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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLASMA CATECHOLAMINES USING 1,2-DIPHENYLETHYLENEDIAMINE AS PRECOLUMN FLUORESCENCE DERIVATIZATION REAGENT

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SUMMARY

A simple, rapid and highly sensitive method for the determination of catecholamines (norepinephrine, epinephrine and dopamine) in human plasma is described which employs high-performance liquid chromatography with fluorescence detection. After cation-exchange chromatography on a Toyopak SP cartridge, the catecholamines and isoproterenol (internal standard) in 500 μl of plasma are converted into the corresponding fluorescent compounds by reaction with 1,2-diphenylethylenediamine in aqueous acetonitrile. These compounds are separated within 8 min on a reversed-phase column, TSK-gel ODS-120T, with isocratic elution using a mixture of water, methanol and acetonitrile containing a Tris—hydrochloric acid buffer (pH 7.0). The detection limit for each catecholamine is ca. 2 fmol in a 100-μl injection volume. N-Methyldopamine can also be used as internal standard.

INTRODUCTION

High-performance liquid chromatography (HPLC), coupled with fluorescence [1—7] or electrochemical [8—12] detection (ED), has recently been introduced for the determination of catecholamines (CAs) in plasma or serum. Although ED is highly sensitive for CAs, many other oxidizable substances present in the sample can be detected concurrently. Therefore rather complicated sample clean-up procedures are required. Fluorescence detection has mainly been based on a post-column derivatization procedure that involves the trihydroxyindole reaction [1—4]. Fluorescence assays can determine l-norepinephrine (NE) and l-epinephrine (E) more sensitively than ED, and have enough selectivity for the amines to give fairly simple chromatograms from plasma samples. However, the methods do not have sufficient sensitivity to assay dopa-

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mine (DA) in plasma. Only a few precolumn derivatization methods for CAs have been reported: they use the reactions between CAs and fluorogenic reagents for amines, Dns chloride [6] and *o*-phthalaldehyde [7]. These methods are neither very sensitive nor selective for CAs.

We have investigated the use of 1,2-diphenylethylenediamine (DPE) for sensitive and selective determination of catechol compounds under mild conditions (pH 6—7, 0—50°C) in the presence of potassium ferricyanide [13, 14]. This reaction is accelerated by glycine or water-miscible organic solvents, and it has been applied to precolumn fluorescence derivatization in HPLC for the determination of CAs in human urine [14].

This study aims to apply precolumn derivatization to the HPLC determination of CAs in humn plasma, where the concentrations are extremely low compared with those of CAs in human urine. Isoproterenol (IP), or N-methyldopamine (NMDA) if necessary, was used as internal standard.

EXPERIMENTAL

Reagents and solutions

NE bitartrate and DA hydrochloride were purchased from Wako (Osaka, Japan). E bitartrate and NMDA hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.), and IP hydrochloride from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of analytical-reagent grade. Deionized and distilled water was used. DPE was synthesized as described previously [13]. Lithium phosphate buffer (0.2 *M*, pH 5.8) was prepared by mixing 8 vols. of a solution containing 0.25 *M* lithium hydroxide and 0.2 *M* phosphoric acid and 1 vol. of 0.2 *M* phosphoric acid. DPE solution (0.1 *M*; apparent pH 6.7) was prepared by dissolving 212 mg of DPE in 10 ml of 0.1 *M* hydrochloric acid. Toyopak SP (strong cation-exchanger, sulphopropyl resin, Na^+, particle size 19—40 μm; Toyo Soda, Tokyo, Japan) cartridge was prepared by pouring 0.5 ml of 20% (v/v) Toyopak SP suspension in water (actual resin volume 0.1 ml) into a polyethylene tube (35 mm × 6 mm I.D.). The top of the cartridge can be connected to a disposable plastic syringe (2—10 ml; Termo, Tokyo, Japan), by which solutions or solvents are passed through the cartridge. The cartridge was washed successively with 1 ml of 2.0 *M* lithium hydroxide (twice), 5 ml of water (twice), 1 ml of 12 *M* hydrochloric acid—ethanol (1:9, v/v) (twice), 5 ml of water (twice) and 1 ml of 0.2 *M* lithium phosphate buffer (pH 5.8) (three times). Weak cation-exchangers, CM-Sephadex C-25 (Na^+; Pharmacia Japan, Tokyo, Japan) and Amberlite CG-50 type I (H^+; Rohm and Haas, Philadelphia, PA, U.S.A.) were washed as described previously [14]. Commercially available cartridges for sample clean-up, Bond-Elut C_{18} and C_8 (300 mg each; Analytichem International, Harbor City, CA, U.S.A.), Sep-Pak C_{18} (375 mg; Waters Assoc., Milford, MA, U.S.A.) and Toyopak ODS (315 mg; Toyo Soda), were washed with 10 ml of water before use. UFO Mini-30 filter for ultrafiltration (molecular weight of exclusion limit, ca. 30 000) was obtained from Toyo Soda. Heparinized blood (2 ml) was taken into a chilled polyethylene tube containing 5 mg of reduced glutathione and centrifuged at 1000 *g* at 4°C for 15 min. The plasma was stored at −70°C until assay.

