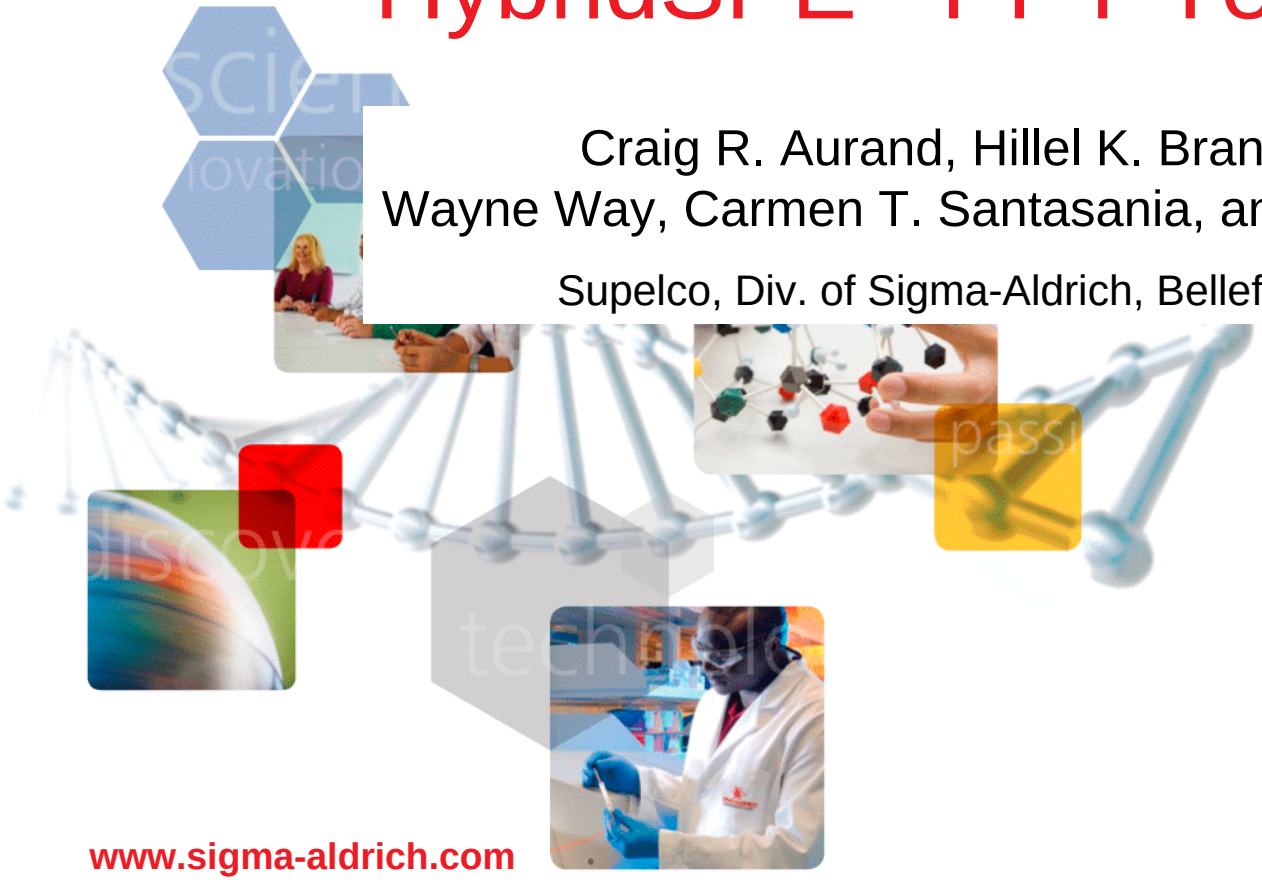
High Throughput Bioanalysis Utilizing Fused-Core™ Particle and HybridSPE® -PPT Technology

Craig R. Aurand, Hillel K. Brandes, Dave S. Bell, Wayne Way, Carmen T. Santasania, and Daniel Vitkuske

