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Analytical Method Development and Validation by RP-HPLC technique: a Review

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ABSTRACT
Chromatography is the most powerful and versatile technique available to the modern analysts. High performance liquid chromatography (HPLC) has emerged as one of the most important analytical technique used in the qualitative and quantitative analysis of drugs worldwide. High-performance liquid chromatography (HPLC) is a highly versatile technique in which analytes are separated by passage through a column packed with micro meter-sized particles. Now a day RP-HPLC is the most widely used separation technique in HPLC. Reasons for this include the simplicity, versatility, and applicability of the reversed-phase method. This is because compounds with different polarities and molecular weights can be processed. Reversed-phase chromatography is used for both analytical and preparative applications in the field of biochemical separation and purification. This review discusses the importance of RP-HPLC in analytical method development and its strategies, along with a brief knowledge of the critical chromatographic parameters that need to be optimized for an efficient method development.

KEY WORDS: Chromatography; HPLC; RP-HPLC; Analytical methods; Chromatographic parameters.

I. INTRODUCTION

The chromatographic method can be simply viewed as a physical separation method that divides the components to be separated into two phases, a stationary phase and a mobile phase[1,2]. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify, and purify the individual components of the mixture[3]. HPLC primarily uses a column containing a packing material (stationary phase), a pump that moves the mobile phase through the column, and a detector to display the retention time of the molecules. A small amount of the sample to be analyzed is introduced into small the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and the composition of both the stationary and mobile phase [4].

II. TYPES OF HPLC

There are five main types of HPLC, depending on the phase system (stationary phase) of the process:

1. Reversed phase Chromatography(RP-HPLC or RPC)

Reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC works on the principle of hydrophobic interactions due to repulsive forces between a polar eluent, the relatively non-polar analyte and a non-polar stationary phase. Analyte binding to the stationary phase is proportional to the contact surface area around the non-polar portion of the analyte molecule upon when bound to the ligand in the aqueous eluent[4-7].

2. Normal Phase Chromatography (NP-HPLC)

Also called Normal phase HPLC, this method separates analytes according to their polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phases. Polar analytes interact with and are retained in the polar stationary phase. The adsorption capacity increases as the polarity of the analyte increases, and theelution timeincreases due to the interaction between the polar analyte and the polar stationary phase[4-8].

3. Size Exclusion Chromatography(SEC)

Size exclusion chromatography, also called as gel permeation chromatography or gel filtration chromatography, separates particles primarily based on their size. It can also be used to determine the tertiary structure and quaternary structures of proteins and amino acids. This method is widely used to determine the molecular weight of polysaccharides[4-7].

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4. Ion Exchange Chromatography

In Ion exchange chromatography, retention is based on the force of attraction between dissolved ions and charged sites bound to the stationary phase. Ions with the same charge are excluded. This form of chromatography is widely used for water purification, ligand exchange chromatography, Ion-exchange chromatography of proteins, and high pH anion exchange chromatography of carbohydrates and oligosaccharides, etc[4-7].

5. Bio-affinity Chromatography

Separation of proteins with ligands byspecific reversible interactions. The ligands are covalently attached to a solid support on the bio-affinity matrix and retains proteins that interact with the column-bound ligands. Proteins bound to bio-affinity column can be eluted in two ways:

i. Biospecific elution: Inclusion of free ligand in elution buffer which competes with column bound ligand.

ii. Aspecific elution: A change in pH, salt, etc. that weakens interaction between the protein and the column-bound substrate. Due to the specificity of the interaction, bio-affinity chromatography can achieve very high level of purification (10 - 1000-fold)in a single step[4-6, 9].

III. RP-HPLC

Reversed-phase chromatography is used for both analytical and preparative applications in the field of biochemical separations and purification. Molecules with some degree of hydrophobicity can be separated with excellent recovery and resolution by reversed-phase chromatography. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its wide range of applications. It is estimated that more than 65% (possibly up to 90%) of all HPLC separations are performed in reversed-phase mode. Reasons forreversed-phase method's simplicity, versatility, and scope. This is because compounds with different polarities and molecular weight can be processed[3-4, 9-10].

