Development of analytical method for catechol compounds in mouse urine using hydrophilic interaction liquid chromatography with fluorescence detection

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Abstract

An analytical method for catecholamines and related compounds using hydrophilic interaction liquid chromatography (HILIC) with native fluorescence detection has been developed. We found that ZIC-cHILIC with phosphorylcholine was suitable for the separation of catechol compounds with good peak shapes among six different HILIC columns (Inertsil Silica, Inertsil amide, Inertsil diol, TSKgel NH₂-100, ZIC-HILIC, and ZIC-cHILIC). Using ZIC-cHILIC, eight catechol compounds (dopamine, epinephrine, norepinephrine, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylglycol, 3,4-dihydroxymandelic acid, and internal standard 3,4-dihydroxybenzylamine) were separated within 15 min. The limit of detection at a signal to noise ratio of 3 was 3.4 - 23.4 nM. An improved sensitivity was obtained as compared to that of reversed-phase liquid chromatography. This was partly attributed to the increase in the fluorescence intensity of the catechol compounds in the acetonitrile-rich mobile phase. Solid phase extraction using a monolithic silica disk-packed spin column with phenylboronate moieties, which have affinity to catechol compounds, was performed for the selective extraction of catechol compounds from mouse urine. Dopamine, epinephrine, norepinephrine, 3,4-dihydroxyphenylalanine, and 3,4-dihydroxyphenylglycol were successfully quantified in mouse urine.

Keywords: catecholamines; ZIC-cHILIC; monolithic silica; phenylboronate

1. Introduction

Catecholamines are monoamine compounds with dihydroxyphenyl groups. Three catecholamine compounds including dopamine (DA), epinephrine (E), and norepinephrine (NE), which are derived from tyrosine and generated in nerve tissue and adrenal glands, play important roles as neurotransmitters and hormones [1]. The analysis of catecholamines is important in the study of physiological regulation systems. Furthermore, the concentration of catecholamines and their metabolites in biological fluids can indicate underlying diseases such as pheochromocytomas and neuroblastomas, which exhibit elevated levels of catecholamines compared to normal [2, 3]. Hence, the assessment of catecholamine concentration in biological fluids is needed for the clinical diagnosis of certain diseases. Simultaneous measurements of catecholamines and their metabolites are also important, since they provide information about the activity of metabolic enzymes [4, 5].

Various methods using high-performance liquid chromatography (HPLC) have been developed for the simultaneous analysis of catecholamines and their metabolites [6, 7]. Reversed-phase liquid chromatography (RPLC) has been used in most studies for the analysis of catechol compounds [8-11]. However, catechol compounds, which are polar and ionizable, are **weakly** retained under RPLC conditions; therefore, simultaneous separation of multiple catechol compounds including catecholamines and their precursors and metabolites is difficult.

Hydrophilic interaction liquid chromatography (HILIC), which was first introduced by A. J. Alpert [12], is a complementary method to RPLC for the separation of polar compounds. Under HILIC conditions, in which a polar stationary phase and an organic-rich mobile phase are employed, polar compounds are strongly retained by hydrogen-bonding, ion exchange, and hydrophilic partitioning between the bulk mobile phase and the water enriched layer on the polar stationary phase [13-15]. Therefore, HILIC conditions are considered to be useful for the separation of catechol compounds [16-18]. However, there are few studies in which HILIC was applied for the determination of catechol compounds in biological samples. Kumar *et al.* determined catecholamines in urine under

HILIC conditions via electrochemical detection [19]. They found that a zwitterionic (ZIC-HILIC) or amide column was more suitable for the separation of catecholamines than bare silica columns in terms of separation efficiency and peak shapes. However, only three catecholamines were measured and the precision of the method was **not sufficient for the determination of catecholamines in biological samples** because of the lack of sensitivity. We found that the fluorescence intensity of fluorescently labeled thiols in the acetonitrile-rich mobile phase (HILIC conditions) was larger than that in the aqueous-rich mobile phase (RPLC conditions) [20]. This technique might be expandable to include other compounds such as catechols. Thus, in this study, we applied fluorescence detection.

