Teaser The article provides an interpharmacopoeial comparison of quality specifications of peptide-based drug monographs and areas for improvement in peptide therapeutics.

Peptide-based therapeutics: quality specifications, regulatory considerations, and prospects

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Exquisite selectivity, remarkable efficacy, and minimal toxicity are key attributes inherently assigned to peptides, resulting in increased research interest from the pharmaceutical industry in peptide-based therapeutics (PbTs). Pharmacopoeias develop authoritative standards for PbT by providing standard specifications and test methods. Nevertheless, a lack of harmonization in test procedures adopted for PbT in the latest editions of Pharmacopoeias has been observed. Adoption of a harmonized monograph could increase further the interest of the global pharmaceutical industry in PbTs. Here, we provide an overview of pharmacopoeial methodologies and specifications commonly observed in PbT monographs and highlight the main differences among the pharmacopoeias in terms of the active pharmaceutical ingredients that they focus on. We also address the prospects for PbTs to mature as a new therapeutic niche.

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Introduction

PbTs have emerged as an important class of molecules, sandwiched somewhere between classical organic entities and high-molecular-weight biopharmaceuticals. In general, peptides are monomers of amino acids ranging between 50 or fewer residues (<5000 Da) linked by a peptide (amide, –CONH–) bond, and lacking a tertiary structure [1–3]. Peptides are distinguished from proteins on the basis of size as proteins comprise one or more polypeptide chains linked to coenzymes and cofactors or to other macromolecules. Till date, More than 7000 naturally occurring peptides have been identified, all of which actively participate in a variety of physiological processes as hormones, neurotransmitters, growth factors, ion channel ligands, antimicrobials, and immunomodulators [4–7]. PbTs have shown remarkable potential in the treatment of a range of

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diseases, including autoimmune disorders, hormonal deficiencies, infections, and metabolic disorders together with diabetes and cancer of various origins [8,9].

The expansion of the therapeutic application of peptides is visible worldwide, with more than 60 peptides marketed in the USA, Europe, and Japan alone; over 150 peptides are in active clinical trials, with 260 peptides currently being tested in humans and 400 peptides in preclinical stages of development [10]. In of drug development, peptides are regarded as an excellent starting point for designing novel drugs, because of their intrinsic characteristics and pharmacological profiles [4]. The selectivity and specificity of these peptides towards cell surface receptors, such as G-protein-coupled receptors or ion channels, triggers their intracellular effects [4]. Moreover, the exquisite selectivity of peptides towards their target and efficacious signaling with cell surface receptors results in high potency with increased safety, efficacy, and tolerability in humans [11]. In addition, their smaller size enables peptides to penetrate deeper into body tissues compared with larger biomolecules. In addition, PbTs are less immunogenic, relatively cheaper than recombinant proteins and antibodies, have greater efficacy, minimal toxicity, and low accumulation in body tissues. Therefore, despite potential limitations, such as their high molecular weight, low systemic absorption, rapid renal and hepatic clearance, and poor membrane permeability, several peptides have entered the market and exhibit significant results against major diseases [11–14].

Peptide-based therapeutics market

The global market for PbTs has increased both in size and economic value over the past few years. Major factors driving the growth of PbTs are their wide applications in metabolic and autoimmune disorders. In terms of value, the global PbT market was valued at US$21.5 billion in 2016 and is expected to grow at a compound annual growth rate (CAGR) of 9.4% by 2025 [15]. The presence of a strong product pipeline has the potential for significant future growth. With the approval of nearly 117 new drugs and therapeutic biological products by the US Food and Drug Administration (FDA) over the past 3 years, biologicals represent a share of 35% of approved new drugs, with PbTs accounting for 8% of the total products introduced onto the market over the same time frame [16]. Table 1 details the peptides approved by the FDA from January 2015 to February 2018 [17].

Thus, with a major paradigm shift towards peptide development and their expansion in various therapeutic indications, the quality assessment of new peptide drugs introduced to the market is crucial to ensure both their efficacy and safety [18].

Regulatory framework and pharmacopoeial considerations

With many peptide drugs already in the market, the prime regulatory challenge is to establish quality control parameters of active pharmaceutical ingredients (APIs) and/or drug substances before the manufacturing of the finished drug products [19]. Several quality tests, in terms of related impurities, high-molecular-weight impurities, amino-acid analysis (AAA), moisture determination, and microbiological testing, can be considered when setting specifications to evaluate the final quality of the product before its release [14]. The release of these peptides by regulators implies the high quality and consistency in manufacturing, as submitted in the marketing authorization dossier for each drug [20].

However, PbTs represent an interesting space in the regulatory landscape, because, depending on the length of peptide, its intended clinical indication, and manufacturing technology, they are sometimes regarded as conventional chemical molecules and, in other cases, as biological entities or biosimilars [21]. The regulations for small-molecule drugs and their impurities are articulated in guidance provided by the International Conference on Harmonization (ICH) [22,23]. In addition, regulatory guidance for biologicals is available and guidance for biosimilars is also being developed. However, currently, there is a lack of guidelines for the regulation of peptide drugs, resulting in a regulatory vacuum, which presents a challenge to peptide manufacturers.

As per the FDA mandate, peptide products with fewer than 100 amino acids and made through chemical synthesis fall into the jurisdiction of New Drug Approval (NDA) pathways, whereas those made through recombinant DNA (rDNA) technologies must go through the Biologics License Application (BLA) process [24]. In addition, any product approved as abbreviated NDA ('ANDA') is considered generic and is also interchangeable, but any product approved through biosimilar pathways will need to prove its interchangeability because of differences in manufacturing technologies between biosimilar peptides and the reference product. During drug development, interchangeability faces additional regulatory hurdles because the biosimilar peptide manufacturer needs to provide sufficient data to demonstrate the pharmacokinetic, pharmacodynamic, efficacy, and safety profiles of the biosimilar peptide with the reference at recommended doses [18].

The European Medicines Agency (EMA) does not distinguish peptides based on size, but focuses instead on the manufacturing technology, whether it is derived through recombinant DNA

TABLE 1

Peptides approved by the FDA from January 2015 to February 2018

Peptide name	Indication	Route of administration	FDA approval	
			Date (MM/DD/YY)	Application No.
Lutetium Lu 177-dotatate	Pancreatic cancer	Infusion	01/26/2018	(NDA) 208700
Semaglutide	Type 2 diabetes mellitus	Subcutaneous	12/05/2017	(NDA) 209637
Abaloparatide	Osteoporosis	Subcutaneous	04/28/2017	(NDA) 208743
Cerliponase alfa	Specific form of Batten disease	Injection	04/27/2017	(BLA) 761052
Etelcalcetide	Secondary hyperparathyroidism	Intravenous	02/7/2017	(NDA) 208325
Plecanatide	Chronic idiopathic constipation	Oral	01/19/2017	(NDA) 208745
Lixisenatide	Type 2 diabetes mellitus	Subcutaneous	07/27/2016	(NDA) 208471
Gallium dotatate GA-68	Diagnostic agent	Intravenous	06/01/2016	(NDA) 208547
Insulin degludec	Diabetes mellitus	Subcutaneous	09/25/2015	(NDA) 203314

(rDNA) technology or chemically synthesized. All applications for marketing authorization for products derived from biotechnology and those considered potentially innovative (e.g., exenatide or Bydureon®) are required to follow a centralized procedure [25]. This procedure enables the marketing authorization holder (MAH) to market the product and make it available to patients throughout European Union (EU) on the basis of a single marketing authorization [26]. For approval of these biosimilar products, the EU has pioneered the regulation of biotherapeutics by establishing a solid framework with comprehensive comparability studies with the reference product [25].

