STABILITY INDICATING REVERSE PHASE-HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTITATIVE ANALYSIS FOR THE ESTIMATION OF BARICITINIB IN PURE SUBSTANCES AND IN MARKETED PHARMACEUTICAL DOSAGE FORM

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***ABSTRACT:** A reverse phase liquid chromatographic method for estimation of Baricitinib in bulk drug and marketed pharmaceutical dosage form was developed and validated. The chromatographic conditions to achieve the highest performance parameters using Symmetry ODS RP C18, 5µm, 15mm x 4.6mm i.d. column with guard filter were optimized. The separation was carried out using a mobile phase containing Acetonitrile and methanol in the ratio of 60:40% v/v pumped at a flow rate of 1.0 mL/min with detection at 275 nm. The method was shown to be linear in 10 – 50 µg/mL concentration range (regression coefficient of 0.9993). The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.08µg/mL and 0.24µg/mL, respectively. The accuracy of the method was assessed by adding fixed amount of pre-analyzed sample to different standard solutions (80%, 100%, and 120% of the tested concentration) in triplicate. The percentage mean recoveries were 99.307% to 100.283% with %RSD values found to be within limits. The method was found to be precise with %RSD value of 0.782% and 0.876% & 0.776% for intraday and interday precision study, respectively. The method specificity and robustness were also established. New and sensitive RP-HPLC method for Quantitative estimation of Baricitinib has been developed, in respect to the reviewed analytical methods.*

***Key Words:** Baricitinib, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.*

I. INTRODUCTION

Baricitinib is an orally bioavailable inhibitor of Janus kinases 1 and 2 (JAK1/2), with potential anti-inflammatory, Immunomodulating and antineoplastic activities. Upon administration, Baricitinib[1] binds to JAK1/2, which inhibits JAK1/2 activation and leads to the inhibition of the JAK-signal transducers and activators of transcription (STAT) signaling pathway. This decreases the production of inflammatory cytokines and may prevent an inflammatory response. In addition, Baricitinib may induce apoptosis and reduce proliferation of JAK1/2-expressing tumor cells. JAK kinases are intracellular enzymes involved in cytokine signaling, inflammation, immune function and hematopoiesis; they are also upregulated and/or mutated in various tumors cell types. Baricitinib[2] is an orally available small molecule inhibitor of Janus kinases that is used to treat moderate-to-severe rheumatoid arthritis and in late 2020 was given emergency use authorization as therapy in combination with Remdesivir for severe COVID-19. Baricitinib is associated with transient and usually mild elevations in serum aminotransferase levels during therapy but has yet to be linked to cases of clinically apparent acute liver injury. Baricitinib[3] is a Janus kinase (JAK) inhibitor. JAKs are tyrosine protein kinases that play an important role in pro-inflammatory signaling pathways. Overactive JAKs have been implicated in autoimmune disorders, such as rheumatoid arthritis. By inhibiting the actions of JAK and JAK2, Baricitinib attenuates JAK-mediated inflammation and immune responses. The IUPAC Name of Baricitinib is 2-[1-ethyl sulfonyl-3-[4-(7H-pyrrolo [2, 3-d] pyrimidin-4-yl) pyrazol-1-yl] azetidin-3-yl] acetonitrile. The Chemical Structure of Baricitinib is below

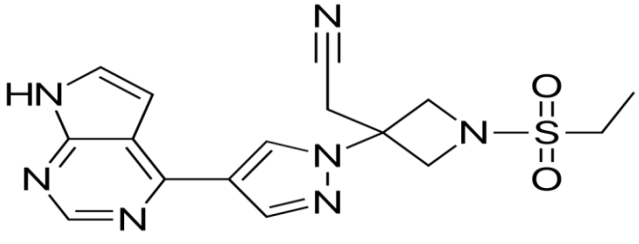

Fig.1. Chemical Structure of Baricitinib

Several methods utilized techniques like HPLC, UV-VIS Spectroscopy, GC, TLC etc. for quantification of Baricitinib have been developed and validated as per ICH Guidelines[34]. Moreover, several HPLC methods for estimation of Baricitinib in present of other active constituents have been established and in respect to these findings a new specific RP-HPLC method for rapid, accurate and precise estimation of Baricitinib in bulk drug and in marketed pharmaceutical dosage form has been developed and validated.

II. MATERIALS AND METHODS

Table-1: Lists of Instruments

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC WATERS** having Empower2 Software with Isocratic and UV-Visible Detector.
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS C_{18}, 250mm x 4.6mm i.d. and 5µm particle size
7.	P^H Analyzer **(ELICO)**
8.	Vaccum Filtration Kit **(BOROSIL)**

Table-2: List of Chemicals

S.No.	Name	Specifications		Supplier/Manufacturer
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem Ltd; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem Ltd; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Hydrochloric acid	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Sodium Hydroxide	99.9%	A.R	Sd fine-Chem ltd; Mumbai
8.	Hydrogen Peroxide	99.9%	A.R	Sd fine-Chem ltd; Mumbai

Method Development

HPLC Instrumentation & Conditions:

The HPLC system[4] employed was HPLC WATERS with Empower2 Software with Isocratic mode of separation with UV-Visible Detector.

