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Analytical method development and validation for the estimation of acalabrutinib in API form and marketed pharmaceutical dosage form by RP-HPLC along with stability studies

Bathula Anusha Yadav [1*], Vijaya Kuchana [2]
[1] Department of Pharmaceutical Analysis, Teegala Krishna Reddy College of Pharmacy, Medbowli, Meerpet (V), Balapur (M), Ranga Reddy, Hyderabad, Telangana, India
[2] Principal and Professor, Department of Pharmaceutical Chemistry, Teegala Krishna Reddy College of Pharmacy, Medbowli, Meerpet (V), Balapur (M), Ranga Reddy, Hyderabad, Telangana, India

* Corresponding Author: **Bathula Anusha Yadav**

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Abstract
A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Acalabrutinib in bulk form and marketed formulation. Separation of Acalabrutinib was successfully achieved on a Develosil ODS HG-5 RP C18, 5 µm, 15 cm x 4.6 mm i.d. column in an isocratic mode of separation utilizing Methanol: Phosphate buffer (0.02 M, pH-3.6) in the ratio of 45:55% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 255nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 12-28 mcg/mL for Acalabrutinib. The correlation coefficient was found to be 0.9995 for Acalabrutinib. The LOD and LOQ for Acalabrutinib were found to be 5.004 µg/mL and 15.164 µg/mL respectively. The proposed method was found to be good percentage recovery for Acalabrutinib, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords: Acalabrutinib, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines

1. Introduction

Acalabrutinib is a member of the class of imidazopyrazines that is imidazo[1, 5-a]pyrazine substituted by 4-(pyridin-2-ylcarbamoyl)phenyl, (2S)-1-(but-2-ynoyl)pyrrolidin-2-yl, and amino groups at positions 1, 3 and 8, respectively[1]. It is an irreversible second-generation Bruton's tyrosine kinase (BTK) inhibitor that is approved by the FDA for the treatment of adult patients with mantle cell lymphoma (MCL) who have received at least one prior therapy. It has a role as an EC 2.7.10.2 (non-specific protein-tyrosine kinase) inhibitor, an antineoplastic agent and an apoptosis inducer. It is a secondary carboxamide, a member of benzamides, a member of pyridines, an aromatic amine, a pyrrolidinecarboxamide, an imidazopyrazine, an ynone and a tertiary carboxamide[2].Acalabrutinib is currently indicated for the treatment of adult patients with Mantle Cell Lymphoma (MCL) who have received at least one prior therapy. It has also been recently approved for chronic lymphocytic leukemia and small lymphocytic lymphoma. Acalabrutinib is a Bruton Tyrosine Kinase inhibitor that prevents the proliferation, trafficking, chemotaxis, and adhesion of B cells. It is taken every 12 hours and can cause other effects such as atrial fibrillation, other malignancies, Cytopenia, hemorrhage, and infection[3]. The IUPAC name of Acalabrutinib is 4-[8-amino-3-[(2S)-1-but-2-ynoylpyrrolidin-2-yl]imidazo [1, 5-a]pyrazin-1-yl]-N-pyridin-2-ylbenzamide. The Chemical Structure of Acalabrutinib is shown in Fig-1.

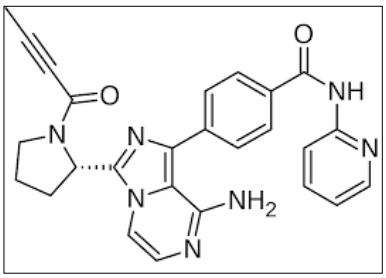

Fig 1: Chemical Structure of Acalabrutinib

Materials and Methods

Table 1: Instruments used

S. No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	LabIndia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table 2: Chemicals used

S. No.	Chemical	Brand Names
1	Acalabrutinib	Local Market
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck
4	Ethanol	Sd fine-Chem ltd; Mumbai
5	DMSO	Sd fine-Chem ltd; Mumbai
6	DMF	Sd fine-Chem ltd; Mumbai
7	Orthophosphoric Acid	Sd fine-Chem ltd; Mumbai

HPLC Method Development

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Acalabrutinib working standard [4] into a 10ml of clean dry volumetric flasks add about 7 ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.1 ml of the above Acalabrutinib stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution

Twenty capsules were taken and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated well. A quantity of powder of Acalabrutinib equivalent to 10 mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC [5] grade methanol was added and the resulting solution was sonicated for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.1 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45 µm) and finally sonicated to degas.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines [13, 14].

