LC-MS Analysis of Highly Sialylated Glycans Using MS Compatible Mixed Mode Chromatographic Separation

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Introduction

- The abundance and structure of highly sialylated glycans can factor into the clearance rate and in-vivo activity of glycosylated biotherapeutics.
- It is challenging to analyze highly sialylated glycans using conventional HILIC/FLR/MS methods.
- We applied a mixed mode (RP/AX) chromatographic separation to improve the chromatographic performance and detection of Fluorescently tagged highly sialylated N-glycans.
- Data reported here were generated from an integrated bench top LC-FLR-MS to characterize N-glycans released from rEPO.

Figure 1. BioAccord™ System with ACQUITY™ Premier System, and the ACQUITY Premier Glycan BEH™ C18AX Column, standards and mobile phase concentrates.

Experimental

Sample

Erythropoietin (EPO) was purchased from European Pharmacopeia (reference material E1515000) and buffer exchanged to Milli-Q™ water prior to glycan release. Additional EPO samples were purchased from StemCell Inc. and reconstituted to a concentration at 1.5 mg/mL (PN 78007). GlycoWorks™ RFMS Kit was used for sample preparations.

System:	BioAccord System with ACQUITY Premier with ACQUITY Premier System
Detection:	ACQUITY Premier FLR Detector (λ_{ex}=265nm, λ_{em}=425nm, 2Hz)
Column(s):	Mixed mode: ACQUITY Premier Glycan BEH™ C18 AX Column, 1.7µm, 2.1×150mm (P/N 186009760) HILIC mode: ACQUITY Premier Glycan Amide Column, 1.7µm, 2.1×150mm (P/N 186009524)
Vials:	QuanRecovery™ w/ MaxPeak™ HPS (P/N 186009186)
Column Temp:	60 °C
Sample Temp.	6 °C
Injection volume:	1 µL
Mobile Phase A:	Mixed mode: Milli-Q H_2O HILIC mode: Milli-Q H_2O, 50mM NH_4HCO_2
Mobile Phase B:	Mixed mode: 100 mM NH_4HCO_2 100 mM formic acid in 40/60 v/v water/acetonitrile HILIC mode: MS-grade acetonitrile

LC Gradient

Mixed mode

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.4	95	5	6
40.00	0.4	77	23	6
40.30	0.4	0	100	6
41.30	0.4	0	100	6
42.00	0.4	95	5	6
49.00	0.4	95	5	6

HILIC mode

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.4	25	75	6
35.00	0.4	46	54	6
36.50	0.2	80	20	6
39.50	0.2	80	20	6
43.10	0.2	25	75	6
47.60	0.4	25	75	6
55.00	0.4	25	75	6

MS settings and Informatics

MS System:	ACQUITY RDa Mass Detector (Figure 1)
Ionization Mode:	ESI Positive
Acquisition Range:	50 - 2,000 m/z
Capillary Voltage:	1.5 kV
Cone Voltage (CV):	45 V
Fragmentation CV:	70 - 90 V

Data Management:
waters_connect™ with UNIFI™ 1.9.4 Software

Results (UPLC-FLR-MS using mixed mode chromatography, RP/AX)

Selectivity (RP/AX vs. HILIC)

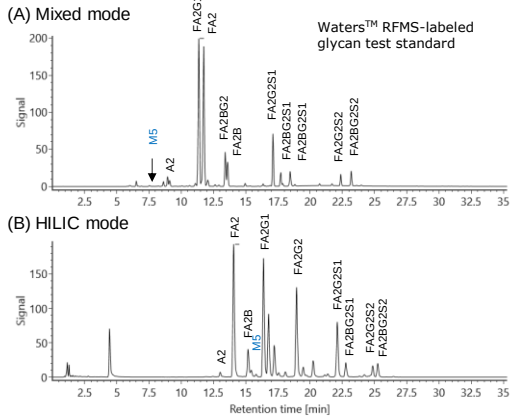

Figure 2. Selectivity differences when comparing (A) mixed mode (RP/AX) separation and (B) HILIC mode separation. Both separations show that Man 5 (M5) is more hydrophilic than the A2 (or G0) glycan.

