REVIEW

HPLC AND ANALYTICAL METHOD VALIDATION – A REVIEW

Asmita Mahapatra*, Chandanam Sreedhar

Karnataka College of Pharmacy, Thirumenahalli, Bangalore-560064, Karnataka, India.

Submitted on: 27.03.18; Revised on: 07.05.18; Accepted on: 16.05.18

ABSTRACT

Validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Chromatographic methods play significant role in the pharmaceutical industry from the drug discovery, development, formulations and quality control. The purpose of writing this review was to compile the analytical method validation and HPLC . Analytic method development, validation, and transfer are key elements of any pharmaceutical development program.

KEYWORDS: HPLC (High Performance Liquid Chromatography), Validation,

Corresponding Author: Asmita Mahapatra
E-mail: i.asmita.mahapatra@gmail.com

Indian Research Journal of Pharmacy and Science; 16(2018)1399-1414;
Journal Home Page: https://www.irjps.in
DOI: 10.21276/irjps.2018.5.1.18

INTRODUCTION

Pharmaceutical analysis is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk drug and pharmaceutical preparations.[1]

Analytical chemistry studies and uses instruments and methods used to separate, identify, and quantify matter. In practice separation, identification or quantification may constitute the entire analysis or be combined with another method. Separation isolates analytes. Qualitative analysis identifies analytes, while quantitative analysis determines the numerical amount or concentration.

Applications:

Analytical chemistry has applications including in forensic science, bio analysis, clinical analysis, environmental analysis, and materials analysis. Analytical chemistry research is largely driven by performance (sensitivity, detection limit, selectivity, robustness, dynamic range, linear range, accuracy, precision, and speed), and cost (purchase, operation, training, time, and space). Among the main branches of contemporary analytical atomic spectrometry, the most widespread and universal are optical and mass spectrometry. Advances in design of diode lasers and optical parametric oscillators promote developments in fluorescence and ionization spectrometry and also in absorption techniques where uses of optical cavities for increased effective absorption path length are expected to expand.[2]

NEED FOR THE DEVELOPMENT OF NEW ANALYTICAL TECHNIQUES

Rapid increase in pharmaceutical industries and production of drug in and around the world bring forward a rise in inevitable demand to seek novel and systematic analytical techniques in the pharmaceutical industries. As a consequence, analytical method development has become the basic activity of analysis. Development in scientific and concrete analytical methods has been resulted from the advancements of analytical instruments. The improvements of the analytical method development and analytical instruments have reduced the time and cost of analysis and enhanced precision and accuracy. Techniques pertaining to analysis are developed and validated for active pharmaceutical ingredients, excipients, related substances, drug products, degradation products and residual solvents etc. Consequently quality control laboratories used these methods to check the efficacy, identity, purity, safety as well as performance of products of the drug.[3]

ANALYTICAL DEVELOPMENT:

Method development is done :

1. For new products

2. For existing products

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. Trial runs are conducted, method is optimised and validated. When alternate method proposed is intended to replace the existing procedure comparitive laboratory data including merits/demerits are madeavailable.[4]

CHROMATOGRAPHY:

Chromatography is a non-destructive procedure for resolving a multi component mixture of trace, minor or major constituent into individual fraction. Different variations may be applied to solids, liquids and gases. While chromatography may be applied both quantitatively , it is primarily a separation tool. Chromatography is relatively a new technique which was first invented by M.Tswett, a botanist in 1906 in Warsaw. In that year, he was successful

in doing the separation of chlorophyll, xanthophyll and several other colored substances by percolating vegetable extracts through a column of calcium carbonate . The calcium carbonate acted as an adsorbent and the different substances got absorbed

to different extent and this gave rise to colored bands at different positions, on the column. Tswett termed this system of colored bands as the chromatogram and the method is chromatography after the greek words 'chroma' and 'graphos' meaning 'color' and 'writing' respectively. [5]

CLASSIFICATION:

In chromatography the mobile phase can be a gas or liquid and the stationary phase may be a solid or liquid. Chromatography can be classified based on natures of phases, principles and modes of separation.

Based on the nature of Stationary and Mobile phase: The different types of chromatography based on the type of stationary phase and mobile phase are:

i. Gas-solid chromatography in which solid is the stationary phase and gas is the mobile phase.
ii. Gas-liquid chromatography in which liquid is the stationary phase and gas is the mobile phase.
iii. Solid-liquid chromatography in which solid is the stationary phase and liquid is the mobile phase.
iv. Liquid-liquid chromatography in which liquid is both stationary and mobile phase.
v. Based on the principle of separation:

The principle of separation involved in chromatography is adsorption or partition of components between mobile phase and stationary phase they are classified as:

i. Adsorption Chromatography: The principle involved in this type of chromatography is adsorption of the components onto stationary phase when the sample solution is dissolved in mobile phase is passed through a column of stationary phase.

