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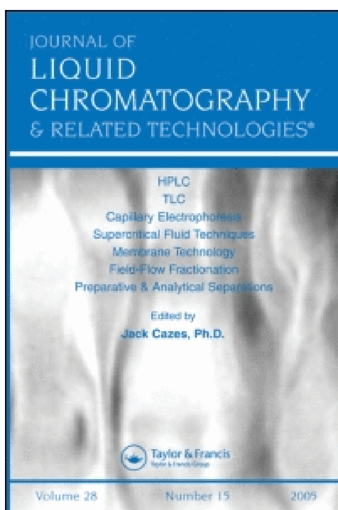

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Measurement of Tissue Sulfhydryls and Disulfides in Tissue Protein and Nonprotein Fractions by High Performance Liquid Chromatography Using Electrochemical Detection

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MEASUREMENT OF TISSUE SULFHYDRYLS AND DISULFIDES IN TISSUE PROTEIN AND NONPROTEIN FRACTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING ELECTROCHEMICAL DETECTION

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ABSTRACT

A specific, sensitive and quantitative method for measuring tissue sulfhydryl (SH) and disulfide levels in nonprotein, protein, and protein-bound fraction has been developed by using HPLC with electrochemical detection. Protein and nonprotein fractions are separated through perchloric acid precipitation. The protein fraction is divided into two aliquots: one which undergoes protein hydrolysis in HCl and the other which undergoes sodium borohydride reduction. The nonprotein, protein and protein-bound fractions generated are then separated by HPLC and the various sulfhydryls (e.g., cysteine, glutathione) and disulfides (e.g., cystine, glutathione disulfide) are measured directly by a dual gold mercury electrode thin layer electrochemical cell. The chromatography takes less than 15 min to separate cystine, cysteine, glutathione, and glutathione disulfide. This assay is more specific and as sensitive as other assays employing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). Furthermore, this procedure allows the simultaneous measurement of SH and disulfides in the nanogram range without complex extractions or derivatizations.

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INTRODUCTION

A growing interest in the biological role of sulfhydryls (SH) and disulfides (SS) has spurred the development of new techniques which measure these compounds. Our interest has been heightened by the possible role SH plays in diseases of the stomach, especially acute gastric mucosal injury (1-3). Unfortunately, simple analytical methods to simultaneously measure various SH and SS compounds in biological materials were not available until recently.

The older colorimetric analytical procedures (4-7) not only do not allow simultaneous measurement of SH and SS, but they are not very specific and sensitive. Other HPLC procedures that use UV or fluorescent detection require pre- or post-column derivitization (8-10). A recent technique detects the SH and SS only in aqueous solutions with a dual gold mercury thin layer cell after their separation by reverse phase HPLC (11). The electrode is designed such that the upstream electrode reduces SS and the downstream electrode oxidizes the generated SH along with any previously existing SH. The output from the downstream electrode is sent to a chart recorder.

We have developed a method of separating the SH and SS in the nonprotein, protein, and protein-bound fractions of tissue (Fig. 1) which can be used to chromatograph these compounds and measure them by electrochemistry.

Reduced and oxidized forms of cysteine and glutathione can thus be measured in tissue samples without extractions or derivatizations.

MATERIALS

Chemicals

L-Cysteine, cystine, coenzyme A, homocystine, glutathione (reduced), sodium borohydride, chloroacetic acid and DL-penicillamine were obtained from Sigma Chemical Company (St. Louis, MO). HPLC grade methanol and disodium EDTA were

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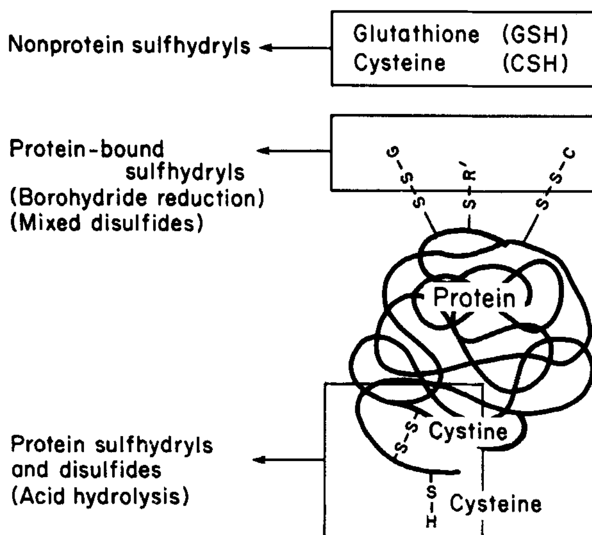

FIGURE 1: Schematic presentation of tissue sulfhydryls and disulfides.

