Journal of Applied Pharmaceutical Science Vol. 13(08), pp 068-083, August, 2023
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2023.109956
ISSN 2231-3354

# Validation steps and parameters of bioanalytical methods using in clinical studies: A narrative review

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**ARTICLE INFO**

Received on: 29/03/2023
Accepted on: 19/07/2023
Available Online: 04/08/2023

***Key words:***
Bioanalytical methods, parameters, therapeutic drug monitoring, validation steps.

**ABSTRACT**

The bioanalytical method certifications (validation) are very essential for therapeutic drug monitoring. It is critical to use well-defined and completely validated procedures to provide reliable results which can be properly interpreted. This review provides practical steps and procedures with bioanalytical methods validation and highlights chromatographic method validations that have been used in the bioanalysis. It also attempts to clarify the nomenclature of certain stages of bioanalytical validation and to describe the overall process used for the drugs measurement and their metabolites in biological matrices and fluids. The stages of this procedure are discussed and correlated with data of scientific literature. A consistent description of key bioanalytical parameters is discussed and investigated with a view to improve scientific standards of bioanalytical methods used in clinical studies.

## INTRODUCTION

Bioanalytical techniques are applied to measure and target biological compounds or drugs and its components in biological fluids such as blood, serum or plasma, urine, and gastric content. These techniques are precise and highly useful and recorded for therapeutic drug monitoring (TDM) (Ermer and Miller, 2006; Viswanathan *et al*., 2007). In clinical practice, the quantitative bioanalysis of drugs and their metabolites provides an important new approach to personalized medicine. It helps the clinician to individualize drug treatment for drugs characterized by a short therapeutic range, and/or decrease the risk of dose-dependent adverse effects (Cremers *et al*., 2016). In addition, the newly available TDM approach has led to several advances that aimed at measuring drug concentrations and relating these results to therapeutic efficacy or secondary effects. Nowadays, several analytical methods were used in routine clinical laboratories, including automated immunoassays (AI), high performance liquid chromatography (HPLC), and liquid chromatography tandem-mass spectrometry (LC-MS/MS) (Mou and Jiang, 2017; Vogeser and Kirchhoff, 2011). However, for such screening in routine bioassay, thin-layer chromatography, gas chromatography (GC) with currently used detectors, and HPLC with a diode array detector (DAD) are mostly applied, but GC-MS is by far the most widely used method in this setting. Although GC-MS is widely applied as the primary reference method for bioanalysis, the intensive work and time-consuming procedures limit their use in the clinical laboratory. On the other hand, the spectrum is largely used in clinical practice, a simple but less selective method. AI is simple to perform with minimal sample preparation and faster (turnaround time). However, it is well recognized that AI is sensitive to cross-reactivity and cannot measure different

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substances simultaneously. Following analytical approaches, there are significant variations between AI methods in terms of patient outcomes as well as reference areas; these differences are responsible for the sensitivity of the antibodies to interference or cross-reaction with structurally similar compounds (Horie *et al*., 2007; Lee *et al*., 2006; Taieb *et al*., 2002). A such bioanalytical validation procedure should support the strength and significance of outcoming results (Boulanger *et al*., 2003; Tiwari and Tiwari, 2010). The initial validation is the first step which must be continuously monitored during the application process to prove its performance (Riley and Rosanske, 1996). Some studies have discussed biomedical validation steps and described protocol for an effective strategy (Ermer and Miller, 2006; Tiwari and Tiwari, 2010). However, bioanalytical method validations require an appropriate statistical analysis to evaluate precision, analytical range, accuracy, sensitivity/specificity, and limit of quantification (LOQ) and detection (LOD). These steps must follow a practical protocol and the obtained results should be compared with predefined quality criteria (Food and Drug Administration, 2016; Peters and Maurer, 2002). According to high importance of bioanalysis assay validation in the field of TDM, some guidance documents related have been published (Capiau *et al*., 2019; van Nuland *et al*., 2020). There is a permanent need for reliable and thoroughly validated bioanalytical techniques in order to detect and measure drugs and other substances in complex mixtures of compounds, such as drugs compositions as well as their metabolites in biological matrix. For several years, the TDM represents an important approach to optimizing clinical care that facilitates the clinician to individualize the treatment in relationship with the patient's physiological profile, control drug doses to achieve a systemic level associated with the desired therapeutic goal, and also reduce the risk of dose-dependent adverse effects (Cremers *et al*., 2016). The role of many TDM assay laboratories is to quantify the concentrations of drugs in a sample and relate this concentration to a therapeutic level published in the literature. The aim of this review was to resume different criteria of bioanalytical method validation and their use in the clinical studies in TDM relation. It was mainly focused on the validation of chromatographic methods that are mostly used. This review paper has also discussed application of solving routine problems related to validation process.

## REGULATION PROCEDURES

Previously, bioanalysts operated under a single Food and Drug Administration (FDA) bioanalytical method validation guideline, but the background differs in regulatory vocabulary that could influence the routine practice of numerous bioanalytical laboratories. The first bioanalytical guideline was published by the FDA (2001). Following this guideline, several draft guidelines have been published in the area of bioequivalence/ bioavailability guidelines is indicated in Table 1, which offer only a brief overview of recommended necessary bioanalytical steps or a recommendation to further bioanalytical standards. The operator is then responsible for using the alternative guidelines to provide complete operational requirements and processes for the performance of the analytical techniques. In this regard, a comprehensive supplementary guidance project has been issued in 2009 from the European Medicines Agency (EMA) (Whitmire *et al*., 2011), which also outlines the requirements for bioanalytical guidance and approaches that parallel those outlined in the workshop papers (Shah *et al*., 2000; Viswanathan *et al*., 2007) or FDA guidance (FDA, 2001). In bioanalytical method validation process, EMA and FDA guidelines are widely used despite some differences in the requirements of each guideline. The guideline provided by the EMA and the FDA on bioanalytical technique validation is largely comparable but not similar. On the basis of the above, there are some differences in the recommended validation parameters. We generally found the format of the FDA guideline clearer and the tables in its supplement very useful. The EMA provides a more precise description of the practical performance of trials. The FDA presents the reports more comprehensively. For bioanalytical method such as liquid chromatography, we also found that the International Conference on Harmonisation (ICH) to be very practical because it combines the advantages of both EMA and FDA guidance to reduce terminology confusion and unnecessary effort to comply with two or more guidelines.

## METHOD DEVELOPMENT

The development of bioanalytical method needs the evaluation and optimization of the different steps such as sample preparation, chromatographic separation, detection systems, quantification, matrix effects and stability of chemical compounds and drugs in the biological matrix.

### Sample preparation

Biological matrix in general, such as blood, serum/ plasma, and urine, due to their complexity and protein concentration, is not suited for direct injection in bioanalytical equipment. A preanalytical step therefore is crucial to prepare the material for the bioanalytical technique (Ashri and Abdel-Rehim, 2011; Nováková and Vlčková, 2009). The objective of sample preparation is to eliminate interfering compounds (including proteins, salts, and lipids) and also to concentrate the analytes. Due to the various physicochemical features of these drugs, selecting the best sample preparation provides a difficulty to methods that quantify drug concentrations.

