Application Note

Biopharmaceuticals

Simple and Efficient Purification of Semaglutide Using the Agilent 1290 Infinity II Preparative LC System

Authors

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Abstract

This application note presents a method for the efficient isolation and purification of Semaglutide from drug products using an Agilent 1290 Infinity II Preparative LC System. Using the automated peak-based triggering setting based on detector signals enabled selective purification of the target compound. This preparative workflow demonstrated high fractionation accuracy over six replicate runs. The collected fractions were re-analyzed under the analytical HPLC conditions, confirming complete separation of Semaglutide from excipients. Through these processes, Semaglutide purified from drug products can be used for subsequent 3D structural analysis and characterization studies by removing the influence of excipients.

Introduction

The recent surge of interest in GLP-1 receptor agonists has spurred the active development of various GLP-1 agonists for the treatment of type 2 diabetes and obesity. Among these, Semaglutide has attracted significant attention as a long-acting peptide that requires only once-weekly administration and has demonstrated not only weight loss benefits but also cardiovascular protection.[1] Currently marketed under brand names such as Ozempic and Wegovy, Semaglutide has established itself as a groundbreaking therapy for type 2 diabetes.

As the GLP-1 therapeutics market grows rapidly, pharmaceutical companies around the world are actively entering the field of generic drug development. Notably, the U.S. Food and Drug Administration recently approved a generic version of liraglutide[2], and development of Semaglutide generics is also progressing swiftly. In response, regulatory authorities are requiring robust evidence of structural and functional equivalence to reference products to ensure quality, safety, and efficacy throughout the generic development process.

This is particularly crucial for peptide-based drugs, where evaluation must go beyond primary sequence analysis to include secondary and higher-order structural assessments. The tertiary structure of peptides is directly related to their biological function and activity, making such analyses essential. Commonly employed techniques for higher-order structure analysis include circular dichroism spectroscopy, nuclear magnetic resonance, and x-ray crystallography, which require samples of high purity and concentration.[3,4]

Considering these challenges, this study introduces a method using an Agilent 1290 Infinity II Preparative HPLC System to efficiently isolate and fractionate Semaglutide from drug products by separating it from excipients such as sodium phosphate dibasic dihydrate, propylene glycol, and phenol. While trifluoroacetic acid (TFA) is commonly used as an acidic modifier for peptide analysis, it can lead to peptide-TFA salt formation during solvent removal in preparative workflows. To minimize this risk, a lower concentration of formic acid was used instead.[5] The system's peak-based automated fractionation capability allowed straightforward collection of the main compound. Semaglutide content in the purified fractions was evaluated under dedicated analytical HPLC conditions, confirming both a high recovery rate and effective removal of excipient.[6] As a result, high-purity Semaglutide suitable for structural and physicochemical characterization was obtained, offering valuable support for future generic drug development and quality control efforts.

Experimental

Instruments

The Agilent 1290 Infinity II Preparative LC System consisted of the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with Pump Head Kit 50 mL (G7161-60023)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B) with 5 mL Sample Loop, SST, 1/8 in (5068-0334)
- Agilent 1260 Infinity III Diode Array Detector WR (G7115A) with Preparative Flow Cell, 3 mm path length (G7115-60001)

The Agilent 1260 Infinity III Prime Bio LC System consisted of the following modules:

- Agilent 1260 Infinity III Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity III Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with InfinityLab Quick Connect Heat Exchanger 1290 Bio Standard Flow and 6-Column Selector Valve (5320-0025)
- Agilent 1290 Infinity III Diode Array Detector (G7117B) with Agilent InfinityLab Bio-Inert Max-Light Cartridge Cell 60 mm (G5615-60017)

Reagents

Formic acid (FA) was purchased from Sigma-Aldrich, trifluoroacetic acid (TFA) was purchased from Merck, and acetonitrile (ACN) was purchased from B&J.

Samples

Semaglutide, Ozempic 1 mg, and Wegovy 2.4 mg were provided by a local customer. Semaglutide was dissolved in 30% ACN at a concentration of 1 mg/mL, while Ozempic and Wegovy were analyzed without further dilution.

