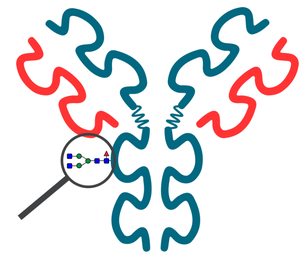
HILIC separation of mAb glycopeptides with UV, fluorescence and MS detection

Alexander Ley[1], Martin Samonig[1], Mauro De Pra[1], and Frank Steiner[1];
[1]Thermo Fisher Scientific, Dornierstraße 4, 82110 Germering, Germany.



ABSTRACT

Peptide mapping is one of the routine methods for biotherapeutics characterization. This technique, combined with mass spectrometry, is utilized in research environments for the determination of the primary sequence of a mAb and the identification of post-translational modifications (PTMs). Glycosylation affects the potency, and efficacy of the biotherapeutic. Therefore it is a significant step in the production process optimization.

After Enzymatic digestion, peptide mapping is typically run by reversed phase (RP) chromatography[1]. However, unlike reversed phase, hydrophilic interaction chromatography (HILIC) offers a very distinct retention behaviour for peptides and glycopeptides. The identification of glycopeptides is greatly facilitated owing to the fact that the glycopeptides elute in a distinct area of the chromatogram with much higher resolution compared to RP. In this study a HILIC-UV/FLD method was developed and glycan identification was performed with LC-MS/MS experiments. HILIC, paired with UV or fluorescence detection, provides a robust and unique detection tool for routine analysis (e.g. glycoform stability studies)—with retention times as qualitative and peak area as quantitative information.

INTRODUCTION

Remsima and Inflectra™ were the first monoclonal antibodies (mAbs) biosimilars to be approved in the European Union. Remsima and Inflectra are both infliximab biosimilars to the originator Remicade™ (Janssen)[2]. During their evaluation, these mAbs had to meet a significant number of strict criteria in order to be granted biosimilarity as per the ICH Q6B guidelines[3]. The evaluation of the glycosolated sites found on the heavy chain of mAbs are among the critical characterizations. Glycosylation affects the potency, and efficacy of the biotherapeutic.

Many conditions during the up- and downstream processing affect the manifestation of PTMs and so this is critical to monitor. Glycosylation is characterized with an array of chromatographic techniques. Depending on the scope of the analysis, glycans may be cleaved from the protein and then analyzed either natively or after fluorescent labelling, mostly with MS or fluorescence detection. Additionally, glycans can be analyzed at glycopeptide level, normally with MS detection, after enzymatic digestion of the protein.

MATERIALS AND METHODS

Sample preparation

A commercially available monoclonal antibody (mAb) infliximab drug product (Hospira UK Limited, Leamington Spa, United Kingdom) was supplied at a concentration of 10 mg/mL in formulation buffer. The sample was digested using the Thermo Scientific™ SMART Digest™ kit followed by an solid phase extraction purification of the digest using the Thermo Scientific™ Pierce™ C-18 Spin Columns.

Instrumentation

The separation was achieved in HILIC mode by using the Thermo Scientific™ Accucore™ 150 Amide HILIC column. The column was operated by the Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system (table 1). Detection was performed using the Thermo Scientific™ Vanquish™ Diode array detector HL with a LightPipe 10 mm standard flow cell or the Thermo Scientific™ Vanquish™ Fluorescence Detector F with a 2 µL micro flow cell coupled to the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer (table 2 and 3).

Table 1. Chromatographic conditions

Column:	Thermo Scientific Accucore Amide 150Å HILIC **2.1 x 150 mm**.
Mobile Phase:	**A:** Acetonitrile : Water 90:10 (v/v) with 10 mM Ammonium Formate **B:** 10 mM Ammonium Formate, pH 4.4 Buffers filtered through 0.2 µm filter membrane before use.
Gradient:	Full & Short as described to the right.
Flow Rate:	0.5 ml/min
Temperature:	50 °C still-air
Injection Volume:	24 – 50 µL of ~ 0.25 µg/µL digested infliximab sample
UV Detection:	280 nm, DAD
FLD Detection:	280 nm excitation 304 nm emission (filter wheel, auto)

Gradient (full)

Time (mins)	B
0	10%
45	60%
46	80%
50	80%
51	10%
70	10%

Gradient (short)

Time (mins)	B
0	25%
1	30%
15	35%
16	80%
20	80%
21	25%
40	25%

Table 2. Mass spectrometer source conditions

Source	Ion Max source with HESI-II probe
Sheath gas pressure	25 psi
Auxiliary gas flow	10 arbitrary units
Probe Heater Temperature	350 ° C
Capillary temperature	320 ° C
S-lens RF Voltage	60 V
Source Voltage	3.5 kV

Table 3. Mass spectrometer conditions

Full MS Parameters		MS^2 Parameters	
Method type	MS2	**Resolution Settings**	15,000 (FWHM at m/z 200)
Full MS mass range	m/z 400-2000	**Target Value**	5e5
Resolution settings	120,000 (FWHM at m/z 200)	**Isolation Width**	2.0 m/z
Target value	3e6	**Signal Threshold**	1e4
Max injection time	100 ms	**Normalized Collision Energy (HCD)**	27
Default charge state	2	**Top-N MS2**	3
SID	0 eV	**Max Injection Time**	250 ms
		Dynamic Exclusion	5.0 s

Data Processing

The data were acquired with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR5 and the Thermo Scientific™ BioPharma Finder™ software, version 2.0, was used for data analysis.