Recently, the FDA's Centre for Drug Evaluation and Research (CDER) released a draft guideline '*ANDAs for Certain Highly Purified Synthetic Peptide Drug Products that Refer to Listed Drugs of rDNA Origin: Guidance for Industry*' that specifically addresses peptides, to assist the MAH to determine whether a synthetic product referring to a previously applied peptide product of rDNA origin should be submitted as an ANDA under Section 505(j) of the Federal Food, Drug and Cosmetic Act (FD&C Act) instead of as a new drug application (NDA) under section 505(b) of the FD&C Act [27]. The FDA also provides guidance on the submission of data for the chemistry, manufacturing, and controls information for synthetic peptide substances [27,28]. By contrast, the ICH provides a general guideline on how to deal with impurities that is widely followed across the world [22,23]. However, these international guidelines are followed in tandem with national guidelines and national pharmacopoeias.

Currently different pharmacopoeias, such as *Indian Pharmacopoeia* (*IP*), *European Pharmacopoeia* (*Ph. Eur.*), *British Pharmacopoeia* (*BP*), *United States Pharmacopoeia* (*USP*), and other international pharmacopoeias, define quality specifications in the form of monographs to which the peptide drug substance and finished product must adhere to. These specifications form a base for establishing quality requirements of individual formulations and are necessary for the proper functioning and regulatory control of peptide drugs [29,30].

In terms of the correct interpretation of a pharmacopoeial monograph, it must be in accordance with general requirements and testing methods, texts or notices pertaining to it as found in the current edition of the pharmacopoeia [31]. For instance, to determine the impurities in peptide products, pharmacopoeias provide limits of impurities, but these requirements are not framed to detect all possible impurities. The tests are designed to fix the limits of impurities that are tolerable and to ensure an absence of impurities that are toxic. *IP* mentions its general tests with an explanatory general chapter 5.5 'Impurities' [32], *USP* mentions tests in a general chapter 'Impurities in drug substances and drug products' [33], whereas *Ph. Eur.* mentions in them in a general chapter 5.10 'Control of impurities in substances for pharmaceutical use' [34].

Peptide characterization

The characterization of peptide therapeutics at each step of manufacturing is necessary to minimize lot-to-lot variation in product quality [35]. The production of peptides is sensitive to the type of manufacturing process used. Varied heterogenicity of production processes is observed even among products originating from the same process [36,37]. Any variability in the quality of the final peptide product is dependent on not only the variability in each manufacturing step, but also the quality of the starting material.

Pharmacopoeial quality specifications comprise a set of appropriate tests that confirm the identity and purity of a product, and ascertain the strength of the active substance, and, when needed, its performance characteristics. Highly characterized reference substances are used in testing to help ensure quality, such as the identity, strength, and purity of peptides [38]. A complete characterization package for peptides includes their physicochemical properties, immunoreactivity, purity, impurity profiling, and potency [39,40]. Table 2 describes some of the analytical procedures that need to be considered for the characterization of peptides. New analytical technologies are continuously being developed and are replacing existing traditional technologies. Furthermore, method validation and relevant stress studies (e.g., acid/base hydrolysis, oxidation, light, heat, and humidity studies) are also needed for the investigation of peptide products [41].

The peptide substances and drug product monographs mentioned in national compendium have critical quality attributes categorized into three subdivisions: identification, tests (purity), and assays. In addition to these critical quality attributes, pharmacopoeial monographs also contains other components, such as description, chemical formula, molecular weight, origin of peptide (if applicable), and storage and labeling. For instance, to provide additional information to stakeholders, pharmacopoeias also mention category and dose in drug substance monographs and usual strength in finished formulations [31].

Compendium tests detailed in the 'identification' section of a monograph confirm the identity of the substance in question. Physical and/or chemical tests and reactions mentioned in the identification section also ensure the specificity of the product. The 'Tests' section is principally directed at limiting impurities in the substance concerned. These limits imposed can be inferred from the conditions of a test or from recovery experiments. The 'assay' section indicates the single specific stability-indicating method used to quantify the active ingredients [42]. In some cases, the same procedure might be used for both the identification and the assay. A comparative overview of peptide drug substances currently described in latest edition of *IP* (2018), *Ph. Eur.* (9.0) and/or *USP-41* is provided in Table 3 [31,43,44].

Here, we discuss monographs for 36 peptide drug substances (*IP*, $N = 21$; *USP*, $N = 26$; *Ph. Eur.*, $N = 31$). We provide routine characterization techniques for peptides in the context of the development of orthogonal methods for the identification of synthetic peptides and to separate them from other closely related structures. Although specifications for drug substance and drug products are exhaustive, we discuss the pharmacopoeial approach of peptide drug substance monographs along with highlighting differences and similarities in pharmacopoeias towards the development of a new peptide not yet described in any compendium.

Monograph requirements

Basic information

The main title of a monograph for a peptide drug substance is the International Non-Proprietary Name (INN) approved by the WHO. Pharmacopoeias also provide subsidiary names and synonyms where they have same significance as the main title of the mono-

TABLE 2

Commonly used analytical tests of quality attributes for peptide products[a]

Class	Product quality attribute
General characteristics	Appearance/color (visual), clarity (turbimetric),pH (potentiometric), particulate matter (optical microscopy, light obscuration, and/or imaging particle analysis), UV/Vis spectral analysis, X-ray diffraction, differential scanning calorimetry, near/far UV circular dichroism spectroscopy, optical rotation
Identification	Intact peptide [HPLC (UV, RI, MS, fluorescence)], peptide mapping, molecular-weight determination (ESI-MS/MALDI-TOF), amino-acid sequencing, immunological identification (ELISA)
Purity and integrity	Purity profile (IEX, RP-HPLC, cIEF), aggregation (SE-HPLC, gel electrophoresis, light-scattering), high order structure (circular dichroism), mass-distribution profile (ESI-MS/MALDI-TOF), oxidation, deamidation and C-terminal lysine (cIEF, peptide mapping with MS and CEX-HPLC), glycation (peptide mapping with MS, HPLC), methylation, isomerization (RP-HPLC), di-sulfide bridges (peptide mapping with UV/MS, SDS-PAGE), enantiomeric purity (HPLC, GC-MS)
Process-related impurities	Residual protein [ELISA, endotoxin (LAL test), western blotting], residual DNA [bioburden (membrane filtration test)], elemental impurities (ICP-MS)
Potency	Potency (cell-based bioassay, animal-based bioassay, ADCC, CDC)

[a] Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; CEX, cation exchange; cIEF, capillary isoelectric focusing; ESI, electrospray ionization; ICP-MS, inductively coupled plasma MS; IEX, ion exchange; LAL, *Limulus* amoebocyte lysate; MALDI, matrix-assisted laser desorption/ionization; RI, refractive index.

graph. In the case of a peptide drug product, the monograph title contains the dosage form of the pharmacopoeial drug substances [e.g., aprotinin injection; calcitonin (salmon) nasal solution; or vancomycin eye drops]. The start of the drug substance monograph also contains the elemental formula, structural formula, molecular mass, physical form, and salt form of the substance.

