



# A Systematic Approach to Address and Resolve Issues Involved in Peptide Quantitation

## Authors

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## Introduction

Quantitative analysis is required to monitor pharmacokinetic profiles of peptide drugs. Quantifying low levels of peptides in the circulation system can be challenging due to short half-lives and to low recoveries from biological matrices. During the LC/MS/MS method development for quantifying the antimicrobial peptide drug, C16G2 (35 amino acid residues) in plasma, we encountered a number of challenges. Concerns included peptide stability, recovery, reproducibility, and unexpected chromatography. A systematic approach was conducted to address individual interfering issues. Different extraction methods were applied to examine the recovery of the peptide. Various protease inhibitors were used to assist in stabilizing peptides. Un-related peptides were pre-coated in tubes and plates to prevent non-specific binding. An on-line column-switching technique was employed to help further separate the unknown interference after peptides were extracted in plasma. We present the results in terms of sensitivity, accuracy, and precision using this systematic approach to peptide quantitation.

## Materials and Chemicals

- A peptide drug (C16G2) was provided by C3 Jian.

Thr-Phe-Phe-Arg-Leu-Phe-Asn-Arg-Ser-Phe-Thr-Gln-Ala-Leu-  
Gly-Lys-Gly-Gly-Gly-Lys-Asn-Leu-Arg-Ile-Ile-Arg-Lys-Gly-Ile-  
His-Ile-Ile-Lys-Lys-Tyr-NH<sub>2</sub>

*MW 4076 Dalton (35 a.a residues with an amidated C-Terminal)*



## Materials and Chemicals (continued)

- Halt™ Protease Inhibitor Cocktail (free EDTA) was purchased from Thermo Scientific. Phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor, was obtained from Sigma.
- Internal Standards
  - Methazolamide, Carbutamide, Glyburide, KRTLRR (a peptide) were used as internal standards during early method development.
  - Verapamil was chosen as a final internal standard for C16G2 quantitative analysis.
- WHWLQL (a peptide) was coated onto 96-well plates to minimize the non-specific binding of C16G2 from the containers.
- C16G2 quantification was performed in hamster, dog, rat, and human plasma (K2-EDTA), obtained from Biochemed.

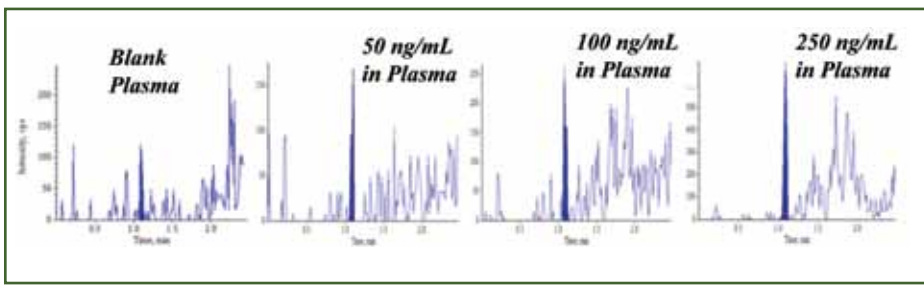
## Observed Issues

ISSUES	EVALUATIONS	OBSERVATIONS
LLOQ (Low Recovery)	Protein Precipitation (PPT)	100 ng/mL
	Solid Phase Extration (SPE)	50 ng/mL
	PPT/SPE	50 ng/mL
	Ultrafiltration (ULT)	100 ng/mL using 100,000 MWCO
Internal Standard (IS)	Methazolamide	non-linear curves
	Carbutamide	non-linear curves
	Glyburide	non-linear curves
	KRTLRR (peptide)	elute in the void volume
LC Column	Synergi™ Polar-RP (2x50mm)	not reproducible
	Metasil C18 (2x50 mm)	ugly peak shape
	XBridge™ C18 (2x50 mm)	not eluted
	Cyano (2x50 mm)	ugly peak shape
	Poroshell C18 (2 x 75 mm)	wide peak shape
Stickiness	Non-specific binding	lose signals in neat solution
Reproducibility	Interferences	inconsistent chromatography

