# A QbD-compatible approach for reliable measurement of sialic acid O-acetylation as a potential Glycosylation Critical Quality Attribute (GCQA) of erythropoietin (EPO) therapeutics

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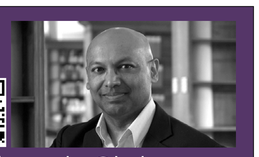

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## Introduction

Sialylation greatly influences the clinical performance of erythropoietin (EPO) drugs, the main effect being on therapeutic efficacy - primarily via modification of serum half life. Consequently, biopharma companies producing EPO products must carefully optimise, accurately measure and tightly control sialylation throughout the lifetime of their drug.

Structure-function relationship (SAR) studies have shown that there are several aspects of glycan sialylation that impact the *in vivo* safety and efficacy profiles of EPO drugs. Clinically relevant glycosylation metrics relating to EPO sialic acids (also known as neuraminic acids) include the following:

- The overall degree of drug sialylation - typically measured as sialic acid residues per EPO molecule,
- Molar distributions of glycans with different numbers of sialic acids,
- Glycosidic linkage types (e.g. Sia$\alpha$2,3 *vs* Sia$\alpha$2,6),
- The topologies (i.e. branching) of the sialic acid bearing glycans,
- The distribution of sialylated glycans over the various glycosylation sites, and
- The presence, number and patterns of acetyl and glycolyl moieties around the neuraminic acid ring. [Ref 1]

## Aims

**Sialic acid O-acetylation** (**SiaOAcet**) is seen in recombinant EPO but, so far, has been relatively unexplored due to lack of suitable analysis tools. To address this gap, we are developing a novel workflow (named **LB-SiaOAcet-EPO**) for studying the patterns of sialic acid O-acetylation in biologic drugs. This is part of Ludger's LongBow™ Glycomics system which is designed for reliable measurements of drug Glycosylation Critical Quality Attributes (GCQAs). LongBow glycomics technology is now being used in preliminary testing of our GlycoShape™ programme. This implements Ludger's QbD framework for realisation of glycoprotein drugs composed of heterogeneous glycoforms with distinct safety-efficacy profiles. At present, our focus is use of GlycoShape to aid QbD development of biosimilars and biobetters therapeutics for cancers and inflammatory diseases. However, the approach is valid for QbD based realisation of any glycoprotein drug. [Refs 2,3]

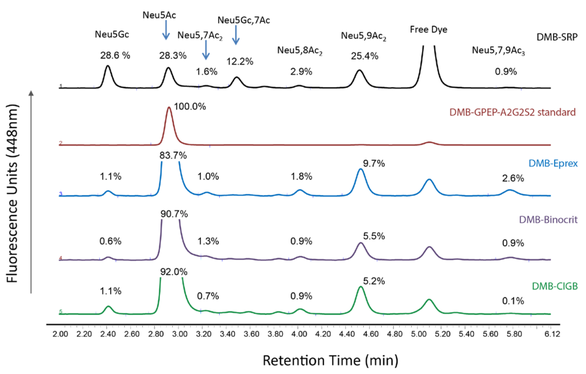
LB-SiaOAcet-EPO is one of a range of LongBow workflows used within the GlycoShape programme. The current version (v1.3) is comprised of three orthogonal glycoanalysis modules - **LB-DMB-EPO**, **LB-ProcLCMSn-EPO**, and **LB-NaNS-EPO**. Together, these provide detailed information on a range of clinically relevant glycometrics for EPO sialylation including the levels, types and patterns of neuraminic acid N- and O-acetylation and N-glycolylation.

This poster gives an overview of the three modules of the LB-SiaOAcet-EPO workflow applied to a preliminary comparability study of the sialylation of three EPO therapeutics - Eprex, Binocrit and CIGB-EPO (an EPO biosimilar).

