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Technical Note 42

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Glycoprotein Oligosaccharide Analysis Using High-Performance Anion-Exchange Chromatography

INTRODUCTION

The development of recombinant-derived glycoproteins for therapeutic use has led to an increasing demand for methods to characterize their carbohydrate structures. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) has been widely used to characterize carbohydrates because the method allows rapid and direct quantification of underivatized samples. HPAE-PAD not only separates oligosaccharides according to charge, but can also resolve oligosaccharides with the same charge according to size, sugar composition, and linkage of monosaccharide units.

Oligosaccharides can be characterized through coelution with standards or through retention time comparison. Empirical relationships between oligosaccharide structure and chromatographic retention for HPAE-PAD have been described.[1] Oligosaccharides separated by HPAE-PAD are frequently collected postcolumn for further structural analysis using mass spectrometry and NMR.

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. A short list of articles in which HPAE-PAD is used for mapping therapeutic glycoproteins is provided in the *Appendix* section.

In this Technical Note, accuracy, precision, linearity, and limits of detection are documented for a commercially available mix of bovine fetuin *N*-linked oligosaccharide alditols using an HPAE-PAD method currently in use for quality control at a biotechnology company. These results are intended as a guide for investigators who need to validate the performance of HPAE-PAD for oligosaccharide mapping.

EQUIPMENT

Dionex DX-500 BioLC® system consisting of:

LC30 Chromatography Module
GP40 Gradient Pump with On-line Degas
ED40 Electrochemical Detector, gold electrode
AS3500 Autosampler or an 8880 Autosampler

PeakNet Chromatography Workstation

GP40 pump performance can be validated using the GP40 Validation Kit (Dionex P/N 50809), while ED40 detector performance can be validated with the ED40 Validation Kit (P/N 49046).

The system should be properly installed according to relevant operating manuals for each component. The accuracy and precision of the autosampler is validated using a 100 µL loop and injecting fourfold 5, 10, 15, 25, 50 µL of a solution of 10 µM glucose onto a CarboPac™ PA10 or PA-100 column followed by elution with isocratic 100 mM sodium hydroxide at 1.5 mL/minute. The coefficient of determination (r^2) generated from a linear regression analysis of a plot of peak area versus volume should indicate linearity ($r^2 > 0.99$). The CarboPac PA-100 performance should be validated by reproducing the test chromatograms provided with the column.

REAGENTS AND STANDARDS

Deionized water, HPLC-grade

Sodium Hydroxide, 50% (w/w) (Fisher Scientific)

Fetuin Oligosaccharide Alditol Standards (Dionex, P/N 43064)

Asialo- and Disialylated Galactosylated Biantennary Oligosaccharide Standards (Oxford GlycoSciences)

CONDITIONS

Column:	CarboPac™ PA-100 Analytical Column (4 x 250 mm) with guard
Flow rate:	1.0 mL/min
Detection:	ED40 Electrochemical Detector, gold electrode
Inj. Vol.:	10 µL
Waveform:	Carbohydrate waveform (reference Dionex Technical Note 21 for most current waveform)
Eluent:	A: 100 mM Sodium hydroxide
	B: 100 mM Sodium hydroxide/0.5 M Sodium acetate

Method:

Time (min)	% A	% B
Initial	99	1
0.0	99	1
0.2	99	1
10.0	90	10
50.0	55	45
50.1	0	100
55.0	0	100
55.1	99	1
70.0	99	1

In this separation method, the initial conditions include 1% eluent B to prevent a baseline disturbance that occasionally occurs when the column is converted from the hydroxide to the acetate form. This method was developed in a quality control laboratory at a biotechnology company. We believe this method will be more reproducible if eluent A is changed from 100 mM NaOH to 100 mM NaOH/5 mM NaOAc and all method times that use 99% A and 1% B are changed to 100% A.

A used, gold electrode that had not been recently polished was used for the experiments in this Technical Note.

