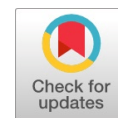


Insights into Therapeutic Peptides and their Quality Control

Ayat Abboud



Abstract: *Therapeutic peptides have been garnering renewed attention in recent years. The manufacturing of peptides and protein-based drugs rapidly expands within the pharmaceutical sector. These compounds may be obtained through solid-phase synthesis or biotechnological methods. Peptides and proteins are delicate molecules that undergo chemical or physical changes such as oxidation of methionine, deamidation of asparagine and glutamine, isomerization, aggregation, and denaturation during production. The alterations could compromise the stability of the drugs, consequently impacting the therapeutic efficacy of the protein or peptide. It is essential to effectively monitor the quality of proteins or peptides to verify the preservation of their biological properties throughout the production process. This review outlines the official guidelines for quality control of peptides manufactured through solid-phase synthesis or genetic engineering. The discussion also covered the frequently utilized separation techniques for evaluating the efficacy of therapeutic peptides.*

Keywords: *Peptides, Analysis, Quality, Synthesis*

I. INTRODUCTION

The number of new therapeutic molecules obtained conventionally by extraction or organic synthesis [1], and submitted to be approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA), has decreased significantly in recent years [2]. In this context, pharmaceutical companies are increasingly moving towards the development of innovative drugs such as proteins and peptides [3]. Indeed, some peptides have been used as therapeutic agents for almost a century in their natural form (for example, insulin and glucagon) [4]. However, until recent years, peptides were slightly used due to their unfavorable pharmacokinetic parameters [5].

Peptides, as drugs, have been attracting renewed interest for some time [6]. To improve their bioavailability, different strategies have been implemented: (i) alternative administration routes for peptide drugs [7], (ii) coupling of the peptide with organic molecules [8], allowing better passage of the intestinal barrier [9], (iii) chemical synthesis processes that improve the stability of the peptide in the body [10].

The production of peptides or protein drugs is booming in the pharmaceutical industry [10]. These compounds can be derived from solid-phase synthesis or biotechnologies [11]. Peptides or proteins are fragile molecules that are subjected, during their production, to chemical or physical modifications (oxidation of methionine, deamidation of asparagine and glutamine, isomerization, aggregation, denaturation, etc.) [12]. These modifications pose problems with the stability of the drugs obtained and have consequences on the therapeutic effect of the protein or peptide [13]. This is why it is necessary to control the quality of the proteins or peptides obtained to ensure that there has been no alteration of the biological properties linked to the production process [14]. Rigorous control of peptide drugs is therefore essential at all stages of their production [15].

This review summarizes the official recommendations concerning the quality control of peptides produced by solid-phase synthesis or genetic engineering. It also discusses the separation methods commonly used to assess the quality of therapeutic peptides.

II. PRODUCTION OF THERAPEUTIC PEPTIDES

The various scientific discoveries in medicine and peptide research have evolved [16]. After using peptides obtained by extraction in therapy, the production of peptides has moved towards other routes, particularly total synthesis, when their amino acid sequence is known [17].

Many of the peptides used in therapy have amino acid sequences that are slightly modified compared to the natural peptides from which they are derived [18]. One of the first “drug peptides” used in therapy was insulin [19]. The first insulin used and obtained by extraction techniques from pig pancreas was marketed in 1924 [20]. This extractive insulin was the only form used in therapy until genetic engineering made it possible to produce insulin [21]. From 1982, the first human insulin, derived from biogenetics, was then marketed. Glucagon followed the same evolution [22].

This was initially obtained by extraction from the pancreas, until 1993, when a form derived from biogenetics was marketed [23]. As the method of obtaining peptides determines the potential impurities that can be obtained together, it is therefore essential to know the methods of peptide production [24].


