Analysis of Catecholamine and Their Metabolites in Mice Brain by Liquid Chromatography–Mass Spectrometry Using Sulfonated Mixed-mode Copolymer Column

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In this study, a simultaneous assay for catecholamines and their metabolites in the brain was established using liquid chromatography–mass spectrometry (LC–MS). To achieve complete separation, a cation-exchange/reversed-phase mixed-mode copolymer resin column containing 0.81 wt% sulfo groups was used for the simultaneous LC–MS assay. The analyzed catecholamines were dopamine (DA), norepinephrine (NE), and epinephrine (E), while the metabolites lacking amino groups were 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG). The metabolites were separated and detected using LC–MS, on columns with and without sulfo groups. However, we could not achieve adequate separation of catecholamines on both columns using a gradient elution of 0–50 (v/v)% methanol containing 0.1 (v/v)% formic acid (FA). When volatile ion-pairing reagents were added to the mobile phase, they improved the retention and detection of catecholamines on the sulfonated mixed-mode column. Under optimized elution conditions, which involved a linear gradient elution of water containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile in 50 mM ammonium formate at 40°C and a 0.20 mL/min rate, all six target molecules were simultaneously detected within 25 min, when using negative mode LC–MS on a sulfonated mixed-mode column. The limits of detection (LODs) for DA, NE, E, DOPAC, HVA, and MHPG were determined to be 20.7, 12.6, 74.6, 1110, 18.7, and 3196 nM, respectively. Moreover, the established LC–MS assay allowed the detection of endogenous DA, NE, and HVA, in normal mouse brain samples at concentrations higher than 20, 9, and 4 pmol/mg, respectively.

Keywords Catecholamine, metabolite, mixed-mode column, cation-exchange/reversed-phase column, liquid chromatography–mass spectrometry

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Introduction

Catecholamines such as dopamine (DA), norepinephrine (NE), and epinephrine (E), which possess a catechol moiety, act as neurotransmitters and mediate various neurophysiological processes (memory processing, motivation, cognition, mood, learning, hormone release, *etc.*).¹ Therefore, it is important to determine the metabolism of catecholamines for clinical diagnosis purposes. It has been reported that catecholamine imbalances have been involved in the onset of neuropsychological dysfunctions.^{1,2} For example, DA and its metabolites, 3,4-dihydroxy phenylacetic acid (DOPAC) and homovanillic acid (HVA), have been closely associated with multiple neuropsychiatric disorders, such as hallucination, depression, schizophrenia, and Parkinson's disease.³ Moreover, 3-methoxy-

4-hydroxyphenylglycol (MHPG), a metabolite of NE and E, has been used as an index for analyzing the pathophysiology of anxiety disorders.⁴ Therefore, catecholamines and their metabolites are often targeted when proposing diagnostic strategies in neurobiology.

Liquid chromatography (LC) coupled with ultraviolet, fluorescence, and electrochemical detection (ECD), as well as mass spectrometry (MS) are common analytical methodologies used for catecholamine assays.⁵ In particular, ECD-high performance LC (HPLC) assays have been widely used for catecholamine assays, since ECD detection is highly sensitive for electrochemically active catecholamines.⁶ However, successful ECD-HPLC assays for catecholamines have been limited by the chromatographic separation of targets and contaminants under isocratic elution.^{5,7,8} Although Sasaki *et al.*⁹ reported the simultaneous assay of seven catecholamine metabolites in urine employing ECD-HPLC using methanol in gradient mode, the strictly controlled elution conditions required a long elution time of >100 min. Using LC–MS or LC-tandem

