Analysis of Sialic Acids in Biopharmaceuticals

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Introduction

Glycosylated proteins—including erythropoietins (EPO), monoclonal antibodies (MAbs), and various hormones constitute a large portion of approved therapeutic biological drugs. Sialic acids are negatively charged monosaccharides present as terminal epitopes on many glycans. The two most common sialic acids in biopharmaceuticals are *N*-acetyl-neuraminic acid (Neu5Ac) and *N*-glycolyl-neuraminic acid (Neu5Gc). Neu5Ac is found in both human and non-human cells. Neu5Gc is synthesized by all mammalian cells except human cells. Neu5Gc has only one oxygen atom difference from its homologue, Neu5Ac, which is sufficient for Neu5Gc to be immunogenic in humans (Figure 1)[1]. A common modification of sialic acids is acetylation. *O*-acetylation reduces the kinetics of enzyme-catalysed desialylation of glycans and could therefore impact clinical efficacy [2]. Since the abundance and type of sialylation influences the clinical performance of therapeutic glycoproteins (serum half-life, immunogenicity, activity, etc.) it is considered a critical quality attribute (CQA). It is therefore a regulatory requirement (ICHQ6B) to characterise sialylation throughout the product lifecycle to monitor the following metrics:

1. ***Total amount of sialic acids – quantitation of Neu5Ac and Neu5Gc (nmoles/mg protein)***
2. ***Amount of Neu5Gc***
3. ***Presence of O-acetylated sialic acids***

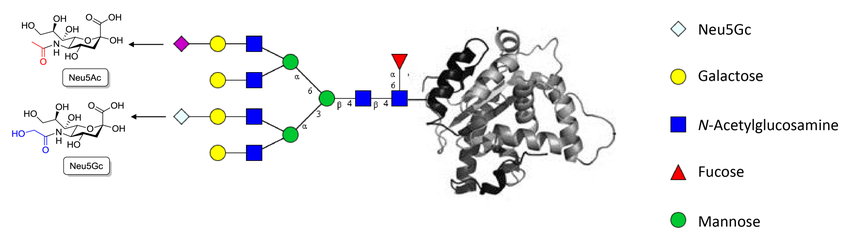


Figure 1: Structural Differences between Neu5Ac and Neu5Gc Sialic acids

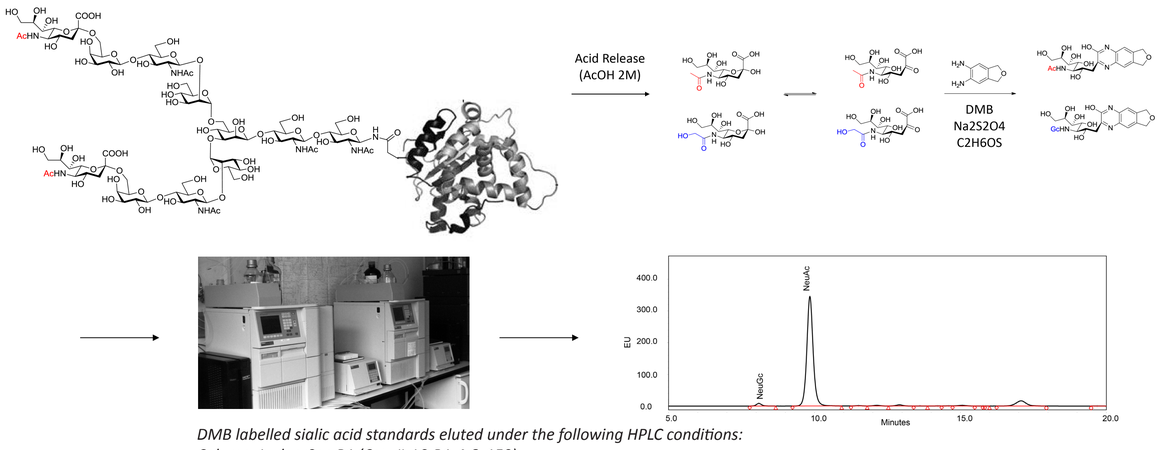
Highlights

The benefits of using Ludger's glycoprofiling scheme and associated products for analysis of sialic acids are:

