Robust LC/MS Application for Bioanalysis of Pain Management Drugs

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Introduction

For efficient therapeutic drug monitoring, it is important for clinicians to have access to fast/ robust analytical methods for accurate assessment of drug efficacy. Industrial trends toward highly specific LC/MS applications over traditional ELISA type immunoassay has resulted in the need for high-speed chromatographic assays along with simplified sample preparation methods. Often the limitation of a bioanalytical technique is based upon the effectiveness of the sample preparation technique. Plasma and serum samples are often suspect to assay irregularities due to matrix induced interferences. In this study a robust bioanalytical method is described utilizing a combination of fast chromatographic separation along with a novel sample preparation platform for the analysis of pain management drug tapentadol and associated metabolites.

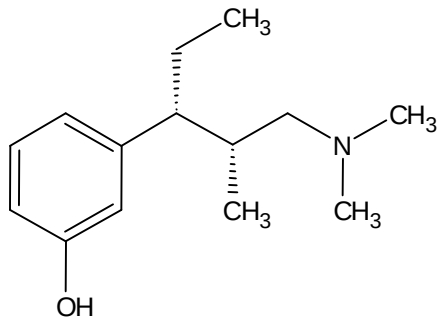
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Method

The focus of this study is to develop a bioanalytical assay for tapentadol and associated metabolites from plasma samples. The goal was to develop a robust analytical technique that facilitates high throughput application with simplified sample processing. The first portion of the study was to develop chromatographic conditions for resolution of tapentadol and associated metablites. The polar basic characteristics of these compounds make them prime targets for HILIC chromatographic separation. Because HILIC mobile phases consist of a high composition of acetonitrile, this can also facilitate the direct analysis of precipitated plasma samples without the need for additional sample solvent exchange. In most cases, the high organic mobile phase also facilitates increased analyte response in ESI+ MS detections.

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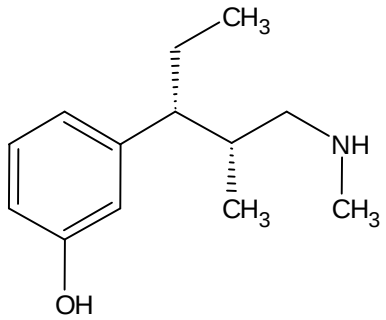
Tapentadol & Metabolites



Tapentadol [*{INN}]

Monoisotopic Mass = 221.177964 Da

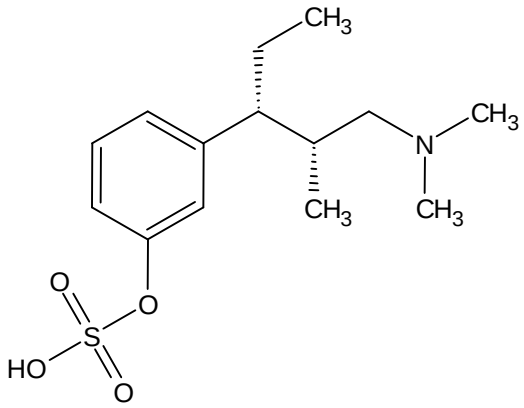
Cerilliant T-058 (as free base)



Desmethyl Tapentadol

Monoisotopic Mass = 207.162314 Da

Cerilliant D-052



Tapentadol-O-sulfate

Monoisotopic Mass = 301.134778 Da

Cerilliant T-061 (as free sulfate)

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Chromatographic Conditions

system:	Agilent 1290, 6210 TOF
column:	Ascentis Express HILIC, 10 cm x 2.1 mm, 2.7 µm (53939-U)
mobile phase:	(A) 5 mM ammonium formate; (B) 5 mM ammonium formate acetonitrile
ratio:	A:B (5:95)
flow rate:	0.6 mL/min
temp.:	50 ºC
injection:	2 µL
MS det.:	ESI+, 100-1000m/z

