Application Note

Biopharma/Pharma

Agilent

Trusted Answers

An In-Depth Analysis of Semaglutide, a Glucagon-Like Peptide-1 Receptor Agonist

Comparative IP-RP analytical outcomes using different column chemistries

Authors

Andrea Angelo P. Tripodi and Andrew Coffey
Agilent Technologies, Inc.

Abstract

Peptide biotherapeutics represent a class of pharmaceuticals that hold significant importance in modern medicine due to their unique properties and diverse therapeutic applications. Peptides are short chains of amino acids, typically comprising fewer than 50 residues, and they play crucial roles in various physiological processes within the human body. With advancements in biotechnology and pharmaceutical research, the development and use of peptide-based therapeutics have surged, offering novel treatment options for a wide range of medical conditions. This application note presents some of the challenges when analyzing a glucagon-like peptide-1 (GLP-1) receptor agonist, semaglutide acetate, comparing different gradient conditions, temperatures, and column chemistries. Furthermore, sequence identification was achieved by LC/MS analysis using an Agilent AdvanceBio Peptide Plus column.

Introduction

GLP-1 receptor agonists (a group of peptide compounds) have gained importance for early-stage therapy of type II diabetes and obesity. Yet, ensuring optimal purity for these peptides presents a significant hurdle. Semaglutide (MW: 4,113.58 Da) is one of the main GLP-1 agonists commercially available today and possesses a fatty acid side chain modification (Figure 1). As with all medicinal substances, the existence of impurities arising from the manufacturing process or during storage holds the potential to compromise its safety, effectiveness, and overall quality. Crude peptides are normally analyzed by HPLC using reversed-phase columns with gradient elution using aqueous acetonitrile (ACN) (typically containing 0.1% trifluoroacetic acid (TFA) as the ion-pair reagent). TFA anions form an ion pair with positively charged peptides, increasing their hydrophobicity and therefore their retention time. However, to identify the impurity peaks in an LC/MS method, formic acid (FA) is the preferred mobile phase modifier, as the weaker acid causes less ion suppression. However, FA is less effective at suppressing nonspecific interactions, and creates a less hydrophobic (and therefore less retentive) ion pair with the peptide. Consequently, resolution when using FA as an ion-pair reagent can be compromised. This application note demonstrates how choosing different column chemistries can greatly enhance the selectivity for certain impurities, increasing the confidence in the ability to detect and quantify modifications present in the sample. Furthermore, the 250 mm long columns used for this work (PLRP-S 100 Å and 300 Å, Polaris and Pursuit) were chosen specifically because they are also available in preparative dimensions.

Figure 1. Structure of semaglutide showing differences to a native GLP-1 fragment sequence.

The characterization of semaglutide was confirmed using the Agilent 6545XT AdvanceBio LC/Q-TOF with an Agilent AdvanceBio Peptide Plus column (2.1 × 150 mm).

Experimental

Reagents and chemicals

All reagents were HPLC-grade or higher.

Analytical equipment

An Agilent 1290 Infinity II LC system consisted of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10 mm Max-Light cartridge cell (G7117-60020)

Method parameters are listed in Table 1.

LC/MS equipment

An Agilent 1290 Infinity II LC system was coupled to the 6545XT AdvanceBio LC/Q-TOF.

Software and data processing

- Agilent OpenLab software suite, version 2.6
- Agilent MassHunter data analysis software, version B.09
- Agilent MassHunter BioConfirm software, version 10.00

Table 1. LC-UV method conditions.

