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RP-HPLC Method Development and Validation of Regorafenib in Pure Form and Pharmaceutical Dosage Form

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ABSTRACT

A new, simple, accurate, precise and robust isocratic RP-HPLC method has been developed and subsequently validated for the determination of Regorafenib in pure form and pharmaceutical dosage forms as per ICH guidelines. The separation achieved on a Symmetry C_{18} Column, 250 mm x 4.6 mm i.d. and 5µm particle size column as a stationary phase and Methanol: Phosphate buffer (pH adjusted to 4.80 with phosphoric acid) in the ratio of 70:30v/v used as mobile phase at a flow rate of 1.0 ml/min. The UV detection was performed at 268nm. The retention time for Regorafenib was found to be 3.544minutes. The detector response was linear in the concentration range of 0-16µg/ml. The respective linear regression equation being Y= 58945.x + 9634 with R2 = 0.999. The percentage of Regorafenib in pharmaceutical dosage form was found to be within the limits. The limit of detection and the limit of quantification were found to be 0.90µg/ml and 2.90µg/ml respectively.

The results of the study showed that, the proposed RP-HPLC method was simple, rapid, precise, accurate and stability indicating, which can be used for the routine determination of Regorafenib in pure form and pharmaceutical dosage forms.

Keywords: Regorafenib, RP-HPLC, Method Development, Validation, Precision, Accuracy, ICH Guidelines.

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INTRODUCTION

Regorafenib (BAY 73-4506, commercial name Stivarga) is an oral multi-kinase inhibitor developed by Bayer which targets angiogenic, stromal and oncogenic receptor tyrosine kinase (RTK). Regorafenib is an orally-administered inhibitor of multiple kinases. It is used for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumours. FDA approved on September 27, 2012. Approved use of Regorafenib[1] was expanded to treat Hepatocellular Carcinoma in April, 2017. Regorafenib is indicated for the treatment of patients with metastatic colorectal cancer (CRC) who have been previously treated with fluoropyrimidine-, oxaliplatin- and irinotecan-based chemotherapy, an anti-VEGF therapy, and, if KRAS wild type, an anti-EGFR therapy. Regorafenib is also indicated for the treatment of patients with locally advanced, unresectable or metastatic gastrointestinal stromal tumor (GIST) who has been previously treated with imatinib mesylate and sunitinib malate.

Regorafenib is a small molecule inhibitor of multiple membrane-bound and intracellular kinases involved in normal cellular functions and in pathologic processes such as oncogenesis, tumor angiogenesis, and maintenance of the tumor microenvironment. In in vivo models, regorafenib demonstrated anti-angiogenic activity in a rat tumor model, and inhibition of tumor growth as well as anti-metastatic activity in several mouse xenograft models including some for human colorectal carcinoma.

The IUPAC[2] Name for Regorafenib is 4-[4-({[4-chloro-3-(Trifluoro methyl) phenyl] carbamoyl} amino)-3-fluorophenoxy]-N-methylpyridine-2-carboxamide. The molecular formula[3] of Regorafenib is $C_{21}H_{15}ClF_4N_4O_3$. The molecular weight is 482.815 g/mol. The chemical structure [4] of Regorafenib shown in following Figure 1.

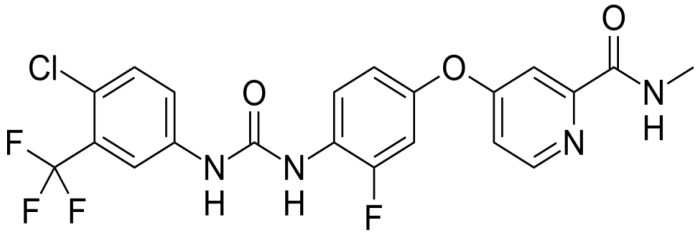

Figure 1: Structure of Regorafenib

MATERIALS AND METHOD

Experimental:

Materials and Methods:

Pharmaceutical grade working standard Regorafenib was obtained from Syncorp Clincare

Technologies Pvt. Ltd. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt. Ltd, Mumbai, India.

Instrumentation:

The analysis was performed using HPLC (Waters-717 series) with UV detector and data handling system Empower2 software, UV-Visible double beam spectrophotometer (Lab india), analytical balance 0.1mg Sensitivity (Lab india), pH meter (Lab india), Vacuum filtration, Ultra sonicator. The column used is Symmetry C18 Column, 250 mm x 4.6 mm i.d. and 5µm particle size with the flow rate 1.0ml/min (isocratic).

