Simultaneous determination of monoamine and amino acid neurotransmitters in rat endbrain tissues by pre-column derivatization with high-performance liquid chromatographic fluorescence detection and mass spectrometric identification

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\textbf{Abstract}

A sensitive and efficient method for simultaneous determination of glutamic acid (Glu), \(\gamma\)-amino-butyric acid (GABA), dopamine (DA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in rat endbrains was developed by high-performance liquid chromatography (HPLC) with fluorescence detection and on-line mass spectrometric identification following derivatization with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC). Different parameters which influenced derivatization and separation were optimized. The complete separation of five neurotransmitter (NT) derivatives was performed on a reversed-phase Hypersil BDS-C\textsubscript{18} column with a gradient elution. The rapid structure identification of five neurotransmitter derivatives was carried out by on-line mass spectrometry with electrospray ionization (ESI) source in positive ion mode, and the BCEOC-labeled derivatives were characterized by easy-to-interpret mass spectra. Stability of derivatives, repeatability, precision and accuracy were evaluated and the results were excellent for efficient HPLC analysis. The quantitative linear range of five neurotransmitters were 2.441–2 \times 10^4 \text{nM}, and limits of detection were in the range of 0.398–1.258 \text{nM} (S/N = 3:1). The changes of their concentrations in endbrains of three rat groups were also studied using this HPLC fluorescence detection method. The results indicated that exhausting exercise could obviously influence the concentrations of neurotransmitters in rat endbrains. The established method exhibited excellent validity, high sensitivity and convenience, and provided a new technique for simultaneous analysis of monoamine and amino acid neurotransmitters in rat brain.

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1. Introduction

Neurotransmitters (NT) widely distribute in central neural system, brain tissues and body fluids of mammals. They consist of amino acid neurotransmitters (AANT), such as glutamic acid (Glu) and \(\gamma\)-amino-butyric acid (GABA), and monoamine neurotransmitters (MANT), such as dopamine (DA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA). The monitoring of NT is an essential tool in elucidating normal and pathological neural system functions [1]. Trace level measurements in the brain are especially important in studying the role of NT in neurophysiology, behavioral effects, pathology, disease diagnosis and control since their changes have been associated with various diseases and disorders such as Parkinson’s disease [2–4], Alzheimer’s disease [5–7], Down’s syndrome, Huntington’s disease [8], schizophrenia, epilepsy and cocaine addiction [9].

Up to now, there have been many reports about relationship between exercise and NT. Blomstrand et al. reported that exhausting exercise caused an increase of MANT (e.g., 5-HT, DA) in hypothalamic area of rats [10]. Hokfelt et al. reported that the concentrations of AANT were a hundred times more than those of MANT in brain [11]. Researches have shown that the concentration changes of Glu, GABA, 5-HT and DA, and the ratio change of Glu/GABA in rat brain were related with the development of exercise fatigue [12,13]. Davis reported that exhaustive exercise resulted in concentration changes of 5-HT and 5-HIAA in whole brain or its part region. Therefore, it was much more accurate to evaluate the formation of exercise fatigue using the ratio of 5-HT/5-HIAA [14].

HPLC or capillary electrophoresis (CE) coupled with various detection methods, such as UV detection [15], fluorescence detection (FLD) [16–21], electrochemical detection (ECD) [22–24,9],...
laser-induced fluorescence detection (LIFD) [25–29] and mass spectrometry (MS) [30–34], have been widely developed for the determination of AANT and MANT. These methods have more or less limitations. UV detection is not sensitive and selective for monoamines and helpless for amino acids. Electrochemical detection tends to lack reproducibility mainly because of hysteretic degradation of the electrode [35]. CE-LIFD is limited for analyzing MANT in biological fluids containing complex mixtures due to the similar electrophoretic behavior of these compounds [36]. HPLC– or CE-MS methods have higher detection limits than FLD or ECD [31,34]. Recently, some new methods have been brought forward for the detection of NT, such as microfluidic electrophoresis chip for AANT [1], intramolecular fluorescence resonance energy transfer detection for MANT [35], microchip electrophoresis for MANT or AANT [29,36–37], monolithic column chromatography coupled with chemiluminescence detection [38]. However, they are just tentative as new techniques for few interested AANT or MANT.

