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NOVEL POST-COLUMN DERIVATIZATION METHOD FOR THE FLUORIMETRIC DETERMINATION OF NOREPINEPHRINE AND EPINEPHRINE

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SUMMARY

A novel method is described in which catecholamines are converted into fluorescent products by heating in alkaline borate buffer. The method was applied to the determination of norepinephrine and epinephrine after separation by high-performance liquid chromatography using a pellicular, strong cation exchanger. The new system is simpler than the system based on the trihydroxyindole reaction. It is suitable for the measurement of catecholamines in the range 0.25—20 ng. The assay of catecholamines in human urine is also described.

INTRODUCTION

Among the chromatographic methods for the determination of catecholamines, high-performance liquid chromatography (HPLC) [1—12] has acquired special importance. Several specific and sensitive reactions [13—16] are readily applicable to the determination of these amines in the eluate. The post-column derivatization system using the trihydroxyindole (THI) reaction [1—4,11,12], which has been most widely used for this purpose, is rather complicated. It consists of three steps, namely oxidation, termination of the oxidation reaction, and rearrangement of the oxidation product under alkaline conditions. Several workers [17—23] have proposed pre-column derivatization using fluorescamine [17—20] or dansyl chloride [21—23]. Although these methods require only simple instruments, pre-column reactions can be tedious. Accordingly, a simple and rapid post-column derivatization method is more suitable for routine use.

The present paper describes a novel post-column reaction in which catecholamines are heated in alkaline borate buffer to give intensely fluorescent products. This procedure, which we named the "borate method", was applied to the determination of norepinephrine and epinephrine after separation on a

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simple prototype high-performance liquid chromatograph. The borate method showed a simplicity and speed comparable with that of the electrochemical method.

MATERIALS AND METHODS

Reagents and standards

Norepinephrine, epinephrine, dopamine hydrochloride and DOPA were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents and alumina (300 mesh) were obtained from Wako (Osaka, Japan). Stock solutions of the catecholamines were prepared by dissolving each amine in 0.01 *M* hydrochloric acid at 100 μg/ml, and appropriate dilution with 0.25 *M* aqueous acetic acid before use.

The mobile phase consisted of 50 m*M* monobasic sodium phosphate solution containing 50 ml of acetonitrile per liter. The reagent for post-column derivatization was 0.5 *M* borate buffer, adjusted to pH 9.7 with sodium hydroxide. All solutions used for the chromatographic studies were filtered through a 0.22-μm micro filter (Fuji Photo Film Co., Tokyo, Japan) and de-gassed.

Separation of the catecholamine fraction from human urine

Daytime specimens of human urine were acidified immediately after collection by adding 1% (v/v) of 6 *M* hydrochloric acid, and stored in a refrigerator [15]. A 5-ml aliquot of the acidified urine was placed in a test-tube and mixed with 1 ml of a 7.5% (w/v) solution of disodium ethylenediaminetetraacetate, and the pH of the mixture was adjusted to 8.5 with 1 *N* sodium hydroxide. To the solution were added 250 mg of alumina, which had been previously treated with 2 *N* hydrochloric acid [24], and the resulting mixture was shaken with an automatic mixer for 5 min. After the mixture had been allowed to stand for 5 min, the supernatant was removed by decantation, and the alumina was washed with three 5-ml portions of water. The washed alumina was then filled into a glass column 4 mm in diameter, and eluted with 0.25 *M* aqueous acetic acid solution; 2.5 ml of the eluate were collected and stored in a refrigerator. A 100-μl aliquot of this solution was injected into the chromatograph.

Chromatographic system

Fig. 1 shows the flow diagram of the chromatographic system. A double plunger pump (Sanuki Industry Co., Tokyo, Japan) was used. Each plunger served independently to deliver the mobile phase and the post-column reagent at constant flow-rates of 0.8 ml/min and 0.4 ml/min, respectively. The mobile phase was pumped through a valve universal injector (Sanuki Industry Co., Tokyo, Japan). A Zipax SCX column (1 m $\times$ 2.1 mm I.D.; DuPont Instruments, Wilmington, MA, U.S.A.) was used for the separations. It was operated at 40°C utilizing a Taiyo thermo unit C-600 (Taiyo Scientific Industry Co., Tokyo, Japan). The column eluate was mixed with the reagent in a mixing T-piece and a PTFE-tubing reaction coil (10 m $\times$ 0.5 mm I.D.) immersed in a water-bath of 75 $\pm$ 0.1°C.

