CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

Oxidized And Reduced Glutathione

Purpose

Reduced glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the major non-protein thiol found in living tissue. Levels of reduced and oxidized glutathione, as well as the ratio between the two, are of considerable interest to biochemists and cell physiologists. These compounds are studied to understand their roles in many cellular processes, such as adaptation to freezing stress in plants, or protection from toxic compounds in animal cells.

Existing Methods

Determination of the disulfides is a more difficult analytical problem than for the thiol, and a variety of approaches have been suggested. In general, these methods involve an initial reaction that reduces the disulfide to a thiol; measurements of thiol levels before and after this reduction yield the disulfide by difference.

LCEC Method

Measurement of reduced glutathione levels by liquid chromatography/electrochemistry (LCEC) was first introduced by Rabenstein and Saetre(1) using a mercury pool electrode of their own design.

At BAS, we offer a dual Hg/Au electrode thin-layer cell that allows detection of both disulfides (GSSG) and thiols (GSH) in a single chromatographic tracing(2,3). The two mercury/gold electrodes are utilized in a series arrangement, with reduction of disulfide to thiol at the upstream electrode, followed by conventional thiol detection downstream(4). This Note describes the application of the dual detector to the determination of GSH and GSSG in human whole blood and in citrus leaf homogenate.

Conditions

System: A BAS 200 liquid chromatograph is ideal for the assay, since it contains the built-in deoxygenation utilities required for dual Hg/Au electrode operation. Otherwise, if O_2 remains in the mobile phase, background currents become prohibitively high and preclude low detection limits and long electrode lifetimes. The 200 is unique in that no gas-permeable (plastic) tubing is used up to the detector; hence it is easy to maintain deoxygenated conditions.

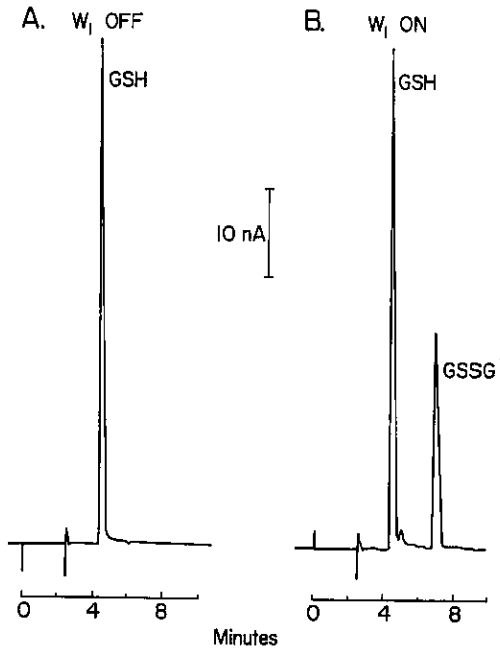

Figure 1. Response of downstream electrode (+0.15 V) to a standard solution containing GSH and GSSG with (A) upstream electrode OFF and (B) upstream electrode ON (-1.0 V); mobile phase, 99% 0.1 M monochloroacetate, pH 3.0/1% methanol; column Biophase ODS, 5 µm.

A BAS 400 system may be adapted to deoxygenated operation by adding user-supplied glassware with mild heating and sparging. Construction details are provided in the BAS manuals(5).

Column: Biophase ODS 5µm (BAS P/N MF6017), 250 x 4.6 mm

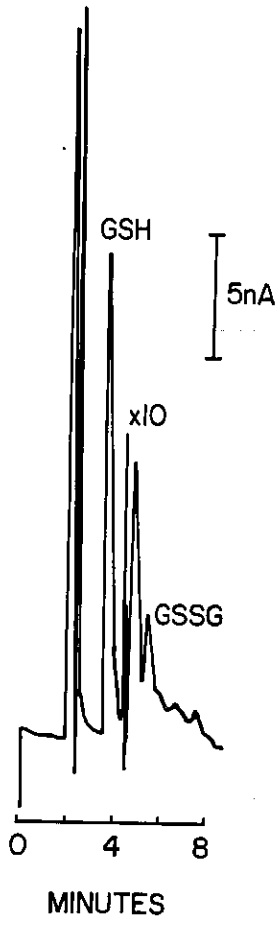

Figure 2. Downstream electrode chromatogram of whole blood filtrate, with upstream electrode ON (-1.0 V). Chromatographic conditions given in F1.

Flow Rate: 1.3 - 1.5 mL/min
Mobile Phase: 0 - 1% methanol, 100 - 99% 0.10 M monochloroacetate (pH 3.0)
Temperature: ambient
Detector Conditions: Upstream Hg/Au electrode: -1.0 V vs. Ag/AgCl, 5 - 10 µA f.s. Downstream Hg/Au electrode: +0.15 V, 5 - 200 nA f.s., as necessary depending on sample.

