

DEVELOPMENT OF REVERSE PHASE ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF SEMAGLUTIDE

A. Ahmed¹, S. B. Zafar¹, S. H. A. Zafar² and M. A. Gondal³

¹Department of Biomedical Engineering, Faculty of Engineering, Science, Technology and Management, Ziauddin University, Karachi, Pakistan

²Bahria University Health Sciences Campus Karachi, Pakistan

³Cholistan University of Veterinary and Animal Sciences Bahwalpur

*Corresponding Author Email: bushra.zafar@zu.edu.pk

ABSTRACT: The biopharmaceutical industry seeks to look for advanced analytical techniques to improve the quality of medicinal products. RP-UHPLC, or reverse phase ultra-high performance liquid chromatography, is a powerful tool for the precise detection of biological compounds. The objective of the study was to develop a precise and sensitive RP-UHPLC method for peptide mapping of semaglutide that resembles a glucagon-like peptide-1 receptor agonist used to treat type 2 diabetes mellitus. The method development phase involved the optimisation of fundamental parameters, including incubation time, column temperature, and enzyme volume to achieve reliable and precise peptide separation. The chromatographic setup comprised of Waters XBridge C18 ODS column. The RP-UHPLC method was established using a 1 mg/mL stock solution of Semaglutide. The results revealed that the optimal conditions established were 6 hours of incubation time with column temperature at 37°C and 20µL of Glu-C endopeptidase. Peptide mapping results demonstrate remarkable results with high accuracy of peak separation and retention time for six specific Semaglutide peptides. This robust analytical approach suggests valuable insights for the characterization of therapeutics in pharmaceutical formulations. Moreover, the potential applicability of this method for analyzing other therapeutic proteins reflects its importance in advancing the pharmaceutical research and development sector.

Keywords: Semaglutide, Peptide mapping, Drug, Diabetes mellitus, Reverse Phase Ultra-High Performance Liquid Chromatography.

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INTRODUCTION

Diabetes mellitus is a serious public health issue that has a profound impact on a person's quality of life and functional capacities. The ongoing increase in diabetes prevalence highlights the need for novel treatments to enhance patient outcomes. Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) have become a promising class of medications for controlling blood sugar levels in diabetic patients (Pratley et al., 2018). The GLP-1RA semaglutide is available both in subcutaneous and oral formulations. Semaglutide has attracted significant attention for managing diabetes because of its high potency and extended half-life (Whitley et al., 2023; Nauck et al., 2021). Semaglutide works by stimulating the GLP-1 receptor, which increases the amount of GLP-1 hormone secreted in the gastrointestinal tract as a response to eating. GLP-1 performs its function by increasing the production of insulin which as a result reduces blood glucose levels (Gitsi et al., 2023). Moreover, if GLP-1 is present in higher concentration it also interacts with the parts of the brain that reduce appetite and signal a feeling of fullness (Marso et al.,

2016; Bakshi and Singh 2002). GLP-1 analogue Semaglutide contains an acyl group with a steric diacid in Lys26.

It is a long synthetic spacer modified to provide stability to dipeptidyl peptidase 4 by including -aminobutyric acid at position 8 (Penmetsa and Sundararajan 2019). Semaglutide is a promising option for those individuals suffering with both diabetes and cardiovascular disease as it can regulate the cardiovascular system along with glucose control (McGuire et al., 2023).

As the pharmaceutical industry explores the potential of Semaglutide-based therapeutics, there comes a critical need for robust analytical methods to confirm the identity and characteristics of this potent compound. Analytical procedures are essential for the detection, separation, and quantification of medicines and the degradants associated with them (Sibony et al., 2023; Piya et al., 2020). For Semaglutide, the improvement of correct and particular analytical strategies is essential to make certain batch-to-batch consistency, stumble on impurities, and determine the stability of the drug substance and product all through manufacturing

(Vanhoenacker et al., 2015). Therefore, in this study, the goal was to develop an analytical strategy for Semaglutide identification by using a selected RP-UHPLC approach for peptide mapping. The validation of the strategies was according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The recommendations will ensure the reliability, accuracy and consistency of Semaglutide-primarily based totally therapeutics.

