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Determination of serotonin, noradrenaline, dopamine and their metabolites in rat brain extracts and microdialysis samples by column liquid chromatography with fluorescence detection following derivatization with benzylamine and 1,2-diphenylethylenediamine

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Abstract

A highly selective and sensitive column liquid chromatographic method for fluorescence determination of serotonin (5-HT), dopamine (DA), noradrenaline (NA) and their related metabolites 5-hydroxyindole-3-acetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) following derivatization with benzylamine and 1,2-diphenylethylenediamine (DPE) is described. The monoamines and the metabolites (20 μl samples) were derivatized in a two-step reaction, initiated with 20 μl of 0.3 M benzylamine in 0.3 M 3-cyclohexylaminopropanesulfonic acid (CAPS) buffer (pH 10.0), (for 5-HT, 5-HIAA, 2 min, 24 °C) and followed by 20 μl of 0.1 M DPE in 0.3 M glycine buffer (pH 10.0), (for DA, NA, DOPAC, 20 min, 50 °C). Both reagents contained 0.02 M potassium hexacyanoferrate(III) and 50% (v/v) methanol. The resulting highly fluorescent and stable benzoxazole derivatives were isocratically separated on a reversed-phase column (150 mm × 1.5 mm i.d., packed with C18 silica, 5 μm) within 45 min. Using fluorescence detection at ex. and em. wavelengths of 345 and 480 nm, respectively, the detection limit (signal-to-noise ratio of 3) for 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC ranged between 0.08 and 5.65 fmol per 20-μl injection (12–847.5 pM in standard solution). The concentrations of monoamines (expressed in μg/g wet weight, mean ± S.E.M., $n = 5$) in tissue extracts from the rat striatum were: 0.45 ± 0.05 (5-HT), 4.27 ± 0.08 (DA), 0.27 ± 0.04 (NA), 0.55 ± 0.06 (5-HIAA), 1.26 ± 0.16 (L-DOPA) and 1.62 ± 0.11 (DOPAC). Microdialysis samples were collected in 20 min intervals from the probes implanted in the striatum of awake rats. The basal monoamine levels (in fmol/20 μl, mean ± S.E.M., $n = 5$) in the dialysates were: 4.1 ± 0.7 (5-HT), 78.4 ± 9.1 (DA), 6.4 ± 0.8 (NA), 785.5 ± 64.5 (5-HIAA) and 5504.5 ± 136.5 (DOPAC). It is concluded that the new fluorescence derivatization protocol provides an excellent means for simultaneous determination of all three monoamines both in the complex samples (e.g. brain homogenates) and also at trace levels, such as those found in the microdialysis samples.

Keywords: Derivatization, LC; Serotonin; Noradrenaline; Dopamine; 5-Hydroxyindole-3-acetic acid; 3,4-Dihydroxyphenylacetic acid

1. Introduction

In the brain, serotonin (5-HT), dopamine (DA) and noradrenaline (NA) play important roles as major neurotransmitters, being involved in control and regulation of such principal functions and behaviours, as mood and emotional control, sleep, vigilance, food intake and temperature regulation. Monitoring the levels of monoamines and their main deaminated metabolites 5-hydroxyindole-3-acetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) has traditionally been implicated for evaluation of therapeutic efficacy of monoamine oxidase (MAO) inhibitors [1–3] and other antidepressants [4–7]. The MAO inhibitors are frequently used for treatment of depression [8–10], panic disorder [11,12], Parkinson's disease [13–15] and Alzheimer's disease [16–19]. The effects of drugs affecting

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extracellular pools of brain monoamines and their metabolites can be elegantly studied by use of microdialysis in awake animals and completed by data obtained from analysis of brain tissue extracts. The neurotransmitters and their metabolites in brain extracts and in microdialysis samples are most often determined by column liquid chromatography with electrochemical detection (LCEC) [20–25]. However, simultaneous determination of 5-HT, DA, NA, 5-HIAA and DOPAC with the LCEC method, though relatively easy and feasible for brain extracts, is barely achievable for analysis of microdialysis samples. This is most likely due to the difficulties associated with poor separation and/or detection of early eluting NA peak and the late eluting peak of 5-HT.

