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Review Article

A Review On: Development and Validation of HPLC in Pharmaceutical Dosage Form

Bharat Dadhich, Rakesh Goyal, Dilip Agarwal

Mahatma Gandhi College of Pharmaceutical Sciences, Jaipur, Rajasthan, India

A B S T R A C T

HPLC is the dominant separation technique in modern pharmaceutical and biomedical analysis because 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 in this method. 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. An analytical procedure is developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. This review gives information regarding various stages involved in development and validation of HPLC method. Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing.

Keywords: HPLC, Method development, Validation.

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***Address for Correspondence:**

Bharat Dadhich, Mahatma Gandhi College of Pharmaceutical Sciences, Jaipur, Rajasthan, India

INTRODUCTION:

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.[1]In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production.[2] HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.[3] The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants. 4High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability.[5] HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. (Figure-1) The technique of HPLC has following features.[6]

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

Need for analytical method[1-4]

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

In brief, the reasons for the development of newer methods of drug analysis are:

- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods for the quantization of the drug in biological fluids may not be available,
- The drug or drug combination may not be official in any pharmacopoeias,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Analytical techniques that are generally used for drug analysis are biological and microbiological methods, radioactive methods, physical methods and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.

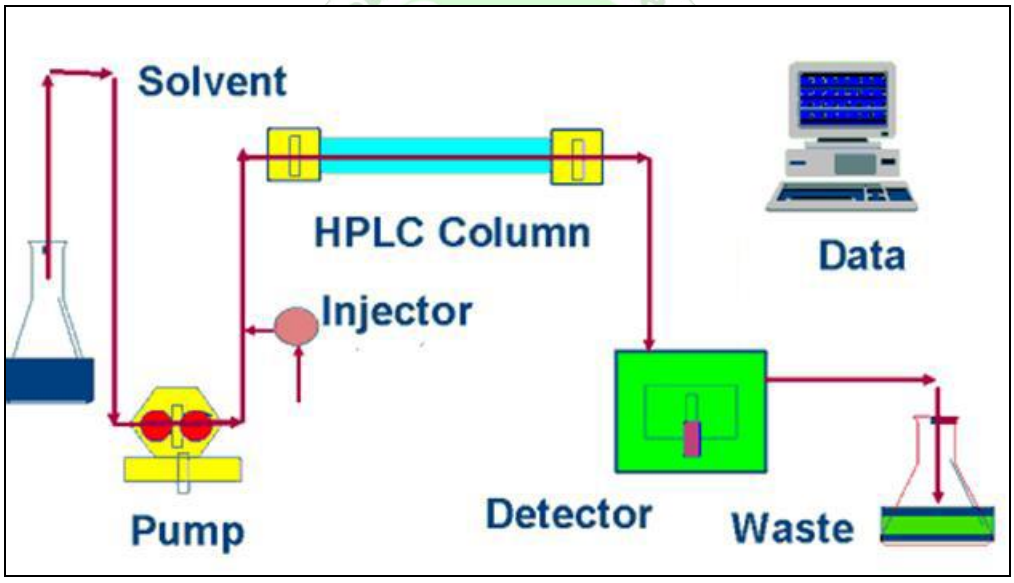

Figure1: Flow Diagram of HPLC

Introduction to HPLC method for analysis of drugs[9-12]

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 in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low volatility
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantitation (less time and less labor)
- Precise and reproducible
- Calculations are done by integrator itself
- Suitable for preparative liquid chromatography on a much larger scale

Types of HPLC

- Mainly two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography.
- In Normal-phase chromatography the stationary bed is

strongly polar in nature (e.g., silica gel), and the mobile phase is non polar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

- Reverse-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained. Above mentioned types cover almost 90% of all chromatographic applications. Eluent polarity plays the highest role in all types of HPLC.

There are two elution mode types: isocratic and gradient. In the first type constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, $C_{8,}$ C_4, etc., (in the order of increasing polarity of the stationary phase).

A schematic diagram of HPLC equipment is given in Figure 1.

