IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

STUDY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Vrushali M. Bhirde[1*],Vipul M. Patil [2], Sachinkumar V. Patil [3]

Ashokrao Mane College Of Pharmacy,Peth-Vadgaon, Shivaji University, Kolhapur.(416112)

Affiliated to Shivaji University, Kolhapur, Maharashtra.

❖ ABSTRACT:

Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the greek words namely chroma (colour) and graphein (to write). The chromatography is very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly High Performance Liquid Chromatography (HPLC) HPLC is the dominant separation technique to detect, separate and quantify the drug. A number of chromatographic parameters were analyzed to optimize the method like sample pre treatment, choosing mobile phase, column, detector selection. The objective of this article is to review the method development, optimization and validation. HPLC method development depends on chemical structure of the molecules, synthetic route, solubility, polarity, pH and pKa values, and functional groups activity etc. Validation of HPLC method as per ICH Guidelines gives information regarding various stages and knowing characteristics like Accuracy, specificity, linearity limit of detection, limit of quantification.

Keywords: Chromatography, High Performance Liquid Chromatography, Instrumentation, Elution, Applications, Mobile Phase.

❖ INRTRODUCTION

High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures upto 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities.(1-7)

HPLC is recognized from traditional ("low weight") liquid chromatography because operational pressures are fundamentally higher (50 bar to 350 bar), while normal liquid chromatography regularly depends on the power of gravity to pass the portable stage through the segment. Because of the small sample amount isolated in scientific

HPLC, column section measurements are 2.1 mm to 4.6 mm distance across, and 30 mm to 250 mm length. Additionally, HPLC segments are made with smaller sorbent particles (2 µm to 50 µm in normal molecule size). This gives HPLC high determining or resolving power (the capacity to recognize components) while isolating mixtures, which makes it a prominent chromatographic method.(8-17)

❖ HISTORY

Preceding HPLC researchers utilized standard liquid chromatographic methods. Liquid chromatographic systems were to an inefficient because of the flow rate of solvents being reliant on gravity. Separations took numerous hours, and some of the time days to finish. Gas chromatography (GC) at the time was more effective than liquid chromatography (LC), in any case, it was trusted that gas stage partition and investigation of extremely polar high atomic weight biopolymers was impossible. GC was ineffectual for some organic chemists due to the thermal instability of the solutes. Accordingly, alternative techniques were hypothesized which would soon bring about the advancement of HPLC. Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and others in the 1960s that LC could be worked in the high-proficiency mode by decreasing the pressing molecule measurement generously beneath the run of the mill LC (and GC) level of 150 µm and utilizing pressure to expand the versatile stage velocity. These expectations experienced broad experimentation and refinement all through the 60s into the 70s. Early developmental exploration started to enhance LC particles, and the innovation of Zipax, an externally permeable molecule, was promising for HPLC technology. The 1970s achieved numerous advancements in equipment and instrumentation. Specialists started utilizing pumps and injectors to make a simple configuration of a HPLC system. Gas amplifier pumps were perfect since they worked at consistent pressure and did not require release free seals or check valves for steady flow and great quantitation.

❖ PRINCIPLE

Chromatography involves the separation of the components of a mixture by virtue of differences in the equilibrium distribution (K) of the components between two phases: the mobile phase and the stationary phase. The principle of HPLC separation is the affinity between non polar stationary phase and polar mobile phase. When a mixture of compound is introduced into the HPLC column, they travel according to their relative affinities towards the stationary phase. The compound which has less affinity towards the stationary phase travels faster. The compound which has more affinity towards the absorbent travels slower and by this method, very smaller particles are used for column preparation which gives a much greater surface area for interaction between stationary phase and molecules flowing past it. This allows a much greater separation of components of mixture.

Figure 1: HPLC- Agilent Instrument

❖ TYPES OF HPLC(27-28)

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis

- **Normal phase chromatography:**

Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

- **Reversed phase chromatography:**

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

- **Size exclusion chromatography:**

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.

- **Ion exchange chromatography:(27-28)**

In Ionexchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligandexchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.

- **Bio-affinity chromatography:**

Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retains proteins with interaction to the column-bound ligands. Proteins bound to a bioaffinity column can be eluted in two ways:

- Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand.
- A specific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (10 - 1000-fold).

