IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 14, Issue 9 Series. I (September 2024), PP. 08-28
www.iosrphr.org

A Comprehensive Guide to Exploring the Process of HPLC Method Optimization, Development, and Validation: Insights into Chromatographic Parameter Variability

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Received 01 September 2024; Accepted 11 September 2024

Abstract: This abstract provides an overview of the comprehensive guide on HPLC method optimization, development, and validation, focusing on insights into chromatographic parameter variability. It emphasizes the importance of HPLC in ensuring the quality, safety, and efficacy of medications through pharmaceutical analysis. The abstract highlights the principles of separation underlying HPLC, the role of instrumentation in enhancing selectivity, and the systematic approach to method development. Additionally, it discusses the guidelines for analytical method validation to ensure the accuracy and reliability of HPLC methods. The abstract concludes by addressing the impact of chromatographic parameter variability on method performance and the importance of managing these factors for consistent and reliable results in pharmaceutical analysis.
Keywords: HPLC method optimization, Pharmaceutical analysis, Chromatographic parameter variability, Method development, Analytical method validation.

I. Introduction:

High-Performance Liquid Chromatography (HPLC)[1] stands as a cornerstone analytical technique in the realm of chemical and biochemical analysis. Its widespread applicability, from pharmaceuticals to environmental monitoring, highlights its pivotal role in both qualitative and quantitative determinations of complex mixtures. The meticulous process of HPLC method optimization, development, and validation, therefore, becomes a subject of paramount importance for researchers and industry professionals alike. This review aims to elucidate the intricate steps involved in these processes, offering a comprehensive guide enriched with insights into the variability of chromatographic parameters.

Optimization of HPLC methods [2] involves fine-tuning a multitude of parameters, each contributing to the overall efficiency, resolution, and sensitivity of the analysis. These parameters include, but are not limited to, the choice of stationary and mobile phases, flow rates, temperature, and detection methods. The interplay between these factors often dictates the robustness and reproducibility of the analytical method. Consequently, understanding their impact and how to manipulate them effectively is crucial for achieving optimal performance. Method development in HPLC is an iterative process that combines empirical data with theoretical principles. It requires a systematic approach to experiment design, guided by an understanding of the chemical properties of the analytes and the behavior of the chromatographic system. This stage often involves the selection of suitable columns, the composition of the mobile phase, and the establishment of gradient programs. Each decision made during this phase influences the separation efficiency and the overall analytical outcome.

Validation of HPLC methods [3-12] is the final, yet critical, step to ensure the reliability and accuracy of the analytical results. This process involves a rigorous assessment of various performance characteristics, including precision, accuracy, specificity, linearity, range, limit of detection (LOD), and limit of quantitation (LOQ). By adhering to established validation protocols, one can ascertain that the method is fit for its intended purpose, providing confidence in the data generated.

Variability in chromatographic parameters presents both challenges and opportunities in HPLC method development. Factors such as column age, solvent quality, and instrument performance can introduce variability, affecting the consistency of the results. However, these challenges can be mitigated through systematic optimization and robust validation strategies. This review will delve into these aspects, offering practical insights and strategies to manage and leverage variability for improved method performance.

The journey from HPLC method optimization to validation is complex yet crucial for accurate and reliable analytical outcomes. By exploring the nuances of chromatographic parameter variability, this review aims to equip practitioners with the knowledge and tools needed to develop and validate robust HPLC methods. Through a detailed examination of each stage, we hope to provide a valuable resource for enhancing the efficacy and reliability of HPLC analyses across various applications.

Selectivity of H PLC-Method Development:

HPLC-method may be used to evaluate the majority of pharmaceuticals because of its speed, specificity, accuracy, and precision, as well as the fact that it can be automated, which removes the need for time-consuming extraction and isolation processes. Some of the benefits include:

Effortless.
Sensitivity has increased
Enhanced clarity
Reliable columns
Suitable for low-volatility compounds
Recovering, managing, and maintaining samples are made simple.

Each module's multiple functionalities may be easily programmed. Before the workday starts, a stable baseline and an equilibrated column may be obtained by starting the detection light and pump in a time-programmable operating sequence.

Excellent reproducibility of retention times.
There is no need for additional hardware to change the injection volume from 0.01 to 100 microlitres.
Analysis of data may be as flexible as needed.
Suitable for avoiding contamination by impurities.
Larger-scale preparative liquid chromatography may be performed using this device.

Column Role:

The HPLC column is the procedure's heart and soul, and it is critical to the separation process. A successful separation can only be achieved if the column is selective, efficient, and reproducible. All of these characteristics are reliant on the quality of the columns and packaging materials produced by the column manufacturer. A completed bonded phase's characteristics are influenced by the silica backbone's metal content and silanol activity throughout the manufacture and bonding process. Only high-quality silica and a precise bonding procedure can guarantee accurate readings. USP L1 and L7 are the most often utilized C_{18} (octadecylsilane) and C_8 (octylsilane) reversed phases.

Initial Mobile Phase Selection:

Reversed-phase HPLC's movable phase is its most critical component. An important factor is which movable phase is employed. If the analyte molecules are ionized, they may either accelerate or inhibit this process, as well as protect the adsorption centers on the adsorbent surface. In the development of an isolation technique, selecting the correct movable step is the second most critical step. An analyte must be dissolved in the movable phase until it reaches a sufficient concentration for detection.

Flow rate role:

Increasing the flow rate has a minimal influence on resolution, but it is achievable. Isocratic separation requires a higher flow rate than gradient isolation. The lowered flow rate will result in lower column back pressure. As a result, the run time rises when the flow rate is lowered to improve the resolution somewhat.

Temperature role:

Temperature is a variable that may affect selectivity, although its effect is minimal. As the temperature rises, the k' decreases for neutral substances, while the effect is less significant for partly ionized analytes. An increase in temperature of 1 o C results in a 1 to 2 percent drop in k' for both neutral and ionic samples, as observed by Snyder et al.

Role of pH:[13]

The selectivity of the separation will be affected by PH, which is another element in the resolution equation. Sample retention increases in reversed-phase HPLC for hydrophobic analytes. A base (B) or acid (HA) gets ionized and so becomes less interacting with the column's binding sites as it becomes hydrophilic (less hydrophobic).

$$HA \rightarrow H^+ + A^-$$

$$B + H^+ \rightarrow BH^+$$

Hydrophobic — Hydrophilic

(More retained on column) — (Less retained on column)

A considerable reduction in K' has occurred as a result, with less ionized analytic remaining on the column. When the pH of the analyte (pKa) is equal to its ionization potential (pI), half ionization occurs. (pI). For retention, it is feasible to assure almost full unionization since most pH-induced fluctuations occur within 1.5 pH units of the pKa value. In reversed-phase HPLC, pH values between 1 and 8 are often referred to as low pH (i.e., pH values between 1-4) and intermediate pH values (i.e., pH values between 5 and 8). (i.e.,4-8). At low pH values, peak tailing may be decreased and technique robustness can be enhanced. To maximize efficiency and analyte retention and selectivity, it's best to operate in the intermediate range.

Chromatographic circumstances influence parameters:

Column efficiency (N)
Capacity factor (K')
Resolution factor (R_S)
Retention Factor (R_f)
Retention Period (Rt)
Relative retention (Rr)
Peak asymmetry factor (As**)**

Column efficiency (N)

Calculating chromatographic column efficiency using theoretical plates (N)

$$\mathbf{N = 5.54\ X t_R^2 / W_h^2}$$

A baseline distance of injection from the peak of interest's highest point to the perpendicular lowered at tR=retention time. Which is the width of the peak of interest at half its peak height, measured in units of tR. Theoretical plate count may be stated in meters (N).

$$\mathbf{N = L / H}$$

Where, L = length of column in meters; H= height of theoretical plates

Several things increase the number of column plates:

Columns that are densely packed
Longer articles
Reduced velocities (but not too low)
Column packing particles that are smaller in size
Higher temperature and lower viscosity in the mobile phase
Molecules of a smaller size

Capacity factor (K'):

The capacity factor is determined by the ratio of solute molecules in the stationary phase to solute molecules in the movable step.

