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SEPARATION AND DETECTION OF SMALL AMOUNTS OF CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The effects of mobile phase pH, flow-rate, column temperature, etc., on the separation of catecholamines by high-performance liquid chromatography (HPLC) on Zipax SCX and Zorbax ODS columns were investigated. Fluorimetric detection methods based on post-column reaction using trihydroxyindole and *o*-phthalaldehyde methods were employed and the results are compared. Optimal reaction conditions ensuring a high sensitivity of the fluorimetric detection were established. Based on the results, a new HPLC system utilizing the trihydroxyindole method has been produced on a trial basis. The favourable detection limits (*ca.* 20–30 pg) achieved permit highly accurate and selective assays for noradrenaline and/or adrenaline in tissues and urine.

INTRODUCTION

Various methods have been used to analyse biological samples for catecholamines. In those methods, the trihydroxyindole (THI) method developed by Lund[1] has been improved by many workers[2–8] and established as a reliable fluorescence analysis technique, which is now widely employed in the clinical field. The catecholamine components are determined without prior separation; they are treated as a mixture and each component is determined from the difference in the yield with suitable selection of pH and type of oxidizing agent, or from the difference in the fluorescence excitation and emission wavelengths. This method, therefore, has the disadvantage that coexisting substances that are detected by the THI method will decrease the quantitative reliability of the data. Mori[9–12] separated catecholamines by high-performance liquid chromatography (HPLC) and determined them with a fluorescent detector by adding THI reagent to the effluent continuously, and demonstrated that the influence of coexisting substances that are detected by this method cannot be neglected. HPLC, which is used extensively for the separation and identification of coexisting compounds, can minimize the influence of the coexisting components and contamination of the catecholamines and hence improve the analytical accuracy.

With the high sensitivity required for the detection of the small amounts of catecholamines in blood and tissues, it is necessary to improve the analytical condi-

tions; for instance, important factors include the optimal operating conditions for separating, optimization of the reaction conditions for the post-column reaction and a high sensitivity of the fluorimetric detector.

In this work we have investigated the operating conditions for separating catecholamines on Zipax SCX and Zorbax ODS columns, the relationship between the sensitivity of detection and the intervals of the reaction process (in post-column reaction), and the choice between non-dispersive and spectrophotometric types of fluorescence detectors. We also investigated a prototype instrument that was produced on the basis of the results. By using this instrument, the levels of catecholamines in blood and tissues could be detected readily.

For compounds with a primary amino group, sensitive post-column reaction has been achieved by fluorimetric detection using *o*-phthalaldehyde reagent. We have also studied this method which can be used for the analysis of catecholamines containing a primary amino group such as noradrenaline.

EXPERIMENTAL

Equipment

The operating conditions were initially investigated by using a Shimadzu–DuPont LC-1 Pr high-performance liquid chromatograph, which was equipped with a UV detector and a column oven. The sample inlet was a SIL-1A variable injector, which can inject samples of volume 1–200 μl by means of a microlitre syringe without stopping the stream of the mobile phase and without the use of a septum.

Reaction/detection unit for THI method

Fig. 1 is a flow diagram of the prototype instrument based on the THI method. Air bubbles and reaction reagents were added to the effluent from the separation column by means of a PRR-2A liquid pump, which feeds reagents by squeezing simultaneously several Tygon tubes (Norton, Akron, Ohio, U.S.A.).

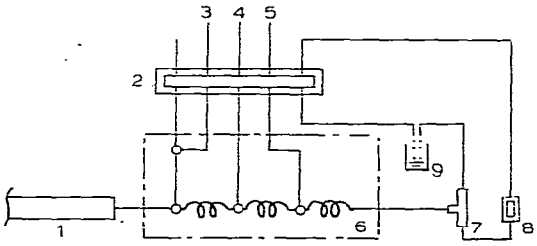

Fig. 1. Flow diagram for THI method. 1 = Column; 2 = squeezing pump; 3, 4 and 5 = reservoirs (reaction reagent); 6 = chemical reaction box; 7 = removal of air bubbles; 8 = fluorescence detector; 9 = waste.

The reaction reagents and the effluent were mixed for reaction in the Pyrex glass coil of the CRB-2A chemical reaction box, which had been specially designed for our experiments. The length of this glass coil, which would determine the reaction time, was decided after repeated experiments.