## 1. LB-DMB-EPO: Quantitate the Sialic Acid Variants

The LB-DMB-EPO module gives the relative levels of the N-acetyl, N-glycolyl and O-acetyl sialic acid variants in each EPO glycoprotein sample. This provides raw data for several important glycometrics relating to the quality of the sialyl residues present in a particular drug batch. These include the **Neu5Gc:Neu5Ac** relative abundance levels as a metric for non-human (Neu5Gc) vs human (Neu5Ac) sialylation (the 5 position being a neuraminic acid ring nitrogen). Neu5Gc:Neu5Ac metric is important because a high value could correlate with (a) reduction of efficacy due to rapid clearance of NeuGc bearing EPO by endogenous anti-NeuGc antibodies (levels of which are vary greatly between individuals) and (b) safety issues (NeuGc is potentially immunogenic and has been implicated in the development of certain cancers). [3]

The data from LB-DMB-EPO also allows calculation of the **SiaOAcet** level - another important glycometric indicating the relative abundance of O-acetylated vs non-O-acetylated sialic acid residues. We propose that SiaOAcet should be: (a) carefully monitored carefully throughout the lifetime of an EPO drug and (b) considered in comparability studies of EPO variants (e.g. innovator drug vs an EPO biosimilar). The reason may be that O-acetylation reduces the kinetics of sialidase catalysed glycan desialylation - and therefore changes in SiaOAcet could lead to alterations in EPO serum half life and therefore clinical efficacy.




### Figure 1: DMB profiles of the three EPO Drugs

Sialic acids were released from EPO glycoproteins by mild acid hydrolysis using conditions that preserve the N-acetyl, N-glycolyl and O-acetylation patterns. The keto groups of the free sialic acids were derivatised with 1,2-diamino-4,5-methylendioxybenzene (DMB) using the LudgerTag™ DMB Analysis Kit (Cat # LT-KDMB-A1).

DMB labelled sialic acids were analysed by uHPLC chromatography using a LudgerSep uR2 UHPLC column (isocratic elution, solvent methanol:acetonitrile:water 7:9:84 v/v) with fluorescence detection ($\lambda_{ex}$ =373nm, $\lambda_{em}$=448nm).

The DMB profile for the sialic acid reference panel (SRP, Ludger Cat # CM-SRP-01) is shown at the top together with assignments and quantities for the different sialic acid variants. The peak at 5.1 minutes is dye-related.

| | Relative sialic acid abundance (%) + CVs | | | | | |
|---|---|---|---|---|---|---|
| | Eprex | | Binocrit | | CIGB | |
| Sialic acid derivative | % sialic acid | CV | % sialic acid | CV | % sialic acid | CV |
| Neu5Gc | 1.14 | 2.52 | 0.58 | 3.45 | 1.03 | 2.57 |
| Neu5Ac | 83.59 | 0.12 | 90.77 | 0.06 | 92.16 | 0.14 |
| Neu5,7Ac2 | 1.47 | 22.11 | 1.09 | 16.13 | 0.61 | 10.07 |
| Neu5,8Ac2 | 1.75 | 2.06 | 0.97 | 4.49 | 0.85 | 1.8 |
| Neu5,9Ac2 | 9.65 | 0.84 | 5.64 | 1.51 | 5.22 | 0.38 |
| Neu5,7,9Ac3 | 2.4 | 6.65 | 0.96 | 3.67 | 0.14 | 10.66 |
| Non O-acetylated | 84.73% | _ | 91.35% | _ | 93.19% | _ |
| O-acetylated | 15.27% | _ | 8.66% | _ | 6.82% | _ |

### Table 1: Relative molar abundances of the sialic acid variants for the three EPO drugs

The data indicates the similarities and differences for the sialic acid acetylation and glycolylation patterns of the three EPO drugs.

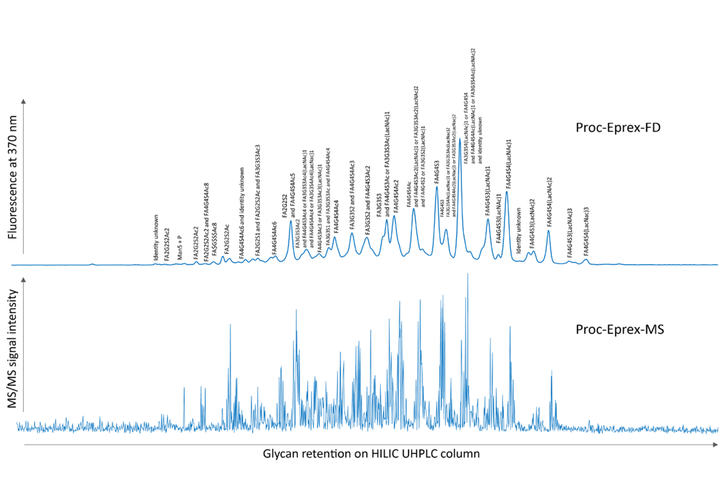
Glycometrics derived from these data include NeuGc:NeuAc