Using this formula, you may calculate how long a solute will remain in solution.

$$\mathbf{(K') = t_R - t_M}$$

Where,

t_R= duration of solute retention

t_M= retention time of an unretained component.

Selectivity may be reduced by having a low Dm value since peak elution is occurring close to the solvent front. For a peak of interest, a DM value of 1 is suggested as a minimum.

To alter how long the test material remains in the sample, it is possible to alter the mobile phase's concentration or composition. Normal-phase columns tend to have shorter retention times when polar solvent concentrations are increased, whereas reversed-phase columns tend to have longer retention times when this concentration is increased.

Resolution factor (R_S):
Here's a method for figuring out how much time passes between two peaks of the same height.

$$R_s = 1.18(t_{R2}-t_{R1})/(W_{b1}+W_{b2})$$

Where,
Each peak's maximum is denoted by a perpendicular line drawn from the injection site to the bottom left and right corners of the perpendicular.
Wb1 and Wb2 are the peak widths obtained at half peak height and are measured in the same units and tR1 units as tR2.

Retention Factor (R_f):
It is possible to calculate how far one molecule travels in a particular solvent using the retention factor, Rf. To evaluate whether an unknown chemical is similar or identical to another, the Rf value may be employed. When the Rf values of two compounds are almost or the same, this suggests that the two compounds are identical or very similar.

$$R_f = D_1 / D_2$$

Where,
D_1 = How far the color traveled after it was applied, calculated by tracing its path from the color's origin to its destination.
D_2 = The total distance traveled by the solvent.

Retention time (R_t):
A column's retention time is the amount of time it takes for a specimen to be eluted from it. The length of retention is measured in minutes or seconds. This computation is also closely related to the distance traveled on paper, which may be calculated in centimeters or millimeters.

$$t_R = t_s + t_m$$

Where t_S is the time of analyte spent in the stationary phase
TM is the time analyte spent in the mobile phase

Relative retention (Rr):
The formula for calculating the relative retention (r) is as follows:

$$r = T_{R2}\text{-}tm / T_{R1}\text{-}tm$$

Where,
t_{R2} = peak retention period of interest
t_{R1} = reference peak retention period
T_m = Unretained component retention time

Peak asymmetry factor (As):
Applying the following formula, you can get the asymmetry factor for a peak.

$$As = W_X / 2d$$

Where,
Wx Peak width at 5% of peak height, measured from the baseline.
d = Peak maximum to peak leading edge distance, expressed in Wx units, for a 5% reduction in height. When a number is more than 2, it might lead to erroneous quantification.

Peak symmetry is influenced by retention and solvent effects, as well as solute incompatibility with the movable step or an excessive void forming at the column's entry. Tailing may occur in reverse-phase chromatography due to leftover silanol groups in the stationary phase (poor peak symmetry).

Reversed-Phase Liquid Chromatography Buffers:
It is possible to make a sensible and acceptable choice for reversed-phase liquid chromatography if you understand the fundamental impact of pH on the retention of ionic analytes and study key C• properties of buffer alternatives (RPC). Solubility or compatibility limits with detection methods, for example, may need a change of this option. The practical aspects of buffer preparation are critical for reproducible and trouble-free operations. A considerable influence will be seen on the retention of analytes at pH levels less than 1.5 pH units below their pKa. As a result, it is possible to comprehend the significance of managing the pH of the mobile phase while developing novel procedures. If the mobile phase is not ionic, the pH does not influence analyte retention.

Method Development Guide:

Defining separation objectives based on sample information.

↓

Special procedures and sample handling are required.

↓

Decide on the detector and the detector settings.

↓

Select a method of payment: preliminary run: determine the ideal separation conditions.

↓

Optimize the circumstances for separation.

↓

Verify the procedure.

Figure No 2: Method validation selection

S.no	Parameters	Recommendations
1	Theoretical plates (N)	> 2000
2	Tailing factor (T)	$\leq$ 2
3	Resolution (Rs)	> 2 between the peak of interest and the closest eluting potential interference
4	Repeatability	RSD $\leq$ 2 %
5	Capacity factor (k^1)	> 2.0

Table No 2. HPLC Parameters limits

Guidelines for Analytical Method Validation:

"When a technique isn't thoroughly validated, "time, money, and resources" are lost.

Method Validation:

It is known as method validation when a method's performance characteristics meet the requirements for its intended application. The complete validation process includes software validation, instrument certification, and system suitability. In the following part, we'll go over some of the analytical features that are often used in method validation. In this diagram, the focus is on liquid chromatography, however in a regulated laboratory, every method must be validated. In the process of method validation, the phrase "eight steps of method validation" is often referred to.

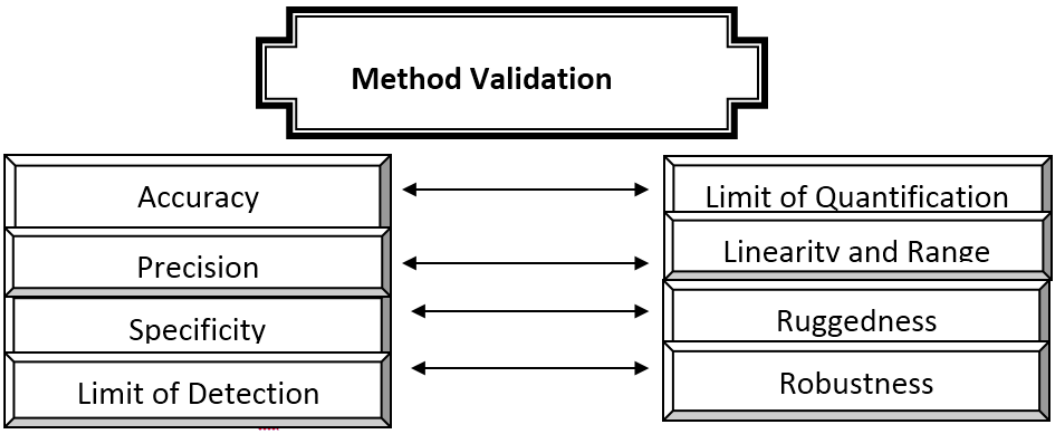

Figure No 3: Method validation

Validation of a quantitative technique in analysis requires consideration of these performance characteristics:

Method parameters	Short description
Precision	The approach may have made a mistake.
Repeatability	Under the best possible circumstances, the measurement of precision.
Inter day Precision	The fluctuation caused by various days, analysts, or equipment is precisely measured in the laboratory.

Robustness	Slight changes in the method's parameters might occur while it is being used (pH, mobile phase composition,)
Reproducibility	As a result of interlaboratory comparisons, precise measurements may be made.
Specificity	Analyte detection when additional chemicals are present
Detection limit	The smallest detectable conc. of sample
Quantification limit	The lowest amount of sample that can be accurately calculated with acceptable bias and accuracy.
Linearity	Method's ability to provide test findings that are proportionate to the specimen's concentration
Range	The method's concentration interval has a proven track record of good performance.
Stability	Analyte concentration in a sample is not affected by time.

Table No 3: Qualitative method validation requires consideration of these performance factors.

