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▲⟨1504⟩ QUALITY ATTRIBUTES OF STARTING MATERIALS FOR THE CHEMICAL SYNTHESIS OF THERAPEUTIC PEPTIDES

SCOPE

This general chapter is intended to provide recommendations on the minimum quality attributes for starting materials used in the manufacture of synthetic therapeutic peptides. Because it is not possible to cover the wide variety of available starting materials that may be used, including amino acids, protected amino acid derivatives, and peptide fragments, the discussion in this chapter will be limited to the most commonly used protected amino acid derivatives (AAD), i.e., 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives (including resin-bound AAD). However, it is intended that the general concepts and guidance provided for this class of starting materials should be applied to all peptide starting materials, where applicable. This chapter is to be used in conjunction with *Quality Attributes of Synthetic Peptide Drug Substances* ⟨1503⟩.

INTRODUCTION

The increased interest in peptides as drug candidates during the past few decades can be attributed to a number of factors, including advances in proteomics and high-throughput screening techniques, which have accelerated the development of new potential therapeutic candidates. Furthermore, the development of efficient synthetic chemical strategies, combined with robust purification processes and more powerful and discriminating analytical methodologies, have facilitated the manufacture of peptides with high-quality standards. The evolution of these synthetic strategies started with the manufacture of relatively short peptides but today, the manufacture of longer peptides, which traditionally have been manufactured by biological processes, is feasible using synthetic chemical techniques.

Because synthetic peptides are not classified as small molecules or biological products [final rule on the *Definition of the Term "Biological Product"* (85 FR 10057, February 21, 2020)], they are excluded from many guidance documents. ⟨1503⟩ provides an overview of the current status regarding the quality attributes of synthetic peptides used as drug substances.

In the manufacture of any active pharmaceutical ingredient (API; this term is used interchangeably with "drug substance"), the term "raw material" is used independently for describing starting materials (SMs), solvents, and reagents. From a quality point of view, the "most critical" raw materials are those that are incorporated as significant structural fragments into the structure of the API. From a regulatory perspective, these critical raw materials are known as SMs because their introduction into the manufacturing process establishes the point at which the production of the API begins.

Special attention must be paid to the quality attributes of these SMs because they have the potential to directly impact the quality of the drug substance. For the manufacture of peptides by chemical synthesis, the starting materials are amino acids, protected amino acid derivatives, and fragments. Hence, this chapter will focus on the quality criteria for the starting materials used in the manufacturing process with a particular emphasis on impurities.

SUPPLIER QUALIFICATION AND EVALUATION OF SYNTHETIC ROUTE

The scope of good manufacturing practices for APIs as described in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines ICH Q7—*Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients* and ICH Q11—*Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities) Questions and Answers (Regarding the Selection and Justification of Starting Materials) Section 5.13* excludes the steps prior to the introduction of the "API starting material" into the manufacturing process. This lack of specific guidance for the manufacture of starting materials does not exclude the responsibility of peptide manufacturers to establish a qualification program for their suppliers and the starting materials they manufacture that is aligned with their process design and their process risk assessment.

It is recommended that the suppliers of starting materials manufacture their products under a quality system that ensures the consistency of batches manufactured and the traceability of the operations carried out to reach the product. The level of the requirements of this quality system should be higher in the case of fragments used as starting materials than for amino acids or protected amino acid derivatives. For more discussion of this topic, refer to ⟨1503⟩.

The implementation of technical agreements or quality agreements for the suppliers of starting materials is strongly recommended. These agreements must consider the obligation for the supplier to inform the customer about changes in the synthetic route used for the protected amino acid derivatives. Peptide manufacturers must assess the impact of these changes on the quality of their final drug substances. Starting material manufacturers are required to provide sufficient information to support regulatory submissions. Publicly available, published synthetic routes for the manufacture of starting materials can be used as supportive information. Implementation of

confidentiality agreements between peptide manufacturers and their suppliers of critical raw materials will be helpful in performing such impact assessments.

Manufacturers of drug substances must assess any changes of starting material supplier or significant changes in the starting material manufacturing process for their impact on the quality attributes of the drug substance. The level of requirements for this assessment must take into consideration whether the starting material is an amino acid, a protected AAD, or a fragment. The existence of a supplier qualification program and a common control strategy at the reception of the starting material (common quality requirements aligned with the assessment of their impact on the quality of the final product) should be adequate. For fragments, as they may be customized, a change of manufacturer should be appropriately justified.

