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A General Review on Bioanalytical Method Development & Validation for LC-MS/MS

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ABSTRACT
Rapid growth in the use of LC-MS/MS for the analysis of drugs in biological matrices has been compelled by the need for timely and high-quality data at every stages in drug discovery and development process: from throughput screening of drug candidates and rapid data generation for pre-clinical studies to almost 'real-time' analysis of clinical samples. A well developed bioanalytical development and its validation plays a pivotal role in achieving the goals. . The aim behind this review is to enlighten the need of validation which provide a practical approach for determining the different parameters like selectivity, specifity, limit of detection, lower limit of quantitation, linearity, range, accuracy, precision, recovery, stability, ruggedness, and robustness to help the perfect studies of pharmacokinetic, toxic kinetic, bioavailability and bioequivalence. Bio-analysis study is for the quantitative determination of drug and their metabolites in biological fluids. Accurate and robust methods for quantitative analysis of drug and their metabolites are important for the successful conduct of pre-clinical, bio-pharmaceutics and clinical pharmacology.

Keywords - *LC-MS/MS bioanalysis , Bio-analytical method development,Validation parameters, sample Preparation LLE, SPE.*

INTRODUCTION
The word “high Bioanalytics” refers to the analysis of the desired analyte in biological fluids. In the present pharmaceutical industry, the bioanalytical methods are playing a crucial role in the quantitative determination of low molecular weight drug molecules and macromolecules. The quantitative determination leads to the evaluation and interpretation of pharmacokinetic, bioavailability, drug-drug interaction, bioequivalence and compatibility studies [1]. Validation of any analytical method ensures that the developed method is reproducible, stable, sensitive, robust, suitable and reliable for its application in blood, plasma, urine, serum and faeces analysis. Bioanalytical validation ensures the high-quality data for regulatory submission as well as for the drug discovery and development [2].

The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of drug products [3] and includes all the procedures demonstrating particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, reliable and reproducible for the intended use [4,5]. The analysis thus carried out must be verified for its alleged purpose and must be validated. An investigation should be performed during each step to determine whether the external environment, matrix or procedural variables can affect the estimation of analyte in the matrix from the time of collection up to the time of analysis [6]. Recent progress in methods development has been largely a result of improvements in analytical instrumentation.

Both HPLC and LC-MS/MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits and demerits. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds but it does not give the high sensitivity as required by some of the potent, low dose drugs and lacks selectivity [7]. The main advantages of LCMS-MS include low

detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Despite their high sensitivity and selectivity LC/MS/MS instruments are limited to some extent due to matrix-induced differences in ionization efficiencies and ion suppression/enhancement effects due to biological matrix. HPLC coupled with UV, PDA or fluorescence detector offers a cost effective bioanalytical method [8,9,10]. Depending on the sensitivity, selectivity and cost effectiveness of the method a choice needs to be made between HPLC AND LCMS-MS.

METHOD DEVELOPMENT

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment [11,12]. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

Sample preparation & extraction procedures for drug and its metabolites from biological samples

The biological media that contain the analyte are usually blood, plasma, urine, serum etc. Blood is usually collected from by different blood withdrawl routes like retro-orbital sinus, tail vein method, dorsal pedal vein (depending on the assay sensitivity and the total number of samples taken for a study being performed). The blood is directly withdrawn into tubes with an anticoagulant, e.g. EDTA, heparin etc. Plasma is obtained by centrifugation at 4000 rpm for 15 min. About 30 to 50% of the original volume is collected [13].

The purpose of sample preparation is to clean up the sample before analysis and/or to concentrate the sample. Material in biological samples that can interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts [14]. A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. General procedures for sample preparation like liquid/liquid extraction, solid-phase extraction (SPE) and protein precipitation.

Liquid-liquid extraction- It is based on the principle of selective extraction of intended analyte present in liquid sample through immiscible organic solvent. LLE is based on differential solubility and partitioning of two immiscible liquid phases in one, it required two immiscible phases, in most of the cases one aqueous and second organic phase, both phases must be immscible [15]. Analyte can be removed from the matrix selectively by choosing a suitable extraction solvent and buffering (pH adjustment of the sample to analyte in unionized form)of sample, if required. The solvents should match the analytes polarity while still being immiscible with water and it should preferably be compatible with the detection method. A large volume of the extraction solvent, compared to the sample, favours partitioning and the enrichment of the analytes is often insufficient [16,17]. The solvents which generally used are tert-butyl methyl ether(TBME), dichloromethane(DCM), ethyl acetate(EA), diethyl ether(DEE), hexane etc or in combination with any other suitable solvent can be used as an extraction solvent.