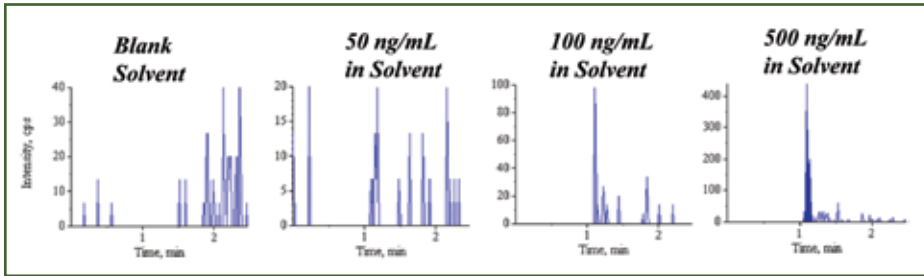


## Examples of Observed Issues

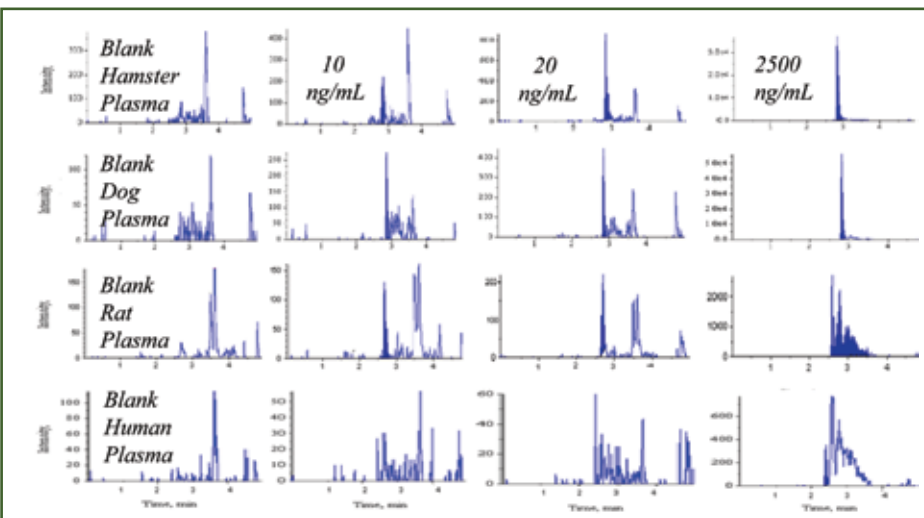
EXAMPLE 1: Poor Recovery Using PPT, SPE, PPT/SPE, ULT



EXAMPLE 2: Non-Specific Binding



EXAMPLE 3: Unexpected Interferences in Rat and Human Plasma



1. Halt™ protease inhibitor was added to plasma prior to sample process.
2. LLE and 2-column LC/MS/MS (no column switching) were used for C16G2 analysis.



## Extraction Methods

EXTRACTIONS	DESCRIPTIONS
<b>Protein Precipitation</b>	<ul style="list-style-type: none"> <li>• Plasma: ACN v:v 1:4</li> <li>• Plasma: ACN, 0.1% formic Acid</li> <li>• Plasma: ACN, 1% NH<sub>4</sub>OH</li> </ul>
<b>Solid Phase Extraction</b>	<ul style="list-style-type: none"> <li>• Pre-condition with MeOH and then H<sub>2</sub>O</li> <li>• Load plasma onto Water Oasis HLB cartridge (60mg)</li> <li>• Wash with 95/5 H<sub>2</sub>O/MeOH, 5% TEA and elute with 90/10 ACN/H<sub>2</sub>O, 5% formic acid</li> </ul>
<b>Ultrafiltration</b>	<ul style="list-style-type: none"> <li>• Plasma diluted with 1% formic acid v:v 1:1</li> <li>• Load plasma onto Millipore centrifuge units (MWCO 10,000 and MWCO 100,000)</li> <li>• Centrifuge at 13,000 rpm and transfer the filtrate</li> </ul>
<b>Liquid/Liquid Extraction</b>	<ul style="list-style-type: none"> <li>• Plasma: HFIP/ACN/TFA v:v:v 49:49:2</li> <li>• Add 50 μL NH<sub>4</sub>OH to each sample</li> <li>• Centrifuge at 13,000 rpm and transfer the organic layer</li> </ul>

