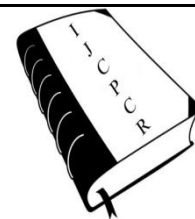International Journal of
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# A REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY USING LC-MS/MS

## Murugan S*, Pravallika N, Sirisha P, Chandrakala K

Department of Pharmaceutical Analysis, Seven Hills College of Pharmacy, Tirupathi-517561, Andhra Pradesh, India.

**ABSTRACT**

Development and validation of bioanalytical method is important to understand the pharmacokinetics of any drug and/or its metabolites. Liquid chromatography-mass spectrometry (LC-MS/MS) is a technique that uses liquid chromatography (or HPLC) with the mass spectrometry. (LC-MS/MS) is commonly used in laboratories for the qualitative and quantitative analysis of drug substances, drug products and biological samples. This article reviews various extraction techniques like liquid–liquid extraction, solid phase extraction and protein precipitation which play important role in sample preparation and detection by LC-MS/MS. 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.

**Key words:** LC-MS/MS bioanalysis, Method development, Method validation.

## INTRODUCTION

Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data [1]. Both HPLC and LCMS-MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds. 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[2]. 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[3-4]. The fundamental parameters for this validation include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability. This guideline provides assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs) and supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation. This guideline also applies to bioanalytical methods used for non-human pharmacology/toxicology studies and preclinical studies. For studies related to the veterinary drug approval process, this guidance applies only to blood and urine BA, BE and PK studies.

## 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,

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Corresponding Author :- **Murugan S** Email:- msm_apcp07@yahoo.com

quantitative or qualitative measurement, precision required and necessary equipment. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

## Sample collection and sample preparation

The biological media that contain the analyte are usually blood, plasma, urine, serum etc. Blood is usually collected from human subjects by vein puncture with a hypodermic syringe up to 5 to 7 ml (depending on the assay sensitivity and the total number of samples taken for a study being performed). The venous blood is 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[5].

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 [6]. 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 principles of differential solubility and partitioning equilibrium of analyte molecules between aqueous (the original sample) and the organic phases. Liquid-liquid extraction generally involves the extraction of a substance from one liquid phase to another liquid phase [7]. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase microextraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME) [8].

## Solid-phase extraction

SPE is a selective method for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Due to many different choices of sorbents, SPE is a very powerful technique [9]. SPE consists of four steps; conditioning, sample loading, washing and elution.

## Conditioning

The column is activated with an organic solvent that acts as a wetting agent on the packing material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms.

## Sample loading

After adjustment of pH, the sample is loaded on the column by gravity feed, pumping or aspirating by vacuum.

## Washing

Interferences from the matrix are removed while retaining the analyte.

## Elution

Disruption of analyte-sorbent interaction by appropriate Typically, sorbents used in SPE consists of 40 μm diameter silica gel with approximately 60 A° pore diameters. To this silica gel, functional groups are chemically bonded, for different modes of action. The most commonly used format is a syringe barrel that contains a 20 μm frit at the bottom of the syringe with the sorbent material and another frit on top, referred to as packed columns. Extraction disks are also placed in syringe barrels. These disks consist of 8–12 μm particles of packing material imbedded into an inert matrix. Disks are conditioned and used in a similar way as packed columns. The major advantage of disks compared to packed columns is that higher flow rates can be applied. Analytes can be classified into four categories; basic, acid, neutral and amphoteric compounds. Amphoteric analytes have both basic and acidic functional groups and can therefore function as cations, anions or zwitterions, depending on pH [10].

## Protein precipitation

Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt or by changing the pH which influence the solubility of the proteins [11]. The samples are centrifuged and the supernatant can be injected into the LC system or be evaporated to dryness and thereafter dissolved in a suitable solvent. A concentration of the sample is then achieved. There are some benefits with the precipitation method as clean-up technique compared to SPE. It is less time consuming, smaller amounts of organic modifier or other solvents are used. But there are also disadvantages. The samples often contain protein residues and it is a non-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may interfere in the LC-system. However the protein precipitation technique is often combined with SPE to produce clean extract.

Methanol is generally preferred solvent amongst the organic solvent as it can produce clear supernatant which is appropriate for direct injection into LC-MS/MS. Salts are other alternatives to acid and organic solvent precipitation. This technique is called as salt-induced precipitation. As the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution [12-13].

**METHOD VALIDATION**

Method validation is a process used to verify/confirm that an analytic method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte. Typical parameters to validate are; include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability. General recommendation for analytical method validation, i.e. for pharmaceutical methods, can be found in The US Food and Drug Administration (FDA) guideline [14].

