Application Note
MAR 2025
AN-850-0147

Structural Characterization of GLP-1 Analogues and Formulations Using Microfluidic Modulation Spectroscopy

- Biosimilars
- mAbs
- ADCs
- AAVs
- Ligand Binding
- ☑ Protein/Peptide Analysis
- VLPs
- Nucleic Acid
- Fusion Proteins
- Enzyme Analysis

- Aggregation
- Quantitiation
- ☑ Structure
- ☑ Stability
- ☑ Similarity

Abstract

Glucagon-like peptide-1 (GLP-1) analogues, such as semaglutide and liraglutide, are widely used in treating obesity and type 2 diabetes due to their enhanced stability and prolonged half-life. Their secondary structure plays a critical role in receptor binding stability, and efficacy, making structural characterization vital for drug development and quality control of these peptides. This application note demonstrates how MMS can be used to quantify peptide concentrations and determine structural differences between unmodified GLP-1 and the commercial GLP-1 analogues in different buffer systems, highlighting the advantages of MMS as a biophysical tool for the development of peptide therapeutics.

Figure 1: In this application note, we discuss the structure/function of GLP-1 (7-37), liraglutide, and semaglutide. The primary sequences of each peptide with all chemical modifications can be seen in the top left part of the figure.[1] Crystal/NMR structures of these peptides indicate they are mostly alpha-helical in nature (shown in the bottom left is liraglutide, PDB ID: 4APD). We analyzed each peptide in the Aurora instrument pictured in the center of the figure and generated HOS bar charts as seen on the right.

Introduction

Glucagon-like peptide-1 (GLP-1) analogues have emerged as a cornerstone in the treatment of type 2 diabetes and obesity, offering significant therapeutic benefits by enhancing glucose-dependent insulin secretion, promoting satiety, and supporting weight loss.[2] Synthetic GLP-1 receptor agonists such as semaglutide, (the active ingredient in Mounjaro®, Ozempic®, Rybelsus®, and Wegovy®) and liraglutide (the active ingredient in Victoza® and Saxenda®) are engineered for improved stability, half-life, and efficacy compared to native GLP-1, making them essential therapeutic agents in metabolic disease management.[3] The modifications can be seen in Figure 2. Liraglutide has an arginine replacing the lysine at position 34 (K34R) and a 16- chain fatty acid attached to a glutamic acid spacer on lysine 26. These small modifications lead to a 13-hour half-life compared to GLP-1 (7-37)'s half-life of 2 minutes. This makes liraglutide ideal for type 2 diabetes single day injections. Semaglutide has the same K34R amino acid modification and a similar fatty acid attached to lysine 26, however, semaglutide also has a modification where alanine 8 is replaced with α-aminoisobutyric acid. This makes it resistant to dipeptidyl peptidase 4 (DPP-4) cleavage and increases the half-life to 1 week.[4] This allows semaglutide to be used in a weekly injection dosage for type 2 diabetes. The rapid expansion of GLP-1 analogues in clinical and commercial pipelines underscores the need for robust biophysical characterization techniques to ensure their structural integrity, stability, and function.

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Introduction, continued

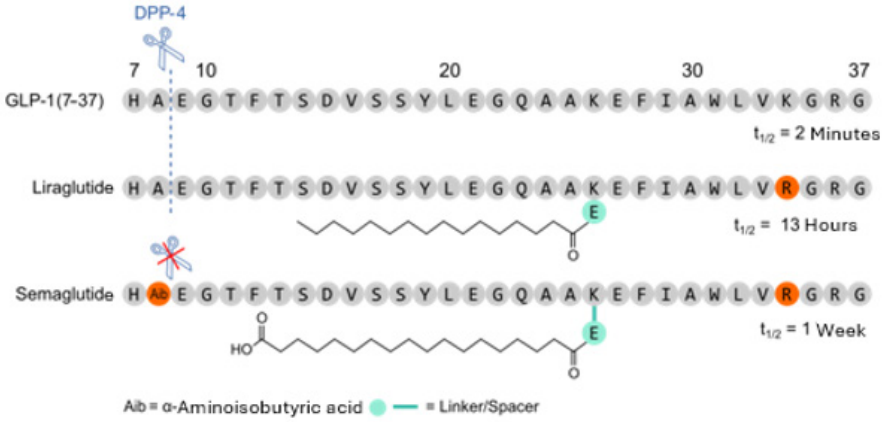

Figure 2: Primary sequences of GLP-1(7-37), liraglutide, and semaglutide, including all chemical modifications and half-lives ($t_{1/2}$). This figure was modified from the original by translating the half-lives to English from German.[1]

