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# Control Strategies for Synthetic Therapeutic Peptide APIs - Part I: Analytical Consideration

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*USP evaluates quality attributes for synthetic peptides.*

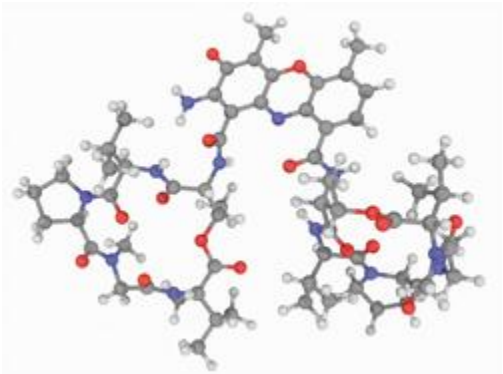

IMAGE: LAGUNA DESIGN/GETTY IMAGES

The United States Pharmacopeial Convention (USP) Therapeutic Peptides Expert Panel was formed in 2013 to evaluate quality attributes for synthetic peptides based on currently available regulatory guidance and expectations. Public quality standards for drug products and drug substances are developed by USP and enforceable by FDA. This series of three articles by the Panel explores the current manufacturing and regulatory landscape and provides a comprehensive overview of quality attributes to be considered for successful synthetic peptide active pharmaceutical ingredient (API) development from manufacturing to lot release. Specifically, the first article covers analytical characterization methods, lot release tests, and points to consider for synthetic peptide API manufacturers entering the market. The second article will focus on quality control of raw materials and impurities resulting from the starting materials used for peptide synthesis. The last article will be devoted to manufacturing processes and impurity control of synthetic peptide APIs.

In 2012, the number of peptide drugs approved by FDA surpassed the number of approved monoclonal antibodies and enzymes (1). These approvals serve to highlight the recent revival of interest in peptides, which have generally been considered to be poor drug candidates due to their low oral bioavailability and propensity to be rapidly metabolized. However, new formulation and conjugation strategies for alternative routes of administration and overcoming short half-lives have emerged, resulting in a larger number of marketed peptide-based drugs, some of which have reached blockbuster status (2, 3). Despite these successes, some challenges still remain. Due to varying sizes and amino acid sequences, synthetic peptides are not easily classified into either small molecule or biologic categories. Therein lie the regulatory challenges with peptides, especially with respect to impurities and bioassay requirements. Understanding these challenges can help shape and create consistency among USP's quality standards for this growing class of drugs.

**Characterization methods**

Comprehensive characterization of a peptide API is required to obtain regulatory approval for marketing; the reference standard is fully characterized to ensure unequivocal identity of the material, its purity and API content. Besides evaluation of appearance, a selection of tests that fall into four categories is performed: identification, assay, purity, and specific tests. The evidence obtained from these studies also may be used in regulatory submissions, for example in Module 3 of the Common Technical Document. Available methods and USP chapters for characterization of peptide APIs are summarized in **Table I**.