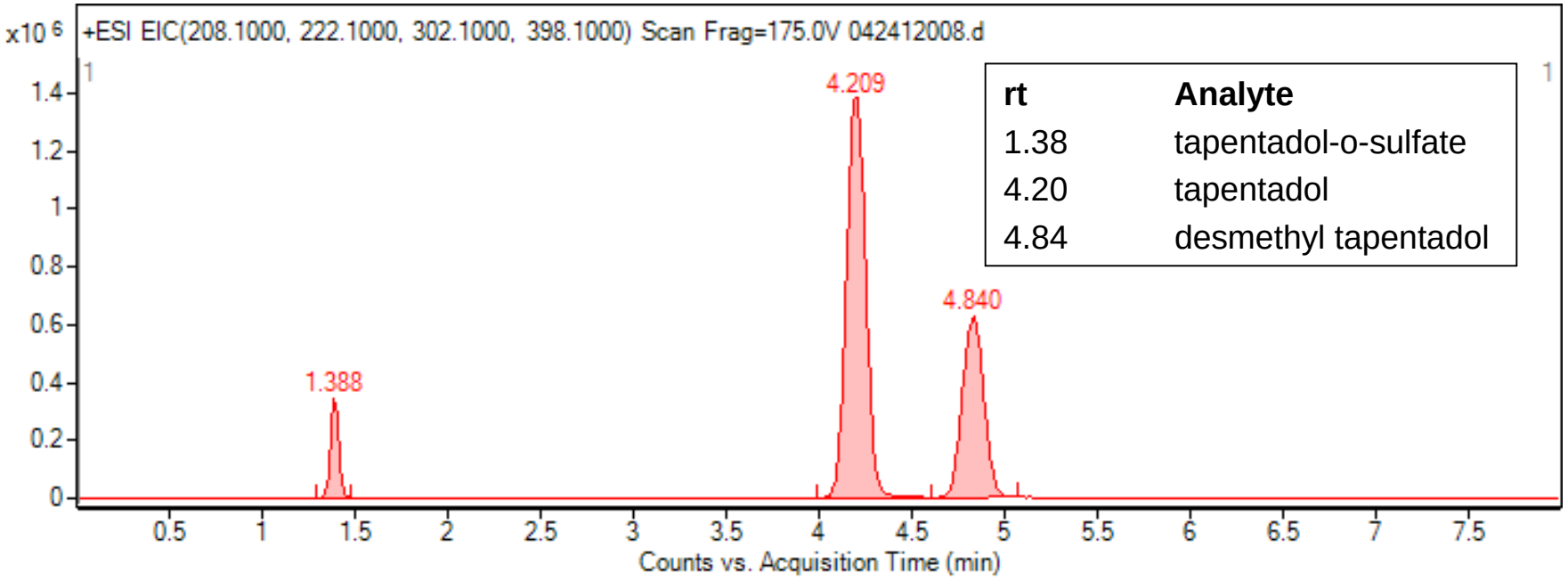
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Figure 1 depicts the efficient separation of tapentadol and associated metabolites under HILIC conditions on the Ascentis® Express HILIC (Si) column. One area that is often missed when developing a HILIC bioanalytical method is the impact of solvent mismatch between the sample composition and mobile phase. For precipitation of proteins from plasma and serum samples, it is common place to utilize a 3:1 ratio of organic crash solvent to sample. As depicted in Figure 2, there is sufficient resolution of tapentadol and associated metabolites but the use of 3:1 organic:sample as a sample solvent resulted in significant peak distortion. For HILIC conditions, the relative high aqueous content (25%) of the sample solvent acts as a strong solvent resulting in poor focusing of the analytes onto the column. To correct for this phenomenon, the organic composition of the sample was increased to 4:1 organic:sample. This increase in organic resulted in better peak shape and a more rugged bioanalytical method.

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Figure 1. Tapentadol and Metabolites on Ascentis Express HILIC