1) Theory of Reversed-Phase Chromatography

The separation mechanism of reversed-phase chromatography relies on hydrophobic bonding interactions between solute molecules in the mobile phase and immobilized hydrophobic ligand, i.e. the stationary phase. Although the actual nature of the hydrophobic bonding interaction itself is a matter of heated debate, but the conventional wisdom is that bonding interactions result from favorable entropic effects. The initial mobile phase binding conditions used in reversed-phase chromatography are predominantly aqueous, exhibiting highly organized water structures surrounding both the solute molecules and the immobilized ligand. Solute binds to immobilized hydrophobic ligand, thus minimizing hydrophobic area exposed to the solvent. Thus, the degree of organized water structure is reduced with a corresponding beneficial increase in system entropy. Therefore, from an energy point of view, it is advantageous to combine hydrophobic moieties, i.e. solute and ligand[11].

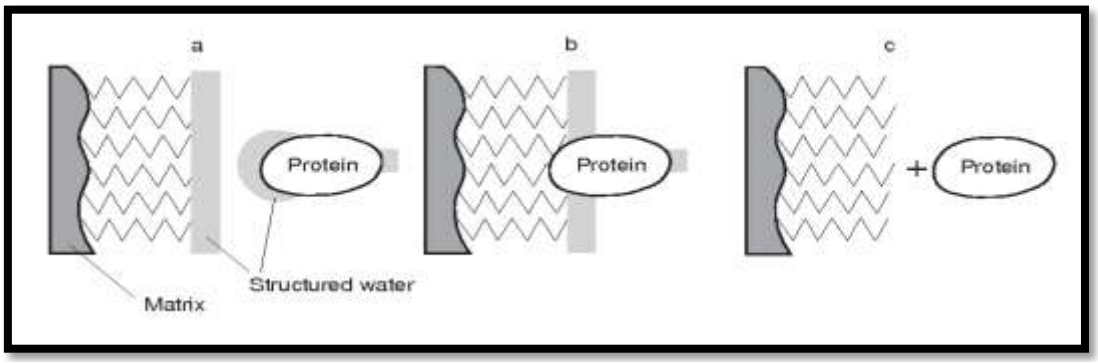

Figure 1: Interaction of a solute with a typical reversed phase medium

Water adjacent to hydrophobic regions is assumed to be more highly ordered than bulk water. When the hydrophobic regions interact, some of these 'structured' water is displaced, leading to an increase in the overall entropy of the system. Reversed-phase chromatographic separations depends on the reversible adsorption/desorption of solute molecules of varying degrees of hydrophobicity on a hydrophobic stationary phase[3-4, 10]. As shown in

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Figure 2, most of reversed-phase separation experiments are performed in a few basicsteps.

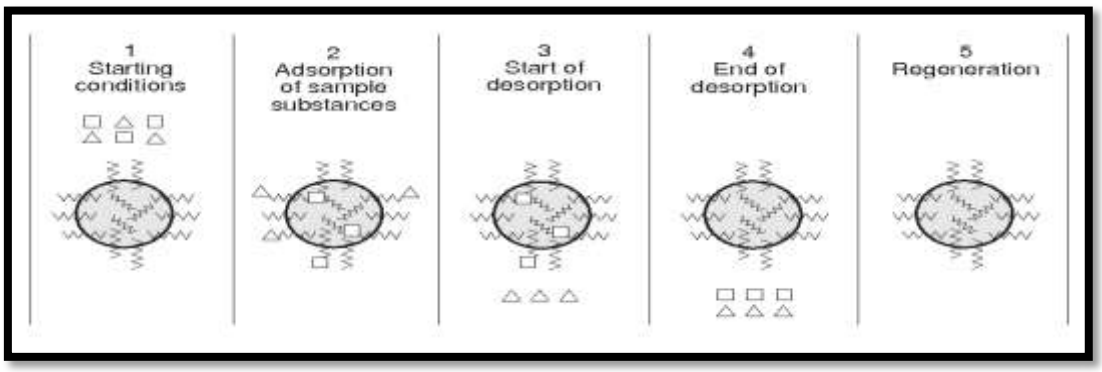

Figure 2: Principle of Reversed-Phase Chromatography with Gradient elution

Selection of Separation Medium: Proper selection of reversed-phase medium is critical to the success of a particular application. Selection should be based on the following criteria[4]:

I. The unique requirements of the application, includingmobilephase scale and conditions.
II. Molecular weight, or size of sample component.
III. Hydrophobicity of sample components.
IV. Class ofsample components.