The most typical extraction techniques presented in Figure 1 are liquid-liquid extraction (LLE) (Blanchard *et al*., 1988; Chang *et al*., 2007; Remane *et al*., 2010), solid-phase extraction (SPE) (Dunér *et al*., 2007; Poole, 2003), and protein precipitation (PP) (Burgess, 2009; Chang *et al*., 2007; Souverain *et al*., 2004). However, during the development of bioanalytical method, the PP and LLE are the major sample preparation techniques for bioanalysis using LC-MS (Ali *et al*., 2008; Raynie, 2006), whereas the PP was made by adding of a precipitating solvent to biological samples using organic solvents such as methanol (MeOH), acetonitrile (ACN), or trichloroacetic acid. In this practical method, the chemical agent used in the sample preparation decreases the plasma's dielectric constant, which improves the attractivity of proteins, resulting in precipitation and protein accumulation (Ryan, 2011). However, LLE showed in Figure 2 may come with some limitations. In case of multiple analytes extraction, it is important to verify that they all have similar partition ratios (similar polarity), as the recovery will not be equivalent. In general, SPE, LLE, and PP are widely applied for sample preparation and enrichment of analytes in biological matrix

**Table 1.** Some international bioanalytical guidance available for bioanalytical method validation.

| Regulatory agency | Document | Year | Guidance | References |
|---|---|---|---|---|
| FDA, USA | Guidance for industry: bioanalytical method validation | 2001 | A comprehensive guidance | FDA (2001) |
| NIHS, Japan | Clinical PK studies of pharmaceuticals | 2001 | Brief description of bioanalytical requirements | Ohno (2001) |
| Association of Southeast Asian Nations | Guidelines for the conduct of bioavailability and bioequivalence studies | 2001 | Adapted from CPMP (EMA) guideline for bioavailability/bioequivalence studies | FDA Thailand (2001) |
| Health Canada | Conduct and analysis of bioavailability and bioequivalence studies | 2002 | Refer to Crystal City 1 report and provide brief description of bioanalytical requirements | Viswanathan (2010) |
| ANVISA, Brazil | Manual for good bioavailability bioequivalence practices | 2003 | Detailed instruction for conducting bioanalysis for bioavailability/ bioequivalence studies | ANVISA (2003) |
| FDA, USA | Bioavailability and bioequivalence studies for orally administered drug products—general considerations | 2003 | Refer to FDA bioanalytical guidance of 2001 | Food and Drug Drug Administration of the United States (2003) |
| State FDA, China | Technique guideline for human bioavailability and bioequivalence studies on chemical drug products | 2004 | Refer to FDA bioanalytical guidance of 2001 and provide brief description of bioanalytical requirements | Bansal *et al*. (2004) |
| Saudi FDA, Saudi Arabia | Bioequivalence requirements guidelines | 2005 | Refer to FDA bioanalytical guidance of 2001 | Saudi Food and Drug Authority (2005) |
| Central Drugs Standard Control Organization, India | Guidelines for bioavailability and bioequivalence studies | 2005 | Brief description of bioanalytical requirements | Ananthakrishnan (2005) |
| KFDA, Republic of South Korea | Guidance document for bioequivalence study | 2008 | Brief description of bioanalytical requirements | KFDA (2011) |
| Health Canada | Conduct and analysis of comparative bioavailability studies (draft) | 2008 | Refer to Crystal City 1 and 3 reports and provide brief description of bioanalytical requirements | Food and Drug Administration (2011) |
| CHMP, EMA, EU | Guideline on validation of bioanalytical methods (draft) | 2009 | Draft for a comprehensive guidance | van Amsterdam *et al*. (2010) |
| CAPA, Egypt | Guidelines for bioequivalence studies for marketing authorization of generic products | 2010 | Brief description of bioanalytical requirements | Shah and Bansal (2011) |
| EMA, EU | Guideline on bioanalytical method validation | 2011 | Refer to guideline on validation of bioanalytical methods of 2009 | Smith (2012) |
| ANVISA, Brazil | Minimum requirements for bioanalytical method validation | 2012 | Refer to manual for good bioavailability bioequivalence practices | ANVISA (2012) |
| EMA, EU | Regulatory-procedural-guideline | 2012 | Reflection-paper-guidance-laboratories-performanalysis-evaluation-clinical-trial-samples | Smith (2012) |
| FDA, USA | Guidance for industry: Bioanalytical Method Validation (draft) | 2013 | Refer to FDA bioanalytical guidance of 2003 | US Food and Administration (2013) |
| EMA, FDA, NIHS | Technical requirements for pharmaceuticals for human use M10: bioanalytical method validation | 2018 | Detailed instruction for conducting nonclinical toxicokinetic /pharmacokinetic (PK) studies and of clinical trials | ICH Guideline (2019) |
| FDA, USA | Guidance for industry: bioanalytical method validation (draft) | 2018 | Current thinking of the FDA guidance for industry: bioanalytical method validation | US-FDA (2018) |

in many bioanalytical laboratories has been summarized in Table 2. Based on previously published research and our own scientific expertise, sample preparation procedures should be identified and improved depending on the objective of bioanalytical method. A suitable technique must be chosen in relation to extraction time, selectivity, number of steps, solvent consumption, and the ability to organize on-line techniques. In this context, sample preparation is frequently the most difficult aspect of developing a bioanalytical procedure. In addition to the above-mentioned sample preparation procedure, affinity chromatography is the single technique that allows purification of an analyte on the basis of biological function or specific chemical structure; this chromatographic technique plays a unique and significant role in separation technology. The use of this separation method allows for highly selective extraction of the target and structurally comparable substances (e.g., a drug and its metabolites) from heterogeneous matrices. However, the

