Journal of Chromatography B, 877 (2009) 3248–3252

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Simultaneous determination of glutamate and aspartate in rat periaqueductal gray matter microdialysates by capillary electrophoresis with laser-induced fluorescence

Hong-Li Chen [a,b], Xiao-Jun Zhang [a], Sheng-Da Qi [a,b], Hong-Xi Xu [c], Joseph J.Y. Sung [d], Zhao-Xiang Bian [a,*]

[a] School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, China
[c] Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong SAR, China
[d] Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China

ARTICLE INFO

Article history:
Received 12 June 2009
Accepted 4 August 2009
Available online 12 August 2009

Keywords:
Microdialysis
Capillary electrophoresis
Laser-induced fluorescence
5-Carboxyfluorescein *N*-succinimidyl ester
Glutamate
Aspartate

ABSTRACT

This study presented a capillary zone electrophoresis (CZE) method for the analysis of trace amount of neurotransmitters glutamate (Glu) and aspartate (Asp) in the microdialysates of rat periaqueductal gray matter (PAG). Glu and Asp were derivatized with the fluorescent agent 5-carboxyfluorescein *N*-succinimidyl ester (CFSE) for the first time and detected by laser-induced fluorescence (LIF) after separation by CZE. The concentration detection limits (S/N = 2) were 6.9×10^{-10} M and 8.1×10^{-10} M for Glu and Asp, respectively. The repeatability (expressed as RSD) of the migration times of CFSE–amino acid were better than 0.5%, and not higher than 1.5% even over the period of 1 month. This method was applied to quantify Glu and Asp in rat PAG microdialysates with the treatment of formalin injection, and the measured basal concentrations of Glu and Asp were $(22.4 \pm 1.6) \times 10^{-6}$ M and $(0.9 \pm 0.1) \times 10^{-6}$ M, respectively.

1. Introduction

Glutamate (Glu) and aspartate (Asp) are two major excitatory amino acids that activate *N*-methyl-D-aspartate (NMDA) receptors in the central nervous system. Studies have found that NMDA receptor play important roles in descending pain modulation [1,2]. It was shown that increased Glu and Asp release are intimately associated with both somatic and visceral nociception [3,4]. However, direct evidence of the immediate and accurate changes of extracellular neurotransmitters such as Glu and Asp in response to noxious nociception is scarce [4,5], therefore inhibiting the deep understanding of the mechanism and the development of therapeutic agents for pain.

Microdialysis has gained wide recognition as a valuable tool for in vivo monitoring neurotransmitters in the extracellular environment of the brain [6]. Capillary electrophoresis combined with laser-induced fluorescence (CE-LIF) detection has been shown to be a technique well suited for the analysis of amino acid neurotransmitters in small-volume microdialysis samples [7–11]. In fluorescence detection with CE, the derivatization of amino acids is performed prior to CE separation in order to achieve good detection sensitivity. The common derivatizing strategies for Glu and Asp are on-line [7–9] and off-line [10,11]. The typical labeling probes for them in the microdialysates are *o*-phthalaldehyde (OPA) [7], naphthalene-2,3-dicarboxaldehyde (NDA) [8], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [9], fluoresceine-5-isothiocyanate (FITC) [10,11], and so on.

Among these fluorescent derivatizing agents, fluorescein derivatives are the most widely used owing to their high absorptivity, near-unity fluorescence quantum yield and good water solubility. FITC, fluorescein dichlorotriazine (DTAF) and carboxyfluorescein succinimidyl ester (CFSE) are structural analogues in that they all react with nucleophiles and then conjugate to the objects almost exclusively through the amino group of lysine residues and the *N*-terminal amino group [12]. Banks and Paquette had concluded that CFSE is a superior derivatization reagent [12] and can quantitatively derivatize nanomolar levels of amino acids [13]. But to our best knowledge, there have been no reports of using CFSE to label Glu and Asp in microdialysates.

