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Questions and Answers (Q&A) for the Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development

<<Scope>>

Q1. Is this guideline applicable to a drug having an amino acid sequence identical to an endogenous substance?

A1. Yes, this guideline is applicable. However, selection of a blank matrix requires special precautions, such as the use of a surrogate matrix or a matrix that has been depleted of the endogenous substance concerned (see Q&A No. 9 and No. 13). Same precautions should be taken for a drug which is indistinguishable by a ligand binding assay (LBA) from the endogenous substance.

<< Reference Standard>>

Q2. What procedures should be followed if the expiration date for the reference standard is yet to be established?

A2. If the expiration date of the reference standard cannot be established, quality of the reference standard should be ensured by other appropriate means, such as setting a re-test date *a priori*.

Q3. What procedures should be followed in renewing the reference standard lot?

A3. Confirm comparability of the current and new reference standard lots by referring to the relevant Certificate of Analysis (CoA), or any appropriate documentation. If no information regarding the lot comparability is available from the CoA or other relevant documentation, it should be confirmed by an LBA.

Q4. Does the reference standard lot have to be the same as the drug substance lot used for dosing in the non-clinical or clinical studies?

A4. Any lot may be used as the reference standard as long as it conforms to the same quality specifications based on information available from a CoA or other appropriate document. In an early stage of non-clinical studies where a quality specifications for a standard material are yet to be established, it is preferable that the reference standard lot is the same as the drug substance lot used for dosing in the non-clinical studies; if this is not the case, the lot comparability has to be confirmed by an LBA.

<<Full validation>>

Q5. What is the difference between the MRD and sample dilution stated in the dilutional linearity section?

A5. The MRD is a dilution factor where samples are diluted with buffer solution to reduce the matrix effect on the analysis: an identical MRD should be applied to all samples, including calibration standards and QC samples. On the other hand, sample dilution is a procedure where samples with high analyte concentrations are diluted with blank matrix or diluted blank matrix to bring the analyte concentration within the calibration range.

Q6. When a sample is analyzed in two or more wells and when there is a large variation in their responses or determined concentrations, what procedures should be followed?

A6. Specify handling procedures for data with a large variation *a priori* in the protocol or standard operating procedure (SOP). Such a variation will compromise the data's reliability.

<<Specificity>>

Q7. Is there any case where specificity evaluation is not necessary?

A7. Specificity is important information for analytical data interpretation. In an LBA, specificity is dependent on the reactivity of the binding reagent. Therefore, if the characteristics of the binding reagent are well known from its development phase, it may not be necessary to repeat the specificity test in validation.

<<Selectivity>>

Q8. Should the use of disease-derived, hemolyzed, or lipemic matrix be necessary?

A8. If such a factor is likely to affect the assay system, consider using a relevant matrix, although this is not mandatory.

<<Calibration curve>>

Q9. What should be used as a blank matrix in analysis of a drug having an amino acid sequence identical to an endogenous substance?

A9. If the presence of the endogenous substance is anticipated in the study sample matrix, a surrogate matrix or a matrix that has been depleted of the endogenous substance can be used. When such matrices are used, their validity should be shown.

<<Accuracy and precision>>

Q10. For QC samples, it is stated that "the mid-level is in the midrange on the calibration curve, and the high-level needs to be at least one-third of the upper limit of quantification (ULOQ) of the calibration curve." Please specify how to set these concentration levels.

A10. The mid-level, in the midrange on the calibration curve/calibration range, generally means a level near the geometric mean of the ULOQ and the lower limit of quantification (LLOQ); a level adjacent to the arithmetic mean may be used for a more balanced QC sample distribution. The high-level, at least one-third of the ULOQ of the calibration curve, is intended to equally distribute QC samples within the range of a calibration curve where the nominal analyte concentrations are plotted on a logarithmic scale. A level around 75% of the ULOQ may be used depending on the overall balance of the QC sample distribution.

Q11. How should the number of replicates be set in each analytical run when assessing the accuracy and precision?

A11. When calculating within-run accuracy and precision, at least triplicate analyses (n=3) per analytical run are required. When calculating between-run accuracy and precision, a single analysis per analytical run would be accepted. An alternative method, such as analysis of variance (ANOVA), is also accepted. When using ANOVA, at least duplicate analyses are set per analytical run to evaluate accuracy and precision.

Q12. Why is a total error required?

A12. The absolute value of the relative error (i.e., accuracy minus 100%) represents the systematic error and precision reflects the random error. Evaluation of the total error allows early elimination of an analytical method having a large variation that could compromise data reliability (DeSilva et al, Pharm. Res., 2003). Although the acceptance criteria in accuracy and precision for LBA are set wider than those for chromatography/small molecules, it is anticipated that, by evaluating the total error, a LBA-based analytical method whose accuracy and precision are both on the edge of acceptability can be eliminated; this should ensure the reliability of the analytical results obtained.

Q13. What are the points to consider in assessing accuracy for the analysis of a drug having an amino acid sequence identical to an endogenous substance?

A13. Use a surrogate matrix or a matrix that has been depleted of the endogenous substance. Alternatively, determine the concentration of the endogenous substance in the blank sample and calculate the accuracy using one of the following formulas.

$$\text{Accuracy (\%)} = \frac{\text{Measured concentration of drug in sample}}{\text{Conc. of endogenous substance + Conc. of reference standard}} \times 100$$

$$\text{Accuracy (\%)} = \frac{\text{Measured conc. of drug in sample – Conc. of endogenous substance}}{\text{Concentration of reference standard}} \times 100$$

<<Dilutional linearity>>

Q14. What is the difference between dilutional linearity and dilution integrity?

