Targeted Analysis of Glycosylation Critical Quality Attributes from Glycoprotein Therapeutics

Paulina A. Urbanowicz*, Jenifer L. Hendel, Maximilianos Kotsias, Radoslaw P. Kozak -- *Ludger Ltd, Culham Science Centre, Oxfordshire, UK*

paulina.urbanowicz@ludger.com

Introduction

Glycosylation is known to greatly affect pharmacological properties of the protein therapeutic, impacting clinical safety and efficacy profile of the drug. Alterations in cell expression systems, extracellular environment and downstream purification strategies can influence the glycosylation of the glycoprotein, changing its pharmacodynamic profile or even introducing the immunogenic epitopes (such as α1-3-linked galactose or *N*-glycocylneuraminic acid). Given this, consistent and human-compatible glycosylation is required for the development of safe biopharmaceuticals and the Glycosylation Critical Quality Attributes (GCQAs) of each glycoprotein drug must be identified and validated to satisfy growing regulatory requirements.

Macro- and micro heterogeneity introduced by glycosylation present many challenges to the analytical characterisation of the glycoprotein drug and its quality control. Therefore, robust analytical strategies are required to accurately and reliably characterize glycosylation. At Ludger we follow optimized approach based on liquid chromatography coupled to mass spectrometry (LC-MS) analysis to study the *N*-glycome of biopharmaceuticals. This workflow, applied to fully characterise and quantify GCQAs from IgG1 monoclonal antibody (mAb) is presented below.

Workflow

Here, we present a combination of two complementary LC-MS based techniques for simultaneous detection, identification and relative quantitation of *N*-glycan species:

1) **LC-ESI-MS/MS analysis of released, procainamide labelled *N*-glycans supported by knowledgeable use of process and system suitability controls** – allowing structure detection and identification
2) **Panel of exoglycosidase digestions** – for additional structure and linkage confirmation.

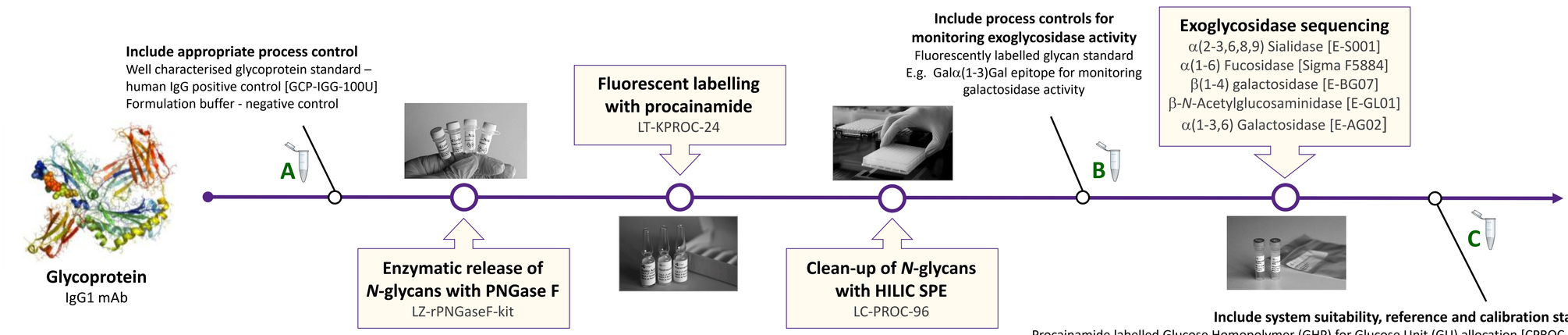


Figure 1. *Workflow for LC-MS analysis of N-glycans released from IgG1 monoclonal antibody.*

Identification of GCQAs

Glycosylation Critical Quality Attributes are those glycosylation features that impact pharmacokinetics and pharmacodynamics of the glycoprotein. They include antigen binding properties, antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), immunogenicity and serum half-life. FDA, EMA and ICH Q6B guidelines specify the GCQAs that must be demonstrated to ensure the safety and potency of commercial drugs before regulatory approval, and those that must become an essential part of a systematic quality control strategy. Below scheme illustrates the most common GCQAs found on glycoprotein therapeutics.

