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Increase Bioanalytical Assay Speed via Phospholipid Removal using HybridSPE™-Precipitation Technology

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Introduction

One of the primary concerns when analyzing small molecules in biological samples via LC-MS/MS is ion-suppression. Ion-suppression is caused by one or more interfering components or species that co-elute with analyte(s) of interest during LC-MS analysis and manifests itself as loss of analyte response. These co-eluting species can affect droplet formation or ionize concurrently resulting in an erroneous decrease (suppression) in signal response. As a result, the phenomenon often leads to poor assay reproducibility, accuracy, and sensitivity. Such deleterious effects are often most notable at the lower limits of quantitation (LLOQ) (1).

Ion-suppression can be caused by a variety of agents including phthalates and plasticizers from plastic ware; common buffers and salts such as TFA; stabilizers (glycerol) and dosing agents (PEG); and plasma anti-coagulants such as lithium-heparin and sodium citrate. One of the principle causes of ion-suppression in bioanalysis is the presence of serum albumin and phospholipids during LC-MS/MS analysis in the positive ion electrospray mode (+ESI). Phospholipids are present in biological plasma at extremely high concentrations (~1 mg/mL) and can suppress analyte response if not removed during sample prep and/or resolved during chromatography (2-4).

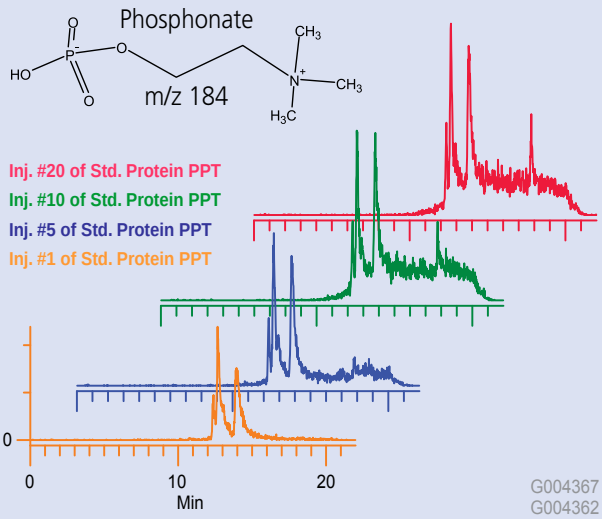
In general, ion-suppression typically occurs during the early and late regions of analysis during reversed-phase chromatography. It is presumed that late elution ion-suppression species are predominately caused by phospholipid contamination (4). As researchers strive to reduce analytical run time (< 5 min.) using ballistic gradients and UPLC conditions, the risk of ion-suppression increases considerably (5). At such ballistic gradients and fast run times, phospholipid contaminants can accumulate on the column and potentially elute uncontrollably during a given run sequence. In Figure 1, blank rat plasma was subjected to protein precipitation by combining rat plasma with 1% formic acid in acetonitrile (1:3, v.v) followed by centrifugation. The resulting supernatant was analyzed by LC-MS using a sub 2 µm C18 column monitoring for m/z 184 (the phosphonate moiety or polar head group of phosphatidylcholine). From the chromatogram, we see that there is a steady accumulation of phospholipids on the column with each successive injection.

Figure 1. Accumulation of Phospholipids on LC Column after Standard Protein PPT of Plasma

column: C18 column, 5 cm x 2.1 mm I.D., 1.8 µm
mobile phase: (A) 13 mM ammonium acetate in water
(B) 13 mM ammonium acetate in acetonitrile

gradient:	Min.	%A	%B
	0	95	5
	5	50	50
	8	50	50
	8.1	95	5
	15	95	5

temp.: 35 °C
flow: 0.2 mL/min.
inj.: 10 µL
det.: MS/MS



In Reporter 26.3, we introduced HybridSPE-Precipitation (HybridSPE-PPT) technology for targeted removal of phospholipids and precipitated proteins for subsequent LC-MS analysis in which we compared its performance against traditional protein precipitation and SPE for the extraction of clenbuterol and clonidine from plasma. In this report we describe how, by removing phospholipids via HybridSPE-PPT, researchers can reduce ion-suppression, prep samples using a 2-3 step procedure, and reduce analytical run time while implementing isocratic conditions.

