High-Performance Liquid Chromatographic Analysis of Catecholamines by Native Fluorescence Measurements

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Abstract: Three kinds of catecholamines and a related compound, dopamine, norepinephrine, epinephrine and dopa were separated completely by high-performance liquid chromatography using a weak cation-exchange gel column and monitored by the native fluorescence measurements. The calibration curve for each catecholamine was a straight line within the range of 0.5 ng to 300 ng. Recoveries of catecholamines in sample solutions were examined by varying the amount of activated alumina as an adsorbent and the concentration of acetic acid as an eluent. The use of 0.40 g of activated alumina and 2 ml of 0.8 M acetic acid resulted in the recoveries of nearly 85% for norepinephrine, epinephrine and dopamine with the coefficients of variation of nearly 2% and 68% for dopa with the coefficient of variation less than 5% from a urine solution containing a known amount of each catecholamine.

Key words: catecholamine, native fluorescence monitoring, HPLC.

(Received 20 August 1980)

Introduction

Recent investigations on the analysis of catecholamines in urine and biological fluids have been carried out by use of high-performance liquid chromatography (HPLC) (Mori, 1975; Higa *et al.*, 1977; Imai *et al.*, 1977; Molnár & Horváth, 1977; Ueda *et al.*, 1977; Imai & Tamura, 1978; Mori, 1978; Anderson *et al.*, 1980). Because of the complexity of the sample matrix, analytical procedures for catecholamines are usually composed of three steps, i.e., purification and concentration, chromatographic separation and detection. The purification and concentration of catecholamines is performed by an adsorption method using activated alumina (Von Euler & Lishajko, 1959; Ueda *et al.*, 1977), ion-exchange resin (Seki, 1976) and aluminum hydroxide or a solvent extraction method (Yoshida *et al.*, 1976). The chromatographic separation is carried out by HPLC using ion-exchange resin (Mori, 1975; Ueda *et al.*, 1977; Okamoto *et al.*, 1978) or bonded-phase column packings (Knox & Jurand, 1976; Crombeen *et al.*, 1978; Asmus & Freed, 1979; Krstulovic *et al.*, 1979). For the detection of catecholamines in the chromatographic effluent, several detection systems are employed, e.g., ultraviolet absorbance detection (Molnár & Horváth, 1977), fluorescence detection with pre-column derivatization (Imai,

1975) or post-column reaction (Von Euler & Lishajko, 1961; Ueda *et al.*, 1977; Okamoto *et al.*, 1978), native fluorescence detection (Miyagawa *et al.*, 1978; Krstulovic & Powell, 1979) and electrochemical detection (Scratchley *et al.*, 1979; Anderson *et al.*, 1980). A large amount of literature on the chromatographic analysis of catecholamines have been published. Among them, the reported data on the recoveries of catecholamines in the adsorption-desorption step with alumina were not satisfactory (Von Euler & Lishajko, 1959; Ueda *et al.*, 1977). In addition, the use of native fluorescence measurements in the HPLC analysis of catecholamines has only been reported recently in a few works and any detailed information is unavailable.

In the present work, the authors examined the calibration curves for the three kinds of catecholamines and a related compound, dopamine, norepinephrine, epinephrine and dopa by use of native fluorescence measurements. The authors determined also the relationship between the recoveries of individual catecholamine investigated, the amount of activated alumina as an adsorbent and the concentration of acetic acid in the eluent.

Experimental

Materials

L-3,4-dihydroxyphenylalanine (L-dopa; Tokyo-Kasei, Japan), Dopamine hydrochloride (DA; Wako, Japan), L-norepinephrine bitartrate (NE; Wako, Japan) and L-epinephrine bitartrate (E; Sigma, U.S.A.), were used without further purification. The individual catecholamine was dissolved in 0.1 M hydrochloric acid and several mixed solutions with different concentrations of catecholamines were prepared. These sample solutions were stored in a freezer before use. A weak cation-exchange resin, Hitachi Gel 3011-C, 10–15 μm (Hitachi, Japan), was used as a column-packing. Activated alumina (200 mesh) was obtained from Wako. All other chemicals were of analytical-reagent grade.

Apparatus

All chromatographic experiments were carried out on a high-performance liquid chromatograph (Hitachi 638) using a UV monitor (254 nm), a Hitachi 650–10 LC fluorescence spectrophotometer and a thermostated stainless-steel column of dimensions 250 $\times$ 4 mm I.D. fed by a thermostated water bath (Haake F3, F.R.G.).

Chromatographic conditions

Hitachi Gel 3011-C was packed in the stainless-steel column by a pressurized slurry method. Eluent was composed of 0.1 M KH_2PO_4 –0.05% H_3PO_4 and degassed by sonicating for 10 min before use. The column was operated at a flow rate of 1.5 $\text{ml}\cdot\text{min}^{-1}$ and the column temperature of 20, 30, 40 or 50°C. Native fluorescence measurements were carried out with an excitation wavelength of 275 nm and an emission wavelength of 325 nm. Some chromatographic parameters, retention time, peak height and peak area,

were printed by use of a computing integrator equipped with a Hitachi 638 Liquid Chromatograph.