Standards

Standard solutions containing either or both reduced and oxidized glutathione were prepared in 0.1% (w/v) Na_2EDTA solution and refrigerated. As a guide, dissolve 20 mg glutathione and 40 mg glutathione disulfide in 100 mL of solution. Resulting GSH and GSSG concentrations are 0.67 mM (0.20 µg/µL). Refrigerate. Note: Subsequent to these experiments, it was found that precision of thiol response on the dual Hg/Au detector was improved by adding a non-retained thiol to each standard and sample. Cysteine is a suitable thiol for the GSH/GSSG assay. The added concentration should be calculated based on the volume of injection; approximately 100 pmole of cysteine should be injected each time for optimum performance.

Internal Standard

If desired, weigh 10 mg of mercaptosuccinic acid (Aldrich). Dissolve into 500 mL of Na_2EDTA solution (0.1% w/v, in H_2O).

Whole Blood Procedure

Blood samples were prepared as follows: 0.8 mL of 0.1% Na_2EDTA solution and 20 µL of fresh blood were combined in a 5 mL glass centrifuge tube, causing the erythrocytes to lyse. Add 20 µL of the 0.67 mM stock solution to one tube to act as a standard. Two hundred microliters of 0.2 M $HClO_4$ was then added and the tube vortexed briefly. After standing 10 minutes to precipitate proteins, the sample was centrifuged 10 minutes at 1600 x g, and the supernatant was filtered through a BAS Microfilter with 0.2 µm RC-58 membranes prior to injection.

Plant Procedure

5g of citrus leaf tissue were chopped and homogenized in 75 mL of methanol. The homogenate was filtered and extracted with petroleum ether to remove chlorophyll. Residual petroleum ether was removed by vacuum aspiration. For LCEC experiments, the homogenate was then evaporated to dryness under nitrogen and reconstituted in an equal volume of mobile phase.

Results

F1 shows chromatograms of a mixture of GSH and GSSG with A) the upstream electrode turned OFF and B) the upstream electrode turned ON. Only GSH is observed when the upstream or "generator" electrode is not on; both GSH and GSSG are observed when both electrodes are on. Each compound is being detected as GSH, but they are presented to the detector resolved in time and are thus detected separately.

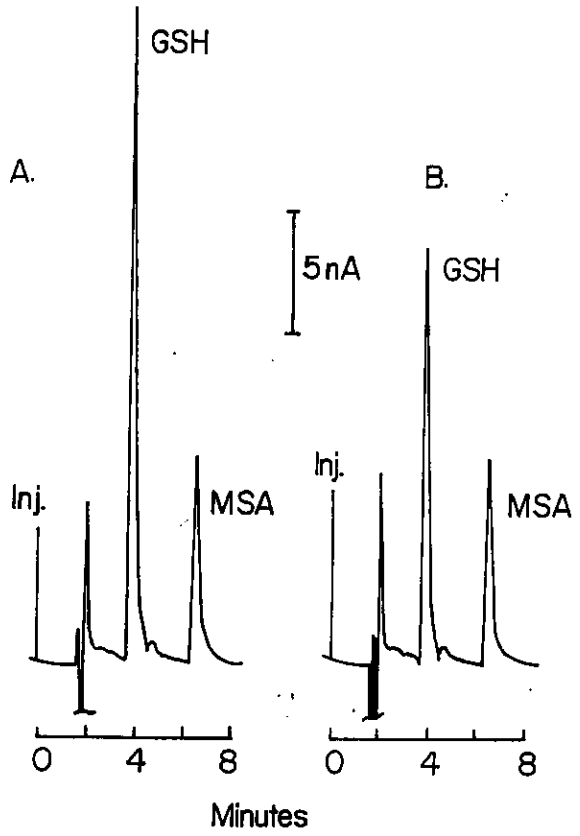

Figure 3. LCEC chromatograms from (A) whole blood (0.315 µg/µL glutathione) and (B) standard (0.2 µg/µL glutathione). Both samples were prepared according to text.

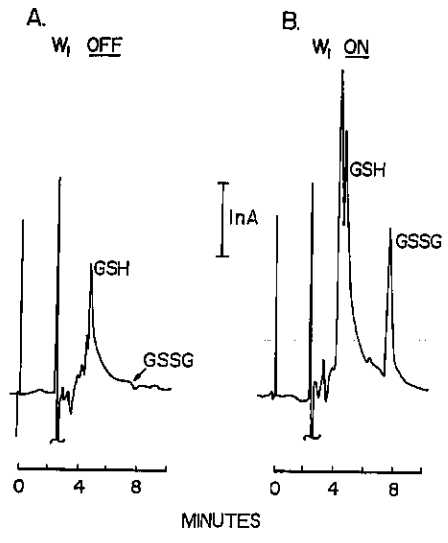

Figure 4. Downstream electrode chromatograms of citrus leaf homogenate with (A) upstream electrode OFF and (B) upstream electrode ON (-1.0 V). Chromatographic conditions as given in F1.