Preparation of Phosphate buffer:

About 6.8 grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. The Ph[5] was adjusted to 4.80 with Ortho phosphoric acid.

Preparation of mobile phase:

Mobile phase was prepared by taking Methanol: phosphate buffer (pH-4.80) (70:30 v/v). Mobile phase was filtered[6] through 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The mobile phase was pumped[7] through the column at a flow rate of 1.0 ml/min.

Standard Preparation for the Analysis

25 mg of Regorafenib working standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution[8] was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Sample Preparation for the Analysis

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drug was transferred to a clean and dry 100ml of volumetric flask and add 70 ml of diluent and the resulted solution was sonicated for 15 minutes by using ultra Sonicator. Then the final volume was make up to the mark with the same diluent. The final solution was filtered through a selected membrane filter (0.45 µm). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flask and volume was made up to 10ml with same solvent system. The prepared solution was injected in six replicates into the HPLC system and the observations were recorded.

Diluent

Mobile phase can be used as diluent.

Study of Spectra and selection of wavelength:

Regorafenib working standard solution was scanned between the range 200-400 nm in 1cm cell against blank. Maximum absorbing wavelength of Regorafenib was selected from spectral data and wavelength selected from spectra of UV spectrophotometer. The λmax for Regorafenib was found to be 268nm. UV spectrum and typical standard chromatogram of Regorafenib are shown in Fig-2.

Optimization of HPLC Method:

The chromatographic conditions[9] were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength and different diluents for sample preparation etc. The selected and optimized mobile phase was Methanol: Potassium dihydrogen orthophosphate buffer (pH-4.80) (70:30v/v) and conditions optimized were: flow rate (1.0 ml/minute), wavelength (268 nm, UV-detector), run time was 7.0 mins and injection volume was 20 µl.

Method Validation

Accuracy:

Recovery study:

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 REGORAFENIB were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery values[10] were calculated. The results were shown in Table-1.

Precision:

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Regorafenib (API). The percent relative standard deviation[11] was calculated for Regorafenib are presented in the Table-2.

Intermediate precision:

Intra-assay & inter-assay:

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations[12] for Regorafenib revealed that the proposed method is precise.

Linearity & Range:

The calibration standard solution of Regorafenib was injected into the HPLC system and the chromatograms were recorded at 268nm and a calibration graph was obtained by plotting peak area versus concentration of Regorafenib. The linearity data is presented in figure-3 and table-4.

Method Robustness:

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile phase (±2%) studied to determine the robustness[13] of the method are also in favour of (Table-5, % RSD < 2%) the developed RP-HPLC method for the analysis of Regorafenib (API).

LOD & LOQ:

The minimum concentration of the analyte can be used to detect the sample by using all experimental conditions and the minimum concentration of the analyte can be used to quantify the sample by using all experimental conditions

The LOD and LOQ were calculated by the use of the equations

$LOD = 3.3 \times \sigma / S$

And

$LOQ = 10 \times \sigma / S$

Where,

σ is the standard deviation of intercept of Calibration plot and S is the average of the slope of the corresponding Calibration plot.

System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability[14] test parameters were established. The data are shown in Table-6.

Estimation of Regorafenib in Tablet Dosage Form

Each tablet contains: 40 mg

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 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.

A duplicate injection[15] of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-7.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

RESULTS AND DISCUSSION

Optimized method

To develop a new, simple, accurate, precise, robust and isocratic RP-HPLC method has been developed and subsequently validated for the determination of Regorafenib in pure form and pharmaceutical dosage forms as per ICH guidelines. The separation achieved on a Symmetry C_{18} Column, 250 mm x 4.6 mm i.d. and 5µm particle size column as a stationary phase and Methanol: Phosphate buffer (pH adjusted to 4.80 with phosphoric acid) in the ratio of 70:30v/v used as mobile phase at a flow rate of 1.0 ml/min. The UV detection was performed at 268nm. The retention time for Regorafenib was found to be 3.544 minutes.

