 min 15% B; 85 min 15%, B; reinject.

Method 2 was developed to provide quantitative analytical data for coenzyme A and dephosphocoenzyme A, which elute as broad peaks with method 1, while retaining the capacity to simultaneously analyze for glutathione and cysteine. A 4.0 x 250 mm Lichrosorp RP-8 10μ analytical column (E. Merck) was used with a Brownley MPLC guard column fitted with an RP-GU 10 μ C-8 cartridge. Solvent A was prepared by dilution of 100 ml methanol, 2.5 ml acetic acid, and 3.4 g tetrabutylammonium phosphate to one liter with water and adjusting the pH to 3.4 with sodium hydroxide or acetic acid. Solvent B was prepared by addition of 2.5 ml acetic acid and 3.4 g tetrabutylammonium phosphate to 100 ml water and diluting to one liter with methanol. The elution protocol (24°C, 1.0 ml per min) with linear gradients was as follows: 0 min, 10% B; 15 min, 50% B; 30 min, 75% B; 40 min, 100% B; 42 min, 10% B; 55 min, 10%, B; reinject. Several different 5 μ C-8 columns were also tested but gave broader peaks for CoA than the column described above.

Method 3 utilizes sodium perchlorate ion pairing to enhance the analysis of cysteamine and WR-1065 derivatives in samples also containing GSH and cysteine. The method was designed for rapid, routine analysis of radioprotective drugs in animal tissue samples and has not been optimized for a wide range of thiol derivatives. The column and guard column used are as described for Method 1.
Solvent A was prepared by diluting 2.5 ml acetic acid, 50 ml acetonitrile, and 7.03 g sodium perchlorate to one liter with water and adjusting the pH to 3.4 with concentrated sodium hydroxide. Solvent B was prepared by addition of 2.5 ml acetic acid and 7.03 g sodium perchlorate to 200 ml water and diluting with acetonitrile to one liter. The elution protocol (linear gradients, 24°C, 1.5 ml per min) was as follows: 0 min, 2% B; 5 min, 13% B; 14 min, 13% B; 16 min, 100%; B; 20 min, 2% B; 35 min, 2% B; reinject.

With the methods described above it is possible to detect 0.1 pmoles of the mBBr derivative of glutathione with a signal-to-noise ratio of 5:1. The response factor is sensitive to solvent and increases as the organic modifier is increased. Most other thiol derivatives have response factors comparable to that for the glutathione derivative but the factor for derivatives of aromatic thiols such as ergothioneine and 5-mercaptop-2-nitrobenzoic acid can be an order of magnitude or more lower. Response factors vary with the equipment used and with the age of the fluorometer lamp and must be determined from standard samples analyzed concurrently with each series of unknown samples. The response factor (integrated area/pmol of standard) has been found to be constant with an increase in concentration for samples containing 0.1 to 200 pmoles of the glutathione derivative.

Analysis of Mung Bean

Figure 2 illustrates the application of HPLC method 1 to the analysis of an extract of mung bean prepared in 50% aqueous acetonitrile containing borate buffer, pH 9. The dominant peak in the reagent blank (Fig. 2A) derives from mBBr and water (Table I). Additional peaks appear in the unknown control (Fig. 2B), the dominant one occurring at ~37 min. This is where syn-(methyl,methyl)bimane, the compound obtained by replacing Br in mBBr by H,
els. In addition to undergoing nucleophilic substitution reactions, mBBr can
serve as an electron acceptor, ultimately being reduced to syn-
(methyl,methyl)bimane. Constituents in cell extracts, especially those from
photosynthetic organisms, appear to include electron donors which can partici-
pate in this reaction and lead to a peak for syn-(methyl,methyl)bimane in the
control sample which is blocked by NEM and DTNB to varying degrees depending
upon the sample. Interpretation of the unknown control is complicated to some
extent by the fact that a few thiol compounds do not react fully with NEM and
DTNB. All of the thiols listed in Table I are blocked by NEM and DTNB with the
exception of ergothioneine (which does not react with either), 2-
mercaptopyridine (which reacts incompletely with DTNB), and thiosulfate (which
is unreactive with NEM and only partially reactive with DTNB).

The reagent blank and unknown control sample are used to identify peaks in
the unknown sample (Fig. 2C) which do not correspond to thiol derivatives. The
nonthiol peaks in the unknown sample are not always of the same intensity as
those in the reagent blank and unknown control samples, presumably because the
reaction of thiol in the unknown sample with mBBr depletes the reagent and
changes the level of formation of other reagent derived peaks. Four significant
peaks represent thiols in Fig. 2C, three of which can be ascribed to cysteine,
\( \gamma \)-glutamylcysteine, and homoglutathione. Analysis of the extract using method 2
(not shown) verified these assignments and also revealed the presence of a low
level of CoA which cannot be analyzed by method 1 because the peak for CoA is
too broad. The fourth peak, eluting at 32 min, does not correspond to any of
our standards and is designated as an unknown (U32). It has been shown that
mung bean contains homoglutathione rather than glutathione. Identification of the thiols in mung bean was relatively straight forward.

