Original Article

A New Stability Indicating High Performance Liquid Chromatography Method for the Estimation of Ruxolitinib in Bulk and Tablet Dosage Form

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ABSTRACT

Background: The present research work described about the systemic development of High-Performance Liquid Chromatography (HPLC) method for the quantitative determination of ruxolitinib in bulk and tablet dosage form. The subsequent validation and degradation study was also performed. **Methods:** The chromatographic Separation was achieved with a HPLC (Waters-717 series) Symmetry ODS RP C_{18}, 250mm x 4.6mm.i.d., 5μm, column with an isocratic mobile phase containing a mixture of acetonitrile: methanol: 1% Ortho phosphoric acid in the volume ratio of 70:25:5. The flow rate of the mobile phase was 1 ml/min and detection wavelength at 258 nm. The developed method was validated according to the ICH guidelines with respect to linearity, accuracy, precision, specificity, detection limits and robustness. **Results:** The precision of the results, stated as the %RSD was below 1.0%. The accuracy of the method demonstrated at three levels in the range of 50%, 100% and 150% of the specification limit. The calibration curve was linear over a concentration range from 5 to 200μg/ml with a correlation coefficient of 0.9997. The recovery of ruxolitinib was found to be in the range of 98 to 101%, whereas the detection limits were found to be 0.09 and quantitation limit was 0.29 μg/ml. Forced degradation study reveals its higher degradation at thermal and peroxide conditions in compare to other degradation condition. **Conclusion:** The present method was validated according to the ICH guidelines and it is applied successfully for the determination of ruxolitinib in tablets.

Key words: Ruxolitinib; HPLC, Method development, Validation, Degradation studies.

INTRODUCTION

Ruxolitinib having a molecular formula $C_{17}H_{18}N_6$, which is chemically known as (R)-3-(4-(7H-pyrrolo[2,3-d] pyrimidin-4-yl)-1H-pyrazol-1-yl)-3-cyclopentylpropanenitrile, belongs to the class of organic compounds[1] known as pyrrolo[2,3-d] pyrimidines, chemical structure shown in Figure 1, used for the treatment of intermediate or high-risk myelofibrosis, a type of myeloproliferative disorder that affects the bone marrow[2] and for polycythemiavera (PCV) when there has been an inadequate response to or intolerance of hydroxyurea.[3] Ruxolitinib is a janus kinase (JAK) inhibitor with selectivity for subtypes JAK1 and JAK2 of this enzyme.[4] Ruxolitinib inhibits dysregulated JAK signaling associated with myelofibrosis. JAK1 and JAK2 recruit Signal Transducers and Activators of Transcription (STATs) to cytokine receptors leading to modulation of gene expression. Today's pharmaceutical industries are looking for new ways to cut cost and shorten time for development of drugs while at the same time improving the quality of their products and analytical laboratories are not exception in this trend. High Performance Liquid Chromatography (HPLC) is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients (API's) and dosage forms.[5] The extensive literature review reveals that there is an availability of one method for the estimation of ruxolitinib in plasma[6] and two HPLC methods were reported. In one reported method[7] the linearity range is 25-150 μg/mL, which can consider narrow. The LOD and LOQ values were found ambiguity, because in different places on the article they reported different values. The reported retention time was 4.28 min, which can be further, minimize to elute ruxolitinib earlier. In another reported method,[8] the reported retention time is 4.8 min which also can be minimize and the linearity level can be expend. The LOD and LOQ values can even modified to more lower level

to improve the sensitivity. The degradation pattern of the ruxolitinib in various stress conditions has not been reported till now. The purpose of search of a novel method is to develop a fast, economic, reliable and validated method.[9] Therefore keeping the all facts in mind the efforts were taken to eradicate all the above reported methods and to develop a novel, fast, reliable and validated method for the estimation of ruxolitinib in bulk and tablet dosage form as per the ICH guidelines.[10]

Experimental

Instrumentation

The analysis of the drug was carried out on a HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER 2 software UV-VIS spectrophotometer PG Instruments Elico SL-159 and matched quartz was be used for measuring absorbance for Ruxolitinib solutions. The pH of the solutions was measured by a pH meter (thermo scientific), sonicator (ultrasonic sonicator), microbalance (Shimadzu ATY 224) and vacuum filter pump were used.