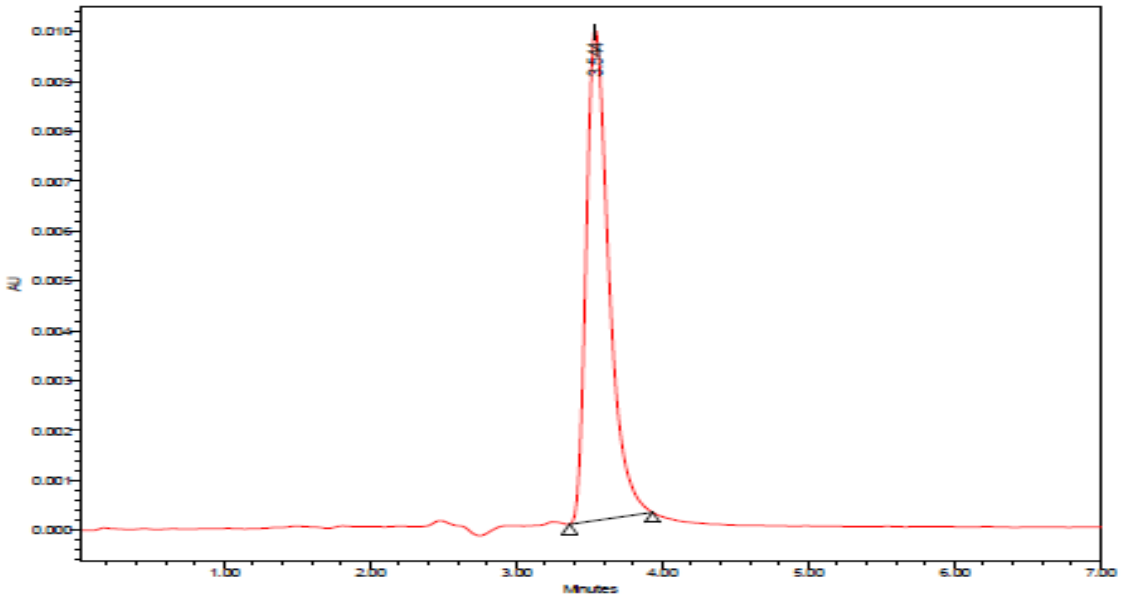

Figure-2: Optimised Chromatographic Condition

Method Validation Results

Accuracy

Table-1: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	8	8.069	485317	100.862	Mean= 100.5413%
S_2 : 80 %	8	7.958	478751	99.475	S.D. = 0.947606
S_3 : 80 %	8	8.103	487312	101.287	% R.S.D.= 0.942503
S_4 : 100%	10	10.048	601947	100.48	Mean= 100.2367%
S_5 : 100%	10	10.073	603395	100.73	S.D. = 0.650103
S_6 : 100%	10	9.950	596176	99.50	% R.S.D.= 0.648568
S_7 : 120%	12	11.985	716127	99.875	Mean= 100.3607%
S_8 : 120%	12	12.116	723840	100.966	S.D. = 0.555257
S_9 : 120%	12	12.029	718706	100.241	% R.S.D. = 0.553262

Precision

Repeatability:

Table-2: Repeatability Readings

HPLC Injection Replicates of Regorafenib	Retention Time	Peak Area
Replicate – 1	3.545	661022
Replicate – 2	3.537	683137
Replicate – 3	3.543	671941
Replicate – 4	3.538	682245
Replicate – 5	3.542	671941
Replicate – 6	3.550	692444
Average	3.5425	677121.7
Standard Deviation	0.004764	11046.13
% RSD	0.134494	1.631336

Intermediate Precision:

Intra-Assay and Inter-Assay:

Table-3: Results of intra-assay & inter-assay

Conc. Of Regorafenib (API) (µg/ml)	Observed Conc. Of Regorafenib (µg/ml) by the proposed method			
	Intra-Day Mean (n=6)	% RSD	Inter-Day Mean(n=6)	% RSD
8	7.76	0.82	8.28	0.98
10	10.16	0.42	9.59	0.23
12	11.68	0.13	12.19	0.33

Linearity and Range:

The calibration curve showed good linearity[16] in the range of 0 – 16 µg/ml, for Regorafenib (API) with correlation coefficient[17] (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y = 58945x + 9634 for Regorafenib.

