Application Note

Biopharma

Comprehensive Characterization of Multiple GLP-1 Analogs

Using an Agilent 6545XT AdvanceBio LC/Q-TOF with electron capture dissociation and ExDViewer software

Authors

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Abstract

GLP-1 analogs are synthetic peptides that mimic the naturally occurring peptide hormone known as glucagon-like peptide-1 (GLP-1). The development of GLP-1 analogs is important due to their potential for managing diabetes and obesity. Characterization of synthetic peptides using Agilent high-resolution LC/Q-TOFs provides critical quality information about GLP-1 analogs. Implementing electron-based fragmentation in LC/MS workflows is an effective technique for sequence and modification analysis of proteins and peptides. This application note describes the characterization of three GLP-1 analogs—liraglutide, semaglutide, and tirzepatide using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell for electron capture dissociation. Agilent ExDViewer is an effective and accessible software tool for analyzing all types of fragmentation data resulting from proteins and peptides. This application note uses ExDViewer's targeted deconvolution workflow to demonstrate in-depth sequence analysis, characterize custom modifications, and highlight powerful tools to visualize fragmentation trends and data quality.

Introduction

GLP-1 is an insulin-stimulating hormone that binds to the GLP-1 receptor.[1,2] In 2019, the first GLP-1 analog was approved by the U.S. Federal Drug Administration (FDA) for type 2 diabetes, and in 2020, GLP-1 analogs were approved for obesity management.[3,4] With the increasing use of GLP-1 analogs, it is important to have precise mass spectrometry (MS) methods for structural characterization, purity, and counterfeit analysis.

LC/MS/MS analysis of synthetic peptides using electron capture dissociation (ECD) provides detailed sequence, modification, and purity information. In contrast to collision-induced dissociation (CID), ECD is considered a "gentler" fragmentation approach that retains modifications such as the fatty acid modifications in GLP-1 analogs. This allows their mapping and characterization, while still effectively cleaving the peptide backbone (Figure 1). Also, secondary fragmentation of amino acid side chains via ECD provides additional evidence of sequence assignments and enables the investigation of amino acid isomers such as aspartate and isoaspartate.[5,6] The Agilent ExD cell and Agilent ExDViewer software provide a powerful approach for the detailed characterization of synthetic peptides with complex structures.

This application note describes LC/MS/MS analysis of three GLP-1 analogs: semaglutide, liraglutide, and tirzepatide. The analysis was performed using a 6545XT LC/Q-TOF equipped with an ExD cell to enable ECD. ExDViewer was used to analyze MS/MS fragmentation spectra using the targeted deconvolution workflow, which considers unique aspects of electron fragmentation such as hydrogen transfer and side chain fragmentation.[7] ExDViewer also has useful tools for visualizing fragmentation trends and understanding data quality in both ECD and CID experiments. By combining ExD hardware and software, these methods enabled fast and in-depth characterization of peptide sequences with nonstandard residues and characterization of custom modifications.

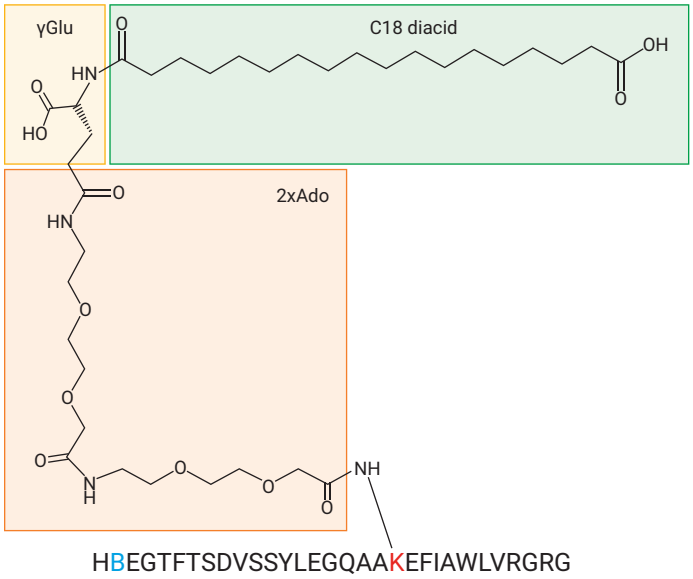

Figure 1. The structure of semaglutide contains the noncanonical amino acid 2-amino isobutyrate in amino acid position 2, indicated by the letter B. Lysine 20 is modified by a C18 fatty acid connected through a bis-aminodiethoxyacetyl (2xAdo) and gamma glutamate linker. Liraglutide and tirzepatide lysine modifications have similar structures with variations in the linker and fatty acid group.

Experimental

Chemicals and standards

- Agilent ESI-L tuning mix (part number G1969-85000) containing 2.5 µg/mL melittin (part number G1997-85001)
- Formic acid, 99.0+%, Optima LC/MS-grade, Cat. no. A-117-50, Fisher Chemical, (part number A-117-50)
- Acetonitrile, 99.9%+ LC/MS-grade, Supelco OmniSolv (part number AX0156-6)
- Liraglutide, Sigma (part number SML3925)
- Semaglutide, AstaTech (part number AT35750)
- Tirzepatide, AstaTech (part number AT40456)

Sample preparation

Each sample was prepared at 10 µM in 15% acetonitrile with 0.1% formic acid. For LC experiments, an equal volume of the three samples was mixed together.

