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Pretreatment and one-shot separating analysis of whole catecholamine metabolites in plasma by using LC/MS

Abstract Catecholamines are biogenic amines that play an important role in the nervous system. Some catecholamines have been used as tumor makers of phenochromocytoma, paraganglioma and neuroblastoma. The analysis of total catecholamine metabolites should be useful for one-shot screening of multiple aspects of diseases; however, it is difficult to do this, because the catecholamine metabolites are divided into three groups: five amines, one amino acid and three carbonic acids. Catecholamines and small molecules were separated from plasma proteins by an internal-surface reversed-phase column (protein-coated octadeyclsilica column) and were analyzed by liquid chromatography (LC)/mass spectrometry (MS) using electrospray ionization time-of-flight MS. Using a reversed-phase column and hydrophilic mobile phases, we succeeded in the separation of nine catecholamines, all of which had similar structures. These nine substances were eluted in the following order: norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine, 3,4-dihydroxyphenylalanine, vanillomandelic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid. The reproducibility of this method was acceptable. The highest coefficient of variation was 7.4%. In addition, various types of compounds were separated from and detected in plasma proteins by applying LC/MS. The plasma direct injection method, which uses an internal-surface reversed-phase column and an ion-pair reagent, allowed us to separate small molecules from plasma proteins. MS detected some compounds that high-performance LC could not succeed in separating and detecting with UV detection. We think that the method can be applied to find new markers in neuroblastoma, by comparing the plasma of patients with that of normal infants. The method can be also used to help in making a diagnosis of other diseases and finding their new makers.

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Introduction

Neuroblastoma is a malignant tumor especially found in infants. The frequency of the incidence is the second highest after that of leukemia. Because of its high frequency and the possibility of curing the disease with early detection, a nationwide neonatal mass screening at 6 months of age was conducted in Japan between 1985 and 2004. Infants with neuroblastoma produce an excess of catecholamines compared with normal infants [1]. Therefore, a considerable amount of vanillomandelic acid (VMA) and homovanillic acid (HVA), the final metabolites of catecholamines, is excreted in the urine [2–7]. The present mass screening observed an increase of the final metabolites by high-performance liquid chromatography (HPLC) [8, 9]; however, this screening method had problems with both the accuracy of metabolite measurement and the determination of a definitive cutoff value. The committee of the Ministry of Health, Labour and Welfare in Japan reached the conclusion that the screening has no effect on mortality from neuroblastoma, and thus it was suspended [10, 11]. Contrary to the negative opinions, some believe that the mass screening actually helped reduce mortality due to neuroblastoma. This disease is still a convalescent unsatisfactory disease and, therefore, mass screening is vital to a reduction of the mortality due to infantile cancers [12–14]. In order to restart the mass screening, the current method, which targets only the final

substances (VMA and HVA) and considers metabolic substances of catecholamine to be insignificant, needed to be reexamined. We analyzed all metabolic substances of catecholamine, including metabolic by-products, and set up an analytical method using liquid chromatography (LC)/mass spectrometry (MS). Urine is the correct sample for the screening, posing the least burden and risk to infants and their parents; however, urine does not contain all of the compounds of the human body and there is also a problem with the stability of components generated by contamination. Blood, on the other hand, contains all of the compounds of the human body; thus, we established a test method for analysis of plasma.

In the plasma, small molecules unite with plasma proteins, especially albumin. For the analysis of small molecules in the plasma, these small molecules needed to be separated from plasma proteins. For the separation, we employed a direct plasma injection analysis, which uses an internal-surface reversed-phase column. Figure 1 shows the characteristics of the column. The outside surface of porous resins was coated with bovine serum albumin (BSA), so that the column did not adsorb the plasma proteins, but still had the reversed-phase characteristics for small molecules. The internal-surface reversed-phase column was selected as a precolumn for the deproteinization and trapping of small molecules [15, 16].

