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Exploration of Related Issues on PK/PD Studies and Bioanalytical Characteristics of Peptides

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Abstract

Peptides play multiple functions in cellular processes and are considered an attractive paradigm for the development of novel drugs and therapeutic approaches. However, the complexity of their pharmacokinetics/pharmacodynamics and physicochemical properties presents challenges in their development. Currently, there is no single analytical method that fully meets the requirements for studying peptide drug pharmacokinetics. Interdisciplinary teams and multiple technical platforms are required to address these challenges. This article explores the pharmacokinetics, bioanalytical methods, challenges, and strategies in the development of peptide drugs. As our understanding of peptide drug pharmacokinetics and bioanalytical characteristics deepens, it will facilitate their development and provide scientific evidence for rational clinical use.

Keywords

Peptides, Drugs, PK/PD, Bioanalytical Characteristics

1. Introduction

Peptides are short chains of two or more amino acids covalently linked by amide bonds. They can be derived from rDNA or from a biological source or made by chemical synthesis. In the absence of clear scientific consensus on the criteria that distinguish proteins from peptides, Food and Drug Administration (FDA) distinguishes proteins from peptides based on size (*i.e.*, number of amino acids) and considers any polymer composed of 40 or fewer amino acids to be a peptide [1].

Peptides, along with proteins, constitute the largest group of mediators in cel-

lular processes, making them crucial for the functionality of many organisms. Consequently, peptides are considered an attractive paradigm for the development of novel drugs and therapeutic approaches [2]. Peptides possess distinct physicochemical properties and therapeutic characteristics compared to proteins and antibodies. In addition to serving as analogs of peptide hormones, peptides can disrupt protein-protein interactions (PPIs), bind to cell surface receptors, and activate or inhibit intracellular signaling pathways (such as receptor tyrosine kinases). These applications endow peptides with broad clinical value, and peptide therapeutics have become a leading sector in the pharmaceutical industry in recent years [3]. Currently, peptide therapeutics have been developed and applied in various fields, including antibiotics/antifungal diseases, viral indications, immune system disorders, cardiovascular diseases, neurological disorders, and cancer [4].

However, the development and optimization of peptide drugs face several challenges, one of which is understanding and optimizing their pharmacokinetic and pharmacodynamic properties *in vivo*. Pharmacokinetic research is the scientific study of the absorption, distribution, metabolism, and excretion processes of drugs in the body, and it is crucial for the development of peptide drugs. Peptide drugs typically have larger molecular weights and complex chemical structures, which can lead to more intricate processes of metabolism and elimination in the body. Understanding the pharmacokinetic properties of peptide drugs can assist in determining the optimal routes of administration, dosages, and dosing frequencies to achieve effective therapeutic outcomes. On the other hand, pharmacodynamic research focuses on the mechanisms of action and effects of drugs in the body. For peptide drugs, understanding their pharmacodynamic properties can aid in identifying their targets, biological activities, and therapeutic effects. This is essential for optimizing the design and development of peptide drugs to ensure their efficacy and safety.

The significance of pharmacokinetic and pharmacodynamic research lies in their provision of crucial information about the behavior of peptide drugs in the body, which guides the development, optimization, and clinical application of these drugs. This article will discuss the advantages of peptide drugs, research progress, challenges and strategies in development, pharmacokinetics/pharmacodynamic, and bioanalysis of peptide drugs.

2. Advantages of Peptide Drugs

Compared to proteins or antibodies, peptide drugs have a smaller molecular weight, which confers them with the potential to penetrate tissues more effectively. Additionally, peptide drugs, including synthetic peptides, generally exhibit lower immunogenicity compared to recombinant proteins or antibodies [5]. As candidate drugs, peptides have advantages over proteins and antibodies, such as lower production costs (compared to synthesis and recombinant approaches) and higher unit activity [6].

In comparison to small organic molecules, peptide drugs possess enhanced potency, selectivity, and specificity. The degradation products of peptides are amino acids, thereby minimizing the risk of systemic toxicity (by reducing drug interactions) [7]. Furthermore, peptides have shorter half-lives and lower accumulation in tissues, thereby reducing the risk of complications caused by metabolites. Most therapeutic peptides are derived from natural peptides and function as receptor agonists. In general, small amounts of peptide agonists are sufficient to activate target receptors [6].

3. Advancements in Peptide Drug Research

The first peptide drug, insulin, was extracted from the pancreas of cows and pigs. In 1954, Vincent du Vigneaud's team achieved a breakthrough by chemically synthesizing peptides, publishing the synthesis of oxytocin and antidiuretic hormone (for which du Vigneaud was awarded the Nobel Prize in Chemistry in 1955). Another significant milestone in the development of peptide drugs occurred in 1963 when Bruce Merrifield proposed the solid-phase peptide synthesis (SPPS) method, which allows for the automated synthesis of peptides by assembling amino acids on a solid support (Merrifield received the Nobel Prize in Chemistry in 1984). The advent of recombinant technology in the 1980s made it possible to produce larger molecular weight peptide drugs in a clean manner. Subsequent technological developments, such as lipidation, conjugation with macromolecules or polyethylene glycol, have enabled peptides to overcome renal clearance issues and increase their plasma circulation time. Display technologies, such as phage display, have facilitated the targeted screening of potential drugable peptides from vast peptide libraries. The Flexizyme technology allows for the incorporation of non-natural amino acids into peptide display libraries, enabling the construction of diverse peptides. Furthermore, the discovery of natural peptides, particularly those derived from venoms, and the development of novel chemical methods have also propelled the advancement of peptide drugs (**Figure 1**) [8].

