Application Note
June 2021
AN-850-0119

Biosimilar Comparison and Accelerated Stability Predictions Based on <2% Secondary Structure Differences Using Microfluidic Modulation Spectroscopy (MMS)

- [x] Research
- [x] Discovery
- [ ] Formulation and Development
- [x] Quality Control
- [ ] Manufacturing

- [x] Aggregation
- [ ] Quantitation
- [x] Stability
- [x] Structure
- [x] Similarity

Introduction

Biosimilar molecules are designed to structurally mimic innovator molecules that have been approved and are available on the market[1]. These are important due to the opportunity they create for competition within the market by creating greater patient access to many therapeutic medicines. The ability to measure even the smallest structural differences between engineered biosimilars and their innovator molecules is vital to ensure successful approval and has utility for a wide range of related applications.

In the first part of this study, Microfluidic Modulation Spectroscopy (MMS) demonstrates that differences in secondary structure of <2% between samples can be confidently detected and differentiated[2] due to highly-reproducible replicate measurements generated by the AQS^{3}pro. Spectral artifacts in the Amide I band which often cause difficulty during analysis using FTIR are automatically corrected[3], making MMS an ideal tool for characterizing biosimilars even in complex backgrounds.

In Part 1, commercially available insulin Humalog® and an in-house Humalog biosimilar prepared by an undisclosed European research facility and were confirmed to have very similar structures, particularly in α-helix and β-sheet, but subtle differences in turn and disordered structure (<2%) were identified.

Sample Name	Storage Temperature (°C)
1a	4
1b	30
2a	4
2b	30
3a	4
3b	30
4a	4
4b	30

Table 1: Storage temperatures for four pairs of accelerated stability test samples

The second part of this study involved four pairs of different insulin biosimilars in a formulation buffer held at 4°C and 30°C for a duration of 8 weeks for use as an accelerated stability study[4,5]. This experiment challenged the MMS technique to predict the relative order of sample stability based on the very small secondary structure differences detected between sample pairs after incubation in order to predict stability over longer time periods. Based on these results, samples were placed in ranked-order by comparison of the magnitude of secondary structure change that was corroborated with supplemental data.

This study demonstrates that MMS can be used for stability studies due to its ability to detect very small structural changes that are invisible to traditional technologies. Additionally, by using similarity analysis, a simple numerical output was generated by AQS^{3}delta analytics allowing samples to be rapidly compared and ranked for facile interpretation of results.

Methods

Part 1 of the study compared two insulin samples: a commercially available insulin Humalog standard (innovator) and an in-house insulin biosimilar molecule. Part 2 samples consisted of four (4) pairs of different insulin biosimilar samples incubated at both 4°C and 30°C for a duration of 8 weeks in an accelerated stability study.

All insulin samples for both parts of the study were prepared directly by dissolving the lyophilized powders in a formulation buffer that contained a polyelectrolyte with undisclosed components. Sample pairs for Part 2 stability studies were prepared originally as one stock for each of the four different biosimilars divided into two equal samples and then incubated at either 4°C or 30°C for duration of 8 weeks (Table 1). The incubation temperature of 30°C represents the temperature just below the first T_m as measured by Differential Scanning Calorimetry (DSC) - data not shown.

Application Note
June 2021

Methods, continued

Data processing was performed using the Data Analysis software package included with the AQS³delta control system. Higher order structure data was fitted using gaussian regression analysis informed by Dong et al[6] for this protein structure type. Similarity analysis was performed using area of overlap comparison to the indicated reference sample between inverted and baselined second derivative spectra, giving a percentage similarity score that was used to rank long-term sample stability among the four pairs of biosimilars.

Results

Part I: Comparison of In-House Insulin and Commercial Humalog Biosimilar

I. Similarity: In the first part of this study, the structural similarity of an in-house insulin standard was compared to a commercial insulin Humalog standard as part of validation of the in-house molecule for potential use as a standard in future experiments. Similarity analysis by area of overlap demonstrated >98.5% spectral similarity between the molecules (Table 2).