Fluorescence intensity of the effluent was measured at 490 nm; excitation of fluorescence was achieved at 400 nm, using an RF-500 LCA spectrofluoromon-

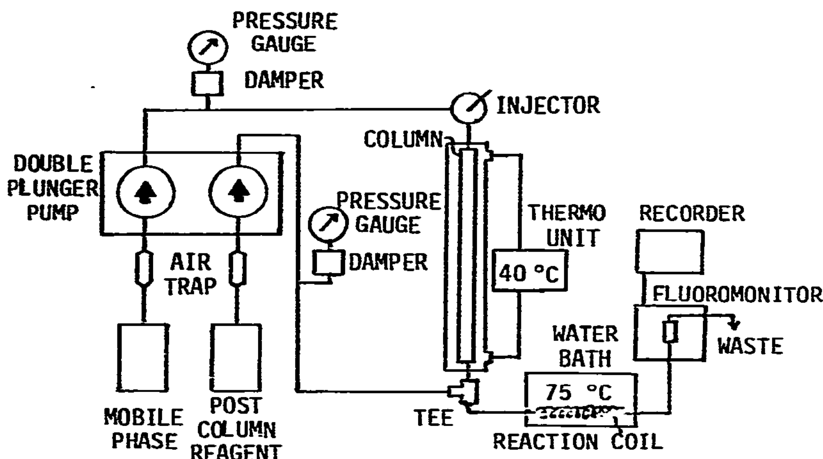

Fig. 1. Flow diagram of the HPLC system used for the separation and fluorescence detection of norepinephrine and epinephrine. Mobile phase, 50 m*M* monobasic sodium phosphate containing 50 ml of acetonitrile per liter (flow-rate, 0.8 ml/min); post-column reagent, 0.5 *M* borate buffer, pH 9.7 (flow-rate, 0.4 ml/min).

itor (Shimadzu Seisakusho, Kyoto, Japan) equipped with a xenon discharge lamp.

Excitation and fluorescence spectra were taken with an RF-510 spectrofluorometer (Shimadzu Seisakusho) which also served for the manual examination of reaction conditions (Fig. 2).

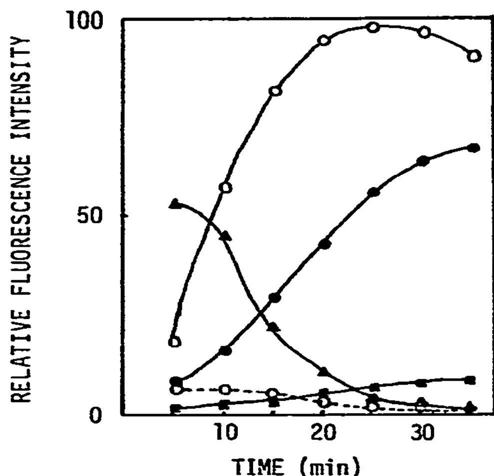

Fig. 2. Reaction conditions for the development of fluorescent products from epinephrine by heating in alkaline buffers. Fluorescence intensity is plotted against reaction time. To 1 ml of 100 ng/ml epinephrine, 3 ml of the buffers listed below were added and the resulting mixture was heated at 60°C for the periods plotted on the abscissa in a thermostatted water-bath. ■——■, 0.5 *M* borate buffer (pH 7.0); ●——●, 0.5 *M* borate buffer (pH 8.0); ○——○, 0.5 *M* borate buffer (pH 9.0); ▲——▲, 0.5 *M* borate buffer (pH 10.0); ○ — — ○, 0.5 *M* phosphate buffer (pH 9.0).

RESULTS AND DISCUSSION

The present study was focused on the development of a simple system for the analysis of norepinephrine and epinephrine, which provide valuable diagnostic information.

Catecholamines show intense fluorescence when heated in alkaline media, but the fluorescence quenches immediately. Addition of boric acid to the reaction mixture prevents quenching and gives stable fluorescence. The conditions of this reaction were examined using epinephrine since its content in biological fluid is normally smaller than that of other clinically important catecholamines such as norepinephrine, dopamine and DOPA. Analytical methods for epinephrine require especially high sensitivity.

