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Note

Fluorimetric determination of catecholamines using glycylglycine as the reagent for post-column derivatization

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The trihydroxyindole method[1–3] and the ethylenediamine condensation method[4–6] are widely used for the fluorimetric determination of catecholamines, but the former method does not permit the determination of dopamine with high sensitivity and the latter is less sensitive than the former.

On the other hand, it has been found that glycinamide reacts with corticosteroids under alkaline conditions at elevated temperature to form fluorescent compound(s)[7], and the yield of fluorescent compound(s) from corticosteroids having a ketolic side-chain could be improved by oxidizing the reactants with hexacyanoferrate(III)[8]. Reaction of catecholamines with glycinamide in the presence of hexacyanoferrate(III) in a borate buffer also yielded fluorescent product(s), and the use of glycylglycine in place of glycinamide increased the rate of the formation of fluorescent compound(s) and reduced the level of the reagent blank fluorescence.

Therefore, the optimal conditions for the fluorimetric determination of catecholamines using glycylglycine were investigated and the method was used successfully for the determination of catecholamines eluted from a column of ion-exchange resin.

EXPERIMENTAL

Reagents

Epinephrine bitartrate, norepinephrine bitartrate, dopamine hydrochloride and isoproterenol hydrochloride were purchased from Yashima Pharmaceutical (Osaka, Japan). Glycylglycine was purchased from Sigma (St. Louis, MO, U.S.A.) and boric acid from Merck (Darmstadt, F.R.G.). Other chemicals were of analytical-reagent grade.

Equipment

A constant-flow pump (Jasco, Model TRI ROTAR III) was used to pump the mobile phase through the chromatographic column. A dual-head pump (Jasco, Model SP-24-2) was used to pump reagent solutions. A spectrofluorimeter (Hitachi, Model MPF-4) was used to obtain excitation and emission spectra, and a fluorimeter equipped with a flow cell (volume 7 μl) was used to monitor fluorescence.

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Reaction of catecholamines with glycylglycine

To 1.5 ml of mixed buffer (0.35 *M* boric acid, 0.12 *M* tartaric acid and 0.5 m*M* disodium ethylenediaminetetraacetate, pH 4.0) containing catecholamines were added 0.5 ml of 1.5% (w/v) glycylglycine solution in deionized water and 0.6 ml of potassium borate buffer (0.5 *M*, pH 9.7) containing 0.01% (w/v) of hexacyanoferrate(III). The final pH of the mixture was 7.4. The mixture was heated in a water-batch at 95°C for 3 min and then cooled to room temperature. Excitation and emission spectra were measured using a Model MPF-4 spectrofluorometer (Hitachi).

Preparation of Amberlite CG-50 column

Amberlite CG-50 (Type 2) was graded according to size and washed and buffered as described previously[9]. 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.

Preparation of Amberlite IRC-50 column

Amberlite IRC-50 (45–50 μm in the Na^+ form) (Amberlite CG-50 of similar particle size could also be used) was prepared and washed as described previously[10], and a suspension of the washed resin was buffered at pH 4.0 with tartaric acid solution (0.2 *M*) and then washed with eluent. The eluent was a mixed buffer of pH 4.0 containing 0.35 *M* boric acid, 0.12 *M* tartaric acid and 0.5 m*M* disodium ethylenediaminetetraacetate. The washed resin was suspended in an equal volume of the

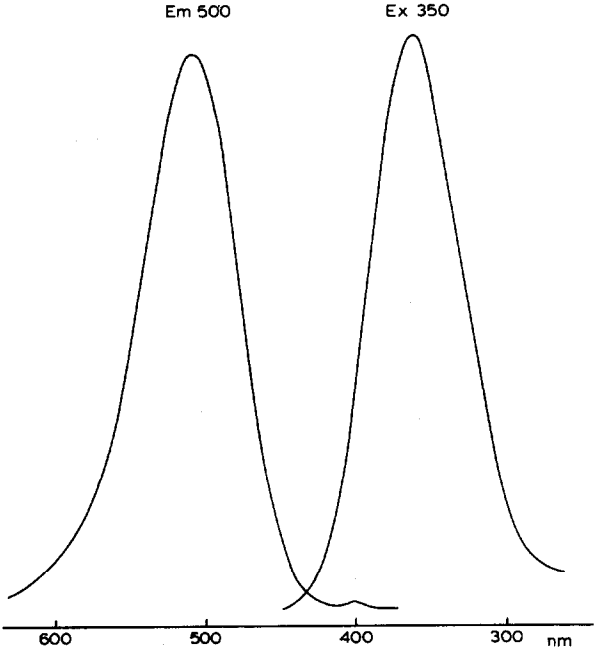

Fig. 1. Excitation and emission spectra of norepinephrine after reaction with glycylglycine under conditions II in Table I.