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MS (MS/MS) assays might improve the poor selectivity of ECD,¹⁰ while the limited application of volatile solvent systems for MS-electrospray ionization (ESI) could cause poor chromatographic separation on common reversed-phase columns. A challenging column separation of catecholamines and their metabolites was performed employing fluorescence derivatization using a cation-exchange/reversed-phase (mixed-mode) column.¹¹ The reported mixed-mode column using pH gradient elution achieved adequate LC separation, while the imidazole-derivatization of catecholamines involved tedious analytical procedures.¹¹ Thus, to establish a convenient and direct assay for catecholamines and their metabolites, an LC-MS-based assay, which used a cation-exchange/reversed-phase mixed-mode column, was proposed in this study. A column packed with ethylstyrene-divinylbenzene copolymer resin featuring attached sulfo groups was used for catecholamine assay, since the column was sufficient for the separation of basic di-peptides.¹²

Experimental

Reagents

We purchased L-NE hydrochloride, E hydrochloride, and MHPG from Sigma-Aldrich Co. (St. Louis, MO), DOPAC from Santa Cruz Biotechnology (Santa Cruz, CA), DA, HVA, ammonium hydroxide, ammonium acetate, and ammonium formate from Nacalai Tesque Inc. (Kyoto, Japan), and tetraethylammonium hydroxide (TEA) from Tokyo Chemical Industry Co. (Tokyo, Japan). In addition, LC-MS grade FA, acetonitrile, and water were obtained from Merck Millipore (Darmstadt, Germany), and LC-MS grade methanol from Kanto Chemical Co. (Tokyo, Japan). All the other chemicals used in this study were of analytical reagent grade.

LC-time-of-flight (TOF)/MS analysis

A standard aqueous mixture of six catecholamine metabolites (DA, NE, E, DOPA, HVA, and MHPG) was prepared, and the concentration of each metabolite was 50.0 μ M. We injected 20 μ L of the standard mixture into an LC-TOF/MS system. The LC separation was performed using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed using either an ethylstyrene-divinylbenzene copolymer column packed with 0.81 wt% sulfo groups (MCI GELTM CHK40/C04, 2.1 mm i.d. $\times$ 100 mm, 4 μ m, Mitsubishi Chemical Co., Tokyo, Japan) or one containing no sulfo groups (MCI GELTM CHP20/C04, 2.1 mm i.d. $\times$ 100 mm, 4 μ m) at 40°C. Separation was facilitated using a linearly gradient elution of mobile phase A to B that was performed over 20 min at a flow rate of 0.20 mL/min. The optimal mobile phase was also investigated and will be discussed further. The TOF/MS experiments were performed using a micrOTOF-II mass spectrometer (Burker Daltonics, Bremen, Germany) in negative ESI mode. We collected MS spectral data in the 50 – 500 m/z range. The ionization conditions were as follows: dry gas (nitrogen) flow, 8.0 L/min; drying temperature, 200°C; nebulizer pressure, 1.6 bar; capillary voltage, 3800 V; capillary exit, –100 V; hexapole radio frequency, 100 Vpp. The target DA, NE, E, DOPAC, HVA, and MHPG were identified at m/z 152.0706, 168.0655, 182.0812, 167.0339, 181.0495, and 183.0652, respectively, using the Bruker Data Analysis software, Ver. 3.2.

Linearity and validation

Under the optimized LC-TOF/MS conditions the linearity,

coefficient of variation (CV), limit of detection (LOD), and limit of quantification (LOQ) were validated for catecholamines and their metabolites dissolved in LC-MS grade water. Quantification of the six targets by LC-TOF/MS on a sulfonated mixed-mode copolymer column was performed using calibration curves for each target. CV was obtained at 50 μ M of each target in intra-day assay. We defined LOD and LOQ as the smallest concentrations yielding signal to noise ratios (S/N) of 3 and 10, respectively. All samples were analyzed in replicates of three for each individual concentration.

