Development and Validation of RP-HPLC Method for Determination of Pirtobrutinib in Bulk and Pharmaceutical Dosage Form

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ABSTRACT:
A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (HPLC) method has been developed for the quantitative analysis of Pirtobrutinib in pharmaceutical dosage form. Chromatographic separation of Pirtobrutinib was achieved on Waters alliance e-2695 HPLC, by using Inertsil ODS (150 x 4.6 mm, 3.5 µ) column and the mobile phase containing 1.8 gms HSA is dissolved in 1lt of HPLC water pH-2.5/OPA & ACN in the ratio of 60:40 % v/v. The flow rate was 1.0 mL/min; detection was carried out by absorption at 271nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Pirtobrutinib were NLT 2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. Additionally, the method was employed for stability studies through forced degradation experiments, revealing that the purity threshold remained below the purity angle across all conditions. The percentage degradation observed for acid, alkali, peroxide, reduction, thermal, Photolytic, and hydrolysis conditions were 11.4 %, 13 %, 14.9 %, 2.0 %, 10.7 %, 2.3 %, and 1.0 %, respectively. Therefore, the developed RP-HPLC method is suitable for the quantification of Pirtobrutinib in routine pharmaceutical operations and analytical laboratories. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Pirtobrutinib.

Key words: HPLC, Pirtobrutinib, ICH guidelines, Degradation studies.

INTRODUCTION:

Pirtobrutinib, a selective, non-covalent Bruton's tyrosine kinase (BTK) inhibitor, has emerged as a promising treatment for various B-cell malignancies. BTK is a key protein in the B-cell receptor (BCR) signaling pathway, and its inhibition has proven beneficial in treating B-cell lymphomas, including chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). Unlike conventional covalent BTK inhibitors (cBTKis) such as ibrutinib, which irreversibly bind to BTK, pirtobrutinib binds reversibly, offering advantages in overcoming resistance associated with mutations in the BTK gene [1].

In a pivotal Phase 1/2 study known as the BRUIN trial, pirtobrutinib demonstrated efficacy in patients with relapsed or refractory B-cell malignancies, particularly in CLL and MCL. The study indicated that pirtobrutinib not only had a favorable safety profile but also was effective in patients who had failed prior therapies, including cBTKis. This is crucial as treatment resistance is a significant challenge in B-cell malignancies, especially for patients who develop mutations in the BTK binding site, which is a common cause of resistance to cBTK inhibitors. In CLL, pirtobrutinib has shown durable responses and prolonged progression-free survival, especially in patients who previously failed ibrutinib and acalbrutinib [2].

The mechanism of pirtobrutinib's action is its ability to target both wild-type and mutant BTK proteins, providing efficacy even in cases where other BTK inhibitors are ineffective due to resistance mutations [3]. This ability to function in resistant cases is especially relevant for patients with high-risk features, such as complex cytogenetics or older age, who typically do not respond well to standard therapies [4]. In MCL, pirtobrutinib has also been effective, with high response rates reported among patients who had failed prior BTK inhibitor therapies, including those with high-risk subgroups [5].

The safety profile of pirtobrutinib is considered favorable compared to other BTK inhibitors. It has a lower incidence of side effects such as atrial fibrillation, bleeding, and hypertension, which are commonly associated

with covalent BTK inhibitors. This makes it a promising treatment option for patients requiring long-term therapy. Most adverse effects reported in clinical studies have been mild to moderate, such as fatigue, diarrhea, and headaches [6].

In addition to its application in CLL and MCL, pirtobrutinib is also being explored for use in other B-cell malignancies. A review by Jensen et al. highlighted the potential of pirtobrutinib across a broader range of B-cell diseases, including Waldenström's macroglobulinemia and diffuse large B-cell lymphoma (DLBCL) [7]. Furthermore, the recent FDA approval of pirtobrutinib for relapsed or refractory MCL underscores its significant clinical impact and therapeutic promise for resistant B-cell malignancies.

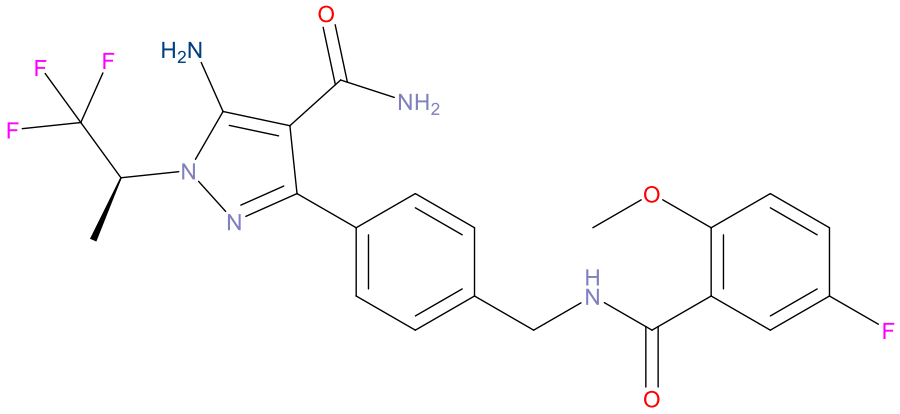


Figure 1: Structure of Pirtobrutinib

A crucial aspect of pirtobrutinib's development is its stability and analytical characterization. Studies focused on the development of robust analytical methods, such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS), have been essential in ensuring the accurate estimation of pirtobrutinib concentrations and identifying its degradants [8]. Such stability-indicating methods are vital for quality control and regulatory approval processes.

Looking ahead, future research will continue to evaluate pirtobrutinib's long-term effects, combination therapy potential, and the identification of biomarkers to predict response. As the understanding of its clinical benefits deepens, pirtobrutinib is poised to become a key player in the evolving landscape of B-cell malignancy treatments [9]. Its approval for use in MCL represents just the beginning, with hopes for broader applications across various B-cell-related cancers in the near future. The comprehensive literature review revealed that to date, no HPLC analytical method have been developed for the quantification of pirtobrutinib. Therefore, there is a significant need for the development of a simple, robust, accurate, and efficient analytical method for its estimation. High-Performance Liquid Chromatography (HPLC) stands out as an effective and sensitive technique for the separation, identification, and quantification of compounds.

MATERIALS AND METHODS

The HPLC system of model acquity manufacturer Waters e 2695 ALLIANCE with quaternary pump, Rheodyne injector with 20 µL loop connected to PDA detector. The analyte was separated on symmetric shield Inertsil ODS (150 × 4.6 mm,3.5 µ) and the mobile phase containing 1.8 gm of HSA dissolved in 1L of HPLC grade water, pH – 2.5/OPA and ACN in the ratio 60:40 % v/v. The flow rate was maintained as 1mL/min and injection volume was 10 µL. The run time was set as 5 min. HPLC grade water and acetonitrile (ACN, HPLC grade) were used throughout the analysis.

Determination of Working Wavelength (λ_{max}):

The maximum absorbance wavelength for the estimation of Pirtobrutinib was determined by scanning the drug solution in a mixture of acetonitrile and HSA pH-2.5/OPA (40:60) using a PDA detector. The wavelength range for scanning was 200–400 nm, with acetonitrile and HSA pH-2.5/OPA (40:60) used as the blank. The absorption curve exhibited at 271 nm, which was selected as the optimal detector wavelength. This wavelength was chosen for accurate drug estimation in the HPLC chromatographic method. Thus, 271 nm was identified as the key detection point for Pirtobrutinib analysis.

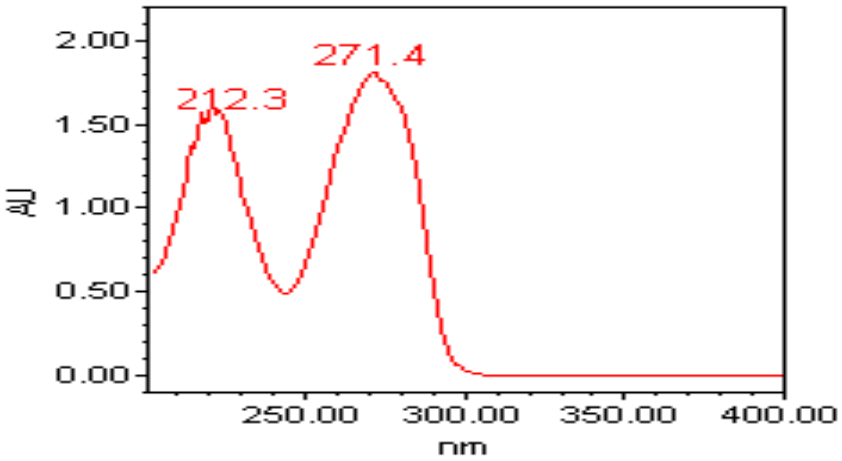


Figure 2: PDA Spectrum of Pirtobrutinib

Chromatographic conditions:
During the selection of chromatographic conditions, numbers of trails were carried out and the best trail was selected for optimized method.

