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Control Strategies for Synthetic Therapeutic Peptide APIs Part III: Manufacturing Process Considerations

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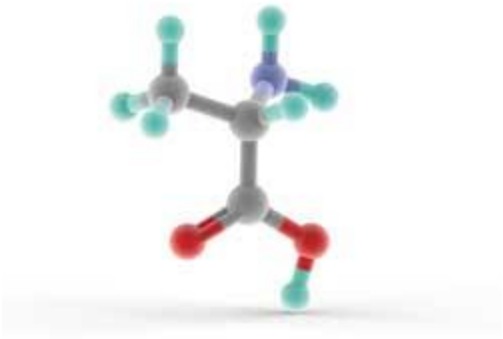
USP's Therapeutic Peptides Expert Panel discusses manufacturing processes and impurity control for synthetic peptide APIs.



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The United States Pharmacopeia (USP) Therapeutic Peptides Expert Panel was formed in 2013 at the direction of the Monographs-Biologics & Biotechnology Expert Committee to evaluate quality attributes for synthetic peptides based on currently available regulatory guidance and expectations. 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 API from development through manufacturing to lot release. The first article (1) covered analytical characterization methods, lot-release tests and points to consider for synthetic peptide API manufacturers entering the market. The second article (2) focused on raw materials used in the chemical synthesis of peptides. This last article in the series is devoted to manufacturing processes and impurity control for synthetic peptide APIs.

In the 1950s, pioneers in the field, such as Bodanszky and Du Vigneaud, produced the first bioactive peptides by purely synthetic methods in solution (3). Synthetic peptide chemistry received a big boost in 1963, when Bruce Merrifield developed the method for synthesis on a solid support (solid-phase peptide synthesis [SPPS]) (4). Nowadays, solid-phase techniques and materials have evolved to the point that sequences exceeding 100 amino acids (AAs) in length have become feasible. Besides various hybrid techniques based on extractive methods and solution- and solid-phase synthesis methods, the scope of synthetic peptide chemistry has been further expanded in the past decade by the development of native chemical ligation techniques, which allow the coupling of unprotected peptide fragments to even larger assemblies (5).

Manufacturing of synthetic peptides
The chemical manufacturing of peptides generally involves the following sequence of operations:

- Assembly of the protected peptide sequence
- Removal of the semi-permanent protecting groups
- Modifications such as disulfide bond formation and fragment couplings
- Purification of the crude peptide by preparative chromatography followed by salt exchange
- Isolation of the final, purified peptide.

Assembly of the protected peptide sequence. The assembly of the peptide backbone involves a series of cycles involving a coupling and a deprotection step. During the coupling step, an activated

AA is coupled, usually in molar excess to ensure complete conversion, to the N-terminal amino acid of the growing peptide chain. This AA is protected by a temporary protecting group on its Nα-amino function and, if the side chain of the AA is reactive under the coupling conditions, it may also be protected by a semi-permanent protecting group. Following coupling, the Nα-amino function of the AA is selectively deprotected, leaving side chain protecting groups on the growing peptide intact and liberating the N-terminus of the growing peptide for further elongation. Removal of excess AA derivative is essential to prevent impurity formation. The same principles apply to both classical solution-phase synthesis and solid-phase synthesis. However, in the latter approach, the growing peptide chain is anchored at the C-terminus to a solid support, which allows removal of excess amino acid derivatives and coupling reagents by washing and filtration.

Removal of the semi-permanent protecting groups. Following assembly of the protected peptide sequence, the semi-permanent protecting groups are removed by acidolysis and the peptide is simultaneously cleaved from the resin support in the case of SPPS. Carbocations originating from the cleaved protecting groups are generated under the harsh conditions of acidolysis, requiring the use of scavengers to minimize impurities resulting from the modification of the sensitive peptide chain. In orthogonal protecting schemes involving only one acidolytic deprotection step at the end of the backbone assembly (Z and Fmoc chemistry), the integrity of the peptide sequence is better preserved than in a protecting scheme, which involves acidolysis in every cycle of the peptide assembly, followed by a harsh acidolysis after the backbone assembly (Boc chemistry).

Modifications such as disulfide bond formation and fragment couplings. A peptide may contain one or more generally intramolecular and well-defined disulfide bonds between Cysteine residues. Formation of disulfide bonds may be achieved during assembly of the peptide backbone, or after the removal of the semi-permanent protecting groups, with some approaches employing orthogonal thiol protecting groups to selectively affect the desired connectivity, particularly when multiple disulfide bonds are required.

Fragment couplings, as well as other modifications of specific functions on the peptide sequence, usually involve the selective deprotection of the functional groups to be modified. Several types of protecting groups for the various AA functional groups have been developed, which are either orthogonal to the normal protecting schemes or display a higher sensitivity towards acidolysis.

