Original Article

Stress Degradation Studies of Riociguat, Development of Validated Stability Indicating Method, Identification, Isolation and Characterization of Degradation Products by LC-HR-MS/MS and NMR Studies

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ABSTRACT

Aim: The present study reports the degradation behavior of new antihypertensive drug Riociguat under various stress conditions as per International Conference on Harmonization guidelines ICH, Q2(R1). **Materials and Methods:** Riociguat was subjected to stress degradation under hydrolytic (acidic, alkaline and neutral), oxidative, photolytic and thermal stress conditions to investigate the inherent stability. A rapid, accurate, precise and robust HPLC method was developed on Waters Symmetry C_{18} Column (150mm X 4.6 mm, 5μ) using isocratic elution of 10 mm ammonium acetate buffer pH 5.7 and acetonitrile in the ratio of 70:30 with the flow rate at 1.0 mL/min.The detection was performed at 254nm. **Results:** The drug was found to be degraded in alkaline and oxidative condition whereas it was stable under acidic, neutral hydrolytic, thermal and photolytic conditions. Two degradation products (DP1, DP2) under alkaline condition and one under oxidative condition (DP3) were characterized by LC-HR-MS/MS with accurate mass measurements. Degradation products (DP1, DP2 and DP3) were isolated by preparative HPLC and were characterized by ^{1}H NMR, ^{13}C NMR, APT and IR Techniques. **Conclusion:** Using spectral data analysis, alkaline degradation product DP1 wascharacterized as 2-(1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-N5-methylpyrimidine-4,5,6-triamine and DP2 was characterized as 2-(1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-6-amino-7-methyl-7H-purin-8(9H)-one while oxidative degradation product DP3was characterized as methyl 2-(1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-4,6-diaminopyrimidin-5-ylmethylcarbamate-N-oxide.The developed chromatographic method was validated in terms of specificity, linearity, accuracy, precision as per ICH guidelines. The robustness of the method was studied with 2-level fractional factorial design 2^4-1.

Key words: Riociguat, Stress degradation, RP-HPLC, LC-HR-MS/MS, Preparative HPLC, NMR.

INTRODUCTION

Riociguat (RIO) (Figure 1a) is the first drug which belongs to the class of soluble guanylate cyclase stimulator.[1,2] Nitric oxide when it binds to soluble guanylate cyclase, results in the synthesis of Cyclic Guanosine Monophosphate (cGMP) which regulates the mechanism of blood pressure. Pulmonary hypertension is characterized by impaired synthesis of nitric oxide and insufficient stimulation of nitric-oxide-s guanylate cyclase- guanosine monophosphate (cGMP) pathway. RIO has dual mode of action.[3] It stimulates sGC to nitric oxide and stabilizes NO-sGC binding thereby it causes relaxes of vascular smooth muscle. It has antiproliferative and antifibrinolytic effects. RIO

Submission Date: 05-04-2019;
Revision Date: 12-06-2019;
Accepted Date: 10-09-2019

DOI: 10.5530/ijper.53.4s.159

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is the first drug used in two forms of hypertension chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension.[4] RIO was approved by USFDA in 2013. It is available as Adempas® by Bayer Healthcare pharmaceuticals.[5] The dose of RIO is starting from 0.5 mg thrice daily.[6] RIO is white, crystalline, non-hygroscopic powder with a molecular weight of 423g/mole and a molecular formula of $C_{20}H_{19}FN_8O_2$. RIO is insoluble in water, slightly soluble in acetone, methanol, freely soluble in dimethylsulphoxide and dimethylformamide.

There are few literatures available for riociguat. Literature has been reported on method development of riociguatin API by UV-visible spectroscopy.[7] There is a literature on method development of riociguatin dosage form by HPLC[8] and LC-MS.[9] Literature has been reported for development of riociguat and metabolite in LC-MS/MS and LC-Q-TOF-MS/MS.[10,11]

Stress testing is an important part of drug development process. Guidelines by International Conference on Harmonization ICH Q3A(R2) and Q3B(R2)[12,13] emphasizes that stress studies should be performed on drug to establish its inherent stability characteristics leading to identification of degradation products.Structural elucidation of unknown degradation products is also required to determine whether degradation products have toxicity. Comprehensive details on degradation behaviour of drugs help in maintaining its quality and pharmaceutical safety.

Robustness testing was performed to obtain information about critical parameters affecting the responses (retention time, peak area, tailing factor, theoretical plates). AQbD plays an important role in developing a robust method as an early risk assessment and helps to identify the critical analytical parameters and to focus on the method development.[14,15] Experimental design is a good alternative approach than traditional approach (OVAT) for proper planning and conduct of study.[16] To study the simultaneous variations of the factors on the considered responses, a multivariate approach DoE with fractional factorial design was applied. In the present work, DoE has been employed to check the robustness of the method.

To the best of our knowledge there are no reports on development of stability indicating method and force degradation studies of RIO by mass spectrometry for identification of degradation products. Aim of the present work is (i) to investigate the degradation behavior of RIO under different stress conditions (hydrolytic, oxidative, photolytic and thermal) and to develop SIAM for separation of RIO from its degradation products (ii) to isolate the major degradation products by preparative HPLC and their characterization by LC-HR-MS/MS, NMR and IR techniques (iii) to predict degradation pathway of RIO(iv) validation of the developed method as per the guidelines by ICH (v) quality by design approach to check the robustness of the developed method using fractional factorial $2^{\wedge 4-1}$ design.