As a matter of fact, pre-column derivatization coupled with HPLC-UV or HPLC-FLD has been successfully used for the determination of MANT and AANT in microdialyses, brain tissue extracts and other biochemical samples [19–21,39,40]. Some fluorescent derivatization reagents have been synthesized and applied in this field including o-phthalaldehyde-2-mercaptoethanol (OPA-2-ME) [35,40], benzylamine and 1,2-diphenylethylenediamine (BA + DPE) [39] and 6-aminquinolynil-N-hydroxysuccinimidyl carbamate (AQC) [19]. Many other derivatization reagents which are not fluorescent ones have also been developed for NT analysis, such as dansyl chloride (Dns-Cl) [41], 5-furoylquinoline-3-carboxaldehyde (FQ) [25], fluorescein-5-isothiocyanate (FITC) [27], naphthalene-2,3-dicarboxaldehyde (NDA) [28] and 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein (DTAF) [29]. However, these pre-column derivatization reagents have more or less limitations in their applications, such as poor stability, low detection sensitivity, tedious analytical procedure, or serious interferences in chromatogram [8,22].

In our previous studies [42,43], we described the synthesis of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) and its applications for analysis of aliphatic amines, mono amino acids and peptides in environmental and biological samples. In this paper, a HPLC–FLD method using BCEOC as pre-column derivatization reagent was developed for the simultaneous determination of AANT and MANT. This method was also used to study the changes of NT in rat endbrains at three states (quiet state, at exercise exhaustion, 12 h after exercise exhaustion) and the relationship between NT concentration and exercise fatigue. To the best of our knowledge, BCEOC is firstly studied for the analysis of NT and shows some advantages including mild derivatization conditions, ease-of-handling and high detection sensitivity.

2. Experimental

2.1. Instrumentation

HPLC separation, MS identification and analysis of samples were performed using Agilent 1100 Series high-performance liquid chromatography/mass spectrometry (HPLC–MSD Trap-SL, a complete LC–MS/MS). All the HPLC system devices were from the Agilent 1100 series and consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD) (model G1321A) and a diode array detector (DAD) (model G1315A). The HPLC system was controlled by HP Chemstation software (Version B.01.01). The mass spectrometer LC/MSD Trap-SL (ion trap) was from Bruker Daltonik (Bremen, Germany) equipped with an electrospray ionization (ESI) source and controlled by Esquire-LC NT software (Version 5.3). Ion source conditions: ESI in positive ion mode, nebulizer pressure 241.3 kPa, dry gas temperature 350 °C, dry gas flow 9.0 L/min and capillary voltage −3500 V. Derivatives were separated on a reversed-phase Hypersil BDS-C18 column (100 mm × 4.6 mm, 5 μm i.d., Dalian Elite Analytic Instruments Co. Ltd., Dalian, China) by a gradient elution. Fluorescence excitation and emission spectra were obtained on a 650–10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Excitation and emission bandpass were both set at 10 nm. A Paratherm U2 electronic water-bath (Hitachi, Tokyo, Japan) was used to control temperature. The mobile phase was filtered through a 0.2 μm membrane.

2.2. Chemicals and materials

1,2-Benzene-3,4-dihydrocarbazole-9-ethyl chloroformate was synthesized in our laboratory [42,43]. Glutamic acid, γ-amino-butyric acid, 5-hydroxytryptamine and 5-hydroxyindole acetic acid were purchased from Sigma (St. Louis, MO, USA). Dopamine was purchased from Fluka (Buchs, Switzerland). Spectroscopically pure acetonitrile was purchased from Merck (Darmstadt, Germany). Water was purified using a Millipore system (Bedford, MA, USA). Other reagents were of analytical grade.

2.3. Preparation of standard solutions

Individual stock solution (1.0 × 10⁻² M) of NT was prepared in acetonitrile/water mixed solution (v/v, 1:1). The mixed standard solutions of NT for HPLC analysis were prepared by diluting the stock solutions with acetonitrile/water to the individual concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.7813, 0.3906, 0.1953, 0.09766, 0.04883 and 0.02441 μM. The BCEOC solution (1.0 × 10⁻³ M) was prepared by dissolving 3.26 mg BCEOC in 10 mL of acetonitrile. The low concentration derivatization reagent solutions were obtained by dilution with acetonitrile. Borate buffer solution (0.2 M) was prepared by dissolving borax in water and pH was adjusted using hydrochloric acid or sodium hydroxide solution to different values in 4.0–11.0 for derivatization. When not in use, all standards were stored at 4 °C in a refrigerator.

