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Determination of thiols and disulfides using high-performance liquid chromatography with electrochemical detection

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Abstract

Low-molecular-mass thiols, such as glutathione (GSH), and their associated disulfides are ubiquitous in nature, and based upon the many known functions of these compounds, their identification and accurate measurement is essential. Our objectives were to develop a simple method for the simultaneous measurement of thiols and disulfides in biological samples using HPLC with dual electrochemical detection (HPLC–DED). Particular emphasis was placed on the applicability to a wide variety of important GSH-related thiols and disulfides, including γ-Glu-Cys, Cys-Gly, their disulfides, and the mixed disulfide of glutathione and cysteine (CSSG), validation on different types of biological samples, maintenance of chromatographic resolution and reproducibility with routine and extended use, and enhancement of assay sensitivity. To this end, optimal HPLC conditions including mobile phase, column, and electrode polishing procedures were established and the method was applied to, and validated on a variety of biological samples. This improved methodology should prove to be a useful tool in studies on the metabolism of GSH and other thiols and disulfides and their role in cellular homeostasis and disease processes.

1. Introduction

Thiols and disulfides play a central role in metabolism and cellular homeostasis. The most abundant low-molecular-mass thiol, glutathione (GSH), is an important antioxidant which is found in high concentrations in nearly all living cells. GSH has many essential functional roles including the detoxification of xenobiotics, free radicals and peroxides, maintenance of protein structure and function, regulation of protein and DNA biosynthesis and cell growth, and maintenance of the immune function [1,2]. Decreased GSH levels have been associated with the pathogenesis of a number of specific diseases such as diabetes [3], alcoholic liver disease [4], AIDS [5] and cataracts [6] and more broadly in carcinogenesis and aging [7].

Although other thiols such as cysteine (Cys) and homocysteine (HCys) are found in lower concentrations in most tissues, they are also involved in a variety of important cellular functions, such as protein synthesis, detoxification, and metabolism [8]. Altered levels of Cys have been implicated in a number of pathological conditions, including Alzheimer's and Parkinson's diseases [9], while altered HCys levels are associated with folate and vitamin B_{12} deficien-

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cies [10], homocystinuria [11], and cardiovascular disease [12]. Finally, a number of recent findings have demonstrated the importance of thiol/disulfide ratios in the regulation of enzymatic activity [13] and assessment of oxidative stress [14].

Given the importance of thiols and disulfides, their accurate measurement in blood and tissues is essential. However, few methods are available for their simultaneous analysis. One HPLC method which utilizes S-carboxy methylation of compounds followed by derivatization with Sanger's reagent is limited by a two-step derivatization and is not able to detect thiols that lack amine groups such as ergothioneine [15]. Another method developed for plasma involves derivatization with monobromobimane and HPLC with fluorescence detection [16]. However, this method does not distinguish between thiol and disulfide forms without re-analyzing samples for disulfides after blocking free thiols with N-ethylmaleimide.

HPLC with electrochemical detection represents an important tool for the analysis of redox-reactive compounds such as thiols and disulfides. Initially, Hg pool electrodes were used for the determination of glutathione and other thiols [17]. This method was later simplified and expanded by the development of a dual Au/Hg amalgam thin layer electrode for the simultaneous determination of GSH and Cys, and their respective disulfides [18]. In a previous report, we described our modification of this method and its application and validation in biological samples [19]. In response to the need to analyze a wider variety of thiols and disulfides in biological samples and an increasing sample load, we have expanded this HPLC–DED method to increase sensitivity and selectivity for a wide variety of biologically important thiols and disulfides.

2. Experimental

2.1. Reagents

L-Cystine, L-cysteine, DL-homocystine, DL-homocysteine, reduced glutathione (GSH), glutathione disulfide (GSSG), Cys-Gly, and γ-Glu-Cys were obtained from Sigma (St. Louis, MO, USA). All other chemicals, of high purity or HPLC grade, were obtained from Aldrich (Milwaukee, WI, USA), Mallinckrodt (Chesterfield, MO, USA), or EM Science (Gibbstown, NJ, USA). Ergothioneine was a gift from D.B. Melville and M.C. Brummel (University of Iowa). Deionized water was prepared using a Millipore Milli-Q System (Bedford, MA, USA).

