ELSEVIER

Journal of Chromatography B, 695 (1997) 59–65

JOURNAL OF CHROMATOGRAPHY B

Dual-enzyme assay of glutamate in single cells based on capillary electrophoresis

Ziqiang Wang, Edward S. Yeung*

Department of Chemistry and Ames Laboratory-USDOE, Iowa State University, Ames, IA 50011, USA

Abstract

A new dual-enzyme on-column reaction method combined with capillary electrophoresis has been developed for determining the glutamate content in single cells. Glutamate dehydrogenase and glutamic pyruvic transaminase were used to catalyze the glutamate reaction. Detection was based on monitoring the laser-induced fluorescence of the reaction product NADH, and the measured fluorescence intensity was related to the concentration of glutamate in each cell. Glutamate dehydrogenase catalyzed the formation of NADH, and glutamic pyruvic transaminase drives the glutamate dehydrogenase reaction by removing a reaction product and regenerating glutamate. The detection limit of glutamate is down to the 10^{-8} M level, which is 1 order of magnitude lower than previously reported detection limits based on similar detection methods. The mass detection limit of a few attomoles is far superior to that of any other reports. Selectivity for glutamate is excellent over most amino acids. The glutamate content in single human erythrocytes and baby rat brain neurons were determined with this method and the results agreed well with literature values.

Keywords: Glutamate; Enzymes

1. Introduction

Biochemical analysis and research has developed to a stage where cellular and subcellular measurement techniques become possible. The capability for analysis at the single-cell or even single-molecule levels could provide insights into cellular and subcellular chemical processes without the necessity of averaging over large cell populations. Considerable efforts have been given to the development of analytical methods for glutamate due to its active role in the human sensory system, in liver function, and most importantly, in the central nervous system (CNS). Glutamate functions as a primary excitatory neurotransmitter in CNS. The transport and communication of glutamate with other active species in CNS was proposed to be an important part of brain and CNS signaling processes [1,2].

HPLC [3–8], GC–MS [9,10], isotope assay [11,12], immunocytochemical methods [13,14] and enzymatic assays [15–17] have all been applied to glutamate measurement with good sensitivity. The detection limits reported with these techniques have reached submicromolar concentrations or subfemtomole amounts in biological samples. Despite the extensive published works, few dealt directly with glutamate function at the cellular or subcellular level due to the limitations of the analytical techniques used. However, the importance of such techniques should be emphasized, because CNS is a complicated biological system extremely heterogeneous in both physical and chemical properties in different sections of the brain, among neighboring cells, and even in different regions of a single cell.

*Corresponding author.

PII S0378-4347(97)00101-1

Capillary electrophoresis (CE) offers several important advantages for the analysis of biological samples. The ability of CE to sample extremely small volumes (subnanoliter), its high separation efficiency, and the existence of extremely sensitive detection schemes make CE compatible with single-cell and subcellular analysis relevant to neurochemical studies. Various methods and applications have been reported in recent years for single-cell analysis by using CE [18–31]. The components typically present in single cells are at the femtomole to zeptomole levels, which makes quantitative detection of these compounds extremely challenging.

Laser-induced fluorescence (LIF) detection, electrochemical detection and immunoassay have all been used to obtain sufficiently low mass detection limits for quantitative determination in individual cells [18,20,32]. In this article we describe a dual-enzyme reaction method combined with CE–LIF for determining the glutamate content in single cells. The enzymes used are glutamate dehydrogenase (GDH) and glutamic pyruvic transaminase (GPT). The reaction scheme is as follows:

$$\text{glutamate} + \text{NAD}^+ \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADH} \quad \text{(GDH catalyzed)} \tag{1}$$

$$\alpha\text{-ketoglutarate} + \text{alanine} \rightarrow \text{pyruvate} + \text{glutamate} \quad \text{(GPT catalyzed)} \tag{2}$$

GPT drives the GDH reaction in the desired direction by removing a reaction product and regenerating glutamate. Detection is achieved by monitoring the fluorescence of the product NADH, and the measured fluorescence intensity is related to the concentration of glutamate. Single human erythrocytes and single rat neurons can be studied in this way.