Our DMB analyses are typically done on a minimum of three glycoprotein replicates for each EPO sample. This gives sufficiently reliable data for preliminary studies.

## 2. LB-ProcLCMSn-EPO: Detailed, Quantitative Structure Characterisation of EPO Glycans

This module is based on analysis of procainamide labelled EPO glycans by FL-HILIC-LC-MS$^n$. Analysis of procainamide labelled N-glycans has been shown to give significantly better performance for detailed, quantitative glycan structure determination than methods based on analysis of 2-aminobenzamide (2-AB) labelled glycans commonly used in the biopharma industry. [Ref 4]

Fig 2 shows typical LC profiles from an automated LB-ProcLCMSn-EPO workflow. This achieves sample processing in 96-well microplates with a Hamilton Microlab Starlet (and other standard lab robots). N-glycans were released using PNGase F (QABio Cat # E-PNG01 or Ludger Cat # LZ-PNGASEF-96) with post-labelling clean-up using a protein binding membrane plate (Cat # LC-PBM-96), procainamide labelling (Cat # LT-KPROC-VP24) and clean-up using a HILIC plate (# LC-PROC-96). Table 3 shows the glycan structure assignments which were made from MS and MS$^n$ fragmentation data while relative quantitations were determined from integration of peaks on the fluorescence uHPLC profiles.



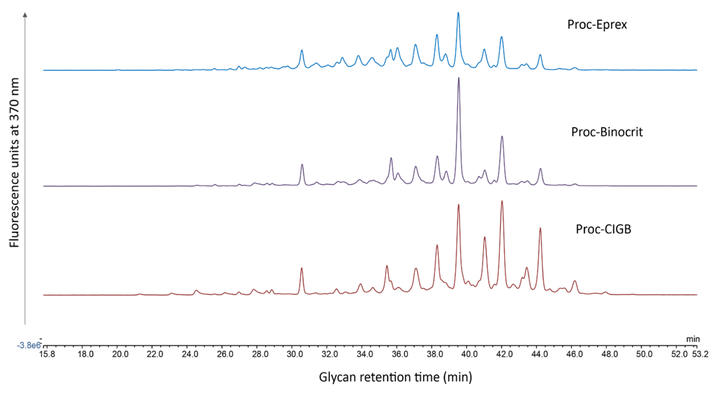

### Figure 2: LC-MS

**Top trace**: UHPLC-FD chromatogram of procainamide (PROC) labelled N-glycans released from Eprex and run on a Waters BEH glycan column on a Dionex UltiMate 3000 UHPLC.

**Bottom trace**: MS-MS CID chromatogram of PROC labelled N-glycans released from Eprex run on a Waters BEH glycan column on a Dionex UltiMate 3000 UHPLC coupled to a Bruker Amazon Speed.

**LC-MS conditions**: Dionex Ultimate 3000 UHPLC using a BEH-Glycan 1.7 µm, 2.1 x 150 mm column. Solvents for both UHPLC and LC-MS: Solvent A: 50 mM ammonium formate pH 4.4 made from Ludger buffer concentrate [LS-N-BUFFX40]; Solvent B: acetonitrile. 70 minute run. $\lambda_{ex}$=310nm, $\lambda_{em}$=370nm. Mass spectrometry analysis was performed by a Bruker AmaZon Speed ETD electrospray mass spectrometer coupled directly after the UHPLC fluorescence detector without splitting. The instrument scanned samples in maximum resolution mode, positive ion setting, MS scan + three MS/MS scans, nebuliser pressure 14.5 psi, nitrogen flow 10 litres/min, capillary voltage 4500 Volts. MS/MS was performed on three ions in each scan sweep with a mixing time of 40 ms.