Apparatus and HPLC conditions

A Beckman 112 solvent delivery module was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Hitachi 650-10 LC spectrofluorimeter fitted with a 18-μl flow-cell operated at an emission wavelength of 485 nm and an excitation wavelength of 345 nm; spectral bandwidths were set at 10 nm in both the excitation and emission monochromators. A TSK-gel ODS-120T column (particle size 5 μm, 150 mm × 4.6 mm I.D.; Toyo Soda) was used. This column can be used for more than 2000 injections when washed with a mixture of acetonitrile—methanol—water (5:1:4, v/v) at a flow-rate of ca. 1 ml/min for 15 min after daily analyses. The mobile phase was a mixture of acetonitrile—methanol—50 m*M* Tris—hydrochloric acid buffer (pH 7.0) (5:1:4, v/v) and the flow-rate was 1.0 ml/min. Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 850 fluorescence spectrophotometer fitted with a 90-μl flow-cell. Spectral bandwidths of 5 nm were used both for the excitation and emission wavelengths.

Procedures for clean-up of plasma sample

Cation-exchange chromatographic clean-up (recommended clean-up procedure). To 0.5 ml of plasma, 25 μl of 10 pmol/ml IP solution (or the solution containing 10 pmol/ml NMDA) and 0.5 ml of 0.2 *M* lithium phosphate buffer (pH 5.8) were added. The mixture was poured into a Toyopak SP cartridge. The cartridge was washed successively with 5 ml of water (twice) and 1 ml of aqueous 50% acetonitrile. The adsorbed amines were eluted with 300 μl of 0.6 *M* potassium chloride—acetonitrile (1:1, v/v) containing 0.6 m*M* potassium ferricyanide. The resulting eluate was used for the derivatization.

Clean-up by alumina treatment. An aliquot (1.0 ml) of the plasma sample and 50 μl of IP solution (10 pmol/ml) were added to 200 mg of acid-washed alumina [15] and the pH of the mixture was adjusted to 8.5 with 1.0 *M* potassium carbonate. The mixture was thoroughly mixed and the alumina was washed with 5 ml of water (three times). The adsorbed amines were eluted with 0.5 ml of 0.3 *M* hydrochloric acid, and the pH of the eluate was adjusted to pH 6.0—6.5 with 1.0 *M* sodium hydroxide. To 150 μl of the resulting solution, 10 μl of 20 m*M* potassium ferricyanide and 150 μl of acetonitrile were added and the mixture was used for the derivatization.

Clean-up by deproteinization with perchloric acid. An aliquot (1.0 ml) of plasma was mixed with 0.5 ml of 2.0 *M* perchloric acid and 50 μl of 10 pmol/ml IP solution. The mixture was centrifuged at 1000 *g* for 10 min. The supernatant was neutralized with 1.0 *M* potassium carbonate to pH 6.0—6.5 and centrifuged at 1000 *g* for 10 min to remove precipitates of potassium perchlorate. The supernatant (150 μl), after the addition of 10 μl of 20 m*M* potassium ferricyanide and 150 μl of acetonitrile, was used for the derivatization.

Clean-up by ultrafiltration. An aliquot (0.5 ml) of plasma was mixed with 25 μl of 10 pmol/ml IP solution. The mixture was placed on a UFO Mini-30 filter and ultrafiltered by centrifugation at 3000 *g* at 4°C for 15 min. An aliquot (150 μl) of the filtrate was mixed with 10 μl of 20 m*M* potassium ferri-

cyanide and 150 μl of acetonitrile, and the resulting solution was used for the derivatization.

