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Journal of Chromatography B, 734 (1999) 1–6

JOURNAL OF CHROMATOGRAPHY B

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On-line derivatization for continuous and automatic monitoring of brain extracellular glutamate levels in anesthetized rats: a microdialysis study

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Received 14 September 1998; received in revised form 28 June 1999; accepted 29 June 1999

Abstract

Glutamate is an important excitatory amino acid in central nervous system. We developed a method for in vivo, continuous and automatic monitoring of brain extracellular glutamate, as well as other amino acids in anesthetized rat. This method involves the use of microdialysis perfusion technique and a high-performance liquid chromatography system equipped with a fluorescence detector. The microdialysate (perfused at a flow-rate of 1 μl/min) was on-line derivatized with *o*-phthaldehyde (perfused at 2 μl/min) through a mixing tee prior to the injection onto the HPLC column. The efficiency of this on-line derivatization was equivalent to that performed with an off-line manner. The effect of cerebral ischemia (2 h) and reperfusion (2 h) in brain cortex of anesthetized rats was monitored using this method. In addition to glutamate, extracellular concentrations of other amino acids, such as aspartate, glutamine, glycine, taurine and γ-aminobutyric acid, were also simultaneously monitored with this on-line method. Since monitoring of extracellular amino acids by microdialysis perfusion is intensively used in neuroscience investigations, this simple and convenient method would be useful in the future applications.

Keywords: Derivatization, LC; Glutamate; Aspartate; Glutamine; Glycine; Taurine; γ-Aminobutyric acid

1. Introduction

Glutamate is an important excitatory amino acid in neuronal transmission [1]. Impaired uptake or unregulated release in brain glutamate may be involved in the pathophysiological progression of various disorders including neuronal degeneration and ischemic insults [2–6]. Brain extracellular glutamate levels in anesthetized or awake animals, or even in human subjects, can be estimated as its level in microdialysates perfused through an implanted probe [7–10]. Glutamate concentration in microdialysate can be analyzed by different methods including high-performance liquid chromatography (HPLC) with fluorescence or electrochemical detection [11–13].

In the HPLC analysis of extracellular amino acid including glutamate, derivatization of the microdialysate with fluorescent reagent such as *o*-phthaldehyde (OPA) or 9-fluorenylmethyl chloroformate (FMOC) is required [14,15]. An on-line method, which has many advantages such as shortened analysis time, simplified sample preparation

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00310-2

and automatic sample injection, can be developed provided the on-line derivatization procedure can be accomplished. Recently, capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection has been developed for on-line monitoring of microdialysate glutamate levels with high temporal resolution [16]. However, the feasibility of an on-line device using an HPLC system, which is a more common and less sophisticated piece of instrument, in such analysis has not been investigated.

Therefore, in the present study, we developed an assay for continuous and automatic monitoring of rat brain extracellular glutamate levels. The method involves the use of microdialysis perfusion and on-line derivatization of microdialysate with OPA prior to the injection on to the HPLC column with fluorescence detection. This device has been used to monitor glutamate concentration profile in rat brain cortex following cerebral ischemia and reperfusion. In addition to glutamate, several other amino acids including aspartate, glutamine, glycine, taurine and γ-aminobutyric acid (GABA) can also been monitored with this method in the rat brain cortex.

2. Materials and methods

2.1. General procedure for microdialysis

The microdialysis system was obtained from Carnegie Medicine Associates (CMA, Stockholm, Sweden). The microdialysis probes (CMA12, membrane length 4 mm) were perfused with Ringer's solution by a CMA-100 perfusion pump at a flow-rate of 1 μl/min.

2.2. Animal preparations

Male Sprague–Dawley rats (280–330 g) were used. The animals were anesthetized with pentobarbital (50 mg/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 process, 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 nose bar positioned 3.3 mm below the horizontal. Following a midline incision the skull was exposed and one burr hole was made on the skull for inserting the microdialysis probe (1.2 mm anterior and 2.5 mm lateral to the bregma and 2 mm from the brain surface). Cerebral ischemia was induced by the ligation of bilateral common carotid arteries (CCA) and unilateral middle cerebral artery (MCA). Reperfusion was performed by unclamping the MCA and CCA.

The microdialysate (1 μl/min) was mixed continuously through a mixing tee (micro tee, CMA) with OPA (2 μl/min) solution. The OPA solution was prepared as follows: OPA stock solution (20 m*M*) was prepared in methanol (10%) containing 0.005% (v/v) β-mercaptoethanol, then the OPA stock solution was mixed with 0.25 *M* sodium borate buffer (1:2). The on-line derivatized microdialysates were then directed into the sampling loop of a on-line injector (CMA 160). The mixture was injected at a 20 min interval. The injection time was 8 s.

