ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 967–973

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Identification and determination of nucleosides in rat brain microdialysates by liquid chromatography/electrospray tandem mass spectrometry

Yongxin Zhu, Philip S.H. Wong, Qin Zhou, H. Sotoyama, Peter T. Kissinger *

Bioanalytical Systems Inc., 2701 Kent Avenue, West Lafayette, IN 47906, USA

Received 8 February 2001; received in revised form 1 May 2001; accepted 7 May 2001

Abstract

A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed for the determination of brain basal nucleosides (inosine, guanosine and adenosine) in microdialysates from the striatum and cortex of freely moving rats. A microdialysis probe was surgically implanted into the striatum or cortex of individual rats and Ringer's solution was used as the perfusion medium at a flow rate of 0.3 or 0.5 μl/min. The samples were then analyzed off-line by LC/MS/MS experiments. The separation of inosine, guanosine and adenosine was carried out on a cyano column using a mobile phase of 10 mM ammonium acetate, 1% acetic acid and 8% methanol at a flow rate of 0.4 ml/min. Analytes were detected by electrospray ionization tandem mass spectrometry in the positive ion mode. The detection limit for inosine, guanosine and adenosine was 80, 80 and 40 pg on column, respectively. With this method, the intercellular basal inosine, guanosine and adenosine concentrations in striatum and cortex of rat were determined.

Keywords: Nucleosides; Brain microdialysis; LC/MS/MS

1. Introduction

Endogenous nucleosides are involved in the regulation and modulation of various physiological processes in the central nervous system (CNS). Adenosine is known to depress the excitability of CNS neurons and to inhibit release of various neurotransmitters presynaptically [1,2]. There is growing pharmacological evidence from several animal models of seizure disorder that adenosine possesses endogenous anticonvulsant activity [3]. Inosine is the major biochemical metabolite of adenosine due to oxidative deamination, which is catalyzed by the cytoplasmic enzyme adenosine deaminase. The determination of these compounds is important for physiological and pharmacological studies. Nucleosides in biological samples have been analyzed by liquid chromatog-

* Corresponding author. Tel.: +1-765-497-8801; fax: +1-765-497-1102.
E-mail address: pete@bioanalytical.com (P.T. Kissinger).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00450-2

raphy (LC) with UV detection [4–7]. Such methods require sample clean-up and have high limit of quantitation and low selectivity. Liquid chromatography/mass spectrometry (LC/MS) provides a powerful tool for the rapid and sensitive determination of a wide variety of organic compounds [8–11]. Recently, the characterization of nucleoside, nucleotide and modified nucleotides by LC/MS has been reviewed [12] and this method has been widely used to determine nucleotides in biological samples [13–16].

Microdialysis has gained wide recognition as an important sampling technique for in vivo physiological and pharmacological studies [17,18]. In vivo microdialysis is a minimally invasive technique that allows continuous sampling of extracellular substances of low molecular weight that diffuse through a semipermeable dialysis membrane probe implanted in an awake freely moving animal. The microdialysis membrane excludes large molecules and therefore samples can be directly injected into an LC system. This technique is particular useful in the determination of extracellular concentrations of metabolites in which rapid cellular uptake and/or degradation constitutes a major limitation to their measurement by traditional techniques. Some important neurotransmitters and neuromodulators, such as acetylcholine and adenosine are among these metabolites. Microdialysis and liquid chromato-

