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Development and method validation of RP-HPLC Method- A Review

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Abstract:
The development of RP HPLC methods is not without difficulties, despite its many advantages. These difficulties include problems with column stability, sample preparation, and optimization. This article describes new techniques that can be used with the RP-HPLC process to design and produce novel pharmaceuticals. Additionally covered are instances of more current developments in hyphenated methods like RP-HPLC. We intend to stimulate ongoing innovation and advancement in the developing field of pharmacy by examining the most recent developments, applications, and difficulties in the creation of RP-HPLC methods.

Keywords: RP-HPLC, LC-MS and API (Active Pharmaceutical Ingredient)

INTRODUCTION:

An essential phase in analytical chemistry is the development of RP-HPLC methods, which entails optimizing a number of parameters to separate and quantify analytes in complicated mixtures as required. Reverse Phase High-Performance Liquid Chromatography, or RP-HPLC, is a prominent chromatographic method for separating polar and non-polar substances according to how hydrophobic they are.[1,2] In pharmaceutical analysis, it is challenging to separate and quantify impurities and APIs without RP-HPLC. It is necessary to develop reliable and accurate RP-HPLC procedures in order to evaluate the drug's efficacy, safety, and quality. Optimizing the separation conditions, selecting the chromatographic parameters, and deciding on the phases that are stationary and mobile are crucial steps in developing analytical procedures utilizing RP-HPLC[3]. Because RP-HPLC can distinguish and measure contaminants from active pharmaceutical ingredients (APIs), the pharmaceutical industry has found it to be essential. Recent advancements in both the stationary and mobile phases have increased the efficiency and selectivity of RP-HPLC. Thanks to advancements in miniaturization, RP-HPLC can now analyze smaller sample amounts faster and at a lower cost. The development of hybrid techniques such as LC-MS has resulted in improved RP-HPLC analytical sensitivity and selectivity [4]. In conclusion, since RP-HPLC is a critical analytical tool in the pharmaceutical industry, developing accurate and dependable RP-HPLC methods is essential to evaluating drugs. Recent advancements in RP-HPLC's efficacy, sensitivity, and selectivity have increased its usefulness as a tool for the analysis of chemical substances in an array of applications[5].The development of new stationary and mobile phases, hyphenated techniques such as LC-MS, and the shrinking of RP-HPLC are several instances of recent developments in the field. Enhanced selectivity and efficiency in newly developed stationary and mobile phases may enable more accurate separation and analysis of complicated chemicals[6]. Smaller sample volumes can be analyzed by miniature RP-HPLC, cutting down on run times and solvent usage. Hybrid approaches offer more sensitive and precise analyte detection by fusing the benefits of mass spectrometry with RP-HPLC.[7]

RECENT ADVANCES IN DEVELOPMENT OF RP-HPLC METHOD

Reverse-phase high-performance liquid chromatography (RP-HPLC) has long been a workhorse for analytical chemists, separating and analyzing complex mixtures. But the world of RP-HPLC isn't stagnant. Recent advances are making this technique even more powerful. Let's delve into the exciting developmentts that are pushing the boundaries of RP-HPLC method development

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Quality-by-design (QbD) approach with Green Chemistry Principles:

QbD is an efficient technique for developing procedures that concentrate on identifying and controlling the causes of variability so as to ensure accurate and superior results. As a reliable procedure, this technology is becoming more and more popular in the progression of RP-HPLC methods.[8] No approach combines RP-HPLC with green Analytical Quality by Design (AQbD) for long-term use to determine BEN (benidipine hydrochloride) and CHD (chlorthalidone), according to the literature. In order to determine BEN and CHD, this study attempted to create a green RP-HPLC by combining analytical quality by design with green chemistry principles. For optimization, a central composite design has been used with the flow rate and 40% ethanol concentration selected as key factors. With a mobile phase of ethanol and potassium dihydrogen orthophosphate (orthophosphoric acid to 3.5) in a ratio of 40:60 v/v at 1 ml/min and a detection wavelength of 230 nm, separation was accomplished using an Agilent Eclipse Plus (C18, 250 mm × 4.6 mm i.d, 5 ?m). The durations of retention for BEN and CHD were 5.1 and 3.1 minutes, respectively. BEN and CHD had concentration ranges of 3.2–4.8 ?g/ml and 5.0–7.5 ?g/ml, respectively. The suggested approach was friendly to the environment and analyzed with green instruments for assessment. Therefore, regular BEN and CHD analysis in pharmaceutical formulations is provided by AQBD and green technologies without influencing the environment. Method variables can be seen through the use of AQbD, leading to reliable and stable procedures that can be effectively used in quality control labs without the need for additional revalidation. A central composite design or other experimental design has been used for the statistical optimization studies. The obtained results determined the drug combination's ideal operating conditions inside the design space region, and these were realistically confirmed by additional runs. Ethanol was utilized as an organic solvent as opposed to a potentially hazardous solvent, in accordance with the GAC principles. Ultimately, the results of the green assessment instruments showed that the process was highly ecologically sustainable and easily adaptable to business and regular monitoring of quality.[9] Another study uses the Analytical Quality by Design (AQbD) technique to build and validate a reverse-phase high performance liquid chromatography method for quick and accurate CDL analysis. Supplies and Procedures: Predefined Analytical Target Attributes (ATPs) served as the basis for the selection of Critical Analytical Attributes (CAAs). Taguchi Design conducted additional screening of Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs) that significantly affected the assigned CAAs. 0.1% orthophosphoric acid (OPA) and methanol in a 35:65% v/v ratio make up the optimum mobile phase. The analysis was done at 240 nm, the column temperature was kept at 30ºC, and the flow rate was kept at 1 mL/min. By selecting the 32-Box-Behnken design, the CAAs were fine-tuned in light of the results. Findings: Within the examined range of 5–30 µg/mL, the area under the curve was determined to be linear (R2 = 0.999). It was discovered that the retention period was 4.18 minutes, with a distinct, abrupt peak. It was discovered that the drug's percentage recovery during the accuracy study fell between 97.06 and 99.29%. It was discovered that the quantification and detection limits were 1.86 µg/mL and 0.61 µg/mL, respectively. Conclusion: An RSD of less than 1% indicated the developed method's precision.[10]

RP-HPLC With Hyphenated Techniques :

Bhole Ritesh et al. The stability-indicating RP-HPLC method for accurately quantifying Dexlansoprazole in bulk materials was successfully developed and verified by this work. The method's special chromatographic conditions included an Acetonitrile and 0.5 mmol Ammonium Acetate (pH 4.5) gradient mobile phase, a 1 ml/min flow rate, and detection at 283 nm. There was also a Kromasil C18 column in operation. With a significant correlation value of 0.997, the approach demonstrated a linear range of 5–30 mg/ml for Dexlansoprazole, and the retention duration was 5.14 minutes. With a limit of quantification (LOQ) of 3.64 mg/ml and a limit of detection (LOD) of 1.2 mg/ml, the method demonstrated excellent sensitivity. The percentage recovery of Dexlansoprazole, which ranged from 98.6% to 102%, demonstrated the accuracy of the method. The developed method was validated according to the guidelines provided by the International Council for Harmonization (ICH), which were adhered to. It addressed a number of variables, including robustness, accuracy, precision, linearity, LOD, LOQ, and solution stability. The method proved stable and capable of effectively separating the degradation products from the analyte peaks, proving its usefulness as a method for stability indication. The structures of the degradants were also established using LC-MS/MS spectra.With the use of this well-established RP-HPLC method, Dexlansoprazole in bulk form can be quantitatively measured.

