APPLICATIONS

Quantitation of Neutral and Sialylated Glycans of Glycoproteins using bioZen™ HILIC Chromatography and Solid Phase Extraction

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Introduction

The glycosylation process is a Critical Quality Attribute (CQA) that must be determined for any therapeutic glycoprotein to ensure safety and mitigate problems. Many recombinant proteins, such as recombinant human erythropoietin (rhEPO), have highly intricate glycosylation patterns consisting of O-linked glycosylation as well as complex, branched N-linked glycosylation with three different N-linked glycosylation sites. The heterogeneity of N-linked glycosylation is mainly attributed to differences in sialylated glycans, which make approaches such as intact mass by high resolution mass spectrometry virtually impossible. This presents a challenge as the presence for sialic acid is critical in the characterization of a sialylated glycoprotein[1].

Simpler glycoproteins, like recombinant mAbs, have only two glycosylation sites on the Fc region with biantennary, neutral glycans which are consistent with the wild type IgG1 glycosylation patterns found in humans. However, depending on the expression system used, there may also be a low abundance of negatively charged sialylated glycans, which can affect target binding as well as decreased efficacy[2].

The established method of characterization of glycosylated glycoproteins uses PNGase F digestion followed by N-glycan labeling and relative quantitation by HPLC-UV or LC-MS/MS. Traditional sample preparation involves intensive manipulation and a loss of sample, but with a HILIC solid phase extraction (SPE) product, such as bioZen-N-Glycan Clean-Up microelution plates, the results are sensitive, effective, and save time. The hydrophilic interaction of HILIC SPE can be a concern and the sialylated glycans can especially be difficult to optimize by HILIC SPE since they are more polar than other fluorophore-labeled glycans. The glycan workflow is outlined in **Figure 1**.

In addition to sample preparation challenges, HILIC chromatography methods are targeted towards neutral biantennary glycans, with little selectivity for sialylated glycans. As such, it is difficult to quantitate both neutral biantennary and sialylated glycans in a single chromatographic run. bioZen Glycan LC Columns offer a unique selectivity to provide higher order separations of released and labeled glycans. Under HILIC-FLR or HILIC-MS conditions, the high efficiency particle of bioZen Glycan excels with increased polar retention and selectivity.

In this study, we look at fluorophore labeling and recoveries of N-linked glycans released serum-derived alpha-1 acid glycoprotein (AGP). This model protein is a suitable surrogate for a complex, highly sialylated glycoprotein with both neutral and sialylated glycans with multiple antennae.

Materials and Methods

Reagents/Chemicals

AGP derived from human plasma was obtained from Sigma Aldrich® (St. Louis, MO, USA). GlycoWorks™ *Rapi*Fluor™ Labeling Module was obtained from Waters® (Waltham, MA, USA).

Experimental Conditions

Glycoprotein sample (AGP) was reconstituted to 2 mg/mL pure water. 15 µg of each sample was denatured by adding 6 µL of surfactant and heating to 90 °C for 3 minutes. 1.2 µL of PNGase F was then added and samples were incubated for 5 min at 50 °C.

Labeling

Released glycans were labeled with 12 µL of labeling reagent solution per sample and incubated 5 minutes at room temperature.

Solid Phase Extaction (SPE)

Microelution 96-Well Plate:	bioZen N-Glycan Clean-up
Part No.:	8M-S009-NGA
Condition:	200 µL Water
Equilibrate:	200 µL Water/Acetonitrile (15:85)
Load:	Labeled pre-treated sample (~400 µL)
Wash:	2x 600 µL Formic acid/Water/Acetonitrile (1:9:90)
Elute:	3x 30 µL 200 mM Ammonium acetate in Acetonitrile/Water (5:95)
Dilute:	100 µL Dimethyl formamide then 210 µL Acetonitrile

HPLC Conditions

Column:	bioZen 2.6 µm Glycan
Dimensions:	150 x 2.1 mm
Part No.:	00F-4773-AN
Mobile Phase:	A: 250 mM Ammonium formate, pH 4.5 B: Acetonitrile

Gradient:

Time (min)	% B	Flow rate (mL/min)
0	70	0.4
38	50	0.4
41.5	0	0.2
44.5	0	0.2
48.1	70	0.2
52.6	70	0.4
60	70	0.4

