Journal of Chromatography, 163 (1979) 329—336
Biomedical Applications

CHROMBIO. 349

ESTIMATION OF CATECHOLAMINES IN HUMAN PLASMA BY ION-EXCHANGE CHROMATOGRAPHY COUPLED WITH FLUORIMETRY

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(Received February 2nd, 1979)

SUMMARY

Estimation of catecholamines in human plasma was made by ion-exchange chromatography coupled with fluorimetry.

Catecholamines in deproteinized plasma were adsorbed onto Amberlite CG-50 (pH 6.5, buffered with 0.4 *M* phosphate buffer) and selectively eluted by 0.66 *M* boric acid. The catecholamine fraction was separated further on a column of Amberlite IRC-50 which was coupled with a device for the automated performance of the trihydroxyindole method (epinephrine and norepinephrine) or the 4-aminobenzoic acid—oxidation method (dopamine). One sample could be analysed within 25 min with either method. The lower detection limits were 0.02 ng for epinephrine and dopamine, and 0.04 ng for norepinephrine.

Plasma catecholamine contents of healthy adults at rest were epinephrine 0.07 ± 0.01 ng/ml (n = 19), norepinephrine 0.27 ± 0.03 ng/ml (n = 19) and dopamine 0.22 ± 0.03 ng/ml (n = 26).

The procedure of adsorption and elution of the plasma catecholamines by ion-exchange resin was simple, the simplicity contributing to constant recovery. The catecholamine fraction could be analysed without evaporation of the eluate. The analytical column could be used for the analysis of more than 1000 samples before excessive back-pressure developed. Our method of continuous measurement of plasma catecholamine fulfils clinical requirements.

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INTRODUCTION

Estimation of catecholamines has been performed by, among others, the trihydroxyindole (THI) method [1—3] and the ethylenediamine condensation method [4]. Recently, several improved methods have been established: high-performance liquid chromatography coupled with fluorimetric detection [5—8] or coupled with electrochemical detection [9], high-performance liquid chromatography of fluorescent derivatives of catecholamines [10], gas—liquid chromatography with electron-capture detection [11], gas chromatography—mass spectrometry [12] and radioenzymatic methods [13—18]. These methods enable picogram amounts of catecholamines to be estimated.

With the exception of some of the radioenzymatic methods [15, 18], catecholamines of biological extracts have to be preseparated by adsorption onto an alumina [19], borate gel [20], or cation-exchange column [21—24]. Furthermore, the catecholamine fraction has to be evaporated and dissolved in a small volume for high-performance liquid chromatography or converted into volatile derivatives for gas—liquid chromatography.

However, it has been reported that catecholamines can be eluted from a column of Amberlite IRC-50, buffered at pH 6.0—6.5, by an aqueous solution of boric acid [25—28]. One of the authors reported that the eluates obtained from Amberlite CG-50 can be separated on a column of Amberlite IRC-50 using a borate-containing buffer as eluant simply by pH adjustment [29]. This method was used for the analysis of urinary catecholamines in combination with a modified ethylenediamine condensation method [30], but it was not sensitive enough for the estimation of catecholamines in plasma. In the present work, an automated THI method [31] and 4-aminobenzoic acid—hexacyanoferrate(III) [4-aminobenzoic acid (PABA)—oxidation] method [32] was used for the fluorimetric detection of catecholamines in eluates of Amberlite IRC-50 columns.

EXPERIMENTAL

Reagents

Epinephrine bitartrate, norepinephrine bitartrate and dopamine hydrochloride were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). 3-Mercaptopropionic acid was obtained from Aldrich Chemical Company (Milwaukee, Wis., U.S.A.). 4-Aminobenzoic acid and other chemicals were of reagent grade and obtained from Yashima Pharmaceutical Co. (Osaka, Japan). Stock solutions of catecholamines were prepared in 0.01 *N* HCl.

Equipment

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

Preparation of Amberlite CG-50 columns

Amberlite CG-50 (type 2) was graded according to size to obtain particles of 90—120 μm. The resin was buffered as described previously [33]. The buffered resin was poured into a tube of 4 mm I.D. with 0.4 *M* phosphate buffer (pH 6.5) and allowed to settle under gravity to a height of 12 cm. The column was closed with a glass-wool plug at the bottom; it had a 10-ml reservoir on the top.