Linearity of the dual mercury/gold detector was demonstrated for GSH over the range of 10-800 pmole injected (slope, 108 pA/pmole; y-intercept, -2.0 pA; correlation coefficient, 0.9994). GSSG also showed a linear response from 10 to 800 pmole (slope, 38 pA/pmole; y-intercept, -0.45 pA; correlation coefficient, 0.9990). Minimum detectable quantities (S/N of 3) were 3.5 pmole for GSH and 5.7 pmole for GSSG.

The chromatogram shown in F2 represents a whole blood filtrate containing 1.1 mM GSH and 1.8 µM GSSG. The sample workup included a dilution of approximately 50-fold prior to injection, as GSH levels were known to be quite high. For better determination of GSSG, it would be preferable to decrease the dilution volume, producing a more concentrated sample. The baseline disturbance at 4.7 minutes occurs in every blood sample; its retention time may be manipulated by mobile phase variations. No internal standard was added.

The single electrode chromatogram in F3 (+0.15 V only) shows the effect of "turning off" disulfide generation upstream and insertion of the mercaptosuccinic acid internal standard.

Measurements of GSH and GSSG levels in plants by current methodology requires considerable analysis time and effort, involving ion-exchange steps and separate enzymatic methods for each compound of interest(7). The dual detector is well- suited to this application; reconstituted homogenates can be directly injected, F4. GSH and GSSG in the homogenate were calculated at 6.5 µM and 1.8 µM, respectively. There is evidence of at least one disulfide in addition to GSSG, since the peak eluting prior to GSH (F4B) is not observed with the upstream electrode turned OFF (F4A). The quality of analytical data provided by this approach is much higher than that obtained with the more lengthy procedures.

Comments

In any experiment involving determination of thiols and disulfides, care must be taken to ensure the stability of thiols under the sampling conditions used. In the case of whole blood, the addition of Na_2EDTA has been shown to prevent thiols from oxidizing to

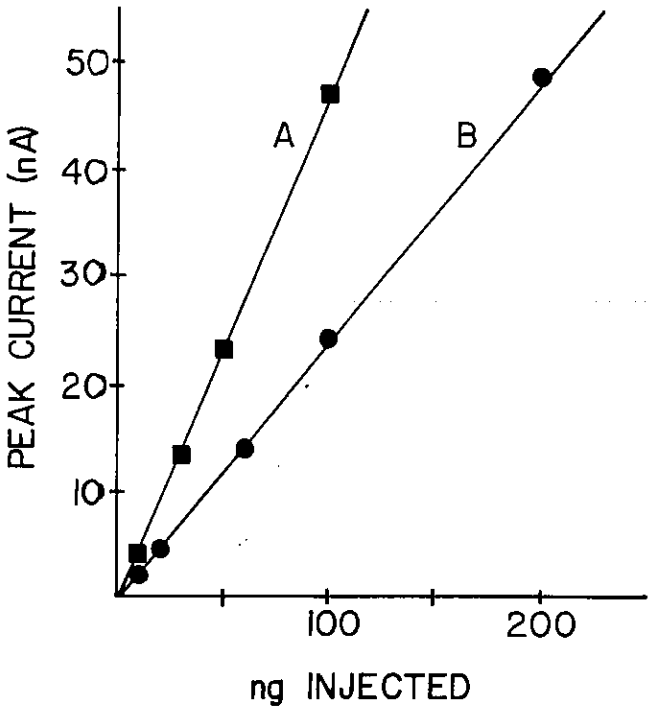

Figure 5. Linearity of LCEC for (A) mercaptosuccinic acid and (B) glutathione.

GSSG(1). With the dual detector, it is very easy to monitor stability under a given set of experimental conditions, as both the thiol and any resulting disulfides can be measured.

The linearity of the LCEC system for glutathione and mercaptosuccinic acid is demonstrated in F5. The sensitivity of electrochemical detection requires that the blood samples be diluted before injection. In this protocol, the dilution was approximately fifty-fold. Lower levels of glutathione could easily be quantitated by decreasing the amount of dilution.

References

1. D.L. Rabenstein and R. Saetre, Anal. Chem. 49(1977) 1036.

2. CAPSULE 192. Optimizing LCEC Surface Chemistry with the Hg/Au Electrode.

3. LCEC Application Note No. 53. Detection of Thiols and Disulfides with Series Dual Hg/Au Electrodes.

4. L.A. Allison and R.E. Shoup, Anal. Chem. 55(1983) 8.

5. LC-4A or 4B Manual. Bioanalytical Systems Inc., Sections 5 and 6.

6. C.L. Guy and J.V. Carter in "Plant Cold Hardiness and Freezing Stress," Li, P.H. and Sakai, A., eds., Academic Press; New York, 1982.