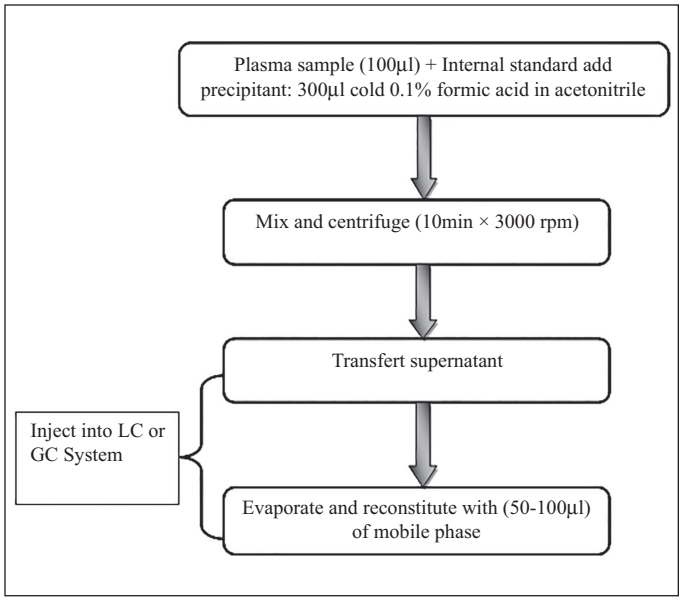


**Figure 1.** Case of PP protocol for plasma samples using organic solvents.

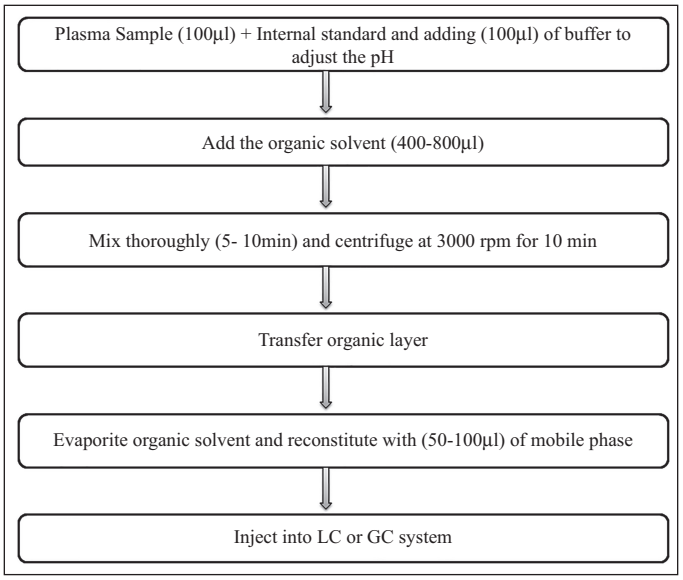


**Figure 2.** Example for LLE steps for plasma samples.

materials used to create this specific preparation sample were frequently cheap, quick, and reproducible; they also had a high capacity and could be recycled and used multiple times.

**Detectors**

For selectivity and sensitivity in the step of bioanalytical method development, the selection of detection tools is extremely important. In some cases, for bioanalysis drugs and their metabolites or biological compounds, methods using ultraviolet DAD (UV-DAD), UV, and mass spectrometry (MS) detector have been applied in the literature. The UV-DAD is an appropriate detector for the identification of this class of drugs and allows a high level of sensitivity for polyunsaturated species. However, UV detection does not distinguish between compounds with similar chromophore groups. More detailed structural information can be taken when an MS is coupled to a UV-DAD. MS is a useful detection for qualitative analysis to identify and confirm the molecular patterns of unknown drugs and is particularly suitable of its high selectivity and sensitivity (Lazaou *et al*., 2000). The benefits of combining liquid chromatography with LC-MS or LC-MS-MS have been demonstrated for many analyses used in various bioanalyses of drugs and their metabolites (Ackermann *et al*., 2002; Lee and Kerns, 1999). The components of an LC-MS system comprise the autosampler, the LC apparatus, and the ionization source that is a part of the mass spectrometer that ionizes compounds and mass spectrometer. In the majority, these components are managed by a single software package. It can be noted that for interfacing LC with MS, there are certain limitations on the mobile phases and flow rate that can be used. The most common solvents of the mobile phase are chemical agents applied to the mobile phase that are used for the chromatographic separation of the sample analysis. During the method development**,** the impact of small changes in the ratio of solvent making up the mobile phase and buffer pH will influence the peak resolution of analytes.

Typical reversed-phase LC systems connected to the MS may use a combination of water/methanol or ACN for the mobile phase. There are restrictions on the components of mobile phase for example, it should be volatile. Typical mobile phase may also contain ammonium acetate, acetic acid, and formic acid. Many papers are available which focus on LC parameters that seem useful in LC-MS analysis (Ackermann *et al*., 2002; Hsieh *et al*., 2003; Tiller *et al*., 2003). In fact, LC-MS and LC-MS-MS are

**Table 2.** Comparison of three sample preparation methods (PP, LLE, and SPE).

| | SPE | LLE | PP |
|---|---|---|---|
| Extraction time (minute) | 10–25 | 10–30 | 10 |
| Solvent volume (ml) | 10 | 10–20 | 0.1–0.5 |
| Simplicity | Moderate | High | High |
| Repeatability | Good | Moderate | Good |
| Cost per analysis | Moderate | Low | Low |
| Commercially available | Yes | - | - |
| Cost of instrument | Moderate | Low | Low |
| Automation | Moderate | Moderate | Low |
| Biological matrix | Serum, plasma and urine | Serum, plasma and urine | Serum, plasma and urine |

used for measurements of newly synthesized substances that are part of a library of compounds. These techniques evaluate that the appropriate product was synthesized and that the purity is adequate for usage in the library. In a subsequent stage, LC-MS is used to examine different physical and chemical characteristics (such as physiological solubility, permeability, and chemical stability) of these new compounds. In addition, a variety of drug metabolite and pharmacokinetic (PK) assays are used in drug discovery to measure the properties of the PK stage of drug molecule, as well as their PK parameters. Several of these tests are dependent on LC-MS or LC-MS-MS for the measurement stage (Tiller *et al*., 2003).

**Selectivity**

During bioanalytical method development steps, the selectivity parameter is a critical criterion for the drug assay and other compounds in biological matrix. This bioanalytical norm is defined as the capacity to detect analyte concentration without interference from sample components; the selectivity for bioanalytical methods must be determined with respect to metabolites of endogenous compounds and known degradation products prior to the validation process. Presumably, the interference merely exists in a trace form and can have a negative effect on the quantification of unidentified compound at concentration approaching to the quantification limit (Vessman, 1996). Selectivity for interference from endogenous compounds in biological matrices could be determined by treating a minimum of diverse sources providing the same blank matrices (Valcárcel *et al*., 2001). A careful analysis of chromatograms over interest peak time windows is necessary to assess selectivity; it must be highlighted that it is not suitable to try a single source of blank matrix (Hartmann *et al*., 1998). However, it is better to test the used blanks and they need to be free from any noise or interference. On the other hand, factors such as the subject's consumption of food and drinks, the ingestion of vitamins additive, the use of nonprescription and prescription drugs other than those tested, and smoking may affect the selectivity criteria. When selecting an inaccurate detection system, serious problems may arise especially when analyte metabolites are undetectable or there are no identifiable known degradation products. In this case, it is necessary to perform the synthesis of specific degradation products and known metabolites, if possible, to validate to verify selection. In the absence of control sample for metabolites or breakdown products, the assays described below may be sufficient to validate selectivity. Biological samples from the patients under treatment could be the best solution; these specimens should be assayed according to the usual chromatographic requirements under varying chromatographic parameters in order to address a large number of potential concerns combining peaks sampling (Dadgar and Burnett, 1995; Peters and Maurer, 2002). If concentrations are sufficiently high and the UV spectrum of the potential byproducts or intermediates change from those of the parent molecule, to provide a purity peak, other multiwavelength detectors, such as a diode array, can be used.

