EJPMR, 2025, 12(11), 435-443 **Review Article** **Impact Factor 7.065**

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

ISSN (O): 2394-3211
ISSN (P): 3051-2573
Coden USA: EJPMAG

A REVIEW OF THE DEVELOPMENT AND VALIDATION OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR ESTIMATING THE AMINO ACID MOLE RATIO IN SEMAGLUTIDE

Swetha Vegesna*, Mishall Masa, Sampath Sri Venkata Sai Kumar, Naga Datta Sasi Mrudula Kothapalli

Department of Pharmaceutical Analysis, School of Pharmaceutical Sciences and Technologies JNTUK Kakinada - 533003, Andhra Pradesh, India.

***Corresponding Author: Swetha Vegesna**

Department of Pharmaceutical Analysis, School of Pharmaceutical Sciences and Technologies JNTUK Kakinada - 533003, Andhra Pradesh, India.

DOI: https://doi.org/10.5281/zenodo.17577864

How to cite this Article: Swetha Vegesna*, Mishall Masa, Sampath Sri Venkata Sai Kumar, Naga Datta Sasi Mrudula Kothapalli. (2025). A REVIEW OF THE DEVELOPMENT AND VALIDATION OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR ESTIMATING THE AMINO ACID MOLE RATIO IN SEMAGLUTIDE. European Journal of Biomedical and Pharmaceutical Sciences, 12(11), 435–443.

Article Received on 17/10/2025 **Article Revised on 07/11/2025** **Article Published on 10/11/2025**

ABSTRACT

Blood sugar regulation and the treatment of type 2 diabetes depend on semaglutide, an agonist of the glucagon-like peptide-1 (GLP-1) receptor composed of a specific amino acid sequence. A precise determination of the mole ratio and amino acid composition is necessary for a peptide-based treatment to be safe, effective, and of high quality. High-Performance Liquid Chromatography (HPLC) has emerged as the most reliable and versatile analytical technique for peptide and amino acid detection due to its exceptional sensitivity, selectivity, and repeatability. This review focuses on the creation and verification of HPLC methods for measuring amino acid mole ratios in semaglutide. It discusses how important amino acid profiling is for tracking degradation, confirming peptide integrity, and figuring out batch-to-batch consistency. The study emphasizes crucial aspects of method development, including the selection of stationary and mobile phases, optimization of chromatographic conditions, and detection technique selection. The validation standards, which address crucial subjects like specificity, linearity, precision, accuracy, sensitivity, and robustness, are also specified using the International Council for Harmonisation (ICH) guidelines Q2(R1).

KEYWORDS: Semaglutide, HPLC, manufacturing machinery, and the Quality Control division.

1. INTRODUCTION

Semaglutide is a synthetic peptide with 34 amino acids. Semaglutide is a member of the pepetide family. Semaglutide, an agonist of glucagon-like peptide-1 receptors (GLP-1 AR), is used to treat type 2 diabetes.[1-2] This work aims to develop and validate an analytical method for determining the amino acid content of semaglutide that satisfies the requirements for the analytical applications for which it is intended.[3-4]

Semaglutide is a drug used to help manage chronic weight and treat type 2 diabetes. It is a member of a class of medications known as GLP-1 receptor agonists, which function by imitating a hormone that the body naturally produces. Various brand names for semaglutide are based on its permitted application and composition.

According to the International Diabetes Federation (IDF), 382 million people worldwide have diabetes, and by 2035, that figure is predicted to rise to 590 million, with up to 95% of cases being caused by type 2 diabetes.[5-6] Insulin resistance, a gradual reduction in glucose-induced insulin production, and abnormally elevated glucagon levels are the hallmarks of type 2 diabetes, which progresses over time and results in elevated blood glucose levels.[7] T2D causes difficulties over time that can be roughly categorized as either macrovascular (atherosclerotic symptoms including myocardial infarction and stroke) or microvascular (neuropathy, nephropathy, and retinopathy).

Since 2009, the European Association for the Study of Diabetes (EASD) and the American Diabetes Association (ADA) have included glucagon-like peptide-

1 (GLP-1) receptor (GLP-1R) agonists in their joint position statements on the treatment of type 2 diabetes. These agonists were first used in clinical practice in 2005.[8– 9] A wide range of the complex pathophysiology of type 2 diabetes is targeted by GLP-1R agonists. As a result, they promote weight loss, enhance glucose homeostasis without increasing the risk of hypoglycemia, and have potentially positive effects on cardiovascular parameters. Naturally, there has been a lot of clinical interest since the release of the GLP-1R agonists. The most common medications used to treat type 2 diabetes mellitus (T2DM) are glucagon-like peptide 1 receptor agonists (GLP-1RAs). GLP-1RAs are advised as first-line therapy, or suitable beginning therapy, for those with type 2 diabetes who have or are at high risk for atherosclerotic cardiovascular disease and heart failure for the following reasons: 1, 2) When feasible, GLP-1RAs are recommended over insulin in individuals with type 2 diabetes. 1, 3) For increased effectiveness and treatment effect persistence, combined therapy with a GLP-1RA is advised if insulin is taken. 1, and 4) GLP-1RAs significantly contribute to weight loss.