Parameter	Value
Agilent 1290 Infinity II Analytical LC Conditions	
Column	(A) AdvanceBio Peptide Plus, 4.6 × 150 mm (B) PLRP-S 8 µm 100 Å, 4.6 × 250 mm (C) PLRP-S 8 µm 300 Å, 4.6 × 250 mm (D) Polaris Amide C18, 5 µm, 4.6 × 250 mm (E) Polaris C18-A, 5 µm, 4.6 × 250 mm (F) AdvanceBio Peptide Mapping, 4.6 × 150 mm (G) Pursuit C18, 5 µm, 4.6 × 250 mm
Mobile Phase	Eluent A1: 0.1% TFA in Water Eluent B1: 0.1% TFA in ACN Eluent A2: 0.1% FA in Water Eluent B2: 0.1% FA in ACN
Flow Rate	1.0 mL/min
Column Temperature	25 °C
Injection Volume	10 µL
Detection	UV, 220 nm
Total Run Time	30 minutes

Table 2. LC-UV gradient optimization.

Gradient	%B	Time (min)
1	25 to 55	0 to 30
2	30 to 55	0 to 30
3	35 to 55	0 to 30
4	30 to 60	0 to 30
5	35 to 60	0 to 30
6	40 to 60	0 to 30

Sample preparation

Semaglutide acetate was purchased from Cayman Chemical and dissolved to 1.0 mg/mL in mobile phase A containing 0.1% TFA. Thermal degradation was performed by heating to 85 °C for 60 minutes.

Results and discussion

Agilent offers a variety of reversed-phase columns and media designed to simplify your synthetic peptide analytical workflows.

To determine the effect of different stationary phases on selectivity for the analysis of semaglutide reference material and semaglutide that had been thermally degraded (see conditions above), several different reversed phase products were screened.

These included columns with fully porous and superficially porous particles, columns with different pore sizes, and columns with different bonding modifications (Table 4).

Three different ACN gradients were evaluated using TFA as the ion-pair reagent in order to determine which gradient would be most suitable for testing all seven column chemistries.

Duplication injections of semaglutide reference material and semaglutide that had been thermally degraded were made on each column in turn, followed by a blank gradient to check for signs of carryover (a potential hazard with peptides that are modified with fatty acid side chain groups).

Table 3. LC/MS data acquisition parameters.

Parameter	Value
Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Dual AJS
Polarity	Positive
Gas Temperature	325 °C
Gas Flow	13 mL/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Acquisition Mode	2.5 Hz
Mass Range	100 to 2,100 *m/z*
Acquisition Rate	5 spectra/s
Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio Peptide Plus 2.1 × 150 mm
Thermostat	7 °C
Solvent A	FA 0.1% in water
Solvent B	FA 0.1% in acetonitrile
Gradient	Time (min) %A %B 0 70% 30% 5 70% 30% 50 45% 55% 51 70% 30% 56 70% 30%
Column Temperature	25 °C
Flow Rate	0.21 mL/min
Injection Volume	0.2 µL

Table 4. Column characteristics.

Product Name	Pore Size (Å)	Particle Size (µm)	Particle Type	Bonding Chemistry	Dimensions (mm)
AdvanceBio Peptide Mapping	120	2.7	Superficially porous	Endcapped C18	4.6 × 150
AdvanceBio Peptide Plus	120	2.7	Superficially porous	Endcapped C18 with charged surface modification	4.6 × 150
PLRP-S	100	8	Fully porous	Polystyrene/divinylbenzene	4.6 × 250
PLRP-S	300	8	Fully porous	Polystyrene/divinylbenzene	4.6 × 250
Polaris Amide C18	180	5	Fully porous	Amide modified C18	4.6 × 250
Polaris C18-A	180	5	Fully porous	C18 Type A	4.6 × 250
Pursuit C18	200	5	Fully porous	Endcapped C18	4.6 × 250

Figure 2 shows the chromatograms from one injection of semaglutide reference material on each of the seven columns overlaid using the gradient described in Table 2. It must be taken into consideration that the two columns packed with superficially porous materials were 150 mm long, compared to the other columns which were all 250 mm long. However, the peak shapes for all columns were sharp and relatively symmetrical. The two polystyrene/divinylbenzene columns gave slightly broader peaks, as would be expected given the larger particle size. The AdvanceBio Peptide Plus column also has a lower bonding density as a result of the presence of the charge surface modification, which accounts for the shorter retention time. The Polaris C18-A and Pursuit C18 columns gave the greatest retention time, which was likely due to higher surface area.

