Trace-Level Analysis of Metanephrines in Plasma by HILIC LC-MS/MS

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Abstract

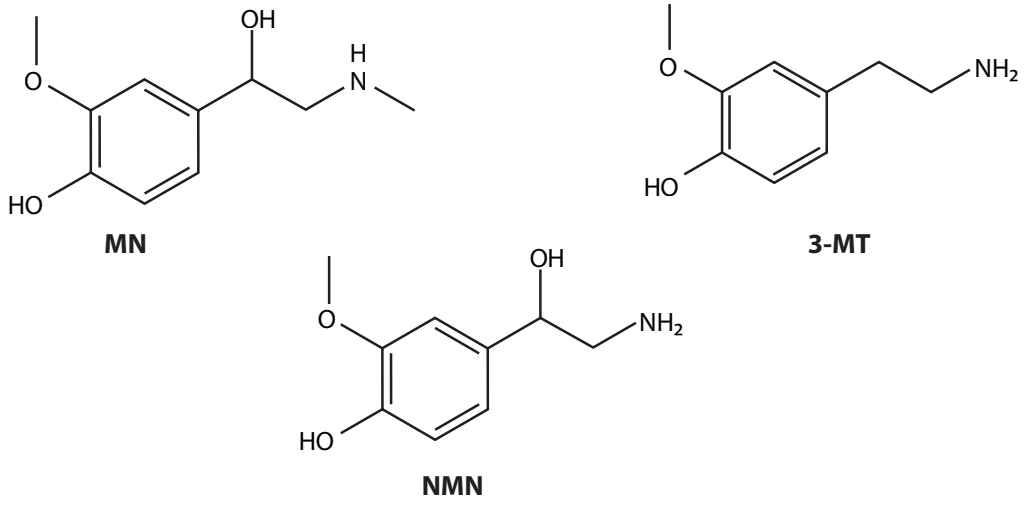
Highly sensitive analysis of metanephrines in plasma is critical in the diagnosis and treatment of pheochromocytoma and paraganglioma. Here, a HILIC LC-MS/MS method was developed using a Raptor HILIC-Si column because this approach provides retention of all target compounds in highly organic mobile phases, thus providing the increased sensitivity needed to reach low detection limits. Data from linearity, accuracy, and precision testing demonstrate that accurate results are consistently obtained—even at trace levels—for these important clinical biomarkers.

Introduction

The metanephrines—metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT)—are methylated metabolites of epinephrine, norepinephrine, and dopamine, respectively (Figure 1). These catecholamine metabolites are released from the adrenal medulla and sympathetic nervous cells and are normally maintained at very low concentrations in the bloodstream. Elevated levels of these compounds in circulation can indicate the presence of pheochromocytoma or paraganglioma, so highly sensitive measurement of in vivo concentrations is critical for correct diagnosis, treatment, and long-term patient monitoring.

Trace-level analysis of metanephrines in plasma using typical reversed-phase LC can be difficult due to limited chromatographic retention and lack of sensitivity. As an alternative, we developed an LC-MS/MS method based on hydrophilic interaction liquid chromatography (HILIC) using a Raptor HILIC-Si column. Combined with a simple solid phase extraction (SPE) procedure, an accurate and precise analysis of these important biomarkers can be achieved in a fast, 5-minute analysis time, making this a beneficial assay for high-throughput clinical labs.

Figure 1: Structures of Metanephrine (MN), Normetanephrine (NMN), and 3-Methoxytyramine (3-MT)



Experimental

Sample Preparation

Internal standard (IS) solution was prepared at 4 ng/mL for metanephrine-d3, and at 8 ng/mL for normetanephrine-d3 and 3-methoxytyramine-d4 in methanol. A plasma sample aliquot (200 µL) was mixed with 10 µL of IS solution and 600 µL of 50 mM ammonium acetate. The mixture was loaded onto an EVOLUTE EXPRESS WCX 96-well plate (30 mg) and washed with 1 mL water and 1 mL methanol:acetonitrile (50:50). The elution was performed twice with 0.9 mL of 5% formic acid in methanol:acetonitrile (50:50) and samples were then evaporated to dryness at 55 °C under a gentle stream of nitrogen. Dried extracts were reconstituted with 100 µL of 100 mM ammonium formate in water (pH 3.0):acetonitrile (10:90) and injected (10 µL) for analysis.