- **INSTRUMENTATION**

The basic HPLC system is consists of following parts

1)Solvent are mobile phase resiovoir

2)High pressure pump

3)Injector

4)Column

5)Detector

6)Data recording and interpretation unit

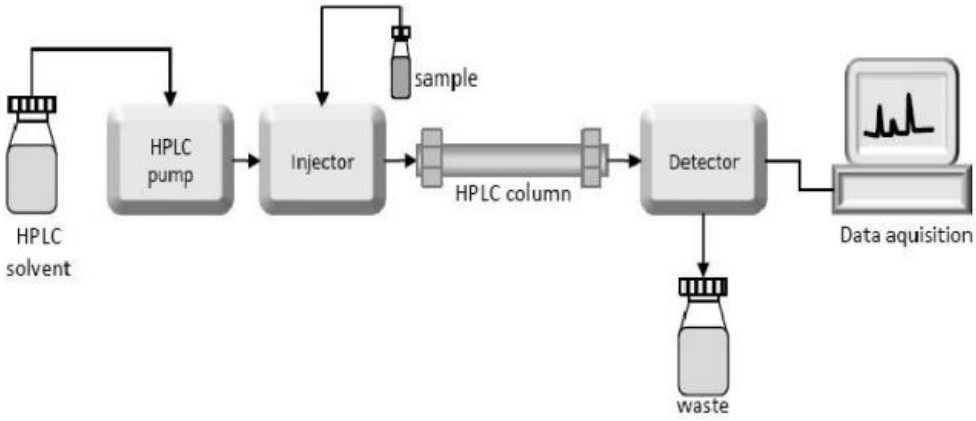

Figure 2 : Parts of HPLC System

Solvent delivery system:

The mobile phase is pumped under pressure at a high pressure at about 1000 to 3000 psi; from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a highpressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity.

Pump:

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. The particle size of the stationary phase is 5-10 µm. So resistance to flow is observed. This is the reason that high pressure is required and this is provided by using pumps. The different types of pumps Include:

- Constant pressure pump which uses a constant pressure to the mobile phase; the flow rate through the column is determined by the flow resistance of the column and any other restrictions between the pump and the detector outlet.
- Constant flow pump generates a given flow of liquid, so that the pressure developed depends on the flow resistance

➢ Constant pressure pumps:

The simplest type of constant pressure pump is the pressurized coil pump or gas displacement pump. Consisting of some form of pneumatic device for the direct pressurization of the mobile phase with an inert gas, give a reliable pulse-free flow and have the advantage of low cost and simplicity. They are however not as accurate as constant volume pumps but can be used where flow accuracy and reproducibility are less critical. But this is now only of historical interest.

➢ Constant flow/volume (Constant Displacement) Pumps:

If a constant flow pump is used, changes in the permeability of the system, caused by settling or swelling of the packing, or viscosity changes in the mobile phase (due to temperature fluctuations or composition changes) are compensated for by pressure changes and the flow rate remains constant. Since flow changes cause nonreproducible retention times, adversely affect resolution, and give unstable base-lines, the constant volume pump provides a more precise analysis.

Solvent degassing system:

The constituents of the mobile phase should be degassed and filtered before use because several gases are soluble in organic solvents. When solvents are pumped at high pressure, gas bubbles are formed which will interfere with the separation process. Numerous methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging, ultra-sonication or purging or combination of these methods

Sample injector:

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. Several devices are available either for manual or auto injection.

a. Septum injectors- for injecting the sample through a rubber septum.

b. Stop flow- in which the flow of the 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 type. This has a fixed volume loop like 20-50 µl or more. The injector has two modes, i.e., load position when the sample is loaded in the

loop and the inject mode, when the sample is injected.

Figure 3: Rheodyne Injector

Column:

- Guard column:

Guard coloum has very small quantity of adsorbent and improves the life of the analytical column. It also acts as a prefilter to remove particulate matter, if any, and other material. Guard column has the same material as that of analytical column. It does not contribute to any separation but is necessarily used before the analytical column to protect & increase lifetime of column. Operator usually slurry or dry packs short guard column regularly with same or similar packing used in analytical column (old column material) are used for guard columns.

- Analytical column:

Columns are typically made of polished stainless steel, glass, and polyethylene or poly ether ether ketone (latest). Column length is around 50 mm to 300 mm and has an internal diameter across of somewhere around 2mm to5 mm. They are generally loaded with a stationary phase with a particle size of 3 µm to 10 µm. 1gm of stationary phase provides surface area ranging from 100-860 sq.m. with an average of 400sq.m. In normal phase mode it contains the silanol groups (hydroxyl group). In the reverse phase mode C18 (Octa Decyl Silane), C8, C4, CN, NH2 columns are used.