**Analyte stability**

All aspects of analyte stability should be clearly defined: patient preparation, sample collection, transportation to laboratory, handling of the sample in laboratory including storage, and stability during all stages of pretreatment (i.e., stability in an organic solvent). Moreover, according to symposium report (Shah *et al*., 2000), the stability of the analyte was determined as follows: the chemical stability of an analyte in a particular matrix, under specific conditions and for specific time intervals. Generally, there are two types of stability: (1) the stability of the analyte during the different stages of pretreatment and (2) the analyte stability in biological sample. The analyte stability depends on its physicochemical characteristics and the conditions of storage and conservation. The stock solutions' stability and individual analytes must be tested under normal laboratory conditions of temperature, humidity, and light for at least 6 hours in comparison with extemporaneously prepared solution. The storage conditions of these solutions must also be clearly established (4°C, −20°C, etc.). Furthermore, the stability of the analytes must be verified during all stages of pretreatment of the sample (stability in the organic solvent, in the dry extracts, on the automatic sample changer, etc.). In pure solutions, the analyte and the internal standard (IS) are considered stable if the deviation from the theoretical concentration does not exceed 2%. A difference up to 5% is tolerated for the stability tests in the dry extracts and on the autosampler. The analyte stability in the matrices is verified by analyzing control-quality samples at three concentration levels (low, medium, and high); then after different storage times and at different temperatures (each level of concentration is evaluated at least three times). In fact, analyte stability throughout the validation stage is a requirement for detection and quantification. This means that the integrity of the chemical is ensured to be preserved throughout the analysis procedure. During the last steps of method development, additional stability tests might be performed (Braggio *et al*., 1996; Viswanathan *et al*., 2007).

**Dilution integrity and matrix effects**

In practice, biological samples are not fully compatible with analytical equipment based on their suitability for analysis. In order to perform clinical and pharmacological survey and control of patients, the analysis screening of their physiological liquids and biomarkers is crucial. In bioanalytical techniques, numerous biological matrices might be encountered and each biological matrix presents a different challenge to the analyst as it may contain components that can influence or interfere with the method (Van Eeckhaut *et al*., 2009). To run across this problem and eliminate or decrease these matrix effects, different measures can be made, such as SPE, LLE, and PP or micro extraction, which is useful as long as the instrumental sensitivity remains adequate (Heller, 2007). Another approach to reduce matrix effects is the optimization of sample preparation and/or chromatographic parameters (Hernandez *et al*., 2005; Niessen *et al*., 2006; Xu *et al*., 2007), by the use of IS to have an idea of the signal threshold. The use of lower flow-rates, flow division, or the need to resort to standard addition are also described (Van Eeckhaut *et al*., 2009). In most cases, matrix effects are directly related to insufficient purification of the sample under study. During analysis, the effects of the matrix can be reduced by injecting smaller volumes or diluting the sample. However, these solutions will clearly influence the sensitivity of the method and are therefore often inappropriate (Antignac *et al*., 2005). Nowadays, LC-MS and LC-MS/MS have

become a powerful analytical tool. These techniques are sensitive, specific, and allow the analysis of traces in complex mixtures. In addition, LC-MS and LC-MS/MS have been increasingly used in routine clinical laboratories during the last two decades, and their characteristics including specificity, sensitivity, and multianalyte potential make it an ideal alternative to immunoassays and ligand binding assay or HPLC to reduce matrix effect (Nicolas *et al*., 2004).

The sample dilution procedure should not affect the accuracy or precision of the method. During the validation stage, the analyst is required to prepare a control sample and dilute to the appropriate concentration.

In the process of bioanalytical method development, the accuracy and precision around the mean value may not exceed 15%. Except for the LOQ, the coefficient of variation (CV) should also not be greater than 20%. In a few cases, a dilution of the samples is recommended, and in this case the dilution should be carried out using the same matrix as the sample, but not necessarily from the same subject.Additionally, if the dilution factor varies or if the samples in the study are at levels higher than the dilution quality control (QC), a redilution would be required.

### Mobile phase effect

The impact of tiny variations in the solvent ratio of the mobile phase (i.e., <2% of the amount of each component) and the pH buffer, if any, should be discussed and documented for bioanalytical procedures such as chromatographic separation (Polson *et al*., 2003). On the other hand, GC analysis and additional factors, including slight variations in oven temperature and gas flow rate, must be considered. Evaluation of the following criteria should demonstrate whether the technique can maintain critical separation in the face of expected invariability from column to column and the mobile phase daily variation (Van Eeckhaut *et al*., 2009).

## VALIDATION PARAMETERS

Validation of bioanalytical methods is an experimental protocol applied to ensure that the bioanalytical performance parameters are suitable for the purpose use. More consistency in validation practice is applied for chromatographic methods used in clinical studies (Dadgar and Burnett, 1995; Lindner and Wainer, 1998; Shah *et al*., 1992, 2000). In general, for chromatographic methods used in biomedical studies, validation procedures in relation to linearity, accuracy, recovery, precision, and LOQ are required. Supplementary parameters that can be tested include the detection limit and the IS and application method. In this section, a correlation between the parameters of validation that must be examined, as well as their acceptance criteria that must be validated are listed in Table 3.

### Calibration curve and linearity

The calibration curve and its linearity are defined as the predicted concentration in the samples, and for chromatographic assays, it is the correlation between the compound quantity in the sample and the relative detector response (ICH Q8, 2005). In chromatographic assay, to establish the linear correlation with concentration as a calibration standard, peak area can be used as a response function. The selected calibration model should correctly describe the relationship between the response function and the analyte concentration. The relationship between the measured *y*-values and the adjusted or residual *y*-value should be calculated using at least five to eight values (excluding blank values) from the predicted range of concentrations. For each range point made on the same day and at different days, from the equation connecting answer and added concentration (Cr added), a concentration is recalculated (Cr calculated) as well as the corresponding relative error (ER% = $[(Cr_{added} - Cr_{calculated})/Cr_{added}] * 100$). For each concentration added, a concentration recalculated average is determined as well as the coefficient of corresponding variation. This coefficient must be below 15%, but 20% of the threshold of quantification remains acceptable (FDA, 2001; Shabir, 2003). Despite the fact that some bioanalysis may involve the application of a nonlinear calibration, it is usual to apply a linear model, based on the principle of parameters estimation using the least squares method. In this approach, the concentration is an independent variable, and the response serves as the dependent variable. The calculation procedure implicitly assumes that the measurement error is the same and is distributed normally for each sample (Hartmann *et al*., 1998). The linearity of the bioanalytical analysis must be demonstrated as follows: the slope of the linear calibration curve differs from 0 in a statistically significant way, the intercept is not statistically different from 0, and the regression coefficient is not significantly different from 1. If a significant nonzero intercept is obtained, it must be highlighted that there is no effect on the method accuracy (Araujo, 2009; Bischoff *et al*., 2007).

