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Research Article

Retrospective Analysis of Bioanalytical Method Validation Approaches in Biosimilar Biological Product Development

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Abstract. Development and validation of a bioanalytical method for biosimilar biological product development (BPD) can be challenging. It requires the development of a bioanalytical method that reliably and accurately measures both proposed biosimilar and reference products in a biological matrix. This survey summarizes the current state of bioanalysis in BPD. Bioanalytical data from 28 biosimilar biologic license applications submitted to U.S. Food and Drug Administration (FDA) up to December 2018 were analyzed. The aim of the analysis was to provide (i) a summary of the bioanalytical landscape for BPD, (ii) a cumulative review of bioanalytical method validation approaches to aid in understanding how a specific method was selected, and (iii) a summary of data regarding bioanalytical bias differences between products. Results show diversity of the bioanalytical approaches used, as well as the observed differences in bioanalytical bias. Our findings highlight the need for understanding the critical aspects of BPD bioanalysis and clarifying BPD bioanalytical best practices, which could help ensure consistent method validation approaches in the BPD community.

KEYWORDS: Biosimilar bioanalysis; Therapeutic biologics; 351(k) BLAs; Bioanalytical method comparability.

INTRODUCTION

Ligand binding assays (LBAs) are generally used for quantification of biologics. Common LBA platforms are the enzyme-linked immunosorbent assay (ELISA), Meso Scale Discovery (MSD), and a microfluidic-based platform named Gyrolab, in which at least one reagent besides the intended analyte is needed to quantify the analyte of interest (1). These reagents are commonly referred to as critical reagents, since the quality, specificity, and stability of such reagents can affect the method performance (1,2). The critical reagents frequently used in LBAs which specifically measure therapeutic biologics are as follows: (a) soluble proteins that therapeutic biologics are intended to bind, (b) monoclonal antibodies raised against therapeutic biologics, and (c) polyclonal antibodies raised against therapeutic biologics. Biosimilar biological development programs (BPD) are not expected to utilize the same bioanalytical methods, nor platforms, as those used in the reference product

Study Highlights

- Among twenty-eight 351(k) BLA submissions received up to December 2018, ELISA remains the most frequently used platform in BPD, likely due to the technology's simplicity. Since most reagents were commercially sourced in these submissions, it may be beneficial for bioanalytical laboratories to qualify and manage the life cycles of critical reagents and include critical reagent information sufficiently in method validation reports.
- Our survey indicated the diversity in conducting the bioanalytical comparability assessment among these submissions and the evidence corroborating the rationale for choosing the single bioanalytical method and the product was not evident. The bioanalytical bias differences between the products were larger than 10% in many cases.
- Evaluating the impact of bioanalytical bias difference on the outcome of PK similarity studies may be important.

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Abbreviations: *ARP*, authentic reference product; *Bias*, %difference from accuracy; *BLA*, biologic license application; *BPD*, biosimilar biological development program; *CRO*, contract research organization; *CV*, coefficient of variation; *ELISA*, enzyme-linked immunosorbent assay; *LBA*, ligand binding assay; *MSD*, Meso Scale Discovery; *PK*, pharmacokinetic; *QC*, quality control; *PBP*, proposed biosimilar product; *USRP*, US reference product; *WHO*, World Health Organization.

development program. This is because bioanalytical methods, including reagents and platforms used in the reference product development program, may not be available to biosimilar product developers. As technology changes and/or improves, critical reagents used in methods and the platforms can be quite different from those used in reference product development programs. Hence, laboratories can choose a bioanalytical method with any platform suitable for measuring different products for BPD. However, the FDA recommends using a single bioanalytical method to measure concentrations of the proposed biosimilar product and the reference product in pharmacokinetic (PK) similarity studies (3).

