COMPREHENSIVE CHARACTERIZATION OF THE N AND O-LINKED GLYCOSYLATION OF A RECOMBINANT HUMAN EPO USING AN OPTIMIZED HILIC STATIONARY PHASE

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Matthew A. Lauber, Stephan M. Koza, Erin E. Chambers
Waters Corporation, Milford, MA

INTRODUCTION

The immunoglobulin G (IgG) modality has paved the way for many efficacious protein-based therapies. At the same time, numerous highly effective patient therapies have also been made possible by the production of recombinant, human hormones and enzymes. For example, erythropoesis stimulating therapeutics, like epoetin (EPO) alpha, have long been available for the treatment of anemia. Such a therapy for increasing patient red blood cell counts was first made possible by the commercialization of Epogen®, which has been available in the US market since its approval by the FDA in 1989. And now, because the landscape of the biopharmaceutical industry continues to evolve and Epogen® patents expired in 2013, EPO drug products are targets for being developed into both international and domestic-market biosimilars.

Epoetin alpha has a relatively small primary structure, yet it has 3 sites of N-glycosylation and 1 site of O-glycosylation (**Figure 1**). Because of its glycosylation, epoetin alpha has a molecular weight between 30 and 40 kDa even though its protein mass amounts to only 18 kDa. Interestingly, the glycosylation of epoetin is very much tied to its potency and serum half life. Two attributes of its glycan profile that are known to show positive correlations with *in vivo* activity include antennarity and sialylation. As a result, it is critical for the glycosylation of an epoetin therapeutic to be well characterized. In addition, the significance of epoetin glycosylation suggests that detailed glycan profiling would be a path toward establishing a viable epoetin biosimilar.

Here, we demonstrate the use of two facile strategies that can be used to detail the N- and O-linked glycosylation of a recombinant, human epoetin (rhEPO). In this work, rhEPO N-glycans were rapidly released, labeled with *Rapi*Fluor-MS and profiled by hydrophilic interaction chromatography (HILIC) using sensitive fluorescence and mass spectrometric detection. Then, in a second, parallel analysis, N-deglycosylated rhEPO was interrogated by intact protein HILIC to elucidate information on O-glycosylation.

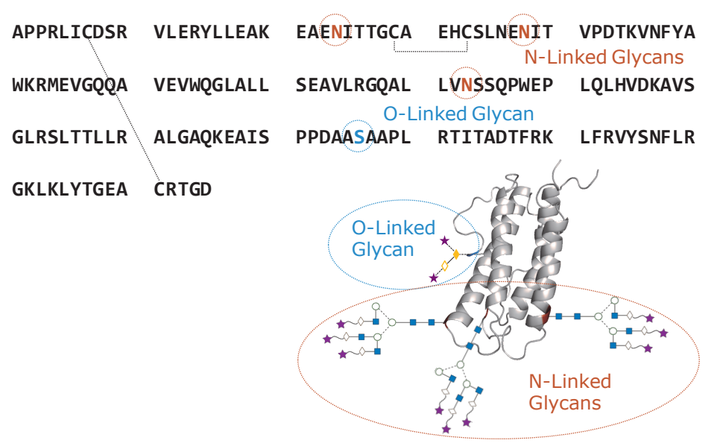

Figure 1. Sequence and Structural Information for Recombinant, Human Epoetin Alpha (rhEPO).

METHODS

SAMPLE DESCRIPTION:
A recombinant, human epoetin alpha expressed from CHO cells (PeproTech, Rocky Hill, NJ) was reconstituted in 50 mM HEPES NaOH pH 7.9 buffer to a concentration of 2 mg/mL. N-glycans were released from rhEPO and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 715004793EN) and the instructions provided in its care and use manual (715004793). To facilitate analysis of O-glycosylation, rhEPO was N-deglycosylated using the rapid deglycosylation technique outlined in the care and use manual of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 715004793).

