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UTILIZING THE LC-MS TOOL BOX IN PROTEIN BIOANALYSIS

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INTRODUCTION

WHEN DESIGNING A METHOD FOR THE QUANTITATIVE ANALYSIS OF PROTEINS IN BIOLOGICAL MATRICES, SEVERAL ASPECTS SHOULD BE CONSIDERED, SUCH AS SAMPLE PURIFICATION, ANALYTE PROCESSING AND LC-MS STRATEGY. TRADITIONAL WORKFLOWS ARE SHOWN IN FIGURE 1. WITH REGARD TO SAMPLE PURIFICATION, THERE ARE MANY APPROACHES AVAILABLE, FROM HIGHLY SELECTIVE (E.G. USING IMMOBILIZED ANTI-IDIOTYPIC ANTIBODIES), MODERATELY SELECTIVE (E.G. PROTEIN A) TO NONE-SELECTIVE PURIFICATION SUCH AS PROTEIN PRECIPITATION FOLLOWED BY PELLET DIGESTION.

THE ANALYTE PROCESSING PORTION INVOLVES THE MODIFICATION OF THE ANALYTE PROTEIN TO THE DETECTION TARGET, SUCH AS IN THE BOTTOM UP APPROACH WHERE ANALYTE PROTEINS ARE DIGESTED TO SUITABLE TARGET PEPTIDES. IN INTACT AND NATIVE PROTEIN LC-MS, ANALYTE PROCESSING CAN BE LESS EXTENSIVE OR NOT PERFORMED AT ALL.

FINALLY, THERE ARE AGAIN MANY LC-MS OPTIONS WITH REGARD TO TYPE OF CHROMATOGRAPHY, THE LC SCALE, MASS ANALYZER AND DETECTION APPROACH. IN THE PRESENT STUDY, TWO LC-MS APPROACHES WERE UTILIZED FOR PROTEIN BIOANALYSIS: BOTTOMUP AND INTACT LC-MS WERE COMPARED ACROSS TANDEM QUADRUPOLE AND HIGH RESOLUTION MASS SPECTROMETERS. INFLIXIMAB (148 KD) AND CYTOCHROME C (12 KD) WERE USED AS REPRESENTATIVE MOLECULES.

METHOD

Infliximab

To assess the bottom up approach, infliximab was spiked into rat plasma, purified using protein A and digested using the ProteinWorks eXpress Digest Kit [1]. Samples were analyzed using an ACQUITY UPLC with the Xevo TQ-S. An ACQUITY UPLC HSS T3 1.7 µm, 2.1 x 150 mm column was used for separation, and the peptides were eluted from the analytical column using an acetonitrile gradient. The unique signature peptides SINSATHYAESVK and DILLTQSPAILSVSPGER were used as detection targets and the suitability of different internal standard tryptic peptides originating from SILu™Mab (Sigma-Aldrich) was investigated.

After spiking the therapeutic protein into mouse serum ultrafiltrate, intact LC-MS analysis of infliximab was performed using either an ACQUITY UPLC with Xevo TQ-S or a Dionex Ultimate 3000 with Q Exactive Orbitrap. In both cases, an Acquity BEH300 C4, 2.1 × 100 mm, 1.7 µm, 300 Å column (Waters) was used for separation, and the proteins were eluted from the analytical column using an isopropanol gradient. For tandem quadrupole analysis, 0.5% m-NBA was added to the mobile phase to provide the analyte ions with more charges.

Cytochrome C

To assess the bottom up approach, cytochrome C was spiked into human plasma, subjected to digestion and extraction using the ProteinWorks eXpress Direct Digest Kit and analyzed using an ACQUITY UPLC with the Xevo TQ-S. To explore the potential sensitivity, intact LC-MS analysis of cytochrome C was performed in Tween 80 0.005%/ maltotriose 0.6% using an ACQUITY UPLC with the Xevo TQ-S.

RESULTS

Infliximab

The bottom up approach resulted in a highly sensitive method for infliximab. The target peptide SINSATHYAESVK yielded the greatest sensitivity with clear signal at 10 ng/mL as shown in Figure 2. The results using DILLTQSPAILSVSPGER as target peptide are summarized in Table 1. For this peptide, the obtained sensitivity was 350 ng/mL, and single digit reproducibility was achieved in a concentration range spanning three orders of magnitude. Application of different tryptic IS peptides from SILu™Mab gave comparable results.

	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
SILUMAB-DTL(IS)	**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
	QC Conc (ug/mL)	Mean Cal. Conc (ug/mL)	Std. Dev.	%CV	Mean Accuracy
DILLTQSPAILSVSPGER*	**0.35**	0.34	0.00	0.89	96.5
SILUMAB-VVSV (IS)	**3.50**	3.65	0.05	1.47	104.2
	35.00	36.51	0.73	2.01	104.3
	350.00	350.80	3.90	1.11	100.2

* Signature

Table 1: Bottom up quantification results of infliximab using DILLTQSPAILSVSPGER as target peptide and DTL and VVSV from SILu™Mab as IS.

The bioanalysis of infliximab was also explored using the intact approach, using two different mass analyzers [2]. An Orbitrap MS spectrum of infliximab containing peaks between m/z 2000 and 6000 is shown in Figure 3. As the Xevo TQ-S has an m/z range up to 2000, more charges had to be added to the infliximab ions. Therefore, m-NBA, which acts as a supercharging agent, was added to the mobile phase. The MS spectrum of infliximab in Figure 4 shows that addition of m-NBA indeed resulted in a shift of the distribution within the available m/z range. In Figure 5, a calibration curve is presented.

