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Simultaneous determination of 5-hydroxyindoles and catechols by high-performance liquid chromatography with fluorescence detection following derivatization with benzylamine and 1,2-diphenylethylenediamine

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Abstract

A highly selective and sensitive method for the simultaneous determination of 5-hydroxyindoles and catechols (serotonin, norepinephrine, dopamine and related compounds) by high-performance liquid chromatography with fluorescence detection is described. The method is based on the two-step precolumn derivatization of 5-hydroxyindoles and catechols with benzylamine (BA) and 1,2-diphenylethylenediamine (DPE), respectively, resulting in highly fluorescent and stable benzoxazole derivatives. The first derivatization with BA proceeds at room temperature (ca. 23 °C) for 2 min in a mixture of 0.3 *M* 3-cyclohexylamino-1-propanesulfonic acid buffer (pH 10.0) and methanol in the presence of potassium hexacyanoferrate(III). The subsequent second derivatization with DPE is carried out at 50 °C for 20 min in the presence of glycine. The resulting fluorescent derivatives of five 5-hydroxyindoles and seven catechols are separated on a reversed-phase column (150×1.5 mm I.D., packed with C_{18} silica, 5 μm) with isocratic elution using a mixture of acetonitrile–15 m*M* acetate buffer (pH 4.5) (34:66, v/v) containing 1 m*M* octanesulfonic acid sodium salt, and are detected spectrofluorimetrically at 480 nm with excitation at 345 nm. The detection limits (signal-to-noise ratio of 3) of the related compounds are 80 amol to 86 fmol for a 20-μl injection.
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Keywords: Derivatization, LC; Hydroxyindoles; Benzylamine; Diphenylethylenediamine; Catechols

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE) and dopamine (DA) have been strongly implicated in the aetiology of affective and neurodegenerative diseases (e.g. Parkinson's disease) of the central nervous system [1–3]. In addition, monitoring the levels of amines and their deaminated metabolites in body fluids and tissue biopsies is often used clinically in the therapy of hypertension [4], carcinomas and other diseases related to the cardiovascular system [5] and adrenal function [6].

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Various methods have been described for the simultaneous determination of 5-HT, NE, DA and their metabolites, namely high-performance liquid chromatography (HPLC) with the electrochemical (LC–ED) [7–12] or fluorescence (LC–FL) [13–15] detection of the native monoamines, and gas chromatography–mass spectrometry (GC–MS) [16–18] of the derivatized amines. Of these methods, the LC–ED technology has been most widely used, both in clinical chemistry laboratories and in experimental neuroscience research. However, the major drawbacks of LC–ED in routine use are its difficult maintenance, noise problems and the requirement of well-trained personnel. On the other hand, LC–FL offers a simpler analytical system, but provides relatively low sensitivity, especially for catecholamines.

We have previously reported benzylamine (BA) and 1,2-diphenylethylenediamine (DPE) as highly sensitive and selective fluorescence derivatization reagents for 5-hydroxyindoles (5-HIs) and catechols, respectively [19–21]. BA and DPE react with 5-HIs and catechols, respectively, in the presence of potassium hexacyanoferrate(III) in weakly alkaline solution to give the corresponding highly fluorescent benzoxazole derivatives (Fig. 1). Thus, highly sensitive and selective HPLC methods have been developed for the individual determination of 5-HIs and catechols using these reagents. Post-column derivatization with BA has been applied to the determination of 5-HT in human plasma [22], in microdialysis samples from the rat [23] and mouse [24,25] and for the on-line detection of 5-HT in combination with pre- and post-column derivatization in microdialysates from rats [26]. On the other hand, the DPE derivatization method has been used for the determination of the catecholamines [NE, epinephrine (E) and DA] in plasma and urine [27,28].

In the course of our previous and present work, we found that BA and DPE react selectively with 5-HIs and catechols, respectively, under mild derivatization conditions. This indicates that the combined use of these reagents might be useful for the simultaneous determination of 5-HIs and catechols.