Purification of the crude peptide by preparative chromatography and salt exchange. Protected peptide fragments have a low propensity to crystallize and, contrary to other classes of compounds, impurities formed in the course of peptide synthesis usually accumulate up to the stage of the crude peptide. This is particularly the case for peptides assembled on a solid support. The crude peptide is, therefore, unlikely to meet the purity specifications set for the API and must be purified. Purification typically involves preparative chromatography, which may comprise sequential purification steps that are based on different retention principles, such as ion exchange or reversed phase. Purification processes developed for peptide APIs typically result from an extensive initial screening of various purification methods on the laboratory scale and rely on a meticulously developed and validated in-process control analytical high-performance liquid chromatography (HPLC) method, which is able to discern actual impurities generated by the synthesis process. This requires an in-depth knowledge of the impurity profile and an understanding of the ability of the selected purification process to purge the impurities.

Following purification, a salt exchange step is typically implemented to remove salts originating from the buffers used during purification to convert the peptide to the desired counterion and set pH. This salt exchange step may be achieved by an additional preparative chromatography step or by use of ion exchange resins.

Isolation of the purified peptide. The isolation of the peptide API usually occurs through lyophilization of the concentrated aqueous peptide solution following purification and salt exchange. Alternatively, precipitation may be used as a more economical process, although this may not be feasible for all sequences and is likely to involve significant development and engineering to ensure that the API meets the desired solid-state properties and is fit for use in the drug product. Although not yet widely applied in peptide manufacturing, other techniques, such as spray drying, may be used.

Types of impurities in synthetic peptides

Several types of impurities may be encountered in synthetic peptides, which either originate from the raw materials, from the manufacturing process, or are formed by degradation during the manufacturing process or during storage (**Figure 1**). The various types of manufacturing process impurities, together with their origins, are presented in **Table I**, while degradation impurities are presented in **Table II**. Identification methods and detectability are indicated in both tables.

Figure 1: Peptide manufacturing flow.

Deletion sequences. Deletion sequences lack one or more amino acid residues. These sequences originate either from incomplete coupling or from incomplete Nα-deprotection steps, especially around so-called “difficult sequences” in SPPS, and hence require careful in-process controls during backbone assembly. The Kaiser colorimetric test (6), typically applied in SPPS to monitor completion of deprotection and coupling reactions, is rapid and straightforward but not always sensitive enough to determine quantitative completion of coupling and especially deprotection reactions. Hence, use of the Kaiser test may not completely prevent the formation of deletion sequences. In such instances, chromatographic analysis can provide quantitative results, but requires a soluble intermediate or, in the case of SPPS, requires cleavage of a resin sample. Sequences lacking the C-terminal residue may arise from incomplete coupling of the first AA residue to a solid-phase resin, when this step is not followed by an efficient capping protocol for residual, active anchoring sites. Removal of a specific deletion sequence impurity during purification is typically more difficult when the missing amino acid is relatively simple (e.g., Glycine or Alanine).

Table I: Potential synthetic peptide process-related impurities.

Impurities	Origin of impurities	Identification method	HPLC detectability
Deletion	Synthesis	LC-MS or LC-MS/MS	+
Insertion	Raw material or synthesis	LC-MS or LC-MS/MS	+
Truncation	Synthesis	LC-MS	+
Diastereomer	Raw material or synthesis	HPLC spiking with synthesized diastereomeric analogs	+/-
Substitution (Leu/Ile)	Raw material	HPLC spiking with synthesized analogs or isolation/AAA	-
Functional group modification	Synthesis or stability	LC-MS or LC-MS/MS	+/-
Disulfide modification	Synthesis or stability	LC-MS or LC-MS/MS	++

LC-MS is liquid chromatography-mass spectrometry. LC-MS/MS is liquid chromatography-tandem mass spectrometry. HPLC is high performance liquid chromatography. AAA is amino acid analysis.
Leu is Leucine. Ile is Isoleucine.

Insertion sequences. Insertion sequences contain one or more "double" AA residues. The presence of either Nα-unprotected AA derivatives or dipeptides in the starting AA derivatives leads to the formation of insertion sequences and can be controlled by setting appropriate specifications for these materials. Alternatively, during peptide backbone assembly, incomplete removal of excess AA derivative prior to the next deprotection and coupling cycle will lead to the formation of insertion sequences. To prevent process-related insertion sequences, following the coupling step, excess activated AA derivative must be inactivated by quenching or extraction prior to the subsequent deprotection step, and the excess of un-activated AA derivative must be removed prior to the next coupling step. Removal of a specific insertion sequence impurity during purification is typically more difficult when the inserted amino acid is relatively simple.

Table II: Potential peptide degradation impurities.

Impurities	Identification method	HPLC detectability
Deamidation of Gln/Asn/C-terminus	LC-MS or LC-MS/MS	+/-
Acetylation of amino functions	LC-MS or LC-MS/MS	++
Disulfide modification	LC-MS or LC-MS/MS	++

Gln is Glutamine. Asn is Asparagine.

