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Review Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION: REVIEW

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ABSTRACT

The development of selective, sensitive and reliable bioanalytical methods for the quantitative evaluation of drugs and their metabolites in biological matrices is crucial for the successful drug development. The data obtained from these methods is required in the pharmacokinetic and toxicokinetic studies of investigational new drug applications (INDs), new drug applications (NDAs) and abbreviated new drug applications (ANDAs). The results of animal toxicokinetic studies and of human clinical trials, including bioavailability and bioequivalence studies requiring pharmacokinetic evaluation are used to make critical decisions supporting the safety and efficacy of a drug. Therefore, it is of paramount importance that the developed bioanalytical methods are well designed, adequately validated and documented to a satisfactory standard to apply in drug analysis in order to obtain reliable results. High pressure liquid chromatography is a versatile analytical tool useful in identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices. So it is advantageous to develop and validate bioanalytical HPLC method for low dose drugs. This article reviews current progress in HPLC based bioanalytical method development and validation of different drugs. So, far drugs like Anti malarials, Omeprazole, Clofarabine, Palonosetron HCl, etc have been analyzed bioanalytically.

***Key words:** Bionalytical method development, HPLC, Validation, Documentation and Application.*

INTRODUCTION

Bioanalytical method includes collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to predicate a quantitative analytical method and is suitable for biomedical applications. A reassurance as to the quality of the method and its reliability is assumed from adopting a number of series of validation experiments and obtaining satisfactory results. Characterization of the stability of analytes in biological samples collected during clinical studies together with that of critical assay reagents, including analyte stock solutions, is recognized as an important component of bioanalytical assay validation. Bioanalytical method validation includes all of the procedures that consist of a specific method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use [1].

Determination of drugs from biological fluids is fundamental to pharmacologic and pharmacokinetic studies, which include determinations of the absorption, distribution, metabolism, and elimination of drugs in animals and humans. A biologic fluid assay provides evidence for studies of correlations of blood tissue levels of drugs and pharmacologic effects (pharmacodynamics), which are desired during drug development [2–4]. Sample preparation for analysis of biological samples is the key factor that determines the extent of recovery of the analyte from the sample matrix. In this framework, LLE is the most widely used method for extraction and separation of analytes from aqueous biological fluids and endogenous interferants. It also provides a simple means of concentrating the analyte by

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evaporation of the solvent. The analyte is isolated by fractioning between the organic phase and the aqueous phase [5, 6].

Method Development: A well choreographed method development is important in drug development. Method development consists of the following:

- Sample preparation
- Separation of analyte and
- Detection of analyte.

Sample preparation:

Sample preparation is key step for analysis of drugs and metabolites in bioanalytical study. Biological samples containing proteins, various endogenous and exogenous substances may interfere with analyte. The main aim of sample preparation is analyte should be free from all possible unwanted substances without significant loss of analyte [7].

Biological matrices used in bioanalysis:

Determination of drug in urine is an indirect method to determine the bioavailability of a drug. Estimation of drug in feces may reflect drug that has not been absorbed after an oral dose or that has been expelled by biliary secretion after systemic absorption. Salivary drug levels indicate the free drug rather than total plasma drug concentration as only free drug diffuses into the saliva. Therefore, the saliva/plasma drug concentration ratio is less than 1 for many occasions.

Measurement of a drug concentration in the blood, serum or plasma is the most direct approach to assess the Pharmacokinetics of the drug in the body. Assuming that a drug in the plasma is in dynamic equilibrium with the tissues, then changes in the drug concentration in plasma reflects changes in tissue drug concentrations [8].

Storage requirements for biological samples: [9]

Biological samples should be frozen immediately upon collection to avoid decomposition or other potential chemical changes in

the drugs to be analyzed, and defrost before analysis. In case drugs are susceptible to plasma esterases, addition of esterase inhibitors, such as sodium fluoride to blood samples immediately after collection, prevent drug from decomposition.

When collecting and storing biological samples, affects from tubing or storage vessels that can contaminate the sample should be avoided. For example, plastic-ware containing the high boiling liquid, bis (2-ethylhexyl) phthalate, and the plunger-plugs of vacutainers contain tri-butoxyethyl phosphate can interfere in certain drug analysis. In the case of faeces, lyophilization of the sample before storage is highly desirable unless prior investigations have revealed little or no reactivity of the drug components with the endogenous intestinal microorganisms.

Preliminary treatment of biological samples:

Separation or isolation of drugs and metabolites from biological samples can be performed for partial purification of biological samples. In this manner, selectivity and sensitivity is an important criteria to detect a particular compound and with minimum interference from components of the more complex biological matrix. The number of steps in a separation procedure should be minimal to prevent loss of drug or metabolite. In most cases, preliminary treatment of a sample is required before the analysis. Analyses are required for drug in samples as distinct as plasma, urine, faeces, and bile. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method. Such factors are lineament and chemical composition of the sample, degree of drug-protein binding, chemical stability of the drug and types of interferences. The pre-treating methods include centrifugation, homogenization and hydrolysis of conjugates.

