Application Note

Pharma

Impurity Profiling of GLP-1 Receptor Agonists by HILIC-MS

Using an Agilent 1290 Infinity III Bio LC System and Agilent InfinityLab LC/MSD iQ

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Abstract

Glucagon-like peptide-1 (GLP-1) receptor agonists are increasingly being used for the treatment of diabetes and obesity. These (bio)synthetic therapeutic peptides are accompanied by several related impurities commonly monitored by reversed-phase liquid chromatography (RPLC). In this application note, HILIC is introduced as an orthogonal methodology and applied to the analysis of Exenatide, Semaglutide, and Tirzepatide. To prevent nonspecific analyte interaction, stainless-steel components in the entire LC flow path are eliminated or deactivated. Molecular weight (MW) information on eluting peaks is readily obtained by hyphenating the biocompatible LC to a compact and stackable single quadrupole mass analyzer.

Introduction

Glucagon-like peptide-1 (GLP-1) receptor agonists are (bio)synthetic peptides mimicking the natural GLP-1 hormone which regulates blood sugar levels by stimulating insulin release, suppressing glucagon, slowing gastric emptying, and reducing appetite. As such, these therapeutics are widely applied in managing type 2 diabetes mellitus (T2DM) and obesity.[1] As native GLP-1 is rapidly degraded by endogenous enzymes, GLP-1 receptor agonists have been engineered to improve therapeutic properties (Figure 1). Semaglutide (Ozempic) differs from natural GLP-1 by two amino acid substitutions and the conjugation of stearic di-acid to lysine via a short spacer, while Exenatide (Byetta, Bydureon) is a synthetic version of Exendin-4, a peptide found in the saliva of the Gila monster, sharing 53% sequence similarity with human GLP-1. The successful introduction of the coagonistic peptide Tirzepatide (Mounjaro), which simultaneously exerts the functions of GLP-1 and the gastricinhibitory polypeptide (GIP) hormone, has set the basis for the next generation of these therapeutic agents. Several peptide-based drugs with diverse properties, pursuing common therapeutic objectives, are being developed, and suitable analytical methods are required to evaluate the quality of these medicines.

Liquid chromatography (LC) in combination with mass spectrometry (MS) is fundamental to profile impurities in (bio)synthetic peptides, which raises concerns about safety, efficacy, and regulatory compliance. These impurities can arise during production or storage and encompass amino acid addition, insertion, deletion, truncation, oxidation, deamidation, and (stereo)isomerization, amongst others, in addition to fatty acid-related impurities.[2] Reversed-phase liquid chromatography (RPLC) is commonly applied to resolve these species. Given the substantial number of structurally related actual and potential impurities, separation modes that offer different selectivities are needed to tackle the analytical challenge.

The current application note introduces hydrophilic interaction liquid chromatography (HILIC) as an orthogonal separation mode to assess GLP-1 receptor agonist impurities. Conditions applied are based on previous work describing HILIC with diode array (DAD) and evaporative light scattering detection (ELSD) for the simultaneous measurement of the active ingredient, product-related impurities, and formulation constituents in GLP-1 receptor agonist drug products.[3] Here, the method is coupled to a single quadrupole MS to confirm the identity of the eluting peptides. To prevent nonspecific interaction of the studied analytes with metal surfaces and metal-contaminated flow paths, low adsorption and biocompatible LC hardware is implemented by means of a deactivated stainless steel HILIC column and an Agilent 1290 Infinity III Bio LC System, the latter coupled to the Agilent InfinityLab LC/MSD iQ.

