# Extraction of Catecholamine Acid Metabolites from Plasma Prior to Analysis Using UHPLC-MS/MS

Adam Senior[1], Lee Williams[1], Alan Edgington[1], Rhys Jones[1], Helen Lodder[1], Geoff Davies[1], Steve Jordan[1], Claire Desbrow[1], Paul Roberts[1], Elena Gairloch[2], Stephanie Marin[2] and Dan Menasco[2]

[1]Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK
[2]Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA

## Introduction

Catecholamine metabolites vanillylmandelic acid (VMA), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5 HIAA) are biomarkers for neuroblastoma and catecholamine-secreting tumors, structures are shown below (***Figure 1***). This poster presents optimization of the method development process to maximize analyte sensitivity in the extraction and quantitation of these metabolites from plasma. Parameters investigated include MRM transitions, chromatography and sample preparation protocols.

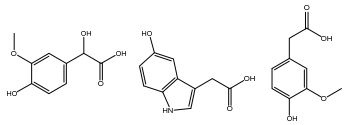

***Figure 1.*** *Structures of a) VMA, b) 5-HIAA, c) HVA*

## Experimental

### Reagents

Standards, ammonium acetate and ammonium fluoride were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). LC/MS grade solvents were from Honeywell Research Chemicals (Bucharest, Romania). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q5 water purifier (Merck Millipore, Watford, UK). Pooled human plasma was from The Welsh Blood Service (Pontyclun, UK) or Golden West Biologicals, Inc. (Temecula CA).

### Sample Preparation

Extractions were developed using supported liquid extraction (SLE) or polymer-based SPE in 96 fixed well plate format. ISOLUTE® SLE+ was used in the 200 µL format (P/N 820-0200-P01) following a load-wait-elute procedure (***Figure 2***).

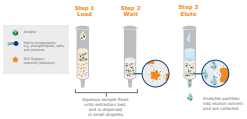


***Figure 2****. Schematic of ISOLUTE® SLE+ Supported Liquid Extraction Procedure.*

A typical SPE procedure incorporating additional wash steps is shown in ***Figure 3***. EVOLUTE® EXPRESS ABN and AX were used in 10 mg formats (P/N 600-0010-PX01 and 603-0010-PX01).


***Figure 3.*** *Schematic of a Typical SPE Procedure.*

Full method optimization was performed for each sample preparation technique with final extraction protocols for each shown in ***Table 1***.

**Post extraction:** Extracts were evaporated at 40 °C and reconstituted in 200 µL of 0.1% acetic acid in 10% ACN prior to injection.

***Table 1.*** *Extraction Protocols.*

| Step | ISOLUTE® SLE+ 200 µL | EXPRESS ABN 10 mg | EXPRESS AX 10 mg |
|---|---|---|---|
| **Condition** | - | MeOH 500 µL | MeOH 500 µL |
| **Equilibration** | - | $H_2O$ 500 µL | 50 mM $NH_4OAC$ pH 6 500 µL |
| **Sample load** | 1:1 100 mM $NH_4OAC$ pH 7 150 µL | 1:3 1% HCOOH (aq) 400 µL | 1:3 50 mM $NH_4OAC$ pH 6 400 µL |
| **Wash 1** | - | 0.1% HCOOH (aq) 500 µL* | $H_2O$ 500 µL |
| **Wash 2** | - | - | MeOH 500 µL* |
| **Wash 3** | - | - | 0.1% HCOOH/DCM 500 µL* |
| **Elution** | 1% HCOOH / EtOAC 1x 250 µL followed by EtOAC 1x 300 µL | 60% MeOH (aq) 400 µL | MeOH/MeCN/$H_2O$ 30:30:40 v/v/v 400 µL |