METHODS

Equipment: A PDA detector at the side of the Waters Arc UHPLC for correct peptide separation and detection throughout the peptide mapping assessment was used. The Empower program has been utilized for statistics interpretation and processing. A temperature-controlled column oven was used to maximize chromatographic overall performance with the aid of preserving strong and correct column temperatures. To reap correct and steady sample injections, the gadget additionally consists of an autosampler with a 50- μ L injection loop.

Chromatographic Conditions: In this study, the peptide mapping assessment includes a Waters Xbridge-C18 ODS column 100% aqueous compatible (4.6mm $\times$ 250mm, 3.5 μ m) with the following chromatographic conditions: a go with the flow rate of 1.0 mL/min, UV detection at 215 nm, and a column temperature set at 37°C. The samples have been maintained at 6.0°C inside the sample tray in the course of the assessment. The cell section consisted of components: Mobile section A containing 0.1% trifluoroacetic acid in water, and Mobile section B containing 0.1% trifluoroacetic acid in 90% acetonitrile. The gradient elution started with 100% Mobile Section A at time 0, located via a linear boom of Mobile Section B from 0% to 35% interior 25 minutes. The gradient persevered with a boom in Mobile Section B to 48% at fifty 4 minutes, and then to 92% at sixty 4 minutes, in advance returning to 100% Mobile Section A at 67 minutes. The elution gradient was modified in addition to reaping 100% Mobile section B at 75 minutes, and it was modified to maintain until 90 minutes. Finally, the gradient again to 100% Mobile section A at 102 minutes, concluding the assessment. A 20 μ L injection amount was modified into used for all samples. These optimized chromatographic conditions allowed for the separation and identification of Semaglutide peptides, offering reliable and precise outcomes interior a walking time of 90 minutes

Preparation of Standard Solution: A solution to the reference standard was made. After preparing 180 μ L of the stock solution, 20 μ L of *Staphylococcus aureus* V-8

Protease (endoproteinase Glu-C) and 300 μ L of diluent were added. After thoroughly mixing the mixture, it was incubated for six hours at 37°C. Next, 10 μ L of the stop solution was added to halt the reaction.

Preparation of Sample Solution: A 1 mg/mL stock of Semaglutide was prepared. The sample was then concentrated using a 3 kDa centrifugal filter unit via centrifugation at 5°C to isolate the Semaglutide. After concentration, one hundred eighty μ L of concentrate was obtained. A pattern turned into accrued and combined with 20 μ L of *Staphylococcus aureus* protease V-eight (endoproteinase Glu-C) and three hundred μ L of diluent. The mixture is blended very well to get remarks on the model's stock. The pattern stock response was then located in a 37°C incubator for 6 hours to go through the important enzymatic digestion. This step permits the semaglutide peptides to go through preferential enzymatic activity. After 6 h, the enzymatic response was turned into became off by including 10 μ L of blocking off technique to the pattern stock response.

RESULTS

Method Optimization: During the improvement phase, diverse experimental parameters have been systematically explored to optimize the peptide mapping of Semaglutide for the use of RP-UHPLC and Glu-C endopeptidase enzymatic degradation. Various incubation times, column temperatures, enzyme volume, and gradient elution conditions, have been systematically varied to reap the maximum dependable and unique results.

Column Time Optimization: The preliminary technique involves the incubation time of the pattern with Glu-C endopeptidase. Multiple incubation times, starting from three hours to 24 hours, have been investigated to perceive the surest length for green peptide degradation.

Column Temperature Optimization: To improve chromatographic decision and sensitivity, the column temperature changed and adjusted at some point in technique development. Temperatures between 37°C and 45°C were explored to determine the temperature that yielded the exceptional height separation.

Enzyme Volume Variation: The quantity of Glu-C endopeptidase used for the enzymatic degradation becomes systematically changed throughout approach development. Volumes starting from 10 μ L to 30 μ L had been tested to become aware of the quantity that furnished premiere peptide cleavage.

