RESEARCH ARTICLE

Design of Experiment Enhanced Development and Validation of RP-HPLC Method for Analysis of Ascorbic Acid and Rutin

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ABSTRACT

This work intends to develop a QbD-based RP-HPLC method and validate rutin (RU) and ascorbic acid (ASC) in combined tablet dosage form. The method is optimized by the central composite design. The independent variables chosen are mobile phase ratio and pH. The dependent variables are retention time for RU (R1), retention time for ascorbic acid ASC (R2), and resolution (R3). Analysis of variance revealed that the method parameters were significant ($p < 0.05$). Waters Alliance-e2695 [C18 (150x 4.6 mm, 3.5)] was used to separate rutin and ascorbic acid using a mobile phase Acetonitrile: Hexane Sulphonic Acid (pH-2.5/OPA) in a 70:30 ratio, the selected wavelength was 215 nm. Method validation and degradation studies were performed ICH guidelines are followed. The approach was determined to be easy, suitable, accurate, precise, and robust for quantitative analysis of ascorbic acid and rutin.

Keywords: Rutin, Ascorbic acid, Quality by Design, Central composite design, Design of Experiment.

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INTRODUCTION

Ascorbic acid (ASC) is chemically known as (5-methyl-2-oxo-1, 3-dioxolen-4-yl) methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl) phenyl] phenyl} methyl imidazol-5-carboxylate.[1] It is a powerful redox agent that fights against bacterial infections, detoxifies reactions, and develops collagen in fibrous tissue, teeth, bones, tissues, etc.[2] Rutin (RU) is chemically known as (quercetin-3-O-rutinoside).[3] It is a flavonoid that is abundant in plants such as passion flowers, buckwheat, tea, apples, and so on; it is an essential nutritional component of foods (Harborne, 1986).[4] RU mostly relieves arthritic pain and includes anti-inflammatory and antioxidant effects.[5] Combining ascorbic acid and rutin is highly beneficial for maintaining normal conditions in the walls of blood vessels and capillaries.[6]

Structures of ASC and RU showed in given below Figure 1.

The quality-by-design approach is becoming a more popular concept for producing and analyzing the quality of pharmaceutical products.[7] Quality by Design is a systematic and risk-based method recommended by the ICH Q8[8,9] and Q9[10,11] principles. Experiment design is a powerful statistical tool for enhancing products/processes and overcoming process/production issues. There are very few analytical techniques are reported for estimating ascorbic acid and rutin.[12,13] However, till date, no QbD-based RP-HPLC technique for Ascorbic acid with Rutin has been disclosed. As a result, the present study was designed for simultaneous QbD-based- RP-HPLC estimation of ASC and RU.

MATERIALS AND METHODS

Instruments and Software

- Waters Alliance-e2695 using a Waters Symmetry C18 (150x4.6mm, 3.5) column with a photodiode array detector.
- Design - Expert software, version 12.0

Materials

Glenmark in Mumbai, India, provided the ascorbic acid and rutin standards. HPLC grade chemicals (Merk India Ltd, Mumbai, India) and water (Milli Q) were purchased. Ruta C 60 tablet was purchased from a local market.

Methods

Preparation of standard and sample solutions

16 mg of Ascorbic acid and 6 mg of Rutin were carefully weighed and placed into a 10 ml volumetric flask with a little amount of diluent, sonicated to dissolve, and volume was brought up to the mark using the same diluent. Pipetted 1 mL of the stock solution into a 10 mL volumetric flask and diluted to the mark with diluent.

