Review

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Comparability of biotherapeutics: characterization of protein vaccine antigens

Advances in biotechnology and analytical tools now permit the application of extensive analytical characterization packages to purified recombinant proteins, a significant progression from the traditional characterization of complex biological products primarily by their manufacturing process. In this article, the authors focus on comparability assessment of biotherapeutics, specifically vaccine protein antigens. Regulatory drivers and analytical approaches used to examine product comparability are discussed and two case studies are described in detail to demonstrate the comparability of pre- and post-change product at the early and late stages of vaccine development. In coming years the number of comparability studies will likely increase due to the greater number of vaccine manufacturers, production at multiple sites and with external partners, and the introduction of innovative process technologies. Comparability studies may be focused on only a few changes, or may be more extensive, to address the impact of multiple process changes at various stages of manufacturing.

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General considerations

» Comparability assessment: regulatory considerations & other drivers

Traditionally, complex biological products such as vaccines presented unique challenges to implementation of even rudimentary characterization packages; thus, the product was defined almost exclusively by its manufacturing process. More recently, advances in biotechnology (e.g., recombinant DNA technology), coupled with the availability of state of the art analytical tools, have permitted the application of more comprehensive and informative characterization packages to products such as purified recombinant proteins. This in turn has allowed manufacturers to assess the impact of manufacturing changes on their products through analytical comparability studies. The results of these studies can, when coupled with appropriate impact assessments, be used to determine whether or not additional nonclinical or clinical studies are required to support continued product licensure.

Comparability studies for commercialized products may be performed under a protocol; that is, a pre-defined, detailed, written plan for assessing the effect of specific manufacturing process changes on the physicochemical and biological attributes of a specific product as they may relate to its safety and efficacy [1–3].

The comparability protocol should outline the proposed manufacturing changes (and their anticipated impacts, where understood) as well as the testing descriptions and scope of the analyses (e.g., in-process samples, drug substance (DS) and drug product (DP) release, stability and characterization testing, and so forth). Where test acceptance criteria are defined, this should be included. Additional acceptance criteria for demonstrating comparability may be defined, taking both the method and the manufacturing variability into consideration.

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Key Terms

Analytical tools: Laboratory methods that are used to determine the concentration, purity, identity and potency of a protein antigen, as well as its higher order structure and function. Normally, a combination of biochemical, biophysical and immunochemical methods are used to build an analytical package for a protein antigen of interest.

Protein antigens: Proteins that induce an immune response in the body, for example, the production of antibodies.

Product comparability: Examination of the process steps and product attributes of a biological product manufactured before and after a process change using pre-defined acceptance criteria for release tests and precision ranges determined for characterization tests. Comparison of the data sets generated for a process stage of interest is used to determine whether pre- and post-changed product qualities are comparable.

Early-stage product development: Product in the preclinical, Phase I or Phase II stage of clinical development.

As stated in ICH Q5E, *"The demonstration of comparability does not necessarily mean that the quality attributes of pre- and post-change product are identical, but that they are highly similar and existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact on safety or efficacy of the product"* [3]. Biocomparability of the product should be demonstrated based on the data derived from, among other things, *"analytical studies that demonstrate that the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components"* [4].

Improvements in the techniques for characterizing products, and controlling the manufacturing process, coupled with greater ability to assess the potential effect of manufacturing process changes on a product, allow comparability exercises to be used with other types of changes, for example a formulation change, or analytical procedure changes [1]. This expanded use of comparability protocols has been recognized in FDA regulations (21 CFR 601.12) and guidances [2,3,5–7]. Lately, several vaccines are formulated using recombinant protein antigens, for instance, Hepatitis B surface antigen, HPV L1 protein. Hence, the guidance and regulations related to recombinant protein-based therapeutic products referred to in this paper applies to vaccines in general.

Since biologics and protein drugs are complex, knowledge of how product attributes affect the safety and efficacy of the product is crucial in designing comparability studies. According to [1–3,8], it is important to have sufficient knowledge of both the manufacturing process and the analytical package, including the analytical procedures, tests, studies and acceptance criteria appropriate to assess the impact of the change on the product quality attributes, and in turn their impact on safety and efficacy. The attributes to be examined are described in the sections below.

