ULTRA HIGH SENSITIVITY MICROFLOW LC/MS QUANTITATION OF AN ADC AND A THERAPEUTIC ANTIBODY USING A KIT BASED APPROACH

Michael Donegan, Mary E. Lame, James Murphy, and Erin E. Chambers
Waters Corporation 34 Maple Street Milford, MA 01757, USA

INTRODUCTION

Monoclonal antibodies (mAbs) and complex modalities like antibody-drug conjugates (ADCs) are particularly attractive as therapeutics due to their target specificity and higher potency. However, protein quantification workflows are complex and laborious, and reliably achieving acceptable quantification limits have proven challenging for experienced and novice bioanalytical scientists alike.

Microflow LC/MS has long been shown to offer significant sensitivity gains over more traditional 2.1 x 50 mm UPLC approaches.[1] The reason for the observed signal gains in microflow lie in the fact that lower flow rates improve the sampling efficiency of the electrospray plume and generate a finer, less disperse spray. Also, as column diameter decreases, peaks elute off of the LC column with a much lower volume and therefore produce peaks with a higher signal-to-noise (S/N) ratio as compared to its UPLC counterpart.

This work demonstrates that a fully standardized, kit-based approach to sample preparation, coupled to a highly sensitive microscale LC/MS platform, can routinely achieve detection limits in the low ng/mL range for therapeutic mAbs and ADCs alike from only 35 µL of sample.

METHODS

Sample Preparation

Infliximab, trastuzumab, and trastuzumab emtansine (T-DM1) were spiked into rat plasma. Protein level affinity purification of the plasma (35 µL), using an anti-human capture, was performed. Resulting purified samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit[2] and provided 3-Step (no reduction or alkylation) or 5-Step protocols (Figure 1).

LC-MS Conditions

LC-MS quantification of signature peptides was performed using a Waters ionKey/MS ™ System. MS conditions are summarized in Table 1. Chromatographic separation was performed with a Waters® BEH C_{18} iKey (150 µm x 100 mm, 1.7 µm). Mobile phase A and B consisted of water and acetonitrile, respectively, each containing 0.1% formic acid. To maximize sensitivity, 20 µL of sample was injected using a trap and elute workflow. Trap conditions involved loading the sample onto a 300 µm x 50 mm BEH C_8 trap column for 2.5 minutes at a flow rate of 20 µL/min. The flow was then reversed into the analytical column for analysis. Resulting tryptic peptides were eluted using a linear gradient (2-40% B) over 7 minutes at a flow rate of 3 µL/min. LC-MS quantification of signature peptides was performed using a Waters Xevo TQ-XS triple quadrupole MS. MS conditions are summarized in Table 1.

