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Analysis of extracellular γ-aminobutyric acid, glutamate and aspartate in cat visual cortex by in vivo microdialysis and capillary electrophoresis-laser induced fluorescence detection

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Abstract

To investigate the influence of a partial sensory deprivation on the extracellular concentration of amino acid neurotransmitters in cat visual cortex, a capillary electrophoresis method was developed for the quantification of γ-aminobutyric acid (GABA), glutamate (Glu) and aspartate (Asp) in in vivo microdialysis samples of cat brain. Microdialysis samples from different regions of area 17 were obtained every 15-min using CMA 12 2-mm probes perfused with synthetic cerebrospinal fluid and derivatized using fluorescein isothiocyanate (FITC). Laser-induced fluorescence (LIF) detection was employed. Good selectivity was obtained with a borate buffer (20 mM, pH 10.25). The whole procedure, including the washing step takes only 15 min. The conditions for derivatization and separation were optimized. The parameters for validation such as linearity, precision and detection limit are also reported. The results are consistent with those of HPLC but, as the sample volumes needed are only 1–5 nl, a much better time resolution can be obtained. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis; Microdialysis; γ-Aminobutyric acid; Glutamate; Aspartate

1. Introduction

GABA, Glu and Asp are considered to be the most important neurotransmitters in the central nervous system (Fagg and Foster, 1983; Tsumoto, 1990). It has been suggested that they are involved in the cortical reorganization that follows after a limited sensory deprivation (Artola and Singer, 1993; Rosier et al., 1995; Anwyl, 1996; Arckens et al., 2000). Quantification of the amino acid neurotransmitters in brain microdialysis samples is conventionally performed using HPLC with electrochemical detection (ED) (Donzanti and Yamamoto, 1988; Smolders et al., 1995; Rowley et al., 1995; Qu et al., 1998). However, HPLC analysis is rather slow (40 min), requires large injection volumes and hence long dialysis sampling times (typically 15 min) resulting in a poor time resolution.

CE is a powerful separation and quantification technique that often provides higher resolving power, shorter analysis time and smaller sample volumes (typically 1–5 nl) than HPLC. Recently, it became a popular method for the determination of amino acids (Lin et al., 1994; Issaq and Chan, 1995; Wan et al., 1995). Coupled to a high sensitive detection system, such as laser-induced fluorescence (LIF), CE is a powerful technique for analysis of small volume microdialysis samples (Hernandez et al., 1993; Dawson et al., 1995; Zhang et al., 1998; Rada et al., 1999).

Abbreviations: Asp, aspartate; ED, electrochemical detection; FITC, fluorescein isothiocyanate; GABA, γ-aminobutyric acid; Glu, glutamate.

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The present paper describes the quantification of GABA, glutamate and aspartate in cat brain microdialysis samples. The samples are first derivatized with FITC, separated by CE and quantified by LIF. The most important parameters governing the detection sensitivity and the separation efficiency were investigated. Quantitative data are reported.

2. Materials and methods

2.1. Instrumental and operating conditions

CE experiments were carried out on a Beckman P/ACE 2020 with LIF detector (488 nm/520 nm, excitation/emission) (Fullerton, CA). The buffer pH was adjusted using 1 M NaOH or 1 M H_3BO_3 before making up to volume. Each day the capillary was flushed during 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 carrousel of the instrument. Samples were injected during 4 s in pressure mode.

2.2. Materials and reagents

All the reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Organics, Geel, Belgium). Milli-Q water (Millipore, Milford, MA) was used for all the experiments. GABA, Asp and Glu were obtained from Sigma (St Louis, MO). Standard stock solutions were prepared at a concentration of 2.5 mM in water and stored at −70°C. Fluorescein isothiocyanate isomer I was obtained from Sigma. Its stock solution was prepared in acetone at a concentration of 10 mM with traces of pyridine (0.002%, v/v) as catalizator for the derivatization reaction and stored at 4°C in the dark. The fused silica capillary was from Polymicro Technologies (Phoenix, AZ): 47 cm (effective length 40 cm) × 50 μm I.D. Every new capillary was conditioned by a 1 M NaOH rinse for 20 min, followed by water for 5 min at 50°C.

