



CODEN [USA]: IAJ PBB

ISSN : 2349-7750

**INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES**

SJIF Impact Factor: 7.187

<https://doi.org/10.5281/zenodo.23345740>Available online at: <http://www.iajps.com>

Review Article

**DEVELOPMENT AND VALIDATION OF HPLC METHODS
FOR QUANTITATIVE ANALYSIS OF ACTIVE
PHARMACEUTICAL INGREDIENTS (APIS) IN COMPLEX
FORMULATIONS****Aashutosh Yeole¹, Waghmare Suresh²**^{1,2}Rashtriya College of Pharmacy, Hatnoor**Abstract:**

High-Performance Liquid Chromatography (HPLC) is a powerful and widely used analytical technique for the separation, identification, purification, and quantitative determination of active pharmaceutical ingredients (APIs) in complex formulations. The technique separates components based on their interactions with the stationary phase and mobile phase and is particularly useful for pharmaceutical compounds that are non-volatile or thermally unstable. The development of an HPLC method involves several important steps, including sample preparation, selection of the appropriate chromatographic mode and column, development of the mobile phase, optimization of chromatographic conditions, selection of a suitable detector, and quantitative analysis. Method validation is performed to establish the reliability and suitability of the developed method by evaluating parameters such as accuracy, precision, linearity, specificity, robustness, and limits of detection and quantification. HPLC is extensively applied in pharmaceutical quality control, drug stability and dissolution studies, as well as clinical, forensic, environmental, food, and flavor analysis. The technique plays an important role in ensuring the quality, purity, safety, efficacy, and consistency of pharmaceutical products.

Keywords: HPLC, Chromatography, Wavelength, Robustness, Manufacturing.

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Please cite this article in press **Aashutosh Yeole et al., Development And Validation Of Hplc Methods For Quantitative Analysis Of Active Pharmaceutical Ingredients (Apis) In Complex Formulations., Indo Am. J. P. Sci, 2026; 13(10).**

INTRODUCTION:

Analytical chemistry determines the substance's qualitative and quantitative makeup. All elements are required in order to interpret the sample material. There are two categories of analytical chemistry: qualitative and quantitative. By identifying the presence or absence of specific components, a qualitative analysis provides information on the sample's characteristics. With the use of equipment, the instrumental techniques of analysis concentrate on measurements to ascertain the substance's chemical makeup and certain physical characteristics. For two terms, chromatography stands for "chromo" (color) and "graphy" (writing). Chromatography is the method used to separate, identify, and determine the chemical components present in complicated mixtures. Similar to spectroscopy, this technique is frequently utilized and has great analytical power for both preoperative and analytical procedures. Using this approach, a column of high grade purity may be produced. This method separates the components of a mixture according to how quickly they pass through a stationary phase (column) using a liquid or gaseous mobile phase.[1]

High performance liquid chromatography

The process in which the components in complex mixtures are separated, identified, and determined is termed chromatography. This technique is widely used like spectroscopy and is a very powerful tool not only for analytical methods but also for preparative methods. Compounds of high-grade purity can be obtained by this method. The late 1960s and early 1970s saw the development of high performance liquid chromatography. It is now widely used for separation and purification in many different fields, including as the food, pharmaceutical, biotechnology, environmental, and polymer sectors. Over the last few decades, HPLC has emerged as the preferred method for analyzing a wide range of chemicals. Its primary benefit over GC is that macromolecules may be used for HPLC analysis since the analyte does not need to be volatile. A little quantity of liquid sample is injected into the mobile phase, a flowing stream of liquid that travels through a column filled with stationary phase particles, to perform HPLC. [2,3]

The degree to which each component is retained in the column determines how the mixture is separated into its constituent parts. Relative solute or stationary phase and solute mobile phase interactions influence this partitioning in HPLC. Reliability and suppleness the capacity to change the stationary and mobile phase composition are the main reasons HPLC has improved in quality.[4] When compared to the traditional LC method,

HPLC is distinguished by:

1. High quality.

2. Tiny (4.6 mm) columns made of titanium, glass, or stainless steel.
3. Very tiny (3, 5, and 10 μm) particles packed in columns.
4. Controlled mobile phase flow and comparatively high inlet pressures.
5. Constant flow sensors that can manage small flow rates and detecting very small amounts.
6. Quick analysis.

