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Analytical Method Validation in HPLC: A Regulatory Perspective Based on ICH Guidelines

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Abstract: *The ongoing processes of method development and validation follow the development of pharmaceutical goods. Existing analytical techniques may need to be modified in response to changes that occur throughout the drug development process. Further validation may be necessary as a result of these methodological changes. If the current methods are inconsistent or unreliable, time-consuming, or too costly, the introduction of new techniques and enhanced instruments in the area of analysis may pave the way for more sensitive, accurate, and precise approaches. Therefore, ongoing efforts to develop and validate novel analytical methods are crucial for the expanding drug development programs.*

Keywords: HPLC, Validation, Development, Precision, Accuracy, Linearity

I. INTRODUCTION

Pharmaceutical analysis plays a crucial role in the quality assurance and control processes for pharmaceutical compounds and their formulations. It offers details on the identification, stability, content, and purity of excipients, active medicinal ingredients, and starting materials. Additionally, it guarantees pharmaceutical items used for therapeutic reasons are safe, effective, and of high quality. The purpose of analytical techniques is to determine the identification, potency, physical attributes, and purity of the medications we take. Techniques are created to facilitate drug testing versus specifications in long-term stability studies, quality release operations, and production. Evaluations of medication performance or safety and characterisation studies may also benefit from it.

High-pressure liquid chromatography is another name for HPLC. Forming column chromatography is crucial, where the stationary phase is made up of tiny particles (2.99–49.9µm pickings) packed in a column with a tiny pore (1.99–4.99mm), one end of which is connected to a source of a pressurized liquid eluent. Ion-exchange partition and adsorption are the three types of high-performance liquid chromatography that are most often used. Along with improved resolution, faster separation, and increased accuracy, precision, and sensitivity, HPLC offers advantages in many other areas. As a result, it has become the most used analytical method for quantitatively analyzing various chemical substances, biomolecules, polymers, and prescription medications.

HPLC is classed into:

1. Reverse phase chromatography
2. Normal phase chromatography
3. Ion exchange chromatography
4. gel permeation or size exclusion
5. liquid-solid chromatography
6. Liquid-liquid chromatography

The literature review contains a number of publications about the development and validation of methods using HPLC. According to ICH criteria, the goal of the current effort is to achieve the best chromatographic parameters that encompass every stage of method development and validation.

Primary Chromatographic Descriptors:

Characteristics of the chromatographic column, system, and necessary separation are typically filed using the following major parameters:

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1. Capacity factor or Retention factor (k)
2. Efficiency (Plate number, N)
3. Resolution (R)
4. Separation factor (Selectivity, α)
5. Tailing factor (T).

$$K = \frac{V_R - V_0}{V_0} = \frac{V_R'}{V_0} = \frac{t_R - t_0}{t_0}$$

The unit-less measure of a compound's or analyte's retention in a certain chromatographic system under specific conditions is called the retention factor (k), and it is defined as
where TR is the analyte retention time, T0 is the retention time of a non-retained analyte, V0 is the volume of the liquid phase in the chromatographic system, and VR is the analyte retention volume.
Conveniently, the retention factor is unaffected by the size of the column or the flow velocity of the mobile phase. Analyte retention is greatly impacted by every other chromatographic condition.

Efficiency (Plate Number, N):
"The measure of the degree of peak dispersion in a particular column, as such, is very essential to the characteristic of the column," is the definition of efficiency. Efficiency is computed as follows and represented as the number of theoretical plates (N):
N=16(tR /w)2
where w is the width of the baseline peak and tR is the analyte retention period.

Resolution (R)
"A measure of the quality of separation of adjacent peaks/bands in a chromatogram; overlapping bands have small R values" is how resolution is defined. Resolution is determined by calculating the breadth and retention duration of two neighboring peaks.

$$\mathrm{R} = 2(\mathrm{t2} - \mathrm{tl})/\mathrm{w1} + \mathrm{w2}$$

where w1 and w2 are the widths of the peaks' baseline, and t1 and t2 are the retention times of the first and second neighboring peaks, respectively. R is less than 0.9999 if dependability is low.