Mobile Phase Optimization

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase [6] was optimized to Methanol and Phosphate buffer (0.02M, pH-3.6) in proportion 45:55% v/v.

Optimization of Column

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d. was found to be ideal as it gave good peak shape and resolution at 1.0ml/min flow [7].

Preparation of Buffer and Mobile Phase

Preparation of Potassium Dihydrogen Phosphate (KH2PO4) Buffer (0.02M) (pH-3.6): Dissolve 2.72172 g of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra-sonication.

Preparation of Mobile Phase

Accurately measured 450 ml (45%) of Methanol and 550 ml of Phosphate buffer (55%) were mixed and degassed[8]in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent [9]. (Stock solution)

Further pipette 0.1 ml of the above Acalabrutinib stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Specificity

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Acalabrutinib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of sample solution

Weight 10 mg equivalent weight of Acalabrutinib sample into a 10mL clean dry volumetric flask and add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1 ml of Acalabrutinib above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay[10] by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity and Range

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (12 ppm of Acalabrutinib)

Take 0.12 ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (16ppm of Acalabrutinib)

Take 0.16ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultra sonicator.

Preparation of Level – III (20ppm of Acalabrutinib)

Take 0.2 ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultra sonicator.

Preparation of Level – IV (24 ppm of Acalabrutinib)

Take 0.24ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate [11] the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (28ppm of Acalabrutinib)

Take 0.28 ml of stock solution in to 10 ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient [12].

Precision

Repeatability

Preparation of Acalabrutinib product solution for precision

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Acalabrutinib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different daysby maintaining same conditions.

Procedure

Analyst 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits[15].

Accuracy

For Preparation of 80% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Acalabrutinib Working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.08 ml of the above Acalabrutinib stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 100% Standard Stock Solution

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1 ml of the above Acalabrutinib stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 120% Standard Stock Solution

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.12 ml of the above Acalabrutinib stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (80%, 100%, 120%) were made under the optimized conditions[16]. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Acalabrutinib and calculate the individual recovery and mean recovery values.

Limit of Detection and Limit of Quantification (LOD & LOQ)

Preparation of 5.004 µg/ml Solution (For LOD)

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.05004 ml of the above Acalabrutinib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 15.164 µg/ml Solution (For LOQ)

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15164ml of the above Acalabrutinib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Robustness

The analysis was performed in different conditions[17]to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard Solution

Accurately weigh and transfer 10 mg of Acalabrutinib working standard into a 10ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1 ml of the above Acalabrutinib stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1 ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of Mobile Phase Organic Composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 50:50, 40:60 instead (45:55), remaining conditions[18]is same. 20µl of the above sample was injected and chromatograms were recorded.

Results and Discussion

Method Development

Wavelength Detection

The detection wavelength [19] was selected by dissolving the drug in mobile phase to get concentration of 10 µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400 nm. The UV spectrum of Acalabrutinib was obtained and the Acalabrutinib show edabsorbance' smaximaat 255 nm. The UV spectra of drug are follows:

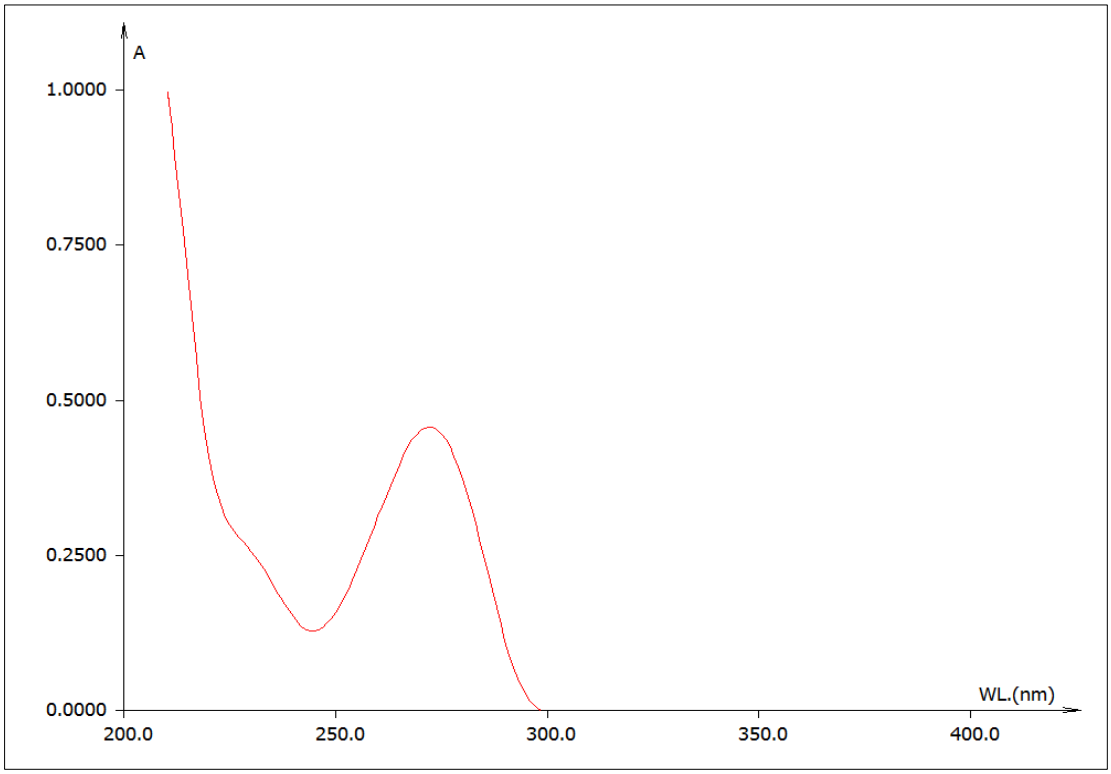

Fig 2: UV Spectrum of Acalabrutinib

Observation: While scanning the Acalabrutinib solution we observed the maxima at 255nm. The UV spectrum[20] has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Optimized Chromatographic Method

Table 3: Optimized chromatographic conditions

Mobile phase	Methanol : Phosphate buffer (0.02M, pH-3.6) = 45:55 v/v
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20 µl
Type of Elution	Isocratic

