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Journal of Chromatography B, 661 (1994) 231–235

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of extracellular glutathione in rat brain by microdialysis and high-performance liquid chromatography with fluorescence detection

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First received 9 June 1994; revised manuscript received 1 August 1994

Abstract

A method for the continuous monitoring of extracellular glutathione (GSH) concentrations in rat brain has been developed. This method involved the in vivo sampling of brain extracellular fluid by microdialysis perfusion and the subsequent analysis by high-performance liquid chromatography (HPLC) with fluorescence detection. Perfusates from the microdialysis probes were directly derivatized with methanolic monobromobimane which acted as the fluorescence tag. Separation of the derivatized perfusate was achieved on narrow-bore reversed-phase C_{18} columns. Recoveries of GSH from the microdialysis probes ranged from 1.5% to 4%. The basal extracellular GSH concentration in rat (Sprague–Dawley) brain cortex was found to be $2.10 \pm 1.78\ \mu M$ (mean ± S.D.) (results of 18 rats). Fluorescence detection and separation on narrow-bore columns provided adequate sensitivity for accurate determination of brain extracellular GSH concentrations in rats. With this method, the extracellular GSH concentrations in the cerebral cortex were found to be significantly elevated upon the onset of cerebral ischemia induced by the ligation of bilateral common carotid arteries.

1. Introduction

Glutathione (γ-glutamylcysteinylglycine, GSH) is an important tripeptide present in most mammalian tissues at a high concentration (0.1 to 10 mM). One of the essential functions of GSH is participation in the detoxification of xenobiotics and numerous harmful cellular compounds [1–3]. GSH also acts as a major tissue antioxidant against oxidative stress in several organs including the brain [4–7]. Additionally, GSH serves important protective functions in damages caused by reactive oxygen species in cerebral ischemia/reperfusion [8–11]. Therefore, vital information could be obtained from measurement of the extracellular brain GSH concentration on which only few data has been reported [12].

Unlike its intracellular abundance, GSH is present in much lower concentrations in extracellular fluids such as plasma and cerebral spinal fluid (i.e. in the micromolar range). Thus, high

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0378-4347/94/$07.00
SSDI 0378-4347(94)00355-6

sensitivity assays are required to determine the GSH concentrations in these fluids. Several HPLC methods employing either fluorescence or electrochemical detection are successful in this respect. Monobromobimane [13,14] and *o*-phthaldehyde (OPA) [15,16] are the most commonly used labeling reagents for fluorescence detection. For electrochemical detection of GSH series Au/Hg dual electrodes have been used [17,18].

Microdialysis provides a minimally invasive method for the continuous monitoring of changes in the amounts of low-molecular-mass metabolites in the extracellular fluids of various organs [13,19]. Thus, microdialysis sampling systems combined with HPLC employing fluorescence or electrochemical detection, are an ideal choice for the determination of the in vivo GSH concentration in brain extracellular spaces. One of the very few reports employing microdialysis and HPLC in the determination of brain extracellular GSH levels employed electrochemical detection [12]. Therefore, we investigated the alternative fluorescence detection mode coupled with microdialysis and HPLC for the determination of extracellular GSH levels in rat brain cortex. Additionally, since GSH is a major antioxidant capable of scavenging the reactive oxygen species generated from cerebral ischemia, we studied the effect of cerebral ischemia on the extracellular GSH concentrations in rat brain cortex.

2. Experimental

2.1. General procedure for microdialysis

The microdialysis system was obtained from Carnegie Medicine Associates (CMA, Stockholm, Sweden). The microdialysis probes (CMA10, membrane length 2 mm) were perfused with Ringer's solution by a CMA-100 perfusion pump at a flow-rate of 0.5 μl/min. Collection of the perfusates was achieved by a CMA-140 fraction collector. Microdialysates of every 10-min perfusion were added directly to 10 μl of 2 m*M* methanolic monobromobimane for derivatization. Recoveries of GSH were measured in vitro using authentic GSH solutions with concentrations comparable to those in vivo. The probe was perfused in corresponding outer medium for 30–60 min before start of the measurements to avoid changes in the relative recovery with time.