Definition and production

The pharmacopoeial definition of a peptide monograph contains a statement relating to the manufacturing process. Wherever applicable, the origin (name and strain of organism) of the peptide is specified along with information about whether it is semisynthetic or derived from a fermentation product. The steps and details of tests carried out during the production process are not within the scope of a pharmacopoeia. Aspects relating to production of the peptide range from extraction from natural sources, chemical synthesis, enzymatic synthesis, rDNA technology, or synthesis in transgenic plants or animals [45,46]. Although the preferred technique for the production of larger peptides, such as insulin and calcitonin, is usually rDNA technology, chemical synthesis is confined to peptides ranging from 5 to 50 amino-acid residues, and enzymatic synthesis for peptides of fewer than ten amino-acid residues [47]. The origins of the peptide drug substances discussed here were: chemical synthesis ($N = 16$), fermentation ($N = 8$), tissue extraction ($N = 4$) and rDNA technology ($N = 7$). However, for some peptide monographs [i.e., calcitonin (salmon) *IP*, calcitonin (salmon) *USP*, colistimethate sodium, oxytocin acetate *IP*, and vasopressin *IP*), two different origins are mentioned in the pharmacopoeias.

Description

This heading is intended to define a qualitative statement about the state (solid, liquid), description (i.e., visual aspects, and organoleptic characters, such as odor and taste), and solubility profile of peptides. These characteristics assist in the preliminary evaluation of product quality. Any change in these characteristics during manufacture or storage should be investigated and appropriate action should be taken. For peptide monographs, a common description statement includes 'white to almost white, hygroscopic powder' and solubility is stated in water, ethanol, methanol, or dilute solutions of acids or alkali.

Identification of peptides

The identification section for peptides should be able to detect the presence of the active drug substance and discriminate other process-related impurities and degradation products that might be structurally similar to the parent peptide. As per ICH Q6A, the identification test should include at least two procedures (physicochemical, biological and/or immunochemical), especially where the assay is a chromatographic procedure. Identification should be specific and unequivocal. However, methods used to determine potency or purity are also used or modified as appropriate for serving identification criteria. As per pharmacopoeial requirements, tests mentioned in the identification section must be able to satisfy the requirements of the test and any failure to meet the requirements of the prescribed identification indicates that product is adulterated or mislabeled [48]. Table 4 describes identity confirmation techniques currently used in *IP*, *USP* and *Ph. Eur.* drug substance monographs.

The identification section of a monograph comprises techniques that verify the size of the molecule, its primary sequence, isoelectric profile, chromatographic properties, and whether the molecule has adopted the correct functional configuration. Typically, this section comprises reverse-phase high-performance liquid chromatography (RP-HPLC), peptide mapping, isoelectric focusing and/or capillary electrophoresis, bioassays, and, in a few cases, nuclear magnetic resonance (NMR) and mass spectrometry (MS) [31,43,44]. A current pharmacopoeial identity test uses liquid chromatography (LC) by RP-HPLC [*IP*, $N = 13$; *USP*, $N = 21$; *Ph. Eur.*, $N = 24$] as the first identification for the separation of intact or digested peptides from a variety of synthetic and biological sources [49]. For secondary identification, the compendia use techniques such as peptide-mapping techniques for drug substances obtained from rDNA technology [*IP*, $N = 07$; USP, $N = 07$; *Ph. Eur.*, $N = 08$], AAA for specificity [*IP*, $N = 03$; *USP*, $N = 03$; *Ph. Eur.*, $N = 10$], spectrometric reactions by infra-red or ultraviolet (UV) [*IP*, $N = 02$; *USP*, $N = 02$; *Ph. Eur.*, $N = 03$], colorimetric reactions [*IP*, $N = 04$; *USP*, $N = 03$; *Ph. Eur.*, $N = 05$], and

TABLE 3

Interpharmacopoeial comparison of peptide drug substances

No.	Peptide	Category	*Indian Pharmacopoeia* (2018)		*United States Pharmacopoeia* (41)		*European Pharmacopoeia* (9.0–9.4)	
			Monograph	Origin	Monograph	Origin	Monograph	Origin
1	Aprotinin	Antifibrinolytic	Vol II; pp. 1256	Bovine tissue	Vol I; pp. 342	Bovine tissue	01/2016:0580; Vol II; pp. 1747 (9.0)	Bovine tissue
2	Aprotinin concentrated solution	Antifibrinolytic	X		X		01/2016:0579; Vol II; pp. 1749 (9.0)	Bovine tissue
3	Atosiban acetate	Oxytocin antagonist	Vol II; pp. 1290	Chemical synthesis	X		X	
4	Bacitracin	Antibacterial	Vol II; pp. 1319	Fermentation	Vol I; pp. 436	Fermentation	01/2008:0465; Vol II; pp. 1789 (9.0)	Fermentation
5	Bacitracin zinc	Antibacterial	Vol II; pp. 1320	Fermentation	Vol I; pp. 440	Fermentation	01/2008:0466; Vol II; pp. 1791 (9.0)	Fermentation
6	Buserelin	Gonadotrophin-releasing hormone	X		X		01/2016:1077; Vol II; pp. 1887 (9.0)	Chemical synthesis
7	Calcitonin (salmon)	Antiosteoporotic	Vol III; pp. 3960	Chemical synthesis/rDNA technology	Vol I; pp. 620	Chemical synthesis/ rDNA technology	01/2008:0471; Vol II; pp. 1906 (9.0)	rDNA technology
8	Capreomycin sulfate	Antituberculosis	Vol II; pp. 1469	Fermentation	Vol I; pp. 670	Fermentation	X	
9	Colistimethate sodium	Antibiotic	Vol II; pp. 1693	Semisynthetic/ fermentation	Vol I; pp.1073	Semisynthetic/ fermentation	01/2008:0319; Vol II; pp. 2165 (9.0)	Semisynthetic/ fermentation
10	Colistin sulfate	Antibacterial	Vol II; pp. 1695	Fermentation	Vol I; pp. 1074	Fermentation	04/2015:0320; Vol II; pp. 2166 (9.0)	Fermentation
11	Desmopressin acetate	Antidiuretic hormone	Vol II; pp. 1773	Chemical synthesis	Vol I; pp. 1182	Chemical synthesis	07/2009:0712; Vol II; pp. 2213 (9.0)	Chemical synthesis
12	Exenatide	Anti-diabetic	X		Vol I; pp. 1667	Chemical synthesis	X	
13	Felypressin acetate	Vasoconstrictor in LA	X		X		01/2008:1634; Vol II; pp. 2454 (9.0)	Chemical synthesis
14	Glucagon	Treatment of hypoglycemia	X		Vol I; pp. 1956	rDNA technology	07/2013:1635; Vol II; pp. 2584 (9.0)	rDNA technology
15	Gonadorelin acetate	Gonadotrophic hormone	X		Vol I; pp. 1977	Chemical synthesis	01/2008:0827; Vol II; pp. 2611 (9.0)	Chemical synthesis
16	Gonadorelin hydrochloride	Gonadotrophic hormone	X		Vol I; pp. 1979	Chemical synthesis	X;	
17	Chorionic gonadotropin	Gonadotrophic hormone	Vol II; pp. 1610	Urine of pregnant women	Vol I; pp. 1981	Urine of pregnant women	01/2011:0498; Vol II; pp. 2613 (9.0)	Urine of pregnant women
18	Goserelin acetate	Gonadotrophic hormone	X		Vol I; pp. 1983	Chemical synthesis	01/2013:1636; Vol II; pp. 2614 (9.0)	Chemical synthesis
19	Gramicidin	Antibacterial	X		Vol I; pp. 1989	Fermentation	01/2008:0907; Vol II; pp. 2616 (9.0)	Fermentation
20	Human insulin	Hypoglycemic	Vol III; pp. 4002	rDNA technology	Vol I; pp. 2171	rDNA technology	01/2011:0838; Vol II; pp. 2768 (9.0)	rDNA technology
21	Insulin (bovine/ porcine)	Hypoglycemic	Vol II; pp. 2292	Pancreas of porcine or bovine	Vol I; pp. 2161	Pancreas of porcine or bovine	Bovine 01/2008:1637; Vol II; pp. 2763 (9.0); porcine 01/ 2008:1638; Vol II; pp. 2774 (9.0)	Porcine or bovine pancreas