Chemicals and reagents

Ruxolitinib was obtained as a gift sample from Syncorp Pvt. Laboratories, Hyderabad, India and acetonitrile were purchased from RFCL, Rankeem Limited. HPLC grade water, glacial acetic acid and acetonitrile were obtained from Rankem, Avantor Performance Material India Limited. High purity water was obtained by using Millipore Milli Q Plus water purification system.

Chromatographic conditions

The method was developed by using an Symmetry ODS RP C$_{18}$, 250mm x 4.6mm .i.d., 5μm column with an isocratic mobile phase containing a mixture of acetonitrile: methanol: 1% ortho phosphoric acid (70:25:5). The mobile phase was filtered through the 0.22μ filter under vacuum filtration. Flow rate of the mobile phase was 1 ml/min. and the eluted compounds were monitored at the wavelength of 258 nm. The sample injection volume was 20μl.

Preparation of 1.0% Orthophosphoric Acid Solution

Take accurately 1ml of orthophosphoric acid and transferred into a 100 ml volumetric flask. Add 30 ml of HPLC Grade water and stir to dissolve the acid solution and then complete the volume up to the mark with HPLC Grade water. The solution is then filtered and degassed on a sonicator for about 15 min to remove air bubbles.

Preparation of Mobile Phase

700mL (700%) of Acetonitrile and 250mL of Methanol (25%) and 50ml of above prepared 1% orthophosphoric acid were mixed well and degassed in ultrasonic water bath for 15 min. The solution was filtered through 0.45 μm filter under vacuum filtration.

Standard Preparation for the Analysis

25 mg of ruxolitinib standard API was transferred into 25 ml volumetric flask, dissolved and make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase to achieve 50μg/ml.

Selection of wavelength

The standard and sample stock solutions were prepared separately by dissolving measured amount of standard API with a suitable solvent in a mobile phase and dilution was made with the same solvent. (After optimization of all conditions) for UV analysis. The samples scanned in the UV spectrum in the range of 200 to 400nm. While scanning the ruxolitinib solution we observed the maxima at 258 nm.

Assay of ruxolitinib in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms were taken and determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask and 15 ml of mobile phase was added than sonicated for 15 min, there after volume was made up to 25 ml with same solvent. Then 0.5 ml of the above solution was diluted to 10 ml with mobile phase. The solution was filtered through a membrane filter (0.45 μm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

Method validation

As per ICH guidelines for the determination of ruxolitinib the described method has been validated for the related substances by HPLC determination.[7]

Linearity

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 5-200μg/mL for ruxolitinib. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

Precision

Precision was determined as repeatability and intermediate precision by analyzing the samples in accordance with ICH guidelines.

Repeatability

The precision of each method was ascertained separately from the peak areas and retention times obtained by actual determination of six replicates of a fixed amount of drug. Ruxolitinib (API). The per cent relative standard deviation was calculated for Ruxolitinib.

Intermediate precision

The intra and inter day variation of the method was carried out and the high values of mean assay and low values of standard deviation and % RSD (% RSD < 2%) within a day and day to day variations for ruxolitinib was calculated.

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100% and 120%) of pure drug of ruxolitinib were taken and added to the pre-analysed formulation of concentration 50μg/ml. From that percentage recovery values were calculated.

LOD and LOQ

The LOD and LOQ of ruxolitinib were determined by using signal to noise approach as defined in ICH guidelines. The LOD and LOQ were assessed at signals to noise ratio of 3:1 and 10:1 respectively by injecting dilute solution of drug was injected into the chromatograph and signal to noise (S/N) ratio was calculated.

Robustness

This parameter is used to measure the capacity of the developed to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was performed by injecting the ruxolitinib standard solution in to the HPLC by altering the flow rate, detection wavelength and also by changing the composition of the organic solvent from the normal chromatographic conditions.

Degradation studies

The forced degradation studies were performed at an initial drug concentration of 50μg/ml in mobile phase and the degradation studies of ruxolitinib was carried out under conditions of acid degradation studies were performed in (1ml of 2N Hydrochloric acid was added and refluxed for 30 mins at 60°C), alkali degradation studies were performed in (1 ml of 2N sodium hydroxide was added and refluxed for 30 mins at 60°C), oxidative studies were performed in (1 ml of 20% hydrogen peroxide (H$_2$O$_2$) was added separately. The solutions were kept for 30 min at 60°C), dry heat degradation studies were performed in (The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation) and photo stability studies were performed in (The photochemical stability of the drug was also studied by exposing the sample solution to UV Light by keeping the beaker in UV Chamber for 1 hrs or 200 Watt hours/m² in photo stability chamber). Neutral degradation studies were performed in (Stress testing under neutral conditions was conducted by refluxing the drug in water for 1hrs at a temperature) Samples were withdrawn at proper time, cooled and neutralized by adding base or acid and subjected

to HPLC analysis after suitable dilution.

