

Protocol

In vivo microdialysis in the visual cortex of awake cat II: Sample analysis by microbore HPLC–electrochemical detection and capillary electrophoresis–laser-induced fluorescence detection

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Abstract

Sampling and monitoring release of excitatory and inhibitory amino acids in the striate cortex of mammals will provide important information for visual system research. Two microbore high performance liquid chromatography–electrochemical detection methods and a capillary electrophoresis–laser induced fluorescence detection were developed to determine the inhibitory amino acid, γ -aminobutyric acid and the excitatory amino acids, glutamate and aspartate in microdialysates of cat striate cortex. In the liquid chromatography method, samples were derivatized using OPA–TBT. Ten microliters of derivatized product was injected onto the microbore column (100 \times 1 mm i.d., C8) for quantitative analysis. Electrochemical detection was employed. In the capillary electrophoresis method, samples were derivatized using fluorescein isothiocyanate and separated in borate buffer within 15 min, then detected by a laser-induced fluorescence detector. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids; GABA

Keywords: In vivo microdialysis; HPLC; Electrochemical detection; Capillary electrophoresis; Laser-induced fluorescence; Amino acid

1. Type of research

- Investigation of neurotransmitter release in the striate cortex of the cat with different visual input manipulation — such as limited central retinal lesion, monocular deprivation and visual stimulation [1,2,5,9,14].
- Determination of excitatory amino acids, aspartate (Asp), glutamate (Glu) and the inhibitory amino acid, γ -aminobutyric acid (GABA), in microdialysates by

microbore high performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) [4,8,12,13].

- Determination of excitatory amino acids, Asp, Glu and the inhibitory amino acid, GABA, in microdialysates by capillary electrophoresis (CE)–laser-induced fluorescence detection (LIF) [3,6,7,11,15].

2. Time required

- Analysis of Asp and Glu in one microdialysis sample by microbore HPLC–ED: 40 min.
- Analysis of GABA in one microdialysis sample by microbore HPLC–ED: 25 min.
- Analysis of Asp, Glu and GABA in one microdialysis sample by CE–LIF: 13 min.

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3. Materials and devices

3.1. Chemicals and reagents

- Asp, Glu, GABA and fluorescein isothiocyanate isomer I were obtained from Sigma (Sigma–Aldrich, Bornem, Belgium).
- Analytical-grade disodium ethylenediaminetetraacetate (EDTA) and HPLC-grade acetonitrile (ACN) were obtained from Merck (Belgolabo, Overysel, Belgium).
- Sodium acetate from Aldrich (Sigma–Aldrich, Bornem, Belgium); tert-butylthiol (TBT) from Acros (Geel, Belgium).

3.2. Microbore HPLC–ED system

- BAS 200A Chromatograph (BAS, West Lafayette, IN, and USA).
- An amperometric detector (integrated in BAS 200A, BAS) with a dual glassy carbon working electrode and Ag/AgCl reference electrode. The cell volume is 0.25 μl with a 16 μm gasket.
- SepStik microbore column (100 \times 1 mm i.d., 3 μm C8, BAS).
- Sample-Sentinel (BAS) was used for automatic sample injection.
- The chromatographic system was controlled by ‘BAS control’ software and the chromatograms were integrated with ‘ChromGraphTM’ software (BAS).

3.3. CE–LIF system

- Beckman P/A CE 2020 (Beckman, Fullerton, CA, USA).
- LIF detector (488 nm/520 nm, excitation/emission) (Beckman, Fullerton, CA, USA).
- The fused silica capillary was from Polymicro Technologies (Phoenix, AZ, USA): 47 cm (effective length 40 cm) \times 50 μm i.d.. Every new capillary was con-

ditioned by a 1 M NaOH rinse for 20 min, followed by water for 5 min at 50°C.

4. Detailed procedure

4.1. Standard preparation

Standard stock solutions were prepared at a concentration of 2.5 mM in Mill-Q water and stored at -70°C . Working solution was freshly diluted every day from stock solution.

4.2. Description of microbore HPLC–ED system

To minimize the dead-volume and provide 10–100 $\mu\text{l}/\text{min}$ flow rate for the microbore column, the HPLC–ED system was connected as shown in Fig. 1.

- A flow splitter (BAS) converts a conventional pump to the microbore regime which provides the low volumetric flow rate required for the microbore column. The splitter consists of tee and restrictor in the form of either a capillary or a packed column. Flow will pass through the restrictor as well as the sample injector and analytical microbore column. The resistance to flow of the restrictor relative to the microbore column determines the split ratio. In this work, the split ratio was 1/16 for using a capillary. Operating the pump at 0.8 ml/min yielded a microbore column flow rate of approximately 50 $\mu\text{l}/\text{min}$.
- The microbore column was coupled directly to the amperometric detector.
- The whole system was maintained at 35°C.
- The dual glassy carbon electrodes were in the parallel position to allow selection of different sensitivity range. The operating potential was set at 750 mV vs. Ag/AgCl.

