Strategies for Phospholipid Removal using Polymer-based SPE

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Introduction

Sample preparation is an essential technique prior to LC-MS/MS analysis of drugs in biological fluid samples as interfering matrix components can mask or interfere with the quantitation of the compound(s) of interest. Generic polymer-based SPE products are extensively used for the extraction of drugs from biological fluids. However, the non-selective nature of these polymers, while advantageous in enabling the simultaneous extraction of a wide range of drug types, can lead to the co-extraction of high levels of unwanted endogenous sample components such as proteins, salts and phospholipids. The optimized surface chemistry of EVOLUTE™ ABN minimizes the amount of co-extracted matrix components giving lower ion suppression as shown in previous work[1] and almost entirely eliminates problems associated with protein retention as shown by the gel electrophoresis image in **Figure 1**. However, as with all generic polymer-based SPE sorbents the retentive nature of phospholipids (outline structures shown in **Figure 2**) is an entirely different challenge. This poster demonstrates the use of a polymer-based SPE sorbent, EVOLUTE ABN, and investigates strategies for the removal of phospholipids through various washing and elution protocols. Where compounds are sufficiently retained on the sorbent, increasing the strength of the wash solvent can significantly reduce the phospholipid content of the extract without affecting recoveries.

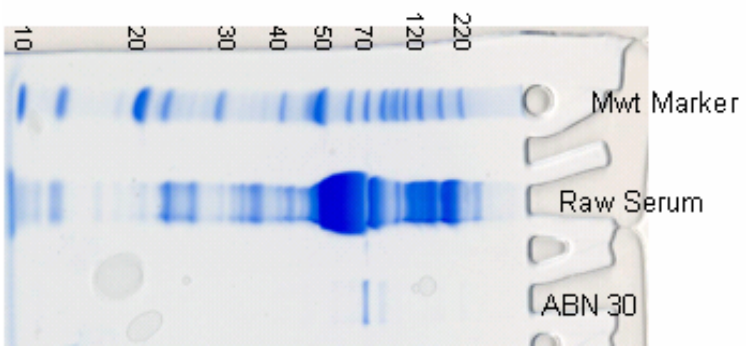

Figure 1. Gel electrophoresis image showing protein removal using the EVOLUTE™ ABN generic method

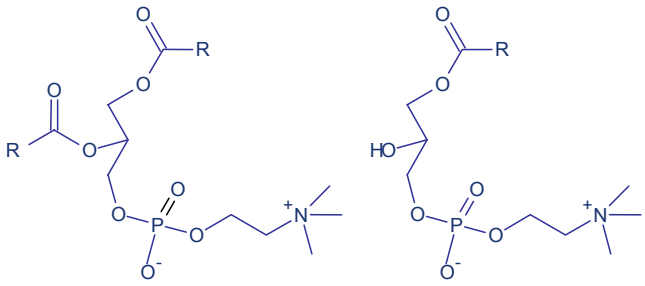

Figure 2. Phospholipid structures

Experimental Procedure

Reagents

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

Solid Phase Extraction Procedure

EVOLUTE™ ABN Generic SPE Method

SPE plate: EVOLUTE ABN 25 mg 96-well plate
Sample Pre-treatment: Plasma sample diluted 1:3 (v/v) with aqueous formic acid (1 %, v/v)
Conditioning: Methanol (1 mL)
Equilibration: Aqueous formic acid (0.1%, v/v, 1 mL)
Sample Load: Sample loaded (400 µL diluted plasma)
Interference Wash: Water/methanol (95:5, v/v, 1 mL)
Elution: Methanol (500 µL)
Post Extraction: The eluate was evaporated to dryness and reconstituted in 0.5 mL of 70:30 (v/v) H_2O/MeOH prior to analysis

Experiment 1: Interference Wash Investigation

Interference Wash: Ranging from 100% Water to Water/Methanol or Water/Acetonitrile combinations up to 60:40, (v/v, 1 mL)
Elution: Either Methanol or Acetonitrile (500 µL)

Experiment 2: Elution Solvent Investigation

Interference Wash: Water/methanol (95:5, v/v, 1 mL)
Elution: Ranging from 100% Methanol or Acetonitrile to Methanol/Water or Acetonitrile/Water combinations up to 60/40 (v/v, 500 µL)

