Application Note

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Sequence Confirmation of Cagrilintide Using the Agilent 1290 Infinity II Bio LC and 6545XT AdvanceBio LC/Q-TOF

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Abstract

Glucagon-like peptide-1 (GLP1) agonists are an emerging and rapidly expanding class of therapeutic agents for the treatment of metabolic disorders. Recent advances in high-resolution mass spectrometry (HRMS) have significantly enhanced the characterization of synthetic peptides by delivering improved sensitivity, mass accuracy, and analytical confidence. This application note demonstrates the robust performance of the Agilent 6545XT AdvanceBio LC/Q-TOF system for comprehensive sequence verification of GLP1 analogues. Cagrilintide is a structurally intricate ~38-amino-acid peptide incorporating a disulfide linkage and a lipophilic fattyacid conjugation. Using high-resolution MS/MS analysis, the sequence of the GLP1 agonist cagrilintide was characterized in both reduced and native forms. The presence and location of the disulfide bond were confirmed through detailed analysis of b- and y-ion fragmentation patterns. This approach enables unambiguous peptide identification and provides a high level of confidence in the structural integrity of the molecule, supporting reliable quality control of complex therapeutic peptides.

Introduction

Peptide therapeutics are an increasingly important class of biopharmaceuticals due to their high specificity, favorable safety profiles, and ability to target complex metabolic pathways. Among these, cagrilintide is a next-generation, long-acting amylin analog under development for the treatment of obesity and related metabolic disorders.[1,2] Derived from the endogenous hormone amylin, cagrilintide plays a role in appetite regulation, gastric emptying, and glucose homeostasis.[1]

Structurally, cagrilintide is an approximately 38-amino-acid peptide containing a disulfide bond and a lipophilic fatty-acid modification (eicosanedioic acid–γ-Glu linker) (Figure 1), which enhances its stability and prolongs systemic exposure.[2] These structural elements introduce additional analytical complexity, requiring robust and high-resolution techniques to confirm sequence integrity and characterize post-synthetic modifications.[3]

Accurate characterization of peptide therapeutics is critical throughout drug development and quality control processes. Key analytical requirements include:

- Verification of molecular weight and intact mass
- Confirmation of disulfide bond formation and structural integrity
- Identification of sequence and modification sites by way of MS/MS fragmentation

Liquid chromatography–mass spectrometry (LC/MS), particularly when coupled with high-resolution quadrupole time-of-flight (Q-TOF) detection, has emerged as a powerful platform for peptide analysis. This technique enables:

- High mass accuracy for intact and reduced species
- Efficient separation of peptide variants
- Detailed structural elucidation through tandem MS (MS/MS)

In this application note, the Agilent 1290 Infinity II bio LC system coupled with the Agilent 6545XT AdvanceBio LC/Q-TOF is used to perform comprehensive characterization of cagrilintide. The workflow includes analysis of both native (oxidized) and reduced forms to assess disulfide bond integrity, followed by MS/MS-based sequence confirmation using fragmentation pattern analysis.

This study demonstrates how the Agilent LC/Q-TOF platform provides a robust, high-performance solution for peptide therapeutics, delivering accurate mass measurements, reliable sequence confirmation, and detailed structural insights essential for biopharmaceutical development and analytical characterization.

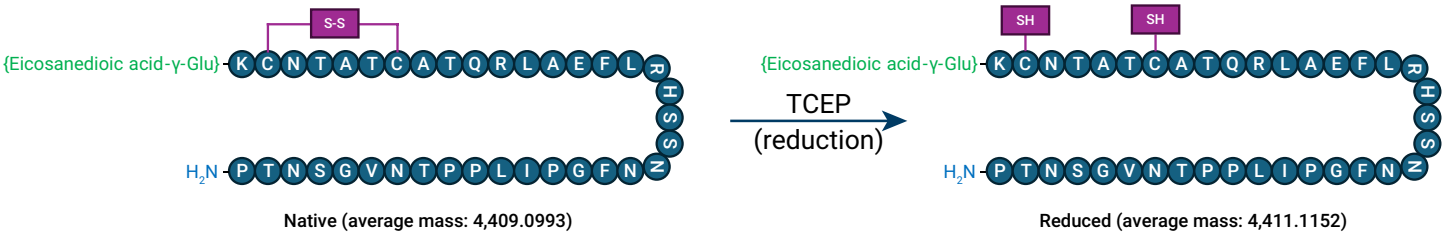

Figure 1. Sequence of cagrilinitide in native and reduced form.

