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Determination of amino acid neurotransmitters in cerebral cortex of rats administered with baicalin prior to cerebral ischemia by capillary electrophoresis–laser-induced fluorescence detection

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Abstract

An efficient, sensitive and rapid analysis of the amino acid neurotransmitters in the cerebral cortex of rats was developed by capillary electrophoresis with laser-induced fluorescence detection and fluorescein isothiocyanate (FITC) derivatization. This method was used to investigate the pharmacological effect of baicalin during cerebral ischemia. Different parameters which influenced derivatization and separation were optimized. The separation of amino acids was carried out in an uncoated fused-silica capillary (57 cm×75 μm I.D.) with a buffer of 15 m*M* borate at pH 9.2 and an applied voltage of 17.5 kV. The detection limits for six amino acids were in the range of 2.1×10^{-11}–6.3×10^{-10} *M*. The changes in the level of amino acid neurotransmitters in brain cortex of three experimental rat groups were studied by this capillary electrophoresis–laser-induced fluorescence detection method. The results show that cerebral ischemia can cause a significant elevation in the concentrations of Glu, Asp, GABA, and Gly in cerebral cortex. Baicalin administration can attenuate the elevations of Glu and Asp induced by cerebral ischemia. This research demonstrates that baicalin may act as a neuroprotectant during cerebral ischemia.

Keywords: Amino acid neurotransmitters; Baicalin

1. Introduction

Elevations in various neurotransmitters and neuromodulators occur during cerebral ischemia [1,2]. The resulting derangements in neurotransmission have been proposed to play a role in the eventual development of tissue damage [3]. In particular, it has been postulated that the large rise in extracellular glutamate and aspartate underlie an excitotoxic mechanism for neuronal cell damage [4,5].

The flavonoid Baicalin, isolated from the dried root of *Scutellariae radix* (Huang Qin in Chinese), has been widely used in traditional Chinese herbal medicine for centuries to treat allergic and inflammatory diseases [6,7]. Research has shown that baicalin has multiple biological activities such as vasodilatory [8], anti-viral [9], anti-thrombotic [10], anti-oxidant [11] and anti-tumor activities [12,13].

Recently, some studies have demonstrated that baicalin has a protective effect against brain edema

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and cerebral ischemic damage. For example, Han [14] reported that derivatives of baicalin could inhibit the neurotoxic action induced by kainic acid (a receptor agonist of glutamic acid). However, there is no report concerning baicalin's effect on the level of amino acid neurotransmitters in brain tissue during cerebral ischemia. In order to investigate the pharmacological effect of baicalin during cerebral ischemia, it is necessary to establish an efficient and sensitive method for the analysis of amino acid neurotransmitters in brain tissue.

HPLC with electrochemical or fluorescence detection has been widely used in the analysis of neurotransmitters [15,16]. Capillary electrophoresis with laser-induced fluorescence detector (CE–LIF) is a powerful analytical technique that provides high resolving efficiency, short analysis time and high detection sensitivity, which make it quite suitable for the analysis of multiple compounds in biological samples. Consequently, CE–LIF has become another choice for neurotransmitter measurement and has been applied to the analysis of amino acid neurotransmitters in brain tissue samples [17] and microdialysis samples [18,19].

In this work, we have investigated whether baicalin could influence the levels of amino acid neurotransmitters during cerebral ischemia. The tissue samples of cerebral ischemia were obtained using the model of middle cerebral artery occlusion (MCAO), a well-established and modified procedure [20]. The rats employed in this experiment were classified into three groups: control (normal), focal cerebral ischemia and baicalin administration prior to cerebral ischemia. A rapid and sensitive method for the analysis of amino acid neurotransmitters in brain cortex was developed using CE–LIF and FITC derivatization and used to analyze the level of amino acid neurotransmitters in the above three groups. Six amino acid neurotransmitters (Gln, GABA, Ala, Gly, Glu and Asp) were determined.

