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A REVIEW: STRATEGY FOR METHOD DEVELOPMENT AND VALIDATION OF HPLC

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ABSTRACT

The process of pharmaceutical drug development requires a suitable technique which helps the scientist to analyse the drug molecule is an accurate, precise, and easiest way. To develop drugs serve their purpose various chemical and instrumental Methods were developed at regular intervals which are involved in the estimation of drugs. For the quantitative and qualitative estimation of drugs in analytical chemistry it is very important to identify the best and easiest method for method development for drug estimation.. This review helps the author to understand the various analytical techniques such as titrimetric, Chromatographic, spectroscopic, electrophoretic, and electrochemical, injection analysis, hyphenated technique as well their corresponding methods that have been applied in the analysis of pharmaceuticals. All these above methods contain different analytical process with a variety of separate techniques. Also, we discuss about the modern trend which are available, and implemented in all these methods to improve the analytical behaviour of these techniques. Method development for the drug component in finished product or in process tests and the sample preparation of drug product and to provide practical approaches for determining selectivity, specificity, limit of detection, limit of quantitation, linearity, range, accuracy, precision, recovery solution stability, ruggedness, and robustness of liquid chromatographic methods to support the routine in process and stability analysis.

INTRODUCTION

Since first initiated by the U.S. Food and Drug Administration (FDA) in its "Pharmaceutical GMPs for the twenty-first century" (Quality by design) has become an important concept for the pharmaceutical industry that is further defined in the International Conference on Harmonization (ICH) guidance on pharmaceutical development as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on technical science and quality risk management".

Currently, increased attention has been paid to Quality by design within the pharmaceutical industry to actively seek out quality using its underlying principles. As analytical techniques and methods are used for the quality control of pharmaceutical compounds and thereby assure patient safety and efficacy during therapy, they have become an essential part of pharmaceutical Quality by design(QbD). The scientific understanding gained during the method development process can be used to method control elements and to manage the risks identified. This approach ensures high likelihood of method success during the product lifecycle. Thus, the validation which is usually performed after method development will serve the purpose of confirming method performance as opposed to identifying potential problem areas. Pharmaceutical analysis plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and finished products. It also plays an important role in building up the quality products through in process quality control as well plays a major role in isolation and characterization of impurities.

ANALYTICAL CHEMISTRY

Analytical Chemistry is a measurement of science consisting of a set of powerful ideas and methods that are useful in all fields of science and pharmaceutical medicine. It seeks ever improved means of measuring the chemical composition of natural and artificial materials. The branch of chemistry, which is both theoretical, and practical science, is practiced in a large number of laboratories in many diverse ways while analytical method, is a specific application of a technique to solve an analytical problem. Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. The disciplinary action of analytical chemistry consists of qualitative and quantitative analysis

CHROMATOGRAPHY

Chromatography is a non-destructive procedure for resolving a multi-component mixture into individual fractions by different process. Quantitative analysis is carried out by measuring the area of the chromatographic peak; hence chromatography can be used for qualitative and quantitative analysis. Chromatography is a new technique which was first invented by M. Tweet, a botanist in 1906 in Warsaw. He used this technique for separation of coloured substances by percolating vegetable extracts through a column of calcium carbonate and he observed colour bands at different position in the column. Tweet termed these colour bands as the chromatogram and the method as chromatography. Chromatography is derived from Greek words Chroma meaning “colour” and graphos means “writing”.

Defination of Chromatography

A separation process that is achieved by the distribution of substance to be separated between two phases as stationary phase and mobile phase, those solutes distributed preferentially in the mobile phase, will move more rapidly through the system than those solutes distributed preferentially in the stationary phase. Thus, the solute will elute in order of their increasing distribution coefficients respect to the stationary phase.

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases that is mobile phase and stationary phase.

Chromatography is mainly divided into two categories:

1. Adsorption Chromatography: Separation is mainly due to the interaction between solute and surface on the adsorbent. In this, stationary phase is solid and mobile phase is liquid.
e.g.: TLC, HPTLC and GC.

2. Partition Chromatography: Separation is based on the partition coefficient of two phases. In this mode, both stationary phase and mobile phase are liquids
e.g.: HPLC, GLC and PC.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

MODES OF CHROMATOGRAPHY

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces are based on the size of the particles in stationary phase.(e.g. Size exclusion chromatography)
Different modes of chromatography are as follows -

- Normal Phase Chromatography
- Reverse Phase Chromatography
- Reverse Phase – ion pair Chromatography
- Ion Chromatography
- Ion-Exchange Chromatography
- Affinity Chromatography
- Size Exclusion Chromatography

1. Reverse Phase Chromatography

Methods can be chosen based on solubility and molecular mass. In most of the cases for non-ionic small molecules ($\mu < 2000$), reversed phase methods are suitable.

