Quantitative Sialic Acid Analysis

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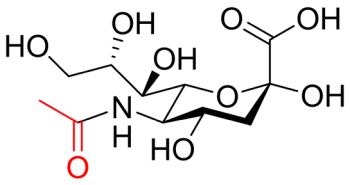
Why is sialic acid analysis important?

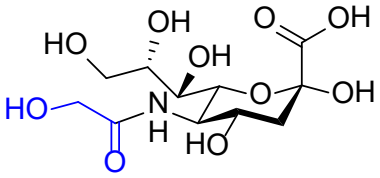
Sialic acids are negatively charged monosaccharides found on the non-reducing termini of glycans. They are important for the stability and 3D conformation of glycoproteins and are involved in many biological interactions. Sialic acids often have a pivotal functional impact: for example sialylation of the N-glycans on IgG increases anti-inflammatory activity; *O*-acetylated sialic acids can change ligand interactions and affect degradation (*O*-acetylated sialic acids including Neu5,9Ac$_2$ are found on EPO); and the presence of sialic acids also increases the serum half-life of glycoproteins by preventing uptake by the asialoglycoprotein-receptor located on liver cells.

A diverse range of sialic acids are found in nature, but the two major sialic acids found on N-glycans and O-glycans in biopharmaceuticals are *N*-acetyl-neuraminic acid (Neu5Ac, or NANA) and *N*-glycolyl-neuraminic acid (Neu5Gc, or NAGA). Humans cannot synthesise Neu5Gc and its presence on a drug can lead to immune reactions such as chronic inflammation. Anti-Neu5Gc antibodies have been detected in normal human sera, and can neutralise any Neu5Gc containing biopharmaceutical, thus lowering the drug's efficacy. It is important to be aware that the choice of cell line can greatly influence the type of sialic acids present on a biopharmaceutical, for instance a large proportion of the sialic acids on mouse IgG are often Neu5Gc.

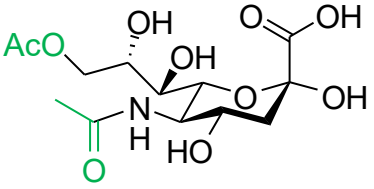
It is therefore imperative, for drug safety and efficacy, to monitor both the level and types of sialic acids during all stages of the product life cycle as well as QC batch to batch consistency. Hence, sialic acid analysis is a regulatory requirement laid out in the ICH Q6B guidelines for characterisation of biopharmaceuticals.



N-acetyl neuraminic acid
Neu5Ac



N-glycolyl neuraminic acid
Neu5Gc



5-*N*-acetyl-9-O-acetyl neuraminic acid
Neu5,9Ac$_2$

What information is obtained by using Ludger Kits and Standards?

- Quantitation of sialic acids (Neu5Ac & Neu5Gc) as nmol/mg protein
- Relative quantitation of the *O*-acetylated Neu5,9Ac$_2$

How to perform quantitative sialic acid analysis using Ludger kits and standards:

Release of Sialic Acids

Sialic acids are released by mild acid hydrolysis from the glycoprotein samples; typically from triplicate 50 µg amounts, although larger amounts may be required for samples with very low levels of sialylation e.g. MAbs. We recommend a number of process controls are also taken through the process: sample buffer or water as negative controls; fetuin glycoprotein (*GCP-FET-50U*) and/or a quantitative glycopeptide (*BQ-GPEP-A2G2S2-10U*) as positive controls. Samples and process controls are incubated with 2M acetic acid at 80°C for 2 hours.

DMB Labelling

To enable sensitive detection the released sialic acids are then fluorescently labelled with 1,2-diamino-4,5-methylenedioxybenzene. 2HCl (DMB) – see figure 1. When the sialic acid ring opens it exposes a ketone group next to the carboxylic acid, the DMB binds across these two adjacent keto groups producing a fluorophore (other monosaccharides do not have these adjacent keto groups, so they are not fluorescently labelled). The acid hydrolysed samples and process controls plus Ludger sialic acid standards: Neu5Ac (*CM-NEUAC-01*), Neu5Gc (*CM-NEUGC-01*), sialic acid reference panel (*CM-SRP-01*) and Neu5,9Ac$_2$ (*CM-NEU5,9,AC2-01*) if using, are incubated with DMB at 50°C for 3 hours.

LC Analysis

The Neu5Ac and Neu5Gc standards are diluted to produce a standard curve for quantitation of these two sialic acids. The sialic acid reference panel (*CM-SRP-01*) and Neu5,9Ac$_2$ are used for sialic acid identification. Samples and controls are analysed by either HPLC using a LudgerSep-R1 column *LS-R1-4.6x150* (table 1), or by UHPLC using a LudgerSep-uR2 column *LS-UR2-2.1x100* (table 2) for faster run times. Typical profiles for the sialic acid reference panel are shown in figure1 (A and B).