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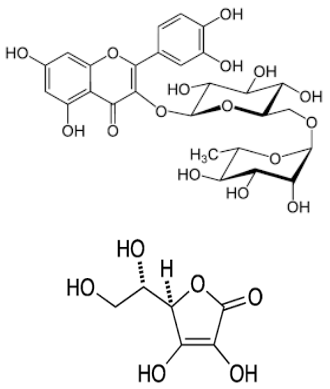

Figure 1: Structure of Ascorbic acid & Structure of Rutin

Sample solution preparation

63.3mg of ASC and RU sample was weighed precisely into a 10mL volumetric flask to that minimal amount of diluent added and sonicated for 30 minutes, centrifuged for 30 minutes to completely dissolve, and volume diluted to the mark with diluent. The solution was then filtered through a 0.45 Injection filter (stock solution). 1-mL of stock solutions was pipetted into a 10 mL volumetric flask and diluted to the specified concentration.

Method Optimization by DoE

At a wavelength of 215 nm, a mobile phase containing a 70:30 combination of acetonitrile and hexane sulphonic acid (pH 2.5 adjusted with OPA) was utilized (Figure 2). The Central composite design was used to optimize. In this case, Mobile phase ratio (X1) and pH (X2) are the independent variables (Factors)., whereas the dependent variables (Responses) are retention time of peak1 (R1), retention time of peak2 (R2), and resolution (R3).

Method Validation

The method was validated as per ICH Q2 [R1] guidelines.[14]

Specificity

The chromatographic separation of ASC and RU with a blank sample and a placebo was observed.

Accuracy

In triplicate injections, a total of 63.3mg of samples were spiked at 50, 100, and 150% concentration levels. The average percentage of recovery was calculated.

Precision

The precision of the method was tested by repeatedly injecting (n = 6) solutions of 160ppm Ascorbic acid and 60 ppm rutin. The %RSD was calculated.

Linearity

The slope, y-intercept, and correlation coefficient (r2) were calculated using least squares regression to determine linearity.

Limit of detection (LoD) and limit of quantification (LoQ)

The drugs' LoD and LoQ are determined by international conference harmonization (ICH) norms.

Robustness

To assess the impact on the procedure, Deliberate changes to the flow rate (FL Minus-0.9ml/min; FL Plus-1.1 mL/min), to the mobile phase (OP Minus-63:37; OP Plus -77:23), and pH (2-3) were made.

Forced degradation study

During the forced degradation studies, the sample was exposed to stress conditions such as acid, alkali, temperature, photolysis, hydrolysis, peroxide, and reduction.[15]

RESULTS

Preliminary Trials for Method Development

Based on preliminary tests (Table 1), the detector wavelength for the HPLC chromatographic process was chosen to be 215 nm.

Method Optimization by QbD

The best separation was noted during preliminary studies with the mobile phase Acetonitrile: Hexane sulphonic acid at 70:30 with pH 2.5. Based on these conditions, factors, and responses were chosen (Table 2).

By incorporating minimum and maximum values of factors into the software. It displayed 13 runs. ANOVA (Table 3) and polynomial equations (Equations-1,2,3) were used to validate responses. The design expert software preferred the quadratic model over the other models because it had the highest least squares regression coefficients for all three responses (R1, R2, and R3). The quadratic model of each response can be represented by the polynomial equation shown below:

$R_1 = 3.78\text{-}2.03 + 0.1690\text{-}0.0172 + 0.4624\text{+}0.0092$ ---- 1

$R_2 = 5.39\text{-}3.33 + 0.1249\text{-}.1375 + 1.38\text{-}0.0665$ ---- 2

$R_3 = 5.52\text{-}2.50 + 0.1257\text{-}0.2275 + 170\text{-}0.2458$ ---- 3

Table 1: Preliminary trails

S. No	*Chromatographic conditions*	*Observation*	*Result*
1	Column: Inertsil ODS (150 mm x 4.6 mm, 3.5 µm). MP: Acetonitrile and 0.1% TFA (80:50) IV:10 µL; FR:1-mL/min; RT :10 minutes WL:215 nm	The resolution was not good	Method rejected
2	Column: Inertsil ODS (150 mm x 4.6 mm, 3.5 µm). MP: Acetonitrile and 0.1% TFA (55:45) IV:10µl, FR:1ml/min, RT :10 minutes WL:215 nm	The resolution was not good	Method rejected
3	Column: Waters Symmetry C18 (150 mm x 4.6 mm, 3.5 µm) MP: Acetonitrile and HSA pH-2.5/OPA (60:40), IV:10 µL, FR:1-mL/min, RT:10 minutes WL:215 nm	Peak splitting was observed	Method rejected