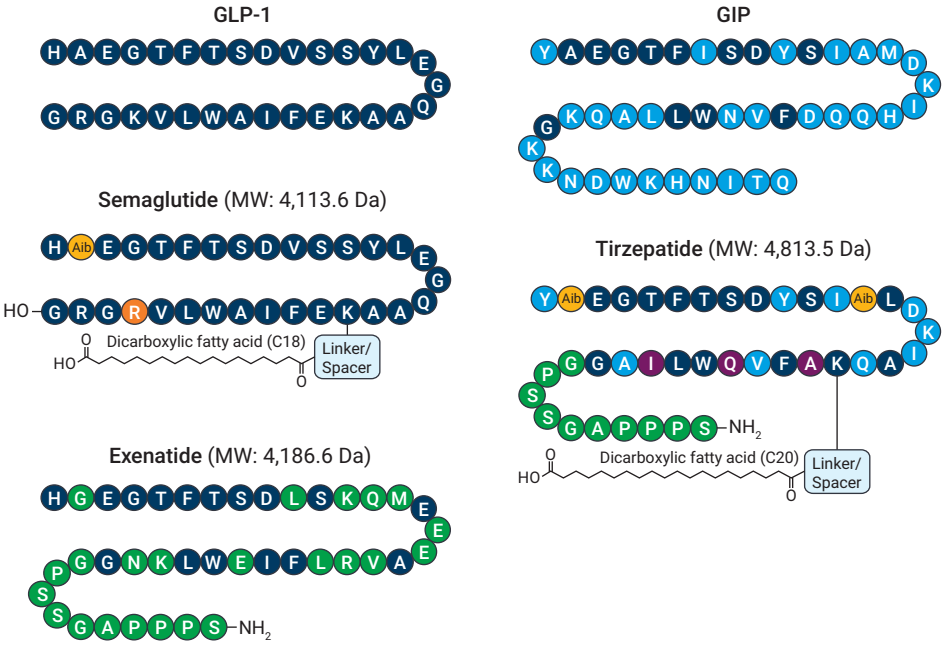

Figure 1. Molecular structures of native GLP-1 and GIP, GLP-1 receptor agonists Semaglutide and Exenatide, and GLP-1 and GIP receptor co-agonist Tirzepatide. Molecular weight (MW) of therapeutic peptides is added.

Experimental

Materials

Water (ULC/MS), acetonitrile (HPLC-S), and formic acid (99%, ULC-MS) were supplied from Biosolve. Ammonium formate (LiChropur), dimethyl sulfoxide (DMSO, Suprasolv), hydrogen peroxide (≥ 30%, for trace analysis), and L-methionine were sourced from Merck. Semaglutide acetate (≥ 95%), Exendin-4 (4886) amide acetate (Exenatide acetate, ≥ 95%), and Tirzepatide sodium salt (≥ 95%) were obtained from Cayman Chemical.

Sample preparation

Semaglutide was prepared in DMSO at 1 mg/mL and heated to dissolve. Exenatide and Tirzepatide were dissolved at 1 mg/mL in 50:50 (v/v) acetonitrile:water.

To induce methionine oxidation, Exenatide was incubated in 0.004% hydrogen peroxide for three hours at room temperature. The reaction was quenched by adding 1 mM L-methionine.

Instrumentation and method

The 1290 Infinity III Bio LC and InfinityLab LC/MSD iQ were used in this study. Details of the instrument configuration can be found in Table 2 and method parameters are summarized in Table 3. Data were acquired and processed in Agilent OpenLab CDS, version 2.7.

Table 2. Details of instrument configuration.

Module	Details
Pump	Agilent 1290 Infinity III Bio High-Speed Pump (G7132A)
Autosampler	Agilent 1290 Infinity III Bio Multisampler (G7137A) with integrated Sample Thermostat
Column Compartment	Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent Quick Connect Bio Heat Exchanger Std (G7116-60071)
Detector (DAD)	Agilent 1290 Infinity III Diode Array Detector (G7117B)
Flow Cell	Agilent Max-Light Cartridge Cell LSS, 10 mm (G7117-60020), aperture not attached
Detector (MS)	Agilent InfinityLab LC/MSD iQ (G6160A)

Table 3. LC-MS method parameters.

Parameter	Value
Column	2.1 × 150 mm, 2.7 µm HILIC-Z in deactivated stainless steel version
Flow Rate	0.4 mL/min
Mobile Phase	A = 100 mM ammonium formate pH 3 B = acetonitrile
Gradient	0–1 min: 90% B 1–19 min: 90–45% B 19–20 min: 45–90% B 20–30 min: 90% B
Column Temperature	40 °C
Autosampler Temperature	10 °C
Injection	1 µL
Needle Wash	10 sec 50:50 (v/v) acetonitrile:water
Detection DAD	280/4 nm, reference 360/100 nm, 2.5 Hz
Detection MS	Mode: ESI positive Gas temperature: 325 °C Gas flow: 11 L/min Nebulizer: 50 psi Capillary voltage: 3500 V Scan range: *m/z* 500–1450 Fragmentor: 120 V Gain factor: 1 Targeted points per second: 1 Hz Scan time: 997 ms Data collection: centroid Diverter valve: 0–1.5 min to waste; 1.5–20 min to MS; 20–30 min to waste

