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A BRIEF REVIEW ON HPLC METHOD VALIDATION

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Absract

It is required to validate the HPLC methods used for analysis of pharmaceutical products. It is a regulatory requirement to verify all analytical methods. High performance liquid chromatography (HPLC) is a modern form of liquid chromatography that uses small cylinders through which the mobile phase is elevated at high pressure. HPLC is become a preferred method of analysis among various analytical methods for pharmaceuticals. Hplc provide rapid analysis, higher sensitivity, high resolution, easy sample recovery, precise and reproducible results. Successful validation requires cooperative efforts of several departments of the organizations including regulatory affairs, quality control, quality assurance and analytical development.

Keywords – HPLC, Validation, analytical methods, regulatory affairs, QC , QA, etc.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The technique of high performance liquid chromatography (HPLC) was developed in the later 1960s and early 1970s from knowledge of the theoretical principles that already had been established for earlier chromatographic techniques in particular for column chromatography. The technique is based on the same modes of separation as classical column chromatography. I.e. adsorption, partition (including reverse phase partition), ion exchange and gel permeation. HPLC differ from column chromatography in that mobile phase is pumped through the packed column under high pressure. The principal advantages of HPLC compared to classical (gravity feed) column chromatography are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with which the separated substances may be quantified.

The other improvement over column chromatography concern the detection method which can be used. These methods are highly automated and extremely sensitive.

- Solvent reservoir : glass / stainless steel, 1 liter
- Sonication or sparging with helium to elimate outgassing.
- Particals in column <50µ m, 500 psi pressure
- Pumps:
    1. Mechanical = constant flow
    2. Pneumatic = constant pressure
        - Gas displacement =highly compressed gas
        - Amplifier type = compressed gas at low pressure impinge on large end
    3. Reciprocating piston type = sapphire pluger

- Gradient analysis : particles sepration,
- Gradient controller : synchronization of pump = mobile phase mixture
- Solvent conditioning chamber : for silica gel column which dissolve below pH 2 and above pH 7 , small column 5-10 cm packed with silica gel after the pump and before the injector , saturation of mobile phase
- Injection device ; septum injection (self sealing rubber , Teflon disk)
- Precolumn / guard column : for thin layer of liquid coated on solid support , tapping particulate matter , 2-10 cm.
- Analutical column : 2-25 cm , 2-4.6 mm diameter , partical size > 10 µm, 6000 psi
- Packing material :
    1. Porous
    2. Pellicular : glass bead , 37-50 m
    3. Totally porous : microparticulate, 3.5,10,20

TYPES of HPLC

1. Partition chromatography.
2. Normal–phase chromatography.
3. Displacement chromatography.
4. Reversed-phase chromatography (RPC)
5. Size-exclusion chromatography.
6. Ion-exchange chromatography.
7. Bio-affinity chromatography.
8. Aqueous normal-phase chromatography.

Basic principle of HPLC

High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds in phases called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic solution, each component in a sample has different distribution equilibrium depending on the

solubility in the phases and the molecular size. As a result, the component move at different speeds over the stationary phase and there by separated by each other. The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase constantly fed into the column inlet at a constant rate by a liquid pump. Sample is injected from sample injector, located near the column inlet. The injected sample enters the column with mobile phase and the components in these samples migrate through it, passing between the stationary phase and mobile phase.

Compound move in the column only it is in the mobile phase and therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower .In this way, component is separated on the column and sequentially elutes from the outlet. A detector connected to the outlet of the column detects each compound eluting from the column. The recorder starts at the time when sample is injected and monitors the separation process and a graph is obtained. This graph is called chromatogram. The time that is required for a compound to elute (called retention time) and the relationship between the compound concentration (amount) and peak area depends on the characteristics of the compound.

Selectivity of HPLC

Most of the drugs can be analysed by HPLC method because of several advantages.

1. Speed (analysis can be accomplished in 20 minutes or less).
2. Greater sensitivity (various detectors can be employed).
3. Improved resolution (wide variety of stationary phase).
4. Reliable columns (wide variety of stationary phase).
5. Ideal for substances of low volatility.
6. Easy sample recovery, handling and maintenance.
7. Easy programming of the numerous functions in each module.
8. Time programmable operation sequence, such as initiating operation of
9. detector lamp and pump to obtain stable baseline and equilibrated column before the work day begins.

- Excellent reproducibility of retention time.

