Application Note

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# Efficient Method Optimization of Semaglutide Analysis Using an Agilent 1260 Infinity II Bio Prime LC System and Blend Assist


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## Abstract

This application note presents an HPLC method development for the purity testing of semaglutide, a type 2 diabetes and obesity treatment. It focuses on the impact of TFA concentration used as an acidic modifier on the separation of impurity peaks and demonstrates easy method optimization using the Blend Assist function of the Agilent 1260 Infinity II Bio Prime LC system.

Highly purified synthetic peptides show limited impurity profiles, requiring high peak-to-peak resolution for the assurance of equivalence. Additionally, a system that is highly robust against acidic modifiers and shows minimal interaction is beneficial. A combination of the Agilent AdvanceBio Peptide Plus column and an Infinity II Bio LC System proved to be an optimal combination for the analysis of modified peptides.

## Introduction

Due to recent advancements in synthetic methods, peptides consisting of fewer than 40 amino acids can now be obtained not only through biological sources using rDNA but also through chemical synthesis. Synthetic peptides exhibit favorable safety, efficacy, and specificity compared to small molecules, while also possessing lower manufacturing costs and immunogenicity compared to biological drugs.[1] The relaxed approval standards outlined in the FDA's guidance have prompted many pharmaceutical companies to actively engage in the development of synthetic peptides.

However, according to the FDA guidance, synthetic peptide substances are required to exhibit impurity levels equal to or lower than those well-identified as the Reference Listed Drug (RLD). The confirmation of the impurity profile mandates proving the safety of impurities exceeding 0.5% of the drug substance.[2] Consequently, the purity test of a drug substance becomes a critical quality attribute (CQA) in ensuring quality, serving as a benchmark for judgment in improving purification or synthetic schemes.[3]

To prevent degradation of semaglutide by DPP-4, modified amino acids were incorporated (for example, Ala at position 8 replaced with alpha-aminobutyric acid and Lys at position 28 substituted by arginine). Additionally, a long fatty acid chain was attached to the Lys at position 20, resulting in a 7-day half-life advantage.[4] However, these modifications increase hydrophobicity, causing interactions with the LC system under analytical conditions, observable as peak tailing or peak splitting.

Varied concentrations of the acidic modifier can change the hydrophobicity and ion-pairing characteristics of the analyte, resulting in changes in specificity.[5] However, the use of a high-concentration acidic modifier can impose harsh conditions for the column and the LC system, potentially compromising the robustness of the analytical system. The AdvanceBio Peptide Plus column demonstrates stability across a wide pH range and maintains stable results even under high concentrations of trifluoroacetic acid (TFA). The AdvanceBio Peptide Plus column consists of modified particles with a charged surface, providing alternate selectivity for peptide analysis. In this application note, the use of the 1260 Infinity II Bio Prime LC, which consists of a bio-compatible flow path (MP35N), resulted in low system surface interaction. Additionally, the AdvanceBio Peptide Plus column demonstrated optimal results in the analysis of various impurities related to semaglutide.

## Experimental

### Instruments

The Agilent 1260 Infinity II Bio Prime LC system comprised the following modules:

- Agilent 1260 Infinity II Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat
- Agilent 1290 Infinity II Multi-Column Thermostat (G7116B) with Quick-Connect Heat-Exchanger 1290 Bio Standard Flow (G7116-60071)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Bio-inert Max-Light cartridge cell, 60 mm (G5615-60017)

### Reagents

TFA was purchased from Merck, and acetonitrile was purchased from B&J.

### Samples

Semaglutide and Ozempic (formulated semaglutide) 1 mg were donated by a local customer. Semaglutide was dissolved in 30% acetonitrile at a concentration of 1 mg/mL, then heated at 80 °C for a day and cooled prior to analysis.