HPLC column

- Agilent AdvanceBio Peptide Mapping 120Å, 2.1 × 150 mm, 2.7 µm (part number 653750-902)

Instrumentation

- Agilent 1290 Infinity II Bio LC System
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF MS with Agilent ExD cell

Software

- Agilent MassHunter acquisition software for LC/Q-TOF, version 11.0
- Agilent ExDControl software, version 3.6
- Agilent ExDViewer software, version 4.6.12

Liquid chromatography methods

The LC methods described in this application note were used for most ECD experiments. However, direct infusion was used for tuning the ExD cell and to investigate charge state-dependent fragmentation. Direct infusion was accomplished using a 500 µL syringe and an infusion rate of 10 to 20 µL/min. PEEK tubing was used to transfer the sample from the infusion syringe to the nebulizer inlet of the Agilent Dual AJS source. Figure 2 shows the chromatographic separation of a mixture of all three GLP-1 analogs.

Table 1. Liquid chromatography methods used for GLP-1 analogs.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent AdvanceBio Peptide Mapping 120Å, 2.1 × 150 mm, 2.7 µm
Mobile Phase A	LC/MS-grade water + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate	0.400 mL/min
Injection Volume	10 µL
Column Temperature	60 °C
Gradient Program	Time (min) %B 0 20 20 60 20.10 80 21.90 80 22 20

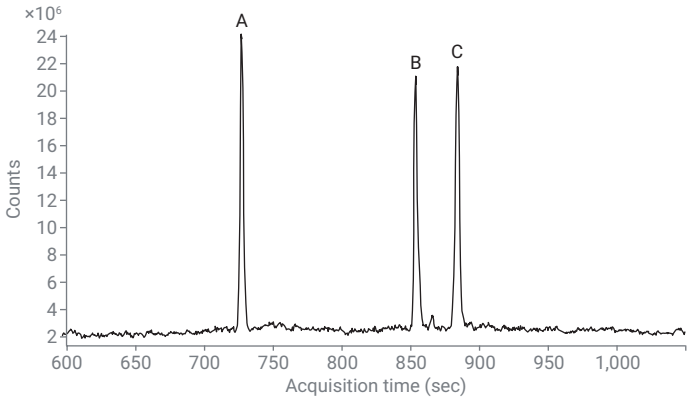

Figure 2. The chromatogram of a mixture of GLP-1 analogs. The peak identities are: (A) semaglutide, (B) liraglutide, and (C) tirzepatide.

Mass spectrometry methods

Table 2. LC/Q-TOF parameters.

Agilent 6545XT Q-TOF MS system	
Ion Source	Agilent Dual Jet Stream ESI source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	10 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	75 V
Acquisition Rate	2 spectra/sec
Acquisition Mode	Extended Dynamic Range (2 GHz)
Isolation Window	Medium (4 amu)
MS Spectrum Range	*m/z* 120 to 3,200
MS/MS Spectrum Range	*m/z* 120 to 3,200

Table 3. Auto MS/MS data acquisition parameters.

Parameter	Value
Precursor Selection	Preferred list for 4+ precursors: – *m/z* 938.2 (liraglutide) – *m/z* 1,028.7 (semaglutide) – *m/z* 1,203.6 (tirzepatide)
Intensity Threshold (Abs)	2,000
Intensity Threshold (Rel)	0.01%
Mass Error Tolerance	20 ppm
Static Exclusion	*m/z* 100 to 400
Isotope Model	Peptides
Use Preferred Ion List Only	False

Targeted ECD MS workflow

The ExD cell lenses and internal filament were controlled by ExDControl software, version 3.6, which operates as a separate application alongside MassHunter acquisition software. An optimal filament current of 2.55 A was determined by an automated filament tune, followed by manual adjustment to optimize melittin ECD fragments. The lens profile for MS1 transmission of intact ions was determined by autotuning on tune mix ions. Melittin ECD fragment ion peaks were used to autotune the lens profile used for ECD in MS2. For LC experiments, an auto MS/MS acquisition method was set up in MassHunter acquisition software with the parameters described in Tables 2 and 3. For direct infusion experiments, a targeted MS/MS acquisition was used to investigate individual charge states.

Data analysis

GLP-1 analog molecular weights were determined using ExDViewer's MS1 deconvolution workflow. Raw .d files were loaded directly into ExDViewer and default parameters were used for deconvolution. MS1 spectra were averaged over each chromatographic peak. Decharged spectra were generated by toggling the spectrum view in the spectrum window. Files were saved as .svg files and imported into Microsoft PowerPoint for customization of the figure elements.

Sequence and modification analysis was accomplished using ExDViewer's targeted deconvolution. First, the GLP-1 analog sequences were defined in the target editor and searched against the MS/MS results. Default parameters were used for fragment matching, except for characterizing lysine modifications. For modification analysis, the ion score threshold was raised to 10 to observe only the highest quality modification containing fragment matches.