2.3. In vivo microdialysis

Microdialysis has been performed as previously described (Qu et al., 2000). In short: under visual control through a surgical microscope and using a stereotactic instrument, four probe guides were permanently implanted in area 17 of one hemisphere of the anesthetized animal and two fixation bars were mounted on the skull to allow fixation of the cat in a stereotactic frame. After recovery from surgery the awake cats were trained to adapt to a fixation of 5 h in a stereotaxis apparatus. Once adapted to that situation, the cats were ready for microdialysis experiments without anesthesia. The day of the experiment, the awake animal was fixed in the stereotactic frame and the probes inserted into the guides. The CMA 12 2-mm probes were perfused with filtered and degassed artificial cerebrospinal fluid (Tamura et al., 1990) (NaCl, 124 mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.3 mM; $CaCl_2$, 2.4 mM; glucose, 5 mM; $NaHCO_3$, 26 mM; pH = 7.4) at a rate of 1 μl/min by a CMA 100 microinjection pump. Dialysis samples were collected at 15-min intervals. The exact localization of the probes in area 17 was examined under the microscope after cresyl violet staining of the appropriate brain section (Qu et al., 2000).

2.4. Derivatization of the microdialysis samples

15 μl dialysis samples were mixed with 1 μl 10 mM FITC-pyridine solution and 1 μl 5.5 M NaOH (this 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. After derivatization the samples were stored in the dark at 4°C.

3. Results and discussion

3.1. Optimization of the derivatization conditions

Since derivatization efficiency greatly depends on the FITC/amino acid ratio, different ratio's (1, 10, 20, 30, 40, 50, 60, 70) were tried. The sample for optimization was an artificial mixture containing a 1×10^{-6} M concentration of the amino acids Asp, Glu and GABA at pH 9.5. 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. At higher ratio's, the derivatization efficiency was still optimal, but the interference with the FITC peak increased. Therefore a FITC/amino acid ratio of 50 was considered as optimal.

As the derivatization can not be carried out at the pH of the artificial cerebrospinal fluid (7.4), 1 μl 5.5 M NaOH was added to 15 μl microdialysis samples to adjust the pH to 9.5.

3.2. 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. The optimization was performed with borate buffer (20 mM), at a voltage of 20 kV and a temperature of 25°C. The pH was varied between 8.75 and 10.5 with steps of 0.25. It was found that at pH 9.25, separation of six amino acid neurotransmitters (GABA, Asp, Glu, ala-

nine, taurine and glycine) could be obtained. But the separation between GABA and 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 separations for alanine, taurine and glycine became worse but they were less important in our study. Therefore pH 10.25 was considered as optimal.

The buffer concentration has an important influence on the electroosmotic flow and current in the capillary. Therefore, keeping the other conditions constant (pH 10.25, 20 kV and 25°C), the buffer concentration was increased from 10 to 40 mM in steps of 10 mM. Increasing the concentration resulted in a better resolution 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. Fig. 1 shows typical electropherograms of a standard mixture and a microdialysis sample of cat brain.

3.3. Validation

Some validation parameters such as linearity, precision and limit of detection were determined for GABA, Glu and Asp. The results are shown in Table 1. Repeatability was checked with a 1×10^{-6} M GABA, Glu and Asp mixture.

3.4. Determination of GABA, Glu and Asp in in vivo microdialysis samples

The method was applied to measure extracellular concentrations of GABA, Glu and Asp in normal, deprived and non-deprived cortex of awake, behaving cats 18–35 days following a binocular central retinal lesion. 260 microdialysis samples were determined. The results of a typical experiment are shown in Fig. 2. As expected extracellular GABA, Glu and Asp levels are

