Effective Strategies for Phospholipid Removal using Polymer-based Solid Phase Extraction (SPE) and LC-MS/MS Analysis

Elena Gairloch[2], Lee Williams[1], Rhys Jones[1], Adam Senior[1], Helen Lodder[1], Geoff Davies[1], Alan Edgington[1], Steve Jordan[1], Claire Desbrow[1], Victor Vandell[2] and Frank Kero[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

Endogenous phospholipids present in biological fluids are a major problem in LC-MS/MS analysis. Due to their strong retention characteristics in reversed phase chromatography, phospholipids tend not to elute as discrete peaks and are often very difficult to separate from analytes of interest. This co-elution often leads to suppression or enhancement in the chromatogram which in turn cause quantitation issues. This poster evaluates the use of polymer-based solid phase extraction (SPE) sorbents, incorporating hydrophobic and various mixed-mode retention mechanisms to address the problems associated with phospholipid removal.

Experimental Procedure

Reagents

Formic acid, acetic acid, ammonium acetate and ammonium hydroxide were purchased from Sigma Chemical Co. (Poole, UK). Blank plasma was obtained from the Welsh Blood Service (Pontyclun, UK). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

Sample Preparation

All extractions were performed in the 25 mg 96-well format processing 100 µL of plasma (n=4) using the documented SPE procedures. All sample pre-treatment was 1:3 plasma:buffer loading 400 µL, while wash and elution volumes were 1 mL unless otherwise stated.

Table 1. *EVOLUTE® ABN SPE protocols*

	*1 EVOLUTE ABN	Modifications
Conditioning	MeOH	
Equilibration	0.1% formic acid (aq)	
Sample Load	plasma:1% Formic acid	
Interference Wash	95:5 (v:v) H_2O/MeOH	*2 0-40% MeOH or MeCN
Analyte Elution	500 µL MeOH	*3 0-50% H_2O in MeOH or MeCN

Table 2. *EVOLUTE® CX SPE protocols*

	*1 EVOLUTE CX	Mods	*4 2% FA Method	Mods
Conditioning	MeOH			
Equilibration	50mM NH_4OAc pH6		2% formic acid (aq)	
Sample Load	plasma:50mM NH_4OAc pH6	*2 plasma:2% formic acid	plasma:2% formic acid	
Interference Wash 1	50mM NH_4OAc pH6		2% formic acid (aq)	
Interference Wash 2	MeOH	*3 **Wash 2**: 2% formic acid **Wash 3**: MeOH	MeOH	
Analyte Elution	5% NH_4OH/ MeOH		5% NH_4OH/ MeOH	*5 5% NH_4OH/ MeCN

Table 3. *EVOLUTE® WAX SPE protocols*

	*1 EVOLUTE WAX	*2 pH6 Method	*3 pH7 Method
Conditioning	MeOH	MeOH	MeOH
Equilibration	H_2O	50mM NH_4OAc pH6	50mM NH_4OAc pH7
Sample Load	plasma:2% formic acid	plasma:50mM NH_4OAc pH6	plasma:50mM NH_4OAc pH7
Interference Wash 1	2% formic acid (aq)	50mM NH_4OAc pH6	50mM NH_4OAc pH7
Interference Wash 2	MeOH	MeOH	MeOH
Analyte Elution	5% NH_4OH/MeOH	5% NH_4OH/MeOH	5% NH_4OH/MeOH

Table 4. *EVOLUTE® AX SPE protocols*

	*1 EVOLUTE AX	Mods	*3 5% NH_4OH Method
Conditioning	MeOH		MeOH
Equilibration	H_2O		5% NH_4OH (aq)
Sample Load	plasma:50mM NH_4OAc pH7	*2 plasma:2% formic acid	plasma:5% NH_4OH
Interference Wash 1	95:5 50mM NH_4OAc pH7/MeOH		5% NH_4OH (aq)
Interference Wash 2	MeOH		MeOH
Analyte Elution	2% formic acid/MeOH		2% formic acid/MeOH

Table 5. *EVOLUTE® WCX SPE protocols*

	*1 EVOLUTE WCX	*2 5% NH_4OH Method
Conditioning	MeOH	MeOH
Equilibration	50mM NH_4OAc pH7	5% NH_4OH (aq)
Sample Load	plasma:50mM NH_4OAc pH7	plasma:5% NH_4OH
Interference Wash 1	50mM NH_4OAc pH7	5% NH_4OH (aq)
Interference Wash 2	MeOH	MeOH
Analyte Elution	2% formic acid/MeOH	2% formic acid/MeOH

Post Extraction: The extracts were evaporated to dryness at 40 °C and reconstituted in 1 mL of 70:30 H_2O:MeOH (v/v).

