RESEARCH ARTICLE

A Quality by Design Approach for Development and Validation of Simple and Robust Reversed Phase (RP) Stability Indicating LC Method for Estimation of Baricitinib and its Impurities

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ABSTRACT
To develop a stability-indicating and quality by design-based RP HPLC method to determine baricitinib and its process and degradation impurities. The separation was achieved using a Unison UK C18 column (Make:Imtakt) with dimensions of 150mm x 4.6mm and a particle size of 3µm.The mobile phase consisted of mobile phase A (10mM Ammonium acetate buffer, pH 5.20 with dilute Glacial Acetic acid solution) and mobile phase B as acetonitrile in gradient mode with an initial ratio of 85:15v/v. The flow rate was set at 1ml/minute and the column temperature was maintained at 35℃. Buffer and acetonitrile in the ratio of 50:50(%,v/v) was used as a diluent. The detection was performed at 220 nm using a photodiode array (PDA) detector with an injection volume of 10 µL and ambient sample temperature. The resolution for baricitinib and ten impurities was found to be greater than 2.0 for any pair of impurities.The method was evaluated according to ICH standards for various validation parameters. Method robustness was assessed using QbD principles through the design of experiments. Forced degradation studies were conducted under stress conditions such as acidic, basic, oxidation, thermal, humidity, photolytic, and hydrolysis. The proposed method is valuable for determining all process and degradation impurities to establish a stability-indicating method.

Keywords: Baricitinib, Method development, Quality by Design, Separation of impurities, Validation parameters, Stress study.

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INTRODUCTION

Rheumatoid arthritis is a persistent condition that affects humans and can be managed through the use of oral medications. One such medication is Baricitinib, which is employed as a second-line therapy for adults with moderate to severe active rheumatoid arthritis that tumor necrosis factor antagonists do not effectively control.[1] Baricitinib functions as a Janus kinase (JAK) inhibitor, targeting intracellular enzymes known as JAKs. These enzymes play a crucial role in transmitting signals from cytokines or growth factor-receptor interactions on the cellular membrane, influencing critical cellular processes like hematopoiesis and immune cell function. By modulating the signaling pathway at the JAKs level, Baricitinib prevents the phosphorylation and activation of signal transducers and activators of transcription.[2] In response to the COVID-19 pandemic, the United States Food and Drug Administration has granted emergency use authorization for baricitinib in the treatment of hospitalized pediatric patients aged 2 to less than 18 years who require supplemental oxygen, non-invasive or invasive mechanical ventilation, or extracorporeal membrane oxygenation.[3] Chemically baricitinib is known as 2-{1-(ethylsulfonyl)-3-[4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl]azetidin-3-yl acetonitrile, Baricitinib is available in oral tablet form under the brand name OLUMIANT in doses of 1 mg, 2 mg, and 4 mg. [4,5] It exhibits limited solubility in water but is slightly soluble in methanol, acetonitrile, and lower pH buffers [6]. After conducting a thorough review of the existing literature,[7-9] it was found that there are limited approaches available for determining baricitinib and its related compounds using HPLC. However, none of these methods utilize a Quality by Design (QbD) approach to determine and quantify baricitinib and its related impurities [1,2,4]. Consequently, this current research aims to establish a method using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) specifically for the analysis of related substances in baricitinib. It is worth noting that the stability-indicating method published thus far has not included any risk assessment parameters. Therefore, this study aims to develop a straightforward, sensitive,

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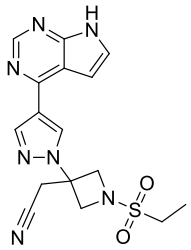
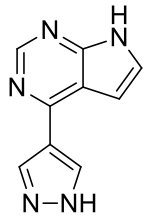
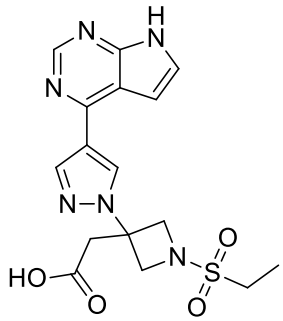
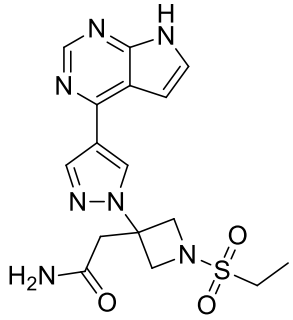
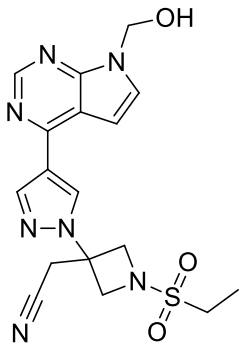
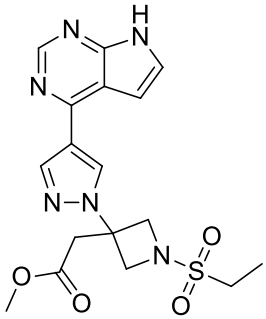
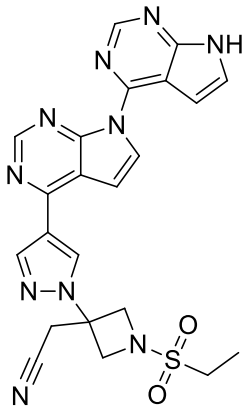
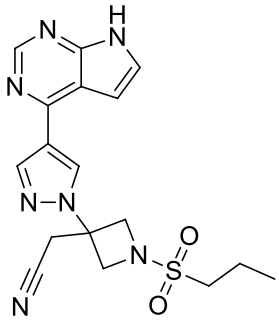
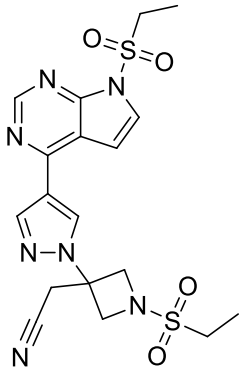
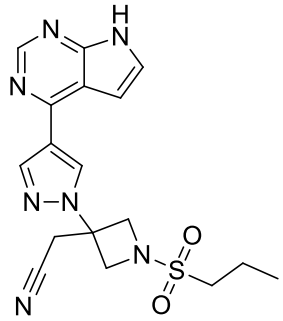
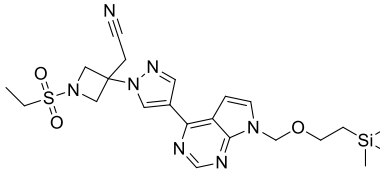
accurate, and robust RP-HPLC method for the analyzing baricitinib and its related impurities. Table 1 provides the molecular structures and International Union of Pure and Applied Chemistry (IUPAC) names for baricitinib and its impurities.

