

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

Carmen T. Santasania, Craig R. Aurand, J.T. Lee, and David S. Bell
Supelco, Div. of Sigma-Aldrich, Bellefonte, PA 16823

Daniel L. Shollenberger
The Pennsylvania State University, Department of Chemistry,
University Park, PA 16802

Abstract

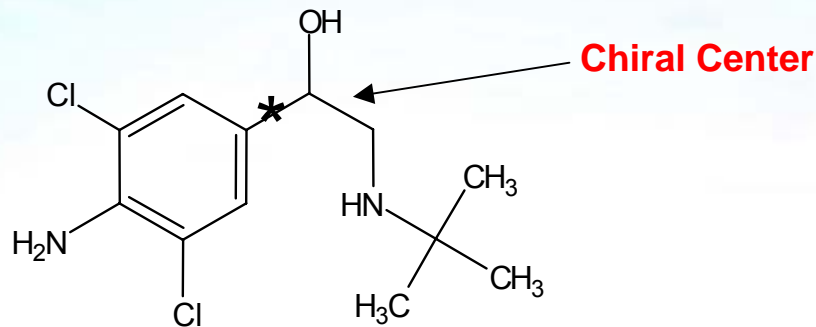
Ion-suppression due to the presence of phospholipids has become a major concern in reversed-phase LC-MS analysis due to trends toward faster chromatographic separations and lower limits of detection. The co-extracted phospholipids are well documented to be detrimental in reversed phase LC-MS applications, 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. Sample preparation techniques used prior to analysis are often time consuming or do not sufficiently remove endogenous phospholipids from plasma samples.

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 protocol along with a newly developed HybridSPE™ protocol designed to simultaneously remove both proteins and phospholipids. Comparisons of sample preparation methods were measured in terms of phospholipids content in the sample extract and the overall effect on signal response of clenbuterol enantiomers. The analyses were performed on the Applied Biosystems 3200 Q Trap Linear Ion trap with MRM transitions to monitor both matrix phospholipids and clenbuterol.

Introduction

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



(+/-)-clenbuterol

- Adrenergic β 2-agonist used for the treatment of pulmonary diseases.
- Administered as a racemic mixture, contains one chiral center.
- Analytical chiral separation of this molecule is useful in determining pharmacological properties and environmental hazards.

Experimental

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.

LC-MS-MS Conditions

Instrument	Agilent 1100 LC
Stationary Phase	CHIROBIOTIC T
Column Dimension	10 cm x 2.1 mm I.D., 5 μ m
Mobile Phase	10 mM ammonium formate in methanol
Flow	200 μ L/min
Temperature	35 $^{\circ}$ C
Injection Volume	5.0 μ L
Detection	Applied Biosystems 3200 Q-Trap
Curtain Gas	35.0 psi
IS Voltage	3,200 eV
Temperature	425 $^{\circ}$ C
Gas 1	45.0 psi
Gas 2	40.0 psi
MRM: Q1	277.2
MRM: Q3	203.1, 168.2
Time	150 ms

