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Journal of Chromatography A, 870 (2000) 405–411

JOURNAL OF CHROMATOGRAPHY A

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Determination of catecholamines in pheochromocytoma cell (PC-12) culture medium by microdialysis–microbore liquid chromatography

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Abstract

An in vitro microdialysis system was constructed for the measurement of catecholamines in pheochromocytoma cell culture medium. The novel microdialysis device is composed of a petri dish, a dialysis membrane and two transmission tubes. The dialysis membrane is located in the space of a petri dish such that it is immersed in the culture medium. Catecholamines contained in the culture medium diffused into a designed dialysis membrane with sufficient recovery (about 60%). Dialysates were collected by a sampling loop and introduced by an on-line injector to a microbore liquid chromatographic system for analysis of catecholamines. This assay yielded a detection limit of 0.2–0.5 pg/injection with acceptable intra- and inter-assay reproducibilities in 5 μl of dialysates. To evaluate the on-line microdialysis system, PC-12 cells were cultured in a petri dish within an incubator. The baseline concentration of dopamine in PC-12 cell culture medium was about 0.29 ng/ml which was elevated to 2.43 ng/ml after treatment with 0.5 m*M* potassium cyanide. In conclusion, the present microassay provides for the sensitive, direct measurement of catecholamines in culture medium while minimizing pretreatment procedures for sample preparation.

Keywords: Pheochromocytoma cells; Catecholamine

1. Introduction

Microdialysis is one of the most widely used techniques for in vivo or in vitro sampling of the chemical substances in extracellular fluids of animal tissues, or cultured cells [1]. Catecholamines and their related compounds play a number of important functions in the peripheral and central nervous systems, and have been implicated in the neurochemistry and physiology of mental diseases and neurological disorders [2,3]. Measurements of catecholamines utilizing high-performance liquid chromatography (LC) and electrochemical detection (ED) [4] are useful in investigating the etiology of neurological disorders in animal models or cell cultures [5–7]. Recently, microbore LC–ED detection has led to sub-femtomolar detection for catecholamines [8–10]. Microdialysis and microbore

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)00910-3

LC–ED technique are complementary tools that are routinely used in this laboratory to study extracellular catecholamines or their metabolites in rat brain [8–10]. In vitro microdialysis studies were also carried out in biological fluids [11] and tissues chambers [12,13]. Dynamic secretion of steroids and peptides in corpus luteum were carried out in a prolonged time by Maas et al. [12]. Similarly, oxytocin stimulates progesterone release from microdialysated bovine corpus luteum was demonstrated in vitro by Miyamato and Chams [13].

Pheochromocytoma cells (PC-12) resemble adrenal chromaffin cells which share many physiological and pharmacological properties of neurons [14–16]. PC-12 cells synthesize, store, and secrete dopamine (DA) and norepinephrine (NE) [15]. On stimulation, PC-12 cells release catecholamines by exocytosis [16]. Many studies have been carried out on PC-12 cells via catecholamine determination to investigate how particular drugs affect the release or uptake of dopamine by PC-12 cells [15–17]. In general, PC-12 cells have been plated in culture dishes and incubated in an incubator. At different times after incubation or certain treatments, the supernatant of cells are removed and analyzed via conventional pretreatment prior to LC–ED analysis. There is no difficulty in the measurement of intracellular catecholamines in PC-12 cells because of their relatively high concentrations (20–200 ng/ml). However, the measurement of extracellular catecholamines in culture medium using conventional LC–ED has been difficult and challenging. First, the extremely low concentration of catecholamines in medium requires an analytical detection limit in low picograms for successful measurement. Second, it is necessary to centrifuge the cells and filtrate cell debris to collect the culture medium, which may cause contamination of PC-12 cell culture system. Third, the detected catecholamine concentrations in the PC-12 cell culture medium are often low which may result in either difficulty in analysis or unreliable data from conventional LC–ED. Fourth, the medium samples require time-consuming pretreatments or complicated extraction prior to analysis by conventional LC–ED assays, which may result in sample losses and increases in the amount of time necessary to complete experiments. Based on Maas et al. and Miyamoto and Schams studies [12,13], we describe an on-line in vitro microdialysis-microbore LC–ED technique for the measurement of catecholamines in PC-12 cell culture medium.