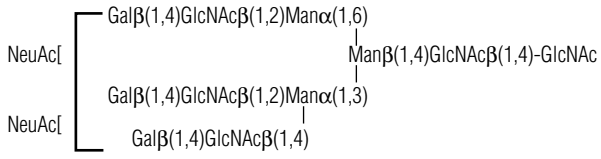
Gradient Optimization

The gradient shown above efficiently separates sialylated fetuin oligosaccharide alditols. Because HPAE separations are by ion exchange, the method is very flexible and gradients should be optimized for the particular separation. The development of two "optimized" gradients, one for the separation of sialylated *N*-glycans and a second gradient for the separation of asialo *N*-glycans, has been reported.[2] For the separation of neutral *N*-linked oligosaccharides, an increase in eluent pH has been reported to improve the separation.[3]

HPAE-PAD commonly uses strongly alkaline eluents. However, there are occasions when neutral or low pH eluents are preferable. For example, low pH eluents provide better resolution of sialylated and phosphorylated oligosaccharides.[4] Use of low pH eluents necessitates the addition of postcolumn base for detection by pulsed amperometry. It has been reported that high pH conditions are required for the optimum separation of fetuin oligosaccharides, while low pH significantly improves resolution of oligosaccharides obtained from orosomucoid, human chorionic gonadotropin, platelet-derived growth factor, and kallikrein.[5] A gradient in which only 0.5 mM sodium hydroxide and 3% sodium acetate (3% acetic acid titrated

Table 1 Major carbohydrate structures of bovine fetuin

1.



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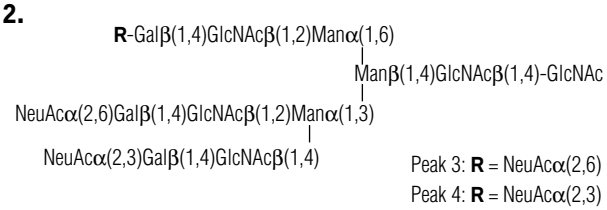

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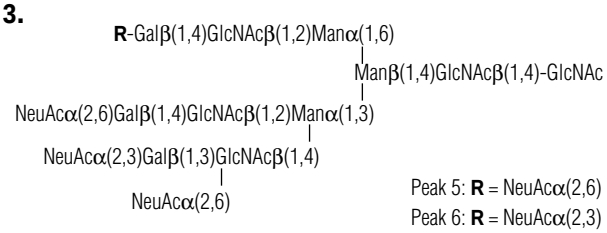

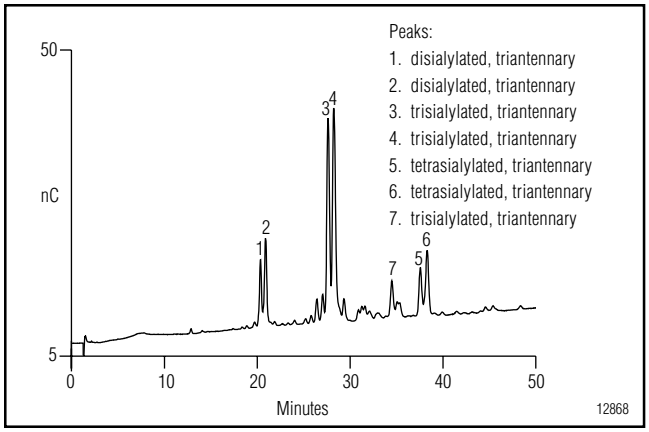

Figure 1 *Fetuin* N*-linked oligosaccharide alditols separated on the CarboPac PA-100 column.*

with 50% sodium hydroxide to pH 5.5) were used for gradient generation, has been shown to be useful for the separation of mono- as well as oligosaccharides.[6]

Reducing vs. Reduced Oligosaccharides

In 100 mM sodium hydroxide, a condition frequently used for HPAE elution, there may be measurable epimerization of terminal GlcNAc to ManNAc. Thus, some highly retained oligosaccharides with GlcNAc or GalNAc at the reducing terminus may suffer epimerization during the course of HPAE-PAD chromatography. Although epimerization is usually insignificant, some investigators choose to reduce oligosaccharides to the corresponding alditol prior to HPAE-PAD chromatography because they have observed better-defined peak shapes.[7–8]