Charge Driven Separation

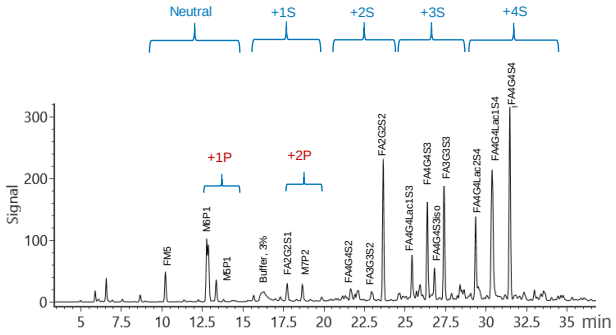

Figure 4A. RP/AX separation of EPO N-glycans. Distinct charge-based separation with hydrophobic separation within each charge grouping. Note that high mannose phosphate glycans have earlier elution time compared to complex type glycans of equivalent charge.

Fragment Ions Provides Additional Structure Information

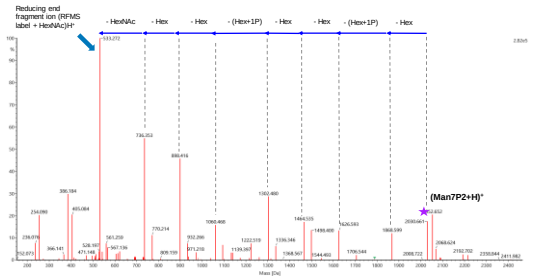

Figure 4B. The BioAccord System can alternately generate spectra of low energy (precursor) ions and higher energy (fragment) ions. The MaxEnt 3 charge deconvoluted spectrum is shown for fragmentation of the high-mannose doubly phosphorylated M7P2 glycan (★=$[M+H]^+$). Consecutive neutral losses from monosaccharides are annotated to confirm the glycan composition.

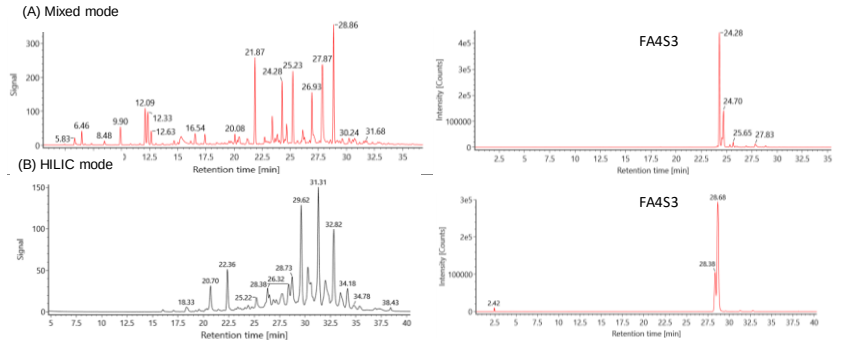

Figure 3. Comparison of EPO released glycan analyses using (A) HILIC mode and (B) mixed mode separation. The charge-based mixed mode separation provides higher resolution (~20% in peak width reduction) for isomeric sialylated glycans. One example is the separation of fucosylated tetra-antennary glycans bearing 3 sialic acids, FA4S3.

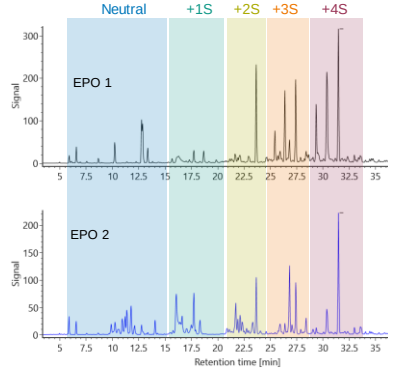

Figure 5. Optimized RP/AX LC method for the analysis of EPO from European Pharmacopeia and StemCell Inc.

Conclusions

- An LC-FLR-MS method was optimized on the BioAccord System with ACQUITY Premier LC System fitted with an ACQUITY Premier Glycan C18 AX Column.
- The new mixed-mode C18 AX column offered more resolving power for complex N-glycans with higher levels of sialic acid (~20%).
- The optimized LC-FLR-MS method enabled distinct charge-based separation and compositional assignments based on the number of sialic acids and mass data from the BioAccord System.