In other instances we have found it necessary to collect individual peaks as
they elute using method 1 and to reinject them using methods 2 and 3 in order to obtain unequivocal assignments. Thiols that cannot be assigned based upon known standards can be purified by methods described elsewhere (this volume [XX]) for structure elucidation using other techniques.

**Quantitative Determinations**

For analytical applications it is important to establish that the methodology selected is capable of quantitatively measuring the thiols of interest in the specific system under study. This is usually done via recovery experiments in which known amounts of the thiols to be measured are added at levels comparable to the endogenous levels during the extraction step. Such recovery studies have shown that the methanesulfonic acid extraction method in combination with HPLC method 3 results in recoveries of the radioprotective drug WR-1065 of at least 80%<sup>14,15</sup>. Application of the aqueous acetonitrile extraction (borate buffer, pH 9.0) in combination with HPLC methods 1 and 2 to analysis of photosynthetic bacteria (*Chromatium vinosum*) was tested for recovery of cysteine, GSH, thiosulfate, and CoA and found to give $\geq$80% recovery (unpublished results). Recovery of GSH, CoA and thiosulfate (0.05 μmoles per g) during extraction of mung bean in acetonitrile (borate, pH 9) was shown to be $\geq$85%. Such results cannot be safely generalized to other thiols or other cell systems and it is essential that recovery experiments be conducted with the specific system of interest if quantitative data are required. The analytical results can sometimes be improved through the use of an internal standard added to the initial extract and used to compensate for mechanical losses during sample processing. Use of a thiol as an internal standard to compensate for losses due to oxidation or other chemical reactions would likely prove unsatisfactory owing to differing rates of reaction for different thiols.
Aside from enzymic degradation, thiol oxidation constitutes the most serious difficulty to be overcome in designing extraction methods that will yield quantitative results. DTPA has been described as a more effective than EDTA in reducing iron mediated formation of peroxide from oxygen and we have generally found less oxidation of thiols during labeling with mBBr in the presence of DTPA than with EDTA. However, even in the presence of DTPA we have found with some cell systems that oxidative loss competes with the bimane labeling reaction, despite the fact that the half life for reaction of GSH with mBBr under typical labeling conditions is only ~20 sec. In such cases it is necessary to exclude oxygen from the sample during the labeling process. When acid extraction methods are used, the acid must be neutralized to permit the labeling reaction to occur at high pH. Large amounts of buffer are often needed for this purpose and it is important that the buffer not contain traces of peroxide which can oxidize the thiol. Such neutralizations should not be conducted with strong base since production of local regions of very high pH can enhance oxidative loss if oxygen is not being excluded.

Following extraction of cells, intracellular thiols can potentially come in contact with cellular disulfides or thioesters with which they might react. Such thiol-disulfide exchange or transacylation reactions can change the thiol composition of the sample. However, these reactions, like the mBBr labeling reaction, occur via the thiolate anion (RS⁻) but are significantly slower than the labeling reaction. Thus, only in the case of an extraordinarily reactive disulfide or thioester are such reactions likely to compete with formation of mBSR.

The methods described here can also be used to determine the thiols that are present in cells in disulfide and thioester forms. For this purpose cells should be extracted in the presence of NEM to block the thiols. It is often
assumed that only disulfides are cleaved by DTT but thioesters can also be
cleaved via transacylation reactions with DTT under conditions similar to those
which lead to disulfide reduction. Methods for selective cleavage of disulfides
and thioesters with accompanying formation of their mBBr derivatives using
hydroxylamine in combination with DTT and NEM have been described.\textsuperscript{18}

\section*{Acknowledgments}

We thank the National Institutes of Health (Grants CA-32333 and CA-39582)
and the National Aeronautics and Space Administration (Grant NAGW-342) for sup-
port of this research.
REFERENCES


16. G. Cohen and P. M. Sinet, in "Chemical and biochemical Aspects of Superoxide and Superoxide Dismutase" (J. V. Bannister and H. A. O.


