HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF FREE CATECHOLAMINES
IN URINE USING 1,2-DIPHENYLETHYLENEDIAMINE AS NEW PRECOLUMN
FLUORESCENT DERIVATIZATION REAGENT

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A simple, rapid and highly sensitive method for the determination of free catecholamines (norepinephrine, epinephrine and dopamine) in urine is described which employs high performance liquid chromatography with fluorescence detection. Catecholamines and isoproterenol added as an internal standard in 100 μ l of urine, after chromatography on a small column of CM-Sephadex C25, are converted into the corresponding fluorescent compounds by the reaction with 1,2-diphenylethylenediamine in aqueous ethanol. These compounds are separated within 7 min on a reversed phase column, TSK-gel ODS-120T, with isocratic elution using aqueous dioxane containing a Tris hydrochloric acid buffer (pH 7.0). The detection limits of norepinephrine, epinephrine and dopamine are 20, 30 and 40 fmol in a 50 μ l injection volume, respectively.

Many methods using high performance liquid chromatography (HPLC) coupled with fluorescence detection have been introduced to the determination of catecholamines (CAs) in biological materials. They are classified into two types, post column derivatization methods such as trihydroxyindole (THI)¹⁾²⁾ and ethylenediamine condensation³⁾ methods, and pre column derivatization methods using fluorogenic reagents for amines, fluorescamine⁴⁾, o-phthalaldehyde⁵⁾ and Dansyl chloride⁶⁾. Though the THI methods have most widely been used because of their high sensitivity to 1-norepinephrine (NE) and 1-epinephrine (E), the sensitivity is not high enough to determine dopamine (DA) at low concentrations. The pre column derivatization methods are not generally very sensitive, and the derivatization reaction is not selective for CAs and so require rather complicated clean up procedures for biological samples.

In the previous paper, we reported that 1,2-diphenylethylenediamine (DPE) reacted sensitively and selectively with catechol compounds in neutral medium in the presence of potassium ferricyanide at ambient temperature to give fluorescent compounds and that this reaction was accelerated by glycine, and thus a fluorimetric method was established for the determination of CAs⁷⁾. The reaction mixture with DA in this method, however, gives two peaks in the chromatogram when subjected to a reversed phase HPLC. Therefore, this reaction cannot be successfully

applied to pre column derivatization of CAs.

Recently, we have found that water miscible organic solvent such as ethanol accelerates the reaction of DPE with CAs, and the reaction mixture gives single peaks for individual CAs and dl-isoproterenol(IP) in a reversed phase HPLC.

This study aims to apply the above findings to the pre column derivatization of CAs and to establish the conditions of rapid separation of their DPE derivatives by HPLC. We also describe the application of this method to the determination of free CAs in urine of normal human, using IP as an internal standard.

EXPERIMENTAL

Reagents and solutions

NE bitartrate and DA hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). IP hydrochloride and E bitartrate were obtained from Nakarai Chemicals(Kyoto, Japan) and Sigma Chemicals(St. Louis, U.S.A.), respectively. All other chemicals were of reagent grade. Deionized and distilled water was used. DPE was synthesized as described previously⁷⁾. Distilled ethanol was used. DPE solution(0.1 M; apparent pH, 7.0) was prepared by dissolving 212 mg of DPE in 10 ml of ethanol - 0.25M hydrochloric acid(6 : 4, v/v). CM-Sephadex C25 (Pharmacia Japan, Tokyo, Japan) column was prepared by packing 0.2 ml of the ion exchanger to a glass tube(130 mm × 4 mm I.D.) in the usual manner. The column was washed successively with 2 ml of 0.1 M sodium hydroxide(once), 2 ml of 2 M sodium chloride(twice), 2 ml of water(three times) and finally 2 ml of 0.2 M phosphate buffer(pH 6.2)(three times). The used column can be regenerated by washing in the same way and is usable more than ten times. Urine(24 h) was collected in the presence of 6 M hydrochloric acid(10 ml), stored at 4°C and used within 2 weeks.

Apparatus

A TSK-gel ODS-120T column(250 mm × 4.6 mm I.D., Toyo Soda, Tokyo, Japan) was used. A Beckman 112 solvent delivery module was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve(50 µl loop) and a Gilson Spectra/gro filter fluorometer fitted with a 45 µl flow cell and 7-60X excitation and 3-72M emission filters. Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 850 fluorescence spectrophotometer fitted with a 20 µl flow cell setting the spectral bandwidths of 5 nm in both the excitation and emission monochromators. Temperature of the HPLC column was controlled by using a Toyo Soda HLC-805 column oven.

Procedure for the fluorescent derivatization and HPLC (Procedure A)

To 0.8 ml of a sample solution, 1.2 ml of ethanol, and 0.1 ml each of the DPE solution and 15 mM potassium ferricyanide solution were successively added. The mixture was allowed to stand at 37°C for 30 min or longer to develop the fluorescence. A 50 µl portion of the mixture was injected into the chromatograph. The mobile phase was a mixture of dioxane and 50 mM Tris hydrochloric acid buffer(pH 7.0)(3 : 2, v/v). The flow rate was 1.0 ml/min. The column temperature was 50°C.

