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Experimental and statistical approaches in method cross-validation to support pharmacokinetic decisions

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ABSTRACT

A case study of experimental and statistical approaches for cross-validating and examining the equivalence of two ligand binding assay (LBA) methods that were employed in pharmacokinetic (PK) studies is presented. The impact of changes in methodology based on the intended use of the methods was assessed. The cross-validation processes included an experimental plan, sample size selection, and statistical analysis with a predefined criterion of method equivalence. The two methods were deemed equivalent if the ratio of mean concentration fell within the 90% confidence interval (0.80–1.25). Statistical consideration of method imprecision was used to choose the number of incurred samples (collected from study animals) and conformance samples (spiked controls) for equivalence tests. The difference of log-transformed mean concentration and the 90% confidence interval for two methods were computed using analysis of variance. The mean concentration ratios of the two methods for the incurred and spiked conformance samples were 1.63 and 1.57, respectively. The 90% confidence limit was 1.55–1.72 for the incurred samples and 1.54–1.60 for the spiked conformance samples; therefore, the 90% confidence interval was not contained within the (0.80–1.25) equivalence interval. When the PK parameters of two studies using each of these two methods were compared, we determined that the therapeutic exposure, $AUC_{(0\text{-}168)}$ and C_{max}, from Study A/Method 1 was approximately twice that of Study B/Method 2. We concluded that the two methods were not statistically equivalent and that the magnitude of the difference was reflected in the PK parameters in the studies using each method. This paper demonstrates the need for method cross-validation whenever there is a switch in bioanalytical methods, statistical approaches in designing the cross-validation experiments and assessing results, or interpretation of the impact of PK data.

1. Introduction

Few pharmacology studies are typically conducted to investigate the pharmacokinetic (PK) parameters during the lead optimization period in the development of a therapeutic biologic. Limited time and availability of reagents during this phase often constrain the initial method so that only readily available reagents are used. Later, methods may be refined after the availability of anti-idiotypic antibodies. Thus, it is common to apply multiple ligand binding assay (LBA) methods to quantify therapeutic biologic concentrations in various preclinical studies. During pilot studies, it is not uncommon to use methods that are not fully validated (i.e., they are qualified with less rigorous validation experiments [mini-validation]) based on levels of accuracy and precision, and minimal stability [1–4]. In contrast, a refined method would be fully validated [1–4]. Method comparison experiments are usually performed during the transition from pilot studies. Before conducting these experiments, goals of cross-validation should be determined based on the need to compare the results generated from two methods, each from a mini-validation and a full validation.

Cross-validation is a comparison of two or more methods that are used to generate data within the same study or across different studies [5,6]. The Conference Report on Bioanalytical Method Validation – A Revisit with a Decade of Progress was published in 2000 and provided a guideline for performing cross-validation when two or more bioanalytical methods are used to generate data within the same study. An original validated bioanalytical method is considered as "reference" and the revised method is the "comparator." On the regulatory side of therapeutic development, the Guidance for Industry on Bioanalytical Method Validation issued by the FDA clearly states the requirement for cross-validation in the following scenarios: (1) when sample analyses within a single study are conducted at more than one site (cross-validation is required in addition to partial-validation) and (2) when data

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generated using different analytical techniques (e.g., LC/MS–MS vs. LBA) in different studies are included in regulatory submissions [5,6]. Nonetheless, there has been no further guidance on cross-validation of methods or on data interpretation. It is not clear to many bioanalytical laboratories whether the changes in capture reagents and/or detection systems represent "major" changes that would require cross-validation, and these issues have not been adequately addressed in the literature [6]. Some statistical approaches on method comparison between original and new methods have been discussed for daily clinical/diagnostic use; however, there have been no publications providing experimental or statistical discussion about pharmaceutical use intended to support PK and/or toxicokinetic (TK) assessments [6,8]. In this paper, we present a case study on cross-validation of two similar LBA differing in the capture reagents and the detection systems. The cross-validation concept was extended to evaluate the equivalence of two LBA methods with different platforms rather than the different analytical techniques using a variance analysis statistical approach.

