Extraction of Catecholamines and Metanephrines from Human Plasma Biotage® Extrahera™ LV-200 and Low Volume SPE Prior to UHPLC-MS/MS Analysis

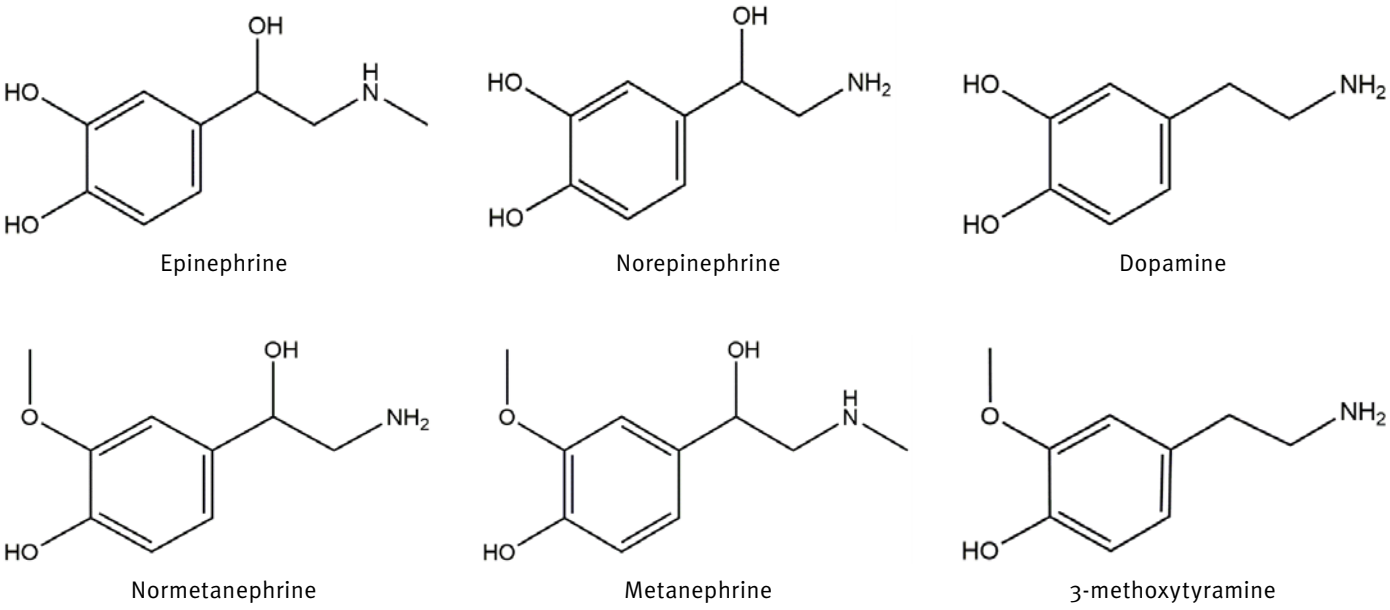


Figure 1. Structures of epinephrine, norepinephrine, dopamine, normetanephrine, metanephrine, and 3-methoxytyramine.

Introduction

This application note describes the extraction of six catecholamines and metanephrines from human plasma using EVOLUTE® EXPRESS WCX 10 mg fixed well plates prior to UHPLC-MS/MS analysis.

Our sample preparation procedure, using weak cation exchange mixed-mode solid phase extraction, delivers high analyte and consistent matrix factors whilst providing low limits of quantitation for all analytes.

The selection of plasma anticoagulant can have a significant effect on analyte stability and matrix interferences. The robust procedure in this application note has similar extraction characteristics for human plasma treated with commonly used anticoagulants.

This application note includes optimized conditions for automated processing of the 10 mg plates (using Biotage® Extrahera LV-200, see appendix for settings) and manual processing (using the Biotage® PRESSURE+ 96 positive pressure manifold). Data generated using both processing systems is shown. Prior to analysis extracts are evaporated using the TurboVap® 96 Dual.

Analytes

Norepinephrine (NE), epinephrine (EP), dopamine (DA), normetanephrine (NE), metanephrine (ME), and 3-Methoxytyramine (3MT).

Internal Standards

Norepinephrine-D_6 (NE-D6), epinephrine-D_6 (EP-D6), dopamine-D_4 (DA-D4), normetanephrine-D_3 (NE-D3), and metanephrine-D_3 (ME-D3). ME-D3 was used as an internal standard for 3-MT.

Sample Preparation Procedure

Format
EVOLUTE® EXPRESS WCX 10 mg Plate, p/n 602-0010-PX01.

Sample Pre-Treatment
Centrifuge plasma samples for 10 min at 6,000 x g before processing further. Add 10 µL of working internal standard and 10 µL working standard diluent or working standard solution to 200 µL plasma. Vortex mix using a medium setting for 5 to 10 s. Pretreat spiked plasma with 200 µL 10 mM sodium citrate pH 7 and vortex mix using a medium setting for 5 to 10 s.

Automated And Manual Processing Conditions
Detailed automated processing conditions using the Biotage® Extrahera™ LV-200 system are included in the appendix.

Plates were processed using a Biotage® PRESSURE+ 96 positive pressure manifold. Each step described below was processed at 1 to 3 psi using the adjustable flow setting. Drying steps were processed at 40 psi using the maximum flow setting.

Condition
Add methanol (500 µL) to each well.

