Effect of Sample Preparation and Chromatographic Separation on Matrix Effects in Quantitative Bioanalysis

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OVERVIEW

- Compare different sample preparation techniques for matrix effects in plasma.
- Compare HPLC vs. UPLC™ for matrix effects when working with protein precipitated (PPT) and solid phase extraction (SPE) extracts from plasma.

INTRODUCTION

The MS response obtained from an analyte solution can differ significantly from that same analyte in a matrix due to ion suppression and interference from co-eluting metabolites and matrix components. Ion suppression results from co-eluting components that compete for ionization capacity and cause errors in accuracy and precision. Metabolite effects occur when a drug-related compound co-elutes with the test compound and then fragments in the ion source of the mass spectrometer to give a product that interferes with the MRM channel for the drug. This will lead to an elevated response for the test compound. These effects can sometimes be addressed by redeveloping the chromatography, but this often increases sample analysis time.

Matrix effects should be evaluated as a part of quantitative LC/ESI-MS/MS method development and validation. Here we investigate the use of mixed-mode solid phase extraction and efficient chromatographic techniques to reduce matrix effects for a range of analytes.

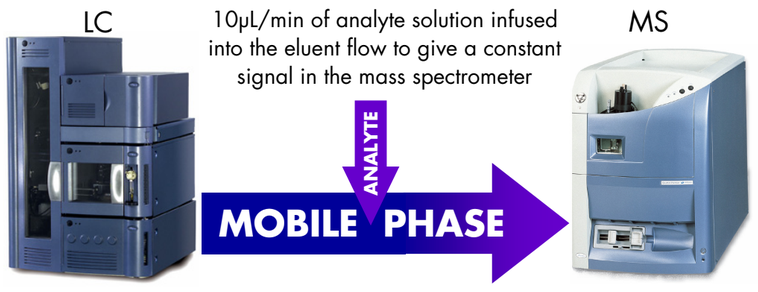
EXPERIMENTAL

Due to the specificity of the detection techniques being used in the following experiments (UPLC™/MS/MS or HPLC/MS/MS), we do not generally see the whole spectrum of endogenous compounds from an injection of matrix.

We have used two different methods of overcoming this:

1) Constant monitoring of the analyte as well as phospholipids and lysophospholipids[1] by using in source CID to form a common fragment ion (details of the MRM function used is included in the MS Conditions section for Terfenadine below).
2) Qualitative matrix effects using a post column infusion experiment (see Figure 1 below)

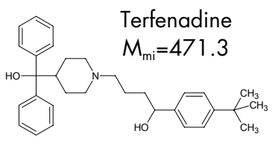
Figure 1—Qualitative matrix effects experiment using post column infusion



An injection of blank extract (solid phase extraction or protein precipitation in this case) is then injected on to the system. Any endogenous components which cause ion suppression of the analyte will appear as negative peaks in the chromatogram.

TERFENADINE EXPERIMENTAL

Terfenadine was selected as a suitable compound to monitor the effect of matrix effects due to its hydrophobic nature. This causes it to co-elute with the first of the phospholipid peaks in the chromatogram. The gradient methods used in these experiments are unusually long to ensure the complete elution of phospholipids from the column.



Terfenadine HPLC Conditions

Waters Alliance® HT
Column: XBridge™ C_{18}, 2.1 x 50mm, 3.5µm
Mobile Phase A: 2mM ammonium acetate in water, pH 9.0 with NH_3
Mobile Phase B: 100% methanol
Flow rate: 0.4 mL/min

Gradient:	Time (min)	%A	%B	Curve
	0.00	95	5	Initial
	1.5	5	95	6
	28	95	5	11
	30	95	5	6

Column Temperature: 40°C
Injection Volume: 5µL

Terfenadine UPLC™ Conditions

Waters ACQUITY UPLC™ System
Column: Waters ACQUITY UPLC™ BEH C_{18}, 2.1 x 50mm, 1.7µm
Mobile Phase A: 2mM ammonium acetate in water, pH 9.0 with NH_3
Mobile Phase B: 100% methanol
Flow rate: 0.65 mL/min