TABLE 3 (*Continued*)

No.	Peptide	Category	*Indian Pharmacopoeia* (2018)		*United States Pharmacopoeia* (41)		*European Pharmacopoeia* (9.0–9.4)	
			Monograph	Origin	Monograph	Origin	Monograph	Origin
22	Insulin aspart	Antidiabetic	Vol III; pp. 4004	rDNA technology	Vol I; pp. 2164	rDNA technology	01/2008:2084; Vol II; pp. 2762 (9.0)	rDNA technology
23	Insulin lispro	Antidiabetic	Vol III; pp. 4008	rDNA technology	Vol I; pp. 2178	rDNA technology	01/2008:2085; Vol II; pp. 2771 (9.0)	rDNA technology
24	Insulin glargine	Antidiabetic	Vol III; pp. 4018	rDNA technology	Vol I; pp. 2167	rDNA technology	01/2014:2571; Vol II; pp. 2766 (9.0)	rDNA technology
25	Leuprorelin acetate	Gonadotrophin-releasing hormone	X		X		01/2008:1442; Vol III; pp. 2883 (9.0)	Chemical synthesis
26	Oxytocin acetate	Uterine stimulant	Vol III; pp. 2829	Pituitary extraction/ chemical synthesis	Vol II; pp. 3132	Chemical synthesis	01/2008:0780; Vol III; pp. 3250 (9.0)	Chemical synthesis
27	Oxytocin concentrated solution	Uterine stimulant	X		X		01/2008:0779; Vol III; pp. 3251 (9.0)	Chemical synthesis
28	Polymyxin B sulfate	Antibacterial	X		Vol II; pp. 3347	Fermentation	01/2008:0203; Vol III; pp. 3366 (9.0)	Fermentation
29	Protamine sulfate	Heparin antidote	Vol III; pp. 3033	Sperm or mature testes of fish	Vol II; pp. 3503	Sperm or mature testes of fish	01/2017:0569; Vol III; pp. 3443 (9.0)	Sperm or mature testes of fish
30	Protirelin acetate	Thyrotropin-releasing hormone	X		X		01/2008:1144; Vol III; pp. 3444 (9.0)	Chemical synthesis
31	Somatostatin acetate	Growth hormone releasing-inhibiting hormone	X		X		04/2014:0949; Vol III; pp. 3613 (9.0)	Chemical synthesis
32	Teriparatide	Bone-forming agent	Vol III; pp. 4053	rDNA technology	Vol II; pp. 3990	rDNA technology	01/2017:2829; Vol III; pp. 3732 (9.0)	rDNA technology
33	Tetracosactide EP (cosyntropin *USP*)	Corticotropic peptide	X		Vol I; pp. 1101	Chemical synthesis	04/2010:0644; Vol III; pp. 3743 (9.0)	Chemical synthesis
34	Tyrothricin	Polypeptide antibacterial	Vol III; pp. 3454	Fermentation	Vol II; pp. 4252	Fermentation	01/2008:1662; Vol III; pp. 3871 (9.0)	Fermentation
35	Vancomycin hydrochloride	Antibacterial	Vol III; pp. 3477	Fermentation	Vol II; pp. 4284	Fermentation	01/2017:1058; Vol III; pp. 3896 (9.0)	Fermentation
36	Vasopressin	Antidiuretic	Vol III; pp. 3482	Chemical synthesis/animal source	Vol II; pp. 4289	Chemical synthesis	X	

TABLE 4

Confirmation techniques currently used in pharmacopoeial drug substance monographs[a,b]

No.	Peptide	*Indian Pharmacopoeia* (2018)	*United States Pharmacopoeia* (41)	*European Pharmacopoeia* (9.0–9.4)
1	Aprotinin	TLC, trypsin	TLC, HPLC	TLC, trypsin
2	Aprotinin concentrated solution	X		TLC, trypsin
3	Atosiban acetate	HPLC, IR	X	
4	Bacitracin	TLC	Composition	1ID, composition; 2ID, TLC
5	Bacitracin zinc	TLC	Composition	1ID (composition); 2ID, TLC
6	Buserelin	X		1ID, HPLC, NMR; 2ID, HPLC, AAA
7	Calcitonin (salmon)	HPLC, AAA, peptide mapping	HPLC	HPLC, AAA, peptide mapping
9	Capreomycin sulfate	UV, sulfate	HPLC, sulfate	–
10	Chorionic gonadotropin	Bioassay	–	Bioassay
11	Colistimethate sodium	TLC, color, sulfate, sodium	IR	TLC, color, sulfate, sodium
12	Colistin sulfate	1ID, HPLC, sulfate; 2ID, TLC, color	HPLC, sulfate, color	1ID, HPLC, sulfate; 2ID, TLC, color, sulfate
13	Desmopressin acetate	HPLC, AAA	MS, HPLC	HPLC, AAA
14	Exenatide	X	HPLC, AAA	X
15	Felypressin acetate	X		HPLC, AAA
16	Glucagon	–	HPLC, peptide mapping	
17	Gonadorelin acetate	X	MS, HPLC, SOR	HPLC, TLC
18	Gonadorelin hydrochloride	X	MS, HPLC	X
19	Goserelin acetate	X	HPLC	NMR, HPLC, AAA
20	Gramicidin	–	UV	1ID, UV, HPLC; 2ID, UV, TLC
21	Human insulin	HPLC, peptide mapping		
22	Insulin	HPLC, peptide mapping		
23	Insulin aspart	HPLC, peptide mapping		
24	Insulin glargine	HPLC, peptide mapping		
25	Insulin lispro	HPLC, peptide mapping		
26	Leuprorelin acetate	X		HPLC, AAA, IR
27	Oxytocin	HPLC, AAA	HPLC, AAA, MS	HPLC, AAA
28	Oxytocin concentrated solution	X		HPLC, AAA
29	Polymyxin B sulfate	–	HPLC	1ID, HPLC; 2ID, TLC
30	Protamine sulfate	Colorimetric	HPLC, bioidentity	SOR, colorimetric
31	Protirelin acetate	X		IR, HPLC
32	Somatostatin acetate	X		HPLC, AAA
33	Teriparatide	1ID, bioidentity, peptide mapping, HPLC; 2ID, SDS-PAGE, CZE	HPLC, peptide mapping	
34	Tetracosactide (cosyntropin)	X	HPLC, AAA	
35	Tyrothricin	1ID, composition; 2ID, TLC	Color	1ID, composition; 2ID, TLC
36	Vancomycin hydrochloride	HPLC	IR	HPLC
37	Vasopressin	HPLC	HPLC, MS	–

[a] X, monograph not present; –, identification not present.
[b] Abbreviations: 1ID, primary identification technique; 2ID, secondary identification technique; IR, infrared spectroscopy; SOR, specific optical rotation.

bioassays [*IP*, *N* = 02; *USP*, *N* = 01]. The monographs of desmopressin *USP*, gonadorelin acetate *USP*, and vasopressin *USP* yield information on molecular weight using MS analysis. Teriparatide *IP* mentions separation by Sodium dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and capillary zone electrophoresis (CZE). 2-ID by traditional techniques, such as thin-layer chromatography (TLC) and paper chromatography, are also identified [*IP*, *N* = 05; *USP*, *N* = 01; *Ph. Eur.*, *N* = 09]. Only where the salt form of a drug substance is used in antibiotic peptides is the nonpeptide moiety identified (e.g., sodium, chlorides, sulfates, and zinc).

