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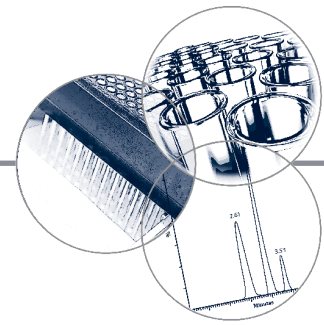

Bioanalytical challenges of biosimilars

Biologics such as monoclonal antibodies and recombinant proteins represent a significant portion of the pharmaceutical market. With many of the first generation biologics' patents expiring, an increasing number of biosimilars will be submitted for approval in the near future. The successful development of a biosimilar requires the demonstration of biosimilarity in terms of efficacy, safety and purity to an innovator-approved product. While regulatory frameworks have been established for the approval of biosimilars in several countries, there is not an established guidance for bioanalytical testing of biosimilars. Although there are regulatory guidances and White Papers on testing requirements for biologics in general, there is a need to address the bioanalytical challenges and solutions that apply specifically to the analysis of biosimilars in biological samples. This paper will focus on components of the PK and immunogenicity assays that are critical to biosimilar drug development.

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Biosimilars are copies of the approved version of a biologic drug. Biosimilar is defined by the US FDA as a biological *"product highly similar to the reference product without clinically meaningful differences in safety, purity and potency"* [101]. Different terms have been applied to biosimilars, such as 'biosimilar products', 'similar biologics', 'follow-on protein products' and 'subsequent-entry biologics'. For the sake of brevity, this paper will use the term biosimilars.

Biologics are manufactured in a living system such as a microorganism, or plant or animal cells. The manufacturing process is highly complicated and is very sensitive to minor process changes (e.g., pH, presence of cytokines and so on) [102]. The manufacturing process includes identifying the correct gene sequence, cloning the gene to the appropriate cell type, fermentation, purification and so on. The process controls for each manufacturing process are unique and proprietary to each manufacturer. It is impossible for a biosimilar manufacturer to copy the identical manufacturing process of the **innovator** without infringing on the innovator's intellectual property rights.

As a result, it is expected that there will be some differences between the innovator and the biosimilar in terms of their process-related purities, physiochemical properties, biological activities and immunochemical properties [102]. Both biosimilar and innovator drugs should be characterized in-depth to gain valuable structural and functional insights [1,2]. While the strategies for the characterization of the drug products are out of the scope of this paper, it should be noted that an understanding of the comparative immunochemical and biological properties of the innovator and the biosimilar is indispensable in developing successful and robust bioanalytical assays.

Several regulatory authorities worldwide, led by the EMA, have issued guidances on the development of biosimilars [101,103,104].These guidances have generated significant responses from the scientific community, in some cases supporting the guidances and in other cases voicing concerns against the guidances [3–6].

The common theme among all guidances is that validated PK and immunogenicity methods, for the determination of both the innovator and the biosimilar, and for the determination of **anti-drug antibodies** (ADA) against both the biosimilar and the innovator in biological samples, are required to demonstrate biosimilarity. While there are regulatory guidances and industry White Papers on the method development and validation of these methods, they do not address the nuances involved in the bioanalytical method development of biosimilars [7–13].

The following sections describe the critical assay parameters and performance characteristics that should be evaluated during bioanalytical method development for biosimilars.

PK assay challenges

Unlike small molecule bioanalysis, where LC–MS/MS methods are commonly used for the measurement of drugs, for biologics LBAs are used to measure the therapeutic level of the biologics in biological samples. The most commonly used LBA method is ELISA with various detection technologies. In general, ELISA assays

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Key Terms

Biosimilars: Generic versions of biologics that are similar to a reference product in terms of safety, purity and potency.

Innovator: Original biotherapeutic licensed or approved by regulatory authorities. It is also known as a reference product.

Anti-drug antibodies: Antibodies generated in response to a drug. This is also referred to as an antitherapeutic antibody or an antiprotein antibody.