2.2. Animal preparations

Male Sprague–Dawley rats (280–330 g) were used. The animals were anesthetized with urethane (1.2 g/kg, i.p.), and body temperature was maintained at 37°C with a heating pad. Polyethylene catheters were inserted into the femoral artery for monitoring the systemic arterial blood pressure (SAP) with a Gould pressure processor. By the same device, the mean systemic arterial pressure (MSAP) and heart rate (HR) were computed electronically. Both common carotid arteries were exposed through a ventral midline incision in the neck, carefully separated from the vago-sympathetic trunks, and loosely encircled with sutures for later ligation. The rat's head was mounted in a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA) with the incisor bar positioned 3.3 mm below the horizontal. Following a midline incision the skull was exposed and one burr hole was made in the skull for inserting the microdialysis probe (1.2 mm anterior and 2.0 mm lateral to the bregma and 2 mm from the brain surface).

2.3. High-performance liquid chromatography

The HPLC system was Hewlett-Packard (Taipei, Taiwan) 1050 Series including a 1050 Series quartenary pump, a 1050 Series autosampler, a 1050 Series on-line degasser, and a 1462A HPLC fluorescence monitor with two holographic diffraction monochromators. Optimal responses for bimane derivatives were observed with excitation and emission wavelengths of 270 nm and 474 nm, respectively. Peak areas were determined using Chem Station Chromatographic Management System.

Separations were achieved on home-packed 5 μm C_{18} columns (150 × 2.0 mm I.D.). Packing materials were Adsorbosphere HS C_{18} (Alltech

Assoc., Deerfield, IL, USA). Prior to preparation of the mobile phases containing tetrabutyl ammonium hydroxide (TBA) as the ion-pairing reagent, the commercially available 40% aqueous solution of TBA was neutralized to pH 6.0 with 85% phosphoric acid. All mobile phases containing TBA had a TBA concentration of 30 m*M*. After the addition of TBA, the pH of these mobile phases was usually adjusted to 3.1–3.4 (apparent) as measured by a glass electrode. In all cases the mobile phases were filtrated through 0.45-μm filters and degassed. Two mobile phases were used. Mobile phase A was 30 m*M* TBA in 25% methanol, while mobile phase B was 30 m*M* TBA in 100% methanol. The following elution profile was used: 0–10 min, isocratic with mobile phase A, 10–15 min, a column wash with 90% mobile phase B to wash the late eluting bimane derivatives, 15–20 min, re-equilibrate with mobile phase A before next injection. The flow-rate was 0.25 ml/min. The injection volume was 10 μl.

For electrochemical detection of the microdialysates from rat brain cortex, the microdialysis perfusing procedure was performed as described above except that the flow-rate was 1.0 μl/min and the microdialysates were collected and injected through a CMA-160 injector. HPLC analysis was performed according to a previously published procedure [17] except that a conventional 5-μm Econosphere C_{18} column (150 × 4.6 mm I.D.) (Alltech Assoc.), PM-80 isocratic pump and LC-4C electrochemical detector (Bioanalytical System, West Lafayette, IN, USA) were used.

3. Results and discussion

An assay involving microdialysis perfusion and HPLC analysis has been developed to determine extracellular GSH concentrations. This assay would be useful to study the role of GSH in cerebral disorders since very little data has been reported on determination of brain extracellular GSH levels. A typical chromatogram obtained from injection of rat brain cortex microdialysates after derivatization with monobromobimane is shown in Fig. 1A. The GSH bimane derivative peak eluted in a relatively clean area.