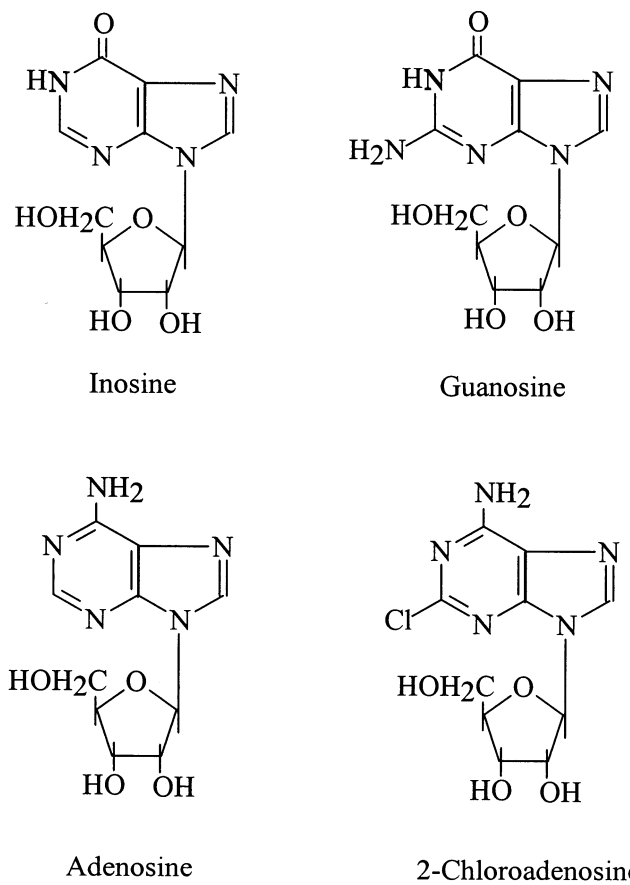

Fig. 1. Structures of inosine, guanosine and adenosine and internal standard 2-chloroadenosine.

