LC/MS/MS Analysis with on-line cartridge for removal of phospholipids from protein precipitation biological fluid samples

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Introduction

Phospholipids are abundantly (at the mg/mL level) present in biological fluids such as blood, plasma, serum, cerebrospinal fluids, among others. They are often co-extracted with a broad range of analytes of interest during sample preparation. The phospholipids present in a sample are notorious in producing various issues in LC/MS-based bioanalysis. They may cause ion suppression or, in rarer cases, ion enhancement, in MS detection. They also tend to buildup on a reversed-phase (e.g. C18 and C8) column, fouling the chromatographic separation and ultimately shortening the column lifetime. Consequently, the accuracy, reproducibility, and sensitivity of the LC/MS bioanalysis may be greatly compromised if the phospholipids are not removed.

We have developed a HybridSPE®-Phospholipid technology for selective and rapid depletion of phospholipids from biological samples prior to LC/MS analysis of small molecules. The technology utilizes the affinity of zirconia particles for selective binding and removal of phospholipids. The technology was introduced a few years ago in two product formats: 96-well filter plates for high throughput sample preparation and cartridges for low sample volume, respectively. Here we introduce a new product format, on-line cartridge, as an alternative option of phospholipid removal and sample preparation. The setup of the on-line cartridges with an LC/MS column is devised and their efficiency in phospholipid removal from protein precipitated plasma samples are evaluated. Their applicability was demonstrated with two sets of compounds of different chemical properties.

Experimental

Material:

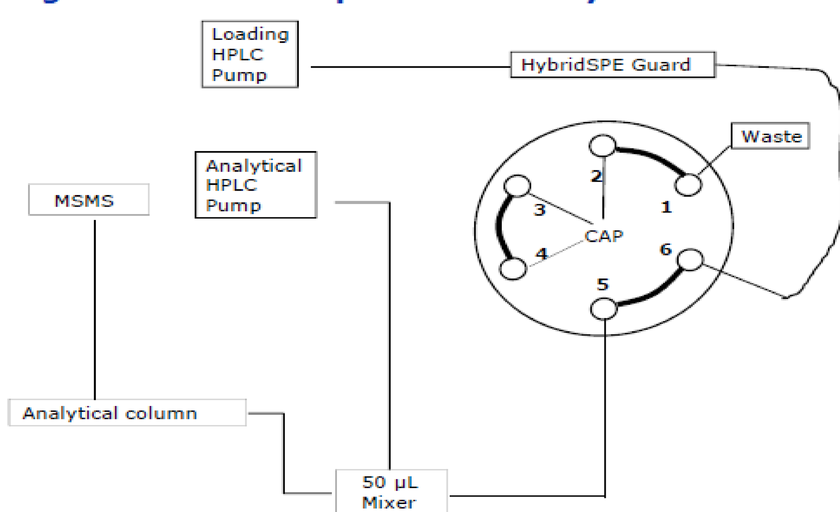
Rat Plasma K2-EDTA (Lampire Cat. No.7306407); Protein precipitation solvent: acetonitrile with 1% formic acid or methanol with 1% (w/v) ammonium formate.

HybridSPE on-line cartridge (2 cm x 4.0 mm I.D.). About 100 mg of material was packed in each cartridge.

Sample Preparation:

The rat plasma spiked with analytes was protein precipitated by vortex mixing the rat plasma with the precipitation solvent at 1:3 ratio. Then the mixture was centrifuged at 10000 rpm x 3 min and the resulting supernatant was collected for LC/MS analysis.

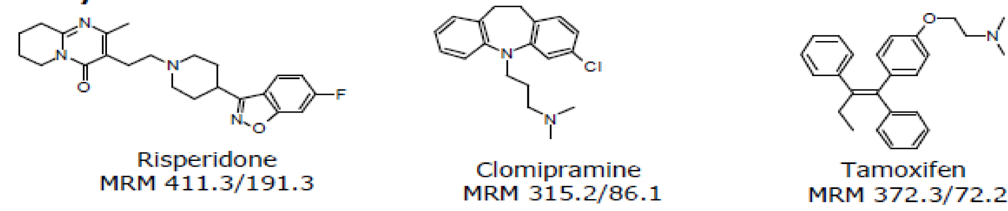
Figure 1. The Setup of On-line HybridSPE Cartridge with LC/MS



