ELSEVIER

Journal of Chromatography B, 704 (1997) 351–358

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of serotonin, catecholamines and their metabolites by direct injection of supernatants from chicken brain tissue homogenate using liquid chromatography with electrochemical detection

Ying Qu, Lieve Moons, Frans Vandesande*

Laboratory for Neuroendocrinology and Immunological Biotechnology, Zoological Institute, K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium

Received 27 May 1997; received in revised form 12 August 1997; accepted 27 August 1997

Abstract

An isocratic liquid chromatographic method with electrochemical detection for the determination of L-3,4-dihydroxyphenylalanine, dopamine, norepinephrine, epinephrine, serotonin, and their major metabolites, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid and 5-hydroxyindole-3-acetic acid in chicken brain tissue is described. Chickens were killed at different ages, the brains were quickly frozen and 300-μm cryostat sections were made. From these sections, two to six tissue micropunches (1 mm in diameter) were punched out from 20 different areas of the hypothalamus and homogenated in 100 μl 0.1 *M* perchloric acid which included 0.01% cysteine as antioxidant. Fifty-μl supernatants were injected directly onto the LC system, separated on a 3-μm Phase II ODS column (100×3.2 mm I.D.) and detected by an electrochemical detector at a potential of +0.75 V. Standard curves, recoveries, analytical precision and detection limits were investigated for each monoamine neurotransmitter and its metabolites. The method was applied to study the influence of food restriction on the concentration of monoamine neurotransmitters in different brain areas, known to be involved in feeding and reproductive behaviour of female broiler chickens. Over 1000 micropunched tissue samples from ad libitum fed and food-restricted female broiler chickens were analyzed. Our results provide a possible role for catecholamines and indolamines in the altered feeding and reproductive behaviour of the broiler chicken.

Keywords: Biogenic amines; Serotonin; Neurotransmitters; Catecholamines

1. Introduction

In the hypothalamus of chicken brain, the control of both reproductive and feeding behaviour seems often to be mediated by identical neurotransmitters, catecholamines and serotonin. These biogenic amines play an important role in the modulation of appetite and food intake and at the same time exert effects on male and female sexual behaviour, and on the secretion of gonadotrophic hormones [1–5]. Recently, we started to study the possible role of catecholamines in the existing contradiction between growth and reproduction in different chicken lines. Adult female chickens of a broiler line (selected for increased muscle growth or meat production) do not lay eggs, or do so only sporadically. By subjecting

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00438-6

these female individuals during a critical period of their development to a strict feeding restriction, they display almost the same reproductive characteristics as those from a laying chicken line (selected for reproductive capacity).

In previous studies we localised the different catecholamines, L-DOPA, serotonin and some related enzymes in the chicken brain by immunocytochemistry [5,6]. In this study we looked for a possible influence of food restriction on the neurotransmitter concentrations in the different brain areas known to be involved in feeding and reproductive behaviour.

In recent years, liquid chromatography (LC) with electrochemical detection (ED) has been widely used for the measurement of these neurochemicals [7], but many of the analytical procedures described involve more than one purification step before LC, resulting in low recoveries and tedious procedures [8,9]. Evaluation of neurotransmitters and metabolite concentrations as mentioned above needs the analysis of thousands of samples. In order to shorten the time of analysis, most methods fail to consider all compounds and are restricted to the determination of catecholamines or serotonin with their respective metabolites [10–12]. The methods published until now can not determine L-DOPA and serotonin in the same run. Therefore we first developed a method by which the simultaneous determination of L-DOPA and serotonin was possible. The method described here is simple, uses a single electrochemical detection and allows (1) direct injection of extracts of homogenized micropunched brain tissue samples and (2) simultaneous determination of L-DOPA, catecholamines, serotonin and their metabolites in only 22 min. In combination with a refrigerated autosampler, the system can be easily run on a 24-h basis without any human supervision. In a second part we describe the application of this method to the physiological problem mentioned above.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile (ACN) and disodium ethylenediaminetetraacetate (EDTA) were obtained from Merck (Belgolabo, Overyse, Belgium). Monochloroacetic acid from Aldrich (Bornem, Belgium). Sodium octyl sulfate from Fluka (Bornem, Belgium). Double sub-boiling distilled perchloric acid (PCA) from Romil (Prosan, Gent, Belgium). The protein assay dye reagent was purchased from Bio-Rad Laboratories (Nazareth, Belgium), while all the following chemicals, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), norepinephrine (NE), epinephrine (E), serotonin (5-HT), and their major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA), deoxyepinephrine hydrochloride (DOE), L-isoprenaline (ISO), L-cysteine, chicken egg albumin (ovalbumin), were obtained from Sigma (Bornem, Belgium). Unless otherwise stated, all reagents were of analytical quality.

