Review Article

A Comprehensive Review on Bioanalytical Method Development and Validation for Pharmaceuticals

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ABSTRACT

Bioanalysis is most widely used for drug discovery and development. Bioanalytical methods have been working for the quantitative estimation of drugs, or its metabolites and biomarkers in its biological samples. The steps involved in bioanalysis comprise of many steps that are from sample collection to sample analysis and then data reporting. The first step involves sample collection from clinical or preclinical studies. After that, the analysis of the sample is done in the laboratory. The second step involves the sample cleanup the cleanup process should be carried out carefully to eliminate the interferences present in the sample matrix. And it increases the performance of the analytical system. The last step involves sample analysis and detection of a separated compound and detection techniques used are HPLC and Liquid chromatography coupled with double mass spectrometry (LCMS-MS) and are used in the bioanalysis of drugs in the body. Each one of these has its advantage and disadvantage. For bioanalysis of small or large molecules, the Chromatographic methods used are HPLC, and Gas Chromatography with LC/MS/MS. The review focuses on an overview of the bioanalytical method, development, validation, and the quality of sample preparations.

Keywords: Bioanalytical method, quantitative applications, HPLC, UPLC, LC/MS/MS.

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INTRODUCTION

Bioanalytical methods are necessary for the measurement of drug concentration and their metabolites in a biological matrix (urine, plasma, saliva, serum, etc.). It is an essential aspect of the progression of a medicinal product. The concentration of biological matrix can be determined by using chromatographic devices, e.g. HPLC (High Performance Liquid Chromatography), LC-MS, etc. Bioanalysis is important in the study of pharmacokinetics, pharmacodynamics, and toxicological evaluation during drug development. The bioanalytical process not only measures small molecules like drugs and metabolites but it also measures large molecules like proteins and peptides. The bioanalytical process is also important in many research areas such as doping control, forensic analysis, and identification of biomarkers for investigative of many diseases. Bioanalytical method validation has an essential role in the Quantitative determination of several types of Analytes in biological matrices and physiological matrices, and this technique can also be useful for human clinical pharmacology and non-human studies. The studies were carried out to determine the results of clinical trials and toxicokinetic including bioequivalence to create critical conclusions supporting the safety and efficacy of a medicinal drug substance or product. Therefore, the practical bioanalytical methods used must be well characterized, completely validated, and documented to reliable results. For analysis of many drugs the technique used can be HPLC or LC/MS/MS. The cost-effective technique of HPLC linked with UV (Ultraviolet), PDA (Photo Diod Array), fluorescence detectors are useful for the identification of many drugs and metabolites but its sensitivity over LC/MS/MS is low. Therefore, a rapid, simple, with high sensitivity and selectivity technique of LC/MS/MS is used for the identification of analytes and impurities. In a large number of laboratories, the LC/MS/MS is practice used for quantitative and qualitative analysis and in many diverse ways. [1-4]

Method Development:

The bioanalytical technique development describes the design, limitation, effective conditions, and suitability of the process for its intended determination and to confirm that the system is optimized for validation.

Steps involved in method development:

1. ***Method selection and complete information of the sample:***

The first step to be done is a literature survey and collection of all the necessary information on drug profile and the pharmacokinetic and physicochemical properties of analytes and the interrelated compounds. And the choice of the internal standard in LC-MS/MS is through chemical structure and chemical properties of analyte and it must be similar. During the selection of internal standard, there must be the same molecule having

different isotopes like deuterium C^{13} and N^{15} in LC-MS/MS. Today in many laboratories the LC-MS/MS has been replaced by HPLC-UV.

2. Selection of initial method conditions:

The setting of initial methods includes diluent selection depending on drug solubility, internal standard and drug metabolites, and comparability with the analytical method. Using the aqueous solution the analyte is quantified and is the lowest concentration. Resolution and run time between the peaks must be taken attention during this phase.

