Extraction of Acidic Catecholamine Metabolites in Plasma Using ISOLUTE® SLE+ Prior to LC-MS/MS Analysis

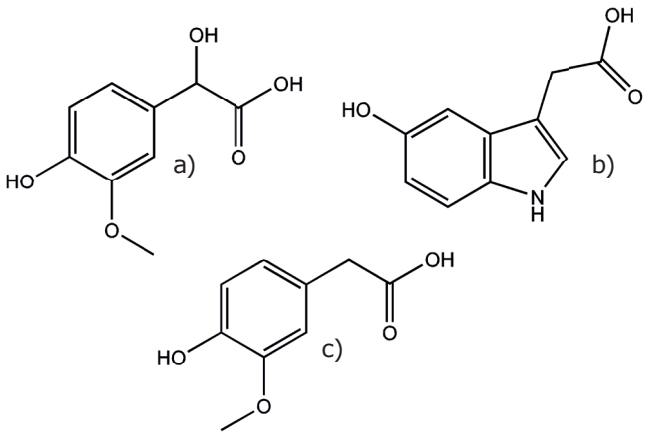

Figure 1. Analyte structures: a) vanillylmandelic acid, b) 5-hydroxyindoleacetic acid, c) homovanillic acid.

Introduction

This application note describes a supported liquid extraction protocol for the extraction of three acidic catecholamine metabolites (vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid) from plasma prior to LC-MS/MS detection. The method described in this application note achieves high reproducible recoveries (>80%, RSD <5%) for these analytes in plasma with linearity >0.995 in the range 2–200 ng/mL.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

Vanillylmandelic acid (VMA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and internal standards (D_3-vanillylmandelic acid, D_5-5-hydroxyindoleacetic acid, D_5-homovanillic acid) obtained from Sigma-Aldrich Company Ltd., Gillingham UK.)

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 200 µL Supported Liquid Extraction plate, part number 820-0200-P01.

Sample Pre-treatment

Add plasma (75 µL) to 100 mM ammonium acetate pH 7 (75 µL) containing an appropriate amount of internal standards. Mix.

Sample Loading

Load 150 µL of the pre-treated plasma (equivalent to 75 µL raw plasma) into each well of the ISOLUTE SLE+ plate. Ensure the surface of the well frit is completely covered by the pre-treated sample. Using a Biotage® PRESSURE+96 Positive Pressure Manifold, apply 2–5 psi of pressure to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

Analyte Extraction

Apply 1% (v/v) formic acid in ethyl acetate (250 µL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of ethyl acetate* (300 µL) and allow to flow under gravity for 5 minutes. Apply pressure (5–10 seconds) to remove any remaining extraction solvent.

***Note:** The 2nd aliquot of elution solvent is NOT acidified with 1% formic acid.

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry 96 (30 °C at 60 L min^{-1}) or TurboVap® 96 (30 °C at 1.0 bar). Reconstitute the extracts with 200 µL 0.1% acetic acid in 10% acetonitrile (aq). Mix thoroughly.

HPLC Conditions

Instrument
Shimadzu Nexera UHPLC

Column
Restek Raptor Biphenyl 2.1 x 100 mm, 2.7 µm analytical column and Biphenyl 2.1 x 5 mm, 2.7 µm EXP® guard cartridge.

Mobile Phase
A: 1 mM ammonium fluoride (aq)

B: Methanol

Flow Rate
0.5 mL min^{-1}

Table 1. Gradient and Divert Valve Settings.

Time (min)	%A	%B	Divert Valve
0.00	90	10	waste
0.50	90	10	MS
0.68	85	15	
1.40	85	15	
3.10	50	50	waste
3.40	10	90	
4.40	10	90	
4.90	90	10	
6.50	90	10	

Column Temperature
40 °C

Injection Volume
10 µL (flow-through needle)

Sample Temperature
15 °C

Table 2. Typical Retention Times.

Analyte	Retention Time (min)
VMA	0.8
5-HIAA	1.8
HVA	2.1

Mass Spectrometry Conditions

Dual polarity ions were acquired using electrospray ionization in multiple reaction monitoring mode and two periods.

