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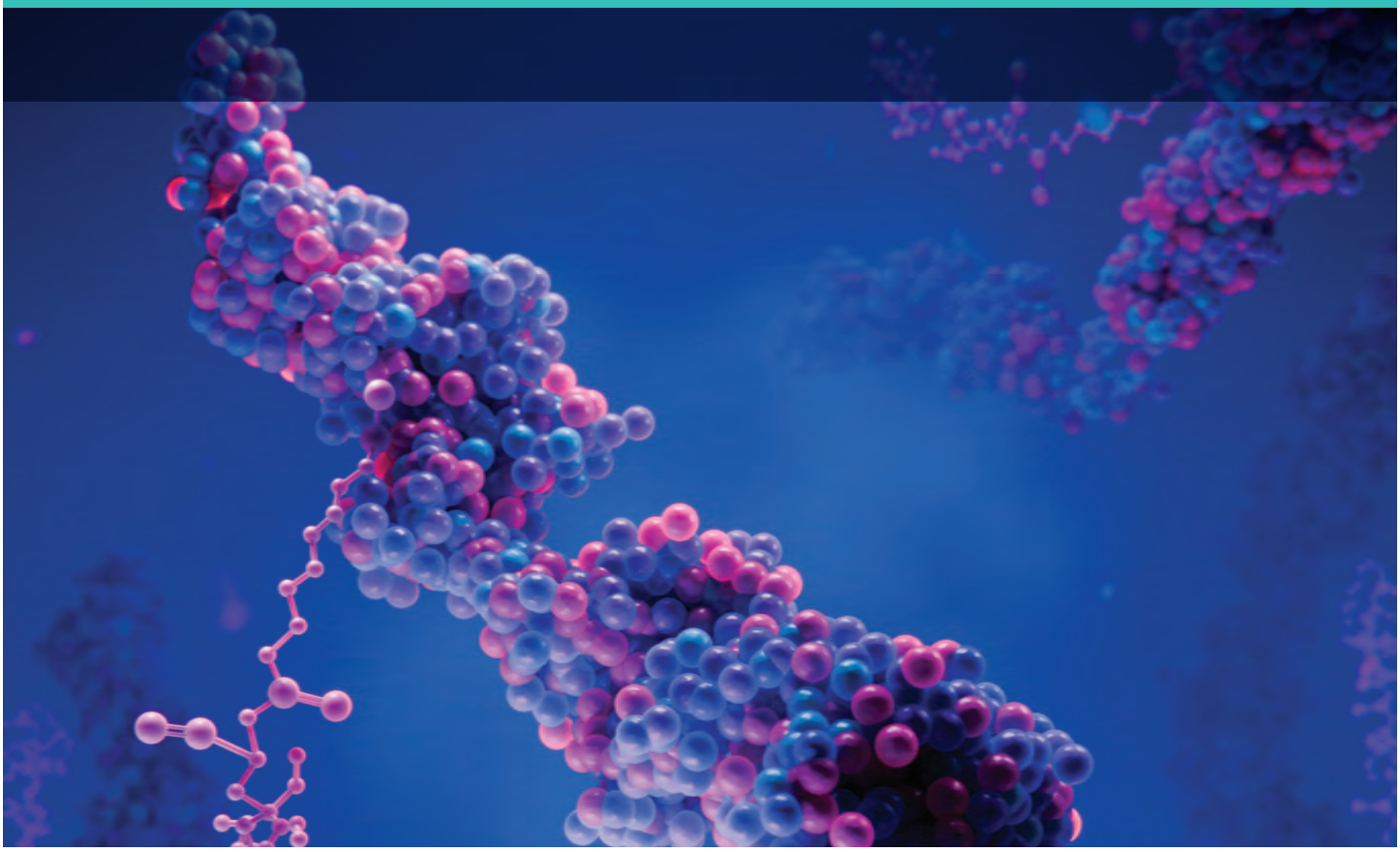
Workflows for GLP-1 Receptor Agonists and Therapeutic Peptides:

Identity, Impurity, Bioanalysis, and Stability Testing



Contents

Introduction

Peptide therapeutics occupy a unique niche between small molecules and biologics, offering tunable pharmacokinetic (PK) and pharmacodynamic (PD) profiles, high target specificity, and generally lower immunogenicity compared to monoclonal antibodies. They degrade into amino acids, minimizing long-term toxicity, and their ability to modulate large protein surfaces makes them ideal for addressing complex biological interactions.

The global peptide drug market is expanding rapidly, with over 110 approved products as of 2024. Metabolic diseases, particularly type 2 diabetes and obesity, remain the largest segment, driven by glucagon-like peptide-1 receptor agonists (GLP-1 RAs). These drugs, such as semaglutide (Ozempic), have transformed diabetes care by improving glycemic control, promoting weight loss, and reducing cardiovascular risk. Next-generation candidates like tirzepatide, which combines GLP-1 and GIP receptor activity, are setting new benchmarks for efficacy. Combination therapies pairing GLP-1 RAs with basal insulin are also gaining traction.

Manufacturing peptides is simpler and more cost effective than producing proteins or antibodies, enabling high purity and consistent quality. Advances in drug design, such as acylation, PEGylation, and fusion strategies, extend half-life and reduce dosing frequency. Delivery innovations and targeted formulations are expanding peptide applications into oncology, immunology, and vaccines.

Despite these advantages, increasing structural complexity and diverse conjugation strategies pose challenges for quality control. Robust analytical workflows are essential to ensure identity, purity, and impurity profiling, as well as bioanalysis for PK/PD studies. Liquid chromatography (LC) and mass spectrometry (MS) remain the cornerstone techniques for characterizing critical quality attributes (CQAs), including sequence confirmation and impurity detection.

This compendium provides practical insights and workflows for GLP-1 RAs and related peptides, addressing analytical challenges from identity verification to impurity characterization and bioanalysis. By leveraging advanced LC/MS technologies and orthogonal separation strategies, scientists can confidently support the development and quality assurance of these transformative therapeutics.

Chapter 1

Raw Material Identification

Application Note

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Verification of Raw Materials for Synthetic Peptide Production with the Agilent Vaya Raman System

Authors

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Agilent Technologies, Inc.

Abstract

Advancements in the manufacturing technology of peptide drug products have enabled large-scale synthetic peptide production, producing high-purity synthetic peptides in bulk. The widespread demand for glucagon-like peptide receptor agonists (GLP-1 RAs) has put pressure on synthetic peptide manufacturing capacity and revealed the need for efficient and scalable tools. The Agilent Vaya Raman raw material identity verification system enables uniquely efficient raw material verification due to its through-container identification capabilities. This application note showcases Vaya's successful through-container identification and differentiation of key building blocks for synthetic peptide manufacturing: fluorenylmethoxycarbonyl (Fmoc)-protected amino acids.

Introduction

Peptides are short chains of amino acids (< 40) that can be powerful therapeutics due to their specificity, selectivity, and potency. Additionally, peptides can be designed to be orally active, allowing for an improved patient experience compared to parenterally administered drugs. Peptides that regulate metabolism, such as GLP-1 RAs, have escalated rapidly in popularity due to their efficacy in treating diabetes and weight management. This growing family of medicine includes dipeptidyl peptidase-4 (DPP4) degradation-resistant peptides such as semaglutide, liraglutide, tirzepatide, and exenatide.

Peptides can be manufactured using recombinant DNA technology or by synthetic means. For manufacture by use of recombinant DNA, raw materials would have similarities to those outlined in a previous application note.1 Production using synthetic means requires a significantly different set of raw materials, involving specialized amino acids, solvents, and reagents.

Synthetic peptide production

The manufacturing of synthetic peptides typically follows two methods: solid-phase peptide synthesis (SPPS) or liquid-phase peptide synthesis (LPPS). SPPS is commonly used for longer or more complex amino acid sequences, such as GLP-1 RAs, involving the sequential assembly of amino acids on a solid support resin.

Raw materials for synthetic peptide synthesis

From a raw material perspective, the crucial starting materials in peptide synthesis are Fmoc-protected amino acids. Fmoc protection is necessary to support the synthesis process and amino acid coupling to produce the specific peptide. In SPPS manufacturing, it is critical that the building blocks and reagents have their identity verified prior to use to ensure a high-quality finished product. Regulatory bodies also globally enforce the verification of raw materials for drug manufacturing to ensure product safety.

Traditionally, Raman handheld devices using conventional backscattering require the container to be opened and sampled, potentially introducing contamination and shortening the shelf life of raw materials. Fmoc-protected amino acids are sensitive to light and air, so identification without opening the container is ideal to help ensure the integrity of the raw material before use in production.

The Vaya Raman is a handheld device that uses spatially offset Raman spectroscopy (SORS). SORS enables the verification of raw materials through transparent or opaque containers such as amber bottles, thick plastic, and paper sacks, eliminating the need to open them. In this application note, the through-container identity verification ability of the Vaya was tested to identify Fmoc-protected amino acids.

Experimental

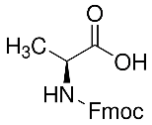
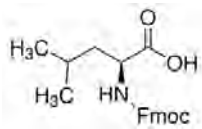
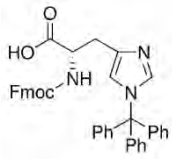
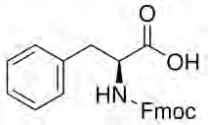
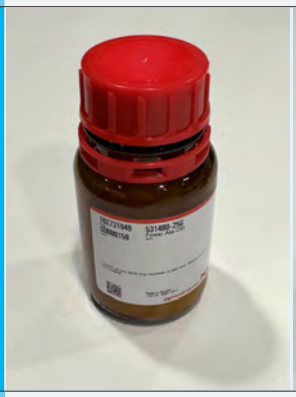
Samples of Fmoc-protected amino acids were supplied by Sigma-Aldrich in white high-density polyethylene (HDPE) or amber bottles. Table 1 outlines the structure and nomenclature of each raw material used in this application note, as well as a picture of the raw materials in their original containers.

Methods were developed on the Vaya for four Fmoc-protected amino acids without opening their respective containers. The guided on-device software provides steps to create methods and ensure models are differentiable. During method development, the software prompts the user to select Container Type for optimal container subtraction. In this case, Glass and Thick Plastic were chosen for amber bottles and white HDPE bottles, respectively.

Once methods were created and validated, Fmoc-protected amino acids could be identified and differentiated through amber bottles or white HDPE in a scan time of less than 40 seconds, delivering a clear and simple Pass/Fail result. A visual representation of analysis using the Vaya is shown in Figure 1.

Figure 1. The Vaya Raman raw material identity verification system being used to verify Fmoc-protected amino acids.

Table 1. Outline of raw materials.

Structure of Raw Material				
Name of Raw Material	Fmoc alanine (Fmoc-Ala-OH)	Fmoc leucine (Fmoc-Leu-OH)	Fmoc histidine TRT (Fmoc-HIS(Trt)-OH)	Fmoc phenylalanine (Fmoc-Phe-OH)
Picture of Raw Material in Native Container				
Container Type Selected on the Device for Method Development	Glass	Thick plastic	Thick plastic	Thick plastic

Results and discussion

Fmoc-protected amino acids show differentiable Raman spectra through both amber bottles and white HDPE. Despite their chemical similarities, Vaya SORS quickly distinguishes and verifies the identity of Fmoc-protected amino acids through the container, preserving the integrity of the raw materials.

In Figure 2, the features at 1,481cm−1 are attributed to the Fmoc-protecting group, and are consistent across the raw material group. The protecting group feature is unique to Fmoc-protected amino acids versus natural amino acids. Additionally, there are features corresponding to the aromatic groups at 1,003, 1,026, 1,582 cm−1, and carbonyl groups can be identified by features at 1,675 and 1,687 cm−1.

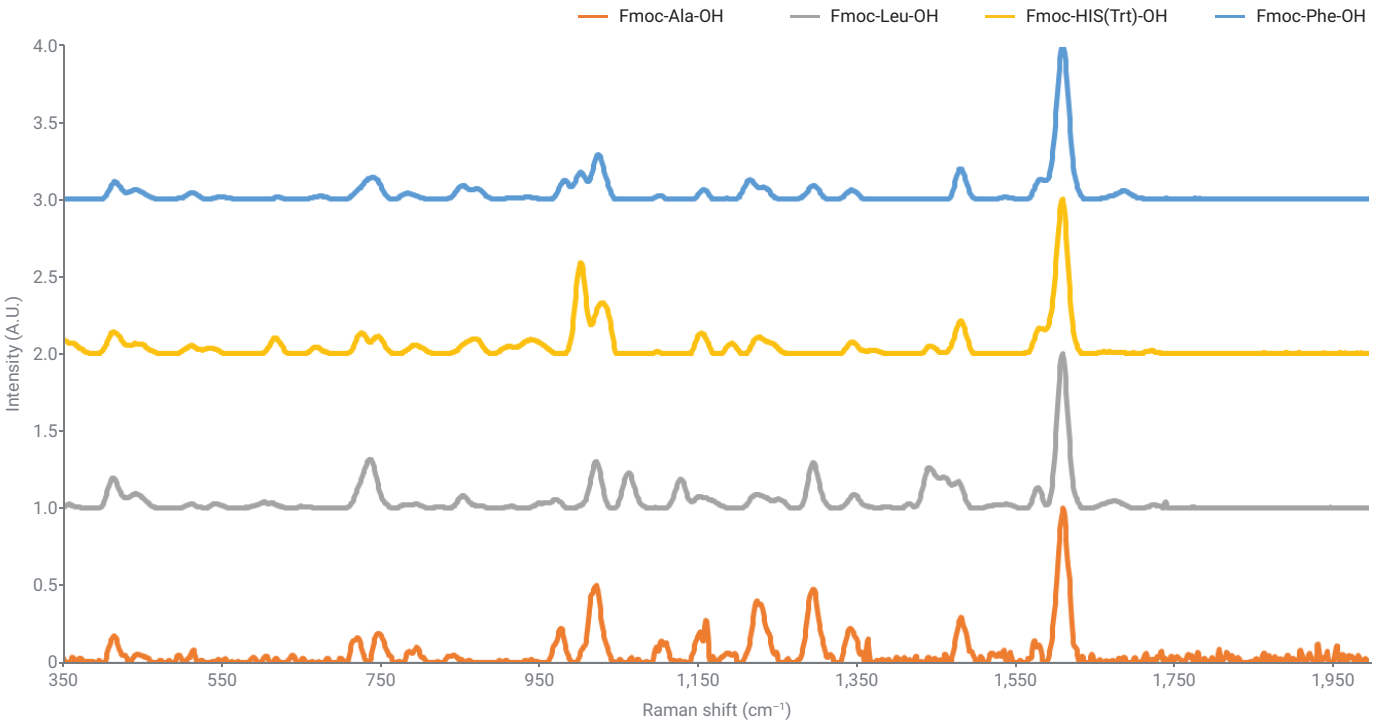


Figure 2. Spectra of Fmoc-protected amino acids.

Conclusion

The increasing demand for peptide biologics, such as GLP-1 receptor agonists, is expected to continue growing and outpacing current manufacturing capacity. To effectively meet demand, scalable solutions like the Agilent Vaya Raman raw material identity verification system introduce efficiencies to avoid bottlenecks that could be faced in raw material identification and regulatory-enforced verification. Additionally, the Vaya's ability to perform nondestructive, noninvasive identification ensures that raw materials are transferred expediently to production while preserving their integrity.

Reference

1. Prullière, F.; Welsby, C. Differentiating Biopharmaceutical Raw Materials Using Spatially Offset Raman Spectroscopy, *Agilent Technologies application note*, publication number 5991-2013EN, **2021**.

Additional resources

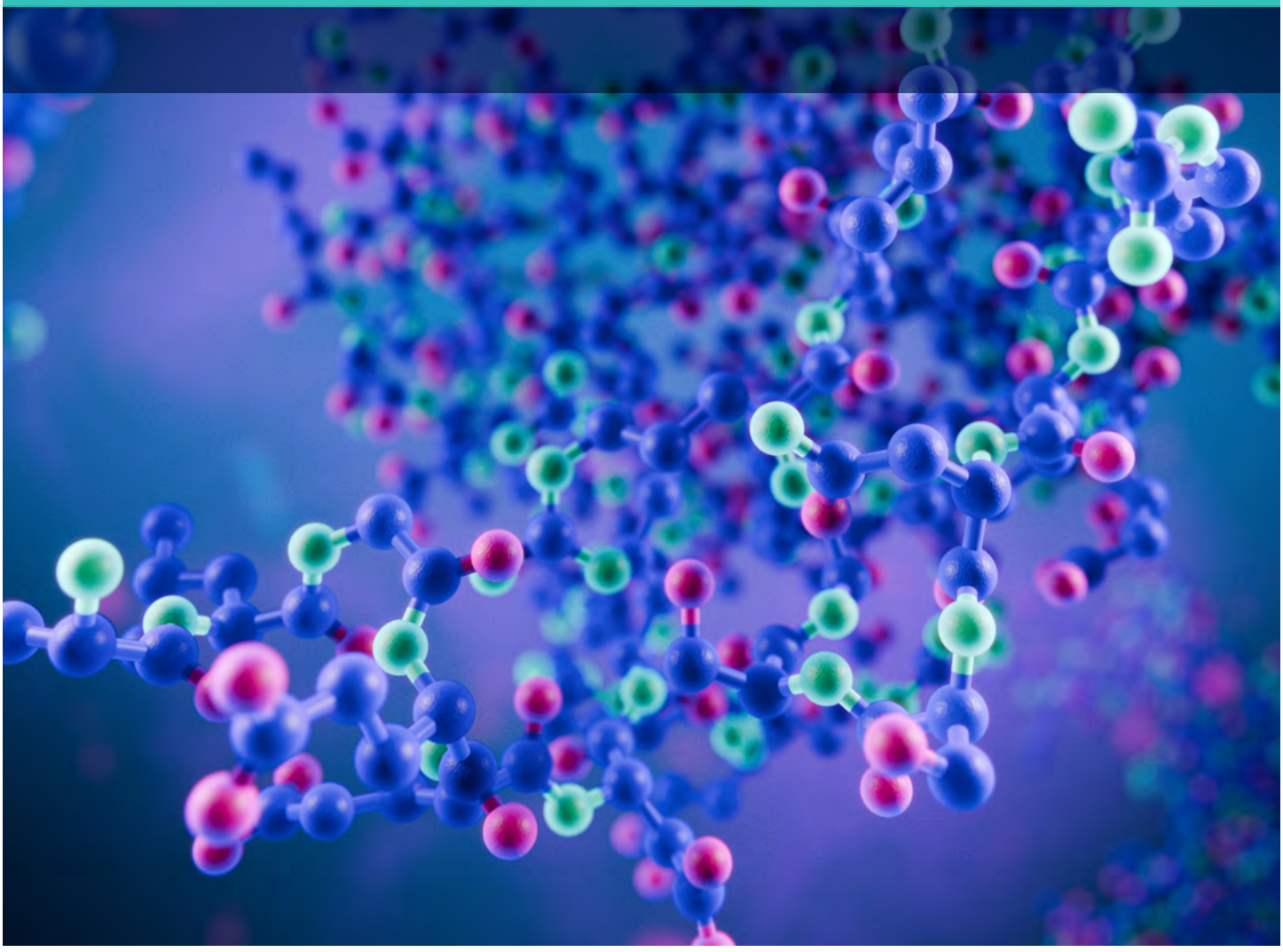
Vaya Handheld Raman Spectrometer

Rapid Testing of Biopharmaceutical Solvents

Differentiating Biopharmaceutical Raw Materials

Chapter 2

Purity and Impurity Analysis



Application Note

Purity Analysis

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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 an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology and an Agilent 1290 Infinity III Bio LC System, the latter coupled to the Agilent InfinityLab LC/MSD iQ.

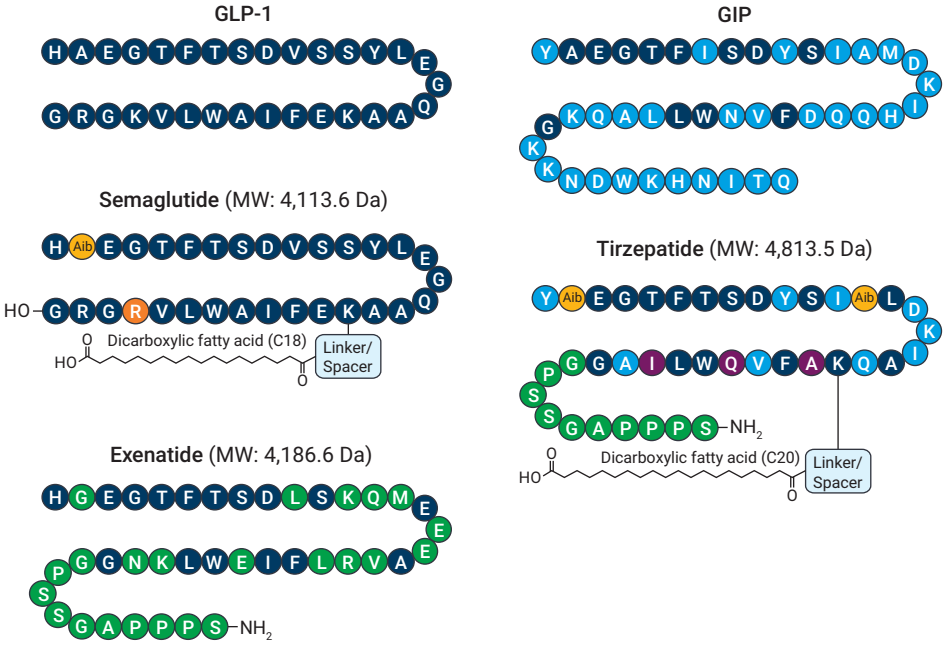

Figure 1. Molecular structures of native GLP-1 and GIP, GLP-1 receptor agonists Semaglutide and Exenatide, and GLP-1 and GIP receptor co-agonist Tirzepatide. Molecular weight (MW) of therapeutic peptides is added.

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	Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, 2.1 × 150 mm, 2.7 µm (p/n 227215-924)
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: 3,500 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 coated 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).

