Application Note

Biopharmaceuticals

Rapid Confirmation of GLP-1 Analog (Liraglutide) Using Agilent InfinityLab LC/MSD iQ

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Abstract

This application note presents the analysis of liraglutide using the Agilent InfinityLab LC/MSD iQ. When rapid molecular weight confirmation is required for GLP-1 analogs, the InfinityLab LC/MSD iQ allows for scanning a wide range, up to 1,450 *m/z*, with high sensitivity. To confirm the intact MS of biopharmaceutical products such as liraglutide, the deconvolution feature in Agilent OpenLab CDS 2.8 was used. For rapid method development, the Agilent InfinityLab Quick Change Valve provides a versatile screening environment for various chromatography columns.

Introduction

The widespread interest in GLP-1-like peptides has led many pharmaceutical companies to venture into the development of GLP-1 analogs. Originally developed as a treatment for type 2 diabetes, GLP-1 analogs have found applications in various fields due to the discovery of additional mechanisms. However, native GLP-1 has an extremely short half-life in plasma, prompting various attempts to extend its half-life. Liraglutide, by incorporating palmitoyl glutamic acid as a side chain, achieves a significantly prolonged half-life compared to native GLP-1.

Peptide drugs, including GLP-1 analogs, refer to those composed of fewer than approximately 40 amino acids. These synthetic peptide therapeutics need to meet the regulatory requirements for pharmaceutical approval. Consequently, the confirmation of manufacturing processes and profiling of impurities resulting from degradation is fundamentally required for drug approval.

A synthetically modified peptide with side chains may include various impurities that share similar characteristics with the target. Therefore, establishing an accurate HPLC evaluation method in the early stages of development is crucial for ensuring a highly refined process. Seamlessly testing various methods for peptide analysis is facilitated by the versatile Agilent Infinity II Flexible pump, which accommodates diverse additive screenings, and the InfinityLab Quick Change Valves, enabling easy online switching of multiple columns.

The InfinityLab LC/MSD iQ can scan up to 1,450 *m/z*, making it suitable for the analysis of peptides exhibiting multiple charges. Additionally, the deconvolution feature in OpenLab CDS 2.8 converts the multiple charged peaks in the MS spectrum to a single neutral mass. The deconvolution feature of OpenLab CDS can be used not only for confirmation in the late stage of biopharmaceutical development, but also for quality control as it satisfies data integrity and compliance.

In this application note, test results were compared based on an acid modifier and column conditions using an Agilent 1260 Infinity II Prime LC and an InfinityLab Quick Change Valve. The intact MS of liraglutide was evaluated using InfinityLab LC/MSD iQ and the deconvolution feature of OpenLab CDS 2.8.

Experimental

Instruments

The Agilent 1260 Infinity II Bio Prime LC system comprised the following modules:

- Agilent 1260 Infinity II Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat
- Agilent 1290 Infinity II Multi-Column Thermostat (G7116B) with Bio-Compatible Standard Flow Heat Exchanger and 6-column selector Bio-compatible valve, 1,300 bar (part number 5320-0025)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Bio-inert Max-Light cartridge cell, 60 mm (part number G5615-60017)
- Agilent InfinityLab LC/MSD iQ (G6160A)

Reagents

Formic acid (FA) and difluoroacetic acid (DFA) were purchased from Sigma-Aldrich, trifluoroacetic acid (TFA) was purchased from Merck, and acetonitrile was purchased from B&J.

Sample

Liraglutide was purchased through Kairos Tech in Korea. Liraglutide was dissolved in water to a concentration of 1 mg/mL, then further diluted with water to concentrations of 100 µg/mL and 10 µg/mL for use in the experiments.