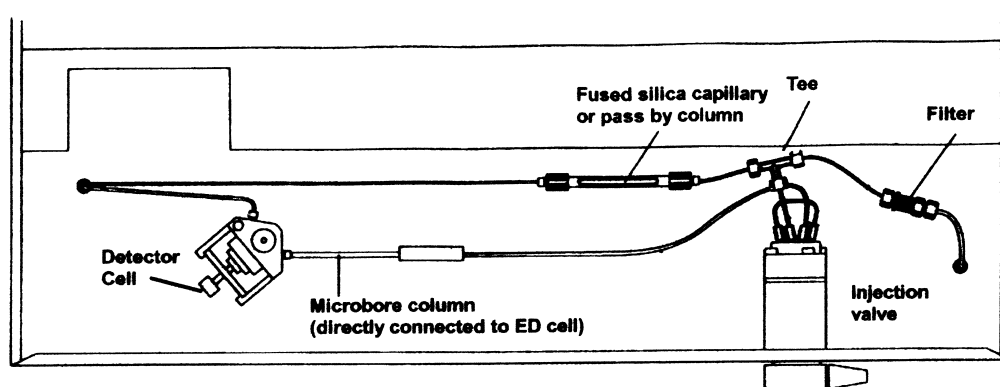


Fig. 1. Schematic diagram of the concentration of microbore column HPLC–electrochemical detection system (from ‘Guide for use: BAS UniJet microbore column’).

4.3. Separation of Asp and Glu by HPLC–ED

To separate Asp and Glu from other amino acids in microdialysate, a solvent switch technique was used and the two mobile phases are:

- Mobile phase A consisted of 17% (v/v) acetonitrile in 0.1 M sodium acetate buffer (pH=6.8), containing 40 mg/l EDTA.
- Mobile phase B contained 90% acetonitrile in the same buffer.
- Mobile phase A and B are delivered according to the following program:
All system flushed by mobile phase A;
After sample injection, at 0.1 min, the system was switched to 100% mobile phase B for 4 min;
At the 4.1 min the system was switched back to mobile phase A for 36 min until next sample injection.

4.4. Separation of GABA by microbore HPLC–ED

An isocratic mobile phase, which consisted of 41% acetonitrile in 0.1 M sodium acetate buffer (pH=5.06, containing 40 mg/l EDTA), was used to separate GABA from the rest of the amino acids in microdialysate.

4.5. Derivatization procedure for HPLC–ED system

- 27 mg OPA was dissolved in 5.0 ml methanol, followed by the addition of 5.0 ml 0.1 M Na₂CO₃ buffer (pH 9.6). This OPA stock reagent was stable for approximately 5 days if kept tightly sealed in a brown bottle at 4°C.
- The working solution was prepared daily by dissolving 2.3 µl of tBT in 1 ml of OPA stock solution. The OPA/tBT reagent was prepared freshly every day and kept at 4°C.
- Three microliters of derivatization reagent were added to 15 µl of microdialysate or amino acids standard mixture which was automatically vortex mixed for 2 min at a 2 bar pressure and automatically injected onto the column by Sample-Sentinel (BAS). The injection volume was 10 µl.

4.6. CE system and separation

- Every new capillary was conditioned by a 1 M NaOH rinse for 20 min, followed by a water rinse for 5 min at 50°C.
- The buffer pH was adjusted to 9.5 using 1 M NaOH or 1 M H₃BO₃ before making up to volume.
- Each day the capillary was flushed for 5 min with 0.1 M NaOH followed by a water wash for 5 min at 50°C.
- Before each analysis, the capillary was washed for 2 min with running buffer.
- Before electrophoresis, the derivatized microdialysis

samples were kept at 4°C in the injection carousel of the instrument. Samples were injected for 4 s in pressure mode.

4.7. Derivatization procedure for EC system

- Fluorescein isothiocyanate isomer I (FITC) stock solution was prepared in acetone at a concentration of 10 mM with traces of pyridine (0.002%, v/v) as catalyzator for the derivatization reaction and stored at 4°C in the dark.
- 15 µl dialysis samples were mixed with 1 µl 10 mM FITC–pyridine solution and 1 µl 5.5 M NaOH that adjusted the pH of artificial cerebrospinal fluid from 7.4 to 9.5. These mixtures were allowed to react for 6 h at 40°C in a water bath. After derivatization, the samples were stored in the dark at 4°C until injection.