The present research shows that cerebral ischemia can result in significant elevations of the concentrations of Glu, Asp, GABA and Gly in cerebral cortex. Baicalin administration can attenuate the elevations of Glu and Asp induced by cerebral ischemia. These results indicates that baicalin may act as a neuroprotectant during cerebral ischemia.

2. Experimental

2.1. Chemicals

Baicalin was provided by the Experimental Center of Shanxi Normal University (Shanxi, China), the purity of which is more than 95%. Unless stated otherwise, all chemicals were of analytical reagent-grade. γ-Amino-*n*-butyric acid (GABA), glutamate (Gln), alanine (Ala), glysine (Gly), aspartate (Asp) and glutamic acid (Glu) were obtained from Shanghai Reagent Corp. (Shanghai, China). Fluorescein isothiocyanate isomer I (FITC) was purchased from Sigma (St Louis, MO, USA). FITC stock solution (9 m*M*) was prepared in acetone, and stored at 4 °C in the dark. Amino acids stock solutions (1 m*M*) were prepared in deionized water and diluted to the desired concentration before use. The buffer pH was adjusted with 1.0 *M* H_3BO_3 or 1.0 *M* NaOH. All solutions were prepared by triple-distilled and deionized water, and filtered through a 0.45-μm filter (Millisolve Kit, Millipore).

2.2. Animal and pharmacological experiments

2.2.1. Animals

Adult male Wistar rats with initial body weight of 250–300 g were used in this study and obtained from Hubei Academy of Medical Science (Wuhan, China).

2.2.2. Control group

Adult male Wistar rats were anesthetized with chloral hydrate (350 mg/kg). Brain cortex tissues (10–20 mg) were removed and weighed accurately. The number of rats was six ($n=6$).

2.2.3. Cerebral ischemia group

Focal cerebral ischemia was induced using the model of middle cerebral artery occlusion (MCAO). In the experiment, the right common carotid artery was exposed through a midline incision in the neck. A nylon suture (20 mm diameter) with its tip rounded by heating over a flame and subsequently coated with poly-L-lysine was induced into the internal carotid artery for a length of 17.5–18.5 mm from the bifurcation. The focal cerebral ischemia of these rats was induced by this procedure. The treated

rats were anesthetized with chloral hydrate 1 h after right MCAO. Brain cortex tissues (10–20 mg) were taken out and weighed accurately. The number of rats was six ($n=6$).

2.2.4. Baicalin administration prior to cerebral ischemia

The rats in this group were administered with baicalin intrasublingually at a dose of 300 mg/kg. Thirty minutes later, focal cerebral ischemia of these rats was induced by the same procedure as in Section 2.2.3. One hour later, the rats were anesthetized with chloral hydrate. Brain cortex (10–20 mg) tissues were removed and weighed accurately. The number of rats was six ($n=6$).

2.2.5. Sample treatment

The brain cortex tissues obtained from the above three groups were minced and homogenized in a certain volume of 15 m*M* borate buffer (pH 9.2) for 20 min (The ratio of tissue weight/buffer volume was 5 mg/1 ml). The homogenates were mixed with an equal volume of chloroform and shaken vigorously (15 min) to deposit proteins. After centrifugation at 20 000 rpm for 20 min, the supernatants were transferred to Eppendorf tubes and stored at −70 °C before use.

2.3. Capillary electrophoresis

2.3.1. Apparatus

A MDQ Capillary Electrophoresis System (Beckman-Fullerton, CA, USA) with a laser-induced fluorescence detector was used for all experiments. The excitation was carried out with an argon ion laser (3 mW) at a wavelength of 488 nm. Separations took place in an uncoated fused-silica capillary [57 cm (50 cm to the detector)×75 μm I.D] (Beckman-Fullerton, CA, USA) at 25 °C. The voltage applied was 17.5 kV. The typical separation buffer was a buffer of 15 m*M* borate at pH 9.2. Sample injection was accomplished by pressure (0.5 p.s.i.) for 5 s. The capillary was washed with 0.1 *M* sodium hydroxide, deionized water and separation buffer before use.