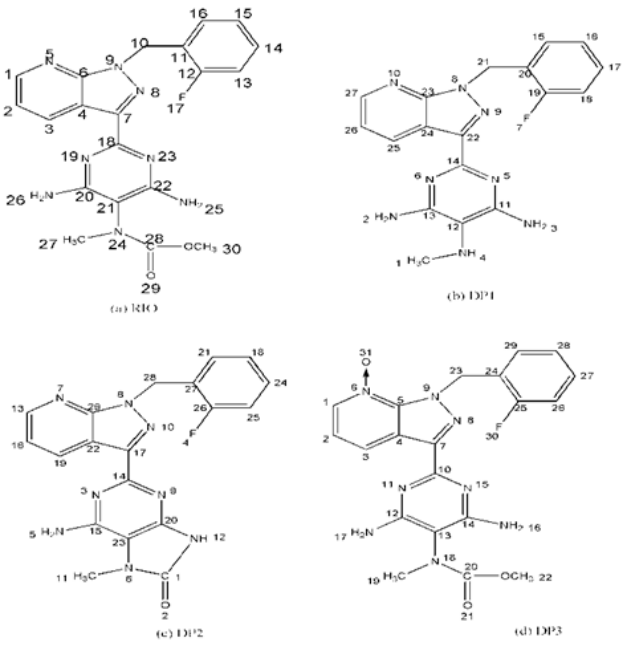

Figure 1: Structures of RIO, DP1, DP2 and DP3.

MATERIALS AND METHODS

RIO standard drug was procured from Angene Chemical Ltd, China. HPLC grade acetonitrile and acetic acid were purchased from Rankem Pvt. Ltd, India. Ammonium acetate of HPLC grade was procured from LobaCheme Pvt. Ltd, Mumbai, India. Hydrochloric acid, sodium hydroxide and hydrogen peroxide were procured from SD Chemical Pvt. Ltd. Mumbai, India.

Equipments and Chromatographic conditions

Degradation study was performed with precision water baths (Thermal Lining Services, Vadodara, India) with a temperature controller. Degradation under photolytic condition was carried in photolytic chamber (Thermolab Scientific Equipments Pvt. Ltd., Vadodara, India) comprising of light bank having four UV (Osram L73) and fluorescent (L20) lamps. Design Expert® 7.0.0 software (DX® 7.0.0) was to obtain DoE design matrix for robustness study.

For HPLC, method development was performed on (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AD pump

(binary) and Shimadzu PDA M20A Diode Array detector with LC solution software), C_{18} Column (Waters Symmetry, 150X 4.6 mm, 5µ). The detection was done at 254nm with flow rate of 1mL/min. Samples were injected through Rheodyne 7725 injector valve with a fixed loop at 20 µL. For HPLC (Preparative), chromatographic separation was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AP pump (binary) and Shimadzu SPD-20A UV-visible detector. Samples were injected through Rheodyne 7725 injector valve. Data acquisition and integration was performed using Class VP software. Promosilcolumn (250X 50 mm, 10µ) wasused for isolation of degradation. The flow rate was kept at 55mL/min. Detection was performed at 254 nm. The gradient programme was (time/% Acetonitrile): 0/30, 45/35, 46/100, 50/100, 55/30, 60/30.

^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance II 400 NMR spectrometer using solvent DMSO d_{6} as solvent and Tetramethylsilane as internal standard. IR spectra were recorded on Shimadzu 8400 s spectrophotometer. For LC-HRMS/MS analysis, system was Q-Extractive plus Biopharma High Resolution Orbitrap Liquid Chromatograph Mass Spectrophotometer (Thermo Fischer Scientific Pvt. Ltd.) equipped with electro spray ionization source in a positive mode. Xcalibur software was used for mass spectroscopic studies.

Preparation of stock and buffer solutions

Stock solution of RIO (1mg/mL) was prepared by weighing accurately 25 mg of RIO in 25 mL of volumetric flask and dissolved in acetonitrile and making up the volume with acetonitrile. From this solution, solutions were prepared in the concentration ranging from 5 to 160µg/mL.

Buffer used in the mobile phase was acetate buffer (10mm) which was prepared by dissolving 77 mg of ammonium acetate in 100 mL of double distilled water and adjusted to pH 5.7 with acetic acid. Mobile phase composed of acetate buffer and acetonitrile in the ratio of 70:30.

Validation of developed HPLC method[17,18]

The developed HPLC method was validated in terms of linearity, sensitivity, precision, robustness in accordance with ICH Q2(R1) guideline and system suitability test.

Robustness: Robustness was evaluated by QbD approach.Four Factor Factorial Designs (FFD) was employed ($2^{\wedge 4-1}$). Factors were varied over at two levels +1 and -1 denoting the maximum level and minimum level of particular factor respectively. The factors considered for development of design were pH of buffer, % organic, flow rate and wavelength. Design expert software was used to predict the percentage contribution of each factor followed by ANOVA statistical analysis, along graphical representation of Pareto charts, perturbation plots, 3Dresponse surface plots and contour plots.

Specificity: Specificity of the method was performed to ensure that there was no on interference from the degradation products, excipients and other impurities. Specificity of the method was done by performing forced degradation study. RIO was subjected to hydrolytic, oxidative, thermal and photolytic conditions as per conditions prescribed by ICH guidelines. For forced degradation study, stock solution of RIO 1mg/mL was prepared in water: acetonitrile (50:50). Stock solutions of RIO were diluted with 1M HCl, water, 0.5 M NaOH, 10% hydrogen peroxide in 1:1 ratio and were kept at 80ºC for 12 hr (1M HCl, water), 60ºC for 4 hr (0.5 M NaOH) and at room temperature for 2 hr (10% hydrogen peroxide) respectively. Effect of dry heat (thermal degradation) was performed on solid state. RIO was spread in a petridish and kept in an oven at 80ºC for 11 days under dry heat conditions. During photodegradation, solid drug powder and solution form was exposed to florescent light (1.25 million lux hours) and UV light (200 Whm^{-2}) in a photostability chamber. All degradation samples (acid, base were neutralized with 1 M NaOH and 0.5 M HCl) were diluted to concentration of 100µg/mL with mobile phase.

Isolation of major degradation products

Three major degradation products DP1, DP2 under alkaline condition and DP3 under oxidative condition were isolated and purified by preparative HPLC. The degradation products were characterized by LC-HR-MS/MS, NMR and IR techniques.