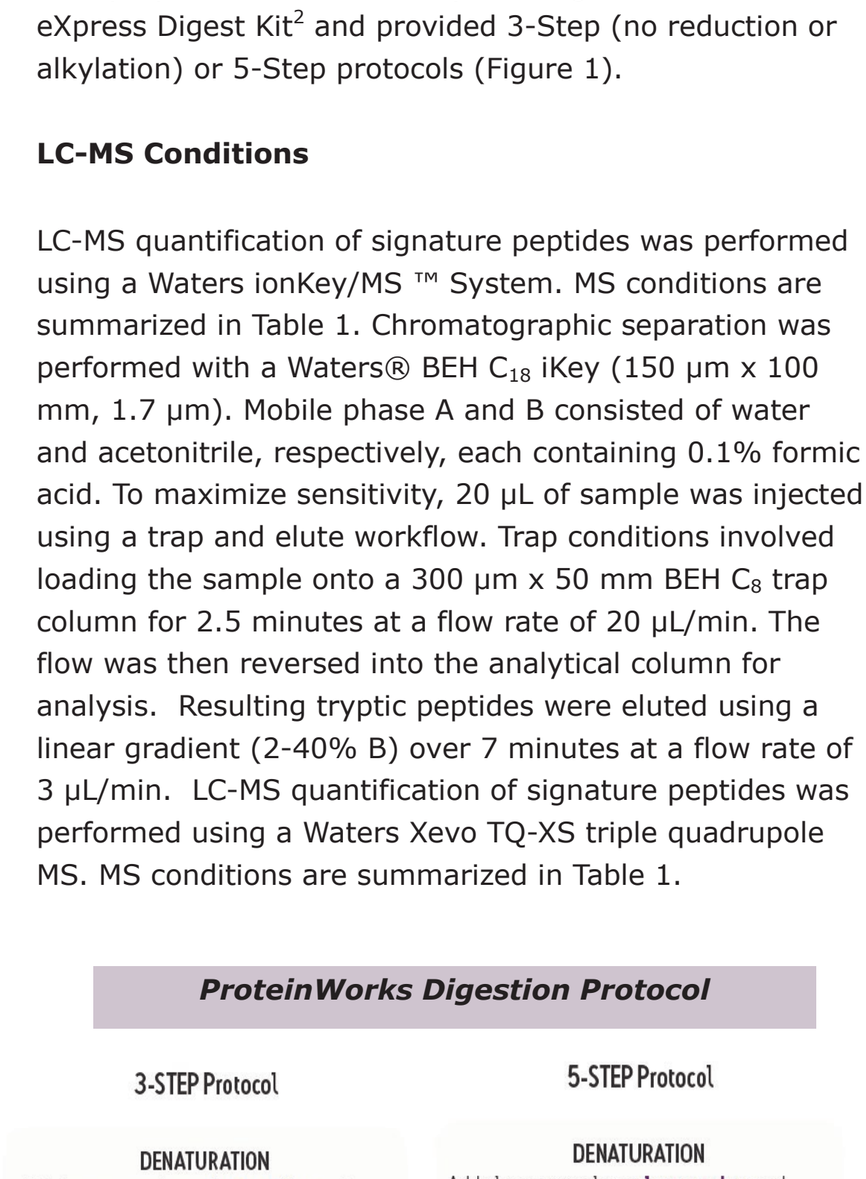

Figure 1. ProteinWorks™ eXpress Digest Kit protocols

RESULTS

I. High Sensitivity Quantification of the mAb, Infliximab Using Affinity Purification and Digestion with ProteinWorks eXpress Digest Kit

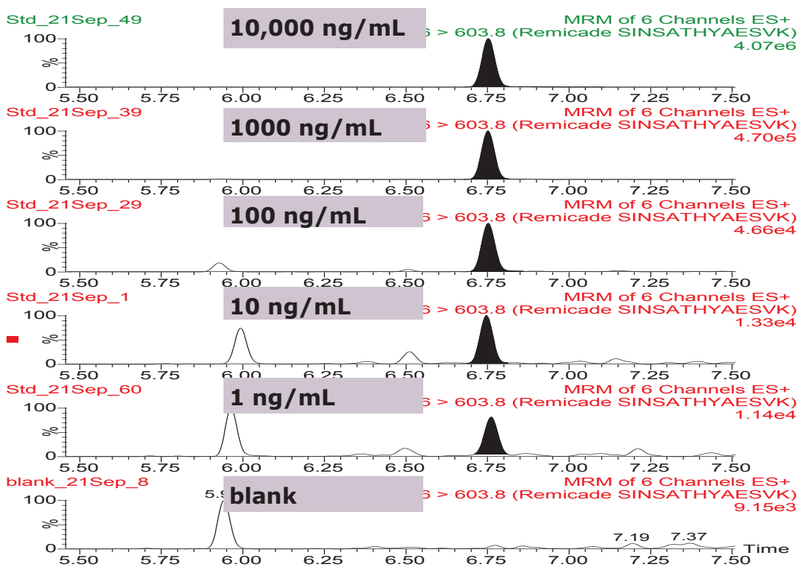

Figure 2: Representative chromatograms highlighting increased concentrations of infliximab in plasma, as compared to blank plasma that was immunopurified and digested (3 -Step) using the ProteinWorks eXpress Digest Kit