Preparation of standard Stock solution:
Accurately weigh and transfer 5 mg of Pirtobrutinib working standard into a 10 mL clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (50 ppm of Pirtobrutinib).

Preparation of sample solution:
Accurately weighed and transfer 17.4 mg of Pirtobrutinib sample into a 10 mL clean dry volumetric flask add Diluent and sonicate it up to 30 min to dissolve, and centrifuge for 30 min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (50 ppm of Pirtobrutinib).

Table 1. Trails in optimization of chromatographic conditions

Trail	Column	Mobile phase	Wavelength	Flow rate	Injection volume	Run time	Observation
Trail 1	Luna Phenyl Hexyl (150×4.6 mm,3.5µ)	ACN+0.1% TFA (70:30)	200-400 nm	1 mL/min	10 µL	10 min	System suitability conditions are not with in the limit.
Trail 2	Luna Phenyl Hexyl (150×4.6 mm,3.5µ)	ACN+0.1% TFA (65:35)	271 nm	1 mL/min	10 µL	2.60 min	Broad peak is observed.
Trail 3	Inertsil ODS (150×4.6 mm,3.5µ)	ACN+0.1% TFA (65:35)	271 nm	1 mL/min	10 µL	10 min	Unknown peak is observed.
Trail 4	Inertsil ODS (150×4.6 mm,3.5µ)	Acetonitrile and HSA pH-2.5/OPA (50:50)	271 nm	1 mL/min	10 µL	10 min	Base line is not sufficient.
Trail 5	Inertsil ODS (150×4.6 mm,3.5µ)	Acetonitrile and HSA pH-2.5/OPA (45:55)	271 nm	1 mL/min	10 µL	10 min	Response of the peak is very high.
Trail 6	Inertsil ODS (150×4.6 mm,3.5µ)	Acetonitrile and HSA pH-2.5/OPA (40:60)	271 nm	1 mL/min	10 µL	10 min	This method is suitable for validation

The Pirtobrutinib peak was observed at 3.439 min with peak area 3876492, tailing factor 1.02. This trial was optimized.

General preparations

Preparation of HSA buffer

Take 1.8 g of HSA dissolved in 1litre of HPLC grade water and pH-2.5 adjusted with OPA. Filter through 0.45 µ nylon filter.

Preparation of Mobile Phase:

Mobile phase was prepared by mixing HSA pH-2.5/OPA and ACN taken in the ratio 60:40. It was filtered through 0.45 µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Preparation of diluent:

Acetonitrile is used as diluent.

Preparation of standard Stock solution:

Accurately weigh and transfer 5 mg of Pirtobrutinib working standard into a 10 mL clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (50 ppm of Pirtobrutinib).

Preparation of sample solution:

Accurately weighed and transfer 17.4 mg of Pirtobrutinib sample into a 10 mL clean dry volumetric flask add Diluent and sonicate it up to 30 min to dissolve, and centrifuge for 30 min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (50 ppm of Pirtobrutinib).

Procedure:

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Pirtobrutinib peak and calculate the % Assay by using the formulae.

SYSTEM SUITABILITY:

Tailing factor for the peak due to Pirtobrutinib in Standard solution should not be more than 2.0. Theoretical plates for the Pirtobrutinib peak in Standard solution should not be less than 2000.

Formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where:

AT = average area counts of test (sample) preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
DS = Dilution of working standard in mL.
DT = Dilution of test (sample) in mL.
WT = Weight of test (sample) taken in mg.
P = Percentage purity of working standard.
LC = Label Claim mg/mL.

METHOD VALIDATION SUMMARY:

SPECIFICITY:

Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drug was specific.

LINEARITY:

Preparation of stock solution:

Accurately weigh and transfer 5 mg of Pirtobrutinib working standard into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (12.5 ppm of Pirtobrutinib):
0.25 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (25.0 ppm of Pirtobrutinib):
0.50 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (37.5 ppm of Pirtobrutinib):
0.75 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (50.0 ppm of Pirtobrutinib):
1.00 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level –V (62.5 ppm of Pirtobrutinib):
1.25 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – VI (75.0 ppm of Pirtobrutinib)
1.50 mL of above stock solutions has taken in different 10 mL of volumetric flasks, dilute up to the mark with diluent.

Range:
The Range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity

Acceptance Criteria:
Correlation coefficient should be not less than 0.999.

Preparation Accuracy Sample solutions:
For preparation of 50 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 8.7 mg of Pirtobrutinib sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (25 ppm of Pirtobrutinib)

For preparation of 100 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 17.4 mg of Pirtobrutinib Sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (50 ppm of Pirtobrutinib)

For preparation of 150 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 26.1 mg of Pirtobrutinib sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (75 ppm of Pirtobrutinib).

Procedure:
Inject the standard solution, Accuracy - 50 %, Accuracy -100 % and Accuracy -150 % solutions.

Acceptance Criteria:
The % Recovery for each level should be between 98.0 to 102.0 %

PRECISION

Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of 3 types.

1. System precision
2. Method precision
3. Intermediate precision (a. Intra-day precision, b. Inter day precision)

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD.

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 50 ppm of Pirtobrutinib).

Acceptance Criteria:

The % RSD for the absorbance of six replicate injections results should not be more than 2 %.

ROBUSTNESS:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 mL/min to 1.1 mL/min.

Standard solution 50 ppm of Pirtobrutinib was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10 %.

B. The variation of Organic Phase ratio. Standard solution of 50 ppm of Pirtobrutinib was prepared and analysed using the varied in mobile phase ratio.

Limit of detection (LOD) and limit of quantification (LOQ):

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH) guidelines.

LOD = 3.3 X σ /S

LOQ = 10 X σ /S

LOD for Pirtobrutinib was found to be 0.45 µg/mL and LOQ for Pirtobrutinib was found to be 1.50 µg/mL.

Acceptance Criteria: s/n value for LOD is 3 and LOQ is 10.

DEGRADATION STUDIES:

Stock Preparation: Weigh 17.4 mg of Pirtobrutinib, dissolve in diluent in a 10 mL volumetric flask, and make up to the mark with the same solvent.

Acid Degradation: Transfer 1 mL of stock solution into a 10 mL flask. Add 1 mL of 1N HCl, heat at 60 °C for 1 hour, neutralize with 1N NaOH, dilute to 10 mL with diluent, and filter through a 0.45 µm syringe filter.

Alkali Degradation: Transfer 1 mL of stock solution into a 10 mL flask. Add 1 mL of 1N NaOH, heat at 60 °C for 1 hour, neutralize with 1N HCl, dilute to 10 mL with diluent, and filter.

Peroxide Degradation: Add 1 mL of stock solution into a 10 mL flask. Add 1 mL of 10 % hydrogen peroxide, heat at 60 °C for 1 hour, cool for 15 minutes, dilute, and filter.

Reduction Degradation: Add 1 mL of stock solution into a 10 mL flask. Add 1 mL of 10 % sodium bisulphite, heat at 60 °C for 1 hour, cool for 15 minutes, dilute, and filter.

Hydrolysis Degradation: Add 1 mL of stock solution into a 10 mL flask. Add 1 mL of HPLC grade water, heat at 60 °C for 1 hour, cool for 15 minutes, dilute, and filter.

Photolytic Degradation: Expose Pirtobrutinib sample to a photo-stability chamber for 3 hours, dilute with diluent, inject into HPLC, and analyze.

Thermal Degradation: Place Pirtobrutinib in a petridish, heat in a hot air oven at 105 °C for 3 hours, dilute with diluent, inject into HPLC, and analyze.

RESULTS AND DISCUSSION

In the optimization of chromatographic conditions for the HPLC method, six trials were conducted to achieve optimal parameters. Initial trials presented variations in retention time, area, USP tailing, and plate count. Trial-1 showed a retention time of 1.192 minutes with an area of 1162502, USP tailing of 1.43, and plate count of 575, indicating the need for further optimization. Trials - 2 to 5 adjusted parameters such as mobile phase

composition, leading to significant improvements in peak symmetry and efficiency. Trial - 5, with a retention time of 3.041 minutes and plate count of 3564, approached acceptable limits but required further refinement for validation.

In Trial-6, all parameters met the required specifications, resulting in an optimized method suitable for validation. This final method displayed a retention time of 3.439 minutes, area of 3876492, USP tailing of 1.02, and a plate count of 7945, confirming enhanced separation and resolution. The optimized conditions used Waters Alliance e-2695 HPLC with a 10 µL injection volume. The mobile phase consisted of acetonitrile and HSA at pH 2.5/OPA (60:40), with an symmetric shield inertsil ODS column (150 x 4.6 mm, 3.5µ), detection at 271 nm, a flow rate of 1 mL/min, runtime of 5 minutes, ambient temperature (25°C), and isocratic mode of separation. This setup demonstrated consistent and reproducible results across validation trials. Figure 3 shows the optimization of chromatographic conditions through trails 1-6.