Results and discussion

In HILIC, polar and ionic solutes are retained and resolved based on their partitioning between the organic mobile phase and an aqueous layer immobilized on the stationary phase, superimposed with other interactions, such as electrostatic or hydrogen bonding, depending on the stationary phase chemistry and mobile phase conditions.[4] HILIC offers an orthogonal separation platform to "golden standard" RPLC for the analysis of therapeutic peptides and associated impurities. The separation of the GLP-1 receptor agonists Tirzepatide, Semaglutide, and Exenatide on an earlier-described HILIC method is shown in Figure 2.[3] UV 280 nm traces displayed as lower wavelengths (e.g. UV 214 nm) are less informative due to aberrant absorbance of the mobile phase. A zwitterionic HILIC phase is used (HILIC-Z) with the superficially porous particles packed in deactivated stainless-steel hardware to prevent nonspecific interactions.[5] Peptide elution is based on the amino acid composition and conjugated fatty acids. As MS-friendly mobile phases are used, the identity of the main peak can be readily confirmed by the MW, calculated from the charge envelope collected over the peak using a quadrupole mass analyzer (Figure 3).

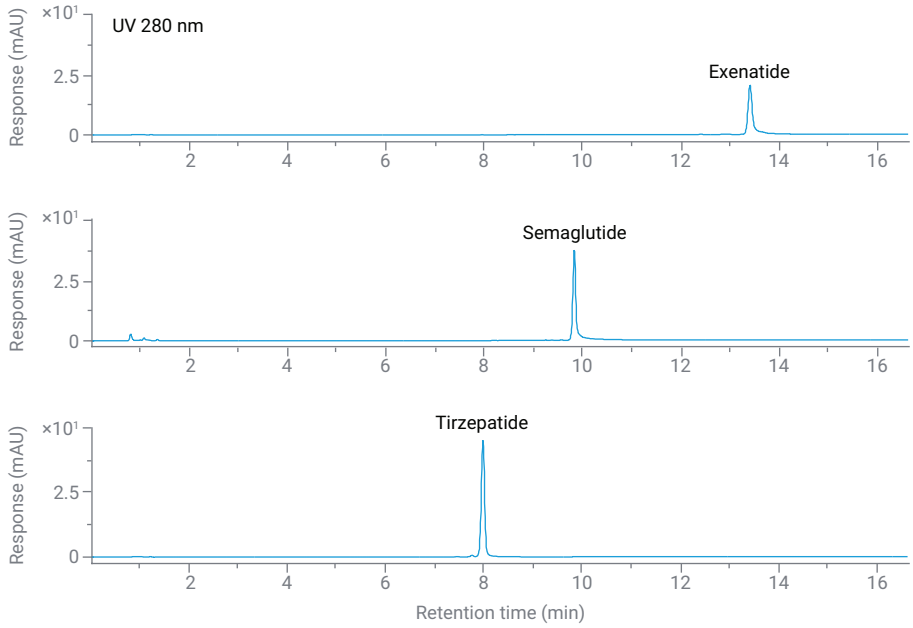

Figure 2. HILIC-UV 280 nm chromatograms of GLP-1 receptor agonists Exenatide, Semaglutide, and Tirzepatide at 1 mg/mL.

