Application Note

Biopharma

Agilent

Trusted Answers

Analytical Characterization of GLP-1 Agonists: Sequence Confirmation by Q-TOF MS/MS

Author

Suresh Babu C. V.
Agilent Technologies, Inc.

Abstract

The role of synthetic peptides in modern therapeutics continues to expand, elevating the importance of rigorous sequence confirmation to ensure product identity, consistency, and purity. This application note demonstrates the robust performance of the Agilent Revident Q-TOF MS for comprehensive sequence verification of GLP-1 agonists. Using high-resolution MS/MS analysis, the GLP-1 agonists liraglutide, tirzepatide, and retatrutide were characterized with 100% sequence coverage across all samples. The rich and highly reproducible MS/MS fragmentation patterns—featuring complete series of b and y ions—enable confident, unambiguous peptide identification and support high-assurance quality control of complex therapeutic peptides.

Introduction

Peptides represent a rapidly growing class of therapeutics used across a wide range of therapeutic applications. Compared with traditional small-molecule therapeutics, peptide therapeutics typically deliver superior target specificity. Ensuring the structural integrity of synthetic peptide therapeutics is essential for verifying amino acid sequence order, chain length, molecular weight, and overall purity. Even subtle sequence deviations such as a single residue substitution or deletion can profoundly affect biological activity and therapeutic performance.[1] Robust sequence confirmation is therefore critical when assessing batch-to-batch consistency and identifying any structural modifications that may arise during development, manufacturing, or storage. Achieving this level of confidence requires advanced analytical platforms capable of delivering high-resolution, comprehensive characterization of synthetic peptides.

Glucagon-like peptide-1 (GLP-1) agonists are an emerging class of therapeutic agents. These synthetic peptides essentially copy the GLP-1 hormone, activating its effects through GLP receptors. These agonists are effective in lowering blood sugar and can also contribute to weight loss. GLP-1 peptides are typically 30 to 40 amino acids in length, with modifications involving a few amino acid substitutions at certain positions and the addition of a fatty acid chain to prolong their half-life. With a robust pipeline of GLP-1 receptor agonists and multi-target receptor agonists currently in development[2], comprehensive sequence confirmation ensures accurate identification and reliable quality characterization of these therapeutic peptides.

Recent advancements in high-resolution mass spectrometry (HRMS) have improved the characterization of synthetic peptides, providing high sensitivity and mass accuracy. Modern Q-TOF MS platforms provide the resolving power needed to distinguish subtle chemical variants and post-synthetic modifications, enabling comprehensive impurity profiling and confident quality assessment of complex peptide therapeutics. Tandem mass spectrometry (MS/MS) enhances structural elucidation by precise verification of peptide primary sequence. Together, HRMS and MS/MS provide a powerful, reliable analytical framework for confirming molecular weight and sequence information.

In this study, we analyzed three GLP-1 agonists—liraglutide, tirzepatide, and retatrutide—to confirm their sequences using tandem MS/MS. The analysis delivered complete sequence coverage for these peptides by comprehensively identifying all b and y ions. The intuitive BioConfirm software interface simplified the sequence data analysis.

Experimental

Reagents and chemicals

Retatrutide, liraglutide, and tirzepatide were purchased from Cayman Chemicals and stored according to the manufacturer's instructions. Formic acid was obtained from Fisher Scientific. LC/MS-grade acetonitrile was obtained from Agilent Technologies. Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system.

Sample preparation

Peptide standards are dissolved in dimethyl sulfoxide to 2.0 mg/mL and further diluted to 1 mg/mL in water.

Analytical equipment

- Agilent 1290 Infinity II high-speed pump (p/n G7120A)
- Agilent 1290 Infinity II multisampler (p/n G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (p/n G7116B)
- Agilent Revident LC/Q-TOF (p/n G6575A)

Software and data processing

- Agilent MassHunter Data Acquisition for LC/TOF and LC/Q-TOF, version 12.0
- Agilent MassHunter BioConfirm software, version 12.1

LC/MS analysis

The LC separation was performed on an Altura Peptide Plus, 2.1 × 150 mm, 2.7 µm (p/n 227215-903) and the raw data were acquired using MassHunter Data Acquisition for LC/TOF and LC/Q-TOF 12.0. (Tables 1 and 2). The data analysis was performed in MassHunter BioConfirm 12.1.

Table 1. Liquid chromatography parameters.

