HALO®

TECHNICAL REPORT

TITLE: IMPURITY ANALYSIS OF COMPOUNDED SEMAGLUTIDE VIA LC/MS WITH HALO® PCS C18

MARKET SEGMENT: PHARMACEUTICAL

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ABSTRACT:

The latest generation of GLP-1 agonists demonstrate remarkable health benefits for patients including weight loss. Recently, there have been supply chain shortages that have limited the commercial supply of these therapeutics. This has allowed compounding pharmacies to begin selling their own formulations. Using an LC/MS methodology developed for Liraglutide on the HALO® PCS C18, we demonstrate that a compounded form of Semaglutide exceeds ANDA guidelines for peptide-related impurities.

INTRODUCTION

Glucagon-Like Peptide (GLP-1) drugs have been commercially available for nearly 20 years with the introduction of Exenatide in 2005. However, it wasn't until recently with the latest generation of therapeutics that sales and benefits have increased dramatically. Compounds like Semaglutide (Ozempic®; Novo Nordisk) and Tirzepatide (Zepbound®; Eli Lilly and Co.) have been engineered to increase both potency and half-life, allowing for a weekly subcutaneous injection as opposed to the previous daily injection.

Semaglutide is an improvement over Novo Nordisk's previous therapeutic, Liraglutide by incorporation of an Alpha-amino butyric acid (AiB) in the 2 position which resists proteolytic degradation by dipeptidyl peptidase-4[1]. The fatty acid chain linker attached to the substituted lysine at position 26 has also been changed to an 18 carbon di-acid improving distribution.

Recently, there have been reports of supply chain shortages for these next-generation GLP-1 agonists which have allowed compounding pharmacies to begin offering custom formulations for sale, circumventing some FDA regulatory guidelines. This creates risk for patients as some peptide-related impurities could be introduced into these compounded formulations that have not been properly evaluated for risks of immunoreactivity, potency, and off-target effects. The current ANDA guidelines for synthetic peptide drug products stipulate that peptide-related impurities should not be more than 0.5% of the Full Length Product (FLP) and justify that such impurities "would not be expected to affect the safety or effectiveness of the proposed generic synthetic peptide as compared to that of the RLD (Reference Listed Drug)"[2]. Additionally, it is stipulated that any "impurities between 0.1-0.5% must be identified, characterized, and justified for not affecting the safety and efficacy"[3].

Impurities can also be introduced through improper handling and storage of peptide therapeutics. Oxidation and deamidation are examples of changes that can occur to a peptide therapeutic. The sale of products such as Semaglutide via compounding pharmacies present concern that these therapeutics may not be properly handled and may introduce impurities via this mechanism that create risk for the patient.

Previously, we demonstrated the utility of our HALO® PCS C18 columns for characterization of a Critical Quality Attribute (CQA) of Liraglutide where the N-terminal histidine can cyclize in the presence of trace levels of formaldehyde[4]. This CQA had also been observed when Liraglutide had been exposed to certain excipients[5]. Semaglutide also contains an N-terminal histidine and we were interested to see if a similar modification could be observed. We confirmed that cyclization of the N-terminal histidine to an imidazopyrimidine ring does occur in Semaglutide. We also obtained a sample of

KEY WORDS:

Semaglutide, GLP-1, Liraglutide, HALO® PCS C18 column, Ozempic®

compounded Semaglutide as well as a sample of Commercial Ozempic® for comparison. We found that the levels of N-Terminal histidine modification in the compounded Semaglutide exceed the FDA ANDA guidelines while the Ozempic® does not. The evidence suggests this modification results as a consequence of improper handling in the production of the compounded product and introduces unnecessary risk to the consumer.

The increasing complexity of these peptide therapeutics have made characterization via liquid chromatography and mass spectrometry an essential tool. However, the basicity and highly hydrophobic nature of these compounds make them a challenge to analyze via routine reverse phase C18-based chromatography. A strong ion pairing agent such as tri-fluoroacetic acid is commonly required. Unfortunately, these strong ion-pairing agents are typically incompatible with mass spectrometry as they cause significant ion suppression. More recently, mixed bonding phases such as the HALO 160 Å PCS C18 which combines a C18 with a positively charged ligand in acidic conditions have shown improved resolving power and peak capacity in weaker ion pairing agents that are more MS friendly such as formic acid.

In this study we show how the HALO® PCS C18, 2.1 x150mm column is superior to a standard C18 column to separate full length product and the N-terminal imidazopyrimidine ring impurity and how this column can be routinely used to monitor for this impurity in any GLP-1 therapeutic that contains an N-terminal histidine.

