

# Introducing HybridSPE™-Precipitation Technology for Pharmaceutical Bioanalytical Sample Preparation

Craig Aurand, An Trinh, Michael Ye and Charles Mi  
an.trinh@sial.com

In pharmaceutical bioanalysis, researchers develop and run various assays to quantitate drugs, pharmaceutical candidates, and their metabolites in biological fluids such as serum and plasma. The data resulting from these assays are used to help determine the pharmacodynamic and pharmacokinetic properties as well as the toxic and therapeutic concentrations of existing and emerging

## Features & Benefits:

- Merges both Protein PPT & SPE
- Offers simplicity & generic nature of protein PPT

### PLUS

- Selectivity approaching SPE via the targeted removal of phospholipids
- 2-3 step generic procedure
- 100% removal of phospholipids & precipitated proteins
- Minimal to no method development
- Available in 96-well and 1 mL cartridge dimensions
- Patent pending technology

pharmaceutical compounds in living cells, tissues, and animals. Although advances in Liquid Chromatography-Mass Spectrometry (LC-MS) technology have reaped overwhelming benefits in terms of increased throughput and sensitivity, good sample preparation

continues to be a critical component of bioanalysis.

The three most common sample prep techniques used in bioanalytical sample prep are protein precipitation (protein PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE). Each technique offers unique advantages and disadvantages that are considered during the method development process. For example, protein precipitation methods are simple (2-3 steps), fast, and often require minimal method development. However, the technique offers minimal selectivity as it only removes gross levels of protein from a sample prior to analysis. In contrast, SPE offers significant benefits in terms of selectivity/sample cleanup, but the technique often requires moderate to extensive levels of expertise and time for adequate method development. In addition, SPE often requires multiple steps (5-8), resulting in increased assay time.

In this report, we introduce a new sample prep platform trademarked HybridSPE-Precipitation (HybridSPE-PPT) in which we merge two predominate techniques in bioanalytical sample prep: protein precipitation and SPE. The end result is a technique that offers the advantages of both approaches while minimizing their disadvantages.

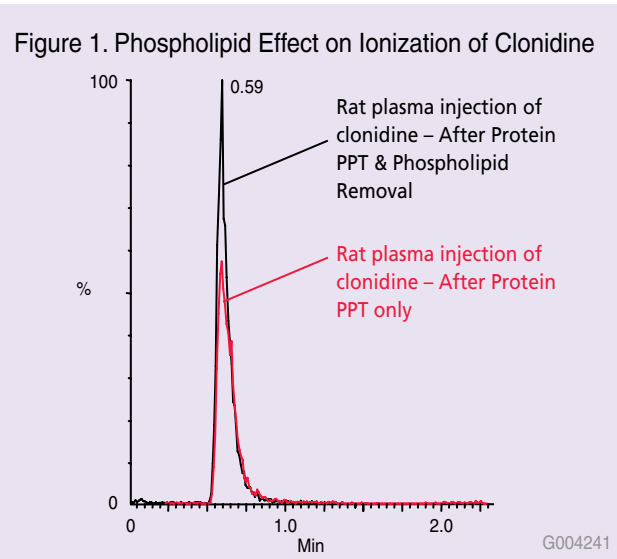
## Ion-Suppression & Phospholipid Contamination

Excessive background from endogenous matrix components has always been a great concern in quantitative bioanalysis, and has become paramount with decreasing analytical run times. In bioanalytical mass spectrometry, the issue of excessive background contributes to the growing problem of ion-suppression.

Ion-suppression is caused by one or more interfering components or species that co-elute with the analyte(s) of interest during LC-MS analysis and manifests itself as a loss of analyte response. These co-eluting species can affect droplet formation or ionize concurrently resulting in an erroneous decrease (suppression) or increase (enhancement) in signal response. Ion-suppression often leads to poor assay reproducibility, accuracy, and sensitivity, and such deleterious effects are often most notable at the lower limits of quantitation (LLOQ) (1).