In the present work, we examined the optimum

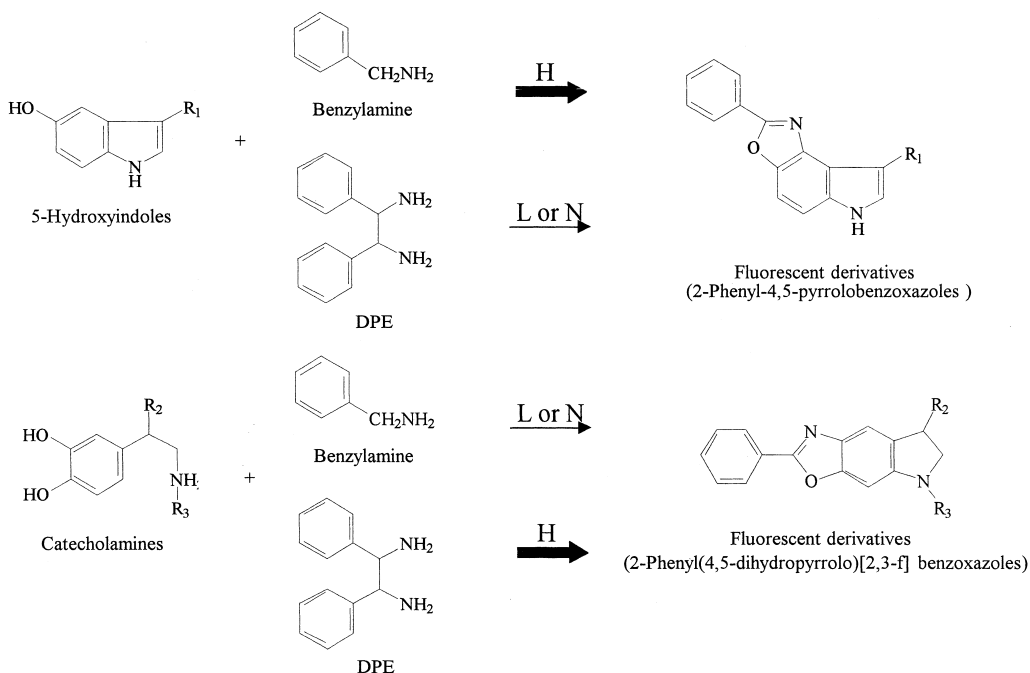

Fig. 1. Derivatization of 5-hydroxyindoles and catecholamines with BA and DPE and their reactivities. H, high reactivity; L, low reactivity; N, no reactivity.

conditions for a dual (two-step) derivatization procedure with the combined use of BA and DPE, and developed a highly sensitive and selective method for the simultaneous determination of 5-HIs and catechols by HPLC with fluorescence detection.

2. Experimental

2.1. Chemicals and solutions

Deionized and distilled water, purified with a Barnstead EASYpure RF (Hansen, Hyogo, Japan) system, was used for all aqueous solutions. 5-HT hydrochloride, 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptophol (5-HTOL) were obtained from Sigma (St. Louis, MO, USA). 5-Hydroxyindole-3-acetic acid (5-HIAA), *N*-acetyl-5-hydroxytryptamine (*N*-Ac-5-HT), NE hydrogentartrate monohydrate, E, DA hydrochloride, 3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylethyleneglycol (DOPEG), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxymandelic acid (DOMA), 3-cyclohexylaminopropanesulfonic acid (CAPS) and glycine were purchased from Wako Pure Chemicals (Osaka, Japan). Potassium hexacyanoferrate(III) was purchased from Kisida Chemicals (Tokyo, Japan). BA hydrochloride was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and was used after purification by recrystallization with absolute ethanol. DPE was purchased from Tosoh (Tokyo, Japan). Other chemicals were of the highest purity available and were used as received. BA hydrochloride (0.3 *M*), 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer (0.3 *M*, pH 10.0) and potassium hexacyanoferrate(III) (20 m*M*) 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 BA derivatization reagent solution was a mixture containing BA–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 room temperature. Stock solutions (0.1 m*M*) of 5-HIs and catechols were prepared in water and kept frozen (−20 °C) in amber-colored test tubes. The solutions were diluted further with water to the desired concentrations before use.

2.2. Derivatization procedure

To 20 μl of an aqueous test solution placed in a 300-μl micro vial (03-CVG, Chromacol, UK) was added 20 μl of the BA derivatization reagent solution. After standing at room temperature for ca. 2 min, 20 μl of the DPE derivatization reagent solution was added to the vial. The vial was tightly sealed and heated at 50 °C for 20 min in a thermo alumibath (ALB-220, Iwaki Glass, Japan). After cooling in ice-water, a 20-μl aliquot of the final reaction mixture was injected onto the chromatograph.