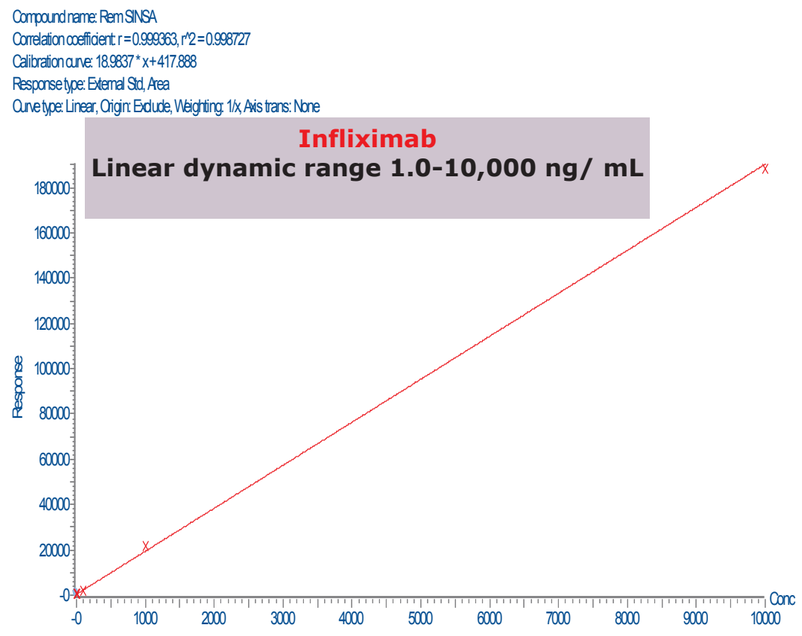

Figure 3. Calibration curve using the signature peptide SINSATHYAESVK to quantify infliximab in rat plasma that was immunopurified, digested and extracted

II. ADC (T-DM1) and mAb (Trastuzumab) Quantification Using Affinity Purification and Digestion with ProteinWorks eXPress Digest Kit .

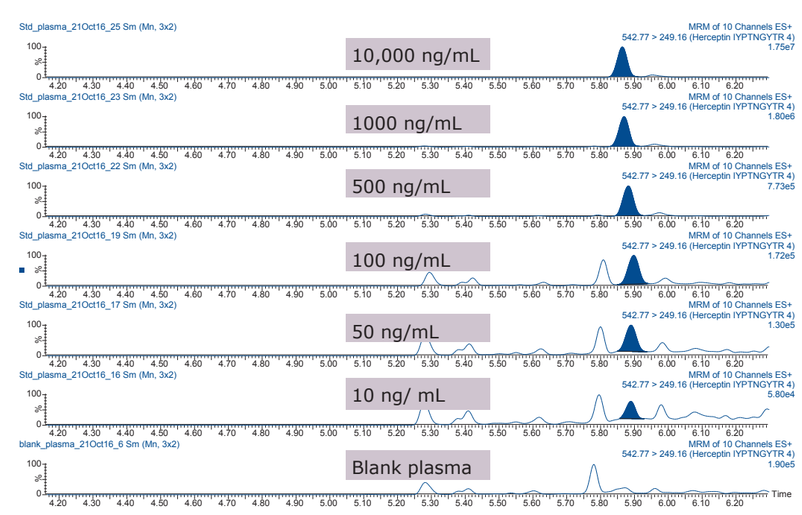

Figure 4: Extracted chromatograms of trastuzumab (using the unique peptide IYPTNGYTR) in rat plasma, that was immunopurified, digested, and extracted using the ProteinWorks eXpress Digest Kit

Protein	Peptide	MRM Transition	Cone Voltage	Collision Energy
T-DM1/Trastuzumb	IYPTNGYTR	542.7/249.2	36	16
		542.7/808.4	12	16
Trastuzumab	FTISADTSK	485.2/721.4	28	22
		485.2/608.3	28	22
Trastuzumab	DTYIHWVR	543.3/597.3	28	24
		543.3/710.4	28	28
T-DM1 miscleavage with small molecule drug attached	FTISADTSKNTAYLQMNSLR	1073.2/547.2	35	38
	GPSVFPLAPSSKSTSGGTAALGCLVK	1149.2/547.2	35	38
Infliximab	SINSATHYAESVK	469.6/603.8	35	15

Table 1. MRM conditions for infliximab, trastuzumab, and trastuzumab emtansine (T-DM1)