MP-Mobile Phase; IV-Injection Volume; RT-Run Time; WL-Wavelength

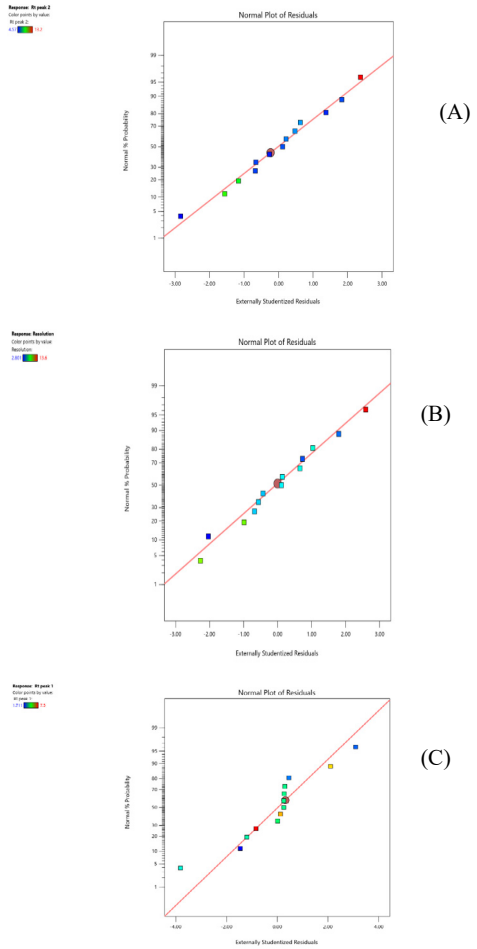

Figure 2: Normal residual Plots of R1, R2, R3 (A) Normal residual plot of R1 (B) Normal residual plot of R2 (C) Normal residual plot of R3

ANOVA found that the p-value for responses R1, R2, and R3 was < 0.2, and the difference between adjusted R^2 and predicted R^2 values was 0.2. As a result, the model is significant. The effect of lack of fit was non-significant. The normal residual plot revealed that there were insignificant deviations in the data and that all of the data was located within the model's line of best fit (Figures 2 A-C). The overall influence of all important

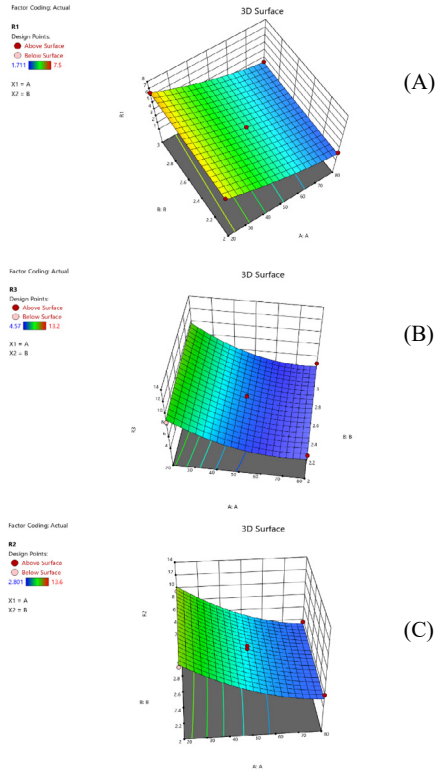

Figure 3: 3D Response surface plots of R1, R2, R3 (A) 3D surface plot of R1 (B) 3D surface plot of R2 (C) 3D surface plot of R3

variables 3D response surface plots indicates the major impact of critical factors on selected responses. (Figures 3 A-C).