Baricitinib has not been officially included in any of the pharmacopeias to date. Therefore, there is a need for a new and reliable method to assess all the impurities related to the process and degradation of the baricitinib drug substance. This study aimed to develop and validate a stability-indicating chromatographic method for determining these impurities in the solid dispersion of baricitinib drug substance. The validation parameters of each impurity were evaluated according to the ICH guideline. [10,11] The current regulatory guidelines emphasize the importance of optimizing or proving the method's robustness using the statistical tool QbD. In this study, the QbD approach was utilized to demonstrate the robustness of the proposed method [12-19]. The QbD-based Design of Experiments (DoE) were conducted using Design Expert software version 13. We are working on the method development of pharmaceutical products using RP-HPLC, we wish to report the robust method of baricitinib and related substance in this paper.

Table 1: Chemical structure of Baricitinib and its impurities [4,5]

Impurity	*Chemical structure*	*IUPAC name*	*Molecular weight (g/mol)*	*Source*
Baricitinib		{1-(ethylsulfonyl)-3-[4-(7Hpyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl]azetidin-3-ylacetonitrile.	371.42	NA
Diamine		4-(1H-pyrazol-4-yl)-7H-pyrrolo[2,3-d]pyrimidine	185.19	Degradation Impurity
Acid		2-(3-(4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)-1-(ethylsulfonyl)azetidin-3-yl)acetic acid	390.42	Degradation impurity
Amide		2-(3-(4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)-1-(ethylsulfonyl)azetidin-3-yl)acetamide	389.43	Degradation impurity

Hydroxy methyl impurity		2-(1-(ethylsulfonyl)-3-(4-(7-(hydroxymethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)azetidin-3-yl)acetonitrile	401.44	Degradation impurity
Methyl Ester		1-(Ethylsulfonyl)-3-[4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl]-3-azetidineaceticacid methyl ester	404.44	Degradation impurity
Dimer		2-(3-(4-(7H-[4,7'-bipyrrolo[2,3-d]pyrimidin]-4'-yl)-1H-pyrazol-1-yl)-1-(ethylsulfonyl)azetidin-3-yl)acetonitrile	488.53	Degradation impurity
Impurity-4		1-(Propylsulfonyl)-3-[4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl]-3-azetidineacetonitrile	385.44	Degradation impurity
Impurity-5		2-(1-(ethylsulfonyl)-3-(4-(7-(ethylsulfonyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)azetidin-3-yl)acetonitrile	463.53	Degradation impurity

Impurity-7		2-(3-(4-(7Hc-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)-1-(butylsulfonyl)azetidin-3-yl)acetonitrile	379.42	Degradation impurity
SEM Impurity		2-(1-(ethylsulfonyl)-3-(4-(7-((2-(trimethylsilyl) ethoxy)methyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)azetidin-3-yl)acetonitrile	501.68	Degradation impurity

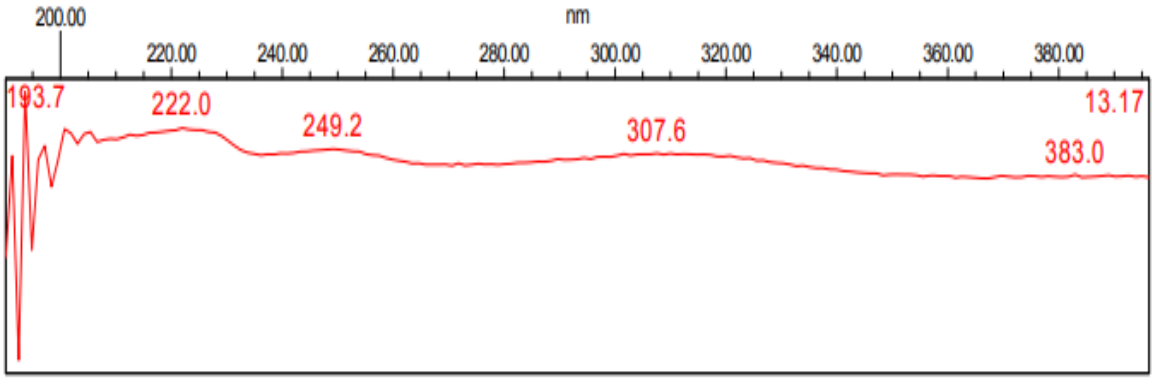


Figure. 1: UV spectrum of baricitinib

Table 2: Gradient program of the HPLC method

Time (min)	*Mobile phase A (%v/v)*	*Mobile phase B (%v/v)*
0.01	85	15
5	85	15
25	25	75
30	25	75
35	85	15
40	85	15

MATERIALS AND METHODS

Materials

Baricitinib, impurity standards, and samples were synthesized from SVAK Life Sciences (Hyderabad, India). Acetonitrile (HPLC grade) and methanol (HPLC grade), analytical grade ammonium acetate, glacial acetic acid solution, acetic acid, sodium hydroxide pellets, and hydrogen peroxide solution (30%) were purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). High purity HPLC grade water was prepared in-house using the Millipore Milli-Q Plus, Merck KGaA (Darmstadt, Germany) water purification system.

Instrument

The HPLC equipment used for the development was Waters Alliance 2695, Waters (India) Private Limited (New Delhi, India) which was equipped with a quaternary pump and a Waters 2996 PDA detector. Data acquisition was carried out by Empower Pro Software Waters (India) Private Limited (New Delhi, India). Also, an analytical balance, Sartorius AG (Germany), an Orion 420A+ pH meter, Thermo Electron Company (Massachusetts, United States) and a bath sonicator, Oscar Ultrasonics (Mumbai, India) were used.

