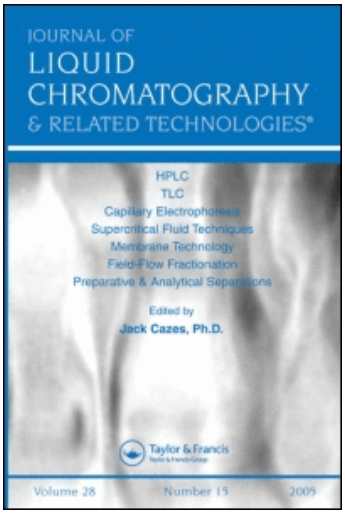

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LIQUID CHROMATOGRAPHIC BEHAVIOR OF BIOLOGICAL THIOLS AND THE CORRESPONDING DISULFIDES

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ABSTRACT

Reverse-phase, ion-exchange, and reverse-phase ion-pairing were evaluated as liquid chromatographic modes for the isocratic separation of cysteine, cystine, glutathione (reduced and oxidized), homocysteine, and penicillamine (reduced and oxidized). A series dual Hg/Au amperometric detector was used to detect both thiols and disulfides. Reverse-phase ion-pairing was determined to provide the most satisfactory performance for a liquid chromatographic separation of the compounds of interest.

INTRODUCTION

There are a number of thiol compounds which are of biological interest. In living systems, glutathione (GSH), cysteine (CSH), and the corresponding disulfides (GSSG and CSSC) are intricately involved in maintaining proper cellular function. The thiol moiety is also present in penicillamine (PSH), a therapeutic agent used as an anti-arthritic and in heavy metal poisoning. One pathway for penicillamine metabolism involves formation of

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disulfides by reaction with another penicillamine molecule or other physiological thiols.

Determination of thiol compounds by liquid chromatography has recently received considerable attention, utilizing various detection schemes. The most common approach for cysteine (1, 2, 6, 8-10), glutathione (1, 2, 7-10), and penicillamine (1-3) has employed strong cation-exchange chromatography with mobile phases of acidic pH. Reverse-phase chromatography with heptane sulfonic acid as an ion-pairing agent has also been used in the separation of cysteine, homocysteine (HSH), and penicillamine (4), and a strong anion-exchange separation has been reported for several thiols, including glutathione and cysteine (9).

Often, measurement of both the thiols and disulfides in a given biological or chemical matrix is desirable, to provide a complete profile of the system. Due to the scarcity of suitable detectors for disulfides, there have been few reports on chromatographic characteristics of underivatized disulfides. Eggli and Asper (6) used strong cation-exchange for a mixture containing cystine (CSSC), cysteine and penicillamine, as did another report on glutathione, cystine, cysteine, and methionine (10). Strong anion exchange with a mobile phase of pH 8.4 was used for GSSG and cystine (5), although the resolution and peak shapes were rather unsatisfactory.

A series dual Hg/Au electrochemical detector has recently been developed in our laboratory for the detection of both thiols and disulfides in liquid chromatographic eluents (11). Briefly, the detector reduces disulfides at an upstream electrode and detects the resultant thiols and any naturally present thiols at the downstream electrode. Using this detector, we now report on the liquid chromatographic behavior of mixtures of selected thiols and disulfides. Strong cation-exchange, reverse-phase, and reverse phase ion-pairing were evaluated as separation schemes for cysteine, cystine, glutathione, homocysteine, penicillamine, glutathione disulfide and penicillamine disulfide.

MATERIALS

Chemicals

L-cysteine, L-cystine, glutathione (reduced), glutathione (oxidized), and D-penicillamine were purchased from Sigma Chemical Co. (St. Louis, MO). D-penicillamine disulfide was obtained from Aldrich Chemical Co. (Milwaukee, WI) and DL-homocysteine was purchased from ICN Pharmaceuticals, Inc. (Cleveland, OH). Sodium octyl sulfate and ethylene diamine tetraacetic acid were obtained from Eastman Kodak Co. (Rochester, NY). All other reagents, including buffer salts, acids, and methanol, were reagent grade.

Apparatus

A Bioanalytical Systems LC-154 liquid chromatograph with dual mercury/gold detector was used. All teflon tubing was replaced with stainless steel to exclude oxygen. The system has been described in detail elsewhere (11). Columns used were BAS Biophase ODS 5μ, 4.6 x 250 mm, and DuPont Zorbax 300 SCX, 4.6 x 250 mm. Detector output was integrated using either an LDC-308 Integrator or Hewlett Packard 3390A Reporting Integrator. Mobile phase flow rates were either 1.5 mL/min or 1.0 mL/min.

