Validation of a Dual Wavelength Size Exclusion HPLC Method with Improved Sensitivity to Detect Aggregates of a Monoclonal Antibody Biotherapeutic

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Abstract

Purpose
The objective of the current study was to validate a dual wavelength SE HPLC UV214, 280 method to enhance the sensitivity for detection of aggregate forms of a monoclonal IgG drug product.

Methods
Concentrated solutions of IgG antibody were chromatographed onto a TSKgel G3000 SWXL SEC column by isocratic elution at 0.7 ml/minute at ambient temperature with a mobile phase composed of 0.2 M Sodium Phosphate, pH 6.8. A Waters Alliance 2695 HPLC with dual wavelength detector was used at 214 nm and 280 nm. The response ratio for the IgG monomer at 214:280 nm was determined by injection of reference standard. In test samples, low abundant aggregate forms of this IgG were best detected at 214 nm when injected at high amounts (300 μg), but the monomer peak was off scale under those conditions. Therefore, the peak area of the monomer at 214 nm was estimated by multiplication of its 280 nm response by the 214:280 ratio determined previously. The percent purity of the IgG sample (300 μg/injection) was then calculated based upon the peak area normalized responses at 214 nm for all forms of the IgG.

Results
Determination of the 214:280 ratio was found to be consistent between 5 to 15 μg/injection with an average ratio response of 14.1 (0.17 % RSD) at 10 μg/injection. The method was found to be precise with intraday and interday results of 1.5% and 1.9% RSD. It was also linear and accurate for the monomer at nominal levels (300 μg/injection) with a correlation coefficient (r) of 1.00 and an average recovery of 99.1% (0.27% RSD). In addition, the method was found to be specific with no interferences detected in the formulation buffer that would interfere with the detection of all forms of the IgG. Sensitivity for quantitation (LOQ) and detection (LOD) was estimated to be 0.66 mg/mL and 0.22 mg/mL. Lastly, the method was found to be robust following purposeful small changes in key method parameters.

Conclusions
The DW SE HPLC method was found to be accurate, precise, linear, specific, sensitive, robust and suitable for its intended use. Sensitivity was increased approximately 6.4-fold by SE HPLC analysis with the use of concentrated IgG samples. This was possible by normalizing the 214 nm response of the IgG monomer using the 214:280 ratio as determined in each analytical run.

Background
The use of dual wavelength SE HPLC to increase the sensitivity to detect protein aggregates was first proposed by investigators at Centocor R&D, Inc (Bond et al. 2010). In this whitepaper, the validation of a DW SE HPLC method for another biotherapeutic IgG monoclonal antibody, produced by Morphotek Inc., is presented. In brief, the method utilizes UV detection of the eluents from SE HPLC at two different wavelengths, 214 nm and 280 nm. The method relies upon the difference in absorptivity of the amide bond at 214 nm versus aromatic residues at 280 nm. Since absorptivity of proteins is generally much greater at 214 nm than at 280 nm, one can detect aggregate forms of proteins more readily at 214 nm. However, detection of low abundant aggregates at 214 nm often results in exceeding the range of the UV detector for the monomer peak. With the monomer off-scale, accurately determining the relative abundance of aggregate(s) vs. the monomer is impossible. This is overcome by determining the 214:280 ratio for the IgG at a lower concentration, where the monomer peak is within the dynamic range of the detector for both wavelengths. Once the 214:280 ratio is established, the peak area of the monomer at 214
nm in more concentrated samples can be determined by multiplying the 214:280 ratio by the peak area response at 280 nm. Once normalized, the monomer peak in concentrated sample preparations detected at 214 nm can now be used to determine the relative abundance of IgG monomer, dimer, aggregates, fragments, and other product forms where they can be readily detected.

Methods

Materials
The test articles used in the method validation consisted of a formulated human monoclonal IgG drug product and a corresponding reference standard; both materials used the same formulation buffer and had an approximate concentration of 5 mg/mL. The formulation buffer, consisting of 10 mM sodium phosphate, 150 mM sodium chloride, 0.01% polysorbate 80, pH 7.2, was used as a control. A concentrated sample of the IgG development material, at 20.4 mg/mL, was also used in some validation experiments. A Bio-Rad molecular weight standard preparation, containing 5 mg of thyroglobulin, globulin, ovalbumin, 2.5 mg of myoglobin and 0.5 mg of vitamin B12, were also used to approximate the apparent MW of the monomer form of IgG antibody.

Sample preparation
For system suitability testing and determination of the 214:280 ratio, formulated IgG reference standard was prepared at a concentration of 165 mg/mL using water and 10 mg (60 µL/injection). Both were analyzed simultaneously at both 214 nm and 280 nm. Formulated IgG samples were analyzed at a nominal concentration of 5 mg/mL and 300 µg (60 µL/injection) amounts were analyzed at 214 nm. The MW standard was resolubilized with 0.5 mL of water and 20 L were injected onto the SEC column with detection at 280 nm. This corresponds to 200 g of thyroglobulin, globulin and ovalbumin, 100 g myoglobin and 20 g of Vitamin B12 injected onto the SE column.

