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Analytical, Bioanalytical, and Stability-Indicating Perspectives of Semaglutide: Advances, Challenges, and Regulatory Expectations for a Long-Acting GLP-1 Analog

Kirtimaya Mishra[1], Diptimayee Jena[1], Debashis Mishra[1*]
[1*]*School of Pharmacy & Life Sciences, Centurion University of Technology & Management, Bhubaneswar, Odisha*
Corresponding Author: Debashis Mishra

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ABSTRACT
Semaglutide (extended release) is a peptide analog for GLP-1. As such, it marks an important advancement in peptide therapies related to diabetes and obesity. The 31 amino acid peptide has been extensively characterized through the use of sophisticated chemical and physical analysis techniques that will allow for the understanding and production of semaglutide. The production of semaglutide will be influenced by fatty acid composition, degradation pathways, low oral bioavailability, and the stability of this compound against abiotic and biotic stressors. This paper will review current analytical methods developed to analyzesemaglutide's characteristics by combining peptide mapping with high-performance liquid chromatography (HPLC) followed by ultra-high performance liquid chromatography (UHPLC) combined with LC-MS, mass spectrometry, circular dichroism, profile of the impurities, stability study on the analyte and pharmacokinetics analyses using analysis techniques established in accordance with regulatory agencies (e.g., ICH) Q2 (R2), Q6B, Q5C and FDA and EMA guidelines.

KEY WORDS: Semaglutdes, UHPLC, ICH, Regulatory agencies, Pharmacokinetices

I. INTRODUCTION

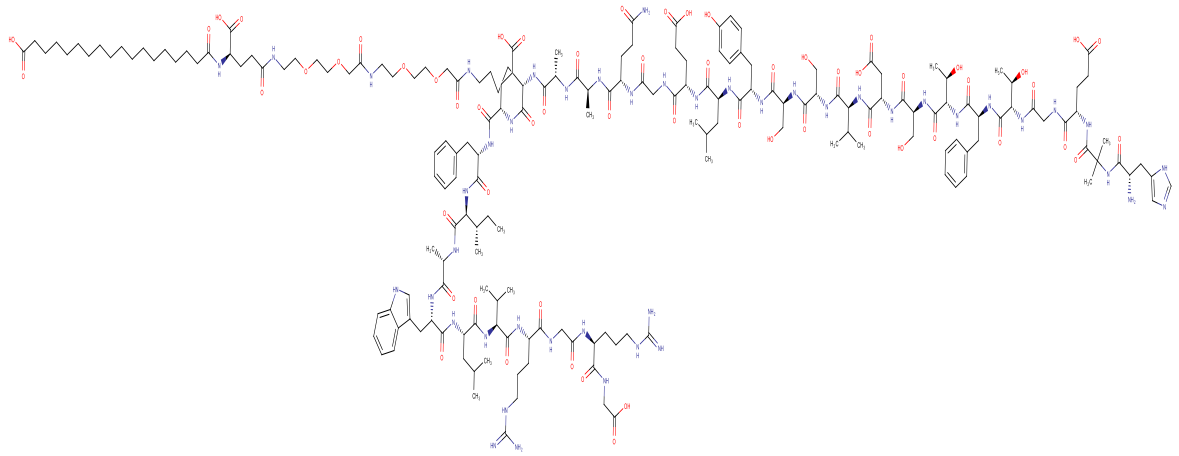

FIGURE 1: THE STRUCTURE OF SEMAGLUTIDE

This review provides analytical insight on semaglutide ($C_{187}H_{291}N_{45}O_{59}$), an established, peptide-based therapeutic, which has undergone an evolution from instability to being a representative of peptide medicine for the past 50 years. This review presents an analytical basis for the development of semaglutide, compares the parameters for semaglutide development relative to the comparison to small molecules [1]. In order to conduct regulatory submissions, semaglutide requires extensive analytical development, as an example of larger molecule-based products. As such, it is important to follow semaglutide's distribution of drug molecules into plasma and distribution within muscle tissues as expected in order to better characterize it in accordance to the above regulatory guidelines [2-4]. When taken with the knowledge that semaglutide has a molecular

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weight of over 4113.6 g/mol and that the maximum UV absorbance for semaglutide is typically 280-293 nm in figure 1 [5] and its low solubility in aqueous solutions, especially in the pH range of 2 to 6, leads to increased analytical complexity, as will be discussed herein. Analyses of semaglutide on the basis of its structure and physicochemical properties require a confirmation of the molecular weight by high-resolution mass spectrometry [6-9]. Needs orthogonal methods for conformational assessment (CD, FT-IR).Fatty acid chain complicates chromatographic retention and MS ionization.Multiple degradation pathways necessitate stability-indicating studies [10-13] in figure 2.

