**1. INTRODUCTION**

**1. 1 INTRODUCTION**

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and Food Drug Administration (FDA) Guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation .It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. The appropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods1.

The developed chromatographic methods further validated as per ICH or USFDA guidelines for all the critical parameters. To access the precision and to evaluate the results of analysis the analyst must use statistical methods. These methods include confidence limit, regression analysis to establish calibration curves. In each analysis the critical response parameters must be optimized and recognized if possible.

Pharmaceutical Analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches like chemistry, physics. And microbiology etc. pharmaceutical Analytical techniques are applied mainly in two areas, quantitative analysis and qualitative analysis, although there are several other applications.

Drugs and pharmaceuticals are chemicals or like substances, which or of organic inorganic or other origin. Whatever may be the origin, we some property of the medicinal agent to measure them quantitatively or qualitatively.

In recent years, several analytical techniques have been evolved that combined two or more methods into one called “hyphenated” technique eg: GC/MS, LC/MS etc. The complete Analysis of a substance consists of four main steps.

The concept of analytical chemistry lies in the simple, precise and accurate measurements. These determinations require highly sophisticated instruments and methods like mass spectroscopy, gas chromatography, HPTLC, HPLC, etc. HPLC method is sensitive, accurate, precise and desirable for routine estimation of drugs in formulations.

Thereby it is advantageous than volumetric methods. Many HPLC methods has been developed and validated for the quantitative determination of various marketed drugs.

Analytical method development and validation places an important role in drug discovery and manufacture of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products majority of analytical development effort goes into validating a stability indicating method. So it is a quantitative analytical method based on the structure and chemical properties of each active ingredient of the drug formulation.

Most of the drugs can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, precision, reproducibility, ease of automation and eliminates tedious extraction and isolation procedures.

On the literature survey, it was found that most of the analytical method available for the above mentioned drug is applicable for quantification in plasma samples, the most widely used method being liquid chromatography-mass chromatography. So it is felt that there is a need to develop accurate, precise analytical methods for the estimation of the drug in solid dosage formulation.

**Newer analytical methods are developed for these drugs or drug combinations of the below reasons:**

* There may not be suitable method for a particular analyte in the specific matrix.
* Existing method may be too error prone or unreliable (have poor accuracy and precision).
* Existing method may be expensive, time consuming, energy intensive and may not be provide sensitive or analyte selectivity, and not easy for automation.
* Newer instrumentation and techniques may have evolved that provide opportunities for improved methods.
* There may be need for an alternate method to confirm, for legal and scientific reasons.

The newly developed analytical methods having their importance in different fields that include, research and development centre (R&D), Quality control department (QC). Approved testing laboratories, chemical Analysis laboratories etc. For analysis of these drugs different analytical methods are routinely being used.

The analytical methods are classified as classical and instrumental. These methods signal measured in those methods was mentioned in following table.

**Table 1: Classification of analytical method**

|  |  |
| --- | --- |
| **Measurement signal** | **Analytical method** |
| **Chromatographic techniques** | |
| Electrical | Gas chromatography (Thermal conductivity detector) |
| Increase in electrical current | Gas chromatography (Flame ionization detector) |
| Decrease in electrical current | Gas chromatography (Flame capture detector) |
| Electromagnetic radiation absorbed | Liquid chromatography (Ultraviolet Light detector, Diode array detector) |
| Electrical | Ion chromatography |
| **Spectrophotometric method** | |
| Emission radiation | Emission spectroscopy (X-ray, UV, Visible), Fluorescence and phosphorescence (X-ray, UV, Visible), radiochemistry. |
| Absorption of radiation | Spectrophotometry (X-ray, UV, Visible, IR)NMR  and Electron spin resonance spectroscopy. |
| Scattering of radiation | Turbidimetry, Nephelometry, Raman spectroscopy |
| Refraction of radiation | Refractometry, Interferometry |
| Diffraction of Light | X-ray and Electron diffraction |
| Rotation of radiation | Polarimetry, Optical rotatory dispersion |
| Mass to charge ratio | Mass spectroscopy |
| **Electro chemical techniques** | |
| Electrical potential | Potentiometry |
| Electrical current | Polarography, Amperometry |
| Electrical resistance | Conductometry |
| **Miscellaneous techniques** | |
| Rate of reaction | Kinetic method |
| Thermal properties | DTA and DSC |
| **Classical methods** | |
| Mass | Gravimetric Analysis |
| Volume | Volumetric Analysis |

