Simultaneous Extraction of Catecholamines and Metanephrines from Plasma Prior to Analysis using LC-MS/MS

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Introduction

Catecholamines and metanephrines are biomarkers used for the detection of diseases such as: hypertension, pheochromocytoma and neuroblastoma. The main target analytes are dopamine, epinephrine, norepinephrine, metanephrine, normetanephrine and 3-methoxytyramine (see ***Figure 1.*** for details). Levels are traditionally analyzed using liquid chromatography with electrochemical detection.

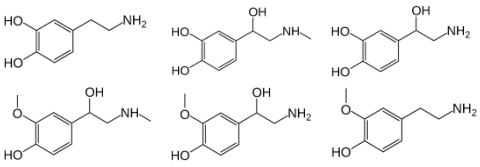

Figure 1. *Structures of dopamine, epinephrine and norepinephrine (top), metanephrine, normetanephrine and 3-methoxytyramine (bottom).*

Experimental

Reagents

Standards were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). Formic acid, ammonium acetate, ammonium formate, ethylene glycol, propan-2-ol, dichloromethane and LC/MS grade chromatographic solvents were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Pooled human plasma was obtained from The Welsh Blood Service (Pontyclun, UK).

All analytes were quantified against deuterated internal standards with the exception of 3 methoxytyramine which was quantified against the metanephrine internal standard.

Sample Preparation

All extractions were performed using polymer-based SPE. EVOLUTE® EXPRESS WCX which was used in the 10 mg 96 fixed well plate format (P/N 602-0010-PX01).

Sample pre-treatment: Increasing the volume of consistently pretreated sample to double that quoted here gave considerable increases in signal making this an attractive option when sensitivity is required over all else.

SPE Optimization: Various extraction strategies were evaluated, including sample load, wash solvent and elution solvent optimization. The final and streamlined protocols are detailed in ***Table 1.***

Table 1. *Extraction strategies.*

Step	Volume	Standard SPE methodology	Load-Wash-Elute methodology
Condition	500 µL	MeOH	-
Equilibration	500 µL	10 mM NH_4OAC pH7	-
Sample load	500 µL	plasma 1:1 0.05% v/v formic acid	
Wash 1	500 µL	10 mM NH_4OAc pH 7	
Wash 2	500 µL	Propan-2-ol	
Wash 3 *	500 µL	Dichloromethane	
Elution	125 µL	85/15 0.1% Water/IPA, 0.1% formic acid overall	

*The plate was fully dried after wash 3.

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

UHPLC Conditons

Instrument: Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany)
Column: ACE Excel 2 C18-PFP 100 x 2.1 mm, 2µm (Hichrom Ltd., Theale, UK).
Guard: Securityguard C18 (Phenomenex, Macclesfield, UK).

Mobile phase: A, H_2O; B, MeOH: both mobile phases contained 7 µL of formic acid and 3 mg of ammonium formate per L.
Flow rate: 0.4 mL/min
Gradient: Isocratic hold at 5% B for 2 min, step to 100% B, hold for 1 minute, step to 0% B hold for 1 minute, resume initial conditions for 3.5 minutes
Column temp: 40 °C **Autosampler temp:** 5 °C
Injection volume: 20 µL

Mass Spectrometry

Instrument: Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, US). Ions (**Table 2.**) were acquired in the positive mode using a Turbo V ESI interface and either MRM or Scheduled MRM transitions.

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_3 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 for all analytes except dopamine and 3-MT. 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 and 3-MT precursor was not observed suggesting only the alkyl OH group is susceptible to dehydration.

Extraction Optimization

EVOLUTE® EXPRESS combines sorbent wettability with optimized SPE components, allowing better flow consistency and in many cases eliminating the need for SPE column conditioning. Therefore the method was replicated without phase conditioning. Results demonstrate that recoveries, although slightly lower, were still precise, see ***Table 3***.

Table 3. *Summary of method recovery and precision.*

Analyte	Full procedure		Load-Wash-Elute	
	Recovery	% RSD	Recovery	% RSD
Epinephrine	86.9	6.0	76.0	5.2
Norepinephrine	75.4	7.3	53.6	11.1
Dopamine	61.0	4.5	51.0	2.8
Metanephrine	93.5	3.1	82.1	1.7
Normetanephrine	80.5	3.0	69.8	2.9
3-Methoxytyramine	80.9	6.8	77.5	8.0

To guard against the potential co-extraction of lipids and to improve the ruggedness of the SPE procedure it was decided to include an additional wash with dichloromethane prior to elution.

As a water immiscible solvent, DCM was an excellent wash step for lipids or other hydrophobic interferences. The wash step that preceded this was IPA, a DCM miscible solvent, which meant a totally dry sorbent bed was not necessary prior to the DCM addition. Finally as the elution step that followed this was not being evaporated it was desirable to minimize the elution volume as much as possible and a drier bed allowed the elution of a smaller volume of sample with better extract composition control.