Off-line derivatization procedure was performed as described elsewhere [7]. Briefly, the sample was added to two volumes of OPA solution and waited for 60 s prior to the injection onto the HPLC column.

2.3. High-performance liquid chromatography

The HPLC system consisted of Hewlett-Packard 1100 series, a 1100 series quaternary pump, a 1100 series autosampler, a 1100 series on-line degasser, and a fluorescence monitor (FL-45, Bioanalytical System, Lafayette, IN, USA) having two holographic diffraction monochromators. Optimal responses for OPA derivatives were observed when excitation and emission wavelengths were at 340 nm and 450 nm, respectively. Peak areas were determined using the Hewlett-Packard Chem Station (1100 series) Chromatographic Management System.

Separations were achieved using an Alltech C_{18} Econosphere (particle size: 5 μm, 150×4.6 mm) conventional column (Alltech Associates, Deerfield,

IL, USA). Ternary gradient elution was used. Mobile phase A consisted of 93 m*M* sodium acetate buffer (pH 6.0) in 7% acetonitrile. Mobile phase B consisted of 10 m*M* sodium acetate buffer in 90% acetonitrile. Mobile phase C was distilled water. Flow-rate was 0.8 ml/min. The elution profile was: 0 min 92% A, 8% B; 3 min 60% A, 40% B; 11 min, 60% A, 40% B; 12 min 30% B, 70% C; 14 min 10% A, 90% B; 17 min, 10% A, 90% B; 17.5 min, 92% A, 8% B.

Schematic representation of this device is shown in Fig. 1. The microdialysate, eluted from an implanted microdialysis probe (usually perfused at a flow-rate of 1 μl/min), was flowing into a microtee to mix with OPA solution perfused from another syringe pump (usually at a flow-rate of 2 μl/min). The mixture was then collected by a loading loop of an on-line injector, which was set to inject at a 20 min interval during which the derivatization can proceed in the loop. To examine the efficiency for on-line derivatization, glutamate solutions of various concentrations contained in the syringe were flowing directly, without going through the microdialysis probe, into the microtee to mix with OPA prior to the HPLC injection.

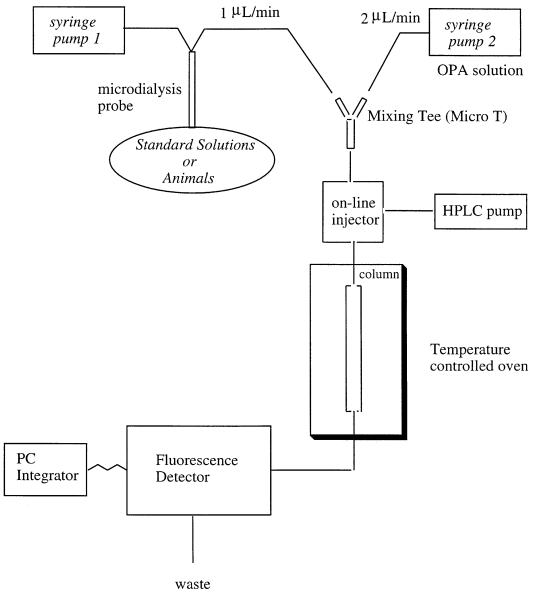

Fig. 1. Schematic representation of the on-line derivatization, injection and HPLC analysis system. The microdialysate flows into a microtee to mix with OPA solution. The HPLC system is equipped with a fluorescence detector.

3. Results and discussion

An assay for in vivo, continuous and automatic monitoring of extracellular glutamate concentration has been developed. This assay involved the use of microdialysis perfusion technique and HPLC system. The microdialysate was on-line derivatized with OPA solution prior to the automatic injection into an HPLC system. Glutamate concentrations determined by this on-line derivation method are similar to those obtained from an off-line derivatization procedure, which was accomplished by the autosampler. These results suggest that the derivatization efficiency are similar between the on-line and off-line derivatization procedure.