Experimental

Reagents and chemicals

Cagrilintide was purchased from MedChemExpress 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

Cagrilintide was initially dissolved in water at a concentration of 1.0 mg/mL. This stock solution was subsequently diluted to 0.2 mg/mL in mobile phase A (0.1% formic acid in water) prior to injection for LC/MS analysis.

Analytical equipment

- An Agilent 1290 Infinity II bio LC system including the following modules:
  - Agilent 1290 Infinity II bio high-speed pump (G7120A)
  - Agilent 1290 Infinity II bio multisampler (G7137A)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF system (G6549AA)

Software and data processing

- Agilent MassHunter data acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1
- Agilent MassHunter Qualitative Analysis version 12.0

LC/MS analysis

The LC separation was performed on an Altura Peptide Plus, 2.1 × 150 mm, 2.7 µm (part number 227215-903) and the raw data were acquired by a MassHunter data acquisition software (Tables 1 and 2). The data analysis was performed in MassHunter BioConfirm.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent 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 1.00 80 20 20.00 40 60 25.00 10 90 25.10 20 20 30.00 80 20
Stop Time	30 min
Column Temperature	40 °C
Flow Rate	0.4 mL/min
Injection Volume	1 µL

Table 2. MS data acquisition parameters.

Parameter	Value
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	270 °C
Drying Gas Flow	11 L/min
Sheath Gas Temperature	375 °C
Sheath Gas Flow	11 L/min
Nebulizer	35 psi
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
MS Range	300–3,200 *m/z*
MS/MS Range	50–3,200 *m/z*
MS Acquisition Rate	2 spectra/sec
MS/MS Acquisition Rate	3 spectra/sec
Isolation Width	Narrow (~ 1.3 *m/z*)
Collision Energy	Charge Slope Offset All 3 1 All 5.3 2 All 4 −1
Maximum Precursors Per Cycle	3
Precursor Threshold	2,000 counts
Active Exclusion	Exclude after 2 spectra, then released after 0.2 min
Isotope Model	Peptides
Sort Precursors	By abundance only

Results and discussion

Intact mass analysis

The cagrilintide was first analyzed for the intact mass. Figure 2 shows the mass analysis of native and reduced forms of cagrilintide. The total ion chromatogram (TIC) shows a single peak for both forms (Figure 2A). The charge state distribution with improved signal-to-noise ratios confirmed detection of cagrilintide in both native and reduced forms (Figure 2B). These results indicate efficient ionization of the peptide using the Agilent dual AJS ESI source. Multiple charge states ($(M+3H)^{3+}$, $(M+4H)^{4+}$, $(M+5H)^{5+}$) were observed, enabling confident mass deconvolution. Deconvoluted spectra (Figure 2C) showed clean mass profiles with well-resolved isotopic distributions, demonstrating high mass accuracy and instrument performance. The observed mass shift (~2 Da) is consistent with reduction of the disulfide bond, confirming correct structural modification.