Figure 3 shows fluorescence HILIC profiles of procainamide labelled N-glycans from the three EPO variants using the LB-ProcLCMSn-EPO workflow. The glycan structure assignments and relative molar abundances determined from the full LC-MSn analyses are given in Table 3. Table 2 shows example glycan structure topologies that are consistent with the MS and MS$^n$ fragmentation data. However, these must be considered as tentative - further analyses (e.g. exoglycosidase sequencing plus MS$^n$ fragmentation of permethylated glycans) would be needed to increase confidence in the detailed structures proposed.




### Figure 3: Comparison of three EPO glycoprofiles

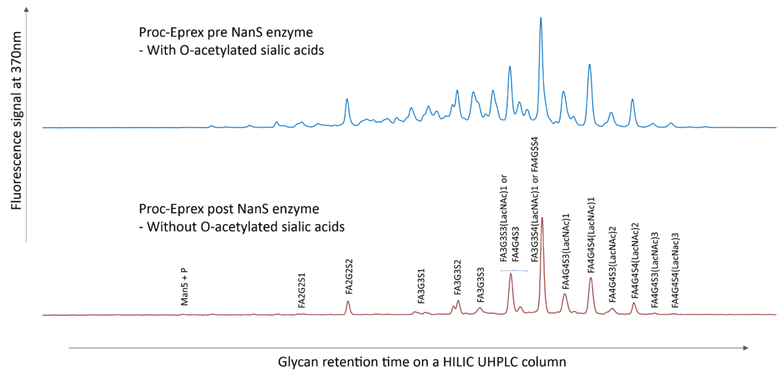
UHPLC-FD chromatograms of PROC labelled N-glycans released from three different EPO biopharmaceuticals and run on a Waters BEH glycan column on a Dionex UltiMate 3000 UHPLC using the automated LB-ProcLCMSn-EPO workflow. Experiment conditions were as per the profile in Fig 2.

Top: Eprex, middle: Binocrit, bottom: CIGB EPO.

Comparison of the three EPOs shows that there is statistically significant variation in the types of N-glycan structures present (including the N-acetyl, O-acetyl and N-glycolyl moieties) and the relative molar abundances of the common glycan species (see Table 3).

## 3. Remove the Sialic Acid O-Acetyl Groups to Simplify the EPO Glycan Map (LB-NanNS-EPO)

The procainamide labelled N-glycans from each EPO variant were de-O-acetylated with sialate O-acetyl esterase (NanS) obtained from the oral pathogen *Tannerella forsythia* (Ludger Cat # LZ-ACASE-KIT). These were then analysed using the LB-ProcLCMSn-EPO protocol. The resulting LC-ESI-MS$^n$ profiles were greatly simplified compared to the untreated glycan samples. Detailed comparisons of the data from the three EPO variants (data not shown) indicated that a large proportion of the differences between the N-glycan profiles of the drugs was due to variation in the sialic acid O-acetylation patterns.

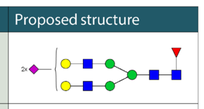
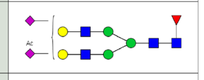
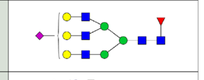
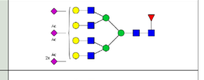
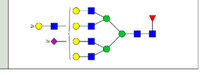



### Figure 4: Comparison of native and de-O-acetylated Eprex N-glycans

UHPLC-FD chromatograms of PROC labelled N-glycans released from Eprex, before and after digestion with an O-acetyl esterase (NanS enzyme), and run on a Waters BEH glycan column on a Dionex UltiMate 3000 UHPLC. Top: Intact procainamide labelled N-glycans with O-acetylated sialic acid structures, Bottom: procainamide labelled N-glycans post O-acetyl esterase with O-acetyl groups removed from sialic acid structures. The decrease in N-glycan peaks following O-acetyl esterase incubation shows a decrease in structural complexity leading to the conclusion that the N-glycosylation of Eprex contains a high amount of O-acetylated sialic acid structures.

