Characterization of Synthetic Peptide Drug and Impurities using High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry (LC/MS)

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Introduction

The use of solid-phase peptide synthesis (SPPS) enables large-scale production of high-purity peptides, with structural modifications improving in vivo stability and efficacy. However, unexpected impurities can cause immunogenicity, making active pharmaceutical ingredient (API) confirmation and impurity control critical quality attributes (CQAs). This study demonstrates rapid API confirmation using liquid chromatography/single quadrupole mass spectrometry (LC/SQ-MS). Method optimization ensured specificity for forced degradation products, while two-dimensional liquid chromatography (2D-LC) facilitated MS-friendly spectral analysis. Peaks obtained under non-MS-friendly conditions were analyzed using 2D-LC to characterize APIs, impurities, and aggregates via deconvolution processing. Additionally, peptide drugs were hydrolyzed into amino acids under acidic conditions and derivatized with ortho-phthalaldehyde (OPA) to determine their amino acid composition.

Analytical Method:

Table 1. LC/MS method for intact mass confirmation

Parameter	Value
Instrument	Agilent 1260 Infinity II Bio Prime LC
Column	Agilent AdvanceBio Peptide Mapping 120Å, 2.1 x 150mm, 2.7 µm
Flow rate	0.4 mL/min
Column temperature	40 °C
Mobile phase	A) 0.1% Difluoroacetic acid B) 0.1% Difluoroacetic acid in ACN
Gradient	Time (min) %A %B 0 70 30 1 70 30 15 10 90 15.1 70 30 20 70 30
Detector	UV 280 nm (DAD HS with Bio-inert Max-Light cartridge cell, 60mm)
MS	Agilent InfinityLab LC/MSD iQ
Ion source	ESI (+)
Source parameters	Gas temperature 325 °C, Gas flow 11 L/min, Nebulizer 45 psi, Capillary voltage 4,500V
Acquisition	Scan 300 – 1,450 m/z (Profile)

Table 2. MS spectral deconvolution settings

Parameter	Value
Software	Agilent OpenLab CDS software version 2.8
Low/High Molecular Weight	2,500 to 8,000
Maximum Charge	10
Minimum Peaks in Set	3

Experimental

Sample Preparation:

Liraglutide was dissolved in water at 1 mg/mL, and Semaglutide was prepared at 1 mg/mL by dissolving it in 30% acetonitrile(ACN). The sample solutions were treated under heat, acid, base, and hydrogen peroxide conditions to generate forced degradation impurities. Ozempic® was transferred into a vial and injected directly without further preparation.

Table 3. 2D-LC-MS instrument method for impurity analysis

Parameter	Value
Instrument	Agilent 1290 Infinity II Bio 2D-LC
^{1}D Column	Agilent AdvanceBio Peptide Plus 2.1 x 250mm, 2.7 µm
^{1}D Flow rate	0.4 mL/min
Injection volume	20 µL
Column temperature	60 °C
^{1}D Mobile phase	A) 0.4% Trifluoroacetic acid B) 0.4% Trifluoroacetic acid in ACN
^{1}D Gradient	Time (min) %A %B 0 70 30 0.5 70 30 65 45 55 70 10 90 75 10 90 75.1 70 30 80 70 30
^{1}D Detector	UV 280 nm (DAD HS with Bio-inert Max-Light cartridge cell LSS 10 mm + aperture)
^{2}D Column	Agilent InfinityLab Poroshell 120 CS-C18, 100Å, 2.1 x 100mm, 2.7 µm
^{2}D Flow rate	0.6 mL/min (Idel flow: 0.05 mL/min)
^{2}D Mobile phase	A) 0.1% Formic acid B) 0.1% Formic acid in ACN
2D-LC Operation mode	Time-based heart-cut MHC
^{2}D Gradient	Time (min) %A %B 0 75 25 10 60 40 ^{2}D run time: 10 min ^{2}D equilibration: 3 min Cycle time: 13 min
ASM Setting	Factor: 3 Flush factor: 2.5
Sample Loop	40 µL
Detector	UV 280 nm (DAD HS with Bio-inert Max-Light cartridge cell, 60mm)
MS	Agilent InfinityLab LC/MSD XT
Ion source	ESI (+)
Source parameters	Gas temperature 325 °C, Gas flow 11 L/min, Nebulizer 45 psi, Capillary voltage 4,500V
Acquisition	Scan 500 – 2,500 m/z (Profile)

Results and Discussion

The Agilent 1260 Infinity II Bio Prime LC system demonstrated flexibility for impurity profiling of synthetic peptides, including Semaglutide and Liraglutide.

Under 0.1% DFA conditions, the InfinityLab LC/MSD iQ provided excellent MS spectra, enabling confirmation of neutral masses through OpenLab CDS deconvolution. This approach was extended to peptide stability testing.

Using 0.4% TFA in 1D for optimal chromatographic resolution and 0.1% formic acid in 2D for MS-friendly conditions, intact masses of low-level impurities were successfully confirmed.

Furthermore, by applying size exclusion chromatography in 1D, 2D-LC was effectively used for aggregate characterization. This enabled clear differentiation between reversible and irreversible aggregates.

Amino acid composition was assessed using OPA derivatization, confirming not only common amino acids but also non-natural residues such as 2-aminoisobutyric acid and aminoethylethanolamine.