Example: Gas-solid chromatography, Thin layer chromatography.

ii. Partition Chromatography : The principle involved in this type of chromatography is partition of the components into stationary phase when sample solution is dissolved in mobile phase is passed through a column of stationary phase.

Example: Gas-liquid chromatography, paper partition chromatography.

Based on modes of chromatography: There are two modes based on the polarity of the stationary and mobile phase used:

i. Normal phase Chromatography: In this mode, the stationary phase is polar and the mobile phase is non-polar.
ii. Reverse phase Chromatography: In this mode, the stationary phase is non-polar and the mobile phase is polar. This is most widely used in pharmaceutical analysis.

Other types of chromatography used for separation of components are:

i. Ion exchange chromatography in which the components are separate by exchange of ions between ion-exchange ions and charged ions.
ii. Gel permeation chromatography or size exclusion chromatography in which the components of a sample are separated according to their molecular sizes by using different gels.
iii. Chiral chromatography in which optical isomers are separated by using chiral stationary phases.[6]

HIGH PERFORMANCE LIQUID CHROMTOGRAPHY:

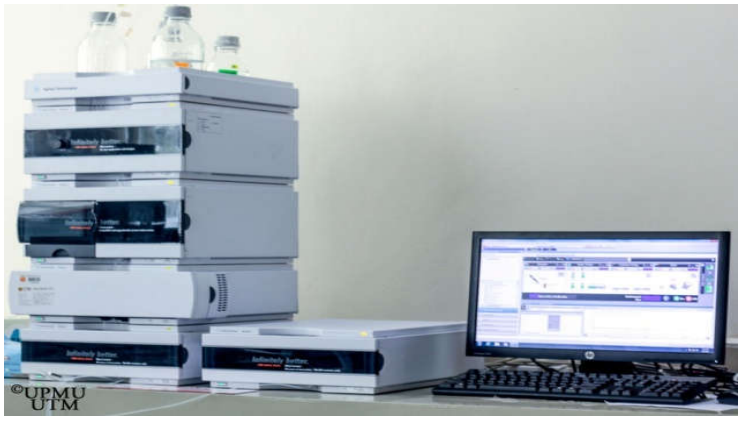

Figure 1: High Performance Liquid Chromatography.

One of the early problems with liquid chromatography was the slow rate at which the analysis took place. Early methods used gravity feed, and it was not uncommon for an analysis to take several days to complete. This led to great delay, but also excessive time on the column inevitably led to the loss of resolution by diffusion, and so on.

Consequently for a number of years liquid was not widely used as means of separating organic compounds. This problem was largely overcome by the advent of high-performance liquid chromatography. In this system pressure is applied to the column forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system.

The action of the pump is critical , since it must not pulsate and mix up the sample being separated in the solvent, causing it to lose resolution. Development of pumps has proceeded quite quickly over the last several years, and now it is possible to achieve good resolution under the conditions required for HPLC.

Principle:

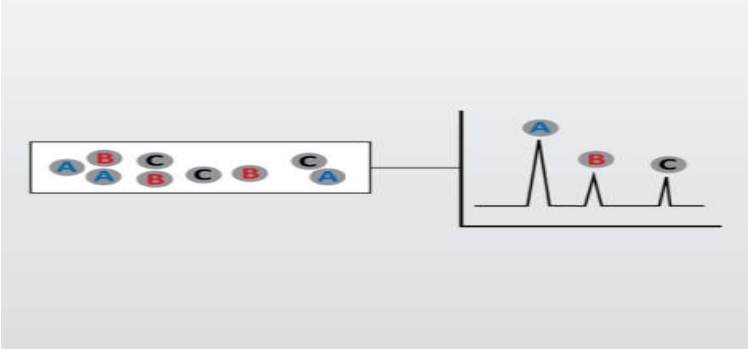

Figure 2: Chromatographic separation of components.

It is known that the resolving power of a chromatographic column increases with the column length and the number of theoretical plates per unit length, although there are limits to the length of a

column due to the problem of peak broadening. A the number of theoretical plates is related to the surface area of the stationary phase it follows that the smaller the particle size of the stationary phase, the better the resolution. Unfortunately, the smaller the particle size,

the greater the resistance to eluant flow. All of the forms of column chromatography so far discussed rely on gravity or lower pressure pumping system for the supply of eluant to the column. The consequences of this is that the flow rates achieved are relatively low and this gives a greater time for band broadening by simple diffusion phenomena. The use of faster flow rates is not possible because it creates a back pressure which is sufficient to damage the matrix structure of the stationary phase, thereby actually reducing eluant flow and impairing resolution. In the past decade there has been a dramatic development in column chromatography technology which has resulted in the availability of new particle size stationary phase which can withstand these pressures and of pumping systems which can give reliable flow rates. These developments, which have occurred in adsorption, partition, ion-exchange, exclusion and affinity chromatography, have resulted in faster and better resolution and explain why HPLC has emerged as the most popular, powerful and versatile form of chromatography. [7]