2. Methods

2.1. Validation plan and documentation

Cross-validation of two LBA methods is often conducted during method validation of the more recent method. A standard operating procedure would typically be followed if there were one in place. Alternatively, a cross-validation plan can be written before the initiation of the cross-validation experiments. We prepared an *a priori* cross-validation plan, which included a detailed background of the methods, experimental design, and selection of test sample sizes for method comparison, as well as restrictive assay conditions to minimize the random error, *a priori* acceptance criteria for bioanalytical equivalence, and a description of the statistical analysis to be performed.

2.2. Bioanalytical methods

Two chronological methods were developed to support PK studies at different stages of drug development. Method 1, applied to an early study, was a chemiluminescence-based LBA in which the therapeutic target protein was used as the capture reagent. Method 2 was a colorimetric enzyme linked immunosorbent assay (ELISA) in which a monoclonal anti-therapeutic antibody was used as a capture reagent. The same detector reagent, horseradish peroxidase (HRP)-conjugated mouse anti-human antibody specific to Fc, was used in both methods. Method 2 was fully validated to support a regulated study according to the FDA guidelines [5] while method 1 was qualified (i.e., mini-validation).

Method 1 Procedure: Microplate wells were coated with biotinylated therapeutic target protein (Amgen Inc., CA). After blocking with 1X PBS with 1% BSA (blocking buffer), standards (STD), quality controls (QC), conformance-spiked samples (made by spiking therapeutic antibody into 100% Cynomolgus monkey serum), blank, and incurred samples were loaded into the wells after pre-treatment at 1:5 with blocking buffer. The therapeutic antibody present in the STD, QC, and samples bound to the immobilized mouse therapeutic target protein. After a wash step, a horseradish peroxidase (HRP conjugated mouse anti-human Fc antibody specific to Fc (Amgen Inc., CA) was added to the wells. The detector antibody was bound to the therapeutic antibody captured in the previous step. After a final wash step, a Pico peroxide substrate (Pierce, Inc. IL) was added to the well. The Pico substrate reacted with HRP and produced a light signal that was proportional to the amount of therapeutic antibody bound by the capture reagent. The intensity of the light output (relative light unit, RLU) was measured using the Molecular Devices Spectra LMAXII 384 Luminometer equipped with SOFTmax Pro software. The conversion of RLU for QC and study samples to concentration was achieved through a computer software mediated comparison to a standard curve assayed on the same plate, which was regressed according to a logistic (Auto-Estimate) regression model with a weighting factor of $1/Y$ using the Watson data reduction package.

Method 2 Procedure: Microplate wells were coated with mouse a monoclonal anti-therapeutic antibody (Clone No. Ab 1.9.1, Amgen Inc., CA). After blocking with 1X PBS with 1 M NaCl, 1% BSA and 0.5% Tween 20 (blocking buffer), STD, QC, conformance-spiked samples (made by spiking therapeutic antibody into 100% Cynomolgus monkey serum), blank, and incurred samples were loaded into the wells after pre-treatment at 1:25 with blocking buffer. The therapeutic antibody present in the STD, QC and samples bound to the immobilized anti-therapeutic antibody. After a wash step, an HRP-conjugated mouse anti-human Fc antibody specific to Fc (Amgen Inc., CA) was added to the wells. The detector antibody bound to the therapeutic antibody captured during the previous step. After a final wash step, a tetramethylbenzidine (TMB) peroxide substrate solution (KPL Inc., MD) was added to the wells. TMB in the substrate solution reacted with the peroxide and, in the presence of HRP, produced a colorimetric signal that was proportional to the amount of therapeutic antibody bound by the capture reagent in the initial step. The color development was stopped by acidification and the intensity of the color (optical density, OD) was measured at 450 nm minus 650 nm using a Molecular Devices Spectra max 340PC microtiter plate reader equipped with SOFTmax Pro. The conversion of OD units for the validation samples and the QC to concentration was achieved through a computer software mediated comparison to a standard curve assayed on the same plate, which was regressed according to a logistic (Auto-Estimate) regression model with a weighting factor of $1/Y^2$ using the Watson data reduction package.

