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Review Article

Strategic Approaches of Controlling Variables in RP-HPLC Method Development and Validation: A Review

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ABSTRACT

High Performance Liquid chromatography is the principal technique in accessing the drug, its various impurities and drug related degradants that can form on synthesis or storage in pharmaceutical products. It results in highly efficient separations and in most cases provides high detection sensitivity. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation. Separation of components in a mixture is based on their interaction between a stationary and a mobile phase based on the properties such as polarity, electric charge (for ionic compounds), pH, functional groups and size of the molecule. Before starting method development there should be good understanding of the chemical structure, polarity. pH, pKa, molecular spectral absorbance electric charges(ionic compounds) of the molecule. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals.

1. Introduction

High Performance Liquid Chromatography is a separation technique in which molecules are separated by differential migration i.e. separation is achieved on the basis of movement of molecule between the stationary phase and mobile phase. HPLC involve two phases (a stationary and a mobile phase) and solution of sample is injected into a column of porous material (stationary phase) and a liquid is pumped at high pressure through the column. The necessary differences in speed of migration are caused by differences in chemical interactions or partitioning between the molecules of the different solutes for the two chromatographic phases [1]. Depending upon the partition behavior of different components, elution at different time take place.

2. Types of Chromatography

Choice of chromatography type depends on analyte retention, solubility and stability. When sample solubility is poor in reversed phase chromatography (non polar samples) normal-phase chromatography (NPC) is a preferred alternatively. Similarly, samples having stability problems in aqueous media are separated by normal phase chromatography using non-aqueous solvents.

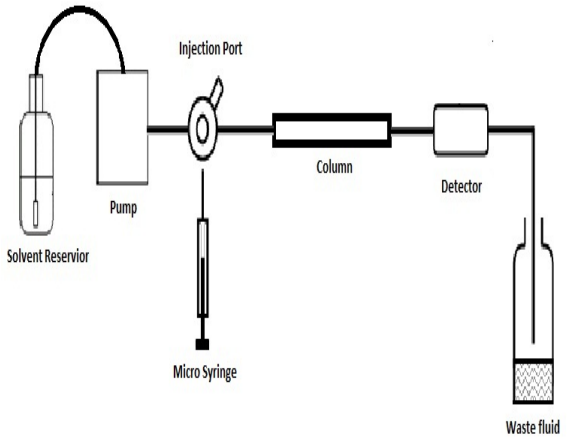

Figure 1: Flow diagram of HPLC

2.1. Normal Phase Chromatography

Normal phase is a type of partition chromatography in which the polarity of the solid

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phase is higher than the mobile phase The solid phase used for normal-phase chromatography is an untreated porous silica-gel column or a column containing silica gel chemically bonded to the surface to polar functional groups, such as the aminopropyl group (NH_2 column) or cynopropyl group (CN column). The mobile phase consists of non polar n-hexane and a polar solvent such as ethanol. The separation of each component differs according to the distribution ratio between the solid phase and mobile phase. The interaction between the solid phase and analyte involve hydrophilic interactions, such as hydrogen bond interactions and electrostatic interactions.

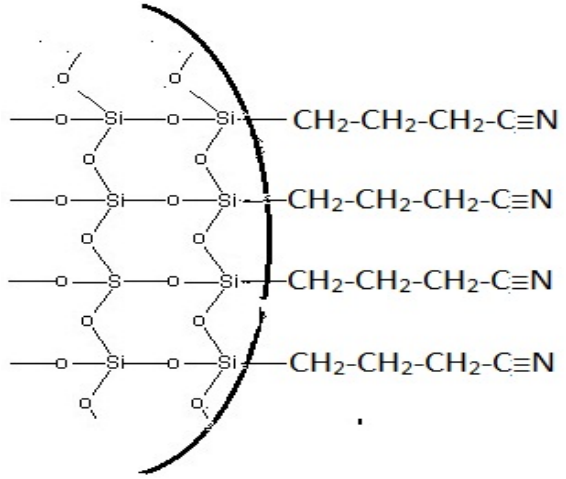

Figure 2: Cynopropyl column bonded with silica

2.2. Reversed Phase Chromatography

Reversed-phase chromatography also known as RP-HPLC is the most commonly used separation mode. Column packing used in RP-HPLC involves a silica gel matrix with chemically bonded alkyl chains. The silica gel matrix offers high number of theoretical plates. Typical alkyl groups that are chemically bonded to the silica gel include the octadecyl group, the octyl group, and the trimethyl group. The longer the alkyl chain, the greater the retaining force.

3. Method Development

Analytical method development play important role in analysis of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products. Successful method development in HPLC not only require good understanding of the chemistry involved in the separation but also require a reliable instrumentation for performing sound chromatographic separations. Fig 04 illustrates factors to be considered during method development.