- Sialic acids are released from glycoproteins using mild acid hydrolysis and are labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) (LudgerTag DMB kit: LT-KDMB-A1). The DMB-labeled sialic acids are then separated by RP-HPLC, and the amount of each sialic acid in the sample can be measured by comparing the response in the sample to a standard curve of each sialic acid
- Analysis in conjunction with several standards provides a robust method for quantitation. The standards used are:
  1. Qualitative sialic acid reference panel (SRP) containing a mixture of sialic acids found in humans and animals. This is used as a system suitability standard (CM-SRP-01).
  2. Quantitative Neu5Ac and Neu5Gc standards (NIST Traceable). Serial dilutions are used to make calibration curves to calculate absolute amounts of Neu5Ac and Neu5Gc (CM-NEUAC-01; CM-NEU5GC-01).
  3. A Neu5,9Ac$_2$ standard for relative quantitation (CM-NEU5,9AC2-01).
  4. A qNMR quantified sialylated glycopeptide (GPEP) process standard to assess the efficiency of the release and labeling procedure (BQ-GPEP-A2G2S2-10U).
  5. Fetuin Glycoprotein Standard positive control that contains protein, Neu5Ac and Neu5Gc (GCP-FET-50U).
- Kit and methods are used in-house for contract analysis – Ludger has continuous hands-on experience with this method and use it with many glycoproteins (EPO, MAbs, etc..).

Workflow

Sialic acids are released from glycoproteins by mild acid hydrolysis using conditions that preserve the *N*-acetyl, *N*-glycolyl and *O*-acetylation. The keto groups of the free sialic acids are derivatised with DMB using the LudgerTag DMB Analysis Kit (LT-KDMB-A1). Typical starting amounts are 50 µg of glycoprotein for a highly sialylated sample and 100-500 µg for glycoproteins with low levels of sialylation. The DMB-labeled sialic acids are then analysed by RP-HPLC along with DMB-labeled standards to allow for quantitation.



DMB labelled sialic acid standards 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
Gradient: 0-19 min (100% A); 19-23.5 (gradient to 10% A, 90% B, wash); 24-35 min (gradient to 100% A, re-equilibration)
Excitation wavelength: 373 nm Emission wavelength: 448 nm

Scheme 1: Workflow for Sialic Acid analysis using Ludger's DMB Kit

ICHQ2 Validation

The validation protocol following ICH guidelines Q2 (R1) shows that Ludger's products and standard operating procedures for sialic analysis are suitable for their intended use with both the sialic acid standards and a glycoprotein sample (fetuin). The following validation characteristics were assessed: specificity, accuracy, repeatability (intra-assay precision), intermediate precision, linearity, working range, detection limit, quantitation limit and robustness. Table 1 shows the range, coefficients of determination, and precisions for injections of fetuin (starting from 50 µg). Using the method described, the limit of quantitation (LOQ) and limit of detection (LOD) were determined to be 0.0205 nmol and 0.0068 nmol, respectively, for Neu5Ac. The LOQ and LOD for Neu5Gc were 0.0216 and 0.0071 nmol, respectively.

A common concern is the stability DMB-labeled sample after derivatisation, to address this we monitored the derivatised samples over 72 hours in the autosampler which was maintained at 10°C in the dark. The CV% of peak area over 72h was 1.2% for Neu5Ac and 2.5% for Neu5Gc indicating that under the conditions used, the samples were stable for up to 72h (Figure 2).

Fetuin Glycoprotein (50 µg)							
Sialic Acid	Average Retention Time (min)	Retention Time Precision (RSD, %)	Peak Area Precision (RSD, %)	Range (nmol, µg)	Coeff. Of Determination (R_2)	LOD (nmol, µg)	LOQ (nmol, µg)
Neu5Ac	8.116	0.4	1.2	0.828-18.357	0.997	0.0068	0.0205
				4.2-92.8		0.034	0.1
Neu5Gc	9.877	0.3	2.5	0.021-0.461	0.996	0.0071	0.0216
				4.3-94.5		1.5	4.4

Table 1. Linearity, LOD, LOQ, and Precision (n=9) for DMB Derivatised Sialic Acid Determination

Sample	Hold Time	Injection	Peak Area [uV*sec] Neu5Gc	Neu5Ac
Fetuin	0	1	1014192	48183212
		2	1016870	48214541
		3	990766	48070259
	24h	1	1085945	48969627
		2	1038230	48874526
		3	1001019	48623225
	48h	1	1021355	48831500
		2	1025108	49521162
		3	1034056	49939516
	72h	1	1050861	49775106
		2	1015437	48652440
		3	1005382	48764621
Average			1024935	48868311
STDEV			25334	607432
STD (CV%)			2.5	1.2