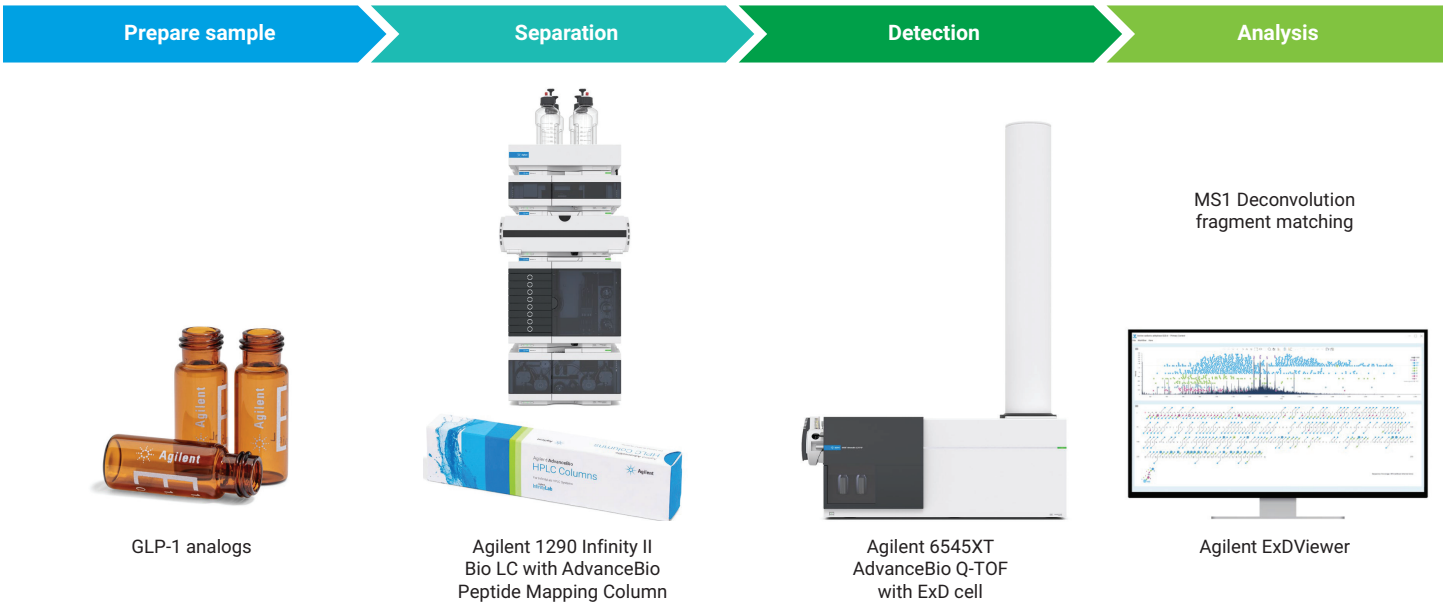

Figure 3. Workflow for the LC/MS/MS analysis of GLP-1 analog synthetic peptides.

Results and discussion

Isotopically resolved spectral deconvolution using ExDViewer

The analysis of GLP-1 analogs using Agilent LC/MS/MS workflows enables the measurement of molecular weight, signal heterogeneity, and sequence in a single experiment. ExDViewer's untargeted deconvolution algorithm effectively processes signals from isotopically resolved spectra, commonly obtained during peptide analysis. MS1 deconvolution allows the determination of the molecular weight of synthetic peptides.

Figure 4 illustrates the MS1 and deconvoluted spectra for each GLP-1 analog from the mixture shown in Figure 2. The deconvoluted spectra reveal that the primary signal from each chromatographic peak corresponds to the GLP-1 analogs, along with some less abundant salt-adducted peaks (Na^+/K^+). Deconvolution of isotopically resolved signals with ExDViewer offers a complementary approach to Agilent MassHunter BioConfirm maximum entropy deconvolution for larger protein ions.