In the human body, catecholamines are formed by the following sequence of reactions: tyrosine→3,4-dihydroxyphenylalanine (DOPA)→dopamine→norepinephrine→epinephrine. They all have physiological activity. After they have performed their roles, DOPA is metabolized and becomes HVA via 3,4-dihydroxyphenylacetic acid (DOPAC). Dopamine also changes into HVA. Norepinephrine transforms into normetanephrine, and epinephrine becomes metanephrine. They then both transform into VMA. These reactions are catalyzed by the following enzymes: tyrosine hydroxylase, DOPA decarboxylase, dopamine-β-hydroxylase, phenylethanolamine-N-methyltransferase, catechol-O-methyltransferase, and monoamine oxidase. Finally HVA and VMA are excreted in the urine (Fig. 2).
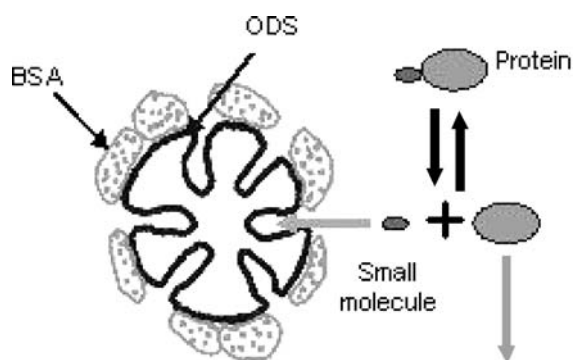

Fig. 1 The characteristics of the internal-surface reversed-phase column. This column was selected as a precolumn for the deproteinization and trapping of small molecules. *BSA* bovine serum albumin, *ODS* octadecylsilica

Materials and methods

Chemicals

DOPA, dopamine, DOPAC, epinephrine, normetanephrine, metanephrine, HVA, VMA and formic acid were obtained from Sigma. Norepinephrine was from Fluka. Methanol, acetonitrile, ammonia solution, ammonium hydrogencarbonate and pentadecafluorooctanoic acid [$CF_3(CF_2)_6$ COOH] were purchased from Kanto Chemical. Trifluoroacetic acid (CF_3COOH), pentafluoropropionic acid (CF_3CF_2COOH) and heptafluorobutyric acid [$CF_3(CF_2)_2$ COOH] were obtained from Aldrich. Nonafluorovaleric acid [$CF_3(CF_2)_3COOH$] was from Tokyo Kasei Kogyo. Methanol and acetonitrile were HPLC grade, and all other reagents were analytical grade. Water was deionized and purifed by a Milli-Q Academic A10 purification system from Millipore.

Sample preparation

Catecholamine metabolites were dissolved in water at a concentration of 1 mg/mL and mixed in one solution to a final concentration of 100 μg/mL to form a standard solution. The standard solution was diluted with fetal bovine serum or human plasma to 10 μg/mL and was used as sample-spiked plasma.

Instrumentation

The HPLC-UV system was a Gulliver series system from JASCO, consisting of a DG-980-50 3 line degasser, an LG-1580-02 ternary gradient unit, a PU-980 Intelligent HPLC pump and a UV-970 Intelligent UV/vis detector. Pretreatment was performed by using the internal-surface reversed-phase column (TSK precolumn BSA-ODS:TOSOH) and a column-packed anion-exchange resin (TOYOPEARL QAE-550:TOSOH). The analysis column was an L-column ODS (150 mm×4.6 mm, 5 μm) from CERI. MS analysis was performed using a Mariner electrospray ionization (ESI) time-of-flight mass spectrometer from Applied Biosystems.