Equilibration
Add 10 mM ammonium acetate pH 6 (500 µL) to each well.

Sample Loading
Add pre-treated plasma (400 µL) to each well.

Wash 1
Add 10 mM ammonium acetate pH 6 (500 µL) to each well to elute aqueous interferences.

Wash 2
Add MeOH:H_2O (80:20 , v/v, 500 µL) to each well to elute neutral organic interferences. On completion, dry the bed for 1 minute.

Wash 3
Add dichloromethane (500 µL) to each well to elute lipophilic interferences. On completion, dry the bed for 5 minutes.

Elution
Elute analytes with 200 µL of water: propan-2-ol (85:15, v/v) containing formic acid (0.1 % v/v) into a 1 mL collection plate (p/n 121-5202). On completion, purge the bed at 12 psi (adjustable flow setting) for 5 seconds.

Post Elution and Reconstitution
Dry the extract in a stream of air or nitrogen using a TurboVap® 96 Dual at 40 °C using a flow rate of 50 L min^{-1} and a plate height of 46 mm (drying time under these conditions is approximately 30 mins).

Reconstitute evaporated samples with H_2O:MeOH (95:5, v/v, 100 µL) containing formic acid (0.1% v/v) and mix thoroughly. Cover with a sealing mat, vortex mix andtransfer to LC vials containing 200 to 300 µL glass inserts, close with an appropriate cap (e.g. LC vials: Supelco p/n 854974; Snap Caps: Supelco p/n SU860093; Inserts: Agilent p/n 5183-2085).

UHPLC Parameters

Instrument
Shimadzu Nexera UHPLC

Column
Avantor® ACE Excel 1.7 C18-PFP (100 mm x 3.0 mm) p/n EXL-1710-1003U, with a RESTEK Raptor ARC-18 2.7 µM (5 mm x 2.1 mm) p/n 9314A0252 guard cartridge.

Mobile Phase
A: 2 mM ammonium formate containing formic acid (0.05% v/v) in water.

B: 0.5 mM ammonium fluoride in methanol.

Flow Rate
0.5 mL min^{-1}

Column Temperature
30 °C

Autosampler Temperature
10 °C

Injection Volume
10 µL

Table 1. UHPLC Gradient.

Time, min	% A	% B	Divert Valve
0.0	98	2	
0.5	98	2	to MS
3.0	70	30	
3.5	5	95	to waste
5.5	5	95	
6.0	98	2	
9.0	98	2	

MS Parameters

Instrument
AB SCIEX Triple Quad 5500 using a Turbo-V source and TurboIonSpray probe in positive ESI mode

IS Voltage (IS)
2500 V

Source Temperature (TEM)
500 °C

Curtain Gas (CUR)
35 psi

Nebulizer Gas (GS1)
50 psi

Heater Gas (GS2)
60 psi

CAD Gas
7

Data Acquisition

Scheduled MRM data acquisition, target scan time 0.40 s, detection window 60 s

Table 2. MRM Parameters.

Analyte	Transition, DA	DP, V	EP, V	CE, V	CXP, V
NE 1	152.1 > 106.9	35.0	5.0	23.5	16.5
NE 2	170.1 > 151.9	42.0	8.0	7.6	19.9
NE-D6	158.1 > 110.9	59.0	4.2	24.6	16.2
EP 1	166.1 > 106.8	102.0	12.0	25.5	13.4
EP 2	184.1 > 165.9	90.0	12.0	15.1	13.4
EP-D6	190.0 > 172.1	90.0	12.0	15.0	17.0
DA 1	154.1 > 90.8	60.0	10.0	31.4	11.3
DA 2	154.1 > 137.1	60.0	10.0	14.5	20.8
DA-D4	141.3 > 94.9	155.0	11.0	26.7	14.8
NM 1	166.1 > 133.9	55.5	3.2	22.0	16.0
NM 2	166.1 > 106.0	55.5	3.2	22.0	16.0
NM-D3	169.1 > 137.1	57.0	12.3	21.8	16.2
ME 1	198.1 > 180.1	56.2	11.0	23.0	17.0
ME 2	180.1 > 119.1	81.6	10.0	24.4	16.0
ME-D3	183.1 > 121.1	81.6	12.0	25.0	14.0
3MT 1	168.2 > 90.9	36.0	11.5	31.5	11.9
3 MT 2	151.2 > 119.0	88.0	11.5	19.3	13.8

Results

Recovery

Extraction recovery was determined using a 120 pg spike in 200 µL sodium citrate plasma. Data are the average of n=7 pre-extraction spikes compared to n=4 post extraction spikes (Figure 2). Recovery was determined for both the analyte and its associated internal standard. Data are tabulated for PRESSURE+ 96 and Extrahera™ LV-200 methods (table 3).

Table 3. Extraction Recovery and Precision for Pooled Gender Human Plasma with Sodium Citrate Anticoagulant.

Method	Analyte	Recovery, %	Precision, % RSD
PRESSURE+ 96	NE	78	7.5
	EP	65	9.8
	DA	75	10.8
	NM	82	6.2
	ME	69	6.8
	3MT	71	6.8
Extrahera™ LV-200	NE	78	10.9
	EP	68	7.8
	DA	78	5.5
	NM	83	7.0
	ME	78	7.3
	3MT	78	7.1

Linearity And Limit Of Quantitation (LOQ)

Extracted analyte linearity was determined using stripped plasma prepared with an in-house procedure, serially diluted in matrix from 500 to 10 pg mL^{-1} with internal standards at 200 pg mL^{-1}. Calibration range was determined where the calibration coefficient r › 0.9975 (r^2 › 0.995). The acceptance criteria used were: accuracy from 90 to 110% (lowest calibrant 80-120%); and precision ‹ 10% RSD (lowest calibrant ‹ 15%). LOQ determined where signal/noise was › 10:1, estimated.