Supelco, Div. of Sigma-Aldrich, Bellefonte, PA 16823 USA



www.sigma-aldrich.com

T410086

SIGMA-ALDRICH®

Introduction

The trend in high throughput bioanalysis is to utilize shorter, faster chromatographic separations with minimal sample preparation for increased sample throughput. Often this approach compromises the quality of data that is obtained from these studies due to insufficient sample preparation and poor chromatographic conditions. In this presentation recent technological advances in chromatographic separation media and sample preparation technique are employed for the advancement in bioanalytical throughput. Combination of these new technologies result in higher quality data along with simpler bioanalytical methods.

Introduction (contd.)

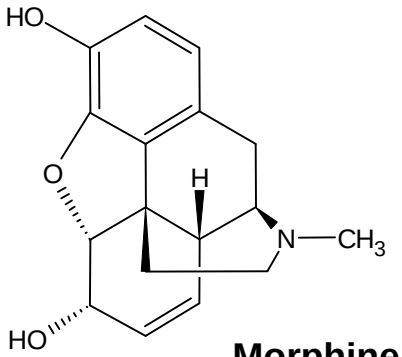
When dealing with biological samples, fast chromatographic separation don't always translate into fast bioanalytical methods. Often extracted sample matrix from minimal sample preparation results in the need for longer run times through additional column volumes or gradient elution. In some cases, changing from reversed-phase separation to HILIC/ANP is desired for matrix elution, increase ionization of analyte and sample compatibility.

In this study, morphine and morphine 3-β glucuronide spike plasma is utilized as a model sample for the evaluation of fast chromatographic separations and the impact sample matrix can have on the overall bioanalytical method. The separation of morphine and the 3-β glucuronide is conducted using both reversed-phase conditions along with HILIC/ANP chromatography.

Experimental

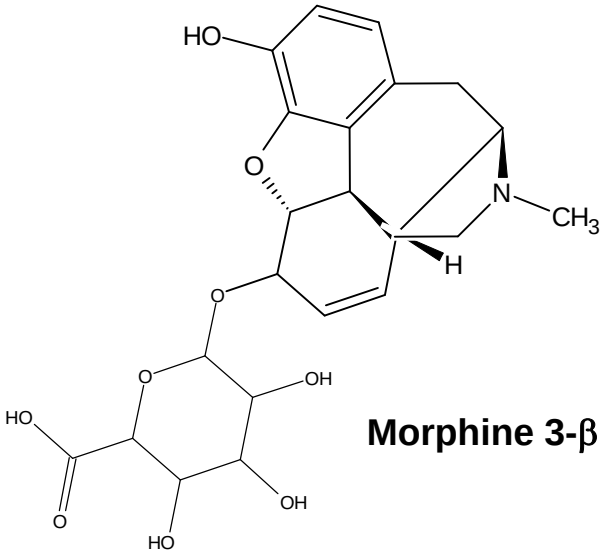
The goal of each method was separation of morphine and the 3-β glucuronide in less than 1 minute using standard 5 x 2.1 column dimensions. The Fused-Core particle of the Ascentis Express line was utilized in both reversed-phase and HILIC/ANP conditions because of the high efficiency at elevated flow rates. Analyte detection was performed using accurate mass of time of flight (TOF MS). Sample matrix impact was conducted by monitoring representative lyso and glycerophospholipids. In the first example standard protein precipitation is utilized with reversed-phase chromatographic separation as the initial approach for the bioanalytical method.

Morphine Structures



Morphine

Monoisotopic Mass = 285.136493 Da



Morphine 3-β glucuronide

Monoisotopic Mass = 461.168581 Da

Phospholipid Matrix Monitoring

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

Sample Protein Precipitation (PPT)

Rat plasma spiked with morphine and morphine 3-β glucuronide was prepared by crashing 100 µL of plasma with 300 µL 1% formic acid acetonitrile. Sample was vortexed for 1 minute followed by centrifugation for 2 minutes at 15000 rpm. Resultant supernatant was collected, evaporated and reconstituted in 10:90 acetonitrile:water. Final concentration of morphine and morphine 3-β glucuronide 600 ng/mL and 300 ng/mL respectively.