Figure 4: Coloumns

Detector:

Several ways of detecting are used when a substance has passed through the column. A detector used depends upon the property of the compound to be separated.

- UV detector:

This is the most commonly used type of detector as it can be rather sensitive, has a wide linear range, is relatively unaffected by temperature fluctuations and is also suitable for gradient elution. It records compounds that absorb ultraviolet or visible light. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time. Absorption takes place at a wavelength above 200nm, provided that the molecule has at least:

(a) A double bond adjacent to an atom with a lone electron pair

(b) Bromine, iodine or sulfur;

(c) A carbonyl group or a nitro group

(d) Two conjugated double bonds

(e) An aromatic ring;

The mobile phase we use, on the other hand, should absorb little or no radiation. Absorption of radiation by solutes as a function of concentration, c, is described by the Beer-Lambert law:

A=Ect

Where A = absorbance=path length of the cell and E= molar absorptivity, which is a constant for a given solute and wavelength.

Figure 5: Schimadzu SPD20 UV-Detector

- Refractive Index (RI) detectors

Refractive index (RI) detectors are nonselective and often used to supplement UV models. These detectors sense the difference in refractive index between the column eluent and a reference stream of pure mobile phase. They are the closest ones in HPLC to a universal detector, as any solute can be detected as long as there is a difference in RI between the solute and the mobile phase. They record all eluting zones which have are RI different to that of the pure mobile phase.

- Fluorescence detectors

Compounds that have fluorescence or of with fluorescing derivatives can be obtained are picked up with high sensitivity and specificity by this detector. The sensitivity may be up to 1000 times greater than with UV detection. Light of a suitable wavelength is passed through the cell and the higher wavelength radiation emitted is detected in a right-angled direction. The light intensity and hence the sensitivity are increased by using a relatively large cell (20ml or greater). Simple units have a fixed excitation wavelength for which band width must not be too narrow and a fixed wavelength range for fluorescent light detection.

- Electrochemical (Amperometric) detectors

Electrochemistry provides a useful means of detecting traces of readily oxidizable or reducible organic compounds with great selectivity. The detection limit can be extraordinarily low and the detectors are both simple and inexpensive. The potential between the working and reference electrodes may be selected. The working electrode is made up of glassy carbon, carbon paste or amalgamated gold. Frequently a silver/silver chloride electrode is used as the reference

- Conductivity detectors

This is the classical ion chromatography detector and measures the eluate conductivity, which is proportional to ionic sample concentration (provided that the cell is suitably constructed). Its sensitivity decreases as the specific conductivity of the mobile phase increases. The active cell volume of 2ml is very small. Good conductivity detectors have automatic temperature compensation (conductivity is highly temperature-dependent) and electronic background conductivity suppression.

- Light scattering detectors

The evaporative light-scattering detector (ELSD) is an instrument for the non selective detection of non volatile analytes. The column eluate is nebulized in a stream of inert gas. The liquid droplets are then evaporated, thus producing solid particles which are passed through a laser, LED, or polychromatic light beam. The resulting scattered light is registered by a photodiode or photomultiplier. Volatile buffers can be prepared with formic, acetic and tri fluoro acetic acid; all these compounds must be of high purity. “Gradient grade” is not necessarily pure enough for ELSD detection. The nebulizer gas is usually nitrogen, helium, or compressed air.

- Photo diode array detectors (PDA) detectors

A photodiode array detector is a lined array of discrete photodiodes on an integrated circuit (IC) chip for spectroscopy. It is placed at the image plane of a spectrometer to allow a range of wave length to be sensed concomitantly. PDA can be programmed for any wavelength range and all the compounds that absorb at this range can be identified in a single range. It can also analyze peak purity by matching spectra within a peak. The resulting spectra are 3-D plot of Response Vs Time Vs Wavelength.

Other detectors:

- Photoconductivity Detectors

These are sensitive, selective detectors for organic halogen and nitrogen compounds. The eluate is split up as it leaves the column. One half passes through the reference cell of a conductivity detector and the other half is irradiated with 214 or 254nm UV light whereupon suitable sample molecules become dissociated into ionic fragments. The ensuing high level of conductivity is recorded in the measuring cell.

- Infrared Detectors

Every organic molecule absorbs infrared light at one wavelength or another. When an IR detector is used, the mobile phase chosen must not be self-absorbent at the required wavelength. Hexane, dichloromethane and acetonitrile are suitable mobile phases for ester detection whereas ethyl acetate is not. The sensitivity is no greater than that of refractive index detectors.