Figure 1. Clenbuterol XIC for Spiked Plasma after Protein Precipitation

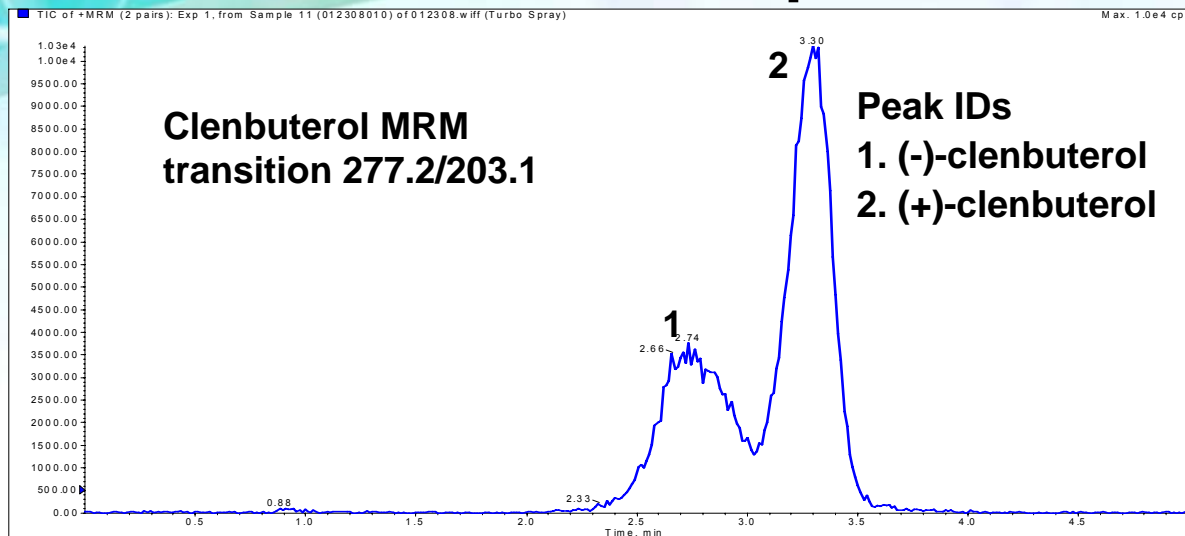


Figure 1a. Phospholipids XIC for Spiked Plasma after Protein Precipitation

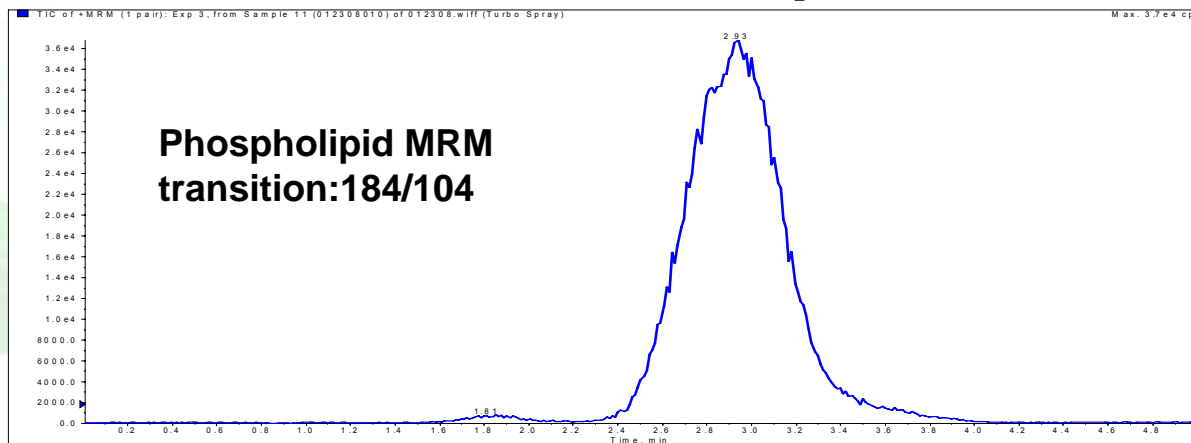
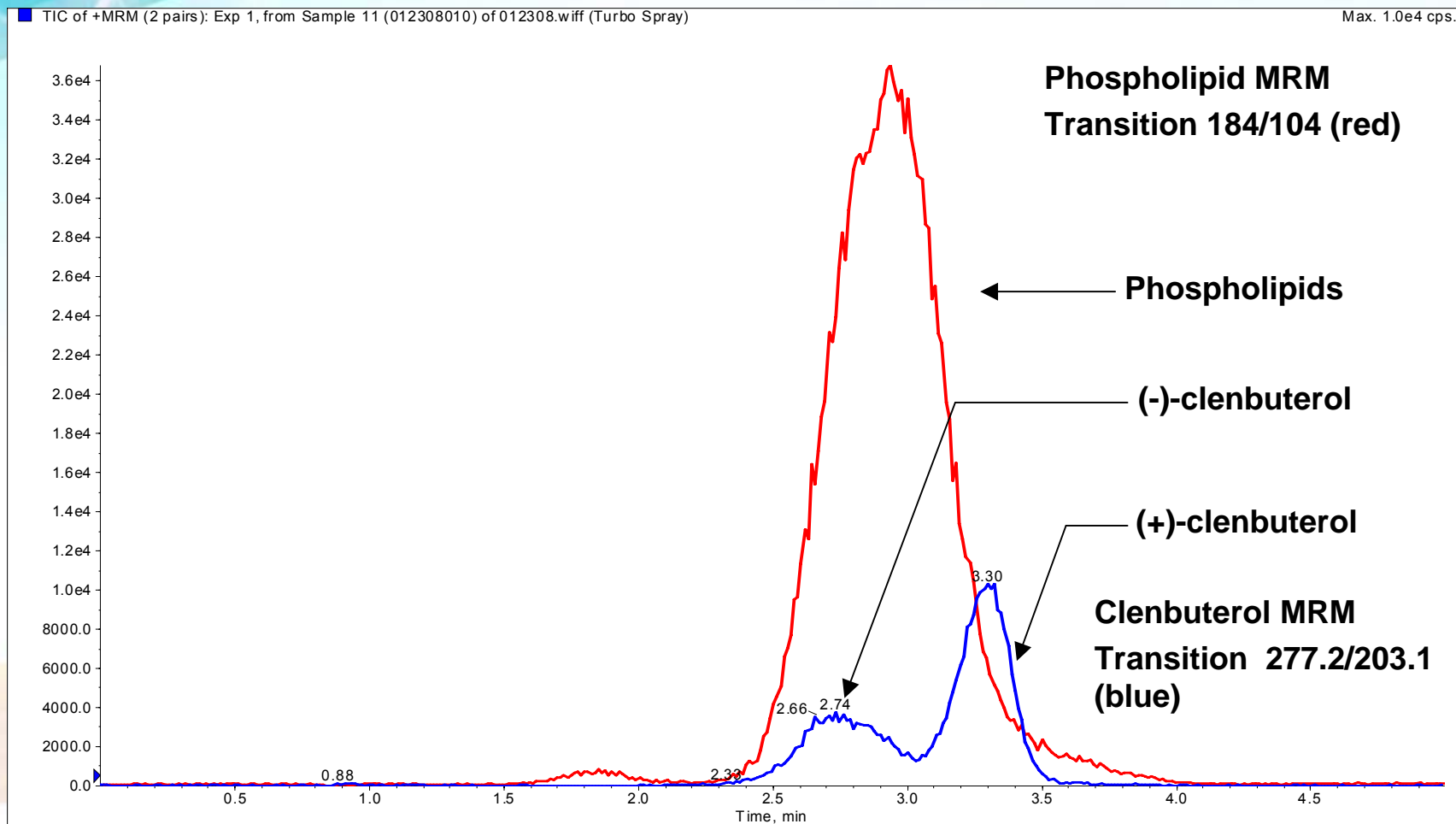