Parallelism: Statistical method for the comparison of potency or binding between a reference product and test product. It calculates relative potency by comparing the dose response curves of the two products.

require one (in case of competitive assays) or two (in case of sandwich assay) binding reagents to measure biologics. The specificity and selectivity of the assay is dependent on the interaction of these binding reagents to the biologic. It is important to evaluate these interactions early in the assay development process.

Establishing biocomparability between the innovator and the biosimilar is a critical first step in PK assay development. Establishment of biocomparability requires the selection of the appropriate assay format and the establishment of equivalent potency between the innovator and the biosimilar. As for all method development efforts, these steps are overlapping, iterative and recursive (Figure 1).

Selecting the assay format

The selection of a robust assay format requires an in-depth understanding of the characterization data. There should be a close collaboration between the bioanalytical scientists and the analytical scientists, so that the characterization data can be fully understood. This will greatly aid the development of a reliable bioanalytical PK assay.

When possible, one assay should be used for the measurement of both the innovator and the biosimilar drugs. In the bioanalytical testing of the biosimilar, the goal is to show that the PK parameters for the biosimilar and the innovator are equivalent. To illustrate equivalency using a PK assay, a common point of comparison is necessary. Using one PK assay will ensure that the comparison is a true representation of the differences in the PK profiles and not the analytical variability between two assays.

The format and the reagents that will most utilize the epitopes of closest similarity, if not identical, between the innovator and the biosimilar should be selected. This will contribute to the development of a PK assay that has low variability, to enable the detection of potential PK differences between the innovator and the biosimilar. The critical reagents should also be selected in a way that can provide the best sensitivity, selectivity and specificity for both the innovator and the biosimilar.

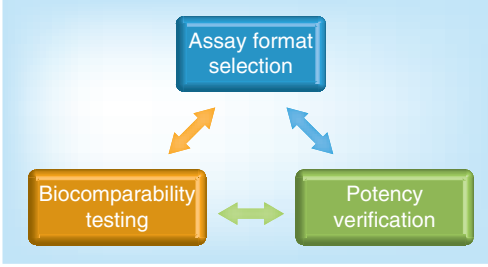

Figure 1. Approaches to method development.

The following scenarios show the impact of different approaches taken for quantitation of the innovator and the biosimilar in biological samples. In these examples, the innovator and the biosimilar are fusion proteins composed of two copies of an endogenous protein and the Fc region of human IgG1 (Figure 2). In these examples, the assay format results in the most comparable measurement of both the innovator and the biosimilar. In addition to comparable measurement of biosimilar and innovator products, other factors such as level of endogenous counterpart, free versus total analyte, the mechanisms of therapeutic protein's absorption, distribution and elimination over time, and so on, should be taken into consideration when choosing an appropriate assay format to support PK studies [14].

Potency of the biosimilar & the innovator product

Potency is the quantitative measure of the biological activity of a drug product. A common source of variability in bioanalytical assays is the difference in the potency between the innovator and the biosimilar. Biological activity-based potency assays have a greater variability and wider acceptance criteria (e.g., 50–150%) than LBA (e.g., 80–120%). The innovator and the biosimilar may show differences in quantitation in a LBA, while demonstrating equivalence in a potency assay. It is important to investigate different lots of both the innovator and the biosimilar to establish inter-lot variability early in the assay development process.

Parallel line analysis may be performed by comparing dose response curves from the biosimilar and the innovator drug products [15]. If the curves for biosimilar and innovator are found to be nonparallel, it will indicate that there is a potency difference between the biosimilar and innovator. In the context of biosimilar PK assay development, potency is defined as the ability of a drug/analyte to bind with assay reagents (i.e., capture protein and detection protein). It is a measure of a drug's immunoreactivity to the assay reagents. A WHO or other international public reference standard, when available, may be used as an anchor reference