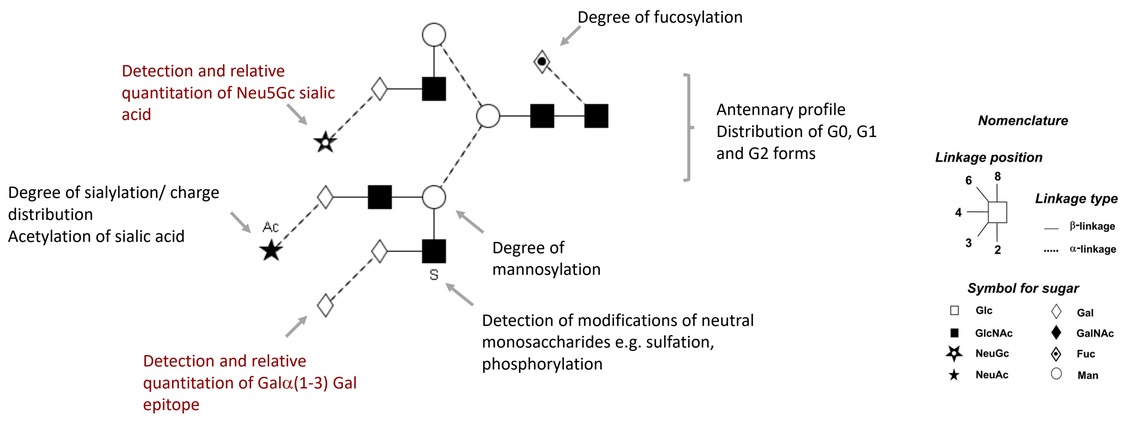
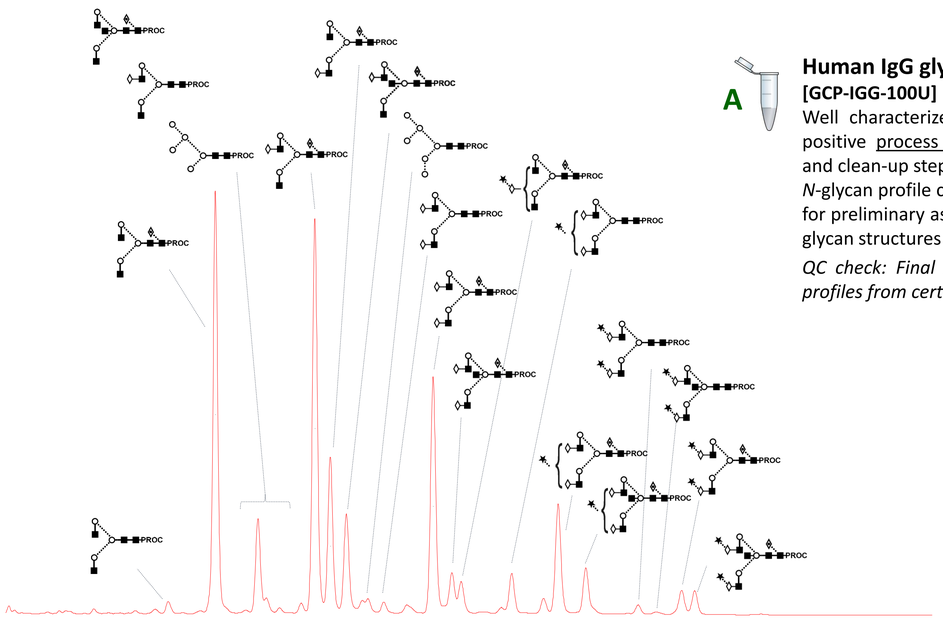


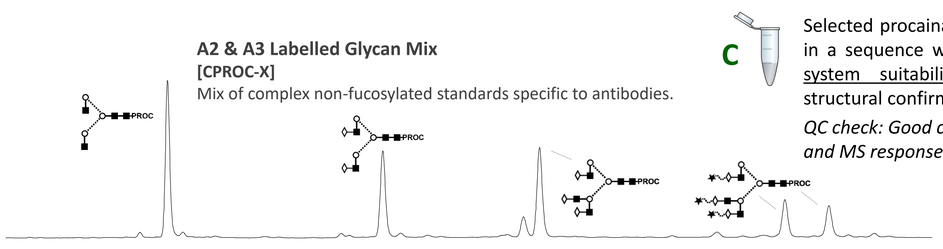
Figure 2. *Common Glycosylation Critical Quality Attributes (GCQAs) recognised by the regulators. Immunogenic epitopes shown in red.*

