CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

Detection Of Thiols And Disulfides

Thiols are detected in an extremely sensitive and selective manner due to their effect on the redox chemistry of the Hg electrode. The detector reaction involves the oxidation of mercury in the presence of a thiol:

$$2\,RSH + Hg \rightarrow Hg(SR)_2 + 2H^+ + 2e^-$$

This reaction occurs at a very "mild" potential by LCEC standards, resulting in a lack of interfering compounds or noisy baselines.

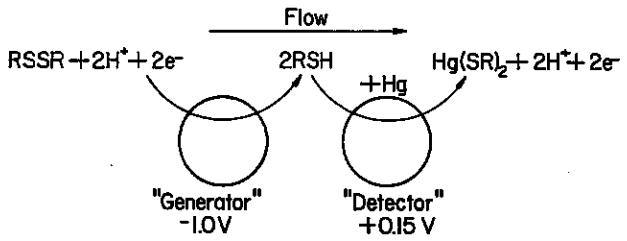

Figure 1. Schematic diagram of dual Hg/Au detector and reactions which occur at each electrode.

Disulfides are easily reduced to thiols but the required negative potentials are too high for the direct monitoring of this reaction. The use of dual Hg/Au electrodes effectively overcomes this problem. Disulfides are reduced to thiols at the upstream electrode, then detected as thiols at the downstream electrode. The upstream electrode is used simply as a "generator" of detectable species; high background current and noise problems are ignored. Only the current at the downstream electrode is monitored to produce the chromatogram, F1.

Assessing A Disulfide

In order for a given disulfide to be successfully detected, the molecule must undergo a facile reduction at the upstream electrode. The efficiency of reduction varies from compound to compound, influenced by such factors as diffusion coefficient,

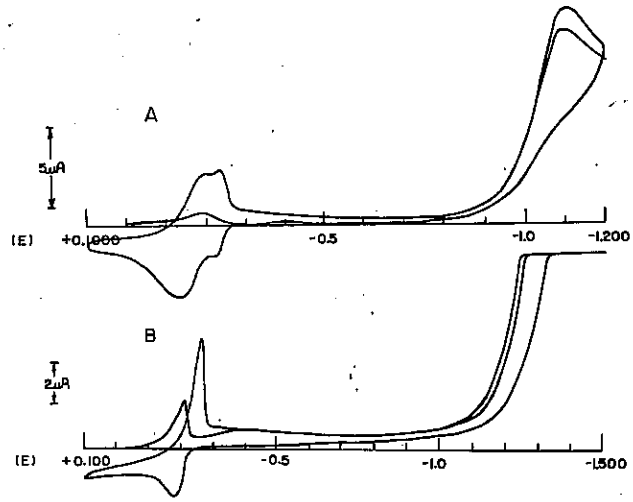

Figure 2. Cyclic voltammograms of A) captopril disulfide (2.6 mM) and B) penicillamine disulfide (2.2 mM). Both compounds are in 50% CH_3CN/50% 0.1 M MCAA, pH 3.0.

reduction potential, and steric shielding of the disulfide functionality. An effective means of approximating a disulfide's performance in this system is cyclic voltammetry. F2 depicts cyclic voltammograms of captopril disulfide and penicillamine disulfide. Captopril disulfide, which is readily detected, shows a reasonably clear diffusion-controlled reduction wave at -1.1 V. This is the reduction that will occur at the upstream electrode in the LCEC system. On the other hand, penicillamine disulfide does not show a reduction wave within the accessible range of potentials, and it is a compound that is difficult to detect. We speculate that steric hindrance is the problem since methyl groups surround the S-S bond in penicillamine disulfide.

LC Hardware

The liquid chromatographic system must thoroughly exclude oxygen from the mobile phase. Deoxygenation of the mobile phase prevents two problems: 1) dissolved oxygen oxidizes thiols on-column, leading to poor reproducibility and 2) reduction of dissolved oxygen causes a high negative background current at both electrodes. This capability is included in all BAS 200 systems. For the BAS 400 series and

