International Journal of Engineering Research & Management Technology
ISSN: 2348-4039
January-February-2023 Volume 10, Issue-1
Email: editor@ijermt.org www.ijermt.org

A STUDY ON HPLC (HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY) ANALYTICAL METHODS

Suresh Babu Jillellamudi , Research Scholar, Dept of Chemistry, Sikkim Professional University

Dr Vinesh Kumar, Professor, Dept of Chemistry, Sikkim Professional University

ABSTRACT

A pump, injector, column, detector, and data system are components of HPLC apparatus. The column where separation takes place is the brain of the system. The mobile phase must be pumped through the column at high pressure because the stationary phase is made up of porous micrometer-sized particles. The solute is injected onto the top of the column to start the chromatographic process. As the analyte and mobile phase are pushed through the column, the components separate. Each component eventually emerges from the column as a peak (or narrow band) on the recorder. Depending on the detector being utilised, it may be necessary to detect the eluting components selectively or universally. A chromatogram, which is displayed on a chart recorder or computer screen, shows the detector's reaction to each component. Computer, integrator, and other data processing tools are routinely used to gather, store, and analyse the chromatographic data.

KEY WORDS: High-Performance Liquid Chromatography, Analytical Methods.

INTRODUCTION

In normal phase chromatography, separation happens when analyte interacts with a less polar mobile phase, such as hexane or heptanes, and a polar stationary phase (often a cyano or amino linked stationary phase). Based on their polarity, analytes are held on the column to varying degrees; the more polar the analyte, the more retained on the column it will be. Its principal use is for the separation of substances that are hydrophobic but soluble in non-polar solvents.

REVERSED PHASE LC

Reverse phase chromatography, often known as RP-HPLC, is a popular HPLC method for the examination of many different chemicals. In this method, the mobile phase is aqueous or somewhat polar in nature, whereas the stationary phase is non-polar.

In RP-HPLC, the mobile phase, which can be water, methanol, or acetonitrile, etc., is somewhat polar while the stationary phase is often a non-polar hydrocarbon. The solutes in RP-HPLC are eluted in the order of decreasing polarity. By applying an organo chloro silane reagent to the surface of the silanol group, stationary phases for RP-HPLC are created. The fundamental working principle of RP-HPLC is retention by contact of the stationary phase containing non-polar hydrocarbon chain with sample molecules.

PRINCIPLE OF SEPARATION

Depending on the kind of stationary phase employed, HPLC is based on the principles of adsorption, partition, ion exchange, or size exclusion. A liquid mobile phase and a solid stationary phase are used in high performance liquid chromatography (HPLC). Differences in the relative distribution ratio of the solutes between the two phases cause the separation of the components of a solution. Over 90% of HPLC separations—the vast majority—are carried out in reversed phase mode. Organic molecules are divided in reversed phase separation according to their hydrophobicity. The level of lipophilicity and retention in the column are correlated. Polar solutes elute later than non-polar lyophilic ones in normal phase HPLC, and vice versa in reverse phase HPLC, according to the elution sequence.

HPLC INSTRUMENTATION

1. A delivery mechanism for solvent
2. Mobile Phase
3. Pumping mechanism
4. The sample injection device
5. Chronological column
6. Detector

SOLVENT RESERVOIR

This serves as a container for the mobile phase's contents. Different solvent compositions are added to the reservoir, and pumps are used to allow the solvents to flow through the column. To achieve the separation objectives, the solvent quantities are changed over time based on the complexity of the sample mixture.