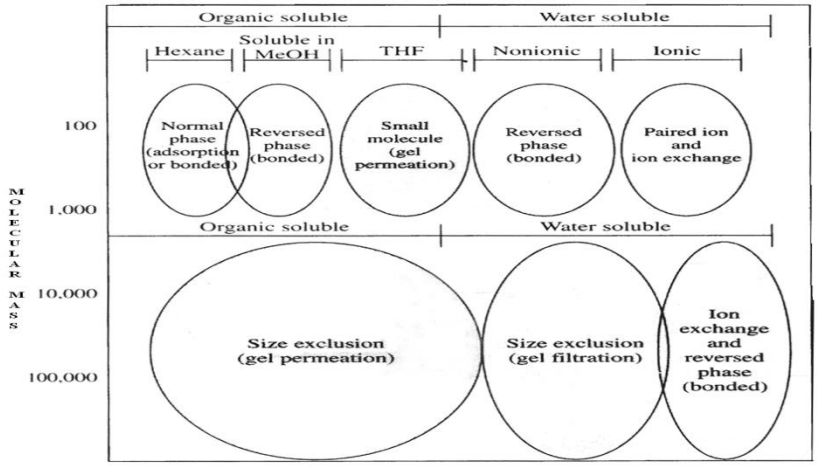

Fig.1 Selection of LC Modes

In 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds.

A large number of chemically bonded stationary phases based on silica are available on commercial scale. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-divinyl benzene copolymer) are slowly gaining ground which give slow result.

In reverse phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be disrupted when a solute is dissolved. Only higher polarsolutes can interact with the water structure and non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms (C18) it is the most popular stationary phase used in pharmaceutical industry for estimation. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use C18 HPLC columns. The solvent strength in reverse phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reverse phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The retention time in reverse phase chromatography increases with increasing amount of water in the mobile phase.

2. Adsorption Chromatography /Normal Phase Chromatography

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end of reaction. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

- Dipole-induced dipole,
- Dipole-dipole,
- Hydrogen bonding,
- π-Complex bonding

The situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The absorption strengths and hence k' values (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatics < organic halogen compounds < sulphides < ethers< esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on steric factors. If a molecule has verious functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as the amino propyl, cyan propyl and diol phases is useful alternatives to silica gel as stationary phase in normal phase chromatography.

The amino propyl and cyan propyl phases provide opportunities for specific interactions between analyse and the stationary phases and thus offer additional options for the optimisations of separations. Other advantages of bonded phases lie in their increased homogeneity of the phase surface.

Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or triethylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

HPLC SYSTEM

The components of a basic High Performance Liquid chromatography (HPLC) system are shown in the simple diagram below:

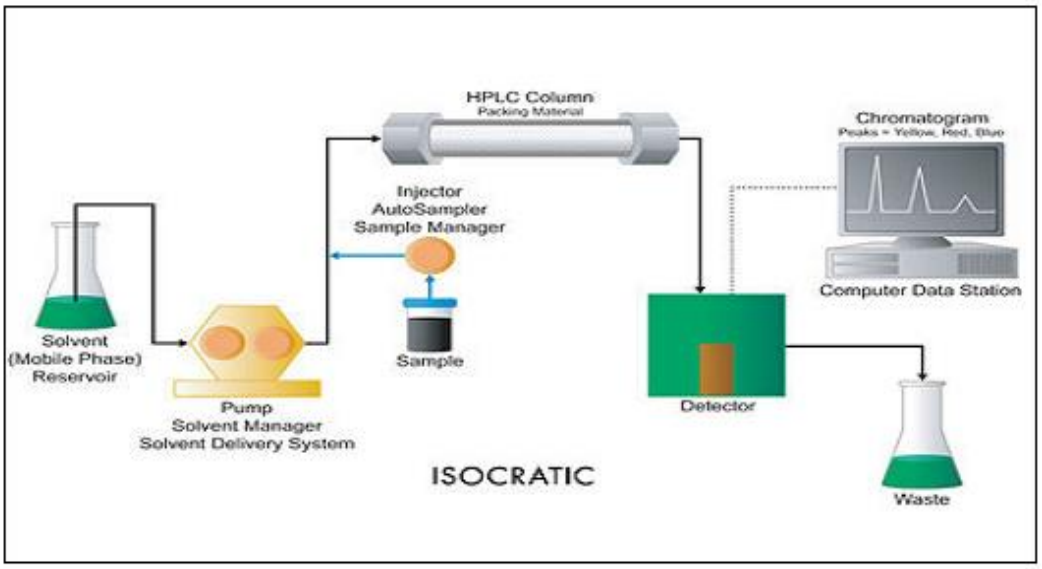

Fig. 2. HPLC System

The importance of Chromatography increasing rapidly in pharmaceutical analysis of drug. The exact differentiation identification and quantitative determination of structurally closely related compounds. Another important field of application of chromatographic methods is the purity testing of finish products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards of country.