*Halt™ protease inhibitor was added to plasma prior to sample process.*

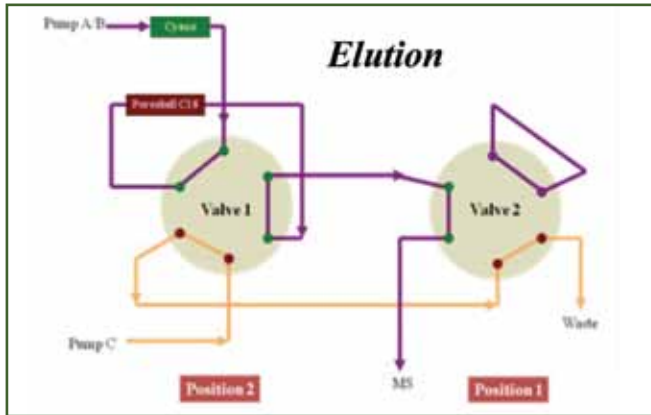
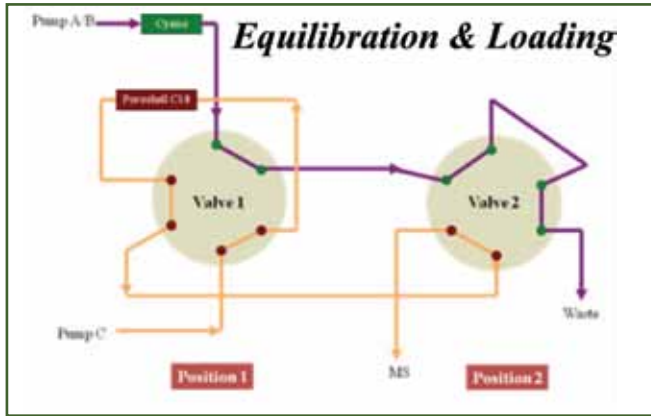
### LC CONDITIONS: NO COLUMN SWITCHING

LC COLUMNS	MOBILE PHASES	GRADIENTS		
Synergi™ Polar-RP (2 x 50 mm)	A: 100% H <sub>2</sub> O, 1% formic acid B: 100% ACN, 1% formic acid	<b>Time (min)</b>	<b>%B</b>	<b>Flow Rate (mL/min)</b>
Metasil C18 (2 x 50 mm)		0.01	15	0.5
XBridge™ C18 (2 x 50 mm)		3.0	40	0.5
Cyano (CN) (2 x 50 mm)		3.1	90	0.5
Poroshell C18 (2 x 75 mm)		4.0	90	0.5
CN/Poroshell C18 (2 x50 & 2 x75 mm)		4.1	15	0.5
		5.0	15	0.5



## Extraction Methods (continued)

### LC CONDITION: COLUMN SWITCHING



CYAN (2 x 50 mm) AND POROSHELL C18 (2 x 75 mm)				
Time (min)	Pump A/B	Switching Valve Position	Pump C	Switching Valve Position
	%B		%C	
0.01	15	1	100	2
0.8	15	1	100	2
0.9	45	2	100	1
1.9	45	2	100	1
2.0	90	2	100	1
2.9	90	2	100	1
3.0	15	1	100	2
3.5	15	1	100	2