* Corresponding author. Tel.: +852 34112905; fax: +852 34112929.
E-mail address: bzxiang@hkbu.edu.hk (Z.-X. Bian).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.08.006

2. Experimental design

2.1. Materials and reagents

5-Carboxyfluorescein *N*-succinimidyl ester (CFSE), Glu, Asp, sodium tetraborate and boric acid were obtained from Sigma (St. Louis, MO, USA). The artificial cerebrospinal fluid (aCSF) consisted of 148 mM NaCl, 4.0 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.2 mM Na_2HPO_4 and 0.3 mM NaH_2PO_4 (pH 7.40) [14]. Standard solutions of 5 mM Glu and Asp were prepared in aCSF and stored at −20 °C before use. The working solutions of Glu and Asp were obtained by diluting the stock solutions with aCSF.

2.2. Apparatus

The electrophoretic system consisted of an automatic P/ACE™ MDQ system (Beckman-Coulter, Fullerton, CA, USA) equipped with a LIF detector. The excitation was performed at 488 nm, and a 520 nm band-pass filter was used for emission. Data collection and processing were performed using the System Gold software package (Beckman). The separation was carried out on a 60.2 cm (50 cm to the detector) × 50 μm i.d. (375 μm o.d.) fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China). Capillary flushing was performed by applying pressure of 20 psi (1379 mbar) at the capillary inlet.

2.3. Collection of microdialysis samples

Adult (300–320 g) Sprague–Dawley rats were obtained from the Experimental Animal Centre, The Chinese University of Hong Kong. Rats were housed in cages and maintained in rooms with temperature kept at 23 ± 2 °C and an alternating 12:12 h light–dark cycle. All of the experimental protocols were carried out with the approval of the Committee on Use of Human & Animal Subjects in Teaching and Research of Hong Kong Baptist University and according to the Regulations of the Department of Health, Hong Kong, China.

One day prior to the microdialysis experiments, a guide cannula (Locking Intracerebral Guide and Stylet, Bioanalytical Systems Inc., USA) was implanted in each rat under chloralhydrate (3.5 mg/kg, i.p.) anesthesia at the following stereotaxic coordinates: 0.6 mm lateral to the midsagittal suture, 6.7 mm posterior to bregma and 5.0 mm ventral to the surface of the skull [15]. The guide was secured with dental acrylic cement (Bioanalytical Systems Inc., USA) and 2 mm screws (Bioanalytical Systems Inc., USA) to the skull. For preventing postoperative inflammation, marbofloxacin (0.2 mg/100 g, i.p.) was used once after surgery.

The microdialysis sampling was conducted after rats had 1-day postoperative recovery in individual cages. A microdialysis probe (Bioanalytical Systems Inc., USA) with a dialysis membrane (2 mm long, 320 μm o.d., molecular cut-off 7000 Da) was inserted through the guide cannula. Rats were kept in BAS Raturn system (Bioanalytical Systems Inc., USA) for collection of dialysate samples throughout the experiment. Microdialysis was performed by perfusing the dialysis probe with aCSF at a rate of 1 μL/min through a syringe drive (Bioanalytical Systems Inc., USA). Following a wash-out period of 60 min after the insertion of the probe, five baseline dialysate samples (3 μL each) were collected in 3 min periods by an autosampler (Bioanalytical Systems Inc., USA) with temperature controlled at 4 °C. After the fifth sample was collected, formalin (50 μL of 5% formalin, diluted in physiological saline) was subcutaneously injected into the dorsal surface of the right hind paw of the rat [4]. After that, eight more samples were collected. Samples were immediately stored at −20 °C, until derivatization.

And then intralesional localization of the microdialysis probe at PAG was checked in each animal through histology according to the standard procedures of hematoxylin–eosin staining, thus to make sure the position of probe.

2.4. Derivatization procedure

Fresh stock solution of CFSE at 5 mM was made in *N*,*N*-dimethylformamide and used immediately. 5 μL of standard, blank or microdialysis samples (2.5 μL microdialysates + 2.5 μL aCSF) were derivatized at room temperature by adding 1 μL 5 mM CFSE and 4 μL 10 mM borate (pH 8.50, adjusted by 1 M HCl). Derivatization was allowed to proceed overnight in the dark. After derivatization, all solutions were diluted 10-fold with ultrapure water, then stored at −20 °C until use.