2.2. Chromatography

Chromatography was carried out using a Bioanalytical System PM-48 pump, Rheodyne 7125 injector, dual LC-4B amperometric detector, dual Au/Hg working thin layer electrode, and Ag/AgCl reference electrodes (BAS, West Lafayette, IN, USA). The working electrodes were in the series configuration with the upstream electrode set at a potential of −1.0 V to reduce disulfides to their corresponding thiols and the downstream electrode at +0.15 V, for thiol detection. A Hitachi (Danbury, CT, USA) D-2500 Chromato-Integrator was used for peak integration.

Optimal separation was achieved using a 5-μm Inertsil ODS 2 silica column (250 mm × 4.6 mm I.D.) (GL Sciences, obtained through Alltech, Deerfield, IL, USA) with a mobile phase of 93.25% (v/v) 0.1 *M* monochloroacetic acid (MCA), 5% methanol, 1.75% N,N-dimethylformamide (DMF) and 2.25 m*M* heptanesulfonic acid adjusted to a final pH of 2.8 with NaOH. The mobile phase was continuously sparged with helium to displace oxygen. Samples were run isocratically at a flow-rate of 1.0 ml/min. Overnight and when samples were not being run the flow was continued at a reduced rate of 0.2 ml/min.

Since it has been suggested that metal contaminants can cause interactions with thiols resulting in peak tailing (20), most of the metal components in our system were eliminated. All stainless-steel tubing, the injector loop, column, column frits, and connections were replaced with Peek components (Upchurch, Oak Harbor, WA, USA). While EDTA is effective at chelating

metal ions, it was not routinely used in the mobile phase because it greatly decreased the life span of the working electrode. Finally, in order to prevent the accumulation of contaminants on the column, the mobile phase was not recirculated.

The resultant profiles were quantified with authentic external standards based on peak areas. Stock solutions of standards were prepared as follows: 1.0 m*M* solutions of ergothioneine, cysteine, GSH, homocysteine, γ-Glu-Cys, Cys-Gly, Cys-Gly disulfide, and GSSG in 3 m*M* EDTA; 1.0 m*M* solutions of cystine and homocystine in 10 m*M* NaOH. Stock solutions were stable for one month when stored at 4°C. Working standards were prepared daily from these stock solutions by diluting in HPLC mobile phase which had been adjusted to pH 2. Metaphosphoric acid (MPA) was added to give final acid concentrations similar to those of processed samples.

2.3. Routine maintenance

With time, degradation of the Hg amalgam occurred and was accompanied by a marked loss in sensitivity. This can be exacerbated by many factors such as air bubbles and certain mobile-phase additives such as EDTA. When there is a loss of sensitivity, the electrode must be resurfaced by first dissolving the old mercury amalgam with 6 *M* nitric acid. The gold is then polished to a mirror-like finish using a four-step polishing procedure with the following abrasives and lubricants: (1) Carbimet 600 grit silicon carbide paper wetted with water; (2) 6-μm Metadi II diamond polishing compound on a nylon polishing pad wetted with Metadi Fluid (water-based extender); (3) 1-μm Metadi II diamond polishing compound on a Texmet polishing pad wetted with Metadi Fluid; (4) 0.05-μm aluminum oxide suspension (Gamma Micropolish Alumina 3B) on a Microcloth polishing pad wetted with water. All polishing supplies were obtained from Buehler (Lake Bluff, IL, USA). Between successive polishing steps, the electrode is rinsed and ultrasonicated for 1 min in distilled water. After drying the electrode, an amalgam is prepared by applying a drop of triple distilled mercury (BAS). After approximately 2 min, excess mercury is removed and the amalgam is allowed to equilibrate overnight. If a large decrease in sensitivity occurs repeatedly with a working electrode, the gold contacts are cleaned with sulfuric acid.