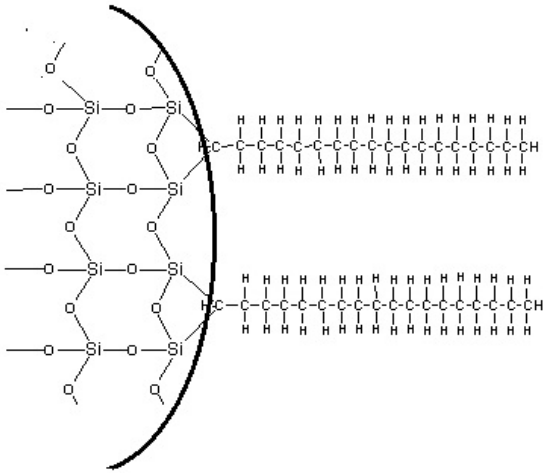

Figure 3: C 18 Column silica with bonded octadecyl alkyl chain

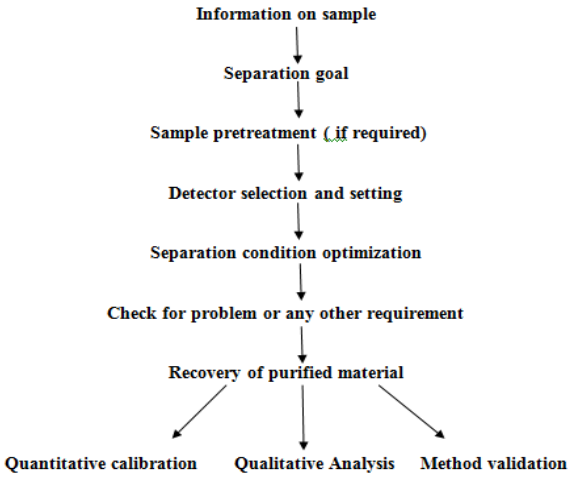

Figure 4: Steps in method development

3.1 Things to be known before Method Development

3.1.1 Nature of the Sample

Before starting method development information of the sample such as chemical structure, pKa, log P, pH, solubility profile, UV spectra, concentration range and number of compounds in the sample must be known. These properties of a drug molecule play an important role in method development. Polarity is a physical property of a compound and it helps to decide the solvent and composition of the mobile phase. Polar component has greater solubility in polar solvent

but show poor solubility or insolubility in non polar solvent. Solvent used in sample preparation must solubilize all the components of the sample without any interaction with any of the components of sample.

pH and pKa plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

$pH = - \log 10 [H_3O^+]$

The acidity or basicity of a substance is measured on pH scale. The concentration of hydrogen ions can be indicated as $[H^+]$ measured on pH scale between 0-14. Selecting a proper pH for ionizable analyte often leads to symmetrical and sharp peaks in HPLC necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. When optimizing mobile phase pH, it is necessary to know the approximate *pKa* values of the various sample components This information allows mobile phase composition to be restricted to a useful range of pH. When an acid is more than 2 pH units above its pKa, it will be >99% ionized and if 2 pH units below its pKa it will remain >99% unionized. Bases are ionized below their pKa and non-ionized above their pKa. As unionized form is less polar (more hydrophobic), and thus more strongly retained in a reversed-phase system. Thus, at low pH, acids will be more retained whereas bases will be more retained at high pH [2-4].

The dissociation constant (pKa) is characteristic of a particular compound, and it tells how readily the compound gives up a proton. pKa value of any compound can be estimated by its chemical structures [5,6] For the specific equilibrium between a monoprotic acid, HA and its conjugate base A^-

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

Equilibrium is measured by the equilibrium constant, K_{eq}.

$Keq = [H_3O^+][A^-]/[H_2O]\,[HA]$

Now in dilute solutions of acid, the acidity constant Ka.

$Keq = [H_3O^+][A^-]/\,[HA]$

In logarithmic form it is expressed as

$pKa = -\log 10\ Ka.$

Corelation between pH and pKa is expressed by following equation

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

At half-neutralization [A−]**/**[HA] = 1 since log(1) = 0, the pH at half-neutralization is equal to pKa. Conversely, when pH = pKa, the concentration of HA is equal to the concentration of A^- or we can say that the pKa of an acid is the pH at which it is exactly half dissociated.

3.1.2 Separation Goals

Before starting objective of separation should be clear i.e. quantitative analysis, detection of undesired substance, characterization of unknown sample components or isolation and purification of compounds [7]. If goal is quantitative analysis level of accuracy and precision required in separation must be known. Sometimes it is necessary to separate all sample components but its not necessary to separate these degradants or impurities from each other. Complete separation of a sample by means of a single HPLC run is a difficult task than the separation of a smaller subset of sample components. A particular compound may be present in different sample types (raw material, one or more formulations) therefore we must know for how many sample matrices method be developed. When a large number of samples must be processed at the same time, run time becomes more crucial. Shorter run time can be achieved by compromising with resolution by shortening the column or increasing flow rate. When the number of samples for analysis is more than 10 run time should be limited to 10-12 minutes

3.1.3 Sample Preparation and Detection

Samples can be obtained in following way

- Solutions ready for injection
- Solutions that require dilution, adding up an internal standard, buffering or other volumetric manipulation
- Samples require extraction procedure
- Samples that require pretreatment to remove interferences to protect the column or equipment from damage

Detector used in HPLC for the detection and quantification of separated compounds must be able to sense all the components of the sample. UV detector is first choice because of applicability for most of the samples. UV spectra can be

obtained from literature, obtained directly by using UV spectroscopy or obtained during separation using PDA detectors.

