BIOANALYTICAL METHOD DEVELOPMENT BY HPLC – A REVIEW

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ABSTRACT
The present review describes about method develop and validate a bioanalytical method in plasma. A HPLC method with UV-visible detection by employing C18 column can be used do estimate the drugs present in plasma with 10 mM ammonium acetate buffer and methanol as mobile phase. The developed method has to validate as per the USFDA guidelines for bioanalytical method validation.

Keywords: Bioanalytical method, Recent Analytical Techniques, HPLC, Plasma.

INTRODUCTION
Analytical chemistry is defined as the science and art of determining the composition of materials in terms of the elements of composition contained. In this, it is of prime important to gain qualitative and quantitative information of the substance and chemical species [1]. Quality assurance plays a central role in determining the safety and efficacy of medicines. Highly specific and sensitive analytical methods are applied to hold the key to the design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetics and in drug metabolism studies, both of which are fundamental to the assessment of bioavailability and the duration of clinical response [2].

Bioanalysis
Bioanalysis is defined as the quantitative determination of active pharmaceutical ingredient(s) and its metabolite(s) in biological fluids. Assays must distinguish between drug and its metabolites. A number of trials have been made for the analytical methods that distinguish drugs from their metabolites in biological fluids. Drug metabolism reactions can be divided into phase-I and phase-II reactions. Phase-I typically involves oxidation, reduction and hydrolysis reactions. In contrast, phase-II transformations entail coupling or condensation of drugs or their phase-I metabolites with common body constituents (e.g., sulphate, glucuronic acid). Except for reduction processes, most of the phase-I and phase-II reactions yield metabolites that are more polar and hence more water-soluble than the parent drug. Methods of measuring drugs in biological matrices are required for new drug discovery and development, clinical pharmacokinetics, bioavailability and bioequivalence studies and basic biomedical research. The major challenge in bioanalysis is to separate the analytes from the matrix of the biological samples [3].

Various biological matrices employed in bioanalysis
The matrix usually complicates detection of the analyte or its metabolite in biological samples. Because of this, various types of cleanup procedures are employed to effectively separate the analyte from the endogenous biological material. The ultimate sensitivity and selectivity of the assay method may be limited by the efficiency of the cleanup methodology [3].
The most common samples obtained for bioanalysis are blood and urine. Faeces are also utilized, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other matrix that is utilized is tissue, which gives information about the distribution of a particular analyte in the tissue. In most of the bioanalytical procedure, plasma or serum obtained from blood is employed. Serum is obtained by allowing the blood to clot followed by centrifugation. The amount of serum obtained is about 20% to 30% of the original volume of blood. Plasma is obtained by centrifugation of a blood sample collected in a tube containing anticoagulant (e.g. heparin, EDTA, citrate). The amount of plasma obtained is generally 50% of the original volume of blood. Though serum is devoid of proteins associated with the clotting process than plasma, the later is generally preferred because of its greater yield from blood. Urine and faeces samples are often providing the greatest source of metabolites and they are mostly used to find out the metabolites [3].

Storage requirements for biological samples [4]

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterases, the addition of esterase inhibitors, such as sodium fluoride, to blood samples immediately after collection helps to prevent drug decomposition. When collecting and storing biological samples, artefacts from tubing or storage vessels that can contaminate the sample should be avoided. For example, plastic-ware frequently contains the high boiling liquid, bis (2-ethylhexyl) phthalate, and the plunger-plugs of vacutainers contain tri-butoxyethyl phosphate, which 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 is performed in order to partially purify a sample. In this manner, an analyst can obtain the selectivity and sensitivity needed to detect a particular compound and can do so with minimum interference from components of the more complex biological matrix. The number of steps in a separation procedure should be minimum 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 diverse 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 can be selected. Such factors are texture 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 usually employed for eliminating the cells in the blood or involved after protein precipitation to remove proteins. Samples are placed in tapered centrifuge tubes and spun at high velocity. After centrifugation, the clear supernatant is transferred to a new centrifuge tube. Centrifugation is usually performed using a centrifuge at 4°C 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 to a considerable extent on the biotransformation. Therefore, it may be 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 can be extracted from the biological matrix. A non-specific acid hydrolysis can be accomplished by heating a biological sample for 30 min at 90 °C to 100 °C in 2N to 5N hydrochloric acid. Upon cooling, the pH of the sample can be adjusted to the desired level and the metabolite can be removed by solvent extraction. Particularly stable conjugates sometimes require hydrolysis in an autoclave [4].