Separation Factor (Selectivity):
"The ability of the chromatographic system to discriminate between two different analytes present in the sample" is what is meant by selectivity (α). "The ratio of corresponding capacity factors" is how it is defined.

$$\alpha = \mathrm{k2/k1} = \mathrm{tR2} - \mathrm{t0/tR1} - \mathrm{t0}$$

Tailing Factor (T):
"The measure of peak symmetry" is the tailing factor (T). It has a value that rises with deeper tailing and mimics completely symmetrical peaks. Under some circumstances, values below unity could be shown. The accuracy decreases with increasing peak asymmetry.
It is stated as

$$\mathrm{T} = \frac{\mathrm{w0.05}}{\mathrm{2f0.05}}$$

Where, w0.05 is the width of the peak at 5 % height, f0.05= half of the peak width at 5% peak height. Ideally, the T value should be ≤ 1.999.

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The Objective of the Research Study:

The launch of a wide range of medications and various pharmaceutical formulations is expanding in the market at an unsustainable pace in the current scenario. To maintain the high quality of the drug formulations, it is essential to identify one's medications and pharmaceutical dosage forms utilizing precise, sensitive, and accurate analytical procedures.

1 To carry out an initial evaluation of the GTH drug.
2 To develop the RP-HPLC method for the estimation of GTH in bulk and formulation.
3 To validate the evolved RP-HPLC method for the estimation of GTH in bulk and formulation according to the ICH Q2 (R1) guidelines.

Strategy for Method Development in HPLC:

Three major steps are involved in strategy for method development:

- Consider analyte chemistry.
- Optimized selectivity.
- Use of Scouting gradient.

Consider Analyte Chemistry:

Retention occurs when an analyte interacts with the HPLC stationary and mobile phases. There may not be any separation in the absence of this interaction. As a result, some of the decisions you make about the whole HPLC process, such as column selection and mobile phase design, will be influenced by reviewing certain basic chemistry concepts related to your analytes. The polarity of your analytes is of particular significance. For retention and separation under reversed-phase HPLC conditions on hydrophobic stationary phases (such as C18, C8), nonpolar analytes are perfect. A more polar stationary phase might be needed for polar or ionizable analytes. To achieve separation, for instance, cyano or amino, or maybe an unusual chromatographic mode in conjunction with hydrophilic interaction liquid chromatography, normal phase, or mixed-mode. In order to obtain retention and prevent peak shape issues and tailing with bases, it is crucial to appropriately adjust the pH of the mobile phase for ionizable analytes, which may be difficult to keep below reversed-phase HPLC settings while in their ionized state. Designing a mobile phase may greatly benefit from an understanding of analyte pKa. To make the analyte unbiased, the mobile phase should be two pH units below or above this value, depending on whether the analyte is an acid or a basic. Screening a range of pH levels and analyzing the effects on resolution and retention may be helpful.

Optimize Selectivity:

Selectivity is the chromatographic characteristic that affects HPLC the most out of all those that influence decision making. Specific technique factors, such as temperature, column size, or the composition of the mobile phase, may change each of the three criteria (specificity, accuracy, and efficiency). Selectivity is most affected by the stationary phase of the column, however. Thus, screening columns with unique phase chemistries is the second piece of advice for developing HPLC methods. The fact that orthogonal stationary phase chemistries may also reveal underlying impurity peaks that would not have been picked up if best a similar column chemistry had been attempted is just one of the many benefits of this method, which also allows you to quickly identify the best stationary phase in your separation. Temperature, solvent components, and mobile phase pH are other method characteristics that might influence selectivity.

Table 1: HPLC detector uses

Detector	Types of Molecules	General Use, Comments
RI (Refractive Index Detector)	Carbohydrates, Polymers	Molecules that do not have a UV chromophore.
UV/Vis, PDA	Organic molecules, Biomolecules, except carbohydrates.	PDAs are typically used for any molecule that absorbs light between 190 and 800nm when the molecule

