Impact of Ion-Suppression Due to the Presence of Phospholipids on the Enantiomeric LC-MS Analysis of Clenbuterol

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Abstract

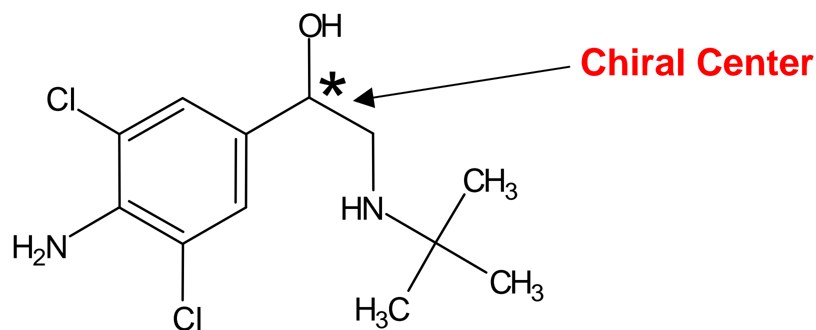
Ion-suppression, due to the presence of phospholipids, has become a major concern in reversed-phase LC-MS analysis due to trends toward faster chromatographic separations and lower limits of detection. The co-extraction of phospholipids are well documented to be detrimental in reversed phase LC-MS applications, but these observations can often be more dramatic in enantiomeric separations. Enantiomeric separations are carried out using mobile phases high in organic content, resulting in coelution of phospholipids with the analytes when analyzing biological matrices.

Abstract (contd.)

This study examines the impact of co-extracted phospholipids from a rat plasma matrix on the enantiomeric LC-MS analysis of clenbuterol. The analysis was performed using a chiral stationary phase containing a macrocyclic glycopeptide covalently bound to silica. Spiked rat plasma samples were prepared using a standard protein precipitation (PPT) protocol along with a newly developed HybridSPE™-PPT protocol designed to simultaneously remove both proteins and phospholipids. Comparisons of sample preparation methods were measured in terms of phospholipid content in the sample extract and the overall effect on signal response of clenbuterol enantiomers.

Introduction

- Demonstrate qualitatively the effect of ion suppression on an enantiomeric separation using standard PPT.
- Establish quantitative data for the effect of ion suppression on analyte response and correlate that data to analyte concentration.
- Demonstrate the use of HybridSPE-PPT protocol to eliminate phospholipids from a biological sample.
- Use enantomeric separation of a racemic mixture of clenbuterol as the test compound.