Recently, we have described several methods using discrete derivatization protocols based on either benzylamine or 1,2-diphenylethylenediamine (DPE) reagents for fluorescence detection of 5-hydroxyindoleamines and catecholamines, respectively [26–31]. These chemistries, though rather similar in terms of final reaction products (substituted benzoxazoles), differ mostly in reaction conditions (time and temperature) and the fluorescence yield, particularly for 5-HT and DA. Thus, 5-HT reacts rapidly (within 2 min) with benzylamine already at room temperature [26], whereas DA-benzylamine derivative yielded poor fluorescence signals even at more vigorous reaction conditions (50 °C, 20 min) [32]. On the other hand, both DA and NA form a strong fluorescence derivatives following reaction with DPE, although this reaction also requires longer reaction time and higher temperature [33,34]. Interestingly, NA reacts both with DPE and benzylamine, as described elsewhere [35]. In the most recent study, we have found that 5-HT, DA, NA can be simultaneously derivatized with benzylamine and DPE in a two-step reaction in the presence of potassium hexacyanoferrate(III) and in weakly alkaline media [36]. The factors affecting the fluorescence yields were studied and the reaction and separation conditions were optimized for microbore liquid chromatographic (LC) determination of monoamine standards [36].

In the present study, we show the applicability of the microbore LC method for determination of 5-HT, DA, NA, 5-HIAA and DOPAC in tissue extracts and microdialysis samples from the rat brain.

2. Experimental

2.1. Chemicals and solutions

Deionized and distilled water, purified with a Barnstead EASYpure RF (Hansen Co., Hyogo, Japan) system, was used for all aqueous solutions. 5-HT, 5-hydroxytryptophan (5-HTP), 5-hydorxytryptophol (5-HTOL), clorgyline and phenelzine were obtained from Sigma (St. Louis, MO, USA). 5-HIAA, *N*-acetyl-5-hydroxytryptamine (N-Ac-5-HT), NA, adrenaline (A), DA, 3,4-dihydroxyphenylalanine (L-DOPA), DOPAC, 3-cyclohexylaminopropanesulfonic acid (CAPS) and glycine were purchased from Wako Pure Chemical Co. (Osaka, Japan). Potassium hexacyanoferrate(III) was purchased from Kisida Chemical Co. (Tokyo, Japan). Benzylamine hydrochloride was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and was used after repeated purification by recrystallization in absolute ethanol. DPE was a kind gift from Tosoh (Tokyo, Japan). Other chemicals were of the highest purity available and were used as received. Benzylamine hydrochloride (0.3 M), CAPS buffer (0.3 M, pH 10.0) and potassium hexacyanoferrate(III) (20 mM) solutions were prepared in aqueous 90, 90 and 50% (v/v) methanol, respectively. DPE (0.1 M) and glycine (0.3 M) solutions were prepared in 0.1 M hydrochloric acid and water, respectively. The benzylamine derivatization reagent solution was a mixture containing benzylamine–CAPS–potassium hexacyanoferrate(III) solution–methanol (2:6:3:24, v/v). The DPE derivatization reagent solution was a mixture of DPE–glycine solutions (2:1, v/v). The reagent solutions were stable for at least 2 weeks at 4 °C, however the purity of the reagents was strongly dependent on the overall laboratory environment, e.g. on purity of the glassware or a co-storage with other evaporating chemicals. In order to achieve and maintain the highest possible sensitivity and reproducibility of the HPLC method it was critical to evaluate the purity of the reagents and the chromatographic system on a regular basis. Thus, blank samples of water and Ringer solution were derivatized with benzylamine and DPE reagents and sequentially analysed by HPLC to evaluate the occurrence of interfering peaks. Typically, the presence of interferences in the chromatogram indicated the contaminated benzylamine reagent, which was then subjected to repeated recrystallization in absolute ethanol. In addition, the quality of water and other chemicals was critical to avoid contamination of the HPLC system. Standard solutions of 5-hydroxyindolamines and catecholamines were prepared in water and kept frozen (−20 °C) in amber-coloured test tubes.

