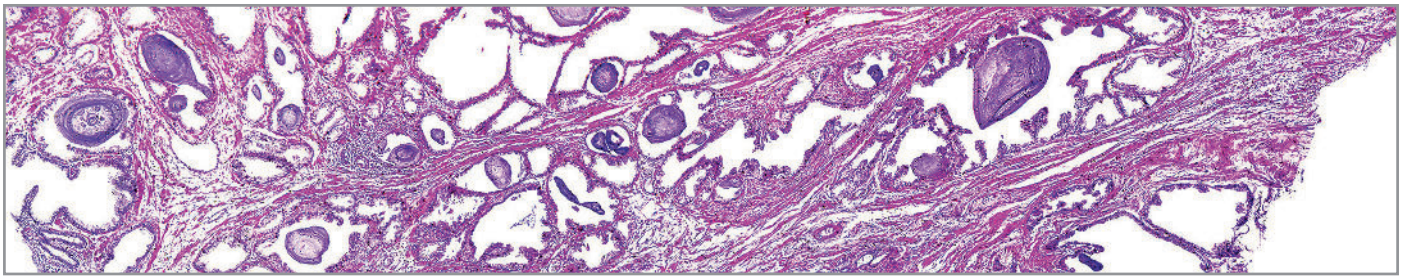


Simultaneous Analysis of Catecholamines and Metanephrines in Urine by LC-MS/MS

By Shun-Hsin Liang and Frances Carroll

Abstract

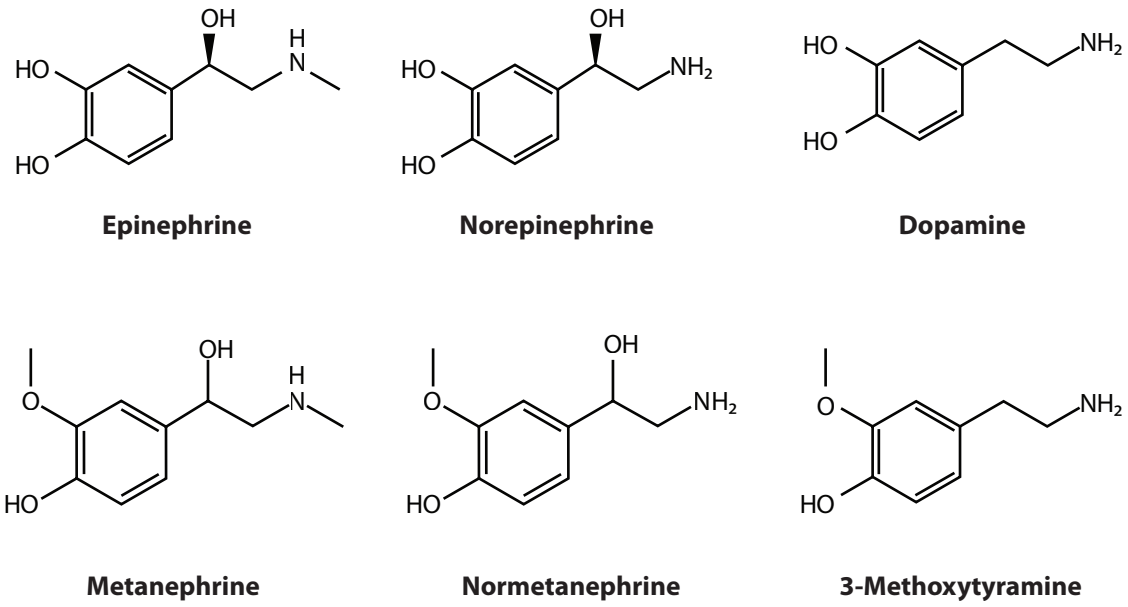
Clinical diagnosis of pheochromocytoma and paraganglioma is often based on the analysis of catecholamines (epinephrine, norepinephrine, dopamine) and metanephrines (metanephrine, normetanephrine, 3-methoxytyramine) in urine. Analysis of these polar compounds using reversed-phase LC can be difficult due to limited chromatographic retention, which results in poor separation of the analytes from closely eluting matrix interferences. This method overcomes these problems by combining a simple solid phase extraction procedure with the consistent and accurate chromatographic performance of a Raptor Biphenyl column.

