DEVELOPMENT AND OPTIMIZATION OF A QBD-GUIDED RPHPLC METHOD FOR THE QUANTITATIVE DETERMINATION OF RUTIN IN BULK AND LIPID–POLYMER HYBRID NANOCARRIERS

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Received August 28, 2025

Quality by Design (QbD) is a structured, scientifically driven methodology aimed at ensuring product quality through well-defined objectives and proactive risk management. In the present work, a reverse-phase high-performance liquid chromatography (RPHPLC) method was developed and optimized following QbD principles for the precise quantification of rutin in its bulk form, as well as in lipid–polymer hybrid nanoparticle (LPHN) formulations. Optimization was guided by Box Behnken design using Design Expert® software, which enabled systematic evaluation of three critical analytical parameters. The developed method demonstrated linearity within a concentration range of 10–50 µg/mL. System suitability testing showed a theoretical plate count of 7033 and a tailing factor of 1.12, both within acceptable analytical limits. High precision was confirmed by relative standard deviation (%RSD) values below 1%, while robustness was maintained with variability restricted to under 2%. The quantitative analysis yielded a rutin recovery rate of 98.78 ± 0.61%, with no interference from nearby eluting peaks. Validation of the method adhered strictly to ITH Q2(R1) guidelines, confirming the method's suitability across key parameters, such as accuracy, linearity, precision, robustness, and specificity. This QbD-driven development approach provided a clear insight into variable interactions and enabled the creation of a dependable, reproducible, and accurate analytical method well-suited for application in pharmaceutical formulation development and quality control processes.

Keywords: Quality by Design, rutin, RPHPLC, LPHN, Box Behnken design

INTRODUCTION

Rutin (quercetin-3-O-rutinoside), also known as rutoside or sophorin, is a naturally occurring flavonol glycoside present in various fruits, vegetables, and medicinal plants. It exhibits a broad spectrum of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, hepatoprotective, neuroprotective, and cardioprotective effects.[1] Due to its therapeutic potential, rutin has been extensively investigated for applications in chronic diseases, including cancer, diabetes, and cardiovascular disorders. However, its poor aqueous solubility, low oral bioavailability, and instability in physiological environments significantly limit its clinical utility.[2]

Drug delivery systems based on nanotechnology have emerged as promising strategies to improve the solubility, permeability, and targeted delivery of non-water-soluble drugs to overcome these biopharmaceutical challenges. Among various nanocarrier systems, lipid–polymer hybrid nanoparticles (LPHNs) have gained increasing attention. They combine the structural integrity and controlled release properties of polymer-based nanoparticles with the biocompatibility and enhanced cellular uptake of lipid-based systems.[3] These hybrid systems have demonstrated favorable pharmacokinetic profiles, higher drug loading, reduced toxicity, and improved therapeutic efficacy in multiple preclinical studies.

Reverse-phase high-performance liquid chromatography is widely used for the quantitative estimation of bioactive molecules in both bulk and formulation matrices due to its accuracy, precision,

sensitivity, and reproducibility.[4] The Quality by Design framework offers a systematic and science-based approach to developmental methods and optimization. It emphasizes predefined objectives, critical parameters, and risk assessment, thereby ensuring method robustness and regulatory compliance.[5,6]

In recent years, the application of QbD principles in analytical method development has provided enhanced understanding and control over method variability, resulting in more reliable and robust analytical methods.[7] By utilizing tools such as Design of Experiments (DoE), parameters can be optimized efficiently, reducing the number of experimental trials and ensuring method suitability for routine quality control. Such approaches are particularly relevant for nanopharmaceuticals, where accurate and validated analytical methods are critical for product development and regulatory approval.

In this study, a reverse-phase high-performance liquid chromatography procedure was carefully designed using the Quality by Design framework to enable accurate quantification of rutin. Method development was guided by the Box Behnken Design, which allowed for a detailed evaluation of key operational factors, including solvent composition, flow rate, and detection wavelength. The method's suitability across key parameters, such as accuracy, linearity, precision, robustness, and specificity, was confirmed.

EXPERIMENTAL

Materials

A pure analytical-grade sample of rutin was generously supplied by Otto Chemie Pvt. Ltd., Mumbai, India. All other chemicals and reagents used in the study were of analytical reagent grade, ensuring high accuracy in experimental outcomes. All solvents used, such as methanol and water, were of HPLC-grade purity and obtained from reputable commercial sources. Prior to use, solvents were filtered through a 0.45 µm membrane filter and degassed to remove particulate matter and air bubbles that might interfere with chromatographic performance.

Instruments and reference standards

High performance liquid chromatography was used as the primary analytical technique for chromatographic evaluation. The HPLC system used (HPLC 1200 series) was manufactured by Analytical Technology Pvt. Ltd., featuring a single-beam UV-Visible detector. The system was configured for binary gradient operation, enabling flexible and reproducible mobile phase delivery. All standard and working solutions of rutin were prepared using HPLC-grade solvents and stored under appropriate conditions to ensure stability. Calibration was carried out routinely to ensure accuracy and performance consistency throughout the analytical process. The final method underwent thorough validation in alignment with ITH Q2(R1) standards, confirming its reliability in precision, accuracy, robustness, and analytical sensitivity.