Calibration Standards and Quality Control Samples

Charcoal stripped human plasma (BioreclamationIVT) was fortified with metanephrine, normetanephrine, and 3-methoxytyramine to prepare calibration standards and QC samples. The linearity ranges were 0.051-20.28 nmol/L (10-4,000 pg/mL) for metanephrine; 0.14-21.83 nmol/L (24-4,000 pg/mL) for normetanephrine; and 0.060-23.92 nmol/L (10-4,000 pg/mL) for 3-methoxytyramine. Three QC levels were prepared at 40, 400, and 2,500 pg/mL for all three analytes. The fortified standard and QC samples were prepared using the SPE procedure described above.

LC-MS/MS analysis of metanephrines in plasma 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 HILIC-Si (2.7 µm, 50 mm x 2.1 mm; cat.# 9310A52)	
Mobile phase A:	100 mM ammonium formate in water, pH 3.0	
Mobile phase B:	Acetonitrile	
Gradient	Time (min)	%B
	0.00	90
	5.00	90
Flow rate:	0.3 mL/min	
Injection volume:	10 µL	
Column temp.:	30 °C	
Ion mode:	Positive ESI	

Table I: Analyte Transitions for Plasma Free Metanephrines LC-MS/MS Analysis

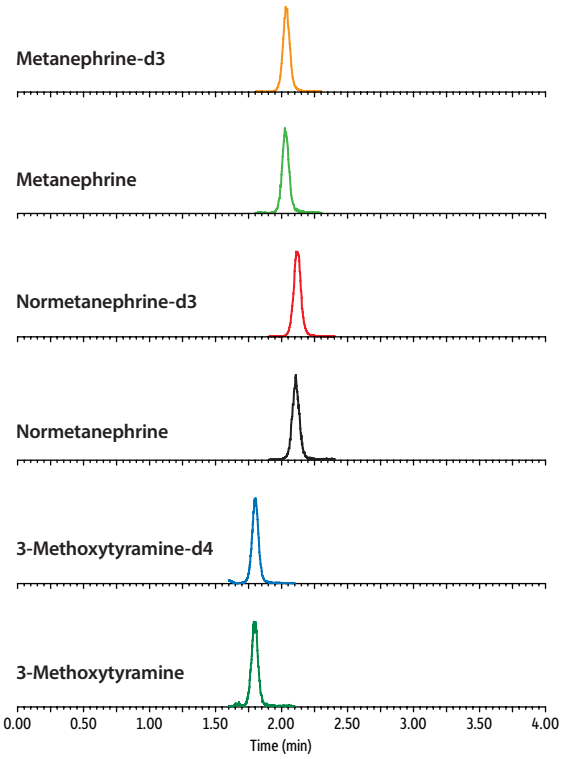
Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Quantifier
Metanephrine-d3	183.00	151.15	—
Metanephrine	179.94	148.22	165.01
Normetanephrine-d3	169.00	136.96	—
Normetanephrine	166.00	134.02	121.01
3-Methoxytyramine-d4	155.07	122.93	—
3-Methoxytyramine	151.00	119.00	91.02

Results and Discussion

Chromatographic Performance

Using a HILIC approach and Raptor HILIC-Si column, good retention was obtained for all analytes. A fast, 5-minute chromatographic analysis (Figure 2) was achieved with injection of reconstituted sample extract. No chromatographic interferences were observed in the analysis of blank plasma samples (Figure 3), indicating that the simple SPE procedure was effective and specific. In addition, the isocratic elution with HILIC showed consistent results over multiple injections indicating that the method was robust (Figure 4).

