Application Note

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Size Exclusion Chromatography-Based Identification of Liraglutide Oligomeric Forms

Using the Agilent 1290 Infinity II bio LC and Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

This application note presents the size exclusion chromatography (SEC) method to study the oligomeric states of liraglutide. Agilent AdvanceBio SEC columns, coupled with LC/UV and LC/Q-TOF, achieved optimized performance for the separation and detection of liraglutide oligomers. Our results reveal a pH-dependent micelle-like oligomer formation, with liraglutide existing as higher-order oligomers at lower pH compared to higher pH. The SEC/UV method conditions were effective in detecting transitions between liraglutide's oligomeric states. Additionally, LC/Q-TOF provided high-resolution, deconvoluted mass data for complex higher-order oligomers. These findings are invaluable for optimizing GLP-1-based pharmaceutical manufacturing development.

Introduction

Synthetic peptides are emerging as powerful biotherapeutic agents. Peptide drugs vary in size from a few amino acids to over 50 amino acids and can play a crucial role in regulating various physiological processes. GLP-1 is a 30 or 31 amino acid peptide hormone that plays a key role in glucose homeostasis.[1] Native GLP-1 has a short half-life due to rapid degradation, which limits its ability to exert biological activity. Liraglutide is an analog of GLP-1 that mimics the human GLP-1 hormone. The conjugation of a fatty acid to liraglutide extends the half-life of this analog by enhancing its binding to albumin.

The conjugation of a hydrophobic lipid chain to a peptide leads to the acquisition of amphiphilic properties by the peptide, and these amphiphilic peptides tend to self-assemble into oligomers.[2] Assemblies of peptide amphiphiles have gained significant attention in research. This self-assembly can result in inconsistent therapeutic effects; hence, it is important to ensure the safe and effective development of lipidated peptide drugs. Specifically, liraglutide has been shown to exhibit a pH-dependent oligomeric transition.[3] Various analytical methods, including ultracentrifugation, circular dichroism, light scattering, and size exclusion chromatography (SEC), have been employed to characterize liraglutide's oligomer states.

In the present study, we focus on SEC/UV and SEC/MS to characterize liraglutide oligomeric states. SEC conditions were optimized to identify oligomeric states under different pH conditions.

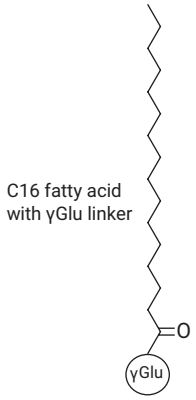

Figure 1. Liraglutide structure.

Experimental

Reagents and chemicals

Liraglutide was purchased from MedChemExpress (Monmouth Junction, NJ) and stored according to the manufacturer's instructions. Sodium dihydrogen phosphate, sodium phosphate dibasic heptahydrate, arginine, and ammonium acetate were procured from Sigma (St. Louis, MO); LC/MS-grade acetonitrile (ACN) was obtained from Fisher Scientific (Waltham, MA). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Billerica, MA).

Sample preparation

Sodium phosphate solutions at pH 6.4, 7.0, and 8.0 were prepared by mixing sodium dihydrogen phosphate and disodium hydrogen phosphate. Liraglutide was dissolved to 2.0 mg/mL in pH 6.4 and 8.0 sodium phosphate solutions. For LC/MS experiments, liraglutide (2.0 mg/mL) was prepared in 100 mM ammonium acetate. The liraglutide samples were incubated in a multisampler for three days and subjected to LC/MS analysis each day. SEC/UV and SEC/MS parameters are shown in Tables 1 and 2.

SEC/MS analysis

Table 1. Parameters for SEC/UV analysis.

Parameters	Value
Column	Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5350)
Mobile Phase	150 mM sodium phosphate pH 7.0 or 150 mM sodium phosphate:acetonitrile:0.1% arginine (9:25:65), pH 8.0
Flow Rate	0.8 mL/min
Injection Volume	5 µL
Column Temperature	30 °C
UV Detection	280 nm

Table 2. Parameters for SEC/MS analysis.