2. Regulatory importance of stability indicating methods

An analytical technique called a Stability Indicating Method (SIM) is capable of separating, identifying, and measuring the active pharmaceutical ingredient (API) from its breakdown products, contaminants, and excipients. Because semaglutide is a biologically active peptide medication that is susceptible to aggregation, oxidation, deamidation, and hydrolysis, stability studies are essential.

2.1. ICH Q1A (R2): New Drug Substances and Products Stability Testing Goal: Offers recommendations for creating stability tests that show how a drug's quality changes over time in response to different environmental conditions (temperature, humidity, light).[10]

Importance for semaglutide

Semaglutide is important because it guarantees the determination of its shelf life and storage conditions (e.g., refrigeration required for peptide stability).

To determine the degradation routes, stress testing is necessary (acid/base hydrolysis, oxidation, heat degradation, photostability).

To distinguish semaglutide from degradation products (such as oxidized or shortened peptide fragments), SIMs are necessary.

2.2. ICH Q2 (R1): Analytical Procedure Validation

Goal: Offers recommendations for analytical method validation parameters.

Importance for semaglutide: Guarantees that the stability analytical technique is verified for:

Specificity: Able to differentiate degradants from intact semaglutide, which is important for peptides that are susceptible to several degradation pathways.

Accuracy & Precision: Accurate measurement of semaglutide at low concentrations.

Linearity & Range: Fits well with dosage levels in formulations (such as injectable pens).

LOD & LOQ: Identifies traces of contaminants or degradants.

Robustness: The technique is still effective even with small changes in pH, mobile phase, and column aging.

Prior to regulatory approval of stability data, a validated SIM is required.[11]

3. Role of HPLC in peptide analysis

In pharmaceutical and biochemical research, High-Performance Liquid Chromatography (HPLC) is essential for both qualitative and quantitative peptide analysis. Peptides are intricate biomolecules that frequently need to be purified, identified, and quantified using high-resolution analytical techniques. HPLC is the gold standard method for peptide analysis because of its effectiveness, repeatability, and compatibility with various detection systems.[12]

3.1. Separation and purification

Peptides can be extracted from complicated mixtures such digested proteins, biological matrices, and crude synthesis products using high performance liquid chromatography (HPLC). Since the hydrophobicity of peptides varies greatly depending on the amino acid composition, reversed-phase HPLC (RP-HPLC), which uses hydrophobic stationary phases (usually C18 columns), is the most widely used technology for peptide separation. Resolution and peak sharpness are enhanced by gradient elution with acetonitrile and trifluoroacetic acid (TFA).

3.2. Identification and Characterization

By detecting minute structural variations such amino acid substitutions, truncations, or post-translational modifications, HPLC helps identify and describe peptides. It enables peptide structure clarification and molecular weight verification when used in conjunction with mass spectrometry (LC–MS). HPLC-based peptide mapping is a crucial method for confirming sequence integrity and identifying deterioration or contaminants in peptide medications such as semaglutide.[13]

3.3. Quantitative analysis and purity testing

RP-HPLC is commonly used for quantitative analysis of peptides, using UV detection at 210–220 nm, which corresponds to peptide bond absorption. This aids in formulating test concentration, potency, and purity. In order to enable precise formulation and stability investigations.[14] HPLC is also helpful in confirming the amino acid mole ratio following peptide hydrolysis.

3.4. Stability and Degradation Studies

Under stressful circumstances (oxidation, temperature changes, pH fluctuations), peptide medications are vulnerable to breakdown. The development of stability-indicating HPLC techniques ensures product safety and

effectiveness over the course of its shelf life by detecting degradation products.[15] Ion-exchange chromatography and RP-HPLC stability studies have been utilized for semaglutide in order to determine breakdown pathways and guarantee regulatory compliance (ICH Q1A, Q2(R1)).

