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Chapter

Bioanalytical Method Development and Validation: A Review

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Abstract

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 to sponsors. 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 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 and validation, validation parameters, sample extraction technique, stability, good laboratory technique, recent trends

1. Introduction and history

When we draw attention on bioanalytical method development and validation, from last three decades, there was major progress in this field. Various agencies namely US Food and drug administration (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) had done collective efforts at different timings to regulate and harmonize bioanalytical method development and validation. The

very first workshop was held in Arlington, VA, December 3–5, 1990 which was collectively organized by AAPS, U.S. Food and Drug Administration, the International Pharmaceutical Federation (FIP), the HPB, and the AOAC and the report of that was published in Pharmaceutical research and four other journals. This workshop clearly points out two important phases of bioanalytical method development and validation that are, an analytical method development in which all parameters of the bioanalytical method should be developed including assay definition, and actual application of a bioanalytical method for Bioavailability, Bioequivalence and Pharmacokinetics studies. Draft guidance on bioanalytical methods validation was issued by the FDA in January 1999.The second AAPS/FDA bioanalytical workshop was held in January 2000. The workshop has resulted in a report 'Bioanalytical method validation—A revisit with a Decade of Progress'. This workshop also forms the basis of FDA guidance on bioanalytical method development and validation, in May 2001. A separate workshop was held 2000 to discuss validation principles for macromolecules. To address the need for guiding principles for the validation of bioanalytical methods for macromolecules, the AAPS Ligand—Binding Assay, Bioanalytical focus group developed and published recommendations for the development and validation of ligand-binding assays in 2003. Current FDA guidance and bioanalytical methods validation workshop white paper was published in 2006. The third AAPS-FDA bioanalytical workshop was held on May 1–3, 2006, in Arlington, VA, concluded with several recommendations to achieve the above goals and objectives related to bioanalytical method development and validation. There was EMA Draft guidance on validation of bioanalytical methods held on April 15–16, 2010. GCC, EBF, CFDA, ANVISA had taken the wide range of efforts to discuss various practical problems of bioanalysis in this decade along with the industry. The regular workshops, conferences of these organizations create interest and improve knowledge in bioanalysis field.

In May 2018, U.S. Department of Health and Human Services, Food and drug administration, Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM) were published guidance for industry regarding bioanalytical method development and validation [1].

2. Need of bioanalytical method development and validation

Sponsors are applying for investigational new drug application (IND), new drug application (NDA), Abbreviated new drug application (ANDA) to FDA. To fulfill the formalities, they have to submit human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies, requiring pharmacokinetic (PK) evaluation including non-human pharmacology and toxicology studies and preclinical studies, for this purpose there is a need to develop and validate bioanalytical method. Generally, for industrial use, the bioanalytical methods are developed in biological matrices such as blood, serum, plasma, or urine [2]. The new guidance was having influence of chromatographic assays (CCs) and ligand binding assays (LBAs), as these types of assays can quantitatively determine the drugs and their metabolites, therapeutic proteins and biomarkers in biological matrices such as blood, serum, plasma, urine and tissues. The guidance document also includes the public comments on the revised draft published in 2013. It also provides recommendations for the development, validation and in-study use of bioanalytical methods. The recommendations can be modified with proper supporting documents according to the specific type of bioanalytical method. The advances in scientific and technical factors were included in the guidance. For the successful conduct of nonclinical, biopharmaceutics and clinical studies, the validated analytical methods which provide quantitative data of analytes including drugs and biological products and

biomarkers in given biological matrix are critical ones. 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. The level of validation should be proper for intended purpose of the study which is stated by fit-for-purpose. The most crucial studies submitted in an NDA, BLA or ANDA which helps in regulatory decision making for approval, safety such as BE or pharmacokinetic studies should contain validated bioanalytical methods. The analytical laboratory, which is involved in conducting toxicology studies for regulatory submissions, should follow 21 CFR 58 and GLP [2].

3. 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 [2].

4. Validation and acceptance criteria

4.1 Background

The main purpose of bioanalytical method development is to clearly define the design, operating conditions, limitations and suitability of the method for its intended purpose. It also ensures that the method is optimized for validation. Before starting development of bioanalytical method, the sponsor should perform the detail study of analyte including physicochemical properties, in vitro and in vivo metabolism and protein binding etc.