Chromatographic conditions

The chromatographic experiments were performed on an HPLC system with a PDA detector. The detector λ_{max} was 220 nm. The column used for chromatography was Imtakt Unison UK C18, ACI Sciences Sdn (M) SDN BHD (Malaysia) 150 mm x 4.6 mm; particle size 3 µm. The optimum separation was achieved using a gradient mode. Mobile phase A was 10 mM ammonium acetate buffer, pH

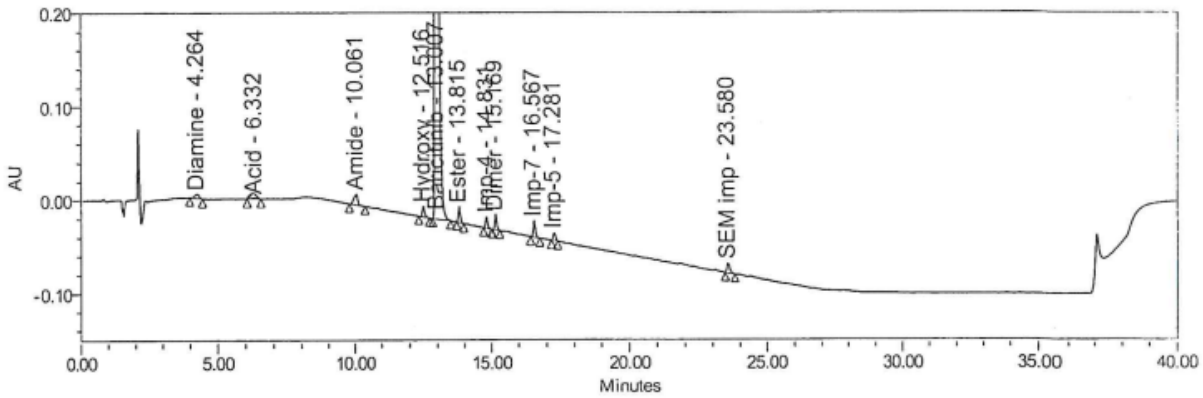


Figure. 2: Typical chromatogram of baricitinib and its impurities

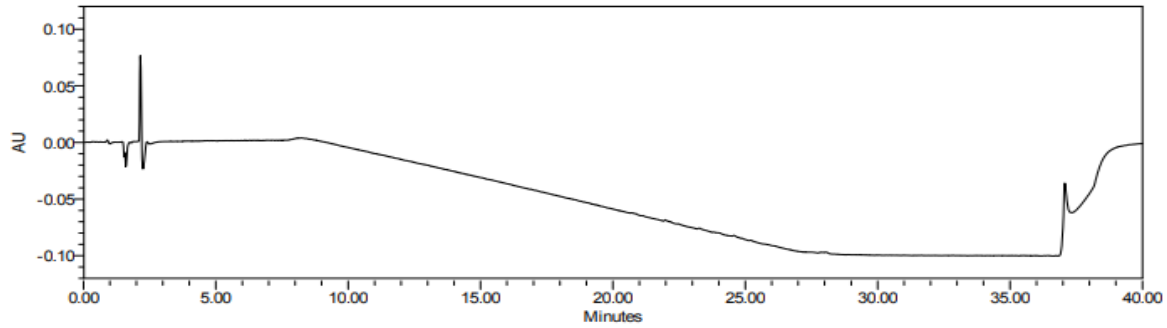


Figure. 3: Typical chromatogram of blank

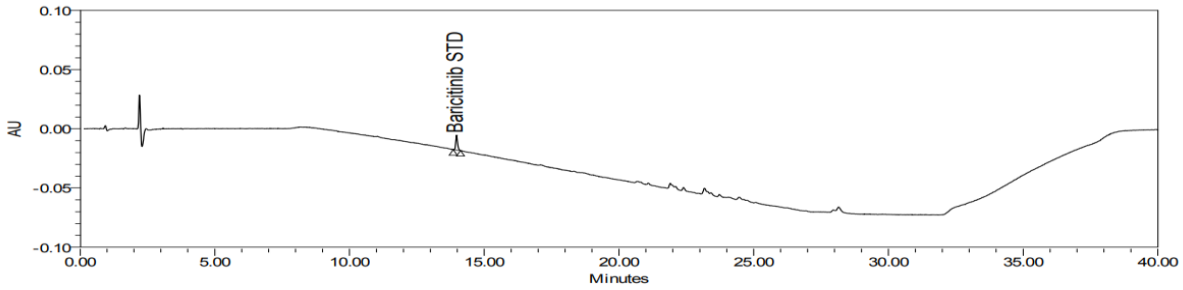


Figure. 4: Typical chromatogram of diluted standard

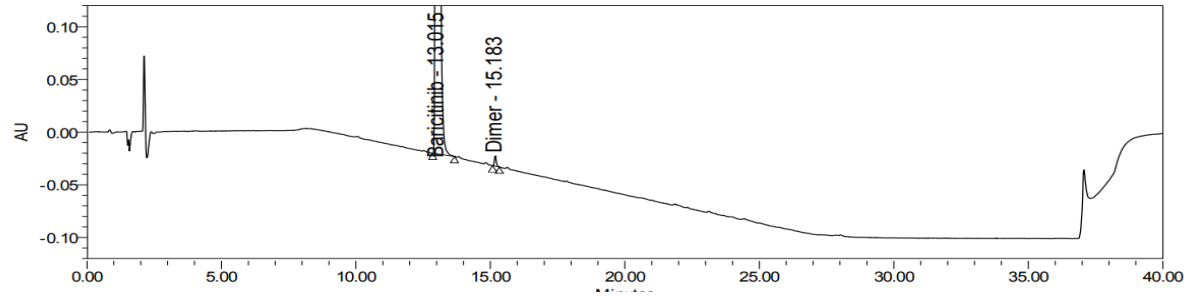


Figure. 5: Typical chromatogram of sample

5.20 with a dilute glacial acetic acid solution, and mobile phase B was 100% acetonitrile with an initial ratio of 85:15 %v/v. The flow rate was 1.0 mL/min. The gradient programme is presented in table 2. The column temperature was maintained at 35°C. The injection volume was 10 µL. Buffer: acetonitrile in the ratio of 50:50 %v/v was used as a diluent.