PREPARATION OF SAMPLES AND SOLUTIONS

Eluents

0.1 M Sodium Hydroxide

It is essential to use high-quality water. It should be of high resistivity (18 MΩ-cm or better) and should contain as little dissolved carbon dioxide as possible. Biological contamination should be absent. Additionally, borate, a water contaminant that can break through water purification cartridges (prior to any other indication of depletion of the cartridge), should be removed by placement of a BorateTrap™ cartridge (Dionex Corporation) between the pump and injection valve. The use of plastic tubing in the high-purity water system should be avoided or minimized, as plastic tubing often supports microbial growth, which can be a source of carbohydrate contamination.

It is extremely important to minimize contamination with carbonate, a divalent anion at pH ≥12, because it binds strongly to the columns and interferes with carbohydrate chromatography to cause a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 1990 mL of water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

0.5 M Sodium Acetate/0.1 M Sodium Hydroxide

Dispense approximately 800 mL of water into a 1-L graduated cylinder. Add a stir bar and begin stirring. Weigh out 41.0 g of anhydrous, crystalline sodium acetate. Add the solid sodium acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt dissolves, remove the stir bar with a magnetic retriever. Using a plastic pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide, and add it to the acetate solution. Rinse the pipette by drawing up the acetate solution into the pipette and dispensing it back into the graduated cylinder several times. Add water to the solution to a final level of 1000 mL. Replace the stir bar and stir briefly to mix. Vacuum filter through a 0.2 µm nylon filter. This may take a while, as the filter may clog with insolubles from the sodium acetate. This eluent should also be kept blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

RESULTS AND DISCUSSION

Chromatography of Fetuin Oligosaccharides

The separation of bovine fetuin oligosaccharides by HPAE-PAD is shown in Figure 1. The carbohydrates of bovine fetuin have been intensively studied.[9–13] HPAE-PAD has been used to characterize ten sialylated oligosaccharides from bovine fetuin. Structures corresponding to peaks 1–6 in Figure 1 are shown in Table 1. Peak 7 is a trisialylated triantennary complex oligosaccharide. Under alkaline conditions the technique resolves these species not only by sialic acid content, but also according to the combination of α(2,3)- and α(2,6)-linked sialic acids within each charge class.[11] Oligosaccharides with the greatest proportion of α(2,6)- to α(2,3)-linked sialic acids are the least-retained. The neutral components of the oligosaccharides also influence separation. Of the oligosaccharides studied, those containing a Galβ(1,3)GlcNAc sequence are retained more strongly than those with Galβ(1,4)GlcNAc.