One of the major causes of ion-suppression in bioanalysis is the presence of phospholipids during LC-MS or LC-MS-MS analysis in the positive ion electrospray mode (+ESI) (2). Phospholipids are the second largest lipid component in biological matrices after triglycerides, and are typically present in extremely high concentrations in biological plasma samples. Figure 1 compares the LC-MS chromatograms of two clonidine spiked rat plasma samples processed by protein precipitation (100 µL spiked plasma + 300 µL 1% formic acid in acetonitrile) alone and protein precipitation followed by phospholipid removal. The black trace chromatogram shows the response of clenbuterol

(continued on page 4)



(continued from page 3)

after protein precipitation and phospholipid removal. The red trace chromatogram was subjected to protein precipitation only. By removing phospholipid interferences prior to analysis, response for clonidine was nearly doubled.

### How Does HybridSPE-PPT Technology Work?

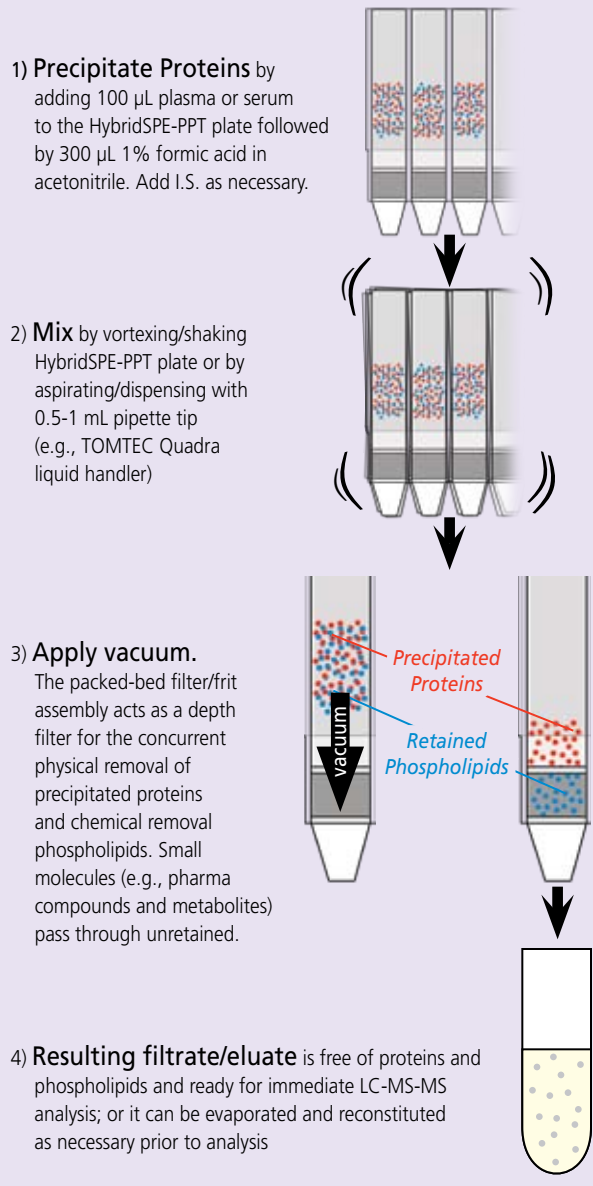
HybridSPE-PPT technology is a simple and generic sample prep platform designed for the gross level removal of endogenous protein and phospholipid interferences from biological plasma and serum prior to LC-MS or LC-MS-MS analysis. Biological plasma or serum is first subjected to protein precipitation 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 96-well plate or cartridge which acts as a chemical filter that specifically targets the removal of endogenous sample phospholipids. The 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. The phospholipid retention mechanism is based on a highly selective Lewis acid-base interaction between the proprietary zirconia ions functionally bonded to the HybridSPE-PPT stationary phase and the phosphonate moiety consistent with all phospholipids. The resulting eluent is ready for immediate LC-MS or LC-MS-MS analysis.

An alternative “In-Well Precipitation” method is available for the HybridSPE-PPT 96-well version in which biological plasma/serum is first added to the 96-well plate followed by acidified acetonitrile (precipitation agent). After a brief mixing/vortexing step, vacuum is applied to the 96-well plate. Figure 2 visually depicts the HybridSPE-PPT process (“In-Well Precipitation”) and describes how phospholipids are removed.

