A study of the impact of sample preparation techniques on matrix effects in LC-MS/MS

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Introduction

Sample preparation is essential prior to the LC-MS analysis of drugs in biological fluid samples, as matrix components can mask or otherwise interfere with the analysis of the compound(s) of interest. Typical biological fluid samples can contain both endogenous material (e.g. proteins, phospholipids) and exogenous compounds (e.g. dose vehicles such as PEG 400 or Tween 80), all of which can cause significant ion suppression or enhancement.

Generic sample preparation techniques including protein precipitation, supported liquid extraction and generic SPE, which can be applied to a wide range of analytes without extensive method development, are widely used in bioanalytical sample preparation. However, the degree of sample clean-up provided by each technique can vary significantly. For each assay, the technique selected is based upon key criteria including assay sensitivity requirements, matrix effects, ease of use, compatibility with high throughput analysis as well as cost.

This presentation aims to compare the effect of different sample preparation techniques (as listed below) on their ability to remove endogenous phospholipids (phosphatidylcholine and lysophosphatidylcholine) which cause ion suppression:

- 'Solvent first' Protein precipitation
- Supported liquid extraction (SLE)
- Non-polar SPE (polymer-based sorbent)
- Non-polar SPE (silica-based sorbent)
- Mixed-mode SPE (silica-based sorbent)

Experimental Procedure

Human plasma samples prepared using each of the sample preparation techniques listed were analyzed by LC-MS. Selected phospholipid ions were monitored in order to investigate the amount of phospholipid remaining in the sample after clean-up, and hence evaluate the effectiveness of each technique in removal of phospholipids from plasma samples.

Post-column infusion using propranolol was used to identify the areas of the chromatogram most affected by matrix effects due to the phospholipids.

Sample Preparation

100 µL of pooled human plasma was extracted using standard methodology for each technique (details below). Following sample preparation, the extracts were evaporated to dryness, and reconstituted in appropriate mobile phase for subsequent analysis. All sample preparation procedures were carried out using 96-well format sample preparation plates.

Biotage

a) Protein Precipitation
ISOLUTE® Array PPT+ (p/n 120-2040-RP)
1. Add 300 µL acetonitrile to each well.
2. Add 100 µL plasma to each well.
3. Allow to stand for 2 minutes.
4. Apply vacuum at -20 "Hg and collect filtrate.

b) Supported Liquid Extraction (SLE)
ISOLUTE SLE+ 200mg (p/n 820-0200-P01)
1. Mix 100 µL of plasma with 100 µL H2O and apply to each well.
2. Apply a short pulse of vacuum to initiate flow.
3. Allow to stand for 5 minutes, and dry with a short pulse of vacuum.
4. Apply MTBE (1 mL) and elute under gravity for 5 minutes. Apply vacuum (2 minutes) to completely remove extraction solvent.

c) Non-polar SPE (silica based sorbent)
ISOLUTE Array C2, C8, C18 25 mg/1 mL (320-0025-RP, 290-0025-RP 220-0025-RP respectively)
1. Condition each well with methanol (1 mL).
2. Equilibrate with ammonium acetate buffer (0.05 M, pH 6, 250 µL).
3. Load plasma sample (100 µL diluted 1:1 (v/v) with ammonium acetate buffer (0.05 M, pH 6)).
4. Wash with buffer:methanol (95:5, v/v, 250 µL).
5. Elute with methanol / 1M ammonium acetate 99.5:0.5 v:v (250 µL).

d) Non-polar SPE (polymer-based sorbent)
EVOLUTE™ ABN Array 25 mg/1 mL (p/n 600-0025-RP)
1. Condition each well with methanol (1 mL).
2. Equilibrate with 0.1 % formic acid (1 mL).
3. Load plasma sample (100 µL diluted 1:3 (v/v) with 1% formic acid).
4. Wash with water:methanol (95:5, v/v, 1 mL).
5. Elute with methanol (500 µL).

