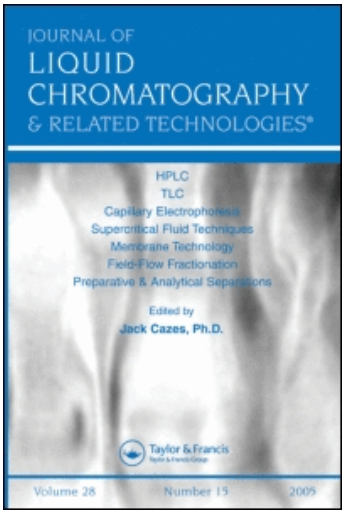

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SIMULTANEOUS MEASUREMENT OF ASCORBIC ACID AND GLUTATHIONE: APPLICATION OF MICRODIALYSIS AND ON-LINE HPLC WITH Au/Hg ELECTRODE IN ANESTHETIZED RAT LIVER

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ABSTRACT

An assay for simultaneous measurement of glutathione and ascorbic acid, two mutually related important aqueous antioxidants in the biological matrix, is reported. This assay involved an HPLC system equipped with an electrochemical detector and Au/Hg electrode. A microdialysis perfusion system was coupled with this LC-ECD system for on-line and continuous determination of glutathione and ascorbic acid levels in extracellular fluids of anesthetized rat liver. Good reproducibility for both ascorbic acid and glutathione was achieved when standard glutathione and ascorbic acid solutions were used.

Additionally, the effects of global liver ischemia on the hepatic extracellular ascorbic acid and glutathione levels were investigated. The simultaneous monitoring of ascorbic acid and glutathione levels is important since glutathione and ascorbic acid are mutually related in many biological functions.

INTRODUCTION

Glutathione and ascorbic acid are important aqueous antioxidants that may play protective roles in various physiological disorders.[1-4] These two antioxidants are related to many physiological functions. In addition to their interdependence on the antioxidant potential, glutathione depletion can induce glycogenlysis-dependent ascorbate synthesis in murine hetpatocytes;[5] Glutathione deficiency in mouse liver increases the synthesis of ascorbic acid,[6] while the ascorbic acid synthesis causes glutathione consumption.[7] Thus, simultaneous determination of glutathione and ascorbic acid levels can be used to represent the biological matrix's capability to defend against oxidative stress, and may provide valuable information regarding the bilateral roles of glutathione and ascorbic acid in biological functions.

Many analytical methods, such as an HPLC system equipped with absorbance, fluorescence, or electrochemical detector, are effective for analyzing glutathione and ascorbic acid individually.[8-11] An electrochemical detector with a Au/Hg electrode or glassy carbon electrode can be used in an HPLC system for analysis of glutathione[9] or ascorbic acid,[11] respectively. Therefore, for simultaneous determination of biological glutathione and ascorbic acid, either the Au/Hg electrode or carbon electrode can be considered. Rose and Bode[12] successfully used a porous carbon electrode to simultaneously analyze ascorbic acid and glutathione in rat liver homogenate. However, carbon electrodes may not be sensitive enough to measure low concentrations of glutathione. From a review of the literature, there have been no studies done to test the usefulness of the Au/Hg electrode for simultaneous determination of glutathione and ascorbic. Thus, the first objective of the present study was to investigate the possibility of using Au/Hg electrode for simultaneous determination of glutathione and ascorbic acid.

Microdialysis perfusion is a recently developed method for in vivo sampling of organ extracellular fluids from anesthetized or awake animals.[13,14] In earlier studies, we used a microdialysis perfusion system, in combination with an LCEC system and Au/Hg or glassy carbon electrode, to monitor in vivo glutathione[15,16] or ascorbic acid levels.[17] Due to low glutathione levels in microdialysates, glassy carbon was considered inadequate for this study. The second objective of the present study was to investigate the efficacy of a combination of HPLC-EC system and Au/Hg electrode-electrochemical detector

for the simultaneous monitoring of liver extracellular glutathione and ascorbic acid levels in anesthetized rats. Furthermore, the effects of global liver ischemia and reperfusion on anesthetized rat liver were investigated.

