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

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Background: Catecholamines are biogenic amines playing an important role in the nervous system. Part of 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 multi aspects of diseases. However it is difficult to conduct, because the catecholamine metabolites are divided into three groups: five amines, one amino acid and three carbonic acids.

Method: Catecholamines and small molecules were separated from plasma proteins by an internal surface reversed phase column (protein-coated ODS column) and analyzed by LC/MS using ESI-TOF/MS.

Result: Using a reversed phase column and hydrophilic mobile phases, we succeeded in the separation of nine catecholamines, all of which structures are similar. These nine substances were eluted in the following order: Norepinephrine, Epinephrine, Normetanephrine, Dopamine, Metanephrine, DOPA, VMA, DOPAC and HVA. The reproducibility of this method was acceptable. The highest C.V. was 7.4%. In addition, various types of compounds were separated from and detected in plasma proteins by applying LC/MS.

Conclusion: Plasma direct injection method, which uses an internal surface reversed phase column and ion-pair reagent, allowed us to separate small molecules from plasma proteins. MS detected some compounds that HPLC could not succeed in the separating and detecting with UV detection. We think that the established method can be applied to find new markers in neuroblastoma, comparing the plasma of patients to that of normal infants. The method can be also used to help for making a diagnosis of other diseases and finding their new makers.

Keywords: neuroblastoma, Catecholamine, Internal surface reversed phase column , LC/MS

DOPA, 3.4-Dihydroxy-phenylalanine; DOPAC, 3.4-Dihydroxy-phenylacetic acid; HVA, Homovanillic acid; VMA, Vanillomandelic acid; and ESI-TOF/MS, electrospray ionization-time of flight/mass spectrometory.

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, the nation-wide neonatal mass-screening at six months of age was conducted in Japan between 1985 and 2004 in Japan. Infants with Neuroblastoma produce an excess of catecholamines compared to normal infants (1). Therefore, a considerable amount of VMA and HVA, the final metabolites of catecholamines, are excreted into the urine (2, 3, 4, 5, 6, 7). The present mass-screening observed an increase of the final metabolites by HPLC (8, 9). However this screening-method had problems with both accuracy of metabolite measurement and the determination of a definitive cut-off value. The committee of 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, the mass-screening is vital to a reduction of the mortality due to infantile cancers (12,13,14). 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 an insignificant subject, needed to be reexamined. We analyzed all metabolic substances of catecholamine, including metabolic by-products, and set up an analytical method using LC/MS. The urine is a proper sample for the screening, posing the least burden and risk to infants and their parents. However, the urine does not contain all of the compounds of the human body and also has a problem with the stability of components generated by contamination. The blood, on the other hand, contains all of the compounds of the human body. Thus we established a test method for analysis of the 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. Fig. 1 shows the characteristics of the column. The outside surface of porous resins was coated with BSA, so that the column did not adsorb for the plasma proteins, but still had the reverse-phase characteristics for small molecules. The internal surface reversed phase column was selected as a precolumn for the deproteinization and a trapping of small molecules(15,16).

FIG. 1

In the human body, catecholamines are formed by the following sequence of reactions: Tyrosine→3.4-Dihydroxy-phenylalanine (DOPA)→ Dopamine→Norepinephrine→ Epinephrine. They have physiological activity respectively. After they performed their roles, DOPA is metabolized and becomes Homovanillic acid (HVA) via 3.4-Dihydroxy-phenylaacetic acid (DOPAC). Dopamine also changes into HVA. Norepinephrine transforms into Normetanephrine, and Epinephrine becomes Metanephrine. They then both transforms into Vanillomandelic acid (VMA). These reactions are catalyzed by the following enzymes: (1)Tyrosine-hydroxylase, (2)DOPA-decarboxylase, (3)Dopamine-β-hydroxylase, (4)Phenylethanolamine-N-methyltransferase, (5)catechol-O-methyl-tranceferase, (6)monoamine-oxidase. Finally HVA and VMA are excreted to the urine (Fig. 2).