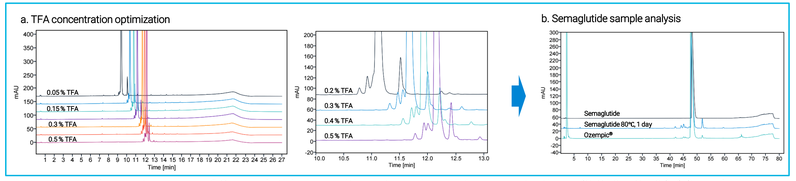

Figure 1. UV chromatograms of Semaglutide impurities at varying TFA concentrations (0.05%–0.5%) (a), and UV chromatograms of Semaglutide API, heat-treated Semaglutide at 80°C, and Ozempic® under 0.4% TFA conditions (b)[1]

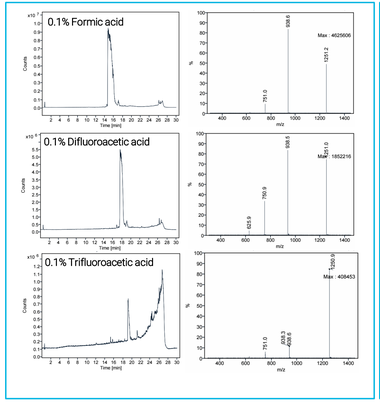

Figure 2. Total ion chromatogram (TIC) and MS spectrum of Liraglutide by type of acidic modifier[2]

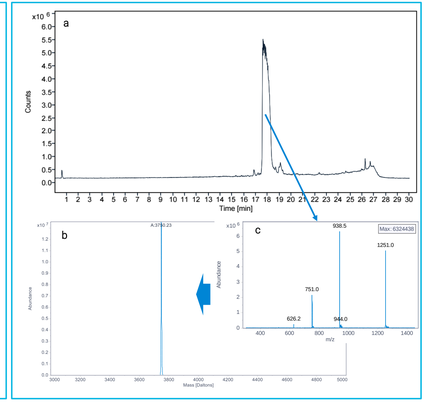

Figure 3. TIC (a), raw mass spectra (c), and deconvoluted mass spectra (b) of Liraglutide under 0.1% DFA condition using Agilent InfinityLab LC/MSD iQ[2]

Results and Discussion

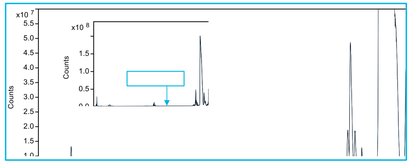

Figure 4. TIC of forced degradation impurities of Liraglutide under acidic conditions obtained with 0.1% DFA using Agilent InfinityLab LC/MSD XT[3]

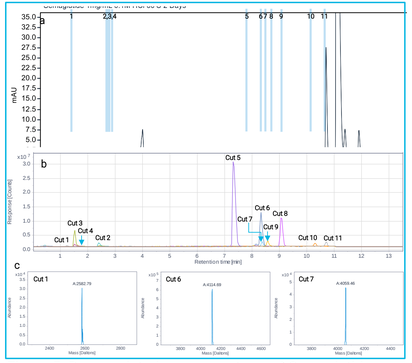

Figure 5. Deconvoluted mass spectrum of Semaglutide impurities confirmed using 0.4% TFA as the 1D condition (a) and 0.1% formic acid and Agilent InfinityLab LC/MSD XT as the 2D condition (b).[4]

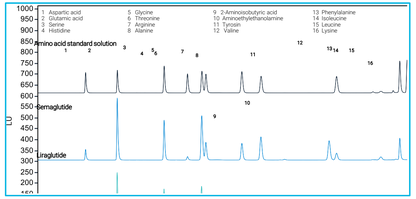

Figure 6. FLD chromatogram of amino acid analysis using the OPA derivatization method.[5]

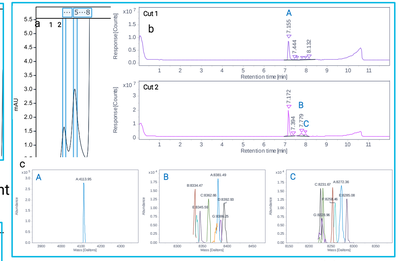

Figure 7. 1D UV Size exclusion chromatogram (a) and 2D TIC (b) deconvoluted mass spectrum (c) of reversible and irreversible aggregates of Semaglutide in Ozempic® confirmed by 2D-LC/MS[6]

Conclusions

- Using Single Quadrupole and 2D-LC, we were able to confirm not only the mass of synthetic peptides but also their impurities and aggregates.
- The Agilent 1260 Infinity II Bio Prime LC was utilized to optimize a UV-based purity test method and for amino acid analysis.

References

[1]Chae-Young, Ryu. Efficient Method Optimization of Semaglutide Analysis Using an Agilent 1260 Infinity II Bio Prime LC System and Blend Assist. *Agilent Technologies application note*, publication number 5994-7414EN, 2024.

[2]Chae-Young, Ryu. Rapid Confirmation of GLP-1 Analog Using Agilent InfinityLab LC/MSD iQ. *Agilent Technologies application note*, publication number 5994-7415EN, 2024.

[3]Chae-Young, Ryu. Peptide Drug Stability Analysis Using Agilent InfinityLab LC/MSD and OpenLab CDS Deconvolution. *Agilent Technologies application note*, publication number 5994-7500EN, 2024.

[4]Chae-Young, Ryu, Confirmation of Peptide-Related Impurity Intact Mass Using Agilent 1290 Infinity II Bio 2D-LC and InfinityLab LC/MSD XT. *Agilent Technologies application note*, publication number 5994-7654EN, 2024.

[5]Chae-Young, Ryu, Amino Acid Composition of Peptide Drug Using Agilent 1260 Infinity II Prime LC. *Agilent Technologies application note*, publication number 5994-7749EN, 2024.

[6]Chae-Young, Ryu, Comprehensive Aggregate Profiling of Liraglutide and Semaglutide Using 1290 Infinity II Bio 2D-LC and InfinityLab LC/MSD XT. *Agilent Technologies application note*, publication number 5994-7740EN, 2024.

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