Gradient:	Time (min)	%A	%B	Curve
	0.00	95	5	Initial
	1.5	5	95	6
	14	95	5	11
	15	95	5	6

Column Temperature: 40°C
Injection Volume: 5µL

Terfenadine MS Conditions

Instrumentation—Waters Micromass® Quattro Premier™ tandem quadrupole MS, ESI+

Capillary Voltage	0.5kV
Source Temperature	120°C
Desolvation Temperature	380°C
Desolvation Gas Flow	1000L/hour
Cone Gas Flow	50L/hour
Collision Gas Pressure	3.5e-3 mbar (argon)

MRM Transitions:

Compound	Precursor	Product	Dwell time	Cone	Collision Energy
Phospholipids	184.3	184.3	100ms	90V	3eV
Terfenadine	472.4	436.6	100ms	40V	26eV

Inter-channel delay	10ms
Inter-scan delay	10ms

Terfenadine Sample Preparation

Protein Precipitation (PPT)

- 100µL human plasma + 300µL methanol used to crash proteins
- The resultant solution was centrifuged for 5 minutes at 13,000rpm
- 100µL of supernatant was transferred to an autosampler vial and diluted with 100µL of a 10ng/mL solution of terfenadine in water prior to injection

Solid Phase Extraction (SPE)

- Water OASIS® MCX 30mg Cartridge
- Condition with 1mL methanol
- Equilibrate with 1mL water
- Load 1mL of sample (100µL human plasma + 1000µL water)
- Wash 1 1mL 2% formic acid in water
- Wash 2 1mL methanol
- Elute 500µL 5% NH_4OH in methanol
- 100µL of extract is transferred to a auto sampler vial and diluted with 100µl of a 10ng/ml solution of terfenadine in water prior to injection

Solution standards were prepared in 1:1 Methanol/Water.