The development of peptide drugs is unstoppable, with the number of approved peptide drugs steadily increasing over the past six decades, and their market accounting for approximately 5% of the global pharmaceutical market. In 2021, the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) approved two new peptide drugs. In the same year, the European Medicines Agency (EMA) approved six peptide drugs, four of which were innovative drugs. Among the 50 new drugs approved by the US FDA's Center for Drug Evaluation and Research (CDER) in 2021, six were peptide drugs. Currently approved peptide drugs are mostly receptor agonists, with indications primarily in the fields of endocrinology, metabolism, oncology, and the central nervous system [8].

Although the development of peptide drugs in China started relatively late, it has experienced rapid growth. There are already over a hundred domestic peptide companies in the country. In terms of generic drugs, the patents for several

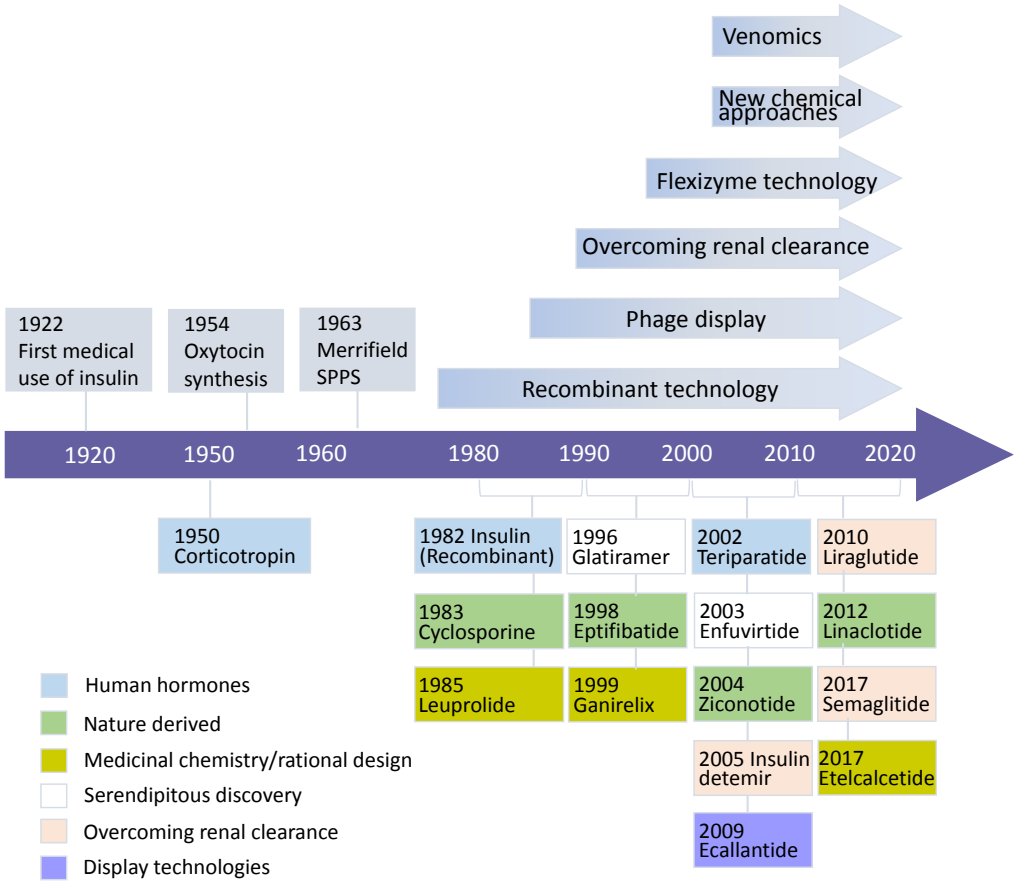

Figure 1. The development history of peptide drugs and representative peptide drugs [8].

blockbuster peptide drugs have expired, and domestic companies have begun investing substantial resources in generic research. China has also made significant progress in the development of innovative peptide drugs. In terms of market distribution, there are more than 40 marketed peptide drugs in China, and there are over 80 ongoing research projects for innovative peptide drugs, covering seven major areas including immunology, gastrointestinal tract, anti-tumor, orthopedics, obstetrics, diabetes, and cardiovascular diseases. The market sales of peptide drugs in China are growing rapidly.

