PRACTICAL CONSIDERATIONS FOR PROTEIN BIOANALYSIS

Mary E. Lame, Erin E. Chambers, Kelly Doering, and Russell Mortishire-Smith
Waters Corporation

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INTRODUCTION

Proteins and peptides represent a growing class of therapeutics due to their target specificity, lower toxicity and higher potency. Historically, protein biologics, in particular monoclonal antibodies (mAbs) have been quantified using ligand binding assays (LBAs). Recently there has been a trend towards increased analysis using LC/MS which offers the benefits of multiplexing, improved specificity, broader linear dynamic range and faster method development times. In addition, LC/MS avoids common LBA shortcomings such as cross reactivity and anti-drug antibody effects in the assay. However, quantification of proteins by LC/MS is not without its challenges. There is no single standardized workflow and the multitude of workflow options can make it difficult to know where to start to obtain optimal results.

Common steps in the protein bioanalytical workflow are demonstrated in Figure 1. For example, in the drug discovery phase, one can choose to use either generic human peptides or unique surrogate peptides to represent the drug. There are also many ways to introduce an internal standard: labeled protein, labeled peptides, and extended tag labeled peptides are just a few of the options. Furthermore, one can choose to do direct digestion of the plasma/serum sample, generic or specific affinity purification prior to digestion, or pellet digestion which incorporates a protein precipitation step. Finally, during the digestion step alone, the ratio of enzyme to substrate, the duration, temperature and source of enzyme may all need to be optimized.

This work aims to provide practical method development guidance and comparative data on the above topics for those endeavoring to develop LC/MS assays for quantification of proteins in biological matrices. The outcome of this work resulted in an optimized prototype reagent kit, which was used for the quantitative analysis of several mAbs in plasma.

METHODS

Sample Preparation

Human or animal plasma containing the antibody drugs of interest and a labeled antibody IS (SiluMab) were denatured, reduced, alkylated and digested using trypsin. In some cases, the antibody drugs were isolated from other plasma components prior to digestion using an agarose-based Protein A clean-up step. Precipitation using various types and ratios of organic solvents was also tested in an effort to reduce the endogenous background prior to digestion. In particular, peptides arising from human serum albumin were monitored to compare the effectiveness of each treatment in albumin removal. After digestion, the resultant peptides were separated from digest reagents and phospholipids using mixed-mode cation exchange SPE.

LC/MS Conditions

LC/MS peptide quantification was performed using a Waters Xevo TQ-S triple quadrupole MS. Chromatographic separation was achieved using an ACQUITY UPLC system with a 2.1 X 150 mm BEH C18 1.7 µm. Mobile phase A and B were water and acetonitrile, respectively, each containing 0.1% formic acid by volume. A linear gradient from 10-55% B over 6 minutes at a flow rate of 300 µL/min was used.

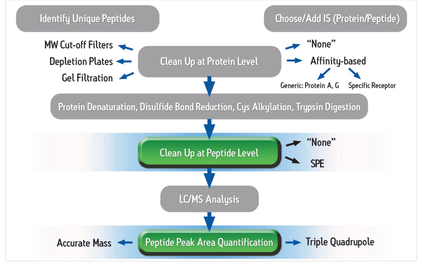

Figure 1 : Common steps in the protein bioanalytical workflow.

RESULTS

Protein Level Clean-Up

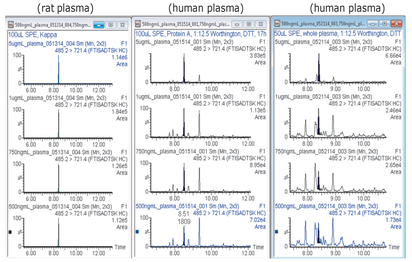

Figure 2: Effect of protein-level purification on MS background and sensitivity for a specific trastuzumab peptide.

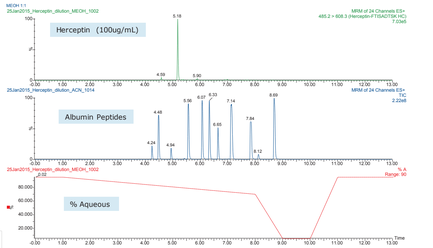

Figure 3: TIC Chromatogram of multiple human serum albumin peptide interferences and XIC of the signature peptide of trastuzumab.

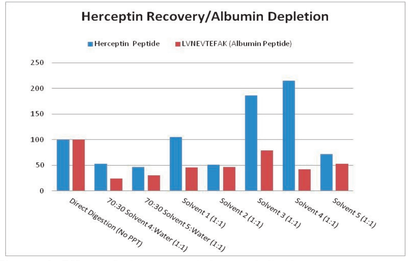

Figure 4: Effect of plasma protein precipitation pre-treatment on albumin depletion and trastuzumab peptide recovery.

Digestion Optimization

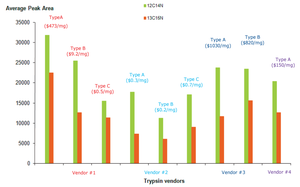

Figure 5: Evaluation of trypsin type and vendor.