3.5. Versatility of HPLC Modes in peptide Analysis

Various chromatographic modes are employed based on the analytical goal:

For peptide mapping and hydrophobic separation, use reversed-phase HPLC (RP-HPLC). For the analysis of charge variants, use Ion Exchange Chromatography (IEX-HPLC). Size Exclusion Chromatography (SEC-HPLC): For estimating molecular size and detecting aggregates. For polar peptides and modified amino acids, use Hydrophilic Interaction Liquid Chromatography (HILIC).[16]

Because of its adaptability, HPLC serves as a multipurpose instrument for peptide-based drug research and development, guaranteeing that compounds such as semaglutide fulfill therapeutic expectations and regulatory quality standards.

4. AMINO ACID SEQUENCE OF SEMAGLUTIDE

H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala Lys(AEEAc-AEEAc-γ-Glu-carboxyheptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-Gly-OH is the amino acid sequence for semaglutide. It is an analogue of human glucagon-like peptide-1 (GLP-1) with a number of significant changes.

Position 8 substitution: Alpha-aminoisobutyric acid (Aib) is used in place of alanine to boost resistance to the enzyme DPP-4's breakdown.

Position 26 acylation: At position 26, two units of an octanoic acid moiety (AEEA) and a glutamic acid spacer bind a fatty diacid (a C18 chain) to the lysine. This alteration increases semaglutide's half-life in the body by enabling it to attach to albumin.

Position 34 substitution: Arginine takes the role of lysine.

5. PHYSICO-CHEMICAL PROPERTIES OF SEMAGLUTIDE

5.1.1. Molecular Weight and Structure

With a chemical formula of $C_{187}H_{291}N_{45}O_{59}$ and a molecular weight of around 4113.58 Da, semaglutide is a synthetic peptide made up of 31 amino acids.[17]

There are three main differences between it and human GLP-1:

Ala8 is substituted with α-aminoisobutyric acid (Aib) to stop DPP-IV from breaking it down enzymatically, while Lys26 is substituted with arg. A glutamic acid spacer attaches a C18 fatty diacid chain to Lys20, improving reversible binding to albumin and extending half-life.[18]

Because of these structural changes, semaglutide has a long systemic circulation and good metabolic stability, enabling once-weekly dosing.

5.2. Solubility: Semaglutide has a low solubility in methanol and ethanol and a high solubility in water and aqueous buffers (pH 7–8). Multiple polar amino acid residues give it hydrophilic peptide behavior, but the C18 fatty acid that is linked gives it mild amphiphilic qualities that allow for strong albumin binding (>99%).[19]

Semaglutide is usually stabilized in saline or isotonic phosphate buffer in pharmaceutical formulations to preserve peptide integrity and solubility.

5.3. Stability: Semaglutide is sensitive to environmental stressors such heat, light, oxidation, and excessive pH, yet it exhibits good stability at physiological pH.

Conditions of stability: Temperature: 2-8 °C, pH 7.4.

5.3.1. Degradation pathways

Oxidation, hydrolysis, and deamidation, especially at the residues of methionine and asparagine.[20]

5.3.2. Formulation consideration

Repeated freeze-thaw cycles or exposure to high temperatures (>30°C) might cause peptide aggregation. As a result, stability-indicating HPLC techniques are required to track degradation products and verify semaglutide quality during production and storage.

Property	Description
Molecular Formula	$C_{187}H_{291}N_{45}O_{59}$
Molecular Weight	~ 4113.58 Da
Appearance	White to off – white amorphous powder
Solubility	Water dissolves it freely, while methanol and ethanol very marginally dissolve it.
Isoelectric Point (pl)	~ 4.9
Stability Range	Stable at PH 7.4, 2-8°c; susceptible to light, heat, and oxidation.

6. Analytical Challenges in HPLC Estimation of Semaglutide

Because of its peptide composition, structural alterations, and amphiphilic qualities, semaglutide analysis by HPLC poses a number of special difficulties. Developing reliable, accurate, and repeatable HPLC techniques for estimating amino acid mole ratios requires an understanding of these difficulties.

6.1.1. Complexity of Peptides

The 31-amino-acid peptide semaglutide has both hydrophilic and hydrophobic residues. In reversed-phase HPLC (RP-HPLC), its lengthy peptide chain and amphiphilic nature may cause strong interactions with the stationary phase, which could lead to peak broadening, tailing, or poor resolution.[21] Furthermore, shortened sequences or closely similar contaminants may co-elute, making measurement more difficult.