Structural characterization of GLP-1 analogues is critical for understanding their secondary structure, which directly influences receptor binding, bioactivity, and formulation stability. Minor conformational changes can impact drug efficacy, immunogenicity, and degradation pathways, necessitating precise analytical tools for quality control and formulation development. Traditional methods such as circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopy have been used to study peptide secondary structures, but these approaches often lack the required sensitivity, reproducibility, or the ability to analyze peptides in complex formulations. Microfluidic Modulation Spectroscopy (MMS) presents a powerful alternative for secondary structure analysis of GLP-1 analogues, offering high sensitivity reproducibility, and the ability to analyze samples in native and formulated conditions.

By leveraging mid-infrared spectroscopy with real time buffer referencing, MMS enables quantitative secondary structure determination, even in low-concentration and complex buffer environments. This application note explores the role of MMS in characterizing GLP-1 analogues, demonstrating its advantages in formulation development, stability assessment, and batch-to-batch consistency monitoring for next-generation peptide therapeutics.

Methods

Synthetic GLP-1 (7-37), semaglutide, and liraglutide (powder, purity > 99.8%) were purchased from MedChemExpress. For PBS solutions, all three peptides were prepared in 1x PBS from 0.1 to 2 mg/mL. The semaglutide and liraglutide formulations were prepared according to the formulations of commercial Ozempic® and Saxenda®, respectively. Briefly, 2.7 mg of semaglutide powder was dissolved in 1 mL of formulation buffer containing disodium phosphate dihydrate (1.34 mg), propylene glycol (14 mg), and phenol (5.5 mg) at pH 7.4. For liraglutide, 6 mg of powder was dissolved in 1 mL of formulation buffer containing disodium phosphate dihydrate (1.42 mg), propylene glycol (14 mg), and phenol (5.5 mg) at pH 8.15. The differential absorbance spectra of each sample against its buffer reference were measured across the amide I band region (1712 – 1588 cm^{-1}) using the Aurora. Triplicate measurements were collected and averaged for each sample.

The data were analyzed using the RedShiftBio Analytics software package. First, the raw differential absorbance was converted to absolute absorbance by normalizing for concentration, macromolecular displacement of buffer, and optical pathlength. The second derivatives of the absolute absorbance spectra are automatically generated along with the "similarity plot" by inverting and baseline subtracting the second derivative plot. Gaussian curve fitting is then applied to the similarity plot to generate the higher-order structure (HOS) bar chart.

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Results

A full MMS characterization of a GLP-1 (7-37) concentration series (0.1, 0.5, 1, and 2 mg/mL) is presented in Figure 3. The raw differential absorbance data (Figure 3A) shows concentration-dependent signals, with the highest at 2 mg/mL. The concentration-normalized spectra, known as absolute absorbance spectra (Figure 3B), show increased noise level at 0.1 mg/mL, while all the other samples overlay very well. The system calculates the fitted concentrations, which were slightly lower than expected, in the normalization process reported in Figure 3C. The fit concentrations were 1.66, 0.78, 0.37, and 0.059 mg/mL. Possible reasons for lower concentrations include incomplete dissolution of the lyophilized powder or small impurities affecting the dry weight but not the protein signal. However, the excellent linearity ($R^2 > 0.9999$), highlights the ability of MMS to accurately calculate peptide concentration. Finally, the higher order structure (HOS) is calculated and presented in the bar chart in Figure 3D. As the concentration gets lower, the error bars increase. We recommend running samples at a concentration of at least 0.5 mg/mL for more accurate HOS readings. The major secondary structure component for GLP-1 is alpha helix, however, it appears to be concentration-dependent and the lower concentrations have less alpha helix and more turn structure quantified. This is consistent with a previous study demonstrating that the helicity of GLP-1 increases with concentration due to helix bundle formation at higher concentrations.[5]