RESULTS

Method Development

In this present study different chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Various parameters such as mobile phase composition, column, column temperature, detection wavelength, pH of mobile phase and diluents were optimized. Various proportions of buffer and solvents (water, acetonitrile and methanol) were evaluated in order to achieve suitable composition of the mobile phase. Choice of retention time, tailing, theoretical plates and run time were the major tasks while developing the method. Finally ruxolitinib eluted with good peak shape and retention time and tailing was passed using a mixture of acetonitrile: methanol: 1% Ortho phosphoric acid in the volume ratio of 70:25:5 with a flow rate of 1 mL/min. The retention time obtained for ruxolitinib is 2.574 min. Quantification was achieved at UV detection at 258 nm based on peak area, shown in Figure 2. The method was validated as per ICH guidelines. The optimised chromatogram was shown in Figure 3.

Method validation

The present developed method was successfully applied for the estimation of drug in marked dosage form and the average percentage assay was found 99.59%, with relative standard deviation 0.19 which is considered within the limit and shows the suitable application of developed method for the estimation of ruxolitinib in dosage form. The assay chromatogram was depicted in Figure 4. And result was shown in Table 1. The linearity was determined for six concentrations and the correlation coefficient was found to be 0.999 for ruxolitinib which is within the specified limits. It showed that the developed method followed Beer-Lambert's law within the range of 5–200μg/ml, shown in the Figure 4. And it follows linear regression equation. The least squares method was used to establish the regression line and the curves were linear. The linearity data was shown in Table 2 and linearity graph was shown in Figure

5. Both precision and accuracy were determined with standard quality control samples. And known samples of ruxolitinib prepared in triplicates at three different concentration levels 80%, 100%, 120%, covering the linearity range and the % recovery was found to be 99.21 to 99.93 which is within the acceptable limit and the % RSD was not more than 2.0%, indicates the proposed method is highly accurate. For the study of repeatability under precision six working sample solutions of 50 μg/mL are injected and the % Amount found was calculated and %RSD was found to be 0.095 and the results of precision were shown in Table 2. For the intermediate precision the % amount found was calculated and %RSD was found 0.6 and the results of precision indicated that the developed method was found precise. And the minimum variation in % RSD indicates the present method was precise. LOD and LOQ are based on analyte concentration of signal to noise ratios of 3:1 for LOD and 10:1 for LOQ respectively. And the LOD and LOQ were found to be 0.09μ/ml and 0.29μg/ml respectively it proves the effectiveness and sensitivity of the developed method. The ability of this method to separate and accurately measure the peak of interest indicates the specificity of the method. Robustness study of the method was carried out by changing three parameters from the chromatographic conditions such as changes in mobile phase composition (±5%), changes in flow rate (±0.1ml/min) and detection wavelength (±2 nm) and the % RSD of the tailing factor was calculated found to be less than 2.0. And the results were proved the method was robust as shown in Table 3. Degradation studies of ruxolitinib were performed under the influence of acid, alkali, Oxidation, thermal, photolytic and water were found that almost all stressed condition leads to degradation. But thermal and peroxide condition leads to highest degradation shows 30.71 % and 27.58% degradation respectively. The details of the % degradation for other conditions were represented in Table 4. The force degradation study indicated that, whatever the degradation pattern, but the ruxolitinib was eluted its specific peak at its own retention time. The chromatograms shown in Figure 6.

