Chromatography Of Biological Thiols And Disulfides

Modern liquid chromatography ("HPLC" or "LC") is an ideal venue for the separation of polar thiols and disulfides of biological origin. Few scientific papers, however, have dealt with the simultaneous separation of thiols and disulfides from a single injection of sample, this in part being due to the lack of a good disulfide detector.

The development in our laboratory of a dual Hg/Au electrochemical detection cell applicable to the simultaneous detection of thiols and disulfides now provides a relatively simple method to detect disulfides.

Utilizing this ability, BAS staff studied the chromatographic behavior of a few selected thiols (cysteine, glutathione, homocysteine, penicillamine) and disulfides (cystine, glutathione disulfide, penicillamine disulfide) via strong cation-exchange, reverse-phase, and reverse-phase ion-pairing techniques.

Materials And Methods

Both the BAS 200 and BAS 400 liquid chromatographs are appropriate for this work. Any BAS 200 designated by MF-200-X2X11X contains the necessary mobile phase deoxygenation and dual detector hardware. A BAS 400 system must contain 2 BAS LC-4B electronics modules and also be supplemented by an in-house- constructed reflux manifold for deoxygenation.

Conditions

A BAS Biophase ODS 5 µm, column (4.6 x 250 mm, P/N MF-6017) was used for reverse-phase and reverse-phase ion-pairing studies. A DuPont Zorbax 300 SCX column (4.6 x 260 mm) was used for the strong cation-exchange studies. The mobile phase flow rate was either 1.0 or 1.5 mL/min.

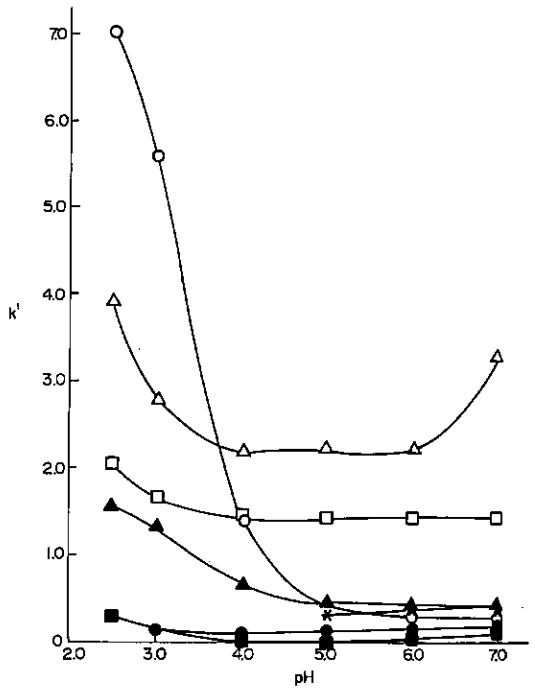

Figure 1. Effect of mobile phase pH on the capacity factors (k') of thiols and disulfides using reverse-phase chromatography. Biophase ODS 5 µm column; mobile phase, 0.1 M phosphate buffer; flow rate, 1.5 mL/min. Cysteine (●), cystine (■), reduced glutathione (▲), homocysteine (✳), oxidized glutathione (○), reduced penicillamine (□), and oxidized penicillamine (Δ).

Mobile Phase: Deionized distilled water is used to prepare all mobile phases. Mobile phases are filtered with 0.2 µm Nylon-66 membranes (Rainin Instrument Co., Inc.). With the BAS 200, mobile phases are refluxed at 35°C and continuously sparged with helium for 10-15 minutes in order to remove dissolved oxygen.

Standard Solutions: Thiols and disulfides for standard solutions are obtained from the following sources: Sigma Chemical Co., L-cysteine, L-cystine, glutathione (reduced), glutathio(oxidized),

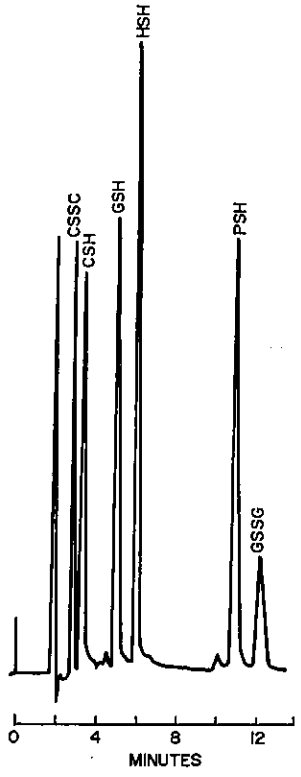

Figure 2. Chromatogram for the reverse-phase ion-pairing separation of the thiol and disulfide mixture under optimum conditions. Biophase ODS 5 µm column; mobile phase: 96% 0.1 M MCAA buffer, pH 3.0, 4% methanol, with 1.0 mM sodium ocytl sulfate; flow rate, 1.5 mL/min.

D-penicillamine; Aldrich Chemical Co., D-penicillamine disulfide; ICN Pharmaceuticals, DL- homocysteine. Sodium octyl sulfate and ethylenediaminetetraacetic acid were purchased from Eastman Kodak Co.

Individual stock solutions are prepared to a 1 mM concentration in deionized, distilled water containing Na_2EDTA at 1 g/L. A 0.1 mM cystine stock solution is prepared in dilute NaOH and acidified with dilute $HClO_4$ just prior to injection. Stock solutions are prepared weekly and stored at 4°C. Standards prepared by dilutions of stock are prepared every two to three days and stored at 4°C.

Detector: Dual Hg/Au surfaces are prepared as described in the BAS literature. Reduction of thiols is carried out at -1.0 V at the upstream electrode. The downstream electrode (+0.15 V) is the only detector monitored, since it contains both thiol and disulfide peak information.

