Quality by design (Qbd) approach to develop stability indicating HPLC method for estimation of rutin in chitosan-sodium alginate nanoparticles

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Rutin is a flavonoid glycoside, mainly consists of phenolic compounds, responsible for many biological activities. The objective of the present study was to develop and validate a precise, simple, robust, rapid and reliable reverse phase high -performance liquid chromatography (RP-HPLC) technique by using Qbd approach for evaluating the rutin in nanoparticles. Central composite design (CCD) was employed for optimizing the experimental conditions of RP-HPLC method. Buffer pH, methanol content in the mobile phase composition, flow rate, and wavelength were selected as independent variables whereas retention time, peak area, and asymmetry factor was selected as dependent variables. The retention time, peak area and asymmetric factor of rutin by using optimized independent variables were found to be 3.75 min, 1014.79 mV, and 1.26 respectively. The limit of detection and limit of quantitation values were found to be 0.005 µg/mL and 0.15 µg/mL respectively. For confirming linearity, accuracy, precision, and robustness, the optimized assay condition was validated as per ICH guidelines. The proposed method, which was optimized by QbD approach was found to be a suitable method for analyzing the rutin in chitosan-sodium alginate nanoparticles.

Keywords: Rutin, Chitosan, sodium aliginate, nanoparticles, RP-HPLC, central composite design, QbD

INTRODUCTION

Rutin also called as sophorin, rutoside and chemically (3,3,4,5,7-pentahydrohyflavone-3-rhamnoglucoside) depicted in Figure 1 is a flavonoid glycoside and mainly consists of phenolic compounds, responsible for many biological activities (Janhvi, Sonali, Suryakant, 2014). Flavonoids represent a large class of phenolic compounds and largely distributed in plants such as vegetables, nuts, fruits, green seeds, tea, cocoa, soy and beverage products (Ahmad *et al.,* 2017). Rutin (quercetin-3-*O*-rutinoside) was most abundant bioactive flavonoid called as vitamin P. Rutin

activates the liver enzymes involved in gluconeogenic, lipid metabolic processes and inhibit the aldose reductase enzyme activity, leads to decrease in glucose level (Salib, Michael, Eskande, 2015; Vankudri *et al.,* 2016). Literature survey revealed that, the different analytical methods such as HPLC (Kardani *et al.,* 2013; Hooresfand, Ghanbarzadeh, Hamishehkar, 2015; Habib, Omar, Mohamed, 2016 and Ramaswamy *et al.,* 2017), HPTLC (Doshi, Une, 2016), UV- Visible spectrophotometry (Kokalj *et al.,* 2017), and capillary electrophoresis methods (Chen, Zhang, Ye, 2000) have been developed for estimating the rutin from pharmaceutical formulations. Amongst all available analytical methods LC method is the most suitable chromatographic process for establishing the analytes from different formulations (Hepsebah, Padma, Kumar, 2014; Cvetkovic *et al.,* 2015).However, no research has been

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performed to optimize the chromatographic conditions by using QbD approach for estimating the rutin from chitosan sodium alginate nanoparticles so far. Commonly, optimization of RP-HPLC methods are carried out by trial and error approach where large numbers of experiments are required to perform the work. To overcome these pitfalls, RP-HPLC method development can be performed for the estimation of rutin by using QbD approach. Response surface methodology (RSM) is used after the preliminary screening of experimental factors that significantly affect the responses using factorial designs (Rakic *et al.,* 2014; Bezerra *et al.,* 2008). RSM offers in detail information of interaction between factors on separation of analytes because the experimental variables and chromatographic response can be examined by performing small number (Narahari *et al.,* 2018). The objective of the current research work was to design and validate an RP-HPLC method by CCD and quantification of rutin in chitosan sodium alginate nanoparticles.
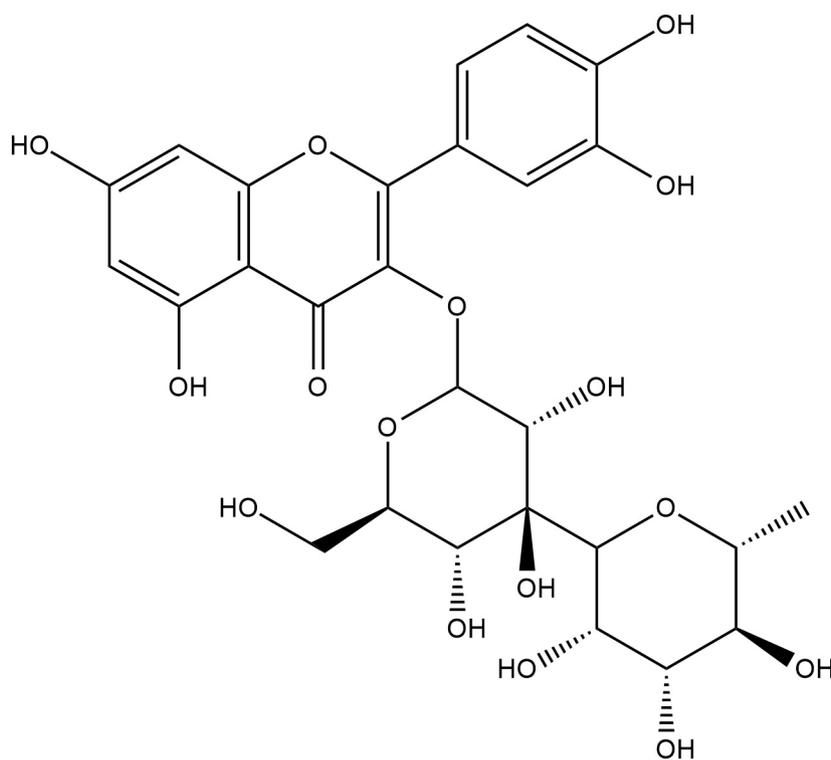

