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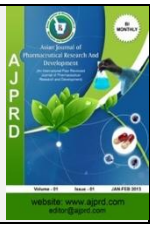

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Review Article

Method Development and Validation for Simultaneous Estimation of Pharmaceutical Dosage Form by HPLC

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A B S T R A C T

Many different strategies of high performance liquid chromatographic method development are used today. This review describes a strategy for the systematic development of High performance liquid chromatographic (HPLC) methods. HPLC is an analytical tool which is able to detect, separate and quantify the drug, its various impurities and drug related degradants that can form on synthesis or storage. It involves the understanding of chemistry of drug substance and facilitates the development of analytical method. A number of chromatographic parameters were evaluated in order to optimize the method. An appropriate mobile phase, column, column temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as degradants and impurities. Forced degradation or alternatively referred as stress testing and it demonstrates specificity when developing stability indicating methods, especially when little is known about potential degradation products. Force degradation studies are helpful in development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances (e.g. excipients).

Key words: High Performance Liquid Chromatographic, Chromatographic Parameters, Impurities.

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INTRODUCTION:

High Performance Liquid Chromatography, or HPLC, is the most common analytical separation tool and is used in many aspects of drug manufacture and research. HPLC is fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity makes it ideal for analysis of many drugs in both dosage forms and biological fluids. High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC is able to separate macromolecules and ionic species, labile natural products, a wide variety of other high molecular- weight polyfunctional groups and polymeric materials[1].

HPLC is presently used in pharmaceutical research and development in following areas:

1. Qualitative and quantitative analysis of unknown mixtures – determining what is there, and how much.
2. Separation of mixtures for later analysis – preparative HPLC.
3. To purify synthetic or natural products.
4. To characterize metabolites.
5. To assay active ingredients, impurities, degradation products and in dissolution studies.
6. In pharmacokinetic and pharmacodynamic studies[2-3].

Most of the drugs in multicomponent 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[4-5]:

- 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).
- Easy sample recovery, handling and maintenance.
- Instrumentation tends itself to automation and quantitation (less time and labour).

Basic principle of HPLC[6, 7, 8]

High Performance Liquid Chromatography (HPLC) is separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles a mobile phase designates the liquid flowing over particles. Under a certain dynamic condition, each component in a sample has difference distribution equilibrium depending on solubility in the phases and or molecular size. As a result, the components move at different speed over the stationary phase and thereby separated from each other. The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phases. Compound move in the column only when is in the mobile phase. Compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination.

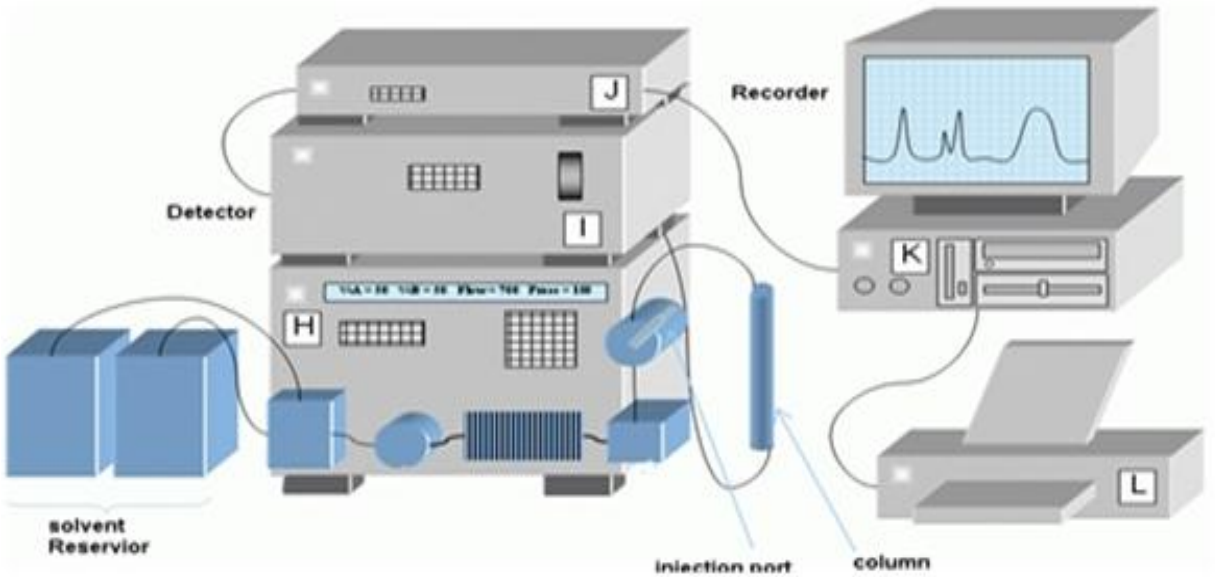


Figure: 1 Diagram of HPLC [9]

Mode of Separation [10, 11]

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography.

Normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

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 nonpolar 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 nonpolar 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).

METHOD DEVELOPMENT IN HPLC [12, 13]

Everyday many chromatographers face the need to develop a high-performance liquid chromatography (HPLC) separation. Method development and optimization in liquid chromatography is still an attractive field of research for theoreticians (researchers) and attracts also a lot of interest from practical analysts.

HPLC method development follows a series of steps which are summarized as follows:

The overall goal in method development is to optimize the resolution for the desired analyte(s) in the shortest possible time. When you start out thinking about method development, it's a good idea to review the key drivers of resolution.

A typical method development scheme has the following steps, which will guide our content in this section:

1. Choose the mode.
2. Choose the column and column packing dimensions.
3. Choose the stationary phase chemistry.
4. Choose the mobile phase solvents.
5. If the mode requires it, adjust the mobile phase pH.
6. Run some initial isocratic or gradient experiments to define boundary conditions.
7. Optimize the experimental conditions.

Aim of Separation

- Is the aim quantitative analysis of components of interest, detection of impurities, characterization of unknown sample components or isolation of pure substances?
- Are all the sample components to be resolved-separation of all impurities/ degradation products for reliable assay?
- For quantitative analysis, level of precision/accuracy required.
- Number of samples to be analyzed at a given time.
- Type of different sample matrices for which the method is expected to be applied.
- Type of HPLC system (isocratic or gradient).

Information about Sample

- Origin (especially important with biological samples).
- History (Sampling, storage, sample preparation).
- Number of components present in the sample.
- Chemical and physical properties
    - Molecular weight
    - Structure
    - Acid/Base properties (pKa value)
    - Solubility behavior.
- UV spectrum of the analyte.
- Nature of sample (solid, liquid, semisolids).

Selection of Sample and standard preparation:

Sample and standard preparation is an essential part of analytical cycle in HPLC. Any variation for the sample and standard preparation will contribute to the total variance ofthe analysis method results. Aim of sample preparation is to provide a reproducible and homogenous solution suitable for injection into column. It should be-

- Reasonably free from possible interferences.
- Solvent used for the preparation of the sample should be compatible with mobile phase and should not significantly affect the retention and resolution of the analyte.
- Should not damage the column.
- Compatible with proposed HPLC method.

Common sample preparation procedures are Filtration, Centrifugation, Solid- Phase extraction, Sonication, Derivatization, Liquid- Liquid extraction.

Mode of Chromatography[14, 15,16]

The first thing you should do is to choose the HPLC mode. The mode is generally decided by the type and solubility of the analyte(s) of interest, its molecular weight (MW), the sample polarity and the availability of the appropriate stationary phase and column. There are mainly two types of mode of separation.

Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C_{18} bonded.