**Table I: Tests used for characterization and quality control of peptide APIs*.**

| Test | Method | Comments |
|---|---|---|
| **Definition (Characters)** | | |
| Appearance* | Visual inspection | |
| Structure | | Sequence in 3-letter code as minimum; ChemDraw structure preferred for shorter sequences (≤15 amino acids [AAs]) |
| **Identification** | | |
| HPLC co-elution with reference standard* | <621> | Method same as for related substances |
| Mass spectrometry* | <736> | Monoisotopic mass ± 1.0 mass units |
| Amino acid analysis* | <1052> | Hydrolysis protocol should be specified |
| MS-MS sequencing | <736> | May be complicated for longer sequences |
| NMR spectroscopy | <761> | Complex interpretation for longer sequences |
| Peptide mapping | <1055> | For longer sequences (>20 AAs); complementary to MS-MS |
| Enantiomeric purity | | Chiral amino acid analysis |
| N-terminal sequence analysis by Edman Degradation | <1045> | Complicated analysis, especially for sequences which are N-terminally blocked; largely superseded by LC-MS-MS |
| Infrared spectroscopy | <197, 851> | Limited use for peptides |
| Higher order structure | Circular dichroism, NMR, FTIR | For investigation of secondary or tertiary structure in aqueous solution |
| Bio-identity | ELISA | Not a routine test for most quality control laboratories; may be required for longer, complex sequences |
| **Assay** | | |
| HPLC assay* | <621> | Method same as for related substances; based on comparison with a reference standard |
| Peptide content by amino acid analysis* | <1052> | Hydrolysis protocol must be validated, only stable amino acids should be included in calculation |
| Peptide content by nitrogen analysis* | CHN analysis, | Direct analysis by elemental analysis or Kjeldahl or using HPLC with a chemiluminescence nitrogen detector (CLND) |
| UV spectroscopy | <197, 851> | Only useful for products containing Trp, Tyr, or Phe |
| Quantitative NMR | <761>, <1761> | A stable internal standard is required |
| **Impurities** | | |
| Peptide-related substances* | <621> | Specific method for substance; must be validated for both process-related impurities and degradation products; limits for total and individual impurities should be specified |
| Residual solvents* | <467> | May be limited to those used in the final step of the manufacturing process |
| Heavy metals* | <(231), 232, 233> | Requirement if metal catalysts used in the process; may be required because of contact with metals in processing equipment |
| Residual trifluoroacetic acid (TFA)* | <503.1>/ <1065> | Only required if TFA used during the manufacturing process; <1065> used for peptides which are not soluble in acid |
| Residual fluoride* | <1065> or ion-selective electrode | Only required if hydrofluoric acid (HF) used during the manufacturing process (Boc-chemistry) |
| Other small molecule impurities | Impurity dependent | Non-peptide impurity limits are required to follow the ICH Q3A guideline; potential genotoxic impurities require additional evaluation |
| **Specific tests** | | |
| Counterion content* | <503> (Acetate) <1065> (Others) | <1065> may be required for acetate if peptide not soluble in acid; titration with $AgNO_3$ may be used to determine chloride |
| Water content (Karl Fischer)* | <921> | Coulometric titration (method Ic) most commonly used |
| Specific rotation | <781> | Information only of limited utility |
| Ellman test | | Only required if the reduced form(s) of a peptide containing disulfide bonds cannot be determined using the method for related substances |
| Bioburden* | <61, 62> | Often required for APIs used in the manufacture of parenteral drug products |
| Bacterial endotoxins* | <85> | Requirement for APIs used in the manufacture of parenteral drug products |
| Mass balance* | N/A | Calculation |

*Recommended test to be performed as part of lot release. HPLC is high-performance liquid chromatography. MS is mass spectroscopy. NMR is nuclear magnetic resonance. FTIR is fourier transform infrared.

**Table I:** Tests used for characterization and quality control of peptide APIs*

**Identification.** For routine identification of peptides, a high-performance liquid chromatography (HPLC) method is recommended. This method should be capable of distinguishing between the peptide and its closely related impurities and, therefore, usually is identical to the method used to determine impurities. The preferred identification procedure involves a comparison of retention times between the main peak in the standard and samples, as well as a coinjection of an equal mixture of both, with a requirement to obtain a single peak.

Identification by mass spectrometry (MS) involves determination of the monoisotopic mass, which should be within ±1.0 mass units of theoretical. For peptides larger than 2 kDa, the use of high resolution instruments may be necessary and determination of the average mass may be appropriate for larger molecules. The sample concentration and solvent should be specified if sample introduction to the instrument via direct infusion is used. MS cannot distinguish between isomers (e.g., isoleucine and leucine) or D- and L-amino acid substitutions.

Information on amino acid (AA) composition can be obtained from amino acid analysis (AAA). The hydrolysis protocol should be established, because those factors may significantly impact the recovery of certain amino acids.

Further characterization should involve determination of
the peptide sequence using MS-MS. At a minimum, fragmentation by collision-induced dissociation (CID) should be used, but if sequence coverage is poor, alternative fragmentation techniques such as electron-transfer dissociation (ETD) may provide better quality data. The latter technique is particularly useful for peptides that contain a significant number of basic residues and are highly positively charged. Furthermore, peptides containing multiple disulfide bonds lend themselves well to ETD, which can selectively cleave S-S linkages, something CID is not capable of. Prior to MS analysis, the disulfide bonds may be reduced using suitable reducing agents, such as dithioerythritol (DTE), dithiothreitol (DTT), or tris(2-carboxyethyl)phosphine (TCEP). The linear peptide can then be subjected to MS/MS, which will allow confirmation of sequence but not disulfide bond assignment.

For most peptides, nuclear magnetic resonance (NMR) spectroscopy should be attempted, although interpretation of spectra may be challenging for peptides containing more than 10 AAs. If comprehensive structure elucidation by NMR is not possible, then peptide mapping may be used as an alternative. The proteases Trypsin or Lys-C are recommended for peptides containing a sufficient number of Arg and Lys residues, whereas for peptides containing Glu residues, Glu-C is useful. The resulting peptide fragments are separated by HPLC and identified by MS. Peptide mapping, without prior peptide reduction, is a potential approach for disulfide bond assignment. For routine use, peptide mapping can involve comparison with a standard.