2.4. Collection and processing of tissue samples

Male Wistar rats (8 months old) were obtained from Charles River Labs (Kingston, NY, USA). Animals were anesthetized with diethyl ether, and exsanguinated by cardiac puncture. Whole blood was collected into syringes containing 100 μl of 0.05 *M* disodium EDTA and processed by the addition of 4 vol. of ice-cold 5% (w/v) metaphosphoric acid. Liver, lung, kidney, pancreas, stomach, testis, spleen, brain, heart, and colon were immediately removed, rinsed in ice-cold saline solution (0.9%), weighed, and homogenized (10% w/v) in 5% MPA. Homogenates were centrifuged at approximately 14 000 *g* for 2 min and the resulting acid-soluble supernatants were removed and diluted twenty-fold with HPLC mobile phase which had been adjusted to pH 2.00. Blood supernatants were run without further dilution. All samples were analyzed within 2 h of processing.

2.5. Processing of coffee samples

A 5% (w/v) boiling water extract was made of ground Columbian coffee (Wechsler, Moonachie, NJ, USA). The extract was diluted two-fold with 5% MPA, centrifuged at 14 000 *g* for 2 min, and the supernatant analyzed by HPLC.

3. Results

In order to optimize chromatographic resolution, a number of HPLC columns were tested. The best results, based on thiol and disulfide resolution, column life span and the extent of non-specific thiol binding, were obtained with an ultra high-purity 5-μm C_{18} column. While other C_{18} columns provided adequate resolution, ex-

cessive thiol binding to the column matrix often occurred, apparently due to metal ions and sulfate contaminants in the silica. Polymeric based reversed-phase columns were tested (Hamilton PRP-1 and PRP-3) as a means of preventing thiol binding, but resolution was too low to allow for useful separation of thiols and disulfides.

Modifications were also made to the mobile phase to enhance chromatographic resolution. DMF was added to increase peak sharpness and selectively alter retention times of later-eluting analytes such as GSSG. Methanol had a weaker effect on retention time than acetonitrile and therefore was better suited for minor adjustments in chromatographic profiles. A pH of 2.8 was selected to allow for adequate ionization of analytes without reducing column life span.

As a result of these changes in chromatography, twelve biologically important thiols and disulfides were separated within 20 min (Table 1). With the upstream electrode turned on, both thiols and disulfides are detected while only thiols are detected with the upstream electrode turned off (Fig. 1). Due to the high peak resolution, complete integration of most closely eluting analytes was possible. While er-

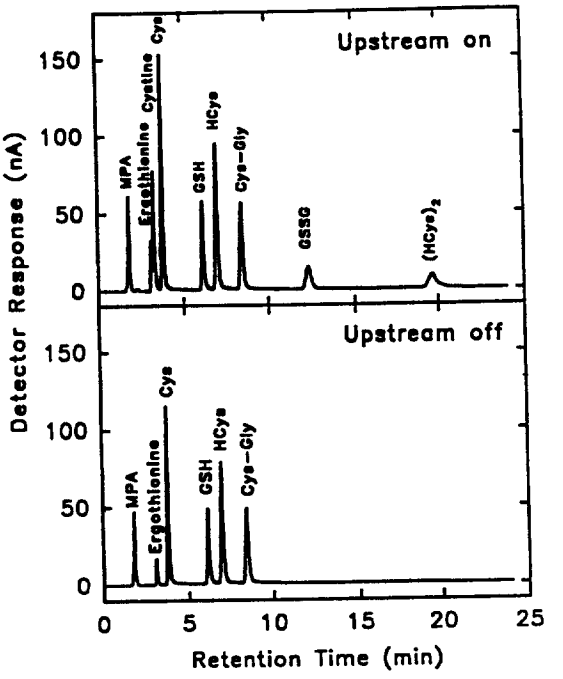

Fig. 1. HPLC–DED profiles of a standard thiol and disulfide mixture. Standards containing 25 μM of each thiol and disulfide were prepared and analyzed as described in the text. Sample volumes were 50 μl. Peaks were labeled as follows: MPA (metaphosphoric acid), and $(HCys)_2$ (homocystine). The lower panel is a chromatographic profile of the standard with the upstream electrode not activated.