HILIC/ANP sample was analyzed directly without the need for evaporation and reconstitution.

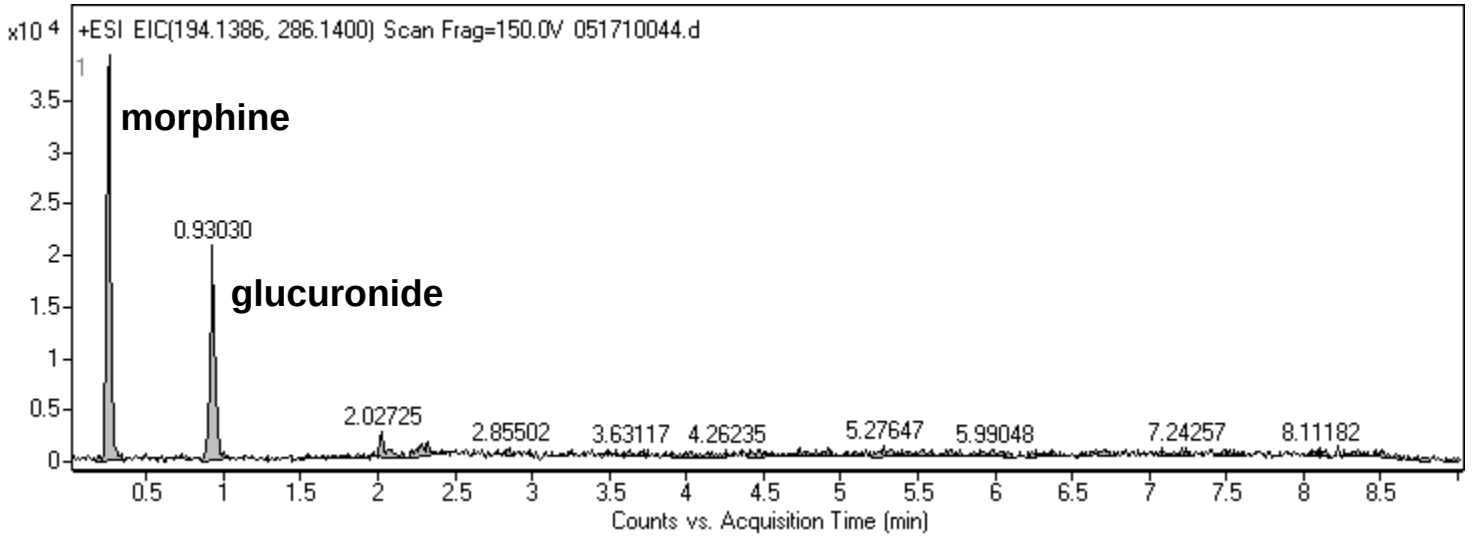
Reversed-Phase Chromatographic Conditions