4. Challenges and Strategies in Peptide Drug Development

Several characteristics of peptide drugs hinder their application in the field of therapeutics. The bottlenecks associated with peptide therapeutics can be summarized as follows [2]: 1) Low bioavailability and short half-life in the body. Firstly, the hydrophilic nature of peptides makes it difficult for them to cross physiological barriers and penetrate cell membranes. Additionally, proteases in the digestive system and blood can rapidly degrade peptides, while hepatic and renal metabolism can quickly clear circulating peptides within minutes, resulting in short half-lives and rapid clearance rates. Often, frequent dosing is required to maintain therapeutic blood concentrations, but this can lead to fluctuations in

drug levels. 2) Linear peptides are highly flexible, allowing them to undergo random twisting and flipping, resulting in high conformational flexibility. This can lead to poor biologically specific distribution and sometimes poor selectivity for peptide receptors, thereby causing drug side effects. 3) The denaturation, aggregation, adsorption, or precipitation of peptide drugs can affect their storage and use. The disruption of secondary or tertiary structures of peptides is associated with denaturation, which makes denatured peptides more susceptible to chemical reactions and difficult to restore their activity. Intermediate products generated during denaturation can aggregate, leading to the formation of aggregates. Additionally, the surface adsorption of peptides presents challenges during storage and use. The denaturation, aggregation, and adsorption characteristics of peptides can all affect the blood concentration and functional efficacy of peptide drugs.

Facing the degradation by gastrointestinal, blood, and tissue proteases, as well as rapid clearance by the liver and kidneys, the biological stability and half-life of peptide drugs are urgent issues to be addressed. Currently, various techniques have been applied to improve the *in vivo* retention time, bioavailability, and half-life of peptide drugs. Methods to enhance the *in vivo* stability of peptide drugs include peptide PEGylation, fusion with the constant region Fc segment of human IgG, binding to stable and abundant humanized proteins such as albumin and scaffold proteins, and encapsulation in nanoparticles or microspheres [2]. For example, PEGylation modification of peptides can increase the volume, hydrodynamic radius, and molecular weight of peptide drugs, avoiding renal clearance and reducing degradation by peptidases outside the peptide chain, thus enhancing the *in vivo* stability of peptides. Additionally, although PEGylation modification may affect the functionality of peptides, it may reduce their immunogenicity. Fusion of the constant region Fc domain of human IgG with peptides allows them to escape degradation by binding to the neonatal Fc receptor (FcRn). Peptide-Fc fusion drugs such as AMG 531 (developed by Amgen) and protein drugs like Enbrel®, Amevive®, and Orencia® (all fused with Fc region) have been successfully marketed [9].

Chemical modification of peptides and other techniques have also been widely applied. This approach aims to increase the protease resistance and enhance the in vitro stability of peptides by reducing their degradation by proteases and peptidases in tissues and serum [2]. Chemical modification methods for peptides include C-terminal amidation, N-terminal acetylation, incorporation of non-natural amino acids or phosphorylated amino acids, and cyclization of linear peptides through disulfide bonds. For example, cyclization through disulfide bonds reduces the high conformational flexibility of linear peptides and improves their proteolytic stability [10]. Additionally, enhancing the in vitro stability of peptides can be achieved by adding stabilizers with different mechanisms of action. For instance, the addition of sugars (such as sucrose, maltose, trehalose, or glucose), salts (such as potassium phosphate, ammonium sulfate, or sodium citrate), or sodium heparin can reduce peptide aggregation, modulate solubility,

and increase peptide stability [11]. Chelating agents, such as EDTA, can form complexes with metal-dependent proteases/peptidases, thereby inhibiting peptide degradation [12]. Moreover, non-ionic surfactants (such as Pluronic F68) can stabilize peptides and proteins and prevent self-aggregation. On the other hand, anionic (SDS) and cationic (cetyltrimethylammonium bromide) surfactants can facilitate peptide transmembrane permeation, thus promoting peptide absorption [13].

5. Pharmacokinetics of Peptide Drugs

Due to the degradation by proteases and peptidases, peptide drugs typically have a short half-life. Endogenous peptides (often with hormonal activity) have extremely short elimination half-lives, and the main focus as potential therapeutic agents lies in regulating their endogenous concentrations and functions. For example, glucagon-like peptide-1 (GLP-1) can lower blood glucose levels in diabetic patients and is a potential treatment for type 2 diabetes. However, the combined effects of factors such as susceptibility to enzymatic degradation, high biological activity, hepatic metabolism and distribution, and slow renal clearance contribute to the short half-life of GLP-1 in the body. These factors limit the stability and persistence of GLP-1 *in vivo*, necessitating the implementation of specific strategies to enhance its pharmacological activity and stability, such as the use of GLP-1 analogs exenatide and liraglutide, with half-lives of 2.5 and 13 hours respectively, to prolong its duration of action [14].

5.1. Absorption

5.1.1. Injection

Intravenous administration can bypass hepatic and gastrointestinal enzymatic first-pass metabolism, making it the most common route of administration for peptide drugs [15].

In addition to intravenous administration, other common routes of parenteral administration for peptide drugs include subcutaneous injection and intramuscular injection. Similar to intravenous administration, subcutaneous and intramuscular injections can bypass hepatic and gastrointestinal enzymatic degradation of peptide drugs. However, due to the inability to avoid first-pass metabolism by proteases and peptidases in the interstitial space at the injection site and degradation in the lymphatic system [16], the bioavailability of peptide drugs via subcutaneous and intramuscular routes is lower than that of intravenous administration [15]. There is a significant difference in the systemic bioavailability of peptide drugs between subcutaneous and intramuscular injections. Differences in blood and lymphatic circulation at the injection sites can influence the pharmacokinetic characteristics of peptide drugs [17].

