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Determination of extracellular glutathione in livers of anaesthetized rats by microdialysis with on-line high-performance liquid chromatography

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Abstract

An on-line analytical system for the continuous in vivo monitoring of extracellular glutathione (γ-glutamyl-cysteinylglycine)(GSH) concentrations in the livers of anaesthetized rats was developed. Microdialysates perfused through implanted microdialysis probes were collected with a loading loop of an on-line injector for direct and automatic injection into a high-performance liquid chromatographic system equipped with an electrochemical detector with Au–Hg electrodes. This method shortened the analysis time and circumvented the sample preparation process which is essential for accurate determination of GSH levels in biological samples. Additionally, this method provided continuous and real-time monitoring of extracellular GSH levels. Basal extracellular GSH concentrations in the livers of anaesthetized rate were found to vary over a wide range (from 4.16 to 76.5 μM). The method was applied to study the effect of global liver ischaemia on extracellular GSH concentrations and it was found that extracellular GSH levels in livers increased immediately with the onset of ischaemia and remained elevated for the 30-min ischaemic period. Ensuing reperfusion did reduce the GSH increase; however, the GSH levels did not return to the basal value.

1. Introduction

Glutathione (GSH) is an important tripeptide that is present in high concentrations in most mammalian tissues. GSH acts as a major antioxidant in addition to various essential biological functions [1–3]. Among different organs, the liver has a particularly high tissue GSH content, and the hepatic export of GSH is responsible for 85% of the GSH content in plasma [4,5]. GSH exhibits important protecting functions against ischaemic damage in liver by acting as a scavenger of reactive oxygen species [6–8]. Therefore, the determination of the GSH efflux from liver cells would provide valuable information for understanding the involvement of GSH in hepatic disorders such as ischaemia. Monitoring of the hepatic export of GSH can be accomplished by measuring the GSH content in the sinusoidal efflux from either an isolated perfused liver or from the liver of an anaesthetized animal [9,10]. However, frequent sampling and relatively large blood volumes, which are needed in monitoring the in vivo sinusoidal GSH efflux of an anaesthetized animal, make the continuous and long-term monitoring of hepatic

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GSH export a very difficult task for small animals such as rats. Additionally, attempts to monitor the in vivo hepatocyte efflux directly and continuously has seldom been reported, probably owing to the difficulty in sampling extracellular fluids in livers.

Microdialysis perfusion provides a minimally invasive method in continuous monitoring for the changes in low-molecular-mass metabolites in extracellular fluids of brains [11,12] and livers [13–15]. However, this technique has not been used to determine the extracellular GSH levels in livers. Microdialysis perfusion should be an ideal approach for sampling the extracellular fluids of livers for the determination of in vivo hepatocyte efflux of GSH. Microdialysis sampling can be directed connected to an HPLC system through an on-line device. The combination of HPLC with on-line microdialysis perfusion has been achieved for determining metabolites in extracellular fluids sampled from brains [16,17]. This combination provides minimal analysis time and automates the sample collection and injection.

Several HPLC methods employing either fluorescence [18,19] or electrochemical detection [20,21] are widely used in measuring physiological GSH concentrations, and these methods could be employed to determine the GSH content in microdialysates. Fluorescence detection requires a precolumn sample derivatization step and electrochemical detection [20,21] requires the sample to be deproteinized prior to the analysis. Hence both of these methods preclude the facile application to an on-line microdialysis HPLC system. With the use of microdialysis perfusion, which usually allows low-molecular-mass metabolites to be removed from sample matrix, an on-line HPLC system for GSH analysis could be connected for continuous sample collection and automatic injection.

The specific aim of the studies described here was to establish an in vivo continuous monitoring method for liver extracellular GSH levels in anaesthetized rats. This system involved the automatic on-line injection of microdialysates into an HPLC system equipped with an electrochemical detector containing Au–Hg electrodes. Additionally, this method was applied to investigate the effect of global liver ischaemia on extracellular GSH concentrations in livers of anesthaetized rats.

2. Experimental

2.1. Chemicals

GSH and urethane were purchased from Sigma (St. Louis, MO, USA), monochloroacetic acid from Merck (Darmstadt, Germany), heptanesulfonic acid from Tokyo Kasei Kogyo (Tokyo, Japan) and analytical-reagent-grade acetonitrile and methanol from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical-reagent grade and deionized distilled water was used throughout.