A chosen method should be validated to quantify both products consistently with the FDA's guidance for bioanalytical method validation (4). To select a single method, most sponsors follow a "two-staged, one-assay" approach recommended by Marini *et al*. (5). According to the authors'recommendations, comparing bioanalytical methods during method development could lead to a single method being used to quantitatively measure the proposed biosimilar product and the reference product during pre-study and in study method validations (5). As expected, validation of a single bioanalytical method that can accurately and precisely quantify at least two products leads to greater complexity (6,7). The selection criteria used in determining a single bioanalytical method during method development is unclear, and the data from method development are not included in method validation reports. In the context of BPD, all three types of critical reagents mentioned previously may have different binding characteristics to a biosimilar product, versus a reference product, while a high degree of cross-reactivity may exist. A slight variation in binding characteristics due to differences in binding affinity between the proposed biosimilar product and the reference product to the same critical reagents may lead to alterations in method performance. As the results, accuracy (presented as %bias or relative error) and precision (presented as inter-assay coefficient of variation (CV)) between multiple products may be different in a single bioanalytical method. In addition, LBAs normally have narrow dynamic ranges and those ranges may be truncated further when a method is validated to achieve better accuracy and precision (8). Also, the assay variabilities are often larger at the upper and lower ends of the quantification range. Therefore, it is important to thoroughly assess comparability of a bioanalytical method with multiple products—from lower limit of quantification to upper limit of quantification using both standard calibration curves and quality controls—prior to selecting a single method.

Between March 2015 and April 2019, 19 biosimilar products received FDA approval (https://www.fda.gov/drugs/biosimilars/biosimilar-product-information). The biologic license application (BLA) for these biosimilars contained one or more PK similarity studies, which contributed to the totality of evidence to support a determination of biosimilarity (9). It is important to note that the PK similarity assessment is highly dependent upon the quality of bioanalytical data for quantifying the circulating concentrations of the evaluated products in humans. Given the continued growth in the number of BPD programs and 351(k) BLA approvals, a survey of the bioanalytical method landscape in BPD programs was conducted. In this article, we present (i) a high-level summary of the bioanalytical landscape for BPD products, (ii) a cumulative survey of the approaches used for bioanalytical method validation, and (iii) observed typical data for bioanalytical bias and inter-assay CV between products.

METHODS

Bioanalytical Landscape in BPD

We examined the bioanalytical method landscape and validation information from 28,351(k) BLA submissions received by the FDA up to December 2018. Of 28 BLAs, 17 products were monoclonal antibodies, seven were recombinant proteins, and four were modified recombinant proteins. For each submission, we conducted the retrospective survey of bioanalytical methods used to quantify therapeutic biologics in a biological matrix. This survey collected information about the bioanalytical methods, including method validation reports (also termed pre-study validation reports), bioanalytical study reports (also termed in-study validation reports), and any respective amendments used in the quantification of therapeutic biologics in a biological matrix. Information collected from each method validation package included: bioanalytical sites where method validations and PK similarity studies were conducted, assay platforms, critical reagent types and their sources, method validation approaches conducted for each validation parameter, and information about how a single bioanalytical method was selected.

Bioanalytical Method Comparability Assessment in BPD

Bioanalytical method comparability assessment in this context is defined as the comparison of both standard curve calibrators (STDs) and quality controls (QCs) prepared from all products, as well as the concentration of QCs prepared from all products, which are then back-calculated against each standard curve calibrator. Complete assessment of bioanalytical method comparability generally involves three parts: (1) comparison of performance of STDs prepared from all products, (2) analysis of the mean bias of back-calculated QCs (prepared from each product) from each STD, and (3) comparison of bias differences between the QCs of all products. To address whether the method validations included adequate bioanalytical method comparability assessment, we documented the approach used to determine comparability from each submission. First, our survey sought to understand whether the comparison of standard curve calibrators prepared from all products was included in the method validation study. Next, we collected information on any additional data that may have been obtained using the standard curve calibrators prepared from each product and/or if an overlay of standard calibration curves was included in the method validation reports or elsewhere in the submission. For those BPD programs that only conducted a comparison assessment using QCs from each product (e.g., the proposed biosimilar product, the US-licensed reference product, and when applicable, a third comparator product), we gathered information on the processes each laboratory used to conduct the bioanalytical method comparability assessment for QC. We also examined whether all products included in PK similarity studies were included in such validation parameters as selectivity, stability, and dilution linearity.

Bioanalytical Bias and Inter-Assay %CV Difference Between Products

For those BPD programs that included QCs from different products (e.g., proposed biosimilar products, US-licensed reference products, and non-US-licensed comparator products) during

accuracy and precision assessment, we collected data on mean inter-assay bias and inter-assay precision (%CV) for each QC level of each product. Here, bioanalytical bias refers to the percentage of inter-assay bias from the nominal concentration or relative error (%RE) in the back-calculated concentration of the QC samples obtained from the accuracy and precision assessment. The bioanalytical %CV refers to the percentage of inter-assay precision in the back-calculated concentrations of the QC samples, also obtained from the accuracy and precision assessment.