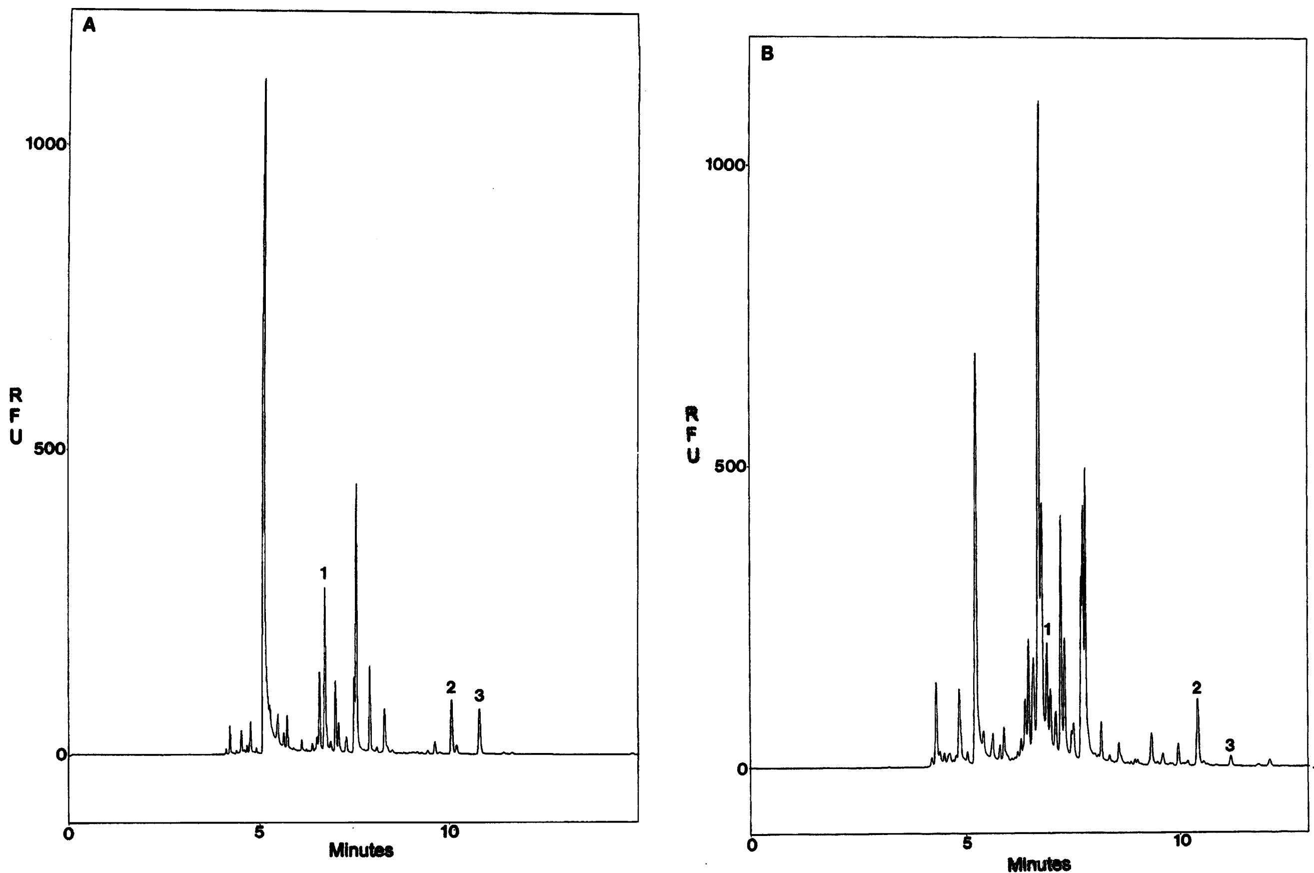

Fig. 1. Electropherograms of (A) standard sample and (B) in vivo microdialysis sample of cat brain. Running buffer: 20 mM borate buffer adjusted to pH 10.25; capillary uncoated fused silica, L, 47 cm, l, 40 cm, 50 µm I. D.; voltage: 20 kV, capillary temperature: 25°C. 1. GABA; 2. Glu; 3. Asp.

Table 1
Validation data for CE of GABA, Glu and Asp

Parameter	Estimated value
Repeatability ($n = 6$)	
Migration time	RSD[b] = 0.52% (GABA)
	RSD = 0.40% (Glu)
	RSD = 0.48% (Asp)
Corrected peak area[a]	RSD = 7.8% (GABA)
	RSD = 4.7% (Glu)
	RSD = 5.4% (Asp)
Linearity (Number of concentrations for each amino acid = 6)	
GABA: 0.05–8 μM	$r = 0.998$
Glu: 0.1–8 μM	$r = 0.993$
Asp: 0.1–8 μM	$r = 0.996$
Limit of detection (S/N = 3)	
GABA	0.001 μM
Glu	0.012 μM
Asp	0.019 μM

[a] Corrected peak area = peak area/migration time.
[b] RSD = relative standard deviation.

very high during the initial 60 min of sampling to drop later to a stable baseline release. To visualize the neuronal origin of GABA, Glu and Asp present in the dialysates, the probe was flushed with 65 mM KCl in artificial cerebrospinal fluid. This resulted in a local extracellular increase of potassium ions causing a depolarization of the neurons and a sudden increase of extracellular neurotransmitter concentrations.

These results are fully in agreement with our previous data obtained HPLC-ED (Qu et al., 2000). The concentrations of Asp, Glu and GABA in microdialysates of normal cat area 17 are listed in Table 2.

4. Concluding remarks

We developed a CE method in combination with LIF detection for the measurement of the amino acid neurotransmitters, GABA, Glu and Asp in in vivo microdialysis samples. The method is easy and reproducible. The separation is much faster than by HPLC (Qu et al., 1998) and the small sample volumes needed allow a much better time resolution during the microdialysis experiments.