Mobile phases

Mobile phase A

- Preparative condition: 0.05% FA in water
- Analytical condition: 0.4% TFA in water

Mobile phase B

- Preparative condition: 0.05% FA in ACN
- Analytical condition: 0.4% TFA in ACN

Columns

- Agilent Prep 100Å C18, 21.2 × 50 mm, 5 µm (part number 446905-702)
- Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm (part number 695775-949)

Methods

Table 1. Agilent 1290 Infinity II Preparative LC method parameters.

Parameter	Value
Column	Agilent Prep 100Å C18, 21.2 × 50 mm, 5 µm
Flow	20 mL/min
Injection Volume	1,000 µL
Mobile Phase	A) 0.05% FA B) 0.05% FA in ACN
Gradient	Time (min) %A %B 0 80 20 1 80 20 4 50 50 4.5 0 100 5.5 0 100 5.6 80 20 7 80 20
Detector	UV 280 nm
Fraction Collection	Peak-based, detection mode: Threshold and slope – UV threshold: 3 mAU – UV up slope: 3 mAU/s – UV down slope: 2 mAU/s

Table 2. Agilent 1260 Infinity III Prime Bio LC method parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, 2.7 µm
Flow	0.6 mL/min
Column Temperature	40 °C
Injection Volume	Varies with Semaglutide concentration
Mobile Phase	A) 0.4% TFA B) 0.4% TFA in ACN
Gradient	Time (min) %A %B 0 80 20 0.5 80 20 15 40 60 20 10 90 22 10 90 22.1 80 20 27 80 20
Detector	UV 280 nm (DAD HS with Bio-Inert Max-Light Cartridge Cell 60 mm)

Software

Agilent OpenLab CDS, version 2.8

Results and discussion

Analysis of Semaglutide and impurities under TFA conditions

Semaglutide was dissolved in 30% ACN at a concentration of 1 mg/mL for analysis, while Ozempic and Wegovy samples were analyzed without further preparation. Under high-TFA mobile phase conditions (0.4% TFA), the Semaglutide peak was observed at 11.7 minutes in both the Semaglutide API and the drug products, Ozempic and Wegovy. However, a peak eluting at approximately 1.6 minutes was detected only in Ozempic and Wegovy samples (Figure 1). This early-eluting peak corresponds to excipients present in the drug product, which tend to elute rapidly under low-ACN conditions. In contrast, the Semaglutide peak appeared at a much later retention time, clearly separated from other excipients under an ACN gradient, confirming the suitability of these conditions for preparative purification.