Chromatographic conditions

Mobile phases for the pretreatment column were water containing 1% of formic acid (solvent A) and 200 mM ammonium hydrogencarbonate buffer/acetonitrile (5:95 v/v) (solvent B). The internal-surface reversed-phase column was equilibrated with solvent A at a flow rate of 0.5 mL/min, and samples were injected into the HPLC system. Following the removal of plasma proteins and other hydrophilic compounds, the mobile phase was changed to solvent B by switching a six-port valve. Catecholamine metabolites and hydrophobic compounds were eluted, and the fractions were collected. Then, the fractions were evaporated. The residue

Fig. 2 The pathway of synthesis and metabolites of catecholamines: *1* tyrosine hydroxylase, *2* 3,4-dihydroxyphenylalanine (*DOPA*) decarboxylase, *3* dopamine-*β*-hydroxylase, *4* phenylethanolamine-*N*-methyltransferase, *5* catechol-*O*-methyltransferase, *6* monoamine oxidase. *DOPAC* 3,4-dihydroxyphenylacetic acid, *HVA* homovanillic acid, *VMA* vanillomandelic acid
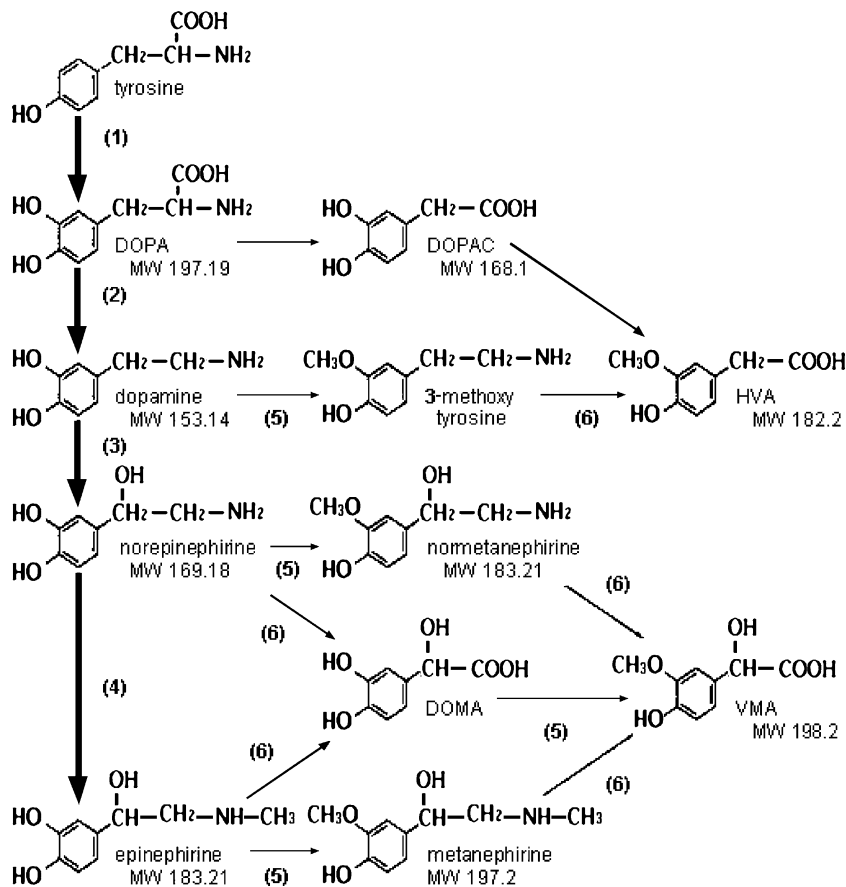
was dissolved in 100 μL of the mobile phase, and 20 μL was injected into the LC/MS system. The two mobile phases used for the analysis of these treated samples were solvent A and methanol containing 1% of formic acid (solvent C). Separation was conducted at a flow rate of 0.2 mL/min. For the separation of amines, the concentration of solvent C was 5% for the first 18 min. In order to elute VMA, DOPAC and HVA the concentration of solvent C was raised to 45% for the next 12 min. The UV detector was set to monitor 280 nm. The mass spectrometer was used in the positive-ion ESI mode.