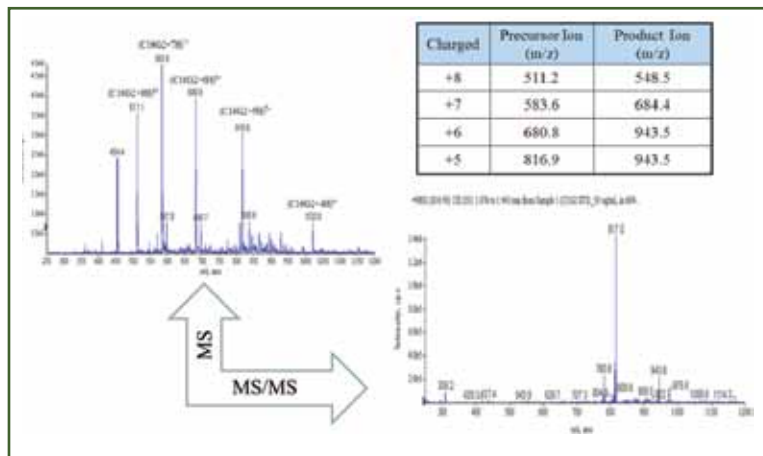
A: 100% H<sub>2</sub>O, 1% formic acid  
 B: 100% ACN, 1% formic acid  
 C: 15/85 H<sub>2</sub>O/ACN, 1% formic acid



## Results and Discussion

- Many issues, such as poor recovery, non-specific binding, reproducibility, and interference from biological matrixes, were present when developing an LC/MS/MS analytical method for C16G2 peptide quantitation.
- It was necessary to add a protease inhibitor (Halt™ protease cocktail inhibitor) to all plasma before the extraction of C16G2. The addition of PMSF did not have an impact on C16G2 stability in plasma.
- The liquid/liquid extraction appeared to be superior over other methods (protein precipitation, solid phase extraction, and ultrafiltration) for the extraction of C16G2. However, this is empirical for peptide sample preparation. Different peptides may work out better with different extraction methods.
- A two-column (Cyano and Poroshell C18) LC/MS/MS without using a column switching technique provided acceptable quantitative C16G2 analysis in hamster and dog plasma.
- Unexpected interferences and poor chromatography of C16G2 were noted in rat and human plasma using the two-column LC/MS/MS.
- The accuracy and precision of C16G2 in rat and human plasma were achieved within 20% bias and 20% CV when a column switch technique was applied using Cyano and Poroshell C18 columns.

