Selective Depletion of Phospholipids in Bioanalysis using HybridSPE-PPT Technology

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The approach of this study was to evaluate the performance of recently developed sample prep platform HybridSPE(TM) Precipitation(HybridSPE-PPT)for the purpose of matrix removal and analyte recovery from spiked plasma samples. The goal of the study was to demonstrate how the HybridSPE-PPT platform can increase bioanalytical throughput by both decreasing sample preparation time and improving chromatographic analysis and detection relative to traditional protein precipitation methods.

Often the major concern in developing bioanalytical methods is addressing the effect of endogenous biological matrix components on the detection of desired analytes. The impact of matrix effects in bioanalysis has been well documented [1,2]. In the majority of cases, co-extracted interferences directly affect the quantitation of analytes due to ionization effects induced by the extracted matrix. This extracted matrix can impact the chromatographic analysis, but more often is a result of chromatographic build up that leads to irregularities. Chromatographic build up of phospholipids is often observed when performing standard protein precipitation techniques [3,5]. Adequate removal of phospholipids from the analytical column often result in the need for gradient elution under reversed-phase separations resulting in prolonged analysis. Even ballistic conditions often used with modern UHPLC instruments are inadequate to remove phospholipids that a have accumulated on the column. In most cases gradient elution is not required for resolution of desired analytes, but instead required only to elute extracted matrix from the analytical column.

The approach of this study was to utilize a simplified two-step sample preparation procedure using the HybridSPE-PPT to allow for sufficient removal of endogenous matrix components from plasma samples containing loratadine and metabolite. Performing a more thorough sample clean up prior to analysis would enable faster chromatographic analysis and thus increase the overall sample throughput. To evaluate this approach, plasma samples were prepared using standard protein precipitation techniques and compared against the HybridSPE-PPT platform for the evaluation of analyte recovery and overall matrix removal. Hydrophilic Interaction Chromatography(HILIC)/ Aqueous Normal Phase (ANP) chromatographic conditions were developed for the separation of loratadine, the metabolite desloratadine, along with monitoring of matrix elution.

Plasma samples prepared using the HybridSPE-PPT platform are sufficiently depleted of phospholipids, enabling the use of isocratic chromatographic separation resulting in dramatic increase in sample through put. A comparative application for loratadine and metabolite from plasma using a standard protein precipitation technique required an analysis time of 20 minutes to elute phospholipids from the analytical column using a standard protein precipitation technique. The combination of HybridSPE-PPT platform with the HILIC/ANP chromatographic mode reduced analysis time to less than 40 seconds. This approach greatly increased sample throughput with minimal impact on analyte recovery and no sample prep method development.

Samples:
Rat plasma stabilized with K2EDTA was acquired from Lampire Biological Laboratories, (Pipersville PA). Reference standards for loratadine and desloratadine metabolite were acquired from Sigma Aldrich Corporation(St.Louis, MO), see Figure 1. Rat plasma samples were spiked at 80, 200 and 400ng/mL with reference standard.

Extraction:
Spiked rat plasma samples were prepared using a standard protein precipitation method along with the HybridSPE-PPT(Supelco, Bellefonte PA) platform. To a 2 mL polypropylene centrifuge tube, 300µL of plasma was added followed by 900µL of 1% formic in acid acetonitrile. Samples were vortexed for 2 minutes to facilitate precipitation of proteins and then placed into a centrifuge at 15000rpm for 3 minutes to form a solid protein pellet. The resulting supernatant was collected and analyzed directly for the standard protein precipitation method.

The HybridSPE-PPT platform method consisted of adding 300µL of spiked plasma followed by 900µL of 1% formic acid acetonitrile to a 2mL polypropylene centrifuge tube. The samples were vortexed for 2 minutes and then placed into a centrifuge at 15000rpm for 3 minutes to form a solid protein pellet. A 400µL aliquot of supernate was collected from each sample and added to the HybridSPE 96well plate. Vacuum was applied at 10" Hg for 4 minutes and the resulting eluent was collected and analyzed directly.