Extraction procedures for drugs and metabolites from biological samples [5,6]

After pre-treating biological material, the next step is usually the extraction of drugs from the biological matrix. All separation procedures use one or more treatments of matrix-containing solute with some fluid. A simple step for removing the proteins present in the biological matrices is called as protein precipitation. If the extracting component is liquid and the matrix is a solid (e.g., lyophilized faeces, tissues), it is called as liquid-solid extraction (solid-phase extraction). If the extraction involves two liquid phases, it is liquid-liquid extraction.

Protein precipitation or Denaturation

Biological materials such as plasma, faeces contain significant quantities of protein, which can bind to a drug. The drug has to be freed from protein before further manipulation. Protein denaturation is important, because the presence of proteins, lipids, salts and other
endogenous material in the sample can cause rapid deterioration of HPLC columns and also interfere the assay. Protein denaturation procedures include the use of acids (tungstic acid, trichloroacetic acid and perchloric acid), salts (ammonium sulfate), heat or organic solvents (methanol and acetonitrile). Acetonitrile is mainly preferred because it produces a flocculent precipitate and not the gummy mass obtained with methanol. Acetonitrile also gives a clearer supernatant and may prevent the drug entrapment that can be observed after methanol precipitation. Ultra-filtration and dialysis procedures have also been used to remove proteins from biological fluids. These procedures are not widely used because they are slow.

Solid-phase extraction
Solid phase extractions occur between a solid phase and a liquid phase, either phase may initially contain the drug substance. Among the solids that have been used successfully in the extraction (usually via adsorption) of drugs from liquid samples are XAD-2 resins, charcoa, alumina, silica gel and aluminum silicate. Sometimes the drugs are contained in a solid phase, such as in lyophilized specimens. Liquid-solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water. Factors governing the adsorption and elution of drugs from the resin column include solvent polarity, flow rate of the solvent through the column, and the degree of contact the solvent has with the resin beads. In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alteration in the lipophilic/hydrophilic balance (HLB) within the solute or solvent mix, and not within the resin, affects adsorption of the solute. Biological samples can be prepared for cleanup by passing the sample through the resin bed where drug (metabolite) components are adsorbed and finally eluted with an appropriate solvent. The solid phase extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug losses and emulsion formulation. It is possible however, that strong drug-protein binding could prevent sufficient adsorption of the drug to resin.

Liquid-liquid extraction
Liquid-liquid extraction is probably the 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.
- The technique is simple, rapid and has a relatively small cost factor per sample.

- The extract containing the drug can be evaporated to dryness and the residue can be redissolved in a smaller volume of a more appropriate solvent. In this manner, 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 (e.g., 100 µL to 150 µL of solvent), thereby extending the sensitivity limits of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

Partitioning or distribution of a drug between two liquid phases can be expressed in terms of a partition coefficient, usually called as log P. A partition coefficient is constant only for a particular solute, temperature and pair of solvents used. 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 extractions 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 influence partition coefficient and hence recovery of drugs in liquid-liquid extraction are choice of solvent, pH and ionic strength of the aqueous phase. 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. It is highly desirable to select an organic solvent that shows great affinity for the drug analyzed, yet leaves 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. It is generally accepted that 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.

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 nonpolar
solvent and therefore, extractable from an aqueous environment. Analysis that estimates a known drug or metabolite, the proper pH for extraction can be calculated from the Henderson-Hasselbalch equation using the pKa of the compound. If the species to be analyzed is unknown, the pH must be approximated based on the chemical nature of the suspected agent. Third factor influencing extractability of drugs from biological samples is 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 nonpolar or organic phase. The technique is commonly called as "salting out".

Either mechanical or manual tumbling, rocking or vigorous shaking of the samples can accomplish mixing of the aqueous-organic phases. 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 leads into 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 an inorganic perchlorate and chloride) [5,6].

