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Determination of biological thiols by high-performance liquid chromatography following derivatization by ThioGlo maleimide reagents

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Abstract

The importance of thiols has stimulated the development of a number of methods for determining glutathione and other biologically significant thiols. Methods that are currently available, however have some limitations, such as being time consuming and complex. In the present study, a new high-performance liquid chromatography (HPLC) method for determining biological thiols was developed by using 9-Acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran (ThioGlo™3) as a derivatizing agent. ThioGlo™ reacts selectively and rapidly with the thiols to yield fluorescent adducts which can be detected fluorimetrically (λ_{ex}=365 nm, λ_{em}=445 nm). The within-run coefficient of variation for glutathione (GSH) by this method ranges from 1.08 to 2.94% whereas the between-run coefficient of variation for GSH is 4.31–8.61%. For GSH, the detection limit is around 50 fmol and the GSH derivatives remain stable for 1 month, if kept at 4°C. Results for GSSG and cysteine are also included. The ThioGlo™ method is compared to our previous method in which *N*-(1-pyrenyl)maleimide (NPM) is used to derivatize thiol-containing compounds. The present method offers various advantages over the currently accepted techniques, including speed and sensitivity.

Keywords: Thiols; ThioGlo maleimide

1. Introduction

Thiol compounds have important biological functions. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH), the most abundant low molecular mass thiol, is widely distributed in living cells and is involved in many biological reactions. Oxidative stress can result from depletion of antioxidants due to malnutrition and/or excess production of Reactive Oxygen Species (ROS) [1]. Glutathione is an antioxidant which protects cells against oxidative stress. During oxidative stress, it has been shown that depletion of GSH along with an increase in its oxidation product, glutathione disulfide (GSSG), levels occurs. Therefore, it is usually preferred to determine both levels and report the ratio of GSH to GSSG in free radical-related research.

Other important bio-thiols studied include Homocysteine (Hcy) and *N*-acetylcysteine (NAC). Hcy is generated from the metabolism of essential amino

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acid methionine. It has recently received significant attention because hyperhomocysteinemia is a risk factor for some cardiovascular diseases such as atherosclerosis and thrombosis [2,3]. It is suggested that Hcy exhibits its role in the pathogenesis of atherosclerosis through the mechanisms involving oxidative stress-induced damage [4]. NAC is a precursor of GSH and is an antioxidant itself. NAC has long been used as a mucolytic agent for treatment of chronic bronchitis and is an effective antidote for acetaminophen poisoning [5,6].

In addition, many radioprotectors and other pharmaceutical agents are thiol-containing compounds. Therefore, determining thiol status becomes very important in understanding the basic biochemical response of cells when exposed to ROS and toxic substances. Numerous methods, including enzymatic, chromatographic, and flow cytometric approaches, have been described to determine the thiol content in biological samples [7–9]. High-performance liquid chromatography (HPLC) is the most popular method for determining thiol content. Previous methods, however, have some disadvantages, including being time consuming and having complicated derivatizations. Therefore, developing a new method for determining thiols has been the focus for many researchers. Recently, 9-Acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran (ThioGlo™3, $C_{25}H_{15}NO_6$, mol. wt. 425.4) maleimide reagent has been shown to have a very high affinity for thiol groups and, once it is bound to any thiol-containing compound, the reaction product fluoresces (Fig. 1A). Previous studies used ThioGlo™3 to identify thiol compounds in intact cells under fluorescent microscopy [10]. The purpose of this study is to develop a method for determining thiol in biological samples by HPLC, using ThioGlo™3 as a derivatizing agent.