A summary of the method formats is described in Table 1, and assay acceptance criteria for each method are listed in Table 2.

2.3. Statistical methods/approaches

2.3.1. Sample size selection

The statistical estimation for sample size described in Table 3 was generated using nQuery Advisor 5.0 sample size determination software from Statistical Solution Inc. (Saugus, MA). Sample sizes were generated for different precision (computed as %CV) and equivalence intervals assuming no initial bias between the methods. Table 3 was used to determine the test sample size in cross-validation of two methods based on the inter-assay precision in relation to the desired equivalence interval. A common practice of equivalence interval of 80–125 was used to determine if two methods were equivalent within 90% confidence interval. If the imprecision of the 2 methods is different, the higher precision of the two methods was used to determine the number of samples to be analyzed. Based on the inter-assay precision for Method 1 and desired equivalence interval of 80% to 125%, 30 samples were tested.

2.3.2. Equivalence analysis for methods 1 and 2

All data analysis was performed using SAS V9.1.3 on a Windows Professional operating system. An analysis of variance with terms for sample and assay was performed on the log responses. A 90% equivalence confidence interval was computed for the ratio of the different estimated concentrations using an estimate statement in the log-transformed value analysis, computing a confidence interval of the differences, and back-transforming (exponentiation) the difference and confidence interval to get the ratio and its confi-

Table 1
Method summary.

	Method 1	Method 2
Coating reagent	Biotinylated recombinant mouse receptor	Mouse anti-therapeutic X monoclonal antibody
Detection reagent	Anti-human IgG Fc-HRP	
Detection mechanism	Chemiluminescence	Colorimetric
Pre-treatment	1:5	1:25
Format	Heterogeneous	
Standard curve fit	4 Parameter logistic	
Weighting factor	$1/Y$	
Assay range (ng/mL)	0.977–500	0.25–20
Pre-study assay characterization	Qualification (3 runs)	Validation (7 runs)

Table 2
Assay performance during validation and qualification and acceptance criteria.

		Method 1	Method 2
		Qualification result from 3 runs	Validation result from 7 runs
Quality controls	%Bias	4–15	−8 to −1
	Intra-assay precision	≤2	≤8
	Inter-assay precision	≤6	≤13
	Assay acceptance criteria		
Quality controls	4- 6- X, X % from nominal	≤25	≤20
Samples	Precision of instrument response	≤25	≤15

X represents the total error (%bias + inter-assay %CV) according to FDA guideline for that measurement which comprises of both the systematic bias and the random error.

dence limit. Means, standard deviations, and ratios of the geometric means of the assayed values were generated.

2.4. Bioanalytical cross-validation experimental design

Standards and quality controls (QC) for both methods were prepared using the same lot of test article and same pooled lot of Cynomolgus monkey serum. The accuracy and precision of standards and QC preparations were verified by running an assay in their respective methods before cross-validation.

2.4.1. Experiment 1 for LBA comparison

Equivalence analysis was performed using 30 incurred samples from a pharmacokinetic study. Samples were randomly selected excluding those with anti-therapeutic antibodies. The concentration ranged from 2.7 to 96 ng/mL with 20 samples at <6 ng/mL and 10 samples between 6 and 96 ng/mL. The sample identities were blinded and then provided to the analyst with designated dilution factors for each sample required for method 2. Samples with the reported biological therapeutic concentration less than the upper limit of quantification (ULOQ) of method 2 at 20 ng/mL were analyzed neat (undiluted), while those with reported biological therapeutic concentrations greater than ULOQ were diluted to 1:10 in the matrix before analysis. All 30 samples were analyzed neat in method 1 in the same manner as previous samples.

2.4.2. Experiment 2 for LBA comparison

Equivalence analysis was performed using 30 conformance (spiked) samples. The nominal concentrations ranged from 1.5 to 200 ng/mL and were prepared by spiking the therapeutic antibody into the standard matrix. The sample identities were blinded and provided to the analyst with the appropriate dilution factor for each sample required for method 2. Similar to incurred samples, samples with the reported therapeutic concentrations less than 20 ng/mL were analyzed neat (undiluted) in method 2, while those with the reported therapeutic concentrations greater than 20 ng/mL were diluted to 1:10, 1:50 or 1:100 in the matrix before analysis. All 30 samples were analyzed neat in method 1.

