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HPLC: A SIMPLE AND ADVANCE METHODS OF SEPARATION AND VALIDATION

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ABSTRACT

High performances liquid chromatography (HPLC) is an essential analytical tool in assessing drug in pharmaceutical product. The present study focuses on the various steps, parameter involved in HPLC condition. HPLC process development is important in case drug discovery, drug development and pharmaceutical product. Now a day reverse phase chromatography is most commonly used separation techniques in HPLC. Reversed phase chromatography has found both analytical and preparative application in the area of biochemical separation and purification. The article designing HPLC method development and validation.

KEYWORD: High performance liquid chromatography, formulation and Development, validation, chromatography, HPLC etc.

INTRODUCTION

Introduction Analytical chemistry may be defined as the science and the art of determining the composition of materials in terms of the elements of composition contained. In this it is of prime importance to gain the qualitative and quantitative information of the substance and chemical species. i.e., it find out what a substance is composed of and exactly how much.

Analytical chemistry is divided into two branches such as-

A) Qualitative analysis

B) Quantitative analysis

A **qualitative analysis** gives us the information about the nature of sample by knowing about the presence or absence of certain components.

A **quantitative analysis** provides numerical information as to the relative amount of one or more of this component.

HPLC is one among must useful tools available for quantitative analysis. Reverse Phase Chromatography refers to the use of a polar mobile phase with non-polar stationary phase in contract to normal phase being employed with a non-polar mobile phase HPLC is always used in injection with another analytical tool for quantitative and qualitative analysis.

CHROMATOGRAPHY

Chromatography according to USP can be defined as a procedure by which solute are separated by a differential migration process in a system consisting of two or more phases, one of which move continuously in a given direction.

Chromatography is laboratory techniques for the separation and isolation of a mixture.one phase is mobile phase (liquid or gas) and another is stationary phase (solid).

The mixture is dissolved in a solvent is called mobile phase, which carries it through a structure holding another material called stationary phase.

The various constituent of the mixture travel at different speeds, causing them to separate.

PRINCIPAL OF CHROMATOGRAPHY

The chromatographic separation involved in HPLC is the result of interaction of sample with both stationary phase and mobile phase.

The principal involved in HPLC can be either adsorption and partition chromatography.

Adsorption chromatography

When the stationary phase is a solid and mobile phase is liquid or gaseous phase, it is called Adsorption Chromatography.

Examples: Thin layer chromatography, Column Chromatography, Gas-solid chromatography.

Partition Chromatography

When the stationary phase and mobile phase are liquid, it is called Partition Chromatography.
Example: Paper partition chromatography, Gas-liquid chromatography.

THEORY OF CHROMATOGRAPHY

The theory behind the liquid chromatography can be explained under two categories

- Plate theory
- Rate theory

Plate theory

According to Martin & Synge a chromatography system basis for the plate theory is the 'theoretical plates'. The separation efficiency of the column can be expressed in terms of number of theoretical plates. a theoretical plate represents single equilibrium step occur during separation process. It is considered as a discrete continuous horizontal layer in a column, in which the equilibrium of solute take place between mobile phase and stationary phase. the greater the number of theoretical plate /equilibrium step, the greater the resolving power of the column.'

The number of theoretical plates is denoted by 'N'. The plate height 'H' is expressed by the length of column 'L' divided by 'N'.

$H=L/N$

Hence to get more number of theoretical plates (N) the height of (H) of the plate should be as short as possible. 'H' is the new term which replaced the old term'HETP' (Height equivalent to the theoretical plates).

Rate theory

This theory considers the dynamics of the solute particles as it passes through the void space between the stationary phase particles in the system as well its kinetic as it is transferred to and from the stationary phase. The processes at work inside a column takes account of the time taken for the solute to equilibrate between the stationary phase and mobile phase. The resulting band shape of a chromatographic peak is therefore affected by the rate of elution.it is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanism which contribute to band broadening, we arrive at the Van Demeter equation for plate height;

$HETP= A+B/u +Cu.$

Where,

u is the average velocity of the mobile phase.

A, B, C are factors which contribute to band broadening .the rate theory based on three terms: path –dependent diffusion.

A-Eddy diffusion

The mobile phase moves through the column which is packed with stationary phase. When the packing in a chromatography column is not uniform, so that two identical analyst migrates differently because one had further to travel than the other. This will cause broadening of the solute band, because different paths are of different lengths.

