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# A REVIEW ARTICLE ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD DEVELOPEMENT AND VALIDATION

**Sneha Patel[1]*, Mitali Dalwadi[2], Dr. Chainesh Shah[3] and Dr. Umesh Upadhyay[4]**

[1]PG Scholar, M. Pharm (Q.A.), Sigma Institute of Pharmacy, (Faculty of Pharmacy), Sigma University, Bakrol, Vadodara-390019.
[2]Assistant Professor, Sigma Institute of Pharmacy, Sigma University, Bakrol, Vadodara-390019.
[3]Professor, Sigma Institute of Pharmacy, Sigma University, Bakrol, Vadodara-390019.
[4]Dean of Sigma Institute of Pharmacy, Sigma University, Bakrol, Vadodara-390019.

***Corresponding Author: Sneha Patel**
PG Scholar, M. Pharm (Q.A.), Sigma Institute of Pharmacy, (Faculty of Pharmacy), Sigma University, Bakrol, Vadodara-390019.

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## ABSTRACT
High-Performance Liquid Chromatography (HPLC) is an essential analytical technique used in pharmaceuticals, environmental science, and food safety. This review paper discusses the critical aspects of HPLC method development, focusing on optimization techniques such as column selection, temperature control, pH adjustments, and flow rate optimization. It highlights the differences between gradient and isocratic elution methods, as well as various detection techniques, including UV and mass spectrometry. Furthermore, the validation of HPLC methods is explored, emphasizing regulatory requirements and key parameters such as precision, accuracy, specificity, and robustness. The applications of HPLC in pharmaceutical analysis, environmental monitoring, and food safety are examined, along with the challenges faced in method development and future directions in the field, including emerging technologies like Ultra-High-Performance Liquid Chromatography (UHPLC). This comprehensive overview underscores the significance of HPLC in analytical science and its evolving role in meeting the demands of modern research and industry.

**KEYWORDS**: HPLC, Separation, Development.

## 1. INTRODUCTION[1]
Different factors in method development in high performance liquid chromatography is necessary to ensure accuracy, precision, and reproducibility of results. The comprehensive strategy in method development mainly deals with choosing appropriate chromatographic conditions on a stationary phase, mobile phase composition, and wavelength selection. Another important requirement is that of method validation, guaranteeing that the developed method will fit pre-established criteria regarding its accuracy, precision, specificity, and robustness. International Council for Harmonisation guidelines codify such activities. In these regards, the ICH enforces that both the development and validation of an HPLC method are critically important to guarantee the reliability of the results obtained. Column chemistry, temperature control, and gradient programming are other pivotal factors that influence the efficiency of separation and resolution of the analytes and, as such, become very important parts in method optimization. When followed, these protocols not only guarantee homogeneous performance in a given laboratory instrument but also provide a basis for method transfer between different laboratories and instruments.

### 1.1 PRINCIPLE[2,3]
HPLC is a separation technique in which a small volume of liquid sample is injected into a column packed with tiny particles called the stationary phase. Individual components of the sample move down the column due to a liquid, which is called the mobile phase, forced through the column by high pressure delivered by a pump.

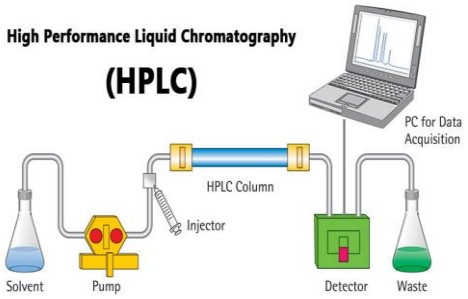


**Figure 1: Instrumentation of high-performance liquid chromatography.**

### 1.2 TYPES OF HPLC[4,5]

High-Performance Liquid Chromatography (HPLC) is one of the most versatile and applied techniques in analytical chemistry. The process of separation in HPLC techniques is categorized under four major groups

- **Normal phase HPLC:** This method uses a polar stationary phase (like silica) and a non-polar mobile phase; it's appropriate for the separation of water-sensitive compounds, geometric isomers, and chiral compounds.