The same approach was taken to evaluate each column using different ACN gradients with FA as the ion-pair reagent. FA is a weaker acid than TFA and also considerably less hydrophobic. The optimum gradient was therefore different compared to the TFA separations. Figure 3 shows chromatograms from each of the columns tested, and there are noticeable changes. Firstly, the peak shape is inferior on several columns as a result of interactions between basic side chain residues of the peptide and residual acidic silanols on the surface of the silica stationary phase, leading to broader peaks and increased tailing. However, the AdvanceBio Peptide Plus, Polaris Amide C18, and Polaris C18-A all gave good peak shapes. The key difference with these materials is the presence of residual positive charge in the bonding chemistry, which helps prevent undesirable secondary interactions that result in increased tailing on other columns.

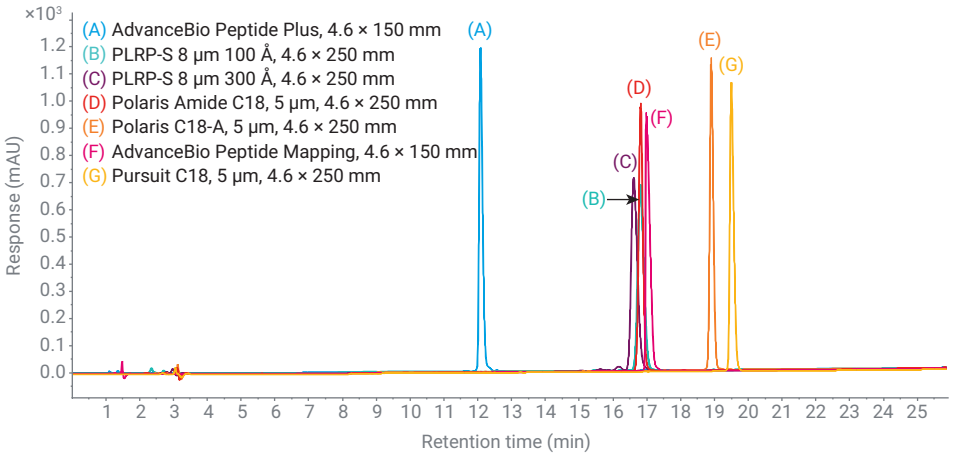

Figure 2. Comparison of analytical LC-UV chromatograms of semaglutide under 0.1% TFA conditions using gradient 5 (Table 2).

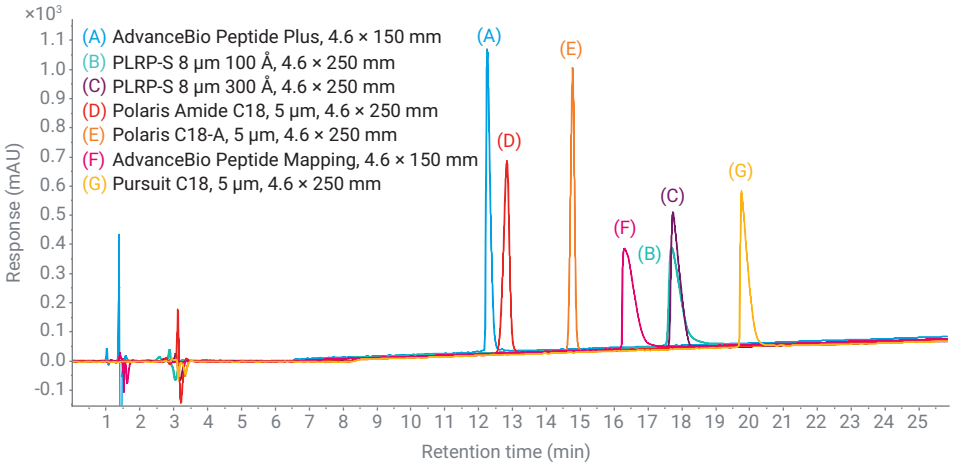

Figure 3. Comparison of analytical LC-UV chromatograms of semaglutide under 0.1% FA conditions using gradient 2 (Table 2).