TERFENADINE RESULTS

Figure 2—Phospholipid transition for Solution, SPE and PPT samples

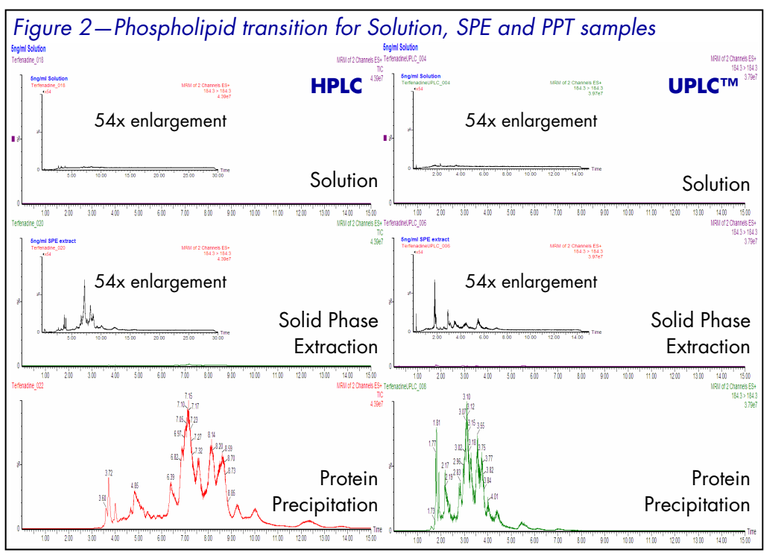


Figure 3—Comparison of SPE and PPT extracts by HPLC

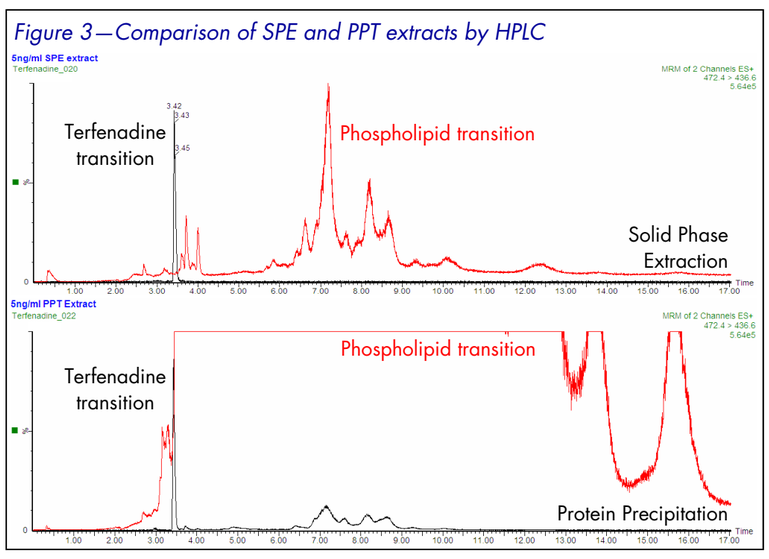
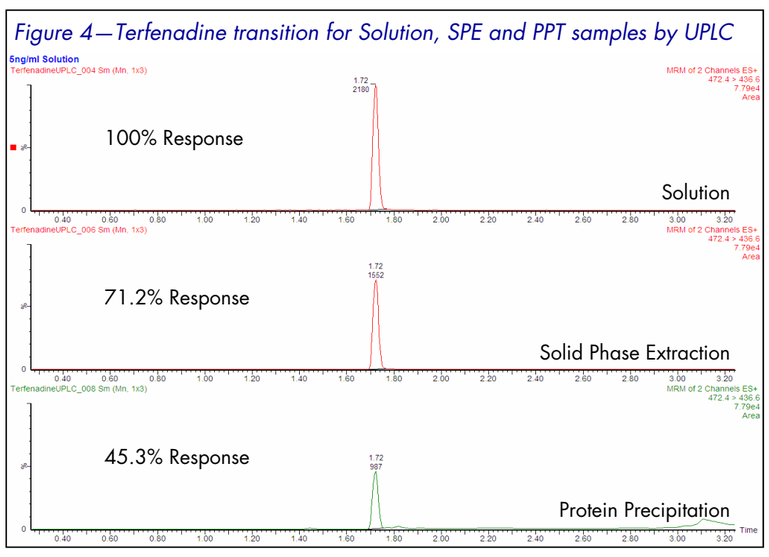
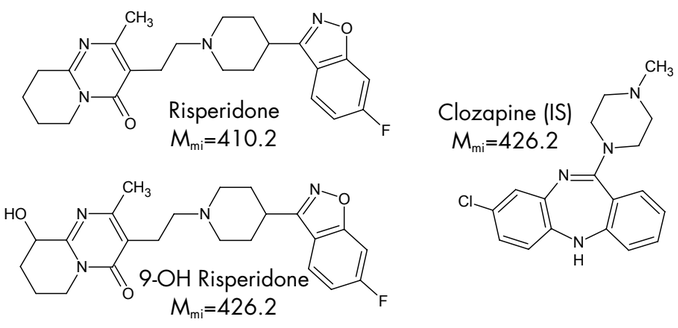


Figure 4—Terfenadine transition for Solution, SPE and PPT samples by UPLC



RISPERIDONE EXPERIMENTAL

As part of a bioanalytical method validation study for the determination of risperidone and its major metabolite (9-OH risperidone), post column infusion experiments were carried out to assess the level of matrix effect observed.



Risperidone HPLC Conditions

Waters Alliance® HT
Column: Waters X-Terra® MS C_{18}, 2.1 x 50mm, 3.5µm
Mobile Phase A: 2mM ammonium acetate in water, pH 9.0 with NH_3
Mobile Phase B: 100% methanol
Flow rate: 0.3 mL/min

Gradient:	Time (min)	%A	%B	Curve
	0.0	50	50	Initial
	0.5	50	50	6
	2.0	0	100	6
	3.5	50	50	11
	5.5	50	50	6

Column Temperature: 40°C
Injection Volume: 5µL

Risperidone UPLC™ Conditions

Waters ACQUITY UPLC™ System
Column: Waters ACQUITY UPLC™ BEH C_{18}, 2.1 x 50mm, 1.7µm
Mobile Phase A: 2mM ammonium acetate in water, pH 9.0 with NH_3
Mobile Phase B: 100% methanol
Flow rate: 0.6 mL/min