### Columns

- Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm (part number 695775-949)
- Agilent AdvanceBio Peptide Plus 2.1 × 250 mm, 2.7 µm (part number 693775-949)

### Software

Agilent OpenLab CDS 2.7

## Methods

**Table 1.** Method parameters.

| Parameter | Value |
|---|---|
| Column | TFA concentration screening:<br>AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm<br><br>Shallower gradient condition:<br>AdvanceBio Peptide Plus 2.1 × 250 mm, 2.7 µm |
| Flow | 0.4 mL/min |
| Column Temperature | 40 °C |
| Injection Volume | 5 µL (TFA screening), 10 µL (shallower gradient) |
| Mobile | A) Water<br>B) Acetonitrile<br>C) 1% TFA in water<br>D) 1% TFA in acetonitrile |
| Gradient – TFA Concentration Screening | Time (min) %(A + C) %(B + D) Flow rate (mL/min)<br>0 80 20 0.6<br>0.5 80 20 –<br>15 40 60 –<br>20 10 90 –<br>22 10 90 –<br>22.1 80 20 –<br>27 80 20 0.6 |
| Gradient – Shallower | Time (min) %(A + C) %(B + D) Flow rate (mL/min)<br>0 65 35 0.4<br>0.5 65 35 –<br>60 55 45 –<br>65 40 60 –<br>70 10 90 –<br>75 10 90 –<br>75.1 65 35 –<br>80 65 35 0.4 |
| Detector | UV 280 nm (DAD HS with Bio-inert Max-Light cartridge cell, 60 mm) |

# Results and discussion

### Blend Assist method settings for screening TFA concentration

To screen for the optimum TFA concentration using Blend Assist, the mobile phase was configured, and a gradient timetable was applied (Figure 1). Subsequently, the final concentration was adjusted and saved, ranging from 0.05% to 0.5%.