### Recovery

The response of a process spiked matrix standard is calculated as a percentage of the response of a pure standard that has not been pretreated on the sample to estimate the recovery of a bioanalytical technique. It indicates that the method gives a response for the total analyte concentration in the sample (Karnes *et al*., 1991). It can be proven more clearly by evaluating the outcomes of extracted samples at low, medium, and high enriched matrix concentrations with nonextracted standards that reflect 100% recovery in replicates of at least six (Buick *et al*., 1990; Lang and Bolton, 1991). In addition, if an IS is used, its recovery should be measured at the method's concentration level. Although analyte recovery does not have to be 100%, the level of analyte and IS recovery should be accurate, precise, and reproducible. It should also be given (Gao *et al*., 2011; Lang and Bolton, 1991) by absolute recovery (Sonawane *et al*., 2014).

$$\text{Absolute recovery} = \frac{\text{Response of analyte spiked matrix}}{\text{Response of analyte of pure standard}} \times 100.$$

### Quantification and detection limit

The LOD is the lowest amount of analyte that can be detected but not quantified (Singh *et al*., 2008). The LOD estimate is vulnerable to error since some bioanalytical laboratories simply measure the lowest quantity of a reference solution that can be detected while others assess the lowest concentration that can be found in the biological sample. The LOQ for individual analytical methods is the smallest amount of analyte in a sample that can be quantitatively determined with adequate precision and accuracy (Murugan *et al*., 2013b; Sonawane *et al*., 2014). On the other hand, for validation of the bioanalytical method, the LOQ can estimated by using the relation LOQ = 10 σ/*S*, where σ represents the response's standard deviation and *S* is the calibration curve's

**Table 3.** Parameters of bioanalytical methods validation (adapted from Bischoff *et al*., 2007; FDA, 2001).

| Parameters validation | Short description | Acceptance criteria |
|---|---|---|
| Selectivity | Ability to measure desired analyte in a complex mixture | Absence of interfering signals |
| Linearity | Proportionality of measured value to concentration | Statistical model fit; acceptance accuracy and precision data |
| Accuracy | Agreement between measured and real value | Bias within ±15% of nominal value (±20% near LOQ). |
| Precision | Agreement between a series of measurements | Precision within 15% RSD |
| LOQ | Lowest amount of analyte that can be measured or quantified | Compliance with accuracy and precision criteria near LOQ; see above; alternatively, S/N ≥ 10. |
| LOD | Lowest amount of analyte that can be detected | Compliance with identification criteria; alternatively, S/N ≥ 3 |
| Recovery | Reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method | 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 |

slope. σ is calculated using the intercept, residual standard deviation, regression line, and standard deviation of the blank response (Araujo, 2009). The most commonly applied approaches for estimating LOD are basically the same as those described for the LOQ except for the approach using precision data, which cannot be used here for obvious reasons. In fact, it should be noted that all these approaches only evaluate the pure response of the analytes (Hartmann *et al*., 1998; Lang and Bolton, 1991; Shah *et al*., 2000).

A simple visual analysis could be adequate for a noninstrumental method. With regard to chromatography methods which have a constant background noise, it can be estimated according to the signal-to-noise ratio.

In such conditions, the LOD will be determined by the concentration at which the signal-to-noise ratio of response equals 3. While, for spectrophotometric techniques, LOD is calculated using the relation 3.3 σ/*S*, where σ is the standard deviation of the response and *S* is the slope of the calibration curve. The standard deviation of the response can be obtained. For LOD, an S/N or *k*-factor equal to or greater than 3 is generally selected either by calculating the residual standard deviation of the regression line or by measuring the standard deviation of the blank response or by computing the standard deviation of the intercept of the *Y*/*x* regression line, which is the estimate standard error (Walfish, 2006).

## Precision

The precision of a bioanalytical method is defined as the expression of the degree of dispersion between a series of measurements obtained from a multiple samples of the same homogeneous sample under the specified conditions (ICH Q8, 2005). The agreement between replicate measurements of the same sample provides the precision, which is a measure of the random error. Its concept is the replicate values' relative standard deviation (RSD) or percentage CV (CV%) (Peters et al., 2007).

$$\text{CV\%} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100.$$

Precision may be regarded as having a batch component in the assay or repeatability which is described as the ability to replicate the same operator; this is frequently referred to as intra-assay precision: employing the same tools and materials rapidly. In practical application, the ability to repeat the same methodology under different conditions is examined, for example, change of analyst, reagents, or equipment. On subsequent occasions, for example across several weeks or months, it is covered by batch precision and reproducibility, also known as interassay precision. For the validation of new bioanalysis methods for routine use in clinical studies, it is suggested that precision is evaluated at four unique concentrations with six replicates, on four separate steps. This procedure generates data for individual analytes to be analyzed using one-way analysis of variance, which determines the method's intra-assay and interassay precision at each concentration. To be acceptable, both measures should be within ±20% at each concentration (Shah *et al*., 1992, 2000).

## Accuracy

The accuracy of a method is defined as the closeness of agreement between the test result and the accepted standard value (ICH Q8, 2005). It is obtained by determining the percentage difference (bias %) between the average calculated concentrations and the corresponding nominal concentrations. The accuracy of a bioanalytical method is a measurement of the systematic error or bias and is expressed as the agreement between the measured value and the real value. Accuracy is best reported as percentage bias which is calculated as follows (Wahlich and Carr, 1990):

$$\text{Bias \%} = \frac{\text{Measured value-True value}}{\text{True value}} \times 100.$$

For examining the correlation between the measured and nominal concentration of the analytes in the spiked substance matrix samples, the accuracy of a bioanalytical method is subsequently established at any concentration. During the certification of a novel bioanalytical technique, the estimated precision will be obtained from the measured concentrations, i.e., from four unique concentrations with six replicates, on four separate steps (4 × 6 × 4 experiments). All findings, excluding those that might be rejected for analytical reasons, including chromatography methods, and the accuracy of the method must be ±15% at each concentration (Deming *et al*., 1988; Karnes *et al*., 1991).

## Internal standard

During the validation and routine use, the IS is critical in bioanalysis to improve precision and accuracy.

Before sample clean - up, the IS combined with the sample in a predetermined amount, exposing it to the same conditions as the analyte before sample preparation/extraction to allow for losses and errors introduced during the process (Shah *et al*., 2000). The correct IS should have similar chemical properties to match those of the analyte of interest. In LC-UV and LC-MS analysis, the compound of interest and the corresponding IS provide the ideal situations (Xu *et al*., 2007). In the same step of sample preparation protocol, a precise quantity of a known IS is added to the sample in order to pursue any procedural loss of the sample which will be accompanied by an equivalent loss of IS. The ratio of the detector response (peak height or area) for the drug and the IS is then used in the calibration and quantification. Furthermore, the calibration curve is created using the ratio between the analyte peak area at each calibration level and the IS peak area at the same range. For these reasons, scientists prefer to use an IS that is structurally similar to the measured drug. One drug may be used as an IS as long as this drug is not a part of the patient's therapeutic treatment. Guidelines are available for the correct application of the IS in the determination of drugs in biological samples (Stokvis *et al*., 2005).