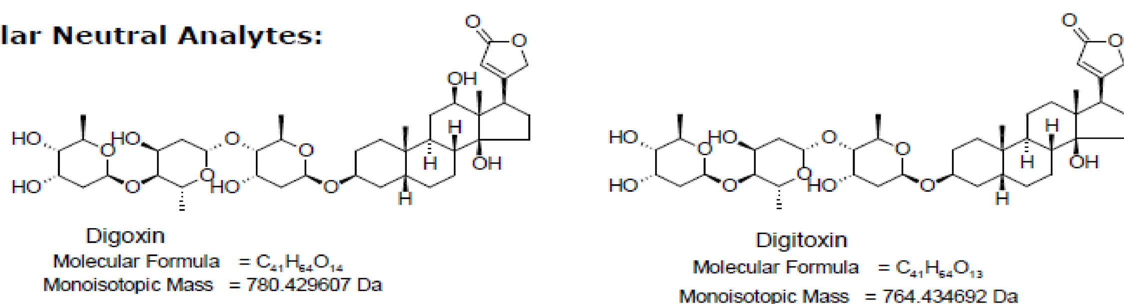
The setup in Figure 1 consists of two pumps, one for HPLC separation, and the other for loading protein precipitated samples and washing the HybridSPE on-line cartridges. A 50 µL tee is employed for mixing of sample loading mobile phase and the HPLC separation mobile phases. The 2-position switching valve allows for washing the cartridges once the samples are loaded onto the HPLC column.

Two Sets of Analytes Tested in the Study

Set 1: Basic Analytes:



Set 2: Polar Neutral Analytes:



LC/MS Conditions

LC/MS Method for Set 1 and 2 Analytes

HPLC column: Ascentis® Express C18, 5 cm x 2.1 mm I.D., 2.7 µm
mobile phase: [A] water; [B] 90% acetonitrile, each with 10 mM ammonium formate
gradient: 0%B for 4 min, to 80% B in 2 min, held for 1.5 min
flow rate: 0.3 mL/min
column temp.: 35 ºC
on-line cartridge: Supel™ Genie HybridSPE (2 cm x 4.0 mm I.D.)
sample loading pump flow: 0.1 mL/min
sample loading solvent: 80% acetonitrile with 50 mM ammonium formate
injection: 1 µL
detection: MS, ESI(+), MRM mode
instrument: Shimadzu™ LCMS-8030 with 2DLC setup

Analyte MRM Transitions and Parameters

Analyte	Ion-P	Ion-D	Dwell	Q1	CE	Q3
Risperidone	411.3	191.1	50	-29	-26	-13
Clomipramine	315.2	86.1	50	-25	-18	-17
Tamoxifen	372.3	72.1	50	-13	-25	-28
Digoxin	798.5	651.5	50	-26	-15	-24
Digitoxin	782.5	635.5	50	-26	-15	-24

LC/MS Method for the Monitoring of Phospholipids

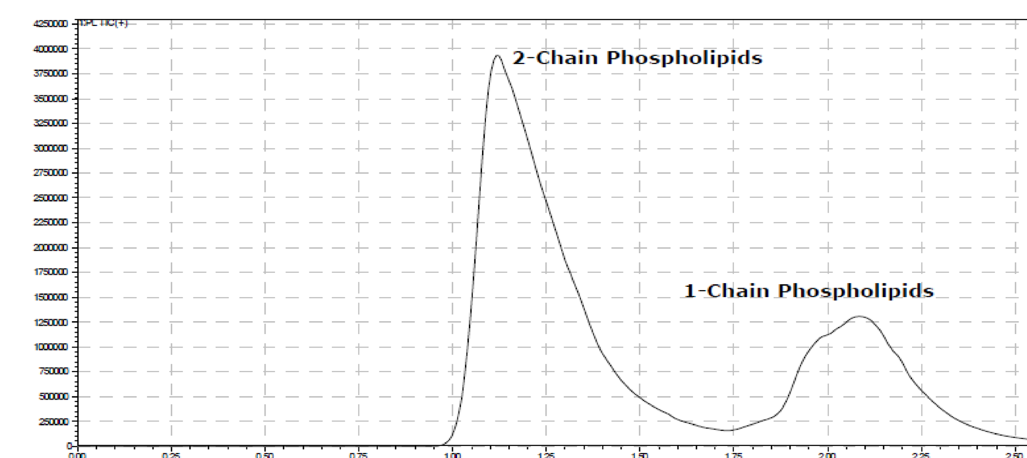
HPLC column: Ascentis Express OH5, 5 cm x 2.1 mm I.D., 2.7 µm
mobile phase: [A] water; [B] 90% acetonitrile, each with 10 mM ammonium formate
isocratic: 85% B
flow rate: 0.3 mL/min
column temp.: 35 ºC
on-line cartridge: Supel Genie HybridSPE (2 cm x 4.0 mm I.D.)
sample loading pump flow: 0.2 mL/min
sample loading solvent: 90% acetonitrile with 10 mM ammonium formate
injection: 1 µL
detection: MS, ESI(+), MRM mode
instrument: Shimadzu LCMS-8030 with 2DLC setup