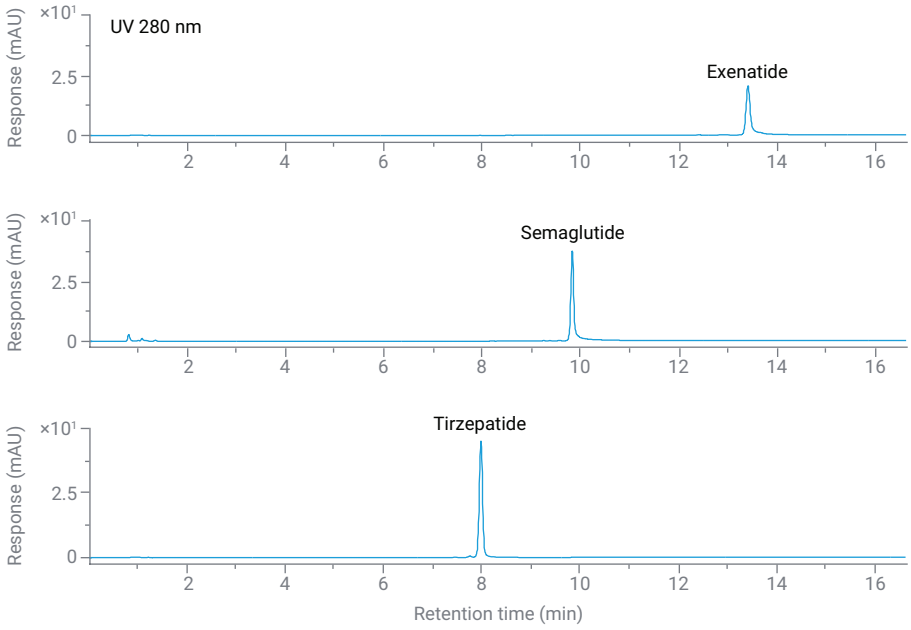

Figure 2. HILIC-UV 280 nm chromatograms of GLP-1 receptor agonists Exenatide, Semaglutide, and Tirzepatide at 1 mg/mL.

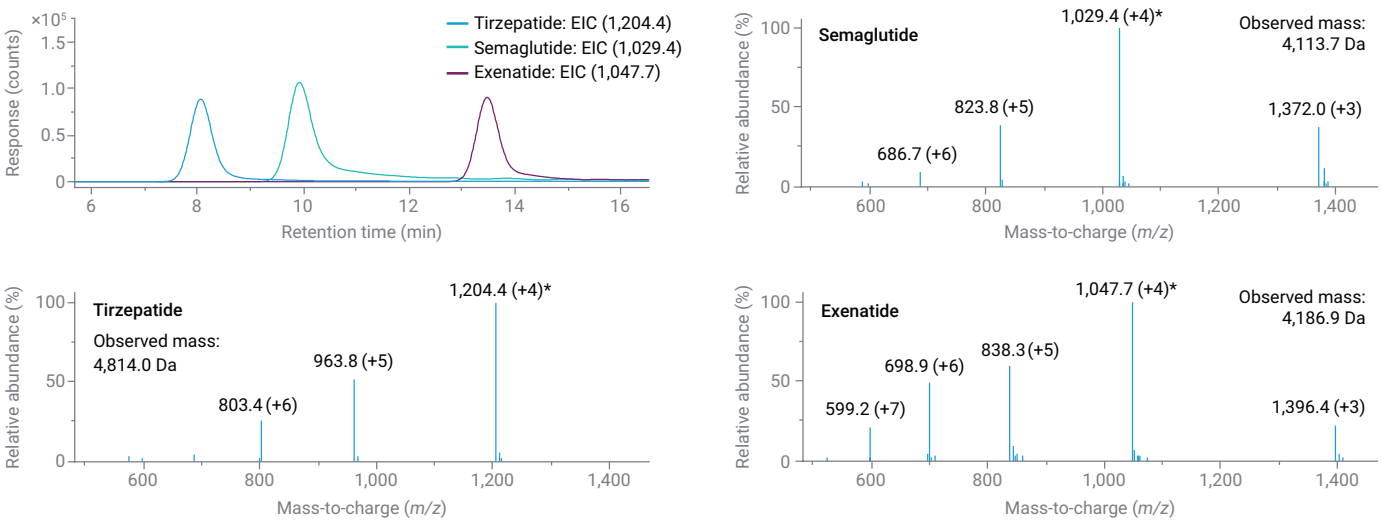

Figure 3. Overlaid extracted ion chromatograms (EIC) of the base peak (*) and single quadrupole mass spectra of Tirzepatide, Semaglutide, and Exenatide (1 mg/mL). Charge state is added between brackets.

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.

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 His-Cyclic-Semaglutide (+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.

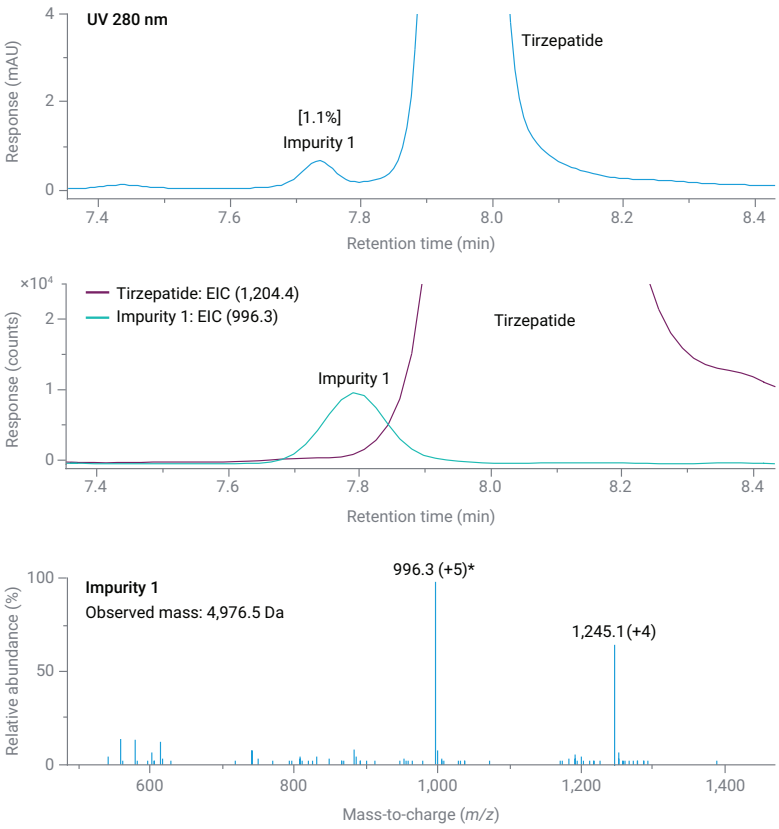

Figure 4. Zoomed HILIC-UV 280 nm chromatogram of Tirzepatide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of Tirzepatide (*m/z* 1204.4) and impurity 1 (*m/z* 996.3) corresponding to the addition of Tyr. The MS spectrum of the impurity is shown; the spectrum of the main peak is shown in Figure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

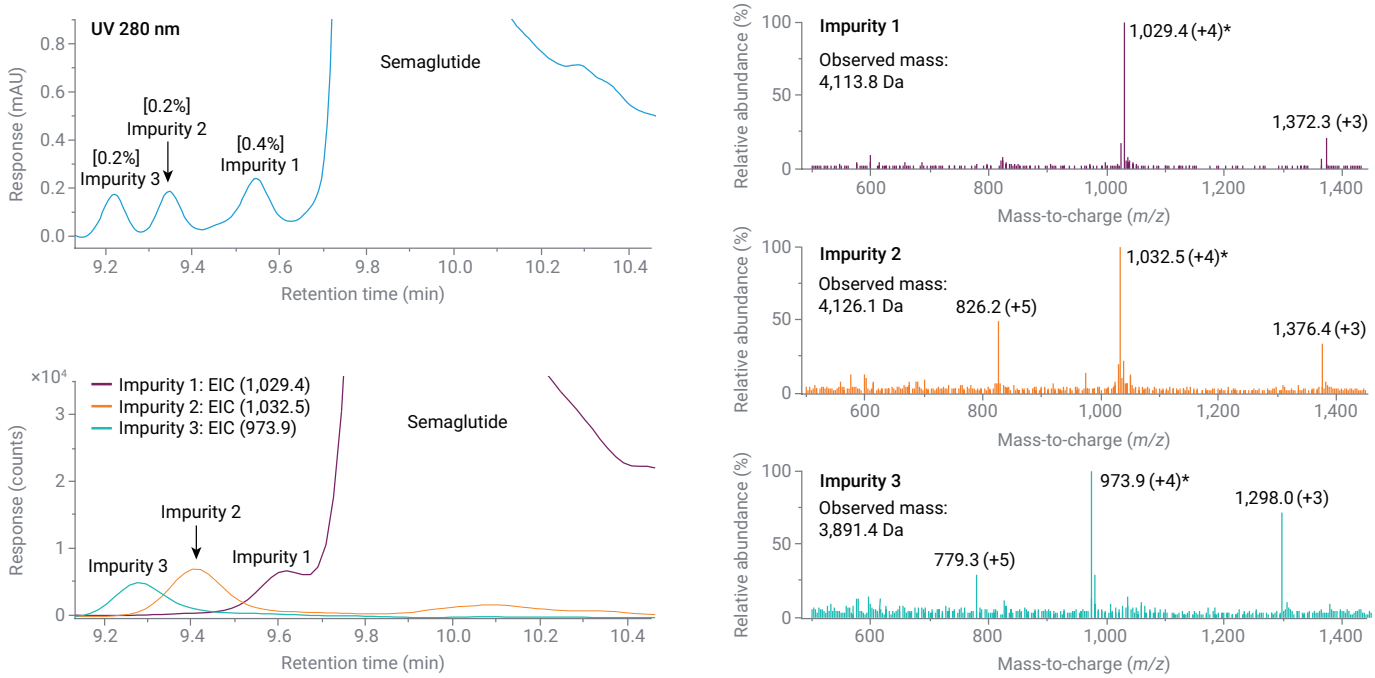

Figure 5. Zoomed HILIC-UV 280 nm chromatogram of Semaglutide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of Semaglutide impurity 1 (*m/z* 1029.4), impurity 2 (m/z 1032.5), and impurity 3 (*m/z* 973.9). The MS spectra of the impurities are depicted on the right. Common *m/z* ions between 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.

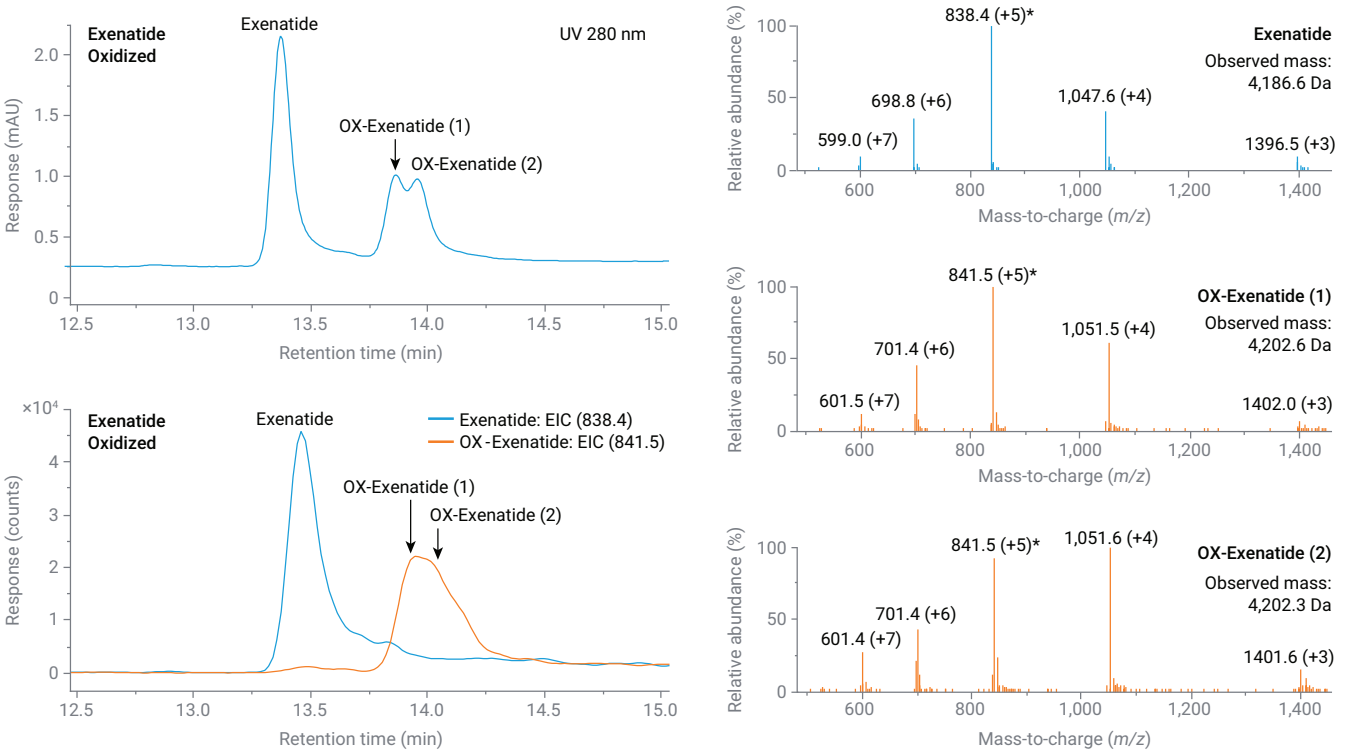

Figure 6. Zoomed HILIC-UV 280 nm chromatogram of H2O2-stressed Exenatide (0.25 mg/mL) and overlaid extracted ion chromatograms (EIC) of Exenatide (*m/z* 838.4) and oxidized-Exenatide (*m/z* 841.5). The MS spectra of the observed peaks is presented on the right. (*) Base peak selected for EIC. Charge state added between brackets.

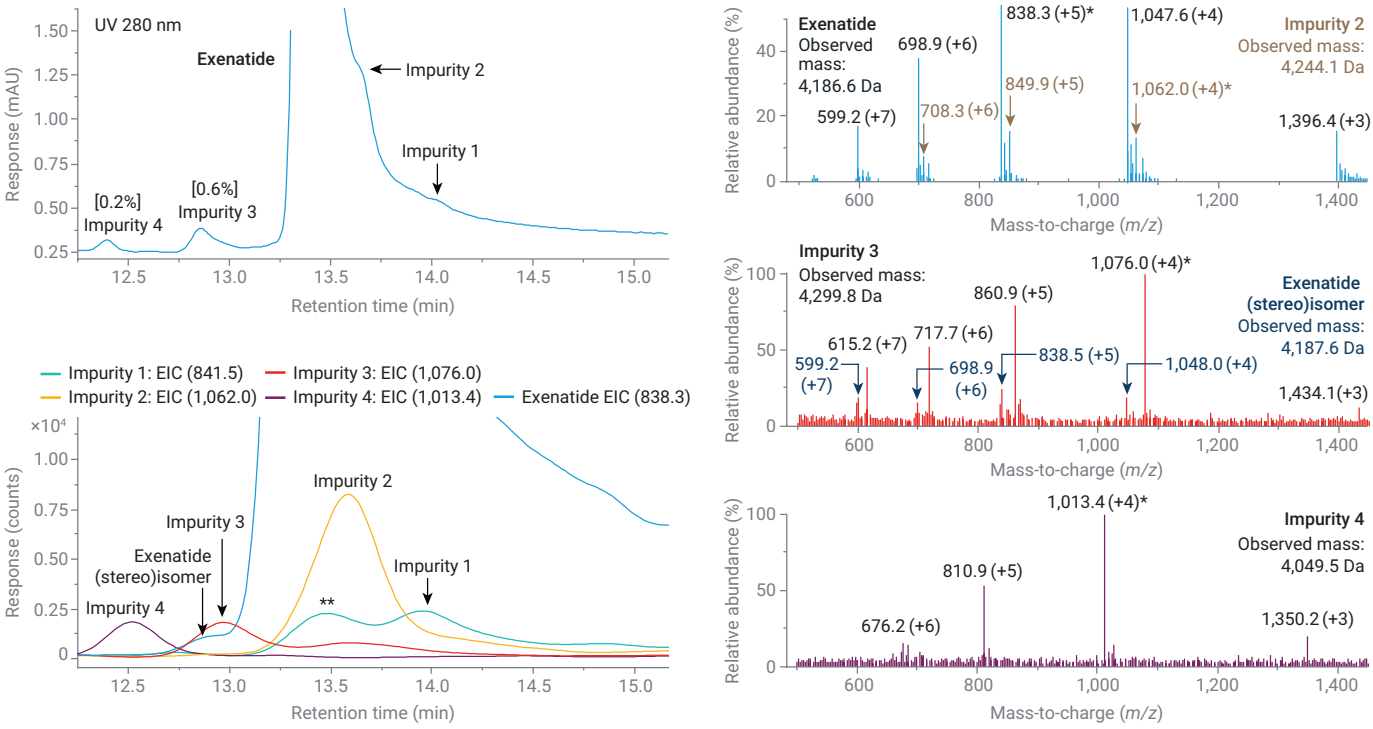

Figure 7. Zoomed HILIC-UV 280 nm chromatogram of Exenatide (1 mg/mL) and overlaid extracted ion chromatograms (EIC) of impurity 1 (*m/z* 841.5, Met oxidation), impurity 2 (*m/z* 1062.0, addition of Gly), impurity 3 (*m/z* 1076.0, addition of Leu/Ile and *m/z* 838.3, (stereo)isomer), and impurity 4 (*m/z* 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.

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.

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.

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 fine-tuned 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.

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Application Note

Purity Analysis

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HILIC Analysis of GLP-1 Receptor Agonists

Using an Agilent 1290 Infinity III Bio LC with DAD and ELSD

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Agilent Technologies, Inc.

Abstract

GLP-1 receptor agonists are widely applied in managing diabetes and obesity. This application note describes HILIC analysis of these therapeutics on a low-adsorption and biocompatible LC flow path. Using DAD and ELSD, the active ingredient, product-related impurities, and formulation constituents can be simultaneously measured. The benefit of eliminating or deactivating all stainless-steel components in the LC flow path is demonstrated.

Introduction

Glucagon-like peptide-1 (GLP-1) receptor agonists represent a class of peptide-based therapeutics used to treat type 2 diabetes mellitus (T2DM) and, more recently, obesity. They mimic the action of the naturally occurring GLP-1 hormone that stimulates insulin release, reduces glucagon secretion, slows gastric emptying, and lowers appetite.1 As native GLP-1 has limited clinical utility due to rapid clearance, marketed products have been engineered in several manners to extend half-life (e.g. amino acid substitution, fatty acid conjugation) (Figure 1).

By way of example, Semaglutide, the active ingredient in the top-selling drug Ozempic, deviates from natural GLP-1 by two amino acid substitutions, in addition to the presence of stearic di-acid conjugated to lysine via a short spacer. Optimal glucose control in T2DM often requires multifaceted therapeutic strategies. Consequently, pharmaceutical companies have developed combination products such as Xultophy, containing the GLP-1 receptor agonist Liraglutide and Insulin degludec, or co-agonistic peptides such as Tirzepatide (the active ingredient of Mounjaro) simultaneously exerting the functions of GLP-1 and gastric-inhibitory polypeptide (GIP).

Given the global rise in T2DM and obesity, there is a high demand for synthetic peptides targeting GLP-1 and other incretin receptors. There is also the need for proper analytical methodologies to assess the quality of these medicines. Reversed-phase liquid chromatography (RPLC), size exclusion chromatography (SEC), and mass spectrometry (MS) are commonly used to determine different attributes associated with the active pharmaceutical ingredient (identity, purity, impurities, content). In the present study, hydrophilic interaction liquid chromatography (HILIC) is introduced as a complementary methodology to study various features of the drug product (DP). In combination with diode array (DAD) and evaporative light scattering detection (ELSD), the simultaneous measurement of the therapeutic peptide and various formulation constituents in the DP is enabled. These include inorganic ions, sugars and amino acids added as buffering or tonicity agents, stabilizers, and antioxidants. To prevent nonspecific interaction of metal-sensitive constituents with metal surfaces and metal-contaminated flow paths, low adsorption and biocompatible LC hardware is implemented by means of an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology and an Agilent 1290 Infinity III Bio LC System.

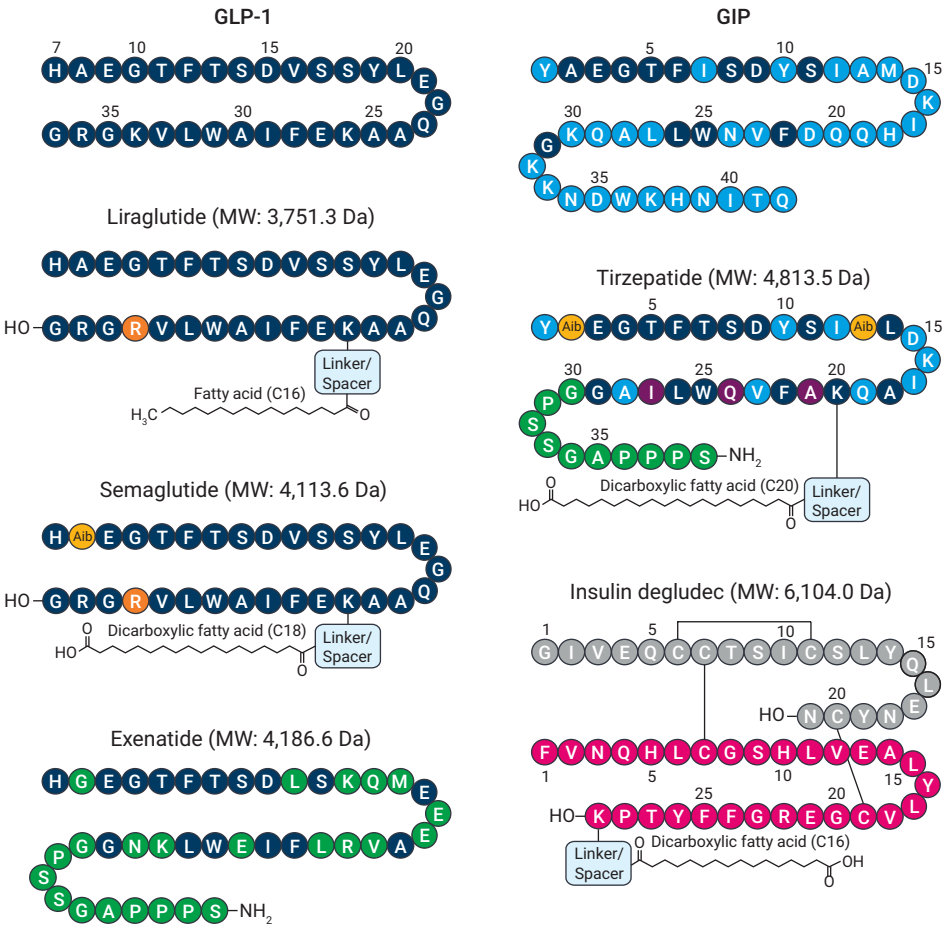

Figure 1. Molecular structures of native GLP-1 and GIP, GLP-1 receptor agonists Liraglutide, Semaglutide, and Exenatide, GLP-1 and GIP receptor co-agonist Tirzepatide, and Insulin degludec. Molecular weight of therapeutic peptides is added.