**1.2 CHROMATOGRAPHY3**

Techniques related to chromatography have been used for centuries to separate materials such as dyes extracted from plants. Russian botanist Tswett is credited with the discovery of chromatography. In 1903 he succeeded in separating leaf pigments using a solid polar stationary phase, It was not until 1930s that this technique was followed by Kuhn and Leder as well as Reichstein and van Euw for the separation of natural products. Martin and synge were awarded the Nobile prize for their work in 1941 in which they described liquid-liquid chromatography. Martin and synge applied the concept of theoretical plates as a measure of chromatographic efficiency. The term “chromatography” (Color-Writing derived from the Greek for Color-chroma and Write-Graphing).

**CHROMATOGRAPHY IN THE PHARMACEUTICAL WORLD**

In the modern pharmaceutical industry, chromatography is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities. Drug discovery and development. The goal of the drug discovered is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). The main functions of drug development are to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening, and clinical trails. Throughout this drug discovery and development paradigm, rugged analytical HPLC separation methods are developed, at each phase of development to analyses of a myriad of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout. This drug development life cycle. This requires a thorough understanding of HPLC principles and theory which have solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization.

**1.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

**1.3.1 Brief Historical prospective of chromatography:**

The historical development of liquid chromatography has been extensively reviewed and can be traced as far back as they early 1900, where the Russian botanist Zwett used a variant of liquid chromatography to separate some colored plant substances.

The focus was on modern development in HPLC, a term that was coined in late 1960s with the advent of more sophisticated instrumentation, better engineered separation columns, and reliable and highly efficient stationary phases and packaging materials.

These technological advances have been, In part, fuelled, by the need to separate an increasingly large variety of differing compounds classes encountered as API s, e.g. Antibiotic, sulphonamides nucleosides, fat soluble vitamins neutral and non polar compounds. Additional challenges include developing faster and more consistent HPLC methods requiring higher flow rates, while maintaining peak shape, peak symmetry and efficiencies. Another important analytical challenge is the desire to detect and accurately quantify low levels of impurities at level present in API materials.

High-pressure liquid chromatography quickly improved with the development of column packing materials. Additional convenience of on-line detectors became rapidly a powerful separation technique and is today called as High-performance liquid chromatography (HPLC)

* One of the early problems with liquid state chromatography was the slow rate at which analysis took place. Early methods use gravity feed, and it was not uncommon diffusion and soon.
* This problem was largely overcome by the advent High-performance liquid Chromatography (HPLC). In this system the pressure is applied to the column forcing the mobile phase through at much higher rate.
* For an analysis to take several days to complete. This led not only to great delay but also the excessive time on the column and thus inevitably led to loss of resolution by

**Table: 2. Different types of chromatographic techniques**

|  |  |  |
| --- | --- | --- |
| **S.NO** | **Basic principle involved** | **Type of chromatography** |
| 1 | Techniques by  Chromatographic bed shape | Column chromatography |
| Paper chromatography |
| Thin layer chromatography |
| 2 | Techniques by physical state of mobile phase | Gas chromatography |
| Liquid chromatography |
| 3 | Affinity chromatography | Super critical fluid chromatography |
| 4 | Techniques by separation mechanism | Ion Exchange chromatography |
| Size Exclusion chromatography |
| 5 | Special techniques | Reversed phase chromatography |
| Two-dimensional chromatography |

**HPLC:**

In High-performance liquid chromatography, mobile as well as the stationary phase compete for the distribution of the sample components. In case of HPLC, separation is based on adsorption and partition. Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as silica gel, alumina or porous glass beads and a non-polar mobile phase such as heptanes, octane or chloroform are used in adsorption chromatography.

