Analysis of Glutathione, Glutathione Disulfide, Cysteine, Homocysteine, and Other Biological Thiols by High-Performance Liquid Chromatography Following Derivatization by N-(1-Pyrenyl)maleimide

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The compound N-(1-pyrenyl)maleimide (NPM) reacts with free sulfhydryl groups to form fluorescent derivatives. A new method for measurement of glutathione and other biological thiols utilizing reverse-phase high-performance liquid chromatography to separate and quantify these derivatives is described. Separation and quantification of glutathione, cysteine, homocysteine, cysteinylglycine, and γ-glutamylcysteine derivatives are achieved. The method allows for the measurement of glutathione disulfide by masking free glutathione with 2-vinylpyridine, reducing glutathione disulfide with glutathione reductase, and measuring the resulting glutathione. Coefficient of variations for the various thiols measured by the NPM method range from 1.5 to 8.8%. The lower detection limit is around 50 fmol of glutathione. NPM derivatives are shown to be stable for 2 months at 4°C. Between 94.2 and 97.2% of glutathione and/or glutathione disulfide added to a sample is recovered using the NPM method. The NPM method is compared to the monobromobimane high-performance liquid chromatography method and the Tietze assay by measuring glutathione in homogenates from five different cell lines. The newly developed method offers some advantages over the currently accepted techniques, including specificity, speed, sensitivity, and ease of use. © 1995 Academic Press, Inc.

The tripeptide glutathione (γ-glutamylcysteinylglycine) is distributed widely in the body and serves several functions (1). Due to its numerous and important functions, interest in glutathione is increasing. Measurement of glutathione and other thiols is complicated by their instability in aqueous solution and their tendency to oxidize to disulfides. Consequently, assays for glutathione must be rapid and specific in order to ensure accurate measurements. Often, the simultaneous measurement of glutathione (GSH) and glutathione disulfide (GSSG) is desired. Glutathione disulfide usually exists as a small fraction of total glutathione (GSH + GSSG) under normal conditions, but increases under conditions of oxidative stress. Therefore, assays for glutathione should be able to measure both GSH and GSSG and be sensitive to small amounts of GSSG. Various methods for glutathione analysis have been published, but most current methods have the disadvantages of being either time consuming or labor intensive. The compound N-(1-pyrenyl)maleimide (NPM) forms fluorescent derivatives with compounds containing a free sulfhydryl group according to the reaction shown in Fig. 1. We have utilized this compound in conjunction with reverse-phase high-performance liquid chromatography (HPLC) to measure GSH in various biological samples. We have also measured GSSG in the samples by blocking free GSH by reaction with 2-vinylpyridine, reducing GSSG with glutathione reductase, and measuring the resulting GSH by forming NPM derivatives. In addition to performing experiments to show the efficacy and define the limits of the new method and to demonstrate the stability of the NPM derivatives, the NPM method is compared to the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) glutathione reductase recycling assay developed by

1 To whom correspondence should be addressed. Fax: (314) 341-6033.
3 Abbreviations used: GSH, glutathione; GSSG, glutathione disulfide; NPM, N-(1-pyrenyl)maleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); mBBr, monobromobimane.

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Tietze (2) and the monobromobimane (mBBr) HPLC method (3). The newly developed method is rapid and sensitive and compares favorably with the Tietze assay and the mBBr method while offering some distinct advantages.

MATERIALS AND METHODS

**Chemicals and reagents.** Acetonitrile, water, methanol, acetic acid, and phosphoric acid (all HPLC grade), monobasic sodium phosphate, disodium EDTA, and di-basic sodium phosphate were purchased from Fisher (St. Louis, MO). NPM and 2-vinylpyridine were obtained from Aldrich (Milwaukee, WI). GSH, GSSG, cysteine, homocysteine, cysteinylglycine, γ-glutamylcysteine, 5-sulfosalicylic acid, DTNB, and NADPH were from Sigma (St. Louis, MO). GSH and GSSG were obtained as SigmaUltra grades with purity claims of 98–100%. mBBr was purchased from Calbiochem (La Jolla, CA) under the name Thiolyte MB. Glutathione reductase was from Boehringer–Mannheim.