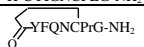
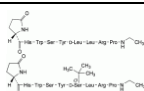
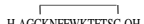
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Table 1: Examples of Commercial Therapeutic Peptides or in Clinical Development

Peptides	Structure	Route of Administration	Indications
Cu-DOTATATE	8 amino acid long peptide	Intravenous	Radioactive diagnostic agent
Setmelanotide	8 amino acid cyclic peptide	Subcutaneous	Genetic obesity
Insulin	2 polypeptide chains (A: 21 amino acids, B: 30 amino acids)	Subcutaneous	Diabetes
Oxytocin		Intramuscular intravenous	Uterine contraction
Desmopressin		Nasale or intravenous	Reduce the production of urine.
Glucagon human	H-HSQGTPTSDYSKYLDSRAQDFVQWLMNT-OH	Subcutaneous intramuscular	Hypoglycaemia
Leuprolide Buserelin		Subcutaneous Subcutaneous	Anticancer (prostate cancer) Treatment of precocious puberty Endometriosis
Tetracosactide (synthetic peptide Of ACH)	H-SYSMEHFRWGKPVGKRRPVKVYP-OH	Intravenous	Diagnostic purposes
Somatostatin		Intravenous	Acute bleeding
Bacitracin	Cyclic peptide	Oral, cutaneous	Antibiotic (Negative Gram)
Gramicidin	Multiple peptides X-GADIAvVvWYIWIW-ethanolamine X: V ou I, Y: W, F, ou Y	Nasal	Antibiotic
Pexiganan	Gly-Ile-Gly-Lys-Phe-Leu-Lys-Lys-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Leu-Lys-Lys-NH2	Cream	Infected diabetic foot ulcer (phase III)
Iseganan	Synthetic structural analog of naturally occurring Protegrin-1	Bains de bouche Aerosols	Antibiotic
Opebacanb	21 kDa recombinant fragment of human	IV	Crohn disease
Peptides PR1	VLQELNVTV	-	Vaccines

A. Recombinant DNA (rDNA)

Recombinant DNA (rDNA) technology involves transfecting a plasmid or virus containing the gene coding for a peptide or protein into a suitable microorganism or cell line [25]. This DNA is then expressed, producing the protein [26]. The desired substance is then recovered by extraction and purification [27]. The cell or microorganism that does not yet contain the vector is called the host, and the stable association of the two, used for production, is called the host-vector system [28]. Using such cells, producing large quantities of the protein or peptide is thus possible [29].

B. Chemical Synthesis

The synthetic peptides have been increasing steadily in recent years [30]. It is mainly carried out in the solid phase. Solid phase chemical synthesis requires several steps: activation of the -COOH function of the amino acid to be introduced into the peptide [31], protection and deprotection of the side functions (-OH, -SH, -COOH, -NH₂, -HN-(C=N)-NH₂, -NH), and specific protection and deprotection, between each coupling step [32], of the -NH₂ function involved in the next peptide bond, without modifying the side protective groups [33]. The first synthetic peptides had low stability in plasma, and therefore low bioavailability [34]. Different chemical synthesis strategies have been implemented to improve the bioavailability of synthetic peptides, particularly their plasma half-life [35]. These strategies are (i) cyclization or substitution of a natural amino acid by an amino acid of D configuration (which is not natural) [36], (ii) addition to the peptide of a carbohydrate chain or a PEGylated chain [37]. These chemical modifications improve the stability of the peptide in plasma and the resistance to degradation by peptidases [38].

III. THERAPEUTIC PEPTIDES

The growing interest in peptides in the pharmaceutical industry is related to the many advantages that peptides have over proteins and antibodies. Indeed, being smaller, they are less immunogenic [39]. They often represent the smallest functional part of the protein, thus offering better selectivity and efficacy [40]. In addition, since the degradation products of peptides are amino acids, the risks of systemic toxicity are minimized [41]. Peptide-based drugs cover many clinical indications, including bacterial infections, pain, hypertension, and cancer [42]. Table I presents examples of peptides for therapeutic purposes.