FIGURE 1 - Chemical structure of rutin.

MATERIAL AND METHODS

Material

HPLC Instrumentation and conditions

The RP-HPLC method development and validation studies were performed on a Shimadzu LC-20AT, SPD-20A HPLC instrument, Japan with UV detector and spinchrom LC solution software with Rheodyne manual injector system. The separation was accomplished by isocratic elution mode. The mobile phase consists of a blend of methanol: phosphate buffer (pH corrected to 3.2 with 80% ortho phosphoric acid) in the ratio of 60:40 v/v was pumped at a flow rate of 1 mL/min. 20 μL and 254 nm were used as an injection volume and as a wavelength respectively. The mobile phase and samples were filtered using 0.45 μm membrane filter and degassed using an ultra-sonicator (FAST CLEAN Model No 1510) before injecting in HPLC system. The

chromatographic separation of drugs was carried on an Inertsil C_{18} column reverse phase (250 mm×4.60mm, 5 μm particle size). The chromatographic system operations like Chromatogram productivity, peaks integration and calculation, retention times, system suitability etc, and recording of data was carried out using the spinchrom software.

Software

Design Expert® 11.0. (Stat-Ease Inc., Minneapolis) was employed to set an Experimental design, and to perform data analysis and desirability function for optimizing RP- HPLC method.

Experimental Design

Response surface methodology (RSM) is used for exploring problems where several independent variables such as buffer strength, flow rate, organic phase and wavelength effect on responses (e.g. retention time, peak area and asymmetric factor). The levels of different variables are optimized by using RSM technique to attain the best system performance. In the experimental design, quadratic models can accurately explain all the responses values of the chromatographic conditions. For calculation of quadratic regression model coefficients, each variable having at least three distinct levels must be studied in order to calculate of quadratic regression model coefficients and thus, a Central Composite Design (CCD) was employed in this optimization study (Rakic et al., 2014) Experimental condition was optimized by considering four chromatographic factors and three response levels, depicted in table I.

TABLE I - Experimental factors and levels used in the central composite design

Factor Code	Name of the factor	Level		
		Low (-)	Medium (0)	High (+)
A	pH	3.2	5.2	7.2
B	Mobile Phase Ratio	45	52.5	60

(continuing)

TABLE I - Experimental factors and levels used in the central composite design

Factor Code	Name of the factor	Level		
		Low (-)	Medium (0)	High (+)
C	Flow rate	0.8	1	1.2
D	Wavelength	254	264	274

Chemicals and Reagents

Working standard of rutin was procured from Yucca Enterprises, Mumbai, India. HPLC grade methanol and water were used throughout the study. 0.45 μm membrane filter was used for vacuum filtration of mobile phase. All chemicals and reagents used were of HPLC grade purchased from Merck India Ltd, India; High-purity water was prepared using Millipore purification system.

Preparation of standard stock solution

10 mg of rutin was dissolved in 10 mL of phosphate buffer solution to get 1000 μg/mL. 1 mL from the stock solution was taken and made up to 10 mL with phosphate buffer to get 100 μg/mL. Different concentrations of solutions were prepared from primary stock solution.

Selection of wavelength

Standard solutions of rutin were scanned in UV spectral range of 200-400 nm individually. Lambda max of the UV spectra was determined and fixed as detection wavelength.

Preparation of sample solution

5 mL of RCSANPs (rutin loaded chitosan-sodium alginate nano particles) dispersion equivalent to 23 mg of drug was taken in 25 mL volumetric flask containing 23 mL of DMSO and ultrasonicated for 15 minutes for complete extraction of drug. The solution was filtered using whatman filter paper and transferred 1 mL of filtrate to 10 mL volumetric flask. The filtrate was appropriately diluted

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by mobile phase and prepared different concentrations of 50, 200 and 350 µg/mL solutions.