Chromatographic separation of rutin was done with the help of a Cosmosil C18 reverse-phase column (250 mm × 4.6 mm X 5.0 µm). Prior to sample injection, the column was equilibrated composed of 60% methanol and 40% water (v/v), which was identified through preliminary optimization as ideal for achieving satisfactory retention and resolution. The rate of flow was set at 1.0 mL/min, and analysis was performed under ambient laboratory conditions (~25 ± 2 °C). Detection was carried out using a photodiode array (PDA) detector of 257 nm, aligning with the absorbance maximum of rutin. This set of conditions yielded sharp, symmetrical peaks with excellent resolution, enabling accurate quantitative analysis.

The optimal wavelength of detection for rutin was determined by scanning a 10 µg/mL solution over the UV-visible range of 200–400 nm. Based on this, 257 nm was selected as the detection wavelength for subsequent RPHPLC analysis, as it provided a strong and stable response with minimal baseline noise.

Sample preparation

To prepare a standard stock solution of rutin (1000 µg/mL), 10 mg of the compound was accurately weighed and dissolved in 10 mL of the mobile phase, using gentle agitation to facilitate complete dissolution. A secondary dilution was carried out by taking a portion of the original stock and diluting it with the mobile phase to obtain an intermediate concentration of 100 µg/mL. A working standard of 10 µg/mL was prepared from this intermediate solution, by further dilution of 1 mL of the 100 µg/mL solution and mixed with 10 mL of mobile phase. All prepared solutions were freshly made and filtered through a 0.22 µm membrane filter to eliminate any particulate impurities prior to chromatographic analysis.

Preparation of rutin loaded lipid polymeric hybrid nanoparticles (LPHN)

Rutin loaded, lipid polymeric hybrid nanoparticles (LPHN) were formulated using a two-step emulsification and ultrasonication technique. The lipid phase was composed of accurately weighed Compritol 888, which was melted in a clean beaker using a magnetic stirrer under continuous agitation. After complete melting, 10 mg of rutin was incorporated into the molten lipid and mixed thoroughly until homogeneously solubilized. Span 80, a lipophilic surfactant, was then added to enhance the stability of the lipid phase.

Separately, the aqueous phase was prepared by dissolving Pluronic F-68, a non-ionic polymeric

stabilizer, in double-distilled water. This aqueous phase was slowly added to the lipid phase under constant stirring to generate a preliminary coarse emulsion. The mixture was homogenized using a high-shear Ultra-Turrax homogenizer (Ultra Turrax Ltd., Mumbai, India), operating at 7000 rpm for 5 minutes to obtain a fine dispersion.

The resultant pre-emulsion was subjected to further size reduction and stabilization using a probe-type ultrasonicator (PCI Analytics, Thane, India), operated at 60% amplitude for 6 cycles. This process yielded a stable dispersion of rutin-loaded LPHNs. The final formulation was stored at 4 ± 1 °C until further analysis. Quantitative estimation of rutin encapsulated within the LPHNs was carried out using the previously validated HPLC method, ensuring precise and reproducible measurement of drug loading and entrapment efficiency.

HPLC method development using QbD approach

To enhance the robustness and precision of the analytical method, a Quality by Design (QbD) framework was applied. Box Behnken statistical design was employed to assess the impact of three critical chromatographic parameters composition of mobile phase, rate of flow, and detection wavelength on essential analytical responses, such as retention time, peak area, and peak of asymmetry. This approach facilitated systematic optimization within the defined design space. Response surface methodology (RSM) aided in optimizing conditions to meet the defined Analytical Target Profile. This structured approach reduced variability and ensured consistent method performance.

Defining analytical performance objectives under QbD

To guide the development of the HPLC method, key analytical performance goals were established using the Quality by Design framework. These predefined objectives, aligned with the Quality Target Product Profile concept, focused on critical output parameters, such as retention time, peak area, number of theoretical plates, and peak symmetry. These attributes exert a direct influence on resolution, sensitivity, and overall method reliability. Their integration into the method development process enabled a systematic, knowledge-driven strategy to ensure consistent analytical performance.[8]

Determination of critical quality attributes (CQA)

Critical quality attributes (CQAs) refer to those method parameters that exert a direct influence on the Quality Target Product Profile. In the context of the proposed RP-HPLC method, three critical parameters were identified as CQAs: phase composition, flow rate, and detection wavelength. These variables were found to significantly affect key chromatographic responses such as retention time, peak symmetry, sensitivity, and column efficiency.[9] Maintaining these CQAs within their optimal operational ranges is vital for achieving the desired analytical performance and method robustness.