In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either polar or non-polar. If the stationary phase is non-polar, it is called normal phase partition chromatography. If the opposite case holds, it is called reversed-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar compounds. In reverse phase partition chromatography, the opposite behavior is observed.

**1.3.2 TYPES OF HPLC TECHNIQUES:**

Based on modes of chromatography:

* Normal phase chromatography
* Reverse phase chromatography

Based on principle of separation:

* Adsorption chromatography
* Ion exchange chromatography
* Size exclusion chromatography
* Affinity chromatography

Based on elution technique:

* Isocratic separation
* Gradient separation

Based on the scale of operation:

* Analytical HPLC
* Preparative HPLC

**Ion Exchange chromatography:** Due to differences in the affinity of ions for the in exchange.

**Size Exclusion chromatography:** Due to differences in molecular weight and size of the molecules to be separated.

**Affinity chromatography:** Separation is based on a chemical interaction specific to the target species. The more popular revered phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvents(s).

**Chairal chromatography:** Separation of the enantiomers can be achieved on chairal stationary phases by the formation of diastereomers.

**Analytical HPLC:** only analysis of the samples is done. Recovery of the samples for reusing is normally not done.

**1.3.3 MOST COMMONLY USED METHODS IN HPLC:**

**Normal phase chromatography:**

For a polar stationary bed like silica we need to choose a relatively non-polar Mobile phase. This mode of operation is termed as Normal phase chromatography. Here the least polar component elutes first, and increasing the mobile phase polarity leads to decrease in elution time. Non-polar solvents like pentane, Hexane, isooctane, cyclohexane, etc. are more popular. It is mainly used for separation of nonionic, non-polar to medium polar substances.

**Reverse phase chromatography:**

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes. The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the reverted, the chromatographic separation carried out with such silica is referred to as Reverse- phase chromatography. Here the most post components elutes first. Increasing mobile phase polarity leads to decrease In elution time. Common solvents used in this mode include Methanol /Acetonitrile /Isopropanol etc. Mostly used for separation of ionic and polar substances. The parameters that govern the retention in reversed phase system are the following:

1. The chemical nature of the stationary phase surface.
2. The type of solvents that compose the mobile phase.
3. pH and ionic strength of the mobile phase.

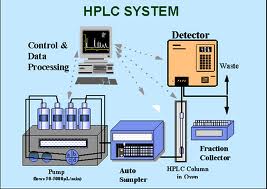
**Isocratic elution:** A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition).

**Gradient elution:** The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution.

1.3.4 **INSTRUMENTATION OF HPLC2:**

The mobile phase components HPLC instrument and their working functions are described below.

* Mobile phase and reservoir
* Solvent degassing system
* Pump
* Injector
* Colum
* Detector
* Data system



**Figure1: Schematic diagram of HPLC instrumentation**

**I.MOBILE PHASE AND RESERVIOR4:**

The most common type of solvent reservoir is a glass bottle. The mobile phase is pumped under pressure from one of several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous buffers. The following points should also be considered when choosing a mobile phase:

* The essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.
* Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.
* The mobile phase should have a pH 2.5 and Ph 7.0 to maximize the lifetime of the column.
* Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible minimizes the absorbance of buffer.
* Use volatile mobile phase when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220nm.

Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous Buffers. Physical properties of some HPLC solvents were summarized in

**Table: 3.Physical properties of common HPLC solvents**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Solvent** | **MW** | **BP** | **RI**  **(25OC)** | **UV**  **λ Cut-off(nm)** | **Density**  **g/ml(25Oc)** | **Viscosity**  **CP(25OC)** | **Dielectric**  **Constant** |
| Acetonitrile | 41.0 | 82 | 1.342 | 190 | 0.787 | 0.358 | 38.8 |
| Dioxane | 88.1 | 101 | 1.420 | 215 | 1.034 | 1.26 | 2.21 |
| Ethanol | 46.1 | 78 | 1.359 | 205 | 0.789 | 1.19 | 24.5 |
| Ethyl  Acetate | 88.1 | 77 | 1.372 | 256 | 0.901 | 0.450 | 6.02 |
| Methanol | 32.0 | 65 | 1.326 | 205 | 0.792 | 0.584 | 32.7 |
| CH2CI2 | 84.9 | 40 | 1.424 | 233 | 1.326 | 0.44 | 8.93 |
| Isopropanol | 60.1 | 82 | 1.375 | 205 | 0.785 | 2.39 | 19.9 |
| n-propanol | 60.1 | 97 | 1.383 | 205 | 0.804 | 2.20 | 20.3 |
| THF | 72.1 | 66 | 1.404 | 210 | 0.889 | 0.51 | 7.58 |
| a :The wavelength at which the absorbance of 1cm is 1.0 | | | | | | | |

**II. SOLVENT DEGASSING SYSTEM:**

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45µfilters, vacuum degassing with an air-soluble membrane, helium purging ultra signification or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase5.

**III. PUMP:**

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The degree of flow of control also varies with pump expense. More expensive pumps include such state of the art technology as electronic feedback and multithreaded configurations. It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

**IV. INJECTOR:**

Sample introduction can be accomplished in various ways. The simplest method. Is touse an injection value in more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors in liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. Sample introduction techniques can be used with a syringe an injection valve.

**V. COLUMN:**

The heart of the system in the column. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C8 or C18 column and determine if it provides good separations. Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. Diol, cyano and amino groups can also be used for reverse phase chromatography. Typical HPLC columns are 5, 10, 15, and 25cm in length and are filled with small diameter (3, 5 or 10µm) particles. The internal diameter of the columns is usually 4.6mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed.

**VI. DETECTOR:**

The detection of UV light absorbance offers both convenience and sensitivity for molecules. When a chromophore is present, the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents. The most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, Buffers and Excipient. Other method of detection can be useful are required in some instances6.

1. Solute specific detectors (UV-Vis, Fluorescence, Electrochemical, Infra-red, Radio activity)

2. Bulk property detectors (Refractive index, Viscometer, conductivity)

3. Desolvation detector (Flame ionization etc)

4. LC-MS detectors

5. Reaction detectors

VII. **DATA SYSTEM:**

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention.

**PERFORMANCE CALCULATIONS8:**

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention

2. Theoretical plates

3. Capacity factor

4. Resolution

5. Peak asymmetry

6. Plates per meter

The following information furnishes the parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: where the terms w and t both appear in the same equation they must be expressed the same units)

**System suitability parameters:**

The theory of chromatography has been used as the basis for system-suitability tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

**1. Relative retention:** The time elapsed between the injection of the sample components in to the column and their detection is known as the retention time (Rt).

**α = (t2-ta) / (t1-ta)**

Where,

α =Relative retention

t1= Retention time of the one peak measured from point of injection.

t2 = Retention time of the second peak measured from point of injection.

ta = Retention time of an inert peak not retained by the column, measured from point of injection.

**2. Theoretical plates:**

**n =16 (t R / w) 2**

Where,

n =Theoretical plates

tR = Retention time of the component

W = width of the base of the component peak using tangent method.

**3. Capacity factor:** The capacity factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column.

**K1 = (t2/t a)-1**

Where,

K1 = Capacity factor

ta = Retention time of an inert peak not retained by the column, measured from point of injection.

**4. Resolution:** the gap between two peaks

**R =2 (t2- t1) / (w2-w1)**

Where,

R =Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1)

W2 = Width of the base of component peak 2

W1 = Width of the base of component peak 1

**5. Peak asymmetry**

**T =W0.05/ 2f**

Where,

T = Peak asymmetry, or tailing factor.

