The stages of drug development begin with the novelty in a drug molecule that has showed therapeutic index to for the treatment of disease [1, 2]. Active pharmaceutical ingredient is defined as synthesis and characterization of novel molecules and analysis of API is the requisite to ensure their safety and efficacy. The compound which is entitled to become the drug molecule undergoes safety tests and a series of experiments to prove its pharmacokinetic parameters and can be considered safe and successful. After successful completion of stages of pre-clinical and clinical trial drug is launched in the market to patients.

There are various aspects in the drug development. Finished products may contain unidentified foreign matter particles [3]. The foreign matter particle has to be identified and its sources should be defined to prevent further contamination. It is required to provide an efficient detection and identification procedure of foreign matter from the dosage forms by utilization of analytical techniques.

The drugs which are marketed may have different dosage forms. Information on pharmaceutical development provides the scientific rationale for formulation development and appropriate suitable dosage form. There is information on pharmaceutical development by regulatory guidance [4], detailed information is provided by toxicological assessments of excipients. Reactions may take place in solid dosage form when drug substance is reactive and may be increased by physical and chemical interaction of drug with excipients. In some cases excipients encourages degradation of drug substance. Primary and secondary amine can react with lactose, glucose and maltose to form glycosylamines [5].

In pharmaceutical research and development, determination of API, drug intermediates, drug formulations, related substances, biological samples and their metabolites by analytical methods are required. Analytical assay methods were included in official monographs beginning from the official pharmaceutical analysis with the objective to characterize the quality of bulk drug materials by setting their limits of active ingredient content.

The reasons for new method development of drug analysis are

1. The drug or their combinations may not be official in any pharmacopeias

2. Due to patent regularity, there may not be availability of proper analytical procedure of the drug in the literature.
3. Due to interference by formulation excipients, there may not be availability of analytical methods drug in particular formulation.
4. The existing analytical procedures may require expensive reagents and solvents. It may also require inconvenient extraction and separation procedures which may not be reliable.
5. There may not be availability of bio-analytical methods for the quantification of the drug.
6. There may not be availability of analytical methods for a drug in combination with other drugs.

1.1. Analytical methods

1.1.1. Stability indicating assay methods (SIAMs)

Stability Indicating Assay methods are analytical procedures used to measure quantitatively reduction in the amount of active pharmaceutical ingredient (API) in drug product because of degradation. As per the stability guidelines by US-FDA and stability guideline draft in 1998, stability indicating method is a validated quantitative analytical procedure that can be used to detect how the stability of drug substances and drug product changes with time. Stability indicating method accurately measures the changes in active ingredient without interferences from other degradation products, impurities and excipients.

Stability indicating methods are of two types – specific SIAM and selective SIAM [6].

Specific Stability Indicating Assay Method is defined as method which is able to measure the drug(s) in presence of all degradation products, additives and excipients, which are expected to be present in the formulation.

Selective Stability Indicating Assay Method is defined as method that is able to measure unequivocally the drug(s) and all degradation products in presence of additives and excipients, which are expected to be present in the formulation. Selective SIAM is a procedure which is the selective to the drug as well as its degradation products (separates all of them qualitatively) and is also specific to all the components (measures them quantitively).

Chromatographic methods can be specific and selective SIAM while UV methods can be specific indicating assay method.

Regulatory status of stability indicating assay methods

Guidelines related to stability testing of API and drug products have been issued by organizations like International Conference on Harmonization (ICH), Food and Drug Administration (FDA) and World Health Organization (WHO). Regulatory mandate for stability testing in the United States is mentioned in 21 CFR Part 211 Section 166, which indicates that there shall be written testing program designed to assess the stability characteristics of drug products. ICH guidelines are put into practice by countries Japan, European and United States.

According to ICH guidelines Q1A(R2) [7], the objective of stability testing is to furnish information on how the quality of a drug substance or drug product may be affected by environmental factors like temperature, humidity and light with time , to specify retesting period of the drug substance, to determine the shelf-life of drug product and suggest the appropriate storage conditions. By stress testing of drug substance, possibly degradation products can be identified which helps in establishing degradation pathways and intrinsic stability of molecule. Stress testing is performed on a single batch of the drug substances to study the effect of change in temperature (by 10ºC), humidity (75% RH), oxidation and photolysis on the drug substance. ICH Q1B [8] guideline emphasizes on light testing to be performed which is an essential part of stress testing. The inherent photo stability properties of new drug substances and products should be established to check that light exposure does not result in unacceptable change. Photo stability testing should be performed on a single batch, if some variations and changes are made in product formulation and packaging photo stability testing should be repeated. ICH guideline Q3A [9] and Q3B [10] focuses that documental evidence related to validation of analytical procedures to be provided and its suitability for the identification, detection and quantitation of degradation products.

ICH Q5C [11] focuses on stability testing on new drug substances and products in relation to biotechnological products in which the active components are proteins or polypeptides biotechnological products are dependent on non-covalent as well as covalent forces. ICH guideline Q6A [12] states that that under universal tests or criteria for drug substances and drug products there is a requirement of stability indicating assay methods. These products are sensitive to environmental factors such as temperature changes, oxidative, light, ionic content and shear. ICH Q7(A) [13] focuses on good manufacturing practice (GMP) for the manufacturing of active pharmaceutical ingredients for managing quality. The objective of this guideline is to ensure that APIs meet the requirements for quality and purity.

Necessity of stability testing and stability indicating assay methods is also provided by European Committee for Proprietary Medicinal \products, World Health Organization (WHO) [14] , United States Pharmacopeia (USP), Canadian Therapeutic Product Directorate’s guidelines, ICH Q7A guideline (Good Manufacturing Practices for Active Pharmaceutical Ingredients)

Steps in Development of Stability Indicating Assay methods to meet regulatory requirements

As per the requirement by regulatory documents to perform stability indicating assay method,

Following are the steps which are to be followed to conduct the forced degradation study [6] :

(i) Critical structure of drug substance to assess the likely decomposition route(s)

Information can be obtained from study of function of functional groups present in drug. Functional groups like esters, lactams, amides, lactones [15] undergo hydrolysis. Functional groups like thioethers and thiols [16] undergo oxidation. Alkenes, aryl halides, aromatic nitro group undergo photodecomposition [17]. New drugs which are congener of existing drug molecules, their degradation behavior can be studied with the literature of reported drugs in series.

There are the cases where congeners may have different degradation behavior. In 2-methyl-5-nitro imidazole series of drugs. Tinidazole and secnidazole undergoes degradation in alkaline conditions to main nucleus 2-methyl-5-nitro-imidazole [18,19] while metronidazole undergoes degradation to ammonia and acetic acid.

(ii) Gathering of information on physicochemical properties

Data on physicochemical parameters pKa, solubility, maximum wavelength, Log P is required to be collected. Information on pKa helps in selection of pH of buffer in mobile phase since slight change in pH within ± 1.5 units of the pKa value may cause changes in retention time [20] . Information on log *P* helps in separation of degradation products and drug on a particular stationary phase. Log P and pKa value can be calculated theoretically using commercial software like ACD chemsketch, chemdraw. Information of solubility parameters in aqueous, organic and HPLC solvents helps in selection of sample solvents and mobile phase. HPLC analysis is based on UV detector, analysis is carried out at maximum wavelength or at wavelength where all components show good absorbance information on maximum wavelength and extinction of the drug and degradation products in different solvents is the requisite .This is applicable when known degradation products are available in pure form. In case if the drug is new and degradation pathway is not established, drug is subjected to stress studies , changes in the spectrum are observed, first in reaction solution and then in mixture of all the solution. For analysis of wavelength dual wavelength detector or photodiode array detector can be used.

(iii) Stress or forced degradation studies

For development of stability indicating assay method, force degradation study is performed for generation of degradation products. Guideline is suggested by ICH Q1A the conditions to be utilized are (a) 10ºC increments above the accelerated temperatures (b) humidity where suitable (75% or greater) (c) hydrolysis across wide pH range (d) oxidation (e) photolysis.

Guidelines are provided on the practical conduct and issues related to stress testing under variety of conditions prescribed by ICH [21]. Flowchart for performing degradation of drug substance and drug product under various stress conditions is shown in Fig. 1.1.