Table 1
Retention times and assay sensitivity of thiols and disulfides measured by HPLC–DED

Thiol/disulfide	Retention time (min)	Relative retention time (min)	Minimum detectible quantity (pmol)	Standard curve slope (nC/nmol)
Ergothioneine	3.06	0.53	0.50	96.8
Cystine	3.17	0.55	0.10	232
Cysteine	3.72	0.64	0.02	432
CSSG	4.73	0.82	ND[a]	ND[a]
GSH	5.80	1.00	0.40	384
Homocysteine	6.63	1.14	0.40	848
γ-Glu-Cys	6.82	1.172	0.20	640
Cys-Gly	7.91	1.36	0.20	688
γ-Glu-Cys disulfide	10.44	1.80	ND[a]	ND[a]
GSSG	12.06	2.08	0.50	197
Cys-Gly disulfide	18.28	3.15	0.50	612
Homocystine	18.43	3.18	1.00	700

[a] Not determined.

gothioneine (3.06 min) was not completely resolved from cystine (3.17 min), it can be easily differentiated by turning the upstream electrode off, since cystine is not detected under this condition. However, this is generally not necessary since ergothioneine is found in very low concentrations in most tissues. Cys-Gly disulfide (18.28 min) and homocystine (18.43 min) were also not completely resolved, but this can be rectified, if required, by decreasing the concentration of organic modifiers in the mobile phase. Although not included in the standard mixture, the mixed disulfide of GSH and Cys (CSSG) (4.73 min) and Glu-Cys disulfide (10.44 min) were completely resolved from other components. Finally, slight variations in retention times did occur, particularly with late-eluting analytes, depending upon the age of the column.

Because many thiols and disulfides are found in very low concentrations in tissues and body fluids, emphasis was placed on increasing sensitivity of the assay. In addition to the new column and mobile phase, changes in the electrode polishing procedure also produced a significant increase in sensitivity and decrease in background noise. These changes also decreased the overall time required to refinish an electrode from about 1/2 h to 10 min.

The high sensitivity of this assay is exemplified by the low minimum detectable quantity (MDQ) values obtained for each analyte, as determined at a signal-to-noise ratio of 2:1 (Table 1). With a 50-μl injection loop, values ranged from 0.02 pmol for Cys to 1.0 pmol for homocystine. The response of the detector for each analyte is also presented in Table 1 as the slope of the standard curve. All standard curves were constructed over a range of concentrations from 6.25 μM to 100 μM with a mean coefficient of determination (r^2) value of 0.98.

Sample pH is also an important factor in optimizing chromatography, based on changes in the net charge of the analytes. In the range of pH 2.5–3.4, the retention time of GSH decreased from 5.80 min to 5.57 min and two ionic forms of GSH were evident at pH 2.8 (Fig. 2). Thus, it was important to adjust sample pH <2.5 to ensure uniformity of retention times. In most

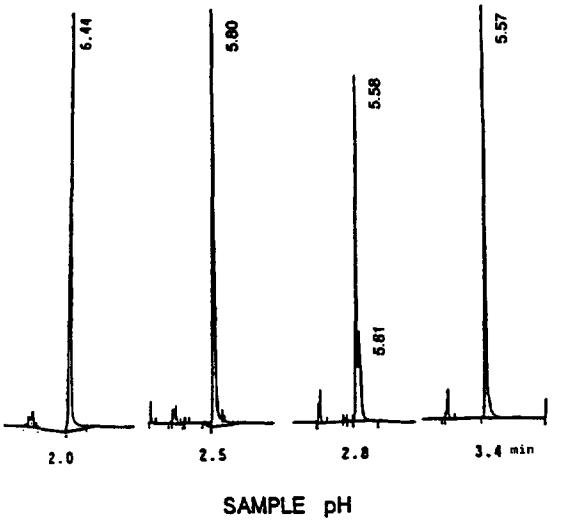

Fig. 2. Effect of sample pH on GSH retention time. A GSH standard was prepared in mobile phase which was adjusted to pH 2.00. pH was adjusted with NaOH. HPLC conditions were as described in the text.

cases, adjustment of biological samples is unnecessary since they are processed by acid extraction. Mobile phase pH was not adjusted to pH 2.5 because of the instability of the column matrix at low pH.