A. Hormones Peptides

Many hormones in our body, whose role is decisive, are peptides such as oxytocin, vasopressin, glucagon, insulin, and adrenocorticotrophic hormone (ACTH) [43]. Some pathologies are linked to an insufficiency of secretion of one or the other of these hormones [44]. It is therefore essential to provide, in a medicinal form, the hormone that is the subject of such a deficiency [45]. Among the hormones currently available on the pharmaceutical market, we can cite insulin (treatment of diabetes), oxytocin (to accelerate childbirth), desmopressin (therapy of diabetes), glucagon (treatment of hypoglycemia), ACTH (Cushing's syndrome). Table I shows some examples of peptide hormones used in treatment [46].

B. Antimicrobial Peptides

Bacterial resistance to conventional antibiotics is emerging as a major public health problem today. Faced with the emergence of resistant germs, research has focused, over the last 25



years, on a new class of peptides produced by living organisms that exhibit antimicrobial activities [47]. For example, bacitracin and gramicidin were the first peptide antibiotics isolated and marketed [48]. They are considered one of the key elements of the innate immune defense, but they are still called non-adaptive [49]. They have a broad spectrum of antibacterial, antifungal, and antiviral activity. These are a set of cationic peptides (containing a high proportion of Lys and Arg residues) and amphiphilic peptides, from 12 to 100 amino acids, presenting a great structural diversity [49]. On the structural level, they are classified into three families: linear peptides forming α -helices, peptides comprising β -sheets, and peptides containing a high percentage of Pro. The expression of these antimicrobial peptides can be constitutive or inducible by an infectious or inflammatory stimulus due to the presence of cytokines, bacteria, or molecules constituting the bacterial wall such as polysaccharides [50]. Two major classes of antimicrobial peptides present in mammals have been particularly studied. Cathelicidins are cationic or amphipathic peptides that contain an α -helix or " β -Hairpin" structure (two β -sheets). They are synthesized in mammals by polymorphonuclear neutrophils. These peptides have immunostimulant and anti-inflammatory effects [51]. Defensins are peptides of about thirty amino acids, comprising three β -sheets and disulfide bridges. They are secreted by cells constituting the immune system such as polymorphonuclear neutrophils and macrophages. Defensins have been isolated from epithelial cells and respiratory mucous membranes. Defensins have also been shown anti-viral (herpes virus) properties [52].

Other antimicrobial peptides, such as cecropins and magainins, are extracted from insect and frog epithelium (strongly cationic peptides, forming α -helices). Cecropins have antiviral properties. These are directed more specifically against enveloped viruses by making their envelope more permeable by a detergent-like effect or by forming pores. Magainins have antibacterial activity linked to their ability to permeabilize the bacterial membrane [53].

C. Vaccine Peptides

Determination of the peptide sequences of antigens allows the synthesis of epitopes likely to induce the formation of neutralizing antibodies [54]. Vaccines have been synthesized for immunobiological protection against bacterial toxins such as diphtheria and cholera. However, immunotherapy is an important approach, not only for infections but also for other diseases such as type 1 diabetes [55], AIDS, and bacterial or viral infections [42]. Peptide vaccination also represents a promising perspective for anticancer therapy [44]. The mechanism of action of this therapy would be based on the immunogenic character of the peptide capable of inducing an immune reaction directed against the cancerous pathology and preventing a recurrence [56]. This theory is based on the epitopes present only on the surface of cancer cells [52]. Clinical trials have shown promising results when multiple epitopes are included in a single vaccine [50]. These vaccines consist, for the most part, of long-chain polypeptides forming a stable linear complex [57]. This vaccine strategy has been successfully used in cervical cancer treatment linked to papillomavirus infection [54]. Indeed, these long-chain

peptides containing multiple epitopes stimulate the production of T lymphocytes directed specifically against these tumor cells.