Preparation of mouse brain tissue samples

The brain tissue samples used in this study were obtained from three 9-week old male Institute of Cancer Research (ICR) mice, weighing 30 – 40 g each (CrI:CD1 (JCR), Charles River Japan, Kanagawa, Japan). All mice were housed for one week at a controlled temperature of $21 \pm 1^\circ\text{C}$, humidity of $55 \pm 5\%$, and exposed to light from 8:00 AM to 8:00 PM. The mice were fed rodent diet CE-2 (CLEA Japan, Tokyo, Japan) and were allowed water *ad libitum*. Animal experiments were conducted in accordance with the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology. The Ethics Committee on Animal Experiments of Fukuoka University approved all experimental protocols (permit number 1715152). All the mice fasted for 16 h, and they were sacrificed after being anesthetized using 25% urethane (Sigma-Aldrich Co.). After decapitating the mice, their entire brains were removed and weighed. Brain samples were immediately frozen in liquid nitrogen and stored at -80°C until they were subjected to LC-TOF/MS analysis. The brain samples were mashed using a biomasher (Nippi Inc., Tokyo, Japan), and lyophilized to dryness. We accurately weighed 50 mg brain powder and dissolved it in 3 mL of 0.1 (v/v)% FA solution. The solution underwent sonication for three 30 s cycles at output control 3 using a Sonifier 250 (Branson Ultrasonics, CT, USA), followed by two 60 s homogenization cycles at 20000 rpm using a Polytron PT2500E homogenizer (Kinematica, Luzern, Switzerland). These processes were performed while maintaining the samples on ice. The obtained homogenate was then centrifuged at $14000 \times g$ for 15 min at 4°C . The supernatant was filtered through an Amicon Ultra-3K filter (Millipore Co., Billerica, MA), followed by evaporation to dryness. Each dried brain sample was dissolved in water prior to LC-TOF/MS analysis.

Results and Discussion

The metabolism of catecholamines in the brain is depicted in Fig. 1A. Dopamine (DA) derived from tyrosine (or phenylalanine) is a precursor of monoamine neurotransmitters (NE and E), and it also enters the DOPAC and HVA (an end-product of DA metabolism) production pathway.¹³ MHPG is also known to be an end-product of the NE/E metabolic pathway.¹⁴ Therefore, the simultaneous determination of catecholamines and their metabolites including the two end-products would allow us to better understand the overall brain homeostasis. Although an ECD-HPLC assay for DA, NE, and E,¹⁵ and an immunoassay for HVA as inflammatory marker¹⁶ have been proposed so far, no reports have been published on a simultaneous assay for all six metabolites due to the poor separation capacities of common reversed-phase LC columns.

Fig. 1 (A) Metabolism of catecholamines and (B) ethylstyrene-divinylbenzene copolymer columns used in this study. We targeted DA, NE, E, DOPAC, HVA, and MHPG and their chemical structures in the metabolic map. Columns containing none or 0.81 wt% sulfo groups were used throughout the study.

Application of sulfonated mixed-mode copolymer column for the separation of catecholamines and their metabolites

To gain more insight into the elution profiles of the six catecholamine metabolites on reversed-phase HPLC columns, we used a column packed with ethylstyrene-divinylbenzene copolymer resin (Fig. 1B), presenting a linear elution gradient ranging from 0 to 50 (v/v)% methanol containing 0.1 (v/v)% FA. As illustrated in Fig. 2A, 50.0 μ M solutions of all six metabolites were detected according to their corresponding m/z values using negative mode LC-TOF/MS. However, the elution profiles were clearly divided into two groups: DA, NE, and E, which featured amino groups, were not retained on the reversed-phase column; while DOPAC, HVA, and MHPG, featuring no amino groups, exhibited significant retention on the column.