In order to examine the usefulness of this modified method, we have applied it to a number of biological samples. Representative chromatographic profiles for acid-soluble extracts of rat kidney, liver, heart, lung, pancreas, spleen, testis, stomach, and brain are shown in Fig. 3. Table 2 shows the mean values of glutathione and cysteine, and their respective disulfides for all rat tissues analyzed. GSH was the most abundant analyte found in all tissues accounting for 60–90% of total thiols and disulfides. A 3.5-fold range of total GSH levels was observed ranging from 1.86 μmol/g tissue in brain to 6.6 μmol/g tissue in liver. In those tissues containing GSSG, the levels are low, representing at most 3.4% of total glutathione in liver. The GSH-related dipeptide Cys-Gly was detected only in pancreas and kidney, and levels were low, representing only about 0.3% of total thiols and disulfides. CSSG was detected in pancreas, spleen, kidney, and liver. All samples were analyzed immediately after processing as thiol oxidation occurs

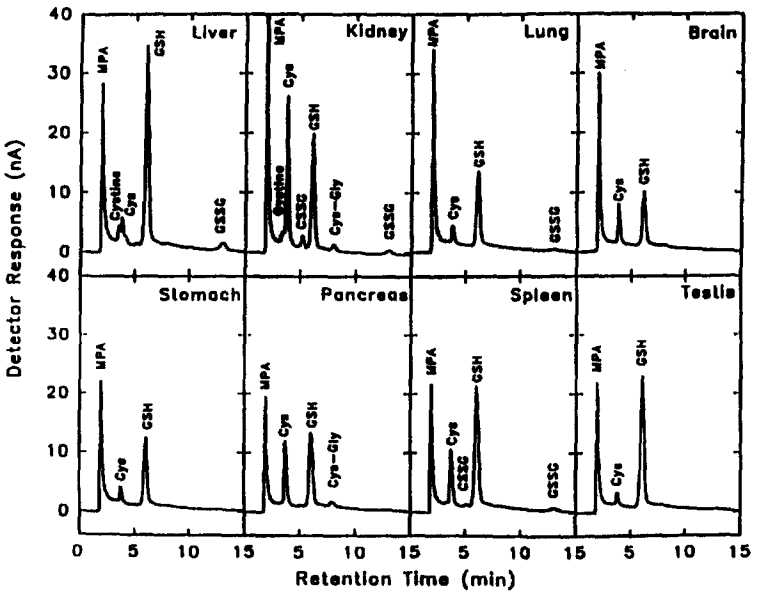

Fig. 3. Chromatographic profiles of acid-soluble biological extracts. Tissues obtained from male Wistar rats were processed and analyzed as described in the text. Acid-extracted samples were diluted twenty-fold with mobile phase adjusted to pH 2.00 and injection volumes were 50 μl.

with storage of acid extracts at −20°C in certain tissues. Recovery experiments were conducted on different tissues for GSH and other metabolites. Complete recovery was obtained in all cases.

To exemplify the utility of this method, thiol/disulfide profiles were examined in a wide variety of samples including foods, beverages, plants, and medicinal preparations. For example, a representative thiol/disulfide profile of coffee ex-

Table 2
Thiols and disulfides in different tissues of the rat

Tissue	Concentration[a] (μmol/g tissue or ml blood)						
	Cystine	Cys	Cys + Cystine (μeq. Cys/g or ml)	Cys-Gly	GSH	GSSG	GSH + GSSG (μeq. GSH/g or ml)
Blood	0.115	0.064	0.254	–	0.834	0.022	0.878
Brain	–	0.179	0.179	–	1.86	–	1.86
Colon	–	0.031	0.031	–	1.19	0.033	1.26
Heart	0.012	0.058	0.082	–	1.62	0.040	1.70
Kidney	0.160	1.603	1.923	0.159	2.59	0.076	2.79
Liver	0.230	0.112	0.572	–	6.38	0.114	6.60
Lung	–	0.158	0.158	–	1.88	0.025	1.93
Pancreas	0.122	0.372	0.616	0.070	1.65	0.013	1.67
Spleen	–	0.460	0.460	–	4.26	0.063	4.38
Stomach	–	0.127	0.127	–	1.98	–	1.98
Testis	–	0.089	0.089	–	4.69	–	4.69

[a] Values are means of 3–5 rats.