Mobile phases

- Mobile phase A
  - 0.1% Formic acid in water
  - 0.1% Trifluoroacetic acid in water
  - 0.1% Difluoroacetic acid in water
- Mobile phase B
  - 0.1% Formic acid in acetonitrile
  - 0.1% Trifluoroacetic acid in acetonitrile
  - 0.1% Difluoroacetic acid in acetonitrile

Columns

- Agilent AdvanceBio Peptide Mapping 120 Å, 2.1 × 150 mm, 2.7 µm (part number 653750-902)
- Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm (part number 695775-949)
- Agilent InfinityLab Poroshell 120 Aq-C18, 2.1 × 150mm, 2.7 µm (part number 693775-742)

Software

Agilent OpenLab CDS 2.8

Methods

Table 1. Agilent 1260 Infinity II Bio Prime LC method parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping 120Å, 2.1 × 150 mm, 2.7 µm
Flow	0.4 mL/min
Column Temperature	40 °C
Injection Volume	5 µL
Mobile	A) 0.1% Acidic modifier B) 0.1% Acidic modifier in acetonitrile
Gradient – Column Screening	Time (min) %A %B 0 70 30 1 70 30 15 10 90 15.1 70 30 20 70 30
Gradient – Optimization	Time (min) %A %B 0 80 20 1 80 20 20 40 60 25 10 90 25.1 80 20 30 80 20
Detector	UV 280 nm (DAD HS with Bio-inert Max-Light cartridge cell, 60 mm)

Table 2. Agilent InfinityLab LC/MSD iQ data acquisition parameters.

Parameter	Value
Ion Source	ESI (+)
Source Parameters	
Gas Temperature	325 °C
Gas Flow	11 L/min
Nebulizer	45 psi
Capillary Voltage	4,500 V
Acquisition	
Scan range	300 to ~1,450 *m/z*
Fragmentor	150 V
Scan time	997 ms (1 Hz)
Storage	– Method optimization: Centroid – Deconvolution: Profile

Results and discussion

Basic analysis of liraglutide using TFA as an acid modifier

In the analysis of peptides using HPLC, TFA is commonly used. Liraglutide was dissolved in water to create a concentration of 100 µg/mL, and it was analyzed under conditions of 0.1% TFA in water and 0.1% TFA in acetonitrile to observe the liraglutide peak and UV spectrum. These analysis conditions were used as a basis for screening columns and acid modifier conditions for the confirmation of API and impurities.

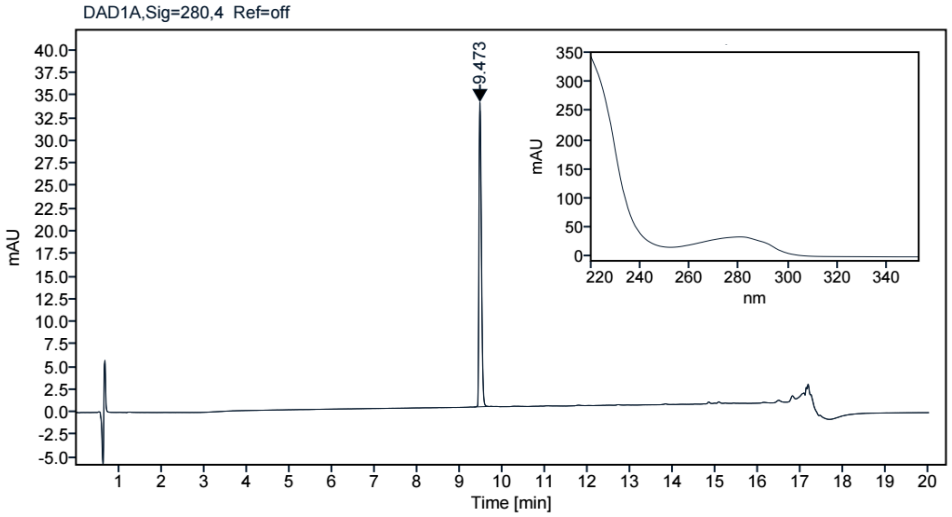

Figure 1. Chromatogram and UV spectrum of liraglutide 100 µg/mL under TFA conditions.

Screening of the column and acidic modifier

The Agilent column portfolio offers various column chemistries for peptide analysis. The results listed are based on analyses using AdvanceBio Peptide Mapping, Peptide Plus, and Poroshell 120 Aq-C18 columns, each possessing distinct characteristics that can yield different selectivities depending on the substance. Therefore, the choice of an appropriate column depends on the desired outcome through the analysis of the target compound.

