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SIMPLE METHOD FOR THE SIMULTANEOUS DETERMINATION OF NORADRENALINE, DOPAMINE AND SEROTONIN BY STEPWISE ELUTION FROM A SHORT COLUMN OF WEAK CATION-EXCHANGE RESIN

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SUMMARY

A simple method for the simultaneous determination of noradrenaline, dopamine and serotonin using a short column of Amberlite CG-50 is described. Noradrenaline and dopamine were eluted from the column with phosphate buffers containing 1.5 and 4.0% boric acid, respectively, and then serotonin was eluted with 1.0 *N* HCl. Catecholamines were determined by a modification of the ethylenediamine condensation method using potassium ferricyanide as oxidant and isobutanol for extraction of the fluorophores. Serotonin was measured by the acidic *o*-phthalaldehyde method. The method was applied to the simultaneous determination of noradrenaline, dopamine and serotonin in discrete regions of rat brain.

INTRODUCTION

In studies of the physiology and pharmacology of noradrenaline, dopamine and serotonin in the central nervous system, it is useful to be able to measure these amines simultaneously. Various methods have been developed for this purpose; solvent extraction[1–4], alumina adsorption[3–5] and weak or strong cation-exchange chromatography[5–11] have been used to purify amines for fluorometric analysis. Weak cation-exchange resins have several advantages over other methods for purification of amines for fluorometric analysis: the amines can be completely separated from their precursor amino acid and acid metabolites; strong acid or organic solvents are not required to elute the amines and the fluorogenic contamination of the eluate is very low. However, unlike strong cation exchangers, weak cation exchangers have the disadvantages that chromatography is affected by the ionic strength of a sample applied, and that it is difficult to achieve sharp separation of each amine on a small column.

Mattock and Wilson[12] reported the specific elution of catecholamine from a weak cation exchanger with aqueous boric acid solution. The separation of dopamine and noradrenaline with boric acid solution was described by Minard and Grant[13], and Seki and co-workers[14–17] studied extensively the chromatographic separation of cate-

cholamines on weak cation-exchange resins, using eluents containing boric acid. The method reported by Minard and Grant involved the use of a relatively long column and a fraction collector; their alternative procedure using a shorter column is not appropriate for sensitive assay, because the sample is diluted by elution from the column.

This paper describes a simple method for separation of the main biogenic amines (noradrenaline, dopamine and serotonin) on a small column of Amberlite CG-50 with buffers of low ionic strength containing boric acid and with HCl, and determination of individual amines by fluorometry. Catecholamines and serotonin were determined by a modification of the ethylenediamine condensation method developed by Seki[17], and the acidic *o*-phthalaldehyde method, respectively. This procedure was successfully applied to the estimation of the three biogenic amines in discrete regions of rat brain.

EXPERIMENTAL

Materials

Ethylenediamine was distilled three times and isobutanol once. Amberlite CG-50 (Type II) was converted into the Na^+ form and graded by the sedimentation method[18] to obtain particles of 95–120 μm. The resin was cycled with 4 *N* HCl, acetone and 4 *N* NaOH and washed extensively with water after each solvent; finally it was equilibrated with 0.1 *M* sodium phosphate buffer, pH 6.0. *o*-Phthalaldehyde was specific grade material for fluorescence analysis (E. Merck, Darmstadt, G.F.K.). Sulphuric acid was superspecific grade material (Wako, Osaka, Japan). Other chemicals were obtained from Nakarai Chemicals (Kyoto, Japan) and were used without further purification. Water was distilled twice in an all-glass apparatus.

Methods

Preparation of brain extract. Male albino rats (Wistar strain) weighing *ca.* 300 g were killed by microwave irradiation (4.5 kW, 2.0 sec) of the head using a microwave applicator (Muromachi Kikai, Tokyo, Japan). Brains were removed and their cerebral cortex, hippocampus, striatum, midbrain including hypothalamus, pons-medulla oblongata and cerebellum were separated essentially according to Glowinski and Iversen[19].