**Chromatographic methods**

Chromatography is a separation technique that 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 a solute adhering to the surface of a polar solid (adsorption), a solute dissolving in a liquid (partition), and a solute passing through or impeded by a porous substance based on its molecular size (exclusion) [6,7].

**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. This has resulted 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 constructed from the materials that are inert to all mobile phases. Materials commonly used are glass, stainless steel, teflon and sapphire. The pump must be capable of generating 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 x 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 demineralized. The most widely used organic modifiers are methanol, acetonitrile and THF. Methanol and acetonitrile have comparable polarities but the latter is an aprotic 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 nonflammable 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

Detectors for HPLC fall into two categories, viz., differential and selective. Differential detectors or bulk property detectors provide a differential measurement of a bulk property that is possessed by both the solute (analyte) and the mobile phase. These detectors are generally nonspecific and respond to a wide range of compounds (e.g., refractive index detectors). The solute property or selective detectors measures a property of the sample (analyte), which is not possessed by the mobile phase (e.g., ultraviolet and fluorescence detectors).

The ideal HPLC detectors would possess high sensitivity, produce reproducible and predictable responses, give a response to any analyte, response is linear over a wide range of sample concentration and it should be rugged and not sensitive to change in temperature or mobile phase composition.

Modes of separation [6-8]

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 bonding 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 pairing 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. \( C_8 \) and \( C_{18} \) packing are popular for ion pair separations because they provide strong dispersive interactions with ion pairing agents. Other reversed phase columns such as \( C_4 \) and phenyl are also used with this mode. Cationic ion pairing agents are cetyltrimethylammonium, tetrabutyl ammonium phosphate or sulfate (TBAP or TBAS) and tetraetham 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.

**Method development [6]**

Analytical chemistry deals with methods for determining the chemical composition of samples. A compound can often be measured by several methods. The choice of analytical methodology is based on many considerations such as chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements (quantitative or qualitative) and the number of samples. Qualitative method yields information of the chemical entity of the species in the sample and a quantitative method provides information regarding the relative amounts of one or more of the analytes in the sample. Qualitative information is required before a quantitative analysis can be performed. A separation step is usually a necessary part of both a qualitative and a quantitative analysis.

**Steps in method development**

It starts with the documentation of the developed studies. All the data related to these studies are established and recorded in laboratory notebook.

**Analytical standard characterization**

a. All the known information about the drug or analyte and its structure is collected such as its physical and chemical properties, toxicity, purity, hygroscopic nature, solubility and stability.

b. The standard analyte is obtained. Necessary arrangement is made for proper storage.

c. When multiple compounds are to be analyzed in the sample matrix the number of components is noted, data is assembled and the availability of standards for each one is determined.

d. Only the methods, which are compatible with sample stability, are conceded.

**Method requirements**

The objectives of method are defined. The required quantitation limits, linearity, range, accuracy and precision are defined.

**Choosing a method**

a) If any reported methods from the literature are adaptable to the current laboratory setting and future needs is determined.

b) Using information in the literature and prints, methodology can be adapted. The methods are modified wherever necessary; acquire additional existing methods for analyte and sample.

c) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar and chemical properties are investigated and are worked out.

**Instrumental setup and initial studies**

a) The required instrument is set up. Installation, operational and performance of instrumentation using laboratory standard operating procedure are reviewed.

b) Always new consumables (solvents, filter and gases) are used.

c) The analyte standard in a suitable injection/introduction solution and in known concentration and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix.

d) Feasibility of method with regards to the analytical figures of merit obtained is evaluated.

**Optimization**

During optimization, one parameter is changed at a time and set of conditions are isolated rather than using a trial and error approach and it should be done from an organized methodological plan and every step is documented in case of dead ends.
**HPLC method development [6,7]**

A systematic approach to develop HPLC method requires knowledge about the nature of the sample and characteristics of the analyte. Various physicochemical properties such as, molecular weight, polarity, ionic character and solubility parameter of the analyte give valuable information to develop the method. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase to be employed. In reversed phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. Therefore, when separating mixtures containing acids and bases by reverse phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results. The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reverse phase chromatography. Gradient can be started with 5-10 % of organic phase in the mobile phase and the organic phase concentration (acetonitrile or methanol) can be increased up to 100%. Separation can then be optimized by changing the initial mobile phase composition and slope of the gradient according to the chromatogram obtained from preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition.