rt	Analyte
1.38	tapentadol-o-sulfate
4.20	tapentadol
4.84	desmethyl tapentadol

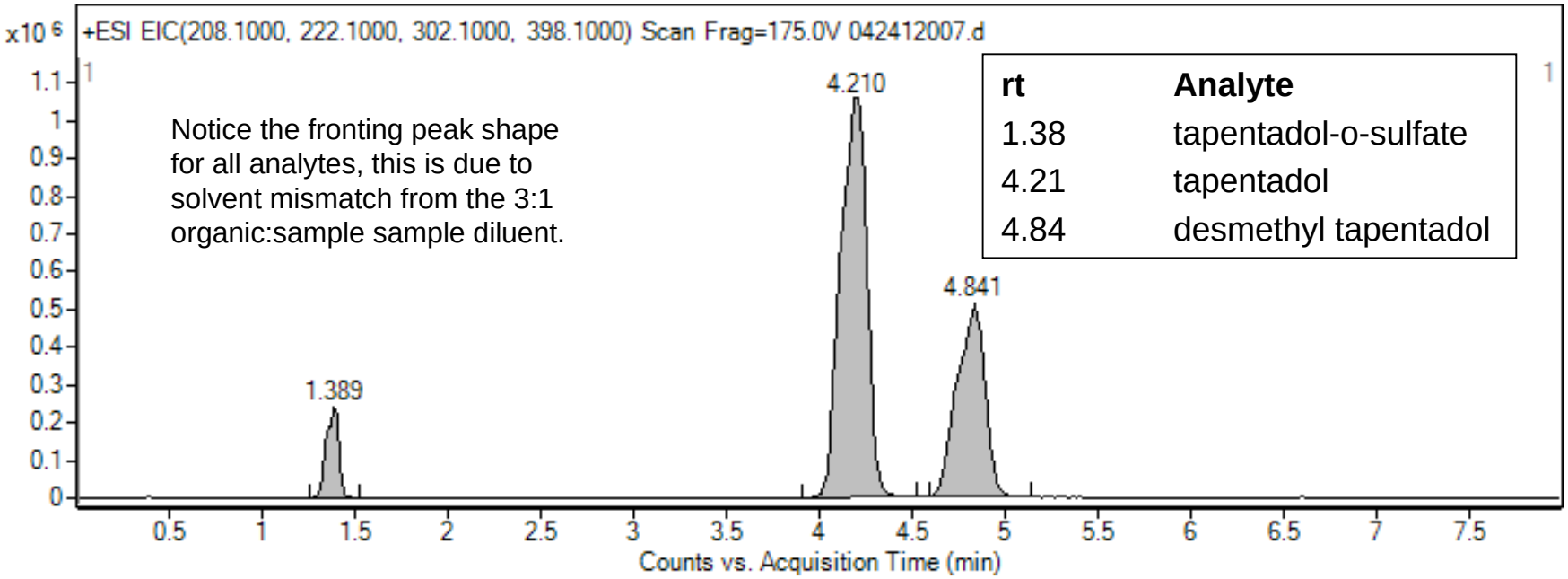
5 mM ammonium formate (95:5 acetonitrile:water)

0.6 mL/min, 50 °C

Standard concentration at 300 ng/mL in 80:20 (1% formic acid acetonitrile:water) 4:1 (organic:sample)

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Figure 2. Tapentadol and Metabolites on Ascentis Express HILIC



Notice the fronting peak shape for all analytes, this is due to solvent mismatch from the 3:1 organic:sample sample diluent.

rt	Analyte
1.38	tapentadol-o-sulfate
4.21	tapentadol
4.84	desmethyl tapentadol

Standard concentration at 300 ng/mL in 75:25 (1% formic acid acetonitrile:water) 3:1 (organic:sample)

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Method

Once the proper chromatographic and sample conditions had been optimized, the second portion of this study was to identify the sample processing. Often when dealing with plasma samples, endogenous matrix such as phospholipids can cause irreproducibility in quantitation due to ionization-suppression effects. Co-elution of endogenous matrix with target analytes can result in arbitrary decrease response in target analytes, thus decreasing the overall accuracy of the method. In this study the targeted depletion of phospholipid matrix interference was achieved using novel HybridSPE®-Phospholipid 96-well Plate. Plasma samples spiked with tapentadol and associated metabolites were prepared using the HybridSPE-Phospholipid sample prep technique and assayed using the HILIC method. With the knowledge from the chromatographic method development, a 4:1 ratio of oranic:sample was used to facilitate the protein precipitation.

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Sample Preparation

Standard Solutions:

Standard solutions were prepared from a stock standard in (4:1) 1% formic acid acetonitrile:water at a level of 10, 50, 100, 200, 300, 500 ng/mL.

An internal standard of 375 ng/mL of tapentadol d-3 was prepared in 1% formic acid acetonitrile. This was then used as the protein precipitation solution.

