RESEARCH ARTICLE

Development and Validation of RP- HPLC Method for Baricitinib using Quality by Design Approach and its Application to Stability Studies

Mannurkar M. M.*, Hamrapurkar P. D.

Prin. K. M. Kundnani College of Pharmacy, Cuffe Parade, Mumbai

Received: 16th July, 2020; Revised: 27th August, 2020; Accepted: 17th October, 2020; Available Online: 25th March, 2021

ABSTRACT

The present paper reports a simple, sensitive, precise, and robust reverse phase high performance liquid chromatography (RP-HPLC) method using quality by design (QbD) approach and has been developed and validated for analysis of baricitinib in bulk drug. Design of experiment (DoE) using a Box Behnken design approach was employed for method development and optimization where the critical method variables like mobile phase pH, gradient time, flow rate, and the interaction effects on the drug response parameters i.e., retention time, NTP, asymmetry factor were evaluated.

Method: The optimal chromatographic separation was carried out by gradient elution mode on a ZORBAX ODS 250x4.6mm, 5 um column using ammonium formate buffer (pH 7): acetonitrile (ACN) as mobile phase at 25℃ with a flow rate of 1-mL/min and injection volume of 10ul. Quantitation was achieved using UV detection at 251nm on Waters Alliance 2695 system with a PDA detector.

Result: The retention time for baricitinib was found to be 8.14 minutes. The calibration curve was linear over a range of 1-3 ug/mL with limit of detection (LoD) and limit of quantitation (LoQ) values found to be 0.1 ug/mL and 0.5 ug/mL, respectively. The percent recovery was found to be within an acceptable limit of 98-102%. Forced degradation studies were carried out under acid, base, oxidative, photolytic, and thermal conditions indicating the well-resolved peak of drug and degradation products.

Conclusion: The optimized chromatographic method was validated as per ICH Q2 (R1) guidelines and proved to be accurate, precise, specific, linear, and robust; also, all the parameters were within acceptance criteria. Forced degradation studies showed that the method developed was specific and can be employed for monitoring the stability of Baricitinib.

Keywords: Baricitinib, Box Behnken design, Forced degradation, QbD approach, RP-HPLC, Stability-indicating.

International Journal of Pharmaceutical Quality Assurance (2021); DOI: 10.25258/ijpqa.12.1.6

How to cite this article: Mannurkar MM, Hamrapurkar PD. Development and Validation of RP- HPLC Method for Baricitinib using Quality by Design Approach and its Application to Stability Studies. International Journal of Pharmaceutical Quality Assurance. 2021;12(1):40-47.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Rheumatoid arthritis is a chronic disease affecting over 1% of the world population, which can be treated using oral drugs. Baricitinib is one such drug used as second-line therapy for moderate to severe active rheumatoid arthritis in adults whose disease is not well controlled using medications called tumor necrosis factor (TNF) antagonists. It is an inhibitor of Janus kinase (JAK) and acts by blocking the enzyme subtypes JAK1and JAK2. It is sold under the brand name Olumiant and baricinix.

The IUPAC name of baricitinib is 2-[1-Ethylsulfonyl-3-[4-(7*H*-pyrrolo [2,3-d] pyrimidin-4-yl) pyrazol-1-yl] azetidin-3-yl] acetonitrile Figure 1. The molecular formula is $C_{16}H_{17}N_7O_2S$. It has a molecular weight of 371.4 g/mol. It is very poorly soluble in water, methanol, and acetonitrile but it is soluble dimethyl sulfoxide.[1-3]

In the literature survey, it was found that there is a method available on bioanalytical studies for simultaneous estimation of baricitinib

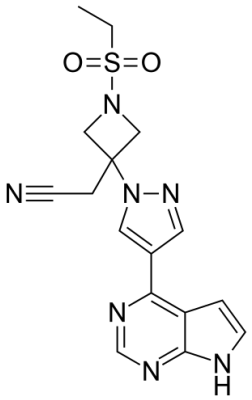

Figure 1: Chemical structure of Baricitinib

**Author for Correspondence: mrunali271997@gmail.com*

with methotrexate using liquid chromatography with tandem mass spectrometry (LC-MS),[4] there is a reverse-phase stability-indicating HPLC method of related substances of baricitinib[5] and a spectroscopic method for the estimation of Baricitinib.[6]

Hence, the literature survey reveals that no method has been developed for the estimation of baricitinib through a QbD approach. The reported stability-indicating method has not reported any risk assessment parameters. So, an attempt has been made to develop a simple, sensitive, precise, and robust RP-HPLC method for baricitinib in bulk drug through the QbD approach and its application to stability studies.

