Enhancing sequence coverage and identification of liraglutide catabolites with CID and EAD fragmentation

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This technical note describes a streamlined workflow for the in vitro characterization of liraglutide peptide catabolites using the ZenoTOF 8600 system. By leveraging complementary CID and EAD fragmentation, the workflow delivers enhanced sequence coverage and greater confidence in structural assignment, enabling more comprehensive characterization of peptide catabolites (Figure 1).

Synthetic therapeutic peptides represent a growing class of biotherapeutics. GLP-1 receptor agonists are modified versions of native human GLP-1, transforming the treatment landscape for type 2 diabetes mellitus (T2DM), obesity, and weight loss management.[1] Characterization of peptide catabolites across diverse masses and abundance levels remains challenging due to limited MS/MS sensitivity from TOF duty cycle constraints. In addition, CID fragmentation is often dominated by b and y ions, resulting in incomplete sequence coverage, ambiguous localization of modifications or cleavage sites, and reduced confidence in catabolite assignment.[2]

The ZenoTOF 8600 system enhances MS/MS sensitivity with Zeno trap[3], while EAD provides complementary c′ and z· ion information beyond traditional CID fragmentation.[4] This combination increases sequence coverage and confidence in assigning peptide catabolites, including low-level metabolites.

Key benefits for catabolite identification using ZenoTOF 8600 system

- **Enhanced structural confidence:** CID and EAD deliver complementary fragment ions that expand sequence coverage and strengthen confidence in liraglutide catabolite assignment.
- **Improved duty cycle and sensitivity:** The Zeno trap improves TOF duty cycle, increasing MS/MS sensitivity for confident identification of low-level peptide catabolites.
- **Streamlined workflow:** Improve insights into structure-metabolic stability relationships by leveraging an end-to-end workflow unifying data acquisition to processing on a single LC-HRMS platform.

