High Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF Mass Spectrometer

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Goal

Report on the benefits of a fast analytical platform employing highly efficient chromatographic peptide separations in combination with fast and high resolution quadrupole Thermo Scientific™ Orbitrap™ mass spectrometry technology as a tool for fast peptide mapping of biopharmaceuticals to obtain analytical information about correct sequence, glycosylation and post-translational or artificial modification of recombinant monoclonal antibodies.

Introduction

Monoclonal antibodies (mAbs) are the major element in the fastest growing sector of biopharmaceuticals within the pharma industry. By 2016, eight of the top ten drugs will be therapeutic proteins. Their manufacture is accomplished in bacterial or eukaryotic expression systems, requiring extensive purification of the target product. During drug development and production the quality of biotherapeuticals needs to be closely monitored. Various analytical methods have been used to study quality attributes such as structural integrity, aggregation, glycosylation pattern or amino acid degradation. Because of their high information content and versatility, characterization methods based on high-performance liquid chromatography and mass spectrometry are among the most powerful protein characterization methods. Proteins can be enzymatically digested to obtain peptides enabling their analysis by means of peptide mapping experiments. Here, we report a fast and sensitive approach by combining enzymatic digestion, fast chromatographic separation, high resolution mass spectrometry and rapid data processing to handle the large amount of samples in diverse biopharma workflows. In this study we have analyzed two commercially available drug products: Rituximab (trade names MabThera® and Rituxan®) and Denosumab (trade names Prolia® and XGEVA®) .

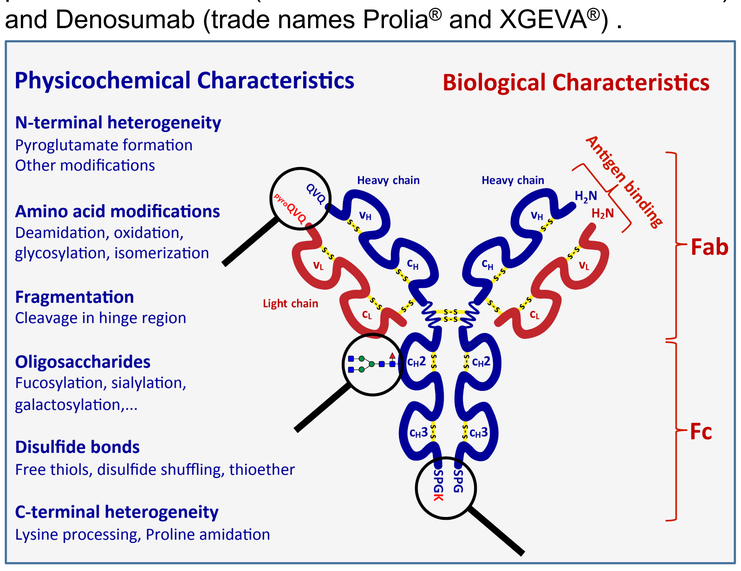

FIGURE 1. General structure of mAbs and their biological and physico-chemical characteristics.

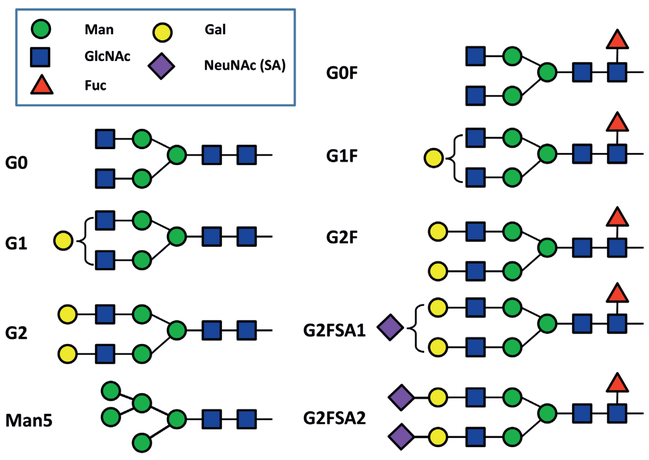

FIGURE 2. Nomenclature of carbohydrate structures commonly observed on antibodies.

