

Analytical Method Development and Validation for the Determination of Riociguat in Bulk Form and Marketed Pharmaceutical Dosage Forms by RP-HPLC

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Abstract—An Analytical, accurate, precise, efficient and simple RP-HPLC method has been developed and validated for the determination of Riociguat in bulk and marketed pharmaceutical dosage forms. The mobile phase used for the chromatographic runs consisted of Acetonitrile and Phosphate buffer (0.01M, pH-3.2) in the ratio of 30:70% v/v. The Chromatographic Separation was achieved on a Symmetry C₁₈ ODS (4.6mm × 250mm) 5µm particle size column using isocratic mode. Drug peak was well separated and were detected by a UV detector at 246 nm. The developed method was linear at the concentration range 6–14 µg/ml for Riociguat. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. Riociguat Limit of detection (LOD) and Limit of Quantification (LOQ) were 0.487µg/ml and 1.477µg/ml respectively. As a result, the suggested method with excellent specificity, accuracy, precision, linearity and robustness as well as economical was useful for the regular quality control analysis of Riociguat in bulk and pharmaceutical dosage forms.

Index Terms—Riociguat, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.

I. INTRODUCTION

Riociguat is a stimulator of soluble guanylate cyclase indicated for the management of persistent or recurrent chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension. It is used for

treatment of chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension¹. It has a role as a soluble guanylate cyclase activator and an antihypertensive agent. It is a pyrazolopyridine, an amino pyrimidine, an organofluorine compound and a carbamate ester. Riociguat is indicated for the treatment of adults with persistent/recurrent chronic thromboembolic pulmonary hypertension (CTEPH), (WHO Group 4) after surgical treatment, or inoperable CTEPH, to improve exercise capacity and WHO functional class². Riociguat is indicated for the treatment of adults with pulmonary arterial hypertension (PAH), (WHO Group 1), to improve exercise capacity, WHO functional class and to delay clinical worsening. Efficacy was shown in patients on Riociguat monotherapy or in combination with endothelin receptor antagonists or Prostanoids³. Studies establishing effectiveness included predominately patients with WHO functional class II–III and etiologies of idiopathic or heritable PAH (61%) or PAH associated with connective tissue diseases (25%). The IUPAC Name of Riociguat is methyl N-[4, 6-diamino-2-[1-[(2-fluorophenyl) methyl] pyrazolo [3, 4-b] pyridin-3-yl] pyrimidin-5-yl]-N-methyl carbamate. The Chemical Structure of Riociguat is as follows

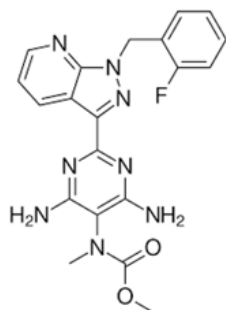


Fig-1: Chemical Structure of Riociguat

II. MATERIALS AND METHODS

Materials and Instruments:

The following are the list of instruments/Equipments, chemicals/reagents and standards to perform the HPLC Analysis of the drug Riociguat.

Table-1: List of Equipments

S. No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – V is spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C ₁₈ Column, 250 mm x 4.6 mm and 5µm particle size
7.	P ^H Analyser (ELICO)
8.	Vacuum Filtration Kit (Labindia)

Chemicals and Reagents:

Table-2: List of Chemicals used

S. No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Method Development:

HPLC Instrumentation & Conditions: The HPLC system⁴ employed was HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.

Standard Preparation for UV-Spectrophotometer Analysis:

The Standard Stock Solutions – 10 mg of Riociguat standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions⁵ were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Riociguat, so that the same wave number can be utilized in HPLC UV detector for estimating the Riociguat⁵.

Selection of Wavelength:

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm.

Selection of Chromatographic Methods:

The proper selection depends upon the nature of the sample, (ionic or ion stable or neutral molecule) its molecular weight and stability. The drugs selected are polar, ionic and hence reversed phase chromatography was selected⁶.

Optimization of Column:

The method was performed with various columns like Hypersil C₁₈ column, X- bridge column and X-terra (4.6 ×150mm, 5µm particle size), Symmetry C₁₈ ODS (4.6mm×250mm) 5µm particle size Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Mobile Phase Optimization:

Initially the mobile phase tried was Water: Methanol and Water: Acetonitrile and Methanol with TEA Buffer with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Phosphate buffer (0.01M, pH-3.2) in the ratio of 30:70 respectively⁷.

