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MODIFIED ETHYLENEDIAMINE CONDENSATION METHOD AND ITS APPLICATION IN THE ANALYSIS OF CATECHOLAMINES BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A modified ethylenediamine condensation method is described in which catecholamines dissolved in a borate buffer are converted into fluorescent products by oxidation with hexacyanoferrate(III) in the presence of ethylenediamine under alkaline conditions. The method was applied successfully in the fluorimetric determination of catecholamines eluted from a column of weakly acidic ion-exchange resin with a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediaminetetraacetate. An analysis of human urine for catecholamines is described.

INTRODUCTION

The ethylenediamine condensation method has been widely used for the determination of catecholamines[1]. The method has been automated[2], and coupled with a high-speed liquid chromatograph[3–5].

On the other hand, an ion-exchange chromatographic method for the separation of catecholamines was developed in which a boric acid-containing buffer was used as the eluent and a weakly acidic ion-exchange resin as the stationary phase[6]. An attempt was made to measure fluorimetrically catecholamines in an eluate from the ion-exchange column using the ethylenediamine condensation method, but dopamine and norepinephrine could be determined only with low sensitivity.

Since this failure seemed to be due to a low rate of oxidation of dopamine and norepinephrine by air oxygen in the presence of borate ion, oxidation of catecholamines with a solution of hexacyanoferrate(III) in the presence of ethylenediamine was tried with success. The modified ethylenediamine condensation method could be automated and used to measure catecholamines eluted from a column of Amberlite IRC-50 with a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediaminetetraacetate. The determination of catecholamines extracted from human urine is also described in this paper.

EXPERIMENTAL

Reagents

Epinephrine hydrogen tartrate was purchased from Nakarai Pharmaceutical Co. (Kyoto, Japan) and norepinephrine hydrogen tartrate, dopamine hydrochloride and ethylenediamine dihydrochloride from Yashima Pharmaceutical Co. (Osaka, Japan). Other chemicals used were of reagent grade. Stock solutions of catecholamine base were prepared in 0.01 *M* hydrochloric acid. Fluorescent impurities contained in ethylenediamine dihydrochloride were removed by passing a 0.5 *M* solution of ethylenediamine dihydrochloride through a column of activated charcoal (15 × 1.5 cm).

Equipment

A syringe-type pump (Jasco, Model FLC-150) was used to pump buffer solution through the chromatographic columns. Peristaltic pumps (Mitsumi Scientific Industry, Models SJ-1211 H and L) were used to pump air and reagents into a gas-segmented-flow reaction detector. A spectrofluorimeter (Jasco, Model FP-4) equipped with a flow cell prepared from a quartz tube (4 mm I.D.) was used to measure fluorescence. pH was measured at 20° using a TOA Electronics (Tokyo, Japan) Model HM-5A glass electrode pH meter.

Human urine

Daytime specimens of urine were acidified immediately after collection by adding 1% (v/v) of 6 *M* hydrochloric acid and stored in a refrigerator. Just before analysis, they were filtered.

Preparation of Amberlite CG-50 column

Amberlite CG-50 (type II) was converted into the Na^+ form and graded according to size by the sedimentation method[7]. The fraction of size range 85–120 μm was collected and washed on a glass filter with ten volumes of the following reagents in turn: 4 *M* hydrochloric acid, water, 1 *M* sodium hydroxide solution, water and 0.4 *M* phosphate buffer (pH 6.5). The buffered resin was poured into a tube with phosphate buffer of pH 6.5 (0.4 *M*) and allowed to settle under gravity to a height of 12 cm (the tube was 20 × 0.5 cm I.D. with a 10-ml reservoir); the column was washed with 2 ml of water before use[8].

Preparation of Amberlite IRC-50 column

Amberlite IRC-50 (40–55 μm and 50–60 μm in the Na^+ form) was prepared and washed as described previously[9], and suspensions of the washed resins (Na^+ form) were buffered at pH 6.3 with a succinic acid solution (0.5 *M*) and then washed with eluent. The eluent was a mixed buffer of pH 6.3 containing 0.12 *M* succinic acid, 0.35 *M* boric acid and 0.002 *M* disodium ethylenediaminetetraacetate. The washed resin was suspended in 2 volumes of the eluent. The suspension of the finer resin was poured into a short chromatographic tube (15 × 0.8 cm), and a suspension of the coarse resin was poured into a longer tube (24 × 0.8 cm) and allowed to settle under gravity. Then both chromatographic tubes were fitted with a column adjuster and the bottom of the short tube was connected to the longer one. The temperature of the column was kept at 42° and, after the eluent had been pumped through the

column at a rate of 0.75 ml/min for several hours, the height of the resin column in the top tube was 9 cm and in the bottom tube 19 cm.