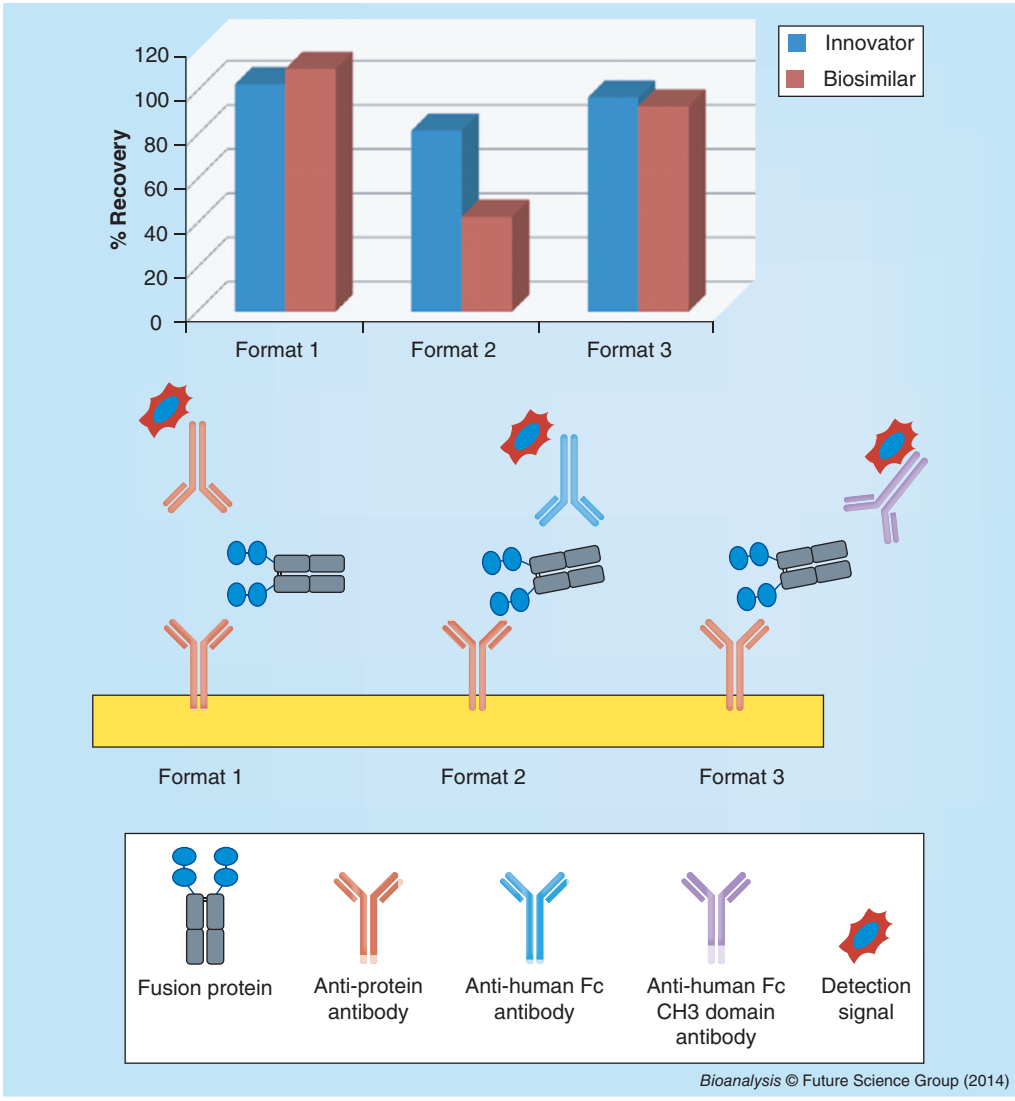

Figure 2. Assay format and its impact on measurement of drug (innovator and biosimilar). Format 1: Both the capture antibody and detection antibody used are targeted against the endogenous protein. This format will not be specific for the intact drug and endogenous proteins will cause crossreactivity. Format 2: The capture antibody is directed against the endogenous protein and the polyclonal detection antibody is targeted against the entire anti-human Fc region. This format is able to detect the intact drug, but shows great variability in quantitating the innovator and biosimilar. This format will show nonparallelism between innovator and biosimilar. Format 3: The capture antibody is directed against the endogenous protein and the monoclonal detection antibody is targeted against the CH3 domain of the Fc region. This format is able to measure the intact drug and is able to quantitate the innovator and the biosimilar similarly.

calibrator for calibrating both the innovator and the biosimilar.