4.8. Data preparation

- The microdialysis data in both HPLC and CE were presented as absolute values in pmol neurotransmitter/15 min collection. The baseline release of Asp, Glu and GABA were measured by the microdialysates obtained between 90 and 180 min after probe insertion. Four to six data points were recorded for each probe.

5. Results

Fig. 2 shows the chromatogram of a baseline release of Asp and Glu in the visual cortex of an awake cat. The Asp and Glu method was applied to investigate the influence of limited sensory deprivation on the extracellular concentrations of Glu and Asp in deprived and non-deprived cortex of awake, behaving control or experimental cats 18–35 days following retinal lesions. Over a period of two years, 1170 microdialysis samples have been measured. The results provided a biochemical basis for the cortical reorganization in adult animals after retinal lesion [10].

Fig. 3 is the chromatogram of a baseline release of GABA in the visual cortex of an awake cat. In this method, 12 amino acids — Asp, Glu, asparagine, histamine, glutamine, serine, arginine, glycine, threonine, tyrosine, alanine, taurine were eluted earlier than GABA in the front peak. Another seven amino acids — isoleucine, lysine, leucine, tryptophan, methionine, phenylalanine and valine were eluted later than GABA. The GABA method was only applied to measure the extracellular concentration of GABA in a normal cat.

Fig. 4 shows a typical electrophorogram of a baseline release of Asp, Glu and GABA in the visual cortex of an awake cat. The method was applied to measure extracellular concentrations of GABA, Glu and Asp in normal, deprived and non-deprived cortex of awake, behaving cats

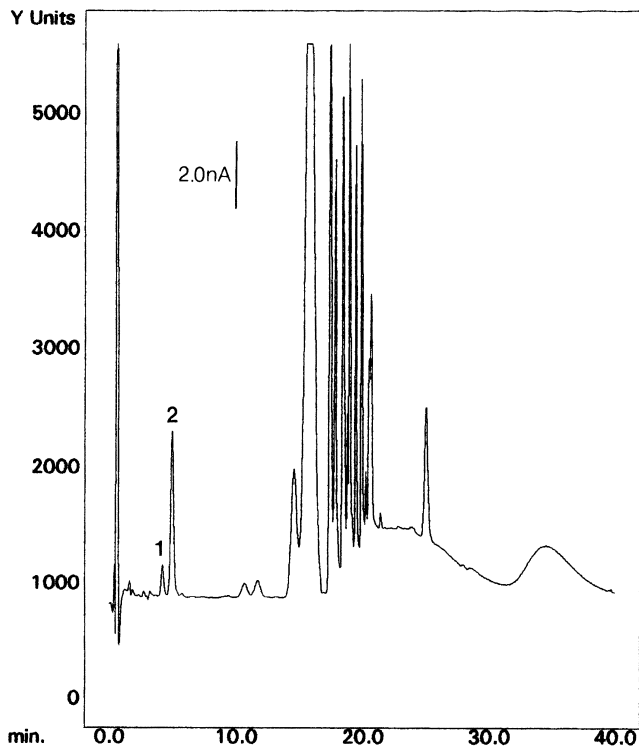


Fig. 2. Chromatogram of a baseline release of Asp and Glu in the visual cortex of awake cats: sensitivity range of detector: 20 nAFS; peaks: 1=Asp, 2=Glu.