Centrifugation is used for eliminating the cells in the blood or to make the sample free from proteins. Samples are placed in tapered centrifuge tubes and rotated at high velocity. After centrifugation, the clear supernatant is transferred to a new centrifuge tube. Centrifugation is usually performed using a centrifuge at 4ºC to avoid decomposition of the analyte. For samples containing insoluble proteins, such as muscle or other related tissues, a homogenization or solubilizing step, using 1N hydrochloric acid, may be required before treating the sample further. A solid sample such as faeces can be homogenized with a minimum amount of methanol. Homogenization is usually performed with a blade homogenizer or tissue homogenizer. The presence of drug metabolites as conjugates, such as glucuronides and sulfates in biological samples cannot be ignored. The effect of a drug depends on a considerable extent on the biotransformation. Therefore, it is important to isolate the actual conjugates. Samples containing either glucuronide acetals or sulfate esters are usually pre-treated using enzymatic or acid hydrolysis. The unconjugated metabolites that result from the hydrolysis procedure are less hydrophilic than their conjugates and usually extracted from the biological matrix. A non-specific acid hydrolysis is accomplished by heating a biological sample for 30 min at 90 ºC to 100 ºC in 2N to 5N hydrochloric acid. Upon cooling, the pH of the sample is to be adjusted to the desired level and the metabolite can be removed by solvent extraction. stable Sometimes conjugates require hydrolysis in an autoclave [9].

Separation of an analyte :

Extraction of analyte from biological matrix is carried out by [10]

(a) Solid-phase extraction (SPE)
(b) Protein precipitation (PP) and
(c) Liquid-liquid extraction (LLE).

(a)Solid Phase Extraction (SPE):

SPE is an effective technique for isolation and concentration of analyte in trace amounts from sample matrices. In SPE the level of interferences can be reduced and final sample volume is minimized to maximize the sensitivity of analyte. High recovery of analyte can be obtained by using a small plastic disposable column or cartridge packed with 0.1 to 0.5 g of sorbent which is RP material (C18 or C8). The components of interest may either preferentially adsorbed on the solid, or they may remain in the liquid phase. If the desired analyte is adsorbed on the solid phase, it can be selectively desorbed by washing with an appropriate solvent. If the component of interest remains in a liquid phase, it can be recovered through concentration, evaporation and or recrystallization. Extraction by SPE is more time consuming and it is difficult with high density materials [11].

(b)Protein precipitation (PP):

PP can be employed for extraction of hydrophilic and hydrophobic substances. It is a simple technique for extraction of the analyte from blood or plasma. The main requirement for this technique is, the analyte should be freely soluble in reconstituting solvent. In this technique, the sample is prepared by protein precipitation by using

Acids: trichloroacetic acid and perchloric acid
Organic solvents: methanol, ethanol, acetone and acetonitrile
Salts: ammonium sulphate.

After precipitation the sample is centrifuged, analyte is present in supernatant. Among the solvents, methanol is preferred as it produces clear supernatant which is suitable for direct injection. The limitation of PP is, it may clog the column.

(c) Liquid-liquid extraction:

Liquid-liquid extraction is most widely used technique because of the following advantages:

- The analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination.
- This technique is simple, rapid and relatively cheap.
- The extract containing the drug can be evaporated to dryness and the residue can be redissolved in a smaller volume of an appropriate solvent. So, the sample becomes more compatible with a particular analytical methodology in the measurement step, such as a mobile phase in HPLC determinations.
- The extracted material can be redissolved in small volumes of solvent (e.g., 100 μL to 150 μL), which increases the sensitivity limits of an assay.
- It is possible to extract more than one sample simultaneously.
- Through multiple or continuous extractions, quantitative recoveries (90% or better) of the drugs can be obtained.

Distribution of a drug between two liquid phases is expressed in terms of a partition coefficient, and it is called as log P. It is constant for a particular solute, temperature and pair of solvents. By knowing the log P value for the extracted drug and the absolute volumes of the two phases to be utilized, the quantity of drug extracted after a single extraction can be obtained. In multiple extraction methodology, the original biological sample is extracted several times with fresh volumes of organic solvent until as much drug as possible is obtained. Because the combined extracts now contain the total extracted drug, it is desirable to calculate the number of extractions necessary to achieve maximum extraction.

Factors affecting partition coefficient:

Factors that effects the partition coefficient and recovery of drugs in liquid-liquid extraction are

Choice of solvent,
pH and
Ionic strength of the aqueous phase.

Choice of solvent:

In almost all the cases, one of the liquid phases is aqueous because of the nature of a biological sample. The second liquid is selected by the analyst. The selected organic solvent should show greater affinity toward the drug to be analysed and should leave contaminants or impurities in the aqueous or biological phase. The solvent should be immiscible with an aqueous phase, should have less polarity than water and should solubilize the desired extractable compound to a larger extent. It should also have a relatively low boiling point so that it can be easily evaporated if necessary. Other considerations are cost, toxicity, flammability and nature of the solvent. If larger numbers of samples are to be extracted, the volume of solvent needed per sample can affect the overall cost of the assay procedure. Diethyl ether and Chloroform are the solvents of choice for acidic and basic drugs respectively, especially when the identity of the drugs in the samples is unknown. In these cases, any chemically neutral drugs are

extracted into either solvent depending on their relative partition tendencies.

pH:

Proper pH adjustment of a biological sample permits quantitative conversion of an ionized drug to an un-ionized species, which is more soluble in a non polar solvent and therefore, extractable from an aqueous environment, the proper pH for extraction can be calculated from the Henderson-Hasselbalch equation using the pKa of the compound for analysis of a known drug or metabolite. If the species to be analyzed is unknown, the pH must be approximated based on the chemical nature of the suspected agent.