Based upon desirability the optimized method was selected. (Table 4)

Optimized Chromatographic Conditions

The optimized chromatographic conditions are shown below. Based on these optimized conditions the standard chromatogram was shown in Figure 4.

- Detector: Waters HPLC with an autosampler and PDA detector.
- Mode of separation: Isocratic mode
- Injection volume: 10 µL

Table 2: Factors

Factor	*Name*	*Type*	*Minimum*	*Maximum*	*Coded low*	*Coded high*	*Mean*	*Std. Dev.*
A	Mobile Phase	Numeric	7.57	92.43	-1 ↔ 20.00	+1 ↔ 80.00	50.00	24.49
B	pH	Numeric	1.79	3.21	-1 ↔ 2.00	+1 ↔ 3.00	2.50	0.4082

Table 3: Statistical summary

Response	*Source*	*F-Value*	*p-Value*	*Adjusted R^2*	*Predicted R^2*
Retention time for peak 1	Model	296.89	<0.0001	0.9920	0.9717
	Lack of fit	5.55	0.0656		
Retention time for peak 2	Model	53.76	<0.0001	0.9565	0.8510
	Lack of fit	4.64	0.0863		
Resolution	Model	33.44	<0.0001	0.9311	0.7578
	Lack of fit	5.49	0.0668		

Table 4: Desirability table

S. No.	*A*	*B*	*R1*	*R2*	*R3*	*Desirability*	
1	80.000	2.000	2.074	3.387	4.572	0.937	Selected
2	79.722	2.000	2.084	3.391	4.562	0.936	
3	80.000	2.108	2.103	3.410	4.645	0.932	

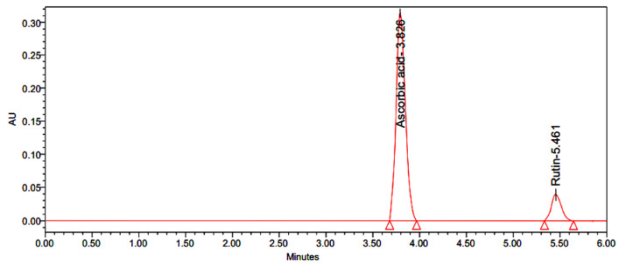

Figure 4: Chromatogram of ASC and RU (Standard)

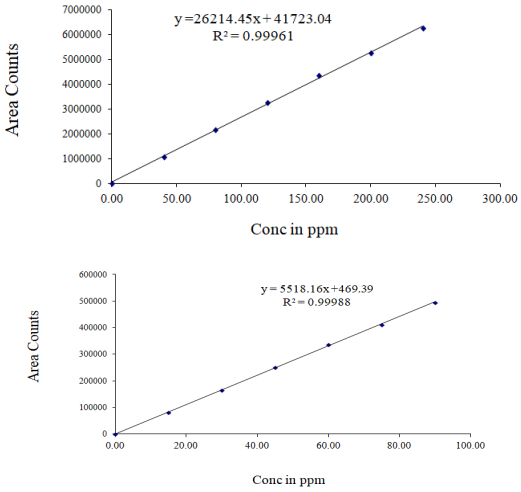

Figure 5: Calibration curves for RU & ASC

Table 5: Summary of validation parameters

Parameters	*Ascorbic acid*	*Rutin*	*Limits*
Linearity range (µg)	40-240 µg/mL	15-90 µg/mL	R^2 0.999
Regression coefficient	0.99961	0.99988	
Regression coefficient (y=mx+c)	y = 26214.45x +41723.04	y =5518.16x + 469.39	
Assay (%Mean assay)	99.9%	99.8%	98–102%
System suitability (%RSD)	0.13	0.12	%RSD<2
Specificity	Specific	Specific	No interference of peaks
System precision (%RSD)	0.13	0.12	%RSD<2
Method precision (%RSD)	0.28	0.77	%RSD<2
Intermediate precision (%RSD)	0.22	0.66	%RSD<2
Accuracy (%recovery)	99.87	99.70	98-102%
LoD	0.48 µg/mL	0.18 µg/mL	--
LoQ	1.6 µg/mL	0.60 µg/mL	--
Flow minus(0.9 mL/min)	0.377	0.091	%RSD<2
Flow plus(1.1 mL/min)	0.261	0.355	
pH change low	0.361	0.765	
pH high	0.567	0.437	
Organic phase plus (77:23)	0.197	0.050	
Organic minus (63:37)	0.601	0.606	

