LC/MS/MS Analysis Using On-line Cartridges for the Removal of Phospholipids from Protein Precipitated Biological Fluid Samples

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Online SPE offers a sample preparation solution performed entirely 'online' the LC instrument, enabling a hands free workflow. This technique is ideal for increasing reproducibility and eliminating the risk of human error as the most critical, labour intensive steps of the process are now automated. In this study, online SPE is used to remove phospholipids from plasma. Phospholipids are notorious for causing ion suppression and other matrix effects when analysing biological samples, and so their removal is critical in some cases. After performing online SPE, recovery and reproducibility data was reported and indicates online SPE is a reliable alternative to traditional sample preparation techniques, such as manual solid phase extraction and protein precipitation.

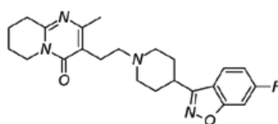
Introduction:

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

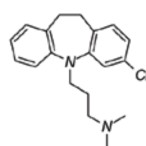
HybridSPE-Phospholipid technology has been developed for selective and rapid depletion of phospholipids from biological samples prior to LC/MS analysis of small molecules. The technology utilises the affinity of zirconia particles for selective binding and removal of phospholipids. The technology was initially designed for off-line analysis. This article describes the application of an on-line cartridge as an alternative option of phospholipid removal and sample preparation. The setup of the on-line cartridges with a LC/MS column is devised and the efficiency for phospholipid removal from protein precipitated plasma samples has been evaluated. Applicability of the system was demonstrated with three sets of compounds with different physiochemical properties.

Three sets of analytes

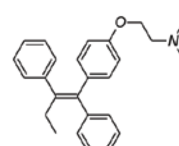
Set-1: basic analytes



Risperidone

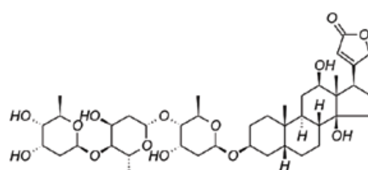


Clomipramine

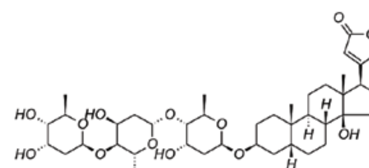


Tamoxifen

Set-2: polar neutral analytes

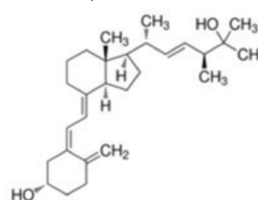


Digoxin

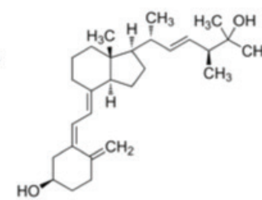


Digitoxin

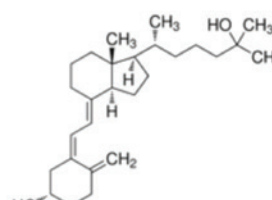
Set-3: non-polar neutral Analytes



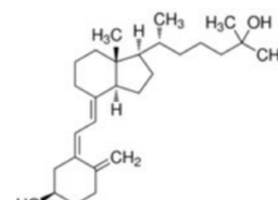
25-Hydroxyvitamin D_2



3-epi-25-Hydroxyvitamin D_2



25-Hydroxyvitamin D_3



3-epi-25-Hydroxyvitamin D_3

Figure 1. Chemical structures of the analytes.

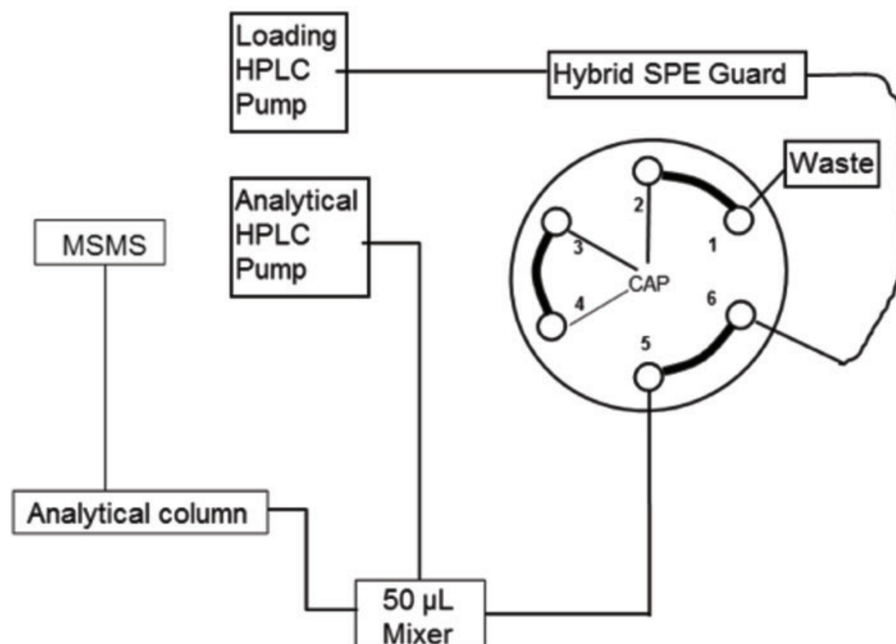


Figure 2. The setup of on-line HybridSPE cartridge with LC/MS.