Therefore, we concluded that a common reversed-phase column was sufficient for the retention of neutral catecholamine metabolites using an acidic mobile phase containing 0.1 (v/v)% FA due to their hydrophobic interactions. By contrast, no catecholamines were retained on the reversed-phase column (Fig. 2A) probably due to the presence of polar cationic groups in their structures. Ion-exchange interactions on the column were, thus, expected to be useful for achieving the retention of catecholamines featuring amino groups. Tsunoda and Imai¹⁷ reported the advantage of using cation-exchange columns for pre-extracting catecholamines from plasma. Thus, in this study a partially sulfonated (0.81 wt%) ethylstyrene-divinylbenzene copolymer column was used for the separation of catecholamines, since in our previous report it was found that the mixed-mode

Fig. 2 Elution profiles of catecholamine metabolites on either copolymer column containing (A) none or (B) 0.81 wt% sulfo groups using negative mode LC-TOF/MS. Identical mixtures of six catecholamine metabolites, each at a concentration of 50.0 μ M, were assayed using both columns. Linear elution was performed using 0 to 50 (v/v)% methanol in 0.1 (v/v)% FA at a flow rate of 0.20 mL/min at 40°C. The other LC-MS conditions are described in the Experimental section.

Fig. 3 Elution profiles of catecholamines on a sulfonated mixed-mode column using negative mode LC-TOF/MS. Eluting a mixture of 50.0 μ M each of DA, NE, and E was performed using water containing 0.1 (v/v)% FA to 50 (v/v)% methanol containing either (A) 10 mM ammonium hydroxide, (B) 1 mM TEA, (C) 10 mM ammonium acetate or (D) 10 mM ammonium formate at a flow rate of 0.20 mL/min and 40°C. The other LC-MS conditions are described in the Experimental section. N.D. = not detected.

Fig. 4 Elution profiles of catecholamines on a sulfonated mixed-mode column using LC-TOF/MS as a function of the concentration of ammonium formate. Elution of a mixture of 50.0 μ M DA, NE, and E was performed using water containing 0.1 (v/v)% FA to 50 (v/v)% methanol containing (A) 1, (B) 10, and (C) 50 mM ammonium formate or (D) water containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile containing 50 mM ammonium formate at a flow rate of 0.20 mL/min and 40°C. The other LC-MS conditions are described in the Experimental section. N.D. = not detected.

copolymer column exhibited good retention capacity for basic di-peptides due to ionic and hydrophobic interactions.¹² As illustrated in Fig. 2B, a good separation of the three metabolites (DOPAC, HVA, and MHPG) was observed on the mixed-mode column, similar to the non-sulfonated column (Fig. 2A). By contrast, when the mixed-mode column was used, the catecholamine peaks were no longer detected. Considering the reported strong ionic interaction of small amines with the cation-exchange stationary phase,¹⁸ the absence of the catecholamine peaks could have been caused by their high retention or methanol containing 0.1 (v/v)% FA not eluting them on the mixed-mode column.

Elution of catecholamines on sulfonated mixed-mode copolymer column

Provided that no catecholamines were detected using MS (see Fig. 2B) due to the high retention on the sulfonated mixed-mode column, further elution experiments using LC-TOF/MS were performed using volatile cationic additives (ammonium hydroxide and TEA) for the cation-exchange column and volatile ion-pairing MS reagents (ammonium formate and ammonium acetate) as mobile phase B. The concentration of each additive in the methanol mobile phase was 10 mM, except for TEA, its concentration being 1 mM. As illustrated in Fig. 3, cationic additives, such as ammonium hydroxide and TEA, or ammonium acetate failed to completely elute the catecholamines by weakening their ionic interaction with the cation-exchange

column, while 10 mM ammonium formate successfully eluted the three catecholamines from the sulfonated mixed-mode column. It was reported that volatile ion-pairing reagents might form additive ion complexes such as $[M+CH_3COO]^-$ and $[M+HCOO]^-$ in negative ESI mode, therefore causing the intensity of the MS signals to decrease.¹⁹ In this study, no $[M+HCOO]^-$ complex ion was observed when water containing 0.1 (v/v)% FA to 50 (v/v)% methanol containing 10 mM ammonium formate was used as elutant (data are not illustrated). Thus, the low MS signal intensity of each catecholamine (Fig. 3) suggested that catecholamines were still retained on the sulfonated mixed-mode column due to their strong ionic interactions.