HPLC Conditons

Instrument: Waters 2795 Liquid Handling System (Waters Assoc., MA, USA)
Column: Zorbax Eclipse XDB C18 3.5 µm analytical column (50 x 2.1 mm id) (Agilent Technologies, Berkshire, UK).
Guard Column: C8 guard column (Agilent Technologies, Berkshire, UK)
Mobile Phase: 0.1% formic acid (aq) and MeCN at a flow rate of 0.25 mL/min
Gradient: 70%, 0.1% formic acid (aq) and 30% MeCN increasing to 90% MeCN over 6 minutes. The high organic mobile phase was held for a further 2 minutes then returned to the initial starting conditions
Injection Volume: 10 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 selected ion recording mode (SIR)
Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C

Table 1, page 3 shows the phospholipid ions monitored and the respective mass spectrometer parameters.

Table 1. Quattro Ultima Pt mass spectrometer parameters.

	SIR Mass	Dwell Time (s)	Cone Voltage (V)
Phospholipids	496	0.1	55
	520	0.1	55
	522	0.1	55
	524	0.1	55
	760	0.1	55
	786	0.1	55
	806	0.1	55

Results

Figure 3 shows the TIC for all phospholipids monitored and the corresponding post column infusion analyte trace after protein precipitation. The top trace shows the retention of the phospholipids while the bottom trace illustrates areas of suppression directly associated with the phospholipid elution. The protein precipitation trace was chosen as it represents the worst case scenario of all sample preparation products used for phospholipid cleanup.