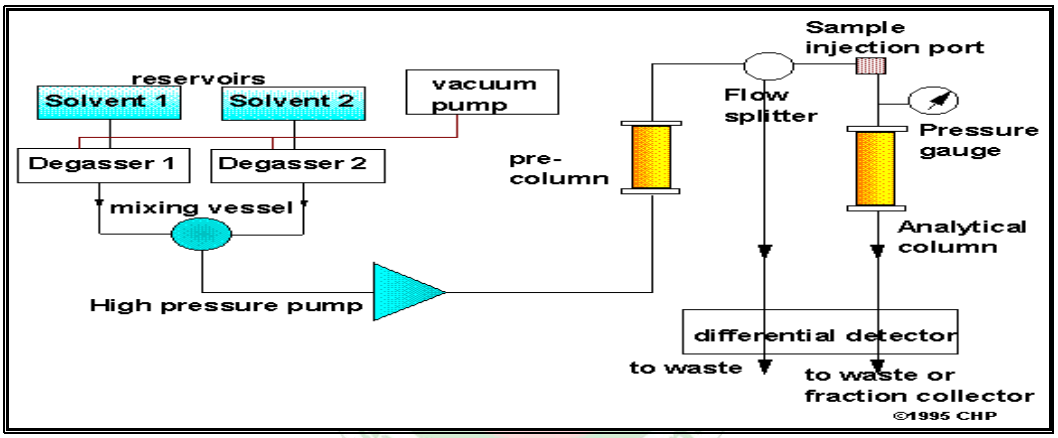

Figure 1: A schematic diagram of HPLC equipment

Various components of HPLC are[9, 10]

a) Solvent delivery system, including pump,
b) Sample injection system,
c) Chromatographic column,
d) Detector,
e) Strip chart recorder,
f) Data handling device and microprocessor control.

a) Solvent delivery system

- **Pumps**

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity.

Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system.

These are:

- Displacement pump:
- Reciprocating pump:
- Pneumatic or constant pressure pump:

b) Sample injection system

The design characteristics divided HPLC injectors into four types.

Type 1 Injectors

It use a completely filled sample loop to determine the injected volume. These simple, reliable devices are six-port rotary valves. A syringe is used to push or suck an excess of sample into a sample loop, filling it completely. Highly precise injections are achieved because the loop volume determines the injected volume.

Type 2 Injectors

It use a microsyringe to transfer sample into the loop. The sample size is always smaller than the loop volume, so it is the syringe which determines the injected volume. No sample is trapped or wasted, but the precision is not as high as type 1.

Type 3 Injectors

It use both complete and partial filling methods, but trap some sample. The loop is loaded by inserting the syringe into the needle port and dispensing the contents. The syringe is left inserted in the port until after the valve is switched. The switching action inserts the loop into the

stream without exposing the syringe to high pressure. In the injection position the syringe is removed and some sample remains trapped in a connecting passage of the injector. There are three consequences of this trapped volume: sample is wasted, the injector must be flushed after each injection and the syringe reading is in error by the amount of trapped volume.

Type 4 Injectors

It also uses both methods, but does not trap sample. This type is similar to type 3 injector but it does not contain a connecting passage between syringe needle tip and sample loop. It therefore not trap sample and there is no sample waste, no syringe reading error and no need to flush between injections, except in trace analysis.

c) Chromatographic column

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 µm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

- Porous, polymeric beds
- Porous layer beds
- Totally Porous silica particles (dia. <10 µm)

These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 µm are usually dry packed. While particles of diameter <20 µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

d) Detectors[13-16]

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. Detectors are usually of following types as described in Table 1.

Table 1: Types of detectors

Detector	Analytes	Comments
UV-visible	Any with chromophores	Has a degree of selectivity and is useful for many HPLC applications
Fluorescence	Fluorescent compounds	Highly selective and sensitive. Often used to analyze derivatized compounds
Refractive Index (RI)	Compounds with a different RI to the mobile phase	Virtually a universal detector but has limited sensitivity
Electrochemical	Readily oxidized or reduced compounds, especially biological samples	Very selective and sensitive
Evaporative Light Scattering (ELSD)	Virtually all compounds	A universal detector which is highly sensitive. Not selective
Mass Spectrometer (MS)	Broad range of compounds	Highly sensitive and is a powerful 2nd dimensional analytical tool. Many modes available. Needs trained operators

e) *Recorder*

It is a electromechanical instrument which transforms the chromatographic signal into a graphical record.

