

Troubleshooting Analyte Recovery when Using HybridSPE-Precipitation Technology

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Introduction

HybridSPE™-Precipitation (HybridSPE-PPT) is a new sample prep platform for pharmaceutical bioanalysis. The technology merges the simplicity of protein precipitation with the selectivity of SPE for the selective removal of proteins and phospholipids from biological plasma. Removal of these two key interferences greatly reduces the risk of ion-suppression during LC-MS-MS analysis for improved assay sensitivity and reproducibility. In Reporter issues 26.3 and 26.5, we provided an overview of HybridSPE-PPT and offered application examples that illustrate the benefits of the technology. In this article, we discuss non-phospholipid specific interactions between the HybridSPE phase and the sample that could potentially lead to low recovery of certain basic and acidic chelator compounds. We conclude our discussions with strategies for improving the recovery of such problematic compounds.

How does HybridSPE-PPT work?

When using 96-well HybridSPE-PPT, 100 μ L of plasma is added to the individual wells followed by 300 μ L precipitation agent (1% formic acid in acetonitrile). After a brief mixing step to adequately precipitate endogenous proteins, vacuum is applied to the plate. As the sample flows through the packed-bed/filter-frit assembly, both proteins

and phospholipids are concurrently removed. Proteins are physically removed by low porosity filters whereas phospholipids are chromatographically removed by the stationary phase. The resulting eluent is free of both phospholipids and proteins, and can be directly analyzed via LC-MS-MS.

The HybridSPE stationary phase is a patent pending zirconia coated silica that is highly selective towards phospholipids. Retention is based on a Lewis acid-base interaction between the empty zirconia d-orbitals (Lewis acid) and the electron pair of the phosphate moiety (Lewis base) inherent of all phospholipids. Phosphate is a very strong Lewis base and will preferentially interact with zirconia over other Lewis bases (Figure 1).

The Importance of Formic Acid

Most acidic pharmaceutical compounds contain carboxyl (-COOH) groups. When processing acidic compounds using HybridSPE-PPT, the HybridSPE Zr-Si stationary phase will likely co-retain acidic compounds along with phospholipids, resulting in low absolute recovery. To rectify the situation, formic acid is added to the precipitation agent and becomes part of the sample during HybridSPE processing. Formic acid is a stronger Lewis base than most -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 phosphates, phospholipids preferentially retain on the HybridSPE-PPT phase.

In this application, we process three acidic compounds (ketoprofen, naproxen, and flunixin) and two neutral compounds using HybridSPE-Precipitation. The analytes were spiked into plasma at the level of 20 ng/mL. 100 μ L of plasma was precipitated with 300 μ L of one of two reagents prior to HybridSPE-PPT: 1) 1% formic acid in acetonitrile or 2) neat acetonitrile. The resulting HybridSPE-PPT eluent was analyzed by LC-MS-MS (MRM) using an Ascentis

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Figure 1. HybridSPE-PPT 96-well Schematic and Phospholipid Retention Mechanism