**Robustness**

Following the ICH guidance, the robustness of an analytical method is a measurement of its ability to remain stable through making minor adjustments to the input technique parameters and gives an indication of its reliability in daily use (McPolin, 2009; Murugan *et al*., 2013a; Sonawane *et al*., 2014). Robustness may also be described as the capacity to reproduce the technique in multiple laboratories or under various operating conditions without unexpected variations in the result(s) obtained, and a robustness test as a practical tool for evaluating the robustness of bioanalysis.

**Carryover**

The carryover is a serious problem that may affect the accuracy and precision of a bioanalytical technique. It is more significant in bioanalytical methods using LC-MS-MS, where the dynamic range is very large and therefore affects the reduced values of accuracy and precision. The carryover is mostly induced by the presence of residual analyte of the sample with a high concentration previously tested during the assay. It not only affects the following sample in the series, though depending on the concentration of the prior sample, but may also have an impact on many following samples. Carryover can also be random, as late elution residues from the chromatographic column may affect samples later in the analysis (Hughes *et al*., 2007). Despite the fact that carryover tests are crucial for method validation, they are not mentioned in the FDA or Agência Nacional de Vigilância Sanitária (ANVISA) guidelines. However, the EMA recommends that carryover should be evaluated by testing series of blank samples after an injection of LOQ. Although acceptance criteria are not included in the EMA guidelines, carryover is generally considered not significant if the area of the blank sample is less than 20% of the analyte peak area in LOQ samples and 5% of the average peak area of the IS in calibration curve standards. If significant carryover is observed, blanks should be injected (the level of carryover affects the number of blanks) during analysis of the study sample for appropriate concentration value estimation. Some analytes tend to adhere to the metallic or polymeric components of the system and may be very difficult to remove. In some cases, an autosampler of a different design may provide injections without transfer effects. However, transfer also depends on the maintenance status of the autosampler and its history of use (Selinger *et al*., 2014).

**Acceptance criteria**

The common approach employed runs acceptance criteria as described in the following. For the calibration curve, at minimum six calibration standards making up at minimum 75% of the total number of measurement standards should be within 15% of the nominal value. In the case of LOQ, the difference can be 20%. This requires that if eight calibration values are extracted, at least six (75%) should be used to develop the calibration curve. If nine standards are extracted, at least seven (78%) must be suitable for the calibration curve to be acceptable (Selinger *et al*., 2014). QC samples are the absolute tool for accepting or rejecting a batch of samples. The "4-6-15" rule is widely accepted, which indicates that six QCs at three concentrations levels in duplicate should be extracted with a batch of test samples (<100), four of these six should be within 15% of the nominal value, and each QCs level must be reflected in these acceptable QCs. In some cases, supplementary acceptance criteria are also included. These, for example, may be as follows:

- The coefficient of determination ($r^2$) of the calibration curve at least 0.99.
- Absence of interference in drug-free samples.
- Concordance of the absolute maximum area or height of an IS.
- Specific QCs, such as hydrolysis QCs, of the test involved.
- Using a dilution QC.

**Template validation**

On the basis of the validation criteria previously mentioned, it is necessary to follow a sequence of experimental approaches, to take into account all criteria performance appropriately, and to document during full validation and specific acceptance that the method requirements are fulfilled. The evaluation of these parameters allows the exact statement of the analytical procedure as it should be applied in the validation procedure. In order to develop a valid template for the validation of other bioanalytical methods in this field, the following sequence of experiments is required are detailed in Table 4.

**New approaches**

Before routine bioanalysis, validation of each bioanalytical method in all analytical laboratories is a required step. However, there are many approaches assisting the analyst to suitably conclude that a technique could that an approach, such as accuracy profile, can be determined to be valid.

The concept of total measurement error serves as the basis for the accuracy profile approach, i.e., the combined systematic error measured by bias and random error measured by RSD (Hubert *et al*., 2003, 2004, 2007). The criteria for the total error are the accuracy of the result. What is required is to

**Table 4.** Method validation template.

| Validation day | Experimental approach | Purpose of validation step | Number of samples |
|---|---|---|---|
| 1 | System suitability | Verify retention times and sensitivity of the LC-MS/MS system | 5 |
| | Blank (pooled matrix) | Quality of processing, carryover | 4 |
| | Zero sample | Impact of IS | 1 or 2 |
| | Calibration standards, at least six levels | Agreement with the calibration model | 6 × 1 or 6 × 2 |
| | LLOQ | Precision and accuracy at LLOQ | 6 |
| | Individual blanks | Specificity | 6 × 1 |
| | Lipidemic blank (if plasma or blood) | Specificity in lipidemic matrix | 1 |
| | Hemolyzed plasma (if plasma) | Specificity in hemolyzed plasma | 1 |
| | QC.1, QC.2, and QC.3, extracted for recovery | Extraction recovery | 3 × 3 |
| | QC.1, QC.2, and QC.3, unextracted, for recovery | Extraction recovery | 3 × 3 |
| | Above the ULOQ-QC | Integrity of dilution | 5 |
| | QC.2 | Autosampler stability | 3 |
| 2 | System suitability | Verify retention times and sensitivity of the LC-MS/MS system | 5 |
| | Blank (pooled matrix) | Quality of processing, carryover | 4 |
| | Zero sample | Impact of IS | 1 or 2 |
| | Calibration standards, at least six levels | Agreement with the calibration model | 6 × 1 or 6 × 2 |
| | LLOQ | Precision and accuracy at the LLOQ | 6 |
| | QC.1, QC.2, and QC.3 | Precision and accuracy over the calibration range | 3 × 6 |
| | QC.1 and QC.3, in the presence of extracted blank | Matrix effect in six individual matrices | 2 × 6 × 3 |
| | QC.1 and QC.3, neat | Matrix effect | 2 × 3 |
| | QC.2 | Autosampler stability | 3 |
| 3 | System suitability | Verify retention times and sensitivity of the LC-MS/MS system | 5 |
| | Blank (pooled matrix) | Quality of processing, carryover | 4 |
| | Zero sample | Impact of IS | 1 or 2 |
| | Calibration standards, at least six levels | Agreement with the calibration model | 6 × 1 or 6 × 2 |
| | LLOQ | Precision and accuracy at the LLOQ | 6 |
| | QC.1, QC.2, and QC.3 | Precision and accuracy over the calibration range | 3 × 6 |
| | QC. 1 and QC.3 | Processing stability at a selected temperature | 2 × 3 × 3 |
| | QC.1 and QC.3 | Freeze-thaw stability | 2 × 3 |
| | QC.1 and QC.3 | Interference experiments | 2 × 3 |
| | QC.2 | Autosampler stability | 3 |
| | QC.1 and QC.3 | Interference by comedication | 2 × 3 |

provide confidence following the validation process that each of the routine test results the laboratory will achieve in the future will be sufficiently precise. Therefore, to achieve this aim, rather than a complete set of statistical tests, one statistical decision process is used by the accuracy profile approach, i.e., a waiting acceptance range determined using the validation standards' concentration levels. This range indicates an area where, mainly, a defined fraction of the population of results is expected to be found (i.e., β expectation limits). In this regard, an accuracy profile can be created by using different concentration levels (Hubert *et al*., 2004, 2007; Rozet *et al*., 2007). Then this profile is provided to the "a priori" acceptance limits, which are set at ±5% or 15%. This value is a standard limit used in the framework assessment of active substances in pharmaceutical product formulations.