RESULTS AND DISCUSSION

Method development and optimisation

An attempt has been made to develop stability indicating RP-HPLC method for determination of RIO in presence of its degradation products. From the literature (Table 1), it is evident that reported methods are based on determination of RIO by spectrophotometric, HPLC, LC-MS methods which were summarized in the comparative study given in Table 1 and compared with the present method. Compared to the reported methods in the literature, detection wavelength of 254 nm was selected due to its sensitivity for all degradation products. For separation of degradation

Table 1: Comparison of previously reported methods for RIO with present method.			
Mobile Phase /Reagent	**Wavelength**	**Method**	**Reference**
Methanol	323	UV	7
0.2% TFA and acetonitrile (60:40)	254nm	HPLC	8
0.1% formic acid and acetonitrile (15:85)	---	LC-MS/MS	9
Ammonium formate (pH 6.8) and acetonitrile in gradient elution	---	LC-MS/MS	10
0.1% formic acid and acetonitrile in gradient elution	---	LC-Q-TOF-MS/MS	11
Ammonium acetate buffer pH(5.7) and acetonitrile (70:30)	254 nm	HPLC	Present method

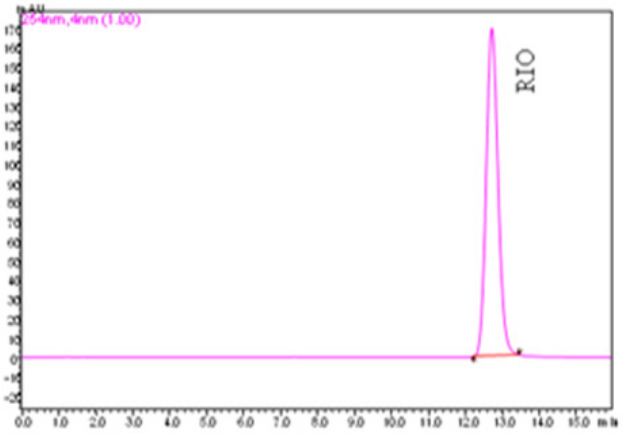

Figure 2: Chromatogram of RIO 100µg/mL.

products from RIO, mobile phase with 0.1% formic acid and ammonium formate buffer in pH 3, 4 were tried but degradation products were co-eluting with RIO and were eluting fast.Ammonium acetate buffer in the pH range 4-6 was tried, 10 mm ammoniumacetate pH 5.7 was found to be suitable for resolution of degradation products and retention time of RIO. Acetonitrile was found to be better in terms or resolution and peak shape if RIO. Method was developed with mobile phase containing 10 mm ammonium acetate and acetonitrile in the ratio of 70:30 on Waters Symmetry C_{18} column with flow rate of 1.0 mL/min. RIO eluted at 12.44±0.049 min with run time of 20 min. (Figure 2).

Method Validation

For system suitability parameters, solution of RIO was injected six times into the HPLC system. RIO eluted at 12.44 ± 0.049 min. Tailing factor was less than 2 and theoretical plates were greater than 2000. The results of system suitability parameters of RIO are shown in Table 2.

The developed method was validated for various parameters as per ICH guidelines. For linearity, good correlation was observed between peak area and concentrationwith regression coefficient r^2 0.9997 in the concentration ranging from 5-160µg/mL. Limit of detection was found to be 0.046µg/mL and Limit of quantification was found to be 0.139µg/mL. Precision of the method was performed by repeatability and inter-day precision in the concentration from 5-160 µg/mL. %RSD value was found to be less than 2%. The developed method was precise. Accuracy of the method was performed by standard addition method. Standard solution of RIO was added at three concentration levels (50, 100 and 150%) were added to the sample solutions and analysis were done in triplicate (n=3). % Recovery were found to be in the range of 99.11-100.05% and %RSD was found to be less than 2. The developed method was found to be accurate. The results of validation parameters are summarized in Table 3.

Table 2: System suitability and validation parameters of RIO.	
System suitability parameters	**Values**
Retention Time (min ± SD)	12.44 ± 0.049
Tailing Factor ± SD	1.124 ± 0.003
Theoretical Plates ± SD	7866.378 ± 372.177
Validation Parameters	**Values**
Linearity range(µg/mL)	5-160
Regression Equation	y=49420x-35097
Correlation coefficient	0.9997
LOD (µg/mL)	0.046
LOQ (µg/mL)	0.139
Intra-day precision (%RSD)	1.229
Inter-day precision (%RSD)	1.418
%Recovery±SD	99.11%±0.80-100.05%±0.36

Robustness –QbD- a fractional factorial method DoE strategy was used to carry out robustness study as summarized in Table 3. The robustness was adjudged by employing Res III FFD. The design matrix used consisted of 2^4-1 with factors being varied over two levels viz. minimum and maximum. Total of four Critical Method Parameters (CMPs) were selected viz., pH, % organic, flow rate, wavelength each varied at two lev-

Table 3: Fractional factorial design for robustness testing using factors and obtained responses.

Factors				Responses			
A: pH	B: % Organic	C: Flow rate	D: wavelength	Retention Time	Area	Tailing Factor	Theoretical Plates
5.9	32	0.9	252	12.95	1682854	1.123	7863
5.5	32	1.1	252	12.54	1685283	1.118	7864
5.5	28	0.9	252	12.94	1683827	1.093	7864
5.5	28	1.1	256	12.52	1682742	1.122	7863
5.5	32	0.9	256	12.94	1683823	1.117	7863
5.9	28	1.1	252	12.62	1683826	1.116	7864
5.9	32	1.1	256	12.48	1682384	1.122	7864
5.9	28	0.9	256	12.98	1684721	1.113	7864

els. Four Critical Quality Attributes (CQAs) taken were retention time, area, tailing factor and number of theoretical plates.

