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Structural Characterization of Multiple GLP-1 Receptor Agonists using Electron Capture Dissociation

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Introduction

Over the past few years, the demand for GLP-1 receptor agonists for managing type 2 diabetes and obesity has surged.

As new GLP-1 receptor agonists emerge, characterizing primary sequence and modification identities early in the development cycle is crucial.

In this study we demonstrate in-depth LC-MS/MS sequence and modification characterization of multiple GLP-1 receptor agonists.

Electron capture dissociation (ECD) facilitated the detailed characterization of GLP-1 receptor agonists by generating sequence-informative fragments while retaining modifications[1].

ExDViewer was applied to analyze fragmentation patterns of GLP-1 receptor agonists with non-standard building blocks and modifications. (Figure 1)

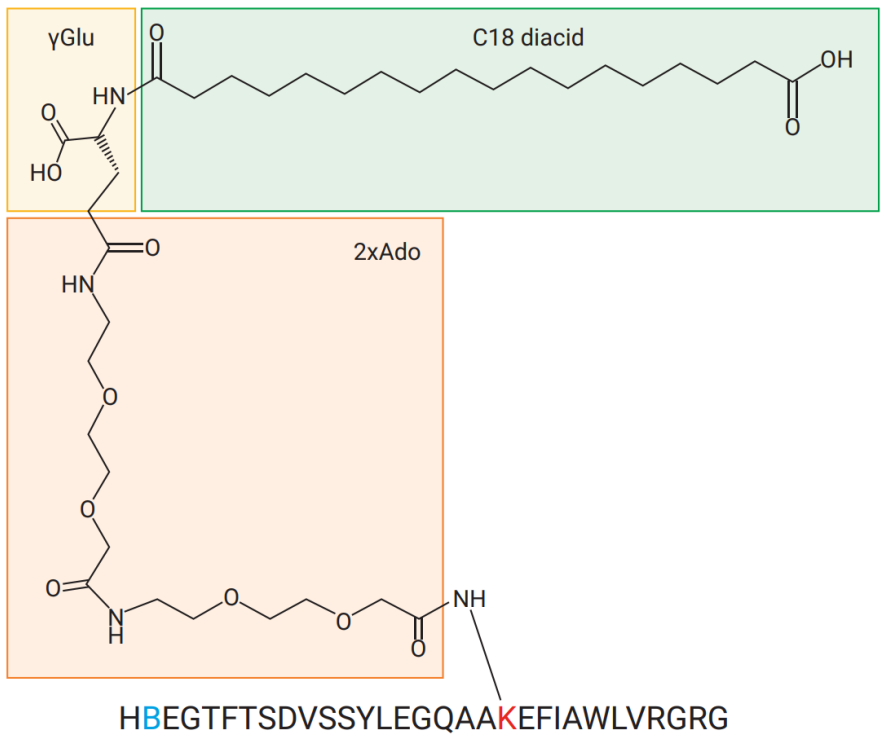

Figure 1. The structure of semaglutide. The non-standard 2-amino isobutyrate amino acid is indicated by the letter B. The structure of the fatty acid containing modification on lysine 20 is highlighted.

Visualizing fragmentation patterns

Observed MS/MS data were compared to theoretical structures of three GLP-1 receptor agonists. Automatically generated data summary figures helped visualize fragmentation pathways, aiding in determining method parameters like collision energy.

Lastly, we demonstrate how ExDViewer identifies isomeric amino acids such as Asp/isoAsp through automatic annotation of isomer-specific fragment ions.

Experimental

Sample preparation

Samples were prepared at 10µM in 15% acetonitrile with 0.1% formic acid. For LC experiments, GLP-1 analogs were mixed at equal molar concentrations.

Instrumentation

The Agilent 1290 Infinity II Bio LC System with the Agilent AdvanceBio Peptide Mapping column were used for LC separations. Chromatography methods are described in a recent application note (See ref. 1).

Direct infusion was used to investigate fragmentation patterns of individual charge states. No internal reference mass was used for calibration.

All GLP-1 analogs were analyzed using the Agilent 6545XT AdvanceBio LC/Q-TOF MS equipped with an ExD cell to enable ECD fragmentation. (Figure 2)

Figure 2. An instrument schematic showing the Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an ExD cell for electron fragmentation.

