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 1A). 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 (ICH/Q8) to characterise sialylation throughout the product lifecycle to monitor the following metrics:

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

Workflow

1) Qualitative Standards: Sialic Acid reference Panel (SRP) and Neu5,9Ac2

The sialic acid reference panel (SRP) contains Neu5Gc, Neu5Ac, Neu5,7Ac, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7,8,9Ac3. SRPs are used as a system suitability standard. The DMB profile for the sialic acid reference panel is shown in Figure 3A. The Neu5,9Ac2 standard is used to determine the relative abundance of O-acetylated vs non-O-acetylated sialic acid residues (Figure 38).

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).

3) Quantitative Process Standard sialylated glycopeptide (GPEP)

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

Application to Biopharmaceutical Samples

The analysis of sialic acids from EPO and Immunoglobulin G glycoproteins 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-acetylated sialic acids (Table 2). This information can be used in QC to monitor batch-to-batch variation, or for comparability studies.

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 sialylation, thus larger amounts of starting material are required (Figure 7, Table 3).

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.


Table 1: Glycometrics for Sialic Acids in EPO

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<tr>
<th>Acid</th>
<th>LOQ</th>
<th>LOD</th>
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<tbody>
<tr>
<td>Neu5Ac</td>
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<td>0.0071</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>0.0216</td>
<td>0.0020</td>
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Table 3: Glycometrics for Sialic Acids in IgG

<table>
<thead>
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Figure 1: Structural Differences between Neu5Ac and Neu5Gc Sialic acids

Figure 2: Sample stability after derivatisation. The Concentration of the derivatised samples over 72 hours in the autosampler which was maintained at 10°C, is shown in imbedded table

Figure 3: Overlay of spectra from serial dilutions of Neu5Ac (9.8 min) and Neu5Gc (8.1 min) standards (Right) used for calibration curves (Left)

Figure 5: DMB profile of EPO in comparison to SRP and Neu5,9Ac2 (Glycopeptide Analysis)

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