ANALYTICAL SOLUTION FOR *N*-LINKED GLYCANS OF GLYCOPROTEINS: RESOLUTION AND QUANTITATION

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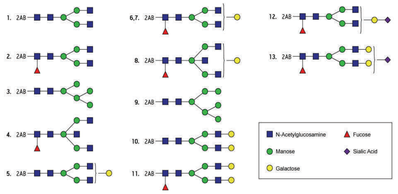
INTRODUCTION

Glycosylation is a post-translational modification of proteins. The sugar chains on monoclonal antibodies, IgGs, can mediate biological activity, and are therefore associated with the safety and efficacy attributes of many biopharmaceuticals. The relative amounts of the individual glycan structures must be monitored at all stages of research and development.

We have developed a glycan analysis solution for these applications. *N*-linked glycans are released from IgGs and are labeled with 2-aminobenzamide (2-AB). The derivatized oligosaccharides are separated by HydrophILic Interaction Chromatography (HILIC) using a 1.7 micron particle UPLC column. A fluorescence detector with a low volume flow cell is specific for the 2-AB label, and enhances sensitivity beyond the predicted improvement from narrow peaks. The instrument used in this study has a low dwell volume and the capability of operating at high pressure (up to 15,000 psi), thus it provides the operating characteristics to realize the resolution, sensitivity and speed benefits of the 1.7 micron particle packing material.

Fluorescent labelling is an important element of this sensitive assay. To obtain maximum sensitivity, excess reagent needs to be removed prior to UPLC. We have used a HILIC mode SPE separation to remove excess 2-aminobenzamide (2-AB), and optimized the MassPREP™ HILIC µElution Plate protocol for the IgG glycans shown below.



METHODS

Labeling Glycans with 2-AB

Released, dry glycans were derivatized with the fluorescent tag 2-aminobenzamide. The 2-AB reagent and sodium cyanoborohydride were dissolved in DMSO/Acetic acid.[1] After heating for two hours, the labeled glycans and excess reagent were diluted 10x with acetonitrile. The acetonitrile solution can then be loaded onto the HILIC µElution Plate. Up to 750 µL or 100 µg can be loaded into each of the 96 wells.

Condition
200 µL Milli-Q Water
↓
Equilibrate
2 x 200 µL 90% Acetonitrile
↓
Slowly **Load** Sample
constituted in 90% Acetonitrile
Volume: 150 µL
↓
Wash
2 x 200 µL 90% Acetonitrile
↓
Elute
2 x 50 µL
10 mM Ammonium Tris-citrate*
↓
Inject 2-AB labeled glycans directly

* Contains up to 25% Acetonitrile

MassPrep™
HILIC µElution Plate

Chromatographic Separation Conditions

Instrument: ACQUITY UPLC pumps, injector and FLR.

Column: ACQUITY UPLC BEH Glycan Separation Technology 1.7µm, 2.1 x 150 mm
Eluent A: 100 mM Ammonium Formate, pH 4.5
Eluent B: Acetonitrile
Temperature: 60 °C
Fluorescence: λex = 330 nm, λem = 420 nm
Sample amount: 15 pmol
UPLC Gradient:

Time (min)	Flow rate (mL/min)	% A	% B
Init	0.5	25	75
46.5	0.5	40	60
48	0.25*	100	0
49	0.25*	100	0
50	0.5	25	75
63	0.5	25	75

*Flow rate lowered during aqueous regeneration

RESULTS AND DISCUSSION

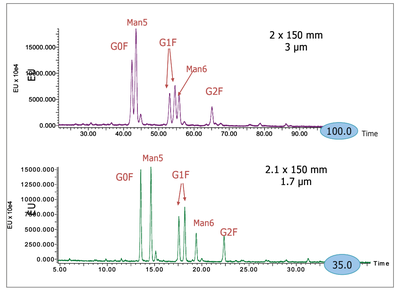


Figure 1. Improvements in separation were realized by the introduction of the Glycan Separation Technology column with 1.7 µm particles. Gains were made including higher resolution, shorter analysis time, and selectivity specific to the separation of G0F and Man5.

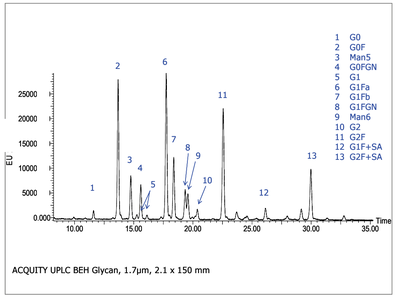


Figure 2. The separation of labeled glycans released from human IgG is the most common method for the structural characterization of the glycoprotein biopharmaceuticals. This example includes both neutral and charged oligosaccharides in a range of chain lengths as well as positional and branching isomers. Note that Man5 and Man6 are present in this example, although not normal components of human IgG glycans.

HILIC mode chromatography utilizes aqueous/acetonitrile gradients from low to high water content. Oligosaccharide separations in HILIC mode (sometimes referred to as normal phase or NP) take advantage of the selectivity provided by the hydrophilic amide stationary phase. The sub-2 micron particle size improves resolution and decreases run time. Separation of glycans from a murine IgG1, shown in Figure 1, demonstrate these improvements. The more complicated mixture of oligosaccharides from human IgGs, shown in Figure 2, contains sialylated, complex, and bisecting GlcNAc structures. High mannose structures were also present in this mixture.