INSTRUMENTATION:

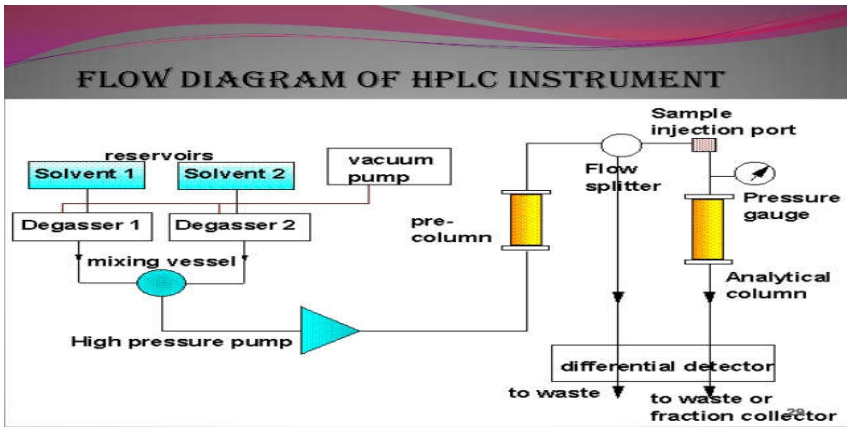

Figure 3: Flow diagram of HPLC Instrument.

The general instrumentation for HPLC incorporates the following components shown in the figure:

1. There is a solvent reservoir for the mobile phase.
2. The mobile phase must be delivered to the column by some type of pump. To obtain separation either based on short analysis time or under optimum pressure, a wide range of pressure and flow is desirable. The pumping system must be pulse free or else have a pulse dapper to avoid generating baseline instability in the detector.
3. Sampling valves or loops used to inject the sample into the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of mobile phase to eliminate an unnecessary solvent peak.
4. Ahead of the separation column there may be a guard column or an in-line filter to prevent contamination of the main column by small particulates.
5. To measure column inlet pressure a pressure gauge is inserted in front of the separation column.
6. The separation column contains the packing needed to accomplish the desired HPLC

separations. These may be silica for adsorption chromatography, bonded phases for liquid-liquid chromatography, ion-exchange functional groups, bonded to the stationary support for ion –exchange chromatography, gels of specific porosity for exclusion chromatography, or some other unique packing for a particular separation method.

7. A detector with some type of data handling device completes the basic instrumentation.

MOBILE PHASE DELIVERY SYSTEM:

The mobile phase must be delivered to the column over a wide range of flow rates and pressures. To permit the use of a wide variety of organic and inorganic solvents, the pump, its seals, and all connections must be made of materials chemically resistant to the mobile phase. If the system is not pulse free, some form of pulse damping is needed. A degasser is needed to remove dissolved air and other gases from the solvent. A pump should operate to at-least 100 atm, a pressure suited to less expensive chromatographs. However, 400 atm is a more desirable pressure limit. For many analytical columns only moderate flow rates of 0.5-2 ml/min need to be generated. Microbore columns require flow rates as low as a few microlitres per minute. The ease with which solvents may be changed is an important consideration in gradient elution work or when scouting for the optimum solvent. The pump should have a small holdup volume. [8]

PUMPS:

The pumping system is most important feature in HPLC . The high resistance to solvents flow offered by columns packed with small particles can be overcome by the pumping systems. The pumps should be resistant to the solvents and can operate at constant pressure or constant displacement.Various types of pumps used in HPLC are:

1. **Reciprocating pump:**

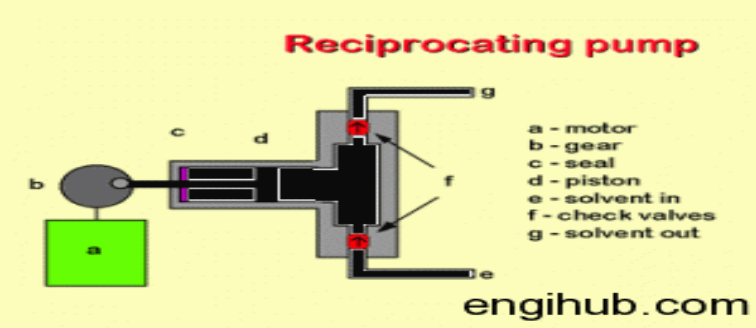

Figure 4: Reciprocating Pump

Reciprocating piston type pumps are relatively inexpensive and permit a wide range of flow rates. In this pump, a small motor-driven piston moves rapidly back and forth in a hydraulic chamber. By means of check valves, on the backward stroke, the piston sucks the solvent from reservoir. At this time, the outlet to the column is closed. On the forward stroke, the pump pushes the solvent to the column inlet and inlet from reservoir is closed. A wide range of flow rates can be obtained by altering the stroke volume or stroke frequency during each cycle.

