Application Note

Biosimilars

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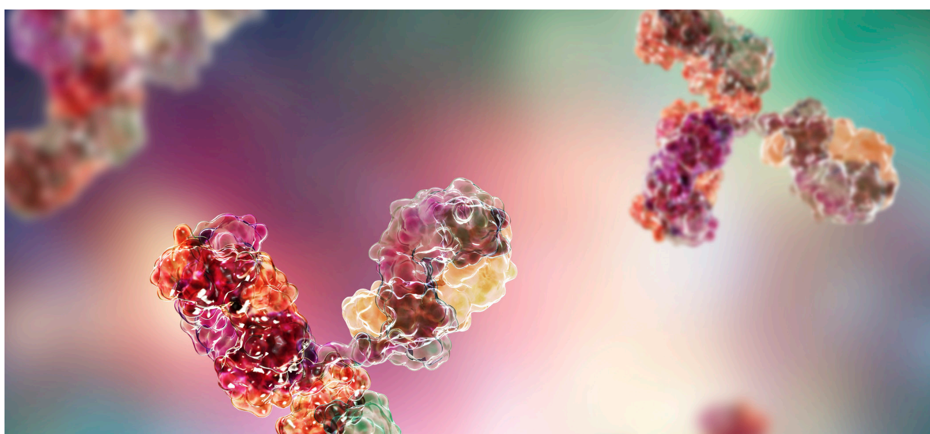
Conformational Studies Using Second Derivative UV-Vis Absorption Spectroscopy

Comparing an innovator and biosimilar monoclonal antibody pair



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Introduction

Monoclonal antibodies (mAbs) are gaining a lot of attention due to their therapeutic value in human health (*1*). Biosimilars are a copy of the innovator mAb for a particular disease and, due to patent expiry of the innovator, these molecules are rapidly expanding in the market (*2*). Some of the methods used to study protein conformational stability (*3*) and show comparability between innovators and biosimilars include UV spectroscopy, circular dichroism, and fluorescence (*4*). This study used an Agilent Cary 60 UV-Vis spectrophotometer to analyze an innovator (rituximab) and biosimilar pair. Data were subsequently interrogated using the proprietary Cary WinUV software, and a detailed comparison was done using second derivative absorption spectra. The results not only highlight the intrinsic capabilities of the Cary 60 as an analytical solution for this application, but also demonstrate how the collected data can provide information about the microenvironment of the aromatic amino acids in the protein globular structure, and therefore about the stability of the mAbs under various test conditions.

Benefits of UV-Vis spectroscopy

UV-Vis spectroscopy is a mature and reliable technology that provides insight into gross protein structure. It is widely available, and requires no expensive reagents or consumables. Importantly, UV-Vis spectrophotometry is also nondestructive, therefore, samples that are measured may be recovered. This key attribute makes UV-Vis an ideal orthogonal tool for use prior to further analysis by techniques such as LC or mass spectroscopy.

The Cary 60 UV-Vis spectrophotometer is a robust and efficient analytical tool. The optical design, based on a Xenon flash lamp, is unique in preventing sample photodegradation, and aids productivity by removing the need for instrument warm-up time. These features ensure that the Cary 60 UV-Vis spectrophotometer is routinely used to yield accurate, precise, and repeatable data in a range of application areas

Materials and methods

L-phenylalanine, L-tryptophan, and L-tyrosine were obtained from Agilent Technologies. Rituximab innovator and biosimilar were obtained from a local pharmacy. All other chemicals were obtained from Sigma-Aldrich.

Sample preparation

The antibodies were prepared in Tris HCl buffer (pH 6.0) at a concentration of 500 mg/mL. Guanidine hydrochlorate (GdnHCl)-induced denaturation of the mAb was performed using GdnHCl concentrations ranging from 0 to 6 M. The samples were prepared by mixing appropriate amounts of protein stock (10 mg/mL) and solutions containing 0 to 6 M GdnHCl in Tris HCl buffer (pH 6.0). L-phenylalanine, L-tryptophan, and L-tyrosine were prepared in water.