Methods

All mobile phases were prepared using deionized distilled water and filtered with 0.2 μ Nylon 66 membranes (Rainin Instrument Co., Inc., Woburn, MA). Mobile phases were refluxed at slightly above room temperature and continually purged with nitrogen to remove dissolved oxygen.

Stock standard solutions of individual compounds were prepared to a concentration of 1 mM in deionized, distilled water containing 1 g/L Na_2EDTA. The cystine stock solution was prepared in dilute NaOH at a concentration of 0.1 mM. All stock solutions were stored at 4°C and prepared weekly. Dilutions and

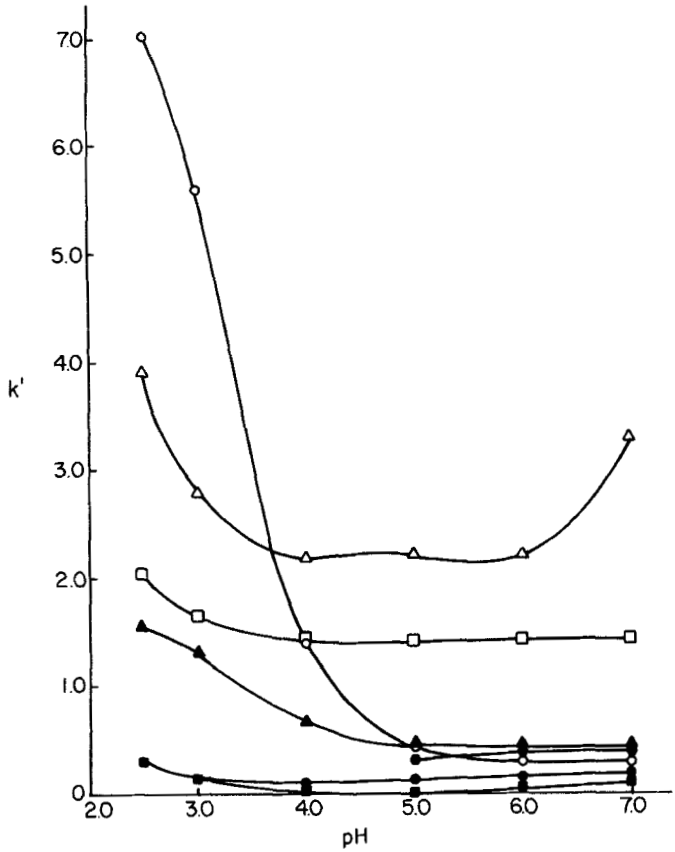

FIGURE 1. Capacity factors (k') of thiols and disulfides as a function of mobile phase pH. Mobile phase consisted of 0.1M phosphate buffer adjusted to the desired pH. A Biophase ODS 5 μ column was used with a flow rate of 1.5 mL/min. Curves represent cysteine (●), cystine (■), reduced glutathione (▲), homocysteine (●), oxidized glutathione (O), reduced penicillamine (□), and oxidized penicillamine (△).

mixtures of standard solutions were prepared every two or three days from the stock solutions. Cystine solutions were acidified using dilute $HClO_4$ prior to injection. Injections were made by overloading a 20 μL or 100 μL loop with deoxygenated standard solution.

The dual Hg/Au electrodes were prepared as described elsewhere (11). The flow channel was defined with the two electrodes in the series arrangement. The upstream electrode was held at -1.0V and the downstream electrode was monitored to obtain the chromatographic tracing.

RESULTS AND DISCUSSION

Reverse-Phase Chromatography

The behavior of the thiols and disulfides of interest was first investigated by using a reverse-phase column (Biophase ODS, 5μ) and varying the pH of the 0.1 M phosphate mobile phase over the range from 2.5 to 7.0. Figure 1 illustrates the measured capacity factors (k') versus mobile phase pH. At acidic pH values, reasonable retention is obtained for GSH, GSSG, PSH and PSSP, but they are observed to elute earlier with increasing pH. This is as expected from the ionizable functional groups in the molecules, which include carboxylic acids ($pK_a \simeq 2$), amines ($pK_a \simeq 8$-9) and thiols ($pK_a \simeq 9$-11). In regions of intermediate pH (4-7), the majority of these compounds exist as zwitterions, with little or no retention on the hydrophobic column. As the pH decreases, the carboxylic acid groups are becoming increasingly protonated, with a concomitant increase in retention. The maximum capacity factor for the longest retained compound, however, is only 7.0, demonstrating that these small, relatively polar molecules are not retained to any great extent, and limiting the flexibility of the separation. Cystine and cysteine are not retained or resolved at any available pH on an ODS column.