### How are Phospholipids Selectively Removed?

Once the plasma/serum sample is subjected to protein precipitation via the addition of 1% formic acid diluted in acetonitrile, it is passed through the HybridSPE-PPT packed bed. The packed bed consists of a proprietary zirconia coated silica particle. The zirconia sites exhibit Lewis acid (electron acceptor) properties that will interact strongly with Lewis bases (electron donor). Phospholipids structurally consist of a polar head group (zwitterionic phosphonate moiety) and a large hydrophobic tail (two fatty acyl groups that are hydrophobic). The phosphate group inherent with all phospholipids acts as a very strong Lewis base that will interact strongly with zirconia atoms functionalized on the particle surface (Figure 3).

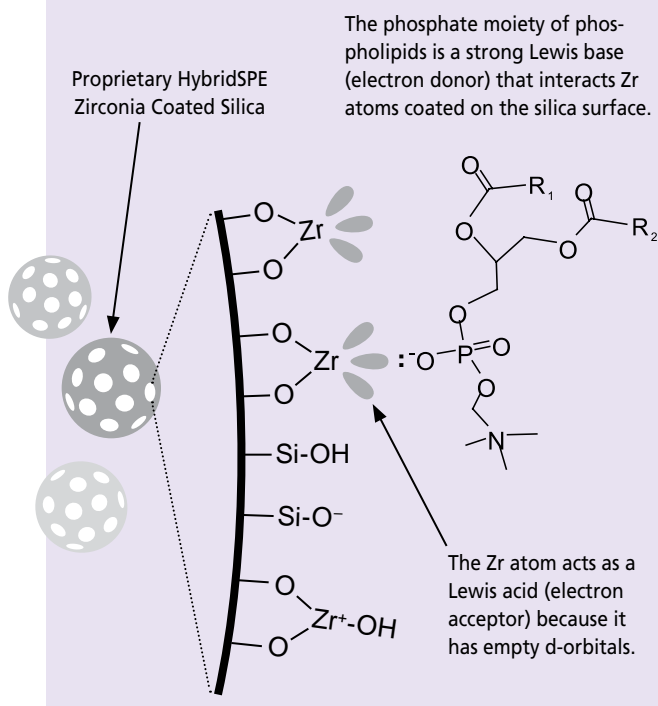
Figure 2. HybridSPE-PPT “In-Well” 96-well Precipitation Method and Phospholipid Removal



Note: The presence of  $\geq 1\%$  formic acid in the acetonitrile precipitation agent is critical because:

- 1) Formic acid is a stronger Lewis base than most carboxyl ( $-\text{COOH}$ ) groups found in acidic pharmaceutical compounds. As a result, formate ions will tie up the phase’s zirconia ions minimizing retention of acidic analytes of interest. Because formate is not a strong enough Lewis base to displace the phosphate moiety found in phospholipids, phospholipids preferentially retain on the HybridSPE-PPT phase.
- 2) The low pH environment induced by formic acid neutralizes residual silanol activity on the silica surface thereby eliminating secondary cation-exchange interaction with basic compounds of interest.

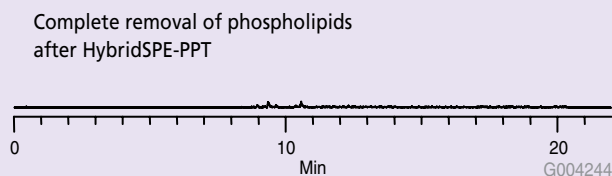
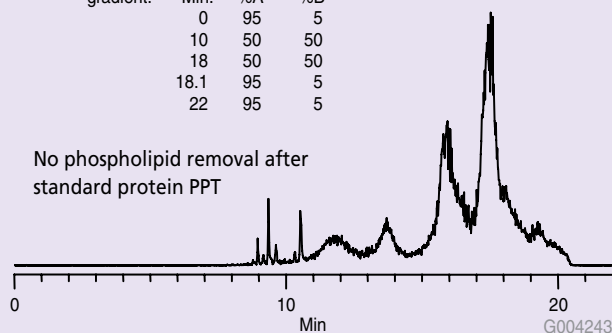
**Figure 3. Lewis Acid Base Interaction Between Hybrid SPE Zirconia Ions and Phospholipids**



