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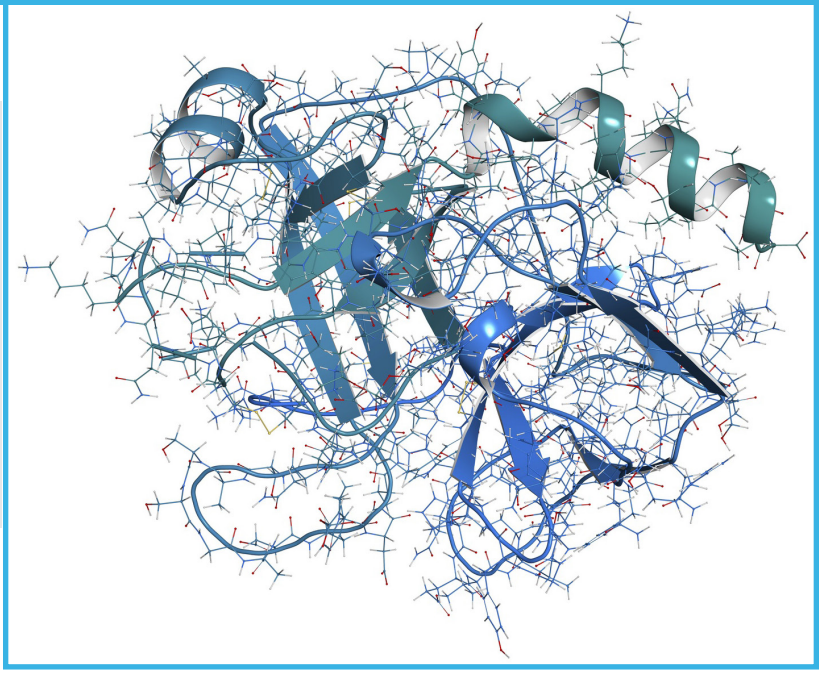
TECHNICAL REPORT: AMT_TR_BIO_25

# TITLE: LC/MS/MS CHARACTERIZATION OF LIRAGLUTIDE WITH HALO® PCS C18

MARKET SEGMENT: BIOCLASS

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## ABSTRACT

The GLP-1 agonist peptide, Liraglutide received generic status in late 2024. The unique properties of Liraglutide make characterization via HPLC and LC/MS challenging. We demonstrate a LC/MS/MS workflow using the HALO® PCS C18 column to characterize a sample of Liraglutide identifying several impurities including an N-terminal cyclization that can form even under gentle handling conditions.


## INTRODUCTION

Glucagon-Like Peptide-1 (GLP-1) analog agonist drugs have demonstrated remarkable benefits to treat illnesses such as type-2 diabetes and obesity and pharmaceutical companies are making rapid progress to improve the potency and half-life of these compounds.

Liraglutide was first introduced in 2010 by Novo Nordisk under the brand name Victoza®. Liraglutide differs from native GLP-1 peptide by a single amino acid substitution at position 34 from lysine to arginine (Figure 1). Additionally position 26 has an amide linkage to the lysine sidechain that attaches a C16 fatty acid. These modifications improve the half-life from approximately 10 mins of native GLP-1 to 13 hours thereby increasing the drugs interaction time with albumin and improving distribution.

Liraglutide has a high homology to glucagon, a generic peptide drug commonly used to stimulate the release of glucose from glycogen in the liver. The quality assurance process used in the manufacturing of glucagon is described in the US Pharmacopeia monograph and provides an excellent template for QA characterization of Liraglutide. Liraglutide, like glucagon, is typically produced recombinantly in yeast, with the C16 fatty acid linked via acylation and the final product is purified prior to QA analysis for Liraglutide. However, the HPLC method described in the glucagon monograph uses a phosphate buffer at pH 2.7 on an L1 column over 45 minutes and therefore is incompatible with LCMS characterization which is something that will likely be necessary for Liraglutide manufacturing. Most common strong ion pairing agents such as trifluoroacetic acid or phosphate are not compatible with mass spectrometry owing to ion suppression or instrument fouling issues. Thus, if MS is required for characterization, a weaker ion pairing agent such as formic acid must be used. The use of formic acid reduces the risk of ion suppression of the analyte, but frequently sacrifices the quality of the separation of basic compounds on standard C18 columns which is exhibited by peak tailing. For increasingly complex QA analysis of biologics, the use of stronger ion pairing agents may be beneficial for routine HPLC-UV analysis, but the method must be changed when MS characterization is warranted. Ideally, a single common method would be preferred for QA and characterization.

