BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF RIOCIGUAT IN HUMAN PLASMA BY USING RP-HPLC

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Abstract:

The main purpose of this current work is bio analytical method development and validation for the evaluation of riociguat in human plasma by using RP-HPLC. The separation was by RP-HPLC technique - utilizing Phenomenex C18 column (250×4.6mm) 5µm, Waters Alliance –e 2695 system and Quaternary Pump system. Mobile phase were developed for evaluation of riociguat in human plasma is 0.1 % Formic acid: Methanol (20:80%v/v). Retention Time was found to be 4.00 min in run time of 10 min. Correlation coefficient r^2 for both standard in mobile phase and in plasma was greater than 0.999. The Riociguat recovery was 98.9% to 99.3% respectively indicates the accuracy of the method. LOD and LOQ indicate the sensitivity of the method. The % RSD values were less than 2% for both interday and intraday which shows the method is precise. The results of current study comply with ICH guidelines. Procured results show that proposed technique can be easily and favorably applied for routine assessment of riociguat in human plasma.

Key words: Riociguat, ICH, RP-HPLC, Validation.

Introduction:

Riociguat is chemically Methyl N-[4, 6-Diamino-2-[1-[(2-fluorophenyl) methyl]- 1H-pyrazolo [3,4-b]pyridin-3-yl]-5-pyrimidinyl]-N- methyl carbaminate. Molecular formula (C20H19FNO2)[1]. It's used as antihypertensive agent and mode of action of riociguat is stimulating soluble guanylate cyclase (sGC), an enzyme in the cardiopulmonary system and the receptor for nitric oxide (NO). When NO binds to sGC, the enzyme catalyzes synthesis of the signaling molecule cyclic guanosine monophosphate (cGMP). Bio-analysis is the strategy used to determine the concentration of drug and metabolites in the biological matrices like plasma, serum, cerebrospinal fluid, urine, saliva etc. Bioanalytical method development and validation are utilized to build up, that a quantitative analytical method can be connected for the biochemical process. Validation involves documentation of laboratory investigations that the method is suitable and reliable for the intended applications.[5] It is utilized for the assessment of bioavailability and bioequivalence studies, quantitative evaluation of drug and metabolites, new drug development, clinical pharmacokinetics, research process and therapeutic drug monitoring. Bioanalytical techniques are constantly undergoing changes and improvements that they are the cutting edge of technology[6].The quantitative evaluation of drug and metabolites, new drug development, clinical pharmacokinetics, research process and Bio analytical technique approval incorporates every one of the methodology and checks needed to demonstrate the dependability of a strategy for quantitative assurance of grouping of an analyte. For logical strategy approval, US_FDA has given a few rules in ICH. The present study portrays the improvement and acceptance of a delicate, particular, fast, straight forward and cost effective HPLC Bioanalytical strategy for Riociguat in human plasma as indicated by ICH Q2 (R1) and EMA guidelines [10-13.] Various methods reported for quantitative analysis of Riociguat in Lc-Ms/Ms[6], Stability Indicating -Forced Degradation Studies Using LC-MS[7], human metabolite M-1 in human plasma by stable-isotope dilution LCMS/MS[8], quantification of morachalcone A in rabbit plasma using high performance liquid chromatography[9]. Since the present study portrays the improvement and acceptance of a delicate, particular, fast, straight forward and cost effective HPLC Bioanalytical strategy for Riociguat in human plasma as indicated by ICH Q2 (R1)[11] and EMA guidelines[10-13]. The objective of validation of Bioanalytical procedure is to demonstrate that it is suitable for the intended purpose and it will be beneficial for the researchers.