2.4. Derivatization procedure

Derivatization reaction scheme is shown in Fig. 1. The BCEOC–NT derivatization proceeded in acetonitrile/water solution in basic medium. 50 μL solution of mixed standard NT, 95 μL acetonitrile, 200 μL borate buffer (pH 9.0) and 150 μL BCEOC solution were orderly and successively added into a 2-mL vial. The vial was then
sealed and allowed to stand for 10 min at 40 °C in a thermostatic water-bath. After derivatization, to the solution was added 5 µL 50% acetic acid/water until the final pH 7.0. Then 10 µL derivatized sample solution was directly injected into the HPLC system for analysis.

2.5. High-performance liquid chromatography

HPLC separation of NT derivatives was carried out on the reversed-phase Hypersil BDS-C18 column by a gradient elution. Eluent A was 30% of acetonitrile consisting of 30 mM ammonium formate buffer (pH 3.7); B was 100% of acetonitrile. The gradient elution program was as follows: 0 min = 15% B, 5 min = 15% B, 15 min = 25% B, 25 min = 100% B and 30 min = 100% B. Before injecting the next sample, the column was equilibrated with the initial mobile phase for 10 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ<sub>ex</sub> 333 nm and λ<sub>em</sub> 390 nm, respectively. The derivatives were quantified by fluorescence detector, and identified simultaneously through HPLC retention time of standards and on-line ESI/MS structure identification.

2.6. Animals

Male Wistar rats were purchased from experimental animal center of Lu Nan Pharmacy Group Joint-stock Ltd. (180–220 g). Exercise training and endbrain samples separation were performed by Prof. Liu Hongzhen (College of Sports Education, Qufu Normal University). Twenty-four rats were adaptively trained on electric drive treadmills for 1 week (rate 10 m/min, training time 20 min/d). Then, the rats were separated at random into three groups (Group A, B and C, eight rats of each group) by avoirdupois. Endbrain tissues were collected at three states corresponding to three groups as follows: quiet state without exercise (Group A, the control group), at exercise exhaustion (Group B, judgment standard: the rats could not hold up to exercise; stimulation and drive were of no effect; when the rats were taken down from electric drive treadmills and upturned, they could not turn over by themselves), 12 h after exercise exhaustion (Group C).

2.7. Extraction of NT in rat endbrain tissues

Male Wistar rats were decapitated at corresponding states of three groups. The whole brain were immediately taken out and washed by ice-cooled physiological salt solution. Endbrains were separated on a glass plate. To a hard glass tube, 1.2 mL 0.1 M ice-washed by ice-cooled physiological salt solution. Endbrains were three groups. The whole brain were immediately taken out and separated on a glass plate. To a hard glass tube, 1.2 mL 0.1 M ice-washed by ice-cooled physiological salt solution. Endbrains were taken down from electric drive treadmills and upturned, they could not turn over by themselves, 12 h after exercise exhaustion (Group C). The supernatant was centrifuged (18,000 rpm for 30 min at 4 °C). The supernatant was separated and added 20% NaOH solution to neutralize the excess amount of HClO4 to pH 8.0–9.0. The resulting solutions were made up to total volume of 2.0 mL with deionized water and stored at −80 °C until HPLC analysis.

2.8. Quantitative analysis

Quantitative conversion of NT from rat endbrains to their BCEO-labeled derivatives was guaranteed by using an excess of BCEO. All NT were quantified using external standard method with fluorescence detection at 390 nm. The calibration curves for each BCEO–NT derivative were obtained by linear regression plotting peak area versus injection amount.

2.9. Data statistic

All the data statistic work was accomplished with SPSS 13.0. The results were expressed in the form of “average ± standard deviation” (X ± S). Data were analyzed with independent sample T-test and one-way analysis of variance. Significant difference level was P < 0.05.

3. Results and discussion

3.1. Fluorescence spectra of BCEO–NT derivatives

Maximum excitation and emission wavelengths which were set in HPLC fluorescence detector were important for the detection sensitivity of NT derivatives. To determine the fluorescence spectra of five NT derivatives, every BCEO–NT sample was prepared by individual derivatization and purification on a solid-phase extraction column (ODS C18, Dalian Elite Analytic Instruments Co. Ltd., Dalian, China), and scanned on the 650–105 fluorescence spectrophotometer. The maximum excitation and emission wavelengths for five BCEO–NT derivatives were 333 and 390 nm, respectively. The representative fluorescence excitation and emission spectra of BCEO–GABA was shown in Fig. 2.

