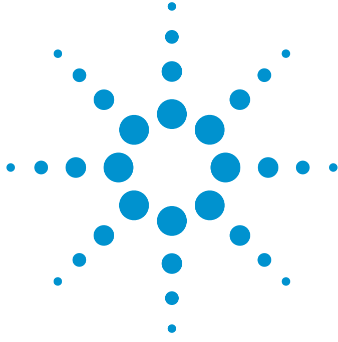

Plasma Catecholamines by LC/MS/MS

Using Agilent Captiva ND^{Lipids}, BondElut PBA SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS

Application Note

Clinical Research

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Abstract

We developed a highly sensitive and specific LC/MS/MS method to quantitate norepinephrine, epinephrine, and dopamine in plasma. An Agilent Captiva ND^{Lipids} filtration and a selective solid phase extraction procedure was used to clean up plasma. The method achieved the required functional sensitivity and quantitated analytes over a sufficiently wide dynamic range. Reproducibility was excellent for all compounds (CV <5 %). All calibration curves displayed excellent linearity, with $R^2 > 0.9997$.

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Introduction

An efficient sample preparation procedure was developed for the simultaneous extraction of norepinephrine, epinephrine, and dopamine (Figure 1) in plasma. Calibrators were created by spiking clean plasma with various concentrations of each analyte. Liquid chromatography/triple quadrupole mass spectrometry (LC/MS/MS) is ideal for the rapid analysis of multiple analytes such as these. The chromatographic system used an Agilent Pursuit pentafluorophenyl (PFP) column and a mobile phase of methanol and water containing 1 mM ammonium fluoride. Quantifier MRM transitions were monitored, and deuterated internal standards were included for each analyte to ensure accurate and reproducible quantitation.

Experimental

LC method

The LC system consisted of an Agilent 1290 Infinity LC Binary Pump, well-plate sampler with thermostat, and a temperature-controlled column compartment. If a LC system with different delay volume is used, the gradient may need to be adjusted and verified to reproduce the same chromatography.

MS method

The Agilent MS/MS system consisted of an Agilent 6460 Triple Quadruple Mass Spectrometer with Agilent Jet Stream technology and Agilent MassHunter Software B.07.00.

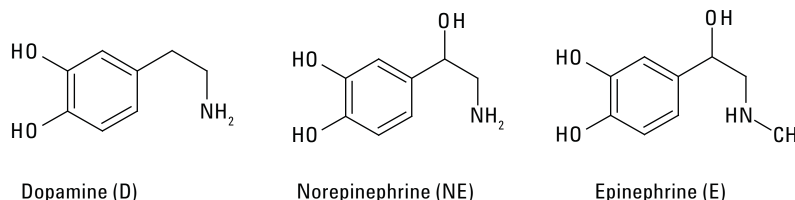

Figure 1. Structures of studied catecholamines.

LC conditions

Parameter	Value
Column	Agilent Pursuit PFP, 2 × 150 mm, 3 µm (p/n A3051150X020)
Guard column	Agilent Pursuit PFP MetaGuard, 200Å, 2 mm, 3 µm (3/pk, p/n A3051MG2)
Mobile phase	A) 1 mM Ammonium Fluoride in water B) methanol
Column temperature	40 °C
Autosampler temperature	4 °C
Injection volume	20 µL
Needle wash	1:1:1:1 MeOH:ACN:IPA:H_2O + 0.1 % formic acid (flush port for 20 seconds)
Flow rate	0.3 mL/min
Gradient	Time (min) %B 0 0 3.8 0 4.0 95 10.0 95 10.1 0 13.0 0

MS conditions

Parameter	Value
Ion mode	Agilent Jet Stream ESI+
Gas temperature	300 °C
Drying gas (nitrogen)	5 L/min
Nebulizer gas (nitrogen)	50 psi
Sheath gas (nitrogen)	400 °C
Sheath flow	12 L/min
Capillary voltage	2,500 V
Nozzle voltage	0 V
Q1/Q3 resolution	0.7 unit
Dwell time	50 ms
Delta EMV	200 V

Chemicals and reagents

Calibrators were purchased from Cerilliant, Round Rock, TX, Cambridge Isotopes Laboratories, Tewksbury, MA, and Medical Isotopes, Pelham, NH. DC Mass Spect Gold plasma MSG3000 was purchased from Golden West Biologicals, Temecula, CA. Endocrine plasma controls 0010 and 0020 were from ChromSystems. Burdick & Jackson LC/MS-grade methanol and reagents were from VWR and Sigma-Aldrich.