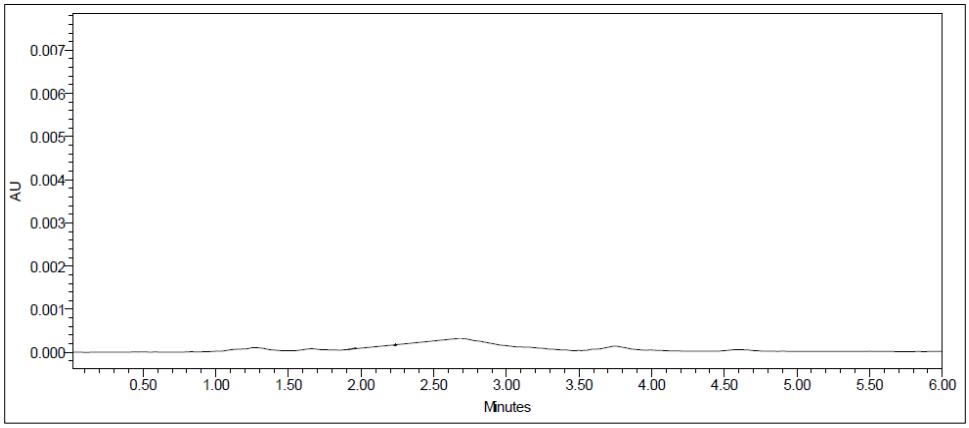

Fig 3: Chromatogram of Blank Solution

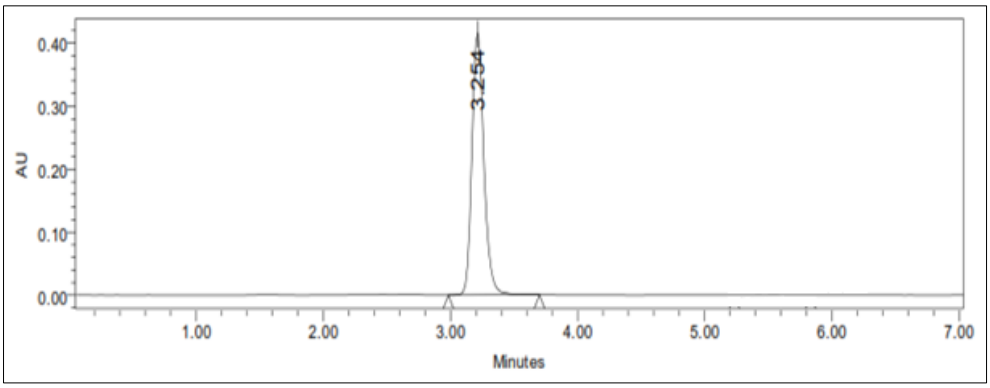

Fig 4: Chromatogram of Acalabrutinib in Optimized Chromatographic Condition

Method Validation

Following ICH requirements [25, 30], the evolved method was validated (Q2) and the parameters "specificity, accuracy, precision, linearity, robustness, the limit of detection (LOD) and limit of quantification (LOQ)" were evaluated.

System Suitability: System suitability 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 that can be evaluated as such. Following system suitability test parameters [21-22] were established. The data are shown in Table-4 & 5.

Table 4: Data of System Suitability Test

S. No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	3.253	284568	7368	1.26
2	Injection 2	3.254	285684	7295	1.25
3	Injection 3	3.215	283659	7346	1.27
4	Injection 4	3.297	284754	7394	1.29
5	Injection 5	3.253	283695	7425	1.25
6	Injection 6	3.213	284578	7385	1.27

Mean			284489.7	7368.833	1.265
S.D			752.5617		
%RSD			0.26453		

Table 5: System suitability results for Acalabrutinib (Flowrate)

S. No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Acalabrutinib = 0.12
2	Theoretical plate	N > 2000	Acalabrutinib = 7258
3	Tailing Factor	(Tf)< 2	Acalabrutinib = 1.25

Specificity

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing three drugs was also prepared. Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method was specific.

Observation: In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific [23].

Linearity: To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 0-28 µg/ml for Acalabrutinib. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20 µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[24] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs: The resultant areas of linearity peaks are plotted against Concentration.

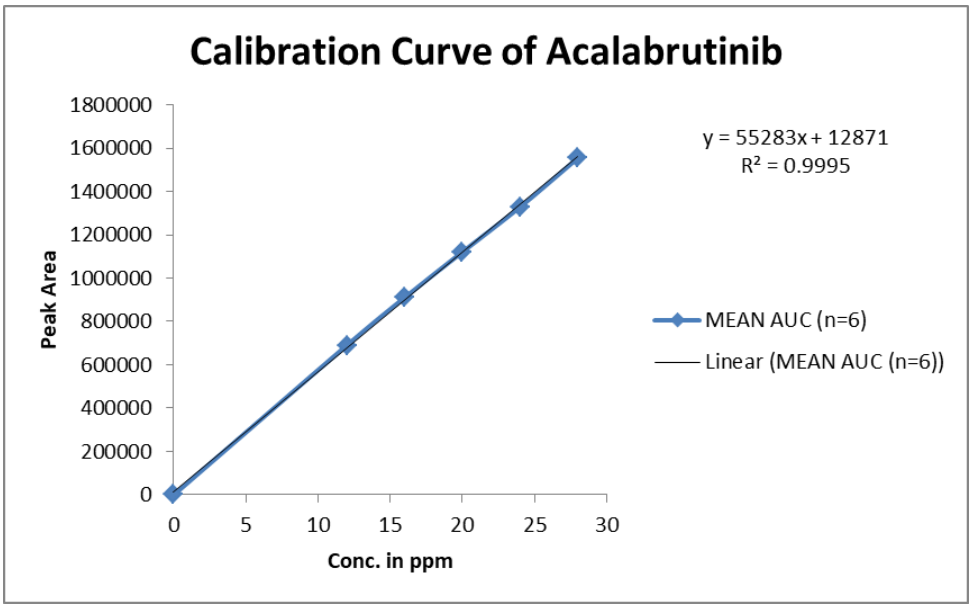

Fig 5: Standard Curve for Acalabrutinib

Observation: Linearity range was found to be 0-28 µg/ml for Acalabrutinib. The correlation coefficient was found to be 0.9995, the slope was found to be 55283 and intercept was found to be 12871 for Acalabrutinib.