System suitability:

Before putting samples through the system, the operator must check each day that the HPLC equipment and method can deliver results of acceptable quality.

As a way to guarantee that the approach can provide findings with appropriate accuracy and precision, system suitability experiments are used.

System appropriateness criteria are often determined after the completion of method development and validation.

Specificity / Selectivity:

Many people confuse the words "selectivity" with "specificity." Strictly speaking, a technique that only detects one analyte is said to be specific, while a method that detects many chemical entities, some of which may or may not be distinct, is said to be selective.

It is a selective approach if the answer is different from all other replies. The word selectivity is more suitable since there are relatively few techniques that solely react to one analyte.

Without any interference from other components, the analyte will be clear. The extraneous peaks from stress tests or the addition of recognized substances should be shown to be baseline resolved from the original analyte in a typical HPLC Chromatogram or profile.

Accuracy:

The closer a measurement is to the genuine value, the more accurate it is. It is possible to accurately measure a sample's "true value" using a procedure with great precision. Recoverability studies are the most common method for determining accuracy, however, there are other methods.

A comparison with a standard reference

Recovering the analyte that was spiked into a non-matriculated sample

Analyte addition procedure.

Precision:

"The degree of agreement among individual test findings when the process is repeatedly performed to several samplings of a homogeneous sample" is one definition of precision. According to ICH, accuracy may be broken down into three distinct categories:

Repeatability

Inter-day precision and

Reproducibility

It is the ability to execute consistently over a short period that defines a method's repeatability. As long as all measurements (standards included) are consistent, a method is said to have intermediate precision. The Precision in various laboratories is often examined in collaborative research or method transfer studies to answer the issue of repeatability.

Linearity & Range:

Calibration plots of response vs concentration may be used to test the linearity of a technique by how closely they resemble a straight line in shape and behavior. If you want to assess linearity, you may utilize single or multiple analyte concentration measurements. A linear least-squares regression is used to further analyze the

data. With this plot, the necessary information on Linearity may be seen in terms of slope, intercept, and correlation coefficient.

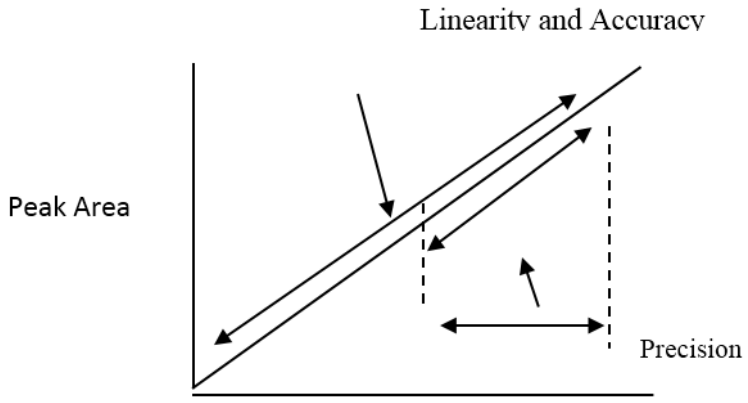

Figure No 4: Graph for linearity and accuracy

Range: Between the largest and lowest values of the varieties, as measured by the difference.

Range= largest value – smallest value

Limit of Detection:

In most cases, these limits are imposed on compounds in the medication substance or medication roduct [18] that are linked to one other. There are other requirements regarding to release and stability of drug substances and drug products that are specified in the specifications. The analyte detection limit is the lowest quantity of analyte in a sample that can be detected but not quantified. To detect low-level compounds, UV detectors are ineffective because of the likely aging of detector lamps or the wide range of noise levels produced by different manufacturers.

The proportion of the analyte's area count that is declared to be its detection limit is a simple approach to evaluate whether extraneous peak detection is achievable. The detection limit may be established in several ways if the process is non-instrumental.

Observational Insights
Using the Signal-to-Noise ratio
Based on the Response's Standard Deviation (SD) and Slope.

The following is an example of how to represent the LOD:

$$LOD = 3.3\ \sigma / S$$

Where, σ = the response's standard deviation
S = the slope of the Calibration curve
The slope S may be determined using the analyte's calibration curve.

Limit of Quantification:

It is important to know how low a concentration of an analyte can be reliably detected in a sample to get a reliable result in terms of precision and accuracy. It is possible to determine the quantitation limit in a variety of methods, depending on the nature of the process.

Based on Visual Evaluation
Based on Signal-to-Noise Approach
Based on the SD of the response and the slope

The following is an example of how to represent the LOQ:

$$LOQ = 10\ \sigma / S$$

Where, σ = the standard deviation of the response
S = how steep the incline of the calibration curve

Robustness:

"A measure of its ability to stay unaffected by tiny, but purposeful alterations in technique parameters" has been established by the ICH. Variation of critical technique parameters consistently and measurement of their influence on separation is a recommended practice.

Ruggedness:

When the same samples are tested in multiple laboratories, by different analysts, with different equipment, and so on, the findings are often cited as proof that the analytical technique's operational and environmental factors did not influence the results. As the name suggests, ruggedness measures how well test findings may be reproduced despite the wide range of conditions that might exist across laboratories and analysts.

Stability:

HPLC requires stable samples, standards, and reagents to provide repeatable and trustworthy findings. To analyze even one sample, the chromatographic analysis may need 10 or more runs, requiring reference doses to construct an analytical curve and several injections of the item being tested. Standard and sample solutions must be stable for at least a few hours even if they are separated for only 10 minutes. Automated analysis of more than one sample may need overnight runs to improve lab efficiency. Such techniques need higher stability in the solution. Stability refers to a drug substance or drug product's ability to maintain its claimed properties, such as potency, quality, purity, and identity, throughout testing or expiration dates .

Tests on the stability of active chemicals or pharmaceutical goods are designed to offer evidence of how their quality is affected by external conditions including temperature, humidity, and light. The stability of a product may also be affected by elements connected to it.

Formulas used:

Correlation coefficient(r):

A correlation coefficient is a numerical measure of some type of linear correlation, meaning a statistical relationship between two variables

Regression equation:

Regression equation= I + aC

$$a = \text{slope} = \frac{Y2 - Y1}{X2 - X1}$$

I = Intercept = regression – a C

As a percentage of mean absorbance.

Standard Deviation:

$$S = \sqrt{\Sigma (X - X^{!})^{2}/N - 1}$$

Where X = observed values

$X^{!}$ = Arithmetic mean = ΣX/N

N = Number of deviations

'S' may be expressed more conveniently in terms of the percent of the estimated average of the analytical range, which is more useful for a practical understanding. As a percentage of the standard deviation, it's termed a covariance (C.V.) or a percent RSD.

C.V OR %RSD = 100* S/ $X^{!}$

Average = Sum of No. of Observations/Total No. of Observation

% Relative Standard Deviation = Standard Deviation × 100

Recovery or Practical Conc = Sample area Average / Standard Area ×std. conc.

% Recovery =Standard concentration*100/Sample concentration amount (Label C)

% Assay of Drug = s*Calculated or Sample Weight/ Label claim

Learning the basics of method development LOQ = 10. * Standard Deviation of Area of Linearity/Slope

Development of a Method and Its Validation:

Learning the basics of method development

Drugs are entering the market at a faster rate than ever before. These medications might be whole new substances, or they could be a modification of an existing substance. The introduction of medicine onto the market and its inclusion in pharmacopeia sometimes take place at different times. These include reports of novel toxicities; patient resistance to medicine; and the availability of newer and better drugs by competing pharmaceutical companies, among others. In certain cases, pharmacopeias do not provide standards or analytical procedures. As a result, new analytical procedures for these medications are now required. As outlined in the

ICH recommendations, the development of analytical procedures must be done by good manufacturing practices (GMPs) and good laboratory practices (GLP) (Q2A and Q2B).

