

HPAE-PAD Analysis of Glycoprotein Oligosaccharides

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ABSTRACT

Knowledge of a glycoprotein's oligosaccharide content is often important to understanding the function of the glycoprotein. Due to the inherent microheterogeneity of a glycoprotein's attached oligosaccharides, a high resolution separation technique is required to fully characterize the number of oligosaccharide structures and their relative amounts. Since the first report of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) for characterizing a glycoprotein's released oligosaccharides 20 years ago,¹ the neutral and charged oligosaccharide content of many glycoproteins, including recombinant therapeutic glycoproteins, have been characterized with this technique.

In this paper we show how a newer HPAE column designed for oligosaccharide analysis improves existing HPAE-PAD methods for oligosaccharide determination. We use this column to separate oligosaccharides released from a variety of standard glycoproteins. Improved resolution allows the identification of minor oligosaccharide peaks not clearly observed in earlier HPAE-PAD experiments. Despite using a lower flow rate, these separations require less time per injection and a lower concentration of sodium acetate eluent than previous methods. Therefore, if oligosaccharides are purified for subsequent analyses (e.g. MS), there is less acetate to remove. We also show that by using exoglycosidase treatments of the released oligosaccharides with HPAE-PAD we can understand the types of oligosaccharide structures present before more definitive analyses (e.g. NMR) are performed. In addition to updating the column in the HPAE-PAD assay we also report on standard operating conditions for the best day to day reproducibility and long term success for HPAE-PAD oligosaccharide analysis.

MATERIALS

Deionized water, Type 1 reagent-grade, 18.2 M Ω -cm resistivity
Sodium acetate, HPLC grade (CH₃COONa, Aldrich, P/N 71185; Dionex, P/N 059326)
Sodium hydroxide, 50% (w/w) (NaOH, Fisher Chemicals, P/N SS254-500)
Bovine Fetuin (Calbiochem)
Human Polyclonal IgG (Sigma-Aldrich)
Neuraminidase, recombinant (cloned from *Clostridium perfringens*) (New England BioLabs)
PNGase F, glycerol-free (New England BioLabs)
 β -Galactosidase (*S. pneumoniae*) – (Oxford Glycosystems – currently available from other sources)
N-acetylneuraminic acid (Neu5Ac) (Ferro Pfanstiehl Laboratories – see Dionex Technical Note 41)
N-glycolylneuraminic acid (Neu5Gc) (Ferro Pfanstiehl Laboratories – see Dionex Technical Note 41)
Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)
Filter unit, 0.2 μ m nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter for eluent preparation

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EQUIPMENT

ICS-3000 chromatography system (Dionex, Sunnyvale, CA) consisting of:

SP Single Gradient Pump module

DC Detector/Chromatography module with single or dual heating zone and 6-port injection valve

ED Electrochemical Detector equipped with cell containing disposable Au working electrode and a combination pH–Ag/AgCl reference electrode

AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray

Chromeleon® 6.8 Chromatography Workstation

Centrifuge (Eppendorf® 5400 series)

SpeedVac® evaporator

Heated water bath

Vacuum pump (for eluent preparation)

CHROMATOGRAPHY

Human Polyclonal Oligosaccharide Separations

Column: CarboPac® PA200 Analytical, 3 x 250 mm (P/N 062896)
CarboPac PA200 Guard, 3 x 50 mm (P/N 062895)

Eluents*: A: 100 mM Sodium hydroxide
B: 100 mM Sodium hydroxide, 0.5 M sodium acetate

Method: 0–5 min 99% A, 1% B
5–60 min 1 to 36% B

Flow Rate: 0.5 mL/min

Column Temperature: 30 °C

AS Tray Temperature: 15 °C

Inj. Volume: 9 µL

Inj. Loop: 10 µL

Detection: PAD, conventional or disposable Au WE

Waveform: See Table 1.

Run Time: 75 min (return to initial conditions for 15 min prior to injection)

* For proper preparation of eluents for HPAE-PAD, please see Dionex Technical Note 71.²

Table 1: Waveform A, Four-Potential Carbohydrate Waveform⁴

Time (sec)	Potential (V) (Ag/AgCl reference)	Gain*	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

* These parameters are not used on older model Dionex chromatography systems.

SAMPLE PREPARATIONS

PNGase F Digestion – 200 µL of 10 mg/mL IgG (in water) were treated with 20 µL 10X G7 buffer (included with the PNGase F purchase) and 20 µL PNGase F, which had been diluted 1:100 with water. This sample was incubated at 37 °C for 20 h, microfuged, and the supernatant analyzed directly.

Neuraminidase Digestion – 20 µL of the PNGase F-digested IgG sample above were treated with 20 µL of 25 mM sodium acetate buffer pH 5.5, and 2 µL neuraminidase. This sample was incubated at 37 °C for 20 h and the supernatant analyzed directly.