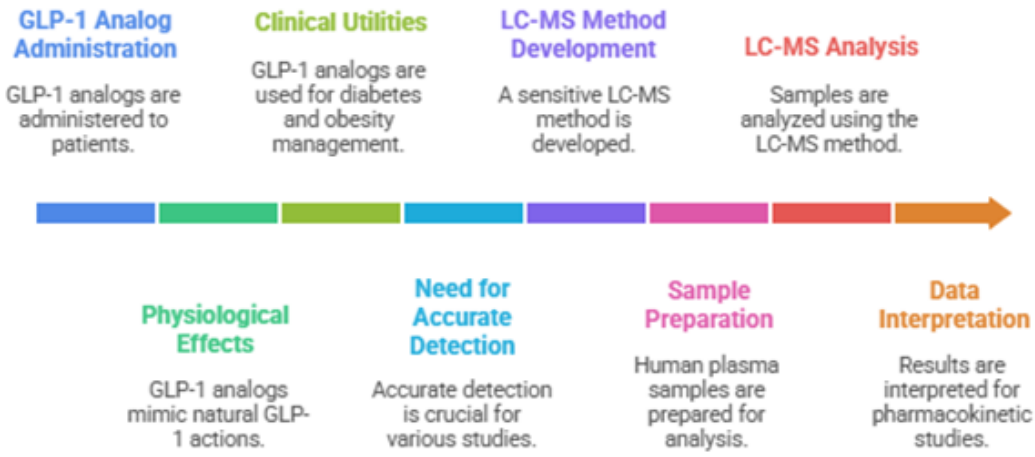

FIGURE 2: GLP-1 ANALOG DETECTION AND QUANTIFICATION PROCESS

II. ANALYTICAL TECHNIQUES FOR SEMAGLUTIDE CHARACTERIZATION

2.1 UV SPECTROSCOPY

UV spectroscopy has been utilized primarily as a method to analyse small molecules; however, it is helpful in supporting the characterisation of drugs containing amino acids, such as semaglutide [14]. Semaglutide contains a peptide backbone and contains aromatic amino acids such as tryptophan and tyrosine, and therefore it has measurable absorbance throughout the ultraviolet (UV) region of the spectrum [15]. The maximum wavelength of absorbance occurs for the peptide bond at approximately 214 nm and for aromatic residues at approximately 280 nm [16]. The usable range of wavelengths provides a means for the preliminary determination of the purity and quantity of peptide drugs like semaglutide and to monitor their chromatographic separation through the use of UV spectroscopy [17-19]. In a quality assurance situation, the combination of UV detection and reversed phase high-performance liquid chromatography (RP-HPLC) is a typical means to monitor semaglutide elution, to determine that the drug is manufactured consistently between batches and identify gross abnormalities in the purity profile of the peptide drug [20]. The spectral properties of semaglutide using only UV detection are not sufficient to definitively identify the complete structure due to its lack of molecular specificity. The presence of the hydrophobic fatty-acid side chain on semaglutide and the many complex modifications that result in the formation of semaglutide do not produce distinguishable UV spectral signals to allow for the definitive identification of truncated peptides, oxidized forms or deamidated impurity forms by UV detection alone [21].In order to overcome the limitations of UV spectroscopy, it is employed in conjunction with techniques such as LC-Mass Spectrometry (LC-MS), peptide mapping, and stability-indicating HPLC; UV spectroscopy provides information on total degradation kinetics when monitoring a chromatogram for either total loss of signal and/or appearance of new peaks resulting from degradation of the drug (i.e. forced degradation studies); while LC-MS confirms the identity of any degradation products, which may include several different species, and quantifies their concentration [22-24]. Although there are certain