Preparation of the Amberlite IRC-50 column

Amberlite IRC-50 (45—60 μm, Na^+) was prepared and washed as described previously [33]. A suspension of the washed resin was equilibrated at pH 6.3 with a succinic acid solution (0.02 *M*). Then it was washed with eluant A (Table I) and poured into two serially connected chromatographic tubes. The tubes were fitted with column adjusters and then the same eluant was pumped through the columns at a flow-rate of 0.8 ml/min for several hours at 42°. The total height of the resin was 25 cm (10 cm in the shorter column and 15 cm in the longer column). Column B, for the separation of dopamine, was packed in the same way using eluant B (Table I). The resin height was 11 cm.

For the preparation of the eluants see Tables I and II.

Preparation of the catecholamine fraction from human plasma

Heparinized blood was drained into chilled tubes containing 5 m*M* reduced glutathione and centrifuged immediately at 4° at 1500 *g* for 15 min. The plasma was stored at −20°. Frozen plasma (1.0—2.0 ml) was mixed with 1.0 ml of 1 *N* HCl and thawed. Then it was deproteinized by addition of 1.0 ml of 2.0 *M* perchloric acid and centrifuged at 1500 *g* for 15 min. The supernatant

TABLE I

COMPOSITION OF THE ELUANTS

Eluant	pH*	Solutions to be mixed**
A	6.4	1 and 2
B	6.3	3 and 4

*pH was measured at 20° with a pH meter manufactured by TOA Electrics (Tokyo, Japan) using a Model GS-135 electrode.

**Succinate—boric acid—NaOH solutions listed in Table II.

TABLE II

COMPOSITION OF THE SOLUTIONS USED FOR THE PREPARATION OF THE ELUANTS

Solution	Composition (*M*)			
	Succinic acid	Boric acid	Disodium EDTA	NaOH
1	0.08	0.66	0.002	0.3
2	0.08	0.66	0.002	0.1
3	0.12	0.35	0.002	0.3
4	0.12	0.35	0.002	0.2

was transferred to a 20-ml beaker, and the protein precipitate was mixed with 2 ml of 0.6 *M* perchloric acid and centrifuged again. The supernatants were combined, chilled on ice and then 0.5 ml of a solution of 5% (w/v) disodium EDTA and 0.5% (w/v) ascorbic acid was added. The mixture was adjusted to pH 6.2 with 1.0 *M* potassium carbonate. The supernatant was applied to the Amberlite CG-50 column. The potassium perchlorate precipitate was mixed with 2 ml of disodium EDTA solution (pH 6.2, 0.005 *M*) and chilled on ice for several minutes. The washings were also added to the column.

The column was washed with 4 ml of deionized water and with 1.0 ml of 0.66 *M* boric acid; 2.0 ml of 0.66 *M* boric acid solution were used for the elution of the catecholamines. The eluate was collected in a tube with 0.07 ml of 1.0 *N* HCl which contained disodium EDTA (0.005 *M*) and sodium dihydrogen phosphate (0.05 *M*). It was diluted with succinate buffer (0.08 *M* succinic acid, 0.002 *M* disodium EDTA, pH 6.3) to 4.0 ml and stored in a refrigerator until analysis ("sample solution").

Chromatographic separation of samples

One millilitre of the sample solution was applied to column A for the detection of epinephrine and norepinephrine, and 1 ml to column B for the detection of dopamine. Elution of each column was carried out with the corresponding eluant at a flow-rate of 0.7 ml/min. When the back-pressure exceeded 30 kg/cm^2 (column A) or 5—10 kg/cm^2 (column B), the columns were repacked.

Fluorimetric determination

THI method. A gas-segmented flow reaction detector was assembled from commercial parts (Technicon Instruments Co., Tokyo, Japan) and Pyrex coils, as shown in Fig. 1. Pyrex coils were made by winding Pyrex tubes of 4 mm O.D. The eluate from column A was segmented by air, mixed with 0.1% (w/v) hexacyanoferrate(III) and heated at 42° (mixing coil: 15 turns). Finally, it was mixed with a solution of 4 *M* sodium hydroxide and stabilizing agents: 2% (w/v) 3-mercaptopropionic acid, 7.5% (w/v) sodium sulfite and 0.3% (w/v)

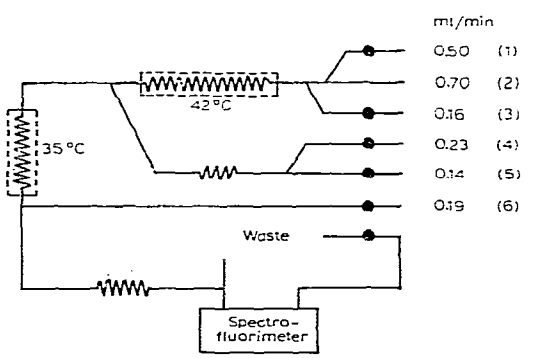

Fig. 1. Schematic diagram of the gas-segmented flow reaction detector using the trihydroxyindole method. Flow-lines: 1, air; 2, column eluate; 3, 0.1% (w/v) hexacyanoferrate(III); 4, 4 *M* sodium hydroxide; 5, stabilizing agents; 6, 6 *M* acetic acid. The pH of the waste was 5.2.

ascorbic acid. After equilibration at 35° (mixing coil: 16 turns), 6 *M* acetic acid was added to the stream and mixed (mixing coil: 8 turns) and debubbled. Fluorescence was measured at 500 nm (excitation at 405 nm). The slit width was 20 nm for excitation and 40 nm for emission.

