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# Simultaneous determination of dopamine and 3,4-dihydroxyphenylacetic acid in mouse striatum using mixed-mode reversed-phase and cation-exchange high-performance liquid chromatography

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ABSTRACT

The measurement of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) is useful for an index of dopamine turnover. We developed a simultaneous determination method for dopamine and DOPAC with high-performance liquid chromatography-fluorescence detection. Mixed-mode reversed-phase and cation-exchange column (CAPCELL PAK CR column) which contained C18 silica particles and sulfonic acid cation-exchange particles was used for the separation of 3,4-dihydroxy-L-phenylalanine, catecholamines (norepinephrine, epinephrine, and dopamine) and DOPAC. The mobile phase was optimized for factors such as pH and counter ion concentration. With a mobile phase of 15 mM sodium acetate buffer (pH 4.5), separation was achieved within 22 min. The developed method was applicable to the determination of dopamine and DOPAC in mouse striatum. The concentrations of dopamine and DOPAC in mouse striatum were $4.98 \pm 0.66$ and $1.00 \pm 0.11$ μM, respectively ($n = 10$).



## 1. Introduction

Dopamine works as a neurotransmitter or hormone in mammals and has an important role in motor control, motivation, learning, memory and signaling reward [1]. Several diseases such as schizophrenia [2] and Parkinson's disease [3] have been linked to defective dopamine neurotransmission. Dopamine is metabolized in 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase. Comparing the ratio of dopamine to DOPAC can exhibit metabolic or neurotransmitter utilization information [4–6]. Hence, the simultaneous determination of dopamine and DOPAC is significant.

The determination methods for catecholamines, namely, norepinephrine, epinephrine and dopamine [7–9], and for DOPAC and homovanillic acid (HVA) [10] have already been developed. Dopamine and DOPAC can be determined with these methods. However, these demand significant time and samples. Hence, the simultaneous determination of dopamine and DOPAC in a single run is preferable.

Mixed-mode reversed-phase and ion-exchange high-performance liquid chromatography (HPLC), which accommodate the hydrophobic alkyl chains and ionic functional groups, have been used to separate a complex mixture of ionizable and ionic organic compounds. Mixed-mode HPLC can be achieved by connecting two columns with ODS and cation-exchange stationary phase [11], by mixing two types of stationary phases of reversed-phase and ion-exchange stationary phases in a column [12,13], and by chemically binding an ionizable group in an alkyl chain on silica based support [14,15]. Recently, new mixed-mode reversed-phase and cation-exchange columns (CAPCELL PAK CR) were developed, in which C18 silica particles and strong cation-exchange (SCX) particles are mixed inside. The CR column is available with different mixing ratios that were not possible to obtain by connecting two columns: SCX:C18 = 1:50, 1:20 and 1:4. We found that CR column is useful for the simultaneous determination of dopamine and DOPAC. In this study, the simultaneous determination method for dopamine and DOPAC was developed, and the method was applicable to the determination of dopamine and DOPAC in mouse striatum.

## 2. Materials and methods

### 2.1. Reagents

All reagents were obtained from commercial sources and, unless noted otherwise, were reagent grade or better. Norepinephrine, epinephrine, dopamine, and 3,4-dihydroxyphenylacetic acid (DOPAC) were purchased from Sigma (St. Louis, MO, USA). Ethylenediamine was from Aldrich. Acetonitrile and ethanol, both of HPLC grade, were purchased from Wako Pure chemicals (Osaka,

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Japan). Imidazole was obtained from Merck (Darmstadt, Germany). The water used was purified on a Milli Ro-Milli Q system (Nihon Millipore, Tokyo, Japan).

### 2.2. Instruments

The system consisted of a NANOSPACE series (Shiseido, Tokyo, Japan) with addition of a thermostatically controlled bath (ASB 200D, JASCO, Tokyo, Japan) and an integrator (807-IT, JASCO). Two 2001 pumps, a 2003 autosampler, a 2004 column oven, a 2011 six-way valve, and a 2013 fluorescence detector were arranged.

### 2.3. Chromatographic conditions

For the mixed-mode determinations, the columns CAPCELL PAK CR 1:50, 1:20 and 1:4, 150 mm × 2.0 mm i.d. with 5 μm particles (Shiseido) were used. For the reversed-phase mode, the column was CAPCELL PAK C18 MGII, 150 mm × 2.0 mm i.d. with 5 μm particles (Shiseido). The mobile phases were 10–30 mM sodium acetate buffer (pH 3.5–5.5). The flow rate was 200 μl/min. The column temperature was 35 °C. The fluorescence detection was made at an emission wavelength of 320 nm with excitation at 280 nm.