MATERIALS AND METHODS

Drug and Chemicals

The active pharmaceutical ingredient Baricitinib was procured from Cipla Limited, Mumbai as a gift sample. HPLC water was prepared using a Millipore Milli Q plus purification system. Ammonium formate AR grade (SDFCL), dimethyl sulfoxide (DMSO) HPLC grade from Merck, Acetonitrile, and Methanol HPLC grade (Qualigens, Thermo Fisher Scientific Pvt. Ltd.) were purchased.

Instrument

The liquid chromatography (LC) equipment used for the development was Waters Alliance 2695 equipped with a quaternary pump and a Waters 2996 photodiode array (PDA) detector. Data acquisition was carried out on Empower Pro software. Also, analytical balance (Citizon CY 204), pH meter (Thermo Electron Company Orion 420A+), and a sonicator (Ultra sonicator) were used.

The design expert software 12.0.9.0 free trial version was used to plan the experiment's design.

Preparation of Mobile Phase A: A total of 0.63 gm of ammonium formate was weighed accurately and transferred in 1000 mL beaker volume was made up with water and then sonicated further the pH was adjusted to 7.0 with dilute ammonia.

Mobile Phase B: Acetonitrile

Diluent: Methanol

Preparation of Blank: Transfer 1.0 mL of dimethyl sulfoxide into 10 mL volumetric flask and makeup to the mark with methanol (Solution A). 1 mL of solution A was diluted to 10 mL with methanol (Solution B). Further, 1 mL of solution B was diluted to 10 mL with diluent (Solution C). Solution C was used as a blank.

Preparation of standard stock solution: 10 mg of Baricitinib was accurately weighed and transferred to a 10 mL volumetric flask. The sample was dissolved in 1mL of DMSO and volume was made up to the mark with methanol (1000 µg/mL) (Solution A). 1 mL of solution A was diluted to 10 mL with methanol (100 µg/mL) (Solution B). Further, 1 mL of solution B was diluted to 10 mL with methanol (10 µg/mL) (Solution C). Solution C was used as a working solution to prepare further dilutions for carrying out validation procedures.

Solution Preparation for Degradation Studies: About 2 mL of solution A of baricitinib was taken separately and treated with acid, base, oxidative conditions separately at different Concentrations. After degradation, the solution was neutralized with the same molar concentration solution for acid, base hydrolysis. All the solutions were diluted to obtain 200ppm of baricitinib. Later it was injected into the LC system.

ANALYTICAL METHOD DEVELOPMENT

Initial Method Development

1. Selection of Analytical wavelength –

The 10 µg/mL solution of baricitinib was scanned in the wavelength range of 200-800nm. The maximum absorbance was found at three wavelengths 225 nm, 251 nm, and 310 nm respectively of which 251 nm was selected for quantitation.

2. Choice of Column and Mobile phase-

The drug was eluted using stationary phases like C8, C18, and various mobile phases containing Trifluoroacetic acid, Ammonium acetate, and Ammonium formate with different pH 2–7.5, and organic modifiers like methanol and acetonitrile were tried.[7]

Design of Experiment

The screening phase using Design Expert 12 software was performed by employing 3 factor- 3 level Box–Behnken design to study the interaction and quadratic effects of critical factors on the specified response variables. In the present study Box-Behnken design (BBD) comprising 15 experimental runs with 12 factorial points and 3 center points per block was used. This design was specifically selected as it has fewer runs than 3 level factorial designs. Based on the risk assessment, the critical method parameters (CMPs) or method variables were identified as pH of the mobile phase, the flow rate, and gradient time.[8] The method responses also called critical quality attributes (CQAs) selected were retention time of the drug, Number of theoretical plates (NTP), and the asymmetry factor.[8] The responses obtained after carrying out the 15 experimental runs were fed back into DoE software.

Validation of Optimized Method[9-11]

1) Specificity

Specificity was evaluated by injecting a blank solution or placebo and recording the chromatogram. Peak purity was also established to check for spectral difference, implying that two or more peaks are co-eluting.

2) System suitability

System suitability tests are run to ensure that the instrument can adequately perform intended application on a daily basis. This test was performed by injecting 6 replicates of working solution (2 ug/mL) of baricitinib, and the mean obtained was then checked to meet the acceptance criteria of system suitability parameters.

3) Limit of Detection and Limit of Quantitation

To determine LoD and LoQ Signal to noise ratio method was used. It was performed by comparing the measured signal of

the analyte (known low concentration) with that of the baseline noise. The S/N ratio for LoD should be at least 3:1 and for LoQ should be at least 10:1

4) Linearity

Linearity was evaluated over the range of 50–150% of the working concentration 2µg/mL in six replicates (1 to 3 µg/mL) for Baricitinib. The Calibration curve was plotted for Response (Area) vs. Concentration (Amount). The correlation Coefficient (r2) and y-intercept were obtained from the graph.

