# Simultaneous Extraction of Catecholamine and Metanephrines from Urine Prior to Analysis using LC-MS/MS

Alan Edgington[1], Adam Senior[1], Lee Williams[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] & 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

Catecholamines and metanephrines continue to be important biomarkers related to various neurological issues. This poster discusses the impact of optimization of various parts of the method development process to maximize assay performance while delivering a combined assay for the analysis of urinary catecholamines and metanephrines. This included optimization of sample loading volume, pH and ionic content to allow for the efficient measurement of a wide range of analyte concentrations.

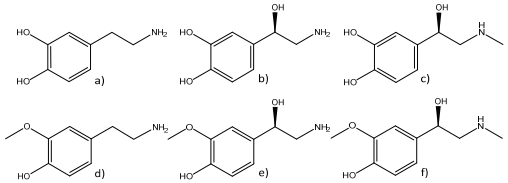


***Figure 1.*** *Structures of a) dopamine, b) norepinephrine, c) epinephrine, d) 3 methoxytyramine (3-MT), e) normetanephrine, f) metanephrine*

## Experimental

### Reagents

Standards were obtained from LGC (Teddington, UK). Formic acid, ammonium hydroxide, hydrochloric acid, ammonium acetate, ammonium formate, propan-2-ol (IPA) and LC/MS grade chromatographic solvents were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). 18.2 MΩ.cm water was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Urine was obtained from healthy human volunteers.

### Sample Preparation

All extractions were performed using polymer-based SPE in a 96 fixed-well plate format. EVOLUTE® EXPRESS WCX was evaluated using a 10 mg bed mass.

**Sample pre-treatment:** 75 µL urine was pre-treated with aqueous buffers varying volume and concentration before mixing thoroughly. Pre-treated sample (150 µL) was applied to each well of the plate.

**SPE Optimization:** Various extraction strategies were evaluated, investigating effect of pH control, wash solvent and elution solvent optimization. ***Table 1.*** details the final extraction protocols.

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

| Step | Volume | Standard SPE | Load-Wash-Elute SPE |
|---|---|---|---|
| Condition | 500 µL | MeOH | - |
| Equilibration | 500 µL | 10 mM $NH_4OAc$ | - |
| Sample load | 150 µL | 75 µL urine 1:2 250 mM $NH_4OAc$ | |
| Wash 1 | 500 µL | 10 mM $NH_4OAc$ | |
| Wash 2 | 500 µL | IPA | |
| Elution | 125 µL | 0.1% formic acid 85/15 aq/IPA | |

**Post extraction:** Due to the high aqueous content of the elution solvent the evaporation step was eliminated. A cap mat was applied to the collection plate for direct injection onto the LC-MS/MS system.

### Biotage® Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocols were transferred to an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The Extrahera™ platform is shown in ***Figure 2***. Full processing conditions for each technique are available on request.

***Figure 2.*** *Biotage® Extrahera™ automated sample preparation platform*

### UHPLC Conditions

**Instrument:** Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Germany)
**Column:** ACE Excel 2 C18-PFP 100 x 2.1 mm, 2µ (Hichrom ltd., UK)
**Mobile phase:** A, 0.25 mM ammonium formate and formic acid (aq); B, 0.25 mM ammonium formate and formic acid in MeOH
**Flow rate:** 0.4 mL $min^{-1}$
**Gradient:** Isocratic hold at 5% B for 1.3 min, step to 95% B, hold for 3 min, resume initial conditions for 3 min
**Column temp:** 40° C
**Injection volume:** 7.5 µL

### Mass Spectrometry

**Instrument:** Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, US). Ions acquired in positive mode using a Turbo V ESI interface using either MRM or Scheduled MRM transitions (***Table 2.***).
**Ion Spray Voltage:** 5500 V
**Source Temperature:** 700° C
**Curtain Gas:** 35 psi
**Gas 1 and Gas 2:** 50 psi