For the reagent blank, 20 μl of water in place of the test solution 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 a 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 C_{18} silica (L-column); particle size 5 μm] was purchased from Chemicals Evaluation and Research Institute (Tokyo, Japan). Separation of the BA–DPE derivatives of 5-HIs and catechols was achieved with isocratic elution using a mixture of acetonitrile–15 m*M* acetate buffer (pH 4.5) (34:66, v/v) containing 1 m*M* octanesulfonic acid sodium salt. The flow-rate was 100 μl/min. The column temperature was ambient (20–23 °C).

3. Results and discussion

3.1. HPLC conditions

The optimum HPLC conditions were examined by using a standard solution containing five 5-HIs and seven catechols (Table 1).

A typical chromatogram obtained with a standard

Table 1
5-HIs and catechols used for determination of optimum HPLC conditions

Compound	Retention time (min)	Detection limit (fmol)
5-HTP	4.7	0.40
NE	6.1	0.08
DOMA	7.5	86.8
5-HT	9.9	0.20
L-DOPA	11.3	5.65
E	14.6	0.05
5-HIAA	16.7	0.24
5-HTOL	21.9	0.15
N-Ac-5-HT	23.1	0.15
DA	26.0	0.13
DOPEG	37.5	0.23
DOPAC	41.2	0.68

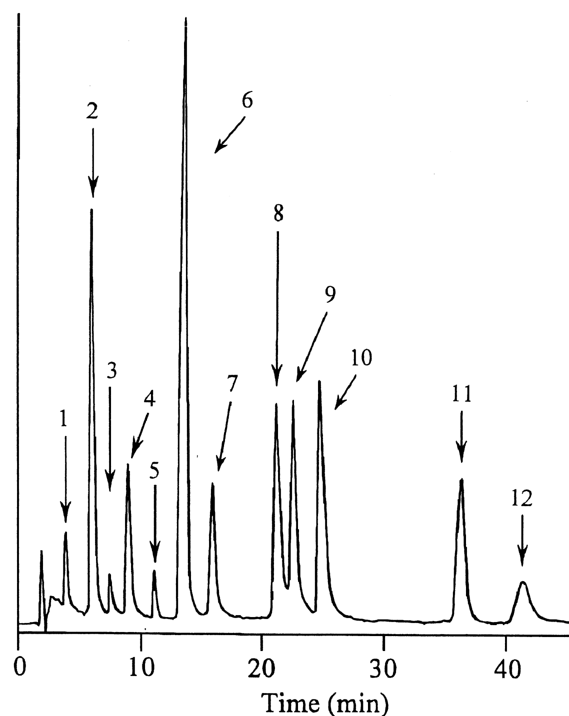

Fig. 2. Chromatogram of a standard solution of five 5-HIs and seven catechols. Peaks (amounts on column): 1=5-HTP (50 fmol), 2=NE (50 fmol), 3=DOMA (5 pmol), 4=5-HT (50 fmol), 5=L-DOPA (500 fmol), 6=E (50 fmol), 7=5-HIAA (50 fmol), 8=5-HTOL (50 fmol), 9=*N*-Ac-5-HT (50 fmol), 10=DA (50 fmol), 11=DOPEG (50 fmol), 12=DOPAC (50 fmol).

solution containing the 12 substances is shown in Fig. 2. A good separation of the BA–DPE derivatives was achieved within 45 min on a microbore reversed-phase column by isocratic elution with a mixture of acetonitrile–15 m*M* acetate buffer (pH 4.5) (34:66, v/v) containing 1 m*M* octanesulfonic acid sodium salt; the individual substances gave single peaks. The retention times of the compounds are shown in Table 1.

3.2. Reactivity of 5-HIs and catechols with BA and DPE

The reactivities of 5-HIs and catechols with BA and DPE were examined by using a standard solution of five 5-HIs and seven catechols. As shown in Fig. 3 (represented by 5-HT, 5-HIAA, NE and E), BA reacted readily with all 5-HIs tested under mild reaction conditions, but not with catechols (Figs. 3A and 4A). BA gave only small peaks for NE and DA and no peaks for the other catechols such as DOPEG and DOPAC. On the other hand, DPE afforded intense peaks for most catechols, including NE and DA (Fig. 3B). However, the reactivity of DPE with 5-HIs was very low. Namely, the simultaneous determination of 5-HIs and catechols was hindered by the fact that BA and DPE are selective for the derivatization of 5-HIs and catechols, respectively.