2. Experimental

The microdialysis microbore LC–ED was comprised of a micropump (CMA-100, CMA, Stockholm, Sweden), an on-line injector (CMA-160), a Microtech LC-pump (Micro-tech Scientific, Sunnyvale, CA), a BAS-4C electrochemical detector (Bioanalytical Systems, Inc., West Lafeyette, IN), a Beckman I/O 406 interface (AI-406), Beckman System Gold Data Analysis Software (Beckman Instrument Inc., Taiwan Branch), and a microbore reversed-phase column filled with Inertsil ODS-2 (GSK-C_{18}, 5 μm ODS, 1.0×150 mm I.D., GL Sciences Inc., Tokyo, Japan). The potential for the glassy carbon working electrode was set at+0.75 V, with respect to a Ag/AgCl reference electrode [18]. Chromatograms were recorded on the Beckman I/O 406 interface, and analyzed via the Beckman System Gold Data Analysis Software. To increase the detection sensitivity of the microbore LC system, a very thin spacer (16 μm) was used instead of a conventional one (51 μm) to create a sub-microliter thin-layer electrochemical cell. Very slow flow-rates were used to minimize pulse fluctuations and noise.

NE, EPI, DA, DOPAC, ethylenediaminetetraacetic acid (EDTA), 1-octanesulfonate (SOS), NaOH, and *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanosulfonic acid] sodium salt (HEPES) were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (CH_3CN) and tetrahydrofuran (THF) were obtained from E. MERCK (MERCK-Schuchardt, Darmstadt, Germany). Monochloroacetic acid ($CH_2ClCOOH$) was purchased from BDH Laboratory Supplies (BDH Laboratory Supplies, Poole, UK). Unless otherwise stated, all reagents were of analytical quality.

PC-12 cells were grown in NUNC T75 flasks (Naperville, IL) in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 5% horse serum and 10% fetal bovine serum (FBS), and maintained in a 10% CO_2 humidified incubator at 37°C as previously described [19]. PC-12 cells were cultured on 35 mm petri dish

with DMEM. The working buffer was prepared by dissolving 0.17 g HEPES in 25 ml liquid medium of DMEM Base, which contained L-glutamine, phenol red, and sodium pyruvate. Then, the pH of the buffer was adjusted to 7.4.

The standard stock solutions of NE, EPI, DOPAC, and DA were prepared at a concentration of 2 μg/ml in 0.1 *M* perchloric acid and stored at −70°C in the dark. For the daily preparation of a standard mixture, a portion of these stock solutions was thawed at 4°C and diluted to the appropriate concentration of 20 ng/ml with a solution containing 10^{-7} *M* ascorbic acid in 0.1 *M* HCl.

The buffer consisted of 9.60 g monochloroacetic acid, 0.16 g sodium 1-octane sulfonate, and 10 mg EDTA, adjusted to pH 3.0 with 1 *M* sodium hydroxide. The final volume of the mixture was adjusted to 1 l with doubled distilled water. The mobile phase was prepared by mixing 50 ml acetonitrile and 950 ml phosphate buffer. The solution was filtered through a nylon filter under reduced pressure and degassed with helium for 15 min. The flow-rate was 80 μl/min, and the column pressure was maintained at ca. 12.4 MPa.