Estimation of Riociguat in bulk and pharmaceutical dosage form:

Procedure

Preparation of Mobile Phase:

Accurately measured 300 ml (300%) of HPLC Grade Acetonitrile and 700 ml of Phosphate buffer (70%) were mixed and degassed in a digital ultra sonicator for 15 minutes and then filtered through 0.45 μ filter under vacuum filter.

Preparation of 0.01M Potassium dihydrogen orthophosphate Buffer Solution:

About 1.36086grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water. The pH was adjusted to 3.20 with diluted orthophosphoric acid⁸.

Diluent Preparation:

Accurately measured 300 ml (300%) of HPLC Grade Acetonitrile and 700 ml of Phosphate buffer (70%) were mixed and degassed in a digital ultra sonicator for 15 minutes and then filtered through 0.45 μ filter under vacuum filter.

Assay

Preparation of the Riociguat standard solution:

Preparation of standard solution: (Riociguat)

Accurately weigh and transfer 10 mg of Riociguat, working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent.

Further pipette 0.1ml of Riociguat from stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

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 guidelines⁹⁻¹³.

Preparation of Sample Solution:

Take average weight of Tablet and crush in a mortar by using pestle and taken weight 10 mg equivalent weight of Riociguat 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.

Procedure:

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

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula¹⁴:

%ASSAY =

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

Analytical Method Validation

Validation is a process of establishing documented evidence which provide a high degree of assurance that specific activity will consistently produce a desired result or product meeting its predetermined specification and quality characteristics.

System Suitability

System suitability⁶ is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. A system suitability evaluation usually contains its own set of parameters. For chromatographic assays, these may include tailing factor, resolution, precision, capacity factor time and theoretical plates¹⁵.

Accuracy:

For preparation of 50% Standard stock solution:

Further pipette 0.05ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% Standard stock solution:

Further pipette 0.1ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% Standard stock solution:

Further pipette 0.15 ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

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 Riociguat and calculate the individual recovery⁷ and mean recovery values¹⁶.

Acceptance criteria:

The %RSD for each level should not be more than 2.

Precision:

Repeatability

Preparation of Riociguat for Precision:

Further pipette 0.1 ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent

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 limits¹⁷.

Ruggedness

To evaluate the intermediate precision⁸ of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

Day 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.

Day 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 limits¹⁸.

The % RSD for the area of five standard injections results should be not more than 2%.

Linearity:

Preparation of Level – I (6µg/ml of Riociguat):

Further pipette 0.06 ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – II (8µg/ml of Riociguat):

Further pipette 0.08 ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – III (10µg/ml of Riociguat):

Further pipette 0.1ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent¹⁹.

Preparation of Level – IV (12µg/ml of Riociguat):

Further pipette 0.12ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – V (14µg/ml of Riociguat):

Further pipette 0.14ml of Riociguat from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

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 coefficient²⁰.

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

Limit of Detection:

The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

Limit of Quantitation

The quantification limit is generally determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

III. EFFECT OF VARIATION OF FLOW RATE

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 recorded²¹.

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Phosphate Buffer was taken in the ratio and 70:30, 75:25 instead of 65:35, remaining

conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Forced Degradation Studies:

The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient.

Acid Degradation Studies: To 1 ml of Riociguat stock, 1 ml of 2N HCl was added and refluxed for 30 min at 60 °C. The resultant solution was neutralized with 1 ml 2N NaOH and makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl was injected into the HPLC system, and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation Studies: To 1 ml of stock solution of Riociguat 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 °C. The resultant solution was neutralized with 1 ml 2N HCl and makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. The sample of 20µl was injected into the system, and the chromatograms were recorded to an assessment of sample stability²².

Oxidation Degradation Studies: To 1 ml of stock solution of Riociguat 1 ml of 20% hydrogen peroxide (H₂O₂) was added separately. The solution was kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl solution was injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Dry Heat Degradation Studies: The 1 ml of standard drug solution was placed in the oven at 60°C for 6h to study dry heat degradation. For HPLC study, the resultant solution was makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered through a 0.45µm membrane filter. A sample of 20µl solution was injected into the

system, and the chromatograms were recorded for the assessment of sample stability.