- Radioactivity Detectors

These are used especially for detecting the b-emitters H3, C14, P32, S35 and I131. The scintillator required for this relatively weak radiation is either added as a liquid between the column and the detector or is contained as a solid in the cell.

Hyphenated HPLC detectors

LC-MS using thermospray–new popularity (pharmaceuticals)

Evaporative light scattering -polymers

LC-FTIR

LC-plasma emission or ICP-MS

- Recorders and Integrator

Recorders: the signals from the detector after amplification (if necessary) are recorded as a series of peaks, each one representing a compound in the mixture. Baseline and the peaks are recorded with respect to time. Retention time for all the peaks can be found from the recordings. The area under the peak is proportional to the amount of substance passed through detector, and this area can be calculated automatically by the computer linked to the display.

Integrator: improved version of recorders by which signals from the detector are gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

Derivatization:

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not adequate for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been extensively used. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives are 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 out let and the detector. Derivative preparation carried out before separation is called precolumn derivatisation and afterwards is called post column derivatisation. This allows optimization of the separation process.

❖ **Application**

HPLC finds application in the fields of pharmacy,environmental, clinical, and forensic and also in foodindustry. The information that can be obtained by HPLCincludes resolution, identification and quantification of acompound. It also aids in chemical separation, molecularweight determination and purification of mixture ofcompounds.

Chemical Separations is based on the fact that certain compounds have different migration rates for a particular column and mobile phase. Thus separations of individual components are achieved.

Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound shows a characteristic peak under certain chromatographic conditions such that the pure desired compound can be collected or extracted without incurring any other undesired compound.

Molecular weight determination allows determining the molecular weight of chemical substance, pharmaceutical substances, proteins, etc.

Identification usually is the assay of compounds that are carried using HPLC. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels so that pure compounds are identified easily.

Other applications of HPLC includes:

HPLC industry Applications3There is a wide variety of applications throughout the process of creating a new drug from drug discovery to the manufacture of formulated products that will be administered to patients. This Process to create a new drug can be divided into 3 main stages

1. Drug discovery 2. Drug development 3. Drug manufacturing.
   LC-MS is the best tool for compound identification and characterization. It may be used as a measurement tool during high throughput screening. Preparative HPLC is also used to isolate and purify hits and lead compounds as required. Eg: a combinatorial synthesis. The ability to prove purity of enantiomeric molecules is a standard in pharmaceutical assays, for which HPLC is suitable.

➢ **Pharmaceutical applications(33-36)**

Tablet dissolution study of pharmaceutical dosages form.Shelf-life determinations and stability studies. Identification of active pharmaceutical ingredients of dosage forms.

Assay of pharmaceutical formulation and analyzing impurities.

Quality control.

Research and development.

➢ **Environmental applications(37-40)**

Detection of phenolic compounds in drinking water. Identification of diphenhydramine in sedimented samples.

Bio-monitoring of pollutant.

➢ **Forensics(41-43)**

Quantification of the drug in biological samples.

Identification of anabolic steroids in serum, urine, sweat, and hair.

Determination of presence of cocaine and metabolites in blood.

Forensic analysis in textile industry.

- **Clinical(44-47)**

Quantification of ions in the human urine.

Analysis of antibiotics in the blood plasma.

Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders.

Detection of endogenous neuropeptides in extracellular fluids.

- **Food and Flavor(48)**

Ensuring the quality of soft drink and drinking water.

Analysis of alcohol and its derivatives.Sugar analysis in fruit juices.

Analysis of polycyclic compounds in vegetables.

Trace analysis of military high explosives in agricultural crops.

Screening for pesticides and insecticides in fruits.

- **Pharmaceutical impurity profiling analysis**

1. Structure elucidation of impurities with LC/MS.

2. Rapid condition scouting for method development.

3. Using a fast LC method for higher sample throughput.

- **Pharmaceutical drug discovery analysis**

Developing a fast, generic method for rapid resolution Liquid chromatography with quadrupole MS detection. Fast, generic LC/MS method enables drug analysis in less than one minute.

- **Recent applications**

Analytic method development9-17 and validation are key elements of any pharmaceutical development program.HPLC analysis method is developed to identify, quantity or purifying compounds of interest. HPLC helps a lot in stability studies of drug formulations. HPLC helps a lot in stability studies of atropine, antibiotics, & biotechnologybased drugs like insulin, streptokinase, etc.

