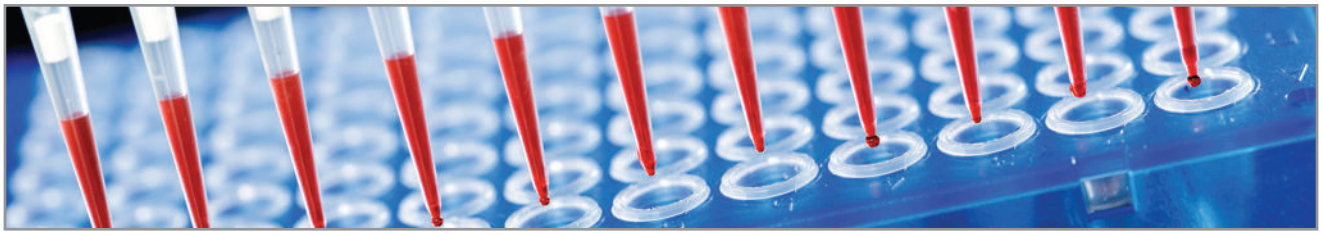


Overcoming the Effects of Highly Organic Protein Precipitation Extracts on LC Peak Shape Using Direct Injection

By Sharon Lupo and Frances Carroll

Abstract

Protein precipitation is frequently used to minimize matrix impact when analyzing biological samples. However, the effects of highly organic protein precipitation sample extracts on LC peak shape can negatively impact accurate quantification. Dilution or further sample preparation steps are often used to minimize these effects; however, here we show that direct injection of sample extracts is a viable option that can be used to prevent peak distortion, while avoiding the time and variability associated with additional sample preparation.

Introduction

Protein precipitation (PPT) extraction is widely used to prepare biological samples for LC analysis because it is fast, simple, and inexpensive. PPT removes proteins from blood, plasma, and serum samples, reducing matrix interferences and preventing column clogging. A typical PPT protocol utilizes a 3:1 ratio of organic solvent to biological sample, which produces a highly organic protein precipitation sample extract that contains approximately 75% organic solvent. Poor chromatography can result when the extract is injected into a reversed-phase LC system with a weaker mobile phase. The effects of the highly organic protein precipitation sample on LC peak shape include peak fronting and splitting, which can negatively impact quantitation. In contrast, if the injection solvent is sufficiently weaker than the mobile phase, the sample will concentrate at the head of the column and a symmetrical peak shape will result. Often, when protein precipitation is used, additional steps, such as evaporation and reconstitution or dilution with a weaker solvent (e.g., water), are performed prior to analysis in order to minimize the effects of the highly organic protein precipitation samples on LC peak shape. These additional steps can reduce peak distortion, but they also are time-consuming and can introduce additional error into the analysis. In this article, we will assess the viability of direct injection using a smaller injection volume in order to minimize the effects of highly organic protein precipitation extracts on LC analyses. Performance will be evaluated by comparing direct injection to dilution in terms of peak shape, intensities, and recovery values for five antiretroviral (ARV) drugs in human plasma.

Experimental

Sample Preparation

To assess the effects of highly organic protein precipitation extracts on LC, calibration standards and quality control samples were prepared by fortifying human plasma with five ARV drugs: lamivudine, zidovudine, nevirapine, efavirenz, and ritonavir. Calibration standards were prepared across a 10–5,000 ng/mL range and quality control samples were fortified at 10; 30; 2,500; and 4,500 ng/mL. For extraction, a 100 µL aliquot of fortified human plasma was protein precipitated with 300 µL of acetonitrile containing isotopically labeled internal standard (nevirapine-d3, 100 ng/mL) in a 1.3 mL 96-well plate (cat.# 26494). The plate was then sealed with a mat (cat.# 26498) and centrifuged for 10 minutes at 4,300 rpm and 8 °C. A portion of each sample extract was diluted 10x with mobile phase A, and another portion remained undiluted.

LC-MS/MS Analysis

All samples (10x diluted and undiluted extracts) were analyzed using a Shimadzu Nexera UHPLC equipped with a SCIEX API 4500 MS/MS under the conditions shown below. The analyte transitions that were monitored are presented in Table I.

