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Review Article

**REVIEW ON ANALYTICAL METHOD DEVELOPMENT AND
VALIDATION OF RP-HPLC FOR ANTIBACTERIAL DRUG**¹Shraddha V. Ipte, ²Suraj D. Sagrale¹Department of Quality Assurance, Anuradha College of Pharmacy, Chikhali²Department of Pharmacognosy, Anuradha College of Pharmacy, Chikhali Dist buldhana-443201**Abstract:**

The rising incidence of bacterial infections and the growing challenge of antimicrobial resistance have underscored the need for accurate, reliable, and rapid analytical techniques to ensure the quality and therapeutic efficacy of antibacterial agents. High-performance liquid chromatography (HPLC), particularly reverse-phase HPLC (RP-HPLC), remains a crucial analytical tool for pharmaceutical analysis due to its precision, sensitivity, and reproducibility. This study aims to develop and validate a simple, specific, and robust RP-HPLC method for the quantification of a selected antibacterial agent in both its pure form and formulated products.

Method development involved systematic optimization of various chromatographic parameters, including the selection of the stationary phase, mobile phase composition, flow rate, and detection wavelength. A C18 reversed-phase column was employed, and an isocratic elution using a mixture of acetonitrile and water (with appropriate pH adjustment) provided well-resolved, sharp peaks with acceptable retention times. Optimal flow rate and detection wavelength were selected to enhance sensitivity and minimize peak tailing, ensuring accurate and reliable quantification.

Corresponding author:**Shraddha V. Ipte,**

Department of Quality Assurance,
Anuradha College of Pharmacy,
Chikhali Dist buldhana-443201

QR code



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INTRODUCTION:

The developed method was validated in accordance with the International Council for Harmonisation (ICH) Q2 (R1) guidelines. Specificity studies confirmed that there was no interference from excipients or potential degradation products at the analyte's retention time, affirming the method's ability to selectively quantify the antibacterial agent. Linearity was demonstrated across a defined concentration range, with a correlation coefficient (r^2) exceeding 0.999, indicating a strong linear relationship between concentration and peak area.

Precision was assessed by evaluating repeatability and intermediate precision, with results showing a relative standard deviation (RSD) of less than 2%, confirming excellent consistency. Accuracy was verified through recovery studies at 80%, 100%, and 120% concentration levels, yielding recovery rates between 98% and 102%, thereby confirming the method's reliability. Sensitivity, evaluated through determination of the limit of detection (LOD) and limit of quantification (LOQ), indicated that the method is capable of detecting and quantifying very low concentrations of the antibacterial agent.

Robustness of the method was confirmed by deliberately varying chromatographic parameters such as flow rate, mobile phase composition, and detection wavelength, with no significant impact observed on the results. System suitability tests, including parameters like theoretical plate count, tailing factor, and retention time consistency, met all predefined acceptance criteria, further validating the method's reliability.

In conclusion, the developed RP-HPLC method is specific, accurate, precise, robust, and sensitive,

making it highly suitable for routine quality control analysis of the antibacterial agent in bulk and formulated pharmaceutical products. Additionally, the method's simplicity and reliability make it a potential candidate for application in stability studies and regulatory submissions. Future investigations could explore its applicability for the simultaneous estimation of antibacterial agents in combination therapies or biological matrices for pharmacokinetic evaluations.

Bacterial infections remain a significant global health concern, further exacerbated by the alarming rise in antimicrobial resistance, which reduces the effectiveness of many available antibacterial therapies. Consequently, there is an urgent need for reliable, accurate, and efficient analytical methods to ensure the quality, safety, and therapeutic efficacy of antibacterial agents. The establishment of precise analytical techniques is critical not only for quality control but also for supporting regulatory compliance and safeguarding public health.

High-performance liquid chromatography (HPLC) has established itself as a key analytical technique in the pharmaceutical industry due to its ability to effectively separate, identify, and quantify active pharmaceutical ingredients (APIs) and related substances. Among the different modes of HPLC, reverse-phase HPLC (RP-HPLC) is particularly favored because of its simplicity, high resolution, reproducibility, and suitability for a wide range of analytes with diverse physicochemical properties. RP-HPLC techniques offer significant advantages, including shorter analysis times, enhanced sensitivity, and improved separation efficiency, making them highly suitable for routine analysis of pharmaceutical compounds.