RESULTS

The separation of a tryptic digest of infliximab was obtained with a 45 minute gradient and a total analysis time of 75 minutes, including column wash with high buffer content and re-equilibration at initial conditions. Figure 1 shows a chromatogram with an overlay of 5 subsequent injections of infliximab tryptic digest, with the glycopeptide region highlighted between 28.0 and 38.0 minutes. Note the separation of the two structural isomers A2G1Fa and A2G1Fb at approximately 32.0 to 32.5 minutes. The reproducibility was assessed using the retention time standard deviation (SD) and relative standard deviation (RSD) of all glycopeptides automatically calculated by Chromeleon CDS. The relative standard deviation was below 0.05% for all 4 main glycopeptides (Table 4). All retention time SDs were in the range of maximal 0.017 min and minimal 0.013 min. This data shows excellent flow delivery precision of a long shallow gradient.

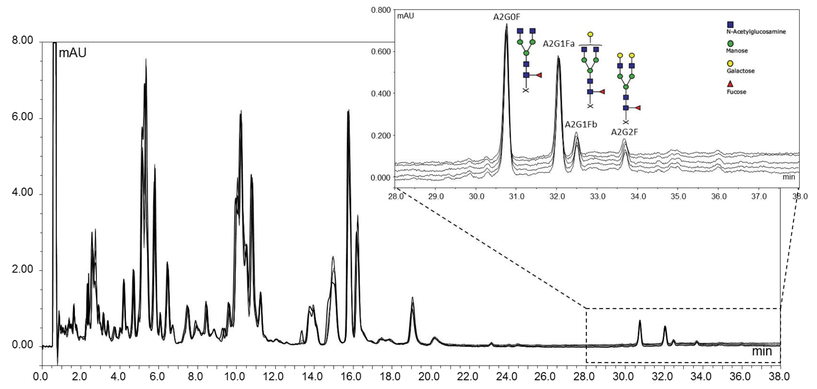


Figure 1. UV chromatogram overlay of 5 subsequent (24 µL) injections of infliximab tryptic digest, highlighting the excellent retention time precision and the distinct glycopeptide region.

Table 4. Retention time precision for 5 subsequent (24 µL) injections of infliximab tryptic digest, using the full gradient.

Peak	Average Retention Time (min)	Standard Deviation RT (min)	Relative Standard Deviation (RT)
A2G0F	30.761	0.013	0.04%
A2G1Fa	32.058	0.016	0.05%
A2G1Fb	32.503	0.017	0.05%
A2G2F	33.701	0.015	0.04%

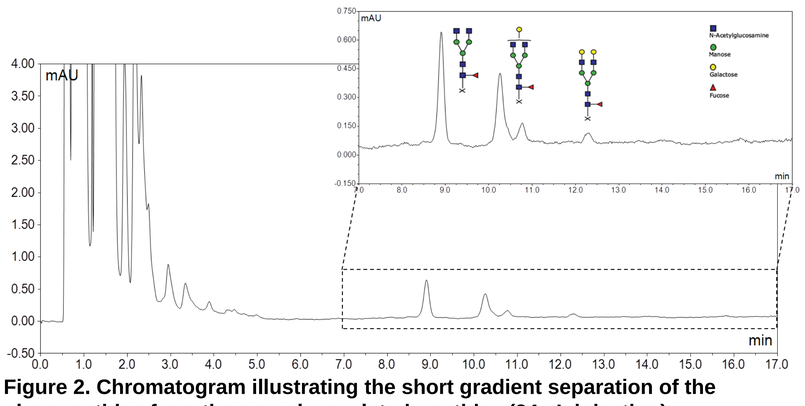


Figure 2. Chromatogram illustrating the short gradient separation of the glycopeptides from the non-glycosylated peptides (24 µL injection).

An optimized, short gradient method (Table 1) was also developed to enable the efficient separation of the glycopeptides in the corresponding region of the chromatogram with a significant loss in resolution of the non-glycosylated peptides. Figure 2 shows non-glycosylated peptides eluting between 0.0 and 7.0 minutes followed by the glycopeptide region starting at 7.0 minutes. The short gradient allows the glycosylation to be characterized in a shorter time, saving time and solvents, without a loss in resolution.