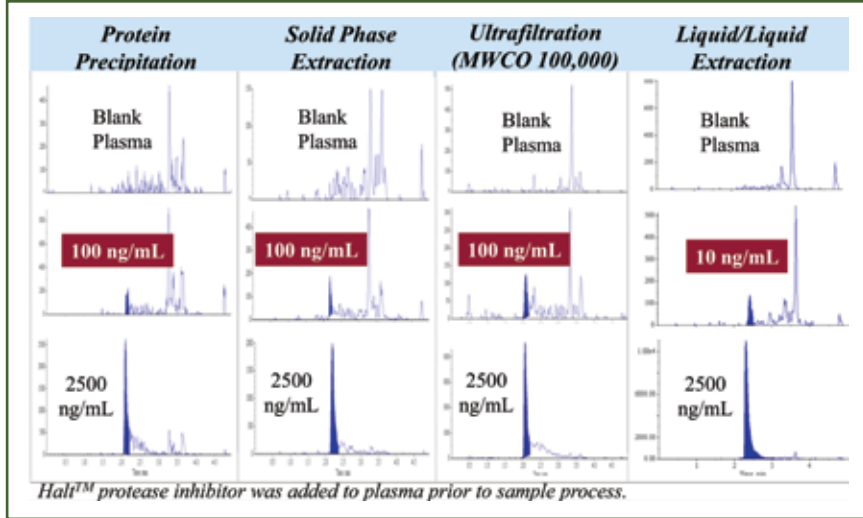
### C16G2 MS AND MS/MS MASS SPECTRA



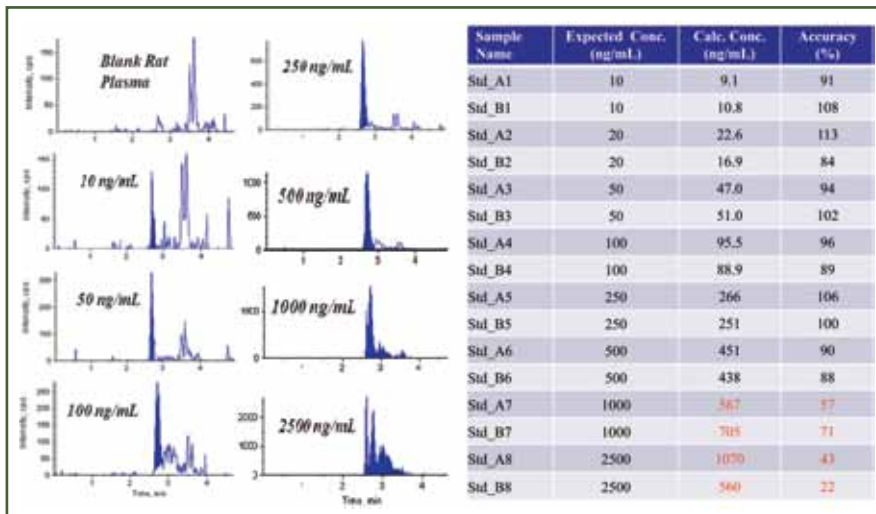


## Results and Discussion (continued)

### C16G2 LLOQ IN RAT PLASMA: RECOVERY COMPARISONS USING DIFFERENT EXTRACTION METHODS



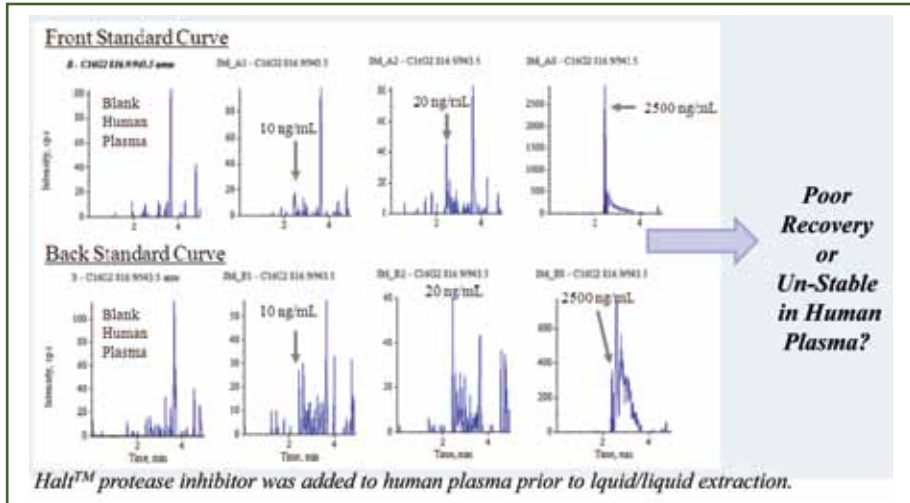
### LOSE ACCURACY IN RAT PLASMA USING LLE AND 2-COLUMN LC/MS/MS