3.2. Method Optimization

The decisive components for a HPLC method are sample preparation (% organic, pH, sample injection size, sample age) analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age), and analyte physicochemical properties (molecular structure, pH, pKa and polarity) [7-12]. During the preliminary method development stage, all individual components should be investigated intensively before the final method optimization. The effect of change in variables changes the method performance. These findings than used to streamline the final method optimization [13-15].

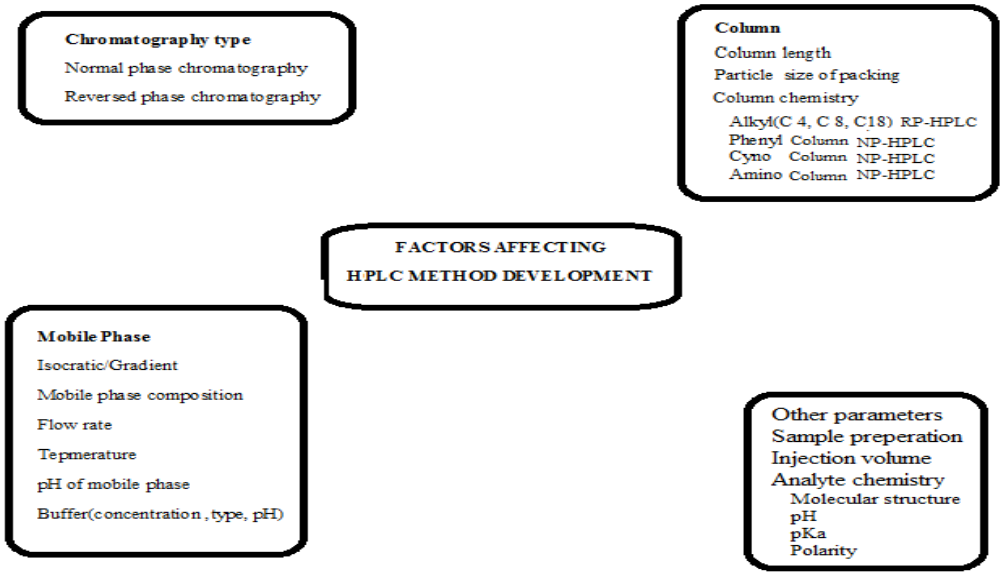

Figure 5: Factors affecting HPLC method development

3.2.1 Selection of Method

Selection of method is based on polarity of the analyte to be analyzed. For neutral and nonionized (comparatively nonpolar) components normal phase chromatography is used as polar components in the mixture when passed through column will retain longer than the nonpolar components and nonpolar ones will pass more quickly through the column. Therefore Normal phase chromatography is suitable for quick separation of comparatively nonpolar compounds. For separation of ionized or polar compounds reversed phase chromatography is used as polar compounds will elute faster than the nonpolar compounds because of the comparatively polar mobile phase than the stationary phase.

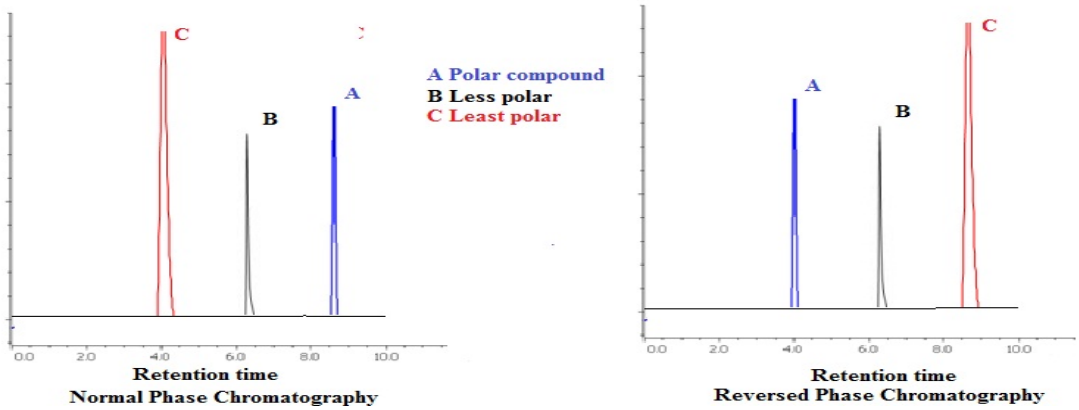

Figure 6: Effect of polarity on retention of analyte

3.2.2 Selection of Column

In HPLC column play significant role in optimum separation of the sample. Selection of column is based on the hydrophobic retaining force on the target compounds. Compounds with longer retention do not elute through the column within appropriate time require column with weaker retaining force conversely, compounds that elute through the column fast require column with stronger hydrophobic retaining force. A change in column length, particle size, or flow rate can result in acceptable final separation and resolution [16-18]. The life of silica-based bonded-phase columns is directly depends on the types of support used, mobile phase pH, type of buffer and organic modifier used.