## APPLICATION OF THE BIOANALYTICAL METHOD IN THE ROUTINE ANALYSIS OF DRUGS

During the routine development of bioanalytical methods, often attempts have been made to find suitable separation and ionization efficiencies for the analytes. To improve LC-MS techniques, scientists often employ stationary phase selection, mobile phase strength, and available ionization sources. Furthermore, experimental variables such as solvent composition and extraction procedures can affect chromatographic performance but also affect the ionization efficiency of analytes when chromatographic techniques are switched to an ionization source before detection by MS (Crepier, 2018). In general, there are many case studies has been included in Table 5 which used to measure drugs and their metabolites in biological matrix for TDM

**Table 5.** Some case studies methods for drugs analysis TDM.

| Analyte | Biomatrix | Sample volume | Sample preparation | Stationary phase | Extraction solvent | References |
|---|---|---|---|---|---|---|
| Antifungal drugs | | | | | | |
| Voriconazole | Serum | 10–50 µl | PP | C18 column | ACN | Keevil *et al*. (2004) |
| Posaconazole | Plasma | 100 µl | PP | C-18A column | ACN | Shen *et al*. (2007) |
| Posaconazole | Plasma | 50 µl | SPE | C18 column | - | Cunliffe *et al*. (2009) |
| Iodiconazole | Plasma | 400 µl | LLE | C18 column | Methanol–water–formic acid (v/v/v) | Gao *et al*. (2009) |
| Antiviral drugs | | | | | | |
| Nelfinavir, indinavir, ritonavir, saquinavir, amprenavir, lopinavir and M8 | Plasma | 250 µl | LLE | Symmetry C18 | Methanol | Frerichs *et al*. (2003) |
| Efavirenz, nevirapine, zidovudine, stavudine, abacavir, lamivudine, zalcitabine, didanosine, indinavir, nelfinavir, ritonavir, atazanavir, saquinavir, lopinavir and amprenavir | Urine | 50 µl | LLE and PP | C18 column | ACN | Jung *et al*. (2007) |
| Amprenavir, atazanavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine | Plasma | 100 µl | LLE | C18 column | Methanol and ACN (v/v) | Ter Heine *et al*. (2007) |
| Amprenavir, atazanavir, lopinavir, ritonavir, nevirapine, darunavir, etravirine and rilpivirine | Plasma | 100 µl | PP | C18 column | 500 µl of ACN | Else *et al*. (2010) |
| Ribavirin and viramidine | Plasma | 100 µl | PP | C18 column | ACN and sulfuric acid | Liu *et al*. (2006) |
| Anticonvulsants | | | | | | |
| Valproate | Plasma | 200 µl | SPE | C18 column | 50 µl of hydrochloric acid | Matsuura *et al*. (2008) |
| Topiramate | Plasma | 500 µl | PP | C18 column | 1 ml of ACN | Contin *et al*. (2001) |
| Carbamazepine | Plasma | 500 µl | LLE | µ-Bondapak C18 | ACN, methanol and formic acid (0.1%) (10:70:20, v/v). | Mowafy *et al*. (2012) |
| Levetiracetam | Plasma, serum, or saliva | 100 µl | PP | C18 column | 100 µl of ACN | Guo *et al*. (2007) |
| Lamotrigine and its metabolites | Plasma | 200 µl | PP | C18 Symmetry | 350 µl of ACN | Beck *et al*. (2006) |
| Gabapentin | Serum | 200 µl | PP | C8 column | 800 µl cold (4°C) ACN | Carlsson and Reubsaet (2004) |
| Gabapentin | Plasma | 200 µl | PP | C8 column | ACN | Wattananat and Akarawut (2009) |
| Phenytoin | Plasma | 50 µl | PP | C18 column | Methanol | Zhang *et al*. (2008) |
| Ethosuximide | Plasma | 200 µl | SPE | C18 column | - | Bhatt and Shah (2010) |
| Antibiotics | | | | | | |
| Rifampicin and clarithromycin | Plasma | 10 µl | PP | C18 column | Methanol and ACN (4:21, v/v) | de Velde *et al*. (2009) |
| Tobramycin | Serum | 20 µl | PP | C18 column | ACN | Keevil *et al*. (2003) |
| Azithromycin | Plasma | 100 µl | PP | Phenomenex Luna CN column | Methanol | Liu *et al*. (2007) |
| Azithromycin | Plasma | 200 µl | LLE | C18 column | Methyl tert-butyl ether–hexane (50:50, v/v) | Chen *et al*. (2006) |
| Erythromycin | Plasma | 200 µl | LLE | C18 column | 5 ml of diethyl ether | Gu *et al*. (2006) |