HALO 160 Å PCS C18 columns are a positively charged reversed-phase stationary phase designed for high-quality separations of basic compounds such as peptides and proteins in the presence of weak ion-pairing agents such as formic acid. This allows for development of a separation method that is LCMS compatible without sacrificing peak capacity. With PCS C18 a single separation methodology that can be used for HPLC-UV detection in QA and for LCMS characterization is possible, increasing confidence of identification and monitoring of impurities.


## KEY WORDS:

GLP-1, Liraglutide, LC/MS/MS, HALO© PCS C18, superficially porous particles, Fused-Core®

Figure 2 demonstrates the separation capability of HALO PCS C18 in weak ion pairing agents analyzing chemically stressed Glucagon. Baseline separation of all four deamidation products, confirmed via LCMSMS, are achieved in a 12.5 minute method[1]. This reduces the method runtime compared to that described in the USP monograph by nearly 75%.

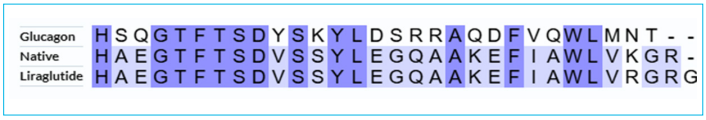


**Figure 1:** Homology alignment of Liraglutide with Glucagon and Native GLP-1 peptide

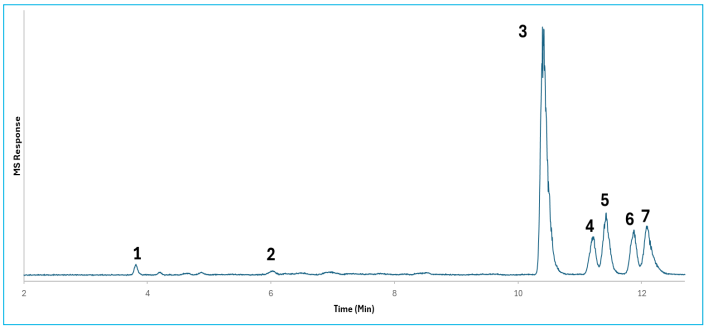


**Figure 2:** Separation of chemically stressed Glucagon

TEST CONDITIONS:
**Column:** HALO® PCS C18, 2.7 µm, 2.1x150 mm
**Mobile Phase A:** $H_2O$ + 0.1% Formic Acid;
**Mobile Phase B:** Acetonitrile + 0.1% Formic Acid;
**Gradient Conditions**: 0 min – 20% B; 15.0 min – 25%B; 15.5 min – 80%B; 17.0min – 80%B; 17.1min – 20%B; 22.0 min – 20%B

PEAK IDENTITIES: 1) Glucagon Fragment, 2) Glucagon Fragment , 3) Glucagon, 4) $Asp^{28}$-Glucagon, 5) $Glu^{3}$-Glucagon, 6) $Glu^{20}$-Glucagon, and 7) $Glu^{24}$-Glucagon

In this study we adapted the methods used to investigate Glucagon to Liraglutide using the HALO® PCS C18 column. We were able to characterize a number of potential impurities including an N-terminal cyclization that appears to be base catalyzed in the presence of trace levels of formaldehyde. The use of the HALO® PCS C18 column allows for rapid separation of these impurities from the primary analyte in the presence of formic acid and is compatible with both HPLC-UV and LCMSMS workflows.

## EXPERIMENTAL /SAMPLE PREPARATION

Research grade Liraglutide was obtained from Cayman Chemical (Ann Arbor, MI; item #24727) and solubilized in 10mM Tris pH 8.0 at a concentration of 0.2 mg/ml. Samples were aliquoted and frozen at -20°C. Solvents and additives were obtained from Sigma Aldrich (St. Louis, MO). A Shimadzu Nexera X2 HPLC system (Columbia, MD) was coupled to a Thermo Q-Exactive HF (Waltham, MA).