The synthetic route for the amino acid derivative used for the manufacture of synthetic peptides potentially could result in different impurities. Impurities can be classified into three main categories:

1. AAD-related impurities originating from the amino acids
2. AAD-related impurities originating from the AAD manufacturing process
3. Non-AAD-related impurities

The first two categories are considered to be critical because they can increase the level of related impurities in the drug substance. The third category refers to process impurities (for example, solvents, elemental impurities, and nitrosamines) that do not generally interfere with the manufacture of peptides especially for solid-phase synthesis; however, their levels must be assessed to determine whether their presence poses a risk to the safety of the product and the health of the patients.

AAD-RELATED IMPURITIES ORIGINATING FROM AMINO ACIDS

One potential cause for critical impurities in the protected amino acid derivatives is related to contaminants of the unprotected amino acids used to manufacture those AADs. Amino acid-related impurities, such as stereoisomers and foreign amino acids, especially, may become critical AAD-related impurities after the introduction of the protecting groups. This will be discussed in this section.

For the sake of simplicity within this chapter, the term “amino acid” shall be limited in its meaning to the 20 proteinogenic (“natural”) α-amino acids and their stereoisomers.

Amino Acid Enantiomers

Of the 20 proteinogenic α-amino acids, 19 have an asymmetrical center at their α-carbon. The only achiral exception is glycine. The chiral proteinogenic amino acids have the L-configuration in the Fisher projection that is equivalent to the *S*-configuration in International Union of Pure and Applied Chemistry (IUPAC) nomenclature according to Cahn–Ingold–Prelog sequence rules.

Most chiral amino acid isomers exist in two forms that are enantiomers to each other, namely the *S*- and *R*-enantiomers (or L- and D-, respectively). Exceptions are isoleucine and threonine as discussed below.

Due to their abundance in nature, the *S*-amino acids are the ones that are most frequently required for peptide synthesis. They are commercially available in excellent optical purity (<0.1%–0.5% D-content) as *R*-amino acids as their most likely isomeric contaminants are rare in natural sources. For the same reason, *R*-amino acids are much more difficult to obtain in high optical purity when needed for peptide synthesis.

Amino Acid Diastereomers

Isoleucine and threonine have two asymmetrical centers: one at the α-carbon and one at the β-carbon. Hence, four isomeric configurations exist and lead to the two enantiomeric pairs *SS*/*RR* and *SR*/*RS*, respectively. These two pairs of enantiomers are diastereomers of each other. Proteinogenic isoleucine and threonine have the *SS*-configuration; whereas their enantiomers have the *RR*-configuration. The *SR*/*RS*-diastereomers of isoleucine and threonine are called *allo*-isoleucine and *allo*-threonine, respectively. The diastereomers are also the most likely isomeric impurities of isoleucine and threonine due to inversion at the α-carbon.

Foreign Amino Acids

Amino acids are normally manufactured from natural protein sources and must be separated from all other amino acids present. Hence, amino acids may be contaminated with residual foreign amino acids that could carry over into the AAD and finally be incorporated into the target peptide. Therefore, foreign amino acids are critical impurities of amino acids and must be controlled and limited if relevant. In particular, contamination with isomeric impurities such as isoleucine in leucine is especially challenging.

Control of the Critical Impurities

The critical amino acid-related impurities may be controlled either directly in the amino acids or in the subsequently manufactured AAD. In most cases, separating foreign or isomeric amino acids is easier at the unprotected stage than at the protected stage as AAD properties are mainly determined by the bulky protecting groups, i.e., the Fmoc group in Fmoc chemistry. Therefore, it is recommended that AAD manufacturers should control these critical impurities directly in the amino acids used for manufacturing the AADs. Peptide manufacturers who purchase the AADs should ensure appropriate quality by:

1. Verifying that their AAD manufacturers appropriately control the critical impurities in their starting amino acids
2. Including the resulting critical impurity derivatives into their AAD specification and incoming material testing

The stereoisomers of both amino acids and their protected derivatives can be quantitated by chiral chromatographic methods. Chiral gas chromatography (GC) analysis with pre-column derivatization and flame ionization detector (FID) detection is recommended for unprotected amino acids, whereas HPLC on chiral reverse-phase (RP)-columns is the recommended technique for Fmoc amino acid derivatives.

Chiral GC analysis is also frequently used for AADs. In this case, the protecting groups must first be removed by hydrolysis. Because the hydrolysis may lead to epimerization, it must be performed in deuterated solvents (deuterium chloride/deuterium oxide), in which case molecules that epimerize during hydrolysis will have the α-hydrogen replaced by deuterium. By using the chiral GC method with mass detection, the isomers that epimerized during hydrolysis will be disregarded. However, the accuracy of this method is generally limited to a standard deviation (SD) of 0.1%. For the determination of very low isomer levels, chiral RP-HPLC is the more accurate technique.

Foreign amino acids may also be determined together with the stereoisomers by the methods described above. Of course, the usual methods for amino acid analysis may also be applied to screen for foreign amino acids.