2.3.2. Precolumn derivatization of standard amino acid neurotransmitters

Precolumn derivatization of standard amino acid neurotransmitters was conducted in microvials. A final volume of 0.5 ml was used for each experiment. After appropriate amounts of the amino acid solutions were diluted and mixed in 5 m*M* borate buffer (pH 9.6), FITC was added to give a final concentration 30 times greater than the total concentration of amino acids. Typically, the mixed solution was reacted at room temperature (20 °C) in the dark for 16 h. The derivatization solutions were then stored in the dark at 4 °C before use.

2.3.3. Precolumn derivatization of amino acid neurotransmitters in brain cortex

Fifty microliters of supernatant obtained by procedures discussed in Section 2.2 were mixed with 400 μl of 5 m*M* borate buffer (pH 9.6) and 50 μl of 9 m*M* FITC solution. The mixture was then allowed to react at room temperature (20 °C) in the dark for 16 h. Prior to analysis, derivatization solutions were diluted 10-fold in running buffer.

2.4. Statistical analysis

Conventional statistical methods have been used to calculate mean values (mean±SD) and relative standard deviations (RSD) of the mean. Statistical differences for amino acid neurotransmitters between different experimental groups were determined by Student's *t*-test. $P<0.05$ was accepted as denoting a statistically significant difference.

3. Result and discussion

3.1. Optimization of the derivatization and separation conditions

Although the derivatization conditions are based on the methods previously reported [17–19,21,22], this experiment aimed at achieving the best possible compromise between higher derivatization efficiency and fewer side reaction peaks. Several parameters affecting derivatization were optimized, including

concentration and pH of the buffer, amount of FITC, reaction temperature and time.

In order to maintain a background electrolyte compatible with that in the separation capillary, borate buffer was chosen as derivatization buffer. Our experiment showed that the concentration of borate buffer had little influence on derivatization efficiency. To reduce electric migration diffusion in capillary and improve resolution, a buffer of 5 m*M* borate was selected as the derivatization buffer.

The influence of buffer pH on derivatization efficiency was studied. Six 10^{-6} *M* standard amino acids were derivatized with FITC in borate buffer (5 m*M*) at various pH values. The mixed solutions were reacted at room temperature (20 °C) in the dark for 16 h. Fig. 1 showed the effect of borate buffer pH on derivatization efficiency. At pH 9.6, the derivatization efficiency was higher and side reaction peaks were relatively fewer. Therefore, a buffer of 5 m*M* borate at pH 9.6 was chosen as the derivatization buffer.

Different ratios of FITC/amino were tried. At a ratio of 30, higher derivatization efficiency was obtained with fewer side reaction peaks. Therefore, a FITC/amino acid ratio of 30 was considered optimal.

In previous reports, the derivatization temperature and time of amine compounds with FITC were classified into two kinds. One was at room temperature (20–25 °C) in the dark for 12–16 h [19,21,22]. The other was at an elevated temperature (40–45 °C) with traces of pyridine for 4–6 h [17,18]. Until now, there has not been a consensus about which derivatization condition is better. In the present work, two parallel experiments were carried out to compare the derivatization efficiency of 10^{-6} *M* standard amino acids with FITC. The first test was carried out at room temperature (20 °C) in the dark for 16 h and the second at 40 °C with pyridine (0.002%, v/v) for 6 h. Higher derivatization efficiency and well-defined electrophoretic peaks of analytes were obtained in the first test. In contrast, more interfering peaks appeared in the electropherogram in the second test. Perhaps pyridine impurities produced the additional FITC derivatives and the higher reaction temperature accelerated side reaction. So, derivatization at room temperature (20 °C) in the dark in the absence of pyridine was chosen as optimum condition.