EXPERIMENTAL

Chemicals

Glutathione, ascorbic acid, and urethane were purchased from Sigma (St. Louis, MO, U.S.A.). Monochloroacetic acid was obtained from Merck (Darmstadt, Germany). Heptane sulfonic acid was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Reagent-grade acetonitrile and methanol were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). All other chemicals were reagent-grade and deionized distilled water was used.

General Procedure for Microdialysis

The microdialysis system was obtained from Carnegie Medicine Associates (Stockholm, Sweden). Microdialysis probes (CMA/20) were purchased from CMA. The probe length was 24 mm. Membrane of the probes was made of polycarbonate, with the length and diameter of 4 mm and 0.5 mm, respectively. Molecular mass cut-off for the membrane was 20,000 Da. The probe was perfused (2 μL/min) with a CMA-100 perfusion pump in corresponding outer medium for 30-60 min with Ringer's solution before starting the measurement to avoid the changes of relative recovery with time. The microdialysates were collected for every 10 min period with a 19.6 μL-loading-loop of a CMA 160 on-line injector. Time length for injection of the collected microdialysates into the HPLC system was 8 s.

Animal Preparation

Male Sprague-Dawley rats weighing 350-450 g were used. The animals were fasted for 12 h prior to the experiments and were anesthetized with an intraperitoneal injection of urethane (ethyl carbamate)(1200 mg/kg). A tracheal tube was inserted to facilitate breathing. Throughout the experiments, body temperature was maintained between 36-38° with a heating pad. Polyethylene cannulas (PE-50) were inserted into the femoral artery for continuous monitoring of heart rate and blood pressure. All animals were heparinized (400 IU/kg body weight). A midline laparotomy was performed, and the liver hilum was exposed. The microdialysis probe was implanted and perfused with Ringer

solution at a flow-rate of 2 μL/min. Hepatic ischemia was induced by clamping the entire hepatic pedicles (hepatic artery, portal vein, and common bile duct) for 30 minutes using a vascular clamp. During the ischemic period, 1 mL of saline was infused i.v. at 15-min intervals to maintain hemodynamic stability and to replace water loss due to portal stasis. Reperfusion of the liver was achieved by unclamping the hepatic pedicle.

HPLC Instrumentation for Glutathione and Ascorbic Acid Analysis

The HPLC system consisted of an HP 1050 series quaternary pump (Hewlett-Packard, Taiwan Branch, manufactured in Waldbronn, Germany), a CMA on-line degasser (CMA 260) and a BAS LC-4C electrochemical detector with dual Au/Hg electrodes (Bioanalytical System, West Lafayette, IN, U.S.A.). An Alltech 5 μm Econosphere C-18 cartridge column (150 x 4.6 mm ID) (Alltech Associates, Deerfield, IL, U.S.A.) was used for the separation. The mobile phase was composed of 0.1 M monochloroacetic acid, 2 mM heptane sulfonic acid (sodium salt) and 0.046 M sodium hydroxide in 2% acetonitrile (final apparent pH 3.0). Flow-rate was 0.8 mL/min. The settings for the electrochemical detector (working potential: +0.15 V vs. Ag/AgCl) were described previously.[15] Data collection and analysis were performed with a Chem Station Chromatographic Management System (Hewlett-Packard, Taiwan Branch).

RESULTS AND DISCUSSIONS

An electrochemical detector with a Au/Hg electrode can be used to determine glutathione concentration. We also investigated the possibility of using Au/Hg electrodes to measure ascorbic acid. Standard mixtures of ascorbic acid and glutathione solutions were injected into the HPLC system equipped with an electrochemical detector and Au/Hg electrode (Figure 1). Linear response was obtained from injections of various concentrations of standard ascorbic acid and glutathione mixtures. These results demonstrate the potential of Au/Hg electrode for simultaneous monitoring of glutathione and ascorbic acid concentrations.