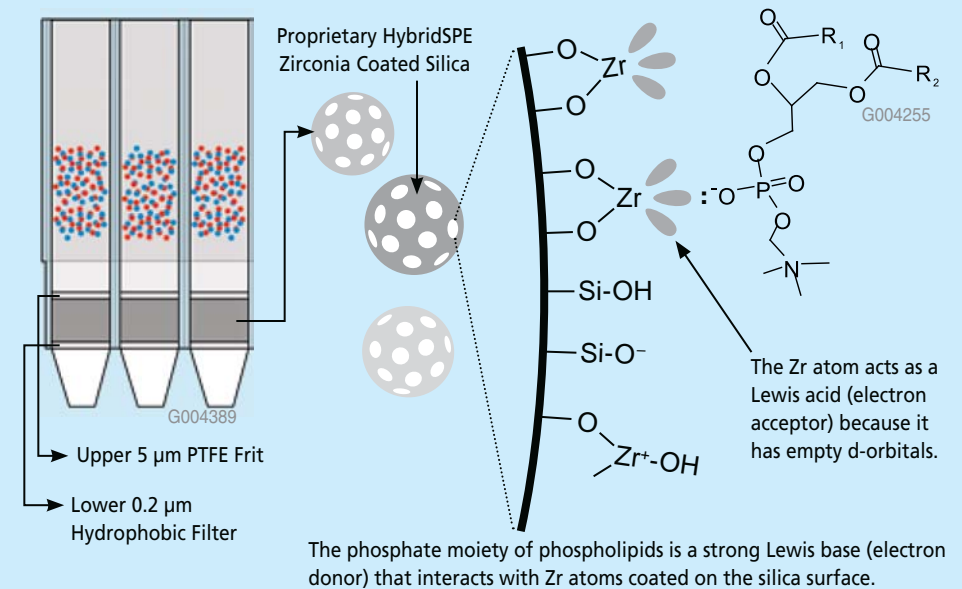
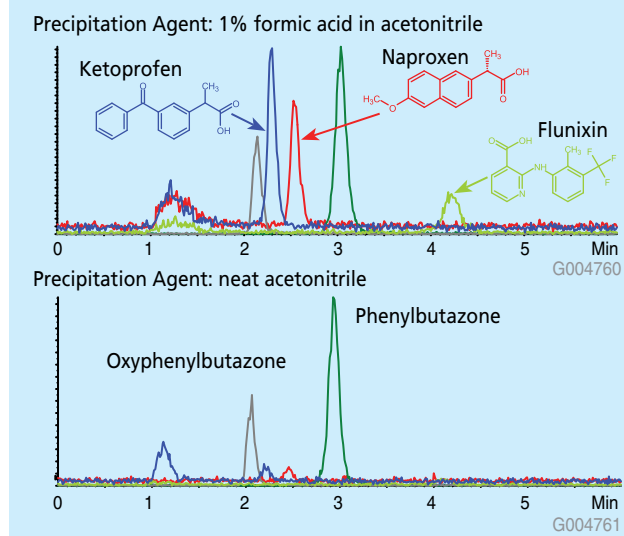


Figure 2. Comparison of Precipitation Agents (with and without formic acid) using HybridSPE-PPT



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RP-Amide column. Figure 2 compares the results between the two precipitation agents. From the results described in Figure 2, complete loss in recovery was observed for the three acidic compounds (ketoprofen, naproxen, and flunixin) when formic acid was not added to the precipitation agent. When formic acid is added to the precipitation agent, greater than 88% absolute recovery was observed for each of the acidic compounds (data not shown). In contrast, the two neutral compounds, phenylbutazone and oxyphenylbutazone were unaffected by the presence of formic acid resulting in high recovery under both conditions.

Troubleshooting Recovery of Chelator and Acidic Chelator Compounds

In our research thus far, we have found that certain chelator and acidic chelator compounds retain exceptionally strong on the Zr-Si stationary phase used in HybridSPE-PPT resulting in low absolute recovery (< 40%) when using the recommended primary HybridSPE-PPT method (100 μ L plasma + 300 μ L formic acid in

Table 1. Relative Retention Strength of Lewis Bases to Zirconia

Lewis Base	Relative Retention Strength on Zirconia
Hydroxide	Strongest ↑ Weakest
Phosphate	
Fluoride	
Citrate	
Sulfate	
Acetate	
Formate	
Chloride	

acetonitrile). Such chelating compounds can be identified as having functional groups with oxygen atoms in the alpha and beta positions. To improve recovery, a Lewis base stronger than formate is required as a modifier in the