The stability of the amino acid derivative at 4 °C in the dark was studied over a period of 48 h. No significant change in the corrected peak area for amino acid derivative was found (RSD<4%), indicating favorable stability of the derivative.

On the basis of the above results, the optimum

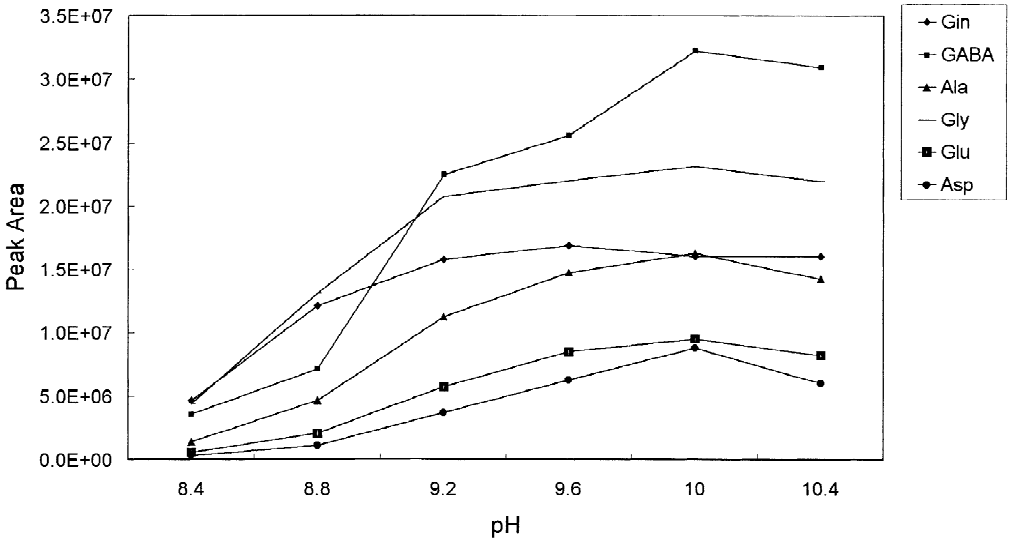

Fig. 1. The effect of derivatization buffer pH on peak area of amino acids. Separation condition as in Section 2.3.1.

