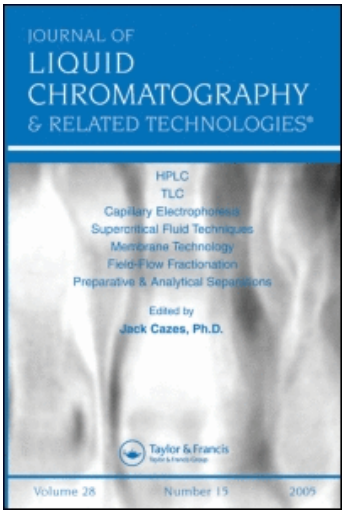

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Liquid Chromatographic Determination of Glutathione with Electrochemically Pretreated Glassy Carbon Electrode

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LIQUID CHROMATOGRAPHIC DETERMINATION OF GLUTATHIONE WITH ELECTROCHEMICALLY PRETREATED GLASSY CARBON ELECTRODE

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ABSTRACT

Simple electrochemical pretreatment of a glassy carbon electrode used as a working electrode in an electrochemical detector has been found to enhance the analytical capability of the detector for the determination of glutathione by lowering the required operating potential as well as by increasing the maximum oxidation current. The electrochemical pretreatment of the electrode was made in a 0.2 M phosphate buffer (KH_2PO_4-KOH, pH 6.5) at +1.9 V *vs.* Ag/AgCl for 2 min. The minimum detectable quantity of glutathione was found to be about 100 pg, when eluate from a reversed-phase column was amperometrically monitored by aid of the detector with the electrochemically pretreated glassy carbon electrode. Amount of glutathione in a rat lens was determined by the chromatographic method developed in this study.

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INTRODUCTION

Progress in almost any area of biochemistry depends importantly on reliable methods. Recent advances in electrochemical detectors for high-performance liquid chromatography (HPLC) make them logical choice for substances easily oxidizable (1). Glutathione (GSH), which is a compound of biological and clinical interest (2), is also easily oxidizable. HPLC coupled with electrochemical detection (ED) was first proposed for thiols by Rabenstein and Saetre (3), who used a mercury pool electrode. Allison and Shoup (4) determined thiols and disulfides by using HPLC-ED with amalgamed gold electrode. Mefford and Adams (5) developed a method for the determination of GSH in mammalian tissues by HPLC-ED with graphite rather than mercury or its amalgamed gold for the working electrode and reported that the minimum detectable quantity of GSH was about 5×10^{-12} moles under their chromatographic conditions. Mefford and Adams (5) used a 0.02 M H_3PO_4 (pH 2.1) as a mobile phase. Recently, we have found (6) that electrochemical pretreatment of glassy carbon (GC) electrodes in phosphate buffer solutions tends to enhance their activity for oxidation of electroactive biomaterials such as methionine, tyrosine, uric acid, and GSH. On the basis of our finding, we aimed to add a method for the determination of GSH in biological samples by HPLC-ED with electrochemically pretreated GC electrode.

In this article, we have developed a method for the determination of GSH by HPLC-ED. In the electrochemical detector, the GC electrode which was polished and then electrochemically pretreated according to the procedure recently optimized (6) was used as a working electrode. Feasibility of the HPLC-ED method developed in this study is demonstrated by showing a chromatogram

obtained by injecting an aliquot of simply deproteinized rat lens-extract onto a reversed-phase column.

MATERIALS AND METHODS

All the experiments were conducted at 25 ± 1°C, unless otherwise stated.

Chemicals in preparing buffer solutions were of analytical reagent grade from Wako Pure Chemicals, Osaka, Japan. Any other chemicals used in this study were the same ones as used in our previous reports (7). All buffers and aqueous solutions were prepared with glass-distilled deionized water. A portion (10.0) mg) of GSH was dissolved in 10 ml of a 0.2 M KCl solution. The GSH solution was placed at -80°C and appropriate dilution of the stock solution was done with a 0.2 M KCl solution just before use.

