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Note

Method for the fluorimetric estimation of dopamine

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Fluorimetric determination of dopamine has been performed by the trihydroxyindole method [1, 2] and the ethylenediamine condensation method [3, 4] after extraction of catechol compounds from biological extract by alumina adsorption. The ethylenediamine condensation method was automated and coupled with a high-performance liquid chromatograph [5, 6], and fluorescent derivatives of dopamine and norepinephrine have been separated by high-performance liquid chromatography (HPLC) on silica gel [7] or a synthetic gel [8]. By these methods, dopamine in both tissue extract and urine could be successfully estimated, but they were not sufficiently sensitive for the determination of picogram amounts of dopamine present in human plasma.

On the other hand, one of us [9] has reported that catecholamines could be estimated fluorimetrically by oxidizing them with hexacyanoferrate(III) in the presence of ethylenediamine in an alkaline borate buffer at 75°. Oxidation of dopamine dissolved in a borate buffer (pH 9.0) containing a primary amine or amino acid with hexacyanoferrate(III) at 80° yielded also fluorescent product(s), and among the compounds tested, *p*-aminobenzoic acid gave the best result. When the method was automated and used to measure dopamine eluted from a column of Amberlite IRC-50 with a mixed buffer of pH 6.3, 0.1 ng of dopamine could be detected at a signal-to-noise ratio of 10, and by this method, dopamine in human plasma could be estimated.

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EXPERIMENTAL

Reagents

Epinephrine hydrogen tartrate was purchased from Nakarai Pharmaceuticals (Kyoto, Japan), norepinephrine hydrogen tartrate, deoxyepinephrine hydrochloride, dopamine hydrochloride and *p*-aminobenzoic acid were purchased from Yashima Pharmaceuticals (Osaka, Japan). Other chemicals were of reagent grade. Stock solutions of catecholamine base were prepared in 0.01 *M* HCl.

Apparatus

A constant flow pump (Jasco, Model TRI ROTAR) was used to pump buffer through a chromatographic column. Peristaltic pumps (Atto, Model SJ-1211 H and L) were used to pump air and reagents into a gas-segmented-flow reaction detector. A spectrofluorimeter (Jasco, Model FP-550) equipped with a flow cell (square cross section, 4 × 4 × 20 mm internal dimension) was used to measure fluorescence. Samples were injected into the column by a motor-driven injector (Kyowa Seimitsu, Auto-Injector Model KUH-6000).

Preparation of Amberlite CG-50 column

Amberlite CG-50 (type 2) was graded according to size, washed and buffered as described previously [10]. 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 × 0.4 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—55 μm in sodium ion form) was prepared and washed as described previously [11], and suspension of the washed resin (sodium ion form) was buffered at pH 6.3 with a succinic acid solution (0.5 *M*) and then washed with an eluent. The eluent is a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediamine tetraacetate. The washed resin was suspended in an equal volume of the eluent and the suspension was poured into a chromatographic tube (15 × 0.8 cm I.D.), and allowed to settle under gravity. Then the tube was fitted with a column adjuster and the eluent was pumped through the column at the rate of 0.8 ml/min for several hours at 42°. The height of the resin column was 12 cm.

Preparation of catecholamine fraction from human plasma

Heparinized blood was drained into a chilled tube containing 5 m*M* reduced glutathione and centrifuged immediately in the cold at 1500 *g* for 15 min. The plasma was divided into 1.0 ml portions and stored at −20°. A frozen plasma sample (1.0 ml) was mixed with 1.0 ml of 0.1 *M* HCl and thawed. Then it was deproteinized with 1.0 ml of 2.0 *M* perchloric acid and centrifuged in the cold at 1500 *g* for 15 min. The supernatant was transferred to a 20 ml beaker, the precipitated protein was mixed with 2 ml of 0.5 *M* perchloric acid and centrifuged again. The supernatant was combined, chilled in an ice bath, and after 0.5 ml each of 5% (w/v) disodium ethylenediamine tetraacetate and

0.5% (w/v) ascorbic acid solution were added, was neutralized to pH 6.2 with a 1 *M* potassium carbonate. The supernatant was added to a column of Amberlite CG-50, the precipitate of potassium perchlorate was washed with 2 ml of cold 0.05% (w/v) disodium ethylenediamine tetraacetate (pH 6.2) and the washing was also added to the column of Amberlite GC-50. The column was washed with 4 ml of deionized water and then with 1.0 ml of 2/3 *M* boric acid solution, then another 2.0 ml of the boric acid solution were used to elute catecholamines from the column. The eluate was collected in a test-tube containing 0.07 ml of a solution of 1 *M* HCl containing sodium dihydrogen phosphate (0.5 *M*) and disodium ethylenediamine tetraacetate (0.005 *M*). The eluate in the test-tube was adjusted to pH 6.3 with 1 *M* HCl containing sodium dihydrogen phosphate (0.5 *M*) and disodium ethylenediamine tetraacetate (0.005 *M*) and diluted to 4.0 ml with a succinate buffer of pH 6.3 (0.08 *M*), and stored in a refrigerator.