(+/-) clenbuterol

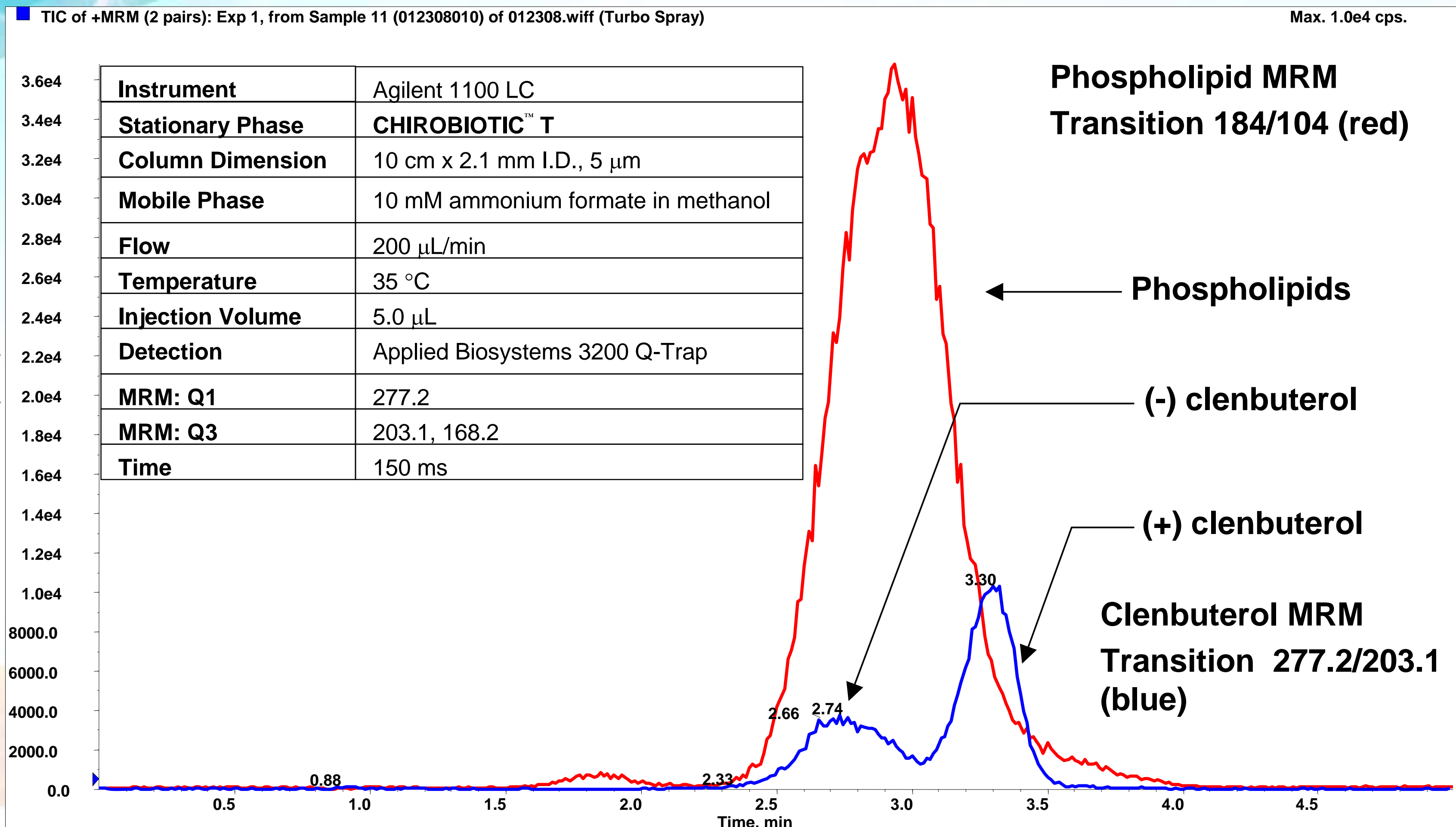
Experimental

PPT Sample Preparation Methods

Protein Precipitation:

- Rat plasma samples were spiked at a concentration of 50 ng/mL with (+/-) clenbuterol standard.
- A 1 mL aliquot of spiked plasma was combined with 3 mL of 1% formic acid in acetonitrile and agitated for 30 sec. The mixture was centrifuged for 3 min. at 15,000 rpm.
- The supernate was collected and analyzed directly.

Figure 1. Overlay of Phospholipid and Clenbuterol XICs after Standard PPT



Instrument	Agilent 1100 LC
Stationary Phase	CHIROBIOTIC™ T
Column Dimension	10 cm x 2.1 mm I.D., 5 µm
Mobile Phase	10 mM ammonium formate in methanol
Flow	200 µL/min
Temperature	35 °C
Injection Volume	5.0 µL
Detection	Applied Biosystems 3200 Q-Trap
MRM: Q1	277.2
MRM: Q3	203.1, 168.2
Time	150 ms