3. Analyzing the analytical method in aqueous standards:

Before working towards a method in a biological matrix, the aqueous standard of the bioanalytical method is checked first and then the calibration curve is plotted for aqueous standard with a least of four concentrations with lowest and highest. The highest standard concentration will depend on C_{max} and the lowest standard concentration shall depend on a preliminary study. The correlation coefficient can be determined by injection of every single calibration curve standard and the range should not exceed more than 0.99.

4. Development and optimization of sample processing method:

The instrument parameters must be checked with the aqueous standards to ensure that it remains stable during validation, prepare a matrix sample. For method development the detailed study through a literature survey is done for Analyte, physicochemical properties, and internal standards, to set and optimize the bioanalytical technique.

5. Analyzing the analytical method in the biological matrix:

When there are less recovery and reproducibility in the technique of liquid-liquid extraction, then the preferred technique is solid-phase extraction to enhance sensitivity, precision, recovery and low interference, and established bioanalytical method is checked for matrix samples for accuracy, precision and recovery and is important before confirming the process for pre-validation. At least 3 aliquots of matrix samples of each Lower Quality Control, Higher Quality Control and Lower Limit of Quantification (LLOQ) are analyzed with one set of the extracted calibration curve standards counting matrix blank and zero standard (blank with only internal standard) and the results shall be compared for the recovery with aqueous quality control (QC) samples of the equivalent concentration.

6. Pre-validation:

When the process of this validation is analyzed to be reliable, formulate a complete procedure for sample preparation with complete details, contributory conditions, and method conditions, to continue for pre-validation. [3, 5, 6.]

Methods used for Sample preparation

Before the start of the analysis, the cleanup of the sample (sample preparation) must be done. Sample preparation is an essential portion of the analysis. The purpose of sample preparation is to produce an interference-free sample aliquot.

Liquid-Liquid Extraction (LLE)

For aqueous and biological sample preparation LLE technique is most widely used. This technique is used to isolate analytes from interferences by partitioning the analytes between two immiscible phases (aqueous and organic). Based on the partition coefficient the analyte mixture gets separated in two immiscible solvents. Today the advanced and developed techniques of LLE have replaced traditional LLE. The advanced technique of LLE includes liquid-phase microextraction (LPME), support membrane extraction (SME), and single drop- liquid-phase microextraction (DLPME). From the biological sample, the basic and acidic drugs can be extracted with more extraction efficiency. LLE process is useful especially to remove salts but it utilizes more time and is of the environmental cost, is due to toxic solvents are used are in excess. And in numerous cases, the analyte with a different polarity like drug + metabolites is not fit for required extraction from the same sample.[3, 6, 7]

Solid Phase Extraction (SPE)

The SPE is the best and popular technique in drug analysis designed with high efficiency, reproducibility, and cost-effective and its principle involves the partitioning of the analytes between two phases. It is mostly used to separate analytes of interest from a solution by sorption on a variety of sample matrices. By use of an appropriate solvent the analyte is either strongly retained (K>>1) or un-retained (K=0) on the stationary phase and the retention is based upon the affinity of the analyte on the stationary phase. And after the desired Analyte gets retained, interferences are washed from the cartridge and that results in the analyte-free from interferences. The cartridge type is still most popular and uses a sorbent of 50-200mg to separate essential Analytes from a complex matrix. In recent year new designs of SPE is also available such as µSPE and flat disks [3,10,11.]

SPE TYPES

1. Reversed-phase:

Here polar or moderately polar sample matrix (mobile phase) includes mid- non-polar Analytes using non-polar sorbent (stationary phase). Carbon-based media are used for packing in the reversed-phase such as C_4, C_8, C_{18}, and phenyl bonded. The reversed-phase consists of polymer-based sorbents. In which it involves the conditioning of organic solvent (e.g. methanol) with aqueous solvent (e.g. water).