The 100 mM $NH_4H_2PO_4$ buffer solution pH 10 was prepared by dissolving 5.75 g of $NH_4H_2PO_4$ in 500 mL of water, and adjusting to pH 10 with NH_4OH 30 %.

Sample preparation

Calibrators (Cerilliant) were prepared with clean plasma matrix (Golden West Biologicals). Isotopically-labeled internal standards (Cerilliant) were used, and their concentrations adjusted so that their responses were in the middle of the desired calibration curve ranges. ChromSystems plasma controls were used. *IMPORTANT: Plasma should be kept frozen at –80 °C until sample analysis*. Stabilizer solutions consisting of EDTA 0.5M and sodium metabisulfite (317 mg/mL) were used to ensure catecholamine stability once thawed.

Table 1. MRM transitions.

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)	CAV (V)
Norepinephrine	152.1	107	115	16	5
Norepinephrine-D6	176.1	111.1	65	24	5
Epinephrine	184.1	151	70	20	5
Epinephrine-D6	190.1	172.1	70	8	5
Dopamine	154.1	119	75	16	3
Dopamine-D4	158.1	95.1	75	28	3

Pretreatment of samples

Add 2 % v/v of stabilizer solutions to plasma samples, calibrators and controls.

The sample preparation consists of two steps:

Step 1 - Protein precipitation and lipid removal

1. Add 1.5 mL of cold 0.5 % formic acid in acetonitrile to an Agilent Captiva ND^{Lipids} cartridge (3 mL, p/n A5300635).
2. Add 50 µL of internal standards solution mix.
3. Add 750 µL of pretreated samples.
4. Mix 3–5 times with a 1.5 mL empty pipette tip.
5. Wait 5 minutes.
6. Place under vacuum at 7" Hg for 2 minutes, then at 15" Hg until dry.
7. Use filtrate for Step 2.

Step 2 - Solid Phase Extraction (SPE)

1. Use filtrate from Step 1.
2. Add 2 mL of 100 mM $NH_4H_2PO_4$ buffer, pH 10.
3. Condition SPE cartridge (Agilent BondElut PBA, 100 mg, 3 mL, p/n 12102127) sequentially with:
   - 1 mL acetonitrile
   - 1 mL 5 % formic acid in methanol
   - 1 mL 100 mM $NH_4H_2PO_4$ buffer, pH 10
4. Add samples.
5. Wash sequentially with:
   - 2 mL 1 % NH_4OH in 95 % methanol
   - 2 mL 1 % NH_4OH in 95 % acetonitrile
   - 2 mL 1 % NH_4OH in 30 % acetonitrile
   - Dry at full vacuum for 5 minutes.
6. Elute with 3 × 500 µL of 5 % formic acid in methanol.
   At the end, apply a vacuum at 5" Hg for 60 seconds.
7. Evaporate under nitrogen flow at 35 °C.
8. Reconstitute with 100 µL of 0.1 % formic acid in water.

Note: The use of silanized glassware is recommended for optimum recoveries.

Data analysis

Agilent MassHunter Quantitative Data Analysis Software (B.07.00) was used for data analysis. A 1/x weighting factor was applied during linear regression of the calibration curves. The quantitation using MassHunter Quantitative Software was performed by comparing chromatographic peak area ratio to a known concentration of the internal standards.

Results and Discussion

Chromatographic separation of all analytes (Figure 2) was achieved with a PFP column. An extra wash was done at the end of every run for superior ruggedness. Although not measured with this method, the separation of epinephrine and normetanephrine, and the separation of metanephrine and 3-methoxytyramine are especially critical since these compounds share common fragments. Without proper separation by retention time, these compounds can cause interferences, leading to inaccurate quantitation. Figure 3 shows the separation achieved with these compounds.

To study matrix effects and recovery efficiency of the SPE extraction procedure, three sets of solutions, A, B, and C were prepared and analyzed. These mixes contained all three analytes and their internal standards at nine different concentrations. Solution A was spiked into 0.2 % formic acid in water, and injected. Solution B consisted of clean extracted plasma, then spiked (post-extraction). Solution C consisted of spiked clean plasma, then extracted (pre-extraction).

