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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF GLUTATHIONE AND ITS THIOL AND DISULFIDE DEGRADATION PRODUCTS

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method for quantitation of picomole levels of glutathione, glutathione disulfide, cysteine, cystine, cysteinylglycine, cysteinylglycine disulfide and cysteine glutathione-mixed disulfide in biological samples is described. The compounds were separated isocratically on a reversed-phase column by ion-pair chromatography. The mobile phase consisted of an aqueous buffer containing 0.1 *M* monochloroacetic acid and 3.3 m*M* 1-heptanesulfonic acid (pH 2.60)—methanol—N,N-dimethylformamide (96.5:3.0:0 5). After chromatographic separation, the disulfides were reduced by a potential (—1.0 V) from a battery, with subsequent detection of all thiols by electrochemical oxidation (+0.15 V) with a dual gold—mercury electrode. Thiol and disulfide concentrations were determined in tissue extracts (liver and kidney) and fluids (bile and plasma) from control rats and rats treated with acivicin, an inhibitor of γ-glutamyltranspeptidase. A marked increase in biliary glutathione concentration was observed in treated animals with a corresponding decrease in cysteine and cysteinylglycine concentrations. The results demonstrate that this method is useful for measuring glutathione and its degradation products in tissues and fluids.

INTRODUCTION

There are several high-performance liquid chromatographic (HPLC) methods presently available for the quantitation of thiols and disulfides based on a variety of detection and separation techniques. These methods include: UV detection with pre-column derivatization and specially prepared analytical columns [1], recycling post-column reaction on an anion-exchange column [2], fluorometric detection of monobromobimane derivatives separated on a

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reversed-phase column [3], separation on a reversed-phase column with detection by post-column reaction with Ellman's reagent [4] and HPLC on cation-exchange [5] or reversed-phase columns [6—12] with electrochemical detection (ED).

Liquid chromatography (LC) with ED, utilizing a dual gold—mercury electrode, is both extremely sensitive and specific for thiol and disulfide determination [5, 8, 12]. Previous methods determined cysteine (Cys), glutathione (GSH) and homocysteine (Hcy) and their disulfides [8, 10]. However, these methods fail to eliminate interference by co-eluting compounds such as the cysteine glutathione-mixed disulfide (CYSSG) and γ-glutamylcysteine (γ-Glu-Cys) without additional preparation or analyses [8, 10].

In this paper, we describe alternative equipment for performing reductions at the gold—mercury electrode that eliminates some of the expense involved for this analysis when performed as previously reported [8—11]. We also developed chromatographic conditions that allow GSH and its degradation products to be quantitated in one assay without co-eluting compounds. The versatility of our method was demonstrated by quantifying thiols and disulfides in liver, kidney, plasma and bile. Finally, we demonstrate the applicability of this method to the study of GSH metabolism. Acivicin is a potent inhibitor of γ-glutamyltranspeptidase (γ-GT) which is responsible for the hydrolysis of GSH to cysteinylglycine (Cys-Gly) [13]. Bile samples from control and acivicin-treated rats were analyzed for thiol and disulfide concentrations.

EXPERIMENTAL

Chemicals

Cystine, cysteine, homocysteine and reduced and oxidized glutathione (GSSG) were obtained from Sigma (St. Louis, MO, U.S.A.). Cysteinylglycine disulfide was purchased from Vega Biochemicals (Tucson, AZ, U.S.A.). Cysteine glutathione-mixed disulfide was a gift from Dr. D.J. Reed (Oregon State University, Corvallis, OR, U.S.A.) and γ-glutamylcysteine disulfide was a gift from Dr. G.A. Hazelton (University of Louisville Medical Center, Louisville, KY, U.S.A.). Acivicin was a gift from Upjohn (Kalamazoo, MI, U.S.A.).