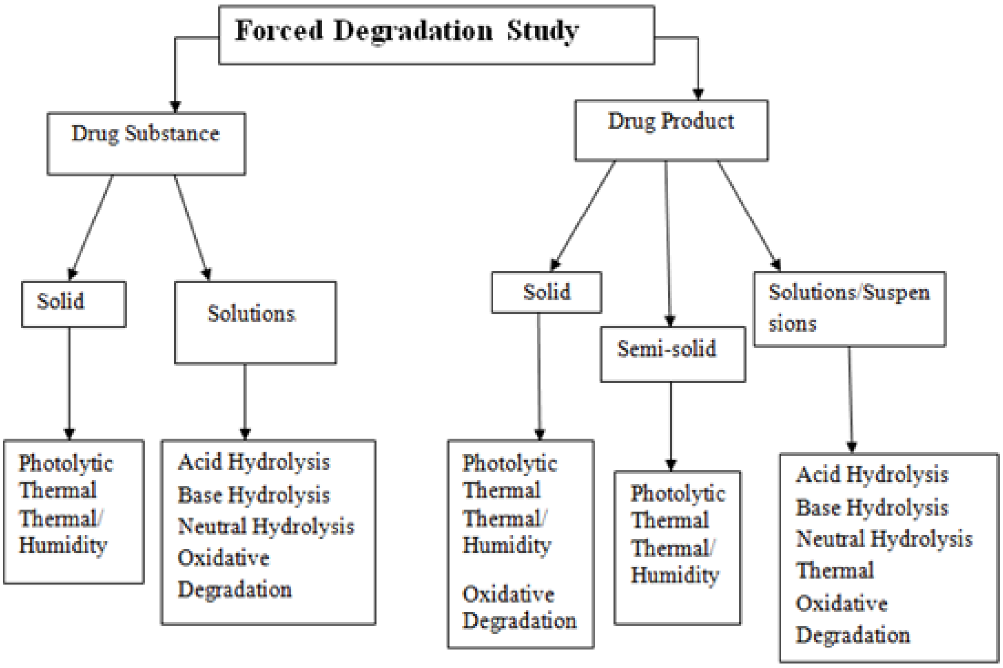


Fig. 1. 1 - Flowchart describing various stress conditions used for degradation of drug substance and drug product [6]

Degradation conditions and sample conditions

Hydrolysis

Hydrolysis is the most common degradation chemical reaction which takes place in pH ranging from acidic, neutral to basic. Hydrolysis is a chemical process by which breakdown of compound takes place by addition with water. Hydrolytic study takes place under acidic and basic conditions in which catalysis of ionizable functional groups takes place in the molecule. During this process there is generation of primary degradation products in the desirable range. For acid hydrolysis reagents used are hydrochloric acid or sulphuric acids ranging from 0.1 –1 M for acid hydrolysis. For basic hydrolysis, reagents used are sodium hydroxide or potassium hydroxide ranging from 0.1-1M. If the compounds are insoluble in water, then co-solvents are used to dissolve them in HCl or NaOH. If there is degradation is not observed at room temperature, then the degradation are preformed at elevated temperature (50-70ºC).

Oxidation conditions

For performing degradation in oxidative condition, hydrogen peroxide is the most commonly regents. Other oxidizing agents which may be used are metal ions, oxygen and radical initiators (azabisiisobutryonitrile, AIBN). Solutions when treated with 0.1-3% hydrogen peroxide at room temperature and at neutral pH up to seven days or maximum, 20% degradation could generate relevant degradation products. Mechanism of oxidative degradation of drug substance takes place by electron transfer mechanism to form reactive anions. Drugs containing functional groups amines, sulfides and phenols on oxidation form N-oxides, hydroxylamine, sulphones and sulphoxide [22]. Drugs containing labile hydrogen like benzylic carbon, allylic carbon, tertiary carbon or alpha positions with respect to hetero atom when subjected to oxidation form hydroperoxides, hydroxide or ketone [23].

Photolytic conditions

The photo stability testing of drug substances must be carried out to check that on light exposure there is any change in drug substance or drug product. To conduct photostability studies, drug substance is exposed to UV or florescent conditions to generate primary degradation products. According to ICH guidelines, samples of drug substance and solid/liquid drug product should be exposed to a minimum of 1.2 million lux hours and 200 Wh/m^2 light. For photolytic degradation commonly accepted wavelength of light is in the range of 300-800 nm. Light stress conditions can cause photo-oxidation by free radical mechanism. Drugs containing functional groups carbonyl, nitro aromatic, N-oxide, aryl chloride, weak C-H bonds, O-H bonds, sulfides and polyene are liable to photodegradation [24].

Thermal conditions

Thermal degradation (dry heat and wet heat) should be performed at harsh conditions than recommended by ICH Q1A accelerated testing conditions. Samples of drug substances and drug products in solid state should be exposed to dry and wet heat, drug products in liquid dosage form should be exposed to dry heat. Studies may be performed at higher temperature for a short period [25]. Effect of temperature on thermal degradation of a substance is calculated through Arrhenius equation : k=Ae-Ea/RT, where k is the specific reaction rate, A is the frequency factor, Ea is the energy of activation, R is gas constant(1.987 cal/deg mole) and T is the absolute temperature. Thermal degradation study is carried out at 40-80ºC.

Techniques used according to literature reviews

Titrimetric and spectrophotometric

The objective of these methods is the analysis of drug along with matrix of excipients, additives, degradation products, impurities and drugs in combinations. Literature has been reported on stability indicating method first order derivative spectroscopy for determination of furazolidone [26] and acetazolamide [27]. Major limitation in these methods is specificity few literatures are available related to this.

Chromatographic methods

Compared to conventional methods chromatographic methods are more accurate, sensitive, provide effective separation of multiple components of stability samples. Chromatographic methods which have been used are thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), gas chromatography (GC), HPLC, capillary electrophoresis (CE).

Although TLC is the simple technique it has some limitations in terms of variability and non-quantitative. Literature has been reported on thin layer chromatographic examination of the degradation of centrubiridine [28] in aqueous solutions. This technique is used in initial degradation studies to study the number of degradation products and in isolation where preparative TLC is preferred.

HPTLC has the advantage over TLC in terms of reliability, fast and accuracy for quantitative drug analysis. This technique minimizes analysis time and cost per analysis, number of sample can be run simultaneously with small quantity of mobile phase. Its limitation is availability of instrument in every laboratory.

GC is used in stability indicating method but is not applicable to those samples which are non-volatile and thermally unstable. If the drug is subjected to increase in temperature to increase its volatility there may be degradation or racemization. Few literatures are available for GC analysis. Literature has been reported on stability indicating gas liquid chromatographic method development for determination of acetaminophen and aspirin in suppositories.

HPLC is the most widely techniques used in stability indicating methods. Non-volatile and thermally unstable samples which cannot be detected by GC can be detected by HPLC. It has good sensitivity and specificity compared to other chromatographic methods.

(iv) Separation studies on stressed samples

Degradation samples obtained from stress testing are checked to study the number and type of degradation products formed under various stress conditions. For separation of peaks, mobile phase like water –methanol or water-acetonitrile are tried due their compatibility with LC-MS, preparative LC and are buffer free. Between methanol and acetonitrile, methanol is usually the preferred choice due to its low cost. Mostly degradation products are more polar compared to drug, for separation of degradation products, the drug peak may be extended to 15 min for separation. For separation other factors can be considered like detection of wavelength, injection volume and flow rate.

(v) Final method development and optimization, kinetic studies

Focus on resolution is given to the degradation products whose relative retention times are close to each other. PDA spectral analysis or LC-MS analysis is required to confirm if the degradation products are same or different. There may be situation where mass of degradation product in different degradation eluting at same retention time may be different. Then better resolution is needed chromatographic separation is performed for separation of co-eluting peaks.

(vi) Degradation kinetics study

Stability of pharmaceutical product [29] is defined as “extent to which product maintains its physical, chemical, microbiological, toxicological, protective specifications throughout its period of storage and use.”

There are advantages of stability studies

- Development of optimum formulation
- Finding the optimum storage conditions (temperature, light and humidity)
- Selecting proper container for dispensing
- Predicting the shelf life of drug
- Anticipating drug excipients interaction
- Stabilization of drug against degradation

Factors affecting drug stability:

- Temperature

  Stability of drug may be affected by increase in temperature may cause oxidation, reduction and hydrolysis reaction
- pH

  Acid and alkaline pH affect the rate. Most drugs are stable between pH 4 and 8. Weakly acidic and basic drugs show good solubility when they are ionized and decompose faster when they are ionized. pH can have serious effect on decomposition. A little change in 1 pH unit change can produce tenfold change in rate constant.