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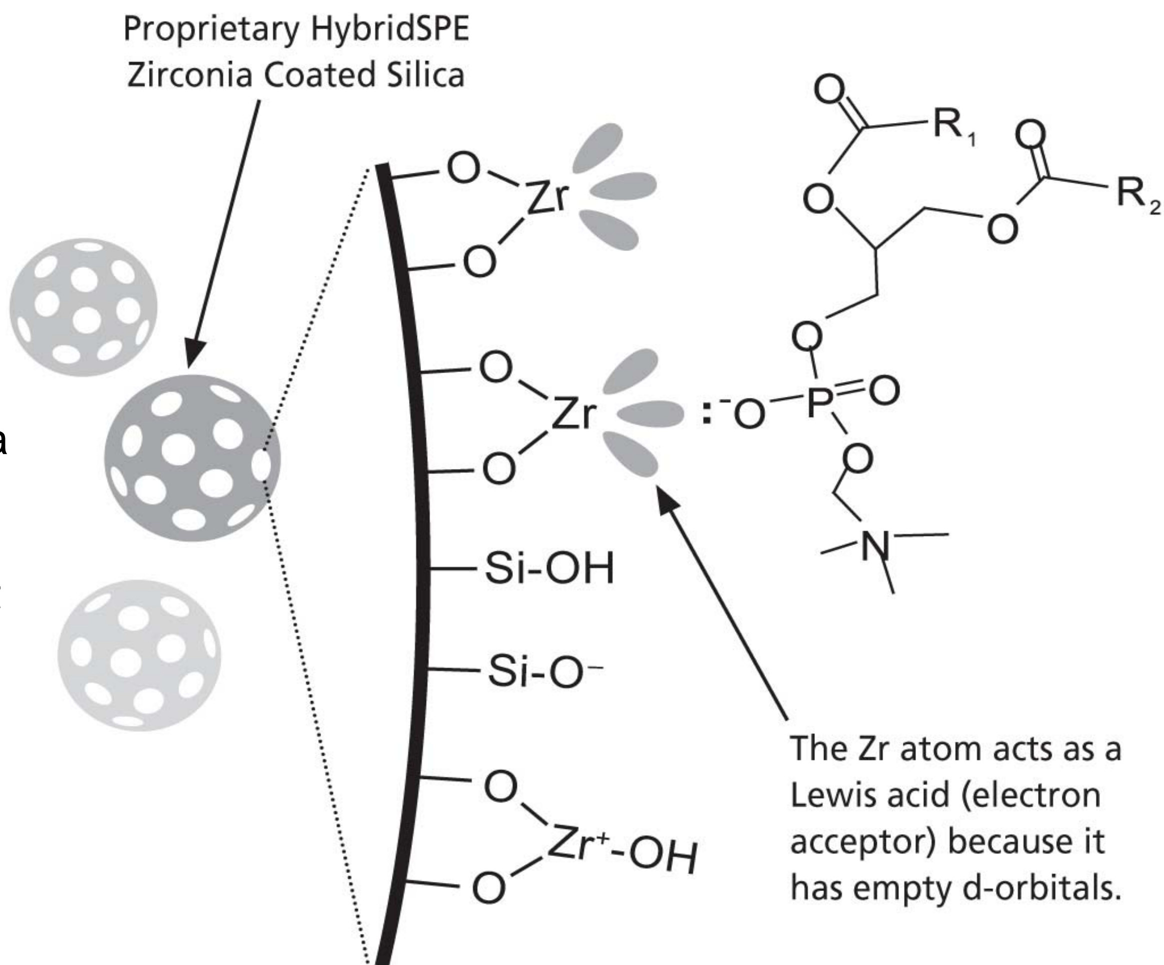
- Figure 1 shows the overlay of extracted ion chromatograms for clenbuterol and phospholipid transitions after standard PPT. The blue trace represents the extracted ion chromatogram for the clenbuterol transition of 277.2/203.1 of (+) and (-) enantiomers at a 50 ng/mL spiked concentration. The red trace represents the extracted ion chromatogram of the transition 184/104 for phospholipids. This transition represents fragmentation of the polar head group of the phospholipids from its long hydrocarbon tail.
- The clenbuterol enantiomers and phospholipids elute in the same retention window. The co-retention of phospholipids caused severe ion-suppression of the (-) clenbuterol enantiomer.