β-Galactosidase Digestion – 20 µL of the PNGase F and neuraminidase digested IgG sample and 40 µL of the PNGase F digested IgG sample were treated with 5 µL and 10 µL of a 1 mU/µL galactosidase solution (vial containing 40 mU of lyophilized product was reconstituted in 40 µL 25 mM sodium acetate buffer pH 5.5), respectively. These samples were incubated at 37 °C for 25 h and the supernatants analyzed directly.

RESULTS AND DISCUSSION

Figure 1 shows a comparison of asparagine-linked (*N*-linked) oligosaccharides released from bovine fetuin and separated on the CarboPac PA100 and CarboPac PA200 columns. The smaller bead size of the PA200 resin results in more efficient peaks and therefore better oligosaccharide resolution compared to the PA100 column. The oligosaccharides are also eluted with less sodium acetate and due to the lower flow rate (0.5 mL/min rather than 1.0 mL/min), the efficiency of online desalting with carbohydrate membrane desalter should be improved. There is less eluent used and less waste generated. We have observed the same resolution improvement for *N*-linked oligosaccharides released from transferrin, alpha-1-acid glycoprotein, and ribonuclease B.

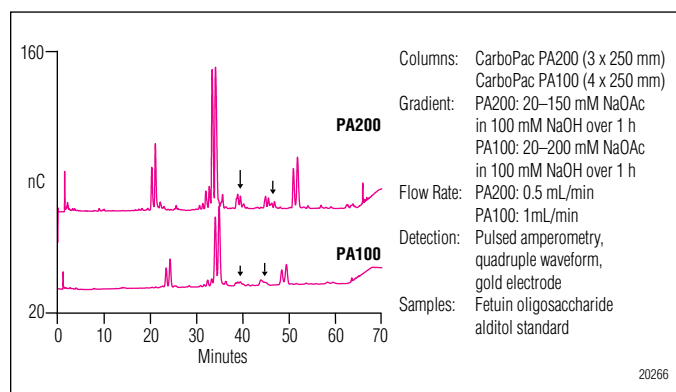


Figure 1. Fetuin oligosaccharides profiles: CarboPac PA200 vs CarboPac100.

The improvement in oligosaccharide resolution is more dramatic when the PA100 and PA200 separations of *N*-linked oligosaccharides released from human polyclonal IgG are compared (Figure 2). The improved resolution of the oligosaccharides between 5 and 15 min is especially beneficial for the oligosaccharides released from recombinant monoclonal antibodies, which have less heterogeneity than human polyclonal IgG.⁴ The majority of the *N*-linked oligosaccharides from human IgG are known to be biantennary.

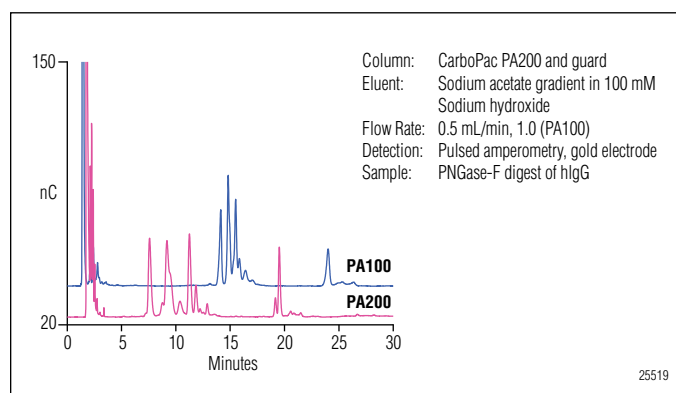


Figure 2. Human polyclonal IgG *N*-linked oligosaccharides CarboPac PA200 vs CarboPac PA100 column.

HPAE-PAD used in combination with exoglycosidases can yield information about oligosaccharide structure. Treating the PNGase F digest of human polyclonal IgG with neuraminidase reveals that the later eluting peaks (18-22 min) contained Neu5Ac (Figure 3). The retention times of those peaks suggest they each contained a single Neu5Ac. The disappearance of small peaks between 27 and 29 min suggest that they are disialylated oligosaccharides.

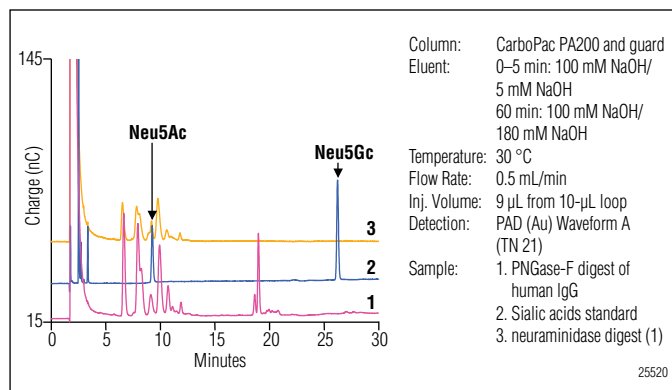


Figure 3. Monitoring release of sialic acids from human polyclonal IgG *N*-linked oligosaccharides by HPAE-PAD.