Figure 2: Human Plasma Fortified with Metanephrine, Normetanephrine, and 3-Methoxytyramine (50 pg/mL Calibration Standard)



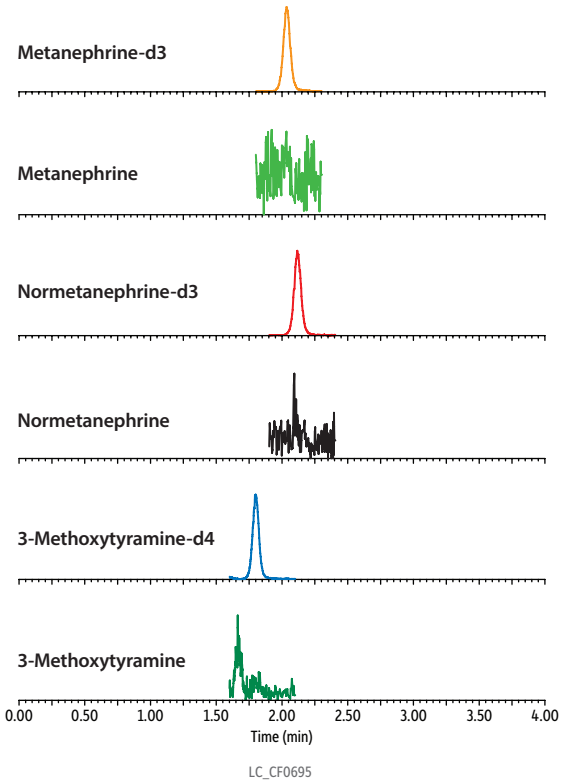
Peaks	t_R (min)	Conc. (pg/mL)	Precursor Ion	Product Ion
1. 3-Methoxytyramine-d4 (IS)	1.80	400	155.07	122.93
2. 3-Methoxytyramine	1.80	50	151.00	119.00
3. Metanephrine-d3 (IS)	2.03	200	183.00	151.15
4. Metanephrine	2.03	50	179.94	148.22
5. Normetanephrine-d3 (IS)	2.11	400	169.00	136.96
6. Normetanephrine	2.11	50	166.00	134.02

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 30 °C
Sample
Diluent: Mobile phase A:mobile phase B (10:90)
Inj. Vol.: 10 µL
Mobile Phase
A: Water, 100 mM ammonium formate, pH 3.0
B: Acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.3	10	90
5.00	0.3	10	90

Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Notes Solid Phase Extraction Procedure:
Charcoal stripped human plasma was fortified with analytes at 50 pg/mL (0.25, 0.27, and 0.30 nmol/L for metanephrine, normetanephrine, and 3-methoxytyramine, respectively). Internal standard (IS) was prepared at 4 ng/mL for metanephrine-d3 and at 8 ng/mL for normetanephrine-d3 and 3-methoxytyramine-d4 in methanol. The plasma sample (200 µL) was mixed with 10 µL of IS solution and 600 µL of 50 mM ammonium acetate solution. The mixture was loaded in an EVOLUTION EXPRESS WCX 96-well plate (30 mg) and washed with 1 mL water and 1 mL methanol:acetonitrile (50:50). The elution was performed twice with 0.9 mL of 5% formic acid in methanol:acetonitrile (50:50) and evaporated to dryness at 55 °C under a gentle stream of nitrogen. Dried extract was reconstituted with 100 µL of diluent and injected (10 µL) for analysis.

