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Bioanalytical Method Validation: A Quality Assurance Auditor View Point

Rama Rao Kalakuntla* and K.Santosh Kumar**.

*Quality Assurance Auditor, Apotex Research Pvt.Ltd, Bangalore, India.

** Research Scientist, Ranbaxy Research Laboratories, Gurgaon, India.

Abstract:

Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for a quantitative measurement of analytes in a given biological matrix is selective, sensitive, reliable and reproducible for the intended use. Any method developed for the analysis of analytes in biological fluids must yield consistent results despite the variations in conditions during the course of a project. An ideal bioanalytical method should include all of the probable effects that are going to occur during the routine analysis of study samples. It may not be possible to test each and every condition that is going to be encountered during the subject sample analysis. However, it should include all the applicable regulatory specified validation parameters and should assure the integrity of the study data. Some of the proposals were made to the validation procedure to encounter the possible situations in the routine study sample analysis. An attempt has been made to understand and explain the bioanalytical method validation for chromatographic assays from the quality assurance auditor viewpoint. A good understanding of the background and principles of the bioanalytical method validation will help the quality assurance personnel to perform their duties in a most effective and focused manner.

Introduction:

Bioanalytical method validation (BMV) employed for the quantitative determination of drugs and their metabolite in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic study data [1]. Why we need to validate a bioanalytical method is not a question anymore, tremendous progress has been made in this area. Like any other assays, bioanalysis is also affected by many of the factors, including but not limited to the variation in the matrix, presence of endogenous biochemicals or chemicals, differences in chromatographic conditions or techniques etc. by performing the validation of the bioanalytical method, it must be assured that the method will yield reliable and reproducible results over a period of time. Quality assurance department plays an important role in the flow chart of the bioanlytical lab, indeed bioequivalence centre. Quality assurance personnel have to assure the management and/or regulatory agencies that the validation of the bioanalytical method has been done as per the standard operating procedures (SOPs) of the organization and as per applicable regulatory guidelines. To do so, quality assurance personnel must understand the basic principles and underlying concepts of a bioanalytical method validation. As per the "Good Laboratory Practice" principles of the Organization for Economic Cooperation and Development (OECD), individuals appointed to Quality assurance functions should have the ability to understand the basic concepts underlying the activities being monitored [2].

As per the Guidance for Industry, "Bioanalytical Method Validation" guidelines form FDA, the analytical laboratory conducting pharmaceutical/toxicology and other preclinical studies for regulatory submissions should adhere to FDA's Good Laboratory Practices and to sound principles of quality assurance

throughout the testing process [3]. In this article, an attempt has been made to understand and explain the issues and concepts of BMV.
Chromatographic method like high performance liquid chromatography (HPLC) and Gas Chromatography (GC) have been widely used for the bioanlysis of small molecules, with liquid chromatography coupled to triple quadrapole mass spectrometry (LC/MS/MS) being the single most commonly used technology.
Essential parameters for validation of a bioanalytical method include Accuracy, Precision, Linearity, Selectivity, Sensitivity, reproducibility and Stability. The other important parameters include dilution integrity, matrix effect, and extraction efficiency. An attempt has been made to explain the background of each of the validation parameter.

Validation Parameters:

1. Selectivity:

Selectivity or specificity should be evaluated to assess the interference at the retention time (RT) of the analyte and internal standard (IS) with predetermined method conditions. If the single method assesses one or more two analyte simultaneously, the interference should be evaluated separately for each analyte individually. At least sox lots of blank matrix are processed as per the method, and after the run select the lot, which contain no or less interference among the all lots and spike to prepare the limit of quantification (LOQ) as per the method. Process six samples of LOQ with the addition of internal standard as per the method and inject. Then assess the interference in all the lots of blank batches against the mean response of the LOQ at the RT of the analyte and IS. The acceptance criteria followed should be as per the internal SOP. For chromatographic assays, the peak response in blank matrix at the retention time of analyte should be not more than 20% of the mean response of the LOQ samples and the peak response at the RT of the IS should be no more than 5% of the mean response of the IS of the LOQ. QA Auditor at this stage should ensure that only blank matrix lots which were showed no significant interference will only be used for further usage in the validation. An acceptance criteria should be set in the SOP for the number of batches, like out of all screened lots, 80 %(or any specification, which is acceptable as per applicable guidelines) should be acceptable. If the acceptance criteria are not met then failure should be investigated.

