

## Development of Separation Methods for GLP-1 Synthetic Peptides Utilizing a Systematic Protocol and MaxPeak™ High Performance Surface Technology

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### Abstract

GLP-1s are synthetic peptide drugs used to treat type-II diabetes and obesity. Recently, GLP-1s, such as semaglutide, have risen in demand. Currently, there is not a single method capable of separating and identifying a full panel of the GLP-1s currently being prescribed today. In this application, we developed a reproducible HPLC-UV/MS method that covers a variety of GLP-1s currently on the market. We also show the capability of this method to separate and identify related impurities. Finally, we demonstrated the benefits of using MaxPeak™ High Performance Surface (HPS) Technology, when compared to traditional stainless-steel systems, for synthetic peptide analysis.

### Benefits

Successful separation methods for synthetic peptides can be generated with a systematic protocol as outlined in the MaxPeak Premier Reversed-phase Column Screening Kit.

Empower tools such as PDA peak purity processing and mass spectra data from the

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ACQUITY™ QDa™ can provide confidence in impurity identification.

MaxPeak HPS Technology was shown to improve the chromatographic peak area, height, and tailing of synthetic peptides and impurities compared to traditional stainless-steel systems.

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## Introduction

Glucagon-Like Peptide-1 Agonists (GLP-1s) are a class of synthetic peptide medications that are prescribed for the treatment and management of obesity and type-II diabetes<sup>1</sup>. Recently, drugs such as semaglutide, have boomed in popularity as a weight management treatment after success in clinical trials.<sup>2</sup> Given the prevalence of GLP-1s, it is important that the quality control for this class of pharmaceuticals is supported by versatile, sensitive, and reproducible chromatography methods. While there are chromatographic methods for some of the common GLP-1s, to our knowledge, there is not a single method to separate an updated panel of this class of synthetic peptides.<sup>3-6</sup> Further, the U.S. Food and Drug Administration (FDA) recently released Product Specific Guidelines (PSG) for some of the synthetic peptides on the market.<sup>7</sup> In this presentation, the FDA states the importance for impurity analysis of synthetic peptides.

Here we address these needs and developed a single HPLC-UV/MS method for the analysis of a variety of GLP-1s utilizing a systematic protocol for method development in combination of Waters™ MaxPeak HPS. This technology has previously been shown to mitigate undesirable metal and peptide interactions leading to improvements in chromatographic separation parameters such as chromatographic peak area count, tailing and retention time reproducibility.<sup>8,9</sup>

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## Experimental

### Stock Standard Preparation

Dulaglutide, Exenatide, Glucagon, Lixisenatide, and Tirzepatide were purchased from Selleck Chem (Houston, TX). Liraglutide and Semaglutide (acetate) were purchased from Cayman Chemical (Ann Harbor, MI). Stock concentrations varied based on solubility and amount of material purchased (Table 1). Any salt factors were

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taken into consideration during preparation. Some sonication was used to dissolve standards into solution.

Stock standard	Concentration	Diluent
Dulaglutide (DUL)	1 mg/mL	DMSO
Exenatide (EXE)	0.5 mg/mL	10 mM Ammonium Formate, pH 8.2
Glucagon (GLU)	1 mg/mL	DMSO
Liraglutide (LIRA)	0.5 mg/mL	DMSO
Semaglutide (SEMA)	0.5 mg/mL	10 mM Ammonium Formate, pH 8.2
Tirzepatide (TIRZ)	0.5 mg/mL	DMSO

*Table 1. List of stock chemicals, abbreviations, concentrations, and diluents. (DI Water is Deionized water. DMSO is Dimethyl-Sulfoxide).*

Stocks were stored at 2 °C–8 °C. Given the physical properties of DMSO, stocks solidified in cold storage. Thawed stocks were allowed to equilibrate to ambient room temperature prior to standard preparation.

## Standard Preparation

Stocks were diluted using 0.1:0.5:99.4 Trifluoroacetic Acid: Acetonitrile: Deionized Water (standard diluent). Stocks were combined and prepared so that all analytes were at a 100 µg/mL concentration (GLP-1 Drug Panel).

