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HPAE-PAD Analysis of *N*-linked Oligosaccharides from Glycoproteins Using Dual Eluent Generation Cartridge Mode

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ABSTRACT

Purpose: To demonstrate Dual Eluent Generation Cartridge (EGC) capability and performance for the analysis of *N*-linked oligosaccharides released from glycoproteins.

Methods: This study uses Thermo Scientific™ Dionex™ EGC 400 MSA and Thermo Scientific™ Dionex™ EGC 400 KOH Eluent Generator Cartridges with a Thermo Scientific™ Dionex™ CarboPac™ PA200 column set in the 1 mm format on a Thermo Scientific™ Dionex™ ICS-6000 HPIC™ system to separate *N*-linked oligosaccharides released from glycoproteins.

Results: This method delivers similar resolution of uncharged IgG oligosaccharides (glycans) and a commercially available mix of bovine fetuin *N*-linked oligosaccharide alditols compared with traditional HPAE-PAD separations using sodium hydroxide/sodium acetate eluents, but simplifies operation and improves the precision and accuracy while requiring no eluent preparation. This method for oligosaccharide analysis requires no sample derivatization, and is orthogonal to CE and HILIC methods.

INTRODUCTION

Understanding and characterizing protein therapeutic glycosylation is important with growing evidence that glycosylation impacts biological efficacy, pharmacokinetics, and cellular toxicity.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is an effective tool for determining glycans present in glycoprotein therapeutics. In the pharmaceutical and biotechnology industries, HPAE-PAD mapping techniques for glycoprotein carbohydrate structures have been used: 1) during initial characterization to separate and identify the oligosaccharide structures present; 2) to monitor consistency of glycosylation and identify changes that may have resulted from alterations in cell culture conditions or during the manufacturing process; and 3) to monitor changes in glycosylation that occur as a result of expression in different cell lines.[1]

A new operating mode for HPAE-PAD systems, Dual Eluent Generation Cartridge (Dual EGC) mode, is available on the Dionex ICS-6000 HPIC system to support the analysis of complex carbohydrates (Figure 1). This mode of operation replaces the manual preparation of the sodium hydroxide/sodium acetate eluents required for analyzing complex carbohydrates by HPAE-PAD with electrolytic generation of potassium hydroxide (KOH)/potassium methanesulfonate acid (KMSA) eluent using MSA and KOH eluent generators installed in series (Figure 2). This enables analysts to run gradient methods for oligosaccharide separations using an isocratic pump and ensures excellent reproducibility and accuracy in eluent preparation.

Figure 1. Overview of Dionex ICS-6000 HPIC System Dual EGC Mode, 1mm column.

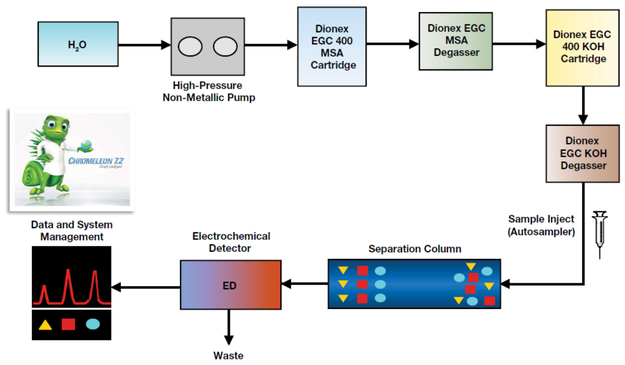

Figure 2. Ion chromatography system Dual EGC mode workflow.

MATERIALS AND METHODS

Sample Preparation

IgG oligosaccharides from human serum were prepared by with PNGase F followed. Typical digestions incubated 1 mg of protein with 4000 units of PNGase F at 37 ºC for 20 h in 0.5 mL of solution. Samples were directly injected after PNGase F digestion. Fetuin oligosaccharide alditol standards were purchased from Thermo Fisher Scientific.