Columns used for separation are indicated below:

**Columns:** HALO 160 Å PCS C18 2.7 µm, 2.1x150 mm
**Part number:** 92112-717

HPLC Method Conditions:
**Mobile Phase A:** LCMS grade $H_2O$ + 0.1% Formic acid
**Mobile Phase B:** LCMS grade Acetonitrile + 0.1% Formic acid
**Flow Rate:** 0.3 ml/min
**Column Temperature:** 60°C
**Pressure:** 230 bar
**Sample Solvent:** 10mM Tris pH 8.0

| Long Gradient: | Time | %B |
|---|---|---|
| | 0.0 | 2 |
| | 5.0 | 2 |
| | 6.0 | 25 |
| | 60.0 | 50 |
| | 70.0 | 95 |
| | 70.1 | 2 |
| | 75 | 2 |

| Fast Gradient: | Time | %B |
|---|---|---|
| | 0.0 | 2 |
| | 2.0 | 2 |
| | 3.0 | 25 |
| | 9 | 50 |
| | 12 | 95 |
| | 12.1 | 2 |
| | 15 | 2 |

MS Method Conditions:
**Mass Spectrometer:** Thermo Q-Exactive HF
**Ion Mode:** Positive Electrospray
**Sheath Gas Flow Rate:** 20
**Aux Gas Flow Rate:** 10
**Sweep Gas Flow Rate:** 1
**Spray Voltage:** 4kV
**Capillary Temp:** 320°C
**S-Lens RF Level:** 60 V
**Aux Gas Heater Temp:** 275°C
**MS1 Resolution:** 120,000
**AGC Target:** 3e6
**Maximum IT:** 200ms
**Scan Range:** *m/z* 300-2000
**In-Source CID:** 10eV

Initial evaluation of a 500ng injection of Liraglutide using a long gradient method shows two prominent peaks in the Total Ion Chromatogram. The principal peak at 43.82 min represents native Liraglutide eluting at approximately 40% Acetonitrile. An unexpected secondary peak from a potential impurity is seen at 46.06 min. Figure 3 shows the +3 charge state of the primary and secondary peaks.

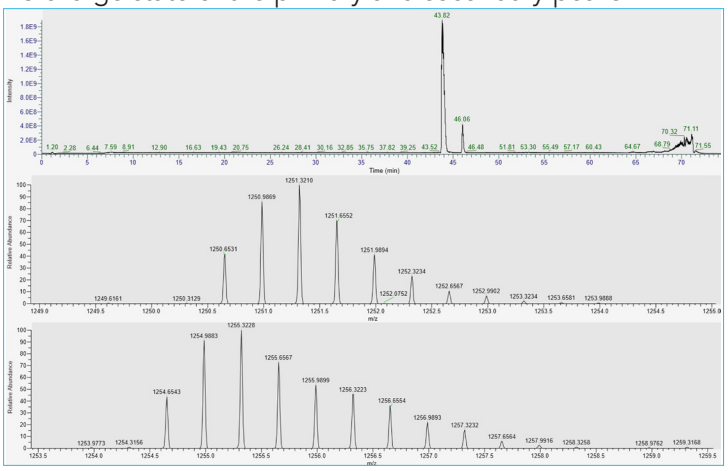


**Figure 3:** Long gradient survey of Liraglutide. 1. Shows the 3+ charge state of Liraglutide, 2. Represents an impurity of exactly +12 Da shift in absolute monoisotopic mass

Deconvolution of the 3+ charge states show that this impurity is exactly +12 Da. MS/MS data localized it to the N-terminus. A literature survey suggests this is a result of cyclization of the N-terminal Histidine in the presence of formaldehyde[2]. Additional research on the impact of excipients on Liraglutide modification suggests that this N-terminal histidine is highly sensitive to formaldehyde impurities[3]. It is believed that our use of the Tris buffer to solubilize Liraglutide contained trace formaldehyde and generated the cyclized impurity.
A buffer in the 7.5-8.5 pH range is necessary to solubilize most GLP-1 peptides as they have limited solubility in more acidic conditions. Similarly, the majority of detergents and chaotropes are incompatible with MS methods. Despite our conservative approach in solubilization and storage, cyclization still occurred. It may be necessary to add an additive to the solution to scavenge trace formaldehyde. Table 1 lists the identified impurities found in the research grade Liraglutide sample.

