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A Comprehensive Review on High-Performance Liquid Chromatography (HPLC)

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ABSTRACT

Chromatography is described as a group of methods used to separate components in a mixture. There are two phases to this technique: stationary and movable phases. The difference in the partition coefficients of the two phases serves as the basis for the separation of constituents. The word "chromatography" comes from the Greek words "chroma" (colour) and "graphein" (to write). For measuring pharmaceutical and environmental sample quality and quantity, high performance liquid chromatography (HPLC) is a crucial technique. It is the most adaptable, secure, dependable, and quick chromatographic approach for determining the quality of medicinal ingredients. The analytical chemistry method of high-performance liquid chromatography (HPLC; formerly known as high-pressure liquid chromatography) is used to separate, recognise, and quantify each component in a mixture. One type of liquid chromatography, HPLC, uses a liquid as the mobile phase. The most used kind of HPLC is reversed-phase HPLC. In a reversed-phase system, the stationary phase is relatively non-polar while the mobile phase is relatively polar. A solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system are components of HPLC instrumentation. The column where separation takes place is the brain of the system. HPLC can be used to identify, quantify, and resolve a compound, among other pieces of information.

INTRODUCTION

Chromatography refers to the methods used to separate, recognise, and quantify the chemical components present in complicated mixtures. Similar to spectroscopy, this method is widely used and extremely effective for both analytical and preparative methods. By using this technique, high-grade pure compounds can be produced. Chromatography's definition is as follows: “It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase”. [1]

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

In order to separate, recognise, and quantify the active chemicals, high-performance liquid chromatography (also known as high pressure liquid chromatography, or HPLC) is a particular type of column chromatography. [2] The primary components of HPLC are a stationary phase (column holding packing material), a pump that circulates one or more mobile phases through the column, and a detector that displays the retention periods of the molecules. The interactions between the stationary phase, the molecules being studied, and the solvent(s) being utilised affect retention time. [3] After being added in a small quantity to the stream of mobile phase, the sample for analysis is delayed by particular chemical or physical interactions with the stationary phase. The kind of analyte and the makeup of the stationary and mobile phases have an impact on the amount of retardation. The retention time is the amount of time it takes for a particular analyte to elute, or exit the column. The majority of miscible mixtures of water or organic liquids are utilised as solvents (the most common are methanol and acetonitrile). [2, 3] Gradient elution refers to the separation process used to change the mobile phase composition during the analysis. [4] According to the analyte's affinity for the present mobile phase, the gradient separates the analyte mixtures. The nature of the stationary phase and the analyte determine the choice of solvents, additives, and gradient.

Principle: In a separation column between a stationary and a mobile phase, the purification happens. A separation column contains a granular substance with incredibly small porous particles as the stationary phase. On the other hand, the mobile phase is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop, which is a tiny tube or a stainless steel capillary. As a result of

interactions with the stationary phase, the various components of the sample are retained to variable degrees, which cause them to migrate across the column at various rates. After leaving the column the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer. At the end of this operation a chromatogram in the HPLC software on the computer is obtained, which allows the identification and quantification of the different substances. [5, 6, 7]

History: Prior to HPLC, researchers used conventional liquid chromatographic techniques. Because the flow rate of solvents depends on gravity, liquid chromatographic techniques are inefficient. Separations take many hours, and maybe even days, to complete. Despite the fact that liquid chromatography (LC) at the time was more efficient, it was assumed that gas stage partition and research of extremely polar high atomic weight biopolymers were both impractical. GC was ineffective for some organic chemists because the solutes were thermally unstable. As a result, it was predicted that alternative methods would soon lead to the advancement of HPLC.

Cal Giddings, Josef Huber, and others predicted in the 1960s that LC could be operated in the high-efficiency mode by reducing the pressing molecule measurement significantly below the standard LC (and GC) level of 150 μm and using pressure to increase the versatile stage velocity, building on the original work of Martin and Synge from 1941. Throughout the 1960s and into the 1970s, these expectations underwent extensive research and improvement. Early research on improving LC particles began, and the development of Zipax, an externally permeable molecule, was encouraging for HPLC technology. Many improvements in machinery and instrumentation were made throughout the 1970s. Injectors and pumps were first used by experts to construct a straightforward 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.