- Moisture

  Water catalyses chemical reaction like oxidation, hydrolysis and reduction reaction
- Light

  Stability of drug is affected by light by thermal effect or light energy which can cause oxidation
- Oxygen

  Exposure of drug formulation to oxygen affects stability

Reactions kinetics [30] is the study of rate of chemical change and the way in which this rate is influenced by the conditions of concentration of reactants, products, solvent and temperature. It is a powerful tool for explaining the mechanism by which chemical reaction takes place.

One of the common applications of kinetics in pharmacy is the study of rates of drug degradation in pharmaceutical products and determination of proper shelf life and storage conditions for these products. The stability of the active ingredient of a drug is the main factor in the rational drug design and analysis of pharmaceutical dosage forms. Harsh chemical degradation of the active ingredient may result in loss of active ingredient from the dosage form, formation of toxic product and decrease in bioavailability.

Rate of reaction

Rate of reaction or degradation rate is the velocity with which the reaction occurs. Rate of the reaction is expressed as ratio of change in concentration of reactant to a change in time.

$$\text{Rate} = dc/dt$$

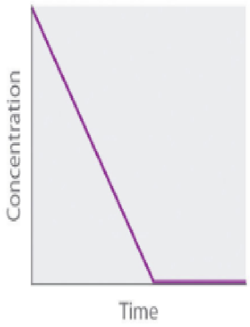
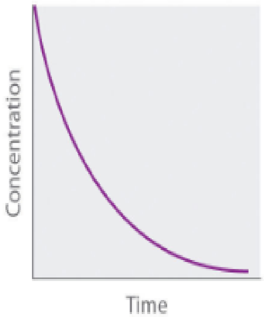
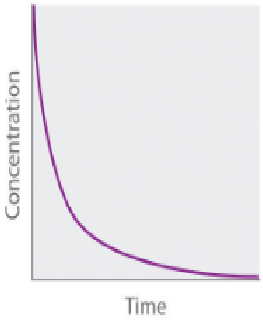
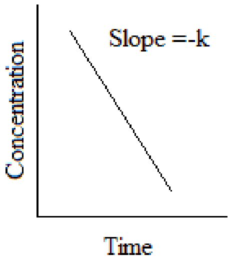
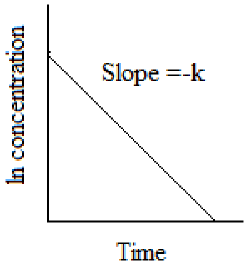
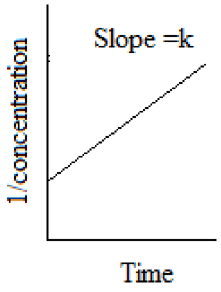
Where dc is the change (increase or decrease) in concentration over the time period dt.

Rate of chemical reaction is expressed as mol $L^{-1}s^{-1}$.

Order of a reaction – Order of a reaction is the way in which concentration of a drug or reactant in a chemical reaction affects the rate. Or it may be defined as sum of the powers of concentrations in the rate law.

Rate α $[A]^a$, where a is the order of reaction. The details of order of reaction is described in Table 1.1. [31]

Table 1. 1 – Orders of reaction

	Zero Order	First Order	Second Order
Differential Rate Law	Rate = $-\Delta A/\Delta t = k$	Rate= $-\Delta A/\Delta t = k[A]$	Rate= $-\Delta A/\Delta t = k[A]^2$
Concentration versus time			
Integrated rate law	$[A] = [A]_0 - kt$	$\ln[A] = \ln[A]_0 - kt$	$1/[A] = 1/[A]_0 + kt$
Straight line plot to determine rate constant			
Relative rate versus concentration	[A],M Rate 1 1 2 1	[A],M Rate 1 1 2 2	[A],M Rate 1 1 2 4

	3 1	3 3	3 9
Half-life	$t_{1/2}$= $[A]_0/2k$	$t_{1/2}$= 0.693/k	$t_{1/2}$=1/k $[A]_0$
Unit of k, rate constant	mole $L^{-1}s^{-1}$	s^{-1}	L $mole^{-1}s^{-1}$

The concept of Activation Energy was introduced by Swedish Scientist Svante Arrhenius in 1889. Activation energy is defined as minimum amount of energy required for a reaction to takes place. It is expressed as Ea and unit of activation energy is kJ/mole. An equation was proposed by Arrhenius for activation energy

$$k = A\ e^{-Ea/RT}$$

where k is the rate constant, Ea is the activation energy, R is the gas constant (8.3145 J/Kmol), T is the temperature expressed in Kelvin. A is the frequency factor with unit of $Lmol^{-1}s^{-1}$. If the rate constant is known activation energy can be calculated from equation.

Activation energy can be calculated graphically. The equation for activation energy can be put in the form of straight line by converting to natural logarithm as

$$\ln k = -Ea/RT + \ln A, \text{ which can be rearrange as :}$$

$$\ln k = 1/T(-Ea/R) + \ln A$$

Plot of ln k versus 1/T gives the equation of straight line. Where slope is –Ea/R. From the slope of the graph Ea can be obtained (Fig. 1.2.).

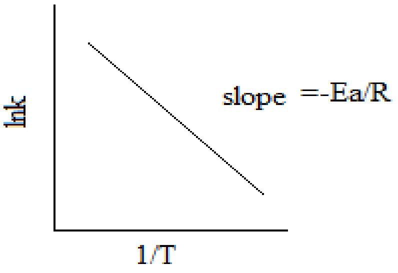


Fig. 1. 2 - Activation energy plot

(vii) Identification and characterization of degradation products and preparation of standards

There are advantages of structural characterization of impurities and degradation products

- Helps in understanding nature and origin so that they can be controlled during drug synthesis or formulation development. For example, there is interaction of drug with counter ion used to form the salt e.g. amlodipine free base was found to react with maleate counter ion of amlodipine maleate salt [32].
- Once characterization is done, synthesis of impurities or degradation products can be performed for availability of pure standard which can be used for spiking study, setting of validation parameters like LOD, LOQ, response factor and calibration curves, in vivo and in vitro qualification studies to establish safety.
- Side effects of drug substances and products can be explained. E.g. allergic reactions by aminopenicillins are due to polymeric degradation products formed in drug products.
- Genotoxic potential of drug can be evaluated by review of the available literature or through computational toxicology
- Correlation of specific impurity or degradation product whether it is metabolite
- Drug degradation pathway and mechanism establishment
- To include impurities and degradation products in compendia monographs
- To include into library of spectral data for chemical compounds
- To create structure-searchable drug degradation databases [33]
- To include mapping of drugs in environment by emphasizing on their major degradation products
- To understand the reactions leading to the formation of impurities or degradation products (mostly in case of photo degradation), which may result in new chemical/drug leads.

There are two approaches for identification and characterization of degradation products. One of the approaches is conventional approach. The conventional approach includes separation and isolation/enrichment synthesis, analysis by spectral techniques since isolation of impurity is essential before characterization. The impurities or degradation products are separated by High Performance Liquid Chromatography (HPLC), Ultra High Performance Liquid Chromatography (UHPLC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Gas Chromatography technique. The impurities or DPs are detected by UV detectors or fluorescence.

Other approach is use of hyphenated techniques. Hyphenated techniques are the mixture of two different analytical techniques with the aid of proper assistance. Chromatographic techniques are associated with spectroscopic techniques. Hyphenated techniques include mixture of separation-separation, separation-identification and identification –identification techniques. These technique include LC-MS (Single Quad), LC-MS-MS (Triple Quad), LC-MS-TOF, LC-Orbitrap, LC-NMR. They provide necessary information related to structural elucidation of impurities or degradation products.

Approaches to develop Stability Indicating Assay Method

1.1.2. Development of HPLC method

High-performance liquid chromatography (HPLC) is a separation technique based on solid stationary phase and liquid mobile phase.

Separations are achieved by partition, adsorption or ion-exchange processes, depending upon the type of stationary phase used.

Compounds to be analyzed are dissolved in suitable solvents and most separations take place at room temperature. Thus, most drugs, being nonvolatile or thermally unstable compounds can be chromatographed without decomposition or necessity of making volatile derivatives.

