

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

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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.

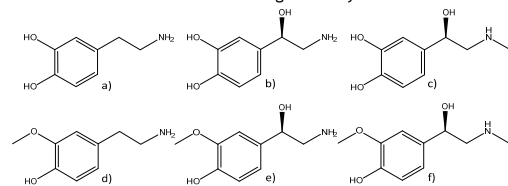


Figure 1. Structures of a) dopamine, b) norepinephrine, c) epinephrine, d) 3-methoxytyramine (3-MT), e) normetanephrine, f) metanephrine

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 ₄ OAc	-
Sample load	150 µL	75 µL urine 1:2 250 mM NH ₄ OAc	-
Wash 1	500 µL	10 mM NH ₄ OAc	-
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 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	Q1P, V
epinephrine	166.1 > 107.1	145	8	24	16
D ₅ epinephrine	172.1 > 112.1	148	8	24	16
norepinephrine	152.1 > 107.1	25	2	22	25
D ₅ norepinephrine	158.1 > 111.1	25	2	22	25
dopamine	154.1 > 91.1	50	9	29	13
D ₅ dopamine	158.1 > 95.1	50	9	29	13
metanephrine	180.1 > 148.0	25	8	22	16
D ₅ metanephrine	183.1 > 151.0	25	8	22	16
normetanephrine	166.1 > 134.0	25	9	20	16
D ₅ normetanephrine	169.1 > 137.0	25	9	20	16
3-methoxytyramine	151.2 > 90.9	110	9	26	11

Results

Mass Spectrometer Optimization

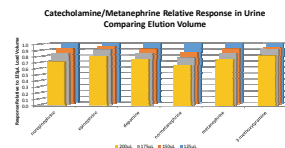
Source parameters were optimized to enhance the production of dehydrated precursor ions to increase signal sensitivity of epinephrine and norepinephrine. At least a two-fold increase in signal was observed when using a suitably optimized MRM transition for epinephrine and norepinephrine with the transitions 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 NH₄OAc as a pre-treatment buffer was determined by comparing pH of urine diluted with increasing volumes of 50 mM NH₄OAc. 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.