Data analysis

Custom amino acids and lysine modifications with fatty acids were defined in ExDViewer's building block and modification editors.

The ExDViewer targeted deconvolution workflow was used to search expected structures against ECD and CID MS/MS fragmentation spectra. Default settings for fragment matching were used.

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Results and Discussion

Isotopically resolved deconvolution of GLP-1 analogs

Intact mass determination is one of the first steps in synthetic peptide characterization. Here, we show that ExDViewer's MS1 deconvolution algorithm works well for isotopically resolved GLP-1 receptor agonist signals.

A mixture of three GLP-1 receptor agonists were separated with liquid chromatography and their intact masses measured. The monoisotopic masses for semaglutide, liraglutide, and tirzepatide were determined to be 4,112.12 Da, 3,749.95 Da, and 4,811.53 Da. (Figure 3)

ExDViewer's deconvolution algorithm is effective for isotopically resolved peptide and protein signals. This algorithm is complimentary to the maximum entropy deconvolution in Agilent MassHunter BioConfirm for larger, non-isotopically resolved ions.

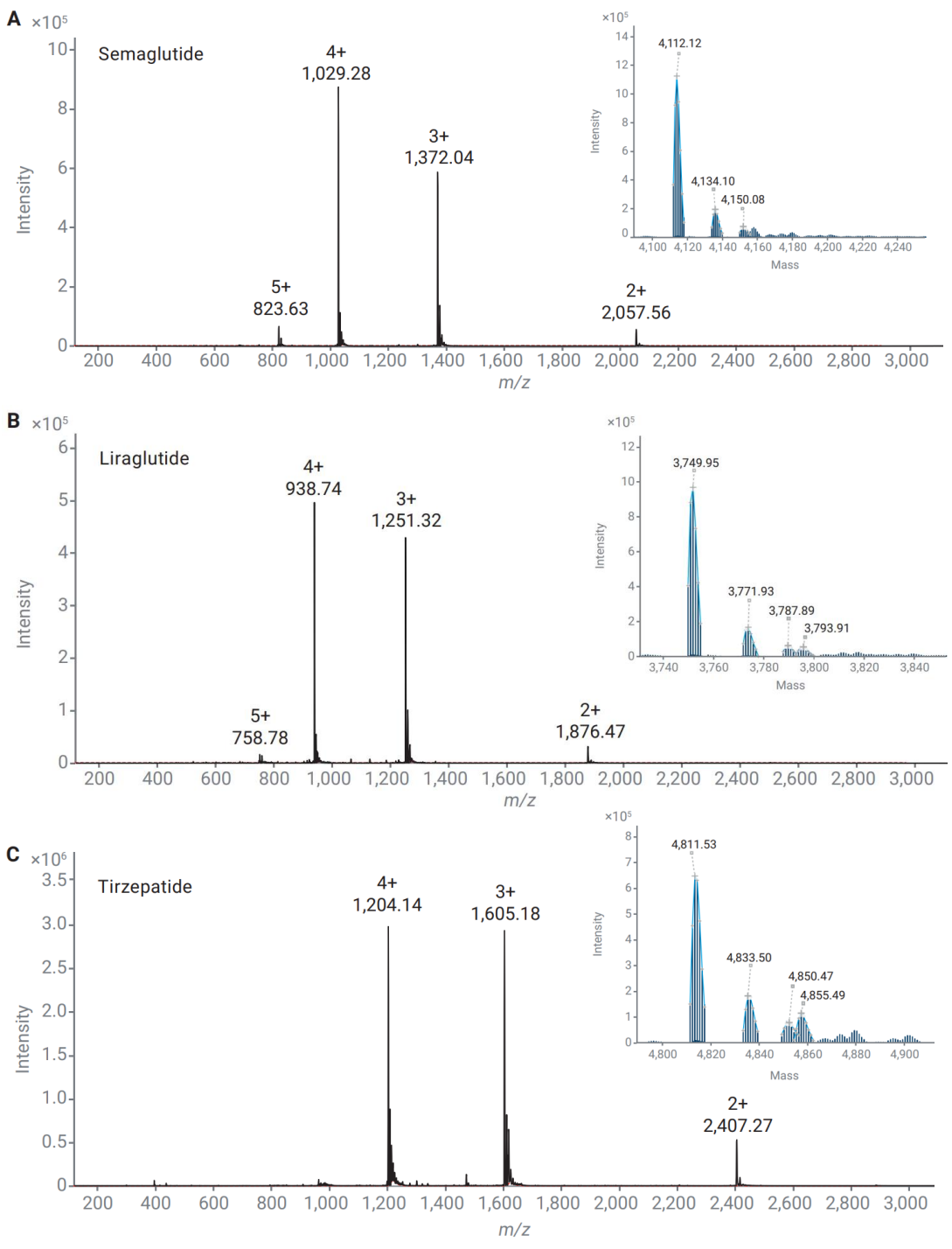

Figure 3. The intact mass spectra for A) semaglutide, B) liraglutide, and C) tirzepatide. The insets show the deconvoluted, zero charge mass for each GLP-1 receptor agonist.

Visualizing method suitability: charge state-specific fragmentation pathways

Synthetic peptides with non-standard amino acids and modifications can challenge fragment matching algorithms. We overcome this issue by building the custom amino acid and modification chemistry into the target search using ExDViewer. We then compared the observed MS/MS spectra to the expected structures for each peptide.

The ECD fragments detected for the 4+ precursor of each GLP-1 receptor agonist resulted in 100% sequence coverage. Precursor fragmentation pathways were visualized using the ion intensities graph which is useful for guiding method development such as precursor selection and optimization of collision energy.

For example, the 4+ precursor yielded the most intense ECD/CID type ions while minimizing unassigned ions. In contrast, the 3+ precursor remained mostly intact under ECD conditions, but fragmentation was improved with 35V of added collision energy. However, the 5+ semaglutide precursor was prone to over-fragmentation with supplemental collision energy. (Figure 4)