Additional restrictive assay conditions in the validation plan included the analysis of both incurred samples and/or the conformance samples on the same day by the same qualified analyst for each method.

2.5. PK data assessment

The objective was to compare PK data from Study A and B, which used method 1 and 2, respectively. PK parameters were derived from a noncompartmental analysis using WinNonlin (Enterprise version 5.1.1). Area under the curve up to 168 h ($AUC_{(0\text{-}168)}$) and maximal concentration (C_{max}) from Study A and B were compared.

Table 3
Sample size selection for test samples in relation to assay imprecision and required equivalence interval.

Inter-assay average CV**	Sample size (N) in relation to equivalence interval		
	Equivalence interval (80–125)	Equivalence interval (85–118)	Equivalence interval (90–111)
5%	4	6	8
7.5%	6	8	14
10%	8	10	22
12.5%	10	16	34
15%	12	20	46
17.5%	16	28	62
20%	20	34	82
22.5%	24	44	102
25%	30	52	126

** For sample size selection, largest inter-assay %CV between two methods was used.

3. Results

3.1. Assay performance for qualification and validation

To validate method 2, a total of 7 accuracy and precision runs were performed using 5 levels of quality control (QC) samples at nominal concentrations of 0.25, 0.75, 6.5, 15 and 20 ng/mL. To qualify Method 1, a total of 3 accuracy and precision runs were performed using 3 levels of QC samples at nominal concentrations of 2, 20 and 200 ng/mL. Assay ranges were 0.977–500 ng/mL and 0.250–20 ng/mL for methods 1 and 2, respectively. The assay performance determined during validation or qualification is listed in Table 2. Run acceptance criteria were set according to the guidelines [3,7].

3.2. Assay performance for method comparison

A total of two runs, one for incurred sample and another for conformance samples, were performed. Each run consisted of a standard curve and two sets of QCs, each at 3 levels. QCs from method 1 had a bias ranging from 14% to 24% between 3 QC levels, whereas QCs from method 2 had a bias ranging from −4% to 9% (Table 4). The imprecision among four replicates (two from each run) was less than 7% CV for both methods (Table 4).

3.3. Equivalence analysis in incurred and conformance samples

Analysis of variance was performed to compare the two sets of incurred and conformance sample data determined from method 1 and method 2 and to compute confidence intervals of the ratios. The observed concentrations are shown in Fig. 1A for incurred samples and 1B for conformance samples. The geometric mean concentrations were 23.4 and 14.3 for incurred and spike conformance samples, respectively. The mean concentration ratio of method 1 to method 2 was 1.63, and the 90% confidence interval was (1.55, 1.72). Therefore, the 90% confidence interval is not contained within the (0.80, 1.25) equivalence interval (Table 5). The result of the equivalence analysis for the incurred samples showed that there were significant differences between the two methods ($p < 0.0001$ for all samples, $N = 30$).

The results of the equivalence analysis for the conformance samples also showed that there were significant differences between the two methods ($p < 0.0001$ for all samples, $N = 30$). The geometric mean concentrations were 43.9 and 27.9 for method 1 and 2, respectively. The ratio of geometric mean concentration between the two methods was 1.57 and the 90% confidence interval was (1.54, 1.60). Therefore, the 90% confidence interval is not contained within the (0.80, 1.25) equivalence interval. The results of the equivalence analysis for both groups of samples showed that there were significant differences between two methods ($p < 0.0001$ for all samples, $N = 30$).

3.4. Dilution effect assessment

Since the dynamic range of method 2 is narrower than that of method 1, dilution was required to bring high-concentration samples into the working range. Therefore, the dilution effect was examined to determine whether it was the cause of non-equivalence. The results in Fig. 2 show that there was no significant difference in the ratios of method 1 to method 2 at different dilution factors for incurred and spiked conformance samples.