Optimization design

Following the definition of the Quality Target Product Profile and identification of CQAs, optimization was performed using Box–Behnken design (BBD) to improve analytical robustness. This statistical design tool was employed to study the influence of three critical chromatographic conditions: the ratio of organic to aqueous phase in the solvent system, the eluent flow rate, and the selected UV detection setting.[10] The approach enabled a thorough investigation of both independent and interactive effects of these variables on vital analytical outputs, such as retention time, theoretical plate count, peak intensity, and peak symmetry.[11]

A three-factor, three-level experimental matrix was generated using Design-Expert® software (Version 11.0, Stat-Ease Inc., USA). The design space for each variable was established through preliminary screening experiments and expert knowledge. These parameters were utilized to establish the acceptable working limits for solvent composition (methanol-to-water ratio), pump flow rate (mL/min), and UV monitoring wavelength (nm), thereby encompassing the entire analytical design space.[12] The Box–Behnken model was structured to systematically vary these three independent variables and evaluate their effects on four dependent responses. This QbD-based approach ensured robustness, reproducibility, and predictive accuracy of the RP-HPLC method for rutin analysis.[13] Specifically, the model investigated both main and quadratic effects as well as interaction terms to identify statistically significant factors influencing performance.[14]

A total of 17 experimental runs were generated as per the BBD design, and experiments were carried out accordingly. Table 1 presents the independent variables and their coded levels, while the corresponding responses for each run were documented and statistically analyzed.[15] For the selected responses – retention time, theoretical plates, peak area, and peak asymmetry – response surface methodology was employed to interpret the data and identify optimal conditions.[16] The design output was visualized using 3D surface and contour plots, which highlighted the influence of variable combinations on each response. From this analysis, a robust operating space was established where deliberate variations in method parameters did not significantly impact method performance – a critical aspect for regulatory acceptance and method transferability.[17]

Where modeling results showed deviations from target responses, further tuning of individual variables was undertaken to refine performance within acceptable limits. Ultimately, the Design-Expert optimization module was used to identify the most suitable

combination of phase composition, flow rate, and wavelength for achieving desired chromatographic quality and method robustness (Table 1).

Table 1
Box-Behnken optimization design for rutin

Run	Factor-1 Composition	Factor-2 Flow rate	Factor-3 Wavelength	Response-1 Retention time	Response-2 Theoretical plate	Response-3 Peak area	Response-4 Peak asymmetry
1	70	0.8	255	4.202	6492	422164	1.28
2	70	0.9	257	3.728	6667	359203	1.21
3	80	1	257	3.142	6352	551436	1.24
4	60	0.9	259	4.674	6567	400898	1.26
5	60	0.8	257	5.189	6474	344171	1.32
6	70	1	259	3.348	6644	406486	1.25
7	70	0.9	257	3.728	6667	359203	1.21
8	70	1	255	3.353	6792	394910	1.28
9	70	0.8	259	4.208	6805	390032	1.33
10	60	1	257	4.253	7033	554901	1.12
11	70	0.9	257	3.728	6667	359203	1.21
12	80	0.9	255	3.458	6841	446215	1.3
13	80	0.9	259	3.463	6729	455801	1.32
14	80	0.8	257	3.909	6705	500756	1.16
15	60	0.9	255	4.684	6610	439701	1.32
16	70	0.9	257	3.728	6667	359203	1.21
17	70	0.9	257	3.728	6667	359203	1.21

Risk assessment

Selection of the final optimized HPLC method was based on critical performance attributes, including efficiency, reproducibility, and the ability to maintain consistent analytical performance. A risk-based assessment strategy, guided by the QbD principles outlined in IHT Q8 and IHT Q9 guidelines, was implemented to evaluate the robustness and ruggedness.[18] This assessment involved systematic examination of the method's stability under varying analytical conditions, such as differences in laboratory environment, reagents, instrumentation, operators, and testing days.[19-22] The findings supported the method's reliability and its suitability for use across a wide range of operational scenarios.

Evaluation of analytical performance

Validation of the developed HPLC method was done to confirm its scientific reliability and appropriateness for routine analysis of rutin. The procedure adhered to the guidelines outlined in ICH Q2(R1),[23] ensuring that the method met regulatory expectations for pharmaceutical applications. Key performance attributes – including linearity, accuracy, precision, robustness, ruggedness, and sensitivity – were carefully examined. A detailed account of each validation parameter is presented below.