As illustrated in Fig. 1, a sampling device is composed of a petri dish (B and E), two transmission tubes (A), and a dialysis membrane (D). The petri dish had a receiving space with an open top. The cover contained with two holes separated by a predetermined distance. This microdialysis system is similar to those reported by Mass et al. [12] and Miyamato and Chams [13]. The top ends of the two transmission tubes jutted out through the holes in the cover. The bottom ends of the transmission tubes were located in the receiving space of the main body, and curved such that the longitudinal central line of the bottom ends and the longitudinal central line of the transmission tubes formed a predetermined angle. The outer wall surface of the transmission tubes and the inner wall surface of the holes of the cover were secured by sealing material. The dialysis tube (D) was made of a dialysis membrane (Spectrum, 20 mm length, 150 μm outer diameter with a cut-off at nominal molecular weight of 13 000, Laguna Hills, CA), and was immersed in the PC-12 cell culture medium (C) as shown in Fig. 1.

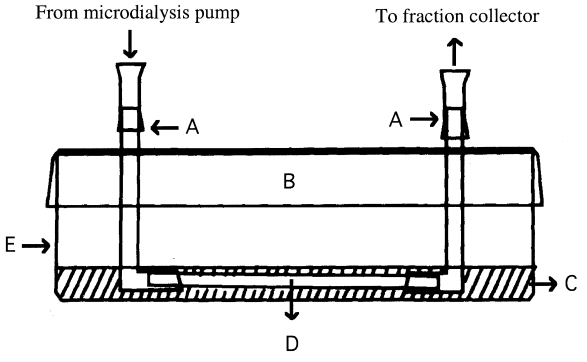

Fig. 1. A schematic diagram of a novel dialysis petri dish. (A): Transmission tubes; (B): Petri dish cover; (C): Culture medium; (D): Dialysis membrane (20 mm in length); (E): Petri dish body.

The sampling device was perfused with the working buffer at 2 μl/min by a micropump (CMA-100). Microdialysates were introduced by an on-line injector (CMA-160) and analyzed by a microbore LC–ED at 30 min intervals. Following a 3 h baseline collection, KCN was introduced to give a final concentration of 0.5 m*M* KCN in the culture medium. PC-12 cells under KCN-induced hypoxia were incubated for an additional 5 h. The equipped on-line injector (CMA-160) was used to collect microdialysates. Five μl of aliquots of microdialysates were automatically assayed for catecholamines by a microbore LC–ED while the petri dish was kept in an incubator.

Prior to the experiments, in vitro recovery was performed using a standard mixture containing catecholamines, to determine the recoveries of all analytes and the dead volume (ca. 36 μl) of the microdialysis system. Experimental data were recorded and analyzed via the Beckman System Gold Data Analysis Software.

3. Results and discussion

Fig. 2A shows a typical chromatogram of a standard mixture (ca. 0.02 ng/ml each) containing NE (3.6 min), EPI (4.8 min), DOPAC (6.9 min), and DA (9.4 min). Analysis was completed within 10 min. Fig. 2B and C show typical chromatograms of microdialysates obtained from PC-12 cell culture medium. All components under study were well resolved. Retention times of NE, DOPAC, and DA in Fig. 2A, B, and C were identical. In the preliminary