How does HybridSPE-PPT work?

HybridSPE – PPT technology is a simple and generic sample prep platform designed for the gross level removal of endogenous protein and phospholipid interferences from biological plasma and serum prior to LC-MS or LC-MS/MS analysis. Biological plasma or serum is first subjected to protein precipitation via the addition and mixing of acidified (with formic acid) acetonitrile. Precipitated proteins are then removed by centrifugation and the resulting supernatant is loaded on the HybridSPE-PPT

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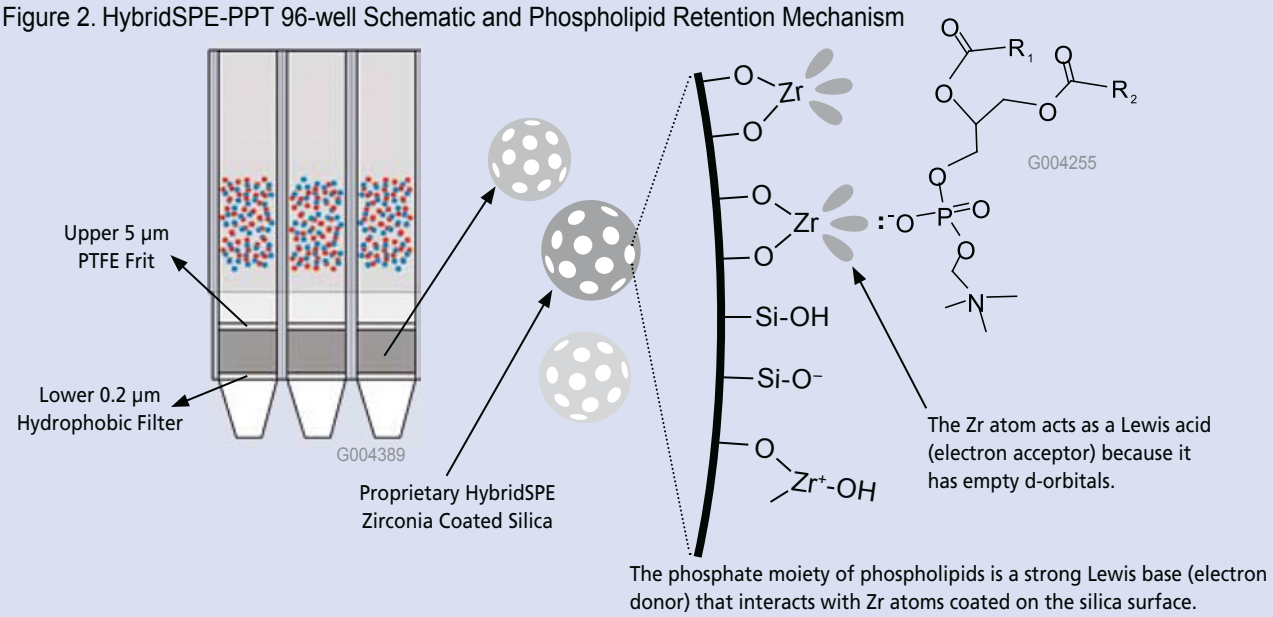
Figure 2. HybridSPE-PPT 96-well Schematic and Phospholipid Retention Mechanism



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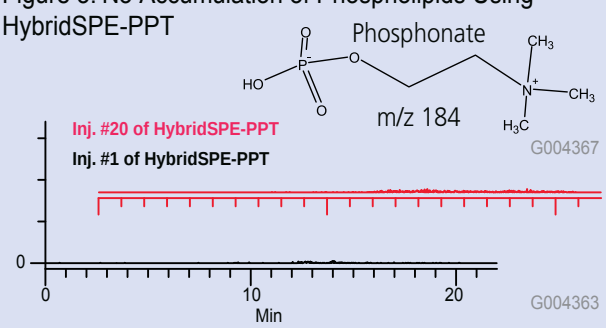
cartridge that acts as a chemical filter that specifically targets the removal of endogenous sample phospholipids.