Example calibration curves are demonstrated in Figure 3. Extracted ion chromatograms from potassium EDTA plasma spiked at 80 pg mL^{-1} overlaid with the stripped blank are demonstrated in Figure 4. Method performance data are tabulated for two matrices using each method (Tables 4 and 5).

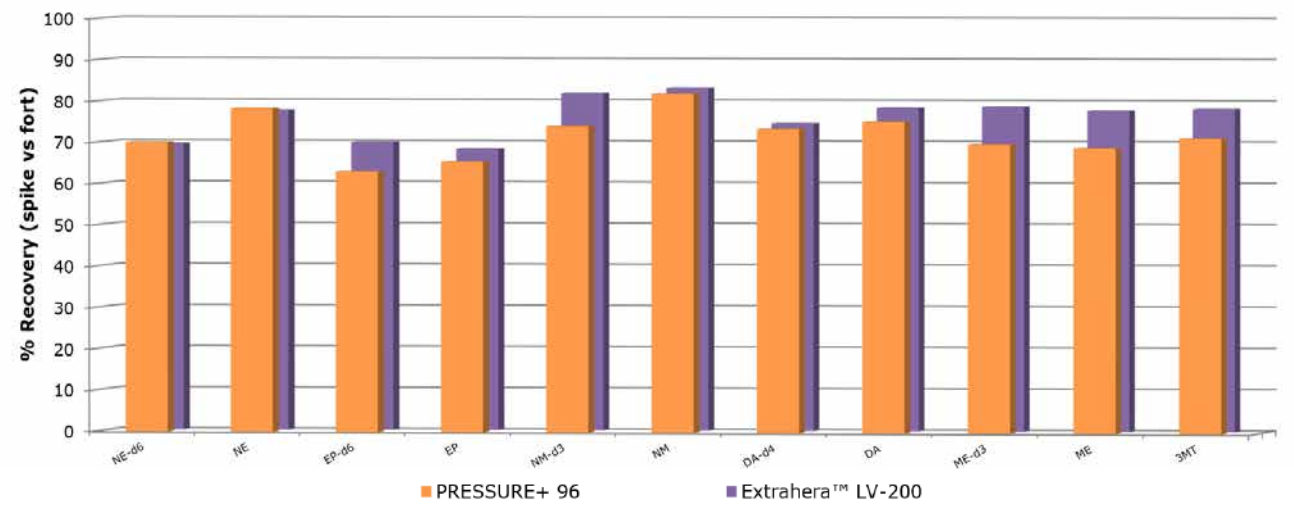


Figure 2. Recovery of catecholamines and metanephrines using the conditions described in this application note. Data is shown for each method (PRESSURE+96 and Extrahera™ LV-200).