Method Development and Design of Separation Method on HPLC[17,18,19,20]

Methods for analyzing drugs in multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. Method development and design of separation method depends on selection of best mobile phase, detector, column length and diameter, buffer, pH of buffer, type of stationary phase etc.

a) *The Best Mobile Phase*

In reverse-phase chromatography, the mobile phase is more polar than the stationary phase. Mobile phase in these systems is usually mixtures of two or more individual solvents with or without additives or organic solvent modifiers. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes. The polarity of organic modifier and its proportion control the rate of elution of the components in the mobile phase. The rate of elution is increased by reducing the polarity. The simple alteration of composition of the mobile phase or of the flow rate allows the rate of the elution of the solutes to be adjusted to an optimum value and permits the separation of a wide range of the chemical types. First isocratic run followed by gradient run is preferred.

Since the mobile phase governs solute-stationary phase interaction, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this

reason, strong acids, bases and halide solutions should be avoided.

- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in column and eventually be detrimental to the results. HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.
- Only volatile buffers should be used in LC/MS.

b) *The Best Detector*

The next consideration should be the choice of detector. There is little use in running a separation if detector one uses cannot "see" all the components of interest, or conversely, if it "sees" too much. UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Unfortunately UV-visible detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromophores, such as aromatic rings, for UV-visible detection.

c) *The Best Column Length*

Many chromatographers make the mistake of simply using what is available. Often this is a 250 × 4.6 mm C_{18} column. These columns are able to resolve a wide variety of compounds (due to their selectivity and high plate counts) and are common to most laboratories. While many reverse phase separations can be carried out on such column, its high resolving capabilities are often unnecessary, as illustrated in Figure 4. Method development can be streamlined by starting with shorter columns; 150, 100 or even 50 mm long. This is simply because they have proportionally shorter run times.

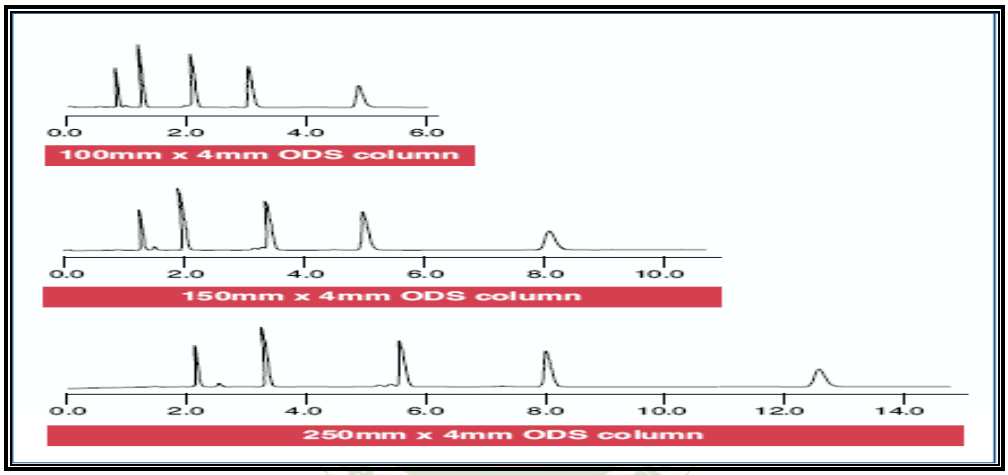

Figure 2: Effect of Column length

d) *The Best Stationary Phase*

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C_8 phase (reversed phase) can provide a further time saving over a C_{18}, as it does not retain analytes as strongly as the C_{18} phase. For normal phase applications, cyano (nitrile) phases are most versatile.

e) *The Best Internal Diameter*

By selecting a shorter column with an appropriate phase, run times can be minimized so that an elution order and an optimum mobile phase can be quickly determined. It can also be advantageous to consider the column internal diameter. Many laboratories use 4.6 mm ID columns as a standard, but it is worth considering the use 4.6 mm ID columns as an alternative. These require only 75 % of the solvent flow that a 4.6 mm column uses. This translates to a 25 % solvent saving over the life of the column and can be even more significant if a routine method is developed for such a column.