Preparation of solutions

Standard and impurity stock solutions

Stock solutions were prepared with a concentration of 1000 µg/mL for baricitinib in diluent, and a stock solution of impurities was prepared separately (diamine, acid, amide, hydroxy impurity, ester, dimer, impurity-4, impurity -5, impurity -7 and SEM at a concentration of 100 µg/mL of the first stock in 20 volumes of methanol and 80 volumes of diluent.

Sample solutions

Baricitinib test solutions were prepared at a concentration of 1000 µg/mL in a diluent, sonicated in a bath sonicator, Oscar Ultrasonics (Mumbai, India) for 5 min to dissolve, and further analysed by HPLC.

Stress degradation studies

The samples specificity was determined by analysis spiked with process and degradation related impurities at the ICH specification limit of 0.15%. No interference was observed at the retention time of the analyte and related impurities. Stress studies were also performed at a concentration of 1000 µg/mL. Forced degradation was performed under stress conditions of UV light (254 nm wavelength), heat (at a temperature of 105°C), humidity (80%RH/25°C), hydrolysis, acid (2N HCl at 60°C), base (2N NaOH at 60°C) and oxidation (10% H_2O_2 at 60°C) to evaluate the capability of the proposed method to separate baricitinib and all impurities including process and degradation products. For thermal and photo stress studies, the study period was 24 h, whereas for acid, base, and hydrolysis it was 1 h, and for oxidation it was 2 h. The purity of each peak was checked using a PDA detector and the purity angle was found to be less than the purity threshold, which directly demonstrated that the peak is pure. The mass balance of each condition-stressed sample was calculated by adding the content of baricitinib, known impurities, and unknown impurities expressed in % w/w.

Table 3: System suitability parameters

Peak Name	*RT (min)*	*RRT*	*USP Resolution*	*USP tailing factor*	*USP NTP*	*%RSD*
Diamine	4.264	0.33	NA	0.93	6940	0.80
Acid	6.332	0.49	6.34	0.90	7600	1.21
Amide	10.061	0.77	11.64	1.01	16880	0.95
Hydroxy impurity	12.516	0.96	11.65	1.49	117373	0.25
Baricitinib	13.007	1.00	3.14	1.11	99594	0.36
Ester	13.815	1.06	5.27	1.33	166061	0.39
Impurity-4	14.831	1.14	7.13	1.38	168812	0.88
Dimer	15.169	1.17	2.42	1.31	209586	1.20
Impurity-7	16.567	1.27	10.05	1.37	208380	1.75
Impurity-5	17.281	1.33	4.70	1.18	194946	0.95
SEM	23.580	1.81	36.12	1.50	311464	1.77

Table 4: LOQ, LOD, regression, correlation coefficient and precision

Peak Name	*LOD (µg/ml)*	*LOQ (µg/ml)*	*Regression equation (y)*		*Correlation coefficient (R)*	*Method Precision %RSD*	*Intermediate precision %RSD*
			Slope (b)	*Intercept (a)*			
Diamine	0.11	0.35	255544.245	-637.000	0.9997	2.13	1.86
Acid	0.12	0.40	85307.111	54.231	0.9990	3.52	3.12
Amide	0.08	0.28	555611.993	-3450.211	0.9986	1.06	1.25
Hydroxy impurity	0.10	0.32	319015.767	-1376.390	0.9997	1.22	2.54
Baricitinib	0.09	0.30	293452.973	-668.038	0.9992	-	-
Ester	0.11	0.33	432177.504	-913.682	0.9995	1.66	1.54
Impurity-4	0.09	0.30	334102.730	-658.598	0.9995	2.98	3.58
Dimer	0.08	0.28	398305.855	-126.068	0.9999	1.86	2.14
Impurity-7	0.09	0.30	472101.024	-150.363	0.9999	2.12	1.93
Impurity-5	0.12	0.40	233219.643	-115.804	1.0000	3.53	2.88
SEM	0.09	0.31	377180.285	-2548.813	0.9990	1.08	1.24

Table 5: Relative response factor (RRF)

Impurity Name	*RRF*
Diamine	1.15
Acid	3.44
Amide	0.53
Hydroxy impurity	0.92
Baricitinib	1.00
Ester	0.68

Impurity-4	0.88
Dimer	0.74
Impurity-7	0.62
Impurity-5	1.26
SEM	0.78

Table 6: Results of accuracy and method precision

Accuracy Level	*Diamine*	*Acid*	*Amide*	*Hydroxy Impurity*	*Ester*	*Impurity-4*	*Dimer*	*Impurity-7*	*Impurity-5*	*SEM*
LOQ	95.6	109.5	105.6	105.5	106.8	99.8	105.5	97.2	101.2	105.6
	96.4	108.2	107.6	107.6	105.5	97.6	106.4	97.4	99.8	103.5
	97.2	110.4	104.9	108.2	107.6	98.6	106.1	98.5	98.9	104.8
Mean	96.4	109.4	106.0	107.1	106.6	98.7	106.0	97.7	100.0	104.6
100%	104.6	103.5	99.4	99.4	101.5	97.5	98.8	99.8	97.9	101.1
	103.2	104.5	97.6	97.6	100.5	99.9	97.6	97.7	98.5	100.6
	106.2	103.6	96.8	96.8	99.9	101.3	99.8	98.9	97.5	100.4
	103.6	102.9	99.1	99.1	98.6	100.2	97.5	96.8	96.7	99.7
	101.8	101.5	97.5	101.5	99.1	99.6	96.8	96.8	98.5	101.2
	105.3	104.6	95.6	95.6	101.8	101.2	99.4	98.8	98.4	98.9
Mean	104.1	103.4	97.7	98.3	100.2	100.0	98.3	98.1	97.9	100.3
150%	104.8	107.7	99.7	96.5	104.4	104.2	101.2	104.4	102.1	101.2
	101.6	104.5	97.8	99.4	103.9	102.9	103.5	103.9	101.7	103.2
	105.9	103.7	98.4	97.6	101.8	101.7	103.4	102.9	102.4	102.5
Mean	104.1	105.3	98.6	97.8	103.4	102.9	102.7	103.7	102.1	102.3

