Sialylation: A Critical Glycosylation Quality Attribute for Biopharmaceuticals

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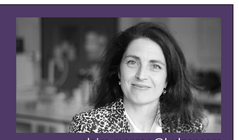

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Introduction

Over the last two years the vast majority (27 of 28) of the FDA approved therapeutic biologics have been glycoprotiens (1). These glycosylated therapeutics are comprised of glycoprotein hormones, cytokines, clotting factors and monoclonal antibodies. Sialic acids are terminal, negatively charged monosaccharides present on many *N*- and *O*-glycans. Biopharmaceuticals often contain two main types of sialic acid; *N*-acetyl-neuraminic acid (Neu5Ac, purple box figure 1) and *N*-glycolyl-neuraminic acid (Neu5Gc, blue box figure 1). Neu5Ac is found in both human and non-human cells, whereas Neu5Gc not present on human glycoproteins and is immunogenic (2). The biopharmaceuticals efficacy, serum half-life and immunogenicity are impacted by both the abundance and the type of sialylation (including *O*-acetylation) (3). Consequently, sialylation is a glycosylation critical quality attribute (GCQA). Sialic acid analysis is in the quality guidelines for registration of biopharmaceuticals (ICHQ6B) and should be performed throughout the product lifecycle

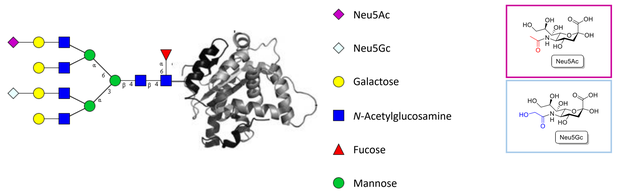
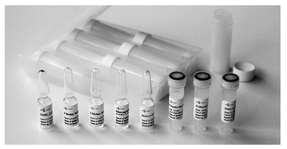


Figure 1: Structural differences between Neu5Ac and Neu5Gc.

Ludger's DMB kit for Sialic Acid Analysis and Standards

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



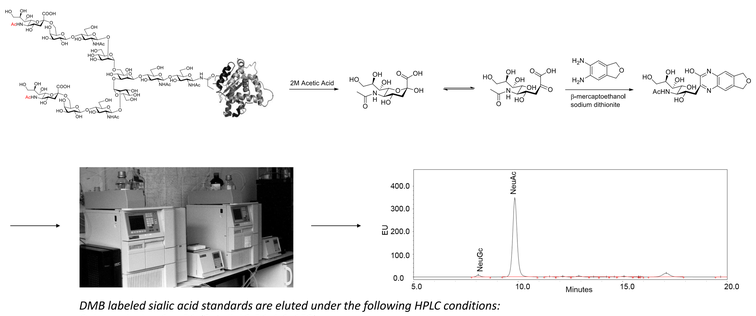
The kit contains the following components:
Reagents: DMB Dye, 2M Acetic Acid, Mercaptoethanol in Acetic acid (1.4 Molar) , Sodium Dithionite
Quantitative Standards: N-acetylneuraminic acid (1 nmol), N-glycolylneuraminic acid (1 nmol)
Reference Standards: Sialic Acid Reference Panel containing Neu5Ac, Neu5Gc, Neu5,7Ac$_2$, Neu5Gc,9Ac, Neu5,8Ac$_2$, Neu5,9Ac$_2$ and Neu5,7,(8),9Ac$_3$

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 α-keto acid of the free sialic acids are derivatised with 1,2-diamino-4,5-methylendioxybenzene (DMB). Typical starting amounts are 50 µg of glycoprotein for a highly sialylated sample and up to 200 µg for a glycoprotein with low levels of sialylation. The DMB-labeled sialic acids are then analysed by RP-HPLC (Scheme 1; HPLC and (U)HPLC Running conditions in Table 1).