TABLE I

RELATIVE FLUORESCENCE INTENSITY OF REACTION PRODUCTS OF CATECHOLAMINES

Reaction conditions: I, reagent and reaction time as described under Experimental, concentration of catecholamine in the reaction mixture $1 \cdot 10^{-8}$ mol/l and fluorescence intensity of norepinephrine taken as 100; II, glycylglycine solution (1.5%) containing zinc sulphate (2 mmol/l) used, other conditions as for I.

Compound	*Reaction conditions*	
	I	*II*
Norepinephrine	100	106
Epinephrine	98	107
Dopamine	23	46
Isoproterenol	93	93

eluent and the suspension was poured into a chromatographic tube (30 × 0.8 cm I.D.) and allowed to settle under gravity. The tube was fitted with a column adjuster and the eluent was pumped through the column at a rate of 1.0 ml/min for several hours at 50°C. The height of the resin column was 22 cm.

Separation of catecholamine fraction from human urine

A 4-ml of portion of filtered urine was mixed with 0.5 ml of a 5% solution of disodium ethylenediaminetetraacetate, 0.5 ml of a 1% solution of ascorbic acid and 800 ng of isoproterenol in 1.0 ml of 0.01 *M* hydrochloric acid, and the pH of the mixture was adjusted to 6.1 with 1 *M* sodium hydrogen carbonate solution. The mixture was then applied to an Amberlite CG-50 column, which was washed with 6 ml of deionized water and then with 2 ml of 2/3 *M* boric acid solution. A further 2 ml of boric acid solution were used to eluate catecholamines from the column, the

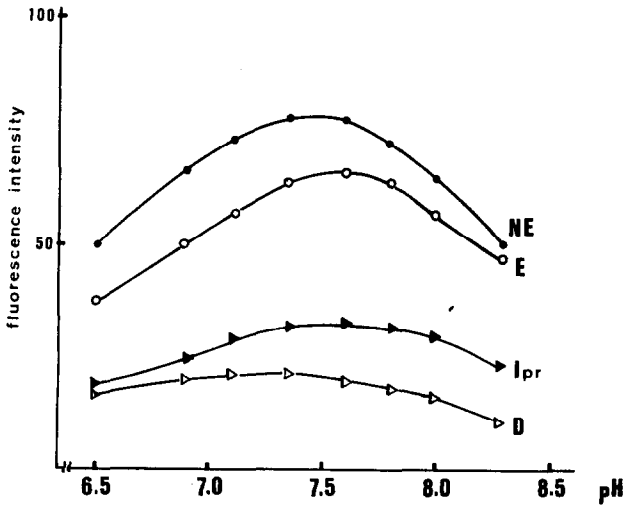

Fig. 2. Relationship between peak response and pH of waste fluid from the detector. Amount of each catecholamine used, 10 ng. NE, norepinephrine; E, epinephrine; Ipr, isoproterenol; D, dopamine.

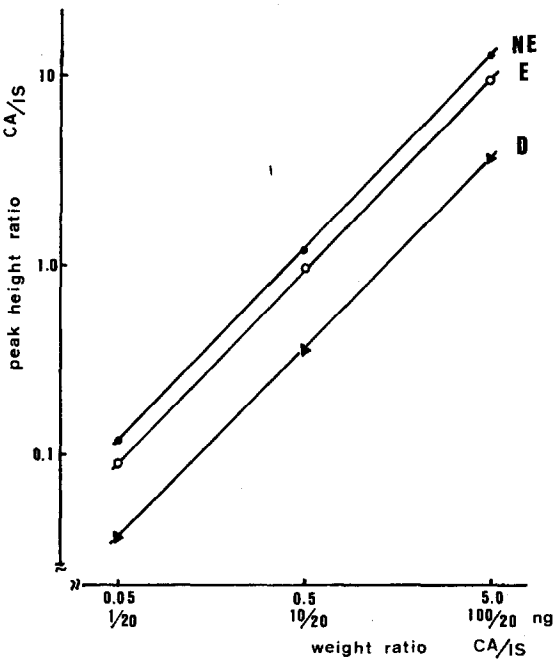

Fig. 3. Calibration graph for catecholamines (Ca) using isoproterenol as an internal standard (IS). Symbols as in Fig. 2. Abscissa, amount of catecholamines analysed with 20 ng of isoproterenol; ordinate, peak-height ratio of epinephrine, norepinephrine and dopamine to isoproterenol.

eluate being collected in a test-tube containing 0.9 ml of 0.1 *M* tartaric acid. The eluate in the test tube was adjusted to pH 4.0 with 0.1 *M* tartaric acid solution and diluted to 4.0 ml with tartrate buffer (0.1 *M*, pH 4.0) containing ethylenediaminetetraacetate (2 m*M*). The catecholamine fraction was stored in a refrigerator.