In the present study, it was found that ZIC-cHILIC columns, which have **the** zwitterionic functional group phosphorylcholine (Table 1), offered the best separation of catechol compounds among six kinds of HILIC columns (**Inertsil Silica, Inertsil amide, Inertsil diol, TSKgel NH₂-100, ZIC-HILIC, and ZIC-cHILIC**), and eight catechol compounds, specifically DA, E, NE, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxymandelic acid (DHMA), and internal standard 3,4-dihydroxybenzylamine (DHBA) (Fig. 1), were separated on a ZIC-cHILIC column within 15 min under isocratic conditions. Furthermore, solid phase extraction (SPE) selective towards catechol compounds from mouse urine was performed using a monolithic silica spin column modified with phenylboronate, **by which SPE can be performed simply by centrifugation**. Catechol compounds in mouse urine were successfully quantified using SPE, separation on ZIC-cHILIC, and native fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

Dopamine (DA), epinephrine (E), norepinephrine (NE), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxymandelic acid (DHMA), 3,4-dihydroxybenzylamine (DHBA), and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate, dipotassium hydrogenphosphate, formic acid, phosphoric acid, and methanol were obtained from Wako (Osaka, Japan). Acetic acid was obtained from Kanto chemical (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Preparation of mouse urine sample

Urine samples were obtained from 3 male C57BL/6J mice (6-7 months old). Sample pretreatment was performed on a spin column (GL Sciences, Tokyo, Japan). The sample preparation procedure was based on a previous report [21]. First, 200 μ L of 1 % aqueous acetic acid was poured into the column, and then centrifugation was carried out. To activate the column, 200 μ L of 100 mM phosphate buffer (pH 8.0) was infused into the column, followed by centrifugation. The urine sample (200 μ L) and 1 M phosphate buffer (pH 8.0; 50 μ L) were poured into the pre-activated spin column, followed by centrifugation. The column was then washed with 200 μ L of 100 mM phosphate buffer (pH 8.0)-methanol (50/50, v/v) by centrifugation. Finally, the adsorbed catechol compounds were eluted with 1 % acetic acid (200 μ L) by centrifugation. After dilution with acetonitrile, 5 μ L of the eluate was injected into the HPLC system. All centrifugations were performed at 10,000 rpm for 1 min.

2.3. HPLC apparatus and chromatographic conditions

The HPLC system was composed of a pump (PU-2080 Plus, JASCO, Tokyo, Japan), a column oven (860-CO, Jasco), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan). The column temperature was 35 °C and catechol compounds were detected by fluorescence with excitation and emission wavelengths of 280 and 320 nm, respectively. Chromato-Pro (Run Time Corporation, Kanagawa, Japan) software was used to analyze the chromatograms. Inertsil SIL (150 mm × 3.0 mm i.d., 5 µm, GL Sciences), Inertsil Diol (150 mm × 4.6 mm i.d., 5 µm, GL Sciences), Inertsil Amide (150 mm × 3.0 mm i.d., 5 µm, GL Sciences), TSKgel NH₂-100 (150 mm × 2.0 mm i.d., 3 µm, Tosoh, Tokyo, Japan), ZIC-HILIC (150 mm × 2.1 mm i.d., 5 µm, Merck, Germany), and ZIC-cHILIC (150 mm × 2.1 mm i.d., 3 µm, Merck) were used. The mobile phase was acetonitrile-10 mM ammonium formate buffer (pH 2.5) (75/25, v/v) and the linear velocity was set at 58 mm/min.

The effect of acetonitrile content, buffer pH, and concentration of salt in the buffer were examined with ZIC-cHILIC column. The primal mobile phase was acetonitrile-10 mM ammonium formate buffer (pH 2.5) (75/25, v/v) and the flow rate was 0.2 mL/min.

2.4. Calculation of the log D value

The log D values of the catechol compounds were calculated with SPARC software [22] under the following conditions: organic solvent, acetonitrile; ionic strength of the water phase, 0; and temperature, 25 °C.

