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Analytical Method development, Method Validation and Technology Transfer using HPLC/UPLC

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Abstract
High pressure, or high performance, liquid chromatography (HPLC) is the method of choice for checking purity of new drug candidates, monitoring changes during scale up or revision of synthetic procedures, evaluating new formulations, and running control/assurance of the final drug product. Impurities and degradation products to be separated are frequently not known and must be elucidated as part of the method development process. The developed HPLC methods need to meet stringent validation requirements before they are utilized for any pharmaceutical evaluations. Selectivity and detectability optimization have been the primary goals in HPLC separations for most of time; however, HPLC scientists who are not trained in the physical sciences are reluctant to use theoretical considerations in method development. Our philosophy for method development is always based upon many considerations. It varies with Product characteristics, experimental conditions and regulatory requirements. Agreement on what is required of the method should be obtained before method development begins. Performance characteristics of method requirements must be extensively studied before fixing of methods and there should be an approach always for continuous improvement. Method validation and method transfer requires a systematic approach. Peoples feel validation and transfer only as a test of the acceptability of method using prefixed conditions and acceptance criteria's. However the real motive of method validation and transfer process is to challenge the method and determine limits of allowed variability for the conditions and specification values for meeting regulatory requirements while listening "voice of customer".

Keywords: HPLC, Method development, validation, Transfer, Regulatory, acceptance criteria

1. Introduction

1. What is Method

An analytical method is set of instrument parameters and chromatographic conditions which defines state of instrument during data collection. An analytical method also defines what should be selected for generating quality and authentic data.

2. Need of Method development and Validation

The pharmaceutical industry, as a vital segment of the health care system conducts research, manufactures and markets pharmaceutical and biological products for the treatment and diagnosis of diseases. The development of the pharmaceuticals brought a revolution in human health. These pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role. This review highlights the role of the analytical instrumentation and the analytical methods in assessing the quality of the drugs. The review highlights a variety of analytical techniques such as titrimetric, chromatographic, spectroscopic, electrophoretic, and electrochemical and their corresponding methods that have been applied in the analysis of pharmaceuticals.

The investigations on the pre drug discovery are based on knowing the basic cause of the disease to be treated, the information on how the genes are altered that cause the disease, the interaction of proteins and the affected cells and changes brought by these affected cells and how they affect these cells. Based on these facts a compound is developed which interacts with the affected cells and finally could become the drug molecule or active pharmaceutical ingredient (A.P.I) Drug discovery and Development, understanding the R&D process.

3. Method Development and Validation Characteristics

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products. The efficient development and validation of analytical methods are critical elements in the development of pharmaceuticals. Success in these areas can be attributed to several important factors, which in turn will

contribute to regulatory compliance. Experience is one of these factors—both the experience level of the individual scientists and the collective experience level of the development and validation department. Analytical methods must be validated to provide reliable data for regulatory submissions. These methods are essential for a number of purposes, including testing for QC release, testing of stability samples, testing of reference materials and to provide data to support specifications.

4. Introduction To validation process

Analytical method validation is the process of establishing through experiments that a method is suitable for intended use. Now a days, Analytical method validation and technology transfer is an important regulatory requirement for pharmaceutical analysis, as it provides documented evidence and assurance that the methods are suitable for determination of identity, strength, Quality, Purity and Potency of drug substances and drug products.

Prior to starting any practical work, a written validation protocol must be finalized while describing all different validation characteristics to be evaluated with the expected acceptance criteria, reference to or description of the analytical test method, and how the validation will be performed. While performing experimental, unexpected incidents are bound to occur. An established procedure should be followed that describes the handling of incidents and deviation during method validation. Any problem or failure should be investigated and well documented. After finalizing the practical work, a validation report should be prepared that summarizes all the results obtained. Individual values as well as summary tables, linearity plots, together with representative chromatograms should be provided. Any deviations observed should be commented on and the appropriate conclusions proving that the method is suitable for its intended use are drawn.

5. Strategy: How to Validate

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a stepby-step instruction format. This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use; ability to be automated and to be controlled by computer systems; costs per analysis; sample throughput; turnaround time; and environmental, health and safety requirements.