Table 7: Summary of stress testing results

Impurity	*Time (h)*	*Temperature (°C)*	*Assay (% w/w)*	*RS by HPLC % degradation*	*Mass balance (% assay + % degradation products)*	*Impurity formation*
Control sample (untreated)	-	-	100.6	0.24	100.8	NA
2N HCl (acid degradation)	3	60	94.6	4.52	99.1	Acid and dimer
2N NaOH (alkali degradation)	3	60	84.6	13.21	97.8	Acid, dimer and ester
Oxidation by 10% H_2O_2	3	60	97.5	1.30	98.8	Dimer impurity
Thermally treated	24	105	100.2	0.22	100.4	No significant degradation observed
UV treated (254 nm)	24	25	100.5	0.25	100.7	
Humidity treated	24	80%RH/25°C	99.8	0.23	100.0	
Hydrolysis treated	2h	25	100.9	0.19	101.1	

Table 8: Peak purity results

Degradation condition	*Acid*		*Dimer*		*Ester*		*Baricitinib*	
	Purity angle	*Purity threshold*	*Purity angle*	*Purity threshold*	*Purity angle*	*Purity threshold*	*Purity angle*	*Purity threshold*
Acid degradation	0.254	0.563	0.177	0.442	-	-	0.298	0.681
Base Degradation	0.324	0.655	0.581	1.212	0.568	0.895	0.314	0.725
Oxidation degradation	-	-	0.344	0.819	-	-	0.415	0.642

RESULTS AND DISCUSSION

Selection of detection wavelength

Analysis was performed by using a PDA detector for the selection of wavelengths and to check the interference of the peaks. The wavelength for analysis and quantification was selected based on the UV spectrum of the analyte peak. The analyte peak shows three UV maxima at about 222 nm, 249 nm, and 307 nm. From that, 222 nm showed the maximum absorbance of the analyte peak. Reference UV spectra have been provided in fig. 1. Thus, the

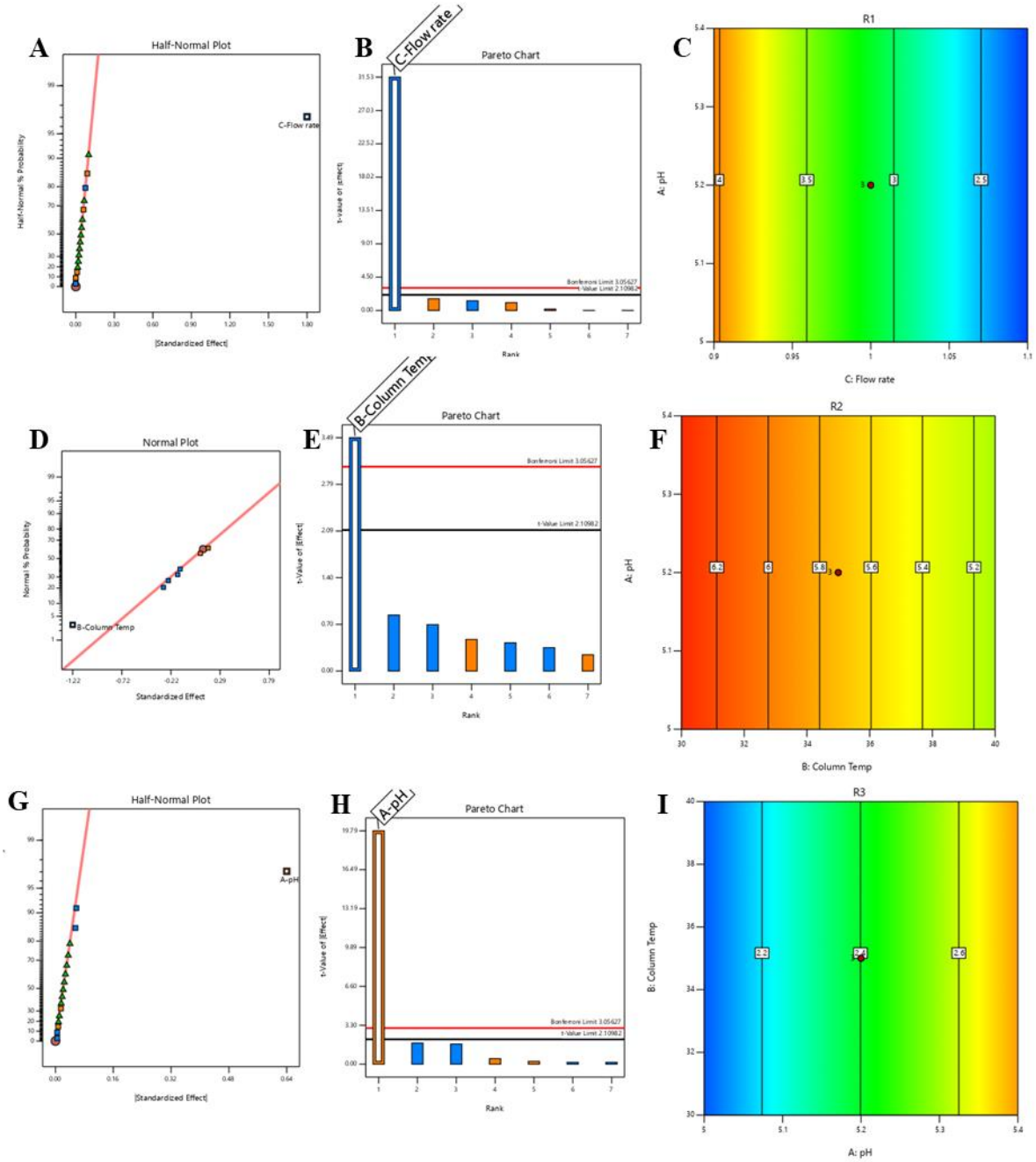


Figure. 6: Half-normal plots, Pareto charts and 2D contour plots for responses R1 (A, B and C), R2 (D, E and F) and R3 (G, H and I)

detection wavelength was selected as 220 nm for related substance analysis.