### 2.4. Optimization of fluorescence derivatization conditions

The effects of the length of the reaction coil and the temperature of the fluorescence derivatization were investigated under the following HPLC conditions. The separation column used was CAPCELL PAK CR 1:20 (150 mm × 2.0 mm i.d.). The composition of the mobile phase was 15 mM acetate buffer (pH 4.5) and the composition of the fluorogenic reagent was 105 mM ethylenediamine and 175 mM imidazole in acetonitrile/ethanol/water (80/10/10, v/v/v). The flow rates of the mobile phase and the fluorogenic reagent were fixed at 200 and 192 μl/min, respectively. Twenty picomoles each of dopamine and DOPAC were injected into the HPLC system. The fluorescence detection was made at an emission wavelength of 495 nm with excitation at 417 nm. The temperature of the reaction coil (1.6 mm o.d. × 0.25 mm i.d. × 2.5–10 m), which was kept in a thermostatically controlled bath, was controlled at between 70 and 95 °C.

### 2.5. Animal experiments

All experiments were conducted according to the Japanese Pharmacological Society guide for care and use of laboratory animals. C57Bl/6J male mice (8–10 weeks old; CLEA Japan, Tokyo, Japan) were decapitated after ether anesthesia, and their brains were rapidly removed and placed in ice-cold, artificial cerebrospinal fluid (aCSF) of the following composition: 124 mM NaCl, 3 mM KCl, 1.24 mM $KH_2PO_4$, 1.4 mM $MgSO_4$, 2.2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, equilibrated with 95% $O_2$ and 5% $CO_2$. Coronal slices (400-μm thickness) were cut in chilled modified aCSF of the following composition: 127 mM NaCl, 1.6 mM KCl, 1.24 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, with a cooled VIB3000 vibratome (VIBRATOME, St. Louis, MO). Then the striata were dissected from the slices. The tissues were homogenized in 0.1 M perchloric acid and centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatant fluids were used for the measurements.

### 2.6. Validation of the method

Standard stock solutions were prepared by dissolving dopamine and DOPAC in 0.1% trifluoroacetic acid. Dopamine or DOPAC (each 0, 0.5, 1.5, 5, 15, or 50 pmol) were injected into HPLC. Linearity of the assay was assessed using 6-point calibration curves (0, 0.5, 1.5, 5, 15, or 50 pmol). Peak area ratios of dopamine and DOPAC to internal standard (*N*-methyl dopamine) were plotted against the corresponding concentrations and linear regression was performed using a $1/x$ weighted linear regression method.

To 10-μl aliquots of mouse striatum sample, dopamine and DOPAC (each 0, 1, 2 and 4 pmol) were spiked and analyzed by the HPLC. The accuracies were expressed as the reciprocal percentages of the amount of dopamine or DOPAC recovered from the spiked samples to the expected values. Both intra- and inter-day precision were calculated by analyzing the same mouse striatum samples five consecutive times or five successive days, respectively. The precision was estimated based on the coefficients of variation (CV, %).

## 3. Results and discussion

### 3.1. Optimization of chromatographic conditions

For simultaneous determination of dopamine and DOPAC, four different kinds of columns, namely a CAPCELL PAK CR 1:50, 1:20 and 1:4 (SCX:C18), and a CAPCELL PAK C18 MG II (reversed-phase), were investigated. Catechol compounds which are 3,4-dihydroxy-L-phenylalanine (L-DOPA), catecholamines (norepinephrine, epinephrine, and dopamine), *N*-methyl dopamine, and DOPAC were chosen for optimization in this study.

The optimum column that best suited the separation of the compounds mentioned above was first investigated. Fig. 1 shows the retention times of catechol compounds with a mobile phase of 15 mM sodium acetate buffer (pH 4.5). The retention times for only basic compounds, namely catecholamines and *N*-methyl dopamine, were changed depending on the column used while those of L-DOPA and DOPAC were almost the same. With C18 column, most compounds were not retained in the column, and mobile phase additives such as ion-pairing reagents have been necessary for separation of these compounds. When the ratio of SCX particles to C18 particles was increased, the retention times of amines were increased because of the interaction of amines with sulfonic group of SCX particles. This allowed increased retention of amines without ion-pairing reagents. The best separation was performed with CAPCELL PAK CR 1:20, and this column was used for further investigations.