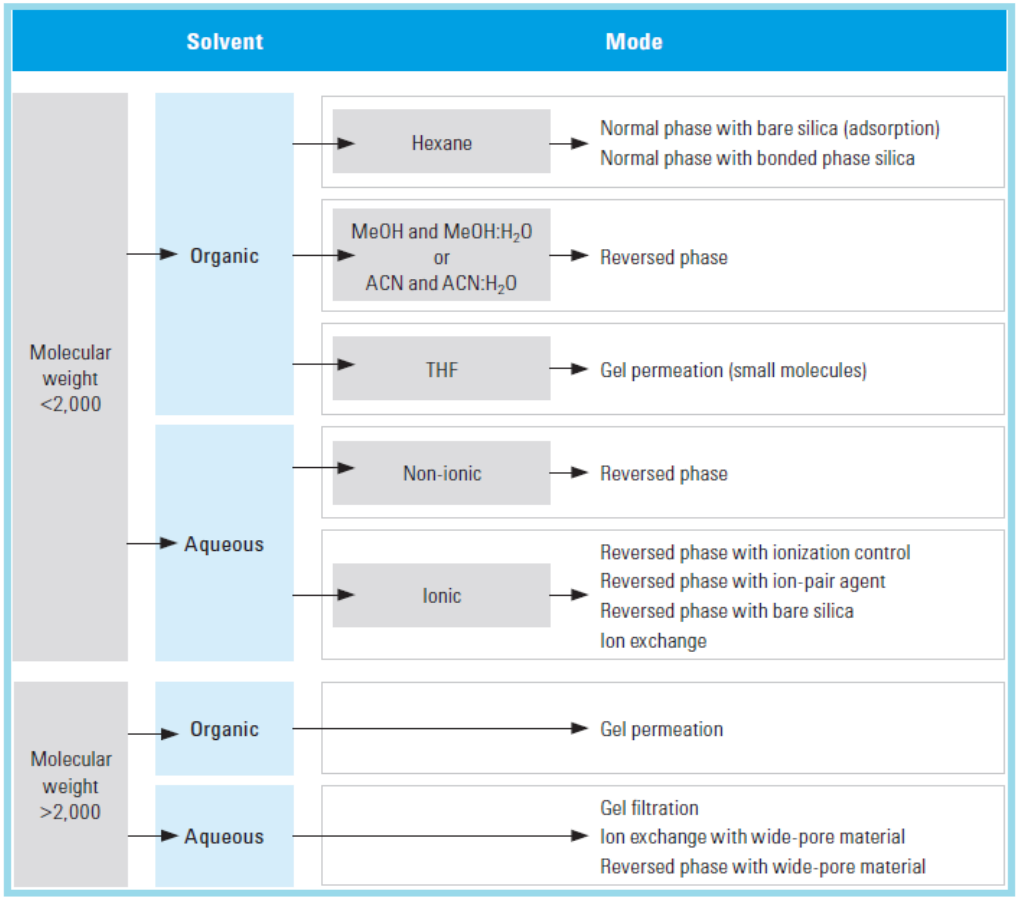
Normal phase is a potential candidate, particularly if the separation of isomers is required for low/medium polarity analytes. For inorganic anion/cation analysis, ion exchange chromatography is best. Size exclusion chromatography would normally be considered for. analysing high molecular weight compounds (2000).

Selection of normal phase or normal phase depend on many factor but main factor is given below: Polarity of analyte, Molecule weight of analyte.

Table: 1 Mode of separation depends on polarity of analyte. [14]

Mechanism of Separation	Use for analytes that are:	Loading Solvent	Eluting Solvent
Normal Phase (Adsorption)	Slightly to moderately polar	Low polarity (P'), e.g. hexane, $CHCl_3$	High polarity (P'), e.g. methanol, ethanol
Normal Phase (polar bonded phase)	Moderately to strongly polar	Low P', e.g. hexane, $CHCl_3$	High P', e.g. methanol, ethanol
Reversed Phase (nonpolar bonded phase-strongly hydrophobic)	Hydrophobic (strongly nonpolar)	High P', e.g. H_2O, CH_3OH/H_2O, CH_3CN/H_2O	Low P', e.g. hexane, $CHCl_3$
Reversed Phase (nonpolar bonded phase-intermediate hydrophobicity)	Moderately nonpolar	High P', e.g. H_2O, CH_3OH/H_2O, CH_3CN/H_2O	Intermediate, e.g. Methylene chloride, ethyl acetate
Reversed Phase (nonpolar bonded phase-low hydrophobicity)	Slightly polar to moderately nonpolar	High P', e.g. H_2O to moderate P', e.g ethyl acetate	High P', e.g acetonitrile, methanol
Polymeric Reversed Phase (Hydrophilically Modified)	Acidic, basic, neutral	Water or buffer	High P', e.g. acetonitrile, methanol

Table: 2 Modes of separation depend on M.W of analyte.[14]



Column Length and stationary Phase

Column is also referred to as the heart of the HPLC separation process. Stable, high performance column is essential requisite for rugged and reproducible method. For the better separation column length, its stationary phase, age of the column should be considered.

Column Length: For many years the column sizes most often recommended for analytical method development were 4.6 x 150 mm or 4.6 x 100 mm with a 5 µm particle size. If more resolution was needed, a 4.6 x 250 mm column was recommended. But with the range of modern choices available analytical method development with 4.6 x 100 mm columns with 3.5 µm or 2.7 µm superficially porous particles are the recommended starting point. During method development, choose the column id (for example 2.1 or 3.0 mm) to accommodate additional application objectives (such as sensitivity, solvent usage) or compatibility with certain instrument types (capillary, nano, or prep columns).Nano, capillary or microbore columns are used when increased sensitivity is required or when the sample is extremely limited.