2. Experimental

2.1. Reagents and chemicals

L-Glutamic acid (monosodium salt), L-alanine, β-NAD^+, NADH, Tris–phosphate, L-glutamate dehydrogenase (GDH, 36 units per mg protein, from bovine liver), glutamic pyruvic transaminase (GPT, 80 units per mg protein, from porcine heart), all amino acids used in the interference study, sodium chloride and sodium phosphate (monobasic) were purchased from Sigma (St. Louis, MO, USA). All experiments were carried out in 10 m*M* Tris–phosphate buffer with pH adjusted to around 7.4.

Enzyme solutions were prepared by diluting enzyme extracts with buffer solution to the 10^{-8} *M* level. All solutions were prepared daily to prevent possible contamination and changes due to bacterial growth.

2.2. Capillary electrophoresis instrumentation

Capillary electrophoresis experiments were carried out using an in-laboratory system [18,20]. 20 kV was applied to a 20 μm I.D., 360 μm O.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) from a power supply (0–40 kV, EH series; Glassman High Voltage, Whitehouse Station, NJ, USA) for separation of the enzyme reaction products. The total capillary length was 64 cm and the effective separation length was 45 cm. The LIF detection system consisted of an argon ion laser (Model 2045, Spectra-Physics, Mountain View, CA, USA) with wavelength of 305 nm. The detection window was made by removing a 3 to 5 mm section of polyimide coating on the capillary. A 1 cm focal length quartz lens (Melles Griot, Irvine, CA, USA) was used to focus the laser beam into the detection region. The fluorescence signal emitted from NADH was collected by a 20× microscope objective (Edmund Scientific, Barrington, NJ, USA). A 456 nm interference filter was placed in front of the photomultiplier tube (PMT, Model IP28, Hammamatsu, Bridgewater, NJ, USA) to exclude scattered light. The output signal from the PMT was acquired by ChromPerfect software (Justice Innovations, Palo Alto, CA, USA) at 5 Hz and stored in an IBM/PC computer for subsequent data analysis.

2.3. Human red blood cells

Human red blood cells were obtained from a normal adult male and sampled fresh each time before the experiment. The cells were washed according to the following procedure [23]. After centrifugation, the supernatant serum was siphoned off and a 135 m*M* NaCl and 20 m*M* sodium phosphate

solution at pH 7.4 (PBS) with a volume equivalent to 4–6 times the volume of red blood cells was added. The mixture was then shaken gently and centrifuged again (at 200 *g* for 3 min), followed by removal of the supernatant. This siphoning–mixing–centrifuging cycle was repeated at least 6 times before use. For easy manipulation at the cell injection stage, further dilution might be necessary if the red blood cell number density was found to be too high.

2.4. Rat neuron cells

Hippocampi were removed from 18-day old rat embryos and washed with Earls balanced salt solution (EBSS) for 5 min before incubation in 0.25% trypsin solution for 15 min. The tissue was then washed for 5 min prior to stopping the enzyme action with trypsin inhibitor. The tissue was washed again in EBSS for 5 min before a 1-min incubation in serum-containing medium. Medium was removed and fresh medium was added to the tissue at a rate of 1 ml per 6 hippocampi. The hippocampi were then dissociated by gentle trituration with a 5-ml glass serological pipette until no visible clumps remained.

The dissociated cells were prepared for freezing by adding enough dry sterile dimethylsulfoxide (DMSO) to produce a 9% solution and mixed thoroughly. This cell solution was divided into 250-μl aliquots in freezing vials and placed at 40°C for 15 min. The vials were then positioned in a freezing box which allows gradual freezing and then placed in a −80°C freezer.

Before the experiment, the sample vial was taken out of the freezer and thawed in a 40°C water bath. 2 ml of PBS solution was added to the vial and then centrifuged gently (200 *g*) for 10 min. The supernatant solution was then poured off carefully, and 2.0 ml of fresh PBS solution was added to the neuron cells and shaken thoroughly before use.