Introduction

Pheochromocytomas and paragangliomas are neuroendocrine tumors that are characterized by the release of elevated levels of catecholamines. The Endocrine Society, the American Association for Clinical Chemistry, and the European Society for Endocrinology have all released clinical practice guidelines for the diagnosis and management of these diseases and recommend two major diagnostic tests: (1) plasma free metanephrines, which is highly sensitive, and (2) 24-hour urinary collection of catecholamines and metanephrines, which is highly specific.

The target analytes for urinary analysis are epinephrine (EPI), norepinephrine (NE), and dopamine (DA), as well as their respective methylated metabolites, metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT) (Figure 1). Although reversed-phase LC-MS/MS has been the method of choice for this analysis, challenges still remain because these polar analytes are difficult to retain and this, in combination with the presence of matrix interferences, can result in inconsistent chromatographic performance. To solve these problems, a simple and fast solid phase extraction (SPE) procedure was developed, followed by LC-MS/MS analysis using a Raptor Biphenyl column. The method is accurate and robust for the simultaneous analysis of catecholamines and metanephrines in urine and, as such, is suitable for high-throughput clinical diagnostics.

Figure 1: Chemical Structures



Experimental

Solid Phase Extraction

A 200 µL aliquot of urine sample was mixed with 10 µL of internal standard solution (1 µg/mL in methanol) and 600 µL of 250 mM ammonium acetate solution. The mixture was loaded onto the EVOLUTE EXPRESS WCX 96-well plate (30 mg) and washed with 1 mL water and 1 mL methanol:acetonitrile (60:40). The elution was performed with 200 µL of water:methanol (95:5) solution containing 5% formic acid and then 2 µL was injected for analysis.

Calibration Standards and Quality Control Samples

DC Mass Spect Gold Urine (Golden West Biologicals) was fortified with six analytes to prepare calibrated standards and QC samples. The linearity ranges were from 0.5-250 ng/mL for epinephrine; 1-1000 ng/mL for norepinephrine; 5-1500 ng/mL for metanephrine and 3-methoxytyramine; and 10-2000 ng/mL for dopamine and normetanephrine. Three QC levels were prepared at 2.5, 25, and 75 ng/mL for epinephrine and norepinephrine; 25, 75, and 750 ng/mL for metanephrine and 3-methoxytyramine; and 75, 750, and 1500 ng/mL for dopamine and normetanephrine. The fortified standard and QC samples were subjected to the SPE procedure described above.

Urine Analysis

To confirm that the method could accurately measure the urinary catecholamines and metabolites, two levels of urine samples (Bio-Rad Lyphochek quantitative urine controls) were analyzed with the established SPE and chromatographic methods. The normal urine was fortified with 10 ng/mL of epinephrine, norepinephrine, 3-methoxytyramine, and 100 ng/mL of dopamine, metanephrine, and normetanephrine. The abnormal urine was fortified with 50 ng/mL of epinephrine and norepinephrine and 200 ng/mL of dopamine, metanephrine, normetanephrine, 3-methoxytyramine. Accuracy was determined using the measured concentration difference between the blank and fortified urine.

LC-MS/MS analysis of catecholamines and metanephrines in urine was performed on an ACQUITY UPLC instrument coupled with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows and analyte transitions are provided in Table I.