Preparation of RCSANPs and sample solution

Preparation of RCSANPs

RCSANPs were prepared by solvent diffusion method. 120 mg of rutin, 200 mg of poloxamer 188 were added in 20 mL of (18 mg/mL) sodium alginate solution in distilled water and adjusted pH to 5.1 with 0.1M HCl. 2 ml of (4 mg/mL) $CaCl_2$ solution was added to sodium alginate solution and stirred under magnetic stirring at 1000 rpm for 30 min. After 30 minute, 4 ml of (12 mg/mL) chitosan solution in 1% acetic acid (adjusted pH 5.4 with 0.1M NaOH) was added to sodium alginate solution and stirred for 2hrs. The resultant dispersion was ultrasonicated for 5 min to obtain nanoparticles (Clementino, Sonvico, 2018).

Forced degradation study

For forced degradation study, samples were prepared and exposed to acidic, alkaline, and oxidant media, and also for thermal and photolytic conditions. For acidic degradation study, 0.01 N HCl was added to drug at room temperature and performed degradation study for 3h till neutralizing the mixture. Alkaline degradation study was performed by adding the drug content to 0.05 N NaOH, equilibrated at room temperature and performed degradation study for 3 h till neutralizing the mixture. For oxidation degradation study, 30% v/v H_2O_2 was added to the drug at room temperature and performed degradation study for 3 h. For thermal degradation study 80°C of temperature was employed to a solid drug for 72 h. For photolytic degradation study UV light was exposed to the drug content for 72 h. After completion of all treatments, the stress content solutions were allowed to equilibrate at room temperature and diluted with mobile phase appropriately to prepare 10 µg/mL concentrations.

Validation

The developed HPLC method was validated for system suitability, linearity, accuracy, precision, robustness, Limit of Detection (LOD), Limit of Quantification (LOQ) in accordance with ICH guidelines.

Linearity

A calibration curve was constructed from seven different rutin standard concentrations within the range of 5-35 µg/mL by diluting with suitable mobile phase. The calibration curve was plotted between rutin concentration versus peak area and calculated regression equations.

System suitability study

20 µL of rutin standard solution (10 µg/mL) in six replicates were injected and calculated the %RSD of the consequent peak areas.

Recovery

For the determination of recovery method, standard (pure rutin) was added to sample solution at three different levels such as low quality control (LQC), medium quality control (MQC) and high quality control (HQC) by spiking method. The sample was quantified by proposed method and determined the peak areas.

Precision

Intraday precision

Intraday precision was determined by injecting three different concentrations (LQC, MQC and HQC) for three times at the interval of 1hr in the same day. %RSD was determined for the consequent peak areas.

Inter day precision

Inter day precision was determined by injecting three different concentrations (LQC, MQC and HQC) for two days in a week. %RSD was calculated for the resultant peak area.

Limit of detection (LOD) and Limit of quantification (LOQ)

Based on the standard deviation of the response and the slope from the linearity graph the Limit of detection (LOD) and Limit of Quantification (LOQ) were determined. The formula of LOD and LOQ is given as below

$$LOD = 3.3 \times \sigma/S; \quad LOQ = 10 \times \sigma/S$$

Where,

σ = standard deviation of the peak area

S = slope of calibration curve

Robustness

The robustness of the developed HPLC method was analyzed by varying the system suitability parameters like flow rate (±0.2 mL/min) and wavelength (±2 nm).

RESULTS AND DISCUSSION

In the present research work, the chromatographic factors were selected based on initial experiments, previous information from the literature, and physicochemical properties of rutin such as solubility and PKa. Response surface methodology (RSM) is used to analyse the problems associated with several independent variables (e.g. pH, flow rate, mobile phase ratio, and wave length) and optimize the level of independent variables for obtaining the best systemic performance. In the experimental design, RSM can explain the quadratic model response for all values in chromatographic conditions. Coefficients of quadratic regression model is calculated by studying each design variable at least at three distinct levels and thus, a Central Composite Design (CCD) was used for optimization study. The CCD design was employed to set the optimum flow rate, mobile phase pH, strength of mobile phase and wave length in order to optimize chromatographic responses such as Rt, Peak area and asymmetric factor. Chromatographic factors and levels were selected and used to optimize experimental condition. Thirty experiments were constructed by using four independent variables (A, B, C and D) in association with three observed responses as depicted in table II (Kalariya *et al.,* 2014).

