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# BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DRUGS IN HUMAN PLASMA BY CHROMATOGRAPHIC METHOD: A REVIEW

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## ABSTRACT

Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetics and toxic kinetic studies. For various types of drug approval processes at different timings to regulate and harmonize bioanalytical method development and validation is required. Gas chromatography, high-pressure liquid chromatography, LC and GC, combined with mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS are used for quantitative analysis. Techniques such as high pressure liquid chromatography (HPLC) and liquid chromatography

coupled with double mass spectrometry (LCMS-MS) can be used for the bioanalysis of drugs in body. Bioanalytical method development and validation can be performed with various validation parameters by using LC-MS/MS and other analytical techniques. Also, there are various stability guidelines and procedures were set which are useful for bioanalysis. The present review is having a special concern on regulatory and practical perspectives to researchers for development and validation of the bioanalytical method.

**Keywords: Bioanalytical method development, Sample extraction Techniques, Instrumentation for bioanalytical method development, Validation parameters**

## INTRODUCTION

Bioanalysis is the method used to determine the concentration of drugs, their metabolites and or endogenous substances in the biological matrices such as blood plasma, serum, cerebrospinal fluid, urine and saliva. Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetics and toxic kinetic studies. It helps in carrying studies like pharmacodynamics, toxicology, pharmacokinetics, bioequivalence, therapeutic drug monitoring (TDM) and clinical studies. Initial stages these studies are done only to find out over dosage conditions and in toxicological studies. When concentration of drug in biological matrix is known, then pharmacokinetic parameters are calculated from that. Bioanalytical studies are important in drug discovery and development. Bioanalysis is an essential part in drug discovery and development **[1]**. For various types of drug approval processes like INDs, NDAs, ANDAs, veterinary drug approval, the data related to bioanalytical method development and validation is needed. Various agencies namely US FDA, American association of pharmaceutical scientists (AAPS), Health protection Branch (HPB), Association of analytical chemists (AOAC), Center for Veterinary Medicine (CVM), U.S. Department of Health and Human Services Food and drug Administration, Center for Drug Evaluation and Research (CDER), European Medicine Agency (EMA), China Food and Drug administration(CFDA), European Bioanalytical Forum (EBF), Global CRO council (GCC), ANVISA (Brazil), Japan Bioanalytical Forum (JBF) had done collective efforts at different timings to regulate and harmonize bioanalytical method development and validation. Regulatory guidance documents are available as a result of the involvement of various official agencies. Bioanalytical method development and validation can be

performed with various validation parameters by using LC-MS/MS and other analytical techniques. Also, there are various stability guidelines and procedures were set that are useful for bioanalysis **[2]**. The selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are very much critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacology studies. High pressure liquid chromatography (HPLC) is most commonly and widely applied analytical techniques because of its highly selective and high reliability, especially in the pharmaceutical, environmental, forensic, clinical, and food department **[3, 4]**.

The present review is having a special concern on regulatory and practical perspectives to researchers for development and validation of the bioanalytical method.

**BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION**

Bioanalysis is related to the analysis of analytes (drugs, metabolites, biomarkers) in biological samples and it involves various steps from sample collection to sample analysis and data reporting. Method development includes sample preparation sampling, separation, detection and evaluation of the results and finally conclusion.

The first step is sample collection from clinical or preclinical studies, then sending the samples to laboratory for analysis. Second step is sample clean-up (sample preparation) and it is very important step in bioanalysis. In order to reach reliable results, a robust and stable sample preparation method should be applied. The role of sample preparation is to remove interferences from sample matrix and improve analytical system performance. Sample preparation is often labor intensive and time consuming. Last step is to the sample analysis and detection. For separation and detection, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is method of choice in bioanalytical laboratories. This is due to high selectivity and high sensitivity of the LC-MS/MS technique. In addition, the information about the analyte chemical structure and chemical properties is important to be known before the start of bioanalytical work. These validated methods provide important data related to safety and effectiveness of drugs and biological products. The validated method addresses the key questions related to specificity, accuracy and precision, sensitivity, sample collection, handling, storage of analyte. There is need for partial or cross validation when there are changes to a validated method **[5]**.

**SAMPLE EXTRACTION TECHNIQUES OF BIOANALYTICAL METHODS**

Some of the following methods are:

A. Extraction method

B. Protein precipitation

C. Chromatography method

D. Ligand binding assay (LBA).

There are various methods of extraction as follows.