DXL was shown to be susceptible to both acidic and oxidative degradation when its degradation behavior was examined under different conditions. Nonetheless, the drug held up well in thermal, photolytic, and alkaline environments. Using the MS spectra, it was feasible to determine probable structures and chemical formulae after

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oxidative stress materials were subjected to LC-MS analysis. The established stability-indicating RPHPLC method was confirmed by ICH Q2 guidelines. The standards were met by the validation parameters in terms of precision, accuracy, specificity, and robustness.[11] Another review of the literature revealed that the RP-HPLC-UV technique, which was created and validated for the assessment of related chemicals (p-chlorophenol and unknown impurities) of the skeletal muscle relaxant chlorzoxazone, was straightforward, dependable, and stability-indicating. A separate RP-HPLC-mass spectrometry (MS)/MS method for the estimation of 2-amino-4-chlorophenol impurity has also been developed and validated, as the molecule contains a potentially genotoxic impurity that requires more sensitivity in quantification. The International Council for Harmonization Q2(R1) was followed in the validation of both methods (RP-HPLC-UV & RP-HPLC–MS/MS). The methods were found to be robust, linear, specific, precise, accurate, and robust with limit of quantification values established with respect to 100% of test concentration, 0.018% w/w of p-chlorophenol by RP-HPLC-UV, and 2 ppm of 2-amino-4-chlorophenol by RP-HPLC–MS.[12]

UHPLC:

A more recent method called ultra-high performance liquid chromatography (UHPLC) employs greater pressures and smaller particle sizes than conventional HPLC. Higher sensitivity, faster analysis times, and higher resolution are the outcomes of this. [13] An additional investigation was conducted in 2023.This study's primary goal was to ascertain the degree of pesticide contamination present in samples of frequently consumed fruits and vegetables that were gathered from three distinct Gujarat state, India, locations. The modified QuEChERS method was utilized to gather and extract samples (n = 312), and the presence of residues from 52 pesticides (organochlorine, organophosphate, carbamate, and synthetic pyrethroids) was measured using GC-MS/MS and UHPLC-QTOF/MS. Twelve of the 52 pesticides had residues found, with Bifenthrin, Fenvalerate, and Cypermethrin being found in the majority of samples. According to the findings, the Maximum Residue Limit (MRL) was not reached by 98.8% of tomato, 97.5 % of bananas, 90% of eggplant, 88.8% of pomegranates, 83.8% of oranges, 75% of okra, or 66.3% of green chili samples. Based on the consumption of vegetable and fruit samples, an acute and chronic dietary risk assessment was computed for the Indian adolescent and adult population. The findings indicated that most samples had HQ (Hazard Quotient) and HI (Hazard Index) values less than 1. The commodities under investigation were deemed safe for human consumption based on the overall results. To safeguard customers, monitoring studies might be conducted, though.[14] All things considered, the demand for more sensitive, effective, and environmentally friendly procedures that can manage complex combinations is what has driven these latest developments in RP HPLC method development. To remain at the forefront of analytical chemistry, researchers and practitioners in the field should be aware of these changes and modify their approaches accordingly.

APPLICATIONS:

- **Development of the RP HPLC Method:** Applications RP HPLC is a popular analytical method with many uses across several sectors. Here are a few instances:

- **Pharmaceutical industry:** RP HPLC is a crucial instrument for the development and discovery of new drugs. Active pharmaceutical ingredients (APIs) in formulations are quantified, contaminants are identified, and drug purity analysis is performed using it.

- **Food and beverage industry:** Food additives, preservatives, and pollutants in food and beverages are analyzed using RP HPLC. Nutritional components like vitamins and amino acids can also be determined using it.

- **Environmental analysis:** Pollutants include industrial chemicals, insecticides, and herbicides are analyzed in environmental samples using RP HPLC. The analysis of organic compounds in soil and water samples is another application for it.

- **In forensic science,** drugs of abuse are analyzed using RP HPLC in biological samples including blood and urine. The analysis of hazardous chemicals in post-mortem samples is another application for it.