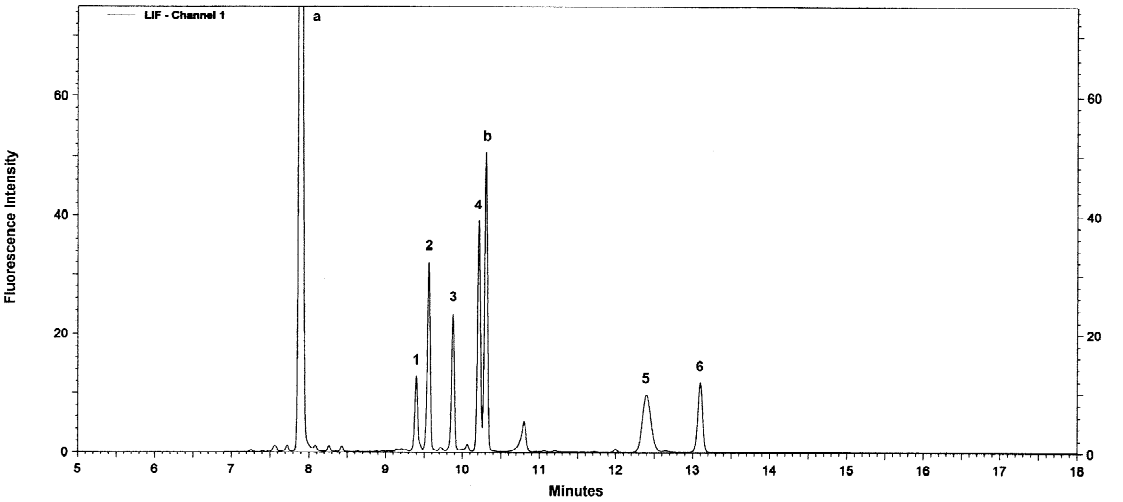

Fig. 2. Electropherogram of standard amino acids. Electrophoretic conditions: buffer of 15 m*M* borate at pH 9.2; voltage: 17.5 kV; capillary temperature: 25 °C; injection 5 s at 0.5 p.s.i. Amino acid concentrations: GABA, Ala, Glu, Asp 4.0×10^{-7} *M*, respectively; Gly and Gln 2.0×10^{-7} *M*, respectively. (1) Gln, (2) GABA, (3) Ala, (4) Gly, (5) Glu, (6) Asp, (a) FITC, (b) fluorescein amine.

derivatization conditions were: a buffer of 5 m*M* borate at pH 9.6, FITC/amino acid ratio of 30, and derivatization at 20 °C in the dark for 16 h.

Borate buffer was used as background electrolyte for the separation of amino acid neurotransmitters in this work, for borate buffer provides a stable electrosmotic flow and low background noise. The concentration and pH of borate buffer, separation voltage and temperature were also optimized. The optimum separation condition was a buffer of 15 m*M* borate at pH 9.2 with a separation voltage of 17.5 kV and a temperature of 25 °C.

Fig. 2 shows typical electropherogram of standard amino acid solution.

3.2. Analytical characterization

The reproducibility (expressed in term of relative standard deviation, RSD) test was carried out with 10^{-7} *M* standard amino acid. Derivatization conditions were a buffer of 5 m*M* borate at pH 9.6, FITC/amino acid ratio of 30, derivatization at 20 °C for 16 h. Electrophoretic separation conditions were a buffer of 15 m*M* borate at pH 9.2 with a separation voltage of 17.5 kV and a temperature of 25 °C. The RSDs for the various amino acids are summarized in Table 1. The RSDs of the migration times are less than 0.5% and the RSDs of the peak areas are typically in the range of 2.2–4.1%.

Table 1
Migration time, RSD of migration time and peak area, regression equation, linear range and correlation coefficients

Analyte	Migration time (min)	RSD(%) (n=6)		Regression equation	Linear range (μmol/l)	Correlation coefficients
		Time	Area			
Gln	9.38	0.28	2.4	$y=3.8\times10^{12}x+690\,053$	0.01–1.0	0.998
GABA	9.54	0.34	2.2	$y=1.2\times10^{12}x+786\,421$	0.01–1.0	0.999
Ala	9.84	0.29	4.0	$y=3.2\times10^{12}x+108\,800$	0.01–1.0	0.996
Gly	10.17	0.33	4.1	$y=6.5\times10^{12}x+818\,853$	0.03–1.0	0.995
Glu	12.36	0.48	2.4	$y=1.5\times10^{12}x+71\,844$	0.05–5.0	0.997
Asp	13.07	0.44	2.2	$y=1.1\times10^{12}x+400\,084$	0.05–5.0	0.995

x, concentration of amino acid (mol/l); y, peak area of amino acid derivatives in electropherogram.

The linearity of the present method was determined by analyzing standard solutions containing known concentrations (with six different amounts of analytes) ranging from 1.0×10^{-8} to 5.0×10^{-6} *M*. From the electropherogram, the peak area is plotted against the concentration of the amino acid to obtain the calibration graph for each amino acid. The results are shown in Table 1. The correlation coefficient of each amino acid was 0.995 or better.

Limits of detection for each amino acid were estimated taking three times the standard deviation of the peak areas obtained from these solutions and calculating the corresponding concentration with the regression equation. The results are listed in Table 2. The detection limits were in the range of 2.1×10^{-11}–6.3×10^{-10} *M*, which are equal to or lower than that of previous reports [17–22].