AAD-RELATED IMPURITIES ORIGINATING FROM THE AAD MANUFACTURING PROCESS

Several types of impurities related to the AAD production process may be found in protected amino acids used for peptide synthesis. They can be divided into several groups based on their origin and potential impact on the quality of the peptide drug substance.

Partially Protected and Unprotected Amino Acids

The first group is the unprotected and partially protected amino acids. These impurities can be formed during the AAD production process or by degradation on storage or transportation. In general, the incomplete protection of functional groups causes a significant change in polarity compared to the fully protected AAD. Therefore, partially protected AADs can normally be removed easily from the target AAD but, nevertheless, these impurities should be controlled through the specification. Partially protected AADs can be divided into three subgroups.

PARTIAL LOSS OF THE SIDE CHAIN PROTECTING GROUP

In peptide synthesis, fully protected amino acids are used to avoid formation of impurities due to various possible side reactions of the unprotected functional groups. However, the presence of a small amount of side chain unprotected amino acid derivatives will not usually result in significant impact on the purity of the peptide (depending on the amino acid, the target peptide, and the synthesis conditions). Therefore, the acceptance criteria for such impurities should be set based on careful evaluation of the particular production process during development. Because amino acid derivatives are frequently protected by an Fmoc group at the N-terminus, these impurities can be easily controlled by regular HPLC methods and identified by liquid chromatography-mass spectrometry (LC-MS).

PARTIAL LOSS OF THE FMOC GROUP

These impurities can have a critical impact on the quality of the drug substance since their presence will lead to formation of double insertion impurities due to double coupling of the amino acid. Moreover, depending on the coupling conditions, the free amino groups may cause further Fmoc cleavages in the coupling mixture thus increasing the impurity concentration autocatalytically. If the amino acid also contains suitable side chain protection, they can be controlled by RP-HPLC method and identified by LC-MS.

UNPROTECTED AMINO ACIDS

Similar to the previous type of impurities without the Fmoc group, these impurities are also critical for the quality of the drug substance, since they can lead to double insertion due to double coupling and also side chain derivatization. Most of the amino acids are very polar and don't have a chromophore to enable sensitive detection by UV; consequently, they elute at the beginning of the run on an RP-HPLC column and are difficult to detect. Therefore, in most cases regular HPLC methods are not adequate for their determination and the development of specific methods is required, usually based on pre-column derivatization. Thin-layer chromatographic (TLC) methods may also be appropriate for this purpose if a limit test is sufficient. In general, free amino acids should not be present in AADs since they can be removed readily from the respective Fmoc derivative.

β-Alanine (Ala) Impurities

The second group of impurities originating from the manufacturing process are derived from the side reaction that takes place during the introduction of the Fmoc-protecting group. If this is accomplished using Fmoc-succinimide (Fmoc-OSu) as the reagent, Fmoc-β-alanine (Ala) and Fmoc-β-Ala-amino acid (AA) impurities may be formed. Because these impurities can be formed in all Fmoc-protected amino acid derivatives, without strict control of the starting material, many potential impurities derived from the addition of β-alanine can be formed at different positions during the synthesis of the peptide sequence. Some of these impurities are very difficult to separate from the target peptide. Control of the presence of these impurities in the AAD and limiting them to acceptable levels is critical for the reduction of β-alanine contamination to acceptable levels during the production of peptide drug substances using Fmoc chemistry. Levels of Fmoc-β-Ala and Fmoc-β-Ala-AA can be controlled by RP-HPLC techniques through use of the appropriate Reference Standard with parallel identification by LC-MS methods, if required.

Other Process-Related Impurities

Additional impurities may be found in the AAD, which are related to the manufacturing process. Such impurities can be dipeptides (for example, Fmoc-glycine-glycine) or derivatives of the AAD formed due to side reaction during the process, especially if Fmoc-chloride is used to introduce the Fmoc protection group. In most cases, such impurities can be controlled by HPLC methods and identified by LC-MS. The acceptable limit of each impurity should be defined based on its ability to form potential impurities in the drug substance and the relative ease of removal of the impurities during purification.

NON-AAD IMPURITIES

Control of non-AAD impurities in starting materials is very important to assure the safety, quality, and efficacy of the resulting peptide drug substances. The major potential non-AAD related impurities in the starting materials are:

- Residual solvents and reagents used in the preparation of starting materials
- Elemental impurities
- Residual allergens and melamine
- Bovine spongiform encephalopathy-transmissible spongiform encephalopathy (BSE-TSE) prions
- Nitrosamines and other genotoxic impurities

Residual Solvents and Reagents Used in the Preparation of Starting Materials

The most commonly used solvents in amino acid and Fmoc amino acid manufacturing processes are Class 2 and Class 3 solvents such as acetonitrile, acetic acid, triethylamine, dioxane, dichloromethane, chloroform, tetrahydrofuran, methanol, etc. The quality of these solvents must be controlled to eliminate the presence of any Class 1 solvent contaminants. For this reason, the manufacturers of all solvents should limit the content of benzene unless they can certify that solvents are benzene-free.

