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Application News

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Bioanalysis / LCMS-8060

Modified Ion-Pairing Reagent LC/MS/MS Method for Analysis of Catecholamines in Cell Cultures

Peiling Hou, Shu Qing Chan*, Jie Xing

❑ Introduction

The catecholamines, including tyrosine, L-DOPA, dopamine and norepinephrine are well known neurotransmitters. The rates of synthesis and release of these neurotransmitters (Fig. 1) are investigated in various types of cell cultures for the studies of various neurological disorders such as depression, schizophrenia and Parkinson's disease [1]. However, there are always challenges for the quantitative determination of catecholamines in biological samples, because these small and very polar molecules are poorly retained on a reversed-phase C18 column. The major disadvantages of existing analytical methods include short retention time, difficulty in separating from each other and potential interference from endogenous compounds present in matrices. There is demand to develop a simpler and rapid LC-MS/MS method for quantitative determination of these catecholamines in cell cultures, which serve as neurotransmitters and are considered important to assess dysfunction of the dopaminergic system for monitoring diseases and therapies.

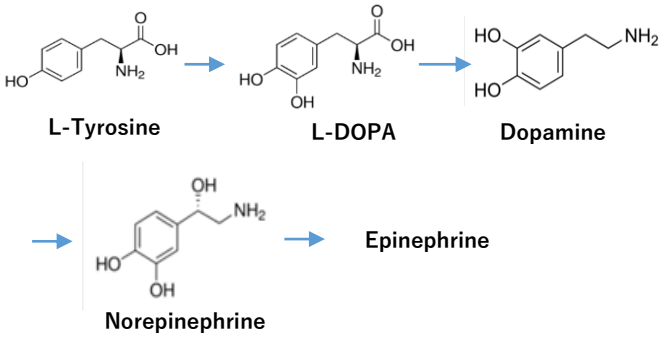

Figure 1. Chemical structures of catecholamines and their metabolic pathway monitored in this study

❑ Experimental

Chemicals and analytical conditions

L-tyrosine, L-DOPA, dopamine, norepinephrine, formic acid (analytical grade), 1-heptane sulfonic acid (HSA, reagent grade) were purchased from Sigma-Aldrich. Catecholamine standards were weighed and dissolved in the Milli-Q water (stock solution, 1000 µg/mL) and diluted to different working solutions ranging from 5 to 2500 ng/mL. The calibration standards were prepared in diluent (MeOH to 1.1 mM ascorbic acid at 1:9, v/v) to contain combined L-tyrosine, L-DOPA, dopamine and norepinephrine at concentrations of 0.25, 0.5, 2.5, 5, 25, 50 and 250 ng/mL. HSA solution was also spiked into the calibration standards at 30 mM as the final concentration for the preparation of calibration and QC standards. The standard solutions were transferred into HPLC vials and 20 µL was injected to Shimadzu LCMS-8060 for analysis. The analytical conditions are shown in Table 1.

Table 1. Analytical conditions of Catecholamines on LCMS-8060

Column	Shim-pack Velox Biphenyl (100 mm. x 2.1mm I.D., 1.8µm)
Flow Rate	0.3 mL/min
Mobile Phase	A : 0.05% formic acid (FA) in milli-Q water B : 0.05% formic acid in methanol (MeOH)
Elution Mode	Gradient elution, LC program 7 minutes 5%B (0.01 min to 0.50 min)→8%B (3.00 min to 4.00 min) →15% B (5.50 min to 6.00 min) →5%B (6.50min)
Oven Temp.	40 ºC
Injection Vol.	20µL

Interface & Temp.	ESI, 250 ºC
MS Mode	MRM, Positive
Heat Block temp.	400ºC
DL temp.	250ºC
Nebulizing gas flow	Nitrogen, 3.0 L/min
Drying gas flow	Nitrogen, 10.0 L/min
Heating gas flow	Zero air, 10 L/min
CID gas	270 kPa (Ar)

*Student for internship training program from School of Chemical and Life Sciences, Singapore Polytechnic, Singapore

MRM optimization of catecholamines

The multiple reaction monitoring (MRM) transitions for L-tyrosine, L-DOPA, dopamine and norepinephrine were optimized on LCMS-8060. The results are shown in Table 2.