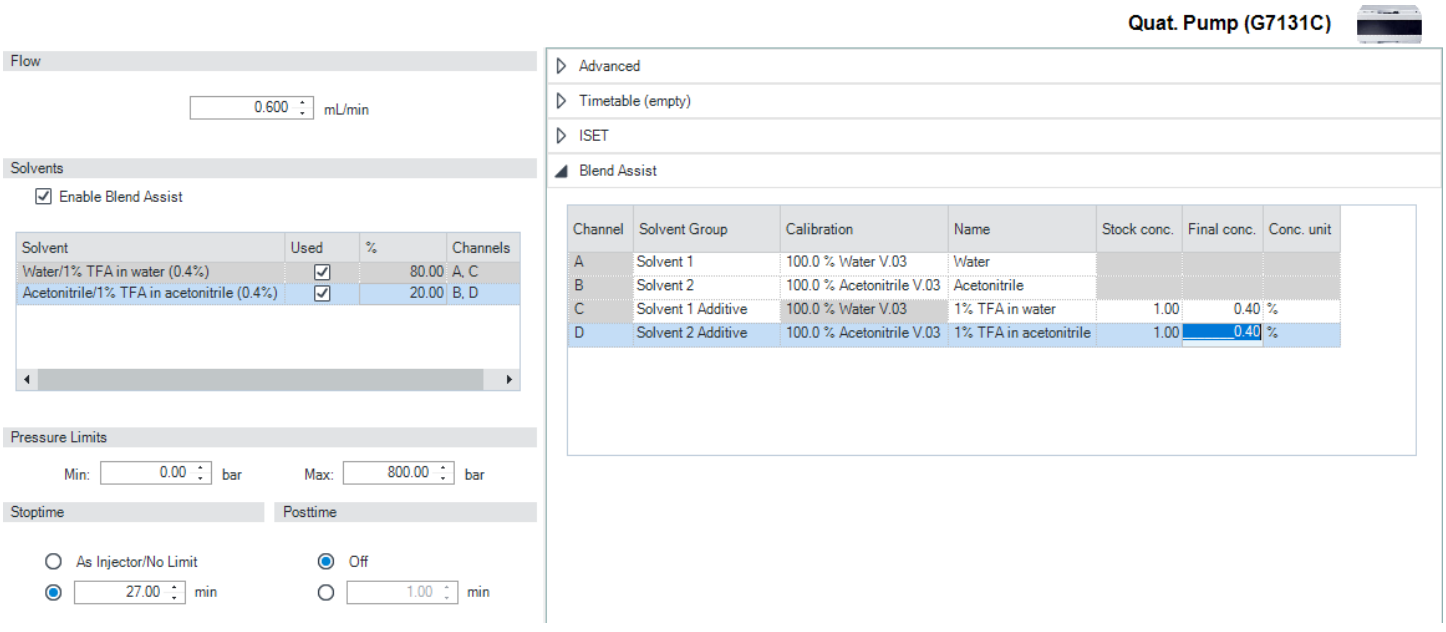


**Figure 1.** Example of Blend Assist settings for configuring the 0.4% TFA condition using 1% TFA.

## Verification of semaglutide impurity profile based on TFA concentration

Using the Blend Assist feature of the Flexible pump, TFA concentrations were manually varied to obtain diverse data. Many columns exhibited degradation due to high TFA concentrations. However, the AdvanceBio Peptide Plus column demonstrated stable performance even at 0.5% TFA concentration. To assess the resolution during an impurity separation, a sample of semaglutide solution heated to 80 °C for one day was used in the analysis. As the TFA concentration increased from 0.05% to 0.50%, it was observed that the separation of impurity peaks visually increased. In Figure 2, the highest resolution was observed at the 0.40% and 0.50% TFA conditions. Based on these conditions, additional tests were conducted to further increase resolution.

## Obtaining maximum peak resolution

Comparing impurity profiles with the RLD is a crucial CQA. A solution of semaglutide was prepared by diluting it to 1 mg/mL with 30% acetonitrile and heating it at 80 °C for one day; this solution was compared with the pre-approved drug, Ozempic. To ensure maximum resolution, a flow rate of 0.4 mL/min was set, and an AdvanceBio Peptide Plus column (2.1 × 250 mm, 2.7 µm) was used. The 1260 Infinity II Bio Flexible pump demonstrated stable retention times even under shallower gradient conditions of approximately 0.17%/min. Despite the strong acidic condition of 0.4% TFA concentration, it consistently exhibited stable peak shapes. As shown in the results in Figure 3, the impurity profile, previously observed through simple TFA concentration screening, was detected with higher resolution.