The solvent delivery is continous with reciprocating pumps. There is no restriction on the reservoir size or operating time. These pumps may produce a pulsating flow of mobile phase. This can be overcome by using dual piston reciprocating pumps.

1. **Displacement pumps:**

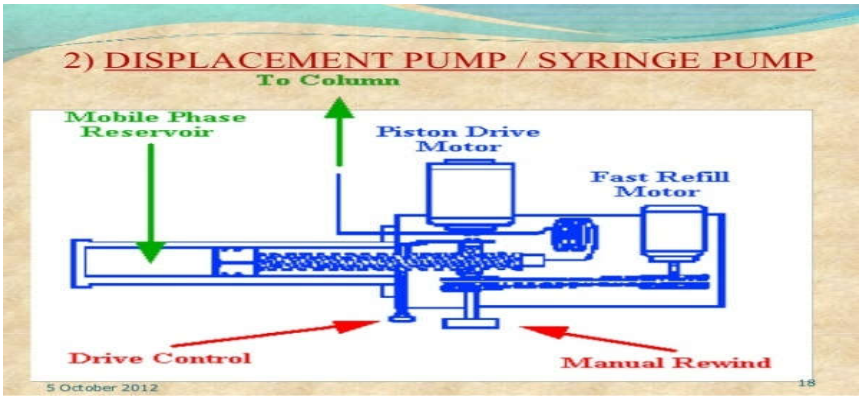

Figure 5: Displacement Pump

These pumps work through positive solvent displacement by a piston mechanically driven at a constant rate. The piston is driven by a screw-feed drive through a gear box by a digital stepping motor. The rate of flow of mobile phase is controlled by changing the voltage of the motor. The solvent chamber has only a finite capacity and must be refilled. By employing these pumps high pressures can be achieved without pulses.

Pulse pumpers:

The detectors used in HPLC are sensitive to variations in flow rates. Pulse dampers are used to dampen the pulses and achieve a constant flow rate. Pulse dampers use a compressible fluid separated from mobile phase by a flexible inert diapharm. In this type, the compressed fluid expands when the pump piston retracts and maintain system pressure and constant solvent flow.

Injection of Sample:

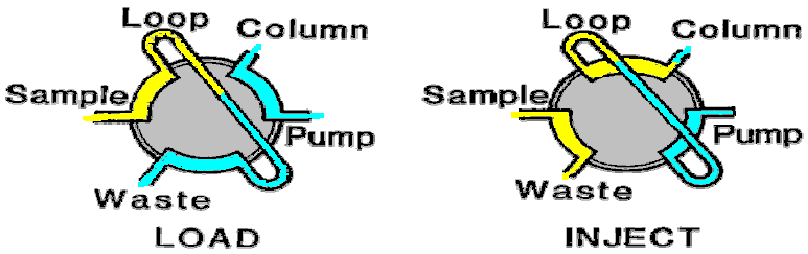

Figure 6: Mechanism of injection

Sample is injected into the pressurised column as a narrow plug. The injection system should not contain any void volume.

Microsampling injectors values are the mostly used sampling devices. The calibrated sample loop is filled with sample solution by means of an ordinary syringe. A rotation of valve rotor places the sample filled loop into the mobile phase stream without interruption of flow.

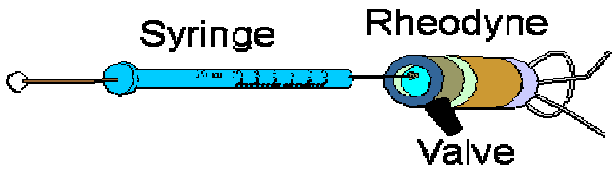

Figure 7: Rheodyne injection

COLUMNS:

Columns are constructed of gas-lined metal tubing or high quality stainless steel, polished internally to a mirror finish. Straight columns are preferred and are operated in vertical position. Standard columns are 4-5 mm in diameter and 10-30mm in length and provide good compromise between efficacy , sample capacity and the amount of packing and solvent required. Different types of columns used are:

1. Radial compression columns:

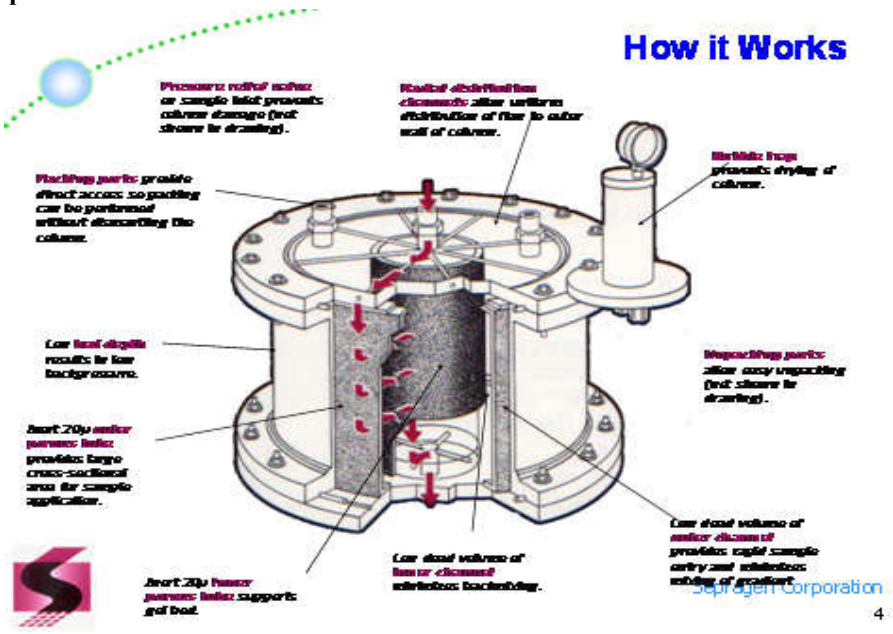

Figure 8 : Radial compression column

Although, the response is decreased when the column diameter is increased , the wider –diameter radial compression columns have some benefits. A decrease in operating pressure allows to decrease analysis time by increasing solvent flow. The radial compression module applies hydraulic pressure to compress a flexible wall cartridge radially held within a plastic holder. Separation is performed when the cartridge is under compression. Compression diminishes the voids and channels between packing particles and cartridge walls and increase the column efficiency. After separation, the cartridge can be decompressed, removed from the module and can be reused without losing efficiency. The consumption of costly solvents for column purging can be reused without losing efficiency. The consumption of costly solvents for column purging can be eliminated by these columns.

2. Narrow-Bore columns:

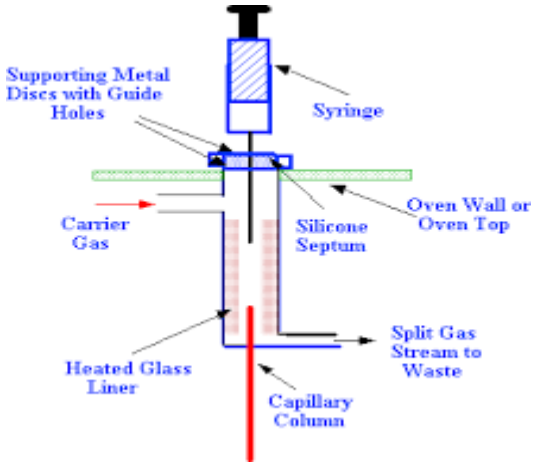

Figure 9: Narrow- Bore column

By decreasing the internal diameter of the column, the signal of sample component is increased without change in column velocity of mobile phase and time taken for analysis. In narrow –bore columns, there is better homogenecity in packing density of the column and small temperature gradient across the column as the frictional heat produced is dissipated better.

3. Short,Fast columns:

A short column packed with 3µm particles can solvent costs, increase sample output and deliver higher sensitivity rates than conventional length columns. These columns are used when the analysis is to be done in a short time as in quality control work.

4. Guard columns and In-line filters:

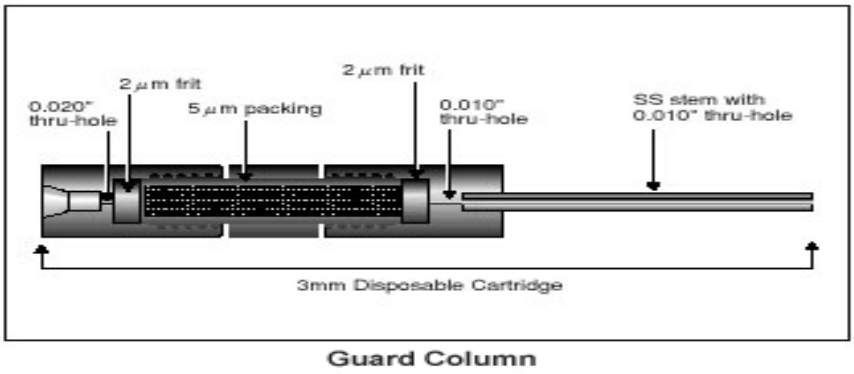

Figure 10: Guard column

The life of analytical columns can be insertion of guard columns a head of analytical column. These act as both chemical and physical filters. These columns are relatively short and contain stationary phase similar to analytical column. They protect the analytical column from particular contamination that comes from the mobile phase or from degrading sample injection valves. These extend the lifetime of expensive columns by preventing the contamination of upper layers of analytical column. These can be repacked , replaced or reconditioned.

Column packing :

Three forms of packing material are generally used for HPLC basing on their rigid solid structure. They are:

1. Microporous supports where microporous ramify through the particles of about 5-10µm in diameter.

2. Pellicular supports where porous particles are coated onto an inert solid core such as glass bead.
3. Bonded phases where the stationary phase is chemically bonded onto an inert support.