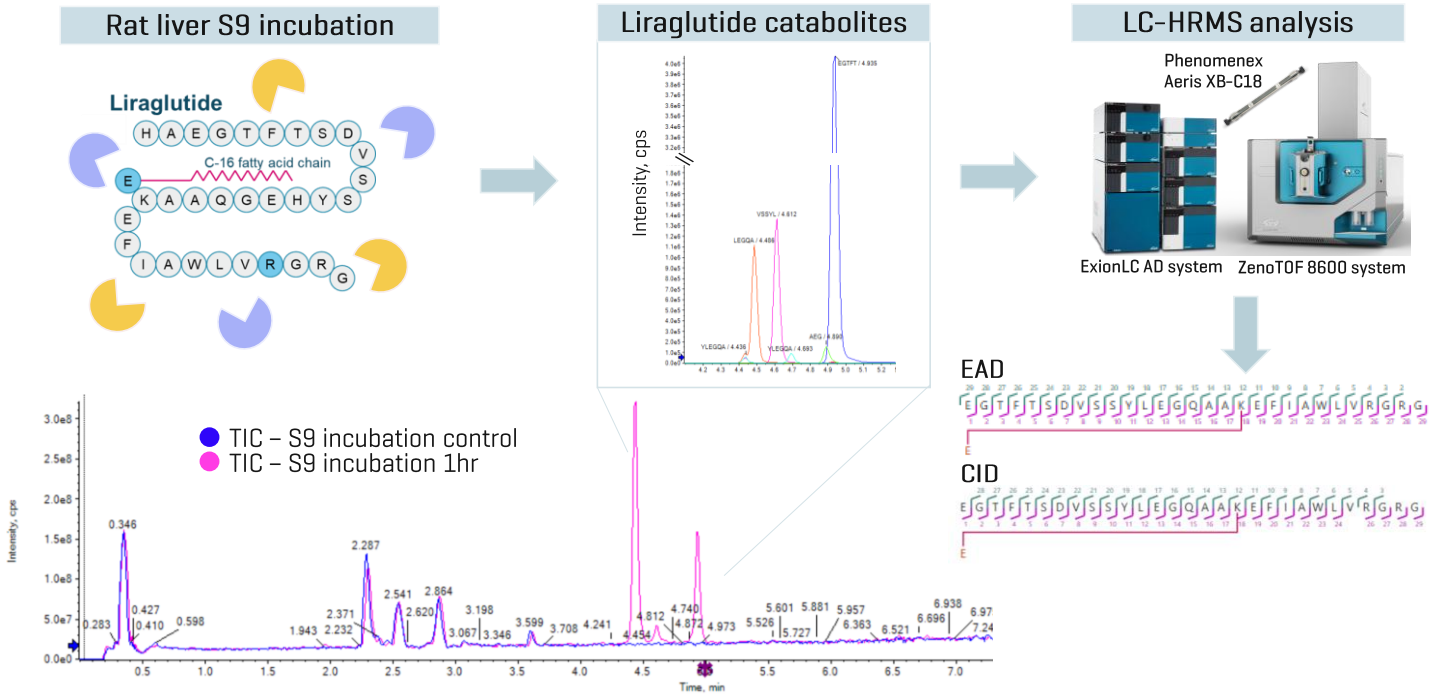


Figure 1. Overview of the analysis of liraglutide catabolites using the ZenoTOF 8600 system. Both CID and EAD fragmentation were utilized for the comprehensive sequence coverage and confident identification of liraglutide peptide catabolites.

Introduction

Comprehensive elucidation of drug metabolism pathways is critical for assessing drug safety and therapeutic efficacy as catabolites can contribute to pharmacological activity, toxicity, and drug–drug interactions. Accurate identification and structural characterization of peptide catabolites are therefore essential components of drug metabolism and pharmacokinetic (DMPK) studies.

Synthetic therapeutic peptides represent an important and growing class of biotherapeutics as their structures can be engineered to improve target specificity, metabolic stability, and pharmacokinetic properties. This technical note uses liraglutide, a GLP-1 receptor agonist, as a model compound. Liraglutide is a modified analog of native human GLP-1 designed to preserve GLP-1 receptor activity while overcoming the short half-life of the endogenous peptide, which is rapidly degraded by enzymes such as dipeptidyl peptidase-4 (DPP-4) and neutral endopeptidase (NEP).[5] Structurally, liraglutide contains a Lys34-to-Arg substitution and a C16 fatty acid attached to Lys26 through a glutamic acid spacer. These modifications improve enzymatic stability, promote plasma protein binding, and extend systemic exposure.

However, the same structural modifications that improve therapeutic performance also increase the complexity of catabolite identification. Peptide catabolism often involves enzymatic backbone cleavage, producing related catabolites that can differ by only a few residues, charge states, or structural modifications. For lipidated peptides such as liraglutide, confident assignment requires not only identifying peptide cleavage sites but also determining whether key structural features, such as the lipidated side chain, are retained or lost.

