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Nota de aplicación

Rapid and Simultaneous Analysis of Plasma Catecholamines and Metanephrines Using Mixed-Mode SPE and Hydrophilic Interaction Chromatography (HILIC) for Clinical Research

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Abstract

This Application note demonstrates the extraction and analysis of plasma catecholamines and metanephrines using Oasis WCX µElution Plates and an ACQUITY UPLC BEH Amide Column in HILIC mode.

Benefits

- Retention and baseline resolution of monoamine neurotransmitters and metanephrines without the need for ion-pairing reagents
- Rapid, simultaneous quantification of plasma metanephrines and catecholamines
- 5x analyte concentration without the need for evaporation and reconstitution
- Linear, accurate, and precise results down to 10 pg/mL

Introduction

Clinical researchers are often interested in measuring elevated concentrations of plasma catecholamines and their O-methylated metabolites (metanephrines). However, these compounds (in particular, norepinephrine, epinephrine, and dopamine) can be a challenge to analyze via reversed-phase LC-MS/MS due to their polarity. As a result, many research laboratories still analyze this panel using ion-pairing reagents and electrochemical detection (ECD). While reversed-phase LC-MS/MS has been used successfully, challenges still exist due to ion-suppression from matrix components, insufficient retention, and inadequate separation of normetanephrine and epinephrine.

Hydrophilic interaction chromatography (HILIC) is increasingly becoming a method of choice for the analysis of polar compounds.[1-6] Expanding upon earlier published methods,[6-7] this application note describes the extraction and analysis of monoamine neurotransmitters and metanephrines from plasma. HILIC-based chromatographic separation is achieved using a Waters ACQUITY UPLC BEH Amide Column. Waters Oasis WCX 96-well µElution Plates are used to extract these compounds from plasma. The use of mixed-mode weak cation exchange solid-phase extraction (SPE) plates, in combination with the amide column for HILIC chromatography and the Waters Xevo TQ-S mass spectrometer, result in a rapid, robust method with

excellent linearity, accuracy and precision, as well as minimal matrix effects.

Experimental

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 100 mm
Column temp.:	30 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	95:5 Water:ACN containing 30 mM NH_4HCOO, pH 3.0
Mobile phase B (MPB):	15:85 Water:CAN containing 30 mM NH_4HCOO, pH 3.0
Needle washes:	Strong and weak needle washes were both placed in MPB

The gradient ramp is shown in Table 1 and includes an initial hold, followed by a shallow ramp and an increase in flow rate to re-equilibrate the column. The entire cycle time is 4.0 min.

Gradient

Time (min)	Flow (mL/min)	%A	%B
0	0.6	0.0	100.0
1.0	0.6	0.0	100.0
2.0	0.6	10.0	90.0
2.1	1.0	10.0	90.0
2.5	1.0	30.0	70.0
2.6	1.0	0.0	100.0
3.9	1.0	0.0	100.0
4.0	0.6	0.0	100.0

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the methods section.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI Positive
Capillary voltage:	0.5 kV
Cone voltage:	Compound specific (see Table 2)
Desolvation gas:	900 L/hr
Cone gas:	150 L/hr
Desolvation temp.:	550 °C
Source temp.:	150 °C

Data were acquired and analyzed using UNIFI Software

Combined stock standards containing 10-µg/mL of dopamine (DA), 3-methoxytyramine, (3-MT) metanephrine (MTN), and normetanephrine (NMT) and 50-µg/mL of norepenephrine (NE) and epinephrine (EP) were prepared in methanol containing 0.1% ascorbic acid to prevent oxidation. A combined internal standard stock solution composed of 10-µg/mL D3-metanephrine, D3-normetanephrine, D4-dopamine, D6-epinephrine, and D6-norepinephrine, was also prepared in methanol containing 0.1% ascorbic acid. Working internal standard solutions were prepared daily in 5% MeOH with 0.1% formic acid at a concentration of 2.5 ng/mL.

Human plasma (sodium heparin) was obtained from Biological Specialty Corporation (Colmar, PA). Pooled plasma (6 lots) was used to prepare calibration and quality control samples.