Tissues (50–200 mg) were homogenized in 4.0 ml of 3% perchloric acid containing 0.2% L-ascorbic acid and 0.2% disodium ethylenediaminetetraacetic acid using a Polytron (Kinematica) operating at the maximum setting for 20 sec at 0°. The precipitate was removed by centrifugation (10,000 *g*, 15 min, at 4°) and the supernatant was adjusted to pH 5.8–6.2 by careful addition of 4.0 or 0.1 *N* KOH in an ice-bath. Potassium perchlorate was removed by centrifugation (10,000 *g*, 15 min, at 4°) and the supernatant was applied to a column of Amberlite CG-50 (9.5 × 0.4 cm I.D. with a 10-ml reservoir), which had been equilibrated with 0.1 *M* sodium phosphate buffer, pH 6.0.

Separation of catecholamines and serotonin on Amberlite CG-50. The column was washed with 10 ml of water. The breakthrough fraction and the washing contained acidic and neutral compounds. After further washing with 3.5 ml of 0.01 *M* sodium phosphate buffer, pH 6.0, containing 1.5% boric acid, noradrenaline and adrenaline were eluted with 3.5 ml of the same solution (noradrenaline fraction) and then dop-

amine was eluted with 3.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.0, containing 4.0% boric acid (dopamine fraction). The column was then washed with 4.0 ml of 0.1 *N* HCl, and serotonin was finally eluted with 2.0 ml of 1.0 *N* HCl. Sodium phosphate buffers (0.01 and 0.1 *M*) containing boric acid were prepared by mixing NaH_2PO_4 (0.01 and 0.1 *M*) with NaOH (0.01 and 0.1 *M*) both containing boric acid.

Determination of noradrenaline and dopamine. Noradrenaline and dopamine were analyzed fluorometrically by a modification of the ethylenediamine condensation method. The noradrenaline fraction (3.5 ml) was mixed successively with 0.5 ml of 10% ethylenediamine (adjusted to pH 9.5 with HCl), 0.25 ml of 2.0 *N* KOH and 0.1 ml of 10% potassium ferricyanide (freshly prepared) the dopamine fraction (3.0 ml) was mixed with 0.5 ml of ethylenediamine solution (pH 9.5), 0.25 ml of 4.0 *N* KOH and 0.1 ml of 0.5% potassium ferricyanide. The mixture was heated in a water-bath at 70° for 10 min (for noradrenaline determination) or at 60° for 10 min (for dopamine determination) and then cooled in tap water for 10 min. Then the fluorophores were extracted with 3.0 ml of isobutanol and the fluorescence of the isobutanol phase was measured with a Hitachi Model MPF-2A fluorospectrophotometer at excitation wavelengths of 410 and 400 nm and emission wavelengths of 510 and 500 nm for noradrenaline and dopamine, respectively. A tissue blank was prepared by the same procedure, but with 0.1 ml of 1.0% L-cysteine instead of potassium ferricyanide.

Determination of adrenaline. For the application of this method to the samples containing substantial amounts of adrenaline compared with noradrenaline, such as adrenal medulla, heart, spleen or urine, etc., adrenaline in the noradrenaline fraction was determined with the trihydroxyindole method[20] at pH 3.5. To 3.5 ml of the noradrenaline fraction, 0.1 ml of 1.0 *N* acetic acid and 0.05 ml of 0.25% potassium ferricyanide were added, and after keeping the mixture for 3–5 min at room temperature, 2.0 ml of 0.05% L-ascorbic acid in 5.0 *N* NaOH were added and the fluorescence was measured within 10 min at 540 nm using an excitation wavelength of 405 nm. With this method 20 ng of adrenaline could be measured.

The fluorescence in the noradrenaline fraction resulting from the ethylenediamine condensation is derived from both noradrenaline and adrenaline fluorophores. To calculate the noradrenaline content in the noradrenaline fraction, the fluorescence of the adrenaline fluorophore must be subtracted from that of the noradrenaline fraction.

Determination of serotonin. Serotonin was determined by the acidic *o*-phthalaldehyde method[21,22]. To 2.0 ml of the serotonin fraction, 10 μl of thioglycolic acid, 0.1 ml of 0.04% *o*-phthalaldehyde in ethanol and 1.0 ml of conc. sulphuric acid were added. The mixture was heated in boiling water for 15 min, then cooled in tap water for 30 min. Its fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 480 nm. A tissue blank was prepared by adding 20 μl of 0.5% $NaIO_4$ before the reaction.