Gradient Elution Development: The gradient elution situations for RP-UHPLC had been fine-tuned to gain green separation of the Semaglutide peptides. Different gradient profiles and elution schemes were examined to

discover the maximum appropriate one for correct peptide mapping, as shown in (Figure 1).

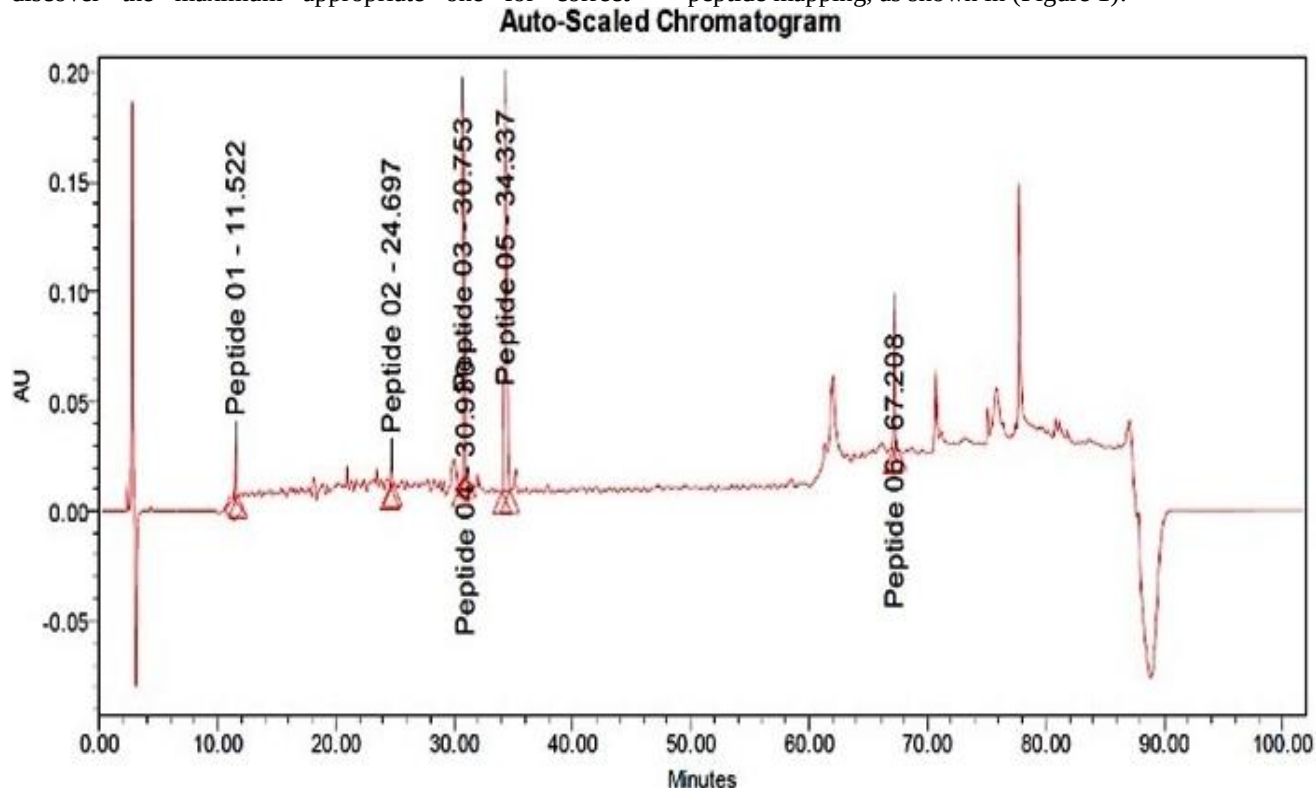


Figure 1: Chromatogram of Optimized Sample

After extensive optimization, the following conditions were identified as providing the most reliable and specific results:

Table 1: Peptide Mapping Results for Semaglutide (Development Phase).