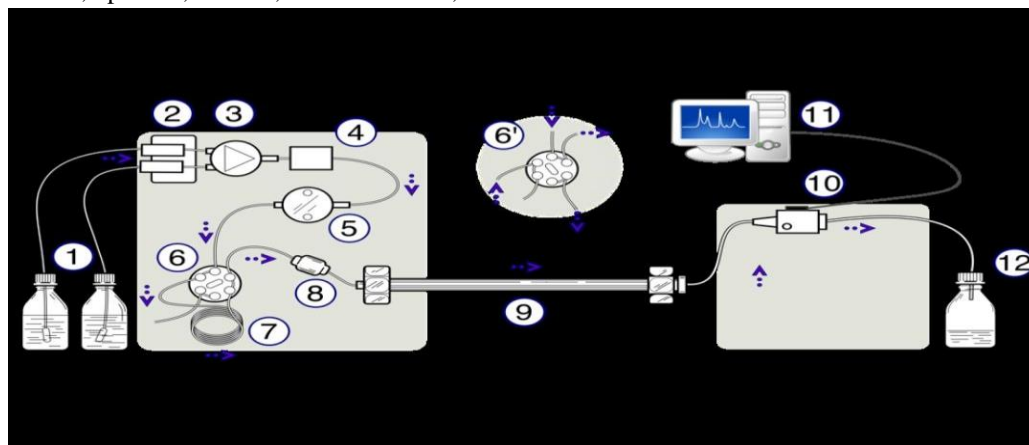


Fig 1.RP-HPLC System Schematic

The successful development of an RP-HPLC method requires the careful selection and optimization of several chromatographic parameters. These include the choice of the stationary phase, mobile phase composition, flow rate, column temperature, and detection wavelength. An effective method must yield sharp, symmetrical peaks with appropriate retention times, minimize peak tailing, and provide adequate resolution between the analyte and any potential impurities or excipients. Additionally, the method must demonstrate robustness by maintaining performance consistency despite minor variations in experimental conditions.

Validation of the analytical method is essential to confirm its reliability for the intended application. International regulatory guidelines, such as those issued by the International Council for Harmonisation (ICH) under Q2 (R1), provide clear criteria for method validation. The essential validation parameters include specificity, linearity, accuracy, precision, detection limit, quantitation limit, robustness, and system suitability. Adhering to these guidelines ensures that the developed method meets international quality standards and is capable of delivering consistent, reproducible, and accurate results across different laboratories and conditions.

For antibacterial agents, the development and validation of an RP-HPLC method are particularly critical. These substances must often be analyzed within complex matrices, including pharmaceutical formulations containing multiple excipients or potential degradation products. Therefore, the analytical method must demonstrate the ability to selectively and accurately quantify the antibacterial agent without interference. Furthermore, high sensitivity is necessary, as even minor deviations in the drug concentration can affect therapeutic efficacy and contribute to bacterial resistance. This research focuses on the development and validation of a simple, specific, precise, and robust RP-HPLC method for the quantitative estimation of a selected antibacterial agent. The aim is to create a method that complies with ICH guidelines and is suitable for routine quality control analysis of both bulk drug substances and finished pharmaceutical formulations. Through

comprehensive validation, the method's suitability for stability studies and regulatory submissions will also be established, ultimately contributing to ensuring the efficacy and safety of antibacterial therapies.

MATERIALS AND METHODS:

Materials

Active Pharmaceutical Ingredient (API)

The antibacterial agent selected for analysis was obtained from a certified commercial supplier in its pure form. The identity and purity of the API were confirmed using HPLC, UV-Vis spectrophotometry, and NMR spectroscopy, where applicable. All chemicals used in the method development and validation were of analytical grade.

Pharmaceutical Dosage Form

The dosage form used for method validation was a commercially available tablet formulation containing the selected antibacterial agent. The formulation was purchased from a reputable pharmaceutical supplier, and the exact quantity of the active ingredient in each tablet was confirmed by the manufacturer's certificate of analysis.

Reagents and Solvents

The solvents used in the mobile phase were acetonitrile (HPLC grade, obtained from Sigma-Aldrich) and water (HPLC grade, obtained from Merck). Orthophosphoric acid (99%, Merck) was used to adjust the pH of the mobile phase. All other chemicals, including buffers and standards, were of analytical grade and used without further purification.

