

Liquid Chromatographic Method and Validation for Quantitative Estimation of Acalabrutinib from Bulk Form and Marketed Tablet Formulation

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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 μ m, 15cmx4.6mm i.d.column in an isocratic mode of separation utilizing Methanol : Phosphate buffer (0.02M, 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 12-28mcg/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 μ g/mL and 15.164 μ g/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.

I.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. 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 therapy¹. 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 pyrrolidine carboxamide, an imidazopyrazine, an ynone and a tertiary carboxamide. Acalabrutinib is currently indicated for the treatment of adult patients with Mantle Cell Lymphoma (MCL) who have received at least one prior therapy². It has also been recently approved for chronic lymphocytic leukemia and small lymphocytic lymphoma. The IUPAC Name of Acalabrutinib is 4-[8-amino-3-[(2S)-1-but-2-ynoyl pyrrolidin-2-yl] imidazo [1, 5-a] pyrazin-1-yl]-N-pyridin-2-yl benzamide³. The Chemical Structure of Acalabrutinib is as following

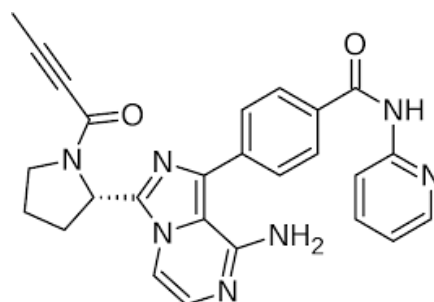


Fig-1: Chemical Structure of Acalabrutinib

After performing an extensive literature review³¹⁻³², an attempt was made to develop a smooth plain

sailing, unambiguous, valid, speedy, and decisive strategy for estimating Acalabrutinib in bulk form and marketed pharmaceutical dosage form by using RP-HPLC.

II. EXPERIMENTAL WORK

Table-1: Instruments used

S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detectors.
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	Synpharma Research Lab, Hyderabad
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 into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

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

Preparation of SampleSolution:

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 10mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC 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 (0.45µm) and finally sonicated to degas.

Procedure:

Inject the samples by changing the chromatographic conditions⁴ and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines¹³⁻¹⁴.

Mobile Phase Optimization:

Initially the mobile phase⁵ tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase 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.

Preparation of Buffer and Mobile Phase:**Preparation of Potassium Dihydrogen Phosphate (KH₂PO₄) Buffer (0.02M) (pH-3.6):**

Dissolve 2.72172g 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 filtration⁶ 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 in digital ultra sonicator 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 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 solution into a 10ml 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 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.

Preparation of Sample Solution:

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

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

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay⁷ 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 7ml 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 (12ppm of Acalabrutinib):

Take 0.12ml 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 ultrasonicator.

Preparation of Level – III (20ppm of Acalabrutinib):

Take 0.2ml 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 – IV (24ppm 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 the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (28ppm of Acalabrutinib):

Take 0.28ml 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.

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.

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 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.

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 0.08ml 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 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 solution into a 10ml 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 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 0.12ml of the above Acalabrutinib stock solution into a 10ml 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. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Acalabrutinib and calculate the individual recovery⁸ and mean recovery values.

Limit of Detection and Limit of Quantification (LOD & LOQ):**Preparation of 5.004µg/ml Solution (For LOD):**

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

Preparation of 15.164µg/ml Solution (For LOQ):

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 0.15164ml 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 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 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 solution into a 10ml 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 1ml/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 are same. 20µl of the above sample was injected and chromatograms were recorded.

Forced Degradation Studies

The purpose of stability testing⁹⁻¹² is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity,

and light, and to establish a re-test period for the drug substance or a shelf life for the drug.

III. RESULTS AND DISCUSSION

Selection of Wavelength:

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

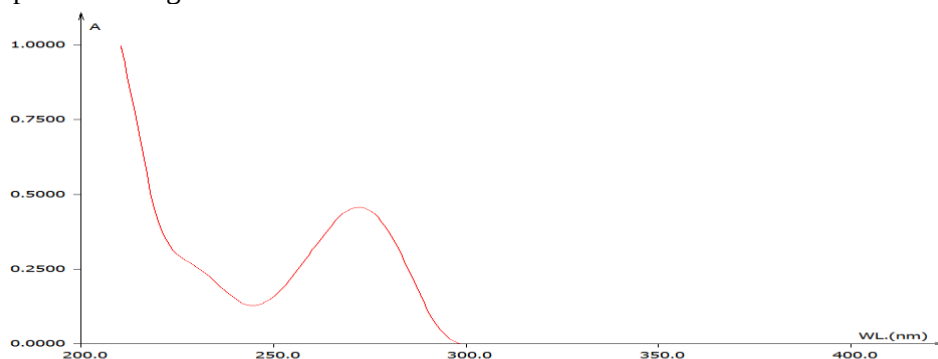


Fig-2: UV Spectrum of Acalabrutinib (255nm)

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

Method Development

Several concurrent trials developed the proposed method to establish the preferred chromatographic conditions, which would be helpful to conduct a complete validation study. The optimal conditions considered for mobile phase were 45:55% v/v ratio of Methanol and 0.02M potassium dihydrogen orthophosphate buffer at pH 3.6 and Develosil ODS HG-5 RP C₁₈, 5 µm, 15cmx4.6mm i.d. Column¹⁶ were used, which gave a sharp symmetric peak, minimum tailing factor with short run time for Acalabrutinib at a flow rate of 1.0 mL/min and detection wavelength 255 nm was optimized. Sample of 20 µL was injected into HPLC System¹⁷ and the different optimization of chromatographic parameters is depicted in Table 3. The retention time for Acalabrutinib was found to be 3.210 minutes (Figure 3).