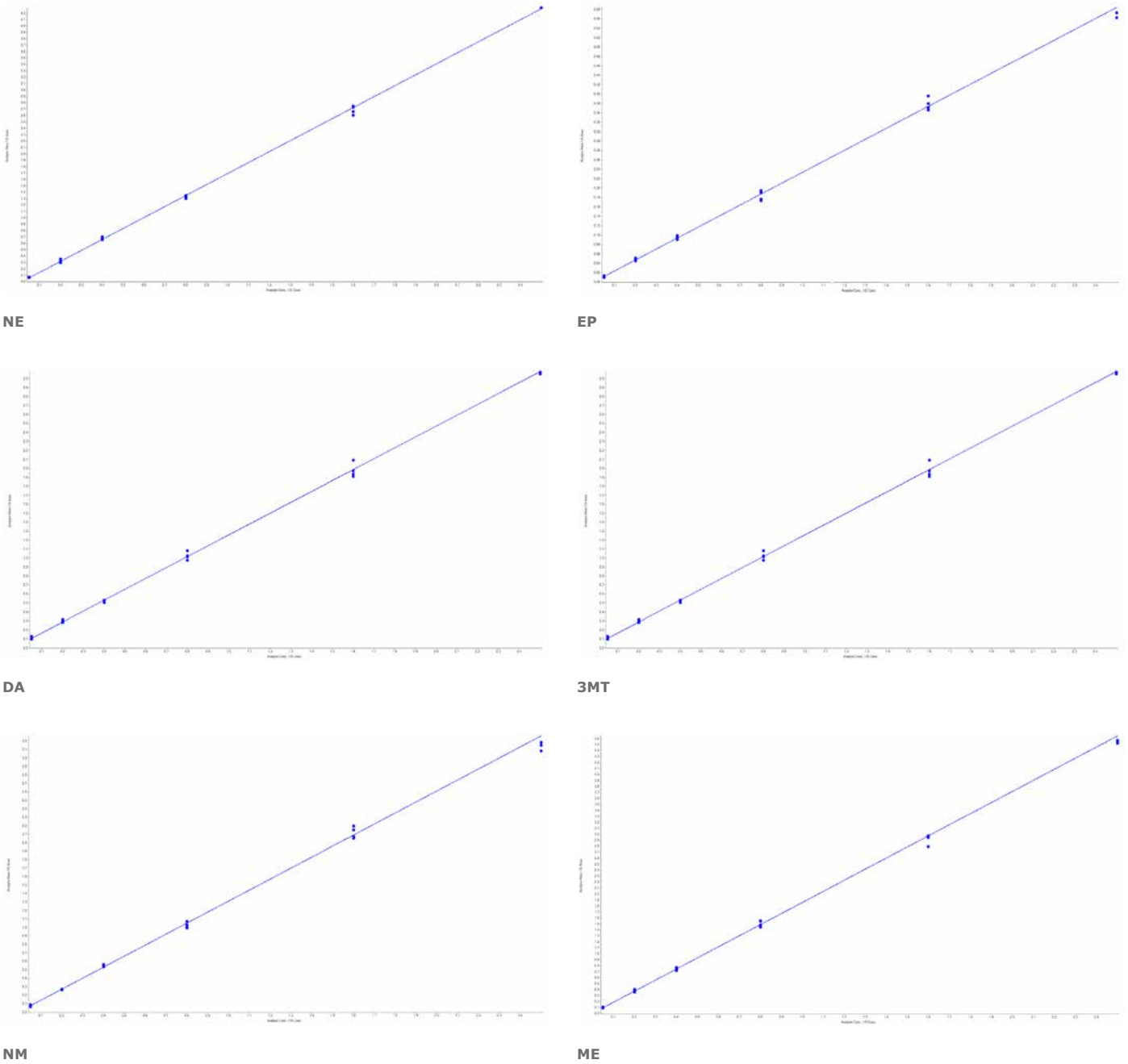
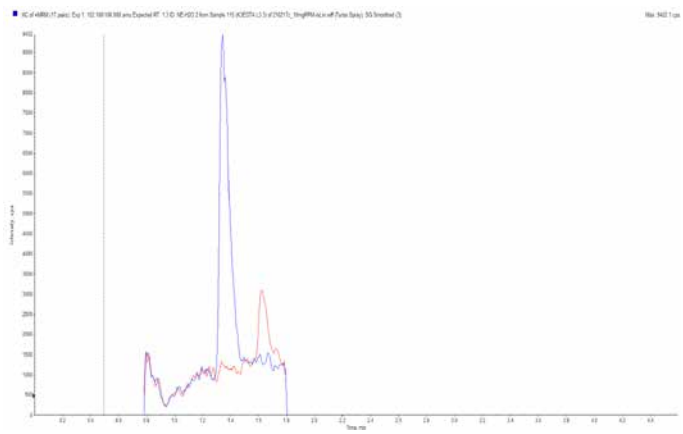
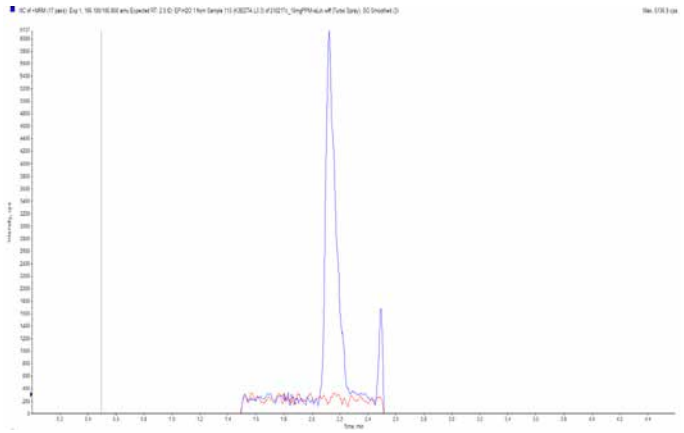


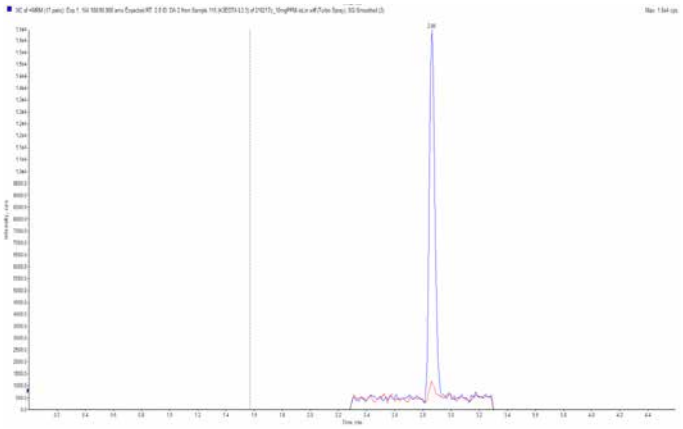
Figure 3. Extracted Matrix Calibration Curves (plasma with potassium EDTA anticoagulant) 10 to 500 pg mL^{-1} (IS 200 pg mL^{-1}).



