Application Note

Potency Assessment and Physicochemical Testing of Interferon-β-1a Using USP Reference Standards

usp Biologics™

Introduction

Interferon beta 1A (IFN-β-1a) is a widely used medication for the treatment of multiple sclerosis (MS), a debilitating chronic autoimmune disease in which immune cells mistakenly attack myelin, a nerve cell insulator, leading to disrupted nerve signal transmission. IFN-β-1a is a complex protein consisting of 166 amino acids that is produced through biological processes; the protein forms into a specific three-dimensional shape that is critical to its activity. IFN-β-1a reduces inflammation by increasing anti-inflammatory cytokines, decreasing pro-inflammatory cytokines, and altering the immune response's intensity by reducing the immune-attack against myelin. The IFN-β-1a molecule has a sugar group attached to it which categorizes it as a glycosylated protein[1]. To maintain its three-dimensional structure, the molecule contains a disulfide bond[2] that connects two parts of the amino acid chain together.

A glycosylated protein sample will not contain identical molecules but is a mixture of heterogenous molecules. Differences may arise due to aggregation, deamidation, or oxidation of the glycosylated protein, degradation leading to multiple fragments of the glycosylated protein, alterations to the sugar moiety by the presence of different glycoforms, or the disulfide bond breaking. Small amounts of these modifications or processes may not affect the performance of the IFN-β-1a product. When these variants of the desired product have properties comparable to those of the desired product with respect to activity, efficacy, and safety, they are considered product-related substances[3]. However, product-related impurities are molecular variants arising during manufacture and/or storage which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety[3] and therefore must be controlled.

It is possible for IFN-β-1a produced by different manufacturers to have different amounts of product-related substances and impurities. When shipping and storing IFN-β-1a products, there are many possibilities of conditions that add to the potential for change in the chemical structure of the IFN-β-1a product that could impact the activity, efficacy, and safety of the product. These concerns highlight the need for physical and activity IFN-β-1a standards that manufacturers can assess their product against.

The United States Pharmacopeia (USP) has highly characterized physical reference standards that can be used to validate system suitability of analytical methods and equipment as well as for quality control of drug substance batches. USP Reference Standards are used as calibrators and comparators when testing samples to determine if the sample has acceptable quality. Through use of USP Reference Standards, product quality can be evaluated to give assurance to proper manufacturing, storage, and supply chain management.

USP has developed 2 Reference Standards for IFN-β-1a. The **Interferon Beta-1A Reference Standard** (USP Catalog # 1342401) has been thoroughly evaluated using multiple analytical techniques in a multiple laboratory collaborative study. The **Interferon Beta-1A for Bioidentity Reference Standard** (USP Catalog# 1342412) has been tested using a bioidentity assay that shows it has acceptable activity by signaling nearby cells to heighten their anti-viral defenses. The two products are formulated to be fit for purpose: the Interferon Beta-1A Reference Standard is provided in a liquid form at a concentration greater than 1 mg/ml whereas the Interferon Beta-1A for Bioidentity Reference Standard is provided in a lyophilized formulation format at a lower concentration to support more consistent bioassays without excessive dilution steps that could lead to errors.

Chemical Identity Testing of Interferon Beta-1A

It is important to prove the identity of the active pharmaceutical ingredient (API) in a pharmaceutical product using analytical test methods when the product is transferred between manufacturing steps or shipped to new locations. During manufacturing, identity testing is performed on the drug substance before it is formulated to ensure the proper molecule is going into the formulation. Identity testing is also performed when receiving the product at a new location to ensure the correct product was shipped. Identity testing can also be used to detect counterfeit product that may enter the supply chain. Test methods for identity testing require the use of a reference standard for comparison to prove the test product is the same as stated on the label.

Peptide mapping is a technique used to identify Interferon Beta-1A. USP provides a peptide mapping-based identity method for IFN-β-1a products and the IFN-β-1a Reference Standard. Briefly, the IFN-β-1a molecules in the test sample and in a sample of the USP Reference Standard are digested into fragments (i.e., peptides) using an enzyme which makes it easier for the analytical method to detect differences between the sample and the Reference Standard. Starting with the same concentration of each, the sample and standard fragments are separated based on their hydrophobicity (i.e., water repulsion) using high-performance liquid chromatography (HPLC). The fragments pass through the HPLC with different retention times based on their hydrophobicity. Accurate measurement of the retention times of the peptide fragments creates a "fingerprint" (i.e., chromatogram) of the IFN-β-1a test sample. The retention time is different for each fragment, seen as peaks, in **Figure 1**.