» Considerations for manufacturing process changes

Characterization of biological/biotechnological products is described in [9] and should address physicochemical properties, biological activity, immunological properties, purity, impurities, contaminants and quantity. These attributes should be included for substantiating the comparability established from other methods. In addition, the robustness of the process (i.e., the ability of the product to remain unaffected by process changes) should be taken into consideration [1–3].

Expression system

Protein vaccine antigens can be produced by pathogen cells or expressed recombinantly in a host cell that is prokaryotic or eukaryotic in origin. For example, prokaryotic (*Escherichia coli*) and eukaryotic insect, avian or mammalian cells are used. It is expected that the expression construct of recombinant protein antigen will encode the same primary amino acid sequence as its corresponding pathogen-produced native protein, although sequence modifications that do not adversely impact safety or efficacy (e.g., genetic detoxification) can be considered. In addition, it is essential to ensure that the primary structure of the target antigen is unaffected by a modified expression system. In addition, the impact of process- and product-related substances and impurities, as well as post-translational modifications on the product safety, purity and efficacy, have to be assessed during vaccine development. The characterization of the expression construct and its genetic stability can be demonstrated according to ICH Q5B [10].

Manufacturing process design

Manufacturing process changes are normal expectations of a product in clinical development stages, and can also occur with products in the post-licensure stage. Process changes can include changes in facilities (design and location), raw materials, process scale, equipment, purification scheme design and work flow, chromatography chemistries and media, as well as sterilization and inactivation steps. The nature and scope of the manufacturing process changes need to be taken into account when assessing impact of the changes, as well as when designing the comparability strategy. Where a written comparability protocol or report is used, it should summarize the actual process changes made and assess the likely impact.

Manufacturing steps

An in-depth understanding of all steps in the manufacturing process should be established during product development. Characterization tests, process controls, and specification (release) tests that emerge from product and process knowledge gained during development may be used to assess the effect of any process change. The use of quality-by-design and risk assessment facilitates the consistent manufacturing of a high-quality product [11,12].

» Product characterization

Physicochemical properties

Physicochemical properties of the protein antigen include all relevant characteristics: content, purity, identity, primary, secondary and tertiary structure, post-translational modifications, and biological activity. According to ICH Q6B, it is important to understand and characterize the heterogeneity of the recombinant product and the ranges of variability of different isoforms [9]. A range of available biochemical and biophysical methods is typically applied to address physicochemical properties.

Often more than one analytical procedure is used to evaluate the same quality attribute. For example, analytical ultracentrifugation and size exclusion chromatography with multi-angle light scattering detection can both be used to determine the presence, nature and quantity of protein oligomers and aggregates in the nanometer range, whereas laser diffraction and microflow imaging can detect particles in the micrometer range [13].

Some physicochemical methods can assess multiple product attributes simultaneously. For example, peptide mapping by liquid chromatography coupled with mass spectrometry (LC–MS) of protein fragments can confirm identity of the target antigen or process-related impurities (e.g., host cell proteins), as well as product-related impurities (e.g., protein fragments) [9,14].

While analytical procedures used in the product specifications need to be validated prior to submission for licensure [9], there are no regulatory requirements for all characterization tests used for product comparability assessments to be validated. For early-stage product development, analytical methods used for clinical lot release, stability and in-process control testing are typically qualified (i.e., formally demonstrated to be fit for their intended use), whereas product safety tests are validated. By Phase III or prior to process validation, all product specification tests are validated. Regardless of whether an analytical method used for product comparability is a specification test or not, it must be scientifically sound, reliable and reproducible. Such tests may be qualified or validated if needed at later stages of product development.

Overall, the characterization tests selected to support comparability exercise have to be of an appropriate sensitivity and specificity to demonstrate whether or not the pre-change and post-change products are comparable.