Figure 3: Blank Human Plasma



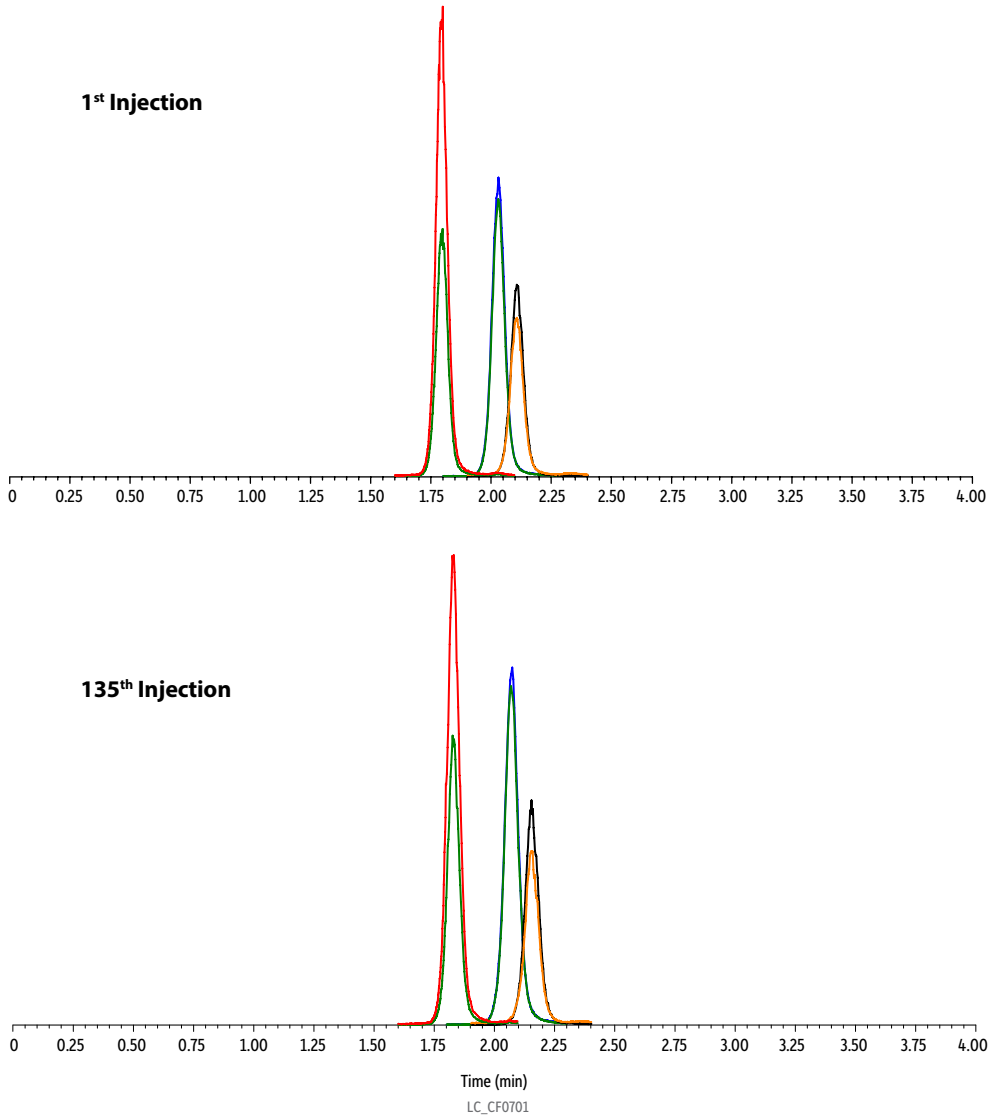
Peaks	t_R (min)	Conc. (pg/mL)	Precursor Ion	Product Ion
1. 3-Methoxytyramine-d4 (IS)	1.80	400	155.07	122.93
2. 3-Methoxytyramine	-	-	151.00	119.00
3. Metanephrine-d3 (IS)	2.04	200	183.00	151.15
4. Metanephrine	-	-	179.94	148.22
5. Normetanephrine-d3 (IS)	2.12	400	169.00	136.96
6. Normetanephrine	-	-	166.00	134.02

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 30 °C
Sample
Diluent: Mobile phase A:mobile phase B (10:90)
Inj. Vol.: 10 µL
Mobile Phase
A: Water, 100 mM ammonium formate, pH 3.0
B: Acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.3	10	90
5.00	0.3	10	90

Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Notes Solid Phase Extraction Procedure:
Internal standard solution (IS) was prepared at 4 ng/mL for metanephrine-d3 and at 8 ng/mL for normetanephrine-d3 and 3-methoxytyramine-d4 in methanol. Charcoal stripped plasma (200 µL) was mixed with 10 µL of IS solution and 600 µL of 50 mM ammonium acetate solution.The mixture was loaded to the EVOLUTION EXPRESS WCX 96-well plate (30 mg) and washed with 1 mL water and 1 mL methanol:acetonitrile (50:50). The elution was performed twice with 0.9 mL of 5% formic acid in methanol:acetonitrile (50:50) and evaporated to dryness at 55 °C under a gentle stream of nitrogen. Dried extract was reconstituted with 100 µL of diluent and injected (10 µL) for analysis.

Figure 4: Robust Column Performance for the Analysis of Metanephrines in Plasma



Peaks	Conc. (ng/mL)	Precursor	Product Ion	Product Ion	1st Injection (t_R)	135th Injection (t_R)
1. 3-Methoxytyramine	1	151.00	119.00	91.02	1.80	1.83
2. Metanephrine	1	179.94	148.22	165.01	2.03	2.07
3. Normetanephrine	1	166.00	134.02	121.01	2.11	2.15

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 30 °C

Sample
Diluent: Mobile phase A:mobile phase B (10:90)
Conc.: 1 ng/mL
Inj. Vol.: 10 µL

Mobile Phase
A: Water, 100 mM ammonium formate, pH 3.0
B: Acetonitrile

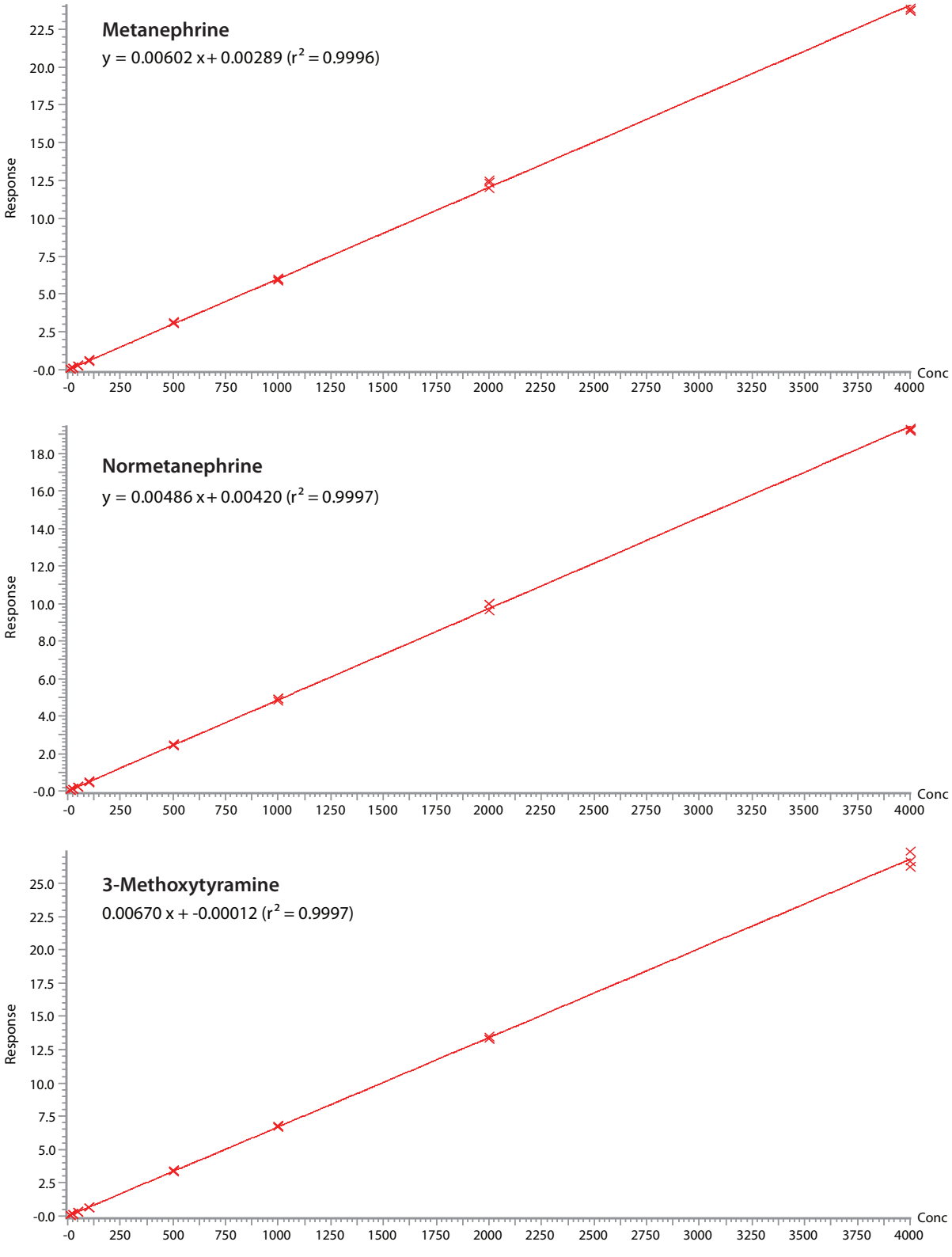
Time (min)	Flow (mL/min)	%A	%B
0.00	0.3	10	90
5.00	0.3	10	90

