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ANALYSIS OF HEPATIC REDUCED GLUTATHIONE, CYSTEINE AND HOMOCYSTEINE BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A high-performance liquid chromatographic method employing a mercury-based electrochemical detector and a cation-exchange column is described for the simultaneous measurement of reduced glutathione, cysteine, and homocysteine in liver homogenates. Sample preparation involves precipitation of protein with perchloric acid, removal of perchlorate by precipitation as its potassium salt and dilution with mobile phase. Mercaptoethylglycine is used as the internal standard. Using this procedure, the sum of the individual hepatic thiols agreed well with the total thiols determined with Ellman's reagent. Comparisons were made with (a) control rats, (b) rats depleted of hepatic thiols by pargyline pretreatment, and (c) rats administered L-cysteine.

INTRODUCTION

Interest in the role of reduced glutathione (GSH) in drug-induced hepatotoxicity [1] and the possible sparing effect of sulfhydryl-containing therapeutic agents and prodrug forms thereof on hepatic GSH stores [2—4], prompted us to evaluate and modify the high-performance liquid chromatographic—electrochemical detection method (HPLC—ED) for the simultaneous measurements of GSH, cysteine and homocysteine in liver homogenates. The application of HPLC—ED to the quantitation of biologically relevant sulfhydryl substances was first described by Rabenstein and Saetre [5] using a mercury-based detector. Although their initial report was limited to standard solutions, they [6—8] and others [9, 10] have subsequently used HPLC—ED

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for the determination of cysteine and homocysteine in plasma; D-penicillamine in whole blood, red blood cells, plasma and urine; and GSH in whole blood.

The HPLC—ED procedure was adapted for measurement of GSH and cysteine in brain and liver homogenates by Mefford and Adams [11]. They used graphite rather than mercury for the working electrode. Although graphite or glassy carbon can be used as an electrode for oxidizing thiols, mercury is the preferred electrode material for thiol detection. The operating potential of the latter is significantly lower [12]. Differences in the operating potential are based on the half-reactions which occur at their respective electrode surfaces, viz., $2\ RSH \rightarrow RSSR + 2H^+ + 2e^-$ (carbon), and $2\ RSH + Hg \rightarrow Hg(SR)_2 + 2H^+ + 2e^-$ (mercury). Consequently, electrochemically active substances such as uric acid and ascorbate are detected and can interfere with thiol analysis using graphite but not mercury electrodes [12].

In our initial attempts to use cation-exchange HPLC—ED for quantitation of thiols in protein-free supernatants prepared from rat liver with perchloric acid and other protein precipitants, a rapid and apparent irreversible loss of column affinity for thiols was observed with these biological samples, but not with identically prepared standards. In this report, we describe a modified HPLC—ED method for simultaneous separation and quantitation of the individual sulfhydryl compounds in liver homogenate using the mercury-based detector. Chromatographic anomalies with liver-derived samples have been eliminated by changing the sample preparation procedure. The method requires minimal sample preparation, and utilizes mercaptoethylglycine (MEG) as an internal standard.

MATERIALS AND METHODS

Chemicals

Reduced glutathione, L-cysteine, pargyline · HCl all from Sigma (St. Louis, MO, U.S.A.), and DL-homocysteine from ICN Pharmaceuticals (Cleveland, OH, U.S.A.) were used as received. Mercaptoethylglycine was prepared as previously described [13].

Animals

Male, random-bred rats of Sprague–Dawley descent from BioLab, (St. Paul, MN, U.S.A.) weighing 200—260 g were used. Food was withdrawn 16 h before sacrifice by decapitation. The livers were perfused in situ through the portal vein with 20 ml of isotonic saline, then excised and weighed.