1. It is used in inorganic chemistry for separating anions & cations.

2. It is used in forensic science for the separation of phenyl alkylamines (morphine and its metabolites) from blood plasma, and for the detection of poisons or intoxicants such as alcohol, carbon monoxide, cholinesterase inhibitors, heavy metals, hypnotics, etc.

3. It is used in environmental studies for analyzing the pesticide content in drinking water

4. It is utilized in food analysis for separating watersoluble and fat-soluble vitamins from variety of food products, fortified food and animal feed.

5. It is also used for determining antioxidants and preservatives present in the food.

6. It is used in the cosmetic industry for the assay and quality control of various cosmetics like lipsticks, creams, ointments, etc.

7. It is used for separating various components of plant products with bear structural resemblanceEg: Analysis of cinchona, digitalis, ergot extracts and licorice.

8. It is used in the agrichemical industry for the separation of herbicides.

❖ **Advantages**

HPLC has many advantages over other methods of chromatography. It has made significant contribution to the growth of analytical science and its diverse application in pharmaceuticals, environmental, forensics, foods, polymers and plastics, clinical fields etc.

HPLC provides a highly specific, reasonably precise, and fairly rapid analytical method for a plethora of complicated samples.

HPLC is capable of tackling macromolecules.

It is profoundly suitable for most 'pharmaceutical drug substances'.

It offers an efficient means of analysis pertaining to 'labile natural products'.

HPLC allows the dependable analysis of a good number of products including biochemical, metabolic products, nonvolatile substance, polar compounds etc.

Preparation and introduction of sample is easy and simple in HPLC.

Resolution of compounds and speed of separation is high.

HPLC software is capable of reporting precise and accurate results.

Sensitivity of detectors used is high.

A large number of stationary phases and columns can be used to suit different ranges of application.

Recording and storage of information is easy.

The columns operated carefully under controlled conditions without overloading can be resed for significant period of time.

HPLC coupled with mass spectrophotometers and FT- IR system have improved efficacy.

Along with hyphenated techniques HPLC have been used to analyse impurities in pharmaceutical formulations.

❖ **Disadvantages**

HPLC is considered one of the most important techniques of the last decade of the 20th century. Despite of the several advantages there are certain limitations also. Limitations include price of columns, solvents and a lack of long term reproducibility due to proprietary nature of column packing. Others include:

Complexity of separation of certain antibodies specific to the protein.

The cost of developing an HPLC apparatus for assay or method of separation of individual components is tremendous.

Due to the speed of the HPLC and its reliance on the different polarities; two compounds with similar structure and polarities can exit the chromatographic apparatus at the same time (co-elution). This is difficult in detecting compounds.

Low sensitivity of some compounds towards the stationary phase in the columns is difficult.

Certain compounds get absorbed or react with the chemicals present in the packing materials of the column.

Sometimes the pressure may get too high or low that the column cannot withstand or separation may not takes place.

Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry.

Resolution is limited with very complex samples.

Newer trends with better efficacy have been established.

❖ CONCLUSION

The literature review of HPLC was done vigilantly and it was found to be one of the most widely used system among the chromatographic techniques. The choice of detection approach is critical to guarantee that all the components are detected accurately. Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. The review focuses on the principle, types , instrumentation, application, advantages and disadvantages of HPLC. The pump delivers the mobile phase from a reservoir and on to a column packing material that typically consists of 3-5 µm silica particles. Sample solutions are injected using a pressure and leak resistant injector onto the mobile phase just before the column. It follows isocratic or gradient elution techniques and the substance eluted from the column are detected using one or more detectors. The UV detectors are the standard detector in pharmaceutical quality and control. Thus, HPLC is used in the determination of drug substances and their metabolites in biological material. It is used for screening of compounds in pharmaceutical preparation and detecting the impurities present. Also used in the identification of active ingredients. Using HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in the same.

REFERENCE

1. Rogatsky E. Modern high performance liquid chromatography and HPLC 2016 International Symposium. J Chromatogr Sep Tech. 2016;7:e135.

2. Mulubwa M, et al. Development and validation of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for determination of tenofovir in small volumes of human plasma. J Chromatogr Sep Tech. 2015;6:300.

3. Santini DA, et al. Development of a high performance liquid chromatography method for the determination of tedizolid in human plasma, human serum, saline and mouse plasma. J Chromatogr Sep Tech. 2015;6:270.