A constant flow pump (Model 655, Hitachi, Tokyo, Japan) was employed throughout this study. Eluate from a 250- x 4.6-mm i.d. stainless steel tube packed with Fine SIL C_{18} (particle size, 5 μm; Jasco, Tokyo, Japan) was amperometrically monitored by aid of an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan). In the electrochemical detector, a GC electrode (GC-20, Tokai Carbon Co., Ltd., Tokyo, Japan) was used as a working electrode and electrochemical potential was always positive at the GC electrode (oxidation mode) relative to the Ag/AgCl reference electrode. The GC electrode was short length of 5-mm diameter rod. A stainless wire was served as an auxiliary electrode.

It has been recognized (8, 9) that somehow pretreatment of GC electrodes is necessary to observe their reproducible and well-defined electrochemical behavior. Therefore, the GC electrodes used in this study were polished according to the procedure of

Engstrom (8) with some minor modifications. Finally, the surface of the GC electrodes was polished to mirror-like on 0.3 μm alumina. The electrodes thus polished were then thoroughly sonicated in the glass-distilled deionized water for 10 min to eliminate such possibility of alumina catalysis as reported by Zak and Kuwana (10). The polished electrode was inserted in tight-fitting polychlorotrifluoroethylene (diflon) tube. The surface of the GC electrode inserted in the diflon tube was electrochemically further treated by using a system shown in Fig. 1 accorting to the procedure recently optimized (6). Electrochemical pretreatment of the polished GC electrodes was performed with a potentiostat (Model HA-301, Hokuto Denko Ltd., Tokyo, Japan) as follows. Each polished GC electrode was anodized in a 0.2 M phosphate buffer (KH_2PO_4-KOH, pH 6.5) at +1.9 V *vs.* Ag/AgCl for 2 min. When the detector sensitivity was lowered, the GC electrode was re-polished and electrochemically re-treated as described above. We have found (6) that the effectiveness of the electrochemical treatment is dependent upon the electrochemical species undergoing electrolysis as well as upon the electrochemical conditions. The mobile phase used in this study was a mixed solution (0.2 M KCl : 0.2 N HCl : H_2O = 50 : 10.6 : 139. 4, volume ratio), whose pH was 2.0. The value for pH of the mobile phase was adjusted to 2.0 by adding an appropriate volume of H_2O into a mixed solution (0.2 M KCl : 0.2 N HCl = 50 : 10.6, volume ratio), when necessary. In this study, use of a phosphate buffer system as a mobile phase was abandoned due to the fact (6) that the excessive deposition of phosphorous ions on the electrode surface during the course of electrolysis in an electrolyte solution containing phosphorous ions tends to decrease the electrode response for GSH oxidation. In order to eliminate this possible complication,

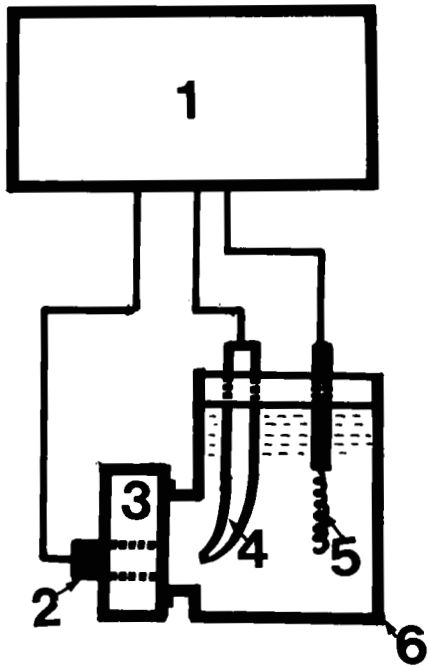

Figure 1. Schematic diagram of the electrochemical treatment: 1, potentiostat; 2, glassy carbon electrode; 3, diflon tube; 4, Ag/AgCl electrode; 5, platimum wire electrode; 6, electrochemical cell (volume, 50 ml). The glassy carbon electrodes inserted into the diflon tube was contacted with an electrolyte solution as shown in this figure.

the mobile phase without phosphorous ions was used in this study. The flow rate was 1.0 ml/min. The column temperature was 25 ± 1°C.