Types of HPLC methods

1. **HPLC in reverse phase** -In the field of biochemical separation and purification, reversed phase chromatography has been used both analytically and before surgery. A certain amount of hydrophobicity in molecules can be separated with outstanding recovery and resolution using reversed phase chromatography. Water-organic is used as the mobile phase, while the stationary phase can be C18 (ODS), C8, phenyl, TrimethylSilane (TMS), or cyano. It is the primary option for the majority of samples, particularly non-ionized or neutral chemicals that dissolve in organic mixes of water.[5]
2. **Normal Phase HPLC**- The mobile phase in normal-phase chromatography is nonpolar, while the stationary phase is polar. The converse is true in reversed phase, when the mobile phase is polar and the stationary phase is nonpolar. Silica or organic moieties with cyano and amino functional groups are common stationary phases for normal-phase chromatography. In this case, cyano, diol, and amino silica are examples of organic solvent combinations that may be utilized as the stationary phase and as the mobile phase and columns, respectively. For preoperative scale HPLC and isomer mixtures, it is the primary choice. For lipophilic materials that are difficult to dissolve in water-organic combinations, it is the second choice.[6]

Analytical method development:

Pharmaceutical discovery, development, and manufacturing heavily rely on the development and validation of analytical methods. These techniques are employed to guarantee the efficacy, identification, purity, and performance of pharmaceutical items. When creating techniques, there are several things to take into account. They first gather data on the physicochemical characteristics of the analyte (pK_a , $\log P$, and solubility) and identify the best detection method for analysis (i.e., the appropriate wavelength for UV detection). Validating a stability-indicating HPLC-method takes up most of the analytical development effort. Separating and quantifying the primary active

ingredient, any reaction contaminants, any accessible synthetic intermediates, and any degradants is the aim of the HPLC process.[7]

The following steps are involved in developing a method:

1. A calibrated and qualified instrument
2. Methods that are documented
3. Trustworthy benchmarks
4. Skilled analysts
5. Integrity and sample selection
6. The analysis need to be inexpensive and time-efficient.
7. The analysis's correctness must adhere to Pharmacopoeia norms.
8. The selected approach needs to be exact and focused.
9. Establish the HPLC parameters.
10. Optimization of the method.

Purpose

Drug analysis makes it possible to identify, characterize, and determine the medicine in mixes, such as biological fluids and definite quantitative forms. The main goal of analytical techniques in method and medication development is to generate information about efficiency, which is closely related to the need of a notable dose), impurity (associated with the drug's safety profile), bioavailability (which encompasses important drug attributes such as crystal type, uniformity, and release), stability (which shows the degradation products), and the influence of manufacturing parameters to ensure consistent drug merchandise assembly. [8]

The goal of internal control is to examine and create a genuine and correct product through a set of procedures intended to prevent and identify faults at various production stages. Requiring a decision on whether to unharness or reject a product depends on one or more types of management activities. The goal of internal control is to examine and create a genuine and correct product through a set of procedures intended to prevent and identify faults at various production stages. Requiring a decision on whether to unharness or reject a product depends on one or more types of management activities.

Offering a simple and analytical approach for a variety of complex formulations might be a topic of the highest significance. The demand for novel analytical techniques has rapidly increased due to the rapid growth of pharmaceutical businesses and ongoing medication manufacture in various parts of the world. Sample details, separation objectives, unique HPLC method requirements, sample handling, etc. Select the detector and its parameters. Select the LC technique, do a preliminary run, determine the ideal separation conditions, Improve the circumstances for separation, Look for issues or if a specific process is needed. Retrieve the material that has been purified. Calibration using quantitative

methods qualitative approach verify the procedure before releasing it to a regular lab. [9,10]

Steps for the development of the method.