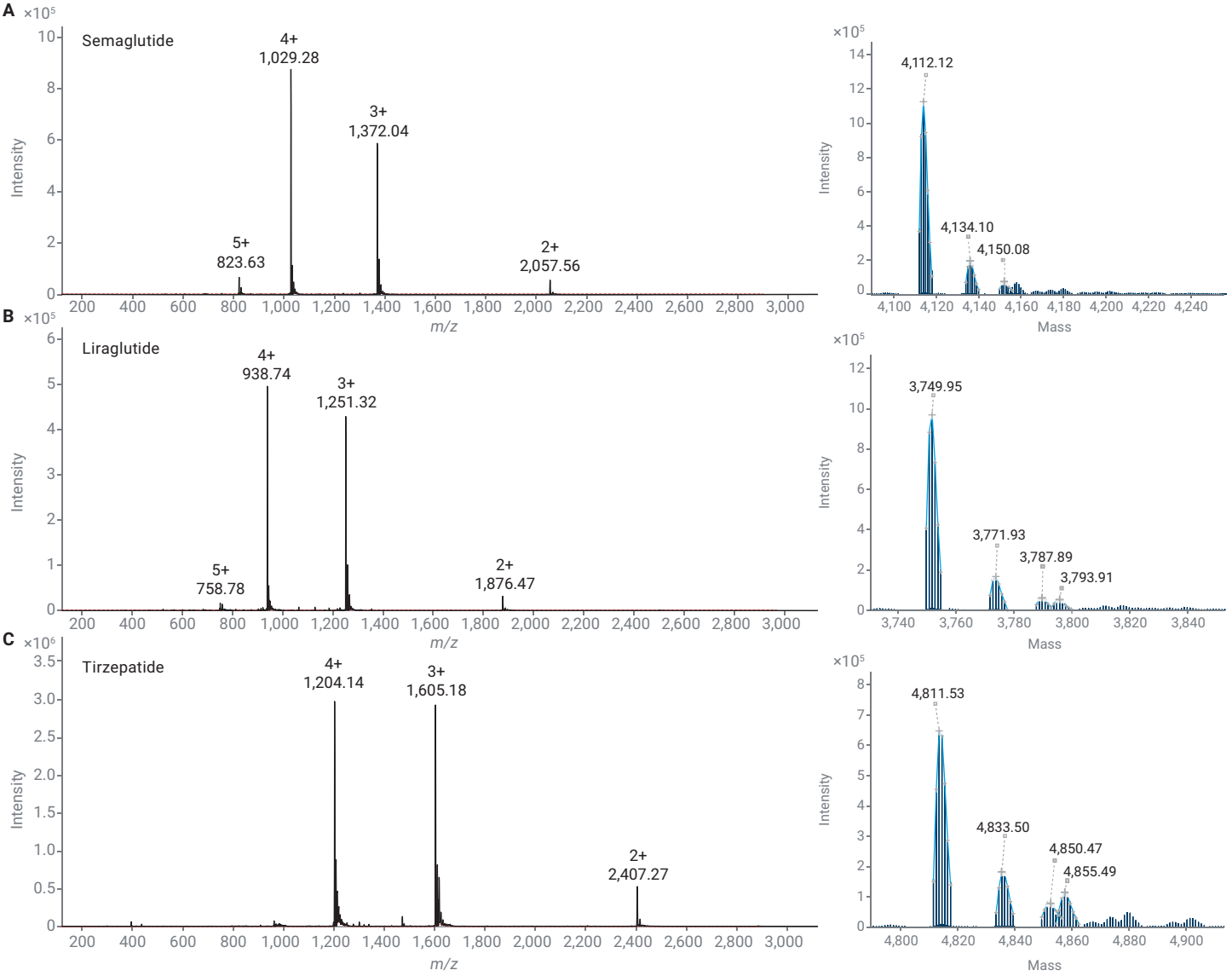

Figure 4. The MS1 spectra (left) and corresponding deconvoluted spectra (right) of the LC-separated GLP-1 analogs (A) semaglutide, (B) liraglutide, and (C) tirzepatide. The monoisotopic masses are annotated in the deconvoluted mass spectra.

Sequence analysis with noncanonical amino acids

Gas phase fragmentation using ECD induces precise breaks in the peptide backbone, which yields clean and reproducible fragmentation spectra that are easily interpreted using ExDViewer. Semaglutide and tirzepatide contain noncanonical amino acids, which can be defined as custom amino acids in the Building Block editor within ExDViewer. In the featured analysis, the noncanonical amino acid is defined as 2-amino isobutyrate, abbreviated as the single-letter code B. Using a single targeted MS/MS spectrum collected at 2 Hz, 100% sequence coverage was obtained for the 4+ precursors of liraglutide, semaglutide, and tirzepatide. Multiple CID and ECD fragment ion types (a,b,c,d and w,y,z) were detected, providing complimentary evidence for sequence confirmation (Figure 5).

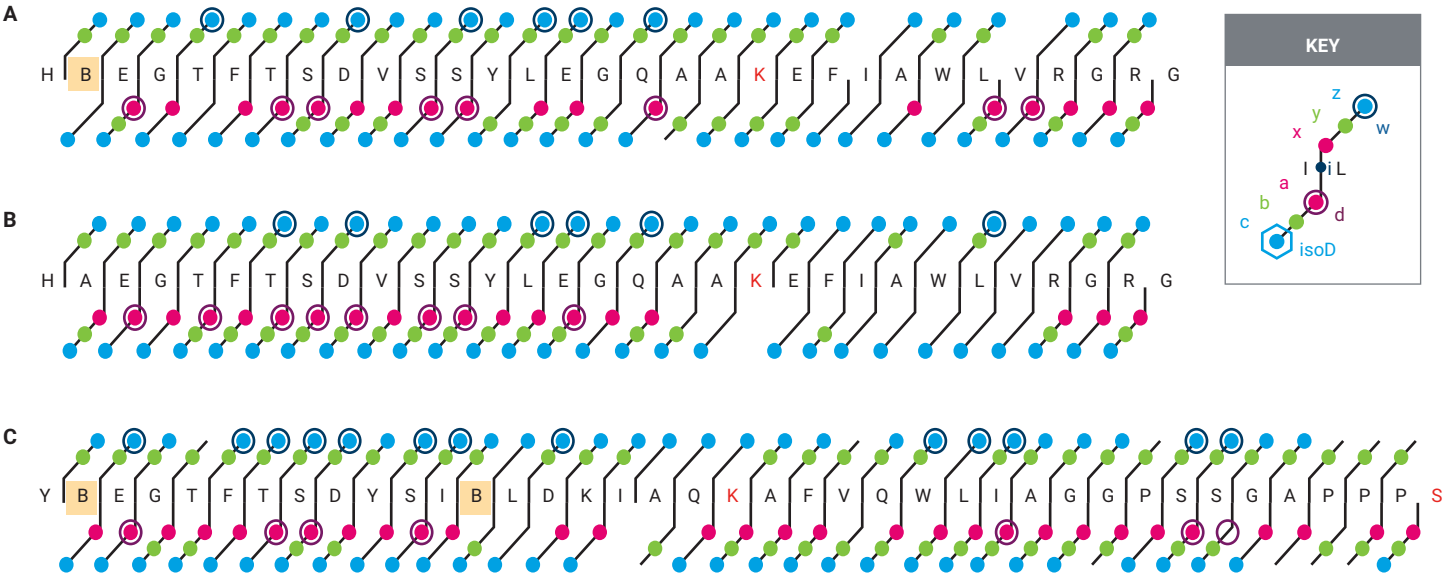

Figure 5. The sequence coverage maps for (A) semaglutide, (B) liraglutide, and (C) tirzepatide. The nonstandard B amino acid is highlighted in yellow, while the modified lysine is indicated in red text. Several complimentary c/z and b/y-type ions were identified. Default parameters were used for Agilent ExDViewer fragment matching.