2.2. General procedure for microdialysis

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

2.3. 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 anaesthetized with an intraperitoneal injection of urethane (ethyl carbamate) (1200 mg/kg). A tracheal tube

was inserted to facilitate breathing. Throughout the experiments, the body temperature was maintained between 36 and 38°C with a heating pad. Polyethylene cannulas (PE-50) were inserted into the femoral artery for continuous monitoring of heart rate and blood pressure, and were inserted into the femoral vein for administration of saline. All animals were heparinized (400 IU/kg body mass). A midline laparotomy was performed and the liver hilum was exposed. The microdialysis probe was implanted and perfused with Ringer's solution at a flow-rate of 2 μl/min. Hepatic ischaemia was induced by clamping the entire hepatic pedicles (hepatic artery, portal vein and common bile duct) for 30 min using a vascular clamp. During the ischaemic period, 1 ml of saline was infused i.v. at 15-min intervals to maintain a haemodynamic stability and to replace water lost due to portal stasis. Reperfusion of the liver was achieved by unclamping of the hepatic pedicles.

2.4. HPLC instrumentation for GSH determination

When an electrochemical detector was used for GSH analysis, the HPLC system consisted of an HP 1050 Series quaternary pump (Hewlett-Packard, Waldbronn, Germany), a CMA 260 on-line degasser and a BAS LC-4C electrochemical detector with dual Au–Hg electrodes (Bioanalytical Systems, Lafayette, IN, USA). An Alltech (Deerfield, IL, USA) 5-μm Econosphere C_{18} cartridge column (150 mm × 4.6 mm I.D.) was used for separation. The mobile phase was composed of 0.1 *M* monochloroacetic acid, 2 m*M* sodium heptanesulfonate and 0.046 *M* sodium hydroxide in 2% acetonitrile (final pH 3.0). The flow-rate was 0.8 ml/min. The settings for the electrochemical detector (working potential 0.15 V vs. Ag/AgCl) were described previously [20]. Data collection and analysis were performed with a Chem Station Chromatographic Management System (Hewlett-Packard).

When a fluorescence detector was used for GSH analysis, the HPLC system and analytical procedure were as described previously [21] except that an Alltech C_{18} Econosphere cartridge column (150 mm × 4.6 mm I.D.) was used. Briefly, collected microdialysate was derivatized with two volumes of 50 m*M* sodium phosphate buffer containing 50 μM dithiolthreitol and 2 m*M* EDTA. The mixture was added to an equal volume of 2 m*M* methanolic monobromobimane for derivatization prior to injection into the HPLC system. The excitation and emission wavelengths for the fluorescence detector were 270 and 474 nm, respectively.

3. Results and discussion

3.1. Determination of GSH by on-line injection into the HPLC system

HPLC with Au–Hg dual-electrode electrochemical detection has been commonly used in determining biological GSH levels [20,22], yet there have been few literature reports discussing microdialysis sampling for GSH determination. Landolt et al. [23] used microdialysis and HPLC with electrochemical detection to monitor brain extracellular GSH levels in cerebral ischaemia; however, they used off-line sampling for detection. We tested the combination of microdialysis and HPLC systems with an on-line injector for the determination of GSH. Microdialysates perfused through probes implanted in standard GSH solutions (200 μM) were collected and automatically injected into the HPLC system. The results are shown in Fig. 1. The GSH levels reached a plateau 30 min after the probe implantation, and this indicated that the system has been equilibrated. Additionally, these results demonstrated that the on-line collection–injection system was working successfully for the determination of GSH in microdialysates perfused through the probes.

3.2. Determination of extracellular GSH levels in livers of anaesthetized rats

Owing to the intracellular abundance of GSH and the tissue damage resulting from the probe insertion, the GSH levels in the microdialysates from the initial collections after probe implanta-

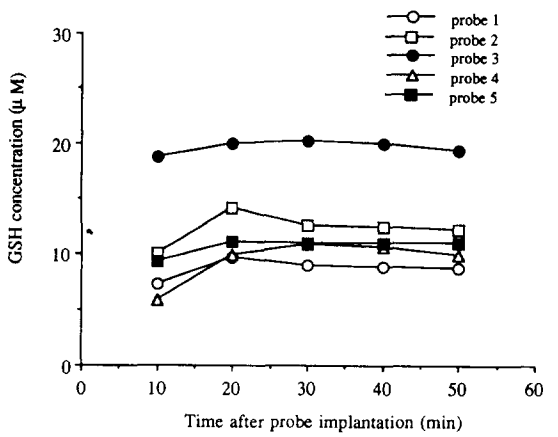

Fig. 1. GSH concentrations in microdialysates collected every 10 min after probe implantation in standard GSH solution (200 μM).