The sample components, which had been derivatized into fluorescent substances, were allowed to flow into an FLD-2A non-dispersive fluorescence detector or an RF-500LCA fluorescence spectromonitor, which was specially adjusted to ensure low noise levels for the analysis of catecholamines.

Reaction/detection unit for o-phthalaldehyde method

As the reaction based on the *o*-phthalaldehyde method proceeds very rapidly, taking less than 20 sec in contrast to about 4 min required by the THI method, the construction of the reaction unit is different from that for the THI method. Fig. 2 shows the flow diagram. As the reaction reagents can be mixed to give a single solution, prior to the operation, a compact plunger-type liquid pump was used to feed the reaction reagent mixture. A CRB-1B chemical reaction box equipped with a temperature controller was used to mix the reagent with the effluent from the LC column. The sample components that had been derivatized into fluorescent compounds were detected by an FLD-1 non-dispersive fluorescence detector.

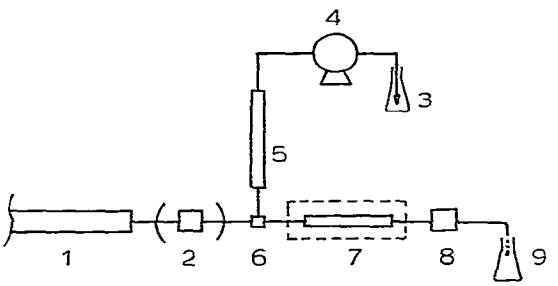

Fig. 2. Flow diagram for *o*-phthalaldehyde method. 1 = Column; 2 = detector (*e.g.*, UV); 3 = reservoir (reaction reagent); 4 = pump; 5 = resistor; 6 = mixing cell; 7 = chemical reaction box; 8 = fluorescence detector; 9 = waste.

Preparation of mobile phase and reaction reagent

Sodium dihydrogen orthophosphate (NaH_2PO_4) and disodium hydrogen orthophosphate (Na_2HPO_4) of special grade were used as the mobile phases for the Zipax SCX and Zorbax ODS columns (DuPont, Wilmington, Del., U.S.A.), respectively. The pH values of these solvents were adjusted by adding orthophosphoric acid or perchloric acid.

A Horiba (Kyoto, Japan) pH meter (precision ± 0.01 unit) was used for pH measurements.

Preparation of reaction reagent for THI method

The fractions from the LC column are first oxidized, using a 0.05–0.1 % solution of potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$. The time required for this oxidation depends on pH, and it is therefore necessary to add buffer solution. This buffer solution was prepared to contain potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate in the proportions 1:1 to 1:2. It is also necessary to add a reducing agent before the alkalinization process in order to stabilize the fluorescent substances produced, and a 0.05–0.1 % solution of ascorbic acid was

used for this purpose. Ascorbic acid itself, however, also decomposes gradually into a fluorescent substance, which will increase the noise level and decrease the signal-to-noise ratio, so it is necessary to use a freshly prepared solution. Sodium hydroxide solution was used for the alkalinization process.

Preparation of reaction reagent for o-phthalaldehyde

A 0.08% solution of *o*-phthalaldehyde containing a small amount of 2-mercaptoethanol was used as the reaction reagent[13]. As this reagent reacts with primary amino radicals at pH >8, it is necessary to adjust the pH of the solution to a suitable value. For this adjustment, a buffer solution consisting of 0.3–0.4 *M* boric acid (H_3BO_3) and sodium hydroxide was used. The buffer solution was used to dissolve the reaction reagent. The pH was measured with a pH meter.

All of these reagents were purchased from Wako (Osaka, Japan).

RESULTS AND DISCUSSION

Operating conditions for separating catecholamines

Separation with Zipax SCX column

Separation with a Zipax SCX column (1 m × 2.1 mm I.D.), which is a strongly acidic cation exchanger of the pellicular type, was investigated. The column was packed by the slurry method at *ca.* 400 $kg \cdot cm^{-2}$. The typical separation on this column is that 3,4-hydroxyphenylalanine (DOPA) is eluted first, then noradrenaline (NA, also called norepinephrine), adrenaline (A, also called epinephrine) and dopamine (DA) in that order. The operating conditions were selected so as to give the best separation for DOPA and NA and for A and DA, both pairs of which are difficult to separate.