In HPLC and LC/MS applications, the commonly used acidic modifiers are FA and TFA. The low-pH mobile phase not only reduces the impact of end-capping on the column but also aids in maintaining the pH of the mobile phase, assisting in a stable formation of the analyte. Hence, the type and concentration of acidic modifiers influence peak shape. Specifically, TFA exhibits a lower pKa compared to FA, forming ion pairs that reduce the second interaction with the column, resulting in minimal peak tailing and high resolution. However, TFA will suppress ion formation in MS and is not easily removed during analysis. DFA, with weaker ion pair

characteristics than TFA, can be used for peptide MS analysis to achieve high sensitivity and resolution in MS while minimizing the issues associated with TFA.

The results demonstrate variations in peak capacity and symmetry factor based on the use of three types of columns and three types of acidic modifiers (Figure 2). It showed the best results when analyzed using the AdvanceBio Peptide Mapping column under TFA conditions. When FA was used as the mobile phase, the symmetry factor was significantly lower, making it difficult to expect high resolution, and the peak height was lowered due to the wide peak width.

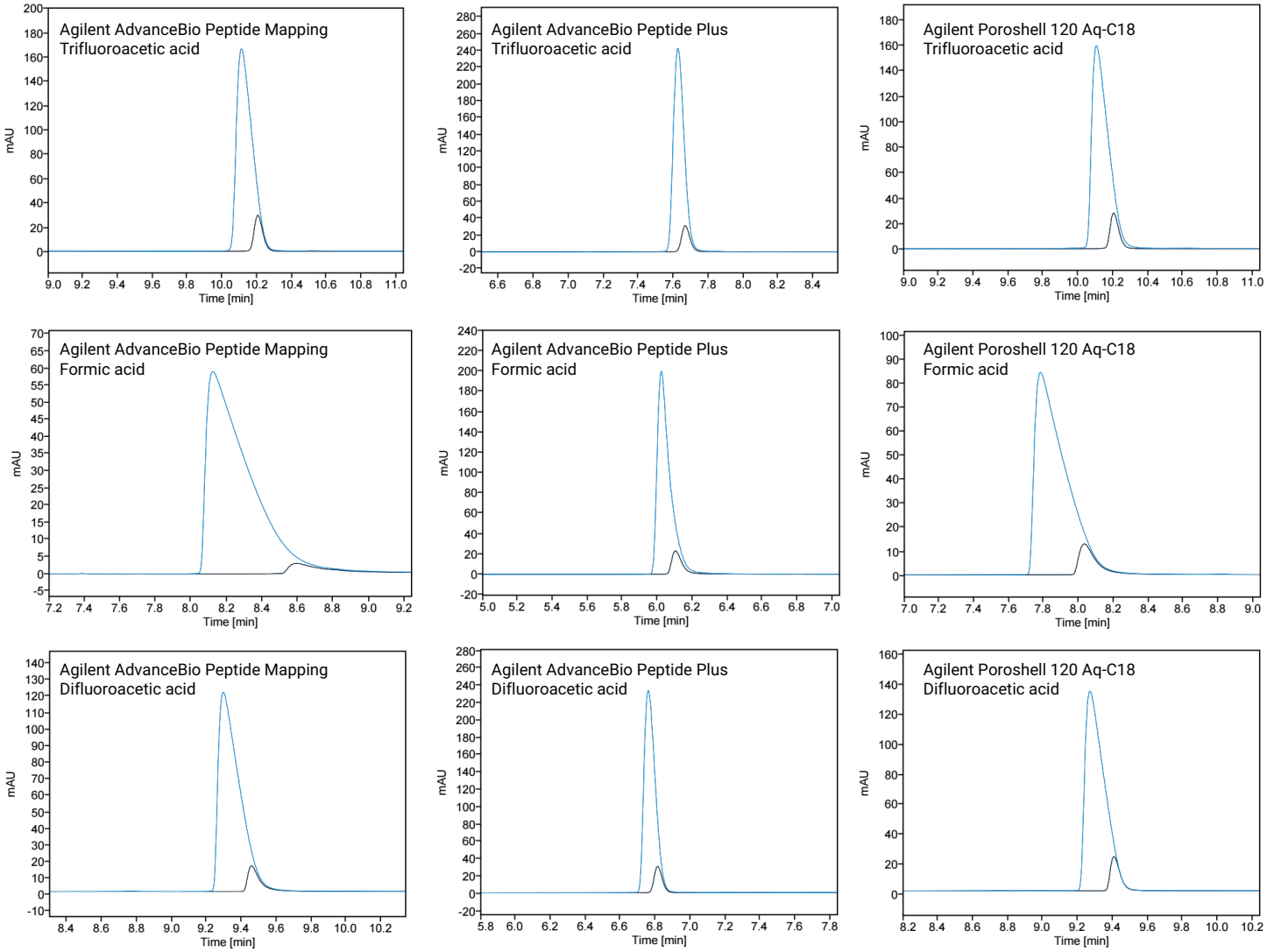

Figure 2. Chromatograms of liraglutide (1 mg/mL and 100 µg/mL) under formic acid, trifluoroacetic acid, and difluoroacetic acid conditions using Agilent AdvanceBio Peptide Mapping, Peptide Plus, and Poroshell 120 Aq-C18 columns (blue: 1 mg/mL, black: 100 µg/mL).

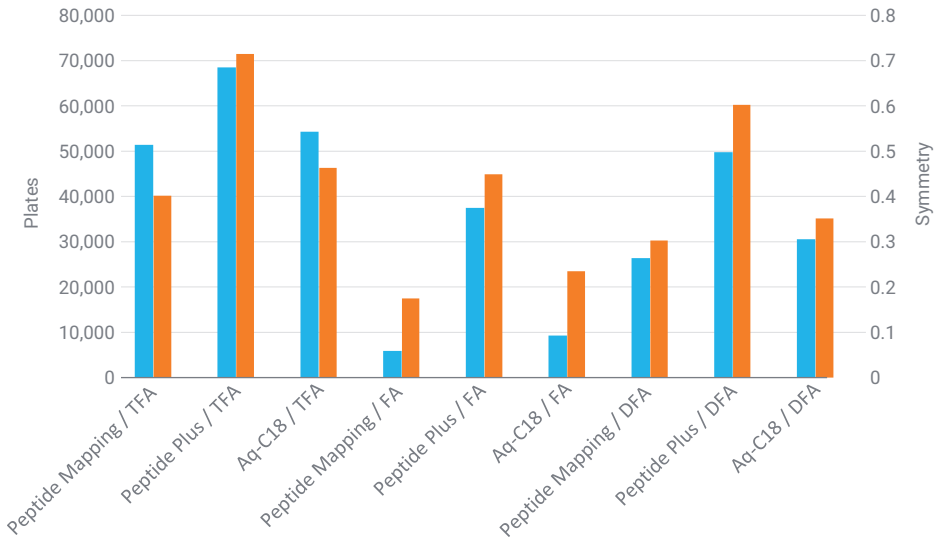

Figure 3. Results of plates and symmetry for each condition (left axis, blue: plates, right axis, orange: symmetry)

Condition screening for peak resolution optimization

AdvanceBio Peptide Plus in combination with TFA exhibited the best results; however, there are concerns regarding MS compatibility and specificity for impurities. Except for the FA condition, which showed the least favorable outcome for peak resolution, gradient conditions were fine-tuned to improve peak resolution. The diversity of impurities was superior with the AdvanceBio Peptide Mapping and TFA combination. However, considering MS analysis, the combination of AdvanceBio Peptide Mapping and DFA could also be a favorable choice for better peak resolution (Figure 4).