Abundant amino acid side chain fragments are commonly produced using the ExD cell, which provides additional information about isomeric amino acids such as leucine and isoleucine. Secondary fragmentation of amino acid side chains from ECD radical ions are referred to as w-ions.[4] ExDViewer automatically annotates side chain evidence, simplifying the analysis of amino acid isomers.[7] Ions specific to isoaspartate formation, an important modification for assessing protein quality, are also annotated.[6,7] In Figure 6, the w_{23}^{2+} ion is annotated, providing additional evidence of aspartate in this position. Hovering over the w_{23}^{2+} ion opens an informative tooltip that describes the percentage hydrogen transfer, mass, side chain loss formula, score, and number of matched isotopes.

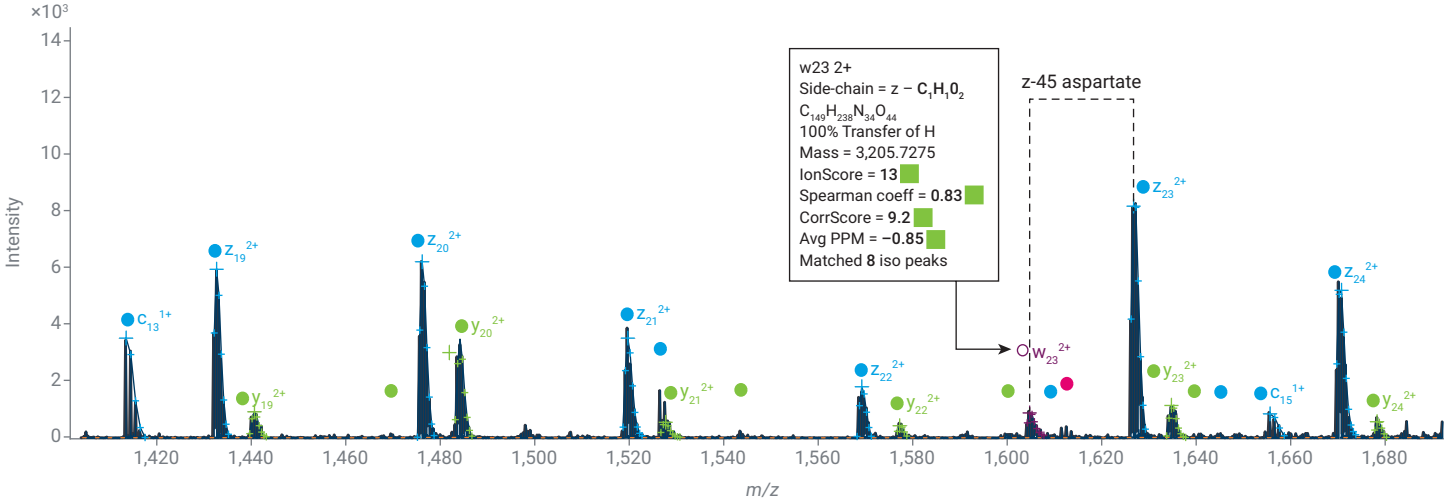

Figure 6. A representative fragmentation spectrum from liraglutide showing the automatically annotated fragment evidence for isomeric amino acids such as aspartate and isoaspartate. Aspartate is identified by the loss of a CHO_2 group from the z_{23}^{2+} ion.

Visualizing fragmentation trends with ECD and CID

Automatically generated plots in ExDViewer illustrate fragmentation trends resulting from various experimental conditions. Visualizing fragmentation trends is a valuable way to assess data quality. For example, the ion intensities graph shows the signal detected for each ion type, including internal and unassigned ions. These graphs are also useful for evaluating the suitability of method parameters such as collision energy. In Figure 7, the ion intensity graphs are used to demonstrate the charge state-dependent fragmentation patterns of semaglutide using ECD or a combination of CID and ECD.

With zero collision energy, ECD effectively achieved 100% sequence confirmation for the 5+ and 4+ semaglutide precursors. However, the 3+ precursor was more challenging to fragment using ECD alone. Adding 35 V of supplemental collision energy helped improve sequence coverage from 57 to 93% for the 3+ precursor (Figures 7C and 7F). Notably, the summed fragment ion abundance for 3+ semaglutide was similar with and without CID, indicating that the increased sequence coverage upon adding CID reflects a different fragmentation pathway rather than a general increase in fragment ion abundance. Therefore, combining ECD with low-level activation can be used as a tool to achieve more

