IgG Oligosaccharide Analysis by HPAE-PAD

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Overview

Purpose: Develop a method to analyze and identify N-linked oligosaccharides from polyclonal IgG and monoclonal antibodies (MAbs).

Methods: High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) was used with a shallow gradient of sodium hydroxide to resolve neutral glycans, followed by an acetate gradient to resolve charged oligosaccharides. Oligosaccharides are identified based on comparison to known standards in combination with exoglycosidase digestions. These identified glycans are compared against maltose and maltose oligomers to assign a retention index that is characteristic of the oligosaccharide of interest.

Results: N-linked oligosaccharides found on polyclonal IgG were identified and compared against a retention time ladder using two Thermo Scientific™ Dionex™ ICS-5000 Ion Chromatography systems. To evaluate the ruggedness of this approach, the values obtained from comparison to the retention time ladder were used to aid in identification of oligosaccharides in an unknown MAb.

Introduction

Antibodies are among the largest class of glycoproteins studied as potential therapeutic proteins. Understanding and characterizing the glycosylation of these products as a potential critical quality attribute is of importance, with evidence that glycosylation may impact biological efficacy, pharmacokinetics, and cellular toxicity. Protein expression systems and reactor conditions can impact glycosylation, leading to potentially undesirable glycosylation. For example, high-mannose species may be present, which are not typical of human antibody glycosylation. The presence of these species in the Fc domain has been linked to increased serum clearance of IgG antibodies.1 HPAE-PAD is an effective tool for analyzing glycans present in glycoprotein therapeutics and has previously been used to evaluate oligosaccharides in MAbs with sodium hydroxide concentrations of <100 mM.2 In the work presented here, a method for analyzing glycans commonly found on antibodies is described.

Methods

Sample Preparation

Samples were prepared by digesting protein solutions with PNGase F followed by exoglycosidase digestion as previously described.3 Typical digestions incubated 1.1 mg of protein with 4000 units of PNGase F at 37 °C for 20 h in 1 mL of solution. Samples were directly injected after PNGase F digestion.

Liquid Chromatography

Dionex ICS-3000 or ICS-5000 system including:

- DP Dual Pump module
- DC Detector/Chromatography Compartment
- AS-AP Autosampler
- ED Electrochemical Detector
- Electrochemical Cell
- Disposable Gold Electrode
- Reference Electrode

The Thermo Scientific™ Dionex™ Chromleon™ Chromatography Data System (CDS) software was used for system control and data processing.

Columns: Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard (3 x 50 mm) Dionex CarboPac PA200 Analytical (3 x 250 mm)

Eluent A: Deionized water
Eluent B: 100 mM Sodium Hydroxide (NaOH)
Eluent C: 200 mM Sodium Acetate in 100 mM NaOH
**Overview**

**IgG Oligosaccharide Analysis by HPAE-PAD**

Antibodies are among the largest class of glycoproteins studied as potential therapeutic proteins. However, the presence of these glycoproteins is limited in the human population, which are not typical of human antibody glycosylation. The presence of these oligosaccharides can influence the pharmacokinetic properties of the protein, leading to a potential loss of efficacy or increased toxicity. Therefore, it is important to characterize the glycosylation profile of potential therapeutic proteins.

To resolve the neutral oligosaccharides, a shallow 50–100 mM NaOH gradient was used with a 200 mM sodium acetate in 100 mM NaOH from 50–60 min. Return to 50 mM NaOH from 60–60.1 min. Equilibration at 50 mM NaOH from 60.1–75 min.

**Sample Preparation**

Samples were prepared by digesting protein solutions with PNGase F followed by exoglycosidase digestion as previously described. Typical digestions incubated for 20 h in 1 mL of solution.

**Methods**

**Gradient:** 50–100 mM NaOH from 0–30 min, 100 mM NaOH from 30–35 min, 0–200 mM sodium acetate in 100 mM NaOH from 35–50 min, 200 mM sodium acetate in 100 mM NaOH from 50–60 min.

**Flow Rate:** 0.5 mL/min

**Inj. Volume:** 5 µL (partial loop)

**Temp:** 30 °C

**Detection:** PAD, Au (disposable, carbohydrate-certified), standard quad. waveform

**Background:** 18–22 nC

**Noise:** 60 pC

**System Backpressure:** ~3000 psi

**Results**

**IgG Oligosaccharide Separation**

To resolve the neutral oligosaccharides, a shallow 50–100 mM NaOH gradient was evaluated. This gradient allows the resolution of many neutral oligosaccharides, including high-mannose species (Figure 1). Identification was confirmed by exoglycosidase digestions, such as the fucosidase digestion shown in Figure 1.