A14. Dilution integrity is tested to confirm that the dilution procedure has no impact on the measured concentration, while dilutional linearity is tested to confirm not only dilution integrity, but also the absence or presence of a hook effect or prozone.

<<Cross validation>>

Q15. What is the rationale for setting the acceptance criteria as “the mean accuracy at each level should be within ±30% deviation of the theoretical concentration, in principle”?

A15. The guideline requires the mean accuracy to be within ±20% deviation of the theoretical concentration for analytical method validation. The acceptance criterion was relaxed to 30% for cross validation to accommodate additional factors, i.e., intra- and inter-laboratory precision. When study samples within a single study are to be analyzed at multiple laboratories, measures should be taken to minimize inter-laboratory variation in study sample analyses, such as by defining criteria for handling of study samples and reference standards in the relevant protocol or SOP, separate from the analytical method validation.

<< Study sample analysis >>

Q16. Should a calibration curve analysis be required for each plate?

A16. In principle, each plate should contain a set of calibration standards. If, however, assay integrity is demonstrated during the validation process, it is also acceptable to apply a single calibration standard set to multiple plates as far as each plate contains a set of QC samples.

Q17. Is it not necessary to evaluate parallelism?

A17. Parallelism is defined as an established parallel relationship between a dose-response curve from a study sample dilution series and a curve from a calibration standard series, with no difference among back-calculated

concentrations for multiple dilutions of a study sample. As of the issuance of this guideline, domestic and international knowledge has neither accumulated nor discussion yet matured regarding cases in which parallelism was not established, causes for failing to establish parallelism, and the extent of impact the failure might have on pharmaceutical development. Therefore, evaluation of parallelism is not necessarily required for all analytical methods. However, if parallelism is an intrinsic issue for an LBA-based bioanalytical method and is likely to cause a problem based on the nature of the analyte or method or data accumulated in the course of pharmaceutical development, scientifically valid evaluation and assessment of the impact on measured concentrations should be considered to the extent possible.

<<QC samples in study sample analysis>>

Q18. What should be considered in the arrangement of calibration standards and QC samples within a plate?

A18. It may sometimes occur that a certain pattern is inevitably seen in the assay results due to sample arrangement within a plate (e.g., edge effect); in such cases measures should be taken to mitigate the impact on the analysis results. Measures include the arrangement of calibration standards, QC samples, and study samples on a plate and the number of replicates for each sample prepared.

<<ISR>>

Q19. How should I perform ISR in toxicokinetic studies?

A19. In a GLP toxicokinetic study, ISR should be performed once per matrix for each animal species. If an analytical method is modified or analysis is performed in a different laboratory, ISR should be performed again.

In addition, ISR can be performed during a bioanalytical method validation using study samples obtained from a non-GLP study such as a dose-finding study performed before a GLP toxicokinetic study. In this case, the study design, including dose and regimen, should be comparable to that of the GLP study.

Q20. How should I perform ISR in clinical trials?

A20. ISR should be performed in representative clinical trials whose pharmacokinetic data as a primary endpoint. To evaluate the validity of an analytical method in an early stage, ISR should be performed as early as possible in the process of pharmaceutical development.

In a clinical trial with a different population of subjects with altered matrix composition, ISR should be performed again. In a bioequivalence study which serves pharmacokinetic data as the primary endpoint, ISR should be performed in the study.

Q21. If study samples obtained from clinical trials are already available at the time of analytical method validation, can I use the samples for ISR?

A21. If you have already obtained study samples from a clinical trial at the time of analytical method validation, you can use the samples for ISR. For example, a metabolite is added to the analyte(s), or reanalysis is performed with an improved analytical method after a failure to meet ISR acceptance criteria. However, an informed consent must be obtained from each subject who provides the study samples. The procedures of ISR and related items should be predefined.

Q22. If overall results meet the ISR acceptance criteria, but the assay variability of a specific sample exceeds the threshold of ±30%, is it required to reanalyze the samples to correct first value?

A22. ISR is intended to confirm the validity of an analytical method using study samples. Therefore, reanalysis of individual study samples is not required to correct the first value, even though the assay variability exceeds the threshold of ±30% when overall result meets the ISR acceptance criteria.

Q23. Where in a report is appropriate to provide ISR results?

A23. When the ISR is performed in the study sample analysis, ISR results should be reported in a study sample analytical report to prove the validity of an analytical method. When the ISR is performed in the analytical method validation, ISR results should be reported in a validation report.

Q24. In non-clinical studies, it is often the case that the sample volume is not sufficient for ISR evaluation. What procedures should be followed in that case?

A24. The study plan should always assume ISR, even in non-clinical studies. Even if the volume becomes insufficient in some samples due to reanalysis or for other reasons, ISR can still be performed, for example, by using samples from other time points. ISR may also be performed by utilizing samples from a preliminary study in which samples were collected under comparable conditions. In any case, even in non-clinical studies, every practical effort should be made to evaluate the reproducibility of the measured concentrations by ISR.

<< Critical reagents >>

Q25. Should an expiration date be established for critical reagents?

A25. Expiration date is not necessarily required for a critical reagent, as long as the quality is ensured by evaluation of data from calibration standards and QC samples during the period the reagent is used.