Based on sodium acetate buffer, the influence of buffer concentrations and pH were studied. Fig. 2 depicts the influence of buffer concentrations of sodium acetate buffer (pH 4.5) on the retention of catechol compounds. As expected, the increase in buffer concentration decreases the retention of amines and has no effects on the retention of neutral compounds such as L-DOPA and DOPAC. In the case of ODS column, the concentration of the buffer usually did not affect retention of the analytes significantly. This result

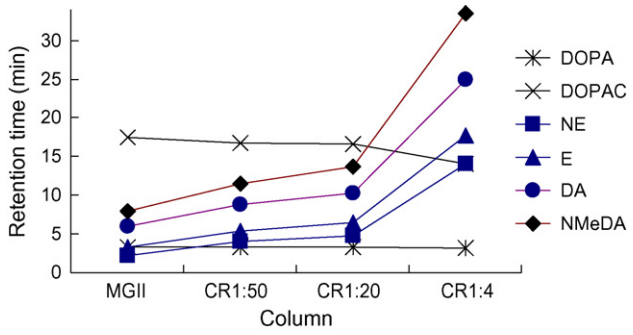


**Fig. 1.** Comparison of retention times of six catechol compounds with four kinds of columns (CAPCELL PAK CR 1:50, 1:20 and 1:4 (SCX:C18), and CAPCELL PAK C18 MG II).

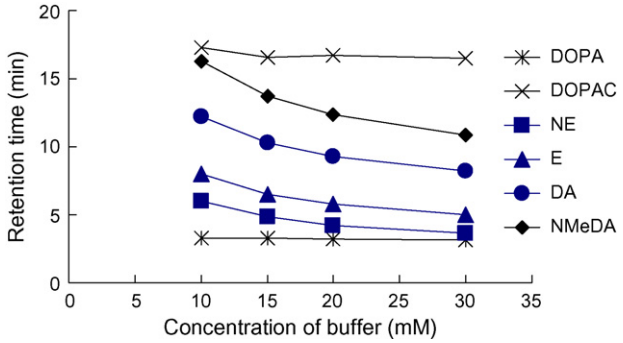


**Fig. 2.** Effects of buffer concentrations on the retention times of six catechol compounds.

suggests that the retention of analytes can be substantially altered by changing the concentration of the buffer with mixed-mode reversed-phase and cation-exchange columns.

Effects of pH on the separation of catechol compounds within the range of pH 3.5–5.5 are shown in Fig. 3. The retention of only DOPAC was dramatically reduced as pH was increased. This was due to the increasing degree of ionization of DOPAC because the carboxylic acid group on DOPAC is deprotonated at pH 4.5 and 5.5 ($pK_a = 4.2$).

From the data in Figs. 2 and 3, 15 mM sodium acetate buffer (pH 4.5) was chosen for the mobile phase composition for the separation of all six catechol compounds.

### 3.2. Optimization of fluorescence derivatization conditions

Catechol compounds selectively reacted with ethylenediamine to produce the fluorescent compounds, and this post-column reaction was used for the selective determination of catechol compounds, including dopamine and DOPAC.

Since the composition of the mobile phase was changed from that of the previous reports for catecholamines [7,8,16] and nitrocatecholamines [17,18], the conditions of the fluorescence derivatization reaction were re-investigated to obtain the optimum condition. The length of the reaction coil in the flow system at 80 °C was first examined. The length of the reaction coil was set at 7.5 m as the optimum condition since it gave the maximum fluorescence intensity (data not shown).

The effect of the temperature of the thermoreactor was investigated with the optimum length (7.5 m). With the increase of the temperature, the recorder responses for dopamine, DOPAC and DOPA increased. At temperatures higher than 80 °C, the responses for norepinephrine and epinephrine were not changed. Hence, the optimum temperature for the fluorescence derivatization reaction was selected as 95 °C.