Chromatographic separation and fluorimetric determination of catecholamines

A 0.1-ml aliquot of sample solution was injected into the column of Amberlite IRC-50, the eluent was pumped at a rate of 1.0 ml/min and the eluate was mixed with a mixture of reagents A and B. Reagent A was a solution of glycylglycine (1.5%, w/v) containing boric acid (0.05 *M*) and zinc sulphate (2 m*M*) and reagent B was potassium borate buffer of pH 9.4 (0.5 *M*) containing hexacyanoferrate(III) (0.01%, w/v). Each reagent was pumped at a flow-rate of 0.4 ml/min and the mixture of reagents and eluate (waste fluid) was heated at 95°C for 2 min in a PTFE tube (20 m × 0.5 mm I.D.) immersed in a water-bath, then cooled to room temperature in a cold water-bath. The fluorescence was monitored with a fluorimeter (Jasco, Model FP-115).

RESULTS AND DISCUSSION

The reaction of glycylglycine with catecholamine oxidized with hexacyanoferrate(III) proceeded rapidly at pH 7–8. Heating for 3 min was sufficient to obtain maximum fluorescence intensity, and it remained unchanged for at least 10 min.

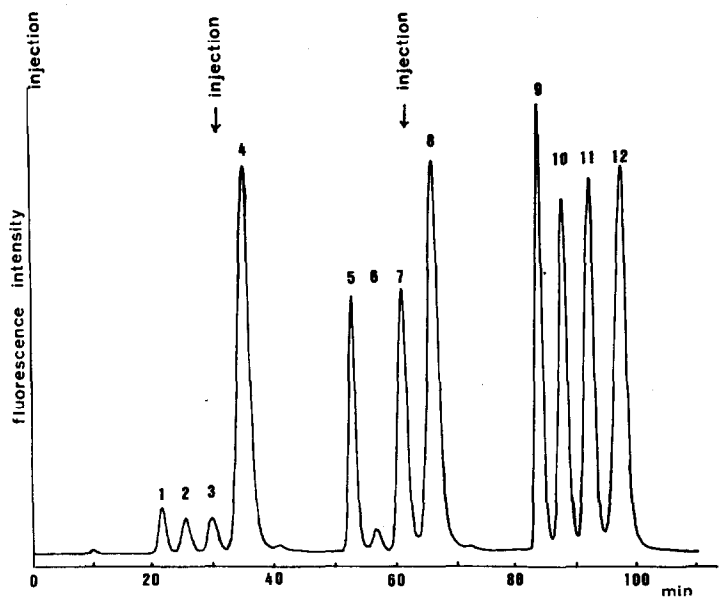

Fig. 4. Elution patterns of standards and urine sample. Peaks: 1 = Ne (1 ng), 2 = E (1 ng), 3 = D (3 ng) and 4 = Ipr (20 ng) as standard compounds; 5–8 = NE, E, D and Ipr in a urine sample; 9 = NE (10 ng), 10 = E (10 ng), 11 = D (30 ng) and 12 = Ipr (20 ng) as standard compounds (for abbreviations, see Fig. 2). Samples were injected at 0, 31 and 62 min.

Alanylglycine gave also fluorescent compound(s) with catecholamines under similar conditions, and the formation of fluorescent compound(s) from catecholamines and glycylglycine is considered to be due to the condensation of one of the oxo groups of the oxidized catecholamines with the amino group of glycylglycine followed by the addition of an amide nitrogen to the other oxo group and dehydration to form a cyclic derivative.

The excitation and emission spectra of four catecholamines examined did not differ appreciably, and had excitation and emission maxima at 350 and 500 nm, respectively (Fig. 1).

The relative fluorescence intensity of the reaction mixture of catecholamines prepared by a manual method is shown in Table I. Addition of zinc sulphate in the reaction mixture doubled the fluorescence intensity of the products derived from dopamine without increasing the reagent blank fluorescence. The intensity of the reagent blank fluorescence was equal to an intensity corresponding to a concentration of $5 \cdot 10^{-9}$ *M* dopamine or $1.26 \cdot 10^{-9}$ *M* norepinephrine in the reaction mixture.

The relationship between peak height and pH of waste fluid is shown in Fig. 2. Each catecholamine had a different pH optimum, and we chose pH 7.4 for the determination of catecholamines by this method. The reaction time was 2 min, which was long enough for dopamine to give a maximum response. Longer reaction times increased the peak response of epinephrine and norepinephrine by about 10%.

Isoproterenol could be used as an internal standard and a linear relationship between the peak-height ratio and the amount of catecholamines injected into the chromatograph was observed from 1 to 100 ng. (Fig. 3).

The elution patterns of standards and a urine sample are shown in Fig. 4. 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 7.2 ± 0.51 (S.D.), 62 ± 0.79 and 217 ± 1.53 ng, respectively. When 100 ng of epinephrine, 100 ng of norepinephrine and 400 ng of dopamine were added to a urine sample and the procedure described above was carried out, the recovery was 95–105% for all catecholamines.

The results obtained indicate that the proposed fluorometric method is suitable for the on-line fluorimetric assay of catecholamines in clinical and biological samples.

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