4. Lin G, et al. Determination of sodium tanshinone iia sulfonate in rat plasma by high performance liquid chromatography and its application to pharmacokinetics studies. Pharm Anal Acta. 2015;6:383.

5. AL-Jammal MKH, et al. Development and validation of micro emulsion high performance liquid chromatography(MELC) method for the determination of nifedipine in pharmaceutical preparation. Pharm Anal Acta. 2015;6:347.

6. Myron P, et al. Tributylamine facilitated separations of fucosylated chondroitin sulfate (fucs) by high performance liquid chromatography (HPLC) into its component using 1-phenyl- 3-methyl-5-pyrazolone (pmp) derivatization. J Chromatogr Sep Tech. 2015;6:256.

7. Tang M, et al. HPLC analysis of monomer release from conventionally and high temperature high-pressure polymerised urethane dimethacrylate intended for biomedical applications. J Chromatograph Separat Techniq. 2014;5:227.

8. Akan JC, et al. Determination of organochlorine, organophosphorus and pyrethroid pesticide residues in water and sediment samples by high performance liquid chromatography (HPLC) with UV/visible detector. J Anal Bioanal Tech. 2014;5:226

9. Parbhunath OL, et al. Optimization and validation of a reverse-phase high performance liquid chromatography assay with ultra-violet detection for measuring total l-ascorbic acid in food and beverage products. J Anal Bioanal Tech. 2014;5:201

10. Szterk A, et al. Comparison of various detection systems coupled to high performance liquid chromatography for determination of tocopherols in meat. The influence and comparison of the most popular sample preparation method. J Anal Bioanal Tech. 2013;S2:005.

11. Lories IB, et al. High performance liquid chromatography, TLC densitometry, first-derivative and first-derivative ratio spectrophotometry for de-termination of rivaroxaban and its alkaline degradates in bulk powder and its tablets. J Chromatograph Separat Techniq. 2013;4:202.

12. Chierentin L and Nunes Salgado HR. Development and validation of a simple, rapid and stability-indicating high performance liquid chromatography method for quantification of norfloxacin in a pharmaceutical product. J Chromat Separation Techniq. 2013;4:171.

13. Srinivasarao K, et al. Validated method development for estimation of formoterol fumarate and mometasone furoate in metered dose inhalation form by high performance liquid chromatography. J Anal Bioanal Tech. 2012;3:153.

14. Sun H, et al. A Rapid and effective method for simultaneous determination of residual sulfonamides and sarafloxacin in pork and chicken muscle by high performance liquid chromatography with accelerated solvent extraction–solid phase extraction cleanup. J Chromat Separation Techniq. 2012;3:154.

15. Virkar PS, et al. Development and validation of a high performance liquid chromatography method for determination of telmisartan in rabbit plasma and its application to a pharmacokinetic study. J Anal Bioanal Tech. 2012;3:133.

16. Gugulothu DB, et al. A versatile high performance liquid chromatography method for simultaneous determination of three curcuminoids in pharmaceutical dosage forms. Pharmaceut Anal Acta. 2012;3:156.

17. Devika GS, et al. Simultaneous determination of eprosartan mesylate and hydrochlorthiazide in pharmaceutical dosage form by reverse phase high performance liquid chromatography. Pharm Anal Acta. 2011;2:122.

18. Harmita, et al. Optimation and validation of analytical method of cotrimoxazole in tablet and plasma in vitro by high performance liquid chromatography. J Bioanal Biomed. 2012;4:26-29.

19. Nardulli P, et al. A combined HPLC and LC-MS approach for evaluating drug stability in elastomeric devices: a challenge for the sustainability in pharmacoeconomics. J Pharmacovigilance. 2014;2:157.

20. Hafez HM, et al. Development of a stability-indicating HPLC method for simultaneous determination of amlodipine besylate and atorvastatin calcium in bulk and pharmaceutical dosage form. Pharm Anal Acta. 2014;5:316.

21. Shintani H. Immobilized enzyme column combined with HPLC and column switching method for the analysis of complicated matrix such as body fluids. PharmaceutReg Affairs. 2014;3:e142.

22. Murthy TGK and Geethanjali J. Development of a validated RP-HPLC method for simultaneous estimation of metformin hydrochloride and rosuvastatin calcium in bulk and in-house formulation. J Chromatogr Sep Tech. 2014;5:252.

23. Arayne MS, et al. Monitoring of pregabalin in pharmaceutical formulations and human serum using UV and RPHPLC techniques: application to dissolution test method. Pharm Anal Acta. 2014;5:287.