Ionic strength:

Addition of highly water-soluble ionized salts, such as sodium chloride, to an aqueous phase creates a high degree of interaction between the water molecules and the inorganic ions in solution. Fewer water molecules are free to interact with the unionized drug. Therefore, solubility of the drug in aqueous phase decreases, thereby increasing the partitioning or distributing in favor of the non polar or organic phase. The technique is commonly called as "salting out". Mixing of the aqueous-organic phases can be achieved either by mechanical or manual tumbling, rocking or vigorous shaking of the samples. The percent recovery of a drug versus time and/or type of mixing should be investigated for each biological sample. In many cases, vigorous shaking of a sample should be avoided because it results in emulsification, which can further interact during centrifugation. Emulsification is often observed when organic solvents are used at basic pH whereas certain organic solvents such as n-hexane and diethyl ether are less emulsion-prone. Certain types of amphoteric drugs that possess extreme water solubility are not amenable to classic solvent extraction. In these cases, other types of analytical methodology such as ion pairing must be adopted (e.g. cationic drugs such as quaternary ammonium ions can ion pair with various anions such as alkyl and arylsulfonate and inorganic perchlorate and chloride).

Chromatographic Methods:

Chromatography is a technique, according to which separation is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture into its various components. The affinities or interactions can be classified in terms of adsorption, partition and a solute passing through or impeded by a porous substance based on its molecular size (exclusion).

High performance liquid chromatography (HPLC):

HPLC is directly derived from classic column chromatography, in which a liquid mobile phase is pumped under pressure rather than by gravity flow through a column filled with a stationary phase. It results in a sharp reduction in separation time, narrower peak zones, and improved resolution. The mobile phase is placed in a solvent reservoir for pumping into the system. A solvent system is usually degassed by vacuum treatment or sonication before use. Most of the drugs in biological sample can be analyzed by HPLC method because of the advantages, such as speed, greater sensitivity (various detectors can be employed), improved resolution (wide variety of stationary phases), reusable columns (expensive columns but can be used for many samples), ideal for the substances of low volatility, easy sample recovery, handling and maintenance, instrumentation lends itself to automation and quantitation (less time and less labour), precise and reproducible, calculations are done by integrator itself and suitable for preparative liquid chromatography on a much large scale.

Components of HPLC:

The major components of HPLC system are pumps (solvent delivery system), injector (manual or auto), analytical columns, detectors and recorders and/or integrators. Other miscellaneous components are mixing unit, gradient controller, solvent degasser and guard column. Recent models are equipped with computers and software for data acquisition and processing.

Pumps:

Pumps must be made up of the materials which inert to all mobile phases. Materials commonly used are glass, stainless steel, teflon and sapphire. The pump must be able to generate pressure up to 5000 psi at flow rates of up to 3 mL/min for analytical operations. The solvent flow from the pump should be pulse less or should be dampened in order to remove pulses. Since the presence of pulses in the solvent flow may cause superior results with some detectors. HPLC pumps can be classified into two groups according to the manner in which they operate, viz., constant flow rate pumps and constant pressure pumps.

Mobile phase selection:

Various parameters are considered for the selection of a mobile phase, such as viscosity. Compressibility, refractive index, UV cutoff, polarity, vapour pressure and flashpoint. The viscosity generally increases with the number of carbons in the solvent. Straight chain alcohols show a very pronounced relationship of this nature. For example, to achieve 1 mL/min flow rate in a 4.6 × 250 mm column packed with 5 μm octadecyl silane material, a pressure of 1500 psi is required with methanol. Solvents of low viscosity are needed to be compatible with the limitations of the pump. Also as viscosity increases, the efficiency of the system, as measured by the number of theoretical plates decreases. The sensitivity of the detection is related to the difference between the respective refractive indices, i.e. the greater the difference, greater is the sensitivity. The UV cutoff is defined as the wavelength below which the solvent absorbs more than 1.0 absorbance unit. Polarity of a solvent is the measure of dielectric constant or the ability to elute a particular type of compound. The vapour pressure of a solvent plays an important role in mobile phase selection. Solvent reservoir could easily change in composition due to the evaporation of one of the more volatile constituents. The flammability of the mobile phase is a safety consideration. Careful attention should be paid to adequate ventilation and waste solvent disposition. The mobile phase in HPLC has a great influence on the retention of the solutes and the separation of component mixtures. The primary constituent of the mobile phase used in reverse phase HPLC is water. Water miscible solvents such as methanol, ethanol, acetonitrile, dioxan, tetrahydrofuran (THF) and dimethylformamide (DMF) are added to adjust the polarity of the mobile phase. The water should be of high quality, either distilled or demineralised. The most widely used organic modifiers are methanol, acetonitrile and THF. Methanol and acetonitrile have comparable polarities but the latter is an aportic solvent. This factor may be important if hydrogen bonding plays a significant role in the separation. When inorganic salts and ionic surfactants are used, the mobile phase should be filtered before use since these additives frequently contain a significant amount of water insoluble contaminants that may damage the column. Reverse phase mobile phases are generally noninflammable due to high water content. Degassing is quite important with reverse phase mobile phases.