2. Normal phase:

Here polar Analytes in non-polar matrices using polar sorbents. The commonly used polar sorbent include Silica, amino, and alumina as a base of all bonded phase. In this phase, the packing that may needs conditioning with a non-polar solvent with subsequent elution of compound is carried with polar solvents. And components like basic pH and functional groups are bonded by silica. However, polar material is irreversibly bonded on a silica surface, and in this case, amino may be used.

3. Ion exchange

Ion- exchange retains charged compounds or removes ionic interferences. The retention mechanism of anions and cations are mainly on resin by exchanging the anion/cation in the sample. These resins are generally silica-based packing and it has a stable pH range and it loses exchange capacity with increasing organic solvent content in the sample .[6, 8,10]

Table 1: Evaluation of sample techniques

Parameter	PPT	LLE	SPE
Workability	Less	More	More
Selectivity	Bad	Good	V. good
Ion suppression	High	Low	Low
Automation	Low	Low	High
Analyte suitability	Hydrophilic	Lipophilic	Hydrophilic and lipophilic
Cost requirement	Less	More	More

4. Protein Precipitation (PPT)

It is a simple extraction process compared to LLE and SPE. Protein precipitations are mainly for high analyte concentration. Proteins are precipitated out by the addition of a required amount of a water-miscible organic modifier (acetonitrile, acetone or methanol), and in many procedures the salts of (aluminum chloride), metal ions (zinc sulfate) or by use of different pH range which impact the solubility of the protein (acid such as trichloroacetic, perchloric, metaphosphoric and tungstic). The advantage of this precipitation is that it can eliminate undesirable plasma protein from plasma fluid as a cleanup technique compared to SPE. The requirement of organic modifier and other solvent is in a smaller amount, it is less time-consuming. And it needs a lower temperature to touch greater purification. And for the hydrophilic and hydrophobic compounds, a simple extraction method can be applied but in certain cases, the PPT can be followed by LLE and SPE for greater efficiency [6, 9,12].

Bioanalytical Method Validation

The validation of a method is a part of GLP (Good Laboratory Practice) study and it confirms the quality of the analytical technique.

Types of method validation

Full validation

It is important when the bioanalytical method is advanced and implemented for the first time. And it must be for a new drug entity. And it is essential for revised assay if metabolites are added to an existing assay for quantification.

Partial validation

It's a reform of previously validated bioanalytical procedures. The changes required for reform of typical bioanalytical methods are:

- ✓ Transfer of Bioanalytical method between laboratories or analysts
- ✓ The analytical method can be changed
- ✓ The matrix within species can be changed
- ✓ Sample processing procedures can be changed
- ✓ Species within matrix
- ✓ Relevant concentration range can be changed
- ✓ Instruments platform can be changed

Cross-validation

It involves a judgment of validation parameters to create documents within the same study or across different studies when more than two bioanalytical techniques are used. For example, cross-validation would be done by reference methods with the revised bioanalytical method, the judgment must be done in both ways. [6, 12,]

Parameters for method validation:

a) ***Selectivity:***

The analytical method can measure and differentiate the analyte in the presence of other components in the sample. This could include metabolites, impurities, degrades, which may be expected to be present matrix components. Analysis of blank samples of the appropriate biological matrix (plasma, urine, or other matrices) should be obtained at least six sources.

All blank samples should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ).

b) Sensitivity:

Sensitivity is measured utilizing the Lower Limit of Quantification (LLOQ) is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision.

c) Calibration curve:

The simplest model must be used to define the relationship between the experimental response value and a known concentration of the standard curve and it can be measured with acceptable accuracy and precision. A calibration curve must be prepared by spiking the matrix with a known concentration of the analyte in the same biological matrix. And this sample study may involve more than one analyte. In case that matrix may be difficult to obtain, in such cases appropriate calibration matrix must be selected and then a comparison of obtained response must be done from both matrix. The concentration of standards must be selected based on the concentration range estimated in a particular study. A calibration curve should contain a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample with internal standard), and 5-9 non-zero samples covering the expected range, including the LLOQ.

d) Recovery:

The recovery of an Analyte in an assay is the detector response obtained from an amount of the Analyte added to and extracted from the biological matrix, compared to the detector response found for the true concentration of the pure authentic standard.