Drug product evolution necessitates a constant stream of new method development. Depending on the stage of drug development, a technique should have a specific objective and purpose. It is possible to concentrate on API behavior during early medication development. Preclinical safety assessments, pre-formulation investigations, and prototype product stability studies should all be supported by these tools. Analytical methodologies are improved and extended when new API and medicinal product informationbecomes available. The procedures must be simple and reliable while still adhering to all applicable regulations. When a technique is being developed, scouting experiments are usually conducted before formal validation trials to determine the approach's performance boundaries. The development of a stability-indicating approach may involve forced degradation experiments. A wide variety of substances, including acids, bases, peroxide, heat, and light, may degrade API. By doing so, the technique can distinguish and quantify degradation products while also offering insight into the degrading processes themselves. To assess API deterioration in the presence of formulation excipients, a stability-indicating process may be used to expose the manufactured medicinal product to heat and light.

A New Approach Is Necessary:

There are several motivations for the creation of a new analytical technique.

It's possible that the sample matrix doesn't include any analytes for which a technique might work.

Current techniques may be inaccurate, artifact- and contamination-prone, or generally untrustworthy.

These techniques By doing so, the technique can distinguish and quantify degradation products while also offering insight into the degrading processes themselves. To assess API deterioration in the presence of formulation excipients, a stability-indicating process may be used to expose the manufactured medicinal product to heat and light. may be prohibitively expensive, time-consuming, and/or environmentally damaging in certain situations, or they may not be easily mechanized.

Analyte selectivity and sensitivities may be compromised by existing approaches.

Legal or scientific considerations may necessitate the requirement for an alternate approach to verify analytical data initially gathered by current procedures.

Method Development Using HPLC:

It is important to pick the optimal chromatographic conditions for regular analysis of any medication in method development, such as the best column, the best mobile phase, and the detection wavelength. HPLC method creation requires a lot of sample information, such as the number of components, pKa values, UV-visible spectra, solubility in various solvents, concentration ranges, and other characteristics of the sample. This information is required before developing a method, such as the choice of chromatography method according to the sample properties, a sample that is HPLC-analysed under conditions where all compounds elute quickly, and optimization of the HPLC method in terms of analysis times, resolution levels, selectivity, and sensitivity.

Analyte Standard Characterization:

Structure, toxicity, purity, and stability of the analyte are all taken into account in the collection of data. Storage and disposal information are established together with the availability of the pure standard analyte. The number of components to be studied in a sample matrix is recorded, and the availability of standards for each component is confirmed if more than one component is to be examined.

Literature Search and Prior Methodology:

There is a thorough search of the existing literature for all relevant information about the analyte. Determine the availability of data on synthesis, physical and chemical characteristics, solubility, and suitable analytical procedures. Refer to books, magazines, and compilations of regulatory agency compendia like the USP/NF or BP. An automated/computerized literature search should also be employed, such as the Chemical Abstracts Service (CAS). Compilation of data, findings, reports, memoranda, and publications related to earlier analyte analysis must be done inside the organization.

Choosing a Suitable Method:

The approaches are altered and changed based on the existing literature and prior methodologies. To take advantage of the most recent methodologies and technology, sample preparation and instrument conditions are implemented. Work from analogy to examine substances with comparable structure and characteristics if existing techniques for the analyte are not available. A chemical with an analytical technique comparable to the analyte of interest is almost always available.

Optimization:

Choice of method:

Normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography, and ion exchange chromatography are the most often used chromatographic methods. First, try reversed-phase chromatography for organic molecules, then normal-phase chromatography, and finally Ion-Pair Chromatography in the reverse phase before finishing with Ion-Exchange Chromatography if the former doesn't work. If the latter doesn't work, then go back to reverse-phase chromatography.

Choice of Movable Phase:

In reversed-phase chromatography, the mobile phase selection is crucial for the medicine analysis. For buffers, K2HPO4 and KH2PO4 can be used in the whole UV range; freshly distilled THF is suitable for HPLC above 240 nanometers; THF, ammonium acetate, and the EDTA buffer may also be used in the upper UV range; however, EDTA can only be used in the lower UV range.

You may utilize gradient elution to determine how much organic material you need in your mobile phase. Gradient reversed-phase chromatography is the most user-friendly method for aqueous sample combinations. To elevate the organic phase concentration (Methanol and Acetonitrile) from 10% to 100% in 20-60 minutes, a gradient of 10% organic phase is used. To fine-tune the composition of the first mobile phase, the chromatogram from the pre-run may be employed. It is possible to compute the mobile phase's beginning composition based on where and at what concentration the chemicals of interest were eluted from the mobile phase.

Changes in the mobile phase's polarity may affect the elution of drug molecules. There are two ways in which polarity affects a mobile phase's elution strength: strong and weak. If ionic samples (acid or basic) are present in an un-dissociated state, they may be separated. Proper pH selection may decrease ionic sample dissociation.

To avoid ionization, the mobile phase's pH must be chosen such that the compounds are not. The organic phase concentration in the mobile phase may be reduced by 5 percent if the retention durations are too short. The organic phase concentrations must be increased by 5 percent increments if the retention durations are excessively lengthy.

To maintain consistent retention time and selectivity during acid/base separation, a buffered mobile phase is required. To reduce peak tailing, buffered salts hide silanol groups and prevent protonated silanol groups from ion-exchange interactions. Potassium is superior to sodium because of its stronger counter ion (Na+). Potassium phosphate is used to make buffers of various pH values. Acidic or amphoteric substances may be checked with a few drops of triethylamine or ammonium acetate if band tailing is seen. A 100mM H_3PO_4 buffer with a pH of 2.3 is used to treat weak to medium acidic or basic compounds in their ionized state. A pH of 4.0 is used to treat weak to medium acidic or basic compounds in their non-ionized form. Water is used as the aqueous eluent to treat neutral chemicals. Water is the initial step in analyzing an unknown substance, followed by an acidic buffer and a neutral buffer.

When preparing the movable phase, the difference in partial pressure of each solvent should be taken into account when combining the solvents. Unless an intermediate solvent can be employed, a solvent system that is miscible with both the prior movable phase and the new movable phase must be used.

Choice of Column:

Normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography, and ion exchange chromatography are the most often used chromatographic methods. repeatable approach needs a stable, high-performance column with exceptional selectivity and efficiency. These qualities are determined by the capillary quality and the packing materials used by the capillary manufacturer.

Column length:

Longer columns are used to boost the clarity of the text.
Stricter back pressure and lesser solvent usage are all advantages of using shorter columns for equilibration.

Column internal diameter:

For increased specimen loading, larger diameter columns are utilized.
Narrow columns are employed to maximize sensitivity while minimizing mobile phase usage.

Particle shape:

Spherical particle columns are used when back pressure stability and increased efficiency are required.
If you require a lot of surface area and a high capacity, irregular-shaped particle columns are the ideal option.

Particle size:
Columns with particle sizes ranging from 3 to 4 nm are appropriate for complicated mixes containing comparable components. It is feasible to obtain quick, high-resolution separations with a short column (10-50 mm) and tiny particle size. For structurally diverse chemicals, larger (5-10 m) particle-size columns are preferable. For preparative separations, columns with big particles of 15 - 20 m are utilized.

Surface area:
These columns are chosen for their capacity, resolution, and long-term retention.
Faster equilibration is achieved by using packing columns with a smaller surface area.

Carbon load:
For increased column capacities and resolution, high-carbon load columns are used. Faster analysis times may be achieved by using columns with a reduced carbon load.