e) Mixed-mode SPE (silica-based sorbent)
ISOLUTE Array HCX 25 mg/1 mL (p/n 902-0025-RP)
1. Condition each well with methanol (1 mL) .
2. Equilibrate with ammonium acetate buffer (0.05 M, pH 6, 250 µL).
3. Load plasma sample: (100 µL diluted 1:1 (v/v) with ammonium acetate buffer (0.05 M, pH 6)).
4. Wash with:
(i) ammonium acetate buffer (0.05 M, pH 6, 250 µL),
(ii) acetic acid (1 M, 250 µL), dry sorbent with 30sec pulse of vacuum,
(iii) methanol (250 µL).
5. Elute with methanol:NH4OH (95:5, v/v, 2 x 100 µL).

HPLC Conditions
Instrument: Waters 2795 Separations module.
Column: Zorbax Eclipse XDB-C18. (50 x 2.1mm, 3.5 µL), Agilent
Guard Column: Zorbax Eclipse XDB-C8. (12.5 x 2.1, 5 µL)
Injection volume: 10 µL. The entire column effluent was directed into the MS.

Table 1: Gradient

Time	A = 0.1% Formic in water	B = acetonitrile	Flow rate mL/min
0	70	30	0.25
6	10	90	0.25
8	10	90	0.25
8.1	70	30	0.25

MS Conditions
Instrument: Waters Quattro Ultima Pt triple quadrupole MS equipped with an electrospray source.
Source Temp: 100°C
Desolvation Temp: 350°C
Post column infusion: analyte (propranolol) concentration of 1 ng/µL, infused at a rate of 5 µL/min.

Table 2 : SIR Conditions

Analyte	SIR Mass	Dwell Time (s)	Cone Voltage (V)
Propranolol (PCI Analyte)	260.1	0.1	55
Phospholipid ions	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

(i) Matrix effects due to phospholipids

The phospholipids investigated in this presentation were found to have a significant suppression effect on propranolol. This is most apparent in the 5.5–8.0 minute range, shown on the mass chromatograms below (**Figure 1**). The phospholipid peaks in this area are shown on the TIC trace (top, consisting of the ions listed in **Table 2**). The mass chromatogram for propranolol (bottom, m/z 260.1) shows significant suppression correlating to the retention times observed for the phospholipid peaks.

