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## Note

# Chromatographic separation of catecholamines on a weakly acidic ion-exchange resin

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Ion-exchange chromatography of catecholamines has been achieved on both weakly acidic and strongly acidic ion exchangers by using buffer solutions or dilute hydrochloric acid as eluent[1–4]; selective elution of catecholamines from a column of weakly acidic cation-exchange resin with boric acid solution has also been reported[5–8].

On the other hand, epinephrine has been separated from norepinephrine by using a chromatographic system developed for separating the dansyl derivatives of amino acids and of amines. This system involves a mixture of citrate buffer (pH 5.60, 0.1 *M*) and organic solvents as eluent and buffered Amberlite IRC-50 as the stationary phase[9]. To improve this system, the effects of various mixtures of buffer and organic solvents on the separation of epinephrine from norepinephrine were tested, with buffered Amberlite IRC-50 as the stationary phase. The best separation was achieved by using an eluent of acetate buffer (pH 5.60, 0.2 *M*) containing boric acid ($\frac{2}{3}$ *M*) and ethylenediaminetetra-acetic acid (5 m*M*) mixed with *tert.*-butanol, tetrahydrofuran and 2,2′-thiodiethanol in proportions of 800:160:40:1 by volume.

## EXPERIMENTAL

### *Materials*

Epinephrine hydrogen tartrate was purchased from Nakarai (Kyoto, Japan), and norepinephrine hydrogen tartrate, isoproterenol hydrochloride and dopamine hydrochloride were purchased from Yashima (Osaka, Japan); other compounds and organic solvents were of reagent grade, and 2,2′-thiodiethanol was of the grade used with amino acid analyzers. Stock solutions of catecholamines corresponding to 1 mg of the catecholamine base per ml were prepared in 0.01 *M* hydrochloric acid.

### *Human urine*

Daytime specimens of urine were acidified immediately after collection by adding 1 % (by volume) of 6 *M* hydrochloric acid and stored in a freezer; just before analysis, they were thawed and filtered.

### *Ion-exchange resin*

Amberlite IRC-50 (A.G.) was pulverized, graded according to size and

TABLE I

COMPOSITION OF ELUENTS

| *Eluent* | *Composition* | *Proportions* |
|---|---|---|
| A | Citrate buffer, pH 5.60 (0.1 *M*)–tetrahydrofuran–ethyl methyl ketone–acetone | 14:1:3:3 |
| B | As for eluent A | 20:1:2:4 |
| C | Mixed buffer, pH 5.60 (0.2 *M* acetic acid, $\frac{1}{3}$ *M* boric acid, 5 m*M* ethylenediaminetetra-acetic acid)–*tert.*-butanol–tetrahydrofuran–2,2'-thiodiethanol | 800:160:40:1 |

washed as described previously[10]; the fractions of size ranges 40–55 and 60–70 $\mu$m in the wet $Na^+$ form were used. Suspensions of the washed resin ($Na^+$ form) were buffered at pH 5.60 with citric acid or acetic acid solution and then washed with the eluent described in Table I.

Amberlite GC-50 (type II) was converted into the $Na^+$ form and graded according to size by the sedimentation method[11]; the fraction of size 85–120 $\mu$m was collected, washed as described previously and buffered at pH 6.50 by washing it with 0.4 *M* phosphate buffer of pH 6.50 (see refs. 5 and 10).

*Preparation of columns*

*Amberlite IRC-50 columns.* After being washed with the eluent described in Table I, the buffered resin, with the eluent used to equilibrate the resin, was poured into a column and allowed to settle under gravity. Columns packed with resin equilibrated with eluent A or B could be used without further treatment; those packed with the smaller sized particles of resin were washed with 500 ml of eluent C under an air pressure of 1 kg per $cm^2$ at 32°. The column dimensions are shown in Table II.

*Amberlite CG-50 column.* The buffered resin was poured into a tube with phosphate buffer of pH 6.50 (0.4 *M*) and allowed to settle under gravity to a height of 12 cm (the tube was 20 cm long × 0.5 cm I.D., with a 15-ml reservoir); the column was washed with 2 ml of water before use.

*Chromatographic separation of synthetic mixtures*

*Eluent A or B.* A 0.3-ml sample of the amines in the eluent to be used for chromatographic separation was applied to the column of Amberlite IRC-50, elution was carried out, and the eluate was collected in fractions of 14 drops each. To each

TABLE II

CONDITIONS FOR CHROMATOGRAPHIC SEPARATION ON AMBERLITE IRC-50

In all instances, the column length was 260 nm.

| *Figure* | *Eluent* | *Column diameter (mm)* | *Particle size of resin (μm)* | *Temperature of column (°C)* | *Flow-rate (ml/h)* |
|---|---|---|---|---|---|
| 1a | A | 5 | 60–70 | 22 | — |
| 1b | B | 5 | 60–70 | 22 | — |
| 2 | C | 8.7 | 40–55 | 32 | 14 |
| 3 | C | 8.7 | 40–55 | 32 | 14 |

fraction were added 2 ml of carbonate buffer (1 *M*, pH 10.0) and 0.2 ml of Folin–Ciocalteu phenol reagent, and, after 2 h, the absorbance of each fraction was measured at 600 nm.

*Eluent C.* Stock solutions of each amine were mixed and diluted with the eluent to give an amine-base concentration of 1–3 $\mu$g per ml. Then 1.5 ml of the solution was mixed with 0.8 ml of buffer solution of pH 5.60 (acetic acid 0.2 *M*, boric acid $\frac{2}{3}$ *M* and ethylenediaminetetra-acetic acid 5 m*M*), and the mixture was applied to the Amberlite IRC-50 column. The sample was forced into the column by air pressure at 1 kg/cm$^2$, then elution was carried out with eluent C at 32° under the same pressure. The flow-rate was 14 ml per h and the eluate was collected in 1.3-ml fractions. Each fraction was mixed with 0.2 ml of the eluent and 0.6 ml of 1 *M* hydrochloric acid, and the fluorescence was measured at 315 nm, with excitation at 280 nm, by using a Hitachi fluorescence spectrophotometer (model MPF-2A).