Figure 2. Overlay of Phospholipid and Clenbuterol XICs



Results of Protein Precipitation Method

- Figure 1 shows the extracted ion chromatogram for the clenbuterol transition of 277.2/203.1 of (+) and (-) enantiomers after protein precipitation at 50 ng/mL. The polarity was determined by analysis of a standard solution on a commercial chiralizer.
- Figure 1a shows the extracted ion chromatogram of the transition 184/104 for phospholipids. This transition represents fragmentation of the polar head group of phosphatidylcholine from its hydrocarbon tails.
- Figure 2 shows the overlay of extracted ion chromatograms for both Figures 1 and 1a. The clenbuterol enantiomers and phospholipids elute in the same retention window. The co-retention of phospholipids caused severe ion-suppression of the (-)-clenbuterol enantiomer.

Results of Protein Precipitation Method (contd.)

HybridSPE 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 μ L aliquot of the supernatant was then passed through a 1 mL, 30 mg bed HybridSPE cartridge at a flow rate of 1 drop/sec.
- The eluant was collected and analyzed directly.

HybridSPE Protocol

- Combines the simplicity of protein precipitation with the selectivity of solid phase extraction to remove phospholipids.
- Enables particulate removal of a filtration plate with the added chemical filtering of solid phase extraction.
- Selective extraction of phospholipids is achieved using a novel zirconia-coated particle technology. This high selectivity towards phospholipids is achieved by utilizing the Lewis acid/base interaction between the phosphate group of the phospholipids and the zirconia surface.
- The zirconia-coated particle exhibits a weaker Lewis acidity than pure zirconium oxide enabling highly efficient extraction of phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.