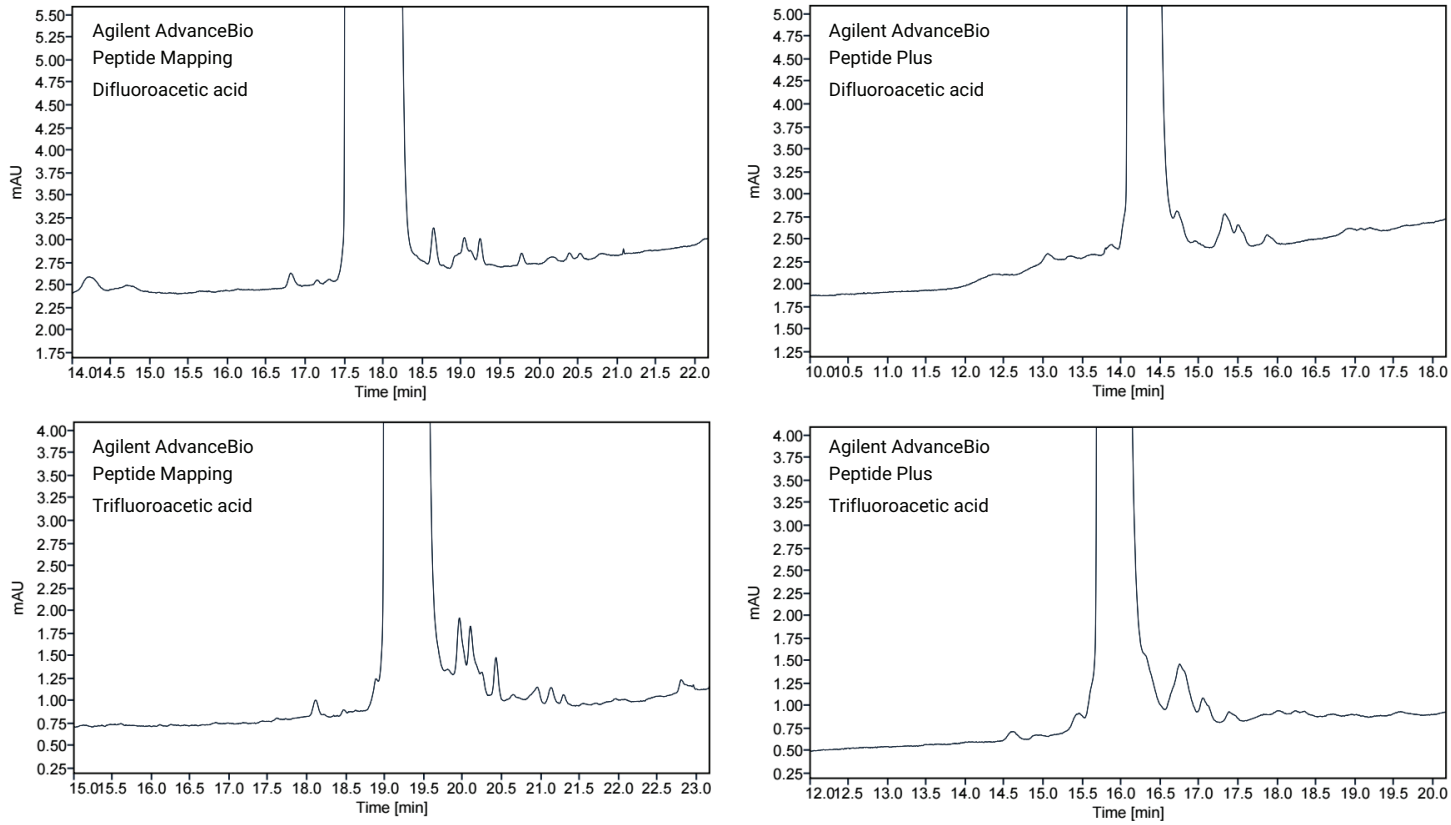

Figure 4. Chromatograms obtained by analyzing liraglutide (1 mg/mL) using Agilent AdvanceBio Peptide Mapping and Peptide Plus columns with DFA and TFA.