For further identification of the glycan structures a 100 µL loop was installed and injections of 50 µL were loaded onto the column. This allowed more peaks to be resolved with UV and sufficient sensitivity for MS1 and MS2 identification. The corresponding UV/FLD traces and extracted ion chromatograms (XIC) are shown in figure 3. 18 peaks were identified with MS and all 18 glycopeptide could be confirmed with MS2 experiments (table 5). All peaks could also be detected with UV/FLD, however quantification is limited to the four most abundant peaks in the chromatogram.

Glycopeptides are easily characterized by MS/MS experiments, owing to the fact that High-Energy Collision Dissociation (HCD) fragmentation is very specific and removes the labile glycans completely. This allows conclusive characterization in the MS2 experiment. However, UV/FLD can also used when in depth characterization is not needed, for instance in stability studies. The data interpretation of these experiments is based on retention time as qualitative and peak area as quantitative information.

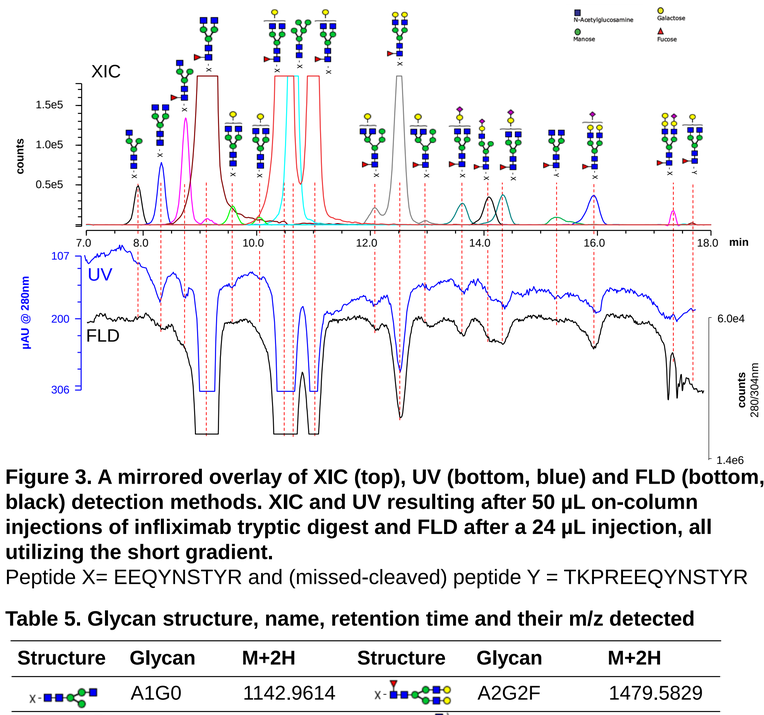


Figure 3. A mirrored overlay of XIC (top), UV (bottom, blue) and FLD (bottom, black) detection methods. XIC and UV resulting after 50 µL on-column injections of infliximab tryptic digest and FLD after a 24 µL injection, all utilizing the short gradient.
Peptide X= EEQYNSTYR and (missed-cleaved) peptide Y = TKPREEQYNSTYR

Table 5. Glycan structure, name, retention time and their m/z detected

Structure	Glycan	M+2H	Structure	Glycan	M+2H
	A1G0	1142.9614		A2G2F	1479.5829
	A2G0	1244.5010		A2G1M4F	1479.5839
	A1G0F	1215.9895		A2Sg1G0F	1552.1024
	A2G0F	1317.5295		A1Sg1F	1450.5619
	A2G1	1325.5273		A2Sg1G0F	1552.1024
	A2G1	1325.5273		A2G0Fmc	1558.6803
	A2G1F	1398.5566		A2Sg1G1F	1633.1296
	M5	1203.4742		A2Sg1Ga1F	1714.6578
	A2G1F	1398.5566		A2G1Fmc	1720.7315
	A2G1M4F	1479.5839			

CONCLUSIONS

- The Vanquish Flex UHPLC system combined with UV/FLD detection and coupled to the Q Exactive HF, provide a robust LC-MS setup to characterize and monitor glycosylation on the peptide level of mAbs.
- Separation of intact glycopetides utilizing HILIC provides a novel chromatographic technique. The resolution of this method is fully comparable to HILIC methods commonly used for released and labeled glycans.
- A similar glyco-profile was achieved, with a much simplified sampler preparation effort (when compared to released labeled glycan analysis).
- The effective glycopeptide separation from other non-glycosylated tryptic peptides of a monoclonal antibody (mAb) has been shown with the use of the Accucore 150 Amide HILIC column. The column provides an effective mode to separate the glycoforms based on their hydrophilic interactions.

REFERENCES

1. Thermo Scientific Application Note 1123: Increased Long-term Stability of Peptide Mapping using the Vanquish UHPLC System. Germering, Germany, 2015.
2. http://www.ema.europa.eu/ema/index.jsp%3Fcurl=pages/news_and_events/news/2013/06/news_detail_001837.jsp%26mid=WC0b01ac058004d5c1
3. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use - http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf

TRADEMARKS/LICENSING