**A. Extraction method**

**1. Liquid-liquid extraction** (LLE)

Liquid-liquid extraction is to separate analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE often is aqueous and second phase an organic solvent. More hydrophilic compounds prefer the polar aqueous phase; whereas more hydrophobic compounds will be found mainly in the organic solvents. Analyte extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted in to the aqueous phase can often be injected directly on to a reversed-phase column. The technique is simple, rapid and has relatively small cost factor per sample when compared to others **[6]**. The extraction containing drug can be evaporated to dryness and the residue reconstituted in a smaller volume of an appropriate solvent (preferably mobile phase). Near quantitative recoveries (90%) of most drugs can be obtained through multiple continuous extractions. Nowadays liquid extraction replaced with advanced and improved methods like liquid phase micro extraction and supported membrane extraction, single drop liquid phase micro extraction **[7]**.

**2. Solid phase extraction**

SPE is method for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Solid phase extraction includes four steps; conditioning, sample loading, washing and elution.

I. Conditioning: The column prepared 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.

II. Sample loading: After adjustment of pH, the sample is enterd on the column by gravity feed, pumping or aspirating by vacuum.

III. Washing: Interferences from the matrix are removed while retaining the analyte.

IV. Elution Distribution of analyte: sorbent interactions by suitable solvent, removing as little of the remaining interferences as possible. Generally, sorbents used in SPE consists of 40 μm diameter silica gel with around 60 A0 pore diameters. To this silica gel, functional groups are chemically

bonded. 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. Extractions disks are placed in syringe barrels. These disks consist of 8-12 µm particles of packing material fixed 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 easily applied **[8, 9]**.

**B. 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 and further cleanup of the sample can be carried out by using micro centrifuge at very high speed. Preliminary evaluation of lower limit of quantification to be done after fixing the sample processing technique. Using the biological matrix with lowest interference, prepare at least three aliquots at each concentration level,

Washing pattern of auto injector needle to be evaluated to avoid carryover of previous injections to next injections. Biological samples such as plasma, feces and saliva contain significant quantities of protein and it can bind a drug easily. Acidic drugs bind more strongly to plasma proteins than do neutral or basic drugs. Proteins, salts, lipids and other endogenous materials can cause rapid deterioration of HPLC column.Protein precipitation is used in routinely analysis to remove proteins. Precipitation can be induced by the addition of an organic modernizer, a salt or by changing the pH which influence the solubility of the proteins. The samples are centrifuged and the supernatant can be inserted into the HPLC system or be evaporated to dryness and dissolved in a suitable solvent. There are some aids with precipitation method as cleanup technique compared to SPE. It is less time-consuming, little amounts of organic modifier or other solvents are used. But there are also disadvantages; the samples often contain protein particles and it is a no-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may restrict in the reversed phase HPLC - system. However, the

protein precipitation technique is often combined with SPE to produce clean extract. Methanol is generally favored solvent among the organic solvents as it can produce clear supernatant which is appropriate for direct addition into HPLC. Salts are other alternative to acid 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 **[10, 11]**.

**C.Chromatographic method**

Reference standards: Analysis of drugs and their metabolites in biological fluids is performed using calibration Standards and quality control samples (QCs) spiked with reference standards. The purity of the reference standard used to prepare spiked samples can affect study data. For this reason, Authenticated analytical reference standards of known identity and purity must be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, a predictable chemical form (free base or acid, salt or ester) of known purity can be used **[12]**.

Three types of reference standards are usually use

• Certified reference standards (e.g., USP compendial standards).

• Commercially-supplied reference standards obtained from a reputable commercial source.

• Other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source, expiration date, lot number, documentations of analyses when existing, and/or internally or externally generated evidence of identity and purity should be unfurnished for each reference and internal standard (IS) used. If the reference or IS expired, stock solutions made with this lot of standard should not be used unless purity is reestablished **[13, 14]**.

**D. Ligand binding assay**

Numerous of the bioanalytical validation parameters and principles discussed above are also applicable to microbiological and LBA. These types of assays have a variety of design configurations that possess some unique features that should be considered during method validation. Key reagents Key reagents, such as reference standards, antibodies, tracers, and matrices should be characterized appropriately and stored under defined conditions. Assay reoptimization or validation may be important when there are changes in key reagents. For example: Labeled analytes (tracers), Binding should be reoptimized and Performance should be verified with standard curve and QCs.