Sample preparation

Pooled plasma samples (250 µL) were pre-treated with 250-µL of 50-mM NH_4CH_3COO and 50-µL of an internal standard working solution (2.5 ng/mL). Pre-treated samples were loaded in individual wells of an Oasis WCX 96-well µElution Plate that had been conditioned with 200-µL of MeOH and 200-µL of H_2O. After loading the samples, wells were washed with 200-µL of 20-mM NH_4CH_3COO followed by 200-µL of 50:50 ACN:IPA. The 96-well plate was then dried under vacuum for 30 s to remove as much solvent as possible from the sorbent bed. The target compounds were eluted from the plate with 2 x 25-µL aliquots of 85:15 ACN:H_2O containing 2% formic acid into an 700-µL 96-well sample collection plate (p/n 186005837). 15-µL of the eluate was injected onto the UPLC-MS/MS System.

Results and Discussion

The structures of all compounds are shown in Figure 1 along with their individual logP values, demonstrating the highly polar nature of many of these compounds. Table 2 shows the retention times and individualized MS parameters of each compound, including MRM transitions, cone voltage, and collision energy.

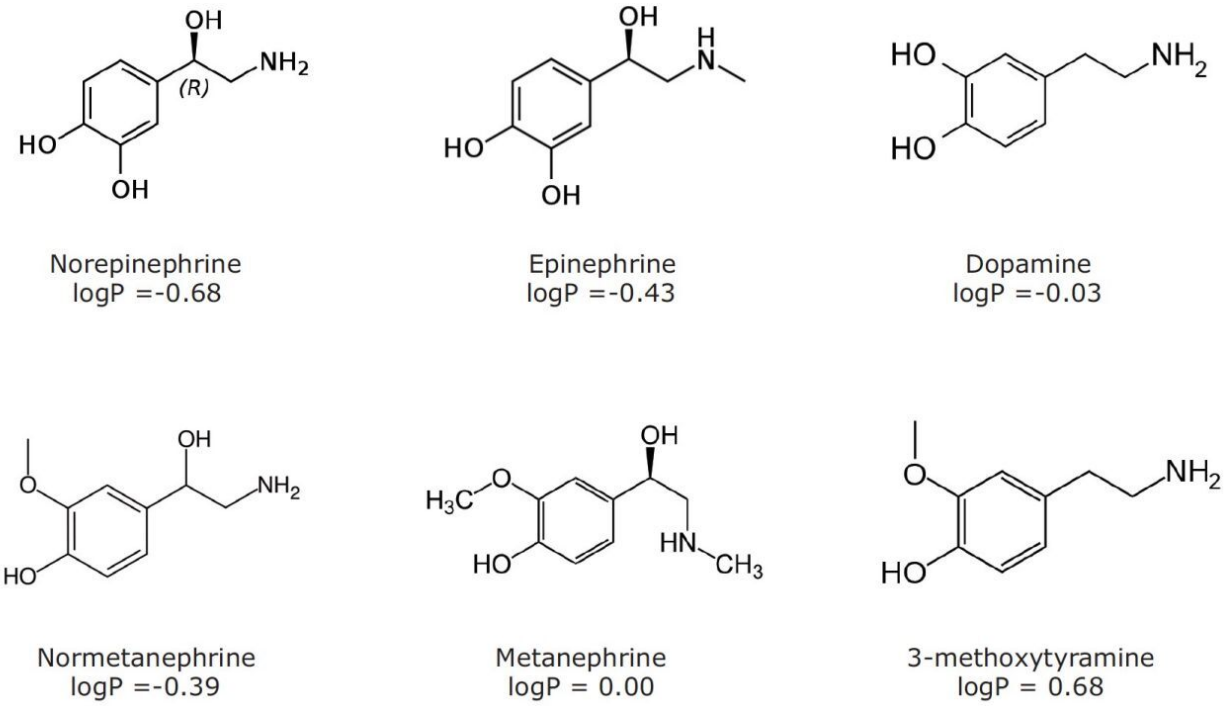

Figure 1. Names, molecular structures, and logP values of catecholamines and metanephrines.

Analyte	RT (min)	MRM transitions *m/z*	Cone voltage (V)	Collision energy (eV)
3-Methoxytyramine	0.84	151.1>91.2	17	22
		151.1>119.2	17	18
Metanephrine	0.91	180>165.1	35	16
		180>148.1	35	20
Normetanephrine	1.17	166.1>134.1	50	16
		166.1>149.1	50	10
Dopamine	1.25	137.1>91.1	50	18
		154.1>137.2	29	10
Epinephrine	1.40	184.1>166.1	15	8
		166.1>107	15	18
Norepinephrine	1.98	152>135.2	46	14
		152>79.2	20	20

Table 2. Mass spectral parameters used for analysis of catecholamines and metanephrines.