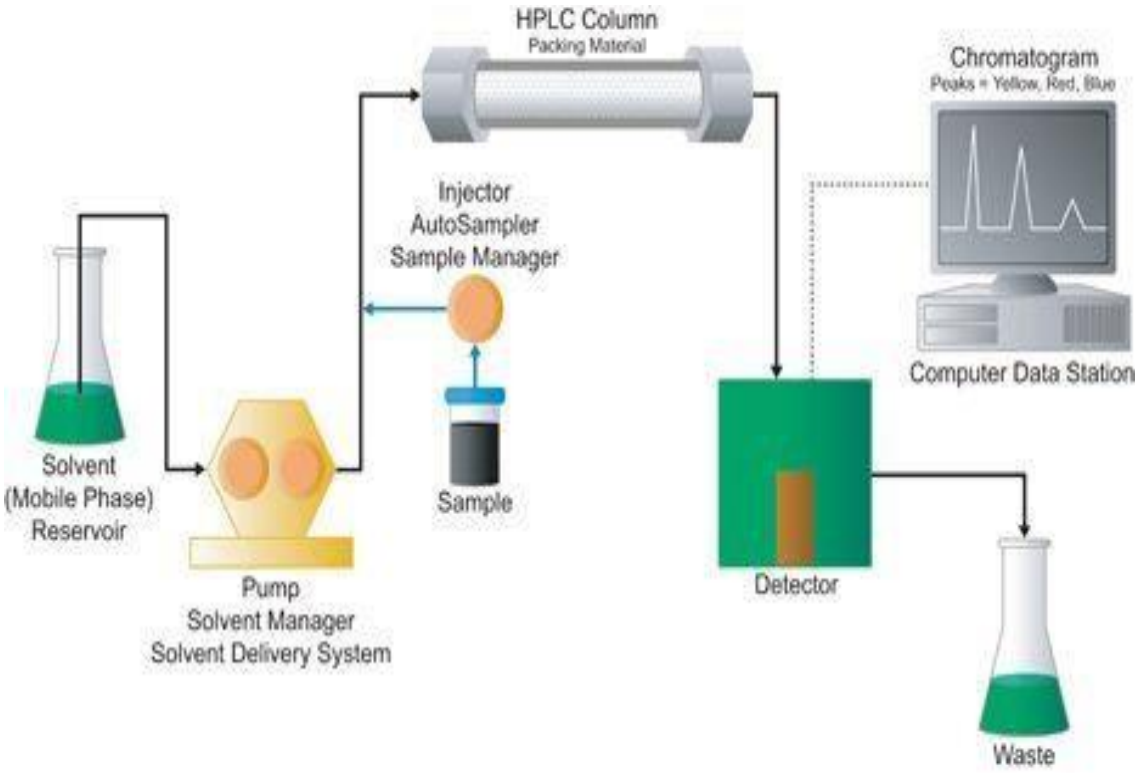

Figure 1: High Performance Liquid Chromatography (HPLC) system

PUMP

The stationary phase's particles are only a few microns in size and cover more of the column's surface. In order to assist the passage of solvents from the reservoir to column and ultimately to the detector, high pressure of roughly 1000 to 6000 psi will be utilised. This is because it offers higher resistance to the flow of solvent. The characteristics of the column, the make-up of the mobile phase, and the flow velocity all play a role in maintaining pressure for the separation. For separation, mechanical and pneumatic pumps with constant flow rates are typically used. The pumps also use check values and pulse dampers to perform properly.

SAMPLE INJECTOR

For various purposes, the sample is injected into the column using devices like septum, stop flow, and rheodyne injectors. The most common injector, which has two operating modes including load and inject, is the rheodyne. Other injectors, which have some restrictions, are not used. The sample injector must deliver sample injections in the volume range of 0.1-1.0 mL under high pressure and can be either manual (using a syringe) or automated (Auto sampler).

COLUMNS

The main component of chromatographic separations is the column since it affects the effectiveness of the separation. Guard columns may not be used since they don't help with separation because analytical columns are typically used for separations. Depending on the technique and mode of separation, different columns are accessible and being used. To sustain high pressure, the column is typically constructed of thick glass or stainless steel tube. The columns typically have an inside diameter of 4 to 10 mm, are 10 to 30 cm long, and contain stationary phase with particles no larger than 25 m in diameter. A 5 mm internal diameter column produces good results due to a compromise struck between efficiency, sample capacity, packing density, and solvent usage.

Table 1: Types of columns

S. No.	Type	Properties (Internal Diameter & Length)
1	Analytical	1.0-4.6 mm & 15-250 mm
2	Preparative	> 4.6 mm & 50-250 mm
3	Capillary	0.1 to 0.1 m & Different lengths
4	Nano	< 0.1 mm

DETECTOR

Depending on the type of sample being separated from and evaluated, several detectors are utilised for HPLC separations. When choosing the detector, the analyte's attribute is taken into account. The column's effluents reach the detector and are picked up there. In table 1.4, many detector types are illustrated.

RECORDERS

These are utilised to record the detector responses following any necessary amplifications as well as the baseline and all peaks that were obtained with regard to time. All peaks' retention times are documented, however the individual peaks' areas cannot be determined.

INTEGRATORS

These are upgraded versions of recorders that can handle certain data and record individual peaks along with

retention time, peak area, peak area percentage, etc. Integrators offer more peak data than recorders do.