System suitability is evaluated by comparing the resolution between the AP6(ox) peak (representing amino acids 34-45 of Interferon Beta-1A) at half height and the AP5 peak (representing amino acids 124-134 of interferon Beta-1A). The USP Interferon Beta-1A RS and the example chromatogram provided in the reference standard certificate are used to identify the peaks. The resolution should be not less than 2.0. The individual peaks in each sample should have similar retention times and peak height, the latter of which is because the initial concentrations are the same.

The test sample chromatogram must not have any new peaks that are not seen in the Reference Standard chromatogram. If all these criteria are met, this proves the IFN-β-1a test sample identity is the same as the Reference Standard and is correctly labelled as IFN-β-1a.

Biological Identity (Bioidentity) Testing of Interferon Beta-1A

Proving the chemical identity of IFN-β-1a is important but equally important is proving the IFN-β-1a product is biologically active, which is dependent on its three-dimensional structure. When the identity of a product is proven by testing its specific biological activity, that testing can be referred to as bioidentity testing. Test methods for bioidentity testing require a Reference Standard to use for comparison to demonstrate product has the same biological activity.

To assess the biological identity of IFN-β-1a products USP provides a bioidentity test method and reference standard (described in **Figure 2**; for full method details, please refer to the Certificate for the USP IFN-β-1a for Bioidentity Reference

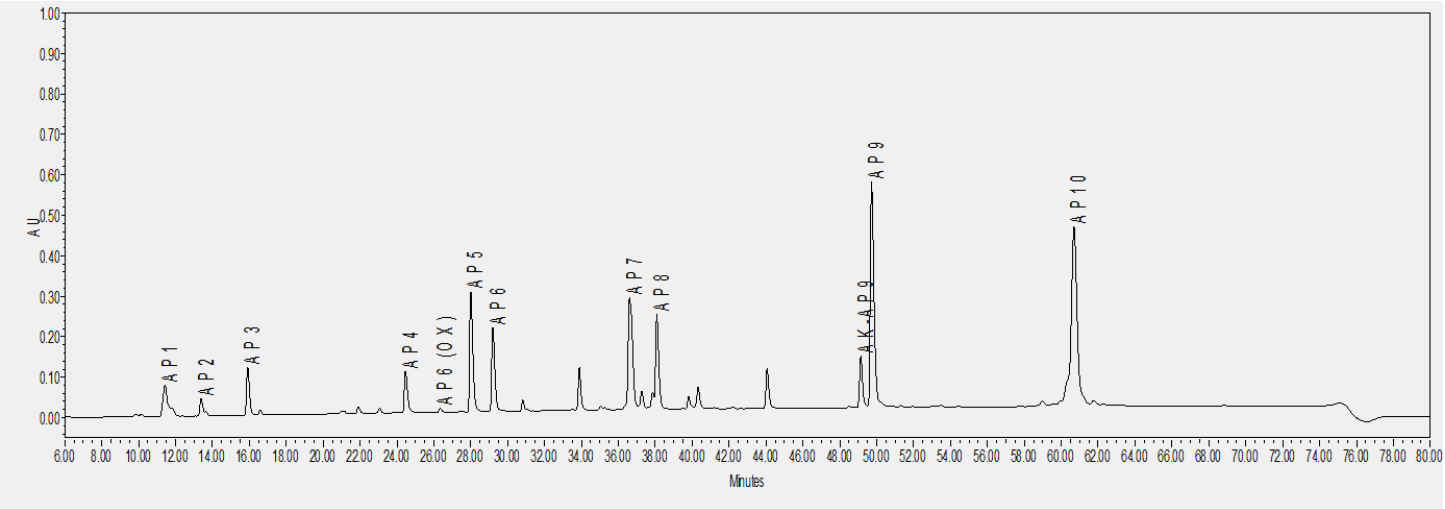

Figure 1. Peptide Map of USP IFN-β-1A Reference Standard[5]