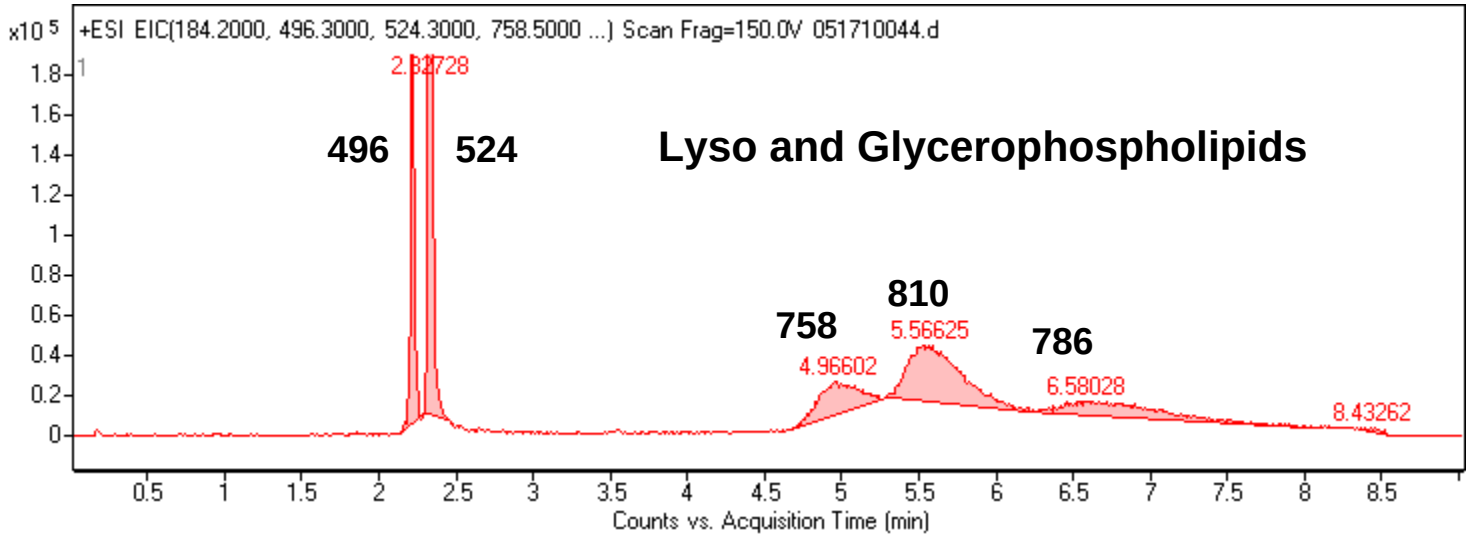
column:	Ascentis Express C18, 5 cm x 2.1 mm, 2.7 µm
mobile phase A:	10 mM ammonium formate pH 3.6
mobile phase B:	10 mM ammonium formate (90:10 acetonitrile:water) S_wpH 4.2
flow rate:	0.6 mL/min.
temp.:	50 °C
det.:	ESI+, 1.5 spectra/sec, 6309 transients/spectra
inj.:	1.0 µL
instrument:	Agilent 1200SL Rapid Resolution, Agilent 6210 TOF LC/MS

gradient:

Min	**%A**	**%B**
0	100	0
1	100	0
2	0	100
8	0	100
8.01	100	0
10	100	0

Ascentis Express C18





Results

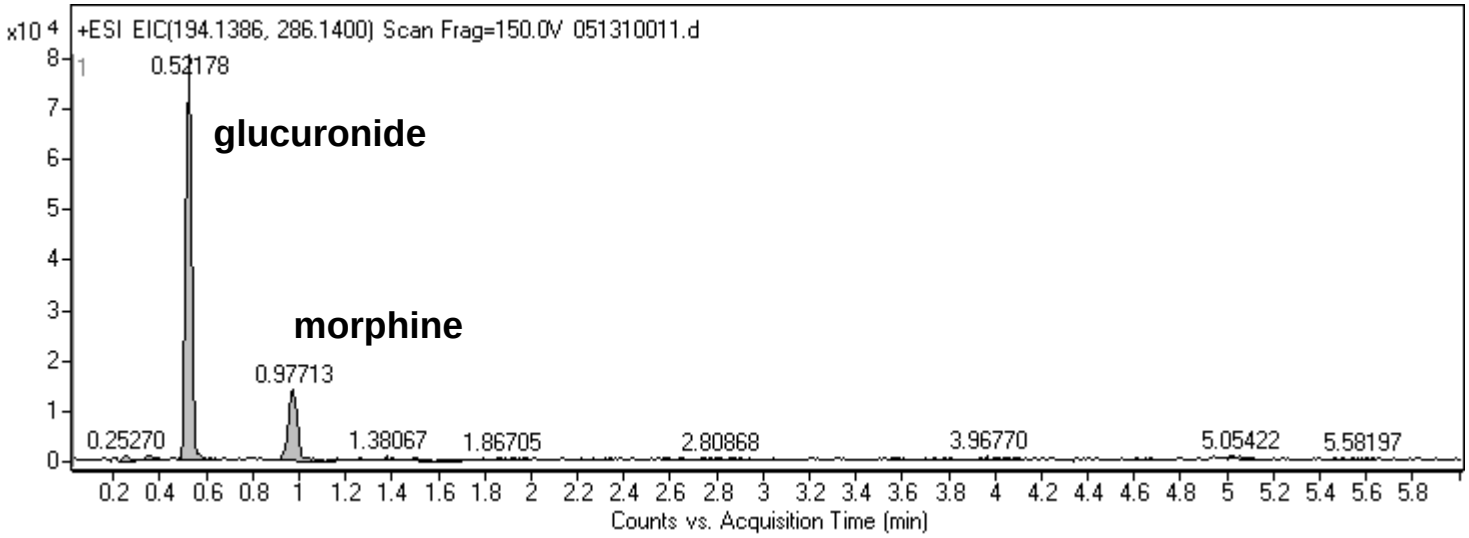
Though the chromatographic separation of morphine and the 3-β glucuronide is completed in less than 1 minute under the reversed-phase conditions, additional gradient elution is necessary to elute the PPT phospholipid matrix from the column. Under these conditions, phospholipids are eluted based upon order of hydrophobicity. The high organic content needed for full elution resulted in a total analysis time of 10 minutes to wash and equilibrate the column.

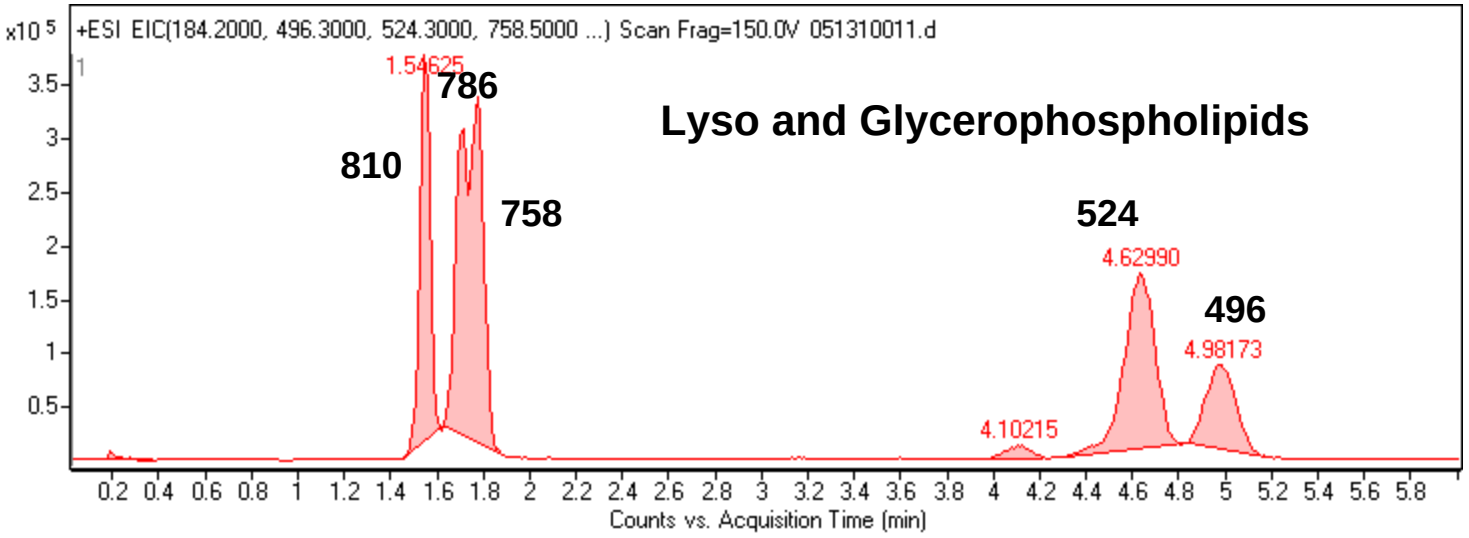
Instead of performing additional sample preparation, a common trend would be to evaluate a separate mode of chromatography to speed up the analysis. Using HILIC/ANP separation would result in minimized retention of the hydrophobic matrix and possible increase analyte response.