The procedures related to extracting the analyte from biological matrix and its detection are the important tasks in method development.

Following are the parameters for method development:

- Reference standards
- Critical reagents
- Calibration curve
- Quality control samples(QCs)
- Selectivity and specificity
- Sensitivity

- Accuracy
- Precision
- Recovery
- Stability of the analyte in the matrix

The developed method should be suitable for analysis of study sample and that is proved on the basis of bioanalytical method validation results. In case if there is new drug entity, its metabolites or biomarkers or any revisions to existing method, the full validation is necessary. The detailed written description like protocol, SOP's should be established. The detailed description of parameters, environment, matrix, collection of sample should be included. Any parameter and results draws any conclusion should be documented and presented in detailed report. Each analyte should be validated in biological matrix [1].

Three types of validation are full validation, partial validation, cross validation. When there is completely new drug entity under investigation, bioanalytical method is developing for the very first time, any small change in laboratories, instrument, software, matrix, (from rat plasma to mouse plasma or matrix within species like human plasma to human urine) in that case validation can range from as little as one assay accuracy and precision determination to a nearly, full validation. In Cross validation, comparison is done in between two bioanalytical methods. For example, data generated using different analytical techniques like LC-MS-MS vs. ELISA in different studies are included in a regulatory submission [3, 4].

4.1.1 Validation parameters

According to FDA guidance following are common method validation terms.

4.1.1.1 Reference standards and critical reagents

The reference standard should be authenticated with known identity and purity to prepare the known concentrations. The reference standard should be identical to the analyte under study but if not possible then the established chemical form like free base, free acid or salt with known purity can be used. For commercially available reference standards, they should be with certificate of analyses (CoA) as per requirement of USP standards. In certain cases, if there is no CoA for internally or externally generated standards, then detail information and evidences regarding purity, source, and lot number should be provided. If expired reference standards are available in that case CoA is necessary or there should be regeneration of identity and purity standards. In case of internal standards, there is no need of CoA, if it is not interfering with analyte. The critical regents should be properly characterized and documented for identity, purity and stability. These critical reagents include antibiotics, labeled analytes and matrices.

In case there is change in critical reagent like lot-to-lot change or switches to another reagent then there is need of assay validation [1].

4.1.1.2 Calibration curve

The proper quantitation range should be selected for assay and calibration standards based on expected concentration range in the particular type of

study. While studying Ligand Binding Assays (LBAs) there should be anchor points outside the range of quantification in addition to calibration standards. However, these anchor points should not be the part of the acceptance criteria. In most of the LBAs the calibration curves are nonlinear therefore more calibration standards are needed to finalize the calibration range for LBAs than for Chromatographic assays (CCs).

The response-error relationship for LBA standard curves is variable function of the mean response. The concentration-response relationship should be explained with simple model including weighting scheme and regression equation. The reproducible calibration curve should be obtained. The biological matrix should be same throughout the study. The calibration curve should be generated for each analyte if sample contains more than one analyte. In case of surrogate matrices, there should be proper justification and validation of the calibration curves [1].

4.1.1.3 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. For evaluation of performance of method and stability of analyte, QCs are helpful. While determining the precision and accuracy of the method, the performance QCs are included. The stability QCs are useful to determine stability under stress condition. The calibration standards and QCs are prepared from separately. The calibrators and QCs should be prepared in lots of blank matrix that is free of interference or matrix effects [1].

4.1.1.4 Accuracy

The degree of closeness of the observed concentration to the nominal or known true concentration. It is typically measured as relative error (% RE) [5, 6].

4.1.1.5 Precision

Measurement of scattering for the concentrations obtained for the replicate sampling of a homogeneous sample. It is typically measured as coefficient of variation (%CV) [5, 6].

4.1.1.6 Selectivity

The ability of the bioanalytical method to measure and differentiate the analyte in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants or matrix components [5, 6].

4.1.1.7 Sensitivity (LLOQ, Lower limit of quantitation)

The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy [5, 6].

4.1.1.8 Standard curve

The relationship between the experimental response value and the analytical concentration [5, 6].

4.1.1.9 Linearity

The ability of the bioanalytical procedure to obtain test results that are directly proportional to the concentration of an analyte in the sample within the range of the standard curve [5, 6].

4.1.1.10 Quantification range

The range of concentration, including the LLOQ and ULOQ (Upper limit of Quantitation) that can be reliably and reproducibly quantified with suitable accuracy and precision by a concentration-response relationship [5, 6].