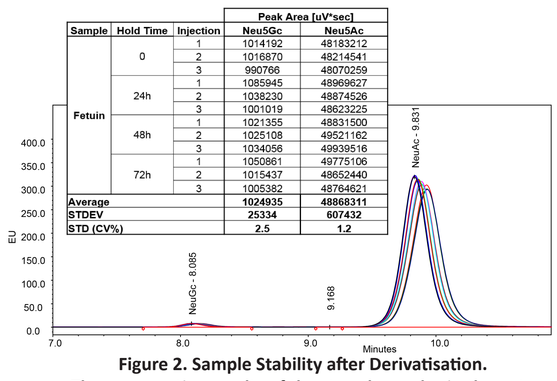


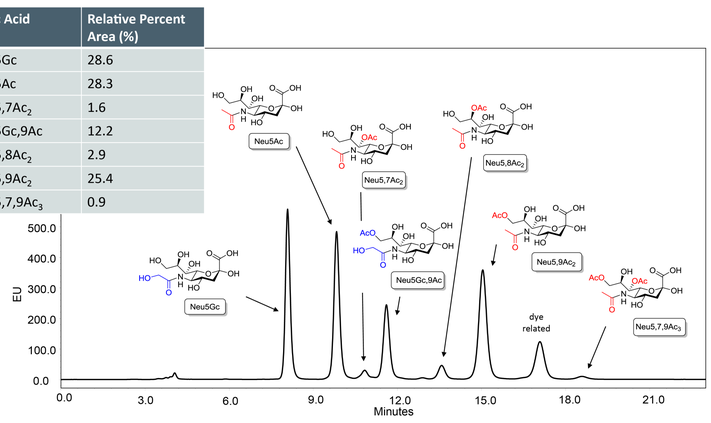
Figure 2. Sample Stability after Derivatisation. The comparative results of the stored samples in the auto sampler at 10°C in the dark are shown in imbedded table

Workflow

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

The sialic acid reference panel (SRP) contains Neu5Gc, Neu5Ac, Neu5,7Ac$_2$, Neu5Gc9Ac, Neu5,9Ac$_2$, and Neu5,7,(8),9Ac$_3$. SRP is used as a system suitability standard. The DMB profile for the sialic acid reference panel is shown in Figure 3A. The Neu5,9Ac$_2$ standard is used to determine the relative abundance of *O*-acetylated vs non-*O*-acetylated sialic acid residues (Figure 3B).

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



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

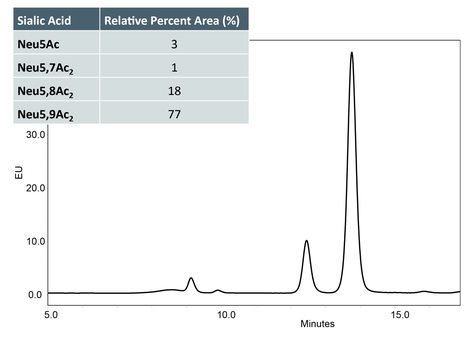


Figure 3: A) DMB-labeled sialic acid reference panel profiled on an LS-R1 HPLC column; B) Neu5,9Ac$_2$ Standard

2) Traceable Quantitative Standards (Neu5Ac, Neu5Gc)

The Neu5Ac and Neu5Gc standards are quantitative. Preparation of serial dilutions of the Neu5Ac and Neu5Gc standards enables quantitative analysis by reference to standard curves (Figure 4).

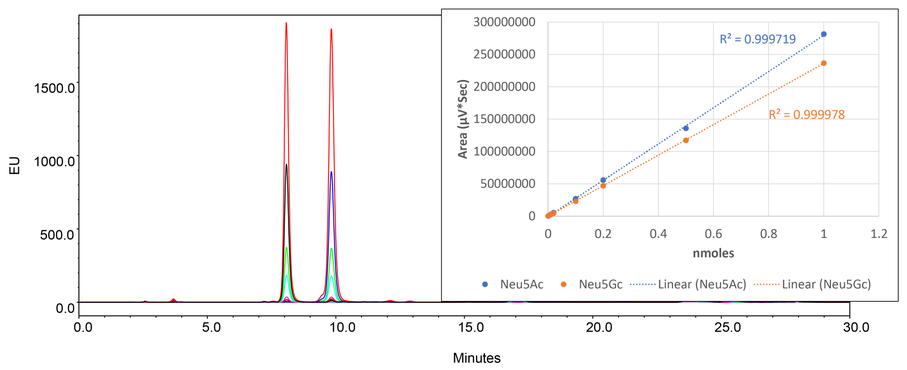
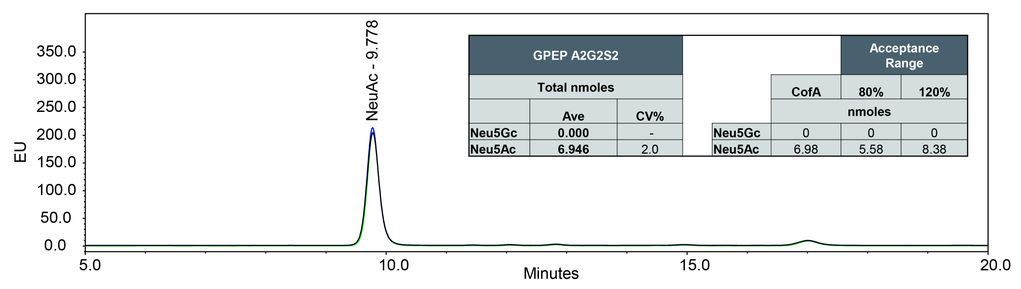


