Analytical Method Development and Validation by Reverse Phase – High Performance Liquid Chromatography: An Overview

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Abstract: **A simple, precise, accurate, specific and RP-HPLC method was developed for determination of drug in pharmaceutical formulation. Analytical method development and validation play important roles in the drug discovery, drug development and manufacture of pharmaceuticals. It involves detection of the purity and toxicity of a drug substance. The present study focuses on the various steps, parameters involved in HPLC condition. A number of chromatographic parameters were analysed to optimize the method like sample pre-treatment, choosing mobile phase, column, detector selection. Analytical method validation confirms the scientific firmness of measurement or characterization and is necessary throughout the regulatory submission process. Various validation parameters like accuracy, specificity, precision, linearity, LOD, LOQ, ruggedness, and robustness, System suitability also are listed concerning ICH Guidelines.**

Keywords: **RP-HPLC, Analytical, Validation, Mobile phase, ICH guideline**

Introduction to Reverse Phase -High Performance Liquid Chromatography Principle:

RP-HPLC is a popular method of chromatography that separates the mixture of parts and removes impurities by introducing the sample mixture into the HPLC column they move according to their relative correlation to the non-polar stand position. The most closely related part of the adsorbent is slower than the part with less compliance to the standing phase. As we know, no two parts are alike in relation to a fixed phase, the parts are separated. [(2)]

In reverse-phase chromatography (RP-HPLC), standing phases, such as immobilised hydrophobic ligands and cellular hydrophilic phases, are used to classify or separate particles based on variations in their hydrophobicity.

Furthermore, gradient elution rather than isocratic elution is used in the phase-shifted chromatography of biomolecules.

The part where the mixture of the analyte passes over the vertical phase particles with holes large enough to enter, when the interaction with the sub-hydrophobic area extends itself from the cell distribution phase. Both hydrophobic and polar interactions affect the type and quality of the contact between sample particles and the standing phase. Each analyte experiences a threshold level of organic solvent concentration in the eluent as it increases, which releases the analyte from the hydrophobic stationary-phase surface and allows it to swell from column to phase in a continuous flow cell.

Introduction to the analysis of the analysis method:

The selection of an analysis strategy is influenced by a variety of variables, including the chemical properties of the sample and the sample matrix, the intelligence and depth of analysis, the type of measurements, that is, the accuracy or precision, and the variety of samples. The quality method offers details on the chemicals and identifies the different kinds of samples. The quantitative method gives numbers that illustrate how valuable one or more analysts are in comparison to other analysts in the sample.(1)

The development of an analysis approach is based on previous or current literature that employs nearly equivalent tests. In addition to the necessity for upkeep or other strategic requirements, the development of any new or improved approach typically sews the delivery processes and tools to the present analyst. And an effective approach for method improvement should only necessitate as many trials as are required to produce the desired outcome. There are a number of phases involved in developing an HPLC methodology, including sample preparation, measurements,

Sample preparation and collection: The first cell phase is the ideal time for sample dissolution. If stability or melting issues prevent this from happening, you can add salt, acetic acid, or fomic acid to the sample to make it more soluble. The goal of sample adjustment is to obtain an aliquot of a sample that has been released from interference. It will not the column and is ideal for the planned HPLC procedure. This is due to the fact that th e solvent sample will melt within the cellular phase without affecting sample processing or storage.(8)

Measurement : There are two ways to measure a particular analyte:

- Separation step
- Detection step

Parameters used for Assay Validation

The validation of the assay procedure was carried out as per ICH guidelines using the following parameters.

Specificity

Testing without analytes in the presence of components that can be anticipated to occur is specified. Generally, that could involve matrix, dirt, and other debris. To ensure the analyst's identification, this can be done. Testing of composite materials, heavy metals,

residual solvent content, etc. are some examples of the hygiene tests that are used to confirm the correct contamination of the contaminant content prior to the analysis of individual analyses.

Accuracy:

The statistical variability and consistency of consistency between measurements acquired from multiple samples of the same sample under predetermined conditions are shown by this kind of analysis. A common name for accuracy is the average relative deviation. Three categories of accuracy can be distinguished: multiplication, average accuracy, and multiplication.

Limit of Detection

The lowest value of the analyst in the sample that can be achieved, but not really the measurement, as a particular value under explicit test conditions is the definition of the acquisition limit for each analysis procedure.

$DL = \frac{3.3\sigma}{s}$

Where,

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σ = the standard deviation of the response

S = the slope of the calibration curve

Limit of Quantification:

The quantitative limit or parameter test for low level analysts in the sample can be determined with acceptable accuracy and precision under the specified test conditions, and is used primarily in determining contaminants and / or corrosive products.