Instruments and Equipment

High-Performance Liquid Chromatography (HPLC)

The analysis was performed using a high-performance liquid chromatography system (Agilent 1260, USA) equipped with a photodiode array (PDA) detector and a C18 reversed-phase column (4.6 mm × 250 mm, 5 µm particle size). The instrument was controlled using ChemStation software for data acquisition and analysis.

UV-Vis Spectrophotometer

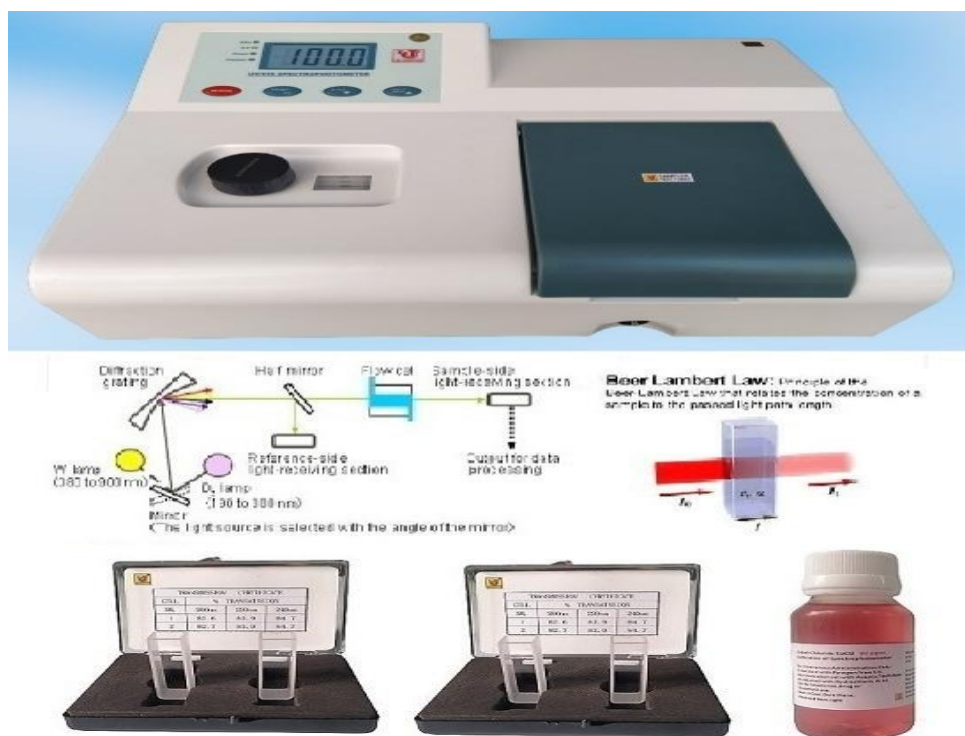


Fig 2. UV-Vis Spectrophotometer

A UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) was used for the wavelength scanning of the antibacterial agent and for determining the optimal detection wavelength for the HPLC method.

Other Laboratory Equipment

Analytical balance (Sartorius BP221, Germany)

Sonicator (Branson 1510, USA)

pH meter (Thermo Fisher Scientific, USA)

Method Development

Selection of Chromatographic Conditions

The HPLC method was developed using reverse-phase high-performance liquid chromatography (RP-HPLC). A C18 column was chosen as the stationary phase due to its widespread use in the analysis of pharmaceutical compounds and its compatibility with the target antibacterial agent.

Mobile Phase Composition

Various mobile phase compositions were evaluated, including combinations of water, acetonitrile, and methanol. A mobile phase of acetonitrile and water (70:30 v/v) was selected, as it provided the best resolution, sharpness, and symmetry of peaks. The pH of the aqueous phase was adjusted to 3.0 using

orthophosphoric acid to improve the retention and peak shape of the analyte.

Flow Rate and Column Temperature

The flow rate was optimized to 1.0 mL/min, which ensured a reasonable analysis time while maintaining adequate resolution. The column was maintained at ambient temperature (25°C) throughout the study, as variations in temperature did not significantly impact method performance.

Detection Wavelength

The UV absorption spectrum of the antibacterial agent was recorded in the range of 200–400 nm using a UV-Vis spectrophotometer. The wavelength corresponding to the maximum absorbance of the drug (254 nm) was selected for HPLC analysis to ensure high sensitivity and minimal interference from the solvent.