2.5. Electrophoretic procedure

The capillary was treated prior to its first use by flushing 10 min with 1 M NaOH, 0.1 M NaOH, ultrapure water and the running buffer, respectively. The separation procedure was as follows: 25 mM borate + 120 mM boric acid as the running buffer (pH 8.50), an applied voltage of +25 kV (the cathode on the outlet), hydrodynamic injections of samples (5 s at 0.5 psi), and the capillary temperature at 25 °C. Between each analysis, the capillary was flushed according to the following sequence: ultrapure water (1 min), 1 M HCl (1 min), ultrapure water (1 min), 1 M NaOH (1 min), 0.1 M NaOH (1 min), ultrapure water (2 min), and then the running buffer (3 min). This rinsing procedure ensured that the intra-day and inter-day repeatability of migration time of CFSE–amino acids were lower than 0.5%, and not higher than 1.5% even over the period of 1 month.

2.6. Quantification validation

Calibration standards for the quantification validation contained Glu (2×10^{-8} M to 2.5×10^{-5} M) and Asp (4×10^{-8} M to 2.5×10^{-5} M). Calibration plots were represented by drawing the peak area *vs.* concentration of the analyte ($n = 10$). Regression equations were calculated by the least squares linear regression method. The concentration detection and quantification limits were calculated as the analyte concentrations that give rise to peak areas with a signal-to-noise ratio of 2 and 10, respectively. Intra-day and inter-day repeatability were determined using a standard mixture solution of Glu and Asp at 3×10^{-6} M and brain microdialysates. The accuracy of the method was twice calculated from the analysis of standard solutions, microdialysates and microdialysates spiked with known quantities of standard.

2.7. Statistical analysis

The data collected in this experiment are expressed as mean ± S.E.M. The differences between with and without formalin injection treatment were compared by using the independent *t*-test, and the *p* values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Optimization of the derivatization conditions

Through the preliminary experiment, borate was selected as the buffer in labeling Glu and Asp with CFSE. Derivatization conditions in terms of borate solution pH, borate concentration, the molecular ratio of CFSE *vs.* amino acid and the derivatization volume were

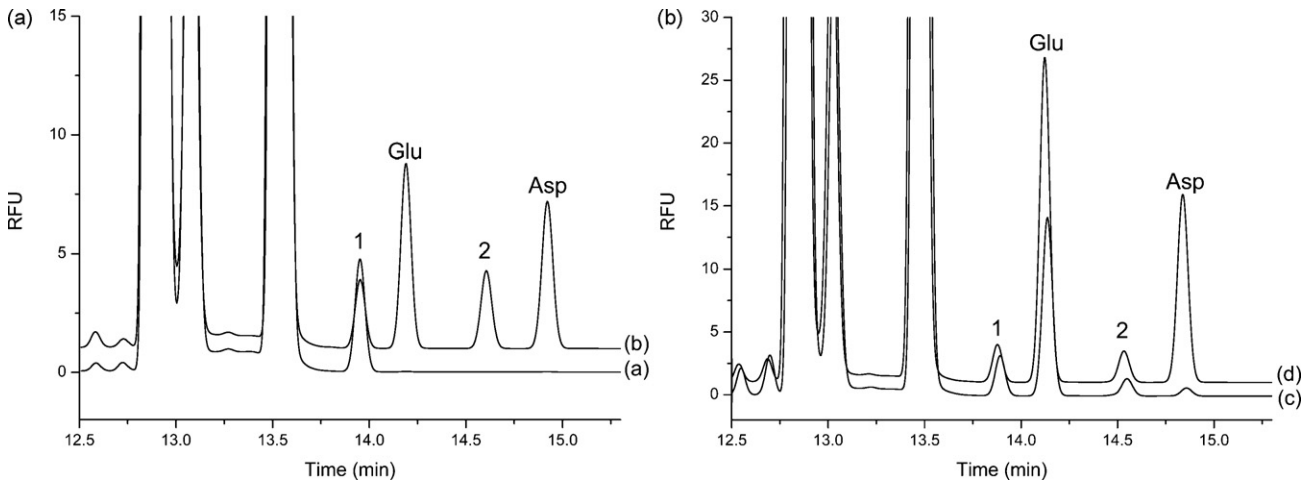

Fig. 1. The typical electropherograms of blank (a), 1.0×10^{-6} M standard mixture of Glu and Asp (b), microdialysate sample (c) and microdialysate spiked with 5.0×10^{-6} M standard mixture of Glu and Asp (d). Glu, the peak of CFSE-Glu; Asp, the peak of CFSE-Asp; 1 and 2, the unknown interfering peaks from CFSE excess. Electrophoretic conditions: separation capillary, 60.2 cm (50 cm to the window) × 50 μm i.d. (375 μm o.d.); pressure injection, 5 s at 5 psi; running buffer, 25 mM borate + 120 mM boric acid (pH 8.50); separation voltage, 25.0 kV; LIF detection, ex. 488 nm/em. 520 nm.

then investigated to obtain the maximum sensitivity (expressed as peak area).