- **Biotechnology industry:** Products such as recombinant proteins and monoclonal antibodies are analyzed for proteins, peptides, and nucleic acids using RP HPLC. All things considered, RP HPLC's sensitivity and adaptability make it a useful instrument for a variety of uses. It is now a routine procedure in many sectors due to its exceptional precision and accuracy in separating and quantifying complicated combinations of substances.[15,16] Common difficulties in developing RP HPLC methods Even though RP HPLC methods are widely used and

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effective, developing new methods is not without its difficulties. The following are some typical difficulties that practitioners and researchers could run into:

- **Selectivity of stationary phases:** Choosing the best stationary phase for a given sample can be difficult, particularly when working with complicated mixes. This problem can be solved with the use of mixed-mode or alternate selectivity columns.

- **Parameter optimization:** It might take a lot of time and trial and error to optimize an RP HPLC method's parameters, such as column temperature, flow rate, and mobile phase composition. Automation and intelligent software can assist speed up this process.

- **Interference caused by sample matrices:** Sample matrices can cause interference that lowers sensitivity and selectivity during separation and detection. Techniques for preparing samples, including solid-phase extraction, can aid in removing matrix influence.

- **Column degradation:** A variety of factors, including sample matrix effects and column overload, can cause columns to deteriorate with time. This problem can be mitigated with regular column replacement and maintenance.

- Regarding potential future paths, it is anticipated that the following domains will witness sustained advancement and enhancement in the development of RP HPLC methods:

- **Column technology:** It is anticipated that the use and development of novel column technologies, such as coreshell particles and monolithic columns, would expand.

- **Design of stationary phases:** In an effort to increase efficiency and selectivity, researchers are always investigating novel stationary phase chemistries and designs.

- **Automation and artificial intelligence:** With the potential for more effective and efficient optimization techniques, the application of automation and AI in the development of RP HPLC methods is anticipated to grow.

- **Miniaturization:** RP HPLC systems that are smaller, like microfluidic chips, may operate more quickly, sensitively, and portablely.[17,18]

- **New Obstacles in the Development of RP HPLC Methods Scholars and practitioners should be aware of the following new issues with RP HPLC technique development:**

- **Analysis of large biomolecules:** RP HPLC is frequently used to analyze tiny compounds, however because of their size, complexity, and hydrophobicity, large biomolecules like proteins and peptides can be difficult to analyze using this method. To meet these problems, new sample preparation methods and column technologies like size exclusion chromatography and protein digestion are being developed.

- **Analysis of chiral compounds:** In many industries, such as medicines, agrochemicals, tastes, and fragrances, the separation of chiral compounds—molecules that exist in two or more mirror-image forms—is essential. Although chiral separations can be achieved with RP HPLC, chiral stationary phases or derivatization procedures are frequently needed, which can be expensive and time-consuming.

- **Analysis of polar chemicals:** Because highly polar molecules like carbohydrates and organic acids have limited retention on RP columns, RP HPLC is not a good method for analyzing them. To overcome this difficulty, substitute techniques such hydrophilic interaction chromatography (HILIC) are being developed.

- **Analysis of trace contaminants:** Although RP HPLC is frequently used to analyze impurities in medications and other items, its sensitivity to small amounts of impurities may be restricted. Improved sensitivity and selectivity for trace contaminants can be achieved by the application of high-resolution mass spectrometry (HRMS) and other cutting-edge detection techniques.[19,20]

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Table 1: Recent Advances In RP-HPLC Method Development [21]

Sr.No.	Technique	Overview	Benefits
1	Ultra-high performance liquid chromatography (UHPLC)	Utilizes columns packed with smaller particles (typically < 2 μm) and higher pressures (up to 1000 bar) for faster separations and higher resolution	Improved speed, resolution, and sensitivity
2	Monolithic columns	Consist of a single piece of porous material, providing higher flow rates and faster separations	Improved speed and resolution, reduced backpressure
3	Stationary phase coatings	Modify the surface of the column to provide improved selectivity and/or reduced non-specific adsorption	Improved selectivity and sensitivity
4	2D-LC	Combines two complementary separation modes (e.g., size	Improved resolution and selectivity for complex samples
		exclusion and RP) to provide higher resolution and selectivity	selectivity for complex samples