Incubation time	Column temperature	Enzyme Volume (μ L)	Peak Separation	Peptides Identify
3 hours	37°C	10	Poor	Incomplete
6 hours	37°C	20	Optimal	Complete
12 hours	40°C	25	Improved	Complete
24 hours	45°C	30	Moderate	Cocomplete

The outcomes in (Table 1) illustrate the modern development in peptide identity and top separation with growing incubation time, column temperature, and enzyme quantity. The most fulfilling circumstance of 6 hours of incubation at 37°C with 20 μ L enzyme quantity led to entire peptide identity and first-rate top separation, which became ultimately used for the advanced method.

Developed Method Results; The advanced RP-UHPLC approach for Semaglutide peptide mapping, following the optimized situations from the approach improvement phase, efficiently recognized six peptides that matched the Semaglutide standard. Each peptide's retention time, molecular weight, and feature fragments were recorded and compared to the anticipated results as shown in (Figure 2 & Figure 3).

After a couple of iterations and optimizations,

the very last technique changed into established. The Semaglutide pattern changed into incubated with 20 μ L of Glu-C endopeptidase for six hours at 37°C. The RP-UHPLC evaluation changed into finishing the use of a Waters XBridge C18 ODS column (250x4.6 mm, 3. five μ m) 100% aqueous well suited with a column temperature of 37°C. The gradient elution profile furnished green peptide separation within a time of 102 minutes.

The evolved technique verified robustness and specificity in figuring out Semaglutide peptides accurately. The optimized conditions, cautiously decided on via systematic trials, make certain dependable and constant results, contributing to the hit peptide mapping evaluation of Semaglutide in pharmaceutical formulations.