Rationale for identity methods

HPLC is the primary method used for the identification, separation, and quantification of peptides and related degraded impurities [50]. The separation utilizes differences in peptide hydrophobicity/hydrophilicity (RP-HPLC, normal phase), net

charge (ion-exchange chromatography), or size exclusion chromatography (SEC). Among the 36 PbTs discussed here, 77% of compendium monographs include RP-HPLC for use in identification, in contrast to other methods, which appear in 23% of monographs. RP-HPLC involves the adsorption of peptides during a hydrophobic stationary phase based on amino acid sequences and molecular conformations using a gradient elution. UV detectors are widely used for the detection of peptides in far-UV (210–220 nm) and for detection of aromatic side chains of phenylalanine, tyrosine, and tryptophan (250–290 nm) [51]. As an approach to the refinement of these methods, *Ph. Eur.* monographs of insulin and its analogs comprise a combination of two analytical methods: LC and peptide mapping. Peptide mapping is a method for the direct analysis of sequences, especially for products obtained by rDNA technology [52].

Electrophoresis (e.g., SDS-PAGE and capillary electrophoresis) is used for the size-based identification and separation of peptides and also gives relatively reliable information about the molecular mass of the peptide [53–55]. Recently, CZE has been recognized as a more reliable and robust technique for the analytical characterization, product development, and quality control of peptides [56]. Pharmacopoeias use the CZE techniques by combining different separation techniques with an appropriate degree of orthogonality [57].

AAA is used to determine the amino acid composition or content of peptides. It allows the quantitation of free amino acids, as well as amino acids released from peptides. It might not complement the latest sophisticated spectrometric instruments but is presented as an alternative [58]. In the monographs of leuprorelin and its nonapeptide analogs buserelin, desmopressin, felypressin, and goserelin, AAA is considered as an identification test by *Ph. Eur.*, but is not described in *IP* or *USP*.

Pharmacopoeias are now interested in replacing with, or additionally including, ^{1}H NMR or ^{13}C NMR spectroscopy for the structural elucidation of peptides [59]. Identification by NMR spectrometry applies to peptides comprising up to 15 amino acids or, for instance, where a peptide comprises unnatural amino acids, based on the analysis of its amino acid composition or sequence [60]. Currently, MS and LC-MS are primarily used in quality control analyses. These techniques provide more detailed information about a peptide and its related impurities, and sample requirements for analysis are comparatively low [61]. With rapid developments in technology, *USP* has switched the method used for the identification of synthetic peptides, such as desmopressin acetate *USP*; gonadorelin acetate *USP*; gonadorelin hydrochloride *USP* and vasopressin *USP*, from conventional techniques to MS.

Tests

During peptide synthesis, many side-chain-protecting groups, scavengers, or activated functional groups are added to the peptide to prevent undesired side-chain reactions. Given that these chemical reactions are not 100% complete, these side chains remain attached to the parent peptide, leading to formation of closely related peptide impurities [62]. The impurities encountered in synthetic peptides originate either from raw materials, during the manufacturing processes, formed by degradation products, or during storage of the product. However, those arising from fermentation process result from media components, residual proteins or residual DNA, degradation products, and aggregates, either deaminated or oxidized [63,64]. Identification of these closely related peptides is detailed in the 'Tests' section of pharmacopoeias. These tests provide information about the extent of known potential or actual impurities and do not guarantee freedom from all possible impurities. The chemical tests that reveal levels of particular impurities or class of impurity are augmented by physical tests, such as specific optical rotation, light absorbance, refractive index, and so on. In addition, the monographs also contain nonspecific tests, such as sulfated ash and loss on drying [32].

Related peptide impurities

The test for related substances is provided in pharmacopoeial monographs to limit impurities and degradation products in the final compound. In a few cases, specific tests might also be provided where a particular impurity arising from the manufacturing process or from degradation needs to be limited on the grounds of toxicity. In drug product monographs where degradation over time is an issue, the same analytical test is used (i.e., 'stability indicating is also purity-indicating'). Most pharmacopoeial monographs feature gradient RP-HPLC with UV detection for related substance tests [31,43] because of its selectivity, high sensitivity, low limit of detection, quantitation, and robustness. When HPLC is coupled with MS, it allows the identification and characterization of impurities based on molecular weight [65]. In its general monograph 'Substances for pharmaceutical use', the *Ph. Eur.* mentions the thresholds for the reporting, identification, and qualification of organic impurities in peptides obtained from chemical synthesis as >0.1%, >0.5%, and >1.0%, respectively. Specific thresholds can be applied for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects [44].

In pharmacopoeias, monographs for peptide drug substances and products include specifications along with acceptance criteria for impurities. The impurities can be specified or unspecified, depending upon their presence and concentration levels [42]. The limit of these impurities should be greater than the limit of detection, and the format for reporting is given as 'Not more than' (NMT) followed by the threshold [42]. For peptide drug substances, pharmacopoeias include the acceptance criteria as NMT 0.5% for each identified specified impurity, NMT 0.3% for unidentified impurities, and NMT 1.0% for total impurities. The impurity limits of residual solvents and inorganic impurities, if present, are mentioned in specific drug substance monographs. In peptide drug products, the acceptance criteria for each identified specific degradation product is NMT 1.0%, each unidentified degradation product is NMT 0.5%, and total degradation product is NMT 2.0% [32].

Concerning the comparison of impurity profiling in pharmacopoeias, the total sum of impurities varies from 1% to 17%. The largest limit for total impurities was observed in the bacitracin *Ph. Eur.* and colistin sulfate *Ph. Eur.* monographs. Attention has to be paid to differences in pharmacopoeias. For example, both *Ph. Eur.* and *USP* include a gonadorelin acetate monograph; however, *Ph. Eur.* specifies individual impurities and total impurity as NMT 2% and NMT 5%, respectively, whereas USP has relaxed the limits to NMT 1% and NMT 2%, respectively. Table 5 provides an overview

TABLE 5

Impurities observed in pharmacopoeial drug substance monographs[a]