Preliminary manual examination of the fluorescence reaction was carried out by heating epinephrine solutions in various buffers. Fig. 2 shows fluorescence intensity plotted against the time of heating at 60°C; this temperature gave the maximum fluorescence intensity in the manual procedure. Borate buffer (pH 9.0) gave the most intense fluorescence, whereas slow development of fluorescence was observed at lower pH and the fluorescence was unstable at higher pH. Heating in phosphate buffer (pH 9.0) yielded only poor fluorescence. Borate seems to stabilize the fluorescent product(s) by formation of complexes. The concentration of borate in the range 0.1—0.5 *M* does not seriously affect fluorescence intensity.

Fig. 3 illustrates the excitation and fluorescence spectra of catecholamines heated in the borate buffer (pH 9.0). The excitation and fluorescence maxima are listed in Table I. The excitation and emission maxima of dopamine and DOPA appear at significantly shorter wavelengths than those of norepinephrine and epinephrine.

HPLC of catecholamines in biological fluids on strong cation-exchange columns such as Zipax SCX has been investigated by several workers [1,3,10, 25]. A recent paper [12] reported the determination of norepinephrine and epinephrine using the trihydroxyindole reaction. Fifteen minutes were needed

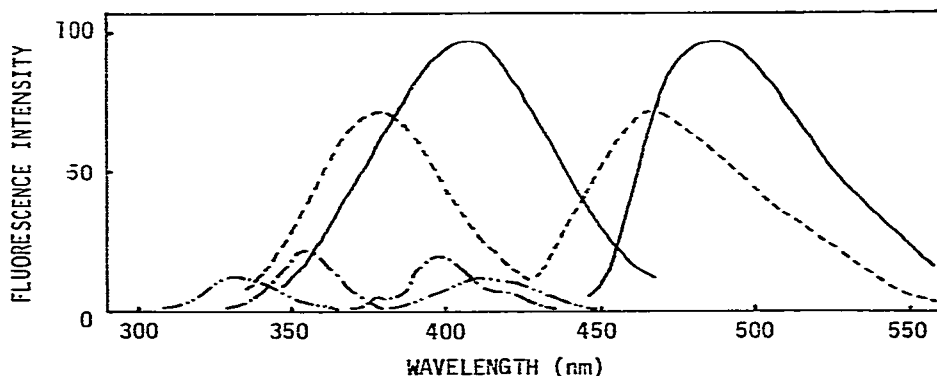

Fig. 3. Excitation and fluorescence spectra of the reaction products of norepinephrine (- - -), epinephrine (——), dopamine (— · —) and DOPA (— · · —). To 1 ml of 100 ng/ml catecholamine, 3 ml of 0.5 *M* borate buffer (pH 9.0) were added and the resulting mixture was heated at 60°C for 25 min in a thermostatted water-bath.

TABLE I

EXCITATION AND EMISSION WAVELENGTHS OF THE REACTION PRODUCTS OF CATECHOLAMINES

Catecholamine	Wavelength (nm)	
	Ex.	Em.
Norepinephrine	380	465
Epinephrine	405	490
Dopamine	353	398
DOPA	330	412

for the elution of a Zipax SCX column with 30 m*M* monobasic sodium phosphate solution containing 60 g of acetonitrile per liter at a flow-rate of 0.8 ml/min. In the present method, the time for the determination was reduced to 10 min using 50 m*M* monobasic sodium phosphate solution containing 50 ml of acetonitrile per liter for the elution.

The post-column reaction conditions were examined using the chromatographic system shown in Fig. 1. Unless otherwise stated, the sample volumes injected were 100 μl and the amount of catecholamines are expressed as weight in a 100-μl sample. The pH of the eluate was maintained constant by delivering 0.5 *M* borate buffer (pH 9.7) to the column effluent. The ratio of flow-rate of mobile phase and borate buffer was 2:1. The reaction mixture passed through the PTFE reaction coil within 1.5 min, the coil being placed in a water-bath (Fig. 1). Fig. 4 shows the fluorescence intensity plotted against the temperature of the water-bath, the maximum fluorescence being at 75°C.