If the parallel line analysis indicates nonparallelism, a root-cause investigation should be performed. One possible cause of nonparallelism may be that the innovator and the biosimilar do not have the same immunoreactivity towards the assay reagents (e.g., capture antibody, detection antibody and so on). The epitopes on the biosimilar that react to the assay reagents should be evaluated and an alternative assay format may be pursued.

The lack of **parallelism** may also indicate that the biosimilar is not adequately similar to the innovator and might affect its efficacy (e.g., if the capture reagent is the same as the target of the innovator) [16]. This situation would necessitate an investigation of the manufacturing process as the source of the difference between the innovator and the biosimilar.

Biocomparability testing

Despite best efforts during the manufacturing process, it is expected that there will be some structural and immunochemical differences between the biosimilar and the innovator compound. It is important to evaluate these differences and their impact on their binding characteristics to the assay reagents. A suggested approach for the comparison between the innovator and the biosimilar is illustrated in Figure 3 [17]. In this approach, the two drugs are compared with each other in the same run. Each run should include a set of innovator calibrators, a set (five levels including LLOQ and ULOQ) of innovator QC samples, a set of biosimilar calibrators, and a set (five levels including LLOQ and ULOQ) of biosimilar QCs. The calibrators and QCs for the innovator and the biosimilar should first be evaluated separately. Each set of calibrators and their corresponding QCs should meet the predefined acceptance criteria. Once these predefined criteria have been met, the equivalency between the innovator and the biosimilar can then be evaluated. They may be considered equivalent if the following conditions are met:

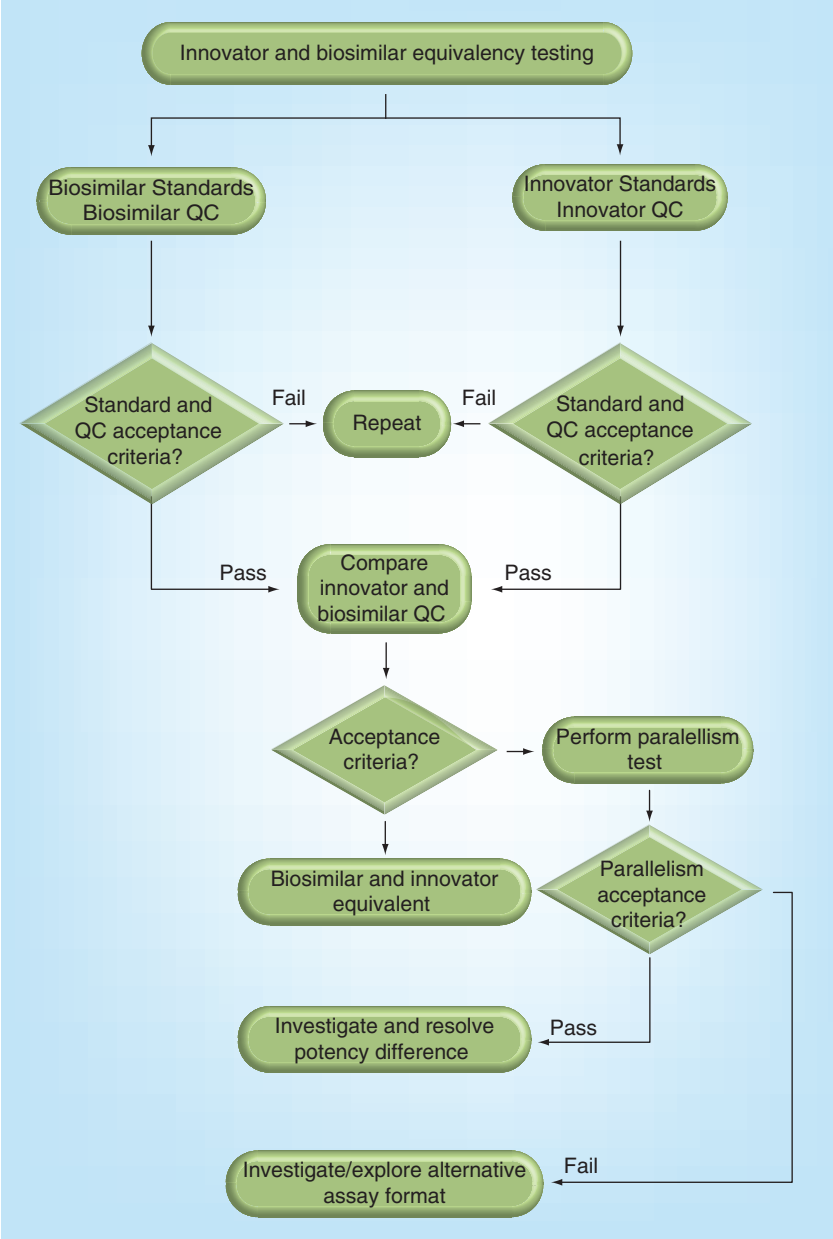

Figure 3. Biocomparability testing.
Adapted from [17].

- The biosimilar QCs meet a predefined acceptance criteria (e.g., ±20% bias [25% bias at LLOQ]) when evaluated against the innovator calibration curve;
- The innovator QCs meet a predefined acceptance criteria (e.g., ±20% bias [25% bias at LLOQ]) when evaluated against the biosimilar calibration curve;
- The percentage difference from the mean between the innovator QCs and the biosimilar QCs do not exceed ±20% (25% at LLOQ).

In addition, any trend in bias between the innovator and the biosimilar should also be evaluated. The innovator and the biosimilar may be considered equivalent if no significant bias or trend is detected. This will indicate that both are equally immunoreactive toward the assay reagents. The equivalency should be established during the initial phases of method development, prior to evaluating other assay parameters (e.g., selectivity, dilutional linearity, matrix effect and so on).

If biocomparability cannot be established, two assays may be used: one for the measurement of innovator and one for biosimilar, with appropriate scientific justification [18]. In this case, all samples should be run in both assays. Robust statistical measures should be developed for meaningful comparison of data from two assays. Data interpretation and acceptance criteria will need to be addressed and documented prior to sample analysis.

Impact of ADA on PK assessment

It is possible that the presence of ADA can have an impact on the PK assessment [19]. A further complicating factor is separating the effects of normal assay variability from ADA interference. Several factors should be kept in mind during

the PK assay development and during the PK sample analysis.

For PK method development, it is critical to understand the characteristics of the reagents being used. For example, if the capture reagent being used is the same as the target, it is possible that neutralizing antibodies could cause interference. Possible ADA interference can be verified by using an ADA positive control (PC) and fortifying it in PK validation samples. The selection of this PC should be carefully considered so that it reflects, as closely as possible, the potential antibody population in test samples.

During sample analysis, the PK results can be correlated with the ADA results. A drop in PK with a corresponding positive response in ADA analysis can indicate possible interference. Another possible approach could be to look at the subjects that were positive for neutralizing antibodies. Individual results (e.g., a positive PK result for predose samples, failure of incurred sample reproducibility and nonparallelism of PK samples) should be carefully evaluated and could indicate ADA interference.

Immunogenicity assay challenges

Immunogenicity testing is a critical component of the safety and efficacy assessment of biosimilars. Biosimilar guidances require that immunogenicity be monitored by tracking the rate of incidence, time for antibodies to form, the persistence of antibodies, the magnitude of the response and the type of response [101,103,104].