* The plate was fully dried between (immiscible) steps

### UHPLC Conditions

**Instrument:** Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany)
**Column:** Restek Raptor biphenyl 2.7 µm 100 x 2.1 mm + guard (Thames Restek, High Wycombe, UK)
**Mobile phase:** A, 1 mM $NH_4F$ (aq); B, MeOH. **Flow rate:** 0.5 mL/min
**Gradient:** Isocratic hold 10% B, 0.5 min; linear to 15% B, 0.2 min; hold, 0.7 min; linear to 50% B, 1.7 min; linear to 90% B, 0.3 min; hold, 1 min; resume initial conditions, 1.6 min.
**Column temp:** 40 °C **Auto-sampler temperature:** 15 °C
**Injection volume:** 10 µL

### Mass Spectrometry

**Instrument**: Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, US). Ions (***Table 2***) were selected from the most intense precursor and acquired in positive or negative polarity using a Turbo V ESI and either MRM or Scheduled MRM transitions.
**Ion Spray Voltage:** 1500-2000 V
**Source Temperature:** 600° C
**Curtain Gas:** 40 psi
**Gas 1:** 50 psi **Gas 2:** 60 psi

***Table 2.*** *MRM Parameters*

| Analyte | Transition | IS, V | DP, V | EP, V | CE, V | CXP, V |
|---|---|---|---|---|---|---|
| **VMA** | 197.1 > 137.1 | -1500 | -50 | -10 | -28 | -21 |
| **VMA-$D_3$** | 199.9 > 139.9 | -1500 | -50 | -10 | -28 | -16 |
| **5-HIAA** | 192.0 > 145.7 | +2000 | +80 | +13 | +33 | +22 |
| **5-HIAA-$D_5$** | 197.0 > 150.1 | +2000 | +70 | +12 | +34 | +19 |
| **HVA** | 181.0 > 136.4 | -1500 | -50 | -10 | -11 | -18 |
| **HVA-$D_5$** | 186.1 > 142.0 | -1500 | -60 | -10 | -12 | -19 |

## Results

### Chromatographic Optimization

Separation performance for a variety of columns including C18, PFP, biphenyl and HILIC were investigated (data not shown). Optimal separation was achieved using the biphenyl column and parameters described above in ***UHPLC Conditions***.

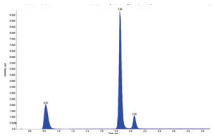

***Figure 4.*** *Overlaid XICs for VMA ($t_R$ 0.82), 5-HIAA ($t_R$ 1.85) and HVA ($t_R$ 2.05) at an equivalent concentration of 10 ng/mL matrix.*

### Evaporation Optimization

Evaporation losses were minimal when reconstituting in acidified aqueous solutions (***Figure 5***). Acidification promotes the solubility of the metabolites. However, acid concentration above 2% is detrimental to analyte recovery (data not shown). The optimal solvent for reconstitution was 0.1% acetic acid in 10% MeCN (***Figure 5***), RSDs were consistently below 4% (n=6).

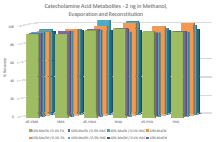

***Figure 5.*** *Evaporation of 2 ng Catecholamine Acids from MeOH and Reconstitution in Aqueous Solvents.*

### ISOLUTE® SLE+ Optimization

Initial experiments determined acidified polar non-halogenated solvents to be the most effective for analyte extraction (***Figure 6***). Slight pigment and/or matrix residue was observed. However, loading/elution volume reduction and wash experiments were undertaken to improve this.

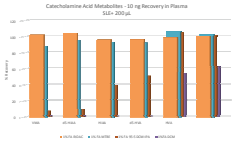

***Figure 6.*** *Extraction Solvent Comparison, 10 ng acid metabolites.*

Decreasing the load volume to ¾ of the bed capacity reduced extract pigmentation. Incorporating an acidified DCM wash demonstrated cleaner extracts with good recovery, at the expense of precision (***Figure 7***).

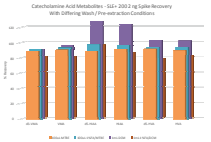

***Figure 7.*** *Acid Metabolites Recovery Varying Wash/Modifier Conditions.*

### EVOLUTE® EXPRESS ABN 10 mg Optimization

Initial extraction optimization focussed on wash/elution solvents. However, significant matrix suppression was observed (data not shown). ***Figure 8*** demonstrates the effect of increased aqueous content on recovery.