Selection of column:

Most column packing is based on a silica matrix. These packing are rigid and can withstand pressure in excess of 10,000 psi. Silica particles are also available in a wide range of porosity. Since the surface of silica contains silanol groups, organic moieties may be chemically bonded to the surface for bonded phase chromatography. Other rigid solids, which can be used as support materials are particles of polystyrene cross-linked with divinyl benzene. The polymers are more resistant to chemical attack than silica particles and may find more uses with mobile phase of high and low pH values. The size of the packing material has a major effect on the resolving power of the system. As the particle size decreases, the height equivalent to theoretical plates also decreases. However, as the diameter of the particles decreases, the resistance of solvent flow increases. Particle diameter of 3-10 μm is used in analytical applications while particles of 37-50 μm can be used for preparative scale HPLC where high solvent flow rates are required.

Detectors:

One of the widely used detectors in HPLC is

UV detector: which is capable of monitoring several wavelengths concurrently; this is possible only by applying a multiple wavelength

scanning program. If present in adequate quantity, UV detector assures all the UV-absorbing components are detected.

Photodiode array (PDA) detector: is a lined array of discrete photodiodes on an integrated circuit (IC) chip for spectroscopy. It is placed at the image plane of a spectrometer to allow a range of wavelengths to be sensed concurrently. When a variable wavelength detector (VWD) is used a sample must be injected numerous times, with changing wavelength, to be sure that all the peaks are detected. In the case of PDA, when it is used a wavelength range can be programmed and all the compounds that absorb within this range can be identified in a single analysis. PDA detector can also analyze peak purity by matching spectra within a peak. PDA detector finds its application in the method development of Iloperidone in pharmaceuticals.

Refractive index detector: is the detector of choice when one needs to detect analytes with restricted or no UV absorption such as alcohols, sugars, carbohydrates, fatty acids, and polymers. Decent trace detection performance is secured through a low noise. This detector is having the lowest sensitivity among all detectors but suitable at high analyte concentrations.

Electrochemical detector: responds to the substances that are either oxidizable or reducible and the electrical output results from an electron flow triggered by the chemical reaction.

Fluorescence detector: one of the most sensitive detectors among the LC detectors is fluorescence detector. Typically its sensitivity is 10–1000 times higher than that of the UV detector for strong UV absorbing materials used as an advantage in the measurement of specific fluorescent species in samples. One of the most important applications of fluorescence is the estimation of pharmaceuticals.

Over a certain period of time most workers used the reversed-phase mode with UV absorbance detection whenever appropriate, because this provided the best available reliability, analysis time, repeatability and sensitivity.

Table No. 1: List of Bio-analytical methods [12]