Results and discussion

Separation of catecholamine metabolites

We developed a method for the HPLC separation of catecholamine metabolites. The catecholamine metabolites were divided into two groups: five amines and one amino acid, and three carbonic acids. In order to separate the five amines and one amino acid, the structures of which were similar, the hydrophilic mobile phase needed to be used. In this case, we used 1% of formic acid/methanol (5:95 v/v) and achieved a good separation. Figure 3 shows the LC and MS chromatograms. The elution order was norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine and DOPA. The peaks of norepinephrine were at m/z 152 ($[M+H-H_2O]^+$) and m/z 170 ($[M+H]^+$). Epinephrine was

detected at m/z 184 ($[M+H]^+$), normetanephrine was detected at m/z 166 ($[M+H-H_2O]^+$) and m/z 184 ($[M+H]^+$), dopamine was detected at m/z 154 ($[M+H]^+$), and metanephrine and DOPA were detected at m/z 180 ($[M+H-H_2O]^+$) and m/z 198 ($[M+H]^+$).

DOPAC, HVA and VMA have a strong affinity with ODS. To accelerate the elution of these three substances, the concentration of the organic mobile phase was raised from 5 to 45%. As a result, all procedures were carried out within 60 min. The elution order of carbonic acids was VMA, DOPAC and HVA (Fig. 4). The peaks of DOPAC were at m/z 169 ($[M+H]^+$), m/z 186 ($[M+NH_4]^+$) and m/z 191 ($[M+Na]^+$). HVA was detected at m/z 183 ($[M+H]^+$), m/z 200 ($[M+NH_4]^+$) and m/z 205 ($[M+Na]^+$). No peak was found for VMA. It is suspected that because VMA has a hydroxyl group close to the carboxyl group, the pK_a is lower compared with that of other carbonic acids. Thus VMA could not be ionized well with the positive ESI mode.

By trapping the catecholamine metabolites on the internal-surface reversed-phase column, we separated catecholamine metabolites and small molecules from plasma proteins. Plasma proteins ran through the column without being trapped, because of the size exclusion and the coated BSA. We set the elution time of proteins by the HPLC-UV and Lowry method (absorbance of 700 nm). At a flow rate of 0.5 mL/min and 100 μL of sample volume, plasma proteins were eluted from the column within 3 min (Fig. 4a,b). Under these conditions the internal-surface ODS did not hold catecholamine metabolites well enough,