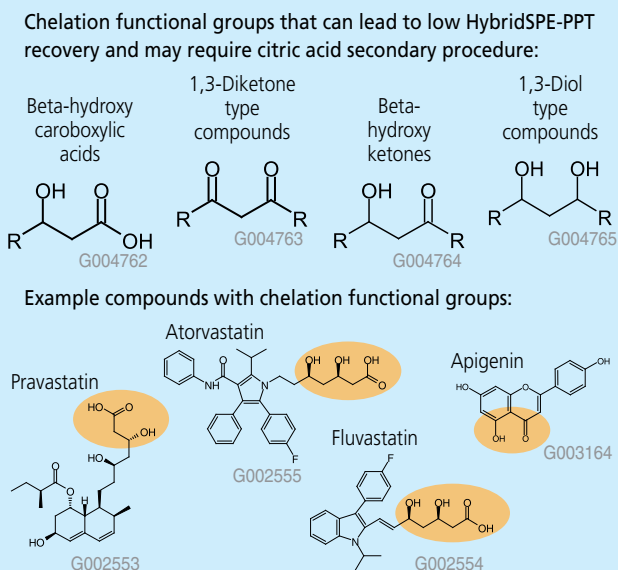
precipitation agent. Table 1 lists various Lewis bases and their relative retention strength on zirconia. From our experience, replacing formic acid with citric acid and adding a simple conditioning step can significantly increase the recovery of certain chelator and acidic chelator compounds. Figure 3 describes specific chelation functional groups and lists example compounds with such functional groups. Details for the secondary procedure we recommend are described in Table 3. By using the secondary procedure described in Table 3, recovery for chelator compounds can improve from <40% to 65-95%. Mechanistically, citric acid is a stronger Lewis base than formic acid, inhibiting retention of chelator compounds. However, citric acid is not a strong enough Lewis base to displace retention of phosphates (i.e., phospholipids).

Troubleshooting Recovery of Basic and Neutral Compounds

Although the primary retention mechanism for HybridSPE is based on Lewis acid-base interactions between Zr ions on the stationary phase and negatively charged functional groups in the sample (e.g., phosphate moiety of phospholipids), secondary interactions derived from the silica surface can retain basic and neutral compounds resulting in poor recovery. These secondary interactions with silica surface include: 1) weak cation exchange and 2) HILIC interactions.

To disrupt any weak-cation exchange interactions between the silica silanol groups (Si-O⁻) and basic compounds (e.g., contains amine functional groups), formic acid should be substituted with ammonium formate. The primary procedure we recommend uses formic acid as part of the precipitation agent. As a result, H⁺ is the resulting counter-ion used to

Figure 3. Low Recovery Chelation Functional Groups with Example Compounds



neutralize exposed silanol groups on the HybridSPE-PPT phase (Si-O⁻ => Si-OH). For some basic compounds, H⁺ is not a strong enough counter-ion to inhibit cation-exchange retention between silanol groups and basic compounds. This results in poor recovery. By replacing formic acid with ammonium formate, a stronger ammonium counter-ion (NH₄⁺) is employed. Ammonium ions are sufficient in counter-ion strength to inhibit most (if not all) basic compounds from interacting with silanol groups.

For more polar compounds, secondary HILIC interactions (e.g., hydrogen bonding) may occur between basic/neutral analytes of interest and the silica surface. These secondary HILIC interactions can be disrupted by substituting acetonitrile with methanol as the precipitation agent. To further minimize potential secondary HILIC interactions, the sample needs to be 25% aqueous prior to HybridSPE-PPT processing. Therefore, combining 100 µL plasma with 300 µL organic precipitation agent is recommended for HybridSPE-PPT. For smaller plasma volumes (e.g., 20-50 µL), the sample should be diluted with DI water to maintain a

Table 3. Summary of Recommended Primary and Secondary Procedures for 96-well HybridSPE-PPT

Primary Procedure (suitable for 80% of applications):

Recommended for most applications (basic, neutral, acidic analytes)

1. To each well, add 100 µL plasma followed by 300 µL 1% formic acid in acetonitrile. Mix the sample well (e.g., vortex).
2. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.
3. If low recovery is observed, proceed to Secondary Procedures.