LC-HRMS has become a central tool for DMPK studies because accurate-mass detection, isotope pattern recognition, and high-resolution MS/MS fragmentation provide the structural information needed to assign peptide catabolites and localize cleavage sites. Commonly used CID-based fragmentation provides useful peptide structural information but can be limited by incomplete or uneven sequence coverage. CID spectra are often dominated by b- and y-type ions, and some regions of the peptide sequence may fragment poorly, making it difficult to confidently localize cleavage sites or distinguish closely related catabolites.[2]

The ZenoTOF 8600 system addresses these challenges by combining high-resolution accurate-mass detection, enhanced MS/MS sensitivity through the Zeno trap, and complementary fragmentation using EAD.[3,4] EAD provides an additional fragmentation approach that uses tunable electron kinetic energy to generate complementary backbone fragment ions, including c′ and z· ions, expanding sequence coverage beyond CID alone.

By combining CID and EAD fragmentation, this workflow provides more comprehensive structural information for liraglutide catabolites, improving confidence in sequence assignment, cleavage-site localization, and characterization of lipidated catabolites in biological matrices. The observed liraglutide cleavage sites were consistent with reported GLP-1 catabolic pathways, supporting the biological relevance of the in vitro characterization workflow.[5,6]

Methods

Sample preparation for incubation in rat S9 fractions: Liraglutide at a 10 µM starting concentration was incubated in rat S9 fractions at 37°C for 1 hour. Samples were removed from the incubation and quenched with acetonitrile at a 2:1 (v/v) ratio. Samples were vortexed for 30 seconds and centrifuged at 12,000 rcf for 12 mins at room temperature. Samples were diluted with 1:5 (v/v) with water containing 0.1% formic acid.

Sample preparation for enzyme incubation: Liraglutide at a 10 µM starting concentration was incubated in phosphate buffer (pH 7.4) containing 2 µg/mL DPP-4 or 2 µg/mL NEP at 37°C for 1 hour. Sample extraction and dilution were carried out as mentioned under “sample preparation for incubation in rat S9 fractions”.