Procedure for the determination of urinary CAs (Procedure B)

To 100 μ l of a urine sample, 100 μ l of 1 nmol/ml IP solution and 1 ml of 0.2 M phosphate buffer(pH 6.2) were added. An aliquot of the mixture (1 ml) was poured on to the CM-Sephadex C25 column. The column was washed three times with 1 ml of water, and the adsorbed amines were eluted with 0.8 ml of 2.0 M sodium chloride. The eluate(0.8 ml) was treated by the procedure A. The amount of CAs were calculated based on an internal standard method. For calibration of the ratios of the peak heights due to individual CAs to that due to IP, 100 μ l of urine sample was replaced with 100 μ l of a standard solution of CAs(1 nmol/ml each) and the same procedure was carried out.

RESULTS AND DISCUSSION

HPLC conditions

Figure 1 shows the chromatograms obtained with a standard solution of CAs and IP, and with water for reagent blank. The DPE derivatives of these amines can be completely separated within 7 min(Table 1). The DPE derivatives in the eluates show the fluorescence excitation and emission maxima around 355 and 470 nm, respectively(Table 1).

Aqueous organic solvents, methanol, ethanol, acetonitrile, tetrahydrofuran, dioxane, and their mixtures, were examined as mobile phase for reversed phase HPLC on TSK-gel ODS-120T. The peaks for E and DA can be most successfully separated when aqueous dioxane is used. The concentrations of dioxane in the mobile phase affect the retention times of the peaks(Fig. 2). With higher concentrations of dioxane, the peaks of the DPE derivatives shift to shorter retention times; 60% was selected for their rapid separation. Dioxane is very viscous and so the column should be kept at high temperature to reduce the

column pressure and to obtain adequate retention times of the peaks. A temperature of 50°C was employed tentatively(the column pressure was 140 kg/cm²).

The pHs(6.0-8.0) and the concentrations(10-100 mM) of Tris hydrochloric acid buffer have no effect on the retention times and heights of the peaks; 50 mM Tris hydrochloric acid buffer of pH 7.0 was employed in the procedure. The Tris hydrochloric acid buffer can be replaced with water, but the peaks slightly broaden.

The detection limits for the compounds in a standard mixture were 10 fmol each for NE and IP, and 15 fmol each for E and DA per 50 μ l of injection volume.

Derivatization

Water miscible organic solvents, methanol, ethanol, acetonitrile, dioxane,

Fig. 1 Chromatograms of (a) DPE derivatives of CAs and IP, and (b) reagent blank

Concentrations of CAs and IP:
0.1 nmol/ml each Peaks: 1;
reagent blank, 2; NE, 3; E, 4;
DA, 5; IP

Table 1 Retention times, fluorescence excitation and emission maxima and relative peak heights of the DPE derivatives of catechol compounds

Compound	Retention time / min	Excitation maximum / nm	Emission maximum / nm	Relative peak height ^a
NE	3.8	350	468	100
E	4.8	360	470	65
DA	5.5	350	480	63
IP	6.5	354	475	96
1-DOPA	4.6	344	482	32
3,4-Dihydroxybenzyl-amine	3.2	336	448	70
3,4-Dihydroxyphenyl-ethyleneglycol	3.6	337	454	51
3,4-Dihydroxyphenyl-acetic acid	3.3	343	450	24

Portions(0.8 ml) of 1 nmol/ml solutions of catechol compounds were treated according to the procedure A. a, The peak height of DPE derivatives from NE was taken as 100.

2-methoxyethanol and acetone, accelerate the derivatization reaction. Of these solvents, ethanol and acetonitrile are most effective; ethanol was selected in the standard procedure for convenience. Maximum and constant peak heights are attained at ethanol concentrations in the reaction mixture in a range of 50 to 70 % (v/v) for NE, E and IP, and in a range of 60 to 70 % (v/v) for DA (Fig. 3); ethanol concentration of approximately 60 % (v/v) was selected as optimum. Figure 4 shows the effect of reaction time at 37°C on the peak heights for CAs and IP. The peak heights reach maximum and constant values after standing for 30 min or longer. Other conditions of the derivatization are optimum⁷⁾. The DPE derivatives in the reaction mixture were stable for at least 8 h even in daylight.