A liquid chromatography instrument (Fig. 1.3) consists of reservoir containing the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector and a data collection device such as computer, integrator or recorder. Stationary phase consists of columns with densely packed particles of silica. Compounds are exchanged between stationary phase and mobile phase through silica. Computer receives signal from the detector and determines retention time of compounds, control chromatographic settings and operations, thus providing for long periods of unattended operation.

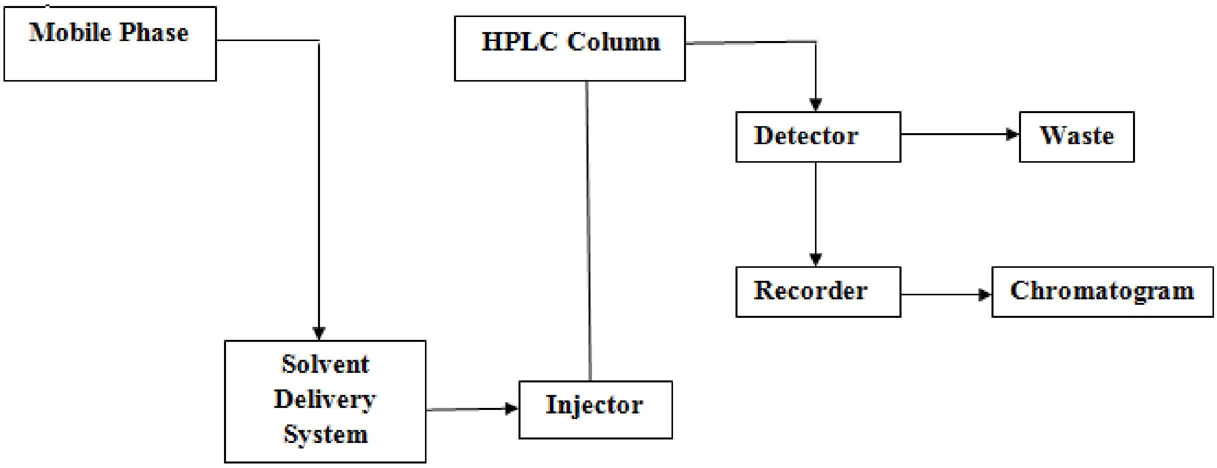


Fig. 1. 3 - Block diagram of HPLC [34]

- *System suitability in HPLC*

This is an integral part of liquid chromatographic method for assuring adequate performance of the system. Because of normal variations in equipment, supplies and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable.

Following parameters are important

System Suitability Parameters and Recommendations:

Capacity Factor (k’): The peak should be well separated from void volume and other peaks in method development. Capacity factor should be greater than 2.

Repeatability: RSD $\leq$ 1% for N $\geq$ 5 is desirable

Relative retention: It is not essential till resolution between given peak and other closest eluting peak (may be impurity, excipient, degradation product, internal standard) is greater than 2 .

Tailing Factor (T): T of < 2

Theoretical Plate (N): In general should be > 2000.

If there’s variation in results due to change in column performance, results of system suitability from these experiments helps in determination of limits for system suitability criteria.

This approach facilitates the investigation of the worst case state of affairs, which reflects minimum performance standard used to ensure that the chromatography is not adversely affected.

Factors which may be affected by the changes in chromatographic conditions are:

- *Resolution* – Resolution (Fig. 1.4) describes the separation power of the complete chromatographic system relative to the particular components of the mixture.

  The resolution, Rs, of two neighboring peaks is defined as the ratio of the distance between two peak maxima. It is the difference in the retention times between two peaks divided by combined width of elution peaks. For effective separation of two peaks, value of resolution Rs is 1.5. Resolution is calculated by the formula given in Fig. 1.4.

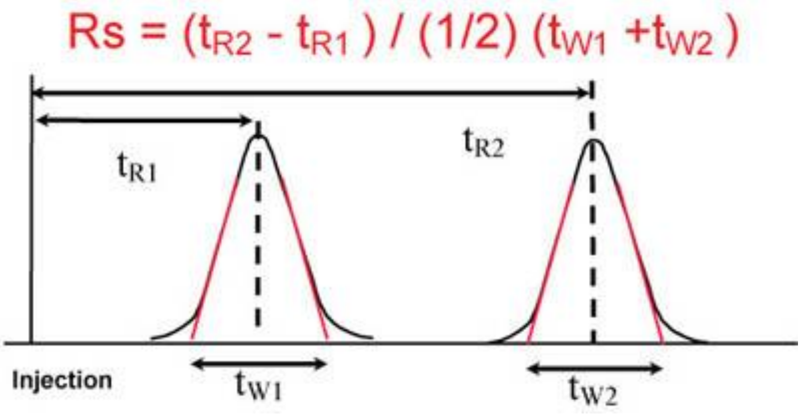


Fig. 1. 4 - Peak resolution Rs

  where t_{R1} and t_{R2} are the retention times of components 1 and 2 and t_{W1} and t_{W2} are peak width of components 1 and 2. There are three fundamental parameters that influence the resolution of a chromatographic separation:

  There are three factors which may affect the resolution of a chromatographic separation:

  - Capacity factor (k’)
  - Selectivity (α)
  - Column efficiency (N)

  These parameters provide with different means to achieve better resolution, as well as defining different problem sources

- *Capacity factor (k’)*-Capacity factor (Fig. 1.5) is the ratio of the reduced retention volume to the dead volume. Capacity factor, k’, is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase.

With capacity factor retention time of an analyte on the chromatographic column can be measured. The ideal value of k’ ranges from 2-10. Capacity factor can be calculated from the formula given in Fig. 1.5.

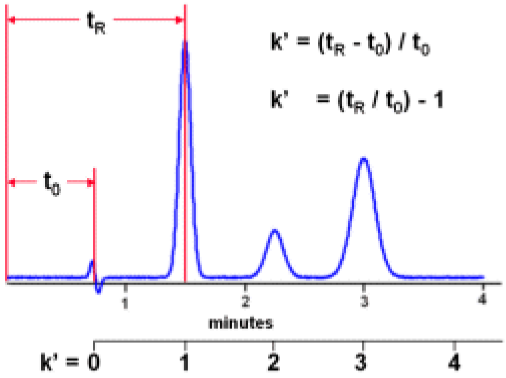


Fig. 1. 5 - Capacity Factor k’

where t_R is the retention time of the peak and t_0 is the dead time of column

Capacity Factors changes are typically due to:

- Variations in the mobile phase composition
- Changes in column surface chemistry (due to aging)
- Changes in operating temperature

In most chromatography modes, capacity factor (k’) changes by 0 percent for a temperature change of 5ºC

Capacity Factor (k’) values are sensitive to:

- Solvent strength
- Composition
- Purity
- Temperature
- Column chemistry
- Sample

- *Selectivity (α)*-The selectivity (or separation factor), α, (Fig.1.6.) is a measure of relative retention time of two components in a mixture. Selectivity is the ratio of capacity factors of both peaks and the ratio of its adjusted retention times. Selectivity is the ability of chromatographic system to chemically differentiate between sample components. Selectivity depends on nature of analyte, mobile and stationary phases. Selectivity is always greater than one. If selectivity is equal to one, it indicates that two peaks are co-eluting , their retention factors values are identical.

The ideal value of α is 2.It can be calculated by using the formula,

$$\mathbf{A} = V_2 - V_1/V_1 - V_0 = k_1'/k_2'$$

where, V_0= the void volume of the column,

V_1 and V_2 = retention volumes of the second and first peak respectively.