Different Modes of Separation of HPLC

- Normal phase mode
- Reverse phase mode
- Reverse phase ion pair chromatography
- Ion exchange chromatography
- Affinity chromatography
- Size exclusion chromatography (gel permeation and gel filtration chromatography)

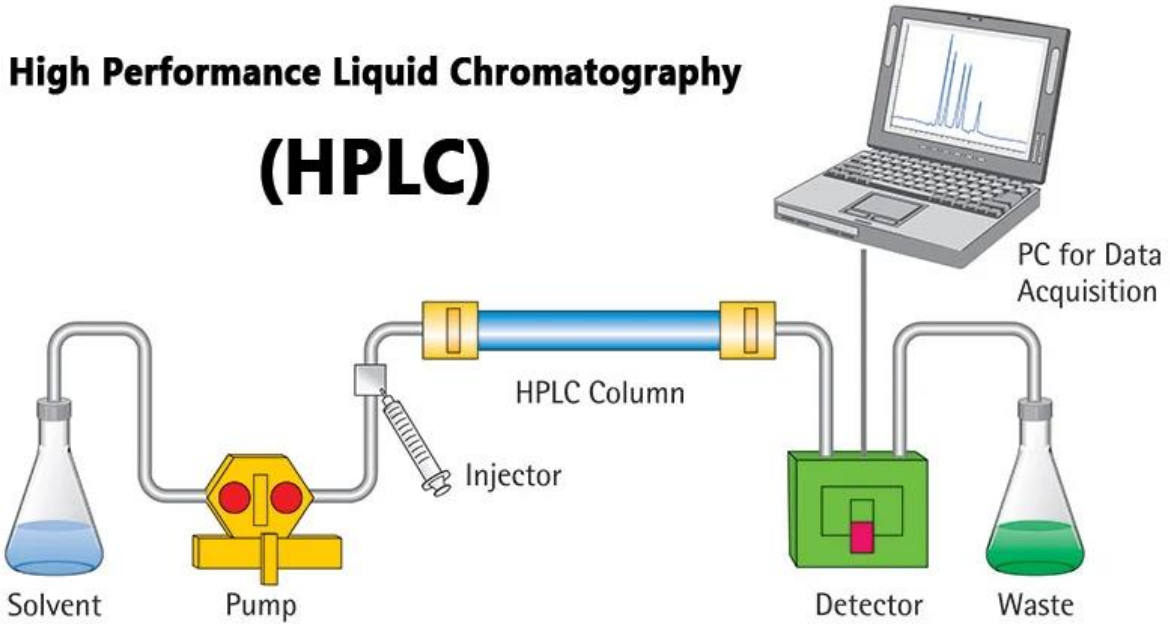
Instrumentation for HPLC

- A solvent reservoir for the mobile phase to be delivered to column over a wide range of flow rates and pressure. A degasser is needed to remove dissolved air and other gases from the solvents
- A pump to deliver the mobile phase to the column. The pumping system must be pulse free. A pump should be able to operate to at least 100atm(1500psm), pressure suited to less expensive chromatography. However, 400atm (600 psi) is a more desirable pressure limit. For many analytical columns only moderate flow rate of 0.5 to 2 ml per minute needed to be generated.
- Sampling valves or loops are used to inject the sample in the flowing mobile phase just at the head of the separation column.
- At the head of the separation column, there may be a guard column or a inline filter to prevent contamination of the main column by small particulates.
- The separation column contains the packing needed to accomplish the desired HPLC separation. These may be silica's for adsorption chromatography, bonded phases for liquid-liquid chromatography, ion exchange functional groups bonded to stationary support for ion exchange chromatography, gels of specific porosity for exclusion chromatography, or some unique packing for a particular separation method.
- Most column lengths ranges from 10 – 30 cm, short, fast columns are 3– 8cm long with an internal diameter of 4 -5 mm. Particle diameter lie in the range 3 -5µm, occasionally up to 10µm or higher for preparative chromatography.
- A detector with some type of data handling device, completes the basic instrumentation.

- The various detectors are
- UV visible photometers
- Refractive index detector
- Flourimetric detector
- Conductivity detector
- Amperometric detector
- PDA

- Detector electronic integrators and computing integrators are widely used today in HPLC for measuring peak areas. These devices automatically sense peaks and print out the areas in numerical forms. With the help of peak areas and height values, the peak width can be calculated (considering the peak as a triangle) and it can also be used for the calculation of number of theoretical plates.

DIAGRAM OF HPLC



Quantitation methods in HPLC

Peak heights or peak area measurements only provide a response in terms of detector signal.