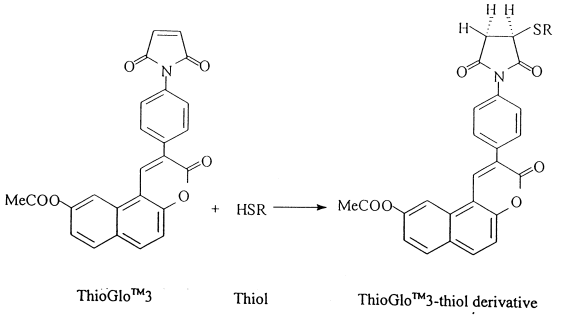

Fig. 1. (A) Reaction of ThioGlo™3 with compounds containing sulfhydryl functional groups. (B) Reaction of NPM with compounds containing sulfhydryl functional groups.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, acetic acid, water, and phosphoric acid (all HPLC grade) were purchased from Fisher (St. Louis, MO). Glutathione, cysteine, homocysteine, glutathione disulfide, glutathione reductase and NADPH were purchased from Sigma (St. Louis, MO). NPM and 2-vinylpyridine were obtained from Aldrich (Milwaukee, WI). ThioGlo™ was obtained from Covalent Associate Inc. (Woburn, MA).

2.2. Animals and cells

Fisher 344 rats (weighing 200–250 g) were anesthetized with metofane and sacrificed; samples of their brains, kidneys, lungs, and livers were obtained. The samples were maintained at −70°C until analyzed by the ThioGlo assay and the NPM method. Chinese hamster ovary (CHO) cells were propagated in Ham's F-12 culture media, supplemented with 10% fetal calf serum (FCS) and maintained at 37°C in 5% CO_2/95% air. Cells were harvested after trypsinization and centrifuged at 1000×g for 5 min.

Thiol levels in CHO cells were determined by both the ThioGlo assay and the NPM method.

2.3. Derivatization of thiols

Tissue samples (liver, lung, kidney and brain) from Fisher 344 rats or Chinese hamster ovary (CHO) cells were homogenized in a serine-borate buffer (SBB: 100 m*M* Tris–HCl, 10 m*M* Borate, 5 m*M* Serine, 1 m*M* Diethylenetriamine penta acetic acid, pH=7.00). Subsequently, 250 μl diluted aliquots of standards or samples were derivatized with 750 μl 0.5 m*M* ThioGlo™ in acetonitrile and 1 m*M* NPM in acetonitrile, respectively. At this point, the pH of the mixture should be 7 or greater in order to facilate the derivatization reaction. After incubation at room temperature for 5 min, the samples are then acidified with 4 μl 2 N HCl to stop the reaction. The final pH of the solution should be around 2.5, which is important for stabilizing the derivatives. The derivatized samples were filtered through a 0.2 μm acrodisc and then injected onto the HPLC system. Tissue or cell protein concentrations were determined by the Bradford method [11].

2.4. Determination of glutathione disulfide (GSSG)

Subsequently, 16 μl of 6.25% 2-vinylpyridine (in absolute ethanol) were added to an 84 μl diluted sample. The mixture was incubated at room temperature for 60 min in order to block the thiol group of the GSH already present. Then, 95 μl of 2 mg/ml NADPH in nanopure water and 5 μl of a 1:50 diluted glutathione reductase were added to reduce GSSG. An aliquot of 100 μl and 150 μl H_2O was immediately derivatized with 750 μl 0.5 m*M* ThioGlo™ and 1 m*M* NPM, respectively as detailed above. GSSG standards were derivatized at the same time.

2.5. HPLC system

The HPLC system (Shimadzu) consisted of a Model LC-10A pump, a Rheodyne injection valve with a 20-μl filling loop and a Model RF 535 spectrofluorophotometer operating at an excitation wavelength of 365 nm and an emission wavelength of 445 nm for ThioGlo derivatives. The HPLC column (Astec, Whippany, NJ) is 100×4.6 mm I.D. and was packed with 3 μm particles of C_{18} packing material. Quantitation of the peaks from the HPLC system was performed with a Chromatopac Model CR 601 integrator (Shimadzu). The mobile phase was 30% water, 70% acetonitrile, with 1 ml/l acetic acid and 1 ml/l phosphoric acid. The ThioGlo™ derivatives were eluted from the column isocratically at a flow-rate of 0.5 ml/min. The excitation and emission wavelengths for the NPM assay were 330 and 375 nm, respectively. The mobile phase was 35% water, 65% acetonitrile, with 1 ml/l acetic acid and 1 ml/l phosphoric acid. The flow-rate was 0.5 ml/min.