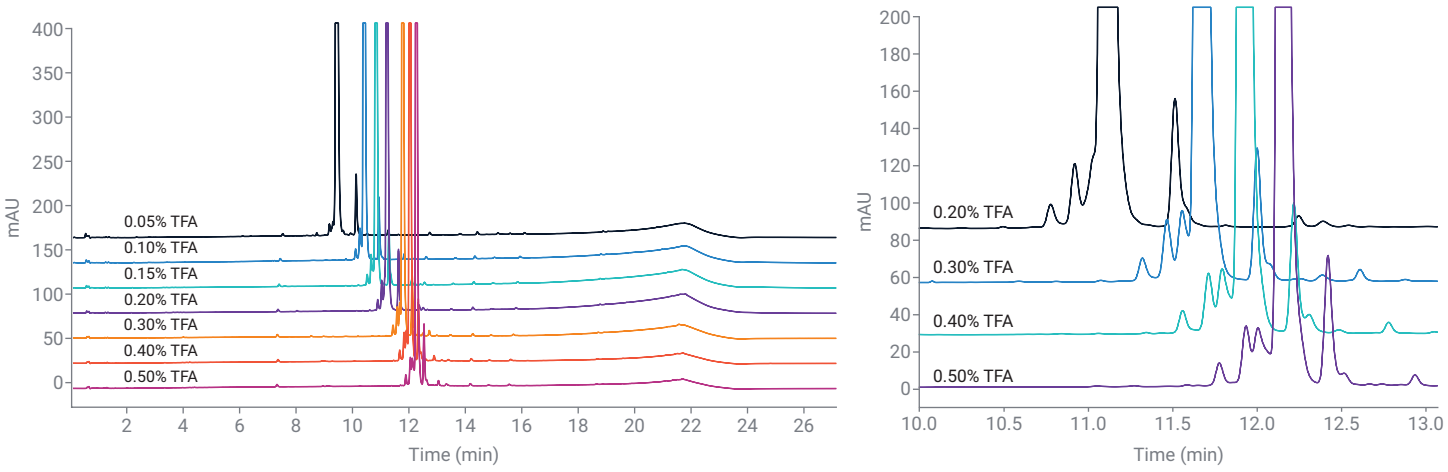


**Figure 2.** Chromatogram obtained by setting the TFA concentration using Blend Assist to a range of 0.05% to 0.50%.

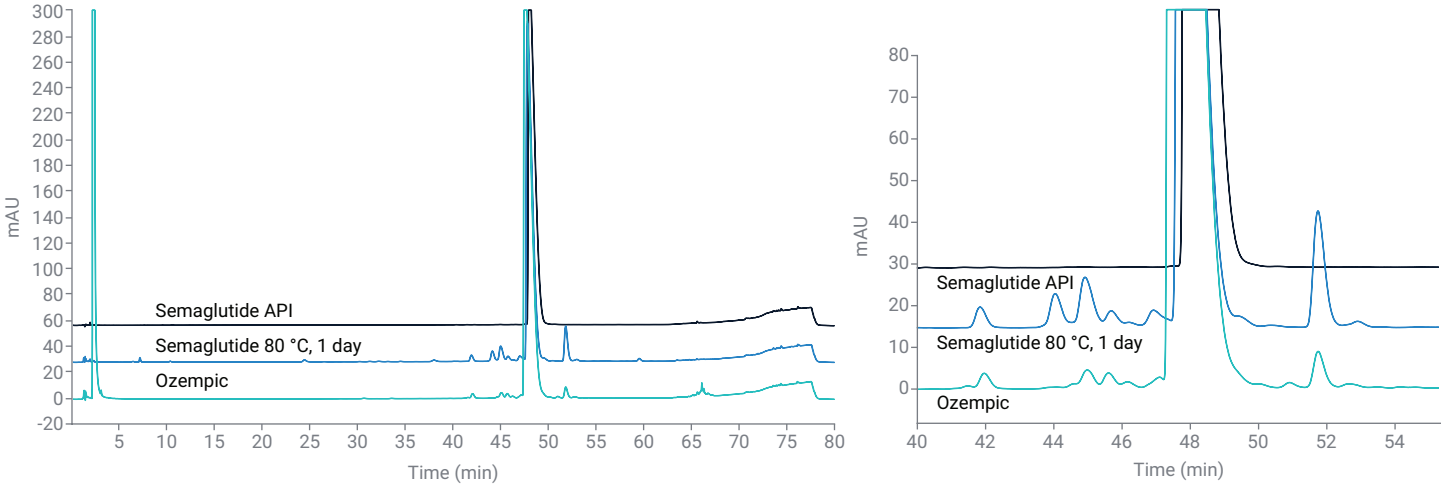


**Figure 3.** Chromatograms of semaglutide API, sample heated at 80 °C, and Ozempic (all prepared at a final concentration of 1 mg/mL) analyzed under 0.4% TFA conditions using an Agilent AdvanceBio Peptide Plus 2.1 × 250 mm, 2.7 µm column.

In Figure 4, detection down to very low concentrations (below 0.01%) was achieved by use of the Max-Light cartridge cell, 60 mm. Through method screening, increasing peak resolution allows for more rational impurity profile management.