f) *Gradient Programming*

The fastest and easiest way to develop a method is to use a mobile phase gradient. Always start with a weak solvent strength and move to a higher solvent strength. To begin, use a very fast gradient (e.g.10 minutes) and then modify the starting and finishing mobile phases to achieve a suitable separation. Of course the choice of solvents and buffers may need to be modified during method development (Different HPLC instruments will give different results for the same gradient, so if a method is to be validated for use by several different laboratories, isocratic methods are recommended). Optimizing the mobile phase for an analysis will help to improve the separation. A number of factors depend upon the solvents chosen.

g) *Retention*

Analytes may be too strongly retained (producing long run times). If this occurs, the solvent strength should be increased. In reverse phase analysis this means a higher % of organic solvent in the mobile phase.

h) ***Poor Separation***

Analytes often co-elute with each other or impurities. To overcome this, the analysis should be run at both higher and lower solvent strengths so the best separation conditions may be determined. Varying solvents may help - try methanol instead of acetonitrile for reversed phase analysis. Using buffers and modifying the pH (within the column's recommended pH range) can also assist the separation. When the optimum conditions have been achieved, improving the resolution is often just a case of changing to a longer column and/or one with a smaller particle size to increase the column efficiency. (For reversed phase analysis, having started with a 100mm C8 column there is also the option of trying C18 columns to get better resolution. The important point is having used a short column for this stage of the development a lot of time was saved).

i) ***Peak Shape***

This is often a problem, especially for basic compounds analyzed by reversed phase HPLC. To minimize any potential problems always use a high purity silica phase such as Wakosil II. These modern phases are very highly deactivated so secondary interactions with the support are minimal. Buffers can be used effectively to give sharp peaks. If peak shape remains a problem, use an organic modifier such as triethylamine, although this should not be necessary with modern phases like Wakosil. One point often forgotten is the effect of temperature changes on a separation. To maximize the reproducibility of a method, it is best to use a column heater to control the temperature of the separation. A temperature of 35 – 40°C is recommended.

j) ***Buffer selection***

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, it retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

When separating acids and bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, "less acidic" reverse-phase columns are recommended and amine additives for the mobile phase may be beneficial. Optimum buffering capacity occurs at a pH equal to the p^{Ka} of the buffer. Beyond that, buffering capacity will be inadequate.

Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure 5). To be most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

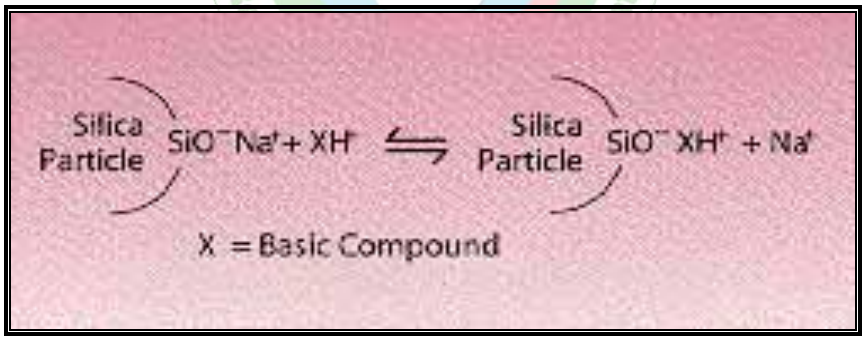

Figure 3: Peak Tailing Interaction

Table 2: Commonly used Buffers for reversed phase HPLC

Buffer	P^{Ka} (25 °C)	Maximum Buffer Range	UV Cutoff (nm)
Phosphate, $p^{K}{}_1$ H_2PO_4	2.1	1.1-3.1	< 200
Phosphate, $p^{K}{}_2$ HPO_4^{2-}	7.2	6.2-8.2	< 200
Phosphate, $p^{K}{}_3$ PO_4^{3-}	12.3	11.3-13.3	< 200
Citrate, $p^{K}{}_1$ $C_3H_5O(COOH)_2(COO)^{1-}$	3.1	2.1-4.1	230
Citrate, $p^{K}{}_2C_3H_5O(COOH)_1(COO)^{2-}$	4.7	3.7-5.7	230
Citrate, $p^{K}{}_3C_3H_5O(COO)^{3-}$	6.4	4.4-6.4	230
Carbonate, $p^{K}{}_1$ HCO_3^{1-}	6.1	5.1-7.1	< 200
Carbonate, $p^{K}{}_2$ CO_3^{2-}	10.3	9.3-11.3	> 200
Formate	3.8	2.8-4.8	210
Acetate	4.8	3.8-5.8	210
Ammonia	9.3	8.3-10.3	200
Borate	9.2	8.2-10.2	N/A