The use of DCM or any immiscible solvent was also easy to incorporate on the Biotage® Extrahera™ automated sample preparation platform. The instrument was designed to be able handle both water miscible and immiscible solvents. It could also be easily programmed to divert water immiscible waste into a separate container for separate disposal. The recently developed high flow head meant that the SPE sorbent could be dried incredibly efficiently prior to the elution step. Alternative water immiscible solvents may also be used however the SPE bed may need to be thoroughly dried before as well as after the final wash step.

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

Chromatographic Optimization

Target analyte peak shape and height were maximized using a low concentration of formate buffer with a small volume of formic acid. In the aqueous mobile phase this gave a pH of approximately 4.0. The C18-PFP column was finally selected based on its superior retention, separation and peak shape. It was noticed over a large number of injections that the initial method developed for catecholamines was susceptible to a build-up of co-extracted lipids which could cause an increase of back pressure or decrease of column chromatographic performance over time.

As well as using a DCM wash to tackle this issue a C18 guard cartridge was used prior to the PFP column. Due to the high polarity of the analytes this had no marked effect on the chromatography of the analytes but did serve as an additional barrier as insurance against any possible analytical column damage. However no significant increase in back pressure or clear drop in chromatographic performance was observed once the DCM wash was incorporated in to the method.

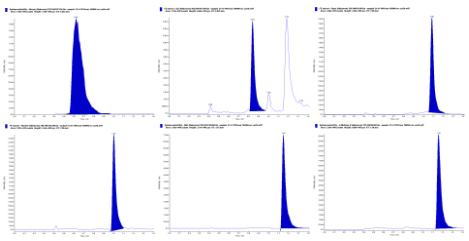

Figure 3. XIC's for norepinephrine, epinephrine, dopamine (top), metanephrine, normetanephrine and 3-methoxytyramine (bottom).

Post Extraction

The importance of avoiding an evaporation and reconstitution step was demonstrated (**Figure 3.**) where each analyte was diluted in injection solvent and analysed. Samples were either run immediately (where their signal was set at 100%) or evaporated for two hours before being reconstituted in the same volume and composition of solvents.

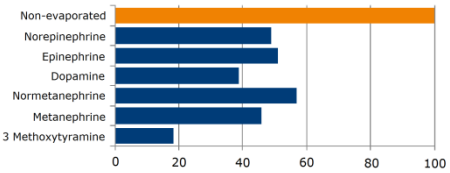

Figure 3. Measurement of Catecholamines and Metanephrines evaporative recoveries where the non-evaporated level was normalized to 100%

Evaporation ultimately demonstrated significant analyte losses, increased imprecision and potentially a variable lower limit of quantification depending upon the protocol. Acidification or the addition of ethylene glycol had previously been found not to eliminate catecholamine losses and so the method had to rely on the direct injection of the elution extract. This created a challenge of producing an elution solvent that was not only efficient at removing the analytes from the SPE sorbent but also gave acceptable peak shapes when injected on a highly aqueous LC system.
When eluting from a dry bed it was measured that on average approximately 95% of the available analyte was eluted in the first 125 µL. Increasing the elution volume further only resulted in dilution of the final analyte concentration.

Calibration curves were constructed in human plasma from 20-1280 pg/mL. The Load-Wash-Elute procedures demonstrated linear recoveries and coefficients of determination greater than 0.99 as detailed in ***Figure 4***.

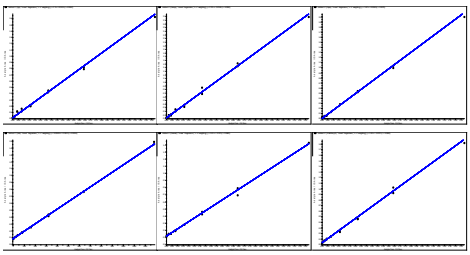

Figure 4. Calibration lines for norepinephrine, epinephrine, dopamine (top), metanephrine, normetanephrine and 3-methoxytyramine (bottom) using Load-Wash-Elute methodology.

Conclusions

- We demonstrate that EVOLUTE® EXPRESS WCX 10mg 96 well plates can be used to extract catecholamines and metanephrines from pooled human plasma in a highly sensitive, linear and rugged assay.
- The addition of a water immiscible solvent wash significantly reduced the extraction recoveries of any lipid interferences.
- Good recoveries and precision were demonstrated whether the EVOLUTE® EXPRESS WCX material was used in the standard SPE processing or modified Load-Wash-Elute protocols.
- Features of the Biotage® Extrahera™ including the high flow head and the ability to separate different washes make this an ideal instrument for performing this method.

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