Photo Degradation Studies: The photo stability of the drug was studied by exposing the stock solution to UV light for 1day or 200Watt-hours/m² in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) solution and filtered with 0.45µm membrane filter. A sample of 20µl solution was injected into the system, and the chromatograms were recorded for the assessment of sample stability.

Water Degradation Studies: To 1 ml of stock solution of Riociguat, 1 ml of distilled water was added. The solution was kept aside for 30 min at 60 °C. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl was injected into the HPLC system, and the chromatograms were recorded for the assessment of sample stability²³.

IV. RESULTS AND DISCUSSION

Method Development:

Selection of Wavelength:

The UV spectrum of Riociguat was obtained and the Riociguat showed absorbance's maxima at 246nm. The UV spectra of drug are follows:

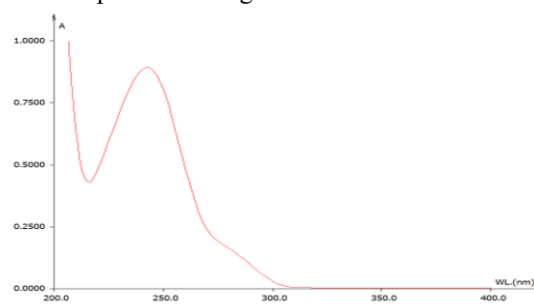


Fig-2: UV Spectrum of Riociguat (246nm)

Observation: While scanning the Riociguat solution we observed the maxima at 246nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – V is spectrophotometer model UV-2450.

Optimized Chromatographic Conditions:

Mobile phase: Acetonitrile: Phosphate buffer (0.01M, pH-3.2) (30:70% v/v)

Column: Symmetry C18 ODS (4.6mm×250mm) 5µm particle size

Flow rate: 1.0 ml/min

Wavelength: 246 nm

Column temp: Ambient

Injection Volume: 20 µl

Run time: 10 minutes

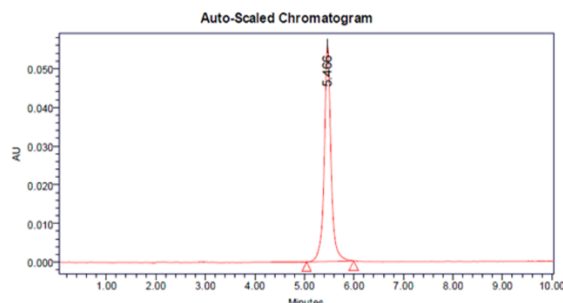


Fig-3: Optimized Chromatographic Condition

Method Validation:

According to the FDA, “Analytical method validation is the process of demonstrating that an analytical procedure is suitable for its intended purpose. The methodology and objective of the analytical procedures should be clearly defined and understood before initiating validation studies²⁴.

System Suitability:

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. System suitability tests are an integral part of chromatographic methods, and are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed.

Table-3: Observation of System Suitability Parameters

S.No.	Parameter	Riociguat
1	Retention Time (min)	5.466
2	Theoretical Plates	6967
3	Tailing factor	1.12
4	Peak Area (AUC)	647856

The system suitability parameters were found to be within the specified limits for the proposed method.

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 to measure accurately quantities Riociguat in drug product.

%ASSAY =

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

The % purity of Riociguat in present in the marketed pharmaceutical dosage form was found to be 99.85%.

Linearity:

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares²⁵.

Table-4: Chromatographic Data for Linearity Study of Riociguat

Concentration µg/ml	Average Peak Area
6	468784
8	615798
10	768759
12	925748
14	1078765

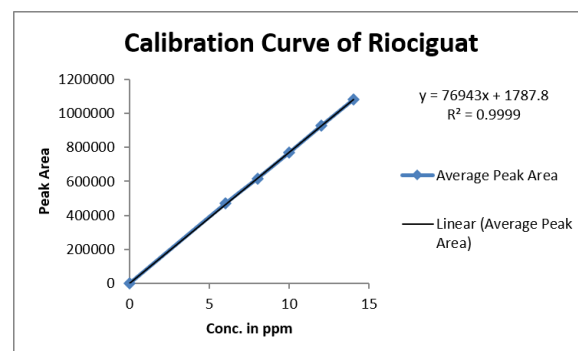


Fig-4: Calibration Curve of Riociguat

Linearity Plot:

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

$$Y = mx + c$$

$$\text{Slope (m)} = 76943$$

$$\text{Intercept (c)} = 1787$$

$$\text{Correlation Coefficient (r)} = 0.99$$

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 76943. 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 conditions²⁶.