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		in the mixture absorbs at different wavelengths or when λ maxis unknown. PDAs can be used for determining peaks.
Fluorescence	Aromatic compounds which Exhibit fluorescence. Carbohydrates, Polymers	Generally used for applications that requires Extremely high sensitivity.
ELSD (Evaporative Light Scattering Detector)	Organicmolecules, Biomolecules	Molecules that do not have a UV Chromophore can be used with gradients, generally more sensitive than an RI detector.
MS(Mass Spectrometer)	Organic molecules, Bio molecules	Can be used to detect and determine the mass of any molecule that can be ionized and is within the Mass range of the specific MS.
MS/MS	Organic molecules, Bio molecules	As above but allows more detailed structural studies to be performed.
ECD (Electrochemical Detector)	Carbohydrates are many other organic molecules whose redox potential is different from the mobile.	Can provide extra sensitivity and sensitivity for molecules that are not readily detected by devices.
Conductivity	Cations and Anions	Used for ion chromatography.

Use A Scouting Gradient:

A linear gradient from 5–10% C to 100% is known as a scouting gradient. Give a certain amount of time (15 minutes is typical). To ensure that all of the sample additives were eluted, the elution intensity is maintained at 100% C for a few minutes. Acetonitrile and water or buffer, assuming there are ionizable analytes, are often the preferred eluents for reversed-phase gradient elution. For usage in LC-mass spectrometry, the use of hazardous buffers, such as ammonium formate, will result in a technique that complements mass spectrometry.

Numerous details on the mobile phase composition, column chemistry, and evaluation method needed for the separation may be found in the obtained chromatogram. The assessment requires a more powerful C solvent or a considerably less retentive column if chemicals are eluted throughout more than 20% of the gradient duration after the gradient ends.

Steps Concerned in Method Development:

- Understanding the Physicochemical Properties of the drug molecules.
- Choice of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation
- Method optimization
- Method validation

Method Development:

Optimized Chromatographic Conditions:

Optimized chromatographic conditions were designed for the column, mobile phase, wavelength, flow rate, temperature, injection volume, diluent, and run duration.

Chemicals and Reagents: Every chemical and reagent used is of HPLC quality. Prior to use, all solvents and solutions were degassed and filtered through membrane filters with pore sizes of 0.45 µm. The solubility of the substances it

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dissolves in is another crucial factor in the development of HPLC methods. The solubility of medicinal ingredients, their contaminants, and degradation products should all be taken into consideration when selecting the solvent. To improve the analyte's peak form, the solvent and mobile phase should be compatible. Use only HPLC-grade solvents and take solvent purity into account. While reversed-phase HPLC necessitates polar solvents like water, acetonitrile ethanol, or methanol, normal phase HPLC systems employ non-polar solvents like hexane, diethyl ether, dichloromethane, isopropyl alcohol, iso-octane, etc. The solvent's physicochemical characteristics influence the selection of the mobile phase. The following characteristics are thought to be crucial for choosing a certain solvent: toxicity, chemical inertness, polarity, and miscibility with other solvents. The solvent's capacity to elute a component from the column is indicated by the polarity index.

Instruments: For the development and validation of methods, an analytical balance, HPLC, filter membrane, sonicator, and vertex machine are all necessary.

Preliminary analysis of drug:

Description: The color and texture of the drug's API were examined.

Solubility: The drug's API was placed in test tubes and tested for solubility in buffer, DMSO, methanol, acetone, ethanol, water, and acetonitrile.

Melting Point: A capillary tube containing the drug's API was placed in a melting point equipment, and the results of the analysis were recorded.

Preparation of Mobile Phase:

A suitable diluent is used to create the mobile phase, which is then filtered through a 0.45µm membrane filter and sonicated for 15 minutes to degas the solutions. Standard stock solutions and Drug Product Stock Solutions are then prepared using the diluent. One of the most important factors is the choice of mobile phase, which promotes interactions between the solute and stationary phase. When choosing the mobile phase, caution must be used; strong acids, strong bases, and halide solutions must be avoided. In the mobile phase, buffers are often used to get reliable chromatographic results. Ionic analyte retention is managed with the use of buffers. In ionic form, the analyte typically elutes rapidly, becomes polar, and spends less time on the stationary phase. The pH of the buffer is crucial in regulating the ionic analytes' selectivity. Generally speaking, basic analytes get ionized when buffer pH falls, while acidic analytes become ionized and more polar when buffer pH rises. At least 1.0 pH units should separate the chosen mobile phase's pH from the analyte's pKa value. This aids in regulating peak shape and run-to-run consistency while confirming that the analytes are either 100% ionized or 100% non-ionized. It always employs a buffer in the mobile phase's aqueous phase, which makes the procedure more robust.