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limitations to this technique, UV spectroscopy continues to provide a useful method for routine quantification, method validation and assessment of suitability of a system for use in an analytical study in table 1. UV Detection has also been employed in methodology studies for the determination of the drug's dissolution properties as well as during formulation and process development, to provide a faster, non-destructive method of determining semaglutide concentrations in circumstances where a higher level of classification and specificity are not required. The use of UV-based HPLC for manufacturing large amounts of material can be used to maintain uniformity of peptide content and provide early identification of most significant impurities found at or near the same wavelengths as the UV wavelengths of the drug [25-29]. Thus, UV spectroscopy will continue to be an important baseline tool along with newer and more sophisticated approaches such as mass spectrometry to best characterize the physical and chemical characteristics of semaglutide and other novel pharmaceuticals within a complete orthogonal analytical framework [30-32].

TABLE 1 IDENTIFICATION OF UV SPECTROPHOTOMETER OF DIFFERENT DATAS

Slno	Drugs	Solvent	Methods	λmax	Linearty R^2	References
1	Semaglutide	0.01N potassium dihydrogen ortho phosphate	Zero order-Linearty, accuracy, Precision, LOD and LOQ	293.80	0.9996	33
	Semaglutide	0.01N Potassium dihydrogen ortho phosphate	First order-0.01N Potassium dihydrogen ortho phosphate	254.28	0.9993	
	Semaglutide	Sodium acetate pH 5	Zero order-Sodium acetate pH 5	293.20	0.9997	
	Semaglutide	Sodium acetate pH 5	First order-Sodium acetate pH 5	254.27	0.9997	

2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

REVERSE-PHASE HPLC

- Most widely used method for assay and related substances.
- C18 columns with TFA or FA mobile phases.
- Gradient elution is required due to the hydrophobic C18 chain.

CHALLENGES

- Late retention due to lipophilic modification.
- Peak broadening due to peptide secondary structure.
- TFA suppresses MS sensitivity; FA is better for MS-compatible assays.

2.3 LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY (LC–MS AND LC–MS/MS)

LC–MS plays an indispensable role in the analytical evaluation of semaglutide, serving as the primary tool for identity confirmation, sequence verification, peptide mapping, impurity characterization, in-vivo bioanalysis, and identification of stability-related degradation products. Different MS platforms are employed based on analytical intent: Q-TOF systems are widely used for detailed peptide and impurity mapping, triple quadrupole LC–MS/MS systems are preferred for sensitive and quantitative pharmacokinetic studies, and Orbitrap mass spectrometers provide ultra-high mass accuracy for structural confirmation. Semaglutide generally shows several charge states during electrospray ionization, which are generally M + 4 to M + 8, and the fatty acid chain prolongs the retention of the m/z values in chromatography. The fragmentation

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profiles obtained following fragmentation in MS/MS provides verification the semaglutide peptide maintains a consistent amino acid sequence and maintains molecular integrity. Peptide mapping is an essential LC–MS approach for establishing the full structure of Semaglutide, with the production of pre-determined patterns of fragmentation as a result of enzyme digestion of proteins using proteases (i.e., Trypsin, Asp-N, and Glu-C), and use of LC-MS/MS or RP-HPLC to analyse the mixture following enzymatic digestion will enable one to achieve near complete sequence coverage of Semaglutide in table 2. The peptide mapping results from LC-MS-Peptide Mapping allow us to confirm the structure's integrity of semaglutide, validate substitution of specific amino acids, and qualitatively and quantitatively identify any PTMs (i.e. deamidation and oxidation) or any Degradation modifications. Therefore, Peptide Mapping using LC-MS will result in conclusive evidence supporting the stability, quality, and consistency of Semaglutide through the development and lifecycle. [34].