- **Reverse phase HPLC:** This is the most common mode of HPLC because it uses a nonpolar stationary phase and a polar mobile phase, such as water mixed with methanol. It can be effectively used in the separation of compounds into polar and non-polar parts.

- **Ion Exchange HPLC:** A stationary phase charged used in the separation of ionic compounds according to their charge.

- **Size-exclusion HPLC:** It actually separates molecules according to size. Generally, larger molecules are eluted out first, being too big to enter the pores within the stationary phase, and then the smaller ones follow.

**Table 1: Comparison of normal and reversed phase HPLC.**

| Type | Mobile phase | Stationary phase | Elution order |
|---|---|---|---|
| Normal phase | Non-polar (hexane, toluene, methanol) | Polar (silica or chemically modified Si such as-O-$(CH_2)_3$-CN) | Least polar first, most polar last |
| Reversed phase | Polar (Water + miscible organic solvent) (acetonitrile, ethanol, methanol) | Non-polar (chemically-modified silica, C8, C12, C18 groups) 5µm diam. Particle size | Most polar first, least polar last |

### 1.3 METHOD DEVELOPMENT PROCESS

#### 1.3.1 SELECTION OF MOBILE AND STATIONARY PHASES[6,7]

- **Polarity Matching:** In such cases, the nature of the analytes forms a determinant factor for the polarity of the mobile phase. Non-polar solvents for non-polar compounds could include hexane or acetonitrile. For polar analytes, the solvents used are of a polar nature, such as water or methanol.

- **pH Control:** For ionizable compounds, the pH for the mobile phase is very important. Most of the time, buffers are generally added into the mobile phase to keep the pH within a certain range.

- **Solvent Strength:** The ratio of the solvent components can be adjusted to vary retention time slightly for resolution.

- **Additives:** In these cases, additives such as salts and acids can be added to improve peak shape and resolution by reducing peak tailing or even changing the ionization of the analyte.

#### 1.3.2 CHOICE OF STATIONARY PHASE

- **Column Material:** Silica-based columns are the most common; however, polymer-based columns are also available for particular applications.

- **Particle Size:** While reducing particle size will increase surface area and, hence increase resolution, it also increases backpressure.

- **Pore Size:** The pore size should be commensurate with the molecular size of the analytes. The typical range for small molecules is 60–100 Å.

- **Bonded Phase Chemistry:** C18 (L1) (Octadecyl) is used the most, while C8, phenyl, and other bonded phases are available to make the separation selective for dissimilar classes of compounds.

#### 1.3.3 SAMPLE PREPARATION[8]

- **Representative Sample:** It must be representative in nature and derived from the matrix or type of analyte that one intends to analyse. This is most important in case the method is to be applied for a wide range of similar samples.

- **Sample Complexity:** Take into account the co-components likely to be present in any interfering manner within your sample. The result will be the selection of a mobile phase, stationary phase, or any techniques useful in sample preparation.

- **Levels of Concentration:** The concentration that the analyte takes up in the sample to ensure appropriate sensitivity and dynamic range of the method; in trace analyses, the analyte concentration of the samples needs to be very low.

- **Stability and Storage:** Note the conditions of storage and assess the sample stability. The stability of the sample will have an effect on the reproducibility and reliability of the method, some samples need to be stored at low temperatures or protected from light.

- **Compatibility with the Method:** The sample matrix should be compatible with the HPLC method employed which includes the solvent system and detector. For example, very viscous samples may require dilution prior to injection.

- **Regulatory and Standard Requirements:** If the method is to be used for regulatory compliance, obtain samples that are compliant with guidelines, for example, from the FDA or ICH.

### 1.3.4 OPTIMIZATION TECHNIQUES[9,12]

- **Column Selection:** The selection of a proper column is the first step in the development of an HPLC method. Some major issues that should be addressed are.

- **Column Chemistry:** The C18 column chemistry is perhaps one of the most common and diverse types of column chemistries in use today; however, depending on the nature of the analytes, other column chemistries such as C8, phenyl, and cyano may be more appropriate.