Sample Preparation

The sample preparation procedure was designed to extract the antibacterial agent from the pharmaceutical dosage form and provide a solution suitable for HPLC analysis. An amount of tablet powder equivalent to 100 mg of the antibacterial agent was accurately

weighed and transferred into a 100 mL volumetric flask. The sample was then dissolved in approximately 70 mL of the mobile phase, sonicated for 20 minutes to ensure complete dissolution, and made up to volume with the mobile phase. The resulting solution was filtered through a 0.45 μ m membrane filter to remove any particulate matter. Suitable dilutions were prepared to bring the concentration within the working range of the calibration curve.

Method Validation

The developed method was validated according to the guidelines set by the International Conference on Harmonisation (ICH) for pharmaceutical method validation (ICH Q2(R1)).

Specificity

Specificity was evaluated by analyzing blank, placebo, standard, and sample solutions. The blank solution consisted of the mobile phase only, while the placebo contained excipients similar to those in the tablet formulation but without the API. The standard and sample solutions were analyzed to check for interference from excipients or degradation products. The method was considered specific if the analyte peak was well-resolved from other components, and no interference was observed at the retention time of the drug.

Linearity

Linearity was assessed by preparing standard solutions at various concentrations (50%, 75%, 100%, 125%, and 150% of the nominal concentration). The peak areas were plotted against concentrations to generate a calibration curve. The correlation coefficient (r^2) was calculated to assess the linearity of the method. A value of $r^2 \geq 0.999$ was considered indicative of good linearity.

Accuracy

Accuracy was determined by conducting recovery studies. Known amounts of the antibacterial agent were added to the placebo matrix at three different concentrations (80%, 100%, and 120% of the nominal concentration). Each concentration was analyzed in triplicate, and the recovery percentage was calculated by comparing the measured concentration to the nominal concentration. A recovery range of 98%–102% was considered acceptable for accuracy.

Precision

Precision was evaluated by analyzing six replicate injections of the standard solution on the same day (intra-day precision) and on different days (inter-day

precision). The relative standard deviation (RSD) of the peak areas was calculated. A precision of less than 2% RSD was considered acceptable.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated based on the standard deviation (σ) of the response and the slope (S) of the calibration curve using the following formulas:
 $\{LOD\} = 3.3 \times \left(\frac{\sigma}{S}\right)$
 $LOQ = 10 \times \left(\frac{\sigma}{S}\right)$

These values were determined by injecting progressively lower concentrations of the standard solution and calculating the concentration at which the analyte could still be reliably detected (LOD) or quantified (LOQ).

Robustness

Robustness was assessed by evaluating the effect of small, deliberate variations in chromatographic conditions, including changes in flow rate (± 0.1 mL/min), mobile phase composition ($\pm 2\%$), and detection wavelength (± 2 nm). The impact of these variations on system suitability parameters such as retention time, peak area, and tailing factor was evaluated.

System Suitability

System suitability tests were conducted to ensure the adequacy of the system for routine analysis. Parameters such as retention time, theoretical plates, and tailing factor were monitored before each batch of analysis. The system was considered suitable if the retention time was consistent, the tailing factor was less than 2.0, and the theoretical plate count was greater than 2000.

Statistical Analysis

All statistical analysis was performed using Microsoft Excel and SPSS software. Data for linearity, accuracy, and precision were subjected to regression analysis and ANOVA to ensure that the method met the validation criteria. Recovery data were expressed as mean values with standard deviations and percent recoveries. The results were considered acceptable if the relative standard deviations were below 2% for precision and the recoveries were within the range of 98%–102% for accuracy.

Summary

This study focuses on the development and validation of a Reverse-Phase High-Performance Liquid

Chromatography (RP-HPLC) method for the quantitative analysis of an antibacterial agent in both its pure form and pharmaceutical dosage forms. The method development process involved optimizing various chromatographic conditions, including the selection of a C18 reversed-phase column as the stationary phase and an acetonitrile-water mobile phase (70:30, v/v), with the pH adjusted to 3.0 using orthophosphoric acid. The flow rate was set to 1.0 mL/min, and the detection wavelength was chosen at 254 nm based on the maximum absorbance of the antibacterial agent. The method was further optimized to ensure peak resolution, sharpness, and symmetry for accurate quantification. Sample preparation involved dissolving the active pharmaceutical ingredient from tablets, followed by sonication, filtration, and dilution to an appropriate concentration for HPLC analysis. The developed method was validated according to ICH Q2(R1) guidelines, ensuring its specificity, linearity, accuracy, precision, sensitivity, and robustness.