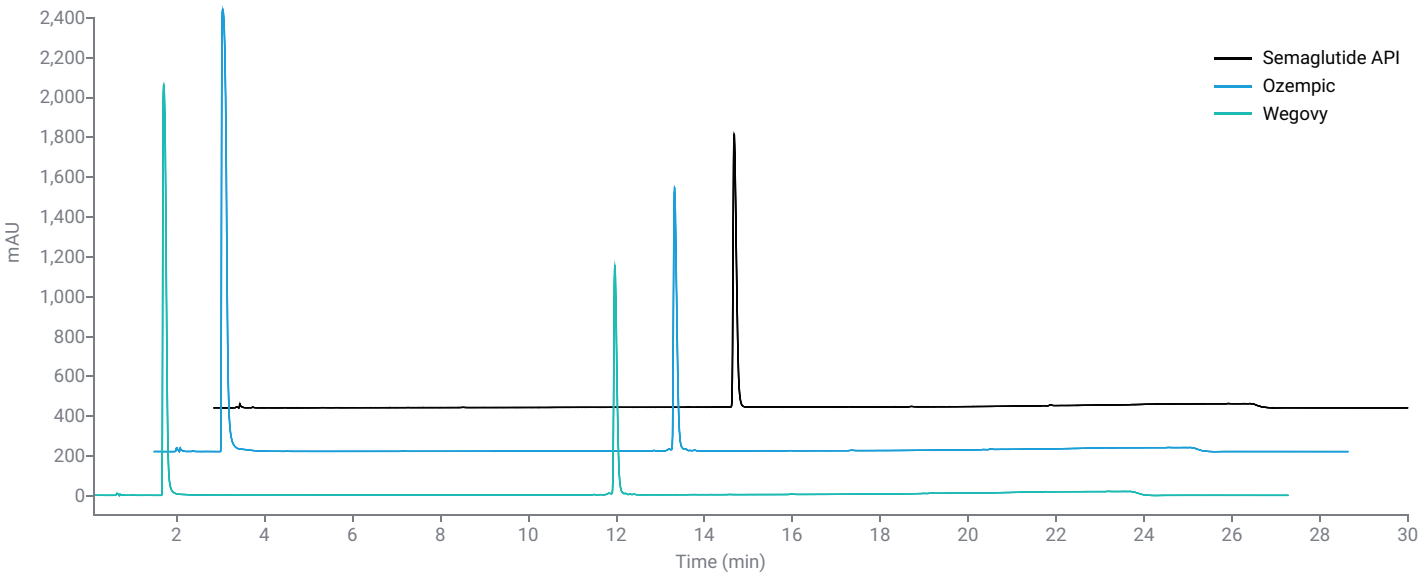

Figure 1. Chromatograms of 1 mg/mL of Semaglutide solution, Ozempic, and Wegovy under 0.4% TFA using the Agilent Infinity III Prime LC and Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm column.

Preparative HPLC conditions under TFA and FA-based mobile phases

To evaluate the suitability of mobile phase conditions for purification of Semaglutide using preparative HPLC, high concentration TFA, low-concentration FA, and acid-free conditions were compared (Figure 2). In peptide analysis, TFA can act as an ion-pairing agent, resulting in reduced peak width and improved resolution compared to FA.[7] However, TFA is prone to forming salts during the drying process of collected fractions, and the absence of an acidic modifier makes it difficult to detect the Semaglutide peak (Figure 2B). To address these limitations, a minimal amount of acidic modifier was introduced to facilitate its removal after purification. A preparative HPLC method was therefore established using 0.05% FA in the mobile phase, under which Semaglutide was stably separated from excipients.

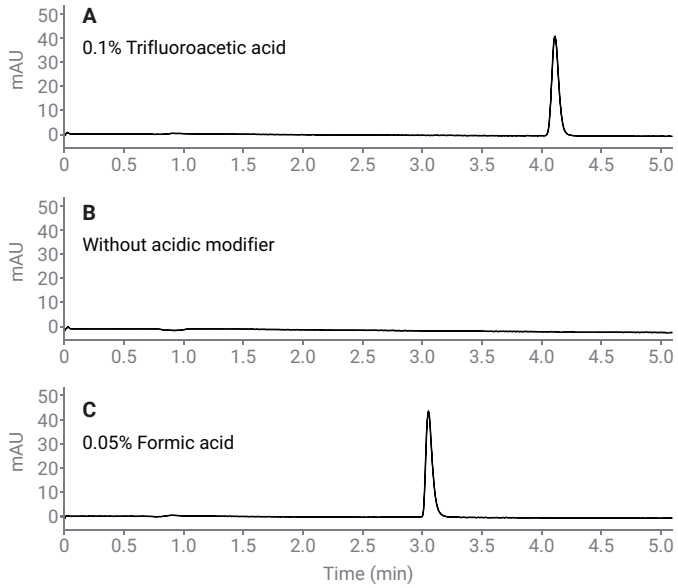

Figure 2. Chromatograms of Semaglutide (1 mg/mL) under 0.4% TFA (A), without acidic modifier (B), and 0.05% FA (C) using an Agilent 1290 Infinity II Preparative LC System and Agilent Prep 100Å C18, 21.2 × 50 mm, 5 µm column.

Preparative HPLC analysis for Semaglutide purification

Preparative analysis was performed under mobile phase conditions with 0.05% FA, optimizing flow rate and gradient conditions to maintain separation efficiency between the main compound and excipients while facilitating rapid elution. The sample injection volume was approximately 200 times higher than that used for assay analysis, increasing the sample processing capacity to 1,000 µL per injection. The fraction collection trigger was set based on peak elution between 2 and 4.5 minutes using a peak-based method. Automated fractionation was carried out according to UV signal thresholds and up/down slope criteria (Figure 3).