A number of controls are also taken through the process:

i. Positive process control glycoprotein: Fetuin glycoprotein (GCP-FET-50U)
ii. Positive process quantitative control glycopeptide: GPEP (BQ-GPEP-A2G2S2-10U)
iii. Negative process control: Water
iv. Negative process control: Sample buffer



DMB labeled sialic acid standards are eluted under the following HPLC conditions:
Column: LudgerSep R1 (Cat. #: LS-R1-4.6x150)
Flow: 0.5 ml/min; Temperature: 30 °C
Solvent A: methanol: acetonitrile: water (7:9:84); Solvent B: acetonitrile
Excitation wavelength: 373 nm Emission wavelength: 448 nm

Scheme 1: DMB Release and Labelling Workflow

Time (min)	Flow mL/min	%A	%B
0	0.5	100	0
19	0.5	100	0
19.5	0.5	10	90
23.5	0.5	10	90
24	0.5	100	0
30	0.5	100	0

Table 1. 30 min running method for HPLC analysis using a LudgerSep-R1 column (4.6 x 150 mm, 3 µm particles) LS-R1-4.6x150.
Injection volume = 25 µL

Qualitative Standards: Sialic Acid reference Panel (SRP) and Neu5,9Ac$_2$

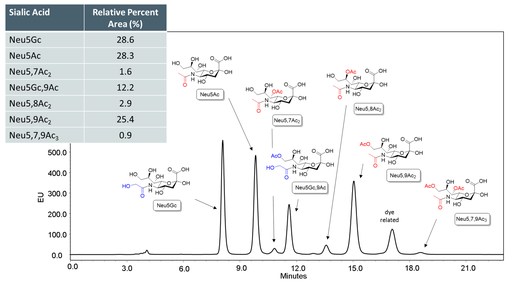
A sialic acid reference panel (SRP) containing a mixture of sialic acids found in humans and animals is used as a system suitability standard. The sialic acid reference standard contains Neu5Gc, Neu5Ac, Neu5,7Ac$_2$, Neu5Gc9Ac, Neu5,9Ac$_2$, and Neu5,7,(8),9Ac$_3$.

The acceptance criteria for this standard are that the HPLC profiles from the SRP at the start and end of the sample set should overlap with minimal drift in retention time (e.g. ± 0.1 min.). The DMB profile for the SRP is shown in Figure 2A.

The purified Neu5,9Ac$_2$ standard is used to determine the relative abundance of O-acetylated vs non-O-acetylated sialic acid residues (Figure 2B).

A)

Sialic Acid	Relative Percent Area (%)
Neu5Gc	28.6
Neu5Ac	28.3
Neu5,7Ac$_2$	1.6
Neu5Gc,9Ac	12.2
Neu5,8Ac$_2$	2.9
Neu5,9Ac$_2$	25.4
Neu5,7,9Ac$_3$	0.9



B)

Sialic Acid	Relative Percent Area (%)
Neu5Ac	3
Neu5,7Ac$_2$	1
Neu5,8Ac$_2$	18
Neu5,9Ac$_2$	77

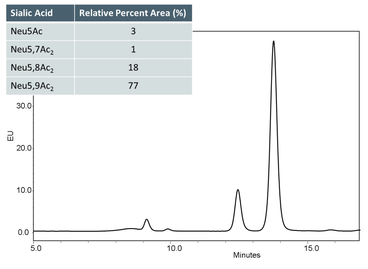


Figure 2: A) DMB-labeled sialic acid reference panel profiled on an LS-R1 HPLC column; B) Neu5,9Ac$_2$ Standard

Quantitative Standards (Neu5Ac, Neu5Gc)

The quantitative standards are NIST-F and USP traceable Neu5Gc and Neu5Ac monosaccharides dispensed to approximately 1 nmole (exact values in C of A for each batch). Preparation of serial dilutions of the Neu5Ac and Neu5Gc quantitated standards provide calibration curves (Figure 3A). The peak areas of Neu5Ac and Neu5Gc in test samples are compared to the calibration curves to provide quantitative data on the amount of each sialic acid.

The Ludger acceptance criteria using in-house SOPs is that the calibration curve should give R^2 values of >0.99 for both Neu5Gc and Neu5Ac (Figure 3B).