IV. QUALITY CONTROL OF THERAPEUTIC PEPTIDES

Peptides have great therapeutic potential covering a wide range of clinical indications. From a pharmaceutical point of view, peptides are situated between classical organic substances and very high molecular-weight biopharmaceutical products, such as proteins. In addition, they can be obtained either by chemical synthesis, genetic engineering, or extraction from animal organs or fermentation media [46]. These particularities make their development complex when establishing a control monograph. Various analytical or biological methods could be used to assess the quality of peptides. These methods are indicated in general or specific recommendations, allowing the characterization and dosage of peptides and the determination of their biological activity or the detection and quantification of their organic or inorganic impurities. Most general guidelines concerning the quality of active substances and pharmaceutical products exclude peptides. Only published guidelines by the Code of Federal Regulations (CFR) or the ICH, Q7 (Good Manufacturing Practice Guide for active pharmaceutical ingredients) include the quality of peptides [55].

However, the diversity of sources of therapeutic peptides leads to different levels of recommended requirements to guarantee their quality. Thus, there are specific guidelines for synthetic peptides (FDA, "Guidance for industry for the submission of chemistry, manufacturing, and controls information for synthetic peptide substances" and others specific for products from biotechnology (ICH, Q6B, ("Test procedures and acceptance criteria for biotechnological/biological products))). Recently, in 2006, a technical guide was published by the European Directorate for the Quality of Medicines & Health (EDQM). It describes the requirements for establishing the monograph of a synthetic peptide or a rDNA polypeptide for pharmaceutical use ("Technical guide for the development of monographs of synthetic peptides and recombinant DNA proteins"). According to this technical guide, two aspects affecting the structure of the monograph differentiate synthetic peptides from peptides from biotechnology: their small size, typically less than 5000 Da or g/mol, and the fact that they may have chemical structures that do not exist in the natural state of proteins or peptides.

Table II compares the recommendations of the European Pharmacopoeia (Ph. Eur.) concerning the monograph of a synthetic peptide and that of a recombinant peptide or protein for pharmaceutical use. We note that the monograph of a therapeutic peptide (whatever its method of obtaining), as for any monograph of an active substance, is essentially composed of three sections: Identification, Tests, and Dosage. It may include other parts depending on the origin of the peptide: the physicochemical characteristics (appearance and solubility) for



synthetic peptides, production, labeling, and storage. The particularities of synthetic peptides have a double consequence on monograph development compared to recombinant peptides. Firstly, the characterization of the product is generally sufficient using the range of physicochemical tests available such as solubility and appearance of the synthetic peptide in the solid state, without the need for a biological assay. Spectroscopic methods such as infrared, and nuclear magnetic resonance may be necessary for identification, to complement conventional methods based on analysis of the amino acid composition or sequencing.

Table 2: Recommendations of Ph. Eur. to Edit Monographs of Synthetic Peptides or Produced by rDNA

European Pharmacopoeia		
	Synthetic Peptides	rDNA Proteins/Peptides
Definitions	<ul style="list-style-type: none"> - Elementary formula - Molecular mass - Physical form - Identity and biological activity of the substance and, possibly, of its natural analog - Mode of production - Structural formula - Content Specifications <ul style="list-style-type: none"> - Salt form - Any chemical modification such as esterification or amidation. 	<ul style="list-style-type: none"> - Formula of the monomer - Molecular mass - Physical form - Identity and biological activity of the substance and its natural analog - Mode of production - Primary sequence (amino acids) of the protein chain - Specific biological activity Production
	-	-
Production	_____	<ul style="list-style-type: none"> - Appropriate details of the production process - Specific biological activity if not covered by an assay described in the monograph - Procedures used to reduce or eliminate infectious agents - Possible use of stabilizers and auxiliary substances - Production process validation procedures.
Characteristics	<ul style="list-style-type: none"> - Appearance of the synthetic peptide in the solid state - Solubility 	_____
Identification	<ul style="list-style-type: none"> - Amino acid composition analysis - Reversed-phase liquid chromatography - Spectrum: infrared (IR) or nuclear magnetic resonance (NMR) 	<ul style="list-style-type: none"> - Bioassay - Peptide mapping - N-terminal sequence analysis - C-terminal sequence analysis - Reversed-phase liquid chromatography - Size exclusion chromatography - Isoelectric focusing or capillary electrophoresis - For glycoproteins, carbohydrate side chain analysis Tests
Tests	<ul style="list-style-type: none"> - Related peptides - Bacterial/pyrogenic endotoxin test - Appearance, optical rotation, 	<ul style="list-style-type: none"> - Purity - Determination of dimers and higher molecular weight impurities