Whenever acidic or basic samples are to be separated it is strongly advisable to control mobile phase pH by adding a buffer. pH of the buffer is adjusted before adding organic phase. The buffer selected for a particular separation should be used to control pH over the range of ~ pKa ± 1.0. The buffer should transmit light at or below 220nm so as to allow low UV detection. Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks in the chromatogram of all the compounds. By a slight change of the mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

**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 [8,9].

**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 LS 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 $1/x^2$.

Validation
According to USFDA “validation is the process of establishing evidence, which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes”. According to cGMP of validation requirement, qualification and calibration must be carried out. Qualification is to verify whether the instruments are working normally. Calibration is to measure the deviation from the standard and carry out the necessary correction. Validation of an analytical method is required for the following reasons:
• Setting standards of evaluation procedures for checking complaints and taking remedial measures
• Retrospective validation is useful for trend comparison of results compliance to cGMP/cGLP
• Closer interaction with pharmacopoeia harmonization particularly in respect of impurities determination and their limits
• For taking appropriate action in case of non-compliance
• To provide high degree of confidence that the same level of quality is consistently built into each unit of finished product from batch to batch
• The consistency and reliability of validated analytical procedure is to produce a quality product with all the quality attributes, thus providing indirect cost saving from reduced testing or re-testing and elimination of product rejection
• Enables scientists to communicate scientifically and effectively on technical matters

Bioanalytical method validation [10]
Published methods of analysis are often modified to suit the requirements of the laboratory performing the assay. These modifications should be validated to ensure suitable performance of the analytical method. When changes are made to a previously validated method, the analyst should exercise judgment as to how much additional validation is needed. During the course of a typical drug development program, a defined bioanalytical method undergoes many modifications. The evolutionary changes to support specific studies and different levels of validation demonstrate the validity of an assay's performance.

Types of method validation
Method validation can be classified into three categories, viz., full validation, partial validation and cross validation.

Full Validation
Full validation is important when developing and implementing a bioanalytical method for the first time, for a new drug entity and when metabolites are added to an existing assay for quantification.

Partial validation
Partial validations are modifications of already validated bioanalytical methods. 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,
• Bioanalytical method transfers between laboratories or analysts
• Change in analytical methodology (e.g., change in detection systems)
• Change in anticoagulant in harvesting biological fluid
• Change in matrix within species (e.g., human plasma to human urine)
• Change in sample processing procedures
• Change in species within matrix (e.g., rat plasma to mouse plasma)
• Change in relevant concentration range
• Changes in instruments and/or software platforms
• Limited sample volume (e.g., pediatric study)
• Rare matrices
• Selectivity demonstration of an analyte in the presence of concomitant 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. 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 the ways.

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. Cross validation should also be considered when data generated using different analytical techniques (e.g., LC-MS/MS versus ELISA) in different studies are included in a regulatory submission.

Validation parameters [10]

The fundamental parameters for a bioanalytical method validation are selectivity, accuracy, precision, recovery, linearity, range and stability. Measurements for each analyte in the biological matrix should be validated.

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

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. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference. The mean response of the blanks at the corresponding retention time of the analyte should not exceed 20% of the mean peak response at the LLOQ of the analyte.

Accuracy and precision

The 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 is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should 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 should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Recovery

The recovery of an analyte in an assay is the detector response obtained from the 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 pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.
**Linearity and range**

Linearity of an analytical procedure is its ability (with in a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Range of an analytical procedure is the interval between the upper and lower concentration (amount) of an analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations 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 six to eight non-zero samples covering the expected range, including LLOQ.

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 20% deviation of the LLOQ from nominal concentration
- 15% deviation of standards other than LLOQ from nominal concentration.

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. Excluding the standards should not change the model used.

**Stability**

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

**Freeze and thaw stability**

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70°C during the three freeze and thaw cycles.

**Short-term temperature stability**

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

**Long-term stability**

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

**Stock solution stability**

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least six hours. 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.
Post-preparative stability (Stability in auto-sampler)

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. Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used.

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.

REFERENCES