Figure 3. Clenbuterol XIC for Spiked Plasma after Protein Precipitation and HybridSPE

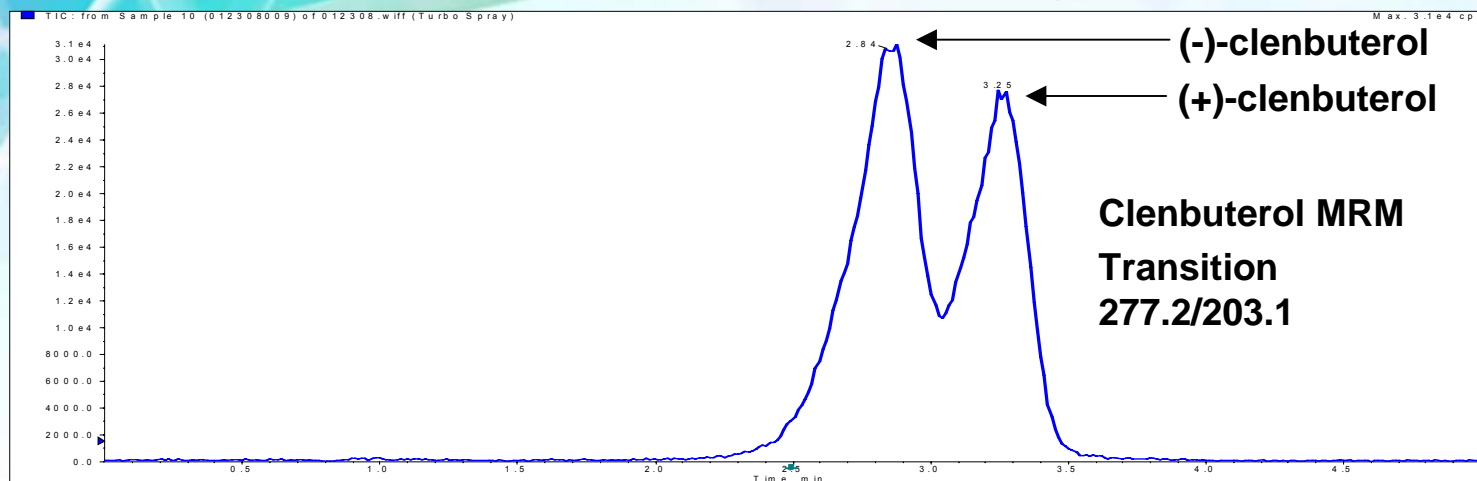


Figure 3a. Phospholipid XIC for Spiked Plasma after Protein Precipitation and HybridSPE