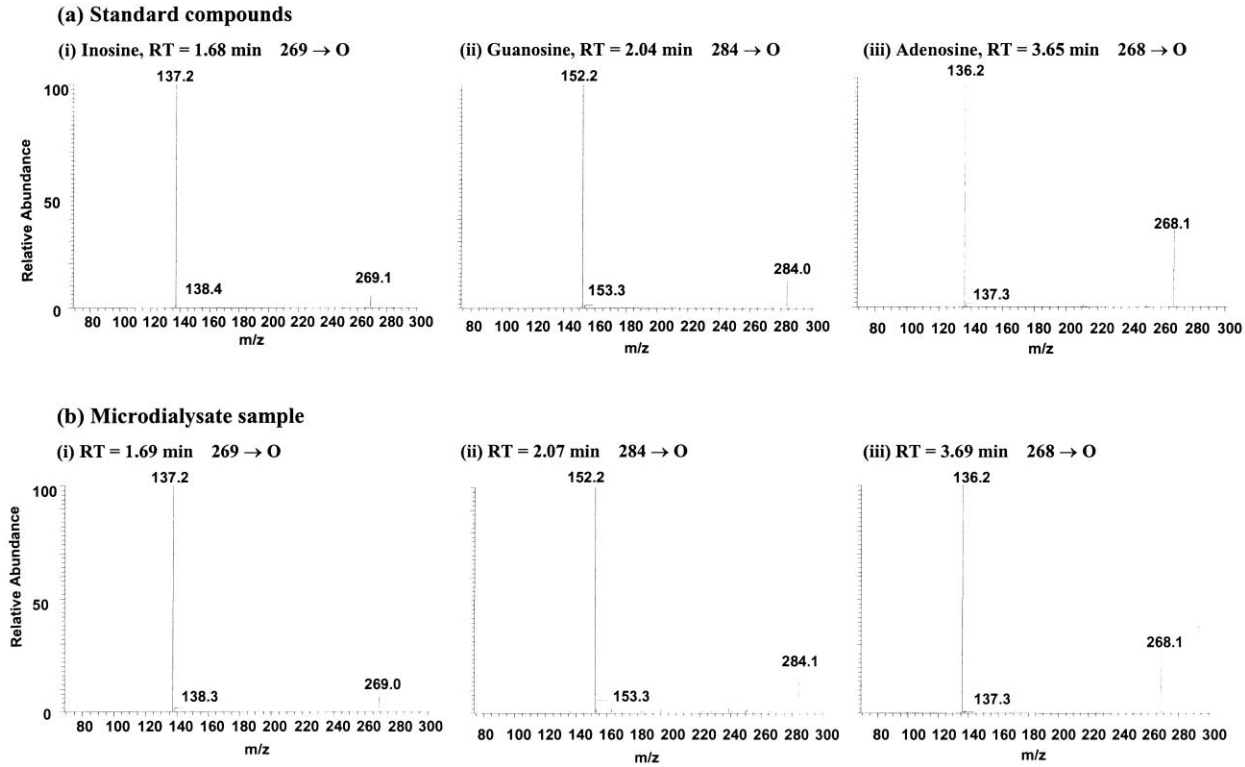

Fig. 2. Full scan MS/MS spectra of (a) authentic standard of inosine (200 ng/ml), guanosine (200 ng/ml) and adenosine (200 ng/ml) and (b) microdialysate sample.

graphic methods are often used for the determination of extracellular nucleoside concentration [19–23]. Recently, microdialysis with mass spectrometric detection has emerged as an important tool in the identification and determination of neurotransmitters in rat brain [24]. The use of microdialysis sampling coupled with LC/MS/MS has been reported as an efficient method to determine melatonin and metabolites in blood [25].

In this paper, we report the use of LC/MS/MS with electrospray ionization (ESI) for the separation and determination of basal inosine, guanosine and adenosine in microdialysis samples from rat brain. Also, full scan MS/MS experiments were used to confirm the identities of the compounds within the brain microdialysates. The goal of the present study was to explore the modern ion trap technology and in vivo microdialysis for the determination of brain microdialysate concentrations of endogenous nucleosides.

2. Experimental

2.1. Materials

Inosine, guanosine, adenosine and 2-chloroadenosine were from Sigma (St. Louis, MO). All other chemicals were analytical reagent grade, from Sigma or Burdick & Jackson (Muskegon, MI). Ringer's solution was prepared as follows: 147 mM Na^+, 2.0 mM Ca^{2+}, 4 mM K^+, 155 mM Cl^-, pH 6.0.

2.2. Microdialysis sampling

Male Sprague-Dawley rats weighting between 350 and 450 g were used for all experiments. All surgical procedures were performed according to Animal Use Statements approved by the BAS Institutional Animal Care and Use Committee. Rats were anesthetized with an intraperitoneal

injection of 1 ml/kg of KX (10 ml ketamine (100 mg/ml) + 1 ml xylazine (100 mg/ml)), after which a guide cannula (MD-2251, BAS, West Lafayette, IN) was stereotaxically implanted into the caudate nucleus-putamen (stratum) at the following coordinates: 2.6 mm anterior from bregma, 2.5 mm lateral from midline and 3.2 mm ventral from surface of the dura mater and the cerebral cortex at the following coordinates: 4.0 mm anterior, 0.8 mm lateral from bregma, 0.8 mm ventral from dura mater according to A Stereotaxic Atlas of the Rat Brain [26]. The animals were allowed to recover from surgery for approximately 3 days. On the day of the experiment, the guide cannula dummy stylet was removed and replaced with a brain microdialysis probe with a 4-mm membrane (MD-2204, BAS). Ringer's solution was perfused through the probe at 0.3 μl/min for the dialysis in cortex and 0.5 μl/min for the dialysis in striatum via a syringe pump (MD-1001, BAS). A lower flow rate would result in a higher concentration recovery. The dialysate fractions were automatically collected into a fraction collector (HoneyComb™, BAS) and maintained at 4 °C. For method development purposes the collection time for each sample was 60 min. During microdialysis sampling, the awake freely moving rat was housed in an interactive rodent containment system which accommodates the animal's movement while preventing the microdialysis tubing from