Successful acceptance of the validation parameters and performance criteria, by all parties involved, requires the cooperative efforts of several departments, including analytical development, QC, regulatory affairs and the individuals requiring the analytical data. The operating procedure or the Validation Master Plan (VMP) should clearly define the roles and responsibilities of each department involved in the validation of analytical methods.

6. Parameters of Validation

The process of validation of analytical method is adopted to confirm that the employed analytical procedure for a specific tests meet the intended requirements. The parameters for method validation have been defined in different working groups of national and international committees and are described in the literatureGuidelines from the ICH, Pharmacopoeias, Various regulatory agencies, FDA, Organizational Standard Operating procedures etc., can provide a framework for validations of pharmaceutical methods. Results from the method validation can be considered to judge its quality, reliability as well consistency pertaining to analytical results.

A tabular summary which details the validation characteristics that should be applied for different types of methods is included in the ICH guidelines. This table is extended in the draft FDA guidelines on 'Analytical Procedures and Method Validation' to include specific tests and robustness, and is reproduced below, see Table.

Type of Analytical procedures / Characteristic		Identification	Testing of Impurities		Assay Dissolution	Specific Test
			Quantitative	Limit	Content/ Potency	
Specificity		+1	+	+	+5	+4
Precision	Repeatability	-	+	-	+	+4
	Intermediate Precision	-	+2	-	+2	+4
Limit of detection		-	-3	+	-	-
Limit of Quantitation		-	+	-	-	-
Accuracy		-	+	-	+	+4
Linearity		-	+	-	+	-
Range		-	+	-	+	-
Robustness		-	+	-3	+	+4
Stability Indicating		+	+	+	+	+

Note

- Signifies that this characteristic is not normally evaluated.
+ Signifies that this characteristic is normally evaluated.

1) Lack of specificity for an analytical procedure may be compensated for by the addition of a second analytical procedure
2) In cases where reproducibility has been performed, intermediate precision is not needed.
3) May be needed in some cases.
4) May not be needed in some cases.
5) Lack of specificity for an assay for release may be compensated for by impurities testing.

The validation characteristics required by current regulatory guidelines to be examined during validation are defined in this

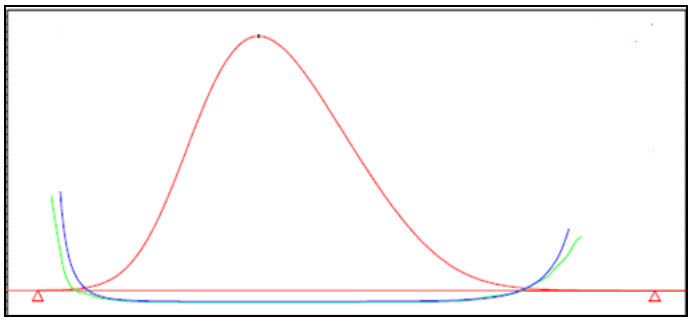
section. Where appropriate, a discussion is provided together with typical acceptance criteria for the different validation characteristics.

A) Specificity VS Selectivity

"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, degradation products, matrix, etc. The lack of specificity of an individual analytical method may be compensated for by an additional analytical method."

The terms selectivity and specificity are often used interchangeably, the term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate Selectivity and specificity are measures of the reliability of measurements in the presence of interferences. Where the measurement stage is non-specific, method development should indicate which analytes do not interfere. There will be cases where chemical interferences can be identified for a particular method but the chances of encountering them in real life may be improbable. The analyst has to decide at what point it is reasonable to stop looking for interferences. These parameters apply to both qualitative and quantitative analysis. The selectivity of a method is usually investigated by studying its ability to measure the analyte of interest in test portions to which specific interferences have been deliberately introduced (those thought likely to be present in samples). Where it is unclear whether or not interferences are already present, the selectivity of the method can be investigated by studying its ability to measure compared to other independent method/techniques.