k) Selection of pH

The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized. For this reason, operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases. The pKa value [acid dissociation (ionization) constant] for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. A more rugged mobile phase pH will be at least 1 pH unit different from the analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography. Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

System Suitability Tests for Chromatographic Methods[21,22]

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample" that is a mixture of main components and expected by-products. List of the terms to be measured and their recommended limits obtained from the analysis of the system suitability sample are given below.

Definition

The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, column, and analyst) is suitable for the intended application. The USP Chromatography General Chapter states:

"System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The test 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."

Table 3: System suitability parameters and recommendations

Parameters	Recommendation
Capacity Factor (k')	The peak should be well resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD ≤ 1 % for N > 5 is desirable
Relative Retention	Not essential as long as the resolution is stated
Resolution (R_s)	R_s of > 2.0 between the peak of interest and the closest eluting potential interferents (impurity, excipient, degrade product etc.)
Tailing Factor (T)	T of ≤ 2
Theoretical Plates(N)	In general should be > 2000

The parameters that are affected by the changes in chromatographic conditions are:

a) Resolution (R_s)
b) Capacity factor (k')
c) Selectivity (α)
d) Column efficiency (N)
e) Peak asymmetry factor (A_s)

a. Resolution (R_s)

Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.

The resolution, R_s, of two neighboring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5.

It is calculated by using the formula,

$$R_s = Rt_2 - Rt_1 / 0.5 (W_1 + W_2)$$

Where,

Rt_1 and Rt_2= The retention times of components 1 and 2

W_1 and W_2= Peak width of components 1 and 2.

There are three fundamental parameters that influence the resolution of a chromatographic separation:

- Capacity factor
- Selectivity
- Column efficiency

b. Capacity Factor (k')

Capacity factor is the ratio of the reduced retention volume to the dead volume.

Capacity factor, k', is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained

by a column during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$k' = V_1 - V_0 / V_0$

Where,

V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The values of k' of individual bands increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in water/ organic mobile phase. Typically an increase in percentage of the organic phase by 10% by volume will decrease k' of the bands by a factor of 2-3.

Adjusting capacity factor (k')

Good isocratic method usually have a capacity factor (k') in the range of 2 -10 (typically 2 – 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively broad peak and unacceptably long run time. If the shift in k' value is observed with both analyst and the column test solution, the

Problem is most likely due to change in column, temperature or mobile phase composition. This is true if the shift occurs gradually over series of run.

Capacity factor (k') values are sensitive to:

- Solvent strength
- Composition
- Purity
- Temperature
- Column chemistry
- Sample

c. Selectivity (α)

The selectivity (or separation factor),α, is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components.

This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of α is 2. It can be calculated by using formula,

$\alpha = V_2 - V_1 / V_1 - V_0 \quad = k_1' / k_2'$

Where,

V_0 = the void volume of the column,

V_1 and V_2 = the retention volumes of the second and the first peak resp.

Adjusting selectivity (α)

When trouble shooting changes in selectivity (α), the approach is similar to the approach used in the capacity factor. When selectivity (α) is affected, the corrective action depends on whether the problem is mobile phase or column related. Be sure to compare results obtained with the test solution to those observed when the column was new.

Selectivity (α) values are sensitive to:

- Changes in mobile phase composition (pH ionic strength)
- Purity
- Temperature

d. Column Efficiency/ Band broadening

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Column with N ranging from 5,000 to 1,00,000 plates/meter are ideal for a good system.

Efficiency is calculated using the formula,

$N = 16\ Rt^2 / W^2$

Where,

Rt = is the retention time and W = is the peak width.