It is interesting to note that penicillamine disulfide does not reduce as readily as the other disulfides at the upstream

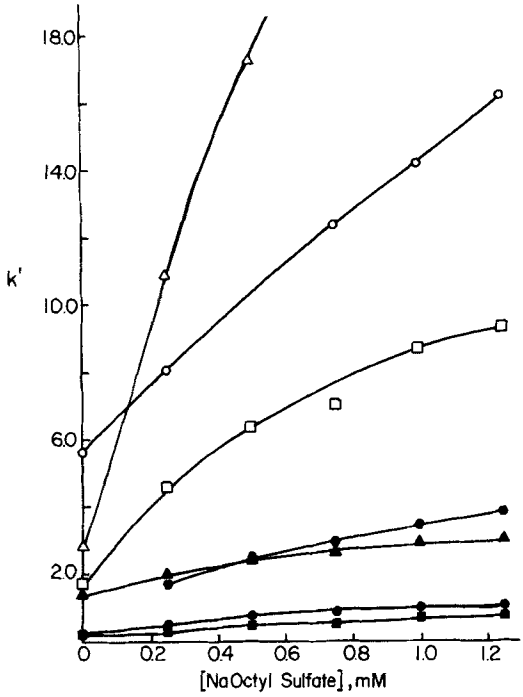

FIGURE 2. Capacity factors (k') of thiols and disulfides as a function of concentration of sodium octyl sulfate. A Biophase ODS 5 μ column was used with 0.1 M monochloroacetate buffer, pH 3.0, flow rate 1.5 mL/min. Curves labeled as in Figure 1.

electrode. Response for PSSP as measured at the downstream electrode is approximately one-hundred fold less than that for cystine and glutathione disulfide, except at pH 5.0, where the response for PSSP is comparable to other disulfides.

Reverse-Phase Ion-Pair Chromatography

A more useful set of mobile phase conditions for the thiols and disulfides was that using sodium octyl sulfate as an ion-pairing reagent with a C18 column. The mobile phase consisted of 0.1 M monochloroacetate (MCAA) buffer, adjusted to pH 3.0 to

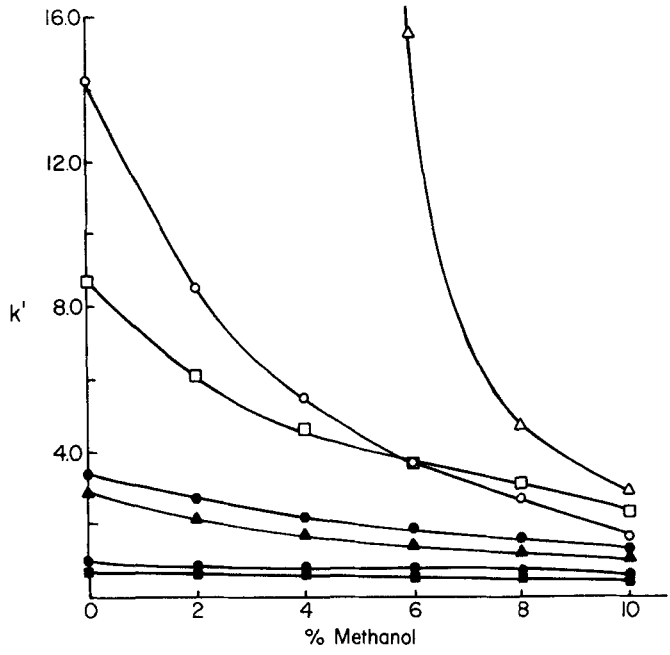

FIGURE 3. Capacity factors (k') of thiols and disulfides as a function of concentration of methanol in mobile phase. Sodium octyl sulfate concentration was fixed at 1.0 mM in 0.1M monochloroacetate buffer, pH 3.0, with a Biophase ODS column. Curves labeled as in Figure 1.

ensure that the compounds carried a net positive charge. In Figure 2, k' is plotted versus concentration of sodium octyl sulfate, demonstrating the large increase in retention which can be effected using this approach. Each of the compounds underwent similar shifts in retention, although penicillamine disulfide was changed to a markedly greater extent than the others. The ion-pairing was successful in resolving cysteine and cystine from one another.