Blank peaks are preferably absent or if present should not interfere with other peaks of interest. Placebo peaks in drug product methods should be separated from other relevant peaks. Unspecified degradation products and degradation impurities should be separated from the main component(s), and from other specified impurities/degradation products for drug product methods. Synthesis impurities are not specified in drug product methods, but are controlled by the drug substance methods. In drug substance methods, the unspecified degradation products and impurities should therefore also be separated from the specified impurities. Specified impurities should be separated from the main component(s), from the specified degradation products, and from each other for drug substance (DS) methods, and preferably from each other in drug product (DP) methods. Specified degradation products should be separated from the main component(s), from the specified impurities and from each other. Peak purity of the main components should be assessed preferentially by either another chromatographic method or a specific spectral analysis identification method like, for example, UV-photo diode array detection or MS detection. When peak purity of the main components is assessed with LC-UV-DAD, the UV-spectra in the front, middle, and the tail of the main compound peak should be comparable. Peak purity of the main components may also be performed with LC-MS. Similarly, the MS-spectra in the front, middle, and the tail of the main compound peak should be comparable. The identity of all impurities and degradation products should be confirmed by spiking experiments using certified reference material of synthesized impurities, or using LC-MS or LC-DAD on impurities in representative sample batches. Matching retention time windows, relative retention time windows, or UV- and MS-spectra confirm the identity of all compounds. If the impurities or degradation products are unavailable, specificity may be demonstrated by comparing the test results to a second well-characterized procedure. In addition, stress studies should demonstrate that impurities and degradants from the active pharmaceutical ingredient and drug product excipients do not interfere with the quantitation of the main product. When criteria for specificity are not met this often indicates that the method is not sufficiently developed. As a consequence, it is likely that the criteria for accuracy, precision, and linearity may also not be fulfilled. Lack of specificity may be compensated by other supporting analytical methods but finally the test method(s) should be able to ensure the identity of an analyte (identification test method), ensure that the method allows an accurate statement of the content of impurities (purity test methods), provide an exact result, which allows an accurate statement on the content or potency of the analyte in the sample (assay test methods).



• Represent Peak of interest • Peak purity angle • Peak purity threshold	Characteristics of a "Pure Peak" - No interference from blank - Purity angle < Purity Threshold - Can be confirmed using Photo diode detector, LCMS, LC-NMR too

B) System Suitability Criteria

"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 tests are based upon the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such."

System suitability may be finalized as combination of two or more parameter like Precision, Resolution, Peak to Valley Ration, Tailing, Symmetry Factor, Number of Theoretical Plates(Column Performance), S/N Ratio (Instrument Performance),Retention Time or Relative Retention Time.

A System Suitability Test should contain:

For Assay: Precision + one or more other parameter

For impurity test: Resolution + Precision + one or more other parameter.

The preceding list of possible system suitability tests is by no means exhaustive. If all of these tests were run for every method, there would be no time to run actual samples. It is up to the method developer or analyst to determine which set of tests will provide the most assurance that the method is running as expected. The number of tests and specific results will depend upon the application.

C) Precision

Precision is the degree of agreement among individual test results when the method/equipment is applied repeatedly to multiple samplings of a homogeneous sample/standard. Precision is usually expressed as relative standard deviation (%RSD) (=coefficient of variation). It may be a measure of reproducibility (which expressed precision between labs) or repeatability (same procedure within the same lab). ICH recommends that repeatability is demonstrated utilizing a minimum of nine determinations covering the specified range for the procedure (e.g. three concentrations/three replications each) or a minimum of six determinations at 100% of the test concentration. The ability of equipment to repeatedly generate reliable data shall be demonstrated during method validation.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

Repeatability (For System and methodology)

It is measured by multiple injections of the same reference solution and expressed as the relative standard deviation for the measured peak areas. The system precision considers the lowest variation of the analytical system, while analysis repeatability is related to all aspects of the test method including sample preparation. The analysis repeatability (within day or intra-assay precision) is determined by analyzing a representative sample batch at the target sample concentration six times or with a minimum of nine determinations covering the specified range of the test method.Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

The inherent variability of a method which is observed when subjected to typical operational variables found within the laboratory. Such variables include different analysts, different reagents, and different dates of testing. Method intermediate precision is measured when aliquots from the same homogeneous source sample are separately prepared and assayed by different analysts on different days using different reagents (i.e., separate working samples are prepared), but are tested using the same equipment.