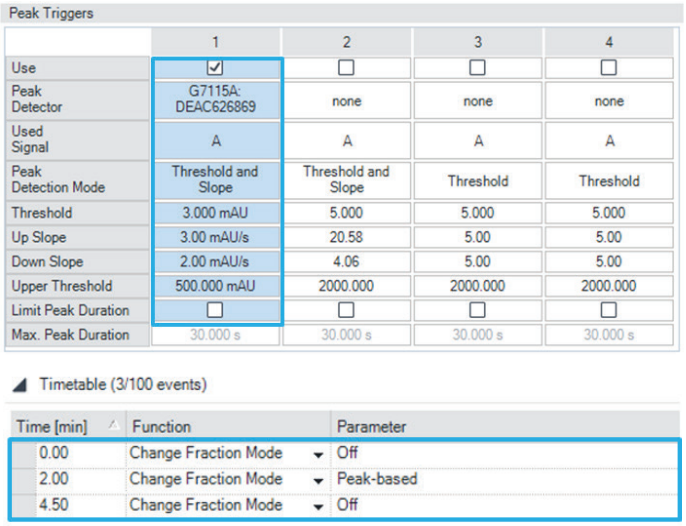

Figure 3. Fraction collector settings for preparative purification. Fractions were collected based on peak detection within the 2 to 4.5-minute window defined in the timetable, with triggering determined by preset slope and threshold values.

As a result, complete separation between Semaglutide and the excipients was achieved in both Ozempic and Wegovy samples (Figure 4). Peaks 1 and 2, corresponding to the excipient and Semaglutide respectively, were clearly resolved and collected in separate time windows. In the case of Ozempic, six repeated 1 mL injections resulted in %RSDs of 0.12% for peak retention times, and 1.08% for peak areas of each peak (Figure 5, Table 3).

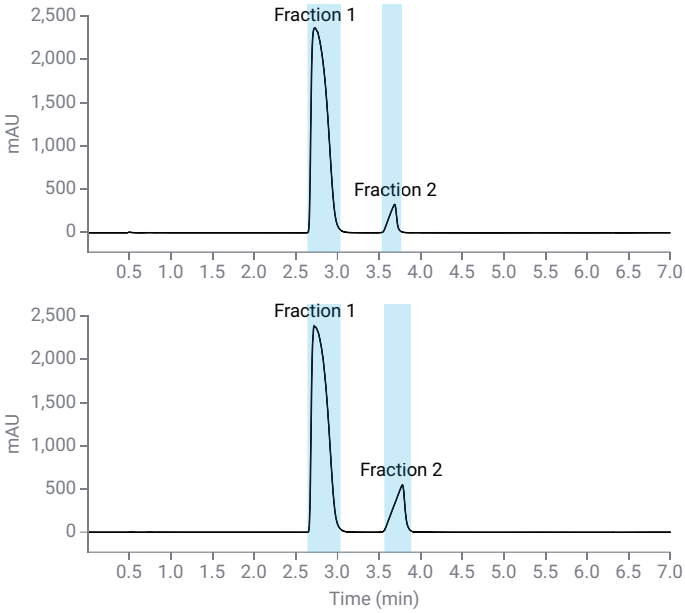

Figure 4. Chromatograms of the preparative purification for Ozempic and Wegovy. Two fractions were collected based on UV-triggered peak detection. Fraction 1: excipient; Fraction 2: Semaglutide. Blue bars represent periods of fraction collection.

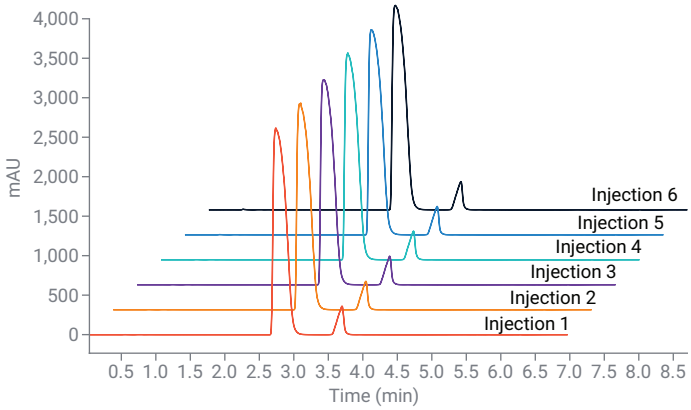

Figure 5. Chromatogram overlay (UV 280 nm) of six consecutive runs of Ozempic.