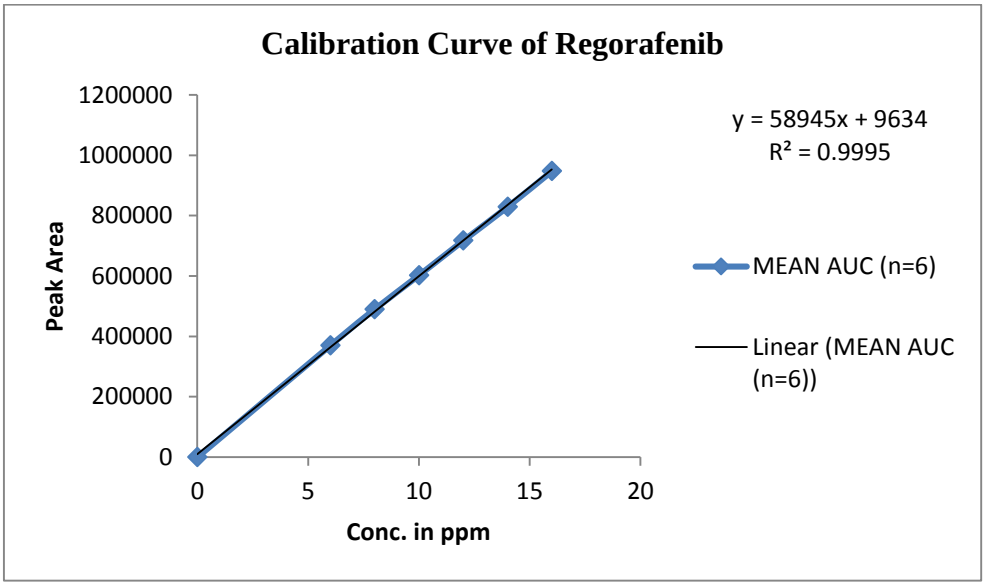

Figure-3: Calibration curve of Regorafenib (API)

Table-4: Linearity Results

Conc.(µg/ml)	Mean AUC (n=6)
0	0
6	370200
8	490231
10	602707
12	717538
14	829248
16	947852

Method Robustness:

Table-5: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.52
Flow (0.9 ml/min)	0.56
Temperature (27^0C)	0.52
Temperature (23^0C)	0.49
Wavelength of Detection (270 nm)	0.97
Wavelength of detection (266 nm)	0.98

LOD and LOQ:

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified[18] (LOQ) were found to be 0.90 & 2.90 µg/ml respectively.

System Suitability Parameter

Table-6: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.41
2	Asymmetry	T ≤ 2	Regorafenib=0.25
3	Theoretical plate	N > 2000	Regorafenib=3985

Estimation of Regorafenib in Tablet Dosage Form

Table-7: Recovery Data for estimation Regorafenib in Nublexa Tablets

Brand name of Tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg)found by the proposed method (n=6)	Assay %(±SD)
Nublexa (40mg) (Silverline Medicare Private Limited)	40	39.91 (±0.498)	99.51 (±0.343)

RESULTS AND DISCUSSION:

The amount of drug found in Regorafenib Tablets was found to be 39.91 (±0.498) mg/tab for Regorafenib & % assay[19] was 99.51 (±0.343).

CONCLUSION

The result shows that the developed method is a sensitive and selective RP-HPLC method has been developed and validated for the determination of Regorafenib in pure form and Pharmaceutical dosage form. The UV detection was performed at 268nm. The Retention time of Rilpivirine was found to be 3.544 minutes. The Percentage Standard Deviation (%RSD) of the Rilpivirine was and found to be 1.63%. The detector response was linear in the concentration range of 0-16µg/ml. The respective linear regression equation being Y= 58945.x + 9634 with R2 = 0.999. The percentage recovery values of Regorafenib in pharmaceutical dosage form were found to be within the limits. The limit of detection and the limit of quantification for Regorafenib were found to be 0.90µg/ml and 2.90µg/ml respectively. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility, accuracy. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Regorafenib in different marketed formulations. The developed method was validated as per ICH guidelines for accuracy calculated as % recovery was in the range of 98.0% to 102.0%. The statistical analysis of the data showed that the method is reproducible and selective for the estimation of Regorafenib in pure form and marketed Pharmaceutical dosage form during routine analysis. The results of the study showed that, the proposed RP-HPLC method was simple, rapid, precise, accurate which can be used for the routine determination of Regorafenib in pure form and marketed pharmaceutical dosage forms.

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