Solid-phase extraction- It is based on adsorption (or) Partitioning on to solid sorbent (Absorbent)selective retardation of analyte using solid sorbent under specific conditions. SPE based on the selective adsorption mechanism. If the targeted analyte are adsorbed on the solid phase, they can selectively be removed or eluted by using an appropriate elution solvent. SPE occur between a solid phase and a liquid phase. SPE is more efficient separation process than LLE. It is easier to obtain a higher recovery of analyte. It employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed with 0.1 to 0.5g o sorbent. The sorbent is usually reversed phase SPE (RP-SPE) assembles both LLE and reversed phase HPLC in its separation characterstics [17]. In SPE, liquid sample is added to the cartridge and wash solvent is selected so that the analyte is either stongly retained (k>>1) or unretained (k=0). When the analyte is strongly retained, interferences are eluted or washed from the cartridge so as to minimize their presence in the final analyte fraction. The analyte is then eluted in a small volume with strong elution solvent, collected, and either ijected directly or evaporated to dryness followed by dissolution in the mobile phase. In the opposite case,

where analyte is weakly retained, interferences are strongly held on the cartridge and the analyte is collected for the further treatment [18].

Protein precipitation- Protein precipitation is the simple method of extraction as compared to the LLE and SPE. This can be carried out by using the suitable organic solvents which has good solubility of the analyte and protein precipitating properties. Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. After protein precipitation the supernatant obtained can be injected directly in to the HPLC or it can be evaporated and reconstituted with the mobile phase [19].

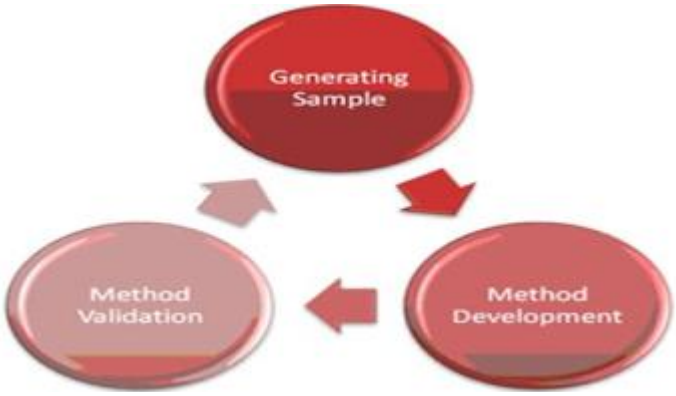

Fig 1: General steps of method development & validation

BIOANALYTICAL METHOD VALIDATION

Bioanalytical method validation includes all of the procedures required to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. The most widely employed bioanalytical techniques include , mass spectrometry based methods (such as GC-MS and LC-MS), tandem mass spectrometry based methods (such as LC-MS-MS) and ligand- based assays (such as RIA and ELISA). Many of the principles, procedures, and requirements of bioanalytical method validation are common to all types of analytical methodologies.

Bio-analytical method validation or bio-analysis of drug has its own value which will depend upon analytes nature and technology which use for the method development and validation. A reliable and reproducible methods and techniques are always very demanding for the drugs and its metabolites studies for bioavailability (BA), bioequivalence (BE) and pharmacokinetic (PK) parameter for conducting the pre clinical studies [20-21]. A perfect approach to reach the drugs and its metabolites best efficacy and side effects value can be known by the accurate and sensitive method development and validation. In respect of economy and market demand the chief and best bio-analytical methods are adopted for routine analysis [22]. In bio-analytical method validation different types and levels are come which must be need to understand basic requirement in the process. Here all types are defined in very specific manner.

Full Validation- Full validation of bioanalytical methods is important:

- During development and implementation of a novel bioanalytical method.
- For analysis of a new drug entity.
- For revisions to an existing method that add metabolite quantification.

Partial Validation- Partial validations evaluate modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Typical bioanalytical method modifications or changes that fall into this category include but are not limited to:

- Bioanalytical method transfers between laboratories or analysts.
- Change in analytical methodology (e.g., change in detection systems).
- Change in anticoagulant in harvesting biological fluid (e.g., heparin to EDTA).
- Change in matrix within species (e.g., human plasma to human urine).
- Change in sample processing procedures.
- Change in species within matrix (e.g., rat plasma to mouse plasma).
- Change in relevant concentration range.

Cross-Validation- Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation in which an original validated bioanalytical method serves as the *reference,* and the revised bioanalytical method is the *comparator.* The comparisons should be done both ways, when sample analyses within a

single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter-laboratory reliability [23,24].

Need & importance for analytical methods to be validated

The main objective of a method validation is to check the reliability of a particular method for evaluating an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. Moreover, if an anticoagulant is used, validation should be performed using the same anticoagulant as for the study samples [25]. Generally a full validation should be performed for each species and matrix concerned for the standardization o the procedure by which an analyte is analyzed.

PARAMETERS OF VALIDATION- [26-32]

1. System suitability

System suitability should be confirmed prior to each run to ensure the performance of the instrument for that particular bioanalytical method. The validity of the analysis is done on routinely basis and checked by evaluation of calibration curves and QC sample in each analytical run. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch or before any re-injection. The Signal to Noise ratio should be more than or equal to 5 for LLOQ QC sample.