Results

Determination of 214:280 nm Ratio
The optimal conditions for determining the 214:280 ratio were determined using nine replicate injections of reference standard IgG at 5.0 µg, 7.5 µg, 10.0 µg, 12.5 µg, and 15.0 µg per injection. The 214:280 ratio was assessed for the monomer peak in all injections. This data is shown in Table 1. Response was linear throughout the range tested (Figure 1).

For determination of the 214:280 ratios in all subsequent analytical runs, a 0.167 mg/mL preparation of the formulated IgG reference standard was used. The 214:280 ratios were determined in this manner for each analytical run. A representative chromatogram is shown in Figure 2 (a and b).

Table 1. Evaluation of 214:280 nm ratio at various levels of IgG

<table>
<thead>
<tr>
<th>Sample Concentration (mg/mL)</th>
<th>µg/Injection</th>
<th>Mean Area Monomer 214 nm</th>
<th>Mean Area Monomer 280 nm</th>
<th>214:280 ratio</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.083</td>
<td>5.0</td>
<td>7,460,057</td>
<td>573,335</td>
<td>13.5</td>
<td>0.1</td>
</tr>
<tr>
<td>0.125</td>
<td>7.5</td>
<td>11,175,529</td>
<td>841,938</td>
<td>13.3</td>
<td>5.1</td>
</tr>
<tr>
<td>0.167</td>
<td>10.0</td>
<td>14,867,638</td>
<td>1,054,199</td>
<td>14.1</td>
<td>0.2</td>
</tr>
<tr>
<td>0.208</td>
<td>12.5</td>
<td>18,886,640</td>
<td>1,332,263</td>
<td>14.2</td>
<td>2.6</td>
</tr>
<tr>
<td>0.250</td>
<td>15.0</td>
<td>23,411,076</td>
<td>1,596,037</td>
<td>14.7</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Repeatability and Intermediate Precision of Sample
Repeatability was determined by 10 injections of IgG sample at nominal concentration of 5 mg/mL (300 µg/injection) with detection at 280 nm. The resultant precision for injection repeatability was 0.1 % RSD for the IgG monomer.

Intermediate precision was performed in triplicate preparations at ± 20% nominal (4 mg/mL, 5 mg/mL, and 6 mg/mL) by two different analysts using separate sample preparations. The precision results are shown in Table 2. The % RSD for a single day analysis was 1.5% and the overall precision between the two analysts was 2.2%. Representative chromatograms for IgG sample injections are shown in Figure 3.

Figure 1. Linearity of Dilute Preparations of IgG Monomer for Determination of 214:280 Ratio Procedure
Linearity of Sample Preparation
Preparations of IgG sample ranging from 2.5 mg/mL to 7.5 mg/mL (150 µg/injection to 450 µg/injection) were analyzed at both 214 and 280 nm in triplicate (example shown in Figure 3). All relevant IgG related peaks were evaluated to include the monomer, dimer, aggregate, and fragment peaks observed in these chromatograms. The resultant linear regression analyses for all IgG components are shown in Figure 4. All IgG components were found to behave in a linear fashion and intercept near the origin (Figure 4, panels A, B, D, E and F) except the monomer when measured at 214 nm (Panel C). For monomer, the 214 nm monomer peak response displayed considerable bias and did not pass near the origin. This bias is due to maximizing of the response of the monomer, the most abundant peak in the IgG, as expected.

Accuracy
Accuracy was assessed at multiple concentrations of IgG in triplicate, ranging from 2.5 mg/mL to 7.5 mg/mL of product. At each level tested, the amount of IgG monomer detected at 280 nm was compared to the theoretical dilution using linear regression as shown in Table 3. Precision for the monomer peak did not exceed 1.7 % RSD at all concentrations. Additionally, molecular weight of the IgG determination of the apparent monomer by comparison to elution of known MW standards is shown in Figure 5.

Specificity, Robustness and Reagent Stability
Specificity was evaluated by analysis of duplicate preparations of formulation buffer. No peaks were found to be present in the placebo formulation buffer that could interfere with the IgG monomer or other primary forms such as dimer, aggregates, and fragments (data not shown).

Robustness was evaluated by purposeful changes (±5%) to critical steps in the SE HPLC method. These included adjustments of ±5% in concentration and pH of the SE HPLC mobile phase nominally at 0.2 M Sodium Phosphate, pH 6.8. Additionally, robustness was challenged using ±5% change in injection volume and testing an additional lot of column. All such purposeful changes did not affect the performance of the method to any significant degree.