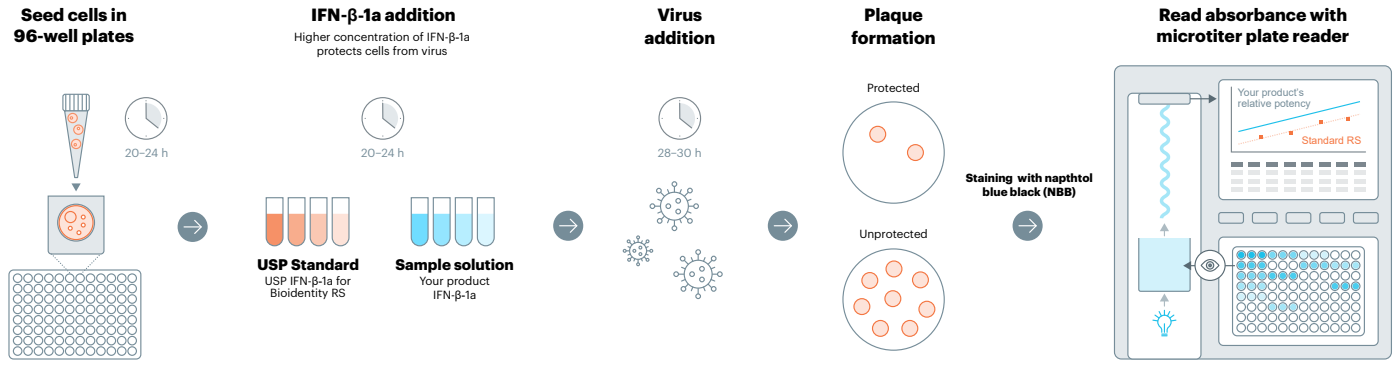

Figure 2. Interferon Beta-1A Cell-based Bioidentity Test method

Standard). The USP bioidentity test compares the protective effect provided to cells by IFN-β-1a against a viral cytopathic effect, in this case encephalomyocarditis virus (EMCV), with that of an appropriate reference preparation calibrated in International Units. The reason is interferons inhibit viral replication by stimulating innate immune responses, inducing interferon-stimulated genes, and creating an antiviral environment within host cells.

Purity Testing of Interferon Beta-1A

When relying on a biological system to manufacture a protein therapy, product-related impurities as well as process-related impurities are things to watch out for as they may have deleterious impacts on the therapy (such as stability and efficacy) or the patient (e.g. inducing negative immunological responses). Products that are oxidized result in a type of impurity that can promote inflammatory conditions in the bowel and have been linked to the onset of carcinogenic processes[5]. Oxidation of IFN-β-1a can occur resulting in an impurity with the amino acid methionine being converted to methionine sulfoxide (MetO)[4]; MetO can cause tissue proteins to misfold or otherwise render them dysfunctional. The peptide map obtained in the chemical identity testing of

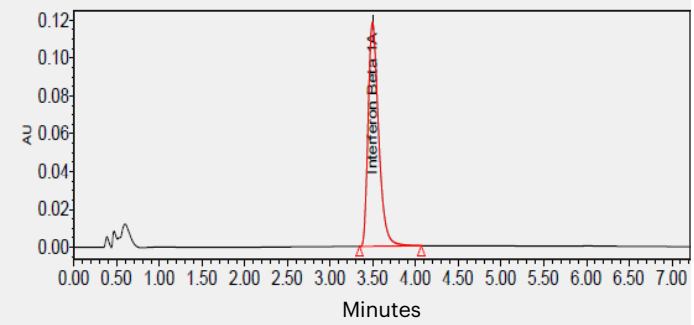

Figure 3. IFN-β-1a Assay Chromatogram[5]

Interferon Beta-1A is also used to determine the amount of oxidized IFN-β-1a in the product. In the IFN-β-1a peptide map shown in **Figure 1**, the peak labelled AP6 is a normal IFN-β-1a fragment and the peak labelled AP6(OX) is an oxidized IFN-β-1a fragment. The USP ***Interferon Beta-1A*** Reference Standard is used during the test to identify the AP6 and the AP6(OX) peaks.

Analytical Test of Interferon Beta-1A Strength

An important element of an IFN-β-1a product is its concentration, also referred to as strength. If the patient is administered IFN-β-1a below the expected dose, the product may not have the intended therapeutic effect. Alternatively, if the patient receives a higher dose of IFN-β-1a than anticipated it could be harmful to the patient. It is of utmost importance that the strength of the IFN-β-1a in the container match the amount stated on the label of the container. The USP provides an analytical method and a Reference Standard suitable for determining the strength of IFN-β-1a product.