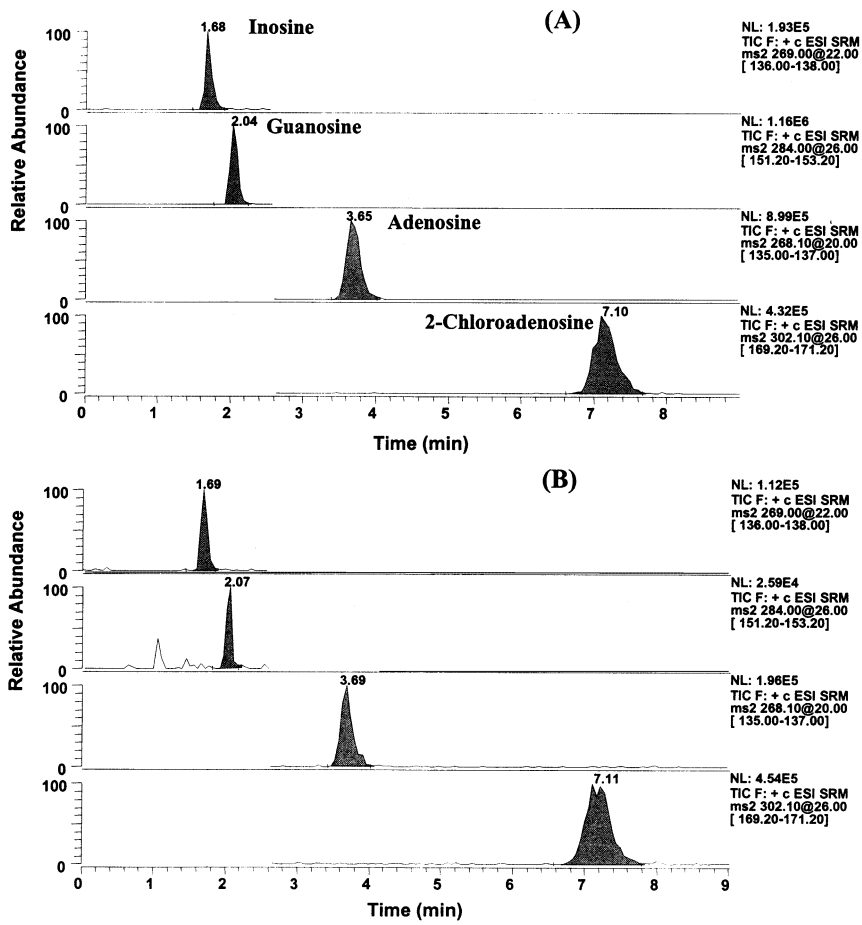

Fig. 3. SRM chromatograms of (A) mixture of four standard compounds, inosine (250 ng/ml), guanosine (250 ng/ml), adenosine (250 ng/ml) and internal standard 2-choloroadenosine (200 ng/ml) and (B) microdialysate sample spiked with internal standard.

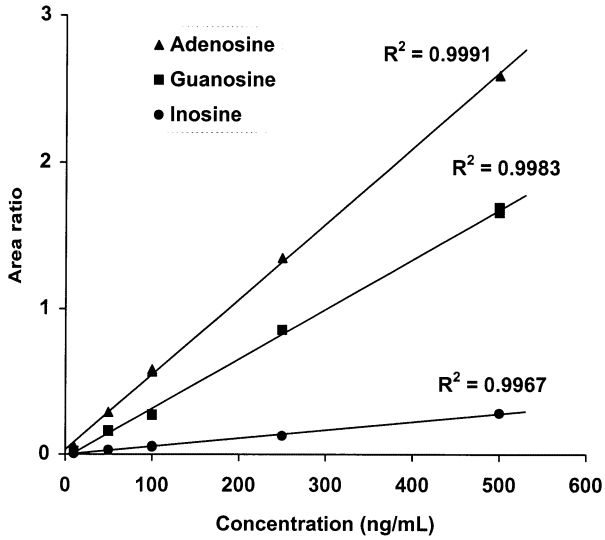

Fig. 4. LC/MS/MS calibration curve for inosine (m/z 269→137), guanosine (m/z 284→152) and adenosine (268→136).

becoming twisted (Raturn™, BAS) [27]. Microdialysis samples (20 μl) were subsequently injected into the LC/MS system. For the quantification of basal nucleosides, microdialysates were mixed with the internal standard (IS) 2-chloroadenosine (200 pg/ml) in a 5:1 ratio.