Column Packing

Silica support packing is the most popular packing material for packing due to its ideal physical properties. This permits the formation of efficient packed beds that are stable under high pressures and longer run. Silica based packing provide column with higher efficiency low back pressure and longer lifetime [19]. Loosely packed column, chemical interaction on the support of column and plugged column due to adsorbed sample components decreases the life of column. Silica based column should not be used at pH above 8 because at such higher pH silica beds gets dissolve due to attack on bond between silane Si-O-Si group.[20-21]

Analyte retention is maximum with bonded alkyl chain of greater number (C18 > C8 > C4 > C3> C1). Stability and life of column also depends on number of carbon in bonded alkyl chain. Long alkyl chain column is more stable than the short alkyl chain column

Table 1: Different types of column in HPLC

Column chemistry	Type of chromatography	Retention
C18(octadecyl silation)	Reversed phase	Highly nonpolar stationary phase Strong interaction with nonpolar components, High retention,
C 8 (octyl silation)	Reversed phase	Comparatively less retention
C4(4 carbon alkyl chain)	Reversed phase	Low retention
C1(trimethylsilyl)	Reversed phase	Least retention
Phenyl	Reversed phase	Moderate retention
CN(Cyno)	Reversed phase/Normal phase	Weak retention for non polar components used for separation of carbohydrate
OH	Normal phase	More polar stationary phase than Cyno
Amino	Normal phase	Highly polar stationary phase

Column diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. Loading capacity of sample is directly related to internal diameter of column. Column with smaller diameter has low load capacity (small amount of sample can be injected) and less solvent (mobile phase) consumption with improved separation. For example column used for analytical purpose have an internal diameter of 4.6 mm and are used for quantitative analysis of sample using UV, PDA detectors. Column with larger diameter has larger load capacity therefore large amount of sample can be loaded in one injection. These columns are also known as semi preparative column are usually seen in industrial applications such as the separation and purification of sample for further use. Narrow-bore columns (1-2 mm) are used when more sensitivity is desired either with fluorescence detection, UV–Visible detectors or with other detection methods like liquid chromatography – mass spectrometry. Capillary columns having a size within 0.3 mm are used with alternative detection means such as mass spectrometry. These columns are usually made from fused silica capillaries, rather than the

stainless steel tubing that are employed by larger columns [22].

Particle size and Column length

Most HPLC separation columns contain small spherical silica particles which come in many sizes with 5 µm being the most commonly used. Column with small particle size packing has greater interactive surface area to interact with analyte this in turns improve overall efficiency of the column. Analyte resolution equation is expressed as

R - 1/4(√N) * (k'/ k'+1) * (α- 1)

Efficiency retention selectivity

The equation comprises three terms: selectivity, retention capacity, and efficiency. Each of these terms is affected by the specific components of an analytical method. A column's particle size, in particular, affects the efficiency term of the resolution equation. A smaller particle size is capable of providing better resolution with no increase in run time, or faster separations with no loss in resolution. An increase in column length gives improved resolution and selectivity. A decreases in flow rate increases column length as well as separation.

Column Particle Shape

For reproducible performance column particles must be of uniform spherical shape with uniformity in particle size. As particle size decreases, percentage of fines and irregular shaped particles increases. This variation in particle shape directly affects performance and consistency of the column. A column packed with uniform spherical beads result in uniformity in packing and voids between particles. The flow of a packed HPLC sample column using perfectly spherical particles has a very predictable performance when used to separate molecules

Column Temperature

An increase in column temperature result in shorter run time sharper peaks better sensitivity and lower column back pressure. In normal phase chromatography change in column temperature have a minor change in band spacing [23]. In reversed phase chromatography change in temperature result in notable change in capacity factor as well as resolution between components of ionic samples [24] but a minor change in neutral samples. A small increases in temperature result in decrease retention or capacity factor (k) so temperature change is an effective parameter for changing band spacing and improving resolution. Changing temperature for effective method development is convenient as it do not require change of column and mobile phase composition.

Pump Pressure Ability of pump used in HPLC is measured on its ability to produce consistent and reproducible flow rate**.** Modern HPLC systems like UPLC works at much higher pressure and therefore are able to use much smaller particle sizes(less than 2 µm) in the columns [25].

3.3 Mode of Separation

3.3.1 Isocratic Mode

In isocratic condition eluent composition as well as movement of compounds running through the column remains constant. Interaction of eluent (mobile phase) and stationary phase with the compound being separated are also constant throughout the run. Therefore isocratic separation gives more predictable and reproducible result. However the numbers of compounds separated in single run is limited when compared to gradient mode. Isocratic separation should be started with strong solvents to check for the samples detection. Then, separate the samples can be achieved by controlling the retention time by changing mobile phase composition. Increasing the concentration of strong elution efficiency solvents results in shorter retention time and decreasing in concentration of them results in longer retention time.