HILIC/ANP Chromatographic Conditions

column:	Ascentis Express HILIC, 5 cm x 2.1 mm, 2.7 µm
mobile phase:	10 mM ammonium formate (90:10 acetonitrile:water) S_wpH 4.2
flow rate:	0.6 mL/min.
temp.:	50 °C
det.:	ESI+, 1.5 spectra/sec, 6309 transients/spectra
inj.:	1.0 µL
instrument:	Agilent 1200SL Rapid Resolution, Agilent 6210 TOF LC/MS

Ascentis Express HILIC





In the HILIC/ANP conditions on the Ascentis Express HILIC, separation of morphine and the 3-β glucuronide is completed in less than 1 minute. Using the bare silica column resulted in increased retention of the PPT phospholipid matrix due to ion-exchange of the permanently charged polar head group of the phospholipids and the silanol surface. Elution order of the phospholipids is a combination of ion-exchange, steric hindrance of the lipid chain and overall orientation and partitioning between the organic rich mobile phase and the aqueous rich particle surface.

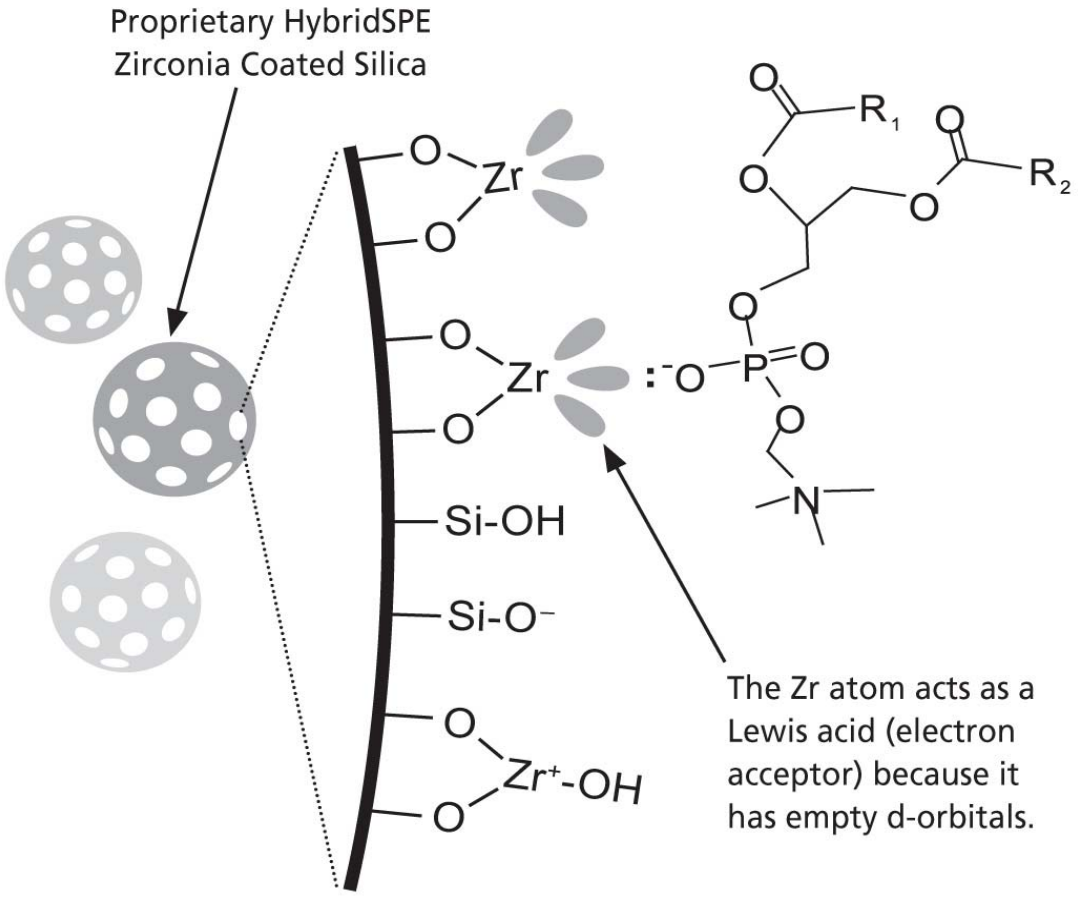
Without additional sample preparation, the total analysis time for this method would be over 6 minutes. Any shorter would result in subsequent overlap with phospholipid matrix from the previous injection causing reproducibility issues in quantitation.