Experimental

Materials

Water (ULC/MS) and acetonitrile (HPLC-S) were supplied by Biosolve. Ammonium formate (LiChropur), dimethyl sulfoxide (DMSO, Suprasolv), hydrogen peroxide (≥ 30%, for trace analysis), D-mannitol, sucrose, L-methionine, sodium chloride, sodium phosphate monobasic, and zinc acetate dihydrate were sourced from Merck. Glycerol was purchased from Thermo Fisher Scientific. Semaglutide acetate (≥ 95%), Exendin-4 (48 to 86) amide acetate (exenatide acetate, ≥ 95%), Liraglutide acetate (≥ 95%) and Tirzepatide sodium salt (≥ 95%) were obtained from Cayman Chemical. Ozempic and Xultophy were received from Novo Nordisk and Byetta from Astra Zeneca (Table 1).

Sample preparation

Semaglutide and Liraglutide were 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. Further dilutions were done in water prior to injection. Byetta, Ozempic, and Xultophy were applied to the column. The excipient solutions (glycerol, mannitol, sucrose, NaCl, NaH_2PO_4, and zinc acetate) were prepared in 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 Agilent 1290 Infinity III LC System (stainless-steel (SST)) and the 1290 Infinity III Bio LC System (BIO) were used in this study. Details of both configurations 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 1. GLP-1 receptor agonist DPs used in the current study.

DP	Active Ingredient	Preparative Runs
Byetta	Exenatide (300 µg in 1.2 mL)	Mannitol (43 mg/mL), metacresol (2.2 mg/mL), glacial acetic acid, and sodium acetate trihydrate (pH 4.5)
Ozempic	Semaglutide (2 mg in 1.5 mL)	Propylene glycol (14 mg/mL), phenol (5.5 mg/mL), disodium phosphate dihydrate (1.42 mg/mL), hydrochloric acid, and sodium hydroxide (pH 7.4)
Xultophy	Liraglutide (10.8 mg in 3 mL) and Insulin degludec (300 units in 3 mL)	Glycerol (19.7 mg/mL), phenol (5.7 mg/mL), zinc (55 µg/mL), hydrochloric acid, and sodium hydroxide (pH 8.15)

Table 2. Details of the instrument configurations used.

Parameter	Analytical Runs	Excipients
Pump	Agilent 1290 Infinity III High-Speed Pump (G7120A)	Agilent 1290 Infinity III Bio High-Speed Pump (G7132A)
Autosampler	Agilent 1290 Infinity III Multisampler (G7167B) with integrated Sample Thermostat	Agilent 1290 Infinity III Bio Multisampler (G7137A) with integrated Sample Thermostat
Column Compartment	Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Heat Exchanger, standard flow (G7116-60015)	Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Bio Heat Exchanger, standard flow (G7116-60071)
Detector (DAD)	Agilent 1290 Infinity III Diode Array Detector (G7117B)	
Flow Cell	Agilent Max-Light Cartridge Cell, standard, 10 mm (G4212-60008)	Agilent Max-Light Cartridge Cell, LSS, 10 mm (G7117-60020), aperture not attached
Detector (ELSD)	Agilent 1290 Infinity III Evaporative Light Scattering Detector (G4261B)	

Table 3. LC-MS method parameters.

Parameter	Value
Columns	Agilent InfinityLab Poroshell HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924) Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, 2.1 × 150 mm, 2.7 µm (p/n 227215-924)
Flow Rate	0.4 mL/min
Mobile Phase	A = 100 mM ammonium formate pH 3 B = acetonitrile
Gradient	Time %B 0–1 min 90 1–19 min 90 to 45 19–20 min 45 to 90 20–30 min 90
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 ELSD	Evaporator temperature: 50 °C Nebulizer temperature: 50 °C Gas flow rate: 1.0 SLM Data rate: 80 Hz Smoothing: 30 PMT Gain: 1.0

Results and discussion

HILIC retains and resolves polar and ionic solutes and offers a different selectivity in comparison to RPLC. Separation is based on the hydrophilic partitioning of solutes 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).[2] Since its inception 35 years ago, HILIC has successfully been applied for the analysis of a wide range of substances including amino acids, peptides, (glyco) proteins, sugars, (oligo)nucleotides, organic acids, and inorganic ions, among others.

The analysis of HILIC-amenable solutes commonly found as excipients in GLP-1 receptor agonist DPs is presented in Figure 2. Chromatographic conditions are based on earlier work describing the use of a HILIC-Z column and ELSD for the measurement of inorganic anions and cations.[3] The same method allows for the separation of the synthetic GLP-1 receptor agonist peptides Exenatide, Semaglutide, Liraglutide, and Tirzepatide, as illustrated in Figure 3. Peptide elution is based on the amino acid composition and conjugated fatty acids.

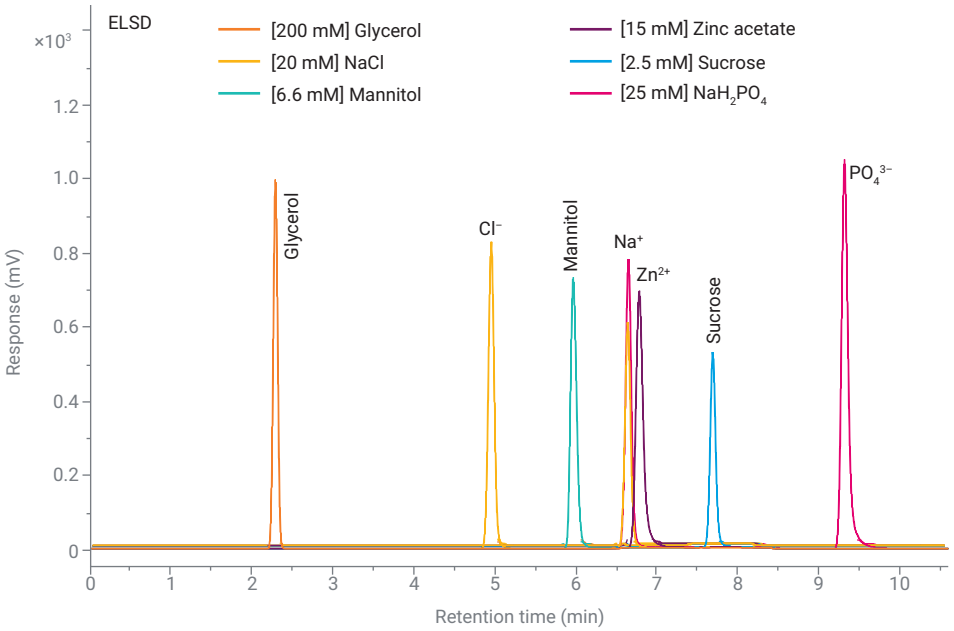

Figure 2. Overlaid HILIC-ELSD traces of various formulation constituents. Concentration has been varied to reach similar detector intensities. Acetate is not measurable by ELSD due to its volatility. Experiments were performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

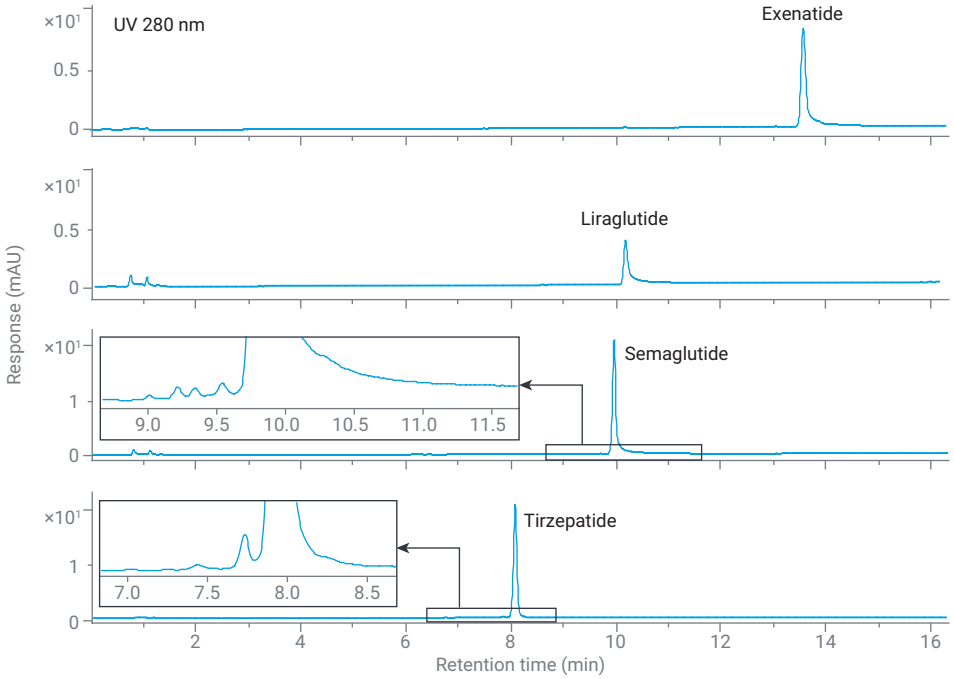

Figure 3. HILIC-UV 280 nm chromatograms of GLP-1 receptor agonists Exenatide, Liraglutide, Semaglutide, and Tirzepatide at 0.5 mg/mL. Experiments were performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

The HILIC-Z method with sequential DAD and ELSD detection opens possibilities to simultaneously assess the active ingredients and formulation constituents in GLP-1 receptor agonist DPs, as shown in Figure 4. The ELSD data provide a read-out of cosolvents (glycerol), tonicity agents (mannitol), and buffer/pH adjustment substances (Na-phosphate, HCl, NaOH) while the UV data allow detection of the synthetic peptide(s) and its impurities, as well as the unretained preservative (phenol, metacresol). The unretained preservative is not detectable by ELSD given its volatility. Note the saturating signal for mannitol, which is present at 43 mg/mL in Byetta.

Data shown thus far have been generated on a low-adsorption flow path using an Altura Poroshell HILIC-Z column with Ultra Inert technology and biocompatible 1290 Infinity III Bio LC. The rationale is that phosphate- and carboxylate-containing analytes tend to adsorb to metal surfaces or interact with leached metals, resulting in peak broadening and diminished recovery.[4] This phenomenon can, to a certain extent, be mitigated by passivating the flow path or adding metal chelators to the mobile phase. A superior alternative is the elimination or deactivation of stainless-steel surfaces.

The impact of the latter strategy on the current study's samples is presented in Figures 5 and 6. Peak shape and recovery of inorganic phosphate, as observed in an NaH_2PO_4 standard and Ozempic, are drastically affected when evolving from a stainless-steel to a low-adsorption flow path (1290 Infinity III LC and stainless-steel HILIC-Z column versus the 1290 Infinity III Bio LC and Altura HILIC-Z column with Ultra Inert technology). Furthermore, peak area precision is substantially improved when opting for the 1290 Infinity III Bio LC and Ultra Inert HILIC-Z column (data not shown). Tirzepatide and Semaglutide elute equally well on both configurations, while Liraglutide and Exenatide have a more distorted peak shape on the stainless-steel setup. Despite the latter, near complete recovery is obtained for the active ingredient. The detection of product-related impurities is considerably better using the low-adsorption flow path, as illustrated for oxidized Exenatide eluting as a postpeak. This species remains undetectable in the tail of the main peak in DP and displays a deformed retention in H_2O_2-stressed Exenatide on a stainless-steel flow path.
A partially resolved peak doublet is observed on HILIC as a result of the formation of methionine sulfoxide S- and R-diastereomers.

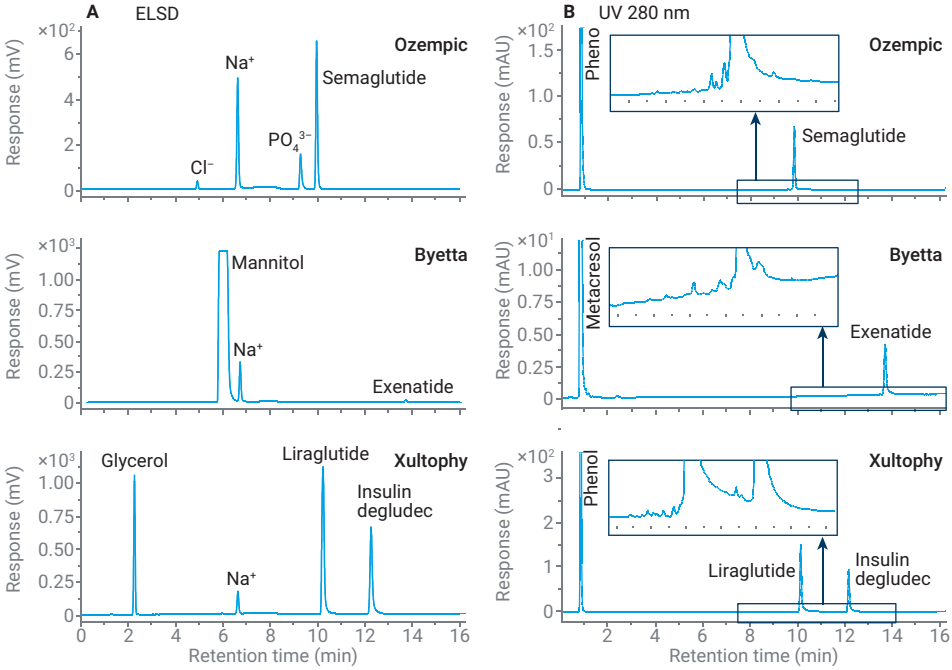

Figure 4. (A) HILIC-ELSD and (B) UV 280 nm chromatograms of Ozempic, Byetta, and Xultophy. Experiments performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio LC System.

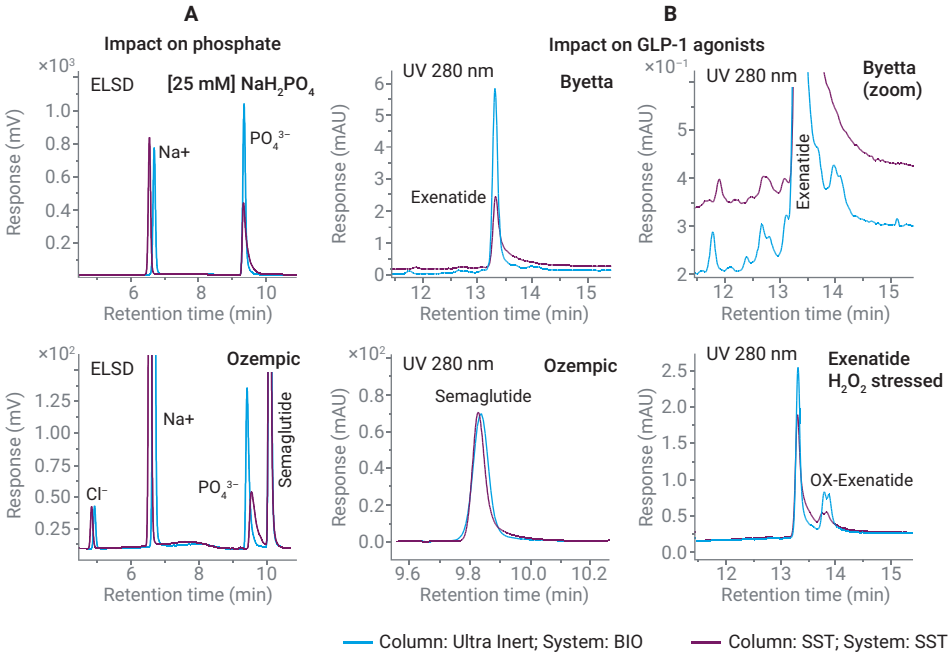

Figure 5. (A) HILIC-ELSD chromatograms of a 25 mM NaH_2PO_4 standard and Ozempic obtained on a low-adsorption and stainless-steel flow path. (B) HILIC-UV 280 nm chromatograms focusing on Semaglutide (Ozempic), Exenatide (Byetta), and oxidatively stressed Exenatide (0.25 mg/mL) elution on a low-adsorption and stainless-steel flow path. Chromatograms have been aligned (retention time) on the main peak to facilitate interpretation. BIO: Agilent 1290 Infinity III Bio LC, SST: Agilent 1290 Infinity III LC (Stainless-steel (SST)). Ultra Inert column: Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, SST column: Agilent InfinityLab Poroshell HILIC-Z with stainless-steel column hardware.

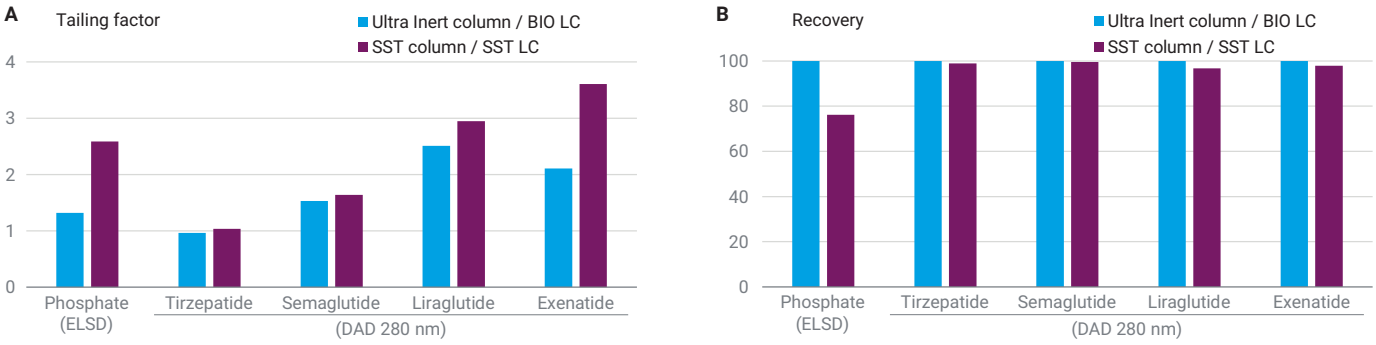

Figure 6. (A) Peak tailing factor and (B) recovery (peak area relative to low adsorption flow path) for 25 mM NaH2PO4 (see Figure 5) and Tirzepatide, Semaglutide, Liraglutide, and Exenatide at 0.5 mg/mL. BIO: Agilent 1290 Infinity III Bio LC, SST: Agilent 1290 Infinity III LC (Stainless-steel (SST)). Ultra Inert column: Agilent Altura Poroshell HILIC-Z with Ultra Inert technology, SST column: Agilent InfinityLab Poroshell HILIC-Z with stainless-steel column hardware

Conclusion

This study demonstrates the simultaneous measurement of GLP-1 receptor agonist active ingredient, product-related impurities, and excipients by HILIC in combination with DAD and ELSD. To prevent nonspecific interactions of solutes with metal components in the flow path, an Agilent Altura HILIC-Z column with Ultra Inert technology and Agilent 1290 Infinity III Bio LC System were used. A generic method was presented here which can demonstrably be fine-tuned to further optimize resolution of specific species.

Reference

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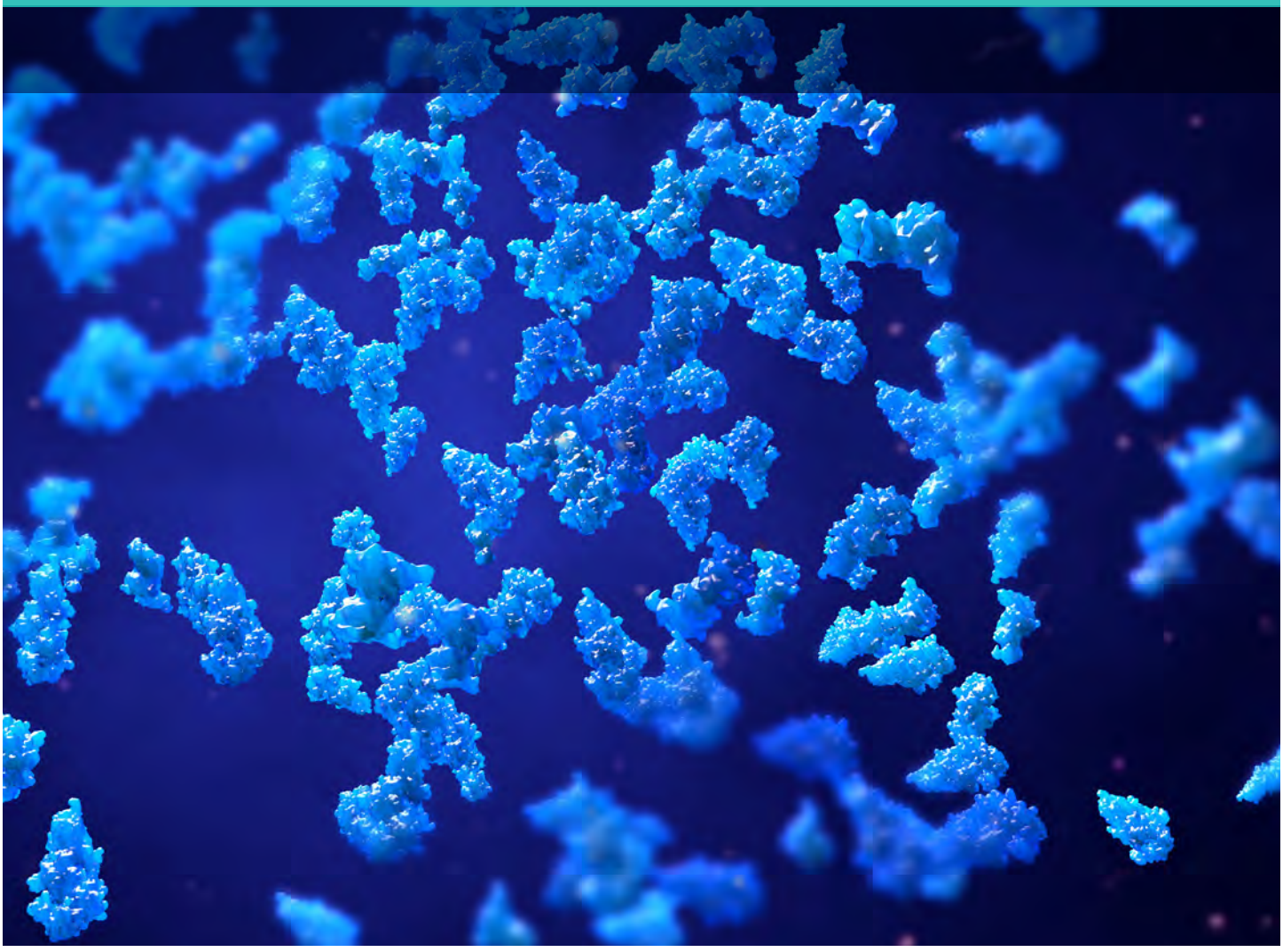
3. Analysis of Metals, Halides, and Inorganic Ions Using Hydrophilic Interaction Chromatography. *Agilent Technologies application note,* publication number 5991-8602EN, **2017**.
4. Guimaraes, G. J.; Bartlett, M. G. Managing Nonspecific Adsorption to Liquid Chromatography Hardware: a Review. *Anal. Chim. Acta* **2023**, *1250,* 340994.