6.1.2. Chemical Modifications

Hydrophobicity and albumin-binding qualities are enhanced by the C18 fatty acid conjugation and the substitution of α-aminoisobutyric acid (Aib) at position 8. The composition, pH, and organic solvent gradient of the mobile phase must be carefully optimized because these changes can change retention duration and decrease solubility in purely aqueous mobile phases.[22]

6.1.3. Sensitivity to degradation

Under stressful circumstances, semaglutide is susceptible to oxidation, deamidation, hydrolysis, and aggregation. Different hydrophobicity or charge properties of degradation products can cause them to co-elute with the parent peptide, which could result in imprecise quantification.[23] Therefore, to distinguish intact semaglutide from its degradation products, an HPLC approach that indicates stability is necessary.

6.1.4. Detection Challenges

UV Detection: Peptide bonds absorb between 210 and 220 nm, although co-eluting contaminants or additives in the mobile phase (like TFA) may interfere with detection.

Derivatization: To increase detectability, hydrolyzed amino acids could need to be derivatized either before or after the column, which would complicate the procedure.[24]

MS Detection: Although coupling with mass spectrometry increases specificity, it necessitates solvent and buffer compatibility, which may restrict the selection of mobile phases.

6.1.5. Column Selection and Peak Resolution

Because semaglutide is amphiphilic, bigger peptide molecules are better accommodated by C18 reversed-phase columns with wide pore diameters (≥300 Å). Poor resolution and insufficient peptide interaction might be caused by small pores or conventional silica columns.[25] Achieving reproducible separation also requires temperature control, flow rate modification, and gradient tuning.

7. Importance of Amino Acid Mole Ratio Determination

The percentage of each amino acid in a peptide molecule is shown by the amino acid mole ratio. Determining this ratio precisely is essential for therapeutic peptides like semaglutide in order to guarantee structural integrity, biological activity, and regulatory compliance.

7.1.1. Confirmation of Peptide Identity

The precise types and proportions of amino acids, in accordance with the peptide's planned structure, are confirmed by amino acid mole ratio analysis. The therapeutic efficiency of semaglutide is maintained via precise composition verification, which guarantees that the functional domains in charge of GLP-1 receptor binding stay intact.[26]

7.1.2. Quality Control and Purity Assessment

One important quality control metric is the mole ratio of amino acids. It is able to identify:

- Peptide chain truncation or incomplete synthesis.
- Chemical changes like deamidation or oxidation.
- The existence of contaminants or degradation products linked to the procedure.

This guarantees consistency from batch to batch, which is essential for peptide treatments and regulatory approval.[27]

7.1.3. Support for Stability Studies

Peptides are susceptible to degradation under stress conditions, such as pH extremes, heat, light, and oxidation. By measuring the amino acid mole ratio after hydrolysis, researchers can:

- Identify residues prone to degradation.
- Quantify changes in peptide composition over time.
- Develop stability-indicating HPLC methods to monitor degradation products.[28]

7.1.4. Analytical Method Development and Validation

Determining the amino acid mole ratio is crucial to creating reliable HPLC techniques. It offers:

- Reference data to confirm the method's precision and accuracy.
- Proof that the processes of hydrolysis and derivatization are finished.
- A method for identifying minute chemical changes or sequence variants that can impact the effectiveness of treatment.[29]

7.1.5. Regulatory Significance

Peptide medications must be thoroughly characterized by regulatory bodies like the FDA and EMA. Determining the amino acid mole ratio:

- Verifies identification and structural integrity.
- Guarantees the medication product's consistency and purity.

- Provides the stability, safety, and efficacy documentation needed for approval.[30]

Researchers can ensure treatment reliability and satisfy regulatory requirements by combining mole ratio studies with HPLC.

8. Analytical Techniques for Amino Acid Mole Ratio Determination in Semaglutide

Step	Technique/ Method	Purpose	Key features/ Notes
Peptide Preparation	Hydrolysis (6N HCL,110°c,24h)	separates pepetide into its component amino acids.	Sensitive residues must be handled carefully to prevent deterioration.
Derivatization	Pre-column derivatization (OPA, FMOC, PITC)	increases the sensitivity of detection	chosen according to the detecting technique (fluorescence, UV)
Separation	RP-HPLC	Based on hydrophobicity, separation	Optimized mobile phase, gradient elution, and C18 column
Optional Seperation	IEX-HPLC/ HILIC	Separation for variations based on charge/polarity	For complicated mixtures, RP-HPLC is complementary.
Detection	UV(210-220nm), Fluorescence, MS/MS	Identification and Quantification	MS for high specificity, UV for routine assay
Data Analysis	Amino acid mole ratio calculation	Verifies the content of peptides	Compare to the semaglutide mole ratio in theory.
Validation	ICH Q2(R1) guidelines	LOD, LOQ, linearity, accuracy, and precision	Validation of stability-indicating methods

9. Why Regulators Require SIMs for Semaglutide

Patient Safety: Peptide degradation products may result in immunogenic responses or a reduction in effectiveness.