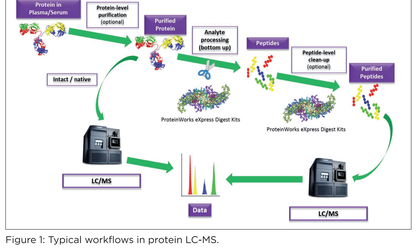

Figure 1: Typical workflows in protein LC-MS.

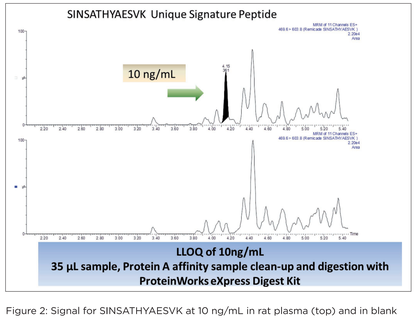

Figure 2: Signal for SINSATHYAESVK at 10 ng/mL in rat plasma (top) and in blank rat plasma (bottom).

Using the intact approach, the sensitivity for infliximab was comparable or slightly less than in the bottom up approach. Figure 6 shows a chromatogram for infliximab spiked into mouse serum ultrafiltrate at 100 ng/mL, as obtained with an Orbitrap. It is assumed that an even higher sensitivity for infliximab could be obtained if the intact LC-MS analysis was preceded by a purification step such as immobilized TNFα, as it was readily feasible to obtain < 100 ng/mL sensitivity when intact infliximab was analyzed in solvent.

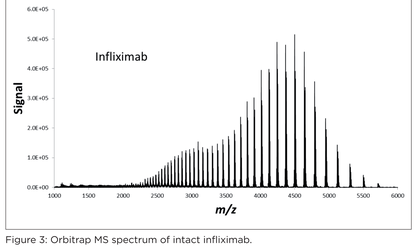

Figure 3: Orbitrap MS spectrum of intact infliximab.

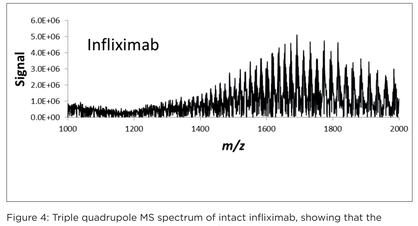

Figure 4: Triple quadrupole MS spectrum of intact infliximab, showing that the charge state distribution shifted to lower m/z due to the addition of m-NBA to the mobile phase.

Cytochrome C

For cytochrome C, it was difficult to find a sensitive target peptide in the bottom up approach, thus the detection limit achieved was significantly lower than using the intact approach (0.5 µg/mL vs. 10 ng/mL for intact). Although the intact LC-MS analysis was performed in solvent, the obtained detection limit offers an outlook to sensitive detection in biological matrices when a selective purification is applied prior to LC-MS analysis.

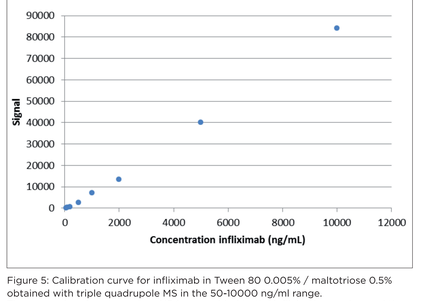

Figure 5: Calibration curve for infliximab in Tween 80 0.005% / maltotriose 0.5% obtained with triple quadrupole MS in the 50-10000 ng/ml range.
The super-charging reagent, m-NBA, was added to the infliximab samples, and the signal was composed of the sum of ten MRMs.

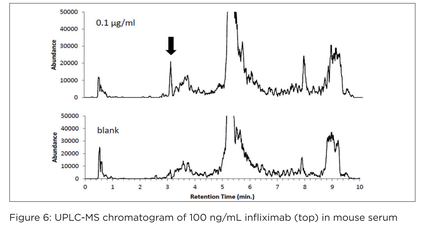

Figure 6: UPLC-MS chromatogram of 100 ng/mL infliximab (top) in mouse serum ultrafiltrate and blank mouse serum ultrafiltrate (bottom), obtained with Orbitrap. The signal was composed of the sum of nine m/z ranges.

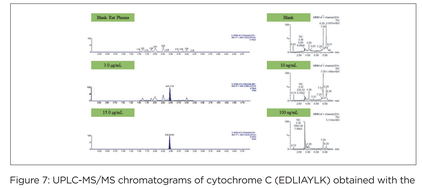

Figure 7: UPLC-MS/MS chromatograms of cytochrome C (EDLIAYLK) obtained with the bottom up approach in rat plasma (left) at different concentrations and UPLC-MS/MS chromatograms obtained with the intact approach in Tween 80 0.005% / maltotriose 0.6% (right) at different concentrations (transition m/z 728 => 120, 300 ms dwell time).

CONCLUSION

- Both bottom up protein LC-MS analysis and intact protein LC-MS can be valuable approaches to obtain high sensitivity in protein bioanalysis
- In this study, higher sensitivity for infliximab was obtained with the bottom up approach
- For smaller proteins such as cytochrome C, intact analysis might afford greater sensitivity, and sample preparation is much simpler
- Depending on the sensitivity required, both the bottom up and top down approaches from the LC-MS tool box should be considered for protein quantification

REFERENCES
1 Lame M, Yang H, Naughton S, Chambers E. High Sensitivity Quantification of Infliximab in Rat Plasma Using a Fast, Standardized Kit-Based Approach. Waters Application Note 720005535EN. (2016)
2 Buscher BAP, Toersche JH, van Holthoon FL, Kleinnijenhuis AJ. Comparison of Triple Quadrupole and Orbitrap Mass Spectrometry for Quantitative Bioanalysis of Intact Proteins. J Res Anal 1, 3-10 (2015).

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