The development process is followed with the appropriate paperwork. All information pertaining to these research has to be entered into an electronic database or a lab notebook. Characterization of the analytic standard [11]

- a) All relevant data on the analyte's structure and physio-chemical characteristics, such as optical isomerism and solubility, are gathered.
- b) The standard analyte (about 100% pure) is acquired. It is necessary to set up the freezer, refrigerator, and desiccators for optimal storage.
- c) When there are several components in the sample matrix that need to be examined, the number of components is indicated appropriately, the data is presented, and the accessibility of the standards is calculated.
- d) When paired with sample stability, techniques such as spectroscopy, HPLC, GC, MS, etc., are taken into account.

Method requirement

Calculating the actual sample recovery percentage and showcasing quantitative sample analysis It estimates the percentage recovery of a spiked, authentic reference medication in a sample matrix devoid of analytes. It is necessary to demonstrate optimization for repeatability of recovery (average $\pm$ standard deviation) across samples. Since the results may be replicated to identify with high accuracy, a 100% recovery is not required at this time.

The physicochemical properties of drug molecule:

When developing a technique, a medicinal molecule's physio-chemical characteristics are crucial. The physical characteristics of the drug molecule, such as its solubility, polarity, pKa, and pH scale, must be studied in order to build a technique. The concept of a molecule's polarity is frequently used to explain its solubility. Solvents that are polar, like water, and non-ionic, like benzene, do not mix. Generally speaking, compounds with comparable polarity are soluble in one another because "like dissolves like." The solubility of the analyte determines the choice of diluent. It is important that the analyte dissolves in the diluent and doesn't react with any of its constituents.[12]

Set up HPLC conditions

A partially neutralized acid that is resistant to pH scale shifts might be a buffer. Typically, salts like NaCl or Na-lactate are used to somewhat neutralize the acid. The buffer's capacity to withstand pH scale fluctuations is known as its buffering capability. (i)

As the morality of the buffer salt/acid solution rises, buffering capability will also rise. (ii) The buffering capacity increases as the buffered pH approaches the pKa. (iii) The capacity for buffering is the amount of sodium hydroxide needed to raise pH by 1.0 is indicated as well. When developing a reversed-phase chromatography (RPC) technique, it is crucial to take into account the effects of pH on analyte retention, the type of buffer to employ, its concentration, solubility in the organic modifier, and its influence on detection[13].

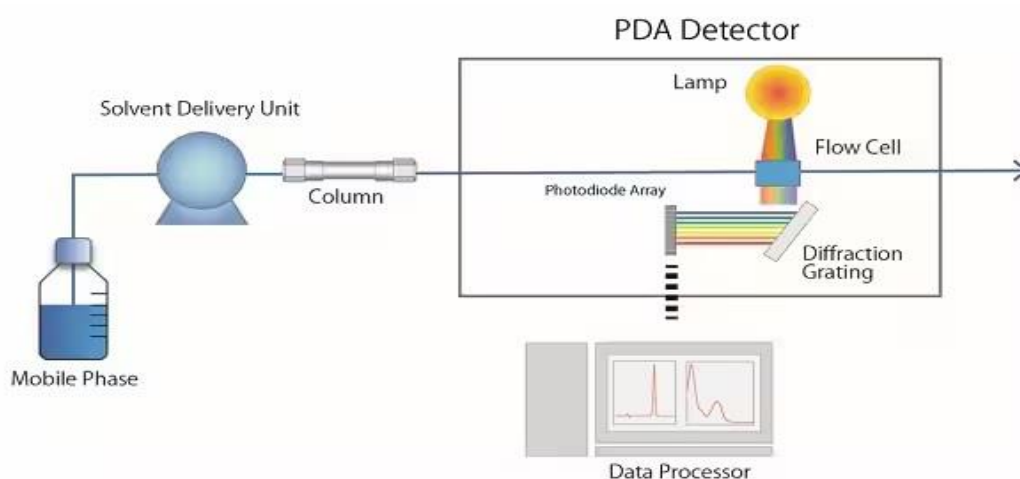
General considerations during buffer selection:

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
3. Ammonium salts are generally more soluble in organic/water mobile phases.
4. TFA can degrade with time, is volatile, absorbs at low UV wavelengths.

5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier.
6. This growth will accumulate on column inlets and damage chromatographic performance.