| *Retention Time* | *Ion Mass (Da)* | *Charge State* | *Monoisotopic Mass* | *Modification* |
|---|---|---|---|---|
| *37.89* | *942.2437* | *+4* | *3764.9456* | *Oxidation* |
| *39.13* | *942.2422* | *+4* | *3764.9396* | *Oxidation* |
| *41.64* | *946.2425* | *+4* | *3780.9408* | *Double Oxidation* |
| *41.64* | *1266.6541* | *+3* | *3796.9374* | *Triple Oxidation* |
| *41.95* | *1255.9869* | *+3* | *3764.9388* | *Oxidation* |
| *42.55* | *1261.3184* | *+3* | *3780.9333* | *Double Oxidation* |
| *43.82* | *938.2413* | *+4* | *3748.9371* | *Native Liraglutide* |
| *43.82* | *1257.9794* | *+3* | *3770.9163* | *Sodium Adduct* |
| *44.54* | *923.9884* | *+4* | *3691.9244* | *Glycine Truncation* |
| *46.06* | *941.2470* | *+4* | *3760.9588* | *N-terminal cyclization* |

**Table 1:** List of Identified Impurities in Research Grade Liraglutide Sample

Identification of these impurities is important in pharmaceutical manufacturing to ensure purity and safety of the product. It is common to characterize and report impurities down to 0.1% abundance of the primary product. By performing a thorough characterization via LCMS, manufacturers have a template to refer to during the QA process if an impurity arises. This template will provide a better understanding of tracking impurities in a particular lot and help to determine the source of the impurity. The N-terminal cyclization impurity is an excellent example. This could indicate that formaldehyde was introduced somewhere into the manufacturing process.

The HALO® PCS C18 column allows for rapid separation of basic compounds, maintaining excellent peak shape in ion pairing agents such as formic acid. This allows for shorter run times, and greater sample throughput. Figure 4 shows a UV trace at 280nm of a 12 minute method with baseline separation of Liraglutide and its N-terminal impurity In 0.1% formic acid. On more conventional C18 columns, this separation could be difficult to achieve without resorting to MS incompatible ion pairing agents such as TFA.

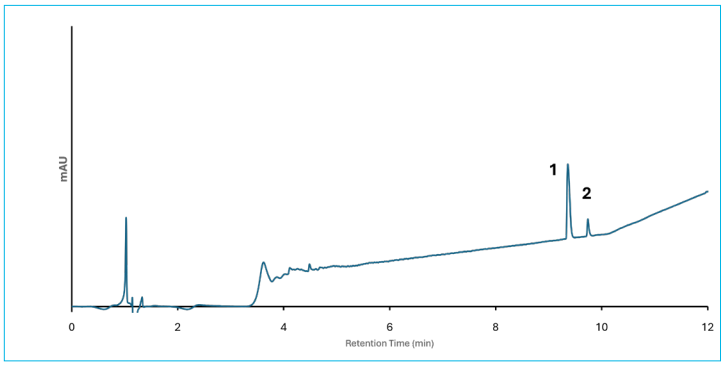


**Figure 4:** 280nm UV trace of fast separation of 1) Liraglutide and 2) N-terminal cyclization impurity on HALO® PCS C18.

## CONCLUSION:

The HALO® PCS C18 column provides excellent separation of basic compounds such as peptides and proteins including complex biologics such as Liraglutide in weak ion pairing conditions _compatible with mass spectrometry. HALO® PCS C18 allows for rapid impurity characterization by both UV/VIS and MS detection systems. The ability to produce excellent separation profiles in ion pairing agents such as formic acid also reduces the environmental footprint by eliminating the need for stronger ion pairing fluorinated agents such as TFA .

## REFERENCES:

1. *LC/MS/MS Characterization of Liraglutide with HALO® PCS C18*

2. *Badgujar D, Bawake, S, Sharma, N. A Comprehensive Study on the Identification and Characterization of Major Degradation Products of Synthetic Liraglutide Using Liquid Chromatography-High Resolution Mass Spectrometry. J. Peptide Sci. 2025 Jan; 31(1):e3652*

3. *Sheikh, AR., Vitore, JG., Bhalekar, VS., Jain, S., Kukreja, D., Giri, T., Sharma, N., Benival, D., Shah, RP. Reactivity of N Terminal Histidine of Peptides Towards Excipients/Impurity of Excipients: A Case Study of Liraglutide Excipient Compatibility Study. J. Pharmaceutical Sciences 113(2024). pp3246-3254.*