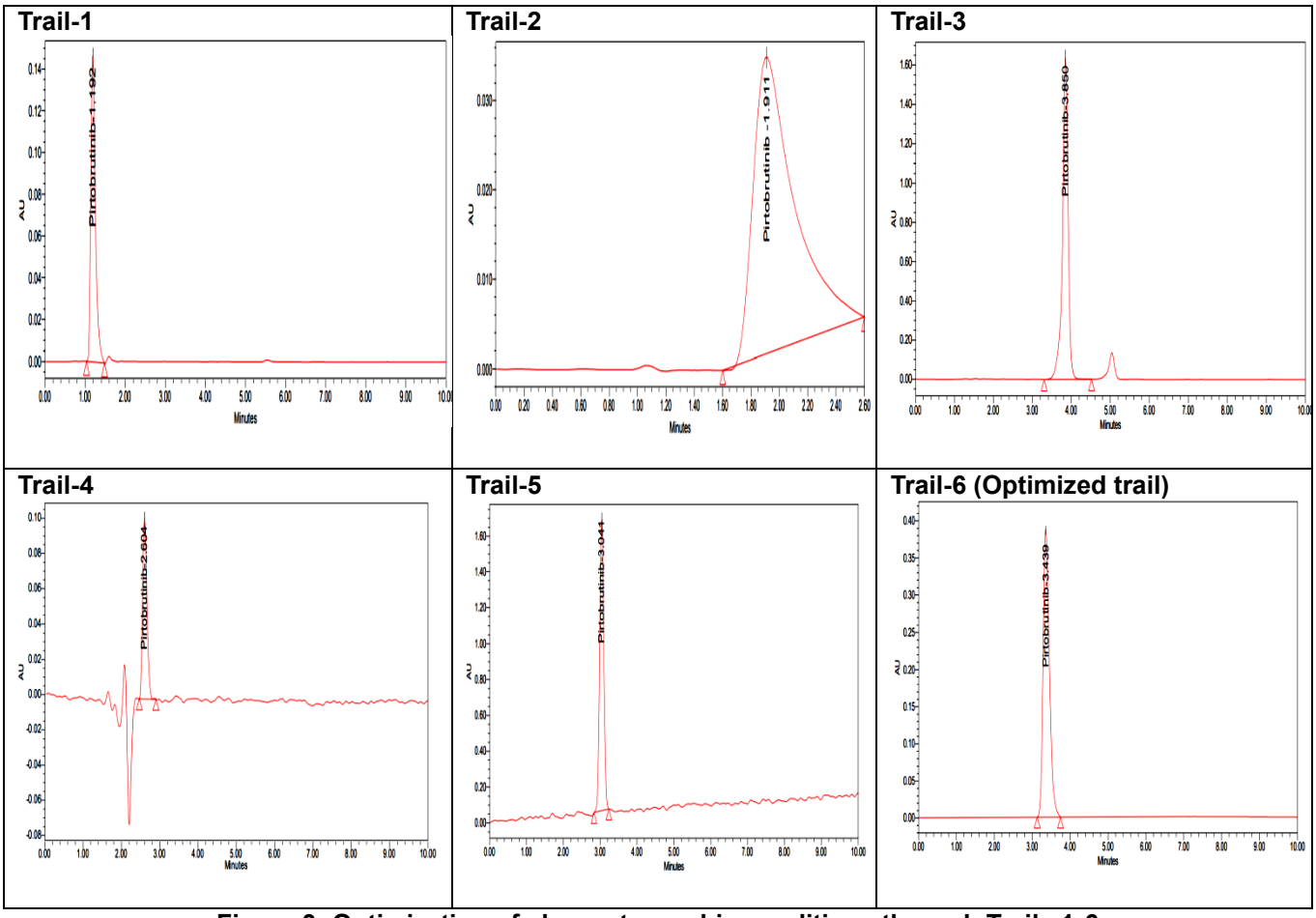


Figure 3: Optimization of chromatographic conditions through Trails 1-6

ANALYTICAL METHOD VALIDATION (HPLC)

The method was validated for its linearity range, accuracy, precision and specificity. Method validation was carried out as per ICH guidelines [10-17].

Table 2. Optimized chromatographic conditions and system suitability parameters of pirtobrutinib

PARAMETERS	OBSERVATION
Instrument used	Waters Alliance e-2695 HPLC
Injection volume	10 µl
Mobile Phase	Acetonitrile and HSA pH-2.5/OPA (40:60)
Column	Inertsil ODS (150 x 4.6 mm, 3.5µ).
Detection Wave Length	271nm
Flow Rate	1 mL/min
Runtime	10 min
Temperature	Ambient (25 °C)

Mode of separation	Isocratic mode
System suitability parameters for Pirtobrutinib	
Retention time	3.439
Plate count	7945
Tailing factor	1.02
% RSD	0.18

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines. System suitability parameters are optimized chromatographic conditions are shown in table-2

Specificity:

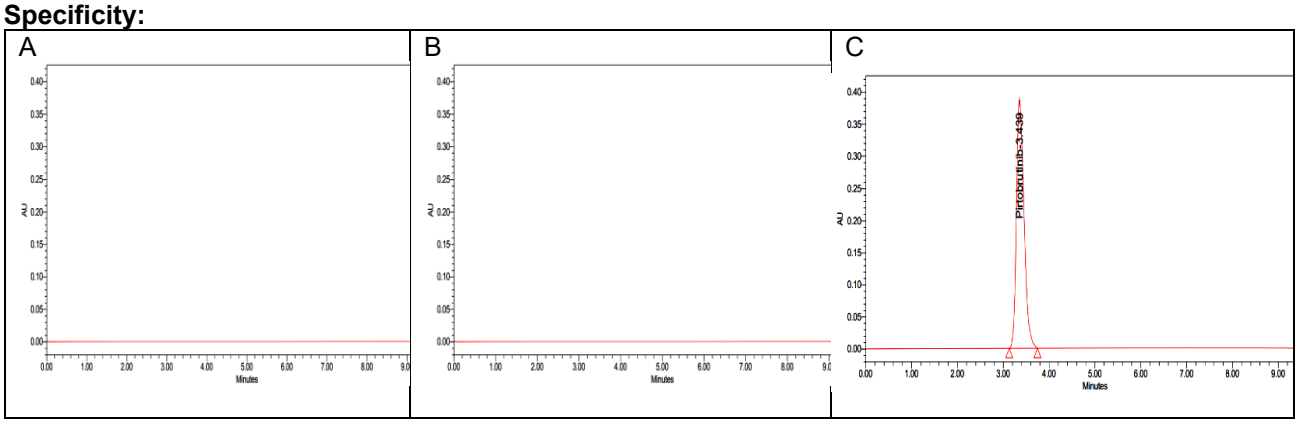


Figure 4. Typical chromatograms of A) Blank B) Placebo C) Standard

Retention times of Pirtobrutinib were 3.439 min. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific. Typical chromatograms of blank, placebo and standard are shown in Fig.4.

Linearity:

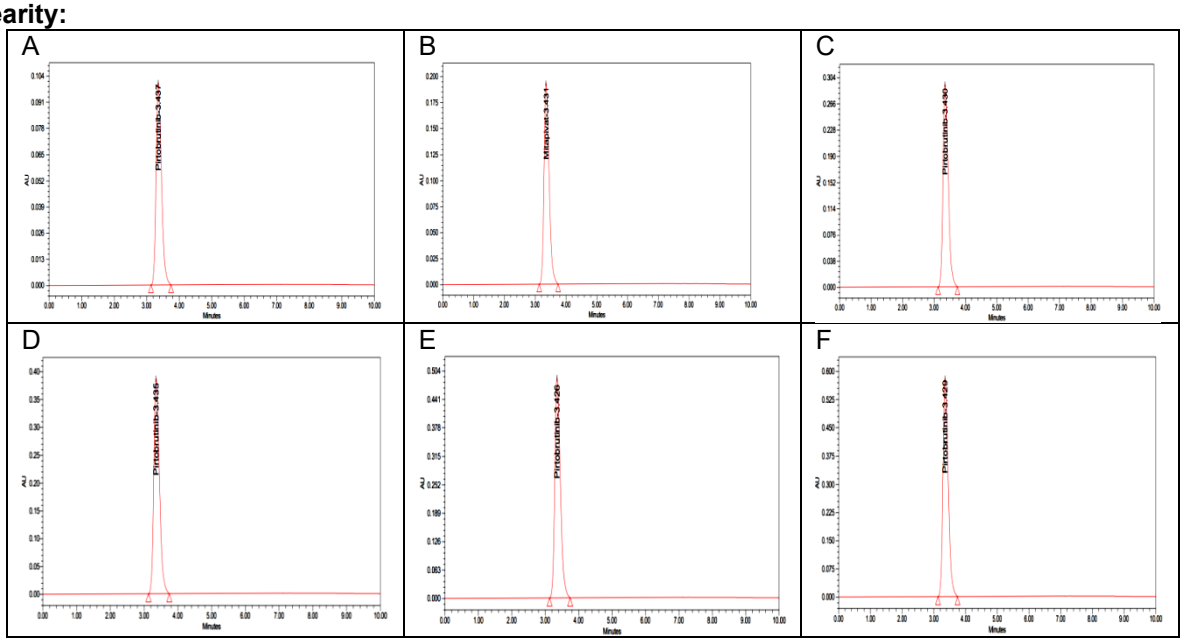


Figure 5. Chromatograms indicating the linearity: A) 25 % B) 50 % C) 75 % D) 100 % E) 125 % F) 150 % concentrated solutions of selected drug.