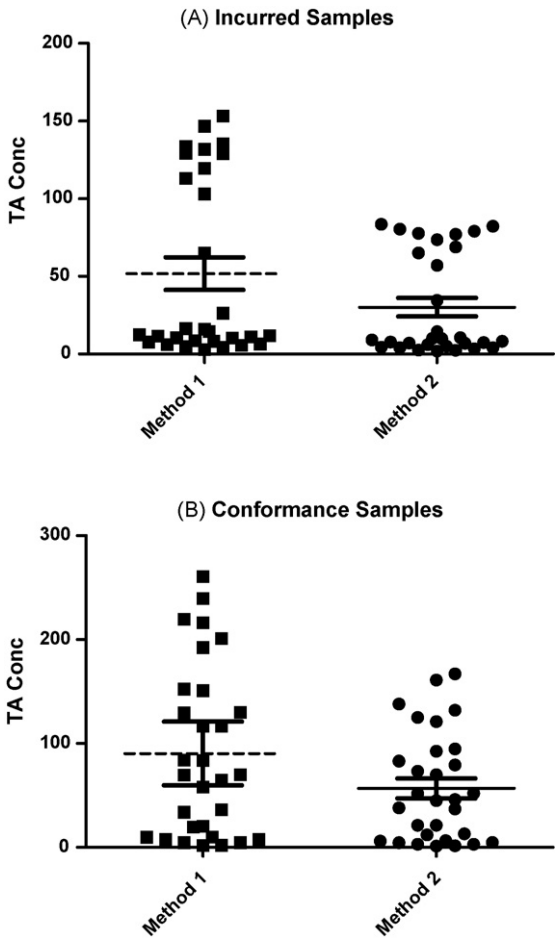

Fig. 1. Quantification of therapeutic antibody level (TA) during bioanalytical cross-validation experiment using incurred samples from a PK study (A) and spiked conformance samples (B) in method 1 and 2. The ratio of geometric mean concentration of method 1 (dotted line) to method 2 (straight line) was 1.63 and 1.57 for incurred and spike conformance samples, respectively. The data represented the individual result values in ng/mL, mean concentration for each sample group with standard deviation.

3.5. Pharmacokinetics

The concentration-time profile from study A was higher than that from study B (Fig. 3), and the resulting exposures, $AUC_{(0\text{-}168\,h)}$ and C_{max}, from study A were approximately twice those from study B (Table 6). The magnitude of difference in PK exposure was approximately the same as the comparative ratios from equivalence analysis on the incurred and conformance samples from bioanalytical cross-validation. However, the variability

Table 4
Performance of Assay QCs during cross-validation experiments.

	Method 1			Method 2		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Nominal concentration (ng/mL)	2.00	20.0	200	0.750	6.50	15.0
Mean concentration (ng/mL)	2.49	23.3	228	0.744	6.22	16.3
%CV between 4 replicates	7	3	2	3	1	7
% Bias	24	17	14	−1	−4	9

Table 5
Cross-validation summary for incurred and conformance samples.

	90% Confidence interval		Method 1: method 2 ratio of mean concentrations
	A priori acceptance criteria	Results	
Incurred samples (n = 30)	Within (0.80, 1.25)	1.55, 1.72	1.63
Spiked conformance samples (n = 30)	Within (0.80, 1.25)	1.54, 1.60	1.57