Biological activity

Functional assays serve multiple purposes in the characterization of protein antigens. They can be used to complement biochemical and biophysical methods and are a measure of the function of the protein product. Some products may exhibit multiple biological activities; in this case, a range of assays may be required to establish comparability. Functional assays may have limitations such as high variability, and thus may not be sensitive to changes in the product. This is particularly the case for *in vivo* assays (e.g., animal immunogenicity). These limitations must be taken into account during assessment of the robustness of the quality and meaning of data from functional assays to support comparability upon process changes.

Immunochemical properties

The immunological properties of protein antigens should be characterized using immunochemical procedures such as enzyme-linked immunosorbent assay (ELISA) or western blot [9]. Analytical tests such as surface plasmon resonance and microcalorimetry are used to characterize the kinetics and thermodynamics of antigen–antibody binding. Technologies such as biolayer interferometry (ForteBio Octet®), surface plasmon resonance (Biacore™), and ELISA use specific antibodies to monitor the integrity and identity of protein epitopes. These parameters can be related to the biological activity and the protein antigen's higher order structure, for example, through the use of neutralizing or conformational antibodies.

Potency

Potency is obviously a key product attribute, but may not be established for a specific product until a relatively later stage of clinical development. In the absence of an established validated potency test alternative, readouts (e.g., content, biological activity) may be applied as discussed above.

Impurities

Product- and process-related impurities have to be identified, quantified and characterized as per ICH Q6B [9]. Process-related impurities include host cell DNA, host cell proteins, cell culture components (antibiotics, media components), impurities introduced by downstream processing steps (reagents, residual solvents, extractables and leachables), endotoxins and bioburden. Absence of adventitious agents and endogenous viral contamination is ensured by screening raw materials, intermediates and finished products, and by robust virus removal and inactivation processes [15]. The goal of the comparability exercise is to demonstrate that the levels of impurities are either comparable, or that post-change product has lower levels of impurities. However, in cases when the level of impurities increase, toxicological studies or other risk assessments may be performed to assess potential biological effects and the overall product safety profile.

Key Term

Late-stage product development: Product in Phase III or post-marketing stage. In this Review, a drug product that is entering a Phase III efficacy and manufacturing consistency trial is referred to as a late-stage product.

» Product stages

Typically, comparability studies are performed at the level of the DS stage, unless the scope of the manufacturing changes includes steps that fall downstream of this stage (e.g., formulation). In such cases, the finished DP must be incorporated into the comparability study. However, it should be noted that at the finished DP stage, fewer analytical methods may be available, due to factors such as presence of a complex matrix (e.g., adjuvants, multiple protein antigens [also referred as multiple valences]; lower concentration of active substance; and/or product presentation [e.g., lyophilization]) [16]. These are all typical considerations for vaccines. A toxicology assessment may be needed to evaluate the effect of formulation, product degradation and primary packaging on product safety [3,8]. Toxicology studies are normally performed at an early stage of product development, for example, at preclinical stage prior to Phase I safety trial, and potentially later on after significant changes in the manufacturing process, as discussed in Case Study 2.

» Stability

Stability studies are performed on representative batches of the pre- and post-change product using previously developed physicochemical and functional assays that are stability-indicating. Forced degradation studies are performed in parallel to establish degradation profiles and provide direct comparison between pre- and post-change product. These studies include multiple stress conditions, such as elevated temperature, freeze–thaw, chemical stress (e.g., low and high pH or oxidation, white light and UV light exposure, agitation, that create incremental product degradation over a limited time period [17]. Stability results from comparability studies that reveal potential product differences upon process changes may require additional investigation. In addition to providing information regarding lot comparability, a similar (or better) accelerated stability profile post-change can potentially be used to project shelf life, based on that of the pre-change product.

Case studies: comparability principles in practice

Comparability study design and choice of analytical methods may facilitate product development by examining and addressing the effects of process changes. These studies may also reduce time and cost if focused on a specific product stage and product attributes that may experience the most significant impact due to process changes.