3.3.2 Gradient Mode

Gradient elution is employed for complex multi component samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). Peak widths differ depending on the rate of the eluent composition variation (gradient slope). Organic solvent composition is changed continuously in order to get shorter separation time with great changes in retention time by slight changes in organic solvent composition

3.4 Mobile Phase Composition

In HPLC, retention or capacity factor of the compound to be analyzed depends on type of organic solvents, composition or solvent strength [26]. Methanol and acetonitrile are extensively used for HPLC. In reversed phase chromatography, retention of the compound in the column decreases as the % of nonpolar (organic) mobile phase increases while in normal phase chromatography retention increases as the % of nonpolar (organic) mobile phase increases. Mobile phase can be a mixture of water and organic phase such as methanol, acetonitrile and the strength of mobile phase is expressed in % organic or non polar phase. Initially method development can be started with 100% organic phase and gradually decreasing % organic phase and increasing water % until effective separation of the compounds is achieved.

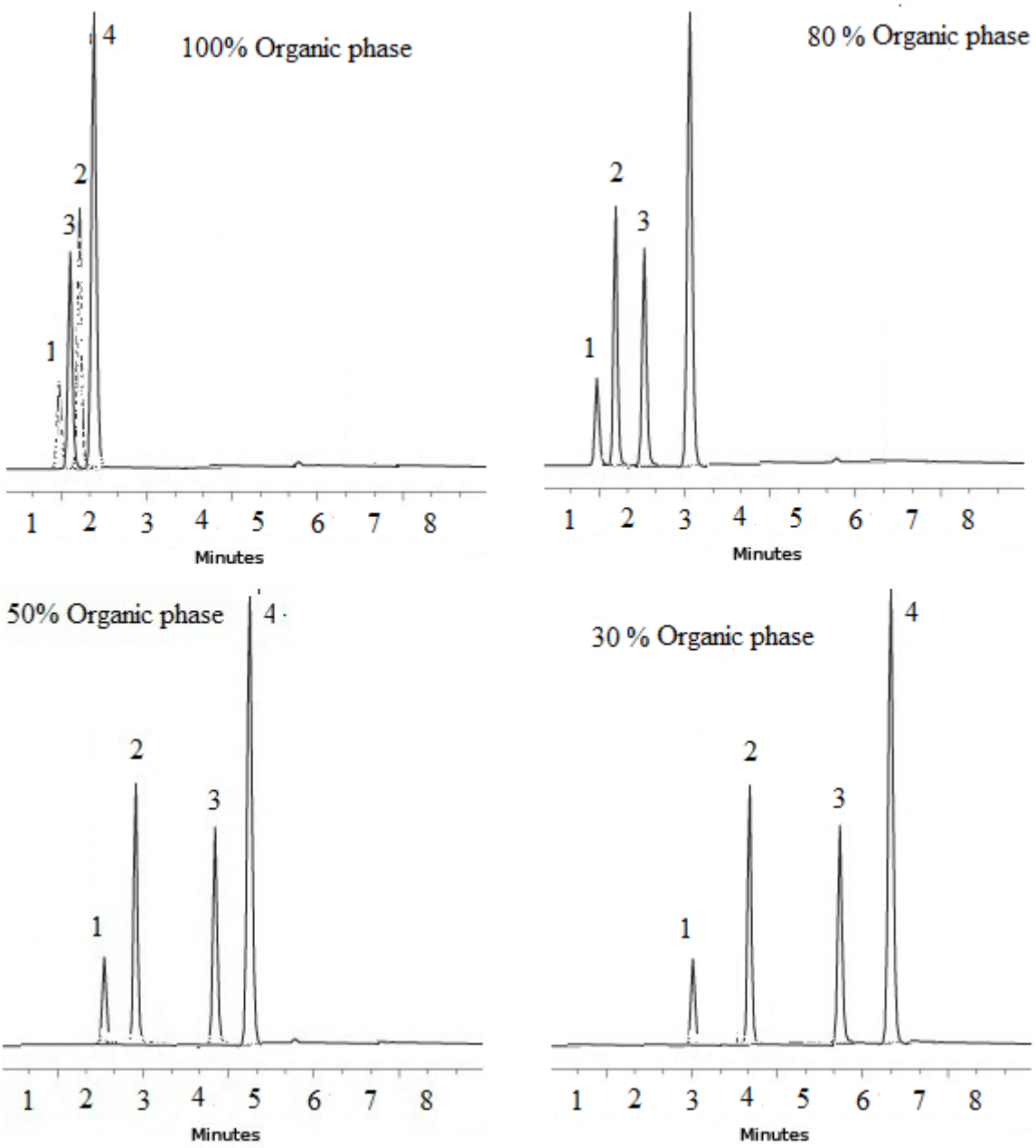

Figure 7: Effect of % Organic phase on retention of compounds

In RP-HPLC a decrease in retention can be achieved by using less polar mobile phase, polar column and by increasing column temperature While an increase in retention can be achieved by using more polar mobile phase, less polar column and a decrease in column temperature.