Reproducibility: Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).If data to support reproducibility are available, intermediate precision assessment is not required. Reproducibility is usually tested during the method transfer activity. The acceptance criteria for reproducibility are implicitly broader than that for intermediate precision.

D) Limit of Detection (LOD) and Limit of Quantitation (LOQ)

As a part of establishing method sensitivity, first consideration comes about determining limit of detection referred as LOD and determining limit of Quantitation referred as LOQ for the method.

The minimum detectable amount of analyte often referred to as the limit of detection (LOD) is the smallest concentration that can be detected reliably. The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it.

The minimum quantitatible amount often known as the limit of Quantitation (LOQ) is the concentration that can be quantitated reliably with a specified level of accuracy and precision. The Quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation products.

There are many approaches for establishing LOD and LOQ for the methods. Every approach has its good aspects and limitation or challenges. Most popular approaches are:

a) Visual Evaluation: Sample with known concentration are injected and peaks are visually inspected for reliable detection. Concentrations are finalized in such a way that peaks are easily visible and can be marked properly. The lowest concentration where all peaks of interest are visible, can be integrated and area & peak heights are reproducible is determined as Limit of detection. RSD for area of six injections should be less than or equal to 30 %.

Once LOD is established LOD x 3.3 times is the concentration which can be selected as Limit of Quantitation. RSD for area of six injections should be less than or equal 10%. Accuracy at LOQ should be established by spiking the impurities at LOQ level in qualified samples and recovery obtained for the area should be within 70-130 %.

b) Standard deviation of the response based on the slope of the calibration curve:

A specific calibration curve is plotted like linearity curve using samples containing an analyte in the range of the limit of detection and Limit of Quantitation. The residual standard deviation of a regression line, or the standard deviation of y-intercepts of regression lines, may be used as the standard deviation. Values obtained for limit of detection and Quantitation must be checked for relative standard deviation for the peak area. Recovery should be checked at that concentration for LOQ. If these concentrations are not able to fulfill predetermined criteria for LOD and LOQ, concentration may be increased or decreased based on previous experiment to meet %RSD and recovery requirements.

c) LOD and LOQ based on Signal to noise ratio

Impurity/Degradants and API Standard solutions are injected at 100 % target concentration and signal to noise ratio are

determined based on hit and trials. A concentration which is able to generate a peak with signal to ratio value between 2-3 can be considered as LOD. Three consecutive injections at that concentration should always produce S/N ratio 2-3.
A concentration (approximately 3.3 times that of LOD) which is able to generate a peak with signal to ratio value near 10 can be considered as LOQ. Three consecutive injections at that concentration should always produce S/N ratio nearly 10.Later precision (%RSD ≤ 10 for six injections) and recovery (between 70-130) should be checked at that concentration.

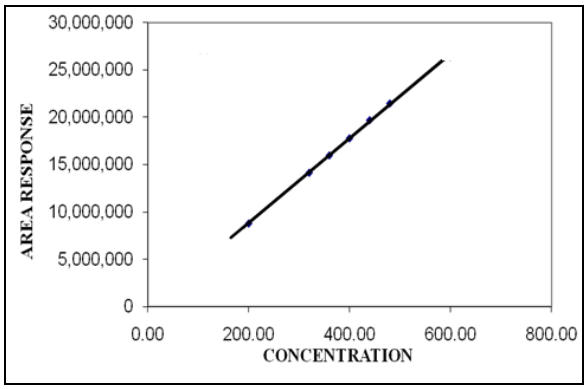
E) Accuracy

"The accuracy of an 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. The accuracy of an 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. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:
1. Comparison to a reference standard.
2. Recovery of the analyte spiked into blank matrix.
3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined.