- Mobile Phase: Acetonitrile: HSA pH-2.5/OPA (70:30)
- Column: Waters Symmetry C18 (150x4.6 mm, 3.5 µ)
- Detection Wavelength: 215 nm
- Flow Rate: 1-mL/min
- Runtime: 6 minutes
- Temperature: 25ºC

Method Validation

The optimized method was validated using different parameters as per ICH guidelines. Results were summarized in Table 5

System Suitability

The suggested method's system suitability was proven by the %RSD of various parameters such as plate count, retention time (Rt), and tailing factor.

Specificity

With placebo and blank, there was no interference in the chromatographic separation of Ascorbic acid and Rutin.

Accuracy

The %recovery was assessed by analyzing the sample at three different concentration levels, i.e., 50, 100, and 150%. The obtained results demonstrated that the validated method is accurate.

Precision

%RSD peak areas for ASC and RU were found to be 0.22 and 0.66, respectively. The %RSD values of system precision and method precision are within limits i.e., <2%, indicating that the method is precise.

Table 6: Degradation

Ascorbic acid				*Rutin*		
Degradation results	*%Deg*	*Purity angle*	*Purity threshold*	*% Deg*	*Purity angle*	*Purity threshold*
Control	0	0.599	7.724	0	1.656	6.522
Acid	12.7	0.584	7.728	11.8	1.627	6.524
Alkali	11.9	0.583	7.732	10.5	1.634	6.537
Peroxide	14.0	0.571	7.739	13.1	1.648	6.587
Reduction	1.4	0.576	7.714	2.5	1.685	6.575
Thermal	10.4	0.558	7.784	3.9	1.666	6.579
Photolytic	3.5	0.582	7.721	2.8	1.671	6.553
Hydrolysis	0.7	0.524	7.736	1.9	1.664	6.565

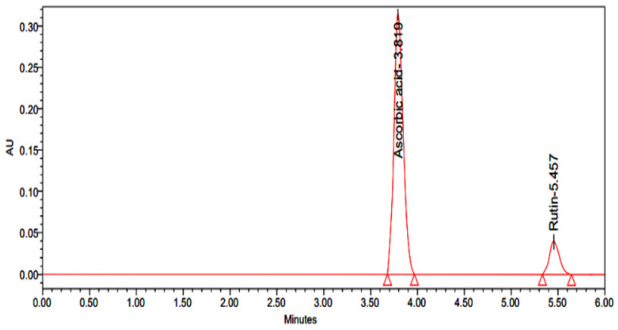

Figure 6: Sample chromatogram

Robustness

During robustness investigation, we found changes in flow rate (0.9-1.1), the ratio of the organic phase (OP Minus-63:37; OP Plus -77:23), and pH varied from 2-3 did not affect results such as retention time, plate count etc.

Linearity

Rutin showed linearity concentrations ranging from 15 to 90 µg/mL and ascorbic acid concentrations ranging from 40 to 240 µg/mL. The correlation coefficient values are 0.99988 and 0.99961 respectively (Figure 5).

LoD and LoQ

Ascorbic acid LoD and LoQ values were 0.48 and 1.6 µg/mL, respectively, whereas Rutin was 0.18 and 0.60 g/mL. It shows the sensitivity of the method.

Study of forced degradation

The result of degradation studies is depicted in the table. The highest degradation was in peroxide i.e., 14% and the lowest degradation was in hydrolysis i.e., 0.7%. The method validation and degradation parameters were summarized in Table 6.