Recovery pertains to the extraction efficiency of an analytical method within the limit of variability. Recovery of the Analyte need not be 100%, but the level of recovery of an Analyte and the internal standard should be consistent, precise, and reproducible. Recovery experiments should be done by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with un-extracted standards that represent 100% recovery.

e) Accuracy:

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

f) Precision:

The precision is the closeness of individual measures of an Analyte (i.e. degree of scattering) among a series of measurements obtained from multiple aliquots of the same homogenous sample under the prescribed conditions. The acceptance criteria for precision at each concentration level must not exceed 15% of the coefficient of variation (CV). And acceptable deviation for LOQ is 20%.

g) Matrix effect:

There may be problems brought by any ion suppression or enhancement of ionization of Analytes by the effect of matrix components in the biological samples that may result in an increase or decrease in analyte response. To determine each analyte and the IS, the matrix factor must be quantitatively measured for each different matrix lots, by calculating the ratio of the Analyte peak area with matrix (determined by studying blank matrix spiked after extraction with Analyte), to the peak area without matrix (analyte solution is pure). The stable isotope-labeled IS normalized Matrix factor is calculated by dividing the Matrix factor of the Analyte by the Matrix factor of the IS. The Coefficient of variation of the IS-normalized matrix factor measured using 6 different matrix lots and it should not be more than 15%. At low and at high concentration levels it must be done for a maximum of 3 times the LLOQ and close to the ULOQ (Upper Limit of Quantification).

h) Stability:

The stability of Analyte in a biological fluid is a function to ensure that every step was taken during the storage of analytes, chemical properties of the drug, matrix/solution in which it is stored, and the container system does not affect the concentration of Analyte. The stability procedure must be ensured at every step to evaluate the stability of the Analytes during sample preparation, sample analysis, sample collection, and handling, after long-term (Store at the freezing storage temperature) and for short term stability (it is at room temperature, benchtop) storage, and after process through freeze and thaw cycles and the analytical process do not affect the concentration of analytes. Conditions used in the stability experiment must reflect conditions likely to be met during actual sample handling and analysis. The method must also comprise an evaluation of analyte stability in stock solution.

i) Ruggedness:

It needs to be done to check the susceptibility of a method by using a different instrument and column of the same dimension and same make and material. And it can be supportive during the method development/pre-validation phase. During one of the precision and accuracy batch, it can be evaluated by injecting using a different column. Also, the precision and accuracy batch should be processed by the different analysts (person) to evaluate the ruggedness of the extraction method [6, 11,13, 14, 15, 16]

Quantitative Applications of Bioanalytical Method

Table 2: Applications of LC-MS/MS in drugs by analysis utilizing different sample preparation techniques.