QUANTIFICATION IN HPLC

QUALITATIVE RESEARCH

- Determination of contaminants and drug compounds
- Purity determination
- Retention intervals employed in the analysis

QUALITATIVE RESEARCH

- To calculate the amount of each component or combination of components in a mixture.
- By comparing the peak areas of the standard and sample, performance is achieved.

ANALYTICAL TECHNIQUES

Analytical techniques are created during the drug development stage to evaluate the purity and quantity of the active pharmaceutical components, intermediates, and finished products. Rapid and cost-effective analytical procedures must be developed for the new drug products, both pharmacopoeial and non-pharmacopoeial drugs, that are introduced to the market.

The following are various steps in establishing a new method:

- Choosing an appropriate analytical mode
- Literature evaluation
- Examining the type of sample
- Detector wavelength selection
- Choosing a column
- Diluent selection and extraction technique
- Picking and enhancing the mobile phase
- The power of the buffer
- Calculating pka and pH
- Improving the composition of the mobile phase

- Choosing a solvent delivery method
- Picking the test volume and concentration
- Choosing a flow rate
- Column temperature selection
- Creation of this solution's stability

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

Ultra Performance Liquid Chromatography (UPLC) is a recently developed method that uses solvent. A unique design feature of the UPLC chromatographic system allows it to resist significant system back pressure. The HPLC to UPLC system transfers the quality control samples of diverse pharmaceutical formulations. Although the separation on a UPLC system is carried out under extremely high pressure (up to 100 Mpa), neither the analytical column nor other chromatographic system components are negatively impacted. The UPLC process keeps separation efficiency constant or perhaps increases it. Chromatographic resolution, speed, and sensitivity analysis all see improvements.

UPLC SYSTEM

High flow rates are also made possible by elevated-temperature chromatography because it lowers the viscosity of the mobile phase, which greatly lowers column backpressure [11]. The porous polymerized material used in monolithic columns has a lower flow resistance than that of traditional particle-packed columns.

PRINCIPLE

While HPLC columns are normally filled with particles ranging in size from 3 to 5 m, the UPLC is founded on the idea of using stationary phase made up of particles smaller than 2 m. The van deemter, an empirical formula that explains the relationship between linear velocity and planar height, governs the fundamental idea driving its evolution.

$$H = A + B/v + Cv$$

INSTRUMENTATION OF UPLC

To produce better findings, ultra-performance liquid chromatography can operate more quickly, sensitively, and precisely at a significantly wider range of linear velocities, flow rates, and backpressures.

The elements of the Acquity UPLC system are:

UPLC SOLVENT MANAGER

The binary solvent manager, sampler manager, which also includes the column heater, detector, and ideal sample organiser make up the Acquity UPLC system.

Binary solvent manager: The binary solvent manager delivers a parallel binary gradient using two separate serial flow pumps. The system's solvent is moved about by a high pressure pump called the binary solvent management. At analytical flow rates, it delivers stable (pulse-free) solvent flow. The binary solvent management can deliver solvent at flow rates of up to 2 mL/min when the pressure is decreased to 9000 psi from 15,000 psi (approximately 1000 bar) (621 bar). The solvent manager can immediately pump two solvents.

SAMPLE MANAGER WITH THE HEATER IN THE COLUMN:

Acquity's sample manager pulls samples from microtitre plates or vials and injects them into the chromatographic flow stream. A locating mechanism accesses sample sites and collects samples there using probes. The column heater is likewise managed by the sample manager. It is possible to reach column temperatures of up to 65 oC.

Column heater: The foot print of the column heater, which has a modular design, is the same as that of the sample manager. As a result, it functions as the sample manager's top cover and is attached to it.

Microtiter or vial plates are stored in the optional sample manager, which also transports them to and from the sample managers to automate processing and boost throughput.

SAMPLE INJECTION (II)

The insertion of the sample is crucial in UPLC. The design and hardening of conventional injection valves, whether automated or manual, prevents them from operating under high pressure. The injection procedure must be largely pulse-free and the device's swept volume must be as small as possible to prevent band spreading in

order to protect the column from excessive pressure fluctuations. To fully take advantage of the speed that UPLC offers, a quick injection cycle time is necessary, which in turn calls for a large sample volume. To improve sensitivity, low volume injections with less carryover are also necessary. For biological samples, direct injection techniques are also available [12].