tion were very high, and decreased sharply with time (Fig. 2). The extracellular GSH level in the liver usually reached an equilibrium 60 min after the probe implantation. Fig. 3A is a typical chromatogram obtained from the injection of 10-min microdialysate from hepatic extracellular fluids. The chromatographic analysis time for a sample was 9 min, during which both the cysteine and the GSH peaks were eluted. Identification of the peak positions of cysteine and GSH was accomplished when this chromatogram was compared with that obtained from the injection of standard cysteine and GSH mixtures. We measured the extracellular GSH levels for ten rats, and found that they varied over a wide range (from 4.16 to 76.5 μM; mean ± S.D. 25.12 ± 27.46 μM).

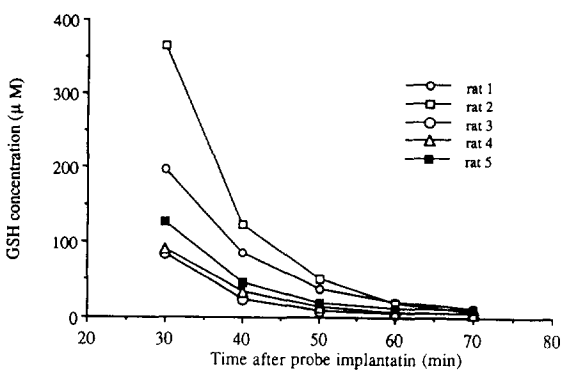

Fig. 2. Extracellular GSH concentrations after probe implantation into the liver. GSH levels in microdialysates for the first and second 10-min periods were very high and are therefore not shown.

In this study, an on-line injection device was used for the automatic injection of the collected microdialysates. This combination offers several advantages. It minimized the sample preparation time and the contact of GSH with air. Prolonged sample preparation and exposure of GSH to air can induce GSH oxidation, which might affect the accuracy of GSH determination [24]. Additionally, the simple and direct collection–injection device automated the sample collection, injection and analysis and thus greatly shortened the time needed for GSH determination. This HPLC system with on-line injection cannot be applied if a fluorescence detector is employed owing to the need for derivatization, although fluorescence detection offers adequate sensitivity. Continuous and accurate monitoring of GSH in the liver extracellular space of anaesthetized animals can be achieved, which is of significance because the liver has been known actively to transport the GSH out of hepatocytes. Hence this method could be applied in the future to related studies targeting the liver GSH status.

3.3. *Identification of the GSH peak*

To identify the GSH peak in the chromatogram obtained from injection of microdialysate collected from the rat, we used another HPLC system equipped with a fluorescence detector to clarify the validity of the GSH peak. With the electrochemical detector off, eluate that had a retention time the same as the presumed GSH peak was collected. The collected samples were re-injected into the HPLC–electrochemical detection (ED) system to confirm that the collected sample indeed contained the substance with a retention time identical with that of GSH. The collected sample was then derivatized with methanolic monobromobimane, which acted as a fluorescent tag. The derivatized material was injected into another HPLC system equipped

