Plasma Metanephrines and 3-Methoxytyramine by LC/MS/MS

Using Agilent SampliQ WCX SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS

Application Note

Clinical Research

Authors

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Abstract

We developed a highly sensitive and specific LC/MS/MS method to quantitate metanephrine, normetanephrine, and 3-methoxytyramine in plasma. A solid phase extraction (SPE) procedure was used to extract the analytes and remove plasma interferences. The method achieved the required functional sensitivity, and quantitated analytes over a sufficiently wide dynamic range. Reproducibility was excellent for all compounds (CV < 6 %). All calibration curves displayed excellent linearity, with $R^2 > 0.9996$.

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Introduction

An efficient solid phase extraction (SPE) sample preparation procedure was developed for the simultaneous extraction of metanephrine, normetanephrine, and 3-methoxytyramine in plasma (Figure 1). 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 0.2 % formic acid. Quantifier and qualifier MRM transitions were monitored, and deuterated internal standards were included for each analyte to ensure accurate and reproducible quantitation.

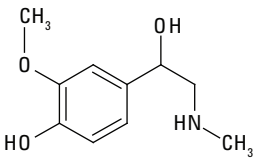
Experimental

LC method

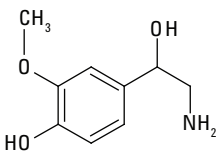
The LC system consisted of an Agilent 1290 Infinity LC Binary Pump, a well-plate sampler with a thermostat, and a temperature-controlled column compartment. If a LC system with different delay volume is used, the gradient may need to be adjusted and verified to reproduce the same chromatography.

MS method

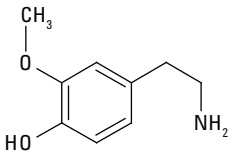
The Agilent MS/MS system consisted of an Agilent 6460 Triple Quadruple Mass Spectrometer with Agilent Jet Stream technology and Agilent MassHunter Software B.07.00.



Epinephrine (MN)



Normetanephrine (NMN)



3-Methoxytyramine (3-MT)

Figure 1. Structures of metanephrines and 3-methoxytyramine.

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) 0.2 % Formic acid in water B) 0.2 % Formic acid in 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	Flow rate (mL/min)
	0	2	0.3
	0.5	2	0.3
	1.5	60	0.3
	4.0	60	0.3
	4.01	95	0.3
	6.0	95	0.3
	6.1	95	0.5
	7.5	95	0.5
	7.6	2	0.3
	10.5	2	0.3

MS conditions

Parameter	Value
Ion mode	Agilent Jet Stream ESI+
Gas temperature	350 °C
Drying gas (nitrogen)	5 L/min
Nebulizer gas (nitrogen)	40 psi
Sheath gas (nitrogen)	400 °C
Sheath flow	11 L/min
Capillary voltage	3,000 V
Nozzle voltage	0 V
Q1/Q3 resolution	0.7 unit
Dwell time	40 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 10 mM $NH_4H_2PO_4$ buffer solution pH 6.5 was prepared by dissolving 575 mg of $NH_4H_2PO_4$ in 500 mL of water, and adjusting to pH 6.5 with NH_4OH 30 %.

Sample preparation

Standard calibrators were prepared by spiking DC Mass Spect Gold plasma with each analyte. Serial dilutions in DC Mass Spect Gold plasma were used to achieve the remaining standard calibrator concentrations. The SPE procedure was as follows:

1. Sample pretreatment: to 0.5 mL plasma, add 50 µL internal standards mix and 0.5 mL of 10 mM $NH_4H_2PO_4$ buffer, pH 6.5.
2. Condition SPE cartridge (Agilent SampliQ WCX, 30 mg, 1 mL, p/n 5982-3513) with 1 mL methanol and 1 mL 10 mM $NH_4H_2PO_4$ buffer, pH 6.5, sequentially.
3. Add pretreated samples.
4. Wash with 1 mL H_2O, 1 mL methanol, and 1 mL 0.2 % formic acid in acetonitrile, sequentially, then dry at full vacuum for 5 minutes.
5. Elute with 2 × 250 µL of 2 % formic acid in acetonitrile, then apply a 5-in Hg vacuum for 60 seconds.
6. Evaporate under nitrogen flow at 40 °C.
7. Reconstitute with 100 µL of 0.2 % formic acid in water. Transfer to an autosampler vial and inject onto the LC/MS/MS system.

Table 1. MRM transitions.