2. Selectivity/specificity

Selectivity is a term defined as the ability of an bioanalytical method to differentiate and quantify the analyte in the presence of other components in the sample & specificity is a term which is defined as the ability of the bioanalytical method to produce a signal only for the analyte of interest and not for other interfering components. Generally, the procedure followed for the evaluation of selectivity & specificity is to compare the response of an analyte in the biological sample at the lower limit of quantification (LLOQ) with blank matrix sample. It is recommended to take blank matrix from at least six different sources and compare it with the spiked LLOQ in the matrix.

3. Accuracy & Precision

Accuracy is defined as the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte and it is determined by replicate analysis of samples containing known amounts of the analyte (i.e., QCs). Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy.

Precision is defined as The closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix and it is measured by using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

$$\% \, Bias = \left(\frac{measured\ value - true\ value}{true\ value}\right) \times 100$$

$$\% \, CV = \left(\frac{SD}{Mean}\right) \times 100$$

4. Calibration curve/Linearity range

It represents the relationship between the response of the instrument and the known concentration of the analyte. A calibration curve should be performed for each analyte in the sample. The calibration standards can contain more than one analyte. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The calibration curves were constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS), and eight concentrations covering the expected range including the LLOQ. The drug-to-IS peakarea ratio was plotted against the respective standard drug concentration to obtain the graph and the linearity was evaluated by weighted (1/x) least-squares regression analysis. The acceptance criteria for each calculated standard concentration was no more than 15% deviation from the nominal value, except for the LLOQ, for which 20% was acceptable. Unknown sample peak–area

ratios were then interpolated from the calibration curve to provide concentrations of unknown samples.

5. Recovery

It is defined as the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent. The procedure involves by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and IS should be consistent, precise, and reproducible.

$$\text{Recovery } \% = \frac{\text{Calculated concentration}}{\textit{Spiked concentration}} \times 100$$

6. Stability

Validation studies should determine the analyte stability after the freeze-thaw cycles, short term and long-term storage. The stability of the analyte should be evaluated in the stock and working solutions using solutions at or near the highest and lowest concentration levels under the actual solution storage conditions. According to EMA guidelines it is recommend to evaluate the stability of at least 3 replicates per QC concentration level before and after the stability storage. The acceptance criteria suggest that the mean accuracy of the measurements at each level should be within ±20% deviation of the theoretical concentrations , while USFDA draft guidelines suggest that the stability study sample results should be within ±15% of the nominal concentrations.

7. Matrix effect

It is defined as the effect or the response produced by any undesired analyte which have an interference with the analyte response. Generally, it is caused by the matrix components or the metabolites which get eluted along with the analyte. Matrix effect can also be caused when molecules co-eluting with the compounds of interest alter the ionization efficiency of the electrospray interface. Usually, the matrix effect is assessed either by post extraction addition method or the post-column infusion method.

Matrix effect can be evaluated by spiking analyte at two concentration levels (low and high QC) in six different lots of blank matrix and reading against freshly prepared CC. The matrix effect is calculated by matrix factor (MF). Matrix factor can be calculated by the following formula:

$$\text{Matrix Effect} = \frac{\text{Analyte (or IS) response in spiked blank extract}}{\text{Analyte (or IS) response in neat solution}}$$

8. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Bioanalytical validation methods	**US FDA guidelines**
Selectivity (specificity)	Analyses of blank samples of the appropriate biological matrix (plasma, urine or other matrix) should be obtained from at least six sources. Each blank should be tested for interference and selectivity should be ensured at LLOQ
Accuracy	Should be measured using a minimum of six determinations per concentration. Minimum of three concentrations in the range of expected concentrations is recommended for determination of accuracy. The mean should be ±15% of the actual value except at LLOQ, where it should not deviate by ±20%. This deviation of mean from the true values serves as the measure of accuracy
Precision	Precision should be measured using a minimum of five determinations per concentration. Minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the CV except for the LLOQ, where it should not exceed 20% of the CV
Recovery	Recovery experiments should be performed at three concentrations (low, medium and high) with unextracted standards that represent 100% recovery
Calibration curve	Should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ
LLOQ	Analyte response should be five times the response compared to blank response. Analyte peak should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%
Freeze–thaw stability	Analyte stability should be determined after three freeze–thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at intended storage temperature for 24 hours and thawed at room temperature. When completely thawed, refreeze again for 12–24 hours under same conditions. This cycle should be repeated two more times, then analyze on third cycle. Standard deviation of error should be <15%. If analyte is unstable, freeze at -70°C for three freeze–thaw cycles
Short-term stability	Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature for 4–24 hours and analyzed. Percent deviation should be <15%
Long-term stability	At least three aliquots of each of low and high concentrations at same conditions as study samples. Analyze on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis
Stock-solution stability	Stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. Percent deviation should be <15%

QC samples	QC samples in duplicates at three concentration levels (one near the 3× LLOQ, one in mid range, one close to high end) should be incorporated at each assay run. At least four out of every six should be within 15% of the respective nominal value. Two of the six may be outside of 15% but not both at the same concentration. Minimum number QCs should be at least 5% of total number of unknown samples or six total QCs, whichever is greater

Table No. 1: US FDA guidelines for bioanalytical method validation

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