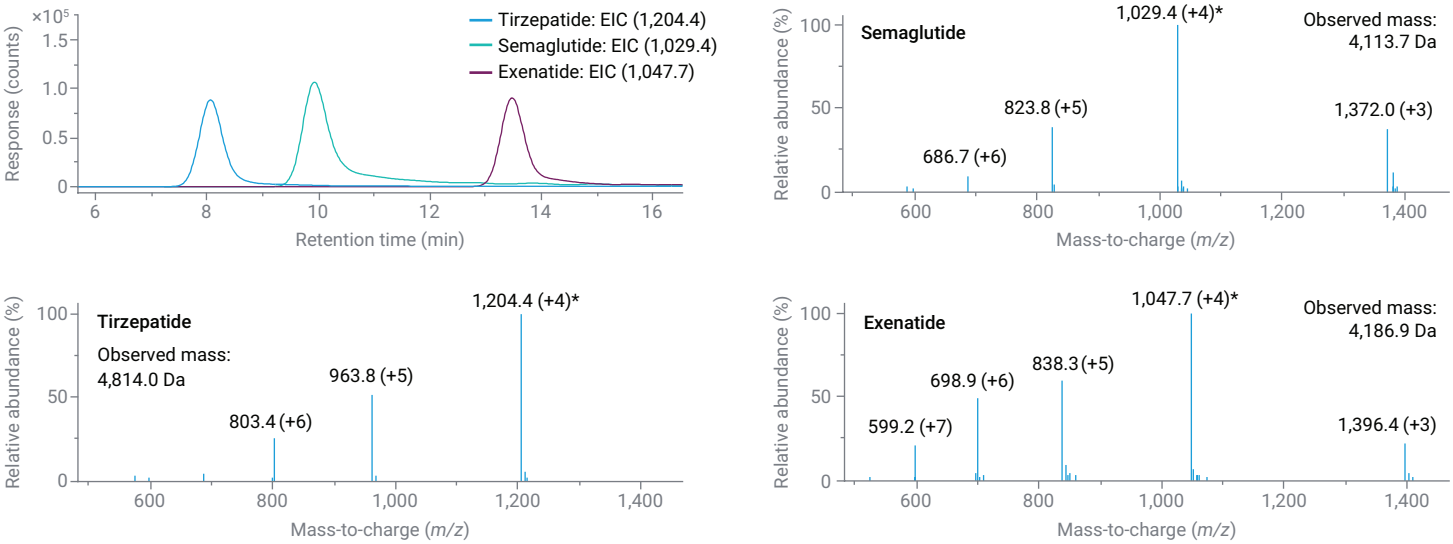

Figure 3. Overlaid extracted ion chromatograms (EIC) of the base peak (*) and single quadrupole mass spectra of Tirzepatide, Semaglutide, and Exenatide (1 mg/mL). Charge state is added between brackets.

Equally so, product-related impurities can be studied at low levels using the described HILIC-MS platform. Based on UV 280 nm absorbance, impurity 1 is present at 1.1% level in Tirzepatide as illustrated in Figure 4. At this level, regulatory agencies concord on the requirement for identification and qualification, i.e. establishing biological safety.[2,6] The MS data reveal a mass difference of +163 Da, corresponding to the addition of tyrosine (Tyr), which renders Tirzepatide more hydrophobic explaining the earlier elution on HILIC. Amino acid additions/insertions result from the excess use of coupling reagents during solid-phase peptide synthesis and their incomplete removal after the condensation reaction.