Among the *N*-linked oligosaccharides known to occur on human IgG are those that have branches that terminate in a β -linked galactose. We treated the PNGase F digest of human polyclonal IgG with β -galactosidase to reveal these structures. Figure 4 shows that many of the neutral *N*-linked oligosaccharides of human IgG have at least one terminal galactose. This also suggests that the two major monosialylated oligosaccharides are biantennary, where one has one branch with a terminal *N*-acetylglucosamine and the other branch with a terminal Neu5Ac (1st peak at about 18.5 min), and second was terminal galactose and terminal Neu5Ac on its two branches (2nd peak at about 19 min). Other studies⁴ suggest that both structures contain core fucose and that will elute earlier than the same structure without core fucose.⁵ The peak eluting just after the main peak at around 6 min in the degalactosylated sample may be the defucosylated version of the main peak.

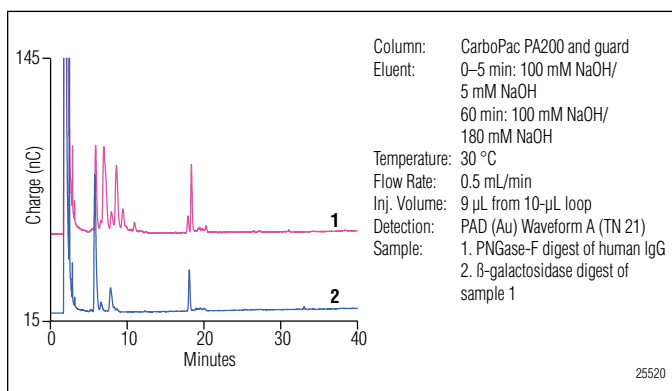


Figure 4. Degalactosylated *N*-linked oligosaccharides from human polyclonal IgG separated on CarboPac PA200 column.

If we first treat the PNGase F digest of human polyclonal IgG with neuraminidase and then treat it with β -galactosidase (Figure 5), we get a similar result as Figure 4 with the notable absence of the sialylated oligosaccharides.

Table 2 lists some of the actions we believe are important to have the best reproducibility and method ruggedness for HPAE-PAD assays.

Table 2: Standard Operating Procedures for Successful HPAE-PAD Assays	
Procedure	Explanation
Prepare eluents according to Dionex Technical Note 71.	Improperly prepared eluents are the number 1 source of HPAE-PAD problems.
If possible, have sodium hydroxide (e.g. 100 mM) in the sodium acetate mobile phase.	Acetate eluents without hydroxide are easily contaminated and can contaminate the whole chromatography system.
Monitor reference electrode pH and replace the reference electrode if the value is off by more than a few tenths of a pH unit.	Reference electrode failure will cause the wrong waveform to be applied causing a change in sensitivity and possible working electrode damage.
Use the waveform in Table 1	This waveform provides the best long term reproducibility of electrode response.
Use disposable Au working electrodes	These electrodes are more reproducible electrode-to-electrode than the conventional electrodes and do not require electrode polishing.
Establish a system suitability standard	This allows you to check the system if you suspect a problem. For oligosaccharide analysis, an inexpensive maltodextrin or inulin standard might be appropriate.

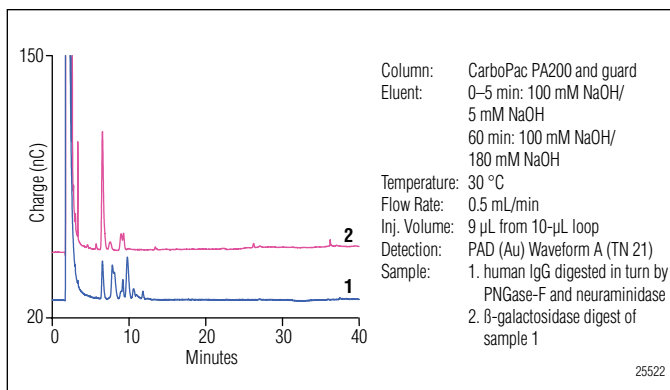


Figure 5. Desialylated and degalactosylated N-linked oligosaccharides from human polyclonal IgG separated CarboPac PA200 column.

CONCLUSIONS

1. The CarboPac PA200 is the best choice for oligosaccharide analysis by HPAE-PAD as it provides the best resolution, uses less eluent, and generates less waste.
2. Exoglycosidase treatments aid in the understanding of HPAE-PAD oligosaccharide profiles of glycoproteins.

REFERENCES

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