Separation of catecholamine fraction from human urine

A 4-ml portion of filtered urine was mixed with 0.5 ml of a 5% solution of disodium ethylenediaminetetraacetate and 0.5 ml of a 1% solution of ascorbic acid, and the pH of the mixture was adjusted to 6.1–6.2 with 1 *M* sodium hydrogen carbonate solution. The mixture was then applied to an Amberlite CG-50 column, the column was washed with 6 ml of deionized water and then with 2 ml of 2/3 *M* boric acid solution, then a further 2 ml of the boric acid solution were used to elute catecholamines from the column, the eluate being collected in a test-tube containing 0.07 ml of 0.5 *M* sodium dihydrogen phosphate solution in 1 *M* hydrochloric acid. The eluate in the test-tube was adjusted to pH 6.3 with a solution of 0.5 *M* sodium dihydrogen phosphate in 1 *M* hydrochloric acid and diluted to 4 ml with a succinate buffer of pH 6.3 (succinic acid 0.12 *M*, disodium ethylenediaminetetraacetate 0.002 *M*). The catecholamine fraction was stored in a refrigerator.

Chromatographic separation of samples

A 1-ml volume of a solution of the amines in the eluent to be used for chromatography or 1.0 ml of a catecholamine fraction prepared as described above was added to the column of Amberlite IRC-50. The sample was forced into the column by an air pressure of 1 kg/cm^2, then elution was carried out with the eluent at a flow-rate of 0.75 ml/min.

Fluorimetric determination of catecholamines

A gas-segmented-flow reaction detector was assembled from commercial parts and Pyrex coils. The Pyrex coils were made by winding 4-mm Pyrex tubing around a brass tube of 14 mm O.D. As shown in Fig. 1, eluate from a column was fed to the detector and segmented by air, mixed with 0.5 *M* ethylenediamine dihydrochloride (mixing coil, 10 turns), 1.5 *M* sodium hydroxide (mixing coil, 8 turns), 0.3% (w/v) hexacyanoferrate(III) (mixing coil, 10 turns), heated at 75° (heating coil,

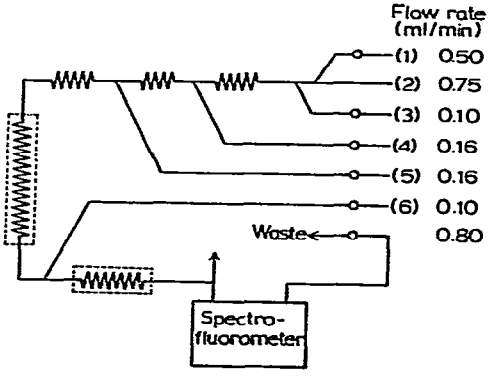

Fig. 1. Schematic diagram of the gas-segmented-flow reaction detector. The pH of the waste was 9.5. (1) Air; (2) eluate; (3) 0.5 *M* ethylenediamine dihydrochloride; (4) 1.5 *M* NaOH–0.1% (w/v) Triton X-405; (5) 0.3% (w/v) hexacyanoferrate(III); (6) 10% (w/v) $NaSO_3 \cdot 7H_2O$.

30 turns) and finally mixed with a 10% (w/v) solution of sodium sulphite heptahydrate and warmed at 42° (heating coil, 15 turns). Then bubbles were removed from the stream and the fluorescence was measured at 510 nm, with excitation at 400 nm.

RESULTS AND DISCUSSION

Three catecholamines could be separated from each other and determined fluorimetrically within 70 min (Fig. 2). The elution pattern was reproducible and the peak heights of 120 ng each of epinephrine, norepinephrine and dopamine were 71.8 ± 0.76, 72.4 ± 0.74 and 48.5 ± 0.89, respectively (five determinations). As shown in Fig. 3, the catecholamine fraction prepared from human urine gave three