Procedure for the fluorescence derivatization

To the sample solution obtained by the clean-up procedure, 50 μl of the DPE solution were added and the mixture was allowed to stand at 37°C for 40 min. The reaction was stopped by cooling the mixture in ice-water. A 100-μl aliquot of the mixture was injected into the chromatograph. The amounts of CAs were calculated based on the internal standard method.

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatogram obtained by application of 300 μl of a standard mixture of CAs, IP and NMDA. The DPE derivatives of these amines can be completely separated within 14 min, and no peaks arose from substances in the reagent blank prepared by treating water in place of the standard mixture. IP is normally employed as an internal standard in the present method and so the elution can be terminated within 8 min. The DPE derivatives in the eluates show the fluorescence excitation and emission maxima around 350 and 480 nm, respectively (Table I).

Aqueous methanol, acetonitrile, ethanol, tetrahydrofuran, dioxane and their mixture containing Tris—hydrochloric acid buffer, were examined as possible mobile phases for HPLC. Aqueous 70% methanol containing 50 m*M* Tris—hydrochloric acid buffer (pH 7.0) did not give very satisfactory resolution of the peaks for E and DA and required relatively long elution times (retention time for NMDA, 17.5 min), whereas aqueous 50% acetonitrile containing the above buffer afforded a good separation of the peaks but took a long time (re-

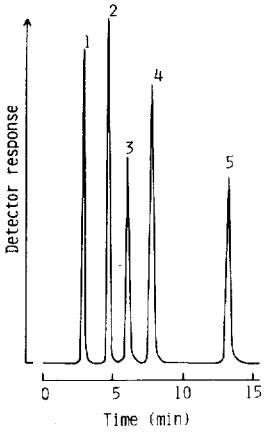

Fig. 1. Chromatogram of DPE derivatives of CAs, IP and NMDA. A portion (0.3 ml) of a standard mixture of CAs, IP and NMDA (10 pmol/ml each) in the eluent for the clean-up procedure with Toyopak SP cartridges was treated according to the procedure for the derivatization and HPLC. Peaks: 1 = NE; 2 = E; 3 = DA; 4 = IP; 5 = NMDA.

TABLE I

k' VALUES, FLUORESCENCE EXCITATION AND EMISSION MAXIMA AND RELATIVE PEAK HEIGHTS OF DPE DERIVATIVES OF CATECHOL COMPOUNDS

Portions (0.3 ml) of 1 nmol/ml catechol compounds dissolved in the eluent for the clean-up procedure with Toyopak SP cartridge were treated according to the procedure for derivatization and HPLC.

Compound	k' Value	Excitation maximum (nm)	Emission maximum (nm)	Relative peak height*
NE	1.9	345	470	91
E	3.6	350	480	100
DA	5.0	345	485	60
IP	6.7	365	480	81
NMDA	12.2	355	495	55
1-DOPA	0.6—9**	340	460	4**
DOPEG	1.5	340	460	11
DOPAC	0.6—7**	335	455	6**
	8.8	340	455	14
α-Methyldopa	6.1	350	495	14

*The peak height of DPE derivative from E was taken as 100.
**A broad peak was obtained and the height at the top was measured.

tention time for NMDA, 20.3 min). Consequently, a mixture of acetonitrile—methanol—Tris—hydrochloric acid buffer (5:1:4, v/v) was used for rapid and complete separation of CA.

The pH values (6.0—7.5) and concentrations (10—1000 m*M*) of Tris—hydrochloric acid buffer had no effect on the retention times and heights of the peaks for CAs, IP and NMDA; 50 m*M* Tris—hydrochloric acid buffer (pH 7.0) was employed in the recommended procedure. The buffer can be replaced with water, but the heights of the peaks were lowered with broadening of the peaks.

Derivatization

We have previously reported that many water-miscible organic solvents, especially ethanol and acetonitrile, accelerate the derivatization reaction [14]. Ethanol in the reaction mixture gave broad peaks that had retention times of 1.2—3.3 min even when thoroughly purified ethanol was used, and these peaks overlapped with the NE peak. Acetonitrile did not give any peaks in the chromatogram and so this was used in the procedure.

The acetonitrile concentration affected the peak heights for CAs, IP and NMDA (Fig. 2). Maximum peak heights were attained at the following concentrations (v/v) in the reaction mixture: 30—40% for NE; 40—50% for E, DA and IP; 60% for NMDA. An acetonitrile concentration of ca. 50% (v/v) was tentatively selected in the procedure.