FIG. 2

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 ($CF_3(CF_2)_6COOH$) were purchased from KANTO CHEMICAL. Trifluoroacetic acid (CF_3COOH), Pentafluoropropionic acid (CF_3CF_2COOH), Heptafluorobutyric acid ($CF_3(CF_2)_2COOH$) 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 Milli-Q^R Academic A10 purification system from Millipore.

Sample preparation

Standard solution of catecholamine metabolites was dissolved in water at a concentration of 1 mg/mL and mixed all in one solution to a final concentration of 100 μg/mL. The mixed standard solution was diluted by foetal bovine serum or human plasma to be 10 μg/mL and used as sample-spiked plasma.

Instrumentation

The HPLC-UV system was Gulliver Series from Jasco, consisting of DG-980-50 3-Line-Degasser, LG-1580-02 Termary Gradient Unit, PU-980 Intelligent HPLC Pump, and 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 L-column ODS (150x4.6 mm, 5 μm) from CERI. MS analysis was performed using a Mariner™ ESI-TOF/MS from Applied Biosystems.

Chromatographic Conditions

Mobile phases for the pretreatment column were (A) water containing 1 % of formic acid and (B) 200 mM Ammonium Hydrogencarbonate buffer/acetonitrile (5:95 v/v). The internal surface reversed phase column was equilibrated by 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 was dissolved in 100 μL of mobile phase, and 20 μL was injected into the LC/MS system. Two mobile phases used for the analysis of these treated samples were (A) water containing 1 % of formic acid, and (C) methanol containing 1 % of formic acid. 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 minutes. In order to elute VMA, DOPAC and HVA the concentration of solvent (C) was raised to 45% for the next 12 minutes. The UV detector was set to monitor 280nm. The MS 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 five amines and one amino acid, of which structures are similar, the hydrophilic mobile phase needed to be used. In this case, we used 1% of formic acid/methanol (5:95 v/v) and accomplished a good separation. Fig. 3 shows the LC and MS chromatograms. The elution order was Norepinephrine, Epinephrine, Normetanephrine, Dopamine, Metanephrine and DOPA. The peaks of Norepinephrine were m/z152 ($[M+H-H_2O]^+$) and m/z170 ($[M+H]^+$). Epinephrine was detected m/z184 ($[M+H]^+$), Normetanephrine was detected m/z166 ($[M+H-H_2O]^+$) and m/z184 ($[M+H]^+$), Dopamine was detected m/z154 ($[M+H]^+$), Metanephrine and DOPA were detected m/z180 ($[M+H-H_2O]^+$) and m/z198 ($[M+H]^+$).

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 minutes. The elution order of carbonic acids was VMA, DOPAC and HVA (Fig. 4). The peaks of DOPAC were m/z169 ($[M+H]^+$), m/z186 ($[M+NH_4]^+$), and m/z191 ($[M+Na]^+$). HVA was detected m/z183 ($[M+H]^+$), m/z200 ($[M+NH_4]^+$), and m/z205 ($[M+Na]^+$). No peak was found in VMA. It is suspected that because VMA had a hydroxyl group close to the carboxyl group, the pKa was lower compared to other carbonic acids. Thus VMA could not ionize well with the ESI positive mode.

FIG. 3

By trapping of catecholamine metabolites to 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 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 three minutes (Fig. 4-a,b). Under these

conditions the internal surface ODS did not hold catecholamine metabolites enough, because they were ionized. In order to increase the affinity of ODS, we used an volatile ion-pair reagent(17,18). With the reagent, Trifluoroacetic acid (CF_3COOH) ,Pentafluoropropionic acid (CF_3CF_2COOH), Heptafluorobutyric acid ($CF_3(CF_2)_2COOH$) , Nonafluorovaleric acid ($CF_3(CF_2)_3COOH$) and pentadecafluorooctanoic acid ($CF_3(CF_2)_6COOH$) were tested (Fig.4-c). We accomplished a good separation using 0.1 mM pentadecafluorooctanoic acid/ 1 % formic acid (Fig. 4-d). With this mobile phase, ion-pair reagent was not removed by evaporation. We placed a column packed strong anion exchange resin between the switching six-port valve and UV-Detector. As a result, the ion-pair reagent was adsorbed to anion exchange resin and removed.