Parameter	Agilent 1290 Infinity II LC System
Column	Altura Peptide Plus, 2.1 × 150 mm, 2.7 µm
Sample Thermostat	10 °C
Mobile Phase A	0.1% FA
Mobile Phase B	0.1% FA in ACN
Gradient	Time (min) %A %B 0.00 80 20 20.00 40 60 20.10 20 80 21.90 20 80 22.00 80 20
Stop Time	22 min
Column Temperature	55 °C
Injection Volume	0.4 µL (MS)
Flow Rate	0.4 mL/min

Table 2. MS data acquisition parameters.

Parameter	Agilent Revident LC/Q-TOF (G6575A)
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nebulizer	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	1,000 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
MS Range	100–3,200 *m/z*
MS/MS Range	50–3,200 *m/z*
MS Acquisition Rate	5 spectra/sec
MS/MS Acquisition Rate	3 spectra/sec
Isolation Width	Narrow (~1.3 *m/z*)
Collision Energy	3.6*(*m/z*)/100–4.8
Maximum Precursors per Cycle	5
Precursor Threshold	1,000 count
Active Exclusion	Enabled
Isotope Model	Peptides
Sort Precursors	By abundance only, >+3, +3, +2

Results and discussion

In this study, the GLP-1 agonists retatrutide, tirzepatide and liraglutide were chosen for sequence confirmation analysis. Table 3 shows the amino acid sequences of these peptides.

- Retatrutide, a highly modified 39-residue triple-agonist peptide, presents a well-known analytical challenge due to its multiple noncanonical residues and a heavily proline-rich C-terminal region. The peptide includes α-aminoisobutyric acid (Aib) at positions 2 and 20, and α-methyl-L-leucine (αMeL) at position 13. A C20 fatty diacid moiety is conjugated to the lysine residue at position 17.
- Tirzepatide is another proline-rich 39-amino acid linear peptide that functions as a dual agonist. The modifications include Aib at positions 2 and 13, C-terminus amidation, and a C20 fatty diacid is attached to a lysine residue at position 20.
- Liraglutide is a 31-amino acid peptide GLP-1 analogue. It is designed by replacing the lysine at position 34 with arginine and a C16 fatty acid chain is attached to the lysine at position 26.

Table 3. Peptide sequences of GLP-1 agonists.

GLP-1	Sequence
Retatrutide	Y-{Aib}-QGTFTSDYSI-{α-Me-Leu}-LDK-{Lys(AEEA-γGlu-C20 diacid)} AQ-{Aib}-AFIEYLLEGGPSSGAPPPS-NH2
Tirzepatide	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20diacid-gamma-Glu-(AEEA)2-Lys}-AFVQWLIAGGPSSGAPPPS
Liraglutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl]}-EFIAWLVRGRG

High-resolution LC/Q-TOF MS/MS analysis was performed to confirm the complete amino-acid sequence of these GLP-1 agonists. Retatrutide produced strong precursor signals suitable for auto MS/MS analysis. The resulting MS/MS data demonstrated that the Q-TOF platform delivered 100% sequence coverage with improved fragmentation across the entire peptide backbone (Figure 1). The acquired spectra exhibited high signal intensity and clear detection of both b and y ion ladders, including regions adjacent to modified and proline-rich residues. A total of 38 y ions and 37 b ions $(M+H^+)$ were identified, generating a comprehensive fragmentation pattern that supports unambiguous sequence interpretation (Figure 2). The corresponding BioConfirm score of 25.0 reflected the high quality of the MS/MS data and confirmed accurate peptide identification. The presence of both b and y ions in the region flanking proline residues indicates more uniform energy distribution and efficient fragmentation along the peptide chain. This improved fragmentation behaviour enables confident assignment of amino acid residues.

Tirzepatide, another structurally complex GLP-1 agonist, was analyzed under identical conditions. The peptide generated strong precursor ion signals and high-quality MS/MS spectra despite its modifications and C-terminal proline cluster (Figure 3). The Q-TOF instrument produced 38 y ions and 36 b ions $(M+H^+)$ with 100% sequence coverage, demonstrating its ability to deliver better fragmentation even for peptides where CID efficiency is traditionally limited. The improvements in fragmentation directly translated to higher BioConfirm scores (25.0), indicating increased spectral confidence and accurate residue-level annotation throughout the full sequence.

Liraglutide, which includes a long-chain fatty-acid modification but has a less proline-dense structure than the other agonists, also produced high-quality MS/MS spectra (Figure 4). The data yielded 29 b ions and 29 y ions $(M+H^+)$, enabling 100% sequence coverage and yielding a BioConfirm score of 25.0. The high-abundance MS/MS fragmentation provided clear identification and peptide sequence confirmation.