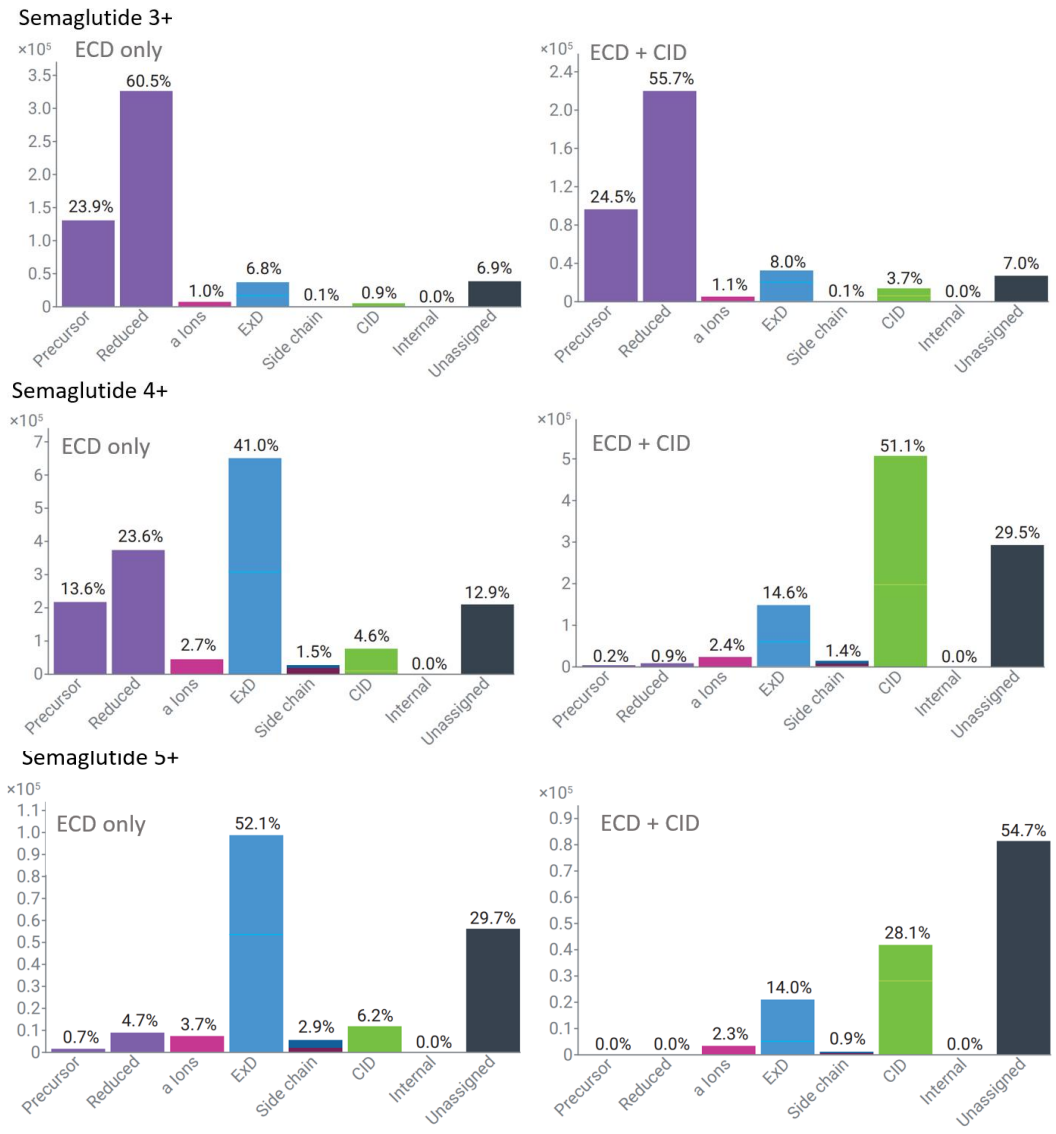

Figure 4. MSMS ion intensities graphs for semaglutide 3+, 4+, and 5+ precursors. The columns are; precursor/reduced precursor ions (purple), a ions (pink), ExD ions (blue), side chain ions (navy), CID ions (green), and unassigned ions (black). Internal ions were not considered.

Results and Discussion

Monitoring isoaspartate formation

An advantage of ECD is its ability to inform on isoaspartate formation via unique c+57 and z -57 fragments[2]. We use ExDViewer to monitor for the unique isoaspartate ions which are automatically annotated in the m/z spectrum.