2.5. Introduction of cell and on-column reaction

Cell injection into the capillary was done under a microscope. The single-cell injection protocol was reported earlier [20]. A drop of cell suspension was placed on a plastic slide and was examined under the microscope with a magnification of 100×. The first several millimeters of the capillary coating at the injection end was removed, and the tip of the capillary was immersed into the droplet on the slide so that the opening of the tubing could be clearly seen. The buffer vial with the other end of capillary immersed into it was sealed by an air-tight septum with a 20-ml syringe attached. By manually controlling the syringe, the cell could be drawn into the capillary, which can be confirmed with the microscope. As soon as a single cell was introduced into the capillary it was allowed to settle down and adhere to the inner wall of capillary. Then the capillary was immersed into the enzymes+ cosubstrates solution to electrokinetically inject the reactants. The injection time was set at 10 s and the voltage was 20 kV. The cell was lysed in seconds due to the osmotic shock, and the contents were released for reaction with the enzyme solution. After this injection step was completed the capillary was placed back into the standard running buffer. The power supply remained off to provide a 15 min incubation period prior to separation and detection.

3. Results and discussion

3.1. Characteristics of enzymatic essay and optimization

In the dual-enzyme assay scheme used in this study, glutamate dehydrogenase (GDH) catalyzes the formation of NADH from NAD^+ during the oxidation of glutamate. This reaction has been used as a standard assay method for glutamate determination from biological samples [33,34] and has been shown to have good selectivity for neurochemical measurements of glutamate [35,36], with a large linear response range and a low detection limit. The only drawback is that this reaction alone is thermodynamically unfavorable for the production of NADH. The equilibrium constant with NADH as the reaction product is only approximately 10^{-14} [37]. The thermodynamically favored products of GDH catalysis are glutamate and NAD^+. Because of this, in practical applications, the GDH reaction is often coupled with a second enzyme reaction which consumes one of the products of the GDH reaction, driving the reaction in the desired direction and improving the response. Glutamic pyruvic transamin-

ase (GPT) is one of the enzymes that is often adapted for this purpose [15,38,39].

Several parameters were studied to optimize the reactions, including concentrations of the co-substrates NAD^+ and alanine, and concentrations of the enzymes, pH and buffer species. For a 6.25 μM glutamate solution, maximum response was approached for a NAD^+/glutamate ratio of 500 and above. A similar dependence was also found for alanine; the ratio of alanine/glutamate for maximum response is about 2000. The concentration level of glutamate in the capillary for cell studies would be at 10^{-7} M due to dilution. Therefore, in all subsequent experiments, the concentrations of NAD^+ and alanine were kept at $5 \cdot 10^{-5}$ M and $2 \cdot 10^{-4}$ M, respectively. For enzyme concentrations, it was found that when the levels were increased from 10^{-8} M to 10^{-7} M, the signals did not increase significantly; in some cases they even decreased. So, both GDH and GPT concentrations were kept at 10^{-8} M for all experiments. We found that NADH fluorescence signal was more stable and GPT retained higher activity in Tris buffer, while GDH was more stable in phosphate buffer. As a result, Tris–phosphate was chosen as a compromise. pH of all solutions, including the CE running buffer, was kept around 7.4. This is because it approximates physiological conditions of living cells, and because it is close to the pH for maximum enzyme activities.

It is important to control the incubation time in order to provide reliable quantitation. Fortunately, reaction does not occur until the cell lyses, and 15 min is a long period that can be reliably controlled to ±1%. For a constant incubation time, standard solutions can be used to establish the calibration curve.

3.2. Interference study

All 20 amino acids which may exist in cells were studied for possible interferences. The results are shown in Table 1. It can be seen that this method has very high specificity; only glutamine and lysine give about 1–2% signal response compared with the same concentration of glutamate. A possible reason for the glutamine signal is the partial hydrolysis of glutamine, which produces glutamate. The reason for the presence of a small lysine signal is unknown.