<table>
<thead>
<tr>
<th>Analyst</th>
<th>Sample Concentration (mg/mL)</th>
<th>Amount Analyzed (µg/injection)</th>
<th>Mean Monomer Peak Area Response at 280 nm</th>
<th>Mean Normalized* Monomer Peak Area Response at 214 nm</th>
<th>%RSD of Normalized* Peak Area Response at 214 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>4.0</td>
<td>240</td>
<td>28,487,290</td>
<td>366,360,987</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>350</td>
<td>34,607,168</td>
<td>447,601,492</td>
<td>1.3</td>
</tr>
<tr>
<td>2nd</td>
<td>4.0</td>
<td>240</td>
<td>27,513,393</td>
<td>353,610,364</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>350</td>
<td>33,816,287</td>
<td>434,773,489</td>
<td>0.8</td>
</tr>
<tr>
<td>Both</td>
<td>4.0</td>
<td>240</td>
<td>NA</td>
<td>360,228,245</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>350</td>
<td>NA</td>
<td>441,217,491</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>390</td>
<td>NA</td>
<td>532,276,980</td>
<td>1.7</td>
</tr>
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</table>

*Monomer peak area response calculated by multiplication of the 280 nm monomer peak area by 214/280 ratio determined in the same analytical run using injections of 10 µg of IgG Reference Standard

Table 2. Intermediate Precision for IgG Monomer
Triplicate preparations of the formulated reference standard were also evaluated for stability at 2-8 °C for up to 48 hours and were found to be stable under those conditions.

**Sensitivity**

Sensitivity of the method was determined by evaluation of IgG samples ranging from 2.5 mg/mL to 7.5 mg/mL in triplicate. The Limit of Quantitation (LOQ) and Limit of Detection (LOD) were determined using the following equations:

\[
LOD = 3.3 \times \text{Standard Deviation of Intercepts/Average of Slopes},
\]

\[
LOQ = 10 \times \text{Standard Deviation of Intercepts/Average of Slopes}.
\]

Since purified preparations of the various IgG forms, dimers, aggregates, and fragments were not available, the IgG monomer was used as a surrogate for evaluation of sensitivity of all the known IgG forms.

The IgG monomer response for concentrated sample preparations at 280 nm was normalized using the 214:280 ratio as per this method. For comparison, the IgG response at 214 nm without normalization was also evaluated. This was done to determine differences in sensitivity using both analytical approaches. It was determined that use of the 214:280 ratio to normalize IgG responses in concentrated samples provided an approximate 6-fold increase in sensitivity as shown in Table 4.

![Figure 4. Linearity for Peak Area Responses of the IgG monomer (at both 280 nm and 214 nm) as well as Dimer, Aggregate, and Fragments at 214 nm Observed in IgG Sample Preparations](image)

![Linearity of IgG Sample at 280 nm](image)

\[
y = 6,862,491x + 554,411 \\
R^2 = 1
\]

![Linearity of IgG Sample at 214 nm Following Normalization vs. 214:280 ratio](image)

\[
y = 86,094,608 + 20,468,765 \\
R^2 = 1
\]

![Linearity of IgG Sample at 214 nm (not normalized vs. 214:280 ratio)](image)

\[
y = 6,358,829 + 112,167,520 \\
R^2 = 1
\]

**References**

Table 3. Accuracy of Monomer at 280 nm

<table>
<thead>
<tr>
<th>Sample Concentration (mg/mL)</th>
<th>Amount analyzed (µg/injection)</th>
<th>% Accuracy</th>
<th>% RSD</th>
</tr>
</thead>
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<tr>
<td>2.5</td>
<td>150</td>
<td>97.6</td>
<td>1.65</td>
</tr>
<tr>
<td>4.0</td>
<td>240</td>
<td>98.2</td>
<td>1.52</td>
</tr>
<tr>
<td>5.0</td>
<td>300</td>
<td>98.9</td>
<td>1.43</td>
</tr>
<tr>
<td>6.0</td>
<td>360</td>
<td>99.1</td>
<td>1.27</td>
</tr>
<tr>
<td>7.5</td>
<td>430</td>
<td>98.6</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Table 4. Results for Evaluation of Sensitivity for IgG by detection of the Monomer Peak at 280 nm versus the same peak when normalization was not performed.

<table>
<thead>
<tr>
<th>IgG Monomer</th>
<th>LOD mg/mL</th>
<th>Fold Increase</th>
<th>LOQ mg/mL</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>280 nm after normalizing using the 21÷280 ratio</td>
<td>8.18</td>
<td>6.3</td>
<td>0.54</td>
<td>6.4</td>
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<tr>
<td>214 nm without normalization</td>
<td>1.14</td>
<td>3.45</td>
<td></td>
<td></td>
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</table>

Figure 4 (Continued). Linearity for Peak Area Responses of the IgG monomer (at both 280 nm and 214 nm) as well as Dimer, Aggregate, and Fragments at 214 nm Observed in IgG Sample Preparations

Figure 5. Verification of IgG Monomer MW by Comparison to Known MW Standards