End capping:
For increased column capacities and resolution, high-carbon load columns are used. Differential selectivity is achieved by managing secondary contacts in non-end-capped columns.

Choice of Detector:
Sensors monitor the substances once they have been separated on the column by the liquid chromatography system's eyes. The detector of choice must be sensitive enough to detect even the smallest changes in the concentrations of all the components in the specimen. There are a few requirements for the detectors: they must be very sensitive with a large linear dynamic range, apply to the majority of solutes, be non-destructive, and respond quickly.

Further Optimization:
After selecting a good method, movable phase, column, and detector, additional tuning may be done to get a well-developed technique.

For shorter analysis time
Employ an isocratic strategy.
The ideal mobile phase composition may be determined using the gradient run. If the necessary resolution is achieved, then a shorter column may be used.

For better resolution
A longer column has been used. Particles as tiny as 3 – 4 m may be handled by the stationary phase.

For better selectivity and sensitivity
Phenyl, CN, and other stationary station phases are examples of these.
The use of ion-forming chemicals for pH control
Acetonitrile may be substituted with methanol or THF.
Measurement of the substance's absorption peak
Higher and narrower peak widths may be attributed to factors such as elution gradients and microbore columns.

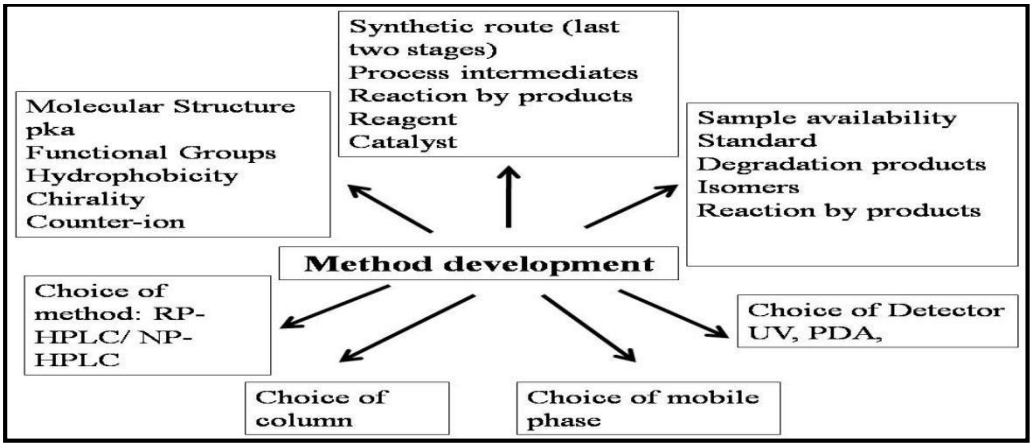

Figure No 5: Outline of the process involved in method development

Parameters Affecting Changes in Chromatograph:

The various parameters affecting the changes in chromatographic conditions are

Rate of flow, Temperature, pH, Ion pair reagent, Column efficiency, Capacity factor, Resolution, Retention period, Peak asymmetry

Effect of Flow Rate:

The flow rate affects the HPLC column's efficiency. It may be a great tool for enhancing the quality of images. Faster eluent flow speeds up the time it takes to run a column, reducing diffusion and improving separation (less band broadening). Analytes must equilibrate between the stationary phase and the mobile phase before the maximum flow rate may be exceeded. Run times rise when column back pressure decreases as a result of decreased flow rates.

Internal diameter of column (mm)	Standard flow rate (µL/ min)
4.6	1000
2.1	200
1.0	50
0.30	4

Table No 4: Flow rates for columns with different internal diameter

Effect of Temperature:

The solubility and diffusivity of a material increases in direct proportion to its temperature. Temperature has an impact on retention, peak form, column efficiency, and overall analysis time due to the thermodynamics and kinetics of adsorption processes. Temperature control provides an additional advantage in addition to improved repeatability. Liquid chromatography makes use of temperature to enhance separations. At higher temperatures, the peaks will be more distinct and will elute more quickly. System pressure is affected by temperature. As the temperature increases, the viscosity of a flowing fluid decreases. If the HPLC system pressure is too high for a specific solvent, the column temperature may be adjusted to 40°C or even 60°C. Temperature increases to limit the lifetime of columns, and certain columns may not be able to sustain temperatures more than 600C.. Increased temperatures and smaller-diameter packing can meet the high throughput needs of the pharmaceutical industry.

Role of pH:

Changing pH has little impact on certain preparations. HPLC retention durations may be altered by altering the pH, which alters the ionization potential and, as a result, can alter the amount of polarity present in the sample solution. As the sample mixture's components are reacted to, their retention times are likewise altered. This alters the selectivity since the peaks may co-elute at a given pH and so the sequence in which they are eluted will vary. To accurately separate ionizable substances by RP-HPLC, the buffer pH must be chosen carefully and consistently. Choosing an incorrect pH for ionizable analytes results in wide, towering, or split asymmetric peaks.

The analyte's pKa should be taken into account when making a buffer selection. Peak shape may be improved by using a buffer that is 2 pH units above or below the pKa value of the sample. Returning to the equation of Henderson-Hassel.

$$pH = pKa + \log ([A^-]/ [HA])$$

It is possible to acquire a decent peak shape only when the analyte is in a single form, which is 99 percent of the time.

Role of Ion-Pair Reagent:

They cannot be employed in reverse-phase/high-performance liquid chromatography because they have ionic or polar characteristics. Before choosing between RP-HPLC and RP-HPLC with ion-pairing, one must first determine the nature of the analyte. Ion pairing is used in RP-HPLC with and without ion pairing. Although the ion pair reagent employed in the RP-HPLC mobile phase boosts the ionic sample selectivity of the movable phase for the latter technology, This interaction is possible because of the hydrophobic surface of the ionic pair reagents and their opposite-charged nature relative to the analyte of interest. The counter-ion and the eluent's

ions form ion pairs during the stationary phase. Separation is made simpler by the variable water retention of analytes.

Only if reversed-phase HPLC fails to provide enough separation is an ion-pair reagent recommended. These extra experimental criteria, such as how to pick an appropriate ion-pair reagent and its concentration, must be taken into consideration when utilizing an IPR reagent. If an ionic analyte is being studied, reversed-phase HPLC should be used first before ion-pair reversed-phase HPLC is attempted. For the separation of charged analytes in RP-HPLC, ionic suppression is applied. Non-ionized analytes are obtained by adjusting the pH of the movable phase. However, it involves substantial technique development and is only suited for simple mixes when the PKa's of the analytes are close together.

Column Efficiency (N):

A chromatographic column's efficiency is expressed in terms of the number of theoretical plates (plate number), N, that the column can hold.

$$N = 16\left(\frac{t_r}{w}\right)^2$$

Gaussian curve inflection points are marked by drawing tangents to the sides and projecting them to intersect the baseline as in. the amount of time that a substance remains in the body after injection, expressed as a time constant (tr).

It is also possible to describe column efficiency in terms of the theoretical plate height (HETP), which is comparable to the height of a plate (or h)

$$h = \frac{L}{N}$$

Where L is the length of the column, and N is the number of theoretical plates.