DETECTORS:

The separated components in the elute form can be detected by means of their bulk property as solute property.

a) Bulk Property Detectors:

These detectors compare an overall change in physical property of mobile phase with and without an eluting solute. The physical property used is refractive index. These detectors are relatively insensitive and require good temperature control.

b) Solute Property Detectors:

These detectors response to a physical property of the solute that is not exhibited by the mobile phase. Properties such as absorbance, fluorescence and electrochemical property are used. These detectors are highly sensitive even in nanograms of sample or less. Precolumn and postcolumn derivatisation expand their applicability.

UV-Detectors:

These detectors operate in the ultraviolet region spectrum. Commercial spectrophotometers serve as excellent detectors and only modification required is the installation of small volume flow cell in the cell compartment. Detector cell volumes on the order of 8µl per centimeter of optical path length are acceptable. A quartz collimating lens focusses the radiation on the sample and reference cells.

UV-detectors of various types are:

a. Fixed-Wavelength Detector:

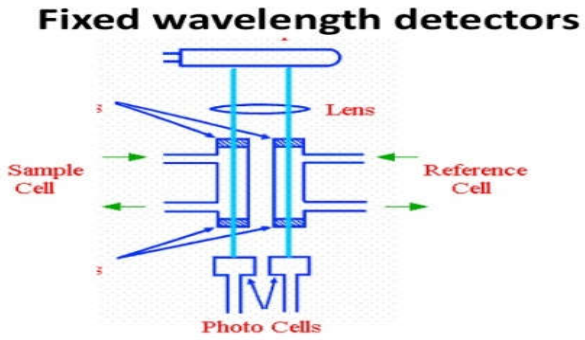

Figure 11: Fixed wavelength detector

A medium pressure mercury lamp which produces maximum light intensity at one or discrete wavelengths produced is used and the wavelength of 254nm is used by using filter.

b. Variable –Wavelength Detector:

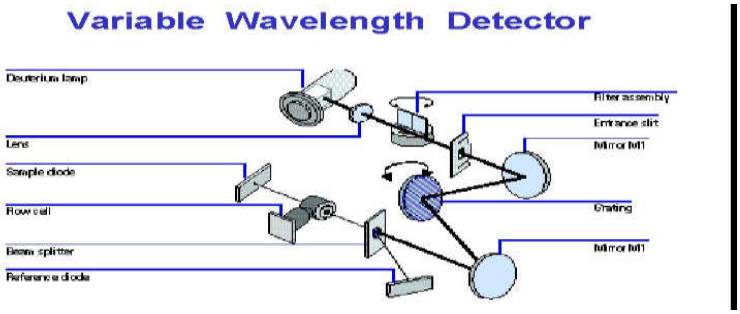

Figure 12: Variable – Wavelength detector

It is a relatively wide-bandpass. UV-Visible spectrophotometer coupled to chromatographic system. It offers selection between UV and visible wavelengths. To obtain a complete spectrum, the

flow of eluant is stopped to trap the component of interest in the detector cell and the UV-Visible spectral region is scanned.

c. Scanning-Wavelength Detector:

Diode array detectors are used to obtain a real-time spectrum. The diode array works in parallel, simultaneously monitoring all wavelengths.

d. Simultaneous Monitoring:

Instead of scanning the complete spectrum, some instruments can be configured to monitor signals at wavelength intervals permitting multicomponent detection in complex samples.

Flourimetric detectors:

For substances that are fluoroscent,these detectors are more sensitive than photometric detectors. The fluoroscence is collected at the right angles to the excitation beam. A concave mirror, is placed around the flow cell to collect fluoroscence . By using micro flow cells, scattered radiation from excitation source is selectively removed with cutoff or band pass filters, components are detected by photomultiplier tube.

Refractive index detector:

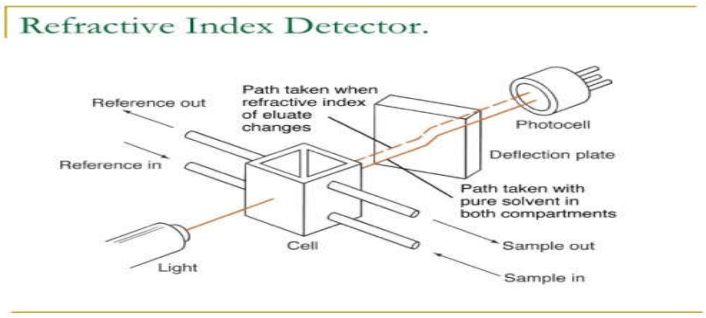

Figure 13: Refractive index detector.