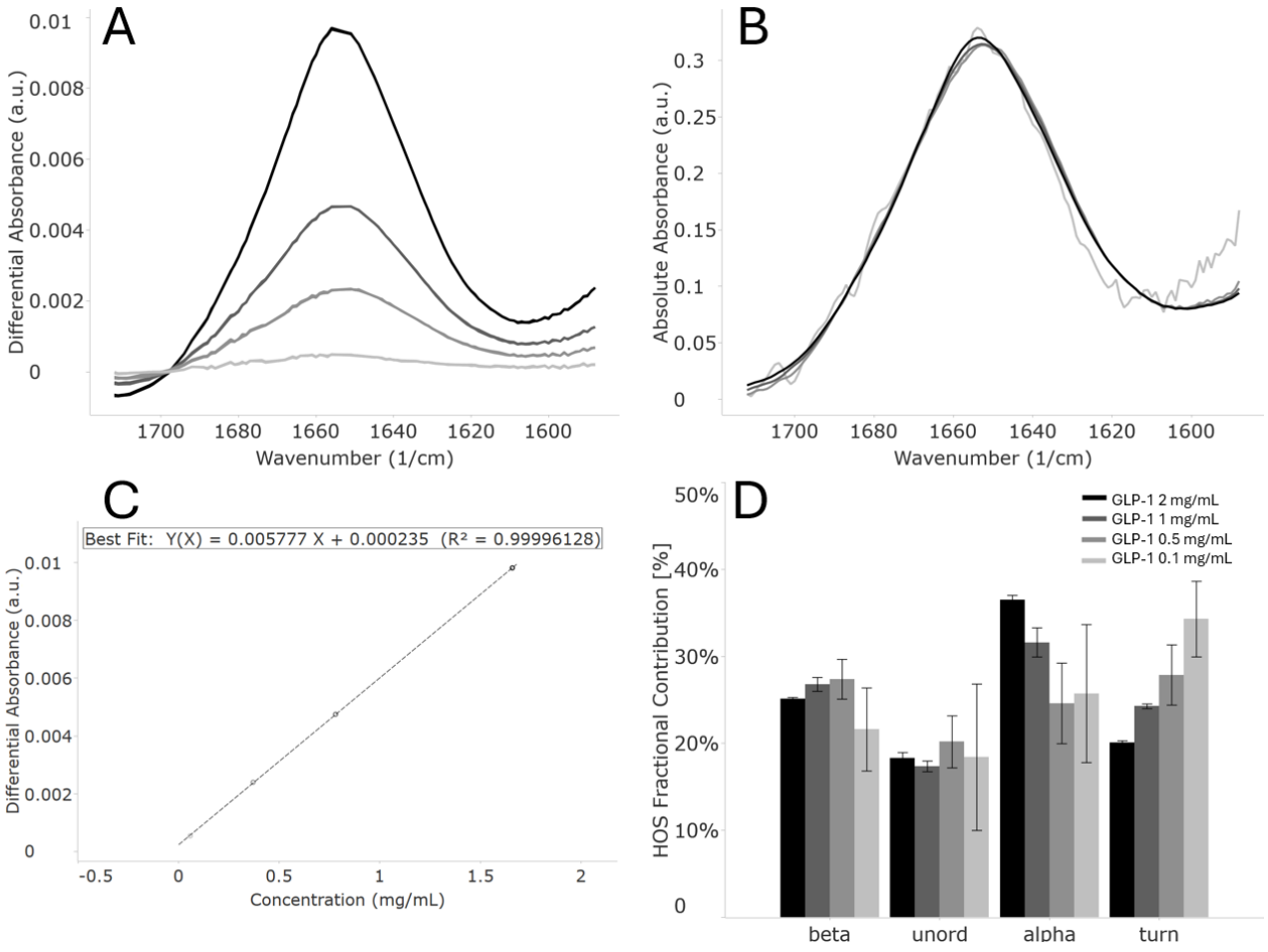

Figure 3: GLP-1 (7-37) MMS analysis including 2, 1, 0.5, and 0.1 mg/mL. (A) The raw differential absorbance plot shows the signal is proportional to the concentration, where the highest concentration, 2 mg/mL (in black), has the highest signal, as expected. When normalized for concentration, all the traces overlap, as seen in the absolute absorbance plot (B). The software automatically calculates the concentration and will generate the quantitation plot in figure (C). The HOS bar chart (D) shows GLP-1 is mostly alpha-helical, however, as the concentration decreases, so does the alpha helix content and in its place, turn structure increases. Additionally, as the concentration decreases, the error bars increase due to higher noise.