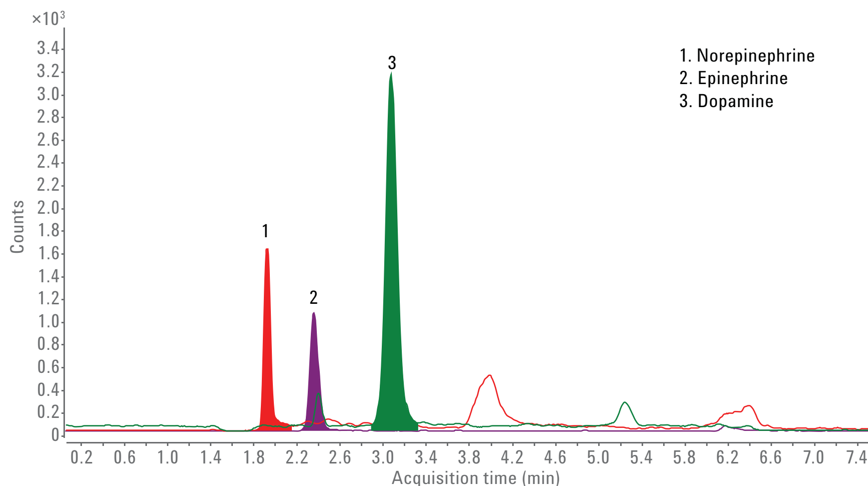

Figure 2. Chromatography of catecholamines.

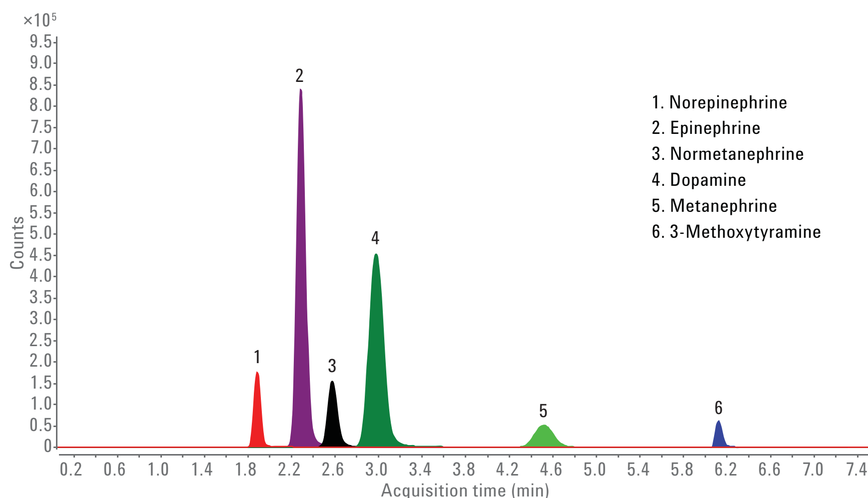

Figure 3. Chromatography of catecholamines, metanephrines, and 3-methoxytyramine.

Matrix effects and recovery efficiencies were calculated as follows:

Matrix effect % = B/A × 100

Recovery efficiency % = C/B × 100

A = Neat standard solutions
B = Plasma extracted then spiked (post-ext)
C = Spiked plasma then extracted (pre-ext)

Matrix effects varied from 42 to 119 %, and recovery efficiencies varied from 56 and 59 % (Table 2). Therefore, matrix effects were observed, but were compensated for by the internal standards, and gave acceptable recovery efficiencies as demonstrated in Tables 3 and 4.

Calibration standards were extracted over a series of three days to establish inter-day precision and accuracy. All three analytes had acceptable accuracies, and the coefficient of variation values were less than 5 % for all concentrations within the linear range (Table 3). ChromSystems controls were extracted over a series of three days, and three times during one day to establish inter- and intra-day, and coefficient of variation values were less than 4 % (Table 4). The method had excellent linearity within the measured range of 5 to 2,500 pg/mL, with an R^2 value greater than 0.9997 (Figure 4).

Table 2. Matrix effects and recovery efficiencies of the SPE procedure.