**W0.05 =** Distance from the leading edge to the tailing edge of the peak, measured at a point 5 % of the peak height from the baseline.

f= Distance from the peak maximum to the leading edge of the peak.

**6. PLATE PER METER:**

**N =n/L**

Where,

N = plates per meter.

L = column length in meters.

**Advantages:**

* HPLC separations can be accomplished in a minutes, in some cases even in seconds.
* High resolution of complex sample mixture into individual components.
* Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for Gas Liquid chromatographic (GLC) analysis due to non-volatility or thermal-instability.
* Quantitative analyses are easily and accurately performed and errors of less than 1 % are common to most HPLC methods.
* Depending on sample type and detector used, it is frequently possible to measure 10-9 g or 1 ng of sample. With special detectors, analysis down to 10-12 pg has been reported.
* As HPLC is versatile, it can be applied to wide variety of samples like organic, inorganic, high molecular weight liquids, solids and ionic-nonionic compounds.

**Disadvantages:**

* HPLC instrumentation is expensive and represents a major investment for many laboratories.
* HPLC cannot handle gas samples.
* HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.
* Only one sample can be analysed at a time.

Finally, at present there is no universal and sensitive detector.

**1.4 ANALYTICAL METHOD DEVELOPMENT9:**

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trail runs are conducted, method is optimized and validated.

When alternate method proposed is intended to replace the existing procedure, comparative laboratory data includes merits /demerits should be made available.The important factors, which to be taken into account to obtain reliable quantitative analysis, are

1. Careful sample and sample preparation

2. Appropriate choice of the column

3. Selection flow rate

4. Selection of detector wavelength

5. Selection of column temperature

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. Analyte standard characterization.

1. All known information about the analyte and its structure is collected i.e., physical and chemical properties.
2. The literature for all type of information related to the analyte is surveyed.
3. Using the information in the literatures and prints, methodology is adapted. The methods are modified where ever necessary.
4. The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory SOP,s are verified.

**HPLC method development is based on few basic steps which include:**

|  |
| --- |
| 1.Information on sample, define separation Goals |

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|  |
| --- |
| 2. Need special HPLC procedure, sample pre-treatment, etc? |

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|  |
| --- |
| 3.Choose detector and detector setting |

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|  |
| --- |
| 4.Choose LC method; preliminary run, estimate best separation |

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|  |
| --- |
| 5.Optimize separation conditions |

↓

|  |
| --- |
| 6.Check for problems or requirement for special procedures |

↓

|  |  |  |
| --- | --- | --- |
| 7a.Recover purified material | 7b.Quantitative  calibration | 7c Qualitative method |

↓

|  |
| --- |
| 8.Validate method for release to routine laboratory |

**Figure2. Steps in HPLC method development**

**Method goals:**

Analytical method goals are often defined as method acceptance criteria for peak resolution, precision, specificity, sensitivity. For instance, pharmaceutical methods for potency assays of an API typically require the following:

* Minimal sample work-up (extra and inject if possible
* Robust method that doesn’t require extensive execution.
* Low cost per analysis.

**Table 4: separation goals in HPLC method development10**

|  |  |
| --- | --- |
| **Goals** | **Comment** |
| Resolution | Precise and rugged quantitative analysis requires that Rs be  greater than 1.5. |
| %RSD | Precision of retention time and peak area, <1-2%RSD. |
| Range | Linearity in the range of 50-150% of the lab label claim. |
| Analysis time | ῀5-30min ( ῀60min for complex samples) |
| Separation time | <5-10min is desirable for routine procedures. |
| Quantification | ≤2 %( %RSD) for assays, ≤5% for less demanding analyses, ≤15% for trace analyses. |
| Pressure | <150 bar is desirable, <200bar is usually essential(new column assumed) |
| Peak height | Narrow peaks are desirable for large signal/ noise ratios |

**Sample analyte information:**

The information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If data not available (e.g., Pka solubility), separate studies should be initiated as soon as possible. The sample related information is summarized in Table4.