The peak heights for NE, E and IP reached maximum and constant values after standing at 37°C for 30 min, whereas the heights for DA and NMDA did not reach stable maxima for the standing time of 30 min or longer (Fig. 3):

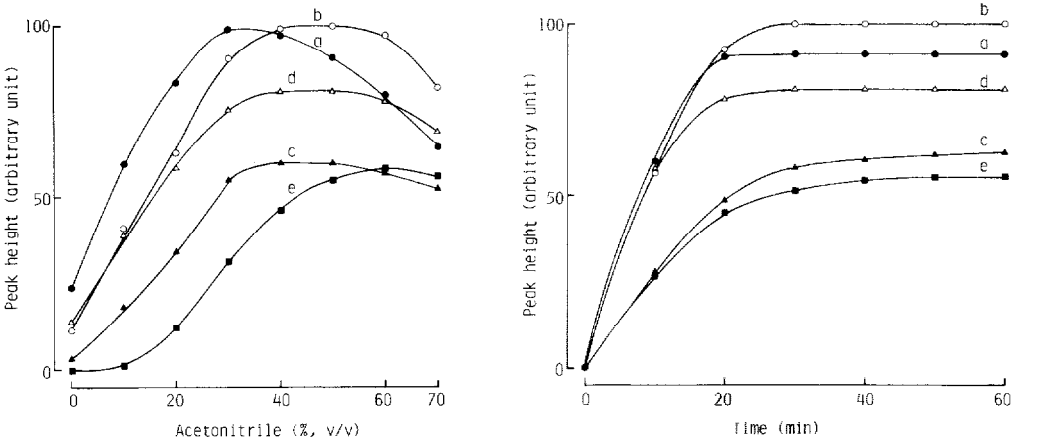

Fig. 2. Effect of acetonitrile concentration in the derivatization reaction mixture on the peak heights from CAs, IP and NMDA. Portions (0.3 ml) of a standard mixture of CAs, IP and NMDA (10 pmol/ml each) dissolved in potassium chloride–potassium ferricyanide (0.3 *M* and 0.6 m*M*, respectively) were treated as recommended with various concentrations of acetonitrile. Curves: a = NE; b = E; c = DA; d = IP; e = NMDA.

Fig. 3. Effect of standing time at 37° C on the peak heights from CAs, IP and NMDA. Portions (0.3 ml) of the standard mixture in Fig. 1 were treated as recommended for various times. Curves: a = NE; b = E; c = DA; d = IP; e = NMDA.

40-min warming was used in the recommended procedure, and the reaction of DA and NMDA was stopped by cooling the reaction mixture at 0°C. Other conditions of the derivatization are optimal [13, 14]. The DPE derivatives in the reaction mixture were stable for at least 8 h, even in daylight.

Determination of CAs in plasma

DPE also reacts with other catechol compounds of biological importance, such as 1-DOPA, 3,4-dihydroxyphenylethyleneglycol (DOPEG), 3,4-dihydroxyphenylacetic acid (DOPAC) and α-methyldopa under the conditions of the derivatization reaction. The DPE derivatives of these compounds could be separated under the HPLC conditions, and the individual eluates had fluorescence excitation and emission spectra similar to those of CAs, although their intensities (peak heights) were low (Table I). These compounds may interfere with the determination of CAs if they are present in plasma in high concentrations. For the removal of these compounds from plasma samples, chromatography on a cartridge of strong cation-exchanger (Toyopak SP) and on commercial reversed-phase cartridges for clean-up, alumina treatment, deproteinization with perchloric acid and ultrafiltration through a membrane filter were examined. The cartridges of reversed-phase chromatography did not retain CAs and IP and could not be used for clean-up. The other clean-up procedures could be used in principle for the purpose, and the chromatograms thus obtained with normal plasma are shown in Fig. 4a–d.