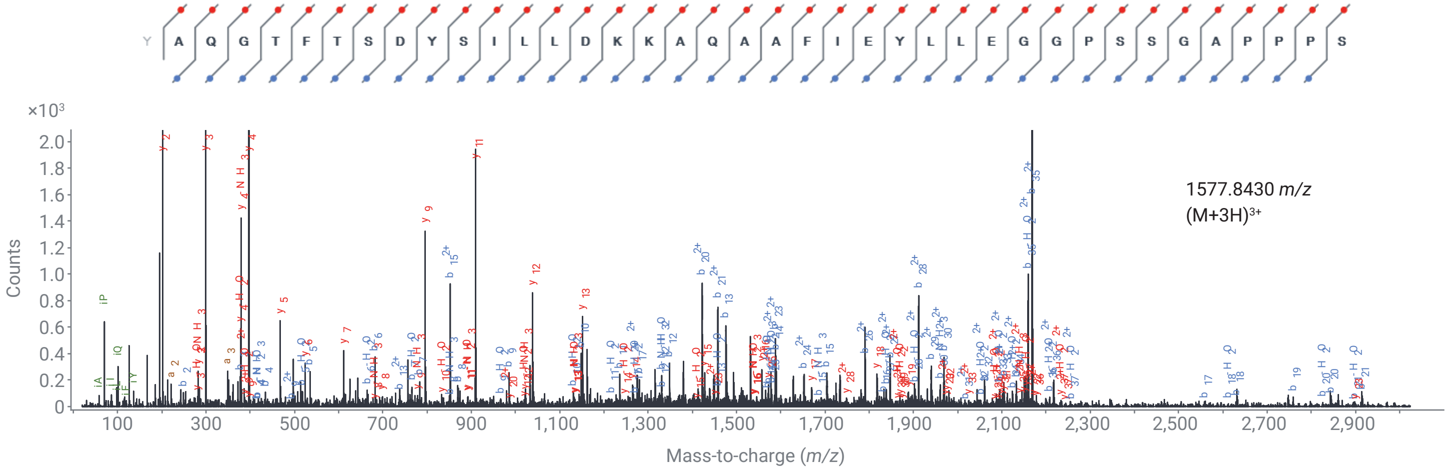
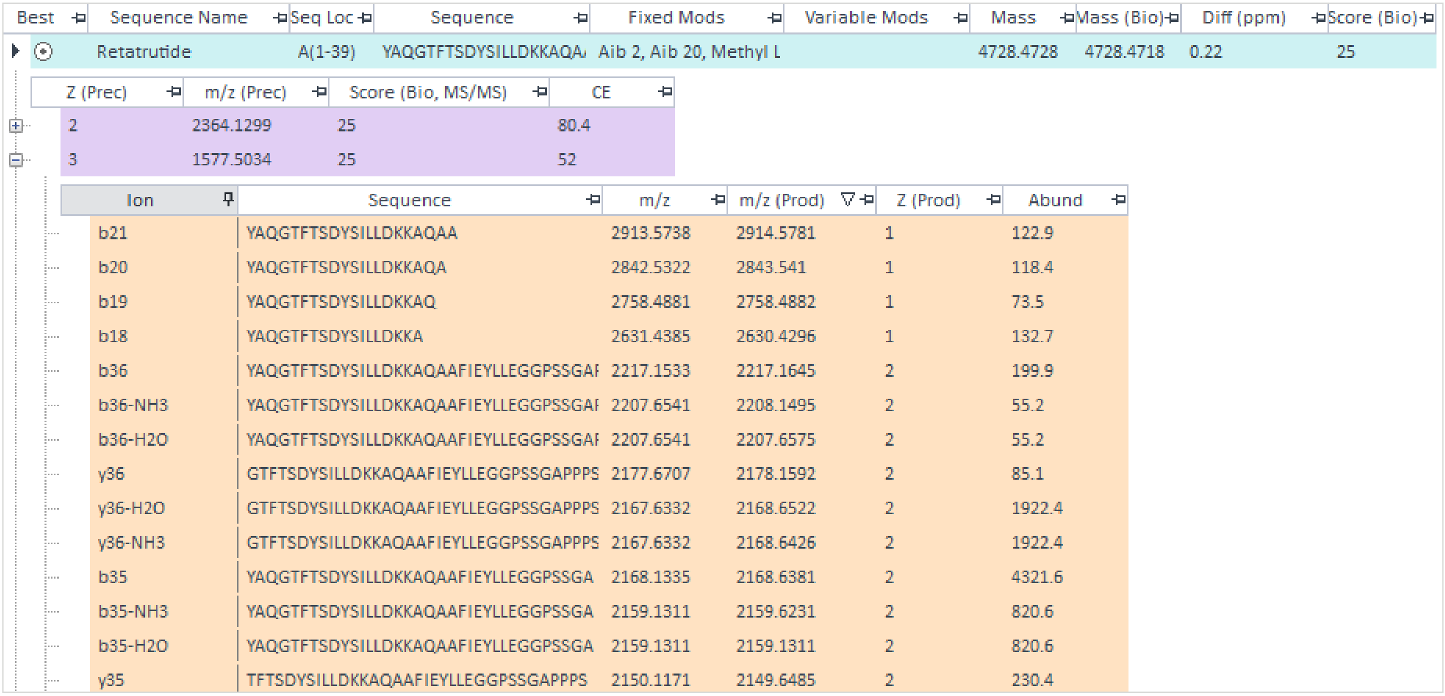