This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for quantization are

1. Normalized peak area method.
2. External standard method.
3. Internal standard method.
4. Method of standard addition.

a) Normalized peak area method - The area percent of any individual peak is referred to as normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method the response factor for each component is identified.

b) External Standard method - This method includes injection of both standard and unknown, and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (RF) can be determined for each standard as follows.

RF = Standard area (peak height) / Standard Concentration

The external standard approach for most samples in HPLC that do not require extensive sample preparation.

c) Internal Standard method - Internal standard is a different component from the analyte but one that is well resolved in the separation. The internal standard should be chosen to mimic the behavior of the sample

component. One of the main reasons for using an internal standard is for samples requiring significant pretreatment or preparation.

d) Method of standard addition - The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is more often used in trace analysis. An important aspect of the method of standard addition is that the response prior spiking additional analytes should be high enough to provide a reasonable S/N ratio (<10), otherwise the result will have poor precision. (Vogel's 2003).

Limitations of HPLC

High-performance liquid chromatography (HPLC) is a powerful analytical technique used in chemistry and biochemistry to separate and identify compounds in complex mixtures. However, like any analytical method, HPLC has its limitations. In this article, we will discuss some of the most significant limitations of HPLC and how they can be overcome.

1. Sample size: One of the main limitations of HPLC is the sample size required. Typically, a few microliters of sample are needed for analysis, which can be a problem for samples that are limited or expensive. However, sample size can be increased by using sample concentrators or by increasing the sensitivity of the detector.
2. Resolution: HPLC is a technique that separates components in a mixture based on their physical and chemical properties. While HPLC can provide high resolution, it can be difficult to achieve complete separation of all the components in a complex mixture. In these cases, alternative techniques such as gas chromatography (GC) or mass spectrometry (MS) or our solution GC-UV is to be more appropriate.
3. Detection limit: The detection limit of HPLC is another limitation, as it is not as sensitive as other analytical techniques such as GC or UV.
4. Column lifetime: HPLC columns are consumable items, which means that they need to be replaced periodically. The lifetime of a column depends on the type of column, the sample matrix, and the sample conditions. Column lifetime can be extended by using proper column care, such as cleaning and storage, but this requires additional time and effort.
5. Cost: HPLC are an expensive technique, as it requires specialized equipment and columns. In addition, the cost of columns and other consumable items can add up over time. This can make HPLC less accessible for researchers with limited budgets.

VALIDATION

Validation is a key process for effective quality assurance. "Validation is established documented evidence, which provides specific a high degree of assurance that a process of equipment will consistently produce a product or result meeting its predetermined specifications and quality attributes".

Definition - USFDA defines validation as "established documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes".

EUGMP defines validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material activity or system actually lead to expected result".

AUSTRALIAN GMP defines validation as "the action of proving that any material, process, procedure, system, equipment or mechanism used in manufacture or control can and will be reliable and achieve the desire and intended result".

Importance of validation

1. As the quality of the product cannot be always assured by routine quality control because of testing of statistically insignificant number of sample.

2. The validation should provide adequacy and reliability of a system or product to meet the predetermined criteria or attributes, providing high degree of confidence that the same level of quality is consistently build into each of finished product from batch to batch.

3. Retrospective validation is useful for trend comparison or results complaints to cGMP to cGLP.

4. For taking appropriate action in case of non-compliance.

Objectives of validation

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality and purity they purport or are represented to possess.

1. Assurance of quality.

2. Government regulation.

Types of validation

The following are frequently required to be validated on a pharmaceutical process

1. Equipment validation

2. Process validation

3. Cleaning validation

4. Analytical method validation

5. Facility validation including utilities

ANALYTICAL METHOD VALIDATION

Method validation is a process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical testing of a pharmaceutical product is necessary to ensure the purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system.