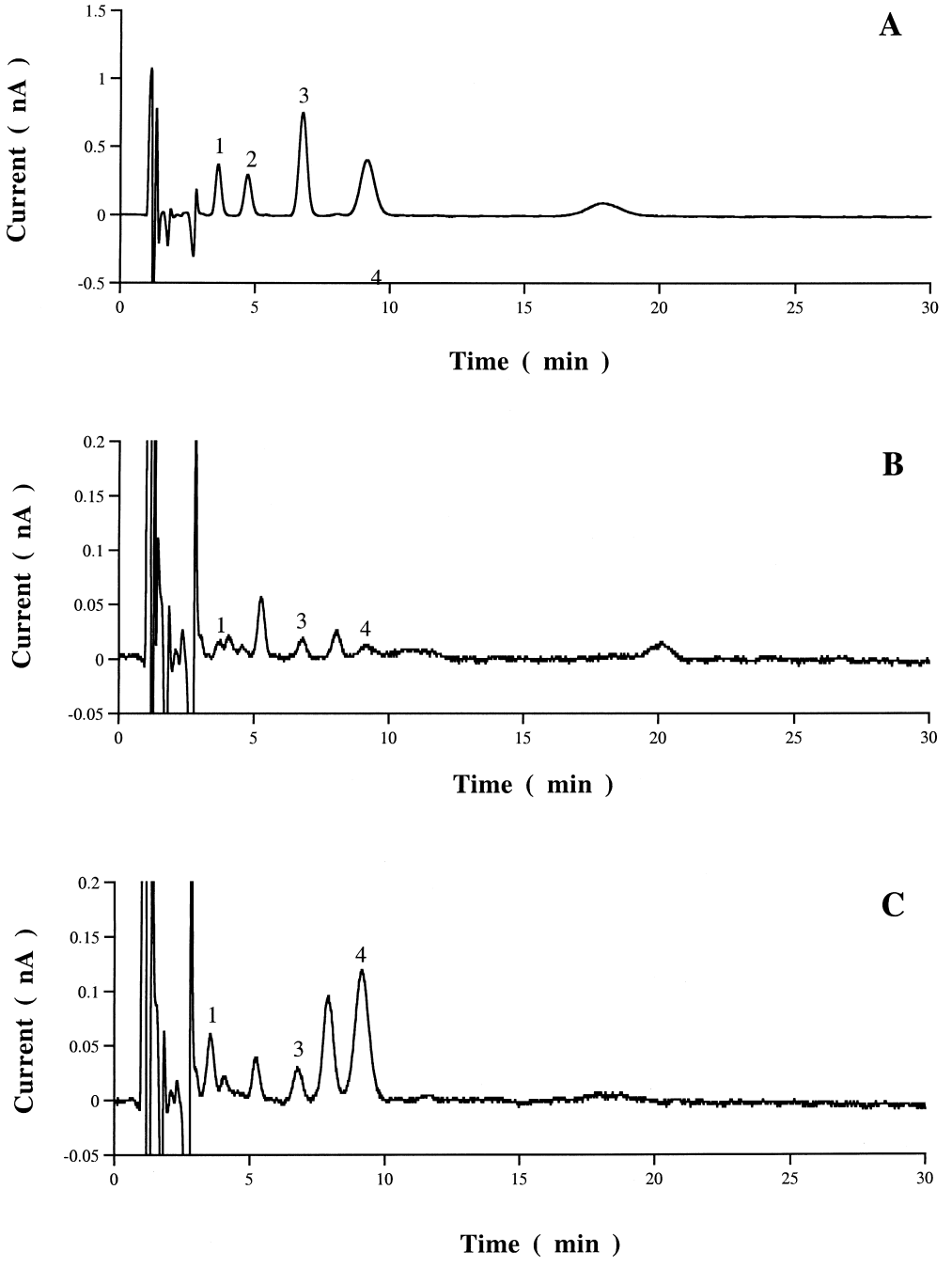

Fig. 2. Typical chromatograms obtained from (A) a standard mixture containing (1) NE, (2) EPI, (3) DOPAC, and (4) DA; (B) a microdialysis of baseline; and (C) a microdialysate after KCN-induced hypoxia.

experiments, EPI was always undetectable in culture medium. which was in agreement with other investigators [20]. The baseline concentrations of NE, DOPAC, and DA in PC-12 cell culture medium were 0.77, 0.55 and 0.29 ng/ml, respectively. Several unknown peaks appeared within 30 min. In order to prevent carry-over peaks from previous runs, a complete run was set at 30 min.

In general, the amounts of each injected analyte were linearly related to chromatographic areas obtained from standard mixtures ($R^2 \geq 0.999$) over a large range of concentrations (1– 2000 pg, Table 1) in microbore LC–ED. However, calibration curves were constructed with three standard mixtures (containing ca. 1, 10, and 100 pg of catecholamines, $n=3$) prior to LC–ED analyses on the day of experiments. Concentrations of NE, DOPAC, and DA in dialysates were determined by these calibration curves.