Analytical column:	Raptor Biphenyl (2.7 µm, 150 mm x 2.1 mm; cat.# 9309A62)	
Mobile phase A:	0.2% Formic acid in water	
Mobile phase B:	0.2% Formic acid in methanol	
Gradient:	Time (min)	%B
	0.00	5
	2.50	25
	3.00	95
	3.01	5
	5.00	5
Flow rate:	0.3 mL/min	
Injection volume:	2 µL	
Column temp.	30 °C	
Ion mode:	Positive ESI	

Table I: Analyte Transitions

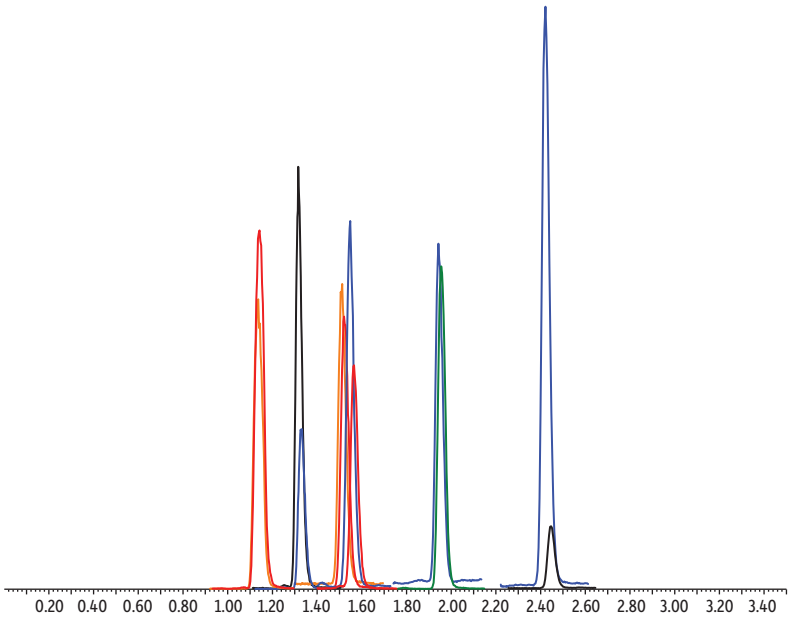
Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
Norepinephrine-d6	158.16	111.26	—
Norepinephrine	152.07	106.91	134.97
Epinephrine-d6	172.16	111.86	—
Epinephrine	166.07	135.00	107.02
Normetanephrine-d3	169.00	136.96	—
Normetanephrine	166.00	148.99	134.02
Dopamine-d4	141.16	94.69	—
Dopamine	136.97	64.66	91.09
Metanephrine-d3	183.00	151.15	—
Metanephrine	179.94	165.01	148.22
3-Methoxytyramine-d4	155.07	122.93	—
3-Methoxytyramine	151.00	91.02	65.05

Results and Discussion

Chromatographic Performance

The analysis of normal human urine (Bio-Rad Lyphochek quantitative urine control, level 1) demonstrates that a fast 5-minute chromatographic analysis is achieved with direct injection of the elution solution that was obtained from the simple SPE procedure (Figure 2). The Raptor Biphenyl column provided adequate retention such that all target analytes could be quantified with no observed influence from matrix interferences.

Figure 2: Analysis of Catecholamines and Metanephrines in Urine



Peaks	t_R (min)	Precursor Ion	Product Ion
1. Norepinephrine-d6	1.14	158.16	111.26
2. Norepinephrine	1.14	152.07	106.91
3. Epinephrine-d6	1.32	172.16	111.86
4. Epinephrine	1.33	166.07	135.00
5. Normetanephrine-d3	1.51	169.00	136.96
6. Normetanephrine	1.52	166.00	148.99
7. Dopamine-d4	1.55	141.16	94.69
8. Dopamine	1.56	136.97	64.66
9. Metanephrine-d3	1.94	183.00	151.15
10. Metanephrine	1.96	179.94	165.01
11. 3-Methoxytyramine-d4	2.42	155.07	122.93
12. 3-Methoxytyramine	2.45	151.00	91.02

Column Raptor Biphenyl (cat.# 9309A62)
Dimensions: 150 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 30 °C
Standard/Sample
Conc.: Endogenous level
Inj. Vol.: 2 µL
Mobile Phase
A: Water, 0.2% formic acid
B: Methanol, 0.2% formic acid