## TABLE I

Retention times of Thiol-mBBr Derivatives and Reagent Derived Products

<table>
<thead>
<tr>
<th>Compound, Abbreviation</th>
<th>Retention time (min)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Thiols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetyl cysteine, NAC</td>
<td>24</td>
<td>19.2</td>
</tr>
<tr>
<td>coenzyme A, CoA</td>
<td>43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.9</td>
</tr>
<tr>
<td>coenzyme M, CoM</td>
<td>13</td>
<td>21.4</td>
</tr>
<tr>
<td>cysteamine, CyA</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9</td>
</tr>
<tr>
<td>cysteine, Cys</td>
<td>8</td>
<td>5.8</td>
</tr>
<tr>
<td>cysteinylglycine, CG</td>
<td>17</td>
<td>5.1</td>
</tr>
<tr>
<td>dephospho coenzyme A, dpCoA</td>
<td>41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7</td>
</tr>
<tr>
<td>dithiothreitol, DTT</td>
<td>48</td>
<td>20.5</td>
</tr>
<tr>
<td>ergothioneine, Ergo</td>
<td>28</td>
<td>7.2</td>
</tr>
<tr>
<td>glutathione, GSH</td>
<td>16</td>
<td>14.3</td>
</tr>
<tr>
<td>d-glutamyl cysteine, d-GC</td>
<td>17</td>
<td>10.1</td>
</tr>
<tr>
<td>y-glutamyl cysteine, y-GC</td>
<td>12</td>
<td>15.1</td>
</tr>
<tr>
<td>homocysteine, hCys</td>
<td>18</td>
<td>8.5</td>
</tr>
<tr>
<td>homoglutathione, hGSH</td>
<td>22</td>
<td>12.7</td>
</tr>
<tr>
<td>2-mercaptoethanol, 2-ME</td>
<td>38</td>
<td>16.0</td>
</tr>
<tr>
<td>2-mercaptopuridine, 2-PySH</td>
<td>57</td>
<td>26.7</td>
</tr>
<tr>
<td>methanethiol, MeSH</td>
<td>48.5</td>
<td>20.9</td>
</tr>
<tr>
<td>2-nitro-5-mercaptopenzoic acid</td>
<td>40</td>
<td>25.3</td>
</tr>
<tr>
<td>pantetheine, Pant</td>
<td>44</td>
<td>18.8</td>
</tr>
<tr>
<td>penicillamine</td>
<td>29</td>
<td>12.5</td>
</tr>
<tr>
<td>4'-phosphopantetheine, 4'-p-Pant</td>
<td>33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.5</td>
</tr>
<tr>
<td>sulfole, H&lt;sub&gt;2&lt;/sub&gt;S</td>
<td>4</td>
<td>18.5</td>
</tr>
<tr>
<td>sulfite, SO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>32</td>
<td>19.1</td>
</tr>
<tr>
<td>thioglycolic acid, TG</td>
<td>7</td>
<td>21.1</td>
</tr>
<tr>
<td>thiosulfate, SSO&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WR-1065</td>
<td>2.2</td>
<td>12.0</td>
</tr>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water-mBBr, unbuffered</td>
<td>21,41</td>
<td>9.0, 11.4, 16.2, 9.4, 14.5</td>
</tr>
<tr>
<td>N-ethylmorpholine pH 8</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>HEPPS pH 8</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>potassium chloride pH 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43</td>
<td>none</td>
</tr>
<tr>
<td>potassium phosphate pH 8</td>
<td>4</td>
<td>16.8, 17.7</td>
</tr>
<tr>
<td>sodium acetate pH 8</td>
<td>37</td>
<td>18.1</td>
</tr>
<tr>
<td>sodium borate pH 9</td>
<td>56, 57, 58</td>
<td>27.3, 28.3</td>
</tr>
<tr>
<td>sodium methanesulfonate pH 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>sulfosalicylic acid pH 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Tris-methanesulfonate pH 8</td>
<td>12</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Broad or unsymmetrical peak. <sup>b</sup>Peaks listed for water-mBBr also occur for other aqueous reagents. All reagents tested at 1 M. Major peaks are underlined, others are less intense. <sup>c</sup>Buffered with 10 mM HEPPS.
CAPTIONS TO FIGURES

Fig. 1. HPLC chromatograms of thiol standard samples: (A) method 1; (B) method 2; (C) method 3. Peaks present in corresponding reagent blank samples are designated R. Other abbreviations are defined in Table 1.

Fig. 2. HPLC chromatograms obtained for mung bean extracted in aqueous acetonitrile containing borate buffer, pH 9.0 (see Preparation of Samples). (A) reagent blank; (B) unknown control; (C) unknown. Peaks designated R and C represent peaks present in the reagent blank and unknown control samples, respectively. U32 designates an unidentified thiol derivative eluting at 32 min. Other abbreviations are given in Table I.
RELATIVE FLUORESCENCE

Cys
γ-GC
C
C
U32
C
C
C
C
h GSH
R
R
R