5) Accuracy

Accuracy was determined based on the recovery of known amounts of analyte. This was performed by spiking analytes in blank matrices. The accuracy was assessed using a minimum of 9 determinations over a minimum of 3 concentration levels at 50, 100 and 150% of the working level. The % recovery was obtained by putting the values of areas of peak obtained in the calibration curve equation to obtain the values of concentration recovered.

6) Precision

Precision was considered at three levels 50%, 100%, and 150% of working concentration (1, 2, and 3 µg/mL), with a minimum of 6 determinations at each level. Intraday precision was performed on the same day at different time intervals and Interday precision was carried out on two consecutive days.

7) Solution Stability

The stability of the drug solution was evaluated for three different levels of working concentration in replicates of 3. The samples were stored at a refrigerated temperature of 10–15°C. The analysis was performed at initial time 0 and then after 6, 9, and 24 hours.

Forced Degradation Studies

Forced degradation was carried out to prove that the method is stability-indicating and show the established method's specificity. Stress degradation studies of baricitinib were carried out under hydrolysis (acid and base), oxidation, photolytic, and thermal condition.[12,13] The drug was treated with 0.1N HCl for 15 min at room temperature to perform acid degradation, 0.1 N NaOH for 15 min. at room temperature for base degradation and 10% H_2O_2 for 6 h at room temperature for peroxide degradation. The standard drug solution was heated at 85°C for 6 hours to study heat degradation. The drug was exposed to sunlight for 8 hrs to study photolytic degradation. The chromatograms of forced degradation studies of baricitinib are shown in Figure 12 and 13.

RESULTS AND DISCUSSION

Method Development

Selection of Column and Mobile Phase

C8 column showed broad peak shape, and poor retention of the analyte as the analyte is hydrophobic in nature it showed very good retention on the reverse phase C18 column. The method development was initiated using methanol as an organic modifier, but it showed baseline noise, high backpressure, and is more viscous than acetonitrile so acetonitrile was preferred over methanol as an organic modifier. Trifluoroacetic acid showed drug ionization also interferes with LC-MS signals; ammonium acetate showed high baseline noise so ammonium formate was preferred.

Software Aided Method Optimization

The design of the experiment was established at 3 levels for 3 factors. The 15 experiments with 12 factorial points and 3 center points were analyzed and summarized in Table 1.

The response factors were analyzed using quadratic equations, 2D contour plots, 3D response surface maps, predicted vs. actual plots, and ANOVA table.

Table 1: Box Behnken design for the screening of method parameters

Sr. no.	*Run order*	*Factor 1 A: pH*	*Factor 2 B: gradient time for %5B to %100B*	*Factor 3 C: flow rate*	*R1: retention time*	*R2: NTP*	*R:3 Asymmetry factor*
1	1	7	10	0.8	7.49	33506	1.17
2	2	6.5	12	0.8	9.46	39389	1.35
3	3	6.5	12	1.2	7.1	26567	1.16
4	4	7.5	12	1.2	8.1	28790	1.26
5	5	7	14	0.8	11.25	48216	1.56
6	6	7.5	10	1	6.9	30531	1.11
7	7	7	14	1.2	9.57	34238	1.4
8	8	7	12	1	8.124	40543	1.2
9	9	7	12	1	8.1	41003	1.19
10	10	7.5	12	0.8	9.48	39387	1.49
11	11	6.5	14	1	10.2	38891	1.38
12	12	7.5	14	1	10.6	43275	1.53
13	13	6.5	10	1	6.2	32649	1.05
14	14	7	10	1.2	5.4	27635	0.97
15	15	7	12	1	8.12	40172	1.14

The classical polynomial quadratic equation in terms of coded factors for each selected CQAs estimating regression coefficients are shown in the following equations[14]:

$$\text{Retention time} = 8.11+0.2650A+1.95B-0.9388C-0.0750AB+0.2450AC+0.1025BC + 0.2339\ A^2+ 0.1264\ B^2+0.1864C^2 \quad \text{....equation (1)}$$

$$\text{NTP} = 40572.67+560.87A+5037.37B-5408.50C+1625.50AB+556.25AC-2026.75BC - 3300.83A^2-935.33\ B^2-3738.58\ C^2 \quad \text{....equation (2)}$$

$$\text{Asymmetry factor} = 1.18+0.0562A+0.1962B-0.0975C+0.0225AB-0.010AC +0.01BC +0.0654\ A^2+0.0254\ B^2+0.0729\ C^2 \quad \text{....equation (3)}$$

The independent variables A, B, C indicate the average response of varying one factor at a time from its low to a high level. The interaction term (AB, BC, and AC) shows how the response changes when two factors are concurrently altered. The polynomial quadratic terms (A^2, B^2, and C^2) were added to examine nonlinearity.