TABLE II - Optimization method parameters for central composite design

Run	Factors				Responses		
	A: pH	B: Mobile Phase Ratio v/v	C: Flow rate mL/min	D: Wavelength nm	Y1: Retention time min	Y2: Peak area mv	Y3: Asymmetry factor
1	5.2	52.5	0.8	264	5.64	1192.3	1.80
2	5.2	52.5	0.8	264	5.7	1192.3	1.80
3	7.2	60	1	254	4.17	1130.34	2.10
4	3.2	45	0.6	274	7.12	952.045	1.26
5	9.2	52.5	0.8	264	5.2	1410.68	2.71
6	5.2	52.5	0.8	264	5.7	1191.3	1.80
7	5.2	52.5	0.8	264	5.7	1191.3	1.80
8	3.2	60	1	274	5.53	1112.27	1.42
9	5.2	67.5	0.8	264	4.92	1177.46	1.93
10	5.2	52.5	1.2	264	4.87	1185.55	1.62

(continuing)

TABLE II - Optimization method parameters for central composite design

Run		Factors				Responses	
	A: pH	B: Mobile Phase Ratio v/v	C: Flow rate mL/min	D: Wavelength nm	Y1: Retention time min	Y2: Peak area mv	Y3: Asymmetry factor
11	5.2	52.5	0.8	264	4.74	1190.3	1.80
12	7.2	60	0.6	254	5.1	1311.55	2.49
13	5.2	52.5	0.4	264	6.4	1275.56	1.99
14	1.2	52.5	0.8	264	6.06	900.23	0.92
15	5.2	37.5	0.8	264	6.39	1184.47	1.69
16	7.2	45	0.6	274	6.34	1377.74	2.17
17	3.2	60	0.6	274	6.37	1108	1.52
18	5.2	52.5	0.8	244	4.69	1163.48	1.83
19	3.2	45	1	254	5.35	1038.24	1.30
20	3.2	60	1	254	3.75	1014.79	1.26
21	7.2	45	1	274	5.72	1381.16	2.12
22	3.2	45	0.6	254	6.24	1046.43	1.39
23	7.2	60	1	274	5.21	1311.52	2.21
24	7.2	45	0.6	254	5.65	1392.43	2.37
25	7.2	45	1	254	4.96	1297.3	2.13
26	3.2	45	1	274	6.31	1038.4	1.28
27	7.2	60	0.6	274	5.85	1394.18	2.47
28	5.2	52.5	0.8	284	6.5	1246.26	1.78
29	3.2	60	0.6	254	5.24	1109.07	1.56
30	5.2	52.5	0.8	264	5.65	1191.02	1.80

Effect of variables on Peak area (Y1)

The effects of the independent variables (A, B, C and D) on the peak area (Y1) are depicted in table II. The positive regression coefficient for A and D, indicate, increase in peak area by increasing A and D. The negative regression coefficient for B and C indicates decrease in peak area with increase in B and C. However, the negative regression coefficient for AB and AC was observed and it clearly indicates, decrease in peak area with the simultaneous increasing in AD, BD and CD. Wave length had not shown any significant effect on peak area (p>0.05), while the other variables (% ratio of methanol, flow rate, and pH) had significant effect (p<0.05). The increase in peak area with respect to pH may be due to faster ionization of drug at acidic pH of Mobile phase.

Peak area = +1191.42+133.24A -1.92 B-22.81C +20.86D -35.63AB -21.48 AC+20.68AD -21.27BC +24.08 BD+24.39 CD -9.74 A2 -3.36 B2 +9.04C2 +2.61D2

Effect of variables on retention time (Y2)

The effect of the independent variables (A, B, C and D) on the retention time (Y2) are depicted in table II. The results clearly indicate that the retention time decreases with an increase of flow rate, strength of mobile phase and pH of mobile phase. The significant positive regression coefficient for D indicates the increase in retention time with an increase in wavelength. However, the positive regression coefficient for AB and AC was observed and it clearly indicates, increase in the retention time with the simultaneous increase in the pH and mobile phase ratio as well as pH and flow rate other than negative regression coefficient was observed for AD. However negative coefficient for BC indicates the decrease with a simultaneous increase in mobile phase ratio and flow rate. Enhanced solubility and faster elution may lead to a decrease in retention time on increasing pH and flow rate.

Retention time = +5.52 -0.1929A -0.3921B -0.4154C +0.4837D +0.1119AB +0.0719AC -0.0944AD -0.0556 BC+0.0881BD +0.0681CD +0.0151 A2+0.0214 B2+0.0164C2 +0.0064D2

Effect of variables on asymmetric factor (Y3)

The effects of the independent variables (A, B, C and D) on the asymmetric factor (Y3) are depicted in table II. The positive coefficient for A and B and negative coefficients for C and D were observed in case of asymmetric factor equation. Thus, it clearly indicates, the asymmetric factor increases with an increase of A and B and decrease of C and D. The asymmetric factor was decreased by increasing the flow rate and wavelength, and by decreasing the pH and mobile phase ratio. However mobile phase ratio and flow rate shown negative influence in the asymmetric factor. Thus it clearly indicate that alone mobile phase ratio may not attribute to minimize the asymmetric factor but flow rate can cause significant decrease in asymmetric factor. pH played a major role in optimizing the asymmetric factor. Increase in pH cause increase in asymmetric factor. Significant decrease in asymmetric factor was observed while considering both pH and flow rate.