Table 1: Assay of marketed formulation by RP-HPLC method.			
Brand Name of Ruxolitinib	**Labelled amount of Drug (mg)**	**Mean (±SD) amount (mg) found by the proposed method (n=6)**	**Assay % (±SD)**
Jakavi Tablets (15mg) (Million Health Pharmaceuticals)	15mg	14.86 (±0.498)	99.59 (±0.343)

Table 2: Summary of validation parameters.	
Parameters	**Ruxolitinib at 258nm**
Beer's law limit in μg/ml	5 to 200
Molar Absorptivity	10.05×10^3 L mol^{-1} cm^{-1}
Regression equation	Y= 0.0663X + 0.0119
Intercept	0.0119
Co-relation co-efficient	0.999
LOD μg/ml	0.09
LOQ μg/ml	0.29
Precision (% RSD)	0.095062

Table 3: Robustness study.	
Change in parameter	**% RSD (*n*=3)**
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Less Organic	0.54
More Organic	0.63
Wavelength of Detection (256 nm)	0.91
Wavelength of detection (260 nm)	0.93

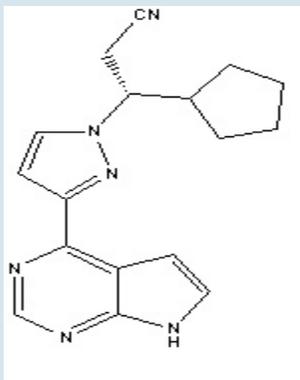| Table 4: Stress degradation study.				
Stress condition	**Time**	**Assay of active substance**	**% degradation**	**Mass Balance (%)**
Acid Hydrolysis (0.1 M HCl)	4Hrs.	98.63	1.37	100.0
Basic Hydrolysis (0.I M NaOH)	4Hrs.	89.51	10.49	100.0
Thermal Degradation (60⁰C)	6 Hrs.	69.29	30.71	100.0
UV (254nm)	24Hrs.	96.34	3.66	100.0
3 % Hydrogen peroxide	24Hrs.	72.42	27.58	100.0

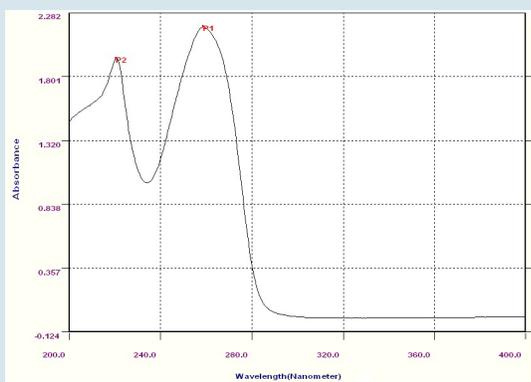**Figure 1:** Chemical structure of ruxolitinib.

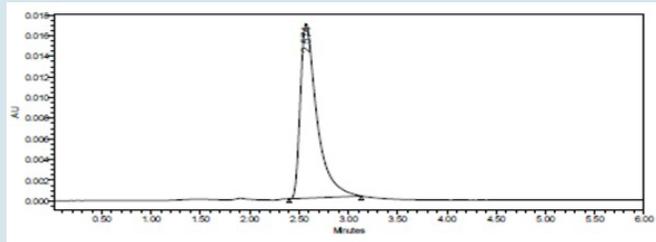**Figure 2:** UV spectrum of ruxolitinib.

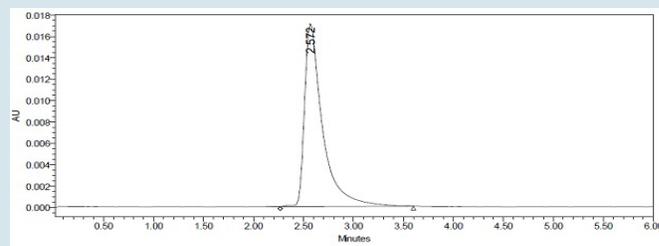**Figure 3:** Optimised chromatogram of ruxolitinib.

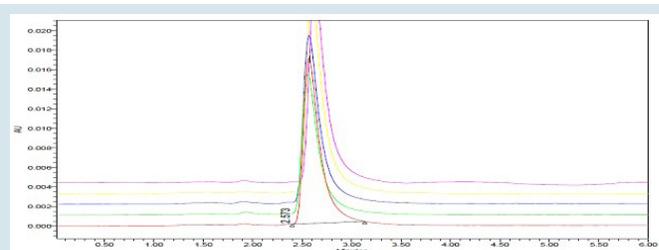**Figure 4:** Assay of marketed formulation.

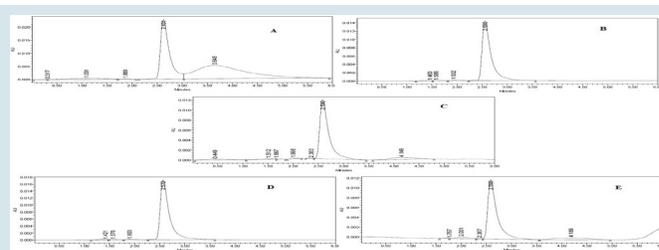**Figure 5:** Linearity chromatogram of ruxolitinib.