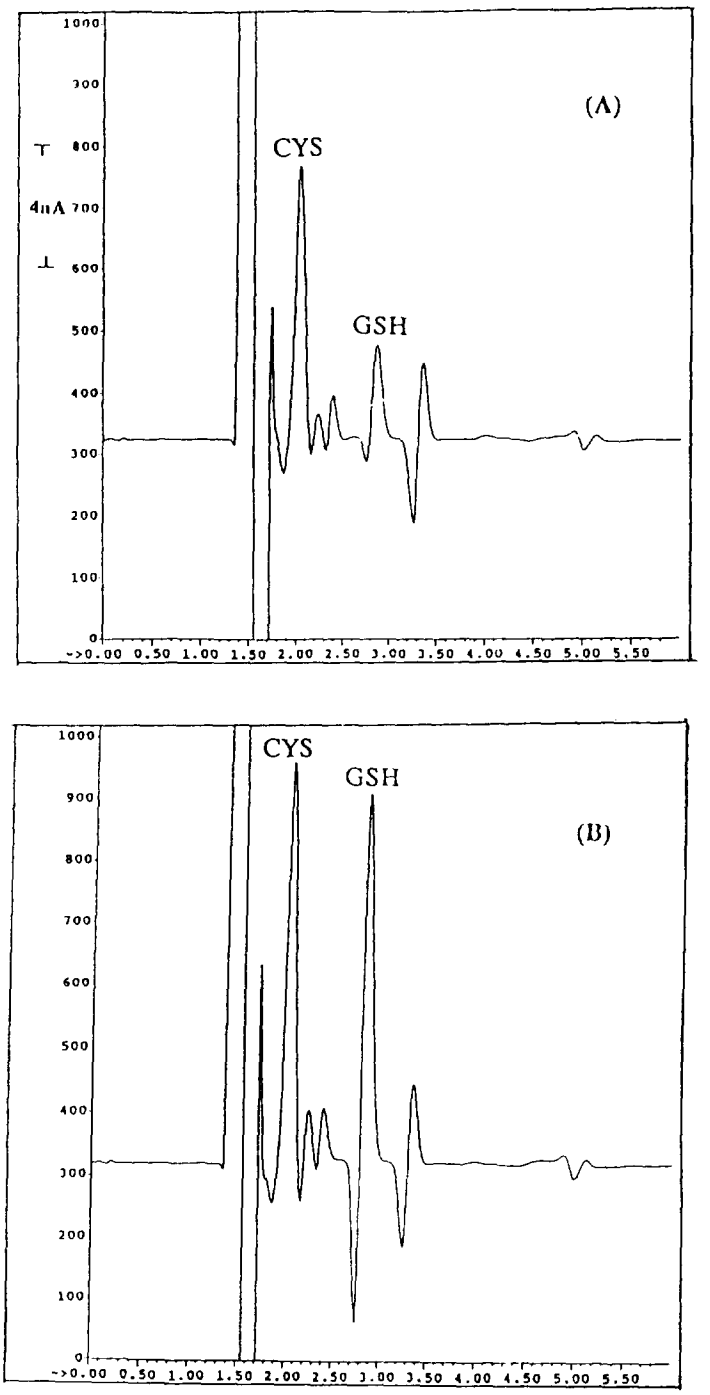

Fig. 3. Chromatograms obtained from the injection of microdialysates (A) before and (B) after the onset of ischaemia induced by the ligation hepatic pedicles (portal vein, hepatic artery and bile duct).

with a fluorescence detector. As shown in Fig. 4, comparison of the chromatograms obtained from injections of authentic GSH solution (Fig. 4A) and blank solution (Fig. 4B) clearly indicated the presence of the GSH peak. Fig. 4C shows the chromatogram obtained from the injection of the derivatized eluate collected from the HPLC–ED system. It is evident from Fig. 4C that a GSH peak is present that has a retention time identical with that in Fig. 4A. Although the identification of the GSH peak in this HPLC–fluorescence system was also achieved by comparing the chromatograms from injections of samples and standard GSH solutions, when this result was combined with the corresponding results from the HPLC–ED system, the validity of the GSH peak was reasonably proved.

3.4. Effect of liver ischaemia on extracellular GSH levels

We applied the method to study the effect of global liver ischaemia on extracellular GSH levels. Global liver ischaemia was achieved by clamping of the hepatic artery, portal vein and bile duct. The liver extracellular GSH concentrations increased rapidly after the onset of ischaemia. A typical chromatogram obtained from injection of microdialysate collected for the first 10-min dialysis period after hepatic ischaemia is shown in Fig. 3B. On comparing Fig. 3B with Fig. 3A, which was obtained from an injection of microdialysate from previous basal extracellular fluids of the same animal, it is evident that the GSH peak is significantly higher