Table 3. Linearity for Pirtobrutinib

S.NO	**Pirtobrutinib**	
	Conc.(µg/mL)	**Peak area**
1	12.50	875421
2	25.00	1945782

3	37.50	2941524
4	50.00	3866924
5	62.50	4691233
6	75.00	5592189
Regression equation	y = 75226.67x+23724.75	
Slope	75226.67	
Intercept	23724.75	
R^2	0.99934	

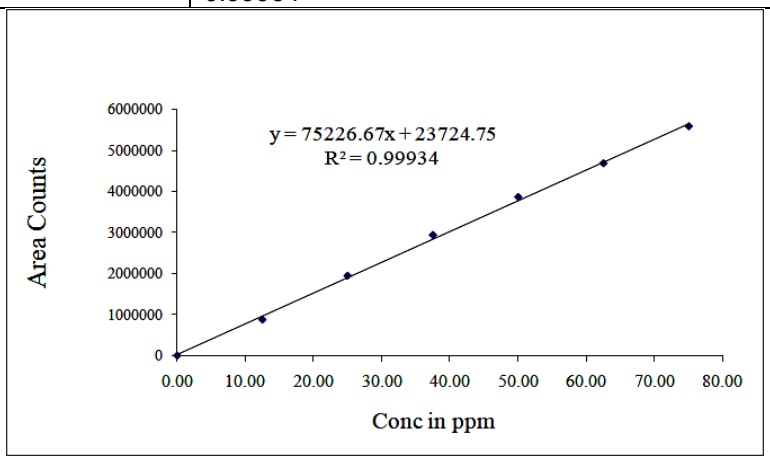


Figure 6: Calibration curve for Pirtobrutinib at 271nm

Linearity in HPLC is the ability of an analytical procedure to produce test results that are directly proportional to the concentration of an analyte in a sample. The linearity data is presented in Table 3, the chromatograms indicating linearity are shown in Figure 5, and the calibration curve for Pirtobrutinib is depicted in Figure 6.

PRECISION:

Table 4. System Precision

S. No	Concentration Pirtobrutinib (µg/mL)	Area of Pirtobrutinib
1.	50	3876492
2.	50	3865210
3.	50	3869145
4.	50	3838702
5.	50	3854813
6.	50	3870654
Mean		3862503
S.D		13705.460
% RSD		0.36

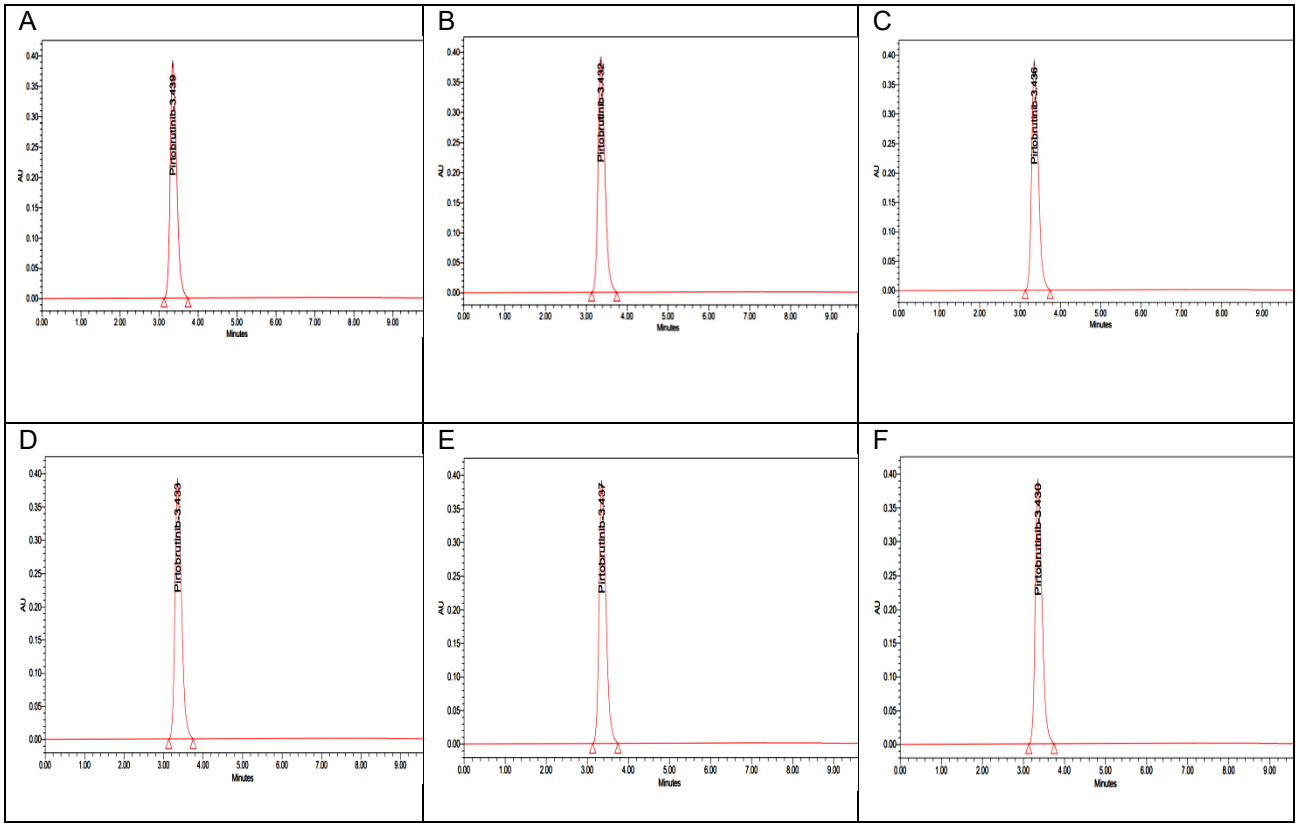


Figure 7. System Precision Chromatograms (A-F)

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD was calculated for Pirtobrutinib % RSD obtained as 0.36 for Pirtobrutinib. As the limit of Precision was less than "2" the system precision was passed in this method. Table 4 and Table 5 shows the system precision and method precision respectively, and the Fig. 7 represents system precision chromatograms. Method precision for Pirtobrutinib is represented in Table 5 and the Figure 8 shows the repeatability chromatograms. Intermedicate precision is represented in Table 6. Interday (Day-1) and Interday (Day-2) chromatograms are shwon in Fig.9 and Fig.10 respectively.

Repeatability:

Table 5: Method Precision for Pirtobrutinib by HPLC method

S. No.	Area for Pirtobrutinib
1	3848795
2	3851207
3	3865423
4	3872649
5	3855381
6	3862413
Average	3859311
SD	9127.838
% RSD	0.24