Discussion: methods Of Manipulation

Reverse-Phase Chromatography. The presence of ionizable functional groups, including carboxylic acids, amines, and thiols, makes it possible to alter the retention times by changing the pH of the mobile phase. As the pH of the mobile phase is increased from an acidic pH of 2.5 to pH 7.0, retention times of GSH, GSSG, PSH, and PSSP will decrease accordingly (F1). The decrease in the capacity factor as the pH increases is explained by the fact that at low pH values the carboxylic acids are mostly protonated and thus retain on the hydrophobic column. As the pH increases from 4 to 7, the compounds exist as zwitterions which spend less time in the stationary phase.

Using pH to alter chromatographic behavior of thiols is limited by the fact that none of these small, relatively polar compounds are strongly retained on the reverse-phase column. In fact, cystine and cysteine are not separable from the void volume at any available pH.

Reverse-Phase Ion-Pair Chromatography. The use of reverse- phase ion-pair chromatography for the separation of thiols and disulfides allows the user more flexibility in terms of developing suitable chromatographic conditions for his analysis than does reverse-phase chromatography.

Suppose one uses a reverse-phase column and an acidic mobile phase (pH of 3.0) so that the thiols and disulfides carry a net positive charge. Three different variables can be manipulated to change capacity factors.

The first variable is the concentration of the ion-pairing reagent. As its concentration increases, a substantial increase in retention time is seen for all the compounds. Although the compounds show similar changes in retention time at various ion-pairing concentrations, the retention time of penicillamine disulfide is somewhat more sensitive to ion-pairing con-

centration changes. Cystine and cysteine can be resolved using ion-pairing.

The second variable is the percentage of methanol in the mobile phase. While use of ion-pairing in the mobile phase allows for the resolution of cysteine and cystine, the ion-pairing also causes the rest of the thiols and disulfides to show correspondingly longer retention times. Adding methanol to the ion-pairing system pushes the strongly retained compounds such as PSSP, GSSG, and PSH off the column more quickly while having little effect on the separation of earlier-eluting cystine and cysteine.

The third variable is the ionic strength of the mobile phase. Changes in the molarity of the mobile phase do not have as marked an effect on the retention times of the thiols and disulfides as do ion-pairing or methanol concentration changes. Therefore, changes in the ionic strength of the mobile phase are useful in making small adjustments in the total chromatographic picture.

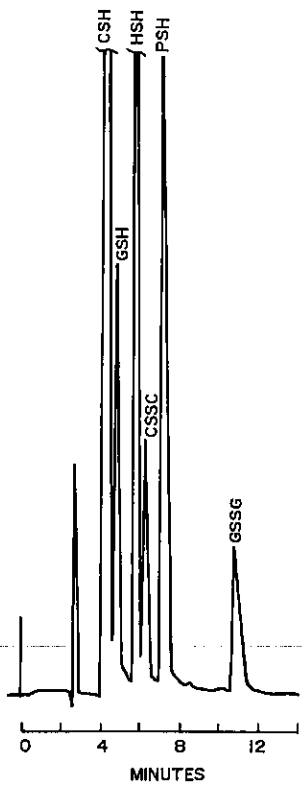

Figure 3. Strong cation-exchange separation of the thiol and disulfide mixture. Zorbax SCX column; mobile phase: 18.4 mM ammonium citrate and 60.7 mM phosphoric acid, pH = 2.45; flow rate 1.0 mL/min.

With careful manipulation of these three variables in reverse-phase ion-pair chromatography, the chromatographic behavior of the thiols and disulfides may be custom-fit to the demands of the particular assay. For the mixture of thiols and disulfides investigated for this report, the optimum chromatographic conditions were found to be 96% 0.1 M MCAA buffer, pH 3.0, 4% methanol, with 1.0 mM sodium octyl sulfate on a Biophase ODS 5 µm column (F2).

Ion-Exchange Chromatography. Much of the chromatography of thiols and disulfides which has been developed to date has utilized cation-exchange. Manipulations of two variables while using this technique effect large changes in the chromatographic behavior of these compounds.

Major changes may be effected in the cation-exchange chromatography of thiols and disulfides by changing the pH of the mobile phase. For example, at pH 2.5 the compounds are separated but not completely resolved (F3); raising the pH to 3.5 causes all the compounds to elute in the void volume.

Changes in the ionic strength of the mobile phase may also be used to alter the chromatographic separation of the thiols and disulfides. As the ionic strength of the mobile phase is decreased, the capacity factors increase. At k' values of approximately 5, peak shape broadens, and the peaks show severe tailing. Thus, while cation exchange chromatography might be successfully developed for a few selected thiols and/or disulfides, it will be more difficult to establish favorable chromatographic conditions for a mixture of many thiols and disulfides.

Related Bas Publications
Thiols

CAPSULE 192. Preparation and Use of Mercury Film Electrode.

CAPSULE 194. Measuring Penicillamine in Plasma and Urine

Disulfides and Thiols

CAPSULE 193. Detection Of Thiols And Disulfides

CAPSULE 235. Oxidized and Reduced Glutathion.

CAPSULE 234. Captopril and Captopril Disulfide in Urine.

CAPSULE 196. Determination of Thiols and Disulfides in Plasma and Urine

Reference

1. *L.A. Allision, and R.E. Shoup, Anal. Chem., 55(1983) 8.*

2. *L.A. Allison, J. Keddington and R.E. Shoup, J. Liq. Chromatogr., 6(1983) 1785.*

3. *L.A. Allison, "Series Dual Hg/Au Electrodes for the Simultaneous Determination of Thiols and Disulfides," Current Separations, Bioanalytical Systems Inc., 4(3)(1982).*