Chemicals for the mobile phase were obtained from the following sources: 1-heptanesulfonic acid (HSA) and monochloroacetic acid (MCA) were from Kodak (Rochester, NY, U.S.A.); ultrapure N,N-dimethylformamide (DMF) and ultrapure perchloric acid (70%) from Alfa Products (Danvers, MA, U.S.A.), HPLC-grade methanol from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other reagent-grade chemicals were obtained from Fisher Scientific. The chemicals from these sources were found to have the fewest interfering impurities.

All standards were prepared daily from stock solutions stored in 0.25 *M* perchloric acid at −80°C. γ-Glu-Cys disulfide and Cys-Gly disulfide were reduced according to the method of Butler et al. [14]. Briefly, 1.0 ml of disulfide (10 μmol/ml) and 1.0 ml of dithiothreitol (50 μmol/ml), both dissolved in 50 m*M* Tris—HCl (pH 8.50), were pipetted into a 10-ml volumetric flask. A gentle stream of nitrogen gas was mixed with the solution for 60 min.

The pH was then adjusted to 2.0 with 6 *M* hydrochloric acid and the volume adjusted to 10 ml. The final concentration of reduced thiol was 2 μmol/ml and was stored at −80°C. Prior to use, 1.0 ml of stock solution was extracted twice with 5 ml of ethyl acetate to remove the dithiothreitol. Samples were centrifuged between extractions. Thiols remained reduced at room temperature for approximately 12 h.

HPLC apparatus and chromatography

HPLC was performed using a Waters Model 6000A pump and a WISP 710B injector (Waters Assoc., Milford, MA, U.S.A.). The separation was carried out with an Alltech C_{18}, 5-μm Adsorbosphere HS stainless-steel column (250 mm × 3.9 mm) with a thermoregulated water jacket (Alltech Assoc., Deerfield, IL, U.S.A.) which was maintained at 35°C. The analytical column was protected by a guard column (50 mm × 4.6 mm) obtained from Alltech Assoc. The guard column was packed with Pellicular C_{18} bonded silica (40 μm; Alltech Assoc.). Compounds were eluted at a flow-rate of 1.0 ml/min. Quantitation was based on integrated peak areas. Integration was performed by an IBM 9000 computer with the Chromatography Applications Program (version 1.3; IBM Instruments, Danbury, CT, U.S.A.). Response factors for each compound were computed daily from standard curves.

MCA buffer consisted of 0.1 *M* MCA and 3.30 m*M* HSA adjusted to pH 2.60 with a freshly made concentrated sodium hydroxide solution (sodium hydroxide solutions developed electrochemically active species upon storage). The mobile phase was MCA buffer—methanol—DMF (96.5:3.0:0.5). Buffers were prepared with deionized water obtained from a Milli-Q System (Millipore, Bedford, MA, U.S.A.) and filtered through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to use. Stainless-steel tubing was used between the mobile phase reservoir and pump to prevent oxygen from entering the mobile phase because oxygen interferes with the reductive mode. The mobile phase was purged of oxygen by refluxing at 50–55°C and sparging continuously with helium. A water-cooled condenser, with a bubble trap at its exit, was attached to the mobile phase reservoir. This prevented the mobile phase composition from changing and oxygen from entering the system.

Electrochemical detector

The detector cell (Fig. 1) consisted of a dual gold—gold electrode bottom, two TG-5M gaskets and a stainless-steel auxillary top (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The inlet tubing (0.16 mm O.D., 0.018 mm I.D. stainless steel) was obtained from Supelco (Bellefonte, PA, U.S.A.) and was attached to the low dead-volume inlet on the steel auxillary top with a Kel-F fingertight fitting (Chrom Tech, Apple Valley, MN, U.S.A.). The positioning of the inlet tubing was critical. The steel tubing must not be in contact with the steel-auxillary top, and yet be close enough to minimize the dead volume. Positioning of the tubing was checked with an ohm meter. There should be 1 MΩ or greater resistance between the tubing and the steel auxiliary electrode top. Typically, the inlet tubing was inserted into the Kel-F fitting until 1 mm or less space remained before it reached the bottom of the Kel-F fitting (Fig. 1B). The reference electrode and reference cell container (Bio-