EXPERIMENTAL/SAMPLE PREPARATION

Research grade Semaglutide was obtained from Cayman Chemical (Ann Arbor, MI; item#29969) and was solubilized in 10mM Tris pH 8.0 at a concentration of 0.2mg/ml. Samples were aliquoted and stored at -20°C. A syringe containing compounded Semaglutide at a concentration of 1mg/ml was donated from a prescribed patient. Similarly, an unused Ozempic® injector pen containing Semaglutide at a concentration of 2.68mg/ml was donated from a prescribed patient. Both the compounded Semaglutide and Ozempic® were diluted to 0.2mg/ml using LC/MS grade water. 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, 2.1x150mm

Part number: 92112-717

HALO 160 Å ES-C18 2.7µm, 2.1x150mm

Part number: 92122-702

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:

Research grade Semaglutide: 10mM Tris-HCl pH 8.0

Compounded Semaglutide: unknown

Ozempic®: Phosphate buffer, Propylene Glycol

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

Similar to our previous observation of solubilizing Liraglutide in Tris-HCl buffer that contained trace amounts of formaldehyde, we observed two peaks in the MS Total Ion Chromatogram (TIC) in a sample of research-grade Semaglutide solubilized in 10mM Tris-HCl at pH 8.0 as shown in Figure 1. Peak 1 represents the FLP Semaglutide and peak 2 represents the +12 da N-terminal histidine modification in Figure 1 and all subsequent figures. A similar +12 dalton shift in the deconvoluted spectra of peaks 1 and 2 is observed and is shown in Table 1. The N-terminal modification was confirmed via MS/MS and is not shown.

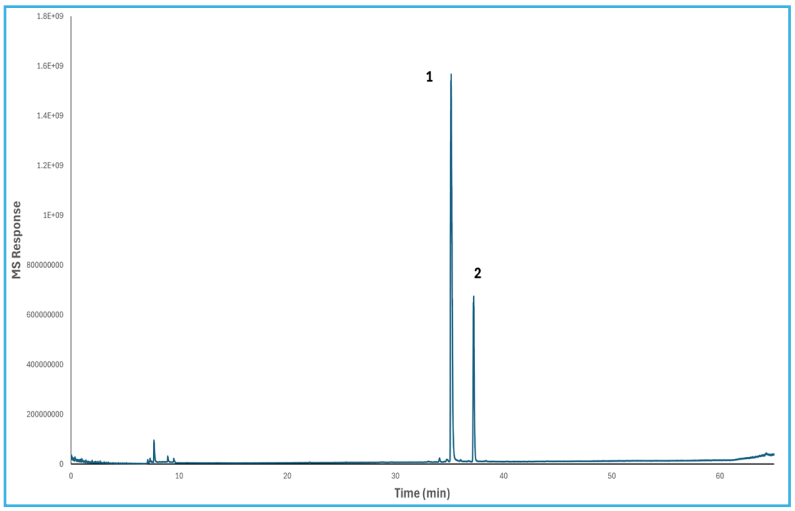

Figure 1: Long gradient survey of 100ng research grade Semaglutide.

	Deconvoluted mass	*Predicted mass*	*ppm mass error*
Semaglutide FLP	*4111.1027*	*4111.1154*	*3.09*
N-ter His modification	*4123.1047*	*4123.1154*	*2.60*

Table 1: Deconvoluted masses of peaks 1 and 2 from Figure 1 compared to theoretical monoisotopic mass.

A 100ng sample of compounded Semaglutide as well as 100ng of Ozempic® were analyzed in identical conditions. The TIC's are represented in Figures 2 and 3 respectively, and a zoomed-in comparison of the amount of N-terminal histidine modification is represented in Figure 4.

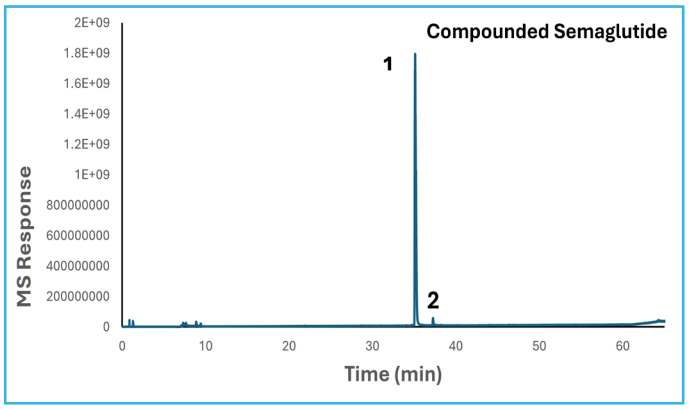

Figure 2: TIC of 100ng compounded Semaglutide.