Figure 2A shows the chromatography of all compounds from a 20 pg/mL calibration standard using the

ACQUITY UPLC BEH Amide Column. Previous work6 had shown that 30 mM NH_4HCOO and 15% water in MPB resulted in an ideal balance of ionic strength and solubility, enabling the resolution and peak shape seen in Figure 2A. One important feature of this separation is the resolution between NMT and EP. These two compounds have the same molecular formula and can interfere with each other if not adequately separated. Figure 2A demonstrates the baseline separation of these compounds in HILIC mode, enabling their unambiguous identification and quantification. Figure 2B shows the HILIC chromatography of an unspiked plasma sample, demonstrating the ability to determine endogenous concentrations of 3-MT, MTN, NMT, DA, EP, and NE (7.0, 31.7, 70.6, 0.0, 29.4, and 360.9 pg/mL, respectively).

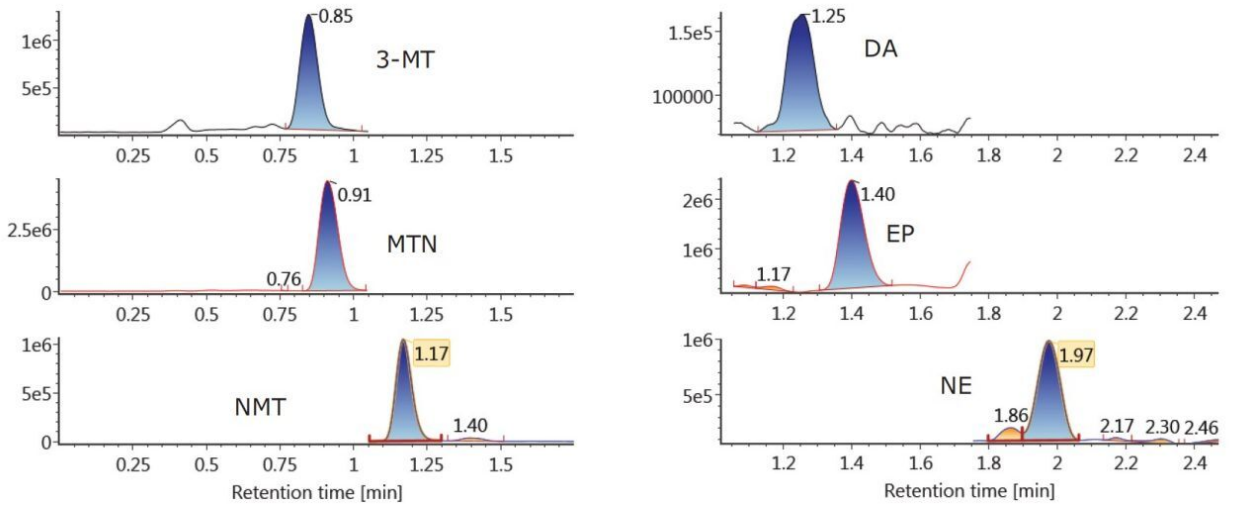

Figure 2A. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative calibration standard spiked at 20 pg/mL (100 pg/mL for EP and NE).