Sample preparation

A 0.5-g section of the lower median lobe was removed from the liver and homogenized in 2.5 ml of a cold 0.3 *M* perchloric acid—5 m*M* EDTA solution. The homogenate was centrifuged at 12,000 *g* for 15 min resulting in a clear supernatant. Of the protein-free supernatant 1 ml was mixed with an equal volume of a solution containing 0.15 *M* dipotassium hydrogen phosphate, 0.01 *M* tripotassium citrate and 5 m*M* tetrasodium EDTA. The mixture was maintained at 0°C for 30 min and then centrifuged for 15 min at 12,000 *g* to remove the potassium perchlorate precipitate. For analysis, 0.1—0.4 ml of the

perchlorate-free supernatant depending on the anticipated levels of thiols, 0.1 ml of 1 m*M* mercaptoethylglycine (internal standard in 5 m*M* EDTA, and 0.1 ml of 0.2 m*M* N-acetylcysteine were added to 1.4—1.7 ml of mobile phase for a final volume of 2.0 ml. After bubbling with nitrogen to remove dissolved oxygen, 20 μl of the final solution were injected onto the column.

HPLC system

Separation of the sulfhydryl compounds was carried out using a 50 × 0.2 cm glass column dry packed with Zipax® SCX (Dupont, Wilmington, DE, U.S.A.). A precolumn (5 × 0.2 cm) with the same packing was used. The precolumn was changed after every 500 biological samples. The mobile phase composed of 0.01 *M* citric acid and 0.01 *M* disodium hydrogen phosphate was adjusted to pH 2.1 with metaphosphoric acid (ionic strength equalled approximately 0.038 *M*), then filtered through a 0.45-μm Millipore® filter (Bedford, MA, U.S.A.) and degassed under vacuum for 10 min before use. An oxygen-free eluent was maintained by keeping the mobile phase reservoir at 50—55°C and by continuously flushing the head space of the reservoir with nitrogen, deoxygenated by bubbling through a chromous chloride solution (Fisher Scientific, Fairlawn, NJ, U.S.A.).

The samples were injected onto the column using a 20-μl loop sample injector (Rheodyne, Model 7125, Berkeley, CA, U.S.A.). A flow-rate of 0.7 ml/min was maintained using a Milton Roy Model 396 minipump (Riviera Beach, FL, U.S.A.). The electrochemical detection system consisted of an LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and a thin-layer transducer (Model TL-6A, Bioanalytical Systems) composed of a Au/Hg working electrode and a glassy carbon auxillary electrode. The potential of the working electrode was 0.0 V versus an Ag/AgCl reference electrode [5]. Between injections a 10-sec cleansing voltage (—0.20 V) was applied to the working electrode [5]. Failure to apply the cleansing voltage between samples resulted in a gradual decline in detector response. The peak areas were integrated using a Hewlett-Packard Model 3390A integrator (Palo Alto, CA, U.S.A.).

Spectrophotometric method

Total sulfhydryls were determined using Ellman's reagent according to the method of Buttar et al. [14]. The same homogenization procedure was used as described under Sample preparation.

Calculations and statistical analysis

Standards containing GSH, cysteine and homocysteine prepared in 0.3 *M* perchloric acid—5 m*M* EDTA were carried through the sample preparation procedure. The following equation was used for calculating the results:

$$\text{Concentration of unknown} = \frac{\text{(Peak area ratio of unknown/MEG)} \times 6.0\ (= \text{dilution factor}) \times \text{concentration of external standard}}{\text{Peak area ratio of external standard/MEG}}$$

The results were expressed as mean ± S.E.M. of triplicate samples unless indicated otherwise. Statistical analyses were carried out using the Student's

t test, paired or unpaired, as appropriate. Differences were considered significant for p values < 0.05.

RESULTS

Homogenization media and chromatography

A typical chromatogram for a combined standard containing GSH, cysteine and homocysteine is shown in Fig. 1A. The citrate—phosphate mobile phase, pH 2.1, is a modification of that used by others [5, 9, 11] with a Zipax SCX column for separation of sulfhydryl compounds. N-Acetylcysteine was included to mask a negative peak [5] which interfered with the electronic integration of the GSH peak area. MEG was present as the internal standard. The retention times for GSH, cysteine, homocysteine and MEG were 1.7, 2.6, 3.8 and 6.8 min, respectively.