**Table 5: sample and analyte information**

|  |  |
| --- | --- |
| **Sample/analyte** | **Information** |
| Sample | Number of components  concentration range of analytes |
| Analyte (s) | Chemical structure, molecular weight and functional groups  Pka  Solubility  Chromophore, wavelength (max)  Chairal centers, isomers  Spectral data (MS,NMR, IR, and UV)  Stability and toxicity |
| Others | Purity of reference standard materials |

**1. Careful sampling and sample preparation:**

Before beginning method development, it is need review what is known about the sample in to define the goals of separation. The sample related information that is important to summarized in

Table: The chemical composition of the sample can be providing valuable clues for the best choice of initial conditions for an HPLC separation.

* Number of compounds present
* Molecular weight of compounds
* Pka values of compounds
* UV spectra of compounds
* Concentration range of compounds in samples of interest

**2. Separation goals**

The goals of HPLC separation need to be specified clearly, which include

* The use of HPLC to isolate purified sample components for spectral identification Or quantitative analysis
* It may necessary to separate all degradants or impurities from a product for reliable content assay or not
* In quantitative analysis, the required levels of accuracy and precision should be known
* Whether a single HPLC procedure is sufficient for raw materials or one or more different procedures are desired for formulations
* When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important. Knowledge on the desired HPLC equipment.
* HPLC equipment, HPLC experience and academic training do to operators have

**3. Sample preparation:** samples come in various forms

* Solutions ready for injection
* solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
* solids must be dissolved or extracted
* Samples that require pretreatment to remove interference and / or to protect the column or equipment from damage

**4. Appropriate choice of the column:**

The selection of the column in HPLC is somewhat similar to the selection of column in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interaction and hydrogen bond information.

Column plays the important role in achieving the chromatographic separation.

The following parameters should be considered while selecting a column:

1. length and diameter of the column
2. packing material
3. size and shape of the particles
4. pore size, surface area and end capping
5. percentage of carbon loading

Columns with silica as a packing material used widely in normal phase chromatography, where the eluent (mobile phase) is non-polar consisting of various organic solvents and the stationary phase is polar. The silanol groups on the surface of the silica give it a polar character.

In reverse phase chromatography a wide variety of columns is available covering a wide range of polarity by cross linking the silanol groups with alkyl chains like C6, C8, C18 and Nitrile groups (-CN), phenyl groups (-C6H6) and amino groups (-NH2)

ORDER OF THE SILICA BASED COLUMNS

I------Non polar------Moderately polar------Polar------I

C18<C8<C6< Phenyl < Amino < Cyano <Silica

**5. Selection of flow rate.**

Flow rate is selected based on the follows:

* Retention time
* Column composition
* Separation impurities
* Peak symmetry

Preferably flow rate shall not be more than 2.5 ml/min. a flow rate that gives least retention times, good peak symmetries, least back pressure and better separation of impurities from API peak shell be selected.

**6. Selection of detector wavelength:**

Selection of detector wavelength is a critical step in finalization of the analytical method. To determine the exact wavelength standard API is injected into chromatographic system with photo Diode array detector and the wavelength, which gives higher response for the Compound

**7. Selection of column temperature:**

Ambient temperature is always preferred as a column temperature. However if the peak Symmetry could not be achieved then the column temperature can be varied between 300To 800 c. if a column temperature above 800c is found necessary, packing material which can Withstand to that temperature shall be chosen. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions.

**1.5 ANALYTICAL METHOD VALIDATION11,12:**

Method validation can be defined as (ICH) “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting is predetermined specifications and quality characteristics”.

Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

**VALIDATION DEFINITION:**

FDA defines validation as “Establishing documented evidence, which Provides a high degree of degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes.

**OBJECTIVE OF METHOD VALIDATION:**

The objective of validation is to form a basis for written procedure for production and control, which are designed to assure that the drug products have the identity, Quality, and purity.