Selection of detector

The detector is a crucial component of HPLC. The chemical makeup of the analytes, possible interference, the necessary detection limit, the detector's availability, and/or cost all influence the choice of detector. A versatile dual-wavelength absorbance detector for HPLC is the UV-Visible detector. This detector provides the high sensitivity required for common UV-based applications, such as chemical analysis and low-level impurity detection. Advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions is provided by the PDA Detector. High spectrum sensitivity and natural action are provided by its integrated software package and optical advancements. The Refractive Index (RI) Detector is the best option for analyzing components with little to no UV absorption because of its great sensitivity, stability, and repeatability.[14]



PDA Detector Used in HPLC
Credit@Shimadzu

Fig no 1: Selection of detectors in HPLC

Column selection

The column is the central component of an HPLC system. As a technique is developed, dynamical a column can get the greatest results for analyte resolution. Typically, spherical colloid beads coated with the hydrophobic stationary part are packed into the column housing to make stylish reverse part HPLC columns. A chlorosilane reacts with the hydroxyl radical teams present on the colloid surface to introduce the stationary portion into the matrix. In terms of extraction, potency, property, and capability issues, the stationary part's character generally performs the best.

The stationary component is supported by a variety of matrices, including silicon oxide, polymers, and aluminum oxide. The most used matrix for HPLC columns is silicon oxide. Silicon oxide matrices are robust, easily derivatized, manufactured to a uniform spherical size, and do not collapse while under pressure. Silicon oxide is chemically stable in low pH scale systems and most organic solvents. The ability of silicon oxide solid supports to disintegrate at pH values below seven is one of its drawbacks. Columns supported by silicon oxide have been created recently for usage at high pH levels.[15]

Mobile part

The mobile part effects resolution, property and potency. In reverse part action, the mobile part consists of associate degree binary compound buffer and a non-UV active water miscible organic solvent. The result of the organic and binary compound part and therefore the proportions during which they're mixed can have an effect on the analysis of the drug molecule. choice of the mobile-phase and gradient conditions depends on the ionogenic nature of the analyte and therefore the property of the analytes within the mixture severally. There are several uses for the binary compound buffer. The mobile component lowers peak tailing and protonates free silanols on the column at low pH. Basic analytes are protonated at low enough pH levels; after ionization, the analyte can rinse farther fast but with better peak morphology. Acidic analytes can remain dead in buffers with a low enough pH scale. Boosting retention. On the other hand, ionized acidic chemicals can rinse sooner and neutral basic compounds can be further retained at higher pH scales.[16]

Isocratic or gradient separations:

Analyte-eluent and analyte-stationary-phase interactions are consistent throughout the run due to isocratic, constant eluent composition, which also implies equilibrium conditions in the column and the actual velocity of chemicals flowing through the column are constant. Despite the low separation power (the number of compounds that might be resolved), this increases the predictability of isocratic separations. The peak capacity is modest, and the resulting peak widens as the component is held on the column for a longer period of time.[17]

Based on type of analysis:

1. Qualitative analysis:- The number of components, the presence of contaminants, the identification of the chemical, etc. Values for the retention time are calculated.

2. Quantitative analysis:- It is decided how many individuals or several components make up a blend. For this, the peak areas of the standard and sample are compared.

Sample preparation for method development

The drug substance under investigation has to dissolve in a solution (dilution). Solutions should be prepared in amber flasks during the process's early manufacture before it is known that active ingredients are included. The is stable at room temperature and does not degrade under normal experimental settings. A 0.22 or 0.45 μm pore filter is often recommended for the removal of particulate matter from the sample solution. A crucial stage in creating the system the researcher will be analyzing is sample preparation.[18]

Method optimization

After appropriate separation, the experimental circumstances should be planned to yield the different partitions and sensitivity. A systematic and planned investigation of factors, such as pH (if ionic), the ratio and components of the mobile phase, the gradient, the flow rate, the temperature, the amount of the sample, the injection volume, and the solvent form elements, will be used to achieve test stability.

Method validation

The techniques were verified in accordance with the guidelines for analytical method validation provided by the International Conference on Harmonization (ICH). Any new or updated drug must undergo validation to guarantee that it can yield results that are repeatable, reliable, and efficient. The process by which laboratory tests have established the analytical method's performance requirements is known as validation. Fulfill the requirements of the intended analytical use.