The absorption of drugs through subcutaneous and intramuscular injections is highly molecule-dependent. After subcutaneous or intramuscular injection, peptide drugs can enter the systemic circulation through capillaries or lymphatic vessels. As the molecular weight of the drug increases, the proportion of drug

absorption through convective transport into the lymphatic system increases. Lymphatic absorption is the primary absorption pathway for therapeutic protein macromolecules. After subcutaneous injection, drugs with a molecular weight below 1 kDa are primarily absorbed by capillaries, while proteins with a molecular weight of 16 - 22 kDa or higher are mainly absorbed through the lymphatic system. The molecular weight of most therapeutic peptides falls between 1 and 10 kDa, and they are generally absorbed through both the lymphatic system and blood vessels, although the extent of absorption through convective transport into the lymphatic system is usually limited. Diffusion into capillaries is the primary absorption mechanism for the majority of peptide drugs [14].

5.1.2. Non-Invasive Administration

Non-invasive routes of administration for peptide drugs include oral, buccal, pulmonary, intranasal, and transdermal routes. These routes offer convenience, gentler administration, and improved patient compliance compared to injection. However, peptide drugs generally possess hydrophilicity and multiple charged moieties, which prevent passive diffusion across epithelial cells. Moreover, their molecular weight is typically greater than 700 Da, making it difficult for them to be absorbed through the paracellular pathway (limited to compounds smaller than 200 Da). As a result, most peptides exhibit poor permeability, and when the peptide molecular mass exceeds the threshold of 700 Da, their bioavailability sharply decreases. This presents a significant challenge for oral or other non-invasive routes of peptide drug administration [14].

Currently, there are relatively few orally available peptide drugs on the market, and they possess unique chemical characteristics. One example is cyclosporine, which is a cyclic peptide consisting of 11 amino acids. The cyclic structure of cyclosporine helps protect it from proteolytic degradation, and its lipophilicity facilitates its absorption through the intestinal mucosa [18]. The unique chemical properties of cyclosporine have improved its oral bioavailability to 30% (Sandimmune® Oral). Another formulation of cyclosporine (Neoral®) utilizes microemulsion technology, resulting in a bioavailability of approximately 40% [19]. Additionally, modifications of the amino acid backbone, chemical conjugation with hydrophobic or targeting ligands, and the use of absorption enhancers can promote the oral absorption of peptide drugs. For example, oral somatostatin analogs employ the Eligen® technology, forming complexes with absorption enhancer SNAC. This approach not only protects somatostatin analogs from gastric acid degradation but also facilitates their rapid absorption in the stomach, thereby improving their oral bioavailability.

Oral administration of peptide drugs allows absorption through the rich capillary and lymphatic networks in the buccal and sublingual regions, bypassing hepatic and gastrointestinal first-pass metabolism.

Pulmonary inhalation of peptides offers the advantages of non-invasive administration, a large absorption surface area, high vascular density at the administration site, rapid drug absorption, and bypassing the first-pass effect of the

liver [14]. However, the alveolar lining fluid, alveolar macrophages, and alveolar epithelium form barriers to peptide absorption, hindering drug penetration into the systemic circulation and cellular uptake [18].

Nasal inhalation of peptides shares similar advantages with pulmonary inhalation: a large surface area, abundant blood vessels, avoidance of hepatic first-pass metabolism, and rapid drug absorption. Nasal epithelial cells have relatively high permeability, allowing the penetration of larger molecules (with a molecular weight threshold of approximately 1000 Da) [14]. However, the bioavailability of peptide drugs via nasal inhalation remains low. For example, the nasal peptide drugs Miacalcin®, Synarel®, and DDAVP® exhibit a bioavailability of approximately equal to or less than 3% [18].

Transdermal administration of peptides offers the advantage of bypassing hepatic and gastrointestinal first-pass metabolism while providing sustained drug release into the systemic circulation. The main drawback of transdermal administration is that most lipophilic drugs have difficulty penetrating the skin, limiting its applicability to only a few drugs. Ultrasound (low-frequency ultrasound) and iontophoresis (iontophoresis) are two commonly used methods to enhance skin permeability. Transdermal administration is particularly suitable for delivering peptide drugs at extremely low doses into the systemic circulation, including GnRH analogs and vasopressin [20].

5.2. Distribution

The speed and extent of drug distribution are determined by tissue permeability and range, tissue distribution within organs, and tissue excretion of the drug. For a specific drug, the rate and extent of its distribution depend on its physicochemical properties (such as size, charge, lipophilicity), protein binding, and dependence on carrier-mediated transport. The distribution of small molecule drugs is primarily driven by passive diffusion driven by concentration gradients. On the other hand, large molecule drugs such as protein drugs mainly enter the interstitial space through convective transport from the vascular system, distribute within the interstitial space, and eventually undergo transport out of the tissue into capillaries. Peptides, being intermediate in size between small molecules and large molecules, can exhibit both diffusion and convective transport in biological systems.