Column Temperature:	60 °C
LC System:	ACQUITY® UPLC® H-Class Bio

Figure 1.
Glycan Workflow

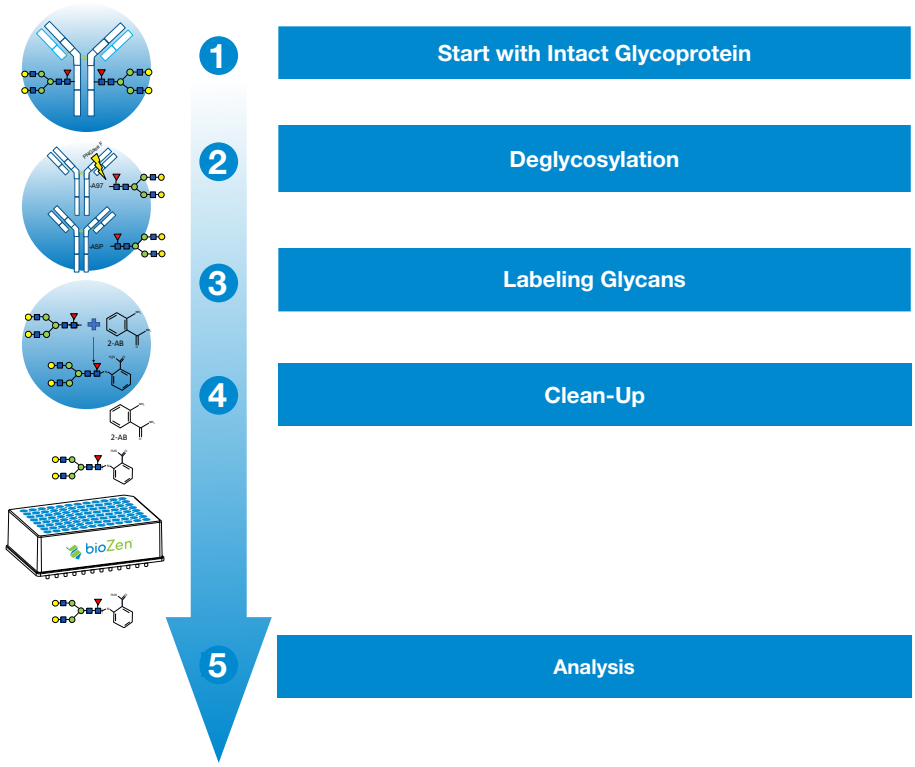

Figure 2.
Labeled Glycans from AGP

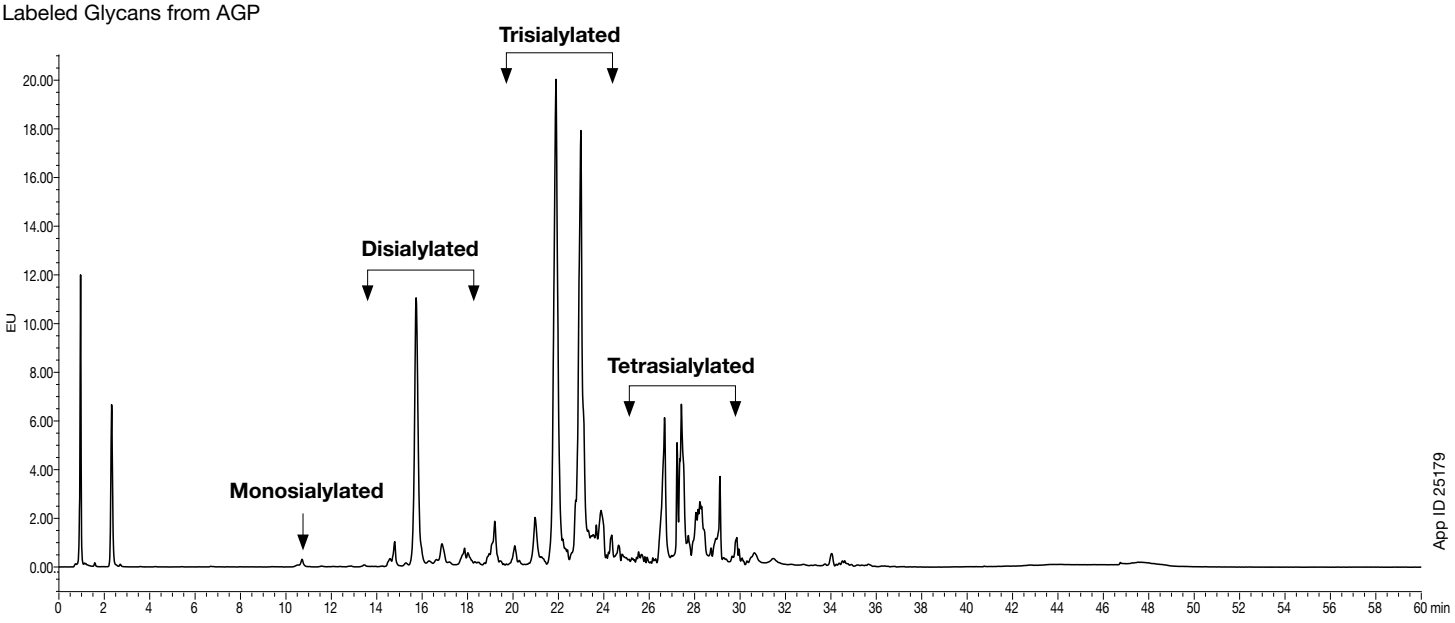

Table 1.
Sum of Peak Areas for Z-value Calculation

Glycan	Sum of Peak Area (%)	Multiplication Factor	Charge Number Share
Asialo	0.28	0	0
Monosialo-	13.41	1	13.41
Disialo-	4.44	2	8.88
Trisialo-	52.49	3	157.47
Tetrasialo-	29.4	4	117.6
Total peak area	**100%**		**Z-value= 297.36**

Results and Discussion

Although most glycoproteins analyzed are predominantly sialylated, the contribution and characterization of neutral biantennary glycans is critical, as the relative amount of sialylation can potentially impact pharmacokinetics and immunogenicity. Here we see good retention and separation for biantennary neutral glycans, allowing for more accurate relative quantitation.

Separation and identification of sialylated glycans is challenging, as they can have up to four antennae with four sialic acids. The common concentration of 50 mM Ammonium formate in mobile phase A (i.e. the strong solvent in HILIC) had to be increased up to 250 mM to obtain optimal chromatography. After gradient adjustments and mobile phase changes, the glycans are partitioned into neutral, mono-, di-, tetra-, and sialylated species.

For sialylated glycans, instead of peak areas, Z-values were used for comparison. Z-value takes a weighted average of contributing charge shares for different levels of sialylation and could reveal a bias or difference in recovery of different forms of sialylated