LC	
Column	Agilent AdvanceBio SEC 120 Å, 2.1 × 150 mm, 1.9 µm PEEK (p/n PL1980-3250PK)
Thermostat	10 °C
Mobile Phase	20 mM ammonium acetate
Flow Rate	0.07 mL/min
Injection Volume	5 µL
Column Temperature	30 °C
MS	
Source	Agilent Dual Jet Stream ESI
Polarity	Positive ion mode
Drying Gas Temperature	150 °C
Drying Gas Flow	10 L/min
Nebulizer	30 psi
Sheath Gas Temperature	150 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor	250 V
Acquisition Mode	Data were acquired in extended dynamic range (2 GHz)
Mass Range	*m/z* 100 to 10,000
Acquisition Rate	1 spectra/s

Instrumentation

The Agilent 1290 Infinity II bio LC system was used, including:

- Agilent 1290 Infinity II bio high-speed pumps (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option no. 101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent InfinityLab bio-inert Quick Connect heat exchanger, standard flow (G7116-60071)
- Agilent 1290 Infinity II diode array detector (DAD; G7117B) equipped with a biocompatible Agilent Max-Light cartridge cell LSS, 10 mm (G7117-60020)

Analysis was performed using the Agilent 6545XT AdvanceBio LC/Q-TOF equipped with the Agilent Dual Jet Stream ESI source.

Software

- Agilent OpenLab CDS, version 2.8
- Agilent MassHunter acquisition software for LC/MS systems, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1
- SEC/UV analysis

Results and discussion

The SEC analysis of amphiphilic molecules, such as liraglutide, requires a biocompatible instrument configuration. The 1290 Infinity II bio LC system with an AdvanceBio SEC column provides a metal-free flow path to prevent any interference caused by the instrument itself. To identify the oligomeric states of liraglutide, the SEC workflow comprising the 1290 Infinity II bio LC, AdvanceBio SEC column, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software, version 12.1, was used (Figure 2). The 6545XT AdvanceBio LC/Q-TOF is designed specifically to analyze native protein complexes with its extended mass range.

SEC/UV analysis

The first step in SEC analysis is to assess column performance and instrument suitability to evaluate column efficiency and help ensure accurate results. For SEC/UV studies, the AdvanceBio SEC 130 Å column is recommended and chosen for the analysis of liraglutide oligomeric states. Figure 3 shows the protein standard separation on the AdvanceBio SEC 130 Å column using a phosphate buffer system. The calibration curve fits the polynomial function. The protein peaks elute in the expected retention time and demonstrate optimal column separation efficiency. Using this plot, the molecular weight of an unknown sample can be determined from its elution volume.

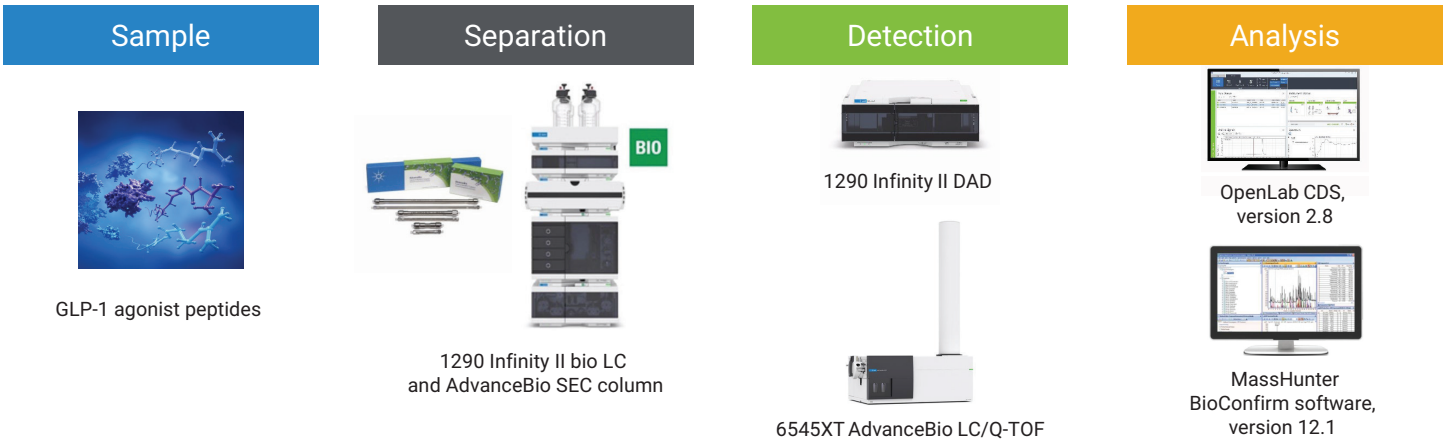

Figure 2. SEC workflow.