TABLE 2 LC MS DATA'S OF SEMAGLUTIDE

Sl.no	Drug	Stationary Phase	Mobile Phase	Detector	M/Z	References
1	Semaglutide	SB C18	A: (Acetonitrile: Methanol (50:50) v/v) and Pump B: (0.2% Formic acid in Milli Q Water (v/v))	XB-C8	1371.2	35

III. IMPURITY AND DEGRADATION PROFILING

Semaglutide impurity profiles consist of degradation-related and process-related impurities and highlight the difficulties associated with the manufacture of peptides and stability, particularly long-term. For example, process impurities include truncated peptides resulting from incomplete chain elongation, misfolded peptides formed during synthesis/refolding, and incomplete products due to partial acylation as well as PEG-related fragments introduced from raw materials or processes used to make the product. In addition, semaglutide can degrade in multiple pathways during storage and distribution, leading to many degradation products that cause issues with potency and safety, including: deamidated asparagine to aspartic acid, oxidized methionine and tryptophan side chains; hydrolysis of peptide bonds; β-elimination; and removal of fatty acids attached to semaglutide precursors. To differentiate intact semaglutide from its broad impurity profile, it is imperative to develop robust stability indication methods (SIMD). To provide an indication of the most common degradation pathways, forced degradation studies are the basis for development of SIMD, and are essential in defining/selecting SIMD methods. Stress conditions for forced degradation studies include: temperature (40 °C to 80 °C), hydrolytic, chemically-derived, oxidative, photostability and mechanical agitation [36-39].

IV. BIOANALYTICAL CONSIDERATIONS

Due to its high binding affinity to albumin, Semaglutide poses a significant number of bioanalytical difficulties. This leads to poor recoveries when using conventional protein precipitation techniques as well as significant sample handling requirements for accurate assessment of LC-MS/MS data. Thus, advanced techniques are used for preparing samples for analysis; for example, the use of acidified methanol with liquid-liquid extraction or solid-phase extraction with hydrophilic–lipophilic balance ("HLB") cartridges, or immunocapture methods to achieve more reproducible and selective extraction of Semaglutide before LC MS/MS analysis. In order to provide accurate LC-MS/MS results, peptide charge-state dependent transitions (MRM) must be carefully defined. Additionally, when determining the concentration of Semaglutide, a stable-isotope-labelled internal standard is utilized to help correct for matrix effects, analytical variability, and to establish sensitivity between µg/mL and mg/mL [40].

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TABLE 3 THE USING OF DRUGS IN PLASMA

Sl.no	Drug	Stationary Phase	Mobile Phase	Detector	Column	Column Temperature	Injection volume	Flow rate	M/Z	Plasma	References
1	semaglutide	C18	0.1% Formic acid in water 0.1% Formic acid in acetonitrile	Xevo TQ-XS	ACQUITY UPLC Peptide CSH C18 100 Å, 1.7 um 2.1 mm x 50 mm (p/n: 186005296)	65^{0}C	10 µl	0.3 ml/min	1029.27	Rat	41
2	Semaglutide	C18	Methanol Acetonitrile	Xevo TQ-XS	SB C18	30^{0}C	10 µl	0.8 ml/min	1029.3	Humane	42

The pharmacokinetic profile of semaglutide is noted for a very long elimination half-life of around 165 hours; thus, when conducting bioanalytical and/or toxicokinetic studies, an extended sampling period will be required. Semaglutide has a high percentage of albumin binding, which will result in a non-linear pharmacokinetic profile, resulting in systemic exposure from a given dose and a difference in dose proportionality in table 3. There are also differences between oral and injectable forms: the routes of administration impact absorption and bioavailability; therefore, there is a need to consider the specific bioanalytical methods and study designs based on the formulation being studied [43-47].

V. ANALYTICAL CHALLENGES IN ORAL SEMAGLUTIDE

However, the addition of sodium salcaprozate (SNAC) – an absorption enhancer – to the oral formulation of semaglutide adds further complexity to the analytical evaluation of semaglutide, in addition to needing validated analytical methods for quantifying not only semaglutide but also SNAC and evaluating any possible drug-SNAC interaction that will impact the oral bioavailability of semaglutide. Accordingly, the conduct of complete gastric pH stability studies is required because of the very highly acidic nature of the gastric conditions that semaglutide will encounter following oral dosing. Furthermore, conducting solubility mapping at the most relevant pH ranges and the performance of in vitro permeability assessments (e.g.,PAMPA and Caco-2 assays) will provide insight into the absorption behaviour of semaglutide and will enable correlation between the in vivo exposure with the performance of the formulation[48].To effectively develop oral formulations of semaglutide, researchers and regulators must be cautious in developing appropriate dissolution and drug release characteristics because the peptide drug's susceptibility to instability during manufacture and storage could make standard dissolution approaches ineffective for developing oral doses. Instead, many methods utilize a buffer solution at pH 1.2 that closely approximates the environment within the stomach in an attempt to decrease peptide degradation. In addition to buffers, other critical aspects in this area include the use of modified versions of the current standard dissolution equipment developed by the U.S. Pharmacopeia (USP) and the use of small quantities of biorelevant dissolution fluids that more accurately replicate the human physiological condition where semaglutide is distributed throughout the body. The use of these techniques allows for the creation of performance-based specifications that establish the stability of each formulation/version produced, confirm batch-to-batch reproducibility, and support an empirical basis for establishing the validity of in vitro/in vivo comparisons[49-52].