The modern form of column chromatography has been called high performance, high pressure, and high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

The essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures.

The systems used are often described as belonging to one of four mechanistic types, adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first.

The various components of a HPLC system are herewith described.

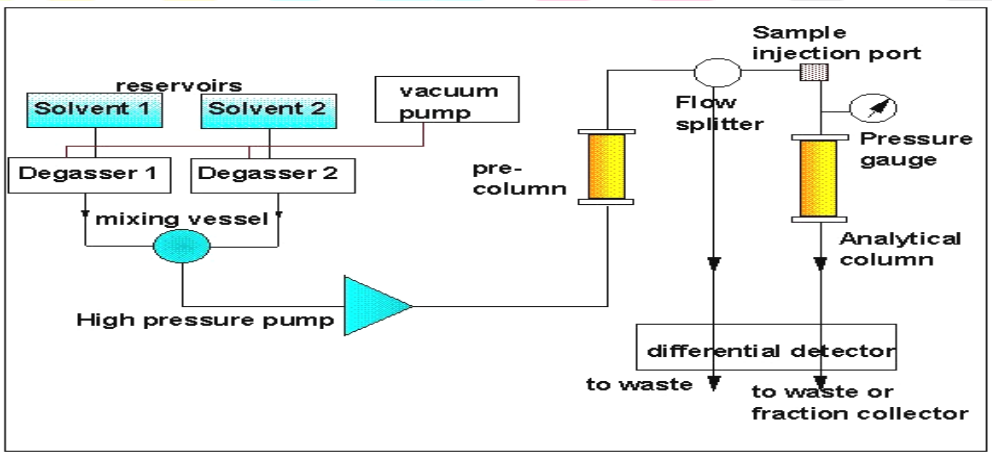

Fig.3. Instrumentation (Components) of HPLC System

SYSTEM COMPONENTS

1. SOLVENT DELIVERY SYSTEM

The mobile phase is pumped under high pressure from one or several reservoirs and flows through the column at a constant rate. In column micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity of the stationary phase and the nature of the sample components. In normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents mobile phase. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and also toxicity.

The most important component of HPLC in solvent delivery system is the pump, because its pressure performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used because as this system gives less baseline noise, good flow rate reproducibility etc.

The function of the pump in HPLC is to pass mobile phase through the column at a controlled and constant flow rate. Features of an ideal pumping system include:

- Generating pressure from 6000 psi to 10000 psi.
- Pulse free output.
- Flow rates ranging from 0.1 to 10 ml/min.
- Flow control and reproducibility of 0.5% relative or better.
- Corrosion resistant components.

These are types of pumps commonly used:

1.1 Reciprocating pumps

Reciprocating pumps is one of the most popular which is use now days. It usually consist of a small motor driven piston moves rapidly back and forth in a hydraulic chamber which as capacity range varying from 35-400µl as per system. By means of check values, the backward stroke of piston it sucks the mobile phase from solvent reservoir at this time the outlet to the separation column is closed. By forward stroke the pump pushes mobile phase out to the separating column and inlet to the reservoir is closed.

1.2 Displacement pumps (Syringe-Type pumps)

It is work through positive solvent displacement at a constant rate by a mechanically driven piston. It usually consists of large syringe like chambers equipped with a plunger that is activated by a screw driver mechanism powered by stepping motor. The rate of solvent delivery to system is controlled by changing the voltage of the motor. It is also produce a flow that tends to be independent of viscosity and back pressure.

1.3Pneumatic pumps

The mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressor gas in pneumatic pump system. This kind of pump inexpensive and gives pulse free flow. They suffer from limited

capacity, pressure output, dependence of flow rate on solvent viscosity and column backpressure. In addition, they are not amenable to gradient elution and they are limited to pressures less than about 2000 psi.

2. SOLVENT DEGASSING SYSTEM

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

3. GRADIENT ELUTION DEVICES

HPLC columns may be run isocratically with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during run. Gradient elution is a means of overcoming the problem of dealing with a complex mixture of solutes.

4. SAMPLE INTRODUCTION SYSTEMS

Several devices are available either for manual or auto injection of the sample. Different devices are

a) Septum injectors

b) Stop flow (on line)

c) Rheodyne injector (Loop valve type)

a. **Septum injectors:** For injecting the sample through a rubber septum. This is not common, since the septum has to withstand high pressure.

b. **Stop flow:** In which the flow of mobile phase is stopped for a while and the sample is injected through a valve device.

c. **Rheodyne injector (Loop valve type):** It is the most popular injector. This has a fixed volume loop like 20µl or 50µl or more. Injector has two modes, i.e., load position when the sample is loaded in the loop and inject mode when the sample is inrequired

5. COLUMNS

The columns are made with 316-grade stainless steel (a Cr-Ni-Mo- steel, relatively inert to chemical corrosion). The inside of the stainless steel column tube should be as smooth as possible, so the tubes are precision drilled or electrqo-polished after manufacture. The common dimensions are 6.35 mm external diameter, 4.6 mm internal diameter and up to 25 cm long of column. The columns can be packed with different partical diameter like 10, 5, 4 or 3 µm.