Effect of column temperature. The effect of column oven temperature (T) is shown in Fig. 3. The salt concentration and the pH of the mobile phase were kept constant. The retention times decrease with increase in column oven temperature and the retention times vary linearly with $1/T$. The separation factor, R_s, decreases slightly with increase in column temperature. The retention times of DOPA and NA, which are eluted early, do not change noticeably with change in the column oven temperature, but the retention times of A and DA are temperature dependent.

The analytical time when using a Zipax SCX column is dependent on the column temperature: when the column temperature is low, the analytical time will be long, whereas when the column oven temperature is high, analyses can be carried out rapidly. When an HPLC instrument with a hot air circulation system is used, it is best to set the column oven temperature at 40°, because the ambient temperature may be as high as 30° in summer even when the column oven heater is left turned off.

Effect of salt concentration in the mobile phase. In the separation of catecholamines on a Zipax SCX column, the concentration of the salt in the mobile phase has a considerable effect on the capacity factor {k' = [(retention time) − (non-sorbed time)]/(non-sorbed time}, to the same extent as in analyses with another cation-exchange column. The separation is more dependent on the salt concentration than on the column temperature.

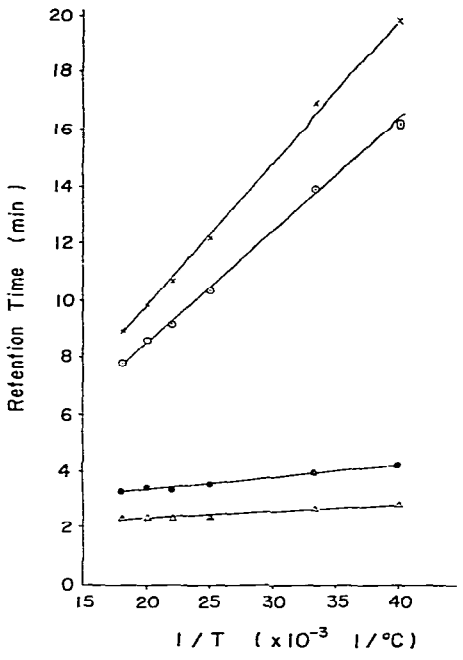

Fig. 3. Effect of column temperature. Column, Zipax SCX (1 m × 2.1 mm I.D.); mobile phase, 0.08 *M* NaH_2PO_4 (pH 4.3); detector, UV photometer (254 nm); flow-rate, 0.56 ml·min^{-1}. Sample: △, DOPA; ●, noradrenaline; ○, adrenaline; ×, dopamine.

The relationship between the capacity factor and the salt concentration (with the other operating conditions, such as the column oven temperature, pH and flow-rate, being kept constant) is presented in Fig. 4. When the salt concentration is low, the analytical time is long and good resolution between A and DA can be achieved. On the other hand, when the salt concentration is high, the analysis can be carried out rapidly but the resolution is poor. From these results, a mobile phase containing about 0.1 g-ion·l^{-1} of Na^+ is preferred in this instance.

Effect of flow-rate. The effects of the flow-rate of the mobile phase on the number of theoretical plates and the resolution are shown in Fig. 5. The results indicate that in order to obtain a resolution of $R_s = 1.0$ for A and DA, it is necessary to adjust the flow-rate to below about 0.65 ml·min^{-1}. It can be concluded that when 0.1 *M* sodium dihydrogen orthophosphate solution (pH 4.3) is used as the mobile phase, the optimal flow-rate is about 0.6 ml·min^{-1}.

When the amount of one of the sample components is extremely small, and a higher separation efficiency is required, it is better to change the flow-rate. The broken line in Fig. 5 shows how the number of theoretical plates changes with the flow-rate: it decreases with an increase in flow-rate, as expected from the theory[14].

Effect of pH of the mobile phase. A Zipax SCX column can be used with a mobile phase within the pH range 2–9, and the effect of pH on the separation was studied in this range. The pH was adjusted by adding perchloric acid to the phosphate, the Na^+ concentration of which had been adjusted to be constant. No noticeable changes in the retention times or the separation factor were observed. From these