HPLC Conditons

Instrument: Waters 2795 Liquid Handling System (Waters Assoc., Milford, MA, USA).
Column: Luna Phenyl-Hexyl 5 µm analytical column (50 x 2.0 mm id) (Phenomenex, Cheshire UK).
Mobile Phase: 0.1% formic acid aq and 0.1% formic acid/MeCN at a flow rate of 0.3 mL/min.
Gradient: The gradient conditions were set to 60%, 0.1% (v/v) formic acid aq and 40% MeCN increasing to 100% MeCN over 6 minutes. The high organic mobile phase was held for 3 minutes and initial starting conditions resumed at 9.1 minutes.
Injection Volume: 5 µL
Temperature: Ambient

Mass Spectrometry

Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the MRM mode using the 184 Da product ion.

Previous phospholipid experiments (full scan, SIR and precursor ion scanning) identified the most abundant phospholipid ions subsequently used in these MRM experiments.
Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.7 x 10^{-3} mbar
Collision Energy: 16 eV

Results

Figures 1 and 2. demonstrate the amount of phospholipid removal comparing various wash and elution protocols using EVOLUTE® ABN.

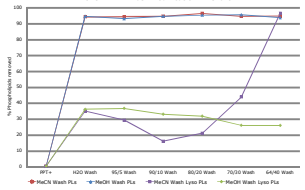

Figure 1*. Line graph comparing various wash protocols for lyso PL and PL removal using EVOLUTE® ABN.*

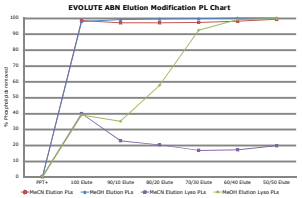

Figure 2. *Line graph comparing various elution protocols for lyso PL and PL removal using EVOLUTE® ABN.*

Figures 3-6. demonstrate PL removal using various protocols on each of the mixed-mode ion exchange SPE polymers. In all cases the generic methods have been used, alongside typical modifications encountered. Both the MeOH interference wash step and the elution step are shown for each method.

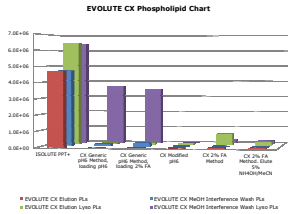

Figure 3*. Total PL levels comparing ISOLUTE® PPT+ and various protocols using EVOLUTE® CX.*

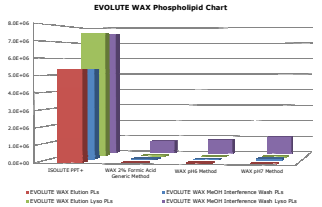

Figure 4*. Total PL levels comparing ISOLUTE® PPT+ and various protocols using EVOLUTE® WAX.*

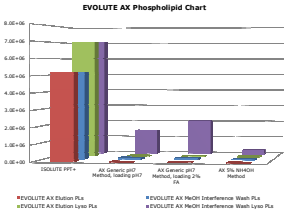

Figure 5*. Total PL levels comparing ISOLUTE® PPT+ and various protocols using EVOLUTE® AX.*

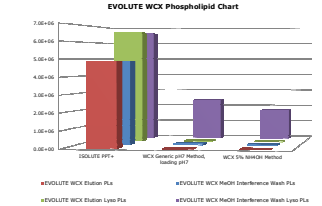

Figure 6*. Total PL levels comparing ISOLUTE® PPT+ and various protocols using EVOLUTE® WCX.*

Conclusions

- EVOLUTE® ABN as with all hydrophobic polymers suffers from retention of lyso PLs. Modification of the generic method from a wash and elution standpoint results in substantially lower levels of lyso PLs in the final extract. 40% MeCN in the wash solvent or 30% H_2O in MeOH as the elution solvent demonstrates > 90% PL removal.
- EVOLUTE® CX shows good removal of larger molecular weight PL using all protocols. However, slightly higher levels of lyso PLs are observed when using a 2% formic acid method.
- All EVOLUTE® mixed mode ion exchange SPE polymers show excellent removal of PLs using a variety of protocols.

Biotage