4.1.1.11 Recovery

The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method [5, 6].

4.1.1.12 Matrix factor

A quantitative measure of the matrix effects due to suppression or enhancement of ionization in a mass spectrometric detector [5, 6].

4.1.1.13 Stability

The chemical or physical stability of an analyte in a given matrix under specific conditions for given time intervals [5, 6].

4.1.1.14 Reproducibility

Ability of the method to yield similar concentration for a sample when measured on different occasions [5, 6].

4.1.2 Stability study

Special focus is given on stability study. Various stability parameters can be explained as below [5, 6].

4.1.2.1 Stock solution stability

The stock solution stability of drug sample and internal standard should be evaluated at room temperature for minimum of 6 h. The stock solutions are kept at frozen or refrigerated over the period. Then these stock solutions are evaluated by comparing with the response of freshly prepared stock solutions. The stock solution stability should be performed at least for one concentration in duplicate.

4.1.2.2 Post preparative (extracted samples or autosampler tray) stability

This stability is determined for extracted samples. To cover expected run time for the analytical batch and to allow delayed injection due to some instrument malfunctioning or long term storage of the samples, this stability is determined for

~48 to 96 h. This stability is determined on QC samples which are kept for measurable time at the autosampler tray. These samples are analyzed with fresh standards.

4.1.2.3 Benchtop stability

The QC samples at minimum of two concentration levels are kept at room temperature for 4–24 h. It covers the time to extract the samples. The concentrations are analyzed by comparing with their nominal values. The samples are analyzed in replicates generally in triplicate.

4.1.2.4 Freeze-thaw stability

This stability is determined at a minimum of two concentration levels. The samples are frozen overnight for −20 or −70°C. Then it is removed and thawed at room temperature. After that, the samples are frozen again at the same temperature for 12–24 h and again thawed. This freeze thaw cycle is repeated for two more times. After completion of third cycle the samples are analyzed. If more degradation is observed than normal values then the first and second freeze-thaw cycle is repeated and the step in which instability occurred is determined. The freeze-thaw cycle can be extended as per requirement.

4.1.2.5 Freezer storage stability

The freezer storage stability should be carried out at nominal freezer storage temperature during the validation process. The long term stability should be carried out and properly documented as per the procedure discussed below.

4.1.2.6 Postvalidation long-term stability

This stability is performed after validation. The two QC samples in the matrix low and high concentration level are analyzed by keeping at long-term storage temperature and analyzed in triplicate. The postvalidation long-term stability should be performed in regular intervals starting from 1, 3, 6, 9 and 12 months in accordance with the length of stability required. The long-term stability of incurred samples at storage data should be assessed with stored in vivo samples. The results should be incorporated in the original report or separate report should be prepared.

4.1.2.7 Matrix stability

At lower temperature, there may be denaturation of matrix proteins. Therefore matrix stability should be validated. For that purpose, additional stability should be carried out at lower temperatures for sample matrix [7].

4.1.2.8 Bioanalysis of hemolyzed samples

As per 2009 EMA draft guidance and the 2003 ANVISA guideline hemolyzed samples should be analyzed during method validation but practically that will difficult at the time of method validation, therefore, it was recommended to perform at the time of method development. Data reliability and reproducibility should be monitored according to an internal standard (IS) and incurred sample reproducibility (ISR) response. Also one can apply standard addition or standard dilution for further investigation of data reporting. However, the final recommendation about

hemolyzed sample is that there is no standard approach for testing of these types of samples, so there should be the least impact on method development or validation.

4.1.2.9 Whole blood stability evaluation

The immediate spinning down of aliquot of whole blood containing the drug taken immediately following preparation (time zero) followed by the spinning down of another aliquot following the stability period. The whole blood stability should be performed during method validation. However, there are various approaches for determination of whole blood stability. In case of large molecule, it is not applicable [7].

4.1.2.10 Dilution effects

The integrity of the dilution should be monitored during validation by QC samples above the ULOQ with like matrix to bring to within quantitation range, if the method measures the diluted samples. There should be proper demonstration of accuracy and precision of these diluted QCs [1].