At the top of the column, there is a distributor for directing the injected sample to the center of the column and then a stainless steel gauze or frit on top of the packing. At the lower end there is another frit to retain the packing, and then, for the 4.6 mm type, a reducing union and a short length of 0.25 mm I.D. Thetubing is connect the column to the detector. Materials used other than stainless steel for columns include glass, glass lined steel tube and polyethene or other inert plastics.

Three forms of column packing material are available based on a rigid solid structure below

a. **Micro porous:** It supports micro porous ramify through the particles which are generally 5-10µm in diameter.

b. **Pellicular:** It supports where porous particles are coated onto an inert solid core such as a glass bead of about 40µm in diameter.

c. **Bonded phases:** Bonded phases where the stationary phase is chemically bonded onto an inert support.

5.1 Analytical columns

The majority of liquid chromatography columns range in length from 5 to 30 cm. The inner diameter of liquid columns is often 4 to 5 mm. The most common particle size of packing is 5 to 10 µm. The LC Columns of this type contain 40000 - 60000 plates per meter.

5.2 Preparative columns

Preparative columns are typically 2-5 cm in diameter and 25 cm long with packing of 15-100 µm diameters. Columns for large-scale work can be 20-30 cm in diameter and 60 cm long, using flow rates up to 1000 cc min-1. The commercial systems can be used isocratically or with gradients, and allow small-scale development and preparative separation to be done using the same system.

5.3 Guard columns

The short length guard column is introduced before the analytical column to increase its life. Guard columns usefull to removes particulate matter, contaminants from the solvents and also sample components that bind irreversibly to stationary phase. The composition of the guard column packing should be closely similar to that of analytical columns but partical size major than analytical column.

Column	Phase	Solvents	Application
C18	Octadecyl	ACN,MeOH,H_2O	General,nonpolar
C_8	Octyl	ACN,MeOH,H_2O	General,nonpolar
Phenyl	Styrl	ACN,MeOH,H_2O	Fattyacids,doublebond
Cyano	Cyanopropyl	ACN,MeOH, H_2O,THF	Ketones,aldehydes
Amino	Aminopropyl	ACN,MeOH, H_2O,THF, $CHCl_3$,CH_2Cl_2	Sugars,anions
Diol	Dihydroxyhexyl	ACN,MeOH, H_2O,THF	Proteins
SAX	Aromatic quaternaryamine	SALTBuffers,ACN,MeOH, H_2O	Anions
SCX	Aromaticsulfonicacid	SALTBuffers,ACN,MeOH	Cations
DEAE	Alkylether,ethyl 2°amine	SALTBuffers,ACN,MeOH, H_2O	Proteincations

Table.2. Various types of columns and their applications

6. DETECTORS

The function of the detector in HPLC is to monitor the mobile phase emerging from the column. After analysing of sample output of the detector is an electrical signal that is proportional to someproperty of the mobile phase or the solutes.LC detectors are basically of two types. Property detectors responds to the mobile phase bulk property such as refractive index, dielectric constant or density. The solute property detectors responds to some property of solutes, such as UV absorbing, diffusion current, fluorescence are not possessed by the mobile phase.

Most common HPLC detectors

- UV-Visible absorbance detector (UV-VIS)
- Photo-diode array detector (PDA)
- Fluorescence detector
- Electrochemical detector (ECD)
- Refractive Index detector (RI)
- Mass detectors (MS)
- Conductometric detector
- Chiral detector (Polarimetric & circular dichrosim)
- Evaporative light scattering detector (ELSD)

6.1 UV-Visible absorbance detector

In this there are three types of detectors are available: a fixed wavelength detector, a variable wavelength detector and scanning wavelength detector.

a. Fixed Wavelength Detector

It uses a light source which emits maximum intensity at one or several discrete wavelengths. This type of detector offers a minimum noise. Many compounds including nucleic acids have absorption bands which encompass the 254nm wavelength. By using medium pressure mercury lamp wavelengths of 254,280,313,334 and 365nm can be selected by using narrow bandpass interference filters. Placing a phosphor converter between the mercury lamp and lens produces an emission band that peaks at 280nm which is suitable for detecting proteins. The operations in the visible region can be accomplished by using quartz iodine lamp and appropriate interference filters.

b. Wavelength Detector

It has relatively wide band pass. It offers a wide range selection of UV and visible wavelengths with an increased cost. To obtain a complete spectrum the eluent flow should be stopped to trap the component of interest in the detector cell while UV-Visible spectral region is scanned.

c. Scanning Wavelength Detector

To obtain real time spectrum for each parallel, simultaneously monitoring all wavelengths. Solute as it elutes, solid state diode arrays are required.