Chromatography: Sample separation was performed using an ExionLC AD system (SCIEX) at a 0.4 mL/min flow rate on a Phenomenex Aeris XB C18 (2.1 x 100 mm, 1.7 µm, 100 Å) column. A 10- minute gradient was run using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The conditions for mobile phase B are summarized for rat S9 incubation and enzyme incubation (Table 1). The column temperature was maintained at 40°C. An injection volume of 5 µL was used for analysis. A mixture of equal volumes of acetonitrile, methanol, and water was used as the needle wash solvent.

Table 1. LC gradient conditions.

Time (min)	S9 incubation Mobile phase B (%)	Enzyme incubation Mobile phase B (%)
0.0	30	3
0.5	30	3
7.8	65	80
7.9	95	95
8.9	95	95
9	30	3
10	30	3

Mass spectrometry: The samples were analyzed using the data-dependent acquisition (DDA) method with Zeno CID DDA and Zeno EAD DDA (2 separate experiments) on the ZenoTOF 8600 system (SCIEX). Table 2 summarizes the source and gas conditions, and Table 3 summarizes the Zeno DDA method conditions.

Table 2. Source and gas parameters.

Parameter	Value
Polarity	Positive
Ion source gas 1	50 psi
Ion source gas 2	60 psi
Curtain gas	44 psi
Source temperature	500°C
Ion spray voltage	3000 V
CAD gas	7

The CID DDA data of the peptide was acquired using dynamic collision energy (CE) as shown in Figure 2. Dynamic CE ensures that both low-mass and high-mass ions receive appropriate activation energy, preventing over-fragmentation of small m/z species while adequately fragmenting large m/z species.

Table 3. Zeno DDA parameters on the ZenoTOF 8600 system.

Parameter	Value
Workflow	Peptide
TOF MS start-stop mass	100 - 1800 Da
TOF MS accumulation time	0.05 s
Maximum candidate ions	5
TOF MS/MS start-stop mass	100 – 1800 Da
TOF MS/MS accumulation time	0.075 s
Collision energy (CID)	Dynamic CE (see Figure 2)
Electron kinetic energy (EAD)	8 eV
Electron beam current (EAD)	5000 nA

Dynamic CE for MS/MS: True

CE Equation: CE = (slope) * (m/z) + (intercept)

Charge state of zero: Applied when charge state is unknown

Charge	*Slope*	*Intercept*
0	0.049	-1
1	0.05	5
2	0.049	-1
3	0.048	-2
4	0.05	-2
5	0.05	-2
6	0.05	-2
7	0.05	-2
8	0.05	-2
9	0.05	-2
10	0.05	-2
11	0.05	-2
12	0.05	-2

Maximum CE (V): 80

Minimum CE (V): 5

Figure 2. Dynamic CE equation and parameters. Dynamic CE applies a linear equation that scales the fragmentation energy with the size and charge of the molecule.

Data processing: Zeno CID DDA and Zeno EAD DDA data were acquired using SCIEX OS software, version 4.0. The Molecule Profiler software, version 1.3 (SCIEX) integrated into SCIEX OS software was used to process the data. Peptide catabolite characterization was performed using an automated algorithm integrated into Molecule Profiler software.

Identification of liraglutide peptide catabolites

Automated structural assignment was performed using Molecule Profiler software, where the ppm error for all fragment ions was observed to be <10 ppm, enabling confident identification of peptide catabolites.

Figure 3 shows a summary of the liraglutide catabolite investigated in rat S9 fraction incubations. Among 16 catabolites detected with charge states spanning +2 to +4, 2 examples with the highest peak area were highlighted in this technical note. For all the selected catabolites, the fatty acid chain was observed to be intact. All selected catabolites were reported in the literature.[5]

To further confirm the catabolite assignments observed in rat S9 incubations, liraglutide was incubated with DPP-4 and NEP enzymes in phosphate buffer. Comparison of the enzymatically generated catabolite profiles with those obtained from rat S9 incubations provided orthogonal confirmation of the proposed biotransformation pathways as shown in Figure 4.