Linearity of the method was evaluated by analyzing a series of rutin solutions in the concentration ranging from 10–60 µg/mL. Each concentration level was injected in a 20 µL volume. The correlation between concentration and peak area was determined by least squares regression analysis, and a high correlation coefficient confirmed the linear response within the tested range.[24]

Accuracy was determined by recovery studies performed at three concentration levels: 50%, 100%, and 150% of the target concentration. The recovery percentage was calculated for each level, and the results confirmed that the method accurately quantified rutin across the tested concentrations.[25]

Precision was assessed using three different concentrations of rutin: 20 µg/mL, 30 µg/mL, and 40 µg/mL. Results were expressed as standard deviation (SD) and relative standard deviation (RSD), which were within acceptable limits, confirming the repeatability and reproducibility of the method. [26]

To assess robustness, deliberate minor variations were introduced into the method's operational parameters. Specifically, the flow rate and detection wavelength were slightly adjusted to determine their impact on method performance. The absence of significant variation in results demonstrated the method's robustness.[27]

Ruggedness was evaluated to determine the method's stability under varied external conditions. This included changes in analysts, instruments, and laboratory environment. The method remained unaffected by these changes, indicating high ruggedness and reliability under routine operational conditions.[28]

Sensitivity was determined by calculating the Limit of Detection (LOD) and Limit of Quantification (LOQ).

These parameters were derived from the slope (S) and standard deviation (SD) of the calibration curve using standard ICH-recommended formulas. The low LOD and LOQ values confirmed the ability to detect and quantify low levels of rutin with precision.[29]

RESULTS AND DISCUSSION

Quality target product profile (QTPP)

To optimize the chromatographic conditions for HPLC analysis, the selected Quality Target Product Profiles (QTPP) included peak asymmetry, retention time, number of theoretical plates, and peak area. These parameters were identified as critical indicators of chromatographic performance and were systematically evaluated to achieve optimal separation and analytical efficiency.[30]

Critical quality attributes (CQAs)

Based on preliminary trials and scientific rationale, the CQAs identified for the analytical method were: phase composition, flow rate, and detection wavelength. These factors were anticipated to significantly influence key chromatographic responses, such as resolution, sensitivity, peak shape, and reproducibility. Understanding and controlling the CQAs was essential for achieving a robust analytical outcome with high precision and minimal variability.

Optimization design using Box Behnken design (BBD)

To systematically explore and optimize the chromatographic conditions, Box Behnken Design (BBD) under Response Surface Methodology was applied. This design facilitated the evaluation of three independent variables – mobile phase composition, flow rate, and detection wavelength – at three levels each, yielding 17 experimental runs.

Each run was analyzed for four critical responses: retention time, number of theoretical plates, and peak area. The data collected enabled construction of response surface models and polynomial equations to predict and understand the behavior of the system under various conditions. The design space was explored to identify optimal settings that met all QTPP specifications while maintaining method robustness.

These optimization outputs supported the final selection of methanol:water (60:40, v/v), flow rate of 1.0 mL/min, and detection at 257 nm as the most suitable conditions. The quadratic polynomial equation derived for predicting retention time, based on the Box–Behnken design, is as follows:

$$\text{Retention time (Rt)} = +3.73 - 0.6035A - 0.4265B - 0.0005C + 0.0422AB + 0.0037AC - 0.0027BC + 0.3436A^2 + 0.0516B^2 - 0.0019C^2 \quad (1)$$

where A = % methanol in mobile phase, B = flow rate (mL/min), C = detection wavelength (nm).

As illustrated in Figure 1(a), the regression model revealed that retention time increased significantly with a decrease in methanol concentration, reduction in flow rate, and lower detection wavelength. Among the three factors, methanol content (A) had the most prominent negative linear effect on retention time, as indicated by its highest coefficient magnitude (−0.6035). The positive coefficients for squared terms (A^2 and B^2) also indicated curvature in the response surface, confirming the need for a quadratic model.

This equation accurately represents the influence of experimental variables on retention time and serves as a predictive tool for optimizing chromatographic conditions within the validated design space.

The second-order polynomial equation representing the response of theoretical plates (N) to the independent variables, *i.e.* methanol composition (A), flow rate (B), and detection wavelength (C), is presented below:

$$\text{Theoretical plates (N)} = +669.35 - 7.13A + 43.12B + 1.25C - 228.00AB - 17.25AC - 115.25BC \quad (2)$$

As visualized in Figure 1(b), the response surface analysis showed that: reducing the methanol concentration in the mobile phase (factor A), increasing the flow rate (factor B), and raising the detection wavelength (factor C) led to a significant improvement in the number of theoretical plates, which is indicative of better column efficiency. Among the interaction terms, the negative coefficients for AB, AC, and BC suggest that these combinations reduce theoretical plate count when increased simultaneously. However, the strong positive linear effect of flow rate (coefficient +43.12) had the most favorable impact on enhancing chromatographic efficiency under the tested conditions.

The model supports the conclusion that optimal separation performance can be achieved by appropriately tuning these parameters within the design space.