F) Linearity and Range

The linearity of a test method is its capability to produce test results which are directly proportional to concentration of that particular component in solution. Range of the method is defined as highest and lowest concentration level between which methods are capable of producing linear test results with accuracy and precision.
To determine Linearity and range of the method prepare a series of solution by diluting Standard Stock solutions or separate weighing and mixing of all analytes using predetermined sample preparation procedures in validation protocol. Minimum 5 different concentration levels should be prepared within specified range.
For the assay of drug substance or drug product minimum five concentration levels should be selected between 80-120 % of specified test concentration in methodology. For related substances method minimum 6 concentration levels should be selected between LOQ-150 % of specified known and unknown impurities amounts in specifications. Analyze these concentrations using defined testing methodology and plot a linearity curve between area obtained for peaks or absorbance and concentration of that particular component in the respective solution. Calculate slope and intercept from the plot and determine the correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares.



G) Solution Stability

It is always essential that solutions (for Standards, impurity standards and test sample) should be enough stable so that these can be used for a certain period of time. It is recommended to establish a solution stability matrix during method feasibility studies so that estimation can be done for solution stability to avoid usage of any degraded solution while running long validation sequences, also it will help you to avoid repetitive preparation of impurity and reference standard solutions. However final solution stability matrix should be established during validation. Different approaches for establishing solution stability can be employed as per organisation practises:

a) First approach is to establish solution stability for solution at minimum three time points ideally covering 0 Hours, 24 Hours and 48 hours at room temperature and Refrigerator conditions.

If your solutions are stable upto 48 hours you can establish more time points covering 72 hours and 120 hours.
If Solutions are not stable upto 48 hours you can establish 0 hours, 6 hours,12 hours, 24 hours, 36 hours.
If solutions are not even stable upto 24 hours, a hourly based (or every two or 3 hours based on rum time of the method) approach should be employed.

b) Establish your system suitability and keep injecting your solutions on same system every hour (or every two or 3 hours based on rum time of the method) while bracketing every 4-5 time points (information based on feasibility data) with system suitability solution till your desired time points.

Acceptance Criteria

H) Robustness

An analytical method is saib to be robust if
"Small deliberate changes in chromatographic conditions of a method does not impact final output of the method".

Robustness of method is confirmed by deliberately doing small changes in parameters like flow rate, column compartment temperature, wavelength, gradient, pH of Mobile phase, amount of organic phase content etc. but not limited to. All these components or chromatographic conditions are deliberately modified and impact of these changes is studied on system suitability criteria. Extent of change depends upon purpose for which method is developed and validation is performed and utility of the method in future. Different guidelines and pharmacopoeia defined limits based upon indent use of method in future. However it is responsibility of organisations and individual users to justify parameters they are studying and extent of changes they are finalizing for method validation.

Any components is changed and final output of method is observed to be impacted or deviated, then method may be modified according to the changes observed or method is declared to be sensitive to the condition. If you are not improving these conditions due to some reasons then comment should be provide and justified in validation report and finally it must be included in Standard Test procedure before it is released for marketed product release or transferred to quality control lab of the organisation.

Following are the parameters which are generally selected for measuring robustness of the methods but not limited to:

- Mobile phase pH : ± 0.2 units
- Flow rate : ± 10-20 %
- Column oven Temperature : ± 5ºC
- Mobile phase organic composition : ± 10-30 %
- Wavelength of detection : ±2 units

Acceptance criteria

- Predetermined system suitability criteria should be achieved (like RT, RRT, Resolution, Theoretical Plates, Symmetry factor of peaks, % RSD of injections etc.)
- All unknown and known impurities should be separated from each other and main component; in spiked sample.

Organisations and individual most of time have concerned about only system suitability criteria during robustness study however it is recommended to check accuracy of the method at least with one preparation at 100 % target concentration for API as well as impurities.

Conclusion

Method development, feasibility & validation and technology transfer to Quality control play a vital role in drug substance and drug products analysis. These activities completed with no barriers insure that method is ready for its intended use. Methods used should stand against acceptance criteria mentioned in Protocol plan. Completion of these activities ensures that may be able to be used for releasing of materials for patient safety. Data generated from methods for monitoring, release and stability will be trusted if methods are reliable and activities completed are as per regulatory and patient safety requirements.

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