2. Matrix Effect:

The recent 3rd bionalytical workshop proposed determination of matrix factor as a way of assessing the matrix effect. Since ionization of analytes will be affected by presence of endogenous components in biological matrix, it could be either suppression or enhancement.

Matrix Factor (MF) can be calculated as,

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in the abscence of matrix ions}}$$

Where peak response could be peak area, peak height, peak area ration or peak height ratio according to the method. Matrix Factor equal to 1 indicates no matrix effect, matrix factor less than 1 indicates suppression and greater than one indicates enhancement [4].

The MFs can be determined for the analyte and IS separately and a ratio of 2 factors yields the IS normalized MF for the analyte. Because of the similarities in chemical properties and elution times of the stable isotope labeled internal standards relative to the analytes, the MFs for an analyte and its stable isotope labeled IS are usually similar. The IS normalized MFs using stable isotope labeled IS are therefore usually close to unity for the bionalytical samples. Thais has a very positive influence in reducing the variability of the assay due to matrix effects and makes the use of stable isotope labeled internal standards very desirable in mass spectrometry(MS) based assays. It is recommended that matrix factor or IS normalized MF being determined in six different lots of matrices. The variability in matrix factors as measured by coefficient of variation (%CV) should be less than 15% [4].

3. Sensitivity:

The lowest standard (LOQ) should be accepted as the limit of quantification of the method. This test should be performed to prove the reproducibility for samples at limit of quantification level. Sensitivity should be evaluated by using at least 5 replicates of the samples at the limit of quantification. The sample used for this evaluation should be independent of calibration standards. The accepted limits for LOQ should be ±20% for accuracy and ≤20% for precision. In addition, signal to noise ratio(S/N) will also be calculated to evaluate the noise level. A minimum recommended S/N ration could be 5:1, however acceptance criteria for the signal to noise ratio depend on the individual method.

4. Preparation of Calibration/ Quality control standards:

Calibration standards can be defined as a biological matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves, from which the concentrations of analytes in QCs and in unknown study samples are determined. In the same way, quality control standard (QC) is also a spiked sample used to monitor the performance of a bionalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch [3]. Calibration/quality control standards preparation plays an important role in the outcome of the method performance. For the preparation of master stocks, an adequately characterized standard reference material must be available, from which the other dilutions may be prepared which will be used for the spiking of calibration/QC standards [5]. Documentation of the characterization (certificate of analysis) must be available to the bionalytical laboratory when this material is used for method validation. Procedure defined in the analytical method should be followed for the preparation of calibration/QC standards. A QA Auditor must audit the process of preparation of calibration/QC standards for compliance and ensure that any special precautions indicated in the certificate of analysis and/or analytical method is followed during the process.

5. Accuracy, Precision and Linearity:

Accuracy and Precision should be assessed by analyzing at least 3 validation batches including both intra

and inter day runs. Both within and between run precision and accuracy should be assessed. Each validation batch must consist of at least 6 to 8 non zero calibration standards, one standard blank (matrix blank) and standard zero(matrix blank with internal standard) and 6 replicates of quality control standards at each limit of quantification (LOQQC), low (LQC), middle (MQC) and high (HQC) levels. Limit of quantification QC (LOQQC) should be prepared at the lower limit of quantification of the method and low-level QC (LQC) should be prepared at above the limit of quantification, but no more than 3 times of the limit of quantification. Middle level QC (MQC) should be prepared at the middle of the calibration curve range; ideally, it should be at the geometric mean of the low and high QC concentrations. The high QC level (HQC) concentration should be near the upper calibration curve range. Ideally, it should be at around the 70 to 80% of the highest calibration curve standard, care should be taken that QC concentration at low, middle and high levels should not represent any of the calibration curve standards.

After the analysis of validation batches, regression model should be determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit. After confirming the regression, determine the mean concentration, standard deviation, precision and accuracy at each LOQQC, LQC, MQC and HQC concentration level. The acceptance criteria for the accuracy and precision should be defined in the SOP. The normal acceptance criteria will be, the between and with in batch CV for low, middle and high QC levels should be ≤15% and for the LOQQC level should be ≤20% and the between and with in batch mean concentrations should be with in ± 15% of the nominal values at low, middle and high QC concentration and should not deviate by more than ± 20% at the LOQQC concentration.