The HybridSPE-PPT 96-well version contains a series of low porosity hydrophobic filters/frits, the packed-bed filter/frit assembly acts as a depth filter facilitating the concurrent removal of both phospholipids and precipitated proteins during the extraction process. As a result, plasma can be first added to the well plate (upper PTFE frit keeps plasma from dripping through prematurely) followed by acidified acetonitrile (precipitating agent). After a brief mixing/vortexing step, vaccum is applied to the HybridSPE-PPT plate and the resulting filtrate / eluate can be analyzed directly. The phospholipid retention mechanism is based on a highly selective Lewis acid-base interaction between the proprietary zirconia ions functionally bonded to the HybridSPE stationary phase and the phosphonate moiety consistent with all phospholipids. The resulting eluent is ready for immediate LC-MS or LC-MS/MS analysis. Figure 2 visually depicts the HybridSPE-PPT 96-well plate.

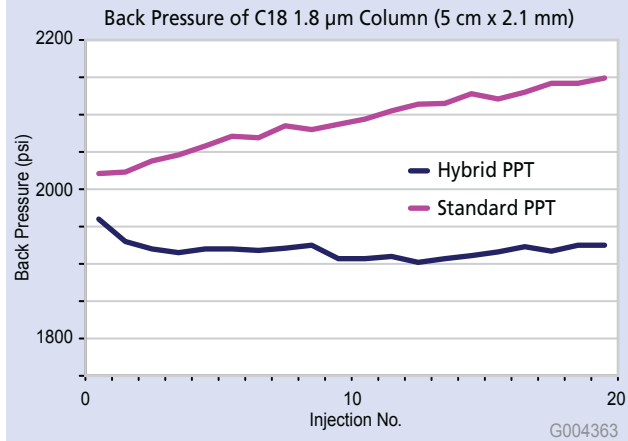
Figure 3. No Accumulation of Phospholipids Using HybridSPE-PPT



HybridSPE-PPT – 100% Removal of Phospholipids

In this study, 100 µL of blank rat plasma was applied to a HybridSPE-PPT 96-well plate followed by 300 µL 1% formic acid in acetonitrile. The plate was vortexed briefly and vacuum was applied. The resulting filtrate / eluate was analyze directly using the analytical conditions described in Figure 1. The results are depicted in Figure 3. By processing biological plasma samples using the HybridSPE-PPT approach, complete removal of phospholipids were achieved. Unlike standard protein precipitation (Figure 1), no accumulation of phospholipids was observed using the HybridSPE-PPT approach. In addition, because the HybridSPE-PPT 96-well acts as both a depth filter and a chemical filter to remove particulate matter and phospholipids concurrently, backpressure was significantly reduced (Figure 4).

Figure 4. Stabilization of Backpressure Using HybridSPE-PPT



Reduce LC-MS Run Time using HybridSPE-PPT

In this study, plasma samples spiked with verapamil, normethyl verapamil, and methoxy verapamil at the level of 10 ng/mL were subjected to protein precipitation by diluting the samples with 1% formic acid in acetonitrile (1:3, v/v) and analyzed by LC-TOF/MS (Figure 5). From these results, we see that although verapamil and its metabolites are eluted early in the run (~90 sec.), contaminating phospholipids are strongly retained and do not elute off from the column until after 10 min. Therefore, shorter run times (e.g., < 5 min.) pose a great risk to on-column phospholipid accumulation / contamination.

Because HybridSPE-PPT depletes phospholipids from the plasma sample (Figure 3), it is not necessary to run long gradient conditions to "wash off" contaminating phospholipids evident when using alternative sample prep techniques. Rat plasma spiked with verapamil and metabolites (10 ng/mL) were extracted using the HybridSPE-PPT method described for Figure 3 and analyzed by LC-TOF/MS using Ascentis Express C18. In Figure 6, we demonstrate the utility of combining HybridSPE-PPT and Ascentis Express column technology. Less than 90 second run time was achieved under isocratic conditions; and because HybridSPE-PPT was employed during sample prep, risk of phospholipid ion-suppression and column accumulation was eliminated.