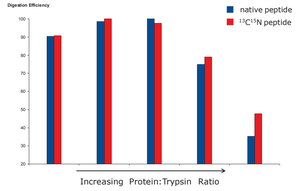

Figure 6: Serum digest protein:trypsin ratio optimization results for a specific trastuzumab

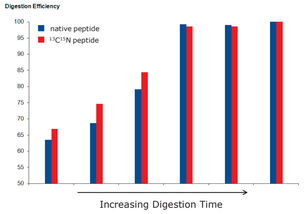

Figure 7: Serum digest time optimization for a specific trastuzumab peptide.

Peptide Level Clean-Up

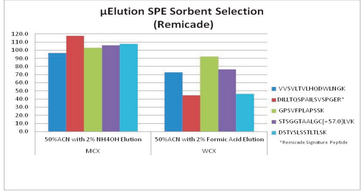

Figure 8 : Serum digest clean-up using a generic mixed-mode cation exchange SPE provides high recovery for generic and surrogate peptides of the monoclonal antibody, infliximab.

Quantification of Infliximab

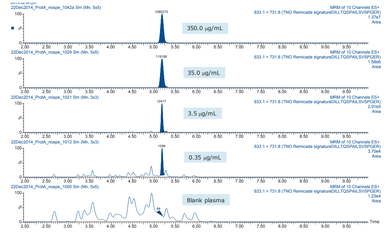

Figure 9: Infliximab QC Chromatograms for the generic peptide VVSVLTVLHQDWLNGK in plasma digest.

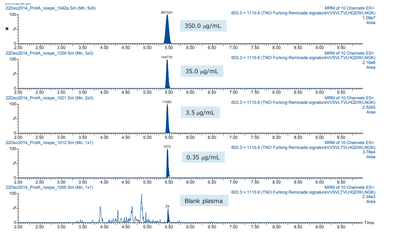

Figure 10: Infliximab QC Chromatograms for the signature peptide DILLTQSPAILSVSPGER in plasma digest.

Peptide	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
GPSVFPLAPSSK	0.35	0.38	0.00	1.05	109.7
	3.50	3.87	0.07	1.90	110.7
	35.00	36.49	0.61	1.67	104.2
	350.00	-	-	-	-
	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
STSGGTAALGC[+57]LVK	0.35	0.38	0.01	3.39	109.4
	3.50	3.62	0.22	6.20	103.5
	35.00	35.01	3.29	9.40	100.0
	350.00	353.43	4.85	1.37	101.0
	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
DSTYSLSSTLTLSK	0.35	0.37	0.00	0.27	105.7
	3.50	3.80	0.08	2.22	108.5
	35.00	37.53	0.61	1.64	107.2
	350.00	347.51	2.50	0.72	99.3
Peptide	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
VVSVLTVLHQDWLNGK	0.35	0.35	0.01	3.89	100.9
	3.50	3.91	0.04	0.95	111.6
	35.00	37.03	1.59	4.28	105.8
	350.00	347.27	13.57	3.91	99.2
	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
DILLTQSPAILSVSPGER*	0.35	0.33	0.02	5.80	93.2
	3.50	3.79	0.02	0.49	108.2
	35.00	39.58	0.17	0.44	113.1
	350.00	350.02	3.09	0.88	100.0

* Signature Peptide

Table 1. Infliximab QC statistics following generic affinity capture and tryptic digestion in plasma.

DISCUSSION

Common challenges of protein quantification in biological matrices by enzymatic digestion are: limited sample volume, sensitivity, time it takes to complete a digestion experiment, as well as cost. Therefore, it is important to assess/optimize all parts of the workflow that will facilitate speed and result in sensitive and robust methods for protein quantification.

- Generic affinity purification at the protein level increased sensitivity for humanized antibodies by 10-20X in preclinical species. (Figure 2).
- Interfering peptides from albumin often co-elute with the signature peptides from the therapeutic protein (Figure 3). Incorporating an optimized precipitation step increased surrogate peptide signal by 2-3X and reduced the abundance of albumin derived peptides (Figure 4).
- Protein bioanalytical quantification, which used targeted MRM peptides analysis, may allow one to use less purified, and significantly more affordable trypsins (Figure 5).
- Protein:protease ratio and digestion time must be optimized to ensure maximum digestion efficiency, while minimizing the amount of trypsin required (Figures 6 and 7).
- Serum digest clean-up, using a generic mixed-mode cation exchange SPE method, provided high recovery for both generic and unique peptides from trastuzumab, bevacizumab, and infliximab whilst removing digest reagents and phospholipids (Figure 8).
- Figures 9 (generic peptide) and 10 (signature peptide) contain representative spectra for QC's samples containing the monoclonal antibody infliximab at 0.35, 3.5, 35.0 and 350.0 µg/mL.
- Linearity, accuracy and the precision of quantification based on generic or unique human tryptic peptides were equivalent in preclinical species. Average LLOQ values were 100 ng/mL. Representative QC statistics are shown in Table 1.

CONCLUSION

This work evaluates and compares analytical options for each step of several common protein bioanalysis workflows. These data enable scientists to understand the magnitude of the effect each choice has on data sensitivity and specificity, thus allowing for more efficient method development based on study need. The data also suggest that a few standardized workflows could satisfy the requirements of a preclinical environment. Using a prototype reagent kit for protein bioanalysis: saved time, simplified the workflow, and allowed standardization for the sensitive, accurate and robust quantification of infliximab, trastuzumab, and bevacizumab.

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