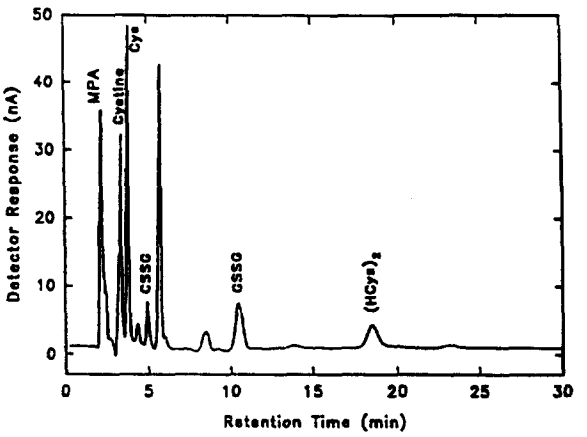

Fig. 4. Chromatographic profile of coffee extract. Aqueous extract of Columbian coffee 5% (w/v) was diluted with MPA and analyzed as described in the text.

tract, made in the same proportion as typical brewed coffee, is shown in Fig. 4. Analytes were identified by direct comparison with authentic standards, and by spiking samples with standards. Cysteine appears to be the most abundant analyte in coffee, with a total Cys of 17.6 μM/ml extract ($n = 4$). Glutathione is found mostly in the oxidized form at 4.12 μM/ml. A number of major peaks including those eluting at 5.54 and 8.53 min are as yet unidentified, but appear to be disulfides based upon their disappearance when analyzed with the upstream electrode turned off.

4. Discussion

Our HPLC–DED method for the determination of thiols and disulfides has a number of advantages including minimal required sample preparation, and wide applicability in biological samples [19]. This method has now been expanded and modified in order to prevent degeneration of chromatographic resolution and nonspecific binding of thiols to the column, both of which occur with extended use. In addition, changes were made to increase sensitivity and validate the method for a number of additional GSH-related thiols and disulfides that were previously not studied. These compounds include γ-Glu-Cys, Cys-Gly and their respective disulfides and CSSG, all important metabolites of GSH.

Previously, we reported that HPLC–DED analysis resulted in comparable values for glutathione as obtained by other methods [19,21]. Likewise, for the rat tissues examined in this study, similar values were obtained by HPLC–DED for total GSH (GSH + GSSG) when compared to the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)/GSSG reductase recycling method [22] ($r = 0.996$), with an average ratio of HPLC–DED/DTNB value of 1.02.

In addition to analytical methods, sample processing procedures also represent a critical factor in the analysis of thiols and disulfides due to the oxidation of thiols or the enzymatic reduction of certain disulfides [21,23]. To prevent a possible loss of analytes during processing, all procedures were preformed as quickly as possible and at 0–4°C. In addition, all tissues were homogenized directly into acid to stabilize thiols and disulfides. Using these sample processing procedures, complete recovery of glutathione which was added in known amounts to actual samples was obtained for blood and other tissues [19,21,24].

The resultant HPLC–DED profiles obtained from various rat tissue extracts, as well as other types of samples, demonstrate the applicability of this highly sensitive method for the measurement of thiols and disulfides in biological samples. The mean values found in our studies are very similar to those reported in the literature. For example, both the glutathione and cysteine contents observed for blood, colon, lung, liver, kidney, heart, and stomach were in close agreement with those reported previously by Potter and Tran in the rat [25]: for GSH, $r = 0.99$, and for Cys, $r = 0.97$.

As demonstrated with coffee, this method can also be used to obtain a complete profile of thiols and disulfides in a wide variety of samples. This important new application can be used in conjunction with other methods of analysis such as mass spectroscopy to identify unknown compounds. Considering the growing importance of glutathione and other related compounds in areas such as cancer chemopreventive agents, diabetes, cardiovascular disease, and AIDS, this

unique analytical method should be of great use in the determination of thiol/disulfide status, and the identification of new thiol/disulfide compounds.

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