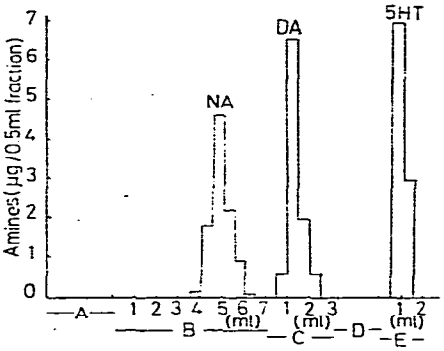
Determination of protein. Protein concentration was determined by the method of Lowry *et al.*[23] using bovine serum albumin as a standard.

RESULTS

Separation of noradrenaline, dopamine and serotonin on Amberlite CG-50

Fig. 1 shows the chromatogram of noradrenaline, dopamine and serotonin on

the Amberlite CG-50 column. When the ionic strength of the elution buffer was increased, dopamine was eluted too close to noradrenaline to give good separation. Use of phosphate buffers containing borate in place of aqueous boric acid solution reduced the elution volume of each fraction by more than half. The best separation was obtained at pH 6 between pH 5.8 and 6.8. Change in the ionic strength of the sample applied had the same effect as change in ionic strength of the equilibration buffer, but the addition of 0.2 mmole of NaCl to tissue extract did not affect the separation of noradrenaline and dopamine. The separation of noradrenaline and dopamine was complete and 1 μg of noradrenaline or dopamine did not affect the determination of 10 ng of another catecholamine. The recoveries of authentic 100 ng samples of noradrenaline, dopamine and serotonin added to the homogenate were 85 $\pm$ 1.5, 87 $\pm$ 2 and 82 $\pm$ 2% (mean $\pm$ S.E.M., $n = 6$) respectively.



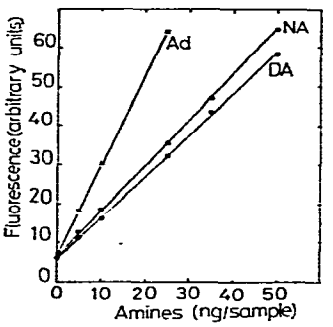


Fig. 1. Elution profile of noradrenaline (NA), dopamine (DA) and serotonin (5HT) from a short column of Amberlite CG-50. Ten micrograms of each amine were added to the tissue homogenate. Deproteinization, neutralization with KOH and elution from the column were carried out as described under *Methods*. Solutions used for elution: A, 10 ml of water; B, 0.01 *M* sodium phosphate buffer, pH 6.0, containing 1.5% boric acid; C, 0.1 *M* sodium phosphate buffer, pH 6.0, containing 4.0% boric acid; D, 4 ml of 0.1 *N* HCl; E, 1 *N* HCl.

Fig. 2. Standard curves for noradrenaline (NA), adrenaline (Ad) and dopamine (DA) determined by the modified ethylenediamine condensation method. The condensation reaction and fluorometric determination were as described under *Methods*. Adrenaline was treated similarly to noradrenaline.

Determination of catecholamines by a modification of the ethylenediamine condensation method

The standard curves for noradrenaline, adrenaline and dopamine determined by the modified ethylenediamine condensation method are shown in Fig. 2. The fluorescence intensities obtained with 5 ng samples of noradrenaline and dopamine were trice that of the blank, therefore 5 ng of these amines could be measured.