Scope of process validation

When applied to all stages of product manufacture, validation is one of the most expansive and complex fields. The extent of validity in a variety of disciplines is as follows: Analytical Equipment, facilities, manufacturing processes, product design, cleaning, operators, raw materials, packaging materials, instrument calibration, and process utility services [19]

Stage 1

The pre-validation qualification stage encompasses all activities that define and enhance product studies, pilot batch testing formulation, scale-up analysis, product study exchange, market-scale innovation for groups, establishing stability conditions, the completion of pharmaceutical formulations in-process, equipment maintenance, master records, and process limit approval.

Stage 2

This includes the procedure's validation stage. Its goal is to guarantee that a vital process parameter's installed limit is not exceeded. is important and that even under the worst-case scenarios, quality results may be produced.[20]

Stage 3

Also known as the validation maintenance stage, it calls for a constant examination of all procedure-related archives, including validation of the review reports, to make sure that no alterations, failures, mistakes, or changes have been made to the production process and that all standard operating procedures (SOPs) pertaining to change control procedures have been found.

Type of process validation

Three types of validation exist: prospective, concurrent, and retrospective, Re-validation .

Prospective validation

Documented evidence that a device accomplishes its goals based on methodologies is known as prospective validation. The purpose of the prospective validation is to demonstrate that the process operates in accordance with a master plan or methodology for pilot product trial validation. There are no limitations on the quantity of analytical techniques that can be used. The equipment/facilities list, which includes calculation and observing/recording gear, provides a comprehensive explanation of the approach and summarizes the primary processing phases to be assessed for usage in combination with its calibration condition.

Concurrent validation

End-product output current testing and in-process tracking of critical processing stages may offer recorded proof that the manufacturing process is under control. It is comparable to the anticipated, public market pricing, with the exception that the operational business will sell the product during the qualifying runs.[21]

Retrospective validation

Such procedures can be validated by historical evidence. Among the steps are the preparation of a particular protocol and the publication of the data review's findings, which will result in a recommendation and a conclusion. Retrospective validity is predicated on the fact that genuine in-process needs for those attributes must align with the pharma product's end product requirements.

Re-validation

It is an aspect or phase that has been replicated and validated. Among the modifications in revalidation are shifts in the source of the active raw material manufacturer.

Changes in crude materials and the source of active producers of crude materials. Packing material changes. Internal plant/facility modifications.

Accuracy

The degree to which the assessed value resembles the authorized or real price is known as accuracy. The difference between the observed mean value and accuracy is known as accuracy. It is computed by applying the technique to samples that have been treated with known analyte concentrations. These can be contrasted with blank and standard answers to verify that there is no involvement.

Specificity

The capacity to detect the analyte differently in the presence of substances that may be predicted to be present, such as excipients, degradation products, and contaminants, is known as specificity. Segregation is not always necessary, specificity just examines the target ingredient without being influenced by other creatures that could be present, etc. It is necessary for the peak purity value to exceed 0.999.[22]

Robustness

It is defined as the assessment of an analytical method's resistance to small but deliberate changes in the method's parameters (such as pH, mobile phase structure, temperature, and experimental settings) and provides an indication of the method's effectiveness when used regularly

How HPLC used in quantitative analysis of active pharmaceutical ingredients (APIs) in complex formulations.

Components are separated by HPLC according to how they interact with the mobile phase (solvent system) and stationary phase (column packing material). Disparities in size, charge, or polarity are what cause the separation. This allows for the efficient separation, detection, and quantification of APIs and other excipients in a formulation.