2.3. LC/MS/MS system

The LC/MS/MS system was equipped with a BAS PM-80 pump coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with an ESI source. The analytical column was a Zorbax SB-CN, 5 μm, 150 × 2.1 mm (Hewlett-Packard, USA). The mobile phase composition was 10 mM ammonium acetate, 1% acetic acid and 8% methanol. The apparent pH of the mobile phase was 3.5. The flow rate was set at 0.4 ml/min. Samples were injected by an autosampler (Sample Sentinel, BAS), which was set at 10 °C and fitted with a 20 μl loop. The mass spectrometer was operated in ESI positive ion mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 80 and 20 units, respectively. The spray voltage and the capillary temperature were set at 5.0 kV and 300 °C, respectively.

For structural confirmation of the nucleosides in rat brain microdialysate, full scan MS/MS experiments were performed. The mass range of ions was scanned in order to detect all product ions of the selected parent ions and compared to the authentic compounds.

For the determination of nucleosides, the analytes were monitored using the selected reaction monitoring (SRM) mode, which is more specific for the analysis. The parent ions of inosine (m/z 269), guanosine (m/z 284), adenosine (m/z 268) and IS (m/z 302) were mass-selected while the product ions m/z 137, 152, 136 and 170 were monitored for inosine, guanosine, adenosine and IS, respectively. Two segments were used and each had two scan events. In the first segment, two events of m/z 269→137 and m/z 284→152 were used to monitor inosine and guanosine for the first 2.5 min, while in the second segment, two events of m/z 268→136 and m/z 302→170 were used to monitor adenosine and the IS for the next 6.5 min. Collision-induced dissociation of the parent ions with helium gas was performed at 22, 26, 20 and 26% collision energy for inosine,

Table 1
Concentration (ng/ml) of nucleosides from rat brain microdialysates

	Striatum			Cortex		
	Rat 1	Rat 2	Rat 3	Rat I	Rat II	Rat III
Inosine	N/D	13.0 ± 1.0	N/D	446 ± 36	123 ± 14	628 ± 74
Guanosine	7.7 ± 0.7	22.6 ± 1.4	19.5 ± 1.9	27.3 ± 0.5	12.2 ± 0.2	21.6 ± 1.4
Adenosine	137 ± 2	231 ± 10	129 ± 11	N/D	35.2 ± 2.7	3.1 ± 0.4

The values in the table are relative concentrations, without in vivo calibration, the length of the probe membrance was 4 mm, the flow rate for striatum and cortex was 0.5 and 0.3 μl/min, respectively. Mean ± S.D., $n = 3$; N/D, not detectable.

guanosine, adenosine and IS, respectively and with an isolation width of 2.0 amu.

3. Results and discussion

3.1. Identification of nucleosides in rat brain microdialysate

In a full scan LCMS study of a microdialysate sample, three chromatographic peaks, monitored at m/z 269, 284 and 268 eluted at 1.69, 2.07 and 3.69 min, respectively. These three ions were further analyzed by full scan MS/MS experiments for the identification of the three target compounds (inosine, guanosine and adenosine). The structures of inosine, guanosine and adenosine are shown in Fig. 1. Standard solutions of inosine, guanosine and adenosine were prepared in Ringer's solution and injected separately into the LC/MS system. Full scan MS/MS spectra of these standard solutions were taken and then compared with those of the components of the microdialysate samples. Fig. 2 shows the MS/MS spectra of both the standard solutions and microdialysis samples. The parent ions of inosine, guanosine and adenosine are m/z 269, 284 and 268, respectively. Their respective fragment ions are m/z 137, 152 and 136 corresponding to the base of each nucleoside. A match between the MS/MS spectra of a standard solution and the dialysate sample confirms that the peaks eluting at retention times of 1.69, 2.07 and 3.69 min are inosine, guanosine and adenosine, respectively. A standard mixture containing four compounds (inosine, guanosine, adenosine and 2-chloroadenosine) was injected (20 μl) into the LC/MS system. They were monitored using the SRM mode. Inosine was monitored by scanning m/z $269 \rightarrow 137$, guanosine by m/z $284 \rightarrow 152$, adenosine by m/z $268 \rightarrow 136$ and 2-chloroadenosine by m/z $302 \rightarrow 168$. Fig. 3(A) shows the SRM ion chromatograms of the mixture. A microdialysate sample spiked with IS was also analyzed in the same way and Fig. 3(B) shows the SRM ion chromatograms. This shows that all four compounds can be monitored in a single experiment without any interference.