FIG. 4

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 its volume was 100 μL. The evaluation was done based on the peak area of chromatogram. The value of recovery as to VMA was rather low due to weak retention, but those for others were more than 90%. The values of reproducibility as to Norepinephrine, Epinephrine and VMA, which are retained weakly in the pretreatment column, were rather high, but others were within an acceptable level less than 6% C.V. Since the retention times of these compounds fluctuate in the pretreatment process depending on the pre-saturated ion-pair concentration, the dispersion of reproducibility in the compounds seemed to be caused by the concentration of ion-pair reagent in the internal surface reversed phase column. The detection limit at signal-to-noise ratio=3 were around 1 ~1.5 μg/mL. In order to improve sensitivity, micro-LC/MS and Quadruple mass filter detection can be adopted.

TABLE. 1

Analysis of the total catecholamine metabolites in plasma

100 μL of sample-spiked plasma was analyzed by the present method. Fig. 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 detector. Fig. 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).

FIG. 5, FIG. 6

Conclusion

For analysis of the metabolic process of catecholamines released from cells such as nerve cells, we established an analytical method of all catecholamine metabolites using 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 ion-pair reagent in pretreatment process. In addition, MS was able to identify some compounds that could not be separated or detected by HPLC. The recovery were almost 90%, the reproducibility were less than 7% C.V. and the detection limit at signal-to-noise ratio=3 were around 1～1.5 μg/mL. This established method can be applied to help finding new markers in Neuroblastoma, by comparing the plasma of patients to that of normal infants. The method can be also used for making a diagnosis of other diseases or finding their new markers.

Acknowledgment

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Fig.1 The characteristics of the internal surface reversed phase column. Tthis column was selected as a precolumn for the deproteinization and a trapping of small molecules.

Fig. 2 The pathway of synthesis and metabolite about catecholamines

(1)Tyrosine-hydroxylase, (2)DOPA-decarboxylase, (3)Dopamine-β-hydroxylase, (4)Phenylethanolamine-N-methyltransferase, (5)catechol-O-methyl-tranceferase, (6)monoamine-oxidase

Fig. 3-a The LC chromatogram. The elution order was Norepinephrine, Epinephrine, Normetanephrine, Dopamine, Metanephrine, DOPA, VMA, DOPAC, and HVA. Flow rate: 0.2mL/min. The sample concentration: 10 μg/mL. The sample volume: 20 μL. Detection: UV280 nm.

Fig. 3-b MS-TIC(ESI-TOF/MS; positive mode)

Fig. 3-c Norepinephrine m/z 152($[M+H-H_2O]^+$), m/z 170 ($[M+H]^+$)

Fig. 3-d Epinephrine m/z 184 ($[M+H]^+$)

Fig. 3-e Normetanephrine m/z 166 ($[M+H-H_2O]^+$) m/z 184 ($[M+H]^+$)

Fig. 3-f Dopamine m/z 154 ($[M+H]^+$)

Fig. 3-g Metanephrine, DOPA m/z 180 ($[M+H-H_2O]^+$) m/z 198 ($[M+H]^+$)

Fig. 3-h DOPAC m/z 169 $[M+H]^+$ m/z 186 $[M+NH_4]^+$ m/z 191 $[M+Na]^+$

Fig. 3-i HVA m/z 183 $[M+H]^+$ m/z 200 $[M+NH_4]^+$ m/z 205 $[M+Na]^+$

Fig. 4-a The LC chromatogram of Sample-spiked FCS. ODS did not hold catecholamine metabolites.

Fig. 4-b The change of proteins concentration. Plasma proteins ran dowm within 3 minutes.

Fig. 4-c The effect of ion-pair reagent. 0.1 mM pentadecafluorooctanoic acid/ 1 % formic acid gave a good separation.

Fig. 4-d A:Injection→3 min. B: After column switting

Fig. 5-a The LC chromatogram of sample-spiked plasma. We investigated a variety of compounds in plasma and succeed a clear separation by HPLC-UV detector as to standard compounds.

Fig. 5-b MS-TIC (ESI-TOF/MS; positive mode)

Fig. 5-c The MS chromatogram. Standerd compounds were detected their peaks respectively.