### Table 2: Example Glycan Structures

This shows tentative structures for glycans identified from the MS and MS$^n$ fragmentation data of the LB-ProcLCMSn-EPO glycoanalysis module. We counsel caution when using such assignments. They are the best estimates of structures consistent with the experimental data. Refinement of the structures and an increase in our confidence of their accuracy would require further relevant orthogonal glycoanalyses.

| Notation | Proposed structure |
|---|---|
| FA2G2S2 |  |
| FA2G2S2Ac |  |
| FA3G3S1 |  |
| FA4G4S4Ac4 |  |
| FA4G4S3(LacNac)2 |  |

### Table 3: Summary Glycan Structure Assignments and Quantitation for Eprex

This information was obtained from the results of the LB-ProcLCMSn-EPO glycoanalysis module. The structure information was derived from the MS and MSn data while the FL-HILIC profiles furnished relative molar abundances of the glycan species.

Supplementary Table 3. N -glycans contained in Eprex.

| UHPLC Peak ID | Eprex Batch 1 Average (4 replicates) | | | ESI-LC/MS | | | | | | | | | | | | | | | | Eprex Batch 2 Average (5 replicates) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Composition | | | Neu5Ac (S) | | | | $H^+$ adducts | | | | | | | | | | |
| | GU value | % Area | RSD % | Possible glycan | Hex (H) | HexNAc (N) | Fuc (F) | 0 OAc | 1 OAc | 2 OAc | potential sulphate or phosphate | $[M/2]^+$ calculated | $[M/2]^+$ observed | $[M/3]^+$ calculated | $[M/3]^+$ observed | $[M/4]^+$ calculated | $[M/4]^+$ observed | $[M/5]^+$ calculated | $[M/5]^+$ observed | % Area | RSD % |
| 1 | 6.51 | 0.06 | 49.30 | Identity unknown | | | | | | | | | | | 922.98 | | | | | - | - |
| 2 | 6.63 | 0.02 | 67.10 | FA2G2S2Ac2 | 5 | 4 | 1 | 1 | 0 | 1 | 0 | - | - | 1337.02 | nd | 891.69 | 891.41 | - | - | 0.13 | 4.41 |
| 3,4 | 6.78, 6.88 | 0.22 | 16.20 | Man5+P | 5 | 2 | 0 | 0 | 0 | 0 | 1 | 1534.57 | 1534.62 | 767.79 | 767.42 | 512.19 | 511.62 | - | - | 0.08 | 5.22 |
| 5 | 7.90 | 0.24 | 10.75 | FA2G2S2Ac2 | 5 | 4 | 1 | 1 | 0 | 1 | 0 | | | 1337.02 | 1336.56 | 891.69 | 891.09 | | | 0.26 | 3.57 |
| 6 | 7.17 | 0.12 | 12.82 | FA2G2S2Ac2 | 5 | 4 | 1 | 1 | 0 | 1 | 0 | | | 1337.02 | nd | 891.69 | 890.96 | | | | |
| | | | | FA4G4S4Ac6 | 7 | 6 | 1 | 0 | 0 | 4 | 0 | | | | | | | | 1060.20 | - | - |
| 7 | 7.28 | 0.25 | 12.09 | FA4G4S4Ac6 | 7 | 6 | 1 | 0 | 0 | 4 | 0 | | | | | | | | 1060.28 | - | - |
| 8,9 | 7.41, 7.50 | 1.12 | 11.30 | FA2G2S2Ac | 5 | 4 | 1 | 1 | 1 | 0 | 0 | - | - | 1316.02 | 1316.07 | 877.68 | 877.43 | - | - | 0.17 | 2.47 |
| 10, 11 | 7.63, 7.73 | 0.78 | 7.03 | Man6+P | 6 | 2 | 0 | 0 | 0 | 0 | 1 | | | 848.81 | 848.427 | | 848.427 | | | 0.22 | 3.25 |
| | | | | FA4G4S4Ac6 | 7 | 6 | 1 | 0 | 2 | 2 | 0 | | | | | 1385.18 | 1385.05 | 1039.14 | 1039.21 | | |
| 12,13 | 7.84, 7.91 | 1.30 | 5.81 | FA2G2S2Ac | 5 | 4 | 1 | 1 | 1 | 0 | 0 | - | - | 1316.02 | nd | 877.68 | 877.44 | - | - | 0.17 | 2.47 |
| | | | | FA3G3S3Ac3 | 6 | 5 | 1 | 1 | 1 | 1 | 0 | | | | | 1124.43 | 1123.78 | 843.58 | 843.46 | 657.06 | nd |
| | | | | FA2G2S1 | 5 | 4 | 1 | 1 | 0 | 0 | 0 | - | - | 1149.47 | 1149.02 | 766.65 | 766.13 | - | | 0.35 | 2.56 |
| 14 | 8.11 | 1.57 | 7.39 | FA4G4S4Ac6 | 7 | 6 | 1 | 0 | 2 | 2 | 0 | | | | | 1385.18 | 1385.22 | 1039.14 | 1039.21 | - | |
| 15 | 8.41 | 4.33 | 5.94 | FA2G2S2 | 5 | 4 | 1 | 2 | 0 | 0 | 0 | - | - | 1295.01 | 1294.05 | 863.68 | 863.17 | - | | 3.05 | 1.44 |
| | | | | Identity unknown | | | | | | | | | | | | | | | 1028.68 | - | |
| 16 | 8.57 | 2.78 | 5.63 | FA4G4S3Ac4 or FA3G3S3Ac4(LacNAc)1 | 7 | 6 | 1 | 0 | 2 | 1 | 0 | - | - | - | | 1260.15 | 1260.17 | 945.36 | 945.50 | 756.49 | nd |
| | | | | FA3G3S3Ac2 | 6 | 5 | 1 | 1 | 0 | 1 | 0 | | | | | 1110.43 | 1109.81 | 833.07 | 832.87 | - | |
| | | | | FA4G4S4Ac4 or FA3G3S4Ac4(LacNAc)1 | 7 | 6 | 1 | 1 | 2 | 1 | 0 | | | | | 1357.18 | 1357.55 | 1018.14 | 1018.21 | 814.71 | nd |