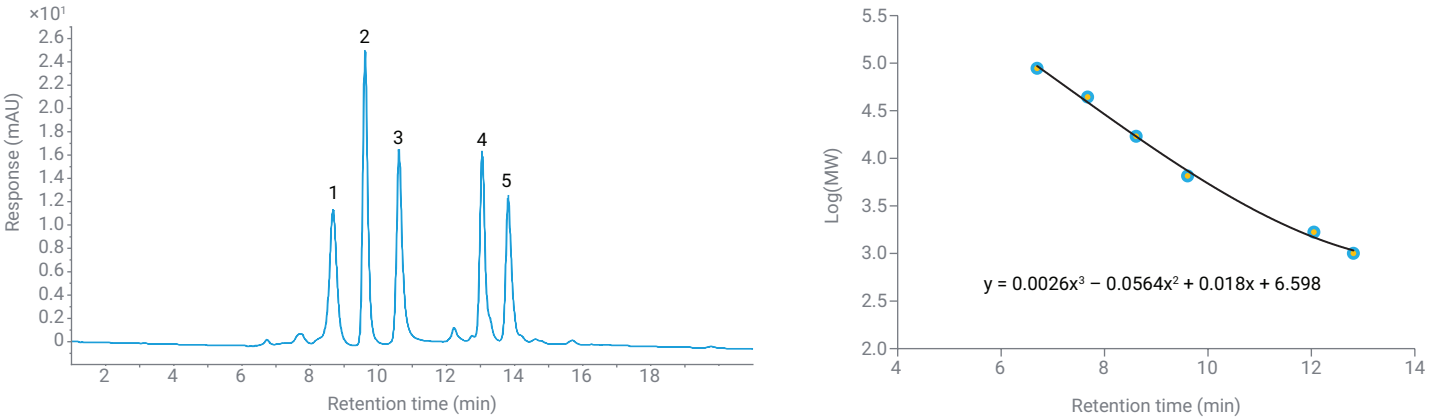

Figure 3. SEC/UV chromatogram and calibration curve of protein standards on the Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. The AdvanceBio 130 Å protein standard includes the following analytes: (1) ovalbumin, (2) myoglobin, (3) aprotinin, (4) neurotensin, and (5) angiotensin II.

Understanding GLP-1 oligomers is crucial for developing effective GLP-1-based therapies. To analyze and detect liraglutide oligomers, the AdvanceBio SEC column coupled to the 1290 Infinity II DAD was employed with UV absorbance at 280 nm. Liraglutide was dissolved in two pH conditions (pH 6.4 and 8.0), and different mobile phase buffers were tested to achieve optimized separation with minimal secondary interactions. Liraglutide forms a higher-order oligomer complex (~ 12-mer) at pH 6.4 and a lower-order oligomer complex (~ 3-mer) at pH 8.0 (Figure 4). Although these micelle-like species do not follow SEC behavior,[3] the estimated oligomeric state may help in understanding liraglutide oligomerization. The observed self-assembled species under different pH conditions align with published results.[4,5]

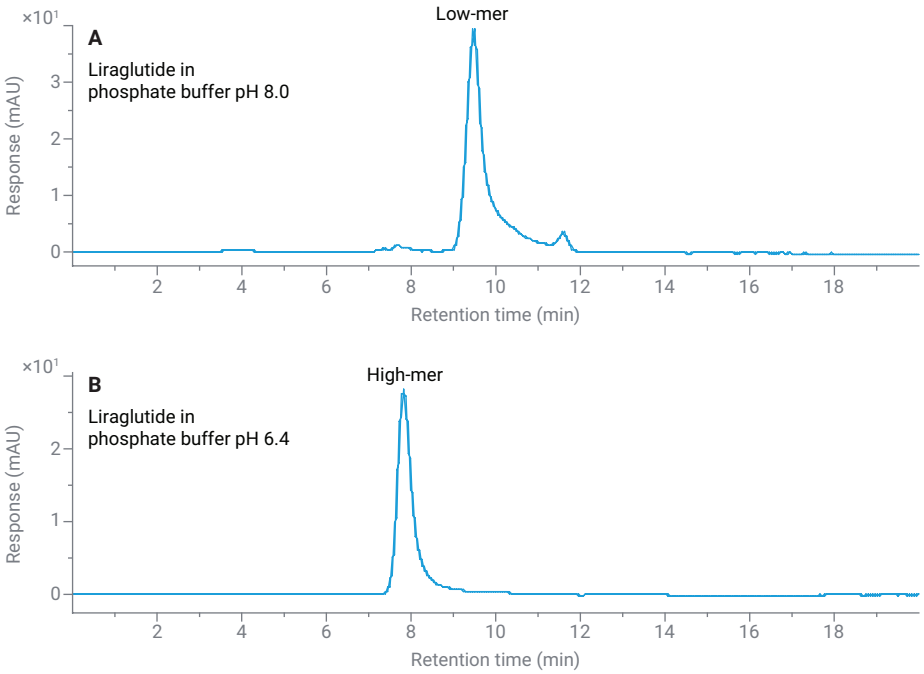

Figure 4. SEC/UV chromatogram of liraglutide prepared at pH 6.4 and pH 8.0. (A) Mobile phase consisting of 150 mM phosphate:acetonitrile:0.1% arginine (9:25:65), pH 8.0. (B) Mobile phase consisting of 150 mM phosphate, pH 7.0.