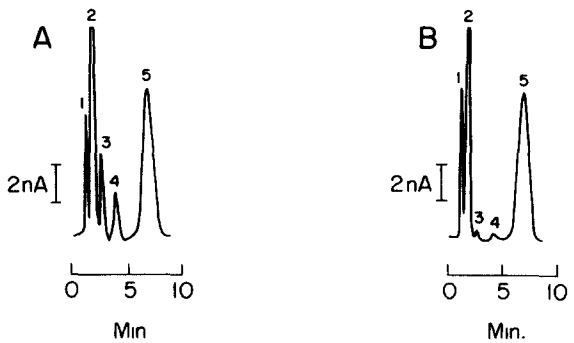

Fig. 1. HPLC—ED chromatograms of biologically significant sulfhydryl compounds in (A) a standard solution and (B) a sample prepared from rat liver. Peaks: (1) N-acetylcysteine; (2) GSH; (3) cysteine; (4) homocysteine; and (5) mercaptoethylglycine. N-Acetylcysteine (1) and mercaptoethylglycine (5) were added for analytical purposes (see text). The amounts of standard thiol injected were: GSH and mercaptoethylglycine, 1.0 nmol and cysteine and homocysteine, 0.05 nmol.

A number of deproteinizing agents including trichloroacetic acid (TCA), metaphosphoric acid, perchloric acid, sulfosalicylic acid and phosphotungstic acid were evaluated for their compatibility with the chromatography of standards. Of these, metaphosphoric acid (1.5%), TCA (5%), and perchloric acid (10%) appeared compatible. Standards in metaphosphoric acid could be injected directly onto the column, whereas standards in TCA and perchloric acid required prior diethyl ether extraction and perchlorate precipitation with tripotassium citrate, respectively, for unaltered chromatographic characteristics.

The chromatograms of the sulfhydryl compounds in protein-free supernatants from rat liver homogenates prepared with metaphosphoric acid, TCA and perchloric acid were compared. Before chromatography, the TCA samples were extracted with diethyl ether and perchlorate was precipitated from the perchloric acid samples by the addition of a potassium phosphate—citrate solution. The biological samples prepared in metaphosphoric acid and TCA caused a rapid deterioration of the cation-exchange column. One to five 20-μl

injections caused a pronounced decrease in retention times of all thiols. The loss of column affinity for the thiols in metaphosphoric acid and in diethyl ether-extracted TCA biological samples was not observed with standards. The original retention times could not be restored by washing the column with water, 1 *M* trisodium phosphate or 1 *M* orthophosphoric acid or a combination thereof. No column deterioration was observed with biological samples prepared with perchloric acid as described. Fig. 1B shows a typical chromatogram of the sulfhydryl compounds from liver.

Standard curves

The standard curves for GSH, cysteine, homocysteine and MEG were linear over a range of 0—1.0 nmol injected. Using MEG as the internal standard, the peak area ratios of individual standards prepared over a concentration range of 0—2 m*M* (and carried through the sample preparation procedure described in Materials and methods) versus the theoretical amount of standard injected, viz., 0 to 1.0 nmol, also gave linear plots.

Recovery and sample storage

The recoveries of GSH, cysteine and homocysteine standards carried through the sample preparation procedure were 100%. The calculated recoveries for externally added GSH, cysteine and homocysteine from a pooled biological sample were 96, 94 and 77%, respectively (Table I).

The effect of storage at −70°C on the GSH content of protein-free and perchlorate-free supernatants prepared from rat liver homogenates is shown in Fig. 2. The relative GSH content of the biological samples and of the identically prepared GSH standards were similar up to eleven days of storage. A minimum recovery of 89% was observed. Although the presence of perchlorate did not significantly affect the recovery of GSH in either the stored biological samples or GSH standard solutions, it is prudent to store biological samples perchlorate-free.

TABLE I

RECOVERY OF GSH, CYSTEINE AND HOMOCYSTEINE FROM PROTEIN-FREE SUPERNATANTS OF RAT LIVER HOMOGENATES

Protein-free supernatants were prepared from liver homogenates from two rats. To 5-ml aliquots of the supernatants (n = 4) were added 3.0 nmol GSH, 1.5 nmol cysteine or 1.5 nmol homocysteine. The protein-free supernatants with and without additions were analyzed for sulfhydryl content by HPLC—ED. The results were compared against data obtained using a combined standard solution of GSH, cysteine and homocysteine. Other experimental details are given under Materials and methods.