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 , 5 µm, 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

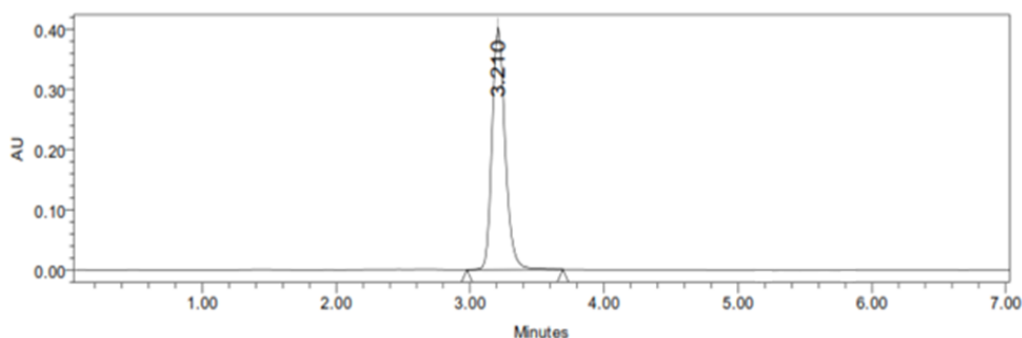


Fig-3: Optimized Chromatographic Condition

Conclusion: This trial shows proper plate count, peak and baseline in the chromatogram. It's Pass the all system suitability parameters. So, it's optimized chromatogram.

Validation of Analytical Method

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 parameters¹⁸ 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	$T \leq 2$	Acalabrutinib = 0.12
2	Theoretical plate	$N > 2000$	Acalabrutinib = 7258
3	Tailing Factor	$(Tf) < 2$	Acalabrutinib = 1.25

Specificity:

Specificity¹⁹ 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. The chromatograms representing the peaks of blank, Acalabrutinib and the sample containing the three drugs were shown in following figures respectively.

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.

Linearity: To evaluate the linearity²⁰, 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 μ g/ml for Acalabrutinib. The prepared

solutions were filtered through Whatman filter paper (No.41). From these solutions, 20 μ l injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve²¹ was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs: There sultant area so flinearity peaks are plotted against Concentration.

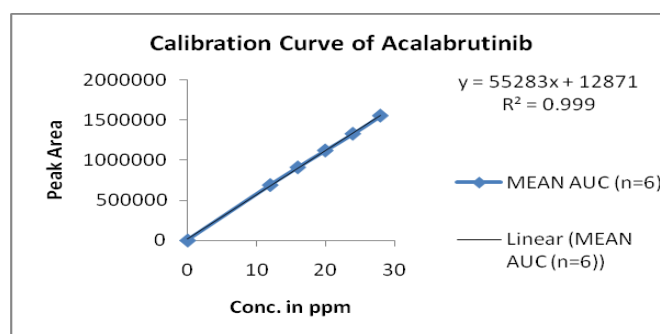


Fig-4: Standard Curve for Acalabrutinib

Observation: Linearity range²² was found to be 0-28 μ g/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.(μ g/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.

$$Y = mx + c$$

$$\text{Slope (m)} = 55283$$

$$\text{Intercept (c)} = 12871$$

$$\text{Correlation Coefficient (r)} = 0.9995$$

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 values²³. Accuracy at different concentrations (80%, 100%, and 120%) was prepared and the % recovery was calculated.

Table-7: Accuracy Results of Acalabrutinib

Sample ID	Concentration (μ g/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
S ₂ : 80 %	8	7.843532	446485	100.637	

S ₃ : 80 %	8	8.19449	465887	100.73	% R.S.D.= 0.471753 Mean= 100.6646667% S.D. = 1.166369295R.S.D.= 1.158667
S ₄ : 100 %	10	9.892661	559767	99.41	
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 precision²⁴ 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 were calculated for Acalabrutinib are 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 precision²⁵ (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 limits²⁹⁻³⁰.

Intra Day (Day-1)/Analyst-1:

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		

Inter Day (Day -2/Analyst-2)

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	3.21	296571	7745	1.24
6	Acalabrutinib	3.254	298698	7658	1.25
Mean			295805		
Std.Dev.			1819.33		
%RSD			0.61505		

Robustness: Robustness²⁶ 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

LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD²⁷ 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 1.165µg/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 LOQ²⁸.

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

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 flasks 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:

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 Capsule(Natco Pharma Limited)	200mg	199.247mg	99.563%

Discussion:

The amount of drug in Acabrunat Capsule was found to be 199.247 (± 0.378) mg/tab for Acalabrutinib & % Purity was 99.563 (± 0.487)%.

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 determine the total fate of the drug that is likely to happen after long time storage, within a very short time as compared to the real time or long term stability testing. The different types of forced degradation pathways/studies are studied here are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

The results of the forced degradation studies³⁰ indicated the specificity of the developed method that has been developed. Acalabrutinib was 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 (0.1N NaOH)	24Hrs.	83.215	16.785	100.00
Thermal Degradation (60 °C)	24Hrs.	90.311	9.689	100.00
UV (254nm)	24Hrs.	81.322	18.678	100.00
3% Hydrogen Peroxide	24Hrs.	73.514	26.486	100.00

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 C₁₈, 5µm, 15cmx4.6mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good. 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 1.0 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 which can help in the analysis of Acalabrutinib in different formulations.

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