**Figure 4. Efficiency of Phospholipid Removal of HybridSPE-PPT Technology (575656-U) - MRM 184 and 104 m/z**

column: Ascentis Express C18, 5 cm x 2.1 mm ID (53822-U)  
 instrument: Agilent 1100  
 mobile phase: (A) 10 mM ammonium acetate  
 (B) 10 mM ammonium acetate in acetonitrile  
 temperature: 35 °C  
 flow rate: 0.5 mL/min.  
 detection: ABI 3200 QT; ESI(+), MRM (184/104 m/z)  
 inj. vol.: 5 µL

gradient:	Min.	%A	%B
	0	95	5
	10	50	50
	18	50	50
	18.1	95	5
	22	95	5



To demonstrate the efficiency of phospholipid removal using HybridSPE-PPT technology, 100 µL of blank rat plasma was subjected to protein precipitation via the addition of 1% formic acid in acetonitrile followed by 1 min. of vortex and centrifugation. A second set of rat plasma samples were processed using the HybridSPE-PPT procedure described in Figure 2. The resulting supernatant (standard protein PPT) and filtrate/eluent (HybridSPE-PPT) was analyzed via LC-MS specifically monitoring for phospholipids (184 /104 m/z). These transition ions represent trimethylammonium-ethyl phosphate MS fragment consistent between the major phospholipids (e.g., phosphatidylcholine) found in plasma (2). In Figure 4, samples processed using HybridSPE-PPT resulted in 100% removal of phospholipids from 100 µL of rat plasma. In contrast, standard protein precipitation yielded high levels of phospholipid contamination which can potentially co-elute with analytes of interest or build up on the column and elute uncontrollably during a given injection sequence. This is especially problematic as analysts strive for shorter analytical run times through the use of smaller column dimensions and particle sizes.

### Comparison of HybridSPE-PPT, Protein Precipitation, and SPE

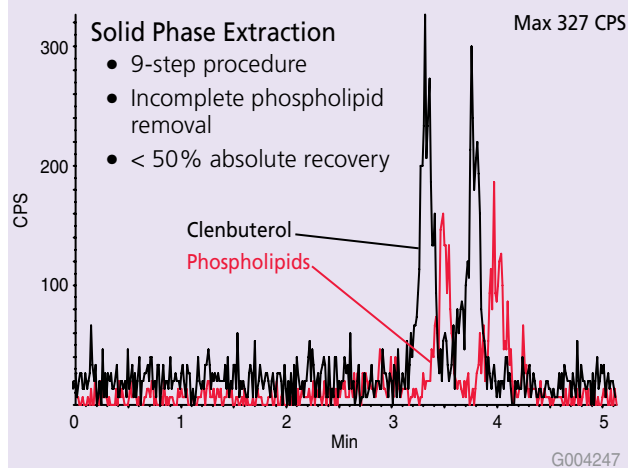
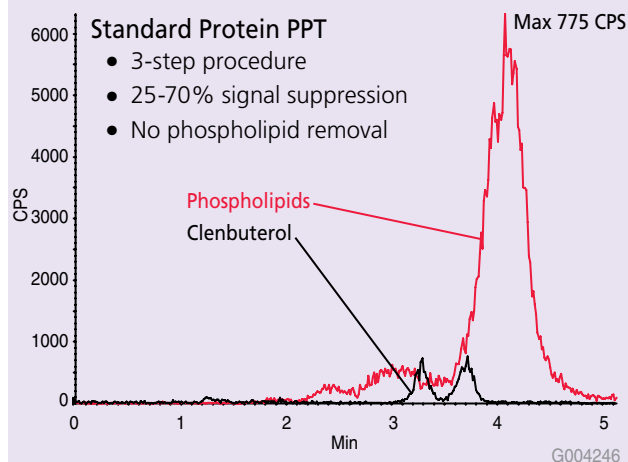
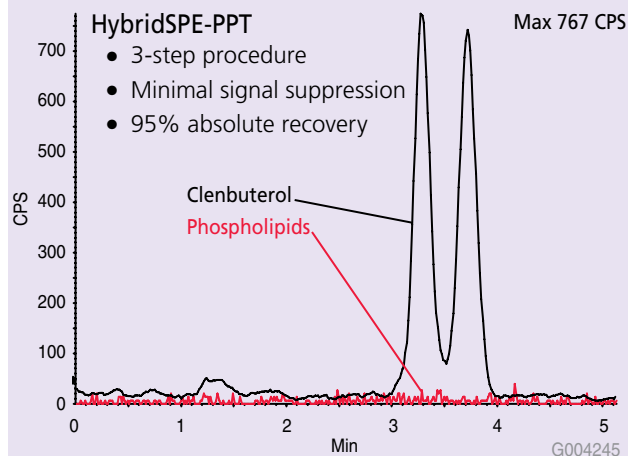
In this application example, rat plasma samples were spiked with clenbuterol (R(-) and S(+)) enantiomers at the level of 10 ng/mL and extracted using three different procedures: HybridSPE-PPT, Protein PPT, and a 9-step SPE procedure optimized for trace level clenbuterol analysis. The analysis was performed using a chiral stationary phase containing a macrocyclic glycopeptide covalently bound to silica and detection via MS-MS. Comparisons of sample preparation methods were made in terms of the amount of phospholipids in the sample extract and the overall effect on signal response of clenbuterol enantiomers. Absolute recovery was determined against an external standard.