Diffusion is the passive movement of molecules or particles from an area of higher concentration to an area of lower concentration. It occurs due to random thermal motion and is influenced by factors such as concentration gradient, molecular size, and the presence of barriers or obstacles. Peptides, despite their larger size compared to small molecules, can still undergo diffusion to some extent. However, their larger size may limit the rate of diffusion compared to smaller molecules. Convective transport, on the other hand, involves the movement of molecules or particles through a fluid medium, such as blood or interstitial fluid, driven by bulk flow or fluid motion. This transport mechanism can

facilitate the movement of larger molecules, including peptides, over longer distances and at a faster rate compared to diffusion alone. Convective transport relies on various physiological processes, such as blood circulation, lymphatic drainage, and interstitial fluid flow, which can carry peptides throughout the body. Thus, peptides, as intermediate-sized molecules, can engage in both diffusion and convective transport in biological systems. The relative contributions of these transport mechanisms depend on the specific characteristics of the peptides and the physiological context in which they are present [17].

The distribution volume of peptides is generally small, limited to the extracellular space. Peptide drugs administered intravenously typically exhibit a biphasic concentration-time distribution. The central compartment (V1) is usually around 3 - 8 L, slightly larger than the plasma volume, with good perfusion in tissues (especially in the kidneys and liver) and high permeability of capillary membranes. The peripheral compartment has poor perfusion. The steady-state distribution volume (Vss) of peptide drugs is typically slightly larger than the interstitial fluid volume (approximately 15 liters in a 70 kg individual) [21]. For example, the glycoprotein IIb/IIIa inhibitor eptifibatide (heptapeptide) has a V1 of 9.2 L [22], while the somatostatin analog octreotide (octapeptide) has a V1 of 5.2 - 10.2 L and a Vss of 18 - 30 L [21]. Additionally, binding to endogenous proteins can affect the distribution of therapeutic peptides. For instance, 65% of octreotide binds to lipoproteins, and over 98% of GLP-1 analog liraglutide binds to plasma proteins.

5.3. Metabolism and Excretion

The metabolic pathways of peptide drugs usually mirror those of endogenous peptides, with the metabolized amino acids being recycled in the endogenous amino acid pool for de novo synthesis of proteins/peptides [23]. Blood, liver, kidneys, and small intestine are likely important sites for the degradation of peptide drugs due to their abundance of various proteases and peptidases. However, since proteases and peptidases are ubiquitous in the body, peptide degradation can occur in various tissues throughout the body.

For most peptide drugs, the liver is not the primary metabolic pathway. However, some smaller molecular weight peptide drugs are primarily metabolized in the liver. For example, the proteasome inhibitor bortezomib, a dipeptide used to treat multiple myeloma, undergoes oxidative metabolism in the liver. A recent study has shown that patients with moderate or severe liver impairment have reduced clearance of bortezomib, and therefore, the initial dosage should be reduced for these patients. Similarly, cyclosporin A, an undecapeptide, is almost completely eliminated through liver metabolism [17].

For the majority of peptides, the amount excreted through non-metabolic pathways such as renal and biliary excretion is negligible. However, peptides with a molecular weight below 10 kDa can generally undergo glomerular filtration and then be degraded at the brush border of proximal tubular cells. Peptide

drugs typically have a molecular weight below 10 kDa, and their renal clearance closely approximates the glomerular filtration efficiency [17]. The phenomenon of very short half-lives observed for most peptides cannot be explained solely by glomerular filtration rate but rather appears to be a result of rapid degradation. However, for peptides with protease resistance, renal clearance may be the primary route of excretion. For example, exenatide, a 39-amino acid analog of GLP-1, which is resistant to degradation by dipeptidyl peptidase-4, is primarily excreted through glomerular filtration and enzymatic degradation at the renal tubular cell membrane. Its half-life in the human body is approximately 2.5 hours, significantly longer than that of GLP-1 (2 minutes) [24].

6. Biological Analysis of Peptide Drugs

6.1. PK/PD Studies

With the maturation of proteomics and the development of peptide drugs, various quantitative analysis methods have also been developed. Accurate and reliable quantitative analysis methods play a crucial role in drug development and research. Currently, bioanalytical methods for protein or peptide drugs include isotopic labeling techniques, *in vivo* imaging techniques, immunoassays, chromatography, capillary electrophoresis, and more. Isotopic labeling techniques offer stable measurement processes, extremely low detection limits, and simple operation procedures. Particularly, radiolabeling methods are suitable for studying tissue distribution and excretion of protein or peptide drugs. *In vivo* imaging techniques are applicable for studying drugs at the tissue and cellular levels in live organisms. Immunoassays are one of the highly sensitive and accurate methods for protein and peptide analysis. Compared to Liquid chromatography-mass spectrometry (LC-MS/MS) techniques, immunoassays can achieve higher sensitivity and are more amenable to automation [25]. LC-MS/MS has become one of the important platforms for bioanalytical studies of biologic therapeutics due to its strong specificity, rapid method development, and multiplex analysis capabilities [26].