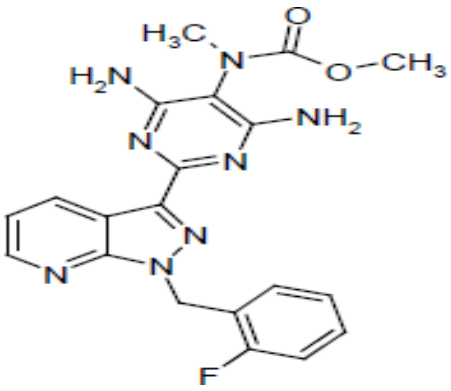

Figure no 1: Structure of Riociguat

MATERIALS AND METHODS

Chemical and Reagents:

The active pharmaceutical ingredients were benevolently acquired Cipla Pvt Limited, Bangalore, and India. The pharmaceutical formulations were purchased from the local market which contains Riociguat 0.5mg, required chemicals, reagents, acetonitrile, methanol used are of HPLC grade.

Preparation of stock solution

Standard stock solution (1mg/mL) of was prepared by accurately weighing 10 mg Riociguat transferring into 10mL of volumetric flask and dissolving up to 10mL with mobile phase, from which 1mL of this stock solution was taken and diluted it up to 100mL with human plasma to get working stock solution containing 10µg/mL of Riociguat concentration were injected for analysis and chromatogram.

Development of chromatographic conditions:

After 5 trials of different mobile phase, the mixture of 0.1% formic acid and methanol in the ratio of 20% v/v in pump A and 80% v/v in pump B was found out as most adaptable mobile phase for riociguat separation. Then mobile phase was pumped with a flow rate of 1ml/min. Prior to the injection of sample equilibrate the column with mobile phase. The detection of the sample at 322nm and run time was set for 10 min.

Validation parameters

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

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. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Detection Limit

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.

Quantization Limit

The quantization limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantization limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

RESULT AND DISCUSSION

Optimization of chromatographic conditions

Separation was done by using Waters, Waters Alliance – e2695, HPLC Software Empower 2, phenomenex C_{18} (250 × 4.6 mm, 5 µm) column on a liquid chromatographic system with LC20AD solvent delivery system, UV-2489 photodiode array detector and rheodyne injector with 20µL loop volume. Mobile phase consists of the mixture of 0.1% formic acid and methanol in the ratio of 20% v/v in pump A and 80% v/v in pump B was found to be the most suitable mobile phase for ideal chromatographic separation of riociguat. It was pumped with a flow rate of 1.0 ml/min through the column. The column was equilibrated by pumping the mobile phase for 30min prior to the injection of the drug solution. The detection of the drug was monitored at 322 nm. The run time was set at 10 min.

Analysis of marketed formulation

Ninety micro liters of drug-free plasma was spiked with 10µL of standard Riociguat. The spiked sample was vortexes for 30secs followed by addition of 1mL of methanol and re- vortexing for 3 min. The sample was centrifuged at 12,000 rpm for 10 minutes at 4°c and an aliquot of 800 µL from supernatant was evaporated in a turbovap for 20minutes, it is then reconstituted with 200µL of cold acetonitrile, from this an aliquot of 20µL was injected into HPLC for quantification. Riociguat was injected for analysis and chromatogram, was recorded. The chromatogram is shown in Figure no 3.

