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Bio-analytical method validation and its importance in Pharma research - A Review Article

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Abstract

Bioanalytical method based on a variety of physico-chemical and biological techniques such as chromatography, immunoassay and mass spectrometry, must be validated prior to and during use to give confidence in the results generated. It is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine is reliable and reproducible for the intended use. The present manuscript focuses on the consistent evaluation of the key bioanalytical validation parameters is discussed: accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery and stability. These validation parameters are described, together with an example of validation methodology applied in the case of chromatographic methods used in bioanalysis, taking in account to the recent Food and Drug Administration (FDA) guidelines and EMA guide

Keywords: Bioanalytical method, Method development, chromatography.

1. Introduction

The development of sound bioanalytical method(s) is of paramount importance during the process of drug discovery and development, culminating in a marketing approval. The objective of this paper is to review the sample preparation of drug in biological matrix and to provide practical approaches for determining selectivity, specificity, limit of detection, lower limit of quantization, linearity, range, accuracy, precision, recovery, stability, ruggedness, and robustness of liquid chromatographic methods to support pharmacokinetic (PK), toxicokinetic, bioavailability, and bioequivalence studies. Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, PK, and toxicokinetic studies. Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacology studies. The process by which a specific bioanalytical method is developed, validated, and used in routine sample analysis can be divided into three stapes as below [1,2].

1) Reference standard preparation,
2) Bioanalytical method development and establishment of assay procedure and
3) Application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

2. Selectivity

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample. Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analysed and evaluated for interference. Use of fewer sources is acceptable in case of rare matrices. Normally, absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the analyte and 5% for the internal

standard. It may also be necessary to investigate the extent of any interference caused by metabolites of the drug(s), interference from degradation products formed during sample preparation, and interference from possible co-administered medications. Co-medications normally used in the subject population studied which may potentially interfere should be taken into account at the stage of method validation, or on a study specific and compound specific base. The extent of back-conversion should be established and the impact on the study results discussed. It is acknowledged that this evaluation will not be possible early during drug development of a new chemical entity when the metabolism is not yet evaluated. However, it is expected that this issue is taken into account and a partial validation is performed if relevant as further knowledge regarding metabolism of the active substance is gained during drug development. It is recognized that in some cases it is very difficult to obtain the metabolites of interest. Alternatively, back-conversion of a metabolite can be checked by applying incurred sample reanalysis. However, in this case potential back conversion during sample processing cannot be ruled out [3,4]

3. Carry-over (AICO)

Carry-over should be addressed and minimised during method development. During validation carryover should be assessed by injecting blank samples after a high concentration sample or calibration standard at the upper limit of quantification. Carry over in the blank sample following the high concentration standard should not be greater than 20% of the lower limit of quantification (LLOQ; see below) and 5% for the internal standard. If it appears that carry-over is unavoidable, study samples should not be randomized. Specific measures should be considered, tested during the validation and applied during the analysis of the study samples, so that it does not affect accuracy and precision. This could include the injection of blank samples after samples with an expected high concentration, before the analysis of the next study sample[5].

4. Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ is considered being the lowest calibration standard (see Accuracy and Precision). In addition, the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample. The LLOQ should be adapted to expected concentrations and to the aim of the study. As an example, for bioequivalence studies the LLOQ should be not higher than 5% of the C_{max}, while such a low LLOQ may be not necessary for exploratory pharmacokinetic studies.

5. Calibration curve

The response of the instrument with regard to the concentration of analyte should be known, and should be evaluated over a specified concentration range. The calibration standards should be prepared in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte. There should be one calibration curve for each analyte studied in the method validation and for each analytical run. Ideally, before carrying out the validation of the analytical method it should be known what concentration range is expected. This range should be covered by the calibration curve range, defined by the LLOQ being the lowest calibration standard and the upper limit of quantification (ULOQ), being the highest calibration standard. The range should be established to allow adequate description of the pharmacokinetics of the analyte of interest. A minimum of six calibration concentration levels should be used, in addition to the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS).

Each calibration standard can be analysed in replicate. A relationship which can simply and adequately describe the response of the instrument with regard to the concentration of analyte should be applied. The blank and zero samples should not be taken into consideration to calculate the calibration curve parameters. The calibration curve parameters should be reported (slope and intercept in case of linear fit). In addition, the back calculated concentrations of the calibration standards should be presented together with the calculated mean accuracy values (see definition of Accuracy below). All the available (or acceptable) curves obtained during validation, with a minimum of 3 should be reported. The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must fulfill this criterion [6,7].

6. Accuracy

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the quality control samples (QC samples). The QC samples should be

spiked independently from the calibration standards, using separately prepared stock solutions, unless the nominal concentration(s) The QC samples are analysed against the calibration curve, and the obtained concentrations are compared with the nominal value. The accuracy should be reported as percent of the nominal value.

A) Within-run accuracy Within-run accuracy should be determined by analysing in a single run a minimum of 5 samples per level at a minimum of 4 concentration levels which are covering the calibration curve range: the LLOQ, within three times the LLOQ (low QC), around 30 - 50% of the calibration curve range (medium QC), and at least at 75% of the upper calibration curve range (high QC). The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

B) Between–run accuracy in the between-run accuracy, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.[9,10]

7. Precision

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). Precision should be demonstrated for the LLOQ, low, medium and high QC samples

A) Within-run precision For the validation of the within-run precision, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples in a single run. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

B) Between –run precision For the validation of the between-run precision, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%

8. Dilution integrity

Dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria, i.e. within ±15%. Dilution integrity should cover the dilution applied to the study samples.[11]

9. Matrix effect

Matrix effects should be investigated when using mass spectrometric methods, using at least 6 lots of blank matrix from individual donors. Pooled matrix should not be used. For each analyte and the IS, the matrix factor (MF) should be calculated for each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalized MF calculated from the 6 lots of matrix should not be greater than 15 %. This determination should be done at a low and at a high level of concentration (maximum of 3 times the LLOQ and close to the ULOQ). The overall CV calculated for the concentration should not be greater than 15 %.[12,13]

10. Stability study

The following stability tests should be evaluated:

- Stability of the stock solution and working solutions of the analyte and internal standard,
- Freeze and thaw stability of the analyte in the matrix from freezer storage conditions to room temperature or sample processing temperature
- Short term stability of the analyte in matrix at room temperature or sample processing temperature
- Long term stability of the analyte in matrix stored in the freezer
- The calibration curve and coefficient of variation is shown in diagram of with their regration it should not more then **0.999** to get perfect calibration curve.(14,15)

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