Sr.No.	Drug	Matrix	LC column	Sample preparation	LLOQ	Ref.
1	Dapoxetine	Human plasma	ACE C_8 (50 mm × 4.6 mm × 5 µm)	LLE	5 ng/ml	17
2	Fisetin	Rat plasma	Nucleoden C_{18} (250 mm × 4.6 mm × 5 µm)	PPT	3.18 ng/ml	18
3	Chlorogenic acid	Human plasma	Zorbax C_{18} (50 mm ×2.1 mm,1.8 µm)	PPT	10 ng/ml	19
4	Rufinamide	Mouse plasma	LichroCART purospher star pre column C_{18} (55 mm × 4mm,3 µm)	PPT and LLE	0.1 µg/ml	20
5	Bepridil	Rat plasma	Hypersil BDS C_{18} HPLC (100 mm × 4.6mm,5 µm)	PPT	1 ng/ml	21
6	Paromomycin	Human plasma	Acquity UPLC HSS T_3 Analytical column (150 mm × 2.1 mm,1.8 µm)	PPT	5 ng/ml	22
7	Tegoprazan and its major metabolite M1	Dog plasma	Cadenza CW-C_{18} (2.0 mm × 75 mm,3 µm)	PPT	50 ng/ml, 5 ng/ml for Tegoprazan and M1, respectively	23
8	Motesanib	Rat plasma	Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm,1.7 µm)	LLE	5.0 ng/ml	24
9	Erdafitinib	Human plasma	Eclipse plus C_{18} column (3.0 mm× 150 mm,5 µm)	SPE	3.0 ng/ml	25
10	Niraparib and its metabolite M1	Human plasma	SunFire C_{18} column (50 mm × 2.1 mm,5 µm)	PPT	1 ng/ml	26
11	Pantoprazole	Human plasma	Zorbax SB-C_{18} column (4.6 mm × 75 mm,3.5 µm)	PPT	10 ng/ml	27
12	Irbesartan	Rat plasma	Hypersil Gold C_{18} column (4.6 mm × 250 mm,5 µm)	SPE	0.2 µg/ml	28
13	Cabozantinib	Human plasma	Xbridge C_{18} (50 mm × 4.6 mm,5 µm)	LLE	5.0 pg/ml	29
14	Erlotinib and Tamoxifen	Rat plasma	Acquity UPLC BEH C_{18} column (100 mm × 1.0 mm,1.7 µm)	SPE	2.0 ng/ml	30
15	Niacin and Nicotinuric acid	Human plasma	Phenomenex Gemini NX (100 mm × 4.6 mm,5 µm)	SPE	10.068 ng/ml and 10.157 ng/ml for (NIC) and (NIA), respectively	31
16	Lansoprazole	Human plasma	Thermo hypurity advance (50 mm × 4.6 mm,5 µm)	SPE	4.50 ng/ml	32
17	Rizatriptan	Human plasma	ACE C_{18} column (150 mm × 4.6 mm,3 µm)	LLE	50 ng/ml	33
18	Dapagliflozin or saxagliptin with Metformin	Human plasma	Zorbax C_{18} (50 mm × 4.6 mm,5 µm)	LLE	5 ng/ml, 2 ng/ml and 10 ng/ml for DAP, SAX, and MET respectively	34
19	Mirabegron and its metabolites	Human plasma	Inertsil C_8-3 (50 mm × 2.1 mm, 3 µm)	SPE or LLE	0.5 ng/ml -1 ng/ml	35
20	Venlafaxine and its 5 metabolites	Rat plasma	Agilent SB-phenyl (50 mm × 4.6 mm, 3.5 µm)	LLE	0.2 ng/ml – 15 ng/ml	36
21	Rapamycin	Rabbit plasma	Reverse phase C_8 xterra (50 mm × 4.6 mm,5 µm)	PPT	2.3 ng/ml	37
22	Milnacipran	Rat plasma	Zorbax SB – CN (75 mm × 4.6 mm,3.5 µm)	LLE	1 ng/ml	38
23	Trifluridine with its metabolites and Tipiracil	Rat plasma	Fused silica capillary (650 mm × 550 mm,50 µm)	PPT	100 ng/ml 200 ng/ml	39