The precision of the assay for glutathione and ascorbic acid was determined. From the results on three consecutive days, the intra-day coefficients of variation (n=6) were 1.1%, 4.6%, and 3.6% for ascorbic acid (18.9 mM), cysteine (16.4 mM), and glutathione (16.4 mM), respectively, while the inter-day coefficients for three consecutive working days were 3.6%, 4.2%, and 4.3% for ascorbic acid, cysteine, and glutathione, respectively.

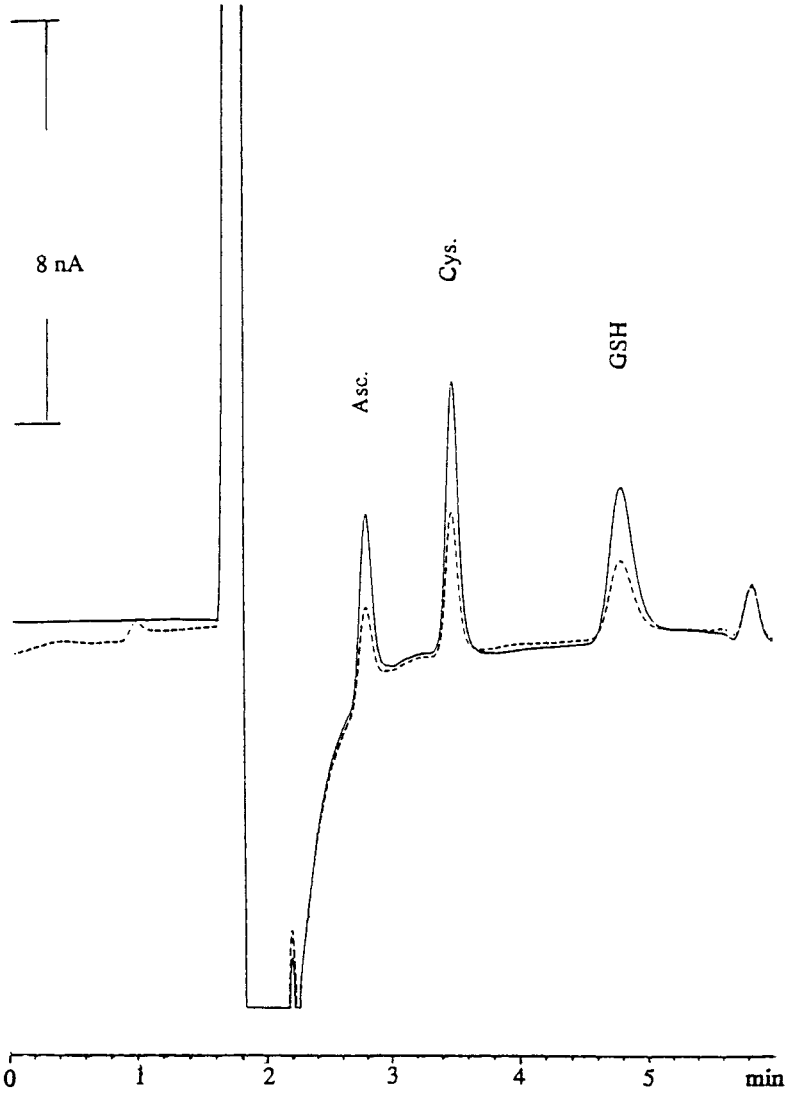

Figure 1. Typical chromatogram obtained from injection of a mixture of ascorbic acid (4.7 μM and 2.4 μM for solid line and dashed line, respectively), glutathione (4.2 μM and 2.1 μM) and cysteine (4.2 mμ and 2.1 μM). Asc: ascorbic acid; Cys: cysteine; GSH: glutathione.