Representative chromatograms of each the sample prep techniques are depicted in Figure 5. From the results indicated in Figure 5, phospholipid contamination levels were highest for protein precipitation resulting in signal suppression levels 70 and 25% for the R(-) and S(+) enantiomers of clenbuterol, respectively. For the SPE procedure, phospholipid contamination was still evident after multiple wash steps, and overall absolute recovery was less than 50%. In contrast, HybridSPE-PPT offered 100% removal of phospholipids resulting in absolute recovery levels of 95%.

(continued on page 6)

Figure 5. Comparative Extraction and LC-MS-MS of 10 ng/mL Clenbuterol (R(-) and S(+)) enantiomers in Rat Plasma

column: Chirobiotic T, 10 cm x 2.1 mm, 5  $\mu$ m (12018AST)  
 instrument: Agilent 1100  
 mobile phase: 10 mM ammonium formate in methanol  
 temperature: 30  $^{\circ}$ C  
 flow rate: 0.3 mL/min.  
 detection: ABI 3200 QT; ESI(+), MRM: 184/104 m/z (phospholipids)  
 and 277.2/203.1 (clonidine)  
 inj. vol.: 10  $\mu$ L



## HybridSPE-PPT Plate and Vacuum Manifold



(continued from page 5)

## Conclusion

In this report, a new sample prep platform specifically designed for pharmaceutical bioanalysis was introduced. The technique, trademarked HybridSPE-Precipitation or HybridSPE-PPT, merges the simplicity of protein precipitation with the selectivity of SPE for the targeted removal of endogenous proteins and phospholipids from biological plasma for subsequent LC-MS analysis. Examples applications demonstrate the chromatographic impact of phospholipids and how its presence can result in signal suppression during MS quantitation. When compared with traditional sample prep techniques such as protein precipitation and solid phase extraction for the extraction of clenbuterol enantiomers from rat plasma, HybridSPE-PPT offered complete phospholipid removal resulting in excellent recovery, minimal signal suppression, and improved S/N ratios. In contrast, lower recovery and higher signal suppression was evident using the traditional sample prep techniques such as protein precipitation and solid phase extraction.

## References

1. King et al., J Am Soc Mass Spectrom 11 (2000), 942-50.
2. Little et al., Journal of Chrom B, 833 (2006), 219-230

## + Featured Products

### Description

#### HybridSPE-Precipitation

96-well Plate, 50 mg/well, pk. 1  
 Cartridge, 30 mg/1 mL, pk. 100

### Cat. No.

575656-U  
 55261-U

## ! Related Information

For more information on HybridSPE-Precipitation technology, please visit [sigma-aldrich.com/hybridspe-ppt](http://sigma-aldrich.com/hybridspe-ppt)