Figure 5. Phospholipid Contamination from Standard Protein PPT Requires Increased Run Time (> 10 min.)

column: Ascentis Express C18, 5 cm x 2.1 mm I.D., (53822-U)
instrument: Agilent 1200 RR
mobile phase: (A) water:acetonitrile, pH 2.55 adjust with formic acid (30:70)
(B) 13 mM ammonium formate diluted in 95% methanol
flow: 0.6 mL/min.
temp.: 35 °C
inj.: 1 µL
det.: TOF/MS
Phospholipids (750-850 m/z range)
Verapamil (455.305 m/z)
Normethyl verapamil (441.280 m/z)
Methoxy verapamil (485.323 m/z)

gradient:

Min	%A	%B
1.0	100	0
2.5	0	100
10	0	100

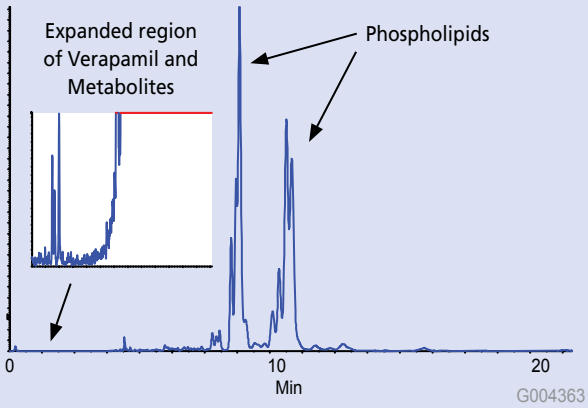
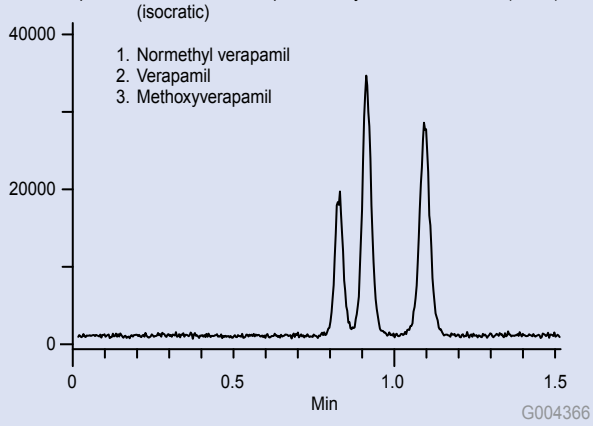


Figure 6. Less than 90 Sec. Run Time Achieved Using Ascentis Express C18 and HybridSPE-PPT for Verapamil and Metabolites in Rat Plasma

Conditions identical to Figure 5, except for mobile phase
Mobile phase: water:acetonitrile, pH 2.55 adjust with formic acid (30:70) (isocratic)



Conclusion

In this report, we discussed the ion-suppression impact of phospholipids and how traditional sample prep techniques such as protein precipitation do not remove this common matrix interference. As researchers strive for shorter run times using ballistic gradients, the risk of column phospholipid accumulation grows considerably. By using HybridSPE-PPT, the risk of phospholipid contamination is eliminated. As a result, shorter run times are possible. As a demonstration, HybridSPE-PPT was combined with Ascentis Express C18 technology for the extraction and analysis of verapamil (and metabolites) from plasma. By combining the two techniques, less than 90 second run time was achieved under isocratic conditions.

References

1. King et al., J Am Soc Mass Spectrom 11 (2000), 942-50.
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3. Little et al., Journal of Chrom B, 833 (2006), 219-230
4. Shen et al., Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 359–367
5. Xu et al., Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 342–355

Featured Products

Description	Cat. No.
HybridSPE-Precipitation	
96-well Plate, 50 mg/well, pk. 1	575656-U
Cartridge, 30 mg/1 mL, pk. 100	55261-U
Ascentis Express C18	
5 cm x 2.1 mm I.D., 2.7 µm	53822-U

Related Information

For more information on HybridSPE-Precipitation and Ascentis Express Column Technology, please visit ***sigma-aldrich.com/hybridspe-ppt*** and ***sigma-aldrich.com/express***, respectively.