While both chromatographic methods demonstrate fast and efficient separation, in neither case did this translate into a fast bioanalytical method. Retention of sample matrix resulted in increase analysis time for complete elution of the phospholipids. Additional sample preparation is required to speed up this analysis. Though traditional SPE methods or online sample prep could be utilized, these techniques require method development or additional pumps and columns.

In this study, a simplified technique using HybridSPE-PPT was utilized for simple and thorough removal of phospholipid matrix interference. This technique required no additional processing time over traditional protein precipitation. Both reversed-phase and HILIC/ANP methods were evaluated using this sample prep technique.

HybridSPE-PPT, Phospholipid Interaction with Zirconia-Coated Particle

- The high selectivity towards phospholipids 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, enabling highly efficient extraction of phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.
- Modifier (1% formic acid) mitigate Lewis acid base interaction between chelation/ acidic compounds.

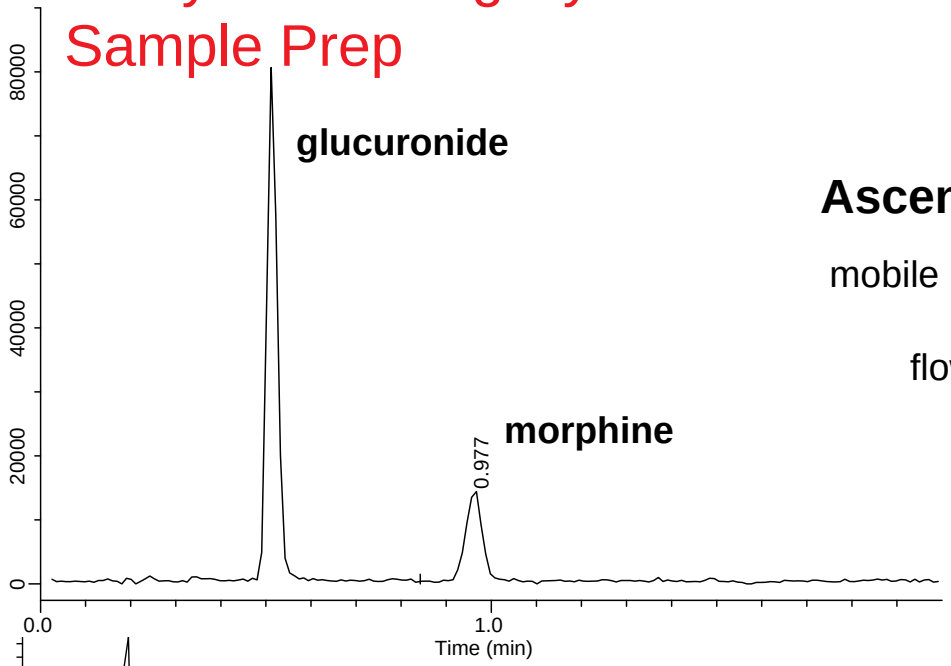


HybridSPE-PPT Sample Preparation

Rat plasma spiked with morphine and morphine 3-β glucuronide was prepared by adding 100 µL of plasma into the HybridSPE-PPT 96 well plate followed by 300 µL 1% formic acid acetonitrile. Sample was agitated for 1 minute followed by filtration by applying 10” Hg for 2 minutes. The resultant supernatant was collected and analyzed directly for HILIC/ANP separation. Final concentration of morphine and morphine 3-β glucuronide 600 ng/mL and 300 ng/mL respectively.