Selection of mobile phase and stationary phase

Various buffers of pH ranging from 2.0-7.5 containing orthophosphoric acid, trifluoroacetic acid, ammonium acetate, and phosphate buffers by using different stationary phases C18, C8, and organic modifiers like methanol and acetonitrile were utilized to develop the method. For method development, a spiked solution of baricitinib was used and initial experiments were conducted based on the available literature using a USP L1 column (250 mm × 4.6 mm, 5 µm particle size) from Waters (India) Private Limited (New Delhi, India) with the mobile phase composed of orthophosphoric acid buffer pH 3.0 solution and methanol in 60:40 %v/v. But in this method, hydroxyl and ester impurities are merged with the baricitinib peak and impurity-4 and dimer impurities are co-eluted with each other and all impurities are eluted within a 10 min run time only. Hence, a gradient method trial was taken with 80:20 %v/v of the initial mobile phase ratio with the L1 column (250 mm × 4.6 mm, 5 µm particle size) and found that only the hydroxyl impurity peak was merged with the analyte peak. To check the effect of pH on the resolution of impurities, we were tried using different buffers at different pH ranging from 2.0 to 7.5 without changing the ionization pH range of pKa ± 1.5. For optimizing the pH of the buffer, an HPLC

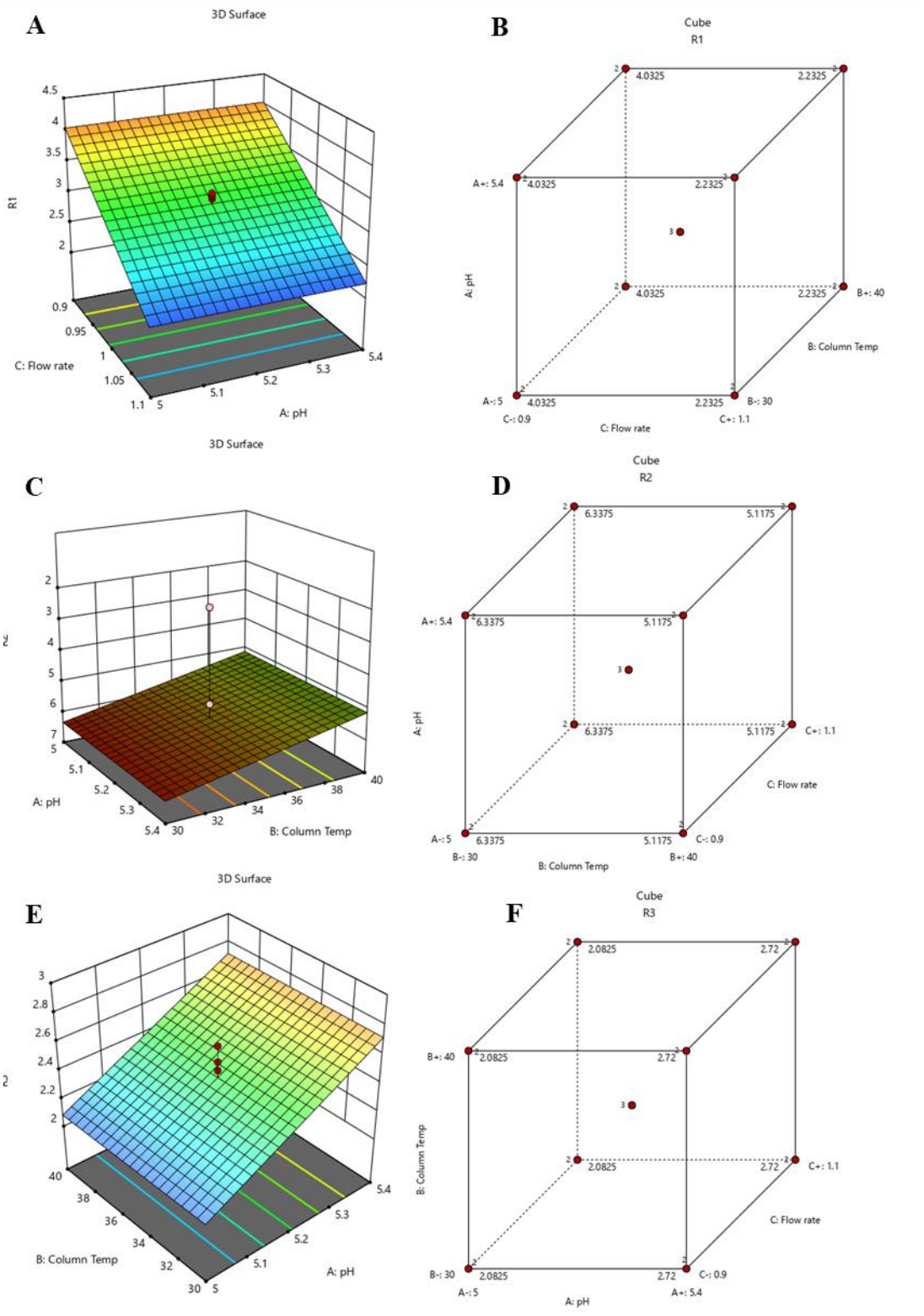


Figure. 7: 3D surface plots and 3D cube plots for responses R1 (A and B), R2 (C and D) and R3 (E and F)

column, Imtakt Unison UK C18, ACI Sciences Sdn (M) SDN BHD (Malaysia) 150 mm x 4.6 mm; particle size 3µm was selected. Spike solution was injected with the 10 mM ammonium acetate buffer, having different pH as mobile phase A and acetonitrile 100 %v/v) as mobile phase B with gradient elution and it was observed that at pH below 5.0, hydroxyl impurity and baricitinib were co eluting each other with less than 2.0 resolution, and above 5.5 impurity-4 and dimer impurity were co-eluting each other with less than 2.0 resolution.However, separation

was achieved for all the impurities and baricitinib at pH 5.2 ± 0.05. Hence, based on the method, it was concluded that it was sensitive to pH.