24. Praveen C, et al. Method development and validation for simultaneous estimation of ethinyl estradiol and drospirenone and forced degradation behavior by HPLC in combined dosage form. Pharmaceut Anal Acta. 2013;4:231.

25. Abdulla SA, et al. Validated HPLC method for the determination of nisoldipine. Pharm Anal Acta. 2013;S1:004.

26. Sawsan Mohammed AH, et al. Effects of blood collection tubes on determination vitamin-A by HPLC. J Chromat Separation Techniq. 2013;4:184.

27. Abidi,S.L. High-performance liquid chromatography of phosphatidic acids and related polar lipids. J. Chromatogr. 1991; 587: 193-203.

28. Hearn M.T.W. Ion-pair chromatography on normal and reversed-phase systems. Adv. Chromatogr. 1980; 18: 59–100.

29. Sree Janardhanan V, Manavalan R, Valliappan K, HPLC Method for The Simultaneous Determination of ProtonPump Inhibitors with Domperidone In Human Plasma Employing Response Surface Methodology, International Journal of Pharmacy and Pharmaceutical Sciences, Vol 4 suppli, 2011, 309-317.

30. Venkatesan P, Sreejanardhanan V, Chandrasekaran M, Valliappan K, Improved HPLC method with the aid of chemometric strategy Determination of Loxoprofen in Pharmaceutical formulation, Acta chim slov, 59, 2012, 242-248.

31. SreeJanardhanan V, Manavalan R, Valliappan K, Chemometric technique for the optimization of chromatographic system: Simultaneous HPLC determination of Rosuvastatin, Telmisartan, Ezetimibe and Atorvastatin used in combined cardiovascular therapy, Arabian Journal of Chemistry, King Saud University, 2011.

32. Revathi M, Indira M Y RP-HPLC Method for estimation of furosemide in Rabbit Plasma; Journal of chemical Pharmaceutical Research, 10(2), 2018, 1-5.

33. Reolon J.B, Brustolin M, Hass S.E, Bender E.A, Malesuik M.D, Colome Development and validation or High Performance Liquid Chromatography Method for Simultaneous determination of acyclovir and curcumin in polymeric micro particles, Journal of Applied Pharmaceutical Vol 8 (01),

2018,136-141.

34. Namrata P, Bhavika C, Mashru R.C, RP-HPLC Method development and validation of simultaneous estimation of Gallic acid and Oleanolic acid in antihyperlipidemicpolyherbal tablets; Journal of Pharmacognosy and Phytochemistry 7(5), 2018, 209-216.

35. Malviya R, Bansal V, Pal O.P. and Sharma P.K High performance liquid chromatography, Journal of Global Pharma Technology, ISSN 2010, 0975 – 8542.

36. Masoom R S, Zaid A. A and Nafisur R Analytical techniques in pharmaceutical analysis, Arabian Journal of Chemistry, Elsevier, 2012, S1409-S1421.

37. Narayudu Y Comparative Study of New Trends in HPLC: A Review, International Journal of Pharmaceutical Sciences Review and Research, 2013, ISSN 0976 – 044X.

38. Snehalakshmi R.P. A Review on Chromatography with High Performance Liquid Chromatography (HPLC) and its Functions, Research and Reviews: Journal of Pharmaceutical Analysis, Volume 4, Issue 1, March, 2015, E-ISSN: 2320-0812.

39. Prathap B, A Review - Importance of RP-HPLC in Analytical Method Development, International Journal of Novel Trends in Pharmaceutical Sciences, Volume 3, 2013, ISSN: 2277 –2782.

40. Santoshkumar B, A Review: HPLC Method Development and Validation, International Journal of Analytical and Bioanalytical Chemistry, Volume 5, Issue 4, 2015, Pg no. 76-81, ISSN-2231-5012.

41. Vibha G, Development and validation of HPLC method - a review, International Research Journal of Pharmaceutical and Applied Sciences,2012, Volume 2, Issue, 4, 2012, Pg no.17-25, ISSN-2277-4149.

42. Mukthi T, A Review on High Performance Liquid Chromatography (HPLC), Research & Reviews: Journal of Pharmaceutical Analysis, Volume 5, Issue 2, 2016, ISSN: 2347-2340.

43. Azim M S, HPLC method development and validation, international Research Journal of Pharmacy, Volume 4, Issue 4, 2012, Pg no.39-46, ISSN 2230-8407.

