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MATERIALS SCIENCES

VALIDATION OF A DUAL WAVELENGTH SIZE EXCLUSION HPLC METHOD WITH IMPROVED SENSITIVITY TO DETECT PROTEIN AGGREGATES OF A MONOCLONAL ANTIBODY BIOTHERAPEUTIC

APPLICATION NOTE

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

By J. Tompkins[1], T. Spurgeon[1], R. Tobias[1], J. Anders[1], E. Butler-Roberts[2], and M. Adomaitis[2]

[1]EAG Laboratories and [2]Morphotek, Inc.

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 and 280 nm. The response ratio for the IgG monomer at 214:280 nm was determined by injection of 10 g 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 % 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 inter day results of 1.5% and 1.9% RSD, respectively. It was also linear and accurate for 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), respectively. 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, respectively. 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, and robust and, therefore, suitable for its intended use. Sensitivity was increased approximately 6.4-fold by SEHPLC analysis with the use of concentrated IgG samples. This was made possible by normalizing the 214 nm response of the IgG monomer by 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, Bond et al. (2010), at Centocor R&D, Inc. In this presentation, 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, such as tryptophan and tyrosine, 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 maximizing the capacity of the UV detector for the monomer peak. With the monomer o-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.

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OBJECTIVE

The objective of this work was to validate a more sensitive DW SE HPLC method for detection and relative quantitation of low abundant dimers, aggregates, and fragment forms of this IgG antibody.

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.

Equipment: The primary equipment used in this project included a Waters 2695 Alliance HPLC with a 2487 Dual Wavelength detector and an Empower 2 chromatographic data acquisition system. A Tosoh TSK gel G3000 SWxl 7.8 × 300 mm, 5 m column was also used.

Sample preparation: For system suitability testing and determination of the 214:280 ratio, formulated IgG reference standard was prepared at a concentration of 165 g/mL using water and 10 g (60 L/injection) was analyzed simultaneously at both 214 nm and 280 nm. Formulated IgG samples were analyzed at a nominal concentration of 5 mg/mL and 300 g (60 L/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.

The mobile phase consisted of 0.2 M Sodium Phosphate at pH 6.8. The flow rate was 0.7 mL/min and the injection volumes were 60 L for IgG material and 20 L for the MW standard. A needle wash solution of water was used and column temperature was ambient. Detection was done at 214 and 280 nm with a 16 nm slit width. Run time was 30 minutes per injection.

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 were assessed for the monomer peak in all injections. The data are shown 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.

Sample Concentration (mg/mL)	µg/Injection	Mean Area Monomer 214 nm	Mean Area Monomer 280 nm	214:280 ratio	% RSD
0.083	5.0	7,746,057	573,535	13.5	0.1
0.125	7.5	11,175,529	841,938	13.3	5.1
0.167	10.0	14,867,638	1,054,149	14.1	0.2
0.208	12.5	18,886,640	1,332,263	14.2	2.6
0.250	15.0	23,411,076	1,596,037	14.7	2.9

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

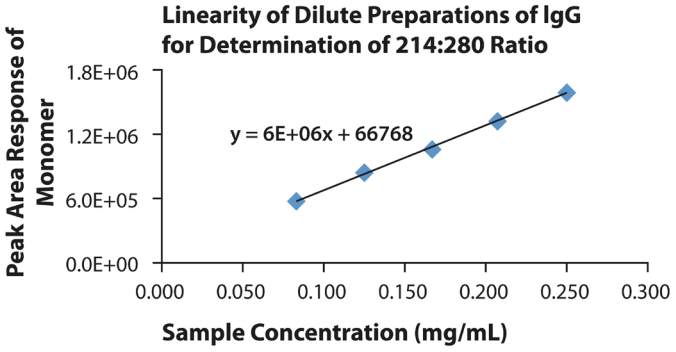

Figure 1. Linearity of Dilute Preparations of IgG Monomer for Determination of 214:280 Ratio Procedure