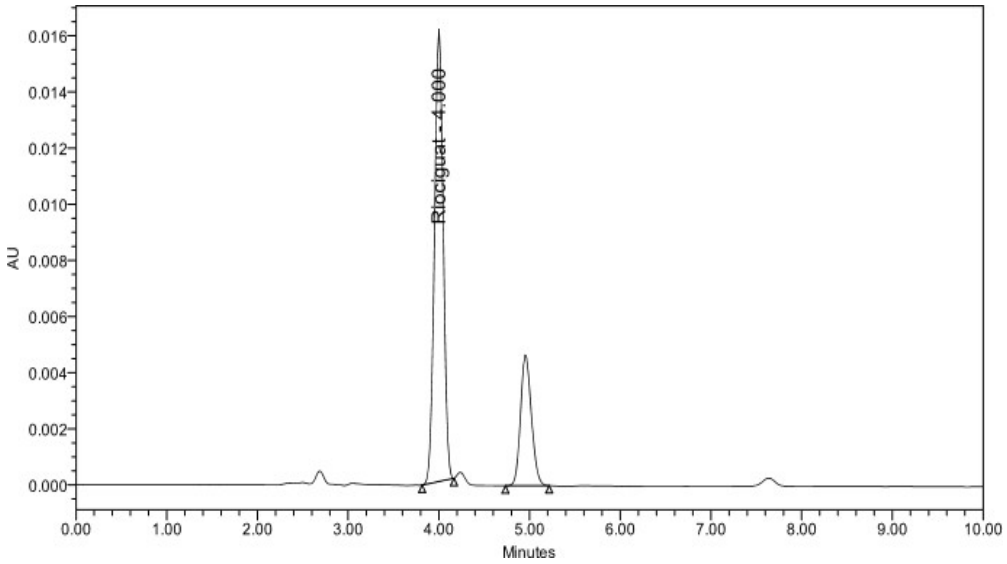

Figure no 2: chromatogram of Riociguat in human plasma

Validation parameters

The optimized method was validated by estimating linearity, precision, accuracy and specificity which was done in complies of ICH.

Linearity:

The calibration plots were constructed by plotting peak area against respective concentrations. Linearity was obtained by analysis of serial dilution sample in the range of 5-25 µg/ml. Calibration curve of riociguat in spiked human plasma suitable aliquots of the working stock solution ranging from 0.5, 1, 1.5, 2, and 2.5 mL were taken into disposable polypropylene micro centrifuge tubes with flat caps. The solution was shaken for 5 min on vortex; the mixture was shaken again on a vortex mixer for 1 min, and centrifuged for 10 min at 12000 rpm in a micro centrifuge at 4°c an aliquot of 800 µL from supernatant was evaporated in a turbovap for 20minutes, it is then reconstituted with 200µL of cold acetonitrile, From this an aliquot of 20µL was injected into HPLC for quantification were taken from solution was taken series of standard solution 5, 10, 15, 20, and 25µg/ml for calibration curve. Calibration graph is shown in Figure

no.3 The linearity range was found to be 5-25 µg/ml. The regression equation was found to be y = 41139×-83443 with a coefficient correlation (r^2) 0.999. Since the outcome is roughly near the valid esteem, the strategy is demonstrated as profoundly huge. (Table no; 1).

Concentration (µg/ml)	peak area
5	126692
10	317582
15	532691
20	754603
25	936663

Table no.1: Calibration curve of Riociguat in human plasma

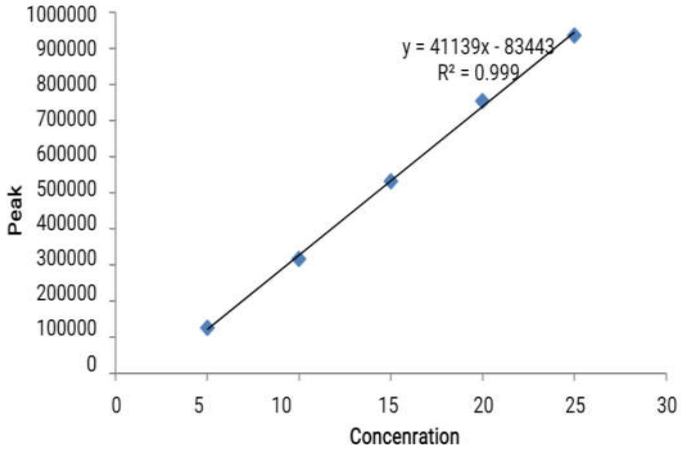

Figure no 3: Calibration curve of Riociguat in human plasma

Precision:

Precision was dictated via conveying three reproduces of concentration 10, 15, 20 µg/ml and performed intraday and interday studies. The % relative standard deviation (% RSD) was viewed as less than 1%. For Intraday precision study, assessment was completed by infusing a standard solution at different time stretches and % RSD of Riociguat was found to be 0.53 %, 0.48 % and 0.72% displayed in (Table no 2) where between day accuracy was did in successive days with % RSD of 0.94%, 0.87% and 0.64% shown in (Table no 3). The %RSD can be reached up to 2%. Since the result is under 1% shown in (Table no 4) it was viewed as agreeable, which demonstrates strategy is exact.