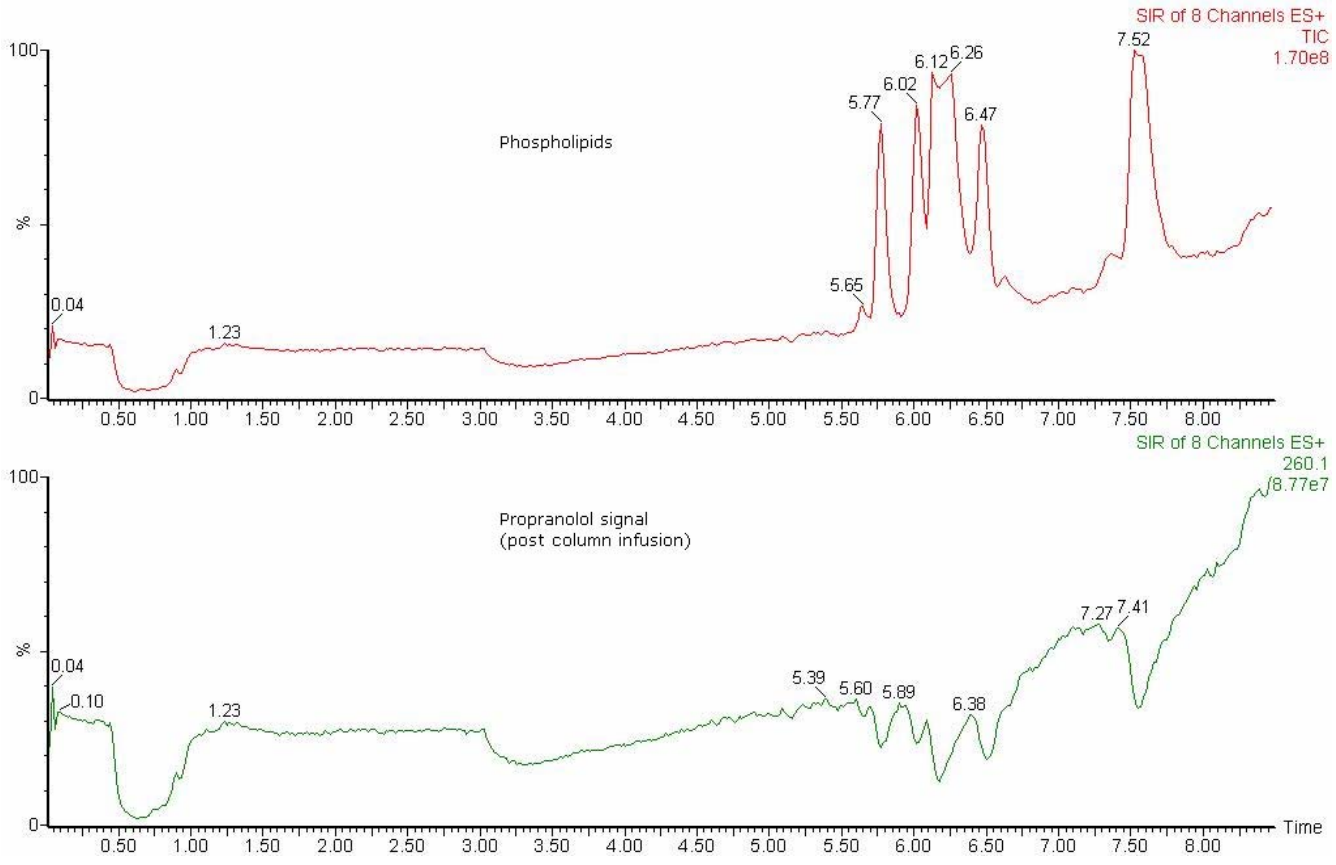

Figure 3. TIC for selected phospholipid ions, with corresponding trace for propranolol (post column infusion, m/z 260.1). Sample: human plasma prepared using protein precipitation (ISOLUTE PPT+)

Experiment 1 Results: Interference Wash Investigation

The interference wash solvent combinations used in this study were; 100% H_2O, 95:5, 90:10, 80:20, 70:30 and 60:40 H_2O/organic (v/v). Methanol and ACN were evaluated. The two washing protocols were employed followed by elution with either MeOH or ACN, effectively giving four combinations. **Figure 4** shows the peak areas of the phospholipids in the final elution using the various washing protocols. When using MeOH combinations in the interference wash, even at 40% there is no reduction in the phospholipid levels in the elution. This trend is the same when using MeOH or ACN as the elution solvent. When replacing MeOH in the interference wash step with ACN at 40%, phospholipid levels are reduced by greater than 50%. The elution solvent doesn't have any effect here as both MeOH and ACN give almost identical results.

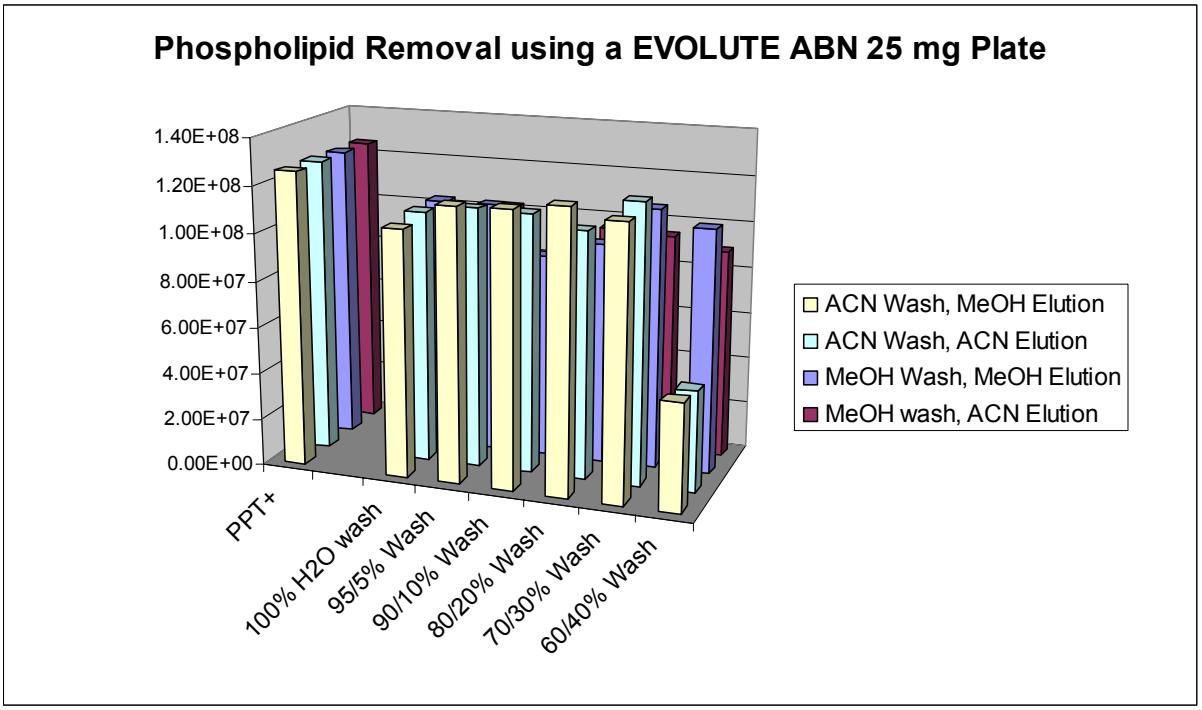

Figure 4. Chart showing phospholipid peak areas with various washing protocols and elution solvent combinations compared to protein precipitation using ISOLUTE PPT+