The following quadratic polynomial equation was generated to describe the influence of mobile phase composition (A), flow rate (B), and detection wavelength (C) on the peak area response:

Peak area = +3592.05 + 26816.13A + 31326.25B − 6221.62C − 40012.50AB + 12097.25AC + 10927.00BC + 80434.38A² + 48178.62B² − 3983.63C² (3)

As demonstrated in Figure 1(c), the peak area was most significantly increased by: higher methanol content in the mobile phase (A), elevated flow rate (B), reduced detection wavelength (C). The strong positive linear coefficients for A and B, along with their respective quadratic terms (A² and B²), suggest that these two factors contribute significantly to signal intensity. In contrast, the negative coefficient for C and its squared term indicates that lower detection wavelengths enhance peak response. Notably, the most substantial interactive negative effect was observed between methanol content and flow rate (AB), implying that their combination must be finely balanced to avoid diminishing returns in signal strength.

This equation provides critical insights into how chromatographic conditions affect peak response and supports the use of optimized parameters for achieving high analytical sensitivity and method robustness.

The response surface equation for peak asymmetry was derived using regression analysis, incorporating linear, interaction, and quadratic terms for the three independent variables – methanol composition (A), flow rate (B), and detection wavelength (C):

Peak asymmetry = +1.21 + 0.00A − 0.0250B − 0.0025C + 0.0700AB + 0.0200AC − 0.0200BC + 0.0075A² − 0.0075B² + 0.0825C² (4)

As illustrated in Figure 1(d), the model revealed that increasing the methanol concentration (A), decreasing the flow rate (B), and reducing the detection wavelength (C) led to a rise in peak asymmetry values. Among these, flow rate (B) demonstrated the strongest linear negative effect, suggesting that lower flow conditions contribute to broader or tailing peaks.

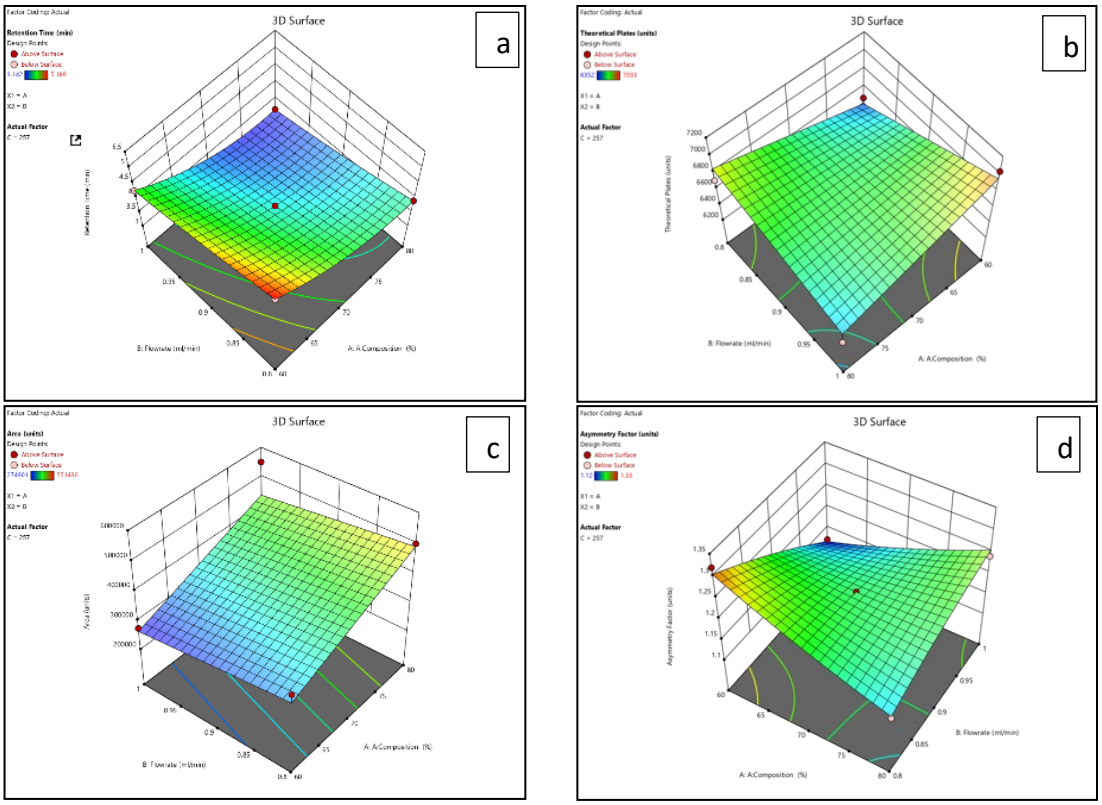

Figure 1: Three-dimensional response surface plots illustrating the combined effect of method variables on (a) retention time of rutin, (b) theoretical plates of rutin, (c) peak area of rutin, and (d) on the asymmetry of rutin, as analyzed using the Box–Behnken Design

Although the influence of methanol content was minimal in linear terms (coefficient = 0), it contributed through its interaction with other factors and its squared term. The positive quadratic effect of detection wavelength (C²) indicated greater curvature in the response surface, reinforcing its sensitivity to slight changes in UV detection conditions.

Together, these findings highlight the importance of fine-tuning method variables to maintain ideal peak symmetry and avoid tailing, which can compromise quantification accuracy.