A	B	C

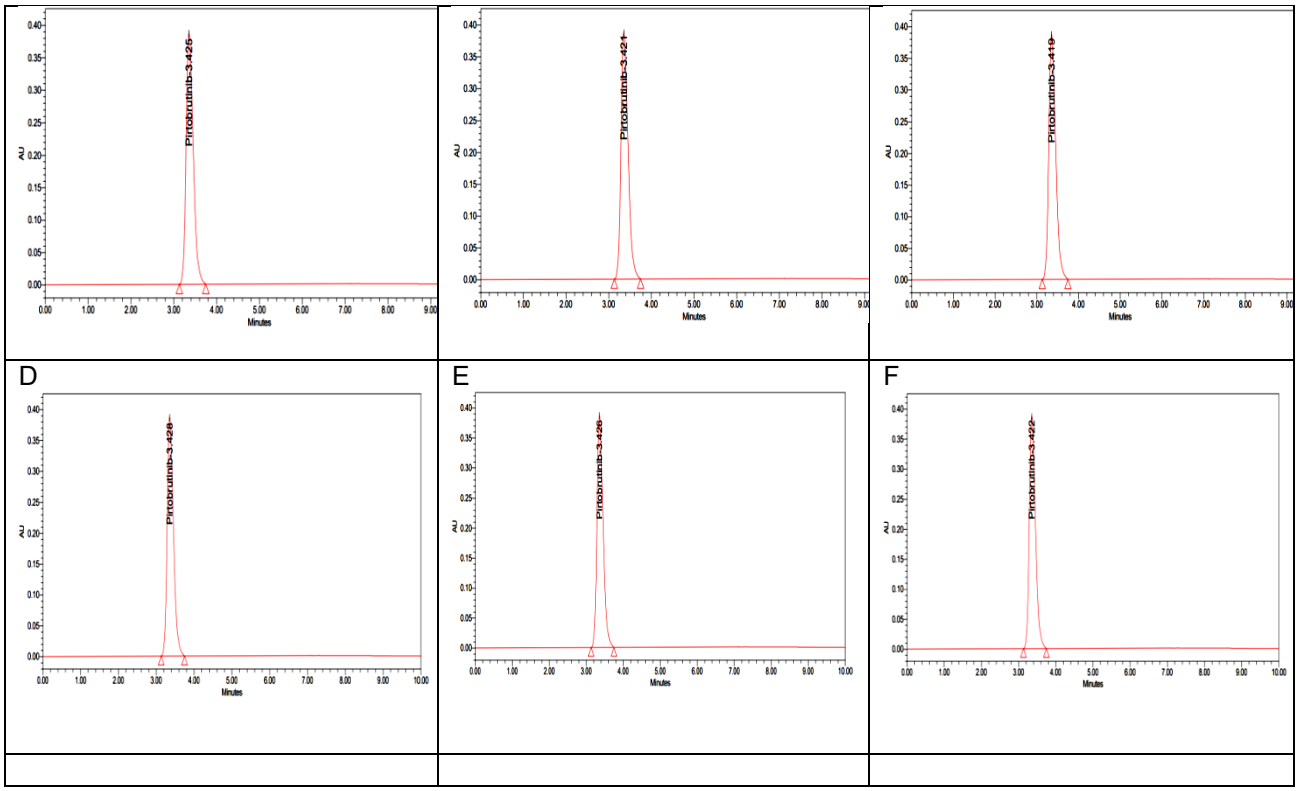


Figure 8. Repeatability Chromatograms (A-F)

Intermediate precision (Day_ Day Precision):

Table 6. Intermediate Precision (Day variation) for Pirtobrutinib by HPLC method

S. No.	Day 1 Area	Day 2 Area
1	3856912	3854278
2	3849714	3816329
3	3856290	3830418
4	3880554	3866732
5	3874216	3829501
6	3854687	3813784
Average	3862062	3835174
Standard Deviation	12299.398	21110.844
%RSD	0.32	0.55

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

A	B	C

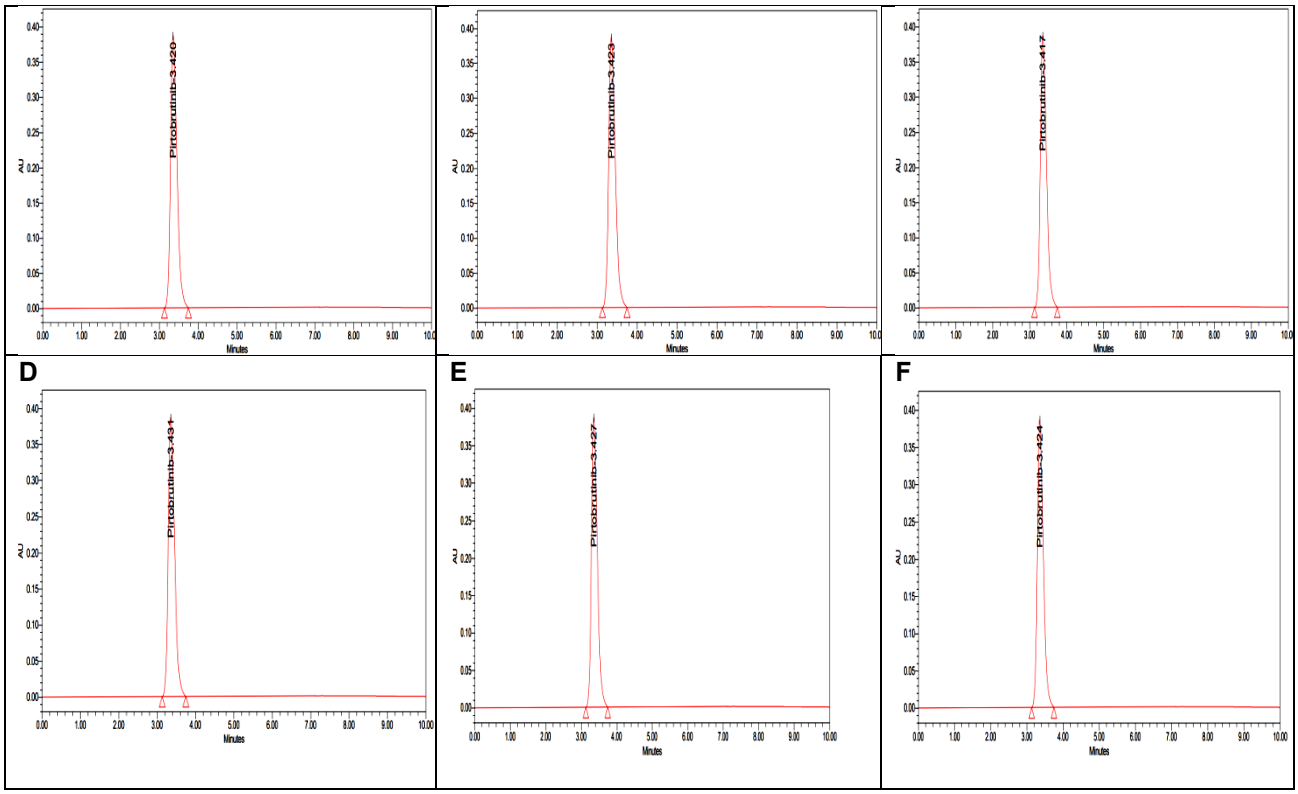


Figure 9. Inter day precision chromatograms - A-F (Day-1)