3.5 Mobile Phase pH

In reversed-phase high performance liquid chromatography, pH and ionic strength of the aqueous mobile phase is important in developing rugged methods which is not affected by small variations in conditions. With ionic compounds, retention of typical species shows significant changes with pH [27,28]. It is very important to control pH in reversed phase systems to stabilize retention and selectivity. The typical pH range for reversed-phase on silica-based packing is pH 2 to 8 and it is better to start method development with this pH range for most of the samples including basic compounds and typical weak acids. pH of the mobile phase can be controlled by using buffer. A buffer is an aqueous solution containing a weak acid and its conjugate base or a weak base and its conjugate acid. A buffered solution's pH changes very little when a small amount of strong acid or base is added to it. It is used to prevent any change in the pH of a solution, regardless of solute. Buffer solutions are used as a means of keeping pH at a nearly constant value. Choice of buffer is typically governed by the desired pH. It is important that the buffer has a pKa close to the desired pH since buffers control pH best at their pKa. For reproducibility, the pH used should be ± 1 pH unit above or below the dissociation constant (pKa) of the compound being separated, however buffer with a pKa value ±2 units of the desired mobile phase pH gives acceptable result. Polymer-based HPLC columns have outstanding stability and can be used from pH 1 -14. For all separations, measure and adjust pH on the aqueous component, before mixing with organic modifiers, to give the most accurate and reproducible results. However, if pH is not controlled properly, pH can be a source of many problems. Since most compounds analyzed by RP-HPLC contain one or more acidic or basic functional groups, most mobile phases require pH control. For this reason, buffers are widely used.

In reversed phase chromatography retention is more for hydrophobic (unionized) compounds as compared to hydrophilic (ionized) form. Acids lose a proton (and become ionized) as pH increases; bases gain a proton (and become ionized) as pH decreases. As pH increases, RPC retention for an acid decreases and retention for a base increases [29]. Sample ionization and retention exhibit a characteristic S-shaped plot

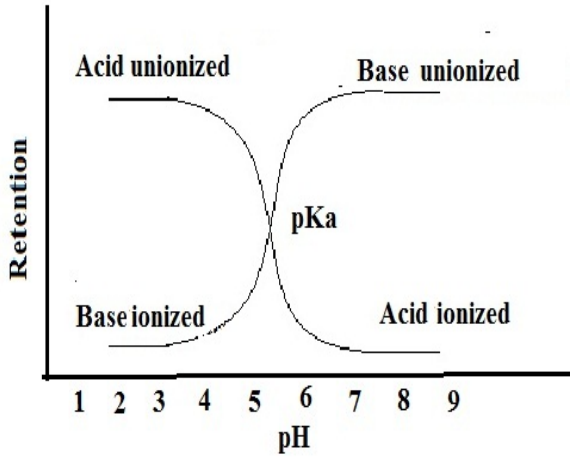

Figure 8: Effect of pH on retention of acid and base

At the midpoint of this retention-pH curve the pH is equal to the *pKa* value of the compound
Ionizable compounds, such as acids or bases, cause significant changes in retention time and selectivity with changes in Ph. Compounds which are non polar or neutral are insignificantly affected by mobile phase pH and have minimum effect on retention time.

3.6 Buffer Properties

Ideally buffer should be transparent in UV region and should not absorb UV light below 220 nm. Other properties like buffer solubility, stability, interaction with equipment parts, sample, column, and volatility are the significant factors and should be taken care while choosing the right buffer. The choice of buffer depends on working mobile phase pH and it should be in ±1 range of pKa of buffer.

Table 2: Common buffers used in RP- HPLC.

Common Buffers	pKa	Useful pH range	UV cutoff
TFA(Trifloroacetic acid)	0.5	Less than 1.5	210 nm
Sulphonate	1.8	1-2.8	
Phosphate	2.1	1.1-3.1	Less than 200 nm
	7.2	6.2-8.2	
	12.3	11.3-13.3	
Citrate	3.1	2.1-4.1	230 nm
	4.7	3.7-5.7	
	5.4	4.4-6.4	
Formate	3.8	2.8-4.8	210 nm
Acetate	4.8	3.8-5.8	210 nm
Ammonia	9.2	8.2-10.2	200 nm
Borate	9.2	8.2-10.2	-----
Diethylamine	10.5	9.5-11.5	Less than 200 nm

3.6.1 Buffer Solubility

Solubility of buffer in mobile phase is a crucial factor in RP-HPLC method development. Buffer should be fairly soluble in mobile phase so as to avoid precipitation of buffer in HPLC lining and column. Precipitation of buffer leads to improper result. Methanol-water mobile phases provide higher solubility than acetonitrile- water or THF-water solution; therefore methanol is the first choice as organic solvent. . As the % organic phase increases the rate of decrease in solubility is more for acetonitrile as compared to methanol and at 90% organic phase it is 5mM for methanol while buffer is insoluble at 90% acetonitrile concentration. Illustrated in the Table 3 for the solubility of potassium Phosphate buffer in commonly used HPLC solvent [29]

Table 03: Solubility of Potassium Phosphate buffer in commonly used HPLC solvent

% Organic phase	Methanol	Acetonitrile
50	More than 50 mM	More than 50 mM
60	More than 50 mM	45
70	35	20
80	15	5
90	5	0

The choice of salts also can make a significant difference in solubility. Ammonium salts of phosphate are much more soluble in ACN than are the potassium salts. Acetate is much more soluble than phosphate. Potassium salts are more soluble than sodium salts because of solubility differences therefore it is better to use potassium salt of the buffer.