Table 2. MRM transitions and parameters of catecholamines on LCMS-8060

Compound	MRM		Q1 Pre Bias (V)	C.E. (V)	Q3 Pre Bias (V)
	Precursor	Product			
L-DOPA	198.15	181.15	-19	-12	-22
		152.15	-20	-15	-20
		107.10	-20	-29	-20
Dopamine	154.15	137.15	-15	-14	-14
		91.10	-15	-23	-18
L-Tyrosine	182.20	165.15	-11	-12	-17
		136.15	-23	-15	-14
		91.10	-11	-29	-18
Norepinephrine	170.20	107.10	-16	-21	-22
	152.15		-17	-21	-20

Pretreatment of cell cultures samples

A simple procedure was used for cell culture media and cell lysis sample treatment before LC-MS/MS analysis. 50 µL of cell media or cell lysis sample was mixed with 125 µL of diluent (MeOH and 1.1 mM ascorbic acid at 1:9, v/v) and vortexed for 30 sec. Ascorbic acid was added as anti-oxidant to prevent potential degradation of catecholamines after the collection. 75 µL of 200mM HSA solution was added to the diluted sample and vortexed for 30 sec, followed by centrifugation at 15,000 x *g* for 10 min. The sample was filtrated using syringe 0.22 um filter, transferred into 1.5 mL vial for LC-MS/MS analysis.

❑ Results and Discussion

Modified ion-pairing chromatograph method

Due to the small molecular size and very polar nature, catecholamines are poorly retained on a reversed-phase C18 column. This may cause difficulty to separate catecholamines from a large number of endogenous compounds in biological samples and leads to ion suppression in ESI ionization on LC-MS/MS.

Several analytical approaches have been used to overcome the separation difficulties such as HILIC and ion-pairing chromatography. However, LC-MS/MS methods based on both separations exhibit low sensitivity for analysis of catecholamines in cell culture samples due to ion suppression. Recently, an alternative approach ion-pairing chromatography has been successfully applied for different types of highly polar compounds [2,3]. These studies show that the ion-pairing reagent can be added to only the extract solution rather than in the mobile phase as in the traditional ion-pairing chromatograph.

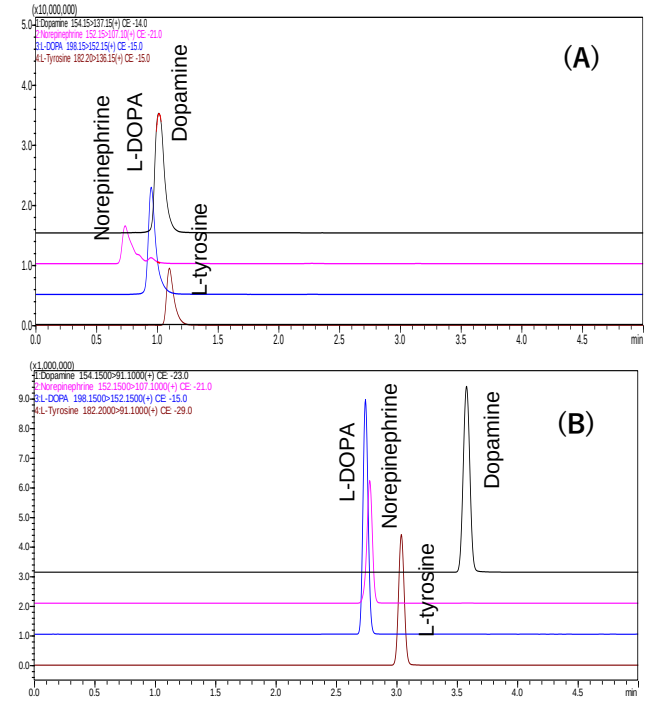

Figure 2. MRM peaks of four catecholamines at 50 ng/mL in mixed standard samples; (A) without use of HSA and (B) with adding 30 mM of HSA as ion-pair reagent.

In this study, this modified ion-pairing chromatography method with adding 1-heptane sulfonic acid (HSA) only in the extract samples, not in the mobile phase, was established and used for quantitation of catecholamines in cell culture samples on LC/MS/MS.

As shown in Figure 2A, the four catecholamines eluted close to the solvent front with little separation without using HSA as ion-pairing reagent either in mobile phase or in mixed standard solution. By adding HSA to the mixed standard sample (30 mM), good retention and improved separation for L-tyrosine, L-DOPA, dopamine and norepinephrine were achieved (Figure 2B). The obvious advantage of this modified ion-pairing chromatograph method by adding the reagent only to the sample is the reduction of ion suppression and contamination in LC-MS/MS analysis.