Table 2
The concentration of Asp, Glu and GABA in microdialysates of normal cat area 17

Amino acids	Concentration (pmol/15 min collection)	Number of probes	Number of measurements
Asp	3.62 ± 0.60	7	20
Glu	16.34 ± 1.56	7	20
GABA	1.30 ± 0.15	7	14

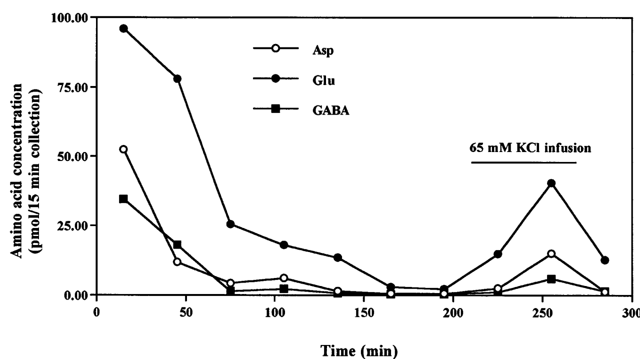

Fig. 2. Extracellular GABA, Glu and Asp concentrations as measured during a typical microdialysis experiment.

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References

Anwyl R. Cortical Plasticity: The Role of Amino Acid Receptors in Synaptic Plasticity. Oxford: Bios Scientific, 1996:9–34.

Arckens L, Schweigart G, Qu Y, Wouters G, Pow DV, Vandesande F, Eysel UT, Orban GA. Cooperative changes in GABA, glutamate and activity levels: the missing link in cortical plasticity. Eur J Neurosci 2000;12(12):4222–32.

Artola A, Singer W. Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation. Trends Neurosci 1993;16:480–7.

Dawson LA, Stow JM, Dourish CT, Routledge C. Analysis of glutamate in striatal microdialysates using capillary electrophoresis and laser-induced fluorescence detection. J Chromatogr A 1995;700:81–7.

Donzanti BA, Yamamoto BK. An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. Life Sci 1988;43:913–22.

Fagg GE, Foster AC. Amino-acid neurotransmiters and their pathways in the mammalian central nervous-system. Neuroscience 1983;9:701–19.

Hernandez L, Tucci S, Guzman N, Paez X. In vivo monitoring of glutamate in the brain by microdialysis and capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr A 1993;652:393–8.

Issaq HJ, Chan KC. Separation and detection of amino acids and their enantiomers by capillary electrophoresis — a review. Electrophoresis 1995;16:467–80.

Lin BL, Colon LA, Zare RN. Dual electrochemical detection of cystine in capillary zone electrophoresis. J Chromatogr A 1994;680:263–70.

Qu Y, Arckens L, Vandenbussche E, Geeraerts S, Vandsande F. Stimultaneous determination of total and extracellular concentrations of the amino acid neurotransmitters in cat visual cortex by microbore liquid chromatography and electrochemical detection. J Chromatogr A 1998;798:19–26.

Qu Y, Arckens L, Vandesande F, Vandenbussche E. Sampling extracellular aspartate, glutamate and γ-aminobutyric acid in striate cortex of awake cat by in vivo microdialysis: surgical and methodological aspects. Brain Res 2000;866:116–27.

Rada P, Tucci S, Teneud L, Paez X, Perez J, Alba G, Garcia Y, Sacchettoni S, del Corral J, Hernandez L. Monitoring gamma-aminobutyric acid in-human brain and plasma microdialysates using micellar electrokinetic chromatography and laser-induced fluorescence detection. J Chromatogr B 1999;735:1–10.

Rosier AM, Arckens L, Demeulemeester H, Orban GA, Eysel UT, Wu YJ, Vandesande F. Effect of sensory deafferentation on immunoreactivity of GABAergic cells and on GABA receptors in the adult cat visual cortex. J Comp Neurol 1995;359:476–89.

Rowley HL, Martin KF, Marsden CA. Determination of in vivo amino acid neurotransmitters by high performance liquid chromatography with o-phthalaldehyde sulfite derivatization. J Neurosci Methods 1995;57:93–9.

Smolders I, Sarre S, Michotte Y, Ebinger G. The analysis of excitatory, inhibitory and other amino-acids in rat brain microdialysates using microbore liquid chromatography. J Neurosci Methods 1995;57:47–53.

Tamura H, Hicks TP, Hata Y, Tsumoto T, Yamatodani A. Release of glutamate and aspartate from the visual cortex of the cat following activation of afferent pathways. Exp Brain Res 1990;80:447–55.

Tsumoto T. Excitatory amino acid transmitters and their receptors in neural circuits of the cerebral neocortex. Neurosci Res 1990;9:79–102.

Wan H, Andersson PE, Engstrom A, Blomberg LG. Direct and indirect chiral separation of amino acids by capillary electrophoresis. J Chromatogr A 1995;704:179–93.

Zhang L, Chen H, Hu S, Cheng J, Li Z, Shao M. Determination of the amino acid neurotransmitters in the dorsal root ganglion of the rat by capillary electrophoresis with a laser-induced fluorescence-charge coupled device. J Chromatogr B 1998;707:59–67.