Among the various models, polynomial model was suggested by the design with the highest least square regression value for all responses as compared to other models. The model was examined using lack of fit test, which indicted insignificant lack of fit value corresponding with higher *p*-value as compared to the model F-value. Graphical interpretation in the form of Pareto charts, perturbation plots, 3-D response surface plots and contour plotsshowed the correlation of effect of factors on the responsesretention time, area, tailing factor and theoretical plates of RIO.

Pareto charts and perturbation plots indicated (Figure 3a-d and 3e-h) that none of the factors had significant effect on responses. The model was evaluated for the effect of individual factors on the responses in the form of 3-D response surface plots and contour plots. The 3-D response surface plots showed that when flow rate and wavelength were kept constant at 1.0ml/min and 254 nm respectively, there was no considerable variation in responses (retention time, area, tailing factor and theoretical plates). 3-D response surface plots and contour plots indicated that the effect of all the responses are independent of the factors pH, % Organic, flow rate and theoretical plates (Figure 4a-d and Figure 4e-h).

Further model was validated by the application of Analysis of Variance (ANOVA) to all response variables to examine the significance of the model, which showed that all the responses achieved are insignificant differences in their values (Table 4).

Equations obtained from the model were as:

Retention Time Y1 = +12.65 +0.012 *A -0.11* C -0.068* D -0.047 *A* C

Area Y2= +1.683E+006-111.00* A -249.00* C-139.75* D +408.00 * A * C -254.25 * A * D

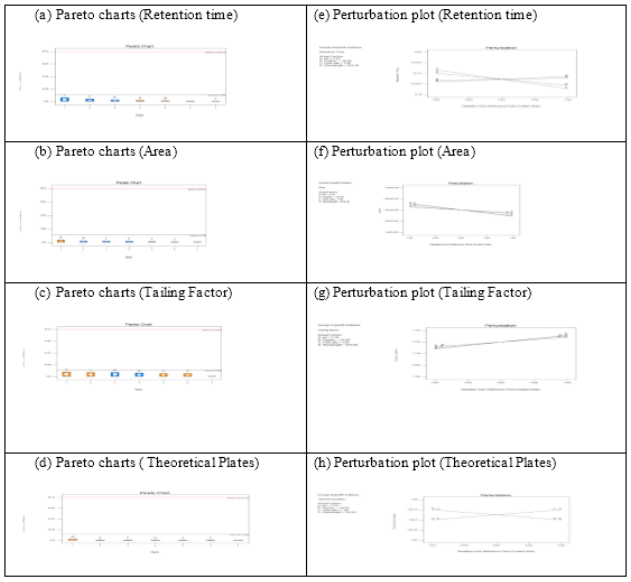

Figure 3: Pareto charts (a-d) and perturbation plots (e-h) showing effect of factorson responses.

Tailing factor Y3 = +1.12 +4.500E-003 * B +4.000E-003* C

Theoritical plates Y4 = +7863.63 +0.12 *A -0.13 *B-0.13 *D +0.38 *A*D

From the equations, positive sign indicates synergistic effect, negative sign indicates antagonistic effect in the polynomial equation. From the table of ANOVA, responses Y1, Y2, Y3 and Y4 indicated that predicted values for all the factors are under satisfactory value. Model *p* value >0.05 indicatesthat factors had non-significant on the responses resulting in a robust method.

Selectivity: Selectivity of the developed method was determined by peak purity analysis of all chromatographic peaks by using PDA detector. All peaks were well separated from each other with optimum resolution and peak purity was found to be greater than

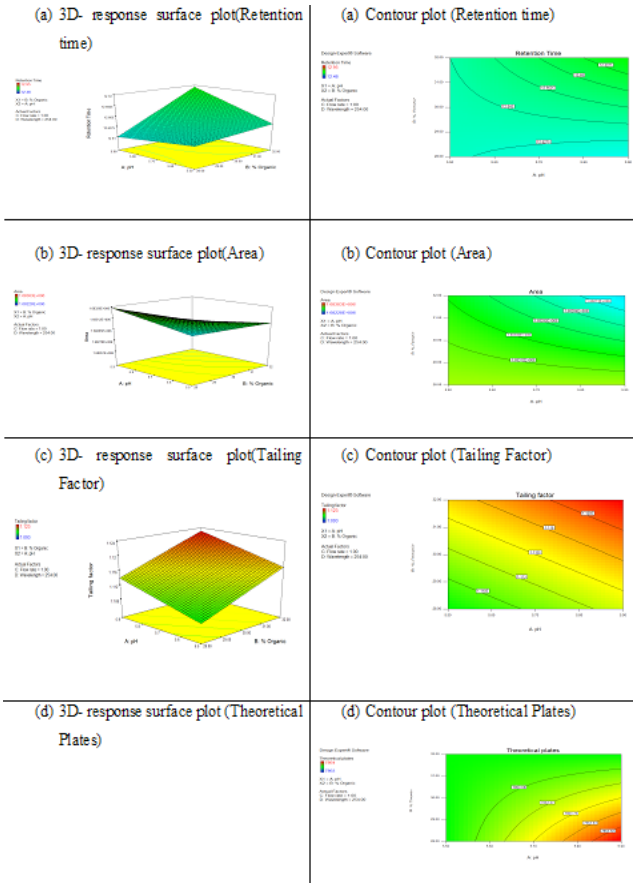

Figure 4.3: D response surface plots (a-d) and contour plots (e-h) showing effect of factors on responses.

purity threshold. Hence the developed method was selectively stability indicating.

Forced degradation study

Hydrolytic degradation: No degradation was observed when RIO was subjected to acidic hydrolysis 1M HCl at 80ºC for 12 h and neutral hydrolysis at 80ºC for 12 hr. RIO showed 12.8% degradation in 0.1M NaOH at 60ºC for 4 h. In alkaline degradation two degradation products were formed DP1 (10.6%) and DP2 (2.2 %) at retention time of 11.4 min and 9.4 min (Figure 5a).

Oxidative condition: RIO showed 9.8% degradation in 10% hydrogen peroxide at room temperature for 2 hr. One degradation product DP3 was formed at retention time of 4.1 min (Figure 5b).