Detector MS/MS
Ion Mode: ESI+
Mode: MRM

Linearity

Using 1/x weighted linear regression, all three analytes showed acceptable linearity with r^2 values of 0.999 or greater, and deviations of <10% (Figure 5). The established limits of quantitation (LOQ) were 0.051 nmol/L (10 pg/mL), 0.14 nmol/L (24 pg/mL), and 0.060 nmol/L (10 pg/mL) for metanephrine, normetanephrine, and 3-methoxytyramine, respectively.

Figure 5: Calibration Curves



Accuracy and Precision

Precision and accuracy testing was performed on three different days. Method accuracy was demonstrated by recovery values that were within 5% of the nominal concentration for all QC levels. The %RSD was 0.2–4.5% and 1.1–4.2% for intraday and interday comparisons, respectively, indicating good method precision (Table II).

Table II: Accuracy and Precision of QC Samples for the Analysis of Metanephrines in Plasma

	QC Level 1 (40 pg/mL)			QC Level 2 (400 pg/mL)			QC Level 3 (2,500 pg/mL)		
Analyte	**Average Conc. (pg/mL)**	**Average Accuracy (%)**	**%RSD**	**Average Conc. (pg/mL)**	**Average Accuracy (%)**	**%RSD**	**Average Conc. (pg/mL)**	**Average Accuracy (%)**	**%RSD**
Metanephrine	39.5	98.7	3.39	411	103	1.10	2,480	99.2	2.89
Normetanephrine	39.3	98.2	4.21	401	100	1.92	2,440	97.3	2.34
3-Methoxytyramine	40	100	1.81	407	102	2.07	2,470	98.9	2.02

Conclusion

As demonstrated here, using HILIC for the analysis of metanephrines in plasma is a viable approach that provides excellent method performance at clinically relevant concentrations. The Raptor HILIC-Si column ensures good retention of metanephrine, normetanephrine, and 3-methoxytyramine, which is generally difficult to achieve at low detection limits using reversed-phase chromatography. Accurate results were consistently obtained without matrix interference even at trace levels. With a fast, simple sample preparation procedure and 5-minute analysis time, the established method provides a reliable high-throughput assay for the clinical diagnosis of pheochromocytoma and paraganglioma.

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