Gradient:	Time (min)	%A	%B	Curve
	0.00	50	50	Initial
	0.25	50	50	6
	0.75	0	100	6
	1.25	50	50	11
	1.50	50	50	6

Column Temperature: 50°C
Injection Volume: 5µL

Risperidone MS Conditions

Instrumentation—Waters Micromass® Quattro Premier™ tandem quadrupole MS, ESI+

Capillary Voltage	1.0kV
Source Temperature	120°C
Desolvation Temperature	350°C
Desolvation Gas Flow	800L/hour
Cone Gas Flow	50L/hour
Collision Gas Pressure	3.5e-3 mbar (argon)

MRM Transitions:

Compound	Precursor	Product	Dwell time	Cone	Collision Energy
Risperidone	411.3	191.3	30ms	40V	30eV
9-OH Risperidone	427.4	207.2	30ms	40V	30eV
Clozapine (IS)	327.1	270.3	30ms	35V	25eV

Inter-channel delay	10ms
Inter-scan delay	10ms

Risperidone Sample Preparation

Protein Precipitation (PPT)

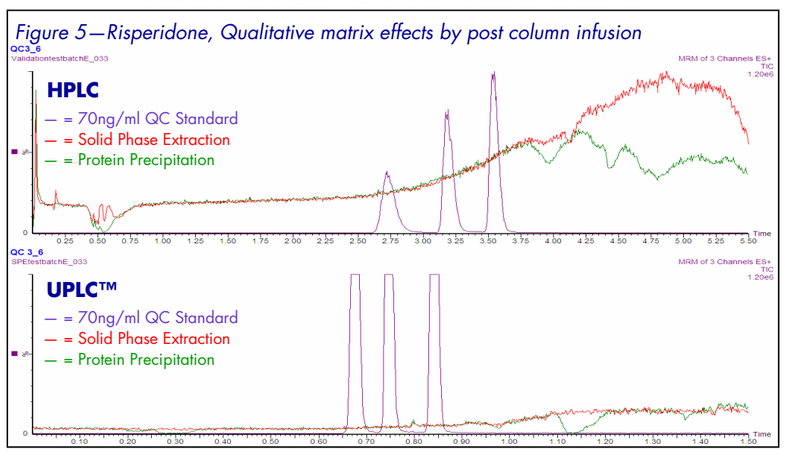
- 100µL human plasma + 500µL methanol used to crash proteins
- The resultant solution was centrifuged for 5 minutes at 13,000rpm
- 100µL of supernatant was transferred to an autosampler vial and diluted with 100µL of water prior to injection

Solid Phase Extraction (SPE)

- Water OASIS® MCX 30mg Cartridge
- Condition with 1mL methanol
- Equilibrate with 1mL water
- Load 1mL of sample (100µL human plasma + 1000µL water)
- Wash 1 1mL 2% formic acid in water
- Wash 2 1mL methanol
- Elute 500µL (2x250µL) 5% NH_4OH in methanol
- 100µL of extract is transferred to a auto sampler vial and diluted with 100µl of water prior to injection

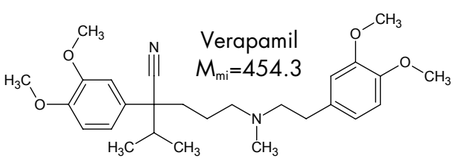
RISPERIDONE RESULTS

Figure 5—Risperidone, Qualitative matrix effects by post column infusion



VERAPAMIL EXPERIMENTAL

A post column infusion experiment was also carried out with verapamil by HPLC and UPLC™ using rat plasma.