Table 6: Linearity Readings for Acalabrutinib

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Linearity Plot

The plot of Concentration (x) versus the Average Peak Area (y) data of Acalabrutinib is a straight line.

Y = mx + c

Slope (m) = 55283

Intercept (c) = 12871

Correlation Coefficient (r) = 0.9995

Acceptance/Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 12871. These values meet the validation criteria.

Accuracy

Inject the three replicate injections of individual concentrations (80%, 100%, 120%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Acalabrutinib and calculate the individual recovery and mean recovery values[26].

Accuracy at different concentrations (80%, 100%, and 120%)

was prepared and the % recovery was calculated.

Table 7: Accuracy results of Acalabrutinib

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.064107	458679	99.867	Mean= 100.4113% S.D. = 0.473694346 % R.S.D.= 0.471753
S_2 : 80 %	8	7.843532	446485	100.637	
S_3 : 80 %	8	8.19449	465887	100.73	
S_4 : 100 %	10	9.892661	559767	99.41	Mean= 100.6646667% S.D. = 1.166369295R.S.D.= 1.158667
S_5 : 100 %	10	9.978655	564521	100.868	
S_6 : 100 %	10	10.19623	576549	101.716	
S_7 : 120 %	12	11.85907	668476	99.878	Mean= 100.4637% S.D. = 0.51154309 % R.S.D. = 0.509181
S_8 : 120 %	12	12.16785	685546	100.69	
S_9 : 120 %	12	12.18644	686574	100.823	

Observation: The mean recoveries were found to be 100.411, 100.664 and 100.463% for Acalabrutinib. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision of each method was ascertained separately from the peak areas obtained by actual determination of six replicates of a fixed amount of drug Acalabrutinib. The percent relative standard deviations[27] were calculated for Acalabrutinibare presented in the Table-8.

i) Repeatability

Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 8: Repeatability Results of Acalabrutinib

HPLC Injection Replicates	AUC for Acalabrutinib
Replicate – 1	285479
Replicate – 2	284571
Replicate – 3	286954
Replicate – 4	283261
Replicate – 5	285964
Replicate – 6	284259
Average	285081.3
Standard Deviation	1318.666
% RSD	0.462558

Observation: The repeatability study which was conducted on the solution having the concentration of about 20µg/ml for Acalabrutinib (n=6) showed a RSD of 0.462558% for Acalabrutinib. It was concluded that the analytical technique showed good repeatability.

ii) Intermediate Precision/Ruggedness

To evaluate the intermediate precision[28](also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure

Analyst 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Table 9: Results of Ruggedness for Acalabrutinib (Analyst-1)

S.No.	PeakName	RT	Peak Area	Theoretical Plates	Tailing Factor
1	Acalabrutinib	3.253	284568	7368	1.26
2	Acalabrutinib	3.254	285684	7295	1.25
3	Acalabrutinib	3.215	283659	7346	1.27
4	Acalabrutinib	3.204	286598	7457	1.22
5	Acalabrutinib	3.202	287965	7635	1.29
6	Acalabrutinib	3.297	285698	7459	1.28
Mean			285695.3		
Std.Dev.			1508.898		
%RSD			0.528149		

Table 10: Results of Ruggedness for Acalabrutinib (Analyst-2)

S. No.	Peak Name	RT	Peak Area	Theoretical Plates	Tailing Factor
1	Acalabrutinib	3.297	294754	7394	1.29
2	Acalabrutinib	3.253	293695	7425	1.25
3	Acalabrutinib	3.213	294578	7385	1.27
4	Acalabrutinib	3.297	296534	7584	1.23
5	Acalabrutinib	3.210	296571	7745	1.24
6	Acalabrutinib	3.254	298698	7658	1.25
Mean			295805		
Std. Dev.			1819.334		
%RSD			0.615045		

Observation: Intraday and inter day studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

Robustness: Robustness[29] is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness of a method is done by varying the chromatographic parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method.