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Results, continued

We tested semaglutide using MMS following the same sample preparation and analysis as GLP-1. As seen in Figure 4, semaglutide shows high quality data for the concentration series tested between 0.1-2 mg/mL with fit concentrations of 1.78, 0.87, 0.44, and 0.11 mg/mL. Notably, the HOS is much more consistent at and above 0.5 mg/mL and appears more alpha-helical in nature compared to GLP-1 (7-37).

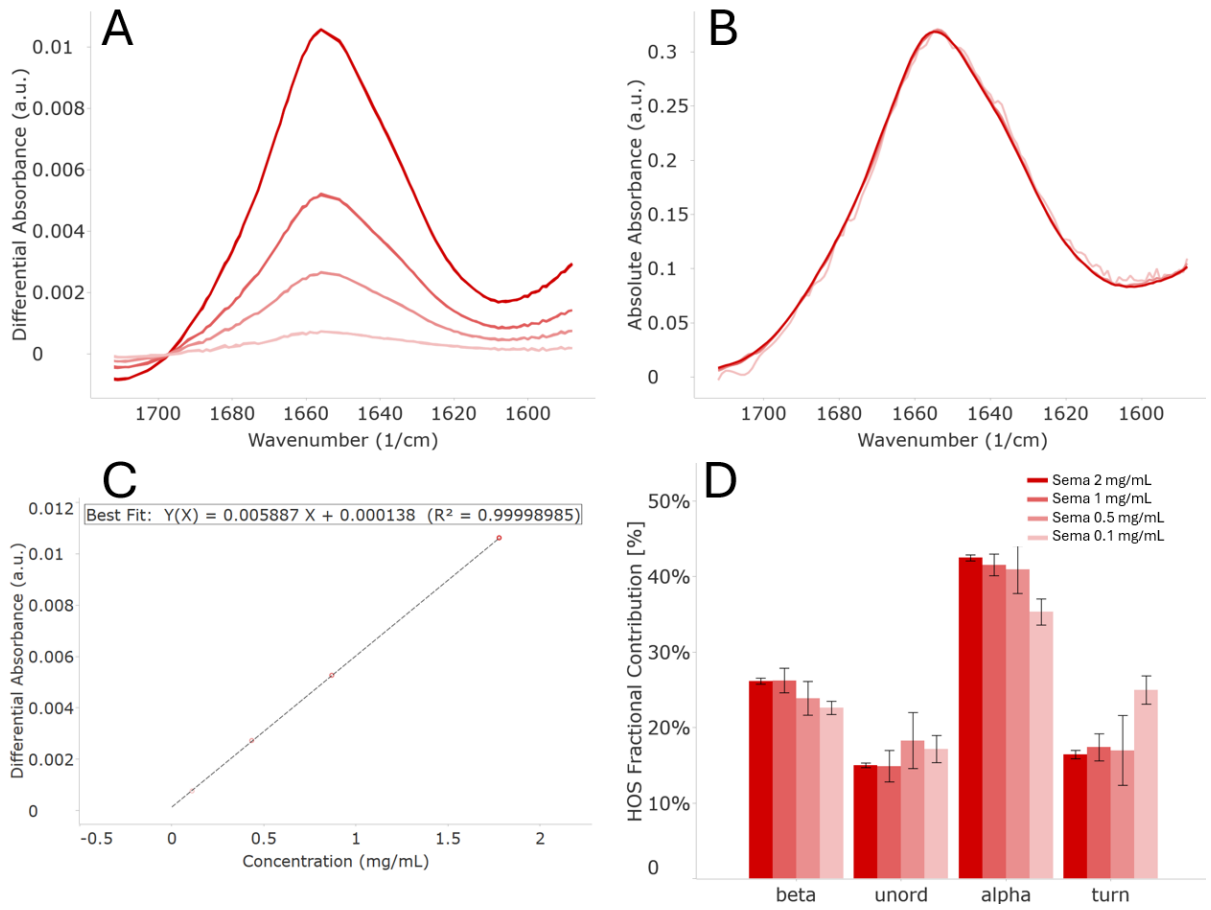

Figure 4: Semaglutide MMS analysis including 2, 1, 0.5, and 0.1 mg/mL. (A) The raw differential absorbance plot shows the signal is proportional to the concentration, where the highest concentration, 2 mg/mL (in bright red), has the highest signal, as expected. When normalized for concentration, all the traces overlap, as seen in the absolute absorbance plot (B). The software automatically calculates the concentration and will generate the quantitation plot in figure (C). The HOS bar chart (D) shows semaglutide is mostly alpha-helical and consistent at all concentrations with the exception of the 0.1 mg/mL sample . This sample shows less alpha helix and more turn structures. However, this sample also has the highest spectral noise and the HOS calculation can be affected by that.