Test Method(s)

Conditions:		
IC System:	Thermo Scientific Dionex ICS-6000 HPIC system (analytical format)	
Tubing:	Dionex IC PEEK Viper Fittings Kit, Dionex ICS-6000 Capillary system with Electrochemical Detector (ED) (P/N 088802)	
Columns:	Thermo Scientific Dionex CarboPac PA200 guard column (1×50 mm, P/N 302862) Thermo Scientific Dionex CarboPac PA200 analytical column (1×250 mm, P/N 302861)	
Eluent Source:	Thermo Scientific Dionex EGC 400 MSA Eluent Generator Cartridge in series with Thermo Scientific Dionex EGC 400 KOH Eluent Generator Cartridge	
Eluent:	Potassium Methanesulfonate/Potassium Hydroxide (KMSA/KOH)	
Gradient:	For IgG oligosaccharides analysis: -5 min: Equilibration 0-25 min: 0.3-1 mM KMSA in 65 mM KOH 25-44 min: 1- 28 mM KMSA in 65-90 mM KOH 44-46 min:28-42 mM KMSA in 90 mM KOH 46-55 min: 42 mM KMSA in 90 mM KOH 55-60 min: 100 mM KMSA in 100 mM KOH 60-75 min: 0.3 mM KMSA in 65 mM KOH	For Fetuin oligosaccharide alditol analysis: 0-50 min: 15-64 mM KMSA in 136 mM KOH 50-60 min: 80 mM KMSA in 90 mM KOH 60-65 min:100 mM KMSA in 100 mM KOH 65-80 min: 15 mM KMSA in 136 mM KOH
Flow Rate:	0.063 mL/min	
Injection Volume:	0.4 µL (full loop)	
Temperature:	30 ºC (column and detector compartments)	
Backpressure:	~3310 psi	
Detection:	Pulsed amperometric, Gold on PTFE Disposable Working Electrode, Ag/AgCl reference, 1 mil gasket	
Background:	~30 nC	
Noise:	60 - 80 pC	

Carbohydrate 4-Potential Waveform for the ED

Time (s)	Potential (V)	Gain Region	Ramp	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

Data Analysis

Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2.8.

RESULTS

Separation of human IgG *N*-linked oligosaccharides

Figure 3 shows separation of human IgG oligosaccharides with traditional HPAE-PAD separations using sodium hydroxide/sodium acetate eluents.[2] Figure 4 shows the majority of the separation of IgG oligosaccharides are with a multi-step gradient of electrolytically generated potassium hydroxide (KOH)/potassium methanesulfonate acid (KMSA). Both neutral and negatively charged IgG oligosaccharides are eluted and separated using KOH/KMSA eluents. Neu5Ac presence can be confirmed by neuraminidase treatment showing disappearance of the putative Neu5Ac-containing oligosaccharide and the appearance of uncharged oligosaccharides as well as a Neu5Ac peak. (Figure 4B). In the methods described here, Neu5Ac elutes between G0 and G1F(1,6).

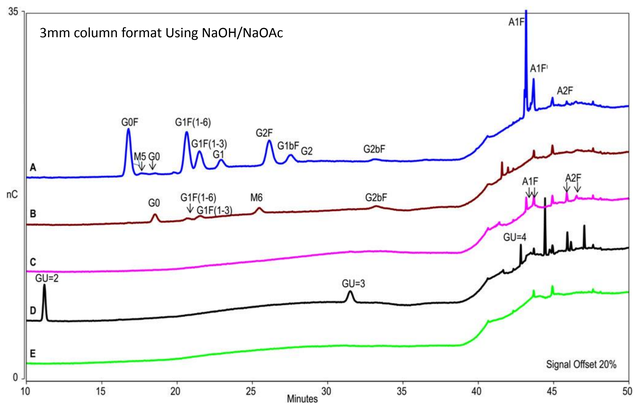

Figure 3. Separation of human IgG *N*-linked oligosaccharides with comparison to neutral and charged *N*-linked oligosaccharide standards and retention index (RI) standards on 3 mm Dionex CarboPac PA200 column using NaOH/NaOAc. Chromatograms: (A) PNGase F digest of human IgG (5 ng protein); (B) mix of oligosaccharide standards (neutral, 5 ng each); (C) mix of oligosaccharide standards (charged, 5 ng each); (D) mix of RI standards (5 ng each) and (E) DI water.

