RP-HPLC Method Development and Validation for the Quantitative Determination of Ruxolitinib in Pure Form and Marketed Pharmaceutical Dosage Form

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Abstract

A novel, simple, accurate, precise, sensitive and specific analytical RP-HPLC method was developed and validated for the quantitative estimation of Ruxolitinib in bulk drugs and pharmaceutical dosage form. Chromatographic separation was achieved on an Symmetry ODS C18 (4.6×250mm, 5µm) analytical column using mobile phase composition of methanol and Phosphate Buffer in ratio of (35: 65 v/v) that was set at a flow rate of 1.0µl/min with detection of 235 nm. The retention time of Ruxolitinib was found to be 3.006min. The drug was analyzed by following the guidelines of International conference on Harmonization (ICH). This drug showing linearity in the concentration range of 6-14µg/ml and the correlation coefficient showing R2 = 0.9996. The % Recoveries showing within the limits. The presentation of the method was validated according to the present ICH guidelines for accuracy, precision and robustness, Linearity, limit of quantification, limit of detection linearity.

Keywords: Ruxolitinib, RP-HPLC, Method Development, Accuracy, Precision

Introduction

Ruxolitinib [1] is a pyrazole substituted at position 1 by a 2-cyano-1-cyclopentylethyl group and at position 3 by a pyrrolo [2, 3-d] pyrimidin-4-yl group. Used as the phosphate salt for the treatment of patients with intermediate or high-risk myelofibrosis, including primary myelofibrosis, post-polycythemia Vera myelofibrosis and post-essential thrombocythemia myelofibrosis. It has a role as an antineoplastic agent and an EC 2.7.10.2 (non-specific protein-tyrosine kinase) inhibitor. It is a nitrile, a pyrrolopyrimidine and a member of pyrazoles. Ruxolitinib [2] is a Kinase Inhibitor and Janus Kinase Inhibitor. The mechanism of action of Ruxolitinib is as a Janus Kinase Inhibitor. Ruxolitinib, formerly known as INCB018424 or INC424, is an anticancer drug and a Janus kinase (JAK) inhibitor. It is a potent and selective inhibitor of JAK1 and JAK2, which are tyrosine kinases involved in cytokine signalling and hematopoiesis. Myeloproliferative neoplasms, such as myelofibrosis and polycythemia Vera, are often characterized by aberrant activation of the JAK-STAT pathway, leading to abnormal blood cell counts and thrombotic complications. By inhibiting JAK1 and JAK2, Ruxolitinib [3] works to block the dysregulated cell signalling pathways and prevents abnormal blood cell proliferation. Due to a large number of patients with myeloproliferative neoplasms who have JAK2 mutations, Ruxolitinib was the first ATP-competitive inhibitor of JAK1 and JAK2 ever developed. Ruxolitinib is an antineoplastic agent that inhibits cell proliferation, induces apoptosis of malignant cells, and reduces pro-inflammatory cytokine plasma levels by inhibiting JAK-induced phosphorylation of signal transducer and activator of transcription (STAT). Inhibition of STAT3 phosphorylation, which is used as a marker of JAK activity, by Ruxolitinib is achieved at two hours after dosing which returned to near baseline by 10 hours in patients with myelofibrosis and polycythemia vera. In clinical trials, Ruxolitinib reduced splenomegaly and improved symptoms of myelofibrosis. In a mouse model of myeloproliferative neoplasms, administration of Ruxolitinib was associated with prolonged survival. Ruxolitinib inhibits both mutant and wild-type JAK2; however, JAK2V617F mutation, which is often seen in approximately 50% of patients with myelofibrosis, was shown to reduce

Ruxolitinib sensitivity, which may also be associated with possible resistance to JAK inhibitor treatment. The IUPAC Name of Ruxolitinib is (3R)-3-cyclo pentyl-3-[4-(7H-pyrrolo [2, 3-d] pyrimidin-4-yl) pyrazol-1-yl] propanenitrile. The Chemical Structure of Ruxolitinib is shown in fig-1.

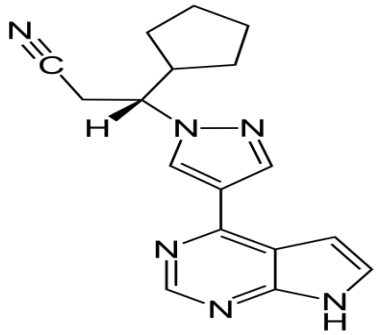

Fig 1: Chemical Structure of Ruxolitinib

Experimental

Materials

Ruxolitinib API and Marketed Formulation were provided by Synpharma Research Lab, Dilsuknagar, Hyderabad. HPLC grade acetonitrile and phosphoric acid, Dipotassium hydrogen orthophosphate, Potassium dihydrogen orthophosphate, Sodium hydroxide, Hydrochloric acid, 3% Hydrogen Peroxide were purchased from SD fine-Chem ltd; Mumbai. HPLC grade water was obtained from SD fine-Chem ltd; Mumbai.