Process controls and system suitability checks

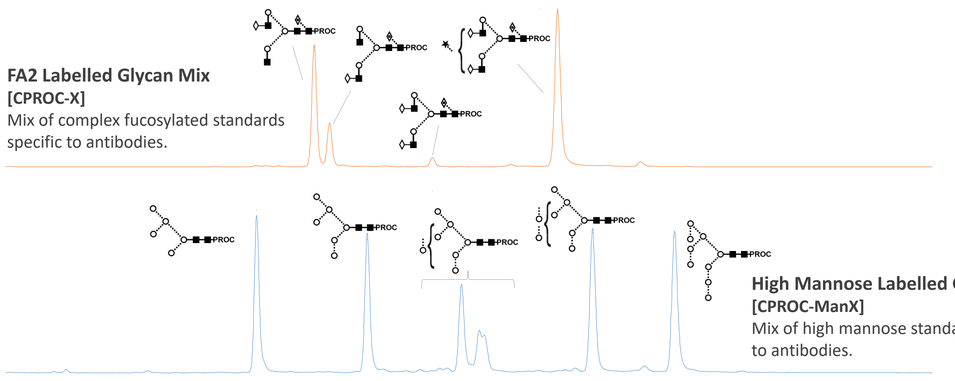
A key component in a well-designed analytical strategy is the inclusion of standards. Well selected process controls and system suitability checks allow not only to assess the functionality of the analytical system and gain confidence in the quality of produced data, but can also help with analysis and interpretation of chromatographic results. Fluorescence profiles of procainamide labelled standards used in the workflow are presented below.



Human IgG glycoprotein
[GCP-IGG-100U]
Well characterized glycoprotein is used as a positive process control for release, labelling and clean-up steps alongside the test samples. *N*-glycan profile can also be used as a reference for preliminary assignment and confirmation of glycan structures in the sample.
QC check: Final N-glycan profile matches the profiles from certificate of analyses.



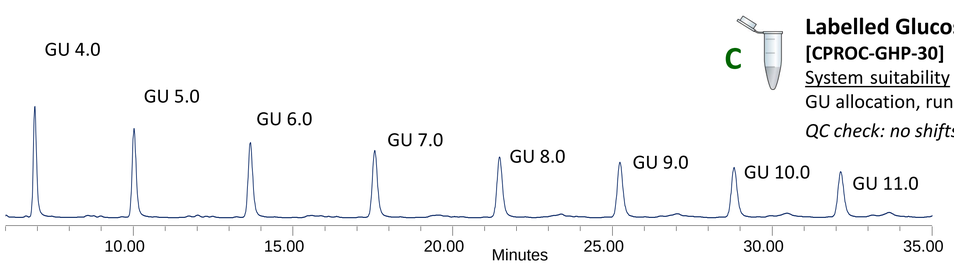
A2 & A3 Labelled Glycan Mix
[CPROC-X]
Mix of complex non-fucosylated standards specific to antibodies.



FA2 Labelled Glycan Mix
[CPROC-X]
Mix of complex fucosylated standards specific to antibodies.

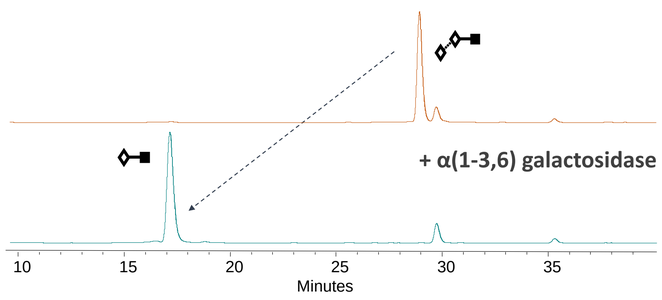
High Mannose Labelled Glycan Mix
[CPROC-ManX]
Mix of high mannose standards specific to antibodies.

Selected procainamide labelled glycans are run in a sequence with test samples as analytical system suitability check and for further structural confirmation (reference standard).
QC check: Good chromatographic resolution and MS response



Labelled Glucose Homopolymer (GHP)
[CPROC-GHP-30]
System suitability and calibration standard for GU allocation, run in front of the test samples.
QC check: no shifts in retention times

Figure 3. *HILIC LC profiles of process control and system suitability/ reference standards used in the N-glycan profiling workflow.*



Labelled Galα(1-3)Gal standard
[CAB-ALPHA-GAL-01]
Used as a process control to assess the specificity of the α-galactosidase.
QC check: shift in retention time of the standard, corresponding to digestion of α, but not β-Gal bond.