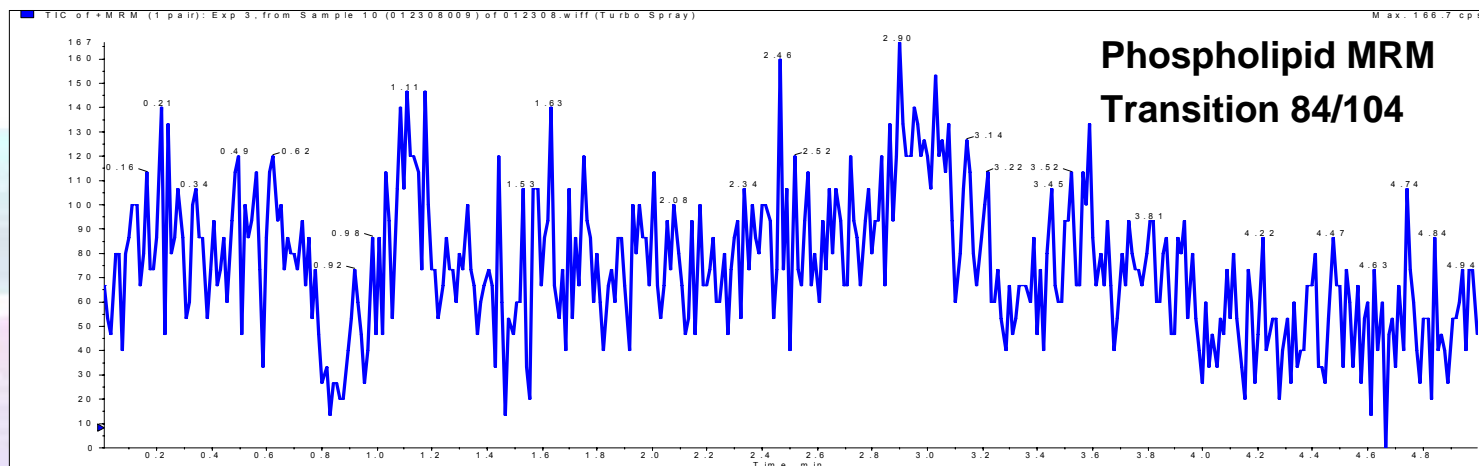
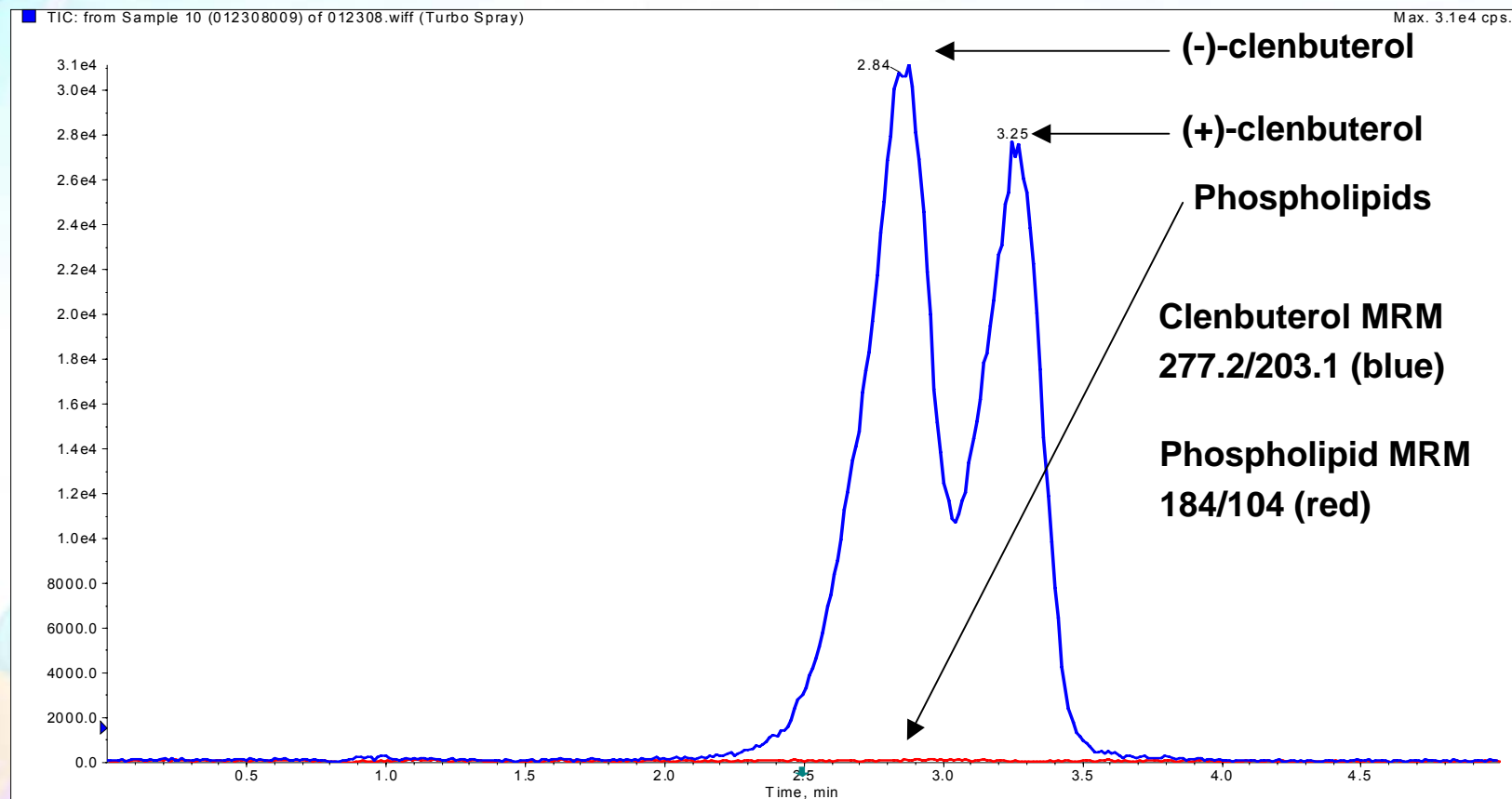


Figure 4. Overlay of Clenbuterol and Phospholipid XICs after Protein Precipitation and HybridSPE



Results of HybridSPE-PPT Cleanup

- Figure 3 shows the extracted ion chromatogram for the clenbuterol MRM transition after being processed using the HybridSPE protocol.
- Figure 3a shows the extracted ion chromatogram for phospholipids. No phospholipids were observed due to depletion from the HybridSPE protocol. Only background noise is observed.
- Figure 4 shows the overlay of extracted ion chromatograms for both Figures 3 and 3a. The removal of phospholipids from the sample has resulted in no ion-suppression of the (-) clenbuterol enantiomer.