Secondary Procedure (acidic & chelator compounds):

Recommended for low recovery chelator and acidic chelator compounds

1. Condition each well with 400 µL 0.5% citric acid in acetonitrile (until flow has ceased).
2. To each well, 100 µL plasma followed by 300 µL 0.5% citric acid in acetonitrile. Mix the sample well (e.g., vortex).
3. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.

Note:

Recovery of chelator compounds can improve from < 40% to 65-95%
Citric acid is a stronger Lewis base than formic acid inhibiting the retention of chelator compounds.

Citric acid is not a strong enough Lewis base to inhibit phosphates (phospholipids) from retaining on the HybridSPE phase.

Secondary Procedure (basic & neutral compounds):

Recommended for low recovery basic and neutral compounds

1. To each well, add 100 µL plasma followed by 300 µL 1% ammonium formate in methanol. Mix the sample well (e.g., vortex).
2. Apply vacuum and collect the resulting eluent for LC-MS-MS analysis.

Note:

Recovery of basic and neutral compounds can improve from < 40% to > 89%
NH₄⁺ (ammonium formate) is a stronger counter-ion than H⁺ (formic acid) inhibiting most basic compounds from interacting with HybridSPE silanol groups (Si-O⁻).
Methanol is a more polar solvent than acetonitrile further inhibiting any potential secondary HILIC interactions between the analyte and HybridSPE silica surface.

Table 2. Improvement of Absolute Recovery when Incorporating Ammonium Formate in the Precipitation Agent

Analyte (% Absolute Recovery)	Standard (no matrix) + 1% formic acid in MeCN	Standard (no matrix) + MeOH	Standard (no matrix) + 1% NH ₄ HCO ₂ in MeOH	Plasma + 1% NH ₄ HCO ₂ in MeOH
Mirtazapine (266/195)	0.0%	13.2%	96.0%	104.0%
Risperidone (411/191)	0.0%	10.4%	99.1%	123.3%
Olanzapine (313/256)	0.0%	13.6%	89.4%	56.4%

final sample volume of 100 µL prior to addition of 300 µL precipitation agent. If greater sensitivity is required after sample dilution, an evaporation and reconstitution step can be added prior to LC-MS-MS analysis.

In this study, 3 basic compounds experienced low recovery when using the primary method (100 µL plasma + 300 µL 1% formic acid in acetonitrile). 100 µL of spiked (20 ng/mL) plasma samples or standard samples (no matrix) were precipitated with 300 µL neat methanol, 1% formic acid in acetonitrile, or 1% ammonium formate in methanol prior to 96-well HybridSPE-PPT processing using the “In-well” precipitation method. Absolute recovery was assessed by reversed-phase LC-MS-MS (Table 2). From these results, significant improvements in HybridSPE-PPT recovery of the basic compounds observed when substituting 1% formic acid in acetonitrile with 1% ammonium formate in methanol as the precipitation agent.

Table 3 summarizes the primary and secondary procedures recommended when optimizing conditions for HybridSPE-PPT.

Conclusion

HybridSPE-PPT technology is a new sample prep platform designed for pharmaceutical bioanalysis. The technology combines the simplicity of protein precipitation and the selectivity SPE by specifically targeting the removal of precipitated proteins and phospholipids. The phospholipid removal mechanism is based on a Lewis acid-base interaction between the phosphate moiety inherent with all phospholipids and the Zr-Si stationary phase. Although a primary method that is suitable for most applications is available, low recovery can occur. In this report, we described the secondary interactions that can take place resulting in low recovery and strategies for how to troubleshoot these recovery issues.

For more information and to download the latest HybridSPE-PPT instruction sheet, please visit our website sigma-aldrich.com/hybridspe-ppt

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Description	Cat. No.
HybridSPE-Precipitation	
96-well Plate, 50 mg/well, pk. 1	575656-U
Cartridge, 30 mg/1 mL, pk. 100	55261-U