3.2. Optimization of derivatization conditions

The main factors affecting derivatization yields were pH value of buffer, reaction time, temperature and concentration of BCEO. Several kinds of basic media were tested including carbonate buffers, phosphate buffers and borate buffers. The maximum peak area was obtained with borate buffer in aqueous acetonitrile. However, high concentration buffers (>0.3 M) yielded a slight reduction of peak area. In the following experiments, 0.2 M borate buffer was used. The effect of pH was investigated in the pH range of 4.0–11.0. Derivatization reaction did not occur in the range of pH < 7.0, while peak areas of five derivatives obviously increased along with the increasing of pH value in the pH range of 7.0–9.0. At higher pH values (>9.0), the derivatives exhibited some hydrolysis and partially...
converted to their mono-substituted ones. Therefore, all subsequent derivatization reaction was performed at pH 9.0. The effects of reaction temperature and time on the derivatization yields were evaluated from 30 to 90 °C and 1–30 min, respectively. Maximal and constant peak areas were achieved when NT standards were derivatized at 40 °C for 10 min. This indicated that BCEOC reacted rapidly and smoothly with NT under mild conditions. In addition, the effect of BCEOC concentration on derivatization yields was investigated. The peak areas of BCEOC-derivatives increased along with the increasing amounts of BCEOC. Maximal peak areas were achieved when the molar reagent was six times in excess. Accordingly, six times excess of molar reagent was taken in our following experiment. To an unknown concentration of real sample, such as the extracted endbrain tissue, complete derivatization was guaranteed by using excess BCEOC until constant peak area for detector responses was reached. After derivatization reaction completed, the solution was neutralized to pH 5.0–7.0 by adding 5 μL 50% acetic acid/water in order to avoid the degradation of NT derivatives.

### 3.3. HPLC separation

Several mobile phases of methanol or acetonitrile aqueous mixtures with different compositions were tested for the separation of derivatives. The results indicated that five NT derivatives could be completely separated on a Hypersil BDS-C18 column when (A) 30% acetonitrile (pH 3.7) containing 30 mM ammonium formate and (B) 100% acetonitrile were used as eluents. The pH value of mobile phase could significantly affect the resolution of five derivatives. Operation at pH 6.0–8.0 of mobile phase A resulted in obviously increase of retention time about 5–11 min for all derivatives. With the optimal pH 3.7, all derivatives were best separated giving appropriate retention time and good peak shape. Furthermore, the acidic eluents could provide hydrogen proton to enhance the ESI ionization efficiency of NT derivatives. Since the ammonium formate itself was volatile, the high dry gas temperature (350 °C) and abundant dry gas flow (9.0 L/min) could effectively reduce its residue in ESI source. Before injected into ESI source, the eluents was split (split ratio = 1:1) by a three-way valve, a diverter designed in Agilent 1100 HPLC–ESI/MS system. This means that only 50% of eluents was injected into ESI source, namely the flow rate was 0.5 mL/min and only half of ammonium formate was used. That was allowable in the service manual of Agilent 1100 HPLC–MS system and did not cause contamination to ESI source. The effect of the concentration of ammonium formate was also investigated to verify whether it caused ion suppression to ESI source. Eluents A containing different concentration ammonium formate (0, 10, 20, 30 and 40 mM) were prepared and tested under the same conditions. The results indicated that MS total ion current intensities of five NT derivatives obviously increased from 0 to 30 mM ammonium formate and leveled off above 30 mM. Moreover, the intensities obtained at 30 mM ammonium formate were about two times stronger than those at 0 mM. Therefore, 30 mM ammonium formate was favorable for MS detection. Under optimum conditions, five NT derivatives were separated with a good baseline resolution as shown in Fig. 3(a). Blank experimental chromatogram obtained by derivatizing the same volume acetonitrile/water solution which was replaced by the mobile phase. Peaks: Glu: glutamic acid; GABA: γ-aminobutyric acid; DA: dopamine; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindole acetic acid; BDC-OH: 1,2-benzo-3,4-dihydrocarbazole-9-ethanol; (BCEOC): bis-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl) carbonate.