Asymmetry factor = +1.81+0.4436 A+ 0.0635 B- 0.0889C- 0.0120D - 0.0033AB - 0.0292AC -0.0058AD - 0.0433 BC +0.0358 BD +0.0392 CD

TABLE III - Statistical parameters obtained from ANOVA

Response	Adjusted R^2	Model P-value	% CV	Adequate precision
Retention time	0.9209	< 0.0001	4.40	18.12
Peak area	0.9981	< 0.0001	0.484	130.78
Asymmetric factor	0.9989	< 0.0001	0.754	213.98

The statistical parameters of Retention time, peak area and asymmetric factors were obtained from ANOVA and are depicted in Table III. The model P values of all response parameters were found less than 0.0001 i.e. P< 0.05, showing that these models are significant. Adjusted R^2 was found in acceptable limits (R^2> 0.9) that indicates that the experimental model is a good fit with polynomial equations. The adequate precision value of all responses was found greater than

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4 which indicate a sufficient signal and thus the model is significant for the quantification process. The coefficient of variation (C.V.) indicates the reproducibility of the model found within the limit for all responses(% C.V < 10) (Beg, Sahai, Gupta, 2003). Effects of variables on various responses were depicted by 3D plot in figure 2.

The chromatographic conditions were optimized for the determination of rutin within a lesser analysis time (< 4 min).The chromatographic column was chosen based on sharp peaks and number of theoretical plates. Separation of drugs was carried on a C_{18} column reverse phase (250 mm×4.60mm, 5 micron particle size). The samples were analyzed using a mobile phase consisting of methanol: phosphate buffer (pH adjusted to 3.2 with 80% ortho phosphoric acid) in ratio of 60:40 v/v was pumped at a flow rate of 1 mL/min. The injection volume was 20 μL and the UV detection at 254 nm. The retention time of rutin was found to be 3.75 min. Typical chromatogram of rutin was depicted in figure 3.
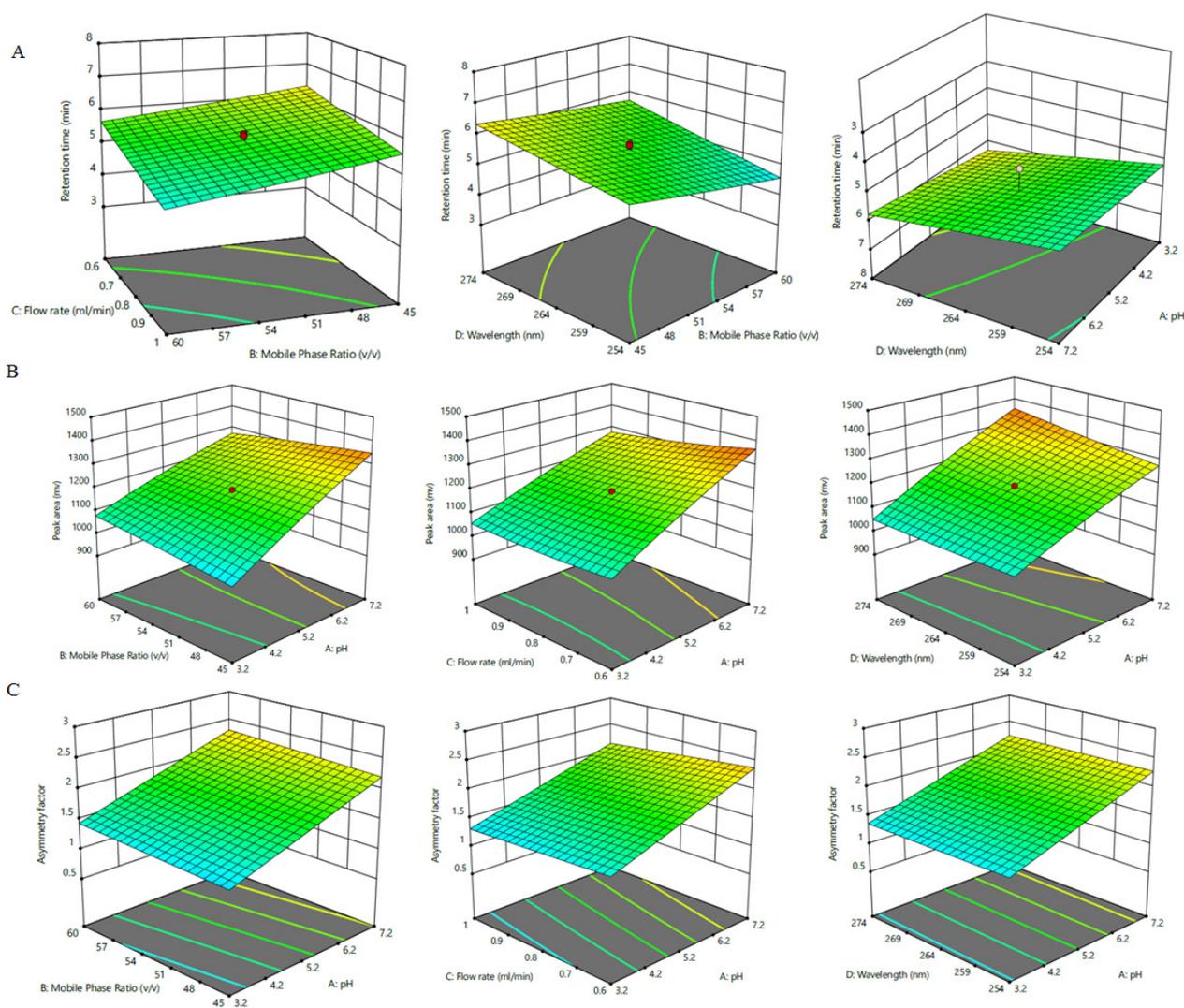