Figure 5 shows one of the catabolites of NEP driven digestion product M41-1 (m/z 790.1, charge state of +2). CID based sequence coverage was incomplete due to the poor fragmentation close to the C-terminal of the peptide, whereas EAD based z ions (such as m/z 216.1224, m/z, 587.3174)

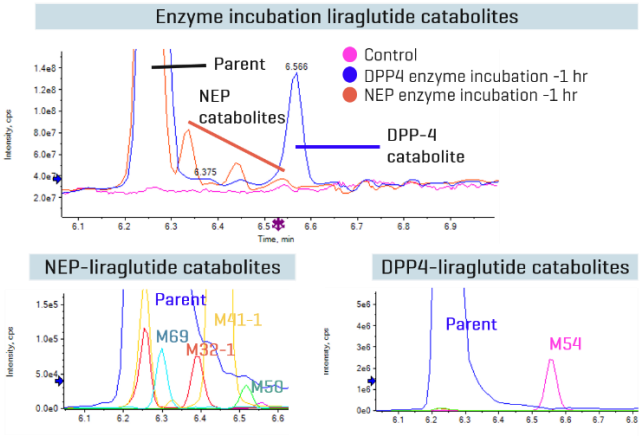
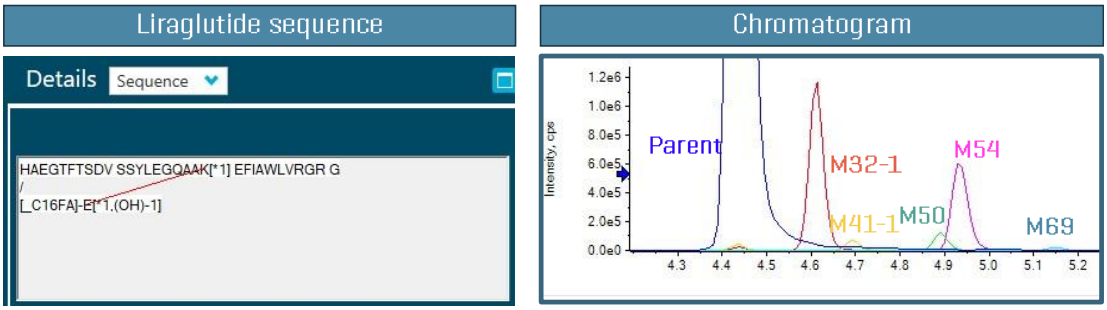


Figure 4. Liraglutide incubations with DPP-4 and NEP enzymes. The top panel shows a representative total ion chromatogram (TIC) of control, DPP-4, and NEP incubations. The bottom left panel shows representative XICs of NEP incubation resulting in several catabolites while catabolite M54 was observed as a major catabolite of DPP-4 (bottom right).

provided fragment information that resulted in improvement in the sequence coverage.

Figure 6 demonstrates the potential of EAD in differentiating isomeric amino acid residues, which is a limitation in CID based applications. EAD can lead to secondary fragmentation of the side chains of leucine (Leu) and isoleucine (Ile) residues, which results in the generation of z-43 fragment ion for Leu and z-29 fragment ion for Ile. The mechanism has been discussed in detail in a previously published technical note.[7,8]



		Peak ID	Name	Formula	Assigned	Average Mass	m/z	Charge	ppm	R.T. (min)	Peak Area
1	✓		Parent [M+4H]4+	C172H265N43O51	✓	3751.10	938.2471	4	3.4	4.98	1.70E+05
2	✓	M24-1	LEGQAAK[*1]EFIAWLVRGRG / [_C16FA]-E[*1,(...	C112H182N28O28	✓	2368.63	790.1304	3	0.7	4.49	1.01E+07
3	✓	M32-1	VSSYLEGQAAK[*1]EFIAWLVRGRG / [_C16FA]-...	C132H210N32O35	✓	2805.16	935.5283	3	-0.3	4.61	1.72E+07
4	✓	M41-1	YLEGQAAK[*1]EFIAWLVRGRG / [_C16FA]-E[*1,...	C121H191N29O30	✓	2531.90	844.4866	3	2.6	4.69	9.50E+05
5	✓	M50	AEGTFTSDVSSYLEGQAAK[*1]EFIAWLVRGRG...	C166H258N40O50	✓	3613.89	903.9805	4	1.5	4.89	2.91E+06
6	✓	M54	EGTFTSDVSSYLEGQAAK[*1]EFIAWLVRGRG /...	C163H253N39O49	✓	3542.79	886.2222	4	2.6	4.93	7.28E+07
7	✓	M69	LEGQAAK[*1]EF / [_C16FA]-E[*1,(OH)-1] [M+2H]...	C65H106N12O19	✓	1359.45	680.3948	2	3.9	5.15	1.37E+05

Figure 3. Overview of the liraglutide catabolites. Liraglutide sequence was generated by defining the link and the C16 fatty acid chain in the Molecule Profiler software modification library. The extracted ion chromatograms (XICs) show the parent and catabolites at the end of 1-hour incubation in rat S9 liver fractions. Peaks were labeled according to the Peak ID in the bottom table. The table on the bottom provides a summary of structural information including formula, average mass, charge state, ppm shift, retention time, and peak area for parent and selected catabolites.