Inorganic buffers are usually relatively stable but some buffers may degrade on standing and may increase their UV absorbance during storage or long-term use (e.g., TFA, triethylamine).

Citrate buffers show interaction with wall of stainless steel column and proper washing must be given. Citrate buffer show UV absorbance at 230 nm and cannot be used for analysis below 230 nm.

3.6.2 Buffer Concentration

Buffer concentration used in method development must be able to effectively control the pH during the separation. Concentration of buffer to be used in separation depends upon buffering capacity and solubility of the buffer in mobile phase mixture. Buffer used in higher concentration will hardly have any change in buffering capacity but may cause problem of buffer precipitation in HPLC lining and column, which result in poor separation and damage to column and HPLC lining. Generally

Buffer concentration in the range of 10-30 mM is adequate for effective separation.

Buffer concentration also has considerable effect on pKa of buffer which inturns can cause a dramatic change in separation.

Table 04: pKa value at different buffer concentration of common buffer used in HPLC[31]

		Equimolar concentration(mol/L)			
Buffer	**pKa**	**0.001**	**0.01**	**0.05**	**0.1**
Acetic acid/acetate	4.76	4.74	4.72	4.69	4.67
Ammonium/ammonia	9.25	9.26	9.28	9.32	9.34
Phosphoric acid/dihydrogenphosphate	2.16	2.15	2.13	2.09	2.07
Dihydrogen phosphate/hydrogen phosphate	7.21	7.14	7.01	6.85	6.76
Citric acid/dihydrogencitrate	3.13	3.12	3.10	3.06	3.04
Dihydrogencitrate/hydrogen citrate	4.76	4.69	4.56	4.40	4.31
Hydrogencitrate/citrate					

The addition of organic modifier to aqueous buffers for preparing mobile phases also affects pH/pKa and buffer capacity. The change in pH/pKa not only depends on % of organic phase used but also on type of buffer used.

Table 5: pKa Values of buffer in Acetonitrile-Water Mixtures [32]

	pKa Value in % Acetonitrile volume						
Buffer	0	10	20	30	40	50	60
Acetic /acetate	4.74	4.93	5.14	5.40	5.62	5.93	6.16
phosphoric/dihydrogenphosphate	2.21	2.38	2.59	2.76	2.97	3.20	3.29
dihydrogenphosphate/hydrogenphosphate	7.23	7.39	7.57	7.78	7.94	8.16	8.27
phthalic/hydrogenphthalate	2.92	3.07	3.23	3.41	3.54	3.71	3.77
hydrogenphthalate/phthalate	5.39	5.73	6.08	6.49	6.85	7.27	7.60
citric/dihydrogencitrate	3.16	3.30	3.46	3.64	3.76	3.94	3.99
ammonium/ammonia	9.l29	9.26	9.18	9.13	9.05	8.99	8.88

3.6.3 Preparation of Buffered Mobile Phase

A small variations in pH can have a remarkable impact on separation and reproducibility therefore a consistent techniques should be there to prepare buffer solution so as to achieve reproducibility[33,34].

Method 1

Solution of an acid and its conjugate base can be prepared by dissolving the acid form of the buffer in water required to obtain the final solution volume and adjusting the pH by adding strong base like NaoH. If buffer solution is made with a base and its conjugated acid the pH can be adjusted by using strong acid and monitored by using pH meter.

Method 2

Buffer solution can also be prepared by using equimolar concentration of both the acid and base form of the solution. To get the final buffer pH, one solution is added to the other while monitoring the pH using pH meter

Method 3

In this method, the exact amount of acid and conjugate base needed to make a buffer of a certain pH can be determined using the Henderson-Hasselbach equation:

pH= pKa+log ([A−][HA])

where pH is the concentration of $[H^+]$, pKa is the acid dissociation constant, and $[A^-]$ and [HA] are concentrations of the conjugate base and starting acid.

4. Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or modified method to ensure that the method is able to give reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The validation plan depends entirely on the particular method and its proposed applications [35-37].

To comply with the requirement of GMP, Pharmaceutical industries should follow the guidelines and parameters as per ICH, FDA and USP [36,38]

1. Specificity
2. Linearity & Range
3. Precision
   - Method precision (Repeatability)
   - Intermediate precision (Reproducibility)
4. Accuracy (Recovery)
5. Limit of Detection (LOD)
6. Limit of Quantification (LOQ)
7. Robustness
8. System suitability

4.1 Specificity

Selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers, and known (or likely) degradation products that may be expected to be present in the sample matrix[39].