A decline in measured column efficiency may be due to

- Age and history of the column.
- Extra column band broadening
- Inappropriate detector setting.
- Change in flow rate and solvent viscosity

You can recognize problems in your separation due to a loss of column efficiency when the width or shapes of all peaks are affected.

Validation of analytical or bio-analytical methods[23,24,25,26]

Types of Method Validation

1) ***Full Validation***

A full validation is required

- If the method is developed and implemented for the first time,
- If a new drug entity is analyzed, or
- If metabolites are added to an existing assay for quantification.

2) ***Partial Validation***

It is performed if validated bioanalytical methods have been modified. It can range from the determination of a within-day accuracy and precision to a nearly full validation.

Typical situations for a partial validation are:

- Method transfers between laboratories and analysts,
- Instrument and/or software platform changes,
- Changes in species within the same matrix,

- Changes in matrix within the same species,
- Change in analytical methodology, and
- Change in sample processing procedures.

3) ***Cross Validation***

In a cross validation two bioanalytical methods for the same analyte are compared. Cross validations are necessary when two or more bioanalytical methods are used to generate data within the same study. They should be conducted with spiked matrix standards and subject samples.

A cross validation should be also considered when

- Sample analyses within a single study are conducted in more than one laboratory,
- Data generated using different analytical techniques in different studies are included in a regulatory submission.

As per the United States Pharmacopoeia (USP), 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 and performance characteristics are expressed in terms of analytical parameters[28].

According to ICH Q2 R1, Typical parameters used in analytical validation are:

- Specificity
- Linearity
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantitation
- Robustness
- Ruggedness

The different parameters of analytical method development are discussed below as per ICH guideline[30].

1) Specificity:

Definition

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Method

- When the impurities are available: Spiking of pure substance (drug substance or drug product) with appropriate levels of impurities/excipients and demonstrate the result is unaffected.
- When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition.
- In chromatographic method: Peak purity test to be done by diode array and mass spectrometry.

Expression/calculation

- Proof of discrimination of analyte in the presence of impurities. e.g. for chromatography chromatogram should be submitted.
- Peak purity test helps in demonstrating that the peak is not attributable to more than one component.
- For assay two results should be compared and for impurity tests two profiles should be compared.

2) Linearity:

Definition

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Method

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation

Minimum of 5 concentrations are recommended

Expression/calculation

Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

3) Range:

Definition

The range of analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Method:

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation:

- Assay of drug/finished product: 80 – 120 % of test concentration.
- For content uniformity: 70 – 130 % of test concentration.
- For dissolution testing: ± 20 % over specified range.
- For impurity: from reporting level to 120 % of specification.

Expression/calculation:

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

4) Accuracy:

Definition

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Method

- Application of procedure to analyze synthetic mixture of known purity.
- Comparison of result with already established procedure.
- Accuracy may be inferred once precision, linearity and specificity have been established.

Recommendation

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

Expression/calculation

- Percent recovery by the assay of known added amount of analyte
- Mean – Accepted true value with confidence interval

5) Precision:

Definition

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Method

Determination of % relative standard deviation (RSD) of response of multiple aliquots

Recommendation

a) Repeatability (Same operating condition over short interval of time):

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates (or)
- At target concentration × 6 determinations

b) Intermediate precision (within laboratory variation):

- Different Days
- Different Analysts
- Different Equipment etc.

Expression/calculation:

Standard deviation, % RSD and confidence interval

6) Detection Limit:

Definition

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Method

1. By visual evaluation

2. Based on S/N ratio

- Applicable to procedure, which exhibit baseline noise.
- Actual lowest concentration of analyte detected in compared with blank response

3. Based on S.D. of response and slope

$LOD = 3.3\ \sigma/s$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Expression/calculation

- If based on visual examination or S/N ratio – relevant chromatogram is to be presented.
- If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.

7) Quantitation Limit:

Definition

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Method

1. By visual evaluation

2. Based on S/N ratio

- Applicable to procedure, which exhibit baseline noise.
- Actual lowest concentration of analyte detected in compared with blank response

3. Based on S.D. of response and slope

$LOQ = 10\ \sigma/s$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Recommendation

Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.

Expression/calculation

- Limits of quantitation and method used for determining should be presented.
- Expresses as analyte concentration.