Sample	Fitted Conc (mg/mL)	Similarity %
Commercial Humalog	3.43	100
In-House Insulin Biosimilar	3.13	98.58

Table 2: Similarity Results for an Insulin Biosimilar vs Commercial Humalog

MMS absorbance data was collected automatically for 3-5 replicates of each sample from both parts of the study using a RedShiftBio AQS³pro system with a 24-well plate configuration. Formulation buffer was used as the reference buffer for background subtraction and was modulated with the samples during analysis at a rate of 1 Hz and 5 psi backing pressure for all measurements.

II. Higher Order Structure (HOS): HOS deconvolution of the two biosimilar molecules identified a small disordered to turn (<2%) transition between the commercial and internal standards, and no significant difference was observed in α-helix and β-sheet content between the pair (Figure 1). Calculated as part of the measurement, the fitted concentrations of both samples were compared to those given to confirm there was no loss due to aggregation.

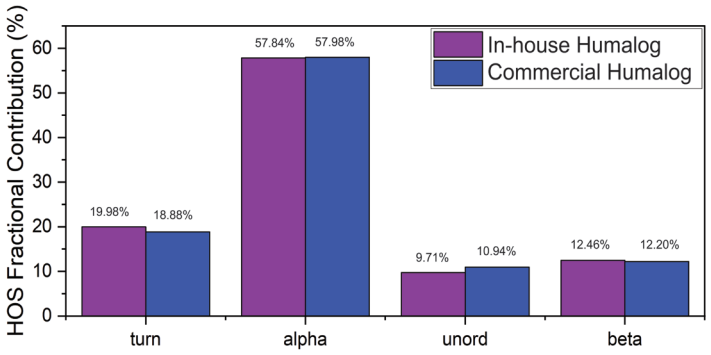

Figure 1: Higher-order structure (HOS) comparison of the commercial Insulin Humalog with an in-house Humalog biosimilar

Part II: Insulin Biosimilar Accelerated Stability Study

I. Second Derivative - Replicate Similarity: For the stability study of four different insulin biosimilar molecules, the similarity across five replicates for a single sample was measured to establish a similarity baseline and variation to use as a reference for the accelerated stability samples in Part 2. Figure 2 shows the reproducibility data for 5 replicates of Sample 1a incubated at 4°C based on the overlay of the second derivative spectra for all replicates.

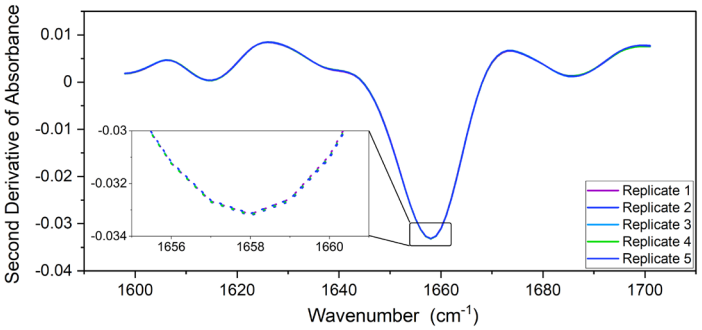

Figure 2: Overlaid second derivative spectra of 5 replicates of sample 1a at 4°C, 6.8 mg/mL

Application Note
June 2021

Results, continued

The similarity calculated between the 5 replicates of Sample 1a resulted in a similarity for each replicate of > 99.48% and an average of 99.59 +/- 0.07% when compared to the first replicate (Table 3). Differences between the 4°C and 30°C stability samples should be greater than this internal variance to be considered significant.

Replicate	Similarity (%)
1	100.00*
2	99.66
3	99.57
4	99.48
5	99.66
Avg of 2-4	99.59
SD	0.07

Table 3: Similarity Results for Replicates of 4°C Stability Sample 1a relative to the first replicate

II. Second Derivative Analysis and Stability: A second derivative plot for 3-5 replicates of the absolute absorbance for all four stability sample pairs at 4°C and 30°C is shown in Figure 3. The plot for Humalog is included for information only. The overlaid second derivative spectra correlate with the higher-order structure analysis results and reveal peaks at 1656 cm^{-1} and 1618 cm^{-1} corresponding to α-helix and β-sheet respectively.