![Fig. 3. Chromatogram of BCEOC-labeled (a) neurotransmitters standard (100 pmol in 10 μL), (b) blank experiment and (c) neurotransmitters standard at 2.441 nM. Chromatographic conditions: column, Hypersil BDS-C18, 100 mm × 4.6 mm, 5 μm i.d.; flow rate = 1.0 mL/min; column temperature 30 °C; excitation wavelength λex = 333 nm; emission wavelength λem = 390 nm. Gradient elution conditions were described in Sections 2.5 and 3.2. Derivatization of blank experiment was performed without neurotransmitter standard solution which was replaced by the same volume acetonitrile/water solution. Peaks: Glu: glutamic acid; GABA: γ-aminobutyric acid; DA: dopamine; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindole acetic acid; BDC-OH: 1,2-benzo-3,4-dihydrocarbazole-9-ethanol; (BCEOC): bis-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl) carbonate.](image)

The overall comparison of BCEOC with reported derivatization reagents for NT analysis including derivatization, separation and detection is given in Table 1. Derivatization reaction for BCEOC with NT was more simple and rapid than those reagents except OPA and AQC. But OPA and AQC were incapable of secondary amines (e.g. 5-HIAA). Moreover, the derivatives of OPA were very unstable as a result of difficulty in quantitation [22]. HPLC separation conditions for BCEOC derivatives including column, mobile phase and elution program were more facile than other methods described in Table 1. And it also brought rapid and complete resolution of NT derivatives. In addition, the fluorescence detector coupled to HPLC was easy to gain, and the higher detection sensitivity of BCEOC–NT derivatives was satisfactory.
The structure identification of NT derivatives was carried out by on-line mass spectrometry with ESI source in positive ion mode. The MS and MS/MS spectra of representative BCEOC-labeled 5-HT derivative are shown in Fig. 4(a) and (b). The cleavage mode of protonated molecular ion of 5-HT derivative is also shown in Fig. 4(c). The molecular ions ([M + H]⁺ ion) and specific fragment ions (MS/MS) of five BCEOC-labeled derivatives are listed in Table 2. All the NT derivatives produced intense molecular ion peaks at [M + H]⁺ ions, which should be attributed to the introduction of an alkalescent nitrogen atom in BCEOC molecular core. The collision-induced dissociation spectra (MS/MS) of molecular ions ([M + H]⁺ ion) produced intense and stable fragment ions at m/z 264.1, m/z 246.1 and m/z 218.1 corresponding to the cleavages of NCH₂CH₂O–CO, NCH₂CH₂–OCO and N–CH₂CH₂OCO bonds, respectively. The selected reaction monitoring, which was based on the [M + H]⁺ ion → m/z 264.1, m/z 246.1 and m/z 218.1 transition, was specific for BCEOC-labeled NT derivatives. There was no detectable signal from blank deionized water sample. Although other endogenous amino compounds present in real samples were presumably co-extracted and derivatized by BCEOC reagent, no interference was observed due to the highly intense molecular ions and the characteristic fragment ions at m/z 264.1, m/z 246.1 and m/z 218.1.

### 3.5. MS identification by ESI/MS

The structure identification of NT derivatives was carried out by on-line mass spectrometry with ESI source in positive ion mode. The MS and MS/MS spectra of representative BCEOC-labeled 5-HT derivative are shown in Fig. 4(a) and (b). The cleavage mode of protonated molecular ion of 5-HT derivative is also shown in Fig. 4(c). The molecular ions ([M + H]⁺ ion) and specific fragment ions (MS/MS) of five BCEOC-labeled derivatives are listed in Table 2. All the NT derivatives produced intense molecular ion peaks at [M + H]⁺ ions, which should be attributed to the introduction of an alkalescent nitrogen atom in BCEOC molecular core. The collision-induced dissociation spectra (MS/MS) of molecular ions ([M + H]⁺ ion) produced intense and stable fragment ions at m/z 264.1, m/z 246.1 and m/z 218.1 corresponding to the cleavages of NCH₂CH₂O–CO, NCH₂CH₂–OCO and N–CH₂CH₂OCO bonds, respectively. The selected reaction monitoring, which was based on the [M + H]⁺ ion → m/z 264.1, m/z 246.1 and m/z 218.1 transition, was specific for BCEOC-labeled NT derivatives. There was no detectable signal from blank deionized water sample. Although other endogenous amino compounds present in real samples were presumably co-extracted and derivatized by BCEOC reagent, no interference was observed due to the highly intense molecular ions and the characteristic fragment ions at m/z 264.1, m/z 246.1 and m/z 218.1.