S. No.	Title	Publisher	Drug name	Drug Category	Method
1.	Bio-analytical method development and validation of atenolol in human plasma by LC-MS.	Asian journal of pharmaceutical and clinical research	Atenolol	Antihypertensive	LC-MS
2.	A simple HPLC Bio-analytical method for the determination of Doxorubicin hydrochloride in rat plasma: application to Pharmacokinetic studies.	Tropical journal of pharmaceutical research march 2014; 13 (3): 409-415	Doxorubicin	Antibiotic	HPLC
3.	Quantitative estimation of Ceftibuten by RP-HPLC method in bulk and capsule dosage form.	journal of pharmacy research 6 (2013)395-400	Ceftibuten	Antibiotic	RP- HPLC
4.	RP-HPLC method for the estimation of Ceftiofurhydrochloride in bulk form.	journal of pharmacy research7 (2013) 246-251	Ceftiofurhydrochloride	Antibiotic	RP- HPLC
5.	Rapid and sensitive HPLC method for the determination of Sirolimus with Ketoconazole as internal standard and its further applications.	International journal of pharmaceutical sciences and drug research 2012; 4(1): 70-73	Sirolimus	Immunosuppressive Agent	HPLC
6.	Bio-analytical method development and validation for simultaneous determination of Linagliptin and Metformin drugs in human plasma by RP-HPLCmethod.	Pharmacophore 2014, vol. 5 (2), 202-218	Linagliptin Metformin	Antidibetics	RP- HPLC
7	Development and validation of Bio-analytical method for determination Quetiapine from human plasma.	International research journal of pharmacy	Quetiapine Fumarate	Antipsychotic	HPLC
8	Development and validation of a sensitive Bio-analytical method for the determination of Sumatriptan in rat plasma by UPLC-MS.	International journal of pharmacy and pharmaceutical sciences, vol 5, suppl 3, 2013	Sumatriptan	Antimaigrane	UPLC-MS
9	Development and validation of spectrophotometric method for estimation of Satranidazole in bulk drugs and formulations.	Asian journal of medicinal and analytical chemistry 01 (01); 2014; 09-11.	Satranidazole	Antiprotozoal	spectrophotometric method
10	Analytical method development and validation for simultaneous Estimation of Lopinavir and Ritonavir by RP-HPLC.	International journal of research and development in pharmacy and life sciences	Lopinavir	Antviral	RP- HPLC
11	Development and validation of analytical method for estimation of Griseofulvin and preservatives in oral suspension.	The pharmaceutical and chemical journal, 2014, 1(1):15-22	Griseofulvin	Antifungal	RP-HPLC
12	Development and validation of analytical method for the estimation of Nateglinide in rabbit plasma.	Journal of Pharmaceutical Analysis 2012;2(6):492–498	nateglinide Antidiabetic		
13	Method development and validation of Ethambutol in human plasmaby using LC-MS/MS.	International journal of research in pharmaceutical and biosciences	Ethambutol	Chemotherapeutic agent	LC-MS/MS
14	A new analytical method development and validation for estimation of Ciprofloxacin in bulk and pharmaceutical dosage form.	Asian j. pharm. Ana. 2012; vol. 2: issue 4, pg 116-117	Ciprofloxacin	Antibiotic	UV spectrophotometeric method
15	Development and validation of new analytical method for the estimation of Tizanidine hydrochloride in bulk and in formulation by UVspectrophotometric method.	International journal of pharma sciences	Tizanidine Hydrochloride	Management of spasticity	UV–spectrophotometric method
16	Spectrophotometric method for simultaneous determination of Olmesartan Medoxomil and	International journal of current pharmaceutical	Olmesartan, Medoxomil and	Hypertension	UV-visible double beam

	Amlodipine besylate from tablet dosage form.	research	Amlodipine besylate		spectrophotometeric method
17	Development and validation of Bio-analytical HPLC method for estimation of Telmisartan in rat plasma: application to pharmacokinetic studies.	J. pharm. sci. 11(2): 121-127, 2012 (December)	Telmisartan	Antihypertension	HPLC
18	Bio-analytical method validation for the Determination of Enalapril in human serum by LC-MS/MS detection.	Paripex - indian journal of research	Enalapril	Pro-drug of enalaprilat (antihypertension)	LC-MS
19	Chromatographic development of validated analytical method for the estimation of Tapentadol and Paracetamol in combined dosage Form.	International journal of advances in pharmaceutics	Tapentadol and Paracetamol	Analgesic	HPLC
20	Development and validation of analytical method for simultaneous estimation of Amoxicillin and Probenecid in bulk and tablet dosage form using HPLC.	Scholars academic journal of pharmacy (SAJP)	Amoxicillin and Probenecid	Analgesic	HPLC

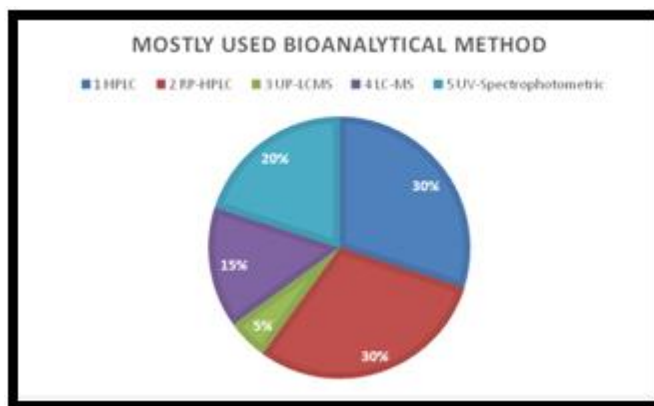

Fig. 1: Mostly used Bio Analytical Method

Modes of separation: [13-15]

HPLC applications can be classified in to six separation modes. They are adsorption, normal bonded phases, reverse phase, ion pair, ion exchange, size exclusion. Adsorption chromatography was the first liquid chromatography mode to be developed. Its primary application is separation of weak to moderately polar organic-soluble compounds. It refers to separations on bare adsorbents such as silica gel or alumina. These adsorbents have polar surfaces and retain solutes by dipole-dipole, dipole-induced dipole, and hydrogen boding forces. The strength of adsorption of a solute is determined by the number and types of functional groups in the molecule. Adsorbents have a rigid structure and their surfaces have a fixed spatial arrangement of strongly polar sites. These properties result in chromatographic selectivity that is unequaled with other HPLC modes.