Viewed from another angle, these facts support that BA and DPE are complementary to each other for the derivatization of 5-HIs and catechols. Further, 5-HIs and catechols gave 2-phenyl-4,5-pyrrolo- and 2-phenyl(4,5-dihydropyrrolo)[2,3-*f*]-benzoxazoles, respectively, independent of the reagents used (Fig. 1) and both derivatives have almost the same fluorescence excitation and emission maxima (Ex. ca. 345 nm; Em. ca. 480 nm). These facts indicate that the combined use of BA and DPE is suitable for the simultaneous derivatization of 5-HIs and catechols.

3.3. Optimization of derivatization conditions

We found that the successive addition of BA and DPE reagent solutions for the first and second step derivatization, respectively, to the test solution is most effective for the simultaneous determination of 5-HIs and catechols (Fig. 3C). When the addition order was reversed (DPE→BA), the peak heights for all substances tested were one-quarter to one-third of

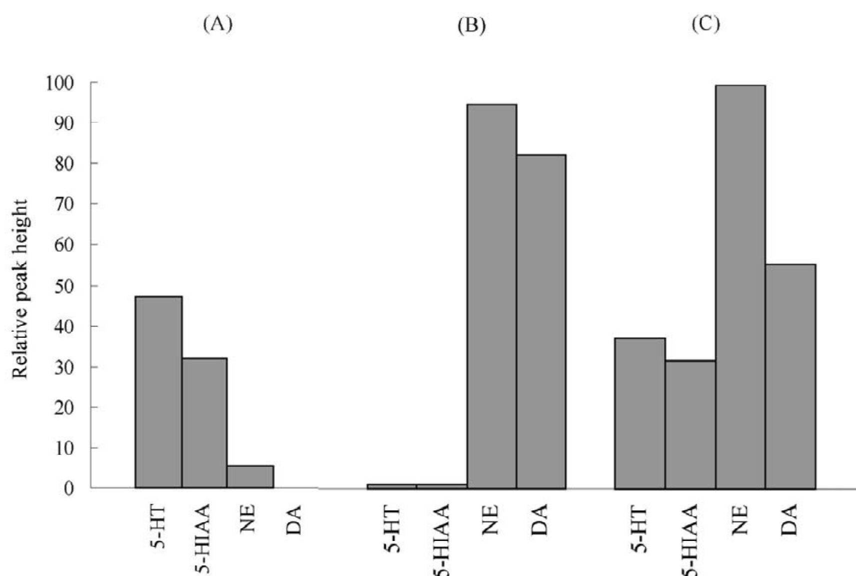

Fig. 3. Reactivity of 5-HIs and catechols with BA and/or DPE. Standard solutions (10 n*M* each) of five 5-HIs and seven catechols were derivatized with (A) BA, (B) DPE and (C) BA and DPE under the respective optimum reaction conditions, which are described in previous and the present work, and then the resulting reaction mixtures were applied onto the present HPLC system.

those obtained with the present method (Fig. 3C). Furthermore, the use of a reagent solution containing both BA and DPE resulted in a decrease of the peak heights for all substances tested to 60–70%. Thus, the two-step derivatization method was employed in the procedure.

The derivatization reaction conditions were examined using a standard solution (10 n*M* each) containing NE, DA, 5-HT and 5-HIAA.

The effects of reaction time and temperature in the first and second step derivatizations on the peak heights were investigated (Fig. 4). In the first step derivatization, the reaction of 5-HIs such as 5-HT and 5-HIAA with BA was completed within 2 min at room temperature (ca. 23 °C) (Fig. 4A). However, BA gave only small peaks for catechols in the first step derivatization. After derivatization, the DPE reagent solution was added to the reaction mixture. The second step derivatization of catechols such as NE and DA with DPE required higher temperatures and longer reaction times, as shown in Fig. 4B–D. As can be seen, there is an optimal window for the reaction temperature of NE; at higher temperature (80 °C) the peak height of the NE-fluorescent product reached the maximum in about 10 min and thereafter rapidly declined. At 50 °C, the maximum NE peak was achieved in 20 min and was stable for at least an additional 40 min. Thus, under optimal reaction conditions of 50 °C and 20 min, the stability of the 5-HT and NE derivatives provided the highest reproducibility of the assay.