Male Sprauge-Dawley rats (24 weeks of age) were decapitated. Immediately after the decapitation, their respective whole lens tissues were removed and placed on dry ice. Each lens tissue was then stored at -80°C just before analysis. Each whole lens (0.045 ± 0.005 g in wet weight) was then homogenized at 4°C for 2 min and

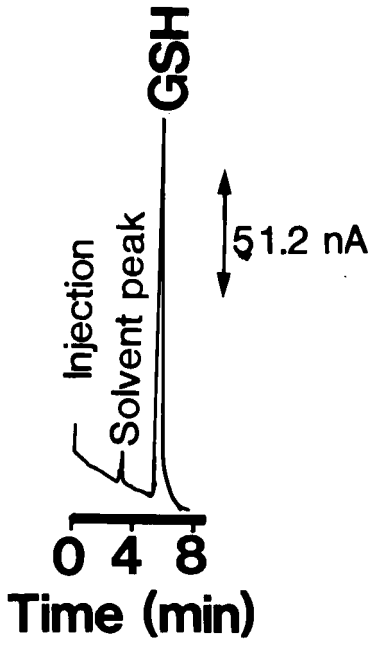

Figure 2. A chromatogram obtained by injecting 50 ng of GSH onto the reversed-phase column. Eluate from the column was amperometrically monitored by aid of the electrochemical detector with the electrochemically pretreated glassy carbon electrode set at +1.1 V vs. Ag/AgCl.

the homogenate was centrifuged at 3,000 g for 10 min at 4°C. The supernatant was filtrated through a 0.45 μm membrane filter and an aliquot (1 μl) of the filtrate was injected onto the column.

Tentative identification of GSH was performed on the basis of retention behavior and co-chromatography with the reference compound.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram obtained by injecting 50 ng of GSH onto the column. Eluate from the column was

amperometrically monitored by aid of the electrochemical detector set at +1.1 V vs. Ag/AgCl. Any other chromatographic conditions were the same ones as described in the experimental section. If the electrochemically pretreated GC electrodes are to be employed in practical chromatographic applications, it is necessary that the detector response for GSH oxidation is sufficiently long-lived to provide reproducible current levels over at least a full day's continuous usage. Immediately after the electrochemical pretreatment of the polished GC electrode as a starting material, its response for GSH was examined as a function of time after the pretreatment. Fig. 3 shows change of the oxidation current levels obtained by repeated injections of 100 ng of GSH as a function of the time. As seen in Fig. 3, the electrochemically pretreated GC electrode response for providing the reproducible current generation of GSH seems to be stabilized within 10 min after the beginning of analysis (electrolysis). In addition, we performed an experiment in the mobile phase alone, without the injection of GSH. When 100 ng of GSH was injected onto the column after the electrolysis in the mobile phase alone for 10 min, under the present chromatographic conditions, the observed current level for GSH oxidation was similar to that obtained for 10 min after the start of analysis shown in Fig. 3. Therefore, analysis of GSH by the HPLC-ED method has been started after the stabilization of the electrochemically pretreated GC electrode in the mobile phase at 1.1 V vs. Ag/AgCl at least for 10 min, when the electrochemically pretreated GC electrode is used for the first time. The activity of the electrochemically pretreated GC electrodes for HPLC-ED has been found to be stable at least 300-time injections of simple GSH.