Truncation sequences. N-terminally truncated sequences may be generated when capping is used as part of the synthesis protocol to prevent deletion sequences that result from incomplete couplings, which is often the case in SPPS. C-terminally truncated sequences, on the other hand, may be generated when quenching is part of the synthesis protocol to prevent insertion sequences. The structure of truncated sequences is usually sufficiently different from the target sequence to allow for efficient removal during purification.

Diastereomers. Diastereomeric sequences contain one or more AA residues in the undesired chiral form. Diastereomers are usually more difficult to remove during purification and typically present a greater separation challenge by analytical HPLC than other types of impurities. Their identification usually depends on HPLC spiking experiments of the peptide product with the synthesized

diastereomeric analogs. Such spiking experiments may also corroborate the suitability of the analytical release method(s).

Diastereomers may originate from the presence of the optical antipode in starting AA derivatives, requiring the establishment of appropriate specification limits for these raw materials. Alternatively, diastereomers may be formed during peptide backbone assembly through epimerization. Several epimerization mechanisms are known that either involve 5(4H)-oxazolone formation during activation of carboxylic functions (during coupling) or direct Cα-proton abstraction under basic conditions. AA residues with a relatively acidic Cα-proton such as Cysteine and Histidine are especially sensitive to epimerization.

Synthetic approaches have been developed to minimize epimerization, the most basic being that the peptide backbone is assembled from the C-terminus to the N-terminus by stepwise coupling of Nα-urethane protected AAs. Some AA derivatives, such as Cysteine and Histidine derivatives, may require special coupling protocols. Concerning fragment couplings, the only safe options to prevent epimerization are the use of a Glycine or Proline residue in the C-terminal position of the fragment being activated in the fragment condensation. All other residues in this position will lead to some degree of epimerization, which can only be minimized by applying special coupling protocols. Furthermore, the esterification of AA derivatives under basic conditions, with the exception of Glycine, will result in some degree of epimerization, the most frequent examples arising from esterification of the first AA to a solid-phase resin. Finally, peptides with a C-terminal Cysteine ester are prone to epimerization during base treatment, and therefore, direct esterification to a solid-phase resin for Fmoc synthesis should be avoided. Numerous coupling reagents have been developed during the years to promote fast and epimerization-minimizing coupling conditions.

Substitution sequences. Substitution sequences occur when one or more AA residues have been substituted by another AA residue, the most common being an Isoleucine ↔ Leucine substitution. Substitution sequences originate from the presence of contaminants in the starting AA derivatives and can consequently be controlled by setting appropriate specifications for these raw materials. The same purification and analytical HPLC challenges described for diastereomer sequences may apply to substitution sequences, especially for the Isoleucine ↔ Leucine substitution.

Modifications of functional groups and disulfide bonds. Several AA side chains are susceptible to modification, either during synthesis or during storage. AAs may undergo rearrangements during coupling (e.g., Asparagine, Aspartic acid, and Glutamine) or may be prone to degradation or electrophilic substitution during acidolysis (e.g., primary amides, Tryptophan, Tyrosine, and Methionine). Alternatively, modifications of functional groups may arise from incomplete removal of protecting groups.

Impurities related to disulfide bond modification include reduced (linear) monomers, oxidized (parallel and anti-parallel) dimers and higher polymers, isomers arising from scrambling of disulfide bonds, and oxidized disulfides (thiosulfinates). The desired isomer is usually obtained under strictly controlled and optimized process conditions. The handling of concentrated solution of the peptide product following the purification process should be carefully controlled to avoid polymerization.

The most common degradation mechanisms include deamidation of Asparagine, Glutamine and the C-terminal amide function, acetylation of amino functions by residual acetate, and disulfide modification (i.e., polymerization).
The identification of critical process parameters, as well as extensive stability studies, provide process and product understanding, allowing for optimization of the manufacturing process and definition of hold-times and storage conditions, thereby contributing to the preservation of the integrity of the target peptide.

Conclusion
Synthetic peptide-related impurities may result from impurities in AA derivatives used as raw materials, from the manufacturing process itself, and from degradation of the peptide during manufacturing or upon storage. Based on a thorough understanding of the peptide, its stability characteristics and its manufacturing process, including identification of raw material attributes and process parameters that affect API quality, an appropriate control strategy for peptide-related impurities can be developed to achieve a manufacturing process that is both economically feasible and also able to yield a peptide API meeting predetermined quality attributes. An understanding of the ability of the purification process to remove peptide impurities can be used to define appropriate impurity specifications for AA derivatives used as raw materials, as well as to define and control critical process parameters during manufacturing. Increased process knowledge may provide an understanding of the ability of the purification process to tolerate variability in the quality of raw materials as well as variability in the crude peptide resulting from peptide backbone assembly, removal of protecting groups, and modification of peptide functional groups.

USP hopes that the work of the Therapeutic Peptides Expert Panel and this series of articles will provide more consistent guidance to help support efforts to create a sustainable platform for the future of peptide-based drugs.

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