Perfecting Pcats – Developing a More Robust UHPLC-MS/MS Method for Plasma Catecholamines

Adam Senior[1], Lee Williams[1], Stephanie Marin[2], Claire Desbrow[1], Elena Gairloch[2], Alan Edgington[1], Katie-Jo Teehan[1], Russell Parry[1], Charlotte Hayes[1], Helen Lodder[1], Paul Roberts[1]

[1]Biotage GB Limited, Distribution Way, Hengoed CF82 7TS, UK
[2]Biotage LLC, 10430 Harris Oaks Blvd., Suite C, Charlotte, North Carolina 28269, USA

Introduction

Achieving desired sensitivity of plasma catecholamines by LC-MS/MS can be problematic. Low LOQs can require a highly sensitive MS instrument. Evaporation of the extracted sample can result in losses during the evaporation step, making concentration of the sample prior to analysis difficult. Removal of matrix components that interfere with analysis or suppress MS response (particularly phospholipids) is necessary for maximum sensitivity and accuracy. This study was conducted to optimize sample preparation and LC-MS/MS conditions to improve recovery and reduce ion suppression for analysis of plasma catecholamines.

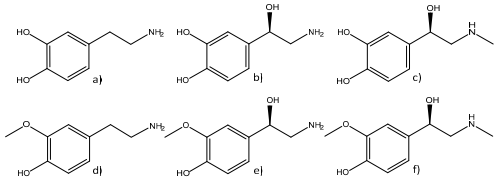

***Figure 1**. Structures: a) dopamine DA, b) norepinephrine NE, c) epinephrine EP, d) 3-methoxytyramine (3MT), e) normetanephrine NM, f) metanephrine ME*

Experimental

Reagents

Standards and reagents were purchased from Sigma-Aldrich Company ltd. (Gillingham UK). HPLC and LC/MS grade solvents were purchased from Rathburn Chemicals ltd (Walkerburn UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Milli-Q Direct-Q 5 water purifier (Merck Life Sciences, Gillingham UK). Plasma and serum was purchased from The Welsh Blood Service (Pontyclun UK), BioIVT (Burgess Hill UK), and Golden West Biologicals, Inc. (Temecula CA).

Sample Preparation

SPE Information

Extractions were performed using Biotage® EVOLUTE® EXPRESS WCX 10 or 30 mg 96 fixed well plate format polymer SPE (602-0010-PX01 or 602-0030-PX01).

All methods follow a typical SPE procedure incorporating additional interference wash steps (***Figure 2***).

***Figure 2.** Schematic of a Typical SPE Procedure*

Matrix Preparation

300 µL plasma was spiked with standards between 10 pg mL^{-1} and 1 ng mL^{-1} before dilution 1:1 with various buffers prior to extraction, investigating pH and components.

SPE Optimization

Wash and elution solvents were optimized. The final extraction protocol is detailed in ***Table 1***.

***Table 1**. Optimized 30 mg WCX Extraction Protocol*

Step	Volume / µL	Details
Condition	1000	MeOH
Equilibrate	1000	10 mM ammonium acetate pH 6.0 (aq)
Load	600	Pre-treated plasma: 1:1 10 mM sodium citrate pH 7
Wash 1	1000	10 mM ammonium acetate pH 6.0 (aq)
Wash 2	1000	80:20 MeOH:H_2O (v/v), dry 1 min
Wash 3	1000	DCM, dry 5 min
Elution	400	0.1% formic acid 15:85 IPA:H_2O (v/v)
Recon	100	0.1% formic acid 95:5 H_2O:MeOH (v/v)

LC/MS Conditions

Instrument: Nexera UHPLC (Shimadzu Europa GmbH, Duisburg Germany), 5500 Triple Quad MS (AB Sciex, Framingham USA)
Column: ACE Excel 1.7 C18-PFP (3.0 x 100 mm)
Mobile phase: A - 2 mM NH_4OOC 0.05% HCOOH / H_2O
B - 0.5 mM NH_4F / MeOH
Gradient: 2% B hold to 0.5 min; ramp to 30% at 3.0 min; ramp to 95% at 3.5 min, hold to 5.5 min; return to 2% at 6 min, hold to 9 min.
Inject volume: 10 µL **Flow rate:** 0.4 mL min^{-1} **Column temp:** 30 °C

Details of MS conditions and MRM transitions available on request.

Results

Chromatographic Separation

C18-PFP demonstrates enhanced selectivity with increased resolution compared to a wide selection of: C18, biphenyl and PFP phases. Some aqueous-compatible C18 phases demonstrate narrower peak width at the expense of a decrease in resolution (data not shown).

Using ammonium fluoride as a mobile phase modifier enhances DA response at the expense of increased NE and EP peak widths. We demonstrate the use of 2 mM ammonium formate with low concentration formic acid (0.05%) to counteract the effect of fluoride on early eluting catecholamines (***Figure 3b***). Formate / HCOOH only and fluoride only are in ***Figure 3a*** and ***3c*** respectively

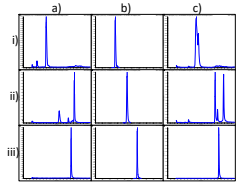

***Figure 3.** Comparison of Chromatography Eluent Modifiers: I) NE, ii) EP, iii) DA*

Evaporative Studies

Experiments comparing analyte recovery spiked post and pre evaporation demonstrate no significant evaporative losses (***Figure 4***).

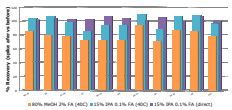

***Figure 4.** Evaporative Effects on Catecholamine Recovery (10 mg WCX)*

The gain in analyte sensitivity using post extraction concentration offsets minimal recovery losses due to analyte evaporation.