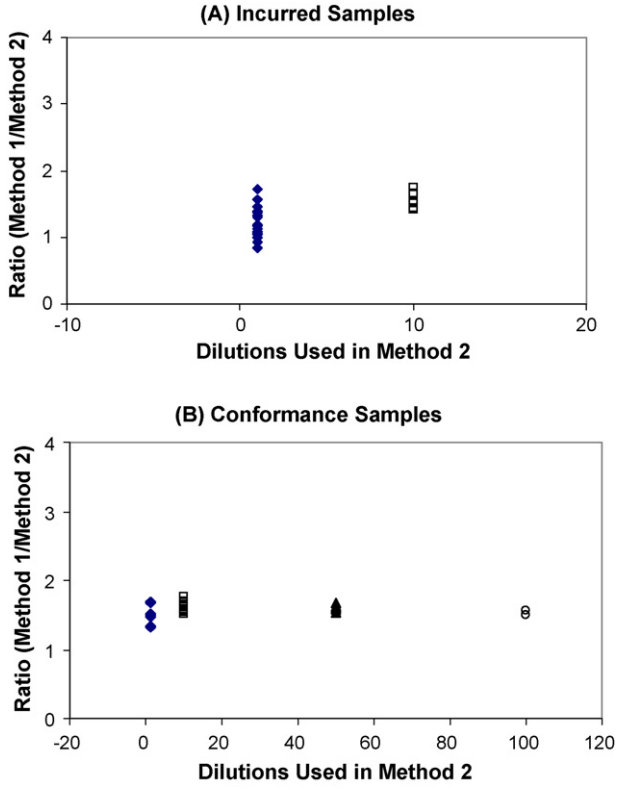

Fig. 2. Effect of dilutions in incurred samples (A) and conformance samples (B) during bioanalytical cross-validation experiment was examined. The result represented the ratio of method 1 to method 2 against the respective dilutions used in method 2. In method 2, dilution factors of 1, and 10 were applied for incurred samples and dilution factors of 1, 10, 50 and 100 were applied for spiked conformance samples. Dilutional linearity was demonstrated previously during validation (data not shown).

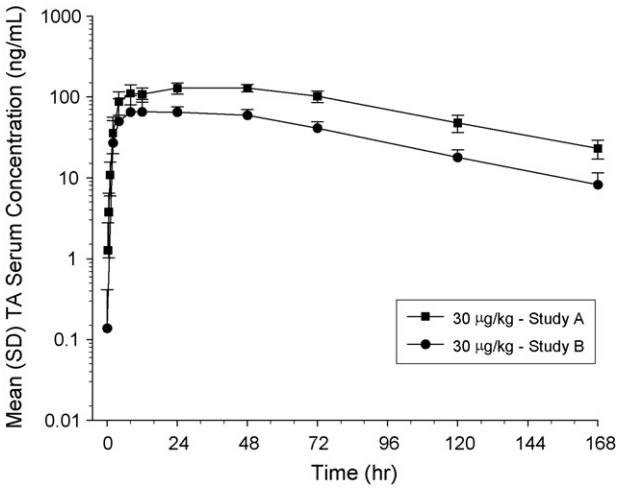

Fig. 3. Comparison of PK profiles between studies where method 1 and method 2 were used. Method 1 was used in quantification of TA level in study A while method 2 was used in study B. Both study A and B had same dose group of 30 μg/kg. TA levels in each dose group were consistently lowered in study B.

of bioanalytical result between individual animal/subject was minimal.

4. Discussion

The conference report published in 2000 and the 2001 FDA guidance stated that cross-validation is required in the following circumstances: when two or more bioanalytical methods are used to generate data within the same study, when sample analyses within a single study are conducted at more than one site, and when data are generated using different analytical techniques (e.g., LC/MS–MS vs. LBA) in different studies are included in a regulatory submission [5,6]. However, there has been no further guidance on how to cross-validate methods or interpret data in the 2007 Conference Report on Quantitative Bioanalytical Method Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays [7]. It is not clear to many bioanalytical laboratories, especially in the pharmaceutical industry when a method change is considered "major" and that would therefore require cross-validation. In an ideal situation, it is desirable to use the same analytical method to support the development of the biological therapeutic throughout the drug development cycle. However, this is not realistic because reagent development time for LBA usually cannot meet the aggressive timelines typical for the early stages of drug development. Initial studies may employ method using critical reagents, including capture and detector reagents, that are readily available, or those that take less time to produce and characterize, while more refined reagents are typically used in later studies. In some instances, "reference" reagent supply may be discontinued and could lead to the development of a new method. Deployment of different capture and detector reagents among different LBA formats/platforms may yield differences in sample quantification that may have an impact on the intended application such as PK parameters. There is no guidance or consensus on method comparison for the same technology, such as LBA used among various studies during the course of drug development.

The data integration and interpretation across studies should take the bioanalytical method changes into consideration and vice versa. Therefore, cross-validation is necessary for the overall integration of data in support of new drug development. The scope of cross-validation in this study comprised not only bioanalytical method comparison but also the evaluation of PK parameters and their impact on data interpretation and conclusions. This case study illustrated that not all LBA are equivalent. Based on the

Table 6
PK comparison between studies.