Recoveries of catecholamines in standard aqueous solution

Sample solutions were prepared by mixing 5 ml of dilute ammonia water with pH 8.5 and 1 ml of a standard solution containing 1 μ g of each catecholamine per ml. The pH of the sample solution was then adjusted again to 8.5. All of the sample solution was passed through a glass column (9 mm I.D. $\times$ 180 mm) containing 0.10 g of activated alumina and washed with 10–15 ml of distilled water. The catecholamines adsorbed in the alumina were eluted with 2 ml of 0.6 M acetic acid and the effluent was collected in a test tube. Similar procedures were carried out for other sample solutions except for the variable amounts of activated alumina in the glass column from 0.10 g to 1.50 g. Ten μ l of the effluent was applied to the liquid chromatograph and the amount of each catecholamine was determined by native fluorescence measurements. The recoveries of catecholamines were obtained by comparing the chromatographic peak areas or heights with those for the standard solution.

The effect of the composition of the solvent on the elution of the catecholamines adsorbed on alumina was examined by the same method as described above except for the use of 0.40 g of activated alumina and a series of acetic acid solutions with different concentrations from 0.2 M to 1.2 M.

Recoveries of catecholamines in urine

One ml of 6 M HCl was added to 50 ml of urine sample and the conjugated catecholamines were acid-hydrolyzed in boiling water for 20 min. After cooling and adding 0.25 g of disodium ethylenediaminetetraacetate, the urine sample was adjusted to pH 8.5 with 7% ammonia water. Then, the solution was immediately passed through a glass column containing 0.40 g of activated alumina. Sample solutions were prepared by mixing 5 ml of the treated urine solution and 1 ml of a standard solution containing 1 μ g of each catecholamine per ml and then the pH of the solution was adjusted to 8.5. All of each sample solution was passed through a glass column (9 mm I.D. $\times$ 180 mm) containing 0.40 g of activated alumina and washed with 10–15 ml of distilled water. The catecholamines adsorbed on alumina was eluted with 2 ml of 0.8 M acetic acid and the effluent was collected in a test tube. The separation and determination of each catecholamine in the effluent was carried out by the same method as described above.

Results and Discussion

Chromatographic separation of catecholamines

Three kinds of catecholamines and a related compound, NE, E, DA and L-dopa, were separated completely by the HPLC system used in the present work and the retention time

decreased for all samples with increasing column temperatures from 20°C to 50°C as shown in Fig. 1. A typical chromatogram for a mixed solution of catecholamines is given in Fig. 2, which shows a well-resolved peak for each component within 20 min at the column temperature of 50°C. These results suggest that the present chromatographic system is applicable to the determination of catecholamines.

Fig. 1.

Fig. 1. Relationship between column temperature and retention times of catecholamines. Eluent: 0.1 M KH_2PO_4 . Flow rate: $1.5 \text{ ml} \cdot \text{min}^{-1}$. Pressure: $46 \text{ kg} \cdot \text{cm}^{-2}$. Detection: native fluorescence measurement (Ex. 275 nm, Em. 325 nm). Sample: 1=L-dopa, 2=NE, 3=E, 4=DA.

Fig. 2.

Fig. 2. A typical chromatogram of catecholamines. A: native fluorescence (Ex, 275 nm, Em. 325 nm). B: UV (254 nm). Column: Hitachi Gel 3011-C, $250 \times 4 \text{ mm}$ I.D. Temperature: 50°C. Other chromatographic conditions same as in Fig. 1. Sample: 1=L-dopa (5 μg), 2=NE (5 μg), 3=E (5 μg), 4=DA (5 μg).

Calibration curves for catecholamines

Although the native fluorescence measurement with excitation at 275 nm and emission at 325 nm is not exclusively selective for catecholamines, it provides a high-sensitive monitoring device (Krstulovic & Powell, 1979; Scratchley *et al.*, 1979). Fig. 3 gives the relationship between the amount of each catecholamine injected into the liquid chromatograph and the corresponding peak area monitored by native fluorescence measurements. The calibration curve for each catecholamine was confirmed to be a straight line with the characteristic slope of it within the range of 0.5 ng to 300 ng. Similar relationships were obtained by use of peak heights instead of peak areas. A clear peak was obtained for each catecholamines even as small as the injected amount of 0.5 ng for L-dopa, NE and E, and 1.0 ng for DA, respectively. These results indicate that the detection of catecholamines by use of native fluorescence give satisfactorily reliable data as a monitor of HPLC.

Fig. 3. Calibration curves for catecholamines by native fluorescence measurements. Chromatographic conditions same as in Fig. 2. Sample: 1=DA, 2=L-dopa, 3=NE, 4=E.

Effect of the amount of activated alumina as an adsorbent and the concentration of acetic acid as an eluent on the recoveries of catecholamines

Fig. 4 gives the recoveries of catecholamines by use of different amounts of activated alumina as the adsorbent and 2 ml of 0.6 M acetic acid as the eluent. For the different kinds of catecholamines investigated, plateaus in recoveries were obtained within the range of 0.25 g to 0.50 g of activated alumina although the recovery of L-dopa was considerably smaller than those of NE, E and DA. From these results it is expected that the use of 0.40 g of activated alumina provides a maximal and constant recovery for each catecholamine.