Chapter 3

Bioanalysis



Application Note

Biopharma/Pharma

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Trusted Answers

Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analog Semaglutide from Plasma

UComprehensive workflow for automated sample cleanup and sensitive quantitation

Authors

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Agilent Technologies, Inc.

Abstract

Sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics are crucial for advancing glucagon-like peptide-1 (GLP-1) receptor agonist medications. This application note presents an automated workflow using LC/MS to quantify the GLP-1 analog semaglutide in human plasma. Our results indicate that this automated LC/MS workflow can enhance assay sensitivity by five times compared to traditional organic solvent precipitation; this assay achieved LLOQ of 0.2 ng/mL and was linear up to 1,000 ng/mL from 100 µL starting material. This workflow can be used for quantitative analysis of GLP-1 therapeutics without the need for specific antibodies. It also provides excellent sensitivity, high specificity, and fast method development, which all play a crucial role in drug discovery and development.

Introduction

In recent years, glucagon-like peptide-1 (GLP-1) receptor agonists have gained significant attention because of their effectiveness to treat obesity and type 2 diabetes. The global market for GLP-1 analogs is projected to grow rapidly, reaching $140 billion by 2030. Among the notable advancements is the peptide semaglutide, which is sold under the brand name "Wegovy" for weight loss and "Ozempic" or "Rybelsus" for diabetes.[1,2] Developing sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics is crucial for advancing these medications.

Traditionally, the plasma concentration of semaglutide is measured by a ligand-binding assay, which requires time to develop antibodies and can lack selectivity and specificity. Liquid chromatography/mass spectrometry (LC/MS), which is used for sequence identification of GLP-1 receptor agonists, has emerged as an alternative methodology to precisely quantify these molecules in biological matrices because of its superior sensitivity, wide dynamic range, and high specificity.

In this application note, we present an automated workflow with LC/MS technologies to quantify GLP-1 analog drugs in human plasma using an Agilent 1290 Infinity II bio LC and an Agilent 6495D triple quadrupole LC/MS (LC/TQ) system (Figure 1).

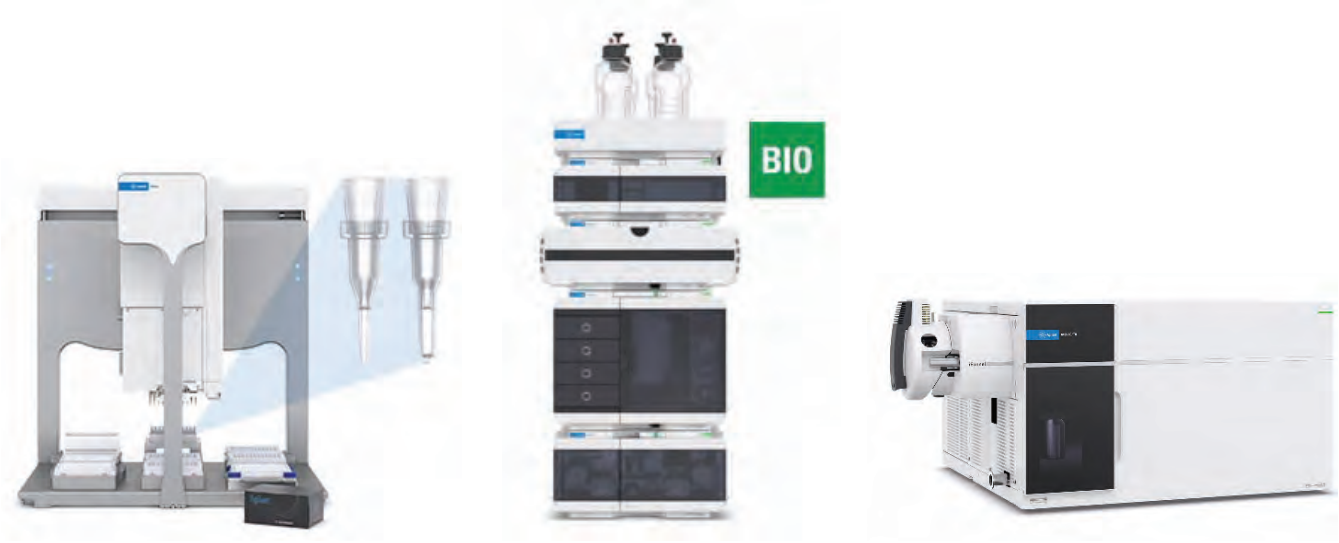

Figure 1. Agilent AssayMAP Bravo protein sample prep platform, 1290 Infinity II bio LC, and 6495D LC/TQ.

Experimental

Materials and methods

Tirzepatide, semaglutide, formic acid (FA), and ammonium formate were purchased from MilliporeSigma (St. Louis, MO, U.S.). Acetonitrile (ACN) and methanol (MeOH) were purchased from Honeywell (Charlotte, NC, U.S.). The 96-well LoBind plates and protein LoBind tubes were purchased from Eppendorf (Hauppauge, NY, U.S.). Also used were Agilent AssayMAP RP-S cartridges.

Instrumentation

The following instrumentation was used:

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- Agilent 1290 Infinity II bio LC system including:
- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116A) equipped with a standard flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495D LC/TQ (G6495DA)

Software

The following software was used:

- Agilent MassHunter Acquisition software (version 12.0)
- Agilent MassHunter Quantitative Analysis software (version 12.0)

Sample preparation

Protein precipitation: A mixture of 150 µL ACN and 150 µL MeOH was added to an aliquot of 100 µL human plasma fortified with different concentration of semaglutide. Also, 5 µL of 1,000 ng/mL tirzepatide was used as the internal standard. The mixture was vortexed for 2 minutes and then spun down at 17,000 g for 10 minutes. A 350 µL volume of each supernatant was transferred to a 96-well plate and dried down under nitrogen gas with heating. Then, 110 µL water was added to each well to reconstitute. The solution was further purified using AssayMAP RP-S cartridges.

AssayMAP automated purification: AssayMAP RP-S cartridges were conditioned with 80% ACN and equilibrated with water. Then, 100 µL of the previously mentioned reconstituted samples were loaded onto AssayMAP RP-S cartridges at 3 µL/min, and cartridges were then washed once with water and once with 10% ACN in 10 mM ammonium formate buffer. The final elution step was carried out by loading 30 µL of 5% FA in 80% ACN to the cartridge at 3 µL/min. Lastly, 30 µL water with 5% FA was added to the final elution and 5 µL was injected into LC/MS for analysis.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II bio UHPLC coupled to an Agilent 6495D LC/TQ with the Agilent Jet Stream Electrospray ion source. Separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization of semaglutide yielded $[M + 4H]^{4+}$ signal at ***m/z*** 1,029.4 as the most intense ion. MRM transitions were optimized and 1,029.4 & 1,238.1 was chosen as the quantifier and 1,029.4 & 136.1 was chosen as the qualifier, and the tirzepatide MRM transition (1,204.4 & 396.2) was chosen. Both peptides' MRM transitions are listed in Table 3 with optimal collision energies.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping 120 Å, 2.1 × 150 mm, 2.7 µM (p/n 653750-902)
Column Temperature	80 °C
Injection Volume	5 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50:50 water:methanol)
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow Rate	0.5 mL/min
Gradient Program	Time %B 0 30 3.0 45 5.0 60 5.1 95 8.0 95 8.2 30 10.0 30
Stop Time	10.0 min

Data processing

All MS data were processed using Agilent MassHunter Quantitative Analysis software.

Table 2. MS acquisition parameters.

Parameter	Value
Ion Mode	Positive
Gas Temperature	270 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	25 psi
Sheath Gas Temperature	200 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,500 V
Nozzle Voltage	400 V

Table 3. Semaglutide and tirzepatide MRM transitions.

Peptide	Precursor Ion	Product Ion	Collision Energy
Tirzepatide	1,204.4	396.2	26
Semaglutide	1,029.4	136.1	42
Semaglutide	1,029.4	1,238.1	30

Results and discussion

Method optimization for GLP-1 analog peptide quantitative analysis

To improve the sensitivity and reproducibility for GLP-1 peptide quantitative analysis, all the sample preparation steps were evaluated to achieve the best results.

1. Organic solvent composition to precipitate proteins was evaluated, including just ACN, just MeOH, and ACN:MeOH (1:1). The ACN:MeOH (1:1) was found to be the best for semaglutide and tirzepatide extraction (Figure 2).
2. Loading buffer was evaluated, including pure water, and water with 0.1% FA and 10%, 20%, or 40% ACN. Pure water was found to be the best loading buffer for GLP-1 peptides.
3. Washing buffer was evaluated, including pure water, water with 0.1% FA, water with 1% FA, and water with 10 mM NH_4COOH pH 10 buffer with or without 10% ACN. The combination of water and 10% ACN in 10 mM NH4COOH pH 10 buffer was found to be the best washing solution.
4. Elution buffers were evaluated, including 10 mM NH_4COOH pH 10 buffer with 30% or 80% ACN, and 80% ACN with 0.1%, 1%, and 5% FA. The 5% FA in 80% ACN produced the best results.

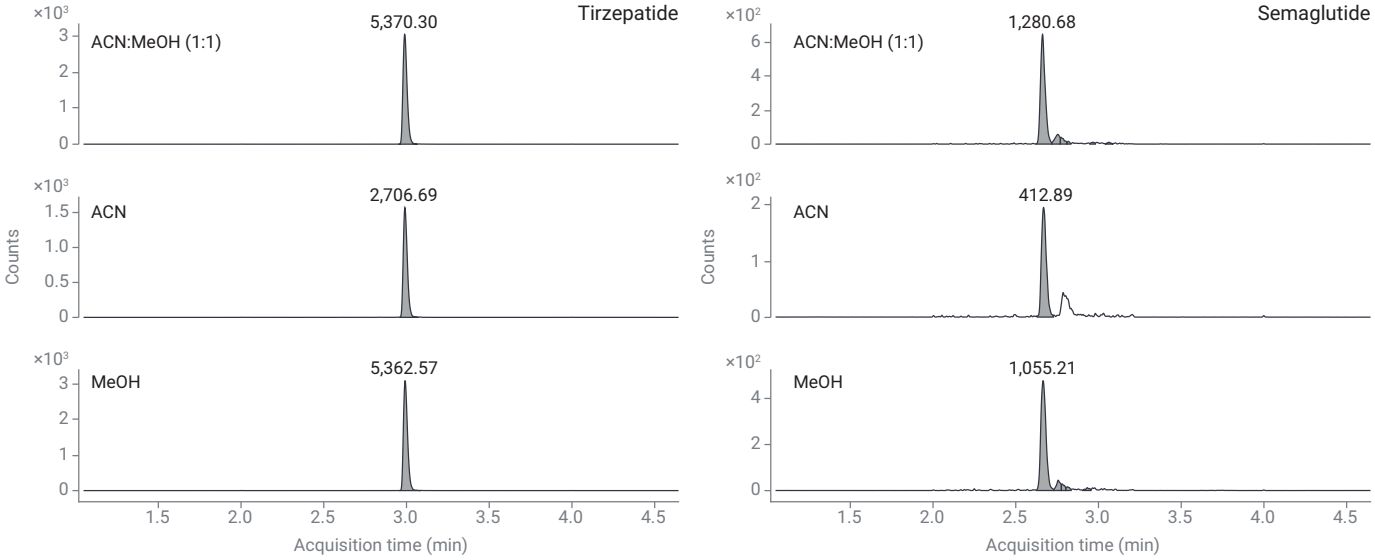

Figure 2. Comparison of organic solvents. Left side is tirzepatide MRM (1,204.4 & 396.2) response after organic solvent precipitation, and right side is semaglutide MRM (1,029.4 & 1238.1) response after organic solvent precipitation.

LC and MS conditions were all optimized, and the peptides' MRM transitions and collision energies were also optimized to achieve the best MS sensitivity for peptide quantitative analysis. The optimized source parameters are listed in Table 2.

Quantitative analysis of semaglutide in human plasma

MassHunter Quantitative Analysis software was used to perform quantitative analysis of calibration curve and QC samples. Overlap chromatograms of blank human plasma and semaglutide low limit of quantification (LLOQ) of 0.2 ng/mL in human plasma are shown in Figure 3A.

Figure 3B shows the overlap chromatograms of protein precipitation alone and further purification using AssayMAP RP-S cartridges, which shows that the AssayMAP RP-S cartridges increased assay sensitivity by five fold. The calibration curve was linear up to 1,000 ng/mL with a linear fit and $1/x^2$ weight, as shown in Figure 3C. Table 3 shows that all calibration points' precision and accuracy are with ± 15% acceptance criteria, demonstrating excellent assay performance.

The intra- and interday analytical precision and accuracy of QC samples were determined from three independent runs performed over three days. The precision and accuracy results for semaglutide in human plasma are shown in Table 4. All levels of QC samples (n = 4) met acceptance criteria of 15%, as recommended by the regulatory agency. The results demonstrated excellent assay performance using automated sample preparation.

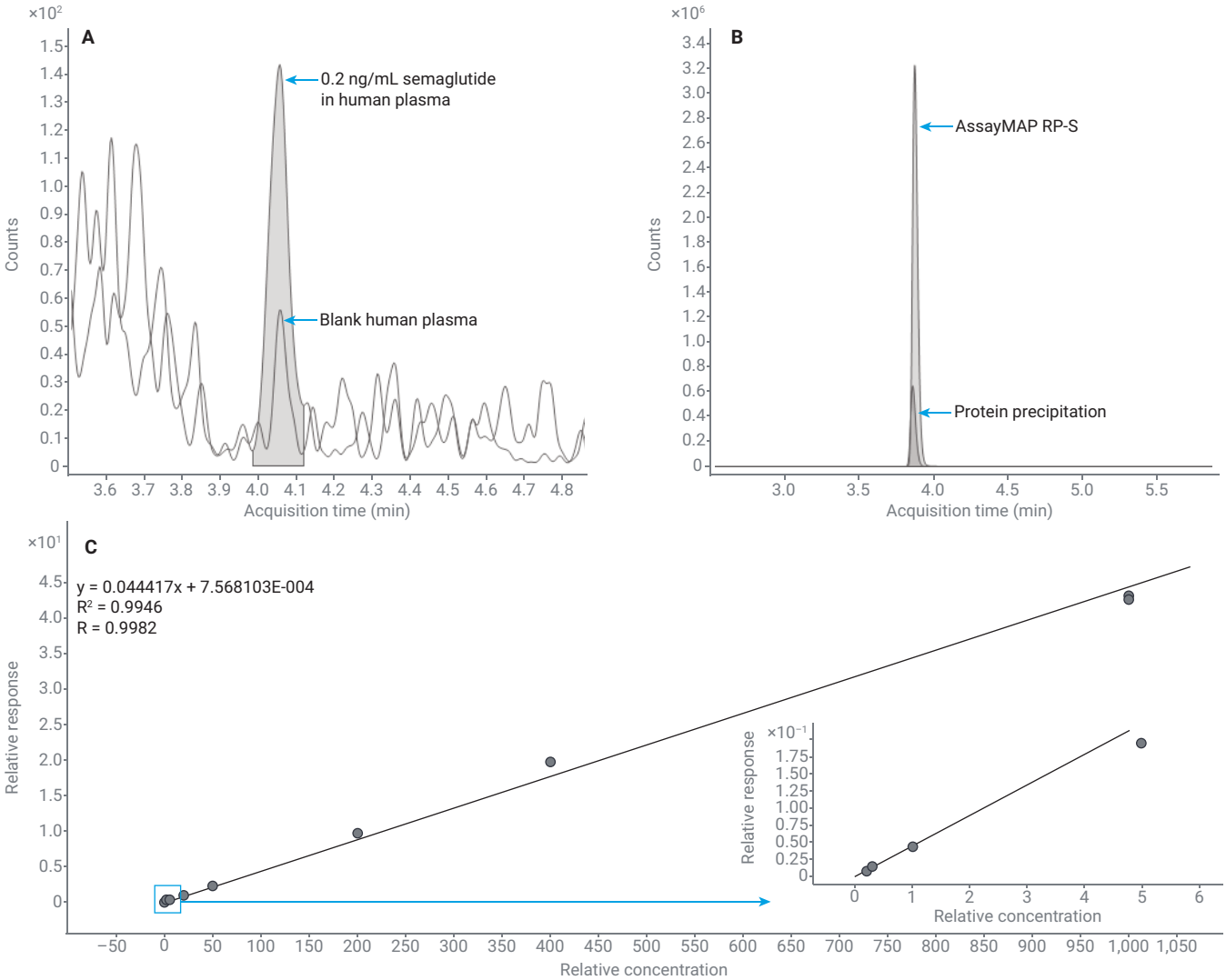

Figure 3. (A) Overlap semaglutide MRM (1,029.4 & 1,238.1) chromatograms of blank human plasma and lowest calibration point. (B) Overlap semaglutide MRM (1,029.4 & 1,238.1) chromatograms of protein precipitation and Agilent AssayMAP RP-S cartridge purification for the same concentration of semaglutide. (C) Calibration curve of semaglutide in human plasma from 0.2 to 1,000 ng/mL. The insert is a zoomed-in snapshot of the lowest four calibration points.

Table 4. Semaglutide calibration curve (n = 3) results summary.

	Concentration (ng/mL)								
	0.2	**0.3**	**1**	**5**	**20**	**50**	**200**	**400**	**1,000**
Run 1	0.223	0.303	0.864	4.250	20.695	49.013	206.798	424.726	985.505
Run 2	0.186	0.279	0.975	4.385	19.75	51.588	216.095	444.826	967.528
Run 3	0.201	0.327	0.916	4.976	18.69	50.559	213.597	434.43	995.441
Mean	0.2034	0.303	0.918	4.537	19.712	50.387	212.163	434.661	982.825
% Bias	1.71	1.00	−8.17	−9.26	−1.44	0.77	6.08	8.67	−1.72
% CV	9.21	7.92	6.05	8.51	5.09	2.57	2.27	2.31	1.44

Table 5. Semaglutide QC samples' (n = 4) precision and accuracy over three runs..

		QC Concentration (ng/mL)			
		0.2 (LLOQ)	**0.5 (Low QC)**	**10.00 (Mid QC)**	**800 (High QC)**
Run 1	Mean	0.197	0.486	8.813	809.632
	% Bias	−1.3	−2.8	−11.9	1.2
	% CV	6.8	4.5	1.7	2.1
Run 2	Mean	0.206	0.484	10.358	821.419
	% Bias	3.1	−3.2	3.6	2.7
	% CV	2.3	5.2	3.0	7.2
Run 3	Mean	0.192	0.520	9.378	871.725
	% Bias	−4.2	3.9	−6.2	9.0
	% CV	7.1	7.3	9.7	3.7
Interday	Mean	0.198	0.497	9.516	834.259
	% Bias	−0.8	−0.7	−4.8	4.3
	% CV	6.7	6.3	8.8	5.5

Conclusion

The developed GLP-1 peptide quantification workflow, combining automation, the Agilent 1290 Infinity II bio LC, and Agilent 6495D LC/TQ system, is an ideal platform for GLP-1 peptide quantitative analysis in biological matrices. Agilent AssayMAP RP-S automated sample cleanup was shown to increase assay sensitivity by five times compared to organic solvent protein precipitation. The developed workflow for semaglutide quantification in human plasma achieved LLOQ of 0.2 ng/mL and was linear up to 1,000 ng/mL from 100 µL starting material, covering a dynamic range of 3.7 order of magnitude. These results are similar or better than reports in the literature. In three individual qualification runs, intra- and interday QC samples' precision and accuracy all met regulatory acceptance criteria, demonstrating excellent assay performance and reproductivity. Another advantage of this workflow is that it is universal and can be applied to many other GLP-1 analogs. It also requires minimal method development time to greatly help drug discovery and development.

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Application Note

Biopharma/Pharma

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Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analog Tirzepatide in Plasma

Using an automated workflow with the Agilent 6495D triple quadrupole LC/MS

Authors

Xi Qiu, Steve Murphy, and David L. Wong

Agilent Technologies, Inc.

Abstract

Sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics are crucial for advancing glucagon-like peptide-1 (GLP-1) receptor agonist medications. This application note presents an automated workflow using LC/MS to quantify the GLP-1 analog tirzepatide in human plasma. Our results indicate that this workflow achieved an LLOQ of 0.05 ng/mL for tirzepatide by using 100 µL biological sample, and was linear up to 1,000 ng/mL. This workflow can be used for quantitative analysis of GLP-1 therapeutics without the need for specific antibodies. It also provides excellent sensitivity, high specificity, and fast method development, which all play a crucial role in drug discovery and development.

Introduction

In recent years, the research and application of GLP-1 receptor agonists have significantly increased due to their effectiveness in treating obesity and type 2 diabetes. The global market for GLP-1 analog is projected to grow rapidly, reaching $140 billion by change to 2030. Developing sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics is crucial for advancing these medications. Liquid chromatography/mass spectrometry (LC/MS) has emerged as an alternative method for analyzing these large molecules due to its high specificity, sensitivity, wide dynamic range, and rapid method development. Additionally, LC/MS can avoid cross-reactivity, enhance productivity, and reduce costs and delays associated with reagent and antigen availability.