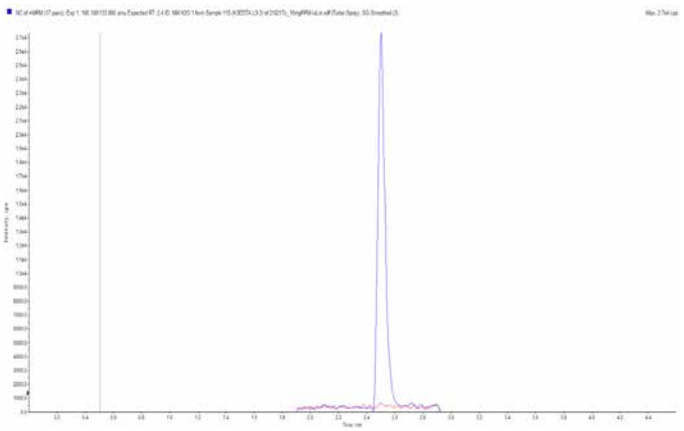
NE



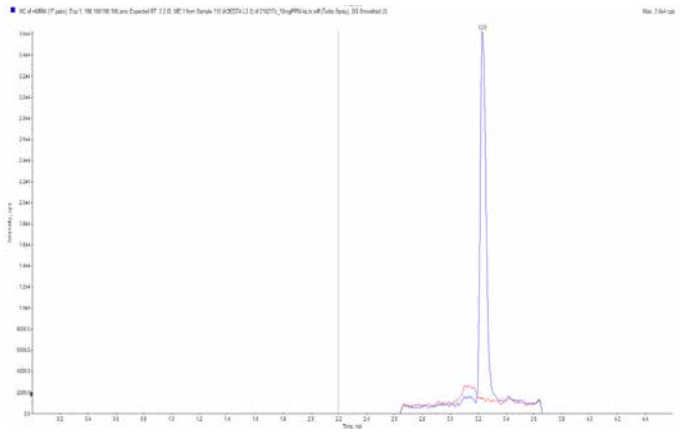
EP



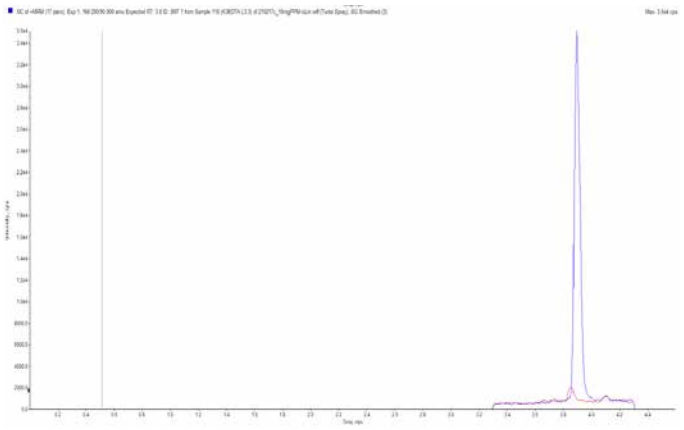
DA



NM



ME



3MT

Figure 4. Extracted Ion Chromatograms, (80 pg mL^{-1} spiked potassium EDTA plasma, overlaid with stripped blank).

Table 4. PRESSURE+ 96 Calibration Performance Data, 10 to 500 pg mL^{-1} (IS 200 pg mL^{-1}).

Matrix	Analyte	Coefficient, *r*	Accuracy, %	Precision, % RSD	Range, pg mL^{-1}	Estimated LOQ, pg mL^{-1}	Estimated LOQ, nmol L^{-1}
Na heparin	NE	0.9994	90-110	< 15	40-500	16	0.09
	EP	0.9994	80-110	< 15	10-500	6	0.03
	DA	0.9995	90-110	< 10	10-500	6	0.04
	NM	0.9994	90-110	< 10	10-500	3	0.02
	ME	0.9996	90-110	< 10	10-500	5	0.02
	3MT	0.9996	80-110	< 10	10-500	2	0.01
K3EDTA	NE	0.9997	90-110	< 10	40-500	20	0.12
	EP	0.9989	90-110	< 15	10-500	6	0.03
	DA	0.9991	90-110	< 10	10-500	7	0.04
	NM	0.9985	90-110	< 15	10-500	4	0.02
	ME	0.9985	90-110	< 10	10-500	5	0.02
	3MT	0.9989	90-110	< 10	10-500	3	0.01

Table 5. Extrahera™ LV-200 Calibration Performance Data, 10 to 500 pg mL^{-1} (IS 200 pg mL^{-1}).

Matrix	Analyte	Coefficient, *r*	Accuracy, %	Precision, % RSD	Range, pg mL^{-1}	Estimated LOQ, pg mL^{-1}	Estimated LOQ, nmol L^{-1}
Na heparin	NE	0.9993	90-110	< 10	40-500	20	0.12
	EP	0.9994	90-110	< 10	10-500	8	0.05
	DA	0.9995	90-110	< 10	10-500	7	0.04
	NM	0.9992	90-110	< 10	10-500	5	0.03
	ME	0.9993	90-110	< 10	10-500	5	0.02
	3MT	0.9997	90-120	< 10	10-500	1	0.01
K_3EDTA	NE	0.9997	90-110	< 10	40-500	20	0.12
	EP	0.9997	80-110	< 15	10-500	8	0.05
	DA	0.9994	90-110	< 10	10-500	13	0.09
	NM	0.9994	90-110	< 10	10-500	2	0.01
	ME	0.9994	90-110	< 10	10-500	3	0.02
	3MT	0.9992	90-110	< 10	10-500	2	0.01

Matrix Interferences

The sample preparation procedure described in this application note results in final extracts that are low in matrix interferences, matrix factors are comparable for both methods. Matrix factor, determined as the ratio of post extraction spike / dilute standard at the same concentration, is shown in Figure 5.