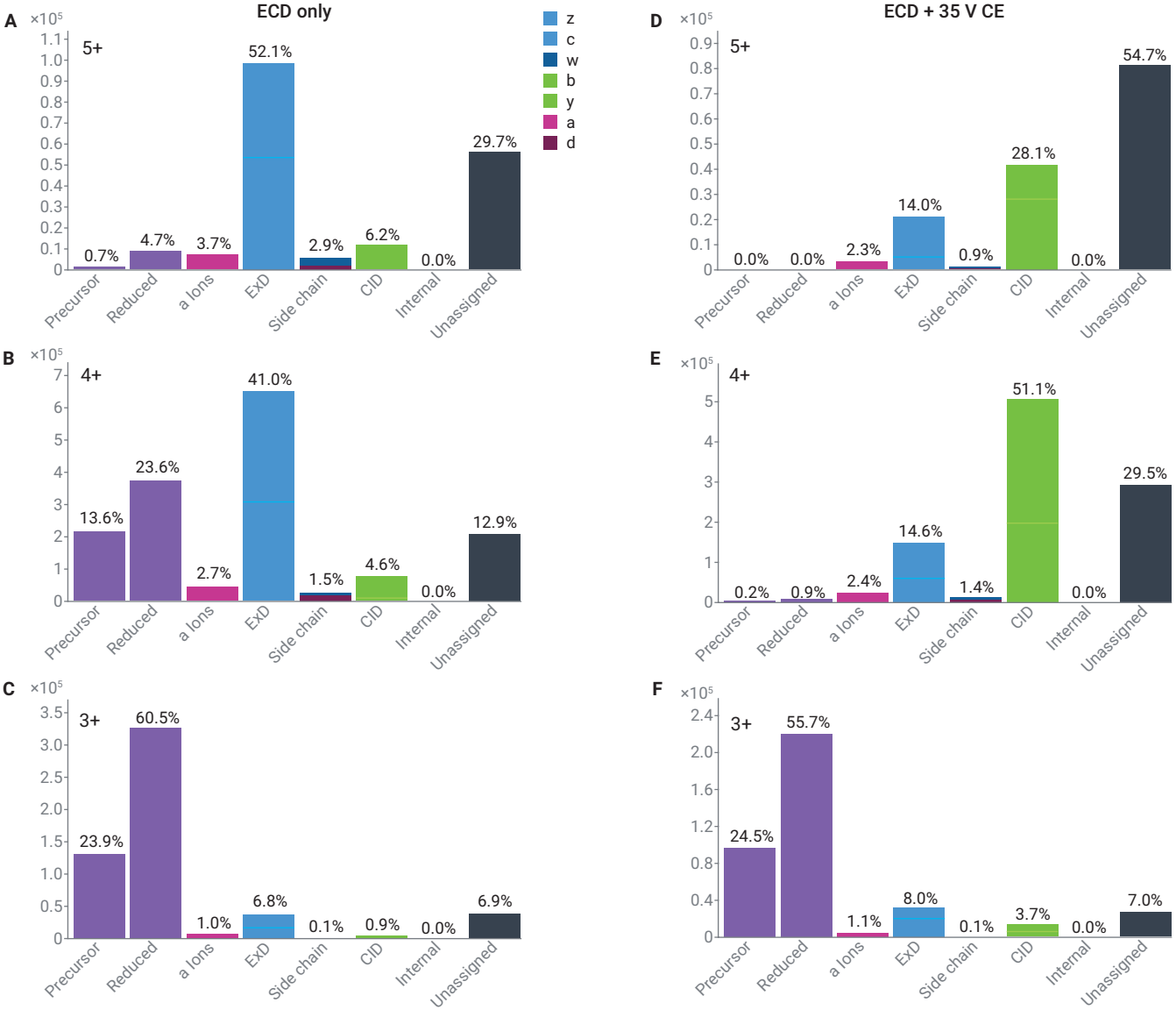

Figure 7. Fragmentation pathways of the semaglutide 3, 4, and 5+ precursor ions. Panels A, B, and C show the ion intensities under ECD-only conditions. Panels D, E, and F show ion intensities resulting from ECD combined with 35 V of collisional activation (ECD+CID). Internal fragment ions were not considered during the analysis of these data sets.

complete sequence coverage even for low-charge peptides. However, adding too much collision energy can result in a high percentage of unassigned ion signals, presumably due to over-fragmentation (Figure 7D).

Characterization of GLP-1 analog modifications

As new GLP-1 analog derivatives are created, it is important to have effective methods to characterize their modifications. Here, ExDViewer was used for fragment analysis to identify the site and structure of synthetic modifications in three GLP-1 analogs. The custom chemistries of the GLP-1 analog modifications were defined in ExDViewer's Modifications Editor.

The ExDViewer fragment matching algorithm assigns each isotope cluster an ion score based on *m/z* and intensity match, along with the surrounding noise levels. The default ion-score setting is effective for many sequence analysis applications. However, for modification characterization, strict spectral quality is essential for pinpointing the modification location. Figure 8 highlights several examples of modification-containing ECD ions detected for tirzepatide, demonstrating the range of ion match quality that can be observed in a fragmentation spectrum.

Comparing ion scores of GLP-1 analogs analyzed with ECD or CID reveals that ECD fragmentation generates more high-quality, modification-containing fragments than CID (Figure 9). High-quality matches on both sides of the modification enhance localization confidence. In many cases, ECD fragmentation alone provides detailed structural information, circumventing the need for collision energy optimization, which saves time in sequence analysis.