The recovery data were obtained by spiking brain cortex homogenate with known quantities of standard solutions of amino acids. The results are shown in Table 2. Within-day and between-day precision for each amino acids was examined using a brain cortex homogenate (treated as in Section 2.3.3) containing Glu (3.50 μmol/l), Asp (3.81 μmol/l), Gln (5.40 μmol/l), GABA (1.71 μmol/l), Ala (1.48 μmol/l), and Gly (3.61 μmol/l), respectively. The precisions expressed as RSD for within-day determinations ($n=6$) and those for between-day determinations ($n=3$) are listed in Table 2.

3.3. Determination of amino acid neurotransmitters in brain cortex homogenates of the three experimental groups

Brain cortex samples of rats were treated as Section 2.2.5. The derivatizations were carried out as Section 2.3.3. CE separations were carried out under the optimal condition listed in Section 3.1. Figs. 3–5 show typical electropherograms for amino acid neurotransmitters in the three groups, which are control, cerebral ischemia and administration with baicalin prior to cerebral ischemia, respectively.

Peaks were identified by comparing migration times and spiking samples with known quantities of standard solutions of amino acids. As marked in Figs. 3–5, six amino acid neurotransmitters were identified. Other amino acids in the brain cortex also led to some electrophoretic peaks. Except for Ala, which was partially resolved with an unidentified constituent in the homogenate of brain cortex, the other five amino acids (Gln, GABA, Gly, Glu and Asp) were well separated from adjacent constituents in the electropherogram.

The measured concentrations of amino acid neurotransmitters in the cerebral cortex of the three groups are shown in Table 3.

Glutamate and aspartate are the major excitatory neurotransmitters in the brain. At high concentrations, they can function as an excitoxin, inducing neuronal injury and death [23]. Extracellular concentrations of Glu and Asp in brain tissues increase rapidly during ischemic episodes, which have been proposed as a significant cause of neuronal cell damage [24]. GABA is an inhibitory neurotransmitter in the brain, which can inhibit the excess activity of nerve cells. Glycine is also an inhibitory neurotransmitter. Although the precise role of glycine in the brain is unclear, it may play a primary neuromodulatory role in the brain [25,26].

Table 3 shows that the concentrations of Glu, Asp, GABA and Gly in the cerebral cortex of the cerebral ischemia group increase significantly compared with the control ($P<0.01$). The increase in Ala is not of statistical significance. In general, these results are in

Table 2
Detection limits, recovery, within-day precision and between-day precision of six amino acids

Analyte	Detection limit (mol/l)	Recovery (%, $n=3$)	Within-day precision (%, $n=6$)	Between-day precision (%, $n=3$)
Gln	6.6×10^{-11}	102.6	2.79	3.12
GABA	2.1×10^{-11}	97.2	1.87	2.38
Ala	8.9×10^{-11}	97.1	2.81	3.78
Gly	1.7×10^{-10}	101.2	2.06	2.72
Glu	4.2×10^{-10}	98.8	1.61	2.09
Asp	6.3×10^{-10}	98.3	2.75	3.71