Thermal degradation: RIO was stable to dry heat at 80ºC for a period of 11 days.

Photolytic degradation: No degradation was observed when RIO in solid state and solution form was exposed to exposed to fluorescent light (1.25 million lux h) and UV light (200Wh/m^2).

Forced degradation study is summarized in Table 5.

Isolation of degradation samples in alkaline and oxidative conditions

Alkaline degradation: For isolation of DP1and DP2 in alkaline condition, DPs were enriched by preparing a solution of 1g of RIO in 80 ml of acetonitrile: water

Table 4: Statistical parameters by ANOVA analysis for the responses.

Parameters	SS	df	MS	F-value	p-value	Model F-value	Model p-value	Prob > F
Response Y1 (Retention Time)								
pH	0.0012	1	0.0012	0.6944	0.557	14.37	0.1992	not significant
% Organic	0.0072	1	0.0072	4	0.295			
Flow rate	0.0882	1	0.0882	49	0.090			
Wavelength	0.0364	1	0.0364	20.25	0.139			
Response Y2 (Area)								
pH	98568	1	98568	0.1906	0.737	0.83	0.6856	not significant
% Organic	393384.5	1	393384.5	0.7606	0.543			
Flow rate	496008	1	496008	0.9591	0.506			
Wavelength	156240.5	1	156240.5	0.3021	0.680			
Response Y3 (Tailing Factor)								
pH	7.2E-05	1	7.2E-05	36	0.105	55	0.1029	not significant
% Organic	0.00016	1	0.0001	81	0.070			
Flow rate	0.00012	1	0.0001	64	0.079			
Wavelength	7.2E-05	1	7.2E-05	36	0.105			
Response Y 4 (Theoretical Plates)								
pH	0.125	1	0.125	1	0.500	2.33	0.463	not significant
% Organic	0.125	1	0.125	1	0.500			
Flow rate	0.125	1	0.125	1	0.500			
Wavelength	0.125	1	0.125	1	0.500			

SS- sum of squares, df – degrees of freedom, MS- Mean square.

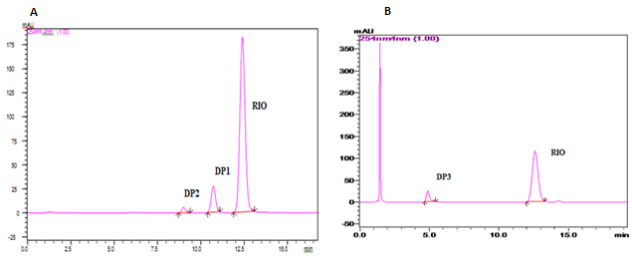

Figure 5: Chromatogram of (a) 0.1 M NaOH at 60ºC for 4 h (b) 10% hydrogen peroxide at RT for 2 h.

(2:1) and 20 mL of 1 M sodium hydroxide. The solution was heated at 60ºC for 24 hr. The solution was neutralized. % degradation was checked by HPLC and was found to contain 50% of DP1 and 15% and DP2.

Oxidative degradation: For isolation of DP3 in oxidative condition, DP3 was enriched bypreparing a solution of 1 g of RIO in 80 ml of acetonitrile: water (2:1) and 20 mL of 30% hydrogen peroxide. The solution was kept at room temperature for 24 h. % degradation was checked by HPLC and was found to contain 10% DP3.

Table 5: Summary of forced degradation study.

Stress Condition	Temperature (ºC)	Time (Hrs)	RT of Degradation Products	% of Degradation Products in API
Acid (1M HCl)	80ºC	12 hrs	---	---
Alkaline(0.1 M NaOH)	60ºC	4 hrs	9.4 min (DP2) 11.4 min (DP1)	12.8%
Neutral	80ºC	12 hrs	--	--
Oxidative (10 % H_2O_2)	RT	2 hr	4.0min(DP3)	9.8%
Thermal	80ºC	11 days	--	--
Photolytic Dry	1.25 million lux h and 200Wh/m²	11 days	--	--
Photolytic Solution		11 days	--	--

Degradation products were isolated and purified by preparative HPLC. Fractions of greater than 95% were pooled together for DP1, DP2 and DP3 separately. Fractions were concentrated on rotary vapor to distill off acetonitrile. The solution so obtained was lyophilized. DP1 was obtained as yellow colored solid while DP2 and DP3 were obtained as white solids.

Structural elucidation of RIO and degradation products

Structural elucidation of RIO

MS/MS spectra: RIO was eluted at retention time of 12.44min. The ESIMS/MS spectrum of RIO shows protonated molecular ion peak $[M+H]^+$ at m/z of 423.1659 (Table 6) and product ions of m/z 391 (loss of methoxy group from m/z 423), m/z 109 (loss of $C_{13}H_{12}N_8O_2$ fromm/z 423) (Figure 6a and 7).

NMR spectra: ^{1}H spectra of RIO shows the presence of methyl group and methoxy groups at 3.03 and 3.39 ppm. Presence of methylene group is indicated by peak at 5.81 ppm. Methyl and methoxy groups are further confirmed in ^{13}C NMR spectra 34.72 and 52.51ppm and methylene group at 43.87 ppm. Two amino groups are pyrimidine ring are indicated in ^{1}H NMR spectra at 6.39 and 6.41 ppm which are absent in D_2O exchange. Protons of pyridine ring are indicated at 9.06, 8.60 and 7.24 ppm. Protons of flurobenzene ring are indicated at 7.12, 7.19 and 7.32 ppm. Presence of carbamate group is indicated in ^{13}C NMR spectra at 155.52 ppm (Table S1).

IR spectra: IR spectra of RIO indicates presence of two primary amino groups at 3508 and 3457 cm^{-1}. Aromatic and methyl groups are merged in the region covering 3099, 3065 and 3035 cm^{-1}. Presence of carbamate is indicated at 1688 cm^{-1} (Table 7).