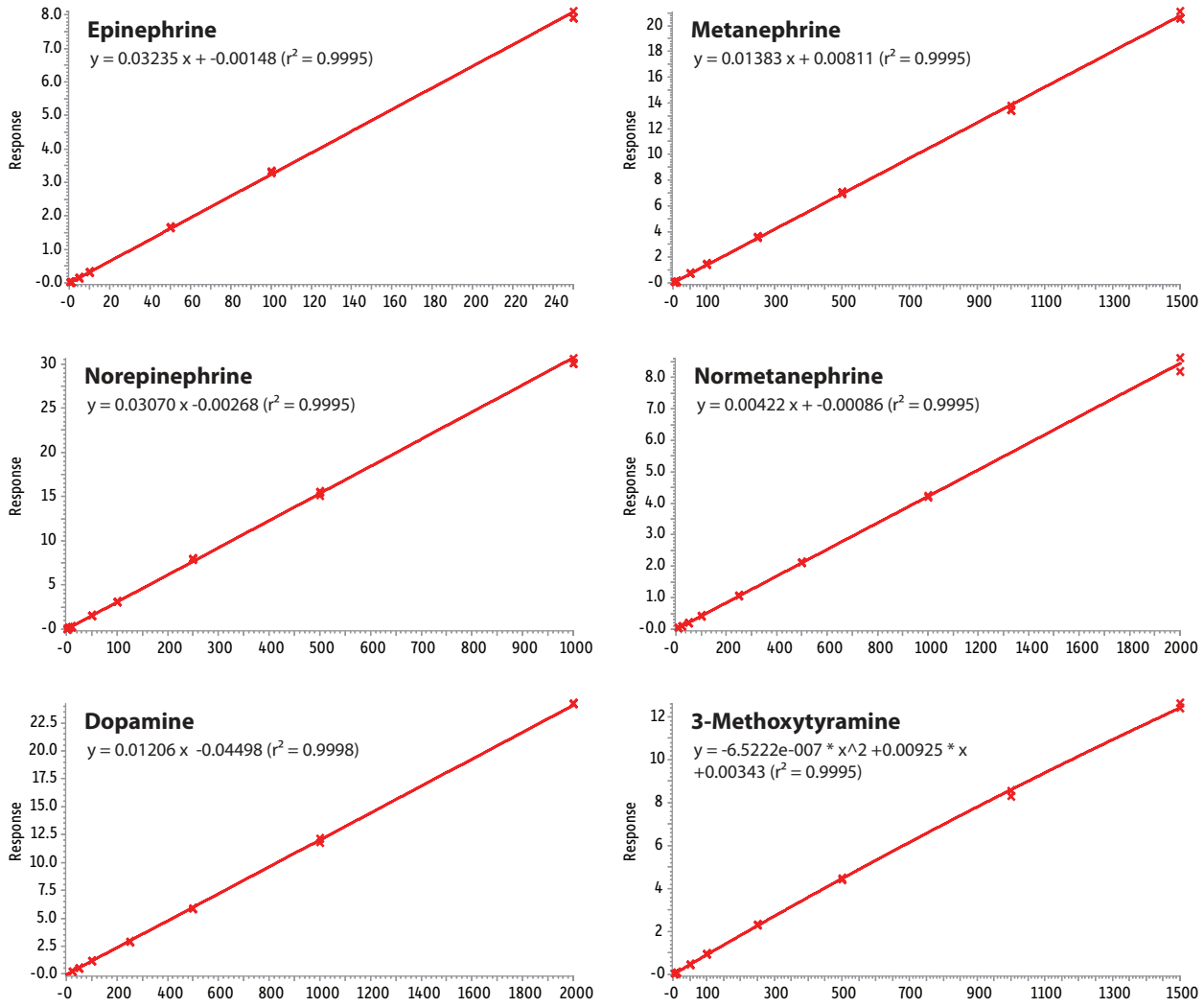
Time (min)	Flow (mL/min)	%A	%B
0.00	0.3	95	5
2.50	0.3	75	25
3.00	0.3	5	95
3.01	0.3	95	5
5.00	0.3	95	5

Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Sample Preparation A 200 µL aliquot of urine sample (Bio-Rad Lyphochek quantitative urine control, normal level) was mixed with 10 µL (1 µg/mL in methanol) internal standard solution and 600 µL of 250 mM ammonium acetate solution. The mixture was loaded onto the EVOLUTE EXPRESS WCX 96-well plate (30 mg), washed with 1 mL water and 1 mL methanol:acetonitrile (60:40), eluted with 200 µL of 5% formic acid in water:methanol (95:5), and injected (2 µL) for analysis.