**HPLC system.** The HPLC system (Shimadzu) consisted of two Model LC-6A pumps, a Model SCL-6B controller, a Rheodyne injection valve with a 20-μl loop, and a Model RF-555 fluorescence spectrophotometer operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The HPLC column (Astec, Whippany, NJ) was 100 × 4.6 mm and packed with 3-μm particle size C_{18} packing material. Quantitation of the peaks from the HPLC system was performed by a Chromatopac Model C-1B (Shimadzu). Although the procedure employed isocratic elution, a two-pump system was used in order to easily achieve slight modifications in the mobile phase to compensate for differences in ambient conditions or for column-to-column differences. Solvent A was 80% water and 20% acetonitrile containing 1 ml/liter acetic acid and 1 ml/liter phosphoric acid. Solvent B was 20% water and 80% acetonitrile containing 1 ml/liter acetic acid and 1 ml/liter phosphoric acid. Solvents A and B were mixed in a chamber before the column and the resulting mixture of solvents A and B was used as the mobile phase. Unless otherwise specified, the NPM derivatives were eluted from the column using 25:75% A:B concentration at a total flow rate of 0.5 ml/min. Peaks were quantified by comparison to standard curves prepared by plotting peak area versus concentration of known standards.

**Measurement of thiols.** Enough water was added to the sample to make a volume of 250 μl. Acetonitrile (250 μl) and 500 μl of a 1.5 mM solution of NPM in acetonitrile were added and the resulting solution was mixed. The pH of the solution at this point is 7 or greater, depending upon the buffer used in the sample. After incubation at room temperature for 5 min, the mixture was acidified with 10 μl of 50% (v/v) acetic acid and filtered through a 0.2-μm pore size nylon filter. The pH of the solution at this point is 5.0. The samples were stored in tightly capped vials at 4°C until they were ready for injection onto the HPLC system. Standard curves for thiols were linear from 0.01 to 4 μM thiol contained in a 20-μl injection of the derivatization mixture or from 0.2 to 80 pmol thiol derivative detected by the HPLC system. There were no significant differences in thiol derivative peak areas when a sample was allowed to incubate from 1 to 60 min. Five minutes was chosen for the incubation time because it was the time necessary to perform pipetting tasks when dealing with a large number of samples. The solution of NPM in acetonitrile appeared to be stable for 2 months when stored at room temperature, giving nearly identical results during the course of storage.

**Measurement of GSSG.** Enough water was added to the sample to make a volume of 98 μl. Neat 2-vinylpyridine (2 μl) was added, and the mixture was allowed to incubate at room temperature for 60 min. A solution of 2 mg/ml NADPH (95 μl) in a 0.1 M sodium phosphate buffer containing 6.3 mM EDTA (pH 7.5) was added. A 5 mg/ml glutathione reductase suspension (5 μl) was added, the solution was mixed, and an aliquot of 100 μl was immediately taken out to measure GSH according to the procedure above. GSSG concentrations were calculated by halving the GSH concentrations obtained.

**Comparison with other methods.** HA1, OC14, O2R95, and OC5 Chinese hamster fibroblast cell lines were plated into 100-mm culture dishes (2.5 to 3.0 × 10⁵ cells/dish) containing 10 ml of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Hy-Clone) and penicillin/streptomycin (100 U/ml, 100 μg/ml) and grown exponentially for 3 days prior to harvest (4,5). The U-87 MG human glioma-derived cell line (ATCC) was plated into 100-mm culture dishes (3 × 10⁵ cells/dish) containing 10 ml of BME supplemented with 10% heat-inactivated (30 min, 56°C) iron-supplemented bovine calf serum (Hy-Clone) and penicillin/streptomycin (100 U/ml, 100 μg/ml) and grown exponentially for 3 days prior to harvest. At the time of harvest, the media were poured off, and the cells were rinsed with cold sa-
line. The cells from each of the three dishes were scraped into a centrifuge tube on ice with cold saline. The dishes were then rinsed with cold saline, with the rinse added to the centrifuge tube. The cells were centrifuged at 400g for 5 min at 4°C. The resulting cell pellet was stored at −20°C overnight. To inhibit γ-glutamyltranspeptidase activity the cell pellets were lysed in 350 μl of serine-borate buffer (6) using four 5-s bursts of a Biosonik III (Bronwill Scientific) sonicator equipped with a microtip at 30% output while keeping the samples on ice. Protein was assayed in the samples by the method of Lowry et al. (7). GSH and GSSG were measured in the samples using the NPM methods outlined above. GSH was measured in the samples using the mBBr HPLC method as previously described (3,8). Total glutathione (GSH + GSSG) was measured in the samples using the Tietze assay as previously described (2,8). All biochemical determinations were blanked to sham-treated samples containing serine-borate buffer without cells.