MS results for each acidic modifier condition

MS results with different acidic modifiers were compared using an AdvanceBio Peptide Mapping column (Figure 5). While the highest sensitivity was exhibited by FA, DFA conditions showed not only sufficient sensitivity but also distinct MS spectra and impurity separation. On the contrary, the lowest MS sensitivity was observed under TFA conditions. Through these results it was observed that various acidic modifiers not only affect the retention time and specificity of peaks but also influence MS sensitivities.

At a concentration of 10 µg/mL, significant differences were observed in the TIC when performing MS (Figure 6). Under DFA conditions, a peak height of 1×10^6 counts was observed, while no MS peak was detected under TFA conditions. This suggests that DFA is more suitable for confirming MS spectra of low-concentration impurities while maintaining chromatographic peak resolution.

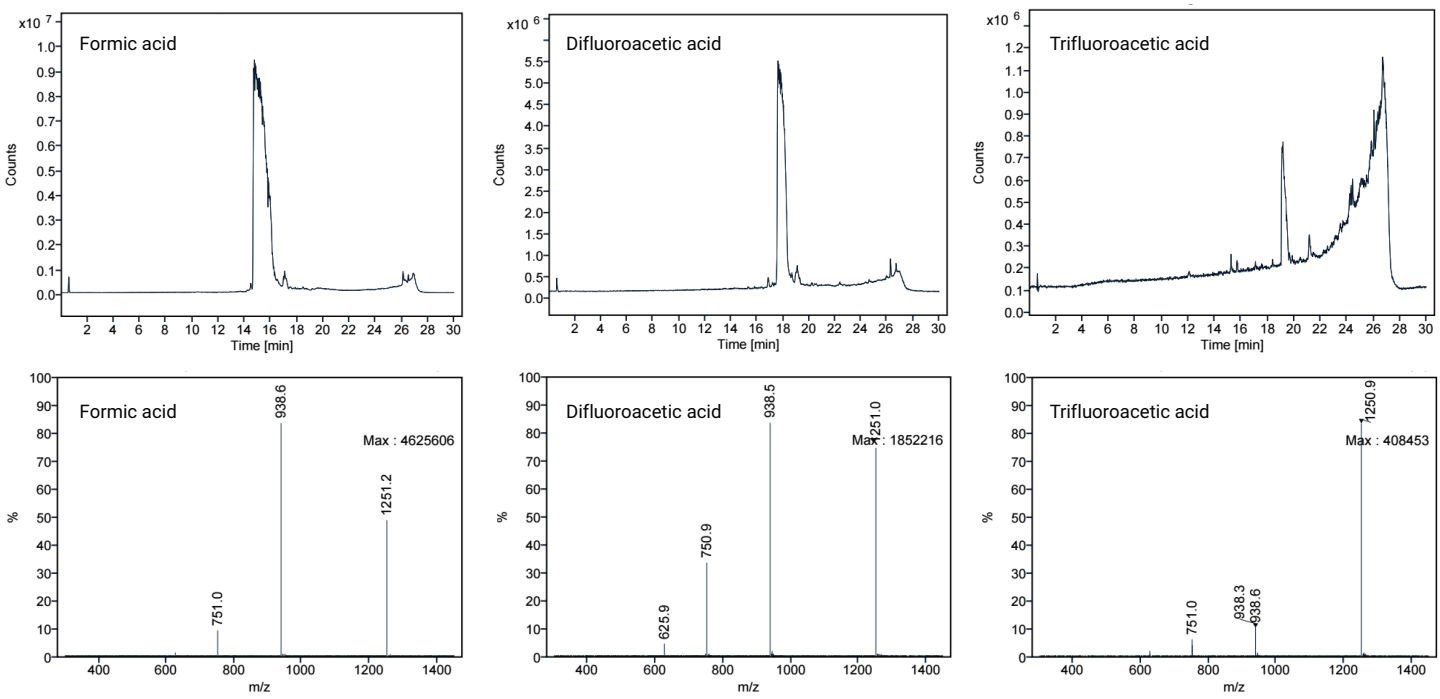

Figure 5. Total ion chromatograms (TIC) and MS spectra of liraglutide (1 mg/mL) using various acidic modifiers with an Agilent AdvanceBio Peptide Mapping column and an Agilent InfinityLab LC/MSD iQ.