The method variables A, B, C, and quadratic terms with a positive coefficient are directly proportional and those with a negative coefficient are inversely proportional to the response. From equation (1, 2, and 3) it was observed that positive signs of factor A and B indicate that retention time, NTP, and Asymmetry factor increases with increase in pH and gradient time whereas, the negative sign of factor C indicates that they all decrease with increase in flow rate.

When all the terms were compared, the coefficient value of variable B (1.95, 5037.37, and 0.1962) was found to be highest, and hence gradient time was considered to be a major contributing factor for the incredible effect on retention time (R1), NTP, and Asymmetry factor.

The 3D surface response and contour plots of the quadratic model given by the design-expert software provide the interactive relationship of two factors on the response by keeping the third factor constant and analyzed to define design space. The 3D surface plots of the interaction effect of pH and gradient time indicate significant model terms ($p < 0.05$) and depicts the effect on the retention time, NTP, and asymmetry factor, which is shown (Figures 2–7) along with predicted vs. actual plot for each response.

The ANOVA results shown in Table 2 suggest that the response surface quadratic model for the three responses proves that the relationship between response and variables is statistically significant. The value of the correlation coefficient (R_2) for all CQAs indicates a perfect fit of the model, which implies that

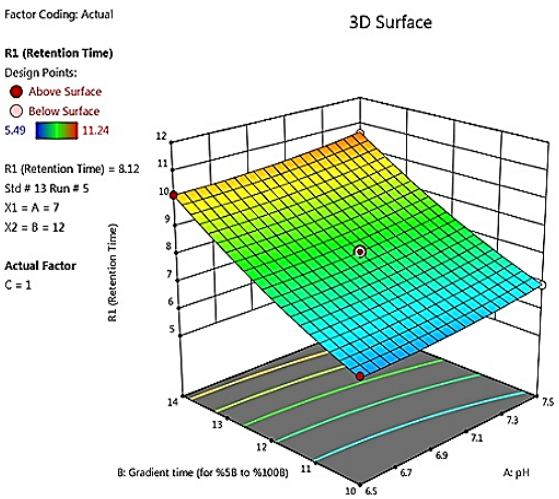

Figure 2: Effect of pH and gradient time on the retention time of Baricitinib

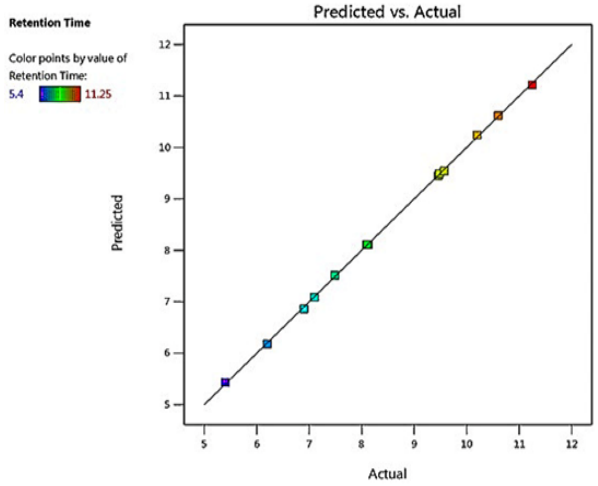

Figure 3: Actual vs. Predicted plot for a Retention time of Baricitinib

Table 2: ANOVA Summary

ANOVA parameters	*R1: Retention Time*	*R2: NTP*	*R3: Asymmetry factor*
R- square value	0.9998	0.9949	0.9928
Adjusted R-square	0.9994	0.9857	0.9798
Predicted R-square	0.9969	0.9271	0.9478
Adequate Precision	178.7485	34.8575	28.2588
Standard deviation	0.0396	750.63	0.0255
C.V. %	0.4715	2.07	2.01
PRESS value	0.1211	4.031E+07	0.0235
F- value	2743.64	108.50	76.46
P-value	<0.0001	< 0.0001	< 0.0001
Model	Significant	Significant	Significant
Lack of fit	15.17	4.75	0.38