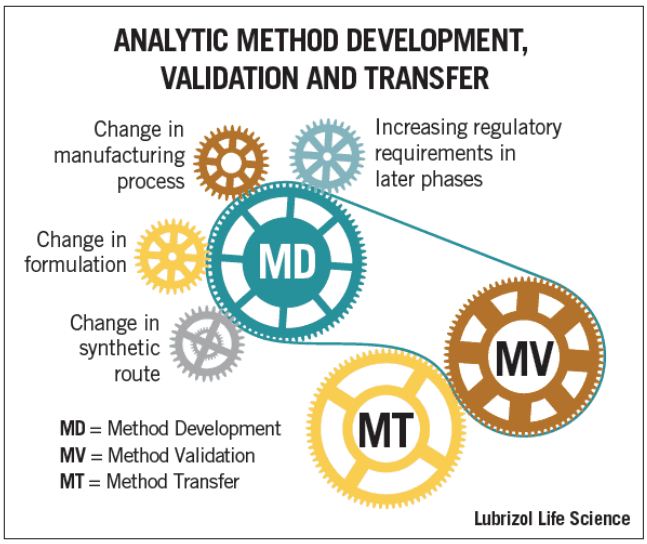
- Validation should be performed in accordance with the validation protocol. The protocol should include procedures and acceptance criteria for all characteristics. The results should be documented in the validation report.
- Justification should be provided when non-pharmacopoeial methods are used. If pharmacopoeial methods are available, justification should include data such as comparisons with the pharmacopoeial or other methods.
- Standard test method should be described in detail and should provide sufficient information to allow properly trained analysis to perform the analysis in a reliable manner.
- As a minimum, the description should include the chromatographic condition (in the case of chromatographic tests), reagents needed, reference standards , the formulae for the calculation of results and suitability tests.

Acceptance criteria of validation parameter for HPLC

S.no	Parameter	Acceptance criteria
1	Accuracy	% Recovery 98-102% % RSD of recovery concentration must be <2
2	Precision	RSD<2%
3	Range	Concentration where data can be reliably detected (80-120%)
4	Specificity	No interference
5	Linearity	Correlation coefficient – NLT 0.999
6	Detection limit	S/N >2 or 3
7	Quantitation limit	S/N>10
8	Ruggedness	Should meet all system suitability parameters
9	Robustness	RSD <2%

Changes to one method during drug development may require modifications to a separate existing analytical method. These modifications in turn may require additional validation or transfer activities, as shown in Figure

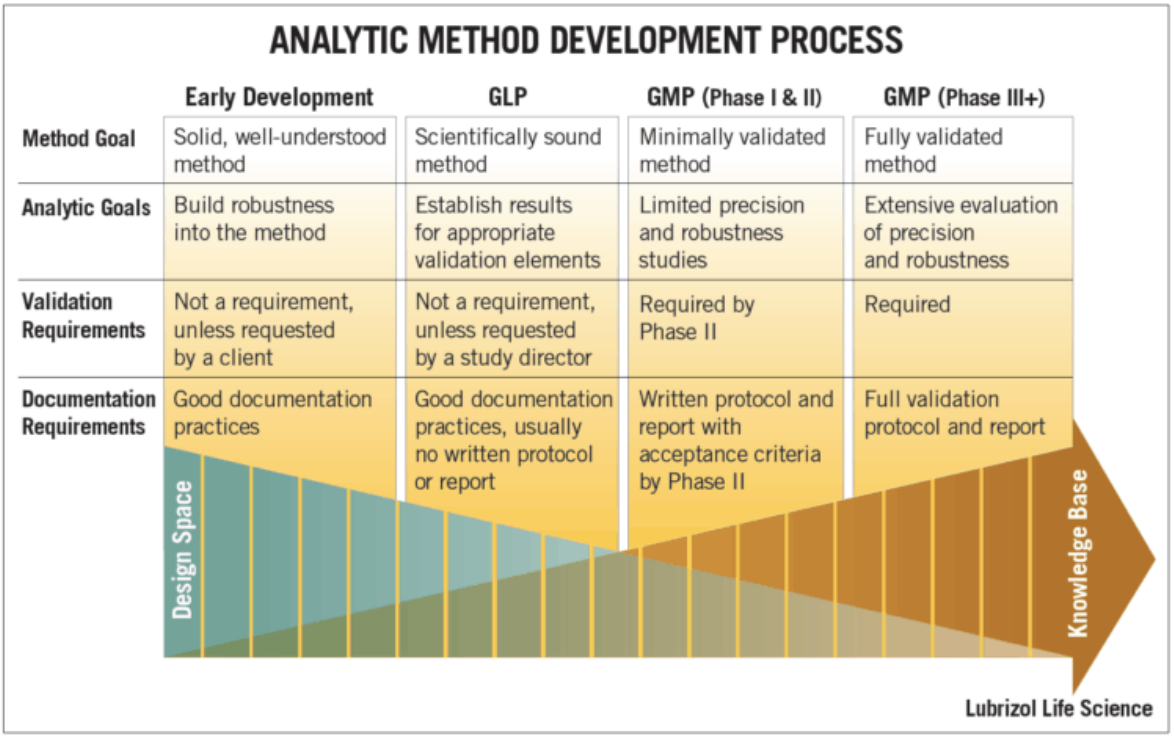
Figure - Analytical Method Development Validation



Method development

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The notion of phase-appropriate method development is a critical one if time, cost and efficiency are concerns. The goal and purpose of the method should reflect the phase of drug development. During early drug development the methods may focus on API behavior. They should be suitable to support pre-clinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines.