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

| Analyte | Transition | DP, V | EP, V | CE, V | CXP, V |
|---|---|---|---|---|---|
| epinephrine | 166.1 > 107.1 | 148 | 8 | 24 | 16 |
| $D_6$ epinephrine | 172.1 > 112.1 | 148 | 8 | 24 | 16 |
| norepinephrine | 152.1 > 107.1 | 25 | 2 | 22 | 25 |
| $D_6$ norepinephrine | 158.1 > 111.1 | 25 | 2 | 22 | 25 |
| dopamine | 154.1 > 91.1 | 50 | 9 | 29 | 13 |
| $D_4$ dopamine | 158.1 > 95.1 | 50 | 9 | 29 | 13 |
| metanephrine | 180.1 > 148.0 | 25 | 8 | 22 | 16 |
| $D_3$ metanephrine | 183.1 > 151.0 | 25 | 8 | 22 | 16 |
| normetanephrine | 166.1 > 134.0 | 25 | 9 | 20 | 16 |
| $D_3$ normetanephrine | 169.1 > 137.0 | 25 | 9 | 20 | 16 |
| 3-methoxytyramine | 151.2 > 90.9 | 110 | 9 | 26 | 11 |

## Results

### Mass Spectrometer Optimization

Source parameters were optimized to enhance the production of dehydrated precursor ions to increase signal sensitivity of epinephrine and norepinephrine. At least a two-fold increase in signal was observed when using a suitably optimized MRM transition for epinephrine and norepinephrine with the transitions also being free of interference. A concomitant increase in the dehydrated dopamine precursor was not observed suggesting only the alkyl OH group is susceptible to dehydration.

### Extraction Optimization

Work previously published demonstrates analyte elution using a solvent with a high aqueous proportion eliminates the need for an evaporative step. The capacity of $NH_4OAC$ as a pre-treatment buffer was determined by comparing pH of urine diluted with increasing volumes of 50 mM $NH_4OAC$. The pH of neat urine, urine diluted 1/4 and 1/8 was **6.9 ± 0.07** units demonstrating minimal urine concentration effects.

As the final extraction method does not incorporate a post-elution evaporation step, it was critical to determine the optimal elution volume for the analytes. Increased elution volume increases the final amount of analyte at the expense of concentration. ***Figure 3.*** compares analyte response relative to 125 µL elution, demonstrating response is inversely proportional to elution volume.

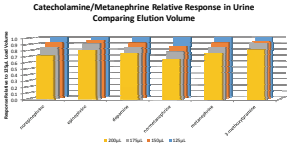


***Figure 3.*** *Relative analyte response with varying elution volume.*

A variety of pre-treatment conditions were investigated at a fixed urine load volume: ratio, buffer concentration and total load volume. ***Figure 4.*** demonstrates an increase in recovery with a combination of a low pre-treatment ratio, moderate buffer concentration and low total load volume. The optimal pre-treatment was chosen to maximize norepinephrine and epinephrine recovery.

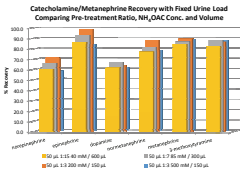


***Figure 4.*** *Pre-treatment effects at fixed urine load.*

The effects of load volume were investigated to determine the extraction efficiency of WCX 10 mg. Varying volumes of urine were pre-treated, altering buffer ratios and concentrations. Analyte responses were corrected for volume loaded. ***Figure 5.*** demonstrates a low urine pre-treatment ratio and load volume combined with moderate buffer concentration give improved analyte response. The optimal method chosen used 75 µL urine pre-treated 1:2 with $NH_4OAC$.