Selection of Analytical Wavelength:

They were scanned in a UV-visible spectrophotometer between 200 and 400 nm in order to determine the proper wavelength for determining the drug solution in the mobile phase. The analytical wavelength, also known as the drug's lambda max, is the wavelength at which the molecule exhibits the most absorption.

Analytical Method Validation:

The process of confirming that the analytical technique's performance characteristics are appropriate for the intended use is known as method validation. Chromatographic techniques must be verified before being used on a regular basis. Using the same matrix as the intended sample, all of the method's variables—including sampling process, sample preparation, chromatographic separation, detection, and data evaluation—should be taken into account in order to provide the most accurate findings. Laboratory investigations are the only way to confirm the validity of an analytical procedure. The report should include documentation of any validation trials used to support assertions or conclusions on the method's validity.

Specificity:

The ability to precisely determine the amount of the target analyte in the presence of other additives that are anticipated to be present in the sample matrix is known as specificity. The degree of influence from such topics is measured by specificity. To ensure a peak response, it may include various active ingredients, excipients, contaminants, and breakdown products. Resolution, plate count, and tailing factor are used in the analysis to determine specificity, and the results are recorded accordingly.

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The capacity to distinguish between compounds with closely comparable structures or by comparison to established reference materials are two ways that specificity is shown for identification purposes. In assay and impurity testing, the resolution of the two nearest eluting substances in the sample serves as an example of specificity.

Typically, the compounds consist of an impurity and main components or active substances. If the sample contains impurities, it must be shown and shown that the presence of spiking items, such as excipients or contaminants, has no effect on the assay. The test findings are compared to a second, well defined and designed technique if contaminants are not present. The two outcomes are compared for the test. The impurity profiles are compared for impurity testing.

Resolution, plate count efficiency, and tailing factor are used to quantify and record specificity in a separation. Modern photodiode array detectors that computationally analyze spectra obtained throughout a peak as a measure of peak homogeneity may also be used to assess specificity. ICH also uses the word specificity and categorizes it into two distinct groups: assay tests and identification tests.

The capacity of an analytical technique to precisely and precisely measure the analyte of interest in the presence of potentially anticipated components in the sample matrix is known as selectivity. An analytical technique is said to be selective if it is able to qualitatively identify the analyte while also separating and resolving the different components of a mixture. It has been noted that the major peaks did not have any diluent or placebo peaks. As a result, the drug estimation chromatographic method was very specific and selective. Studies on specificity showed that the excipients had no effect on the analysis.

High resolution of the separated compounds was seen in the chromatogram, which displayed a clean baseline free from excipient contamination. This suggests that the suggested approach was targeted.

Demonstrating specificity in the test necessitates that the process be unaffected by the presence of excipients or contaminants. This may be accomplished in practice by adding suitable amounts of contaminants or excipients to the drug compounds or product and proving that the test is unaffected by their presence. Specificity may be shown by comparing the test findings of samples containing impurities or degradation products to a second, well-characterized process in the event that the degradation product impurity standards are not available.

The target analyte's specificity was supported by a comparison of the chromatograms of the test, standard, and blank samples. It has been noted that the major peaks did not have any diluent or placebo peaks. Studies on specificity showed that the excipients had no effect on the analysis. The chromatogram showed no excipient interference. This suggests that the suggested approach is targeted.

Linearity and Range:

The capacity of the procedure to provide test findings that are exactly proportionate to the analyte concentration within a certain range is known as linearity. The variance of the regression line's slope is often used to express linearity. The range is the distance between the analyte's higher and lower values that can be found using the written technique with precision, accuracy, and linearity. Typically, the range and the test results produced by the procedure are stated in the same units. According to the ICH recommendations, there must be at least five concentration levels and specific minimum ranges.