Peptide drug substance	Specified impurity	Test (acceptance criteria)		
		Indian Pharmacopoeia (2018)	*United States Pharmacopoeia* (41)	*European Pharmacopoeia* (9.0–9.4)
Aprotinin	Des-Ala-aprotinin	NMT 8.0%		
	Des-Ala-des-Gly-aprotinin	NMT 7.5%		
	N-pyroglutamyl-aprotinin [(5-oxoprolyl) aprotinin]	NMT 1.0%		
	Aprotinin oligomers total	NMT 1.0%		
	Any other impurity	NMT 0.5%		
	Sum of all unknown impurities	NMT 1.0%		
Aprotinin concentrated solution	Des-Ala-aprotinin	X	X	NMT 8.0%
	Des-Ala-des-Gly-aprotinin			NMT 7.5%
	N-pyroglutamyl-aprotinin [(5-oxoprolyl) aprotinin]			NMT 1.0%
	Aprotinin oligomers total			NMT 1.0%
	Any other impurity			NMT 0.5%
	Total impurities			NMT 1.0%
Atosiban acetate	Individual impurity	NMT 1.0%	X	X
	Total impurity	NMT 3.0%		
Bacitracin	Bacitracin impurity E (IP and EP)	NMT 6.0%	–	NMT 6.0%
	Content of bacitracin A	NLT 40.0%		
	Content of active bacitracin (sum of bacitracins A, B1, B2 and B3)	NLT 70.0%		
	Limits of early eluting peptides	NMT 20.0%		
	Limit of bacitracin F	–	NMT 6.0%	–
Bacitracin zinc	Bacitracin impurity E (IP and EP)	NMT 6.0%	–	NMT 6.0%
	Content of bacitracin A	NLT 40.0%		
	Content of active bacitracin (sum of bacitracins A, B1, B2 and B3)	NLT 70.0%		
	Limits of early eluting peptides	NMT 20.0%		
	Limit of bacitracin F	–	NMT 6.0%	–
Buserelin	[2-D-histidine] buserelin (impurity A)	X	X	NMT 2.5%
	Endo-3a-serine-buserelin (impurity F)			NMT 1.0%
	Endo-8a-proline-buserelin (impurity G)			NMT 1.0%
	Any other impurity: [4-D-serine] buserelin; buserelin-(3-9)-peptide; [5-D-tyrosine] buserelin			NMT 0.5%
	Total impurity			NMT 4.0%
Calcitonin (salmon)	Acetylcalcitonin (salmon) (impurity A)	Individual impurity; NMT 3% of total area of all peaks		For each impurity, NMT 3%
	[9-D-leucine] Calcitonin (salmon) (impurity B)			
	Des-22-tyrosine-calcitonin (salmon) (impurity C)			
	O-acetylated calcitonin (salmon) (impurity D)			
	Salmon calcitoninyl glycine (impurity E or calcitonin salmon-related compound-B)	NMT 0.6%		
	[1,7-bis(3-sulfo-L-alanine)] calcitoninylglycine (rDNA) (impurity F)	NMT 0.2%		
	[1,7-bis(3-sulfo-L-alanine)] calcitonin (salmon) (rDNA) (impurity G)	NMT 0.2%		
	Total impurities	NMT 5.0%		
Capreomycin sulfate	N/A	–		X
Colistimethate sodium	N/A	–		
Colistin sulfate	Individual impurity	NMT 4.0%	–	NMT 4.0%
	Total impurity	NMT 23.0%	–	NMT 23.0%
Cosyntropin	Cosyntropin sulfoxide	X	NMT 2.0%	X
	Reduced Trp in cosyntropin		NMT 2.0%	
	Any other individual impurity		NMT 1.0%	
	Total impurities		NMT 5.0%	
Desmopressin/ desmopressin acetate (*USP*)	Individual impurity	NMT 0.5%		
	Total impurity	NMT 1.5%		

TABLE 5 (*Continued*)

Peptide drug substance	Specified impurity	Test (acceptance criteria)		
		Indian Pharmacopoeia (2018)	***United States Pharmacopoeia* (41)**	***European Pharmacopoeia* (9.0–9.4)**
Exenatide	Procedure 1: exenatide related substances: [Glu^{13}]-exenatide; sum of [Asp28]-exenatide and [Met(O)14]-exenatide; unspecified impurities	NMT 0.50%; 0.50%; 0.50%, respectively		
	Procedure 2: *N*-acetyl His^{1}-exenatide			
Felypressin	S^{1},S^{6}-bis [(acetylamino)methyl]-(reduced felypressin) (impurity A)	X	X	NMT 0.5%
	[5-aspartic acid] felypressin (impurity B)			NMT 0.5%
	Bis (reduced felypressin) (1,6′), (1′,6)-bis (disulfide) (impurity C)			NMT 0.5%
	Bis (reduced felypressin) (1,1′), (6,6′)-*bis* (disulfide) (impurity D)			NMT 0.5%
	N^{1}-acetylfelypressin (impurity E)			NMT 0.5%
	[4-glutamic acid] felypressin (impurity F)			NMT 0.5%
	Any other impurity, for each impurity			NMT 0.1%
	Total impurities			NMT 3.0%
Glucagon	Four desamidoglucagons	X	NMT 2.0%	–
	Deamidated forms		–	NMT 0.8%
	Total impurities		NMT 6.0%	NMT 3.0%
Gonadorelin acetate	Individual impurity	X	NMT 1.0%	NMT 2.0%
	Total impurity		NMT 2.0%	NMT 5.0%
Gonadorelin hydrochloride	Individual impurity	X	NMT 2.0%	X
	Total impurity		NMT 3.0%	
Chorionic gonadotropin	–	–	–	–
Goserelin acetate	Decarbamoylgoserelin	X	NMT 1.0%	–
	Goserelin-(1-8)-peptidyl-l-prolinohydrazide (impurity E)		–	NMT 1.0%
	Any other impurity:; ([4-D-serine]goserelin); [6-[O-(1,1-dimethylethyl)-L-serine]] goserelin; [9-D-proline] goserelin; Des-9-L-proline-goserelin; [5-D-tyrosine]goserelin; [2-D-histidine]goserelin; [1-(5-oxo-D-proline)]goserelin; endo-8a,8b-di-L-proline-goserelin; endo-8a-L-proline-goserelin; [4-(O-acetyl-L-serine)]goserelin; [7-D-leucine]goserelin		NMT 0.5%	NMT 0.5%
	Total impurity		NMT 2.5%	
Gramicidin	Individual impurity	X	–	NMT 1%
	Any other impurity; [4-methionine]gramicidin A1; gramicidin A1 3-hydroxypropyl; gramicidin B2; [10-methionine] gramicidin C1; gramicidin A2 3-hydroxypropyl			NMT 2%
Human insulin	A21 desamido insulin	NMT 5.0%	NMT 2.0%	
	Total impurities excluding A21 desamido insulin	NMT 6.0%	NMT 2.0%	
Insulin	A21 desamido insulin (bovine/porcine)	NMT 5.0%	NMT 10%	NMT 3.0%/2.0%
	Total impurities excluding A21 desamido insulin (bovine/porcine)	NMT 6.0%	–	NMT 3.0%/2.0%
	Other insulin-related compounds (USP)	–	NMT 5.0%	–
Insulin aspart	B28isoAsp insulin aspart	NMT 1.0%		
	A21 Asp insulin aspart	NMT 2.0%		
	B3 Asp insulin aspart			
	B3isoAsp insulin aspart			
	Total impurities	NMT 1.5%		
Insulin lispro	A21 desamido insulin Lispro	NMT 1.0%		
	Individual impurity	NMT 0.5%		
	Total impurities other than A21 desamido insulin Lispro	NMT 2.0%		
Insulin glargine	Individual impurity	NMT 0.4%	NMT 0.5%	NMT 0.4%
	Total impurity	NMT 1.0%	NMT 1.5%	NMT 1.0%
Leuprorelin acetate	[4-(O-acetyl-L-serine)]leuprorelin (impurity D)	X	X	NMT 1.0%
	[4-D-serine]leuprorelin (impurity A)			NMT 0.5%
	[2-D-histidine]leuprorelin (impurity B)			NMT 0.5%
	[6-L-leucine]leuprorelin (impurity C)			NMT 0.5%
	Unspecified impurities: [3-D-tryptophane]leuprorelin; [2-D-histidine, 4-D-serine]leuprorelin; [5-D-tyrosine]leuprorelin; [7-D-leucine]leuprorelin; [1-(5-oxo-D-proline)]leuprorelin; [8-[5-N-[imino(1H-pyrazol-1-yl)methyl]-L-; ornithine]leuprorelin; [4-dehydroalanine]leuprorelin			For each impurity NMT 0.5%