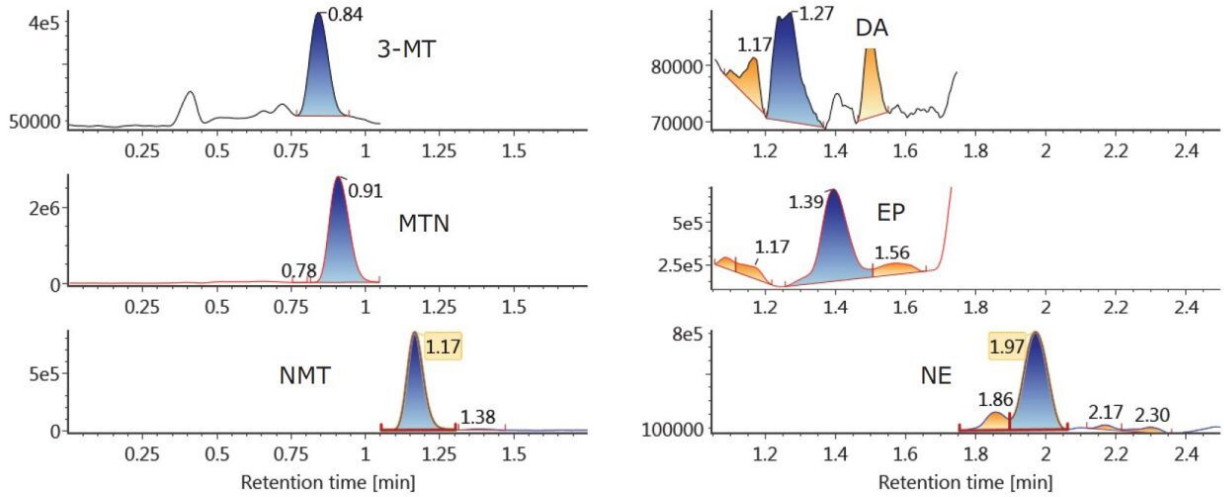

Figure 2B. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative method blank. Endogenous concentrations of all compounds are listed in Table 3.

Recovery and matrix effects

Extraction recoveries and matrix effects are shown in Figure 3. Recoveries ranged from 54% for NE to 90% for DA, with an average recovery of 76.4%. Matrix effects averaged -6.9%. The largest matrix effects were -23% and -22% for NE and DA, respectively, but were negligible for all other compounds. These results highlight another advantage of HIILIC chromatography, the ability to minimize matrix effects when analyzing polar compounds.