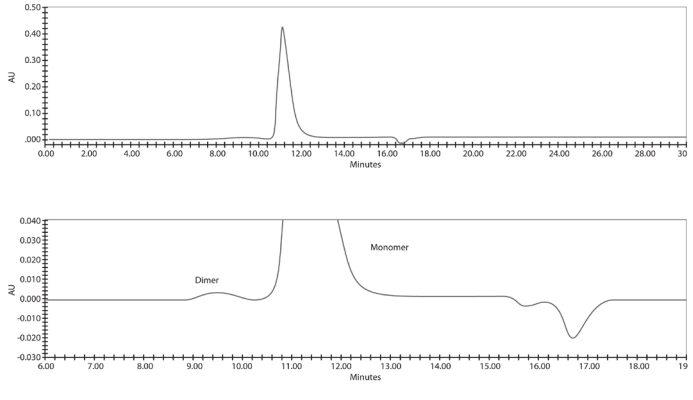

Figure 2(a). 0.167 mg/mL (10 µg/injection) of IgG Reference Standard at 214 nm, On-Scale and Expanded View

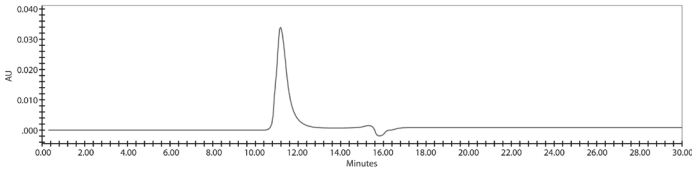

Figure 2(b). 0.167 mg/mL (10 µg/injection) of Iggy Reference Standard at 280 nm

Repeatability and Intermediate Precision of Sample: Repeatability was determined by 10 injections of IgG sample at nominal concentration of 5 mg/mL (300 g/injection) with detection

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Analyst	Sample Concentration (mg/mL)	Amount Analyzed (µg/Injection)	Mean Monomer Peak Area Response at 280 nm	Mean Normalized* Monomer Peak Area Response at 214 nm	%RSD of Normalized* Peak Area Response at 214 nm
	4.0	240	28,487,290	366,380,987	1.1
1st	5.0	300	34,807,108	447,661,492	1.5
	6.0	360	42,080,499	541,206,089	0.3
	4.0	240	27,503,493	353,610,364	0.8
2nd	5.0	300	33,816,287	434,773,489	0.6
	6.0	360	40,705,522	523,347,872	0.2
	4.0	240	NA	360,228,245	2.2
Both	5.0	300	NA	441,217.491	1.9
	6.0	360	NA	532,276,980	1.7

**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 ug of IgG Reference Standard*

Table 2. Intermediate Precision for IgG Monomer

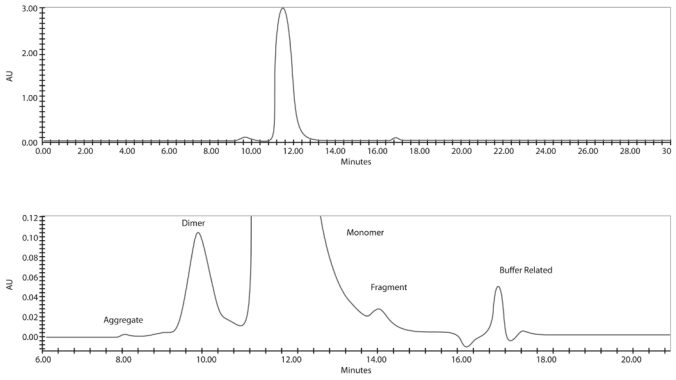

Figure 3. HPLC size exclusion of IgG sample in formulation buffer, 300 µg injected.

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 samples preparations. 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.