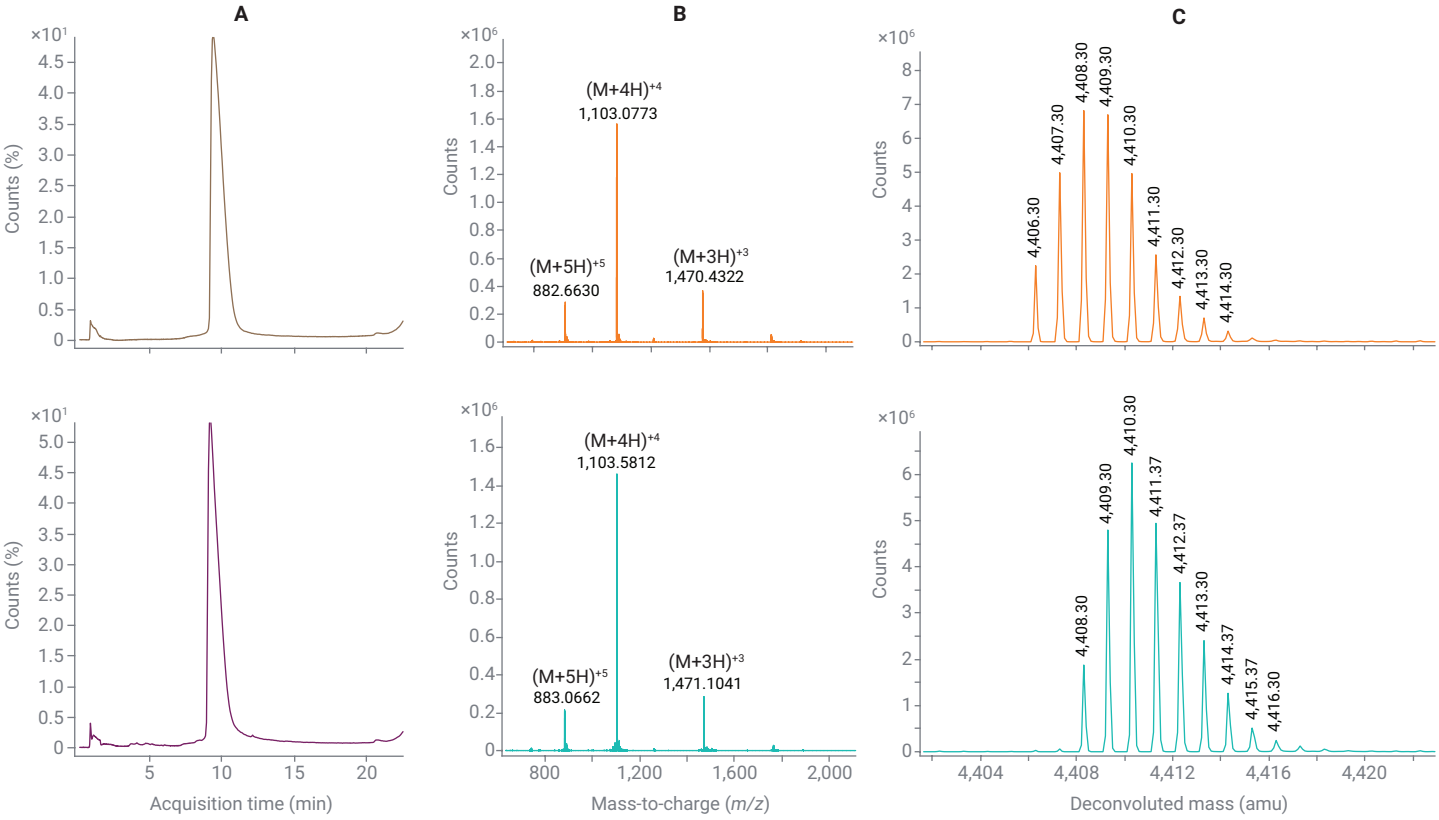

Figure 2. (A) Total ion chromatogram of native (top) and reduced (bottom) cagrilintide. (B) Charge state envelope of native and reduced cagrilintide. (C) Deconvoluted spectra of native and reduced cagrilintide.

MS/MS-based sequence confirmation

High-resolution MS/MS analysis was performed to confirm the sequence and structural integrity of cagrilintide. Fragmentation spectra acquired for both the native (oxidized) and reduced forms showed extensive b- and y-ion series, enabling confident sequence assignment across the peptide backbone.

MS/MS fragmentation of both native (oxidized) and reduced forms generated clear b- and y-ion series (Figures 3 and 4). Representative ions (for example, b_7 and y_{36} ions) were consistently identified in both conditions. The fragmentation ladder enabled sequence confirmation across the peptide backbone, and high-quality spectra supported confident assignment of amino acid sequence. These results confirm the structural integrity and sequence identity of cagrilintide.