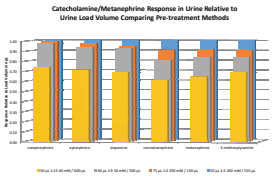


***Figure 5.*** *Relative analyte response corrected for load volume.*

The optimal method gave the best combination of extraction recovery and precision compared to other methods (data not shown). Due to the varying clinical ranges of the analyte suite, linear ranges were required to be demonstrated between 0.1 to 25 ng $mL^{-1}$ and 2.5 to 625 ng $mL^{-1}$. Example calibration curves are demonstrated in ***Figure 6.*** and were constructed for standard SPE and Load-Wash-Elute protocols using manual or Biotage® Extrahera™ processing methods (data available on request).

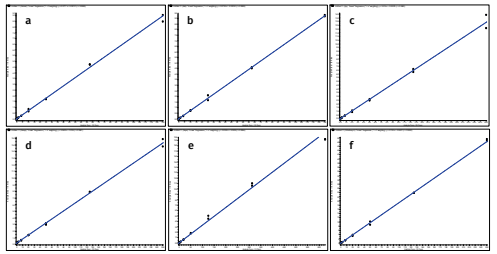


***Figure 6.*** *Calibration curves: a) normetanephrine, b) metanephrine, c) epinephrine, d) norepinephrine, e) dopamine, f) 3-MT.*

Regression coefficients and recovery (%RSD) data were generated for each protocol using optimized extraction conditions, these are tabulated in ***Table*** and ***Table*** .

***Table 3.*** *Linearity Parameters*

| Calibration Parameter | Range, ng $mL^{-1}$ | Regression coefficient, $r^2$ | | | |
|---|---|---|---|---|---|
| Analyte | | Extrahera (STD) | Extrahera (LWE) | Manual (STD) | Manual (LWE) |
| epinephrine | 0.1 – 25 | 0.995 | 0.991 | 0.994 | 0.996 |
| norepinephrine | 1.0 – 250 | 0.997 | 0.996 | 0.999 | 0.998 |
| dopamine | 2.5 – 625 | 0.995 | 0.993 | 0.991 | 0.993 |
| metanephrine | 0.5 – 125 | 0.990 | 0.996 | 0.996 | 0.998 |
| normetanephrine | 0.5 – 125 | 0.995 | 0.998 | 0.993 | 0.997 |
| 3-MT | 1.0 – 250 | 0.997 | 1.000 | 0.998 | 0.998 |

***Table 4.*** *Recovery Parameters*

| Recovery Parameter | Recovery at maximum concentration (%RSD) n=8 | | | |
|---|---|---|---|---|
| Analyte | Extrahera (STD) | Extrahera (LWE) | Manual (STD) | Manual (LWE) |
| epinephrine | 110.4 % (7.4) | 93.7 % (6.0) | 109.3 % (7.0) | 108.3 % (8.5) |
| norepinephrine | 77.4 % (4.9) | 69.5 % (5.8) | 88.8 % (3.5) | 76.1 % (7.0) |
| dopamine | 87.4 % (4.2) | 88.4 % (2.0) | 91.8 % (3.1) | 88.2 % (3.3) |
| metanephrine | 83.5 % (5.8) | 82.4 % (5.7) | 83.3 % (5.0) | 88.4 % (6.8) |
| normetanephrine | 80.7 % (7.4) | 77.2 % (7.2) | 87.9 % (6.6) | 83.6 % (8.6) |
| 3-MT | 78.0% (5.8) | 80.1 % (4.8) | 84.2 % (4.2) | 83.6 % (2.1) |

## Conclusion

- We demonstrate that EVOLUTE EXPRESS WCX 10 mg 96 well plates can be used to extract polar catecholamines and metanephrines from human urine in a highly sensitive, linear assay.
- Good recoveries and excellent precision are demonstrated whether the EVOLUTE® EXPRESS WCX material is used in standard SPE processing or modified Load-Wash-Elute protocols.
- A number of steps were optimized to improve sensitivity of the analytes: pH control throughout the extraction, reducing SPE wash buffer concentration, avoidance of evaporation and reconstitution, incorporation of IPA in the SPE elution solvent and reducing mobile phase buffer concentration.



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