- **Particle Size:** Smaller particles increase resolution but also increase backpressure. Columns of less than 2 µm particles are in use in UHPLC for higher efficiency.

- **Pore Size:** The size of the pores will affect the retention of bigger molecules. For macromolecules, large pores are required, while much smaller pores should be there for smaller organic compounds.

### 1.3.5 TEMPERATURE CONTROL[10]

**Temperature role in HPLC performance**

- **Range of Temperature Impact:** In general, the range of temperature depressed retention times and also improved peak shapes.

- **Selectivity:** For example, temperature variations might result in drastic changes within the selectivity of the separation process.

### 1.3.6 ADJUSTING pH LEVELS[9]

**The pH of the mobile phase will be a critical factor in ionizing the analytes**

- **Ionizable Compounds:** Adjust the pH to best retain. This is generally within a pH of 2 to 8, depending on the pKa of the analyte.

- **Buffer Selection:** Common buffers include phosphate and acetate, but care must be taken to avoid interference in sensitive detection methods like mass spectrometry.

### 1.3.7 FLOW RATE OPTIMIZATION[4]

The flow rate optimization is critical to achieve an appropriate balance between the duration of the analysis and resolution. According to
The Van Demeter Equation provides a means to determine the flow rate that gives the best efficiency.

- **Effect on Analysis Time:** Higher flow rates cut down analysis time but can compromise resolution and peak shape.

### 1.3.8 GRADIENT V/S ISOCRATIC ELUTION[10,11]

- **Gradient Elution:** This method involves gradual increases in the concentration of organic solvent to achieve higher resolution of difficult mixtures. Therefore, this can be applied to samples that have a wide polarity range.

- **Isocratic Elution:** In this process, there is no change in the composition of the solvent throughout the chromatogram. Though simpler, it has the disadvantage of sometimes not being able to provide adequate resolution for some components. Whether to use an isocratic or gradient mode depends on the sample complexity and the required resolution.

### 1.3.9 DETECTION METHODS[4]

**Various HPLC detection methods can be used, including**

- **UV-Vis Detection:** The most common method, suitable for compounds with chromophores.
- **Mass Spectrometry:** Offers high sensitivity and specificity, particularly regarding complex mixtures.
- **Fluorescence detection:** More sensitive but restricted to fluorescent compounds.

## 1.4 VALIDATION OF HPLC METHODS[9,10,11]

The validation procedure is structured methodically, aimed at determining the suitability of an analytical method for its intended application, thus ensuring that it reliably produces accurate and dependable results. High-Performance Liquid Chromatography (HPLC) is an essential analytical technique in the pharmaceutical industry, necessitating comprehensive validation to verify its reliability and compliance with regulatory standards. The different ICH guidelines for the validation of analytical procedures, especially Q2(R1), describe requirements that are almost predictable. Requirements like precision, accuracy, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, and stability parameters are usually recommended. Validation is an integral step in the process of ensuring the reliability of HPLC methods; therefore, it must adhere to recognized regulatory guidelines like ICH Q2(R1)

**Basic Parameters:** These are precision, accuracy, specificity, linearity, the limit of detection, the limit of quantification, robustness, and ruggedness.

- **ACCURACY:** This is a concept relating to the closeness of the observed values to the real value. The main way it is evaluated involves recovery studies, where fixed amounts of the analyte are added into the sample matrix, and afterwards, the percentage recovery is measured.

- **PRECISION:** Precision is the proximity of results which are obtained under identical conditions using the described method. It is expressed quantitatively in terms of repeatability, intra-day precision, and

intermediate precision, inter-day precision. The relative standard deviation is one of the most commonly used statistical measures for describing precision.

- **SPECIFICITY:** Specificity is the ability of the procedure to discern the target analyte in the presence of other components that include impurities or degradation products. The presence of this characteristic is usually determined by carrying out analyses on blank samples alongside containing known amounts of the analyte.

- **LINEARITY:** This is the ability of the method to produce results that are directly proportional to the concentration of the analyte over a given range. The analyte concentration can be plotted against the response obtained and then the correlation coefficient $R^2$ can be calculated by curving the calibration graph.