B-Longitudinal diffusion

The term 'B' in Van Demeter equation represents the longitudinal or molecular diffusion. This types of diffusion occur due the concentration gradients within .the columns. The molecules tend to migrate from concentrated zone to dilute zone. Longitudinal diffusion I the results of material moving from an area of high concentration (the centre of a band on a chromatography column) to an area of low concentration. (The outside edges of the same band) this result band broadening. This is more in case of gas used as mobile phase than liquid. Longitudinal diffusion increase with increasing temperature.

C- Mass transfer

The term 'C' represents the mass transfer.in the column chromatography, generally an equilibrium of the solute established between the mobile phase and stationary phase. The analyst takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of the analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

CLASSIFICATION

HPLC can be broadly classified into two major types

1. Normal Phase chromatography
2. Reversed phase chromatography.

Normal Phase chromatography

The stationary phase is polar and the mobile phase is non polar in nature. Generally silica columns of the non-bonded phase are used as a stationary phase. And non-polar solvent such

as hexane, heptane, iso-octane are generally use gas mobile phase in combination with slightly more polar solvent such as isopropanol, ethyl acetate, chloroform. In this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase.

Reversed phase chromatography

The stationary phase is non polar in nature whereas the mobile phase is polar in nature. generally Octyldecaylsilanol columns are most widely used as stationary phase In additional C8,C14 Columns are also used. In reversed phase chromatography generally water is usually used as the base solvent. Other polar solvent such as Methanol, Actonitrilenorbtetrahydrofuran are added in fixed or varying proportion. The polar compound gets eluted first in this mode and non-polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster.

Ion exchange chromatography

Ion exchange chromatography is a process that allow the separation of ion and polar molecules based on their charge. It can be used for almost any kind of charged molecules including large protein, small nucleotide, and amino acid.

The use of a resin (stationary solid phase) is used to covalently attach anions or cationsont it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resins by electrostatic force.

Ion –pair chromatography

This technique is also referred to as Reversed Phase Ion Pair Chromatography or Soap Chromatography. It may be used for the separation of ionic compounds and this method can also substitute for Ion Exchange Chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (columbic association species formed between two ions of opposite electric charge) with suitable counter ions.

Gel permeation chromatography

This type of chromatography lack an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size.

The pores are normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

The elution in HPLC can be classified into two categories

1. Isocratic elution
2. Gradient elution

1. Isocratic elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic elution.

In isocratic elution, peak width increases with retention time linearly with the number of theoretical plates.

For example, if a method consisting of mobile phase as methanol and water in the ratio of 70:30, the same ratio is maintained for the entire chromatographic procedure in isocratic method.

2. Gradient elution

A separation in which this mobile phase composition is changed during the separation process is described as a gradient elution.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower peaks. This also improves the peak shape and peak height.

For example, initially a composition (methanol :water,70:30) for some time period (10min), is maintained then the polarity is modified by changing the ratio to (80:20) for next 5 min and then to 90:10 for another 5 min.

Changing the composition to bring out the desirable separation is called as gradient elution method.

INSTRUMENTATION

The instrument of a HPLC consist of the following components:

- Solvent reservoirs
- Pumping systems
- Sample injector
- Column
- Detectors
- Data collection Devices