Plasma:

Rat plasma stabilized with K_2EDTA was acquired from Lampire Biological Laboratories, (Pipersville PA). Plasma was spiked directly from stock standard to a level of 500 ng/Ml.

Standard Recovery: apply 500 µL of tapentadol standards prepared at 100 ng/mL in (4:1) 1% formic acid acetonitrile:water to the HybridSPE-Phospholipid well plate. Place on vacuum manifold and apply 10”Hg vacuum for 4 minutes, collect filtrate and analyze directly.

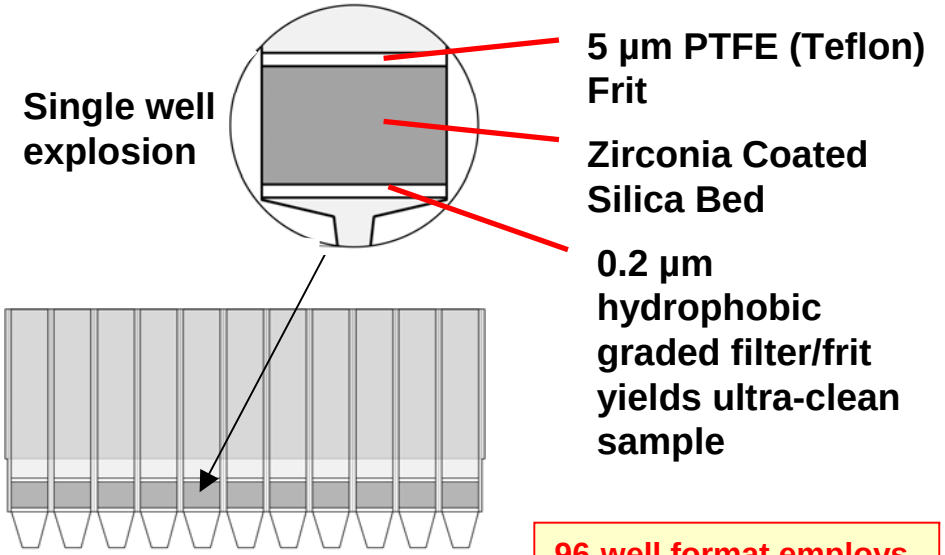
HybridSPE-Phoshoplipid 96-Well Plasma Samples: apply 100 µL of plasma to plate, followed by 400 µL of 1% formic acid acetonitrile. Agitate via vortex for 4 minute, place on vacuum manifold and apply 10”Hg vacuum for 4 minutes. Collect filtrate and analyze directly.

Concentration of final sample work up in both techniques is equivalent to 100 ng/mL.

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Figure 3. Sample Process for HybridSPE-Phospholipid 96-well Plate

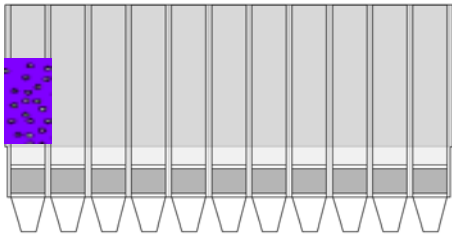




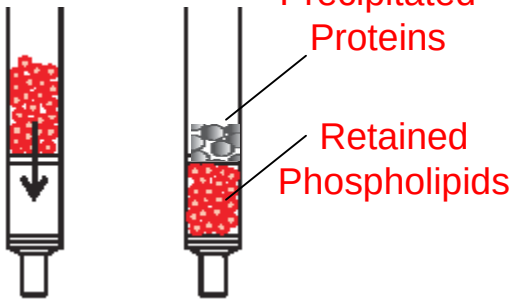
96-well format employs special frits at the top and bottom of the same selective bed; proteins *can be removed on-line* for added speed and convenience.

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In-Well Precipitation Schematic for HybridSPE-Phospholipid …96-well Format



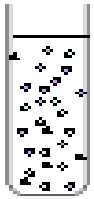




1) Precipitate Proteins: Add 100 µL plasma/serum to the HybridSPE plate followed by 300 µL 1% formic acid in acetonitrile. Add I.S. as necessary. Note: the upper PTFE frit keeps plasma from dripping through packed-bed prematurely.