Fig. 3 a The liquid chromatography (*LC*) chromatogram. The elution order was norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine, DOPA, VMA, DOPAC and HVA. Flow rate 0.2 mL/min, sample concentration 10 μg/mL, sample volume 20 μL, detection 280 nm. **b** Mass spectrometry (*MS*) total ion chromatogram (*TIC*) (electrospray ionization time-of-flight, *ESI-TOF*, MS; positive mode). **c** Norepinephrine m/z 152($[M+H−H_2O]^+$), m/z 170 ($[M+H]^+$). **d** Epinephrine m/z 184 ($[M+H]^+$). **e** Normetanephrine m/z 166 ($[M+H−H_2O]^+$), m/z 184 ($[M+H]^+$). **f** Dopamine m/z 154 ($[M+H]^+$). **g** Metanephrine, DOPA m/z 180 ($[M+H−H_2O]^+$), m/z 198 ($[M+H]^+$). **h** DOPAC m/z 169 $[M+H]^+$, m/z 186 $[M+NH_4]^+$, m/z 191 $[M+Na]^+$. **i** HVA m/z 183 $[M+H]^+$, m/z 200 $[M+NH_4]^+$, m/z 205 $[M+Na]^+$
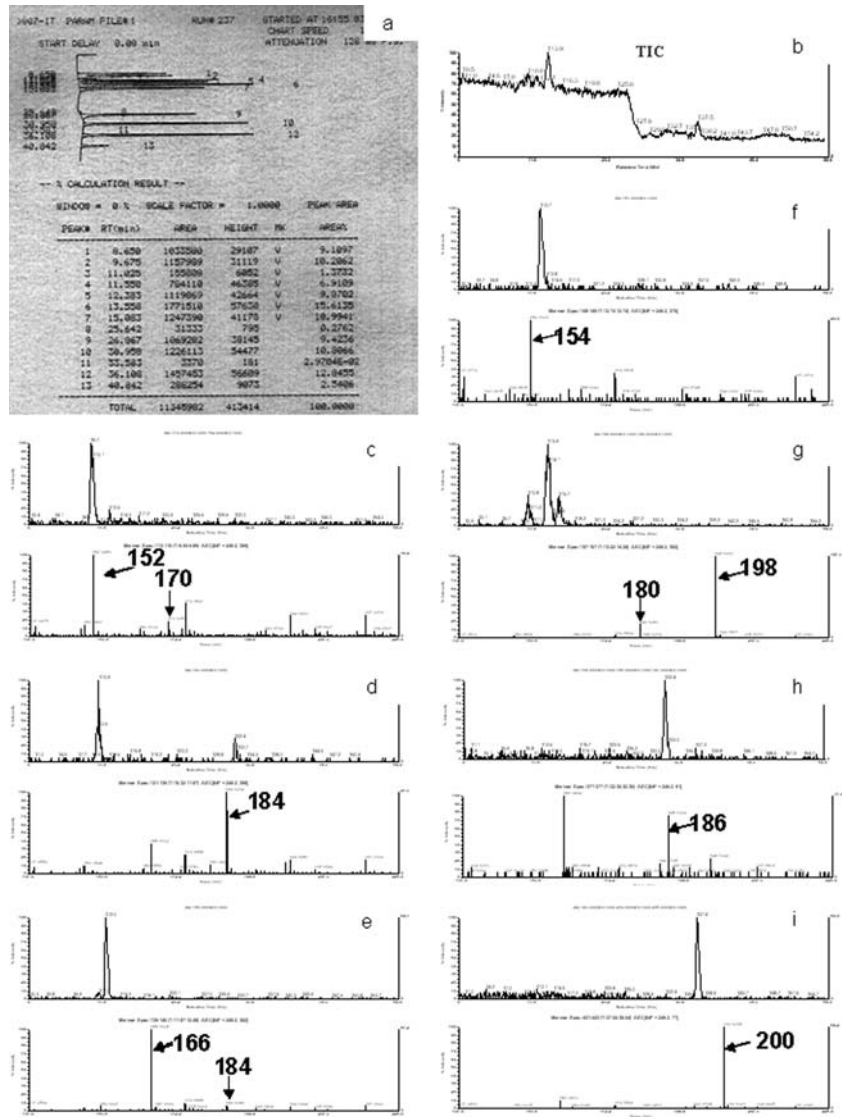