Absorbance measurements and data analysis

High-resolution absorbance spectra were obtained using a Cary 60 UV-Vis spectrophotometer. All samples were analyzed at room temperature in a 3 mL quartz cuvette with a 1 cm path length. Spectra were collected over a 240–350 nm wavelength range using a data interval of 0.5 nm.

Second derivative spectra were calculated using the Savitzky Golay algorithm with a five-point data filter within the Cary WinUV software application. A spline function was applied to the resulting derivative spectra using 99 interpolated points between each raw data point to enhance the resolution to 0.01 nm for monitoring antibody unfolding induced by GdnHCl.

Results and discussion

Second derivative absorption spectroscopy is useful for the analysis of the microenvironment of tryptophan, tyrosine, and phenylalanine residues (W, Y, and F respectively) as an indication of protein structure (*5,6*). The ability of the second derivative spectra to resolve the spectral bands of aromatic amino acids permits monitoring a specific shift in absorbance when the protein changes from an ordered to a disordered state. Figure 1 shows a scan and corresponding second derivative spectra for free L-aromatic amino acids in addition to a monoclonal antibody. When we look at each second derivative absorption spectrum, characteristics of these amino acids and the proteins are clearly identified. The negative peak at 291 nm in the second derivative spectra was selected as an indicator to monitor the changes in the microenvironment of the tryptophan residues in the biosimilar and innovator mAbs.

Second derivative absorption spectra were collected using a Cary 60, and used to compare the structural stability of innovator and biosimilar rituximab by comparing the susceptibility of each to denaturation in the presence of the chemical denaturant GdnHCl. Figure 2 shows the second derivative spectra of innovator and biosimilar at 0 M and 5 M GdnHCl concentration. Examination of the spectra shows a shift in the negative absorption peak around 291 nm (as shown by the arrow in Figure 2), when mAb samples are exposed to GdnHCl. This indicates changes in the microenvironment of tryptophan residues in the mAbs.

The denaturation of the innovator and biosimilar was compared using increasing concentrations of GdnHCl. Figure 3 shows the wavelength shift that occurred when the mAbs were exposed to increasing concentrations of GdnHCl. Both the innovator and bisomilar become denatured at a similar GdnHCl concentration, suggesting that the microenvironment of tryptophan residues within the protein structure are similar. This result confirmed earlier studies using reverse-phase high-performance liquid chromatography (RP-HPLC), size exclusion chromatography (SEC), and peptide mapping to show that this mAb pair is similar in both primary and higher order structure using Agilent HPLC instruments(*7*).

Comparison of the protein structure was also possible by analyzing the microenvironment of tyrosine residues in the protein sequence using derivative spectroscopy to generate an a/b ratio*(8,9)*, where a is the distance between the maximum ($\sim\lambda_{288}$) and minimum ($\sim\lambda_{283}$) absorbance of the negative peak contributed by tyrosine, and b is the distance from a second maximum ($\sim\lambda_{294}$) and minimum ($\sim\lambda_{291}$) contributed by tryptophan absorbance (Figure 4 inset).

Figure 4 shows that the a/b ratio changes as protein structure changes in the presence of increasing concentrations of GdnHCl. At low concentrations, the a/b ratio is approximately 1.05, which is consistent with a protein globular structure[9]. At high concentrations, the a/b ratio increases, indicating that the tyrosine residues are becoming more exposed. The innovator and biosimilar show similar spectral profiles, suggesting a similar microenvironment of tyrosine for both mAbs.