LC Conditions

Solvent A = acetonitrile:methanol:water 9:7:84
Solvent B = acetonitrile
Fluorescence: Excitation λ: 373 nm; Emission λ: 448 nm
Column temp = 30°C; Sample temp = 10°C

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.*

Time (min)	Flow mL/min	%A	%B
0	0.25	100	0
7	0.25	100	0
7.5	0.25	10	90
8	0.25	10	90
8.5	0.25	100	0
15	0.25	100	0

Table 2. *15 min running method for UHPLC analysis using a LudgerSep-uR2 column (2.1 x 100 mm, 1.9 µm particles) LS-UR2-2.1x100. Injection volume = 5 µL.*

LC Data

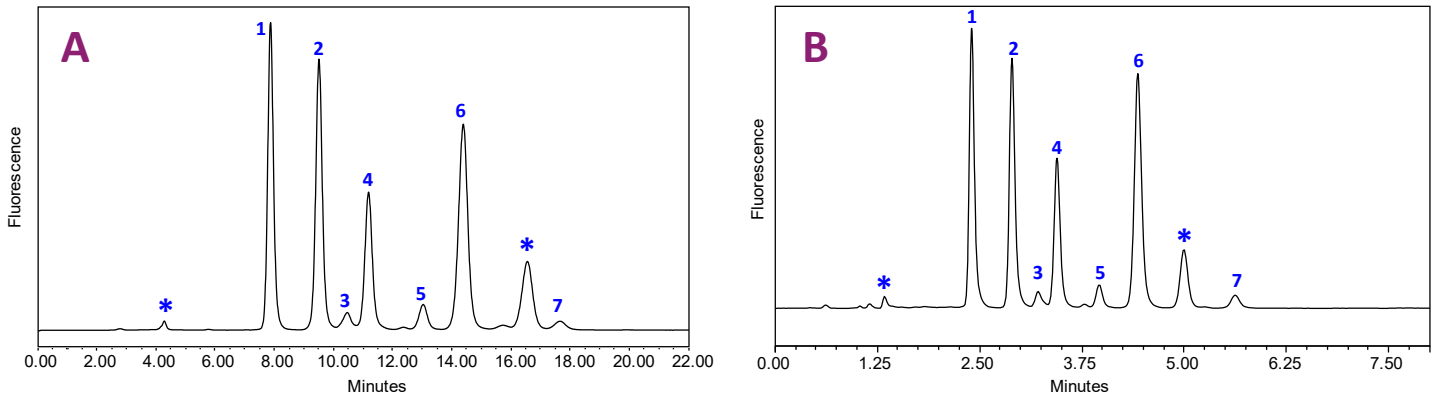
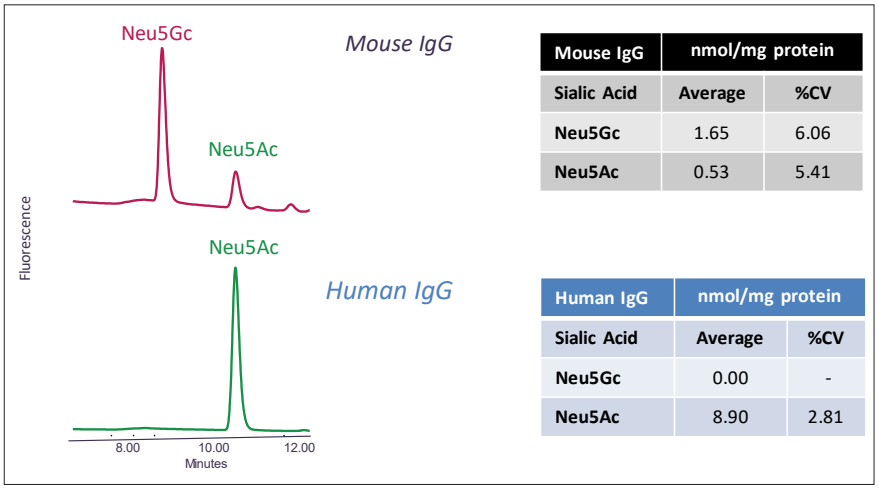


Figure 1: *Chromatogram of DMB Labelled Sialic Acid Reference Panel (CM-SRP-01) run on the LudgerSep-R1 HPLC column (A) or LudgerSep-uR2 UHPLC column (B)*
*Peaks: 1 = Neu5Gc; 2 = Neu5Ac; 3 = Neu5,7Ac$_2$; 4 = Neu5Gc,9Ac; 5 = Neu5,8Ac$_2$; 6 = Neu5,9Ac$_2$; 7 = Neu5,x,xAc$_3$ (where x is an unknown acetyl position); * = Reagent.*