The peaks of CAs and IP (peaks 1–4) were well separated under the HPLC

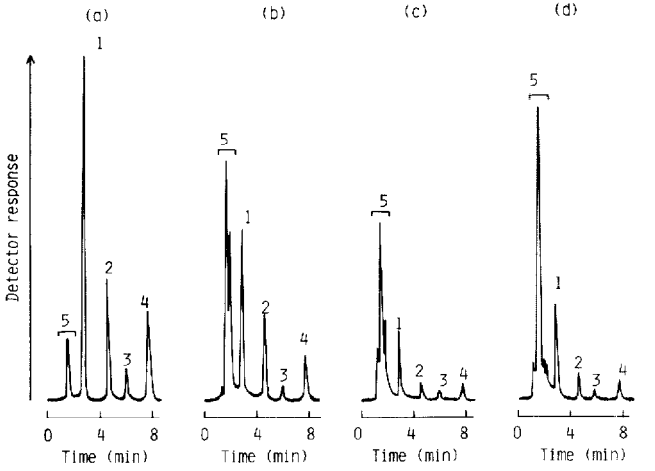

Fig. 4. Chromatograms obtained with a plasma added with IP. Portions (0.5—1.0 ml) of plasma were treated according to the clean-up procedures; (a) chromatography on Toyopak SP, (b) alumina treatment, (c) perchloric acid deproteinization and (d) ultrafiltration through UFO Mini-30 filter, followed by the procedure for the derivatization and HPLC. Peaks (concentrations in pmol/ml in parentheses): 1 = NE (1.72); 2 = E (0.56); 3 = DA (0.21); 4 = IP (0.50); 5 = unidentified peaks.

conditions, but some peaks (peaks 5) were still observed at retention times of ca. 1.5 min. The peaks for CAs (peaks 1—3) could be identified on the basis of their retention times and fluorescence excitation and emission spectra of the eluates in comparison with the standard compounds, and also by co-chromatography with the standards. The eluates of peak 5 in the chromatograms have fluorescence excitation and emission maxima at ca. 340 and 450 nm, respectively, which are characteristic of catechol compounds (Table I). The peaks were not observed when the DPE solution was omitted from the derivatization reaction. These observations suggest the peaks are due to endogenous catechol compounds that remain unremoved, though the compounds could not be identified.

Such catechol compounds in plasma were most effectively reduced by the chromatography on Toyopak SP. The chromatography gave higher recoveries (more than 85% for each amine, as described later) of CAs and IP added in plasma than those achieved in the other clean-up procedures: alumina treatment, ca. 60—70%; acid deproteinization and ultrafiltration, ca. 70—80%. Furthermore, the chromatographic procedure is much simpler than the others and does not cause dilution of the sample. Thus, chromatography on Toyopak SP was selected for the highly sensitive assay of CAs in plasma. Cation-exchange chromatographic clean-up on a CM-Sephadex C25 or Amberlite CG 50 Type I column was tested in the same manner as in the assay for urinary CAs [14], but the recoveries of CAs and IP were much lower (ca. 60% for each amine) than those obtained with the Toyopak SP cartridge.

The ionic form of Toyopak SP affected the recoveries of CAs and IP (Table II). Li^+, Na^+, K^+ and H^+ gave better recoveries, in that order. Therefore, the cation-exchanger (Na^+) was converted into Li^+ by the equilibration with a

TABLE II

RECOVERIES OF CATECHOLAMINES AND ISOPROTERENOL ADDED TO PLASMA

The amounts added to 0.5 ml of plasma, 0.5 pmol each.

Cation form of Toyopak SP	Percentage recovery (mean ± S.D., n=5)											
	Lithium chloride in the eluent*				Sodium chloride in the eluent*				Potassium chloride in the eluent*			
	NE	E	DA	IP	NE	E	DA	IP	NE	E	DA	IP
Hydrogen**	55±2	51±3	85±3	61±2	56±1	52±2	80±3	56±2	66±1	56±1	87±2	63±1
Lithium***	72±2	74±3	90±3	84±2	74±2	76±3	85±3	78±2	87±1	85±1	92±2	87±1
Sodium***	60±2	59±1	68±2	63±2	67±1	65±2	90±3	72±2	68±2	66±2	79±2	71±3
Potassium***	57±2	56±1	73±3	60±1	57±3	54±2	51±1	58±2	62±3	59±2	78±3	63±2

*Aqueous acetonitrile (50%, v/v) containing one of the chlorides in a concentration of 0.3 *M*.

**Toyopak SP cartridge was washed successively with 2 ml of 1 *M* hydrochloric acid and 10 ml of water before use. After the pouring of twice water-diluted plasma, the cartridge was washed successively with 5 ml of water, 2 ml of 0.2 *M* lithium phosphate buffer (pH 5.8), 10 ml of water and 1 ml of acetonitrile—water (1:1, v/v) and then eluted according to the clean-up procedure. The eluate was treated as recommended for the derivatization and HPLC.