| 17 | 8.85 | 1.77 | 5.94 | FA4G4S3Ac3 or FA3G3S3Ac3(LacNAc)1 | 7 | 6 | 1 | 1 | 1 | 1 | 0 | | | | | 1246.14 | 1245.79 | 934.86 | 934.72 | - | |
| 18 | 9.01 | 1.73 | 3.73 | FA3G3S3Ac | 6 | 5 | 1 | 2 | 1 | 0 | 0 | - | - | 1644.13 | nd | 1096.42 | 1096.52 | 822.57 | 822.51 | - | |
| | | | | FA3G3S1 | 6 | 5 | 1 | 1 | 0 | 0 | 0 | - | - | 1332.03 | nd | 888.36 | 888.80 | 666.52 | nd | - | |
| | | | | FA4G4S4Ac4 | 7 | 6 | 1 | 1 | 2 | 1 | 0 | | | | | 1357.18 | nd | 1018.14 | 1017.74 | 814.71 | 814.80 |
| 19 | 9.11 | 3.29 | 5.79 | FA4G4S4Ac4 | 7 | 6 | 1 | 2 | 0 | 2 | 0 | | | | | 1357.18 | 1357.88 | 1018.14 | 1018.48 | 814.71 | 814.83 |
| 20 | 9.40 | 5.16 | 4.22 | FA3G3S2 | 6 | 5 | 1 | 2 | 0 | 0 | 0 | - | - | 1477.58 | nd | 985.39 | 985.14 | 739.29 | 739.85 | | |
| | | | | FA4G4S4Ac3 | 7 | 6 | 1 | 2 | 1 | 1 | 0 | | | | | 1343.17 | 1342.91 | 1007.63 | 1007.23 | 806.31 | nd |
| 21 | 9.65 | 5.73 | 5.63 | FA4G4S3Ac2 | 7 | 6 | 1 | 2 | 0 | 1 | 0 | | | | | 1232.14 | 1232.19 | 924.36 | 923.98 | 739.69 | 739.28 |
| | | | | FA3G3S2 | 6 | 5 | 1 | 2 | 0 | 0 | 0 | - | - | 1477.58 | nd | 985.39 | 985.19 | 739.29 | 739.45 | - | |
| 22 | 10.00 | 6.19 | 7.58 | FA3G3S3 | 6 | 5 | 1 | 3 | 0 | 0 | 0 | - | - | 1623.13 | nd | 1082.42 | 1082.15 | 812.07 | 811.92 | - | |
| | | | | FA4G4S3Ac or FA3G3S3Ac(LacNAc)1 | 7 | 6 | 1 | 2 | 1 | 0 | 0 | | | | | 1218.14 | 1218.20 | 913.85 | 913.70 | 731.28 | nd |
| 23 | 10.14 | 6.92 | 4.90 | FA4G4S4Ac2 | 7 | 6 | 1 | 3 | 0 | 1 | 0 | - | - | - | | 1329.17 | 1329.18 | 997.13 | 997.23 | 797.91 | nd |
| 24 | 10.50 | 10.12 | 2.92 | FA4G4S4Ac | 7 | 6 | 1 | 3 | 1 | 0 | 0 | | | | | 1315.17 | 1315.23 | 986.63 | 986.76 | 789.50 | nd |
| | | | | FA4G4S3Ac2(LacNAc)1 or FA3G3S3Ac2(LacNAc)2 | 7 | 6 | 1 | 2 | 0 | 1 | 0 | | | | | 1353.85 | nd | 1015.64 | 1015.19 | 812.71 | nd |
| | | | | FA4G4S2 or FA3G3S2(LacNAc)1 | 7 | 6 | 1 | 2 | 0 | 0 | 0 | | | 1660.15 | nd | 1107.10 | 1107.18 | 830.58 | 830.22 | - | |
| 25 | 10.95 | 7.88 | 4.25 | FA4G4S3 | 7 | 6 | 1 | 3 | 0 | 0 | 0 | | | | | 1204.13 | 1203.83 | 903.05 | 903.53 | - | |
| 26 | 11.13 | 4.33 | 5.52 | FA4G4S3 | 7 | 6 | 1 | 3 | 0 | 0 | 0 | | | | | 1204.13 | 1204.21 | 903.05 | 902.73 | - | |
| | | | | FA4G4S3AcLacNAc1 or FA3G3S3AcLacNAc2 | 7 | 6 | 1 | 2 | 1 | 0 | 0 | | | | | 1339.85 | nd | 1005.14 | 1005.21 | 804.31 | 804.76 |
| | | | | FA4G4S4Ac2(LacNAc)1 or FA3G3S3Ac2(LacNAc)2 | 7 | 6 | 1 | 2 | 2 | 0 | 0 | | | | | 1450.88 | nd | 1088.41 | 1087.74 | 870.93 | nd |
| 27 | 11.41 | 13.46 | 3.04 | FA3G3S4(LacNAc)1 or FA4G4S4 | 7 | 6 | 1 | 4 | 0 | 0 | 0 | - | - | - | - | 1301.16 | 1300.47 | 976.20 | 976.30 | - | |
| | | | | Identity unknown | | | | | | | | - | - | - | - | | | | 921.71 | - | |
| | | | | FA4G4S4Ac(LacNAc)1 or FA3G3S4Ac(LacNAc)2 | 8 | 7 | 1 | 3 | 1 | 0 | 0 | | | | | 1436.88 | nd | 1077.91 | 1077.68 | 862.53 | nd |
| 28 | 12.00 | 6.06 | 9.79 | FA4G4S3(LacNAc)1 | 8 | 7 | 1 | 3 | 0 | 0 | 0 | - | - | - | - | 1325.84 | 1325.96 | 994.67 | 994.78 | 795.91 | nd |
| 29 | 12.22 | 0.74 | 17.14 | FA4G4S3(LacNAc)1 | 8 | 7 | 1 | 3 | 0 | 0 | 0 | - | - | - | - | 1325.84 | nd | 994.67 | 994.48 | 795.91 | nd |
| 30 | 12.41 | 7.15 | 8.54 | FA4G4S4(LacNAc)1 | 8 | 7 | 1 | 4 | 0 | 0 | 0 | - | - | - | - | 1422.87 | nd | 1067.41 | 1067.55 | 854.13 | 854.56 |
| 31 | 12.70 | 0.36 | 18.06 | Identity unknown | | | | | | | | | | | | | | | 1012.68 | | |
| 32,33 | 12.91, 13.02 | 2.24 | 17.06 | FA4G4S3(LacNAc)2 | 9 | 8 | 1 | 3 | 0 | 0 | 0 | | | | | 1447.45 | nd | 1085.92 | 1028.72 | 868.93 | nd |
| 34 | 13.37 | 2.98 | 16.50 | FA4G4S4(LacNAc)2 | 9 | 8 | 1 | 4 | 0 | 0 | 0 | - | - | - | - | 1544.58 | nd | 1158.69 | 1158.53 | 927.15 | 927.28 |
| 35,36 | 13.88, 14.02 | 0.55 | 21.05 | FA4G4S3(LacNAc)3 | 10 | 9 | 1 | 3 | 0 | 0 | 0 | - | - | - | - | 1569.26 | nd | 1177.20 | 1177.13 | 941.96 | nd |
| 37 | 14.31 | 0.52 | 21.53 | FA4G4S4(LacNAc)3 | 10 | 9 | 1 | 4 | 0 | 0 | 0 | - | - | - | - | - | - | 1249.97 | nd | 1000.20 | 1000.24 |