The yield of CFSE–amino acid was found to be pH-dependent. When aCSF (pH = 7.40) as derivatization buffer was applied, the sensitivity of labeled CFSE-Glu or CFSE-Asp was very low. With pH in the range of 8.10–9.50, the sensitivity began relatively stable. However, when the pH was higher than 9.50, the hydrolysis of CFSE became very significant. The selected optimal derivatization pH was 8.50.

The effect of borate concentration (5–70 mM) on the sensitivity was investigated. With increasing borate concentration, the sensitivities increased and then leveled off. However, when borate was higher than 35 mM, the sensitivities strongly decreased and the obtained peak began broad and tailing. Thus, 10 mM borate was chosen.

The effect of the molar ratio of CFSE *vs.* amino acid between 6.25 and 200 on the sensitivity was also investigated. The fluorescent sensitivity increased nearly in line with the increase in CFSE concentration, without a plateau. But when the ratio was higher than 100, the rate of the increase for CFSE-Glu in peak area became larger than that in peak height, which led to broadening of the obtained peak. Therefore, the optimum ratio was selected as 100. Moreover, the concentrations of Glu and Asp in brain microdialysates were lower than 5×10^{-6} M, so 5×10^{-4} M was chosen as the CFSE concentration.

As for the derivatization volume, the experimental results showed that 10 μL of the derivatization volume can assure enough derivatization and high sensitivity. In summary, the optimum derivatization procedure was as follows: 5 μL of standard solutions were mixed with 1 μL 5 mM CFSE and 4 μL 10 mM borate

Table 1
Quantitative parameters for the analysis of Glu and Asp in standard solutions and brain microdialysates.

		Glu	Asp
Standards			
Calibration range (M)		2×10^{-8} to 2.5×10^{-5}	4×10^{-8} to 2.5×10^{-5}
Intercept *a*		29609.44	31336.30
Slope *b*		4.77×10^{11}	4.06×10^{11}
Correlation coefficient (*R*)		0.9998	0.9999
Intra-day repeatability (RSD)[a]	Migration time	0.3%	0.3%
	Peak height	3.4%	3.2%
	Peak area	3.6%	3.2%
Inter-day repeatability (RSD)[c]	Migration time	0.3%	0.4%
	Peak height	4.7%	4.5%
	Peak area	4.4%	4.0%
Detection limit (mol/L)		6.9×10^{-10}	8.1×10^{-10}
Quantification limit (mol/L)		3.4×10^{-9}	4.0×10^{-9}
Microdialysates			
Intra-day repeatability (RSD)[b]	Migration time	0.2%	0.2%
	Peak height	2.8%	4.3%
	Peak area	3.0%	4.6%
Inter-day repeatability (RSD)[c]	Migration time	0.4%	0.5%
	Peak height	7.7%	8.4%
	Peak area	8.2%	8.0%

[a] Eight replicates.
[b] Four replicates.
[c] Four days, three replicates each day.

