ISSN 2349-7203

IJPPR

INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH

An official Publication of Human Journals

HUMAN

Human Journals

Review Article

June 2020 Vol.:18, Issue:3

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HPLC Method Development and Validation: A Review

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Submission: 26 May 2020

Accepted: 02 June 2020

Published: 30 June 2020

www.ijppr.humanjournals.com

Keywords: Chromatography, RP-HPLC, Validation, Column

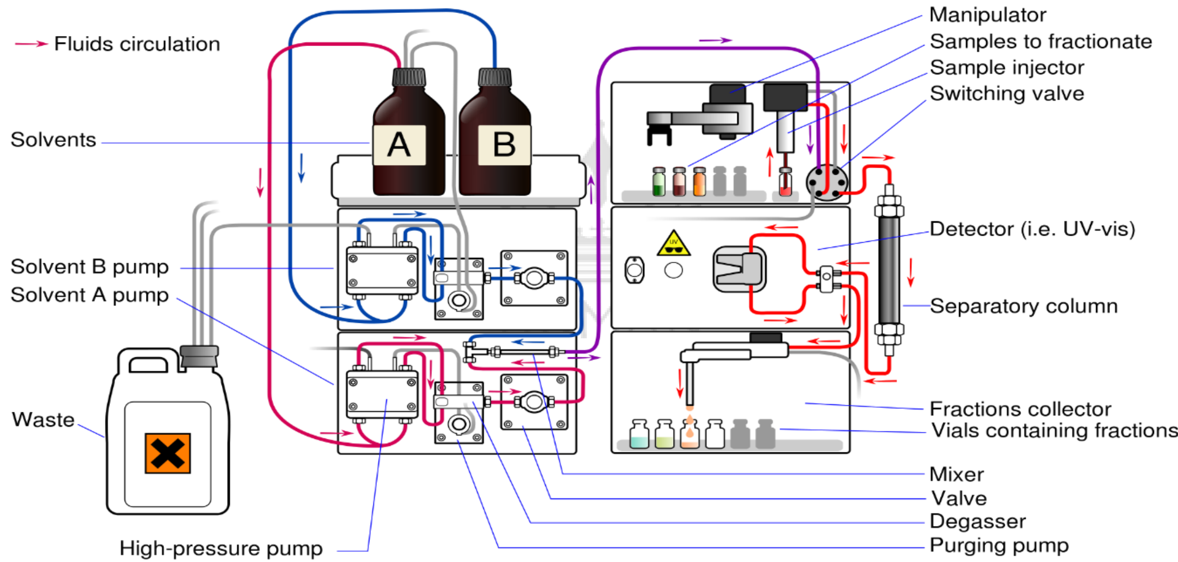
ABSTRACT

Chromatography is probably the most dominant advisory technique available to the modern chemist. Its size emerges from its ability to decide quantitatively numerous specific segments present in combination with a single analytical method. High-performance liquid chromatography (HPLC) is a chromatographic system that can isolate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify, and purify the individual components of the mixture.

INTRODUCTION

High-performance liquid chromatography is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Reversed-phase chromatography has discovered both diagnostic and preparative applications in the area of biochemical split-up and purification. Molecules that possess some degree of hydrophobic characters, such as proteins, peptides, and nucleic acids, can be detached by reversed-phase chromatography with outstanding recovery and determination. Now a day reversed-phase chromatography is the most usually used separation method in HPLC due to its expansive submission range.