Normal phase mode refers to separations on bonded phases with polar functional groups such as cyano, amino or hydroxyl. A polar stationary phase and a relatively nonpolar mobile phase are employed. It is functionally similar to adsorption mode. Nonpolar hexane is a weak solvent and more polar solvents such as methylene chloride or chloroform are strong solvents. Reversed phase mode refers to chromatography on stationary phase having nonpolar or low polarity surfaces with polar eluents. Very polar water is usually the weak solvent and less polar organics such as methanol or acetonitrile are strong solvents. Solutes elute in the order of decreasing polarity. The most significant characteristic of reversed phase is its exceptionally wide range of application. The most common mobile phase used is mixture of water and methanol. Substitutes for methanol are acetonitrile and tetrahydrofuran. In order to sharpen the peak shape, a small quantity of sodium phosphate or sodium acetate is added to the mobile phase. Similarly, addition of 1-2% of THF to acetonitrile or methanol will produce the same effect. Using low pH buffer, the ionization of acidic molecules is suppressed, which will produce the higher retention time and vice versa.

Ion paring agents are ionic compounds with a large hydrophobic moiety. Ion pairing agents are used in solvent extraction. The extraction of an ionized organic compound from water is greatly enhanced by adding an ion-pairing agent of the opposite charge. These are adsorbed by dispersive interactions with the stationary phase. C8 and C18 packing are popular for ion pair separations because they provide strong dispersive interactions with ion pairing agents. Other reversed phase columns such as C4 and phenyl are also used with this mode. Cationic ion pairing agents are cetyltrimethylammonium, tetrabutyl ammonium phosphate or sulfate (TBAP or TBAS) and tetraethyl ammonium phosphate or sulfate (TEAP or TEAS). Anionic ion pairing agents are sodium salt of dodecane sulfonic acid, hexane sulfonic acid, octane sulfonic acid, pentane sulfonic acid, and trifluoroacetic acid.

Ion exchange chromatography (IEC) is used for the separation of ionic materials such as protein, amino acids and inorganic substances. Ion exchange packing has either cationic or anionic functional groups covalently bound to a support. Cationic groups are used to separate anions and vice versa. Eluents used for IEC are aqueous solutions of buffers and salts. The primary advantage of IEC is its ability to separate inorganic ions. Using conductivity detection, very low concentrations can be measured. This technique is also useful for the separation of small water-soluble organic ions. The disadvantages of IEC are lower efficiency separations, peak tailing, and poor column stability.
Size exclusion chromatography (SEC) separates solutes by molecular size. It is also called as gel permeation chromatography (GPC) or gel filtration chromatography. SEC is used for samples that contain high molecular weight compounds and for samples whose components are significantly different in molecular size. It can be used to determine the molecular weight distribution of a polymer.

Quantitative analysis:

In quantitative analysis the goal is to determine the exact amount of analyte molecules. Most often two different analytes of equal concentration give different detector responses in chromatography, therefore the detector responses must be measured for known concentrations of each analyte. A standard curve is a graph showing the detector response as a function of the analyte concentration in the

sample. For the quantification analysis, three methods of calibration are employed, viz., external standard calibration, internal standard method and standard addition method.

External standard calibration:

The external standard calibration method is a simple but less precise method and should only be used when the sample preparation is simple or no instrumental variations are observed. The method is not suitable for use with complicated matrices but is often used in pharmaceutical product analysis characterized by simple matrices and easy sample preparation. To construct a standard curve, standard solutions containing known concentrations of the analyte must be prepared and a fixed volume is injected into the column. The resulting areas or heights of the peaks in the chromatogram are measured and plotted versus the amount injected. Unknown samples are then prepared, injected and analyzed in exactly the same manner, and their concentrations are determined from the calibration plot. The term "external standard calibration" implies that the standards are analyzed in chromatographic runs that are separate from those of the unknown sample.

Internal standard method:

The internal standard (I.S) method is a more accurate method. The internal standard technique can compensate for both instrumental and sample preparation errors and variations (e.g. dilution and extraction). Sample pretreatment steps such as extraction using protein precipitation, often results in sample losses, and a proper internal standard should be chosen to mimic the variations in these steps. Thus, both the accuracy and precision of quantitative data increase if an internal standard is included in the procedure. The internal standard should be similar but not identical to the analyte, and the two should be well resolved in the chromatographic step. The standard curves are obtained from standards of blank samples spiked with different concentrations of the analyte of interest and addition of an I.S at constant concentration. Also to the unknown samples the same constant concentration of the I.S is added. The standard samples are processed parallel with the unknown samples. In the calibration curve, the ratios of analyte to I.S peak area (or height) are plotted versus the concentration of the analyte. A proper internal standard in a bioanalytical method should fulfill the following requirements:

- Be well resolved from the compound of interest and other peaks
- Not be present in the sample
- Be available in high purity (not contaminated with the analyte)
- Be stable
- Should resemble the analyte in all sample preparation steps
- Be of similar structure as that of the analyte
- Be of similar concentration as the analyte.

Most often a compound with similar structure is selected. The internal standard method has become a very popular technique not only in chromatography, but also in quantitative HPLC-MS methods.

Standard addition method:

The standard addition method is often used in cases when it is possible to obtain suitable blank matrices. The approach is to add different weights of analyte to the unknown sample, which initially contains an unknown concentration of the analyte. After the chromatographic analysis, peak areas (or heights) are plotted versus the added concentration. Extrapolation of the calibration plot provides the original unknown concentration of the analyte. A standard addition method that possesses even greater accuracy and precision is obtained if one incorporates an internal standard.