2.6. Detection limit

The detection limit of the ThioGlo assay was achieved by preparing diluted solutions of GSH, derivatizing them with a ThioGlo reagent, and then identifying the concentration of GSH that gave the smallest peak ($S/N=3$) of GSH in the chromatogram.

2.7. Precision

Within-run precision was determined by successively injecting the same sample seven times and comparing the peak areas for the glutathione and cysteine derivatives for the seven injections. The between-run precision was obtained by derivatizing the same sample at seven different times, and running the derivatized samples at different times.

2.8. Relative recovery

Relative recovery was determined by measuring GSH in the sample first, followed by spiking the sample by adding 10 μl of a concentrated standard GSH per 100 μl sample, then redetermining GSH concentration in the sample. Relative recovery of GSSG and cysteine was done by spiking the sample with GSSG and cysteine, respectively. The spiking of GSH, GSSG or cysteine was sufficient to double the original concentrations of these thiol compounds.

2.9. Stability

All of the derivatized samples were kept in sample vials and reinjected periodically onto the HPLC system until significant changes in peak areas were noticed.

2.10. Protein assay

The Bradford method was used to determine the protein content of the tissue and cell samples [11]. Concentrated Coomassie Blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water. Then, 2.5 ml of the diluted dye was added to 50 μl of the sample or standard solution. The mixture was incubated at room temperature for 5 min and the absorbance was measured at 595 nm by spectrophotometer. A standard curve, constructed by using bovine serum albumin (BSA), ranged between 0.1 and 1 mg/ml. The homogenized samples were appropriately diluted before protein determination was performed.

3. Results

Fig. 2 shows chromatograms of the blank (A) and standards (B) obtained by the ThioGlo™3 method. ThioGlo™3 derivatives of GSH, Cys, and Hcy were separated retention times of 6.0, 6.7 and 7.8 min, respectively. The detection limits per 20 μl sample were 50 fmol for GSH, 100 fmol for GSSG, and 50 fmol for Cys. Within-run precisions for GSH, GSSG, and Cys were 1.08–2.94%, 0.85–7.29% and 0.58–4.13%, respectively. Between-run precision was 4.31–8.61% for GSH, 3.02–7.64% for GSSG, and 2.07–7.55% for Cys. GSSG and Cys derivatives are stable for 2 weeks, if kept at 4°C. GSH derivatives can be kept for 1 month at 4°C without breakdown.

Calibration curves (Fig. 3) were plotted by using integrated peak areas as *y*-axis and the standard concentration as *x*-axis. GSH and GSSG had linear ranges of 2.5–1250 n*M* with regression constants of 0.9996 and 0.9986, respectively. Cys had a linear range of 5.0–1250 n*M* and a regression constant of 0.9996.

To demonstrate that the ThioGlo method is applicable to biological samples, rat tissues and CHO cells were homogenized in SBB and analyzed by this method. Thiol levels detected are shown in Table 1. Comparisons of values for GSH, GSSG, and Cys in tissues and cells, using the NPM method are also

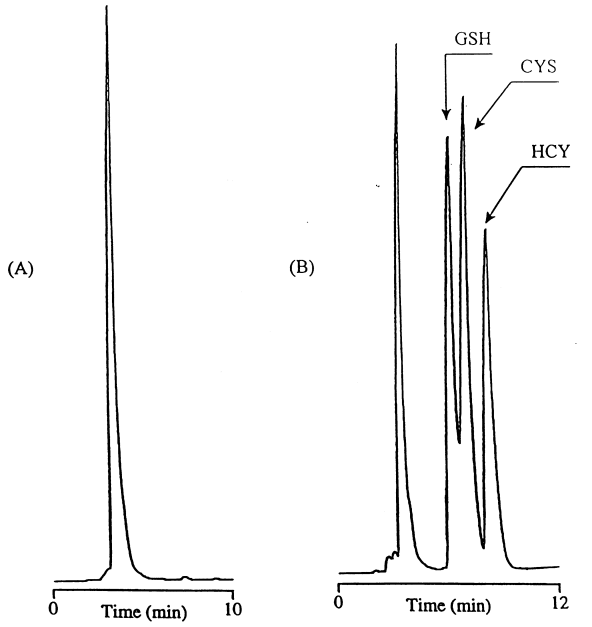

Fig. 2. Chromatogram A is a blank chromatogram prepared by substituting water for a thiol-containing sample. Chromatogram B shows separation of ThioGlo™3 derivatives. GSH, glutathione derivative; CYS, cysteine derivative; HCY, homocysteine derivative.