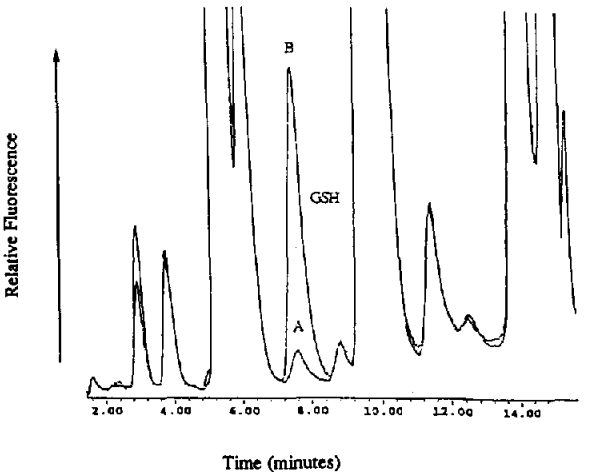

Fig. 1. Overlaying chromatograms from injections before (A) and after (B) the onset of cerebral ischemia induced by ligation of bilateral common carotid arteries.

We examined the effect of concentration on the GSH recoveries with three different probes. The results are shown in Fig. 2. Three different concentrations of authentic GSH (10 μM, 100 μM and 1000 μM) were used. There was no significant variation in GSH recoveries. Recoveries of authentic GSH through the microdialysis probes ranged from 1.5% to 4% (with a microdialysis perfusion rate of 0.5 μl/min), which was relatively low compared to free amino acids such as glutamate or aspartate. This low GSH recovery might be due to the fact that GSH

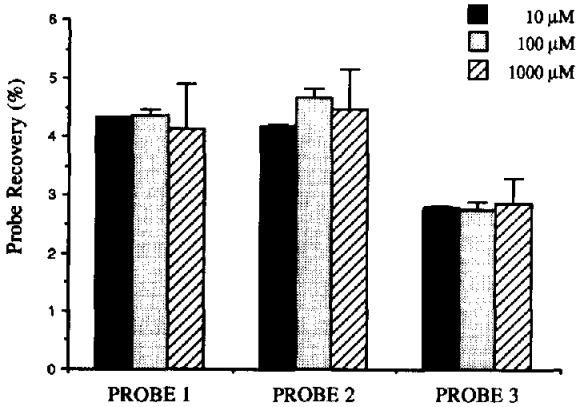

Fig. 2. Effect of concentration on probe recoveries for GSH.

is a relatively large molecule when compared with catecholamines and amino acids. Additionally, the recoveries were inversely related to the perfusion flow-rates (data not shown) which was in accordance with the results obtained for catecholamines and amino acids.

GSH is very abundant in intracellular spaces. Probe implantation could cause lysis of neighbouring cells and the subsequent release of intracellular GSH. To achieve equilibrium of the GSH concentrations after probe implantation, the extracellular GSH levels were monitored for 120 min after probe insertion. These results are shown in Fig. 3. Extracellular GSH levels dropped significantly in the first 20 min, and then leveled off for the next 20 min. Cortex extracellular GSH concentrations reached equilibrium 60 min after probe implantation. Basal extracellular GSH levels were found to be 2.10 ± 1.78 μM (mean ± S.D.) from the experiments of 18 Sprague–Dawley rats. To compare the results obtained by fluorescence with those obtained by electrochemical detection, we measured the corresponding extracellular GSH levels with electrochemical detection in 7 rats, and the results (1.61 ± 0.71 μM, mean ± S.D.) were in agreement with those obtained by fluorescence detection. The only published study discussing extracellular GSH levels in brain used spontaneous hypertensive rats, and our results were in accordance with the published results [12].

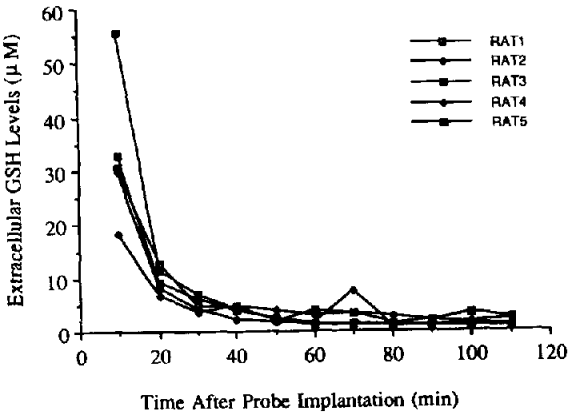

Fig. 3. Extracellular GSH concentrations after probe implantation.