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purchased from Fisher Scientific (Pittsburgh, PA). Cysteamine-HCl and perchloric acid were bought from Aldrich Chemical Company (Milwaukee, WI). Sodium octyl sulfate was obtained from Eastman Kodak (Rochester, NY).

Apparatus and Mobile Phase

A LC-154 liquid chromatograph with tandem LC-4B controllers (Bioanalytical Systems, West Lafayette, IN) was used (11) with a Biophase ODS 5 micron column (250 x 4.6 mm BAS) at a flow rate of 1.5 ml/min. The mobile phase was 96% 0.05M chloroacetic acid (pH 3)/4% methanol, with 300mg/l of sodium octyl sulfate. The mobile phase was filtered through a 0.2 micron membrane filter (Gelman Sciences, Ann Arbor, MI) and degassed under vacuum for 15 min before use. The temperature of the mobile phase was kept at 40°C under reflux. All samples were filtered through 0.2 micron nylon 66 membrane sample filters (Alltech Associates, Deerfield, IL) before injection.

Standards

All stock standard solutions were made weekly in 0.1% disodium EDTA and kept refrigerated. The cystine standard was made in 0.1 N sodium hydroxide and acidified with 0.2 M perchloric acid before injection. All standards were made from the 1.0 mM stock solutions each day of experimentation.

METHODS

Sample Preparation

Fasted female Sprague-Dawley rats (150-200 g) were decapitated. The stomach, duodenum, and liver were rapidly dissected out. The mucosa from glandular stomach and proximal (about 2 cm) duodenum were scraped with a blunt knife and frozen on dry ice. The procedure is outlined in Figure 2. Subsequently, tissues were weighed and homogenized in 3 volumes (x g wet tissue) of cold 0.2 M perchloric acid using an ultra turrax homogenizer (Tekmar Company, Cincinnati, OH) (three 5 sec bursts). The homogenate was frozen using liquid nitrogen, thawed, refrozen, and centrifuged 30 min later at 5,000 rpm for 20 min. The supernatant was pipetted off and filtered. A 20 ul sample of the filtrate was injected into the HPLC for measurement of nonprotein SH: glutathione (oxidized and reduced), cysteine and cystine. The precipitated protein pellet was washed twice with 1.0 ml of 0.2 M perchloric acid by rehomogenization and centrifugation at 5,000 rpm for 15 min to remove any remaining free thiol. All washes were discarded and the washed pellet was rehomogenized a third time with 1.0 ml 0.2 M perchloric acid. The homogenized pellet was then divided into two aliquots for the preparation of the hydrolysates and the protein-bound SH. The aliquot for hydrolysis was put into glass culture tubes. Each aliquot was recentrifuged at 5,000 rpm for 15 min and the supernatant was discarded. To the culture tubes 0.5 ml of 6 M hydrochloric acid was added. The samples were first frozen, the tubes were flushed with nitrogen gas and the tubes were then sealed.

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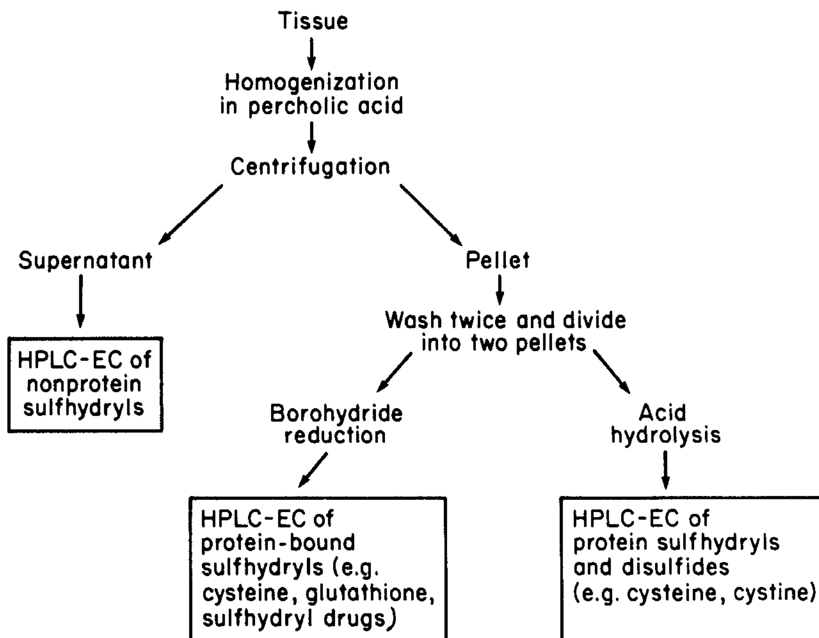

FIGURE 2: A flow chart illustrating the procedure of obtaining the nonprotein, protein and protein-bound fractions.