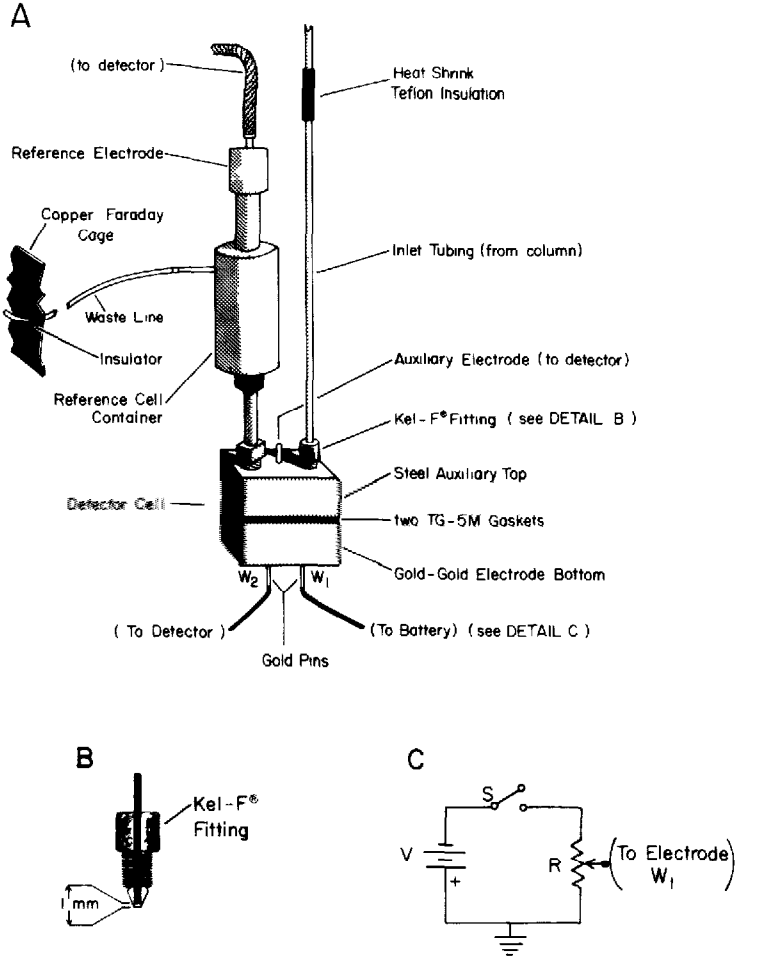

Fig. 1. (A) Diagram of the electrochemical detector cell. (B) Detail showing placement of inlet tube in the Kel-F inlet fitting. (C) Voltage divider circuit used to supply a reducing potential (—1 V) at W_1; R = a wire-wound potentiometer; S = an off—on switch; V = a 6-V battery with the positive pole at ground (see Experimental section).

analytical Systems) were positioned opposite the downstream electrode, W_2 (Fig. 1). Mercury amalgam was prepared according to previous reports [7]. It was important to have all air removed from the detector cell and reference container prior to use. The detector cell and reference electrode were surrounded by a well grounded, copper Faraday cage in order to reduce baseline noise. All lines passing through the Faraday cage were electrically insulated. This included the steel inlet tubing, the plastic waste line and the four electrical leads.

A battery supplied the current to reduce disulfides at the upstream electrode, W_1. The voltage at W_1, with respect to ground, was adjusted to —1.0 V with a simple voltage divider circuit (Fig. 1C). The circuit consisted of a 6-V lantern battery (Eveready, 510S), an off—on switch and a wire-wound potentiometer (Mallory, VW 15K). The electrochemical detection system

consisted of an EC/230 amperometric LC detector (IBM Instruments, Wallingford, CT, U.S.A.) which was used at the downstream (W_2) electrode to measure the oxidation of all thiols. The downstream electrode was set at +0.15 V. A Bioanalytical Systems LC-4 amperometric detector was also found to produce similar results. IBM electrical cables from the detector were adapted for use with the Bioanalytical Systems detector cell by replacing the connectors at the cell end. All connections in the circuit were soldered. The positive pole of the battery was set at ground. The Faraday cage was used as a common ground for the voltage divider and the IBM detector. The battery was typically used for nine weeks before replacement.