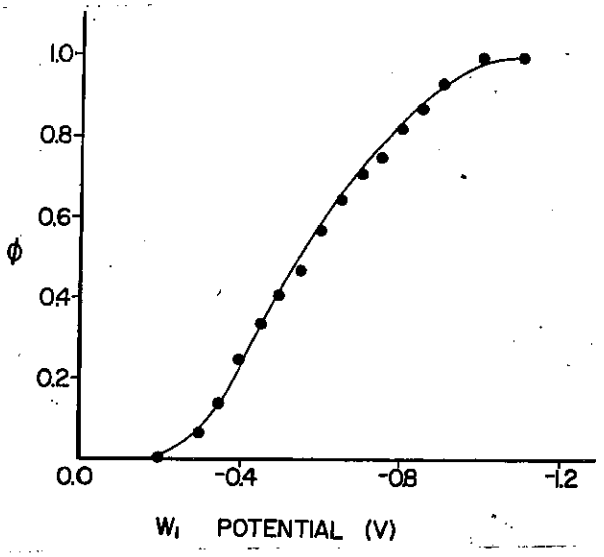

Figure 3. Peak height responses at the downstream electrode (+0.15 V) as a function of applied potential at upstream electrode. Each point represents the mean of two injections, 200 ng of GSSG. ø is defined as the ratio of the peak current to the limiting current.

other LC's, the necessary system modifications are outlined in the BAS manuals. In general, all teflon tubing must be replaced by stainless steel, and the mobile phase is deoxygenated by constant bubbling with nitrogen under reflux conditions at 35 - 50°C.

Either the BAS 200 or two LC-4B controllers are required, with a standard dual connecting cable and one dual gold electrode. The auxiliary electrode must be directly across from the working electrode.

The mercury film is prepared according to the instructions in the BAS 200 Reference Manual or the LC-4B Operations Manual. Briefly, to the clean, dry, polished gold surface is applied a drop of mercury sufficient to cover the entire electrode surface. After 2-3 minutes, the excess mercury is removed with the side of a Pasteur pipette tip or the edge of a computer card into an appropriate waste beaker. Next, smooth the film by polishing it on a dry "kitten-ear" polishing pad as supplied by BAS. The electrode may then be installed using 2 0.005" thick Teflon (MF-1047) gaskets as the spacer.

Following the application of mercury, there is a period of equilibration, during which time the electrode response is rapidly changing and the electrode is unsuitable for quantitative measurements. This stabilization occurs independently of the environment of the electrode and no potential need be applied to achieve it. A purely physical amalgamation process is probably responsible. It is recommended that the surface be allowed to stabilize overnight prior to analytical experiments.

The cell is assembled in the standard manner, taking care that the electrodes are aligned in series relative to the flow of mobile phase. A distinguishing mark on the outside of the block is helpful to permit identification of each electrode. Two 0.005" thick gaskets should be used between the cell halves, to compensate for the additional thickness of the mercury film.

For most applications, potentials of -1.00 V at the upstream electrode and +0.10 to +0.15 V at the downstream electrode are appropriate choices. F3 shows the peak height response for GSSG at the downstream electrode, as a function of applied potential at the upstream electrode. It is necessary to use a potential of -1.0 V at the upstream electrode in order to achieve the maximum response for oxidized glutathione.

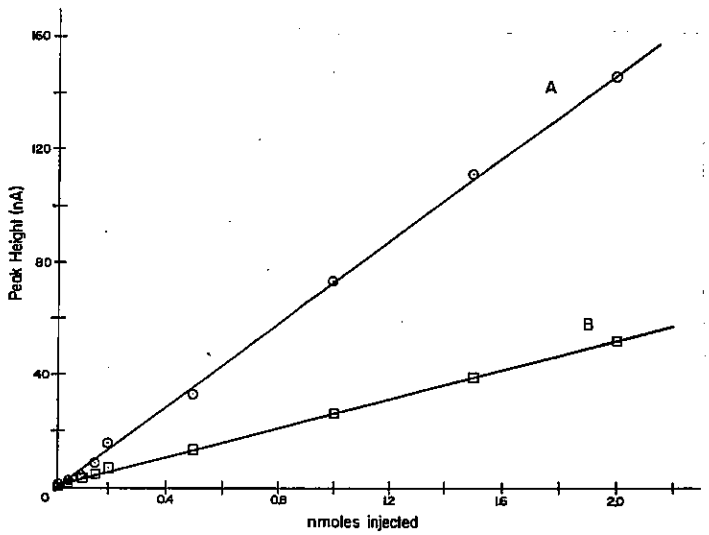

Figure 4. Linealty of downstream electrode response (+0.15 V) for cysteamine (A) and cystamine (B).