Fig. 4 a LC chromatogram of sample-spiked fetal calf serum (*FCS*). ODS did not hold catecholamine metabolites. **b** Change of protein concentration. Plasma proteins ran down within 3 min. **c** The effect of the ion-pair reagent: 0.1 mM pentadecafluorooctanoic acid/1% formic acid gave good separation. **d** *A* Injection→3 min; *B* after column switching
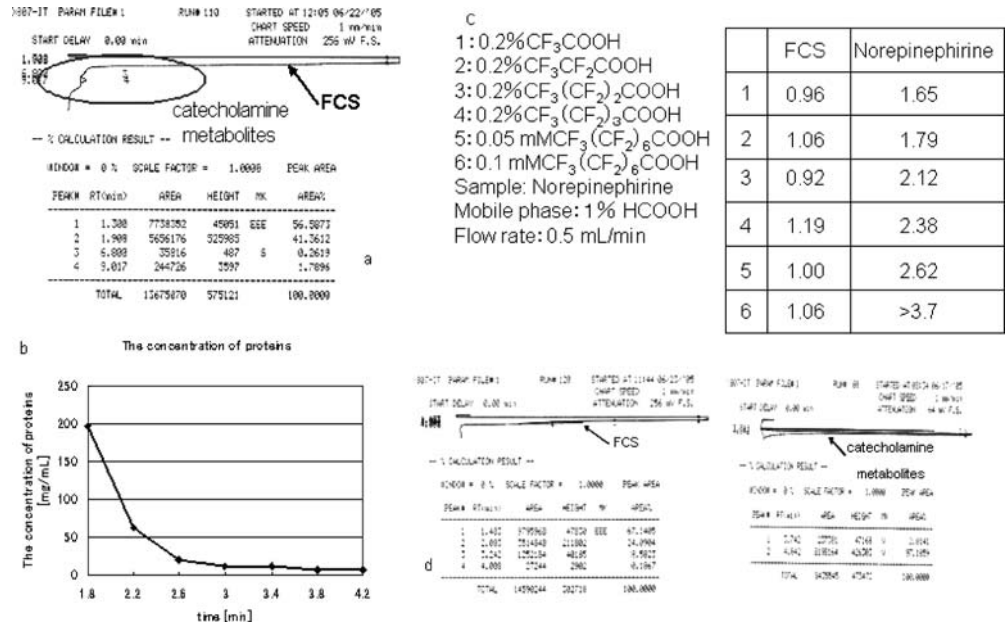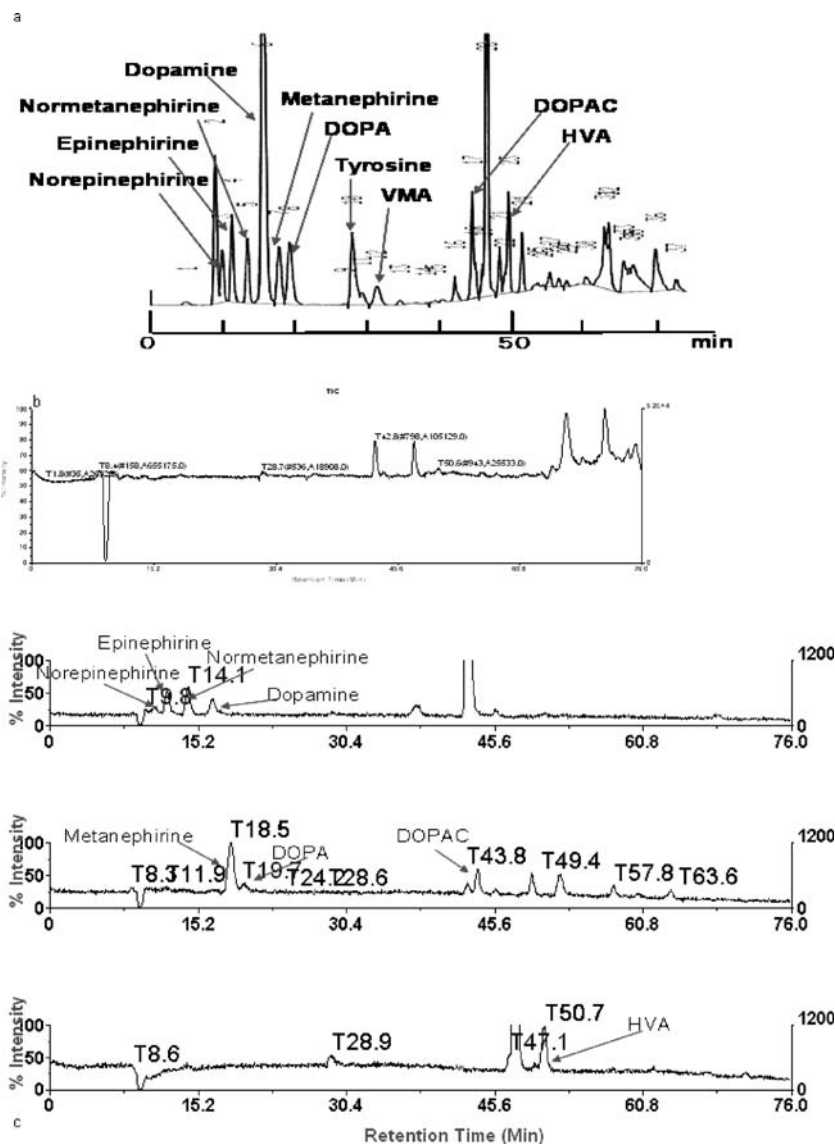
Table 1 The evaluation of reproducibility, recovery and detection limit (*n*=5)