Figure 4 compares the heat-treated, degraded, semaglutide sample run on the AdvanceBio Peptide Plus column with TFA and FA as ion-pair reagents. It is clear that the level of resolution remains excellent in FA. Figure 5 shows the same comparison using the Polaris Amide C18 column. Although the performance in FA is not compromised too much, there has been some loss in resolution compared to TFA.

For this reason, the AdvanceBio Peptide Plus column was chosen for LC/MS analysis using formic acid as the ion-pair reagent.

A further consideration when choosing an appropriate column for peptide analysis and purification is the pore size of the stationary phase. Although peptides are generally very small, with longer sequences or sequences that are modified, it may be beneficial to consider a wider pore size column. Larger pores do not restrict the mass transfer in and out of the pores as much as smaller pore sized columns, which can then lead to sharper peaks. This is best illustrated in Figure 6, which shows the same degraded sample analyzed on the PLRP-S 100 Å column versus PLRP-S 300 Å column. The resolution was clearly improved on the wider pore 300 Å column.

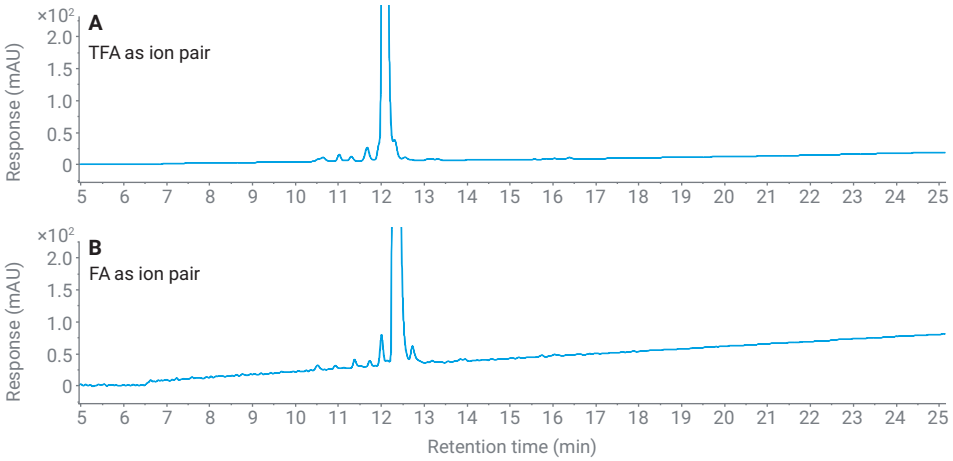

Figure 4. Comparison of heat-treated semaglutide LC-UV chromatograms showing the effect of ion-pair reagents TFA (A) using gradient 5 and FA (B) using gradient 2 with the Agilent AdvanceBio Peptide Plus column.