- **LOD and LOQ:** LOD is the very low concentration that the analyte can be detected reliably, while LOQ is the low concentration of the analyte which can be quantified with acceptable precision and accuracy. These limits are determined using statistical methods based upon the standard deviation of the response and slope of the calibration curve.

- **ROBUSTNESS:** The robustness describes the reliability of the procedure in different operational conditions, for example, pH, temperature, or mobile phase composition. A procedure has this characteristic studied by voluntary changing one or more method parameters and measuring eventual variations of the output.

- **STABILITY:** Stability studies ensure that the integrity of the analyte is maintained throughout different conditions of storage and for a long period. This would involve the stability of the analyte in the sample matrix and the stability of the prepared solutions.

### 1.4.1 Q2(R1) vs. Q2(R2) Comparison[12,14,16,17,18]

**Table 2: Q2(R1) vs. Q2(R2) Comparison.**

| Aspect | ICH Q2(R1) | ICH Q2(R2) |
|---|---|---|
| **Introduction Date** | October 27, 1994 | March 24, 2022 |
| **Scope** | Focused on validation of analytical procedures | Expanded to include multivariate procedures and non-linear methods |
| **Performance Characteristics** | Limited to traditional methods | Introduces new performance characteristics and validation tests, including multivariate calibration |
| **Accuracy and Precision** | Evaluated independently | Allows for combined evaluation of accuracy and precision, enhancing flexibility in validation approaches |
| **Working Range** | Defined as linearity | Replaced linearity with "Working Range" and includes suitability of calibration models |
| **Validation Lifecycle** | Basic validation lifecycle considerations | Emphasizes continuous validation throughout the lifecycle of analytical procedures |
| **Guidance on Statistical Methods** | Minimal statistical guidance | Enhanced guidance on statistical methods, including confidence intervals and risk-based assessments |
| **Alignment with ICH Q14** | Limited alignment with newer guidelines | Improved alignment with ICH Q14 on Analytical Procedure Development, promoting a comprehensive approach |
| **Validation Scope** | General guidelines | More detailed and specific |
| **Focus** | Method development | Method validation |
| **Robustness Testing** | Less emphasis | Greater emphasis |

## 1.5 APPLICATIONS OF HPLC

**HPLC is utilized across various sectors, including[13,15]**

- **Pharmaceutical Analysis:** Employed in drug purity testing, content uniformity, and stability studies.

- **Environmental Analysis:** Detects pesticides, pollutants, and other contaminants in environmental samples.

- **Food Industry:** Analyses food additives, contaminants, and nutritional content.

## 1.6 Challenges and Future Directions[14,15]

The field of HPLC faces several challenges and opportunities for advancement:

- **Emerging Technologies:** Innovations like UHPLC and micro-HPLC are improving separation performances and minimizing solvent consumption.

- **Challenges in Method Development:** Matrix effects, method transfers, and instrumental variability remain some of the key challenges.

- **Future Directions:** The integration of automation, miniaturization, and hyphenated techniques such as for example, HPLC-MS is expected to drive progress in the field.

## CONCLUSION

In developing the HPLC method, there is a step-by-step approach where careful optimization is carried out with a number of variables with a view to ensuring that results obtained from such tests are reliable. The future of HPLC in analytical science is bright, with new improvements in technologies and methodologies that will widen its application in many fields.

Throughout the review, HPLC method development and validation were discussed, with a focus on the most critical parameters impacting the accuracy, precision, sensitivity, and robustness of any given analytical method. The paper is supposed to direct the readers toward systematic method development and profound procedures for method validation to be followed so that reliable and reproducible results may be established for pharmaceutical quality control or environmental analysis. The development of UHPLC technology and coupling with MS took the analytical laboratories to a new horizon. However, issues regarding matrix effect, method transferability, and continuous updates of guidelines by regulatory authorities are issues yet to be understood. Further work is needed in the development of detectors offering higher sensitivity and selectivity, automation of method development, and techniques for the validation of methods that can be more universally adopted. Such efforts will go a long way in ensuring that the development of HPLC as one of the cornerstones of analytical chemistry goes ahead.

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