6. Recovery or Extraction Efficiency:

Recovery can be determined by comparing the detector response of the analyte or IS from an extracted sample to the detector response of the analyte from an unextracted sample representing the 100% recovery. Analytical method determines the type of unextracted sample to be used. Unextracted sample could be a neat drug solution or the blank plasma sample processed and reconstituted with the neat drug solution to represent the 100% recovery. Recovery will be evaluated at low, middle and high QC concentrations, the average recovery of the analyte or IS at three levels could be considered as the recovery. At least 6 samples at each QC level should be injected. Recovery does not need to be very high, but must be consistent, precise and reproducible. However, if the reported recovery is more than 100%, justification for reporting the same need to be mentioned in the validation report. Acceptance criteria for recovery evaluation could be stated in the SOP. Recovery deemed acceptable if %CV is, 15% for %mean recovery between low, middle and high QC levels.

7. Dilution Integrity:

Dilution of the study samples will be performed when the obtained

concentration is exceeding the upper limit of quantification or when there is less sample availability compared to the method requirement. Dilution integrity can be tested for nay dilution ranging from 1:2 to 1:10; preferably, dilution integrity will be evaluated at 1:2 and 1:4. a dilution integrity standard (approximately at a concentration of two times the concentration of 90% ULOQ) will be prepared and frozen for a period of at least 24 hours, on the day of evaluation, the dilution integrity standard could be diluted for 6 replicates each by a factor of 2 times and 4 times using the prescreened blank plasma. Dilution integrity samples will be processed and analyzed against a freshly prepared calibration curve. Acceptance criteria could be the precision and accuracy of the dilution integrity QCs is ≤15% and with in ± 15% of the nominal concentrations respectively.

8. Carry Over:

Carry over test will be performed to observe any carry over form the instrument from one sample to the next sample. To evaluate carry over a blank sample will be placed next to the ULQ standard in the sequence. Calculate the absolute peak response as percentage of the LOQ standard. Carry over should be considered not significant when the response in the blank sample is ≤ 20% of the LOQ sample.

9. Anticoagulant Effect:

Anticoagulant effect will be evaluated if the anticoagulant is different in the plasma used for the preparation of CC, QC samples and study samples. 6 replicates of low and high QC samples will be spiked with the matrix used in the method validation and another 6 replicates of low and high QC samples will be spiked with the matrix that shall be used in the study samples. Calibration standards will be prepared with the matrix used in the method validation and process and analyze all the samples together as per the method. The effect of anticoagulant is deemed nullified of the precision and accuracy of the QC's is ≤15% and with in ± 15% of the nominal concentrations respectively.

10. Stability Evaluations:

Drug stability is a function of the chemical properties of the analyte, the storage conditions, the matrix/solutions in which it is stored, and the container system. The stability tests should reflect the situations likely to be encountered during actual subject sample handling and analysis. The stability of analyte in the matrix during collection and storage of samples should be assessed. The different stability evaluations need to be performed, but not limited to be:

(a) Short-term stability of analyte(s) and internal standard(s) in solution/solvent.
(b) Long-term stability of analyte(s) and internal standard(s) in solution/solvent.
(c) Stability of analyte following sample processing (post preparative stability)
(d) Freeze and Thaw stability.
(e) Short term stability of analyte in matrix at room temperature(bench top stability)
(f) Long-term stability of analyte in spiked samples and incurred samples.

Solutions/samples may be stored at less stressful conditions than the proven stability conditions at which they were tested. For example, if stability is proven at **-20C** then the stability at **-70C** is automatically defined; based on the Arrhenius principle of chemical reactivity, lower rates of reactivity (analyte degradation) at lower temperature. However, the counter argument to the above inference should also be considered valid, since lower temperature may cause denaturation of the matrix proteins, which may affect the protein binding and ability to extract drug from the matrix, here the matrix degradation and not the chemical stability was the issue. Decision based on the above counter arguments should be considered from case to case basis.