Optimization of LC-TOF/MS analysis for catecholamine metabolites using ammonium formate

To improve the poor MS signal intensity of catecholamines when mobile phase B consisted of 10 mM ammonium formate (Fig. 3D), the effect of the concentration of ammonium formate (1, 10, and 50 mM) on the MS signal intensity was investigated. As shown in Figs. 4A – 4C, a slight improvement in MS signal intensity was obtained by increasing the concentration of ammonium formate in mobile phase B. For a concentration of 50 mM of ammonium formate in 50 (v/v)% methanol, a significant elution of each catecholamine was observed, along with corresponding weak MS signal intensities. Considering the strong solvent effect of acetonitrile for MS detection,

Fig. 5 Typical LC-TOF/MS chromatograms of six catecholamine metabolites on a sulfonated mixed-mode copolymer column in (A) a standard solution (50.0 μ M) and (B) mouse brain samples. Brain samples spiked with 0 and 20.0 pmol/mg of each metabolite were subjected to an LC-TOF/MS assay. Linear elution was performed using water containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile containing 50 mM ammonium formate at a flow rate of 0.20 mL/min and 40°C. The other LC-MS conditions are described in the Experimental section. N.D. = not detected.

Table 1 Quantitation of catecholamine metabolites using LC-TOF/MS on a sulfonated mixed-mode column^a

Analyte	RT/ min	Linearity ^b correlation coefficient, <i>r</i>	Linear range/ μ M	LOD		LOQ		CV, % at 50 μ M (<i>n</i> = 3)
				nM	pmol/injection	nM	pmol/injection	
DA	24.2	$y = 6380.3x - 8017.6$ $r = 0.9975$	0.1 - 50.0	20.7	0.41	62.6	1.3	5.6
NE	20.6	$y = 6446.3x - 247.28$ $r = 0.9999$	0.1 - 50.0	12.6	0.25	38.1	0.76	1.1
E	22.8	$y = 8070.3x - 5015.9$ $r = 0.9995$	0.1 - 50.0	74.6	14.9	226	4.5	1.3
DOPAC	17.6	$y = 79.151x - 389.46$ $r = 0.9909$	2.5 - 25.0	1110	22.2	3365	67.3	9.4
HVA	20.0	$y = 4146.6x - 7358.7$ $r = 0.9974$	0.1 - 50.0	18.7	2.4	56.7	1.1	13.1
MHPG	16.6	$y = 27.728x - 29.821$ $r = 0.9999$	2.5 - 25.0	3196	63.9	9686	194	7.2

a. A mixture of six catecholamine metabolites was assayed using a linear elution of water containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile containing 50 mM ammonium formate over 20 min at 0.20 mL/min and 40°C. b. *y* indicates an MS signal peak, and *x* indicates a concentration of target. The other LC-MS conditions are described in the Experimental section.

compared to that of methanol,²⁰ utilizing acetonitrile as solvent for the 50 mM ammonium formate solution used as mobile phase B greatly improved the inferior MS signal intensity obtained when methanol was the solvent on the sulfonated mixed-mode column (Fig. 4D).

When 50 (v/v)% acetonitrile mobile phase B containing 50 mM ammonium formate was utilized, all six catecholamine metabolites were successfully separated and simultaneously detected within 25 min using LC-negative ESI-TOF/MS analysis on the mixed-mode column (Fig. 5A). The observed retention times of DA, NE, E, DOPAC, HVA, and MHPG at the 0.20 mL/min elution rate and 40°C on the sulfonated mixed-mode column were 24.2, 20.6, 22.8, 17.6, 20.0, and 16.6 min,

respectively. As summarized in Table 1, under the above-mentioned elution conditions, the MS peak area of each metabolite provided a linear regression at concentrations between 0.1 - 50.0 μ M of DA, NE, E, and HVA or 2.5 - 25.0 μ M of DOPAC and MHPG ($r > 0.991$). The LOD ranged from 12.6 nM for NE to 3.2 μ M for MHPG, and the CV ranged from 1 to 15% when the concentrations of all six targets were 50.0 μ M.