	Recovery (%)	Mean	Standard deviation	Coeffecient of variation (%)	Detection limit: signal-to-noise ratio of 3 (µg/mL)
Norepinephrine	94.1	305,000	21,400	7.0	1
Epinephrine	94.1	363,000	27,700	7.6	1
Normetanephrine	97.9	294,000	7,900	2.7	1
Dopamine	93.3	341,000	12,300	3.6	1
Metanephrine	93.3	357,000	18,400	5.2	1
3,4-Dihydroxyphenylalanine	94.0	323,000	7,300	2.6	1.5
Tyrosine	97.4	207,000	7,200	3.5	1
Vanillomandelic acid	87.0	145,000	11,000	7.4	–
3,4-Dihydroxyphenylacetic acid	97.7	350,000	11,000	3.1	1.5
Homovanillic acid	98.0	435,000	26,000	6.0	1

Fig. 5 **a** The LC chromatogram of sample-spiked plasma. We investigated a variety of compounds in plasma and achieved a clear separation by high-performance LC-UV detection for standard compounds. **b** MS-TIC (ESI-TOF/MS; positive mode). **c** The MS chromatogram. Standard compounds were detected by their peaks

time	m/z					
8.5	147	149	163	167	172	189
8.6	177					
8.9	266	267	268			
9.6	271					
9.7	266	267				
9.8	172					
9.9	208	234	264	279	280	
10.1	175					
10.2	162	163	227	228	229	
14	204					
15	235					
16	137					
17.5	150	151				
28.8	183					
37.5	166					
41.9	195					
42.8	166	167	168	169		
43.2	181					
43.8	285					
43.9	163	180	282			

time	m/z			
44.8	256			
45.7	167	184		
45.8	186			
46.1	181			
46.7	283	300	301	
47	239			
47.6	188	205	206	207
49.4	180	181	195	
49.5	265			
49.7	222			
50.4	222			
50.7	183	200	201	
50.8	282			
52.3	186	187	277	
52.6	153			
53	256			
53.2	172			
53.3	160			
55.1	181			
56	291			

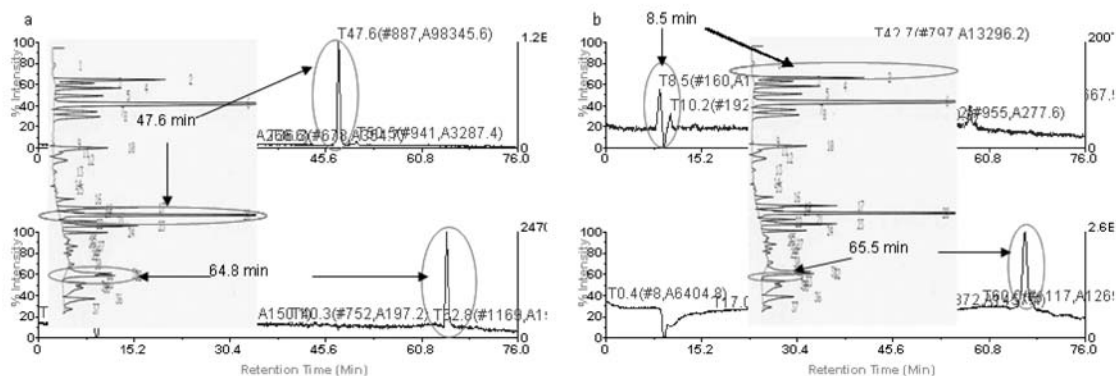| time	m/z						
56.1	239						
57.8	163	180					
59	190						
60.1	191						
60.9	287	288					
63.3	182						
63.6	191						
63.7	208						
64.8	223	224	241	242	263		
65.2	288						
65.5	264	249	250	251	271	272	273
66.7	266	267	268				
67.6	286	287					
68.1	294						
68.8	269	270					
69.9	280						
70.1	299						
70.8	284						
71.3	279	280	281				
72.3	252						

