Catecholamine Analysis: Evaluation of Method Optimization to Improve Sensitivity and reduce Limits of Quantitation using LC-MS/MS

Biotage®

Alan Edgington[1], Adam Senior[1], Lee Williams[1], Victor Vandell[2], Rhys Jones[1], Helen Lodder[1], Geoff Davies[1], Steve Jordan[1], Claire Desbrow[1]
Paul Roberts[1] & Elena Gairloch[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 are biomarkers used for the detection of diseases such as: hypertension, pheochromocytoma and neuroblastoma. The main target analytes are dopamine epinephrine and norepinephrine (see ***Figure 1.*** for details), which are traditionally analyzed using liquid chromatography with electrochemical detection. This poster investigates various parts of the method development process to develop a highly sensitive LC-MS/MS method for their analysis.

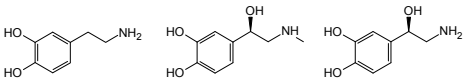

Figure 1. *Structures of dopamine, epinephrine and norepinephrine.*

Experimental

Reagents

Standards were obtained from LGC (Teddington, UK). Formic acid, ammonium hydroxide, hydrochloric acid, ammonium acetate, ammonium formate, ethylene glycol, propan-2-ol 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).

Sample Preparation

All extractions were performed using polymer-based SPE. EVOLUTE® EXPRESS WCX was evaluated in the 10 and 30 mg 96 fixed well plate format.

Sample pre-treatment: 250 µL of plasma was pre-treated by diluting 1:1 with various aqueous buffers and mixed thoroughly. Pre-treated sample (500 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. The final and streamlined protocols are detailed in ***Table 1.*** for extraction using the 10 mg plate format.

Table 1. *Extraction strategies.*

Step	Volume	Standard SPE methodology	Load-Wash-Elute methodology
Condition	500 µL	MeOH	-
Equilibration	500 µL	10 mM NH_4OAC	-
Sample load	500 µL	plasma 1:1 0.05% v/v formic acid	
Wash 1	500 µL	10 mM NH_4OAc	
Wash 2	500 µL	IPA	
Elution	200 µL	85/15 0.1% formic acid 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.

UPLC Conditons

Instrument: Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany)
Column: ACE Excel 2 C18-PFP 100 x 2.1 mm, 2µ (Hichrom ltd., Theale, UK)
Mobile phase: A, H_2O; B, MeOH: both mobile phases contained 7 µL of formic acid and 4 mg of ammonium format per L.
Flow rate: 0.4 mL/min
Gradient: Isocratic hold at 5% B for 1.2 min, step to 95% B, hold for 2 mins, resume initial conditions
Column temp: 40 °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
Norepinephrine	152.1 > 107.1	25	2	22	25
Dopamine	154.1 > 91.1	50	9	29	13

Results

Mass Spectrometer Optimization

Source parameters were optimized to enhance the production of dehydrated precursor ions to increase signal sensitivity of epinephrine and norepinephrine.

Figure 2. demonstrates at least a two-fold increase in signal 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.

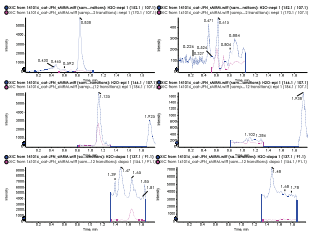

Figure 2. *XICs showing MRMs (blue: dehydrated precursors; purple: protonated molecular precursors).*

Chromatographic Optimization

Chromatographic separation was optimized by comparison of multiple eluent systems with low concentrations of either MeOH or MeCN. Target analyte peak shape and height were maximized using 0.25 mM formate buffer with small volume addition of formic acid. Increasing the concentration of buffer salts and formic acid to 0.1% was detrimental to chromatography and analyte sensitivity, as was use of acetate as a counter-ion. The C18-PFP column was finally selected based on its superior retention, separation and peak shape.

It was noticed that Dopamine peak shape was much improved when IPA was used as a second wash solvent. As a result this was additionally incorporated into the elution solvent and gave large improvements in the peak shape and ultimate assay sensitivity for all three components.

The LOQ could not be accurately quoted due to the absence of catecholamine free plasma. However, a comparison of control plasma containing endogenous levels and the same spiked at 20 pg/mL demonstrates method sensitivity (**Figure 3.**).