Solvent reservoirs

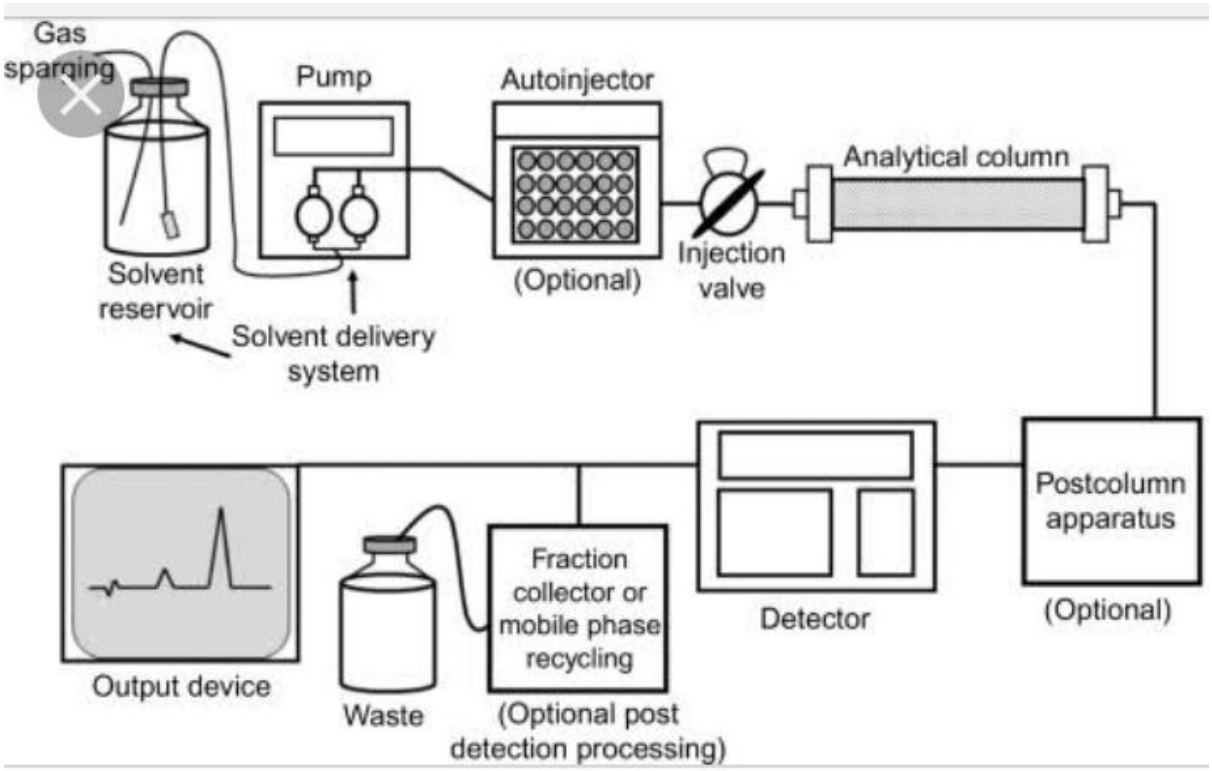
Generally reservoirs made up of glass are used .One or two reservoirs of 500ml capacity or more quantity if required are used. The mobile phase, solvent in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentration are varied depending on the composition of the sample.

Pumping systems

The mobile phase is forced from reservoir to the column using pumps. High pressure is required to push the mobile phase with uniform flow rate.

Based on the mechanism of working the pumps can be classified into-

- Syringe pump /Displacement pumps
- Reciprocating piston pumps
- Constant pressure pumps



Sample injector

The injector can be a single injection or an automated injection system. an injector for an HPLC system should provide injection of the liquid sample within the range of 0.1 -100 ml of volume with high reproducibility and under high pressure (up to 4000psi0 Nowadays the methods are replaced by the incorporation of valves for injection. there are two types of valves.

- External loop valve injector
- Internal loop valve injector

Columns

Columns are usually made of polished stainless steel ,are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. they are commonly filled with a stationary phase with particle size of 3-10um.

Types of columns

- Guard columns
- Analytical columns

Guard column

Guard columns is the column placed before the analytical column. The pressure drop across the precolumn is negligible as compared to that in the analytical column. These columns are otherwise called as pre columns. They are to protect the analytical column s from the impurities and other contaminates from the solvents.

Analytical columns

- Analytical columns is the considered as the heart of the HPLC system. The reasons is that is the part where the separation of the mixture take places.

Type of the analytical columns

- Small-bore columns
- 3x3 columns
- Monolithic columns

Detectors

The HPLC detector, located at the end of the column detect the analyst as they elute from the chromatographic column. HPLC detectors can be classified into two main types,

- Bulk property detector
- Solute property Detector

Bulk property Detector

Bulk property detector measure the changes in the property of combined eluting mobile phase and eluting solute. e.g refractive index and conductivity detectors. They are generally universal in application tend to have poor sensitivity and limited range.

Solute property Detector

Detect the changes in physical and chemical property eluting component of the mobile phase. E.g. spectrophotometric detectors, fluorescence, electrochemical detectors.

Various types of HPLC detectors re mentioned.

Refractive index detectors

Refractive index is a bulk property of column eluent. In thus, there are two chamber separated by glass plates. The solvent passes through one half of the cell and the elate then flows through the other chamber.in this detector, detection depends on solute modifying

overall refractive index of the mobile phase. Bulk property chamber have an inherently limited sensitivity .this detector cab be very useful for detection of non-ionic compounds that either absorb in UV region or fluoresce.

Various types of RI detectors are mentioned here.

- Christiansen effect detectors
- Interferometer detector
- Thermal lens detector
- Dielectric constant detectors

Solubility detector

UV detectors are most commonly used for HPLC. as most organic compounds absorb light in the UV region (190-400nm) and visible region(400-750nm), UV-VIS spectrophotometric detectors are most commonly used detectors in HPLC. fixed wavelength, variable wavelength and diode array detectors are available for detection in these region. Sensitivity of the detector depends on a (1%, 1 cm) value of analyte.