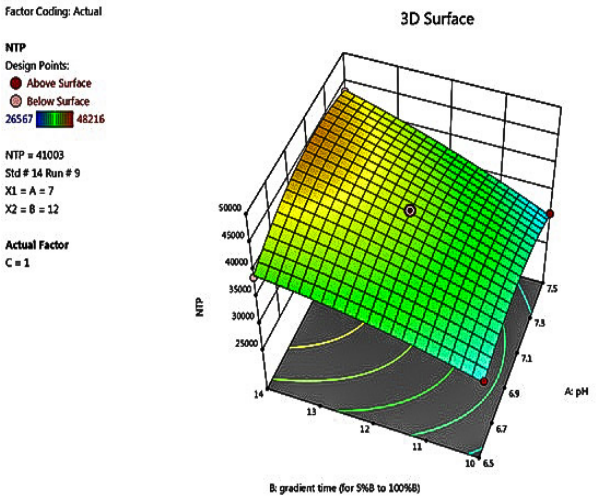

Figure 4: Effect of pH and gradient time on NTP of Baricitinib

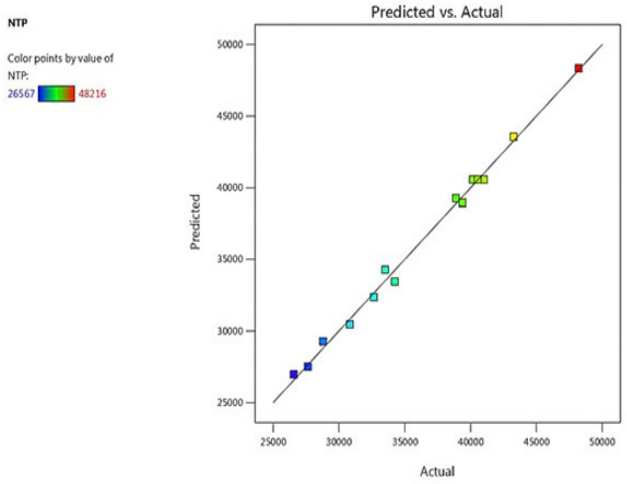

Figure 5: Actual vs. Predicted plot for NTP of Baricitinib

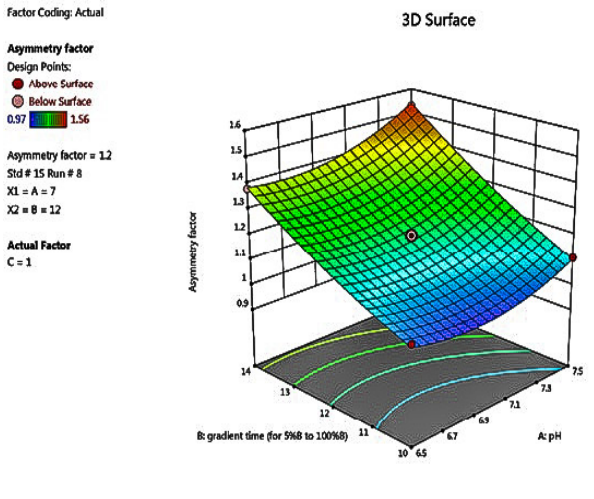

Figure 6: Effect of pH and gradient time on Asymmetry factor of Baricitinib

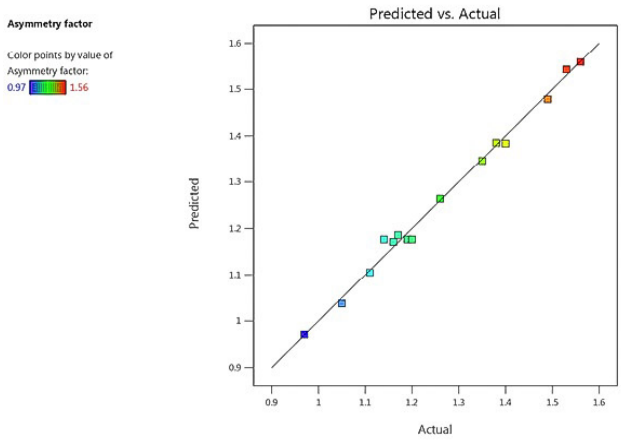

Figure 7: Actual vs. Predicted plot for Asymmetry factor of Baricitinib

the model is valid. The more valuable marker of the variation in response variables was adjusted *R*-squared value, while predicted *R*-squared indicated how well the model could predict future data, relatively high values of adjusted and predicted *R*-squared concluded that the applied statistical model effectively predicted the response. The F-values so high implies the models are significant and there is only a 0.01% chance that F-values this large could occur due to noise. p-values less than 0.0500 indicate model terms are significant. If the value is greater than 0.100 that indicates the model terms are not significant. Adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable and indicates an adequate signal. So that model can be used to navigate the design space.[14,15]

Identification for Optimum Method Condition

The optimum method condition was identified by numerical optimization, simply by setting the criteria for anticipated targets, i.e., maximum theoretical plates, retention time in range 6-9 minutes, and minimum peak tailing factor. The numerical optimization suggested a solution with Desirability function close to one, which was selected as the optimum solution. The optimized method conditions were found to be mobile phase pH 7, gradient time 12 minutes, and flow rate of 1-mL/minutes as shown in Figure 8 with the desirability of 0.665. The graphical optimization showed that the optimized solution was found within operable analytical design space.