Observed mean biases for each QC were subsequently used to compute an absolute bioanalytical bias difference between the products, using the equation below:

$$\%\text{bioanalytical bias difference} = |\,\text{Product 1 mean}\%\text{bias at level 1} - \text{Product 2 mean}\%\text{bias at level 1}\,|$$

The observed mean inter-assay CV for each QC was used to compute the absolute %CV difference between the products, using the equation below:

$$\%\text{CV difference} = |\,\text{Product 1}\%\text{CV} - \text{Product 2}\%\text{CV}\,|$$

All analyses were conducted in R 3.5.1 (10) and used the package "ggplot2" (11).

RESULTS

Bioanalytical Landscape in BPD up to December 2018

A summary of the bioanalytical method landscape and validation information collected from twenty-eight 351(k) BLA submissions is shown in Figs. 1 and 2. The bioanalytical method validations or analysis of study samples collected from the PK similarity studies were conducted by one or more contract research organizations (CROs). In all 351(k) BLAs submitted up to December 2018, three LBA platforms were employed, including 23 colorimetric-based ELISAs, four MSDs, and one Gyrolab (Fig. 1a).

Since critical reagents in LBAs could play a significant role in assessing the bioanalytical method comparability between different products, we sought to understand the type of critical reagents used and sources of these reagents in BPD. Among the 28 methods described above, 86% of capture reagents and 82% of detection reagents were commercially acquired; while 18% of the detection reagents were custom-made and labeled with specific detection agents (Fig. 1b, c). Furthermore, 61% (17 of 28) had detection reagents that were specific to the analytes being measured; however, 11 of those 17 methods used detection reagents targeted against the Fc region of analytes, such as anti-human IgG antibody, if the intended analytes were either monoclonal antibodies or recombinant proteins with a portion from IgG.

Bioanalytical Comparability Assessment in BPD

Next, we determined how the bioanalytical method comparability assessment would be performed. Figure 2a shows the distribution of products used in preparation of standard calibration curves for the method validation studies.

Only two method validation studies included the comparison of standard calibration curves and QCs prepared from more than one product. The remaining studies ($n = 26$) conducted method validation with standard calibration curves prepared from only one product (Fig. 2a). Method development data showing the bioanalytical comparability were neither presented in method validation reports nor provided as supporting documents in all but one submission. In all other cases, the selection criteria used in determining a single PK method during method development is unclear. Among those 26 method validation studies, 50% ($n = 13$) of the laboratories selected the proposed biosimilar products (PBPs) to prepare standard calibration curves, followed by 19% ($n = 5$) for US-licensed reference products (USRPs), 15% ($n = 4$) each for authentic reference products (ARPs, e.g., WHO reference materials) and non-US-licensed comparator products (Fig. 2b). These numbers indicate that PBPs were the primary product choice in preparing standard calibration curves for bioanalytical method validation studies.

In most method validation studies, the standard calibration curves prepared from one product were used to conduct the comparison of five levels QCs prepared from all products. Among the 28 method validation packages, 68% ($n = 19$) of method validation studies included the comparison of five QC levels

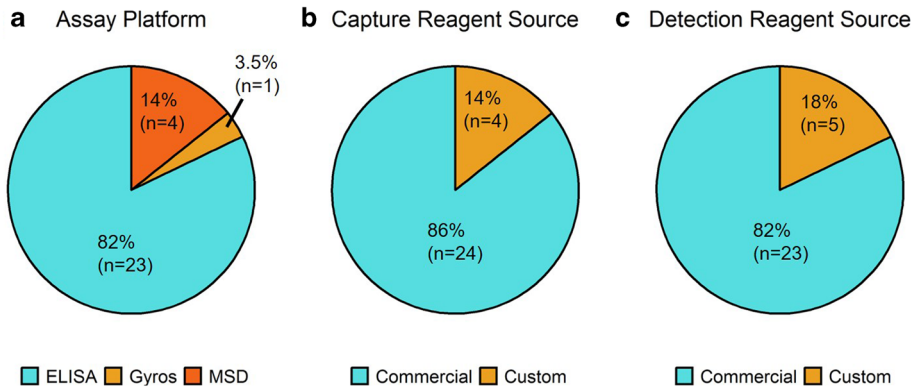

Fig. 1. Survey of bioanalytical landscapes and information in 351(k) BLAs submitted up to December 2018 ($n = 28$ methods). **a** Assay platforms, **b** source of the capture reagents, and **c** source of the detection reagents