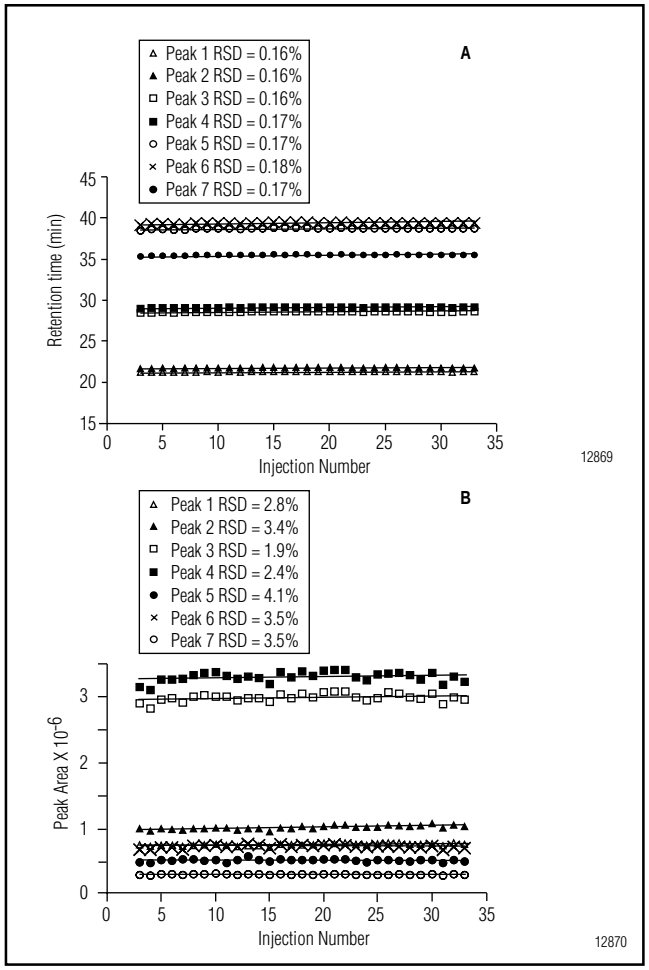

Figure 2 *Retention time reproducibility (A) and peak area reproducibility (B) for fetuin oligosaccharide alditols on the CarboPac PA-100 column.*

Accuracy and Precision

Retention time and peak area stability for the bovine fetuin oligosaccharide separation shown in Figure 1 were assessed by determining retention time and peak area RSDs of 33 consecutive injections of 250 picomoles of the commercial fetuin oligosaccharide alditol mix. The temperature of the CarboPac PA-100 column was held constant at 30 ˚C.

Retention time and peak area RSDs for each of the 7 major peaks in Figure 1 are plotted in Figures 2A and 2B. Data from the first two injections were omitted because it was found that detector response had not stabilized until the third injection. Retention time RSDs were less than 0.2%. Peak area RSDs fell in the range of 1.9% to 4.1%. Repetition of the identical schedule on a second system where the separations were done at ambient temperatures gave retention time RSDs of <0.5% and similar peak area RSDs. Retention time RSDs of <0.5% are similar to those reported for separations of the sialylated and desialylated *N*-linked carbohydrates isolated from recombinant human erythropoietin expressed in BHK cells.[2] The reported RSDs were obtained from multiple runs on different days using two internal standards [*N*-acetylneuraminic acid-Neu5Ac, and $(Neu5Ac)_3$] per run.[2]

Linearity

Detector response for the mix of *N*-linked fetuin oligosaccharide alditols was assessed. Across a broad concentration range (250 picomoles to 5 nanomoles), the peak area response for the seven major oligosaccharides was not linear (Figure 3A). However, over a narrower concentration range (100 picomoles to 1 nanomole), the response approached linearity (Figure 3B; r^2 >0.990 for peaks 1, 2, 3, 4, 6, 7 and r^2 >0.943 for peak 5). Repetition of this set of experiments on a second system generated similar results. HPAE-PAD quantitative analysis of the *N*-linked sialylated oligosaccharides of recombinant erythropoietin expressed in CHO cells has been reported. In that case, the response factors for each oligosaccharide were nearly the same.[14] Relative molar electrochemical responses for di-, tri-, tetra-, and pentasialylated oligosaccharides were reported to be 4.8 ± 14% relative to glucose.[11]

Limits of Detection

Limits of detection were determined for two authentic, commercially available oligosaccharide standards: an asialo galactosylated biantennary standard and a disialylated galactosylated biantennary standard. Low level determinations of the asialo galactosylated biantennary standard are shown in Figure 4A and reveal that limits are approached at low picomole levels. At 1.2 picomole the signal-to-noise ratio is 13.0. Limits of detection for the disialylated galactosylated biantennary standard are shown in Figure 4B. Limits of detection are similarly in the low (<3) picomole range. At 1.8 picomole, the signal-to-noise ratio is 11.8. Baseline drift from 0–50 minutes was found to be approximately 5 nC.

CONCLUSION

We have characterized the accuracy, precision, linearity, and lower limits of detection for an HPAE-PAD oligosaccharide mapping method. These results demonstrate "expected performance" and are intended for the use of investigators who need to validate HPAE-PAD oligosaccharide mapping methods.