Among the notable advancements is the peptide tirzepatide, which is sold under the brand names "Zepbound" for weight loss and "Mounjaro" for diabetes.[1,2] Traditionally, the plasma concentration of tirzepatide is measured by a ligand-binding assay, which requires time to develop antibodies and can lack selectivity and specificity.

In this application note, we present an automated workflow with LC/MS technologies to quantify GLP-1 analog drugs in human plasma using an Agilent 1290 Infinity II bio LC and an Agilent 6495D triple quadrupole LC/MS (LC/TQ) system (Figure 1).

Figure 1. Agilent AssayMAP Bravo protein sample prep platform, 1290 Infinity II bio LC, and 6495D LC/TQ.

Experimental

Materials and methods

Tirzepatide, semaglutide, formic acid (FA), and ammonium formate were purchased from MilliporeSigma (St. Louis, MO, U.S.). Acetonitrile (ACN) and methanol (MeOH) were purchased from Honeywell (Charlotte, NC, U.S.). The 96-well LoBind plates and protein LoBind tubes were purchased from Eppendorf (Hauppauge, NY, U.S.). Also used were Agilent AssayMAP RP-S cartridges.

Instrumentation

The following instrumentation was used:

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- Agilent 1290 Infinity II bio LC system including:
- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116A) equipped with a standard flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495D LC/TQ (G6495DA)

Software

The following software was used:

- Agilent MassHunter Acquisition software (version 12.0)
- Agilent MassHunter Quantitative Analysis software (version 12.0)

Sample preparation

Protein precipitation: A mixture of 150 µL ACN and 150 µL MeOH was added to an aliquot of 100 µL human plasma fortified with different concentration of semaglutide. Also, 5 µL of 1,000 ng/mL tirzepatide was used as the internal standard. The mixture was vortexed for 2 minutes and then spun down at 17,000 g for 10 minutes. A 350 µL volume of each supernatant was transferred to a 96-well plate and dried down under nitrogen gas with heating. Then, 110 µL water was added to each well to reconstitute. The solution was further purified using AssayMAP RP-S cartridges.

AssayMAP automated purification: AssayMAP RP-S cartridges were conditioned with 80% ACN and equilibrated with water. Then, 100 µL of the previously mentioned reconstituted samples were loaded onto AssayMAP RP-S cartridges at 3 µL/min, and cartridges were then washed once with water and once with 10% ACN in 10 mM ammonium formate buffer. The final elution step was carried out by loading 30 µL of 5% FA in 80% ACN to the cartridge at 3 µL/min. Lastly, 30 µL water with 5% FA was added to the final elution and 5 µL was injected into LC/MS for analysis.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II bio UHPLC coupled to an Agilent 6495D LC/TQ with the Agilent Jet Stream Electrospray ion source. Separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization of semaglutide yielded $[M + 4H]^{4+}$ signal at ***m/z*** 1,029.4 as the most intense ion. MRM transitions were optimized and 1,029.4 ➔ 1,238.1 was chosen as the quantifier and 1,029.4 ➔ 136.1 was chosen as the qualifier, and the tirzepatide MRM transition (1,204.4 ➔ 396.2) was chosen. Both peptides' MRM transitions are listed in Table 3 with optimal collision energies.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping 120 Å, 2.1 × 150 mm, 2.7 µM (p/n 653750-902)
Column Temperature	80 °C
Injection Volume	5 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50:50 water:methanol)
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow Rate	0.5 mL/min
Gradient Program	Time %B 0 30 3.0 45 5.0 60 5.1 95 8.0 95 8.2 30 10.0 30
Stop Time	10.0 min

Data processing

All MS data were processed using Agilent MassHunter Quantitative Analysis software.

Table 2. MS acquisition parameters.

Parameter	Value
Ion Mode	Positive
Gas Temperature	290 °C
Drying Gas Flow	15 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	6,000 V
Nozzle Voltage	2,000 V

Table 3. Semaglutide and tirzepatide MRM transitions.

Peptide	Precursor Ion	Product Ion	Collision Energy
Tirzepatide	1,204.4	396.2	26
Semaglutide	1,204.4	299.2	65
Semaglutide	1,029.4	1,238.1	30

Results and discussion

Method optimization for GLP-1 analog peptide quantitative analysis

To improve the sensitivity and reproducibility for GLP-1 peptide quantitative analysis, all the sample preparation steps were evaluated to achieve the best results.

1. Organic solvent composition to precipitate proteins was evaluated, including just ACN, just MeOH, and ACN:MeOH (1:1). The ACN:MeOH (1:1) was found to be the best for semaglutide and tirzepatide extraction (Figure 2).
2. Loading buffer was evaluated, including pure water, and water with 0.1% FA and 10%, 20%, or 40% ACN. Pure water was found to be the best loading buffer for GLP-1 peptides.
3. Washing buffer was evaluated, including pure water, water with 0.1% FA, water with 1% FA, and water with 10 mM NH_4COOH pH 10 buffer with or without 10% ACN. The combination of water and 10% ACN in 10 mM NH4COOH pH 10 buffer was found to be the best washing solution.
4. Elution buffers were evaluated, including 10 mM NH_4COOH pH 10 buffer with 30% or 80% ACN, and 80% ACN with 0.1%, 1%, and 5% FA. The 5% FA in 80% ACN produced the best results.

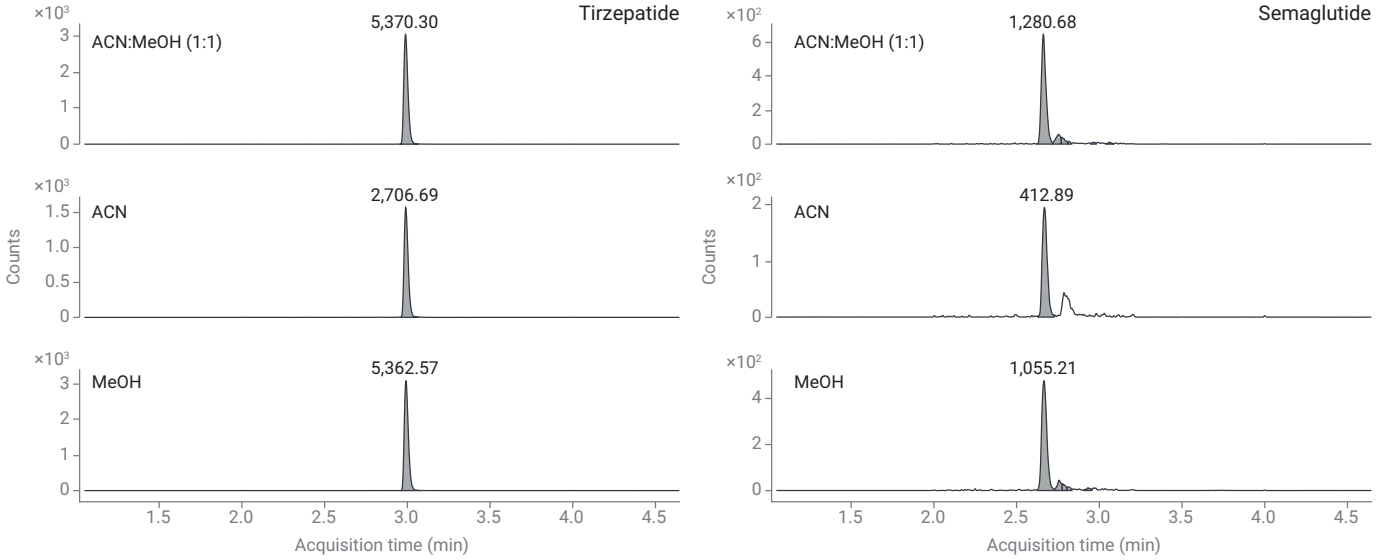

Figure 2. Comparison of organic solvents. Left side is tirzepatide MRM (1,204.4 & 396.2) response after organic solvent precipitation, and right side is semaglutide MRM (1,029.4 & 1238.1) response after organic solvent precipitation.

LC and MS conditions were all optimized, and the peptides' MRM transitions and collision energies were also optimized to achieve the best MS sensitivity for peptide quantitative analysis. The optimized source parameters are listed in Table 2.

Quantitative analysis of tirzepatide in human plasma

MassHunter Quantitative Analysis software was used to perform quantitative analysis of calibration curve and QC samples. Blank human plasma and tirzepatide had a low limit of quantification (LLOQ) of 0.05 ng/mL, as shown in Figure 3A. The calibration curve was linear up to 1,000 ng/mL with linear fit and $1/x^2$ weight, as shown in Figure 3B. Table 4 shows that all calibration points' precision and accuracy are with ± 15% acceptance criteria, demonstrating excellent assay performance.

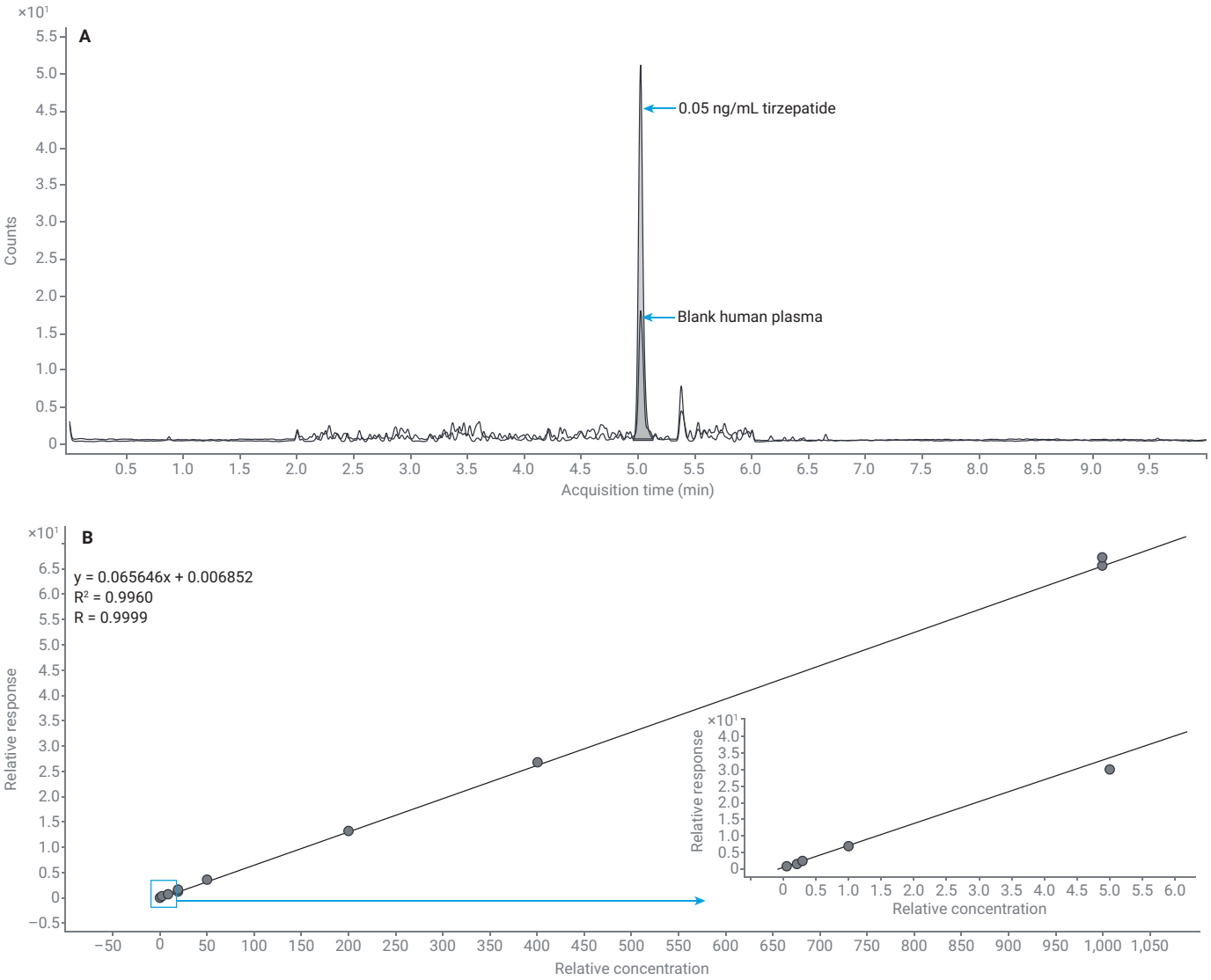

Figure 3. (A) Overlayed tirzepatide MRM (1,204.4 & 396.2) chromatograms of blank human plasma and lowest calibration point. (B) Calibration curve of tirzepatide in human plasma from 0.05 to 1,000 ng/mL. The insert is a zoomed-in snapshot of the lowest five calibration points.

Table 4. Tirzepatide calibration curve (n = 3) results summary.

	Calibration (ng/mL)									
	0.05	**0.2**	**0.3**	**1**	**10**	**20**	**50**	**200**	**400**	**1,000**
Run 1	0.0486	0.183	0.297	1.000	10.324	20.601	51.183	200.032	405.977	1,022.503
Run 2	0.0510	0.179	0.304	0.949	10.326	21.343	50.758	188.363	396.803	941.911
Run 3	0.0512	0.205	0.302	1.109	10.179	19.918	52.995	193.679	385.151	929.260
Mean	0.0503	0.189	0.301	1.019	10.276	20.621	51.645	194.025	395.977	964.558
% Bias	0.51	−5.51	0.36	1.93	2.76	3.10	3.29	−2.99	−1.01	−3.54
% CV	2.92	7.41	1.15	8.02	0.82	3.46	2.30	3.01	2.64	5.24

Table 5. Tirzepatide QC samples' (n = 4) precision and accuracy over three runs.

		QC Concentration (ng/mL)			
		0.05 (LLOQ)	**0.1 (Low QC)**	**5.00 (Mid QC)**	**800 (High QC)**
Run 1	Mean	0.0507	0.0915	5.0159	794.3824
	% Bias	1.3	−8.5	0.3	−0.7
	% CV	9.2	5.3	2.2	1.0
Run 2	Mean	0.0506	0.0997	4.8651	821.4187
	% Bias	1.3	−0.3	−2.7	2.7
	% CV	5.1	5.2	3.2	7.2
Run 3	Mean	0.0518	0.0952	5.350	723.432
	% Bias	3.7	−4.8	7.0	−9.6
	% CV	12.0	8.8	6.5	0.4
Interday	Mean	0.0511	0.0955	5.077	779.744
	% Bias	2.1	−4.5	1.5	−2.5
	% CV	8.5	7.0	5.8	6.8

The intra and interday analytical precision and accuracy of QC samples were determined from three independent runs performed over three days. The precision and accuracy results for tirzepatide in human plasma are shown in Table 4. All levels of QC samples (n = 4) met acceptance criteria of 15%, as recommended by the regulatory agency. The results demonstrate excellent assay performance using automated sample preparation.

Conclusion

The developed GLP-1 peptide quantification workflow, combining automation, the Agilent 1290 Infinity II bio LC, and Agilent 6495D LC/TQ, is an ideal platform for GLP-1 peptide quantitative analysis in biological matrices. The automated LC/MS workflow combines the advantages of both systems and provides excellent assay sensitivity and reproducibility. This workflow achieved an LLOQ of 0.05 ng/mL for tirzepatide by using 100 µL biological sample, and was linear up to 1,000 ng/mL, covering a 4.3 order of magnitude of dynamic range. In three individual qualification runs, the QC samples in all levels met regulatory agency requirements with excellent accuracy and precision. Another advantage of this workflow is that it is universal and can be applied to many other GLP-1 analogs. It also requires minimal method development time to greatly help drug discovery and development.

Reference

1. Mounjaro- tirzepatide injection, solution. DailyMed. 13 May 2022.
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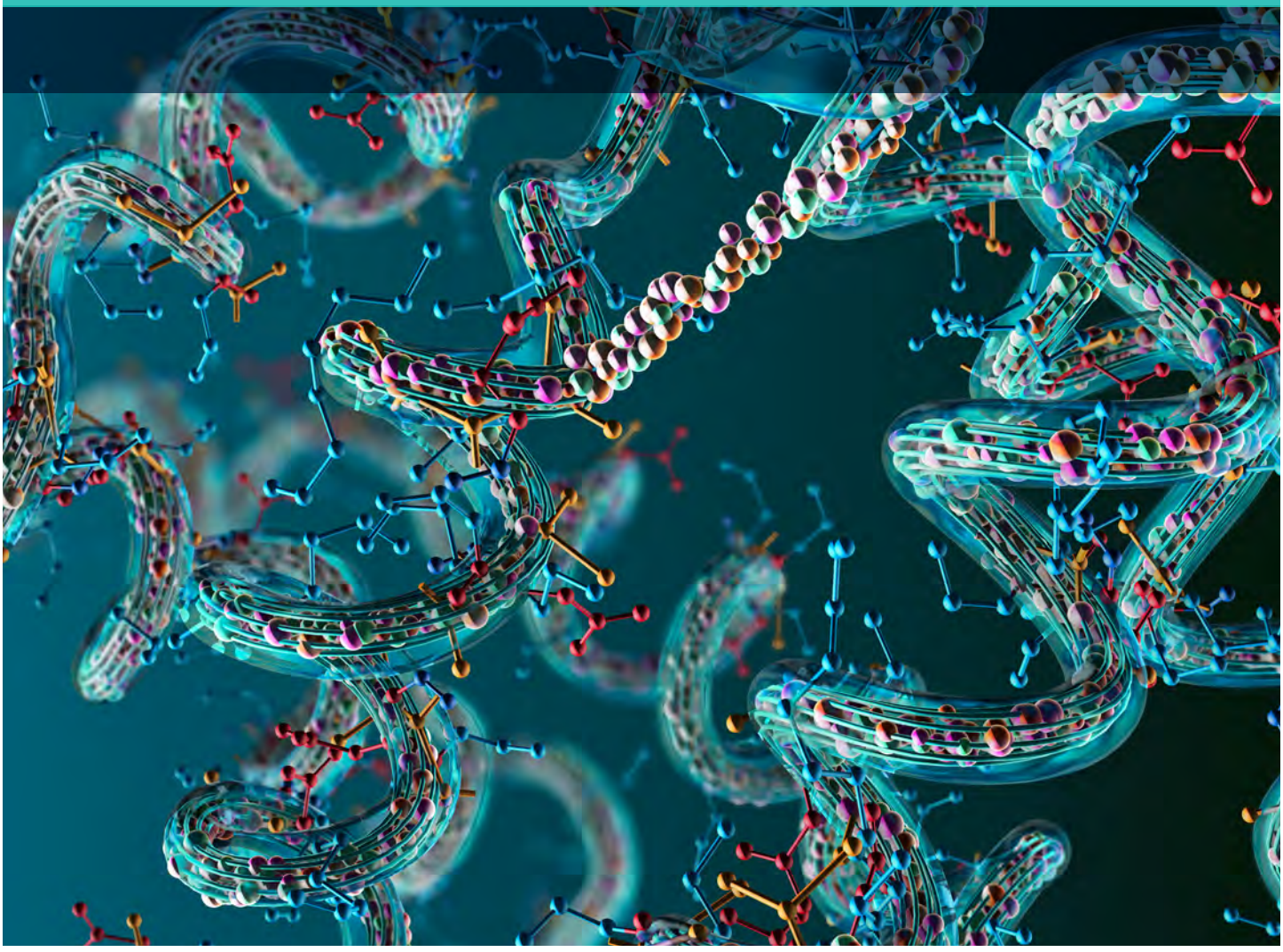
Chapter 4

Stability Testing Studies



Application Note

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Enhanced Peptide Characterization and Stability Assessment

UV-visible second-derivative spectroscopy on an Agilent Cary 3500 Multicell UV-Vis Spectrophotometer

Authors

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Agilent Technologies, Inc.

Abstract

Monitoring the stability of peptide therapeutics is vital in biopharmaceutical development. Traditional ultraviolet-visible (UV-Vis) absorption spectroscopy techniques commonly used in stability studies often do not provide sufficient wavelength resolution, especially for biological molecules such as proteins and DNA which typically have broad peaks. This makes it challenging to detect subtle structural changes, particularly when spectral features overlap. This application note demonstrates the benefits of using UV-Vis second-derivative spectroscopy with the Agilent Cary Multicell 3500 UV-Vis Spectrophotometer to improve peptide characterization and stability assessment. The advanced features of the Cary 3500 Multicell UV-Vis Spectrophotometer, including multicell measurement and built-in derivative calculation, streamline data collection and analysis, making it a valuable tool for peptide stability studies in biopharmaceutical applications.

Introduction

Synthetic peptides are a growing class of biotherapeutics. Glucagon-like peptide-1 (GLP-1) agonists are synthetic peptides that mimic natural GLP-1 hormone and play an important role in metabolic regulation and help in both insulin secretion and the promotion of weight loss.[1] Because the GLP-1 molecule is prone to various degradation pathways, assessing the quality and stability of GLP-1-based therapeutics is crucial to ensuring their effectiveness.

Peptide stability is commonly measured by observing changes in the absorbance spectra produced by ultraviolet spectroscopy.[2] Because it is non-destructive, the technique offers significant workflow advantages. For example, valuable samples remain intact after analysis and can be subsequently analyzed by complementary techniques such as liquid chromatography-mass spectrometry. Additionally, UV-Vis spectroscopy is fast and easy, making it accessible to a wide range of users.

However, conventional UV-Vis absorption spectra can be difficult to interpret when monitoring for subtle changes such as small shifts in peak positions or minor intensity variations, especially for biomolecules exposed to environmental factors like oxidation stress. Poor resolution of spectral features is also a challenge when biomolecule spectra consist of multiple overlapping components. Derivative spectroscopy is a powerful technique that addresses these limitations by enhancing subtle features, allowing resolution of overlapping peaks that would be indistinguishable in a traditional absorption spectrum.

The Agilent 3500 Multicell UV-Vis spectrophotometer with its multicell module is a powerful tool for biomolecule analysis using second-derivative measurements. The instrument allows simultaneous measurement of multiple samples — including standards, samples, and controls — under the same conditions. The Agilent Cary UV Workstation software that controls the Cary 3500 Multicell UV-Vis spectrophotometer includes a built-in equation function that automatically produces second-derivative spectra after absorbance measurement to minimize calculation error and streamline data processing.

This application note demonstrates use of the advanced capabilities of the Cary 3500 Multicell UV-Vis Spectrophotometer and UV-Vis second-derivative spectroscopy to improve the resolution of overlapping absorption peaks collected from peptides and aromatic amino acids.