Figure 1. The MS/MS fragmentation pattern for the retatrutide peptide (z = 3) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series for the sequence.



Best	Sequence Name	Seq Loc	Sequence	Fixed Mods	Variable Mods	Mass	Mass (Bio)	Diff (ppm)	Score (Bio)
○	Retatrutide	A(1-39)	YAQGTFTSDYSILLDKKAQA	Aib 2, Aib 20, Methyl L		4728.4728	4728.4718	0.22	25

Z (Prec)	m/z (Prec)	Score (Bio, MS/MS)	CE
2	2364.1299	25	80.4
3	1577.5034	25	52

Ion	Sequence	m/z	m/z (Prod)	Z (Prod)	Abund
b21	YAQGTFTSDYSILLDKKAQAA	2913.5738	2914.5781	1	122.9
b20	YAQGTFTSDYSILLDKKAQA	2842.5322	2843.541	1	118.4
b19	YAQGTFTSDYSILLDKKAQ	2758.4881	2758.4882	1	73.5
b18	YAQGTFTSDYSILLDKKA	2631.4385	2630.4296	1	132.7
b36	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2217.1533	2217.1645	2	199.9
b36-NH3	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2207.6541	2208.1495	2	55.2
b36-H2O	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2207.6541	2207.6575	2	55.2
y36	GTFTSDYSILLDKKAQAAFIEYLLEGGPSSGAPPPS	2177.6707	2178.1592	2	85.1
y36-H2O	GTFTSDYSILLDKKAQAAFIEYLLEGGPSSGAPPPS	2167.6332	2168.6522	2	1922.4
y36-NH3	GTFTSDYSILLDKKAQAAFIEYLLEGGPSSGAPPPS	2167.6332	2168.6426	2	1922.4
b35	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2168.1335	2168.6381	2	4321.6
b35-NH3	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2159.1311	2159.6231	2	820.6
b35-H2O	YAQGTFTSDYSILLDKKAQAAFIEYLLEGGPSSGA	2159.1311	2159.1311	2	820.6
y35	TFTSDYSILLDKKAQAAFIEYLLEGGPSSGAPPPS	2150.1171	2149.6485	2	230.4

Figure 2. Agilent BioConfirm software display of observed fragment ions.

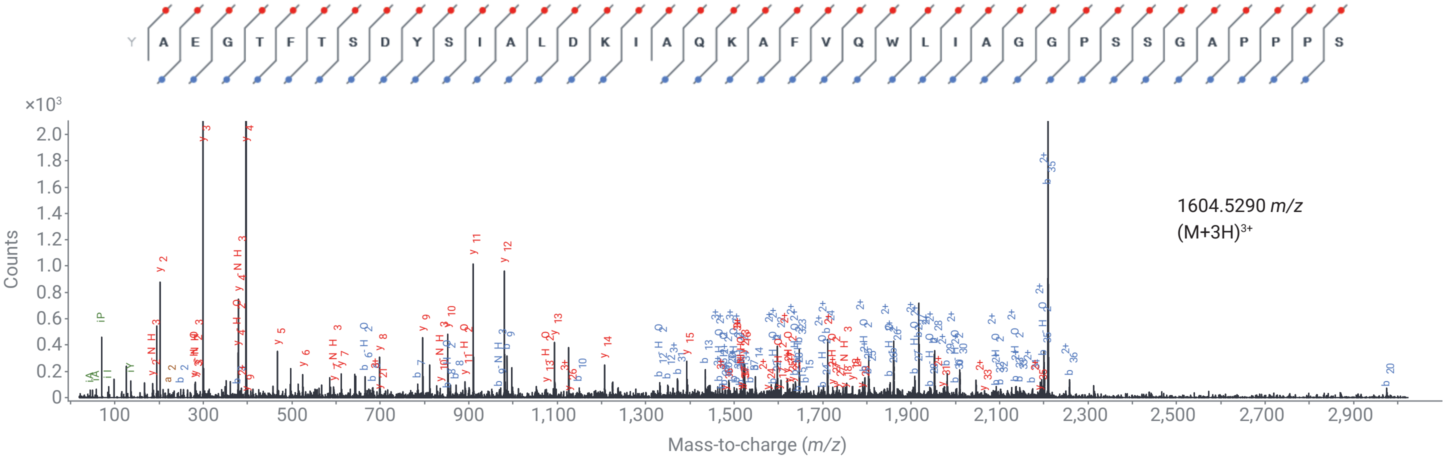


Figure 3. The MS/MS fragmentation pattern for the tirzepatide peptide (z = 3) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series for the sequence.

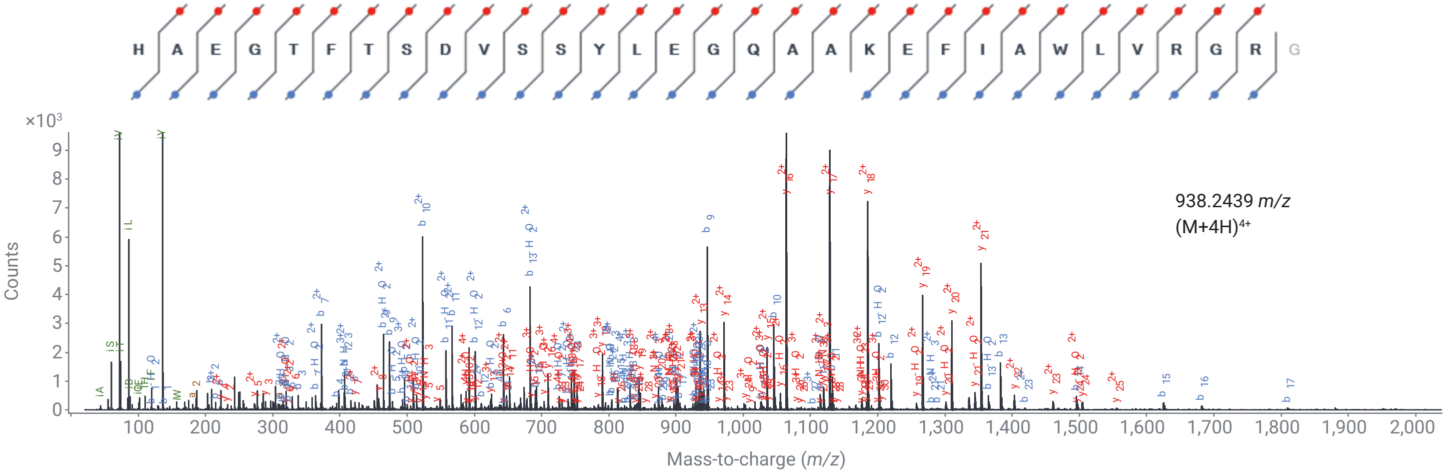


Figure 4. The MS/MS fragmentation pattern for the liraglutide peptide (z = 4) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series for the sequence.

Conclusion

These results demonstrate the suitability of this LC/Q-TOF MS/MS workflow for high-resolution sequence confirmation of GLP-1 agonists. For all three peptides examined, the MS/MS method provided complete sequence coverage together with well distributed fragment ion series This is important for synthetic therapeutic peptides, where sequence identity is a critical parameter in product quality and regulatory confidence.

References

1. Malovichko, G.; Zhu, X. Single Amino Acid Substitution in the Vicinity of a Receptor-Binding Domain Changes Protein–Peptide Binding Affinity. *ACS Omega*. **2017**, *2*, 5445–5452. doi: 10.1021/acsomega.7b00963
2. Melson, E.; *et al.* What Is the Pipeline for Future Medications for Obesity? *Int. J. Obes. (Lond)* **2025**, *49(3)*, 433–451. doi: 10.10.1038/s41366-024-01473-y

www.agilent.com

DE-014875

Printed in the USA, May 26, 2026
5994-9249EN

Agilent
Trusted Answers