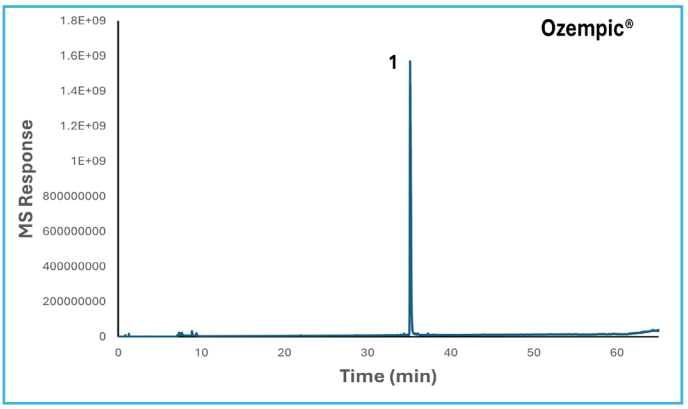

Figure 3: TIC of 100ng Pharmaceutical grade Ozempic®.

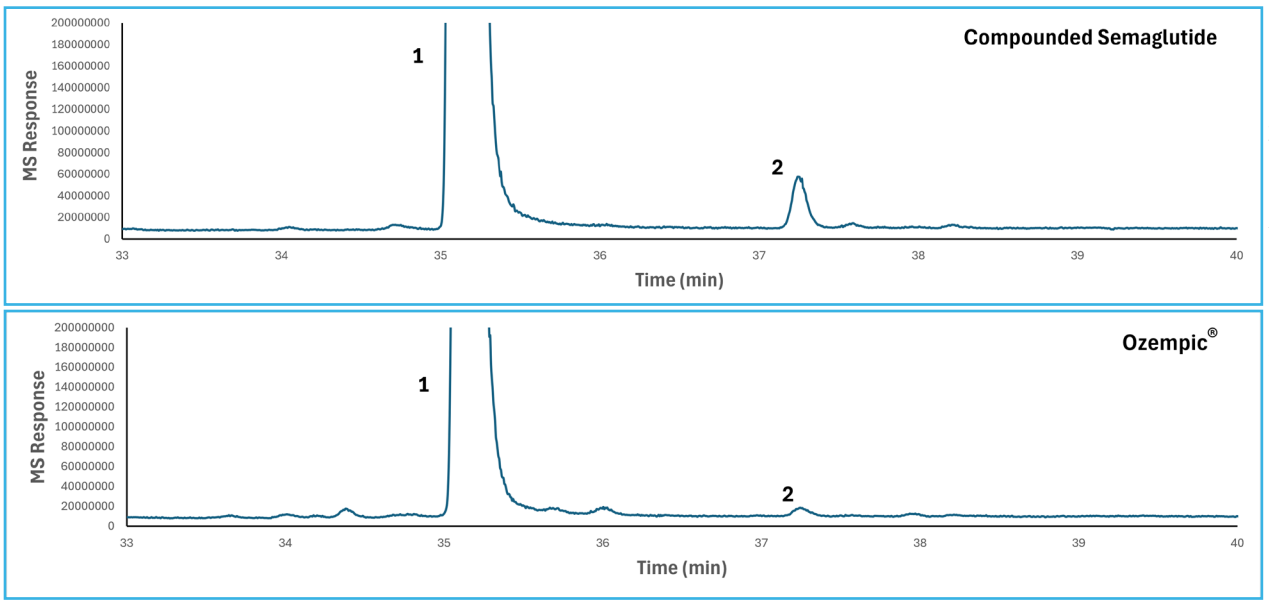

Figure 4: Zoomed-in comparison of compounded Semaglutide vs Pharma-ceutical-grade Ozempic®. The compounded Semaglutide sample shows a significant increase in the N-terminal histidine modification.

As shown in Figure 4, a significant amount of the N-terminal histidine modification is still observable. A much smaller amount is seen in the Ozempic® sample.

To compare relative amounts of each analyte, two different approaches were utilized. The first compared the abundance of the deconvoluted peaks using Thermo Freestyle software and relative amounts of each were computed, including identified Na+ and K+ adducts. The second approach integrated the peaks in the TIC. The relative amounts are shown in Table 2:

	Deconvoluted EIC	*Integrated TIC*
Compounded Semaglutide	*1.42%*	*1.77%*
Ozempic	*0.24%*	*0.47%*

Table 2: Calculated relative amount of modified N-terminal histidine versus FLP and the method by which was computed

As seen in Table 2, the amount of N-terminal histidine modification is 3-4x higher in the compounded sample than seen in the Ozempic® sample. This amount exceeds the FDA guidelines for acceptable levels of impurities in peptide pharmaceuticals and presents a risk to patients. It is unclear whether this particular modification has been investigated in regards to potency and immunogenicity. However, it is likely clear that any existing GLP-1 agonist that contains this N-terminal histidine are at risk of this modification due to improper manufacturing or handling. This includes Liraglutide, Semaglutide, Dulaglutide, Exenatide, Lixisenatide, and potential therapeutics currently in clinical trials as of this writing such as Amycretin.