**TYPES OF ANALYTICAL PROCEDURES:**

1. Identification tests
2. Quantitative tests for impurities content
3. Limit test for control of impurities
4. Quantitative tests of the active moiety in samples of drug substances or drug product or
5. Other selected components(s) in the drug product.
6. Dissolution testing for drug products
7. Particle size determination for drug substances.

**1.6 VALIDATION PARAMETERS (ICH).15**

Typical validation study include system suitability

1. Accuracy
2. Precision
3. Specificity
4. Linearity
5. Detection limit
6. Quantitation limit
7. Range
8. Robustnes

**1. System suitability**

Prior to the analysis of samples of each day, the operator that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation has been completed.

**Table: 6. System suitability parameters and Recommendation**

|  |  |
| --- | --- |
| **Parameter** | **Recommendation** |
| Capacity factor | The peak should be well-resolved from  Other peaks and the void volume,  Generally K>2.0 |
| Repeatability | RSD ≤1% N ≥ 5 is desirable |
| Relative retention | Not essential as long as the resolution is  Stated |
| Resolution | RS of >2 between the peak interest and  The closes to eluting potential  Interference (impurity, excipient,  degradation product, internal standard,  etc,) |
| Tailing factor | T of ≤ 2 |
| Theoretical plates | N> 2000 |

**Non-Interference of placebo:**

The portion of specificity evaluation applies to the finished drug product only.Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte.13,14

**2. Linearity**

The linearity of a method is a measure of how well a calibration plot of response VS concentration approximates a straight line. Linearity can be assessed by performing Single measurement at several analyte concentrations. The data is then processed using a linear least- squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

**3. Precision**

Precision can be defined as “The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample”. A More comprehensive definition proposed by the international conference on Harmonization (ICH) divides precision into three types.

1. Repeatability

2. Intermediate precision and

3. Reproducibility

**Repeatability:** is the precision of a method under the same operating conditions over a short period of time.

**Intermediate precision:** is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.15

**Reproducibility:** examine the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

**4. Accuracy:**

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose “true value” is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies.

There are three ways to determine accuracy:

1. Comparison to a reference standard

2. Recovery of the analyte spiked into black matrix or

3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. e.g., weight/ weight or area percent in all cases with respect to the major analyte.

**5. Specificity/ selectivity16:**

The terms specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method said to be selected. Since there are very few methods that respond to only one analyte, the term selectivity is more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peak either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.16,17

**6**. **Ruggedness:**

The ruggedness of an analytical method is the degree of reproducibility of test results Obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental Conditions that may differ but are still within the specified parameters of the assay. The testing of the ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

For the determination or ruggedness, the degree of reproducibility of test result is determined as a function of the assay variable. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the analytical method.

**7. Robustness:**

The concept of robustness of an analytical procedure has been defined by the ICH as “a Measure of its capacity to remain unaffected by small, but deliberate variations in method Parameters”. A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involve flow rate, column temperature, sample temperature, pH and mobile phase composition.

**8. Stability:**

To generate reproducible and reliable results, the samples, standards, and Reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, One week, and one month, depending on need). Therefore, a few hours of standard and sample solution suitability can required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency such practices add requirements for greater solution stability.

**9. Limit of Detection17:**

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but the necessarily quantitated, under the stated experimental conditions.

* Based on visual Evaluation
* Based on the standard Deviation of the Blank
* Based on the calibration curve
* Based on signal-to-noise: A signal-to-noise ratio of 3 or 2:1 is acceptable

LOD may be expressed as

LOD =3.3σ/s

Where, σ = the standard deviation of the response

S= the slope of the calibration curve

The slope S may be estimated from the calibration of the analyte.

**10. Limit of quantitation:**

Limit of quantitation is the lowest concentration of analyte in a sample that can be Determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible.

* Based on visual Evaluation
* Based on standard Deviation of the blink
* Based on the calibration curve
* Based on the signal-to-Noise Approach: A typical signal-to-Noise is 10:1

LOQ may be expressed as

LOQ = 10σ /s

Where, σ = standard deviation of the response

S= the slope of the calibration curve