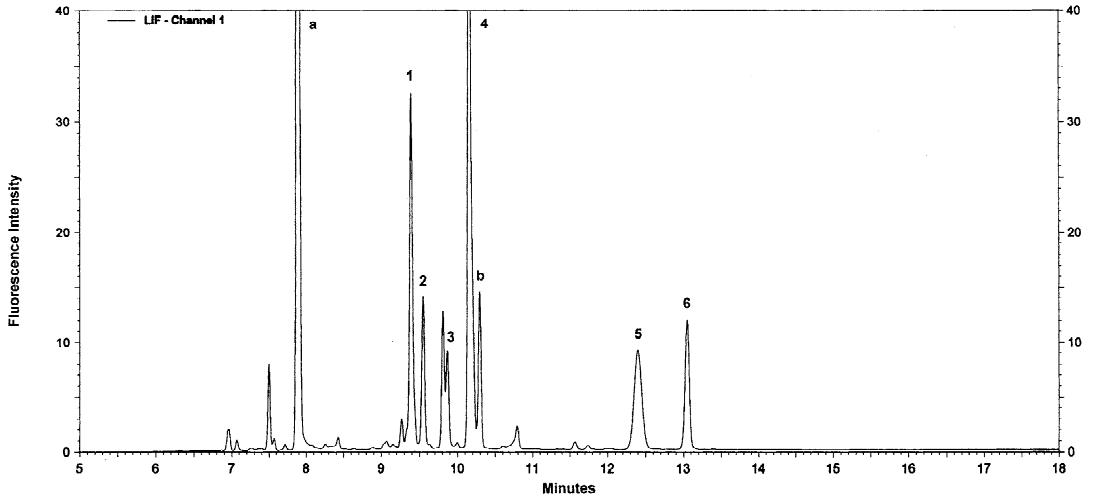

Fig. 3. Electropherogram of the brain cortex homogenate of the control rat. Electrophoretic conditions as in Fig. 2. (1) Gln, (2) GABA, (3) Ala, (4) Gly, (5) Glu, (6) Asp, (a) FITC, (b) fluorescein amine.

agreement with other reports [27,28], which demonstrated that elevations of various neurotransmitters occur during cerebral ischemia. Our results show that compared with the control group, the concentrations of Glu, Asp, GABA and Gly in the cerebral ischemia group increase by 84, 92, 71 and 74%, respectively. The results of the present work show that the increased magnitudes of inhibitory neurotransmitters (GABA and Gly) are low than that of excitatory neurotransmitters (Glu and Asp) after cerebral ischemia occurs. It is noticeable that there is a significant decrease in the concentration of Gln after cerebral ischemia occurred. Perhaps the metabolic equilibrium between Glu and Gln is disturbed when cerebral ischemia occurs. The exact reason for this phenomenon is under investigation.

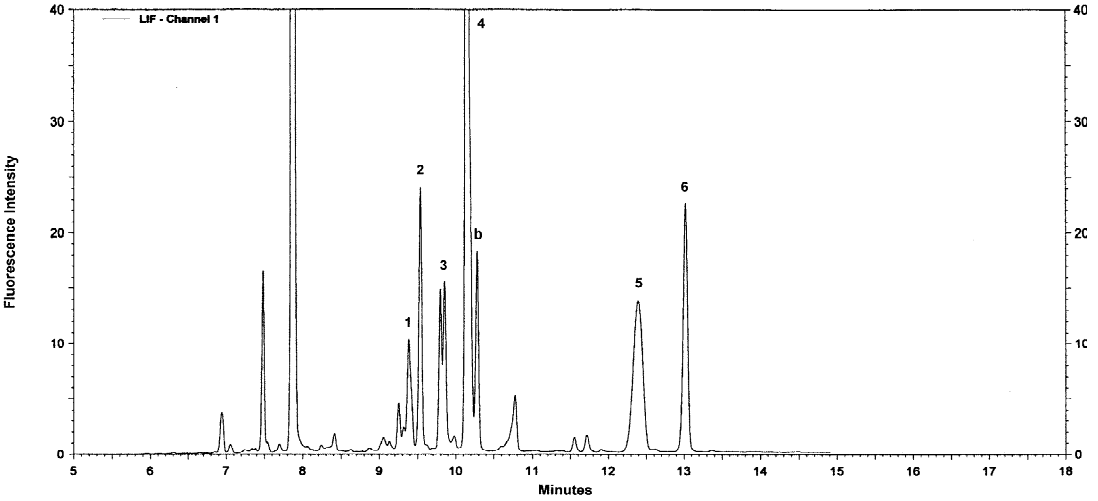

Fig. 4. Electropherogram of the brain cortex homogenates of the cerebral ischemia rat. Electrophoretic conditions as in Fig. 2. (1) Gln, (2) GABA, (3) Ala, (4) Gly, (5) Glu, (6) Asp, (a) FITC, (b) fluorescein amine.