The ethylenediamine condensation product of dopamine was readily oxidized. Thus, when the incubation period was longer than 10 min, the sensitivity decreased and the standard curve became non-linear. The fluorophores of noradrenaline and adrenaline were more stable than that of dopamine. After cooling in tap water and extraction into isobutanol, the fluorophores of noradrenaline and dopamine were stable for at least 1 h. The tissue blank prepared as described under *Methods*, had the

same intensity as the reagent blank. Table I shows the effects of boric acid and potassium ferricyanide on the sensitivity of the ethylenediamine condensation method. As in boric acid solution, catecholamines were stabilized and were hardly oxidized even at higher pH, when potassium ferricyanide was used as oxidant. The fluorescence intensity of the ethylenediamine condensation product of dopamine became as high as that of noradrenaline in the presence of potassium ferricyanide. This enhancement of the fluorescence in the absence of boric acid has been described by Westerink and Korf[24]. Other oxidants, such as manganese dioxide and sodium periodate, did not improve the sensitivity. With potassium ferricyanide, the optimum pH of the reaction for noradrenaline and dopamine was 9.5 and that for adrenaline was 11.0.

TABLE I

EFFECTS OF BORIC ACID AND POTASSIUM FERRICYANIDE ON THE SENSITIVITY OF A MODIFIED ETHYLENEDIAMINE CONDENSATION FOR CATECHOLAMINE ASSAY

Conditions for ethylenediamine condensation without both boric acid and $K_3Fe(CN)_6$; pH 10.4; ethylenediamine concentration 1.3%; incubation time 30 min and incubation temperature 60°. When $K_3Fe(CN)_6$ and/or boric acid was present, the concentrations of both compounds were the same as those described in the text and reaction pH was 9.5. The fluorophores were extracted with isobutanol and the fluorescence of the organic phase was measured. The excitation (Ex) and emission (Em) wavelengths are given.

Catecholamine	*Fluorescence intensities (%)**				*Ex (nm)*		*Em (nm)*	
	Borare − $K_3Fe(CN)_6$ −	*Borate − $K_3Fe(CN)_6$ +*	*Borate + $K_3Fe(CN)_6$ −*	*Borate + $K_3Fe(CN)_6$ +*	−	+	−	+
Adrenaline	100	102	34	108	420	420	530	530
Noradrenaline	65	67	15	68	420	410	490	510
Dopamine	30	58	10	61	420	400	520	500

* The fluorescence intensity of adrenaline in the absence of both boric acid and potassium ferricyanide was taken as 100%.

Fluorescence spectra of the fluorophores

The fluorescence spectra of noradrenaline and dopamine fluorophores obtained by the present method were different from those obtained by the usual ethylenediamine condensation method[25–27]: the excitation and emission peaks of the fluorophore of noradrenaline were at 410 and 510 nm, and those of the fluorophore of dopamine were 400 and 500 nm, respectively (Fig. 3). On the other hand, the fluorescence spectrum of the adrenaline fluorophore did not change when potassium ferricyanide was used.

Amine contents in discrete regions of rat brain

Table II shows the contents of these amines in six discrete regions of rat brain, as determined by the present method. Tissue concentrations of each amine were calculated using 100 ng amounts of the amines added to the homogenate as internal standards. Noradrenaline and serotonin were found in all regions and dopamine in all regions except the hippocampus and cerebellum.

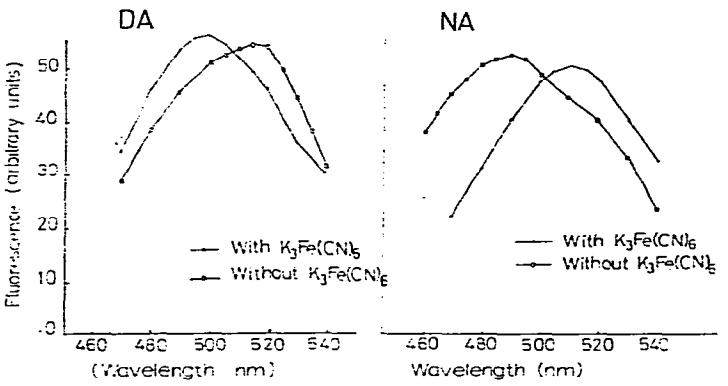


Fig. 3. Fluorescence spectra of ethylenediamine condensation products of noradrenaline (NA) and dopamine (DA), showing the fluorophores produced in the presence and absence of potassium ferricyanide. Without ferricyanide, the condensation reaction was carried out in 1.3% ethylenediamine solution (adjusted to pH 10.4 with HCl), at 60° for 30 min. When ferricyanide was used, the reaction conditions were as described in the text. After incubation, the fluorophores were extracted with isobutanol and the fluorescence spectra were recorded with a Hitachi MPF-2A fluorospectrophotometer. The excitation wavelengths are described in Table I.