5. Sample collection, stability, storage, sample transport

For analysis purpose, some processing is required after collection from an animal or human subject. Harvesting of plasma or serum can be done by centrifugation process and it is kept in frozen condition. The conditions like temperature, centrifugation time and force, maximum from sampling to freezing sample are specified and maintained for proper development of the method. If an analyte is less stable in whole blood than plasma, any delay in processing the sample or poor temperature control could result in analyte loss; in such case, one can apply stabilizers or other special sample handling conditions. The stability of analyte in biomatrix should be defined during validation and analyzed during that period only. To obtain Short-term stability, freeze-thaw stability, long-term stability in bio-matrix (typically at −20 or −70°C), there is need to maintain all parameters like backup capacity of freezer, alarm system for staff, freezer and also all related document, to track sample during collection, storage and stability. Bio-matrix samples are usually frozen in insulated containers with dry ice. The main concern is ensuring that the shipment is still frozen upon arrival. Shipments are usually packed with sufficient dry ice to last for a significantly greater period than the anticipated shipment time. Samples are split into two aliquots at the collection site, for additional security; a set of reserve aliquots can then be safely stored until the first set is received for analysis [8–14].

6. Sample extraction techniques

Sample preparation is most important and critical step in bioanalytical method development and validation. The main task is to remove interferences present in the sample and to make the sample with the higher concentration of analyte, which contributes to the sensitivity of the method [6].

There are various methods of extraction as follows.

6.1 Liquid-liquid extraction

In liquid-liquid extraction, the analyte gets partitioned in between two immiscible phases. Generally, selective partitioning of an analyte of interest is occurring

in between two immiscible solvents and proper extracting solvents plays important role in this step. In LLE, the analyte gets distributed in one immiscible phase and this partitioning also helps to separate interferences. The analyte is dissolved in a suitable solvent. Then the second solvent is added which should be immiscible with the first solvent. The contents in the sample tube are mixed thoroughly and the two immiscible solvents are allowed to separate into two layers. The less dense solvent will be upper layer, while the more dense solvent will be the lower layer. The analyte mixture will be get separated or distribute in two immiscible solvents according to their partition coefficient. The extent of partition of analyte is depends upon the solubility characteristics of the components of analyte in mixture. As there is the partitioning of analyte in between two immiscible layers, the analyte which is soluble in less dense solvent will be at the upper layer and which one more soluble in the denser solvent will be in the lower layer. These two immiscible layers are separated and after separation, the respective analyte is isolated. The hydrophilic compounds are getting soluble in polar aqueous phase and hydrophobic compounds are in the organic solvent. Generally, by evaporation, the analyte in the organic phase is recovered and diluted with mobile phase and then injected into the column while aqueous phase may directly be injected [6].

In LLE analyte is brought in the organic phase, and for that, the required conditions are maintained. In this, there is a direct extraction of biological material with the water-immiscible solvent. The important task is partitioning of analyte in to the organic phase in which aqueous phase is also present [7].

6.2 Solid phase extraction

In Solid Phase Extraction, the partitioning is occurring in between liquid and solid phase. The main advantage of SPE is removing impurities present in analyte, which helps in increasing the sensitivity of the method. The removal of particulate matter from analyte is major output of SPE. In SPE, multiple sampling generally 12–24 with a lower quantity of solvent with automation are major contributing factors. In SPE the recovery of the sample is higher. Small disposable column or cartridge is employed for partitioning. The SPE consists of the medical syringe which is packed with 0.1–0.5 g of sorbent generally C18 silica. Liquid samples are added to the cartridge and wash solvent is selected to either strongly retain or unretain the analyte. To minimize the presence of interferences, this method is advantageous. Even though analyte get retained on the cartridge, the interferences can be eluted or washed, that results in the analyte-free from interferences. Then the analyte is eluted with elution solvent and either directly injected in or evaporated to dryness followed by dilution with the HPLC mobile phase [15, 16].

6.3 Protein precipitation

Protein precipitation is one of the methods to make the matrix interference free. This can be achieved by denaturation and precipitation. Trichloroacetic acid and perchloric acid has a wide choice as a precipitating agent. Various organic solvents like methanol, acetonitrile, acetone, and ethanol are the wide choice for removing plasma proteins and possess compatibility with high performance liquid chromatography (HPLC) as a mobile phase. One part of sample matrix is diluted with three–four parts of the precipitating agent then vortex mixing is carried out. After that centrifugation, filtration is done to remove the protein mass. The supernatant liquid or filtrate obtained is directly analyzed for the analyte of interest. For quantitative analysis, the supernatant can be isolated, evaporated to dryness and then reconstituted with a suitable solvent before analysis [8]. In protein precipitation

method, the analyte should be freely soluble into the reconstituting solvent. Either by converting soluble protein to a nonsoluble state that salting out or by the addition of water miscible precipitation solvent or organic solvents such as acetone, ethanol, acetonitrile or methanol, this technique can be achieved [10, 15].