Structural elucidation of DP1

MS/MS spectra: DP1 eluted at retention time of 11.4 min. ESI/MS/MS spectrum of DP1 shows protonated molecular ion peak at m/z of 365.161 corresponding with elemental composition $C_{18}H_{17}FN_8^+$ (Table 6). It showsfragment ions of m/z 256 (loss of C_7H_5F group from m/z 365), m/z 241 (loss of NH from m/z 256), m/z 214 (loss of C_2H_4N from m/z 256), m/z 109 (loss of $C_{11}H_{10}N_8$from m/z 365), m/z 83(loss of C_2H_2 from m/z 109) (Figure 6b and 7).

NMR spectra: In DP1, there is absence of methyl group and carbamate group. In ^{1}H NMR spectra there are absence of 3 protons which indicates loss of methyl group and other methyl group is shifted towards upfield at 2.51 ppm. There is formation of –NH at 4.00 ppm which is absent in D_2O exchange. ^{13}C NMR spectra of DP1 indicates absent of carbamate group at 155ppm,

Table 6 : Elemental Composition of RIO and its degradation products.					
RIO and its DPs	**Molecular Formula $[M+H]^+$**	**Calculated m/z**	**Observed m/z**	**Error (ppm)**	**MS/MS fragment ions**
RIO	$C_{20}H_{19}FN_8O_2^+$	423.1649	423.1657	-0.08	391, 109
DP1	$C_{19}H_{17}FN_8^+$	365.1561	365.1607	-0.46	256, 241, 214, 109, 83
DP2	$C_{19}H_{15}FN_8O^+$	391.1323	391.1398	-0.75	149, 109
DP3	$C_{20}H_{19}FN_8O_3^+$	439.1598	439.1612	-0.14	422, 390

Table S1: NMR assignments of RIO.				
RIO				
Position	**^1H**	**Chemical Shift(δ ppm)**	**Position**	**^{13}C**
1-3	3H	9.06(d),8.60(d), 7.24(d)	28	155.52 Ester
13-16	4H	7.12(d),7.31(m), 7.19 (d),7.31(m)	1-3	148.89,117.89,133.82 Aromatic -CH-
25	2H	6.39, s, absent in D_2O exchange	4,6	114.65, 155.09 Quaternary Carbon
26	2H	6.41, s, absent in D_2O exchange	7	141.78 Quaternary Carbon
10	2H	5.81, s	11, 12	124.24, 161.04, Quaternary Carbon
27	3H	3.03, s	13,14,15,16	115.52,129.74,124.53, 129.83Aromatic -CH-
30	3H	3.39, s	18,20,21	150.78, 159.50, 100.27, 158.60 Quaternary Carbon
			30	52.51, CH_3
			10	43.87, CH_2
			29	34.72, CH_3

Table 7: IR interpretation of RIO, DP1, DP2 and DP3.							
RIO		**DP1**		**DP2**		**DP3**	
Wave number (cm^{-1})	**Assignments**	**Wave number (cm^{-1})**	**Assignments**	**Wave number (cm^{-1})**	**Assignments**	**Wave number (cm^{-1})**	**Assignments**
3508,3457	N-H (Stretching)	3463,3329	N-H (Stretching)	3515, 3282,	N-H Stretching	3371, 3261 3010	Broad peak covering –N-H, Aromatic C-H Stretching and N-oxide
3099, 3064,3035	Aromatic C-H and Methyl C-H stretching			3153	Aromatic C-H Stretching		
		3266, 3125	Aromatic C-H Stretching	2988, 2967	Methyl C-H Stretching	2747	Methyl C-H Stretching
						1703	Ester C=O Stretching
1688	C=O Stretching	1598, 1568,1459	Aromatic C=C Bending	1711	Carbonyl C=O Stretching	1636	Aromatic C-H bending
1622	Aromatic C=C Stretching			1639	Aromatic C=C stretching	1633	
1602				1602		1522	
1505				1596			

absence of one of the methyl group at 52.52 ppm (Table S2).

IR spectra: An IR spectrum of DP1 indicatesformation of secondary amino group at 3329 cm^{-1}. It also indicates absence of carbamate group at 1688cm^{-1} which is present in RIO.

DP1 is characterized as 2-(1-(2-fluorobenzyl)-1H-pyrazolo [3, 4-b] pyridin-3-yl)-N5-methylpyrimidine-4, 5, 6-triamine (Table 7).

Structural elucidation of DP2

MS/MS spectra: DP2 eluted at retention time of 9.4 min. ESI/MS/MS spectrumof DP2 showed protonated

Table S2: NMR assignments of DP1.				
DP1				
Position	**^{1}H**	**Chemical Shift (δ ppm)**	**Position**	**^{13}C**
25,26,27	3H	7.35(d),8.6(d),9.06(d)	22, 23,24	142.08, 153.09, 114.49 Quaternary carbon
15,16,17,18	4H	7.35(d),7.13(d), 7.31 (t),7.12(d)	25,26, 27	133.82, 115.52,148.8 Aromatic-CH-
2	2H	6.09,s, absent in D_2O exchange	15,16,17,18	129.92,124.52, 129.81, 115.31 Aromatic –CH-
3	2H	6.09,s, absent in D_2Oexchange	19,20	161.05,124.56 Quaternary carbon
21	2H	5.79,s	11,12,13, 14	158.61,106.98, 158.26, 150.78 Quaternary carbon
4	1H	3.39,s-NH,absent in D_2O exchange	21	43.75,CH_2
1	3H	2.51,d	1	33.06,CH_3

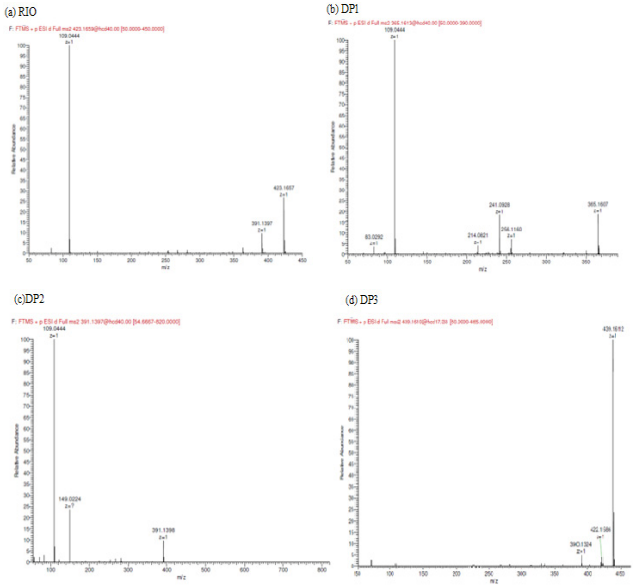

Figure 6: ESI-MS/MS spectra of RIO, DP1, DP2 and DP3.