Quality Assurance: Verifies that during the course of its shelf life, the product retains its identity, potency, strength, and purity.

Regulatory Approval: Without verified stability data produced with a SIM that complies with ICH Q1A and Q2(R1), agencies (FDA, EMA, and CDSCO) will not approve.

Consistency: guarantees consistency between various manufacturing batches.

10. MATERIALS AND METHODS

Accurate Biopharma PVT LTD was the supplier of semaglutide. n-hexane, hydrochloric acid, sodium acetate trihydrate, acetonitrile glacial acetic acid, and analytical-grade, IP/BP/USP equivalent solvents are all available in the lab.

11. Equipment

A type of column chromatography known as high-performance liquid chromatography, sometimes known as high-pressure liquid chromatography, is widely used in biochemistry and analysis to separate, identify, and quantify active chemicals. It is an analytical technique that is frequently used to identify, quantify, and separate each component of a mixture. HPLC is a cutting-edge column liquid chromatography instrument.[31]

The HPLC procedure pushes the solvent at high pressures of up to 400 atmospheres so that the sample can be divided into distinct constituents based on variations in relative affinities. Normally, the solvent flows through the column due to gravity. HPLC typically consists of a detector that measures the retention periods of molecules, a pump that pushes the mobile phase (s) through the column, and a column that holds the packing material (stationary phase).[32]

The interactions between the stationary phase, the molecules under study, and the solvent or solvents used all have an impact on the retention time. Small amounts of the samples to be examined are added to the stream of the mobile phase, and they are slowed by particular physical or chemical reactions with the stationary phase.[33] The type of the analyte and the makeup of the stationary and mobile phases both affect the amount of retardation. The amount of time it takes for a particular analyte to elute is known as the retention time.[34]

A common solvent is any mixture of organic liquids or water that is miscible. During the analysis, the composition of the mobile phase has been altered using gradient elution. Analyte mixtures are separated by the gradient according to the analyte's affinity for the present mobile phase. The selection of solvents, additives, and gradients is influenced by the characteristics of the analyte and the stationary phase.[35]

12. HPLC Method Development

Methods are created for new products when there aren't any formal ones. For current (non-pharmacopoeia) products, alternative approaches aim to increase precision and durability while cutting costs and time.

Comparative laboratory data, including advantages and disadvantages, is provided whenever a different approach is suggested to replace an established practice. The primary active ingredient, any reaction impurities, all available synthetic intermediates, and any degradants are all separated and quantified using the HPLC method.

- The steps involved in method development are as follows:

Being aware of a medication molecule's physicochemical characteristics.
Selecting the conditions for chromatography.
Formulating an analytical plan.

- **Preparation of the sample**

1. Method enhancement
2. Verification of the method

12.1.1. The following steps are involved in method development

1. Being aware of the medication molecule's physicochemical characteristics.
2. Chromatographic condition selection.
3. Creating the analytical methodology.
4. Preparing samples.
5. Optimization of methods.
6. Validation of the method[36]

12.2. HPLC Procedure Analysis Method

The state of chromatography Setting Up the Buffer

Weigh out about 13.6 g of sodium acetate trihydrate, then add 1000 ml of water and stir well to dissolve. To lower the pH to 6.50, use glacial acetic acid or diluted sodium hydroxide.

Phase A of Mobile: Mix thoroughly, then filter using a 0.22µm filter after mixing 70 milliliters of acetonitrile with 930 milliliters of sodium acetate trihydrate solution (Buffer).[37-38] To avoid disturbances to the baseline.

In mobile phase B, acetonitrile and water are mixed 80:20 (%v/v), properly mixed, and then sonicated.

Diluent: Water.

Column: 4.6 x 250 mm, 5µm, Ultsil Amino Acid

Flow Rate: 1.0 ml/min 10µL of injection volume

The column's temperature is 40ºC.

Autosampler temperature: Ambient.

PDA/UV semaglutide detector wavelength: 254 nm, 35 minutes in length Holding on The amino acid times are shown below: (Just for information).

About 4.59 minutes for Asp, 5.22 minutes for Glu, 9.78 minutes for Ser, 10.57 minutes for Gly, 10.98 minutes for His, 12.63 minutes for Arg, 13.76 minutes for Thr, 14.49 minutes for Ala, 15.04 minutes for *Pro, 20.50 minutes for Tyr, 21.90 minutes for Val, 24.44 minutes for *Cys, 25.63 minutes for Ile, 26.07 minutes for Leu, 28.22 minutes for He, and 30.64 minutes for Lys.[39–40]

Note: Since *Pro, *Met, and *Cys are not components of the semaglutide molecule, they are not included in the system suitability solution or the amino acid calculation.