Theory about RP-HPLC:-

Reversed-phase chromatography have originated both systematic and preparative applications in the area of biochemical detachment and refinement. Particles that have some level of hydrophobic character can be detached by reversed-phase chromatography with superb recovery and resolution. The departure mechanism in reversed-phase chromatography depends on the hydrophobic mandatory contact between the solute molecule in the mobile phase and the stopped hydrophobic ligand, i.e. the stationary phase. The actual nature of the hydrophobic obligatory collaboration itself is a substance of heated argument but the predictable wisdom accepts the obligatory collaboration to be the result of a favourable

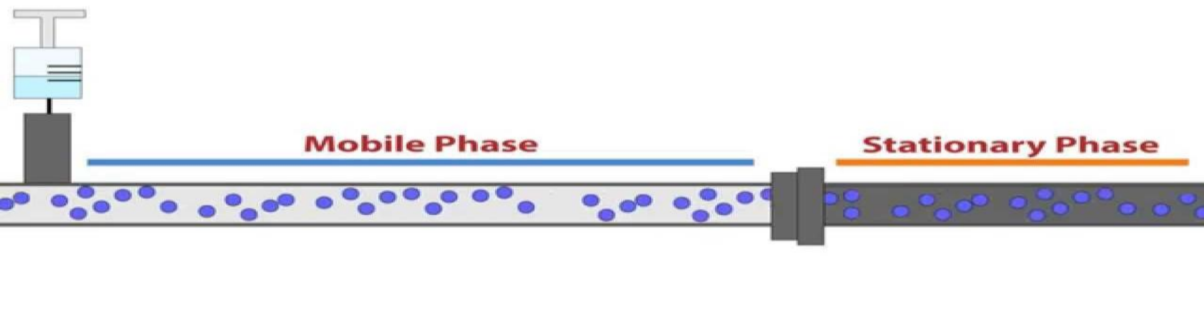
outcome. The initial mobile phase mandatory situations used in reversed-phase chromatography are primarily aqueous which shows a high degree of prepared water structure adjacent to both the solute molecule and the stopped ligand. As solute fixes to the stopped hydrophobic ligand, the hydrophobic area visible to the solvent is diminished. Therefore, the degree of ordered water structure is contracted with a matching constructive rise in system entropy. In this way, it is strategic from an energy point of interpretation for the hydrophobic moieties, i.e. solute and ligand, to a subordinate. Water together to hydrophobic sections is postulated to be more highly ordered than the bulk water. Fragment of this „structured" water is displaced when the hydrophobic spaces co-operate leading to a rise in the global entropy of the system. Departures in reversed-phase fluctuating degrees of hydrophobicity to a hydrophobic stationary phase. The majority of reversed-phase separation experiments are performed in several ultimate steps.

Analytical method development using RP-HPLC:-

Methods of analysis are consistently developed, enhanced, validated, collaboratively studied, and applied. Gatherings of these settled methods then appear in large sets such as USP, BP, and IP, etc. In most cases, anticipated separation can be realized easily with only a few experiments. In other cases, an extensive amount of testing may be needed. However, a good method development strategy should involve only as many experimental runs as are essential to achieve the chosen final result(s). The development of a method of analysis is usually based on former art or remaining literature using nearly the same or similar experimentation. The development of any new or enhanced method usually tailors existing slants and instrumentation to the recent analytes as well as to the final need or requirement of the method.

Method development generally requires choosing the method requirements and determining what type of instrumentation to relate and why. In the HPLC method development period, conclusions about the choice of the column, mobile phase, detectors, and method quantitation must be deliberated. So development includes a reflection of all the parameters relating to any method. Therefore, the development of a new HPLC method includes a selection of the greatest mobile phase, best detector, best column, column length, stationary phase, and best inside diameter for the column. The analytical strategy for HPLC method development contains several stages.

The column/Stationary phase:



The assortment of the stationary phase/column is the leading and the most central step in method development. The development of a sharp and reproducible method is unbearable without the accessibility of a constant, high-performance column. To sidestep problems from irreproducible sample protection during method development, it is key that columns be steady and reproducible. A C8 or C18 column made from particularly purified, less acidic silica and calculated specifically for the departure of basic compounds are usually suitable for all samples and is resiliently suggested. The column is particularly depending on the nature of the solute and the evidence about the analyte. Reversed-phase mode of chromatography enables a catholic range of columns like dimethyl silane (C2), phenyl, cyanopropyl (CN), nitro, amino, etc. Mostly lengthier columns provide better departure due to higher theoretical plate numbers. As the particle size decreases the apparent area available for coating increases. Columns with 5-µm particle size give the best conciliation of adeptness, reproducibility, and consistency.