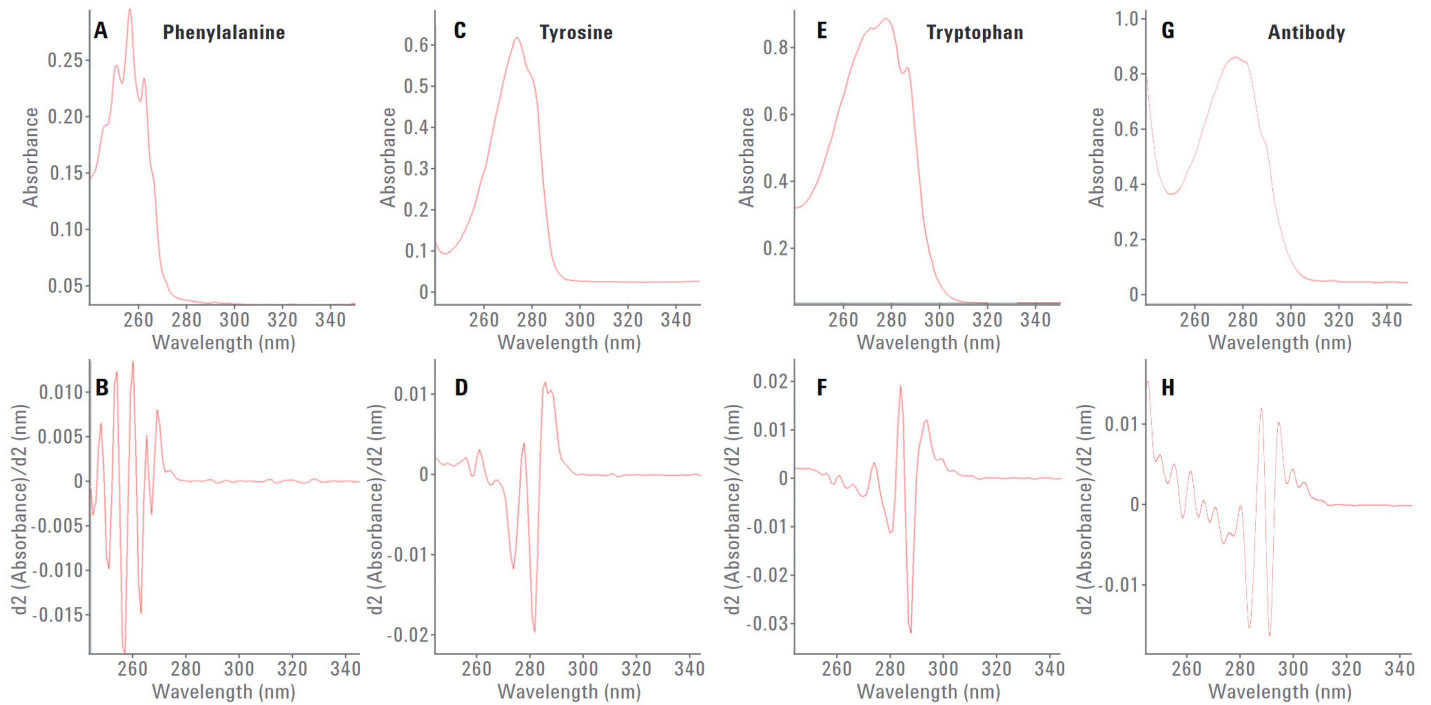

Figure 1. Absorbance and second derivative spectra for phenylalanine (A, B), tyrosine (C, D), tryptophan (E, F), and antibody (G, H).