Results

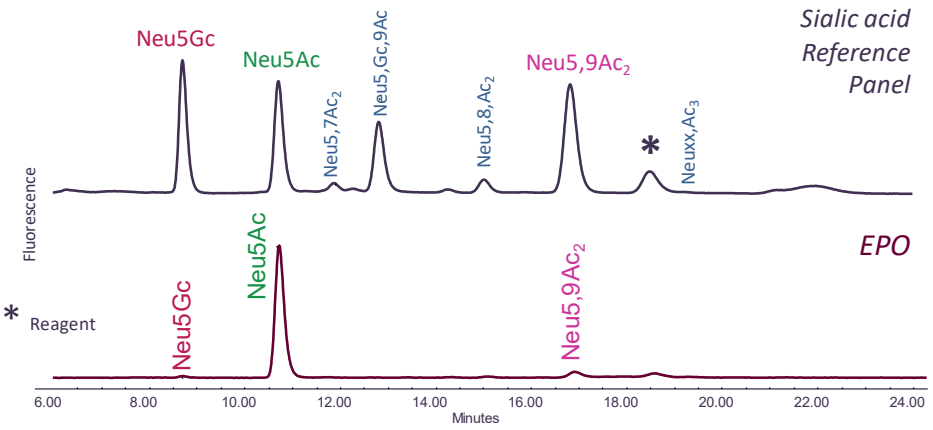
The peak areas of Neu5Ac and Neu5Gc in samples are compared to standard curves to provide quantitative data on the amounts of the sialic acids in your samples and in the process control. Figure 2 shows data for sialic acids released from mouse and human IgGs. The mouse IgG has high levels of the non-human sialic acid Neu5Gc, and in this case the human IgG has 4 times more sialic acids than the mouse IgG. Figure 3 shows data for the sialic acids in EPO. The absolute amounts of the Neu5Ac and Neu5Gc can be determined by comparison to the standard curve. However, EPO also contains the *O*-acetylated sialic acid Neu5,9Ac$_2$. Under aqueous conditions the acetate on the 9 position can migrate to the 8 position, so it is difficult to have a fully quantitative standard for Neu5,9Ac$_2$. The sialic acids are therefore identified by comparison to the retention times of the standards, and the relative proportions of the Neu5Ac, Neu5Gc and Neu5,9Ac$_2$ are determined from their peak areas.



Mouse IgG	nmol/mg protein	
Sialic Acid	Average	%CV
Neu5Gc	1.65	6.06
Neu5Ac	0.53	5.41

Human IgG	nmol/mg protein	
Sialic Acid	Average	%CV
Neu5Gc	0.00	-
Neu5Ac	8.90	2.81

Figure 2: *Identification and quantitation of Neu5Ac and Nue5Gc in mouse and human IgG. Analysis on LudgerSep-R1 HPLC column.*



Neu5Gc & Neu5Ac quantified in nmol/mg protein by reference to standard curves

	nmol/mg protein	
Sialic Acid	**Average**	**%CV**
Neu5Gc	3.61	5.11
Neu5Ac	270.32	5.31

Relative proportions of Neu5Gc, Neu5Ac & $Neu5,9Ac_2$ calculated from % peak areas.

	Relative % Area	
Sialic Acid	**Average**	**%CV**
Neu5Gc	0.99	0.24
Neu5,Ac	93.91	0.02
$Neu59Ac_2$	5.09	0.39

Figure 3: *Identification and quantitation of sialic acids in EPO. Analysis on LudgerSep-R1 HPLC column.*

Product/Ordering Information

Sialic acid Release, and Labelling

LT-KDMB-A1	LudgerTag DMB sialic acid release and labelling kit

Positive Controls

GCP-FET-50U-X4	Fetuin Glycoprotein Standard
BQ-GPEP-A2G2S2-10U	LudgerBioQuant GPEP A2G2S2 glycopeptide standard

Sialic Acid Standards

CM-NEUAC-01	N-acetylneuraminic acid (Neu5Ac or NANA) quantitative standard
CM-NEUGC-01	N-glycolylneuraminic acid (Neu5Gc or NGNA) quantitative standard
CM-NEU5,9,AC2-01	5-N-acetyl-9-O-acetyl neuraminic acid standard (Neu5,9Ac2)
CM-SRP-01	Sialic acid reference panel

LC Analysis:

LS-R1-4.6x150	LudgerSep R1 HPLC Column
LS-UR2-2.1x100	LudgerSep uR2 UHPLC Column