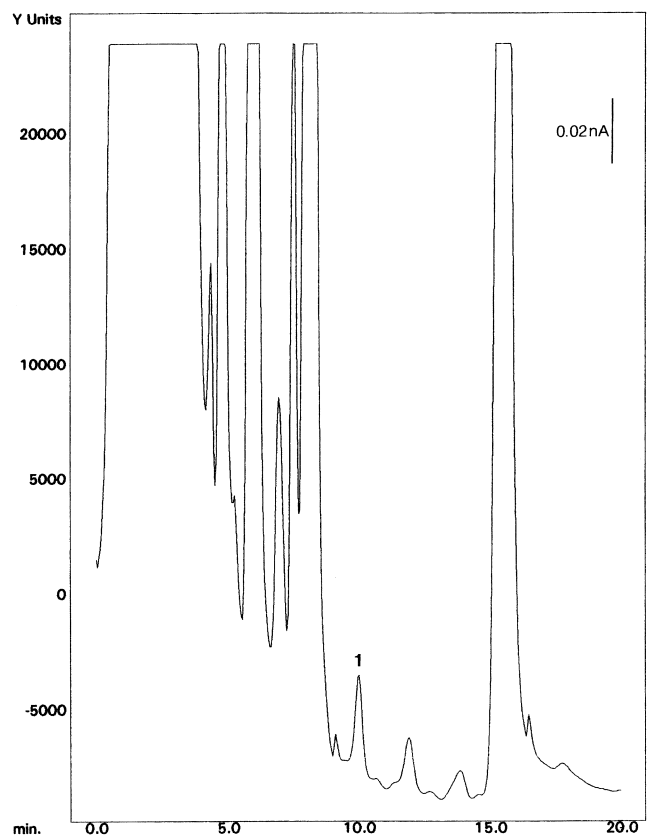


Fig. 3. Chromatogram of a baseline release of GABA in the visual cortex of awake cats: sensitivity range of detector: 0.2 nAFS; peak: 1=GABA.

18–35 days following a binocular central retinal lesion. 260 microdialysis samples were determined.

6. Discussion

6.1. Alternative and support protocols

The validation parameters of the two methods are compared in Table 1. Repeatability was checked with a 1×10^{-6} M GABA, Glu and Asp mixture. The results show that there is no significant difference in linearity, precision and limit of detection between these two methods. However, CE-LIF significantly shortens the time of analysis and decreased the volume of the sample. Theoretically, the CE-LIF method has higher mass sensitivity than the microbore HPLC-ED method. However, due to the limitation of injection volume of CE-LIF (several nl), the detection limit for these two methods is quite similar in practice. These two methods could be an alternative to each other and the application of each method depends on the availability of instruments in the laboratory and the knowledge, as well as the experience of scientists. In the literature, analysis of amino acid neurotransmitters by HPLC methods [4,8,12,13] and CE methods [3,6,7,11,15] have been reported by other laboratories.

6.2. Optimization of CE-LIF method

6.2.1. Optimization of the derivatization conditions

Increasing the FITC/amino acid ratio resulted in a continuous increase of the derivatization efficiency. At a ratio of 50 the maximum efficiency was reached. As the derivatization can not be carried out at the pH of the artificial cerebrospinal fluid (7.7), 1 μ l 5.5 M NaOH was added to 15 μ l microdialysis samples to adjust the pH to 9.5.

6.2.2. Optimization of the separation

The pH is one of the most important parameters that may influence the resolution in CE and small differences may be responsible for good or bad separations. It was found that at pH 9.25, separation of six amino acid neurotransmitters (GABA, Asp, Glu, alanine, taurine and glycine) could be obtained. But the separation between GABA and the interfering peak from the derivatization reaction was critical. A pH increase to 10.25 resulted in an optimal GABA separation without affecting the separation of Glu and Asp.

The buffer concentration has an important influence on the electro-osmotic flow and current in the capillary. Increasing the concentration resulted in a better resolution

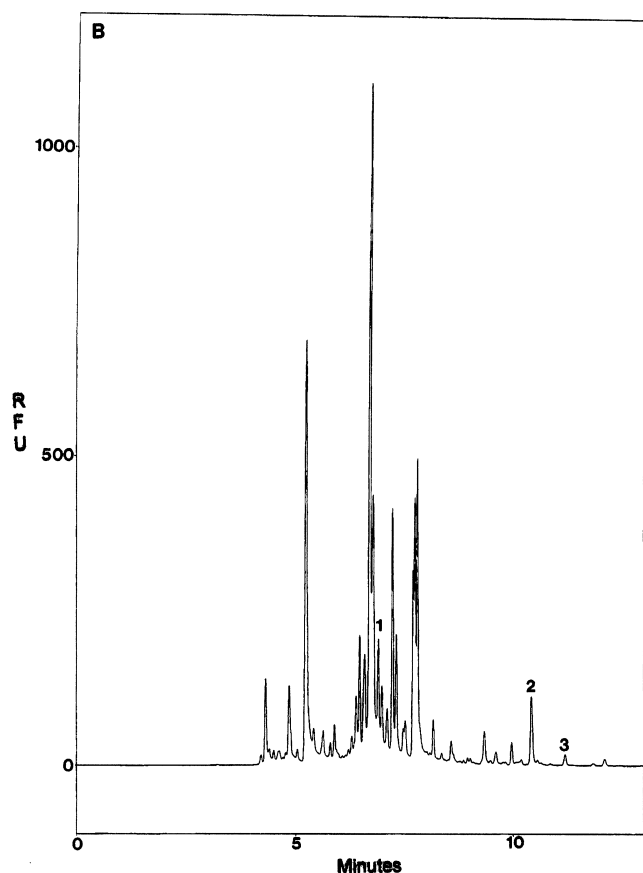


Fig. 4. Electrophorogram of a baseline release of Asp, Glu and GABA in the visual cortex of awake cats: Running buffer: 20 mM borate buffer adjusted to pH 10.25; Capillary uncoated fused silica, L, 47 cm, l, 40 cm, 50 μm i.d.; voltage: 20 kV, capillary temperature: 25°C. Peaks: 1=GABA; 2=Glu; 3=Asp.

of GABA and the interfering derivatization peak but also increased the current, rendering the method less stable. To obtain better repeatability, 20 mM was selected.