6.2 Photo-Diode Array Detector (PDA)

This is a recent one which is similar to UV detector which operates from 190-600 nm. The Radiations of all wavelengths fall on the detector simultaneously and the resulting spectrum is 3-D or three dimensional plot of Response vs. Time vs. Wavelength. The advantage is that the without selecting wavelength detector detects the response of all the compounds.

6.3 Fluor metric Detector

Tapered, square or cylindrical flow cells have been used. The fluorescence of about one-sixth is often collected at a right angle. If a concave mirror is placed around the flow cell and the rear of the cell is reflective, about 75%

of the emission is collected. With all flow cells, scattered radiation from the excitation source is selectively removed with cutoff or bandpass filters placed before the photomultiplier tube.

6.4 Electrochemical (Amperometric) Detector

The electrochemical detection depends on the voltammetric characteristics of solute molecules in an aqueous or aqueous-organic mobile phase. Amperometric transducers measure the current at acontrolled potential as a function of time. The flow cell is a channel in a thin polyfluorocarbon gasket sandwiched between two blocks, one plastic and stainless steel. Along one side of the channel is positioned a working electrodes and downstream to the working electrode as a reference electrode.

6.5 Christiansen Detector

The Christiansen effect detector is a refractive index-dependent detector that passes light through the sample and reference cells to observe the difference in refractive index of the two liquids which are packed with a solid that has the same refractive index as the chromatographic mobile phase. When a sample is eluted from the column and carried through the cell, the refractive index changes indicating a change in the transmission of the light and is measured by means of photo detectors.

7. COLUMN CHARACTERISTICS

7.1 Retention time (tR)

This is the time of emergence of the peak maximum of a component after injection. Retention time is the sum of the times the component spends in the mobile phase (tM) and in the stationary phase. The adjusted retention time (t'R) is the time the component spends in the stationary phase and is given by

t'R = tR - tM

The capacity factor (or ratio) (k) is the ratio of the time the component spends in the stationary phase to the time in the mobile phase

k = t'R/ tM= tR - tM/ tM

Where,

tR= Retention time

t'R = Adjusted retention time

tM = Time of the component in mobile phase

k = Capacity factor

7.2 Retention volume (VR)

This is the volume of carrier gas required to elute one half of the compound from the column as indicated by the peak maximum and is given by

VR = tR× f

Where,

VR = Retention volume

tR = Retention time

f = Flow rate of the carrier gas at the outlet pressure of the column and the temperature of the column.

7.3 Adjusted retention time (V'R)

This allows for the gas hold-up of the column which is due to the interstitial volume of the column and the volume of injector and detector systems. It is given by

V'R = t'R× f

Where,

V'R = Adjusted Retention volume

t'R = Adjusted Retention time

f = Flow rate at the outlet pressure of the column and the temperature of the column

7.4 Specific retention volume (Vg)

This is the net retention volume per g of liquid phase at 0°.

Vg = VN 273/ TWtL

Where,

Vg = Specific retention time

VN = Net retention volume

WtL = Weight of the liquid phase

T = Temperature of the column

7.5 Theoretical plates per meter

n = (5.54Vr2)/ L Wh2

Where,

n = Number of theoretical plates per meter

Vr = Distance along the base line between the point of injection and a perpendiWhere dropped from the maximum of the peak of interest

L = Length of the column in meters

Wh = Width of the peak of interest at half peak height

7.6 Tailing factor (Symmetry factor)

Symmetry factor of as peak was calculated from the following expression:

Symmetry factor = Wx/2A

Where,

Wx = Width of the peak at one twentieth of the peak height.

A = Distance between the perpendicular dropped from the peak maximum and the leading edge of peak at one twentieth of the peak height.

7.7 Resolution (RS)

RS is the measure of how well two peaks are separated for reliable quantification; well-separated peaks are essential for quantification. This is more useful parameter if potential interference peak(s) may be of concern. The closest potential eluting peak to the analyte should be selected RS is minimally influenced by the ratio of two compounds being measured.

RS = 2(tR2-tR1) / (w1+w2)

8. DERIVATIZATION

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives can be formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column outlet and the detector.

STRATEGY FOR METHOD DEVELOPMENT OF HPLC

Selection of suitable chromatography for organic compounds,

- First reverse phase should be tried.
- If not successful, then, normal phase should be taken into consideration.

METHOD DEVELOPMENT AND OPTIMIZATION

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved in terms of resolution or peak shape, plate counts asymmetry, capacity,retention time, detection limits, limit of quantitation, and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow

1. Manual
2. Computer driven

The manual approach comprise varying one experimental variable at a time, while holding all others constant, and recording changes in response. The variables might include flow rates, mobile or stationary phase composition, temperature, detection wavelength, and pH this univariate approach to system optimization is slow,

time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated methods development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications. In addition, they are ableto significantly reducing the time, energy and cost of virtually all-instrumental methods development.