Although improvements in apparatus had a significant role, the history of HPLC is mostly the narrative of the evolution of molecular technology. There has been a consistent trend towards smaller molecules since the introduction of permeable layer particles to increase effectiveness. But when molecule sizes shrank, other problems emerged. The drawback from the unneeded pressure drop is anticipated to be the difficulty of setting up a uniform pressing of extremely fine materials as well as the difficulty of driving versatile liquid through the

segment. To handle the pressure, another cycle of instrument advancement should typically take place every time the molecule size is completely reduced. [8-13]

Operation: The sample mixture that will be separated and combined is added to the stream of mobile section that permeates the column in a unique very small volume (typically microliters). The sample is divided into segments that move through the phase at various speeds that are a result of particular physical interactions with the adsorbent (likewise known as stationary stage). Each element's speed is determined by its compound structure and mobile section composition. Retention time refers to the time at which a chosen analytical elutes (rises up out of the column). A characteristic that is customary for a certain analysis may be the retention time measured under particular circumstances [14–18]. There are numerous types of columns available that are filled with adsorbents with different molecular sizes and surface characteristics ("surface science"). The use of packing materials with tiny molecular sizes necessitates the use of increased operational pressure ("backpressure"), which frequently improves action resolution (i.e. the degree of division between sequent analyses rising up out of the column). Stuff particles may have polar or hydrophobic properties. Basic mobile phases combine water with a variety of natural solvents, including any mixable mixture of water (the most widely recognised area unit acetonitrile and methanol). Certain HPLC systems use mobile phases that are not water-soluble. The aqueous phase of the mobile section might contain acids, (for example, formic, element or trifluoroacetic corrosive) or salts to assist with the separation of the sample parts.

During the chromatographic analysis, the composition of the mobile section may remain unchanged ("isocratic extraction mode") or change ("inclination extraction mode"). When it comes to pattern elements that are no longer wholly exceptional in their predisposition for the stationary stage, isocratic extraction typically succeeds. In gradient extraction, the cellular area's employer frequently varies from low to excessively high eluting tremendous. Analytical maintenance times show the eluting quality of the mobile section, with high eluting quality delivering quick extraction. The stationary stage's stationary stage and the strength of connections between completely separate example pieces ("analyses") determine the structure of the mobile section (also known as eluent) (e.g. hydrophobic connections in turned around stage HPLC). Based on their preference for the stationary and mobile stages, analyses divide between the two during the detachment operation in the sample. This process is similar to what occurs during a liquid-liquid extraction, except that it is continuous rather than stepwise. When the mobile stage becomes more densely packed with acetonitrile in this

situation (during a variable amount of upper eluting quality), extra hydrophobic elements may wash (fall off the column) late. [19-27].

Types of HPLC: Varieties of HPLC often depend on the process's utilisation of a phase system. [3, 4] the following HPLC types are frequently used in analysis-

Normal phase chromatography: This technique, also known as Normal phase HPLC (NP-HPLC), divides analytes according to polarity. Polar stationary phase and non-polar mobile phase are both used in NP-HPLC. The polar stationary phase reacted with the polar analyte and held it. Increased analyte polarity results in stronger adsorption forces, and the interaction of the polar analyte with the polar stationary phase lengthens the elution time.

Reversed phase chromatography: Reversed phases the stationary phase of HPLC (RP-HPLC or RPC) is non-polar, and the mobile phase is aqueous and moderately polar. As a result of repulsive forces between a polar eluent, the comparatively non-polar analyte, and the non-polar stationary phase, RPC works on the theory of hydrophobic interactions. Upon association with the ligand in the aqueous eluent, the analyte's affinity for the stationary phase is proportional to the contact surface area around its non-polar segment.