Phospholipid MRM Transitions and Parameters

Ion-P	Ion-D	Dwell	Q1	CE	Q3
496.4	184.1	50	-12	-30	-12
520.4	184.1	50	-34	-30	-19
522.4	184.1	50	-34	-30	-12
524.4	184.1	50	-20	-30	-12
758.6	184.1	50	-26	-35	-12
782.6	184.1	50	-26	-35	-12
786.6	184.1	50	-28	-35	-12
810.7	184.1	50	-20	-35	-12

Results

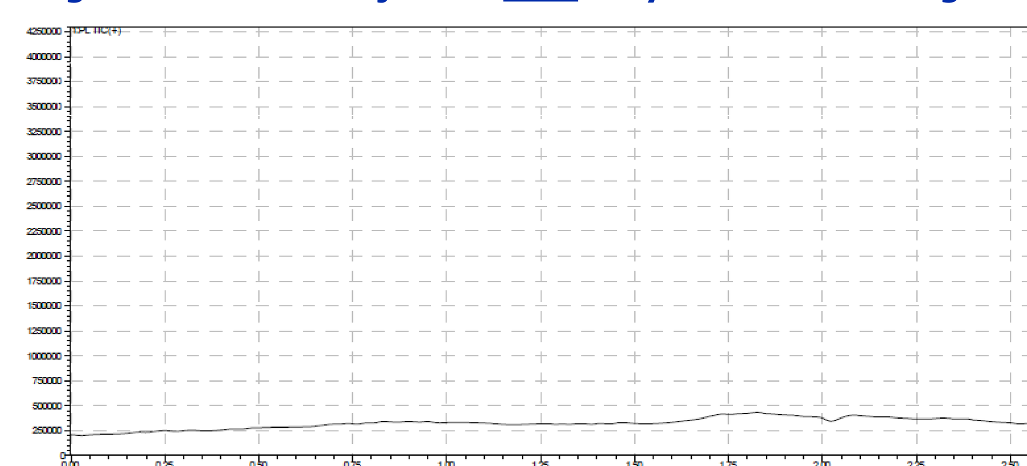
Figure 2A. Phospholipids in Plasma Sample without HybridSPE® Cartridge



The phospholipids in plasma are separated in two broad peaks with high intensities.

- 1.25 million counts of peak height of 1-chain phospholipids
- 4.0 million counts of peak height of 2-chain phospholipids

Figure 2B. #120th Injection with a HybridSPE® Cartridges

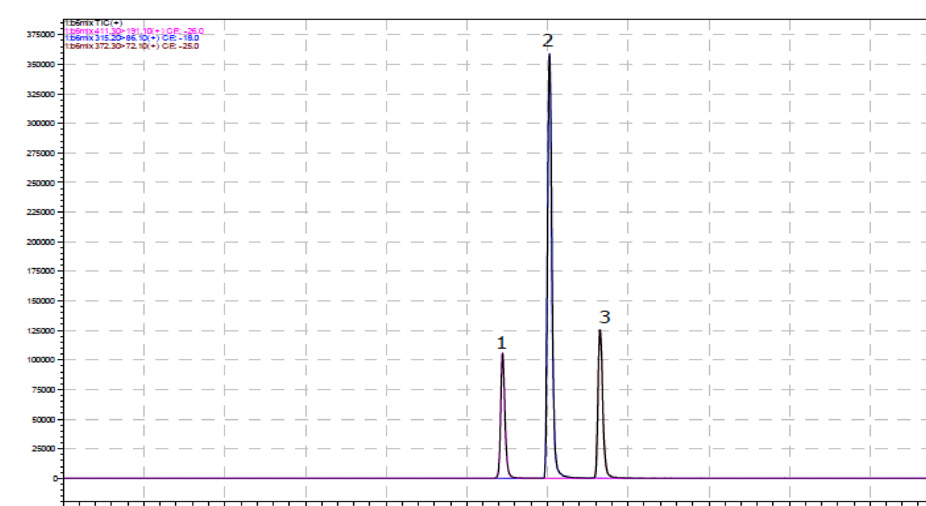


No phospholipid peaks were detected at 120th injection with HybridSPE cartridge.