Further, a separate standard containing only Glucagon was made to demonstrate the use of focused gradients and how it can be used to optimize the separation of impurities. This standard was created by diluting the individual glucagon stocks to a 750 µg/mL concentration using the standard diluent.

## LC Conditions

System set-up	Premier	Traditional Stainless-Steel
LC system:	Arc™ Premier LC System	ACQUITY Arc™ LC System
Column(s):	XSelect™ Premier Peptide CSH C <sub>18</sub> 130 Å, 2.5 µm, 4.6 mm × 150 mm (P/N : 186009909)	XSelect™ Peptide CSH C <sub>18</sub> 130 Å, 2.5 µm, 4.6 mm × 150 mm (P/N : 186007038)
Detection:	Waters™ Arc™ Premier 2998 Photodiode Array Detector, 214 nm AQUITY QDa Mass Detector, ESI Positive mode, scanning from 220.0 amu to 1250.0 amu Cone voltage: 15 V Capillary voltage: 0.8kV	Waters™ 2998 Photodiode Array Detector, 214 nm
Column temp.:	60 °C	
Sample temp.:	25 °C	
Injection volume:	10 µL	
Flow rate:	0.96 mL/min	
Mobile phase A:	Deionized water with 0.1% formic acid	
Mobile phase B:	Acetonitrile with 0.1% formic acid	

## Gradient Table for GLP-1 Panel Screen

### Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.96	99.5	0.5	Initial
2.00	0.96	99.5	0.5	6
22.00	0.96	45.0	55.0	6
25.00	0.96	5.0	95.0	6
26.00	0.96	5.0	95.0	6
28.00	0.96	99.5	0.5	6
40.00	0.96	99.5	0.5	6

## Gradient Table for Glucagon Focused Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.96	80.0	20.0	Initial
2.00	0.96	80.0	20.0	6
18.00	0.96	72.0	28.0	6
21.00	0.96	5.0	95.0	6
23.00	0.96	5.0	95.0	6
25.00	0.96	80.0	20.0	6
30.00	0.96	80.0	20.0	6

## Data Management

Instrument control: Empower™ 3.7.0

Data processing: Empower 3.7.0

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## Results and Discussion

### Method of Separation Using a Screening Gradient

The systematic protocol approach for method development suggests screening a variety of columns and reversed phase mobile phase combinations to determine the most appropriate conditions to optimize chromatographic separations.

Four different columns and mobile phase eluent combinations were investigated as defined in the MaxPeak Premier Reversed-phase Column Screening Kit (Table 2).

Abbreviation of combination	Column used	Mobile phases	
		A	B
XSFA	XSelect Premier Peptide CSH C <sub>18</sub> 130 Å, 2.5 µm, 4.6 × 150 mm	0.1% Formic acid in deionized water	0.1% Formic acid in acetonitrile
XBFA	XBridge Premier Peptide BEH C <sub>18</sub> 130 Å, 2.5 µm, 4.6 × 150 mm		
XSTFA	XSelect™ Premier Peptide CSH C <sub>18</sub> 300 Å, 2.5 µm, 4.6 mm × 150 mm	0.1% Trifluoroacetic acid in deionized water	0.1% Trifluoroacetic acid in acetonitrile
XBTFA	XBridge Premier Peptide BEH C <sub>18</sub> 130 Å, 2.5 µm, 4.6 × 150 mm		

Table 2. The four combinations of columns and mobile phase investigated.

Abbreviations are used to reference the respective experiment results.

A representative chromatogram of each set of conditions clearly demonstrated the difference in retentivity when using different columns and mobile phase combination in Figure 1 for the GLP-1 panel utilized in this work.