Size exclusion chromatography: SEC, also known as gel permeation chromatography or gel filtration chromatography, is a type of chromatography that primarily uses size to separate particles. Also, it is helpful for figuring out the quaternary and tertiary structures of proteins and amino acids. This method is frequently used to determine the molecular weight of polysaccharides.

Ion exchange chromatography: The attraction between solute ions and charged sites bound to the stationary phase drives retention in ion-exchange chromatography. Same-charge ions are not included. This type of chromatography is frequently employed in the purification of water, ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, among other applications. [3, 4]

Bio-affinity chromatography: Separation based on a particular, reversible interaction between ligands and proteins. A bio-affinity matrix has ligands covalently bonded to a solid support that holds onto proteins that interact with the ligands connected to the column. A bioaffinity column can elute proteins bound to it in one of two ways:

- A specific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

- Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand.

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

Instrumentation:

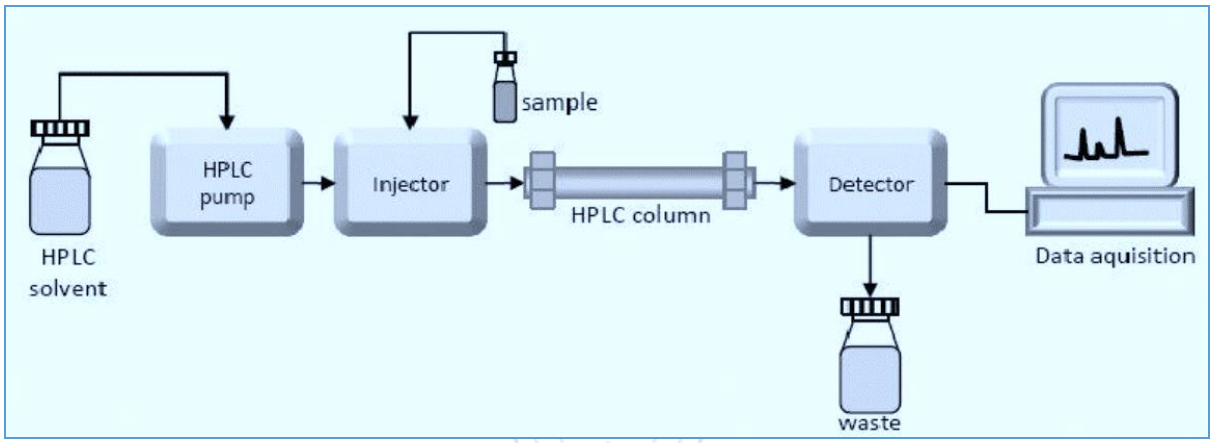

Figure No. 1: HPLC Instrumentation Flow chart

a. Solvent Reservoir: Mobile stage substances are contained in a glass reservoir. The versatile stage, or dissolvable, in HPLC is typically a blend of polar and non-polar liquid segments whose particular fixations are changed relying upon the arrangement of the specimen.

b. Pump: A pump suctions the versatile stage from the dissolvable reservoir and drives it through the framework's column and detector. Contingent upon various components including column measurements, molecule size of the stationary stage, the stream rate and sythesis of the versatile stage, working weights of up to 42000 kPa (around 6000 psi) can be created.

c. Sample Injector: The injector can be a solitary infusion or a mechanized infusion framework. An injector for a HPLC framework ought to give infusion of the liquid specimen inside the scope of 0.1–100 mL of volume with high reproducibility and under high weight (up to 4000 psi).

d. Columns: Columns are generally made of cleaned stainless steel, are in the vicinity of 50 and 300 mm long and have an inside distance across of in the vicinity of 2 and 5 mm. They are normally loaded with a stationary stage with a molecule size of 3–10 µm. Columns with interior distances across of under 2 mm are regularly alluded to as microbore HPLC columns. In a perfect world the temperature of the portable stage and the column ought to be kept steady amid an examination.

e. Detector: The HPLC indicator, situated toward the finish of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized finders are UV spectroscopy, fluorescence, mass-spectrometric and electrochemical indicators.

f. Data Collection Devices: Signals from the indicator might be gathered on outline recorders or electronic integrators that differ in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the identifier to every part and places it into a chromatograph that is anything but difficult to peruse and decipher. [28, 29, 30]

g. Degasser: The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymer membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line, it is more convenient and efficient. Many of new HPLC unit system contain a degasser. [31, 32, 33]

h. Column Heater: The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep the consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50 to 80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room

temperature. There are possibilities that small different of temperature causes different separation results. Thus, columns are generally kept inside the column oven (column heater).