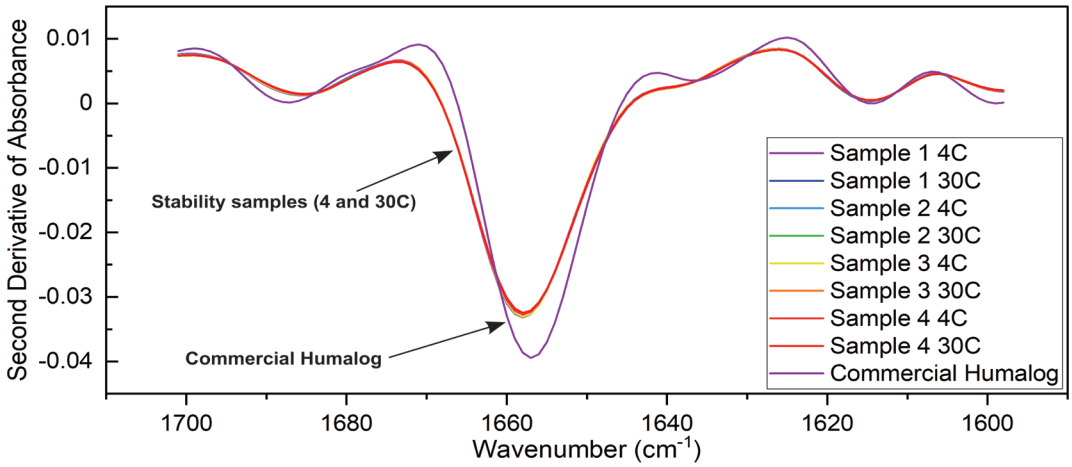

Figure 3: Second derivative spectra of all sample pairs in this study highlighting differences between second derivative spectra of a commercial standard Humalog and four pairs of stability samples incubated at 4 and 30°C

III. Similarity of Stability Samples: Similarity analysis was performed for the four pairs of stability samples and individual comparisons were made using the 4°C sample from each pair as a reference to calculate similarity for the 30°C samples (Table 4).

Sample Name	Storage Temperature (°C)	Nominal Conc. (mg/mL)	Fitted Conc. (mg/mL)	Similarity % vs paired 4C sample
1a	4	7.2	6.77	100.00
1b	30	7.2	6.77	99.10
2a	4	7.2	6.84	100.00
2b	30	7.2	6.84	99.28
3a	4	7.2	6.98	100.00
3b	30	7.2	6.98	99.50
4a	4	7.2	6.70	100.00
4b	30	7.2	6.70	99.36

Table 4: Similarity and Calculated (Fitted) Concentration comparisons relative to the 4°C sample for each pair

Application Note
June 2021

Results, continued

Differences of less than 1% were observed between each 4°C and its related 30°C sample for the series. However, all differences were larger than the replicate-to-replicate average variance of 0.4 +/- 0.07% shown in Table 3, demonstrating that although small, these differences are significant. Sample 3 most retains its structure at higher temperature, with a similarity of 99.50%, whereas Sample 1 shows the most change at 99.10% relative to the 4°C counterparts. Samples 2 and 4 showed structural change between these extremes, with similarities of 99.28% and 99.36% respectively.

IV. Quantitation: Concentrations of each sample were calculated by area of overlap and compared to a standard of known concentration as indicated in Table 4. Calculated sample concentrations were within experimental error of nominal concentrations, and most importantly did not significantly change between sample pairs at the two incubation temperatures indicating that no material was lost due to aggregation for any of the four samples.

V. HOS: Higher-order structure (HOS) plots were generated to show the micro-changes in four secondary structure types between the four formulated modified insulin samples held at 4°C and 30°C for 8 weeks. Figure 4 shows higher-order structure plots for each stability sample pair and indicates small but significant differences between them. The percentage values of each structure type indicate that in all four pairs, the HOS contribution of α-helix decreased between the 4°C and 30°C samples, and the amount of β-sheet increased for all four sets between the 4°C and 30°C samples.