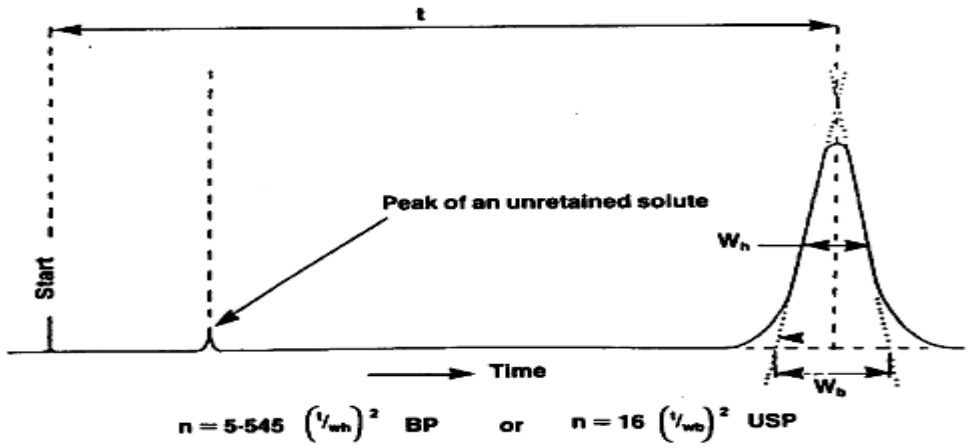

Figure No 6: The number of theoretical plates shown in a picture.

Capacity Factor (kg)

Chemical-specific peak position in the chromatogram is a metric that defines the amount of time it takes for a certain compound to be isolated.

$$k' = \frac{t_r - t_m}{t_m}$$

Where, t_r– retention period of the solute, t_m – retention period of the unreturned compound by the column packing. K influences both the stationary and mobile phases, as well as the column packing quality. The k's value should be between 1 and 10 to provide the optimum chromatographic performance with isocratic separation. It is not possible to achieve satisfactory isocratic separation using the current column and mobile phase, and gradient elution should be attempted if k' > 30 the separation takes too long or the bands become too broad if k' 1.0.

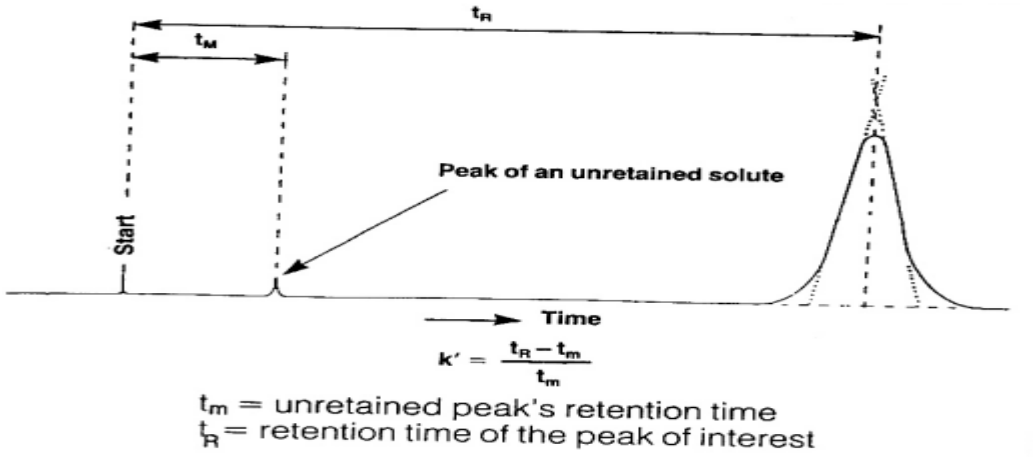

Figure No7: Pictorial representation of capacity factor**.**

Resolution (R_s):
The column's capacity to isolate two various solutes. It is the distance between two peaks in a chromatogram.

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_1 - w_2)}$$

Where, t_{r1},t_{r2} – retention time of two immediately adjacent peaks,
w_1, w_2 – peak widths of two immediately adjacent peaks

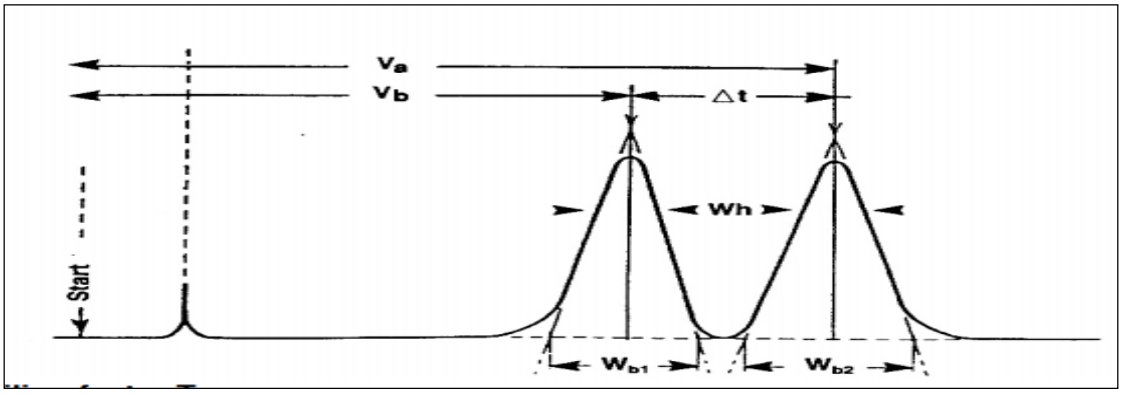

Figure No 8: Pictorial Representation of Resolution

Selectivity factor (α) / Relative Retention:
Distance between two close mountains may be expressed in this manner. The capacity factor is the best method for calculating peak separation since it takes into account the interactions between the components and the stationary phase.

Where,
k'_a, k'_b– peak A and peak B's capacity factors.

For the most part, the quality of separation is more important than the quality of oneness in establishing value. The separation factor is equal to the ratio of the distribution constants. Using the capacity factor, any instrument may provide reproducible findings since the separation factor should be constant for any column and any mobile phase composition at any given temperature.

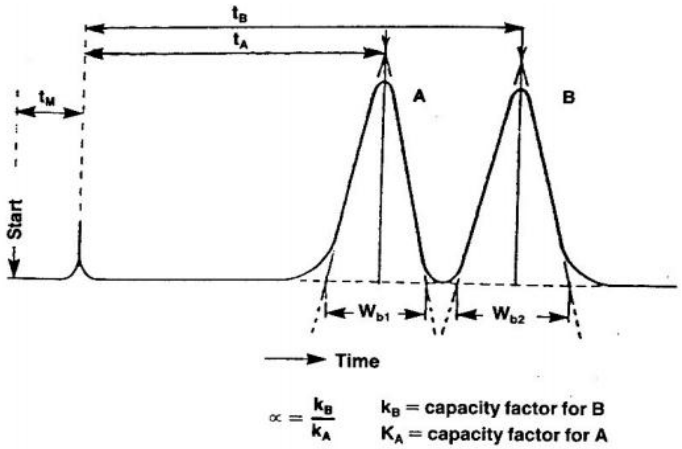

Figure No 9: The selectivity factor is shown in a visual form

Peak Asymmetry (A_s):
The tailing factor is another name for it. The following formula may be used to figure out how much of a peak is asymmetrical.

$$A_s = \frac{W_{0.05}}{2f}$$

Where, at a 5 percent height increase from the baseline, W0.05 peak width
f – The distance between the peak's highest point and the peak's lowest point
It is also calculated from

$$A_s = \frac{b}{a}$$

Where, b - the distance from the peak to the trailing edge of the curve,
a – The distance between the peak's leading edge and its highest point