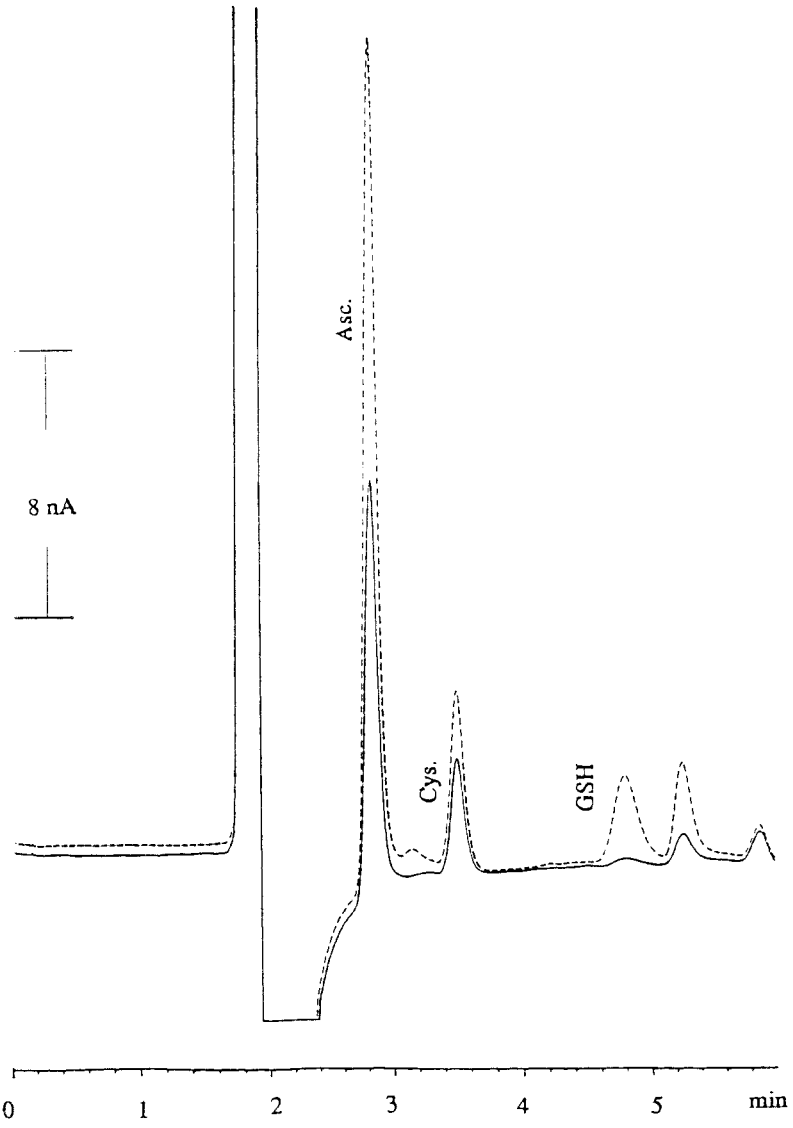

Figure 2. Typical chromatograms obtained from injection of liver microdialysates in anesthetized rat liver before (solid line) and 20 min after (dashed line) global liver ischemia.

Simultaneous determination of ascorbic acid and glutathione can also be performed with a porous carbon electrode.[12] However, the carbon electrode is not sensitive enough to analyze low levels of glutathione, like those in microdialysates from anesthetized rat organs. Therefore, we tested the possibility of using Au/Hg electrode in the analyses of glutathione and ascorbic acid from microdialysates.

Microdialysis perfusates from standard mixtures of glutathione and ascorbic acid solutions were analyzed for the glutathione and ascorbic acid levels on two consecutive days. From the two trials, a linear response was observed between the concentrations of ascorbic acid and glutathione in the corresponding medium and the concentrations in the microdialysates. This linear response is a good indication that the probe recovery remained constant over the concentration ranges used in the experiment. Additionally, this linearity demonstrates that the Au/Hg electrode was operating properly.