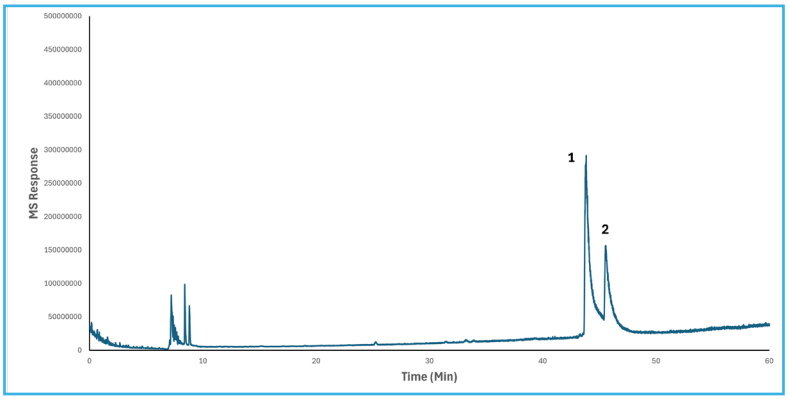

Figure 5: Separation of research-grade Semaglutide via ES-C18. The pairing of C18 with the weak ion pairing agent formic acid causes significant degradation of peak shape.

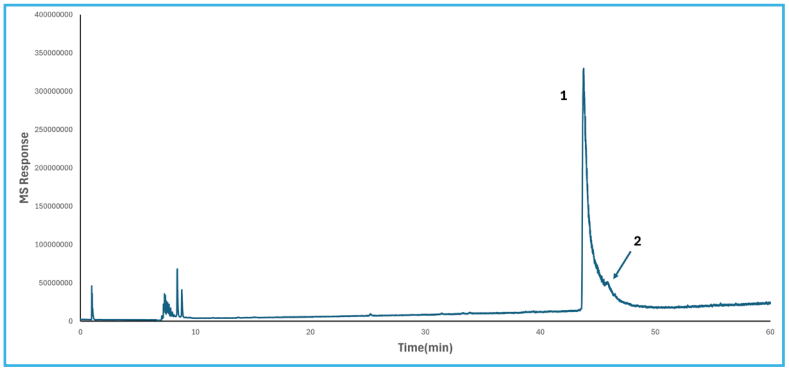

Figure 6: Separation of Compounded Semaglutide via ES-C18.

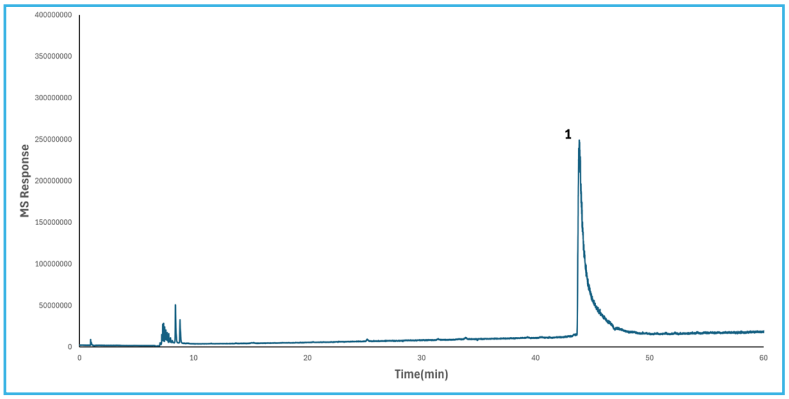

Figure 7: Separation of Ozempic® via ES-C18. Peak tailing prevents observation of any N-terminal modification.

Comparison of PCS C18 to ES-C18

In order to demonstrate the benefits of using a Positively Charged Surface C18 column in the presence of a weak ion pairing agent such as formic acid for LC/MS applications, we re-ran the Semaglutide samples described above on a HALO® ES-C18 column with the same particle size, pore size, column dimensions, and methodology. Figures 5, 6, and 7 to the left show that on a standard C18 column, peak broadening and tailing increase considerably and would likely make identification of the N-terminal histidine modification much more difficult, even with High Resolution MS.

CONCLUSION:

The HALO® PCS C18 maintains excellent peak shape and resolving power with basic compounds in weak ion pairing environments and allows for efficient detection of impurities in various biological pharmaceutical preparations. Using the HALO® PCSC18 column, we identify an impurity in a compounded Semaglutide sample that exceeds FDA guidelines and present a method that can be utilized to screen other compounded Semaglutide samples for impurity risks.

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