Figure- Analytical Method Development Validation



Scouting experiments are frequently performed during method development to establish the performance limits of the method prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, oxidation, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light to evaluate potential degradation of the API in the presence of formulation excipients.

Additional experiments help to define the system suitability criteria that will be applied to future analytical sample sets. System suitability tests are a set of routine checks to assess the functionalities of the instrument, software, reagents, and analysts as a system3. Final method system suitability parameters may be determined from evaluations of method robustness performed under statistical design of experiments. The goal is to identify the critical parameters and establish acceptance criteria for method system suitability.

Elements of Validation

The validation of an analytical method demonstrates the scientific soundness of the measurement or characterization and is required throughout the regulatory submission process. The validation practice demonstrates that an analytical method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to understand the behavior of the method and to establish the performance limits of the method. Resources for information and approaches to method validation are listed in the endnotes

In order to perform method validation, the laboratory should be following a written standard operating procedure (SOP) that describes the process of conducting method validation. The laboratory should be using qualified and calibrated instrumentation with a corresponding operating SOP. There should be a well-developed and documented test method in place and an approved potocol should be in place prior to the execution of any validation experiments. The protocol is a plan that describes which method performance parameters will be tested, how the parameters will be assessed, and the acceptance criteria that will be applied. Finally, samples of API or drug product, placebos, and reference standards are needed to perform the validation experiments

PARAMETERS USED FOR ASSAY VALIDATION

The validations of the assay procedure are carried out using following parameters.

SPECIFICITY:

Specificity is the ability to asses unequivocally analyte in the presence of impurities, degradation, matrix components, etc. which may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

Specificity can be used for identification test, purity test, assays ,etc.

The ICH documents state that, when chromatographic procedure are used representative chromatogram should be represented to demonstrated the degree of specificity (selectivity) and peaks should be appropriately labeled. Peaks purity tests may be useful to show that the analyte chromatographic peaks is not attributable to more than one components.

PRECISION:

Definition - The precision of an analytical procedure express the closeness of the agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or co-efficient of variation of a series measurement.

The precision of an analytical method is determined by assessing sufficient number of aliquot of a homogenous sample to be able to calculate statistically valid stimates of standard deviation of relative standard deviation (i.e. coefficient of variation).

System precision - A system precision is evaluated by measuring the peak response for the six replicable injection of the same standard solution prepared as per the proposed method .The %RSD is calculated and it should not be more than 2%.

Method precision - A method precision is evaluated by measuring the peak response for six replicate injection of six different weigh of sample solution prepared as per proposed method. The %RSD is calculated and it should not be more than 2%.

Determination - The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.

ICH Requirements - The ICH documents recommended that repeatability should be assessed using a minimum number of nine determinations covering the specified range of the procedure (I, e., three concentrations and there replicates of each concentrations or using a minimum of six determinations at 100% of the test concentration).

ACCURACY

Definition - The accuracy of an analytical procedure express the closeness of the agreement between the values which is acceptable either as conventional true value or an accepted reference value and the value found.

Determination - In case of assay of drug in a formulated product ,accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which the known amount of analyte have been added within the range of the method . If it is not possible to obtain all product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare results with those of a second ,well characterized method ,the accuracy of which has been stated or defined. Accuracy

studies for drug substance and drug product are recommended to be performed at the 80,100 and 120% level of label claim as stated in the guidelines for submitting samples and analytical data for method validation .At each recommended level studied ,replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method 14 is. The mean of the replicates, expressed as %label claim, indicates how accurate the test method is.

ICH Requirements - The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentration and three replicates of each concentration).

LINEARITY

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

.Determination - Linearity of an analytical procedure is established minimum of five concentrations. It is established initially by visual examination of plot of signals as a function of analyte concentration of content .If there appears to be a linear relationship ,test results are established by appropriate statistical methods(i.e., by calculation of the regression line by the method of least squares).

LIMIT OF DETECTION (LOD)

Definition - LOD is the lower concentration of the substance that the method can detect but not necessarily quantify. LOD simply indicates that the sample below or above a certain level.