Fmoc amino acids may contain traces of acetic acid, ethylacetate (a potential source of acetic acid), or other potentially reactive solvents such as aldehydes, which can cause chain termination during synthesis. For this reason, it is recommended that very low limits for acetic acid content, as well as the content of other potentially reactive solvents, should be established for all Fmoc amino acids.

Elemental Impurities in Starting Materials

Elemental impurities are important quality attributes for the safety of peptide drug substances. Whereas the processes used for solid-phase synthesis of peptides are likely to eliminate elemental impurities in starting materials, it is important to consider these impurities as part of the risk assessment during development in order to ensure that the drug substance complies with the limits specified in ICH Q3D, *Elemental Impurities* and *USP* chapters *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩.

Residual Allergens and Melamine

Sources of amino acids in starting materials are either synthetic or of plant origin and may be derived from potential allergens such as soybeans, wheat, rye, oat, barley, corn, maize, spelt, kamut, sugar, and alcohol. Because amino acids that are produced from proteins undergo harsh hydrolysis conditions and are further purified, it is extremely unlikely that allergens would pass through the hydrolysis and purification steps. Allergen- and melamine-free statements should be obtained from the starting material vendor and may also be ensured by the quality supply agreement.

BSE/TSE-Free Starting Materials

Only synthetic or plant-derived starting materials should be used in peptide synthesis. Amino acids that originate from or come into contact with animal materials (waste from slaughterhouses, bones, cartilage, gelatin, etc.) should be avoided. The origin of the amino acids should be known and manufacturers of starting materials should provide adequate certification of TSE compliance. Preferably, human- and animal-derived amino acids should be avoided. If use of amino acids of animal origin cannot be avoided, the respective material should meet the requirements of the *European Pharmacopoeia* general chapter *5.2.8. Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Human and Veterinary Medicinal Products*. The requirement for BSE/TSE-free statements should be part of the quality supply agreement.

Nitrosamines and Other Genotoxic Impurities

Chemically synthesized peptide manufacturers are required to assess the risk for the presence of *N*-nitrosamines impurities in their products. Nitrosamines are chemical compounds with potential carcinogenic risk. It is commonly accepted that the formation of *N*-nitrosamine impurities can occur due to the presence of a nitrosating agent together with a secondary or tertiary amine (named as the nitrosatable substance) usually under acidic reaction conditions. Although the most common source of nitrosating agents from a synthetic point of view are nitrite salts, all potential nitrosating agents should be considered (nitrate salts, nitrous acid, nitrous acidum ion, nitryl chloride, nitrosothiol, nitrosyl halides, and alkyl nitrites, among others). Amino acid derivatives used for the manufacturing of chemically synthesized peptides are considered a potential source of *N*-nitrosamines that could be transferred to the drug substance. It is recommended to avoid those manufacturing processes that could lead to the formation of *N*-nitrosamines. The presence of other genotoxic impurities should be carefully considered and evaluated during development, taking into consideration the requirement of ICH M7—*Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk*.

CONCLUSION AND RECOMMENDATIONS FOR AAD SPECIFICATIONS

Based on the considerations outlined above, the most common quality attributes to be used for protected amino acid derivative starting materials are:

- Appearance
- Identification
- Related impurities
    - Total
    - Specified
    - Foreign amino acids
    - Optical isomers (diastereomers and enantiomers)
    - Unspecified
- Other impurities (not related)

- E.g., residual reagents, solvents, elements
- Assay (e.g., by titration)
- Other components as necessary

Limits for specified impurities (e.g., unprotected amino acids, other amino acid derivatives, foreign amino acids, and chiral isomers) are set and justified based on prior batch history and an understanding and risk assessment of the API manufacturing process. The correlation between the content of a specified impurity in the starting material and the content of the related impurity in the API must be considered. For example, will the manufacturing process produce a related impurity in the API and to what extent is the process able to reduce a related impurity that has been formed? For unspecified impurities, batch history and the API specification for unspecified impurities are used as a starting point for the specification in the starting material. Assay by base titration is recommended for verification that the amino acid derivative is supplied as the carboxylic acid form suitable for activation and coupling and that no salts of the amino acid derivative are present. Other impurities (not related) and potential components are included in the specification based on their potential effect on the API manufacturing process and the quality of the final API.▲ (USP 1-Dec-2023)

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