Experimental

Materials

Tryptophan (W), tyrosine (Y), phenylalanine (F), α-melanocyte-stimulating hormone (MSH), melittin, liraglutide, angiotensin II, 30% (v/v) hydrogen peroxide (H_2O_2), and sodium phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

All peptide samples including tryptophan (0.15 mM), tyrosine (0.5 mM), and phenylalanine (4.0 mM) were prepared in sodium phosphate buffer, pH 7.4, at a concentration of 1 mg/mL. For the stress conditions, the peptides were incubated with 0.07% H_2O_2 overnight at room temperature. No other sample preparation before UV-Vis analysis was required.

Instrumentation

Data acquisition was performed by the Cary 3500 Multicell UV-Vis Spectrophotometer equipped with the Cary Workstation software using the parameters listed in Table 1. The multicell design allows for simultaneous measurement of up to seven samples along with a reference sample. Measurements were carried out using an ultra-microvolume rectangular cuvette with a path length of 10 mm (Agilent part number 5062-2496). Spectra were averaged from multiple runs (n = 5) to ensure reproducibility and reduce noise.

Table 1. Agilent Cary 3500 Multicell UV-Vis Spectrophotometer method parameters.

Parameter	Value
Collection Mode	Scan
Scan Range	200 to 500 nm
Averaging Time	0.02 s
Data Interval	1.00 nm
Scan Rate	3,000 nm/min
Spectral Bandwidth	2.00 nm
Derivative	Second derivative
Smoothing Filter	9

Results and discussion

UV-visible spectra of aromatic amino acids

Tryptophan, phenylalanine, and tyrosine are aromatic amino acids that are commonly found in peptides and proteins and have characteristic UV-Visible absorption profiles. Figure 1A shows the UV-Vis zero-order spectra of tryptophan, phenylalanine, and tyrosine. The overlapping peaks in the zero-order spectra make it difficult to distinguish subtle spectral features in the amino acids.

To enhance the resolution and accuracy of the absorption spectra, second-order derivative spectra were analyzed based on the Savitzky-Golay method. Figures 1B, 1C, and 1D show the second-order derivative UV-Vis spectra of tyrosine, phenylalanine, and tryptophan. After the sample measurements were acquired, the second-order spectra were automatically generated using the built-in calculation function in the Cary UV Workstation software. The second-order derivative spectra display peaks with maxima and minima (points of inflection) which can be used to distinguish the different spectral absorbance bands of the various aromatic amino acids, enabling study of the minor spectral changes that occur when biomolecules experience stress conditions. The absorption maxima of the amino acids were:.

- **Tryptophan:** Absorption maximum at approximately 273, 280, and 288 nm
- **Tyrosine:** Absorption maximum at approximately 275 and 282 nm
- **Phenylalanine:** Absorption maximum at approximately 246, 252, 257, 264, and 268 nm

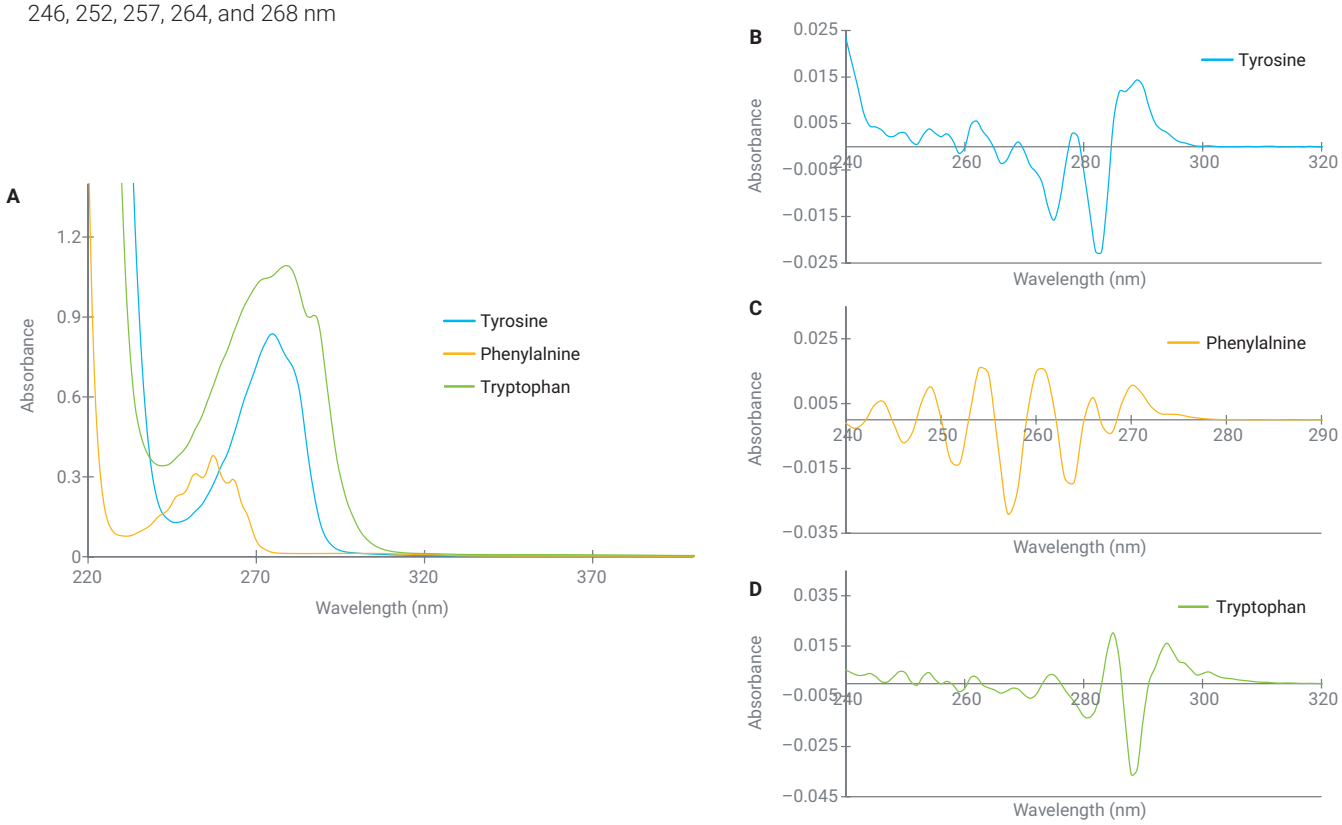

Figure 1. UV-Vis absorption spectra of aromatic amino acids. (A) zero-order and (B, C, and D) second-order derivative UV-Vis absorption spectra.

UV-visible spectra of peptides

To generate different absorption spectra for comparison, the peptides selected for study had varying proportions of aromatic amino acids. Figure 2 shows the second-order derivative absorption spectra of the four peptides melittin, MSH, angiotensin II, and liraglutide. The peak locations in the second-order derivative spectra correlated well with the known absorption features of their constituent aromatic amino acids. Overall, the second-order derivative spectra provided a more resolved view of the peptides' spectral characteristics. The number of aromatic amino acids present in the peptides studied is summarized in Table 2. Melittin showed characteristic peaks at 291, 283, and 275 nm, which were primarily attributed to tryptophan. MSH and liraglutide had peaks at 290, 283, and 266 nm, reflecting contributions from all three aromatic amino acids. Angiotensin II had peaks at 283, 276, 264, 258, and 252 nm, which was consistent with the presence of tyrosine and phenylalanine.

Table 2. Amino acid composition of peptides analyzed.

Peptides	Sequence	Number of Tryptophan (W)	Number of Tyrosine (Y)	Number of Phenylalanine (F)
Liraglutide	HAEGTFTSDVSSYLEGQAAKEEFIAWLVRGRG	1	1	1
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	1	0	0
α-Melanocyte-stimulating hormone (MSH)	SYSMEHFRWGKPV	1	1	1
Angiotensin II	DRVYIHPF	0	1	1
Substance P	RPKPQQFFGLM	0	0	2

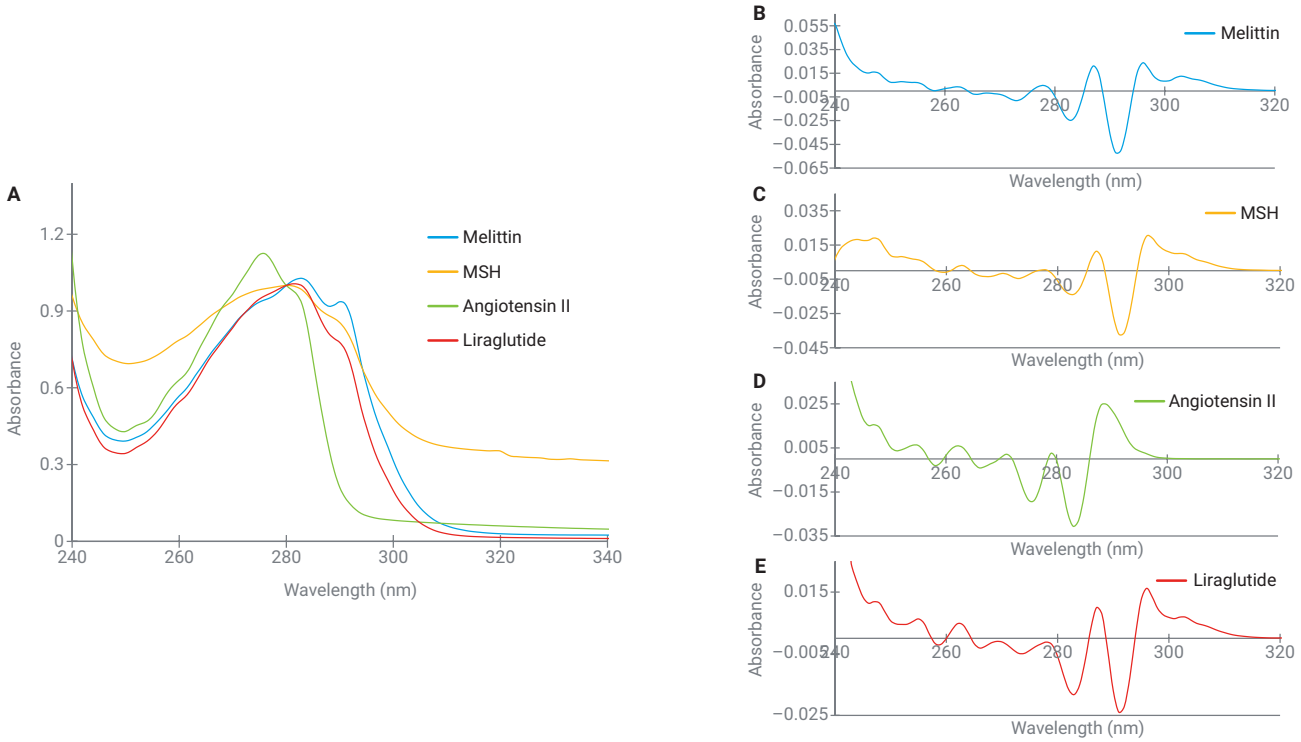

Figure 2. UV-Vis absorption spectra of peptides. (A) zero-order and (B, C, D, and E) second-order derivative UV-Vis absorption spectra.

Monitoring peptide stability under oxidation stress

To assess the utility of derivative spectroscopy for stability monitoring, the peptides were treated with H_2O_2. Figure 3 shows the comparison of second-order derivative UV-Vis spectra of untreated and H_2O_2 treated peptides. Minute changes in peptide wavelength absorbance due to H_2O_2 treatment were effectively captured in the second-order derivative spectra. Angiotensin II peptide lacks the tryptophan residue, so no significant change was observed around the 291 nm region after H_2O_2 treatment. Minimal changes were observed in melittin's second-order derivative spectrum, indicating it is less prone to oxidation. Tryptophan-containing MSH and liraglutide showed a noticeable loss in the magnitude of the 291 nm peak after H_2O_2 treatment. This indicates the compounds' susceptibility to oxidation, which primarily affects tryptophan residues, further demonstrating the specificity of the second-derivative method for detecting tryptophan oxidation. To further confirm the oxidation status of liraglutide, H2O2 treated samples were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). Figure 4 shows the total ion chromatogram (TIC) of H_2O_2 treated liraglutide. The deconvolved mass spectrum confirmed the oxidation forms showing mass shifts of +16 Da and +32 Da that correspond to mono and di oxidation products, respectively.[3]

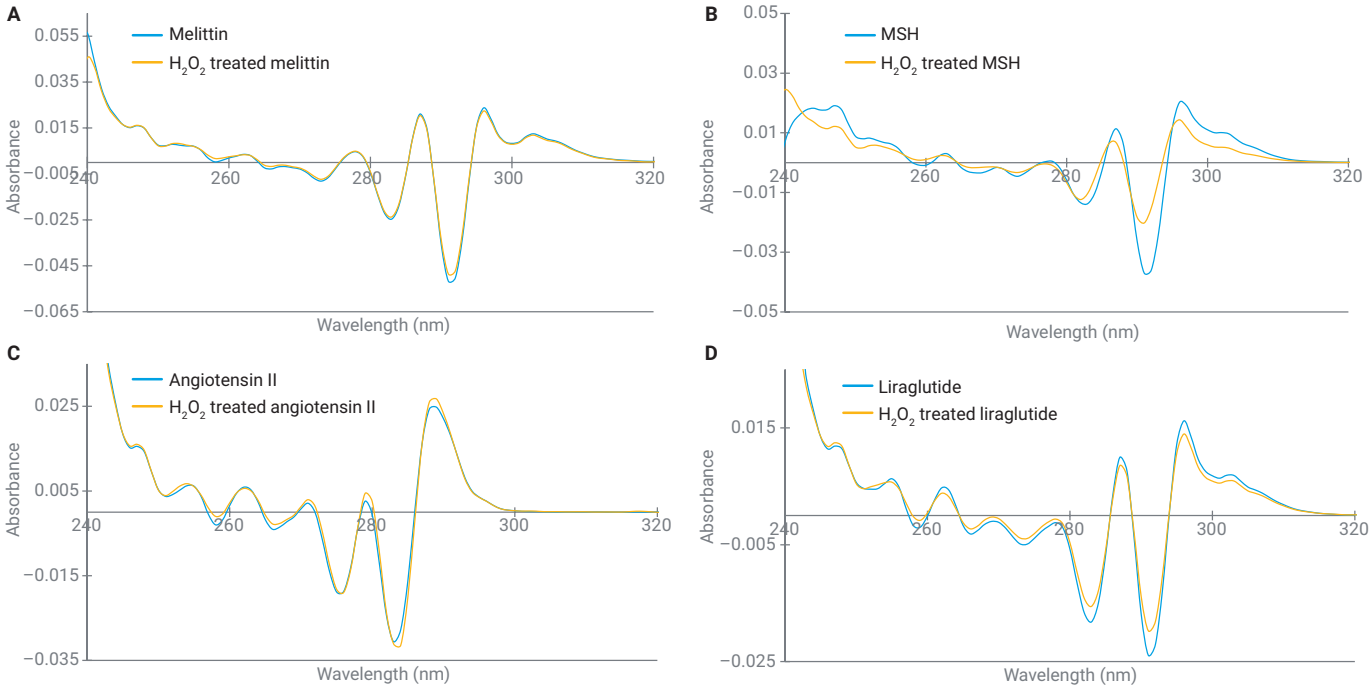

Figure 3. Second-order derivative UV-Vis absorption spectra of untreated and H_2O_2 treated peptides.

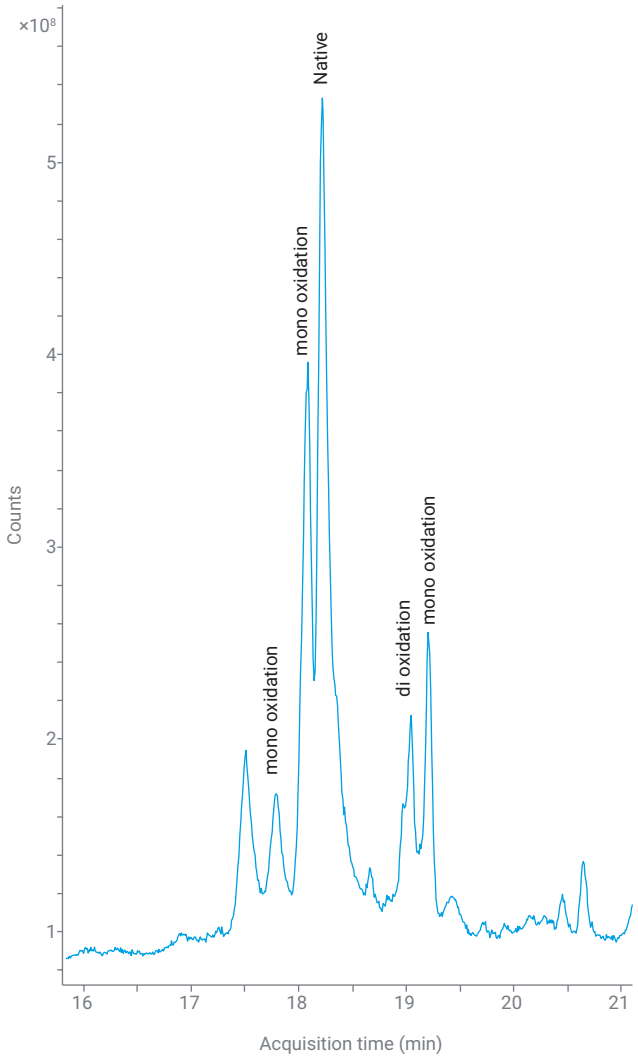

Figure 4. LC/MS total ion chromatogram of oxidated liraglutide.

Conclusion

The results of this study highlight the ability of UV-Vis second-derivative spectroscopy to precisely identify and monitor subtle spectral differences arising from changes in the aromatic residues in peptides, making it an invaluable tool for monitoring peptide stability. The Agilent Cary Multicell 3500 UV-Vis Spectrophotometer's multicell feature reduces measurement time by allowing simultaneous monitoring of eight samples. The built-in calculation feature streamlines analysis with automated calculations, making it easier for users to interpret data and make decisions. This capability is particularly valuable for biopharmaceutical analysis, where accurate and efficient monitoring of peptide stability is crucial for drug development and quality control. Non-destructive, UV-Vis spectroscopy preserves samples for subsequent analyses such as complementary LC-MS analysis. The Cary 3500 Multicell UV-Vis Spectrophotometer is an ideal choice for drug development and quality control (QC) testing laboratories, where speed, ease of use, and the ability to reuse valuable samples are paramount.

Reference

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Application Note

Biopharma

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Impurity Profiling of Tirzepatide Under Stress Conditions

Using Agilent Pro iQ Plus

Authors

Mahsan Miladi, PhD

Agilent Technologies, Inc.

Abstract

The increased demand and use of glucagon-like peptide-1 (GLP-1) agonists prompts a need for robust analytical methods to screen for impurities that may impact their safety and efficacy. Synthetic GLP-1 peptides are susceptible to degradation through pathways such as oxidation under stress conditions.[1] In this study, a high-performance liquid chromatography (HPLC) system coupled with an Agilent InfinityLab Pro iQ Plus mass detector was used to monitor tirzepatide impurities under various pH and storage conditions. This study demonstrates that a high-sensitivity single quadrupole LC/MS system can be used to streamline the detection and monitoring of low-level peptide impurities, making it suitable for implementation in routine quality control (QC) and quality assurance (QA) environments.

Introduction

Glucagon-like peptide-1 (GLP-1) agonists are a class of compounds utilized to treat type-2 diabetes mellitus (T2DM) and obesity. The function of GLP-1 agonists is to lower serum glucose levels and thereby manage metabolism in affected patients. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are both incretin hormones inactivated by dipeptidyl peptidase-4. These hormones stimulate insulin secretion after an oral glucose load through the incretin effect.[2,3] In T2DM, this process can become blunted or absent; however, pharmacological levels of GLP-1 can restore insulin secretion.

The US Food and Drug Administration (FDA) considers the types and amounts of impurities present in a proposed generic drug compared to its reference listed drug (RLD). According to the FDA, a proposed generic synthetic peptide should not contain (i) impurities at levels greater than those found in the RLD nor (ii) any new specified peptide-related impurity that is greater than 0.5% of the drug substance.[4]

Tirzepatide, a dual GIP and GLP-1 receptor agonist, offers promising therapeutic potential but presents analytical challenges due to its susceptibility to chemical degradation, including deamidation, oxidation, and peptide backbone cleavage. Monitoring such degradation pathways is essential for ensuring product integrity, safety, and efficacy.

The Agilent InfinityLab Pro iQ Plus single quadrupole mass spectrometer (MS) offers enhanced sensitivity and dynamic range for low-abundance (sub-ppm) impurity detection. The Agilent 1290 Infinity II LC system coupled with the Pro iQ Plus LC/MS instrument brings a cost-effective mass detection workflow for routine analysis to QC laboratories. This application note details the results from analyzing tirzepatide and its related impurities under varying pH and storage conditions.

Experimental

Chemicals and standards

- Formic acid, 98% (LC-MS grade LiChropur), Millipore Sigma (part number 5.33002)
- InfinityLab acetonitrile (LC/MS grade), 1 × 1 liters, Agilent (part number 5191-5101-001)
- Tirzepatide, AstaTech (part number AT40456)
- Ammonium formate (LC-MS grade LiChropur), Millipore Sigma (part number 70221)
- Ammonium hydroxide, for HPLC, 35% solution in water, Thermo Fisher Scientific (catalog number 460801000).

Sample preparation

Tirzepatide stock solution was prepared at 2 mg/mL in 15% acetonitrile/de-ionized water. Working solutions were diluted to 50 ng/µL in buffer solutions adjusted to pH 5, 7, and 9. Samples were injected directly without additional purification. Aliquots were stored in the autosampler at 5 °C and analyzed on different days after storage for up to seven days.

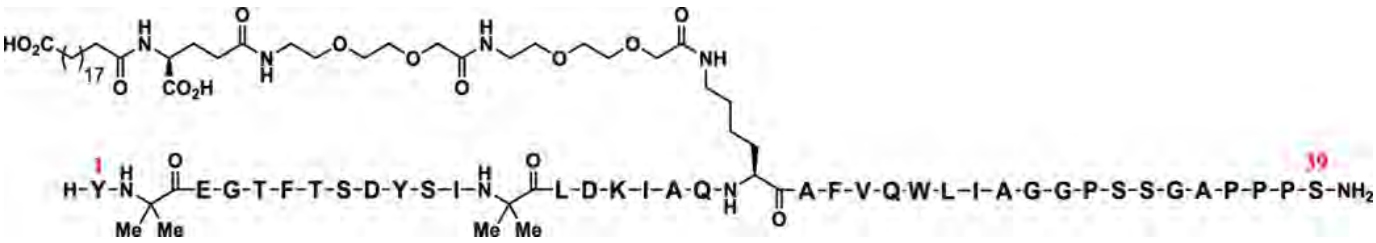

Figure 1. Tirzepatide amino acid composition.[5]

UV-visible spectra of peptides

LC/MS analysis was performed using the Agilent 1290 Infinity II bio LC system coupled with the Pro iQ Plus mass detector. Chromatographic separation was achieved on an Agilent ZORBAX RRHD 300 Å StableBond C18 column. The detailed LC and MS operating parameters are summarized in Tables 1 and 2.