24	Maraviroc	Human plasma	Waters BEH C_8 (50 mm × 2.1 mm,1.7 µm)	PPT	0.5 ng/ml	40
25	Cefpodoxime	Rabbit plasma	RP C_{18} phenomenax 100 (250 mm × 4.6mm,5 µm)	LLE	0.5 µg/ml	41
26	Cefdinir	Human plasma	RP C_{18} waters symmetry shield column (50 mm × 2.1 mm,5 µm)	PPT	5 ng/ml	42
27	Bupropion, hydroxybupropion, erythrohydrobupropion and threohydrobupropion	Human plasma	Chiralpak α_1 acid glycoprotein (AGP) column (100 mm × 2.0 mm,5 µm)	PPT	0.5-2 ng/ml	43
28	Mematine	Human plasma	Zorbax SB-C_{18} (7.5 mm × 4.6 mm,3.5 µm)	LLE	50 ng/ml	44
29	Metaxalone	Human plasma	RP C_{18} chromalopak peerless basic (50mm × 4.6 mm,3.0 µm)	LLE	25.19 ng/ml	45
30	Ramipril and hydrochlorothiazide	Human plasma	Enable C_{18} G column (150 mm × 4.6 mm,5 µm)	LLE	2 ng/ml	46
31	Lorcasarin	Human plasma	Phenomenex luna C_{18} (250 mm × 4.6 mm,5 µm)	SPE	500 ng/ml	47
32	Ciprofloxacin HCl	Human plasma	Phenomenex C_{18} column (4.6 mm,3.5 µm)	LLE	0.5 µg/ml	48
33	Sugammadex	Human plasma	Polaris C_{18}-A PEEK analytical column (50 mm × 4.6 mm,5 µm)	SPE	0.1 µg/ml	49
34	Imatinib, voriconazole, and their metabolites	Rat plasma	Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm,1.7 µm)	PPT	5 ng/ml	50
35	Amphotericin B	Human plasma	Hypersil gold column (50 mm × 2.1 mm,1.9 µm)	PPT	0.125 mg/ml	51
36	Deoxyelephantopin	Rat plasma	Agela-C_{18} analytical column (50 mm × 2.1 mm,1.8 µm)	LLE	13.2 ng/ml	52
37	Canagliflozin	Human plasma	Zodiac C_{18} (100 mm × 4.6 mm,5 µm)	SPE	10.3 ng/ml	53
38	Temozolomide	Human plasma	Kinetex C_{18} column (100 mm × 2.1 mm,2.1 µm)	PPT,SPE,LLE	10 ng/ml	54
39	Rosuvastalin and Irbesartan	Rat human	Agilent eclipse plus ODS (100 mm × 4.6 mm,3 µm)	PPT	1 ng/ml	55
40	Ibuprofen	Human plasma	XDB-C_{18} column agilent (50 mm × 4.6 mm,1.8 µm)	PPT	0.15 µg/ml	56
41	Talazoparib	Rat plasma	Acquity BEH C_{18} (50 mm × 2.1 mm,1.7 µm)	PPT	0.5 ng/ml	57
42	Ulifloxacin, fenbufen, and felbinac	Rat plasma	Kinetex EVO C_{18} (100 × 4.6 mm, 2.6 µm)	SPE	0.05 -0.5 µg/ml	58
43	Bedaquiline	Rat plasma	RP C_{18} column (100 × 4.6 mm)	PPT	3.9 ng/mL	59

DISCUSSION

The major bioanalytical technique is an attempt to understand and explain the bioanalytical studies using a systematic approach starting with information gathering, followed by method development and systematic validation from a basic point of view for determining selectivity, sensitivity, calibration curve, recovery, accuracy, precision, matrix effect, stability, the ruggedness of chromatographic method to support pharmacokinetic, toxicokinetic, bioavailability and bioequivalence studies. Also, various essential sample preparation techniques are discussed in bioanalysis by using a detection techniques HPLC, LC/MS/MS, UPLC (Ultra Performance Liquid Chromatography).

CONCLUSION

In this review, the recent concepts and new progress which has beeen made in many areas comprising various sample preparation tools and separation is covered. The widest use of sample preparation technique is SPE and LLE. Large progression has been included in SPE technology such as new SPE format and selection sorbents. This review has focused on LC-MS/MS and promptly used the technique of high-quality for bioanalysis of small molecules. New ideas and relatively new technology are also covered in this review article that can be used to advance LC-MS/MS.

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