2.5. Fluorometry

To investigate the relationship between solvent and fluorescence intensity of catechol compounds, the fluorescence intensity of 100 µM of catechol solution containing 0 or 75 % (v/v) acetonitrile in water was measured using a FP-6500 spectrofluorometer (JASCO). The shift in the fluorescence intensity of 100 µM DA dissolved in a solution containing 0 to 99 % (v/v) acetonitrile was also investigated.

2.6. Validation

The limit of detection (LOD) and limit of quantification (LOQ) were calculated at S/N (signal to noise ratio) = 3 and 10, respectively. The LOD and LOQ were calculated from the chromatograms of the standards with the following concentrations: DOPAC, 40 nM; DHPG, 10 nM; DHMA, 80 nM; DA, 10 nM; E, 10 nM; DOPA, 10 nM; and NE, 10 nM. Quantification was performed using the relative peak height to DHBA (internal standard), which is not present as an endogenous compound. Eight standard concentrations of the catechol compounds (50-5000 nM or 100-5000 nM for DHMA) were analyzed to obtain a calibration curve. The recovery of the catechol compounds in urine was examined by spiking additional catechol compounds at two different concentrations (3.3 and 6.6 μM for DA, NE, and DOPA; 1.1 and 2.2 μM for E; 4.4 and 8.8 μM for DHPG). The recovery value was calculated as the ratio of the increase in the amount of catechol compounds, measured by the calibration curve, to the amount of spiked catechol compounds. In order to assess the intra- and interday precisions, the same urine sample was measured five times on the same day and on consecutive days, respectively.

3. Results and discussion

3.1. Column selection

It is important to select an appropriate HILIC column depending on analytes since many kinds of HILIC columns are available. Hence, six kinds of HILIC columns (Inertsil SIL, Inertsil Diol, Inertsil Amide, TSKgel NH₂-100, ZIC-HILIC, and ZIC-cHILIC, Table 1) were investigated in the separation of eight catechol compounds using acetonitrile-10 mM ammonium formate buffer (pH 2.5) (75/25, v/v) as the mobile phase. The separation of eight catechol compounds (DA, E, NE, DOPA, DOPAC, DHPG, DHMA, and DHBA) on each HILIC column is shown in Fig. 2. Catechol compounds were strongly retained on Inertsil Amide, ZIC-HILIC, and ZIC-cHILIC, and ZIC-cHILIC gave the best separation of catechol compounds. Accordingly, ZIC-cHILIC was selected for further studies.

3.2. Optimization of mobile phase for the separation of catechol compounds on ZIC-cHILIC column

The retention mechanism of HILIC is attributed to hydrophilic partitions, and hydrogen-bonding and electrostatic interactions. By examining the ratio of acetonitrile to water (for hydrophilic partition), pH, and salt concentration (for hydrogen-bonding and electrostatic interactions) in the mobile phase, the composition of the mobile phase was optimized for the separation of catechol compounds under HILIC conditions.

The primary mobile phase was acetonitrile-10 mM ammonium formate buffer (pH 2.5) (75/25, v/v). First, the effect of acetonitrile content in the mobile phase on the retention time of the analytes was examined. As shown in Fig. 3A, with the increase of acetonitrile content, the retention times of the catechol compounds increased. The peaks were not well separated with 70 % acetonitrile, but the analysis time was too long with 80 % acetonitrile. Thus, 75 % (v/v) acetonitrile was selected as the optimum ratio.