Applications: The identity, quantification, and resolution of a compound are all pieces of information that can be discovered via HPLC. The isolation and purification of chemicals is known as preparative HPLC. This contrasts with analytical HPLC, where learning more about the sample substance is the primary goal. The following are the main applications:

i. Pharmaceuticals: In order to determine API and related compounds in a single run, high-performance liquid chromatography delivers dependable quantitative precision and accuracy in addition to a high linear dynamic range. The dispersion of samples in water or aqueous solutions that have been modified with acetonitrile or methanol is a practical method for solid dosage form manufacturing. There are numerous ways to separate chiral compounds into their individual enantiomers using HPLC. Precolumn derivatization to create diastereomers is one of them. You might also employ custom columns made with cyclodextrins or unique chiral moieties as stationary phases. To put it briefly, reverse phase HPLC the pharmaceutical industry's most widely used option for quantitative analysis is HPLC. The following are typical application areas for pharmaceutical analysis:

- Analytical Method Validation
- To control drug stability.
- Tablet dissolution study of the pharmaceutical dosage form.
- Compound Identification
- Pharmaceutical quality control.
- Stability Studies
- Assay
- Related Substances
- Working Standards.

ii. Foods: In the area of food analysis, high-performance liquid chromatography has brought about a number of desired benefits. Food matrices are typically complex, making analyte extraction a difficult operation. The fact that both desired and unwanted components are

frequently present in trace quantities further complicates issues, and traditional extraction and analysis do not offer the necessary levels of accuracy and precision. Due to the numerous stationary phase and mobile phase possibilities, HPLC provides workable solutions.

Common applications in foods are

- Residual pesticides such as 2, 4-D, and Monochrotophos.
- Fat-soluble vitamins (A, D, E, and K)
- Antioxidants such as TBHQ, BHA, and BHT.
- Residual antibiotics
- Water-soluble vitamins (B-complex vitamins such as B1, B2, B3, B6, Folic acid, Pantothenic acid, B12, Vitamin C)
- Sugars: Glucose, Fructose, Maltose, and other saccharides
- Mycotoxins such as Alfatoxins B1, B2, G1, G2, M1, M2, and ochratoxin
- Steroids and flavonoids
- Cholesterol and sterols
- Amino acids
- Aspartame and other artificial sweeteners.

iii. Manufacturing: Both in the laboratory and in the field of clinical science, HPLC has several uses. Since it is a reliable method for obtaining and ensuring product purity, it is a common approach employed in pharmaceutical development. Despite the fact that HPLC can create products of incredibly high quality (purity), it isn't always the main technique employed in the manufacturing of bulk medicinal ingredients. Only 15.5% of syntheses, according to the European Pharmacopeia, use HPLC. In contrast, it's involved in 44% of the syntheses in the US Pharmacopeia. Given that HPLC can be an expensive procedure when used on a big scale, this might be the result of different time and financial restrictions. Unfortunately, an increase in cost is correlated with an increase in HPLC's specificity, precision, and accuracy.

iv. Research: Research can use similar techniques to find concentrations of prospective medicinal candidates like antifungal and asthma medications. This method certainly works well for observing several species in samples that have been gathered, but it is necessary to utilise standard solutions when trying to identify the species. Purity is crucial in this kind of research, hence it is employed as a way to verify the outcomes of synthesis reactions.

v. Medical: HPLC can be used for medication analysis in medicine, however nutritional analysis is more closely associated with this use. Blood serum is the sample used for the majority of medical HPLC tests, even though urine is the most used medium for assessing drug concentrations. HPLC has been compared against other approaches, especially immunoassays, for the detection of compounds useful for clinical studies. In one instance, the sensitivity of HPLC and competitive protein binding assays (CPBA) for the detection of vitamin D was evaluated. It was discovered that while this CPBA was helpful for identifying vitamin D deficiency in children, its sensitivity and specificity only reached 40% and 60%, respectively, of HPLC's capacity. HPLC is a costly tool, yet it has almost unmatched accuracy.