Five to six injections of five or more standards with concentrations ranging from 80 to 120 percent of the predicted concentration range are used to determine linearity. The answer is immediately proportionate to the analyte concentration in the sample or proportionate to the mathematical calculation that has been correctly stated. The results that had an intercept that was no longer significantly different from zero were subjected to a linear regression equation. It should be shown that the procedure has no influence on accuracy if a significant non-zero intercept is produced. A minimum of 80–120% of the target concentration is required for the test. The reporting level of each impurity to 120% of the specification is the lowest range for an impurity test.

The peak reaction was found to be linear and directly proportional to the drug's concentration. At 0.999, the correlation coefficient was found to be substantially within the recognized range.

Accuracy:

The exactness of an analytical procedure or the degree of agreement between the value discovered and the value that is recognized as either a conventional true value or an acceptable reference value is known as accuracy. It is calculated by spiking samples in a blind trial to determine the percentage of analyte recovered by assay. Accuracy values for the drug substance test are acquired by comparing the findings to either a second, well-characterized technique or the

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examination of standard reference material. The accuracy of the drug product assay is assessed by examining synthetic mixes that have been tampered with with known amounts of components.

The ICH methodological guideline suggests gathering data from at least nine determinations over at least three concentration levels within the designated range (e.g., three concentrations, three replicates each) in order to demonstrate accuracy. Either the difference between the mean and real value with confidence intervals or the percentage recovery of the known, extra amount should be used to report the data. The procedure was found to be accurate since the findings' percentage recoveries indicate that the recoveries are well within the acceptable range.

Precision:

The percent relative standard deviation for a statistically significant number of samples is often used to describe precision, which is a measure of an analytical method's degree of repeatability under typical operating conditions. The ICH states that three degrees of accuracy should be achieved: reproducibility, intermediate precision, and repeatability. When a procedure operates under the same inter-assay precision circumstances over a short period of time, repeatability results. It should be calculated using at least nine determinations that span the procedure's designated range, such as three levels and three repeats each, or from at least six determinations that are 100% of the test or goal concentration. The findings of lab variance brought on by unforeseen circumstances, such as various days, analysts, equipment, etc., are known as intermediate precision. Experimental design should be used to monitor the impact of the distinct variables in order to determine intermediate precision.

Limit of Detection:

The lowest analyte concentration in the sample that can be identified, although not always quantified, is known as the limit of detection. Limit tests determine whether an analyte is above or below a certain threshold. LOD may be computed using the following formula: LOD = 3.3(S.D/A) based on the slope (S) of the calibration curve at values close to the LOD and the standard deviation of the response. The response's standard deviation is calculated using the residual standard deviation of the regression line, which is the standard deviation of the blank. The standard deviation of the regression lines' y-intercept may also be used to calculate it. The process used to calculate LOD must be supported and documented. The created approach should be validated by analyzing a suitable number of samples.

Limit of Quantitation:

The detection of the smallest quantity of analyte in the sample is known as the limit of quantitation (LOQ). It is the parameter that provides the true analyte concentration in a sample that may be ascertained during analysis with a suitable level of precision and accuracy under the method's specified operating parameters. The slope (A) of the calibration curve and the response's standard deviation (S.D.) are used to calculate the limit of quantification (LOQ). LOQ = 10(S.D/A) is the calculation formula. The response's standard deviation is calculated using the standard deviation of the blank (S.D.), the regression line's residual standard deviation, or the standard deviation of the plot's regression lines' y-intercepts. The process for determining LOQ should be supported and documented, much like LOD. To validate the suggested approach, a sufficient number of samples should be evaluated at the limit.

Robustness:

The ability of a technique to withstand minor but intentional changes in its parameters is known as robustness. A number of technique characteristics, such as temperature, ionic strength, pH, and the proportion of organic solvent, are used to assess a method's robustness and assess its impact on the method's outcomes. Depending on the kind of research being conducted, the robustness assessment should be taken into account at the development stage. It should demonstrate the validity of the analysis with regard to intentional changes in the parameters of the approach. A slight modification in the chromatographic conditions at a concentration equivalent to the standard concentration was used to conduct a robustness test, and the percentage change in the findings was computed. Here, resilience was achieved by altering the detector's wavelength, mobile phase ratio, and mobile phase flow rate.

A number of parameters had been purposefully changed in order to evaluate the RP-HPLC method's robustness. A range of flow rates and minor variations in wavelength changes were covered by the parameters. Under such experimental conditions, standard concentration was examined. It was discovered that the chromatograms had not changed much, proving that the developed method was now reliable.