METHOD CONDITIONS FOR RELEASED GLYCAN ANALYSIS:
LC Conditions:

LC system:	ACQUITY UPLC H-Class Bio System
Column Temp.:	60 °C
Flow Rate:	0.4 mL/min
Fluorescence Detection:	Ex 265 / Em 425 nm (2 Hz, Gain =1)
Column:	ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column, 2.1 x 150 mm (p/n 176003702)

Ammonium formate/acetonitrile mobile phases and gradient as described in the GlycoWorks *Rapi*Fluor-MS Care and Use Manual (715004793).

MS Conditions

MS system:	Xevo G2-XS QTof
Analyzer mode:	Resolution Mode (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	600 L/Hr
Acquisition:	700–2000 m/z, 2 Hz

METHOD CONDITIONS FOR INTACT PROTEIN HILIC:
LC Conditions

LC system:	ACQUITY UPLC H-Class Bio System
Column temp.:	45°C
Flow Rate:	0.2 mL/min
Fluorescence Detection:	Ex 280 nm / Em 320 nm (10 Hz)
Mobile Phase A:	0.1% (v/v) TFA, H_2O
Mobile Phase B:	0.1% (v/v) TFA, ACN
Column:	ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column, 2.1 x 150 mm (p/n 176003702)
Gradient:	25 to 35% A over 20 min

MS Conditions

MS system:	Synapt G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	Resolution Mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/Hr
Acquisition:	700–4800 m/z, 1 Hz

For more experimental details and additional results, see Waters Application Note "Comprehensive Characterization of the N and O-Linked Glycosylation of a Recombinant Human EPO" (720005462EN)