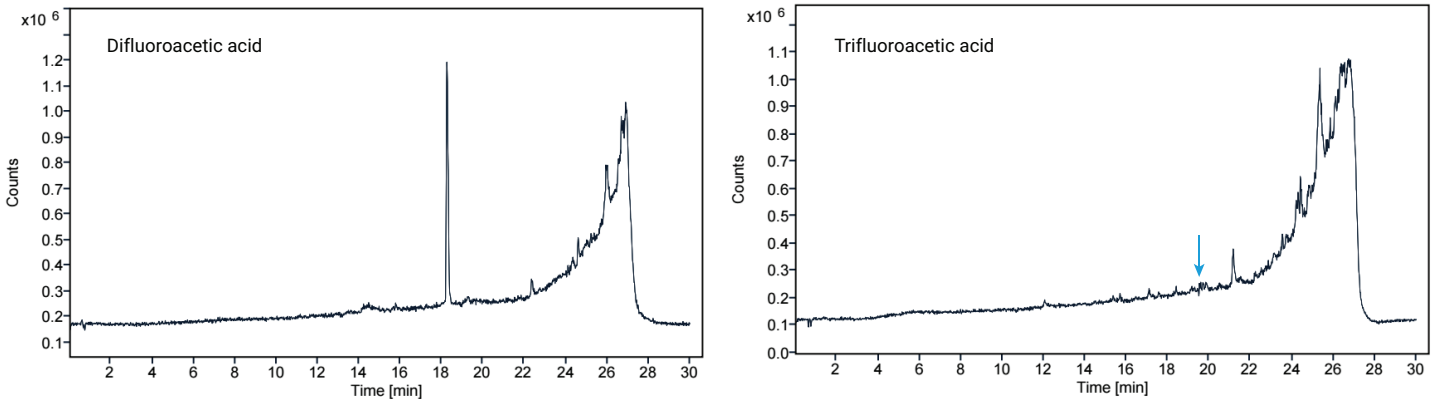

Figure 6. Comparison of TICs between DFA and TFA for liraglutide at 10 µg/mL.

Deconvolution and confirmation of the MS spectrum of liraglutide

Finally, the MS spectrum of liraglutide was confirmed using 0.1% DFA and an AdvanceBio Peptide Mapping column on an InfinityLab LC/MSD iQ (Figure 7). Multiple charges from +3 to +6 were observed, showing results that closely matched theoretical values for the charge states of liraglutide (Table 3).

Table 3. Theoretical and observed values for the multiple charges of liraglutide.

	Theoretical	Observed
$[M+3H]^{3+}$	1,251.3	1,251.0
$[M+4H]^{4+}$	938.7	938.5
$[M+5H]^{5+}$	751.1	750.9
$[M+6H]^{6+}$	626.1	625.9

The MS spectrum obtained with the InfinityLab LC/MSD iQ was deconvoluted using the deconvolution feature of OpenLab CDS 2.8, revealing a mass of 3,750.23, which matches the calculated mass value of 3,749.95 within expected tolerances.

Conclusion

The use of an Agilent 1260 Infinity II Bio Prime LC and Agilent InfinityLab LC/MSD iQ facilitates not only the analytical method setup but also the seamless confirmation of synthetic peptides such as liraglutide. Through the application of an Agilent Flexible Pump and an InfinityLab Quick Change Valve, optimal acidic modifiers and reversed-phase columns were efficiently screened. The results indicated that an Agilent AdvanceBio Peptide Mapping column with trifluoroacetic acid demonstrated excellent performance in UV-based detection, while an AdvanceBio Peptide Mapping column with difluoroacetic acid exhibited high specificity and MS sensitivity in MS-based detection. The deconvolution feature of Agilent InfinityLab LC/MSD iQ and OpenLab CDS 2.8 proved sufficient for confirming the neutral MS of liraglutide.