**Selectivity**

Selectivity exercise is carried out to assess the ability of the bioanalytical method to differentiate and quantify the analyte(s) in presence of other components in the sample. For selectivity, analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) obtained from atleast six sources should be carried out. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ).

**Accuracy**

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

**Precision**

The precision of an analytical method describes 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. Precision should be measured using a minimum of five determinations per concentration. A 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 coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability, which measures precision with time and may involve different analysts, equipment, reagents and laboratories.

**Linearity and range**

A calibration curve is the relationship between instrument response and known concentration of the analyte. The calibration curve should be prepared in the same biological matrix as the samples and a calibration curve should be generated for each analyte. The range of the method is the concentration interval where accuracy, precision and linearity have been validated. The used calibration curve should be the simplest model that adequately describes the concentration-response relationship. The deviation should not exceed more than 20% from the nominal concentration of the LLOQ and not more than 15% from the other standards in the curve.

**Limit of detection**

The limit of detection (LOD) is a characteristic for the limit test only. It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. The detection is usually expressed as the concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb).

**Limit of quantification**

Lower limit of quantification: LLOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. Detemining LLOQ on the basis of precision and accuracy is probably the most practical approach and defines the LLOQ as the lowest concentration of the sample that can still be quantified with acceptable precision and accuracy. LLOQ based on signal and noise ratio (s/n) can only be applied only when there is baseline noise, for example to chromatographic methods. Upper limit of quantification: ULOQ is the maximam analyte concentration of a sample that can be quantified, with acceptable precision and accuracy. The ULOQ is identical with the concentration of the highest calibration standards.

**Recovery**

The recovery of an analyte in an assay is 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 pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

**Matrix effect**

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along with internal standard are prepared and screened [15-17].

### Robustness

According to ICH guidelines, The robustness of an analytical procedure is the 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. Robustness can be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method.

### Stability

The stability of the analyte under various conditions should also be studied during method validation. The conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The following stability conditions are required by FDA and are advisable to investigate;

### Stock solution stability

The stability of the stock solution should be evaluated at room temperature for at least 6 hours.

### Short-term temperature stability

The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed.

### Long-term temperature stability

The stability of the analyte in the matrix should exceed the time period from sample collection until the last day of analysis.

### Freeze and thaw stability

The stability of the analyte should be determined, after three freeze and thaw cycles. Three aliquots of low and high concentration should be frozen for 24 hours and then thawed at ambient temperature.

### Post-preparative stability

The stability of the analyte during stages of the analysis process should be evaluated.

**Figure 1. General solid-phase extraction procedure**



Analyte ▲
Interferences ▢ ✕
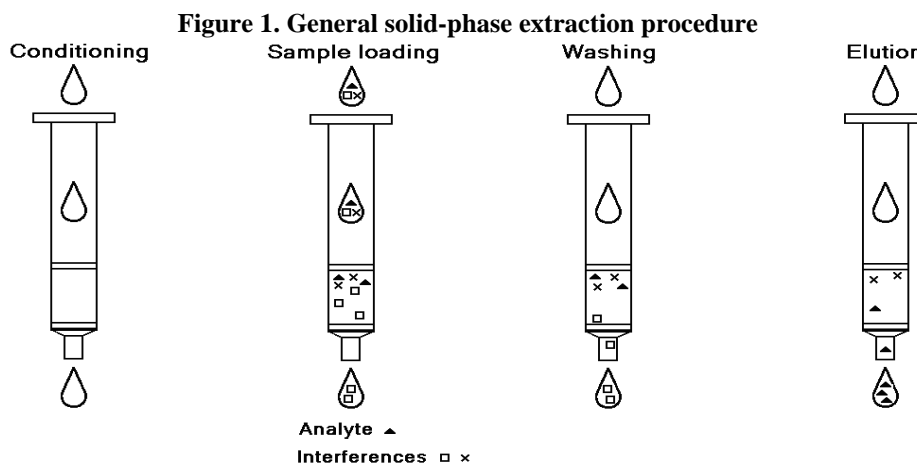
### CONCLUSION

The relatively new concepts and recent progress made in several areas including sample preparation, separation and how to reduce matrix effect discussed in this review article attest to the fact that LC-MS/MS has been used as the technique of choice for bioanalysis of small molecules. The concepts and relatively new technology covered in this review article can be used to enhance LC-MS/MS bioanalytical method development and the matrix effect caused due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

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