Table 11: Result of Method Robustness Test for Acalabrutinib

Parameter used for Sample Analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	283261	3.254	7258	1.25
Less Flow rate of 0.9 mL/min	315864	3.297	7569	1.29
More Flow rate of 1.1 mL/min	298542	3.212	7841	1.41
Less organic phase	279856	3.253	7965	1.27
More organic phase	306985	3.215	7458	1.28

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) &organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-38, % RSD < 2%) the developed RP-HPLC method for the analysis of Acalabrutinib (API).

LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.

L.O.D. = 3.3(SD/S).

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Table 12: Results of LOD

	LOD
SD of Intercept	19518.16286
Slope	55283

Observation: The LOD was found to be 1.165 µg/ml for Acalabrutinib.

LOQ: The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ.

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Table 13: Results of LOQ

	LOQ
SD of Intercept	19518.16286
Slope	55283

Observation: The LOQ was found to be 3.53µg/ml for Acalabrutinib.

Assay of Pharmaceutical Dosage form

Twenty tablets/Capsules were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock arrangement (1 ml) was exchanged to five distinctive 10 ml volumetric flagons and volume was made up to 10 ml with same dissolvable framework (Mobile stage). The readied arrangements were infused in five repeats into the HPLC framework and the perceptions were recorded.

A duplicate injection (Blank Solution) of the standard arrangement likewise infused into the HPLC framework and the chromatograms and peak zones were recorded and figured.

ASSAY

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

Where:

AT = Peak Area of drug obtained with test preparation
AS = Peak Area of drug obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard

The assay was performed as explained in the previous chapter. The results which are obtained are following:

Table 14: Recovery Data for estimation Acalabrutinibin Acabrunat Capsule

Brand name of Acalabrutinib	Labelled amount of Drug (mg)	Amount (mg) found by the proposed method (n=3)	Assay %
Acabrunat Molflu Capsule (200mg) (Natco Pharma Limited)	200mg	199.749mg	99.598%

Result & Discussion: The amount of drug in Acabrunat Capsule was found to be 199.749 (±0.789) mg/tab for Acalabrutinib & % Purity was 99.598 (±0.695) %.

Forced Degradation Studies

Following protocol was strictly adhered to for forced degradation of Acalabrutinib Active Pharmaceutical Ingredient (API). The API (Acalabrutinib) was subjected to keep in some stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. It is one type of accelerated stability studies of the drugs that is used to help us to determining the total fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing [30]. The different types of forced degradation pathways/studies are studied here are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

Results of Degradation Studies: The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Acalabrutinib were stable only in acidic, thermal and basic stress conditions. The results of stability studies are given in the following Table-15.

Table 15: Results of Force Degradation Studies of Acalabrutinib API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	91.326	8.674	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	83.215	16.785	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	90.311	9.689	100.00
UV (254 nm)	24Hrs.	81.322	18.678	100.00
3% Hydrogen Peroxide	24Hrs.	73.514	26.486	100.00

Summary and Conclusion

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Acalabrutinib, 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 Develosil ODS HG-5 RP C_{18}, 5 µm, 15cmx4.6mm 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, water, 0.1N NaOH, 0.1NHCl). The drug was found to be freely soluble in N, N-dimethylformamide, soluble in dichloromethane, very slightly soluble in ethanol (96%). and practically insoluble in water. Solubility in water is increasing with lowering of pH within the physiological range. 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 Acalabrutinib it is evident that most of the HPLC work can be accomplished in the wavelength range of 255 nm conveniently. Further, a flow rate of 1 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 which can help in the analysis of Acalabrutinib in different formulations.