The precision of the assays was tested using a standard mixture and pooled dialysates of PC-12 cell culture medium (Table 2). The intra-assay variabilities were assessed with 12 replicates at 1 h intervals and expressed as coefficients of variation (C.V.,%). The intra- ($n=12$) and inter-assay ($n=6$) variabilities were assessed and the C.V. values of all analytes in dialysates were less than 7%. In general, the C.V.s for DA and NE were lower than the C.V.s for EPI and DOPAC in the chromatographic measurements. The detection limits (signal-to-noise ratio =3) of all analytes in the present assay were between 0.2 and 0.5 pg per injection (or 0.04–0.1 ng/ml).

The concentration of an analyte in the dialysate relates to the concentration of that analyte in the PC-12 cell culture medium. This relation is called the relative recovery and is usually expressed as a percent value. For in vitro microdialysis recovery, the dialysis device was calibrated in a standard solution containing 100 ng/ml of each analyte. The working buffer solution was perfused at flow-rates ranging from 0.3 to 5.0 μl/min. The in vitro recovery ($R_{\text{in vitro}}$%) of each analyte was calculated from the concentration of each analyte (C_{out}) in dialysates divided by the concentration of the analyte in the petri dish (C_{in}). The higher the perfusion flow-rate, the lower the recovery as shown in Table 3. In general, experiments were carried out at 1.0–3.0 μl/min to collect sufficient amount of samples and to overcome the dead volume of the dialysis device. The perfusion flow-rate was fixed at 2.0 μl/min in the present study (recovery values between 56 and 66%). In vitro recovery values of all

Table 1
Correlation of anodic current with the amount of biogenic amines, and their metabolites in standard mixtures (ranging from 1 pg to 2000 pg) measured by the microbore LC–ED system

	Standard curve equation[a]	R^2
NE	$Y=4020X+3$	1.000
EPI	$Y=3865X+14$	0.999
DOPAC	$Y=6360X-5$	1.000
DA	$Y=5880X+18$	1.000

[a] Y = peak area measured; X = amount of analytes in pg.

Table 2
Analytical precision on the stabilities of intra-assay ($n=12$, at 1 h intervals) and inter-assay ($n=6$, in six consecutive working days) of standard mixtures and PC-12 cells dialysates in the microbore LC–ED system

	Coefficient of variation (%)			
	NE	EPI	DOPAC	DA
Intra-assay				
Standard mixture in 0.1 *M* HCl				
(ca. 1 pg each)	3.9	2.4	6.2	2.4
(ca. 50 pg each)	2.3	3.5	4.2	2.1
PC-12 cells pooled dialysates	4.8	N.D.	5.2	3.3
Inter-assay				
Standard mixture in 0.1 *M* HCl				
(ca. 50 pg each)	4.3	5.2	5.3	4.0

Table 3
In vitro recovery values (%) of catecholamines at varied perfusion flow-rates for the novel dialysis device

Flow rate (μl/min)	NE	EPI	DOPAC	DA
0.3	93	99	87	97
0.5	89	92	81	90
1	82	82	73	79
1.5	72	72	63	71
2	65	66	56	64
3	50	51	42	50
5	29	31	27	31

analytes at 2.0 μl/min were also recorded and validated on each dialysis device prior to experiments.

Extracellular catecholamine concentrations in dialysates of PC-12 cells did not change much within an 8 h period as shown in Table 4. However, NE concentrations (0.77 ng/ml at baseline) dramatically increased to 15.8 ng/ml within 5 h after 0.5 m*M* KCN treatment, as shown in Table 4. DOPAC concentrations (0.55 ng/ml at baseline) slowly increased to about 3 fold of baseline (1.84 ng/ml) at 5 h after 0.5 m*M* KCN treatment. DA concentrations demonstrated a transient elevation (18 fold, from 0.29 to 2.43 ng/ml) at the beginning of KCN treatment and drastically decreased to relatively lower levels (about two folds of baseline) within 3 h after 0.5 m*M* KCN treatment. The mechanism by which KCN increased catecholamine levels in the extracellular space probably reflects both an increased release of catecholamines into the medium as well as a decreased removal of these substances from the medium. Removal of substances from the medium is largely due to an uptake mechanism which is an adenosine triphosphate (ATP)-dependent process. KCN-induced hypoxia is known to cause ATP depletion [21]. Therefore, the uptake mechanism is blocked and catecholamine levels accumulate. The entire time profiles of catecholamines obtained from PC-12 cell culture medium before and after KCN-induced hypoxia are shown in Table 4. It is evident that these analytes varied very soon after administration of KCN. In general, increased release of catecholamines was demonstrated during KCN-induced hypoxia. These data are in agreement with those of other investigators [22,23]. However, the detailed mechanism of the release of catecholamines during KCN-induced hypoxia requires further investigation.