Table 2: Applications Of RP-HPLC Method Development In Various Industries [22]

Sr.No.	Industry	Applications
1	Pharmaceutical	Drug development and quality control, impurity analysis, pharmacokinetic studies
2	Food and beverage	Analysis of additives, contaminants, and nutritional components
3	Environmental	Analysis of pollutants, toxins, and metabolites in air, water, and soil
4	Forensic	Analysis of drugs, toxins, and metabolites in biological samples
5	Biotechnology	Analysis of proteins, peptides, and nucleic acids in research and development

CONCLUSION:

All things considered, the evolution of the RP HPLC method will remain crucial to contemporary analytical chemistry, with new advancements and modifications tackling current issues and broadening its scope of use. To sum up, this review has covered current developments, uses, and difficulties in the creation of RP HPLC methods. One of the review's main conclusions is the potential advantages of applying cutting-edge technology like 2D- and UHPLC. Furthermore, we emphasized the wide range of industries in which RP HPLC finds use, including biotechnology, environmental analysis, food and beverage, pharmaceutical, and forensic research. These results have important ramifications for the field of analytical chemistry. More precise, sensitive, and selective tests will be possible with the ongoing development and improvement of RP HPLC techniques, which will enhance both product quality and safety and environmental protection. Moreover, the incorporation of artificial intelligence and machine learning in the process of developing RP HPLC methods holds significant promise for augmenting their velocity and efficacy. In summary, this study underscores the significance of sustained innovation and advancement in the development of RP HPLC methods, and stresses the necessity of interdisciplinary cooperation between scholars and practitioners in both academia and industry to propel the field forward. Through tackling the obstacles and investigating the latest developments in RP HPLC technique development, we may unlock its potential as a potent analytical instrument for contemporary analytical chemistry.