Figure 5 demonstrates the annotated isoaspartate evidence using a model peptide with the sequence REALLYisoDELIGHTFLK where an isoaspartate is identified in amino acid position 7.

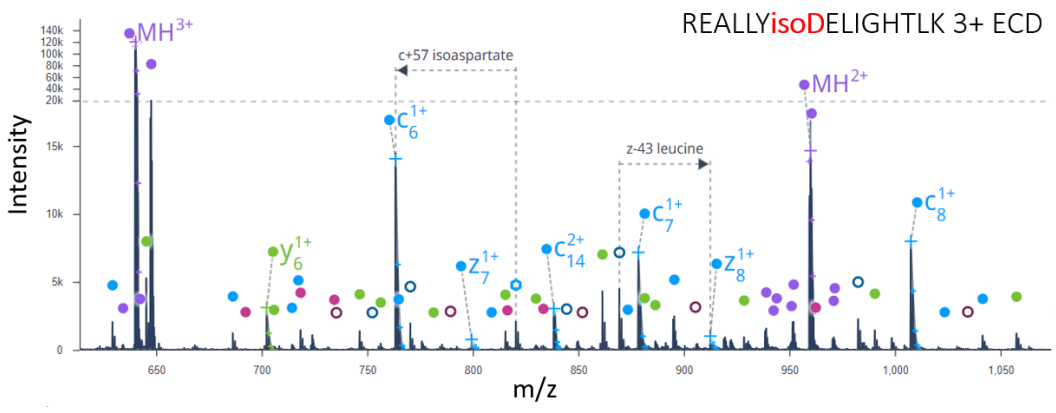

Figure 5. The ECD spectrum of REALLYisoDELIGHTFLK. Ions specific to isomeric amino acids I/L and D/isoD are automatically annotated.

ECD retains sensitive modifications, enabling precise localization without CID energy optimization.