FIGURE 2 - 3D Response surface plots depicting the influence of independent variables on responses.
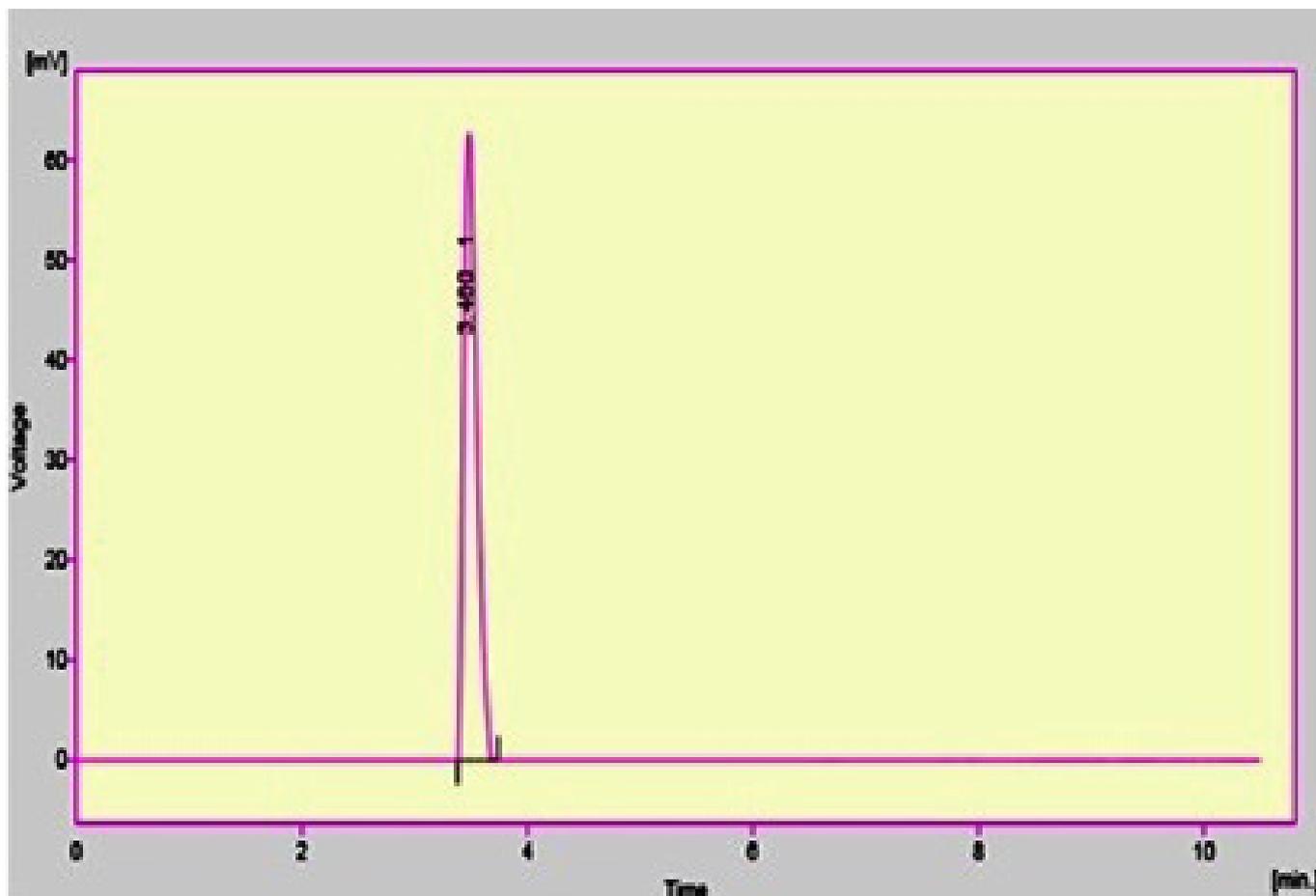
FIGURE 3 - Representative chromatogram of rutin.

Validation

The method was validated for linearity, precision, accuracy, limit of detection, limit of quantification, robustness and system suitability as per the ICH guidelines.