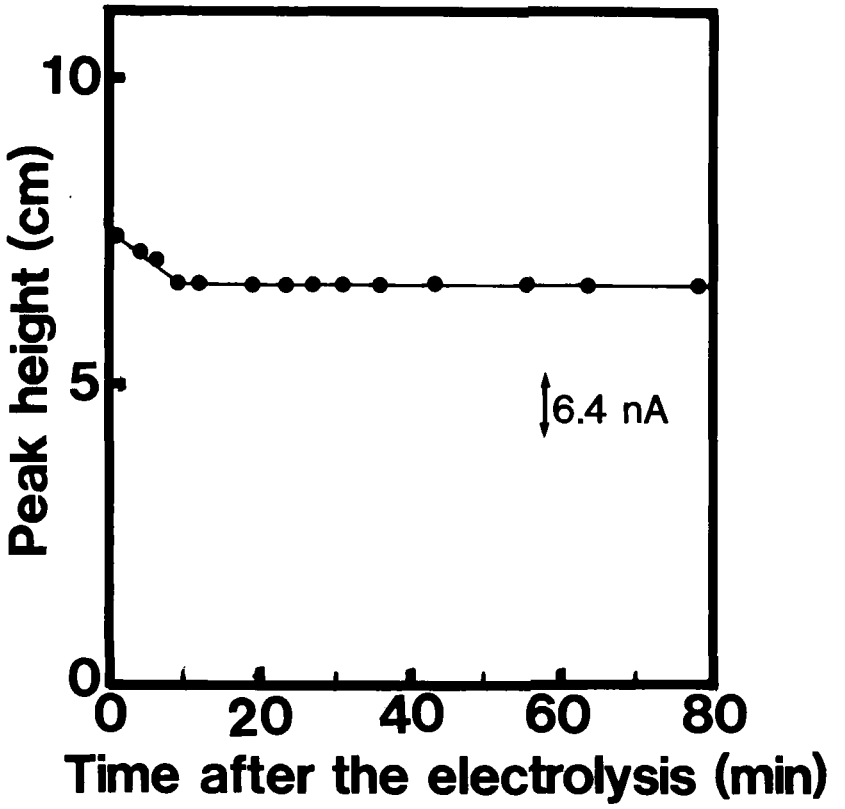

Figure 3. Change of the electrochemically pretreated glassy carbon electrode response for GSH as a function of time after the start of electrolysis for the first time. As seen herein, the electrode response for GSH analysis can be stabilized in the mobile phase for 10 min after the first analytical use.

Fig. 4 (a) illustrates a hydrodynamic voltammogram obtained for GSH in the present chromatographic system by repeated injections of 100 ng of GSH at different electrochemical detector potentials. As seen in Fig. 4 (a), the maximum detector response for GSH analysis was obtained at 1.1 V vs. Ag/AgCl. Therefore, the electrochemical detector was set at 1.1 V vs. Ag/AgCl in Figs.