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

Table 1. The evaluation of reproducibility, recovery and detection limit. (n=5)

	recovery (%)	means	S.D.	C.V (%)	detection limit:S/N=3 (μg/mL)
Norepinephrine	94.1	305000	21400	7.0	1
Epinephrine	94.1	363000	27700	7.6	1
Normetanephrine	97.9	294000	7900	2.7	1
Dopamine	93.3	341000	12300	3.6	1
Metanephrine	93.3	357000	18400	5.2	1
DOPA	94.0	323000	7300	2.6	1.5
Tyrosine	97.4	207000	7200	3.5	1
VMA	87.0	145000	11000	7.4	—
DOPAC	97.7	350000	11000	3.1	1.5
HVA	98.0	435000	26000	6.0	1

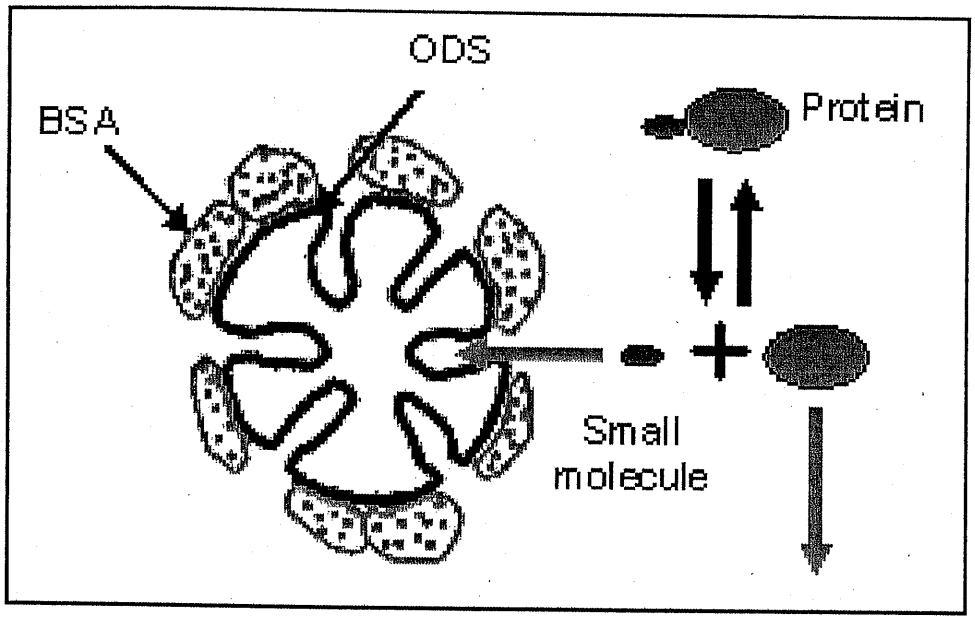


Fig. 1

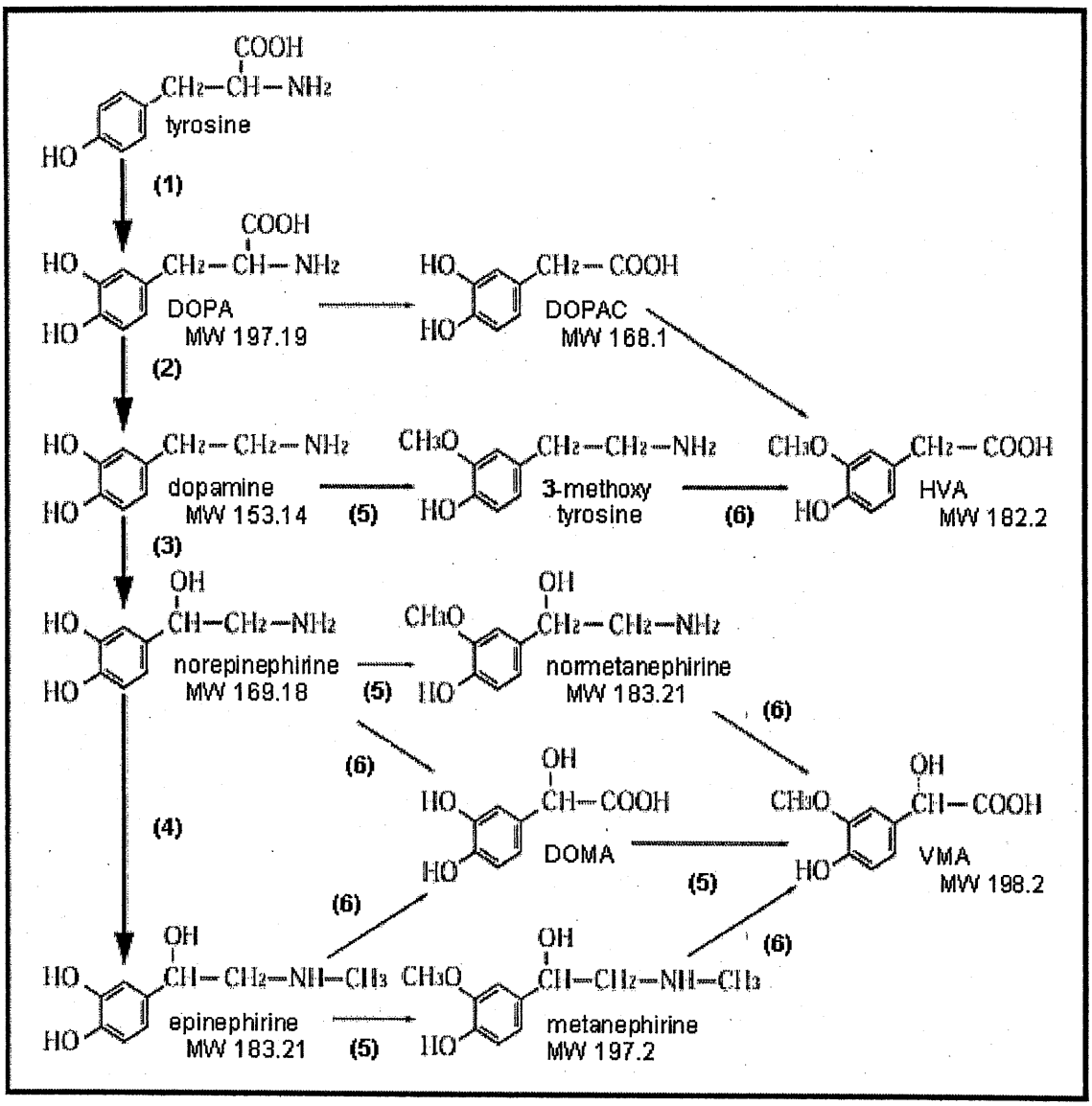

tyrosine
(1)
DOPA
MW 197.19
DOPAC
MW 168.1
(2)
dopamine
MW 153.14
(5)
3-methoxy
tyrosine
(6)
HVA
MW 182.2
(3)
norepinephirine
MW 169.18
(5)
nometanephirine
MW 183.21
(6)
(6)
(4)
DOMA
(5)
VMA
MW 198.2
(6)
(6)
epinephirine
MW 183.21
(5)
metanephirine
MW 197.2