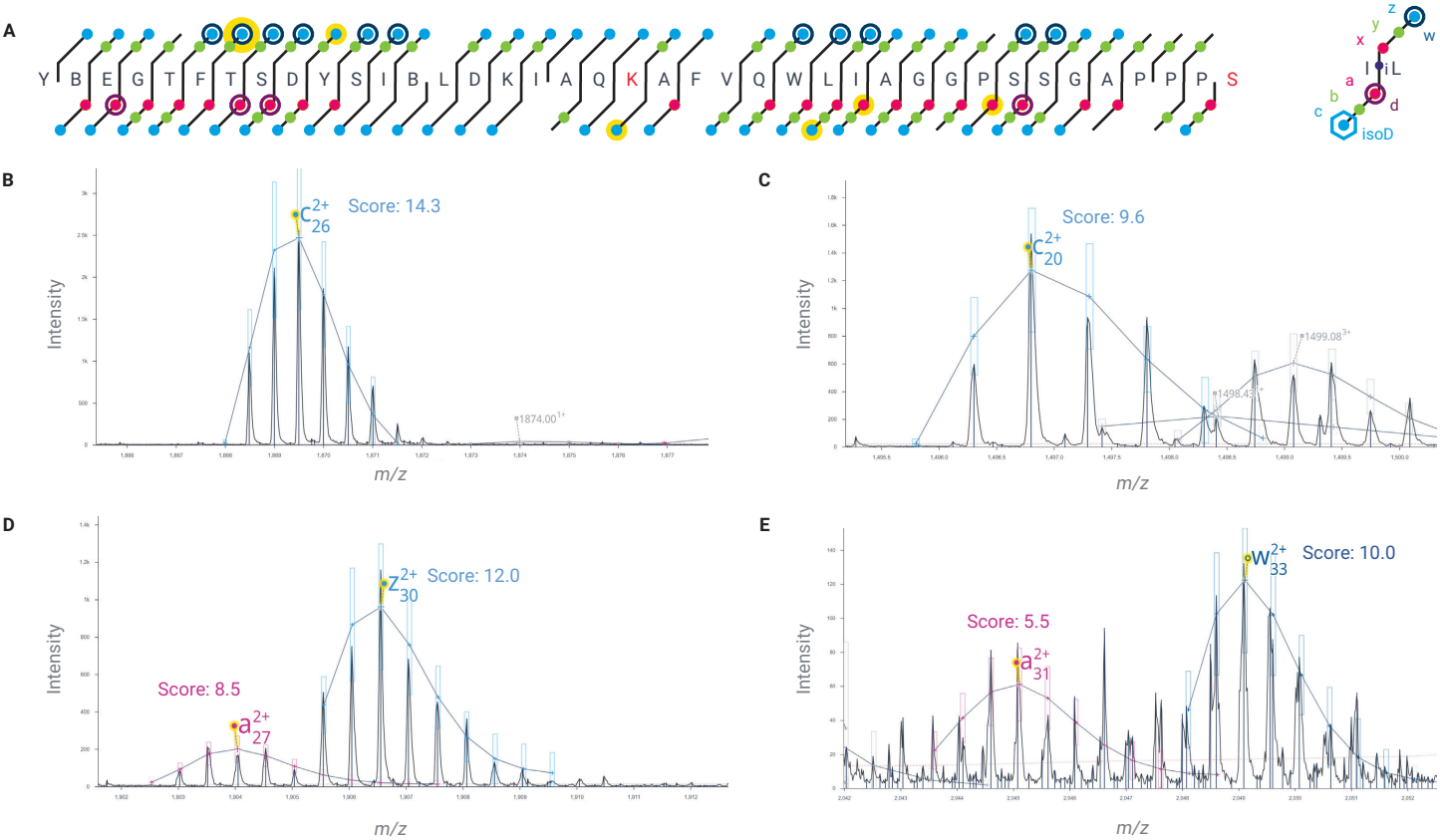

Figure 8. Representative tirzepatide fragments with various ion scores. Each ion is assigned a score based on the *m/z* and predicted intensity match, as well as the surrounding noise levels. (A) Tirzepatide sequence coverage map where only ions with a score of 5 or greater were considered. (B–E) Examples of modification containing fragments and their corresponding ion scores. The modified lysine is indicated in red text. The c_{20}^{2+} ion is a site-specific localizing fragment, while the other highlighted ions are modification-containing peptides fragmented at various positions in the peptide backbone.

Unlike collisional dissociation, ECD is a gentle technique that fragments the peptide backbone while preserving important labile chemical modifications. Comparing ion scores of GLP-1 analogs analyzed with ECD or CID reveals that ECD fragmentation generates more high-quality, modification-containing fragments than CID (Figure 9).

High-quality matches on both sides of the modification enhance localization confidence. In many cases, ECD fragmentation alone provides detailed structural information, circumventing the need for collision energy optimization, which saves time in sequence analysis.