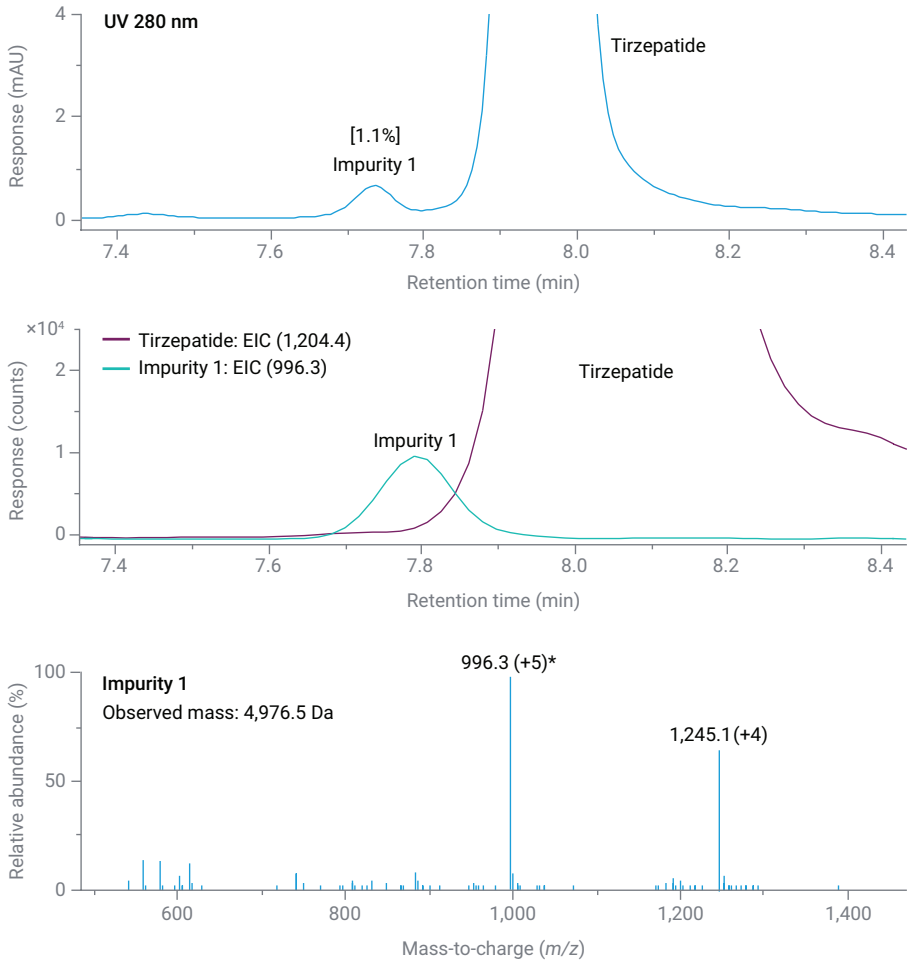

Figure 4. Zoomed HILIC-UV 280 nm chromatogram of Tirzepatide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of Tirzepatide (*m/z* 1204.4) and impurity 1 (*m/z* 996.3) corresponding to the addition of Tyr. The MS spectrum of the impurity is shown; the spectrum of the main peak is shown in Figure 3. (*) Base peak selected for EIC. Charge state (MS) and impurity percentage (UV 280 nm) added between brackets.

Low-abundant impurities (0.2 to 0.4%) are furthermore observed in Semaglutide (Figure 5). Impurity 1 and main peak share common *m/z* values, classifying the former as an isomer (potentially stereoisomer). Amino acid racemization frequently occurs during solid-phase peptide synthesis primarily arising from the use of side-chain-protected amino acids during the coupling process.

Racemization is highly dependent on process conditions, the type of protective groups, and the amino acid. Separation of stereoisomeric impurities can be challenging when using RPLC, often resulting in the implementation of more than one analytical method to cover the target analytical impurity profile. Semaglutide impurity 2 and 3, on the other hand, display a mass shift indicative of HisCyclicSemagludide (+12 Da) and truncated [3-31]-Semaglutide (−222 Da), respectively. The former impurity results from the cyclization of the N-terminal His during the process, while the latter impurity is a product intermediate which, once coupled to a reactive His-Aib-OH fragment via the N-terminal Glu residue, results in the full-length peptide sequence.