HybridSPE-LC/MS conditions for Set-1 and -2 analytes:

Instrument:	Shimadzu LCMS-8030 with 2DLC setup
HPLC column:	Ascentis Express C18 5 cm x 2.1 mm
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:	0.3 mL/min
Column temperature:	35°C
Sample loading flow:	0.1 mL/min
Sample loading solvent:	80% acetonitrile with 50 mM ammonium formate
Injection Vol:	1 µL
Detection:	MS, ESI(+), MRM mode

HybridSPE-LC/MS conditions for Set-3 analytes:

Instrument:	Shimadzu LCMS-8030 with 2DLC setup
HPLC column:	Ascentis Express F5 10 cm x 2.1 mm (Sigma Cat# 53569-U)
Mobile phase:	(A) Water/10 mM ammonium formate; (B) methanol
Gradient:	0% B% for 4 min, to 75% B in 0.5 min, held for 8 min
Flow:	0.3 mL/min
Column temperature:	45°C
Sample loading flow:	0.1 mL/min
Sample loading solvent:	methanol with 10 mM ammonium formate
Injection Vol:	1 µL
Detection:	MS, ESI(+), MRM mode

HybridSPE-LC/MS conditions for phospholipid detection:

Instrument:	Shimadzu LCMS-8030 with 2DLC setup
HPLC column:	Ascentis Express OH5 5 cm x 2.1 mm
Mobile phase:	(A) Water; (B) 90% Acetonitrile, each with 10 mM ammonium formate
Isocratic:	85% B
Flow:	0.3 mL/min
Column temperature:	35°C
Sample loading flow:	0.2 mL/min
Sample loading solvent:	90% acetonitrile with 10 mM ammonium formate
Injection:	1 µL
Detection:	MS, ESI(+), MRM mode
Phospholipid ions:	parent ions 496, 520, 522, 524, 758, 782, 786, and 810, daughter ions are all 184

Experimental: Materials

SupelGenieTM HybridSPE on-line cartridge (2 cm length x 4.0 mm i.d), Rat Plasma K2-EDTA (Lampire); Protein precipitation solvent: Acetonitrile with 1% formic acid or methanol with 1% (w/v) ammonium formate.

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

HybridSPE-LC/MS setup

As shown in Figure 2, the setup consists of two pumps, one for HPLC separation, and the other for loading protein-crashed samples and washing the HybridSPE online 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.

Results and Discussion:

PLs are abundantly (at the mg/mL level) present in biological fluids such as plasma. The HybridSPE on-line cartridges are designed for removal of PLs from more than 100 injections of 1 µL of protein precipitated plasma samples. Figure 3(A) shows the phospholipids in the rat plasma, if not removed, give rise to two broad peaks of high intensity. The two peaks correspond to the PLs containing one and two fatty acyl chain(s), respectively. When the HybridSPE on-line cartridge is set up with the LC/MS (Figure 2), no phospholipid peaks were detected even at the 120th injection of the same rat plasma sample and there was also no issue with PL carryover from the column either (see Figure 3(B)). The results demonstrate the HybridSPE cartridges are capable of elimination of the phospholipids from 120 consecutive injections of 1 µL of protein precipitated plasma samples.

The applicability of the HybridSPE on-line cartridges has been demonstrated with three sets of analytes including basic and neutral classes. As can be seen from Figure 4-6, narrow and symmetrical peaks were observed for all of the tested analytes with a peak width at half height <6 s and tailing factors 0.9-1.3, respectively. Table 1 summarises the recoveries and the reproducibility of the recoveries. For all of the tested analytes, a recovery of 94%-102% was obtained and a reproducibility of 1%-5% was achieved.

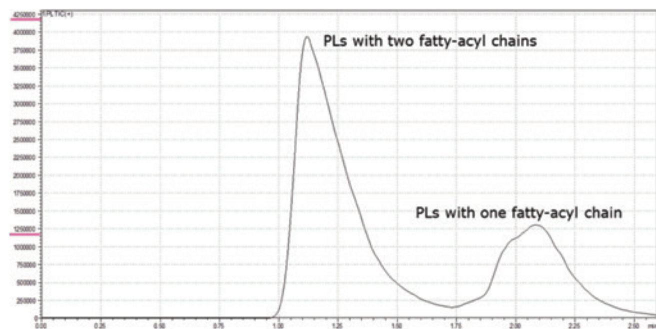


Figure 3 (A)