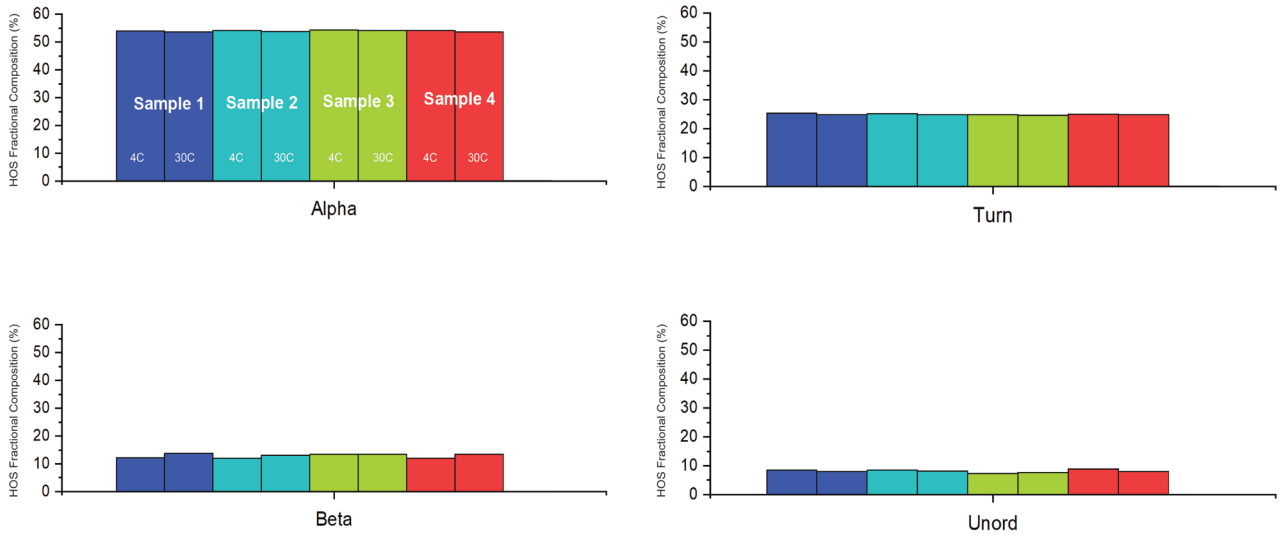

Figure 4: Higher-order structure (HOS) plot for pairs of formulated modified insulin samples held at 4°C and 30°C for 8 weeks

Application Note
June 2021

Results, continued

VI. Percent Change in HOS: Due to the small differences measured, all changes in secondary structure that are shown in Figure 4 between sample pairs for each secondary structure type at both temperatures were calculated and transformed into a differential HOS plot (Figure 5) using the calculation: ((% structure at 30°C – % structure at 4°C)/(% structure at 4°C)) x 100. Differences between samples are accentuated using this method, highlighting those which show greater stability to temperature and the direction of change (positive or negative).