Figure 4. *HILIC LC profiles of process control and system suitability/ reference standards used in the N-glycan profiling workflow.*

Results

Glycan analysis presents several challenges due to the heterogeneous character of these biomolecules. IgG1 contains complex mixture of neutral and sialylated glycan species, several of which co-elute in HILIC LC and/ or have identical MS mass composition. Therefore, three orthogonal methods were adopted to assign glycan structures with high degree of confidence. As a result, a total of 31 distinct structures were identified and quantified. Analysis strategy based on these complementary techniques is presented below.

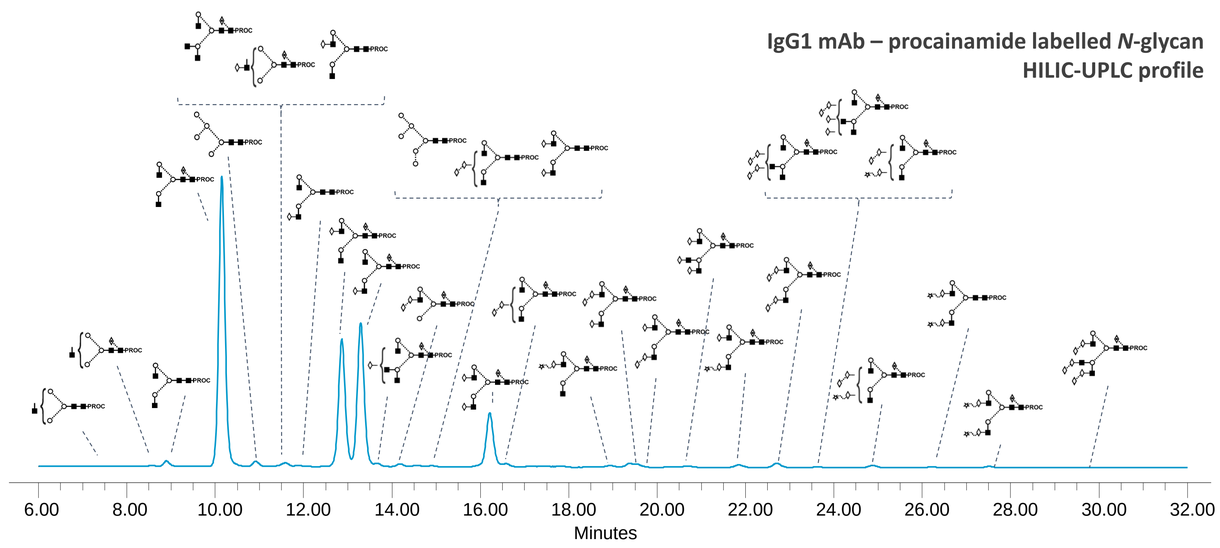


Figure 5. *HILIC-UPLC profile of N-glycans released from IgG1 monoclonal antibody and labelled with procainamide. All detected glycan peaks were assigned with a proposed structure.*

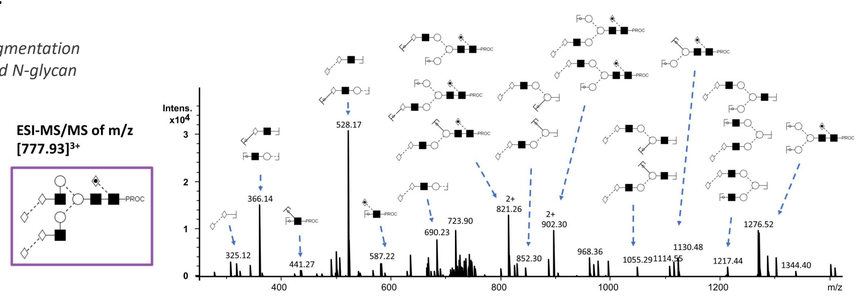
1. Analysis based on GU allocation and profile comparison with reference standards

GHP derived GU values provide preliminary glycan assignments by comparison with the reference standards (see Figure 3) and reported GU values for procainamide labelled glycans in the literature and databases.

2. MS and MS/MS analysis

Electrospray ionisation (ESI) MS analysis is a powerful tool allowing for compositional analysis of dozens of glycan in a single run, thanks to derivatisation with procainamide (MS-sensitive tag). Furthermore, sequential MS/MS fragmentation enables to gain insights into some three-dimensional features lacking, however, the capacity to identify glycan linkages and monosaccharide type.