2) Analytical Method Development using RP-HPLC

The analytical methods are continually developed, improved, validated, co-studied and applied. Combinations of these established methods later emerged in major collections such as USP, BP and IP, etc. In most cases, as expected, the separation can be easily achieved with only a few experiments. In other cases, extensive amount of testing may be required. However, a good method development strategy should only involve the number of experimental runs necessary to achieve the chosen final result. The development of analytical method is often based on prior art or remaining literature using nearly identical or similar experiments. The development of any new or improved method usually involvesadapting existing gradients and instrumentation to the recent analyte, and to the final need or requirement of the method[4, 10-11].

In general, method development involves choosing the method requirements and determining the types of instrumentation involved and why. During the HPLC method development stage, conclusions regarding the selection of column, mobile phase, detectors, and method quantitation must be considered. Therefore development includes reflection of all parameters associated with any method[3-4, 10].

Developing a new HPLC method therefore requires the selection of optimal mobile phase, detectors, best column, column length, stationary phase and best inside diameter for the column. The analytical strategy for HPLC method development involves many steps[3-4, 10].

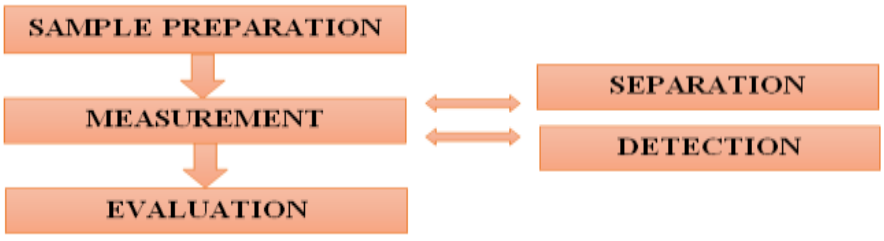

Figure 3: A typical strategy for HPLC method development

➢ **Sample Collection and Preparation:**

The samples are better dissolved in the initial mobile phase. If this is not possible due to stability or solubility issues, formic acid, acetic acid or salts can be added to the sample to enhance solubility. These additives generally do not affect

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the separation as long as the amount of the sample loaded is small compared to the volume of the column. The only effect of large sample loading is the elution of one or two additional peaks into the void volume after sample injection[3-4].

a) Sample preparation: Sample preparation is a critical part of HPLCanalysis to provide reproducible andhomogenous solutions suitable for injection onto the column. The aim of sample preparation is an aliquot of sample that,

- Is relatively free from interferences,
- Will not damage the column, and
- Is compatible with the desired HPLC method i.e, sample solvent dissolve in the mobile phase without affecting sample retention or resolution[3-4].

Sample preparation begins at the sampling point and extends to sample injection onto the HPLC column and includes various operations summarized in **Table 1**. All of these operations form an important part of sample preparation and have a significant impact on the final method accuracy, precision, and usability[3-4].

b) Measurement: The measurement of a given analyte can often be divided into a separation step and a Detection step[3].

c) Separation: Analytes in a mixture should preferably be separated prior to detection. Simple LC consists of a column with a fritted bottom containing the stationary phase in equilibrium with a solvent. The mixture to be separated is loaded on to the top of the column followed by more solvent. The different components inthe column pass at different rates due to difference in their partitioning behavior between mobile liquid phase and stationary phase[4].