- Nano columns for sample sizes below 1 pg used with nL/min flow rates.
- Capillary columns for sample sizes in the range pg to ng with flow rates around 4 µL/min.
- Microbore columns for sample sizes from ng to µg typically operate at flow rates around 40 µL/min.

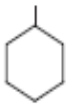
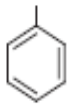
Stationary Phase:

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C_{18} 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.

Aged Column:

An aged HPLC column should be used to develop the initial HPLC conditions. Usually it is more difficult to achieve the required resolution with an aged column (e.g., column with about 200 injections). This will reflect the worst case scenario likely to be encountered in actual method uses, and help the long-term method robustness.

Table 3: Material used for stationary phase

Mechanism of Separation	Typical Phases	Structure(s)
Normal Phase (Adsorption)	Silica	Si-OH
	Alumina	AlOH
	Florisil	Mg_2SiO_3
Normal Phase (polar bonded phase)	Cyano	-CN
	Amino	$-NH_2$
	Diol	-CH(OH)-CH(OH)-
Reversed Phase (nonpolar bonded phase – strongly hydrophobic)	Octadecylsiloxane (C18)	$(-CH_2-)^{17}CH_3$,
	Octylsiloxane (C8)	$(-CH2-)_7CH_3$
	PS-DVB	PS-DVB
	DVB (Polymeric)	DVB
Reversed Phase (nonpolar bonded phase – intermediate hydrophobicity)	Cyclohexyl	
	Phenyl	
Reversed Phase (nonpolar bonded phase – low hydrophobicity)	Ethyl (C2)	$-C2H_5$
	Methyl (C1)	$-CH_3$

Mobile Phase and solvent strength

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Based on knowledge of sample composition and the goals of separation, the first question is: Which chromatographic method is most promising for this particular sample? Selection of the following is then required.

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength. for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforementioned capacity limits. Other factors (such as pH and the presence of ion pairing reagents) may also affect the overall retention of analytes.

General criteria should be considered for the selection of mobile phase:

(a)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 nearby analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

When considering method development with ionizable analytes, it is important to know that non-ionized analytes have better retention than ionized analytes.

If you have acidic analytes, choose a low mobile phase buffered pH to keep the analytes from being ionized. Knowing the pka of the analytes allows you to effectivity choose a mobile phase pH. A buffer is effective at +/- 1 pH units from the pK of the buffering ion, giving you some flexibility in optimizing your mobile phase. Acetate, for example has a pKa of 4.8 and buffers from pH 3.8-5.8. Formate is more acidic and buffers from pH 2.8-4.8. There are additional buffer choices if your acidic analytes would be not being ionizable at lower pH.

If you have basic compounds, the non-ionized form may be at a high pH that is not suitable for the column. But many basic compounds are adequately retained at low pH. While greater retention can be achieved in an nonionized

form, this may not be practical or necessary for all basic compounds.

(b) 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 pKa 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. To be most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

Table: 4 Commonly used Buffers for reversed phase HPLC[14]

	v/v	pK_a at 25 °C	Max. pH Range	UV Cutoff (nm)
Trifluoroacetic acid (TFA)	0.1%	0.3		210
	0.05%	0.3		210
	0.01%	0.3		210
Phosphate, pK_1		2.1	1.1. – 3.1	<200
Phosphate, pK_2		7.2	5.2 – 8.2	<200
Phosphate, pK_3		12.3	11.3 – 13.3	<200
Citrate, pK_1		3.1	2.1 – 4.1	230
Citrate, pK_2		4.7	3.7 – 5.7	230
Citrate, pK_3		6.4	5.4 – 7.4	230
Carbonate, pK_1		6.1	5.1 - 7.1	<200
Carbonate, pK_2		10.3	9.3 – 11.3	<200
Formate		3.8	2.8 – 4.8	210 (10 mM)
Acetic Acid (HAC)	1.0%	4.8	3.8 – 5.8	210
Acetate		4.8	3.8 – 5.8	210 (10 mM)
Ammonia		9.2	8.2 – 10.2	200 (10 mM)
Borate		9.2	8.2 – 10.2	n/a
Triethylamine (TEA)		10.8	9.8 – 11.8	<200
TRIS-HCl		8.3	7.3 - 9.3	205 (120 mM)

Selection of Detector:[15]

Detector is the main part of LC system and measures the compounds after separation on the column. There are various types of detector used like UV detector, fluorescent, conductivity and Refractive index. (Table 1.5) Variable-wavelength ultraviolet (UV) detectors normally are the first choice, because of their convenience and applicability for most samples. For this reason, information on UV spectra can be an important aid for method development. Selection of detectors is based on the following criteria:

- Potential interferences.
- Chemical nature of analytes.
- Limit of detection required.
- Availability and/or cost of detector.