3.3. Glutamate determination of single red blood cell

The electropherogram for glutamate determination in a single human erythrocyte is shown in Fig. 1. The step-like NADH peak shape at 7.5 min is due to the continuous formation of NADH when the injected enzyme solution plug moved along the capillary. The integrated area of the step is proportional to the amount of glutamate. The broad peaks from 3 min to 5.5 min are from the enzymes, as was confirmed by control experiments—injecting only the enzyme solution without glutamate. Enzymes are proteins which usually possess some native fluorescence. The detection limit is about 10^{-8} M ($S/N=3$) or attomole levels of NADH. This means the detection limit for glutamate is also in the same range. The baseline returns to the original level at the end of each run, which indicates that the enzymes and cell contents

Table 1
Interference factors for glutamate determination

Compound	% Response	Compound	% Response
L-Glutamate	100	L-Alanine	0
L-Arginine	0	L-Asparagine	0
L-Aspartate	0	L-Cysteine	0
L-Glutamine	~2	L-Glycine	0
Hydroxy L-proline	0	L-Histidine	0
L-Isoleucine	0	L-Leucine	0
L-Lysine	<1	L-Methionine	0
L-Phenylalanine	0	L-Proline	0
L-Serine	0	L-Threonine	0
L-Tryptophan	0	L-Tyrosine	0
L-Valine	0		

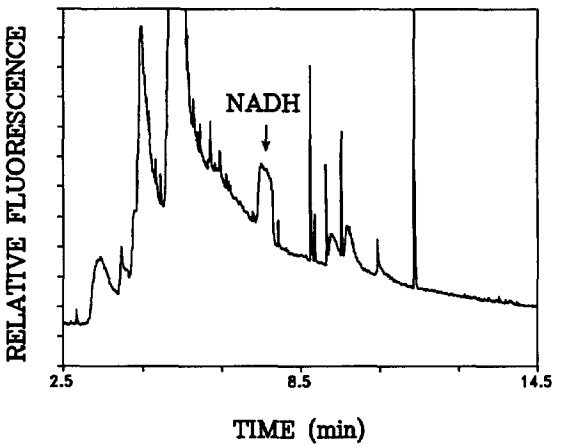

Fig. 1. Electropherogram of the enzymatic assay of a single red blood cell.

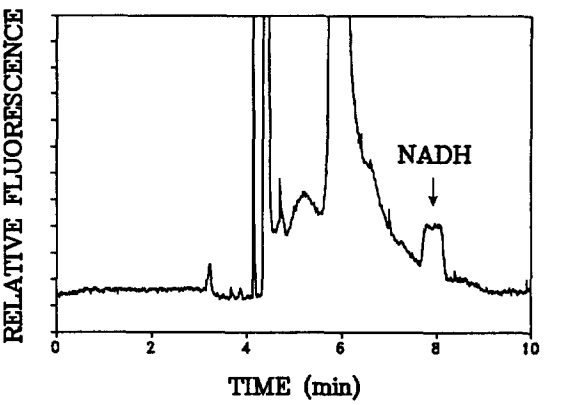

Fig. 3. Electropherogram of the enzymatic assay of a single rat neuron.

are not adsorbed to the capillary walls. A standard series of enzyme assays with glutamate concentrations from $5 \cdot 10^{-8}$ M to $1 \cdot 10^{-5}$ M ($r^2 = 0.991$) is used to calibrate the amount of NADH produced from single red blood cells, and in turn to calculate the amount of glutamate in the cells. The results of analyses of 19 single red blood cells are shown in Fig. 2. According to these 19 runs, the amount of glutamate in single red blood cells ranges from $3.0 \cdot 10^{-17}$ M to $2.8 \cdot 10^{-16}$ M, or a concentration range from 0.33 mM to 3.1 mM. These values reflect real differences among single cells, since the precision for repeated measurements of standard solutions is much higher. Compared to literature values [40,41], which are based on the bulk average, the results here are in good agreement.

3.4. Glutamate determination in single rat neuron cells

The electropherogram resulting from enzyme assay of a single rat neuron is shown in Fig. 3. The results of analyses of 22 single neurons are shown in Fig. 4. According to these results, the amount of glutamate in a single neuron is about $2.0 \cdot 10^{-18}$ M to $3.3 \cdot 10^{-16}$ M, or concentration levels from 3.8 μM to