Distribution of analytical data using weighted linear regression:

Statistical linearity investigations, also called as „lack-of-fit" tests, are only recommended if deviations from linearity are suspected or if the intrinsic response function is unknown. Unweighted linear regression is a constant variability of the y-values over the whole concentration range. A concentration range of more than two orders of magnitude will most probably violate the assumption. Non-constant variability (in homogeneity of variances) can be identified by graphical evaluation or a statistical test, such as the F-test at the upper and lower limit of the range. In order to achieve the same representation for all data, the „weight" of the smaller concentrations must be increased in the regression. This is achieved by using weighting factors in the least-squares regression. Either the reciprocals of the actual variability (variance or standard deviation) or generalized estimates of the error function are used. There can either be an individual model of the specific error function or a suitable approximation may be used taking the respective concentration into account, for example 1/x or 1/x2.

Validation:

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The increased number of biological agents used as therapeutics (in the form of recombinant proteins, monoclonal antibodies, vaccines, etc.) has prompted the pharmaceutical industry to review and redefine aspects of the development and validation of bioanalytical methods for the quantification of this therapeutics in biological matrices in support of preclinical and clinical studies. Bioanalytical method validation employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data [17]. These studies generally support regulatory filings [18]. The quality of these studies is directly related to the quality of the underlying bioanalytical data. It is therefore important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community.

Bioanalytical Method Validation:

Bioanalytical method validation is vital not only in terms of regulatory submission but also for ensuring generation of high quality data during drug discovery and development. BMV assures that the quantification of analyte in biological fluids is reproducible, reliable and suitable for the application[19]. Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance [16, 19] for accuracy, precision, selectivity, sensitivity, reproducibility, and stability.

Chromatographic methods (high-performance liquid chromatography [HPLC] or gas chromatography [GC]) have been widely used for the bioanalysis of small molecules, with liquid chromatography coupled to triple quadrupole mass spectrometry (LC/MS/MS) being the single most commonly used technology [20]. The objective of validation of bioanalytical procedure is to demonstrate that it is suitable for its intended purpose. The most widely accepted guideline for method validation is the ICH guideline Q2 (R1), which is used both in pharmaceutical and medical science [21]. Other guidelines, which are much more detailed, which require more extensive validation and which also have defined strict limits for the most of determined parameters are focused directly toward bioanalysis. They are represented by a "Guideline on Bioanalytical Method Validation" by EMA [17, 22] and "Guidance for Industry, Bioanalytical Method Validation" by FDA [16, 19].

Need of Bioanalytical Method Validation:

1. It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted.
2. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology.
3. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte.
4. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability.

Types of Bioanalytical Method Validation:

Validation is mandatory by the regulatory agencies. The main objective of method validation is to demonstrate the reliability of a particular method developed for the quantitative determination of an analyte in a specific biological matrix.

Bioanalytical method validation is classified into three types,

A. Full validation
B. Partial validation
C. Cross validation

Full validation:

The full validation is an establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte. Full validation is important:

1. When developing and implementing a bioanalytical method for the first time.
2. Fo r a new drug entity.
3. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

Partial validation:

Partial validations are modifications of already validated bioanalytical methods or Modification of validated bioanalytical methods that do not necessarily call for full revalidation. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.

Typical bioanalytical method changes that fall into this category include, but are not limited to:

1. Bioanalytical method transfers between laboratories or analysts
2. Change in analytical methodology (e.g., change in detectionsystems)
3. Change in anticoagulant in harvesting biological fluid
4. Change in matrix within species (e.g., human plasma to humanurine)
5. Change in sample processing procedures
6. Change in species within matrix (e.g., rat plasma to mouseplasma)
7. Change in relevant concentration range
8. Changes in instruments and/or software platforms
9. Limited sample volume (e.g., pediatric study)
10. Rare matrices
11. Selectivity demonstration of an analyte in the presence ofconcomitant medications Selectivity demonstration of an analyte in the presence of specific metabolites

Cross validation:

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies

1. An example of cross-validation would be a situation where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator. The comparisons should be done both ways.
   a. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability.
   b. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MSMS vs. ELISA) in different studies are included in a regulatory submission.

Validation Parameters: [23-25]

The basic parameters for the validation of a chemical assay comprises of all criteria determining data quality such as selectivity, accuracy, precision, recovery, linearity, calibration model, limit of detection (LOD), lower limit of quantification (LLOQ), stability, reproducibility, and ruggedness [3-5 & 29].

1. Selectivity (specificity):

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics [26].

For selectivity, blank samples of the appropriate biological matrix should be analysed from at least six sources. Each blank should be tested for interference of other substances and selectivity should be ensured at LLOQ [27].

2. Accuracy:

Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.

Accuracy should be measured for a minimum of three concentrations in expected range of concentrations using a minimum of 6 determinations per concentration. The mean should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. This deviation of mean from the true value serves as the measure of accuracy [27].

3. Precision:

Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Intraday precision is precision during single analytical run. Inter day precision measures with time, and may involve different analysts, equipment, reagents and laboratories.