Linearity of Sample: Preparations of IgG sample ranging from 2.5 mg/mL to 7.5 mg/mL (150 g/injection to 450 g/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

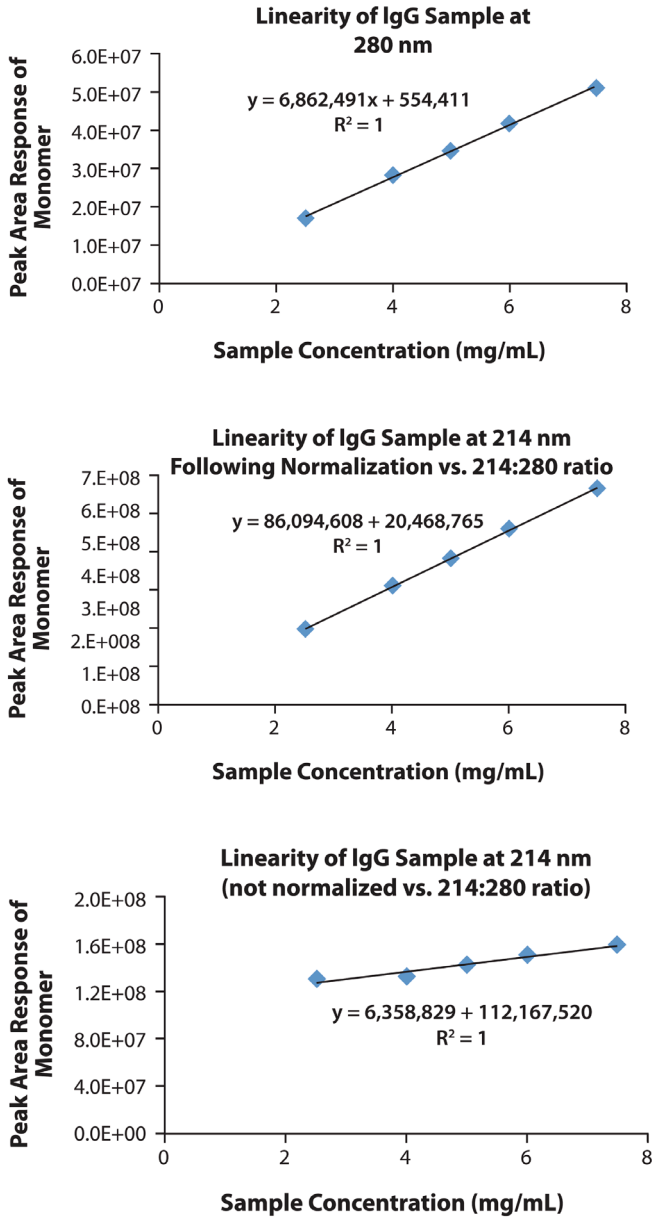

Figure 4. Linearity for Peak Area Responses of the IgG monomer (at both 280 nm and 214 nm) as well as Dimer, Aggregate, and Fragments at 214 nm Observed in IgG Sample Preparations