Assay development

As with the PK assay, for the effective comparison of immunogenic potential of innovator and biosimilar, a common point of comparison is necessary. However, unlike PK samples, the samples used for immunogenicity testing will typically be composed of a heterogeneous mixture of ADA against the drug. The drug is typically used as the capture reagent in an ADA assay. If the biosimilar is used as a capture reagent, it will not be able to bind and detect ADAs that are unique to the innovator drug and vice versa. This will create a risk of generating false negative results. It is important to develop and validate two assays, with each assay being specific to the ADA for the innovator and the biosimilar early in the biosimilar development process. It is acceptable to use one assay using a biosimilar as a capture reagent if early development data shows that both products are comparable in terms of immunogenic profile. In addition, if the historical data from the innovator product used in a population shows a very low incidence of ADA positive samples, and the reference product falls under the low risk category (i.e., clinical consequences of immunogenicity is not severe), it is acceptable to use one assay. Otherwise two assays, with each assay being specific to the ADA for the innovator and the biosimilar, should be used.

However, comparing data (e.g., incidence of positive response, titer of the response, isotype distribution and so on) from two different assays is challenging. This reasoning is very often cited by the proponents of the one assay approach. From a safety and regulatory point of view, the comparison of data should not focus solely on the incidence of positive response, titer or isotype distribution. Instead the comparison should be focused on the clinical impact of the above mentioned ADA response parameters. This might mean that the above parameters could be different between the innovator and the biosimilar. Biosimilarity might still be demonstrated based on the comparison of clinically meaningful immunogenicity data. Moreover, the risk of generating false negative results using one assay far outweighs the complexity in data comparison using two assays.

Positive controls

The key reagent in an ADA assay is the PC. The PC is used to evaluate the sensitivity, specificity, drug tolerance and assay precision of an ADA assay. PCs are usually generated by immunizing animals, preferably nonhuman primates. One PC generated against the biosimilar drug may be used for both assays.

However, it should be noted that if there are structural differences (i.e., significant difference in glycosylation pattern) identified or suspected between the innovator and the biosimilar, two PCs may be necessary to evaluate the assay parameters. The goal of the PC is to mimic the potential immune response in humans as closely as possible. Generation of individual PCs may provide additional information about the two assays, when structural differences are suspected.

If separate PCs are generated against the innovator and the biosimilar, they should be generated in the same species and should be purified using the same purification method. These PCs will not be identical due to the fact that they have been generated by two different animals. There will be differences in the immunoreactivity, affinity and avidity of these

PCs. For additional comparison when assessing assay sensitivity, monoclonal antibodies directed against the immunogenic portion of the drug may be used to compare sensitivity between the two assays.

It is important to note that while there are two assays being developed, attempts should be made to keep the assay conditions the same (i.e., washing buffer used, incubation times, incubation temperatures, concentration of capture and detection reagents, and so on). Depending on the format of the assay, the detection reagents may need to be conjugated. Conjugation should be performed using the same procedure and the same challenge ratio for both the innovator and the biosimilar. The ultimate goal is to develop two assays that are comparable in assay sensitivity, precision, specificity, linearity and drug tolerance.

■ Specificity & characterization of ADAs

The evaluation of the specificity of the ADA assay is important for the comparison of ADA results between the innovator and the biosimilar. Specificity of ADAs should be evaluated using competitive confirmatory assays utilizing both intact drugs (innovator and biosimilar) and a relevant specific domain of the drug. Additionally, potential crossreactivity to endogenous proteins should be considered if the drug contains an endogenous protein sequence [20,21].

ADAs should also be characterized to determine if they are a binding antibody or a neutralizing antibody by using, preferably, a cell-based assay. Additional ADA characterization should be considered within the larger context of the biosimilar testing program. A risk-based approach should be utilized. The type of ADAs detected should also be classified by its isotype if it is indicative of the possible severity of an immune response and indicative of potential safety risk (e.g., IgE isotypes can indicate a possible anaphylactic response).

If ADA response is detected, but no PK changes are observed, additional characterization of the positive response may not be necessary. If the effects of the ADA interference with the PK assessment are demonstrated differently between the innovator and the biosimilar, this would require additional investigation and risk mitigation.