6.4 Solid phase microextraction

Solid Phase Microextraction involves the sampling, extraction, concentration and sample introduction single step which is solvent-free step. The bonded phase which is fused silica fiber coated with polyacrylate, polydimethylsiloxane, carbowax is kept in contact with the sample and exposed to the vapor, also it can be placed in the stream of a gaseous sample to isolate the analyte and concentrate analyte into a range of coating materials. Lastly, the fibers are transferred to analytical instruments like gas chromatography (GC) and GC/mass spectrometry (GC/MS) for separation and quantification of the target analyte with the help of syringe. For routine analysis of volatile and semi-volatile compounds, SPME can be implemented. Exposed fiber has the ability to extract and sample delivery is a key aspect of this method [8–14]. The SPME apparatus is a very simple device. It looks like modified syringe consisting of a fiber holder and a fiber assembly, the latter containing a 1–2 cm long retractable SPME fiber. Analyte in the sample is directly extracted and concentrated to the extraction fiber. The method saves preparation time and disposal costs and can improve detection limits. SPME was also introduced for direct coupling with high-performance liquid chromatography (HPLC) and HPLC-MS in order to analyze weakly volatile or thermally labile compounds not amenable to GC or GC-MS [12].

6.5 Matrix solid-phase dispersion

In Matrix solid-phase dispersion technique solid matrices are used for sample preparation. It is advantageous as the sample requirement is less that is 1 g with low solvent, which is why it is also termed as microscale extraction technique. Near about 98% solvent use is reduced and giving 90% sample turnaround time. In the Conventional extraction of an organic analyte from tissue, the homogenization of small amount of sample tissue with bulk bonded silica-based sorbent has to perform, this can be achieved in mortar and pestle. The structure of tissue is getting disturbed due to mechanical shearing. The sample gets dispersed on the surface of support sorbent, for this, hydrophilic and hydrophobic interaction plays a role which causes the mixture to become semi-dry and free-flowing homogeneous blend of the sample. The sample disruption will be performed due to bound solvent in the sorbent. The sample disperses over the surface of the bonded phase support material to provide a new mixed-phase for isolating analytes from various sample matrices. The interferences and analyte are eluted by transferring in to a pre-fitted SPE cartridge. This technique has recently been applied, using acid alumina, to extract the organic analyte. The two important lacunas with the method are longer analytical time and having a limited limit of determination (LOD) [15, 20, 21].

6.6 Supercritical fluid extraction

Supercritical fluid extraction is generally used for removing nonpolar to moderately polar analyte from the matrix. As per regulatory point of view, there should be need to replace organic solvents and it is advantageous in the sense of environment. The density of the supercritical fluid is like liquid while its viscosity and diffusivity is in the gas and liquid values. By reducing the pressure and by the evaporation, the recovery of supercritical solvent can be obtained. Even though there is increase in the

pressure, if the temperature is maintained above the critical temperature, the liquid phase will not be appeared. To obtain more efficient extraction the density of supercritical fluid should be like liquid which can be obtained by increasing pressure and this step is more advantageous that of organic solvents. Carbon dioxide dissolves many volatile polar compounds, acting good supercritical solvent. This work can be achieved in the presence of trace amounts of polar co-solvents like water and short-chain alcohols. Supercritical fluids can be used to extract analyte from samples [15, 17]. The SFE is fast process. The rate of diffusion of a species in the fluid and viscosity of the fluid determines the rate of mass transfer between a sample matrix and an extraction fluid. The greater the diffusion rate and the lower the viscosity, the greater will be the rate of mass transfer. The SFE can generally be completed in 10–60 min. The solvent strength of a supercritical fluid can be varied by changes in the pressure and to a lesser extent in temperature. Many supercritical fluids are gasses at ambient conditions. Thus recovery of analyte becomes simple compared to organic liquids. Some supercritical fluids are cheap, inert and nontoxic. Thus they are readily disposed-off after an extraction is completed by allowing them to evaporate in to the atmosphere [11].