Sample Pretreatment Optimization

Previous work used 0.05% formic acid sample pretreatment to maintain the loading conditions around pH 6, > 2 units below the pK_a of biologically relevant catecholamines and maintain the WCX resin in its ionized form. Matrix robustness studies (***Table 2***) demonstrate increased precipitation using 0.05% HCOOH when the plasma anticoagulant has an antithrombin mode of action (heparin). The effect was less when using Ca^{2+} chelating anticoagulants e.g. citrate.

***Table 2**. Relative Precipitation with Varying Plasma Pretreatment*

Pretreatment Solution	Observations	Visual Scale (0 to 5, 0 = no ppt)
0.05% formic acid	cloudy suspension	4
50 mM ammonium acetate pH 6	faint suspension	3
50 mM ammonium formate pH 6	v. faint suspension	2
50 mM sodium citrate pH 6	no visible precipitate	0

Matrix precipitation has a detrimental effect on sample preparation, causing well blocking and low precision. We compared the effect of sample pretreatment solution on analyte recovery, ***Figure 5*** demonstrates dilute sodium citrate with neutral or weakly acidic pH maximises analyte recovery with minimal precipitation.

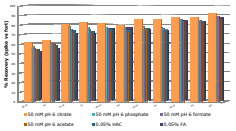

***Figure 5.** Catecholamine Recovery, Lithium Heparin 1:1 with Differing Pretreatment Solutions, 10 mg WCX*

Sample Extraction Optimization

Equilibration and aqueous wash steps are unchanged from previous work on plasma catecholamines. Organic wash optimization demonstrated a small proportion of water in wash 2 enhances catecholamine recovery while maintaining miscibility with following interference washes (***Figure 6***).

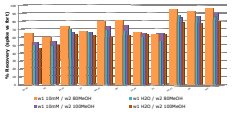

***Figure 6** Catecholamine Recovery, Sodium Heparin 1:1 with Wash 1 and Wash 2 Solvents, 10 mg WCX*

Mild elution conditions using 0.1% HCOOH in 15:85 IPA:H_2O (v/v) demonstrates good recovery (***Figure 7a***) whilst simultaneously maximising final extract cleanliness (***Figure 7b***).

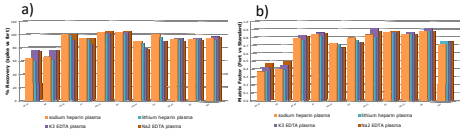

***Figure 7.** Catecholamine Recovery a) and Matrix Factor b), Heparin and EDTA Plasma, Final Extraction Protocol, 300 µL Matrix,30 mg WCX*

Observations continue to demonstrate using DCM as an interference wash enhances chromatographic method robustness through the removal of MS-silent species.

These conditions demonstrate maximum retention of phospholipids by the sorbent (***Figure 8b***). It is possible to increase analyte recovery using 2% HCOOH in 80:20 MeOH:H_2O (v/v) at the expense of higher phospholipid breakthrough (***Figure 8c***).

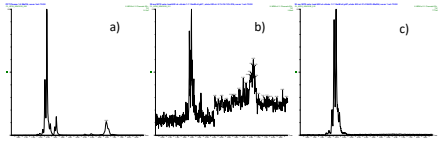

***Figure 8.** Phospholipid Interference Comparing a) Plasma 1:3 ACN, 3.16e7; b) Optimized Extraction Method, 1.64e4; c) 80% MeOH Elution, 7.77e5*

Method Performance

In the absence of catecholamine free plasma, calibration curves were constructed using universal negative serum (***Figure 9***).

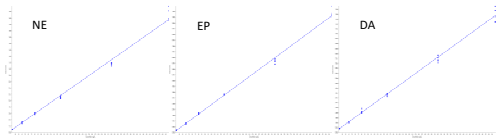

***Figure 9.** Catecholamine Calibration Curves Extracted from Universal Negative Serum (10 pg mL^{-1} to 500 pg mL^{-1}, 300 µL matrix load, WCX 30 mg)*

Method performance data is in ***Table 3***, S/N >10:1, precision <10% (15), accuracy 90-110% (80-120), criteria in parentheses are for the lowest calibration standards. Typical parameters are shown below.

***Table 3.** Method Performance Table (Final Method, 30 mg WCX)*

Matrix	Analyte	Coefficient, *r*	Range, pg mL^{-1}	LOQ, pg mL^{-1}
Sodium heparin	Norepinephrine	0.9979	40-500	40
	Epinephrine	0.9992	10-500	10
	Dopamine	0.9984	10-500	10
Lithium heparin	Norepinephrine	0.9988	40-500	40
	Epinephrine	0.9994	10-500	10
	Dopamine	0.9991	10-500	10
K_2EDTA	Norepinephrine	0.9981	40-500	40
	Epinephrine	0.9987	40-500	40
	Dopamine	0.9983	40-500	40
Na_2EDTA	Norepinephrine	0.9982	40-500	40
	Epinephrine	0.9992	10-500	10
	Dopamine	0.9996	10-500	10

Conclusions

- We demonstrate a SPE method that delivers clean final extracts for LC-MS/MS analysis using mixed-mode weak cation exchange.
- This assay is linear over three orders magnitude and demonstrates LOQ at 10 pg mL^{-1} for clinically relevant analytes.
- Elution without evaporation is feasible with low volume injection. A final evaporation step with minimal losses permits larger injections of more concentrated samples for maximum sensitivity.

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