2.2. Derivatization procedure

To 20 μl of standard solution or brain sample 20 μl of benzylamine derivatization reagent solution was added. The mixture was allowed to react for 2 min at room temperature, thereafter 20 μl of DPE reagent was added and final mixture in a sealed vial was heated at 50 °C for 20 min. A 20-μl portion of the final reaction mixture was injected onto the chromatographic column. For the reagent blank or Ringer solution, the sample was subjected to the same procedure.

2.3. Apparatus and chromatography

Chromatography was performed with an L-7100 (Hitachi, Tokyo, Japan) high-performance liquid chromatograph with L-7480 fluorescence spectromonitor (12 μl flow cell, Hitachi, Tokyo, Japan). The spectromonitor was operated at an excitation wavelength of 345 nm and an emission

wavelength of 480 nm. The column [150 mm × 1.5 mm i.d.; packed with C18 silica (L-column); particle size 5 μm] was purchased from Chemicals Evaluation and Research Institute (Tokyo, Japan). Separation of benzylamine-DPE derivatives of 5-hydroxyindolamines and catecholamines was achieved using a mixture of acetonitrile and 15 mM acetate buffer (pH 4.5) (34:66, v/v) containing 1 mM octanesulfonic acid sodium salt. The flow rate was 100 μl/min. The column temperature was ambient (20–23 °C). The sampling loop, the flow cell of the detector and the injection syringes were cleaned in the ultrasonic bath every third day.

2.4. Animals

Male Sprague Dawley rats (250–350 g, Charles River, Japan) were used in all experiments. Rats were maintained on a 12-h light–dark cycle (light at 7:00 a.m.). Food and water were freely available. A 5-day adaptation period always preceded the microdialysis experiments. All experiments were performed in accordance with general recommendations of Japanese animal protection legislation.

2.5. Brain tissue

Brain tissue samples were weighted and homogenized with a micro homoginezer (Iuchi, Osaka, Japan) in 50 μl of ice-cold 0.1 N perchloric acid containing 10^{-7} M ascorbic acid. The final homogenate was centrifuged at 5200 × *g* for 30 min at 4 °C, then filtered through a 0.22 μm filter (Advantec, Tokyo, Japan). To 5 μl of the filtrate, 2995 μl of distilled water was pipetted and 20 μl of the diluent was injected on the chromatographic column.

2.6. Surgery and brain microdialysis

Rats were anaesthetized with sodium pentobarbital (40 mg/kg) and placed in the stereotaxic frame. Each rat was implanted with a guide cannula in the striatum (rostal-caudal, 3.0 mm; lateral, 0.2 mm; ventral, 3.5 mm, from the bregma and the dural surface) according to the atlas of Paxinos and Watson [37]. After the implantation, the guide cannula was fixed firmly to the skull with to anchor screws and dental cement. At least 3 days after the surgery, the dialysis probes (0.22 mm i.d., 3.0 mm membrane length, molecular weight cut-off 50,000, Eicom Ltd., Japan) were inserted in freely moving rats. The dialysis probes were perfused with Ringer solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 2.3 mM) at a flow-rate of 1.0 μl/min. The dialysates were collected every 20 min.

3. Results and discussion

3.1. Optimal conditions, calibration graph, precision and detection limit

The derivatization reaction for catechols and 5-hydroxyindoles in brain tissue extracts and in microdialysis samples was carried-out at same conditions as optimized and described for individual standards and their mixtures [36] and as summarized in Section 2. Fig. 1A shows a typical chromatogram of 20 μl standard solution containing 10 fmol–10 pmol of each respective compound. Full separation of benzylamine and DPE derivatives was achieved within 45 min on a microbore reversed-phase column and analysis of individual chromatograms revealed that each derivative gave only a single peak. A derivatized blank (water) sample recorded at the highest sensitivity of the detector (attenuation 0) is shown in Fig. 1B. The relationship between the peak heights and concentrations of standard solutions of 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC was linear up to at least 1 pmol per 20 μl injected (150 nM in the standard solution); the linear correlation coefficient was 0.996 ($n = 5$). The relative standard deviation for repeated analysis