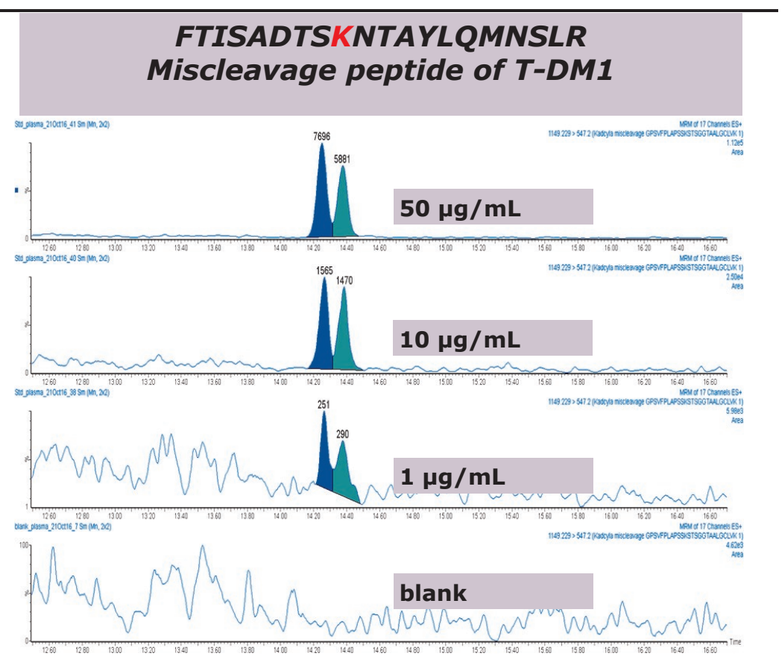

Figure 5: Chromatograms demonstrating the presence, and increase of signal, for the miscleavage peptide of T-DM1 with small molecule drug attached (FTISADTSKN) in plasma.

Peptide	Std curve range (ng/mL)	Weighting	Linear fit (r^2)	Mean % accuracy
IYPTNGYTR	10-50,000	1/x	0.993	98.2
FTISADTSK	50-50,000	$1/x^2$	0.991	99.4
DTYIHWVR	50-50,000	1/x	0.999	100.0

Table 2. Linear dynamic range, weighting, and average accuracy for standard curves of trastuzumab used to quantify the ADC, T-DM1, and trastuzumab in plasma, digested and extracted using the ProteinWorks eXpress Digest kit.

DISCUSSION

As more drug development efforts focus on large molecules such as monoclonal antibodies and ADCs, traditional "small molecule" scientists find themselves challenged not only by the complex and laborious sample preparation workflows but also the need for highly sensitive, and reproducible quantification methods.

- Using only 35 µL of plasma, protein level affinity purification, and digestion using the ProteinWorks eXpress Digest Kit, detection limits of 1 ng/mL were achieved for infliximab (Figure 2) Excellent linearity and accuracy (over 4 orders of magnitude) for infliximab is highlighted in Figure 3.
- The ADC, T-DM1 is an antibody–drug conjugate used to treat breast cancer. Following immunopurification and subsequent digestion, quantification limits between 10-50 ng/mL were achieved. Representative chromatograms are illustrated in Figure 4. Standard curve statistics are summarized in Table 2.
- Following tryptic digestion of TDM-1, there is potential for miscleavage on the lysine residue when it is conjugated with the small molecule drug.[3] For this reason, one would need to be cautious of using the lysine containing peptides such as, FTISADTSK and GPSVFPLAPSSK for accurate quantification of T-DM1. In fact, at higher concentrations of T-DM1, we were successfully able to detect a miscleavage peptide of T-DM1 with the small molecule drug attached, (FTISADTSKNTAYLQMNSLR). This is illustrated in Figure 5.
- The combined use of integrated microscale LC (ionKey/MS) and multidimensional chromatography, specifically a trap and elute strategy, facilitated large sample injection volumes (20µL), ultimately providing substantial gains in sensitivity.
- Including affinity purification and digestion with a commercially available kit, total sample preparation time was <6 hours.

CONCLUSION

The ProteinWorks eXpress Digest Kits were successfully used to quantify the mAb infliximab and the ADC, trastuzumab emtansine. This universal, kit-based approach coupled with a highly sensitive microscale LC/MS platform, allows scientists to achieve both the sensitivity and reproducibility required in discovery studies to make time sensitive and critical project decisions.

References

1. P. D. Rainville, J Langridge, M. Wrona, I. Wilson and R. Plumb. Bioanalysis, 7(11), 1397-1411 (2015).
2. **Waters ProteinWorks eXpress Digest Kits: http://waters.com/proteinworks**
3. **Waters Poster: Library Number: PSTR134864615** In-Depth Characterization of Lysine-Conjugated Antibody-Drug Conjugates (ADCs) by LC/MS Qualitative and Quantitative Analysis

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