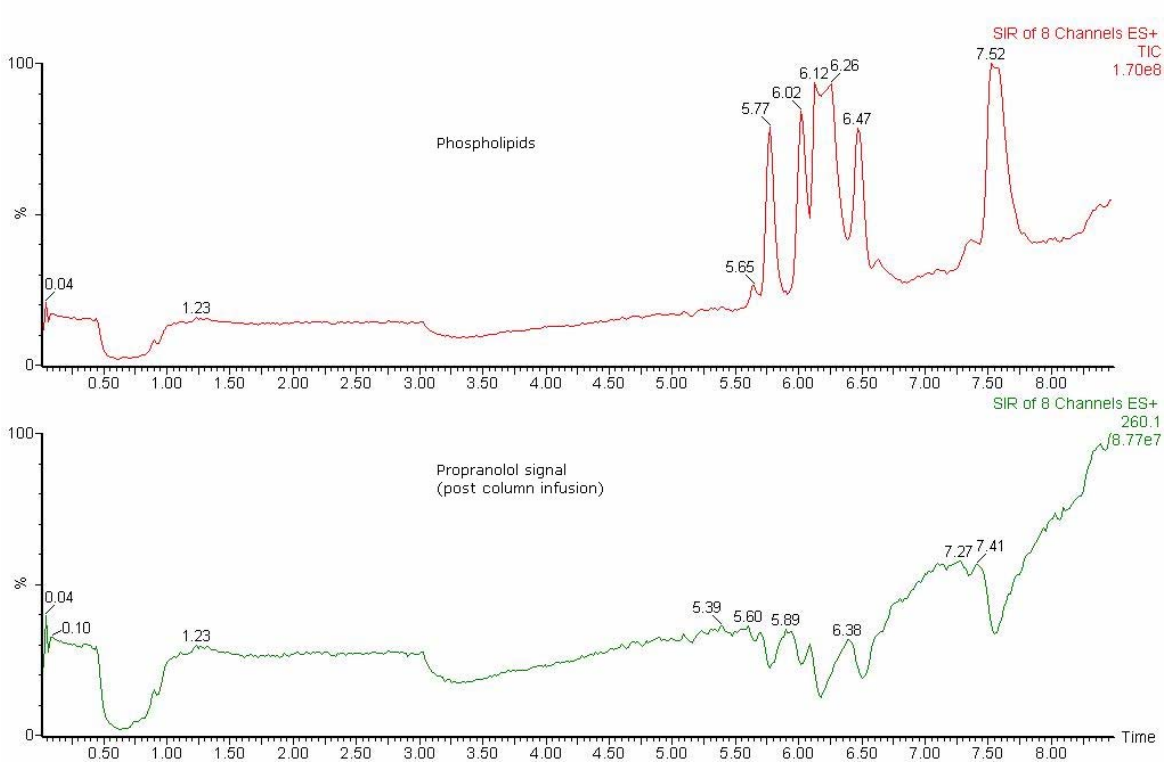

Figure 1. TIC for selected phospholipid ions, with corresponding trace for propranolol (post column infusion, m/z 260.1). Sample: human plasma prepared using protein precipitation (ISOLUTE PPT+) as described above.

(ii) Comparison of effectiveness of different sample preparation techniques in removal of phospholipids from human plasma samples

Figure 2 shows the TIC for residual phospholipids in human plasma samples prepared using each of the sample preparation techniques listed. Significant variation in the effectiveness of the different techniques for removal of phospholipids can be observed. This shown graphically in **Figure 3**.

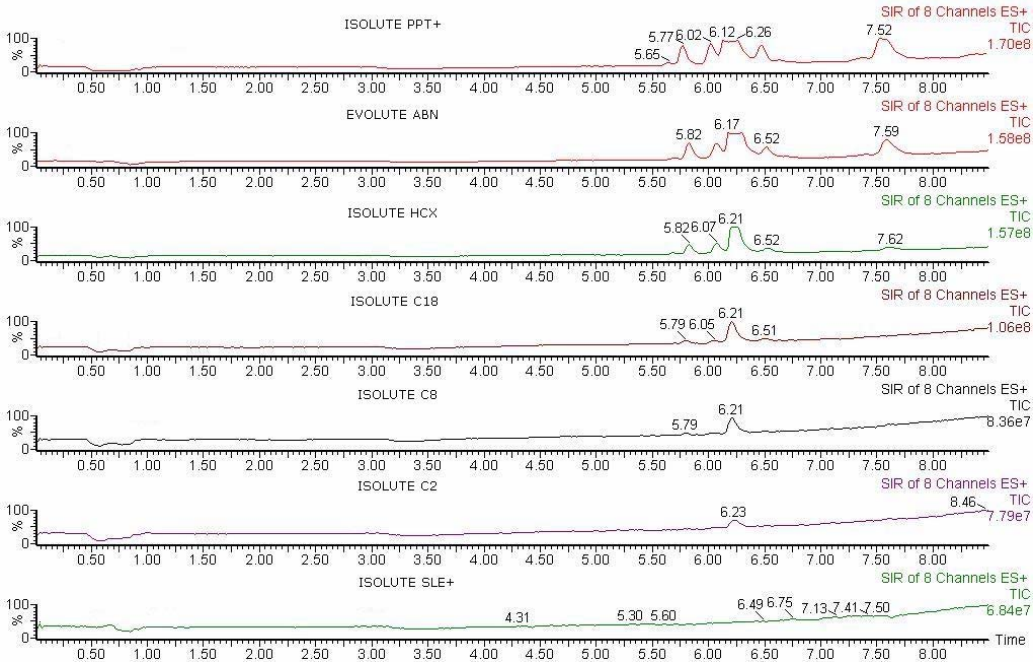

Figure 2. TIC of residual selected phospholipid ions in human plasma prepared using the various sample preparation techniques