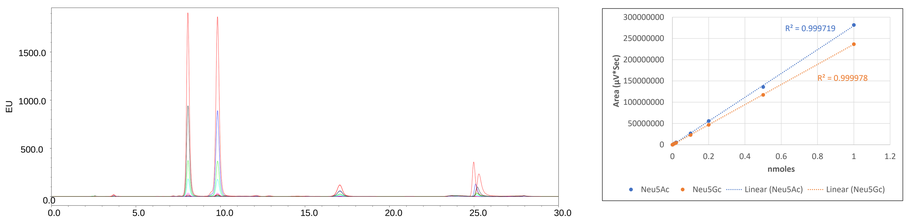
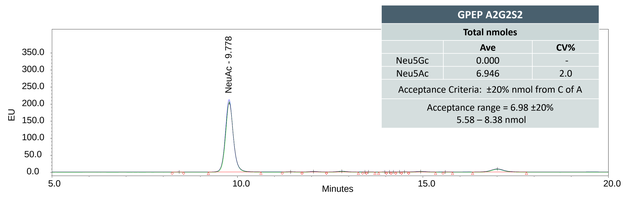


Figure 3: A) Overlay of the spectra from Neu5Ac and Neu5Gc serial dilutions; B) Calibration curves

Quantitative Process Standard sialylated glycopeptide (GPEP-A2G2S2)

The Ludger BioQuant™ GPEP quantitative standard is a purified sialylated glycopeptide standard used as a positive process control. This glycopeptide standard contains a biantennary N-linked glycan terminating in two sialic acids and has been quantified using qNMR. This standard is used to check the efficiency of glycan release, labeling and recovery (Figure 4).

The Ludger acceptance range for GPEP-A2G2S2 analysed using in-house SOPs is 5.6 to 8.4 nmol (which is the amount determined by quantitative NMR ± 20%)



GPEP A2G2S2		
Total nmoles		
	Ave	**CV%**
Neu5Gc	0.000	-
Neu5Ac	6.946	2.0
Acceptance Criteria: ±20% nmol from C of A		
Acceptance range = 6.98 ±20% 5.58 – 8.38 nmol		

Figure 4: Overlay of DMB-labeled Sialic Acid from GPEP-A2G2S2 (Triplicate Analysis)

Validation to ICH Q2 (R1) Guidelines with Fetuin Glycoprotein

The validation protocol followed ICH guidelines Q2 (R1) to show that the Ludger Standard operating procedures for sialic analysis were suitable for their intended use with both the sialic acid standards and a glycoprotein sample (fetuin). According to the ICH guideline Q2 (R1) (for Analytical Validation) the following validation characteristics were assessed: specificity, accuracy, repeatability (Intra-assay precision), intermediate precision, linearity, working range, detection limit, quantitation limit and robustness. Notable findings are listed below.

Range: The working range for starting amount of fetuin glycoprotein that gives a linear response is 5 to 100 µg for Neu5Ac and 25 to 100 µg for Neu5Gc.

Limit of quantitation: Both Neu5Ac and Neu5Gc show a linear relationship between sample amount and signal to noise and are present at levels above the LOQ (10* S/N) across the whole range of amounts (5 to 100 µg).

Stability: A common concern is the stability of DMB-labeled samples after derivatisation, to address this we monitored the samples over 72 hours at 10ºC in the dark. An overlay of the chromatograms of DMB-labeled sialic acids released from fetuin at 0, 24, 48 and 72 hours after derivatisation is shown in figure 5. CVs are less than 1% for retention times and less than 5% for peak areas. CVs for the calculated nmol/mg protein are less than 5% for Neu5Ac and Neu5Gc. From this data we conclude that it is acceptable to store samples in the dark at 10°C for up to 72 hr, provided that the calibration standards have been stored in the same conditions and are analysed at the same time. Additionally we have found that samples stored in the freezer in the dark are suitable for analysis. DMB labelled samples can be frozen for up to 2 days.

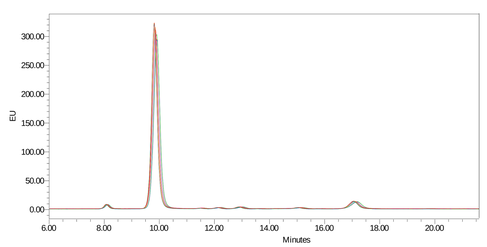


Figure 5: Sample Stability of DMB-Labelled Sialic acids after Derivatisation (overlay of traces from time points: 0, 24, 48, 72 h)

Biopharmaceutical Sialic Acid Analysis to Satisfy Regulatory Requirements

Sialic acid analysis is a regulatory requirement and should be performed throughout the product lifecycle. The key data acquired from this study:

