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**Note**

# Ultramicro method for the determination of picogram amounts of norepinephrine and epinephrine by high-performance liquid chromatography

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The trihydroxyindole fluorometric procedure has been widely used to measure levels of norepinephrine and epinephrine[1]. However, when measuring small amounts of catecholamines, this method lacks sensitivity and specificity. The sensitivity of this method is more than a few nanograms. Although, a radioenzymatic assay procedure is now capable of measuring picogram amounts of catecholamines[2], this method is not routinely used for laboratory work or clinical examinations since it is complex and time-consuming. In 1975, Kissinger *et al.*[3] reported a new method for catecholamine analysis which combined high-performance liquid chromatography (HPLC) with an electrochemical detector. However, even using this, only nanogram levels of catecholamine can be detected. We report a new highly sensitive HPLC method which is capable of determining picogram amounts of norepinephrine and epinephrine simultaneously.

## EXPERIMENTAL

### *Reagents*

Norepinephrine and epinephrine were purchased from Sigma (St. Louis, Mo., U.S.A.). All other chemicals were obtained from Wako (Osaka, Japan). The reagents used were of the highest grade and the solutions were filtered and degassed through a 0.22-$\mu$m membrane filter (Millipore, Bedford, Mass., U.S.A.) immediately before use.

### *Liquid chromatograph and reaction system*

The following equipment was obtained from Shimadzu (Kyoto, Japan): LC-3A pump (flow-rate 0.8 ml/min); SIL-1A injector; Zipax SCX column (DuPont) (1 m × 2.1 mm I.D.); mixing coils, 10-turn coil (0.5 m × 1.8 mm I.D.) and 20-turn coil

(1 m × 1.8 mm I.D.) (Pyrex glass); PRR-2A proportioning pump (flow-rate 0.3 ml/min) (0.8 mm I.D. for 2, 3, 4, 5 in Fig. 1 and 1.6 mm I.D. for 6, Tygon tube); RF-500-LCA spectrofluorophotometer (excitation wavelength 402 nm, emission wavelength 510 nm); square flow cell (120 $\mu$l); strip chart recorder (chart speed 2.5 mm/min). Conditions: column oven temperature, 40°; incubator temperature, 23°; sample size, 10 $\mu$l.

Fig. 1 shows the flow scheme of this system. Samples containing norepinephrine and epinephrine are injected through an injector onto the column for the separation of these two catecholamines with 0.15 *M* $NaH_2PO_4$ as the mobile phase (1 in Fig. 1). The constant flow-rate for elution is 0.8 ml/min at 50 kg/cm$^2$. The column is operated at 40°. To this column effluent, reagents for the trihydroxyindole reaction (2 in Fig. 1, 0.5 *M* phosphate buffer at pH 7.0 containing 0.05% $K_3Fe(CN)_6$ and Brij 35 (0.002 mg/ml); 4 in Fig. 1, 0.05% ascorbic acid and $Na_2S_2O_5$; 5 in Fig. 1, 5 *N* NaOH) are added sequentially at the constant flow-rate of 0.3 ml/min using the proportioning pump. The reactions are carried out in the three mixing coils in the incubator at 23°. Finally the fluorescent products are dispatched to the highly sensitive spectrofluorophotometer.

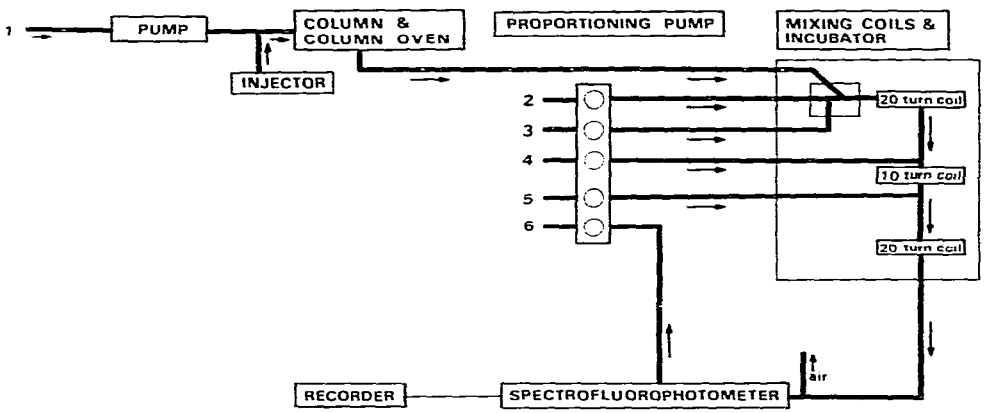


Fig. 1. Flow scheme of the catecholamine analyzing system. 1 = Mobile phase (0.15 *M* $NaH_2PO_4$); 2 = 0.5 *M* phosphate buffer at pH 7.0 containing 0.05% $K_3Fe(CN)_6$ and Brij 35 (0.002 mg/ml); 3 = air; 4 = 0.05% ascorbic acid and $Na_2S_2O_5$; 5 = 5 *N* NaOH; 6 = the waste from the spectrofluorophotometer.