**INSTRUMENTATION FOR BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION**

Gas chromatography, high-pressure liquid chromatography, LC and GC, combined with mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS are used for quantitative analysis. For the quantification of conventional, low molecular weight drugs in biological fluids has shifted dramatically in favor of mass spectrometry-based methods, particularly LC-MS and LC-MS-MS. In the years of 90's there have been tremendous advancements in the field of mass spectrometry with the development of new interfaces, ionization and detection techniques. These advancements resulted in the rapid emergence and widespread commercial use of hyphenated mass spectrometry-based assays, which have largely replaced conventional HPLC, GC, and GC-MS assays **[15]**.

**VALIDATION PARAMETERS**

The developed method should be suitable for analysis of study sample and that is proved on the basis of bioanalytical method validation results. According to FDA guidance following are common method validation terms

- Reference standards
- Critical reagents
- Calibration curve
- Quality control samples (QCs)
- Selectivity and specificity
- Sensitivity
- Accuracy
- Precision
- Recovery
- Stability of the analyte in the matrix

**Reference standards and critical reagents**

During method validation and the analysis of study samples, a blank biological matrix is spiked with the analyte(s) of interest using solutions of reference standard(s) to prepare calibration standards, QCs and stability QCs. Calibration standards and QCs should be prepared from separate stock solutions. However, calibration standards and QCs may be prepared from the same stock solution provided the accuracy and stability of the stock solution have been verified. A suitable internal standard (IS) should be added to all calibration standards, QCs and study samples during sample processing. The absence of an IS should be technically justified. It is important that the reference standard is well characterised and the quality (purity, strength, identity) of the reference standard and the suitability of the IS is ensured, as the quality will affect the outcome of the analysis and, therefore, the study data. The reference standard used during validation and study sample analysis should be obtained from an authentic and traceable source. The reference standard

should be identical to the analyte. If this is not possible, an established form (e.g., salt or hydrate) of known quality may be used. Suitable reference standards include compendial standards, commercially available standards or sufficiently characterised standards prepared in-house or by an external non-commercial organisation.

**Calibration curve**

The quantitation range should be selected for assay and calibration standards based on expected concentration range in the particular type of study. The calibration curve should be generated for each analyte if sample contains more than one analyte. The calibration curve shows the relationship between the nominal analyte concentration and the response of the analytical platform to the analyte. Calibration standards, prepared by spiking matrix with a known quantity of analyte, span the calibration range and comprise the calibration curve. Calibration standards should be prepared in the same biological matrix as the study samples. The calibration range is defined by the LLOQ, which is the lowest calibration standard, and the ULOQ, which is the highest calibration standard. There should be one calibration curve for each analyte studied during method validation and for each analytical run. A calibration curve should be generated with at least 6 concentration levels of calibration standards, including LLOQ and ULOQ standards, plus a blank sample. The blank sample should not be included in the calculation of calibration curve parameters. Anchor point samples at concentrations below the LLOQ and above the ULOQ of the calibration curve may also be used to improve curve fitting. The relationship between response and concentration for a calibration curve is most often fitted by a 4- or 5-parameter logistic model, there are data points near the lower and upper asymptotes, although other models may be used with suitable justification.

**Quality control samples**

For determining precision and accuracy as well as stability, the quality control samples should be used. During method development stage, freshly prepared QCs are recommended. The endogenous concentrations of the analyte in the biological matrix should be evaluated prior to QC preparation (e.g., by replicate analysis). The blank matrices with the minimum level of the endogenous analyte should be used. The concentrations of the QCs should account for the endogenous concentrations in the biological matrix (i.e., additive) and be representative of the expected study concentrations. The QCs used for validation should be aliquots of the authentic biological matrix unspiked and spiked with known amounts of the

authentic analyte. In spiked samples, the added amount should be enough to provide concentrations that are statistically different from the endogenous concentration.

**Selectivity and specificity**

Selectivity is the ability of the method to detect and differentiate the analyte of interest in the presence of other "unrelated compounds" (non-specific interference) in the sample matrix. The matrix can contain non-specific matrix component such as degrading enzymes, heterophilic antibodies or rheumatoid factor which may interfere with the analyte of interest. Selectivity should be evaluated at the low end of an assay where problems occur in most cases, but it is recommended that selectivity is also evaluated at higher analyte concentrations. Selectivity exercise is carried out to assess the ability of the bioanalytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) obtained from at least six sources should be carried out. Each blank sample should be tested for interference and selectivity should be ensured at the lower LOQ (LLOQ).