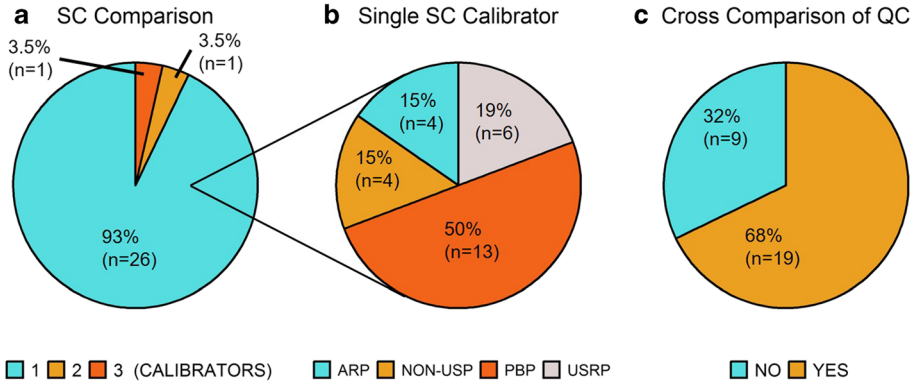

Fig. 2. Summary of bioanalytical method comparability assessment. **a** number of products used to prepare standard calibration curves during accuracy and precision assessment ($n = 28$). **b** Product type used in standard calibration curves ($n = 26$). **c** Cross-comparison of quality control samples ($n = 28$) prepared with at least two products. SC, Standard calibration curve; ARP, Authentic reference product such as WHO reference material; non-USP, non-US-licensed comparator product; USRP, US-licensed reference product; PBP, Proposed biosimilar product; QC, quality control samples

prepared from all products included in their PK similarity studies, while 32% ($n = 9$) comprised neither the comparison of standard calibration curves nor QCs (Fig. 2c). In one case, only three QC levels were used during the accuracy and precision assessment in the method validation (data not shown).

In two different method validation studies that compared standard calibration curves prepared from more than one product (Fig. 2a), the overlay figures representing standard calibration curves, comparing the instrument responses against the concentration of products, provide evidence of comparability of standard calibration curves among products. However, the overlay figure representing standard calibration curves prepared from two or more products was not provided in method validation reports, even though the comparison of standard curve calibrators prepared from all products was conducted during pre-study method validation studies in both cases. The results of our survey on data analysis and bioanalytical method validation approaches used are summarized in Table 1. In six BLA submissions, a figure representing overlay standard calibration curves was presented in an electronic common technical document. In this case, the data were likely collected during method development.

Of 28 method validation packages, 79% included all the products in the assessment of other method validation parameters. The results summarized in Fig. 2 and Table 1 show the diverse approaches used in assessing bioanalytical method comparability between different products during BPD method validation studies.

Bioanalytical Bias and Inter-Assay %CV Difference Between Products

We next examined the bioanalytical inter-assay bias and %CV of QCs from each individual product observed during the accuracy and precision assessments. According to the guidance for industry on bioanalytical method validation (4), a limit of 20% is set for the bias and %CV allowed for individual products used in a study. Each product included within the same submission had bioanalytical bias and %CV that were within the 20% range. However, the data showed the bioanalytical errors could vary between the products with respect to bioanalytical bias. Three example BLAs are presented in Fig. 3 illustrating the mean bioanalytical bias of individual products (products 1 and 2) and the bioanalytical bias difference between two products. Individually, each product in each BLA had a mean bioanalytical bias of < 20% at all five QC levels (Fig. 3a–c), meeting the FDA's regulatory bioanalytical bias requirement of < 20% (4).