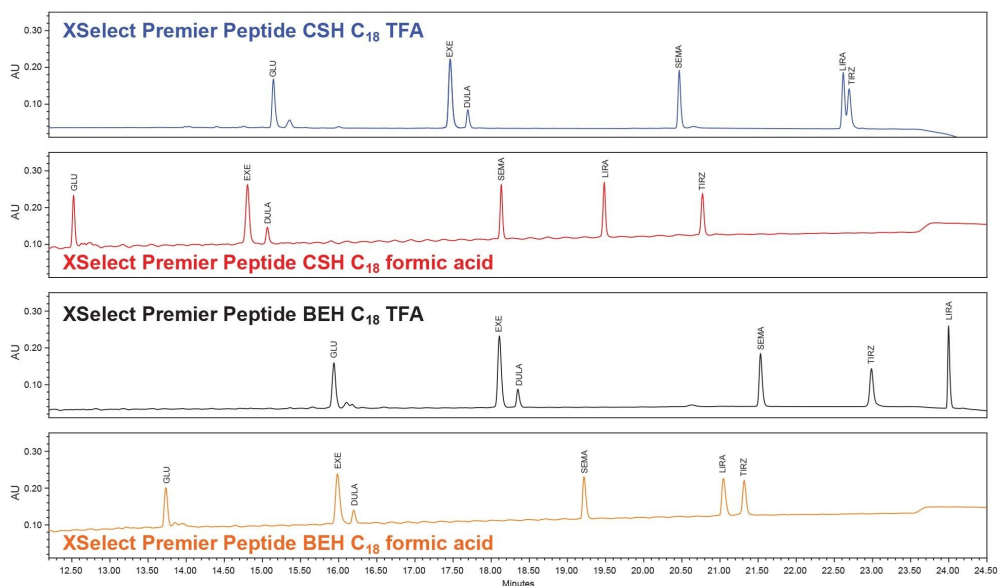


Figure 1. Stacked chromatogram demonstrating the differences of retentivity and selectivity of the GLP-1 standard mix from each of the column and mobile phase combinations.

Based on our screening experiments we selected the XSelect Premier Peptide CSH C<sub>18</sub> Column with the formic acid mobile phases for our method due to the overall speed and resolution these combinations of method conditions produced. We further chose these conditions based on the maximized chromatographic peak height performance of glucagon (Table 3).

Column and condition	Glucagon	Exenatide	Dulaglutide	Semaglutide	Tirzepatide	Liraglutide
XSTFA Premier avg. height	127634.5	187108.2	49745.0	49745.0	108515.5	154190.6
<b>XSFA Premier avg. height</b>	<b>140294.3</b>	<b>159272.1</b>	<b>44573.2</b>	<b>146996.1</b>	<b>112041.9</b>	<b>146932.8</b>
XBTFFA Premier avg. height	118788.4	191861.8	48532.2	142966.2	101143.9	223043.8
XBFA Premier avg. height	107338.5	134415.8	36411.6	114793.9	92747.2	99662.1

*Table 3. A table consisting of the average height for each column and eluent combination. Data was gathered using ten injections of the GLP-1 Drug Panel standard.*

Further, the XSelect Premier Peptide CSH C<sub>18</sub> Column provided better separation of the glucagon desamido impurities when compared to the other column and mobile phase combinations as shown in Figure 2. Glucagon has four common desamido impurities, which are a result of deamination of the peptide's amino acid residues.<sup>4,11</sup>