Elution Mode: Gradient, Mobile phase B: 0% for 0 minutes, 7% for 11 minutes, 12% for 13.9 minutes, 15% for 14.0 minutes, 32.0 minutes for 70%, 100% for 35.0 minutes, 100% for 42 minutes, 0% for 45 minutes, and 0% for 60 minutes.

Blank: Diluent

13. Validation parameters

System Adequacy

Getting the Standard Solution Ready:

Fill a 200 ml volumetric flask with 4 ml of the semaglutide standard stock solution. Use diluent to dilute to the appropriate level and thoroughly mix. (10 ppm semaglutide)

- Acceptance criteria: each amino acid peak's percentage RSD of area from three replicate standard solutions must not exceed 5.0.

13.1. Specificity[41]

Preparation of Standard Solution.

Acceptance criteria

Every amino acid peak that was extracted from the standard solution ought to be distinct from the others. Every peak of an amino acid that was extracted from the standard solution ought to pass the peak purity test.

13.1.2. Linearity and Range

Cover at least five locations with triplicate injections of amino acid standard solutions ranging from 12.5% to 200% of the specified level.

Determine the amino acid mole ratio of semaglutide by doing a regression analysis and reporting the linearity range.

CALCULATION

$$\text{Y – intercept value at 100\% level} = \frac{\text{Y intercept}}{\text{Area of 100\% level}} \times 100$$

Acceptance criteria

Each component should have a linear concentration versus peak area plot with a correlation coefficient (R) of at least 0.99.

Provide the intercept and slope values. The Y intercept should not exceed ±10.0% of the 100% standard solution response.

Table 1: Preparation linearity level.

Level	Percentage of the linearity level	Volume of standard solution in ml to be added (µl)	Diluted to volume (µl) with diluent	Conc. Of amino acids (%)
1	12.5	25	975	12.5
2	25	50	950	25
3	50	100	900	50
4	100	200	800	100
5	200	400	600	200

13.1.3. Precision

Six separate sample preparations of the semaglutide test sample should be prepared, and the technique should be followed for analysis in order to ensure method precision.

Acceptance criteria

In six test preparations, the percentage RSD of each amino acid mole ratio shouldn't exceed 5.0.

13.1.4. Intermediate Precision (Ruggedness)

Six separate sample preparations of the semaglutide test sample should be prepared in order to assess ruggedness. The analysis should be conducted according to the technique, taking into account various analysts, different days, different columns, and different instruments.

Acceptance criteria

- The percentage RSD of each mole ratio of amino acids in the six test preparations should not exceed 5.0.
- From procedure precision and intermediate precision, the cumulative percentage RSD of each amino acid mole ratio from twelve determinations should not exceed 5.0.

13.1.5. Accuracy

- By injecting the Semaglutide standard solution in triplicate at 80%, 100%, and 120% levels and analyzing the results according to the procedure, accuracy will be ascertained.

S.NO	Accuracy Level	Weight of semaglutide standarad in mg (A)	From solution A transfer (µl)	Diluted to volume (µl) with diluents	Conc. 0f amino acids (%)
1.	80%	2.4	200	800	80
2.	100%	3.0	200	800	100
3.	120%	3.6	200	800	120

14. Challenges and Future Perspectives

14.1. Challenges

- Detection limitations: low UV absorbance for certain residues;
- Stability issues: degradation can affect accuracy;
- Chemical modifications: alter hydrophobicity and retention times;
- Peptide complexity: amphiphilic nature affects separation;
- Method reproducibility: variability in hydrolysis and derivatization

14.2. Future Perspectives

- Regulatory harmonization: standardized protocols for amino acid mole ratio analysis;
- Automation: microfluidics and automated derivatization for high throughput;
- Advanced HPLC techniques: UPLC, multi-dimensional chromatography;
- LC-MS integration: simultaneous quantification and identification;
- Bioinformatics: predictive modeling of peptide retention and derivatization.[42]

15. CONCLUSION

It is clear that the suggested techniques can be successfully used in routine analysis for the determination of amino acid mole ratio in semaglutide since they are accurate, precise, straightforward, sensitive, robust, and quick.