Mobile phase: In many cases, the average term used for the mobile phases in reversed-phase chromatography is “buffer”. However, there is little buffering ability in the mobile phase solutions since they frequently contain strong acids at low pH with large kindnesses of organic solvents. Passable buffering volume should be maintained when working nearer to physiological situations. strength in reversed-phase chromatography.

Organic solvents: can be used in reversed-phase chromatography, in preparation only a few are habitually employed. The two greatest generally used organic transformers are acetonitrile and methanol, although acetonitrile is the more prevalent select. Isopropanol (2-propanol) can be employed because of its hard eluting properties but is limited by its high viscosity which results in lower column productivities and progressive back pressures. Both acetonitrile and methanol are less tacky than isopropanol. All three solvents are basically UV

clear. This is a crucial property for reversed-phase chromatography since column elution is classically monitored using UV detectors. Acetonitrile is used almost exclusively when splitting peptides. Most peptides only absorb at low wavelengths in the ultra-violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths.

Ion suppression: The retention of peptides and proteins in reversed-phase chromatography can be modified by mobile phase pH since these particular solutes contain ionizable groups.

The degree of ionization will depend on the pH of the mobile phase. The stability of silica-based reversed-phase media dictates that the operating pH of the mobile phase should be below pH 7.5. The amino groups contained in peptides and proteins are charged below pH7.5. The carboxylic acid groups, however, are defused as the pH is decreased. The mobile phase used in reversed-phase chromatography is generally prepared with strong acids such as astri-fluoro acetic acid (TFA) or ortho-phosphoric acid. These acids maintain a low pH environment and defeat the ionization of the acidic groups in the solute molecules. Variable the concentration of robust acid mechanisms in the mobile phase can change the ionization of the solutes and, therefore, their retention performance.

The major advantage of ion conquest in reversed-phase chromatography is the removal of varied mode retention effects due to ionizable silanol groups remaining on the silica gel exterior. The effect of mixed-mode retention is increased retention times with momentous peak expansion.

pH:

pH plays a key role in realizing the chromatographic separations as it panels the elution assets by controlling the ionization characteristics. Reversed-phase separations are most often achieved at low pH values, commonly between pH 2-4. The low pH results in good solubility of the sample components and ion suppression, not only of acidic groups on the sample.

Molecules, but also of residual silanol groups on the silica matrix. Acids such as tri -fluoro-acetic acid, hepta-fluorobutyric acid, and ortho-phosphoric acid in the concentration chain of0.05 - 0.1% or 50 - 100 mM are commonly used. Mobile phases covering ammonium acetate or phosphate salts are suitable for use at pH closer to objectivity. Note that phosphate buffers are not volatile. It is important to preserve the pH of the mobile phase in the range of

2.0 to 8.0 as most columns do not survive to the pH which is outside this range. This is because the siloxane linkage area cleaved under pH 2.0; while at pH valued above 8.0 silica may dissolve.

Absorbance: An UV-visible indicator is based on the principle of absorption of UV visible light from the effluent emerging out of the column and passed through a photocell placed in the radiation ray. UV detector is usually suitable for gradient elution work. Most compounds absorb UV light in the range of 200-350 A°. The mobile phase used should not interfere in the peak design of the desired compound hence it should not absorb at the detection wavelength engaged.

Selectivity: Selectivity (α) is equivalent to the relative retention of the solute peaks and, unlike efficiency, depends strongly on the chemical properties of the chromatography medium.