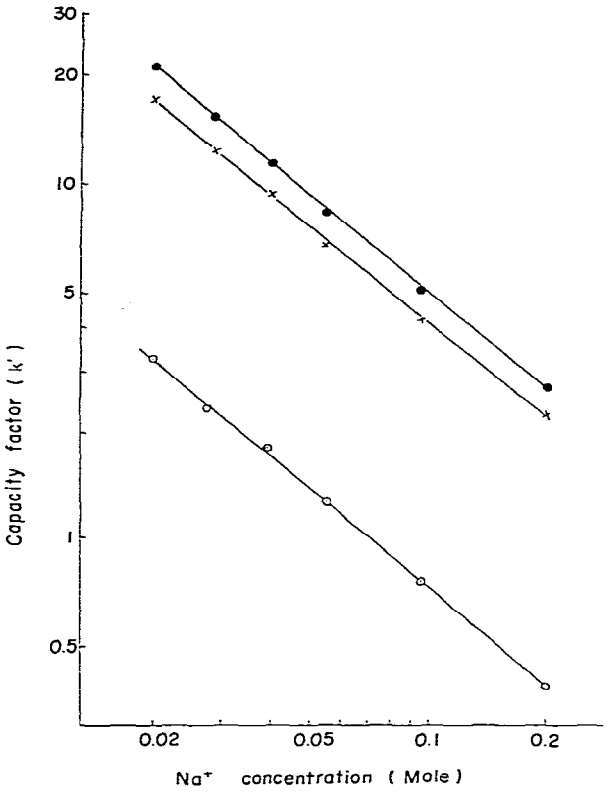

Fig. 4. Effect of salt concentration in mobile phase. Column, Zipax SCX; flow-rate, 0.55 ml·min^{-1}; column temperature, 40°. ●, Dopamine: ×, adrenaline: ○, noradrenaline.

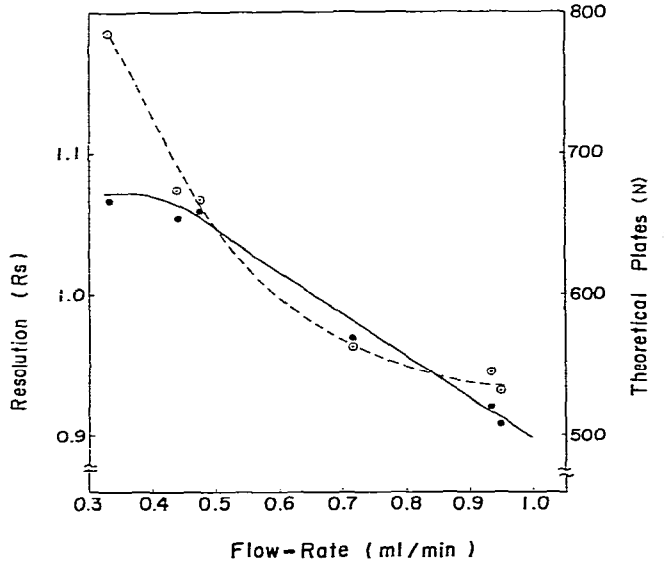

Fig. 5. Effect of flow-rate on resolution (R_s) between adrenaline and dopamine (solid line) and (b) the number of theoretical plates for adrenaline (broken line). Column, Zipax SCX; mobile phase, 0.1 M NaH_2PO_4 (pH 4.3); column temperature, 40°.

results, a mobile phase of any pH value in the acidic region can be used successfully in the analysis of catecholamines with a Zipax SCX column. It follows that a solution of the commonly used sodium dihydrogen orthophosphate, having buffering properties, can be used as the mobile phase (pH 4.3).

Separation with Zorbax ODS column

A Zorbax ODS column can also be used to separate catecholamines, using an aqueous solution as the mobile phase. No effects of salt concentration in the mobile phase were observed. Experiments were carried out with a 15 cm × 4.6 mm I.D.

Effect of column temperature. The relationship between column temperature and retention time is shown Fig. 6. The results show that the retention time decreases considerably with increase in column temperature, and the separation of A and DOPA was particularly affected, the components being hardly separated at 55°. To improve the separation, it is necessary to keep the column at a low, constant temperature, preferably 20--25°.

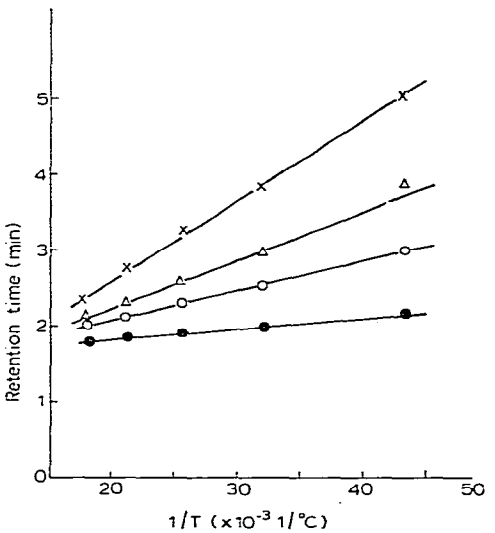

Fig. 6. Effect of column temperature. Column, Zorbax ODS (15 cm × 4.6 mm I.D.); mobile phase, 0.1 *M* NaH_2PO_4 (pH 3.15); flow-rate, 0.72 ml·min^{-1}. Symbols as in Fig. 3.

Effect of pH of the mobile phase. The relationship between the pH of the mobile phase and the retention time is shown in Fig. 7. The retention times of NA and A hardly change with changes in the pH of the mobile phase greatly, being eluted very early when the pH is high. When the pH is lower than 4.5, DOPA is eluted between A and DA; at a pH above 4.5, it is eluted between NA and A. A mobile phase with a pH of about 3 is preferable for the analysis of these four components.

Therefore, in the analysis of catecholamines with a Zorbax ODS column, 0.1 *M* phosphate solution, having buffering properties, is used as the mobile phase, to adjust the pH to about 3 and to keep the column at a low, constant temperature.

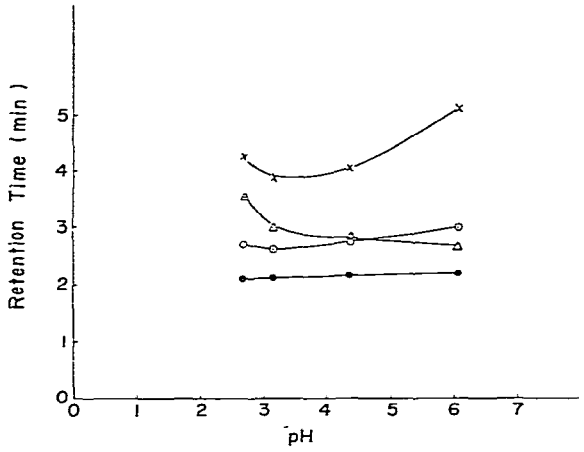

Fig. 7. Effect of pH of mobile phase. Column, Zorbax ODS; mobile phase, 0.1 *M* NaH_2PO_4 (pH 3.0); flow-rate, 0.7 ml·min^{-1}; column temperature, 31°. Symbols as in Fig. 3.

Optimal column and operating conditions for the HPLC system

The number of theoretical plates of a Zorbax ODS column for the separation of catecholamines is higher than that of a Zipax SCX column. In the Zorbax ODS column, however, it is difficult to separate the peaks from the region of the solvent front, and the peaks cannot be separated from those of extraneous components in some instances. On the other hand, optimal operating conditions can be chosen with the Zipax SCX column. On the basis of the results, we chose a Zipax SCX column for use in the new HPLC system.

The optimal operating conditions for such a column, of dimensions 1 m × 2.1 mm I.D., are as follows: a 0.1 *M* solution of sodium dihydrogen orthophosphate (pH 4.3) as the mobile phase, a column oven temperature of 40° and a flow-rate of about 0.6 ml·min^{-1}.

Reaction and detection

Reaction and detection in THI method

Oxidation. The times necessary for the oxidation of catecholamines, adrenaline and noradrenaline, for example, are dependent on pH and also on the type of oxidizing agent used. The oxidizing agent used in our work was potassium hexacyanoferrate(III), which oxidizes catecholamines almost completely within 2 min at a pH of about 6. An excess of potassium hexacyanoferrate(III) (0.1 % solution) is added to a phosphate buffer solution (about pH 7) to adjust the pH at the time of reaction to about 6. Even when this potassium hexacyanoferrate(III) solution was left for a few days, the baseline stability was not affected.

Reduction. If potassium hexacyanoferrate(III), which is an oxidizing agent, should be present when catecholamines are alkalinized, it would decompose the fluorescent substances produced. For stabilization, ascorbic acid solution was used as the reducing agent. It is better to adjust its concentration to be in excess of that of the potassium hexacyanoferrate(III) solution. Our experiments indicated that it

is best to adjust the concentration of the potassium hexacyanoferrate(III) solution to about twice that of the ascorbic acid solution.

Alkalinization. To convert the oxidized catecholamines into fluorescent substances, it is necessary to alkalinize them, for which purpose we used 4 *N* sodium hydroxide solution.

Peak spreading. As the reaction based on the THI method is completed in only about 4 min and the inner diameter of the Pyrex glass coil was restricted to 1.6 mm by a squeezing pump, undesirable broadening of the peaks during the reaction will occur. Therefore, air bubbles were mixed with the stream, taking care not to broaden the peaks. Fig. 8a shows that the air bubbles mixed at the column outlet were removed just before entering the detector cell. The results obtained in the absence of bubbles under the same conditions are shown in Fig. 8b. The spread of the faster eluted peaks was reduced considerably by the air bubbles.

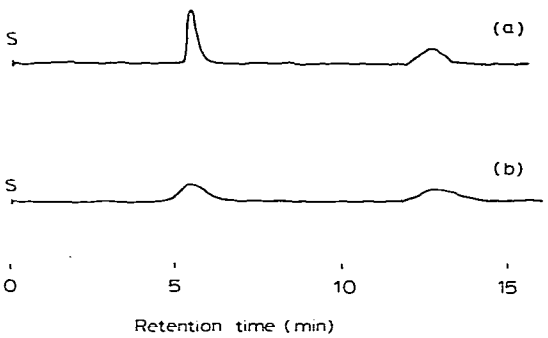

Fig. 8. Effect of air bubbles. (a) Air bubbles mixed with the mobile phase; (b) air bubbles not mixed with the mobile phase. Column, Zipax SCX; mobile phase, 0.1 *M* NaH_2PO_4; flow-rate, 0.6 $ml \cdot min^{-1}$; column temperature, ambient; detection, THI method.

Detection. The fluorescent substances produced as described above were measured with a non-dispersive fluorescent detector or a fluorescence spectromonitor. The volume and the shape requirements of the flow cell were satisfied by using a 120-μl square cell, based on a comparison of some different flow cells, *e.g.*, 17-μl cylindrical and square cells, 50-μl cells, etc. It was found in the measurement of the excitation and emission spectra of A and NA that the optimal excitation and emission wavelengths are 410 and 510 nm, respectively. In the non-dispersive detector, the excitation is effected with a low-pressure mercury lamp with a peak wavelength at about 410 nm and selected by an optical filter. The emission light is filtered in order to cut off wavelengths shorter than about 460 nm.

Reaction and detection in the o-phthalaldehyde method

The reaction of HPLC fractions with *o*-phthalaldehyde is greatly dependent on pH, as shown in Fig. 9. DOPA is the most pH dependent; its reaction is the greatest at pH 10. The reaction rates of NA and DA are almost constant over the pH range 8.8–10.6. The optimal reaction, therefore, is obtained by adjusting the pH to about 10.

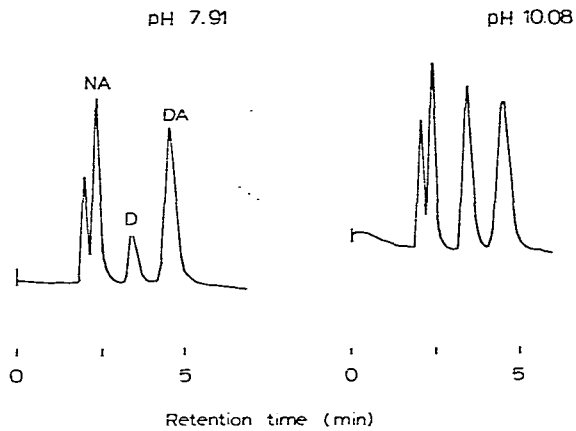

Fig. 9. Effect of pH in the reaction system of the *o*-phthalaldehyde method. Column, Zorbax ODS; mobile phase, 0.1 *M* NaH_2PO_4 (pH 3.0); flow-rate, 0.7 ml·min^{-1}; column temperature, 31°; detection, *o*-phthalaldehyde method.

In the measurement of excitation and emission spectra, the excitation is effected with a low-pressure mercury lamp with a peak wavelength at about 360 nm and the emission light is filtered in order to cut off wavelengths shorter than about 430 nm.

HPLC system using THI method

A prototype of the new HPLC system for the THI method has been produced, based on the above results. A schematic diagram is shown in Fig. 10. The equipment for the stepwise gradient consisted of a motor, valve, timer, etc., which changed the mobile phase from one to a maximum of six. The liquid pump 1 was a reciprocating pump, in which the volume of the cylinder was *ca.* 100 μl. The column oven utilized the hot air circulation system, and was set at 40°. The liquid pump-2 was a PRR-2A, which squeezed simultaneously four Tygon tubes of I.D. 1/32 in. and one of I.D. 1/16 in. The same amounts of potassium hexacyanoferrate(III), ascorbic acid, sodium hydroxide solution and air bubbles were squeezed into the chemical reaction coil. The air bubbles and part of the liquid were removed just before the flow cell. The residual liquid passed through the flow cell and was subsequently returned to

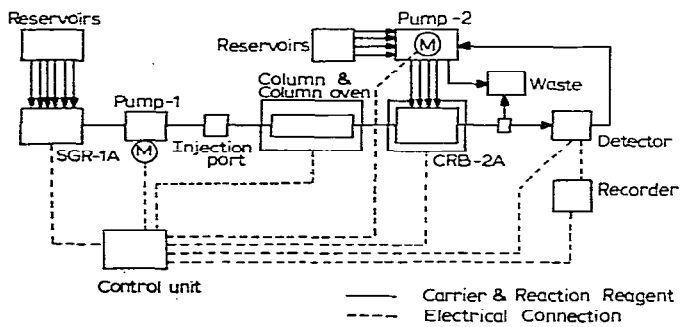

Fig. 10. Schematic diagram of the new HPLC system using the THI method.

the PRR-2A pump. The CRB-2A chemical reaction box consisted of a Pyrex glass coil (I.D. 1.6 mm), Teflon tubing, etc. The intervals of each reaction were determined by the length of the coil. A non-dispersive FLD-2A fluorescent detector was used. All of the instruments were controlled by a control unit.

Application

A typical chromatogram for a catecholamine sample obtained by using the new HPLC system is shown in Fig. 11. The calibration graphs for NA and A under the same operating conditions have a good linearity range and the detection limits were a few tens of picograms (Fig. 12). These results seem to be good enough for the

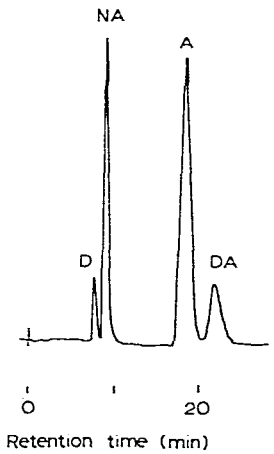

Fig. 11. Typical chromatogram for catecholamines using the new HPLC system. Column, Zipax SCX; mobile phase, 0.1 *M* NaH_2PO_4 (pH 4.3); flow-rate, 0.6 ml·min^{-1}; column temperature, 40°.

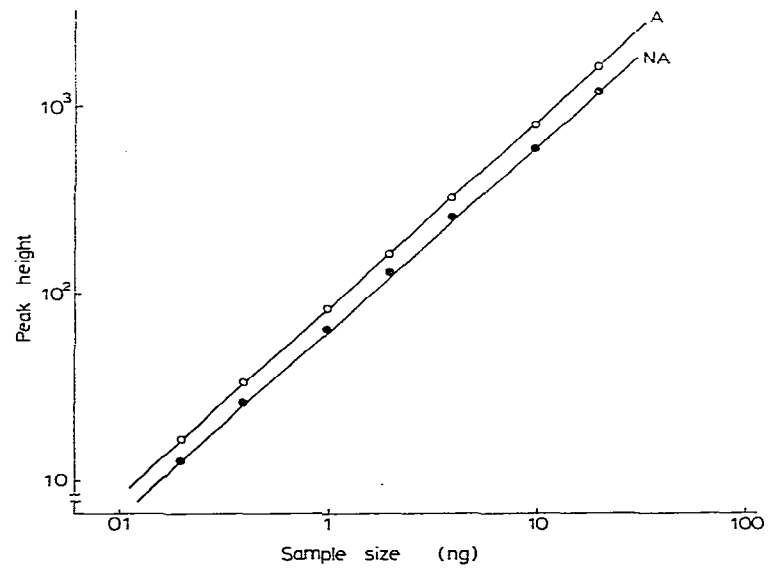

Fig. 12. Calibration graphs for noradrenaline and adrenaline. Operating conditions as in Fig. 11.