The effects of BA, potassium hexacyanoferrate(III), CAPS buffer and methanol concentrations and the pH of the CAPS buffer on the fluorescence intensity (peak heights) are shown in Fig. 5. BA concentrations greater than 10 m*M* in solution gave the maximum peak heights for NE, 5-HT and 5-HIAA (Fig. 5A). However, the fluorescence intensity of DA decreased when using a higher concentration of BA. A BA concentration of 0.3 *M* was adopted for preparation of the reagent. The concentration of potassium hexacyanoferrate(III) affected the peak heights of all substances tested (Fig. 5B). Maximum peak heights were attained at the following concentrations in solution: 15–100 m*M* for NE and 5-HT; 15 m*M* for DA; and 30–100 m*M* for 5-HIAA. 20 m*M* hexacyanoferrate(III) was selected for the procedure. A CAPS buffer afforded maximum peaks for all substance tested at pH 10–11 (Fig. 5D) in the concentration range 0.2–0.3 *M* (Fig. 5C). Thus,

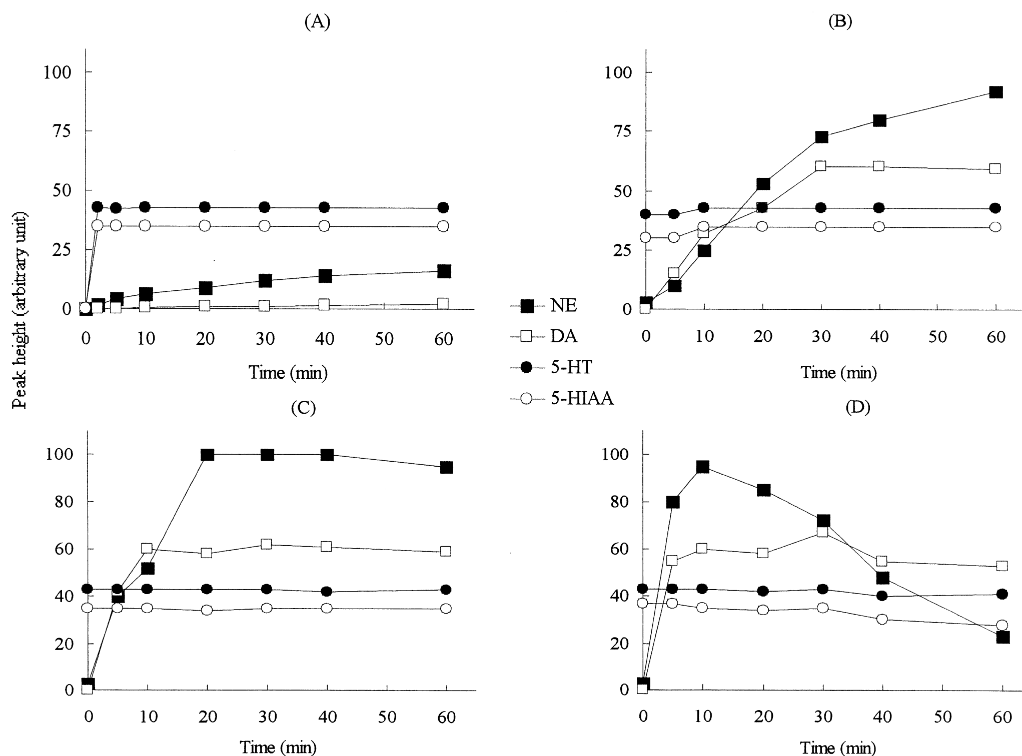

Fig. 4. Effects of reaction time and temperature in the first (A) and second (B–D) step derivatization with BA and DPE, respectively, on the peak heights. Reaction temperature: (A, B) 23 °C, (C) 50 °C and (D) 80 °C. (A) A standard solution of 5-HT, 5-HIAA, NE and DA (10 n*M* each) was treated at 23 °C as in the first step derivatization, and then subjected to the present HPLC procedure without the second step derivatization. (B–D) The reaction mixture in the first step derivatization procedure was treated as in the second step derivatization procedure except that the reaction temperature and time were varied.

0.3 *M* CAPS at pH 10 was taken as the optimal buffer for the derivatization. The methanol concentration also affected the peak heights of all substances tested (Fig. 5E). Maximum peak heights were attained at the following concentrations (v/v) in the final reaction mixture: 40% for NE; 60–70% for DA; 70% for 5-HT; and 20% for 5-HIAA; ca. 40% was employed.