Steps in HPLC Quantitative Analysis

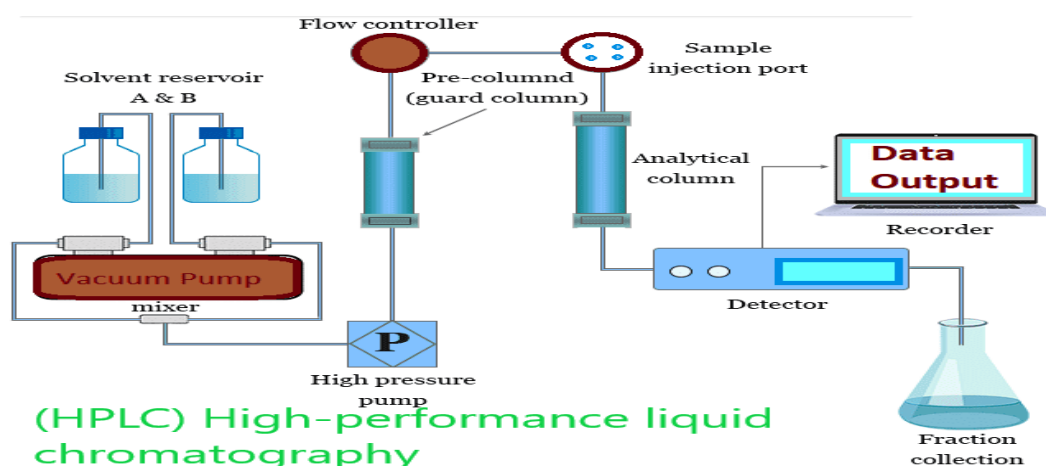


Fig no 2: Steps in HPLC

1. Sample Preparation

A suitable solvent is used to dissolve the complex medicinal composition. The choice of solvent depends on the solubility and chemical properties of the active pharmaceutical ingredient (API) and other components present in the sample. Insoluble excipients, impurities, or particulate matter are frequently removed from the prepared sample by filtration or centrifugation. This step helps prevent blockage of the HPLC column and improves the quality and reliability of the chromatographic analysis. In some situations, further sample cleaning may be necessary to isolate the API from interfering substances. Techniques such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE) can be used to selectively extract and concentrate the API before HPLC analysis.[23]

2. Selection of HPLC Mode

Reversed-phase HPLC (RP-HPLC) is the most commonly used HPLC mode for the analysis of medium-to-high polarity APIs. It generally uses a non-polar stationary phase and a relatively polar mobile phase, allowing effective separation of many pharmaceutical compounds. Normal-phase HPLC (NP-HPLC) is generally used for the analysis of non-polar APIs. In this technique, a polar stationary phase and a relatively non-polar mobile phase are used to separate compounds based on their polarity. Ion-exchange HPLC can be used for APIs that possess ionic characteristics. The separation occurs through interactions between charged molecules in the sample and oppositely charged groups present on the stationary phase. Size-exclusion HPLC is mainly used for the analysis of larger molecules, such as proteins and peptides. Separation is based primarily on the molecular size or hydrodynamic volume of the compounds.[24]

3. Development of Mobile Phase

A suitable combination of solvents, such as water, acetonitrile, or methanol, is selected as the mobile

phase. The composition of the mobile phase is optimized according to the chemical properties of the API and the required separation. The pH and ionic strength of the mobile phase are often controlled using suitable buffers. These parameters are important because they can influence the ionization, separation, retention, and stability of the API during HPLC analysis.[25]

4. Chromatographic Conditions Optimization

Important chromatographic parameters, including gradient elution conditions, flow rate, and column temperature, are optimized to obtain effective separation of the API from other components present in the sample. Proper optimization helps produce sharp, symmetrical, and well-resolved peaks. The detector is selected according to the chemical and physical characteristics of the API. Commonly used detectors include UV/Vis, photodiode array (PDA), fluorescence, and mass spectrometry (MS) detectors.[26]

5. Injection and Separation

A predetermined volume of the prepared sample is injected into the HPLC system. The injection volume is kept constant to ensure reproducible chromatographic results. After injection, the mobile phase carries the sample through the chromatographic column. Different components interact differently with the stationary and mobile phases, resulting in their separation. The API is therefore separated from excipients and other substances present in the medicinal formulation.

6. Detection

After separation, the API is detected as a chromatographic peak at a specific retention time (Rt). The retention time can be compared with that of an authentic API standard to assist in identifying the compound. A UV/Vis detector is commonly used for APIs that contain chromophores because these compounds absorb ultraviolet or visible

radiation at specific wavelengths. The detector measures the absorbance and produces a chromatographic peak corresponding to the compound. A photodiode array (PDA) detector provides spectral information in addition to the chromatographic peak. The spectral data can be used to support peak identification and assess the purity of the API peak. A mass spectrometry (MS) detector provides high sensitivity and selectivity. It can provide information about the molecular mass and structural characteristics of the API, making it particularly useful for the identification and confirmation of pharmaceutical compounds.[27]

7. Quantification

API standards of known concentration are used to prepare a calibration curve. Different concentrations of the standard are analyzed, and the corresponding peak areas are used to establish the relationship between concentration and detector response. The concentration of the API in the sample is calculated by comparing the peak area of the sample with the established calibration curve. The area under the chromatographic peak is generally proportional to the amount of API present, allowing its concentration to be quantitatively determined.