(a) Analyte and IS stock solution stability in solvent/solution:

Prepare analyte, IS master stock solutions, and keep aliquots of the same at refrigerated temperature or as required by the specific method. These samples serve as stability stock solutions. Following the required period of stability, prepare fresh stock solutions of the analyte and IS, these solutions serve as comparison stock solutions. When performing the stability evaluation for the first time, on the day of evaluation remove the stability stock solutions and keep on workbench for a period of at least 4 to 6 hours. After the completion of anticipated time, prepare appropriate equivalent dilutions from both stability and comparison stock solutions. Inject 6 replicates of the stability and comparison stock dilutions, and calculate the mean, S.D and %CV of the peak area response of each of the above stability and comparison stock solutions. % stability shall be calculated as follows:

$$\% \text{Stability} = \frac{\text{Mean response of stability samples}}{\text{Mean response of comparision samples}} \times 100$$

The stability is deemed acceptable if % stability is with in the range of 85-115%. Appropriate correction factor needs to be applied to peak area response of comparison samples to account for difference in the stock weights. Stability exercise needs to be performed at regular intervals to cover anticipated storage period of the stock solutions in the intended study. Keeping the stock solutions on the workbench is required only for the first time evaluation of the stability period; it may not be required for the extended stability evaluations of the same stock solutions. If the acceptance criteria are not met for a particular stability period, then stability evaluation for a shorter period will be performed.

(b) Stability of analyte and IS dilutions:

Stability of dilutions containing analyte (analyte spiking solution), IS (IS spiking solution) and a solution containing both the analyte and IS needs to be evaluated at the specified storage conditions depending on the individual method. The dilutions are prepared from the master stock (stability dilutions) are kept at the specified storage conditions 9normally refrigerated conditions) for a period of time as required. On the day of evaluation, fresh dilutions are prepared from the same master stock, which was used as comparison dilutions. Appropriate intermediate dilutions are prepared from both stability as well as

comparison dilutions and 6 replicates are injected. Mean, S.D. and %CV and %stability are calculated and the acceptance criteria of stock solution stability is applicable to this one. Demonstrating the stability of the dilutions of the analyte, IS and a solution containing both the analyte and IS offers the flexibility to use the solutions for a longer period, rather than preparing them every day.

Matrix Stability:

(c) Stability of analyte following sample processing (Post preparative or auto sampler stability):

Post preparative stability is evaluated to cover the anticipated run time for the analytical batch, and to handle the situations like system malfunctioning etc. where samples are injected after a certain period of time. Process 6 replicates of QC samples at low and high concentrations and place the samples in the auto sampler as per the specifications of the analytical method. After the stability period, the same QC samples (test samples) will be injected along with the calibration standards and another set of freshly extracted QC samples (reference samples). All the QC sample concentrations are back calculated using the calibration curve.

(d) Short term stability of analyte in matrix at room temperature (Bench Top Stability):

Bench top stability evaluation will be performed to evaluate the stability of the samples, which were kept on bench during the extraction process. The anticipated time for the bench top stability (usually 4 to 24 hours) should cover the duration of the time, it takes while extraction process. 6 replicates of QC samples at low and high levels will be removed from the freezer on the afternoon prior to the day of evaluation and left to remain on the bench top until the following day. On the day of evaluation, bench top stability samples (test samples) along with the calibration curve and 6 sets of freshly spiked QC standards at low and high levels (reference samples) will be processed and injected.

(e) Freeze-Thaw Stability:

Freeze-Thaw stability evaluation will be performed to evaluate the stability of the analyte in the matrix after multiple cycles of freezing and thawing. Generally, freeze thaw stability will be performed for three cycles, the number of cycles will be increase or decreased as per the requirement keeping in the mind the stability of the drug tested. 6 sets of low and high level QC samples (test samples) prepared during the bulk spiking should be stored in the freezer for at least 24 hours, after that the same samples are withdrawn from the freezer and allow them to thaw unassisted at room temperature and then refreeze the samples for a minimum of 12 hours. Repeat the same exercise for two more times. While thawing at each cycle, the test QC samples should be uncapped to mimic the situation encountered for the study samples. After the required number of cycles, on the day of evaluation, remove the freeze-thawed test QC samples along with the calibration curve and 6 sets of freshly spiked QC standards at low and high levels (reference samples) and process all the samples and analyze.