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To achieve hydrolysis of the proteins the culture tubes were heated at 105 - 110°C overnight. The samples were evaporated under nitrogen to prevent oxidation. The dry hydrolysate was reconstituted in 3 volumes of wet weight starting tissue with 0.05 M chloroacetic acid (pH 3) and filtered. The sample (20 ul) was then injected into the HPLC to measure the cystine and cysteine content of the protein fraction. In the second aliquot any excess perchloric acid was neutralized with 50 ul of 1.0 N sodium hydroxide. The pellet was then resuspended in 0.75 vol/g wet starting tissue of 0.2 M sodium phosphate (pH 9). Disulfides in the proteins were then reduced by adding 0.1 ml of 0.1% sodium borohydride and incubated for 30 min at 37°C. The reaction was stopped by addition of 1.5 vol/g wet starting tissue of 0.2 M perchloric acid. The reprecipitated

protein was centrifuged at 5,000 rpm for 20 min. The supernatant was filtered and 100 ul was injected into the HPLC to determine the protein-bound SH concentration.

In other experiments cysteamine (30 mg/100g) per os, or penicillamine (60 mg/100g) subcutaneously was given to rats 30 min before autopsy. The concentrations of these agents were measured in the stomach, duodenum and liver.

In recovery experiments, 50 ul of liver nonprotein supernatants received an additional 100 ul of 1.0mM glutathione, 50 ul of 1.0mM cysteine, or 50 ul of 1.0mM cystine. The liver supernatants were injected in triplicate both before and after the addition of the respective standard and the percent recovery was calculated in terms of umoles/ul.

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RESULTS

The chromatography of glutathione and cysteine standards (Fig. 3) exhibited linearity up to 307 ng and 500 ng respectively. The sensitivity of the electrodes was 2.4 ng and 1.9 ng for glutathione and cysteine respectively.

The preliminary experiments determined that perchloric acid was best suited for the precipitation of protein because it showed no interference with the electrode and it gave a very low void peak. Chromatography of the nonprotein supernatant from the gastric mucosa gives a clear separation of cystine, cysteine, and glutathione in less than 10 min (Fig. 4). The pH of the sample injected is around 2.0. This low pH does not interfere with the electrode or the chromatography because the mobile phase is at pH 3 in order for the amine groups to be fully protonated. The chromatography of the components can be increased by increasing the concentration of sodium octyl sulphate in the mobile phase or decreasing the percent methanol in the mobile phase. A larger void peak might require a greater concentration of sodium octyl sulphate to completely resolve cystine from the void. Sodium octyl sulphate effectively adds more negative active sites to the hydrophobic column so that positively charged groups will be slowed down and eluted later.

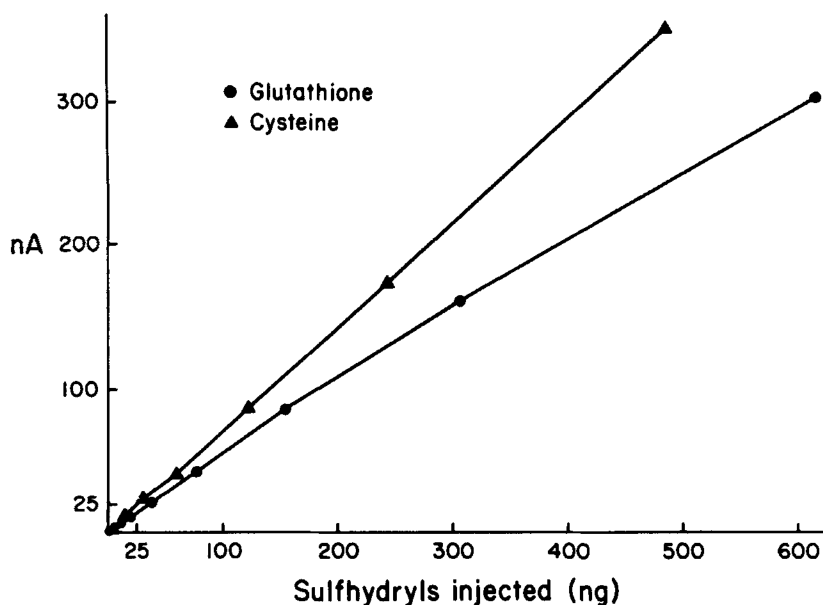

FIGURE 3: Standard curves for glutathione and cysteine showing linearity and sensitivity.