6.1.1. Immunoassay Techniques

Enzyme-linked immunosorbent assay (ELISA) is a widely used and well-established immunoassay technique. It is relatively simple and cost-effective. ELISA can detect various analytes, including proteins, antibodies, and small molecules. Quantitative results can be obtained through colorimetric or fluorescence readouts. Optimized experimental protocols can achieve high sensitivity and specificity. However, ELISA has certain limitations. It has a limited dynamic range, which may affect the detection of analytes with high or low concentrations. Manual steps involved in the assay can introduce variability and require skilled operators. Compared to some newer technologies, ELISA has a longer assay time. Its multiplexing capabilities are limited, typically allowing detection of only a few analytes simultaneously. Electrochemiluminescence (ECL) is an electrochemical luminescence technique that offers high sensitivity and a wide dynamic

range. Compared to fluorescence techniques, ECL has lower background noise. It exhibits high signal stability and longer signal duration. ECL has multiplexing capabilities, enabling simultaneous detection of multiple analytes. It is suitable for high-throughput applications. However, ECL requires specialized equipment, such as an electrochemical reader. The instrumentation and experimental protocols are relatively complex. Quanterix's digital single-molecule array technology (Simoa) is a single-molecule array technology that provides exceptional sensitivity. It can achieve sensitivity at the femtogram level and is widely applied in PK/PD, and biomarker research in preclinical and clinical trials of innovative drugs. However, Simoa also has certain limitations. The technology requires complex instrumentation and experimental procedures, and it is relatively expensive. The sample processing steps can be time-consuming.

ELISA, ECL, and Simoa are three analysis techniques with their own characteristics. The choice of the appropriate technique depends on experimental requirements, including the nature of the analytes, sensitivity requirements, multiplexing capabilities, as well as available resources and equipment considerations. Please refer to Table 1 for the recommended validation parameters for immunoassay method validation.

However, immunoassay methods have limitations. The antibodies used in the analysis may exhibit varying degrees of cross-reactivity with metabolites of the drug, and post-translational modifications are often not recognized. These are particular challenges in protein or peptide therapies, where drug metabolites may or may not be active but structurally resemble the target peptide or protein being detected [25]. Additionally, the quantification range of immunoassay methods is limited. In recent years, with the development of sample preparation techniques, chromatography, and mass spectrometry hardware and software, LC-MS/MS has emerged as an important platform for protein and peptide analysis and can overcome the limitations of immunoassay methods [27].

6.1.2. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS)

For larger peptide drugs, the use of surrogate peptides generated by proteolytic digestion is often preferred for quantitative analysis. The smaller size of surrogate peptides allows for lower sensitivity and specificity in the analysis method. The main steps involved in surrogate peptide-based quantification include sample preparation, proteolytic digestion of proteins or peptides, enrichment of peptide fragments, and liquid chromatography and mass spectrometry analysis.

Table 1. Recommended validation parameters for immunoassay method validation.

Standard Curve	Selectivity
Specificity	Residue
Precision And Accuracy	Dilution Linearity And Hook Effect
Parallelism	Stability

For smaller peptide drugs, direct quantification of the intact peptide itself is typically performed without using surrogate peptides. Additionally, with improvements in the detection capabilities of LC-MS/MS for large molecules, quantification of intact peptides has become a trend to avoid introducing variations and interferences during the proteolytic digestion process. Bronsema *et al.* [28] conducted quantitative analysis of salmon calcitonin using both surrogate peptide-based and intact peptide-based methods. The results showed that the lower limit of quantification (LLOQ) for the undigested samples was about 5 times lower than that of the digested samples. This is because the digestion process increases the complexity of the sample, thereby interfering with the quantification of characteristic peptides. Another reason for using intact peptides for quantification is that surrogate peptides may not fully represent the unprocessed intact peptide molecules, especially the surrogate peptides generated by proteolysis (**Table 2**) [26].

Validation parameters for LC-MS/MS analysis methods include standard curve, selectivity and specificity, precision and accuracy, among others (**Table 3**). Although LC-MS/MS analysis methods serve as an important platform for protein or peptide quantification, they still face various challenges. For instance, during the sample preparation process, it is necessary to remove interfering substances from other sample matrices and enhance the specificity of detection. Factors to consider include protein binding, non-specific binding, peptide solubility, peptide specificity, and achieving reproducible high recovery rates during sample purification processes.

6.2. Immunogenicity Analysis

Immunogenicity of peptide drugs typically refers to the ability of peptide drugs and/or their metabolites to induce an immune response or immune-related events against self or related proteins. Immunogenicity assessment is an important aspect of evaluating the safety of biological therapeutic drugs such as protein drugs and peptide drugs. Repeated or long-term administration often leads to the production of anti-drug antibodies (ADAs) [29]. The generation of ADAs can not only modulate or even eliminate the bioactivity of therapeutic drugs (neutralizing antibodies) but also potentially alter their pharmacokinetic characteristics [17]. Safety issues related to immunogenicity include the potential for hypersensitivity or allergic reactions, tissue cross-reactivity of neutralizing antibodies, and toxicity mediated by immune complexes deposited in specific tissues or organs [30].