Figure 4: Overlay of spectra from serial dilutions of Neu5Ac (9.8 min) and Neu5Gc (8.1 min) standards (Right) used for calibration curves (Left)

3) Quantitative Process Standard sialylated glycopeptide (GPEP)

The Ludger BioQuant GPEP quantitative standard is a purified sialylated glycopeptide standard used as a positive process control. This standard is a complex 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 5).



GPEP A2G2S2				Acceptance Range	
Total nmoles			CofA	80%	120%
	Ave	CV%	nmoles		
Neu5Gc	0.000	-	Neu5Gc 0	0	0
Neu5Ac	6.946	2.0	Neu5Ac 6.98	5.58	8.38

Figure 5: DMB-labeled Sialic Acid from GPEP-A2G2S2 (Triplicate Analysis)

Application to Biopharmaceutical Samples

The analysis of sialic acids from EPO and Immunoglobulin G glycoprotiens illustrate how this analysis supports drug-development and characterisation.

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 [2]. The DMB-labeled sialic acids from an EPO glycoprotein are shown in Figure 6 with the relative levels of the *N*-acetyl, *N*-glycolyl and *O*-acetyl sialic acids (Table 2). This information can be used in QC to monitor batch-to-batch variation, or for comparability studies.

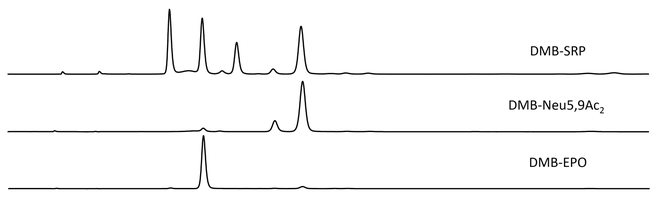


Figure 6: DMB profile of EPO in comparison to SRP and Neu5,9Ac$_2$

EPO (50 µg Glycoprotein)			
Sialic Acid	1. Amount of Sialic Acids on Protein (nmoles/mg protein)	2. Amount of Neu5Gc compared to 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
Neu5,9Ac2	-	-	4.88

Table 2: Glycometrics for Sialic Acids in EPO

Immunoglobulin G (IgG)

Most therapeutic mAbs are IgGs and their levels of sialic acid can impact pharmacokinetics, specificity, and anti-inflammatory activity [3]. Compared to EPO, IgGs have lower levels of sialyation, thus larger amounts of starting material are required (Figure 7, Table 3).

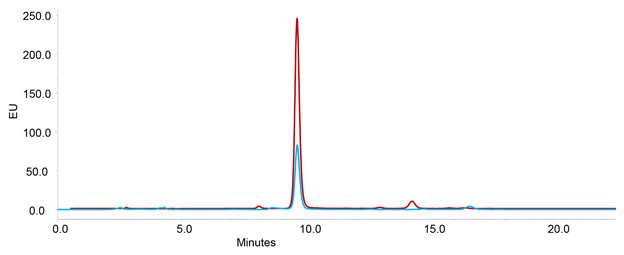


Figure 7: Overlay of DMB profiles of EPO (Red) and IgG (Blue)

IgG (100 µg Glycoprotein)			
Sialic Acid	1. Amount of Sialic Acids on Protein (nmoles/mg protein)	2. Amount of Neu5Gc compared to Neu5Ac (nmoles)	3. Average Relative Percent (%) of Sialic Acids from Peak Areas
Neu5Gc	n.d	n.d.	n.d
Neu5Ac	7.0278	0.70278	100
Neu5,9Ac2	-	-	n.d

n.d. – not detected

Table 3: Glycometrics for Sialic Acids in IgG

Acknowledgements and Citations

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

1. Ghaderi D, Taylor RE, Padler-Karavani V, Diaz S, Varki A. Implications of the presence of N-glycolylneuraminic Acid in Recombinant Therapeutic Glycoproteins. Nature biotechnology. 2010;28(8):863-867.
2. Fukuda, M.N.; Sasaki, H.; Lopez, L.; Fukuda, M. Survival of Recombinant Erythropoietin in the Circulation: The Role of Carbohydrates. Blood 1989; 73:84–89.
3. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 2006;313:670-673.

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