	<ul style="list-style-type: none"> - and absorbance - Acetic acid, loss on drying, water content 	<ul style="list-style-type: none"> - Specific impurities] Determination of sialic acid <ul style="list-style-type: none"> - Bacterial endotoxins/pyrogens
Essay	<ul style="list-style-type: none"> - Content determination is carried out by comparative liquid chromatographic methods against a defined chemical reference substance as a standard. 	Two procedures: <ul style="list-style-type: none"> - Determination of the protein content, carried out by a comparative liquid chromatographic method against a defined chemical reference substance - An <i>in-vivo biological assay</i> was carried out by comparison with the International Standard.
Labeling	Labeling requirements and storage conditions	

A. Control of the Purity of Synthetic Peptides

As for all raw materials, peptides' quality is controlled by a set of tests. These tests cover the relevant organic and inorganic impurities classically present in peptides for therapeutic purposes [55].

The impurities usually present in peptides can be classified into different categories. The first category is inorganic impurities such as heavy metals, and mineral salts. The second category is synthesis reagents and residual solvents (e.g. isopropanol, acetonitrile, TFA, triethylamine, acetic acid). Acetic acid content is determined by reversed-phase partition liquid chromatography. Indeed, there is a monograph in the European Pharmacopoeia concerning the method for determining the acetic acid content [Acetic acid in synthetic peptides, 2.5.34].

The third category is organic impurities. These impurities can appear during the manufacture and (or) storage of the peptide and lead to the production of so-called “related” peptides.

Monographs of synthetic peptides generally include an analysis by reversed-phase liquid chromatography to highlight the presence of related peptides.

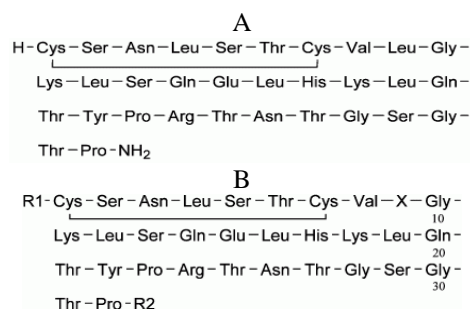
These synthetic impurities are linked to the chemical synthesis process of peptides which presents in different stages of protection/deprotection of the amino acids composing the primary peptide sequence. These can lead to multiple potential organic impurities in the peptides obtained by chemical synthesis. These impurities can also appear during storage (degradation products). Table III presents the different organic impurities that could be found in synthetic peptides [52]. As we can see in Table III, these impurities can be constituted by the deletion or insertion of an amino acid, the replacement of one amino acid by another, the oxidation of certain amino acids, racemization, products of deamidation or acetylation or products that have retained protective groups used during synthesis. Figure 1 presents peptides of interest and their impurities that could be produced during synthesis. The fourth category is high molecular weight impurities that originate from the aggregation of the peptide.