(f) Long term Stability:

Long-term stability evaluations will be performed to demonstrate the stability of the analyte in the matrix for longer duration of time. The anticipated duration for the long terms stability should cover the duration of time form the first sample collection to the last sample analysis of the study. Long-term stability evaluation should be performed on at least three separate occasions to cover the entire stability period. Following the appropriate stability period, 6 sets of low and high concentration QC samples (test samples) will be removed from the freezer and processed along with the calibration curve and 6 sets of freshly spiked low and high concentration QCs (reference samples) and analyze.
The acceptance criteria for the post-preparative stability (auto sampler stability) bench top stability, freeze thaw stability and long-term stability should be, the relative means of back calculated concentrations (test/reference) for both levels tested must be with in 85-115%.

An additional check point may added to the acceptance criteria, that 4 out of 6 QCs of both test and reference samples must be quantifiable and with in specifications. This additional check point is needed, since in some of the cases, both test and reference QC concentrations may be out of the specification of 85-115% of the nominal for accuracy (for example both test and reference QCs failing on either lower side or higher side), even though as we are measuring the test/ reference ratio, the final outcome may give the conclusion as the evaluations is meeting the specifications. To overcome such type of false interpretations a mechanism should be in place in the SOP to address such situations.

Combined evaluation of freeze thaw, bench to and long-term stability:

During the validation, freeze thaw stability, bench top stability and long-term stability evaluations are performed separately to assess the stability for the intended stress conditions. However, the study samples (unknown samples) may undergo the combined effect of all the above-mentioned stress conditions. Therefore, it is advisable to evaluate the so-called combined affect of different stress conditions during the validation phase.
The combined stress conditions effect may be evaluated in the following manner. Prepare 6 sets of QC samples (low and high levels) and stress for required number of freeze thaw cycles and store for the anticipated long term storage period. After the anticipated long-term storage of the freeze thawed samples, stress the same samples for bench top conditions. Then process and analyze the same QC samples along with the calibration curve and 6 sets of freshly spiked QC samples at low and high levels (reference samples). The acceptance criteria may set as like any other individual stability evaluations.

Extended Precision and Accuracy:

Extended precision and accuracy will be evaluated to decide how much maximum number of samples can be analyzed in as single batch of analytical run. Most of the validation batches were small, where as during the routine subject sample analysis; we may process and analyze the samples of tone or more subjects at a

time. This evaluation will helps in getting the preliminary idea of as how many samples can be analyzed in a single batch. For this evaluation, process and analyze at least 30 to 35 sets of low, middle and high QC samples against a single calibration curve. The exact number of QC samples will be decided by keeping in mind the expected size of the analytical batch in the study. Extra extended precision and accuracy batch may not be required if one of the batch analyzed during the validation contains the number of anticipated samples in the study. The acceptance criteria for the extended precision and accuracy batch should be the mean back calculated concentration values for the low, middle and high QC concentrations should be with in ±15% of their nominal concentrations and the CVs for low, middle and high QC concentrations should be ≤15%.

Evaluation of prepared sample reinjection/reanalysis capability:

Prepared samples may be reinjected or reanalyzed when the run sequence was interrupted in between the sequence due to any of the reasons like instrument malfunction, fluctuation in the power supply etc. In these cases, some of the samples are injected and some are not injected. After certain period, when the instrument and other conditions are ready, there may be a situation, whether to continue the sequence from where the run ahs stopped or to reanalyze the whole sequence from the starting including the calibration curve. To take a confidant decision in these type of situations, which may occur during the subject sample analysis, a simple validation exercise could be performed. Process a set of calibration standards and 6 QC sets of low and high level concentrations (test stability samples) and analyze and determine whether the calibration/QC standards are with in the specifications. If the samples are with in the specifications, keep the samples in the auto sampler for the required period of time (usually 24 to 48 hours). Then after the completion of the intended duration inject the whole sequence for the second time. Check for the specifications of the reinjected run. If the reinjected samples are with in the specifications, then it may be concluded that the samples has the reinjection stability. Then quantify the reanalyzed test stability samples with the first injected calibration curve, if the test stability samples are with in the specifications with the first injected calibration curve also, then it may be concluded that the interrupted sequence may be started from where it was interrupted. If the test stability samples are not meeting the specifications when quantified against the first injected calibration curve, it may be concluded that, whenever the re is an interruption of the run sequence, the whole sequence including the calibration curve must be reinjected.

Conclusion:

An attempt has been made to understand and explain the bioanalytical method validation from a quality assurance auditor viewpoint. Some of the proposals were described for inclusion in the validation parameters for the different situations encountered in the study sample analysis.

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