TABLE II

CONCENTRATIONS OF NORADRENALINE, DOPAMINE AND SEROTONIN IN DISCRETE REGIONS OF RAT BRAIN

The figures represent ng/mg protein, mean ± S.E.M. (n = 5). N.D. = Not detected by our method, *i.e.*, less than 5 ng per sample.

Brain region	*Noradrenaline*	*Dopamine*	*Serotonin*
Cerebral cortex	2.68 ± 0.51	4.17 ± 0.78	4.44 ± 0.29
Hippocampus	2.26 ± 0.35	N.D.	4.23 ± 0.24
Striatum	1.80 ± 0.33	75.3 ± 10.0	6.24 ± 0.50
Midbrain + hypothalamus	5.43 ± 0.20	2.60 ± 0.56	7.28 ± 0.34
Pons-medulla oblongata	5.14 ± 0.27	0.65 ± 0.26	5.17 ± 0.42
Cerebellum	1.89 ± 0.19	N.D.	1.77 ± 0.37

DISCUSSION

Various methods have been developed for simultaneous determination of noradrenaline, dopamine and serotonin in discrete regions of the brain. Solvent extraction, alumina adsorption and cation-exchange chromatography have been used for separation and purification of amines for fluorometric analysis[1–11]. Although good separation of amines on a Dowex 50 column was reported by Atack and Magnusson[11], the use of strongly acidic cation exchangers involves strong acid and/or organic solvents for elution, which are unsuitable for unstable biogenic amines and which result in high blank values on fluorometry. Therefore, we used a weak acidic cation exchanger for separation and purification of biogenic amines.

With the procedure described in this paper it is possible to elute the three main biogenic amines in small volumes well separated from each other. The ethylenediamine condensation method gives more stable fluorephores than the trihydroxyindole method for noradrenaline and the dihydroxyindole method for dopamine[28–31], but

it has not often been used because it is less pescific and less convenient than the other two methods. In our method, acidic and neutral catechol compounds are not retained on the column, and the dopamine fraction is essentially pure, but the noradrenaline fraction contains adrenaline in addition to noradrenaline. Since the adrenaline content of rat brain is negligible compared with the noradrenaline content[32], the fluorescence of the noradrenaline fraction was considered to represent that of noradrenaline in the brain; for analysis of adrenaline in brain, the noradrenaline fraction should be assayed by a procedure that distinguishes a low concentration of adrenaline from noradrenaline[16,33].

In our procedure, we increased the sensitivity of the ethylenediamine condensation method in borate solution by using potassium ferricyanide, which produced fluorophores different from those obtained by the usual ethylenediamine condensation method. The chemical structures of the fluorophores were not identified, but the following evidence suggests that the fluorophores were condensation products of aminochrome derivatives and ethylenediamine. (i) Oxidation of catecholamines with potassium ferricyanide before condensation with ethylenediamine gave the same fluorophores as formed in our method. (ii) Pyrocatechol does not give an aminochrome derivative, but yields the same fluorophore as that of noradrenaline in the usual ethylenediamine condensation method[34]. In our method, pyrocatechol gave the same fluorophore as that of the usual ethylenediamine condensation method, and the fluorophore of pyrocatechol was very unstable to oxidation. (iii) Adrenaline yielded the same fluorophore in the presence or absence of potassium ferricyanide. This fluorophore is known as a condensation product of adrenochrome and ethylenediamine[34].

Our method has several advantages over other procedures for fluorometric determination of noradrenaline, dopamine and serotonin in the same sample: (1) amines are separated from their precursors and metabolites on a single column; (2) amines are separated from each other in small volumes of eluents with low blank fluorescence; (3) noradrenaline and dopamine are highly purified in a single step, and both amines are determined with the same reagents and with comparable sensitivity; (4) 10 ng of each amine can be determined accurately; (5) the fluorophores of all amines are fairly stable, so that a large number of samples can be handled at the same time; (6) the method is fairly rapid.

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