8) Robustness:

Definition

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Method

It should show the reliability of an analysis with respect to deliberate variations in method parameters.

In case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

Recommendation

- Robustness should be considered early in the development of a method.
- If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

Expression/calculation

Effect of these changed parameters on system suitability parameters.

9) Ruggedness:

Definition

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions.

Method

Analysis of aliquots of homogenous lots in different laboratories by different analysts under different operational and environmental conditions.

Expression/calculation

% RSD

Note: In the guideline on definitions and terminology, the ICH did not address ruggedness specifically. This apparent omission is really a matter of semantics, however, as ICH chose instead to cover the topic of ruggedness as part of precision, as discussed previously.

10) Stability:

During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phases, standards, and sample solutions. For routine testing in which many samples are prepared and analyzed each day, it is often essential that solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto-samplers.

Stability has not been given due importance in ICH guidelines but the USFDA has discussed stability parameters for biosamples. It is important to determine the stability of an analyte in a particular matrix by comparison with freshly prepared standards.

CONCLUSION

In recent years development of the analytical methods for identification, purity evaluation and quantification of drugs has received a great deal of attention in the field of pharmaceutical analysis. This review describes HPLC method development and validation in general way. A general and very simple approach for the HPLC method development for the separation of compounds was discussed. Knowledge of the physiochemical properties of the primary compound is of utmost importance prior to the any HPLC method development. The selection of 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.

REFERENCES:

1. V. Gupta, A.D. K. Jain, N.S. Gill, K. Gupta, Development and validation of HPLC method - a review , Int. Res J Pharm. App Sci., 2012; 2(4) :17-25
2. Y. Kazakevich, R. Lobrutto, HPLC for Pharmaceutical Scientists, John Wiley & Sons, New Jersey, 2007.
3. S. Ahuja, H. Rasmussen, Development for Pharmaceuticals, Vol.8 Separation Science and Technology, Elsevier, New York 2007.
4. M.S. Azim, M. Mitra, P.S. Bhasin, HPLC method development and validation: A review, Int. Res. J. Pharm.2013; 4(4):39-46.
5. B.V. Rao, G.N. Sowjanya1, A. Ajitha, V.U.M. Rao, Review on stability indicating hplc method development, World Journal of Pharmacy and Pharmaceutical Sciences, 2015; 4(8):405-423.
6. M.S. Charde, A.S. Welankiwar, J. Kumar, Method development by liquid chromatography with validation, International Journal of Pharmaceutical Chemistry, 2014; 04(02):57-61.
7. C.K. Kaushal, B. Srivastava, A process of method development: A chromatographic approach, J. Chem. Pharm. Res.2010; 2(2):519-545.

8. N.Toomula, A. Kumar, S.D.Kumar, V.S. Bheemidi, Development and Validation of Analytical Methods for Pharmaceuticals, J Anal Bioanal Techniques.2011; 2(5):1-4.
9. K. Kardani, N. Gurav, B. Solanki, P. Patel, B. Patel, RP-HPLC Method Development and Validation of Gallic acid inPolyherbal Tablet Formulation, Journal of Applied Pharmaceutical Science.2013; 3(5):37-42.
10. B. Nigovic, A. Mornar, M. Sertic, Chromatography – The Most Versatile Method of Chemical Analysis, Intech 2012; 385-425.
11. T. Bhagyasree, N. Injeti, A. Azhakesan, U.M.V. Rao, A review on analytical method development and validation, International Journal of Pharmaceutical Research & Analysis,2014; 4(8):444-448.
12. A. Shrivastava, V.B. Gupta, HPLC: Isocratic or Gradient Elution and Assessment of Linearity in Analytical Methods, J Adv Scient Res, 2012; 3(2):12-20.
13. V. Kumar, R. Bharadwaj, G.G., S. Kumar, An Overview on HPLC Method Development, Optimization and Validation process for drug analysis, The Pharmaceutical and Chemical Journal, 2015; 2(2):30-40.
14. Validation of Analytical Procedures: Text and Methodology, International Conferences on Harmonization, Draft Revised 2005; Q2 (R1).
15. Validation of Compendial Procedures, United State Pharmacopeia, USP 36 NF, 2010; 27(2).