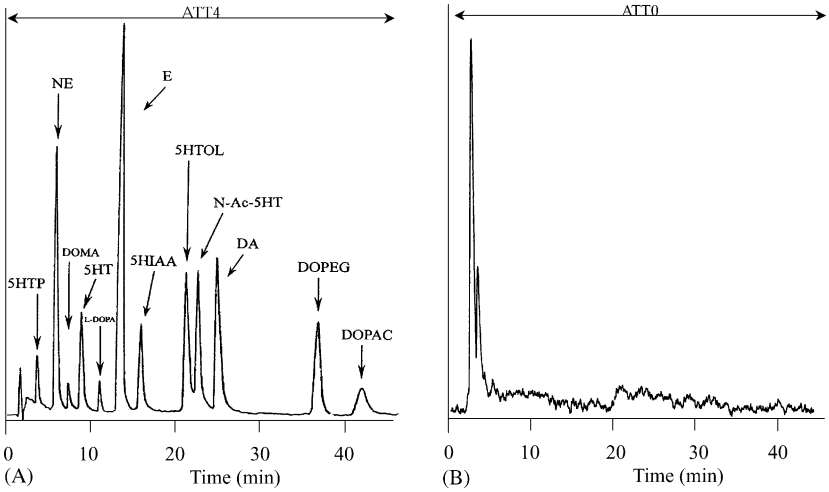

Fig. 1. Chromatograms of (A) a standard solution of benzylamine/DPE-derivatized 5-hydroxyindolamines and catecholamines and (B) a blank (water) sample. A final volume of 20 μl was injected onto the column. Peaks: 5-HTP (50 fmol), NA (50 fmol), DOMA (5 pmol), 5-HT (50 fmol), L-DOPA (500 fmol), A (50 fmol), 5-HIAA (50 fmol), 5-HTOL (50 fmol), N-Ac-5-HT (50 fmol), DA (50 fmol), DOPEG (50 fmol), DOPAC (50 fmol); ATT4: lower sensitivity at attenuation 4, ATT0: highest sensitivity of the detector.

($n = 10$) of 5-HT, DA, NA, 5-HIAA and DOPAC standards at concentrations of 50 fmol/20 μl (2.5 nM in the standard solution), each was 4.2%. Detection limits (signal-to-noise ratio of 3) were calculated from each peak height in a chromatogram obtained with a standard solution (0.3, 0.2, 0.2, 0.4, 9.0 and 0.4 nM for 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC, respectively). The detection limit for 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC was 0.2, 0.13, 0.08, 0.24, 5.65 and 0.24 fmol in an injection volume of 20 μl (30, 19.5, 12, 36, 847.5 and 36 pM in standard solution), respectively; the values show the concentration of the amines in the standard solution prior to the derivatization.

3.2. Analysis of monoamines in brain extracts and microdialysis samples

The applicability of the new fluorescence derivatization method for determination of pharmacologically modified levels of 5-HT, DA, NA, 5-HIAA and DOPAC was evaluated for rat brain extracts following clorgyline and for striatal microdialysates, following phenelzine treatment. Clorgyline and phenelzine are effective inhibitors of MAO-A [38] and mixed MAO-A/B [39], respectively. Chromatograms in Fig. 2A and B illustrate separation of derivatized indoles and catecholamines in typical samples taken from the extract of rat striatal homogenates at (A) basal conditions and (B) 120 min following systemic administration of clorgyline (4 mg/kg i.p.). The individual peaks were identified as fluorescent derivatives of 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC on basis of retention times compared to the standards, by co-chromatography (spiked samples) of samples with standards and by comparing the retention times of the identified peaks and those of the standards at varying elution conditions (25–50% acetonitrile in the mobile phase). The fluorescence excitation (maximum, 345 nm) and emission (maximum, 480 nm) spectra of the identified peaks in the samples were in good agreement with those observed for the pure standard derivatives. In addition, the identified peaks were absent when benzylamine, DPE and/or potassium hexacyanoferrate(III) was not present in the derivatization reagent. This observation supports the conclusion that each of the peaks in Fig. 2 comprises of a single component only, the benzylamine derivatives of 5-HT, 5-HIAA and the DPE derivatives of DA, NA, L-DOPA and DOPAC [36]. The recovery calculated for a spiked tissue extract from the rat striatum was within 94.5–98.5% for 5-HT, DA, NA, 5-HIAA, L-DOPA and DOPAC. The levels of 5-hydroxyindoles and catechols in the rat striatum extracts, expressed in μg/g wet weight (mean ± S.E.M., $n = 5$ rats) were: 0.45 ± 0.05 (5-HT), 4.27 ± 0.08 (DA), 0.27 ± 0.04 (NA), 0.55 ± 0.06 (5-HIAA), 1.26 ± 0.16 (L-DOPA) and 1.62 ± 0.11 (DOPAC) (Table 1). The levels are not quite different from those reported by other workers [40–45].