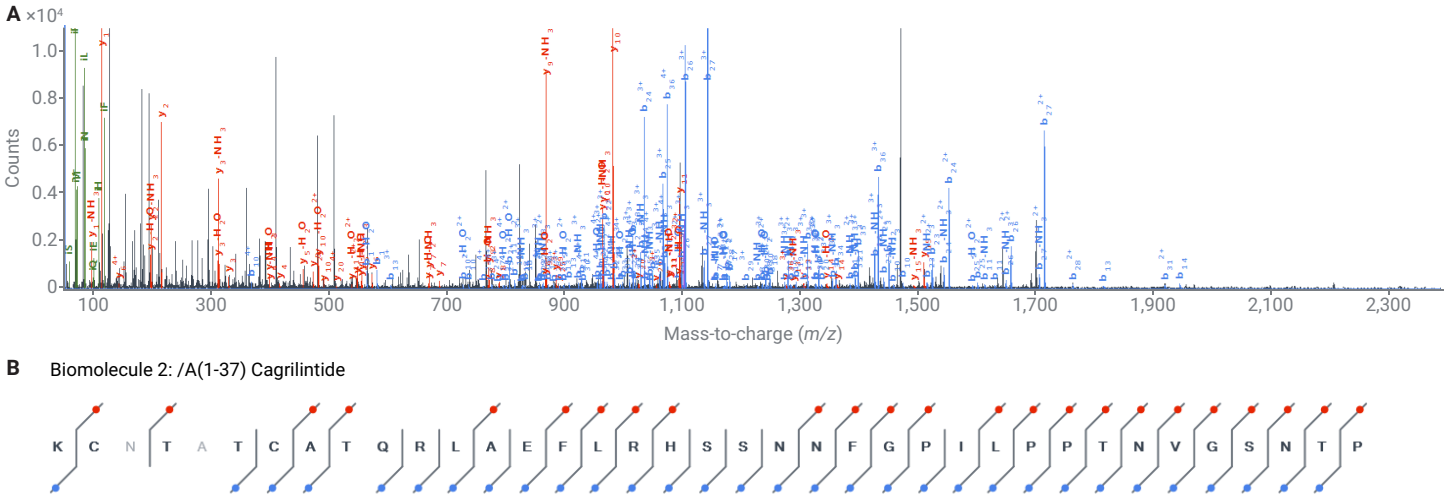

Figure 3. (A) MS/MS spectrum of cagrilintide (native). (B) The fragmentation ladder which annotates the identified b/y series for the sequence.

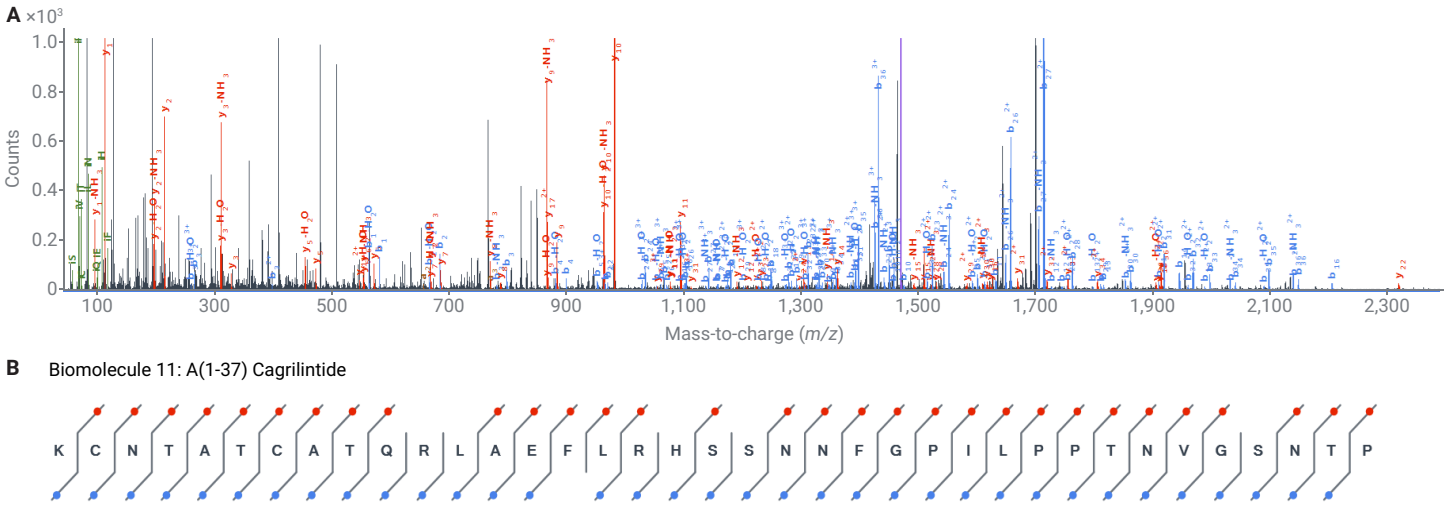

Figure 4. (A) MS/MS spectrum of cagrilintide (reduced). (B) The fragmentation ladder which annotates the identified b/y series for the sequence.

A targeted evaluation of diagnostic fragment ions was carried out by examining the b_7 and y_{36} ion regions (Figure 5), which are particularly informative for assessing structural changes associated with disulfide bond reduction. The expanded spectra display well-resolved peaks with high signal-to-noise ratios, allowing accurate determination of *m/z* values. A corresponding table summarizing the observed fragment masses and their assignments is included with the spectra to facilitate direct comparison between the native and reduced forms.