Since the basal extracellular GSH concentrations in rat brain cortex were in the range of several micromolar, the GSH amount in brain microdialysates after derivatization with monobromobimane would be in the range of nanomolar considering the low recoveries. Detection of concentrations in this low range with conventional fluorescence detection would be a difficult task. Thus, two approaches have been used to enhance the sensitivity of this assay. One approach was to lower the perfusion flow-rate (0.5 μl/min) to increase the GSH recovery, and the other was to use low-dispersion columns (2.0 mm I.D.) instead of conventional columns (4.6 mm I.D.).

Little has been reported on continuous monitoring of extracellular GSH levels in tissues. This may be for two reasons: one is that it is difficult to perform in vivo continuous sampling and the other is that the assay would require high sensitivity since the GSH concentration in the cerebral extracellular space is low. With microdialysis perfusion continuous sampling of extracellular fluids can be performed, however, using microdialysis probes could make the detection of GSH more difficult since the tripeptide GSH does not exhibit a high recoveries through microdialysis probes. Therefore, we chose to use fluorescence detection since it has adequate sensitivity. Since GSH is known to be easily oxidized, eluted microdialysates were added directly to methanolic monobromobimane which minimized exposure of GSH to air and thus provided more accurate measurement of GSH levels. Combination of these two techniques has been shown to be an ideal tool for the continuous monitoring of brain extracellular GSH concentrations.

Extracellular GSH concentrations in rat brain cortex significantly increased upon the onset of cerebral ischemia induced by ligation of bilateral common carotid arteries. Shown in Fig. 1B is a typical chromatogram obtained from a microdialysate after ligation as compared to that obtained before ligation (Fig. 1A). It is evident that the GSH peak was significantly larger after ligation than before ligation. The time profile for extracellular GSH concentrations before and

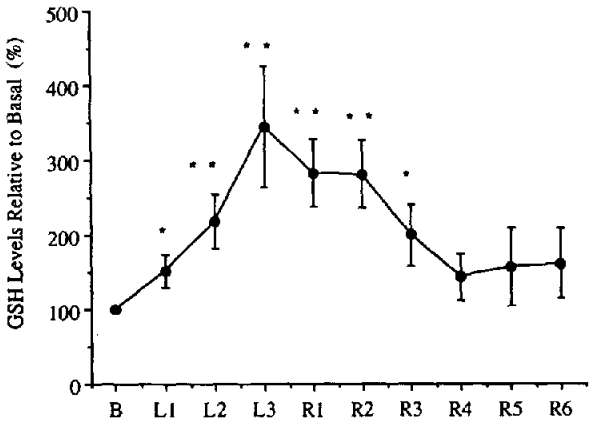

Fig. 4. Time profile for extracellular GSH concentrations in rat brain cortex before and after ligation of bilateral common carotid arteries. Data are represented as the averaging results of 9 rats. B: basal; L1, L2, L3: 10, 20 and 30 min after ligation; R1, R2, R3, R4, R5, R6: 10, 20, 30, 40, 50 and 60 min after reperfusion. Absolute value for 100% GSH levels $1.96 \pm 1.46\ \mu M$ (mean ± S.D.). (*) Statistically different (student's t-test) from basal values with $p < 0.1$, (**) statistically different from basal values with $p < 0.05$.

after ischemia is shown in Fig. 4. There are two possible reasons for the increase in extracellular GSH levels after cerebral ischemia. One is that during ischemia the cellular membrane is ruptured, and GSH is released due to the steep gradient in the GSH concentrations between the intracellular and extracellular spaces. Another possible cause for the GSH release is that increased GSH is needed to scavenge the free radicals that are formed in cerebral ischemia.

In conclusion, we have developed an assay capable of continuously monitoring the brain extracellular GSH levels in rat brain. This assay involved microdialysis perfusion and HPLC analysis with fluorescence detection. The assay has been applied to study the increase in GSH levels after cerebral ischemia/reperfusion.

Acknowledgements

This research was granted by Taichung Veterans General Hospital (TVGH No. 837312). We greatly appreciate the generous offering of the narrow-bore columns used in this study by Dr. Fu-Chou Chen and Miss Yin Shih.

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