Verification of Experiment at Optimum Condition

The optimum method was run, Table 3 shows the actual values were found to be within the 95% confidence interval of predicted values.

Final Optimized Chromatographic Conditions

The chromatographic separation was achieved using Waters Alliance 2695 equipped with (PDA) detector set at 251 nm using Zorbax ODS (250mm x 4.6mm x 5µm) and a mobile phase consisting of Ammonium formate (pH- 7) and acetonitrile. The sample volume of 10 µL was injected into the system that was operated using linear gradient elution at a flow rate of 1-mL/min and run time 25 minutes. The column oven was conditioned at 25°C. The needle was washed using Methanol: Acetonitrile: Dimethyl sulfoxide (45:45:10) before every injection. The gradient program is mentioned in Table 4.

Table 3: Verification of optimized conditions.

Method response	*Predicted*	*95% Confidence interval (low)*	*95% Confidence interval (high)*	*Actual*
Retention time	8.1147	8.05704	8.1723	8.137
NTP	40572.7	39481.3	41664.1	40635
Asymmetry factor	1.1767	1.13954	1.2137	1.2128

Table 4: Gradient Program for baricitinib

Time	*%A (buffer)*	*%B (ACN)*
0.00	95	5
12.00	0	100
15.00	0	100
16.00	95	5
25.00	95	5

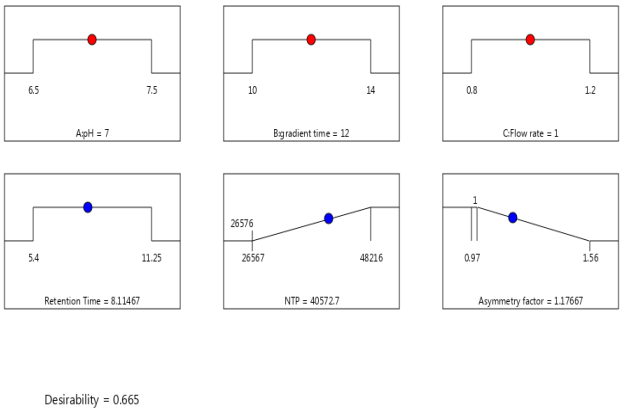

Figure 8: Optimization and prediction of method responses by model

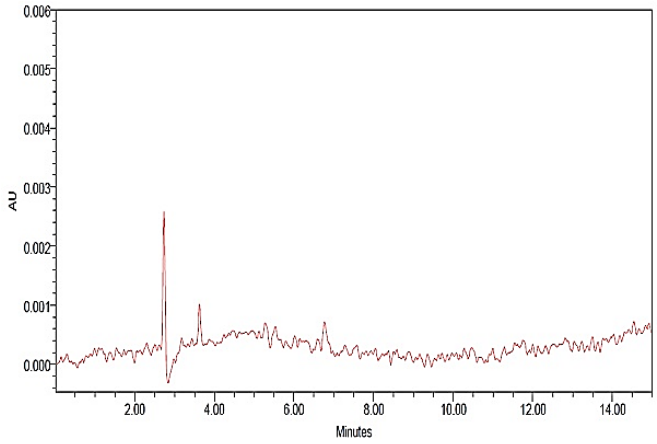

Figure 9: Chromatogram of blank

Validation of Optimized Method

Specificity studies showed that there was no interfering peak at the retention time of the analyte peak as shown in Figure 9. The purity threshold is greater than the purity angle, indicating no co-eluting peak (Figure 10). The calibration curve (Figure 11) shows that the method is linear in the range of 1- 3 µg/mL and an excellent correlation exists between concentration and peak area. The solution stability studies showed that drug solutions were found to be stable for up to 24 hours. The data represented in Table 5 summarizes all the validation parameters.