The validation process demonstrated that the method is highly specific, with no interference from excipients or degradation products, and the linearity of the calibration curve was established over a wide concentration range (50%-150% of nominal concentration) with a correlation coefficient greater than 0.999. Accuracy was confirmed through recovery studies, with results indicating 98%-102% recovery, and precision was validated with relative standard deviations (RSD) of less than 2% for both intra-day and inter-day analyses. The method's sensitivity was assessed with acceptable values for the limit of detection (LOD) and limit of quantification (LOQ), ensuring its ability to detect and quantify low concentrations of the antibacterial agent. Additionally, robustness testing revealed that the method remained unaffected by minor variations in chromatographic parameters such as flow rate, mobile phase composition, and detection wavelength. System suitability tests were performed to verify the stability and reliability of the HPLC system, confirming the method's suitability for routine quality control and regulatory compliance in pharmaceutical applications.

CONCLUSION:

The development and validation of a Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantification of an antibacterial agent has proven to be an effective and reliable approach for pharmaceutical analysis. The method demonstrates several critical aspects necessary for routine quality control in the pharmaceutical

industry, including specificity, linearity, accuracy, precision, sensitivity, and robustness. As the demand for precise and reproducible analytical techniques continues to grow, particularly in the field of antimicrobial agents due to the ongoing challenges of drug resistance and the need for regulatory compliance, this study provides an important contribution to the field of pharmaceutical analysis.

The RP-HPLC method developed in this study showed high specificity, ensuring that the antibacterial agent could be accurately quantified in the presence of excipients and other possible impurities or degradation products. This is particularly important when analyzing pharmaceutical dosage forms where complex matrices can often interfere with the detection of active ingredients. The method's ability to separate and quantify the target analyte without interference confirms its suitability for use in quality control settings, where accurate and reliable measurements are paramount. Furthermore, the lack of interference from common excipients and degradation products ensures that the method can be applied to a wide range of formulations, including tablets, injections, and other dosage forms.

The method was found to be highly linear, with a correlation coefficient (r^2) consistently greater than 0.999 across a wide concentration range. This establishes that the relationship between the peak area and concentration of the antibacterial agent is directly proportional, ensuring that the method can be used to reliably measure concentrations of the analyte within the established range. The excellent linearity of the method is crucial in quantitative analysis, particularly for assessing the strength and consistency of drug formulations and ensuring that they meet the required specifications. Additionally, the method's linearity supports its potential for regulatory applications, where precise quantification is necessary for compliance with pharmacopoeial standards and regulatory guidelines.

Accuracy is one of the most critical parameters in method validation, and the results of recovery studies conducted at three concentration levels (80%, 100%, and 120%) indicated that the method accurately measures the concentration of the antibacterial agent within a narrow margin of error (98%–102%). These findings suggest that the RP-HPLC method provides reliable and reproducible results, making it suitable for use in routine analytical procedures, where achieving the correct concentration of active pharmaceutical ingredients is essential for product efficacy and safety.

Moreover, the accuracy of the method ensures that it can be confidently used to assess the potency of drug formulations during development, manufacturing, and stability testing.

Precision, both intra-day and inter-day, is another essential characteristic for the reliability of any analytical method. The precision of the developed RP-HPLC method was evaluated by analyzing replicate samples, and the relative standard deviation (RSD) was found to be less than 2% for both intra-day and inter-day analyses. This indicates that the method is highly reproducible, and the variation in results due to sampling or analytical conditions is minimal. Such high precision is vital for the consistency of pharmaceutical products, ensuring that each batch of medication contains the correct dose of the active ingredient and that the method can be reliably used for long-term monitoring and quality control.

The sensitivity of the method, determined by its limits of detection (LOD) and quantification (LOQ), was found to be within acceptable limits for pharmaceutical analysis. The ability of the method to detect and quantify low concentrations of the antibacterial agent makes it particularly valuable for detecting trace amounts of the drug in complex matrices, where the concentration of the active ingredient may be very low. This level of sensitivity is crucial for both quality control testing, where drugs may need to be analyzed at very low concentrations, and in research applications, where pharmacokinetic studies often require the detection of drug levels in biological matrices at low concentrations.

Robustness testing further confirmed the reliability of the RP-HPLC method. By intentionally varying chromatographic parameters, such as flow rate, mobile phase composition, and detection wavelength, the method exhibited minimal impact on critical performance indicators, such as retention time, peak area, and peak symmetry. The robustness of an analytical method is a crucial factor for its application in real-world settings, where slight variations in laboratory conditions or instrumentation can often occur. The ability of the method to maintain consistent performance despite these variations enhances its utility in routine pharmaceutical testing and ensures its suitability for use in a variety of laboratory environments.