Fig. 2

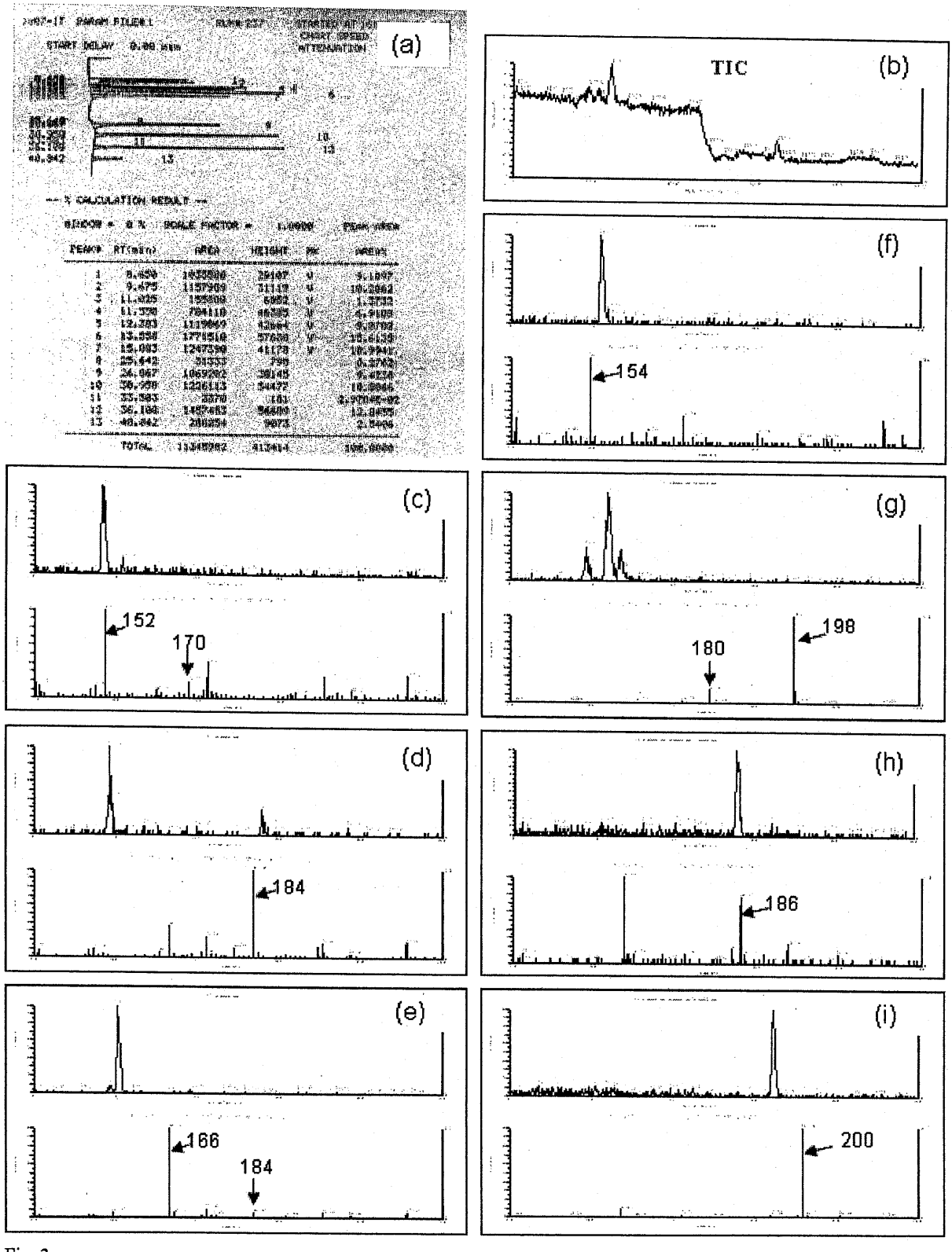
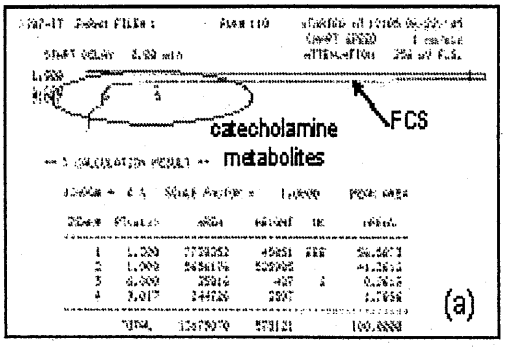
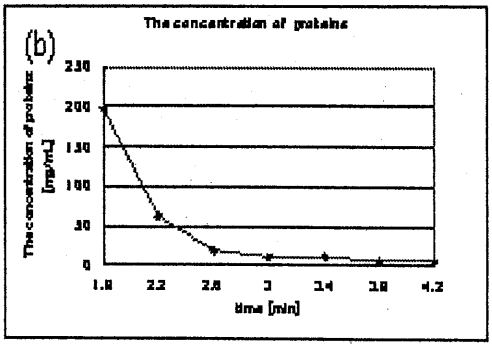