A microdialysis probe was placed in standard glutamate solutions at various concentrations (12.5 μ*M*, 25 μ*M*, 50 μ*M*, 100 μ*M*). Glutamate concentrations in the microdialysates and in the solutions where the probe was placed were determined by the on-line method. Linear responses were observed between the two sets of glutamate concentrations (Fig. 2). The linear response demonstrated that this on-line derivatization device was adequate. Additionally, the slopes in Fig. 2 can be used to estimate the in vitro recovery of microdialysis probe for glutamate. Based on the signal-to-noise ratio of 2, the detection limit for the glutamate analyzed as the OPA derivative was 0.1 pmol per 20 μl of injected volume.

It is well documented that cerebral ischemia can induce accumulation of glutamate in brain extracellular space [5,6]. Thus, the effect of cerebral ischemia on extracellular glutamate levels in brain cortex of anesthetized rat, by using this on-line derivatization method, was investigated. Typical chromatograms from injection of microdialysates, collected from brain cortex of anesthetized rat during the basal (Fig. 3A) and 20 min after cerebral ischemia (Fig. 3B) are shown. Cerebral ischemia, induced by the ligation of bilateral common carotid arteries and unilateral middle cerebral artery, significantly increased the extracellular glutamate levels (Fig. 4). After 2 h of ischemia, the reperfusion of blood flow, performed

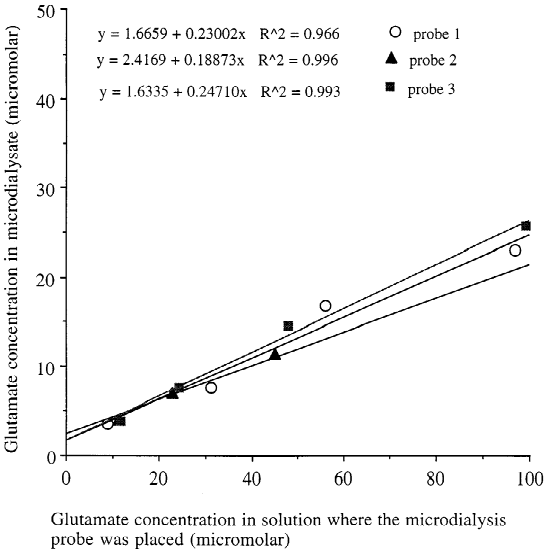

Fig. 2. Effect of glutamate concentrations on probe recovery for glutamate in both on-line and off-line derivatization. Four different glutamate concentrations were sued (12.5 μM, 25 μM, 50 μM, 100 μM).

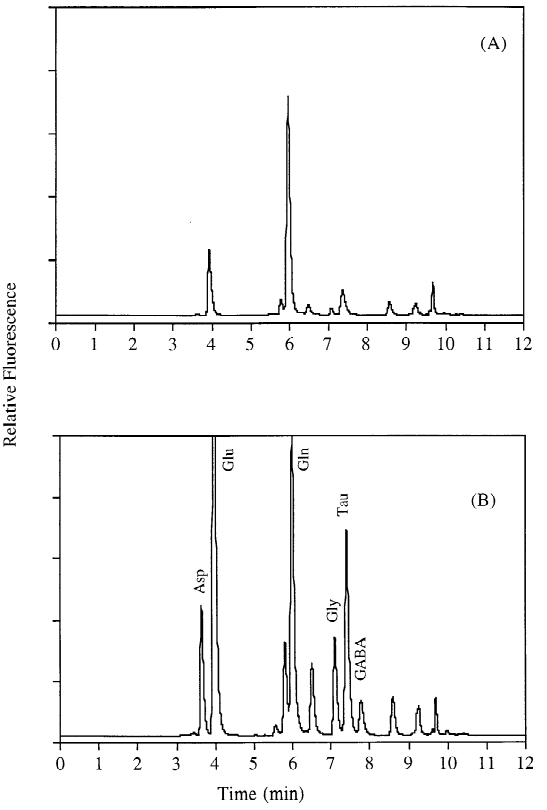

Fig. 3. Typical chromatograms obtained from microdialysates which were derivatized and injected with an on-line manner. Glutamate levels from microdialysates in anesthetized rat brain during basal (A) and ischemia (B) are shown.

by unclamping the blood vessels, the extracellular glutamate levels gradually decreased, but did not return to the basal levels 120 min after the reperfusion (Fig. 4). These results are in good accordance with published results [5,6].