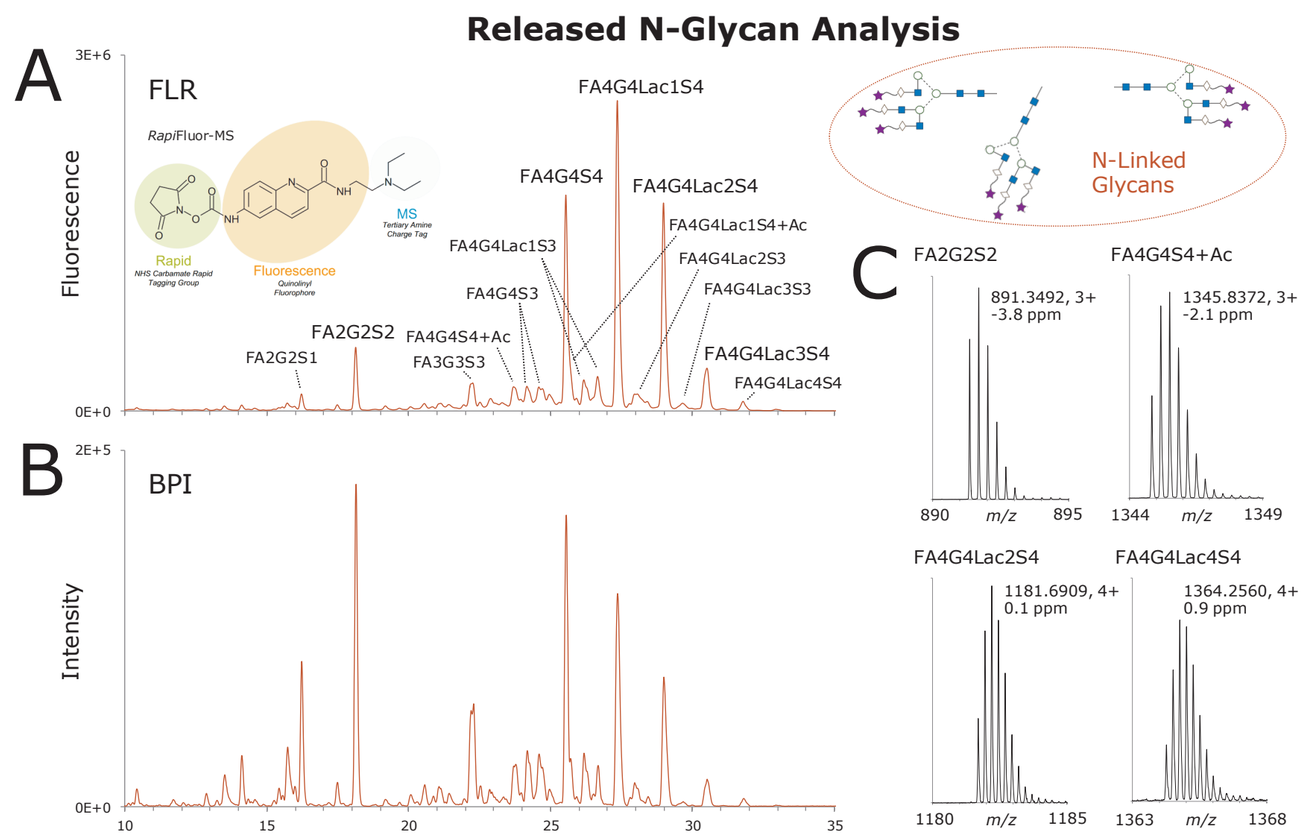

Figure 2. HILIC Profiling of Release N-Glycan Analysis of rhEPO. (A) Fluorescence and (B) base peak intensity (BPI) chromatograms for *Rapi*Fluor-MS labeled N-glycans from rhEPO. Chromatograms obtained for glycans from 0.4 µg protein using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm column. (C) MS spectra for four example N-glycan species. N-glycan assignments are listed according to Oxford notation. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues(Neu5Ac).

RESULTS AND DISCUSSION

Released N-Glycan Analysis of rhEPO Using *Rapi*Fluor-MS Labeling and HILIC Profiling

A profile of the N-glycans from rhEPO can be readily obtained with a new sample preparation strategy involving the novel glycan labeling reagent, *Rapi*Fluor-MS. This sample preparation, based on the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, allows an analyst to rapidly release N-glycans and label them with a tag that provides enhanced sensitivity for fluorescence and electrospray ionization mass spectrometric (ESI-MS) detection. In previous applications, *Rapi*Fluor-MS has been predominately used in the analysis of different IgG samples. Nevertheless, using the protocol from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, an analyst can successfully prepare samples from even heavily glycosylated proteins, such as rhEPO.

*Rapi*Fluor-MS labeled N-glycans have proven to be amenable to hydrophilic interaction chromatography (HILIC). Accordingly, HILIC-fluorescence-MS of *Rapi*Fluor-MS has emerged as a very powerful tool for detailing the N-glycosylation of proteins. To this end, a sample of *Rapi*Fluor-MS N-glycans derived from rhEPO was profiled using HILIC. A recently introduced widepore amide column, the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7µm column, was selected for this work to obtain high resolution N-glycan separations. This column was purposefully designed to facilitate HILIC separations of large molecules, such as glycopeptide and glycoproteins. However, the widepore particle architecture has also been shown to increase the peak capacity of highly branched, tri and tetra-antennary N-glycans by 10-20%, making it an ideal choice for the HILIC profiling of EPO N-glycans, which typically exhibit high antennarity. **Figures 2A and 2B** show the HILIC fluorescence and base peak intensity (BPI) MS chromatograms of the *Rapi*Fluor-MS N-glycans resulting from 0.4 µg of rhEPO. Even with this relatively limited amount of sample, high signal-to-noise chromatograms are obtained. The sensitivity of the fluorescence trace allows for accurate, relative quantitation across the profile. The signal-to-noise of the MS chromatogram is also particularly noteworthy, though it should be noted that MS sensitivity decreases as N-glycan structures become larger. Nevertheless, the quality of these particular data is made possible by use of the *Rapi*Fluor-MS reagent in combination with the Xevo G2-XS QTof, a new generation MS instrument with improved transmission efficiency and sensitivity. This QTof technology provides unprecedented sensitivity as well as high mass resolution, as can be observed in the collection of mass spectra in **Figure 2C** that have been used to support the assignment of various N-glycan species.