Linearity

The linearity of calibration curves of rutin was determined over the concentration range of 5-35 µg/mL. The R^2 value for Rutin was found to be 0.9897.

Precision of method

The mean values of the intra day and inter day precisions were found to be 1.49 and 0.81, respectively. The results indicate that the selected factors were unaffected by small variations. The resultant %RSD values were found to be within the acceptable limit. The results are depicted in Table IV.

Limit of detection (LOD) and limit of quantitation (LOQ)

The minimum concentration level at which the analyte can be detected (LOD) and LOQ were found to be 0.005 µg/mL and 0.15 µg/mL respectively, and is depicted in figure 4.

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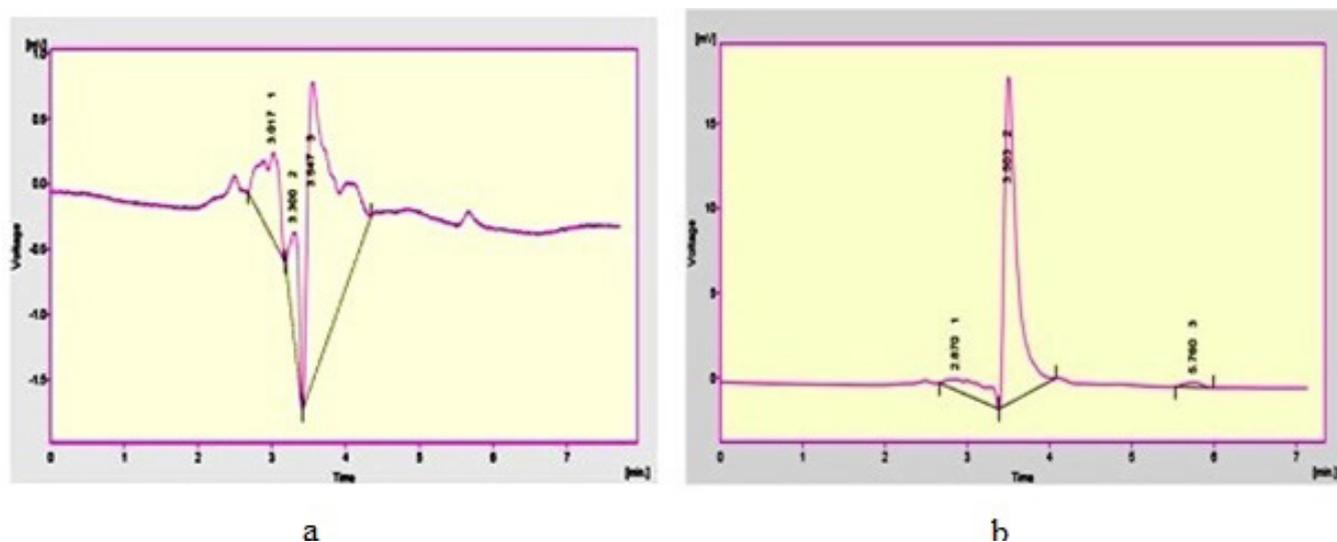
FIGURE 4 - a. LOD b. LOQ chromatograms of rutin.

Accuracy

It was determined by spiking standard solutions with the different concentrations of the formulation at three different levels (LQC, MQC, and HQC). The analyzed sample solutions yielded high recovery values listed in Table IV.

Robustness

The robustness method was determined by varying the operating conditions viz. flow rate ±0.2 mL/min, change in the wavelength ±2 nm. In system suitability studies six replicate injections of one solution was given and %RSD was found to be <2%, listed in Table IV.

TABLE IV - Optimized Chromatographic Conditions

S. No.	Chromatographic Conditions/Validation parameters	Optimized conditions/values
1	Mobile Phase (v/v)	Methanol: Phosphate buffer (pH 3.2) (60:40)
2	Flow Rate	1 mL/min
3	Detection Wave Length	254 nm
4	Retention time	3.400 min
5	Asymmetry	1.465
6	Theoretical Plates	4280

(continuing)

TABLE IV - Optimized Chromatographic Conditions

S. No.	Chromatographic Conditions/Validation parameters		Optimized conditions/values
7	Linearity		5-35 μg/mL; R^2=0.9897
8	System precision (%RSD)		1.45%
9	Precision	Intraday precision	1.534%
		Interday precision	0.523%
10	%Recovery	LQC	98.36%
		MQC	98.08%
		HQC	99.24%
11	LOD (ng/mL)		0.5
12	LOQ (ng/mL)		1.5

Forced degradation studies

Forced degradation studies were carried out by treating the sample under the following stress conditions (Table V, Figure5).

Oxidation

Rutin was extremely prone to degrade under oxidative conditions. Rutin showed seven additional degradation peaks (TableV). Around 83.56% of the drug was degraded under oxidative conditions.