Often the test for strength is described in the USP as an "assay". To perform the USP assay for IFN-β-1a strength, HPLC separation is used (please refer to the USP IFN-β-1A Reference Standard Certificate for full method details). Briefly, a test sample is prepared of the IFN-β-1a product, and a standard is prepared using the USP ***Interferon Beta-1A*** Reference Standard. **Figure 3** shows an example of a chromatogram from the assay of IFN-β-1a. The size of the IFN-β-1a peak in the sample chromatogram is mathematically compared to the size of the IFN-β-1a peak in the Reference Standard chromatogram. The strength of the IFN-β-1a product is calculated using the USP IFN-β-1a Reference Standard peak for calibration. The retention time of the IFN-β-1a product peak must match the retention time of the USP IFN-β-1a Reference Standard peak in the assay to further

demonstrate the identity of the IFN-β-1a in the product. If the strength of the IFN-β-1a product determined by analytical testing matches what is on the product label, it gives assurance the patient is receiving the proper dose.

Glycosylation Pattern of Interferon Beta-1A

The IFN-β-1a molecule should have a single carbohydrate group attached to it, but the population of IFN-β-1a molecules in a sample may have chemically different carbohydrate groups that contribute to the glycosylation pattern of the sample, which can influence the solubility, stability, and biological activity of the sample[4]. USP provides an analytical method suitable for determining the glycosylation pattern of IFN-β-1a products; when compared to a product specification, ensure it will perform as expected in the patient.

In the USP test a HPLC separation method is used to determine the glycosylation pattern for an IFN-β-1a product (for full method details, please refer to the USP IFN-β-1A Reference Standard Certificate). Briefly, to prepare the test sample for the USP glycosylation test the sugar groups are removed from the IFN-β-1a and a chemical label is attached to each carbohydrate group. The chemical label allows the sugar groups to be detected by the HPLC instrument. A sample of the USP ***Interferon Beta-1A*** Reference Standard is prepared in the same way as the test sample. The chemically labelled carbohydrate molecules are separated from each other based on their polarity. **Figure 4** shows an example of a chromatogram from the analysis of the glycan groups obtained from an IFN-β-1a sample. The glycosylation pattern shown in the chromatogram of the IFN-β-1a sample should be similar in glycan types and amount of each glycan when compared to the USP IFN-β-1a Reference Standard chromatogram. If the glycosylation pattern of the IFN-β-1a product is similar to the USP Reference Standard this gives assurance the IFN-β-1a product will perform as expected in the patient.

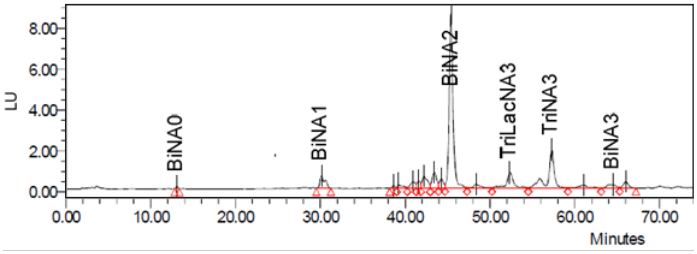

Figure 4. Chromatogram of the Glycosylation Pattern of the USP IFN-β-1a Reference Standard[5]

Conclusions

The USP Interferon Beta-1A Reference Standards provide users with comparators for chemical integrity and biological activity. This is valuable for those who are assessing product they have received or developing production processes. When manufacturing an IFN-β-1a product, these Reference Standards are needed to perform analytical testing to demonstrate acceptable quality.

The USP Interferon Beta-1A reference standards can be used for identification, determination of strength or assay, for measurement of oxidation and other impurities, and as a comparator for analysis of glycosylation. Interferon Beta-1A for Bioidentity RS is used to show the identify of Interferon Beta-1A through its biological activity. In addition to these uses, the USP Interferon Beta-1A Reference Standards support analytical method development, validation, release testing, stability testing, and analytical method transfer. They can serve as system suitability samples for quality control testing as well as a reference model for characterization studies.

References

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5. USP Certificate for Interferon Beta-1A. https://store.usp.org/product/1342401

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