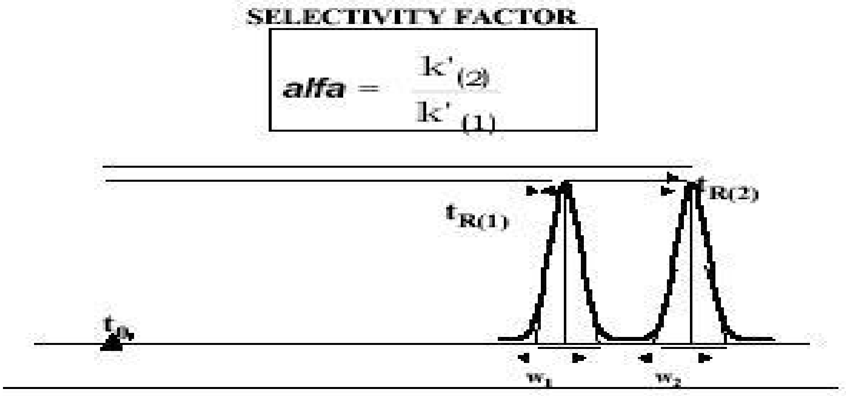


Fig. 1. 6 - Selectivity *α*

Selectivity (α) values are sensitive to :

  - Changes in mobile phase composition (pH ionic strength)
  - Purity
  - Temperature

- *Column efficiency/Band broadening* -Efficiency, N, (Fig. 1.7) of a column is measured by the number of theoretical plates per meter. It is an indirect measure of a peak width for a peak at specific retention time. Similar the band spread, highest is the number of theoretical plates indicating good column and system performance. Columns with N ranging from 5000 to 100000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

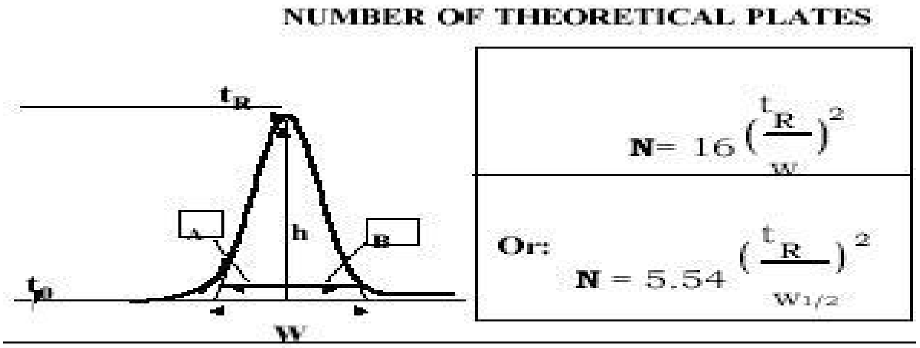


Fig. 1. 7 - Theoretical Plates N

where, t_R is the retention time and W is the peak width

A decline in measured efficiency may be due to:

- Age and history of the column
- Extra column band broadening (such as due to malfunctioning injector or improper tubing ID)
- Inappropriate detector settings
- Change in the flow rate and solvent viscosity

One can recognize problems in separation due to loss of column efficiency when the width and shape of all peaks are affected.

- *Peak asymmetry factor (T_f)*: Peak asymmetry factor, T_f (Fig. 1.8) can be used as criteria of column performance. The peak half width, b, of a peak at 10% of the height, divided by the corresponding front half width, a, gives the asymmetry factor. A new well packed column is acceptable if an asymmetry factor value is between 0.9 to 1.1.

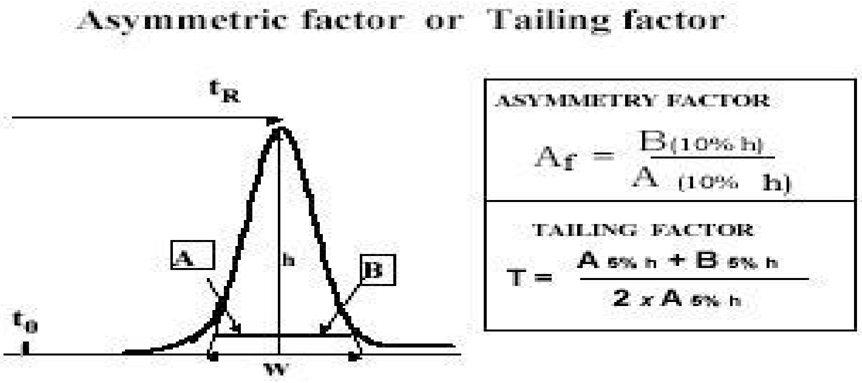


Fig. 1. 8 – Asymmetry factor

In HPLC choice of detection approach is crucial to ensure that all the components are detected. UV detector is the widely used in HPLC analysis. Most compounds absorb UV light in the wavelength ranging from 200-400 nm. There are two types UV detectors- fixed wavelength and variable wavelength.

A photodiode (PDA) 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. PDA allows detection at single wavelength of multiple wavelengths. PDA detector has ability of acquiring peak purity analysis.

In case when analytes are UV-inactive or having low UV absorbance, other detectors are preferred in HPLC which include refractive index detector, electro chemical detector, fluorescence detector, mass detector, ELSD. These detectors are based on the change in the refractive index, oxidizable/reducible, fluorescence absorbance, mass property and volatility of analyte.

Preparative HPLC

Preparative HPLC [35] is used for the isolation and purification of degradation products from active pharmaceutical ingredients, biotechnology, biochemistry and advantageous over conventional methods like liquid liquid extraction, column chromatography and thin layer chromatography. Depending on the application, amount of compound to isolate or purify differs accordingly. It has applications in isolation of enzymes in biotechnology where micropurification is required. In case of identification and structure interpretation of unknown compounds by organic synthesis or from natural products pure compounds are required in the range of milligrams. Applications of preparative HPLC are given in Table 1.2.

Table 1. 2 – Applications of preparative HPLC

Compound amount	**Working area**
µg	• Isolation of enzymes

mg	• Biological and chemical testing • Structural elucidation and characterization of – Side products from production – Metabolite from biological matrix – Natural products
g	• Reference compounds(Analytical standards) • Compounds for toxicological screenings – Main compound in high purity – Isolation of side products
Kg	• Industrial scale , active compounds , drugs

Parameters in method development by preparative HPLC

1. **Adsorption isotherm**

   In preparative HPLC if more than a certain amount of sample is injected onto column the adsorption isotherm becomes non-linear. The peak becomes unsymmetrical, shows strong tailing and capacity factor decreases. This effect is called as concentration overloading. Since the adsorption isotherm is dependent on the compounds the chromatographic system column loadability has to be determined.

2. **Column loading and overloading**

   Large amounts of samples are purified by two methods: concentration overloading and volume overloading. Summary of concentration and volume overloading is given in Table 1.3.

Table 1. 3 – Summary of concentration and volume overloading

Concentration Overloading	Volume Overloading
Determined by solubility of compound in mobile phase	Determined by injection volume
It is based on preparative area of adsorption isotherm	It is based on analytical area of adsorption isotherm
Throughput is determined by	Throughput is determined by column

selectivity	internal diameter
Particle size of stationary phase of low influence	Small particle size require

3. **Method scale up**

   The method optimization and scale–up of an analytical to preparative method is done in three steps

   - Optimization of the analytical method regarding resolution
   - Column overloading on the analytical column
   - Scale up to preparative column

4. **Role of column in preparative HPLC**

   Resolution and selectivity are important factors in preparative HPLC since columns are frequently overloaded and peaks are broad. If selectivity between two sample components is high then one may overload the column to a greater extent. Choice of stationary phase may be critical to provide the best selectivity for the respective compound. Guideline for capacity of preparative column is given in Table 1.4.

Table 1. 4- Guideline for capacity of preparative column

Column ID	$\alpha < 1.2$	$\alpha > 1.5$
4.6 mm	2-3 mg	20-30 mg
9.4 mm	10-20 mg	100-200 mg
21.2 mm	50-200 mg	500-2000 mg

5. **Particle size and column dimension**

   In preparative HPLC, for well resolved samples, larger particles of 7 and 10 µm can be used. Since pressure drop is inversely proportional to the particle diameter squared, larger particle give lower pressure drop that allow higher flow rates which increase the throughput of preparative column.

   Column dimensions are predicted by amount of material per injection that one desires to inject. 4.6 mm i.d. is for small scale preparative HPLC, 7-8 mm i.d. columns for semi preparative HPLC, 21.2 mm i.d. columns for larger scale preparative HPLC.

6. **Choice of mobile phase**

   For preparative HPLC, volatile buffers are preferred since removal will be easier in the final stages of the method and if mass spectrometry is used for confirmation, system will be compatible.

7. **Purification system in preparative HPLC**

   Purification of compound is performed by collection of factions.

   The difference between analytical and preparative HPLC is that in analytical HPLC sample travels directly to waste receptacle after it has left the detector while sample goes to fraction collector in preparative HPLC. Faction collection is performed by following strategies:

   Time based fraction collection – collection of fraction based on pre-defined time intervals or fixed number of fractions within a specified time

   Peak based fraction collector- Collection based on detector signal

   Mass based fraction collection- if a user selected target mass is found by an MSD

1.1.3. **Development of HPTLC method**

HPTLC is the advanced form of TLC.