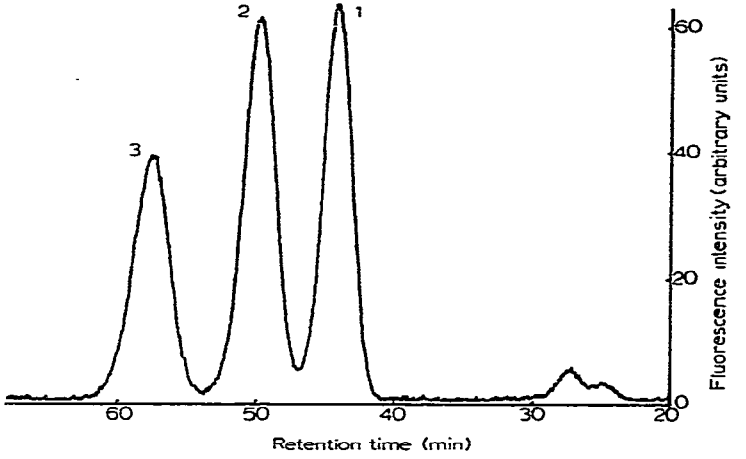

Fig. 2. Elution and fluorimetric determination of catecholamines. A mixture of 100 ng each of epinephrine (1), norepinephrine (2) and dopamine (3) was separated on the column of Amberlite IRC-50 and determined using the detector described in Fig. 1.

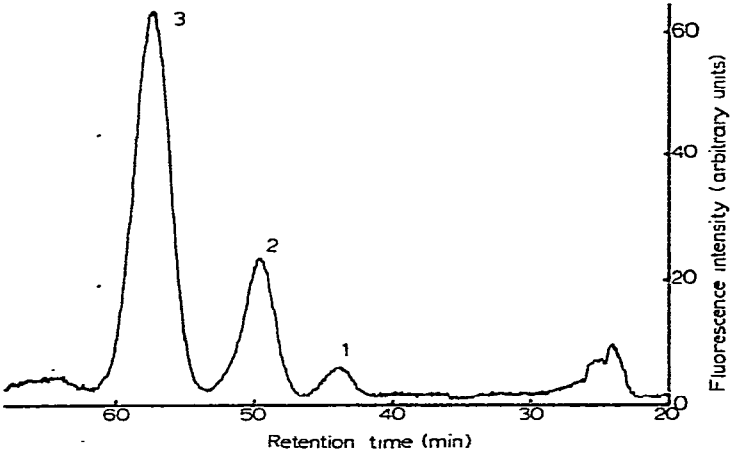

Fig. 3. Elution and fluorimetric determination of catecholamine fraction prepared from human urine. Retention times of peaks (1), (2) and (3) corresponded to those of epinephrine, norepinephrine and dopamine, respectively.

peaks corresponding to epinephrine, norepinephrine and dopamine. Based on six determinations of 4-ml aliquots of the same human urine sample, the mean amounts of epinephrine, norepinephrine and dopamine per millilitre of urine were calculated to be 6.9 ng ± 0.42, 34.1 ng ± 1.6 and 151 ng ± 6.3, respectively. When 100 ng of epinephrine, 200 ng of norepinephrine and 400 ng of dopamine were added to a urine sample before the procedure described above and one quarter of the catecholamine fraction of each sample was analysed, the amounts recovered were 23.5 ng ± 2, 46.4 ng ± 2.3 and 90.8 ng ± 10.2, respectively (five determinations).

A mixed buffer containing a lower concentration of boric acid (0.35 *M*) than the boric acid solution (2/3 *M*) was used as the eluent, because catecholamines are specifically retarded with an eluent containing lower concentration of boric acid, and their separation from impurities will be improved with such an eluent.

A linear relationship between peak height and the amount of amines added to the column was obtained over the range 5–2000 ng for norepinephrine and dopamine and 5–200 ng for epinephrine, when the amines were oxidized and subjected to reaction with ethylenediamine under alkaline condition (pH 9.5, Fig. 4). When catecholamines were treated under less alkaline or neutral conditions, the relative peak heights of these amines changed, as shown in Fig. 5. Although the peak heights of catecholamines were higher at pH 8.65 than at pH 9.5, the dynamic range of norepinephrine was narrower at pH 8.65.