Apparatus and Chromatographic Conditions

Analysis were performed using a Waters HPLC with Empower2 Software with Isocratic with UV-Visible Detector was provided from Synpharma Research Lab, Dilsuknagar, Hyderabad. This Instrument is connected to a Symmetry ODS C_{18} (4.6×250mm, 5µm) supplied with an isocratic pump and an auto-sampling device. The experiments were operated at ambient temperature. The mobile phase[4] was composed of a mixture of Methanol and Phosphate Buffer (35:65%) v/v. The mobile phase flow-rate was 1.0 mL/min. The detection was performed using a UV detector at wavelength 235 nm.

HPLC Method Development

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Ruxolitinib 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 Ruxolitinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

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 [30].

Mobile Phase Optimization

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Symmetry ODS C18 (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution [5] at 1ml/min flow.

Preparation of Buffer and Mobile Phase

Preparation of Potassium dihydrogen Phosphate (KH_2PO_4) buffer (pH-3.6)

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate [6] the solution by vacuum filtration and ultra-sonication.

Preparation of Mobile Phase

Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration [7].

Diluent Preparation

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Ruxolitinib 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 Ruxolitinib 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 [8]. 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 Ruxolitinib 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 Ruxolitinib 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 Ruxolitinib sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent [9] and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1ml of Ruxolitinib 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[10-11] 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

Accurately weigh and transfer 10 mg of Ruxolitinib 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 (6ppm of Ruxolitinib)

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate [12] the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Ruxolitinib)

Take 0.8ml 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 (10ppm of Ruxolitinib)

Take 0.1ml 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 (12ppm of Ruxolitinib)

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 – V (14ppm of Ruxolitinib)

Take 0.14ml 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[13] 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 [14].

Precision

Repeatability

Preparation of Ruxolitinib Product Solution for Precision

Accurately weigh and transfer 10 mg of Ruxolitinib 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 Ruxolitinib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

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

Intermediate Precision:

To evaluate the intermediate precision (also known as Ruggedness [16]) 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 [17].

Accuracy

For Preparation of 50% Standard Stock Solution

Accurately weigh and transfer 10 mg of Ruxolitinib 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 0.05ml of the above Ruxolitinib 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 Ruxolitinib 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 Ruxolitinib stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 150% Standard Stock Solution

Accurately weigh and transfer 10 mg of Ruxolitinib 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 0.15ml of the above Ruxolitinib stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Ruxolitinib and calculate the individual recovery and mean recovery values [18].

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 Ruxolitinib 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 Ruxolitinib 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. 10µl of the above sample was injected and chromatograms[19] 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 40:60, 30:70 instead (35:65), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Results and Discussion
Method Development
Optimized Chromatographic Conditions:
Mobile phase ratio: Methanol: Phosphate Buffer (35:65) V/V
Column: Symmetry ODS C18 (4.6×250mm, 5µm)
Column temperature: Ambient
Wavelength: 235nm
Flow rate: 1ml/min
Injection volume: 10µl
Run time: 8min

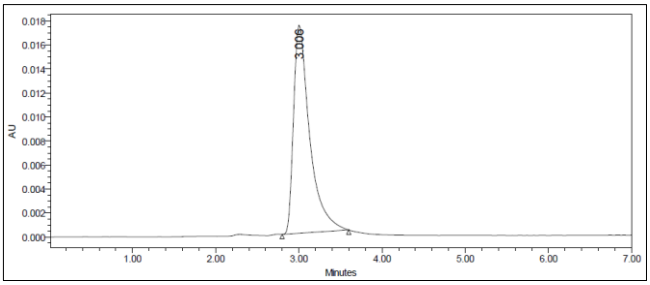

Fig 2: Chromatogram of Optimized Chromatographic

Condition
Method Validation
Method validation [20-22] is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

System Suitability

Table 1: Results of System Suitability for Ruxolitinib

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ruxolitinib	3.008	1652847	185647	6589	1.24
2	Ruxolitinib	3.005	1653658	186254	6587	1.26
3	Ruxolitinib	3.001	1654521	185475	6584	1.28
4	Ruxolitinib	3.000	1653564	186594	6582	1.29
5	Ruxolitinib	3.001	1658745	185684	6895	1.24
Mean			1654667			
Std. Dev.			2355.764			
% RSD			0.142371			

Specificity
The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.
Analytical method was tested for specificity [23] to measure accurately quantitates Ruxolitinib in drug product.

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

Standard area Dilution of standard Weight of sample 100 Label claim
The % purity of Ruxolitinib in pharmaceutical dosage form was found to be 99.86%.