Methods

The two drug products Rituximab and Denosumab were denatured for 30 min in 7 M Urea and 50 mM Tris HCL at pH 8.00. The samples were reduced with 5 mM DTT for 30 min at 37°C, alkylation was performed with 10 mM IAA for 30 min at room temperature and the reaction was quenched by addition of 10 mM DTT. Pierce™ Trypsin Protease (MS Grade) was added and digestion allowed to proceed over night at 37°C. Digests were stopped by addition of TFA to approximately pH 3.00. Thermo Scientific™ Vanquish™ UHPLC system with a 2.1 x 250 mm i.d. Thermo Scientific™ Acclaim™ 120 C18 column and gradients of water and ACN with 0.1% formic acid each were used to separate the peptide mixtures. Five different separation times were applied and compared: 5, 8, 13, 20 and 30 min for the gradient ramping from 2-45% ACN. Flow rates were adapted accordingly using 1.1 (5 min), 1.0 (8 min), 0.6 (13 min), 0.4 (20 min) and 0.4 mL/min (30 min). The Thermo Scientific™ Q Exactive™ HF mass spectrometer (MS) equipped with a HESI-II probe was used for mass spectrometric detection. The data were acquired with Thermo Scientific™ Xcalibur™ 3.0 software in combination with Thermo Scientific SII for Xcalibur 1.1 software. Data analysis was performed using Thermo Scientific™ PepFinder™ 2.0 and FreeStyle™ 1.0 software packages.

Results

Peptide mapping experiments were performed using the Rituximab digest for assessing the sequence coverage for light and heavy chain as well as for identification and (relative) quantification of a specific set of modifications [1, 2]: a) oxidation, b) glycosylation and c) deamidation. For all five gradient times from 30 min down to 5 min, a very good separation was achieved (Figure 3) and resulting sequence coverages of 100% were obtained from all separation times both for light and heavy chain, even for the very short gradient of 5 min. The sequence coverage map (Figure 4) shows the overlap of the different peptides identified in different intensities and in different lengths due to missed cleavages.

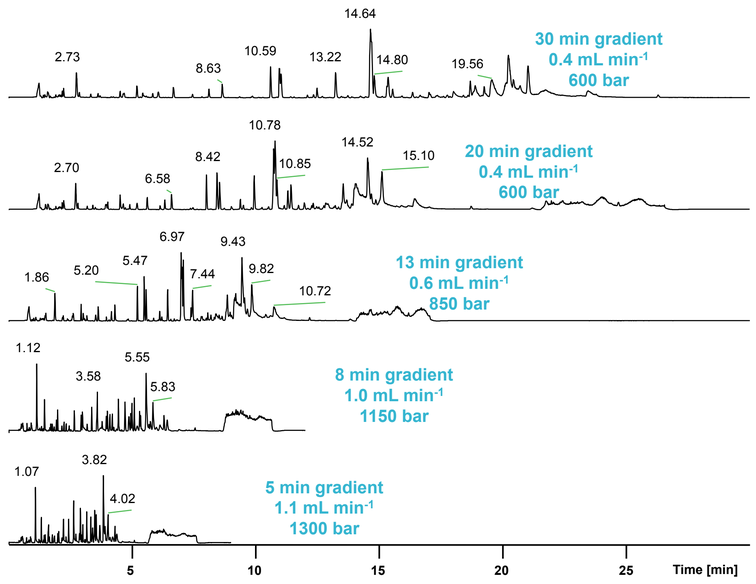

FIGURE 3. Total ion chromatograms obtained from peptide mapping experiments of Rituximab applying gradient lengths of 30, 20, 13, 8, and 5 min. Flow rates and resulting pressures are indicated in the individual traces..

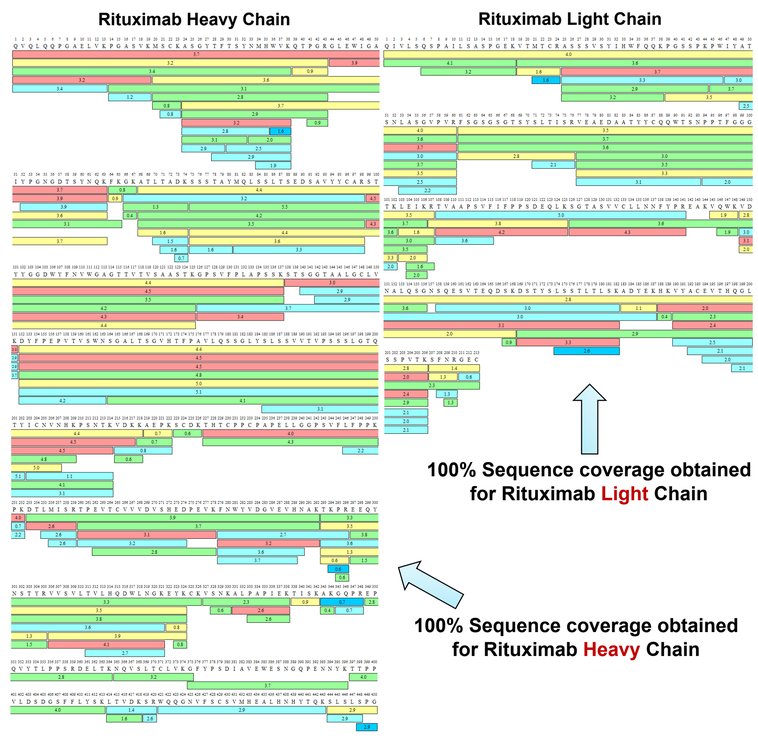

FIGURE 4. Sequence coverage map of Rituximab heavy (left) and light chain (right), obtained using the **5 min gradient** for peptide separation.