Level	Concentration (µg/ml)	Peak area	%RSD
1	10	317584	0.53
		316975	
		314380	
2	15	525571	0.48
		525992	
		521380	
3	20	754511	0.72
		754938	
		754358	

Table no 2: Intraday precision studies of Riociguat

Level	Concentration (µg/ml)	Peak area	%RSD
1	10	303358	0.94
		299681	
		305300	
2	15	523318	0.87
		514671	
		521310	
3	20	753588	0.64
		744331	
		746440	

Table no 3: Interday precision studies of Riociguat

Concentration(µg/ml)	Peak area	%RSD
15	512481	0.97
	502384	
	511870	
	512046	
	503482	
	513488	

Table no 4: Repeatability Riociguat

Accuracy:

Accuracy of the method was determined by replicates (n=3) analysis, carried out using three different concentration levels 80%, 100%, 120%, accuracy was calculated by comparing the difference between the spiked value (theoretical valve) and that actual found value. Results are presented in the tem of % recovery of Riociguat is presented in (Table no 5).

S.No	Level	% Recovery	% RSD*
1	80%	98.9	0.66
2	100%	99	0.45
3	120%	99.3	0.26

Table no 5: Percentage recovery study of Riociguat

Limit of quantification

LOD was determined by standard deviation method and slope of the calibration plot by using the formula 10* σ/S. It was observed to be 1.06µg/ml. As the amount of analyte was found to be less, we can estimate the drug at very low concentration. (Table no 6).

Drugs	Parameters	
	LOD	LOQ
Riociguat	0.17	1.06

Table no 6: LOD and LOQ studies of Riociguat

Limit of detection

LOD was determined by standard deviation method and slope of the calibration plot by using the formula 3.3*σ/S. It was observed to be 0. 0.17µg/ml. since the observed concentration is low, the method is sufficiently sensitive. (Table no 7).

Parameters	Riociguat
Asymmetry Factor	1.6
Theoretical Plate(N)	6524
Retention Time	4.0min

Table no 7: System suitability studies

System suitability study

System suitability parameters like plate number, peak asymmetric factor, capacity factor, selectivity factor, resolution factor is calculated with the help of standard chromatogram (Table no 8).

S No	Parameters	Results
1.	Wavelength	322nm
2.	Linearity range (µg/mL)	5-25µg/ml
3.	Regression equation (Y)	Y = 41139X -83443
4.	Slope (b)	41139
5.	Intercept (a)	83443
6.	Correlation coefficient (r)	0.999
7.	Limit of detection, LOD (µg /ml)	0.17
8.	Limit of quantification, LOQ (µg /mL)	1.06
9.	Precision (Inter-day) % RSD	0.57
10.	Precision (Intra-day) % RSD	0.81
11.	Recovery %RSD	0.45

Table no 8: Validation parameters of optimized method

Conclusion:

The developed RP-HPLC method was found to be simple, precise, accurate and sensitive for the estimation of Riociguat in human plasma. Validation of results according to ICH and EMA carried out revealed high accuracy and good precision Table VIII. The RSD for every one of the parameters are observed to be short of what one, which shows the legitimacy of the technique is reasonably fine. A mixture of 0.1% formic acid and methanol in the ratio of 20% v/v in pump A and 80% v/v in pump B was found to be the most suitable mobile phase for ideal chromatographic separation of Riociguat at a flow rate of 1 ml/min. The wavelength was found to be 322 nm in UV spectroscopy. Retention time was found to be 4.00 min with a run time 10 min. 5-25 µg/ml concentration of Riociguat shows linearity with a correlation coefficient of 0.999. Thus, we can conclude that this method can be easily and conveniently adopted for the quality control analysis of Riociguat in human plasma.