6.7 Column switching

The broad definition of 2D (or multidimensional) chromatography is a 'Selective transfer of analyte of interest from a first column to the second column. (By means of switching valve)' [12]. Column switching is one of the interesting techniques for sample preparation. In this technique, the analyte of interest is retained and separated on HPLC stationary phase while the unretained components are eliminated from the column. In this technique, the component of interest separated at lower retention time is cut and transferred onto another HPLC column for further separation. The important advantage is that the process is automated and whatever transfer of analyte occurs can be determined quantitatively. The analyte gets transferred quantitatively without any loss in concern with the adsorption or degradation [15, 18, 19].

7. Acceptance criteria for method validation

One care must be taken while preparing standard and QC samples, which they should be prepared from same stock solution, also the stability and accuracy of both should be verified before proceeding for actual practical. The selectivity of sample matrix should be verified, can be used throughout the experiment. Standard curve samples can be inserted at any sight in the run.

7.1 Matrix-based standard calibration samples

75% or a minimum of 6 standards, when back-calculated(including ULOQ) should fall within ±15% of nominal, except for LLOQ when it should be within ±20% of the nominal value.

7.2 Quality-control samples

At least five replicates, at a minimum of three concentration levels that are LLOQ, MQC and HQC should be inserted in into each run. The results obtained for QC samples are the basis of acceptance or rejection of the run. At least 67% (4/6) of the QC samples must be within 15% of their respective nominal (theoretical) values; 33% of the QC samples (not all replicates at the same concentration) may be outside the ±15% of the nominal value [3].

7.3 Selectivity

For chromatographic assays, the peak response in the blank matrix at the retention time of analyte(s) should be no more than 20% of the response for the lower limit of quantitation (LLOQ) sample [5, 6].

7.4 Sensitivity

Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision. By analyzing at least five replicates of the sample at the LLOQ on one of the validation days should be performed for determining the accuracy and precision. The samples used should be different from that of calibration curve samples. The accuracy as determined by the relative error (RE %) at this concentration should be within ±20% and the CV should be less than 20% [5, 6].

7.5 Accuracy and precision

Accuracy and precision should be determined for both intra-and inter-runs. They are determined at three concentration levels, which are representing entire calibration range. The mean and CV of observed QC concentrations should be determined to obtain intra-run accuracy and precision. The mean of the observed concentrations should be within ±15% of the nominal at all concentrations of the QC samples. Coefficients of variation (indicating precision) around the mean observed concentration should not exceed 15% at all concentrations. For both intra and inter-run, all QC samples should be considered for calculation including the samples that are failed [5, 6].

7.6 Extraction efficiency

The ratio of the results obtained for analyte from an extracted sample to the results obtained by analyzing unextracted samples. In both cases samples should contain same amount of analyte. The extraction efficiency need not be very high, but it should be consistent, precise and reproducible. One can also determine the extraction efficiency of IS. The ratio of the extraction efficiencies of the analyte and IS provide an IS-normalized extraction efficiency [5, 6].

7.7 Matrix effect

The Matrix effect is the suppression or enhancement of ionization of analyte by the presence of matrix components in the biological samples. The quantitative measure of matrix effect is matrix factor. Matrix factor = peak response in presence of matrix ions/peak response in absence of matrix ions [22]. For determining matrix effect standard curve should be compared with the standard in the buffer to detect matrix effects. Parallelism of diluted study samples should be evaluated with the diluted standard to detect matrix effects [9]. Due to disease conditions, there may be variations in lipid or specific or total protein that should be considered while determining matrix effect. During method development, the impact of hemolyzed and lipemic samples may be assessed. At the time of validation, suppression/enhancement (matrix effect), should be assessed instead of matrix factor [21].

7.8 Recovery

The recovery of analyte should be consistent and reproducible. It is not expected that it should be 100%.The recovery should be determined at three concentrations that are low, medium, and high by comparing analyte results of extracted samples with those of spiked control extracts [21].