RESULTS

A chromatogram showing separation of NPM derivatives of glutathione, cysteine, homocysteine, cysteinylglycine, and γ-glutamylcysteine is given in Fig. 2A. The sample was prepared by derivatizing a solution containing 0.5 μM of each of the five thiols according to the procedure for measurement of GSH. The load onto the column was 10 pmol of each of the five thiols. The peaks for the NPM derivatives are labeled. The peaks were identified by injecting each prepared derivative separately and by spiking the sample with the genuine prepared derivatives of each different compound. Fluorescent yields relative to the GSH derivative peak were GSH, 1.00; γ-glutamylcysteine, 0.87; cysteine, 0.86; cysteinylglycine, 0.56; and homocysteine, 1.06. The chromatogram in Fig. 2B was from a blank sample prepared by adding serine-borate buffer in the place of a thiol-containing sample. As can be seen, unlabeled peaks in chromatogram A also appeared in the blank, chromatogram B. These peaks are thought to arise from reagent impurities or products obtained by the hydrolysis of the reagent. The area of the chromatogram where thiol derivative peaks appeared was free of reagent impurity and reagent hydrolysis product peaks.

Within-run precision for the method was obtained by injecting the same sample seven times consecutively and comparing the peak areas for the glutathione and cysteine derivative peaks obtained for the seven injections. The coefficient of variation obtained for the within-run precision was 1.5% for the GSH derivative peak and 2.3% for the cysteine derivative peak. A between-run precision was determined by derivatizing the same sample seven different times and injecting the resulting seven derivative mixtures independently. The coefficient of variation for the between-run precision was 5.5% for the GSH derivative peak and 8.8% for the cysteine derivative peak.

The lower detection limit of the NPM method was found by preparing dilute solutions of GSH, assaying them for GSH with the NPM method, and identifying
the concentration of GSH that gave the smallest observable GSH derivative peak in the chromatogram. Figure 3 is a chromatogram showing detection of 58 fmol of GSH, approximately the lower detection limit of the NPM method. An attempt to detect 58 fmol of GSH with the mBBR method gave no observable GSH derivative peak in the mBBR chromatogram.

Experiments to determine the stability of NPM derivatives were performed. Derivatives were prepared from a standard solution of GSH in mixtures of 10% water/90% acetonitrile to 90% water/10% acetonitrile. Following the derivatization reaction, these solutions were either acidified with 10 μl of 50% acetic acid or left nonacidified. Aliquots of these solutions of GSH derivatives were injected onto the HPLC system periodically during the course of a 2-month storage at 4°C. Greatest stability of derivatives was obtained by preparing the derivatives in 25% water/75% acetonitrile followed by acidification of the derivative mixture after the derivatization reaction. Derivatives prepared in this manner showed no breakdown during the course of the 2-month storage at 4°C.

Experiments were also performed to determine the appropriate incubation time of GSH and 2-vinylpyridine in the determination of GSSG using the NPM method. A solution of 2 mM GSH was allowed to react with 2-vinylpyridine at the ratio of 2 μl of neat 2-vinylpyridine per 100 μl of solution. Periodically, an aliquot was removed to determine the concentration of free GSH remaining according to the NPM method. Figure 4 shows the time course of the reaction by plotting percentage free GSH remaining versus time of reaction with 2-vinylpyridine. As can be seen from the figure, the reaction is rather slow, but by 60 min 99.9% of the GSH has been blocked by the 2-vinylpyridine.

A separate experiment was performed to determine the time course of the glutathione reductase reaction portion of the GSSG determination. A solution of 1.0 mM GSSG was allowed to be reduced by glutathione reductase according to the GSSG determination procedure. Periodically, an aliquot of this solution was removed to determine GSH concentration using the NPM method. Essentially, all of the GSSG was reduced as soon as the solution was mixed and an aliquot was removed for GSH determination. No differences in GSH derivative peak areas were observed from the time necessary to mix the sample to 45 min glutathione reductase reaction time.