| Eprex Batch 2 Average (5 replicates) | |
|---|---|
| % Area | RSD % |
| - | - |
| 0.13 | 4.41 |
| 0.08 | 5.22 |
| 0.26 | 3.57 |
| | |
| 0.22 | 3.25 |
| | |
| 0.17 | 2.47 |
| 0.22 | 3.25 |
| | |
| 0.17 | 2.47 |
| - | - |
| 0.35 | 2.56 |
| 0.53 | 1.22 |
| 3.05 | 1.44 |
| 0.65 | 2.56 |
| 1.24 | 2.79 |
| 1.47 | 1.87 |
| 0.82 | 4.82 |
| 1.42 | 1.40 |
| 1.68 | 2.15 |
| - | - |
| 3.48 | 1.11 |
| 0.85 | 0.46 |
| 4.40 | 1.92 |
| 2.37 | 1.34 |
| 2.03 | 2.16 |
| 4.98 | 0.91 |
| 6.35 | 3.47 |
| 4.24 | 3.81 |
| - | - |
| - | - |
| 6.85 | 1.01 |
| - | - |
| 0.42 | 0.79 |
| 5.20 | 1.59 |
| 1.38 | 2.53 |
| 8.25 | 2.55 |
| - | - |
| 2.15 | 5.27 |
| 3.15 | 6.28 |
| 0.54 | 2.30 |
| 0.54 | 6.16 |