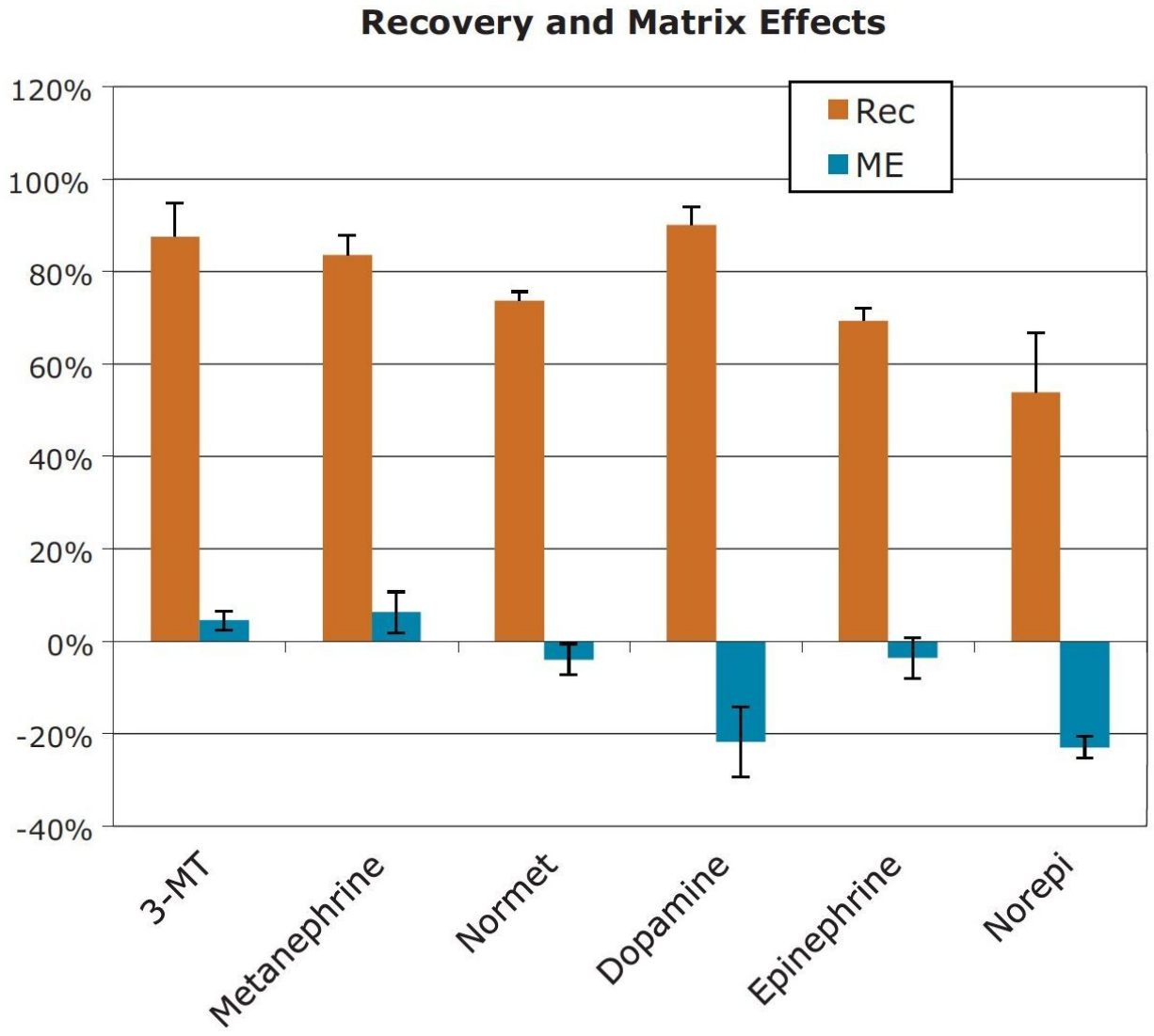

Figure 3. Recovery and matrix effects for catecholamines and metanephrines extracted from urine using Oasis WCX 96-well µElution Plates (N=4). Error bars indicate standard deviations. All compounds were spiked at 100 pg/mL into pooled human plasma.

Quantitative results

Calibration curves and quality control samples were prepared via the standard addition method by spiking pooled plasma samples with known concentrations of analytes. Two ranges of calibration curves were used, reflecting different expected concentrations of various compounds in plasma. Calibration levels for 3-MT, metanephrine, normetanephrine, and dopamine ranged from 10–2,000 pg/mL. Calibration levels for epinephrine and norepinephrine ranged from 50–10,000 pg/mL. After data processing, the endogenous concentrations were extrapolated from the resulting calibration curves. These data were used to correct the

actual calibration concentrations. For example, the plasma sample used for calibration was determined to contain 31.7 pg/mL of metanephrine, so the calibration concentrations were adjusted to 41.7–2031.7 pg/mL. The resulting calibration curves showed excellent linearity, with R_2 values of 0.999 or greater for all compounds. Figure 4 shows representative calibration curves for DA and MTN, both of which have R_2 values greater than 0.999. Table 3 summarizes the calibration data for all compounds. Mean % deviations from expected calibration values were less than 1% for all analytes. In addition, the maximum % deviations from calibration values are listed and show that with the exception of epinephrine, the maximum % deviation for all calibrators was less than 10%. The calculated endogenous concentration of compounds in the pooled plasma used for calibration is also listed, along with the corrected calibration ranges for each compound.

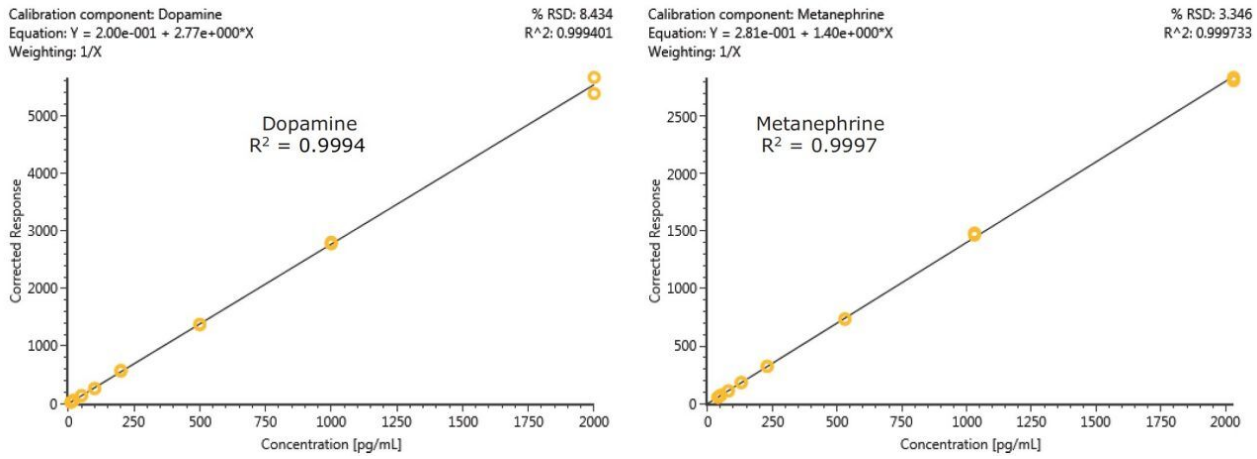

Figure 4. Representative calibration curves for dopamine (DA) and metanephrine (MTN) extracted from plasma samples. The data were fitted with a 1/x weighted linear fit. Basal concentrations for DA and MTN were 0 and 30 pg/mL, respectively.

