Satisfying regulatory requirements using orthogonal methods for glycosylation analysis; An example with an IgG1 monoclonal antibody

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Introduction

Glycosylation is known to greatly impact pharmacokinetics and pharmacodynamics of the glycoprotein, including its 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 glycosylation attributes 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. Here, we present a workflow for the analysis of glycosylation, designed to satisfy the requirements outlined by the regulatory authorities and ensure the potency and clinical safety of the biopharmaceutical. The strategy involves orthogonal techniques: total N-glycan profiling and quantitative analysis of neutral monosaccharides and sialic acids, wherein an IgG1 mAb is used as a model glycoprotein. The methodologies presented can be applied throughout biopharmaceutical's product life cycle.

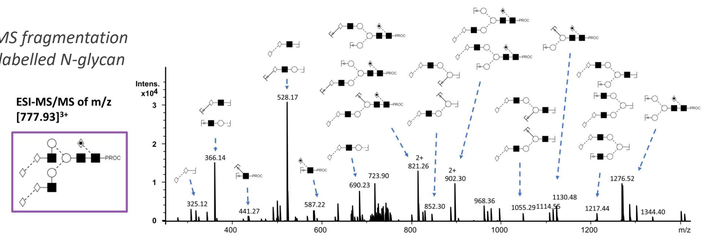
1) Total *N*-glycan Profiling and Characterisation

N-glycan profiling is a universal method for identification and quantitation of the essential glycosylation critical quality attributes (GCQAs) such as **degree of sialylation, core fucosylation, antennary composition, alpha-galactose and Neu5Gc sialic acid content** in a biopharmaceutical. *N*-glycans are enzymatically released from the glycoprotein using PNGAse F, then derivatised with procainamide (PROC). Procainamide labelled glycans are characterised using a combination of LC-ESI-MS/MS and exoglycosidase sequencing.

A. 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.

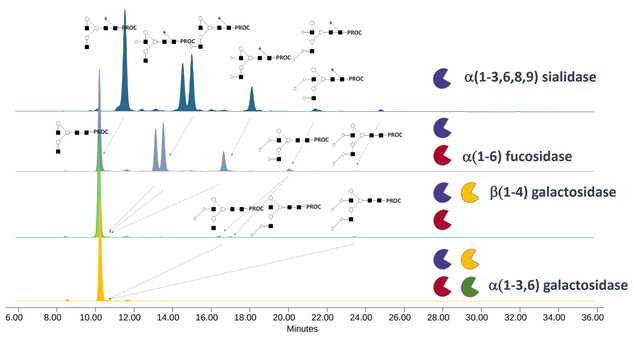
Figure 1. *Example of MS/MS fragmentation patterns of procainamide labelled N-glycan released from IgG1 mAb.*



B. 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 2. *Example of exoglycosidase sequencing performed on IgG1 mAb N-glycans, for confirmation of Galα(1-3)Gal structures. Shifts retention times indicate where structures were digested with specific enzymes.*



Final results

Glycan characterisation 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, two 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, including glycans containing alpha-galactose epitopes and Neu5Gc sialic acids. Final *N*-glycan assignments deducted using presented complementary techniques are presented below.