## Conclusions

The sialylation patterns of three EPO drugs - Eprex, Binocrit and CIGB - were characterised using Ludger's LB-SiaOAcet-EPO workflow. This uses three orthogonal glycoanalysis modules with automated sample prep. Eprex contained the greatest relative abundance of O-acetylated derivatives, mainly due to high levels of Neu5,9Ac2 and Neu5,7,9Ac3. Binocrit had the lowest levels of Neu5Gc, a sialic acid known to evoke an immune response in humans. High levels of O-acetylation, with up to six sialic acids on one glycan structure were seen in CIGB.

We plan to extend the LB-SiaOAcet-EPO workflow to provide even more refined details of the glycan structures and to use it in collaborations to develop (a) biosimilars that more closely match the originator's drug and (b) biobetters with significantly improved serum half lives and, consequently, superior clinical efficacy. The component glycoanalysis modules of LB-SiaOAcet-EPO as well as the complete workflow are being standardised and will be formally validated to GMP level. We are scheduled to incorporate this workflow into our GlycoShape programme to support QbD-based realisation and regulatory submissions for glycoprotein therapeutics.

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And thank you for viewing our poster. If you'd like a copy or want to know more about our LongBow Glycomics workflows or GlycoShape programme then send me (Daryl Fernandes) an email (**daryl.fernandes@ludger.com**) or connect on LinkedIn (**http://uk.linkedin.com/pub/daryl-fernandes/1/637/95**).

Ludger