Next, the effect of buffer pH on the retention was examined (Fig. 3B), and pH 2.5 was selected

as the optimal pH value. When estimating the contribution of partition mechanism to the retention, it is useful to compare the log D value and retention time at various pH values. The log D value is a distribution coefficient between the organic solvent and aqueous solution, which takes into account of the electrolytic dissociation of compounds. Hence, we calculated log D values of DA (basic), DOPAC (acidic), DHPG (neutral), and DOPA (zwitterionic) using SPARC (Fig. 4). Although a shift in the pH range was observed for DOPAC, DHPG, and DOPA (as shown in Figs. 3B and 4), the changes in log D were similar to the changes in the retention times. The shift in the pH range may result from the difference between the pH of the aqueous solution and the apparent pH of the aqueous-organic mixture. This indicates that partition is the main mechanism in the separation of acidic or neutral catechol compounds. As for DA, however, with an increase in pH, the log D increased (hydrophilicity was decreased), while the actual retention strengthened. This behavior indicated that the electrostatic attraction between amines and deprotonated silanol groups on the stationary phase surface at high pH values more strongly influenced the retention mechanism of basic compounds than hydrophilic partition. Furthermore, higher pH values caused increased peak tailing of the catecholamines (data not shown), which also supports the speculation of electrostatic attraction between protonated catecholamines and the underlying deprotonated silanol group on the stationary phase at higher pH values.

Finally, the effect of buffer concentration in the mobile phase was investigated (Fig. 3C). Amine-containing compounds (DA, E, NE, and DHBA) were less retained with increased buffer concentrations. This may be due to the reduction of the electrostatic attraction between positively charged amine analyte and negatively charged phosphate moiety or underlying deprotonated silica on the stationary phase at high buffer concentrations. The peaks were not well separated at high buffer concentrations, and buffer concentrations lower than 10 mM may result in low buffering ability. Hence, 10 mM was selected as the optimal concentration of ammonium formate buffer. As such, the optimized conditions for the separation of catechol compounds using a ZIC-cHILIC column

were determined to be acetonitrile-10 mM ammonium formate buffer (pH 2.5) (75/25, v/v). Fig. 5A shows a representative chromatogram of a standard solution under the optimal conditions. Eight catechol compounds were separated within 15 min with isocratic elution.

3.3. Fluorescence intensity of catechol compounds

The fluorescence intensity of an analyte can be affected by the composition of the solvent [23]. Hence, the relationship between fluorescence intensity of the catechol compounds and acetonitrile content in the solvent was investigated. The fluorescence intensity of DA increased with the increase of acetonitrile content (0 to 99 %). Furthermore, the relative fluorescence intensity of the catechol compounds in the acetonitrile and water (75/25, v/v) mixture to that in water was determined as follows: 6.1 for DA, 4.3 for E, 4.6 for NE, 3.4 for DOPA, 9.5 for DOPAC, 7.0 for DHPG, and 0.2 for DHMA. These results indicated that the detection of catechol compounds, except for DHMA, should be more sensitive under HILIC conditions with the acetonitrile-rich mobile phase than that under RPLC conditions with the water-rich mobile phase.

3.4. Extraction of catechol compounds from mouse urine

The levels of urinary catecholamines and their metabolites are used as *in-vivo* markers of various physiological conditions, and mouse urine was used as an application of the method for biological samples. To extract catechol compounds from urine, SPE using a phenylboronic-acid binding column, which forms a negatively charged complex with the *cis*-hydroxyl groups of catechol compounds, was used.

We investigated the extraction of catechol compounds from mouse urine using a monolithic silica disk-packed spin column with phenylboronate moieties, by which sample loading, washing, and elution can be performed by centrifugation. First, the extraction of catechol compounds (**1 μ M**) was performed in accordance with previous studies [21, 24]. Recovery of catechol compounds from the

standard solution was 87-111%. However, when applied to mouse urine samples, a large amount of impurities was observed at low retention times, and the detection of DOPAC, DHPG, and DHMA was difficult. Since these impurities were considered to be comparatively hydrophobic, it was expected that the impurities could be reduced by washing with a more hydrophobic solution. Hence, we investigated potential wash solutions containing acetonitrile or methanol. As expected, the amount of impurities decreased in the extract washed by the methanol or acetonitrile-containing solution in comparison with that washed by the 100% aqueous solution. As shown in Table 2, the recovery of DOPAC decreased with an increased hydrophobicity of the washing solution. As the recovery of DOPAC using the washing solution containing acetonitrile was low, the methanol-containing washing solution was selected. Accordingly, further experiments were carried out using 100 mM phosphate buffer (pH 8.0)/methanol (50/50, v/v) as the washing solution. Fig. 5B shows the representative chromatogram of mouse urine. Catechol compounds in mouse urine were identified by a comparison of the retention times with those of standards. DHPG, DA, E, NE, and DOPA were detected, while DOPAC and DHMA were undetectable, potentially owing to the interference of impurities in urine, relatively low concentration in urine, and low recovery from SPE. The concentration measured in the present method was approximately equal to those reported in previous studies, which analyzed mouse urine samples using an enzyme immunoassay [25] and alumina SPE and RPLC with electrochemical detection [26].