Steps in the method development of HPTLC [36, 37] are:

- *Selection of stationary phase* – Commercially available precoated HPTLC plates with Silica Gel 60 F254 on either glass or aluminum backing are used. In addition to the silica gel other stationary phases can provide separation and analysis of mixtures by different mechanisms: cellulose layers [normal-phase (NP) partition], polar NP-bonded layers (diol, cyanopropyl and aminopropyl), ion-exchange layers, impregnated layers (argentation TLC for unsaturated organic compounds), thicker preparative layers (0.5 -1 mm) for isolation of larger amount of analytes and chiral layers for analysis of enantiomers.
- *Layer prewashing* - HPTLC plates are prewashed to improve the reproducibility and robustness of the quantitative analysis. Plates are prewashed with either methanol or mixture of methanol and ethyl acetate or mobile phase of the developed method may be used. Prewashed plate should be activated by drying in oven at 120ºc for 20 min. Plates should be equilibrated with laboratory temperature and relative humidity in a suitable container providing protection from dust and fumes.

- *Selection and optimization of mobile phase* -The mobile phase systems that are used are diethyl ether, methylene chloride and chloroform combine individually or together with hexane as the strength adjusting solvent for normal phase and methanol, acetonitrile and tetrahydrofuran mixed with water for strength adjustment in reverse phase [38,39].

  Four level techniques were suggested by CAMAG laboratory for mobile phase selection and optimization. In first level neat solvents (seven or twelve) are used and those solvents are selected which can have average separation power for the desired drugs. At this level, solvents are categorized on the basis of R_f value. In second level decreasing or increasing solvent strength hexane or water is used. Solvent strength in group B is reduced by adding a weak solvent (hexane, heptanes and cyclohexane). If the entire sample runs in the front, the solvent should be diluted in the first trial at least 1:4. For sample components which have higher R_f values and somewhat are separated, for those 1:1 dilution can be tried. The strength of the solvent in group C needs to be increased by adding polar modifier like acetic acid, formic acid, diethyl amine, ammonia and start with 10%. Water may be added if the solvent is mixable. In the third level mixtures of solvents from selected solvents of first or second level which can be optimized by the use of modifier like acids or bases.

  If the R_f values are acceptable, level 4 is preferred in which minor adjustment to the solvent strength is done and use of modifiers to improve the shape of the separated zones and alteration of chamber saturation time. If the separated zones show tailing 0.5 to 1% water is added. On the basis of above trials if analytical goal is not achieved, procedure is repeated on a different stationary phase.

- *Sample application* -Sample application technique determines the quality of separation. There are two ways to transfer sample on HPTLC plate contact application and spray-on techniques. In spot application by contact application technique, the solvent of the sample performs circular chromatography which can cause irregular distribution of the sample components across the spot and after chromatogram development, after method development, the developed spots may be wide and irregular. The spray-on technique avoids any chromatography during sample application even if the sample is dissolved in strong solvents. Band wise application by spray-on technique ensures resolution and detection limits of TLC system since there are homogenous distribution of the sample over the entire length of the band.

- *Chromatogram Development* -HPTLC plates are developed in flat-bottom chambers, twin trough chambers (TTC) or horizontal –development chambers. With the design of TTC chamber development is favoured with low volumes of mobile phase and easy pre-equilibrium of the layer with the vapors of the mobile phase. During development in addition to stationary and mobile phase, gas phase is present which can influence the result of result of the separation. The gas phase depends on the type, size and saturation condition of the chamber during the development. Unsaturated chambers should be avoided due to development of bent solvent fronts and reduced reproducibility. In case of complex separation resolution can be achieved by using two-dimensional development, in which sample mixture is applied to one corner of the HPTLC plate. The plate is developed with the first mobile phase, dried and developed with a second mobile phase in the perpendicular direction.
  - *Detection*- After removal of the mobile phase from the developed plate by heating, zones is detected on the layer by their natural color, natural fluorescence, quenching of fluorescence or UV-absorbing zones or fluorescence zones after reaction with a reagent. Zones with fluorescence or quenched fluorescence are viewed in cabinets that equipped with short wave (254 nm) and long wave (366 nm) UV lamps. Detection under UV light is the first since it is non destructive.

1.1.4. **Validation of analytical methods**

Validation is the necessary for assuring that any procedure, process and systems are working. The development of analytical method is of importance in pharmaceuticals and other industries.

Analytical method validation [40,41] is one of the regulatory requirements. According to the Code of Federal Regulations “the accuracy, sensitivity, specificity and reproducibility of test methods employed by the firm shall be established and documented.”

Management of the quality control unit would definitely want to ensure that the analytical methods that the department uses to release its products are properly validated for its intended use so the product will be safe for human use.

Parameters for method validation for analytical procedures

The analytical procedure refers to the way of performing the analysis. It should describe the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of formulae for the calculation etc. The important validation parameters are:

1. Specificity – specificity is ability to evaluate the analyte accurately in presence of components like impurities, excipients, degradation products and matrix. It is applicable for determining relative resolution, peak purity to check if there is any interference from excipients or degradation products.
2. Accuracy – Accuracy of an analyte procedure is defined as closeness of the test value to the true value. It is determined by comparison to reference standard, recovery of analyte spiked into blank matrix and standard addition of analyte.
3. Precision – The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogenous, authentic samples. The precision of an analytical standard is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

   Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also called as intra-assay precision.

   Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipments etc.
4. Linearity – Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of an analyte. If linear relationship is observed between test result and analyte concentration, by the method of least squares regression equation is obtained. Information on correlation coefficient, y-intercept, slope of regression line and plot of data between test result and analyte concentration is obtained.

   The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

5. Range – Range of analytical procedure is the interval between upper and lower concentrations of analyte in the sample for which it has been demonstrated that analytical procedure has s suitable levels of precision, accuracy and linearity.
6. Limit of detection (LOD) – Limit of detection is the lowest amount of analyte in a sample which can be detected . LOD can be obtained visually, based on the signal to noise ratio approach, based on the standard deviation of the response and slope. Limit of detection is expressed as $3.3*\sigma/S$, where σ is the standard deviation of the intercept and average of slope of the calibration curve.
7. Limit of quantitation (LOQ) – Limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined. LOQ can be obtained visually, based on the signal to noise ratio approach, based on the standard deviation of the response and slope. Limit of quantitation is expressed as $10*\sigma/S$, where σ is the standard deviation of the intercept and average of slope of the calibration curve. The quantitation limit is a parameter of quantitative assays for low levels compound in sample matrices and is for determination of impurities or degradation products.
8. Robustness – The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

   In hplc, typical variations are pH of mobile phase, mobile phase compositions, different column, temperature, flow rate.

1.1.5. Quality by Design and Design of Experiments

Quality by design (QbD) is a concept developed by the quality pioneer Dr. Joseph M. Juran [42]. According to Dr. Juran quality should be designed into a product and that most quality crises and problems relate to the way in which a product was designed in the first place. Janet Woodcock [43] Director for the Center for Drug Evaluation and Research defined pharmaceutical quality as “product that is free of contamination and reproducibility delivers the therapeutic benefit promised in the label to the consumer.” The US Food and Drug Administration (FDA) [44, 45] motivates risk-based approaches and the adoption of QbD principles in drug product development, manufacturing and regulation.

Pharmaceutical QbD has evolved with the issue of ICH Q8(R2) [46] (Pharmaceutical Development), ICH Q9 [47] (Quality Risk Management), and ICH Q10 [48] (Pharmaceutical Quality System).According to FDA [45] QbD is defined as " a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."

There are literatures reported that similar opportunities exist for applying QbD to analytical methods as in case of manufacturing processes. With AQbD, there is development of robust and cost-effective method, which is applicable through the life-cycle of the product, to strengthen the regulatory flexibility in analytical method. It means freedom to change method parameters within a method's design space called as method operable design space (MODR).