Standard & Sample Preparation for Analysis:

10 mg of Baricitinib standard was transferred into 10 ml volumetric flask dissolved & make up the volume up to

the mark with the mobile phase. Further dilution was prepared by transferring about 0.5ml of the above prepared solution into a 10ml volumetric flask and make up to volume with the mobile phase.

Table-3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 40 : 60	1.0ml/min	275nm	Very Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 55 : 45	1.0ml/min	275nm	Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Acetonitrile : Water = 50:50	1.0ml/min	275nm	Tailing peaks	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Water = 70:30	1.0ml/min	275nm	Resolution was not good	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol = 70:30	1.0ml/min	275nm	Tailing peak	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol = 60:40	1.0ml/min	275nm	Nice peak	Method accepted

III. RESULTS AND DISCUSSION

Method Development

Table-4: Summary of Optimised Chromatographic Conditions

Mobile phase	ACN : Methanol: = 60:40
Column	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	275nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic
Retention time	2.570 minutes

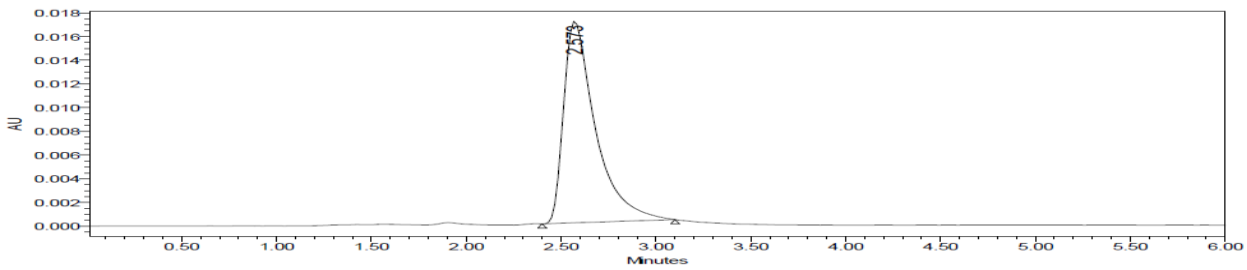

Fig.2. Optimized Chromatographic Condition

Method Validation

1. Accuracy:

Recovery Study: To determine the accuracy[5] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Baricitinib were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery[6] values were calculated. The results were shown in table-5.

Table-5: Readings of Accuracy

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	8	8.036155	156421	100.1601	Mean= 99.3073% S.D. = 0.96244 % R.S.D.= 0.96915%
S_2 : 80 %	8	8.087732	155412	99.49803	
S_3 : 80 %	8	7.989834	153531	98.26378	
S_4 : 100 %	10	10.0029	192210	98.91496	Mean= 99.67104% S.D. = 0.8229 % R.S.D.= 0.82561%
S_5 : 100 %	10	10.06592	193421	99.55066	
S_6 : 100 %	10	10.16476	195320	100.5475	
S_7 : 120 %	12	12.04989	231541	99.6343	Mean= 100.2838% S.D. = 0.629055 % R.S.D. = 0.62727%
S_8 : 120 %	12	12.13228	233124	100.3268	
S_9 : 120 %	12	12.19932	234412	100.8902	

2. Precision:

2.1. Repeatability: The precision[7] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Baricitinib (API). The percent relative standard deviation[8] was calculated for Baricitinib are presented in the table-6.

Table-6: Readings of Repeatability

HPLC Injection Replicates of Baricitinib	Retention Time (Minutes)	Peak Area (AUC)
Replicate – 1	2.572	186125
Replicate – 2	2.570	186651
Replicate – 3	2.573	184858
Replicate – 4	2.570	183813
Replicate – 5	2.574	187216
Replicate – 6	2.573	187611
Average		**186045.7**
Standard Deviation		**1455.199**
% RSD		**0.782175**

2.2. Intermediate Precision:

2.2.1. Intra-Assay & Inter-Assay:

The intra & inter day variation[9] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Baricitinib revealed that the proposed method is precise[10].

Table-7: Results of Intra-Assay & Inter-Assay

Conc. of Baricitinib (API) (µg/ml)	Observed Conc. of Baricitinib (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	6.95	0.69	8.02	0.94
10	9.06	0.95	9.59	0.72
12	11.78	0.99	11.09	0.67

3. Linearity & Range:

The calibration curve showed good linearity[11] in the range of 0 – 50 µg/ml, for Baricitinib (API) with correlation coefficient[12] (r^2) of 0.999 (Fig-3). A typical calibration curve[13] has the regression equation[14] of y = 122700x + 3777 for Baricitinib.