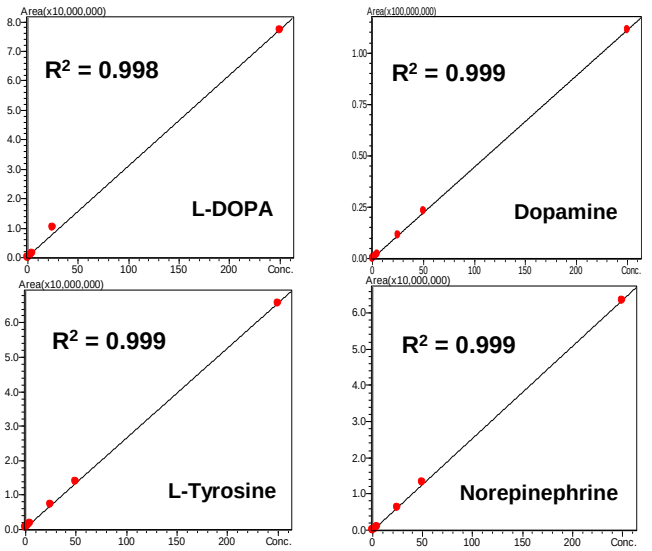

Figure 3. Calibration curves of catecholamines in mixed standard solutions with adding HSA at 30 mM.

Calibration curves

Linearity was evaluated by analyzing calibration standards containing L-tyrosine, L-DOPA, dopamine and norepinephrine at concentrations of 0.25, 0.5, 2.5, 5, 25, 50, 250 ng/mL. The representative calibration curves for all catecholamines are shown in Figure 3. The estimated LOD and LOQ from lowest calibration point (0.25 ng/mL) in mixed standard sample are shown in Table 3.

Table. 3 Linearity, LOD and LOQ of catecholamines in neat standard solutions

Compound	Range (ng/mL)	R^2	Estimated LOD (ng/mL)	Estimated LOQ (ng/mL)
L-DOPA	0.25 – 250	0.998	0.06	0.3
Dopamine	0.25 – 250	0.999	0.06	0.3
L-Tyrosine	0.25 – 250	0.999	0.003	0.03
Norepinephrine	0.25 – 250	0.999	0.4	1.0

Accuracy of analysis

The accuracy and precision tests were performed by testing QC samples at low, medium and high concentrations within their respective calibration ranges. Quality control samples were accurate and precise, ranging from 87.9-113.9% with RSD less than 15% (Data not shown).

Analysis of cell cultures samples

The developed method was applied for the quantitation and monitoring of catecholamines in cell cultures. The chromatographs (shown in Fig. 4) and quantitation results (Table 4) for representative cell media and cell lysis samples were obtained using this developed method. This simple sample treatment method and the established calibration curve were suitable for the cell culture samples with variable concentration levels of catecholamines in this study.

Table 4. Detection and Quantitative results of catecholamines in cell cultures samples

Compound	Cell media (ng/mL)	Cell lysis (ng/mL)
Dopamine	Not detected	Not detected
Norepinephrine	Not detected	5.4
L-DOPA	Not detected	3.4
L-Tyrosine	1113.1	55.7

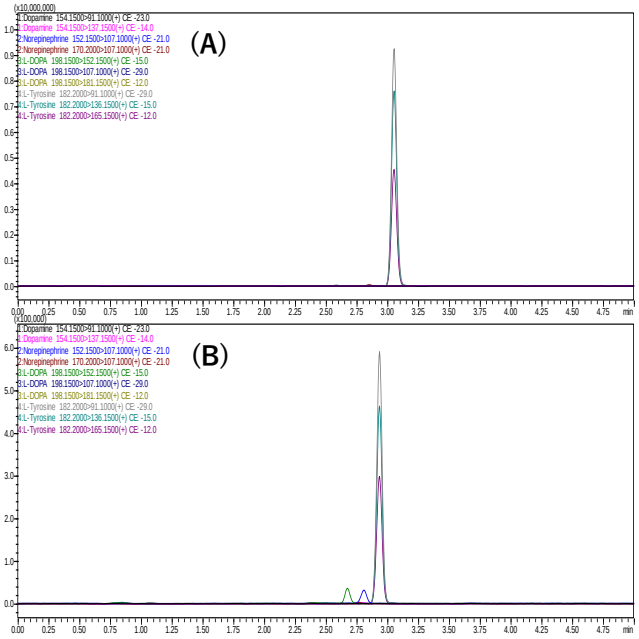

Figure 4. MRM peaks of catecholamines in (A) cell media sample and (B) cell lysis sample

❑ Conclusions

An alternative method of ion-pairing chromatography by adding the reagent, 1-heptane sulfonic acid (HSA), only in the sample was used in LC-MS/MS analysis of four catecholamines, L-tyrosine, L-DOPA, dopamine and norepinephrine in cell culture samples. The optimized concentration of the HSA reagent added to each sample was 30 mM under the conditions, which showed dramatic effects to the retention times and separation selectivity for the four catecholamines. The method was proven to be applicable to cell cultural samples, although slight shifts in retention occurred.

❑ References

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2. M.L. Bergmann, S. Sadjadi, *J. Chromatogr. B* 1057, 2017: 118-3123.
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SHIMADZU (Asia Pacific) Pte. Ltd
79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264,
www.shimadzu.com.sg; Tel: +65-6778 6280 Fax: +65-6778 2050