Then, the recoveries of catecholamines from 0.40 g of activated alumina were examined for a series of eluents with different concentrations of acetic acid. The results are given in Fig. 5. The recoveries of E, NE and DA are practically constant values, near 80% for the eluents with concentration of acetic acid from 0.3 M to 1.2 M. On the other hand, the recovery of L-dopa increased with the increasing concentration of acetic acid and reached a plateau of nearly 60% at the eluent with 0.8 M acetic acid.

Fig. 4.

Fig. 4. Relationship between recoveries of catecholamines and amounts of activated alumina. Eluent: 0.6 M acetic acid (2 ml). Sample: 1=E (1 μ g), 2=NE (1 μ g), 3=DA (1 μ g), 4=L-dopa (1 μ g).

Fig. 5.

Fig. 5. Relationship between recoveries of catecholamines and concentration of acetic acid. Activated alumina: 0.40 g. Volume of eluent: 2 ml. Sample: 1=E (1 μ g), 2=DA (1 μ g), 3=NE (1 μ g), 4=L-dopa (1 μ g).

Table 1. Recovery of catecholamine from urine and its retention time in HPLC analysis

Run	L-Dopa			Norepinephrine			Epinephrine			Dopamine		
	Recovery A.	H.	R. T.	Recovery A.	H.	R. T.	Recovery A.	H.	R. T.	Recovery A.	H.	R. T.
1	62.0	63.0	3.40	83.0	81.5	6.61	81.5	81.0	9.24	88.0	84.0	14.13
2	69.0	70.0	3.40	85.5	85.5	6.10	86.0	86.5	9.21	85.5	85.5	14.09
3	70.5	71.0	3.41	86.0	85.5	6.16	84.0	86.0	9.25	90.5	85.5	14.16
4	67.0	68.0	3.42	85.8	86.0	6.16	84.5	87.0	9.29	84.0	85.5	14.29
5	70.5	70.5	3.14	87.0	86.0	6.10	85.5	87.0	9.17	87.5	85.5	14.14
6	66.5	67.0	3.38	87.0	85.5	6.09	84.5	87.0	9.20	88.5	89.0	14.24
Mean	67.6	68.3	3.40	85.7	84.9	6.13	84.3	85.8	9.23	87.3	85.8	14.18
C. V.	4.76	4.39	0.29	1.72	2.02	0.49	1.87	2.75	0.43	2.62	1.94	0.54

A. : Peak area measurement

H. : Peak height measurement

R. T. : Retention time (min)

C. V. : Coefficient of variation

On the basis of these results, it is concluded that the use of 0.40 g of activated alumina and 2 ml of 0.8 M acetic acid is a reasonable experimental condition for the adsorption of catecholamines from the sample solution and the desorption of them from the activated alumina, respectively.

Recoveries of catecholamines from urine

In order to confirm the practical applicability of the present method to urine samples, 1 μ g of each catecholamine was added to the pre-treated urine solution and the recovery was determined. The results are given in Table 1. Retention time for each catecholamine in the chromatographic separation is also listed in the same table.

Although the recoveries of the different kinds of catecholamines investigated are a little higher than those expected from the data in Fig. 5, this data seems to be reliable judging from the coefficients of variation as small as nearly 2% for NE, E and DA, and less than 5% for L-dopa. The reproducibility in retention time for each catecholamine by HPLC analysis was also satisfactory.

In conclusion, the present method was confirmed to be a useful one for the sample preparation followed by a HPLC analysis of catecholamines. However, there are many components in the extracted solution from biological samples, some of which may be detected by the native fluorescence measurements under the conditions employed here. For the quantitative analysis of catecholamines and related compounds in biological samples, it is required that some HPLC system with higher resolution efficiency is combined with the present method for the sample preparation and detection.

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高速液体クロマトグラフィー・自然蛍光法を用いたカテコールアミンの分析

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要 旨: カテコールアミンおよびその関連物質、ドーパミン、ノルエピネフリン、エピネフリンおよびドーパ、弱陽イオン交換樹脂を用いた高速液体クロマトグラフィーにて分離し、自然蛍光測定によって検出した。自然蛍光測定より得られた各々のカテコールアミンの検量線は 0.5-300 ng の範囲で直線を示した。試料液中のカテコールアミンの回収率に対する吸着剤として活性化アルミナの量そして溶離液として酢酸濃度の影響について検討を加えた。活性化アルミナ 0.4 g と 0.8 M 酢酸 2 ml を用いて、既知量のカテコールアミンを含む尿中からの回収率と変動係数を求めた。ノルエピネフリン、エピネフリンおよびドーパミンの回収率と変動係数はほぼ85%と 2 %, ドーパのそれらはほぼ68%と 5 %以下であった。

J. UOEH (産業医大誌), 2 (4): 439-447 (1980)