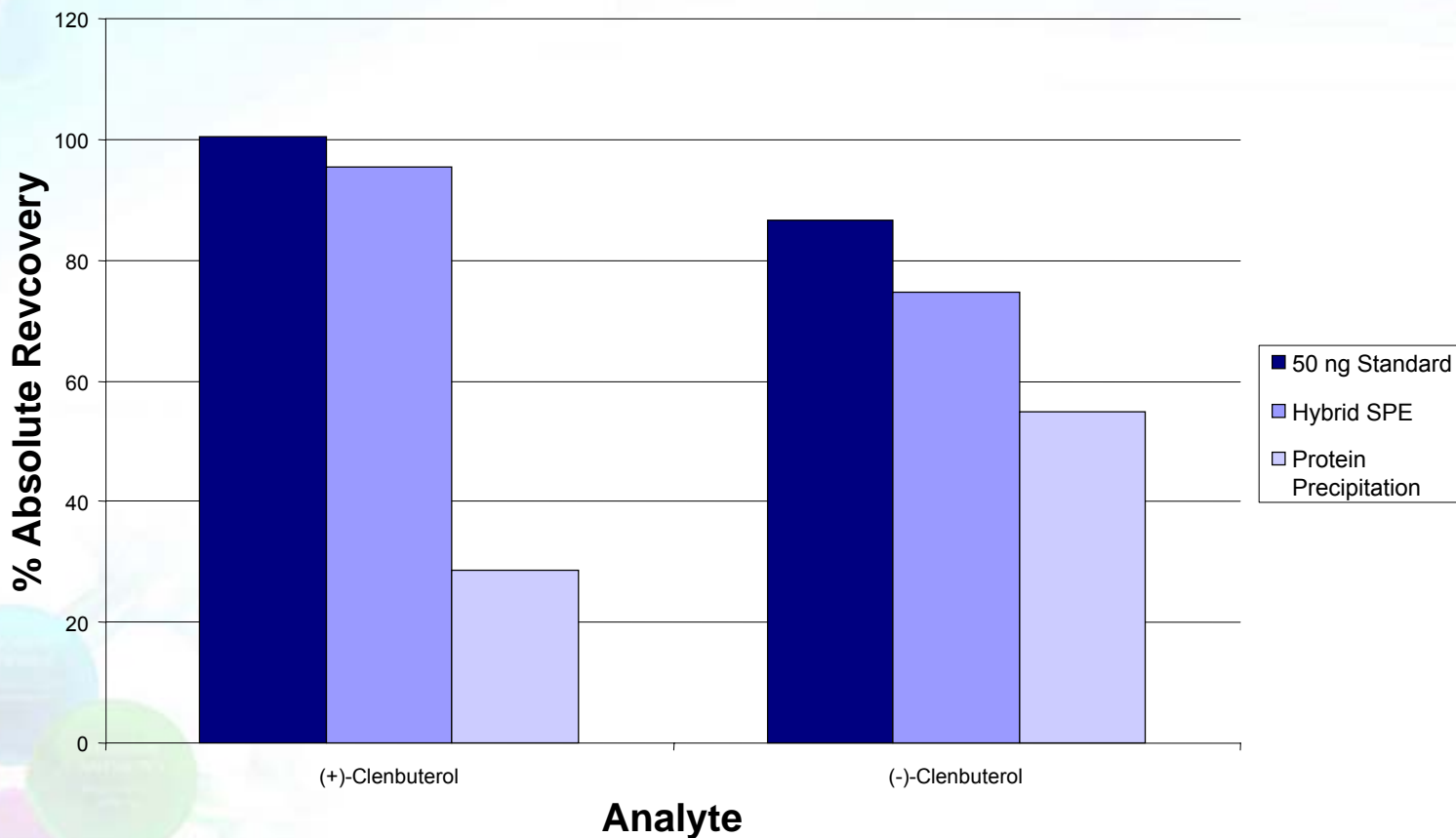
Qualitative Discussion

- Clearly samples prepared using standard protein precipitation resulted in severe ion-suppression of the (-)-clenbuterol enantiomer. Samples prepared using the HybridSPE 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 protein precipitation 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 phospholipids ion-suppression, a quantitative experiment was conducted to evaluate recoveries of the analyte from the two sample prep techniques.