Table 3. %RSD values for the retention times of each peak obtained from six consecutive preparative injections of Ozempic 1 mg, analyzed using an Agilent Prep 100Å C18, 21.2 × 50 mm, 5 µm column.

Number	RT (min)	Start Time (min)	End Time (min)	Area (mAU·s)	Fraction Volume (mL)
1	3.69	3.44	3.88	2,097	5.51
2	3.69	3.49	3.91	2,101	5.46
3	3.69	3.49	3.90	2,133	5.42
4	3.70	3.44	3.91	2,134	5.48
5	3.70	3.47	3.89	2,138	5.50
6	3.70	3.49	3.92	2,156	5.50
Average	3.70			2,126	
%RSD	0.12			1.08	

Re-analysis of fractions for separation evaluation

To confirm the preparative purification process, each collected fraction 2 was pooled and homogenized, then re-analyzed according to the analytical HPLC method described in Table 2. The injection volumes for fractions were adjusted so that the on-column amount of Semaglutide matched the original concentration in the products. This adjustment accounted for differences in initial Semaglutide concentrations, dilution during fraction collection, and the number of injections required, and was intended to visually clarify the separation observed in the chromatograms. As a result, it was confirmed that the excipients and Semaglutide peaks were effectively separated in fractions 1 and 2 in the re-analyzed chromatogram (Figure 6).

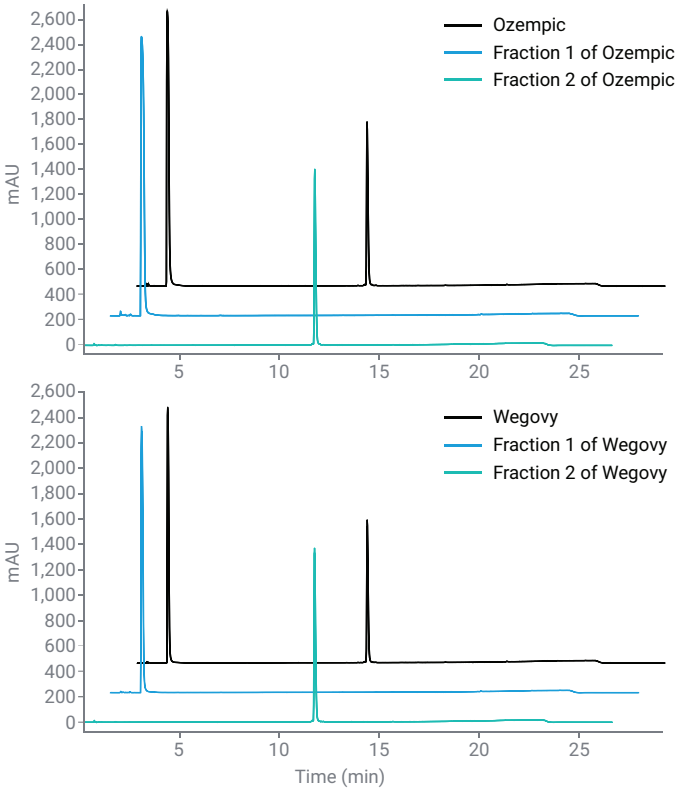

Figure 6. Re-analysis of fractions collected from Ozempic and Wegovy to confirm the clear separation of the excipient and Semaglutide peaks under the analytical HPLC method. Injection volumes of the fractions were adjusted to match the on-column Semaglutide amount of the original drug products.

Conclusion

In this study, an optimized preparative HPLC method employing a suitable acidic modifier and gradient conditions was developed to efficiently isolate semaglutide from drug products. Using formic acid as an acidic modifier minimized the risk of salt formation often associated with TFA, thus enhancing compatibility with structural analyses. Fraction collection was triggered by UV signal thresholds and slope criteria during the 2 to 4.5-minute elution window. For six replicate injections of Ozempic, the retention time and peak area of the Semaglutide peak showed excellent reproducibility, with %RSD values of 0.12 and 1.08%, respectively. Fractions were re-analyzed by analytical HPLC after adjusting injection volumes to match the original Semaglutide content. Clear separation of excipients and Semaglutide was confirmed in both fractions 1 and 2. These results demonstrate that the preparative method is effective for the simple and fast separation of Semaglutide from excipients, and suggests that isolated fractions are suitable for further structural and analytical studies.

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