The present method requires no pretreatment of samples, whereas conventional pretreatment procedures require more than 4 hours to prepare 8–10 samples [24–26]. The cumbersomeness of pretreatment procedures of small volumes of culture medium is still a problem for culture systems. Another advantage of this method is its low detection limits (0.2–0.5 pg/per injection, typically). This on-line method can increase the experimental speed, provide high sensitivity, minimize required sample volume, enhance detection limits, and decrease degradation of the analyzed compounds.

In vitro microdialysis system described by Maas et al. [12] and Miyamoto and Schams [13] was modified. An autosampler and an incubator were used in this study. In conclusion, the dialysis device is

Table 4
Concentrations of NE, DOPAC, and DA in PC-12 cell culture medium dialysates obtained from petri dishes incubated under control or KCN treatment

Time (h)	NE		DOPAC		DA	
	Control	KCN	Control	KCN	Control	KCN
0.5	0.16	1.03	0.25	0.24	0.10	0.18
1	0.48	1.77	0.23	0.31	0.14	0.25
1.5	0.58	1.81	0.21	0.40	0.07	0.18
2	0.73	1.98	0.22	0.58	0.09	0.13
2.5	0.91	1.74	0.42	0.64	0.05	0.19
3[a]	0.57	0.77	0.50	0.55	0.31	0.29
3.5	0.60	3.16	0.45	0.69	0.54	0.53
4	0.53	4.21	0.47	1.02	0.07	2.43
4.5	0.63	5.48	0.60	1.24	0.07	1.51
5	0.50	8.34	0.61	1.20	0.19	0.99
5.5	0.49	9.62	0.55	0.92	0.08	0.61
6	0.41	9.94	0.42	1.08	0.02	0.57
6.5	0.57	12.51	0.78	1.08	0.23	0.65
7	0.51	12.77	0.66	1.52	0.38	0.52
7.5	0.49	15.06	0.78	1.84	0.22	0.78
8	0.73	15.80	0.71	1.84	0.14	0.48

[a] KCN was given at 3 h.

capable of taking samples for examining the extracellular catecholamines with minimal disruption of the normal growth of cells in PC-12 cell culture. The device also avoids contamination of cell culture and minimizes the disturbance on the closed system for a prolonged period of time. Microdialysis samples are free from impurities and macromolecules and can be directly analyzed via LC. Microbore LC–ED has the advantages of signal enhancement and noise reduction. As a result, low detection limits and short analysis times can be obtained. The novel dialysis device described in the present study is relatively simple in construction and is compatible with an on-line injection analyzer. The present study is relatively efficient, cost-effective, and less vulnerable to human error, as compared with conventional studies in which a number of petri dishes are used. Indeed, the present study minimizes pretreatment procedures for sample preparation, decreases possible contamination from sampling of culture medium, and enhances the detection sensitivity of catecholamines in PC-12 cell culture medium. Furthermore, this novel microdialysis device can also be applied to the measurement of chemical substances in other culture systems.

Acknowledgements

This study was supported in part by grants from Taichung Veterans General Hospital (TCVGH-897304C) and the National Science Council (NSC-88-2113M-075A-002) of the Republic of China.