The MMS characterization of liraglutide is shown in Figure 5. We show the raw data (Figure 5A) and the normalized spectra (Figure 5B). The concentration curve (Figure 5C) demonstrates high quality data in this series as the R^2 is > 0.99999 highlighting the ability to calculate concentration accurately even at very low concentrations like 0.1 mg/mL. The fit concentrations for the liraglutide concentration series are 1.46, 0.72, 0.36 and 0.082 mg/mL. The 0.5-2 mg/mL samples have very consistent HOS (Figure 5D) and, not surprisingly, the 0.1 mg/mL sample is more varied. Although MMS with Aurora has no problem quantifying the concentration of samples down to 0.1 mg/mL, we recommend concentrations > 0.5 mg/mL for the most accurate HOS characterization due to large differences in noise affecting the Gaussian curve-fitting.

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Results, continued

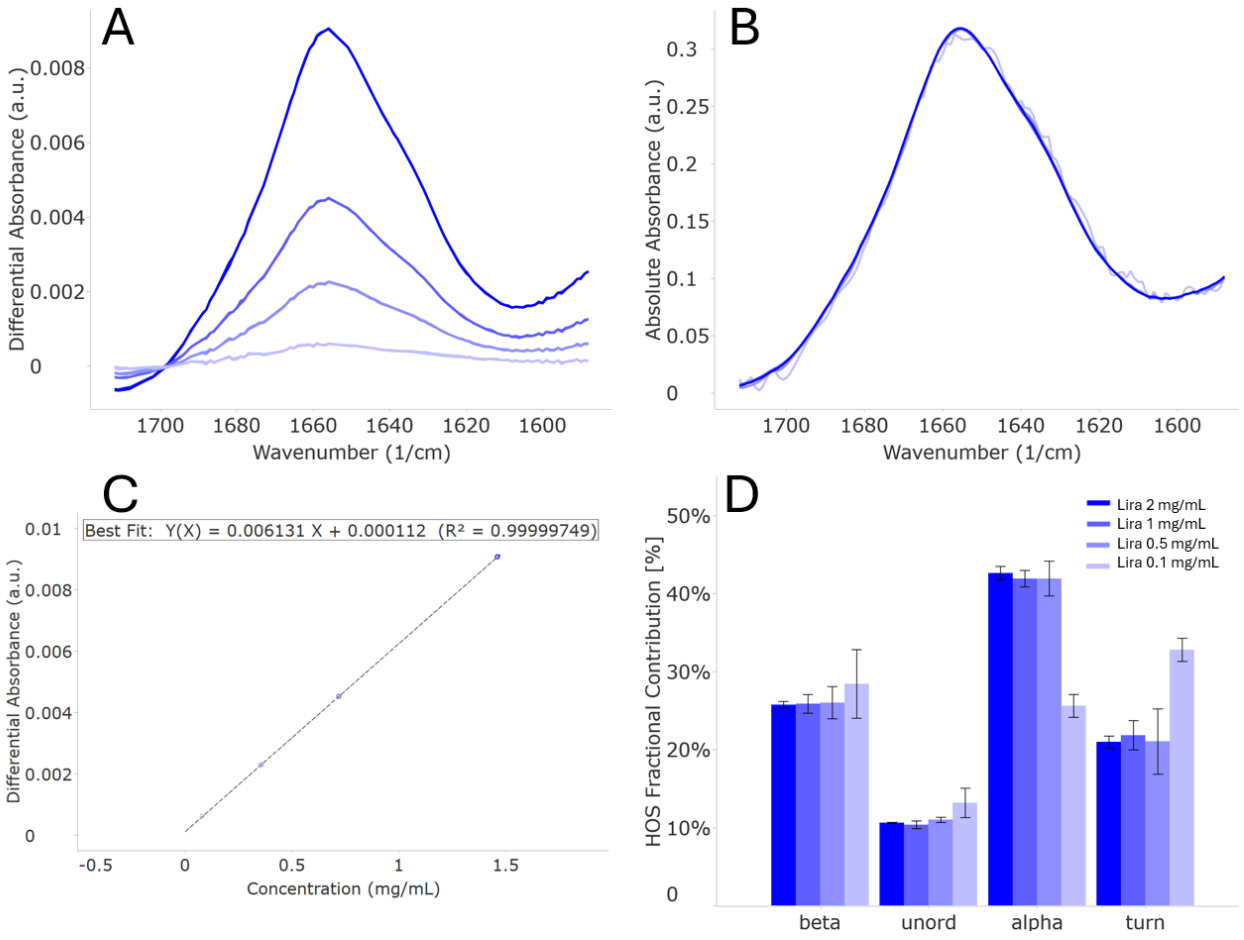

Figure 5: Liraglutide MMS analysis including 2, 1, 0.5, and 0.1 mg/mL. (A) The raw differential absorbance plot shows the signal is proportional to the concentration, where the highest concentration, 2 mg/mL (in bright blue), has the highest signal, as expected. When normalized for concentration, all the traces overlap, as seen in the absolute absorbance plot (B). The software automatically calculates the concentration and will generate the quantitation plot in figure (C). The HOS bar chart (D) shows liraglutide is mostly alpha-helical. Similar to semaglutide, the 0.1 mg/mL sample shows significantly less alpha helix and more turn structures. This could be affected by the higher noise in the spectral signal at concentrations < 0.5 mg/mL.