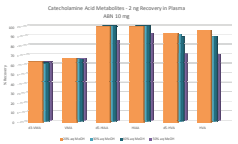

***Figure 8.*** *ABN Methanol Elution Varying Aqueous Concentration.*

***Figure 9*** demonstrates ethyl acetate and MTBE have comparable recovery performance to 60% MeOH (aq) with low matrix suppression and improved phospholipid removal. This work also confirmed the suitability of hexane as a wash solvent for ABN.

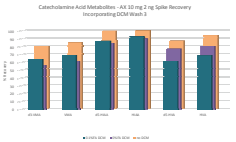

***Figure 9.*** *Method Optimization Incorporating Immiscible Solvents.*

### EVOLUTE® EXPRESS AX 10 mg Optimization

Due to the anion exchange interaction between the sorbent and weak acid groups on the analyte, recoveries were maximized using pre-treatment with 10-50 mM $NH_4OAC$ pH 6 (data not shown). ***Figure 10*** demonstrates using DCM as a 3rd wash with up to 0.1% HCOOH decreases recovery but with the benefit of an improved suppression profile. Previous work on catecholamine extraction demonstrates use of a water immiscible wash solvent extends LC column lifetime.

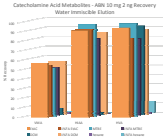

***Figure 10.*** *Recovery of 2 ng Acid Metabolites from Plasma incorporating DCM as a wash step.*

### Phospholipid Removal

***Figure 11.*** compares the efficiency of phospholipid removal using three different sorbents with optimized extraction protocols. Almost complete removal is achieved using SLE+ and AX, ethyl acetate improves PL removal using ABN albeit with decreased VMA recovery.

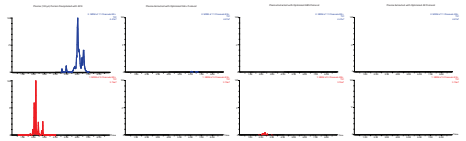

***Figure 11****. Visual Comparison of Phospholipid Removal from Plasma Using Protocols for the Extraction of Acid Metabolites.*

### Method Performance

Calibration curves were constructed in stripped human plasma from 2-200 ng/mL. The optimized procedures demonstrated good recoveries and RSD (n=6) with linearity and coefficients of determination 0.990 or greater. Full data is summarized in ***Table 3***.

***Table 3****. Summary of method performance.*

| Analyte | SLE+ 200 µL | | ABN 10 mg | | AX 10 mg | |
|---|---|---|---|---|---|---|
| | Recovery (RSD) | $r^2$ | Recovery (RSD) | $r^2$ | Recovery (RSD) | $r^2$ |
| **VMA** | 86 (3.0) | 0.998 | 65 (6.8) | 0.994 | 70 (7.9) | 0.996 |
| **VMA-$D_3$** | 85 (3.2) | 0.997 | 60 (4.6) | 0.993 | 64 (7.2) | 0.995 |
| **5-HIAA** | 96 (4.3) | 0.998 | 108 (3.4) | 0.994 | 89 (4.8) | 0.997 |
| **5-HIAA-$D_5$** | 96 (2.3) | 0.998 | 108 (3.0) | 0.996 | 83 (4.0) | 0.998 |
| **HVA** | 103 (2.8) | 0.994 | 85 (3.3) | 0.998 | 74 (6.5) | 0.999 |
| **HVA-$D_5$** | 105 (4.4) | 0.995 | 85 (5.9) | 0.998 | 61 (8.8) | 0.999 |

## Conclusion

- Various strategies were presented for the extraction of acid catecholamine metabolites from plasma using ISOLUTE® SLE+ 200 µL, EVOLUTE® EXPRESS ABN and AX 10 mg 96 well plates.
- The extraction performance of deuterated internal standards is equivalent to native acid metabolites.
- Good recoveries and precision were demonstrate with high S/N below reporting levels (20 ng/mL), using strategies to reduce matrix interferences such as phospholipids.



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