Specific application to animal treatment

Four-week-old, male, Sprague—Dawley rats (Sasco, Omaha, NE, U.S.A.) were anesthetized with sodium pentobarbital [50 mg/kg intraperitoneally (i.p.)] and the bile duct cannulated with polyethylene tubing (PE-10). The body temperature of the animals was maintained at 37°C with a heating lamp controlled by a thermister, rectal probe. Animals were injected with acivicin [100 μmol/kg, 5 ml/kg intravenously (i.v.)] dissolved in saline. Control rats received saline. For the measurement of biliary thiol and disulfide excretion, bile was collected in 30-min periods for 2 h into 1.0 ml of 0.25 *M* perchloric acid to prevent oxidation of the thiols [1, 5, 15, 16] and centrifuged prior to storage.

After the bile samples were collected, blood was taken from the vena cava into heparinized containers and immediately centrifuged. Plasma samples were then diluted 1:1 with 0.25 *M* perchloric acid. Liver and kidney samples were removed and homogenized in nine volumes of ice-cold 0.25 *M* perchloric acid and centrifuged. All samples were stored at —80°C.

Spectrophotometric method

Total GSH was determined enzymatically in 49-day-old rats according to the method of Tietze [17]. Samples were prepared as previously described.

RESULTS AND DISCUSSION

Previous studies have demonstrated that LC—ED is an extremely sensitive method for the determination of thiols and disulfides [5—12]. Several investigators have reported that CYSSG may co-elute with GSH [9, 10]. To solve this problem, investigators have injected samples with the upstream electrode turned off which results in the detection of only reduced thiols. The sample would then be reacted with N-ethylmaleimide and injected on the system again with both electrodes switched on. N-Ethylmaleimide reacts with thiols to form conjugates which are not detected by the electrochemical detector. Thus, only disulfides are then detected [10]. These procedures require additional sample preparation, and they do not eliminate the possibility of other co-eluting oxidized or reduced compounds. We found that γ-Glu-Cys has a similar retention time to GSH using the reported chromatographic conditions [8] as well as after altering the composition of the mobile phase. Thus, the reaction with N-ethylmaleimide would not eliminate the interference due to the γ-Glu-Cys.

Of several columns tried, the Alltech Adsorbosphere HS column provided the best resolution. Chromatography of thiols and disulfides on this column exhibited behavior different from a Biophase column [8] with regard to mobile phase components. Capacity factors (k') for the thiols and disulfides were measured at different mobile phase compositions thereby allowing the selection of an optimal mobile phase. Increasing MCA concentration decreased the elution time of some of the longer retained compounds; 0.1 *M* MCA was chosen owing to the slight decrease in resolution between Cys and cystine when MCA concentration was increased. Reduction in capacity factors for later-eluting disulfides was also observed with increasing DMF concentrations. DMF was used in our system to selectively accelerate elution of weakly basic compounds which reduces tailing in reversed-phase chromatography [18].

Changes in retention times were observed when the system was operated overnight with the automatic injector. At night, the temperature would drop, and a significant increase was observed in retention times. For this reason, a column jacket was installed to maintain optimum temperature. As the tem-

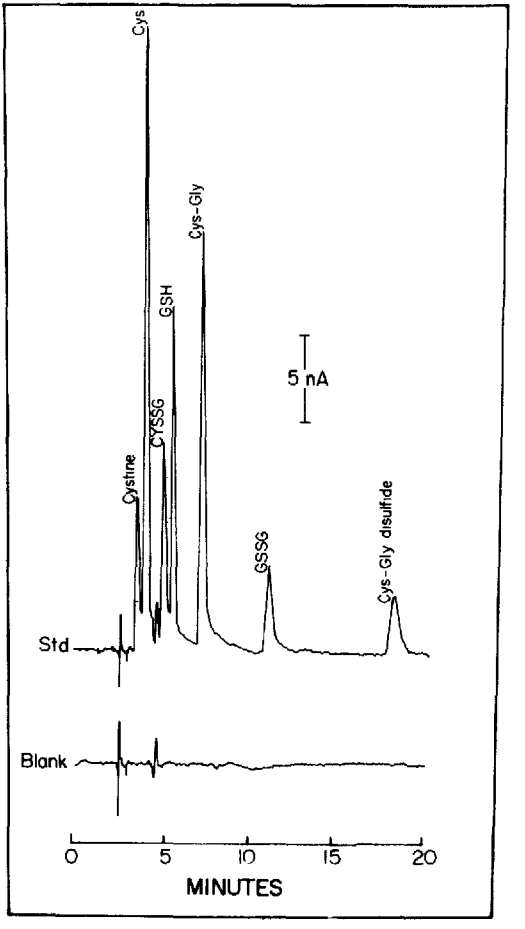

Fig. 2. Representative chromatogram of a standard mixture (250 pmol of each thiol and 400 pmol of each disulfide, total injection 5 μl). Mobile phase: 3.3 m*M* HSA (pH 2.6)—methanol—DMF (96.5:3.0:0.5).

perature increased, a decrease was observed in the k' values. This decreased resolution between Cys and CYSSG and GSSG and Cys-Gly. The effect of pH on k' was very similar to the effects of temperature; pH also had the greatest effect on the disulfides. A pH of 2.6 was selected because it provided optimal separation of CYSSG from both Cys and GSH.

The ion-pairing agent, HSA, was the final variable manipulated in our system. Among the mobile phase components, HSA had the greatest effect on selectivity and thus was used to maximize resolution between the compounds. Disulfides were affected to a greater extent than the thiols. A concentration of HSA of 3.30 m*M* separated the seven compounds of interest without interference from less commonly found compounds such as Hcy and γ-Glu-Cys. Complete resolution of ten thiols and disulfides including Hcy, γ-Glu-Cys and γ-Glu-Cys disulfide, was accomplished by increasing the ion-pairing agent concentration to 8—10 m*M*. However, this mobile phase was not selected because the retention of Cys-Gly disulfide was increased significantly (> 45 min). It may be possible to develop a gradient system that could reduce elution times of the disulfides and resolve all ten compounds in a reasonable time. This could be accomplished by increasing the pH and the organic concentration in a gradient.

Our main interest was to develop a chromatographic system to resolve the compounds formed when GSH is degraded. A typical chromatogram for the mixture of cystine, Cys, CYSSG, GSH, Cys-Gly, GSSG and Cys-Gly disulfide is shown in Fig. 2. The run time was only 20 min. The mobile phase selected gave adequate resolution between GSH and CYSSG and it also produced adequate separation between GSH and other thiols such as Hcy and γ-Glu-Cys. The retention time for both Hcy and γ-Glu-Cys was 5.86 min under these chromatographic conditions (data not shown).

A variety of deproteinizing agents including perchloric acid, metaphosphoric acid, trichloroacetic acid, sulfosalicylic acid and methanol were tested for interfering compounds. Perchloric acid produced the cleanest baseline and a small peak at 4.8 min which did not co-elute with any of the seven compounds of interest (Fig. 2). Little or no oxidation of thiols was observed when samples were prepared in 0.25 *M* perchloric acid and stored at −80°C for seven to ten days (data not shown).

TABLE I

ABSOLUTE RECOVERY OF GSH AND RELATED THIOLS AND DISULFIDES FROM LIVER

Each value represents the mean ± S.E. (n = 4).

Compound	Recovery (%)
GSH	99 ± 5
GSSG	106 ± 1
Cys	103 ± 0.4
Cysteine	99 ± 3
Cys-Gly	96 ± 5
Cys-Gly disulfide	86 ± 2
CYSSG	94 ± 2

TABLE II

REPRODUCIBILITY OF HPLC RETENTION TIMES AND DETECTOR RESPONSES

Capacity factor, $k' = (t_R - t_0)/t_0$ where t_R = retention time of compound and t_0 = retention time for unretained molecules.

Compound	t_R (min)	k'	C V. (n = 5) (%)	C.V. of peak area (n = 5) (%)	
				Standard	Bile sample
Cystine	3.59	0.44	0.3	1.2	N.D.*
Cys	3.87	0.55	0.1	7.1	5.6
CYSSG	5.11	1.04	0.2	4.3	5.2
GSH	5.46	1.18	0 2	6.4	4.8
Cys-Gly	7.33	1.93	0.2	1.3	1.2
GSSG	11.17	3.47	0.1	3.8	4.8
Cys-Gly disulfide	18.37	6.35	0.2	6.1	6.1

*N.D. = undetectable level.

The recoveries of GSH and its related thiol and disulfide degradation products are shown in Table I. GSH was added to liver homogenates to increase its concentration by 250 μM, and the other thiols and disulfides were added to increase their concentration by 125 μM. The recoveries for the externally added compounds were 86—106% (Table I).

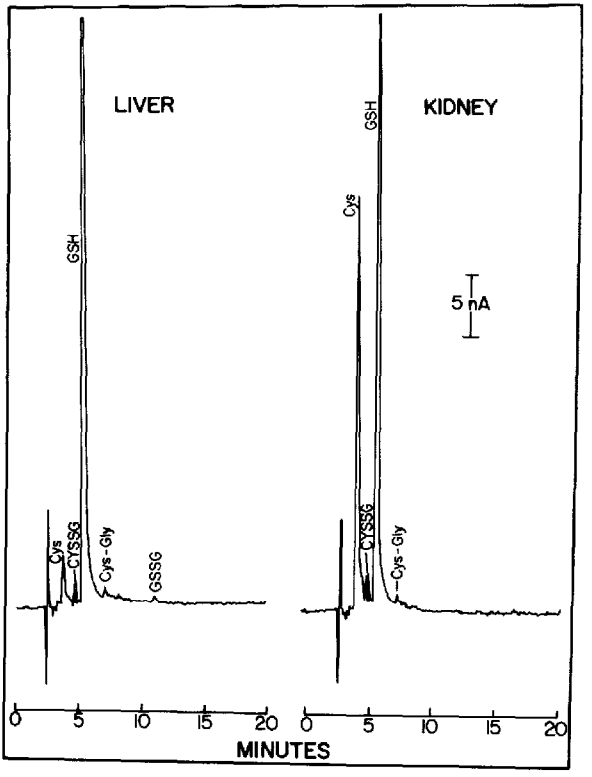

Fig. 3. Typical chromatograms of thiols and disulfides in liver and kidney. Supernatants from tissues were further diluted 1:10 with 0.25 M perchloric acid prior to injection (10 μl).

Retention times and peak areas were extremely reproducible as shown in Table II. The coefficient of variation (C.V.) of peak areas for each compound was less than 7% whether they were analyzed in standards or bile samples (Table II). Hcy and γ-Glu-Cys had a k' of 1.34 and co-eluted; however, both compounds were resolved from GSH. Detector response for all thiols and disulfides was linear over a range of 7.8 to 1000 pmol with the coefficients of correlation being > 0.996. The limits of detection for thiols and disulfides were approximately 3.5 and 6.0 pmol per 10 μl injection, respectively. External standards were injected at the beginning and end of an 8—10 h time period to ensure stable detector responses. The gold—mercury electrode provided consistent responses over 200 injections before resurfacing of the electrode was necessary.

Liver, kidney, plasma and bile samples were analyzed for thiols and disulfides. Representative chromatograms of these biological tissues are shown in Fig. 3 and biological fluids in Fig. 4. There were no peaks present in these chromatograms that could not be accounted for by the standards or perchloric acid. Thiol and disulfide concentrations in various tissues and fluids from 28-day-old rats are presented in Table III. Hepatic GSH and Cys concentrations determined by this method are in agreement with previously published values [19, 20].

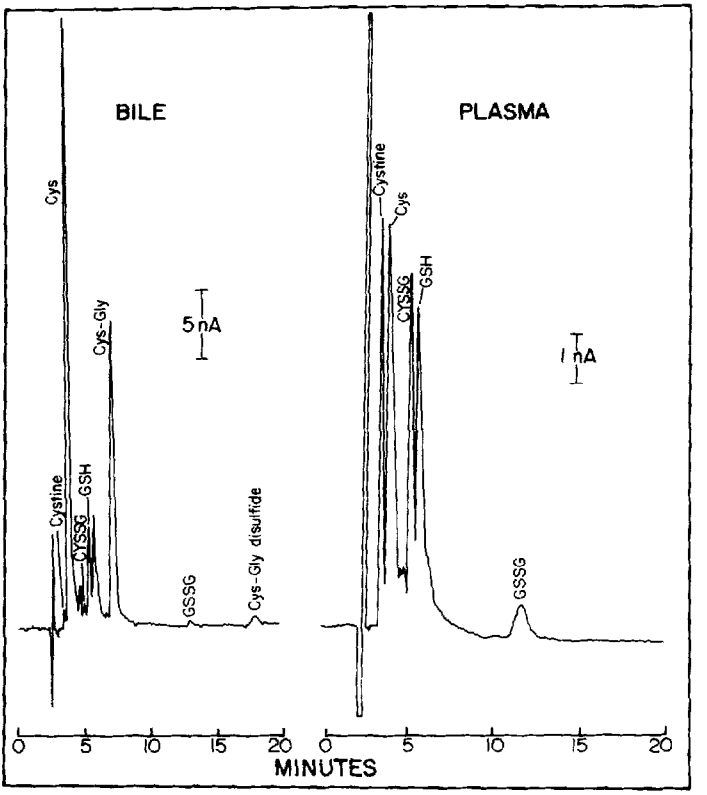

Fig. 4. Typical chromatograms of thiols and disulfides in bile and plasma. Plasma samples were diluted 1:1 with 0.25 *M* perchloric acid and supernatants from bile samples were diluted 1:6 prior to injection (10 μl).

TABLE III

DETERMINATION OF THIOLS AND DISULFIDES IN VARIOUS TISSUES AND FLUIDS

Each value represents the mean ± S.E. (n = 6); N.D. = undetectable level.

Compound	Concentration			
	Liver (nmol/g)	Kidney (nmol/g)	Bile (nmol/kg·min)	Serum (nmol/ml)
GSH	7239 ± 474	1049 ± 940	11.60 ± 4.00	11.9 ± 1.9
GSSG	195 ± 54	62.2 ± 8.7	0.489 ± 0.22	1.80 ± 0.4
Cys	164 ± 24	239 ± 19	26.4 ± 4.4	10.7 ± 1.2
Cysteine	51.9 ± 17	16.4 ± 2.9	1.44 ± 0.49	37.0 ± 2.4
Cys-Gly	29.6 ± 10	19.0 ± 3.7	19.6 ± 2.6	N.D.
Cys-Gly disulfide	N.D.	N.D.	5.73 ± 0.95	N.D.
CYSSG	80.6 ± 32	5.28 ± 9.1	0.444 ± 0.15	7.47 ± 1.1

TABLE IV

COMPARISON OF TISSUE AND FLUID GSH LEVELS DETERMINED BY THE HPLC AND ENZYMATIC METHODS

Each value represents the mean ± S.E. (n = 3)

Sample	GSH concentration	
	HPLC	Enzymatic method
Liver (nmol/g)	6485 ± 364	5718 ± 284
Kidney (nmol/g)	1766 ± 202	1647 ± 39.5
Bile (nmol/kg·min)	130.0 ± 0.050	123.9 ± 3.45
Serum (nmol/ml)	10.29 ± 0.010	10.74 ± 0.090

Tissue samples from 49-day-old rats were prepared as previously described and analyzed for GSH by HPLC and enzymatic [17] methods (Table IV). No significant differences were observed between methods for any of the tissues or fluids.