1. Identity of each sialic acid present
2. Absolute amounts of Neu5Gc and Neu5Ac
3. Relative Percentages of Neu5Gc/Neu5Ac/Neu5,9Ac$_2$ (if applicable)

Erythropoietin (EPO)

EPO is a highly glycosylated protein and its high level of sialylation and accompanying acetylation has a significant effect its therapeutic properties, especially on the circulation half-life of the protein (3). The DMB labeled sialic acids from an EPO glycoprotein (starting amount 50 ug) are shown in Figure 6 with the relative levels of the N-acetyl, N-glycolyl and O-acetyl sialic acids (Table 2). This information is useful for QC to monitor batch to batch variation, or for comparability studies .

Note: When monitoring O-acetylation of sialic acids on products such as erythropoietin (EPO), acetyl esterase (sialate-O-acetylesterase) can be used during characterization. The enzyme removes 9-, 8- and 7-O-acetyl groups from released sialic acids, released glycans or glycoproteins. The use of this enzyme for characterisation of follicle stimulating hormone (FSH) is detailed in Dr. Radoslaw P. Kozak's Poster (P-203).

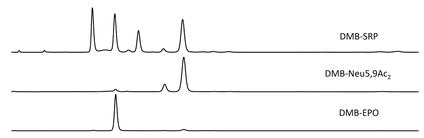


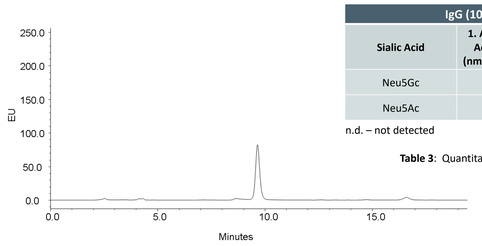
Figure 6: DMB profile of EPO in comparison to SRP and Neu5,9Ac$_2$

EPO (50 µg Glycoprotein)			
Sialic Acid	**1. Amount of Sialic Acids on Protein (nmoles/mg protein)**	**2. Amount of Neu5Gc and Neu5Ac (nmoles)**	**3. Average Relative Percent (%) of Sialic Acids from Peak Areas**
Neu5Gc	4.97	0.25	0.95
Neu5Ac	307.44	15.37	94.17
Neu5,9Ac$_2$	-	-	4.88

Table 2: Quantitative Amount and Relative Percent for sialic acids in EPO

Immunoglobulin G (IgG)

Most therapeutic mAbs are IgGs where the levels of sialic acid can impact the pharmacokinetics, specificity, and anti-inflammatory activity (4). Compared to EPO, IgGs have lower levels of sialyation so larger amounts of starting material are required (e.g. 100ug – 200ug) (Figure 7, Table 3).



IgG (100 µg Glycoprotein)		
Sialic Acid	**1. Amount of Sialic Acids on Protein (nmoles/mg protein)**	**2. Amount of Neu5Ac (nmoles)**
Neu5Gc	n.d	n.d.
Neu5Ac	7.0278	0.70278

n.d. – not detected

Table 3: Quantitative amount of Sialic acids in IgG

Figure 7: DMB profiles of sialic acids released from IgG

Conclusions

As sialylation of therapeutic glycoprotiens is critical to a drug's safety and efficacy, a sensitive, robust and easy to implement method to detect both low and high levels of sialylation is necessary. The benefits of using Ludger's sialic acid analysis workflow are:

- Satisfies the regulatory requirements for biopharmaceutical sialic acid content analysis, providing: 1) the overall degree of drug sialylation (absolute quantitation of sialic acid residues per molecule); 2) Relative quantities of Neu5Ac:Neu5Gc; and 3) relative percentages and identity of O-acetylated sialic acids.
- Utilizes multiple standards to provide a robust analysis method including acceptance criteria to ensure optimal process performance
- Is validated to an ICHQ2 standard.
- Kit and methods are used in-house for contract analysis – Ludger has continuous hands-on experience with this method and use it with many therapeutic glycoproteins that have both low and high levels of sialylation (erythropoietin (EPO), monoclonal antibodies, etc..).

References

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Acknowledgements

The human EPO was a kind gift from Raquel Montesino and Antonio Vallin from the Centre for Genetic Engineering and Biotechnology, Cuba.

Contact for more information

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