Figure 6: Drgradation chromatograms of ruxolitinib. [A]: Acid degradation. [B]: alkaline degdatation. [C] Photolytic degradation. [D]: Thermal degradation. [E] peroxide degradation.

DISCUSSION

The present study involved the utilization of different chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Ruxolitinib eluted with good peak shape and retention time and tailing was passed using a mixture of acetonitrile: methanol: 1% Ortho phosphoric acid in the volume ratio of 70:25:5 with a flow rate of 1 mL/min. The retention time obtained for Ruxolitinib is 2.574 min. The average percentage assay considered within the limit and shows the suitable application of developed method for the estimation of ruxolitinib in dosage form. The linearity of the method as a part of validation was determined for six concentrations and the correlation coefficient was found near to 1 for ruxolitinib which indicate its specified limits. It also indicated that the developed method followed Beer-Lambert's law within the range of sufficient range. The least squares method was used to establish the regression line and the curves were linear. Precision and accuracy were determined with standard quality control samples and it covers the linearity range and the % recovery was found within the acceptable limit and the % RSD was not more than 2.0%, indicated the developed method is highly accurate. In repeatability study the amount found was calculated and %RSD was found to be within acceptable limit. The results of precision study indicated that the developed method was found precise. And the minimum variation in % RSD indicates the present method was precise. And the LOD and LOQ values proves the effectiveness and sensitivity of the developed method. The ability of this method to separate and accurately measure the peak of interest indicates the specificity of the method. In robustness study results the % RSD of the tailing factor was calculated found less than 2.0 and the results were proved the method was robust because no such significant changes were found on deliberate changes in the optimised parameters. Degradation studies results of ruxolitinib indicated that thermal and peroxide condition leads to highest degradation in compare to other stressed condition, but in every case the ruxolitinib chromatogram was found very specific.

CONCLUSION

Based on the empirical evidences, the present method was strongly clammed about the novelty of the developed method over the reported methods. This is the first stability indicating method, which is 'rapid' be-

cause it significantly reduced the total analysis time which is the lowest analysis time required. The method justifies "easy", because the proposed method does not involved use of dual wavelength, gradient techniques. The present method is "stability indicating" as this has been shown less degradation pattern in stressed conditions and good separation of ruxolitinib among the other degraded peaks. The method considers "validated" because all the results of validation parameters were found within the limits as per the ICH Q2B guidelines. Hence the present developed method can be designate as a reliable, validated and highly useful for the routine analytical and quality control study of the ruxolitinib in the tablet dosage form.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RP HPLC: Reverse phase High performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **RSD:** relative standard deviation; **UV:** Ultra violet; **PDA:** Photo diode array; **ICH:** International conference on Harmonization.

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PICTORIAL ABSTRACT

New developed assay chromatogram
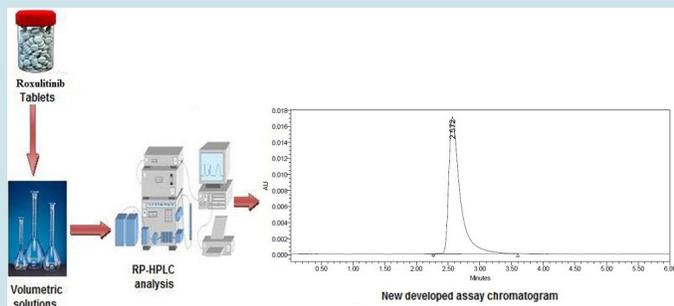
SUMMARY

The present research work described about the systemic development of high-performance liquid chromatography (HPLC) method for the quantitative determination of ruxolitinib in bulk and tablet dosage form. The subsequent validation and degradation study was also performed. The separation was achieved with a HPLC (Waters-717 series) Symmetry ODS RP C18, 250mm x 4.6mm.i.d., 5μm, column with an isocratic mobile phase containing a mixture of acetonitrile: methanol: 1% Ortho phosphoric acid in the volume ratio of 70:25:5. The developed method was validated and applied for marketed dosage form successfully. Forced degradation study reveals its higher degradation at thermal and peroxide conditions in compare to other degradation condition. The present method can be suitability applied for the routine analysis of ruxolitinib.

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