HybridSPE-PPT is a generic sample prep platform designed for the gross level removal of endogenous protein and phospholipid

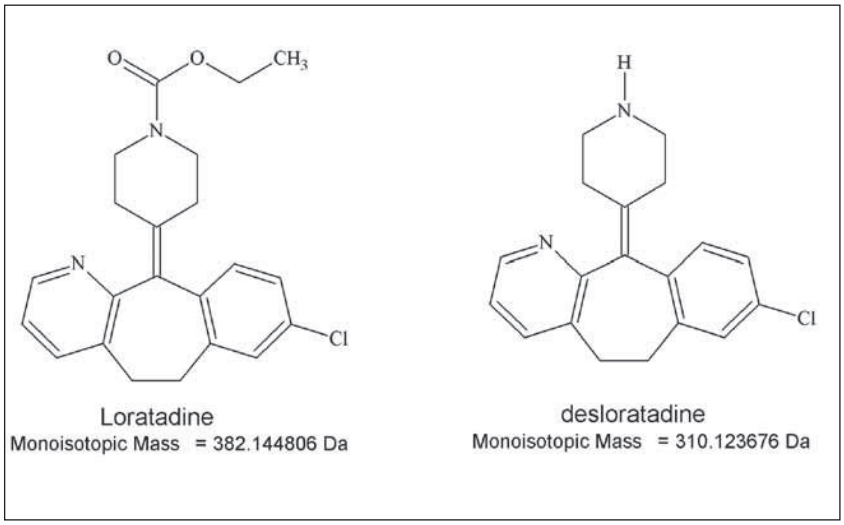

Figure 1. Structure of Loratadine and Desloratadine

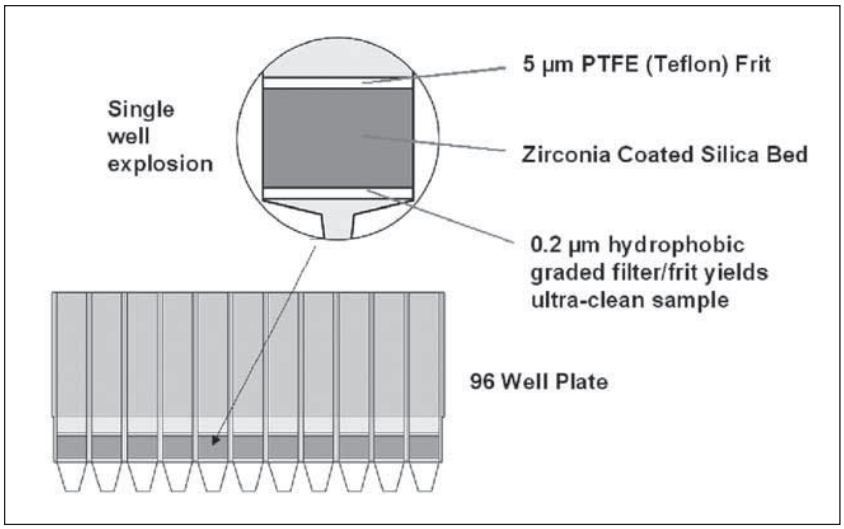

Figure 2. HybridSPE-PPT Plate Configuration

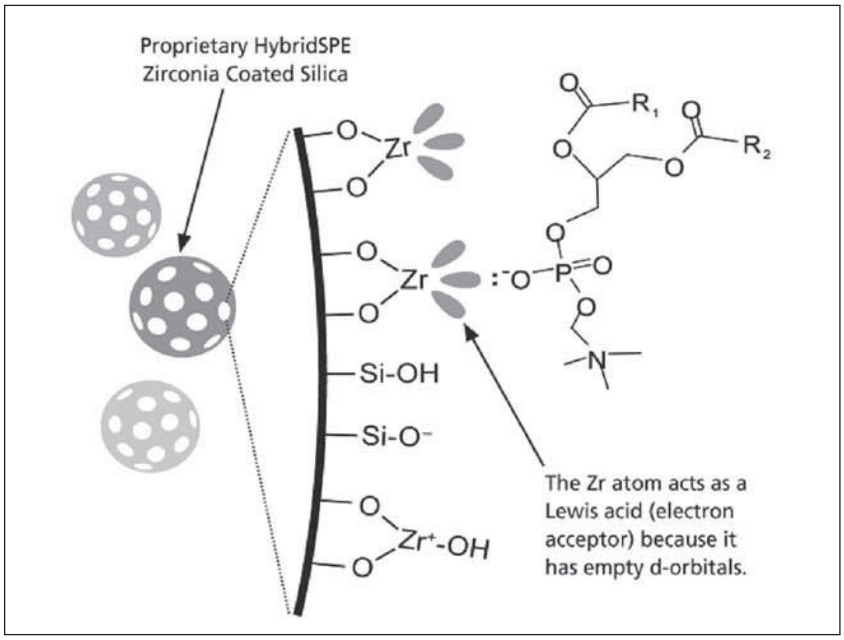

Figure 3. HybridSPE-PPT Particle

interferences from biological plasma and serum prior to LC-MS analysis. It combines the simplicity of protein precipitation with the added selectivity of solid phase extraction resulting in a simplified two-step sample prep process. The HybridSPE-PPT 96-well plate contains a series of low porosity hydrophobic filters/frits and a proprietary zirconia coated silica stationary bed, (Figure2). This packed-bed filter/frit configuration acts as a depth filter to facilitate the concurrent removal of both phospholipids and precipitated proteins during the extraction process. Protein precipitation can be conducted directly in the HybridSPE-PPT plate by first adding plasma to the well plate (upper PTFE frit keeps plasma from dripping through prematurely) followed by acidified acetonitrile (precipitating agent). After a brief mixing/votexing step, vacuum is applied to the HybridSPE-PPT plate and the resulting filtrate / eluent is analyzed directly.