Analytical column: Raptor Biphenyl, 2.7 µm, 50 mm x 2.1 mm (cat.# 9309A52)
Guard column: Raptor Biphenyl EXP, 2.7 µm, 5 mm x 2.1 mm (cat.# 9309A0252)
Mobile phase A: 0.01% acetic acid in water
Mobile phase B:
0.01% acetic acid in methanol

Gradient	Time (min)	%B
	0.00	10
	1.00	60
	2.50	100
	2.51	10
	4.50	10

Flow rate: 0.8 mL/min
Oven/AS temp.: 30 °C/15 °C
Needle stroke: 52 mm for 10x diluted extracts and 45 mm for undiluted extracts
Injection volume: 5 µL for 10x diluted extracts and 0.5 µL for undiluted extracts
Ion mode: Positive ESI

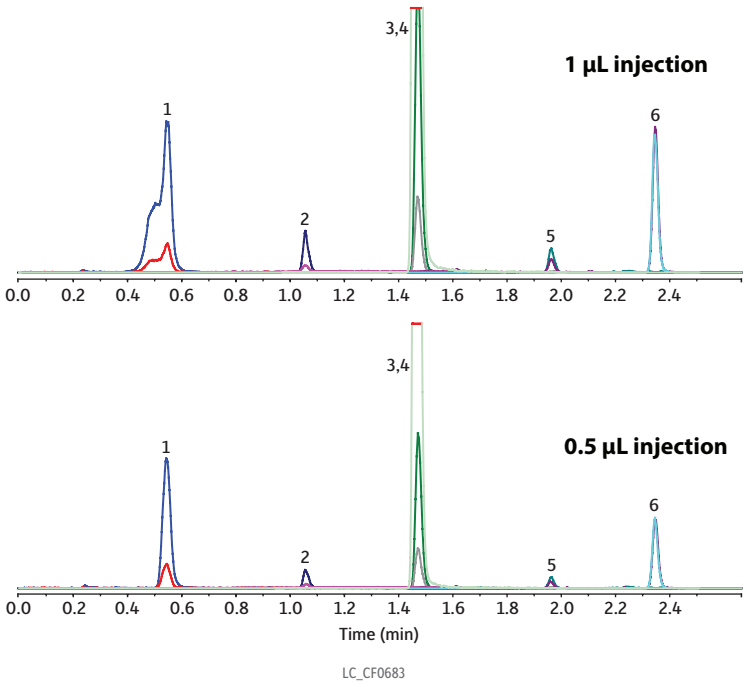
Table I: Analyte Transitions

Peak ID	Analyte	Retention Time (min)	Precursor Ion	Product Ion	Product Ion
1	Lamivudine	0.55	230.0	112.0	95.1
2	Zidovudine	1.06	268.1	127.2	110.1
3	Nevirapine-d3 (IS)	1.46	270.3	229.1	—
4	Nevirapine	1.47	267.3	225.9	196.9
5	Efavirenz	1.96	316.1	243.8	168.1
6	Ritonavir	2.35	721.5	140.1	296.0

Results and Discussion

Injections in solvents that are stronger than the mobile phase carry the sample through the column until the sample becomes fully diluted in the mobile phase. This results in peak broadening or splitting, which negatively impacts quantitation, reproducibility, and sensitivity. The degree of peak distortion is a function of injection volume, column volume, and the difference in solvent strength between the injection solvent and the mobile phase. The smaller the column, the smaller the injection volume required to overcome strong solvent effects. By decreasing the injection volume, PPT extracts can be injected directly and the effects of highly organic protein precipitation extracts on LC peak shape can be minimized or overcome. In Figure 1, we compare the peak shapes obtained from 1 µL and 0.5 µL direct injections of a 25 ng/mL PPT standard. Obvious differences in peak shape are observed for lamivudine, which elutes first in each chromatogram. When the size of the injection volume is reduced from 1 µL to 0.5 µL, a symmetrical peak shape is achieved.

Figure 1: Injection Volume Effects on Highly Organic Protein Precipitation Sample LC Peak Shape (Direct Injection)



Peaks	t_R (min)	Precursor Ion	Product Ion	Product Ion
1. Lamivudine	0.55	230.0	112.0	95.1
2. Zidovudine	1.06	268.1	127.2	110.1
3. Nevirapine-d3 (IS)	1.46	270.3	229.1	—
4. Nevirapine	1.47	267.3	225.9	196.9
5. Efavirenz	1.96	316.1	243.8	168.1
6. Ritonavir	2.35	721.5	140.1	296.0

Column Raptor Biphenyl (cat.# 9309A52)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 30 °C
Sample
Conc.: 25 ng/mL
Mobile Phase
A: Water + 0.01% acetic acid
B: Methanol + 0.01% acetic acid

Time (min)	Flow (mL/min)	%A	%B
0.00	0.8	90	10
1.00	0.8	40	60
2.50	0.8	0	100
2.51	0.8	90	10
4.50	0.8	90	10

Max Pressure: 400 bar
Detector MS/MS
Ion Mode: ESI+
Mode: Scheduled MRM
Instrument UHPLC
Notes Sample Preparation Details: A 100 µL aliquot of fortified human plasma was protein precipitated with 300 µL acetonitrile containing isotopically labelled internal standard in a 1.3 mL 96-well plate (cat.# 26495). The plate was sealed and centrifuged for 10 minutes at 4,300 rpm and 8 °C.