8. Good laboratory practices: an important part of regulatory acceptance

The workshop on EMA draft guideline on validation of bioanalytical methods held on April 15–16, 2010 in Brussels that was jointly organized by the European bioanalysis forum (EBF) and the European federation of pharmaceutical Sciences (EUFEPS). The draft guideline explains how Good Laboratory Practices are essential for bioanalytical method development and validation. For regulatory acceptance of method, the GLP should be followed. The word that is "regulated bioanalysis" should be implemented while practicing the bioanalysis. The internal quality assurance units are responsible for proper procedures, documentation of data and review of all processes. This should be performed in a transparent environment. In addition, the fundamentals' of GLP should be strictly and interestingly supported by both bioanalytical chemist and regulators that contribute in the reliability of bioanalytical results. When there is any new or literature based method, complete validation should be performed. In case there is a change in the matrix, partial validation should be performed. For QC samples, which are separated in two aliquots in such case, cross validation is to perform. Selectivity should be confirmed by using at least six sources of the appropriate blank matrix. In the case of matrix effect at three times LLOQ and additional determinations of at medium and high QC were recommended. Stability should be performed at every stage. The between-run accuracy of QC samples should be within 15% of the nominal value and between-run precision should not exceed 15% [8]. SOPs should be readily available for various activities of bioanalytical method development and validation. Study director or principal investigator should have overall responsibility of the bioanalytical method. In addition, all protocols should be generated according to GLP and any alteration in protocol can be done by issuing amendment [8–14].

9. Recent trends of bioanalytical method development and validation

As per second China bioanalysis forum (CBF), all approved clinical trials that are BE and pharmacokinetic (phase I–IV trials) should be registered and published on CFDA website eng.sfda.gov.cn. CBF expert committee (EC) participated in the first independent BMV draft guidance of the Chinese pharmacopeia [14].

European bioanalytical forum was established in 2006 to focus on the issues on ISR, matrix suppression and metabolite quantification. The EBF-IGM also focuses on Ligand-binding assays (LBAs) and immunogenicity assessment. There were certain observations of the industry as well as FDA during audits creates interest in ISR (Incurred sample reproducibility).It should be included in bioanalytical support. Because there were, different observations and readings were found while performing repeat analysis. In bioanalytical method validation, human methods are considered validated without ever applying to study samples which are the major drawback of FDAs guidance. The EBF provides additional clarification and

recommendations with a view to achieving uniformity in quantitative bioanalytical estimations of various molecules. The ISR reinforces confidence that a method is valid and reproducible. The few important recommendations related to acceptance of ISR suggest that it does not accept or reject a study. Failed ISR should lead to investigation and follow-up. For small molecules, two-thirds of repeats agree within 20%, large molecules within 30%.Incurred sample reproducibility enforces the confidence that a method is valid and reproducible for intended purpose. ISR should be part of method validation in addition to various parameters. It is important to document of robustness and repeatability of the method. It is part of regular process check on laboratory procedures like SOPs and analysis protocols. Whenever the first time in a new matrix whether animal or human, in new population, first patient study, disease state changes in patient population, any major method change, existing method in new laboratory, whenever scientific reasons require retesting of ISR, process check, all BE studies, incidental check in any studies collectively both clinical and nonclinical studies, there is recommendation of ISR by EBF [23].

The 5th Global CRO Council was held on 13 November 2011 in which European medicines Agency (EMA) guidelines were the main issue of discussion. The GCC recommends three major recommendations on incurred sample reproducibility, consultants, and good clinical practice. The ISR should be carried out in separate batches from that of study samples in a very short period finalized on the priority basis of knowledge related to the stability of the analyte and the matrix. The mean of two results that are of original and ISR results and their difference should be determined. They also recommend good clinical practices with respect to method validation, ISR, repeat analysis, Data recording, reporting and retention of data, facilities and equipment maintenance, computerized systems, QA and QC, SOPs and policies [24]. The European medicine agency (EMA) suggests about the stability of sample in the matrix containing all the analyte. Accordingly, Global CRO council recommends stability validation experiments in presence of co-administered compounds for fixed-dose drug combination studies including patient studies [25]. The 2nd Global CRO Council (GCC) for bioanalysis closed forum was held on 15 April 2011 in Montreal, Canada. In this forum they have suggested 10 recommendations on internal standard response, analyte stability, stability of light-sensitive compounds, Incurred sample reanalysis, incurred sample accuracy, whole blood stability evaluation, stability of the Co-administered compounds, rejected evaluations of validation reports, stock solutions used for calibrates and quality control sample preparation, carryover control, which are essential while developing bioanalytical method [26]. The GCC also recommends 20 recommendations on new EMA guidelines on issues like certificate of analysis, internal standard, calibration curve, accuracy, stability, sample stability, matrix effect, presence of excipients, matrix obtained from special populations, study samples, calibration standard and QC samples, acceptance criteria, chromatogram integration, sample reanalysis, reference standards, matrix effect, matrix selection, parallelism, stability, reagents [27].