HybridSPE-PPT Protocol:

- Rat plasma samples were spiked at a concentration of 50 ng/mL with (+/-) clenbuterol standard.
- A 1 mL aliquot of spiked plasma was combined with 3 mL of 1% formic acid in acetonitrile and agitated for 30 sec. The mixture was centrifuged for 3 min. at 15,000 rpm.
- A 400 µL aliquot of the supernatant was then passed through a 1 mL, 30 mg bed HybridSPE-PPT cartridge at a flow rate of 1 drop/sec.
- The eluent was collected and analyzed directly.

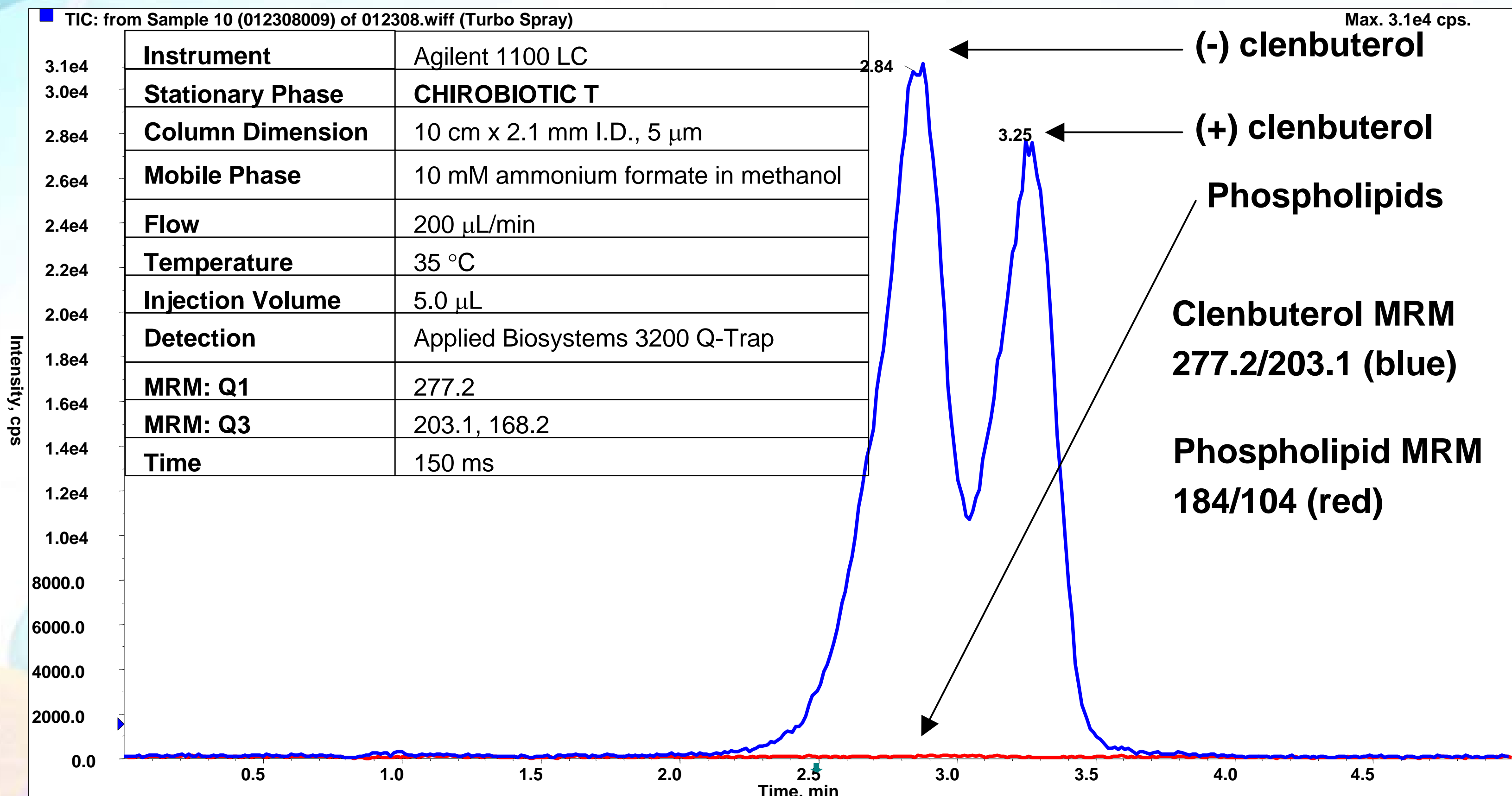
The selective extraction of phospholipids is achieved using a novel zirconia-coated particle technology.