3.2. In vitro recovery of nucleosides

Generally, only free analyte can be recovered from a tissue by microdialysis, which are both a limitation and an advantage of this methodology in drug metabolism studies. The relative in vitro recovery was determined by placing the microdialysis probe into a stirred standard solution containing inosine (400 ng/ml), guanosine (230 ng/ml) and adenosine (100 ng/ml). The solution was perfused at room temperature with Ringer's solution at perfusion rate of 1.0 μl/min. The relative recovery ($\text{recovery}_{\text{in vitro}} = C_{\text{out}}/C_{\text{i}}$) was calculated by comparing the areas under the chromatographic peaks using selected reaction monitoring for inosine, guanosine and adenosine in the dialysate (C_{out}) and in the medium (C_{i}). The relative recovery was found to be about 39, 28 and 36% for inosine, guanosine and adenosine, respectively. The relative recovery indicates that the microdialysis probe provides good extraction efficiencies for nucleosides. This will vary with probe geometry, flow rate, and membrane composition.

3.3. Determination of nucleosides in rat brain microdialysate

Fig. 3(B) shows the SRM mass chromatogram of a rat brain microdialysate sample spiked with the internal standard 2-chloroadenosine. The detection limit for inosine, guanosine and adenosine are 80, 80 and 40 pg on column, respectively. This method is about 30 times more sensitive than UV detection [5]. It has been stated that extracellular adenosine in dialysates from the striatum and nucleus accumbens of the rat can not be detected using UV detection [7]. Calibration curves were constructed by using area ratios of inosine, guanosine, adenosine to IS. Fig. 4 shows the calibration curves of these three compounds in the range of 10–500 ng/ml. The basal striatal and cortical concentration of inosine, guanosine and adenosine in microdialysates from three rats was determined. Table 1 shows that adenosine has a higher concentration in striatum while its metabolite inosine has a higher concentration in cortex. Both adenosine and inosine concentrations show significant differences between striatum and cor-

tex. But guanosine maintains a much closer concentration in both brain regions.

Intracellular adenosine in the brain is inactivated by two main pathways. One of the pathways involves its oxidative deamination to inosine, which is catalyzed by the cytoplasmic enzyme adenosine deaminase. Both biochemical and immunohistochemical studies of adenosine deaminase indicated that specific adenosinsinergic neurons are present in brain, and that this enzyme may serve as a marker for such cells [28,29]. But these studies are insufficient and cast serious doubt on the use of adenosine deaminase as a neurotransmitter/neuron marker. Direct determination of adenosine and its metabolite inosine in a specific brain region by in vivo microdialysis and LC-MS/MS provides a new approach to confirm the location of adenosinergic pathways in the brain.

4. Conclusion

A reverse phase liquid chromatography/tandem mass spectrometry method has been developed for the determination of inosine, guanosine and adenosine in rat brain microdialysate. Structural identification of these nucleoside compounds in microdialysate was confirmed by full scan MS/MS experiments. In vivo microdialysis can be coupled on-line to LC/MS/MS [25] for the continuous monitoring of brain nucleosides. This technique shows promise for studying brain pharmacodynamics of endogenous nucleosides in a freely moving animal.