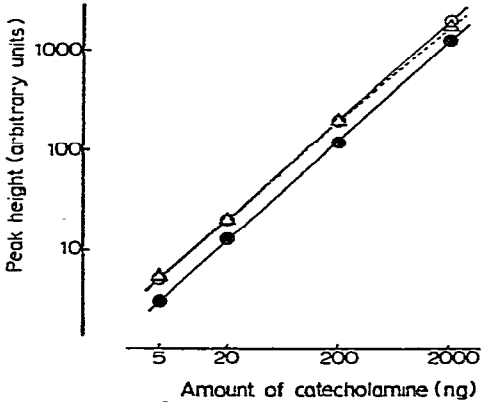

Fig. 4. Relationship between peak height and amount of catecholamines. Catecholamines were separated on the column of Amberlite IRC-50 and determined by using the detector described in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

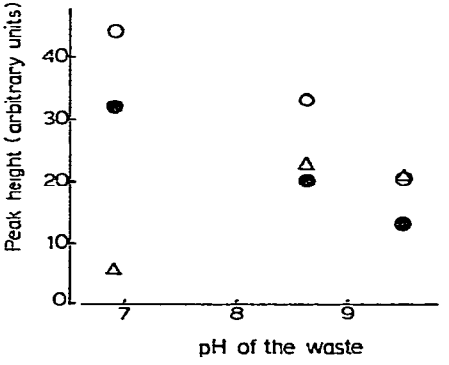

Fig. 5. Relationship between pH of the reaction mixture and the peak height of catecholamines. Catecholamines (20 ng of each) were separated and detected as in Fig. 4; 0.05, 0.75 or 1.5 *M* sodium hydroxide solution was used to keep the pH of the waste at 6.9, 8.65 or 9.5. Other conditions as in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

Oxidation of catecholamines with hexacyanoferrate(III) in the presence of ethylenediamine under alkaline condition was preferable to oxidizing them at neutral pH prior to the addition of ethylenediamine and sodium hydroxide solution, because the latter reaction sequence yielded narrower dynamic ranges for dopamine and norepinephrine. With a different batch of ethylenediamine dihydrochloride, the

relationship of peak height to the amount of catecholamines added to the column changed to some extent, but the dynamic range of catecholamines remained unchanged. The concentration of hexacyanoferrate(III) used to oxidize catecholamines was 0.3%, but epinephrine and norepinephrine could be determined with higher sensitivity by oxidizing them with a more concentrated solution of hexacyanoferrate(III) (Fig. 6). Excess of hexacyanoferrate(III) had to be reduced, because it absorbs the exciting light, and the time for reduction was 2.5 min. The time for the reaction of ethylenediamine with the oxidation products of catecholamines could be reduced to 5 min by keeping the temperature of the reaction coil at 75° and the total time of mixing and reaction was 15 min. Therefore, the modified ethylenediamine condensation method described here can be conveniently utilized for the on-line determination of catecholamines in an eluate from a chromatographic column.

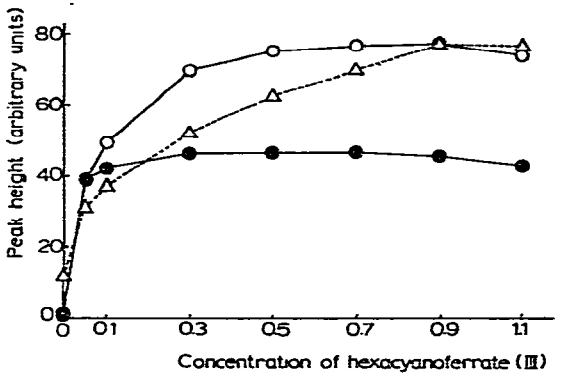

Fig. 6. Relationship between peak height and concentration of hexacyanoferrate(III) (%, w/v) used to oxidize catecholamines. Catecholamines (100 ng of each) were separated and detected as in Fig. 4. A different batch of ethylenediamine was used in this experiment, and except for the concentration of hexacyanoferrate(III), the reaction conditions were as described in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

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REFERENCES

1 H. Weil-Malherbe and A. D. Bone, *Biochem. J.*, 51 (1952) 311.
2 J. K. Viktora, A. Baukal and F. W. Wolff, *Anal. Biochem.*, 23 (1968) 513.
3 A. Kojima-Sudo, *Ind. Health*, 12 (1974) 153.
4 K. Mori, *Jap. J. Ind. Health*, 17 (1975) 170.
5 G. Schwedt, *Chromatographia*, 10 (1977) 92.
6 T. Seki, *J. Chromatogr.*, 124 (1976) 511.
7 P. Decker and H. Höller, *J. Chromatogr.*, 7 (1962) 392.
8 T. Seki and H. Wada, *J. Chromatogr.*, 114 (1975) 227.
9 T. Seki and K. Matsumoto, *J. Chromatogr.*, 27 (1967) 423.