To highlight the differences between these peptides, we overlayed the baseline corrected second derivative MMS spectra of GLP-1 (7-37), semaglutide, and liraglutide (Figure 6A). Each peptide was dissolved in PBS to keep the formulation constant for this comparison. Shown in Figure 6A, there are clear spectral differences between the peptides and those differences can be seen at the major peak around 1656 cm^{-1} and in the region around 1640 cm^{-1}. Those spectral changes lead to large structural changes as seen in the HOS bar chart in Figure 6B, specifically in the alpha helix and unordered structures. This is consistent with literature indicating helicity and structure is important for stability and binding efficacy to the GLP-1 receptor.[6]

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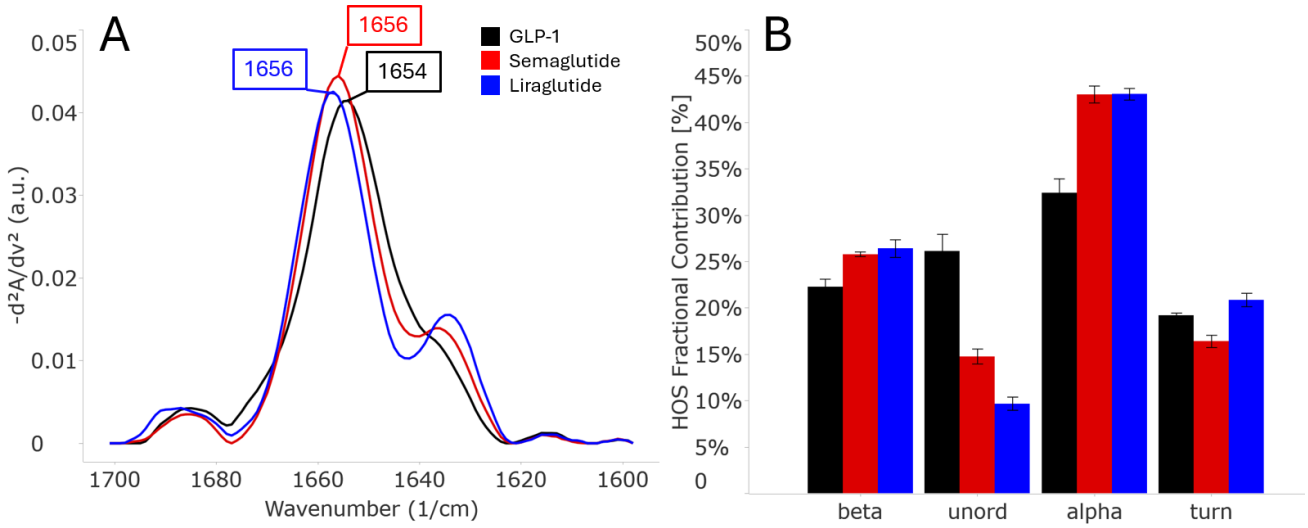

Figure 6: A comparison of the secondary structures of GLP-1 (7-37), semaglutide, and liraglutide each at 2 mg/mL in PBS. (A) Similarity plots show the major peak for these three peptides is unique in the position and intensity. However, all three major peaks fall in the alpha-helical region in the spectrum. This is clear from the HOS bar chart in figure (B) that shows that all three peptides are dominated by alpha-helical secondary structures.