Stock solutions of biogenic amines and internal standards were prepared in 0.1 *M* PCA containing 0.01% L-cysteine at a concentration of 1 mg/ml and stored at −70°C. Working solutions were freshly prepared.

2.2. Apparatus

A model Waters 625 LC system (Waters Assoc., Millipore, Brussels, Belgium) was used in combination with a BAS LC-4C amperometric detector (Bioanalytical Systems, BAS Europe, Belgium). A Waters 715 ULTRA WISP sample processor (Waters) was used for automatic sample injection at 4°C. Chromatographic separations were performed on an 3-μm BAS Phase II ODS analytical column (100× 3.2 mm I.D.) preceded by a 7-μm BAS Phase II ODS pre-column (15×3.2 mm I.D.) (BAS) which effectively minimized the accumulation of particulate matter and residual protein in the samples on the analytical column. The whole system was controlled and the results were calculated by the Maxima 820 chromatography workstation (Waters). An enzyme immunoassay auto reader (Scinic Kuco-21, Scinic Co.) was used for protein determination.

2.3. Chromatographic conditions

The mobile phase consisted of 0.1 *M* monochloracetic acid, 0.65 m*M* sodium octyl sulfate, 0.5

m*M* EDTA in Milli-Q water and the pH was adjusted to 3.05 with 6 *M* NaOH. Following filtration through a GV 0.22-μm filter (Waters), acetonitrile was added to a final volume concentration of 2.9%. The mobile phase was degassed on line through a BAS-LC 26 vacuum degasser (BAS). The separation was performed isocratically at a flow-rate of 1.0 ml/min. The detector potential was at +0.75 V vs. Ag/AgCl. Injection volume was 50 μl.

2.4. Sample preparation

Ad libitum-fed and food-restricted female meat-type chickens were purchased from the Hybro strain developed by Euribrid Company (Aarschot, Belgium) and housed in large rooms. They were divided into two groups. One group was fed ad libitum (AD) and another group was fed on a restricted quantity of food (RF) as recommended by the breeder company. The animals were killed at the age of 4, 10, 16 and 22 weeks. Brains were removed rapidly, frozen on dry ice and sliced into thick (300 μm) cryostat sections. From these frozen sections, two to six micropunches (micropunch size, 300 μm×1 mm in diameter) from 20 different brain areas, known to be involved in feeding and/or reproduction, were punched out and expelled into 100 μl 0.1 *M* chilled perchloric acid containing 500 pg/50 μl DOE, ISO as the internal standards and 0.01% cysteine as antioxidant. The tissue punches were homogenized by using small conical mortars and pestles for 2 min, while kept on ice. The homogenate was centrifuged at 12 000 *g* for 15 min and the supernatants kept at −70°C until use. The tissue pellets were dissolved in 0.1 *M* NaOH and diluted 1:50 with PBS buffer (H_3PO_4–Na_3PO_4 buffer, pH 7.3), for protein content determination using a modification of the Bradford method [13]. Standard protein solutions were prepared in 0.1 *M* PBS buffer containing varying amounts of (0.58–25.0 μg/ml) ovalbumin. A 200-μl volume of the Bio-Rad Protein Assay reagent (diluted 1:20 with Milli-Q water) was added to 50 μl of the standard solutions or samples in a polystyrene microtiter plate. The absorbance at 570 nm was measured after 2 min using an Enzyme Immunoassay Auto Reader. This allowed us to express the results as pg monoamine/μg protein.

2.5. Quantitative method

The concentrations of DOPA, NE, DOPAC, E, 5-HIAA, HVA, DA and 5-HT were calculated based on the response of the standard curves using the internal standard method. The DOE was used as internal standard for DOPA, NE, DOPAC, E, HVA, DA, 5-HIAA, and ISO was used as internal standard for 5-HT. Statistical analyses for differences in mean tissue concentrations were performed using the *t*-test, with $P<0.05$ considered significant.