Table 3: Different Types of Potential Peptide Impurities, [European Pharmacopoeia, 6th Edition]

Origin of Impurity	Modification	Examples
Racemization	Conversion of one amino acid of the form L to D	[2-D-histidine] leuprorelin (Ph. Eur. Impurity B)
	Conversion of one amino acid of the form D to L	[6-L-leucine] leuprorelin (Ph. Eur. Impurity C)
	Conversion of two amino acids of D/L to L/D	[2-D-histidine,4-D-sérine] leuprorelin (Ph. Eur. Impurity F)
Racemization	Conversion Asn to Asp	[5- L-Aspartic acid] desmopressin (Ph. Eur. Impurity A)
	Conversion Gln to Glu	[4- L-Glutamic acid] desmopressin (Ph. Eur. Impurity B)
	Deamidation C-terminal	[9-Glycine] desmopressin (Ph. Eur. Impurity C)
deletion	Deletion of one or two amino acids	des-22-tyrosine-calcitonine (Ph. Eur. Impurity C)
Insertion	Insertion of one amino acid	endo-8a-L-proline- goserelin (Ph. Eur. Impurity J)
	Insertion of two amino acids	endo-8a,8b-di-L-proline- goserelin (Ph. Eur. Impurity I)
Deletion of side N or C	Amino acids deleted from N-terminal	Buserelin-(3-9)-peptide (Ph. Eur. Impurity C)
	Amino acids deleted from C-terminal	5-oxo-L-prolyl-L-histidine (protirelin, Ph. Eur. Impurity C)
Acetylation	Serine O-acetylation	O ⁴ -acetyl goserelin (Ph. Eur. Impurity K)
	Threonine O-acetylation	[12-(O-acetyl-threonine)] somatostatin (R0-CEP 2005-245-Rev 00)
	Lysine N-acetylation	(N-acetyl-lysine) somatostatin (R0-CEP 2005-245-Rev 00)
	N-terminal acetylation	N ¹ -acetyl felypressin (Ph. Eur. Impurity E)
Oxidation	Oxidation of Met	Sulfoxide of tetracosactide (Ph. Eur. Impurity A)
	Oxidation of other amino acids (e.g. Trp, Cys, His)	Leuprorelin with oxidized Trp
	Formation of trisulfide bonds from disulfide bonds	Salmon calcitonin trisulfide
Reduction	Reduction of e.g. Trp and disulfure	Leuprorelin with reduced Trp
Incomplete deprotection	Acetylaminoethyl (Acm) derivatives	S ¹ ,S ⁶ -bis[(acetylamino)methyl]-(felypressin) (Ph. Eur. Impurity A)
Related substances	t-Bu, Fmoc, etc derivatives C-terminal demethylation	t-Bu/Fmoc- leuprorelin N ^{1,9} ,N ^{1,9} -diméthyl-desmopressine (Ph. Eur. Impurity G)
	C-terminal	[9-L-prolinohydrazide] goserelin (Ph. Eur.

	diazene to hydrazide	Impurity E)
	Reactivate lateral chains (e.g. arginine)	[8-[5-N-[imino(1H-pyrazole-1-yl)methyl]-L-ornithine]] leuprorelin (Ph. Eur. Impurity J)
Cyclization	Formation of amide intramolecular bonds	Cyclo-(L-histidyl-L-prolyl-) (Protirelin Ph. Eur. Impurity E)
Oligomers	Associations of covalent intramolecular interactions	(1,6'),(1',6)-bis(disulfide) de la (felypressin) (Ph. Eur. Impurity C)



[Fig.1: Examples from the European Pharmacopoeia, 6th Edition, of Related Substances of Therapeutic Peptides: Salmon Calcitonin (A), and Associated Substances Where Ph. Eur. Impurity A: R1 = CO-CH3, R2 = NH₂, X = L-Leu: Acetyl Calcitonin and Ph. Eur. Impurity B: R1 = H, R2 = NH₂, X = D-Leu: [9-D-leucine] Calcitonin (B)]