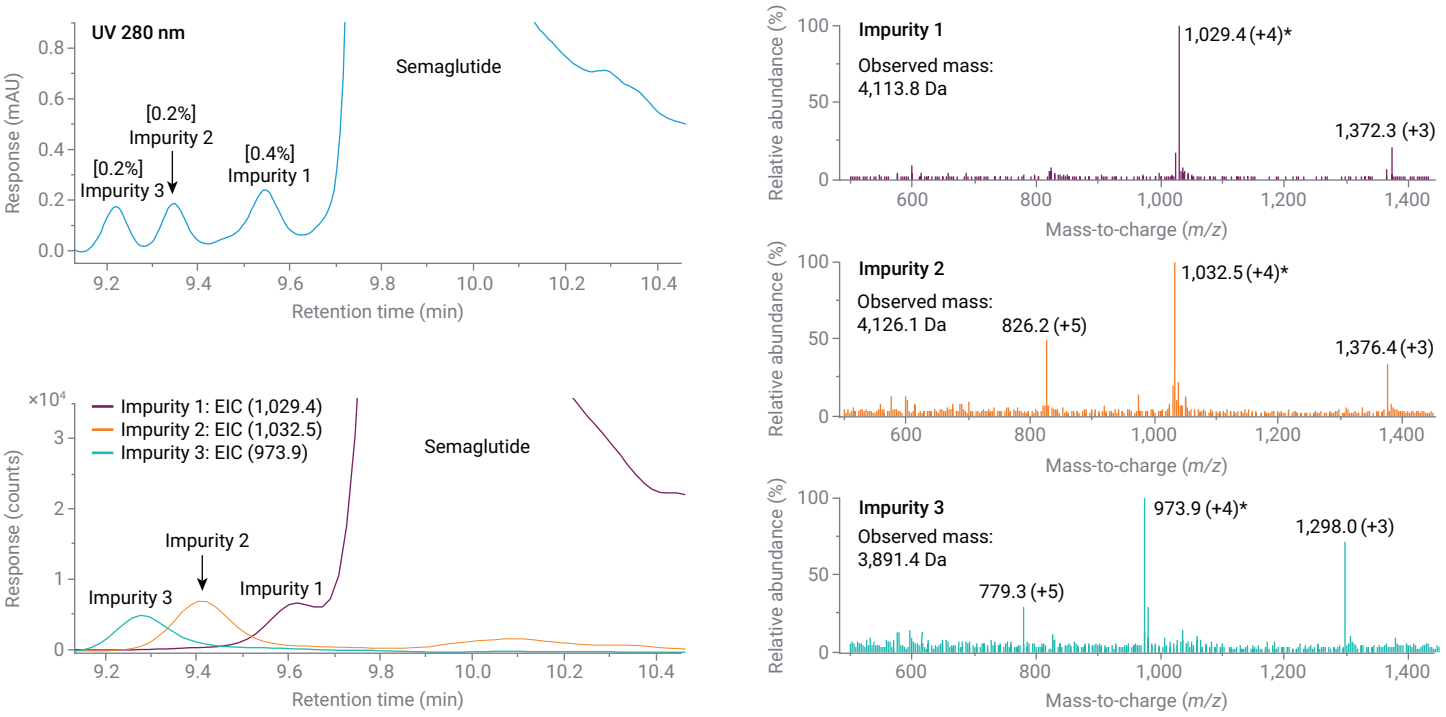

Figure 5. Zoomed HILIC-UV 280 nm chromatogram of Semaglutide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of Semaglutide impurity 1 (*m/z* 1029.4), impurity 2 (*m/z* 1032.5), and impurity 3 (*m/z* 973.9). The MS spectra of the impurities are depicted on the right. Common *m/z* ions between Semaglutide main peak (Figure 3) and impurity 1 are observed, classifying the later one as an isomeric impurity (potentially stereoisomer). Impurity 2 and impurity 3 correspond to His-Cyclic-Semaglutide and truncated [3-31]-Semaglutide, respectively. (*) Base peak selected for EIC. Charge state (MS) and impurity percentage (UV 280 nm) added between brackets.

Forced degradation studies performed during drug development may reveal an additional set of impurities beyond those originating from peptide synthesis. Figure 6 presents the measurement of Met oxidation in H_2O_2-stressed Exenatide as evidenced by the 16 Da mass increment. Of note, a partially resolved peak doublet eluting as a post peak is observed on HILIC due to the formation of Met sulfoxide S- and R-diastereomers. These species might further be resolved by adapting the gradient slope.