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Reference:

1.Khaybullina D, Patel A, Zerilli T. Adempas (Riociguat): A novel, first-in-class therapy approved for the treatment of 2 types of pulmonary hypertension. Am Health Drug Benefits. Pharmacotherapy .2014;39 (11):749-758.

2. The merck index 16th edition online.

3.Kirthi R, Shanmugam R, Shanti Prathyusha M and Jamal Basha D. A review on bio analytical method development and validation by RP-HPLC. *J Glob Trends Pharm Sci* 2014; 5(4): 2265-2271.

4.Goel D. Ticagrelor. The first approved reversible oral antiplatelet agent. *Int J Appl Basic Med Res* 2013; 3(1): 19-21.

5. Lalit V Sonawane, Bhagwat N Poul, Sharad V Usnale, Pradeepkumar V Waghmare and Laxman H Surwas. Bioanalytical method validation and its pharmaceutical application a review. *Pharm Anal Acta* 2014; 5:1000228:1-7.

6.Ramshankar Nayak, Narenderan ST, Lingamallu Venkata Sai Krishna1, Meyyanathan SN, Babu B and Kalaivani M. Analytical Method Development and Validation for the Determination of Riociguat in their Formulations by Lc-Ms/Ms. *Journal of global pharma technology*, 2018; 10 (12):19-23.

7.Vikrant Salode and Game M. D. Development and Validation of Stability Indicating Method for Riociguat with Forced Degradation Studies Using LC-MS**,** *International Journal of Pharmaceutical Sciences and Nanotechnology* 2020, 13 (2): 4831-4844.

8.Mark-jean gnoth, peter-Michael Hopfe, Waldemar czembor. Determination of riociguat and its major human metabolite M-1 in human plasma by stable-isotope dilution LCMS/MS. *Bioanalysis.*2015, 7(2):193-205.

9.Michael Raharja Gani, Isnaeni , Amirudin Prawita, Achmad Fuad Hafid , Aty idyawaruyanti. Bioanalytical method development and validation for quantification of morachalcone A in rabbit plasma using high performance liquid chromatography, *Pak J Pharm Sci* 2018, 1 (31): 311-315.

10. Rushikesh Babasaheb Tanpure, Jyoti B. Wadekar, Analytical Method Development And Validation For Estimation Of Riociguat By Rphplc. International Journal of Research and Analytical Reviews (IJRAR), July 2022, Volume 9, Issue 3, 774-782.

11.Faruk Kocak [a], Mevlut Albayrak [b], MehmetEmrah Yaman [c], Alptug Atila [d], Yucel Kadioglu [e], Omer Araz [f]Determination and pharmacokinetic study of riociguat by UPLC-MS/MS in human plasma**,** Journal of Chromatography B, Volume 1210, 1 November 2022, 123454

12. Milena Rmandić [1], Marija Rašević [1], Jelena Stanković [2], Mira Zečević [1], Biljana Otašević [1], Ana Protić [1], Anđelija Malenović [3]. Development and robust optimization of the RP-HPLC method for the determination of riociguat and its four impurities in tablets using the AQbD approach. J Pharm Biomed Anal, 2025 Jun 15, 258, 116742.

13.Guideline on Bio-analytical method validation: European Medicines Agencies; 2011.

14.ICH Q1A Stability testing of new drugs substances and products. International Conference on Harmonization. Geneva: 2002.

15.ICH Q2 (R1) Validation of analytical procedures methodology. International Conference on Harmonization. Geneva: 2005.