*Continued*

| Analyte | Biomatrix | Sample volume | Sample preparation | Stationary phase | Extraction solvent | References |
|---|---|---|---|---|---|---|
| Vancomycin | Serum | 200 µl | SPE | C8 column | - | Zhang *et al*. (2007) |
| Amoxicillin and clavulanic acid | Plasma | 200 µl | PP | C8 column | ACN | Sonawane *et al*. (2014) |
| Sulfamethoxazole and trimethoprim | Plasma | 250 µl | SPE | C18 column | - | Bedor *et al*. (2008) |
| Cefixime | Plasma | 500 µl | PP | C8 column | Methanol followed by ACN | Meng *et al*. (2005) |
| Clarithromycin | | 25 µl | PP | Hexyl column | ACN (200 µl) | Matsuura *et al*. (2008) |
| Isoniazid and Ethambutol | Plasma | 100 µl | PP | C18 column | ACN (200 µl) | Chen *et al*. (2005) |
| Clindamycin | Plasma | 100 µl | PP | C18 column | ACN (200 µl) | Yu *et al*. (1999) |
| Anticancer drugs | | | | | | |
| Imatinib | Plasma | 200 µl | LLE | C18 column | 800 µl of methanol | Awidi *et al*. (2010) |
| Lenalidomide and flavopiridol | Plasma | 350 µl | PP | C18 column | ACN (1 ml) | Liu *et al*. (2008) |
| Docetaxel and paclitaxel | Plasma | 250 µl | LLE | RP-18 column | 3 ml of methyl-t-butyl ether | Mortier and Lambert (2006) |
| Sunitinib | Plasma | 200 µl | LLE | C18 column | 4 ml of tert-butyl-methyl-ether | Minkin *et al*. (2008) |
| Tamoxifen | Serum | 50 µl | PP | C18 column | ACN (75 µl) | Gjerde *et al*. (2005) |
| Paclitaxel | Plasma | 200 µl | LLE | C18 column | Tert-butyl methyl ether (1.3 ml) | Zhang and Chen (2008) |
| Drugs for cardiovascular system | | | | | | |
| Amiodarone | Plasma | 100 µl | SPE | Hydro-RP | - | Kuhn *et al*. (2010) |
| Digoxin | Plasma | 200 µl | LLE | C18 column | Diethyl ether | Lafuente-Lafuente *et al*. (2009) |
| Rivaroxaban | | 200 µl | PP | C18 column | Methanol (0.5 ml) | Rohde (2008) |
| Angiotensin II receptor blocker | Urine | 400 µl | PP | C18 column | Chloroform, tétrachlorométhane or Dichloroéthane (50 µl) | Soltani *et al*. (2012) |
| Angiotensin II receptor blocker | Plasma | 500 µl | PP | C18 column | Chloroform, tétrachlorométhane or Dichloroéthane (50 µl) | Soltani *et al*. (2012) |
| Hydrochlorothiazide, Triamterene | Plasma | - | PP | C18 column | Deep eutectic solvents | Ramezani *et al*. (2018) |
| Atorvastatin, Metformin, and Metoprolol. | Plasma | 500 µl | PP | C18 column | ACN (500 µl) | Ramezani and Absalan (2020) |
| Atorvastatin, Metformin, and Metoprolol. | Urine | 500 µl | PP | C18 column | ACN (600 µl) | Ramezani and Absalan (2020) |
| Immunosuppressant drugs | | | | | | |
| Sirolimus | Whole blood | 500 µl | PP followed by SPE | C18 column | ACN and zinc sulphate (70:30, v/v) | Taylor and Johnson (1998) |
| Everolimus | Whole blood | 500 µl | PP followed by SPE | C18 column | ACN and zinc sulphate (70:30, v/v) | Salm *et al*. (2002) |
| Tacrolimus, sirolimus, CsA, everolimus | Whole blood | 100 µl | PP | C18 column | Methanol and zinc sulphate (70:30, v/v) | Streit *et al*. (2002) |
| Tacrolimus, sirolimus | Whole blood | 80 µl | PP | C18 column | Zinc sulphate | Wang *et al*. (2005) |
| Biomarker drugs | | | | | | |
| Malondialdehyde | Plasma | 500 µl | PP | C18 column | Hydrochloric acid (200 µl) | Malaei *et al*. (2018); Safavi *et al*. (2018) |

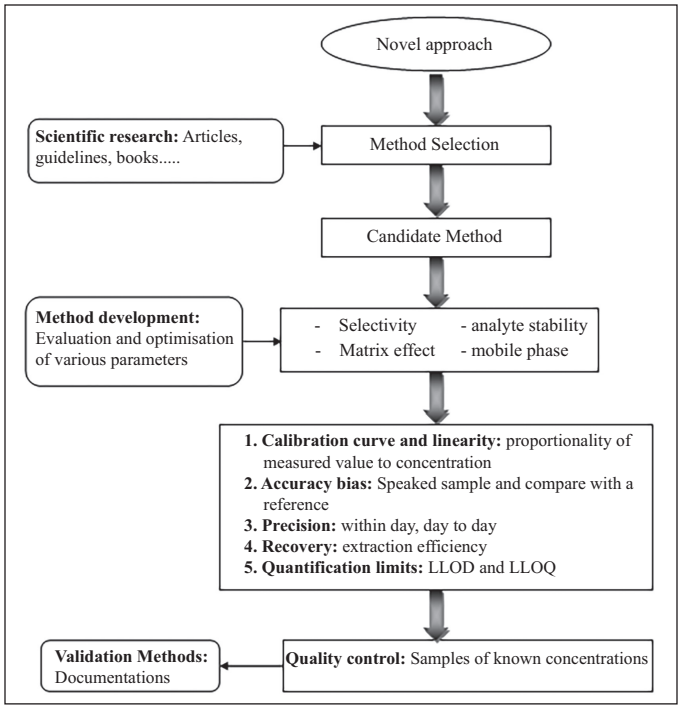


**Figure 3.** Various stages for development and validation of bioanalytical method.

to optimize therapy of critical dose drugs with a short therapeutic range when there is a high risk of both drug overdosing and underdosing. This table describes case studies of bioanalytical assays validation using various preparation samples and different column separation.

## CYCLE VALIDATION

Validation of a bioanalytical method includes a succession of procedures to demonstrate that the method used makes it possible to quantify an analyte in particular matrices for a precise application. Different parameters define the acceptance and validation of bioanalytical method such as accuracy, recovery, selectivity, specificity, repeatability, linearity, the robustness, and finally the stability of the analyte during the different stages of pretreatment and in the matrix (Causon, 1997; Nicolas *et al*., 2004; Shah *et al*., 2000). Validation is a dynamic and adaptable event according to its application and operating conditions. The life cycle of bioanalytical method validation represented in Figure 3 shows that bioanalysis are often described as fixed procedure. This is somewhat the impression given by manuals and other technical standards collections (Feinberg, 2009). However, since any production process, bioanalytical methods are born, evolve, and die. To thoroughly comprehend the significance and importance of validation in the life of bioanalytical technique, it is interesting to describe its life cycle from the moment it is selected until the moment it is abandoned. Finally, a simplified summary of the steps such as full methods validation may cover the experimental plans, which should ideally be applied to the validation of each bioanalytical method.

## CONCLUSION

Overall, this review describes a simple practical guide to the validation of bioanalytical methods used in the research and measurement of drugs and their metabolites for TDM. It also pointed out the critical aspects of methodological development according to the international guidelines. For this reason and the need to meet the regulatory requirements of international standards, it has covered and discussed the essential performance characteristics of the validation procedure for bioanalytical methods. On the other hand, we provide guidance to biomedical laboratory staff and simple approaches to use with a scientific background in order to improve the bioanalytical validation process. Today, the commercial availability of a large number of automated rapid tests may reduce the effort involved in the laborious development and validation of LC- MS/MS methods. If all of the objectives are met, LC-MS-MS will be the most robust and widely used technique for the measurement compounds in clinical studies. Finally, other instruments, such as spectroscopic techniques, are developed for the diagnosis of human diseases from their biological samples which can reduce time and cost. This will improve the treatment rate of patients and prevent adverse clinical result.

## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## FINANCIAL SUPPORT

There is no funding to report.

## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this review.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

## PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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**How to cite this article:**
Abdessadek M, Kharbach M, Ben-Saghroune H, El Amraoui B, Khabbal Y. Validation steps and parameters of bioanalytical methods using in clinical studies: A narrative review. J Appl Pharm Sci, 2023; 13(08):068–083.