DPE and glycine concentrations affected the peak heights of all substances (Fig. 6). DPE gave maximum peaks at concentrations greater than 50 m*M* in solution for all substances tested (Fig. 6A). A 0.1 *M* DPE concentration is recommended for the preparation of the reagent. Glycine was used as an accelerator for the derivatization reaction between catechols and DPE [21]. 0.3 *M* glycine, which provided almost maximum peak heights for all substances, was selected for the recommended procedure (Fig. 6B).

Other indoles (tryptophan, tryptamine, indole-3-acetic acid, 5-methoxy-L-tryptophan, 5-methoxytryptamine and 5-methoxyindol-3-acetic acid) did not yield fluorescent derivatives at a concentration of 10 m*M* under the present derivatization conditions. Other catechols (4-methylcatechol, 3,4-dihydroxycinnamic acid, catechol, pyrogallol, 3,4-dihydroxybenzylamine, 3,4-dihydroxybenzoic acid, 2-hydroxyestrone, 4-hydroxyestrone, protocatechuic acid, protocatechualdehyde and 3,4-dihydroxycinnamic acid) gave only weak fluorescence (less than 1/1000 times the fluorescence intensity from DA) under the present derivatization conditions, and the peaks due

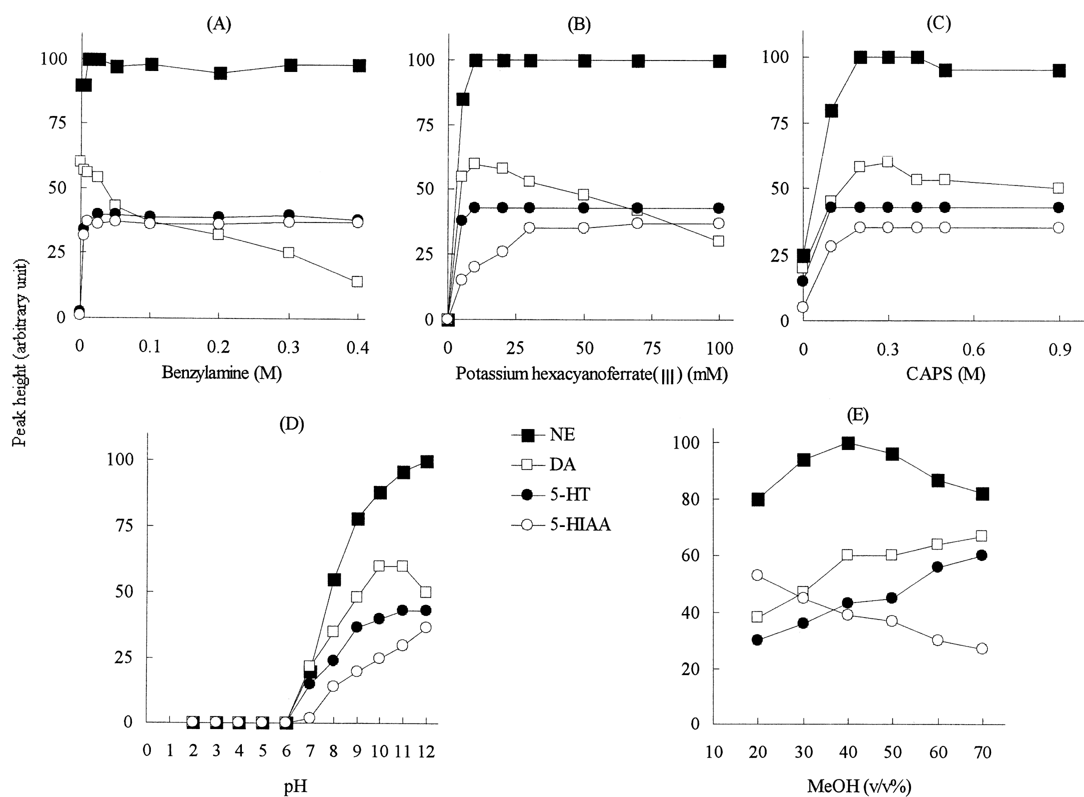

Fig. 5. Effects of (A) benzylamine, (B) potassium hexacyanoferrate(III) and (C) CAPS buffer concentration in solution, (D) the pH of the CAPS buffer and (E) the methanol concentration in the final reaction mixture on the peak heights of 5-HT, NE, DA and 5-HIAA.