- The high selectivity towards phospholipids is achieved utilizing Lewis acid/base interaction between the phosphate group of the phospholipids and the zirconia surface.
- The zirconia-coated particle is not as Lewis “acidic” as pure zirconium oxide, thus enabling highly efficient extraction of phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.



Interaction between a representative phospholipid and the zirconium surface of the HybridSPE-PPT particle via Lewis acid-base interaction.

Figure 2. Overlay of Clenbuterol and Phospholipid XICs after HybridSPE-PPT



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MRM: Q1	277.2
MRM: Q3	203.1, 168.2
Time	150 ms

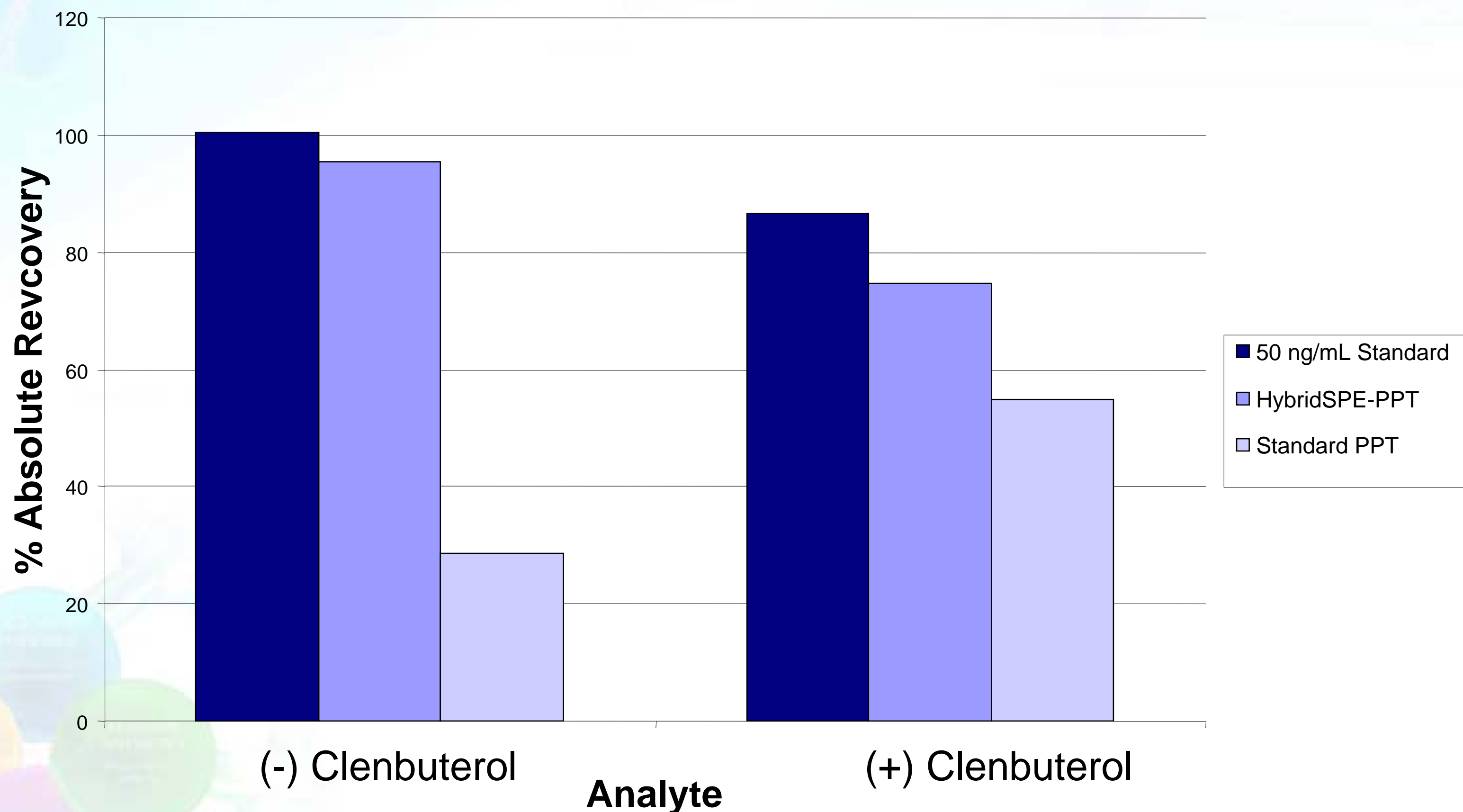
Results

Figure 2 shows the overlay of extracted ion chromatograms for both clenbuterol and phospholipid transitions using the HybridSPE-PPT protocol. The blue trace depicts the extracted ion chromatogram for the clenbuterol MRM transition. The red trace depicts the extracted ion