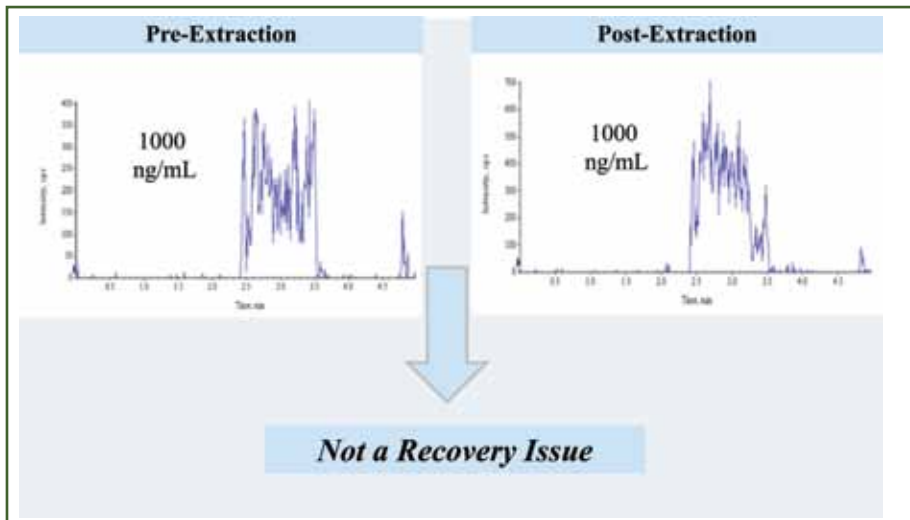


## Results and Discussion (continued)

### UNACCEPTABLE C16G2 QUANTIFICATION IN HUMAN PLASMA USING LLE AND 2-COLUMN LC/MS/MS



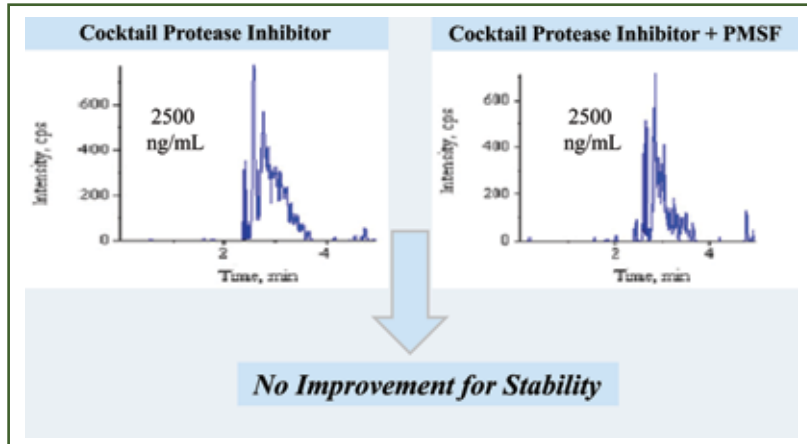
### RECOVERY EVALUATION IN HUMAN PLASMA (LLE AND 2-COLUMN LC/MS/MS)





## Results and Discussion (continued)

### PROTEASE INHIBITOR EVALUATION IN HUMAN PLASMA (LLE AND 2-COLUMN LC/MS/MS)



### WHAT HAPPENED TO C16G2 IN RAT AND HUMAN PLASMA?

Unknown endogenous molecules present in rat and human plasma may interact with C16G2



Removal of unknown endogenous molecules can improve the detection of C16G2 in rat and human plasma