Other application of HPLC includes

Environmental Applications

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

Applications in Forensics

- Quantification of drugs in biological samples.
- Identification of steroids in blood, urine, etc.
- Forensic analysis of textile dyes.
- Determination of cocaine and other drugs of abuse in blood, urine, etc.

Applications in Clinical Tests

- Urine analysis, antibiotics analysis in blood.
- Analysis of bilirubin, biliverdin in hepatic disorders.

- Detection of endogenous neuropeptides in the extracellular fluid of the brain, etc. [34-38]

CONCLUSION

The primary medication and any reactive contaminants must be separated and quantified using the HPLC technique. The mobile phase in HPLC is a liquid. The most used kind of HPLC is reversed-phase HPLC. Reversed-phase refers to a situation in which the stationary phase is relatively non-polar and the movable phase is substantially polar. Consequently, compared to polar chemicals, non-polar compounds will be kept more and have longer retention durations. The stationary phase is relatively polar in normal phase HPLC, while the mobile phase is generally non-polar. Column packing, which involves various chemical and/or physical interactions between the components' molecules and the packing particles, keeps these parts apart from one another. At the departure of a column, a low-through device (detector) that measures their quantity detects these separated components. Principle-wise, LC and HPLC operate in a similar manner, but HPLC has far better speed, efficiency, sensitivity, and ease of use. The output from this detector is known as a "HPLC." Also, it is the most reliable analytical technique frequently used to assess the stability of drug goods and conduct quantitative and qualitative analyses of drug products.

REFERENCES:

1. Ali AH. High- Performance Liquid Chromatography (HPLC): A review. Ann Adv Chem. 2022; 6: 010-020.
2. Martin M., Guiochon, G. Effects of high pressures in liquid chromatography. J. Chromatogr. A, 2005; (1-2)7: 16-38.
3. Liu Y., Lee M. L. Ultrahigh pressure liquid chromatography using elevated temperature. Journal of Chromatography. 2006; 1104 (1-2): 198–202.
4. Abidi, S. L. High-performance liquid chromatography of phosphatidic acids and related polar lipids. J. Chromatogr. 1991; 587: 193-203.
5. Gupta V, Jain A D, Gill N S, Gupta K. Development and validation of HPLC method - a review. International Research Journal of Pharmaceutical and Applied Sciences. 2012; 2(4):17-25.
6. Sonia K, Nappinnai M. Development and validation of HPLC and UV-visible spectrophotometric method for the pharmaceutical dosage form and biological fluid –review. European Journal of Biomedical and Pharmaceutical sciences. 2016; 3(3): 382-391.
7. Sánchez MLF. Chromatographic techniques, European RTN Project, GLADNET, retrieved on 05-09-2013.
8. 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.
9. 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.
10. 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.
11. Shintani H. Immobilized enzyme column combined with HPLC and column switching method for the analysis of complicated matrix such as body fluids. Pharmaceut Reg Affairs. 2014;3:e142.