Sulfhydryl	Concentration (μmol/g wet weight liver)		Found after addition	Percent recovery (%)
	Initial	Calculated after addition		
GSH	3.72 ± 0.07	7.32	7.19 ± 0.05	96
Cysteine	0.059 ± 0.008	1.86	1.75 ± 0.03	94
Homocysteine	0.109 ± 0.005	1.91	1.50 ± 0.04	77

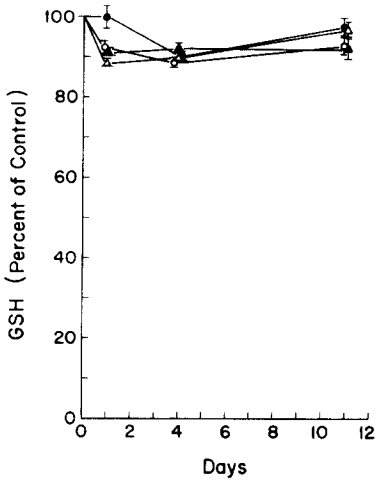

Fig. 2 Effect of storage at −70°C on GSH levels in protein-free and potassium perchlorate-free supernatants prepared from liver homogenates. The livers from two rats were individually homogenized in 0.3 *M* perchloric acid—5 m*M* EDTA, pooled and processed as described in Materials and methods. A GSH standard (3.0 m*M*) was treated identically to the biological samples. The protein-free and perchlorate-free supernatants prepared from the pooled liver homogenate and the GSH standard were stored in 2.0-ml aliquots, deaerated by bubbling with nitrogen and sealed for storage The data represent mean ± S.E.M. of triplicate samples compared to freshly prepared GSH standards. The initial hepatic GSH concentration was 3.26 ± 0.03 μmol per g wet weight tissue. Protein-free supernatants from liver homogenate (○) and corresponding GSH standard (△); perchlorate-free supernatants from liver homogenate (●) and corresponding GSH standard (▲).

Comparison of hepatic sulfhydryls by HPLC—ED and spectrophotometric methods in control and drug-treated rats

Liver homogenates were prepared from isotonic saline (control), pargyline and L-cysteine pretreated rats. The hepatic GSH, cysteine and homocysteine levels were determined in these animals using the described HPLC—ED method (Table II). Pargyline is known to decrease hepatic GSH levels [15] and L-

TABLE II

HEPATIC GSH, CYSTEINE AND HOMOCYSTEINE LEVELS IN RATS PRETREATED WITH SALINE, PARGYLINE OR L-CYSTEINE

The drugs were administered, intraperitoneally, 1 h before sacrifice Levels are given in μmol per g wet weight liver

Drug	*n*	HPLC—ED method				Chemical method	*p***
		GSH	Cysteine	Homocysteine	Total thiols*	Total thiols	
Saline control	6	4 07 ± 0 21	0 050 ± 0 032	0 087 ± 0 006	4 20 ± 0 21	4 67 ± 0 23	N S
Pargyline (1 0 mmol/kg)	6	0 53 ± 0 14 (<0 001)***	0 023 ± 0 007 (N S)	0 099 ± 0 008 (N S)	0 66 ± 0 14 (<0 001)	0 83 ± 0 14 (<0 001)	N S
L-Cysteine (1 25 mmol/kg)	5	5 04 ± 0 33 (<0 05)	0 183 ± 0 025 (<0 01)	0 075 ± 0 010 (N S)	5 30 ± 0 31 (<0 05)	5 61 ± 0 29 (<0 05)	N S

*Equals sum of the individual thiols None of the data are corrected for recovery
**Total thiols for the two methods are compared using the paired Student's *t* test N S = non-significant
***The *p* values in parentheses represent statistical comparisons against the control within each column