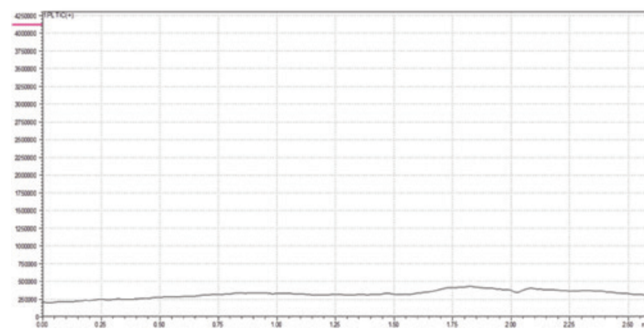
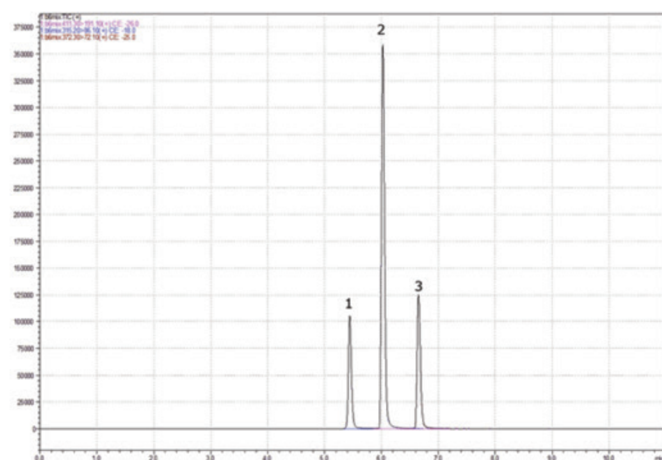


Figure 3 (B)

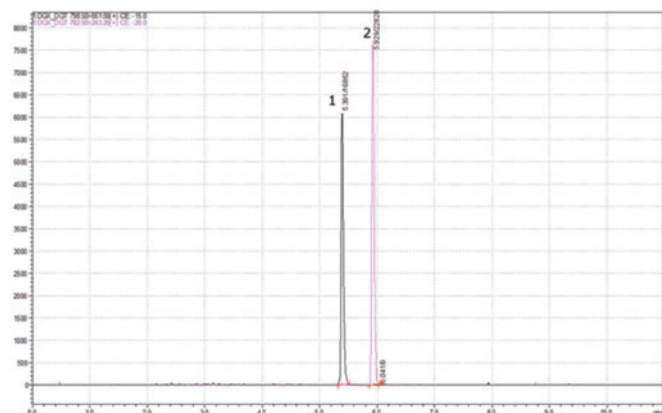
Figure 3. (A) Phospholipids in plasma sample without a HybridSPE cartridge; (B) #120th injection of the same plasma sample with a HybridSPE on-line cartridge set up with LC/MS (see Figure 2 for the setup).



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

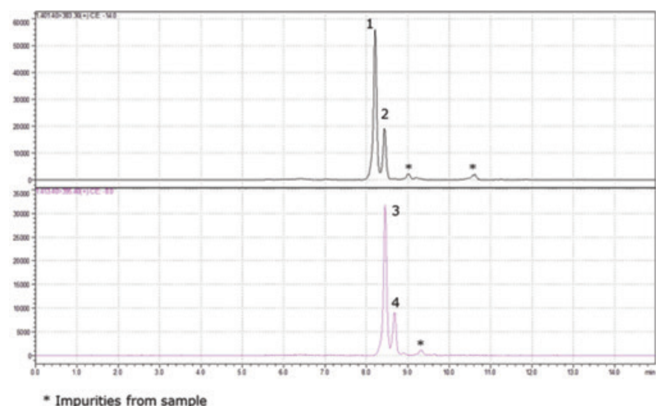
Figure 4. Representative LC/MS chromatogram of basic (Set-1) analytes.



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

Figure 5. Representative LC/MS chromatogram of polar neutral (Set-2) analytes.



Peak	Analyte	Peak width at 50% height (s)	Tailing factor
1	25-OH D3	4.74	0.9
2	3-epi-25-OH D3	4.80	0.9
3	25-OH D2	4.62	1.0
4	3-epi-25-OH D2	5.10	0.9

- All peaks are narrow: <6s peak width at half height
- Both peaks are symmetric: tailing factors 0.9-1.0.
- Baseline is low and clean: no interference peaks

Figure 6. Representative LC/MS chromatogram of non-polar neutral (Set-3) analytes.

Summary

An on-line cartridge for phospholipid removal with LC/MS analysis has been successfully developed. The performance testing demonstrates the on-line cartridges are capable of removing >95% of PLs from a 1 µL of plasma samples even after 120 consecutive injections. Three applications have been established using on-line HybridSPE with LC/MS detection. Recovery of the analytes is 94%-102%, with a reproducibility of 1%-5%. For all of the tested analytes, narrow and symmetric peaks were observed, peak width at half height <6 s and tailing factors 0.9-1.3, respectively.

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
25-OH Vitamin D3	8.2	401.4 / 383.3	102%	1.2
3-*epi*-25-OH-Vitamin D3	8.4	401.4 / 383.3	102%	2.5
25-OH Vitamin D2	8.4	413.4 / 395.4	100%	2.0
3-*epi*-25-OH Vitamin D2	8.6	413.4 / 395.4	99%	4.2

** 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.*