The phospholipid retention mechanism on the HybridSPE-PPT is based on a highly selective Lewis acid-base interaction [4] between the proprietary zirconia ions functionally bonded to the HybridSPE stationary phase and the phosphate moiety consistent with all phospholipids, (Figure 3). The resulting eluent is depleted of proteins and phospholipids and is ready for immediate LC-MS or LC-MS/MS analysis.

Chromatographic Conditions:
Analysis of extracted plasma samples was conducted on an Agilent(Wilmington, DE) 1200SL Rapid Resolution instrument with an Agilent 6210 TOF mass spectrometer. Chromatographic separation of loratadine and desloratadine was conducted on a 3µm Discovery HSF5, 50mm x 2.1 mm (Supelco, Bellefonte PA) under HILIC/ aqueous normal phase (ANP) conditions. The mobile phase consisted of 10mM ammonium acetate in 60:40 acetonitrile water, the pH of the solution was adjusted to pH 5.2 with formic acid. The column oven temperature

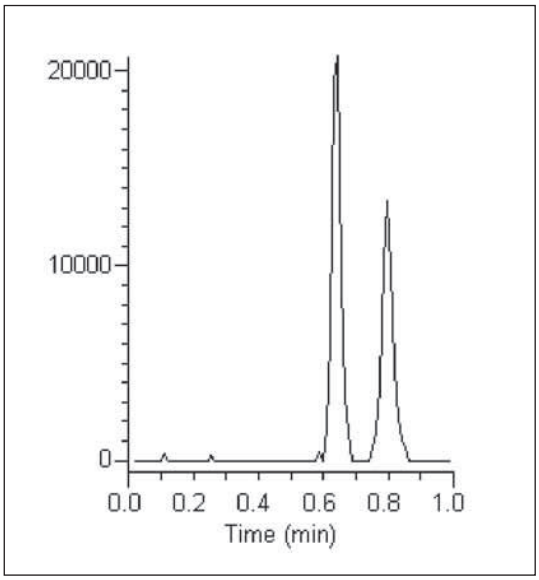

Figure 4. Loratadine and Desloratadine Standard on Discovery HSF5, Extracted Ion Chromatogram 383.1540m/z, 311.1321m/z

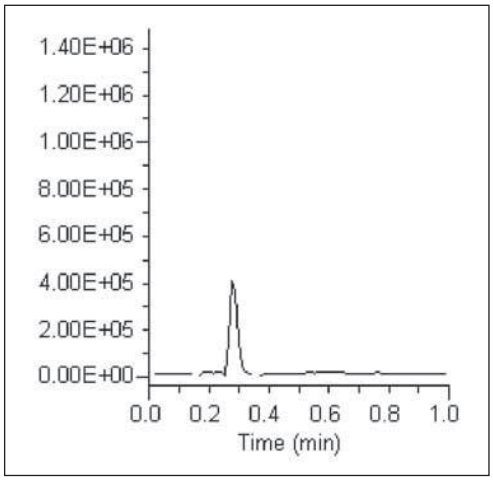

Figure 5. HybridSPE Sample Preparation of Plasma on Discovery HSF5, Phospholipid Monitoring 350-900m/z