Instrument
Sciex Triple Quad 5500 mass spectrometer

Source Temperature (TEM)
600 °C

Curtain Gas (CUR)
40

Source Gas 1 (GS1)
50

Source Gas 2 (GS2)
60

Table 3. MRM Parameters.

Analyte	Transition (Da)	IS (V)	DP (V)	EP (V)	CE (V)	CXP (V)
VMA	197.1 > 137.1	-1500	-50	-10	-28	-21
D_3-VMA	199.9 > 139.9	-1500	-50	-10	-28	-16
5-HIAA	192.0 > 145.7	2000	80	13	45	22
D_5-5-HIAA	197.0 > 150.1	2000	70	12	45	19
HVA	181.0 > 136.4	-1500	-50	-10	-11	-18
D_5-HVA	186.1 > 142.0	-1500	-60	-12	-10	-19

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Results

Extraction Recovery

Extraction recoveries were determined at 40 ng mL^{-1} equivalent to 15 ng when extracting 150 µL of pre-treated plasma.

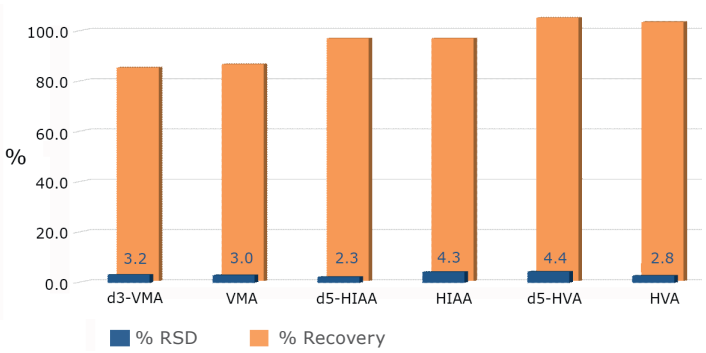

Figure 2. Extraction recovery and RSD (n=6) of acidic catecholamine metabolites (40 ng mL^{-1} spike).

Linearity

Extraction linearity was determined between 2 and 200 ng mL^{-1} from a mixed stock spiked into pooled plasma at 200 ng mL^{-1} then serially diluting in pooled plasma. Each calibration level was extracted in duplicate with a fixed concentration of internal standards. Figure 3 demonstrates representative calibration curves.

Table 4. Linearity data for Catecholamine Acid Metabolites extracted using ISOLUTE® SLE+.

Analyte	Linear Range (ng mL^{-1})	Coefficient (r^2)	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
VMA	5 to 200	0.9987	5	2
5-HIAA	5 to 200	0.9994	5	2
HVA	5 to 200	0.9987	5	2

Phospholipid Removal

Removal of phospholipids was demonstrated by monitoring MRM transitions for the common product ion at m/z 184. Unspiked plasma extracted using the optimized ISOLUTE® SLE+ protocol was compared to plasma extracted using a solvent crash (1:4, v/v) in acetonitrile. Figure 4 demonstrates a significant reduction in lysophospholipids (0 to 4.00 min) and a reduction in phospholipids (4.00 to 8.00 min) when using the optimized ISOLUTE SLE+ protocol compared to a 1:4 solvent crash. A range expansion of x100 and x10 was used to display the ISOLUTE SLE+ extracted plasma lysophosholipids and phospholipids respectively.