Peptides with a molecular weight below 4000 Da are generally considered to have low immunogenicity, but there are exceptions due to the complexity of the human immune response system. For example, the glucose-lowering drug taspoglutide, a human sequence-based GLP-1 analog, was discontinued in development due to severe hypersensitivity reactions (with an incidence rate of less than 1%) [31].

Table 2. Peptide drugs quantified using intact peptides after 2015 [26].

Analyte	MW (kDa)	LLOQ	IS type	Sample Volume (uL)	Injection Volume (uL)	Sample Preparation	LC (Sorbent; L*id; particle size)
PTH 1 - 34	~4.1	15 pg/mL	SIL-IS	150	30 - 40	PPT	C18; 100 mm × 2.1 mm, 1.7 μm
Semaglutide	~3.1	3 ng/mL	Analog	100	NA	PPT	C18; 50 mm × 2.1 mm, 1.8 μm
BNP (1 - 32)	~3.4	1.4 pg/mL	SIL-IS	1000	10	LAC	C18; 50 mm × 150 μm, 1.8 μm
GPL1 (7 - 36), GLP1 (9 - 36), OXM, Glucagon	~3.3 (GPL1(7 - 36)), ~3.1 (GLP1(9 - 36)), ~4.4 (OXM), ~3.5 (Glucagon)	0.78 pM	SIL-IS	500	16	LAC	C18; 50 mm × 150 μm, 1.7 μm
Liraglutide	~3.7	100 ng/mL	Analog	25	5	PPT	C4; 50 mm × 2.1 mm, 1.8 μm
PYY (3 - 36)	~4.0	2 nM	None	30	5	PPT	C12; 50 mm × 2.0 mm, 4 μm
Sermorelin	~3.3	50 pg/mL	Analog	100	1	LAC	C18; 100 mm × 75 μm, 3 μm
Glucagon	~3.4	15 pg/mL	SIL-IS	400	40	PPT + SPE	C18; 100 mm × 2.1 mm, 1.7 μm
GLP1 (7 - 36)	~3.3	25 pg/mL	SIL-IS	400	40	PPT + SPE	C18; 100 mm × 2.1 mm, 1.7 μm
Insulin glargine	~6.1	16 pM (0.1 ng/mL)	SIL-IS	500	20	LAC	C4; 50 mm × 2.1 mm, 1.7 μm
Sun flower trypsin inhibitor (SFTI)	~1.7	0.125 ng/mL	None	30	5	PPT	C18; 100 mm × 2.1 mm, 5 μm
Variegin	~3.6	21.6 nM	SIL-IS	200	20	SPE	C18; 50 mm × 2.1 mm, 5 μm
Glucose-responsive insulin (GRI)*	~7.1	100 pM (1 ng/mL)	Analog	40	10	LA	C4; 50 mm × 2.1 mm, 1.7 μm
Glucose-responsive insulin (GRI)**	~7.1	500 pM (0.4 ng/mL)	Analog	200	10	SPE	C4; 50 mm × 2.1 mm, 1.7 μm
Liraglutide	~3.7	10 ng/mL	Analog	50	5	PPT	C18; 50 mm × 150 μm, 1.8 μm

*in rat and dog plasma; **in human plasma.

Table 3. Recommended validation parameters for LC-MS/MS method validation.

Standard Curve	Matrix Effect
Selectivity And Specificity	Residue
Precision And Accuracy	Limit Of Quantification (Loq)
Extraction Recovery	Dilution Reliability
Batch Capacity	Stability
Reproducibility	

Furthermore, the route of administration of a drug may influence its immunogenicity. Although the route of administration cannot directly alter the immunogenicity characteristics of peptides or proteins, it can increase the probability of immune reactions against immunogenic peptides or proteins. In general, compared to intramuscular or intravenous administration, subcutaneous administration of the same drug increases the likelihood of protein aggregate formation at the injection site, leading to a higher incidence of immune reactions [32].

Methods to reduce the immunogenicity of peptide drugs include avoiding antigenic sequences in the amino acid sequence of the peptide, structural modifications of the peptide (such as glycosylation or PEGylation), and shielding the antigenic epitopes of the drug through steric hindrance, thereby preventing recognition by the immune system [16]. For example, the immunogenicity of vespulakinin 1 (VSK1) was significantly reduced when attached to carbohydrates [33].

The detection strategy for anti-drug antibodies typically involves a multi-tiered analysis approach, including screening assays, confirmatory assays, titer assays, and neutralizing activity assays (**Figure 2**).

The immunogenicity validation parameters include the following (**Table 4**): cut-off value, sensitivity, precision, drug tolerance, etc. These immunogenicity validation parameters are essential to ensure the reliability and accuracy of the immunogenicity assessment for peptide drugs. They help establish the performance characteristics of the assay and determine its suitability for detecting and quantifying ADAs in clinical samples.

The analytical methods used for evaluating immunogenicity and ADA detection include ELISA, surface plasmon resonance (SPR), (electro) chemiluminescence, cytometric bead array (CBA) based on flow cytometry, and cell-based neutralizing antibody assays. Due to the complexity of immune reactions, it is often necessary to combine different techniques to fully assess the immunogenicity of drugs.