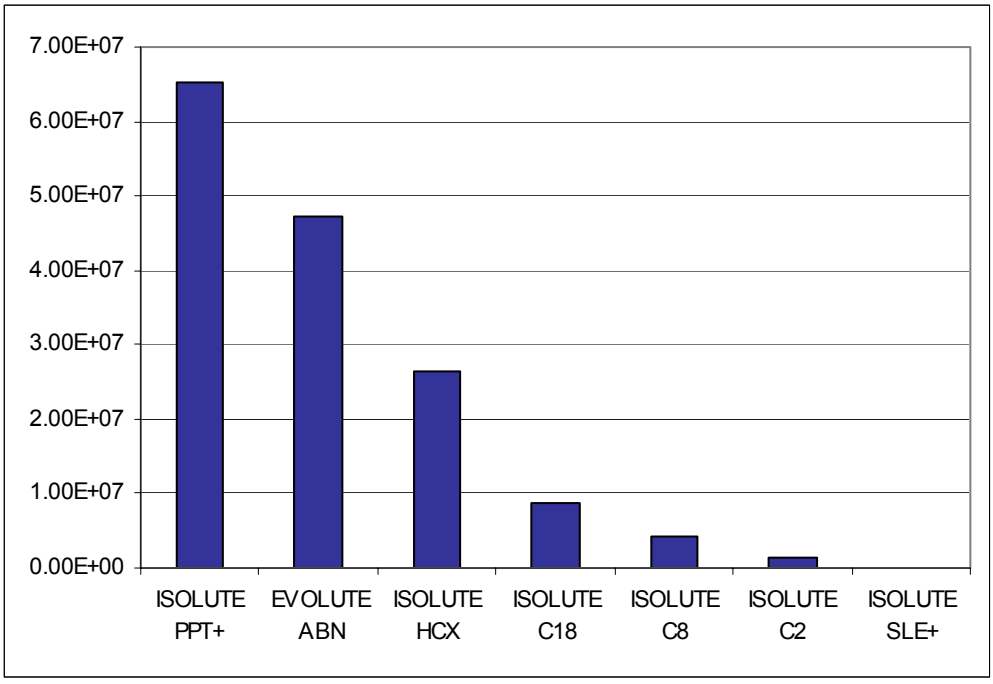

Figure 3. Area count (TIC, 5.5-8.0 minutes) for residual selected phospholipid ions in human plasma prepared using the various sample preparation techniques

Discussion

1. ISOLUTE PPT+ was least effective in removal of phospholipids, as this technique is based on protein removal only.
2. ISOLUTE SLE+ gave the cleanest extract (most effective phospholipid removal). This probably due to the low solubility of phospholipids in the extraction solvent (MTBE) used.
3. Effectiveness of phospholipid removal using non-polar SPE was inversely proportional to the hydrophobic character of the sorbent. Thus EVOLUTE ABN extracts (polymer based sorbent with high hydrophobic character) contained relatively high amounts of phospholipids, whereas ISOLUTE C2, with very low hydrophobic character, gave very clean extracts.
4. ISOLUTE HCX, a mixed-mode sorbent, gave relatively high phospholipid content, despite the rigorous interference elution regime possible. This is believed to be due to interactions of the zwiterionic phospholipids with both the non-polar and cation exchange functional groups. Further work will investigate this result.

Overall Conclusions

Sample preparation techniques can be ranked as follows for their effectiveness in removal of phospholipids from human plasma samples (from most effective to least effective).

- Supported liquid extraction
  - ISOLUTE SLE+
- Non-polar SPE (silica)
  - ISOLUTE C2
  - ISOLUTE C8
  - ISOLUTE C18
- Mixed-mode SPE
  - ISOLUTE HCX
- Non-polar SPE (polymer)
  - EVOLUTE ABN
- Protein precipitation
  - ISOLUTE PPT+

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