The following sections describe two case studies that serve as examples of how comparability principles are applied for protein antigen components of vaccine products in various stages of clinical development. In each case, the comparability testing package is focused on specific product attributes. **Figure 1** summarizes key considerations for a comparability exercise, in terms of product stages and product attributes. The two case studies described below were undertaken by the authors and will be used to demonstrate the application of the considerations discussed above for

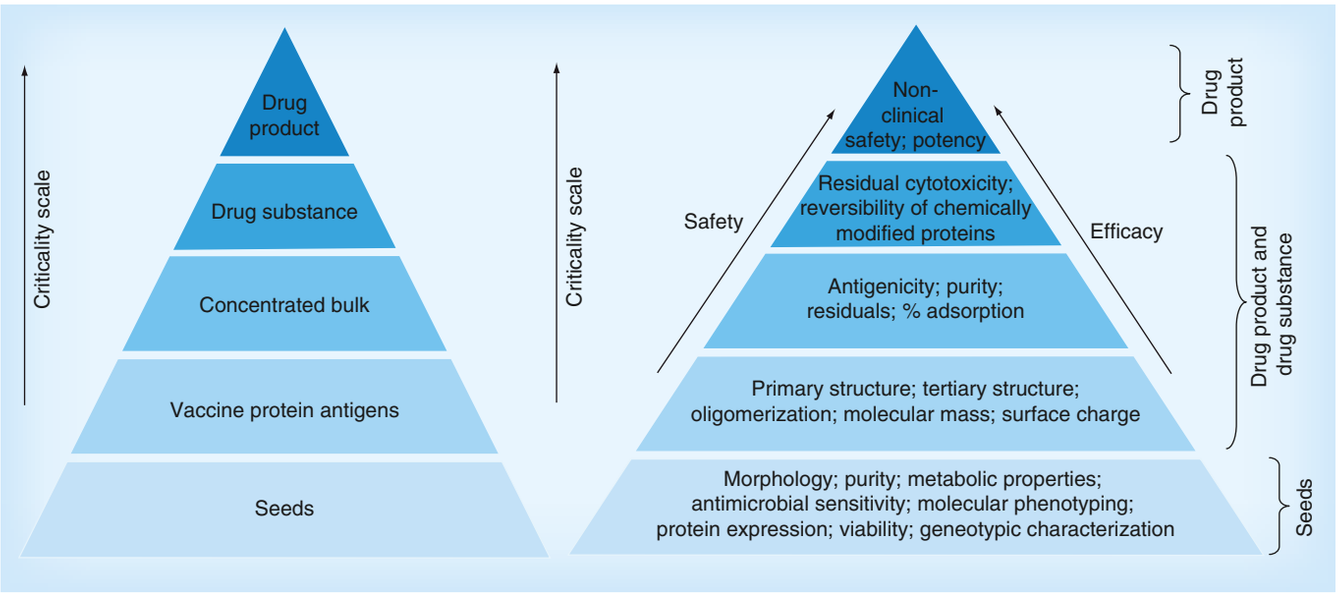

Figure 1. Comparability based on product stages and product attributes.

the comparability of post-change vaccine at early- and late-stage product development.

» Case Study 1: comparability at an early stage of product development

Case Study 1 describes a comparability study performed retrospectively for a product in an early stage of development. Specifically, the impact of a process change incorporating antibiotic-free media for expression in *E. coli* of three individual recombinant protein antigens' purity, impurity profile, higher order structure and stability was assessed.

The objective of this study was to assess comparability between Vaccine X Phase I DS material produced using an antibiotic-free fermentation process and DS material produced using the original fermentation process using antibiotic.

The antigens, X1, X2 and X3, all representative of their respective Phase I manufacturing processes, were expressed as recombinant proteins in *E. coli* from a common expression vector that carries an antibiotic-resistance marker. Current guidelines from regulatory agencies acknowledge the use of certain antibiotics in the manufacturing of viral, plasmid DNA and recombinant protein vaccines, and additionally specify that their clearance in the purification process needs to be demonstrated [9,18]. While monitoring of residual levels of antibiotics can be routinely performed in order to control levels in the product in clinical development, any use of antibiotics in the manufacturing of biologics at an industrial scale may contribute to an increase in antibiotic-containing environmental waste.