Glutamate is an important excitatory amino acid in central nervous system. The use of microdialysis perfusion in combination with either an HPLC or a CE system is commonly used to monitor the extracellular glutamate levels in brain or spinal cord. In most of the investigations, microdialysates have to be derivatized with fluorescent reagents prior to the injection [14,15]. This derivatization is usually performed in an off-line manner. On-line monitoring has several advantages such as simplified sample pretreatment, shorten analysis time and automatic injection. However, the on-line collection and injection device is usually restricted to HPLC systems equipped with either an absorbance detector or an electrochemical detector in which no derivatization is required. For example, the on-line collection and analysis is commonly used in analyzing neurotransmitters such as dopamine and epinephrine, or antioxidants such as ascorbate and glutathione in microdialysates [17–20]. On-line analysis of glutamate in microdialysates can also be achieved provided on-line derivatization is accomplished prior to the HPLC injection. Recently, CE–LIF has been used for the analysis of microdialysates after the on-line derivatization [16]. In that particular system a very high temporal resolution of 6 s has been achieved. The on-line derivatization system described in this report utilized HPLC system with a fluorescence detector, this HPLC system has the characteristics of simplicity and popularity when compared with CE–LIF. Although the temporal resolution of the HPLC on-line system cannot be comparable to that of the CE–LIF system, however, the longer analysis time for HPLC method allows the separation of other amino acids which also act as important regulatory molecules in brain. A microdialysis probe was placed in the standard amino acid mixtures (at four

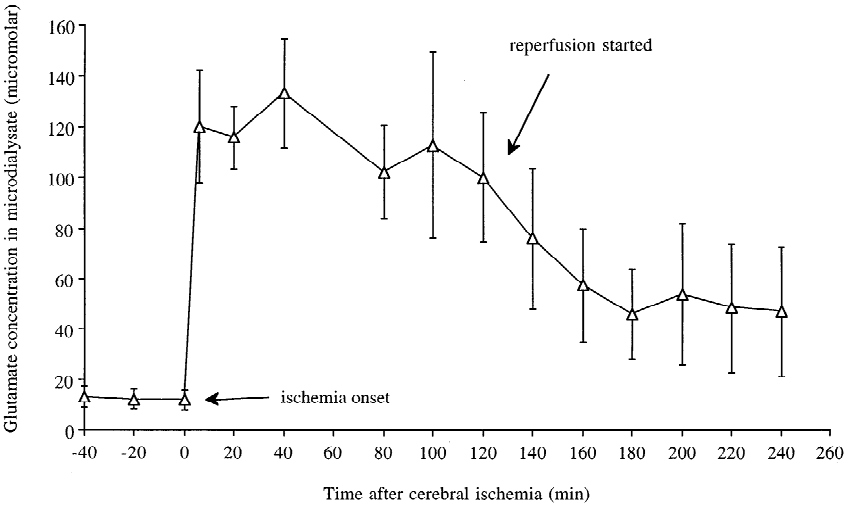

Fig. 4. Glutamate concentration in microdialysates collected from anesthetized brain cortex before and after ischemia; and after reperfusion. Data are represented mean±S.E.M. Data are obtained from the average of 10 rats.

Table 1

	Asp	Glu	Gln	Gly	Tau	GABA
Recovery (%)[a]	30	28	32	20	22	12
Correlation coefficient	0.98	0.99	0.98	0.99	0.99	0.99
Detection limit[b]	0.21	0.1	0.13	0.16	0.18	0.35

[a] The recovery was the slope plotted from the concentrations of amino acids in the microdialysates versus concentrations in the solutions where the probe was placed.

[b] Based on a signal-to-noise ratio of 2, the detection was represented as the pmol per injection at a volume of 20 μl.

different concentrations: 10 μ*M*, 20 μ*M*, 40 μ*M*, 80 μ*M*) containing aspartate, glutamate, glutamine, glycine, taurine and GABA. The amino acid levels in the microdialysates and in the solutions where the probe was placed were determined using this on-line HPLC method, the results are shown in Table 1. Linear responses were observed between the two sets of glutamate concentrations. Additionally, these amino acids can also be identified in the typical chromatograms obtained from injection of microdialysates that were collected before and after cerebral ischemia (Fig. 3A and B, respectively). Thus, this on-line HPLC system would be useful for various research works including the neuroscience investigations when analysis of amino acids are required.

In conclusion, we have developed an assay for in vivo, continuous and automatic monitoring of the brain extracellular glutamate levels in brain cortex of anesthetized rat. This assay has been applied in the investigations of the effect of cerebral ischemia on extracellular glutamate levels in brain cortex of anesthetized rat. This on-line derivatization assay can also be applied to analyze various other amino acids.

Acknowledgements

This research was supported by grants from the National Science Council (NSC 88-2113-M-075A-001) and from the Taichung Veterans General Hospital (TVGH873315C).

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