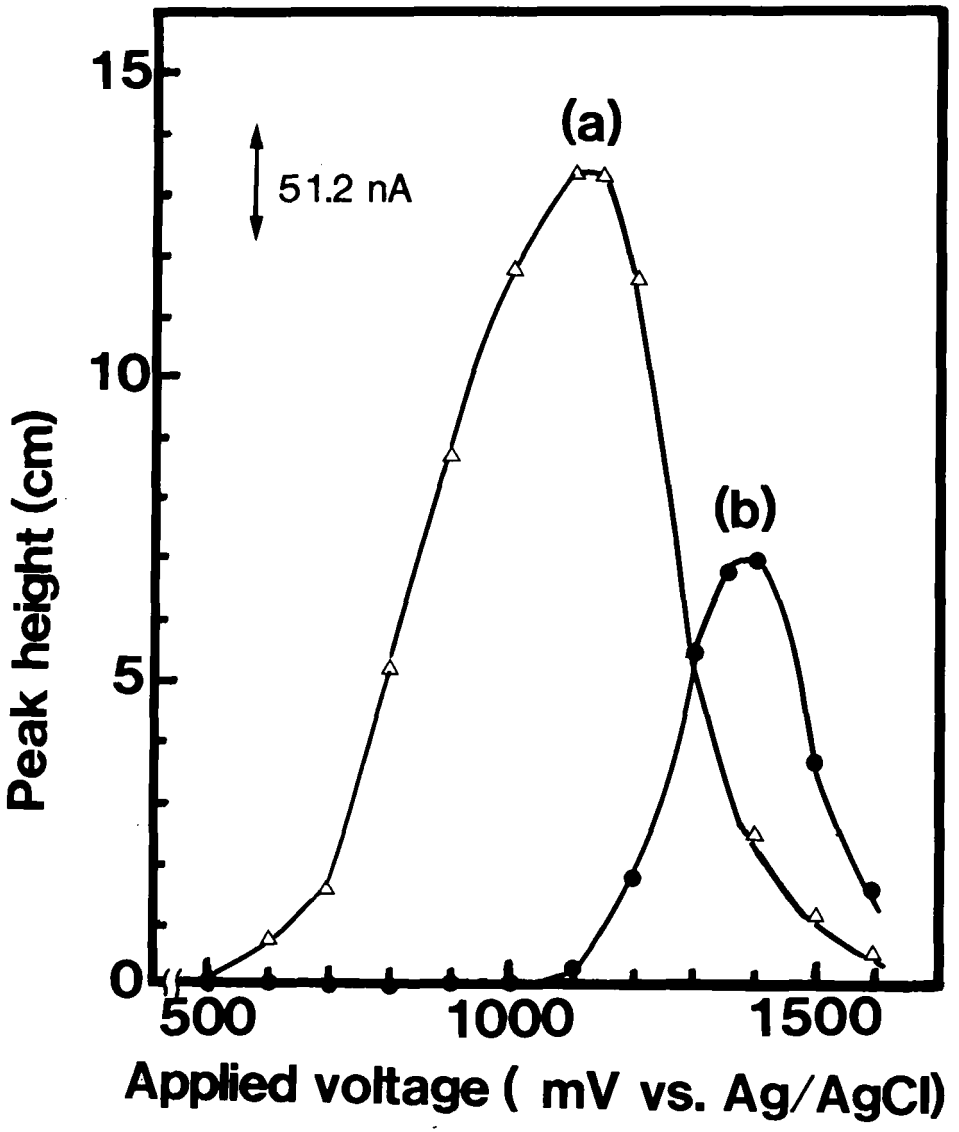

Figure 4. Hydrodynamic voltammograms obtained for simple GSH in the present chromatographic system by repeated injections of 100 ng of GSH at different electrochemical detector potentials. Eluate from the column was electrochemically monitored by aid of the electrochemical detector (a) with the electrochemically pre-treated glassy carbon electrode and (b) the polished but electrochemically untreated one.

2 and 3. As a reference, eluate from the column was also monitored by aid of the electrochemical detector with the polished but electrochemically untreated GC electrode and a hydrodynamic voltammogram was obtained for GSH according to the same procedure as used in Fig. 4 (a)(see Fig. 4 (b)). Comparison of the hydrodynamic voltammorgams (a) and (b) in Fig. 4 has revealed that the electrochemical pretreatment of the polished GC electrodes introduces the lowering of the oxidation potential required for detection of GSH as well as the enhancement of the detector sensitivity for GSH analysis. Fig. 5 represents the typical standard curve for high-performance liquid chromatographic determination of GSH under the same chromatographic conditions as used in Figs. 2 and 3. The minimum detectable quantity is about 100 pg for GSH. The usual detector response has been found to be linear over a range (0.1-500 ng). A non-linear response tends to occur, when more than 500 ng of GSH was injected.

As an example, Fig. 6 shows a chromatogram obtained by injecting an aliquot (1 μl) of the simply deproteinized rat lens-extract onto the column under the same chromatographic conditions as used in Figs. 2 and 3. As seen herein, a GSH peak was observed as a distinguished one. The amount of GSH in the rat lens was estimated to be 1,199 ng/mg tissue. The activity of the electrochemically pretreated GC electrodes for HPLC-ED has been found to be stable at least for 100-time injections of the simply deproteinized rat lens-extracts. GSH standard solutions added to the lens-extract diluted with an appropriate volume of the mobile phase were analyzed with good precision at concentrations comparable to those in various biological samples. The recoveries of GSH in different standard solutions added to twenty different rat lens-extract preparations prepared on four different