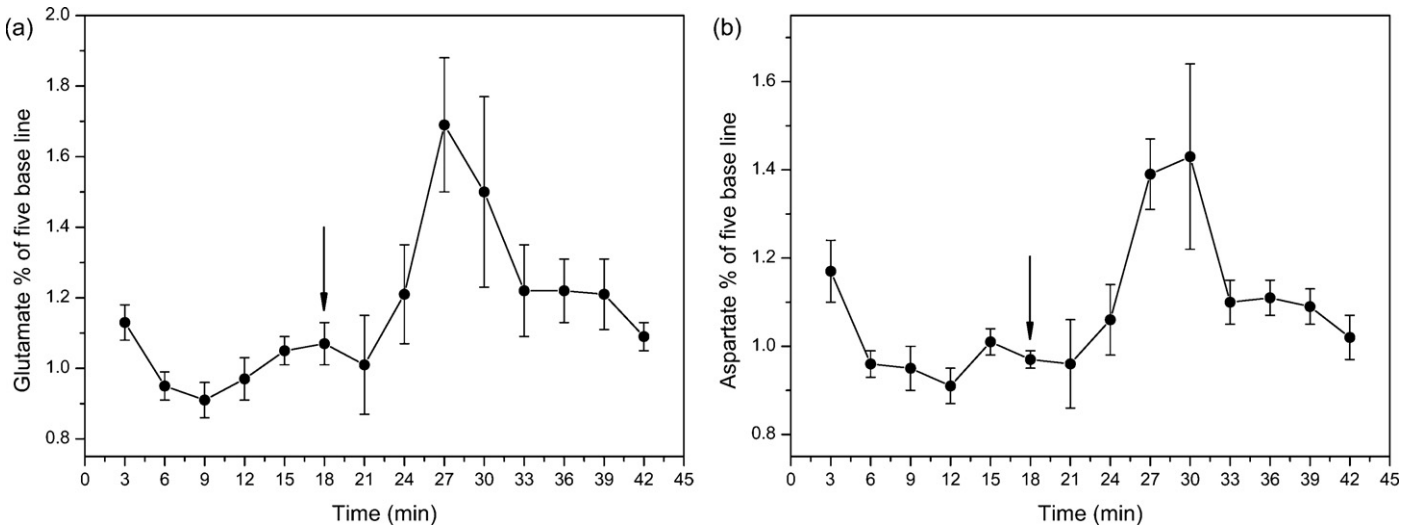

Fig. 2. Percentage of changes of glutamate (a) and aspartate (b) over basal levels in PAG of rats responding to formalin injection. The arrow indicates the start time of formalin injection. Data are presented as mean ± S.E.M. ($n = 5$). $^*p < 0.05$ *vs.* the fifth sample.

(pH 8.50), and allowed to sit overnight in the dark at room temperature.

3.2. Optimization of the separation conditions

The baseline separations of both pair I (CFSE-Glu and the interfering peak 1 labeled in Fig. 1) and pair II (CFSE-Asp and the interfering peak 2 labeled in Fig. 1) are the aim in the optimization of the separation conditions. The data indicated that the composition of borate and boric acid had great influence on the separation of pair I. With increasing the concentration of boric acid in 25 mM borate (gradually decreased pH), the resolution of pair I was gradually improved. The obtained optimal ratio of borate:boric acid was 5:24 and the corresponding pH value was 8.50. In addition, changing the concentrations of running buffer with the same ratio of borate:boric acid (5:24) can seriously affect the resolution of pair II. When buffer concentration was too low, both the two pairs were entirely overlapped. With continuous increase in buffer concentration, the resolution of pair II increased continuously; however that of pair I was basically unchanged. Finally, 25 mM borate and 120 mM boric acid was selected as running buffer.

3.3. Validations

3.3.1. Method validation

The following validation parameters including linearity, limits of detection and quantification, intra-day and inter-day repeatability were determined and shown in Table 1. The RSDs of the migration times were typically better than 0.4%. The RSDs of the peak areas of CFSE–amino acid in standard solutions and microdialysates were in the range of 3.2–4.4% and 3.0–8.2%, respectively.

The limits of detection and quantification were both $<4 \times 10^{-9}$ M, which were lower than the concentrations of Glu and Asp measured in microdialysis samples. In the study, the concentrations of the standard mixture of amino acids which were used to construct the calibration curve were the derivatization concentration, not the dilution concentration injected into the separation capillary. So, the limits of detection and quantification obtained from the calibration curve represented the minimum derivatized concentrations, and these limits were the key to evaluate the analytical potential of a developed LIF method [16].

This method was applied to quantify Glu and Asp in rat PAG microdialysates, and the measured real basal concentrations of Glu and Asp were $(22.4 \pm 1.6) \times 10^{-6}$ M and $(0.9 \pm 0.1) \times 10^{-6}$ M, respectively. Accuracy of the methods and the potential matrix effects were established by the recovery test. The obtained average recoveries were 98% for Glu and 110% for Asp.