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REFERENCES

1. Snyder, L. R., Kirkland, J. J., & Dolan, J. W. Introduction to modern liquid chromatography. John Wiley & Sons.2010
2. Ahuja, S. Handbook of modern pharmaceutical analysis. Academic Press.2011
3. H Mustafa. "Method Development and Validation for the Simultaneous Estimation of Ambrisentan in Tablet Dosage form by HPLC." Tamil Nadu Dr. M.G.R. Medical University, 2016.
4. S Orlandini, B Pasquini, C Caprini, M Del Bubba, et al. "Enantioseparation and impurity determination of ambrisentan using cyclodextrin-modified micellar electrokinetic chromatography: Visualizing the design space within quality by design approach." Journal of Chromatography A, vol. 1438, 2016, pp. 75-84. doi: 10.1016/j.chroma.2016.01.039.
5. VSK Yelamanchi. "Modified and residual determination method development and validations for active pharmaceuticals ingredient by RP HPLC." KL University, 2017.
6. Kirthi, A., Shanmugam, R., Prathyusha, M. S., & Kumar, S. S. (2014). A review on bioanalytical method development and validation by RP-HPLC. Journal of global trends in pharmaceutical sciences, 5(4), 2028- 2037.
7. Pyrzynska, K., & Sentkowska, A. (2015). Recent developments in the HPLC separation of phenolic fod compounds. Critical Reviews in Analytical Chemistry, 45(1), 41-51.
8. Ahmad S, Ganbase M, Deshmukh N, Patil K, Barde L, Deshpande M, Tajane P .Development of validated HPTLC and UHPLC methods for quantification of withanolide in extract and formulation. International Journal of Experimental Research and Reviews 30: 375- 387.https://doi.org/10.52756/ijerr.2023.v30.035(2023)
9. Naveenarani Dharuman, Karunanidhi Santhana Lakshmi & Manikandan Krishnan Environmental benign RP-HPLC method for the simultaneous estimation of anti-hypertensive drugs using analytical quality by design, Green Chemistry Letters and Reviews, 16:1, 2214176, DOI: 10.1080/17518253.2023.2214176(2023)
10. Randive KH, Maste MM, Kempwade AA. Exploring Analytical Quality by Design (AQbD) Enabled RP-HPLC Method for Carvedilol. Indian J of Pharmaceutical Education and Research. 2024;58(2):651-60.
11. Bhole, Ritesh P.; Bonde, Chandrakant G.; Girase, Ghansham; and Gurav, Shaliendra (2024) "Stability Indicating Validated Novel RP-HPLC Method for Dexlansoprazole and LC-MS/MS Study of Degradation Product," Palestinian Medical and Pharmaceutical Journal: Vol. 9 : Iss. 1 , Article 9. Available at: https://doi.org/10.59049/2790-0231.1128(2024)
12. Bhujanga Rao Nagulancha, Vandavasi Koteswara Rao; Stability-indicating method for quantification of potential genotoxic impurity and other organic impurities of chlorzoxazone using hyphenated techniques.SSC Plus Journal. 02 November 2023 https://doi.org/10.1002/sscp.202300138
13. Ankush Bhalerao, Mr.Satish Shelke, Dr. Vijay Borkar; Advances, Applications, and Challenges in RP HPLC Method Development. International Journal of Advanced Research in Science, Communication and Technology (IJARSCT). DOI: 10.48175/IJARSCT-9017; Volume 3, Issue 1, April 2023.
14. Divya Kottadiyil, Tejal Mehta, Rupal Thasale, Sivaperumal. P; Determination and dietary risk assessment of 52 pesticide residues in vegetable and fruit samples by GC-MS/MS and UHPLC-QTOF/MS from Gujarat, India. Journal of Food Composition and Analysis Volume 115, January 2023, 104957
15. Huang, Y., Yang, S., Zhang, H., Yu, Y., Huang, X., & Liu, Z. (2021). RP-HPLC method development and validation for the simultaneous determination of five major bioactive components in Huang-Lian-Jie-DuTang. Journal of Separation Science, 44(4), 852-859. Zeng, J., Li, X., Li, L., Li, J., & Zhou, L. (2020). RP-HPLC method development and validation for the determination of ten pesticides in tobacco. Journal of Analytical Science and Technology, 11(1), 13.
16. Chen, Y., Zhao, J., Li, M., Zhang, X., & Suo, Y. (2019). RP-HPLC method development and validation for simultaneous determination of 11 flavonoids in Houttuynia cordata Thunb. by ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry. Journal of Separation Science, 42(12), 2094-2104
17. Rezaei, Z., & Asadollahi-Baboli, M. (2021). RP-HPLC method development and validation for the determination of four major bioactive flavonoids in onion (Allium cepa) extract. Analytical Chemistry Letters, 11(1), 78-92.

ISSN: 2583-6129
DOI: 10.55041/ISJEM02312

18. S. Saha, S. Bera, P. Banerjee, S. Bose, and A. B. Chatterjee, "Recent advances in the development and application of monolithic stationary phases for reversed-phase liquid chromatography," TrAC Trends Anal. Chem., vol. 123, pp. 115769, 2020.
19. M. I. Alam, N. N. Chowdhury, and M. Z. Rahman, "Recent advances in the development of reversed-phase high-performance liquid chromatography methods for the analysis of small molecules," J. Chromatogr. A, vol. 1626, pp. 461298, 2020.
20. K. Ma, Y. Xiong, Y. Liang, and X. Qiao, "Recent advances in the development of chiral stationary phases for reversed-phase liquid chromatography," J. Sep. Sci., vol. 44, no. 1, pp. 55-72, 2021.
21. B. W. Armstrong, "Recent advances in chiral stationary phases for reversed-phase high-performance liquid chromatography," J. Chromatogr. A, vol. 1511, pp. 113-125, 2017.
22. J. Wang, Y. Luo, J. Hu, J. Cheng, and H. Chen, "Recent advances in the application of deep eutectic solvents in reversed-phase liquid chromatography," J. Chromatogr. A, vol. 1643, pp. 462028, 2021.