Quantitation limit based on the standard deviation of the response and the slope. It can be expressed as,

$$QL = [10\sigma/S]$$

σ =standard deviation of the response

S =slope of the calibration curve (of the analyte)

Linearity and range: The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It is demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure. Linearity is usually expressed as the confidence limit around the slope of the regression line. For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline. The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the method.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test result obtained, the analysis of conditions such as different laboratories, different analysis using different instrument, on different days. Different source of reagent, elapsed assay, times, assay temperature conditions. Ruggedness is the measure of reproducibility of test result under the variation in conditions normally expected from analyst to analyst. The criteria of the ruggedness is the RSD should be not more than 2%

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameter such as percent organic content, pH of the mobile phase, buffer concentration, temperature and injection volume. The RSD should not be greater than 2% as a criterion for robustness.

System suitability

System suitability testing is an integral part of many analytical procedures the tests are based on the concept that the equipment, electronics, analytical operation and samples to be analyzed constitute an integral system that can be evaluated as such. Typically the process involves making five injections of a standard solution and evaluating several chromatographic parameters such as resolution, area % reproducibility, number of theoretical plates and tailing factor.

RP-HPLC Critical parameters:

- Column
- flow rate
- Mobile phase
- Organic solvent
- pH

- Absorbance
- Selectivity
- Viscosity
- Temperature

Column:

A chromatographic column that contains a non-polar stationary phase that is present in solid form and made of silica gel. These are bonded hydrocarbons like C8 and C18, i.e., Octyl ligand and Octadecyl ligand, respectively, [3] and many more listed below [6]

Table 1 shows the Relative use of stationary phases.

Phase	Relative usage (%)
C18 (octadecylsilane)	39
C8 (Octyl)	26
Cyanopropyl	14.5
Phenyl	12
C4 (butyl)	3.7
Hydrophobic interaction	1.8
C2 (ethyl)	1.1
C1 (methyl)	0.8
Other	0.8
Polymers	0.5

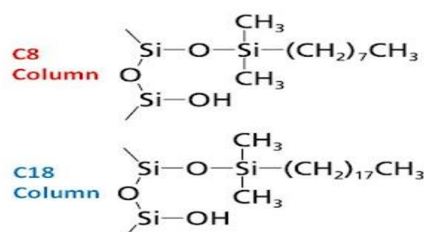

Figure 1 depicts the structure of C8 and C18 hydrocarbons.

Several of the standing parts are often bonded with silica gel. These bounded columns also contain sub-category kinds like embedded polar sections, capped and non-end-capped types, and mixed categories (like phenyl-hexyl). Other packing materials used in reversed-phase chromatography include polymers, polymer coated silica and alumina, inorganic-organic hybrids, coated zirconia, and graphitized carbon. There are benefits and drawbacks to each kind of phase.
The correct solvents are used to clean the column or revive the pollutants, and varied flush volumes are needed for columns of various sizes.

Table 2: Analytical column volume. [5]

Column size (mm × mm)	Void volume (ml)
250× 4.6	2.5
150× 4.6	1.5
150 ×3.0	0.64
150 ×2.1	0.28
50× 4.6	0.50
30× 4.6	0.30
15× 4.6	0.15

In a column washing system of various silica-bonded columns, there is a mobile phase without buffer salt such as:

- 100% methanol
- 100% acetonitrile
- 75% acetonitrile-25% isopropanol
- 100% isopropanol
- 100% methylene chloride

100% hexane.

For example: a minimum of 10 columns for each washing solvent should pass through the column. For analysis analyzes of 250 mm 4.6 mm, analysts can use a standard HPLC flow rate of 1–2 mL / min.
Mobile phase: The word "buffer" is most frequently used to refer to a chromatography cell in the reverse phase. (3)
The mobile section needs to be chosen based on solvent solute separation and solute retention. The analysis in the columns of the retrospective phase is eliminated using a combination of water (water baths) and organic solvents. (4)
There is a decrease in differential pressure or flow rate of different cellular phases (8), and in addition to this note, the volume of the bath should also be maintained when working close to physical conditions.