Detection of endogenous catecholamine metabolites in mouse brain

We utilized the proposed LC-TOF/MS assay on a cation-exchange/reversed-phase mixed-mode column to assay the six

Table 2 Content of catecholamine metabolites in mouse brain samples determined using LC-TOF/MS on a sulfonated mixed-mode column

Analyte	Amount of catecholamine metabolites pmol/mg brain		
	Mouse A	Mouse B	Mouse C
DA	21.6	29.0	22.4
NE	9.2	9.1	9.0
E	N.D. ^a	N.D.	N.D.
DOPAC	N.D.	N.D.	N.D.
HVA	5.7	5.4	4.3
MHPG	N.D.	N.D.	N.D.

The endogenous catecholamine metabolites in brain samples were assayed using a linear elution of water containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile containing 50 mM ammonium formate over 20 min at 0.20 mL/min and 40°C. The other LC-MS conditions are described in the Experimental section. The amount of each metabolite was determined individually in three mouse brain samples. a. N.D. = not detected.

catecholamine metabolites targeted in this study from male ICR mouse brain samples. The mice were not subjected to stress, such as the injection received during urethane anesthetization. As shown in Fig. 5B, DA, NE, and HVA were detected as endogenous catecholamine metabolites in normal mouse brain samples, while the other three metabolites were not detected using the above-mentioned MS detection method (Table 1). This indicated that the urethane-anesthetized mice used in this study might not be exposed to excess nervous stress or promotion of the brain nervous system.^{21,22} The amounts of DA, NE, and HVA detected in the brain samples from the three mice using the aforementioned LC-TOF/MS procedures ranged from 21.6 to 29.0, 9.0 to 9.2, and 4.3 to 5.7 pmol/mg, respectively (Table 2), and were comparable to the reported levels of DA, NE, and HVA (in pmol) in brain tissue samples.²² As shown in Fig. 5B, each of the six metabolites was clearly detected in brain samples spiked with 20.0 pmol/mg of each metabolite, suggesting that the proposed assay system might be applied for the simultaneous assay of catecholamines and their metabolites without interferences from the brain tissue matrices. This indicated that the proposed LC-TOF/MS assay using a sulfated mixed-mode copolymer column allows for simultaneous evaluation of diagnostic catecholamine metabolites, *e.g.*, DA and HVA as tumour marker²³ in fluids such as urine and blood in a single experimental run. However, the recovery of each metabolite in spiked (20 pmol/mg) brain samples was less than 70% (data are not shown). Thus, in future studies more reliable determination of the targets in the brain by the proposed LC-TOF/MS on the mixed-mode copolymer column is needed by using their isotope labeled compounds as internal standard to compensate variable extraction yield of targets from brain tissue.

Conclusions

The present study demonstrated that a partially sulfonated ethylstyrene-divinylbenzene mixed-mode copolymer column could be a useful simultaneous catecholamine assay using LC-TOF/MS. We achieved successful elution or detection of the catecholamine metabolites on the cation-exchange/reversed-phase mixed-mode column using a gradient elution of water

containing 0.1 (v/v)% FA to 50 (v/v)% acetonitrile containing 50 mM ammonium formate as volatile ion-pairing reagent. Using the proposed simultaneous LC-TOF/MS assay, endogenous DA, NE, and HVA were detected in mouse brain samples at concentrations higher than 4 pmol/mg. In conclusion, LC-MS assays combined with sulfonated mixed-mode columns could allow for the simultaneous analysis of catecholamines and their metabolites in brain samples and probably blood and urine samples, too.

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