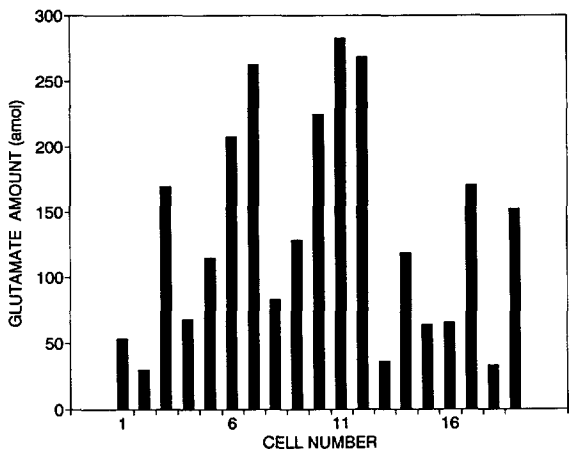

Fig. 2. Glutamate amounts in 19 individual human erythrocytes.

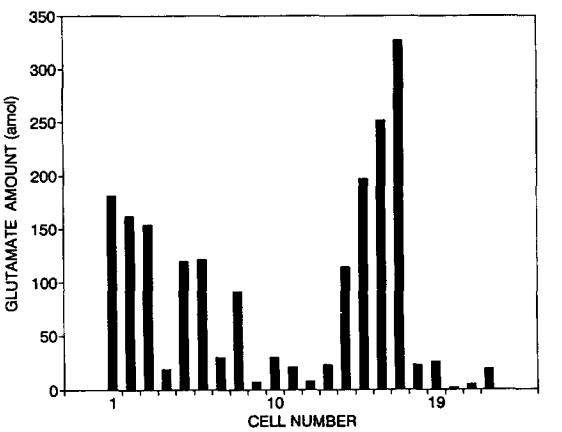

Fig. 4. Glutamate amounts in 22 individual rat neurons.

0.64 m*M* (assuming the diameter of a neuron is 10 μm). Again, large cell-to-cell variations are evident.

No previously reported value of glutamate amount in single rat neurons was found to compare with our results. All data found in the literature about glutamate in rat brain neurons have the unit (μg (or pg)/wet weight of tissue), which is not comparable here. However, it was reported [42] that the average concentration level of glutamate in the rat central neural system is about 1 mmol/kg wet weight of tissue, with the highest concentrations existing in astrocytes (about 10 mmol/kg wet weight), and the lowest value in extracellular fluid (about 1–3 μmol/kg wet weight). The concentration gradient of glutamate across the plasma membrane is about a thousand-fold. Also, from several publications [43–45] which studied the CNS glutamate uptake system it has been suggested that the glutamate uptake rate for neurons is about 10 attomol/min per cell in vivo. Therefore, these data suggest that our results lie in a reasonable range.

Acknowledgments

The authors thank Philip Haydon for providing the rat neuron cells. The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences.