Precision should be measured using a minimum of three concentrations in the expected range of concentrations with five determinations per concentration. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV [27].

4. Recovery:

Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiments should be performed at three concentrations (low, medium and high) with un-extracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible [27].

5. Linearity:

Linearity is the relationship between instrument response to known concentrations of the analyte. Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample.

6. Calibration curve:

It is the relationship between experimental response value and known concentrations of the analyte. A calibration curve should be designed by using the same biological matrix in which the intended study is to be done by spiking the matrix with known concentrations of the analyte. If enough blank sample is not available, e.g. in case of cerebrospinal fluid, 0.9% NaCl can be used as calibration matrix and response from both matrices should be compared.

Concentration of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and 5-8 non zero samples covering the expected range of analyte including LLOQ.

Calibration curve should be designed by applying simplest model that satisfactorily describes the concentration response relationship using suitable weighting and statistical tests for goodness-of- fit.

The following conditions should be met when developing a calibration curve:

15% deviation of standards other than LLOQ from true concentration and 20% deviation of the LLOQ from authentic concentrations.

At least four out of six non zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration [27].

7. Limit of Detection (LOD):

It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.

8. Lower Limit of Quantification (LLOQ):

It is the lowest amount of analyte in a sample that can be detected but necessarily quantitated under the stated experimental conditions with acceptable accuracy and precission.

The lowest standard on the calibration curve should be accepted as LLOQ if the following criteria are met:

The analyte response should be at least five times the response compared to blank response. Analyte peak should be identifiable, discrete and reproducible with a precision of maximum 20% and accuracy of 80-120% [27].

9. Matrix effect:

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along with internal standard are prepared and screened (Cappiello, 2008) (Chiu, 2010) (Patel, 2011).

10. Stability:

It is the chemical stability of an analyte in a given matrix under specific conditions for given time intervals. The aim of a stability test is to detect any degradation of the analyte(s) of interest, during the entire period of sample collection, processing, storage, preparation, and analysis. All but long term stability studies can be performed during the validation of the analytical method. Long term stability studies might not be complete for several years after clinical trials begin. The condition under which the stability is determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis) [27].

a) Freeze-thaw stability:

The influence of freeze/thaw cycles on analyte stability should be determined after at least 3cycles at 2 concentrations in triplicate. At least three aliquots at each of the low and high concentrations should be stored at intended storage temperature for 24 hours and thawed at room temperature. When completely thawed, refreeze again for 12-24 hours under the same conditions. This cycle should be repeated two more times, then analyse on 3rd cycle. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate blank, interference-free biological matrix. Standard deviation of error should be <15%. If analyte unstable freeze at -70oC for three freeze-thaw cycles.

b) Short-term stability:

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature for 4-24 hours and analyse. % Deviation should be <15%.

c) Long-term stability:

At least three aliquots of each of low and high concentrations should be thawed at room temperature and kept at this temperature for 4-24 hours and analyse. Analyse on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis.

d) Stock-solution stability:

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. % Deviation should be <15%. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

e) Post-Preparative Stability:

The stability of processed samples, including the resident time in the auto sampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

11. Reproducibility:

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility only has to be studied, if a method is supposed to be used in different laboratories [29].

12. Ruggedness: (Robustness):

Ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis due to pH, mobile phase composition, temperature etc. Ruggedness should be tested if a method is supposed to be transferred to another laboratory. Ruggedness is not mandatory under full validation, but it would be helpful during the method development as problems that may occur during validation are often detected in advance [28].

Quality control of samples:

QC samples in duplicates at three concentration levels (one near the 3 x LLOQ, one in mid range, one close to high end) should be incorporated at each assay run. Minimum number QCs should be at least 5% of total number of samples in a run or six total QCs whichever is greater [27].

Acceptance criteria:

The results of the QC samples provide the basis for accepting or rejecting a run. At least four out of every six should be within 15% of respective nominal values. Two of the six may be outside of 15% but not both at the same concentration [27].

Repeat analysis:

The guidelines for repeat analysis should be established. The rationale for the repeat analysis should be clearly documented such as sample processing errors, equipment failure, poor separation and resolution of analyte [27].

CONCLUSION

Quantitative determination of active pharmaceutical ingredient and its degradative products in biological fluids is a challenging task for an analyst due to difficulties and uniqueness of the sample. A systematic study of pharmaceutical products on human subjects whether patients or non-patient volunteers in order to discover or verify the clinical, pharmacological (including pharmacodynamics/ pharmacokinetics), adverse effects, with the object of determining their safety or efficacy and correcting plasma level with therapeutic action. HPLC is used for chemistry and biochemistry research analyzing complex mixtures, purifying chemical compounds, developing processes for synthesizing chemical compounds, isolating natural products, or predicting physical properties. It is also used in quality control to ensure the purity of raw materials, to control and improve process yields, to quantify assays of final products, or to evaluate product stability and monitor degradation. Thus the method developed and validated in the present review can be employed to estimate these two drugs simultaneously in all the in vivo studies, along with their metabolites.

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