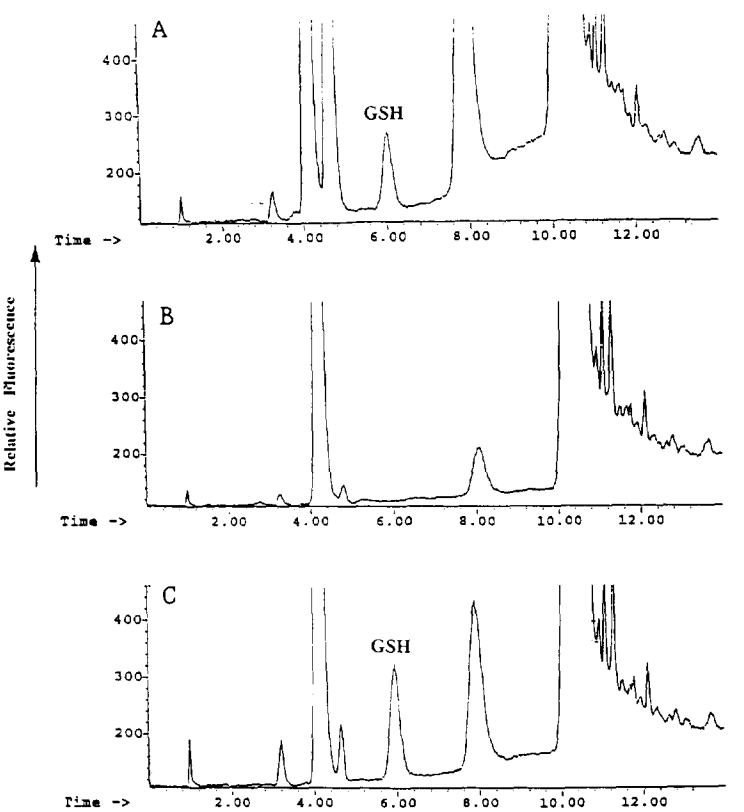

Fig. 4. Chromatograms obtained from injections of (A) derivatized authentic GSH (0.625 μM) solution, (B) derivatized blank solution and (C) derivatized eluate collected from the HPLC–ED system.

in Fig. 3B. The ischaemic period lasted for 30 min, followed by reperfusion, which was accomplished by unclamping the vessels. The time profile for the extracellular GSH levels in liver before and after ischaemia is shown in Fig. 4, which clearly indicates that ischaemia for 10 min elevated the liver extracellular GSH. Although sustained ischaemia for an additional 10 min further increased the liver extracellular GSH levels, the increasing trend was much less sharp than for the first 10-min period of ischaemia. Liver extracellular GSH levels exhibited no significant difference between the second and third 10-min periods of ischaemia. Reperfusion gradually reduced the ischaemia-induced elevation of liver extracellular GSH levels. However, even 30 min after the reperfusion, the GSH levels did not return to the basal values (Fig. 5).

There might be several reasons for the increase in extracellular GSH levels following liver ischaemia. One of the possible causes is that global ischaemia severely damages the integrity of the cellular membrane of hepatocytes and thus intracellular GSH, the level of which is two to three orders of magnitude higher than that in extracellular space, is rapidly released into the latter compartment. The other possible cause is that a large amount of GSH was released to scavenge the reactive oxygen species, which are known to be generated in hepatic ischaemia [6–8]. The elevation of extracellular GSH after ischaemia tended to smooth out after the perfusion of the blood flow.

In conclusion, a method involving microdialysis perfusion, automatic on-line injection and HPLC analysis for the continuous monitoring of extracellular GSH levels in the liver of anaesthetized rats has been developed. This method has been used in global liver ischaemia/reperfusion investigations, which provided evidence for significantly elevated extracellular GSH levels in the anaesthetized rat livers.

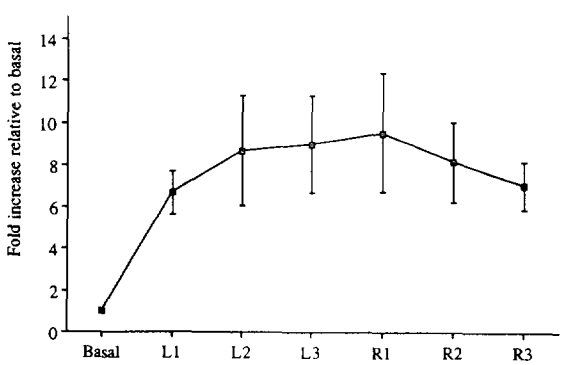

Fig. 5. Time profile for extracellular GSH levels in rat liver after the ligation of hepatic pedicles. Data are represented as the average results for seven rats. L1, L2, L3, 10, 20 and 30 min after ligation, respectively; R1, R2, R3, 10, 20 and 30 min after reperfusion, respectively. The absolute value for 100% GSH varied from 4.16 to 66.27 μM. GSH levels after hepatic ischaemia were significantly different ($p < 0.05$ by Student's t-test) from the basal values.

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