To address concerns regarding potential antibiotic-containing fermentation media waste associated with future large-scale manufacturing of Vaccine X, demonstration lots were produced using the original fermentation process, but without antibiotic. No additional modifications were made to the manufacturing process of the DS. Demonstration lots of DS produced with and without antibiotic were evaluated for comparison.

Comparability was assessed by evaluating a panel of product attributes, as well as stability of the DS lots produced by the two fermentation processes. The following four types of datasets were identified, through a scientific assessment, as important to show comparability:

- Relevant release testing results;
- Results for process-related impurity tests;
- Biochemical and biophysical characterization test results;
- Stability of the DS at the intended storage temperature (2–8°C) as well as elevated temperatures (23–27°C and 35–39°C).

The data generated from products manufactured with and without antibiotic were comparable and within the defined specifications where applicable. For all tests, including characterization tests, where no product acceptance criteria have been defined, the results for the pre- and post-process change lots were judged to be comparable, based on the ranges established during method qualification and a scientific assessment of the testing results.

The conclusion of the part of the comparability study regarding stability was based on a limited number of antibiotic-free batches placed on long-term stability. Elevated temperature studies were also performed [19].

Stability testing for Vaccine X drug substances includes two orthogonal assays for percent purity: SDS-PAGE with densitometry and RP-HPLC with UV detection. Both assays are quantitative and have the additional advantage of compatibility with further approaches (e.g., MS) to investigate degradation products, where required.

Using ANCOVA analysis, a comparison was conducted using results obtained for DS demo lots produced with and without antibiotic, incubated at 23–27°C. The p-values demonstrated that X1 and X2 were comparable for both percent purity by SDS-PAGE and percent purity by RP-HPLC; however, X3 did not show significant similarity between the two lots for percent purity by SDS-PAGE, but did for percent purity by RP-HPLC. To further investigate, additional analyses were conducted using ANCOVA for X3 at 37°C and 2–8°C; both results indicated that the lot stabilities were statistically similar at these two temperatures. While lots produced using antibiotic and the antibiotic-free lots can be considered comparable, due to the limited batches available, it was recommended that additional manufactured batches produced without antibiotic also be placed on stability.

Based on the comparability experimental results and conclusions summarized above, the recommendation was to use the antibiotic-free manufacturing process for the next Vaccine X clinical campaign. Due to the limited number of DS batches available using the antibiotic-free process, it was also recommended that a number of future DS batches be fully characterized and tested in the stability program. Importantly, no comparability results were obtained that suggested an impact of the process change on product safety.

» Case Study 2: comparability at a late stage of product development

Case Study 2 focuses on comparability of representative lots of Phase II and Phase III processes for vaccine Y. The manufacturing changes between Phase II and Phase III were intended to streamline the process through the implementation of new technologies and facilities; to facilitate the industrialization of the vaccine Y; and to maintain the existing product characteristics, or improve the safety and impurity profile of the product. The process refinement that was performed prior to Phase III ensured operability of the overall process at several thousand liters fermentation scale, as well as improvement of the product safety attributes. The process changes include the use of animal-free bacterial seeds, scale-up from several hundred to several thousand liters fermentation, and improved safety by changing the chemical modification step, leading to decrease of chemical modifier levels in the final vaccine product.

Figure 1 shows a criticality scale according to the product stage or attributes for the purpose of comparability. Although it is not strictly the same for every situation, it can be used as a general guideline for comparability of vaccine and recombinant proteins in general. Safety and efficacy are equally important for any product. First a product has to meet the safety requirements before it is even considered for any clinical evaluation. The cytotoxicity assay referred to in the figure is a qualitative test with a pass/fail result and does not provide any additional information for comparability evaluation. In contrast, a potency assay demonstrates the biological activity of the product and it is quantitative in general making it more powerful for comparability evaluation. Thus, while safety and potency are both essential product attributes, the potency assay is ranked as more critical to the comparability evaluation in Figure 1.