Signal factor, determined as the ratio of pre-extraction spike / dilute standard, demonstrates matrix has a minimal effect on analyte response used for recovery (with the exception of norepinephrine). Signal factors are comparable for both methods (see Figure 6).

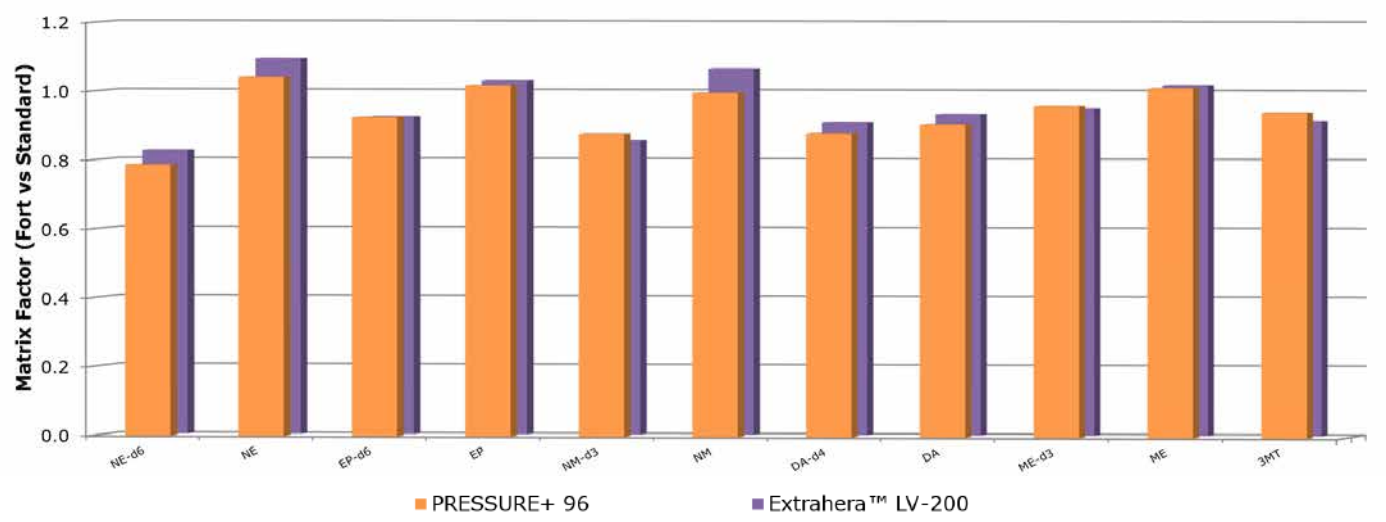


Figure 5. Matrix factors for sodium citrate plasma (PRESSURE+ 96 and Extrahera™ LV-200 methods).

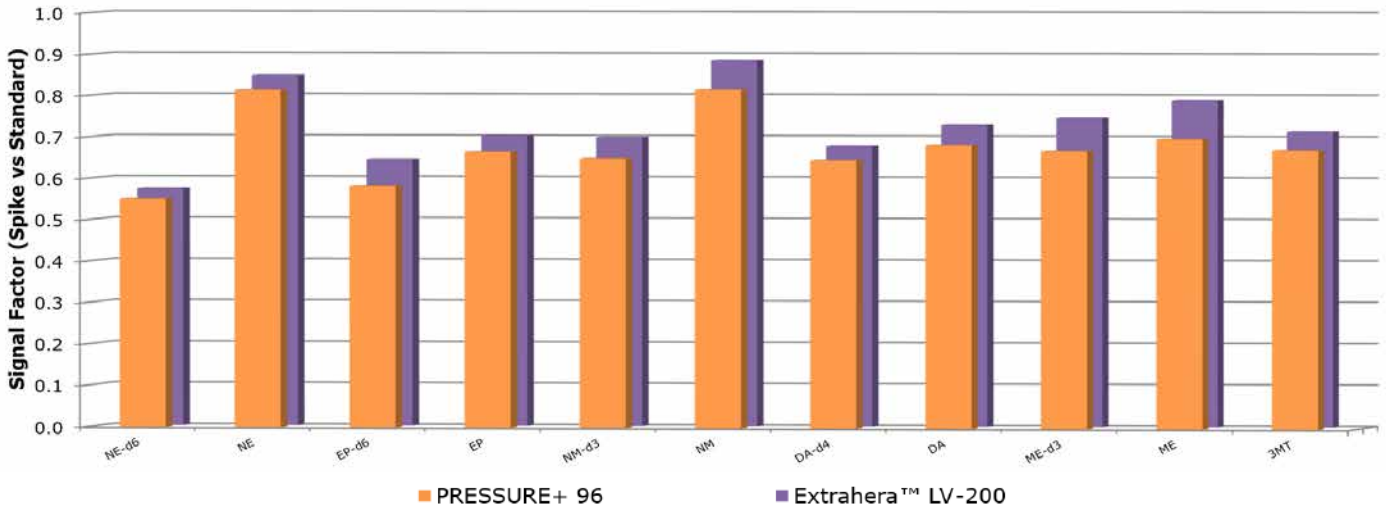


Figure 6. Signal factors for sodium citrate plasma (PRESSURE+96 and Extrahera™ LV-200 methods).

Discussion and Conclusion

The sample preparation method performance data on the previous page was generated using an elution solvent with high water content and an evaporation-reconstitution workflow. We recommend this approach to minimize matrix effects. The concentration step ensures this method meets clinically relevant reference intervals without needing a cumbersome derivatization step. Evaporation time is typically 30 minutes for a 200 µL elution volume.