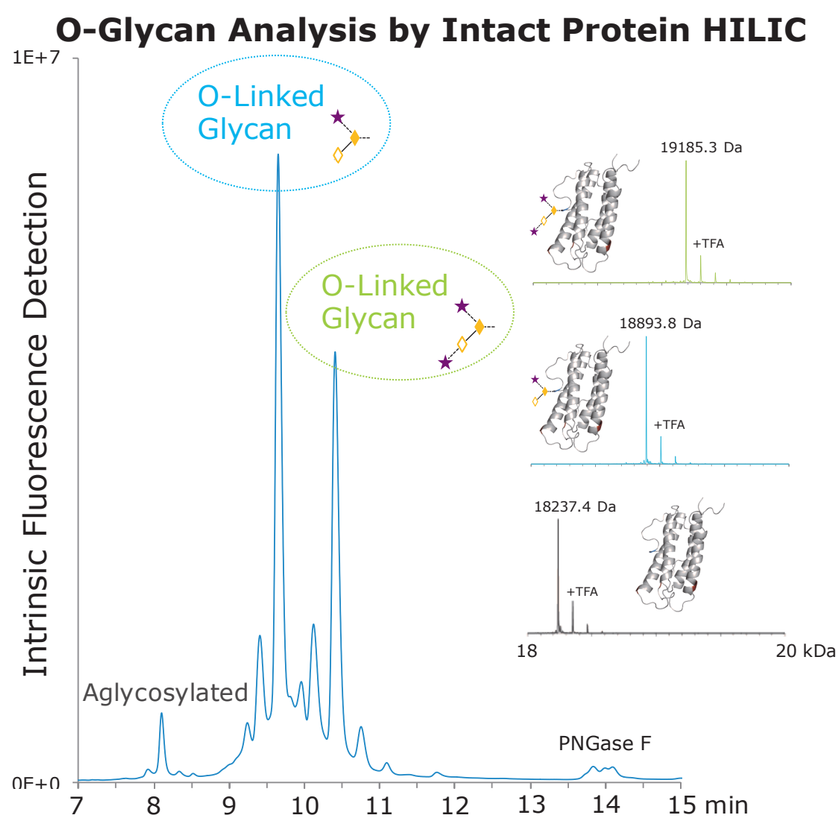

Figure 3. HILIC-Fluorescence-MS Analysis of N-Deglycosylated, Intact rhEPO. (A) Fluorescence chromatogram demonstrating O-linked glycan heterogeneity and occupancy. Chromatograms obtained from 0.7 µg protein. (B) Deconvoluted mass spectra corresponding to three of the major rhEPO proteoforms. Peak identifications, in addition to those denoted here, are tabulated in Figure 4.

Profiling the O-Glycosylation of Intact rhEPO using a Widepore Amide HILIC Separation