The various parameters that include to be optimized during method development,

1. Method requirements
2. Literature survey and prior methodology
3. Selecting a method
4. Mode of separation
5. Selection of stationary phase
6. Selection of mobile phase
7. Selection of detector

1. Method requirements

The requirement of analytical methodology is important to establish the analytical figures of advantage such as linearity, precision, accuracy, LOD, LOQ, specificity, selectivity and range etc.

2. Literature survey and prior methodology

All types of information (Physical properties, chemical properties, solubility, manufacturing related analytical methods etc.) regarding the analyte are obtained by doing literature survey by referencing books, journals, pharmacopoeias etc. Chemical Abstract Service (CAS) automated computerized literature searches are also helpful for making literature survey.

3. Selecting a method

The methodology is developed by using the information obtained from the literature survey. The method is being revised where necessary and also there is a need to other instrumentation to reproduce, modify, validate or improve available methods for samples and analytes. If there is no any established method for analyte in the any literature, then such compounds are searched which are identical in chemical properties and structure of analyte and develop the new method.

4. Selectionof Mode of Separation

In reverse phase chromatography mode, the mobile phase ismore polar comparative to stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor and nature of the matrix is the secondary factor during the selection of the mode of separation.

5. Selection of Stationary Phase / Column

Selection of the stationary phase or column is the first and the most important step in method development. The perfect choice of separation column includes three different approaches

Selection of separation system

1. The particle size and the nature of the column packing
2. The physical parameters of the column i.e. the length and the diameter
3. Some of the important parameters considered while selecting chromatographic columns are

- Length and diameter of the column.
- Packing material.
- Shape of the particles.
- Size of the particles.
- % of Carbon loading
- Pore volume.
- Surface area.
- End capping.

The stationary phase or column is selected depending on the nature of the solute and the information about the analyte in literature. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C_2), butylsilane (C_4), octylsilane (C_8), octadecylsilane (C_{18}), base deactivated silane (C_{18}) BDS phenyl, cyanopropyl (CN), nitro, amino etc.

Generally longer size columns provide better separation or resolution due to higher theoretical plate numbers. As the particle size decreases in column the surface area available for coating increases. The columns with 5-µm particle size and inner diameter is 4.0mm give the best compromise of efficiency, reproducibility and reliability.

Peak shape is equally necessary in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- In accurate plate number and resolution measurement
- Imprecise quantisation
- Degraded and undetected minor bands in the peak tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is necessary for developing a rugged and repeatable method.

6. Selection of Mobile Phase

The primary objective in selection of mobile phase is to achieved optimum separation of all the individual impurities and degradants from each other and from analyte peak

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute – mobile phase and the mobile phase – stationary phase. For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation) Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes; the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength.

The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer
- pH of the buffer
- Mobile phase composition.

a. Buffer, if any and its strength

Buffer and its strength play an important role in deciding the peak symmetries and resolution. Some of the most, commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, etc
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers – Ammonium acetate, Sodium acetate, etc.
- Acetic acid buffers prepared using CH_3COOH.

The elute times also depend on the molar strengths of the buffer. Molar strength is increasingly proportional to retention times of analyte. The strength of the buffer can be increased, if necessary, to achieve the required resolution.

b. pH of the Buffer

The pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics in mobile phase. Experiments were conducted using buffers having different pH to obtain the required resolution or separation of peak.

It is necessary to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkages area cleaved below pH 2.0, while pH valued above 8.0 silica may dissolve.

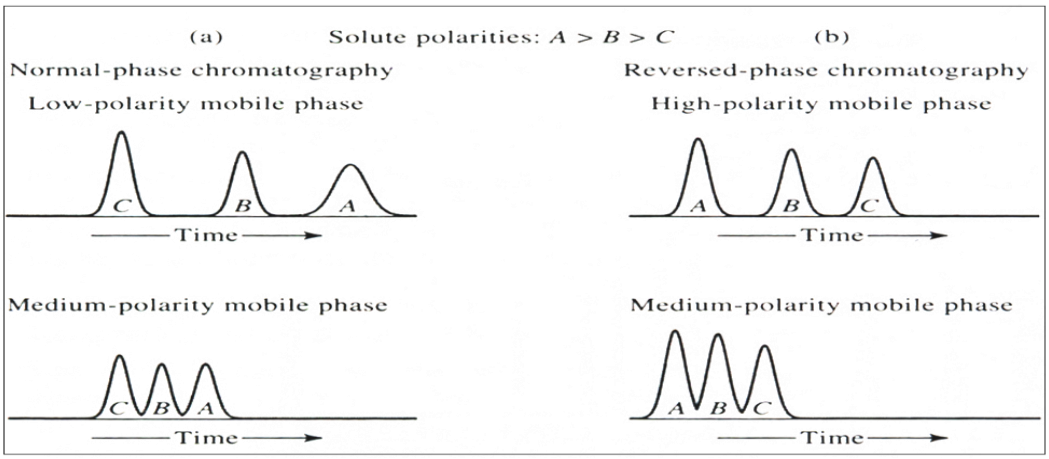

Fig.4. Relationship between Polarity and Elution Times for NP and RP Chromatography