In order to check the recovery of the extraction procedure, brain homogenate samples with and without addition of different known amounts of standard mixture were analysed. The recovery was calculated using the following equation:

$$\text{Recovery} = [(S_m - B)/S] \times 100\%$$

where S = the peak area given by the known amounts of standard mixture; B = the peak area obtained by measuring the amounts of analytes in the brain homogenate without addition; S_m = the peak area obtained by measuring the amount of the analytes in a brain homogenate spiked with the standard mixture.

3. Results and discussions

3.1. Optimization of chromatographic conditions

Liquid chromatography with electrochemical detection (LC–ED) has been widely used for the simultaneous determination of several monoamine neurotransmitters and their metabolites in brain samples. Several one-step analytical methods using direct injection of brain homogenate extracts onto the analytical column have been published [10,11,13,14]. To avoid co-elution and to shorten the separation time, many mobile phases were designed. The method suggested by BAS showed the best performance but we decided to optimize it for our specific needs. We first adjusted the applied potential of detector to +0.75 V. The most flexible parameter for optimization was the composition of the mobile phase. We adjusted acetonitrile concentration, sodium octyl sulfate concentration and the pH. The optimal

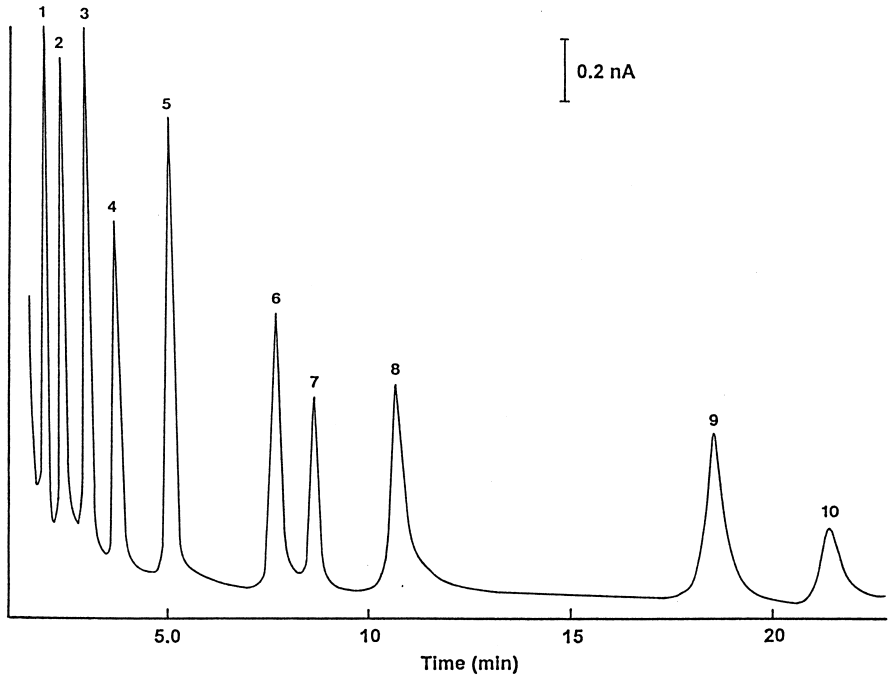

Fig. 1. Chromatogram of a standard mixture of biogenic amines (200 pg/50 μl): (1) L-DOPA; (2) NA; (3) DOPAC; (4) AD; (5) HVA; (6) DA; (7) 5-HIAA; (10) 5-HT; internal standard (400 pg/50 μl): (8) DOE; (9) ISO (sensitivity range, 2.0 nA/V).

mobile phase was that described in Section 2. The typical chromatograms of a standard mixture and a brain homogenate are shown in Figs. 1 and 2, respectively. The identities of the peaks were confirmed by their retention time. Each analysis was completed within 22 min. All components in Fig. 2 were well resolved and identical with those in Fig. 1. Each peak of these eight biogenic amines was verified by addition of a standard mixture which increased its height, but did not change its shape. Some unknown endogenous substances in the brain homogenates also appeared in the chromatogram (Fig. 2). We have been trying to identify them by comparing their retention times with those of other known aminergic neurochemicals (metanephrine (M), normetanephrine (NM), 3-methoxytyramine (3-MT), 4-hydroxy-3-methoxyphenylglycol (MHPG), 5-hydroxy-1-tryptophan (5HTP)), but none accorded with the unknown brain components.