SEC/MS analysis

Native SEC/MS measurements were performed using the AdvanceBio SEC 120 Å column in the *m/z* range 100 to 10,000. The optimized SEC/MS conditions are shown in Table 2. To facilitate mass spectrometry detection, liraglutide was dissolved in ammonium acetate buffer, and oligomer formation was monitored for different time points. Figure 5 shows the formation of oligomeric species over time, and Figure 6 shows the deconvoluted spectra of a sample incubated for three days. The freshly prepared liraglutide shows a higher abundance of lower-order oligomer complexes (2 to 6-mer). However, when the sample is incubated for an extended period, higher-order oligomers are detected.

After three days of incubation, the intensity of the higher-order oligomer charge states increases. As illustrated in Figures 5C and 5D, a mixture of lower-order and higher-order oligomers are detected throughout the incubation period. The deconvolution spectra in Figure 6 (*m/z* 2,500 to 6,000 region) show the distribution of higher-order oligomers (12 to 15-mer). The oligomer distribution of liraglutide can be influenced by the fatty acid chain and pH. Specifically, the hydrophobic and hydrophilic interactions of monomers can be affected by these factors, resulting in the formation of different oligomeric species.[6] Further studies to understand the nature of these interactions and oligomerization are required.

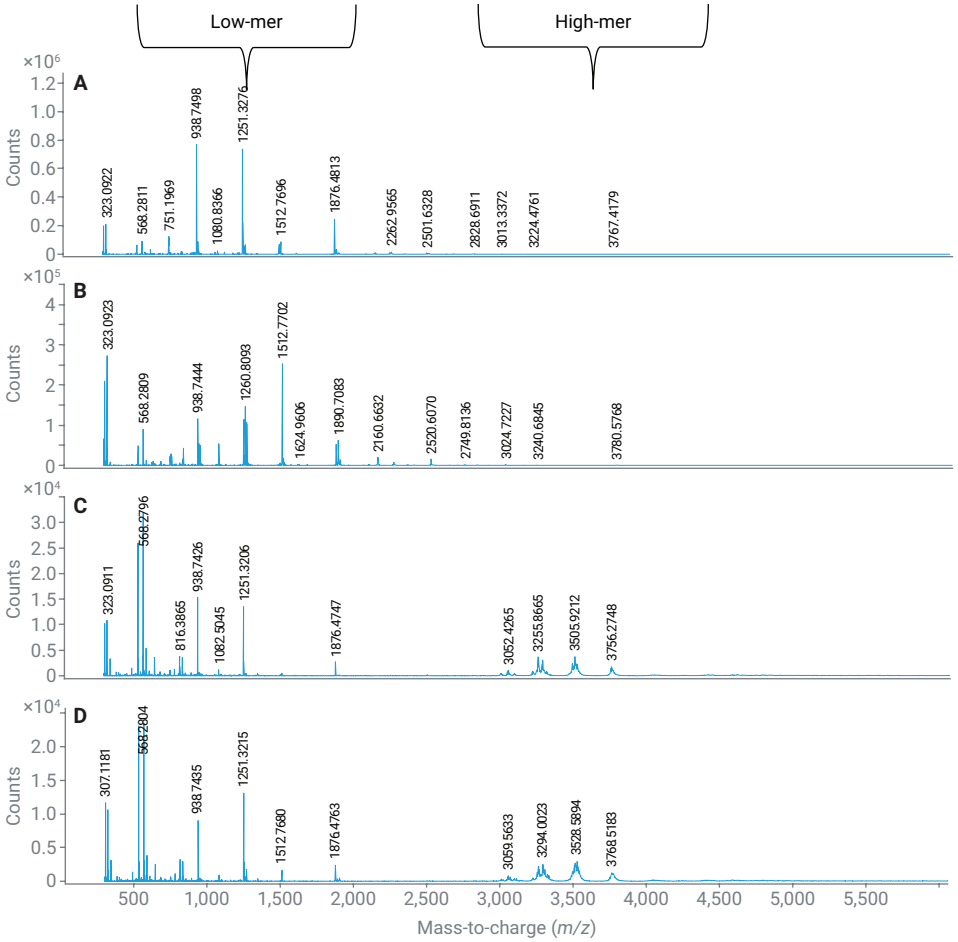

Figure 5. Mass spectra of liraglutide, monitoring the oligomeric states of liraglutide at different timepoints. Spectra for (A) freshly prepared sample, (B) 1-day old sample, (C) 2-day old sample, and (D) 3-day old sample are shown.