Table no. 1: Sample pretreatment options

OPTION	COMMENT
Sample Collection	Obtain representative sample using statistically valid processes.
Sample Storage and Preservation	Use appropriate inert, tightly sealed containers; be especially careful with volatile, unstable, or reactive materials; biological samples may require freezing.
Preliminary Sample Processing	Sample must be in a form for more efficient sample pretreatment (e.g., drying, sieving, grinding, etc.); finer dispersed samples are easier to dissolve or extract.
Weighing or Volumetric Dilution	Take necessary precautions for reactive, unstable, or biological materials; for dilution, use calibrated volumetric glassware.
Alternative Sample Processing Methods	Solvent replacement, desalting, evaporation, freeze drying, etc.
Removal of Particulates	Filtration, solid-phase extraction, centrifugation.
Sample Extraction	Different methods used for liquid samples and solid samples.
Derivatization	Used mainly to enhance analyte detection; sometimes used to improve separation.

d) Detection: It is essential to use reagents and solvents of high purity to ensure minimum detection limits for optimum sensitivity. All organic solvents and many additives, such as ion pairing agents, absorb in the UV range and the detection limit is related to the wavelength. A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles for detecting the analytes after the chromatographic separations. However, only about twelve of them can be used effectively for LC analysis and, of those twelve, only four are in common use. The four dominant detectors used in LC analysis are the UV detector (fixed and variable wavelength), the electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications. The choice of detector depends on the sample and the purpose of the analysis[3].

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IV. CRITICAL PARAMETERS IN RP-HPLC

- **Classifying the Sample:** The first step in method development is to characterize the sample as regular or spherical. Regular samples are a mixture of small molecules (<2000 Daltons) that can be separated using more or less standardized starting conditions. Separations in regular samples respond in predictable fashion to change in solvent strength (%B) and type (Acetonitrile, methanol) or temperature. A 10% decrease in %B increases retention by about threefold, and selectivity usually changes as either %B or solvent type is varied[3-4].

It is possible to isolated many consistent samples just by erratic solvent strength and type. Therefore, RPC method development for all systematic samples (both neutral and ionic) can be carried out primarily in the same way[10].

- **The column/Stationary phase:** Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended[3-4, 10]. Some important factors need to be considered while selecting column in RP- HPLC are summarized in **Table 2.**

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C2), butylsilane (C4), octylsilane (C8), octadecylslane (C18), base deactivated silane (C18) BDS phenyl, cyanopropyl (CN), nitro, amino, etc. Generally longer columns provide better separation due to higher theoretical plate numbers. As the particle size decreases the surface area available for coating increases. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability[3, 10].

V. FACTORS AFFECTING COLUMN EFFICIENCY

Table no. 2: Factors affecting column efficiency

FACTOR(S)	EFFECT ON COLUMN EFFICIENCY
Column Length	• Choose longer columns for enhanced resolution. • Choose shorter column for shorter analysis time, lower back pressure and fast equilibration and less solvent consumption.
Column internal diameter	• Choose wider diameter column for greater sample loading. • Choose narrow column for more sensitive and reduced mobile phase consumption.
Particle shape	• Choose spherical particles for lower back pressure, column stability and greater stability. • Choose irregular particles when high surface area and high capacity is required.
Particle size	• Choose smaller particle (3-4 µm) for complex mixture with similar components. • Choose larger particle (5-10 µm) for sample with structurally different compounds. • Choose very large particle (15-20 µm) for preparative separation.
Pore size	• Choose a pore size of 150 or less for sample with molecular weight less than 2000. • Choose a pore size of 300 or less for sample with molecular weight greater than 2000.
Surface area	• Choose end capped packing to eliminate unpredictable secondary interaction with the base materials. • Choose non-end capped phase for selectivity differences for polar

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	compounds by controlling secondary interaction.
Carbon load	• Choose high carbon loads for greater column capacities and resolution. • Choose low carbon loads for fast analysis.

The column should provide,

→ Reasonable resolution in initial experiments,

→ Short runtime,

→ An acceptable pressure drop for different mobile phases[11].

→

i. Mobile phase:

In many cases, the average term used for the mobile phases in reversed phase chromatography is "buffer". However, there is little buffering ability in the mobile phase solutions since they frequently contain strong acids at low pH with large kindnesses of organic solvents. Passable buffering volume should be maintained when working nearer to physiological situations[12].

ii. Organic solvent:

The organic solvent (modifier) is added to lower the polarity of the aqueous mobile phase. The lower the polarity of the mobile phase, the greater its eluting strength in reversed phase chromatography. Although a large variety of organic solvents can be used in reversed phase chromatography, in practice only a few are routinely employed. The two most widely used organic modifiers are acetonitrile and methanol, although acetonitrile is the more popular choice. Isopropanol (2-propanol) can be employed because of its strong eluting properties, but is limited by its high viscosity which results in lower column efficiencies and higher back pressures[3, 10].