Instrumental parameters such as capillary temperature and applied voltage were also optimized. The optimal electrophoretic parameters were: 20 mM borate buffer, pH 10.25; 20 kV and a capillary temperature of 25°C.

6.3. Troubleshooting of microbore HPLC–ED

Compared to the conventional size of a column, microbore columns provide much higher mass sensitivity, lower sample size requirements, and reduce mobile phase consumption. However, the following phenomena are the common problem in the microbore operating system and can be solved by the following steps:

1. The back pressure remains high. The possible reason and their solutions are listed in Table 2.
2. The retention time of the solvent peak obviously remains longer than usual. The problem might be due to the dead volume, which is induced by the connection between tubing and column. Therefore, as you tighten a fitting, be sure that the tube or column is continually being pushed into its port to avoid dead volume.
3. The retention time of Asp and Glu gradually shifted to the later value. When applying a microbore column with a flow splitter system, an important problem arises from the change in split ratio after a certain amount of injections. Particulate from the sample or injector will be deposited on the column inlet frit and

Table 1
Comparison of two methods

Compound	Method	HPLC–ED	CE–LIF	
Asp	Time of analysis (min)	40	13	
	Repeatability (RSD*)	Peak area	1.33%	5.4%
		Capacity factor or migration time	0.69%	0.48%
	Linearity range	1.0–20.0 μM	0.1–8 μM	
	r^2	0.998	0.996	
Limit of detection ($S/N=3$)	0.001 μM	0.019 μM		
Glu	Time of analysis (min)	40	13	
	Repeatability (RSD*)	Peak area	2.64%	4.7%
		Capacity factor or migration time	0.67%	0.40%
	Linearity range	1.0–20.0 μM	0.1–8 μM	
	r^2	0.997	0.993	
Limit of detection ($S/N=3$)	0.001 μM	0.012 μM		
GABA	Time of analysis (min)	25	13	
	Repeatability (RSD*)	Peak area	1.88%	7.8%
		Capacity factor or migration time	0.83%	0.52%
	Linearity range	0.1–2.0 μM	0.05–8 μM	
	r^2	0.990	0.998	
Limit of detection ($S/N=3$)	0.002 μM	0.001 μM		

*RSD: relative standard deviation.

Table 2

Reasons and solutions for the back pressure remaining high

Possible reasons	Possible solutions
1. Deposition of debris on the filter	1. Clean the on-line filter by sonication
2. Particles block one part of system	2. Determine the source by removing components one at a time, starting with the column and moving upstream. After each step, restart the pump and allow the pressure to stabilize.
3. Particles deposited on the top of the column	3. Reversing the direction of the column and flushed with 40% CAN
4. On-line precipitation	4. Change a new column

the splitter ratio will increase favor the bypass restrictor. Asp and Glu retention times will shift to later values. Therefore a separation condition was optimized as follow: after Asp and Glu were eluted, there is no other component eluted within another 6 min, which allowed possible shifts in the Asp and Glu peaks to longer retention times without influencing the quantification.

- No Asp and Glu peaks, only the solvent peak comes out. Particulate from the sample or injector deposited on the column inlet frit and the splitter ratio is 100% through the bypass restrictor. Clean the system as shown in the “possible solution section of Table 2”.

7. Quick procedure

7.1. Microbore HPLC–ED method

- Prepare OPA stock solution by adding 27 mg OPA in 0.5 ml methanol and 0.5 ml 0.1 M NaCO₃.
- Prepare OPA working solution by adding 2.3 µl TBT in 1 ml OPA stock solution.
- 3 µl OPA working solution was added into 15 µl amino acid standard solution or microdialysate and vortex mixed 2 min, than injected onto microbore HPLC–ED system.

7.2. CE–LIF method

- Prepare FITC stock solution by adding 0.002% (v/v) pyridine in 10 mM FITC acetone solution.
- One µl 10 mM FITC solution and 1 µl 5.5 M NaOH were added in 15 µl amino acid standard solution or microdialysate and reacted in the water bath for 6 h at 40°C, then injected into the CE–LIF system.

8. Essential literature references

References [8,9,15].

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