TABLE 5 (*Continued*)

Peptide drug substance	Specified impurity	Test (acceptance criteria)		
		Indian Pharmacopoeia (2018)	***United States Pharmacopoeia* (41)**	***European Pharmacopoeia* (9.0–9.4)**
	Total impurity	–	–	NMT 2.5%
Oxytocin acetate	Any impurity	–	–	NMT 1.5%
	Total impurity	–	NMT 5%	
Oxytocin concentrated solution	Any impurity	X	X	NMT 1.5%
	Total impurity			NMT 5.0%
Polymyxin B sulfate	Any impurity	X	–	NMT 3.0%
	Total impurity			NMT 17.0%
Protamine sulfate	–	–	–	–
Protirelin acetate	Any impurity; 5-oxo-L-prolyl-D-histidyl-L-prolinamide; 5-oxo-D-prolyl-L-histidyl-L-prolinamide; 5-oxo-L-prolyl-L-histidine; 5-oxo-L-prolyl-L-histidyl-L-proline; (3S,8aS)-3-(1H-imidazol-4-ylmethyl)hexahydropyrrolol[1,2-a]pyrazine-1,4-dione(cyclo(-L-histidyl-L-prolyl-))	X	X	For each impurity, NMT 2.0%
	Total impurity			NMT 3.0%
Somatostatin acetate	Any impurity	X	X	NMT 1%
	Total impurity			NMT 2%
Teriparatide	Total of methionylsulfoxides of teriparatide [comprising Met + O(8) teriparatide, Met + O(18) teriparatide, and Met + O (8,18)] (impurity A,B,C)	NMT 0.5%		
	Largest other individual related impurities	NMT 0.5%		
	Total impurities	NMT 2.5%		NMT 2.0%
Tetracosactide (cosyntropin)	Tetracosactidesulfoxide (Impurity A)	X	–	NMT 3%
	Impurity B		–	NMT 4%
	Unspecified impurities		–	For each impurity, NMT 2.5%
	Sum of impurities other than A		–	NMT 9%
Tyrothricin	–	X	–	–
Vancomycin hydrochloride	Any impurity; monodechlorovancomycin	–	NMT 4.7%	NMT 4%
	Sum of all secondary peaks	NMT 4%	–	–
	Total impurities	NMT 7%	–	NMT 7%
Vasopressin	Total impurities	NMT 5%	NMT 5%	X

[a]Abbreviation: NLT, not less than; NMT, not more than.
[b]X, monograph not present; –, identification not present.

of the typical peptide impurities currently described in *IP*, *USP* and *Ph. Eur.* [31,43,44].

Absorbance and optical rotation

UV absorption spectroscopy is commonly used to determine the concentration and enzyme activity of peptides [66]. Generally, synthetic peptide monographs containing tryptophan residue gives specification on UV absorbance at 278 nm. However, two similar synthetic peptides of goserelin and leuprorelin lack a UV absorbance test, indicating that it is not always necessary for quality control if other modern spectrophotometric methods are available. In a few monographs, optical rotation is also mentioned; however, chiral chromatography could offer the potential to replace optical rotation.

High-molecular-weight impurities

High-molecular-weight impurities are encountered in peptide drug substances arising from protein aggregation. These aggregates differ in origin, type, and size, and can be caused by multiple factors [67]. Regulatory agencies have a particular interest in aggregates that have potential to enhance immune responses and cause adverse effects that might affect safety and efficacy of product. These aggregates are classified into multiple categories, such as dimer, trimer and tetramer structure; fibrilloid and amorphous; covalent, noncovalent and colloids; and native and denatured functionality [68]. Quantification of these peptide impurities is performed using size-exclusion chromatography in nondenaturing conditions (neutral, aqueous buffers) to avoid dissociation and consequent nondetection of noncovalent aggregates.

In monographs of insulin and its analogs, impurities are explicitly analyzed using size-exclusion chromatography. The acceptance limits of total impurities with a molecular mass higher than that of the insulin monomer are harmonized and range from 0.25% to 1%. In some cases (e.g., aprotinin), covalently bound oligomers can also be determined using RP-HPLC.

Moisture determination and counter-ions

The requirement to demonstrate lot-to-lot consistency cannot be fulfilled without showing that the tests for water (residual moisture remaining from lyophilization) and counter-ion analysis (e.g., acetate or hydrochloride) are controlled. These are essential quality attributes for peptide stability. Loss on drying is consistently described in pharmacopoeial nonsynthetic peptides except for vancomycin and vasopressin monographs, where water determination is the preferred method. For synthetic peptides, except for oxytocin *USP* and oxytocin *IP*, residual water is commonly applied test using Karl–Fischer titration.

Depending on the hydrophilicity of the compound, the peptide monographs in pharmacopoeias give single-value acceptance criteria for water and loss on drying tests, and range from NMT 3% to NMT 14%. The counter-ions in pharmacopoeial monographs are determined using LC and acceptance criteria for acetic acid range from ±3% to 13%.

Amino acid analysis

AAA is performed throughout the manufacturing process because it is used to ensure consistency across batches. This technique is used to quantify peptides, to determine the identity of peptides based on their amino acid composition, to support peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect typical amino acids that might be present in the peptide. It involves the hydrolysis of the peptide (usually in acidic conditions) to its individual amino acid residues, followed by chromatographic separation using an amino-acid analyzer [69]. *Ph. Eur.* and *USP* require the use of this purity test in synthetic peptides, as in the gonadorelin and calcitonin (salmon) monographs. As an exception, *IP* mentions the AAA test in the identification section of peptide monographs.

Microbiological attributes

One of the significant factors controlling the quality of peptide therapeutics is the assessment of the microbiological quality of a medicinal product [70]. Testing performed on both peptide drug substances and drug products involves microbial enumeration tests for total aerobic microbial counts (TAMC) and total yeast and mold counts (TYMC) and must adhere to the acceptance criteria of 10^3 colony-forming units (CFU)/g and 10^2 CFU/g, respectively [71]. In cases where the label states that the product is sterile, the peptide monograph should meet the requirements for sterility testing for assurance of product sterility requirements. If intended for parenteral use (i.e., administration by injection, infusion, or implants), a bacterial endotoxin test (BET) is mentioned in the monograph. The limits for BET are based on the maximum recommended human dose of the product per kg of body weight in a single hour period. However, BET can be replaced by a pyrogen test wherever justified [72].

Bioidentity

Bioidentity in the test section is mentioned in seven *USP* monographs, including insulin and its analogs. For calcitonin (salmon) *USP*, the test involves quantification of cAMP produced within the human mammary tumor cell line T-47D. By contrast, the protamine sulfate *USP* monograph involves bioidentity via a titration method and quantitates the number of USP Heparin units in the volume of titrant added at the endpoint per mg of protamine sulfate. For insulin monographs, the quantitative test is determined by a rabbit blood sugar method carried out by subcutaneous injection of insulin standard and samples in rabbits.

Inorganic impurities

IP and *Ph. Eur.* are consistent in mentioning the test for inorganic impurities, such as 'sulfated ash', in peptide monographs to determine the inorganic impurity content of organic substances. Most peptide monographs also mention tests for heavy metals, which can result from starting materials and reagents, leaching of equipment, and residual catalysts. Pharmacopoeial harmonization is observed in the vancomycin hydrochloride monograph, wherein the limit of 30 ppm is prescribed for the heavy metal test. To incorporate modern analytical technologies and to detect important elemental impurities, the *USP* deleted tests for heavy metals from all individual monographs and replaced them with *USP* elemental impurities.