The selectivity, α, for two peaks is given by; $\alpha = k2' / k1' = V2 - V0/V1 - V0 = V2/V1$Where V1 and V2 are the retention volumes, and $k2' / k1'$ is the capacity factors, for peaks 1and 2 respectively, and V0 is the void volume of the column. Selectivity is affected by the surface chemistry of the reversed-phase medium, the nature and composition of the mobile phase, and the gradient shape. Both high column efficiency and good selectivity are important to overall resolution. However, changing the selectivity in a chromatographic experiment is easier than changing efficiency. Selectivity can be changed by changing easily modified conditions like mobile phase composition or gradient shape.[Viscosity: Solvent of deepest possible viscosity should be used to minimize separation time. An added advantage of low viscosity is that great efficiency theoretical plate (HETP) value sare usually lower than with solvents of higher viscosity because mass allocation is earlier .viscosity should be less than 0.5 centipoises, otherwise high pump pressures are compulsory and mass transfer between solvent and stationary phase will be reduced.

Temperature: Temperature can have a reflective outcome on inverted phase chromatography, particularly for low molecular weight solutes such as short peptides and oligonucleotides. The viscosity of the mobile phase used in reversed-phase chromatography decreases with the swelling column temperature. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process, falling solvent viscosity usually leads to more competent mass transfer and, therefore, higher resolution.

Increasing the temperature of a reversed-phase column is principally effective for low molecular weight solutes later they are suitably stable at the elevated temperatures.

Detectors: A large number of detectors are used for RP-HPLC analysis. However, among these, the five main detectors used in LC analysis are the electrical conductivity detector, the fluorescence detector, the refractive directory indicator, mass spectrometry indicator, and the UV detector (fixed and variable wavelength). These detectors are active in over 95% of all LC analytical applications the detector certain should be selected reliant upon some typical property of the analyte comparable UV absorbance, fluorescence, conductance, oxidation, reduction, etc. Appearances that are to be pleased by an indicator to be used in HPLC determination are.

- High sympathy, enabling suggestion analysis.
- Tiny baseline noise to qualify minor detection.
- Short point and noise near.
- Eclectic linear active range (this simplifies quantitation).
- Low deceased volume (minimal peak broadening).
- Cell strategy that removes the changing of the detached bands.
- Insensitivity to deviations in kind of solvent, flow rate, and temperature.
- Operative ease and consistency.
- Tunability, so that revealing can be enhanced for dissimilar compounds.
- Huge linear dynamic collection.
- Non-destructive to section.

APPLICATIONS:- The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. Preparative HPLC refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain information about the sample compound.

Chemical Separations:- It is based on the fact that certain compounds have different migration rates given a particular column and mobile phase, the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification: Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound showed a characteristic peak under certain chromatographic conditions. The migration of the compounds and contaminants through the column needs to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

Identification: Generally assay of compounds is carried using HPLC. The parameters of this essay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels in which the essay will be performed**.**

Validation:-

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality**,** reliability, and consistency of analytical results; it is an integral part of any good analytical practice.

Validation of an analytical procedure is to validate that it is appropriate for its proposed tenacity. A tabular précis of the appearances valid to identification, control of impurities, and assay procedures is comprised. Other analytical procedures may be careful in upcoming additions to this article.

Types of Analytical Procedures to be Validated

The conversation of the validation of analytical procedures is fixed to the four greatest shared.

Types of analytical procedures.

- Identification tests.

- Quantitative tests for impurities relaxed.

- Limit tests for the device of impurities.

- Quantitative tests of the active moiety in samples of a drug substance or drug product or another certain component (s) in the drug product.

While there are several other analytical procedures, such as dissolution difficult for drug products or particle size determination for drug substance, these have not been addressed in the early text on validation of analytical procedures. Validation of these additional analytical procedures is equally important to those listed herein and may be lectured in the following documents.

A brief explanation of the kinds of tests measured in this article is delivered below. Identification tests are planned to certify the character of an analyte in a sample. This is generally realized by the judgment of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard.

- Challenging for impurities can be any quantitative test or a limit test for the impurity in a sample. Whichever test is planned to precisely reproduce the clarity appearances of the sample? Dissimilar validation characteristics are compulsory for a quantitative test than for a limit test.