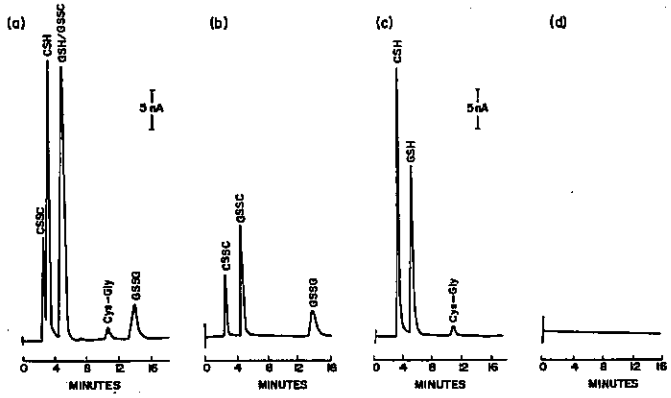

Figure 5. (A) Detection of thiols and disulfides. 2 electrode, no NEM. (B) Disulfides only in same sample, twin electrodes plus NEM. (C) Thiols only in single electrode experiment. Upstream electrode is off. (D) All peaks eliminated single electrode plus NEM.

If you are using discrete LC-4B's the upstream electrode should be connected to the LC-4B designated as W_2, while the downstream should be W1. This permits the upstream electrode to be turned on or off without affecting the operation of the downstream electrode. W_2 may not be used when W_1 is on standby. In a BAS 200 system, the electrodes are independent in operation and the designations may be assigned interchangeably.

Sample Requirements

The precision of detector response for some disulfides is consistently better than that for the corresponding thiols. This phenomenon was determined to be a result of adsorption of thiol onto the upstream mercury surface. It can be effectively eliminated by inclusion of a non-retained thiol within each injection. This thiols "conditions" the upstream mercury surface prior to the peak of interest, resulting in reproducible detector response. This is an important point for obtaining satisfactory results with the dual Hg/Au detector. Provide about 100-200 pmole of an unretained thiol with each injection. Cysteine is ideal if not the analyte of interest. More recently, the extent of adsorption of thiols has been lessened by other mobile phase choices. For a customer interested only in cysteine and cysteine in protein hydrolysates, a pH 2.2 sodium phosphate, mobile phase did not exhibit adsorption problems.

Hg/Au Lifetimes

Often the upstream electrode requires resurfacing before the downstream. This may be evidenced by a falling off in response for disulfides vs. thiols.

Our current recommendation with respect to resurfacing is to simply rinse the old surface with methanol, let it air-dry, and then apply new mercury to it. The excess mercury is removed by polishing on "kitten-ear" cloth as before.

Usually, upon startup of the system with deoxygenated mobile phase, the dual Hg/Au system will require approximately one hour of "warm-up" time.

Linearity

In F4, the linearity of detector response for cysteamine and cystamine is shown over the range of 20 to 2000 pmoles injected. Detection limits (S/N of 3) are usually about 2-5 pmoles thiol injected and 5-10 pmoles of disulfide injected.

Thiol vs. Disulfide

The dual Hg/Au detector offers you a qualitative handle on the chemical structure of the compounds being detected. Reasonable deductions may be made based on both instrumental and chemical experiments. For example, in a mixture of thiols and disulfides (see F5A), all four compounds are detected with both electrodes on. Turning off the upstream electrode and repeating the injection produces a thiols-only chromatogram (F5C).

Chemical manipulations selective for masking thiols can conversely show only the disulfides. Both N-ethylmaleimide (NEM) or iodoacetamide scavenge thiols rapidly. In F5B, reaction of the same mixture with NEM was obviously effective. The combination of both chemical masking of the thiols and instumentatal deactivation of the disulfide reduction step prevented *any* peaks from being seen (see F5D).

References

1. L.A. Allison 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. R. Eggli and R. Asper, Anal. Chim. Acta. 101(1978), 253.

4. L.A. Allison, Current Separations, 4(1982), 38.

Related BAS Publications
Thiols

CAPSULE 192. Preparation and Use of Mercury Film Electrodes.

CAPSULE 194. Measuring Penicillamine in Plasma and Urine.

Disulfides and Thiols

CAPSULE 235. Oxidized and Reduced Glutathione.

CAPSULE 234. Captopril and Captopril Disulfide in Plasma and Urine.

CAPSULE 196. Determination of Thiols and Disulfides in Urine.