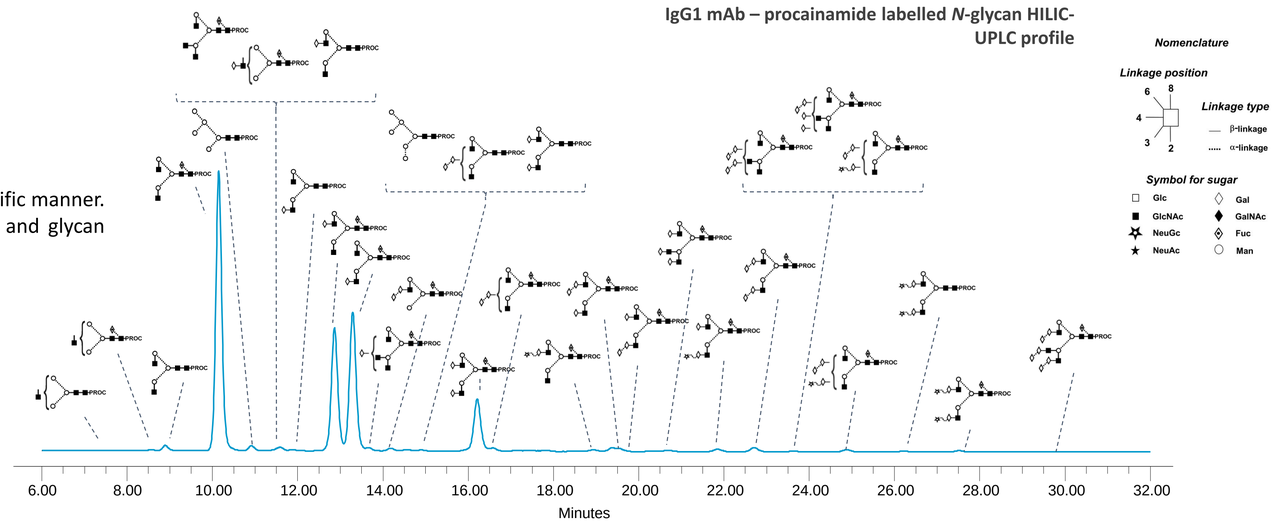


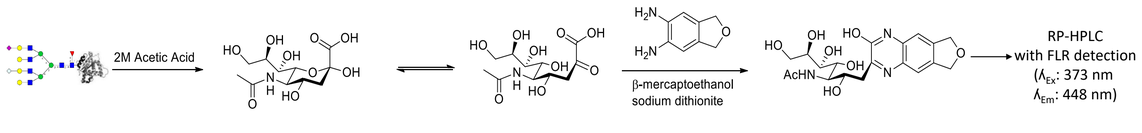
Figure 3. *HILIC-UPLC profile of N-glycans released from IgG1 monoclonal antibody and labelled with procainamide. All detected glycan peaks were assigned with a proposed structure.*

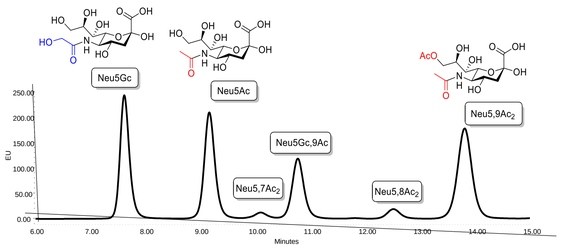
2) Sialic Acid Analysis

Sialylation is integral for the structure and function of many glycoprotiens and is a glycosylation critical quality attribute (GCQA) for biopharmaceuticals. Sialic acid analysis provides data for both the abundance and the type of sialylation (including O-acetylation). Sialic acid analysis workflow designed to satisfy the regulatory requirements for biopharmaceutical sialic acid content analysis to specify the following:

1. **Overall degree of drug sialylation – absolute quantitation of sialic acid residues per molecule (nmol/mg protein)**
2. **Relative quantities of Neu5Ac:Neu5Gc**
3. **Identification and relative percentage of O-acetylated sialic acids**

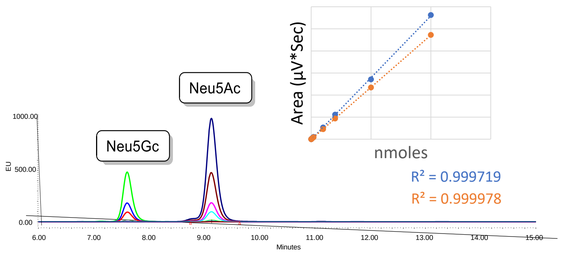
Workflow: Sialic acids are released from glycoproteins by mild acid hydrolysis (2M acetic acid) using conditions that preserve the *N*-acetyl, *N*-glycolyl and *O*-acetyl groups. The free sialic acids are derivatised with 1,2-diamino-4,5-methylendioxybenzene (DMB). The DMB-labeled sialic acids are then analysed by RP-HPLC alongside the reference and quantitative standards.





Reference Standard

*Figure 4. RP-HPLC profile of the **Sialic acid reference panel (SRP)** containing a mixture of sialic acids found in humans and animals.*
Acceptance criteria: the HPLC profiles at the start and end of the sample set should overlap with minimal drift in retention time (e.g. ± 0.1 min.).

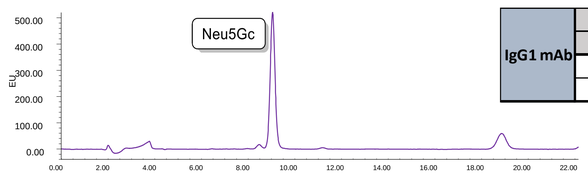


Quantitative Standards

*Figure 5. RP-HPLC profiles of the **Neu5Gc** and **Neu5Ac** - dispensed to approximately 1 nmole. Preparation of serial dilutions provide calibration curves.*
Acceptance criteria: the calibration curves should give R^2 values of >0.99

Quantitative analysis of the sialic acid from IgG1 mAb

The analysis of sialic acids from a monoclonal antibody illustrates how this analysis supports drug-development and characterisation. Most therapeutic mAbs where the levels of sialic acid can impact the pharmacokinetics, specificity, and anti-inflammatory activity.