***Toyopak SP cartridge, washed as described above, was equilibrated with lithium, sodium or potassium phosphate buffer (0.2 *M*, pH 5.8) before use, and the other procedures were the same as recommended except that lithium phosphate buffer for the pH adjustment of plasma was replaced with the corresponding alkali phosphate buffer.

lithium phosphate buffer before use. Lithium phosphate buffer of 0.1—0.25 *M* and pH 5.6—6.0 gave maximum recoveries of the amines: 0.2 *M* and pH 5.8 were optimal. This buffer was also used to adjust the pH of the plasma sample before pouring it into the cation-exchange cartridge.

The amines adsorbed on the cartridge were most effectively eluted with potassium chloride at almost saturated concentration (0.3 *M*) in aqueous 50% acetonitrile (Table II). Elution could not be achieved in the absence of acetonitrile, and its concentration ranging from 50 to 60% gave maximum and constant recoveries of the amines: concentrations above 60% resulted in the deposition of potassium chloride. Elution with lithium and sodium chlorides (0.3 *M* each) in aqueous 50% acetonitrile gave lower recoveries of the amines (Table II).

Linear relationships were obtained between the ratios of the peak heights of CAs to that op IP and the amounts of CAs added in the range 0.02—10 pmol each to 500 μl of plasma. The limits of detection for NE, E and DA in plasma were 7, 7 and 10 fmol/ml (1, 1 and 2 fmol per 100 μl of injection volume), respectively, at a signal-to-noise ratio of 2. The precision of the method was established by repeated determinations (n=10) by using a normal plasma. The

TABLE III

CONCENTRATIONS OF CATECHOLAMINES IN PLASMA FROM HEALTHY PERSONS*

Blood samples were collected at 9:00—10.00 a.m. from healthy volunteers who had a light breakfast at ca. 7:30 a.m.

Sex*	Age	Concentration (pmol/ml)		
		NE	E	DA
M	54	1.78	0.69	0.04
M	38	2.07	0.68	0.05
M	35	3.72	0.19	0.08
M	33	1.07	0.55	0.08
M	30	1.39	0.60	0.04
M	27	3.94	0.20	0.06
M	26	1.07	0.51	N.D.**
M	26	4.48	0.24	0.10
M	26	1.32	0.14	0.05
M	25	1.44	0.77	0.07
M	25	2.06	0.28	0.13
M	24	0.92	0.09	0.04
M	23	2.11	0.67	0.28
M	23	2.78	0.31	0.06
F	33	2.67	0.36	0.10
F	25	0.97	0.29	0.03
F	22	1.12	0.30	0.03
F	21	1.76	0.16	0.04
Mean ± S.D.		2.03 ± 1.08	0.39 ± 0.22	0.07 ± 0.06

*M = male; F = female.
**N.D. = Not detected.

coefficients of variation for NE, E and DA were 1.8, 2.4 and 3.7% at mean concentrations of 1.67, 0.52 and 0.10 pmol/ml of plasma, respectively.

The concentrations of plasma CAs from eighteen healthy persons were assayed by the present method (Table III). The values for NE and E are in fairly good agreement with data obtained by other workers [1—3, 8—10]. However, the DA concentration in plasma measured by the HPLC with ED varied over a wide range of less than 0.05 to 1.0 pmol/ml of plasma for unknown reasons [8—11], and the values obtained by the present method agreed with the lowest values of the reported data [8—10].

IP, an internal standard in this method, has been employed clinically as a bronchodilator in asthma and as a cardiac stimulant in cardiovascular disorders, and the amine in urine or plasma was determined by means of HPLC coupled with fluorescence detection [16] or ED [17]. The present method permitted simultaneous determination of CAs and IP with NMDA as internal standard. The pattern of the chromatogram for CAs and IP was identical with that of Fig. 4a, and the retention time for NMDA was 13.2 min. If necessary, a more rapid separation (within 9 min) of the peaks can be achieved by using a mobile phase consisting of acetonitrile—methanol—50 m*M* Tris—hydrochloric acid buffer (pH 7.0) (3:1:1, v/v).

This method is highly sensitive and simple enough to assay ten samples within 3 h. The method should be useful for biological, biomedical and clinical investigations of CAs.

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