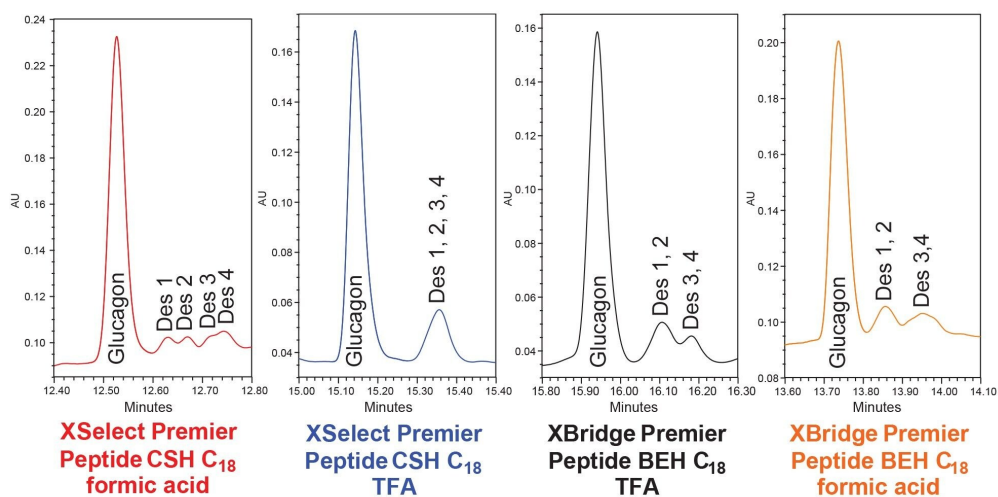


Figure 2. Chromatograms of each of the column and mobile phase combinations, demonstrating the differences in selectivity and retentivity for glucagon and its desamido impurities.

## Focused Gradient to Separate Glucagon Impurities

In Figure 2 we can see that the glucagon desamido impurities are poorly resolved using the initial screening gradient. To improve the separation of these impurities, we used a focus gradient in conjunction with the XSelect Premier Peptide CSH C<sub>18</sub> Column and formic acid mobile phases as it is described in the MaxPeak Premier Peptide Reversed-phase Column screening Kit. The gradient details are presented previously in the gradient table within the method conditions section. In Figure 3 we show the results of the optimized focused gradient for glucagon and associated desamido impurities.



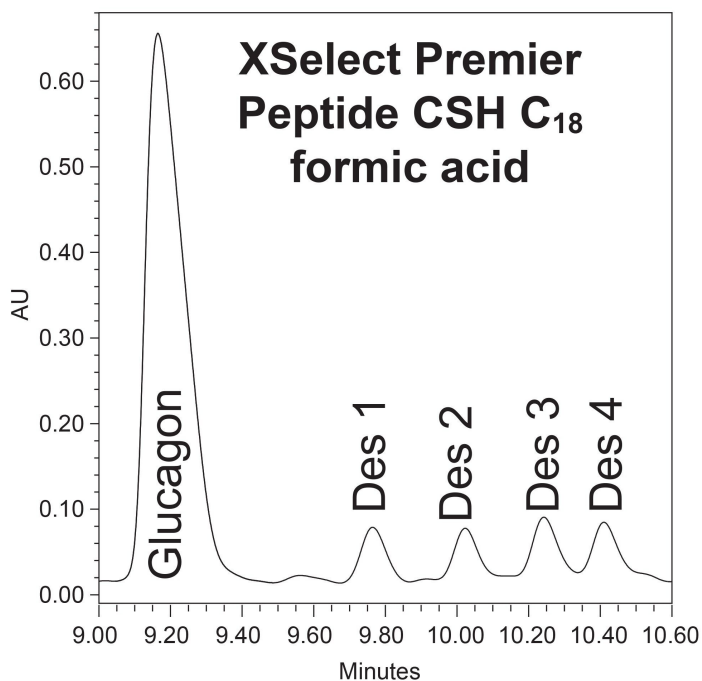
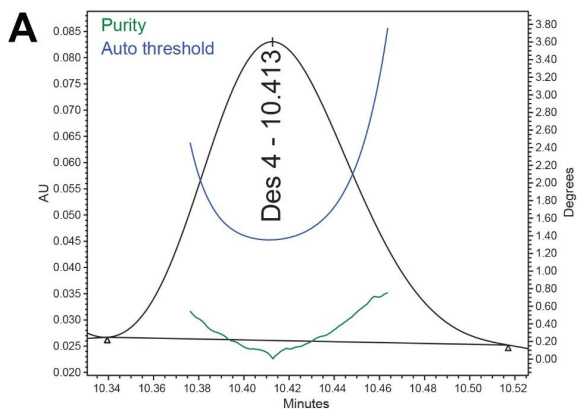


Figure 3. Representative chromatogram showing the results using the focus gradient conditions to separate the glucagon individual standard (750 ug/mL) and associated desamido impurities.