Enantiomeric purity is determined using chiral AAA. The method is based on acid hydrolysis of the peptide, suitable derivatization of the resulting AAs and determination of the optical isomers of the constituent AAs by chiral gas chromatography with mass spectrometric detection (GC-MS). HPLC-based methods of chiral AAA involve pre- or post-column derivatization of AA hydrolysates, for example using Marfey's reagent or o-phthalaldehyde, respectively. Regardless of the hydrolysis procedure employed, the method should be validated to account for potential racemization during hydrolysis or subsequent derivatization. An approach that addresses this issue involves the use of deuterated reagents (e.g., deuterium chloride [DCl])/deuterium oxide [$D_2O$]). Substitution of the α-carbon hydrogen with deuterium allows for correction for racemization when using MS-based detection methods.

Infrared (IR) spectrophotometry is not a useful characterization or identification tool, because spectra tend to be dominated by water vibrations from residual water, and carbonyl bands, arising from the peptide backbone and acetate, if present as a counterion.

The characterization of secondary or tertiary structure in aqueous solution may be relevant for peptides, particularly in the context of determining whether a bioassay is required. The most commonly used methods for this purpose are circular dichroism (CD), fourier transform intrared (FTIR) analysis of amide bond vibration regions, and NMR. For the latter technique, additional information can be obtained using deuterium exchange.

**Assay.** When considering assay, it is important to emphasize that many peptides are quite hygroscopic and should be handled in a controlled-humidity environment (e.g., a glove box). Failure to do so can lead to erroneous assay results because moisture exchange of the peptide material with the environment can be very rapid. This process is especially fast if high surface area lyophilized material is being weighed. Establishing a sorption isotherm under ambient conditions is useful to determine the optimum relative humidity conditions for handling the API.

Assay by HPLC is the preferred approach, assuming that a quantitative standard has been established. In the absence of a quantitative standard, a “gravimetric” method, in which both the standard and the sample are corrected for counterion content and water content (determined as part of the assay method), may be used.

Assay of peptides has traditionally been determined by quantitative AAA, which compares the peak areas of AAs in a hydrolyzed sample with the areas of the peaks for the same AAs in an external standard. The peptide hydrolysis procedure should be validated to ensure good recovery, and only AAs stable to the hydrolysis conditions should be used in the calculation. Some AA sequences containing multiple, adjacent hydrophobic residues, such as Ile, Phe, Leu and Val, may give lower recovery than expected. Because the method involves a relatively complex sequence of operations, the results may be variable.

Assay by nitrogen content is also used routinely, most frequently determined from elemental analysis. Kjeldahl analysis or chromatographic methods with chemiluminescence nitrogen detection may also be used.

Only peptides containing Trp, Tyr, or Phe residues may lend themselves to assay determination using UV absorbance measurement at 280 nm. The molar absorption coefficient can be calculated theoretically based on knowledge of the sequence; however, the method should be independently validated using one of the techniques described previously. At lower wavelengths, common counterions such as acetate may interfere with this assay.

Quantitative NMR is potentially useful for assay determination, although the method requires a stable internal standard with signals that fully resolve from bands associated with the peptide.

**Purity/related impurities determination by HPLC and control strategy**. The purity of a peptide API is usually determined using HPLC. The method should be capable of separating impurities introduced through starting materials, process-related impurities, and impurities resulting from degradation of the peptide. One or more methods may be required to accomplish this objective. When using multiple methods, it is recommended that orthogonal separation mechanisms be used. At least one of the methods should be stability-indicating and methods should be validated per *United States Pharmacopeia* < 1225> Validation of Compendial Procedures. In addition, use of LC-MS compatible methods may be advantageous for the identification of impurities.

For peptides containing hydrophobic residues two reversed-phase (RP) methods may be appropriate, although alternatives, such as mixed mode RP/ion-exchange (IEX) stationary phases, should be considered. For hydrophilic peptides, a RP method or mixed-mode RP/IEX stationary phase may be complemented by an IEX or hydrophilic interaction chromatography (HILIC) type method.

A single HPLC method may be appropriate based on the sequence of the peptide and knowledge of its potential impurities. A control strategy, built on process characterization, should be implemented that accounts for the fate of impurities introduced with starting materials and generated during the

synthesis process and purification of the peptide. Additional, appropriately validated in-process control methods may be used to ensure quality of the final product.

Non-peptide, process-related impurities, such as residual solvents and heavy metals (especially if metal catalysts are used in the process), should also be monitored. To support the overall control strategy, additional methods should be used, such as ion chromatography, inductively coupled plasma (ICP)-MS and NMR. The latter is particularly useful during process characterization, because diffusion experiments allow for rapid differentiation between small-molecule organic impurities and peptide-bound modifications.