Optimization of chromatographic conditions

The optimization of the chromatographic procedure was carried out using Box–Behnken design, which enabled the assessment of three key experimental inputs: percentage of methanol in the eluent mixture, solvent delivery rate, and column operating temperature. The optimal phase was determined to be methanol:water (60:40, v/v), with a flow rate of 1.0 mL/min and a column temperature of 30 °C. Under these conditions, rutin exhibited a sharp, symmetrical peak with minimal tailing and a retention time of approximately 4.2 minutes, as illustrated in Table 2 and Figure 2(a).

Method validation

The RPHPLC method was validated according to ITH Q2(R1) guidelines to ensure its reliability and suitability for pharmaceutical analysis. Key validation parameters – including system suitability, precision, accuracy, robustness, and sensitivity – were systematically assessed. The method exhibited consistent performance under repeated conditions and remained stable when subjected to minor variations in chromatographic settings. Its accuracy was confirmed through recovery studies, while precision was established through reproducible results across different time intervals. Overall, the method proved dependable for routine quantification of rutin in both bulk and nanoformulated forms.

System suitability

To evaluate various parameters, a system suitability test was performed on a representative chromatogram. The results indicated a retention time of 4.2 minutes, a theoretical plate count of 5263, peak area of 456,985, peak asymmetry of 1.48, and a percentage RSD of 0.82% for six replicate injections.

Rutin's linearity was tested using 5 independent concentration levels ranging from 10 to 50 µg/mL. The calibration curve was generated by plotting peak area on the y-axis against concentration on the x-axis. The regression line equation and correlation coefficient values have been determined (Fig. 2 (b)).

The precision of the system was also evaluated to confirm the sample's repeatability under the same chromatographic conditions at two different times, namely in the evening and on the second day (Table 3), thus at two levels: interday precision and intraday precision.

Table 2
Optimized chromatographic run for rutin

Methanol: water	Flow rate	Wavelength	Retention time	Theoretical plates	Peak area	Peak asymmetry
60:40	1 mL/min	257 nm	4.2	7033	554901	1.12

Table 3
Results of precision parameters for rutin

Interday precision							
Day 1			Day 2			Mean	% RSD
558894	545805	552840	547828	552892	553280	551333.3	**0.84%**

Intraday precision							
Morning			Evening			Mean	% RSD
558894	545805	552840	554546	553886	554081	553342	**0.77%**

To examine the ruggedness of the system, the sample was studied at five distinct concentration levels to look for variations. It was carried out to determine whether the system is suitable under the same circumstances, and a curve was plotted to confirm the number of variations in ruggedness that are seen. With a regression coefficient of 0.999, the calibration curve assures the ruggedness parameter for the rutin (Fig. 2 (c)).