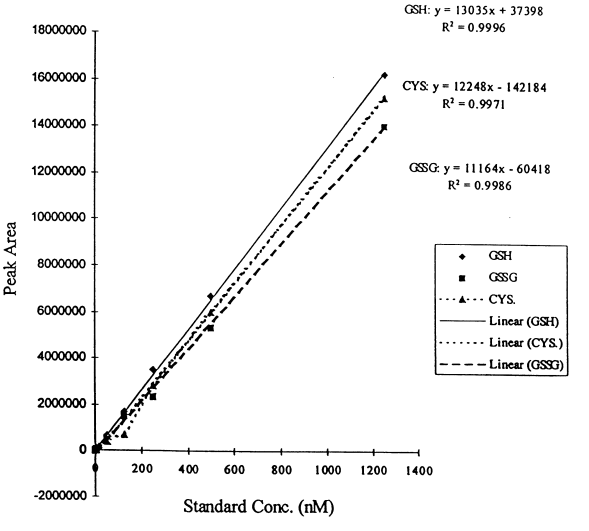

Fig. 3. Calibration curves for GSH, GSSG, and Cys.

Table 1
GSH, GSSG, and Cys measurements using ThioGlo™ assay and NPM assay[a]

	ThioGlo™ assay			NPM assay		
	GSH	GSSG	Cys	GSH	GSSG	Cys
Liver	45.000±4.06	1.230±0.30	ND	40.040±7.73	1.910±0.91	ND
Kidney	0.092±0.035	0.503±0.105	25.93±1.67	0.082±0.041	0.384±0.107	24.47±1.15
Lung	7.770±0.72	3.440±1.54	3.33±0.51	6.320±1.66	2.060±0.51	4.11±1.07
Brain	19.020±0.78	0.880±0.15	ND	18.420±0.94	1.300±0.35	ND
CHO cell	27.410±1.7	0.240±0.12	ND	21.710±0.71	0.290±0.05	ND

[a] All values are mean±SD of nmol/mg protein determined from three separate samples using the two different assays.

included in Table 1. Both methods provided similar results.

4. Discussion

Although there are several methods available for detecting of thiols, such as an enzymatic assay and gas chromatography–mass spectrometry (GC–MS), the most widely-used method is high-performance liquid chromatography (HPLC). HPLC can be coupled with UV, fluorescent, or electrochemical detectors. The HPLC method of Fariss and Reed is one of the most popular HPLC procedures for the quantification of GSH, GSSG, and protein mixed disulfides [12]. Carboxymethylation of the thiol group is initially used to block thiol-disulfide exchange reactions in this method. Free amino groups are then derivatized by fluoro-dinitro benzene to form a chromophoric group. The derivatives are separated on a 3-aminopropylsilane derivatized silica column and detected by UV with nanomole sensitivity. Since any compound having free amino groups is detected, this method is not highly specific to thiol compounds. Sample preparation is also complicated. In addition, thiols lacking free amino groups can not be measured with this approach. HPLC with an electrochemical detector does not require sample derivatization; it is specific and rapid. However, oxygen and temperature can easily interfere with the detector. In addition, sensitivity may decrease as injection times increase. Furthermore, the purity of water affects the baseline [9,13]. Therefore, HPLC with fluorescent detection is preferred by most researchers.