Chromatographic separation of samples

A 1-ml volume of a solution of the amines in the eluent to be used for chromatography, or 1.0 ml of catecholamine fraction prepared as described above was injected into the column of Amberlite IRC-50. Then, elution was carried out with the eluent at a flow-rate of 0.7 ml/min.

Fluorimetric determination of dopamine

A gas-segmented-flow reaction detector was assembled from commercial parts and pyrex coils. Pyrex coils were made by winding 4-mm pyrex tubing around a brass tube of 14 mm O.D. As shown in Fig. 1, eluate from the column was fed to the detector and segmented by air, mixed with 1% (w/v) *p*-aminobenzoic acid solution in 0.1 *M* disodium hydrogen phosphate (mixing coil, 7 turns), 0.8 *M* sodium hydroxide containing 0.1% (w/v) Brij-35 (mixing coil, 7 turns), 0.3% (w/v) hexacyanoferrate (III) (mixing coil, 7 turns), and

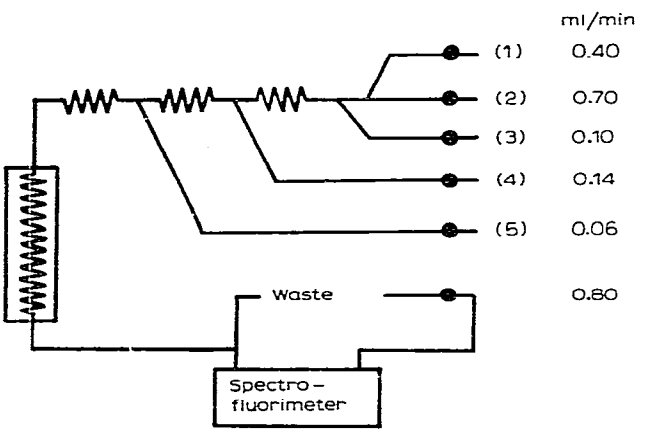

Fig. 1. Schematic diagram of the gas-segmented-flow reaction detector. pH of the waste was 9.0. (1) air; (2) eluate; (3) 1% (w/v) solution of *p*-aminobenzoic acid in 0.1 *M* disodium hydrogen phosphate; (4) 0.8 *M* NaOH—0.1% (w/v) Brij-35, (5) 0.3% (w/v) hexacyanoferrate(III).

heated at 80° (heating coil, 30 turns). Bubbles were removed from the stream and the fluorescence was measured at 520 nm, with excitation at 465 nm. The slit width of both excitation and emission was 20 nm.

RESULTS AND DISCUSSION

p-Aminobenzoic acid was used for the formation of fluorescent product(s) from dopamine in an alkaline reaction medium containing hexacyanoferrate (III) as an oxidizing agent. The method was automated as shown in Fig. 1. Eluate from a column was mixed with the reagents and heated at 80° and the times for mixing and heating were 6 and 5.5 min, respectively. Oxidation was necessary for the formation of fluorescent product(s) from dopamine, and the optimum pH for the reaction was 9.0. Oxidation of dopamine with hexacyanoferrate(III) in the presence of *p*-aminobenzoic acid under alkaline conditions was preferred to oxidation at neutral pH prior to the addition of *p*-aminobenzoic acid and sodium hydroxide solution since the latter reaction sequence has a lower yield of fluorescent product(s). Reduction of hexacyanoferrate(III) was not necessary, because hexacyanoferrate(III) in the reaction mixture absorbed less than 5% of the excitation light.