Fig. 1(a) and (b) presents the typical electropherograms of blank and 1.0×10^{-6} M derivatized standard mixtures of Glu and Asp, respectively. Obviously, both CFSE-Glu and CFSE-Asp were baseline separated from the reagent excess. Fig. 1(c) and (d) presents the typical electropherograms of brain microdialysates and brain microdialysates spiked with 5×10^{-6} M standard mixture of Glu and Asp, respectively.

3.3.2. Samples preservation

All the solutions before and after derivatization, including the standard solutions of amino acid and brain microdialysates, were stored at −20 °C in a refrigerator. The changes in the fluorescent signals of CFSE-Glu and CFSE-Asp were both lower than 10% for up to 50 days. So, the CFSE–amino acid was very stable and suitable for routine determination of Glu and Asp in brain microdialysates.

3.4. Application in rat microdialysates

The periaqueductal gray matter (PAG) is a dense layer of neurons surrounding the aqueduct of Sylvius. Some experimental evidences suggested that it is activated by noxious stimulation [17,18]. Given that Glu and Asp are both excitatory neurotransmitters that activate NMDA receptors, we measured the dynamic changes of the two neurotransmitters in the PAG of rats in response to nociceptive stimulation of formalin injection. It can be seen from Fig. 2(a) and (b), when formalin was injected to the rat paw, the Glu and Asp in rat brain increased significantly, and our results are agreement with that of the literature [4]. Most importantly, the results further confirmed the accuracy and reliability of our method.

4. Conclusion

The proposed accurate and robust method is suitable for the dynamic analysis of extracellular Glu and Asp in the rat brain. Furthermore, it is a way to detect minute changes in amounts of neurotransmitters in the central nervous system instantly; such a method will have wide applications in various areas of brain research.

Acknowledgement

Financial support from Hong Kong Jockey Club Institute of Chinese Medicine (JCICM-4-07) is gratefully acknowledged.

References

[1] M.O. Urban, G.F. Gebhart, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 7687.
[2] M. Xu, C.J. Kim, M.J. Neubert, M.M. Heinricher, Pain 127 (2007) 253.
[3] P.K. Zahn, K.A. Sluka, T.J. Brennan, Pain 100 (2002) 65.
[4] E. Silva, L. Hernandez, Q. Contreras, F. Guerrero, G. Alba, Pain 87 (2000) 131.
[5] N. Dmitrieva, A.J. Rodríguez-Malaver, J. Pérez, L. Hernández, Eur. J. Pain 8 (2004) 245.
[6] T.E. Robinson, J.B. Justice Jr., Microdialysis in the Neurosciences, Elsevier Science, New York, 1991.
[7] M.W. Lada, T.W. Vickroy, R.T. Kennedy, Anal. Chem. 69 (1997) 4560.
[8] S.Y. Zhou, H. Zuo, J.F. Stobaugh, C.E. Lunte, S.M. Lunte, Anal. Chem. 67 (1995) 594.
[9] C.C. Klinker, M.T. Bowser, Anal. Chem. 79 (2007) 8747.
[10] D.M. Zhang, J.M. Zhang, W.Y. Ma, D.Y. Chen, H.W. Han, H.J. Shu, G.Q. Liu, J. Chromatogr. B 758 (2001) 277.
[11] Y.M. Li, Y. Qu, E. Vandenbussche, L. Arckens, F. Vandesande, J. Neurosci. Methods 105 (2001) 211.
[12] P.R. Banks, D.M. Paquette, Bioconjug. Chem. 6 (1995) 447.
[13] S.K. Lau, F. Zaccardo, M. Little, P. Banks, J. Chromatogr. A 809 (1998) 203.
[14] Y.Q. Liang, X.C. Tang, Neurosci. Lett. 361 (2004) 56.
[15] G. Paxinos, C. Watson, Paxinos & Watson the Rat Brain in Stereotaxic Coordinates, Elsevier Academic Press, Burlington, MA, 2005.
[16] M. Molina, M. Silva, Electrophoresis 23 (2002) 2333.
[17] K.A. Keary, C.I. Clement, B. Owler, A. Depaulis, R. Bandler, Neuroscience 61 (1994) 727.
[18] R. Traub, E. Silva, G.F. Gebhart, A. Solodkin, Neurosci. Lett. 215 (1996) 165.