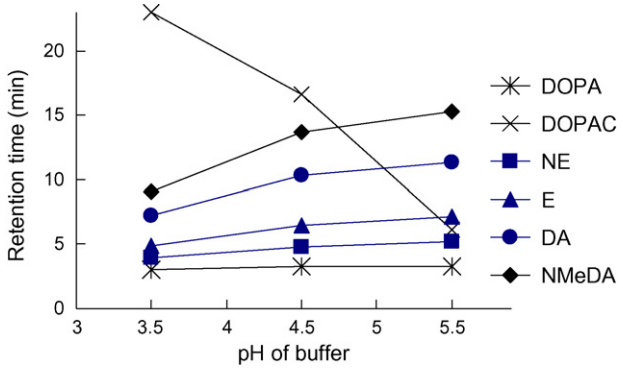


**Fig. 3.** Effects of pH of buffer on the retention times of six catechol compounds.

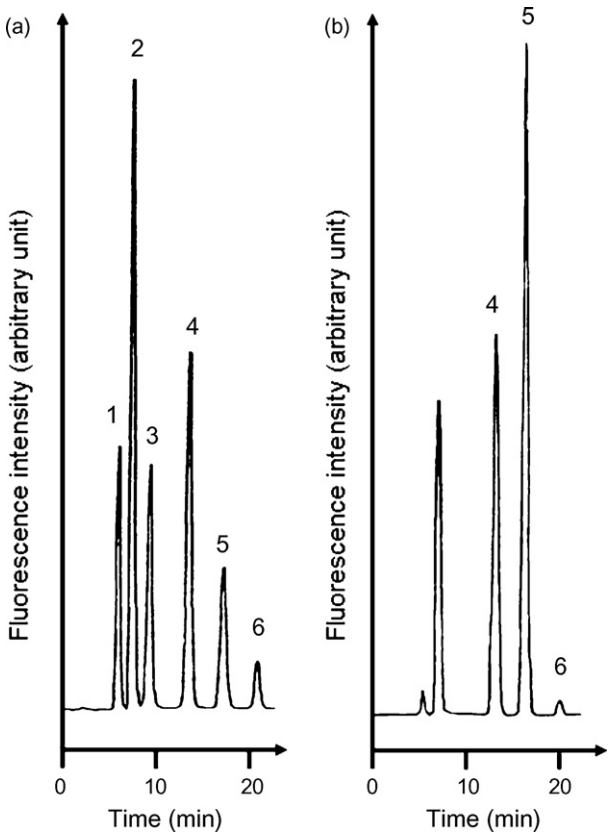


**Fig. 4.** Representative chromatograms obtained (a) from 20 pmol of a standard sample and (b) from mouse striatum sample. Peaks: (1) L-DOPA, (2) norepinephrine, (3) epinephrine, (4) dopamine, (5) *N*-methyl dopamine, and (6) DOPAC.

### 3.3. Chromatogram

With the optimized chromatographic and fluorescence derivatization conditions, chromatogram of six catechol compounds was obtained (Fig. 4(a)). The separation of the compounds could be accomplished in less than 22 min. The striatal dopamine is essential for the appropriate regulation of motor control [19]. Motor impairment in the Parkinson's disease is at least in part due to the deficiency of released dopamine in the striatum. Hence, striatum was chosen as a sample. Fig. 4(b) shows the chromatogram obtained from a mouse striatum sample. The peaks for dopamine and DOPAC were clearly shown with no interference from endogenous compounds, which implied the high selectivity of the proposed method. The concentrations of dopamine and DOAPC in mouse striatum were $4.98 \pm 0.66$ and $1.00 \pm 0.11$ μM, respectively ($n = 10$).

### 3.4. Validation of the proposed method

The calibration curves for dopamine and DOPAC showed linearity in the range of 0.5 pmol (dopamine) or 1.5 pmol (DOPAC) to 50 pmol. The correlation coefficients were 0.999.

The limits of detection (a signal-to-noise ratio of 3) were 100 and 500 fmol for dopamine and DOPAC, respectively. The limits of quantification (a signal-to-noise ratio of 10) were 300 and 1500 fmol for dopamine and DOPAC, respectively. These values were higher than those previously reported [7,10], and were sufficient for the simultaneous determination of dopamine and DOPAC in mouse striatum.

The accuracies for dopamine and DOPAC are summarized in Table 1. The intra-day precision of the method was 3.96 and 4.29% for dopamine and DOPAC ($n = 5$), respectively. The inter-day precision of the method was 4.38 and 5.37% for dopamine and DOPAC

**Table 1**
Accuracy for the determination of dopamine and DOPAC in mouse striatum ($n = 3$).

| | Added amounts (μM) | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 4 |
| Dopamine | | | | |
| Found (μM) | 4.85 | 5.83 | 6.87 | 8.84 |
| Accuracy (%) | | 98.0 | 101 | 99.8 |
| DOPAC | | | | |
| Found (μM) | 1.02 | 1.99 | 2.98 | 4.89 |
| Accuracy (%) | | 97.0 | 97.8 | 96.8 |

($n = 5$), respectively. These suggest that the proposed method is appropriate for a routine assay of dopamine and DOPAC in mouse striatum.

## 4. Conclusion

A simultaneous determination method for dopamine and DOPAC was developed using mixed-mode reversed-phase and cation-exchange column (CAPCELL PAK CR 1:20). The mobile phase variables such as buffer concentrations and pH can be modulated to enhance the selectivity. Without ion-paring reagent, six catechol compounds were retained with simple mobile phase. The method as developed was applicable to the determination of dopamine and DOPAC in mouse striatum, and took less time and samples. The mixed-mode column is useful for the separation of organic compounds with acidic and basic groups, e.g. a drug and its metabolites.

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