- Fixed wavelength UV detector
- Variable wavelength UV detectors
- Multi wavelength UV detectors.

Fluorescence's detectors

Fluorimeric detectors used a HPLC are similar in design to the flurometers and spectroflurometers used for fluorometry. they are sensitive, specific and selective among existing modern HPLC detectors. Fluorescence sensitivity is 10-1000 times higher than UV detectors for strong UV absorbing materials. Mercury excitation source or xenon source and monochromatic to isolate the fluorescence radiation are used.

- Single wavelength excitation fluorescence detectors
- Multi wavelength fluorescence detectors
- Laser induced fluorescence detectors (LIFD)

Transport detectors

A transport detectors consist of a carrier such as metal chain, wire of disc that passes continually through column eluent extracting sample of mobile phase contain solute as a thin film adhering to its surface.

- Moving wire detectors

- Chain detectors

Electrochemical detectors

Electrochemical detector responds to those substances that are oxidizable or reducible. elecrtochemical detector required a three electrodes working electrodes(oxidation or reduction take place), auxiliary electrodes and references electrodes (which compensate any changes in back ground conductivity of mobile phase).this detector can be grouped into two types,

- Dynamic detector
- Equilibrium detector

Electrical conductivity detectors

Electrical conductivity detectors provides universal, reproducible, high sensitivity detection of all charged species such as anions, captions, metals, organic acids, and surfactants. These detectors measure the conductivity of the total mobile phase hence categorized in bulk density detectors. Electrodes are usually made up of platinum, stainless steel or some other noble metal. it is used for determination of alkali and alkaline earth captions.

Liquid light scattering Detectors

These detectors are based on measurement of scattered light. When light is scattered by a polymer or large molecular weight substances present in the column eluent, it is examined by passing through an appropriate sensor cell. There are two types of detectors based on laser principle such as

- Low angle laser light scattering detectors (LALLS)
- Multiple angle laser scattering (malls) Detectors (MALLS)

Aerosol based detectors

The aerosol –based detectors are a family of detectors that have ability to detect an analyse based on differential volatility compared to the mobile phase. ELSD & CNLSD/NQAD are based on light scattering detectors, whereas CAD is based on charged aerosol detection. The aerosol based detectors utilised a three stages process, involving

1. Nebulisation
2. Evaporation
3. Detection

These are generally classified into three types:-

- Evaporating light scattering detectors (ELSD)
- Nano quantity aerosol detectors(NQAD)
- Charged aerosol detectors (CAD)

Chiral detector

Chiral detectors is used for detection of optically active compounds such as amino acids, sugars, terrenes, other compound contain a asymmetric carbon. ORD detectors are based on differences in refractive index and CD detectors differentiate enantiomers by measuring differences between the absorption of right and left –handed circularly polarized light.

- Polari metric detectors
- Circular dichroism detectors.

Pulsed amperometric detectors (PAD)

This techniques was developed to remove the poisons waveform electrode surface during detection. A three steps waveform system called as pulsed amperometric detection is applied to electrodes approximately once ever second. PAD is important methods for separation and detection of Polar aliphatic compounds which have poor detection properties and require derivatization for optical measurement.

Data collection devices

The computer integrates the response of the detectors to each component and places it into a chromatography that is easy to read and interpret.

VALIDATION OF HPLC

The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness

- Ruggedness
- System suitability determination
- Forced degradation studies
- Stability studies

Accuracy

The accuracy of an analytical procedure expresses the closeness of the agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method is the closeness of the measured value to the true value for the sample.

Accuracy criteria for an assay method is that the mean recovery will be 100+2% at each concentration over the range of 80-120% of the target concentration.

Precision

The precision of an analytical procedure express the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation or coefficient of variance of a series of measurements. The first type of precision study is instrument precision or injection repeatability.

Precision criteria for an assay method is that the instrument precision (RSD) will be 1% and the intra assay precision will be 2%.

Linearity

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

Detection limit

The detection limit of an individual analyte procedure is the lowest amount of analyte in a sample which can be detected but not necessarily qualities as an exact value. Detection limit based on the standard derivation of the response and the slope.

Detection limit (or) limit of detection may be expressed as,

DL= [3.3σ/S]

Where,

σ =standard deviation of the response

S =slope of the calibration curve (of the analyte)

Quantitation limit

The quantitation of an analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Quantitation limit based on the standard deviation of the response and the slope. It can be expressed as,

QL = [10σ/S]

σ =standard deviation of the response S =slope of the calibration curve (of the analyte)

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

Range

Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure ash a suitable level of precision, accuracy, and linearity.