Both acetonitrile and methanol are less viscous than isopropanol. All three solvents are essentially UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors. Acetonitrile is used almost exclusively when separating peptides. Most peptides only absorb at low wavelengths in the ultra- violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths[3, 10].

iii. Ion suppression:

The retention of peptides and proteins in reversed phase chromatography can be modified by mobile phase pH since these particular solutes contain ionisable groups. The degree of ionisation will depend on the pH of the mobile phase. The stability of silica-based reversed phase media dictates that the operating pH of the mobile phase should be below pH 7.5. The amino groups contained in peptides and proteins are charged below pH 7.5.The carboxylic acid groups,however,are neutralized asthe pH is decreased. The mobile phase used in reversed phase chromatography is generally prepared with strong acids such as tri-fluoroacetic acid (TFA) or ortho- phosphoric acid. These acids maintain a low pH environment and suppress the ionisation ofthe acidic groups in the solute molecules. Varying the concentrationofstrongacid componentsin the mobile phase can change the ionisation of the solutes and, therefore, their retention behavior[10].
The major benefit of ion suppression in reversed phase chromatography is the elimination of mixed mode retention effects due to ionisable silanol groups remaining on the silica gel surface. The effect of mixed mode retention is increased retention times with significant peak broadening[3].

iv. pH:

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Reversed phase separations are most often performed at low pH values, generally between pH 2-4. The low pH results in good solubility of the sample components and ion suppression, not only of acidic groups on the sample molecules, but also of residual silanol groups on the silica matrix. Acids such as tri-fluoroacetic acid, hepta-fluorobutyric acid and ortho-phosphoric acid in the concentration range of 0.05 - 0.1% or 50 - 100 mM are commonly used[3, 10].

Mobile phases containing ammonium acetate or phosphate salts are suitable for use at pH's closer to neutrality. Note that phosphate buffers are not volatile. It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkage area cleaved below pH 2.0; while at pH valued above 8.0 silica may dissolve[3-4].

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v. Absorbance:

An UV-visible detector is based on theprinciple of absorption of UV visible light from the effluent emerging out of the column and passed through a photocell placed in the radiation beam. UV detector is generally suitable for gradient elution work.Most compounds adsorb UV light in the range of 200- 350 A°. The mobile phase used should not interfere in the peak pattern of the desired compound hence it should not absorb at the detection wavelength employed[4].

vi. Selectivity:

Selectivity (α) is equivalent to the relative retention of the solute peaks and, unlike efficiency, depends strongly on the chemical properties of the chromatography medium.

The selectivity, α, for two peaks is given by;

$\alpha = k2'/k1' = V2-V0/V1-V0 = V2/V1$

Where V1 and V2 are the retention volumes, and k2′/k1′ are the capacity factors, for peaks 1 and 2 respectively, and V0 is the void volume of the column. Selectivity is affected by the surface chemistry of the reversed phase medium, the nature and composition of the mobile phase, and the gradient shape.

Both high column efficiency and good selectivity are important to overall resolution. However, changing the selectivity in a chromatographic experiment is easier than changing the efficiency. Selectivity can be changed by changing easily modified conditions like mobile phase composition or gradient shape[3, 10].

vii. Temperature:

Temperature can have a profound effect on reversed phase chromatography, especially for low molecular weight solutes such as short peptides and oligonucleotides. The viscosity of the mobile phase used in reversed phase chromatography decreases with increasing column temperature. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process, decreasing solvent viscosity generally leads to more efficient mass transfer and, therefore, higher resolution. Increasing the temperature of a reversed phase column is particularly effective for low molecular weight solutes since they are suitably stable at the elevated temperatures[4].

viii. Viscosity:

Solvent of lowest possible viscosity should be used to minimize separation time. An added advantage of low viscosity is that high efficiency theoretical plate (HETP) values are usually lower than with solvents of higher viscosity, because mass transfer is faster. Viscosity should be less than 0.5 centipoise, otherwise high pump pressures are required and mass transfer between solvent and stationary phase will be reduced[3, 10].

ix. Detectors:

A large numbers of detectors are used for RP-HPLC analysis. However, among these the five dominant detectors used in LC analysis are the electrical conductivity detector, the fluorescence detector, the refractive index detector, mass spectrometry detector and the UV detector (fixed and variable wavelength). These detectors are employed in over 95% of all LC analytical applications.