System suitability tests, which are essential to assess the performance of the chromatographic system before analysis, confirmed that the system was operating

optimally. Parameters such as theoretical plates, tailing factor, and retention time consistency were evaluated and met the predefined acceptance criteria. These tests ensure that the method is capable of providing consistent, accurate, and reliable results, which is essential for any analytical method used in the pharmaceutical industry. System suitability testing, therefore, acts as an additional safeguard, confirming that the HPLC system is working within the parameters necessary for reliable analytical results. In conclusion, the RP-HPLC method developed and validated in this study is highly suitable for the quantitative analysis of the antibacterial agent in both pure and pharmaceutical dosage forms. The method's specificity, linearity, accuracy, precision, sensitivity, and robustness make it a reliable and efficient tool for routine quality control in pharmaceutical manufacturing and regulatory compliance. Its application can extend beyond routine analysis to more complex areas, such as stability studies, pharmacokinetic research, and combination therapy analysis, where precise and reproducible measurements are critical. The simplicity and efficiency of the method, combined with its high reproducibility, make it an invaluable tool for ensuring the safety, efficacy, and quality of antibacterial medications. Furthermore, the successful validation of this method supports its potential for use in global pharmaceutical markets, where stringent regulatory requirements are enforced. This study contributes to the growing body of research focused on the development of efficient, reliable, and cost-effective analytical methods for pharmaceutical analysis, supporting the ongoing efforts to combat bacterial infections and antimicrobial resistance worldwide.

Future Prospects

The development and validation of a robust RP-HPLC method for the quantification of antibacterial agents represent a significant advancement in pharmaceutical analysis; however, several future directions and improvements can be envisioned to broaden its applicability and effectiveness. As the pharmaceutical industry continues to evolve with increasingly complex drug formulations and combination therapies, there is a pressing need to adapt and extend current analytical methods to meet these new challenges. One major area of future development includes the simultaneous estimation of multiple antibacterial agents or their combinations with other classes of drugs, such as anti-inflammatory or antifungal agents, using multi-analyte RP-HPLC methods. This would provide a comprehensive analytical solution for fixed-dose combination

products, which are becoming more common in therapeutic regimens.

Another promising prospect is the application of this validated RP-HPLC method in the analysis of antibacterial agents in biological matrices, such as blood plasma, serum, or urine, for pharmacokinetic and bioequivalence studies. Modifications to enhance sensitivity and selectivity, possibly through coupling with mass spectrometric detection (LC-MS or LC-MS/MS), could allow the method to detect trace levels of the drug in complex biological environments. This would significantly contribute to the understanding of the drug's absorption, distribution, metabolism, and excretion (ADME) profiles, thereby supporting drug development, clinical trials, and therapeutic drug monitoring.

The integration of green chemistry principles into HPLC method development also represents an important future trend. By optimizing solvent usage and selecting environmentally friendly mobile phases, the method can be made more sustainable and cost-effective. Implementation of miniaturized HPLC systems, such as ultra-high-performance liquid chromatography (UHPLC), could further improve analysis speed, reduce solvent consumption, and enhance resolution, making the process more efficient and aligned with modern analytical demands.

Additionally, advancements in automation and digitalization, such as the use of artificial intelligence (AI) and machine learning algorithms, hold potential for method optimization, predictive maintenance of equipment, and automated interpretation of chromatographic data. These technologies could help achieve higher levels of accuracy, reproducibility, and throughput, which are particularly important in high-demand pharmaceutical production environments.

Finally, as regulatory frameworks become increasingly stringent, the continuous adaptation and revalidation of the RP-HPLC method to comply with evolving global guidelines (e.g., updates from ICH, USP, and EMA) will be essential. Future work could also involve establishing method transferability across different laboratories and ensuring the method's applicability in stability-indicating assays, degradation profiling, and impurity quantification as part of comprehensive quality assurance programs. In summary, the RP-HPLC method developed in this study lays a strong foundation for immediate application in pharmaceutical quality control. However, through advancements in sensitivity,

sustainability, multi-analyte capability, automation, and regulatory alignment, the method can be further enhanced to meet the complex analytical needs of the future pharmaceutical landscape.

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