The linear response for glutathione was expected since the use of microdialysis perfusion with on-line HPLC system for glutathione has been documented.[15,16] However, the use Au/Hg electrode for the analysis of ascorbic acid levels in animal microdialysates has not been described. Our results suggest that Au/Hg electrode can also be used to analyse the ascorbic acid concentration in microdialysate.

The microdialysis probe was implanted into the liver extracellular space of anesthetized rats. Both the ascorbic acid and glutathione peaks are evident on the chromatogram (Figure 2). From the average of 8 rats, the glutathione and ascorbic acid levels in microdialysates perfused from liver extracellular fluids were (mean* SD) 1.03 ± 0.51 μM and 11.8 ± 6.3 μM, respectively. Simultaneous measurements of glutathione and ascorbic acid in microdialysates from liver extracellular fluids are of value. Hepatic export of the glutathione, which is known to be present in a particularly high concentration in liver, is responsible for over 85% of the glutathione levels in body extracellular fluids such as plasma. Glutathione and ascorbic acid are mutually related in many hepatic physiological functions.[4-6] Therefore, the relationships between the concentrations of ascorbic acid and glutathione are of importance.

Our earlier results showed that global liver ischemia can significantly increase the extracellular glutathione levels in anesthetized rats.[15] In the present study, we simultaneously measured glutathione and ascorbic acid levels in the microdialysates perfused from anesthetized rat liver following global liver ischemia (Figure 3). Glutathione levels rapidly increased after the onset of ischemia. Interestingly, ascorbic acid levels also rapidly increased at the onset of global liver ischemia.

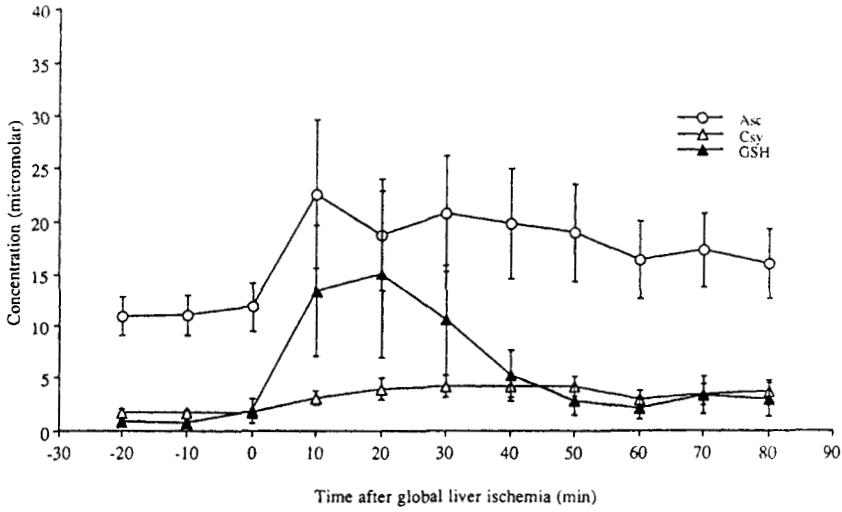

Figure 3. Effect of global liver ischemia on liver extracellular glutathione, ascorbic acid and cysteine levels in anesthetized rat liver. Data are represented as meanem. Results shown are the average from eight rats.

In conclusion, we successfully used Au/Hg electrode in an HPLC-EC system to simultaneously determine ascorbic acid and glutathione (and cysteine) concentrations. This HPLC-EC system was combined with an on-line microdialysis perfusion system to monitor liver extracellular glutathione and ascorbic acid levels in anesthetized rats. Elevation of both ascorbic acid and glutathione was observed in liver extracellular fluids following global liver ischemia.

This HPLC-EC (Au/Hg electrode) system may be useful for future studies targeted at the mutual relationship between ascorbic acid and glutathione in the biological matrix.

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