We found that when evaluating the bioanalytical bias difference between products within a BLA, the difference exceeded 10% in at least one QC level in two of the three BLAs (red line in Fig. 3a–c). Each example shows different patterns of bioanalytical bias difference along the validated assay range. Given this observed significant bioanalytical bias difference between products, we proceeded to compute the bioanalytical bias difference at each QC level, comparing between products for all 28 method validation packages. Among the 28 method validation packages, 19 included comparison of QCs prepared from at least two products, the US-licensed reference product and the proposed biosimilar product. Only data from 18 of those 19 method validation packages were ultimately used for our cumulative analysis, since one method validation package included only three QC levels and did not compare the QCs prepared from different products. Among these 18 method validation packages, 14 included the comparison of QCs prepared from three products; the US-licensed reference product, the proposed biosimilar product, and a non-US-licensed comparator product to support three-way PK similarity studies. Four of these packages contained the comparison of QCs prepared from two products to support two-way PK similarity studies. Figure 4 shows a pairwise comparison of five QC levels prepared from two different products.

The bias differences between a US-licensed reference product and proposed biosimilar product, between a non-US-licensed comparator product and a proposed biosimilar product, and between a US-licensed reference product and a non-US-licensed comparator product are presented in Fig. 4a–c, respectively. Bioanalytical bias differences between the proposed biosimilar products and their respective US-licensed reference products was less than 10% in half of those BLA cases (9 of 18). However, 27% of these BLA cases (5 of 18) had bioanalytical bias differences greater than 15% in at least one QC level (Fig. 4a), indicating larger bioanalytical bias differences. When comparing the

Table 1. Survey of Bioanalytical Method Validation Approaches Used in BPD as of December 2018. A Total of 28 Method Validation Studies Were Surveyed

	Yes	No
If the standard calibrator comparison was conducted (as in Fig. 2a), were the overlay standard curves from all products used in PK similarity provided?	21% (6/28)[a]	100% (2/2)[a]
Were all products used in PK similarity studies included in validation parameters, such as selectivity, stability, and dilution linearity?	79% (22/28)	21% (6/28)

[a] Of these six method validation packages, two included more than one product for preparing standard calibration curves, but overlay standard calibration curves for only one product was shown

bioanalytical bias differences between proposed biosimilar products and their respective non-US-licensed comparator products, 47% of those BLA cases (7 of 15) were less than 10%, and 33% (5 of 15) had bioanalytical bias differences greater than 15% in at least one QC level (Fig. 4b). In fact, two had bioanalytical bias differences greater than 20% in at least one QC level. When comparing the bioanalytical bias differences between non-US-licensed comparator products and their respective US-licensed reference products, 25% of those BLA cases (3 of 12) had bioanalytical bias differences greater than 10% in at least one QC level (Fig. 4c), and only one of the comparisons had bioanalytical bias differences greater than 15%.

We also examined whether there were any differences in inter-assay imprecision between products (Fig. 5a–c). Inter-assay %CV between different products was mostly < 10% in all comparisons. These data indicate that, in most cases, the bioanalytical bias difference between products, versus the inter-assay CV, contributes more to total bioanalytical error differences.

DISCUSSION

Validation of a bioanalytical method that quantitatively measures both proposed biosimilar and reference products in a BPD can be an arduous task (4,7). Several issues, such as differential immunoreactivity of the critical reagents used in the bioanalytical methods to quantify both products, quality of critical reagents, assay platforms, and limited information about the innovator product may confound this effort. While a comparable bioanalytical method performance between different products is desirable during pre-study and in-study method validation studies, the abovementioned issues can challenge many bioanalytical scientists. Our regulatory research highlights these challenges and summarizes the diverse approaches used in the field of biosimilar bioanalysis as of December 2018.

Several perspectives on summary surveys are noteworthy. First, the ELISA remains the most frequently-used platform in BPD, likely due to the technology's simplicity. This is expected, since LBAs provide an efficient, effective, and specific means of quantifying protein analytes in various matrices. Second, most reagents were commercially sourced. While the use of commercial reagents may allow for consistent use of method formats across various CROs for proposed biosimilar products with the same mechanisms of action, bioanalytical laboratories can benefit from understanding the reagent quality and lot-to-lot variability. On the other hand, custom-made reagents may provide additional benefits, as these are known for specificity and selectivity for the products being measured. Thus, it may be beneficial for bioanalytical laboratories to qualify and manage the life cycles of critical reagents and include critical reagent information in method validation reports, since sources and types of reagents utilized in bioanalytical methods may also impact method performance and reproducibility.