A differential refractometer which responds to the change in bulk property of the refractive index between the mobile phase and the common eluent used in these detectors.These detectors operate on one of the two principles:

a) In deflection type, the deflection of a beam of monochromatic light by a double prism is measured. Eluent is passed through one half and the mobile phase passes through the other half. An optical mask confines a beam of light from an incandescent tungsten lamp to the face of reference and standard compartments. The beam is collimated by a lens is passed through the compartments and is reflected back by the mirror through the compartment again.The beam is foccussed on a beam splitter before passing into twin photodetectors. The reference and the sample compartments are separated by a diagonal glass divider.

b) In reflection type refractometer, the change in percentage of reflected light at a glass liquid interface is measured as the refractive index of the liquid changes. In the optical path, two collimated beams from the projector illuminate the reference and sample cells. The cells are made with a Teflon gasket, which is clamped between the cell prism and a stainless steel reflecting back plate. As the light beam is transmitted through the cell interfaces, is passes through the flowing liquid and impinges on the surface of reflecting back plate. This diffuse, reflected light appears as two spots of light that are imaged by lenses onto dual photodetectors. As the ratio of reflected light to transmitted light is function of refractive index of two liquids, the illumination of the cell back plate is direct measure of the refractive index of the liquid in each chamber.This has a limited range.

The various applications of HPLC are:

1. It can be used both for qualitative and quantitative analysis of components in a sample.
2. It can be used for checking purity of components and presence of impurities.

3. It can be used for isolation and identification of mixture of components of both natural and synthetic origin.
4. It can be used in Biopharmaceutical, Pharmacokinetic and stability studies.
5. It can be used for analysis of drugs an metabolites present in body fluids.
6. It can be used to separate closely related compounds efficiently. [9]

Validation

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications.[10]

Reasons For Validation

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality-control system. The second is that current good manufacturing practice regulation requires assay validation.

Steps followed for validation procedures:

1. Proposed protocols or parameters for validations are established.
2. Experimental studies are conducted.
3. Analytical results are evaluated.
4. Statistical evaluation is carried out.
5. Report is prepared documenting all the results.[11]

Objective and Parameters of Analytical Method Validation:

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH guidelines, typical analytical performance and characteristics that should be considered in the validation of the types of methods:

1. Accuracy
2. Precision
3. Specificity
4. Detection Limit
5. Quantification Limit
6. Linearity
7. Range
8. Ruggedness
9. Robustness

Accuracy:

The accuracy is the closeness of the measured value to the true value for the sample. The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e, three concentrations and three replicates of each concentration). Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from regression equation obtained in linearity study. Accuracy was determined from the mean relative error for a set of replicate analysis (i.e. the difference between measured and nominal concentration) for spiked samples. The % Recovery for each level should be between 98.0 to 102.0%.

Precision :

The precision of an analytical procedure expresses the closeness of agreement between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variations of a series of measurements. The ICH documents recommend the repeatability should be assessed using a minimum of nine determinations covering specified range of procedure. Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. The % RSD for precision should not be more than 2%.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra –assay precision.

Intermediate Precision:

Intermediate precision expresses with in laboratories variations: different days, different analyst and different equipment.

Reproducibility:
When the procedure is carried out by different analysts, different laboratories, using different equipment, reagents and laboratory settings, reproducibility is determined by measuring repeatability and intermediate precision. Reproducibility is assessed by means of an inter-laboratory trial.

Specificity
An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities. An ICH document defines specificity as the ability to access unequivocally the analyte in the presence compounds that may be expected to products and matrix components. The definition has the following implications:

Identification test*:*
Suitable identification tests should be able to discriminate compounds of closely related structure which are likely to be present .Ensure identity of an analyte .The analyte should have no interference from other extraneous components and be well resolved from them.

Purity Test:
To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e related substances test, heavy metals, residual solvents etc.

Assay:
To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample.

Detection Limit
It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample. S/N Ratio value should be 3 for LOD solution.

The LOD may be expressed as:
LOD = 3.3 σ / S

Where, σ = Standard deviation of Intercepts of calibration curves
S = Mean of slopes of the calibration curves. The slope S may be estimated from the calibration curve of the analyte.

Determination of Detection Limit
For instrumental and non- instrumental methods detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Quantification Limit (QL)
It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Quantification limit is expressed as the concentration of analyte (e.g- % ppm) in the sample. S/N Ratio value should be 10 for LOQ solution.

The LOQ may be expressed as:
LOQ = 10 σ / S

Where, σ = Standard deviation of Intercepts of calibration curves
S = Mean of slopes of the calibration curves
The slope S may be estimated from the calibration curve of the analyte.

Determination of quantification limit
For instrumental and non- instrumental methods, the quantification limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

Based on Standard Deviation of the blank
Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these response.

Based on the calibration curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residuals SD of regression line or the S.D of intercepts of regression lines may be used as the S.D. The quantitative limit is a parameter of quantitative assay for low levels of compounds in sample matrices, and is use particularly for the determination of impurities or degradation products.

Linearity and Range

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to concentration of analyte in samples. The range of an analytical is the intervals between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated which it has been demonstrated that the analytical procedure has a suitable level of precision accuracy and linearity. Correlation coefficient should be not less than 0.999.