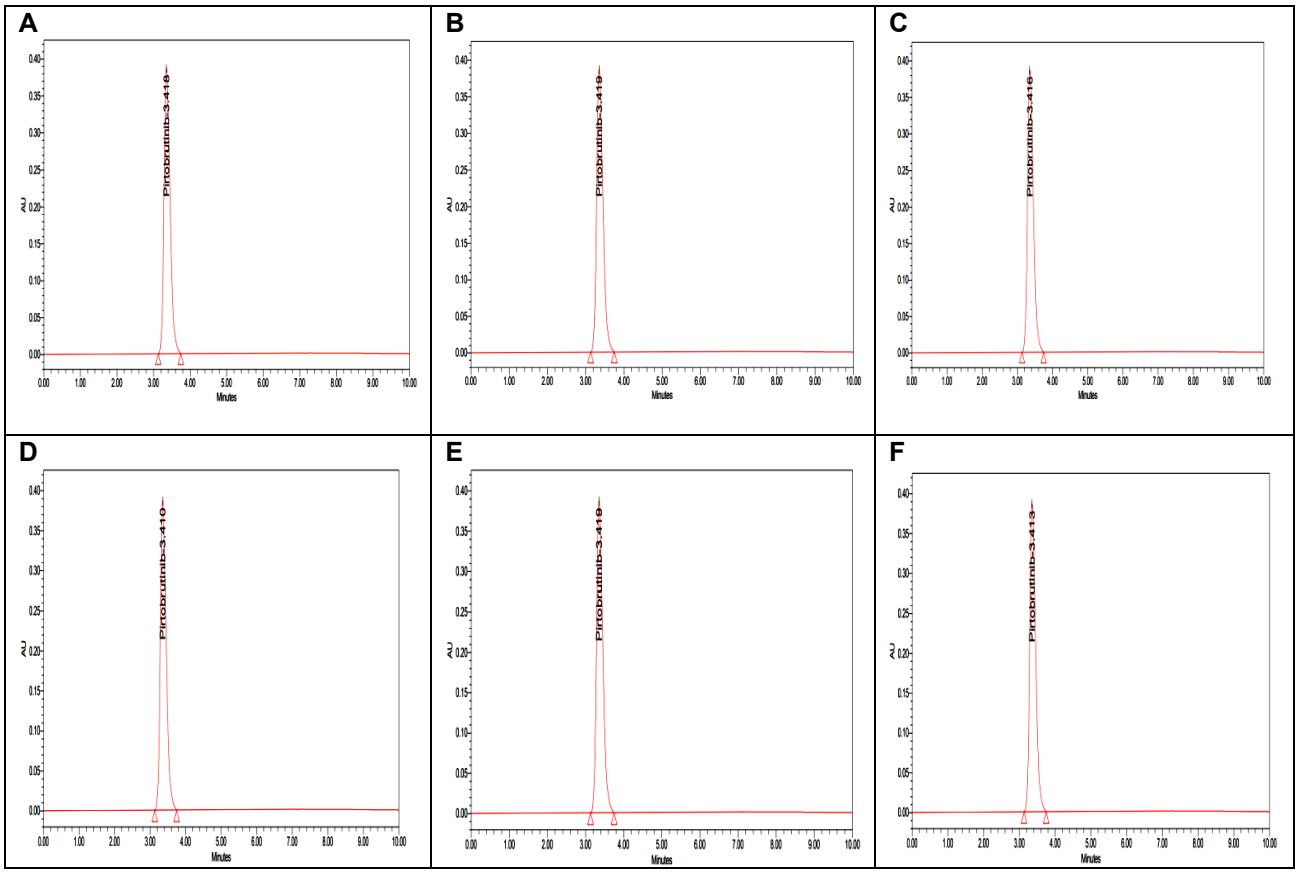


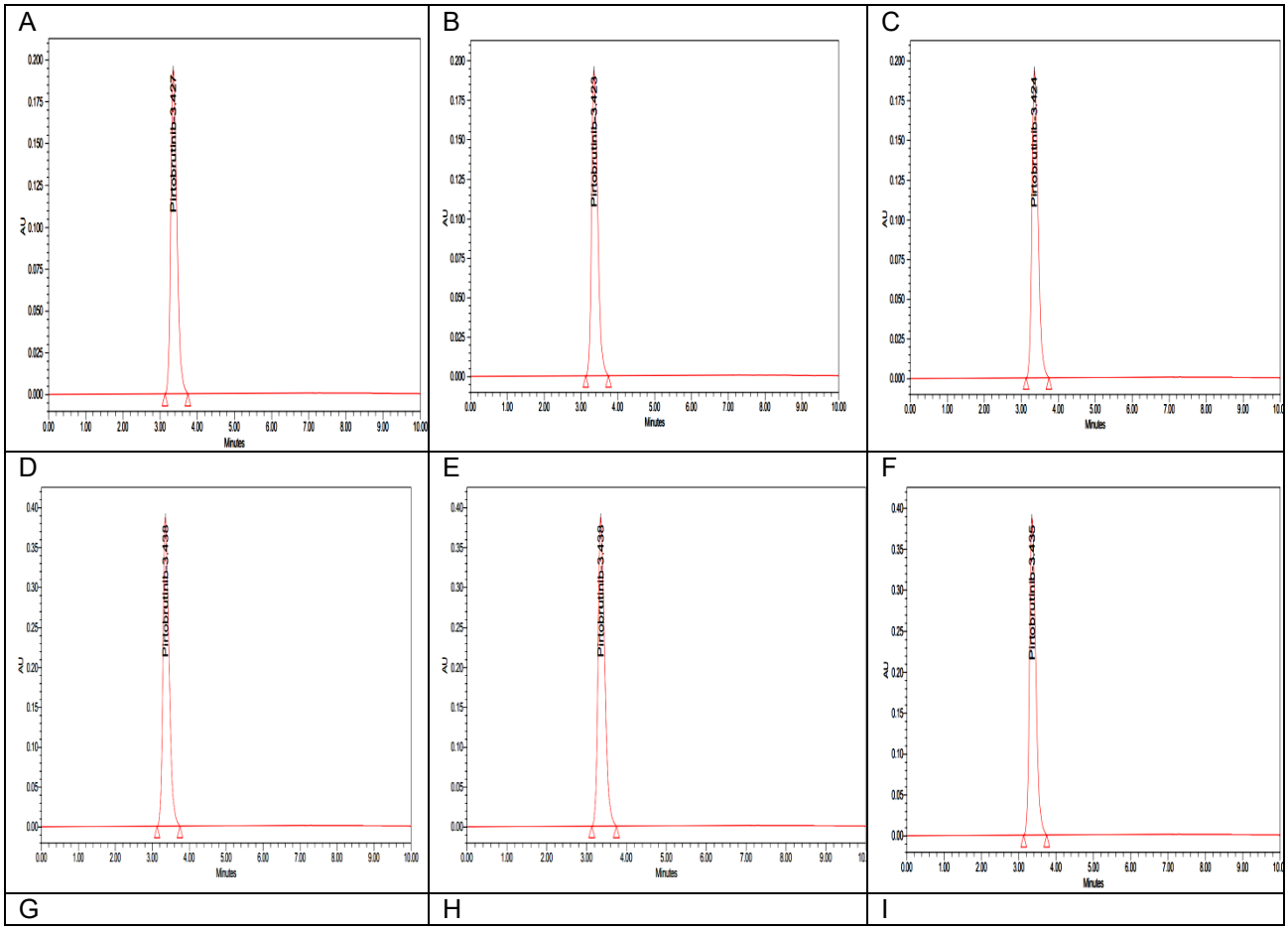
Figure 10. Inter day precision chromatograms - A-F (Day-2).

Accuracy:

Table 7. Accuracy results of Pirtobrutinib by HPLC method

Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
50 %	1942463	2.5	2.515	100.6	100.1
	1924284	2.5	2.491	99.6	
	1931312	2.5	2.500	100.0	
100 %	3857459	5.0	4.993	99.9	100.0
	3871946	5.0	5.012	100.2	
	3859603	5.0	4.996	99.9	
150 %	5812031	7.5	7.524	100.3	100.3
	5814570	7.5	7.527	100.4	
	5806456	7.5	7.516	100.2	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.1% for Pirtobrutinib respectively. Accuracy table is shown in table 7and the accuracy chromatograms are depected in Fig. 11.



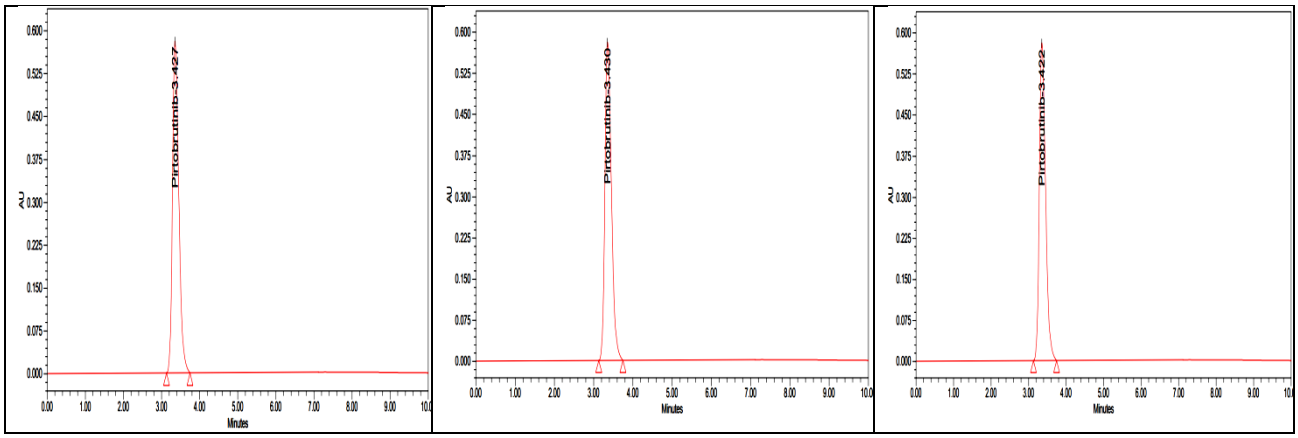


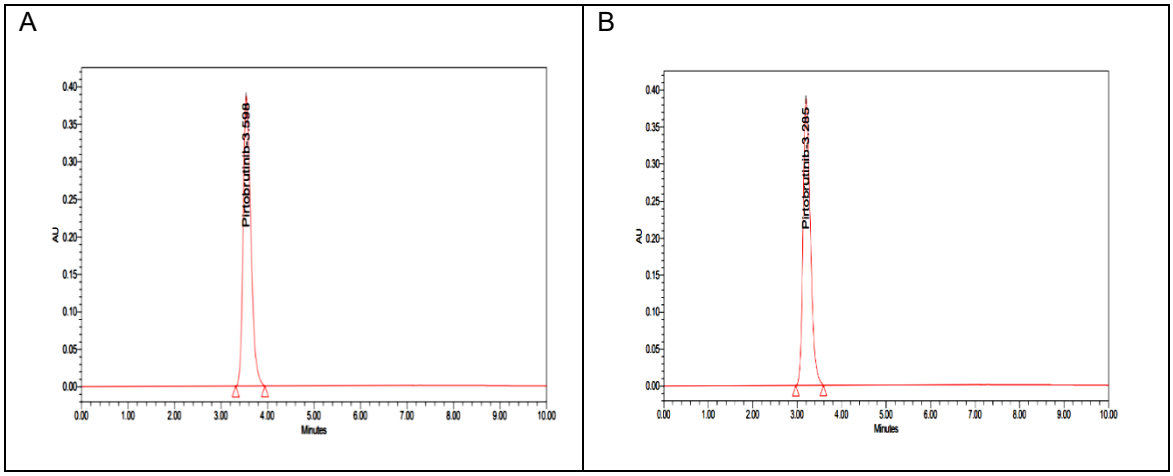
Figure 11. Chromatograms representing accuracy levels at 50 % (A, B, C), 100 % (D, E, F), and 150 % (G, H, I)