### 3.6. Method validation

#### 3.6.1. Linearity, limit of detection (LOD) and its comparison with other methods

Under the optimum experimental conditions, 14 standard NT solutions (0.02441–200 μM) were derivatized and analyzed. The calibration graphs were established with peak area (Y) versus NT injection amounts (X, pmol). Injected amount of each NT was from 24.41 fmol to 200.0 pmol with injection volume of 10 μL, namely the linear range of each NT was 2.441–20000 nM with an 8193-fold concentration range. Linear regression equations, correlation coefficients, limits of detection and limits of quantitation (LOQs) for all NT by HPLC-FLD were shown in Table 2. All NT derivatives gave excellent linear responses over this range with correlation coefficients of 0.9997–0.9998. The LODs of BCEOC-labeled NT were calculated at S/N = 3:1 from the standard chromatogram (Fig. 2(c)) and were in the range of 0.398–1.258 nM. The LOQs of BCEOC-labeled NT were in the range of 2.42–2.46 nM (S/N = 10:1).

LODs of BCEOC–NT derivatives by ESI/MS detection were in the range of 10.5–23.2 nM (10.5 for 5-HIAA, 11.8 for 5-HT, 14.3 for DA, 22.4 for GABA, 23.2 for Glu), which were determined by injecting standard BCEOC–NT derivatives at 48.82 nM and calculated at S/N = 10:1 (R.S.D. < 4.0%, n = 3). The LODs by ESI/MS were higher about 20 times than those by HPLC-FLD. For further comparison of HPLC-FLD and ESI/MS detection, six replicative injections (10 μM,
Fig. 4. MS spectra of representative BCEOC-labeled 5-HT derivative: (a) molecular ion MS, (b) MS/MS and (c) the cleavage mode of protonated molecular ion. Scanning range from 100 to 1000 amu under ESI positive ion mode; derivatives were isolated from a Hypersil BDS-C18 column and into the on-line mass spectrometer.

Table 2
Linearity, LODs and its comparison with reported methods, LOQs, MS and MS/MS of five BCEOC–NT derivatives by HPLC-FLD

<table>
<thead>
<tr>
<th>NT</th>
<th>( Y = AX + B )</th>
<th>Correlation coefficients</th>
<th>LODs(^a) (nM)</th>
<th>HPLC-ECD ( [23,44] ) (nM)</th>
<th>HPLC-MS ( [31] ) (nM)</th>
<th>CE-LIFD ( [25] ) (nM)</th>
<th>HPLC-FRET ( [35] ) (nM)</th>
<th>LOQs(^b) (nM)</th>
<th>MS [M+H](^+)</th>
<th>MS/MS specific fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>( Y = 61.85X + 13.52 )</td>
<td>0.9998</td>
<td>0.687</td>
<td>No</td>
<td>No</td>
<td>10</td>
<td>No</td>
<td>2.45</td>
<td>437.1</td>
<td>418.9, 390.9, 264.1, 246.1, 218.1</td>
</tr>
<tr>
<td>GABA</td>
<td>( Y = 65.12X + 28.05 )</td>
<td>0.9997</td>
<td>0.799</td>
<td>No</td>
<td>No</td>
<td>2</td>
<td>No</td>
<td>2.43</td>
<td>393.2</td>
<td>375.0, 264.1, 246.1, 218.1</td>
</tr>
<tr>
<td>DA</td>
<td>( Y = 77.59X + 67.64 )</td>
<td>0.9998</td>
<td>1.258</td>
<td>2.5</td>
<td>42.4 (6.5 ng/mL)</td>
<td>80</td>
<td>4.65</td>
<td>2.42</td>
<td>443.1</td>
<td>424.2, 368.1, 307.1, 264.1, 246.1, 218.1</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>( Y = 674.1X + 29.99 )</td>
<td>0.9997</td>
<td>0.483</td>
<td>0.6</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>2.46</td>
<td>481.2</td>
<td>462.3, 434.9, 264.1, 246.1, 218.1</td>
</tr>
<tr>
<td>5-HT</td>
<td>( Y = 62.96X + 27.36 )</td>
<td>0.9997</td>
<td>0.398</td>
<td>1.0</td>
<td>4.4 (0.78 ng/mL)</td>
<td>1</td>
<td>0.85</td>
<td>2.44</td>
<td>755.2</td>
<td>711.1, 536.0, 492.0, 264.1, 246.1, 218.1</td>
</tr>
</tbody>
</table>

\(^a\) Linear regression equations; \( Y \): peak area; \( X \): injected amount (pmol).
\(^b\) LODs were calculated at S/N = 3:1, the injection volume was 10 µL.
\(^c\) LOQs were obtained when S/N = 10:1, the injection volume was 10 µL.