Clorgyline (4 mg/kg i.p.), given 120 min before the rats were sacrificed and the brains analysed, caused significant increases in striatal tissue levels of 5-HT, DA and NA to 180, 155 and 220% of saline-treated rats, respectively (Fig. 3). At the same time, the tissue levels of 5-HIAA were reduced to 50% and DOPAC to 30% of the control rats. These results are in good agreement with literature data where MAO inhibition by clorgyline [46] caused marked increases in brain 5-HT and NA, followed by DA, whereas the metabolites 5-HIAA and particularly DOPAC were dramatically reduced. In a parallel experiment, the basal and phenelzine-modulated extracellular levels of 5-HT, DA, NA, 5-HIAA and DOPAC were determined in the dialysates from the rat striatum, as shown in Fig. 4A and B, respectively. The mean basal levels of indoles and

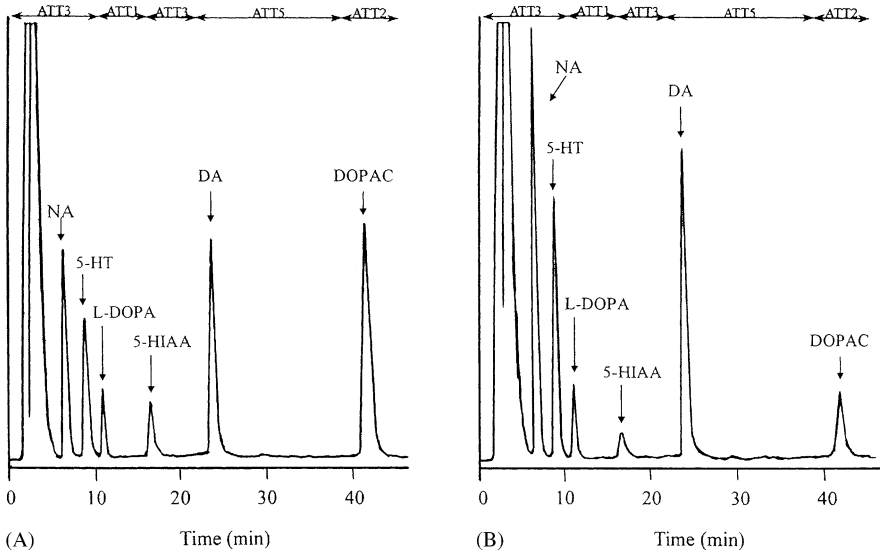

Fig. 2. Typical chromatograms of the tissue extracts from the rat striatum illustrating monoamine levels at (A) basal conditions and (B) 120 min following clorgyline (4 mg/kg i.p.) administration. The estimated concentrations, expressed in μg/g wet weight, were: NA (0.28; 0.61), 5-HT (0.43; 0.81), L-DOPA (1.27; 1.34), 5-HIAA (0.51; 0.24), DA (4.12; 6.01) and DOPAC (1.56; 0.34) for chromatograms A and B, respectively.