The LOD is generally expressed as the concentration of the analyte sample (e.g. percentage or parts per million, etc.).

Determination - For non-instrumental methods, the detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

ICH Requirements - The ICH describes a common approach, which is to compare measured signal from samples with known concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established.

Calculations of LOD for instrument sensitivity

LOD(mg/L)=3×Noise/Signal×Lowest concentration of the linearity samples

Typically acceptable signal-to-noise ratios are 2:1 or 3:1.

LIMIT OF QUANTITATION (LOQ)

Definition - LOQ is the lowest concentration of the substance that can be estimated quantitatively with acceptable precision, accuracy and reliability by the proposed method. LOQ is determined by the analysis of samples containing decreasing known quantity of the substance and determining the lowest level at which acceptable level of accuracy and precision is attained.

Determination - For non-instrumental methods, quantization limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

ICH Requirements - The ICH documents describe a common approach which is to compare measured signals from samples with known low concentration of analytes with those of blank samples.

These quantitation limits should be subsequently validated by the analysis of a suitable numbers of samples known to be near or prepared at the quantitation limit.

Calculations of LOQ values for instrument sensitivity:

LOQ(mg/L)=10×Noise/Signal×Lowest concentration of the linearity samples

RANGE

Definition - The range of an analytical procedure is the interval between the upper and the 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.

Determination - The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ROBUSTNESS

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

Determination - The robustness of method determined by performing the assay by deliberately altering parameters(change in flow rate ±10%,change in mobile phase ratio ±2,change in pH of mobile phase ±0.2,change in wave length detection ±5nm, change in temperature ±1 to 50) that the results are not influence by the changes in the above parameters.

RUGGEDNESS

Definition - The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the samples under a variety of conditions, such as different laborites, different analyst, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days etc.

Determination - The ruggedness of analytical method is determined by the analysis of aliquots from homogenous lots in different laboratories, by different analysis, using operational and environmental condition that may differ but are still within the specified parameters of the assay .The degree of reproducibility of the results is that determined as a function of assay variables. This reproducibility may be compared to the precision of assay under normal condition to obtain a measure of the ruggedness of the analytical method.

SAMPLE SOLUTION STABILITY

Solution stability of the drug substance or drug product after preparation according to the test method should be evaluated. Most laboratories utilize auto samples with overnight runs and the sample will be in solution for hours in the laboratory environment before the rest procedure is completed. This is concern especially for drugs that can undergo degradation by hydrolysis, photolysis, and adhesion to glassware.

SYSTEM SUITABILITY SPECIFICATION AND TESTS

The accuracy and precision of HPLC data collected begin with a well behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose.

It consists of following factors:

- Capacity factor
- Precision\Injection repeatability
- Relative retention
- Resolution
- Tailing factor
- Theoretical plate number

1. Capacity factor (K') , K' = (tR-tO/tf)

The capacity factor is a measure of where the peak of interest is located with respect to the void volume i.e., elution time of the non-retained components.

2. Precision/Injection repeatability (RSD)

Injection precision expressed as RSD (relative standard deviation) indicates the performance of the HPLC which includes the pumping, column and the environmental conditions, at the time the samples are analyzed .It should be noted that sample preparation and manufacturing variations are not considered.

3. Relative retention (α) , α = K'1/K'2

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (Rs) is stated.

4. Resolution (Rs) , Rs = (tR2-tR1)/(1/2)(tw1+tw2)

Rs is a measure of how well two peaks are separated. For reliable quantitation well separated peaks are essential for quantitation. This is a very useful parameter if potential inference peaks (s) may be concern.

Tailing factor , T = Wx/2f

The accuracy of quantitation decreases with increases in peak tailing because of the difficulties encountered by the integrator in determine where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are present by the analyst for optimum calculation of the area for the peak of interest. If the integrator is unable to determine exactly when an upslope for down slope occurs, accuracy drops.

Theoretical plate number (N) , N = 16(tR/tw) 2 = L/H

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatograph. N- Constant for each peak on the chromatogram with a fixed set of operating conditions.

H- Height equivalent of a theoretical plate.

L- Length of column.

Conclusion

HPLC is much faster than other chromatography methods, one of the primary benefits of high performance liquid chromatography methods is the increased speed compared to other chromatography methods. It is the technique of choice when analysis materials for a wide range of organic compounds. . HPLC is become a

preferred method of analysis among various analytical methods for pharmaceuticals. HPLC provide rapid analysis, higher sensitivity, high resolution, easy sample recovery, precise and reproducible results.

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