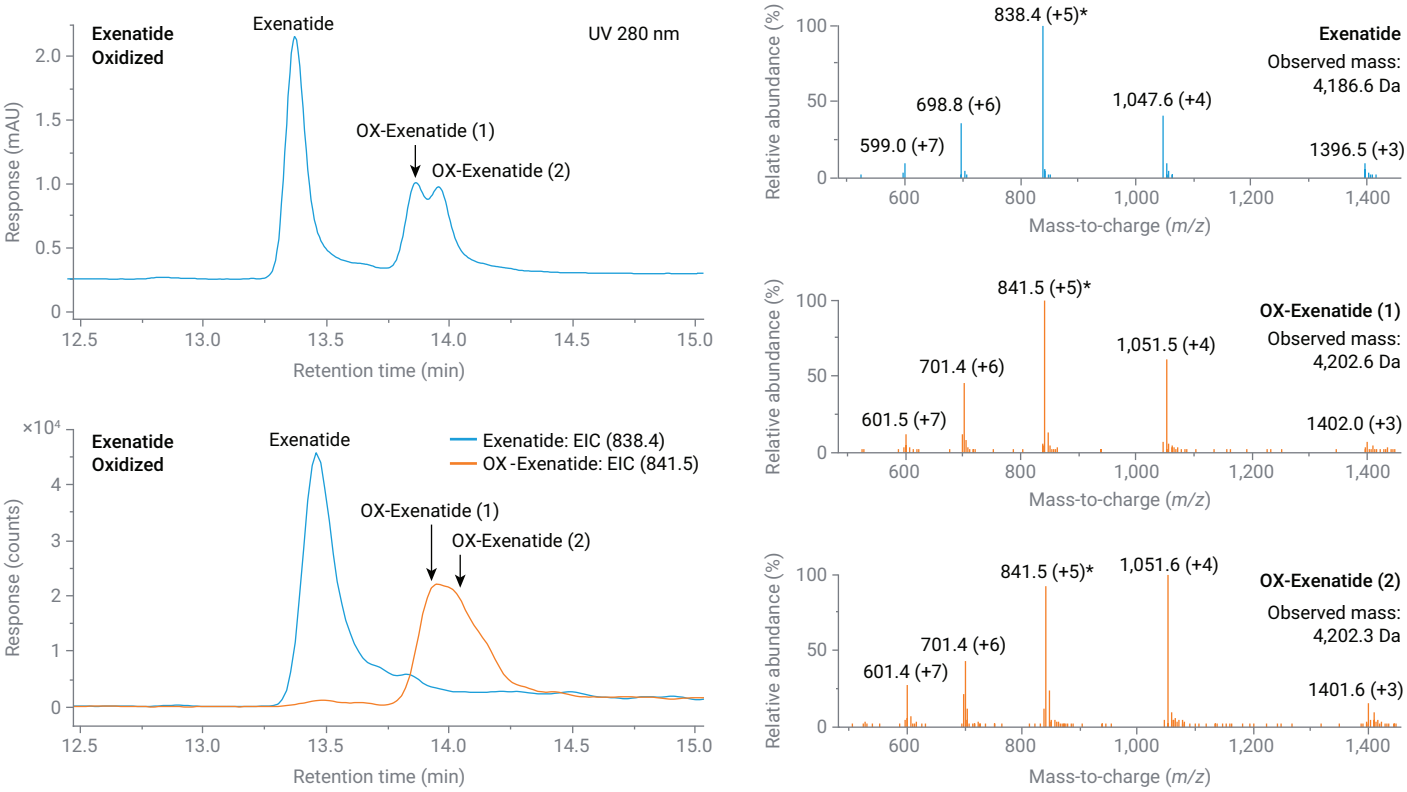

Figure 6. Zoomed HILIC-UV 280 nm chromatogram of H_2O_2-stressed Exenatide (0.25 mg/mL) and overlaid extracted ion chromatograms (EIC) of Exenatide (*m/z* 838.4) and oxidized-Exenatide (*m/z* 841.5). The MS spectra of the observed peaks is presented on the right. (*) Base peak selected for EIC. Charge state added between brackets.

The measurement of selected low-abundant impurities in nonstressed Exenatide is illustrated in Figure 7. The selectivity offered by the single quadrupole mass analyzer facilitates the detection of coeluting impurities or those hidden in the tail of the main peak, such as impurity 1 (Met oxidation, +16 Da) and impurity 2 (Gly addition, +57 Da). Integration of these impurities using the UV 280 nm chromatogram is impractical. The MS spectrum collected at the apex of impurity 3 reveals two coeluting species with one charge envelope corresponding to the addition of Leu/Ile (+113 Da) and the other exhibiting the same ions as the Exenatide main peak, indicative of isomerization (potentially stereoisomer). Impurity 4 displays a clean spectrum with the different charge states in agreement with N-terminal His truncation (–137 Da). This event might result from incomplete removal of protective groups, the incorporation of partially activated amino acids, or inefficient coupling to the resin during peptide synthesis.

Note that the identity of all reported impurities in this application note has been confirmed by Q-TOF MS.