Compound	Precursor (*m/z*)	Product (*m/z*)	Frag (V)	CE (V)	CAV (V)
3-Methoxytyramine*	151.1	91.1	135	20	3
3-Methoxytyramine	151.1	119	135	12	3
3-Methoxytyramine-D4	155.1	95.1	135	24	3
Normetanephrine*	166.1	106.1	105	20	3
Normetanephrine	166.1	121.0	105	20	3
Normetanephrine-D3	169.1	109.1	105	20	3
Metanephrine*	180.1	165.1	120	16	5
Metanephrine	180.1	148.1	120	16	5
Metanephrine-D3	183.1	168.1	120	16	5

* = Quantification transition

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 an Agilent PFP column. The separation of metanephrine and 3-methoxytyramine is especially critical since these compounds share common fragments. Also, although not measured with this method, the separation of epinephrine and normetanephrine is equally critical for the same reason. Without proper separation by retention time, these compounds can cause interferences, leading to inaccurate quantitation. Figure 3 shows the separation achieved with these compounds.

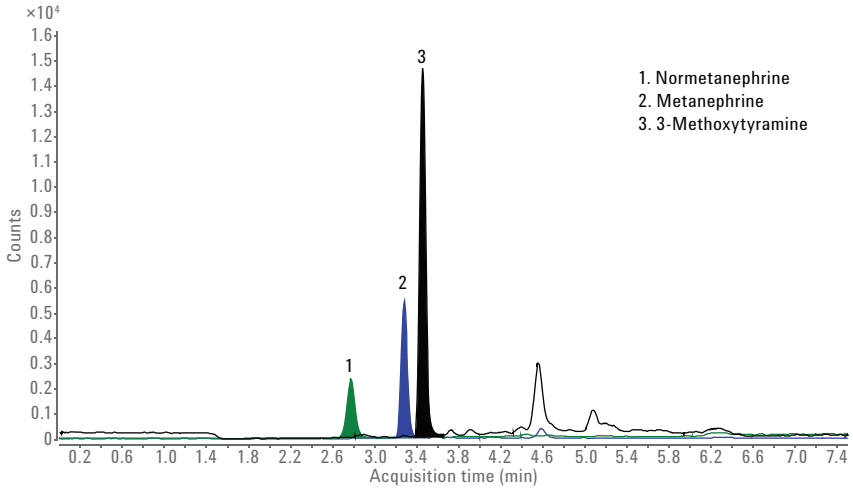


Figure 2. Chromatography of metanephrines and 3-methoxytyramine.

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). 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 36 % to 78 %, and recovery efficiencies varied from 88 % to 104 % (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.

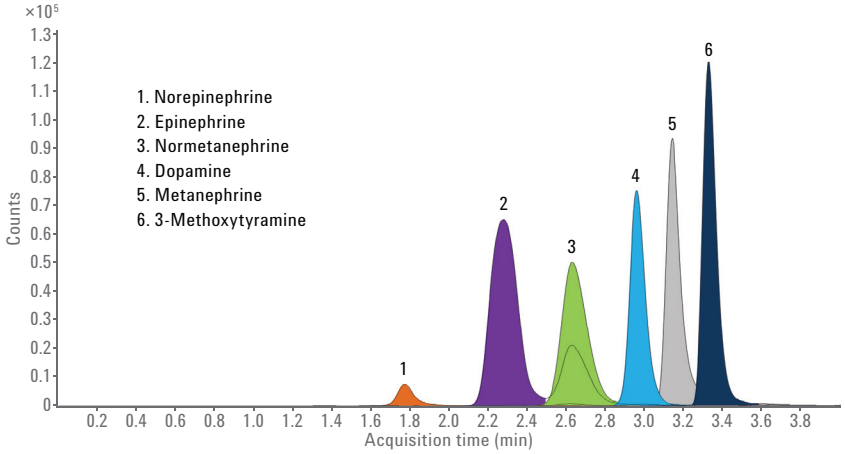


Figure 3. Chromatography of catecholamines, metanephrines and 3-methoxytyramine.

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

	Matrix effects % (n = 9)		Recovery efficiency % (n = 9)	
Compound	Average	SD	Average	SD
3-Methoxytyramine	78.3	7.2	99.1	16.9
Normetanephrine	36.0	13.7	87.7	27.0
Metanephrine	73.0	9.0	104.4	17.3

Table 3. Summary of analyte performance.