Compound	Matrix effect % (n = 3)		Recovery efficiency % (n = 3)	
	Average	SD	Average	SD
Norepinephrine	42.3	1.9	56.3	6.3
Epinephrine	70.1	6.6	56.5	2.4
Dopamine	118.5	21.5	58.7	4.3

Table 3. Summary of the analyte performance.

Compound	R^2 (n = 3)	Concentration (pg/mL)	Concentration (nmol/L)	Accuracy % (n = 3)	Inter-day CV % (n = 3)
Norepinephrine	0.9999	5	0.03	107.6	4.7
		20	0.12	95.4	1.2
		250	1.5	98.6	1.4
		2,500	14.8	100.2	0.3
Epinephrine	0.9998	5	0.03	108.4	2.1
		20	0.11	96.5	1.1
		250	1.4	97.5	1.6
		2,500	13.6	100.6	0.3
Dopamine	0.9997	5	0.03	108.7	3.2
		20	0.13	98.8	2.6
		250	1.6	98.1	0.9
		2,500	16.3	99.6	1.1

Table 4. Results of ChromSystems controls by LC/MS/MS.

Compound	QC level	Measured value intra-day (n = 3)		Intra-day CV % (n = 3)	Measured value inter-day (n = 3)		Inter-day CV % (n = 3)
		pg/mL	nmol/L		pg/mL	nmol/L	
Norepinephrine	0010	240	1.42	3.6	242	1.43	3.0
	0020	1,756	10.4	0.7	1,767	10.4	0.8
Epinephrine	0010	93.4	0.51	1.6	92.3	0.50	1.2
	0020	451	2.46	0.7	449	2.45	0.4
Dopamine	0010	164	1.07	0.9	162	1.06	0.8
	0020	595	3.88	0.5	597	3.90	0.9

Conclusions

We developed a robust analytical method for quantifying epinephrine, norepinephrine, and dopamine in plasma. All three analytes were extracted simultaneously with good recoveries using an Agilent Captiva ND^{Lipids} filtration and solid phase extraction. Chromatographic separation of the analytes using conditions compatible with LC/MS/MS was also developed.

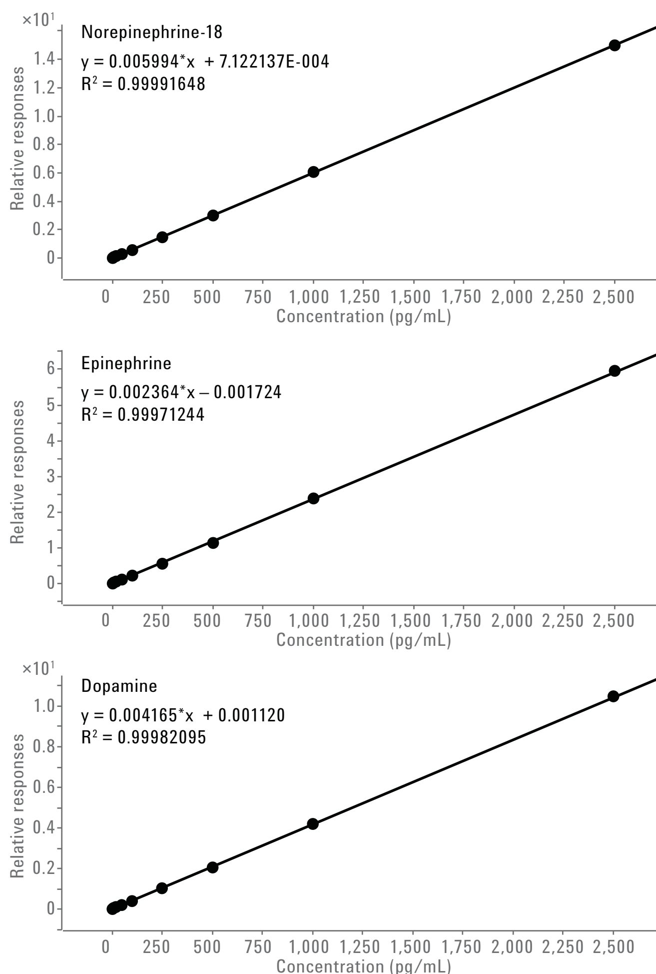

Figure 4. Calibration curves for catecholamines.

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Published in the USA, January 15, 2016
5991-6530EN

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