Considering semaglutide and liraglutide have different formulation buffers, we also compared the peptides as they are formulated in Ozempic® and Saxenda®, respectively (see Methods section for exact formulations). The major difference in these formulations is the pH for semaglutide is 7.4 and liraglutide is 8.15. We also compared the formulated samples to the samples dissolved in PBS at 2 mg/mL. As shown in Figure 7B, both peptides in their formulation buffer (FB) have higher contributions of alpha-helical structure and less turn. Since structural differences can lead to differences in stability and activity, it is important for formulation groups to optimize the buffer conditions. This study demonstrates MMS to be a powerful way of studying protein and peptide structure in optimized formulation buffers, allowing for comparisons across different buffer conditions.

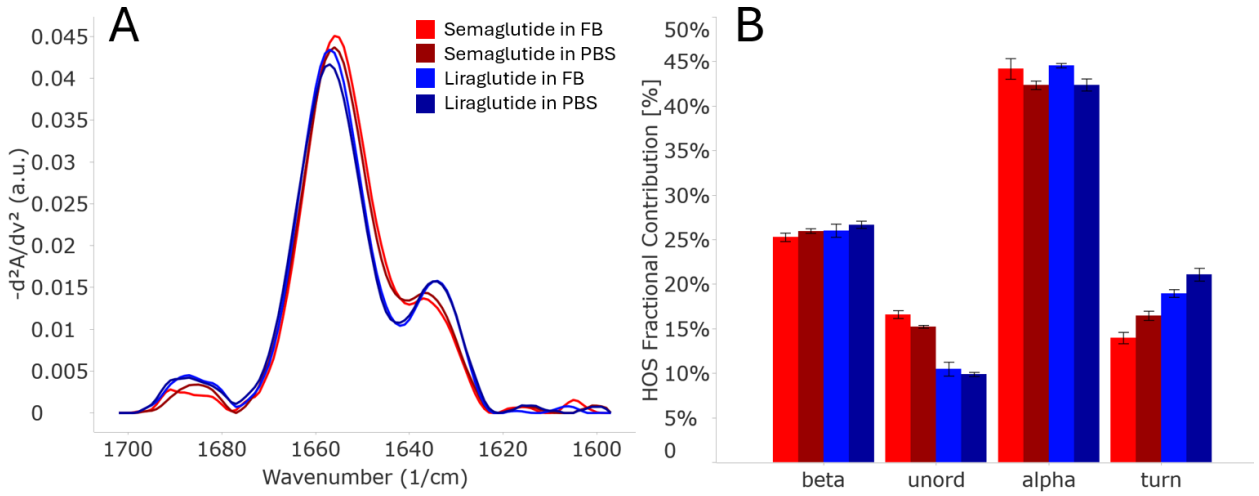

Figure 7: A comparison of the secondary structures of semaglutide and liraglutide in PBS and formulation buffers. (A) Similarity plots show both liraglutide and semaglutide in each formulation are similar, but small differences can be seen in the 1640 cm^{-1} region. When we quantify the spectral changes into the higher-order structures (B), there are clear changes caused by the buffer exchange out of formulation buffer (FB). Each peptide has more alpha-helical structure when in formulation buffer and less turn structure, possibly indicating an increase in stability due to higher native structure.

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Conclusions

In this work we showed that MMS is an exceptional tool for measuring peptide concentration and secondary structure in various buffer and concentration combinations. This application could be particularly impactful for measuring concentrations of other peptides that lack aromatic residues, making A280 ineffective. We also compared the structures of GLP-1 and the chemically similar semaglutide and liraglutide and found large structural differences specifically in the alpha-helical and turn structure percentages. These structure differences and covalent modifications may contribute to the drastically different half-lives of each peptide, where GLP-1 is the least stable and semaglutide is the most stable. Overall, MMS is an ideal tool for looking at peptide concentration and structure in their formulation buffers.

Contributors

Valerie Collins, PhD
Richard Huang, PhD

References

1. Original figure was generated by Benff and is under the Creative Commons Attribution-Share Alike 4.0 International license. Small modifications were made translating the half-lives into English from German https://commons.wikimedia.org/wiki/File:Semaglutide_vs._liraglutide_v01.svg

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