Fig. 6 The ion peaks without catecholamine metabolites detected by ESI-TOF/MS; positive mode. **a** Two peaks detected by both HPLC-UV and MS. **b** Two peaks detected only by MS

because they were ionized. In order to increase the affinity of ODS, we used a volatile ion-pair reagent [17, 18]. With the reagent, trifluoroacetic acid, pentafluoropropionic acid, heptafluorobutyric acid, nonafluorovaleric acid and pentadecafluorooctanoic acid were tested (Fig. 4c). We accomplished a good separation using 0.1 mM pentadeca-fluorooctanoic acid/1% formic acid (Fig. 4d). With this mobile phase, the ion-pair reagent was not removed by evaporation. We placed a column-packed strong anion-exchange resin between the switching six-port valve and the UV detector. As a result, the ion-pair reagent was adsorbed to anion-exchange resin and removed.

Recovery, reproducibility and detection limit

The recovery, reproducibility and detection limit of this method are summarized in Table 1. The sample concentration was 10 μg/mL and the volume was 100 μL. The evaluation was done based on the peak area of the chromatogram. The recovery for VMA was rather low owing to weak retention, but for the other compounds it was more than 90%. The reproducibility for norepinephrine, epinephrine and VMA, which are retained weakly in the pretreatment column, were rather high, but for the other compounds it was within an acceptable level of less than

6% for the coefficient of variation. Since the retention times of these compounds fluctuate in the pretreatment process depending on the presaturated ion-pair concentration, the dispersion of the reproducibility for the compounds seemed to be caused by the concentration of the ion-pair reagent in the internal-surface reversed-phase column. The detection limit at a signal-to-noise ratio of 3 was around 1–1.5 μg/mL. In order to improve the sensitivity, micro-LC/MS and Quadruple mass filter detection can be adopted.

Analysis of the total catecholamine metabolites in plasma

A 100-μL aliquot of sample-spiked plasma was analyzed by the present method. Figure 5 shows the results. We investigated a variety of compounds in plasma and achieved a clear separation by HPLC as shown by UV and MS detection. Figure 6 shows the ion peaks without catecholamine metabolites. Some of the peaks were identified by both HPLC-UV and MS (Fig. 6a), and the rest of them were detected only by MS, indicating that they had no significant UV absorption (Fig. 6b).

Conclusion

For analysis of the metabolic process of catecholamines released from cells such as nerve cells, we established an analytical method for all catecholamine metabolites using a preconcentration column and LC/MS. We succeeded in separating catecholamine metabolites and hydrophobic compounds from plasma proteins in a single separation process by using the internal-surface reversed-phase column and an ion-pair reagent in the pretreatment process. In addition, MS was able to identify some compounds that could not be separated or detected by HPLC. The recovery weas almost 90%, the reproducibility had a coefficient of variation of less than 7% and the detection limit at a signal-to-noise ratio of 3 was around 1–1.5 μg/mL. This method can be applied to help find new markers in neuroblastoma, by comparing the plasma of patients with that of normal infants. The method can also be used to make a diagnosis of other diseases or to find their new markers.

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