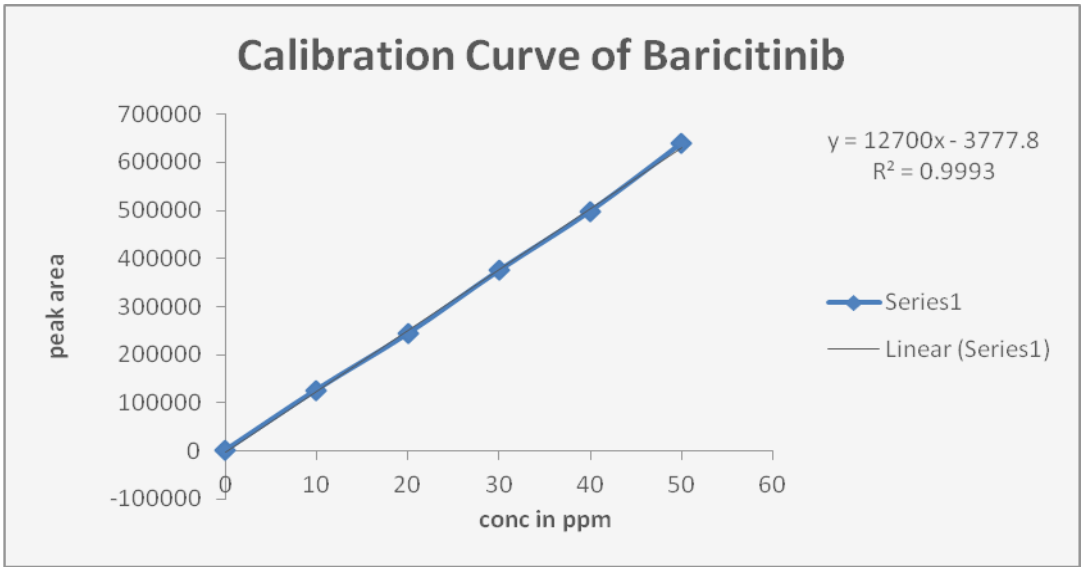

Fig.3. Calibration Curve of Baricitinib (API)

Table-8: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
10	126465
20	243214
30	374782
40	498192
50	639624

4. Method Robustness:

Influence of small changes in chromatographic conditions[15] such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness[16] of the method are also in favour of (Table-9, % RSD < 2%) the developed RP-HPLC[17] method for the analysis of Baricitinib (API).

Table-9: Result of Method Robustness

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.61
Flow (0.9 ml/min)	0.48
Temperature (27^{0}C)	0.63
Temperature (23^{0}C)	0.72
Wavelength of Detection (270 nm)	0.93
Wavelength of detection (280 nm)	0.95

5. LOD & LOQ:

The Minimum concentration level at which the analyte can be reliable detected[18] (LOD) & quantified[19] (LOQ) were found to be 0.668 & 2.004µg/ml respectively.

6. System Suitability Parameter

System suitability[20] testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system[21] that can be evaluated as such. Following system suitability[22-24] test parameters were established. The data are shown in Table-10.

Table-10: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	7.86
2	Asymmetry	T ≤ 2	Baricitinib =0.26
3	Theoretical plate	N > 2000	Baricitinib =4265
4	Tailing Factor	T<2	Baricitinib =1.29

7. Estimation of Baricitinib in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent[25] to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase[26]. The solution was filtered[27] through a membrane filter (0.45 µm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system[28] and the observations were recorded.

A duplicate injection of the standard solution[29] was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-11.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard[30] taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity[31] of working standard

Table-11: Recovery Data for estimation Baricitinib in Barinat 4

Brand Name of Baricitinib	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Barinat 4 (4 mg) (Natco Pharma Ltd)	4mg	3.78 (± 0.687)	99.79 (± 0.247)

Result & Discussion: The amount of drug in Barinat 4 Tablets was found to be 3.78 (± 0.687) mg/tab for Baricitinib & % assay[32] was 99.79 %.

Forced Degradation Studies

Results of Degradation Studies:

The results of the stress studies[33] indicated the specificity of the method that has been developed. Baricitinib was stable in thermal and photolytic stress conditions. The result of forced degradation studies are given in the following table-12.

Table-12: Results of Forced Degradation Studies of Baricitinib

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	81.36	18.64	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	83.37	16.63	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	98.92	1.08	100.0
UV (254nm)	24Hrs.	96.33	3.67	100.0
3 % Hydrogen peroxide	24Hrs.	89.41	10.59	100.0

IV. CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Baricitinib, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS RP C_{18}, 5µm, 15mm x 4.6mm i.d. Column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl). The drug was found to be Soluble in DMSO (61 mg/ml at 25 °C), ethanol (15 mg/ml) with warming, DMF (~5 mg/ml), water (<1 mg/ml at 25 °C), and methanol and acetonitrile. Using these solvents with appropriate composition newer methods can be developed and validated. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Baricitinib it is evident that most of the HPLC work can be accomplished in the wavelength range of 210-300 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 20µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Baricitinib in different formulations.

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