Selection of detectors is based on the following criteria:

Table: 5 Detectors and their applications [15]

DETECTOR	ANALYTES	COMMENTS
UV-visible	Any with chromophores	Has a degree of selectivity and is useful for many HPLC applications
Fluroscence	Fluroscent 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 has a limited sensitivity.
Electrochemical	Readily oxidized or reduced compounds, biological compound	Very selective and sensitive.
Evaporative Light scattering (ELSD)	Virtually all compounds	A universal detector which is highly sensitive.
Mass spectrometer	Broad range of compounds	Highly sensitive and is a powerful 2- dimensional analytical tool.

Method Optimization

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. For the final method optimization following parameters should be considered.

- Shorter analysis time
- Better Resolution
- Better selectivity
- Better Sensitivity

After method optimization, system parameter optimization also considered. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Introduction to Analytical Method Validation

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety, efficacy throughout the all phases of the shelf life. Such monitoring is accordance with the specifications elaborated during product development. Analytical Method Validation is the corner stone of the process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new methods developed are validated.

Method Validation is the process of ensuring that a test procedure performs within acceptable standards of reliability, accuracy and precision for its intended purpose. In short, validation is the act of confirming that a method does what it is intended to do.. A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled. The transfer of a method is best accomplished by a systematic method validation process. The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method.

Type of analytical procedures to be validated

Validation of analytical procedures is directed to the four most common types of analytical procedures.

- Identification test.
- Quantitative test for impurities content.
- Limit test for the control of impurities.
- Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

In our method of validation, we are following last type.

Assay procedures are intended to measure the analyst present in given sample, assay represent a quantitative measurement of the major component(s) in the drug sample.

Objective of validation: Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

Performance characteristics examined when carrying out method validation are

- Accuracy / Precision.
- Repeatability / Reproducibility.
- Linearity / Range.
- Limit of detection (LOD)/ Limit of quantification (LOQ).
- Selectivity / Specificity.
- Robustness / Ruggedness.

Advantages of Validation

- Reliability of analytical results and assurance of quality product.
- Performance capability of the method can be confirmed by analysis using the method.
- Motivation for improvement in quality of work.
- Helps in scientific communication on technical matters.

Disadvantage of Validation

- Increasing cost.
- Need for experienced personnel.

Validation Requirements for Type of Analytical Method

The type of method and its intended use dictates which parameters needed to be investigated, as illustrated in Table 1.6.

- signifies that this characteristic is not normally evaluated.

+ signifies that this characteristic is normally evaluated.

(1) In cases where reproducibility has been performed, intermediate precision is not needed.
(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).
(3) May be needed in some cases.

Table: 6 Validation Requirements for different type of analytical method [20]

Type of Analytical Procedure Characteristics	Identification	Testing For Impurities Quantitation Limit	Assay - Dissolution (Measurement Only) - Content/Potency
Accuracy	-	+ -	+
Precision	-		
Repetability	-	+ -	+
Intermediate Precision	-	+ (1) -	+ (1)
Specificity	+	+ +	+
DetectionLimit	-	- (3) +	-
Quantitation limit	-	+ -	-
Linearity	-	+ -	+
Range	-	+ -	+

Furthermore revalidation may be necessary in the following circumstances:

- Changes in the synthesis of the drug substance.
- Changes in the composition of the finished product.
- Changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes.

The different parameters of analytical method development are discussed below as per ICH guideline:-

(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 chromatogr- aphy 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.

Acceptance criteria: Not specified.

(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 is recommended.

Expression/calculation

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

Acceptance criteria: Not specified.

(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.

Acceptance criteria: Not specified.

(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.

Acceptance criteria: Not specified

(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 replicate (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.

Acceptance criteria: Not specified.

(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 σ/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.
- Sy/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.

Acceptance criteria's/N ratio > 3 or 2:1; not specified in other cases.

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 σ/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.
- Sy/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.

Acceptance criteria: S/N ratio > 10:1; not specified in other cases.

(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.

Acceptance criteria: The method must be robust enough to withstand slight changes and allow routine analysis of sample.

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