**FIGURE 1. Separated neutral IgG oligosaccharides. Note the conversion of Peak 1 to Peak 3 (G0F to G0), Peaks 5 and 6 to Peaks 7 and 8 (G1F isomers to G1 isomers), and Peak 11 to Peak 13 (G2F to G2). Similarly treated neutral standards are shown illustrating G0, G1, Man6, and G2b.**

**Columns:** Dionex CarboPac PA200 Guard (3 × 50 mm) and Dionex CarboPac PA200 Analytical (3 × 250 mm)

**Eluents:**
- A. Deionized water
- B. 100 mM NaOH
- C. 200 mM Sodium Acetate in 100 mM NaOH

**Gradient:** 50–100 mM NaOH from 0–30 min, 100 mM NaOH from 30–35 min, 0–200 mM sodium acetate in 100 mM NaOH from 35–50 min, 200 mM sodium acetate in 100 mM NaOH from 50–60 min.

**Flow Rate:** 0.5 mL/min

**Inj. Volume:** 5 µL (partial loop)

**Temp:** 30 °C

**Detection:** PAD, Au (disposable, carbohydrate-certified)

**Samples:**
- A) IgG oligosaccharides before fucosidase digestion
- B) IgG oligosaccharides after fucosidase digestion
- C) Neutral oligosaccharide standards after fucosidase digestion

**Peaks:**
- 1. G0F
- 2. Man5
- 3. G0
- 4. Neu5Ac
- 5. G1F (1-6)
- 6. G1F (1-3)
- 7. G0bF, G1 (1-6)
- 8. G1 (1-3)
- 9. G0b
- 10. Man6
- 11. G2F
- 12. G1bF
- 13. G2
- 14. G1b
- 15. G2bF
- 16. G2b
Retention Time Ladder and Retention Index Values.

These released oligosaccharides were compared to a ladder of maltose, maltotriose, and maltotetraose, which were assigned arbitrary retention indices of 200, 300, and 400 (Table 1). The retention indices of identified oligosaccharides are shown in Table 1 along with comparative data to previously published work determining abundance of individual glycans. Although the values are not identical, there is great similarity in the relative abundance values. This process was also used to characterize charged glycans during the 0–200 mM sodium acetate in 100 mM NaOH section of the gradient (Table 2).

Table 1. MAb acronym, relative abundance, and structure of neutral oligosaccharides identified compared to published results in polyclonal IgG. The retention index value, determined by comparison to a maltose, maltotriose, and maltotetraose ladder, is used in additional samples for identification (illustrated in Figure 2).

<table>
<thead>
<tr>
<th>MAb Acronym</th>
<th>Published Relative Abundance in IgG (%) Relative to G2F</th>
<th>Retention Index (Maltose-Based Ladder)</th>
<th>Structure (Adapted from CFG)</th>
<th>Relative Abundance for Polyclonal IgG, Sample 1 (% Peak Area Relative to G2F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F</td>
<td>61.5</td>
<td>228</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>G0</td>
<td>0.9</td>
<td>236</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>G1F (1-6)</td>
<td>100</td>
<td>247</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>G1F (1-3)</td>
<td>59</td>
<td>251</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>G0bF</td>
<td>7.8</td>
<td>259</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>G1 (1-6)</td>
<td>5.5</td>
<td>258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 (1-3)</td>
<td>3.6</td>
<td>263</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>G0b</td>
<td>1.0</td>
<td>266</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>G2F</td>
<td>100</td>
<td>274</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>G1bF</td>
<td>13.5</td>
<td>281</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>9.7</td>
<td>290</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>G1b</td>
<td>1.4</td>
<td>291</td>
<td>Not Detected</td>
<td></td>
</tr>
<tr>
<td>G2bF</td>
<td>4.1</td>
<td>316</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>G2b</td>
<td>1.0</td>
<td>341</td>
<td>Not Detected</td>
<td></td>
</tr>
<tr>
<td>Man5</td>
<td>0.3</td>
<td>232</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Man6</td>
<td>Not Listed</td>
<td>269</td>
<td>Not Detected</td>
<td></td>
</tr>
</tbody>
</table>

**Acronyms**: G0, G2, G2F, G1F, G1bF, G0b, Man5, Man6

**Symbols**: N-acetylglucosamine (GlcNAc), Galactose (Gal), Fucose (Fuc), N-acetylleucaminic acid (Neu5Ac), Mannose (Man)
Table 2. Structure, MAb acronym, and relative abundance of charged oligosaccharides identified compared to published results in polyclonal IgG. The retention index value, determined by comparison to a maltose, maltotriose, and maltotetraose ladder, is used in additional samples for identification.

<table>
<thead>
<tr>
<th>MAb Acronym</th>
<th>Published Relative Abundance in IgG (%) Relative to G2F</th>
<th>Retention Index (Maltose-Based Ladder)</th>
<th>Structure (Adapted from CFG)</th>
<th>Relative Abundance for Polyclonal IgG. Sample 1 (% Peak Area Relative to G2F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1F(1-3)</td>
<td>52</td>
<td>404, 409</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A1(1-3)</td>
<td>3.5</td>
<td>405, 410</td>
<td>Not Detected</td>
<td></td>
</tr>
<tr>
<td>A2F</td>
<td>4.4</td>
<td>424, 429</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>0.6</td>
<td>426, 431</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>FA2G1S1</td>
<td>14</td>
<td>403-Shoulder on A1F(1-3)</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Due to base-catalyzed epimerization, unprotected GlcNAc on the oligosaccharide may convert to ManNAc, leading to two peaks for each of the well-retained charged oligosaccharides as seen in Figure 2 and reflected in the retention indices listed in Table 2.