A flow cell with a square cross section was used since it gave lower background fluorescence than a flow cell with round cross section. Excitation and

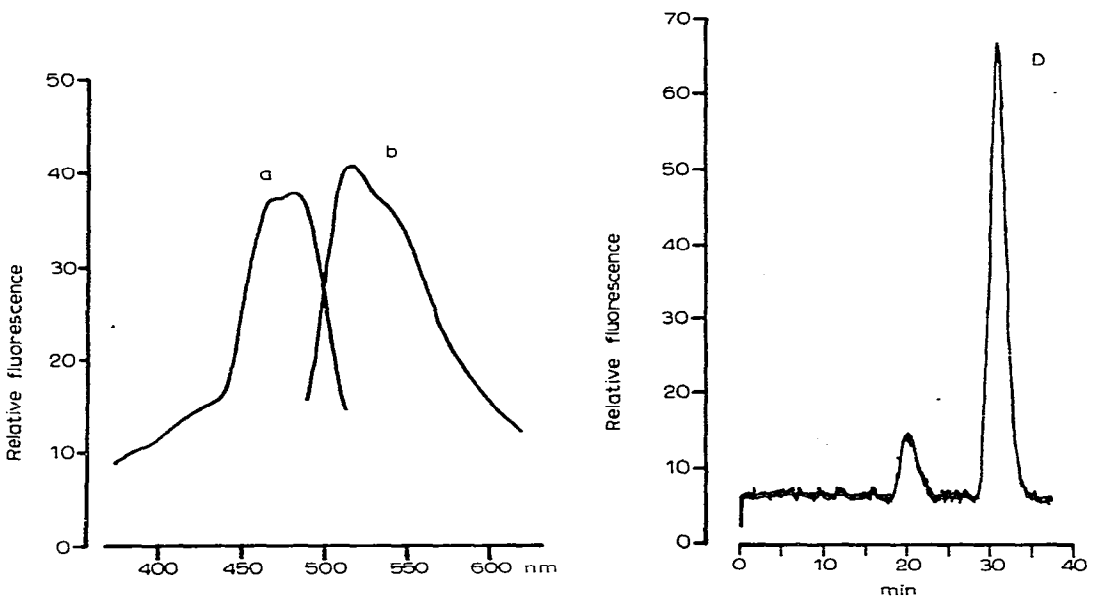

Fig. 2. Fluorescence spectra (uncorrected) of the reaction product(s) of dopamine. A solution of dopamine in the eluent (6 μg/ml) was fed to the detector. Slit width of excitation and emission was 5 nm and 10 nm respectively. (a) = excitation spectrum, emission wavelength was 520 nm; (b) = emission spectrum, excitation wavelength was 465 nm.

Fig. 3. Elution and fluorimetric estimation of dopamine. One nanogram of dopamine was separated on the Amberlite IRC-50 column and estimated using the detector described in Fig. 1. Peak D = dopamine. The first peak = impurity present in sample solution.

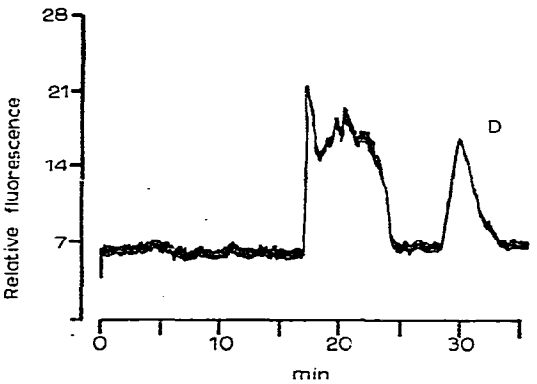

Fig. 4. Elution and fluorimetric estimation of dopamine from human plasma. Retention time of peak D corresponded to that of dopamine.

emission spectra of the fluorescent product(s) from dopamine are shown in Fig. 2. Epinephrine and norepinephrine yielded virtually no products which fluoresce following excitation at 465 nm, but deoxyepinephrine could be measured at a sensitivity of ca. one sixth and dopa at ca. the same sensitivity as that of dopamine. A shorter column than that reported previously, was used in this experiment [9], because it gives a shorter analysis time and higher sensitivity, and deoxyepinephrine, which was not completely separated from dopamine, will not interfere with the estimation of dopamine in human plasma or in urine, since it is not usually found in these samples.

The elution pattern of dopamine was readily reproducible and 1.0 ng of dopamine could be measured at a signal-to-noise ratio of 100 (Fig. 3). A linear relationship between the peak height and the amount of dopamine added to the column was obtained over the range of 0.05—100 ng. As shown in Fig. 4, a peak with a retention time corresponding to that of dopamine was clearly separated from the peaks of impurities present in a catecholamine fraction from human plasma. When 1.0 ng of dopamine was added to plasma samples before deproteinization, the recovery was 87 ± 1.5% (S.E.; 5 determinations). Dopamine content of human plasma taken from 26 individuals (morning; at rest and before breakfast) ranged from 0.60 ng to 0.09 ng, and the mean value was 0.23 ng ± 0.17 (S.D.) per ml.

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