The peak purity for glucagon and the desamido impurities was confirmed with UV and mass spectra data obtained from the PDA detector and ACQUITY QDa Mass Detector (Figure 4a through 4c).



Name	Migration Time (min)	Purity1 Angle	Purity1 Threshold	Area (μV*sec)	Height (μV)	Purity1 Flag
1 Glucagon	9.168	0.120	0.368	4785486	630739	<input type="checkbox"/>
2 Des 1	9.767	0.336	1.454	307275	62482	<input type="checkbox"/>
3 Des 2	10.025	0.395	1.540	256412	56356	<input type="checkbox"/>
4 Des 3	10.245	0.402	1.258	299411	64590	<input type="checkbox"/>
5 Des 4	10.413	0.270	1.350	258058	56956	<input type="checkbox"/>

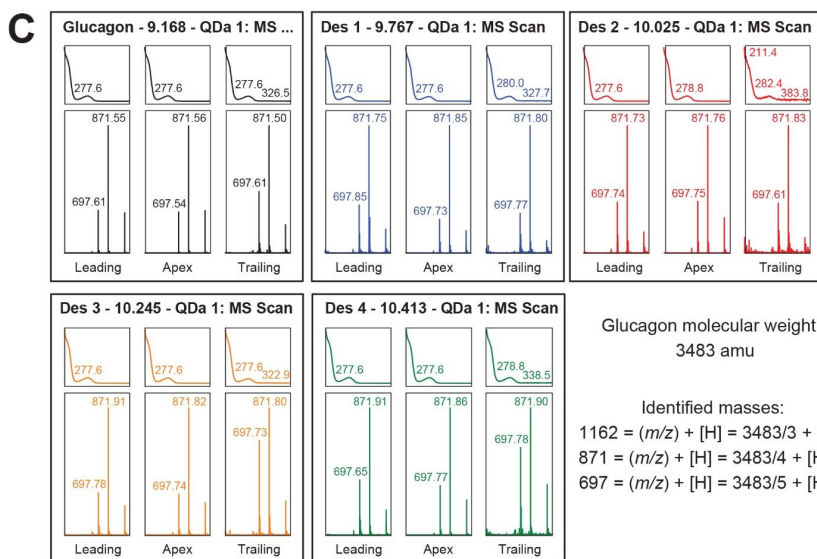


Figure 4a. An example of the UV PDA peak purity of the Des 4 chromatographic peak using the peak purity tools within Empower. Here the purity threshold is below the auto threshold indicating the peak is pure.

Figure 4b. Tabulated data associated with peak purity for glucagon and its desamidos using the peak purity tools in Empower.

Figure 4c. Mass spectral data results from the ACQUITY QDa Mass Detector for a

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representative injection of the glucagon individual standard. Here we show, the leading, apex, and trailing aspects of the MS scan of glucagon and each of its desamido peaks. Distinct ions are shown in each of the plots. The ions are associated with charged states of the glucagon peptide. Based on the mass spectra, and UV spectra, the data suggests each of the desamididos are related to glucagon.

## Comparison of Premier Technology to Tradition Stainless Steel

During our investigation of the different column and mobile phase combinations, we examined the effects that Premier Columns and Systems featuring MaxPeak HPS had on the GLP-1 Drug Panel. The results of some analytes in the panel were compared to results obtained using a traditional stainless-steel columns and systems.

When using a focused gradient on XSelect Peptide CSH C<sub>18</sub> Columns and formic acid mobile phases to separate glucagon and its impurities, we found that MaxPeak HPS increased the area by up to 19%, increased the height by up to 30%, and decreased the tailing by up to 36% (Figure 5, Tables 4 through 6).