TABLE 1. Comparison of the oxidation, deamidation and glycosylation modifications identified in runs obtained from the different gradient times.

			Gradient Time					
			30 min	20 min	13 min	8 min	5 min	
Protein Chain	Modification	Recovery	Abundance	Abundance	Abundance	Abundance	Abundance	σ
Rituximab_LC	Q1+NH3 loss	Good	91.95%	91.17%	89.69%	90.93%	26.57%	28.80%
Rituximab_HC	~Q1+NH3 loss	Good	99.62%	99.67%	99.61%	99.68%	99.69%	0.04%
Rituximab_HC	N33+Deamidation	Good	0.52%	0.51%	0.58%	-	0.51%	0.03%
Rituximab_HC	M34+Oxidation	Good	1.64%	1.54%	1.73%	1.42%	1.45%	0.13%
Rituximab_HC	N301+A1G0F	Fair	4.32%	4.42%	3.83%	3.52%	3.38%	0.46%
Rituximab_HC	N301+A1G1F	Good	1.87%	1.91%	1.72%	3.32%	1.46%	0.73%
Rituximab_HC	N301+A2G0	Good	1.09%	1.02%	1.02%	-	0.98%	0.05%
Rituximab_HC	N301+A2G0F	Good	37.88%	37.11%	38.59%	40.48%	43.12%	2.41%
Rituximab_HC	N301+A2G1F	Good	42.06%	41.89%	43.42%	43.20%	43.35%	0.75%
Rituximab_HC	N301+A2G2F	Good	10.23%	10.17%	9.81%	10.36%	10.05%	0.21%
Rituximab_HC	N301+A2S1G0F	Fair	0.83%	0.86%	-	-	-	0.02%
Rituximab_HC	N301+A2S1G1F	Fair	2.14%	-	-	-	-	-
Rituximab_HC	N301+A3Sg1G0	Fair	1.30%	-	-	-	-	-
Rituximab_HC	N301+M5	Good	1.61%	1.59%	1.66%	1.87%	1.86%	0.14%
Rituximab_HC	N301+Unglycos.	Good	0.54%	0.90%	0.76%	0.83%	0.97%	0.16%
Rituximab_HC	G450+Lys	Fair	3.57%	3.56%	3.92%	3.40%	3.15%	0.28%
							median	0.19%

Table 1 shows the identification and comparison of a subset of monitored modifications across the different separation times applied. The relative abundance of the detected modifications in the five different methods has a standard deviation of 0.19% and shows that important information regarding PTMs can equally and accurately be obtained at all separation times.

Since the quantification of modified peptides performed in PepFinder 2.0 software requires their identification based on MS/MS spectra, special care has to be taken in choosing the appropriate ion injection times in the method setup enabling the acquisition of high quality MS/MS spectra required for a positive identification. This is especially true for glycopeptides analyzed using CID and HCD fragment ion spectra which contain exclusively ions representing sequential loss of glycan residues and no fragment ions representing the decomposition of the peptide as shown in Figure 5.

The identification of peptides and modified peptides using PepFinder 2.0 software is based on the comparison of a simulated and the measured spectrum. The strength of the implemented algorithm for spectra and especially fragment ion intensity prediction is displayed in Figure 5 showing the MS/MS spectrum of the low abundant glycosylated peptide TKPREEQFN*STFR (*=G0) identified in the 5 min run with the typical fragmentation pattern: the two hexonium ions 204 (HexNAc), 366 (Hex-Hex-NAc) and the sequence ladder of the fragmented glycan attached to the intact peptide.