Although antibody detection methods have become more mature, there are still several challenges in the analysis process. For example, some peptide drugs cannot be directly coated on plates due to their small molecular weight. The complex composition of matrices (such as serum) may contain various immunoglobulins that can interfere with the capture of target antibodies, thereby affecting the detection performance. The secondary antibodies used in direct or indirect immune analyses may also exhibit non-specific reactions with non-target antibodies, leading to high background signals. Additionally, linear peptides are highly flexible and can undergo random distortions and flips, making it difficult

Table 4. Recommended validation parameters for immunogenicity method validation.

Cut-Off Value (Screening Cut-Off Value, Confirmatory Cut-Off Value)	Sensitivity
Precision (Screening Precision, Confirmatory Precision, Titer Precision)	Drug Tolerance
Specificity	Selectivity
Hook Effect	Stability
Reproducibility	Robustness

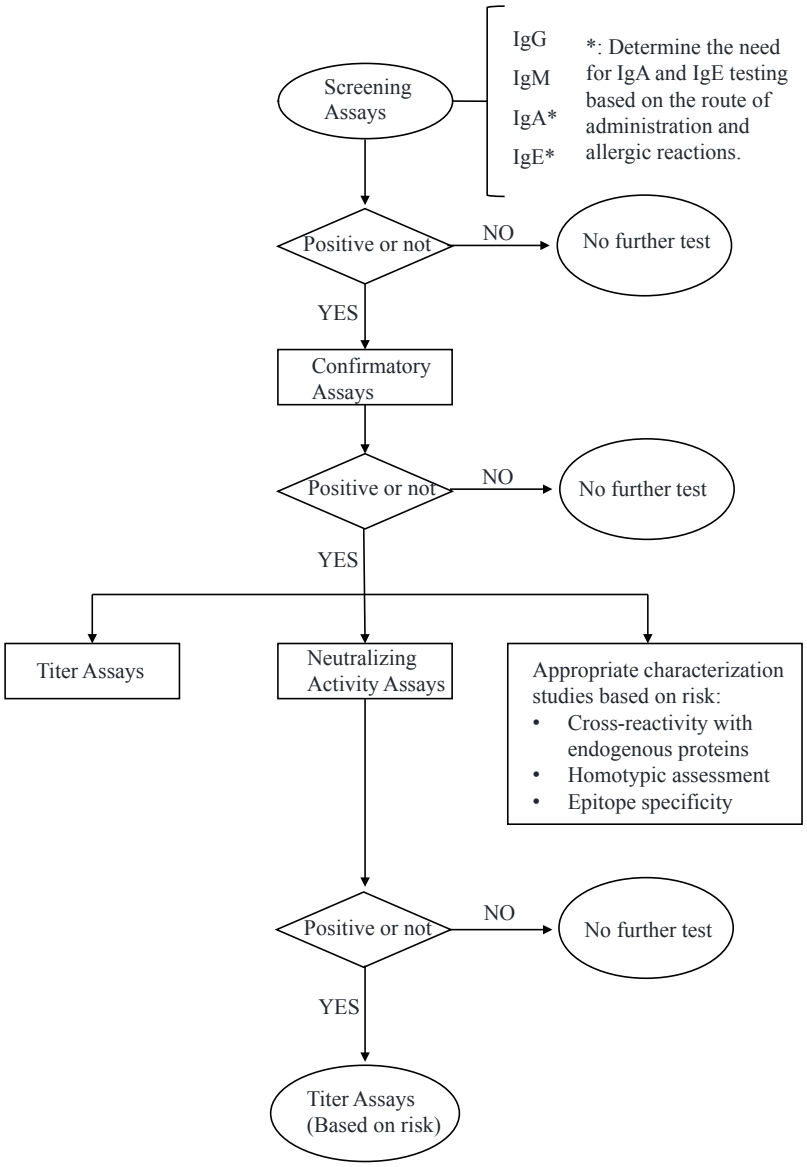

Figure 2. Multi-tiered immunogenicity detection strategy [34].

to analyze using classical immune methods.

These challenges highlight the need for careful consideration and optimization of assay conditions and techniques to ensure accurate and reliable assessment of immunogenicity and ADA detection for peptide drugs.

7. Summary

Peptide drugs have become a hot topic in international pharmaceutical research and development due to their high biological activity and low toxicity. They have undergone significant development and have attracted the attention of domestic pharmaceutical companies, which are actively involved in peptide drug research and development. However, the complexity of the pharmacokinetics/pharmacodynamics and physicochemical properties of peptide drugs undoubtedly poses challenges

to their development. Currently, there is no single analytical method that can fully meet all the requirements for studying the pharmacokinetics of peptide drugs. The pharmacokinetics/pharmacodynamics and bioanalysis of peptide drugs require interdisciplinary teams and multiple technical platforms to complement each other.

As the understanding and refinement of the pharmacokinetics/pharmacodynamics and bioanalytical characteristics of peptide drugs deepen in the industry, it will undoubtedly promote the development of peptide drugs and provide important scientific evidence for rational clinical use. The complexity and challenges associated with peptide drugs necessitate the collaboration of multidisciplinary teams and the integration of various analytical techniques to overcome the hurdles and maximize the potential of these promising therapeutics.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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