UPLC COLUMNS, THIRD (C8 AND C18 COLUMNS)

The ACQUITY BEH UPLC column is frequently utilised (Ethylene Bridged Hybrid). The chemistry in this column can withstand a very low pH range. A superior pH-stable Sub 2 column is more in demand on the market. Different sizes of the C8 and C18 BEH columns are offered.

PHENYL COLUMNS

For excellent selectivity, ACQUITY BEH phenyl columns were skillfully constructed. The majority of the molecules have good peak shapes, and the pH is stable. A trifunctional C6 alkyl ether links the silyl and phenyl rings in this column.

PROTECTING RP-C18 COLUMNS

- The selectivity of the ACQUITY UPLCTM BEH shield C18 columns is comparable to that of the ACQUITY UPLC BEH C8 and C18 phases. The stationary phase of the column has an integrated carbamate group that helps to reduce the peak tailing of the basic compound.

- HILIC (Hydrophilic interaction liquid chromatography) BEH columns are designed for the separation of extremely polar basic sample materials. The unbounded Ethylene Bridged Hybrid particle in the ACQUITY UPLC BEH HILIC is intended to retain and separate polar chemicals. Under UPLC HILIC settings, these special columns are evaluated and tuned to achieve effective, repeatable separations. The chemical stability of the BEH particles leads to longer column life periods and makes them more durable than silica-based HILIC.

DETECTOR TYPES

INFRARED DETECTORS

- Adjustable ultraviolet sensors
- Detectors for evaporative light scattering
- A detector for fluorescence.

ADVANTAGES

- Shortens the runtime and boosts sensitivity
- Offers LC analysis's selectivity, sensitivity, and dynamic range.
- Preserving resolution effectiveness
- A unique separation material with very tiny particle size can speed up analysis.
- Operating costs are decreased
- Lower usage of solvents
- Provides real-time analysis in sync with production procedures.

By offering more data per unit of effort, UPLC boosts productivity in both chemistry and instrumentation. It does this by improving liquid chromatography's resolution, speed, and sensitivity. The biggest benefit is a shorter analytical time, which also meant using less solvent. When compared to traditional HPLC, UPLC can provide considerable gains in speed, sensitivity, and resolution.

METHOD VALIDATION

ANALYTICAL METHOD VALIDATION

The process of proving that analytical processes are appropriate for their intended application and that they support the identity, quality, purity, and potency of the drug material and product is a crucial component of method development (s). Establishing recorded proof that offers a high level of assurance that a particular process will consistently produce a product matching its planned requirements and quality attributes is what it is described as (ICHQ 2B).

Table-2 : Analytical validation parameters

Validation Parameters	**Procedure**

Linearity	Not less than five reference solutions are prepared in the concentration range and the sample are estimated byinterpolation in the linearity plot. The specified limit for the assay is 80 % to 120 % to test concentration and the acceptance criteria is correlation coefficient should be not less than 0.9990
Precision	**Repeatability: S**ystem precision (Injection repeatability) – six replicate injections of standard preparation. **Method precision** (Method repeatability) - Six replicate analysisof samples. **Intermediate precision: S**ix replicates measurements within a laboratory over as longer period of time under differentlaboratory conditions such as two different days, analysis and equipment. **Reproducibility:** it is the measure of precision between the different laboratories. It is beneficial to standardization of methodologies and collaborative studies. % RSD not more than 2 %.
Accuracy	Minimum of three levels (50 %, 100% & 150 %) of testconcentration for assay and each level was performed into triplicate and the recovery should be in between 98 % to 102 %.
Range	Interval between the upper and lower levels of an analyte is considered as range and established with acceptable precision, accuracy and linearity using either a linear or nonlinear response curve.
Ruggedness	Six replicates measurements made by different analysts, laboratories, instruments and days etc.
Limit of Detection	This is the lowest concentration in a sample that can be detected, but not necessarily quantified under given experimental conditions and calculated as concentration yielding a signal-to-

	noise ratio of 3:1.
Limit of Quantitation	The lowest concentration of analyte in the sample, which is determined with acceptable precision and accuracy and it is concentration yielding a signal-to-noise of 10:1.
electivity and Specificity	Detection specifically without interference
Robustness	Measure of ability of a method to remain unaffected by smalldeliberate changes in the operational parameters and effect on system suitability is examined.
System suitability parameters	Capacity factor (> 2), Resolution, Tailing factor (≤ 2), Theoretical plates (N > 2000).