Using a more typical high organic content elution or direct injection of the aqueous elution will reduce turnaround but may have a detrimental effect on performance, respectively increasing the breakthrough of phospholipids in the final elution or raising achievable limits of quantitation. We used 2% formic acid in 80% MeOH (aq) as an alternative elution solvent, evaporation time was typically 20 minutes. It is possible to directly inject the elution solvent from the method in this application note. However, the injection volume must be reduced to 5 µL so as not to cause peak broadening of early eluting analytes (e.g. norepinephrine).

Inclusion of comprehensive wash and associated drying steps enhances assay robustness, reducing system maintenance at the cost of a small increase in turnaround time. During development of this application, column lifespans were typically over 3000 injections (over 500 hours use).

This method provides high, reproducible recoveries of catecholamines and metanephrines in human plasma treated with typical clinical anticoagulants.

The method described in this application note was automated using a Biotage® Extrahera™ LV-200 system. Total processing time using the Extrahera was approximately 60 minutes for 96 samples (excluding evaporation and transfer steps). Results generated using the automated method demonstrate comparable analyte recovery and RSD. Matrix and signal factors are comparable between manual and automated methods. Extrahera™ LV-200 linearity and LOQ are comparable to the manual processing method. The appendix contains software settings required to configure an Extrahera™ LV-200 to run the above method.

Additional Information

Chemicals And Reagents

- Reagents were purchased from Sigma-Aldrich Company Ltd. (Gillingham UK). LC-MS grade methanol and propan-2-ol (isopropanol) were purchased from Rathburn Chemicals Ltd (Walkerburn UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Milli-Q Direct-Q 5 water purifier (Merck Life Sciences, Gillingham UK).
- Stock standard diluent: ascorbic acid, 0.04% w/v in methanol. Dilute ascorbic acid (39.5 to 40.5 mg) in methanol (100 ± 0.5 mL). Prepare daily immediately prior to use.
- Working standard diluent: ascorbic acid, 0.04% w/v in water. Dilute ascorbic acid (39.5 to 40.5 mg) in deionized water (100 ± 0.5 mL). Prepare daily immediately prior to use.
- Pre-treatment solution: 10 mM sodium citrate pH 7, aq. Dilute trisodium citrate (621 to 625 mg) in deionized water (250 ± 1 mL) and titrate to pH 7.0 using formic acid 10%, aq (approximately 80 µL). Prepare fresh daily.
- Equilibration and aqueous wash solution: 10 mM ammonium acetate pH 6, aq. Dilute ammonium acetate (383 to 387 mg) in deionized water (500 ± 2 mL) and titrate to pH 6.0 using formic acid 10%, aq (approximately 80 µL). Prepare weekly.
- Organic wash solution: methanol/water 80:20 v/v. Dilute 20 mL deionized water with 80 mL methanol.
- Elution solvent: water/propan-2-ol (85:15, v/v) containing formic acid, 0.1% v/v. Dilute 15 mL propan-2-ol with 85 mL deionized water. Further dilute 0.1 mL formic acid in 99.9 mL of water/propan-2-ol (85:15 v/v).
- Reconstitution solvent: water/methanol (95:5, v/v) containing formic acid (0.1%, v/v). Dilute 5 mL methanol with 95 mL deionized water. Further dilute 0.1 mL formic acid in 99.9 mL of water/methanol (95:5, v/v).
- Mobile phase A: 2 mM ammonium formate containing formic acid, 0.05% v/v in water. Dilute formic acid (500 ± 1 µL) in deionized water (1000 ± 5 mL). Further dilute ammonium formate (124 to 128 mg) in water containing formic acid, 0.05% v/v (1000 ± 5 mL). Prepare every two days or more frequently if needed.
- Mobile phase B: 0.5 mM ammonium fluoride in methanol. Dilute ammonium fluoride (9.2 to 9.7 mg) in methanol (500 ± 2 mL), sonicate for 10 to 15 minutes to aid dissolution. Prepared fresh every two days or more frequently if needed.

Standards

Cerilliant® standards were purchased from Sigma-Aldrich Company Ltd. (Gillingham UK) at 1.0 mg mL^{-1} in methanol. Deuterated Cerilliant® internal standards were purchased from the same at 100 µg mL^{-1} in methanol.

Extraction Matrices

Gender pooled human plasma was purchased from The Welsh Blood Service (Pontyclun, UK), BioIVT (Burgess Hill, UK), and Golden West Biologicals, Inc. (Temecula, CA). Method linearity and LOQ were determined with plasma prepared using an in-house stripping process. Spike-recovery experiments were performed on unstripped plasma. We recommend plasma is centrifuged for 10 min at 6,000 x g before processing further.