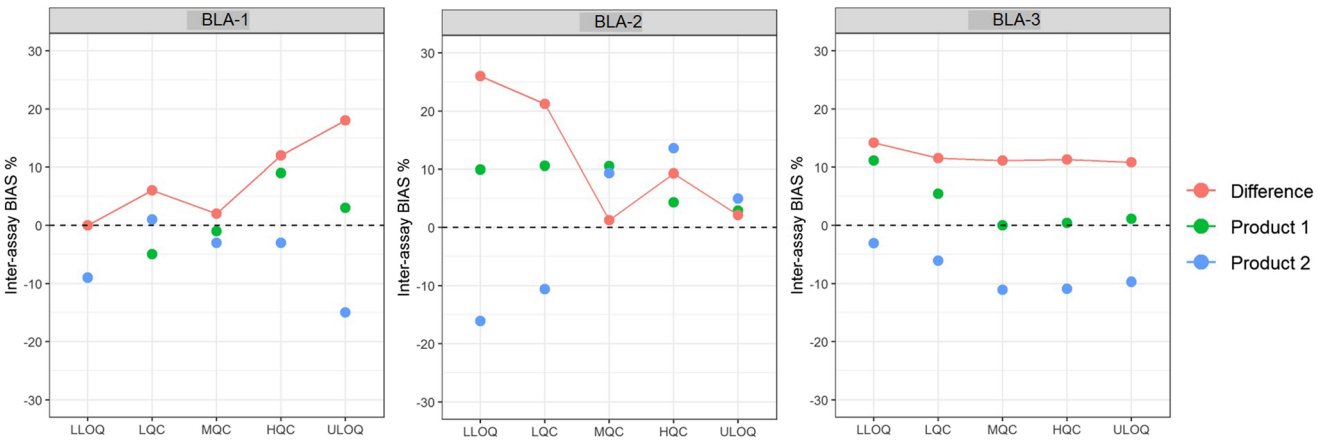

Fig. 3. Three prototypical 351(k) BLA submission examples in which significant differences in bioanalytical bias between products used in the PK similarity study were observed. Green and blue circles indicate the bioanalytical bias of each product from lower limit of quantification to upper limit of quantification. Red circles indicate the absolute difference in bioanalytical bias, with the red line showing the patterns of absolute bias difference