c. MobilePhase Composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to that fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Themost popularly used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments were conducted by trial and error method with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase which gives separation of all the impurities and degradants from each other from analytic peak and which is rugged for variation of both aqueous and organic phase by at least ±0.2 % of the selected mobile phase composition.

7. Selection of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, conductance,oxidation,reduction,fluorescence etc. characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity, facilitating trace analysis
- Negligible baseline noise. To facilitate lower detection
- Large linear dynamic range
- Low dead volume
- Non destructive to sample
- Inexpensive to purchase and operate

All the Pharmaceutical ingredients do not absorb UV light equally, so that selection of detection wavelength is important for individual drug. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is helpful.

For the greatest sensitivity λ_{max} should be used. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity. Here, PDA detector Used.

Stationaryphase	C_8or C_{18}		
Columnlengthandinternaldiameter	250mmx4.6mm		
Particlesize	10or5µm		
Mobilephase	Buffer:Acetonitrile		
%Bufferisocratic	50%		
%Buffergradient	20-80%		
Bufferconcentration	Phosphate50 mM		
pHofmobilephase	3forneutral compounds	3and7.5for ionicacidic	3and7.5for ionicbasic
Modifier	10mMTEA and1%HSA	1%HAS	10mMTEA
Flowrate	1.5-2mL/minutes		
Columntemperature	Ambientto35°C		
Injectionvolume	10–25µl		

Table. Initial HPLC Condition

METHOD VALIDATION

It can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. It is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including:

- Specificity /Selectivity
- System suitability
- Precision
  - Repeatability
  - Intermediate precision
  - Reproducibility
- Accuracy
- Linearity
- Range
- Limit of Detection
- Limit of Quantitation

- Robustness

1. SPECIFICITY/SELECTIVITY

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures as containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc.) is compared with the response of a solution containing only the analyte. Other potential sample components are generated by exposing the analyte to stress conditions sufficient to degrade it to 80-90% purity. For bulk pharmaceuticals, stress conditions such as heat (50|AoC), light (600 FC), acid (0.1 N HCl), base (0.1 N NaOH), and oxidant (3% H_2O_2) are typical. For formulated products, heat, light, and humidity (85%) are often used.

The resulting mixtures are then analysed, and the analyte peak is evaluated for peak purity and resolution from the nearest eluting peak. If an alternate chromatographic column is to be allowed in the final method procedure, it should be identified during these studies. Once acceptable resolution is obtained for the analyte and potential sample components, the chromatographic parameters, such as column type, mobile-phase composition, flow rate, and detection mode, are considered set. An example of specificity criteria for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 1.5 from all other sample components. If this cannot be achieved, the unresolved components at their maximum expected levels will not affect the final assay result by more than 0.5%. An example of specificity criteria for an impurity method is that all impurity peaks that are 0.1% by area will have baseline chromatographic resolution from the main component peak(s) and, where practical, will have resolution from all other impurities.

2. SYSTEM SUITABILITY

It is an integral part of many analytical procedures to determine the overall system performance. In System suitability testing various parameters is to be established for a particular procedure depends on the type of procedure to be validated. Calculating the following values 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 formulae shows 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 in the same units).

Relative Retention

$\alpha = (t_2 - t_a) / (t_1 - t_a)$

Theoretical Plates

$n = 16 (t / W) 2$

Capacity Factor

$K' = (t_2 / ta) - 1$

Resolution

$R = 2 (t_2 – t_1) / (W_2 + W_1)$

Peak Asymmetry

$T = W_{0.05} / 2f$

Plates Per Meter

$N = n / L$

Height Equivalent to Theoretical Plate

$HETP = L/n$

Where,

α = Relative retention.

t_2 = Retention time of the second peak measured from point of injection.

t_1 = Retention time of the first peak measured from point of injection.

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

n = Theoretical plates.

t = Retention time of the component.

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

K' = Capacity factor.

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

W_2 = Width of the base of component peak 2.

W_1 = Width of the base of component peak 1.

T = Peak asymmetry, or tailing factor.

$W_{0.05}$ = 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.

N = Plates per meter.

L = Column length, in meters.