3.2. Tissue protein precipitation

The relative effectiveness of perchloric acid (PCA) concentration in precipitating proteins from tissue homogenates was determined; 0.01–1.0 *M* of perchloric acid was used. Following centrifugation, the residual protein content in the supernatant was determined by a modification of the Bradford method (as in Section 2). It was found that using 0.1 *M* PCA, 5% of original protein content remained in the supernatant. This result indicated that 0.1 *M* PCA is effective in precipitating tissue protein and allowed direct injection of the samples without clogging the column. After hundreds of injections, only small amounts of protein and other impurities slowly precipitated on the column and the solvent front peak became larger and started to overlap with the L-DOPA peak. Therefore, after 200–250 injections, installing a new pre-column and rinsing the analytical column was necessary.

According to McKay et al. [15], the presence of ascorbic acid is the primary cause of a large solvent front peak in brain samples, and a simple enzyme addition (ascorbic acid oxidase) can reduce the solvent front by 80%. We could not, however, confirm this finding with chicken brain extracts. The purity of the PCA and the state of the column seemed the most important factors which influence

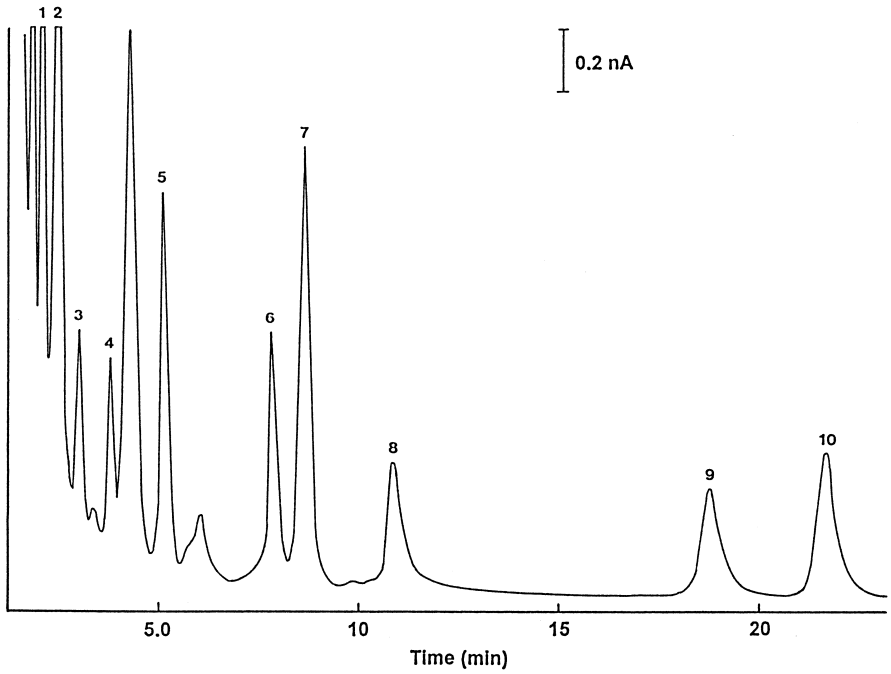

Fig. 2. Typical chromatogram of a brain homogenate (three micropunches) from *area ventralis* (AVT). (1) L-DOPA; (2) NA; (3) DOPAC; (4) AD; (5) HVA; (6) DA; (7) 5-HIAA; (8) DOE; (9) ISO; (10) 5-HT (sensitivity range, 2.0 nA/V).

Table 1
Linear range and correlation coefficient of standard curve, and recovery of biogenic amines in brain tissue

Compound	Practical linear range (pg/50 μl)	r^2	Average recovery (%)	*n*
DOPA	25–150	0.9998	83.02	4
NA	25–300	0.9970	91.64	6
DOPAC	25–250	0.9976	98.73	6
AD	25–350	1.0000	78.46	5
5-HIAA	25–200	0.9999	88.39	5
HVA	25–500	0.9998	96.69	11
DA	25–400	0.9998	85.89	9
5-HT	25–500	0.9999	95.85	8

Table 2
Coefficient of variation (%)[a]

	DOPA	NA	DOPAC	AD	5-HIAA	HVA	DA	5-HT
Standard mixture (150 pg/50 μl)	0.145	0.75	0.355	0.347	0.51	1.81	8.63	1.82
Brain homogenate	—[b]	—	7.11	2.17	6.13	5.72	4.17	4.31
Brain homogenate with spiked standard (100 pg/50 μl)	2.14	0.704	1.20	5.99	8.63	9.37	7.58	2.63

[a] $n=6$.
[b] NE and DOPA concnetrations in this experimental brain area are too low to be measured.

the size of the front peak and resolution of the early eluting component L-DOPA.