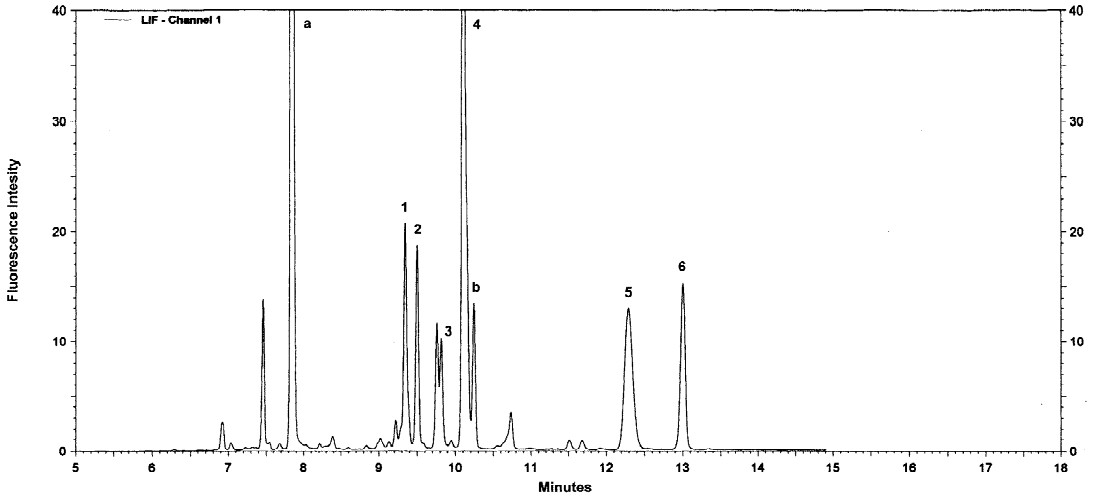

Fig. 5. Electropherogram of the brain cortex homogenate of the rat administered with baicalin prior to cerebral ischemia. Electrophoretic conditions as in Fig. 2. (1) Gln, (2) GABA, (3) Ala, (4) Gly, (5) Glu, (6) Asp, (a) FITC, (b) fluorescein amine.

Compared with the cerebral ischemia group, the levels of Glu, Asp, GABA and Gly in the cerebral cortex of baicalin administration group were reduced significantly ($P<0.01$), and that of Gln increased notably. The above results demonstrate that in the presence of baicalin, the elevations of Glu and Asp evoked by cerebral ischemia are significantly attenuated; this may be a potential mechanism by which baicalin can act as a neuroprotectant during cerebral ischemia.

4. Conclusions

The present work makes use of capillary electrophoresis coupled laser-induced fluorescence to investigate the pharmacological effect of baicalin during cerebral ischemia. Cerebral ischemia can result in significant elevations of Glu, Asp, GABA and Gly in cerebral cortex. Baicalin administration can attenuate the elevations of Glu and Asp induced by cerebral ischemia. Baicalin may have a neuroprotective effect during cerebral ischemia.

Acknowledgements

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Table 3
Measured amounts of amino acid neurotransmitters in the brain cortex tissues of three groups (μg/mg, $n=6$)

Analytes	Control	Cerebral ischemia	Baicalin administration prior to cerebral ischemia
Glu	0.97±0.086	1.77±0.100*	1.10±0.043**
Asp	0.98±0.047	1.88±0.068*	1.20±0.076**
GABA	0.32±0.035	0.56±0.050*	0.42±0.041**
Gln	1.50±0.110	0.41±0.025*	1.01±0.120**
Ala	0.20±0.116	0.28±0.078	0.26±0.063
Gly	0.47±0.081	0.92±0.062*	0.67±0.010**

n, number of rats in each group.
*$P<0.01$ compared with control group.
**$P<0.01$ compared with cerebral ischemia group.

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