Robustness:

Robustness in HPLC assesses the method's reliability against small, deliberate variations in parameters like flow rate, mobile phase composition. It ensures consistent and accurate results under varied analytical conditions. Robustness results are shown in Table 8. Chromatograms showing the effects of flow rate (A: 0.9 mL/min, B: 1.1 mL/min) and organic phase composition in % (C: 36:64, D: 44:56) are shown in Fig.12. Robustness results of Pirtobrutinib is represented in Table 8.

Table 8: Robustness results of Pirtobrutinib

Parameter	Pirtobrutinib					
	Condition	Retention time(min)	Peak area	Tailing	Plate count	% RSD
Flow rate Change (mL/min)	Less flow(0.9 mL)	3.598	3632279	1.05	7832	0.25
	Actual flow(1.0 mL)	3.439	3876492	1.02	7945	0.36
	More flow(1.1mL)	3.285	3930105	0.98	8012	0.59
Organic Phase change	Less Org (36:64)	3.759	3533129	1.12	7741	0.55
	Actual (40:60)	3.432	3865210	1.07	7926	0.36
	More Org (44:56)	3.176	4029870	1.03	8179	0.42



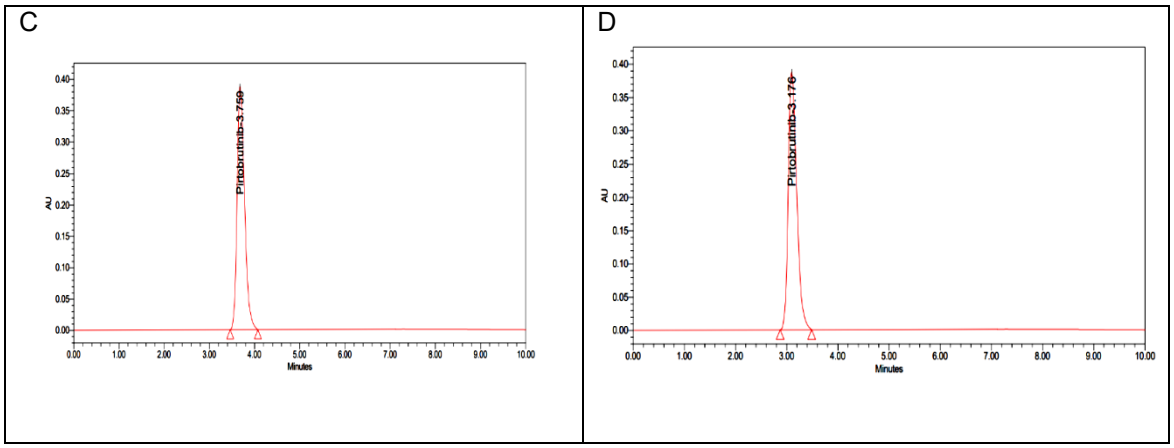


Figure 12. Chromatograms showing the effects of flow rate (A: 0.9 mL/min, B: 1.1 mL/min) and organic phase composition in % (C: 36:64, D: 44:56)

LOD and LOQ (µg/mL):

LOD (Limit of Detection) is the lowest concentration of an analyte that can be detected but not quantified, typically with a signal-to-noise ratio of 3:1. LOQ (Limit of Quantification) is the lowest concentration that can be quantified accurately and precisely, with a signal-to-noise ratio of 10:1. Chromatograms for LOD and LOQ are shown in Fig.13. and the Sensitivity parameters (LOD & LOQ) are shown in Table 9.