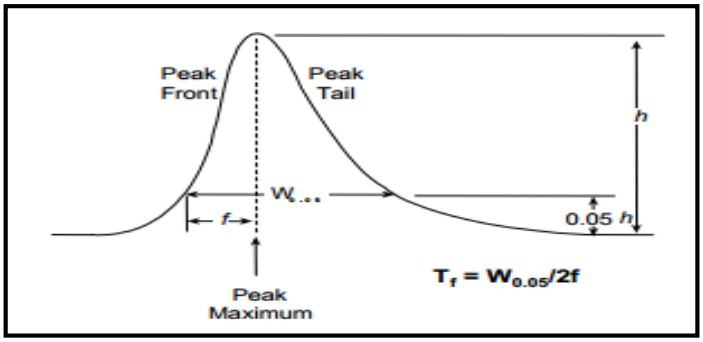

Figure No 10: Figures depicting asymmetrical peaks

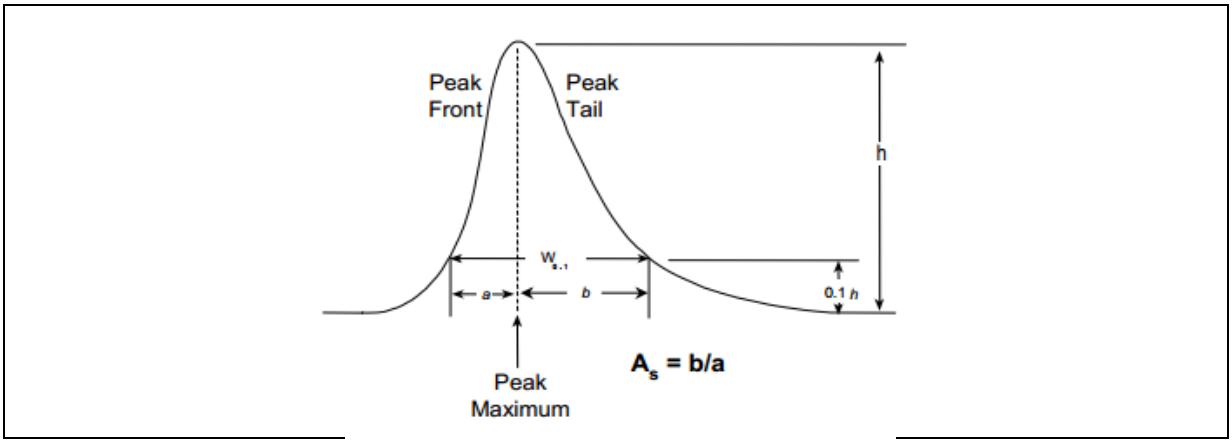

Figure No 11: Peak asymmetry as seen in a picture

Method validation Introduction:

The systematic examination of systems, facilities, and processes to determine if they fulfill their intended tasks sufficiently and consistently, as defined, is an essential component of quality assurance. It is important to note that validation does not enhance procedures but rather validates that the processes have been correctly established and are being appropriately monitored and controlled.

Scientific investigations are performed to validate the design of an analytical technique to ensure that it is acceptable for its intended usage. Pharmacopoeias, or sets of approved test procedures, have been created for many commercially available medications in the United States, Europe, and other countries to ensure compliance with quality and safety regulations. Few analytical processes are explicitly recognized by Section 501 (b) of the Federal Food, Drug, and Cosmetic Act (USP). The USP has approved these compendia procedures. Federal regulations in the United States concern additional validation of analytical techniques (CFR). Pharmaceutical regulations have been standardized throughout Europe, the United States, and Japan. The International Conference on Harmonization has created new verification standards for analytical techniques (ICH). ICH recommendations are mentioned in both current FDA draught guidance publications and in the United States. The validity of a test is determined by its capacity to show specific features of its analytical performance, which are sometimes referred to as its "analytical figs of merit." Validation or revalidation of methods is crucial.

Before its widespread usage and introduction;

For example, if a new instrument with different properties is used, the procedure will need to be tested again.

Any time that a method is altered in a way that goes outside the scope of the method.

Analytical processes are validated in the following four categories: Tests for impurity identification and quantification Limit tests for impurity control; Analysis of active moiety concentrations in various drug substances, drug products, and/or specific drug component(s).

Method Validation (ICH Guidelines)

Accuracy
Precision
3.Repeatability
4.Intermediate precision
Specificity / Selectivity
Limit of Detection
Limit of Quantification
Linearity
Range
Robustness
System Suitability

The vocabulary used in the ICH validation criteria differs from that used in the USP guidelines, but only in two respects. According to the ICH recommendation, system appropriateness does not fall under this category and is instead considered part of method validation.

Accuracy:

Values recognized as either conventional true values or acceptable reference values are used to measure an analytical procedure's degree of accuracy. All aspects of the analytical technique should be checked for accuracy.

The proportion of analyte retrieved by assay, spiking specimens in a blind trial, is how accurately a test is judged.

According to the recommendations for submitting specimens and analytical data for method development, medication substance, and medication product accuracy tests should be conducted at 80, 100, and 120 percent of the label claim levels.

Analytical procedures should be assessed utilizing at least nine determinations covering at least three concentration levels comprising the required range (e.g., three concentrations / three repetitions of the whole analytical process). Look at the percentage recovery or the difference between the mean and an accepted actual value, both of which have 95 percent confidence intervals, to determine the accuracy of a sample.

Accuracy is defined by the RSD (Real Standard Deviation) of the recovery values not exceeding 2.0 percent for all of them.

Precision:

Accuracy is measured in terms of the spread between measurements taken from the same homogeneous sample at different points in time under the same conditions. There are three forms of precision: reproducibility, repeatability, and intermediate precision. It is common to describe the accuracy of an analytical technique in terms of a measurement series' variance, standard deviation, or coefficient of variation (CV). A relative standard deviation (RSD) of no more than 2 percent should be used for the assay of six formulation preparations.

Specificity:

'Specificity,' thus, refers to the analyte's ability to be accurately assessed in the presence of any components present in the sample matrix. Examples include degradations, impurities, matrix, and so forth. To ensure that a peak reaction is attributable to a single component and not the effect of other variables such as these, it measures interference from other active components, excipients, contaminants, and degradation products.

Both identification and assay/impurity tests fall under the category of specificity. To establish specificity, one must discriminate between compounds of comparable structure or use a recognized standard for identification purposes. The resolution of two closely eluting substances shows selectivity in assay/impurity testing. Most of these chemicals are either the primary component or the active ingredient.

Detection limit (LOD):[14]

The detection limit of an analytical technique refers to the least amount of analyte in a sample that can be detected but not necessarily quantified precisely. Analytes may be tested to see whether they are over or below a given threshold.

Signal-to-noise ratios of 2:1 or 3:1 are common for this kind of measurement. LOD may also be calculated using two alternative methods: Calculation techniques and non-instrumental visual approaches: Thin-layer chromatography (TLC) and titrations are two examples of visual non-instrumental procedures. For example, LODs may be determined using standard deviations (SD) and slopes of calibration curves (S) that approach the LOD:

$$LOD = 3.3\left(\frac{SD}{S}\right)$$

Where,

SD- standard deviation

S- Slope

When calculating the response's standard deviation, you may use the blank's standard deviation, the residual's standard deviation, or the standard deviation of the regression line's y-intercepts. There should be documentation of the LOD technique and sufficient samples evaluated at the limit to establish the level's validity.

Quantification Limit (LOQ): The lowest concentration of an analyte in a sample at which it can be properly and precisely quantified using the method's operating parameters is known as the limit of quantitation (LOQ).

Two more possibilities may be utilized to calculate the LOQ: the ICH recognizes a 10-to-1 signal-to-noise ratio as usual.

$$LOQ = 10\left(\frac{SD}{S}\right)$$

Non-instrumental visual approaches and a mathematical technique. The response standard deviation (SD) and the slope of the calibration curve (S) are used to calculate the results.
Where, SD- standard deviation
S- Slope
Most often used are y-intercept and residual standard deviations combined with the standard deviation of the answer itself to figure out how to calculate response standard deviation. Like LOD, a sufficient number of samples should be evaluated at the limit to validate the level, as well as documentation and help.