Figure 6. *Example of MS/MS fragmentation patterns of procainamide labelled N-glycan released from IgG1 mAb.*



3. Exoglycosidase sequencing

Exoglycosidases are enzymes that remove terminal monosaccharides from the non-reducing end of a glycan in a highly specific manner. Use of array of exoglycosidases, supported with LC analysis provides further insights into monosaccharide composition and glycan structure/ linkages, and is often used as an identification technique in its own right.

Figure 7. *Example of exoglycosidase sequencing performed on IgG1 mAb N-glycans, for confirmation of Galα(1-3)Gal structures. Shifts in GU and retention times indicate where structures were digested with specific enzymes.*

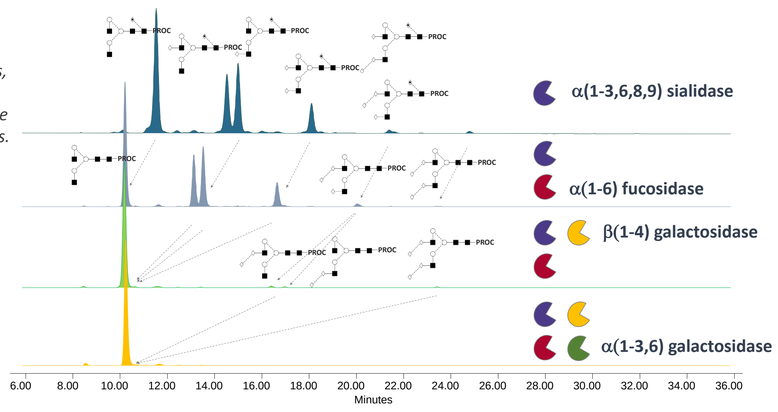


Table 1 shows how the data obtained using the three orthogonal techniques was summarised for a FA2G2Ga2 glycan.

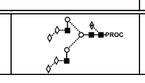
Structure		HILIC-UPLC		ESI-MS/MS						Degree of Certainty			
		GU	% Area	Composition				Mass found	Mass Calculated	GU	Exoglycosidase digestions	m/z at GU	MS/MS at GU
				Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)						
FA2G2Ga2		9.12	0.78	7	4	1	0	$[771.93]^{3+}$	$[771.98]^{3+}$	Y	Y	Y	Y

Table 1. *Example summary of UPLC, MS, MS/MS and exoglycosidase sequencing data that allowed for identification of FA2G2Ga2 glycan.*

Summary

Here we presented an analytical workflow for detailed characterization of *N*-glycans originating from monoclonal antibodies. Three orthogonal glycan identification strategies were followed, supported by use of standards and process controls: 1) Primary identification based on GU value allocation 2) Compositional analysis based on MS and MS/MS and 3) Final structure verification using exoglycosidase sequencing. Furthermore, HILIC LC analysis of procainamide labelled glycans with fluorescence detection supported by MS allowed for glycan relative quantitation.

Sialylation		Glycan type		Other detected GCQAs	
Total sialylation	2.11%	Mono-antennary	1.61%	Core fucosylation	96.49%
Neu5Ac	0.00%	Bi-antennary	95.22%	α(1-3)Gal content	4.22%
Neu5Gc	2.11%	Tri-antennary	2.17%	G2 glycan	0.37%
Neutral	97.89%	High mannose	1.00%		

Table 2. Summary of GCQAs identified and quantified in IgG1 mAb sample.

In summary, presented strategy enabled identification and quantitation of GCQAs in the IgG1 mAb sample, and can be routinely adopted for glycosylation quality control throughout the biopharmaceutical's lifecycle.

References

1. Zhang, P. et al. Challenges of glycosylation analysis and control : an integrated approach to producing optimal and consistent therapeutic drugs. Drug Discov. Today 21, 740–765 (2016).
2. Parekh, R. B. Effects of glycosylation on protein function. Curr. Opin. Struct. Biol. 1, 750–754 (1991).
3. Morgan, C. & Fernandes, D. L. Designing Biobetter Monoclonal Antibody Therapeutics By Glycoengineering. Int. Biopharm. Ind. 1, (2008).
4. Dotz, A. V. et al. Mass spectrometry for glycosylation analysis of biopharmaceuticals. Trends Anal. Chem. 73, 1–9 (2015).

Contact for more information:

If you'd like a copy or want to know more please email me (**paulina.urbanowicz@ludger.com**) or connect with me on LinkedIn.

Ludger
www.ludger.com