3.3. Standard curve and recovery

Standard curves for L-DOPA, NE, DOPAC, E, 5-HIAA, HVA, DA and 5-HT alone or added to brain tissue homogenates were co-linear for all compounds. Consequently, all quantifications can be performed using a standard curve made directly in 0.1 *M* PCA. The linear range of the standard curves for each compound is rather large. The practical linear ranges, correlation coefficients and the recoveries for all neurotransmitters (calculated by the equation mentioned above) are summarized in Table 1, which shows the validity of the method. As the isoindole structure of 5-HT and 5-HIAA is not stable and easily oxidized, linear standard curves for these compounds could only be obtained after addition of L-cysteine as an antioxidant.

3.4. Precision and detection limit

The precision of the method was determined by making six consecutive injections of a standard mixture, a brain homogenate and a brain homogenate spiked with a standard mixture. The coefficients of variation (C.V.) are summarized in Table 2. The detection limits at a signal-to-noise ratio of 3 were between 0.25 and 5.0 pg, as shown in Table 3.

Table 3
The detection limit of biogenic amines

Compound	Detection limit (pg per injection)
DOPA	2.5
NA	2.5
DOPAC	1.0
AD	2.5
5-HIAA	0.5
DA	0.25
HVA	1.0
5-HT	5.0

3.5. Determination of brain tissue samples

We used the present method for the determination of L-DOPA, NE, DOPAC, E, 5-HIAA, DA, HVA and 5-HT in micropunched hypothalamic areas of ad libitum-fed and food-restricted female broiler chickens. Twenty brain areas of each experimental group were compared. The results were published in [16]. In most hypothalamic areas studied, and for most neurotransmitters determined, no concentration difference, resulting from food restriction, was found. However, food restriction did affect the amount of certain biogenic amines in a few hypothalamic

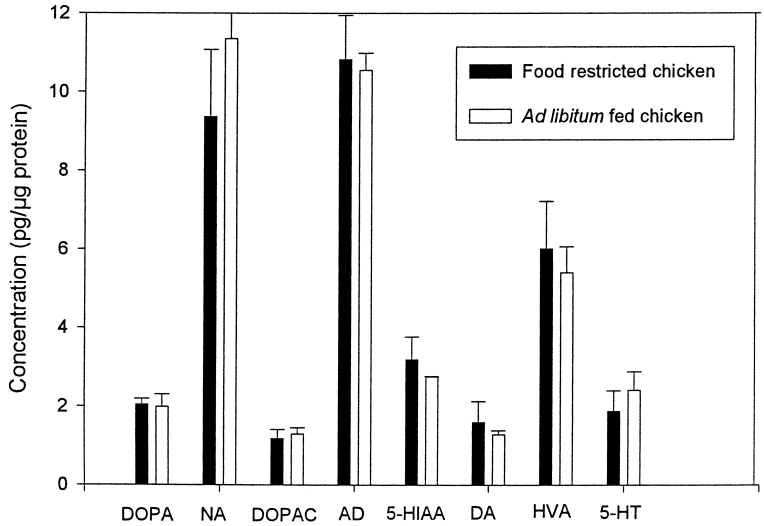

Fig. 3. Concentrations of biogenic amines in the *necleus ventromedialis hypothalami* (VMN) of 16 weeks ad libitum-fed and food-restriced chickens. There is no significant difference between two groups.