44. Pallavi N P, HPLC method development- A Review, Journal of Pharmaceutical Research & Education, Volume 1, Issue 2, 2017, Pg no. 243-260, ISSN 2456-4508.

45. Taleuzzaman M, Ultra Performance Liquid Chromatography (UPLC) - A Review, Austin Journal of Analytical and Pharmaceutical Chemistry, Volume 2, Issue 6, 2015, Pg no. 1-5, ISSN: 2381-8913.

46. Subramani P, An Overview of Liquid Chromatography-Mass Spectroscopy Instrumentation, Volume 5, Issue 2, 2014, Pg no. 47-54.

47. Anastasia Z, An overview of recent advances in HPLC instrumentation, Central European Journal of Chemistry,Volume10, Issue3, 2012, Pg no. 554-569.

48. Michael S, HPLC detectors: A brief review, Journal of Liquid Chromatography & Related Technologies, 33, k 2010, 1130–1150, ISSN: 1082-6076.

49. Thammana M. A review on high-performance liquid chromatography. Research & Reviews: Journal of Pharmaceutical Analysis, 5(2), 2016, 22.

50. Malviya. R., et al., High-performance liquid chromatography: A short review, Journal of Global Pharma Technology, 2, 2010, 22 – 26.

51. Bailey F, Applications of high-performance liquid chromatography in the pharmaceutical industry, Journal of Chromatography A, 122, 1976, 73-84.

52. Heewon Lee, Pharmaceutical applications of liquid chromatography coupled with mass spectrometry (LC/MS), Journal of liquid chromatography & related technologies, 28, 2005, 7-8.

53. Ma Bokai, Gou XinLei, Zhao XinYing, Application of highperformance liquid chromatography in food and drug safety analysis, Journal of Food Safety and Quality, 7, 2016, 4295-4298.

54. Salgado P, Visnevschi-Necrasov T, Kiene R.P, Azevedo I, Rocha A.C.S, Almeida C.M.R, Magalhaes C, Determination of 3-mercaptopropionic acid by HPLC: A sensitive method for environmental applications, Journal of Chromatography B, 992, 2015, 103-108.

55. Ian M Bird, High-performance liquid chromatography: principles and clinical applications, BrMedJ, 299, 1989, 783-

56. Ante M.Krstulovic, Investigation of catecholamine metabolism using high-performance liquid chromatography: Analytical methodology and clinical applications, Journal of chromatography B: Biomedical Sciences and Applications, 229, 1982, 1-34.

57. Ravisankar P, Gowthami S, and Devala Rao G, A review on analytical method development, Indian journal of research in pharmacy and biotechnology, 2 (3), 2014, 1183-1195.

58. Shivani Sharma, Swapnil Goyal, Kalindi Chauhan, A review on analytical method development and validation, International Journal of Applied Pharmaceutics, 10(6), 2018, 8-15.

59. Ravisankar Panchumarthy, Naga Navya Ch, Pravallika D, Navya Sri D, A review on step-by-step analytical method validation, IOSR Journal of Pharmacy, 5 (10), 2015, 07-19.

60. Siro Passi, Paolo Casella, Marcella Nazzaro - Porro, et al, An application of high-Performance liquid chromatography to analysis of lipids in archaeological samples, Journal of lipid research, 22(5), 1981, 778 - 784.

61. Mohammed Ashraf Ali et al, Application of HighPerformance Liquid Chromatography for hydrocarbon group type analysis of Crude Oils, Fuel Science and Technology International, 12, 1994, 21 -23.

62. Ravisankar P, Swathi V, Srinivasa Babu P, Shaheem Sulthana Md, Gousepeer SK: Current trends in performance of forced degradation studies and stability indicating studies of drugs. IOSR Journal of Pharmacy and Biological Sciences 12(6), 2017, 17-36.

63. Cavazzini A, Pasti L, Massi A, Marchetti N, Dondi F., Recent applications in chiral high-performance liquid chromatography, Anal Chim Acta, 706(2), 2011, 205-22.

64. Panchumarthy Ravisankar, S. Anusha, Kommanaboina Supriya, U. Ajith Kumar, Fundamental Chromatographic Parameters, Int. J. Pharm. Sci. Rev. Res, 55(2), 2019, 46 - 50.

65. Ravisankar P, Sai Geethika A, Rachana G, Srinivasa Babu P, Bhargavi J, Bioanalytical method validation: A comprehensive review, Int.J.Pharm.Sci.Rev.Res., 56(1), 2019, 50-58.