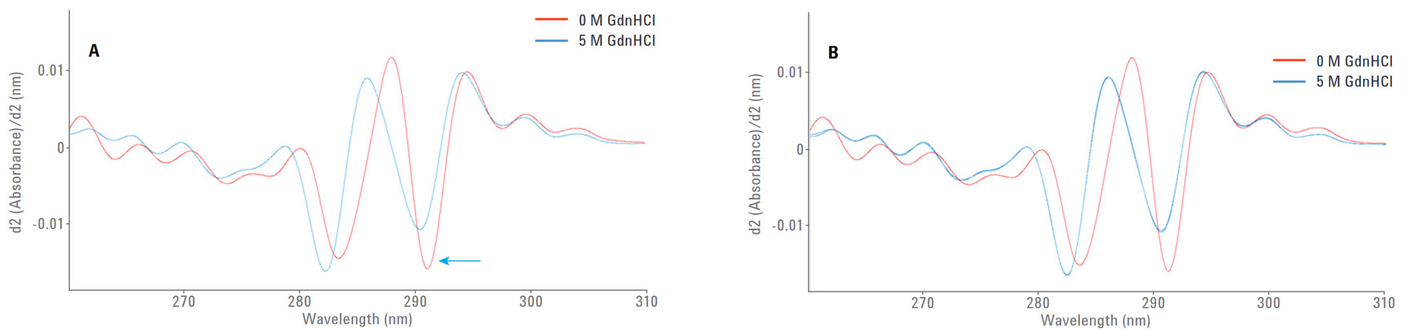

Figure 2. Second derivative spectra for innovator (A) and biosimilar (B) in the presence of 0 M GdnHCl (red trace) and 5 M GdnHCl (blue trace).

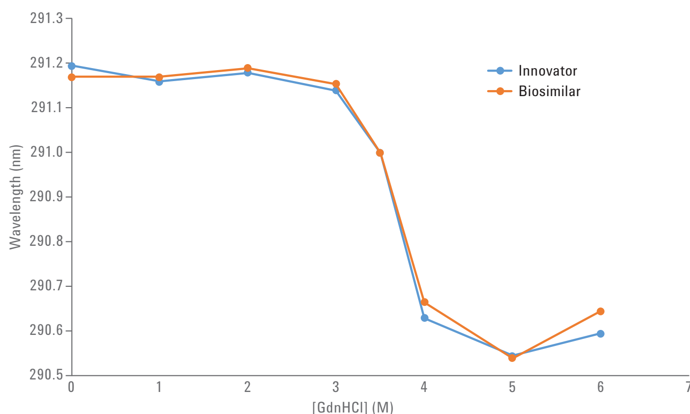

Figure 3. Denaturation profile for innovator and biosimilar against GdnHCl concentration.

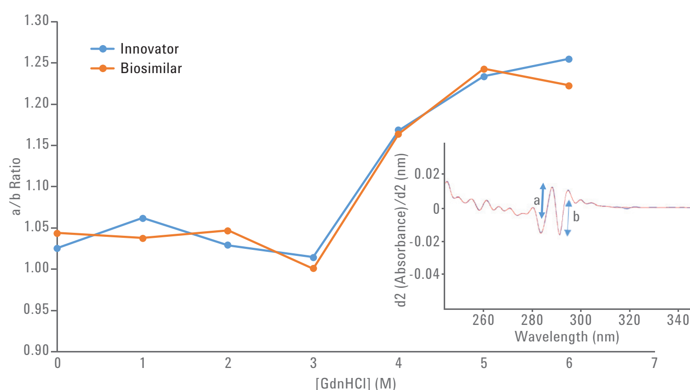

Figure 4. The a/b ratio for innovator and biosimilar at different concentrations of GdnHCl. The inset shows the definitions for a and b.

The capacity of UV-Vis spectrophotometry to yield precise, detailed, and accurate information such as this highlights the potential of the technique. In addition, the potential use of an inexpensive and easy-to-use technique such as UV-Vis spectrophotometry installed in a laboratory as an alternative analytical technique that can alleviate the workload of other critical analytical instrumentation cannot be underestimated.

Conclusion

- The Agilent Cary 60 UV-Vis spectrophotometer provides the resolution and accuracy necessary to compare the protein structure of an innovator and biosimilar mAb pair.
- The studied mAb innovator and biosimilar pair show a similar unfolding pattern towards a chemical denaturant (GdnHCl). This suggests a similar gross protein structure for the mAb pair.
- This study also demonstrates the use of second derivative absorption spectroscopy for comparing innovator and biosimilar mAbs for structural studies.

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