chromatogram for phospholipids. No phospholipids were observed due to depletion using the HybridSPE-PPT protocol, only background noise is observed. The removal of phospholipids from the sample resulted in no ion-suppression of either clenbuterol enantiomer.

Qualitative Discussion

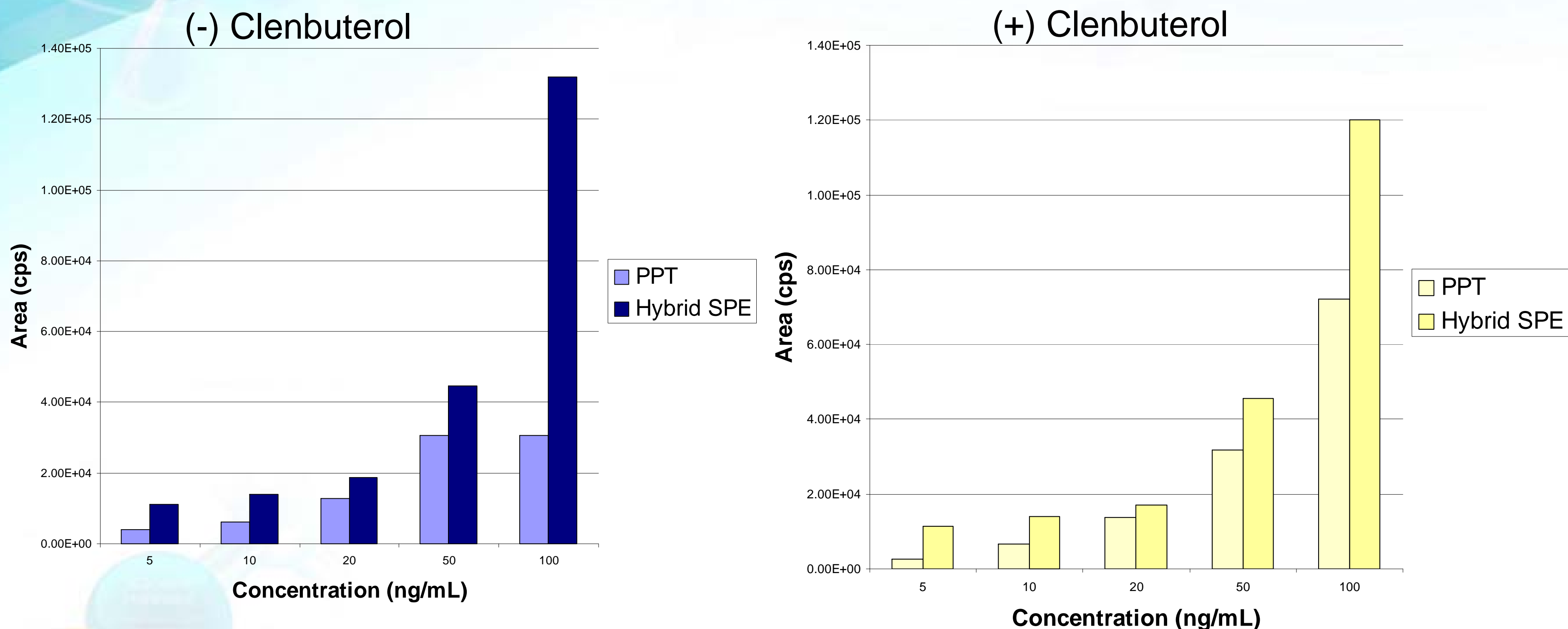
- Clearly, samples prepared using standard PPT resulted in severe ion-suppresion of the (-) clenbuterol enantiomer. Samples prepared using the HybridSPE-PPT resulted in no ion-suppression due to depletion of phospholipids from the sample.
- When comparing the two techniques, there is approximately 60% loss in signal of the (-) clenbuterol in the standard PPT sample due to co-retained phospholipids. This impact was significant, even at the relatively high 50 ng/mL concentration of clenbuterol.
- To further determine the impact of phospholipid ion-suppression, a quantitative experiment was conducted to evaluate recoveries of the analyte from the two sample prep techniques.