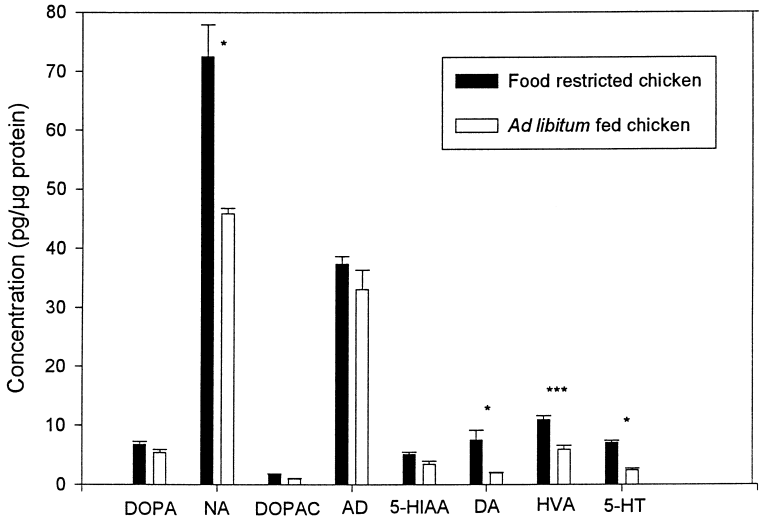

Fig. 4. Concentrations of biogenic amines in the median eminence (EM) of 16 weeks ad libitum-fed and food-restriced chicken. There was a significant increase of NA, DA, HVA and 5-HT in this area in food-restriced chickens (*$P<0.05$, ***$P<0.005$).

nuclei. The most striking difference occurred in the median eminence, where the concentration of NE was largely increased in food-restricted animals at all ages examined. Another significant difference could be found for DA in the region of the nucleus premamillaris in the tuberal hypothalamus where the DA concentration was decreased very drastically in ad libitum-fed chickens from 10 weeks of age. The concentration of the amines present in the various brain areas remained constant during growth to sexual maturity in most of the brain regions, although a decrease or increase in neurotransmitter concentration as a function of age could be distinguished for some monoamines in several brain nuclei. Two typical results, for the nucleus ventromedialis hypothalami and the median eminence, are shown in Figs. 3 and 4.

In conclusion, with the method described in this report, 50 samples could be analyzed in 24 h without supervision. Over 1000 chicken brain tissue samples were determined showing that the present method is simple, efficient and sensitive. Moreover, the method might not be restricted to quantification of neurotransmitters and metabolite concentrations from brain homogenates, but other body fluids might possibly be used. This technique is a suitable tool that meets the requirement of a routine neurochemical research laboratory.

Acknowledgements

This work was supported by grants from the Queen Elisabeth Medical Foundation and the Fund for Scientific Research–Flander (F.W.O. Vlaanderen), Belgium. The technical assistance of Luc Grauwels is gratefully acknowledged.

References

[1] D.M. Denbow, H.P. Van Krey, P.B. Siegel, Pharmacol. Biochem. Behav. 24 (1986) 39.
[2] D.M. Denbow, B.J. Sheppard, Brain Res. Bull. 31 (1993) 121.
[3] M.C. Macnamee, P.J. Sharp, J. Endocrinol. 121 (1989) 67.
[4] M.A. Ottinger, J. Balthazart, Behav. Proc. 14 (1987) 197.
[5] L. Moons, J. Van Gills, E. Ghijsels, F. Vandesande, J. Comp. Neurol. 346 (1994) 97.
[6] L. Moons, E. D'Hondt, K. Pijcke, F. Vandesande, J. Comp. Neurol. 360 (1995) 331.
[7] C.T. Duda, P.T. Kissinger, Methods in Neurotransmitter and Neuropeptide Research, Elsevier, Amsterdam, 1993, p. 41.
[8] G. Eisenhofer, D.S. Goldstein, R. stull, H.R. Keiser, T. Sunderland, D.L. Murphy, I.J. Kopin, Clin. Chem. 32 (1986) 2030.
[9] M. Picard, D. Olichon, J. Gombert, J. Chromatogr. 341 (1985) 445.
[10] O. Magnusson, L.B. Nilsson, D. Westerlund, J. Chromatogr. 582 (1992) 1.

[11] F.C. Cheng, J.S. Kuo, Y. Shih, J.S. Lai, D.R. Ni, L.G. Chia, J. Chromatogr. 615 (1993) 225.
[12] H. Saito, S. Murai, E. Abe, Y. Masuda, T. Itoh, Pharmacol. Biochem. Behav. 42 (1992) 351.
[13] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[14] V.M. Gregory, J. Chromatogr. 615 (1993) 225.
[15] L. Mckay, C. Bradberry, A. Oke, J. Chromatogr. 311 (1984) 167.
[16] L. Moons, Y. Qu, F. Vandesande, 24th Annual Meeting of Society for Neuroscence Abstracts, vol. 19, part 1, 1994, 591.10.