Comparing ECD and CID fragmentation patterns for tirzepatide, we show that ECD generates more modification containing fragment ions. (Figure 7)

Detection of modification containing ions on both sides of the modified residue results in more confident localization through the quality and redundancy of supporting fragment information.

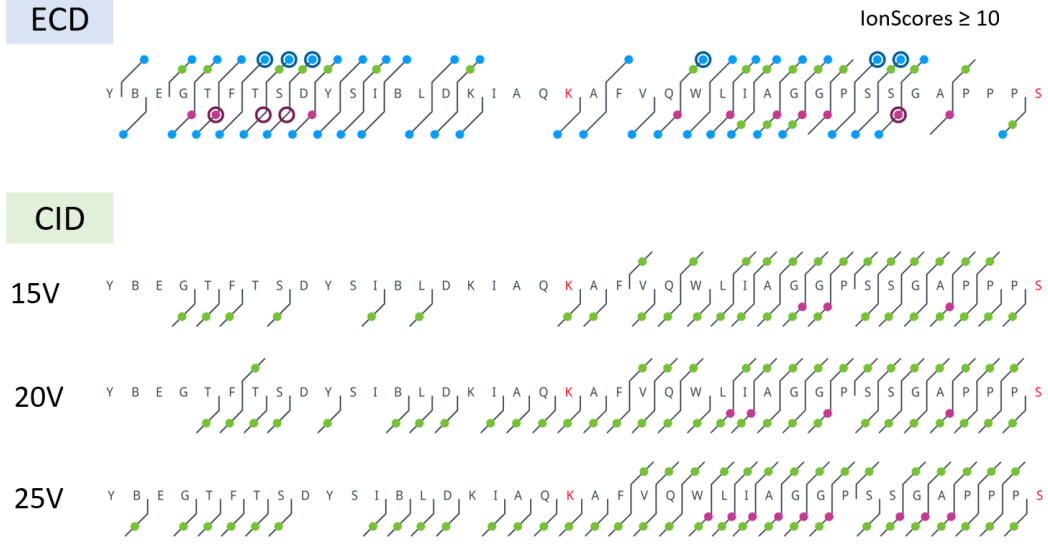

Figure 7. Sequence maps for tirzepatide generated using ECD or CID fragmentation. Only high-quality ions with a IonScore of 10 or greater were considered. The maximum IonScore is 15.

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Figure 6 shows the sequence coverage map of liraglutide where the isomerization of Asp8 was investigated. Table 1 summarizes the fragment ion information detected for this residue showing a lack of fragmentation evidence for isoAsp in position 9.

Figure 6 Sequence map for liraglutide. The *c8* and *z23* ions investigated for isoD formation are highlighted.

Tabel 1. Ion candidate statistics for *c8* and *z23*. Matched ions are highlighted in blue text.

Name	IonScore	PPM	Total Intensity	# Peaks
c8 2+	0.0	6.9	29	2
c8+57(isoD) 1+	0.0	-17.8	26	1
c8+57(isoD) 2+	0.0	15.7	16	1
z23-57(isoD) 3+	0.0	2.7	153	2
z23-57(isoD) 4+	0.0	-0.3	84	1
z23-57(isoD) 2+	0.1	-9.9	428	2
c8 1+	12.1	-4.9	76,870	4
z23 2+	12.2	-4.3	83,563	8

Conclusions

ECD enables comprehensive sequence and modification analysis of GLP-1 receptor agonists.

Fragmentation of amino acid side chains offers additional evidence for identifying amino acid isomers such as aspartate and isoaspartate.

ExDViewer provides powerful tools for visualizing fragmentation data from synthetic peptides with non-standard amino acid chemistry.

References

1- Comprehensive Characterization of Multiple GLP-1 Analogs Using an Agilent 6545XT AdvanceBio LC/Q-TOF with ECD and Agilent ExDViewer software. Agilent Technologies application note, publication number 5994-7994EN, 2025.

2- 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.

Conflict of Interest Disclosure

Authors are employees of Agilent Technologies, which sells the instrumentation used in this analysis. As such, the authors disclose a potential conflict of interest, in respect to their commercial involvement.

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