Figure 3. Absolute Recovery Comparison of Clenbuterol using Standard PPT and HybridSPE-PPT Methods



- In Figure 3, standard solutions prepared with the HybridSPE-PPT showed little loss of analyte to the stationary phase, with no loss of (-) clenbuterol and 13.3 percent loss of (+) clenbuterol.
- Spiked plasma samples processed using the HybridSPE showed 86.7 and 74.7% respective recoveries for the (-) and (+) clenbuterol enantiomers.
- Spiked plasma samples processed using standard PPT showed respective recoveries of 28.6 and 54.9% for the (-) and (+) clenbuterol enantiomers.
- The recovery of (-) clenbuterol using protein precipitation is 51.8% less than the HybridSPE-PPT method.
- To further investigate the effects of phospholipid ion-suppression, an experiment was conducted over a concentration range to determine if ion-suppression has a greater affect at lower levels and may affect limits of quantitation.

Figure 4. Peak Areas of (-/+) Clenbuterol for Standard PPT and HybridSPE-PPT Samples over a Concentration Range



Rat plasma samples were spiked at concentrations of 5, 10, 20, 50, and 100 ng/mL with (+/-) clenbuterol and prepared using both standard PPT and HybridSPE-PPT protocols.

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- Figures 4 show the comparison of peak areas between samples prepared using protein precipitation and the HybridSPE-PPT technique for both clenbuterol enantiomers.
- There is a trend of greater signal suppression at lower concentration ranges.
- This trend is important for samples and applications that require low limits of detection and quantitation from biological matrices.

Conclusion

- A comparison of sample preparation techniques demonstrates the severity of phospholipid ion-suppression on the recovery of (-) clenbuterol when using protein precipitation.
- The HybridSPE-PPT protocol removed phospholipid interference and greatly improves analyte response.
- Quantitation reveals little loss of analyte to the HybridSPE-PPT stationary phase and nearly full recovery of the analyte using the HybridSPE-PPT protocol.
- Experiments demonstrated greater signal suppression at lower concentration levels of analyte, affecting levels of detection and quantitation.