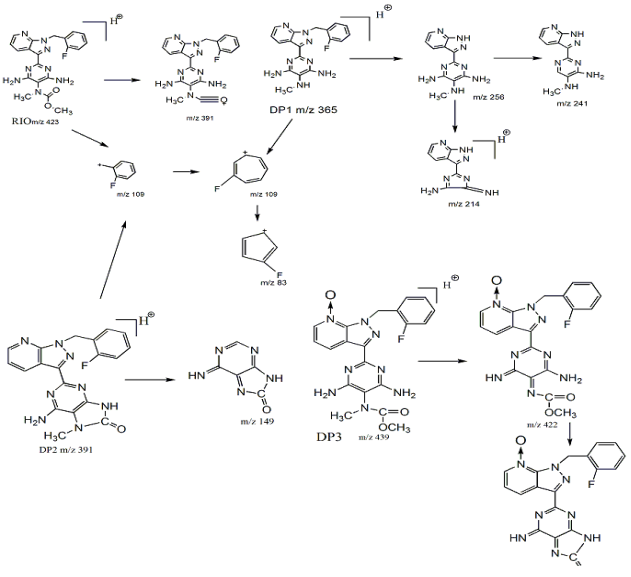

Figure 7: Mass spectral fragmentation of RIO and its degradation products.

molecular ion at m/z 391.1357corresponding with elemental composition $C_{19}H_{15}FN_8O$ (Table 6). It shows fragment ions of *m/z* 149 (loss of $C_{14}H_{12}FN_3$ group from *m/z* 391), m/z 109 (loss of $C_{11}H_{10}N_8$ from *m/z* 391) (Figure 6c and 7).

NMR spectra: ^{1}H NMR spectra shows absence of one of the methyl protons, there is formation of –NH (-NHCO) at 11.6 ppm compared to RIO. ^{13}C NMR spectra of DP2 indicates loss of carbamate group and formation of imidazolinone at 152.52 ppm. Loss of one of the methyl group is indicated by absence of peak at 52.52 ppm (Table S3).

IR spectra: An IR spectrum of DP2 indicates formation of secondary amine at 3282 cm^{-1} and formation of carbonyl group at 1710cm^{-1} (Table 7).

DP2 is characterized as 2-(1-(2-fluorobenzyl)-1H-pyrazolo [3, 4-b] pyridin-3-yl)-6-amino-7-methyl-7H-purin-8(9H)-one.

Structural elucidation of DP3

MS/MS spectra: DP3 is formed in oxidative condition. DP3 elutes at retention time of 4.1min.ESI/MS/MS spectrum of DP3 shows protonated molecular ion at 439.1612corresponding with elemental composition of $C_{20}H_{19}FN_8O_3^+$ (Table 6) shows fragment ions of *m/z* 422 (loss of methane from m/z 439), *m/z* 390 (loss of methanol from *m/z* 422) (Figure 6d and 7).

NMR spectra: The number of protons and number of carbons in DP3 are same in ^{1}H and ^{13}C NMR spectra as that of RIO. In the protons of pyridine ring chemical shift is observed in three protons compared to RIO which indicates that formation of N-oxide has taken

Table S3: NMR assignments of DP2.				
DP2				
Position	**^{1}H**	**Chemical Shift (δ ppm)**	**Position**	**^{13}C**
12	1H	11.6, -NH absent in D_2O exchange	1	152.52, imidazolinone
13, 16, 19	3H	9.06(d), 8.63(d), 7.37 (d)	13,16,19	149.06, 117.98, 133.37 Aromatic-CH-
18,21,24,25	4H	7.22 (d), 7.35(d), 7.24(d), 7.20 (d)	22, 29,17	114.27,152.92,141.39,Quaternary carbon
5	2H	6.76, s, -NH_2 absent in D_2O exchange	18,21, 24	124.56,130.16,129.91, Aromatic –CH-
28	2H	5.80,s	25,26,27	115.55,161.33,124.59, Quaternary carbon
11	3H	3.49,d	14,15,20, 23	150.78, 158.68, 147.8,105.19, Quaternary carbon
			28	43.87 CH_3
			11	28.27 CH_3

Table S4: NMR assignments of DP3.				
DP3				
Position	**^{1}H**	**Chemical Shift (δ ppm)**	**Position**	**^{13}C**
1,2,3	3H	8.65(d), 7.41(s), 8.54 (d)	1,2,3	149.06, 117.47, 134.34 Aromatic –CH-
26,27,28,29	4H	7.18 (d), 7.27(d), 7.23(d), 7.31(d)	4,5,7	114.86, 152.86, 137.25 Quaternary carbon
17	2H	7.2,s,-NH_2 absent in D_2O exchange	10,12,13,14	140.09,158.66, 99.96, 158.76 Quaternary carbon
16	2H	6.8,s,-NH_2 absent in D_2O exchange	24,25,26,27,28,29	124.62, 161.10, 115.59,129.96,123.87,130.21 Aromatic –CH-
23	2H	5.81, s	20	154.99 Ester
19	3H	3.49,3.46,d	22	52.61 CH_3
22	3H	2.90,s	23	43.94 CH_2
			19	34.54 CH_3

place in pyridine ring and there is chemical shift value in these protons (Table S4).