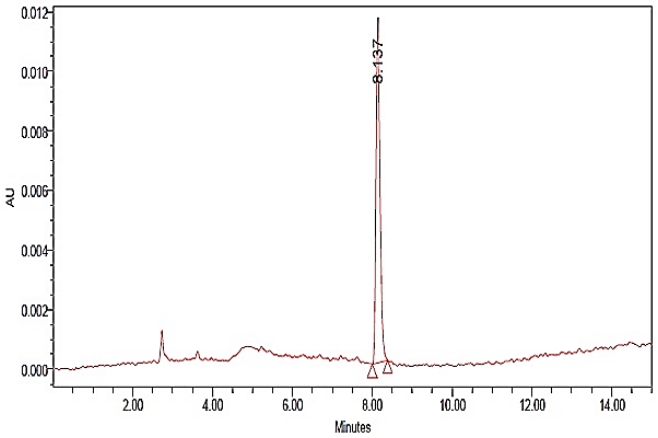

Figure 10: Chromatogram of working solution (2 µg/mL)

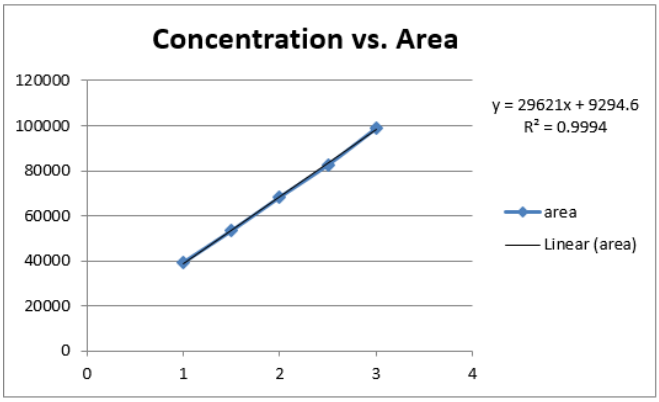

Figure 11: Calibration curve for Baricitinib

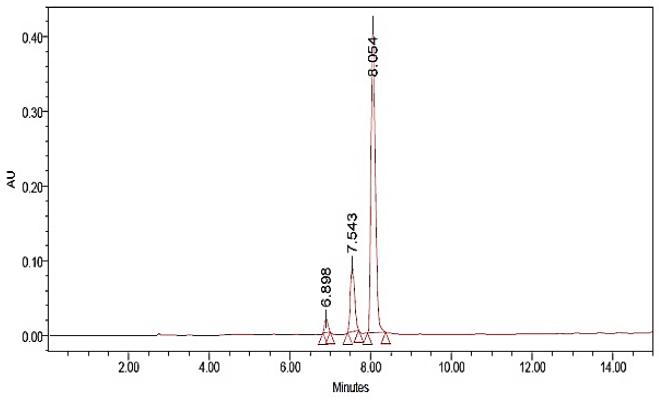

Figure 12: Representative chromatogram for Acid hydrolysis of Baricitinib

					Peak purity	
Peak name	*Rt*	*area*	*USP tailing*	*NTP*	*Purity Angle*	*Purity Threshold*
Baricitinib	8.137	68949	1.213	40635	0.583	0.960

Table 5: Validation Summary of Baricitinib

Validation parameter	*Baricitinib*	*Acceptance criteria*
System suitability	NTP- 43515 %RSD- 0.23% Tailing factor- 1.22	NTP >2000 %RSD <1.0% for 5 replicates Tailing factor ≤ 2.0
specificity	No interference was found at Rt of drug peak	-
LOD	0.1µg/mL	S/N ratio 3:1
LOQ	0.5µg/mL	S/N ratio 10:1
Linearity	R^2 = 0.999 Y=29621x +9294	$R^2 \geq 1$
Range	1µg/mL - 3µg/mL	-
Accuracy(% Recovery)	99.55%	98-102%
Intraday Precision (%RSD)	0.48%	≤ 2%
Interday precision (%RSD)	0.52%	≤ 2%
Solution Stability (%RSD)	1.15%	≤ 2%

Table 6: Percentage of degradation of Baricitinib using various stress conditions.

Degradation condition	*Rt. of degradation product (min)*	*% of degradation*
Acid hydrolysis	6.898,7.543	19.71
Base hydrolysis	6.921,7.563	21.66
Oxidative	No degradation	--
Photolytic	No degradation	--
Thermal	No degradation	--
Total degradation		43.83