REFERENCE

1. Shelke, S., & Singh, N. Analytical method development and validation of impurity profile in semaglutide. African Journal of Biomedical Research, 2024; 27(3S).
2. Manasa, M., & Aanandhi, M. Stability indicating RP-UPLC method development, validation, and dissolution testing of semaglutide. Journal of Pharmaceutical Negative Results, 2022; 13(4): 1459–1464.
3. Zhang, B., Xu, W., Yin, C., & Tang, Y. Characterization of low-level D-amino acid isomeric impurities in semaglutide using RP-UPLC-HRMS/MS. Journal of Pharmaceutical and Biomedical Analysis, 2022; 215: 115164.
4. Bartolomeo, M. P., & Maisano, F. Validation of a reversed-phase HPLC method for quantitative amino acid analysis. Journal of Biomolecular Techniques, 2006.

5. L. Guariguata, By the numbes: new estimates from the IDF Diabetes Atlas for 2012. Diabetes Res Clin pract, 2012; 98.
6. International Diabetes Federation, IDF diabtes atlas sixth edition.
7. S.E. Kahn, R.L. Prigeon, D.K. McCulloch, E.J. Boyko, R.N. Bergman, M.W. Schwartz, et al.
8. D.M. Nathan, J.B. Buse, M.B. Davidson, E. Ferrannini, R.R. Holman, R. Sherwin, et al., Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes, Diabetes Care, 2009; 32: 193-203.
9. S.E. Inzucchi, R.M. Bergenstal, J.B. Buse, M. Diamant, E. Ferrannini, M. Nauck, et al., Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) Diabetes Care, 2012; 35: 1364-1379.
10. ICH Harmonised tripartite guidelines, 2003.
11. ICH Harmonised tripartite guideline, 2005.
12. Mant, C. T., Chen, Y., Yan, Z., Popa, T. V., Kovacs, J. M., Mills, J. B., Tripet, B. P., & Hodges, R. S. HPLC analysis and purification of peptides. Methods in Molecular Biology, 2007; 386: 3–55.
13. Gilar, M., Olivova, P., Daly, A. E., & Gebler, J. C. Peptide mapping by reversed- phase high-performance liquid chromatography with mass spectrometric detection. Journal of Chromatography A., 2005; 1060(1–2): 15–35.
14. Guillarme, D., Ruta, J., Rudaz, S., & Veuthey, J. L. New trends in fast and high-resolution liquid chromatography: A critical comparison of existing approaches. Trends in Analytical Chemistry, 2010; 29(1): 15–27.
15. Douša, M., Grund, J., & Gibala, P. Application of ion-exchange chromatography for separation of peptide stereoisomers. Journal of Chromatography A, 2013; 1304: 90–97.
16. Jandera, P. Stationary and mobile phases in hydrophilic interaction chromatography: A review. Analytica Chimica Acta, 2011; 692(1–2): 1–25.
17. Novo Nordisk. (2020). Ozempic® (semaglutide) injection: Prescribing information. Bagsværd, Denmark: Novo Nordisk A/S.
18. Marbury, T. C., Flint, A., Jacobsen, J. B., & Derving Karsbøl, J. Pharmacokinetics and safety of semaglutide in healthy subjects and patients with type 2 diabetes. Clinical Pharmacokinetics, 2017; 56(12): 1421–1430.
19. Müller, T. D., Finan, B., Bloom, S. R., D'Alessio, D., Drucker, D. J., & Tschöp, M. H. Glucagon-like peptide-1 (GLP-1). Nature Reviews Drug Discovery, 2017; 16(12): 745–772.
20. Gupta, R., Sharma, S., & Singh, R. Development of stability-indicating RP-HPLC method for peptide drugs: A case study on GLP-1 analogues. Journal of Pharmaceutical Analysis, 2022; 12(4): 421–431.
21. Gilar, M., Olivova, P., Daly, A. E., & Gebler, J. C. Peptide mapping by reversed-phase high-performance liquid chromatography with mass spectrometric detection. Journal of Chromatography A., 2005; 1060(1–2): 15–35.
22. Marbury, T. C., Flint, A., Jacobsen, J. B., & Derving Karsbøl, J. Pharmacokinetics and safety of semaglutide in healthy subjects and patients with type 2 diabetes. Clinical Pharmacokinetics, 2017; 56(12): 1421–1430.
23. Gupta, R., Sharma, S., & Singh, R. Development of stability-indicating RP-HPLC method for peptide drugs: A case study on GLP-1 analogues. Journal of Pharmaceutical Analysis, 2022; 12(4): 421–431.
24. Krokhin, O. V., & Spicer, V. Peptide retention time prediction in reversed-phase chromatography: A hydrophobicity-based model for peptides containing common post-translational modifications. Molecular & Cellular Proteomics, 2009; 8(5): 907–918.
25. Gilar, M., Olivova, P., Daly, A. E., & Gebler, J. C. Peptide mapping by reversed-phase high-performance liquid chromatography with mass spectrometric detection. Journal of Chromatography A., 2005; 1060(1–2): 15–35.
26. Gupta, R., Sharma, S., & Singh, R. Development of stability-indicating RP-HPLC method for peptide drugs: A case study on GLP-1 analogues. Journal of Pharmaceutical Analysis, 2022; 12(4): 421–431.
27. Marbury, T. C., Flint, A., Jacobsen, J. B., & Derving Karsbøl, J. Pharmacokinetics and safety of semaglutide in healthy subjects and patients with type 2 diabetes. Clinical Pharmacokinetics, 2017; 56(12): 1421–1430.
28. Gupta, R., Sharma, S., & Singh, R. Development of stability-indicating RP-HPLC method for peptide drugs: A case study on GLP-1 analogues. Journal of Pharmaceutical Analysis, 2022; 12(4): 421–431.
29. Krokhin, O. V., & Spicer, V. Peptide retention time prediction in reversed-phase chromatography: A hydrophobicity-based model for peptides containing common post-translational modifications. Molecular & Cellular Proteomics, 2009; 8(5): 907–918.
30. Gupta, R., Sharma, S., & Singh, R. Development of stability-indicating RP-HPLC method for peptide drugs: A case study on GLP-1 analogues. Journal of Pharmaceutical Analysis, 2022; 12(4): 421–431.
31. Rao BV, Sowjanyal GN, Ajitha A, Uma V, Rao M. A review on stability indicating HPLC method development. World Journal of Pharmacy and Pharmaceutical Sciences, 2015; 4(8): 405-423.
32. Rasaiah JC. Molecular Theory of Solutions By Arieh Ben-Naim (The Hebrew University, Jerusalem, Israel). Oxford University Press: Oxford, New York. 2006. xviii + 380. $64.50. ISBN: 0-19-929970- 6. Journal of the American Chemical Society, 2007; 129(28): 8922–8922.
33. Yadav V, Bharkatiya M. A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION.