IR spectra: IR spectra of DP3 indicates broad peak in region at 3371 cm^{-1} which indicatesformation of N-oxide (Table 7).

DP3 is characterized as methyl 2-(1-(2-fluorobenzyl)-1H-pyrazolo [3, 4-b] pyridin-3-yl)-4, 6-diaminopyrimidin-5-ylmethylcarbamate-N-oxide.

Degradation behavior of RIO

The chemical structure of RIO contains pyridine ring fused with pyrazole ring, flurobenzene ring, pyrimidine ring and N-methyl carbamate. Carbamate group is susceptible to hydrolysis. DP1 and DP2 are formed under alkaline condition. DP1 is formed from RIO by bimolecular addition elimination reaction in which there is addition of hydroxide ion to carbamate group and formation of tetrahedral intermediate.[21] Tetrahedral intermediate loses methoxy group and corresponding acid is formed. Acid undergoes elimination of carboxylate and DP1 is formed. DP2 is formed by loss of methoxy group and as a result there is intra molecular cyclisation between carbonyl group and amino group of pyrimidine ring and formation of DP2. Under oxidative condition, from hydrogen peroxide reagent, there is attack of hydroxide ion on pyrimidine ring, with further loss of proton, there is formation of N-oxide (DP3) (Figure 8).

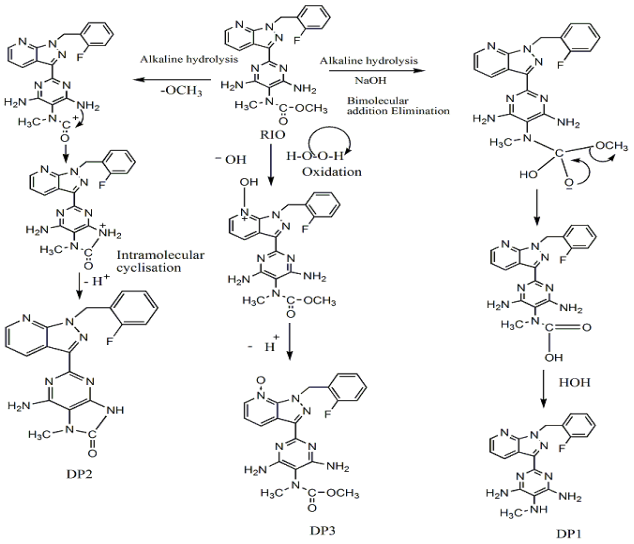

Figure 8: Degradation pathway of RIO.

CONCLUSION

Stability indicating method was developed for determination of Riociguat. Forced degradation studies were performed to evaluate stability indicating method. Degradation was observed in alkaline and oxidative conditions while Riociguat was found to be stable in acidic, neutral hydrolytic, thermal and photolytic conditions. Degradation products were well separated from Riociguat. The developed method was validated as per ICH guidelines. The robustness of the developed method was adjusted by application of QbD using fractional factorial design. Two degradation products in alkaline conditions and one in oxidative condition were isolated by preparative HPLC and characterized by LC-HR-MS/MS, NMR and IR techniques. DP1 is formed from RIO by bimolecular addition elimination reaction while DP2 is formed from intramolecular cyclisation of RIO. DP3 is formed from RIO by oxidation by formation of N-oxide. The structure and degradation pathways for degradation products were proposed on the basis of LC-HR-MS/MS. The method is simple, accurate and fast. It is applicable to assay the drug substance, for long term stability studies and also to kinetic studies.

ACKNOWLEDGEMENT

The authors are thankful to UGC for providing UGC-BSR (RFSMS) fellowship to carry out the present work.

CONFLICT OF INTEREST

Authors declare that there are no conflicts of interest.

ABBREVIATIONS

ML: Milliliter; **DP:** Degradation Product; **HPLC:** High Performance Liquid Chromatography; **LC-HR-MS:** Liquid Chromatography Mass Spectrometry; **NMR:** Nuclear Magnetic Resonance; **IR:** Infrared; **APT:** Attached Proton Test; **ICH:** International Conference on Harmonization; **µL:** Microlitre; **DoE:** Design of Experiments; **QbD:** Quality by Design; **ANOVA:** Analysis of Variance; **PDA:** Photo Diode Array; **ppm:** Parts per million.

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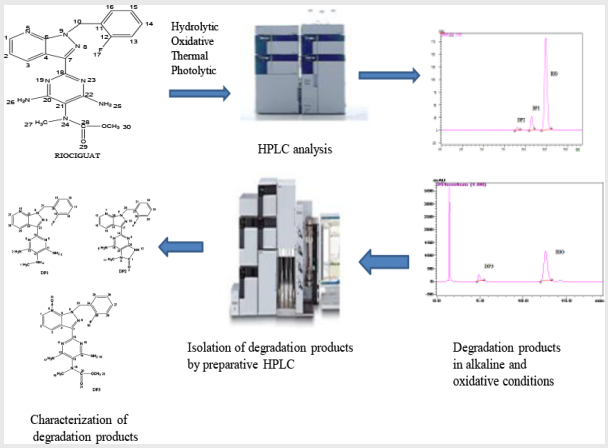
PICTORIAL ABSTRACT



SUMMARY

Stability indicating method was developed for determination of Riociguat and was developed as per ICH guidelines. Riociguat was subjected to ICH prescribed stress degradation conditions. Degradation was observed under alkaline and oxidative conditions. Degradation products were identified, isolated and characterized by LC-HR-MS/MS, NMR and IR techniques.

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Cite this article: Pandya CP, Rajput SJ. Stress Degradation Studies of Riociguat, Development of Validated Stability Indicating Method, Identification, Isolation and Characterization of Degradation Products by LC-HR-MS/MS and NMR Studies. Indian J of Pharmaceutical Education and Research. 2019;53(4s):s630-s641.