PABA—oxidation method. We reported previously [32] that dopamine was converted into fluorescent product(s) by oxidation with hexacyanoferrate(III) in the presence of 4-aminobenzoic acid in an alkaline solution. Picogram amounts of dopamine can be measured by this reaction. The fluorimetric detector was assembled as shown in Fig. 2. The eluate from column B was segmented by air and mixed with 1% (w/v) 4-aminobenzoic acid 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 heated at 80° (heating coil: 30 turns). The bubbles were removed from the stream and fluorescence was measured at 520 nm (excitation at 465 nm). The slit width was 20 nm for both excitation and emission.

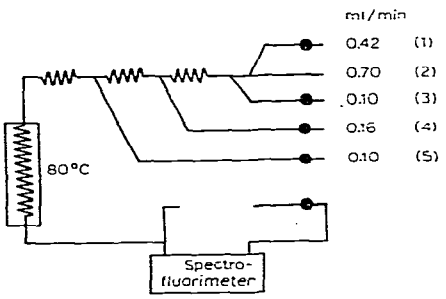

Fig. 2. Schematic diagram of the gas-segmented flow reaction detector using the 4-aminobenzoic acid (PABA)—oxidation method. Flow-lines: 1, air; 2, column eluate; 3, 1% (w/v) solution of 4-aminobenzoic acid in 0.1 *M* disodium hydrogen phosphate; 4, 0.8 *M* NaOH—0.1% (w/v) Brij-35; 5, 0.3% (w/v) hexacyanoferrate(III).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

RESULTS AND DISCUSSION

Chromatographic separation

Pretreatment. Catecholamines in biological extracts were adsorbed onto a weakly acidic ion-exchange resin and selectively eluted by a small volume of boric acid with constant recovery in agreement with previous results [26]. The addition of ascorbic acid and disodium EDTA prevented the oxidation of catecholamines during the procedure. The catecholamines in the eluate were stable below 10° for at least one week with less than 5% decomposition. It is convenient that the catecholamine fraction can be directly applied to the analytical column without evaporation.

Ion-exchange chromatography. As shown in Fig. 3, epinephrine and norepinephrine could be separated within 40 min with eluant A using column A. Eluant A, containing a higher concentration of borate, was used in order to accelerate the elution, but the peak of isoproterenol overlapped that of epinephrine, and the separation of epinephrine from norepinephrine was not

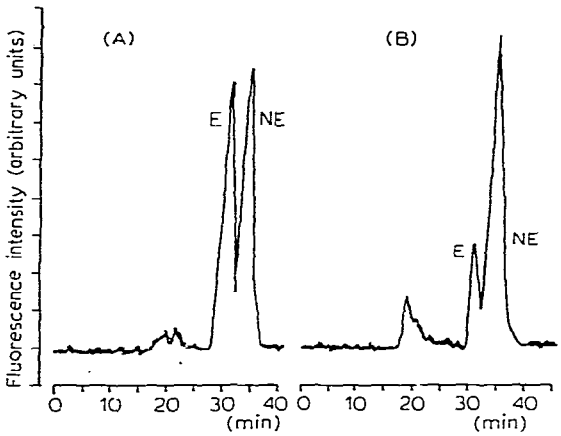

Fig. 3. Elution pattern and fluorimetric determination of epinephrine (E) and norepinephrine (NE). A, Standard sample of epinephrine (0.5 ng) and norepinephrine (1.0 ng). B, Plasma sample.