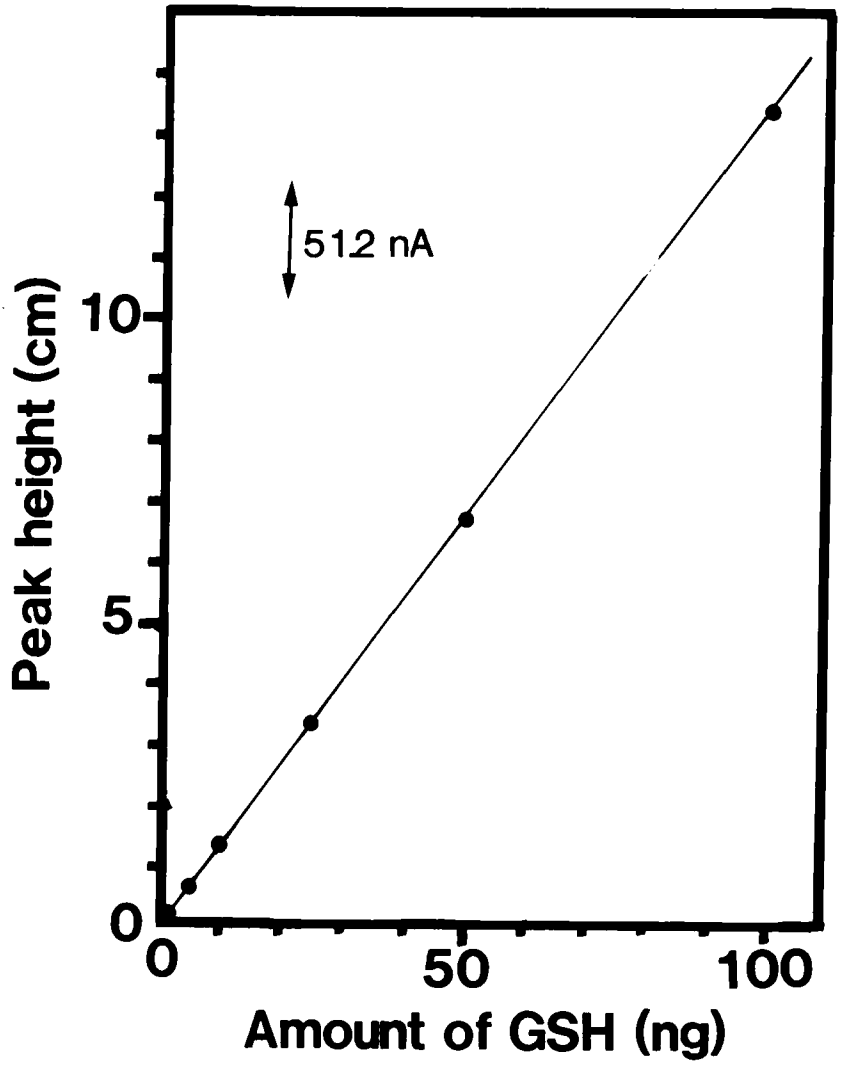

Figure 5. A calibration curve for GSH under the present chromatographic conditions. The calibration graph was daily obtained before and after the chromatographic determination of GSH. Triplicate injections gave standard deviation of peak height and retention times of 0.5 and 1%, respectively.

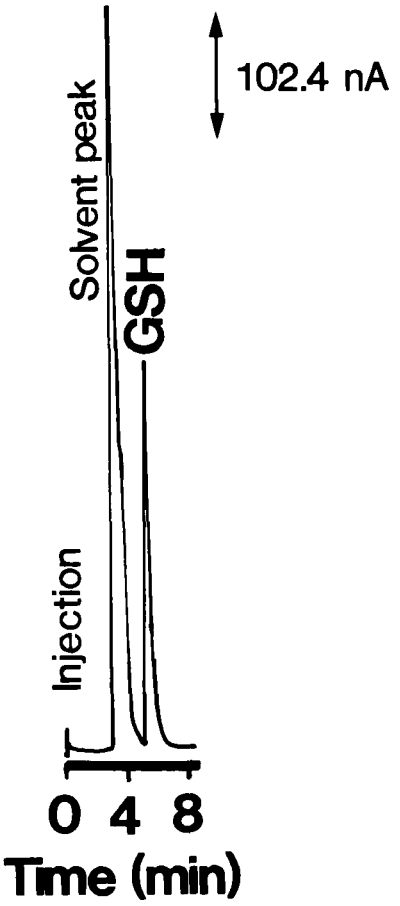

Figure 6. A typical chromatogram obtained by injecting an aliquot (1 µl) of the simply deproteinized rat lens-extract onto the column under the same chromatographic conditions as used in Figs. 2 and 3.

days were found to be 98.5 ± 1% under the present analytical conditions. The electrochemical detector with the electrochemically pretreated GC electrode, whose switch was off for three days, was found to show the same activity for GSH analysis as obtained three days before.