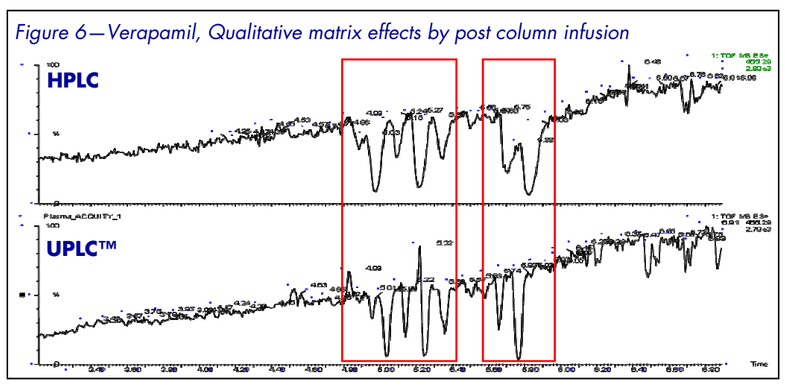


Verapamil LC & MS Conditions

Waters ACQUITY UPLC™ System	Waters Alliance® HT
Column: Waters ACQUITY UPLC™ BEH C_{18}, 2.1 x 50mm, 1.7µm	Column: XBridge™ C_{18}, 2.1 x 50mm, 3.5µm
Eluent A: 0.1% Formic Acid in H_2O	Eluent A: 0.1% Formic Acid in H_2O
Eluent B: 0.1% Formic Acid in MeCN	Eluent B: 0.1% Formic Acid in MeCN
Gradient: 5—95% B in 5 minutes	Gradient: 5—95% B in 5 minutes
Temp: 40°C	Temp: 40°C
Injection Volume: 5µL	Injection Volume: 5µL
Flow Rate: 550µL/min	Flow Rate: 270µL/min

Waters Micromass® LCT Premier™ (OA-TOF) ESI+, chromatograms below are displayed as extracted ion chromatograms at 455.29Da. Sample injected was rat plasma, protein precipitated using 2:1 MeCN/plasma.

Figure 6—Verapamil, Qualitative matrix effects by post column infusion



DISCUSSION

During the course of the above experiments we have demonstrated some of the differences between PPT and mixed–mode SPE as sample preparation techniques.

Figure 2 shows the phospholipid response for 5ng/ml terfenadine standards made up using solvent, SPE extract and PPT extract. The chromatograms are shown on the same intensity scale and demonstrate that while mixed-mode SPE does not remove all traces of phospholipid, far more is removed than with PPT.

Figure 3 shows a comparison of overlaid phospholipid and terfenadine traces by SPE and PPT. Looking at the PPT chromatograms, clearly the amount of phospholipids present in the sample are creating peaks in the terfenadine trace. Using mixed-mode SPE as the sample preparation step, this does not happen and the baseline is free from interferences

Figure 4 shows the relative responses of terfenadine by each sample preparation technique. Taking the solvent standard as 100, in this case there is a 30% drop in signal when SPE is used, however when PPT is used there is a drop of approximately 55% due to the enhanced levels of endogenous components present in the sample.

The risperidone experiment again showed less ion suppression due to fewer residual endogenous components by SPE as compared to protein precipitation.

Figure 5 shows a comparison between UPLC™ and HPLC using realistic bioanalytical run times. Better resolution is achieved between the analytes, IS and endogenous components of the samples when using UPLC™ with a 1.5 minute run time as opposed to HPLC with a run time of 5.5 minutes.

Figure 6 shows the results of a post column infusion experiment using verapamil by HPLC and UPLC™. The highlighted portions of the chromatogram show the same pattern of peaks, however, the UPLC™ trace shows greater resolution of these components when compared to HPLC. This allows more chance of separating the matrix components from the analyte of interest.

CONCLUSIONS

- **PPT is a far less effective method of sample clean up than mixed-mode SPE.**
- **UPLC™ provides better resolution of endogenous materials (vs. HPLC), hence gives a better chance of separating those interferences from the analyte of interest.**
- **HPLC methods can be transferred to shorter UPLC™ methods without introducing matrix effects.**
- **The combination of UPLC™ and mixed-mode SPE facilitate the development of fast and robust LC/MS/MS methods with the potential to greatly increase sample throughput.**
  - For example in the case of the risperidone assay described, a full 96 well plate would take over 9.5 hours to run.
  - the same number of samples run by UPLC™ would take just over 3 hours resulting in a 66% reduction in analysis time.

REFERENCES

[1]James Little, Eastman Chemical Company, Kingsport, TN—Poster entitled "Simple Method to Monitor Lysophospholipids and Phospholipids During LC-MS Method Development via In-Source CID" presented at ASMS 2005

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