The detector selected should be chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction, etc. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are[3-4, 10]:

- High sensitivity, facilitating trace analysis.
- Negligible baseline noise to facilitate lower detection.
- Low drift and noise level.
- Wide linear dynamic range (this simplifies quantitation).
- Low dead volume (minimal peak broadening).
- Cell design that eliminates remixing of the separated bands.
- Insensitivity to changes in type of solvent, flow rate, and temperature.
- Operational simplicity and reliability.
- Tunability, so that detection can be optimized for different compounds.
- Large linear dynamic range.
- Non-destructive to sample.

VI. APPLICATIONS

- Designing a biochemical purification.
- Purification of platelet-derived growth factor(PDGF).
- Purification of cholecystokinin-58 (CCK-58) frompig intestine.
- Purification of recombinant human epidermalgrowth factor.
- Process purification of inclusion bodies.

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VII. CONCLUSION

The development of analytical methods plays an important role in the discovery, development and manufacture of pharmaceuticals. Probably the RP-HPLC is most versatile and sensitive analytical procedure, and is unique in that it can easily handlemulti-component mixtures. When developing methods for pharmaceutical analysis by RP-HPLC, it is essential to have a good working understanding of chromatographic separation and how it varies with sample and different experimental conditions in order to achieve optimum separation. To effectively develop a HPLC method, most effort should be spent in method development and optimization, as this will improve the performance of the final method.

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REFERENCES

[1] Scott RPW: Principles and Practice of Chromatography. Chrom-Ed Book Series, 2003, 1-103.

[2] Ahuja S: Chromatography and Separation Science. Elsevier Science (USA), 2003, 1-16.

[3] Pooja Madhurkar, S.B. Bhawar and M.H. Kolhe, "A REVIEW ON: RP-HPLC." World Journal of Pharmaceutical Research, 2021, 10(8), 1148-1160.

[4] Abhijeet Jadhao, Deepak Ambhore, and Kailash Biyani, "Importance of RP-HPLC in Analytical Method Development: A Review." International Journal of Advanced Research in Science, Communication and Technology, 2022, 2(8), 345-351.

[5] Uday Patond, S.C. Kale, Ashish Gawai, and K.R. Biyani, "A Review on Analytical Method Development and Validation by High Performance Liquid Chromatography Technique." International Journal of Advanced Research in Science, Communication and Technology, 2022, 2(2), 545-557.

[6] M.E. Ghanjaoui, A. Mandil, A. Ait Sidi Mou, and R. Slimani, "High performance liquid chromatography quality control." International Journal of Advanced Chemistry, 2020, 8(1), 160-169.

[7] Siddhata Deshmukh, Ganesh Chavan, Suvarna Vanjari, and Rajendra Patil, "A Review on Analytical Method Development and Validation by High Performance Liquid Chromatography Technique." Journal of Pharmaceutical Sciences and Research, 2019, 11(11), 3599-3605.

[8] Azim Md. Sabir, Mitra Moloy, and Bhasin Parminder, "HPLC method development and validation: A Review." International Research Journal of Pharmacy, 2013, 4(4), 39-46.

[9] Murugan S, Thejaswini N, Zeenathaman S, Syam Vijayakar P, Sai Swathi Varma, and Niranjan Babu, "A Overview on High Performance Liquid Chromatography." American Journal of Biological and Pharmaceutical Research, 2014,1(4), 156-159.

[10] Ravindra Laware, Sudarshan Kakad and Ninad Padalkar, "An overview on development and validation of RP-HPLC method for estimation of drugs in bulk and pharmaceutical dosage forms." World Journal of Pharmaceutical Research, 9(6), 770-782.

[11] Reversed Phase Chromatography: http://wolfson.huji.ac.il/purification/PDF/ReversePhase/AmershamRPCManual.pdf