Figure 2. Oligosaccharides separated on the Dionex CarboPac PA200. Oligosaccharides were identified for a second polyclonal IgG sample and a MAb with the aid of the retention index.

Columns: Dionex CarboPac PA200 Guard (3 × 50 mm) and Dionex CarboPac PA200 Analytical (3 × 250 mm)
Eluents: A. Deionized water  
B. 100 mM NaOH  
C. 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM NaOH from 0–30 min, 100 mM NaOH from 30–35 min, 0–200 mM sodium acetate in 100 mM NaOH from 35–50 min, 200 mM sodium acetate in 100 mM NaOH from 50–60 min. Return to 50 mM NaOH from 60–60.1 min. Equilibration at 50 mM NaOH from 60.1–75 min.
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL (partial loop)
Temp: 30 °C
Detection: PAD, Au (disposable, carbohydrate-certified)

Samples:
A) Polyclonal IgG oligosaccharides, Sample 2
B) A1F and A2F standards
C) MAb oligosaccharides
Ruggedness Testing

Retention of polyclonal IgG oligosaccharides from polyclonal IgG were identified by retention time and retention index across two systems and analysts for three weeks (n = 25 injections). Retention time comparison between systems is good, but for closely eluting peaks may lead to ambiguous identification. The comparison of retention indices showed improved precision, as measured by RSD. This improvement helps to remove ambiguity in oligosaccharide identification.

Table 3. Retention Time (RT) and Retention Index (RI) Precision of IgG oligosaccharides with two chromatography systems.

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>System 1 RT (min)</th>
<th>System 1 RI (Arbitrary Units)</th>
<th>System 2 RT (min)</th>
<th>System 2 RI (Arbitrary Units)</th>
<th>RT RSD (%)</th>
<th>RI RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F</td>
<td>16.76</td>
<td>228</td>
<td>17.39</td>
<td>228</td>
<td>3.8</td>
<td>0.39</td>
</tr>
<tr>
<td>MAN-5</td>
<td>17.58</td>
<td>232</td>
<td>18.28</td>
<td>232</td>
<td>4.0</td>
<td>0.46</td>
</tr>
<tr>
<td>G0</td>
<td>18.52</td>
<td>236</td>
<td>19.17</td>
<td>237</td>
<td>3.7</td>
<td>0.35</td>
</tr>
<tr>
<td>G1F (1-6)</td>
<td>20.63</td>
<td>247</td>
<td>21.40</td>
<td>247</td>
<td>3.9</td>
<td>0.51</td>
</tr>
<tr>
<td>G1F (1-3)</td>
<td>21.45</td>
<td>251</td>
<td>22.27</td>
<td>251</td>
<td>4.0</td>
<td>0.56</td>
</tr>
<tr>
<td>G1F (1-6) / G0bF</td>
<td>22.84</td>
<td>258</td>
<td>23.64</td>
<td>258</td>
<td>3.6</td>
<td>0.34</td>
</tr>
<tr>
<td>G2F</td>
<td>26.10</td>
<td>274</td>
<td>27.07</td>
<td>274</td>
<td>4.0</td>
<td>0.55</td>
</tr>
<tr>
<td>G1F</td>
<td>27.40</td>
<td>281</td>
<td>28.35</td>
<td>281</td>
<td>3.6</td>
<td>0.38</td>
</tr>
<tr>
<td>G2F</td>
<td>32.94</td>
<td>314</td>
<td>34.05</td>
<td>316</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>A1F (1-3)</td>
<td>43.14</td>
<td>404</td>
<td>43.30</td>
<td>404</td>
<td>0.63</td>
<td>0.27</td>
</tr>
<tr>
<td>A1F (1-3)(ManNAc)</td>
<td>43.68</td>
<td>408</td>
<td>43.84</td>
<td>409</td>
<td>0.63</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Conclusion

- Using a combination of an hydroxide gradient and an acetate/hydroxide gradient both neutral and charged oligosaccharides can be well resolved in one method.
- By correlating retention times of oligosaccharides to inexpensive maltose standards identification can be simplified.
- This strategy has been successfully employed with polyclonal IgG and a MAb.
- Comparison of two analytical systems and two analysts suggests a rugged method for IgG oligosaccharide characterization.

References


Acknowledgements

We would like to thank Uday Aich and Zhiqi Hao of Thermo Fisher Scientific for supplying the monoclonal antibody and the related released oligosaccharides.