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameter such as percent organic content, pH of the mobile phase, buffer concentration, and temperature and injection volume. The criteria for robustness are the RSD should be not more than 2%.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test result obtained, the analysis of conditions such as different laboratories, different analysis using different instrument, on different days. Different source of reagent, elapsed assay, times,

assay temperature conditions. Ruggedness is the measure of reproducibility of test result under the variation in conditions normally expected from analyst to analyst. The criteria of the ruggedness is the RSD should be not more than 2%.

System suitability testing

System suitability testing is an integral part of many analytical procedures the tests are based on the concept that the equipment, electronics, analytical operation and samples to be analysed constitute an integral system that can be evaluated as such. Typically the process involves making five injections of a standard solution and evaluating several chromatographic parameters such as resolution, area % reproducibility, number of theoretical plates and tailing factor.

Forced degradation or stress studies

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substances are exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about the degradation pathways and degradation product that could form during storage.

Stability Studies

During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light 29-36 drug product. Storage condition tolerances are defined as the acceptable variations in temperature and RH of storage facilities for stability studies.

1. General case.

Study	Storage condition	Minimum time period.
Long term	25°C ± 2 °C/60% RH ± 5% RH or 30 °C± 2 °C/65% RH ± 5% RH or 30 °C ± 2 °C/75% RH ± 5% RH	12months or 6months
Intermediate	30°C ± 2 °C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2 °C/75% RH ± 5% RH	6 months

2. Active pharmaceutical ingredients intended for storage in a refrigerator.

Study	Storage condition	Minimum time period
Long term	5°C ± 23°C	12 month or 6 month
Accelerated	25°C ± 2 °C/60% RH ± 5% RH or 30 °C± 2 °C/65% RH ± 5% RH or 30 °C ± 2 °C/75% RH ± 5% RH	6 month

3. Active pharmaceutical ingredients intended for storage in a freezer.

Study	Storage condition	Minimum time period
Long term	-20°C ± 5°C	12 month or 6 month

4. Active pharmaceutical ingredients intended for storage below −20°C APIs intended for storage below −20°C should be treated on a case-by-case basis.

FACTOR AFFECTING COLUMNS EFFICIENCY

Factor	Factor affecting column Efficiency
Column length	*Choose longer columns for enhanced resolution *Choose shorter column for shorter analysis time, lower back pressure and fast equilibration and less solvent consumption
Column internal diameter	*Choose wider diameter column for greater sample loading *Choose narrow column for more sensitive and reduced mobile phase consumption
Particle shape	*Choose spherical particles for lower back pressure, column stability and greater stability *Choose irregular particles when high surface area and high capacity is required
Particle size	*Choose smaller particle (3-4µm) for complex mixture with structure compounds *Choose larger particle (5-10µm) for sample with structurally different compounds *Choose very large particle (15-20µm) for preparative separation
Pore size	*Choose a pore size of 150 or less for sample with molecular weight less than 2000 *Choose a pore size of 300 or less for sample with molecular weight greater than 2000
Surface area	*Choose end capped packing to eliminate unpredictable secondary interaction with the base materials *Choose non-end capped phase for selected differences for polar compounds by controlling secondary interaction
Carbon load	*Choose high carbon loads for greater column capacities and resolution *Choose low carbon loads for fast analysis

Table No:- factor affecting in columns efficiency.

APPLICATION OF HPLC

The information that can be obtained by HPLC include resolution, identification, and quantification of a compound. It also aids in chemical separation and purification .the other application of HPLC include:

- **Pharmaceutical application**

1. To control drug stability.
2. Tablet dissolution study of pharmaceutical dosage form.
3. Pharmaceutical Quality control.

- **Environmental Application**

1. Detection of phenolic compounds in drinking water.
2. Bio-monitoring of pollutants

- **Application in Forensics**

1. Quantification of drugs in biological samples
2. Identification of steroids in blood, urine etc.
3. Forensic analysis of textile dyes.

- **Food and flavour**

1. Measurement of quality of soft drinks and water.
2. Sugar analysis in fruit juices.
3. Preservative analysis.

- **Application in Clinical Tests**

1. Urine analysis, antibiotics analysis in blood
2. Analysis of bilirubin, biliverdin in hepatic disorders.
3. Detection of endogenous Neuropeptides in extracellular fluid of brain etc.

CONCLUSION

This review emphasizes the general techniques of HPLC method development and validation. The method development and validation of HPLC was found accurate, precise and reliable as per ICH guideline for estimation of purity and active constituent in pharmaceutical compound. The advantages of HPLC method were high selectivity, sensitivity, economic, less time consuming and low limit of detection so method found to be very effective.

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