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System Suitability:

Tests for system appropriateness are essential to chromatographic procedures. These tests serve to confirm that the system's resolution and repeatability are sufficient for carrying out the analysis. The idea behind system suitability testing is that the tools, electronics, analytical processes, and samples make up a cohesive system that can be assessed as a whole. Making ensuring the whole testing system—including the equipment, reagents, columns, and analysts—is appropriate for the intended use is the aim of the system suitability test.

The process of verifying a system's performance before to or during the study of unknowns is known as system appropriateness. Plate count, tailing factors, resolution, and repeatability percentage are examples of parameters. For six trials, the RSD retention time and area are calculated and compared to the method's requirements. During the examination of a system suitability "sample" consisting of a combination of primary components and anticipated by-products, these characteristics are assessed.

As analysts gain more expertise with the assay, the system suitability test approach should be updated, much like the evolution of the analytical technique. overall chromatographic suitability and system performance consistency.

Chromatograms were examined for many criteria, including retention duration, asymmetry, and theoretical plates, to determine whether or not they adhered to the suggested limit.

Table 2: System Suitability Test Parameters

Parameter	System Suitability
Theoretical Plates (N)	To be greater than 2000
Tailing Factor (T)	Generally, should be less than 2
Resolution (R)	Usually, more than 2
Repeatability	RSD $\leq$ 1% for n $\geq$ 5 is acceptable.
Capacity Factor (k)	Should be greater than 2

Ruggedness:

The USP defines ruggedness as the percentage RSD that represents the degree of repeatability of the findings obtained under a range of situations. Different labs, analysts, equipment, chemicals, days, etc. are some examples of these situations. The ICH did not particularly discuss roughness in the definitions and nomenclature guideline. However, this seeming exclusion is just semantic in nature, since ICH opted to precisely address the subject of roughness, as was previously mentioned.

II. CONCLUSION

The following factors led to the creation of HPLC techniques for drug analysis: 1) When pharmacopeias do not provide an official analytical technique for analyzing a drug or drug combination. 2) When patent rules prevent an analytical technique for an existing medication from being published in the literature. 3) When the drug formulation lacks analytical techniques because of the influence from the formulation excipients. 4) It is discovered that analytical techniques for measuring the analyte in bodily fluids are not accessible. 5) Expensive reagents and solvents can be required for the current analytical methods. Complex extraction and separation processes can also be required. The first step in creating an HPLC technique is always to go through the chromatographic literature to see whether the analysis has been done before and how. This will at least provide a sense of the necessary circumstances and might perhaps eliminate the need for extensive experimental effort. New techniques are being developed for the examination of innovative goods in situations when there are no official procedures available. In addition to saving time, innovative techniques are being developed to test current pharmaceutical and non-pharmacopoeial items with greater sensitivity, accuracy, and precision. Trial runs are used to refine and verify these techniques. In the comparative laboratory data, alternative approaches are suggested and implemented to replace the current technique, taking into account all relevant advantages and disadvantages. Method development is a multi-phase process that might take months to finish, depending on the method's objectives and level of complexity.

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Table 3: Validation of Analytical Procedures

Type of analytical procedure	**Identification**	**Testing for impurities**		**ASSAY Dissolution (measurement only) content/ potency**
Characteristics		**quantitative limit**		
Accuracy	-	+	-	+
Precision Repeatability Inter day Precision	- -	+ +	- -	+ +
Specificity	+	+	+	+
Detection Limit	-	-	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

Worldwide, one of the most important analytical methods for both qualitative and quantitative drug analysis is high-performance liquid chromatography (HPLC). HPLC analysis is used for most medications recommended in official pharmacopeias. Pre-formulation, formulation, manufacturing, quality assurance, and marketing of pharmaceutical products are all related to method development and validation, which are crucial components of drug development programs in the pharmaceutical industry. The process of developing an analytical technique is a complex one that might take many hours or months. The fundamental practical features of HPLC are covered in this review article, along with advice on how to create HPLC analytical techniques. An overview of the requirements and several stages involved in the development of HPLC analytical methods is given in this paper.

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