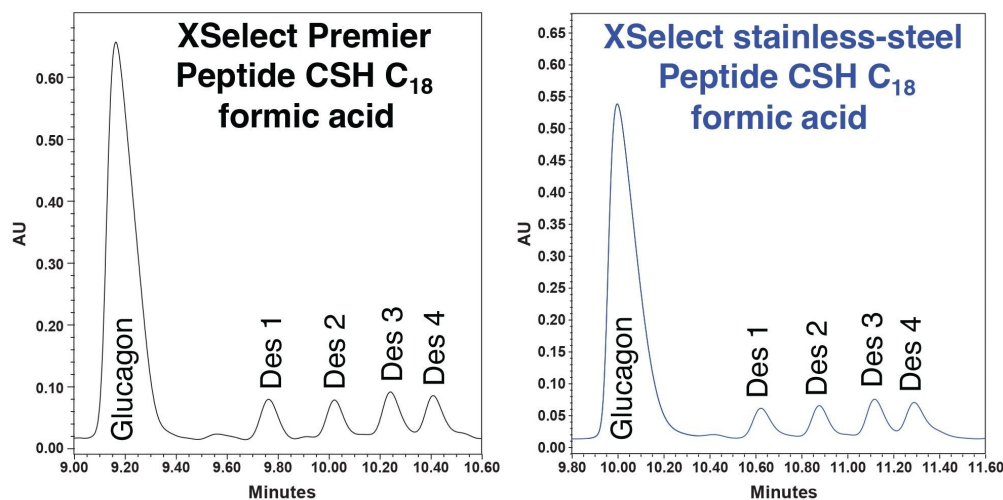


Figure 5. Representative chromatogram of method performance for both MaxPeak HPS Technology and traditional stainless-steel systems for glucagon and related desamididos impurities.

Column and eluent combination	Glucagon	Des 1	Des 2	Des 3	Des 4
XSFA Premier avg. area	4789199.7	297008.6	260446.7	304550.4	267874.7
XSFA Stainless-steel avg. area	4498615.9	261006.8	245176.3	255669.7	278562.0

Table 4. Comparison of the average peak area for 10 injections for glucagon and its desamidos between the two LC systems.

Column and eluent combination	Glucagon	Des 1	Des 2	Des 3	Des 4
XSFA Premier avg. height	641145.1	60307.2	59454.5	68144.7	60924.2
XSFA Stainless-steel avg. height	521724.9	46838.5	48271.1	52229.8	48214.2

Table 5. Comparison of the average peak height for 10 injections of glucagon and its desamidos between the two LC systems.

Column and eluent combination	Glucagon	Des 1	Des 2	Des 3	Des 4
XSFA Premier avg. tailing	1.7	1.1	1.1	1.1	1.1
XSFA Stainless-steel avg. tailing	2.0	1.2	1.2	1.1	1.7

Table 6. Comparison of the average peak tailing for 10 injections of glucagon and its desamidos between the two LC systems.

When using a screening gradient with the XBridge Peptide BEH C<sub>18</sub> Column using trifluoro-acetic acid eluents to separate semaglutide in the GLP-1 Drug Panel mix, we found that MaxPeak HPS increased the area by 20% and increased the height by up to 30% (Figure 6, tables 7 through 8).

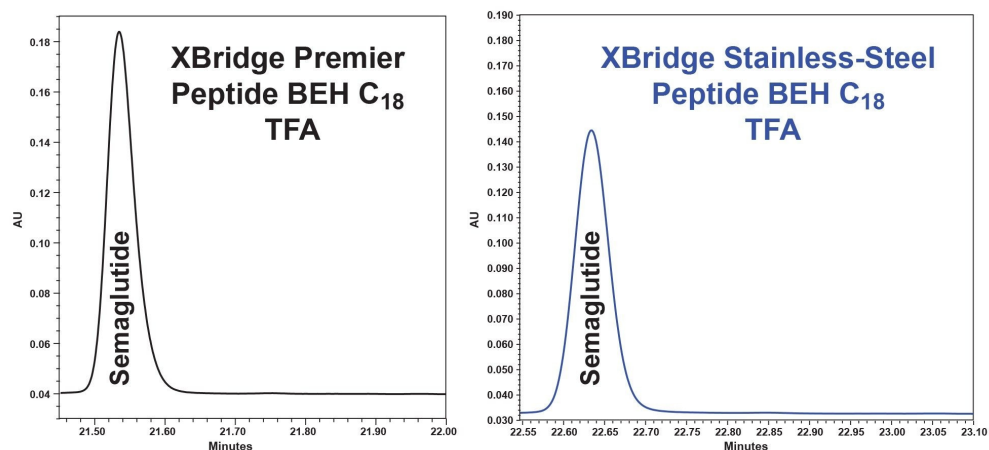


Figure 6. Representative chromatogram of method performance for both MaxPeak HPS Technology and traditional stainless-steel systems for semaglutide.