It is possible that during the course of the glutathione reductase reaction GSH that is being formed from GSSG will react with the excess 2-vinylpyridine that is left following the masking of GSH, giving erroneously low GSSG values. This possibility was examined by varying the glutathione reductase reaction time in the GSSG assay. Most of the GSH formed from GSSG in the glutathione reductase reaction (85%) remained when the reaction was allowed to proceed 5 min in the presence of the excess 2-vinylpyridine that remained following the masking of GSH. When the reaction was allowed to proceed in the presence of excess 2-vinylpyridine only the time necessary to mix and remove an aliquot for GSH determination, essentially all of the resulting GSH is derivatized with NPM. There are no
differences in GSH derivative peak areas obtained from GSSG when the glutathione reductase reaction is performed in the presence or absence of 2-vinylpyridine, provided the reaction is allowed to proceed only as long as is necessary to mix the sample and remove an aliquot for GSH analysis.

Experiments were performed to determine whether known amounts of GSH and GSSG added to a biological sample could be accurately recovered by the NPM method. These experiments were performed by measuring a sample of the HA1 cell line for GSH using the NPM method, spiking the sample by adding 10 μl of a concentrated solution of standard GSH (0.1 to 0.2 mM) per 100 μl of sample, and remeasuring the sample for GSH. This was repeated by spiking with GSSG and also with a mixture of GSH and GSSG. In all cases, the spiking of GSH or GSSG was enough to double the GSH or GSSG concentration of the sample. Expressed as mean ± SD of three separate experiments, recovery of GSH from a GSH-spiked sample was 97.0 ± 7.8%. Recovery of GSSG from a GSSG-spiked sample was 96.4 ± 2.4%. Recovery of GSH from a GSH + GSSG-spiked sample was 94.2 ± 2.1%. Recovery of GSSG from a GSH + GSSG-spiked sample was 97.2 ± 4.5%. To confirm the 98–100% purity claims by the manufacturer of the GSH and GSSG, they were assayed independently by reaction with Ellman’s reagent (9). A solution of GSH was allowed to react with Ellman’s reagent and the absorbance at 412 nm was measured. Assuming an extinction coefficient of 13,600 liter mol⁻¹ cm⁻¹ (9), the concentration of GSH in the mixture was calculated and compared to the concentration calculated from the mass of GSH used assuming 100% pure GSH. The GSH was found to be >99% pure. Similarly, the purity of GSSG was determined by reducing a solution of GSSG to GSH using glutathione reductase and allowing the resulting solution of GSH to react with Ellman’s reagent. The GSSG was found to be >98% pure.

Comparison of values obtained for GSH, GSSG, and total glutathione (GSH + GSSG) in the HA1, OC14, O₂R95, OC5, and U-87 MG cell lines using the NPM method, the Tietze assay, and the mBr HPLC technique is shown in Table 1. GSH values obtained using the NPM method are compared to values obtained using the mBr method, and the values for total glutathione (GSH + GSSG) obtained using the Tietze assay are compared to the sum of the values of GSH and GSSG obtained using the NPM methods. The values shown are nanomole GSH equivalents/mg protein and are mean ± SD for three different measurements of the same cell line sample. The NPM method gave values similar to the other methods, with all comparisons being within 10%. Quantification of other thiols using the NPM method was also achieved. Cysteine values in nmol/mg protein were 1.07 ± 0.70 for HA1, 1.82 ± 0.78 for OC5, 2.64 ± 0.47 for OC14, 2.97 ± 0.46 for O₂R95, and 1.40 ± 0.56 for U-87 MG. γ-Glutamylcysteine values in nmol/mg protein were 0.120 ± 0.0228 for HA1, 0.268 ± 0.0368 for OC5, 0.320 ± 0.00799 for OC14, 0.427 ± 0.0519 for O₂R95, and 0.280 ± 0.0272 for U-87 MG. Cysteinylglycerine values in nmol/mg protein were 0.205 ± 0.022 for HA1, 0.136 ± 0.011 for OC5, 0.154 ± 0.0060 for OC14, 0.283 ± 0.027 for O₂R95, and 0.178 ± 0.023 for U-87 MG. Homocysteine values in nmol/mg protein were 0.152 ± 0.0076 for HA1, 0.131 ± 0.018 for OC5, 0.125 ± 0.015 for OC14, 0.233 ± 0.057 for O₂R95, and 0.318 ± 0.04 for U-87 MG. Figure 5 is a chromatogram from the OC14 cell line.