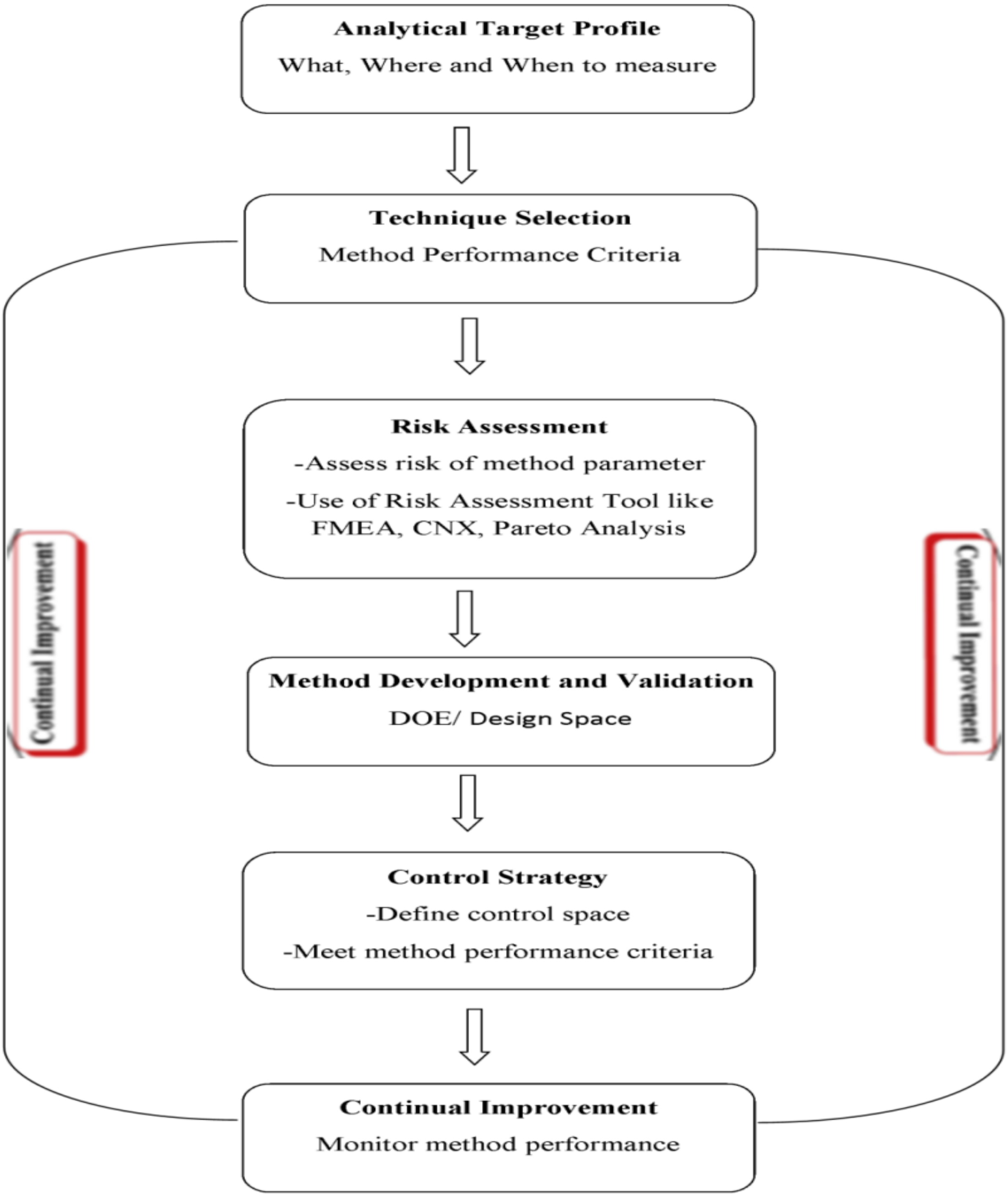


Fig. 1. 9 - Analytical method development in QbD

Aspects covering AQbD (Fig. 1.9) are

Analytical Target Profile (ATP)

It is the tool for method development. It defines the requirement of the method [49,50] which are expected to be measured . An ATP defines what method has to measure (i.e. acceptance criteria) and up to what extent the measurement is required (performance level characteristics e.g. precision, accuracy, range, sensitivity). Preliminary risk assessment should be carried out for method requirements. ATP for analytical procedures consists of selection of target analytes (APIs and degradation products), selection of analytical technique (HPTLC, GC and HPLC) and choice of method requirements.

According to ICH guidelines factors for method requirements are accuracy, precision, robustness and ruggedness. Details information has to be obtained on compound properties like pH, pKa, solubility, stability, UV chromophore.

Critical Quality Attributes

According to ICH guideline Q8, Critical Quality Attribute is a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality.

Critical Quality Attributes for analytical methods consists of method attributes and method parameters. CQA for HPLC (UV or RID) are pH and buffers in mobile phase, organic modifier, column selection. CQA for HPTLC is type of stationary phase, mobile phase ratio, injection volume, saturation time of mobile phase and developmental distance.

Risk Assessment

According to ICH Q9 guideline quality risk management is a systematic process for the assessment, control, communication and review of the risks to the quality of the drug (medicinal) products across the product lifecycle. It provides a link between input process variable and CQA. Techniques used in risk assessment are Fishbone or Ishikawa or cause effect diagram, Pareto analysis, Cause and effect analysis, Failure mode effect analysis. Risk assessment can be carried out in three steps that is risk identification, risk analysis and risk evaluation. Risk factors are classified as

High factors - These factors are fixed during method development process.

Noise factors – These factors are subjected to robustness testing.

Experimental factors- instrumentation and operation methods. These are subjected to ruggedness testing and acceptable range is identified.

Design of Experiments

As per the requirement by ICH Q8 guideline, with respect to design space in method development, to develop robust and cost effective method, method operable design region can be produced. MODR is operated in the range by which critical method input variable gives outcome as per expected in the ATP. MODR is based on science, risk based and multivariate approach to study the effects of factors on method performance. MODR can be performed along with method validation as per the recommendation by FDA. Analytical methods are performed with factorial or fractional factorial or response surface methodology. Steps in Design of Experiment in analytical quality by Design are

Screening – In screening input variables are screened. Critical method parameters for optimization are identified. In screening experiments, critical method parameters should be segregated which are either controlled or in DOE techniques in MODR optimization.

Optimization – This is the stage in which relationship between critical method in variables and output response can be understood by which effect on method performance and ATP can be understood.

Selection of DOE tools

In optimization, statistical techniques are used to derive a mathematical relationship (model). Tool for DOE [51, 52] is selected is based on the number of input of variables, controlled parameters, understanding between result and variable. If effect of input variables and interactions are to be measured, factorial design can be selected for screening and optimized with response surface methodology. If large numbers of input variables are studied without interaction effects, Plackett Burmann method is used.

Method Operable Design Region (MODR) and surface plot- For MODR, contour plot can be generated. Contour plot is 2D response plot in effect on method variables (x-axis) and output response (y-axis) and some instrument configurations are controlled. Contour plot is suitable when the response is nonlinear and method response is having more curvature effect. Using mathematical models, MODR can be selected from contour plot. Actual experimental run is performed to check the predicted value of method response for model validation.

Modal Validation

Predicted values obtained from the method response are to be verified by performing experimental run. For validation of statistical model, regression analysis is performed.

Method Verification/ method validation

Based on the optimized conditions obtained by DoE, method validation is performed s per ICH Q2 (R1) guidelines. Method verification can be performed by using multipoint verification within MODR. E.g. - if the buffer concentration is 10 mM, robustness has to be checked from 5 mM to 15 mM.

Control Strategy

By control strategy, there is assurance that method is performing on a routine basis. It relies on data generated during method development and method verification. Based on this, focus has to be done on high risk factors. If the risks are low and system suitability parameters are checked and verified from time to time.

Life cycle approach

When the method is developed, the method performance is monitored from time to time to the compliance with ATP criteria. This is performed by using system suitability parameters and method related investigations. Continuous monitoring helps analysis to identify any deviating factor in analytical method.

1.2. REFERENCES

1. Siddiqui MR, AlOthman ZA, Rahman N. Analytical techniques in pharmaceutical analysis: A review. Arabian Journal of Chemistry. 2017; 10 (1): S1409-S1421.
2. Valagaleti R, Burns PK Gill M. drug Information Journal. Analytical support for drug manufacturing in the United States –From Active Pharmaceutical Ingredient Synthesis to Drug Product Shelf Life. 2003; 37: 407-438.
3. Pajander J, Haugshoj KB, Bjorneboe K, Wahlberg P, Rantanen J. Foreign matter identification from solid dosage form. Journal of Pharmaceutical and Biomedical Analysis. 2013; 80: 116-125.
4. U.S. Department Health and Human Services, 2003. Food and Drug Administration, Center for drug evaluation and Research, Center for Biologics Evaluation and Research. Guidance for Industry, Drug Product, Chemistry Manufacturing and Control Information, Draft Guidance, Rockville, Maryland,1-62.
5. Serajuddin ATM, Thakur AB, Ghoshal RN, Fakes MG, Ranadive SA, Morris KR, Varia SA. Selection of dosage form composition through drug-excipient

compatibility testing. Journal of Pharmaceutical Sciences. 1999; 88(7): 696-704.