Applications with Different Types of Analytes

The analyte recovery and reproducibility from the on-line phospholipid removal LC/MS methods were evaluated. Two sets of analytes representing basic and neutral compounds were tested with cartridges packed with different lots of HybridSPE material.

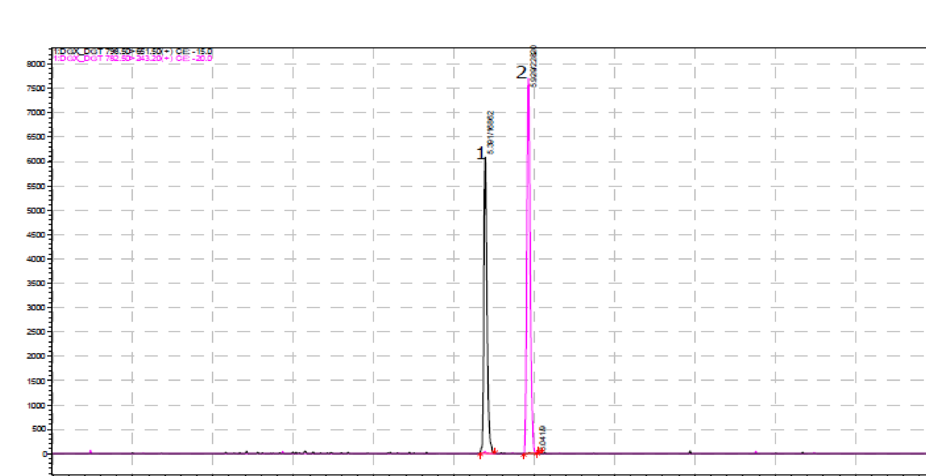
Figure 3. Representative LC/MS Chromatogram of Basic Analytes



Peak	Analyte	Peak width at 50% Height (s)	Tailing Factor
1	Risperidone	3.54	1.2
2	Clomipramine	3.78	1.3
3	Tamoxifen	3.36	1.2

- All peaks are narrow: <4s peak width at half height.
- Both peaks are symmetric: tailing factors 1.2-1.3.
- Baseline is low and clean: no interference peaks.

Figure 4. Representative LC/MS Chromatogram of Neutral Analytes



Peak	Analyte	Peak Width at 50% Height (s)	Tailing Factor
1	Digoxin	2.52	1.3
2	Digitoxin	2.70	1.2

- Both peaks are narrow: <3s peak width at half height
- Both peaks are symmetric: tailing factors 1.2-1.3.
- Baseline is low and clean: no interference peaks.

Table 1. Analyte's Recovery and Reproducibility

Analyte	Retention Time (min)	MRM Quantifier	Recovery* (Avg. n = 20)	Recovery Reproducibility RSD (%, n = 20)
Digoxin	5.4	798.5 / 651.5	96%	4.9
Digitoxin	5.9	782.5 / 243.2	97%	2.2
Risperidone	5.4	411.3 / 191.3	102%	1.5
Clomipramine	6.0	315.2 / 86.1	94%	1.1
Tamoxifen	6.6	372.3 / 72.2	98%	1.4

*The recovery was calculated by comparison of the peak area of the spiked analytes in plasma to those of the neat analytes at the same concentration.

For all of the analytes, including the basic, polar, and non-polar analytes

- A recovery of 94%-102% was obtained
- A reproducibility of 1%-5% was achieved

Conclusion

An on-line cartridge packed with zirconia-coated silica particles has been successfully developed for the on-line phospholipid removal during LC/MS analysis of biological samples.

- Performance testing shows the on-line cartridges are capable of removing >95% of phospholipids from a 1 µL of plasma samples even after 120 consecutive injections.
- Two applications have been established using on-line HybridSPE® with LC/MS detection. For all of the analytes:
  - ✓ A recovery of 94%-102% was obtained
  - ✓ A reproducibility of 1%-5% was achieved
  - ✓ Narrow and symmetric peaks were observed: peak width at half height is <6s and tailing factors 0.9-1.3, respectively.

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