DISCUSSION

Glutathione appears to be involved in several important biological processes, including protection against free radicals formed following exposure to ionizing radiation (1,10,11), protection against oxygen toxicity (1,4,12,13), and metabolism of xenobiotics (1). Due to its numerous and important protective functions, research involving glutathione is increasing. Sensitivity and rapidity are very important considerations for glutathione assays in order for researchers to keep pace with the increased interest in the molecule. Since glutathione is labile in solution, assays must have the capacity to rapidly convert glutathione to a stable and measurable form. Assays also must have the capacity to measure GSSG, which is usually present in small amounts in biological samples under normal conditions, but increases during oxidative stress.

Several methods for glutathione measurement have been published. Earlier methods included assays based upon enzymatic reactions involving glutathione as a co-factor, such as the glyoxylase reaction (14). These assays were insensitive and time consuming relative to more recent techniques. Development of the Tietze assay (2), an acceptable and widely used method for total glutathione determination, afforded sensitivity and specificity by coupling the reaction of DTNB and thiols with the glutathione reductase reaction. More sensitive assays using HPLC have been developed (15–18), but many of these assays still have certain disadvantages. HPLC methods based upon electrochemical detection (19,20) or uv absorbance (21) are not specific for thiol-containing compounds, which creates a very complicated chromatogram. Fluorescent labeling of thiol compounds followed by separation by HPLC offers high sensitivity and specificity for thiol-containing compounds, which simplifies chromatograms obtained by requiring the separation of fewer peaks. This is the basis for some HPLC methods, including the mBr HPLC method, another widely used and acceptable method for measurement of GSH.

The compound NPM reacts with sulphydryl groups to give fluorescent derivatives (22,23). This compound has been used as a postcolumn derivatizing agent, but was
TABLE 1

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>GSH, mBBr assay</th>
<th>GSH, NPM assay</th>
<th>GSSG, NPM assay</th>
<th>GSH + GSSG, Tietze assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1</td>
<td>11.10 ± 0.68</td>
<td>10.87 ± 0.62</td>
<td>0.543 ± 0.137</td>
<td>12.69 ± 2.47</td>
</tr>
<tr>
<td>OC5</td>
<td>19.72 ± 1.17</td>
<td>20.11 ± 2.44</td>
<td>0.283 ± 0.114</td>
<td>19.92 ± 0.91</td>
</tr>
<tr>
<td>OC14</td>
<td>24.93 ± 1.53</td>
<td>23.40 ± 1.85</td>
<td>0.628 ± 0.081</td>
<td>24.21 ± 0.88</td>
</tr>
<tr>
<td>O2R95</td>
<td>27.89 ± 4.23</td>
<td>28.21 ± 0.39</td>
<td>0.511 ± 0.101</td>
<td>28.87 ± 0.55</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>26.36 ± 3.19</td>
<td>23.30 ± 0.81</td>
<td>0.270 ± 0.062</td>
<td>23.23 ± 2.51</td>
</tr>
</tbody>
</table>

*Note. All values are mean ± SD of nmol GSH equivalents/mg protein determined from assaying one sample from each of the cell lines at least three times using the three different assays. GSH values obtained with the mBBr method are to be compared to GSH values obtained using the NPM method. The sum of GSH + GSSG values obtained using the Tietze assay is to be compared to the sum of the values of GSH obtained using the NPM method and GSSG using the NPM method.*

Rejected as a precolumn derivatizing agent because derivatives obtained under the conditions employed were not stable (24,25). The previous study used aqueous solutions of the derivatives, which were determined to be unstable by both the previous and present studies. In the present study, we show that the derivatives are stable for at least 2 months if kept in an acidified solution of 25% water and 75% acetonitrile and stored at 4°C. Hydrolysis of the NPM derivatives can occur at the bonds between the carbonyl groups and the nitrogens of the derivatives (25). Lowering the water content and acidification of the derivative solutions following derivatization is suspected to slow the rate of hydrolysis of the NPM deriva-

tives, making the derivatives more stable than they would be in a nonacidified, purely aqueous solution. This makes it possible to use NPM as a precolumn derivatizing agent, which is preferable since samples can be derivatized immediately after collection, ensuring that no loss of glutathione occurs during sample storage.