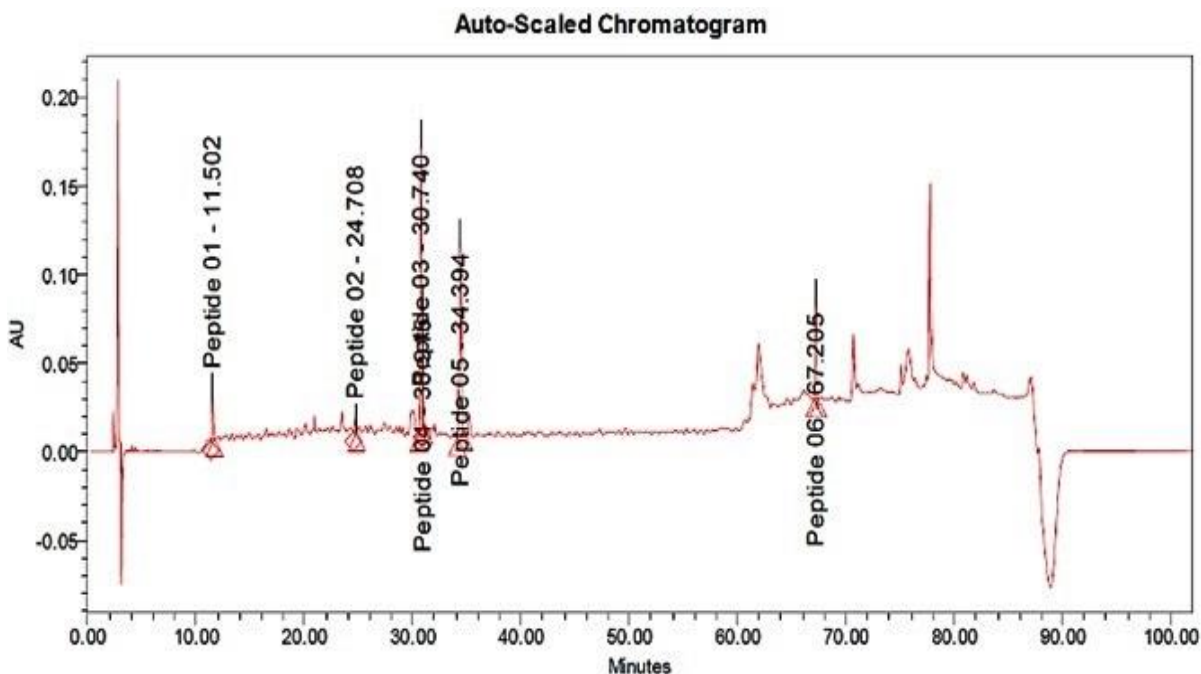


Figure 2: Chromatogram of Reference Standard

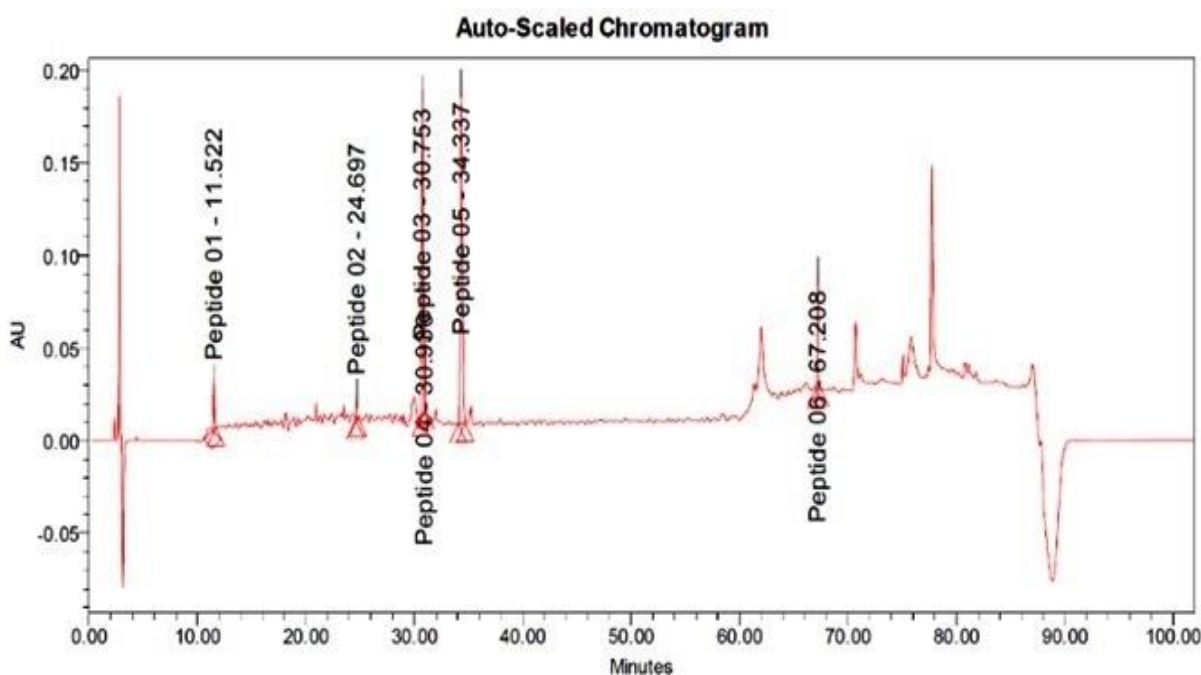


Figure 3: Chromatogram of Semaglutide sample

Table 2: Peptide Mapping Results for Semaglutide (Developed Phase)

Peptide name	Retention Time (min)	Peak Area (Semaglutide)	Peak Area (Standard)	Peak Separation
Peptide 01	11.502	175094	186965	Excellent
Peptide 02	24.708	70850	623643	Excellent
Peptide 03	30.740	1350923	1339856	Excellent
Peptide 04	30.915	411700	474589	Excellent
Peptide 05	34.395	1431017	1381638	Excellent
Peptide 06	67.205	384098	361411	Excellent

The evolved RP-UHPLC approach verified excessive sensitivity, precision, and accuracy in figuring out the precise peptides of Semaglutide. The near matching of retention instances and height regions among the Semaglutide pattern and the usual in addition helps the reliability of the approach as shown in (Table 2).

DISCUSSION

RP-UHPLC (Reversed-Phase Ultra-High-Performance Liquid Chromatography) is a powerful method for identifying and analyzing Semaglutide, a medication used to treat diabetes. Other techniques used for the identification of Semaglutide include HPLC (High-Performance Liquid Chromatography), UPLC (Ultra-Performance Liquid Chromatography), and MS/MS (Tandem Mass Spectrometry). HPLC and UPLC are commonly employed for separating peptide products like Semaglutide, while MS/MS is utilized for structural elucidation and identifying degradation products. RP-UHPLC offers advantages in terms of efficiency, sensitivity, and speed, making it preferable for precise identification and quantification in pharmaceutical research (Gumieniczek and Berecka-Rycerz 2023).