Assay

Assay were calculated for ASC and RU was found to be 99.9 and 99.8% respectively. The chromatogram of sample was showed in (Figure 6)

DISCUSSION

A simple, rapid, and accurate RP-HPLC analytical technique for the simultaneous measurement of ASU and RU was successfully established using the QbD methodology (CCD). Following that, it was validated to correspond with ICH guidelines. Based on preliminary trials the critical factors and their responses (minimum and maximum) were identified. Finally, the method was optimized by response surface methodology, which gave a comprehensive understanding of each factor's relationship with the response as well as any interactions between them. The optimized method was validated according to ICH guidelines. All of the validated parameters satisfied the acceptance criteria. The validated method for determining ASC and RU was shown to be linear, precise, accurate, specific, and robust. Drug substances were subjected to different stress conditions. In all conditions, less than 20% degradation was observed which shows the method is stability indicating. Hence, the established approach appears to be suitable for quality control in the pharmaceutical industry.

REFERENCES

1. Merck Index, Encyclopedia of Chemicals and Pharmaceuticals, 12th ed, Merck & Co Inc., Whitehouse station N.J, USA, 1996, 867 and 8456.
2. Helms. S, Miller. A, Altern Med. Rev 11 (2006) 196–207.
3. Vijayaraj S, Palei NN, Archana D, Lathasri K, Rajavel P. Quality by design (Qbd) approach to develop stability indicating HPLC method for estimation of rutin in chitosan-sodium alginate nanoparticles. Braz J Pharm Sci [Internet]. 2020;56:e18793. Available from: https://doi.org/10.1590/s2175-97902020000318793
4. Harborne, J.B. (1986), *et.al.,* Encyclopedia of Plant Physiology, Springer, Berlin, Vol. 8, pp. 329-95.
5. Marjan NA, Taghi. N, Farivar, Esmail. A, Hamid Reza. S, Mehdi. S, Mina Lotfizadeh, Parisa. B. Effects of rutin on oxidative stress in mice with kainic acid-induced seizure. Journal of Integrative Medicine. 2013;11(5): 337– 342. Available from- https://doi.org/10.3736/jintegrmed2013042.
6. Ensminger. A.H. Food & Nutrition Encyclopedia, second ed.1, 1994 206–210.
7. Amit S. Patil, Anil M pethe. Quality by Design (QbD): A new concept for the development of quality pharmaceuticals' J Pha. Qua. Assu.2013: 4(2):13-19.
8. International Conference on Harmonization (ICH) Q8 (R2), Harmonized tripartite guideline, pharmaceutical development, Geneva, 2009.
9. US Food and Drug Administration. Guidance for industry: Q8 Pharmaceutical Development, US Department of Health and Human Service, FDA, Rockville, MD, May 2006.

10. International Conference on Harmonization (ICH) Q9, Harmonized tripartite guideline, Quality Risk Management, ICH, Geneva, Switzerland, 2005.
11. US Food and Drug Administration. Guidance for Industry: Q9 Quality Risk Management, US Department of Health and Human Service, FDA, Rockville, MD, June 2006.
12. Tamer Z Attia.Simultaneous determination of rutin and ascorbic acid mixture in their pure forms and combined dosage form. Spectrochemical Acta Part A Molecular and Biomolecular Spectroscopy.2016; 169:82-86. Available from- http://dx.doi.org/10.1016/j.saa.2016.06.030
13. Kareem M. Younes, Mai A. Basha and Maissa Y. Salem. Spectrophotometric and chromatographic methods for the simultaneous determination of rutin and ascorbic acid in their pharmaceutical formulation. Der Pharma Chemica, 2014; 6(2):111-121.
14. ICH guideline Q2 (R1): Validation of analytical procedures. In International Conference on Harmonisation, IFPMA, Geneva, 2003.
15. Iram F, Iram H, Azhar Iqbal, Asif Husain. Forced degradation studies. J Anal Pharm Res. 2016;3(6):11–12. Available from-10.15406/japlr.2016.03.00073