A clear and consistent mass shift is observed for both b_7 and y_{36} ion series when comparing native and reduced states. These differences, highlighted in Figure 5, are consistent with the reduction of the disulfide bond, resulting in the addition of hydrogen atoms and the expected increase in mass. Importantly, the fragmentation patterns remain conserved, confirming that the peptide backbone sequence is preserved while the structural modification is localized to the disulfide linkage.

This combination of high mass accuracy, clear fragmentation ladders, and predictable mass shifts provides strong, site-specific evidence for disulfide bond connectivity. The results demonstrate that high-resolution Q-TOF MS/MS enables detailed structural elucidation, supporting unambiguous sequence verification of complex peptide therapeutics.

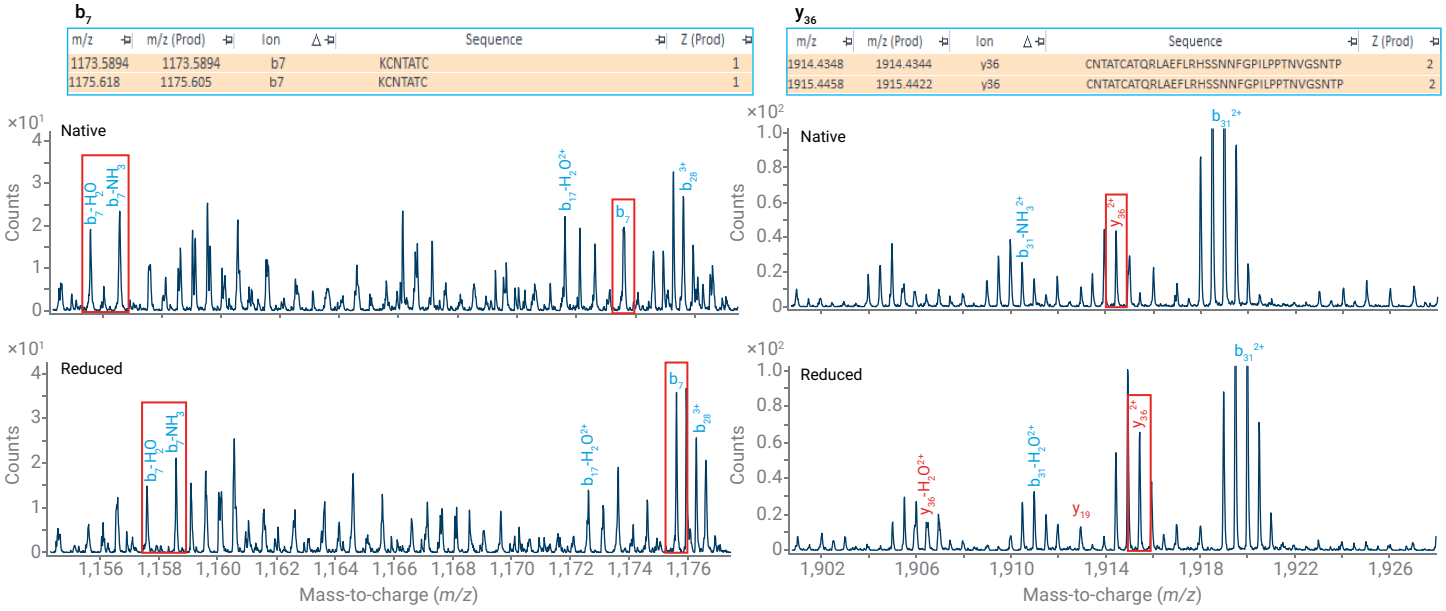

Figure 5. Zoomed-in view of the b_7 and y_{36} MS/MS spectra of cagrilintide in both native and reduced forms. The table associated with the spectra lists the fragment ions assigned to the b_7 and y_{36} series (doubly charged). Differences between the corresponding ions are highlighted with red boxes to clearly indicate +2 Da mass shift associated with disulfide bond reduction.

Conclusion

This study demonstrates that the Agilent 1290 Infinity II bio LC system coupled with the Agilent 6545XT AdvanceBio LC/Q-TOF provides a powerful platform for peptide characterization:

- Accurate intact mass measurement for native and reduced forms
- Confirmation of disulfide bond by way of reduction analysis
- High-confidence sequence confirmation using MS/MS fragmentation
- Robust and reproducible analytical performance

The workflow is well-suited for biopharmaceutical peptide characterization, sequence confirmation, and structural integrity assessment.

References

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