It is important to establish the method and the product variability for a comparability exercise. The method variability can be obtained from analytical method qualification or validation results. The product variability is closely related to the manufacturing process and can be derived from testing multiple lots manufactured using the same process and scale. Based on this knowledge, acceptance criteria for demonstrating comparability can be established. The impact of any observed changes in product attributes that may relate to safety or efficacy, needs to be taken into account in the final conclusion.

To demonstrate comparability between the vaccine Y protein antigens manufactured using Phase II and Phase III processes, two representative lots from each process were selected and analyzed by different analytical methods to assess the characteristics of the antigens and the potential impact on product safety and potency, including the impurity profile. Various attributes such as purity by capillary gel electrophoresis, surface charge by anion-exchange chromatography, size distribution by analytical ultracentrifugation and tertiary structure by differential scanning calorimetry and intrinsic fluorescence were evaluated to demonstrate comparability. In addition, epitope integrity of the antigen proteins by ELISA, potency by an established animal model, and biological activity by an *in vitro* cell-based method were assessed. Furthermore, the stability profiles of representative Phase II and Phase III lots were assessed using established stability-indicating assays to demonstrate comparability.

Based on the results of the experiments described above, the overall conclusion of the study was that the Phase II and Phase III vaccine Y DPs were comparable. Furthermore, the comparability of Phase II and Phase III DP lots is demonstrated by the similar stability profiles observed for these lots. Results from a repeat dose toxicology study demonstrated that Phase III DP is well tolerated and there was no systemic toxicity observed in animals used in the study. Overall, the comparability of Phase II and Phase III DP lots supports the continued clinical use of the Phase III vaccine Y.

Conclusion

The comparability study design is specific to the product and to the stage of product and process development. Studies may be more efficiently performed if focused on the specific product stage, and on product attributes that may experience the most significant impact due to the process changes. In the early stages of product development, process changes may be focused more on the upstream steps, for example the fermentation media components and conditions. The comparability exercise discussed in Case Study 1 examines the effect of one process change only, that is, removal of the antibiotic used during fermentation, and the scope was limited to the individual drug substances. In later stages of product development, process changes may include change of media for the seed, scale-up, change of the equipment, and manufacturing facility. Here, the comparability exercise may be more extensive and examine various stages of the process and attributes of product intermediates, drug substance(s) and DP. It should be noted that characterization testing of the DP may be limited (relative to that of the drug substance) by factors such as product formulation and presentation. Due to the greater criticality of the comparability assessment for the final product (injectable material) (Figure 1), in Case Study 2, the focus was on the lyophilized DP, fol-

lowing reconstitution. For this material, the final concentration of reconstituted DP permitted application of key immunochemical and biophysical assays also used for the intermediate and drug substance.

Future perspective

In the coming years the importance of comparability studies to vaccines will likely increase. Reasons for this include an increased number of vaccine manufacturers, manufacturing strategies that involve multiple internal sites of the manufacturer and/or external companies through outsourcing, and introduction of innovative process technologies such as disposable bioreactors and single-use formulation systems. Comparability studies focused on a few process changes only will be less extensive, while studies assessing multiple process changes at different stages of manufacturing will need to be more extensive.

Overall, a scientifically sound comparability study design and careful choice of analytical methods may facilitate product development by assessing the impact of process changes on product attributes. These studies may also reduce time and cost if focused on a specific product stage and product attributes that may experience the most significant impact due to process changes.

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Executive summary

Background

» Comparability assessments of biotherapeutics are reviewed, with specific focus on vaccine protein antigens.
» Regulatory drivers and analytical approaches used to demonstrate product comparability are discussed.

Manufacturing process changes & product characterization

» Aspects to consider in characterization to support comparability include: expression systems, manufacturing process design and manufacturing steps.
» Aspects of product characterization are discussed, including physicochemical properties, biological activity, immunochemical properties, potency and impurities.

Comparability principles in practice

» Two case studies – one early-stage, one late-stage product – are presented.

Conclusion & future perspective

» Comparability study design and choice of analytical methods may facilitate product development by assessing the impact of process changes. These studies may also reduce time and cost if focused on a specific product stage and product attributes that may experience the most significant impact due to process changes.

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