3.5. Validation of the developed method

Table 3 shows the linear range, LOD, and LOQ. Although the sensitivity was obviously lower than those with chemiluminescence detection [27], it was higher than that obtained using ZIC-HILIC and electrochemical detection (97, 109, and 148 fmol for DA, E, and NE, respectively) [19]. Previously, derivatization was necessary to detect catechol compounds with fluorescence or chemiluminescence, since the native fluorescence of catechol compounds is too weak for the

analysis of biological samples, due to the low concentration. In contrast, catechol compounds were detected without derivatization under HILIC conditions, partly due to the improved fluorescence intensity in the acetonitrile-rich mobile phase as mentioned above. Table 4 shows the precision and recovery in detail. The recovery of DHPG was lower than that of the catecholamines. This may be because the interaction between DHPG and anionic boronate was lower than that between the cationic amines and anionic boronate.

Conclusions

In this study, we developed a method for the selective determination of catechol compounds in mouse urine under HILIC conditions with native fluorescence detection. As far as we know, this is the first report that ZIC-cHILIC was suitable for the separation of catechol compounds. Eight catechol compounds were separated in 15 min with isocratic elution using ZIC-cHILIC. The acetonitrile-rich mobile phase was suitable for the native fluorescence detection of catechol compounds in terms of sensitivity. Following the pretreatment using a spin column with phenylboronate moieties for the selective extraction of catechol compounds from mouse urine, DHPG, DA, E, DOPA, and NE were quantified. **Since the developed method used a simple pretreatment and did not require derivatization,** it should be helpful in clinical diagnosis and for investigating the relationship between catecholamine metabolism and related diseases.

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Table 1 HILIC columns investigated in this study

HILIC column	Functional group	Chemical structure
Inertsil SIL	Bare silica
Inertsil Amide	Amide
Inertsil Diol	Diol
TSKgel NH ₂ -100	Amino
ZIC-HILIC	Sulfobetaine
ZIC-cHILIC	Phosphorylcholine

Table 2 The effect of washing solution on recovery (%) of catechol compounds

Washing solution	DOPAC	DHPG	DHMA	DA	E	DOPA	NE
Buffer ^a	86.7±5.0	110.9±4.4	95.9±7.8	107.1±1.5	105.5±1.7	108.0±1.3	98.7±0.6
Buffer-methanol ^b	74.3±6.0	117.1±6.3	87.7±7.1	110.8±2.3	108.9±2.8	101.8±0.4	97.9±1.5
Buffer-acetonitrile ^c	45.6±3.2	115.5±6.8	81.5±7.5	114.0±2.0	109.9±1.8	86.6±2.2	97.1±0.7

(n=4, mean±SD)

a, 100 mM phosphate buffer (pH 8.0); b, 100 mM phosphate buffer (pH 8.0) / methanol = 50 / 50 (v/v); c, 100 mM phosphate buffer (pH 8.0) / acetonitrile = 50 / 50 (v/v).

Table 3 Linearity, limits of detection (LOD) and limits of quantitation (LOQ) of catechol compounds.

	LOD [nM] $S/N = 3$	LOD [fmol]	LOQ [nM] $S/N = 10$	LOQ [fmol]	Linearity [nM] $r^2 > 0.99$
DOPAC	13	65	43	216	50-5000
DHPG	3	17	11	57	50-5000
DHMA	28	138	92	459	100-5000
DA	4	18	12	61	50-5000
E	5	24	16	80	50-5000
DOPA	8	40	27	134	50-5000
NE	10	48	32	160	50-5000

Table 4 Precision and recovery of catechol compounds in spiked **mouse** urine.