References

[1] T.E. Robinson, J.B. Justice Jr. (Eds.), Microdialysis in the Neurosciences, Elsevier Science, Netherlands, 1991, p. 1.
[2] H. Yao, K. Fukiyama, Y. Takada, M. Fujishima, T. Omae, Jpn. Heart J. 37 (1985) 593.
[3] D.M.A. Mann, P.O. Yates, B. Marcyniuk, Clin. Neuropathol. 3 (1984) 199.
[4] E. Kempf, P. Mandal, Anal. Biochem. 112 (1981) 223.
[5] C.Y. Chai, A.M.Y. Lin, C.K. Su, S.R. Hu, L.S. Kao, J.S. Kuo, D.S. Goldstein, J. Auton. Nerv. Syst. 33 (1991) 35.
[6] C.A. Altar, M.R. Marien, J. F Marshall, J. Neurochem. 48 (1987) 390.
[7] H. Nissbrandt, A. Carlsson, J. Neurochem. 49 (1987) 59.
[8] F.C. Cheng, L.L. Yang, F.M. Chang, L.G. Chia, J.S. Kuo, J. Chromatogr. B 582 (1992) 19.
[9] F.C. Cheng, J.S. Kuo, J. Chromatogr. B 665 (1995) 1.
[10] F.C. Cheng, Y. Shih, Y.J. Liang, L.L. Yang, C.S. Yang, J. Chromatogr. B 682 (1996) 195.
[11] F.C. Cheng, L.L. Yang, F.M. Chang, L.G. Chia, J.S. Kuo, J. Chromatogr. 582 (1992) 19.
[12] S. Maas, H. Jarry, A. Teichmann, W. Rath, W. Kuhn, W. Wuttke, J. Clin. Endocrinol. Metab. 74 (1992) 306.
[13] A. Miyamoto, D. Schams, Biol. Reprod. 44 (1991) 1163.
[14] Y. Shoji-Kasai, A. Yoshida, K. Sato, T. Hoshino, A. Ogura, S. Kondo, Y. Fujimoto, R. Kuwahara, R. Kato, M. Takahashi, Science 256 (1992) 1820.
[15] L.A. Greene, A.S. Tischler, Proc. Natl. Acad. Sci. USA 73 (1976) 2424.
[16] M. Schultzberg, C. Andersson, A. Under, M. Troye-Blomberg, S.B. Svenson, T. Bartfai, Neuroscience 30 (1989) 805.
[17] H.M. Huang, L.S. Kao, J. Neurochem. 66 (1996) 124.
[18] F.C. Cheng, J.S. Kuo, Y. Shih, J.S. Lai, D.R. Ni, L.G. Chia, J. Chromatogr. B 615 (1993) 225.
[19] L.A. Greene, A.S. Tischler, Adv. Cell. Neurobiol. 3 (1982) 373.
[20] F. Bussolino, F. Tessari, F. Turrini, P. Braquet, G. Camussi, M. Prosdocimi, A. Bosia, Am. J. Physiol. 255 (24) (1988) C559.
[21] Y. Cheng, P. Wixom, M.R. James-Kracke, A.Y. Sun, J. Neurochem. 63 (1994) 895.
[22] A.G. Kanthasamy, E.U. Madhu, R.W. Peoples, J.L. Borowitz, G.E. Isom, Toxicol. Appl. Pharmacol. 110 (1991) 275.
[23] G.K. Kumar, J.L. Overholt, G.R. Bright, K.Y. Hui, H.W. Lu, M. Gratzl, N.R. Prabhakar, Am. J. Physiol. 274 (1998) C1592.
[24] J. Odink, H. Sandman, W.H.P. Schreurs, J. Chromatogr. 377 (1986) 145.
[25] M. Picard, D. Olichon, J. Gombert, J. Chromatogr. 341 (1985) 297.
[26] G. Eisenhofer, D.S. Goldstein, R. Stull, H.R. Keiser, T. Sunderland, D.L. Murphy, I.J. Kopin, Clin. Chem. 32 (1986) 2030.