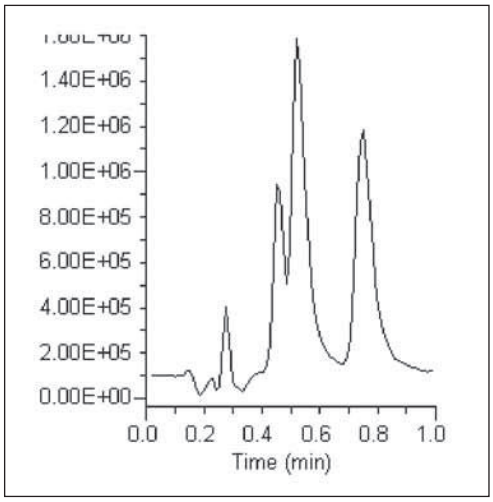

Figure 6. Standard Protein Precipitation of Plasma on Discovery HSF5, Phospholipid Monitoring 350-900m/z

was set to 35°C with a flow rate of 0.6mL/min. A 2uL injection volume was used for all standards and samples. Figure 4 depicts the separation of loratadine and desloratadine conducted using ESI+ ionization mode and accurate mass determination. Accurate mass measurements were maintained by performing simultaneous reference mass correction via dual spray ESI source. Phospholipid matrix was monitored by scanning mass ranges of 350-900m/z for all samples as shown in Figure 5. This region covers the range of molecular ions for the majority of phospholipids.

Results & Discussion:

The selective depletion of phospholipids from spiked plasma samples using the HybridSPE-PPT platform enabled the use of this fast isocratic LCMS method with no observable ion-suppression of analytes. Figure 5 depicts the chromatogram for phospholipid monitoring of samples prepared using the HybridSPE-PPT. Excellent recovery of loratadine and metabolite was observed from the HybridSPE-PPT when compared to external standards. The depletion of phospholipids resulted in no observable ion-suppression of analytes, allowing for more accurate determination across the spike concentration range. The implementation of the HybridSPE-PPT sample prep method resulted in higher analyte response and aided in the overall speed of the finalized method,(Figure7). By depleting phospholipids from the plasma sample prior to analysis, this eliminated the need for lengthy run times or gradient elution and allowed for separation of matrix from the analytes. Samples could then be processed as fast as chromatographically possible.

In the case of the standard protein precipitation method, a significant amount of ion-suppression was observed due to co-retention with extracted phospholipids. Figure 6 depicts the chromatogram for phospholipid monitoring of samples prepared using the standard protein precipitation technique. Even at a high concentration, severe ion-suppression was observed for loratadine, Figure7. Because phospholipids are not removed using the standard protein precipitation method, the fast LCMS method resulted in co-elution of analytes with phospholipids. Under the chromatographic conditions used on the Discovery HSF5 stationary phase, the mechanisms for retention for loratadine and desloratadine are enhanced ANP and ion-exchange. The overlap of the phospholipids with the analytes is a result of high mobile phase flow rate and hydrophobicity of the phospholipids. The large hydrophobic tails of the phospholipids inhibit partitioning from the mobile phase in this system, resulting in minimal retention and thus causing overlap with the analytes.

To evaluate the impact of phospholipid retention, a recently published method [6] on the analysis of loratadine and metabolite was compared to this newly developed method. The published method based the chromatographic separation on a phenyl based stationary phase requiring a long 150mm column to obtain sufficient resolution between loratadine and metabolite. In the example listed above, a 150mm X 2.1mm Ascentis Phenyl column was used to demonstrate the impact of using this type of method. Here the mobile phase of 68:32 acetonitrile:0.2% formic acid water was used with an increased flow rate of 0.4mL/min at ambient temperature. Retention of loratadine was 4.2 minutes under these conditions with minimal resolution from desloratadine (not shown), Figure 8. When monitoring the matrix elution of the phospholipids, a total run time of 15 minutes was required to elute matrix from the injection, Figure 9. Run times less than 15 minutes would result in overlap of phospholipid matrix into the next injection resulting in erratic analyte response.