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Increasing the ion-pairing reagent concentration causes highly positive SH species such as cysteamine and penicillamine to elute much later and exhibit a tailing effect (Fig. 4).

The protein hydrolysates must be evaporated to remove the 6 M HCl which would otherwise destroy the column and effectively strip the electrode surface. Chromatography of a protein hydrolysate from gastric mucosa gives the two expected peaks of cystine and cysteine (Fig. 4).

Sample oxidation is avoided because all the samples are in acidic solutions. SH oxidations occur minimally at low pH and maximally at high pH. Most hydrolysates contain considerable cystine so it is important to dilute the samples in order for the component peaks to chromatograph within the range of the detector.

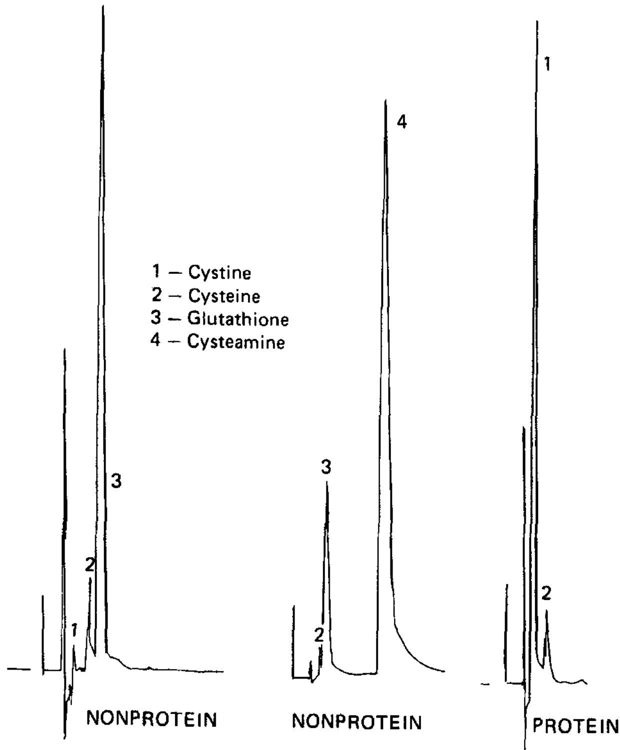

FIGURE 4: Representative chromatograms obtained with reverse-phase high performance liquid chromatography (HPLC) of rat gastric mucosa. A. (left) The nonprotein fractions were detected by the dual gold mercury electrode thin layer cell. B. (center) The nonprotein fraction from gastric mucosa of rat which was given exogenous cysteamine. Note the tailing effect of cysteamine detected in this tissue. C. (right) The protein hydrolysate from rat gastric mucosa. The concentration of cystine and cysteine are 0.33 nM and 0.06 nM, respectively.

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The protein and nonprotein fractions contain considerably higher concentrations of SH than the protein-bound fraction. This fact makes it necessary to increase the injection size of the sample to 100 ul. The peak width increases but the standard curve remains linear. The chromatogram may show multiple void peaks, but the separation of cysteine and glutathione is unaffected by the borohydride reduction (Fig. 5). The borohydride reduction reaction was tested with disulfide standards under identical sample conditions. These conditions proved to reduce the disulfide standards greater than 95%. Furthermore, the concentration of exogenous cysteamine which bound to the protein fraction in the gastric mucosa can be measured in the detectable range (Fig. 5).

Although coenzyme A is in sufficient concentrations to be detected by the electrode, it does not chromatograph using these conditions due to the fact that at pH 3 it has no net charge, causing it to be eluted with the void volume. Homocystine is also not detectable under these conditions because it is not retained; therefore, it is removed with the void.

Recovery experiments were performed to measure the accuracy of detectability. Known amounts of glutathione, cysteine, and cystine were added to nonprotein sulfhydryl liver supernatants. The percent recovery for glutathione, cysteine, and cystine were 103%, 122%, and 106%, respectively.

In all the tissue chromatographed, the detection of the SH and SS was very satisfactory in all three fractions. The concentration of various components in several tissues is shown in Table 1.