Among methods that have been developed using different fluorescent tags, the method developed by Fashey and Newton is the most popular one [14]. This method uses mono bromobimane (mBBr) as a fluorescent probe and can measure many biologically important thiols. It is both specific and sensitive to thiols, but the sample preparation and derivatization are complex and lengthy. Winters et al. (1995) developed a new HPLC method using *N*-(1-pyrenyl)-maleimide (NPM) as a derivatizing agent [15]. The NPM assay is very specific to thiols (Fig. 1B). Its advantages include increased sensitivity, rapid analysis, and ease of use when compared to the mBBr method. However, hydrolysis peaks present at the beginning and the end of elution [15] may interfere with the separation and quantification of thiols, particularly in samples. Another disadvantage of the NPM method is the impurity of the NMP. As mentioned above, the NPM method was developed in our lab and we have used it for several years but after discovering that each batch provides inconsistent results due to its impurity, we decided to develop another method.

ThioGlo™3 reagent is a new fluorescent probe which has little or no fluorescence before reacting with thiols and a high quantum yield after reaction with thiols [16]. This makes it possible to use ThioGlo™3 as a precolumn derivatizing agent for biological thiols (Fig. 1A). The ThioGlo assay was shown to be reproducible with good within-run and between-run precision. This method offered high sensitivity with a GSH detection limit of 50 fmol, using 0.5 m*M* ThioGlo™3. The GSH detection limit was 1 pmol by the mBBr assay and 50 fmol by the NPM method (NPM concentration is 1.0 m*M*). When 1 m*M* ThioGlo™3 was used, it provided an even lower detection limit.

Another advantage of the ThioGlo assay was that it had less hydrolysis interference. ThioGlo™3 was very resistant to hydrolysis in an aqueous buffer.

Some hydrolysis products formed, but since they were nonfluorescent, there were fewer hydrolysis peaks in the chromatograms.

Table 1 demonstrates that the NPM assay and the ThioGlo method showed very similar results. Both methods were easy to perform. The total elution time was less than 10 min for the ThioGlo method and less than 15 min for the NPM assay although NPM elution time significantly changed from one batch to another. Thus, the ThioGlo method is very rapid and more reliable.

Another biologically important thiol, NAC, was also derivatized by ThioGlo™3 and injected onto an HPLC system. The NAC derivative was eluted at a retention time of 2.5 min. Although NAC was eluted shortly after the breakthrough volume, standards showed good linearity (R^2=0.9902, 0–1250 n*M*). The advantage is that it can be eluted together with GSH, Cys, and Hcy by the same mobile phase. Under the circumstances, where only NAC needs to be determined, modified mobile phase compositions may help improve the separation similar to the that of NPM assay [17].

γ-Glutamyl cysteine and cysteinylglycine were also analyzed by the ThioGlo method. Resolutions between γ-glutamyl cysteine and cysteine, cysteinylglycine and Hcy were not complete (results are not shown here). Further research is required to improve the resolution. However, most tissues and cells had very low concentrations of γ-Glu-Cys and Cys-Gly [18]; therefore, this method is an excellent assay for determining GSH, GSSG, Cys, and Hcy. It is sensitive, specific, rapid and easy to use. If γ-Glu-Cys and Cys-Gly need to be determined, gradient elution and/or a longer column may be used.

The lower cytotoxicity makes ThioGlo™3 particularly suitable for intracellular determination of GSH and other thiols in viable cells. The ThioGlo method gives very promising results on GSH, GSSG, Cys, and Hcy analysis. It may also be applicable to other biological thiols (cysteamine, D-penicillamine, ergothioneine, etc.). Future research should focus on how to improve the current separation and extend its usage to detect more biological thiols.

In summary, a new HPLC method has been developed for determining biologically important thiols by using ThioGlo™3 as a derivatizing agent. The ThioGlo assay provides a sensitive, rapid, and simple method for analysis of GSH, GSSG, Cys, Hcy and NAC. The high sensitivity, low cytotoxicity, and resistance to hydrolysis make ThioGlo™3 a distinguished fluorescent probe. Since thiols play an important role in the biological system and thiol status has been associated with signal transduction, gene expression, and pathogenesis of several diseases, the ThioGlo method provides a powerful tool for studying the role of thiols.

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