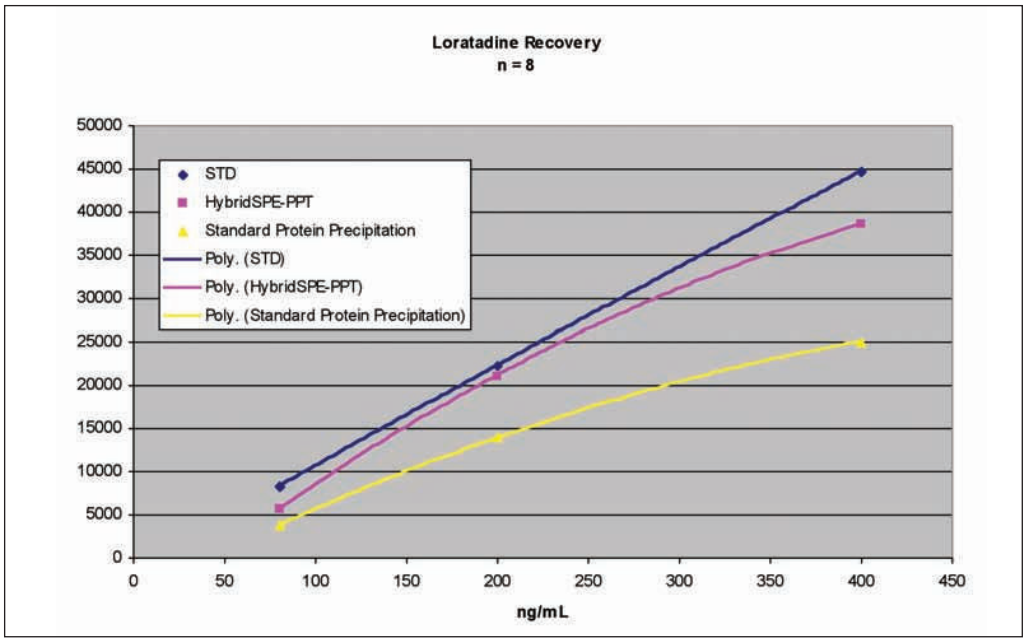

Figure 7. Loratadine Recovery Comparison from Spiked Plasma Samples

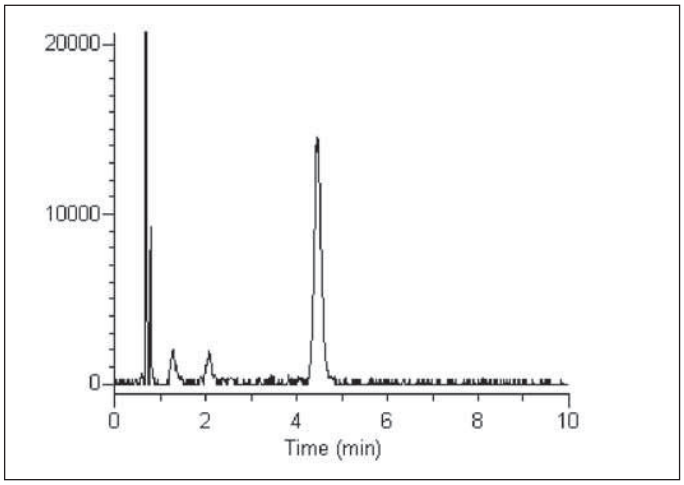

Figure 8. Loratadine Standard on Ascentis Phenyl, Extracted Ion Chromatogram 383.1540m/z

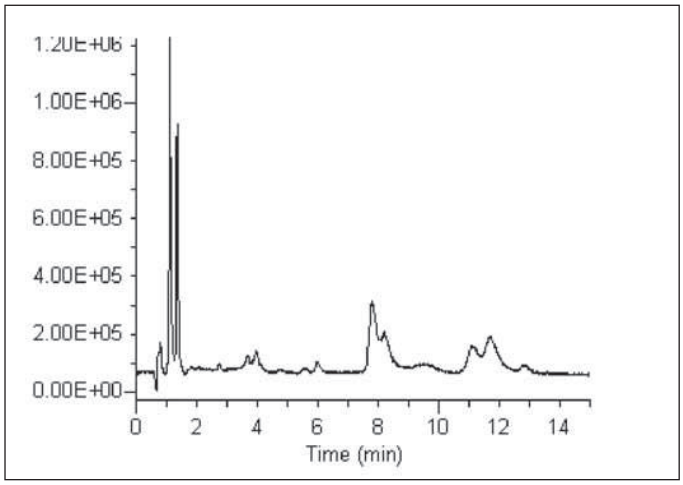

Figure 9. Standard Protein Precipitation of Plasma on Ascentis Phenyl, Phospholipid Monitoring 350-900m/z

Conclusions:

The selective depletion of phospholipids from the plasma sample prior to analysis enabled the use of fast chromatographic separation without the need for lengthy run times or gradient elution to remove matrix from the column. The HybridSPE-PPT platform enabled a simplified sample prep procedure resulting in high analyte recovery while removing phospholipid matrix from the plasma samples. Performing the HILIC/ANP separation on the Discovery HSF5 stationary phase enabled the direct injection of processed samples without the need for evaporation and reconstitution. This approach proved to be advantageous over the traditional protein precipitation sample prep method with reversed phase chromatographic separations resulting in increase analyte response with shorter run times.

References:

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