2) Mix by vortexing HybridSPE plate or by aspirating/dispensing with 0.5-1 mL pipette tip.

3) Apply vacuum. Packed-bed filter/frit assembly acts as a depth filter for the concurrent physical removal of precipitated proteins and chemical removal phospholipids. Small molecules (e.g., pharma compounds and metabolites) pass through unretained.

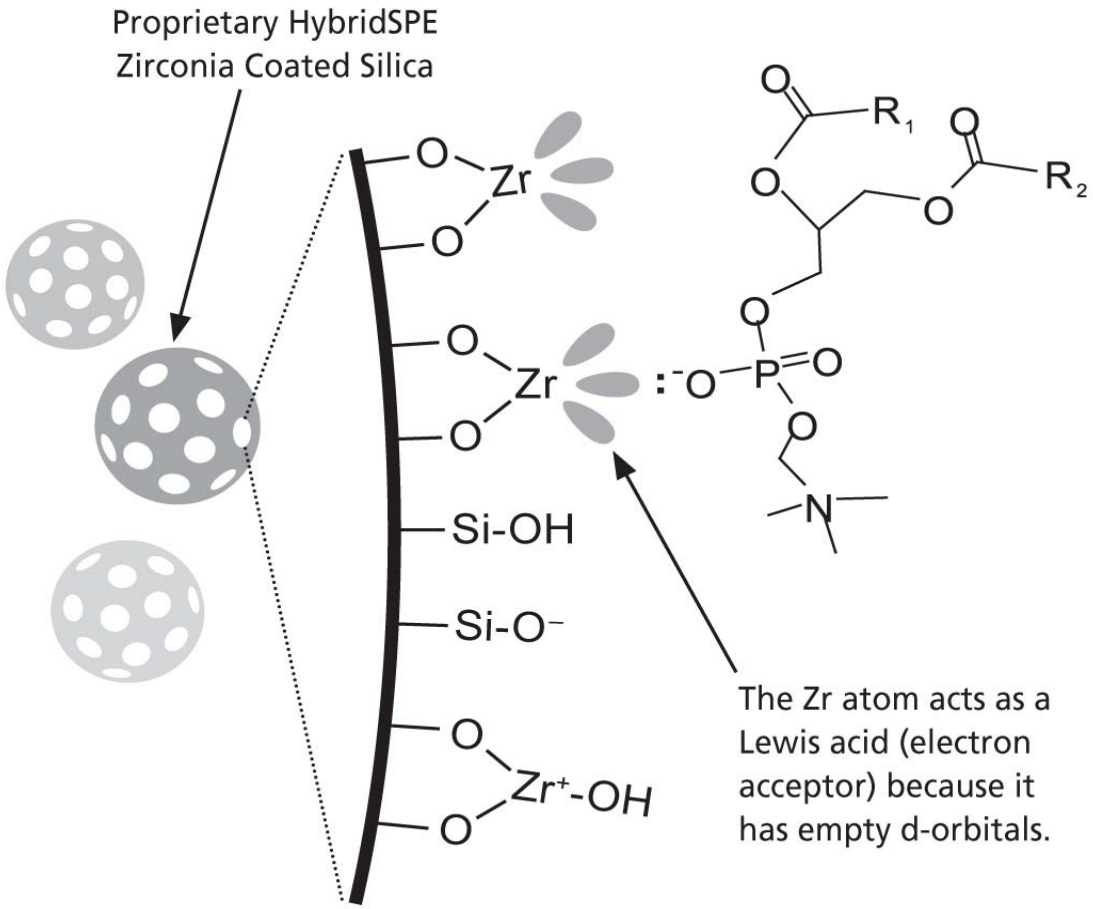


4) Resulting filtrate/eluate is free of proteins and phospholipids and ready for immediate LC-MS/MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis

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- 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.