Linearity

Good linearity was achieved for all compounds using a 1/x weighted linear regression for epinephrine, norepinephrine, dopamine, metanephrine, and normetanephrine, and a 1/x quadratic regression for 3-methoxytyramine (Figure 3). All six analytes had r^2 values of 0.999 or greater and deviations of <10%, except for the lowest concentration standard, which had deviations of <20%.

Figure 3: Calibration Curves



Accuracy and Precision

Precision and accuracy analyses were performed on three different days. The accuracy of the method was demonstrated by the recovery values, which were within 10% of the nominal concentration for all QC levels. The %RSD was 0.2-6.2% and 1.8-3.8% for intraday and inter-day comparisons, respectively, indicating that method precision was also acceptable (Table II).

Table II: Accuracy and Precision of QC Samples

	QC Level 1 (2.5–75 ng/mL)			QC Level 2 (25–750 ng/mL)			QC Level 3 (75–1500 ng/mL)		
Analyte	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD
Epinephrine	2.43	97.3	2.97	26.0	104	2.31	77.9	104	2.19
Norepinephrine	2.44	97.5	2.34	25.3	101	2.92	76.9	103	2.48
Dopamine	74.5	99.4	3.42	787	105	3.85	1,560	104	3.37
Metanephrine	25.4	102	2.46	81.0	108	1.81	774	103	2.00
Normetanephrine	78.4	105	3.36	788	105	2.98	1,570	105	3.52
3-Methoxytyramine	25.7	103	3.39	81.4	109	2.87	764	102	2.94

Urine Analysis

From three sets of analyses of blank and fortified urine samples (BioRad Lyphochek quantitative urine control, level 1 and level 2), the results showed that the recoveries of all fortified samples were within 90-105% of their nominal values (Table III). This demonstrated that the method is suitable for the analysis of catecholamines and metanephrines in urine from human patients at clinically relevant levels.

Table III: Analysis of Fortified Urine Samples

	Level 1					Level 2				
Analyte	Blank Urine (ng/mL)	Fortified Urine (ng/mL)	Calculated Conc. (ng/mL)	Fortified Conc. (ng/mL)	Average Accuracy (%)	Blank Urine (ng/mL)	Fortified Urine (ng/mL)	Calculated Conc. (ng/mL)	Fortified Conc. (ng/mL)	Average Accuracy (%)
Epinephrine	13.0	22.6	9.57	10.0	95.7	91.7	143	51.4	50.0	103
Norepinephrine	42.2	51.3	9.14	10.0	91.4	185	234	49.3	50.0	98.7
Dopamine	77.2	166	90.2	100	90.2	459	655	196	200	98.1
Metanephrine	71.9	163	90.7	100	90.7	516	699	183	200	91.7
Normetanephrine	251	345	94.8	100	94.8	1,310	1,500	188	200	94.3
3-Methoxytyramine	12.2	21.5	9.31	10.0	93.1	418	603	185	200	92.6

Conclusion

As demonstrated here, the Raptor Biphenyl column provides good retention and accurate results for the simultaneous analysis of catecholamines and metanephrines in urine. With a fast, simple sample preparation procedure and just five minutes of chromatographic analysis time, the established method is suitable for high-throughput labs performing analysis of pheochromocytoma and paraganglioma.

RESTEK
Pure Chromatography

Questions? Contact us or your local Restek representative (www.restek.com/contact-us).

www.restek.com

Lit. Cat.# CFAN2800A-UNV