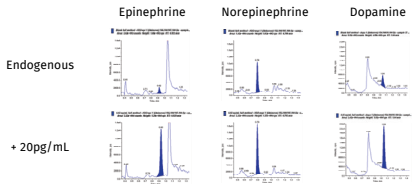

Figure 3. *Peak areas on equivalent scales from blank plasma and plasma spiked at 20 pg/mL.*

Extraction Optimization

Due to the small polar nature of the analytes there was concern about volatility during evaporation. Acidification or the addition of ethylene glycol did not eliminate evaporative losses and subsequent variability as demonstrated in **Figure 4.**

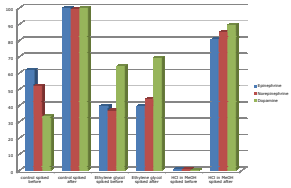

Figure 4. Comparison of evaporation losses with Catecholamines being spiked before and after evaporation with no modifiers (control), 4µL of ethylene glycol and 50 µL of 2% HCl in MeOH.

Sample pre-treatment optimization focused on control of pH below 8 to ensure the amine functional group remained charged whilst maintaining the neutrality of the hydroxyl groups. Additionally, pre-treatment should be at least 1 pH unit away from the approximate pK_a of the WCX polymer in order to maximize binding.

Several interference wash strategies were investigated, modifying aqueous interference washes with either buffer of organic modifier and modifying the nature and eluotropic strength of the organic interference wash. Increasing the buffer concentration showed no significant improvement in extraction recovery but demonstrated results with inferior repeatability (data not shown).

Due to analyte polarity it was possible to tailor the extraction solvent with a high proportion of aqueous, such that we could eliminate the evaporation step. The consistency of the elution solvent had to be such that an injection wouldn't give fronting or tailing on the LC. 85/15% 0.1% formic acid aq/IPA was optimized for the elution solvent.

The extraction was moved from the 30 mg to the 10 mg 96-well plate in order to minimize elution solvent volumes for direct injection. An optimized volume of 200 µL was used.

Additionally, the sorbent needed to be consistently dried across the plate before and following the addition of the eluent so positive pressure was used throughout (Pressure+ manifold, PPM-96).

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 and precision were not significantly affected, see **Table 3**.

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

Analyte	Standard		Load-Wash-Elute	
	Recovery	% RSD	Recovery	% RSD
Epinephrine	88.2	5.0	81.1	2.6
Norepinephrine	81.6	3.7	70.8	4.2
Dopamine	84.3	1.0	77.4	0.8

Calibration curves were constructed in human plasma from 20-1280 pg/mL. Both standard and Load-Wash-Elute procedures demonstrated linear recoveries and coefficients of determination greater than 0.997 as detailed in **Figures 5-6**.

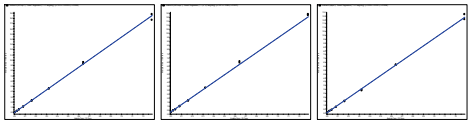

Figure 5. Calibration lines for epinephrine, norepinephrine and dopamine using the standard methodology.

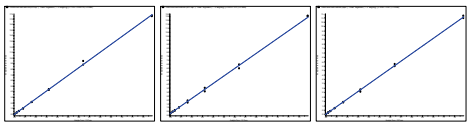

Figure 6. Calibration lines for epinephrine, norepinephrine and dopamine using the Load-Wash-Elute methodology.

Conclusions

- We demonstrate that EVOLUTE EXPRESS WCX 10mg 96 well plates can be used to extract polar catecholamines from pooled human plasma in a highly sensitive, linear assay.
- Good recoveries and excellent precision are demonstrated whether the EVOLUTE® EXPRESS WCX material is used in the standard SPE processing or modified Load-Wash-Elute protocols.
- A comparison of blank (endogenous) and spiked plasma samples demonstrate applicable sensitivity down to or below 20 pg/mL.
- A number of steps were optimized to improve sensitivity of the analytes. This included reducing the buffer strength in the mobile phase, reducing the buffer strength of washes, careful pH control throughout the extraction, avoiding an evaporation step and incorporating IPA in the reconstitution solvent.

This poster was presented at MSACL EU, Salzburg, 2015
Part Number: P137