It should also be noted that reaction of maleimides with glutathione and other amino acid derivatives can produce diastereomers that are theoretically separable using HPLC. No splitting of any derivative peak was noted using the conditions of the present study. Injection of standard GSH derivative gave only one more peak than was noticed in the blank sample. Therefore, it appears that either one diastereomer is the favored product of the reaction or that the two different diastereomers are not separable with the HPLC conditions used in the present study. GSH can be effectively blocked from reaction with NPM by reacting GSH first with 2-vinylpyridine. This has been the basis of a modification of the Tietze assay to measure only GSSG (26). The reaction of NPM with GSH is relatively rapid at neutral to basic pH, with reaction rate increasing with increasing pH to pH 11 (25). Using the conditions in the present study, the reaction is complete within 1 min. The reaction of NPM and GSH is very rapid compared to the reaction of 2-vinylpyridine and GSH. This has allowed us to use 2-vinylpyridine to mask free GSH and measure GSSG with the NPM methodology in standard conditions as well as cell homogenates. The reaction of 2-vinylpyri-
dine and GSH is relatively slow, but is essentially complete within 60 min at room temperature. The glutathi-
one reductase reaction with GSSG to form GSH is shown to be very rapid, and aliquots of the reaction mix-
ture could be taken immediately for NPM derivatization of the new GSH before excess 2-vinylpyridine could re-
act with the newly formed GSH.

The NPM method is shown to be reproducible, with within-run precision ranging from 1.5 to 2.3% and be-
tween-run precision ranging from 5.5 to 8.8%. The method is shown to be very sensitive, detecting as little
as 58 fmol GSH. It is also demonstrated that the NPM method can accurately recover GSH and GSSG added to a biological sample. When measuring cell samples for GSH, the NPM method gave similar results as the mBBr method. When measuring the same cell samples for total glutathione (GSH + GSSG), the NPM methods compare favorably with the values obtained using the Tietze assay, with values agreeing to within 10%. These data show the ability of the NPM method to give results similar to those obtained with two other widely accepted methods for measuring glutathione.

The NPM method offers some advantages over the mBBr method and the Tietze method, the first being specificity. The Tietze assay is typically used to measure only total glutathione, but can be modified to measure oxidized glutathione by masking free GSH with 2-vinylpyridine (8,26). This modification, however, adds steps to a method that is already more labor intensive than the NPM method. Both the NPM method and the mBBr method as modified (27) can measure total glutathione (GSH + GSSG) and reduced glutathione. Also, the mBBr and the NPM methods are able to measure any thiol-containing compound, an advantage over the Tietze assay which can only detect glutathione.

The second advantage of the NPM method is sensitivity. In our hands, the Tietze assay as modified (8) is able to detect 16 pmol of GSH per 50 μl of sample. The mBBr method has a lower detection limit reported to be 1 pmol of GSH (28). The NPM method appears to be the most sensitive of these assays with a lower limit of detection of 58 fmol of GSH.

Another advantage of the NPM method is the speed of analysis. The derivatization reaction of the NPM method is complete within 1 min, whereas the mBBr derivatization reaction is carried out for 20–45 min. All fluorescent material is eluted from the column in 20–30 min in the NPM method, with the column being ready for another injection. The mBBr method requires at least 45–60 min to elute all peaks from the column, wash the column of late-eluting fluorescent material, and re-equilibrate the column (3,8). Therefore, measurement of GSH in a sample requires 25–35 min with the NPM method, while the mBBr requires 65–105 min.

The final advantage of the NPM method is the ease of handling. Use of the Tietze assay requires much sample and reagent handling and pipetting to prepare cuvettes for the spectrophotometer. Since the mBBr and the NPM method utilize HPLC, both methods have the advantage of being easily automated. Monobromobimane solutions used to derivatize are light sensitive and unstable; therefore, derivatizations in the mBBr method must be carried out in the dark (3,8). The NPM method does not have this inconvenience.

In summary, separation and quantification of NPM derivatives of glutathione, cysteine, homocysteine, cysteinylglycine, and γ-glutamylcysteine were achieved.

NPM derivatives were demonstrated to be stable for 2 months when stored at 4°C. The NPM method was able to accurately recover genuine GSH and GSSG added to a biological sample. The NPM method gave similar results as the mBBr method and the Tietze assay when measuring cell homogenates. The NPM method offered the further advantages of increased sensitivity, rapid analysis, and ease of use when compared to the mBBr and the Tietze methods for measuring glutathione.

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