**Accuracy**

Accuracy and precision should be determined by analysing the QCs within each run (within-run) and in different runs (between-run). Accuracy and precision should be evaluated using the same runs and data. Accuracy and precision should be determined by analysing at least 3 replicates per run at each QC concentration level (LLOQ, low, medium, high, ULOQ) in at least 6 runs over 2 or more days. A minimum of three concentrations in the range of expected. 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 **[16]**.

**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 needed. 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 inter day, intraday and different analyst or repeatability, this carried out precision or repeatability measure, which measures precision with time and may involve

different analysts, equipment, reagents and laboratories **[17]**.

**Repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

**Intermediate precision**

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. Intermediate precision expresses within-laboratories variations: Different days, different analysts, different equipment's, etc. **[18]**. The ISO definition used the term "M-factor different intermediate precision," where the M-factor expresses the number of factors (operator, equipment, or time) that differ between successive determinations. Intermediate precision is sometimes also called between-run, between day, or inter-assay precision.

**Linearity and range**

The linearity and range would ascertained on the basis of accuracy (recovery) studies and graph would plotted as per %labeled claim versus response.

**Limit of detection (LOD) and Limit of quantitation (LOQ)**

LOQ is the small amount of analyte present in a sample that can be determined quantitatively with suitable accuracy and precision. Determining LOQ on the basis of accuracy and precision is probably the most practical method and defines the LOQ as the lowest concentration of the sample that can still be quantified with acceptable accuracy and precision. LOQ based on signal and noise ratio can only be applied only if baseline noise, for example chromatographic methods.

**Stability**

Stability evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte. The storage and analytical conditions applied to the stability tests, such as the sample storage times and temperatures, sample matrix, anticoagulant, and container materials should reflect those used for the study samples. Reference to data published in the literature is not considered sufficient. Validation of storage periods should be performed on stability QCs that have been stored for a time that is equal to or longer than the study sample storage periods. 871 Stability of the analyte in the studied matrix is evaluated using low and high concentration stability QCs. Aliquots of the low and high stability QCs are analysed at time zero and after the applied storage conditions that are to be evaluated. A minimum of three stability QCs should be prepared and analysed per concentration level/storage condition/timepoint. The stability QCs are analysed against a

calibration curve, obtained from freshly spiked calibration standards in a run with its corresponding freshly prepared QCs or QCs for which stability has been proven. While the use of freshly prepared calibration standards and QCs is the preferred approach, it is recognised that in some cases, for macromolecules, it may be necessary to freeze them overnight. In such cases, valid justification should be provided and freeze-thaw stability demonstrated. The mean concentration at each level should be within ±20% of the nominal concentration. Since sample dilution may be required for many LBA assays due to a narrow calibration range, the concentrations of the study samples may be consistently higher than the ULOQ of the calibration curve. If this is the case, the concentration of the stability QCs should be adjusted, considering the applied sample dilution, to represent the actual sample concentration range. Stability at room temperature or sample preparation temperature and freeze-thaw stability. In 888 addition, long-term stability should be studied. 889 For chemical drugs, it is considered acceptable to extrapolate the stability at one temperature 890 (e.g., -20°C) to lower temperatures (e.g., -70°C). ICH M10 Guideline 27 891 For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the 892 stability has been demonstrated at -70°C and at -20°C, then it is not necessary to investigate the 893 stability at temperatures in between those two points at which study samples will be stored **[19]**.

## CONCLUSION

To develop bioanalytical methods has become more and more challenging over the past years due to very demanding requirements in terms of method reliability, sensitivity, speed of analysis and sample throughput. HPLC has established itself as a method of choice for routine analysis of biological materials. A development of such method consists of several steps including sample preparation and clean-up step, efficient chromatographic separation, sensitive and selective detection of analytes in complex matrices, a choice of convenient data processing and calibration approach and finally method validation. Each of these steps has its own constraints and challenges. Novel and modern approaches in sample preparation and method validation are required to demonstrate the performance of the method and reliability. Now it is widely accepted that bioanalysis is an integral part of the pharmacokinetic/pharmacodynamic characterization of novel chemical entity from the time of its discovery and leading to its market authorization.

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