Determination of linearity and range

These characteristics are determined by application of the procedure to a series of samples having analyte concentration spanning the claimed range of procedure. When the relationship between response and concentration is not linear, standardization may be providing by means of a calibration curve. The ICH recommends that for the establishment of linearity a minimum of five concentrations normally used.

Ruggedness

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst.

Degree of representative of test results is then determined as a function of the assay variable. The % RSD for Ruggedness should not be more than 2%. By analysis of aliquots from homogenous lots in different laboratories, by different analyst, using operational and environmental conditions that may differ but are still within the specified parameter of the assay variable.

Robustness

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Testing varying some or all condition:
-Column temperature
-PH of buffer in mobile phase
-Reagents and flow rate.

System Suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole.

SYSTEM SUITABILITY PARAMETERS:

Table 1: System Suitability Parameters and Recommendations**:**

Capacity Factors(k')	The peak should be well-resolved from other peaks and the void volume, generally k''<2.0
Repeatability	RSD </= 2% for N >/= 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution(Rs)	Rs of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.
Tailing Factor(T)	T of </= 2
Theoretical plates(N)	In general should be > 2000

System suitability tests are most often applied to analytical instrumentation. They are designed to evaluate the components of the analytical system in order to show that the performance of the system meets the standard required by the method. After the method has been validated an overall system suitability tests should be routinely run to determine, if the operating system is performing properly. System suitability tests are integral part of the gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis .

The following information furnishes the parameters used to calculate the system performance values for the separation of two chromatographic components.

Relative rétention (selectivity):
α = (t2-ta) / (t1-ta)
Where, α = Relative retention.
t1 = Retention time of the first 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.

Theoretical plates:
n = 16 (t / w) 2
Where, n = Theoretical plates.
t = Retention time of the component.
w = Width of the base of the component peak using tangent method.

Capacity factor:
K1 = (t2 / ta) – 1
Where K1 = Capacity factor.

Resolution:
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.

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.

Plates per meter:
N = n/ L
Where, N = Plates per meter.
L = Column length, in meters.
HETP = L / n

Linear fit:
A linear calibration fit determines the best line (linear regression) for a series of calibration points. A minimum of two calibration points is required to determine a linear fit. The equation for calibrating the uncorrected amount is:
[Y = a X + b]
Where, Y = Component area or height.
a = Slop of the calibration line.
X = Uncorrected [12]

CONCLUSION

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. The HPLC methods are the preferred methods of analysis due to their responsiveness. This review guides analysts to validate chromatographic methods in order to comply with regulatory requirements.

ACKNOWLEDGEMENT

The author would like to express deep sense of gratitude and heartfelt thanks to **Dr C. Sreedhar** Head of Dept, Dept of Pharmaceutical Analysis, Karnataka College of Pharmacy, Bengaluru for inestimable encouragement and support with full motivation throughout the review work and without whom this work would have not reached the present status.

REFERENCE

1. Beckett AH, Stenlake JB. Practical Pharmaceutical Chemistry. 4th ed. New Delhi: CBS: Publishers and Distributors; 1997; p. 2.
2. Available on https://en.wikipedia.org/wiki/Analytical_chemistry.
3. Panchumarthy Ravisankar , Ch. Naga Navya, D. Pravallika, D. Navya. A Review on Step-by-Step Analytical Method Validation. Journal Of Pharmacy,2015 Oct ;5(10): 7-9
4. Michael E , Schartz Ira S , Krull. Analytical Method Development and Validation; p. 25-46.
5. Gurdeep R. Chatwal , Sham K Anand. Instrumental Methods of Chemical Analysis. 5th ed. Mumbai: Himalaya Publishing House PVT. LTD: p. 2.55.
6. Prepared by Experienced Academicians. A textbook of Pharmaceutical Analysis. 4th ed. Mehdipatnam: Professional Publications; p. 5.2- 5.3.
7. Gurdeep R. Chatwal , Sham K Anand. Instrumental Methods of Chemical Analysis. 5th ed. Mumbai: Himalaya Publishing House PVT. LTD: p. 2.61-2.62.
8. Hobart H. Willard , Lynne L. Merritt, John A. Dean , Frank A. Settle. Instrumental Methods of Analysis. 7th ed. New Delhi: CBS Publishers & Distributors Pvt. Ltd. ; p. 592-3.
9. Prepared by Experienced Academicians. A textbook of Pharmaceutical Analysis. 4th ed. Mehdipatnam: Professional Publications; p. 5.42-.48.
10. David Harvey. Modern Analytical Chemistry. 1st ed. United States of America: Mc GrawHill Companies, Inc.; 2000; p. 578.
11. Frank Settle. Hand book of Instrumental Techniques for Analytical Chemistry. United States of America: Prentice-Hall, Inc.; 1997; p. 149.
12. Joel K Swadesh. HPLC Practical and Industrial Applications. 2nd ed. United States of America: CRC Press LLC; 2001; p. 2-15.

CONFLICT OF INTEREST REPORTED: NIL ; SOURCE OF FUNDING: NONE REPORTED