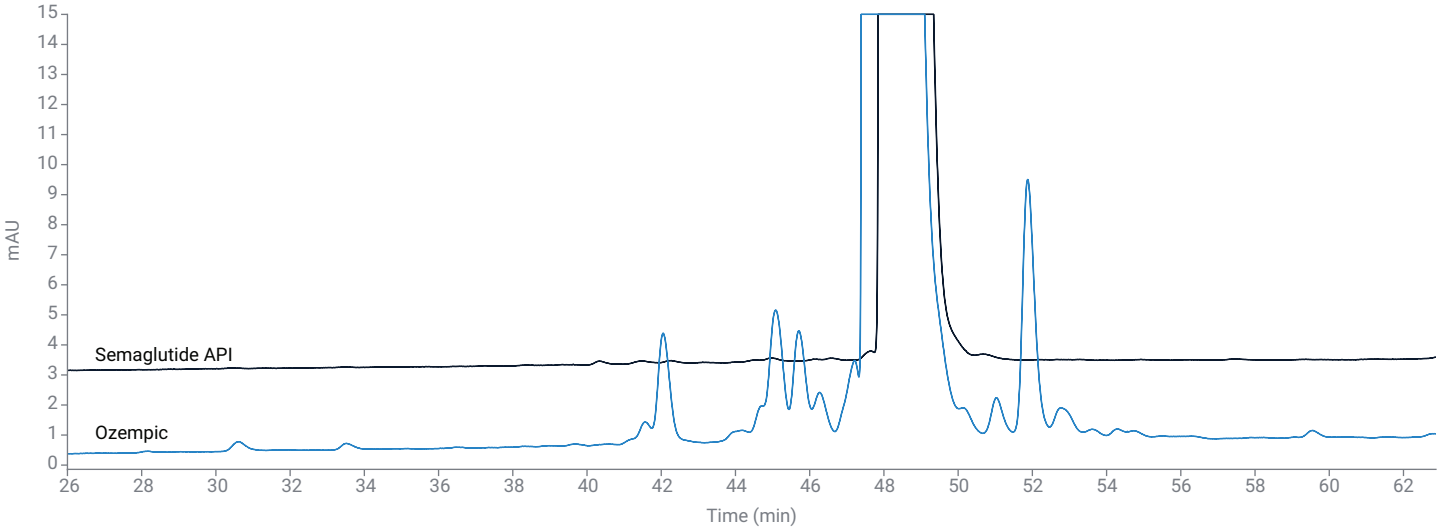


**Figure 4.** Zoomed-in chromatogram of semaglutide API and Ozempic.

## Conclusion

Due to its chemical nature, the separation of semaglutide is influenced by a combination of the LC system and column material. Achieving high resolution requires the use of an Agilent 1260 Infinity II Bio Prime LC in combination with a robust Agilent AdvanceBio Peptide Plus column, along with a system capable of delivering reliable shallow gradient performance. Furthermore, therapeutic peptides, synthesized by chemical processes, show significantly fewer impurities and quantities compared to peptides produced by biological processes. However, in contrast to synthetic small molecule drugs, impurities associated with peptide drugs exhibit characteristics very similar to the drug substance. This poses a significant challenge in the separation to achieve the necessary resolution between the drug substance and the impurities.

This application note describes an example in which the Blend Assist function simplified and helped to improve the resolution between impurities and main peak by automatically adjusting the concentration of the acidic modifier. The superior performance of the 1260 Infinity II Bio Prime LC, providing robustness even under harsh conditions and high resolution, is highlighted. Additionally, the AdvanceBio Peptide Plus column demonstrates high-resolution separation and maintains robust performance even at high TFA concentrations.

## References


1. Stefan, M. Regulatory Challenges of Nanomedicines and Their Follow-on Versions: A Generic or Similar Approach? *Advanced Drug Delivery Reviews* **2018**, *131*, 122–131.
2. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs for rDNA Origin, Guidance for Industry. *U.S. Department of Health and Human Services, FDA, CDER*, **2021**.
3. Guideline on the Development and Manufacture of Synthetic Peptides. *European Medicines Agency* **2023**.
4. Minzhi, Y.; *et al.* Battle of GLP-1 Delivery Technologies. *Advanced Drug Delivery Reviews* **2018**, *130*, 113–130.
5. Melanie, M.; Clarissa, D. Agilent 1260 Infinity II Flexible Pump with Blend Assist. *Agilent Technologies technical overview*, publication number 5991-9088EN, **2018**.


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