glycans. This separation gives easier assignment to Z-values. The AGP sample shown here has an average Z-value of 297, which is relatively close to the value reported by Hemertin et al[3]. The variation that is seen in wild type AGP could account for any discrepancy in reported value. This indicates good recovery of sialylated glycans during sample preparation, with minimal amount of during N-linked glycan release, labeling and clean-up.

Conclusion

The bioZen™ N-Glycan Clean-Up and bioZen Glycan UHPLC column are effective for the analysis of fluorescently labeled N-linked glycans. This analysis is particularly effective for sialylated glycans, for which both sample clean-up after labeling and HPLC analysis can be challenging.

References

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2. Reusch D, Tejada ML. Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology. 2015;25:1325–1334
3. Hermentin, Peter, et al. “The Hypothetical N-Glycan Charge: a Number That Characterizes Protein Glycosylation.” Glycobiology, vol. 6, no. 2, 1996, pp. 217–230., doi:10.1093/glycob/6.2.217.

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Ordering Information

Sample Preparation

bioZen™ Solid Phase Extraction	Format	Sorbent Mass	Part Number	Unit
bioZen N-Glycan Clean-Up	Microelution 96-Well Plate	5 mg/well	8M-S009-NGA	1/box

LC Columns

bioZen Columns (mm)				Biocompatible Guard Cartridges	
Phase	50 x 2.1	100 x 2.1	150 x 2.1	for 2.1 mm	Holder
bioZen 2.6 µm Glycan	00B-4773-AN	00D-4773-AN	00F-4773-AN	AJ0-9800	AJ0-9000

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