TABLE I

REPRODUCIBILITY OF PEAK AREA AND RETENTION TIME

Run No.	*Noradrenaline*		*Adrenaline*	
	Retention time (min)	*Peak area*	*Retention time (min)*	*Peak area*
1	4.89	7266	7.19	27042
2	4.82	6730	7.16	26796
3	4.92	6613	7.22	27308
4	4.91	7244	7.21	26720
5	4.91	6684	7.21	27557
6	5.04	6878	7.44	27842
7	4.98	6823	7.28	27281
8	4.92	6534	7.22	27181
9	4.93	7173	7.23	27996
10	4.91	7060	7.21	26933
Average	4.923	6900.5	7.237	27266
Coefficient of variation	1.10%	3.70%	1.02%	1.48%

analysis of biological samples. Table I shows the reproducibility of the retention time and peak area obtained by using a data processor (Chromatopac-E1A). The coefficient of variation of the peak area for NA is 3.7%, which seems to be the cause of the overlap of the NA and solvent front peaks.

As an example of an application, Fig. 13 shows the chromatogram for catecholamines of sample A, extracted from the cerebra of rats. The pre-treatment before the injection was based on the well known method using adsorption on alumina[15]. The amount of NA in a half of a cerebrum was about 250 ng.

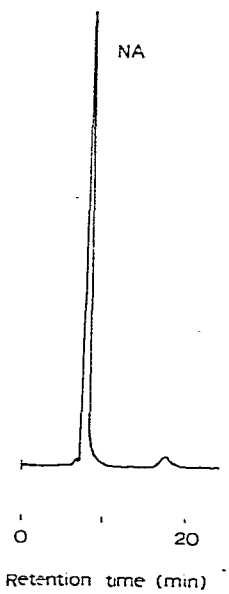

Fig. 13. Separation of sample A using the THI method. Operating conditions as in Fig. 11. Sample: extract from the cerebra of rats.

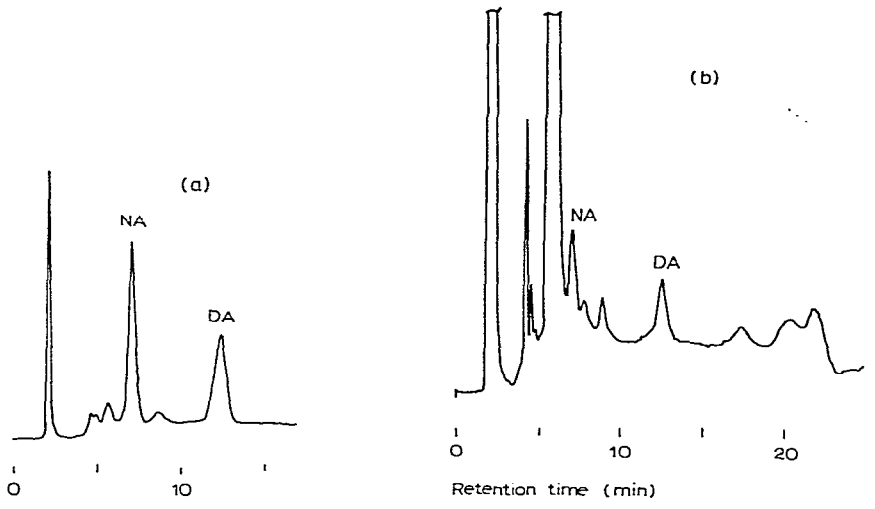

Fig. 14. Separation of sample B using the *o*-phthalaldehyde method. Column, Zipax SCX; mobile phase, gradient system [final concentration 0.5 *M* NaH_2PO_4 (pH 4.3)]; flow-rate, 0.6 ml·min^{-1}; column temperature, 40°; detection, *o*-phthalaldehyde method.

Fig. 14 shows the separation of similar cerebra of rats using the *o*-phthalaldehyde method. In this method, peaks of coexisting components appear. This seems to indicate that the THI method is more selective than the *o*-phthalaldehyde method. It also indicates that higher sensitivity and accuracy in the analysis of catecholamines are possible with the THI method. In the detection of the DA peak, however, the *o*-phthalaldehyde method is more favourable. In Fig. 14, the detection limits of the catecholamines with the *o*-phthalaldehyde method are NA 0.1 ng and DA 0.5 ng. These results indicate a higher sensitivity than that in the work of Froehlich and Cunningham[16], who obtained detection limits of NA and DA of 7.5 and 9.3 ng, respectively.

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