Fig. 3





(c)

1: 0.2% CF_3COOH

2: 0.2% CF_3CF_2COOH

3: 0.2% $CF_3(CF_2)_2COOH$

4: 0.2% $CF_3(CF_2)_3COOH$

5: 0.05 mM $CF_3(CF_2)_6COOH$

6: 0.1 mM $CF_3(CF_2)_6COOH$

Sample: Norepinephirine

Mobile phase: 1% HCOOH

Flow rate: 0.5 mL/min

	FCS	Norepinephirine
1	0.96	1.65
2	1.06	1.79
3	0.92	2.12
4	1.19	2.38
5	1.00	2.62
6	1.06	>3.7

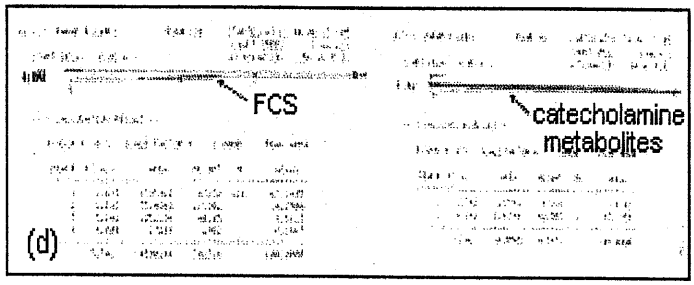


Fig. 4

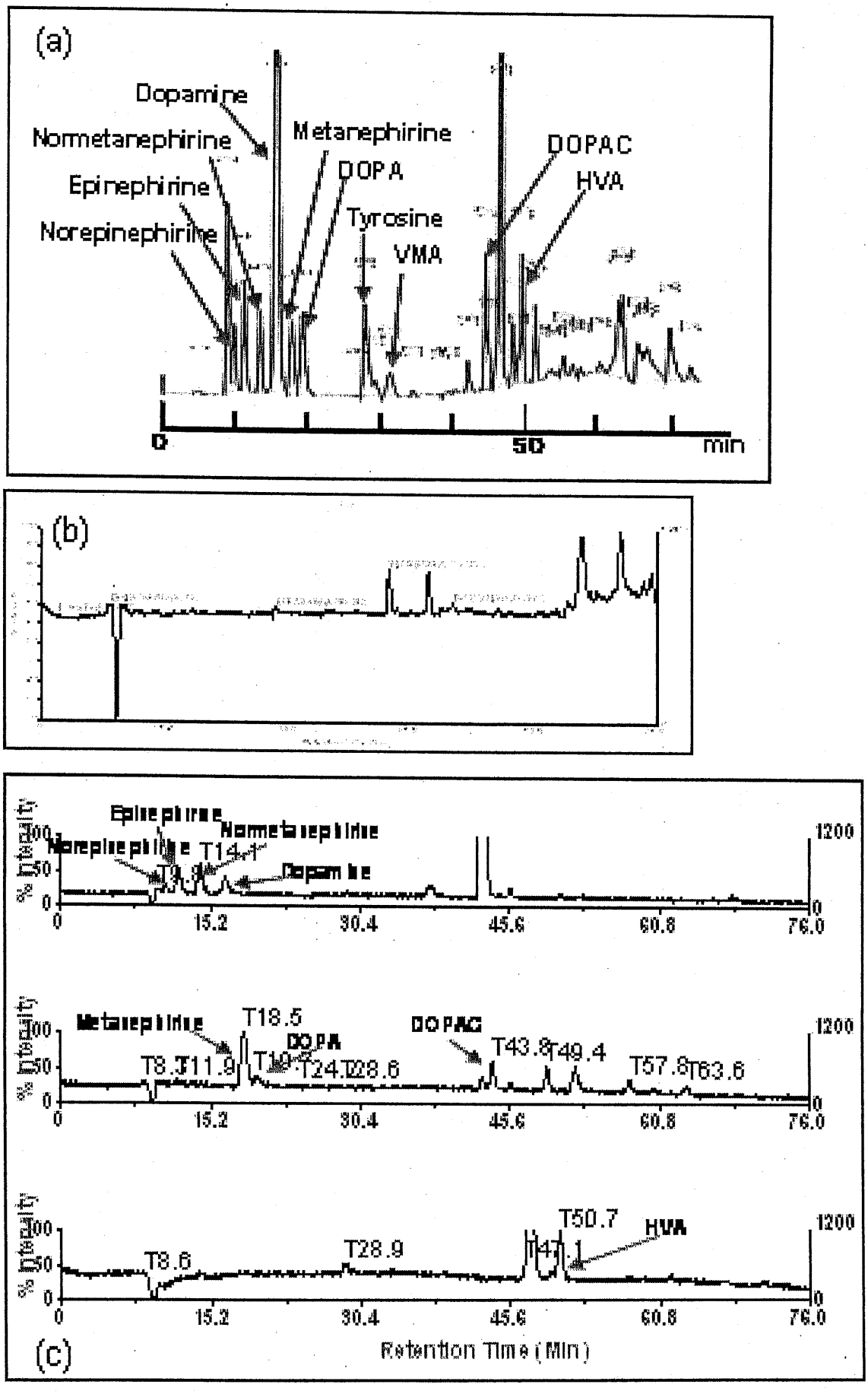

(a)
Dopamine
Normetanephirine
Epinephirine
Norepinephirine