12. 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.
13. Suresh Babu V V, *et al.* Validated HPLC method for determining related substances in compatibility studies and novel extended release formulation for ranolazine. J Chromatograph Separat Techniq. 2014;5:209.
14. Arayne M S, *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.
15. 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.
16. Abdulla S A, *et al.* Validated HPLC method for the determination of nisoldipine. Pharm Anal Acta. 2013;S1:004.
17. Sawsan Mohammed A H, *et al.* Effects of blood collection tubes on determination vitamin-A by HPLC. J Chromat Separation Techniq. 2013; 4:184.
18. Subbaiah P R, *et al.* Method development and validation for estimation of moxifloxacin HCl in tablet dosage form by RP-HPLC method. Pharm Anal Acta. 2010;1:109.
19. Ahir K B, *et al.* Simultaneous estimation of metformin hydrochloride and repaglinide in pharmaceutical formulation by HPTLC Densitometry method. J Chromat Separation Techniq. 2013;4:166.
20. Khodadoust S, *et al.* A QSRR study of liquid chromatography retention time of pesticides using linear and nonlinear chemometric models. J Chromat Separation Techniq. 2012;3:149.
21. Vali S J, *et al.* Separation and quantification of octahydro-1h-indole-2-carboxilic acid and its three isomers by HPLC using refractive index detector. J Chromat Separation Techniq. 2012;3:136.
22. Fayyad M K, *et al.* Effect of temperature, wavelength, ph, ion pair reagents and organic modifiers' concentration on the elution of cystatin c. stability of mobile phase. J Anal Bioanal Techniques. 2010;1:103.
23. Ndorbor T, *et al.* Chromatographic and molecular simulation study on the chiral recognition of *Atracurium besylate* positional isomers on cellulose tri- 3, 5-dimethylphenycarbamate (CDMPC) column and its recognition mechanism. J Chromat Separation Techniq. 2013;4:176.
24. Hua Z, *et al.* Extraction and purification of anthocyanins from the fruit residues of *Vaccinium uliginosum* Linn. J Chromat Separation Techniq. 2013;4:167.
25. Rogatsky E. 2D or Not 2D. Column-switching techniques, multidimensional separations and chromatography: approaches and definitions. J Chromat Separation Techniq. 2012; 3:159.
26. Al-Sagar K A and Smyth M R. Multi-Dimensional column chromatographic method with UV detection, for the determination of propranolol at therapeutic levels in human plasma. Pharmaceut Anal Acta. 2012;3:197
27. Flores HE and Galston AW. Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 1982;69:701-706.
28. Hearn M.T.W. Ion-pair chromatography on normal and reversed-phase systems. Adv. Chromatogr. 1980; 18: 59–100.
29. HPLC – Chemiguide. May 2, 2007. www.chemguide.co.uk.
30. Rao G, Goyal A. An Overview on Analytical Method Development and Validation by Using HPLC. The Pharmaceutical and Chemical Journal, 2016; 3(2): 280-289.
31. Mcpolin Oona. An Introduction to HPLC for Pharmaceutical Analysis. Mourne Training Service. 11-12.
32. Fundamentals of Analytical chemistry- Skoog, West, Holler, Crouch-2009:973.
33. Elshanawane A A, *et al.* Development and validation of HPLC method for simultaneous estimation of brimonidine tartrate and timolol maleate in bulk and pharmaceutical dosage form. J Chromatograph Separate Techniq. 2014;5:230.
34. The European Pharmacopoeia. Fourth ed., Council of Europe, Strasbourg. 2002.
35. Tsai I L, Weng T I, Tseng Y J, Tan H K, Sun H J, Kuo C H. Screening and confirmation of 62 drugs of abuse and metabolites in urine by ultrahigh- performance liquid chromatography-quadrupole time-of-flight spectrometry. J Anal Toxicol. 2013 Nov-Dec; 37(9):642-51. doi: 10.1093/jat/bkt083. Epub 2013 Sep 30. PMID: 24084874.
36. Hearn M T W. Ion-pair chromatography on normal and reversed-phase systems. Advance Chromatography. 1980; 18: 59–100.

37. Siddiqui M R, AlOthman Z A, Rahman N. Analytical techniques in pharmaceutical analysis: A review. Arabian Journal of Chemistry. 2013.
38. Willard H, Merritt L, Dean J, Settle F. Instrumental Methods of Analysis, 7th edn, Wadsworth Publishers, Stamford, CT. 1998.