IgG1 mAb	nmol sialic acid per mg protein			
	Neu5Ac		Neu5Gc	
	Mean	RSD	Mean	RSD
	-	-	0.25	5.24

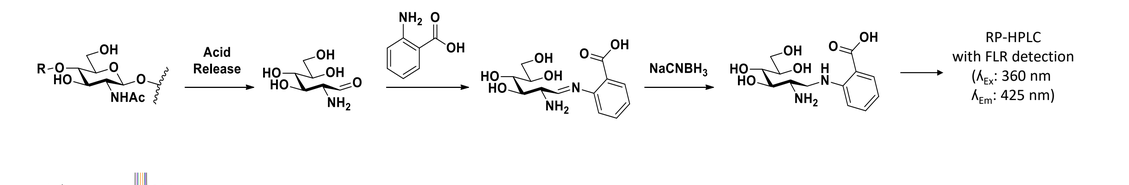
Figure 6 and Table 1. RP-HPLC profile of the sialic acid present in the IgG1 mAb sample, together with the quantitative data for the identified sialic acid.

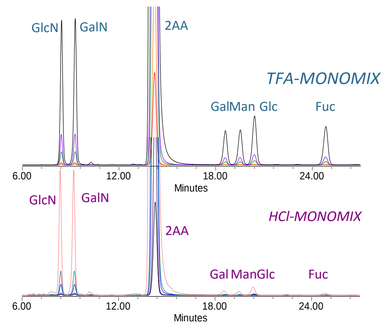
3) Monosaccharide Analysis

Monosaccharide analysis provides absolute or relative quantitation of the neutral (i.e. non-anionic) monosaccharides and information relating to the types of *N*- and/or *O*-glycans present on a glycoprotein. Monosaccharide analysis workflow designed to satisfy the regulatory requirements for biopharmaceutical characterisation to specify the following:

1. **Absolute quantitation of GlcN, GalN, Gal, Man, Glc and Fuc expressed as nmol/mg protein**
2. **Absolute quantitation of Xyl expressed as nmol/mg protein**
3. **Possibility of adoption of the assay to identify and quantify other unique monosaccharides**

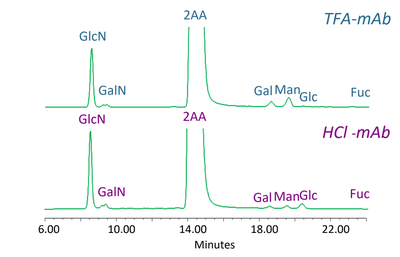
Workflow: Monosaccharides are released from the glycoprotein by acid hydrolysis using 2M trifluoroacetic acid (TFA) (for quantitation of mannose (Man), galactose (Gal), glucose (Glc), fucose(Fuc)) or 6M hydrochloric acid (HCl) (for quantitation of glucosamine (GlcN) and galactosamine (GalN)). Free monosaccharides are derivatised with 2-aminobenzoic acid (2-AA) and analysed alongside the reference and quantitative standards.





Reference and Quantitative Monosaccharide Standards

*Figure 7. RP-HPLC profiles of the monosaccharide mix (**MonoMix**) standard, containing GlcN, GalN, Gal, Man, Glc and Fuc dispensed to 10 nmole each. Preparation of serial dilutions provide calibration curves.*
Acceptance criteria for quantitation: calibration curves should give R^2 values of >0.9
System suitability acceptance criteria: HPLC retention times of the monosaccharides should have less than 0.1 min difference when run at the start, middle and end of the analysis.



Quantitative analysis of the monosaccharides from IgG1 mAb

GlcN and GalN data (from the HCl release):
- majority of the *N*-glycans are biantennary (with 4 GlcN)
- low percentage of *O*-glycans (low, but real GalN value)

Gal, Man, Glc & Fuc data (from TFA release)
- Not all *N*-glycans have 2 Gal (1.8 per site).
- Complex *N*-glycans have 3 Man; indicates low % of high mannose structures present.
- The majority of the *N*-glycans are core fucosylated (1.2 per site)
- Glc is present as a background contaminant (detected in the negative controls)

HCL & TFA release data	nmol/mg of protein		# of monosaccharides per N-glycan site
Monosaccharide	Average	RSD	# per site
GlcN	58	2.1	4.4
GalN	3	2.0	0.2
Gal	24	2.4	1.8
Man	41	1.6	3.1
Glc	5	9.2	0.4
Fuc	6	1.3	1.2

Figure 8 and Table 2. RP-HPLC profiles of the monosaccharides present in the IgG1 mAb sample, together with the quantitative data for each identified monosaccharide.

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).

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