Traces of reagents used in the process, such as trifluoroacetic acid (TFA), which is commonly used in chromatographic purification, and inorganic fluoride [from the use of liquid hydrogen fluoride in products manufactured using t-butyloxycarbonyl- (Boc-) based chemistry], should also be monitored.

**Specific tests.** A number of tests are universally applicable to quality control testing of peptide APIs, of which the most important are counterion content and residual water content. As discussed in the assay section, the hygroscopicity of the peptide may affect the results.

APIs intended for use in injectables require bioburden and endotoxin determination. Finally, a mass balance calculation is recommended which, at a minimum, requires a summation of the percentages of major sample components (i.e., assay [peptide content], counterion content, and water content). The result should approach 100%.

Lot release methods
While it is, obviously, not necessary to perform all of the tests described as part of routine lot release, the testing performed must comply with regulatory requirements for, at a minimum, establishment of identity, purity, and assay for the peptide API. The Panel's recommendations for the test methods to be performed as part of lot release, as well as the quality attributes necessary for inclusion in monographs for this class of APIs, are highlighted with an asterisk in **Table I.**

The use of two separate identification methods is required per the International Conference on Harmonization (ICH), because a single method is rarely unequivocal. HPLC, for example, may be used together with MS; or HPLC together with AAA, based on method complementarity considerations. Other methods may also be necessary. An assay method based on HPLC is preferred, but other methods may also be used if appropriately validated.

Guidance for methods and levels of impurities exist (see references provided in **Table I**) except for peptide-related impurities and TFA, where no generally accepted guidance exists. Levels for these are, therefore, set during the regulatory approval process, based on safety data for the product. Specifications for counterion content, water content, and other specific tests are product-related and are set based on process capability and batch history.

**Points to consider for API manufacturers entering the market**
It should be recognized that USP monograph requirements list the minimum quality attributes that APIs must satisfy. For synthetic peptides, the suitability of a monograph to adequately control the quality of the API must be closely evaluated based on the manufacturing process used. While the HPLC impurities methods described in monographs are demonstrated to be stability-indicating for the subject API and, therefore, should be expected to universally control potential degradation products, the same is not automatically true for process-related impurities. It is necessary that manufacturers demonstrate the suitability of the HPLC impurities methods to control actual and potential synthetic impurities specific to their process.

Other process related residuals may include starting materials, intermediates, solvents, reagents, catalysts, and metals. They may be adequately controlled by the manufacturing process through in-process controls and the design of the process. Synthetic peptide API manufacturers should, therefore, assess the ability of their manufacturing process to control potential process residuals and identify which residuals may be present at levels that require monitoring and control at the final API stage. If the monograph methods are inadequate to control such identified process residuals, then the manufacturer must use additional validated methods, as appropriate. The manufacturer must also establish appropriate limits for process residuals not controlled by the monograph that are justified based on the capabilities of the manufacturing process and a toxicological safety assessment of the process residuals.

**Conclusion**

As a class of APIs, peptides are extremely diverse, not only in terms of their pharmacological properties, but also in terms of their size, AA sequence, and potential for secondary and tertiary structures. Possibly because of this, peptides are specifically excluded from virtually all international guidance documents. Furthermore, while peptides are generally considered to be non-toxic, there is no generally accepted guidance for the requirements for characterization and toxicological characterization of product-related impurities. Limits proposed by the European Medicines Agency (EMA) (4) are wider than those required by the guidance in ICH Q3A, but the former are not recognized by FDA, which therefore, considers the specifications for new peptide APIs on a case-by-case basis.

The USP hopes that, through the work of the Therapeutic Peptides Expert Panel and this series of articles, more consistent guidance may be provided, both for the minimum, acceptable quality attributes for peptide API monographs, and also to industry for the characterization and quality control testing of peptide APIs.

**References**

1. FDA, FDA FY2012 Innovative Drug Approvals[4] (December 2012), accessed Feb. 7, 2014.
2. P. Vlieghe et al., *Drug Discovery Today* 15(1/2) 40-56 (2010).
3. P. Van Arnum, *Pharm. Techno*l. 37(8) 48-50 (2013).
4. Ph. Eur., *General Guidance 2034, Substances for Pharmaceutical Use* (2013).

## References

1. www.pharmtech.com/pharmtech/inside+usp/usp-partners-globally-for-21st-century-medicines/articlestandard/article/detail/817347?contextcategoryid=3436
2. www.pharmtech.com/pharmtech/regulatory+beat/ema-collaborates-with-hta-assessment-networks/articlestandard/article/detail/834618
3. www.pharmtech.com/regulatory+watch
4. www.pharmtech.com/pharmtech/data/articlestandard/pharmtech/102014/837404/www.fda.gov/downloads/aboutfda/reportsmanualsforms/reports/ucm330859.pdf