To further explore the impact of small volume direct injection on the effects of highly organic protein precipitation extracts on LC analyses, differences in recovery values for QC samples were evaluated in comparison to the results for diluted samples. Percent recovery and relative precision values were similar for each preparation across QC levels. Results for efavirenz are shown in Table II. Chromatograms from two separate injections of a 10 ng/mL LOQ calibration standard were compared in Figure 2. In one chromatogram, 0.5 µL of the undiluted standard extract is injected. In the other, the standard extract was diluted 10x in mobile phase A prior to injecting 5 µL. Both techniques result in the same amount of matrix and analyte on column with minimal differences in peak shape, intensity, or observed matrix interferences.

Table II: Comparison of Recovery Values for Diluted and Direct Injection (Undiluted) QC Samples (Efavirenz)

	10x Dilution in Mobile Phase A (n = 6)		Direct Injection (Undiluted) (n = 6)	
Nominal Conc. (ng/mL)	CV (%)	%Recovery	CV (%)	%Recovery
10	7.5	88.7	11.1	96.0
30	3.9	101.1	2.5	97.9
2,500	5.1	101.5	2.6	102.3
4,500	3.4	111.5	2.6	108.9

Figure 2: Comparison of Diluted and Direct Injection (Undiluted) PPT Extract of 10 ng/mL in Human Plasma.

Peaks	t_R (min)	Precursor Ion	Product Ion	Product Ion
1. Lamivudine	0.55	230.0	112.0	95.1
2. Zidovudine	1.06	268.1	127.2	110.1
3. Nevirapine-d3 (IS)	1.46	270.3	229.1	-
4. Nevirapine	1.47	267.3	225.9	196.9
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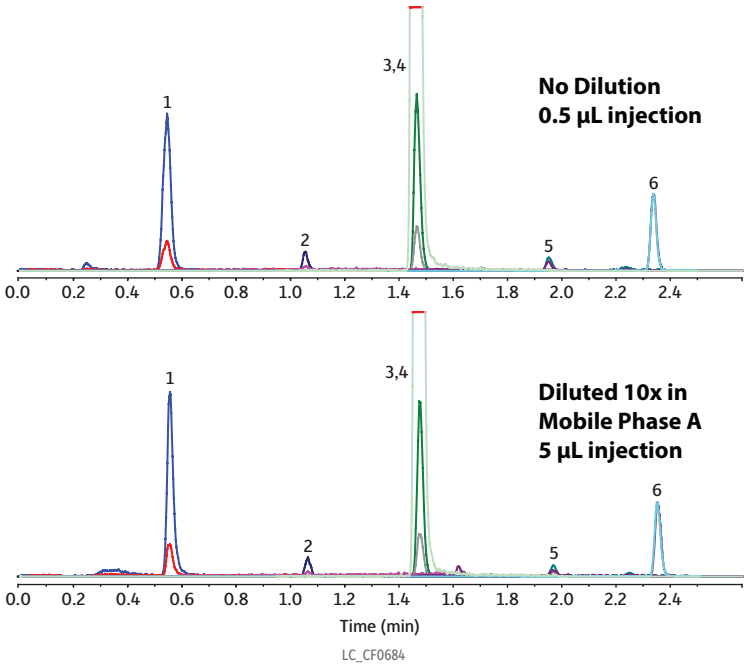

Column Raptor Biphenyl (cat.# 9309A52)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 30 °C
Sample
Conc.: 10 ng/mL
Mobile Phase
A: Water + 0.01% acetic acid
B: Methanol + 0.01% acetic acid

Time (min)	Flow (mL/min)	%A	%B
0.00	0.8	90	10
1.00	0.8	40	60
2.50	0.8	0	100
2.51	0.8	90	10
4.50	0.8	90	10

Max Pressure: 400 bar
Detector MS/MS
Ion Mode: ESI+
Mode: Scheduled MRM
Instrument UHPLC
Notes Sample Preparation Details: A 100 µL aliquot of fortified human plasma was protein precipitated with 300 µL acetonitrile containing isotopically labelled internal standard in a 1.3 mL 96-well plate (cat.# 26495). The plate was sealed and centrifuged for 10 minutes at 4,300 rpm and 8 °C. A portion of each sample extract was diluted 10x with mobile phase A. All samples (10x diluted and undiluted extracts) were injected.

Lastly, column lifetime was evaluated by performing 500 direct injections of undiluted 100 µg/mL PPT extract. It was noted that retention and peak shape remained consistent for all analytes throughout the experiment, although a decrease in peak intensity was observed as the experiment progressed (Figure 3). The most prominent loss in intensity occurred for the early eluting analytes lamivudine and zidovudine, which displayed decreases in peak height of 60% and 80%, respectively; ritonavir (eluting last) was the least impacted with a 25% loss in intensity. Without additional experiments, it is difficult to conclude whether the loss in sensitivity was due to the direct injection of PPT extracts or another variable, such as sample stability.