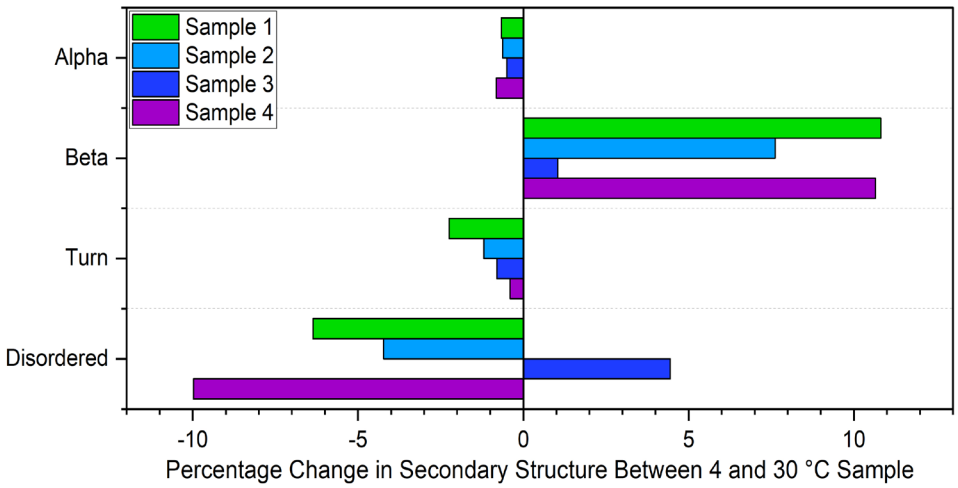

Figure 5: Differential graph between the 4°C and 30°C for each structural population. Left side shows a decrease in the secondary structure type and right shows an increase

By evaluating higher-order structure data as percent change between 4 and 30°C sample pairs, it is evident that sample 3 is the most stable of the series with the least change seen in almost all categories of HOS. This correlates with the % similarity results discussed previously. The smallest increase in β-sheet structure, which commonly indicates the formation of structural aggregates, was also observed in Sample 3 predicting that is least likely to form amyloidogenic species. Unlike the others in the series, Sample 3 showed an increase in disordered structure with incubation that did not develop to form β-structured aggregates, indicating that this mutant does not favour β-sheet formation by sequence in this formulation buffer.

Samples 1 and 4 showed the greatest increase in β-sheet structure on incubation, and are most likely to aggregate as indicated by this data. They also show slightly different behaviour to one another which may have an impact on their respective mechanisms of aggregation. Sample 2 is similar to Samples 1 and 4, but shows a lesser degree of destabilisation, demonstrating that this is the second most stable sample of the series. Stability of this series in decreasing stability was therefore predicted to be 3, 2, 4, 1 by MMS, which correlated directly with previous and proprietary stability data gathered (not shown) and verifies the stability order of Sample 2 and 4.

Application Note
June 2021

Conclusions

MMS is a revolutionary new infrared-based technology that enables more sensitive and reproducible secondary structure information for proteins compared to traditional spectroscopic techniques such as FTIR, even in complex or absorbing buffers. Because measurements are made using a quantum cascade laser in combination with a microfluidic flow cell, the influence of water or other components is automatically corrected alongside any variations in the light source or detector. Measurements can therefore be made in simple and complex formulations with equal ease, across a very wide concentration range (0.1 to >200 mg/mL) with no labelling or other sample adulteration.

Utilising the extremely high sensitivity and reproducibility of MMS, a biosimilar comparison was performed in Part 1 between a commercial and an in-house standard Insulin Humalog sample. Analysis revealed very similar overall structures, but also identified less than 2% differences in turn and disordered structure that may have been obscured with less sensitive technologies. No significant differences were observed in α-helix and β-sheet content between the pair.

A similar detailed comparison of the very small differences observed between 4 related modified insulin sample pairs held at 4°C and 30°C for 8 weeks was used in Part 2 as an accelerated stability study to predict stability over longer time periods. This analysis was performed by comparison of the magnitude of secondary structure change between pairs, and also by spectral similarity analysis that was automatically generated by the AQS^{3}delta analytics package included with the AQS^{3}pro. Sample stability was correctly predicted to be 3, 2, 4, 1 in decreasing order, as corroborated with internal but undisclosed data.

References

1. Nupur N., mAbs, 2018, 10(1): 143-58
2. Kendrick B. et. al., J. Pharma. Sci., 2019, 1-4.
3. Kong J. et. al., Acta Biochimica et Biophysica Sinica, 2007, 39(8): 549-559.
4. Matheus S. et. al., Pharm. Res., 2006, 23(7):1617-27
5. Lin S. et. al., Adv. Drug Deliv. Rev., 2012, 64(5): 461-786.
6. Dong A. et. al., Biochem., 1990, 29: 3303-08.

Author: P. King, Ph.D.

Contact

RedShift BioAnalytics, Inc
131 Middlesex Turnpike | Burlington, MA | 01803
T: 781.345.7300 | F: 781.345.7301 | E: info@redshiftbio.com