Table 1
Amounts of 5-HT, NA, DA and their metabolite in the rat striatum determined by the present and previous methods

Method	Concentration (μg/g wet weight)						Reference	
	5-HT	DA	NA	5-HIAA	L-DOPA	DOPAC		
Our data	0.45 ± 0.05	4.27 ± 0.08	0.27 ± 0.04	0.55 ± 0.06	1.26 ± 0.16	1.62 ± 0.11		Mean ± S.E.M.
HPLC-ECD	0.78 ± 0.02	10.41 ± 0.03	0.29 ± 0.02	0.71 ± 0.03		1.41 ± 0.04	[40]	Mean ± S.E.M.
HPLC-ECD	0.38	3.8	0.37	0.46		1.01	[41]	Mean
HPLC-ECD	0.64 ± 0.03	6.42 ± 0.01		0.46 ± 0.001		2.59 ± 0.36	[42]	Mean ± S.E.M.
HPLC-FD	0.55 ± 0.05	7.56 ± 0.46	0.32 + 0.03				[43]	Mean ± S.D.
HPLC-ECD	0.25 ± 0.03	7.31 ± 0.67	0.13 ± 0.02			3.96 ± 0.35	[44]	Mean ± S.E.M.
HPLC-ECD		15.4 ± 1.22			3.38 ± 0.23	3.3 ± 0.32	[45]	Mean ± S.E.M.

HPLC-ECD; high-performance liquid chromatography with electrochemical detection HPLC-FD; high-performance liquid chromatography with fluorescence detection.

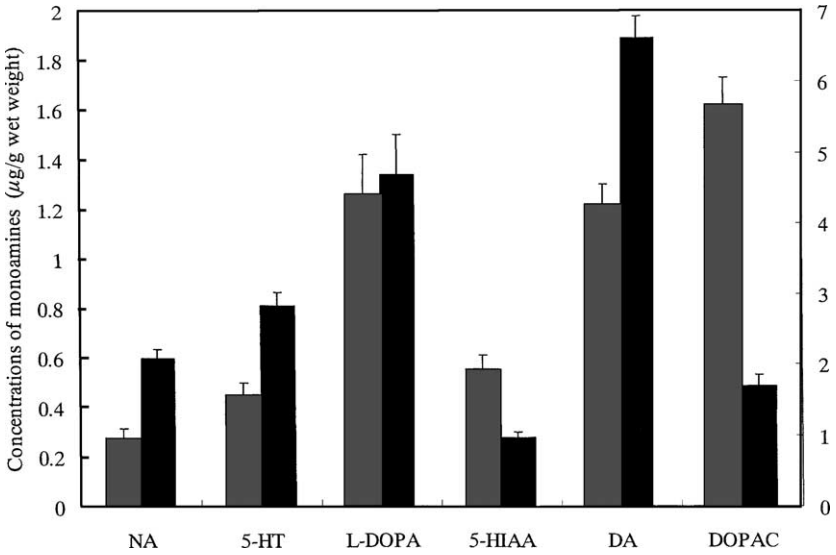

Fig. 3. The effect of MAO-A inhibitor clorgyline (4 mg/kg, 120 min after its i.p. administration) on tissue concentrations of 5-HT, NA, DA, 5-HIAA and DOPAC in the rat striatum. The data are expressed in μg/g wet weight (left axis: 5-HT, NA, 5-HIAA, L-DOPA, DOPAC; right axis: DA) as mean ± S.E.M., $n = 5$ rats.