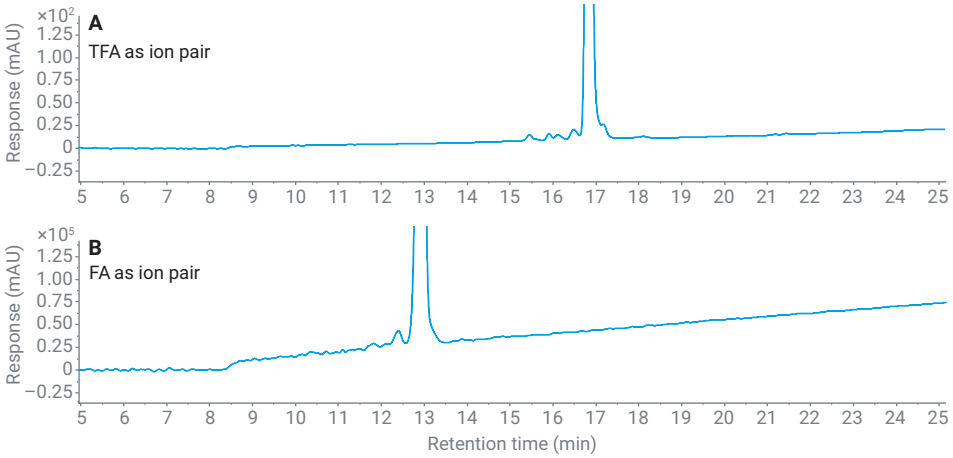

Figure 5. LC-UV chromatogram comparison of heat-treated semaglutide between TFA (A) using gradient 5 and FA (B) using gradient 2 with the Agilent Polaris Amide C18 column.

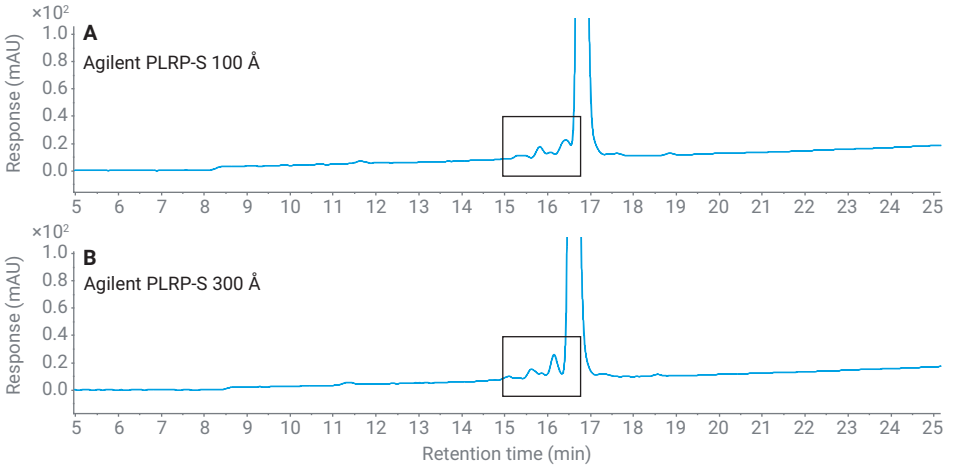

Figure 6. LC-UV chromatogram comparison of heat-treated semaglutide using Agilent PLRP-S columns, 100Å (A) and 300Å (B).

Finally, Figure 7 shows the LC/MS data for the analysis of the semaglutide reference material using the AdvanceBio Peptide Plus column. The analysis is made more complicated by the presence of the fatty acid side chain modification, however, the data clearly confirms the identity of the molecule with the expected $[M + 3H]^{3+}$ at 1,372.05, $[M + 4H]^{4+}$ at 1,029.29, $[M + 5H]^{5+}$ at 823.63, and $[M + 6H]^{6+}$ at 686.53 (Figure 8) corresponding to the full-length amino acids of semaglutide of 4,113.58 Da.

Conclusion

This application note demonstrates that it is always critically important to select the right stationary phase chemistry. Moreover, it is crucial in designing an optimal gradient, keeping in mind the differences between trifluoroacetic acid and formic acid in order to achieve maximum resolution and identification. All Agilent columns used for this work have demonstrated that the analysis of semaglutide can be easily performed and impurities well separated when using TFA as ion-pair reagent. The Agilent AdvanceBio Peptide Plus column stands out by having greater selectivity in both TFA and (most importantly) under FA conditions when compared to the C18 and PLRP-S polymeric stationary phase columns. Finally, LC/MS analysis and sequence confirmation was successfully achieved under formic acid using the Agilent AdvanceBio Peptide Plus column on an Agilent AdvanceBio Q-TOF 6545XT.