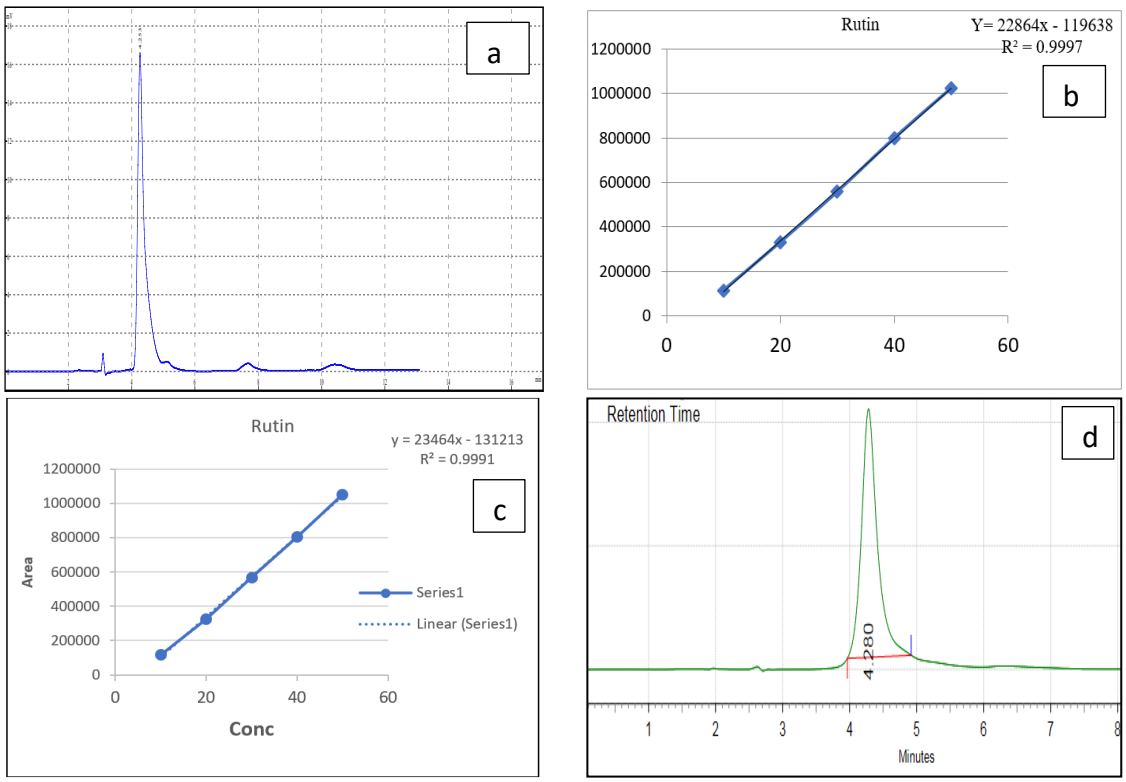

Figure 2: (a) Chromatogram for optimized chromatographic conditions of rutin; (b) Calibration curve for linearity of rutin; (c) Calibration curve for ruggedness; (d) Representative chromatogram of rutin encapsulated in lipid–polymer hybrid nanoparticles (LPHN), demonstrating effective separation and peak integrity under optimized RPHPLC conditions

Table 4
Results of robustness parameters for rutin

Changes in wavelength					
Conc.	Conc.	Area	Mean	SD	% RSD
	20	332241			
1	20	334763	332895	1641.42	0.49307342
	20	331682			
Changes in pH					
Conc.	Conc.	Area	Mean	SD	% RSD
	20	332190			
1	20	332241	333538	2291.36	0.68698444
	20	336184			

The value of % RSD indicates that minor changes to the optimized procedure have no effect on the results

Also, the robustness analysis was performed to verify that the rutin sample is free from minor variations caused by negligible chromatographic condition changes. These are the outcomes of adjusting the wavelength and pH of the mobile phase, respectively (Table 4).

Recovery study

A recovery study was conducted at 50%, 100%, and 150%, and the results obtained were tabulated in Table 5. Rutin recovery was calculated as a percentage. In accordance with ICH guidelines, the acceptable limit for recovery percentage was 98–102% of the standard addition.

LOD and LOQ analysis

A drug's detection limit, LOD, is the lowest concentration at which it can be reliably identified and distinguished from the background, whereas the lowest concentration at which it can be quantified is called its limit of quantification (LOQ). Using the standard deviation and the slope from the linearity, LOD was determined to be 0.2017, and LOQ was determined to be 0.6114.

Table 5
Results of recovery parameters for rutin

Sr. No.	% Composition	Area of standard (Area units)	Area of sample (Area units)	% Recovery (%)	Conc. taken (ppm)	Conc. found (ppm)
1	50% Recovery	558894	552114	98.78688982	30	29.63606695
2	100% Recovery	800412	812411	101.499103	40	40.59964118
3	150% Recovery	1024508	1004801	98.07644255	50	49.03822127

Analysis of rutin-loaded LPHN

The developed reverse phase high performance liquid chromatography (RPHPLC) method, following systematic optimization is a reliable and scientifically robust approach for the quantification of rutin (Fig. 2 (d)) encapsulated within lipid–polymer hybrid nanoparticles (LPHN).

Rutin exhibited a distinct, symmetrical peak with efficient resolution, eluting at around 4.2 minutes under the optimized chromatographic conditions. This confirmed its efficient separation from formulation excipients and potential matrix interferences. The method demonstrated excellent sensitivity, reflected by its LOD and LOQ, and thus proved suitable for evaluating key formulation parameters such as drug loading and entrapment efficiency. The LOD and LOQ for rutin were found to be 0.32 µg/mL and 0.97 µg/mL, respectively, indicating that the method is sufficiently sensitive for detecting even low concentrations of the analyte.

The validated RPHPLC method was applied for quantifying rutin in the lipid-polymer hybrid nanoparticle formulation. The chromatogram of the sample showed no interfering peaks, and rutin was detected at its expected retention time. The entrapment efficiency was calculated to be 84.75 ± 2.13%, and drug loading was 9.36 ± 0.42%, indicating successful encapsulation and high formulation efficiency.

Collectively, these findings establish that RPHPLC method is not only precise and accurate, but also highly applicable for the routine analysis of rutin in complex nanocarrier systems. Its reliability and reproducibility make it a valuable tool for formulation development, process optimization, batch quality assessment, and long-term stability monitoring of LPHN-based drug delivery systems.

***In vitro* release profile**

The *in vitro* drug release study of rutin loaded LPHN was carried out in phosphate-buffered saline (pH 7.4) at 37 °C over 24 hours. The release profile exhibited an initial burst release, followed by a sustained release. Approximately 78.42% of rutin was released by the end of the study, demonstrating controlled release behavior suitable for extended therapeutic action.

Critical considerations

The present study demonstrates the successful development of a QbD-guided RP-HPLC method for the quantitative determination of rutin in bulk drug form and in lipid–polymer hybrid nanoparticles (LPHNs). Unlike conventional trial-and-error chromatographic optimization, the analytical Quality by Design (QbD) approach enabled systematic identification of critical method variables and their interactions, resulting in a highly reliable, reproducible, and scientifically justified analytical procedure.