An air bubble (3 in Fig. 1) is added by the proportioning pump in order to separate solutions without spreading[4]; the air bubble is extracted just before the inlet to the spectrofluorophotometer. Solutions from the outlet of the spectrofluorophotometer are delivered to the waste (6 in Fig. 1) by the proportioning pump.

## RESULTS AND DISCUSSION

A chromatogram obtained for a standard solution containing 30 pg each of norepinephrine and epinephrine is shown in Fig. 2. Under the chromatographic conditions, the lowest limit of the detection for each catecholamine is *ca.* 1 pg.

Fig. 3 shows the standard calibration curve for norepinephrine and epinephrine.

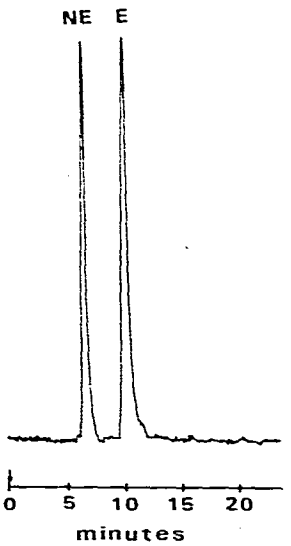


Fig. 2. Chromatogram of catecholamine standard containing 30 pg each of norepinephrine (NE) and epinephrine (E). The arrow shows the point of injection of the samples. Conditions: sample size, 10 $\mu$l; column, Zipax SCX (1 m × 2.1 mm I.D.); mobile phase, 0.15 *M* $NaH_2PO_4$; flow-rate, 0.8 ml/min; column temperature, 40°; spectrofluorophotometer excitation wavelength, 402 nm; emission wavelength, 510 nm.

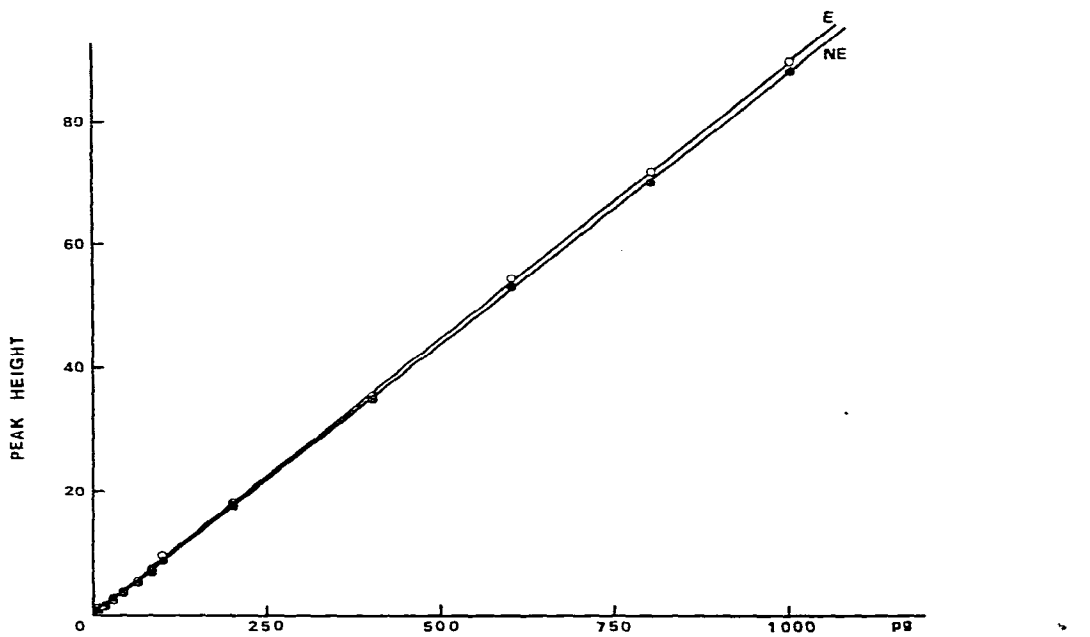


Fig. 3. Standard calibration curve for norepinephrine (NE) and epinephrine (E). Conditions as in Fig. 2.

There is a linear relationship between fluorescence intensity (peak height) and catecholamine concentration between 0 and 1000 pg, the highest concentration tested.

Regarding the coefficient of variation, within-run chromatographic determinations for standard norepinephrine and epinephrine (30 pg) vary by only 0.5% and 0.4%, respectively ($n = 10$).

Anton and Sayre[1] stated that the fluorescence intensity of catecholamine increased in proportion to a rise in NaOH concentration in the trihydroxyindole reaction. However, when concentrations of NaOH higher than 5 *N* were used in this system, the reaction coil was plugged by precipitated salts from the reaction mixture. Therefore, we used 5 *N* NaOH. The best operating conditions to obtain a linear relationship between catecholamine concentration and fluorescence intensity (peak height) were found to be 0.05% ascorbic acid and 0.05% $K_3Fe(CN)_6$, respectively.

With this method, norepinephrine and epinephrine can be determined simultaneously after the complete separation and, moreover, constant blank levels can be obtained by the automatic analyzing system. The sensitivity of this method is sufficient for application of the differential determination of norepinephrine and epinephrine in human plasma.

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