No: not reported.
10 µL) were analyzed. R.S.D.s of peak area were in the range of 1.95–2.86% for HPLC-FLD and 6.84–13.45% for ESI/MS detection, respectively. According to the comparison mentioned above, HPLC-FLD was better than ESI/MS detection.

In addition, LODs of our HPLC-FLD method was compared with those of reported methods (Table 2). It indicated that LODs of the proposed HPLC-FLD method were lower about 2–60 times than those of reported methods. LODs of our BCEOC method were also compared with those of other derivatization reagents in Table 1. LODs of BCEOC method were comparable with those of BA + DPE method [39] and FITC method [27], and were obviously lower than those of other seven reagents.

3.6.2. Stability

A standard solution containing of 50 pmol NT derivatives was analyzed by HPLC-FLD after being placed at room temperature for 0, 1, 2, 4, 8, 16, 24 and 48 h, respectively. R.S.D.s of peak area were 3.2%. Thus the stability of BCEOC–NT derivatives was satisfactory for chromatographic analysis.

3.6.3. Repeatability

Derivatization repeatability was examined by measuring peak area and retention time of six replicative derivatizations of mixed NT at 1.0 µM. R.S.D. values of retention time and peak area were in the range of 0.31–0.46% and 2.36–2.93%, respectively. It indicated that the BCEOC-labeling derivatization reaction was of high reproducibility.

3.6.4. Precision and accuracy

Endbrain tissue of male Wistar rat was collected from the same species as described in Section 2.6 and then was divided into two equal parts. To one part was spiked known amount NT standard which had similar concentration with rat endbrain tissue (Glu, 500 µg/g; GABA, 300 µg/g; DA, 20 µg/g; 5-HIAA, 4 µg/g; 5-HT, 4 µg/g) and to the other part was spiked nothing. Pretreatment and derivatization of endbrain samples were the same as described in Sections 2.7 and 2.4. Six replicative analyses (n = 6) were performed. The precisions were expressed as R.S.D.s (%). The accuracies were expressed as recovery and calculated as follows: recovery (%) = 100(a − b)/c, where a was the measured concentration obtained from the extracted tissue solutions which were spiked standard, b was the concentration of NT in the matrix and c was the added known concentration to the matrix. R.S.D. values of peak area and retention time were in the range of 2.56–3.85% and 0.38–0.74%, respectively. Accuracies (i.e. recoveries, %) were in the range of 91.8–105.6%. The results demonstrated that the pre-treatment and derivatization of our HPLC-FLD method were satisfactory for the quantification of NT in real samples.

### Table 2

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<thead>
<tr>
<th>Method</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCEOC</td>
<td>0.15</td>
</tr>
<tr>
<td>BA + DPE</td>
<td>0.5</td>
</tr>
<tr>
<td>FITC</td>
<td>1.0</td>
</tr>
</tbody>
</table>

3.7. Application to rat endbrain tissues analysis

Under the optimum experimental conditions mentioned above, concentrations of NT in endbrain tissues of Group A, B and C corresponding to three states were determined. The results were doubly identified by chromatographic retention time and on-line MS identification. Typical chromatogram of NT derivatives from rat endbrain is shown in Fig. 5 (peaks as Fig. 3). The concentrations of five NT and ratios of 5-HT/5-HIAA, DA/5-HT and Glu/GABA in endbrains were shown in Table 3. The concentration changes and the ratio changes of AANT and MANT in rat endbrains at corresponding states of three groups were in accordance with the papers reported before [11–14].

4. Concluding remarks

In this study, a new HPLC-FLD-ESI/MS method using BCEOC as pre-column derivatization reagent for the simultaneous determination of MANT and AANT in rat endbrain tissues has been developed and validated. Two most attractive advantages of this method are high detection sensitivity and convenience. All excellent characteristics allow for the development of a highly sensitive and specific method for the quantitative analysis of NT in brain tissues and other biological samples.

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References