Figure 3: Column Lifetime: Effects of 500 Direct Injections of Undiluted 100 ng/mL Protein Precipitated Human Plasma Extracts.

Peaks	t_R (min)	Precursor Ion	Product Ion	Product Ion
1. Lamivudine	0.55	230.0	112.0	95.1
2. Zidovudine	1.06	268.1	127.2	110.1
3. Nevirapine-d3 (IS)	1.46	270.3	229.1	-
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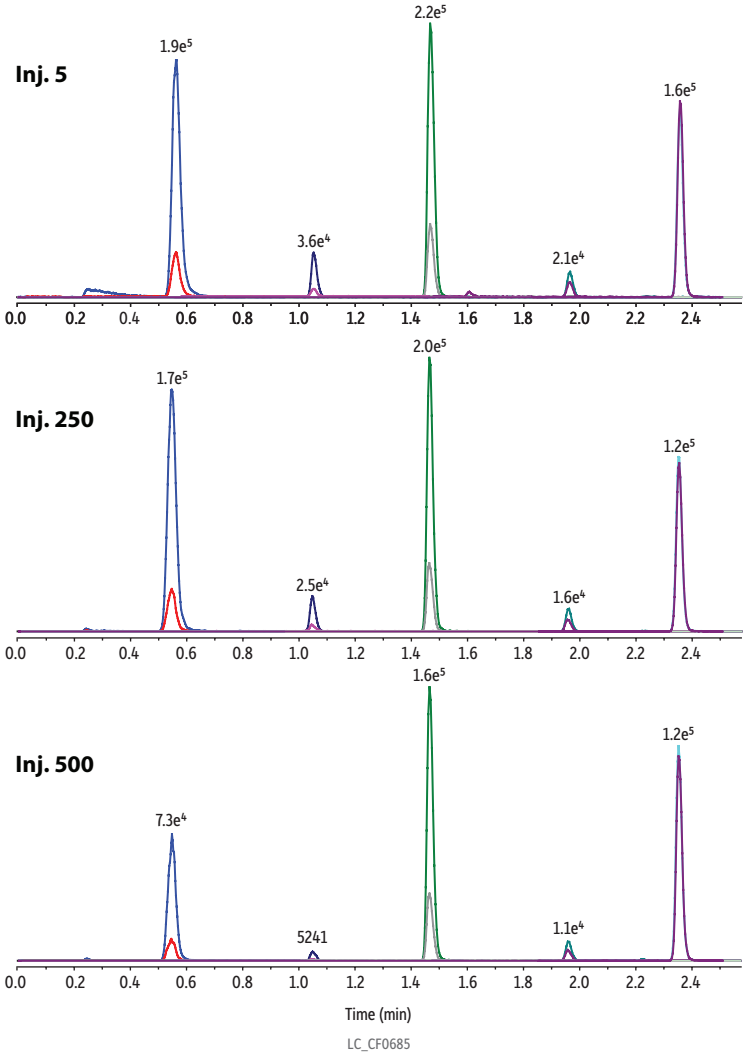

LC_CF0685

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 30 °C

Sample
Conc.: 100 ng/mL
Inj. Vol.: 0.5 µL

Mobile Phase
A: Water + 0.01% acetic acid
B: Methanol + 0.01% acetic acid

Time (min)	Flow (mL/min)	%A	%B
0.00	0.8	90	10
1.00	0.8	40	60
2.50	0.8	0	100
2.51	0.8	90	10
4.50	0.8	90	10

Max Pressure: 400 bar

Detector MS/MS
Ion Mode: ESI+
Mode: Scheduled MRM
Instrument UHPLC
Notes Sample Preparation Details: A 100 µL aliquot of fortified human plasma was protein precipitated with 300 µL acetonitrile containing isotopically labelled internal standard in a 1.3 mL 96-well plate (cat.# 26495). The plate was sealed and centrifuged for 10 minutes at 4,300 rpm and 8 °C.

Conclusion

While PPT is a common and effective sample preparation technique for biological matrices, the effects of the highly organic protein precipitation sample on LC peak shape can negatively impact accurate quantification. These effects can be mitigated by reducing the amount of organic solvent through either dilution of the extracts or direct injection of a smaller extract volume. As demonstrated here, direct injection is a viable option that can be used to prevent peak distortion, while avoiding the time and variability associated with dilution. Note that when using direct injection, the injection volume must be reduced enough to adequately compensate for differences in the specific solvents and the column dimensions that are being used. Prior to replacing sample extract evaporation/recondensation or dilution steps with direct injection, impacts on instrument performance should be assessed.

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