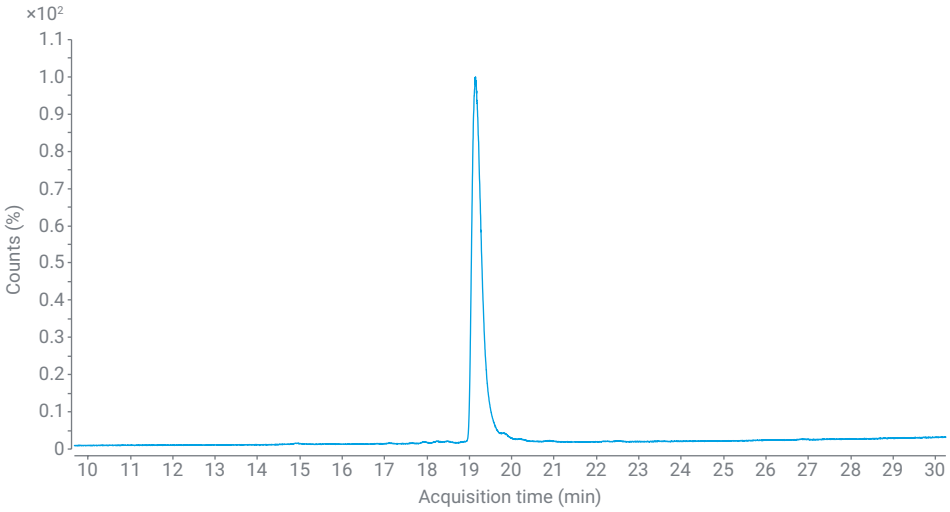

Figure 7. Total ion chromatogram (TIC) result of semaglutide acetate analyzed by LC/MS on an Agilent AdvanceBio Peptide Plus column (for method conditions, see Table 3).

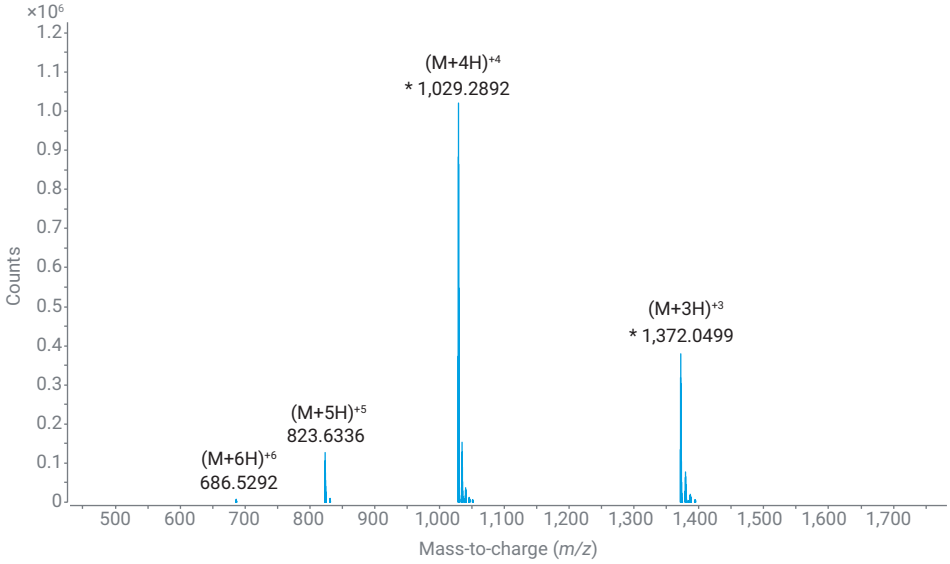

Figure 8. Mass spectrum result of semaglutide acetate analyzed by LC/MS on an Agilent AdvanceBio Peptide Plus column (for method conditions, see Table 3).

www.agilent.com

DE86081012

This information is subject to change without notice.

© Agilent Technologies, Inc. 2024
Printed in the USA, May 8, 2024
5994-7419EN

Agilent
Trusted Answers