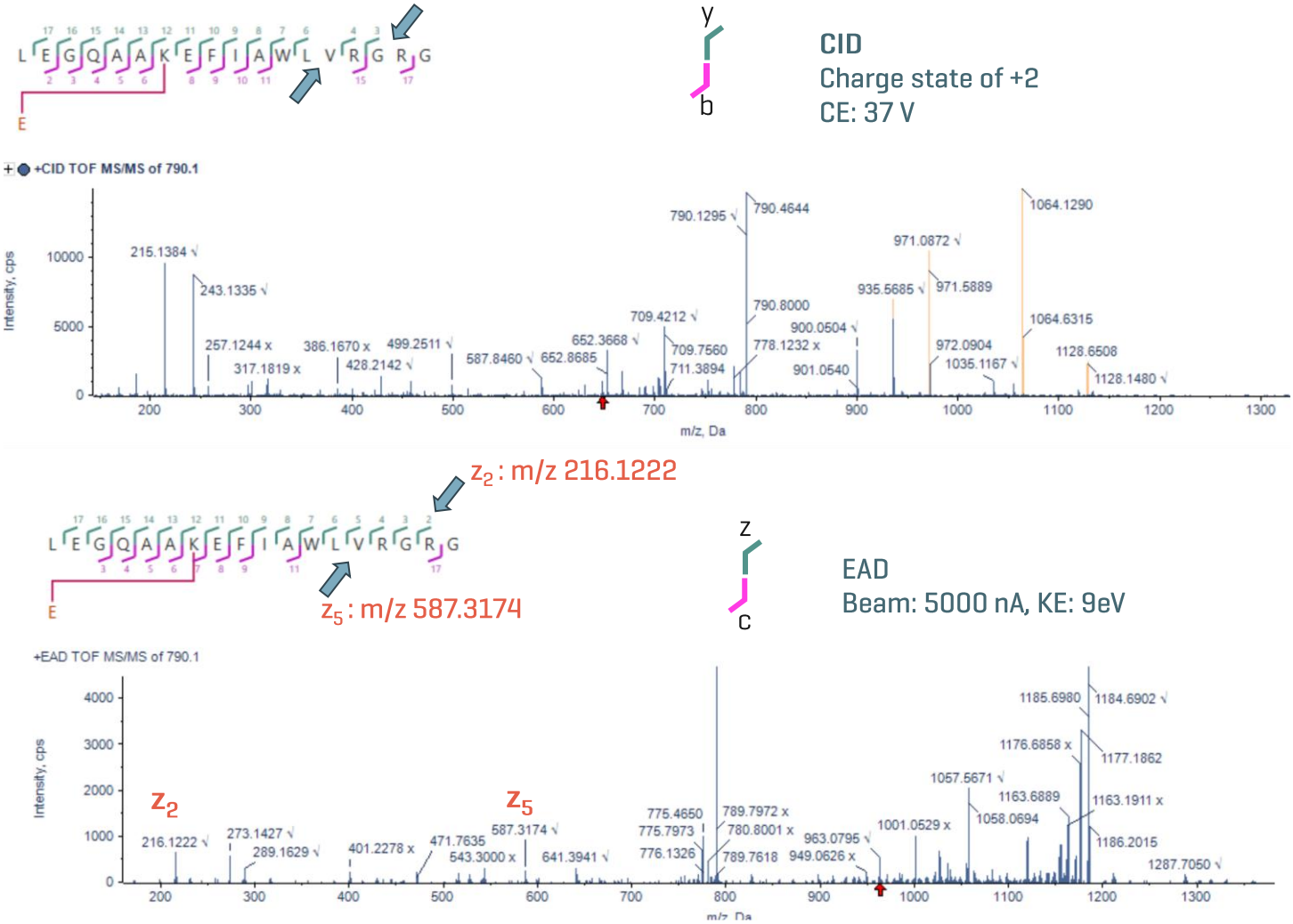


Figure 5. NEP-derived liraglutide catabolite M41-1. CID (top) and EAD (bottom) MS/MS spectra of one of the products generated by NEP digestion during the incubation. The link with E represents γ-glutamic acid spacer with a C16 fatty acid modification. The CID spectrum was acquired using dynamic CE. Based on the charge state and mass of the catabolite, a 37 V was applied as CE to generate the fragments. In this example, EAD generated fragment ions z_2 and z_5 which contributed to a more comprehensive sequence coverage of the M41-1 catabolite. The mass error for z_2 and z_5 ions was 2.2 and 1.2 ppm, respectively. Overall mass error for MS/MS fragments was lower than 10 ppm for both CID and EAD.

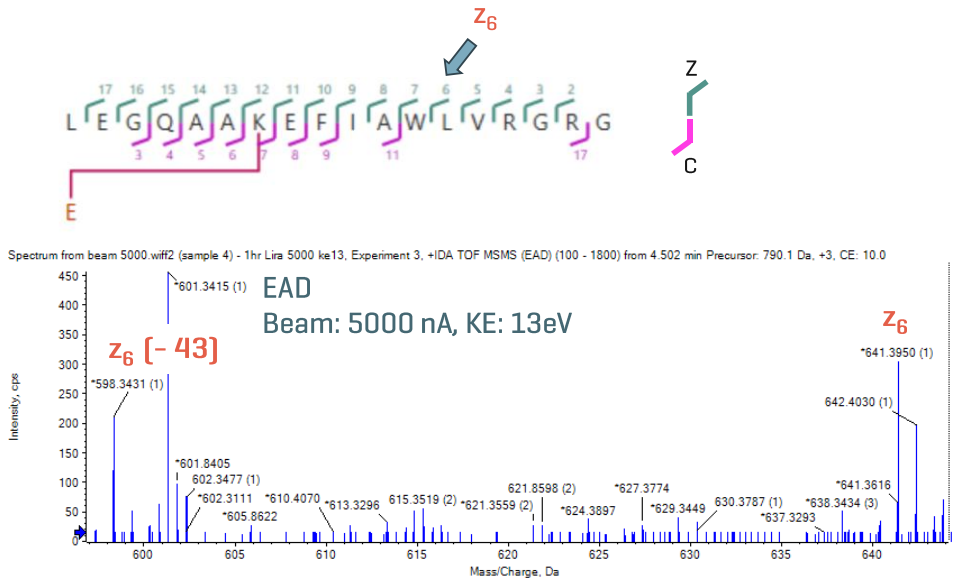


Figure 6. EAD generates diagnostic fragments for the identification of Leu residue on peptide catabolite (m/z 790.1) from rat S9 fraction. EAD generates z ions which can differentiate isomeric peptides through the diagnostic fragmentation of their side chains, such as z – 43 (C_3H_7) and z – 29 (C_2H_5), for Leu and Ile, respectively. Here, the identification of Leu using z_6 (m/z 641.3950) and the location of z-43 fragment ions (m/z 598.3431) of Leu is demonstrated.