Table 1. Liquid chromatography parameters.

1290 Infinity II Bio LC System	
Column	ZORBAX RRHD 300 Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)
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	Standard
Injection Volume	1 µL
Column Temperature	60 °C
Gradient Program	Time %B 0 20 5 48 10 58 11 60 12 80 14 80 14.1 20 15 20

Table 2. MS parameters.

Pro iQ Plus Single Quadrupole LC/MS	
Source	
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Gas Temperature	300 °C
Drying Gas Flow	11 L/min
Nebulizer	30 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Pro iQ Plus	
Fragmentor	95 V
Scan Type	Scan
Scan Time	500 ms
Data Storage	Profile
MS Spectrum Range	*m/z* 500–2,500

UV-visible spectra of peptides

The Pro iQ Plus LC/MS system was operated using Agilent OpenLab CDS 2.8 software, which includes built-in spectral deconvolution capabilities for processing GLP-1 peptide data. The deconvolution algorithm in OpenLab CDS is specifically optimized to simplify complex spectra generated from multiply charged ions, particularly those acquired using unit mass resolution instruments. Table 3 outlines the specific data processing parameters used in this study.

Table 3. Data processing parameters used in OpenLab CDS 2.8 for GLP-1 peptide analysis.

Parameter	Value
Spectrum Extraction Type	Peak apex spectrum
Background Mode	Spectrum at peak start and end
Use *m/z* Range	Disabled
Run Automatic Deconvolution	Enabled
Low Molecular Weight	500
High Molecular Weight	10,000
Maximum Charge	6
Minimum Peaks in Set	3
MW Agreement (0.01%)	5
Absolute Noise Threshold	1,000
Relative Abundance Threshold (%)	10
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	50

Results and discussion

Tirzepatide degradation products were highly dependent on pH and storage duration. The Pro iQ Plus single quadrupole LC/MS system successfully detected trace levels of impurity-related products.

Peptide analysis using OpenLab CDS

OpenLab CDS software provides a unified platform for peptide characterization and impurity assessment. Figure 2 shows the data review for tirzepatide after seven days of storage at 5 °C and pH 7. As shown in Figure 2, impurity peaks were detected at trace levels, eluting close to the main tirzepatide peak. It should be noted that accurate deconvolution relies on acquiring high-quality mass spectra. The Pro iQ Plus mass detector provides such high-quality mass spectra, even for trace impurities.

The software layout includes helpful tools such as chromatogram view, MS spectrum window, and spectral deconvolution results, allowing for efficient review of both the main peptide and any low-level impurities.

The total ion chromatogram (TIC) shows peaks corresponding to tirzepatide and its related impurity products. The mass spectrum at 7.89 minutes confirms the elution of tirzepatide, with the presence of +3, +4, and +5 charge states (Figure 2). The deconvoluted mass spectrum shows a molecular weight of 4,813.1 Da, consistent with the theoretical average mass of tirzepatide (4,813.5 Da) and within the expected mass accuracy (±0.3 Da) for the instrument.

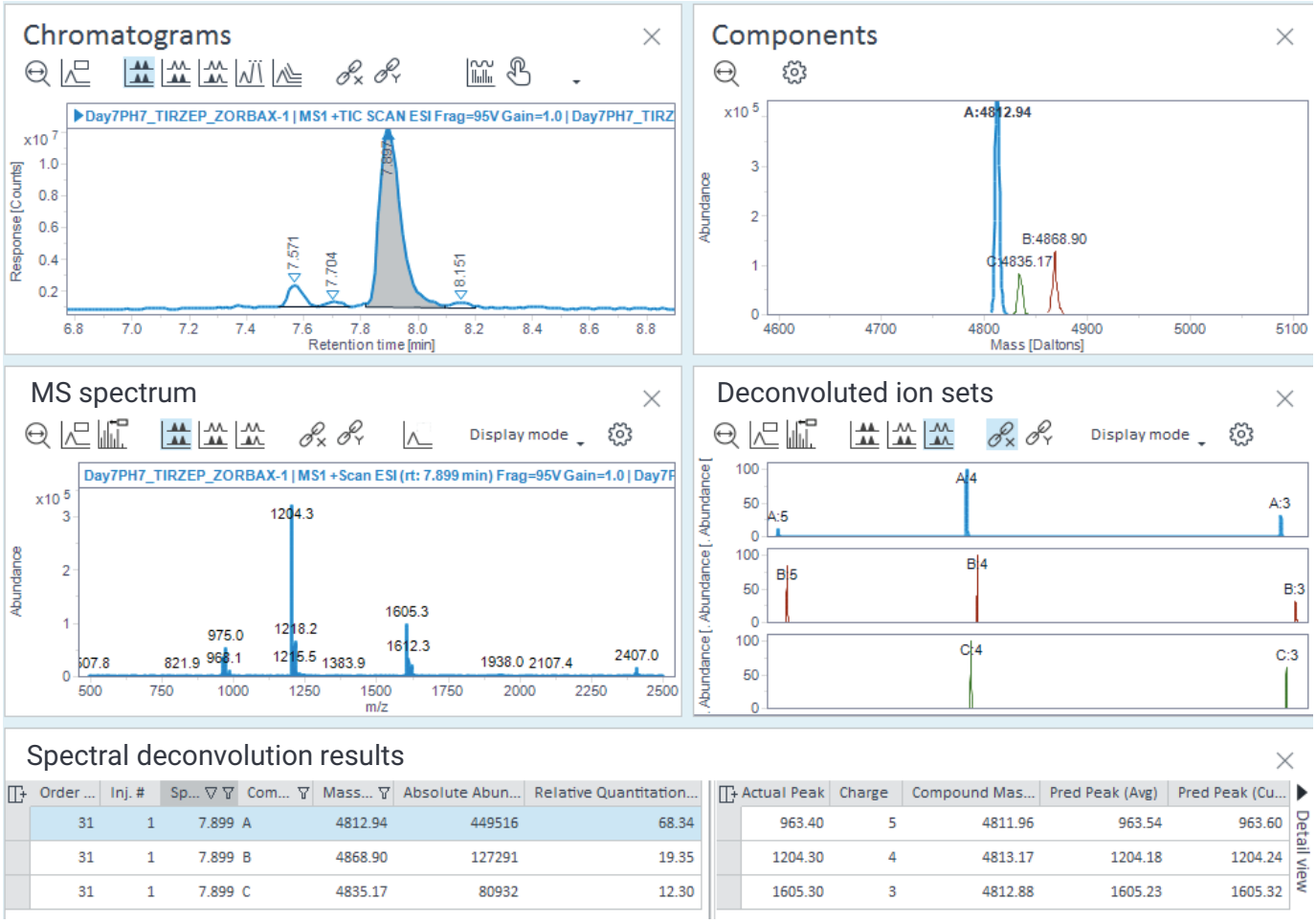

Figure 2. Data review for tirzepatide after seven days of storage at 5 °C and pH 7.

The Pro iQ Plus LC/MS system enables sensitive detection and characterization of low-level impurities in tirzepatide samples. Figure 3 shows the mass spectra of two impurities eluting at 7.57 and 7.71 minutes (as seen in Figure 2), with deconvoluted masses of 4,845.2 and 4,817.9 Da, respectively. These species correspond to mass shifts of +32 and +4.4 Da relative to the theoretical average mass of native tirzepatide (4,813.5 Da). The mass shifts are consistent with oxidative modifications, which are common degradation pathways for peptides under stress conditions. Amino acids such as methionine, tryptophan, and histidine are particularly susceptible to oxidation. Notably, tryptophan oxidation can lead to a +4 Da shift due to specific structural modifications (+O2–CO).[6]

Table 4 summarizes the deconvoluted molecular weights of tirzepatide and its related impurities, including oxidized forms and an unknown impurity.

In addition to the oxidation-related impurities, an unknown species was detected at a retention time of 8.15 minutes. The deconvoluted mass spectrum of this species indicates a molecular weight of ~ 4,689.6 Da, corresponding to a mass difference of +124 Da relative to native tirzepatide. Further MS characterization (that is, MS/MS) is required to identify this impurity.

The high sensitivity and dynamic range of the Pro iQ Plus LC/MS instrument allows for confident detection of both major and trace-level species in a single run. Importantly, the MS successfully detects impurities present at a relative peak area < 2%, highlighting the suitability of the Pro iQ Plus LC/MS for monitoring minor degradants in peptide samples. Furthermore, the integrated spectral deconvolution within OpenLab CDS streamlines data analysis by simplifying interpretation of multiply charged spectra, eliminating the need for external software.

Table 4. Deconvoluted molecular weights of tirzepatide and its related impurities.

GLP-1 Peptide	Native Peptide Molecular Weight (Da)	Impurity Product		
		Oxidation ($+O_2$)	Oxidation (O_2–CO)	Unknown Impurity
Tirzepatide	4,813.1	4,845.2	4,817.9	4,689.6

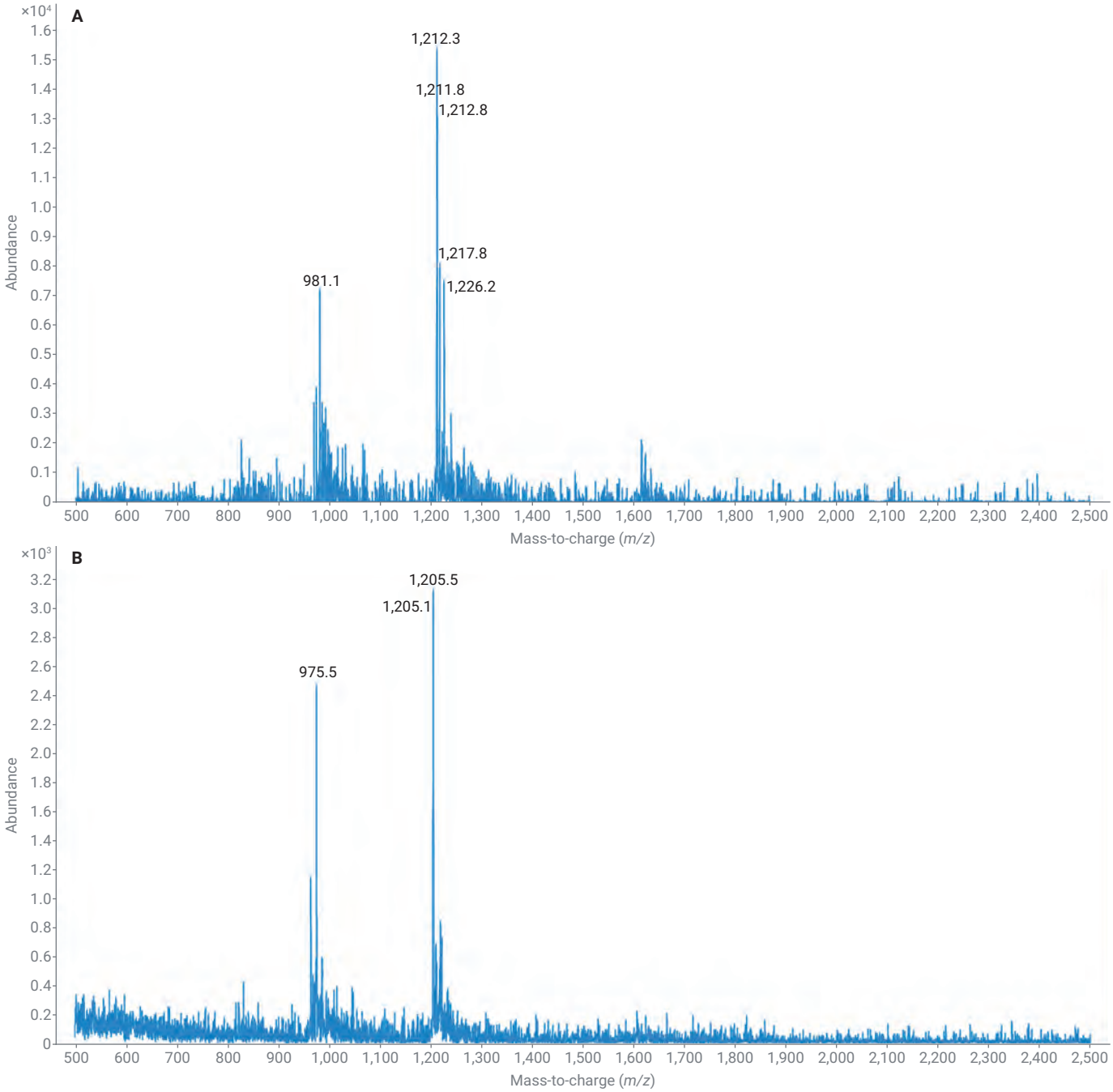

Figure 3. Mass spectra of tirzepatide impurities eluting at retention times 7.57 minutes (Panel A) and 7.71 minutes (Panel B). Mass spectral deconvolution reveals molecular weights of 4,845.2 Da for the impurity in Panel A and 4,817.9 Da for the impurity in Panel B.

UV-visible spectra of peptides

Proper storage and handling of GLP-1 medications are essential to preserve their stability, potency, and overall effectiveness. Inappropriate storage conditions can lead to degradation, reduced therapeutic efficacy, and potential safety risks. Therefore, evaluating how different storage durations and conditions influence the formation of degradation products is critical for ensuring product quality and patient safety.

Figure 4 shows temporal plots for the generation of the oxidation products of tirzepatide at various solution pH values. The data in Figure 4 suggest that tirzepatide is least stable at pH 5 and can undergo significant oxidation, even at 5 °C.

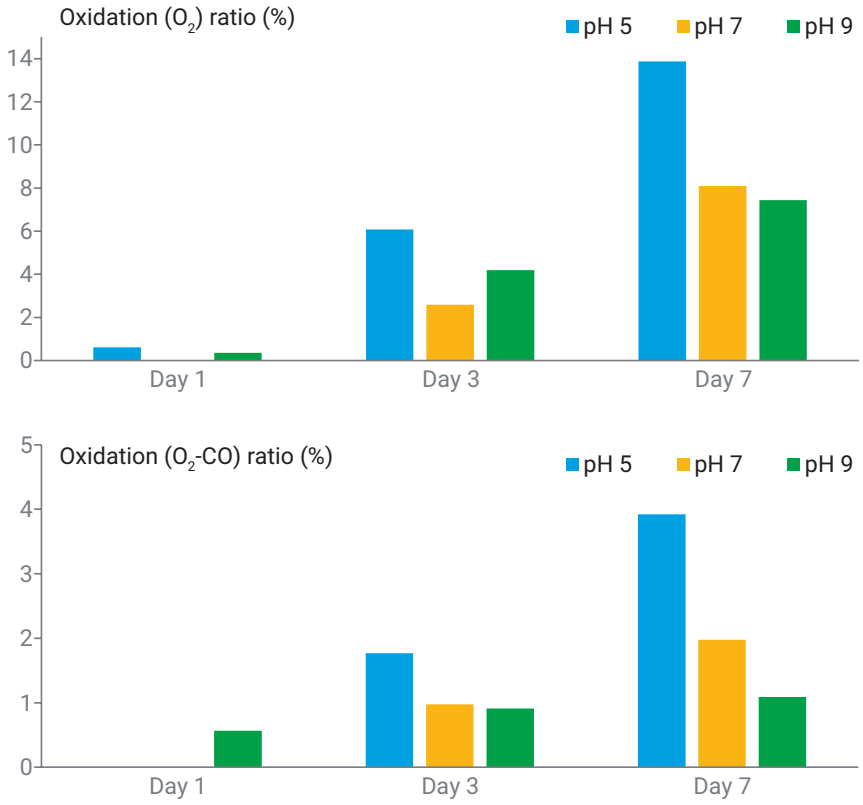

Figure 4. Oxidation ratio of tirzepatide related impurities over time under different pH conditions.

Conclusion

In conclusion, impurity profiling of tirzepatide under stress conditions using the Agilent Pro iQ Plus mass detector demonstrates the effectiveness of this analytical method for detecting and monitoring low-level peptide impurities. Notably, the method was able to detect impurities with relative peak areas less than 2%, showcasing its sensitivity for low-level impurity identification. The study highlights the susceptibility of tirzepatide to degradation through oxidation, particularly under varying pH and storage conditions. These findings underscore the importance of proper storage and handling to maintain the stability and efficacy of GLP-1 medications. The high sensitivity, cost-effectiveness, and simplicity of this single quadrupole LC/MS make it a practical and accessible tool for routine pharmaceutical QC and QA workflows.

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Application Note

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Integrated Structural and Functional Characterization of GLP-1 Analog (liraglutide) Using LC/MS and SPR

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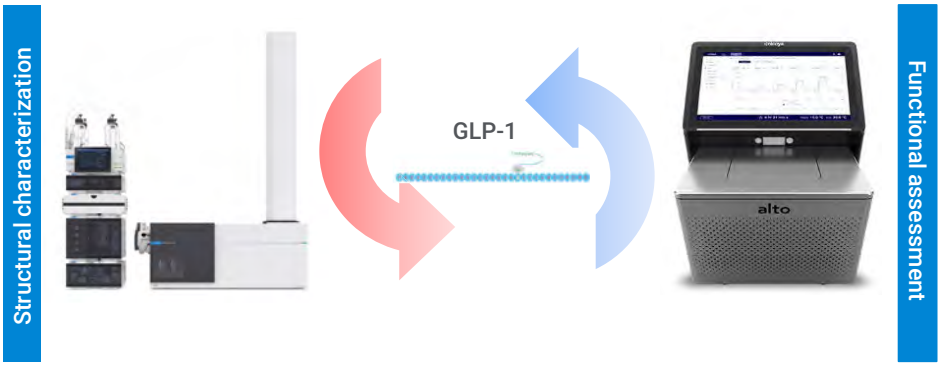
Abstract

Glucagon-like peptide 1 receptor agonists (GLP-1 RAs) are a rapidly expanding class of peptide therapeutics, transforming diabetes and obesity management and now recognized for their growing list of health benefits. As new chemical modifications are introduced into these peptides, early structural and functional characterization becomes essential. This application note demonstrates an integrated workflow using an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and a Nicoya digital surface plasmon resonance (digital SPR) system to link structural integrity with functional receptor binding (in some cases with unexpected results), demonstrating the importance of performing both assays. By combining orthogonal techniques, biopharma developers can correlate chemical modifications and degradation with biological activity, supporting stability, comparability, and impurity profiling studies. This workflow aligns with industry trends toward more comprehensive characterization strategies that accelerate development timelines.



Introduction

GLP-1 RAs have emerged as one of the most impactful classes of peptide therapeutics in modern medicine. Initially developed for glucose control in type 2 diabetes, GLP1 RAs are now widely recognized for their substantial benefits in weight management, cardiometabolic health, and organ-protective effects.[1] Their rapid adoption has accelerated innovative efforts, driving the development of next-generation analogs with extended half-lives, enhanced potency, and improved stability. As manufacturers explore new sequence variants, chemical modifications, and formulation strategies, the need for early and comprehensive characterization becomes essential. These peptides often incorporate lipidation, PEGylation, amino acid substitutions, or other structural changes intended to modulate pharmacokinetics and receptor engagement.2 However, such modifications can also influence peptide folding, degradation pathways, aggregation, or target binding in ways that are not always intuitive. Understanding how structural integrity translates into functional activity is therefore critical for guiding design decisions, optimizing stability, and ensuring product comparability throughout development.

This application note presents an integrated analytical workflow combining an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and a Nicoya Digital Surface Plasmon Resonance (digital SPR) system to provide a holistic assessment of GLP-1 RA structure and function. LC/MS enables detailed structural profiling—including mass confirmation, impurity analysis, and degradation monitoring—while digital SPR delivers real-time, label-free measurements of GLP-1 receptor binding kinetics. When used together, these orthogonal techniques allow developers to directly correlate chemical modifications or degradation products with changes in biological activity. In several cases, this combined approach reveals unexpected disconnects between structural homogeneity and receptor binding behavior, underscoring the value of functional assays alongside structural characterization. By integrating LC/MS and digital SPR, biopharmaceutical scientists can streamline early-stage screening, support stability and forced degradation studies, improve comparability assessments, and gain deeper insight into structure-activity relationships. This workflow aligns with current industry trends toward faster development timelines and more robust characterization strategies, enabling confident decision-making from early discoveries through development and quality control.

Experimental

Reagents and chemicals

Liraglutide was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and stored according to the manufacturer's instructions. Difluoroacetic acid (DFA), Trizma base, Tris-HCl, and 30% (v:v) hydrogen peroxide (H_2O_2), calcium chloride, and formic acid was procured from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade chymotrypsin were obtained from Promega (Madison, WI, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

LC/MS grade acetonitrile (ACN) was obtained from Fisher (Waltham, MA. USA). Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA. USA).

GLP-1R for SPR experiments was procured from Sino Biological (Paoli, PA, USA). All other SPR reagents used were prepared by Nicoya (Kitchener, ON, USA): PBS-T (0.1% Tween 20), pH 7.4 (DSPR-PBST), Carboxyl Surfacing Kit (DSPR-CBX-SURF), 10 mM Sodium Acetate Buffer, pH 4.0 (DSPR-IMB-4.0) and 10 mM Glycine-HCl, pH 1.5 (DSPR-GLYHCl-1.5).

Analytical equipment

An Agilent 1290 Infinity II bio LC system included the following modules:

- Agilent 1290 Infinity II Bio high-speed pump (G7120A)
- Agilent 1290 Infinity II Bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)
- Digital SPR 16-Channel Instrument with Nicosystem Pro Software (DSPR16-PRO)
- 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)

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
- Digital SPR Nicosystem™ User Portal

Sample preparation

Oxidation Protocol: Liraglutide was dissolved to 2.0 mg/mL in 30% ACN. Sonication was applied to aid solubilization until a homogeneous, visually clear solution was obtained. The resulting stock was used immediately for oxidation studies. For oxidative stress, stock solutions were diluted to 0.5 mg/mL and incubated at 2.5% concentrations of the oxidizing agent H_2O_2 overnight at room temperature.