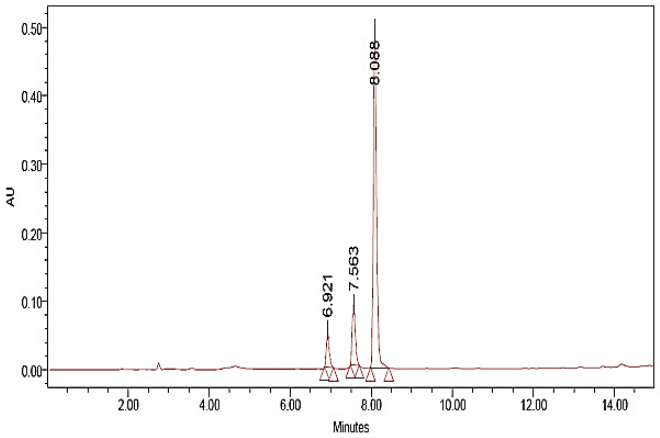

Figure 13: Representative chromatogram for Base hydrolysis of Baricitinib

Forced Degradation Studies

The validated RP-HPLC method was used to perform force degradation studies of baricitinib. The force degradation studies showed that drug is susceptible to acid and base hydrolysis as shown in Table 6. The chromatograms show that peaks of degradants were well resolved and do not interfere with the analyte peak, indicating the method's specificity.

CONCLUSION

The developed and validated RP-HPLC method was found to be sensitive, precise, and accurate. It was validated according to current ICH guidelines. The QbD approach used for method development and optimization led to a reduced number of trials and errors. The developed method can be successfully applied in routine use. Also, the force degradation studies showed that the degradants peak was well resolved and did not interfere in the elution of the analyte.

ACKNOWLEDGEMENT

The authors are grateful to Cipla Ltd., Mumbai for providing the gift sample of the API. The authors would also like to thank Ultra Pure Analytics Pvt. Ltd., Mumbai, for providing the required facilities that helped complete the project.

REFERENCES

1. 1. http://www.drugbank.ca/drugs/DB11817
2. 2. http://pubchem.ncbi.nlm.nih.gov/compound/baricitinb
3. 3. http://www.caymanchem.com/product/16707/baricitinib
4. 4. Veeraraghavan S, Thappali S, Vishwanadha S, Vakkalanka S, Rangaswamy M. Simultaneous Quantification of Baricitinib and Methotrexate in Rat Plasma by LC-MS/MS: Application to a Pharmacokinetic Study. Scientia Pharmaceutica. 2016; 84(2): 347-359. Available from: doi.10.3797/scipharm.1510-08
5. Gandhi S, Kapoor B. Development and Validation of UV Spectroscopic Method for Estimation of Baricitinib. Journal of Drug Delivery and Therapeutics. 2019; 9(4-S): 488-491. Available from: DOI. 10.22270/jddt.v9i4-s.3230
6. Seelam M, Nathani S, Kondraganti L. Development and Validation of a Stability Related Substances of Baricitinib by RP-HPLC and its Degradation. International Journal of Management and Humanities. 2019; 4(2):4-9.
7. Snyder L, Kirkland J, Dolan J. Introduction to Modern Liquid Chromatography. 3rd edition, John Wiley & Sons, Inc., Hoboken, New Jersey. 2010:337-341.
8. Jayagopal B, Shivashankar M. Analytical quality by design- A Legitimate Paradigm for Pharmaceutical Analytical Method Development and Validation. Mechanics, Materials Science, and Engineering. 2017; 9(2):364-373. Available from: Doi. 10.2412/mmse.96.97.276
9. Q2 (R1), Analytical Validation, ICH Guidelines.
10. Suresh R, Anarthanan S, Manavalan R, and Valliappan K. Aspects of validation in HPLC method development for pharmaceutical analysis - comparison of validation requirements

by FDA, USP, and ICH. International Journal of Pharmaceutical Education and Research. 2010; 1(12):123-132.
11. Harshad V. Paithankar H(2013) HPLC Method Validation For Pharmaceuticals: A Review. International Journal Of Universal Pharmacy And BioSciences. 2013; 2(4):229-24.
12. Q1A (R2), Stability Testing of New Drug Substances and Products, ICH Guidelines.
13. Q1B, Stability Testing: Photostability Testing of New Drug Substances and Products, ICH Guidelines.
14. Suryawanshi D, Durgesh Kumar Jha, Shinde U, Amin P. Development and validation of a stability-indicating RP-HPLC method of cholecalciferol in bulk and pharmaceutical formulations: Analytical quality by design approach. Journal of Applied Pharmaceutical Science. 2019; 9(06):021-032.
15. Vanitha C, Satyanarayana SV, Bhaskar Reddy K**.** Quality By Design Approach To Stability-Indicating Reverse-Phase High-Performance Liquid Chromatography Method Development, Optimization, And Validation For The Estimation Of Simeprevir In Bulk Drug. Asian Journal of Pharmaceutical and Clinical Research. 2019; 12(15):93-100.