Determination of CAs in urine

Urine contains various catechol compounds other than CAs which are mainly metabolites of CAs, such as 3,4-dihydroxyphenylethyleneglycol and

Fig. 2 Effect of the concentration of dioxane in the mobile phase on the retention times of DPE derivatives of CAs and IP

Concentrations of CAs and IP: 0.1 nmol/ml each Curves: a; NE, b; E, c; DA, d; IP, e; reagent blank

Fig. 3 Effect of the concentration of ethanol in the derivatization reaction on the peak height

Concentrations of CAs and IP: 0.1 nmol/ml each Curves: a; NE, b; E, c; DA, d; IP, e; reagent blank

Fig. 4 Effect of standing time at 37°C on the peak height

Concentrations of CAs and IP: 0.1 nmol/ml each Curves: a; NE, b; E, c; DA, d; IP, e; reagent blank

3,4-dihydroxyphenylacetic acid⁸). They react with DPE to afford fluorescent compounds (Table 1) and interfere with the assay of CAs if they present in urine at high concentrations. For the removal of these compounds from urine sample, chromatography on small columns of strongly acidic cation exchanger (Dowex 50W×8) and weakly acidic cation exchangers (CM-Sephadex C25, Amberlite CG50 Type I and Dowex CCR2) were tested. A clean up technique using acid-treated alumina⁹ was also examined. A CM-Sephadex C25 column gave most reproducible recoveries of CAs and IP; the recoveries of 0.1 nmol each of the amines applied on to the column as 1.0 ml of their solution in 0.2 M phosphate buffer (pH 6.2) were 71.7 ± 1.5 for NE, 61.5 ± 2.4 for E, 63.7 ± 1.1 for DA and 57.9 ± 2.2 for IP (mean $\pm$ standard deviation (S.D.), $n=12$ each). The compounds having no amino group and l-DOPA in urine sample were completely removed by this chromatography.

Sodium chloride solution as eluent of CAs and IP in the chromatography on the Sephadex column has an effect on the peak heights especially for DA. Sodium chloride at a concentration of approximately 1.5 M in the eluate reduced the peak height for DA to 55 % and those for NE, E and IP to 95 % of those obtained with CAs and IP dissolved in water, respectively. This is large enough to measure DA because the amine occurs at high concentrations in urine.

Figure 5 shows a typical chromatogram obtained with a normal human urine.

Peaks for CAs and IP are successfully separated under the conditions of HPLC. The components of peaks 2-4 were identified on the basis of the retention times and fluorescence excitation and emission spectra of the eluates by comparison with a standard solution of CAs and also by co-chromatography of the standard and urine sample. The peak height for DA is reduced by the presence of sodium chloride in the eluate for the reason described above. Peak 1 in the chromatogram has a retention time of 3.3 min. The peak is due mainly to endogenous compound(s) in urine sample and partially to the reagent blank, and its eluate has fluorescence excitation and emission maxima at 340 and 450 nm, respectively. This suggests that the peak component(s) may be some compound(s) with catechol and amino moieties, though the component(s) could not be identified. Peak 1 did not interfere with the determination of NE (peak 2) when the procedure B was applied to 20 samples of normal urine, and so further clean up of the sample is not necessary.

Linear relationships were observed between the ratios of the peak heights of CAs to that of IP and the amounts of CAs added in the range of 0.01-1.0 nmol each to 100 μ l of urine. The recoveries of NE, E, DA and IP added in the amounts of 0.1 nmol each to 100 μ l of urine, were 69.8 ± 4.9 , 64.5 ± 2.5 , 62.7 ± 4.1 and 58.2 ± 3.7 % (mean $\pm$ S.D., $n=6$ each). The limits of detection for NE, E and DA in urine were 1.0, 1.5 and 2.0 pmol/0.1 ml (20, 30 and 40 fmol/50 μ l of injection volume), respectively, at a signal-to-noise ratio of 2. The precision of the method was established with respect to repeatability by using an urine sample. The coefficients of variation for NE, E and DA were 3.2, 4.8 and 3.0 % at mean concentrations of 0.25, 0.11 and 2.57 nmol/ml urine, respectively.

The amounts of free CAs in 24 h urine from 20 healthy persons (22-53 years old) assayed by this method were 221.9 ± 93.9 nmol (37.6 ± 15.9 μ g) for NE, 83.9 ± 51.9 nmol (16.4 ± 9.5 μ g) for E and 2.15 ± 0.94 μ mol (329 ± 144 μ g) for DA (mean $\pm$ S.D.). These values are in good agreement with data obtained by other workers¹⁰⁾.

This method is highly sensitive and rapid enough to assay 10 samples within 3 h. The THI methods cannot determine DA at low concentrations and the pre column derivatization methods using fluorescamine or o-phthalaldehyde do not allow to determine E, a secondary amine. The pre column derivatization method using Dansyl chloride is liable to form artifact and the Dansyl derivatives are unstable.

Fig. 5 Chromatogram obtained with urine sample according to the procedure B

Peaks: 1; unidentified peak including that of the reagent blank, 2; NE, 3; E, 4; DA, 5; IP Urinary CA concentrations (nmol/ml): NE; 0.31, E; 0.19, DA; 2.25

The present method permits the assay of all the CAs simultaneously and does not require complicated clean up procedure compared with the HPLC with electrochemical detection¹⁰⁾¹¹⁾. This method should be useful for biological and biomedical investigations of human diseases accompanying change in the level of urinary CAs.

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