Research Journal of life science Bioinformatics Pharmaceutical and Chemical Science, 2017; 2(6): 166. DOI-10.26479/2017.0206.12.
34. Kumar Bhardwaj S. A Review: HPLC Method Development and Validation. International Journal of Analytical and Bioanalytical Chemistry, 2015; X(Y)ZZ.
35. Swetha Reddy G. A Review on new Analytical method Development and Validation by RP-HPLC. A Review on new analytical method development. International Research Journal of Pharmaceutical and Biosciences, 2017; 4(6): 41-50.
36. Rao G, Goyal A. An Overview on Analytical Method Development and Validation by Using HPLC. The Pharmaceutical and Chemical Journal, 2016; 3(2): 280-289.
37. Gnanou, J. V., Srinivas, K. K., & Kurpad, A. V. Automated derivatization with o-phthalaldehyde for estimation of amino acids in plasma using reversed-phase high performance liquid chromatography. Indian Journal of Biochemistry & Biophysics, 2004; 41(6): 322–325.
38. Georgi, G., Pietsch, C., & Sawatzki, G. High-performance liquid chromatographic determination of amino acids in protein hydrolysates and in plasma using automated pre-column derivatization with o-phthaldialdehyde/2-mercaptoethanol. Journal of Chromatography, 1993; 613(1): 35–42.
39. Joyce, R., Kuziene, V., Zou, X., Wang, X., Pullen, F., & Loo, R. L. Development and validation of an ultra-performance liquid chromatography quadrupole time of flight mass spectrometry method for rapid quantification of free amino acids in human urine. Amino Acids, 2016; 48(1): 219–234.
40. Prinsen, H., Schiebergen-Bronkhorst, B. G. M., Roeleveld, M. W., Jans, J. J. M., de Sain-van der Velden, M. G. M., Visser, G., et al. Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography coupled with tandem mass-spectrometry. Journal of Inherited Metabolic Disease, 2016; 39(5): 651–660.
41. Chen, Y. C., Wu, H. Y., Lin, L. C., Chang, C. W., & Liao, P. C. Characterizing the D-amino acid position in peptide epimers by using higher-energy collisional dissociation tandem mass spectrometry: A case study of liraglutide. International Journal of Molecular Sciences, 2024; 25(3): 1379.
42. Gupta et al. (2022), Marbury et al. (2017), Gilar et al. (2005), Krokhin & Spicer (2009).