Figure 3 shows the effect on k' of increasing percentages of methanol in the mobile phase. In this manner, the ion-pairing was utilized to increase retention and resolution of cysteine and

cystine, and methanol was added to speed elution of longer-retained compounds. Cysteine and cystine were not significantly affected by addition of methanol, while PSSP, GSSG, and penicillamine were moved in to more reasonable retention times. The chromatogram in Figure 4 illustrates the separation obtained using 1.0 mM sodium octyl sulfate with 4% methanol and a flow rate of 1.5 mL/min. Excluding PSSP, the chromatogram was complete in thirteen minutes and the peak shapes were quite satisfactory.

A third variable which was manipulated in ion-pairing chromatography was the ionic strength of the mobile phase buffer. By changing the molarity of monochloroacetate, the retention times could be correspondingly changed. The effects obtained with ionic strength variations were of less magnitude than those observed when changing sodium octyl sulfate or methanol concentration. Ionic strength, then, can be used to "fine-tune" the reverse-phase ion-pairing separation to achieve the desired chromatographic characteristics. Figure 5 shows a chromatogram of the selected compounds using 0.25 M monochloroacetate buffer, with 1.0 mM sodium octyl sulfate and 6% methanol, flow rate 1.0 mL/min. The chromatogram was complete within 10 minutes, although cysteine and cystine were closer to the void volume response than with 0.1M MCAA, which could pose a problem with real samples.

Based on the described studies, the chromatographic conditions that were adopted for future studies utilized the Biophase ODS 5 μ column with a mobile phase containing 0.1 M MCAA buffer, pH 3.0, 1.0 mMsodium octyl sulfate, with 4% methanol.

Ion-Exchange Chromatography

As the majority of literature work on these compounds has been performed using cation exchange, a column of this type was also investigated. The initial conditions consisted of the Zorbax SCX column with a mobile phase of 18.4 mM ammonium citrate and 60.7 mM phosphoric acid (final pH 2.49), producing the separa-

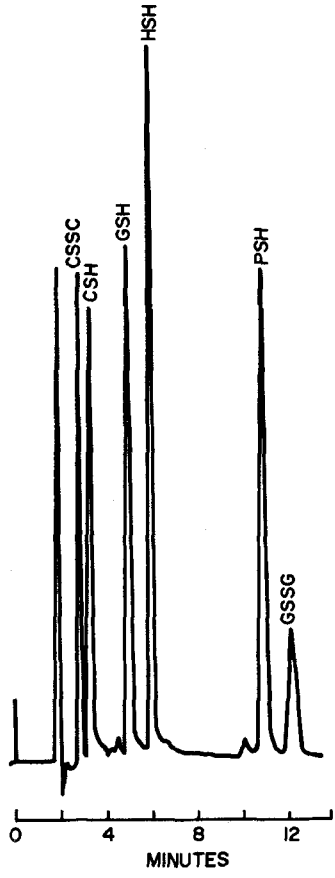

FIGURE 4. Reverse-phase ion-pair separation of thiols and disulfides. Column: Biophase ODS 5 μ; mobile phase: 0.1M monochloroacetate buffer, pH 3.0, 1.0 mM sodium octyl sulfate, 4% methanol.

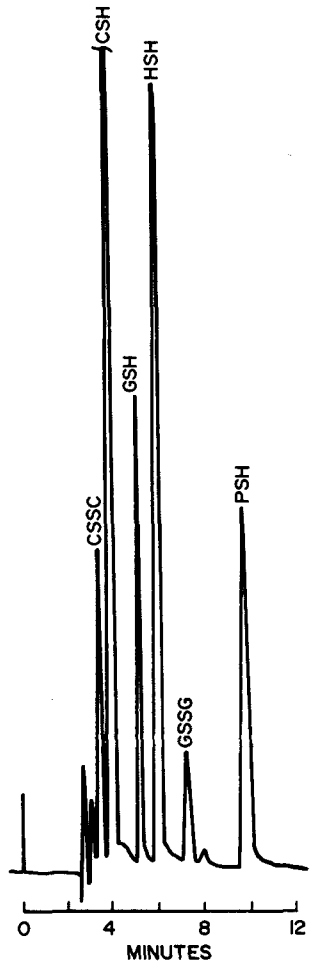

FIGURE 5. Effect of ionic strength on reverse-phase ion-pair separation of thiols and disulfides. Chromatographic conditions as in Figure 4, except monochloroacetate buffer was 0.25M.