Linearity and Range:

An analyte's concentration must be proved to be proportionate to the method's linearity for test results to be obtained. Regression line slope variance is typically used as a measure of linearity. There are a variety of analytes that may be measured, and each analyte has its concentration range. As a rule of thumb, test results and measurement range are provided in the same units.

There must be a minimum of five concentration levels and a set of minimally specified ranges defined. 80 to 120 percent of the target concentration is often required for assays. In an impurity test, the minimum range is the difference between the reported contaminant level and the specification's minimum value.

It is recommended that a linear correlation of at least 0.9 exists between the drug concentration (measured in percent) and the area of the sample.

Robustness:

A technique's robustness is defined as its resistance to even the smallest changes in the parameters of the procedure. Several variables, such as the concentration of organic solvent, pH, ionic strength, and temperature, may be used to assess a method's resiliency. When testing for medications in a specimen under specially altered chromatographic criteria, an RSD of more than 2 percent is not acceptable.

System Suitability:

Procedures using gas and liquid chromatography involve system-appropriateness assessments in the experimental setup. A chromatographic system's resolution and repeatability can only be as good as the chromatographic system it is connected to. Test samples, analytical equipment, and electrical components are all seen as components of a unified system that may be evaluated as a whole at any point in time.

The testing of a system to ensure that it functions as predicted before or after the study of unknowns is known as "system appropriateness." The method's characteristics, such as plate count, tailing factor, resolution, and repeatability, are compared to the measured values. A mix of the system's key components and projected by-products is used to evaluate its suitability.

Parameter	**Limit**
Capacity Factor	k'> 2
Injection precision	RSD < 1% for n ≥ 5
Resolution	R_s> 2
Tailing factor	$A_s \leq 2$
Theoretical plates	N> 2000

Table No 5: Set of Acceptance Criteria for Testing the System Suitability

Method Validation Definitions and Formulas:

Mean/ Average (x_i):

To determine the average, the total of all individual findings is divided by the number of values (n).

$$x_i = \frac{x_1 + x_2 + x_3 \ldots}{n}$$

Where, x_1, x_2, x_3.. = Values of individual outcomes

n = Number of individual outcomes

Standard Deviation (SD):

RMSE is the root mean square deviation of values from their mean.

$$SD = \sqrt{\frac{\sum(x - x_i)}{n - 1}}$$

Where Σ = A total of all the facts

x_i = "Mean," often known as the "arithmetic average"

x = A single value has been recorded.

$x - x_i$ = Values that are out of the norm

n = The number of people who have noticed this

Relative Standard Deviation (RSD):

A measure of standard deviation as a proportion of the mean.

$$RSD = \frac{SD}{x_i} \times 100$$

Where SD = Standard deviation

x_i = Mean or arithmetic average (Σx / n)

Correlation Co-Efficient (R):

Using the correlation coefficient, we may gauge how closely two unrelated occurrences are tied together. It provides a correlation strength and direction range of -1 to +1. A negative correlation indicates that the two variables are moving in opposing directions, as shown by the negative numbers. As a rule, negative correlation coefficients indicate that two variables are moving in the opposite direction. A correlation of +1 or -1 indicates that the two variables are strongly linked.

$$R = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[\sum x^2 - (\sum x)^2]\,[[\sum y^2 - (\sum y)^2]}}$$

Where n = Number of observations

x = First value

y = Second value

Σxy = Sum of products of first and second value

Σx = Sum of first values

Σy = Sum of second values

Σx^2 = Sum of squares of the first value

Σy^2 = Sum of squares of the second value

Linear Regression:

Regression is the study of the connection between two variables. It's used to figure out how closely two variables are related to one another.

The equation for a straight line is

Where b = slope

A =intercept

$$\text{Slope } (b) = [\, n(\Sigma xy) - (\Sigma x)(\Sigma y)] / [n(\Sigma x^2) - (\Sigma x)^2]$$

$$\text{Intercept } (a) = [\, (\Sigma y) - b(\Sigma x)] / n$$

Where	n	=	Number of observations
	x	=	First value
	y	=	Second value
	Σxy	=	Sum of products of first and second value
	Σx	=	Sum of first values
	Σy	=	Sum of second values
	Σx^2	=	Sum of squares of the first value
	Σy^2	=	Sum of squares of the second value

Method optimization begins with the careful selection of the mobile phase, stationary phase, and detection techniques, each tailored to the specific properties of the analytes. Systematic adjustments and fine-tuning of parameters such as pH, temperature, flow rate, and gradient elution are crucial in enhancing resolution, sensitivity, and peak shape. The optimization phase is iterative, necessitating a balance between theoretical knowledge and empirical adjustments to achieve the desired chromatographic performance.

The development of an HPLC method extends beyond optimization, encompassing the establishment of method specificity, accuracy, precision, linearity, range, and robustness. Each of these attributes is integral to the method's ability to consistently produce reliable data. The development phase also involves the integration of advanced techniques such as chemometrics and design of experiments (DoE) to streamline the optimization process and ensure a comprehensive understanding of the method's behavior under various conditions.

Validation is the final, yet equally critical, step in the HPLC method lifecycle. It provides documented evidence that the method performs reliably within the defined parameters. This phase includes rigorous testing for system suitability, accuracy, precision, specificity, detection limits, quantitation limits, linearity, and range. Validation ensures that the method can be confidently applied to real-world samples, yielding accurate and reproducible results across different laboratories and analysts.

Understanding and controlling chromatographic parameter variability is a recurring theme throughout this process. Variability can stem from numerous sources, including instrument performance, environmental conditions, and operator technique. Recognizing and mitigating these sources of variability is essential to maintaining the integrity and consistency of the HPLC method.

In conclusion, the optimization, development, and validation of HPLC methods are foundational to the advancement of analytical chemistry. By embracing a systematic and informed approach to managing chromatographic parameter variability, researchers and analysts can develop robust methods that deliver precise and reliable data. This guide underscores the importance of continuous learning and adaptation in the face of evolving analytical challenges, ultimately contributing to the broader scientific community's efforts to achieve excellence in chromatographic analysis.

II. Conclusion

The journey through HPLC method optimization, development, and validation is intricate, demanding a deep understanding of chromatographic principles. This guide has highlighted the essential aspects of the process, underscoring the importance of managing chromatographic parameter variability to achieve robust and reproducible results. Method optimization involves careful selection and adjustment of the mobile phase, stationary phase, and detection techniques tailored to the analytes' properties. Systematic fine-tuning of parameters such as pH, temperature, flow rate, and gradient elution is crucial for enhancing resolution and

sensitivity. The development phase extends beyond optimization, focusing on method specificity, accuracy, precision, linearity, range, and robustness. Advanced techniques like chemometrics and design of experiments (DoE) are integrated to streamline the process and provide a comprehensive understanding of the method's behavior under various conditions. Validation is the final critical step, providing documented evidence that the method performs reliably. This phase includes rigorous testing for system suitability, accuracy, precision, specificity, detection limits, quantitation limits, linearity, and range, ensuring the method's applicability to real-world samples. Understanding and controlling chromatographic parameter variability is a recurring theme. Variability can arise from instrument performance, environmental conditions, and operator technique. Recognizing and mitigating these sources is essential for maintaining method integrity and consistency. In conclusion, HPLC method optimization, development, and validation are crucial for advancing analytical chemistry. By systematically managing chromatographic parameter variability, researchers can develop robust methods that deliver precise and reliable data, contributing significantly to the field of chromatographic analysis.

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