Table 9: Quality by design tool generated design of experiments runs and responses

Std	*Run*	*Factor 1*	*Factor 2*	*Factor 3*	*Response 1*	*Response 2*	*Response 3*
		A: pH	*B: Column temperature*	*C: flow rate*	*(R1)*	*(R2)*	*(R3)*
5	1	5.0	40	0.9	3.90	6.30	2.08
15	2	5.4	40	1.1	2.20	4.80	2.72
12	3	5.4	30	1.1	2.25	6.51	2.65
4	4	5.4	30	0.9	4.10	6.31	2.69
17	5	5.2	35	1.0	3.21	5.27	2.45
6	6	5.0	40	0.9	4.10	4.99	2.09
14	7	5.0	40	1.1	2.15	4.95	2.11
8	8	5.4	40	0.9	3.99	4.90	2.71
18	9	5.2	35	1.0	3.25	2.28	2.51
11	10	5.4	30	1.1	2.25	6.51	2.77
19	11	5.2	35	1.0	3.30	5.30	2.62
13	12	5.0	40	1.1	2.10	5.01	2.09
7	13	5.4	40	0.9	4.31	5.11	2.85
9	14	5.0	30	1.1	2.25	6.29	2.02
10	15	5.0	30	1.1	2.31	6.38	2.01
3	16	5.4	30	0.9	3.85	6.21	2.75
1	17	5.0	30	0.9	3.91	6.10	2.11
16	18	5.4	40	1.1	2.35	4.88	2.62
2	19	5.0	30	0.9	4.10	6.39	2.15

Table 10: Analysis of variance table (ANOVA)

Responses	*Source*	*Sum of squares*	*df*	*Mean square*	*F-value*	*p-value*	
R1	**Model**	12.96	1	12.96	994.21	< 0.0001	Significant
	C-Flow rate	12.96	1	12.96	994.21	< 0.0001	
	Curvature	0.0369	1	0.0369	2.83	0.1119	
	Residual	0.2086	16	0.013			
	Lack of fit	0.0697	6	0.0116	0.8365	0.5687	Not significant
	Pure error	0.1389	10	0.0139			
	Cor total	13.21	18				
R2	**Model**	5.95	1	5.95	12.18	0.003	Significant
	B-Column temperature	5.95	1	5.95	12.18	0.003	
	Curvature	5.27	1	5.27	10.78	0.0047	
	Residual	7.82	16	0.4887			
	Lack of fit	0.8623	6	0.1437	0.2066	0.9667	Not significant
	Pure error	6.96	10	0.6957			
	Cor total	19.04	18				
R3	**Model**	1.63	1	1.63	391.62	< 0.0001	Significant
	A-pH	1.63	1	1.63	391.62	< 0.0001	
	Curvature	0.0397	1	0.0397	9.57	0.007	
	Residual	0.0664	16	0.0042			

Lack of fit	0.0267	6	0.0044	1.12	0.4172	Not significant
Pure error	0.0398	10	0.004			
Cor total	1.73	18				

Table 11: Results of robustness study

	USP resolution										
Parameter / variation	**Diamine*	*Acid*	*Amide*	*Hydroxy impurity*	*Baricitinib*	*Ester*	*Impurity -4*	*Dimer*	*Impurity -7*	*Impurity -5*	*SEM*
As such	5.46	6.34	11.64	11.65	3.14	5.27	7.13	2.41	10.05	4.70	36.12
Flow rate (mL/min)											
0.90	6.12	6.89	12.21	11.96	3.89	6.14	8.10	3.03	10.89	5.23	38.78
1.10	4.98	5.78	10.78	10.68	2.90	5.10	6.53	2.21	9.33	4.24	33.41
Buffer Ph											
5.0	5.22	6.14	10.12	10.45	3.02	4.98	7.04	2.22	9.89	4.55	35.46
5.4	5.65	6.49	12.01	11.87	3.26	5.52	7.28	2.56	10.55	4.89	38.02
Temperature (°C)											
35	5.56	6.45	11.98	11.91	3.23	5.39	7.34	2.67	10.52	4.96	37.18
40	5.33	6.12	10.79	10.46	3.05	5.04	6.86	2.29	9.68	4.52	35.16

*Resolution from blank peak to diamine

It was thus finalized to use ammonium acetate buffer of pH 5.2 as mobile phase A and acetonitrile (100%v/v) as mobile phase B with a gradient as per table 2, at a flow rate of 1.0 mL/min with a column temperature of 35°C. To get baseline smoothing and reduce the run time, Imtakt Unison UK C18, ACI Sciences Sdn (M) SDN BHD (Malaysia); 150 mm x 4.6 mm; particle size 3 µm was selected. Significant separation (>2.0) for all ten impurities and baricitinib was achieved using these chromatographic conditions. The retention time of baricitinib was 13 min. The typical spike chromatogram of baricitinib and its impurities is presented in fig. 2. Diluent as a blank and diluted standard solution at specification level was injected into the HPLC system, and it was confirmed that no blank interference was observed at the retention time of any of the impurities or the analyte. Typical chromatograms are presented in fig. 3 and fig. 4. As such, a 1 mg/mL sample solution was subjected to routine analysis of impurities as per HPLC chromatographic conditions, and a typical chromatogram was presented in fig. 5.

Method validation of the proposed method

The proposed method was validated concerning system suitability, specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness for the determination of related substances in the solid dispersion of baricitinib by HPLC as per ICH guidelines. The LOD and LOQ for baricitinib and all impurities were determined by a signal to the noise ratio of 3:1 and 10:1 respectively.

System Suitability

The HPLC system was stabilized for 60 min to get a stable baseline. Six replicate injections of standard solution and spiked sample solution were injected into the HPLC system. In the developed HPLC conditions, system suitability parameters like tailing factor, USP theoretical plates, and USP resolution were evaluated for baricitinib and its ten impurities (fig. 2). The USP theoretical plates for all impurities were more than 5000, the USP tailing factor for all the impurities was less than 2.0, and the USP resolution between any pair of impurities was more than 2.0 (table 3).