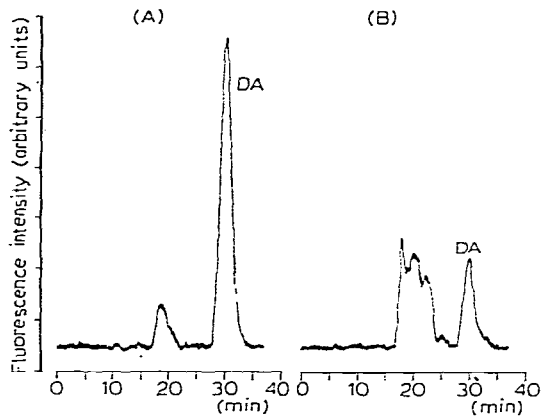

Fig. 4. Elution pattern and fluorimetric determination of dopamine (DA). A, Standard sample of dopamine (1.0 ng). B, Plasma sample.

complete. In a sample containing ten times more norepinephrine than epinephrine, the epinephrine peak was 5% higher than it was expected to be. In a sample with an epinephrine/norepinephrine ratio of 10, the norepinephrine peak was even 20% higher due to tailing. In these cases, repetition using an eluant with lower boric acid concentration is advisable [29].

We used a shorter chromatographic column (column B) to reduce analysis time and to improve sensitivity for dopamine (Fig. 4). The analytical columns (A or B) can be used for the separation of more than 1000 samples.

Fluorimetric determination

Epinephrine and norepinephrine were determined by the THI method and dopamine by the PABA—oxidation method. An automated reaction system for the THI method was assembled according to the description of Martin and Harrison [31] with minor modifications. The reaction was complete within 8 min. Lowering the pH with acetic acid has a favorable effect on stability and

sensitivity of fluorescence [8]. The elution pattern was quite reproducible. Intra-assay variation was 1.6% for epinephrine (1 ng) and 1.5% for norepinephrine (1 ng) (n = 6). Inter-assay variation was 1.5% (1 ng) and 3.7% (0.3 ng) for epinephrine and 2.6% (1 ng) and 6.5% (0.3 ng) for norepinephrine (n = 10). Intra-assay variation for plasma samples was 6.2% for epinephrine and 2.6% for norepinephrine (n = 6). Intra-assay variation for dopamine standard (1 ng) was 1.2% (n = 7) and for plasma samples 2.1% (n = 7). During storage at —20° for three months, the decrease of plasma catecholamine contents was less than 10%.

A linear relationship between peak height and amount of the amines added to the column was obtained over the range of 0.05—500 ng for epinephrine and norepinephrine, and 0.05—100 ng for dopamine. One nanogram of epinephrine and norepinephrine were detected at a signal-to-noise ratio of 100 and 50, respectively. Thus, the lower detection limits were about 20 pg of epinephrine and 40 pg of norepinephrine. One nanogram of dopamine was also detected at a signal-to-noise ratio of 100; so 20 pg of dopamine could be detected. The sensitivity was sufficient for the measurement of catecholamines in 1—2 ml of human plasma.

Over-all recoveries for epinephrine, norepinephrine and dopamine added to plasma are shown in Table III. These recoveries are superior to those obtained by the alumina adsorption method [3, 19]. Probably our method has the following advantages: (1) no troublesome handling of plasma samples; (2) isolation of catecholamines by chromatography on Amberlite CG-50 is simple; and (3) regeneration of Amberlite IRC-50 is not necessary. By continuous measurement of fluorescence, samples could be injected every 25 min before the emergence of epinephrine peak (column A) or dopamine peak (column B).

Normal values are shown in Table IV. Peripheral venous samples of healthy adults at rest were obtained painlessly from an antecubital vein through an indwelling catheter. The mean values of epinephrine (0.07 ± 0.01 ng/ml) and norepinephrine (0.27 ± 0.03 ng/ml) were close to the values obtained by a previous fluorimetric method [3], radioenzymatic methods [13—18] or gas

TABLE III

RECOVERY OF CATECHOLAMINES ADDED TO PLASMA

	Amount added (ng)	Recovery (%, mean ± S.E.)
Epinephrine	0.30	86.7 ± 3.5
Norepinephrine	0.50	95.4 ± 4.0
Dopamine	1.00	86.9 ± 1.5

TABLE IV

VALUES OF PLASMA CATECHOLAMINES OF HEALTHY INDIVIDUALS

	Concentration (ng/ml)	
	mean ± S.E.	Range
Epinephrine	0.07 ± 0.01	0.18—0.03
Norepinephrine	0.27 ± 0.03	0.63—0.06
Dopamine	0.22 ± 0.03	0.60—0.04

chromatography—mass spectrometry [12]. However, the mean value of dopamine (0.22 ± 0.03 ng/ml) was 2—5 times higher than the values obtained by other methods [12, 14, 15]. Even higher than our values were those reported by Pederson and Christensen [17]. Comparison with other fluorimetric estimations of plasma dopamine levels was not possible because no other fluorimetric method was sufficiently sensitive for the measurement of plasma dopamine.

ACKNOWLEDGEMENT

The authors thank Dr. K. Nakao and Professor Y. Kawashima for their interest in this work.

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