Compound	R^2 (n = 3)	Concentration (pg/mL)	Concentration (nmol/L)	Accuracy % (n = 3)	Intra-day CV % (n = 3)	Inter-day CV % (n = 5)
3-Methoxytyramine	0.9997	15.63	0.09	115.4	2.4	1.9
		78.13	0.47	97.6	1.8	1.6
		1,250	7.5	100.4	1.1	1.8
		10,000	59.8	100.0	0.4	1.0
Normetanephrine	0.9996	15.63	0.09	117.3	2.7	4.9
		78.13	0.43	100.0	2.4	2.5
		1,250	6.8	97.1	2.5	1.8
		10,000	54.6	100.4	0.4	0.8
Metanephrine	0.9998	15.63	0.08	116.6	1.7	1.9
		78.13	0.40	96.5	2.5	2.2
		1,250	6.3	100.2	1.1	0.9
		10,000	50.7	100.3	0.6	0.6

Calibration standards were extracted over a series of three days, and three times in one day to establish both inter- and intra-day precision and accuracy. All three analytes had acceptable intra-day accuracies, and inter- and intra-day 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 the coefficient of variation values were less than 6 % (Table 4). The method had excellent linearity, within the measured range of 15.63 to 10,000 pg/mL, with an R^2 value greater than 0.9996 (Figure 4).

Conclusions

We developed a robust analytical method for quantifying metanephrines, normetanephrine, and 3-methoxytyramine in plasma. Offline solid phase extraction for simultaneous extraction of all three analytes from plasma is shown with excellent recoveries. Chromatographic separation of the analytes using conditions compatible with LC/MS/MS was also developed. Typical method performance results were well within acceptable criteria.

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

	Level 1 (n = 3)		Level 2 (n = 3)	
Compound	Measured (pg/mL)	CV %	Measured (pg/mL)	CV %
3-Methoxytyramine	–	–	1,768.9	0.8
Normetanephrine	95.9	5.1	7,369.9	3.0
Metanephrine	85.0	2.2	1,620.8	3.2

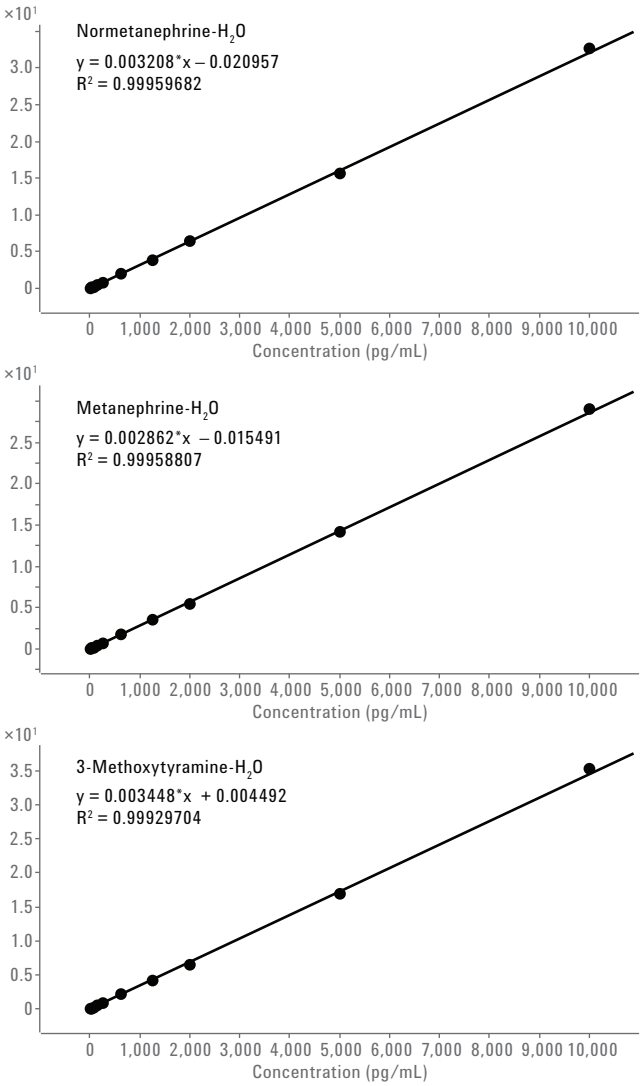


Figure 4. Calibration curves for metanephrines and 3-methoxytyramine.

References

1. Whiting, M. J. Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection. *Ann. Clin. Biochem.* **2009**, *46*, 129–136.

2. Gabler, J. A sensitive and interference-free liquid chromatography tandem mass spectrometry method for measuring metanephrines in plasma. *J. Chromatograph. Separat. Techniq.* **2012**, S2.

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