Lewis Base	rel. Strength on Zirconia
Hydroxide	**Strongest**
Phosphate	
Fluoride	
Citrate	
Sulfate	
Acetate	
Formate	
Chloride	**Weakest**



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

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Phospholipid Monitoring

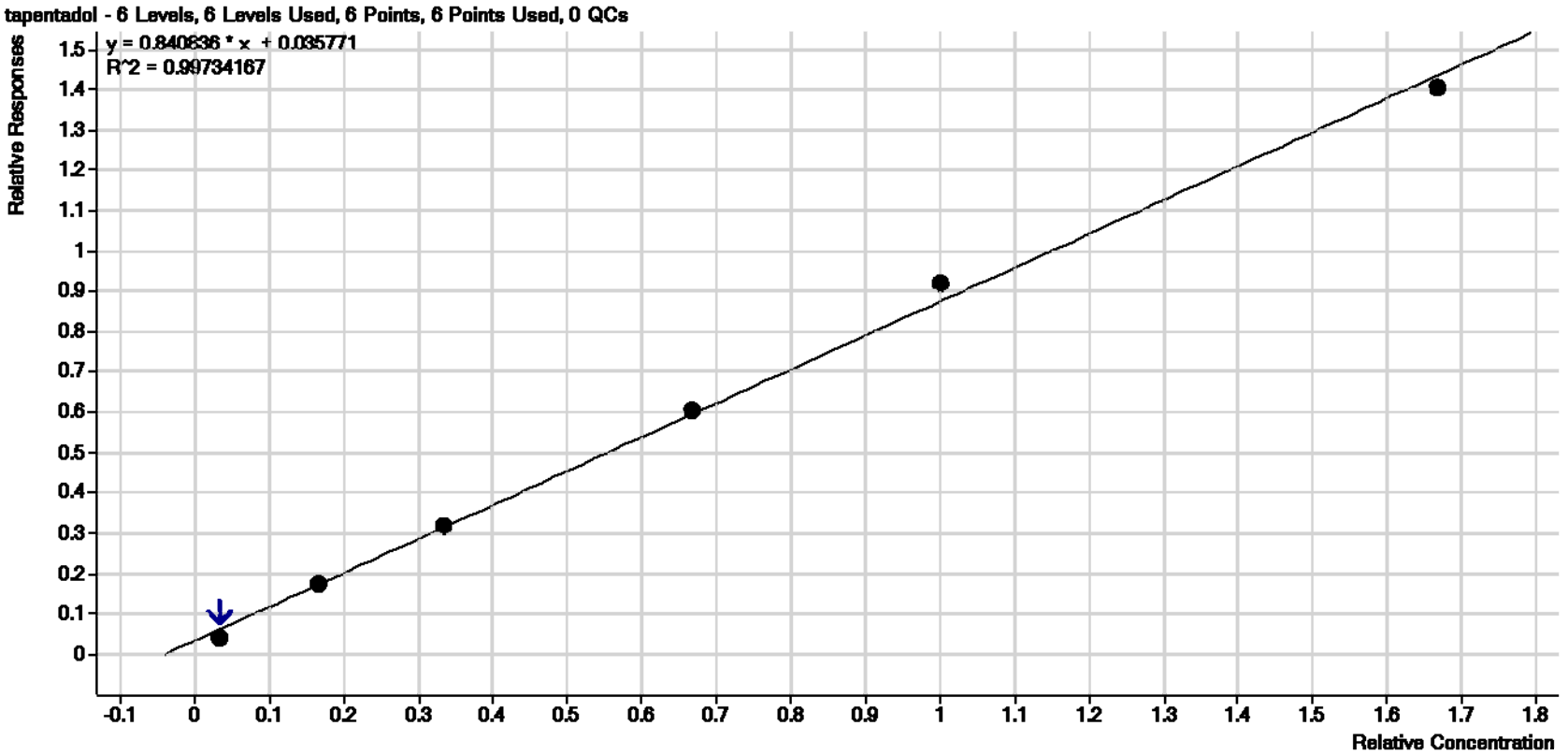
Processed spiked plasma samples were analyzed for target drug and metabolites along with associated matrix interference. Phospholipids were monitored over a range of both lyso and glycero phospholipids.