While it is important to compare the incidence of positive response and to compare the characteristics of the ADAs (i.e., binding vs neutralizing, comparison of isotypes and so on) between the biosimilar and the innovator, it is more important to correlate the ADA results to clinical parameters that are relevant to the clinical risk–benefit assessment.

Future perspective

Robust and reliable bioanalytical assays are critical to establishing 'biosimilarity' between an innovator and biosimilar drug. While there are regulatory and industry guidances on the development of bioanalytical assays, they do not adequately address some of the critical aspects of the bioanalytical assays to support biosimilar drug development. For example, the acceptance limit for the inter-assay variability and total error allowable in these guidances may not be sufficient to support biosimilar development. It may require more stringent acceptance criteria for these parameters, so that the PK differences between the innovator and the biosimilar can be detected. It is recommended that all assay parameters and their acceptance criteria should be evaluated on a case-by-case basis and must be established in consultation with the pharmacokineticist and the statisticians, with appropriate scientific justifications.

Despite best efforts, it is inevitable that biologics manufactured using different processes will yield different products, which cannot always be fully characterized by currently available analytical methods. In some cases, even the different lots from same manufacturing process may be significantly different. This poses a significant challenge in establishing bioanalytical assays. A WHO or other international public reference standard, when available, should be used as an anchor reference calibrator for calibrating both the innovator and the biosimilar. Currently, there are very limited international standards available for biologics. Additional efforts from the international scientific community and collaborative studies with innovator companies are needed to establish international public reference standards for biologics.

Immunogenicity assays are generally not quantitative by nature. It is very challenging to establish similarity of immunogenicity profile based on qualitative assays and when two assays are used to measure ADAs against the innovator and the biosimilar. Analytical variability should be minimized, so that the immunogenicity profile for the innovator and the biosimilar can be sufficiently compared. Any detected differences between the innovator and biosimilar immunogenicity profile should be

assessed based on their relevance to the clinical outcome.

For drugs where a low incidence of ADA is expected, the size of the study may not be large enough to detect a statistically meaningful difference in immunogenicity. In addition, the relationship between the immunogenicity profile (i.e., incidence and magnitude of the ADA response) and the clinical outcome (i.e., safety and efficacy) is uncertain. A risk-based approach should be utilized when evaluating and comparing immunogenicity of a biosimilar to its innovator. Ultimately, postauthorization pharmacovigilance studies will provide more reliable evidence for biosimilarity to the innovator product, with respect to safety and efficacy [22].

In recent years, there have been significant efforts from the scientific community and regulatory authorities in the area of biosimilar development. These efforts are expected to help provide context and discussion around current industry practices to enable better understanding in this area. This article is intended to stimulate scientific discussions and contribute to a better understanding of development of bioanalytical assays to support biosimilars. Future experience with biosimilars will help develop industry best-practices, and enable safe and effective biosimilar development.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Compexity of biologics & biosimilars

- Biologic and biosimilars are large complex biomolecules produced from living cells. Manufacturing processes cannot be duplicated exactly, and small changes can result in a change in safety and efficacy.
- Special considerations must be given to the development of bioanalytical assays for use in biosimilar testing.

PK assay

- One PK assay should be used for both the innovator and the biosimilar, when possible.
- It is crucial to demonstrate biocomparability between an innovator and a biosimilar early in the method development.
- Calibrators should be prepared in either the innovator or the biosimilar. QCs should be prepared in both the innovator and the biosimilar.
- It is important to evaluate anti-drug antibodies (ADA) interference in PK assay, if ADAs are expected to be present in samples. If the effects of the ADA interference with the PK assessment are demonstrated differently between the innovator and the biosimilar, this would require additional investigation and risk mitigation.

Immunogenicity assay

- Two assays should be used for ADA determination – one specific for the innovator and one specific for the biosimilar, early in the biosimilar development. One assay may be utilized to measure ADAs against both products, if early development data shows that both products are comparable in terms of immunogenic profile.
- The ultimate goal is to correlate the ADA response with clinical observations, to use the overall data to evaluate the differences between the innovator and the biosimilar, and its impact on safety and efficacy.

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