Table 9: Sensitivity parameters (LOD & LOQ) by HPLC

Name of drug	LOD(µg/mL)	s/n	LOQ(µg/mL)	s/n
Pirtobrutinib	0.45	3	1.50	10

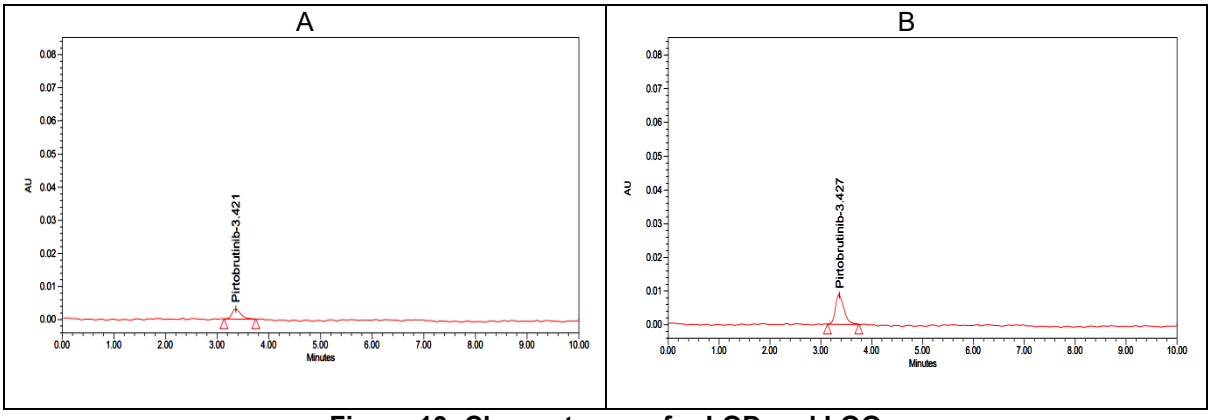


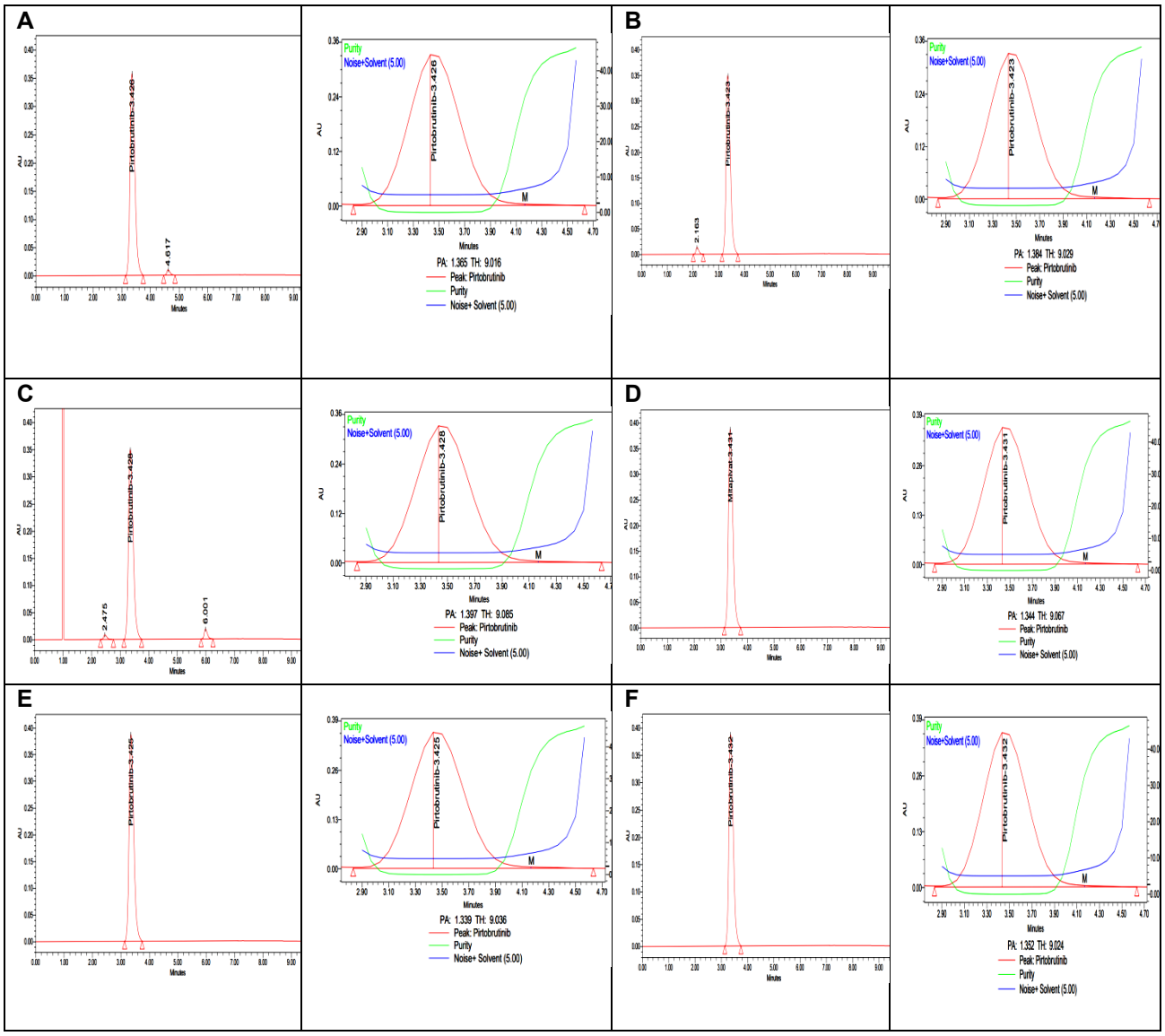
Figure 13: Chromatogram for LOD and LOQ

Forced degradation studies:

Forced degradation studies are essential for assessing the stability of pharmaceutical compounds under various stress conditions. These studies help in understanding the degradation pathways and identifying potential impurities that might form under different environmental factors. In the case of Pirtobrutinib, the forced degradation tests were performed under multiple stress conditions, including acid, alkali, peroxide, reduction, hydrolysis, thermal, and photolytic stress. The chromatograms presented below illustrate the results obtained from each degradation condition. These data provide valuable insight into the stability profile of Pirtobrutinib, which is crucial for its formulation and storage. Table 10 represents the forced degradation results for Pirtobrutinib. Fig.14 shows the chromatograms of forced degradation studies.

Table 10: Forced Degradation results for Pirtobrutinib

	Pirtobrutinib							
	Sample Weight In mg	Area Counts Injections	Mean Area Count	% Label Claim	Purity Angle	Purity Threshold	%Degradation	Pass/ Fail
Control	17.4	3862618	3862618	100	1.352	9.024	0	Pass
Acid	17.4	3421279	3421279	88.6	1.365	9.016	11.4	Pass
Alkali	17.4	3358931	3358931	87.0	1.384	9.029	13.0	Pass
Peroxide	17.4	3285013	3285013	85.1	1.397	9.085	14.9	Pass
Reduction	17.4	3783550	3783550	98.0	1.344	9.067	2.0	Pass
Thermal	17.4	3447942	3447942	89.3	1.305	9.004	10.7	Pass
Photolytic	17.4	3771065	3771065	97.7	1.339	9.036	2.3	Pass
Hydrolysis	17.4	3824178	3824178	99.0	1.347	9.052	1.0	Pass



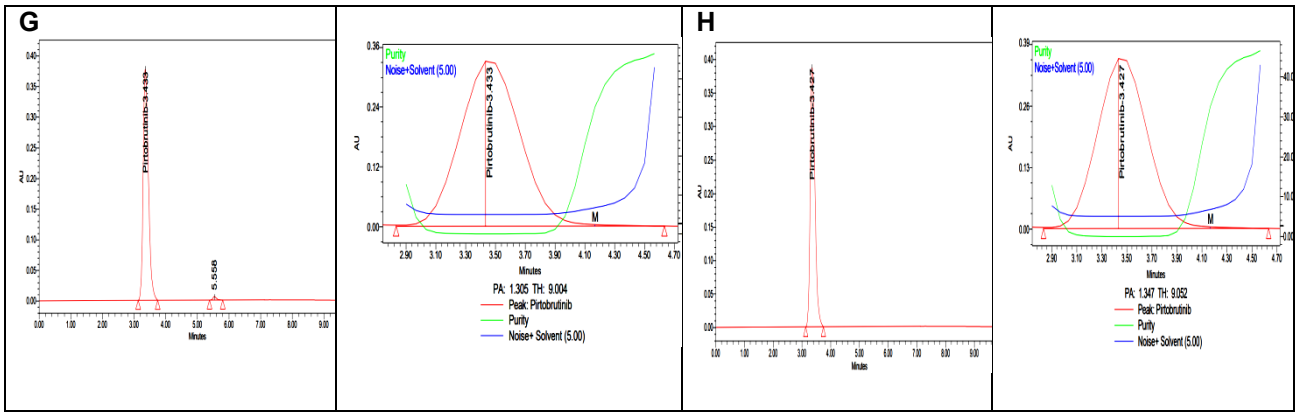


Figure 14. Chromatograms of forced degradation studies: A. Chromatograms of control and purity plot of cntrol degradation, B) Acid degradation C) Alkali degradation D) Peroxide degradation E) Reduction degradation F) Hydrolysis Degradation G) Thermal Degradation H) Photolytic Degradation

Assay:

The assay of Pirtobrutinib involves comparing the sample's chromatographic area counts to those of a standard preparation. Key parameters include the weight of the sample, dilution factors, and standard purity. The formula used allows calculation of the percentage assay based on these values and the results are presented in the following (Table 11) and chromatograms are represented in Fig.15.

Assay of Pirtobrutinib and formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where:

AT = average area counts of test (sample) preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
DS = Dilution of working standard in mL.
DT = Dilution of test (sample) in mL.
WT = Weight of test (sample) taken in mg.
P = Percentage purity of working standard,
LC = Label Claim mg/mL.

Table 11: Assay of Pirtobrutinib

Brand	Sample Area	Avg sample area (n=5)	Std. wt (mg)	Sample wt. (mg)	Label amount (mg)	Std purity	Amount found (µg/mL)	% assay
Jaypirca (Pitrobrutinib)	3857459	3860570	5.0	17.4	50	99.9	4.997	99.9

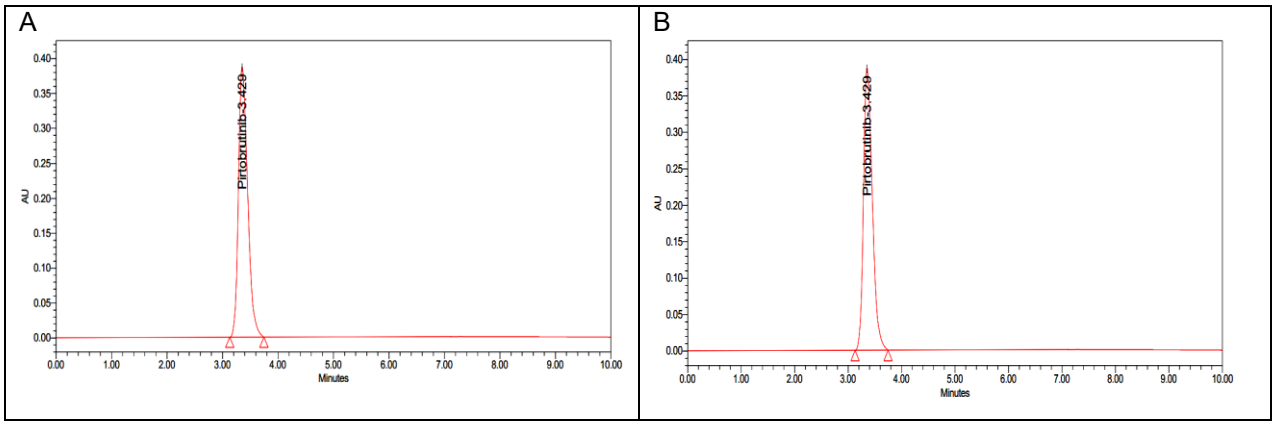


Figure 15. Chromatogram of Assay-A and B.

Conclusion:

The developed stability-indicating HPLC method for the estimation of Pirtobrutinib is simple, rapid, accurate, precise, robust, and economical. The mobile phase and solvents are easy to prepare, cost-effective, and reliable, making the method highly efficient for laboratory use. The results of the method validation, including linearity, precision, accuracy, ruggedness, and robustness, comply with ICH guidelines, confirming the reliability of the developed HPLC method. The retention time of Pirtobrutinib was identified as 3.439 minutes, ensuring a short analysis time without interference from excipients or degradation products. The recoveries of Pirtobrutinib were found to be within acceptable limits (98 -102 %), supporting the accuracy of the method for pharmaceutical dosage forms. Additionally, the method demonstrated no interference from formulation excipients or degradation products, making it suitable for routine quality control and stability analysis. In conclusion, the stability-indicating assay method using HPLC is specific, reproducible, and well-suited for the routine analysis of Pirtobrutinib in bulk and pharmaceutical formulation.

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