The impurity control of synthetic peptides is essential because it has been shown that even small changes in the sequence of peptides by one or two amino acids can modify their pharmacological activity. Thus, receptor agonists can become antagonists, and vice versa [53]. Therefore, organic impurities in therapeutic peptides must meet specific thresholds indicated in the guidelines. Indeed, since the publication of the ICH Q3 recommendation (“Impurities in new drug substances”) which excluded peptides, the specific thresholds of organic impurities in peptides have been the subject of discussions [48]. Thresholds for the declaration, identification, and qualification of related substances for peptides obtained by chemical synthesis have been included in the monograph of the European Pharmacopoeia “Substances for pharmaceutical use” (07/2009: 2034). These impurities are declared, identified whenever possible, and qualified according to the indications in Table-IV, unless otherwise indicated or justified and authorized exception.

Table 4: Thresholds for Declaration, Identification, and Qualification of Organic Impurities in Peptides obtained by Chemical Synthesis, (Monograph, European Pharmacopoeia: Substance for pharmaceutical use, 2034, 07/2009)

Thresholds of Declaration	Thresholds of Identification	Thresholds of Qualification
> 0,1 %	> 0,5 %	> 1,0 %

The purity of a peptide is usually determined using HPLC. The chromatographic method must be able to separate impurities from the therapeutic peptide. Reversed-phase HPLC remains the most



widely used method at present. Ion exchange, ion pairing, or size exclusion chromatography can also be used.

B. Peptide Mapping

A key step in the quality control of proteins or peptides produced by genetic engineering is peptide mapping [40]. Peptide mapping consists of analyzing peptide fragments of a protein obtained after enzymatic or chemical cleavage. In the European Pharmacopoeia, there is a monograph for peptide mapping (Peptide mapping, 2.2.55). Peptide mapping could be performed by the cleavage of certain peptide bonds using chemical agents or enzymes. Cyanogen bromide (BrCN) is mainly used to achieve chemical cleavage. It cuts at the C-terminal of Met producing relatively large fragments. Proteases are used to cleave proteins more or less specifically at certain amino acids. For example, trypsin cuts at the C-terminal side of Arg and Lys. The complex obtained mixture of peptides is then separated, detected, and quantified. In the various monographs of the European Pharmacopoeia describing protein control, cleavage carried out using trypsin is most often proposed. Peptide mapping allows the highlighting of possible errors at the level of expression, mutations, and degradations of a protein. Table V presents examples of chemical and enzymatic cleavage agents proposed by the European Pharmacopoeia for peptide mapping [monograph, European Pharmacopoeia: Peptide mapping, 2.2.55].

Table 5: Examples of Protein Cleavage Agents, from [Monograph, European Pharmacopoeia: Peptide Mapping, 2.2.55]

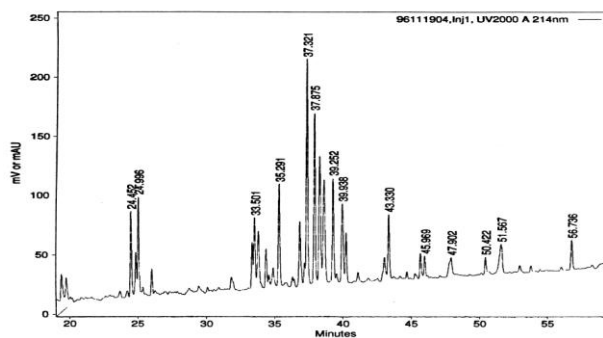
Type	Agents	Specificity Of Cleavage
Enzymatic	Trypsin	C-terminal of Arg and Lys
	Chymotrypsin	C-terminal of hydrophobe residues (Leu, Met, Ala, aromatic compounds)
	Pepsin	Nonspecific Hydrolyse
	Lysyl endopeptidase (Lys-C endopeptidase)	C-terminal of Lys
	Glutamyl endopeptidase	C-terminal of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Endo proteinase Asp-N)	N-terminal of Asp
Chemical	Clostripain	C-terminal of Arg
	Cyanogen bromide	C-terminal of Met
	2-nitro-5-thio-cyano benzoic acid	N-terminal of Cys
	O-iodosobenzoic acid	C-terminal of Trp and Tyr
	Diluted acid	Asp and Pro

Many separation techniques can be used to perform peptide mapping. The choice of a methodology will depend on the protein considered and the physicochemical properties of the peptides produced after digestion of the protein. Table VI presents an overview of the techniques that have proven themselves for peptide separation.