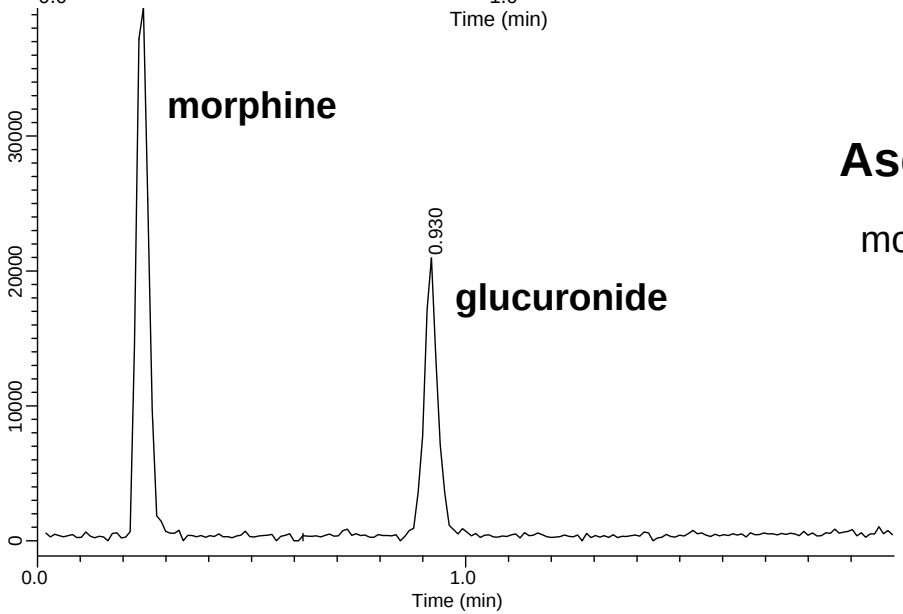
Reversed phase sample was evaporated and reconstituted in 10:90 acetonitrile:water.

Analysis Utilizing HybridSPE-PPT Sample Prep



Ascentis Express HILIC, 5 x 2.1

mobile phase: 10 mM ammonium formate (90:10 acetonitrile:water) S_wpH 4.2
flow rate: 0.6 mL/min
temp.: 50 °C
inj.: 1.0 µL



Ascentis Express C18, 5 x 2.1

mobile phase: 10 mM ammonium formate pH 3.6
flow rate: 0.6 mL/min.
temp.: 50 °C
inj. 1.0 µL

Discussion

Because the phospholipid matrix was depleted using the HybridSPE technique, no matrix interference was introduced on the analytical column eliminating the need for gradient elution or increased column volumes. Using the simplified sample prep technique, the real benefit of the high speed separation was realized. The HybridSPE enabled the use of isocratic elution in the reversed-phase separation, making both HILIC/ANP and reversed-phase methods as a viable approach for a high throughput bioanalytical method.

Summary

When developing bioanalytical methods, special consideration needs to be given for matrix interference and the impact on the chromatographic method. Fast chromatographic separation don't always translate into fast bioanalytical methods. In most cases, just changing chromatographic mode is not sufficient to minimize the impact of matrix interference. Efficient sample preparation is necessary to gain the benefit of the faster chromatographic separations. As demonstrated, using the simplified technique of the HybridSPE minimizes phospholipid matrix interference resulting in efficient and fast bioanalytical methods.