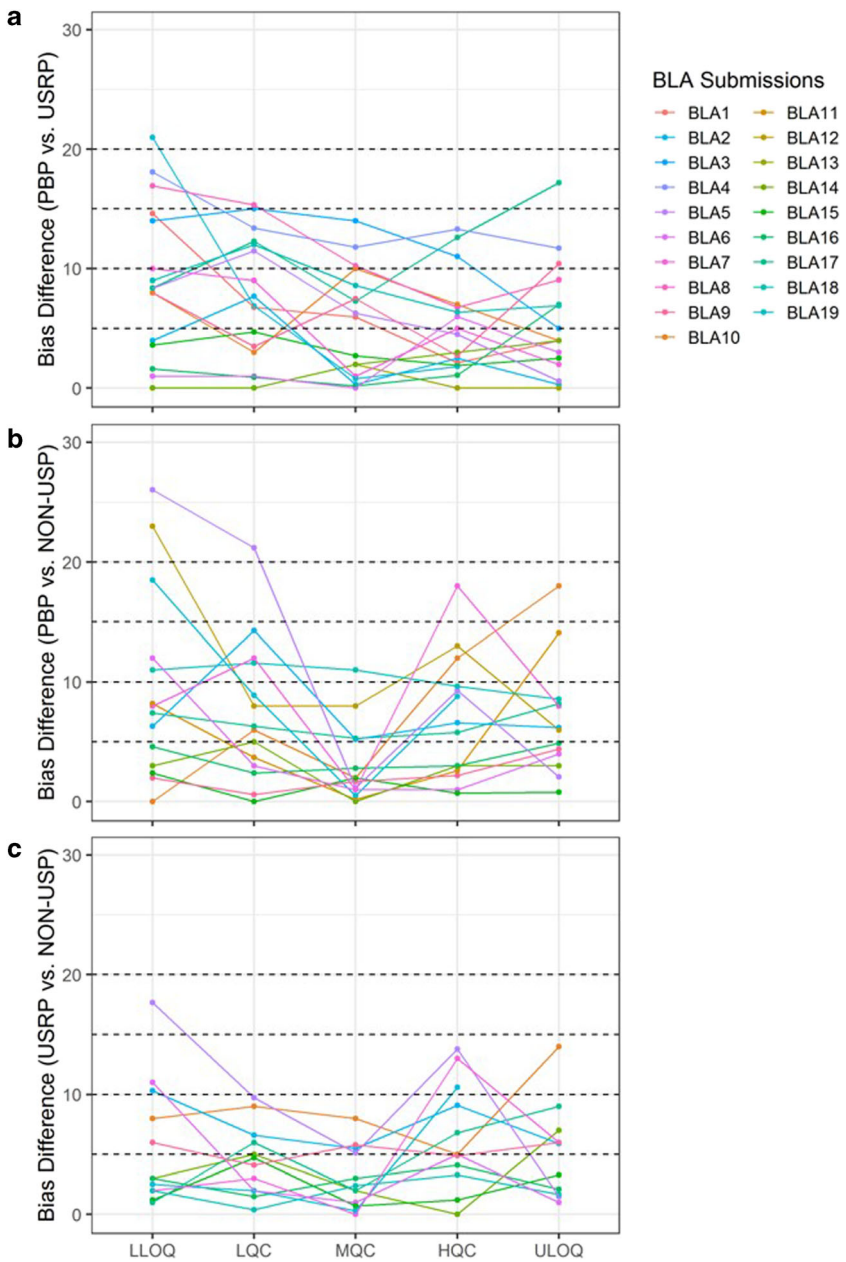

Fig. 4. Bioanalytical bias difference comparing two products; **a** between US-licensed reference product (USRP) and proposed biosimilar product (PBP), **b** between non-US-licensed comparator product (NON-USP) and proposed biosimilar product, and **c** between US-licensed reference product and non-US-licensed comparator product

In this article, we summarized the various approaches used in method validations for 28,351(k) BLAs received up to December 2018. Since such bioanalytical methods are always used in the bioanalysis of PK similarity studies, they play a critical role in generation of concentration data in assessment of PK similarity. This PK similarity data adds to all data and information used to support a demonstration of biosimilarity between a proposed biosimilar product and the US-licensed reference product. Furthermore, the impact of the variation in the method performance can hamper the ability of the PK similarity study results to support a conclusion that there are no clinically meaningful differences between the two products. The bioanalytical method performance characteristics for the reference and biosimilar products are theoretically expected to be similar if the two products are shown to be similar analytically. However, the presence of a matrix effect may impact the bioanalytical method's performance characteristics between the reference and biosimilar products.

Therefore, conducting the bioanalytical comparability assessment provides granularity in understanding the degree of similarity in bioanalytical method performance between different products. These bioanalytical method comparability assessments across products provide scientific justification and supportive data in selecting a single method for quantification of protein therapeutic concentrations collected from PK similarity studies. In many of the cases we surveyed, the evidence corroborating the rationale for choosing the single bioanalytical method and the product was not evident. For