Application of HPLC

1. General Applications- High-Performance Liquid Chromatography (HPLC) has several applications in clinical, forensic, pharmaceutical, food, and environmental fields. It is widely used for the separation, identification, analysis, and purification of different compounds present in complex mixtures. Due to its high accuracy and sensitivity, HPLC is an important analytical technique in various scientific and industrial applications.[28]
2. Pharmaceutical Applications- HPLC is extensively used in the pharmaceutical industry for quality control and analysis of medicines. Major applications include internal quality control, drug stability studies, and dissolution studies. It helps determine the purity, potency, and stability of pharmaceutical products and ensures that medicines meet the required quality standards.[29]
3. Environmental and Forensic Applications- In environmental analysis, HPLC is used for monitoring contaminants and identifying various components present in potable water and other environmental samples. In forensic science, HPLC can be used to measure hormones and drugs present in biological samples. It is also useful for the analysis and identification of textile dyes and other chemical substances involved in forensic investigations.[30]

4. Clinical Applications- HPLC has important applications in clinical analysis, particularly in the examination of biological samples such as blood and urine. It can be used to analyze endogenous compounds, including neuropeptides, and to detect and quantify various substances present in biological fluids. This makes HPLC useful in clinical research, diagnosis, and biochemical investigations.[31]
5. Food and Flavor Applications- HPLC is widely used in the food and flavor industry for the analysis of various food components. It can be used for the determination of preservatives, analysis of sugars in fruit juices, and detection of polycyclic compounds and other contaminants in vegetables. These applications help ensure the quality, safety, authenticity, and composition of food products.[32]

Conclusion:

High-Performance Liquid Chromatography (HPLC) is an essential analytical technique for the separation, identification, purification, and quantitative analysis of pharmaceutical compounds. The development of an effective HPLC method requires careful consideration of sample preparation, column selection, mobile-phase composition, chromatographic conditions, and detector selection. Optimization of these parameters provides efficient separation and reliable detection of APIs in complex formulations. Method validation is an important part of HPLC analysis because it demonstrates that the analytical method is accurate, precise, specific, robust, and suitable for its intended purpose. Quantitative determination of APIs can be achieved using calibration standards and comparison of sample peak areas with the calibration curve. Overall, HPLC provides accurate, dependable, and reproducible results and has wide applications in pharmaceutical quality control, clinical analysis, forensic investigations, environmental monitoring, and food analysis. Therefore, the development and validation of HPLC methods are essential for maintaining pharmaceutical product quality, ensuring regulatory compliance, and supporting the safety and efficacy of medicines.

Acknowledgment:

The authors would like to express their sincere gratitude to Rashtriya College of Pharmacy, Hatnoor, for providing the necessary facilities, laboratory infrastructure, and academic support required to conduct this research. The authors also thank the teaching and technical staff of the institution for their valuable assistance and support throughout the study.

Funding:

The authors received no specific grant, financial support, or funding from any government, commercial, or non-profit organization for the conduct of this research. The study was carried out using the institutional resources available at Rashtriya College of Pharmacy, Hatnoor.

Conflicts of Interest:

The authors declare that they have no conflicts of interest related to the research, authorship, or publication of this manuscript.

Authors' Contributions:

Aashutosh Yeole: Conceptualization, methodology, investigation, experimental work, data collection, data analysis, and manuscript preparation. Waghmare Suresh: Supervision, guidance, validation, interpretation of results, critical review, and revision of the manuscript. Both authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Data Availability:

The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval:

Ethical approval was not required for this study, as the research involved analytical method development and validation using pharmaceutical formulations and did not involve human participants, human biological samples, or experimental animals.

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