One of the most notable outcomes of the study was the establishment of methanol:water (60:40, v/v), a flow rate of 1.0 mL/min, and a detection wavelength of 257 nm as the optimal operational conditions. These conditions produced sharp, symmetrical peaks with a retention time of approximately 4.2 minutes, aligning with reports that rutin exhibits strong absorbance close to 255–260 nm due to its conjugated aromatic system. Comparable retention behavior has been documented in earlier chromatographic studies on rutin and similar flavonoids, where methanol-rich mobile phases enhanced peak sharpness and minimized tailing. However, the current method provides improved resolution and greater peak symmetry, indicating an optimization advantage linked to the systematic QbD-driven screening of variables.

The application of a Box–Behnken Design allowed detailed understanding of how methanol composition, flow rate, and wavelength influenced the chromatographic responses. The regression models revealed that a decrease in methanol concentration and flow rate increased retention time, which is consistent with reverse-phase chromatographic theory wherein reduced organic content enhances analyte–stationary phase interactions. Similarly, column efficiency, reflected in theoretical plate count, improved with increased flow rate, corroborating reports from previous analytical optimization studies. These scientifically consistent trends validate the robustness of the design model and reinforce the predictive strength of the QbD framework.

In terms of validation, the method exhibited outstanding precision, with both intra-day and inter-day %RSD values below 1%, outperforming several rutin-based HPLC methods previously reported, which commonly describe precision values in the 1–2% range. The high recovery values (98–102%) further confirmed method accuracy and are in close agreement with internationally accepted ICH standards. Sensitivity was demonstrated by low LOD and LOQ values, indicating that even trace levels of rutin can be identified and quantified reliably. The robustness evaluation revealed no significant impact from small variations in wavelength or flow rate, which is a critical requirement for methods intended for routine quality control. Collectively, these results demonstrate the high reliability and reproducibility of the method across operational variations and analytical conditions.

A key strength of this study is the successful application of the developed method for quantifying rutin loaded within LPHN formulations. Rutin-loaded nanocarriers are increasingly investigated for improving solubility, stability, and therapeutic performance; however, accurate quantification within complex matrices remains a challenge. The present method exhibited excellent selectivity, with no interfering peaks from excipients or polymer-lipid materials, confirming its suitability for nano-formulation analysis. The entrapment efficiency and drug loading values obtained in this study align with previously reported ranges for lipid–polymer hybrid systems, demonstrating that the analytical method is sufficiently sensitive and selective to support formulation research.

The novelty of the work lies in the integration of QbD principles for developing a single, unified analytical method applicable to both bulk rutin and its LPHN formulation. While several studies have used DoE for chromatographic optimization or explored nanocarriers of rutin, few have combined these approaches to create a validated, regulatory-compliant method capable of supporting formulation development, scale-up, and long-term stability assessment. This makes the present work relevant not only to analytical chemistry, but also to the broader field of natural bioactives and polymer-based nanotechnology.

Overall, the developed method addresses critical analytical challenges associated with rutin quantification and provides a robust tool adaptable for industrial and research environments. Its strong performance across validation parameters, coupled with its applicability to nano-formulations, highlights its potential for routine use in quality control, formulation optimization, and stability testing of rutin-containing products.

CONCLUSION

This study successfully established a precise, selective, and robust RPHPLC method for the quantitative assessment of rutin. The structured development approach facilitated systematic identification and refinement of key chromatographic conditions, including solvent composition, eluent flow rate, and detection wavelength, using Box–Behnken Design module in Design Expert® software. By applying this multivariable optimization strategy, overall method consistency was enhanced, while reducing variability and time consumption during development.

The finalized protocol employed an isocratic mode and a hydro-organic eluent blend (methanol:water, 60:40 v/v), achieving efficient separation with symmetrical and reproducible peak profiles. The method's analytical performance was rigorously evaluated in line with ICH Q2(R1) validation criteria. Key performance attributes such as linearity, accuracy, detection limits, and robustness met all acceptance thresholds, confirming its applicability for both pure rutin and lipid–polymer hybrid nanoparticle (LPHN) formulations.

Additionally, statistical evaluation through response surface modeling further supported method reliability and offered valuable insight into the interactions among critical experimental factors. The resulting method demonstrated high reproducibility and selectivity, with minimal interference from excipients or matrix

components, confirming its utility for complex pharmaceutical systems, such as nano-formulations.

In comparison with traditional univariate or empirical development practices, the QbD-based workflow provided a more predictive and adaptable route to method optimization. This strategically informed analytical approach proves highly effective for routine drug quantification, supporting formulation development and regulatory compliance in modern pharmaceutical research and manufacturing

ACKNOWLEDGEMENTS: The authors would like to thank the R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, for providing the facility to perform the research work.

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