The optimization process during method development allowed for the refinement of key parameters, leading to the selection of optimal conditions. Notably, incubating the sample for six hours at 37°C with 20µL enzyme volume emerged as the most favourable condition. This advanced technique exhibited excellent peak separation and retention time accuracy for all six identified peptides, bolstering its reliability in Semaglutide identification within pharmaceutical formulations (Mhaske and Kumbhar 2023; Blebea et al., 2021; Fical et al., 2021).

In another study, RP-UHPLC was performed to investigate the peptide mapping of Insulin glargine. Similar patterns of specificity and identity were noted which validates the robustness of the current method (King et al., 2020). Similarly, another study exploring peptide mapping of Erythropoietin utilizing different enzymatic cleavage strategies and UHPLC systems corroborated the successful identification of specific peptides, further supporting the reliability of the reported method (Alley et al., 2020). Additionally, prior research on the peptide mapping of bovine serum albumin (BSA) revealed that the use of RP-UHPLC and several proteases exhibited similarities in terms of optimization efforts and height separation. Despite the variations in protein shape and sequence, the effects reflected fulfilment in accomplishing peptide identification (Fekete and Guillaume 2014). This alignment of effects throughout numerous proteins demonstrates the flexibility and applicability of the advanced technique for peptide mapping of numerous pharmaceutical compound (Fekete et al., 2014). Another study reported that peptide

mapping of Trastuzumab with the aid of Hydrophilic Interaction Liquid Chromatography (HILIC) additionally mentioned unique retention instances and top regions for unique peptides. Although the separation method differed from the one used in the reported study, their effects similarly validate the efficacy of the RP-UHPLC approach for peptide mapping (Vanhoenacker et al., 2015).

Peptide mapping of semaglutide for the usage of RP-UHPLC and Glu-C endopeptidase enzymatic degradation has established encouraging findings for the current research. For semaglutide peptides, the present-day approach provided fantastic specificity, peak separation, and identification accuracy. These consequences are per modern-day research on the peptide mapping of various proteins, which facilitates the steadiness and adaptableness of the current approach. The fulfilment of the semaglutide approach offers useful records for pharmaceutical formulation, research, and way optimization (Petry et al., 2023; Wu et al., 2023).

Through this study, it has now no longer most effectively provided a well-advanced technique however additionally tested its ability for broader programs inside the evaluation of different proteins. As researchers and analysts keep discovering peptide mapping techniques, the paintings lay a vast basis for reinforcing the excellent consistency of pharmaceutical formulations.

Future studies endeavours may also discover the use of various proteases or chromatographic strategies to similarly enhance peptide mapping methodologies. By experimenting with a much wider variety of proteases and refining chromatographic separation methods, improvements could be made to the accuracy of peptide mapping analyses. These improvements should result in a more complete and designated characterization of complicated protein structures, assisting in the identification of post-translational changes and capacity impurities. Moreover, investigations into the peptide mapping of different pharmaceutical compounds' past proteins, together with nucleic acids or lipids, can extend the knowledge of organic systems.

Conclusions: The progressed accuracy and intensity of peptide mapping analyses can contribute to the stronger and similarly powerful improvement of recovery proteins, ensuring that the immunogenic or poisonous epitopes are diagnosed and addressed eventually in drug improvement. This, in turn, also can moreover want to result in superior affected person protection and decreased unfavourable reactions eventually in scientific trials and scientific use. With the information of precise molecular developments of numerous recovery agents, scientific practitioners also can moreover want to tailor remedies to sufferers primarily based totally on their genetic make-up and unique physiological responses. This degree of customization also can moreover want to

bring about better remedy achievement expenses and higher affected person consequences, revolutionizing the present-day generation of precision medicine.

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Conflict of interest: The authors declare no conflict of interest.

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