Ordering Information

Part Number	Description	Quantity
602-0010-PX01	EVOLUTE® EXPRESS WCX 10 mg Plate	1
121-5202	Collection plate, 1 mL Square	50
121-5204	Piercable Sealing Cap	50
Automated processing		
417000	Biotage® Extrahera™ LV-200	1
416920SP	Pipette Rack, LV/MV	1
417423SP	Pipette Rack, Short	1
417008	50 µL Clear Tips	960
417009	200 µL Clear Tips	960
Manual Processing		
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold (96 position)	1
Evaporation		
418000	TurboVap® 96 Dual	1

Appendix

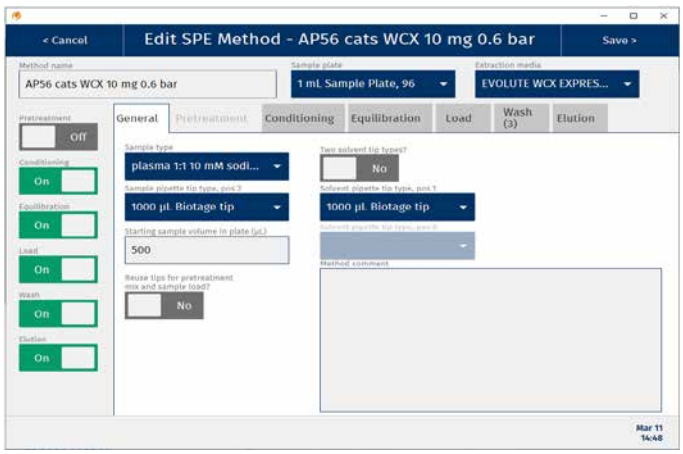
Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™ LV-200 using EVOLUTE® EXPRESS WCX 10 mg plates.

This appendix contains the software settings required to configure Extrahera to run this method. As described in the main body of the application note, analyte recoveries, linearities and LOQs were comparable for both manually processed and automated methods.

Total time for extraction of 96 samples using this method was approximately 60 minutes (excluding post extraction evaporation and transfer steps).

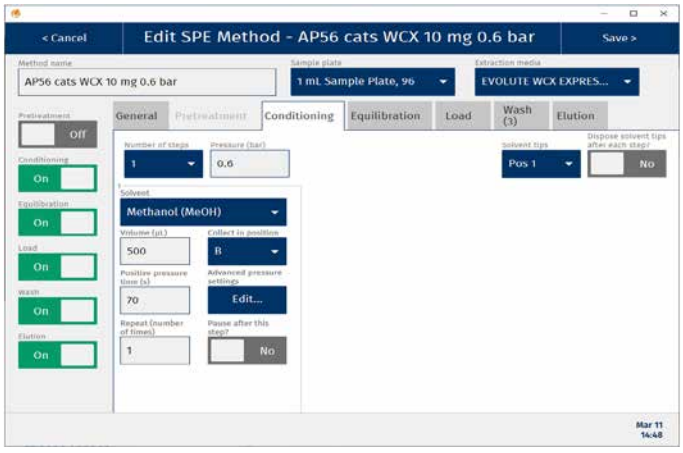
General tab



Settings

Sample type	plasma pretreated 1:1 10 mM sodium citrate pH 7
Sample volume	500 µL

Conditioning tab



Number of steps	1
Pressure (bar)	0.6
Dispose tips	No
Volume (µL)	500
Collect in position	D (W1)
Positive pressure time (s)	70
Advanced pressure settings	
None	

Equilibration tab

Number of steps	1
Pressure (bar)	0.6
Dispose tips	No
Volume (µL)	500
Collect in position	D (W1)
Positive pressure time (s)	80

Advanced pressure settings

None

Load tab

Number of steps	1
Pressure (bar)	0.6
Premix (no times)	Yes (1)
Volume (µL)	400
Collect in position	D (W1)
Positive pressure time (s)	80

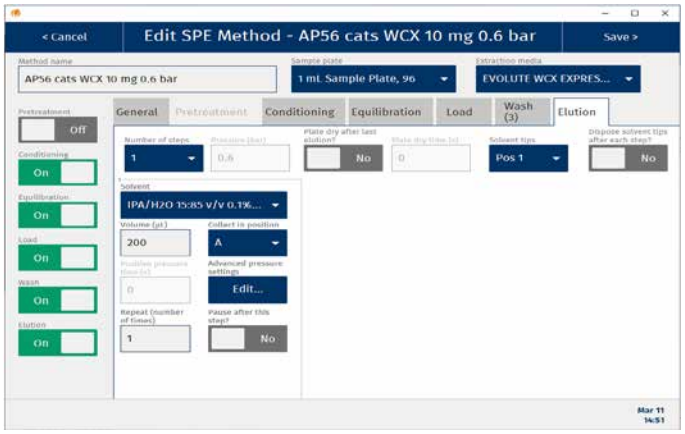
Advanced pressure settings

None

Wash tab

Number of steps	3
Pressure (bar)	0.6
Plate dry after last wash	Yes
Plate dry time (s)	300
Step 1	
Volume (µL)	500
Collect in position	D (W1)
Positive pressure time (s)	70
Advanced pressure settings	
None	
Step 2	
Volume (µL)	500
Collect in position	D (W1)
Positive pressure time (s)	-
Advanced pressure settings	
Number of steps	2
Step 2.1 pressure (bar)	0.6
Step 2.1 time (s)	80
Step 2.2 pressure (bar)	5.0
Step 2.2 time (s)	60
Step 3	
Volume (µL)	500
Collect in position	B
Positive pressure time (s)	60
Advanced pressure settings	
None	

Elute tab



Number of steps	1
Pressure (bar)	0.6
Plate dry after last elution	No
Volume (µL)	400
Collect in position	A
Positive pressure time (s)	1
Advanced pressure settings	
Number of steps	2
Step 1 pressure (bar)	0.6
Step 1 time (s)	60
Step 2 pressure (bar)	4.0
Step 2 time (s)	10

Literature Number: AN963.v2