- Assay procedures are expected to quantity the analyte present in a quantified sample. In the location of this article, the assay characterizes a quantitative measurement of the major section(s) in the drug substance. For the drug product, parallel validation characteristics also relate when assaying for the active or other selected section(s). The same validation characteristics may also apply to assays related to other analytical procedures (e.g., dissolution.

Steps of Analytical Validation

The Three Stages of Process Validation stay:

Stage 1 – Process Design

Stage 2 – Process Validation or Process Qualification

Stage 3 – Continued Process Validation

Stage 1 – Process Design

This is the exploration and progress phase and contains defining a process for manufacturing.

The product. It frequently includes the subsequent:

- Creation of a Quality Target Product Profile (QTPP)
- Identifying Critical Quality Attributes (CQAs)
- Defining Critical Process Parameters (CPPs)
- Conducting risk assessments

From a regulatory point of understanding, a critical section of this stage is a careful and comprehensive record charge. That said, the assembly and appraisal of data as part of this Process Validation step also has business welfares too. This is because you can use the data to improve your production process, create efficacies, and troubleshoot problems.

Stage 2 – Process Validation or Process Qualification

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This stage evaluates/qualifies the process designed previously to certify it can reproduce consistent and reliable stages of quality.

It comprises gathering and estimating data on all facets and phases of the manufacturing process. This includes. The building and services, i.e. ensuring they follow to local regulations as well as pharmaceutical manufacturing regulations.

- The conveyance of raw materials
- Storage of raw resources
- The knowledge, preparation and working performs of production line employees
- Every period of the procedure to turn raw resources into the finished product. This includes having pre-defined sampling points at many periods of the process.
- Finished product packaging, storage, and circulation

- Another convenient component of this step of Process Validation is to develop contingency Strategies for conditions where things go mistaken.

Stage 3 - Continued Process Verification

Continuous Process Verification includes ongoing validation throughout the production of the Profitable product to certify the process designed and qualified in the earlier stages stays to bring steady quality.

One of the key purposes of this period is to notice and resolve process meaning. The point involves product sampling, analysis, and verification at several points in the manufacturing process, and involves the contribution of employees with quality control training. Again, inclusive record-keeping is obligatory at this point, including sorting differences and matters with product quality.

Parameters of Validation:-

1. Linearity

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

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2. Range

The range is the upper and lower limit of the analyte used to analyze the sample at changed concentrations for the resolve of linearity, accuracy, and precision.

3. Precision: -Precision of an analytical procedure expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same.

The homogeneous sample under the prescribed condition.

3. A) Method Precision/ Repeatability

Method precision is performed to validate and evaluate the variations experienced by the first analyst on the instrument.

3. B) Intermediate precision/ Ruggedness

The activity should be performed by a deferent/ second analyst to capture the variation on a different day with different equipment. A statistical comparison is made to the first analyst result.

4. Repeatability

It has been accomplished by repetition analysis of equal concentration.

5. Accuracy

The accuracy of an analytical procedure is the closeness of the test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. It may often be expressed as the recovery by the assay of known, added amounts of analyte.

6. Robustness

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

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7. LOD and LOQ

It has been normally accomplished to regulate whether how much small amount and small discovery appraisals have been acquired for the enhancement method.[9][10]

CONCLUSION

The enlargement of the HPLC method for drugs has conventional significant kindness in current years because of its significance in routine quality control analysis. A HPLC method was invased by the appropriate method of the purpose of sofosbuvir in clean and in its tablet form. For the achievement of the good departure, we have chosen the C-18 column, which is commonly superior as compared to the other columns along with the mobile phase. Analytical methods development plays a significant role in the detection, development, and manufacture of pharmaceuticals. RP-HPLC is perhaps the most worldwide, most delicate analytical procedure and is exceptional in that it easily copes with multi-component mixtures.

While mounting the analytical methods for pharmaceuticals by RP-HPLC, they must have a good practical concern of chromatographic departure to know how it differs with the sample and with fluctuating experimental conditions in order to achieve supreme split-up. To develop HPLC method effectually, most of the exertion should be expended in method development and optimization as this will improvement the final.

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