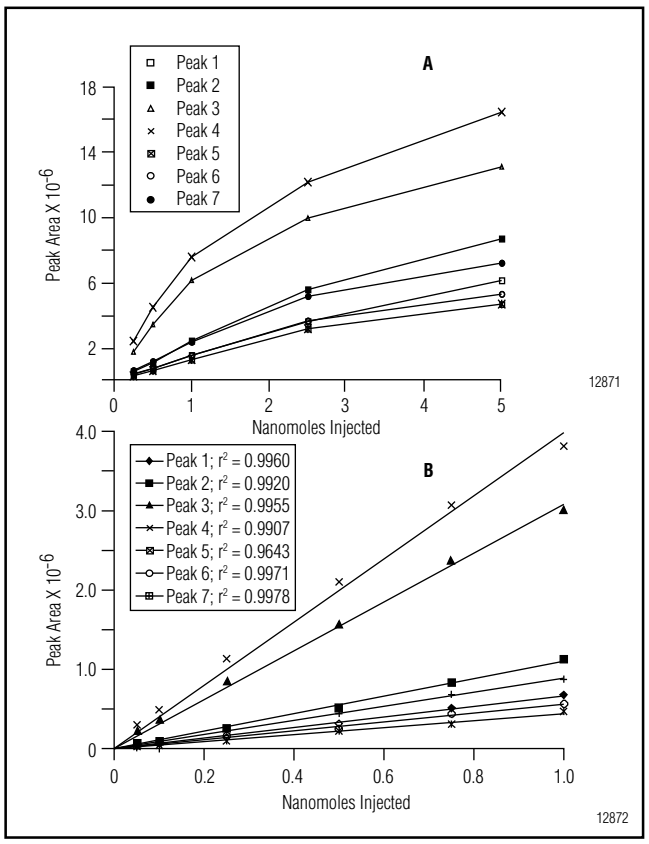

Figure 3 *(A) Detector response for fetuin oligosaccharide alditols over a broad concentration range. (B) Linearity of response for fetuin oligosaccharide alditols from 50 to 1000 picomoles.*

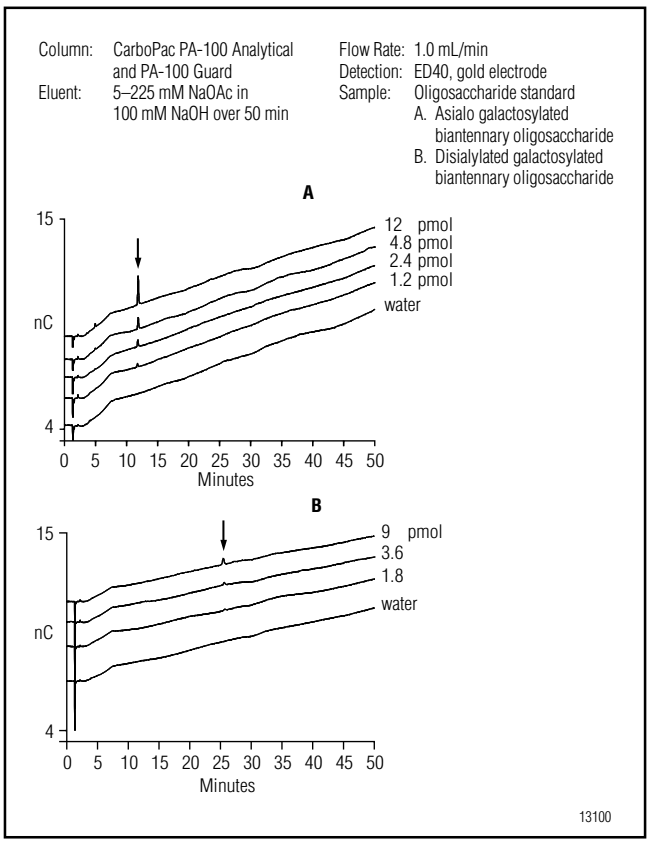

Figure 4 *Oligosaccharide standards—low level determinations.*

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LIST OF SUPPLIERS

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Oxford GlycoSciences
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Tel.: 800-722-2597.

APPENDIX

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