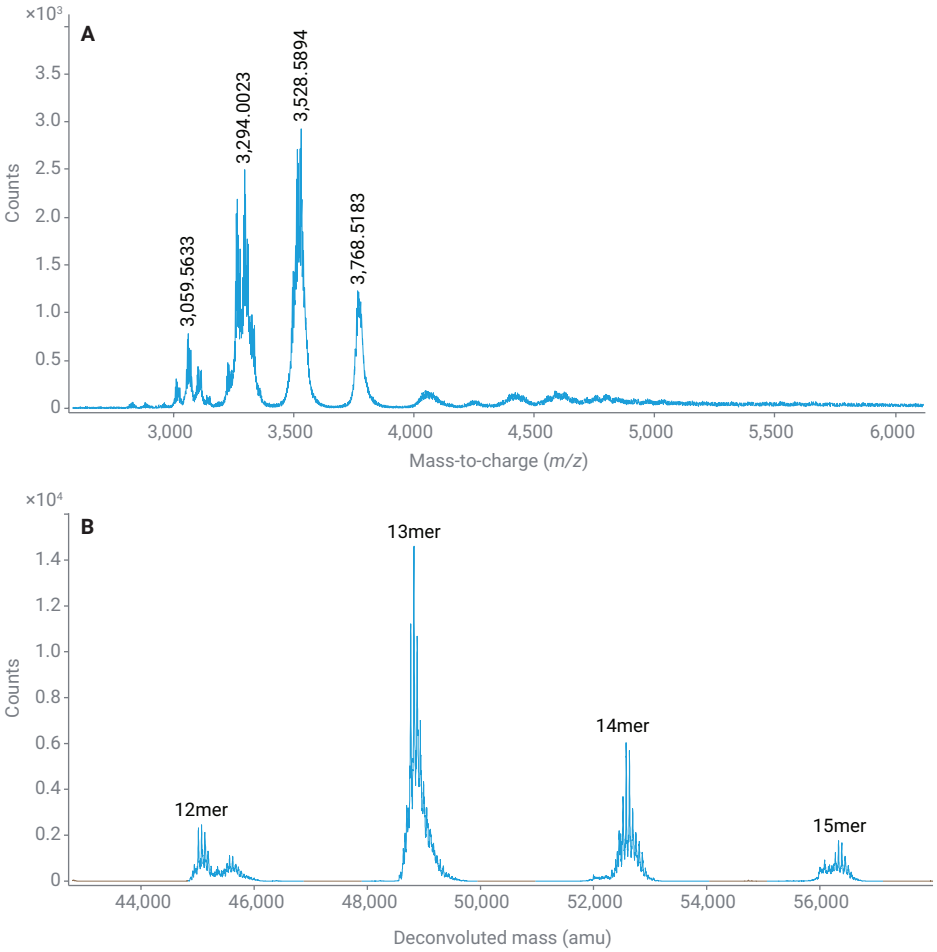

Figure 6. (A) Mass spectra (*m/z* 2,500 to 6,000 region) and (B) deconvoluted mass spectra of liraglutide oligomers in the high mass range (3-day old sample).

Conclusion

Identifying the oligomeric state of therapeutic peptides is crucial for optimizing drug stability and therapeutic performance. In this application note, we demonstrated the use of SEC coupled with LC/UV and LC/Q-TOF to identify the oligomeric states of liraglutide. Notably, liraglutide exhibits pH-dependent oligomeric transitions. The workflow employed in this study is highly suitable for characterizing liraglutide. High-performance separation was achieved using the Agilent 1290 Infinity II bio LC and Agilent AdvanceBio SEC column. The integrated Agilent 6545XT AdvanceBio LC/Q-TOF, along with Agilent MassHunter BioConfirm software, enabled efficient assessment of oligomeric states. The SEC methodology used in this study aids in understanding the self-assembly of peptide amphiphiles and shows great promise for applications in lipidated peptide-based therapeutic development.

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