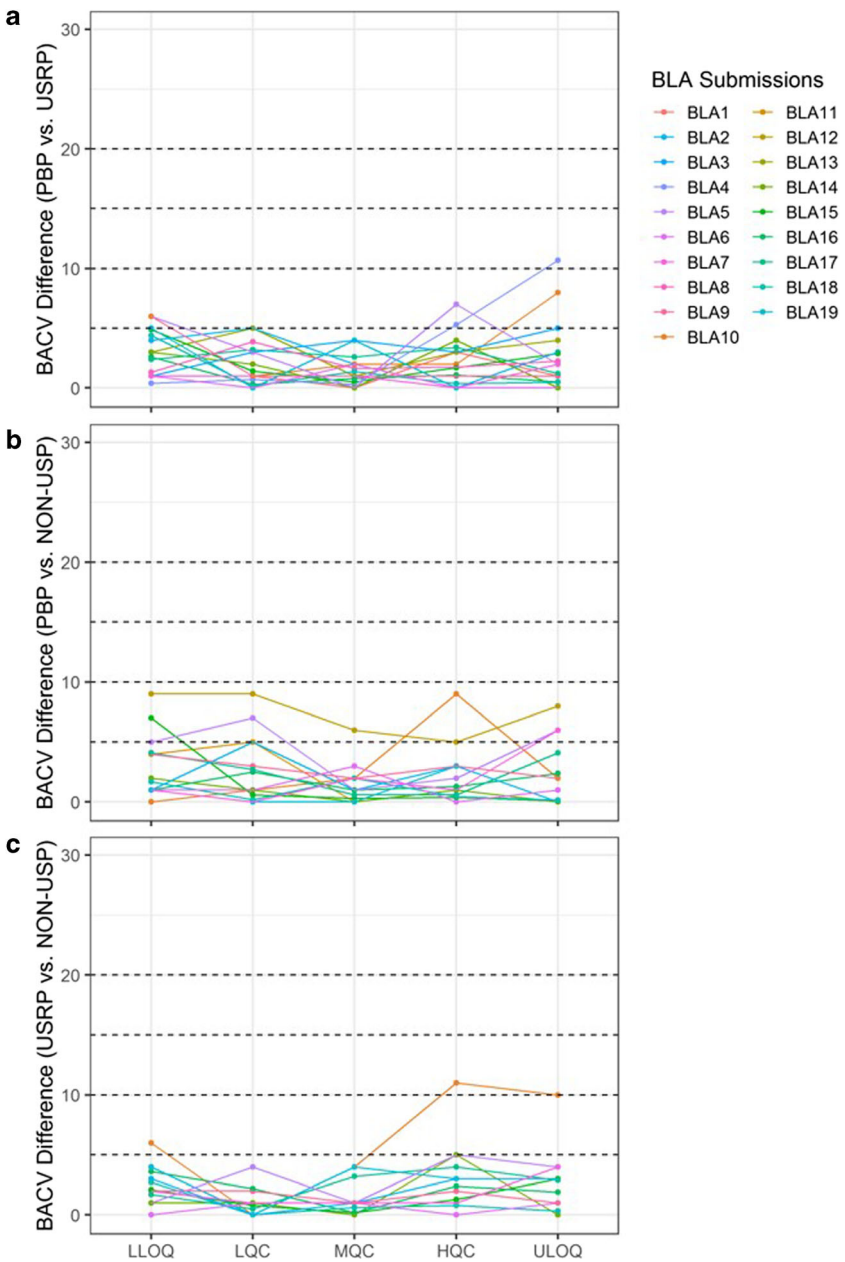

Fig. 5. Bioanalytical difference for inter-assay imprecision between products; **a** between US-licensed reference product (USRP) and proposed biosimilar product (PBP), **b** between non-US-licensed comparator product (non-USP) and proposed biosimilar product, and **c** between US-licensed reference product and non-US-licensed comparator product

example, in most cases, the assessment of bioanalytical comparability using the STDs prepared from different products was not conducted. In addition, assessing bioanalytical comparability using QCs prepared from different products was not performed in 32% of the method validation packages. Not performing bioanalytical comparability assessment using neither STDs nor QCs offers no advantage in BPD because biases and %CV may still exist along the validated range, especially in analyzing the other products that were included in PK similarity studies. Furthermore, bioanalytical method comparability assessments are critical in justifying the selection of a single bioanalytical method and the success of PK similarity assessment.

The various bioanalytical method issues we highlighted may ultimately be related to total bioanalytical errors of inaccuracy and imprecision. An individual product may have an inherent bias in a bioanalytical method. However, in the case of a BPD with at least two products being compared, the understanding of bioanalytical bias difference between two products is important. Our survey of the 18 biosimilar BLAs showed that the bioanalytical bias differences between the products were larger than 10% in many cases, while bioanalytical imprecision between different products remained less than 10% in most comparisons. As what has been previously reported, that bioanalytical bias difference between two formulations can be a significant factor in

meeting PK comparability criteria (12). The absolute difference between the biases of two formulations impacts the propensity for studies to fail the biocomparability assessment (12). Therefore, large bias differences observed bioanalytically between products may impede the ability to demonstrate PK similarity. Evaluating the impact of bioanalytical bias difference on the outcome of PK similarity studies may be important.

In conclusion, given the diversity in bioanalytical approaches used and bioanalytical bias differences observed, a need exists for continued understanding of the critical aspects of BPD bioanalysis. The observations in this survey also indicate knowledge gaps in the field of biosimilar bioanalysis that should be addressed. Ample opportunities are available for clarifying bioanalytical best practices for BPD. We plan to further contribute to this scientific area by developing our perspective on these issues based on our regulatory review experience to date, with the goal of informing and promoting best practices for biosimilar bioanalysis.

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