Study A	Subject	AUC0_168 (hr*ng/ml)	C_{max} (ng/ml)
	N	4	4
	Mean	13300	136
	SD	1840	19.1
	CV%	13.83	14.04
Study B			
	N	4	4
	Mean	5960	79.5
	SD	1010	29.2
	CV%	16.95	36.73
	Study A/study B ratio	2.23	1.71

cross-validation results, there were significant differences between the two bioanalytical methods, and the two methods were not equivalent in measuring concentrations of the biological therapeutic. Bioanalytical results generated from method 1 were used to project human exposure at proposed clinical doses, while those from method 2 were used to determine NOAEL exposure (No Observed Adverse Effect Level). The exposure ratio (NOAEL/clinical dose) represents the exposure/safety margin. The non-equivalence of the bioanalytical methods would require some adjustment to the experimentally determined exposures to appropriately interpret PK results. In this study, the magnitude of bioanalytical method differences is similar to that of PK results. Therefore, an adjustment was made using the approximate ratio.

On the other hand, if non-equivalence of the bioanalytical methods did not have an impact on the exposure/safety ratio it would be reasonable to make a PK decision to proceed without adjustment. If the magnitude of bioanalytical method differences was far smaller than that of PK variability, then the adjustment would be of minimal value. If the magnitude of bioanalytical method differences exceeds the variability of PK results, resolutions can be made in both bioanalytical and PK perspectives. The following possible bioanalytical resolution steps can be considered when comparing an established method with a new one: (1) systemic and random errors of both methods can be assessed using spiked conformance samples; (2) a correction factor for bioanalytical method differences can be determined based on the error assessment and applied to the respective method; and (3) the new method can be redeveloped and validated. The third option would be of use if the new method had larger systemic and random errors than the original method and if the new method were to be used in future studies. If the redeveloped method were equivalent to the original method, a portion of or all study samples could be reanalyzed with the redeveloped assay. In addition to the bioanalytical resolution, the impact on the PK parameters should be assessed by the phamacokineticist. A scientific strategy and justification must be documented in the decision-making processes for PK assessment and its impact on the projected human exposure ratio.

Statistical approaches on method comparison between original and new methods for diagnostic applications have been previously discussed [6,8]. These are the traditional approaches for judging the acceptability of two methods to compare the new method with the existing "reference" method [6,8]. In our study, the identification of the "reference" method, method 2, was in reverse chronological order. The experimental and statistical approaches presented here can also be applied in other types of cross-validation, such as method transfer from one laboratory to another, or use of different instrument platforms within the study or between studies. Another statistical approach for comparison between 2 methods or results would be the Bland-Altman analysis [9,10], *F*-test, *t*-test, least square analysis, correlation coefficients [11] or linear regression for cross-validation between laboratories [8]. Bland-Altman analysis is not a statistical test measured with a *p*-value. It is a process used to assess agreement between two methods of measurement. An important requirement of this analysis for measuring agreement is that the two methods measure the same characteristic using the same scale of measurement. Linear regression is the simplest and most common tool used to examine systemic error. Based on variance across the concentration range, non-weighted or weighted linear regression analysis can be performed. Each statistical analysis has both advantages and disadvantages in comparing the equivalence of two methods and in informing decisions on method acceptability. The statistical analysis used in our study provides *p*-value and 90% confidence limits and therefore is sufficient to compare the equivalence of two methods.

5. Conclusion

We described detailed experimental and statistical approaches that were used to determine whether two LBA methods for PK/TK assessment were equivalent. In this case study the two LBA methods were not statistically equivalent, the magnitude of the difference was reflected in the PK parameters of the respective studies, and an adjustment was made using the appropriate ratio. In summary, this case study demonstrated the need for cross-validation between two bioanalytical methods and the benefits of cross-validation in the assessment of intended purpose. Thus, cross-validation is strongly recommended when reagents and/or assay platforms change and is highly beneficial in the assessment of intended purpose.

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