	R^2	*Mean % Dev.*	Max % Dev.	Endogenous (pg/mL)	Corrected Calibration Range
3-MT	0.9993	0.25%	2.89%	7	17-2007
Metanephrine	0.9997	0.00%	2.50%	32	42-2042
Normetanephrine	0.9998	0.00%	1.72%	71	81-2081
Dopamine	0.9994	-0.33%	4.57%	0	10-2000
Epinephrine	0.9990	0.84%	11.83%	29	79-10079
Norepinephrine	0.9995	0.00%	2.59%	361	411-10411

Table 3. Summary of calibration data for plasma metanephrines and catecholamines. Mean % deviation indicates the average % deviation of all calibration points from their theoretical concentrations. The max % deviation indicates the maximum deviation over the entire calibration range. The calculated endogenous concentrations are listed and used to correct the calibration range.

Quality control samples (N=6) were overspiked at 200, 500, 2000, and 4000 pg/mL for EP and NE and at 40, 100, 400, and 800 pg/mL for the remaining compounds. QC results were accurate and precise (see Table 4). All QC values were within 10% of their target values, and most were within 5%. In addition, all coefficients of variation (%CV) were less than 10%. This demonstrates that the method is linear, accurate, and precise over a calibration range that includes the entire scope of expected values for normal and pathologically elevated samples.

	QC spike concentration											
	40 pg/mL			100 pg/mL			400 pg/mL			800 pg/mL		
	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV
3-MT	99.9%	7.4%	7.4%	99.2%	3.0%	3.1%	105.9%	1.8%	1.7%	93.9%	2.6%	2.8%
Metanephrine	99.9%	2.0%	2.0%	97.6%	0.8%	0.8%	107.3%	1.2%	1.1%	94.6%	1.7%	1.7%
Normetanephrine	99.8%	1.6%	1.6%	96.8%	1.7%	1.8%	104.6%	0.4%	0.4%	93.4%	1.0%	1.1%
Dopamine	97.0%	7.2%	7.4%	91.2%	3.4%	3.7%	103.7%	3.1%	3.0%	95.6%	2.7%	2.8%
	200 pg/mL			500 pg/mL			2000 pg/mL			4000 pg/mL		
	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV
Epinephrine	97.3%	4.3%	4.4%	98.8%	2.2%	2.2%	100.8%	1.4%	1.4%	97.0%	2.6%	2.6%
Norepinephrine	105.1%	7.7%	7.4%	102.6%	8.2%	8.0%	96.7%	1.3%	1.3%	97.1%	4.2%	4.3%

Table 4. Quality control results for plasma catecholamines and metanephrines. Concentrations refer to the spiked concentration. Accuracies were calculated by comparing the result of the sum of the spiked concentration and endogenous calculated values in the plasma sample to the theoretical sum of these concentrations.

Conclusion

The extraction and analysis of plasma catecholamines and metanephrines using Oasis WCX µElution Plates and an ACQUITY UPLC BEH Amide Column in HILIC mode is detailed. Extraction using the Oasis WCX µ Elution Plate resulted in low matrix effects and consistent recoveries for all compounds that translated into excellent analytical accuracy and precision. In addition, the ability to elute the samples in an extremely low volume (50 µL) enabled 5x sample enrichment without the extra time or risk associated with evaporation and reconstitution. The ACQUITY UPLC BEH Amide Column used for HILIC separation resulted in rapid and efficient separation of all compounds, with baseline resolution between normetanephrine (NMT) and epinephrine (EP). It also enabled the analysis of the monoamines, dopamine, norepinephrine and epinephrine. Quantitative results were excellent, with highly linear responses across the entire calibration range and excellent accuracy and analytical precision.

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