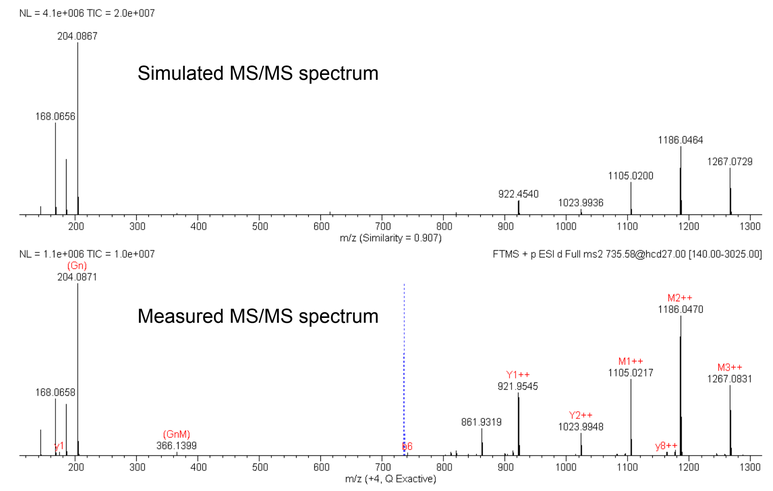

FIGURE 5. Simulated (top) and measured (bottom) MS/MS spectra of the glyco-peptide aa 290-302 (TKPREEQFN*STFR, *=G0) in the 5 min gradient run.

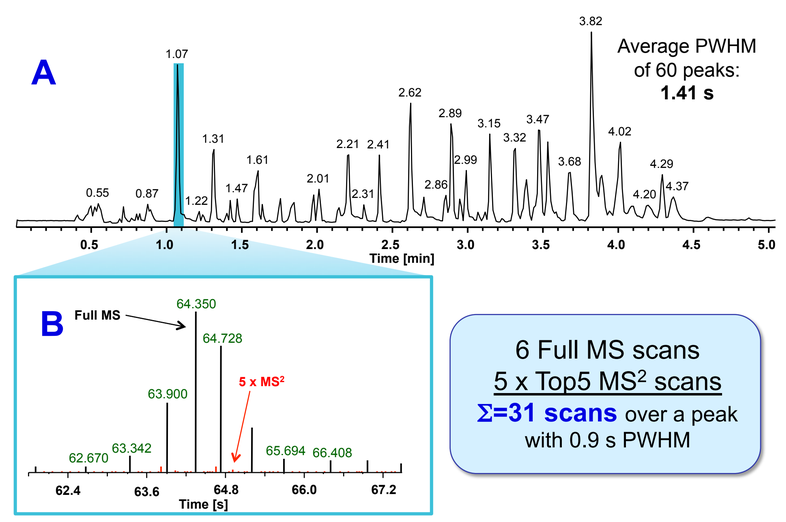

FIGURE 6. A) Total ion chromatogram of a five minute gradient separation of Denosumab and **B)** data point distribution for a Full MS / ddMS² Top5 method during a representative chromatographic peak..

Even with ultra short gradients down to 5 min, as shown in Figure 6A, spectacular separation efficiency and peak widths of less than 1 s were obtained. Figure 6B is highlighting the number of scans obtained across one chromatographic peak. Typically 6 Full MS spectra and 25 (5xTop 5) MS/MS spectra were acquired. The achieved scan speed is key to the success in obtaining full sequence coverage.

Conclusion

- The applied hardware setup chosen for the experiments consisting of column size of 2.1 x 250 mm coupled with Viper™ connectors and flow rates between 0.4 and 1.1 mL/min combined with the HESI-II source on the mass spectrometer delivers a vey robust setup allowing for long term stability and reproducibility.
- The Vanquish UHPLC system delivers outstanding peak widths in combination with high separation efficiency.
- For all gradient times ranging from 5 to 30 min 100% sequence coverage for both light and heavy chains were obtained.
- Sequence variants (C-terminal Lys), glycosylation sites and PTMs (N-terminal pyro-Gln formation) could be confidently identified and quantified, even with gradient times as short as 5 min.
- The accelerated scan speed of the Q Exactive HF delivers sufficient data points per chromatographic peak also for very fast separations obtained with the Vanquish UHPLC system.
- The data presented in this study clearly show the capability to significantly speed up peptide mapping for mAbs enabling the analysis of larger numbers of samples due to much reduced gradient times.

Abbreviations

ACN – acetonitrile, DTT - dithiothreitol, FA – formic acid, IAA –iodoacetamide, mAb – monoclonal Antibody, PTMs – post translational modifications, PWHM – peak with at half maximum, TFA – trifluoroacetic acid.

References

[1] Z. Zhang, Large-scale identification and quantification of covalent modifications in therapeutic proteins. Anal. Chem. (2009) 81, 8354-64.

[2] Shah, X. G. Jiang, L. Chen, Z. Zhang, LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins. J. Am. Soc. Mass. Spectrom. (2014) 25, 999-1011.

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