Experiment 2 Results : Elution Solvent Investigation

The EVOLUTE ABN generic method was followed in this experiment until the elution step. The elution solvents used in this study were; 100% organic, 90:10, 80:20, 70:30 and 60:40 organic/H_2O. The organic solvent used was either MeOH or ACN. **Figure 5, page 5** shows the peak areas of the phospholipids in the final elution using the various combinations above. When using MeOH elution combinations no reduction in phospholipid content are observed until 80:20 MeOH/H_2O is used. Far lower phospholipid levels are seen when moving to 70:30 and 60:40 MeOH/H_2O. When using ACN elution combinations, no reduction in phospholipid levels are observed until 70:30 ACN/H_2O. Both the 70:30 and 60:40 ACN/H_2O give very low phospholipid levels in the final extracts. At 100% organic to 80:20 organic/H_2O combination the ACN elutions give higher phospholipid levels than the corresponding MeOH elutions.

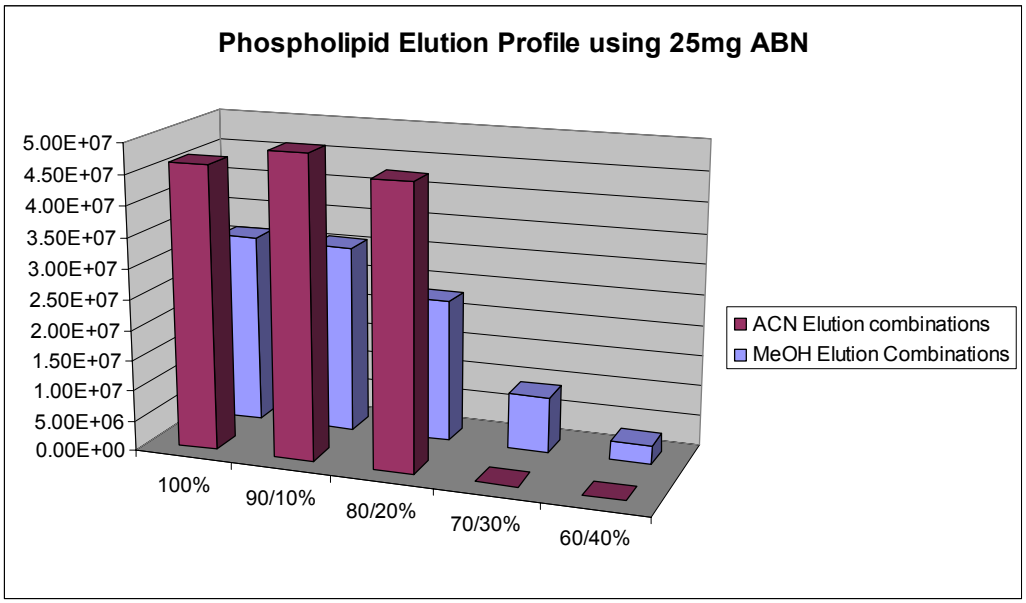

Figure 5. Chart showing phospholipid peak areas with various elution solvent combinations

Overall Conclusions

1. Using an interference wash of 60:40 H_2O/ACN gives greater than 50% reduction in phospholipid levels in the final extract.
2. No reduction in phospholipid levels was observed using an interference wash of H_20/MeOH up to 60:40.
3. Modifying the elution combinations to incorporate H_2O above 20% yielded the greatest phospholipid reductions.
4. MeOH/H_2O combinations in the elution solvent started to show lower levels of phospholipids above 20% H_2O.
5. ACN/H_2O combinations with 30-40% H_2O in the elution solvent gave lowest levels of phospholipids.
6. Overall, modification of the generic method, depending on analyte properties can give significant removal of phospholipids and result in cleaner extracts and more reliable quantitation.

References:

1. A New Polymer-based SPE Sorbent to Reduce Matrix Effects in Bioanalytical LC-MS/MS Presented at ASMS 2005. M.Cleeve *et al*

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