VI. REGULATORY EXPECTATIONS FOR ANALYTICAL PROFILES

Regulatory authorities expect analytical methods for semaglutide to adhere to multiple guidelines from the International Council for Harmonisation (ICH) that apply to biotechnological and peptide-derived products. Method validation

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must follow ICH Q2(R2) to verify that analytical methods are appropriate for the intended application. ICH Q6B describes how to set appropriate specifications for a biotechnological product, such as a peptide drug, and ICH Q5C provides guidance on stability testing of semaglutide, which is further supported by the ICH Q1 series, which indicates that comprehensive studies on the effects of stress and degradation are necessary to understand the stability and other properties of semaglutide. The bioanalysis of pharmacokinetic and toxicokinetic activity should follow the guidelines established in ICH M10, especially for the use of LC–MS/MS to quantify semaglutide in biological fluid matrices [53]. With respect to analytical validation, there is a strong emphasis on specificity, as it is critical to differentiate between intact semaglutide and similar peptides, metabolites, and degradation products. Precision and accuracy must be demonstrated across the entire range of the assay as well; robustness will be critically important for peptide-based methods due to sensitivity to small variations in handling of the sample and the conditions under which they are used. The stability of semaglutide in plasma or serum at different storage and processing temperatures should also be well established through thorough characterization, as regulatory authorities are concerned about carryover control due to issues with trace residues compromising data integrity [54-55].

VII. FUTURE ANALYTICAL TRENDS FOR SEMAGLUTIDE

Advancements in LC-MS technology should greatly improve the analytical characterization of semaglutide. New emerging platform technologies including ion mobility-mass spectrometry provide an additional separation dimension allowing for greater separation of conformational variants and closely related species. Additionally, new high-resolution single-ion monitoring and data independent acquisition strategies exhibit improved sensitivity and selectivity for low level quantitation. As well, new top-down peptide sequencing methodologies allow for the in situ analysis of the intact molecule while retaining structural information that is normally lost using traditional bottom-up methodologies. Collectively, these types of technologies will supplement and expand upon methods currently used to confirm structural identity, profile impurities, and improve the mechanistic understanding of degradation pathways. In conjunction with these achievements in instrumentation, there will also be increasingly important developments taking place concerning nanoscale and automated sample preparation. Examples include microfluidic-based solid phase extraction methods that select samples with smaller volumes to reduce matrix effects and increase reproducibility; and, automated immunocapture systems that will provide an opportunity to increase selectivity and throughput for bioanalytical methods. Anticipating the future with an increase in the availability of biosimilars, expect that there will be increased analytical rigor required as municipalities permit the expiration of patented products. Consequently, there will be a need for an extensive assessment of higher-order structure, charge variant analysis, and comprehensive peptide mapping with 100% coverage when demonstrating comparability between semaglutide biosimilars and reference products[56-58].

VIII. CONCLUSION

The complexity of the semaglutide peptide drug makes it one of the most analytically challenging peptide drugs today from the perspective of a Senior Professor of Pharmaceutical Analysis.Semaglutide has a complex structure, which is characterized by hydrophobic modifications, an extended half-life, and multiple routes of degradation, which all necessitate the use of advanced orthogonal analytics. Therefore, advancement in the development of semaglutide will be dependent upon the implementation of high-resolution analytical technologies, extensive impurity and degradation profiling, and highly sensitive bioanalytical technologies developed in full compliance with regulatory requirements. The continued evolution of semaglutide demonstrates that it is a significant therapeutic advance and exemplifies the increasing sophistication and depth of the science involved in modern pharmaceutical analysis.

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