Linearity
Chromatographic Data for Linearity Study:

Table 2: Data for Linearity of Ruxolitinib

Concentration µg/ml	Average Peak Area
6	1078475
8	1461129
10	1808358
12	2211573
14	2593778

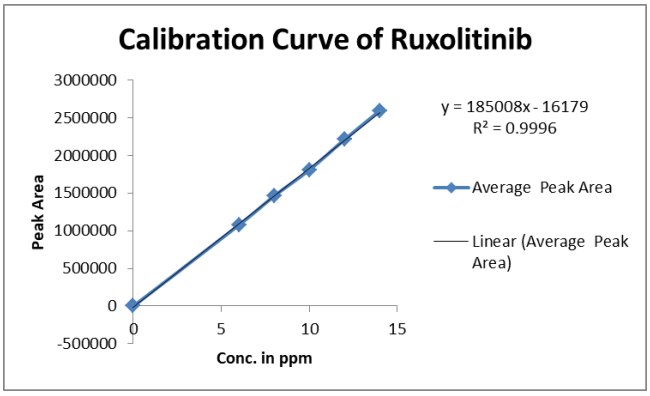

Fig 3: Linearity Curve of Ruxolitinib

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

Y = mx + c
Slope (m) = 185008
Intercept (c) = 16179
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity [24] is verified if the Correlation Coefficient is 0.99 or greater.
Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 0.16179. These values meet the validation criteria.

Precision
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.
Repeatability: Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD [25].

Table 3: Results of Repeatability for Ruxolitinib

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ruxolitinib	3.008	1658954	186958	1.26	6785
2	Ruxolitinib	3.000	1658745	187548	1.27	6854
3	Ruxolitinib	3.013	1659865	189854	1.26	6852
4	Ruxolitinib	3.006	1653254	186985	1.25	6784
5	Ruxolitinib	3.001	1654781	189542	1.24	6895
Mean			1657120			
Std. Dev			2913.592			
%RSD			0.175823			

Intermediate Precision:
The Intermediate Precision[26] consists of two methods:-
Intra Day: In Intra Day process, the 50%, 100% and 150% concentration are injected at different intervals of time in same day.
Inter Day: In Inter Day process, the 50%, 100% and 150% concentration are injected at same intervals of time in different days.

Table 4: Results of Intra-Assay & Inter-Assay

Conc. of Ruxolitinib (API) (µg/ml)	Observed Conc. of Ruxolitinib (µg/ml) by the Proposed Method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
50	49.38	0.56	49.45	0.56
100	100.17	0.71	99.70	0.77
150	150.89	0.89	149.91	0.85

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Ruxolitinib revealed that the proposed method is precise.
Accuracy: Accuracy [27] at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table 5: The Accuracy Results for Ruxolitinib

%Concentration (at Specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	
100%	202187	10	10.054	100.540%	100.72%
150%	297032.3	15	15.181	101.206%	

Limit of Detection for Ruxolitinib
The detection limit [28] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD = 3.3 × σ / s
Where
σ = Standard deviation of the response
S = Slope of the calibration curve

Result
= 1.2µg/ml

Quantitation Limit
The quantitation limit[29] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ = 10×σ/S
Where
σ = Standard deviation of the response
S = Slope of the calibration curve

Result
= 3.6µg/ml

Robustness
The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Ruxolitinib. The method is robust only in less flow condition. The standard of Ruxolitinib was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 6: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Temperature (27^0C)	0.54
Temperature (23^0C)	0.63
Wavelength of Detection (280 nm)	0.91
Wavelength of detection (270 nm)	0.93

Acceptance Criteria: The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Estimation of Ruxolitinib in TABLET Dosage Form
Jakavi 20mg Tablet
Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.
The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.
A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-7.

ASSAY
% Assay=AT/AS×WS/DS×DT/WT×P/100×AW/LC×100
Where:
AT = Peak Area of Ruxolitinib obtained with test preparation
AS = Peak Area of Ruxolitinib 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
Results obtained are tabulated below:

Table 7: Assay of Ruxolitinib Tablets

Brand Name of Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Jakavi 20mg Tablet (Novartis)	20mg	19.853 (± 0.765)	99.476 % (± 0.347)

Result & Discussion: The %Purity of Jakavi 20mg Tablet containing Ruxolitinib was found to be 99.476 % (± 0.347).

Summary and Conclusion
To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Ruxolitinib, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS C18 (4.6×250mm, 5µm) 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). Ruxolitinib was found to be Very slightly soluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride, very slightly soluble in acetonitrile, freely soluble in acetone, in anhydrous ethanol. 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 Ruxolitinib it is evident that most of the HPLC work can be accomplished in the wavelength range of 235 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 10µl 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 Ruxolitinib in different formulations.

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