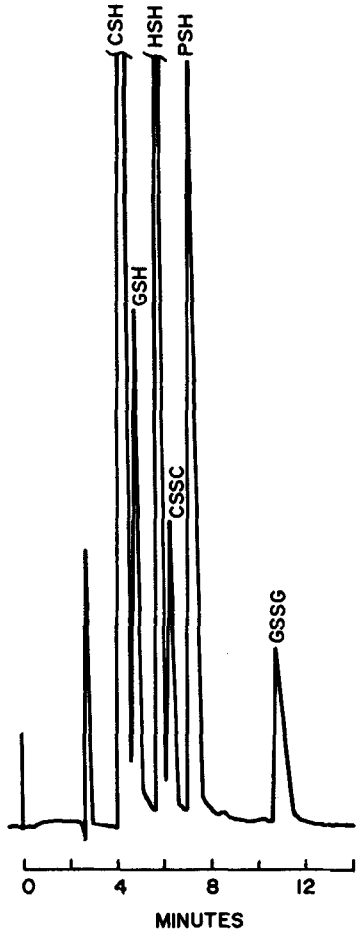

FIGURE 6. Strong cation-exchange separation of thiols and disulfides. A Zorbax SCX column was used, with a mobile phase containing 18.4 mM ammonium citrate and 60.7 mM phosphoric acid, flow rate 1.0 mL/min.

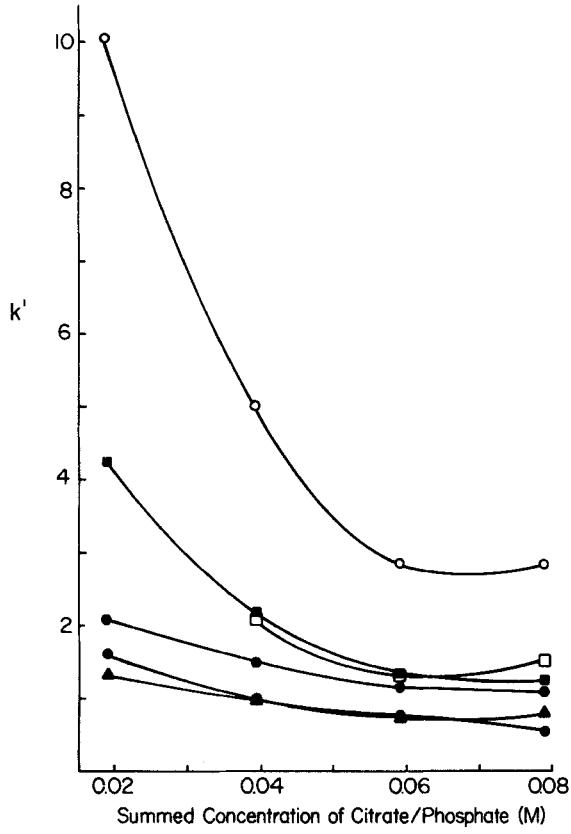

FIGURE 7. Capacity factors (k') of thiols and disulfides as a function of mobile phase buffer concentration. Mobile phase was phosphate/citrate buffer at a flow rate of 1.0 mL/min. with a Zorbax SCX column. Curves labeled as in Figure 1.

tion shown in Figure 6. This separation was considered to be less than optimum, as cysteine/glutathione and homocysteine/cystine were not completely resolved. Increasing the mobile phase to pH 3.5 moved all peaks into the void volume.

The ionic strength was decreased in order to modify the separation; Figure 7 details the effects of ionic strength on k'. When the molarities were 75 and 50% of initial values, several

compounds coeluted, as shown. Peak shapes began to rapidly deteriorate after a k' value of about 5, with broadening and severe tailing.

Cation-exchange chromatography was found in these experiments to be inferior to reverse-phase ion-pair chromatography for applications requiring resolution of all of the thiols and disulfides ion our mixture. It could, however, be optimized to provide satisfactory performance for one or two selected compounds.

CONCLUSIONS

In summary, three types of liquid chromatographic separations were evaluated for the LCEC determination of cysteine, cystine, homocysteine, glutathione (reduced and oxidized), and penicillamine (reduced and oxidized): reverse-phase, reverse-phase ion-pairing and strong cation-exchange. Conditions were found which produced satisfactory performance with both reverse-phase ion-pairing and ion-exchange. The reverse-phase ion-pairing mode was determined to be preferable, based on better peak shapes and the high degree of flexibility possible by varying methanol content, ion-pairing reagent concentration, and ionic strength.

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