6. Bakshi M, Singh S. Development of validated stability-indicating assay methods- a critical review. Journal of Pharmaceutical and Biomedical analysis. 2002; 28(6): 1011-40.
7. Guideline ICH, Stability Testing of New Drug Substances and Products Q1A (R2), in: International Conference on Harmonisation, IFPMA, Geneva ; 2003.
8. Guideline ICH, Stability Testing: Photostability Testing Of New Drug Substances And Products Q1B, in: International Conference on Harmonisation, IFPMA, Geneva; 2003.
9. Guideline ICH. Impurities in new drug substances Q3A (R2), in: International Conference on Harmonisation, IFMPA, Geneva; 2006.
10. Guideline ICH. Impurities in new drug products Q3B (R2), in: International Conference on Harmonisation, IFMPA, Geneva; 2006.
11. Guideline ICH, Quality of biotechnological products: Stability testing of biotechnological / biological products Q5C: in International Conference on Harmonisation (ICH). Developments in biological standardization. 1995
12. Guideline ICH, Test procedure and acceptance criteria for new drug substances and new drug products: Chemical substances Q6A, in: International Conference on Harmonisation, IFPMA, Geneva; 1999.
13. Guideline ICH. Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients. Q7A, in International Conference on Harmonisation (ICH). Current Step. 2000.
14. WHO, Guidelines for Stability Testing of Pharmaceutical Products Containing Well Established Drug Substances in Conventional Dosage Forms, in WHO Expert Committee on Specifications for Pharmaceutical Preparations. Technical Report Series 863, World Health Organization, Geneva, 1996, 65-79.
15. Connors KA, Amidon GL, Stella VJ. Chemical Stability of Pharmaceuticals, Wiley, New York, 1986.
16. Hovorka SW, Schonoeich C. Journal of Pharmaceutical Sciences. 2001 ; 90: 253-269.
17. Anderson NH. Photostability testing: Design and Interpretation of Tests on Drug Substances and Dosage Forms, Taylor and Francis, London, 1996.

18. Wilkins BJ, Cainsford GJ, Moore DE,. Journal of Chemical Society. Perkin Trans. 1987: 1817-1820.
19. Moustafa AA, Bibawy LI. Spectroscopy Letters. 1999; 32: 1073: 1098.
20. Synder LR, Kirkland JJ, Glach JL. Practical HPLC Method Development, Wiley, New York, 2000: 295.
21. Bakshi M, Singh S. Guidance on Conduct of Stress Tests to Determine Inherent Stability of Drugs. Pharmaceutical Technology On-Line. 2000; 24 : 1-14.
22. Gupta A, Yadav JS, Rawat S, Gandhi M. Method development and hydrolytic degradation study of Doxofylline by RP-HPLC and LC-MS/MS. Asian Journal of Pharmaceutical Analysis. 2011; 1(1): 14-8.
23. Aslante KM, Hatajik TD, Lohr LL, Santafianos D,Sharp TR. Solving impurity/ degradation problems: case studies. Separation Science and Technology. 2004; 5: 361-400.
24. Baertschi SW, Thatcher SR. Sample presentation for photostability studies : problems and solutions. Pharmaceutical Photostability and Stabilization technology: Taylor & Francis New York; 2006. P.445.
25. Aslante KM, Ando A, Brown R, Ensing J, Hatajik TD, Kong W et al . The role of degradant profiling in active pharmaceutical ingredients and drug products. Advanced drug delivery reviews. 2007; 59(1): 29-37.
26. Walash MI, eL-Brashy AM, Sultan MA. Stability-indicating first derivative spectrophotometric determination of furazolidone. Acta Pharmaceutical Hungarica. 1994; 64(1): 5-8.
27. Khamis EF, Abdel-Hamid M, Hassan EM, Eshra A, Elsayed MA. A stability indicating first derivative spectrophotometric assay of acetazolamide and its use in dissolution and kinetics studies. 1993; 8(2): 97-101.
28. Baveja SK, Singh S. Thin-layer chromatographic examination of the degradation of centbucridine in aqueous solutions. Journal of Chromatography. 1987; 396: 3317-3344.
29. Patrick JS, Yashveer S. Martin’s Physical Pharmacy and Pharmaceutical Sciences. Philadelphia, Lippincott: Williams & Wilkins, 2006.
30. Bahl A, Bahl BS, Tuli GD. Essentials of Physical Chemistry Bahl Tuli. New Delhi : S. Chand Publishing, 2011.
31. Chemistry library texts – using graphs to determine integrated rate laws.

32. Murakami T, Fukutsu N, Kondo J, Kawasaki T, Kusu F. Application of liquid chromatography-two-dimensional nuclear magnetic resonance spectroscopy using pre-concentration column trapping and liquid-chromatography-mass spectrometry for the identification of degradation products in stressed commercial amlodipine maleate tablets .Journal of Chromatography A. 2008 ; 1181 : 67-76.
33. Available from : http://d3.cambridge soft.com
34. Remington JP, Beringer P. Remington: the Science and Practice of Pharmacy Philadelphia, Lippincott: Williams & Wilkins, 2013.
35. Huber U, Majors RE. Principles in preparative HPLC. Retrieved from https://www.agilent.com/cs/library/primers/Public/5989-6639EN.pdf
36. Sonia K, Bhavyashree B, Lakshmi KS. HPTLC Method Development And Validation : An Overview. Journal of Pharmaceutical Sciences and Research. 2017 ; 9(5) : 652-657.
37. Patel RB, Patel MR, Patel BG. Experimental Aspects and Implementation of HPTLC. Springer. 2011; 41-54.
38. CAMAG, 2010-2011. Basic equipment for modern thin layer chromatography. Switzerland: Camag. Available from: camag.com/downloads/free/brochures/CAMAG-basic equipment-08.pdf.
39. CAMAG, 2010-2011. Instrumental Thin Layer Chromatography. Switzerland: Camag. Available from : camag.com/downloads/free/brochures/CAMAG-TLC-10-11_E.pdf.
40. ICH .Validation of analytical procedures: text and methodology Q2 (R1). International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, IFPMA, Geneva, Switzerland, 2005.
41. FDA. Guidance for Industry: Analytical Procedures and Methods Validation, 2000.
42. Juran JM. Juran on quality by design : the new steps for planning quality into goods and services. New York : The Free Press, 1992.
43. Woodcock J. The concept of pharmaceutical quality. American Pharmaceutical Review. 2004, 1-3.
44. FDA. Pharmaceutical CGMPs for the 21st Century- a risk-based approach. Final report Rockville, MD. 2004.

45. FDA. PAT guidance for industry- a framework for innovative pharmaceutical development, manufacturing and quality assurance. US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Office of Regulatory Affairs, Rockville, MD, 2004.
46. Guideline ICH. Pharmaceutical Development Q8, (R2) ; US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER) : Rockville, MD.2009.
47. Guideline ICH. Quality Risk Management Q9, In: International Conference on Harmonisation of technical requirements for registration of pharmaceutical for human use, 2006.
48. Guideline ICH. Pharmaceutical Quality Systems Q10, In : International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use, 2008.
49. Schweitzer M, Pohl M, Hanna-Brown. Implications and opportunities of applying QbD principles to analytical measurements. Pharmaceutical Technology.2010; 34(2) : 52-59.
50. Elder P and Borman P. “Improving analytical method reliability across the entire product lifecycle using QbD approaches. Pharmaceutical Outsourcing. 2013; 14(4): 14-19.
51. Borman P, Chatfield M, Nethercote P, Thompson D, Truman K. The application of quality by design to analytical methods. Pharmaceutical Technology. 2017; 31(12): 142-152.
52. Burnett KL, Harrington B, Graul TW, Fanalis S, Haddad PA, Poole CF. QbD in Liquid Chromatographic Applications. Elsevier, 2013.