Metanephirine
DOPA
Tyrosine
VMA
DOPAC
HVA
0
50
min
(b)
(c)
% Intensity
Epinephrine
Normetanephrine
Norepinephrine
T14.1
Dopamine
T18.5
Metanephrine
DOPA
DOPAC
T8.3
T11.9
T24.2
T28.6
T43.8
T49.4
T57.8
T63.6
T8.6
T28.9
T50.7
HVA
0
15.2
30.4
45.6
60.8
76.0
1200
Retention Time (Min)

Fig. 5

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	265					
43.9	163	180	262			

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						

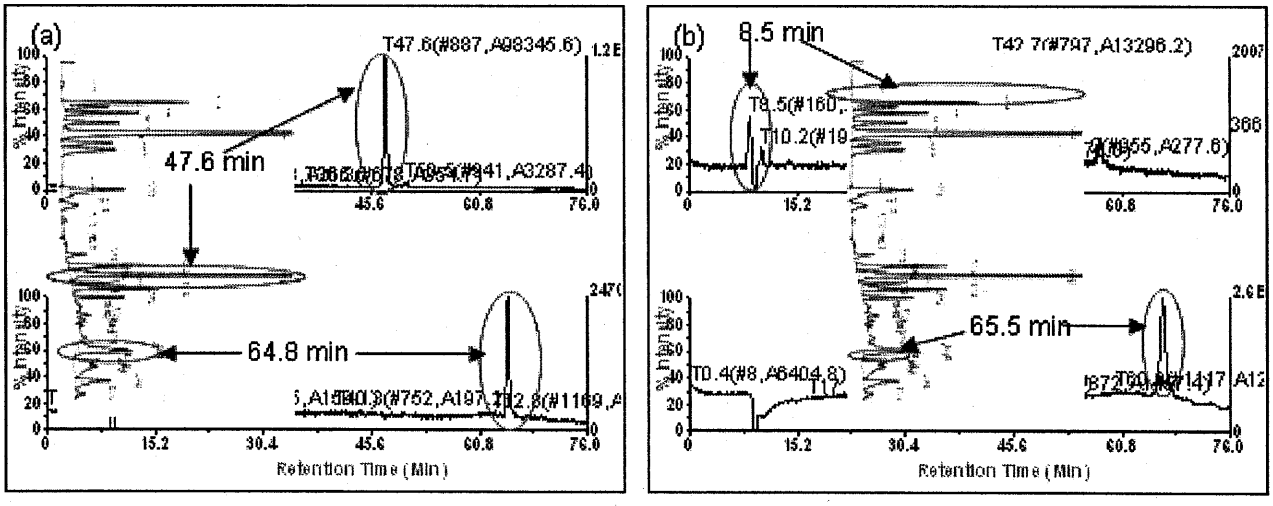


Fig. 6