Column and eluent combination	Semaglutide
XBTF A Premier avg. area	401295.1
XBTF A Stainless-steel avg. area	333857.9

Table 7. Comparison of the average peak area for 10 injections of semaglutide between the two LC systems.

Column and eluent combination	Liraglutide
XSTFA Premier avg. area	441028.1
XSTFA Stainless-steel avg. area	345019.4

Table 8. Comparison of the average peak height for of injections for semaglutide between the two LC systems.

Finally, when using a screening gradient with the XSelect Peptide CSH C<sub>18</sub> Column using trifluoro-acetic acid eluent to separate liraglutide in the GLP-1 Drug Panel mix, we found that MaxPeak HPS increased the peak area by 28%, increased the peak height by 51%, and decreased peak tailing by 13% (Figure 7, tables 9 through 11).

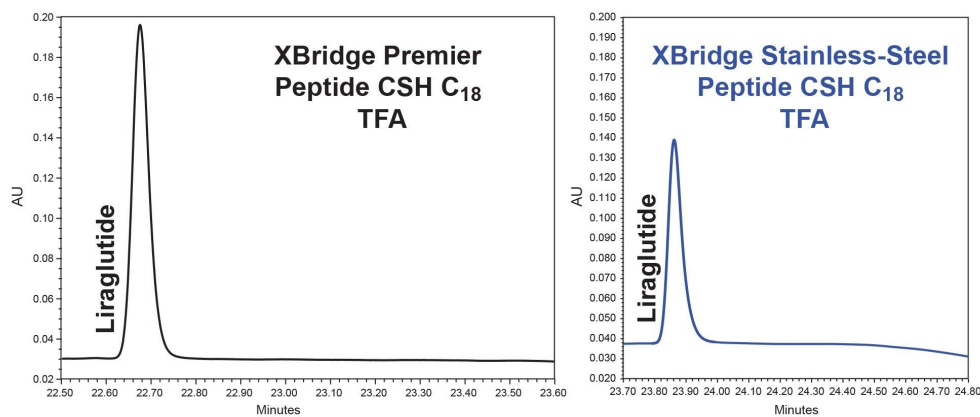


Figure 7. Representative chromatogram of method performance for both the MaxPeak HPS Technology and traditional stainless-steel systems for liraglutide.

Column and eluent combination	Liraglutide
XSTFA Premier avg. area	441028.1
XSTFA Stainless-steel avg. area	345019.4

Table 9. Comparison of the average peak area for six injections of liraglutide between the two LC systems.

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Column and eluent combination	Liraglutide
XSTFA Premier avg. height	164931.0
XSTFA Stainless-steel avg. height	108840.1

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Table 10. Comparison of the average peak height for six injections for liraglutide between the two LC systems.

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Column and eluent combination	Liraglutide
XSTFA Premier avg. tailing	1.2
XSTFA Stainless-steel avg. tailing	1.4

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Table 11. Comparison of the average peak tailing for of six injections for liraglutide between the two LC systems.

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## Conclusion

In this application note, we demonstrated the utilization of the systematic protocol outlined in the MaxPeak Premier Peptide Reversed-phase Column Screening Kit. This method development approach produced quality separations of glucagon like peptides (GLP- 1s), glucagon and associated desamido impurities. Glucagon and desamido impurities were base line separated and detected by both UV and MS detection. The purity of the chromatographic peaks was determined through the Empower Peak purity processing tool, providing information on desamido relatedness to glucagon. The use of MaxPeak HPS Technology provided increases in chromatographic peak area of up to 28%, peak height of up to 51%, and reduction in peak tailing of 13% for a variety of GLP-1s tested.

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