Chymotrypsin Digestion Protocol: Liraglutide peptide samples (200 µg/mL) were dissolved in 100 mM Tris buffer (pH 8.0) containing 10 mM $CaCl_2$. Peptide digestion was performed at 37 °C for 2 hours with a chymotrypsin enzyme to peptide ratio of 1:30 (w/w). The reaction was stopped by acidifying the mixture with formic acid, and LC/MS experiments were then performed.

LC/MS analysis

For the oxidized samples, the LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using a 30-minute gradient as described earlier.3 The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF and data analysis was performed in MassHunter BioConfirm version 12.1 and MassHunter Qualitative Analysis version 12.0.

For the chymotrypsin digested liraglutide, the LC separation was performed on an Agilent Altura Peptide Plus column, 2.1 × 150 mm, 2.7 µm (part number 227215-903) using an 8-minute gradient (Table 1). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (Table 2) and data analysis was performed in MassHunter BioConfirm version 12.1 and MassHunter Qualitative Analysis version 12.0.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Altura Peptide Plus, 2.1 × 150 mm, 2.7 µm (p/n 227215-903)
Sample Thermostat	10°C
Mobile Phase A	0.1% Formic acid in water
Mobile Phase B	0.1% Formic acid in ACN
Flow Rate	0.3 mL/min
Gradient	Time %A %B 0 95 5.00 1.0 95 5.00 5.0 65 35.00 6.0 30 70.00 7.0 30 70.00 8.0 95 5.00
Stop Time	As Pump/Injector
Column Temperature	50 °C
Injection Volume	3.00 µL

Table 2. MS data acquisition parameters.

Parameter	Value
Ion Mode	Positive mode, dual AJS ESI
Gas Temp	325 °C
Gas Flow	13 l/min
Nebulizer	35 psig
SheathGasTemp	350 °C
SheathGasFlow	12 l/min
Capillary Voltage	4,000 V
Nozzle Voltage	1,000 V
Fragmentor	175 V
Skimmer1	65 V
OctopoleRFPeak	750 V
MS Min Range	100 *m/z*
MS Max Range	1,700 *m/z*
MS Scan Rate	3.00 spectra/sec
MS/MS Min Range	50 *m/z*
MS/MS Max Range	1,700 *m/z*
MS/MS Scan Rate	3.00 spectra/sec

SPR Methods

Binding kinetics were measured using a capture-based assay on an Alto 16-channel Digital SPR instrument with Nicosystem Pro software (DSPR16-PRO). Liraglutide samples were immobilized on a 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16) by amine coupling and was interrogated by GLP-1R in solution for kinetic analysis.

1. **Reconstitution:** The lyophilized proteins were reconstituted in sterile water according to the manufacturer's instructions to generate a stock solution.
2. **Normalization:** Carboxyl sensors were normalized with normalization solutions.
3. **Immobilization**: Carboxyl sensors were activated with 200 mM EDC/NHS, followed by immobilization of 10 µg/mL liraglutide diluted in sodium acetate, pH 4.5 onto all even sensors. All sensors were blocked with 1 M ethanolamine to quench the remaining active carboxyl groups.
4. **Conditioning:** All sensors were conditioned with 10 mM Gly-HCl, pH 1.5.
5. **Automated Serial Dilution:** The Alto Digital SPR automatically executed a five-step, threefold serial dilution on the cartridge with running buffer. A 900 nM stock of GLP-1R yielded 3.7 nM, 11.1 nM, 33 nM, 100 nM and 300 nM solutions.
6. **Kinetics:** All sensors were exposed to the lowest GLP-1R concentration, allowed to dissociate in running buffer, and regenerated with 10 mM Gly HCl, pH 1.5. This step was repeated for the remaining four liraglutide concentrations, which then constituted a full round of multi-cycle kinetics (MCK).

Kinetic Analysis

All interactions were automatically referenced in the Nicosystem by subtracting the signal from reference channels (no liraglutide immobilized). The resulting sensorgrams were fitted to a 1:1 Langmuir binding model to extract the association rate constant k_a, dissociation rate constant k_d, and equilibrium dissociation constant k_D.

Results and discussion

Structural Characterization by LC/MS

Liraglutide peptide characterization was performed using the 1290 Infinity II bio LC system with an AdvanceBio Peptide Mapping column, coupled to a 6545XT AdvanceBio LC/QTOF and processed in MassHunter BioConfirm 12.1. The bioinert configuration minimizes peptide-metal interactions, ensuring accurate chromatography and sensitive detection of low-level modifications.

This setup was used to study H_2O_2-induced oxidative degradation of liraglutide. Methionine and tryptophan are the primary oxidation prone residues in peptide therapeutics, and the GLP-1 agonist examined contains a single tryptophan. H_2O_2 is commonly applied in stress testing to generate oxidative variants, which may alter receptor binding.

Figure 1 shows the LC/MS profiles after overnight H_2O_2 exposure. The resulting TICs reveal multiple peaks, indicating formation of several oxidized species that are well resolved on the AdvanceBio column.

Table 3 lists experimental monoisotopic masses obtained through resolved isotope deconvolution in MassHunter Qualitative Analysis 12.0. Deconvolution of the multiply charged ions confirms multiple oxidation states. Mass increases of +16, +32, and +48 Da correspond to mono, di, and trioxidized products, respectively. A +14 Da shift is also observed and attributed to carbonylation.[4]

Tryptophan oxidation commonly yields isomeric products5, and the deconvolved masses allow assignment of these species as summarized in the Figure 1 inset. Reversed-phase LC provided partial separation of the singly, doubly, and triply oxidized isomers.

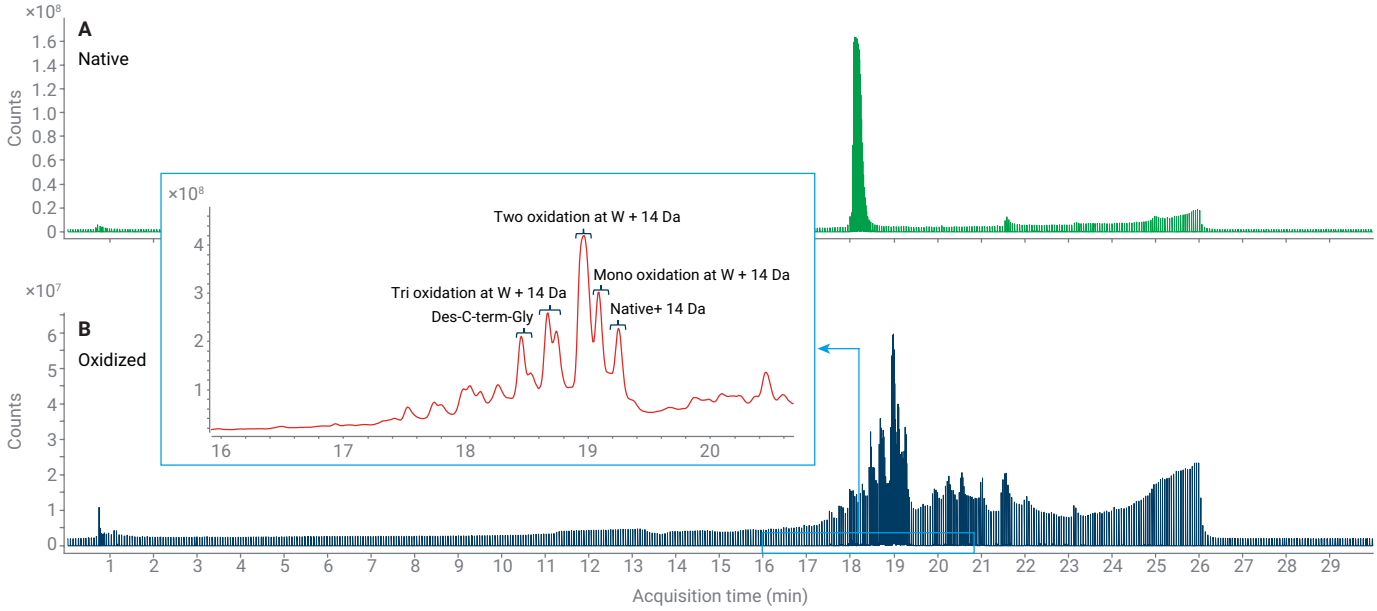


Figure 1. Total Ion Chromatogram: TIC of Native (A) and Oxidized Liraglutide (B). Inset in B shows the assignment of different oxidized species

Table 3. Experimental monoisotopic masses of liraglutide.

Peptide	Sequence	Molecular Weight (monoisotopic)					
		Native	Des-C-term-Gly	Two oxidation at W + 14 Da	Mono oxidation at W + 14 Da	Tri oxidation at W + 14 Da	Native +14/16 Da
Liraolutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl}-EFIAWLVRGRG	3,748.94	3,691.88	3,794.93	3,778.93	3,811.93	3,764.92

To further investigate the structural stability of liraglutide, chymotrypsin digestion was carried out. Chymotrypsin preferentially cleaves peptide chains at the carboxyl side of aromatic amino acids (Tyr, Phe, and Trp) and may also cleave—though less frequently—at selected aliphatic residues. In this study, six fragments were generated from the chymotrypsin digestion of liraglutide, as shown in the TIC in Figure 2. These fragments correspond to C1, C2, C3, C4, and C5 in order of their retention times (Table 4). The fragment designated C4* is attributed to a secondary cleavage site at a leucine residue. Figure 2 inset also shows the assignments of the buffer-related peaks.

The identities of the peptide fragments were confirmed through MS/MS analysis, with representative spectra shown in Figure 3. LC/MS analysis of liraglutide under two different treatment conditions further confirmed structural changes induced by these treatments. Together, these structural characterizations were used in conjunction with SPR studies to establish correlations between structural alterations and functional behavior.

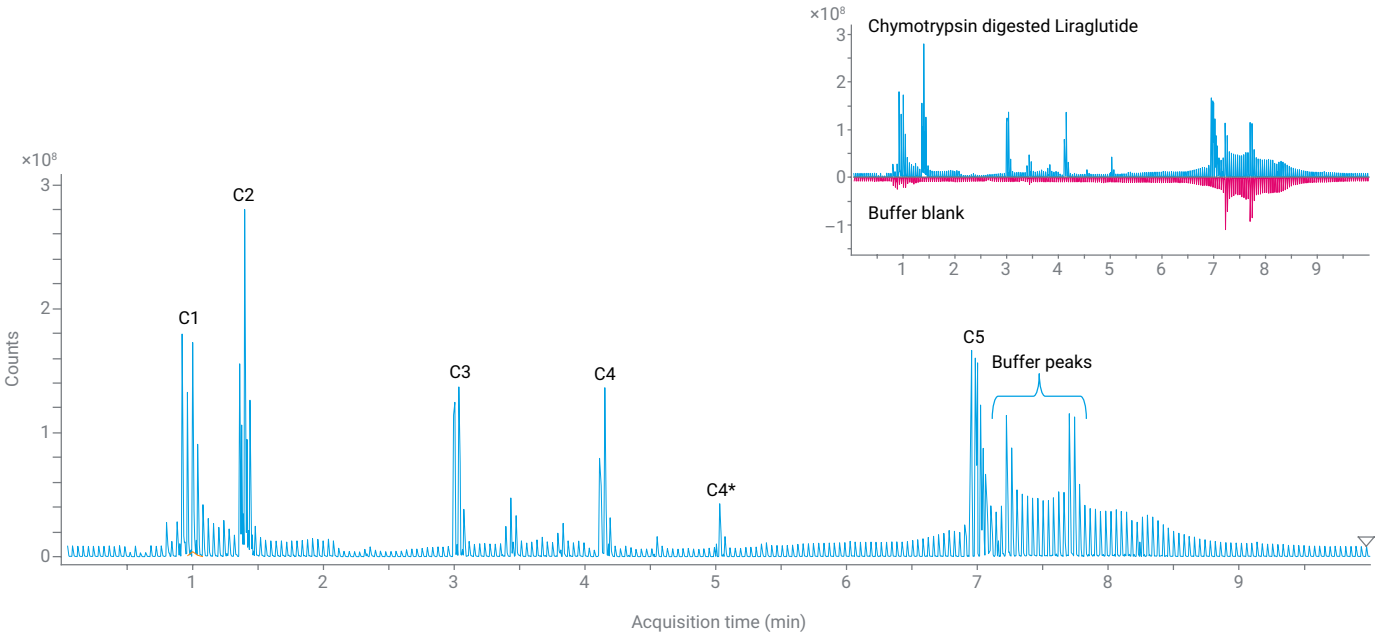


Figure 2. Total ion chromatogram (TIC) of chymotrypsin digested Liraglutide .
Inset shows the mirror plot of chymotrypsin digested liraglutide with Buffer blank.

Table 4. Fragments of chymotrypsin-digested liraglutide.

Fragment	Retention time (min)	Peptide fragment	Observed mass (Da)	Target mass (Da)	Mass error (ppm)
C1	0.968	LVRGRG	656.4044	656.4082	-5.78
C2	1.349	HAEGTF	660.2834	660.2867	-5.03
C3	2.995	TSDVSSY	757.31	757.313	-3.98
C4	4.123	IAW	388.2089	388.2111	-5.67
C4*	5.028	IAWL	501.2926	501.2951	-5.13
C5	6.981	LEGQAA**K**EF	1,358.764	1,358.7697	-4.51

C4* indicates secondary cleavage site of chymotrypsin
K indicates lipidated Lys

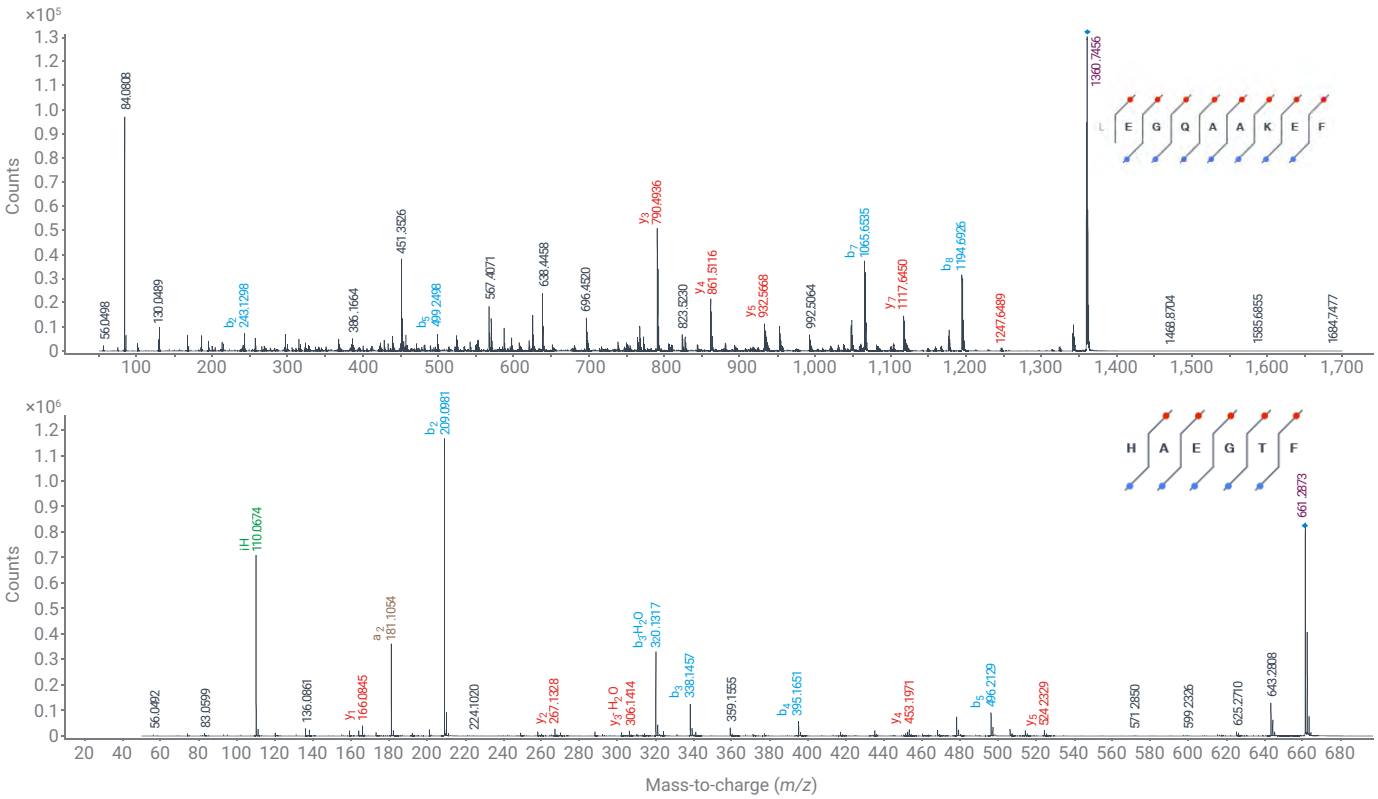


Figure 3. Representative MS/MS spectra of fragments of liraglutide following chymotrypsin digestion

Functional Assessment by SPR

In this study, Digital SPR was used to compare the binding kinetics of liraglutide against GLP-1R using direct amine coupling. The immobilization of ligands on the carboxyl and streptavidin surfaces was optimized to obtain a low ligand density while allowing for the detection of all analyte concentrations sampled. Sensors were regenerated fully using 10 mM Gly-HCl, pH 1.5. Kinetic parameters for each liraglutide sample were calculated using a 1:1 Langmuir fit (Figures 4a-b).

SPR requires sample concentration as an input to determine kinetic parameters. The Cary 3500 UV-Vis platform enables rapid quantitation of biomolecules. In addition to quantitation, its high wavelength resolution enables detection of subtle structural changes for peptide characterization and stability assessments[9]

The kinetics parameters calculated for native and oxidized liraglutide are summarized in Table 5. The K_D values measured were nearly identical, 3.51 nM and 3.68 nM for native and oxidized liraglutide, respectively. The k_a and k_d for each liraglutide sample are also the same, indicating there is no change in liraglutide function upon oxidation. Modification of the tryptophan residue to anchor the lipid moiety, instead of the lysine residue, has been reported to produce no change in binding affinity compared with the lysine anchored lipid species.

These findings suggest that the tryptophan residue does not make a measurable contribution to GLP1 receptor engagement[6]. Digesting liraglutide with chymotrypsin abolished binding affinity for the GLP-1R completely, as shown in Figure 4c. Chymotrypsin cleaves peptide bonds following aromatic amino acids (such as phenylalanine, tyrosine, and tryptophan). Liraglutide contains several such residues and therefore chymotrypsin breaks the backbone in multiple places, resulting in small fragments that cannot maintain the necessary structure to fit into the receptor's binding pocket. Previous studies have established that GLP1 receptor binding is primarily mediated by the N-terminal residues, as reported in earlier publications [7,8].These results demonstrate that some changes in peptide structure result in a change or inhibition of receptor binding, while other structural changes may have no impact on receptor binding. Therefore, Nicoya's Digital SPR is a powerful functional complement to Agilent's dependable LC systems, enabling scientists to link biological function back to structural integrity.

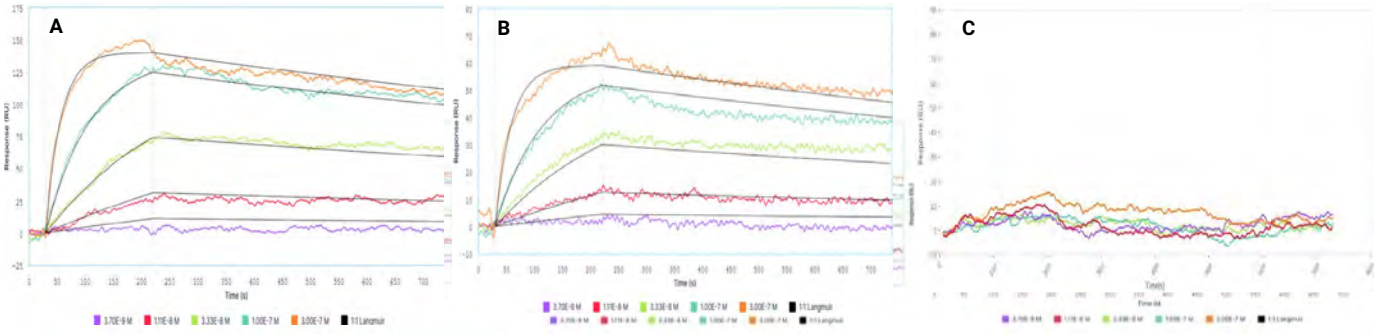


Figure 4. Representative corrected binding data for GLP-1R binding to (A) native liraglutide (B) oxidized liraglutide, and C) liraglutide digested with chymotrypsin using a multi-cycle kinetics (MCK) assay format. The analyte concentrations and associated colors are shown in the figure legends, while the Langmuir 1:1 binding fit is shown in black. A binding fit was not applied to C) as no analyte binding was observed.

Table 5. Binding kinetics of native and oxidized liraglutide with GLP-1R

Sample	k_a (M-1)	k_d (M-1 s-1)	k_D (nM)
Native liraglutide	$1.79 \times 10^5 \pm 4.28 \times 10^4$	$6.26 \times 10^{-4} \pm 2.02 \times 10^{-4}$	3.51 ± 0.77
Oxidized liraglutide	$1.57 \times 10^5 \pm 2.98 \times 10^4$	$5.64 \times 10^{-4} \pm 8.48 \times 10^{-5}$	3.68 ± 0.77

Conclusion

The integrated workflow described in this study—combining LC/MS for structural analysis with SPR for ligand binding assessment—provides an important framework for understanding structure–function relationships in GLP1 Analogs and their receptor.

The results generated using this integrated approach contribute valuable insights to the existing knowledge base for these molecules. The study exposed that cleaving of the peptide caused it to completely lose its function, highlighting the risk of degraded species in the final product. Meanwhile, we showed that oxidation, a common degradation pathway for peptides, had no impact on the function of Liraglutide, perhaps de-risking some manufacture and storage approaches.

Beyond supporting fundamental characterization, this workflow offers meaningful advantages to the biopharmaceutical industry. It can help optimize candidate selection and refinement during both discovery and development.

As GLP-1 RAs continue to expand in therapeutic scope and market impact, advanced analytical solutions will be essential for ensuring patient safety and accelerating access to innovative therapies.

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