Other tests

In addition to the tests described above, other tests, such as appearance, solubility, pH, residual solvents, and tests for other residual materials, are also required for the quality control of peptides. The decision to incorporate these tests is based on the regulatory status of the material, manufacturing process validation status, and knowledge of the synthesis and purification routes.

Assay

For synthetic peptides, pharmacopoeial assays are generally comparative chromatographic procedures, performed using a defined chemical reference substance as the standard. For many peptides, RP-HPLC with detection at 210–220 nm targeting the amide bond is generally used for both assay and related substances and compared with a well-characterized reference substance. Peptide antibiotics obtained from fermentation processes are usually assessed using a microbial assay of the antibiotics. Thus, the content of active moiety is expressed in units of biological activity.

Acceptance criteria are normally expressed in terms of acetic acid-free or anhydrous substances. The permitted limits for such tests are typically asymmetric, the upper limit being 100% + the permitted assay repeatability (usually ± 2.0%), and the lower limit being 100% – (the permitted assay repeatability + the maximum permitted level of impurity). Pharmacopoeial inconsistencies can be seen in the protamine sulfate monographs, for which *IP* and *Ph. Eur.* favor traditional methods (i.e., titration assays), whereas *USP* has developed a LC method that is more accurate and quicker.

Prospects

As biological moieties obtained from animals, peptides constitute an isolated therapeutic niche that continues to grow in prominence in the pharmaceutical research and development sector [10]. Peptides have now been shown to be useful as treatments for a variety of chronic diseases, including obesity, cancer, and diabetes [73,74]. PbTs have already positively impacted the global pharmaceutical market, accounting a market share of US$21.5 billion in 2016 [15]. Although many peptides have entered in clinical trials, technical hurdles to the development of effective PbT, such as lack of harmonization in regulatory requirements,

effective delivery routes for peptides, stability issues, new sources of lead molecules, innovative compositions and cost-effective manufacturing, remain to be addressed [11].

There is an urgent need for the development of a harmonized set of guidelines by regulatory authorities to define the level of impurities that can be present in PbTs. This can address two potential challenges: (i) because of the lack of clear guidance for setting impurities limits, many potential peptide drugs might fall by the wayside because of technical and economical inabilities to meet stringent impurity limits. The availability of regulatory directives will provide guidance to industry on how to set limits of impurities; and (ii) designing the manufacturing processes to produce higher quality products with minimal impurities will significantly affect the economic viability of the resulting products. For instance, if one considers that peptides have low toxicity and the daily dose administered is between 50 mg to 50 mg, requiring limits of individual, unidentified impurities of NMT 0.5% constitutes an element of 'caution' for complex peptides.

For emerging pharmaceutical and biotech companies with limited budgets, the challenge is to obtain the correct balance between expediency and due diligence to capture the market. For complex peptides, the urge to push forward quickly, without duly diligent analytical characterization of the final drug substance, can be risky. The detection of impurities that cannot be removed through chromatographic techniques might require alternative synthetic approaches. Additionally, many HPLCs fail to resolve the enantiomeric and isomeric forms of peptides if the chain is long. Therefore, there is a need to develop analytical techniques to specifically detect degradation products.

Another important area for improvement in the near future is the need for effective and patient-friendly delivery technologies, because poor membrane permeability, accelerated metabolism by intestinal, plasma/cellular esterases and hepatic cytochrome P450 enzymes, and fast plasma clearance rates are associated with the use of PbT [11]. A upsurge in interest in PbT could result once effective platforms for the delivery of peptides into the blood stream are developed [75]. Parenteral administration of therapeutic peptides, which is currently the most widely used delivery method, face several challenges, including fast metabolism, renal clearance, and lower acceptability by patients [76]. Hence, research has shifted to non-injectable delivery methods, including transdermal, pulmonary, nasal, oral, and buccal administration [77–79]. Although initial successes have satisfied the needs of researchers and industry, new formulation methods and modifications are required to further improve the bioavailability without compromising the conformations of biologically active peptides.

There is also a need to identify alternative or new sources of lead molecules for the development of PbTs. Yeast cell surface display methods, ribosomal and other biomimetic cyclic peptide syntheses, and microbiomes will be new avenues providing appropriate starting points or lead molecules [11]. More recently, stable cyclic peptides in plants have been identified, which can be produced in high yield and have been shown to be important lead molecules in peptide-based drug design [80]. Cyclic peptides offer several favorable attributes, such as efficient binding affinity with receptors, target selectivity, and minimal toxicity, which highlight plants as possible new sources of, or production factories for the development of, PbTs [81].

The synthesis of therapeutic peptides requires expensive coupling reagents, resins, and amino acids as starting materials, which makes the manufacturing of peptides uneconomical. Hence, inexpensive methods for their synthesis as well as purification are needed [82]. Chemical synthesis and molecular biology techniques, including recombinant peptide manufacture, might have a crucial role in providing cost-effective methods for the synthesis of PbTs. Chemical synthesis is effectively and economically used for the synthesis of smaller peptide chains (usually $\leq$35 amino acids), whereas recombinant peptide expression is particularly attractive for longer peptides (usually $\geq$40 amino acids).Transgenic animals could also be an ideal source for peptide production and are currently being utilized for the large-scale manufacture of longer PbTs [83]. In addition, the number of peptides entering clinical trials is increasing; methods other than conjugation with antibodies and polyethylene glycol (PEG) should also be identified to optimize their site-specific delivery and half-lives. Although there are many hurdles in all alternative modes of administration, the need to find new or alternative sources of lead molecules, ways to enhance their stability, and cost-effective methods for their synthesis remains. Scientists are also working towards the application of peptides as drug carriers for drug and gene delivery systems. Moreover, the development of peptide-based synthetic vaccines using minimal microbial components that are able to stimulate long-lasting protection against pathogens represents the potential future of vaccination. In addition, the commercial usage of peptide-based antimicrobial peptides also needs to be explored.

Concluding remarks

The role of pharmacopoeias in regulating the control of drug substances, excipients, and drug products used by manufacturers, national control laboratories, and regulatory authorities is huge [30]. Pharmacopoeias provide public standards for quality control by establishing specifications (i.e., qualitative and quantitative characteristics) and acceptance criteria for drugs. They also have an important role in identifying counterfeit and not-of-standard-quality (NSQ) drugs. It is the responsibilities of pharmacopoeias to respond rapidly to any new risk to public health (new impurities, etc.) and analytical technologies. The standards designed by pharmacopoeias should have the ability to evaluate the quality of the final drug substance and finished formulation. The test methods adopted should be robust, based on technologies that are state of the art and available globally and updated regularly.

With no official guidelines for peptide drugs, the quality tests incorporated in pharmacopoeias must be justified. The relevance of the tests mentioned in all three pharmacopoeias (*IP*, *Ph. Eur.* and *USP*), such as absorbance and specific optical rotation, can be questioned. However, there is scope for improvement in, and interpharmacopoeial harmonization across, many peptide monographs. With new emerging peptide identification technologies (using NMR and& LC-MS), decisions should be taken by pharmacopoeias to include bioassays involving animal models for batch-release testing.

Despite these limitations, we speculate that, once all potential barriers are eliminated, the full therapeutic potential of peptides will be utilized for improvement in healthcare systems across the world.

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