Fig. 5A shows a chromatogram of a standard solution containing 5 ng each of norepinephrine and epinephrine which are clearly separated within 10 min. Fig. 5B shows a chromatogram of the catecholamine fraction from human urine after alumina treatment, giving three peaks. Two of them, the second and

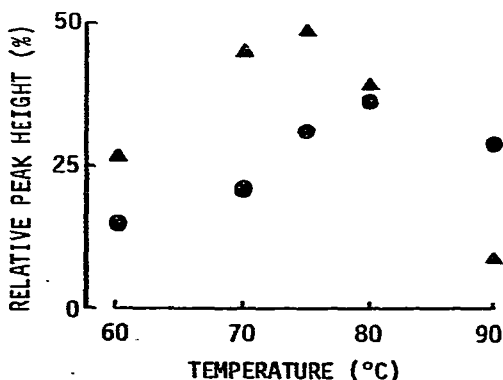

Fig. 4. Fluorescence intensities of the reaction products of catecholamines plotted against the temperature of the water-bath during post-column reaction. Aliquots of 100 μl of a standard solution containing 5 ng of norepinephrine (●) or epinephrine (▲) were injected into the chromatograph.

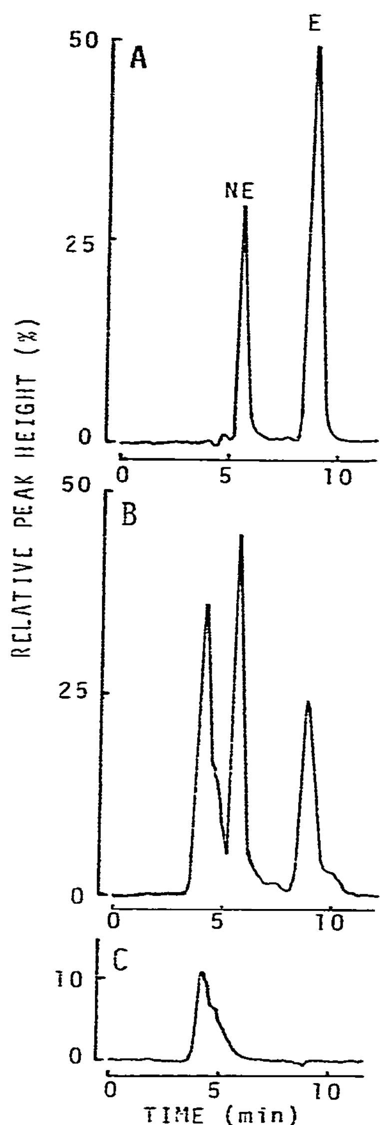

Fig. 5. Chromatographic profile of norepinephrine (NE) and epinephrine (E). (A) Chromatogram obtained by injecting 100 µl of a mixture containing norepinephrine (5 ng) and epinephrine (5 ng). (B) Chromatogram of the catecholamine fraction of human urine under standard conditions. (C) Chromatogram of the catecholamine fraction of human urine. Distilled water was delivered instead of the borate buffer to the column effluent in this experiment.

third peaks, correspond to those of standard norepinephrine and epinephrine. Fig. 5C demonstrates that these two peaks disappear when distilled water is delivered to the chromatograph instead of the alkaline borate buffer. However, the first peak is still observed although its intensity is reduced. This peak has not yet been identified, but it is sufficiently separated from the second and third peaks and does not interfere with the determination of norepinephrine and epinephrine.

There are linear relationships between the peak heights and the amounts of

norepinephrine and epinephrine injected ranging from 0.25 to 20 ng. The coefficients of variation (n=8) for 0.5 ng and 5 ng of standard norepinephrine were 3.3% and 1.9%, respectively, and those for 0.5 ng and 5 ng of epinephrine were 2.4% and 1.6%, respectively.

When the determination was repeated with a 5-ml aliquot of normal human urine, the average norepinephrine (n=5) and epinephrine (n=5) levels were 3.94 μg/dl and 1.19 μg/dl, respectively, with coefficients of variation of 3.6% and 2.2%, respectively. Recoveries of 100 ng of norepinephrine and epinephrine added to 5 ml of human urine were 82% and 88%, respectively.

The "borate method" provides a rapid and reproducible means of determining norepinephrine and epinephrine in human urine. The apparatus for post-column derivatization is simple compared with that necessary for the tri-hydroxyindole procedure. The present method is expected to be useful in the routine analysis of catecholamines in biochemical or clinical laboratories.

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