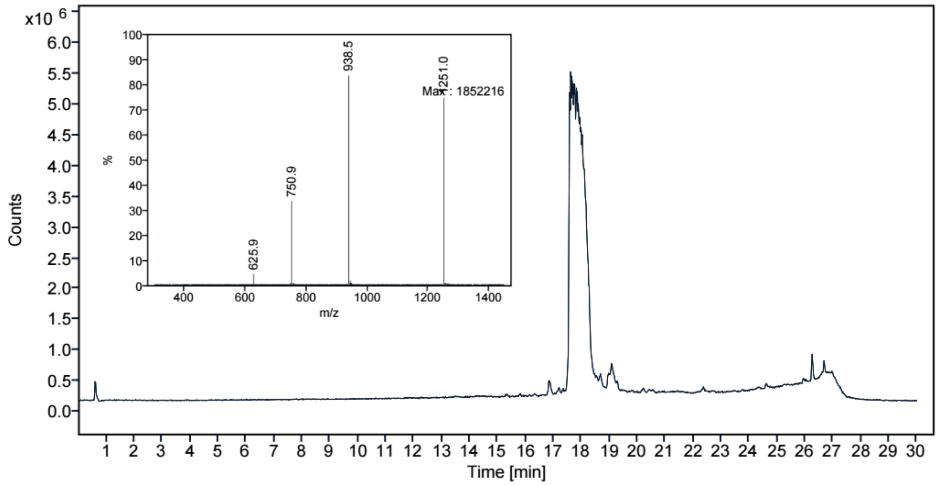

Figure 7. TIC and MS spectrum of liraglutide at 1 mg/mL.

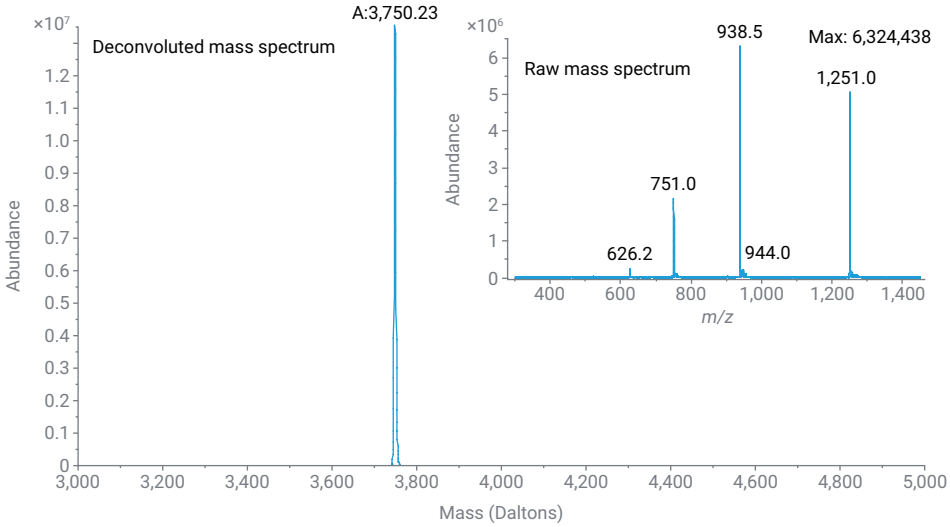

Figure 8. Deconvolution results for liraglutide.

References

1. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs for rDNA Origin, Guidance for Industry. *U.S. Department of Health and Human Services, FDA, CDER*, **2021**.
2. Guideline on the Development and Manufacture of Synthetic Peptides. *European Medicines Agency*, **2023**.
3. Brian, R.; Jarod, G. Unit Mass Spectral Deconvolution for Molecular Weight Confirmation of Large molecules. *Agilent Technologies technical overview*, publication number 5994-6928EN, **2024**.
4. Benedikt, M.; Lucas, W. Simplify Your Method Development Using the Agilent 1260 Infinity II Prime LC System and InfinityLab LC/MSD iQ. *Agilent Technologies technical overview*, publication number 5994-2124EN, **2020**.

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