Table 6: Different Techniques Could be used in Peptide Mapping, according to [Monograph, European Pharmacopoeia: Peptide Mapping, 2.2.55]

Analytical Techniques of Peptides
HPLC-RP
Ion exchange chromatography
Hydrophobic interaction chromatography
Polyacrylamide gel Electrophoresis (PAGE)
Dodecyl sulfate de sodium -PAGE (SDS-PAGE)
Capillary electrophoresis (CE)
High-pressure Paper Chromatography

The separation of peptide fragments after protein digestion can be achieved by chromatographic methods [40] or electrophoretic methods [43]. The mixture of peptides produced after digestion is Often complex and sometimes requires multidimensional systems using complementary separation principles to obtain a complete resolution of the peptides present in the mixture [45]. At the European Pharmacopoeia level, capillary electrophoresis is rarely proposed for establishing peptide maps of peptides or proteins. However, this method is proposed for erythropoietin mapping [monograph, European Pharmacopoeia, 01/2008: 1316]. Most peptide or protein monographs describe reversed-phase liquid chromatographic methods for establishing peptide mapping, this is the case, for example, for insulin, salmon calcitonin, human glucagon, interferon alfa-2, and somatropin. Figure 2 shows the separation of a complex mixture of peptides produced after tryptic digestion of interferon- α 2 by reversed-phase liquid chromatography [monograph, European Pharmacopoeia, 01/2008: 1110].



[Fig.2: Peptide Mapping of Interferon- α 2 by HPLC-Reversed Phase. Column: Nucleosil C18; 150 x 4.6 mm, 5 μ m. Flow Rate: 1mL/min, Mobile Phase: Phase A: Water + 0,1% TFA, Phase B: ACN + 10% Water + 0,1% TFA. Gradient: 0 - 8 min: 100% A, 8-68 min: from 100% A to 40%A, 68 - 72 min: 40% A, 72-75 min: 40 % A to 100% A. Detection: 214 nm. [Monograph, European Pharmacopoeia, 01/2008 :1110]

It should be noted, however, that the peptide map can be produced by other chromatographic modes: ion exchange chromatography, hydrophobic interaction chromatography, and hydrophilic interaction chromatography.

V. CONCLUSION

Peptides have been attracting renewed interest in the pharmaceutical industry in recent years. The compounds can be acquired using solid-phase synthesis or biotechnological techniques. Peptides and proteins are susceptible to chemical or physical modifications. The changes implemented may alter the stability of the acquired medications, thereby affecting the therapeutic effectiveness of the protein or peptide. Monitoring the quality of proteins or peptides is crucial to ensure their biological properties during production. This review presented the official recommendation for ensuring the quality of peptides produced via solid-phase synthesis or genetic



engineering. The commonly employed methods for assessing the effectiveness of therapeutic peptides were presented.

DECLARATION STATEMENT

I must verify the accuracy of the following information as the article's author.

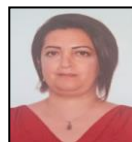
- **Conflicts of Interest/ Competing Interests:** Based on my understanding, this article has no conflicts of interest.
- **Funding Support:** This article has not been sponsored or funded by any organization or agency. The independence of this research is a crucial factor in affirming its impartiality, as it has been conducted without any external sway.
- **Ethical Approval and Consent to Participate:** The data provided in this article is exempt from the requirement for ethical approval or participant consent.
- **Data Access Statement and Material Availability:** The adequate resources of this article are publicly accessible.
- **Authors Contributions:** The authorship of this article is contributed solely.

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