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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

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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, 5µm, 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 5.004µg/mL and 15.164µg/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.

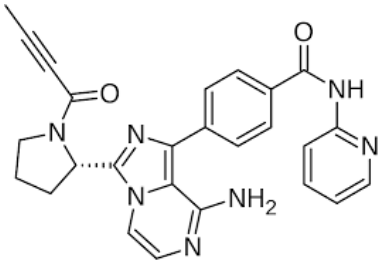

**Fig 1:** Chemical Structure of Acalabrutinib

## 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 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 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 10mg 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 (0.45µm) 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 2.72172g 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 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 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent[9]. (Stock solution)

Further pipette 0.1ml 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 aboveAcalabrutinib stock

solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

**Preparation of Sample Solution**

Weight 10 mg equivalent weight ofAcalabrutinib 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 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 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[11] 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[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 Acalabrutinibworking 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[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 5.004µg/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[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 Acalabrutinibworking 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 Bufferwas 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**

**WavelengthDetection:**

Thedetectionwavelength[19]wasselectedby dissolvingthedruginmobilephase togeta concentrationof10µg/mlforindividualandmixedstandards.Th eresulting solution wasscannedin U.V range from200-400nm. The UV spectrumofAcalabrutinib was obtained and the Acalabrutinib show edabsorbance' smaximaat 255nm. The UV spectra of drug are follows:

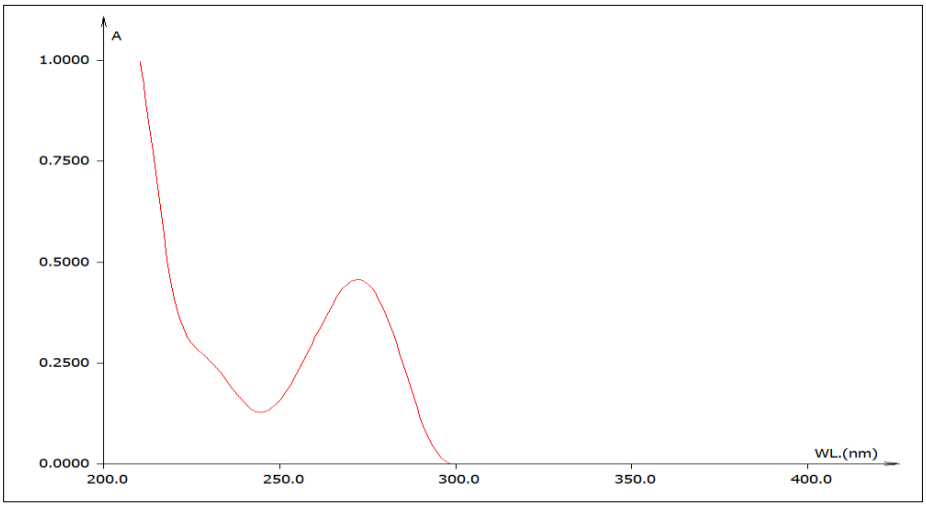


**Fig 2:** UV Spectrum of Acalabrutinib

**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 | 20µl |
| Type of Elution | Isocratic |

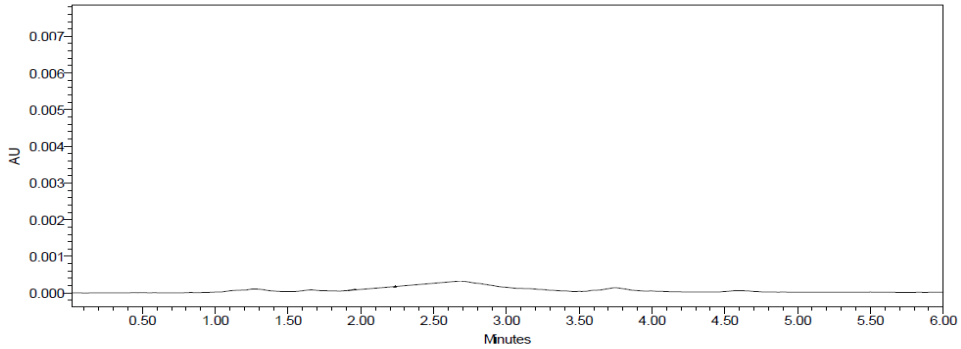


**Fig 3:** Chromatogram of Blank Solution

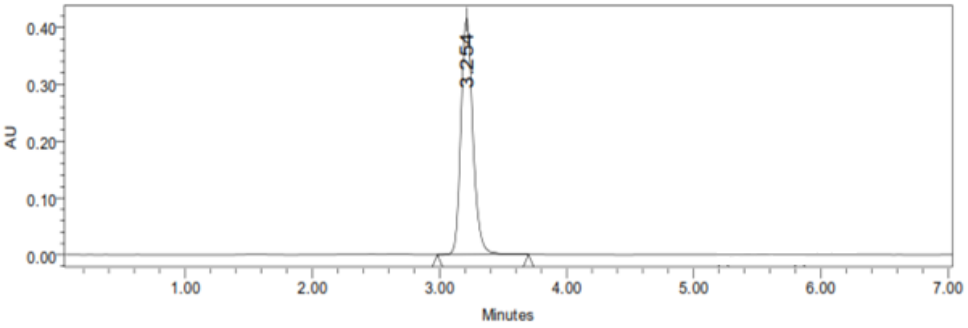


**Fig 4:** Chromatogram of Acalabrutinib in Optimized Chromatographic Condition

### 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 abovechromatograms 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 0-28µg/ml for Acalabrutinib. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20µl 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.

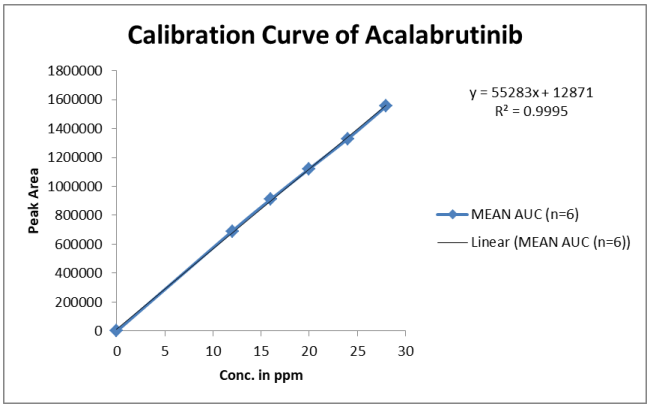


**Fig 5:** Standard Curve for Acalabrutinib

**Observation:** Linearity range was found to be 0-28µg/ml for Acalabrutinib. The correlation coefficient was found to be 0.9995, the slope was found to be 55283and 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. | PeakName | 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 interday 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 1.165µg/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 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 (254nm) | 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 $C_{18}$, 5µm, 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.

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