Catechol compounds (n=5) (added/ μ M)	Intra-day precision				Inter-day precision			
	Concentration (μ M)				Concentration (μ M)			
	mean	SD	C.V. (%)	Recovery (%)	mean	SD	C.V. (%)	Recovery (%)
DHPG								
0	0.86	0.08	8.7	-	0.71	0.07	9.7	-
4.4	3.76	0.24	6.5	66	3.06	0.14	4.4	53
8.8	6.63	0.39	5.8	66	6.41	0.24	3.7	65
DA								
0	1.20	0.06	4.8	-	1.09	0.06	5.3	-
3.3	4.94	0.31	6.3	113	4.23	0.26	6.2	95
6.6	8.18	0.08	1.0	106	7.88	0.61	7.7	103
E								
0	0.08	0.01	9.2	-	0.06	0.003	3.9	-
1.1	1.10	0.09	8.0	93	0.93	0.07	8.0	78
2.2	2.08	0.02	0.7	91	2.09	0.17	8.0	92
DOPA								
0	1.02	0.06	6.1	-	0.93	0.05	4.9	-
3.3	3.66	0.31	8.5	80	3.90	0.19	4.8	90
6.6	6.97	0.53	7.6	90	6.40	0.55	8.7	83
NE								
0	0.97	0.02	2.1	-	0.89	0.06	6.4	-
3.3	4.94	0.38	7.7	120	4.36	0.36	8.3	105
6.6	7.90	0.25	3.1	105	8.22	0.36	4.4	111

Figure captions

Figure 1 Chemical structures of catechol compounds; 1, DA; 2, E; 3, NE; 4, DOPA; 5, DOPAC; 6, DHPG; 7, DHMA; 8, DHBA.

Figure 2 Chromatogram of catechol compounds on six HILIC columns. Peaks: 1, DOPAC; 2, DHPG; 3, DHMA; 4, DA; 5, E; 6, DOPA; 7, DHBA; 8, NE. Column temperature: 35 °C. Mobile phase: acetonitrile-10 mM ammonium formate buffer (pH 2.5) = 75/25 (v/v). Linear velocity: 58 mm/min.

Sample: 4 μ M catechol compounds.

Figure 3 Effect of (A) acetonitrile content, (B) pH of ammonium formate buffer and (C) concentration of ammonium formate buffer on retention time of catechol compounds. Symbols: DOPAC, ●; DHPG, ◇; DHMA, ▲; DA, □; E, ◆; DOPA, ○; DHBA, ■; NE, △. Column: ZIC-cHILIC (150 mm × 2.1 mm i.d., 3 μ m, Merck). Column temperature: 35 °C. Mobile phase: (A) acetonitrile-10 mM ammonium formate buffer (pH 2.5), (B) acetonitrile-10 mM ammonium formate buffer = 75/25 (v/v), and (C) acetonitrile-ammonium formate buffer (pH 2.5) = 75/25 (v/v). Flow rate: 0.2 mL/min. Fluorescence detection: ex 280 nm, em 320 nm.

Figure 4 Log D values of catechol compounds.

Figure 5 Typical chromatograms of (A) standard sample (4 μ M) and (B) mouse urine sample. Peaks: 1, DOPAC; 2, DHPG; 3, DHMA; 4, DA; 5, E; 6, DOPA; 7, DHBA; 8, NE. Column: ZIC-cHILIC (150 mm × 2.1 mm i.d., 3 μ m). Column temperature: 35 °C. Mobile phase: acetonitrile-10 mM ammonium formate buffer (pH 2.5) = 75/25 (v/v). Flow rate: 0.2 mL/min. Fluorescence detection: ex 280 nm, em 320 nm.

Figure 1

1

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Figure 2

Figure 3

Figure 4

Figure 5