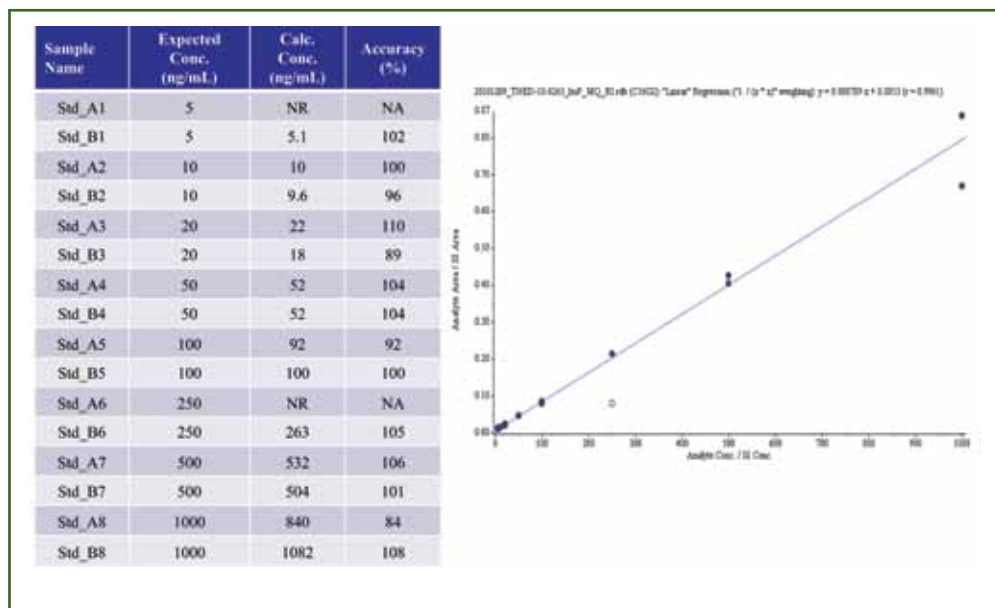
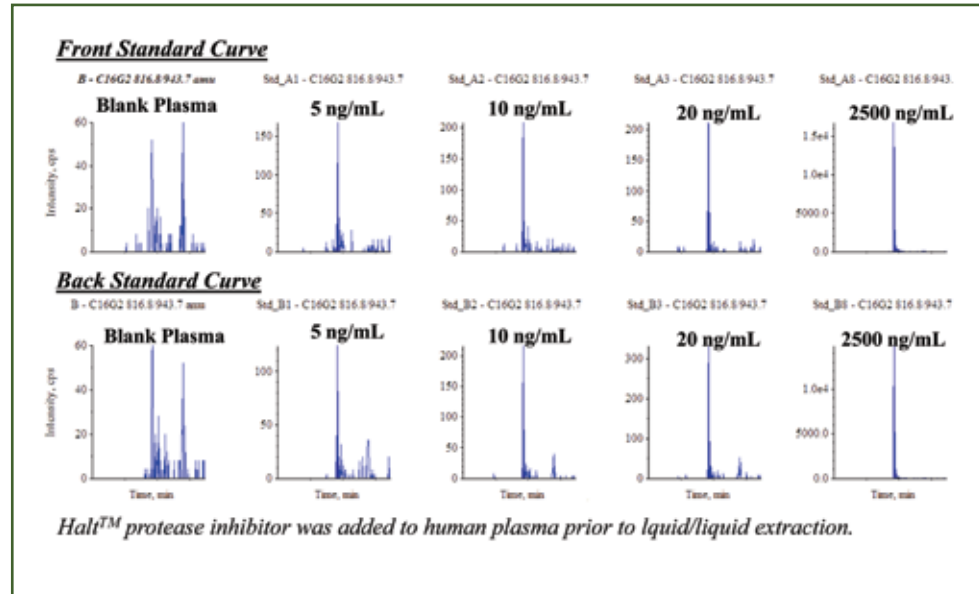


Apply a column switching technique to remove unknown interferences after liquid/liquid extraction



## Results and Discussion (continued)

### C16G2 QUANTIFICATION IN HUMAN PLASMA USING LLE AND 2-COLUMN LC/LC/MS/MS





## Conclusions

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- Protease inhibitor and LLE were required to stabilize and recover C16G2 in plasma.
- LLOQ was achieved at 10 ng/mL using LLE and a 2-column LC/MS/MS method (no column switching) in hamster and dog plasma.
- Use of column switching techniques removed unknown endogenous molecules (interferences) which possibly interacted with C16G2 in rat and human plasma.
- LLOQ of 10 ng/mL was achieved in rat and human plasma using LLE and a 2-column LC/LC/MS/MS method (column switching).