References

[1] R. Coradetti, G. Lo Conte, F. Moroni, M.B. Passani, G. Pepeu, Eur. J. Pharmacol. 104 (1984) 19–26.
[2] C. Schmidt, M.C. Bellingham, D.W. Richter, J. Physiol. 483 (1995) 769–781.
[3] M. Ballarin, M. Herrera-Marschitz, M. Cassa, U. Ungerstedt, Neurosci. Lett. 83 (1987) 338–344.
[4] G. Xu, C.D. Stefano, H.M. Liebich, Y. Zhang, P. Lu, J. Chromatogr. B 732 (1999) 307–313.
[5] G. Luippold, U. Delabar, D. Kloor, B. Muhlbauer, J. Chromatogr. B 724 (1999) 231–238.
[6] D.L. Walters, D.L. Jacobs, J.E. Tomaszewski, S. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955–965.
[7] A. Salem, W. Hope, Eur. J. Pharmacol. 369 (1999) 39–42.
[8] P. Michelsen, G. Pettersson, Rapid Commun. Mass Spectrom. 8 (1994) 517–520.
[9] Y. Takada, M. Yoshida, M. Sakairi, H. Koizumi, Rapid Commun. Mass Spectrom. 9 (1995) 895–896.
[10] P.E. Andren, M.R. Emmett, B.B. DaGue, A.F. Steulet, P. Waldmeier, R.M. Caprioli, J. Mass Spectrom. 31 (1998) 281–287.
[11] P.E. Andren, R.M. Caprioli, Brain Res. 845 (1999) 123–129.
[12] E.L. Esmans, D. Broes, I. Hoes, F. Lemiere, K. Vanhoutte, J. Chromatogr. A 794 (1998) 109–127.
[13] J.V. Weber, K. Sampino, R. Dunphy, D.J. Burinsky, T. Williams, M.G. Motto, J. Pharm. Sci. 83 (1994) 525–531.
[14] N. Takeda, H. Yoshizumi, T. Niwa, J. Chromatogr. B 746 (2000) 51–62.
[15] M. Abedel-Hamid, L. Novotny, H. Hamza, J. Pharm. Biomed. Anal. 22 (2000) 745–755.
[16] J.F. Banks Jr., S. Shen, C.M. Whitehouse, J.B. Fenn, Anal. Chem. 66 (1994) 406–414.
[17] C.E. Lunte, D.O. Scott, P.T. Kissinger, Anal. Chem. 63 (1991) 773A–780A.
[18] W.F. Elmquist, R.J. Sawchuk, Pharm. Res. 14 (1997) 267–288.
[19] B.A. Meyerson, B. Linderoth, H. Karlsson, U. Ungerstedt, Life Sci. 46 (1990) 301–308.
[20] P. Kuang, Y. Tao, J. Shi, J. Tradit. Chin. Med. 17 (1997) 140–147.
[21] A. Dobolyi, A. Reichart, T. Szikra, N. Szilagyi, A.K. Kekesi, T. Karancsi, P. Slegel, M. Palkovits, G. Juhasz, Neurochem. Int. 32 (1998) 247–256.
[22] A. Melani, L. Pantoni, C. Corsi, L. Bianchi, A. Monopoli, R. Bertorelli, G. Pepeu, F. Pedata, Stroke 30 (1999) 2448–2454.
[23] S. Kondoh, S. Nagasawa, M. Kawanishi, K. Yamaguchi, S. Kajimoto, T. Ohta, Neurol. Res. 21 (1999) 682–686.
[24] Y. Zhu, P.S.H. Wong, M. Cregor, J. Gitzen, L. Coury, P.T. Kissinger, Rapid Commun. Mass Spectrom. 14 (2000) 1670–1695.
[25] P.S.H. Wong, K. Yoshioka, F. Xie, P.T. Kissinger, Rapid Commun. Mass Spectrom. 13 (1999) 407–411.
[26] L.J. Pellegrino, A.S. Pellegrino, A.J. Cushman, A Stereotaxic Atlas of the Rat Brain, Plenum Press, New York, London, 1979.
[27] Metabolic Chamber Operation for BAS Raturn™, Current Separations, 18 (1999) 62.
[28] T. Yamamoto, J.D. Geiger, P.E. Daddona, J.I. Nagy, Brain Res. Bull. 19 (1987) 473–484.
[29] J.I. Nagy, T. Yamamoto, H. Uemura, W.P. Schrader, Neuroscience 73 (1996) 459–471.