In 1962, Yamada and Sato (11) published the first paper on GC. GC electrodes exhibit low background currents over a wide range of potentials and are useful for numerous electrochemical applications, as summarized by Gross and Jordan (12). However, electron transfer rates observed for redox processes at these surfaces are often slower than those at surfaces of metal electrodes such as mercury and gold. As a result, for example, GC electrodes often exhibit substantial overpotentials which cause the related oxidants and reductants to take place at potentials significantly in excess of their thermodynamic potentials. For these compounds, detection by a GC electrode cannot provide optimum levles of sensitivity and selectivity and, in extreme cases, can provide not useful quantitation at all. Recently, Henderson and Griffin (13) have proposed a method for the determination of adenosine and other purine metabolites by HPLC-ED with GC. In their study (13), however, they were unable to exceed +1.65 V vs. Ag/AgCl under their chromatographic conditions because of excessive background current generation (greater than 1 μA). We also met the similar problem at the first step of this study. As examplified elsewhere (8, 9), electrochemical pretreatment procedures seem to cause the GC electrode to efficiently and reproducibly catalyze electrochemical reactions and to be relatively convenient to perform. Ravichandran and Baldwin (9) have found enhancement of the electrochemical detector response for NADH, ascorbic acid, and hydrazine by use of electrochemically pretreated GC electrode.

As demonstrated in this study, the electrochemical pretreatment procedure is also relatively simple and rapid. Nevertheless, the electrochemically pretreated GC electrdoe response for GSH oxidation can be increased as compared with the electrochemically untreated one. Pretreatment procedures have been sought that would cause the electrode to efficiently and reproducibly catalize electrochemical reactions and that would be relatively convenient to perform. As described by Ravichandran and Baldwin (9), however, the potential of electrochemical pretreatment procedures to improve the electrochemical detector response has not been seriously considered. It would appear certain that the important need in HPLC-ED is the development of the procedures for electrochemical treatment of GC electrodes also seems to be required for progress in HPLC-ED.

As described above, we have developed a method for the determination of GSH in biological samples by HPLC-ED with the electrochemically pretreated GC electrode demonstrating the determination of the compound in rat lens tissues. Sample treatment prior to chromatography is minimal, required only homogenization, followed by centrifugation. We are now aiming to elucidate the mechanism for enhancing the GSH oxidation rate at the electrochemically pretreated GC electrode.

REFERENCES

1. Kissinger, P.T., "Electrochemical detection in liquid chromatography and flow injection analysis". In: Laboratory Techniques in Electroanalytical Chemistry, Ed. by P.T. Kissinger and W.R. Heineman. New York and Basel. Marcel Dekker, Inc., 1984, pp. 611-635.

2. Meister, A. and Anderson, M.E., "Glutathione", Ann. Rev. Biochem., 52, 711-760, 1983.

3. Rabenstein, D.L. and Saetre, R., "Mercury-based electrochemical detector for liquid chromatography for the detection of glutathione and other sulfur-containing compounds", Anal. Chem., 49, 1036-1039, 1977.

4. Allison, L.A. and Shoup, R.E., "Dual electrode liquid chromatography detector for thiols and disulfides", Anal. Chem., 55, 8-12, 1983.

5. Mefford, I. and Adams, R.N., "Determination of reduced glutathione in guinea pig and rat tissue by HPLC with electrochemical detection", Life Sci., 23, 1167-1174, 1978.

6. Iwamoto, T., Yoshiura, M., and Iriyama, K., to be published elsewhere with any other related experimental results.

7. Iwamoto, T., Yoshiura, M., Iriyama, K., Tomizawa, N., Kurihara, S., Lee, T., and Suzuki, N., "Identification of glutathione in a reversed-phase chromatogram of a lens", Jikeikai Med. J., 32, 245-249, 1985.

8. Engstrom, R.C., "Electrochemical pretreatment of glassy carbon electrodes", Anal. Chem., 54, 2310-2314, 1982.

9. Ravichandran, K. and Baldwin, R.P., "Enhancement of LCEC response by use of electrochemically pretreated glassy carbon electrdoes", J. Liq. Chromatogr., 7, 2031-2050, 1984.

10. Zak, J., and Kuwana, T., "Electrooxidative catalysis using dispersed alumina on glassy carbon surfaces", J. Amer. Chem. Soc., 104, 5514-5515, 1982.

11. Yamada, S. and Sato, H., "Some physical properties of glassy carbon", Nature, 193, 261-262, 1962.

12. Gross, M. and Jordan, J., "Voltammetry at glassy carbon electrodes", Pure & Appl. Chem., 56, 1095-1129, 1984.

13. Henderson, Jr., R.J. and Griffin, C.A., "Electrochemical detection of adenosine and other purine metabolites during high-performance liquid chromatographic analysis", J. Chromatogr., 298, 231-242, 1984.