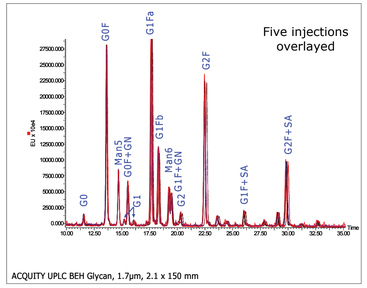


Figure 3. Reproducibility over 20 injections was demonstrated with the Prozyme Human IgG glycans (included Man5 and Man 6). The chromatograms for injections numbered 2, 7, 10, 15, and 20 were overlayed

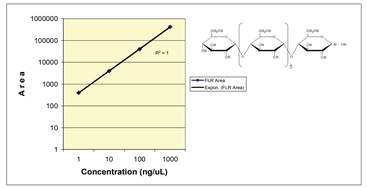


Figure 4. Fluorescence Detection was shown to have a linear response over three orders of magnitude for 2-AB labeled maltoheptaose, including HILIC mode SPE and UPLC-FLR.

Reproducibility of the resolution by Glycan Separation Technology Column and the ACQUITY UPLC Binary Solvent Manager is shown in Figure 3. The 2-AB labeled human IgG glycans were injected over more than 24 hours. The overlay of five fluorescence chromatograms demonstrates the reproducibility of the glycan separation profile.

Using the neutral oligosaccharide 2-AB maltoheptaose, linearity of response was shown to be excellent over three orders of magnitude in the exponential plot, Figure 4. Purification of the 2-AB maltoheptaose by HILIC microelution was also linear.

To extend this microelution method, recovery studies were performed on a mixture of three 2-AB labeled glycans; one high mannose, one complex and one sialylated. See Figures 5 and 6. The glycans were loaded onto the microelution plate in the presence of excess 2-AB labeling reagent. Further SPE studies with a more complex mixture (Figure 7) demonstrate reproducibility. The area for each of six glycans is reported as a percent relative to the total of area counts. The variance due to SPE recovery is decreased in relative percent.

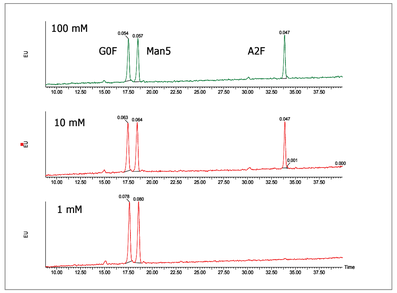


Figure 5. UPLC-FLR chromatograms of 2-AB labeled oligosaccharides, G0F, Man5, and A2F recovered from a MassPREP™ HILIC µElution plate with varying concentrations of ammonium Tris-citrate. Chromatograms shown from the HILIC mode amide-based separation with 1.7 µm partcles.

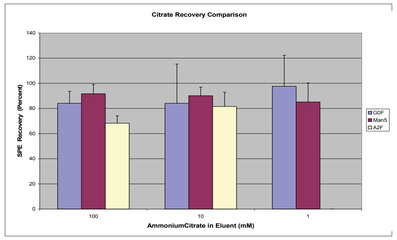


Figure 6. Recovery of 2-AB labeled glycans from a MassPREP™ HILIC µElution plate with varying concentrations of ammonium Tris-citrate.

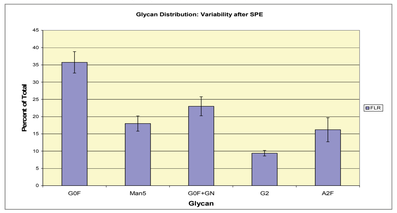


Figure 7. Reproducibility of the fluorescence area percent from a mixture of 2-AB labeled standards after purification by SPE. The values are the percent of the total for these glycans. Six replicate SPE purifications were averaged and the standard deviation is shown. These glycans are typical of N-linked human IgG oligosaccharide structures.

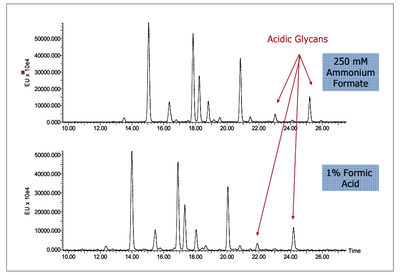


Figure 8. Sialylated, acidic, oligosaccharides can be strongly retained on the Glycan Separation Technology column. High ionic strength, 100 mM or 250 mM ammonium formate at pH 4.5 yielded elution of mono to tetra-sialo glycans after neutral oligosaccharides (data not shown). Increasing the ionic strength of an alternative formic acid buffer to the 250mM range, 1%, also provides excellent retention of sialylated glycans for quantitation. For assays requirement LC/MS both mobile phases provide molecular weight confirmation of monosaccharide.

Mass spectrometric confirmation of glycan MWs is often required. Since high ionic strength is required to retain sialylated glycans, 1% formic acid was compared to 250 mM ammonium formate.

CONCLUSIONS

- **Glycan Separation Technology UPLC columns provide excellent resolution of mouse and human IgG oligosaccharides.**
- **High sensitivity and linearity over 3 orders of magnitude result from the low dispersion instrument and fluorescence flow cell.**
- **The relatively short analysis time increases the throughput of glycan chromatography.**
- **10 mM ammonium citrate is required for elution of the sialylated biantennary structures found on IgGs.**
- **SPE purification by HILIC mode µElution of labeled glycans yields consistent patterns of glycoform distributions.**
- **The combination of SPE sample preparation, the low dispersion instrument, and superior column technology provide a robust complete solution for the quantitative requirements for biopharmaceuticals.**

References

1. Bigge J. C.; Patel T. P.; Bruce J. A.; Goulding P. N.; Charles S. M.; Parekh R. B. *Anal. Biochem.* **1995**, 230, 229–238.

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