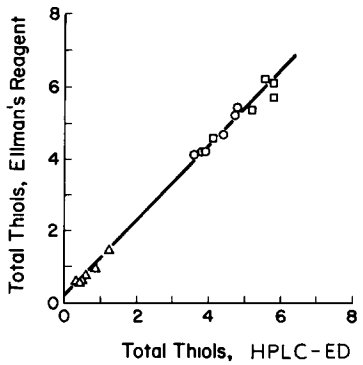

Fig. 3. Comparison of the total hepatic thiols measured by the spectrophotometric versus HPLC—ED method. The data are from Table II. Control (○), pargyline (△) and cysteine (□) treated animals.

cysteine is a biochemical precursor of GSH. In the control animals, GSH constituted nearly 97% of the total hepatic sulfhydryl content, whereas GSH was reduced to approximately 80% of the total in the pargyline-treated animals. Administration of L-cysteine caused a 20% increase in hepatic GSH and 266% increase in cysteine levels, over controls.

In these same groups of animals, the total hepatic sulfhydryl content measured by HPLC—ED, i.e., the sum of GSH, cysteine and homocysteine, was compared with the total sulfhydryl content measured by a spectrophotometric method using Ellman's reagent (Table II). For each group of animals, the total thiols measured by these two methods were not statistically different. Furthermore, the correlation curve comparing these two sets of data was linear with a slope of 1.04 and a correlation coefficient of 0.996 (Fig. 3).

DISCUSSION

Methods development

The attractive features of HPLC—ED and the mercury-based electrode for the measurement of hepatic sulfhydryl substances include (a) facile sample preparation, (b) high detector sensitivity for thiols, and (c) detector insensitivity toward electrochemically inactive substances present in biological samples. The lower operating potential of the mercury compared to the carbon electrode provides additional detector selectivity [12]. Other HPLC procedures utilizing spectrophotometric detectors contain one or more derivatization steps and are characterized by long elution times [16, 17].

Our application of HPLC—ED to the analysis of hepatic thiols is based on the earlier work of others [5, 8, 9, 11]. The resolution of GSH, cysteine and homocysteine was optimized by altering the pH and ionic strength of citrate—phosphate eluents while retaining short elution times. MEG which has a sulfhydryl functional group was selected as the internal standard based on its optimal elution time.

A major problem encountered was finding a protein-precipitating agent which did not alter the chromatographic characteristics. Of those evaluated, metaphosphoric acid, TCA, and perchloric acid appeared compatible when

used with standard thiol solutions. However, the removal of excess reagents, TCA by diethyl ether extraction and perchlorate by precipitation as its potassium salt, was required before analysis. Analysis of protein-free supernatants of biological samples prepared with metaphosphoric acid or TCA — even after extraction with diethyl ether — caused a nearly complete loss of column affinity. The cation-exchange column could not be regenerated with either acid or base. Protein-free supernatant prepared with perchloric acid followed by removal of perchlorate could be injected directly onto the column without column deterioration or changes in the chromatographic characteristics of the thiols when compared with chromatograms of standard thiol solutions.

Hepatic thiol levels and application

The sum of the individual hepatic thiols determined by HPLC—ED, viz., the sum of GSH, cysteine and homocysteine levels, is in good agreement with the total thiols determined spectrophotometrically using Ellman's reagent (Table II, Fig. 3). However, the HPLC—ED values are consistently slightly lower suggesting that the total thiols measured using Ellman's reagent may include an additional sulfhydryl component.

GSH constituted more than 95% of the total thiols in the liver of control rats, whereas GSH still represented about 80% of total thiols in the GSH-depleted livers of pargyline-treated animals (Table II). These results suggest that the measurement of total hepatic thiols using Ellman's reagent represents only an approximation of the actual GSH levels.

The application of this HPLC—ED method may be particularly relevant to studies on the biochemical mechanisms of GSH restoration in experimental animals following GSH-depletion by highly reactive metabolites derived from xenobiotic substances such as acetaminophen [18] and after therapeutic intervention with L-cysteine and/or its prodrug derivatives [2—4, 19].

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