3. PRECISION

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards degree. It is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD) of the repeated measurements.

$$\%RSD = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

The measured standard deviation can be subdivided into three categories

3.1.Repeatability

Repeatability is obtained when the analysis is carried out in one laboratory by one operator using one piece of equipment over a relatively short time span. At least 5 or 6 determinations of three different matrices at two or three different concentrations should be done and the relative standard deviation calculated. The acceptance criteria for precision depend very much on the type of analysis.

3.2.**Intermediate Precision**

Intermediate precision is a term that has been defined by ICH as the long-term variability of the measurement process and is determined by comparing the results of a method run within laboratory(single laboratory). The method's intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. The main of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

3.3. **Reproducibility**

The objective is to verify that the method will provide the same results in different laboratories with different analyst. The reproducibility of an analytical method is determined by analysing aliquots from homogeneous lots in different laboratories with different analysts and by using operational and environmental conditions that may differ from but are still within the specified parameters of the method. Validation of reproducibility is important as per standard guidelines if the method will used in different laboratories.

4. **ACCURACY**

It's an analytical method is the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in different ways. One alternative is to compare results of the method with results from an established reference method as standard. This approach assumes that the uncertainty of the reference method is known and acceptable. Secondly, accuracy can be assessed by analysing a sample with known concentrations, for example, a certified reference standard material, and comparing the measured value with the true value as supplied with the material. If such certified reference material is not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. The concentration should cover the range of concern and should particularly include one concentration close to the quantitation limit. The expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration.

5. LINEARITY

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration of analyte. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels, from 50 to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data. The standards are evaluated using the chromatographic conditions determined during the specificity studies of analyte

Standards should be prepared and analysed as a minimum of three times. The 50 to 150% range for this study is wider than what is requirement by the FDA guidelines. In the final method procedure, a higher range of three standards is generally used, such as 80, 100, and 120% of target; and in some instances, a single standard concentration is used.

Validating over a wider range provides confidence that the routine standard levels are well removed from non-linear response concentrations, that the method covers a wide enough range to incorporate the limits of content uniformity testing, and that it allows quantitation of crude drug samples in support of process development. For impurity methods, linearity is determined by preparing the standard solutions at five different Concentration levels over a range such as 0.05-2.5 wt%.

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of > 0.999 is generally considered as evidence of acceptable fit of the data to the regression line.

6. RANGE

The range of an analytical method is the analyte concentration interval over which acceptable accuracy, linearity, and precision are obtained. In practice, the range is determined using data from the linearity and accuracy of validation studies. Assuming that acceptable linearity and accuracyresults were obtained as described earlier, the only remaining factor to be evaluated is precision. This precision data should be available from the triplicate analyses of spiked samples in the accuracy study of validation.

7. LIMIT OF DETECTION (LOD)

ICH defines the detection limit of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The LOD is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be determine but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method.

$$\mathbf{LOD = 3.3\ (SD)/S}$$

where, SD = Standard deviation of Y intercept

S = Slope

The limit of detection (LOD) was found to be 0.99 µg/ml.

8. LIMIT OF QUANTITATION (LOQ)

ICH defines the limit of quantitation (LOQ) of an individual analytical procedure as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and issued particularly for the determination of impurities and degradation products.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

LOQ = 10 (SD)/ S

Where, SD = Standard deviation Y intercept

S = Slope

The limit of quantitation (LOQ) was found to be 2.98 µg/ml

9. **ROBUSTNESS**

The robustness of an analytical system is a degree of its ability to stay unaffected by small but deliberate variations in method parameters and affords an indication of its reliability during regular utilization, within the case of liquid chromatography, examples of normal versions are:

- influence of versions of pH in a mobile section
- have an impact on of variations in cellular segment composition
- unique columns (special lots and/or providers)
- Temperature
- glide price

The elements selected for all the medicine beneath researched been the waft fee, cellular segment composition, pH of a mobile phase and the use of exceptional lot of LC column. The commentary shall be summarized and crucial parameters will be indexed out within the validation record.

10. RUGGEDNESS

The ruggedness of an analytical technique is the degree of reproducibility of test outcomes received by means of the analysis of the equal samples beneath a diffusion of regular test conditions consisting of different laboratories, distinctive analysts, using operational and different environmental conditions which could vary but are still inside the unique parameters of the assay. Ruggedness is typically expressed as the lack of the influence at the check consequences of operational and environmental variables of the analytical technique of perticular drug.

CONCLUSION

The development of analytical methods for drug identification, purity evaluation, and quantification has received a lot of attention in the field of pharmaceutical analysis in recent years. This review provides a general overview of HPLC method development and validation. This article gives an idea about number of sample preparation, procedure and acceptance criteria for all analytical method validation parameters in wider range. Applications of analytical method and method transfer are also taken into consideration in this article. These several essential method and validation characteristics for analytical methodology have been discussed with a view to improving the standard and acceptance in this area of research.

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