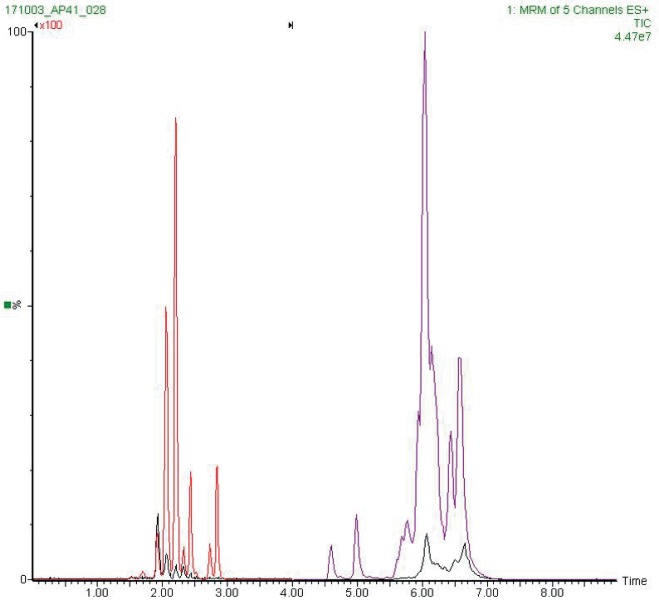

Figure 4. Overlaid TIC for the 184.0 m/z product ion comparing plasma 1:4 (v/v) acetonitrile (red/purple) to plasma extracted using the optimized ISOLUTE® SLE+ protocol (black/green).

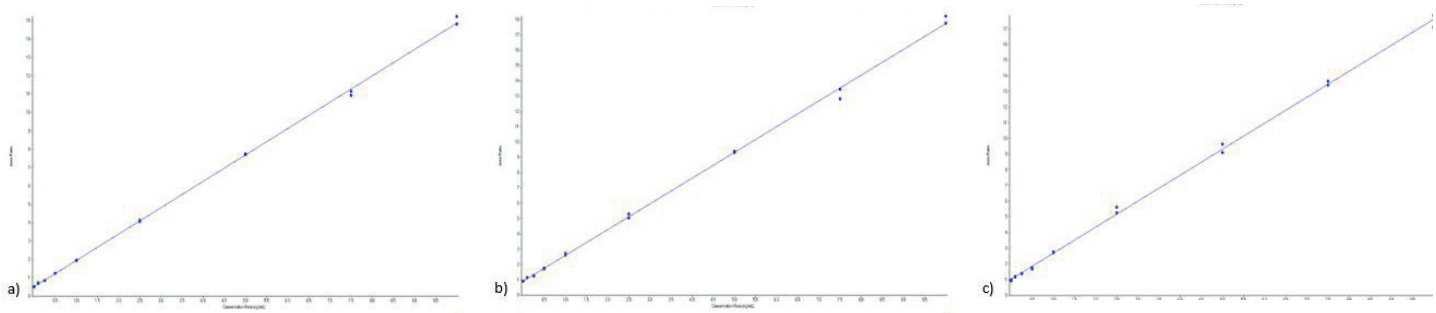

Figure 3. Representative Extracted Catecholamine Acid Metabolite Calibration Curves, 2 to 200 ng mL^{-1} relative to 50 ng mL^{-1} IS: a) VMA, b) 5-HIAA, c) HVA.

Other Chemicals and Reagents

Preparation of Internal Standards

- Prepare a mixed substock of internal standards at a concentration of 5 µg mL^{-1} in methanol. For twenty sample pre-treatment aliquots dilute 30 µL of internal standard substock in 3 mL of 100 mM ammonium acetate pH7 (pre-treatment solution) to give a working concentration of 50 ng mL^{-1} (proportionally increase the substock and dilution volumes to prepare a larger number of samples). A 75 µL aliquot of pre-treatment solvent contains 3.75 ng of each internal standard, equivalent to 50 ng per mL plasma.
- 18.2 MΩ.cm water was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK).
- Solid reagents and chemicals were obtained from Sigma-Aldrich Chemical Co. (Gillingham, UK), HPLC grade or higher.
- Liquid reagents and solvents were obtained from Honeywell Research Chemicals (Bucharest, Romania), HPLC grade or higher.
- Pooled human plasma was obtained from the Welsh Blood Service (Pontyclun, UK).

Ordering Information

Part Number	Description	Quantity
820-0200-P01	ISOLUTE® SLE+ 200 µL Supported Liquid Extraction Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120 V	1
C103264	Turbovap® 96 Evaporator 220/240 V	1
C103263	Turbovap® 96 Evaporator 100/120 V	1

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