LOD and LOQ

LOD and LOQ for baricitinib and all ten impurities were determined by signal-to-noise ratio of 3:1 and 10:1, respectively. LOD and LOQ values were reported in Table 4. A precision at LOQ study was also conducted, which calculated the %RSD for the areas of each Impurity. A linearity study was conducted at LOQ to 200% with six levelsm slope, intercept, and correlation coefficient were calculated for all impurities and standards by the regression equation.

Precision

The precision of the method was determined by method precision and intermediate precision studies. In both studies, the RSD of peak areas for all the impurities was

less than 5.0%. These results demonstrate that the method is precise (table 4).

Relative response factor (RRF)

The slope of each impurity and baricitinib was calculated from six levels of linearity. RRF was calculated by dividing the baricitinib slope by the respective impurity. The RRF for all impurities is reported in table 5. Thus, the developed method was highly efficient, as quantification was done with a diluted standard solution of baricitinib and there was no need to inject all impurity standards. Quantification of impurities was done by multiplying the response factor of the respective impurities.

Accuracy

The accuracy of the method was determined by spiking all impurities at three levels: the LOQ level, the 100% level, and the 150% level of the specification limit (0.15%). Recovery for all ten impurities was found within the range of 80 to 120% as mentioned in table 6.

Linearity and range

The linearity of the method was calculated at six levels ranging from LOQ to 150% to the specification level of 0.20% for all impurities, while for drug substance, a calibration curve was obtained for baricitinib in concentrations ranging from LOQ to 150% to the specification level of 0.10%. The correlation coefficient was found to be greater than 0.990 for all the impurities. The slope, correlation coefficient, and y-intercept values have been reported in table 4, which specifies the linear method.

Specificity and stress testing studies

Each impurity at 0.15% and baricitinib at 0.10% levels were injected individually for the specificity study. A spiked sample was also injected and from diode array detector purity plots, each impurity in the spiked sample was extracted. For the degradation study, baricitinib solution (1000 µg/mL) was injected for each stress testing study. No significant degradation was observed when the solid dispersion of baricitinib was subjected to thermal degradation (at 105°C for 24 h), UV light (at 254 nm for 24 h), humidity degradation (at 80% RH/25°C for 24 h) and hydrolysis degradation conditions. When liquid dispersion was subjected to degradation 1.3% of degradation was observed when the drug was subjected to oxidation (10% H_2O_2 heat at 60°C for 3 h), 13% degradation was observed when the drug was subjected to alkali degradation (2N NaOH heat at 60°C for 3 h) and 4.5% degradation was observed when the drug was subjected to acid degradation (2N HCl heat at 60°C for 3 h) conditions. Major degradation was observed with alkali and acid degradations, which led to the formation of acid, dimer and ester impurities. Results from stress testing studies are reported in table 7. An assay was calculated against the baricitinib standard solution. The mass balance was calculated for each condition of stressed samples and was found to be in the range of 95-105% which confirms that the developed method was stability-indicating. Depending on the chemistry and structure of the molecule it may undergo degradation in a specific strength of acid, base or peroxide at a specific temperature only. Hence, only some specific conditions influence the degradation of the drug substance. Peak purity was checked in the degraded sample, and the purity angle and purity threshold for the known impurity are reported in table 8. This table shows that the purity angle is less than the purity threshold for known impurity peaks formed due to degradation. Mass balance was established by related substance sample. 1mg/mL solution was diluted to an assay concentration of 0.1 mg/mL and subjected to HPLC analysis under similar conditions.

Robustness by quality by design

The QbD based DOE statistical analysis verified the development method's robustness. Based on the method development, critical method parameters (CMPs) were identified. The method uses buffer pH (5.2), column temperature (35°C) and flow rate (1.0 mL/min). The CMPs were changed to positive and negative modes as per the current regulatory guidelines. The pH of the buffer changed 5.0-5.4, the column temperature was changed to 30 ± 5°C and the flow rate changed between 0.90 and 1.10 mL/min. Based on the method of optimization and criticalities three pair's resolutions were chosen for response factors; resolution between hydroxy impurity and baricitinib (R1), resolution between baricitinib and ester impurity (R2) and resolution between impurity-4 and dimer impurity (R3). A two-level randomized full factorial design was constructed with three center points and zero blocks. The design given totals 19 runs. The runs performed as per the design, and the responses were tabulated in table 9.

All the responses were analyzed by considering the significant effects. In the ANOVA (table 10), it is clear that P values were below 0.05 and significant. The lack of fit was insignificant; significant data reflecting the model was passed for all three responses. The difference between predicted and observed R2 was less than 0.2. The design space region met the desired acceptance criteria for all the responses. The Pareto charts, half-normal plot, and 2D contour plots shown in fig. 6. 3D surface plots and 3D cube plots were shown in fig. 7. The response R1 was found to be sensitive to factor 3 flow rate. The response R2 was found sensitive to factor 2 column oven temperature, and the response R3 was found sensitive to factor 1 pH. Based on the results, it was concluded that none of the transmuted conditions impacted the chromatography, and the proven method was robust. The classical robustness study was performed as per the ICH guidelines for analytical method validation, which covers most of the critical variables of the analytical method. To demonstrate the robustness of the developed method, spiked samples were analyzed under altered chromatographic conditions (flow rate ± 10%, pH ± 0.2 units and column temperature ± 5°C). No significant difference in quantification or resolution indicates that the developed method is robust. The results of the robustness study were reported in table 11. Based on the QbD statistical tool's evaluation of robustness and classical robustness analysis data, both showed unique results and confirmed that the method was robust.

CONCLUSION

A simple, accurate, and robust RP-HPLC method has been developed using the QbD approach and validated as per regulatory guidelines for the determination and quantification of process and degradation impurities for the oncology drug baricitinib. The developed RP-HPLC method demonstrated that the method is precise, linear, accurate, rugged, and a proven stability-indicating method that can be used for routine analysis of production samples and to check the stability of samples for industrial-scale manufacturing.

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SOURCE OF SUPPORT

Nil.

CONFLICT OF INTEREST

None

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