Lysophosphatidylcholines:	**m/z**
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine	496.3
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine	524.3

Glycerophosphocholines:	**m/z**
1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine	758.5
glycerophosphocholine 36:2	786.5
1-(9Z,12Z-octadecadienoyl)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine	806.5
1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine	810.5

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Figure 4. Tapentadol Calibration Curve



Analyte	R2	Slope
tapentadol-O-sulfate	0.998916	y= 0.095973x + 0.001221
desmethyl tapentadol	0.998605	Y=0.423365x + 0.015791
tapentadol	0.997341	y=0.840836x + 0.035771

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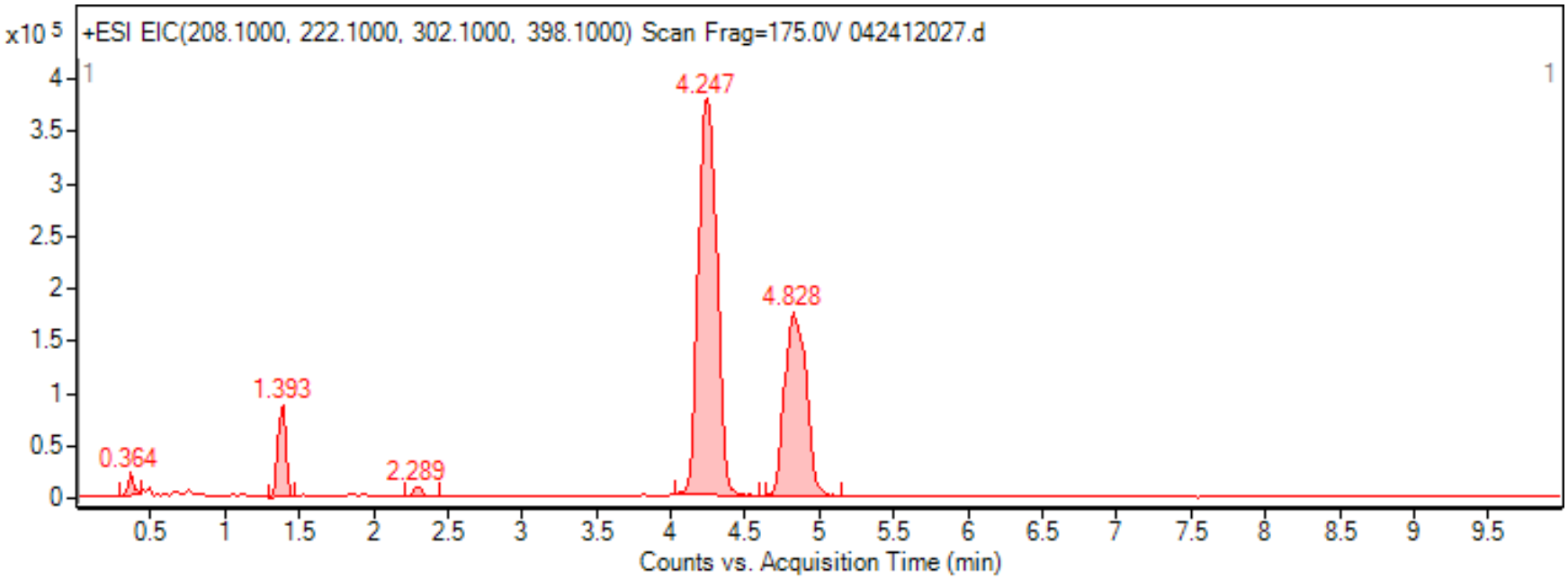
Figure 4 depicts the calibration curve for tapentadol and metabolites, demonstrating a linear response across the calibration range of 10 to 500 ng/mL for all analytes. Standard solutions along with spiked plasma samples were then processed through the HybridSPE-Phospholipid to establish analyte recovery along with sample cleanliness.

Figure 5 depicts the chromatogram for tapentadol and metabolites from the spiked plasma samples, notice there are no interfering peaks that would complicate quantitation. Figure 6 depicts the phospholipid monitoring for the spiked plasma samples processed with the HybridSPE-Phospholipid well plate, not phospholipid interference is detected using this technique.

Absolute recoveries were then measured for both standard solution and spiked plasma samples. Sample replicate of n=8 were prepared for both standard solution and spiked plasma samples.