Acid degradation

Rutin was found to undergo intense acid degradation. Reaction in 0.01 N HCl for 3h resulted in the degradation of the drug, with three additional peaks (Table V, Figure 5). In this condition, 92.86% of the drug was degraded. Degradation study depicts among all the stress conditions the drug was found more degradation in acidic condition
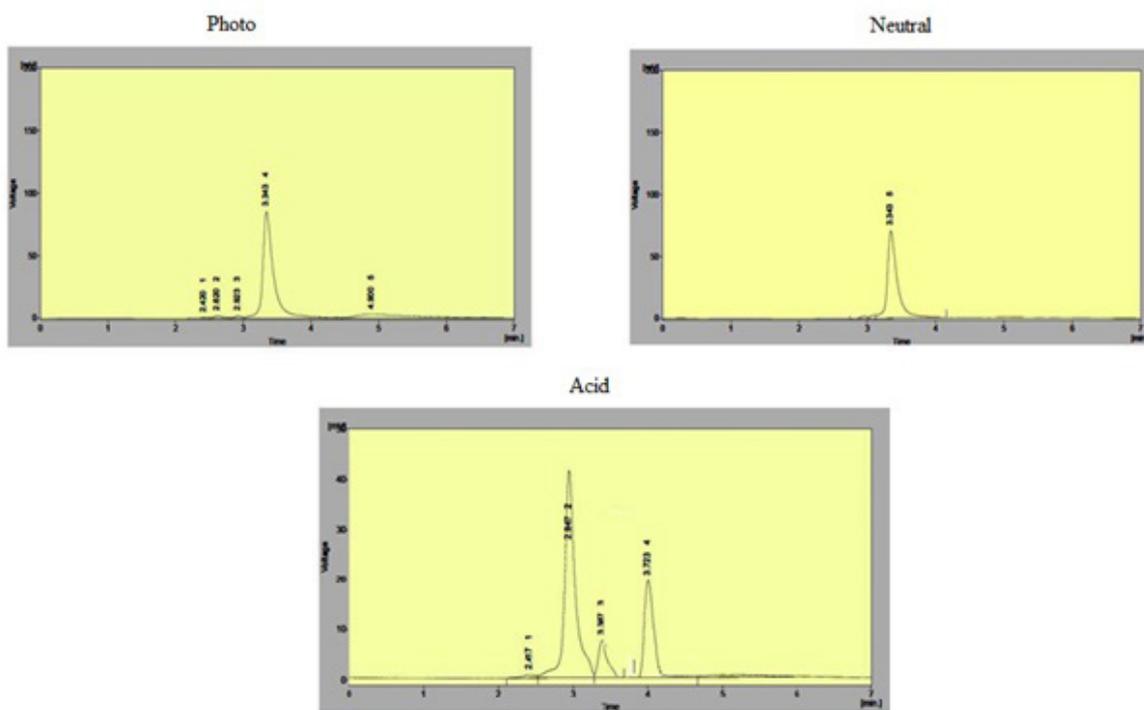
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FIGURE 5 - Representative chromatogram of rutin in various degradation conditions.

Photo degradation

The analyte was stable in photolytic conditions (19.92%) as shown in figure 5. The drug showed three additional peaks (TableV, Figure 5)

Thermal degradation

Under this condition, only 63.44% of the drug was degraded. The drug showed three additional peaks (Table V).

TABLE V - Results of forced degradation studies

Degradation condition	Number of degradation products (*t*R values)	%Drug degraded
Oxidation	7 (2.6,2.9,3.1,3.2,3.6,4.2,5.2)	83.56
Acid	3 (2.4,2.9,3.7)	92.86
Base	7(2.4,2.6,2.9,3.1,3.7,5.0,7.7)	87.11

(continuing)

TABLE V - Results of forced degradation studies

Degradation condition	Number of degradation products (*t*R values)	%Drug degraded
Photo	3 (2.6,2.9,4.9)	19.92
Thermal	3 (2.9, 3.7,4.5)	63.44
Neutral	-	

Assay of rutin in RCSANPs

Validated HPLC method was used for analysis of rutin in RCSANPs. The concentration of rutin in RCSANPs was found to be 2.3 mg/mL. The typical HPLC chromatogram of samples was shown in figure 5. No interfering peaks were observed. The chromatogram clearly indicates that there was no interference from the excipients used in nanoparticles. The RSD values were less than 1%.

CONCLUSION

A new chemometrics assisted stability indicating RP-HPLC method was developed and validated for the determination of rutin with UV detection. Design of Experiment has been employed during the development of the method to minimize retention time and optimise peak area and peak asymmetry. The model equation shown good agreement between predicted values and observed values. The compound eluted within 4 min and thus required less time for the analysis. The method was found to be simple, rapid, accurate, precise and robust and effective for analyzing the rutin in rutin nanoparticles. All the validation parameters showed satisfactory results. The study proved that chemometrics can be effectively coupled with chromatography to enhance separation process. Hence the validated RP-HPLC method can be readily adapted for estimation of rutin in formulations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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