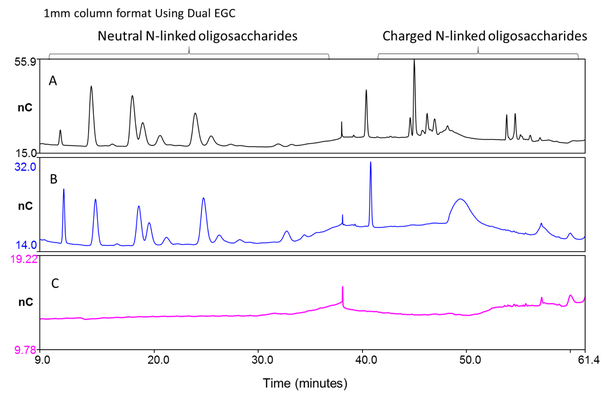

Figure 4. Separation of *N*-linked oligosaccharides released from human IgG before and after neuraminidase digestion on 1 mm Dionex CarboPac PA200 column using Dual EGC. Chromatograms: (A) IgG oligo-saccharides before neuraminidase digestion (500 ng protein); (B) IgG oligosaccharides after neuraminidase digestion (500 ng protein); (C) DI water.

Accuracy and Precision of human IgG *N*-linked oligosaccharides

Retention time and peak area stability for the human IgG oligosaccharide separation shown in Figure 5 were assessed by determining retention time and peak area of three consecutive injections. The results showed good accuracy and precision were achieved in Dual EGC Mode with a 1 mm Dionex CarboPac PA200 column.

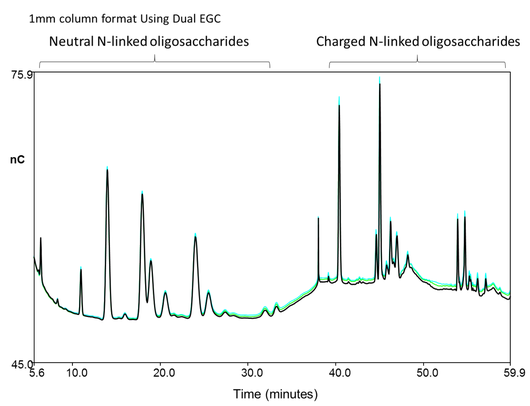

Figure 5. Overlaid chromatograms of three consecutive injections of *N*-linked oligosaccharides released from human IgG.

Separation of Fetuin Oligosaccharides

The separation of bovine fetuin oligosaccharide alditols by HPAE-PAD is shown in Figure 6. The Dual EGC technique resolves these sialylated *N*-glycans from bovine fetuin. It provides performance comparable to traditional HPAE-PAD separations using sodium hydroxide/sodium acetate eluents. (Figure 6).

Columns: Dionex CarboPac PA200, 3 mm (guard + separator)
Dionex CarboPac PA200, 1 mm (guard + separator)

Gradient: Dionex CarboPac PA200, 3 × 250 mm
0–60 min: 20–150 mM NaOAc in 100 mM NaOH
60–65 min: 500 mM NaOAc in 100 mM NaOH
65–80 min 20 mM NaOAc in 100 mM NaOH

Dionex CarboPac PA200, 1 × 250 mm
0–50 min: 15–64 mM KMSA in 136 mM KOH
50–60 min: 80 mM KMSA in 90 mM KOH
60–65 min: 100 mM KMSA in 100 mM KOH
65–80 min: 15 mM KMSA in 136 mM KOH

Flow rate: Dionex CarboPac PA200, 3 × 250 mm: 0.5 mL/min
Dionex CarboPac PA200, 1 × 250 mm: 0.063 mL/min

Detection: Dionex CarboPac PA200, 3 × 250 mm: PAD, Au on PTFE, 2 mil gasket, Ag/AgCl ref.
Dionex CarboPac PA200, 1 × 250 mm: PAD, Au on PTFE, 1 mil gasket, Ag/AgCl ref.