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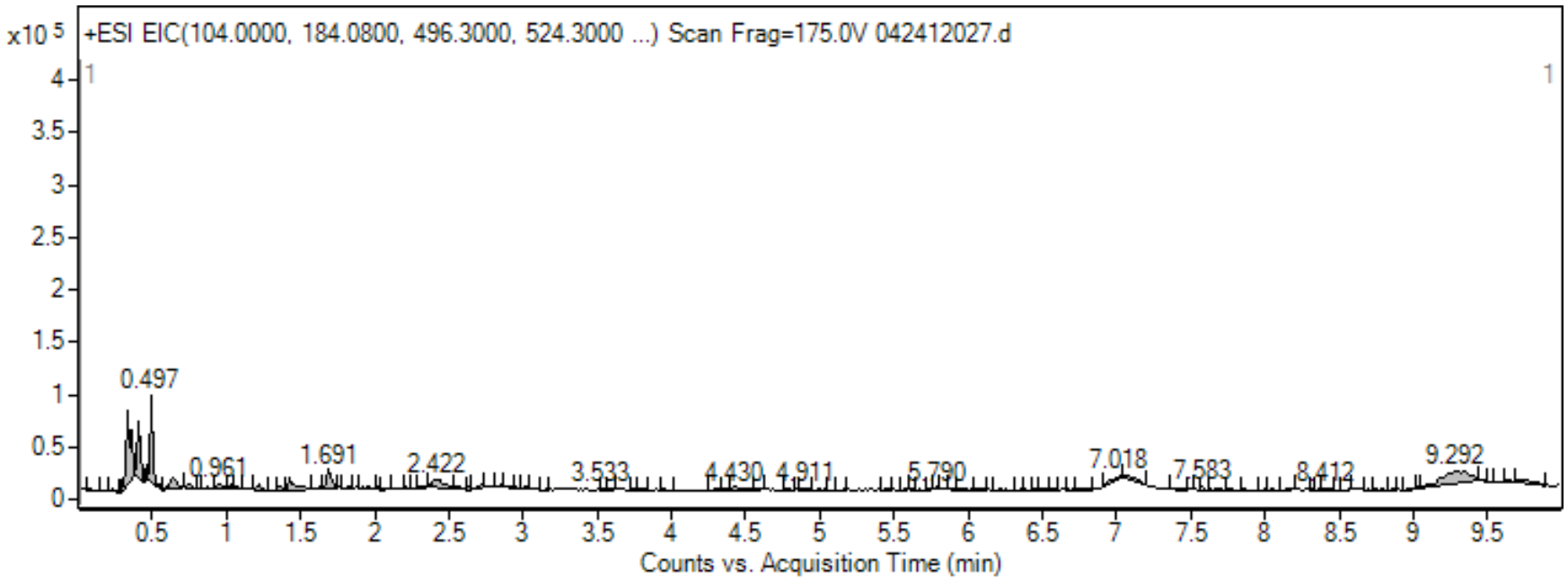
Figure 5. Tapentadol and Metabolites in Spiked Plasma, HybridSPE-Phospholipid Sample Processing



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Figure 6. Monitored Phospholipids in Spiked Plasma, HybridSPE-Phospholipid Sample Processing



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Figure 7. Recovery of Tapentadol and Metabolites from HybridSPE-Phospholipid

	Sample	Tapentadol-O-sulfate	Tapentadol	Desmethyl Tapentadol
Name	Acq. Date-Time	Calc. Conc.	Calc. Conc.	Calc. Conc.
HybridSPE STD 100 ng/mL	Average	72.59	88.55	93.88
	STDEV	0.90	0.89	5.49
	%CV	1.24	1.01	5.85
HybridSPE plasma 100 ng/mL	Average	71.17	85.31	93.00
	STDEV	5.29	1.55	1.57
	%CV	7.43	1.82	1.69

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Conclusions

- The novel sample prep technique of the HybridSPE-Phospholipid enabled a simplified sample processing with no detected phospholipid matrix interference.
- Excellent recovery of tapentadol and metabolites was observed from the HybridSPE-Phospholipid for both standard solutions and spiked plasma samples.
- The HILIC approach for analysis of the tapentadol and metabolites enabled direct analysis of processed plasma samples without the need for additional sample preparation
- The combination of the simple and efficient sample technique along with the unique selectivity of the HILIC chromatographic method resulted in a fast and robust bioanlaytical technique for pain management drugs.

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