4.2 Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity is usually expressed as the confidence limit around the slope of the regression line [36] For preparation of calibration curve minimum 5 concentration are prepared covering the specified range. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line or by the method of least squares. The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the method. The linearity range depends on the purpose of the test method. For example, Linearity range for drug substance or a finished (drug) product and dissolution testing is 80-120% and linearity range for content uniformity is 70-130%

4.3 Precision

The precision of an analytical procedure in HPLC expresses the closeness of agreement (degree of scatter) between a series of measurements or result obtained from repetitive injections of the same homogeneous sample under the prescribed conditions [40]. Precision may be considered at three levels:

4.3.1 Repeatability can be evaluated by assessing

(1) Minimum of 9 results covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or

(2) A minimum of 6 deter at 100 percent of the test concentration.

4.3.2 Intermediate precision (also known as ruggedness) expresses within laboratories variations, as on different days, or with different analysts or equipment within same laboratory.

4.3.3 Reproducibility is assessed by comparing inter laboratory results of method under specified condition. Reproducibility test are done for inclusion of procedures in pharmacopoeias or technology transfer. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation of series of measurements.

4.4 Accuracy (Recovery)

Accuracy is the measure of how close the experimental value is to the true value. It is determined by applying the method to samples to which known amounts of analyte have been added[41]. Accuracy studies for drug substance and drug product are performed at the 80, 100 and 120% levels of label claim as stated in the Guideline for for Methods Validation. These should be analyzed against standard and blank solutions to ensure that no interference. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Recovery data should be taken at least in triplicate, at each level (80, 100 and 120% of label claim) . The mean is an estimate of accuracy and the RSD is an estimate of sample analysis precision.

4.5 Limit of Detection (LOD)

Limit of detection (LOD) of an particular analytical method is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value under defined experimental condition. These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product.

In HPLC Limit of detection can be determined

- by knowing signal to noise (S/N) ratio if analytical procedure show baseline noise. Signal-to-noise ratio is determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 is generally considered acceptable. The signal-to-noise ratio is determined by: s = H/h Where H = height of the peak of the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

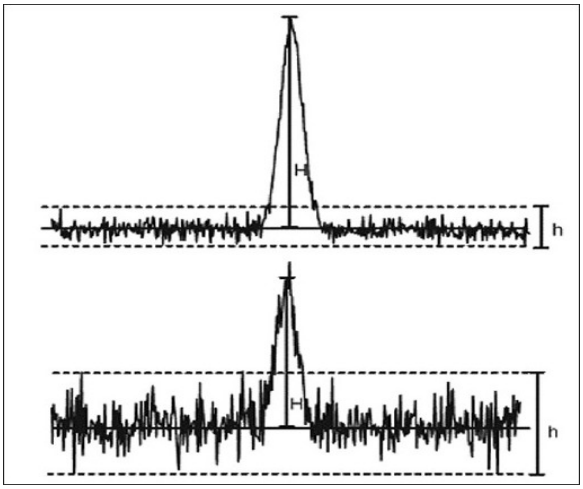

Figure 9: Signal to Noice ratio in HPLC

- LOD can also be determined by method based on the standard deviation of the response and the slope.

LOD =3.3 σ /S

Where σ = the standard deviation of the response it can be determined by plotting specific calibration curve of samples containing the analyte in the range of DL. The residual standard deviation of a regression line may be used as the standard deviation.

S = the slope of the calibration curve estimated from the calibration curve of the analyte.

4.6 Limit of Quantification (LOQ)

The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. In HPLC that exhibit baseline noise, the LOQ is estimated

- from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.
- Quantitation limit can also be determined based on calculation of standard deviation of the response and the slope.

LOQ = 10 σ /S

Where σ = the standard deviation of the response it can be determined by plotting specific calibration curve of samples containing the analyte in the range of LOQ

S = the slope of the calibration curve estimated from the calibration curve of the analyte.

4.7 Robustness: It is the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, flow rate, temperature and different column) .It confers reliability of the method during routine uses. Determination of robustness is a orderly process of varying a parameter and measuring the effect of change in parameter on the method by observing system suitability and/or the analysis of samples.

4.8 System Suitability: System suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the

concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Factors, such as the peak resolution, number of theoretical plates, peak tailing and capacity have been measure buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

Abbreviations

HPLC High Performance Liquid Chromatography
ICH International conference on Harmonization
GMP Good Manufacturing Process
Id Internal Diameter
LC Liquid Chromatography
LOD Limit of Detection
LOQ Limit of Quantitation
Mm Millimolar
NPC Normal Phase Chromatography
ODS Octyl decyl silane
PDA PhotoDiode Array
RP HPLC Reversed Phase Chromatography
THF Tetrahydrofuran
USP United states Pharmacopeia
UV Ultraviolet

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