References

1. Krishna GA. Development and Validation of Novel HPLC Bioanalytical Analysis Method for Acalabrutinib: An Anticancer Drug in Human Plasma. Asian Journal of Chemistry. 2020;32(10):2606-2610.
2. Priyanka P. Development and Validation of RP-HPLC Method for Determination of Acalabrutinib in Bulk and Its Pharmaceutical Formulation. European Journal of Biomedical and Pharmaceutical Sciences. 2019;6(4):465-470.
3. Anusha A. Stability Indicating RP-HPLC Method Development and Validation for the Determination of Acalabrutinib in Bulk Drug and Capsule Dosage Form. International Journal of Bio-Pharma Research. 2019;8(8):2758-2762.
4. Snyder R, Kirkland J, Glajch L. Practical HPLC Method

Development. 2nd ed. John Wiley and Sons International publication; c2011.
5. Ashutoshkar S. Pharmaceutical Drug Analysis. 2nd ed. New Age International Private Limited Publishers; 2005.
6. Beckett H, Stenlake JB. Practical Pharmaceutical Chemistry. 4th ed. C.B.S. Publishers and Distributors, New Delhi; c1988.
7. Williard HH, Merit LL, Dean FA, Settle FA. Instrumental Methods of Analysis. 6th ed. C.B.S. Publishers and Distributors, New Delhi; c1987.
8. Thakekar S, *et al*. Method Development and Validation for Quantitative Estimation of Acalabrutinib in Capsule Dosage Form by RP-HPLC Method. International Journal of Pharmaceutical and Pharmaceutical Research. 2022;23(2):34-46.
9. Kealey D, Haines PJ. Instant Notes on Analytical Chemistry. BIOS Scientific Publishers Limited, UK; c2002.
10. Chatwal GR, Anand SK. Instrumental Methods of Chemical Analysis. 5th ed. Himalaya Publishing House, Mumbai; c2005.
11. Swartz ME. Journal of Liquid Chromatography. 2005;28(7/8):1253-1263.
12. Journal of Chromatography B. 2008 Mar 1;863(2):258-265. Published on Jan 18, c2008.
13. Guideline IH. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1(20):05.
14. ICH Q2A. Validation of Analytical Methods: Definitions and Terminology. International Conference on Harmonization (ICH); c1994.
15. Green JM. A Practical Guide to Analytical Method Validation. Analytical Chemistry News & Features; c1996. p. 305a-309a.
16. Winslow PA, Meyer RF. Defining a Master Plan for the Validation of Analytical Methods. Journal of Validation Technology; c1997. p. 361-367.
17. AOAC Peer-Verified Methods Program. Manual on Policies and Procedures. Arlington, Va., USA; c1998.
18. Patil RS, Ghormade V, Deshpande MV. Chitinolytic enzymes: an exploration. Enzyme and Microbial Technology. 2000;26(7):473-83.
19. Baht GS, Silkstone D, Vi L, Nadesan P, Amani Y, Whetstone H. Exposure to a youthful circulation rejuvenates bone repair through modulation of β-catenin. Nature Communications. 2015;6(1):7131.
20. Williard HH, Merit LL, Dean FA, Settle FA. Instrumental Methods of Analysis. 7th ed. C.B.S. Publishers, New Delhi; c2002.
21. Wilson DH, Yu J, Karian A, Kozlowski J, O'Reilly S. Development and multisite evaluation of an automated assay for B12 on the Abbott AxSYM analyzer. Clinical chemistry. 1999;45(3):428-429.
22. Food and Drug Administration (FDA). Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation. Federal Register (Notices). 2000;65(169):52776-52777.
23. Vibha G. Development and Validation of HPLC Method - A Review. International Research Journal of Pharmaceutical and Applied Sciences. 2012;2(4):22-23.
24. Bliesner DM. Validating Chromatographic Methods. John Wiley & Sons Inc; c2006. p. 88-92.
25. ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology.
26. Gupta V. Development and validation of HPLC method - A Review. International Research Journal of Pharmaceutical and Applied Sciences. 2012;2(4):17-25.
27. Bhardwaj SK. A Review: HPLC Method Development and Validation. International Journal of Analytical and Bioanalytical Chemistry. 2015;accepted 20 November.
28. Vidushi Y, Meenakshi B, Bharkatiya M. A review on HPLC method development and validation. Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences. 2017;2(6):178.
29. Sonawane LV. Bioanalytical Method Validation and Its Pharmaceutical Application- A Review. Pharmaceutica Analytica Acta. 2014;5:3.
30. Guideline IH. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1(20):05.