The 5th Workshop on Recent Issues in Bioanalysis(WRIB) was held in Montreal, Canada, on 13 and 14 April 2011 which was organized by Calibration and Validation Group (CVG).This workshop gives 17 recommendations on various points like alternate detectors, tissue analysis, whole-blood stability evaluation, chromatographic peak integration, Systems cross-validation, stability issues in bioanalytical method validation and the definition of fresh, fit-for—purpose validations, interpretation of guidelines between different auditors, batch failure, effect of anticoagulant counter ions, differences in slopes of the calibration curves on different LC-MS/MS systems, variability of the IS response in analytical results, reinjection versus reanalysis versus nonreportable values, matrix stability for co-formulated and

co-administered drugs, Hemolysis of samples, method transfers and cross-validations, method development data. These recommendations should be studied and implemented for proper regulatory acceptance of bioanalytical methods [28]. The new draft guidance ANVISA (Brazil) was put forth in 2011. Japan bioanalysis forum (JBF) was also put forth in 2011, which has participated for MHLW bioanalytical study group to draft Japanese guidelines for bioanalytical method development. Both ANVISA and JBF guidelines are having the impact of EMA guidelines with no any remarkable difference [29]. In September 2013, FDA released a draft guidance, which includes number of changes in bioanalysis and addition of biomarker assays [30]. In December 2013 there was 8th GCC closed forum to discuss 2013 revised FDA draft guidance. It was recommended that there should be minimum requirements for biomarker assays. It should be extended to validated, qualified or screening assays. In case of full validation, the pharmacokinetic assay approach can be implemented but there will be certain limitations as biomarker kit are involved [31].

10. Conclusion

To evaluate and interpret bioavailability, bioequivalence, pharmacokinetic and toxicokinetic study data, bioanalytical method validation plays an important role. In this, the quantitative estimation of drug and its metabolites in the biological fluid can be performed. There is a need to discuss issues related to bioanalytical method development and validation and need to follow the guidelines, regulatory aspects that are formed during the tenure of last three decades. Nowadays LC-MS/MS plays important role in developing bioanalytical methods including GC-MS and other techniques which are useful for these types of tasks. Various related authorities are having a keen focus on different aspects of bioanalytical method development and validation. If researchers applied all practical aspects of bioanalytical method development and validation for determination of API or certain chemical entity, that is advantageous for regulatory submissions of particular drug component. While developing the bioanalytical method there should be complete clarity about the nature of the analyte, that whether it is a small molecule or macromolecule. There certain differences in principles of bioanalysis for these types of an analyte. For small molecules generally LC-MS and for macromolecules, Ligand-binding assays should be performed. Recent trends related to bioanalytical method development and validation should be followed with GLP requirements for regulatory acceptance of method. There are continuous conferences; workshops are arranged at different sights of the world by CBF, GCC, and other related agencies to discuss, to solve and to improve practical difficulties and additions in the field of bioanalysis.

List of abbreviations

AAPS	American Association of pharmaceutical Scientist
HPB	health protection branch
AOAC	Association of Analytical Chemist
CVM	Center for Veterinary Medicine
CDER	Center for Drug Evaluation and Research
EMA	European Medicine Agency
CFDA	China Food and Drug Administration
EBF	European Bioanalytical Forum
GCC	Global CRO Council
BA	bioanalytical

BE	bioequivalence
LC	liquid chromatography
GC	gas chromatography
MS	mass spectrometry
CBF	China Bioanalytical Forum
JBF	Japanese Bioanalytical Forum
CoA	certificate of analyses
LBAs	ligand binding assays
CCs	chromatographic assays
ISR	incurred sample analysis
IS	internal standard
SOP	standard operating procedure
GLP	good laboratory practices
LLOQ	lower limit of quantitation
ULOQ	upper limit of quantitation
QC	quality control
MQC	middle quality control
HQC	high quality control
QA	quality assurance
SOP'S	standard operating procedure
CV	coefficient of variance
FDA	Food and Drug administration

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