BIOANALYSIS

Drug concentrations, their metabolites, and/or endogenous chemicals can be found in biological matrices like blood plasma, serum, cerebrospinal fluid, urine, and saliva. This is done using a technique called bioanalysis. Studies on bioequivalence, pharmacokinetics, toxicokinetics, and therapeutic drug monitoring all benefit from the use of bio analytical methods for the quantitative assessment of pharmaceuticals and their metabolites in biological fluids. In the process of finding and developing new drugs, bio analytical investigations are crucial [23].

- Bioanalysis can be used to determine a particular drug's therapeutic efficacy. The following steps are involved in bioanalysis.
- The choice and gathering of biological fluid.
- Analyte extraction from the biological matrix is part of sample preparation.
- Analyte detection using various techniques.

CONCLUSION

In order to permit instrumental analysis or to enhance the instrumental analyte signals compared to those obtained from untreated samples, sample preparation is the goal. The extraction of the analyte from the sample matrix, a clean-up step, and/or a pre-concentration step are a few examples of the sample preparation stages. Analytical difficulties arise when hydrophilic chemicals are extracted from these aqueous matrices. Blood is made up of many different substances, including different kinds of proteins, lipids, salts, and suspended cells. After adding an anti-coagulant, the red blood cells can be separated from the plasma by centrifugation. For these types of samples, the most basic sample preparation requires dilution, centrifugation, filtration, and/or evaporation. Because sample preparation can be accomplished using several extraction techniques, this phase in the bio analytical method is more crucial.

REFERENCES

1. Mallikarjuna Rao N and GowriSankar D. Development and Validation ofStability-Indicating HPLC-DAD Method for Simultaneous Determination of Emtricitabine, Elvitegravir, Cobicistat and Tenofovir in their Tablet Dosage Forms, Indian Journal of Pharmaceutical Education and Research. 2016;50(1).
2. Arun Ramaswamy, Anton Smith Arul Gnana Dhas. Development and validation of analytical method for quantitation of Emtricitabine, Tenofovir, Efavirenz based on HPLC. Arabian Journal of Chemistry, 2014.
3. G. Vijay Swaroop, T.E. Divakar. A Novel Stability Indicating RP-HPLCMethod Development and Validation for Simultaneous Estimation of Bictegravir, Emtricitabine, and Tenofovir in Pure and Fixed Dose Combination. Asian Journal of Organic and Medicinal chemistry. 2019, 4(1): 7-13.
4. MD Abdul Sattar and Suneetha Achanta. Analytical Method Development and Validation for the Determination of Emtricitabine and Tenofovir Disoproxil Fumarate Using Reverse Phase HPLC Method in Bulk and Tablet Dosage Form. Journal of pharmaceutical sciences and Research, 2018, 10(15):1207-1212.
5. N.MD. Akram, M. Umamahesh. A New Validated RP-HPLC Method for the Determination of Emtricitabine and Tenofovir AF in its Bulk and Pharmaceutical Dosage Forms. Journal of Chemical and Pharmaceutical Sciences.2017; 10:4.
6. ICH: Q2 [R1], Validation of analytical procedures: text and methodology; 2005.
7. ICH: Q2B. Harmonized Tripartite Guideline, Validation of Analytical procedure: Methodology,

IFPMA, in Proceedings of the International Conference on Harmonization, Geneva, 1996.

8. Ngwa G. Forced degradation studies as an integral part of HPLC stability indicating method development. Drug Delivery Technol 2010; 10:56-9.
9. Cinti F, Moffa S, Impronta F, Cefalo CM, Sun VA, Sorice GP, Mezza T, Giaccari A: Spotlight on ertugliflozin and its potential in the treatment of type 2 diabetes: evidence to date. Drug Des Devel Ther. 2017; 11, 2905-2919.
10. Ghazala K. Dinesh S, Agarwal YP, Neetu S, Avinash J. Gupta AK. Simultaneous estimation of metformin and Sitagliptin in tablet dosage form. Asian J Biochem Pharm Res. 2011; 2, 223-9.
11. Herman GA. Stevens C, Van Dyck K, Bergman A, Yi B, De Smet M, et al. Pharmacokinetics and pharmacodynamics of sitagliptin , an inhibitor of dipeptidyl IV,in healthy subjects: Results from two randomized, double-blind, placebo-controlled studies with single oral doses. Clin Prarmacol Ther. 2005; 78(6), 675-88.
12. Plosker GL, Sitagliptin: A review of its use in patients with type 2 diabetes mellitus, Drugs. 2014; 74(2), 22342.