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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 SIL, Inertsil Amide, Inertsil Diol, TSKgel NH_2-100, 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 3–28 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.

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

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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 NH_2-100, 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 toward 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 three male C57BL/6J mice (6–7 months old). Sample pretreatment was performed on a spin column, MonoSpin PBA (GL Sciences, Tokyo, Japan). The sample preparation procedure was based on a previous report [21]. First, 200 μL of 1% aqueous acetic acid was poured into the column, and then centrifugation was carried out. To activate the column, 200 μL of 100 mM phosphate buffer (pH 8.0) was infused into the column, followed by centrifugation. The urine sample (200 μL) and 1 M phosphate buffer (pH 8.0; 50 μL) were poured into the preactivated spin column, followed by centrifugation. The column was then washed with 200 μL 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 μL) by centrifugation. After dilution with acetonitrile, 5 μL of the eluate was injected into the HPLC system. All centrifugations were performed at 10,000 *g* 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 NH_2-100 (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 0–99% (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 NH_2-100, ZIC-HILIC, and ZIC-cHILIC, Table 1) were investigated in the separation of eight catechol compounds