Table 3: Commonly used mobile phases in RP-HPLC:

Mobile phases	Polarity index	UV-cut off (nm)
Acetonitrile	6.2	190
Isopropanol	4.3	210
Methanol	6.6	205
Tetrahydrofuran	4.2	212-230
Water	9.0	180

Organic solvent:
To lessen the polarity of the aqueous cell phase and increase the solubility of hydrophobic substances, organic solvent is utilised in RP-HPLC. Although acetonitrile is the most preferred choice, methanol with 0.1% acidity and acetonitrile are the two most frequently used converters.
PH
PH plays a special role in chromatographic separation as it regulates elution structures by regulating ionization factors. In order to achieve good sample component melting and ion compression, RP-HPLC is typically performed at low pH levels, typically between pH 2-4. Commonly used acids have a concentration of 0.05-0.1% or 50-100 mm and contain trifluoroacetic acid, heptafluorobutyric acid, and ortho-phosphoric acid. Use pH levels close to neutral, or pH 7, for cell types that contain ammonium acetate or phosphate salt. To mention that phosphate baths don't alter. (8)

Absorbance:
The UV detector is commonly used for casting off analytes, also known as gradient elution work. Most of the compounds adsorb UV light in the range of 200-350 A°. The mobile phase used must not interfere with the peak pattern of the desired compound; therefore, it must no longer absorb at the detection wavelength employed. [(7)]
To speed up separation, a solvent with the lowest practical viscosity must be utilised. A new advantage of low viscosity solvents is that excessive performance theoretical plate (HETP) values are frequently lower than with solvents of higher viscosity because mass transfer occurs more quickly. The viscosity must be considerably lower than 0.5 centipoise. In any other situation, mass transfer between the solvent and stationary phase can be lowered and large pump pressures are necessary.

Temperature:

Temperature will have a significant impact on the retrospective phase's chromatography, particularly for low-molecular-weight solutes like short peptides and oligonucleotides. At rising column temperature, the viscosity of the cell phase employed in retrospective phase chromatography reduces. Lower solvent viscosity frequently leads to efficient mass transfer and higher refinement. Because the lower cells may be more stable at higher temperatures, raising the temperature of the relegated column column is particularly useful in resolving the weight of the lower cells. [(10)]
Detector
According to the detector, it should be chosen based on a few functional analyte variables, including UV absorption, fluorescence, conductance, oxidation, reduction, etc. The analysis makes use of numerous exceptional detectors, including the UV detector, mass spectrometry detector, fluorescence detector, and electrical conductivity detector (fixed and variable length). More than 95% of all LC analysis applications make use of these detectors.

Also, there are a few traits which might be fulfilled with the aid of using a detector to be used in RP-HPLC determination, which are:
- High sensitivity, facilitating trace analysis
- Negligible baseline noise to facilitate decreased detection.
- Low drift and noise level
- Low dead volume (low peak broadening)
- Tunability, so that detection can be optimised for different compounds.
- A broad linear dynamic range
- Non-destructive sampling.
- Operating simplicity and reliability
- unresponsive to changes in solvent type, flow rate, or temperature

Conclusion: Analytical methods development plays important roles in the discovery, development and manufacture of pharmaceuticals. The selection of Column, buffer, detector and wavelength and another conditions composition (organic and pH) plays a dramatic role on the separation selectivity. The advantages of RP- HPLC technique were high selectivity, sensitivity, economic, less time consuming and low limit of detection. In RP-HPLC classification is performed on the basis of molecular polarity. The development of an analysis method requires process knowledge and planning. In the process of verifying important parameters such as accuracy, Specificity, precision , linearity LOD, LOQ specification, are guaranteed to be improved.

References:

1. Method development and validation of pharmaceuticals by different instrumental techniques: a review by Sabyasachi Biswal Sumanta mondal, H K Sandep Kumar, International Journal of Pharmaceutical Science Reviews and research. 2018.
2. HEMA, G. Swetha Reddy, 2017. A review on new analytical method development and validation using RP-HPLC, et al.

3. Amersham biosciences "Reverse phase chromatography principles and methods" page 9-10.
4. Akul Mehta (Dec 27, 2012) "principle of reverse-phase chromatography HPLC/UPLC." Pharmaxchange. Retrieved 10 Jan 2013.
5. Ronald E. Majors, Agilent technologies, Wilmington Delaware, USA, The cleaning and regeneration of reverse phase HPLC column, LC.GC Europe (Jul 2003)
6. R.E Majors, LCGC 15 (11), 1008-1015 (1997).
7. Kar A: pharmaceutical Drug analysis. 1st edition 2001; 565-592.
8. Snyder LR, Kirkland JJ, Glajch JL: Practical HPLC method development. 2nd ed. 2001.
9. Sethi PD: HPLC Quantitative Analysis of Pharmaceutical Formulation. CBS Publishers and Distributors, 1st edition 2001.
10. Amesham bioscience: reverse phase chromatography. Principles and methods, page. 6-8.