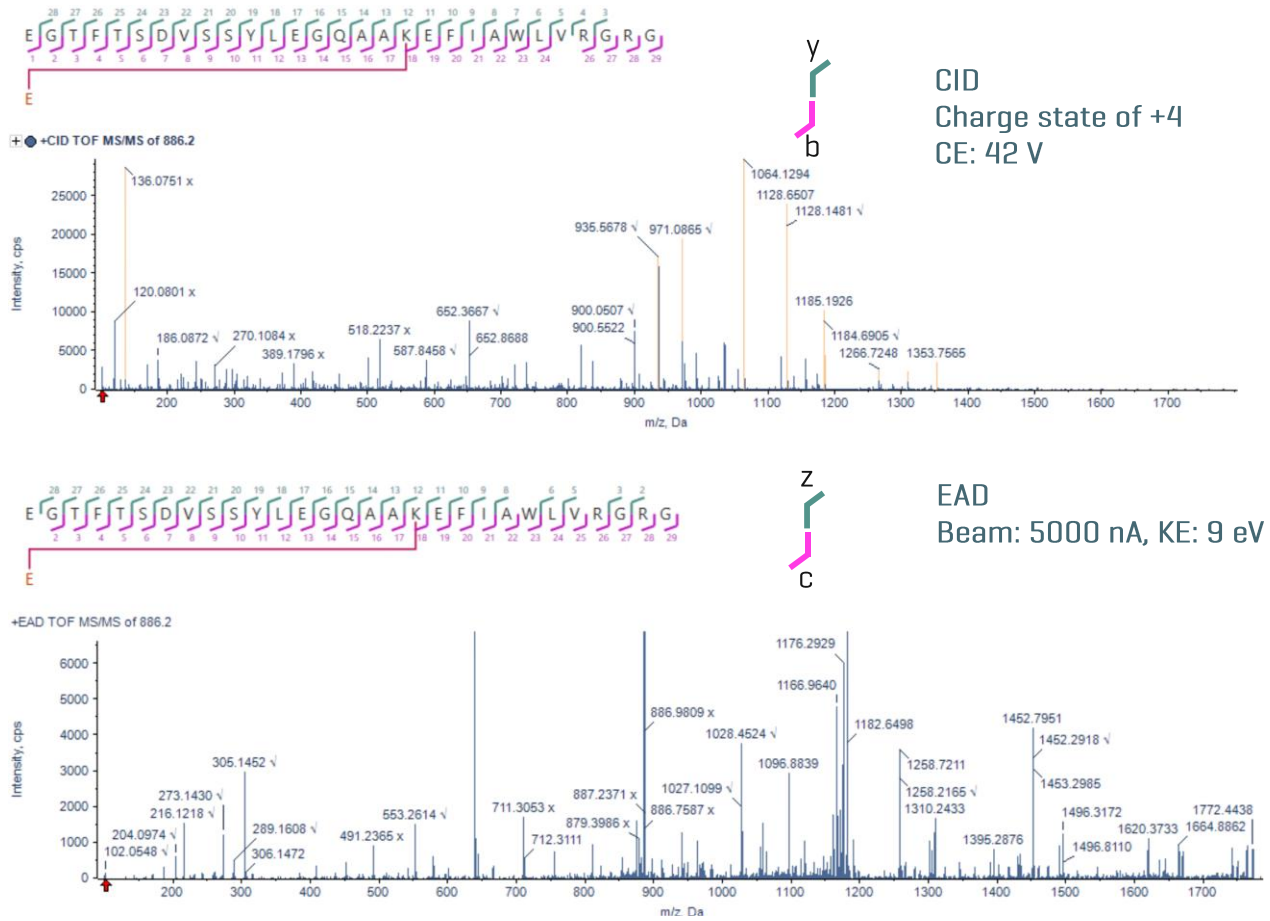


Figure 7. DPP-4-derived liraglutide catabolite M54 (m/z 886.2). CID (top) and EAD (bottom) MS/MS spectra of the dominant catabolite generated by DPP-4 digestion. The link with E represents γ-glutamic acid spacer with C16 fatty acid modification. The CID spectrum was acquired using dynamic CE. Based on the charge state and mass of the catabolite, 42 V was applied as CE to generate the fragments. Together with CID, EAD provided additional c′ and z· fragment ions to facilitate complete sequence coverage of the M54 peptide catabolite. The mass error for MS/MS fragments was lower than 10 ppm for both CID and EAD.

Figure 6 shows a z-43 fragment ion (m/z 598.3431) generated by EAD, indicative of Leu. Figure 7 shows the CID and EAD spectra for the dominant catabolite M54 (m/z 886.2, charge state of +4) produced by DPP-4 digestion.[3] Both CID and EAD fragments provide full sequence coverage of M54 through complementary fragmentation pathways. A near-complete set of b and y ions from CID and c′ and z· ions from EAD enabled extra confidence in structure characterization and assignment of the catabolite.

In summary, CID and EAD spectra both provided complete sequence coverage of peptide catabolites. In addition, EAD-based fragmentation provides additional c′ and z· ions, facilitating enhanced confidence in catabolite identification.

Conclusions

- More comprehensive sequence coverage was achieved by combining complementary CID and information-rich EAD fragmentation on the ZenoTOF 8600 system.
- The identification of catabolites in a wide concentration range with varying charge states was easily achieved with the Zeno trap, enabling enhanced MS/MS sensitivity for both EAD and CID applications.
- Fragments from EAD and CID spectra were identified and processed in a single result file using Molecule Profiler software to achieve more accurate structure assignment of catabolites.
- Informative data was generated using a quick and easy-to-use workflow on a streamlined platform, accelerating the early drug discovery process.

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