Repeatability:

Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-5: Results of Repeatability for Riociguat:

S. No.	Peak Name	Retention time	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Riociguat	5.419	645784	83685	6825	1.05
2	Riociguat	5.405	642589	84932	6849	1.09
3	Riociguat	5.478	643658	85847	6845	1.08
4	Riociguat	5.466	648759	86295	6839	1.09
5	Riociguat	5.493	649657	86587	6895	1.07
6	Riociguat	5.466	647854	87853	6874	1.10
Mean			646383.5			
Std. Dev			2853.319			
%RSD			0.441428			

Intermediate Precision/Ruggedness:

Analyst 1:

Table-6: Results of Intermediate precision for Riociguat

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Riociguat	5.484	636854	84863	6758	1.09
2	Riociguat	5.493	637489	84759	6726	1.08
3	Riociguat	5.406	635762	84685	6749	1.09
4	Riociguat	5.419	636984	84697	6698	1.07
5	Riociguat	5.446	634856	84258	6728	1.08
6	Riociguat	5.452	639689	84753	6699	1.08
Mean			636939			
Std. Dev.			1649.149			
% RSD			0.258918			

Analyst 2:

Table-7: Results of Intermediate Precision Analyst 2 for Riociguat

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Riociguat	5.491	628985	85698	6985	1.09
2	Riociguat	5.482	624879	85479	6899	1.07
3	Riociguat	5.416	625846	85748	6928	1.06
4	Riociguat	5.482	623568	85647	6874	1.09
5	Riociguat	5.495	628985	85246	6984	1.07
6	Riociguat	5.427	628473	85924	6872	1.08
Mean			626789.3			
Std. Dev.			2340.636			
% RSD			0.373433			

Accuracy:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated²⁷.

Table-8: The Accuracy Results for Riociguat

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	386559	5	5.00	100.000%	100.130%
100%	768536	10	9.965	99.650%	
150%	1164522	15	15.111	100.740%	

Limit of Detection

The detection limit 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.

$$LOD = 3.3 \times \sigma / s$$

Were,

σ = Standard deviation of the response

S = Slope of the calibration curve

Result: 0.487 μ g/ml

Quantitation Limit

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

$$LOQ = 10 \times \sigma / S$$

Were,

σ = Standard deviation of the response

S = Slope of the calibration curve

Result: 1.477 μ g/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 Riociguat. The method is robust only in less flow condition. The standard of Riociguat was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count²⁸.

Table-9: Results for Robustness of Riociguat

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	648759	5.484	6845	1.08
Less Flow rate of 0.9 mL/min	635248	5.599	6786	1.09
More Flow rate of 1.1 mL/min	659865	4.576	6528	1.05
Less organic phase	625986	7.415	6689	1.03
More organic phase	615869	3.827	6354	1.01

Forced Degradation Studies:

The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient. The results of forced degradation studies shown in table-10.

Fig-10: Results of Forced Degradation Studies for Riociguat

S. No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	648759	0	100%	100%
2	Acidic	539378.232	16.86	83.14	100%
3	Basic	603540.497	6.97	93.03	100%
4	Oxidative	545217.063	15.96	84.04	100%
5	Thermal	616450.801	4.98	95.02	100%
6	Photolytic	533344.773	17.79	82.21	100%
7	Water	625079.296	3.65	96.35	100%

V. SUMMARY AND CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum

absorbance was found to be at 246nm and the peak purity was excellent. Injection volume was selected to be 20 μ l which gave a good peak area. The column used for study was Symmetry C₁₈ ODS

(4.6mm×250mm) 5µm particle size because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Acetonitrile: Phosphate buffer (0.01M, pH-3.2) (30:70% v/v) was fixed due to good symmetrical peak. So, this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery.

Run time was selected to be 10min because analyze gave peak around 5.466min and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Riociguat target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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