DISCUSSION

Results presented here demonstrate the use of the dual gold mercury electrode to simultaneously measure SH and SS in rat organs. This procedure, which measures the SH and SS concentration in nonprotein, protein, and protein-bound fractions, has many advantages. For instance, by direct measurement of the SH and SS

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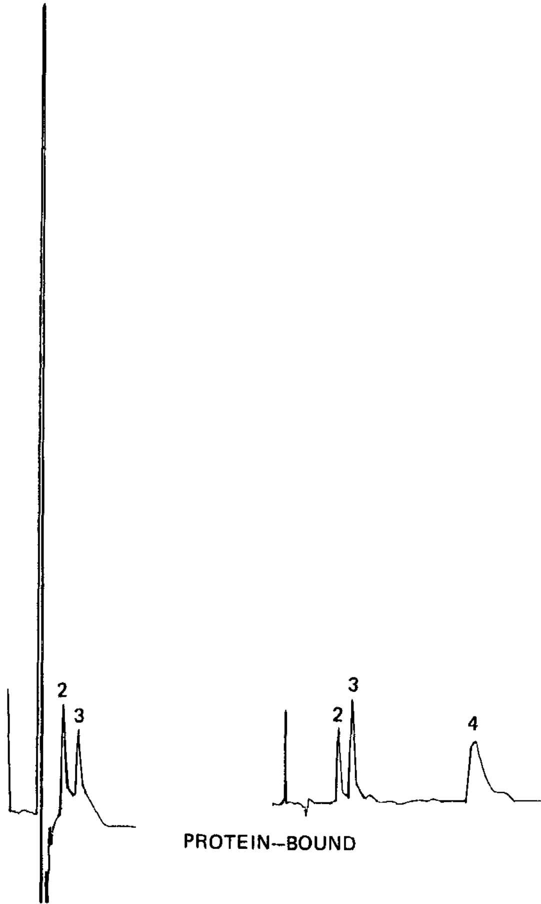

FIGURE 5: Representative chromatograms of the protein-bound fraction of rat gastric mucosa. Injection size is 100 ul. A. (left) The concentration of cysteine and glutathione is 0.75 uM and 0.67 uM, respectively, in the gastric mucosa of a control rat. B. (right) An extra peak of cysteamine is detected in the gastric mucosa of rat which was given exogenous cysteamine.

TABLE 1

Tissue Concentrations of Sulfhydryls and Disulfides

Tissue	Fraction	Cysteine nmoles/g wet tissue	Cystine nmoles/g wet tissue	Glutathione (reduced) nmoles/g wet tissue
Gastric Mucosa	nonprotein	54.8± 8.3	22.5± 2.8	528.8±132.8
	protein	268.5± 57.8	1592.5±255.4	--
	protein-bound	7.6± 2.1	--	16.5± 3.4
Gastric Muscle	nonprotein	93.1± 14.8	26.6± 4.0	1038.0±128.8
	protein	97.0± 21.6	718.3± 27.0	--
	protein-bound	4.1± 1.0	--	2.1± 0.5
Liver	nonprotein	96.2± 6.1	117.0± 26.1	5270.0±165.0
	protein	1190.3±187.4	825.8± 97.2	--
	protein-bound	493.2± 34.2	--	116.5± 3.3

The results are expressed as mean ± SEM derived from measurements in 4-6 rats.

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at a set potential any interferences are avoided, thus giving a result that is more accurate. The absence of the need to derivatize the samples to achieve measurement speeds up analysis by removing steps in the preparation and also decreasing the chromatography time. Furthermore, by utilizing common wet chemical steps with minor modifications, the simplicity of the procedure is increased. This technique is much more specific than colorimetric procedures. A recent derivatization procedure for HPLC requires a separation time of over 30 min (9) while this method separates all the components in less than 15 min. Furthermore, most of the derivatization procedures require gradient elution which adds further equilibration time between sample injections. Our procedure is performed with isocratic elution so samples can be injected sooner together because of no needed reequilibration time. The separation of the various peaks can easily be altered just by changing the methanol concentration in the

mobile phase. If the reagents for the sample preparation interfere with the standard peaks, then slight modification of the mobile phase is usually sufficient to achieve proper separation.

Exogenously administered SH drugs such as cysteamine and penicillamine can also be quantitated in various tissues with this procedure. This could prove useful in studies of SH drug metabolism and its possible reactivity with endogenous SH. Although not every endogenous SH or SS can be separated under these conditions, this technique greatly increases the applications for the use of the dual gold mercury thin layer cell and will prove very useful in all areas of research concerning the role of SH and SS in normal physiologic and biochemical reactions as well as in various diseases. Furthermore, this method may lead to the discovery of unknown SH or SS moieties which exist in living tissue.

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