Samples: 50 µmol/L fetuin oligosaccharide alditol standard

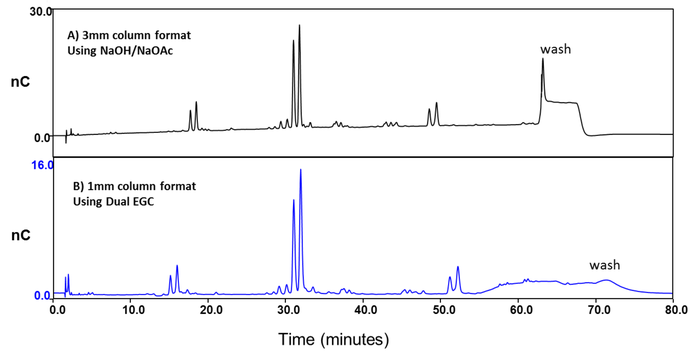

Figure 6. Separation of fetuin *N*-linked oligosaccharide alditols using A) sodium hydroxide/sodium acetate eluents on a 3 mm Dionex CarboPac PA200 column B) KOH/KMSA eluents generated by Dual EGC on 1 mm Dionex CarboPac PA200 column.

Accuracy and Precision of Fetuin Oligosaccharides

Retention time and peak area stability for the bovin fetuin oligosaccharide separation shown in Figure 7 were assessed by determining retention time and peak area of three consecutive injections. The results showed good accuracy and precision were achieved by Dual EGC Mode on a 1 mm Dionex CarboPac PA200 column.

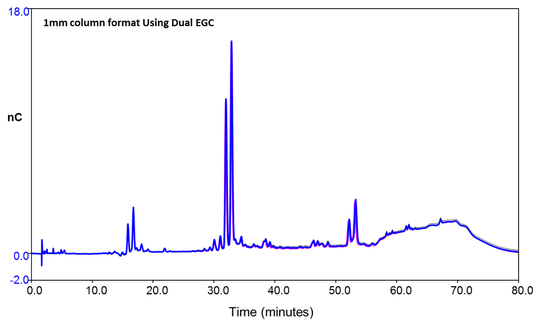

Figure 7. Overlaid chromatograms of three consecutive injections of fetuin oligosaccharide alditols.

CONCLUSIONS

Our results demonstrate the Dual EGC mode achieved “expected performance” for the HPAE-PAD analysis of glycoprotein oligosaccharides and can be used by investigators who need to validate HPAE-PAD oligosaccharide mapping methods. This mode of operation for HPAE-PAD:

- Eliminates Manual Eluent Preparations.
- Improves IC reproducibility.
- Maximizes instrument uptime, minimizing maintenance.
- Eliminates issues with sodium acetate purity.

This oligosaccharide method simplifies operation and improves the precision and accuracy while requiring no eluent preparation, no sample derivatization, and is orthogonal to CE and HILIC methods.

Note: See Thermo Scientific Application Note 72714 for more details on Dual EGC mode.[3]

REFERENCES

1. Thermo Scientific Technical Note 42: Glycoprotein Oligosaccharide Analysis Using High-Performance Anion-Exchange Chromatography. Sunnyvale, CA. 1997.
2. Rohrer, J.S., Basumallick, L., and Hurum, D. C., Profiling *N*-linked oligosaccharides from IgG by high-performance anion-exchange chromatography with pulsed amperometric detection. *Glycobiology*. 2016 Jun; 582-91.
3. Thermo Scientific Application Note 72714 : HPAE-PAD analysis of galactosyl-oligosaccharide containing samples using dual eluent generation cartridges. Sunnyvale, CA. 2018.

TRADEMARKS/LICENSING

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