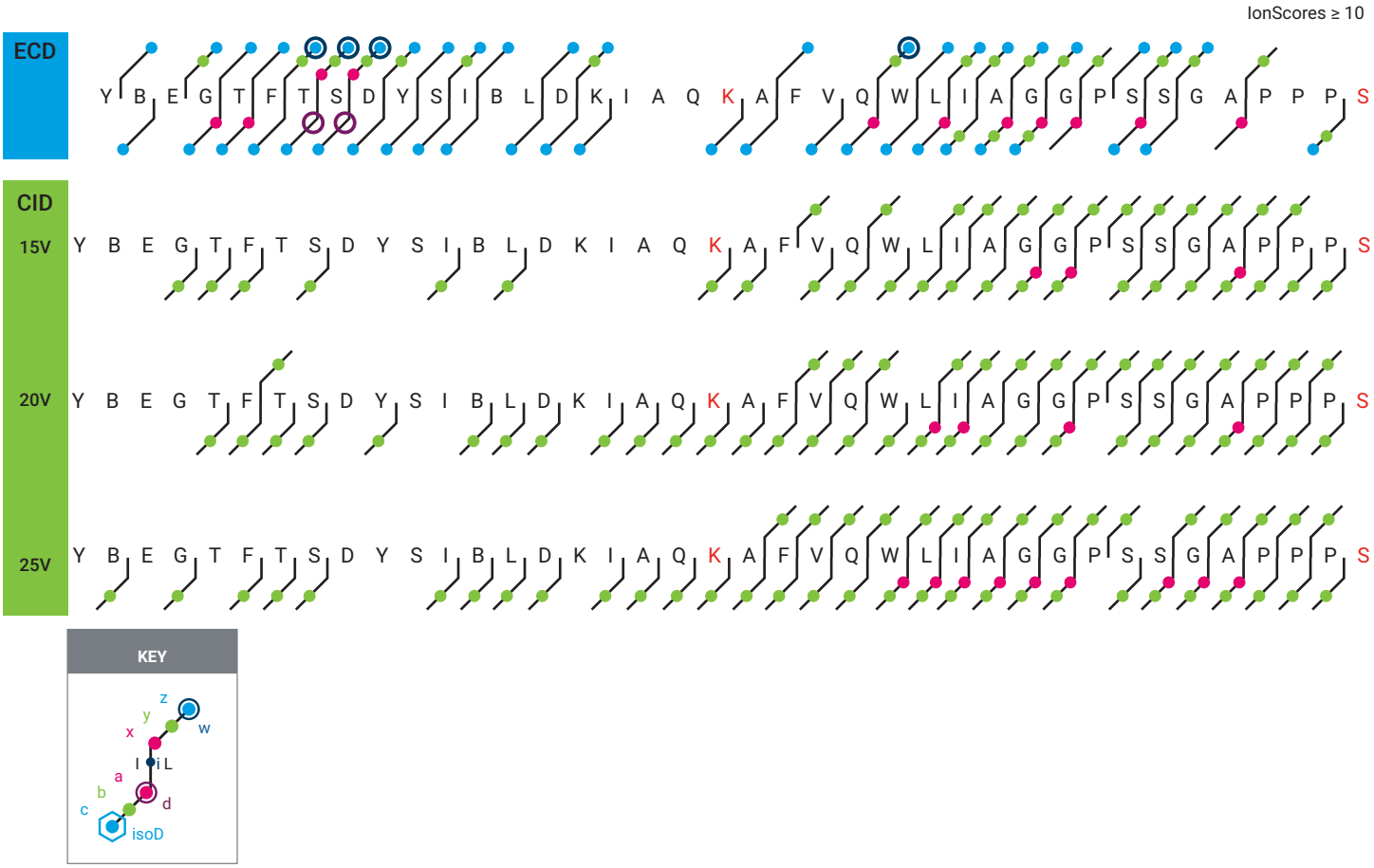

Figure 9. Tirzepatide fragments identified in ECD or CID experiments with ion scores of 10 or greater. ECD provided more high-quality modification-containing fragments, particularly on the N-terminal side of the modification. ECD yielded a more complete ion series with several complimentary ion types. The exception to this is the proline-rich region because ECD does not cleave on the N-terminal side of proline.

Conclusion

This application note demonstrates the comprehensive sequence analysis and localization of modifications in three synthetic GLP-1 analogs by electron dissociation using an Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent ExDViewer for fragment analysis. Fragmentation of amino acid side chains offers additional evidence for identifying amino acid isomers such as aspartate and isoaspartate. High-quality fragment matches from ECD data enable effective modification characterization and localization, saving time as it does not require optimization of collision energy. ExDViewer provides powerful tools for visualizing fragment pathways and data quality for both ECD and CID experiments. The methods presented in this application note provide a framework for the analysis of GLP-1 analogs and should also be used as a starting point for investigating new GLP-1 analog derivatives and impurities using ECD.

References

1. Andersen, A.; Lund, A.; Knop, F. K.; Vilsbøll, T. Glucagon-Like Peptide 1 in Health and Disease. *Nat. Rev. Endocrinol.* **2018**, *14(7)*, 390–403.
2. Holst, J. J. The Physiology of Glucagon-Like Peptide 1. *Physiol. Rev.* **2007**, *87(4)*, 1409–1439.
3. U.S. Food and Drug Administration FDA News Release. FDA Approves First Oral GLP-1 Treatment for Type 2 Diabetes. https://www.fda.gov/news-events/press-announcements/fda-approves-first-oral-glp-1-treatment-type-2-diabetes (accessed 2019).
4. U.S. Food and Drug Administration FDA News Release. FDA Approves Weight Management Drug for Patients Aged 12 and Older. https://www.fda.gov/drugs/news-events-human-drugs/fda-approves-treatment-adults-alzheimers-disease (accessed 2020).
5. Beckman, J. S.; Voinov, V. G.; Hare, M.; Sturgeon, D.; Vasil'ev, Y.; Oppenheimer, D.; Shaw, J. B.; Wu, S.; Glaskin, R.; Klein, C.; *et al.* Improved Protein and PTM Characterization with a Practical Electron-Based Fragmentation on Q-TOF Instruments. *J. Am. Soc. Mass Spectrom.* **2021**, *32(8)*, 2081–2091.
6. Wang, J.; Mukherjee, S.; Zubarev, R. A. Isoaspartate and Neurodegeneration. *Aging* (Albany NY), **2022**, *14(22)*, 8882.
7. Identification of Amino Acid Isomers Using Electron Capture Dissociation in the Agilent 6545XT AdvanceBio LC/Q-TOF System. *Agilent Technologies application note*, publication number 5994-7506EN, **2024**.

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