Quantitative Experiment

- Rat plasma spiked at 50ng/ml clenbuterol was prepared.
- A standard stock solution of 50ng/mL in mobile phase was also prepared.
- Samples and standards were prepared using both standard protein precipitation and the HybridSPE protocol.
- Absolute recovery was determined using external standards for both spiked plasma and standard solutions.

Figure 5. Absolute Recovery Comparison of Clenbuterol using PPT and Hybrid SPE Methods



Recovery Discussion

- Standard solutions prepared with the HybridSPE 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.
- Spike plasma samples processed using protein precipitation showed respective recoveries of 28.6 and 54.9% for the (-) and (+) clenbuterol enantiomers.
- There is a 51.8% less recovery of (-)-clenbuterol using protein precipitation compared to the HybridSPE 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 effect at lower levels that may effect limits of quantitation.

Concentration Effect Experiment

- Rat plasma samples were spiked at a concentrations of 5, 10, 20, 50, and 100 ng/mL with (+/-) -clenbuterol standard.
- Samples were prepared using both HybridSPE protocol and standard protein precipitation.
- A relative recovery comparison was made between the two techniques.
- Charts were prepared to graphically represent the difference in sample prep techniques.

Figure 6. Peak Areas of (-)-Clenbuterol for PPT and Hybrid SPE Samples over a Concentration Range

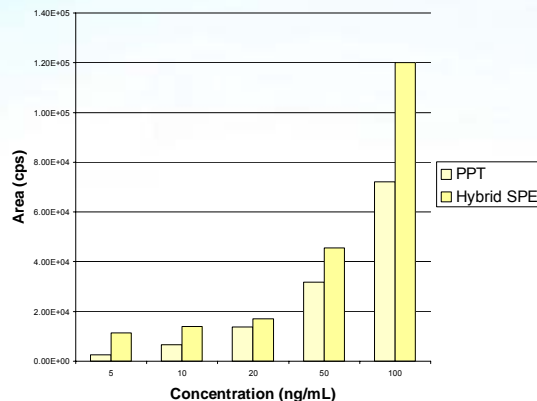


Figure 6a. Peak Areas of (+)-Clenbuterol for PPT and Hybrid SPE Samples over a Concentration Range

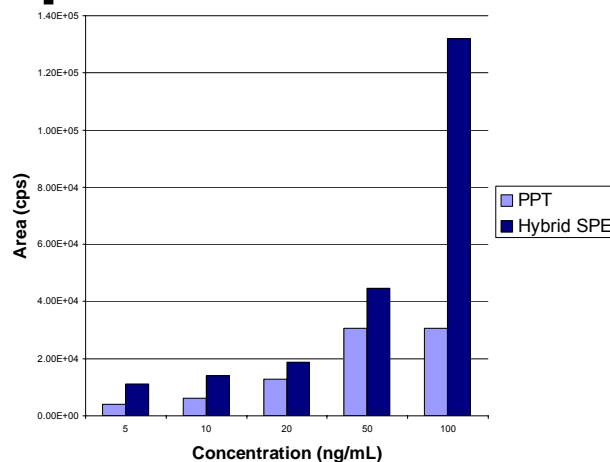


Table 2. Difference in Sample Response between PPT and SPE Samples over a Concentration Range

Concentration (ng/mL)	(-)-Clenbuterol			(+) -Clenbuterol		
	HybridSPE Area	PPT Area	% difference	HybridSPE Area	PPT Area	% difference
5	1.13E+04	2.66E+03	76.5	1.11E+04	4.13E+03	62.8
10	1.41E+04	6.70E+03	52.5	1.39E+04	6.20E+03	55.4
20	1.72E+04	1.38E+04	19.8	1.87E+04	1.29E+04	31
50	4.56E+04	3.18E+04	30.3	4.47E+04	3.06E+04	31.5
100	1.20E+05	7.21E+04	39.9	1.32E+05	3.06E+04	77

Concentration Discussion

- Figures 6 and 6a show the comparison of peak areas between samples prepared using protein precipitation and the HybridSPE technique for both clenbuterol enantiomers.
- The percent difference appears to be concentration dependent.
- 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 protocol removed phospholipid interference and greatly improves analyte response.
- Quantitation reveals little loss of analyte to the Hybrid SPE stationary phase and nearly full recovery of the analyte using the HybridSPE protocol.
- Experiments demonstrated greater signal suppression at lower concentration levels of analyte, affecting levels of detection and quantitation.