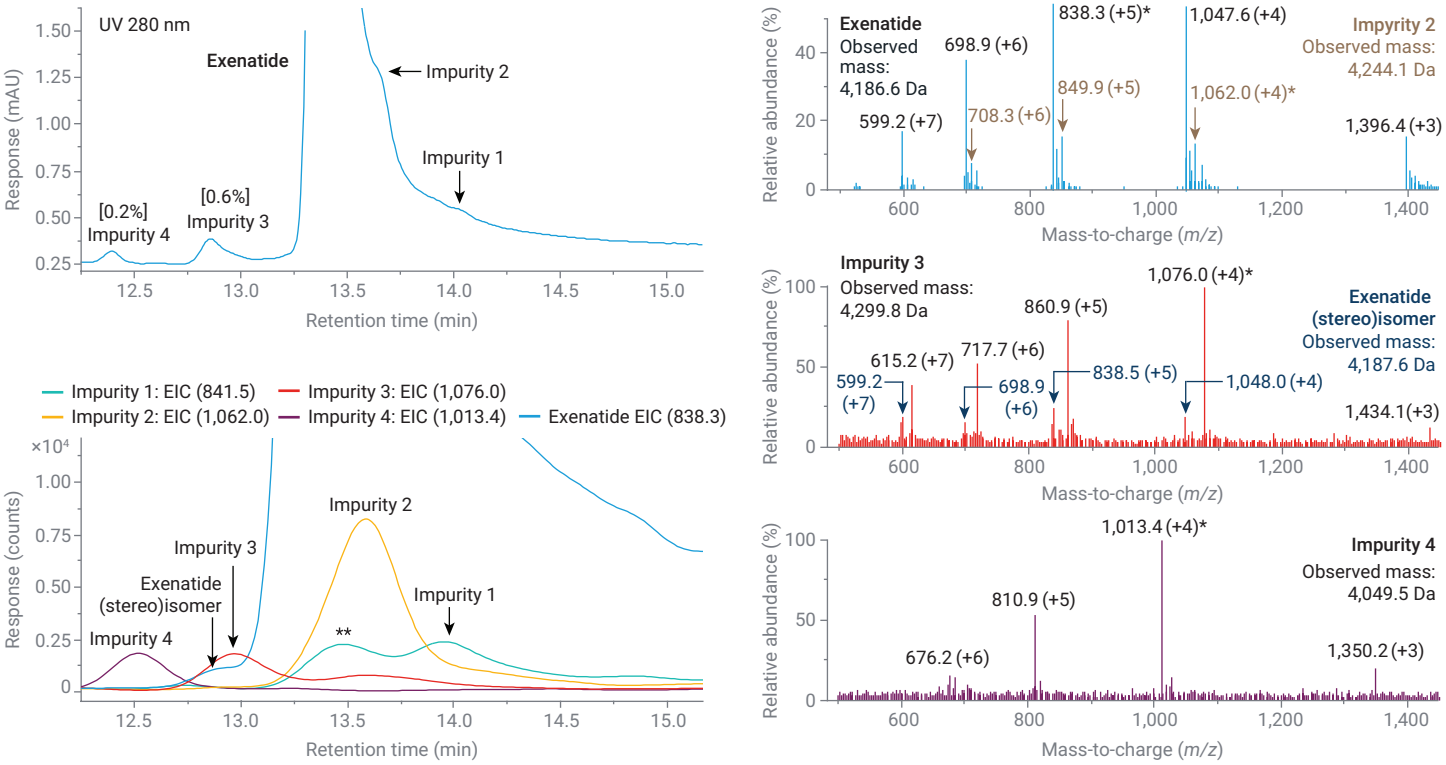

Figure 7. Zoomed HILIC-UV 280 nm chromatogram of Exenatide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of impurity 1 (*m/z* 841.5, Met oxidation), impurity 2 (*m/z* 1062.0, addition of Gly), impurity 3 (*m/z* 1076.0, addition of Leu/Ile and *m/z* 838.3, (stereo)isomer), and impurity 4 (*m/z* 1013.4, N-terminal His truncation). The MS spectra collected at the shoulder of the main peak (impurity 2) and at the peak apex of impurity 3 and 4 are provided on the right. (*) Base peak selected for EIC. (**) In-source oxidation of Exenatide. Charge state (MS) and impurity percentage (UV 280 nm) added between brackets.

Conclusion

A generic HILIC-MS method was presented for the measurement of GLP-1 receptor agonist active ingredients and product-related impurities. The chromatographic conditions can be further finetuned to optimize resolution of specific species or extended to other therapeutic peptides. Moreover, given its orthogonal behavior, the method can be applied in a multidimensional setup in combination with RPLC and solvent modulation. The implementation of a compact and stackable MS based on single quadrupole technology eases therapeutic peptide impurity profiling and can furthermore be an asset to support method development.

References

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DE-005941

Printed in the USA, May 13, 2025
5994-8315EN

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