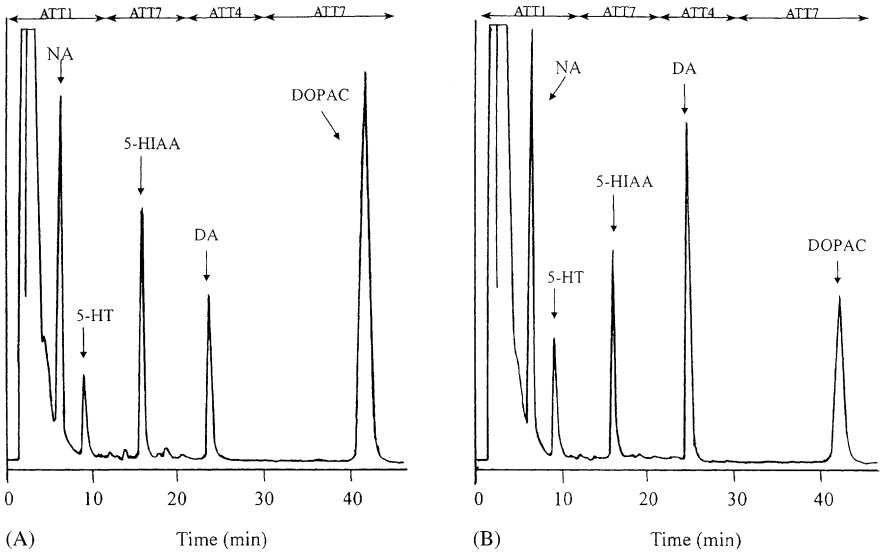

Fig. 4. Representative chromatograms of microdialysis samples from the rat striatum at (A) basal conditions and (B) 100 min following phenelzine (5 mg/kg i.p.) treatment. The estimated concentrations of monoamines and metabolites, expressed in fmol/20 μl, were: NA (6.2; 7.9), 5-HT (4.2; 6.3), 5-HIAA (798.5; 674.5), DA (80.4; 165.7) and DOPAC (5459.5; 2304.2) for chromatograms A and B, respectively.

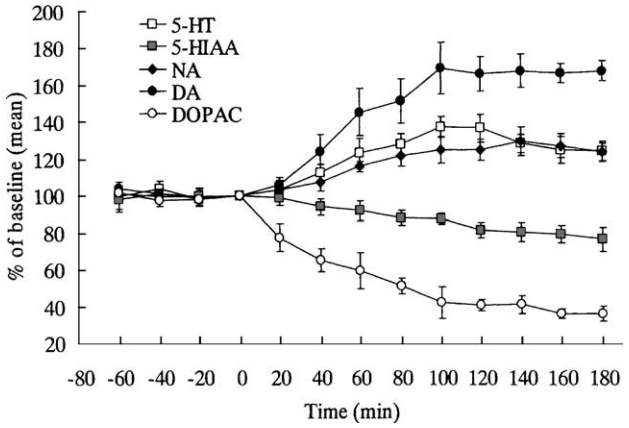

Fig. 5. The effect of MAO-A/B inhibitor phenelzine (5 mg/kg i.p., injected at 0 min) on 5-HT, NA, DA, 5-HIAA and DOPAC levels in the rat striatal microdialysates. The data are expressed as % change of the basal concentrations at time 0 min, mean ± S.E.M., $n = 5$ rats.

catechols in striatal microdialysates, expressed in fmol/20 μl (mean ± S.E.M., not corrected for in vitro probe recovery, $n = 5$) were: 4.1 ± 0.7 (5-HT), 78.4 ± 9.1 (DA), 6.4 ± 0.8 (NA), 785.5±64.5 (5-HIAA) and 5504.5±136.5 (DOPAC). Systemic administration of phenelzine (5 mg/kg i.p.) caused a gradual increase of extracellular 5-HT, DA and NA levels, which at 120 min reached the peak values of 140% (5-HT), 171% (DA) and 121% (NA) of the pre-drug levels (Fig. 5). The corresponding extracellular levels of 5-HIAA and DOPAC decreased to 83 and 41%, respectively. Several earlier microdialysis studies using the LCEC methods [43,46–49] have demonstrated that MAO-A and MAO-B inhibitors cause increases in extracellular DA, NA and 5-HT levels, whereas the metabolites DOPAC and 5-HIAA were decreased. Thus, clorgyline caused significant decreases in extracellular levels of deaminated metabolites and increase in DA both after a single [50,51] or repeated [52] administration. To our knowledge, the present study reports for the first time, the effects of phenelzine treatment on extracellular monoamine levels measured by microdialysis in vivo.

4. Conclusion

The present paper describes a new method for simultaneous determination of 5-HT, DA, NA, 5-HIAA and DOPAC in the rat brain tissue and in the microdialysis samples. A major advantage of using fluorescence derivatization strategy for monoamine detection in LC is a possibility of achieving ultra-high sensitivity in the sub-fmol (10^{-11} M) range for all three monoamines (5-HT, NA and DA), which is virtually unattainable when using conventional electrochemical detection and ion-paired reversed-phase LC. Thus, besides microdialysis samples, the method should be useful for determination of trace levels of monoamines in other minute biological samples such as tissue biopsies (punches), synaptosomes or cell lysates.

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