*Separation of a catecholamine fraction from human urine and its chromatographic analysis*

A 5-ml portion of filtered urine was mixed with 0.2 ml of a 5% solution of disodium ethylenediaminetetra-acetate, and the pH of the mixture was adjusted to 6.5 with concentrated carbonate solution[12]; 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 $\frac{2}{3}$ *M* boric acid solution, then another 2 ml of the boric acid solution were used to elute catecholamines from the column, the eluate being collected in a test-tube containing 0.05 ml of 2 *M* acetic acid. The adsorption, washing and elution was performed at 10–15°. The eluate in the test-tube (pH 6.2–6.4) was adjusted to pH 5.5 with 2 *M* acetic acid and mixed with 0.3 ml of a mixture of *tert.*-butanol, tetrahydrofuran and 2,2'-thiodiethanol (160:40:1 by volume); the solution was then applied to the column of Amberlite IRC-50, and catecholamines were eluted with eluent C as described above.

## RESULTS AND DISCUSSION

Separation of norepinephrine from dopamine was unsatisfactory with eluent

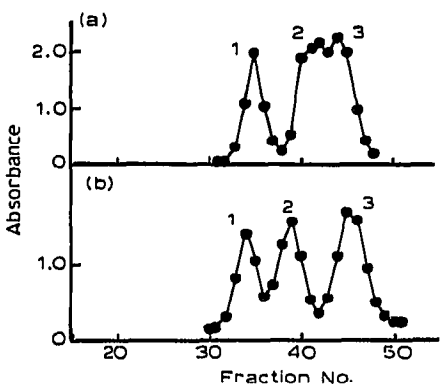
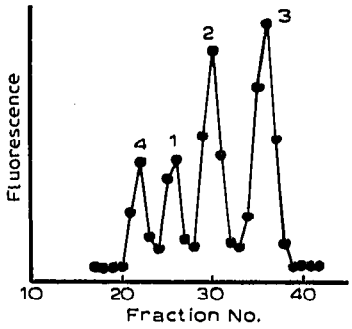



Fig. 1. Elution of catecholamines under the conditions given in Table II. The peaks are those of epinephrine (1), norepinephrine (2) and dopamine (3).



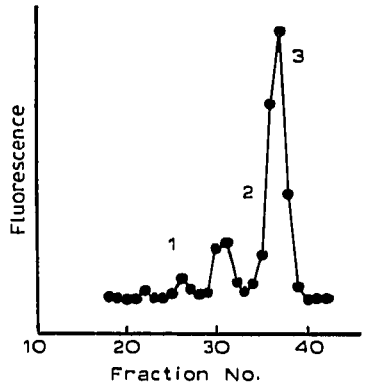




Fig. 2. Elution of catecholamines under the conditions given in Table II. The peaks are those of isoproterenol (4), epinephrine (1), norepinephrine (2) and dopamine (3).

Fig. 3. Elution pattern of the catecholamine fraction from human urine under the conditions given in Table II. The elution volumes of the peaks numbered 1, 2, and 3 coincided with those of epinephrine, norepinephrine and dopamine, respectively.

A (see Fig. 1a), even when the ratio of components in eluent A was changed to 14:1:2:4. Eluent B, which contained less organic solvent than eluent A, gave better separation of these amines (Fig. 1b). However, separation of epinephrine from norepinephrine was unsatisfactory with an eluent that did not contain any organic solvent, so the content of organic solvent in the eluent was varied in order to find conditions for optimum separation of the catecholamines. Ethyl methyl ketone and acetone could be replaced by *tert.*-butanol, and incorporation of borate into the buffer enabled elution of the catecholamines in smaller volumes of eluate without adverse effect on their separation (see Fig. 2). Tetrahydrofuran was included in the eluent in order to prevent formation of bubbles in the column, and 2,2'-thiodiethanol was added to decompose peroxide present in tetrahydrofuran. Under these conditions, recovery of catecholamines from the column was satisfactory and the column could be used repeatedly.

Catecholamines were extracted from human urine by a modification of the method of Routh *et al.*[7]; by using a column of smaller diameter (5 mm) and greater length (12 cm), it was possible to elute catecholamines adsorbed to the resin with 2 ml of $\frac{2}{3}$ *M* boric acid solution. The catecholamine fraction obtained could be applied directly to the Amberlite IRC-50 chromatographic column after adjusting its pH and adding organic solvent. As shown in Fig. 3, three peaks corresponding to epinephrine, norepinephrine and dopamine were obtained. Based on 8 determinations on 5-ml aliquots of the same human urine sample, the mean quantities of epinephrine, norepinephrine and dopamine were calculated as 85 ng $\pm$ 10 (S.D.), 306 ng $\pm$ 36 (S.D.) and 1600 ng $\pm$ 76 (S.D.), respectively. When 400 ng each of epinephrine and norepinephrine, and 1.2 $\mu$g of dopamine were added to urine samples before the procedure described above, the respective recoveries were 94 $\pm$ 6% (S.D.), 95 $\pm$ 5% (S.D.) and 92 $\pm$ 5% (S.D.) (8 determinations).

The special features of the proposed method are as follows: (1) the use of an eluent containing boric acid permits application of the complete catecholamine fraction from 5 ml of human urine to the analytical column, and (2) catecholamines are at no time exposed to a high pH.

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