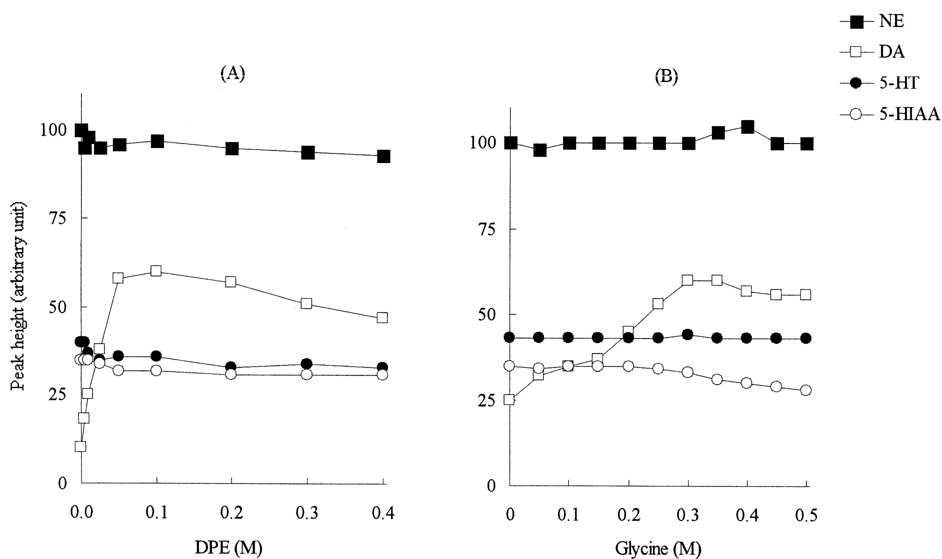

Fig. 6. Effects of (A) DPE and (B) glycine concentration in solution on the peak heights of 5-HT, NE, DA and 5-HIAA.

to these catechols did not appear in the chromatogram at a concentration of 10 mM under the present HPLC conditions.

Other biologically important substances examined did not fluoresce at a concentration of 10 mM. The compounds tested were catecholamine metabolites (normetanephrine, metanephrine, 3-methoxytyramine, vanillylmandelic acid, homovanillic acid and 4-hydroxy-3-methoxyphenylethyleneglycol), 17 different L-α-amino acids, tryptamine, histamine, octopamine, creatine, creatinine, uric acid, putrescine, spermidine, spermine, acetone, formaldehyde, acetaldehyde, *p*-hydroxybenzaldehyde, lactic acid, pyruvic acid, α-ketoglutaric acid, phenylpyruvic acid, oxalic acid, homovanillic acid, acetic acid, D-glucose, D-fructose, D-galactose, D-ribose, D-glucosamine, maltose, sucrose, L-ascorbic acid, uracil, thymine, cytosine, adenine, guanine, cholesterol and cortisone.

3.4. Validation of the method

The relationships between the peak heights and the concentrations of the individual 5-HIs and catechols were linear up to at least 10 pmol per 20 μl injection volume; the linear correlation coefficients were greater than 0.995 ($n=7$) for all substances tested. The within-day precision was established by repeated determination using standard solutions of NE, 5-HT, DA and 5-HIAA (20 and 100 fmol per 20 μl injection). The relative standard deviations did not exceed 4.6% ($n=7$). The detection limits (signal-to-noise ratio 3) are given in Table 1. The detection limits were less than 1 fmol for all substances excluding L-DOPA and DOMA in an injection volume of 20 μl. The sensitivity of the method is much higher than those of previous LC–ED and LC–FL methods for the simultaneous determination of 5-HIs and catechols [7–15]. The reason for the low sensitivity of L-DOPA and DOMA remains unknown: studies are now in progress.

4. Conclusion

The present paper describes a new HPLC method with fluorescence detection for the simultaneous determination of 5-HIs and catechols. This method permits the highly sensitive, selective and simple determination of 5-HT, DA, NE and their related metabolites. Studies on the application to the measurement of 5-HT, DA, NE and their related metabolites in rat brain and its microdialysates are in progress. The method should be useful for investigations into diseases such as depression, panic disorder, Parkinson's disease, heart disease and hypertension.

Acknowledgements

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