γ-GT is the enzyme responsible for the initial hydrolysis of GSH to Cys-Gly [13]. This enzyme is located on the renal brush border and the hepatic biliary tree [19, 20]. Animals were treated with acivicin, a known inhibitor of GSH hydrolysis, to demonstrate the usefulness of this method for the study of GSH metabolism. Preliminary data from our laboratory suggest that acivicin lowers the concentration of the degradation products of GSH in bile in four-week-old rats. Fig. 5 shows typical chromatograms of bile samples taken from control and acivicin-treated animals. Cys and Cys-Gly concentrations were markedly lowered in the bile of acivicin-treated rats, whereas the GSH concentration was increased. This was probably due to inhibition of γ-GT in the biliary tree. A decrease in γ-GT activity would account for the enhanced GSH concentration and the significant decreases in degradation products.

In summary, the sensitivity and specificity of LC—ED for the analysis of thiols and disulfides has been reported in several studies [5—12]. Simultaneous

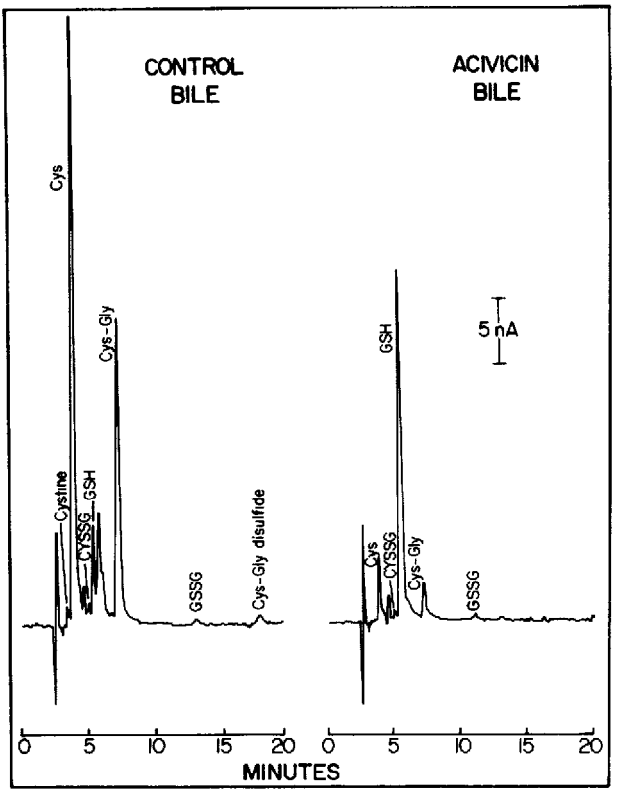

Fig. 5. Typical chromatograms of bile samples from control and acivicin-treated rats. Bile samples (30 min) were collected directly into 1.0 ml of ice cold 0.25 *M* perchloric acid and centrifuged. Supernatants from bile samples were diluted 1:6 with 0.25 *M* perchloric acid prior to injection (10 μl).

analysis of both thiols and disulfides was accomplished after separation by HPLC and detection with a dual gold–mercury electrode. However, several of these studies reported that there were compounds that may co-elute with GSH and that additional preparation and analysis may be required [9, 10]. Furthermore, the initial cost for two amperometric LC detectors may be prohibitive to some laboratories.

In this report we demonstrate similar levels of detection for sulfhydryls accomplished by substituting a 6-V battery for one of the amperometric LC detectors. The battery supplies the reducing potential at the upstream reducing electrode at a much lower cost. We also found that several thiols and disulfides were not resolved by the methods previously reported. The described method resolves GSH from co-eluting compounds such as CYSSG and γ-Glu-Cys. In addition, this HPLC—ED method can be used to resolve and quantitate thiols and disulfides produced during the degradation of GSH including CYSSG, Cys-Gly and Cys-Gly disulfide.

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