References

[1] F. Fonnum, J. Neurochem., 42 (1984) 1–11.
[2] V. Parpura, T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija and P.G. Haydon, Nature, 369 (1994) 744–747.
[3] P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667–1674.
[4] P.L. Lee, Biochem. Med., 10 (1974) 107–121.
[5] L. Zecca, F. Zambotti, N. Zonta and P. Mantegazza, J. Chromatogr., 233 (1982) 307–312.
[6] K.L. Roger, R.A. Philibert, A.J. Allen, J. Molitor, E.J. Wilson and G.R. Dutton, J. Neurosci. Methods, 22 (1987) 173–179.
[7] W.L. Caudill, G.P. Honck and R.M. Wightman, J. Chromatogr., 227 (1982) 331–339.
[8] L.T. Graham and M.H. Aprison, Anal. Biochem., 15 (1966) 487–497.
[9] M. Wolfensberger and U. Amsler, J. Neurochem., 38 (1982) 451–456.
[10] P.L. Wood and D.L. Cheney, in A.A. Boulton and G.B. Baker (Editors), Neuromethods 3: Amino Acids, Humana Press, Clifton, NJ, 1985, pp. 51–89.
[11] J.W. Ferkany, in A.A. Boulton and G.B. Baker (Editors), Neuromethods 3: Amino Acids, Humana Press, Clifton, NJ, 1985, pp. 117–154.
[12] R.F. Butterworth, in A.A. Boulton and G.B. Baker (Editors), Neuromethods 3: Amino Acids, Humana Press, Clifton, NJ, 1985, pp. 81–96.
[13] J.Y. Wu and C.T. Lin, in A.A. Boulton and G.B. Baker (Editors), Neuromethods 3: Amino Acids, Humana Press, Clifton, NJ, 1985, pp. 155–178.
[14] P.L. Nakane and G.B. Pierce, Jr., J. Cell. Biol., 33 (1967) 307–318.
[15] A. Wang and M.A. Arnold, Anal. Chem., 64 (1992) 1051–1055.
[16] D.G. Nicholls, J. Neurochem., 52 (1989) 331–341.
[17] M. Fosse, J. Kolstad and F. Fonnum, J. Neurochem., 47 (1986) 340–347.
[18] Q. Xue and E.S. Yeung, Anal. Chem., 66 (1994) 1175–1178.
[19] E.S. Yeung, P. Wang, W. Li and R.W. Giese, J. Chromatogr., 608 (1992) 73–77.
[20] B.L. Hogan and E.S. Yeung, Anal. Chem., 64 (1992) 2841–2845.
[21] Q. Xue and E.S. Yeung, J. Chromatogr. A, 661 (1994) 287–295.
[22] T.T. Lee and E.S. Yeung, J. Chromatogr., 595 (1992) 319–325.
[23] T.T. Lee and E.S. Yeung, Anal. Chem., 64 (1992) 3045–3051.
[24] R.A. Wallingford and A.G. Ewing, Anal. Chem., 60 (1988) 1972–1975.
[25] A.G. Ewing, J. Neurosci. Methods, 48 (1993) 215–224.
[26] H.K. Kristensen, Y.Y. Lau and A.G. Ewing, J. Neurosci. Methods, 51 (1994) 188–189.
[27] R.T. Kennedy, R.L. St. Claire, J.G. White and J.W. Jorgenson, Mikrochim. Acta, 2 (1987) 37–45.
[28] R.T. Kennedy, M.D. Oates, B.R. Cooper, B. Nickerson and J.W. Jorgenson, Science, (Washington, DC) 246 (1989) 57–63.
[29] B.R. Cooper, J.A. Jankowski, D.J. Leszczyszyn, M.R. Wightman and J.W. Jorgenson, Anal. Chem., 64 (1992) 691–694.
[30] J.B. Chien, R.A. Wallingford and A.G. Ewing, J. Neurochem., 54 (1990) 633–638.
[31] T.M. Olefirowicz and A.G. Ewing, J. Neurosci. Methods, 34 (1990) 11–15.
[32] Z. Rosenzweig and E.S. Yeung, Anal. Chem., 66 (1994) 1771–1776.
[33] H.F. Fisher, Methods Enzymol., 113 (1985) 16–27.
[34] I.A. Olson and C.B. Anfinsen, J. Biol. Chem., 202 (1955) 841–845.
[35] G.S. Ayoub and D.R. Copenhagen, J. Neurosci. Methods, 37 (1991) 7–14.

[36] D.G. Nicholls, J. Neurochem., 52 (1989) 331–341.
[37] S. Subramanian, Biophys. Chem., 7 (1978) 375–378.
[38] M.H. Saier and W.T. Jenkins, J. Biol. Chem., 242 (1967) 91–93.
[39] L. Ernster, Methods Enzymol., 113 (1985) 309–312.
[40] R.B. Pennell, in C. Bishop and D.M. Surgenor (Editors), The Red Blood Cell, Academic Press, New York, NY, 1964, pp. 29–70.
[41] R.H. Mcmenamy, C.C. Lund, G.J. Nevill and D.F. Wallach, J. Clin. Invest., 39 (1960) 1675–1705.
[42] O.P. Otterson, N. Zhang and F. Walberg, Neuroscience, 46 (1992) 513–534.
[43] N.C. Danbolt, Prog. Neurobiol., 44 (1994) 377–396.
[44] A. Schonsbon, H. Fosmarr and L. Hertz, J. Neurochem., 25 (1975) 909–911.
[45] R.D. Gordon and R. Balăzs, J. Neurochem., 40 (1983) 1090–1099.