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

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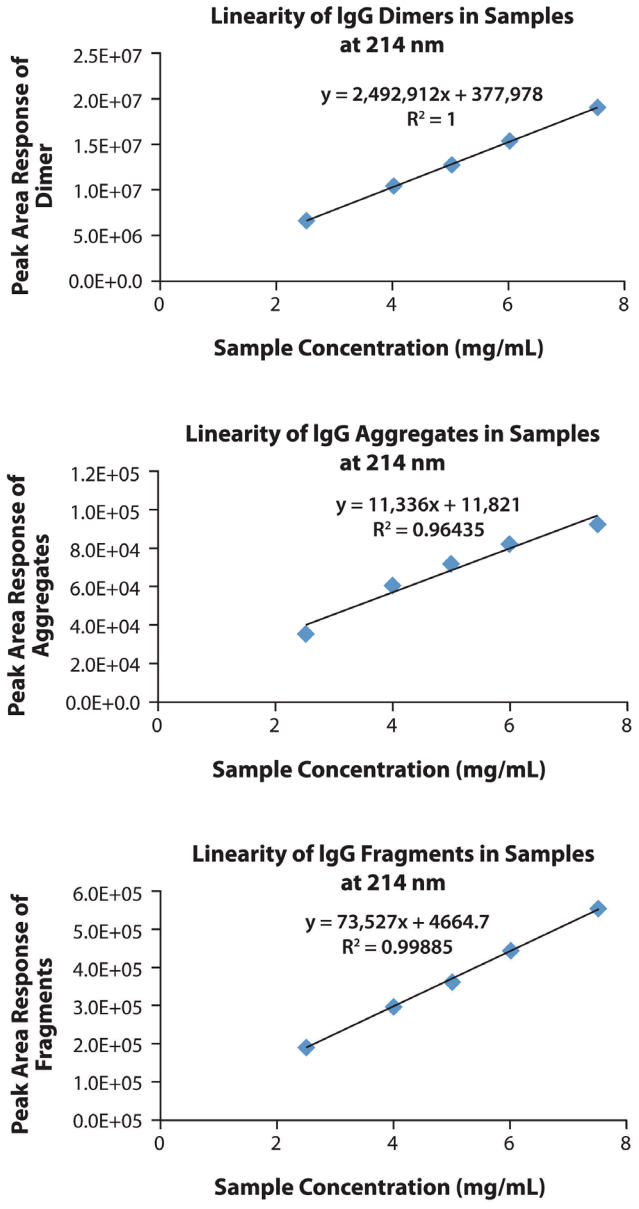

Figure 4 (Continued). Linearity for Peak Area Responses of the IgG monomer (at both 280 nm and 214 nm) as well as Dimer, Aggregate, and Fragments at 214 nm Observed in IgG Sample Preparations

regression as shown in Table 3. Precision for the monomer peak did not exceed 1.7% RSD at all concentrations. Additionally, determination of the apparent molecular weight of the IgG 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).

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

Table 3. Accuracy of Monomer at 280 nm

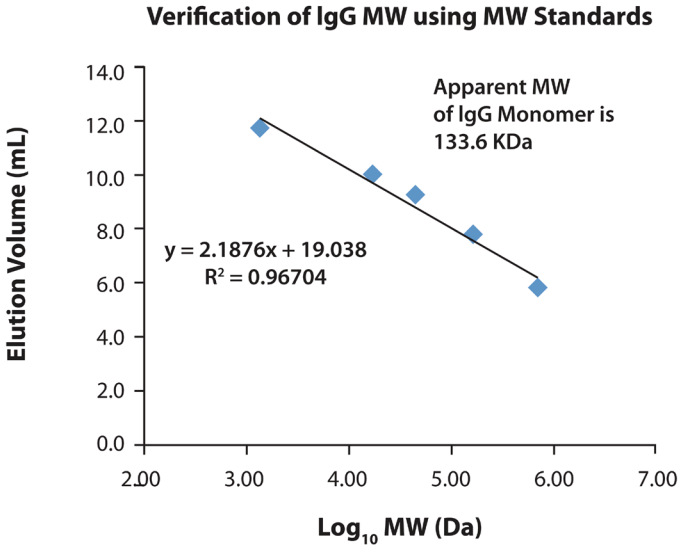

Figure 5. Verification of IgG Monomer MW by Comparison to Known MW Standards

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.

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 equation:

- **LOD = 3.3 x Standard Deviation of Intercepts/Average of Slopes**

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IgG Monomer	LOD		LOQ	
	mg/mL	Fold Increase	mg/mL	Fold Increase
280 nm after normalizing using the 214:280 ratio	0.18	6.3	0.54	6.4
214 nm without normalization	1.14		3.45	

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.

- **LOQ = 10 x 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.

REFERENCES

Bond M.D., et al., "Evaluation of a Dual-Wavelength Size Exclusion HPLC Method With Improved Sensitivity to Detect Protein Aggregates and Its Use to Better Characterize Degradation Pathways of an IgG1 Monoclonal Antibody", Journal of Pharmaceutical Sciences, vol. 99, No. 6, June 2010.

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