O-linked glycans can be challenging to characterize due to the paucity of high fidelity mechanisms to release them from their counterpart proteins. So, rather than attempt a released O-glycan analysis of rhEPO, we looked to develop an alternative characterization strategy. A novel workflow was devised that first involved subjecting the rhEPO to rapid deglycosylation using GlycoWorks Rapid PNGase F and 1% RapiGest SF surfactant. In a 10 minute preparation, a sample of N-deglycosylated intact rhEPO was obtained that could then be profiled via a HILIC separation with a Glycoprotein BEH Amide column. **Figure 3** presents the chromatogram obtained in this analysis using intrinsic fluorescence detection and intact protein HILIC techniques that have been described in previous work. The N-deglycosylated rhEPO analyzed in this study resolved into a series of approximately 10 peaks. Online ESI-MS provided highly detailed information, allowing for proteoforms of rhEPO to be assigned to the various chromatographic peaks. The two most abundant LC peaks were found to be represented by deconvoluted masses of 18893.8 and 19185.3 Da, which are consistent with N-deglycosylated rhEPO that has a C-terminal arginine truncation as well as trisaccharide and tetrasaccharide O-linked glycans modifications, respectively. More specifically, the mass shift observed for the lighter species is indicative of a glycan modification comprised of 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. Meanwhile, the mass shift observed for the heavier species suggests a glycan modification comprised of the same structure with an additional N-acetyl neuraminic acid. Further investigation of the LC-MS data also showed that the proteoform of rhEPO that is aglycosylated with respect to the O-linked glycan eluted with a retention time of approximately 8.2 min. Moreover, these LC-MS data indicated there to be at least two additional O-linked glycoforms and even more C-terminal truncation proteoforms (**Figure 4**).

Figure 4. LC-MS Data Supporting the Identification of Various N-Deglycosylated rhEPO Proteoforms. "–C-term" denotes the C-terminal truncation of the rhEPO; losses of different residues are noted. Hex, HexNAc, and Neu5Ac stand for hexose, n-acetylhexosamine, and N-acetylneuraminic acid. "+O" denotes a mass shift indicative of the addition of an oxygen. "+Ac" denotes an acetylation.

RT (min)	Species	$MW_{Avg, Theo}$ (Da)	$MW_{Avg, Obs}$ (Da)	Mass Error (Da)
8.0	N-deglycosylated, –C-term GDR	18066.5	18065.2	-1.3
	N-deglycosylated, –C-term DR	18123.6	18122.4	-1.2
8.2	**N-deglycosylated, –C-term R**	**18238.7**	**18237.4**	**-1.3**
9.3	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1+Ac**	**18937.3**	**18936.2**	**-1.1**
9.5	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac1	18723.1	18722.3	-0.8
	N-deglycosylated, –C-term DR +Hex1HexNAc1Neu5Ac1	18780.1	18779.1	-1.0
9.7	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1**	**18895.2**	**18893.8**	**-1.4**
9.9	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2+Ac**	**19228.5**	**19227.3**	**-1.2**
10.0	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1 + O**	**18911.2**	**18910.0**	**-1.2**
10.2	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac2	19014.3	19013.7	-0.6
10.5	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2**	**19186.5**	**19185.3**	**-1.2**
10.8	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2 + O**	**19202.5**	**19201.2**	**-1.3**

CONCLUSION

Using an ACQUITY UPLC Glycoprotein BEH Amide Column and *Rapi*Fluor-MS labeling, we have established a released glycan analysis for EPO that affords not only high resolution but also unprecedented sensitivity. Given that N-glycosylation correlates with the half life and activity of an EPO, such information, with its unparalleled quality, would be invaluable in developing a new EPO therapeutic.

EPO is also O-glycosylated; the occupancy and heterogeneity of which could also be critical to demonstrating comparability among different drug substances. Using the Glycoprotein BEH Amide Column, we have outlined a simple sample preparation and subsequent HILIC separation that is capable of profiling these O-glycan attributes on intact rhEPO.

In summary, we have demonstrated the use of two facile strategies that can be used to detail both the N- and O-linked glycosylation of recombinant, human epoetin (rhEPO), a molecule which has been perceived to be challenging to characterize due to its relatively complicated glycosylation.

References

1. Lauber, M. A.; Yu, Y. Q.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Magnelli, P.; Guthrie, E.; Taron, C. H.; Fountain, K. J., Anal Chem 2015, 87 (10), 5401-9.
2. Lauber, M. A.; Koza, S. M.; Chambers, E. E., Comprehensive Characterization of the N and O-Linked Glycosylation of a Recombinant Human EPO. Waters Application Note 720005462EN August 2015.

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS