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A Validated Stability Indicating RP-HPLC Method for the Estimation of an Anti-Cancer Drug Regorafenib in Pure and Pharmaceutical Dosage Form

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Abstract: A simple, economic, accurate, sensitive, specific and precise stability indicating reverse phase high performance liquid chromatographic [RP-HPLC] method for the determination of Regorafenib in pure and tablet dosage from was developed and validated. The chromatographic separation was carried out using Phenomenex Luna-C_{18} column (4.5x250 mm; 5 µm particle size) as a stationary phase and methanol: acetonitrile: water (55:25:20 v/v/v) as a mobile phase. The flow rate of 1 mL/min was used with PDA detection at 275 nm. The retention time of Regorafenib was 2.480 min. RP-HPLC method was developed with linearity range of 40-240 µg/mL of Regorafenib. The correlation coefficient [r^2] was found to be 0.9999. The assay results obtained was in good agreement with the corresponding labeled amount by developed method within range of 98.83 ± 0.6937. Accuracy of the method was confirmed by recovery studies and the recoveries were found to be between 99.61 % and 100.22 %, the corresponding %RSD was found to be 0.2029. Precision, LOD, LOQ, specificity, robustness and ruggedness were performed as per ICH Q2(R1) guidelines and were within the acceptance criteria. This method can be conveniently used to detect the possible degradation product in the dosage form of Regorafenib during stability studies (acidic, alkaline, oxidative, thermal and photolytic). The method proved to be effective on the analysis of stressed marketed tablet formulation.

Keywords: Regorafenib; RP-HPLC; stability indicating method; validation; ICH-guidelines

1. Introduction

Regorafenib (REG, Figure 1), chemically known as 4-[4-[[[[4-chloro-3-(trifluoromethyl) phenyl] amino] carbonyl] amino]-3 -fluorophenoxy]-N -methyl-2-pyridine carboxamide hydrate with empirical formula of $C_{21}H_{15}ClF_4N_4O_3.H_2O$. Regorafenib is an oral multi-kinase inhibitor developed by Bayer, which targets angiogenic, stromal and oncogenic receptor tyrosine kinase. REG shows anti-angiogenic activity due to its dual targeted VEGFR2-TIE2 tyrosine kinase inhibition. It is currently being studied as a potential treatment option in multiple tumor types.[1] REG demonstrated to increase the overall survival of patients with metastatic colorectal cancer.[2-4] Stivarga is being approved with boxed warning altering patients and health care professionals that severe and fatal liver toxicity occurred in patients treated with Stivarga during clinical studies.[4, 5]

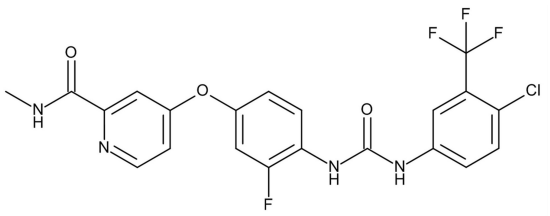

Figure 1. Structure of Regorafenib

Literature review revealed that several methods were reported for the estimation of REG in tablets; it was estimated in bulk and in tablets by RP-HPLC and LC-MS. [6, 7] But to the best of our knowledge and a through literature review indicated that there is no stability-indicating method reported for REG. The present work aims to develop a simple, precise, and accurate stability indicating RP-HPLC method for the estimation of REG in pure and in its tablet formulation through stress studies under a variety of ICH recommended test conditions and to develop a validated stability-indicating assay method.[8-10]

2. Result and Discussion

In the present work, a stability indicating analytical method based on RP-HPLC using PDA detection was developed and validated for assay determination of REG in tablet dosage formulation. The analytical conditions were selected, keeping in mind the chemical nature of REG. The development trails were taken using different conditions. The column selection has been done on the basis of backpressure, peak shape, and theoretical plates and day-to-day reproducibility of the retention

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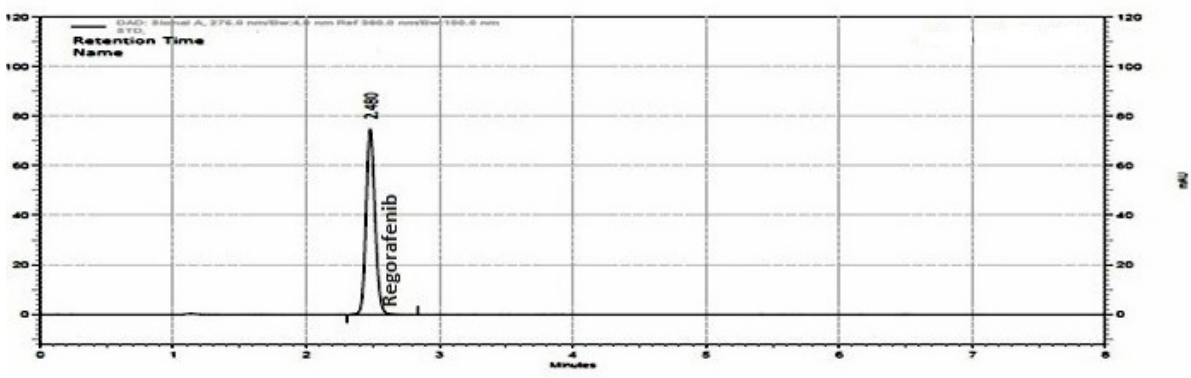

Figure 2. Typical chromatogram of REG

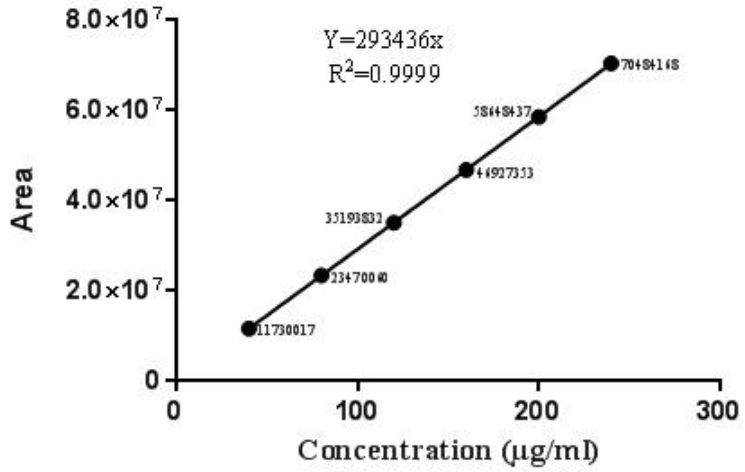

Figure 3. Calibration of curve of REG

Table 1. Estimation of REG in Stivarga® 40 mg Tablets

Standard area	Sample area	*Label claim [mg/tab]	Amount found [mg/tab]	% Assay
46921973	45591759	40 mg	39.43	98.57
46933226	45638169		39.39	98.47
46906638	45657108		39.23	98.08
46900114	45606470		39.42	98.55
46948741	45601147		39.75	99.39
46936942	45649673		39.98	99.95
			Average	98.83
			SD	0.6937
			%RSD	0.7019
			SE	0.2832
			CI[Confidence Interval 99%]	97.78 – 99.87

Table 2. Recovery Studies

Parameters	Amount present [µg / mL]	Amount added [µg / mL]	Peak area	Amount found [µg / mL]	Amount recovered [µg / mL]	% Amount recovered
80%	160	128	82885556	286.82	126.82	99.61
			82986547	287.16	127.16	99.88
			82927481	286.96	126.96	99.72
100%	160	160	92326615	319.49	159.49	100.22
			92102736	318.71	158.71	99.73
			92243018	319.20	159.20	100.03
120%	160	192	101598549	351.57	191.57	100.13
			101496868	351.22	191.22	99.95
			101527915	351.33	191.33	100.01
					Average	99.92
					SD	0.2028
					%RSD	0.2029
					SE	0.0676
					CI [Confidence Interval 99%]	99.62 – 100.09

time. After evaluating all these factors, Phenomenex Luna-C_{18} column [4.5x250 mm; 5 µm] was found to be giving satisfactory results. The selection of mobile phase was done based on chemical structure of the drug. Best results were obtained with methanol: acetonitrile: water [55: 25: 20 v/v/v]. It was found to reduce the longer retention time to attain good peak shape with the flow rate of 1 mL/min at the injection volume was 10 µL. The detection was carried out at 275 nm. The peak retention time of REG was found to be 2.480 min with good baseline stability.

Typical chromatogram of REG was shown in Figure 2. The calibration curve was plotted utilizing the peak area of REG against concentration of the drug. The calibration curve showed linearity, over a concentration range of 40-240 µg/mL for the drug as showed in Figure 3. Regression coefficient [R^2] was found to be 0.9999. The number of theoretical plates obtained was 6454, which indicates the efficient performance of the column. Assay of REG tablets [Stivarga® 40 mg, Bayer Pharma AG, India] using the developed method showed acceptable relative error values. The %RSD for assay of the drug was 0.7019.

2.1. Analysis of Pharmaceutical Formulation

Tablet powder equivalent to 40 mg of REG was weighed and transferred in to 100 mL volumetric flask, 30 mL of diluent was added and sonicated for 15 min and the volume was made up to the mark with diluent. From this solution further dilution was made to get the final concentration of 160 µg/mL. A volume of 10 µL of the final solutions was injected into the system and the chromatogram was recorded. The retention time was found to be 2.491. The content of REG in the tablets [Stivarga® 40 mg, Bayer Pharma AG, India] was computed by putting value of the peak areas (Table 1) in respective standard regression equation obtained from calibration curve.

2.2. Method Validation

2.2.1. Linearity

Linearity was demonstrated from the standard drug solution using six concentration levels for REG. The peak areas were recorded and calibration plot was obtained by plotting peak area versus concentration of REG. The correlation coefficient results revealed that developed analytical method having excellent linearity.

2.2.2. Accuracy

Accuracy was calculated by addition of standard drugs to pre-analyzed sample at 3 different concentration levels and computing percentage recoveries. Standard limit of percentage recovery study is 98-102 % as per ICH guideline. From the studies it was concluded that percentage recovery study of REG complies with standard limit of ICH guideline. The results were shown in Table 2. The percentage recoveries obtained was found to be between 99.61 and 100.22%, which indicated that the method was accurate.

2.2.3. Precision

Repeatability: Six repeated injections of standard and sample solutions containing 160 µg/mL of REG was prepared and the response factor of the drug peaks and %RSD were calculated. The results obtained were presented in Table 3.

Intraday precision: Solution containing 160 µg/mL of REG was prepared from their respective standard stock solution. Analysis was replicated for 3 different times within the same day. The results of intraday precision studies were shown in Table 4. The results revealed that the %RSD of intraday was within the permissible limits of 2%.

Interday precision: Solution containing 160 µg/mL of REG was prepared from their respective standard stock solution. Analysis was replicated for 3 different days. The results of interday precision studies were shown in Table 5. The results revealed that the %RSD of interday were within the permissible limits of 2%.

Table 3. Precision

Standard solution		Sample solution	
No. of Injections	Peak area	No. of Injections	Peak area
1	46969189	1	45996546
2	46971992	2	45989189
3	46994018	3	46431357
4	46982628	4	46489802
5	47024182	5	46891759
6	46893315	6	46425107
Average	46972554	Average	46370627
SD	39829	SD	310340.9
%RSD	0.0847	%RSD	0.6692

SD: Standard deviation; RSD: Relative standard deviation

Table 4. Results of Intraday precision

Parameter (Hours)	Conc. [µg / mL]	Peak area*	% Amt. found*	SD	%RSD
0		46848688	99.82		
3	160	46917447	99.96	0.6465	0.6468
6		46940764	100.02		

*Mean of six determinations

Table 5. Results of Interday precision

Parameter (Days)	Conc. [µg / mL]	Peak area*	% Amt. found*	SD	%RSD
I		46927498	99.99		
II	160	46941956	100.02	0.1396	0.1396
III		46887687	99.90		

*Mean of six determinations

2.2.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were calculated from the standard deviation of the y-intercepts and slop of the calibration curve of REG. The results of LOD and LOQ for REG obtained were presented in Table 6.

Table 6. Results of LOD and LOQ

Slope	Y-Intercept
293259.49	2030.53
293783.11	-32884.35
293264.53	1653
293784.39	-31997.25
293268.95	1809.92
293782.18	-31562.35
Average 293523.80	
SD	18616.4
LOD [µg / mL]	0.2092
LOQ [µg / mL]	0.6342

Table 7. Results of Robustness

Parameters	Retention time*	Peak area*	% Amount found*	SD	%RSD
Flow minus [0.8 mL/min]	2.773	46109514	98.81	0.3300	0.3340
Flow plus [1.2 mL/min]	2.767	46765338	100.02	0.2540	0.2539
nm plus [277 nm]	2.500	47602481	101.14	0.2079	0.2055
nm minus [273 nm]	2.500	46685382	99.93	0.2585	0.2587
Temperature plus [32 ^{0}C]	2.500	46221638	98.86	0.3173	0.3210
Temperature minus [28 ^{0}C]	2.487	47493041	101.13	0.1565	0.1546
Methanol [50]	2.880	46963436	100.06	0.1940	0.1938
Methanol [60]	2.540	47533950	101.18	0.2700	0.2668
Acetonitrile [20]	2.880	46526392	99.24	0.1662	0.1674
Acetonitrile [30]	2.880	47159305	100.27	0.3531	0.3521

2.2.5. Robustness

Variation in the flow rate, mobile phase, nanometer and temperature has been made to the analytical method in order to evaluate and measure the capacity of the method to remain unaffected by such variations. The % RSD was found to be less than 2. The results were shown in Table 7.

2.2.6. Ruggedness

Ruggedness of the method was confirmed by the analysis of formulation by the different analyst. The results were shown in Table 8.

Table 8. Results of Ruggedness

Parameter	Conc. [µg / mL]	Peak area*	% Amt. found*	SD	%RSD
Different Analyst	160	46525064	99.14	0.1698	0.1712

2.2.7. System suitability

System suitability was established to determine the adequate reproducibility of the proposed method. Parameters including asymmetry factor, theoretical plates, repeatability of peak area and retention time were calculated. The results were shown in Table 9.

Table 9. Results of system suitability parameters

Parameters	Results
Theoretical plates [N]	6454
Asymmetry factor	1.1
Retention time	2.480
% RSD of peak area	0.0847
% RSD of retention time	0.5436

2.3. Forced Degradation Study

Forced degradation studies were performed to evaluate the stability indicating properties [Specificity] of the proposed method. REG was subjected to neutral, acid, base, oxidation, thermal and photo degradation to ensure the effective separation of degradation peaks and main peak. From the degradation of these solutions under the stressed condition gave us an idea about the origin of degrading products. Degradants did not show any interference with the elution of drug peak. Hence, the method is stability indicating. The results of degradation studies were shown in Table 10.

3. Conclusion

A simple, sensitive, precise and accurate stability indicating RP-HPLC analytical method was developed for the estimation of REG in pure and tablet dosage form. The method was successfully validated and proved as linear, precise, accurate and robust. Documented evidences of the present work suggested that the developed method was an economical one in terms of lower acetonitrile concentration for the estimation of the drug and can be successfully employed for routine analysis in quality control laboratories.

4. Experimental

Material & methods: REG standard reference was purchased from Spectrum Labs Limited, Hyderabad, India. REG tablets [Stivarga® 40 mg, Bayer Pharma AG, India] were purchased from a local retail pharmacy. HPLC grade methanol and acetonitrile were purchased from E-Merck Specialties Pvt. Ltd, Mumbai and Milli Q water [HPLC Grade] were used for the analysis. The analyses were performed on a Hitachi HPLC system containing D-2000 Elite HSM [English] software and PDA detector set at 275 nm with an isocratic elution at a flow rate of 1 mL/min on a Phenomenex Luna- C_{18} column [4.5x250 mm; 5 µm]. Mobile phase composition of methanol: acetonitrile: water [55: 25: 20 v/v/v] was used.

4.1. Method Development

4.1.1. Chromatographic condition

Mobile phase: Methanol: acetonitrile: water (55: 25: 20 v/v/v)
Diluent: Mobile phase
Column: Phenomenex Luna-C_{18} column (4.5x250 mm; 5 µm)
Column temperature: 30 ^{0}C
Detection wavelength: 275 nm
Injection volume: 10 µL
Flow rate: 1 mL/min
Run time: 8 min

4.1.2. Preparation of mobile phase

The diluent was prepared by mixing 55 mL of methanol, 25 mL of acetonitrile and 20 mL of water and the resulting solution was sonicated for 15 min and it was used as mobile phase.

4.1.3. Preparation of standard stock solution

Quantity of REG equivalent to 40 mg was weighed and transferred in to a 100 mL volumetric flask, 30 mL of mobile phase was added and sonicated for 15 min and the volume was made up to the mark with mobile phase. From this solution further dilution was made to get the final concentration of 160 µg/mL, 10 µL of the

final solutions were injected into the system and the chromatograms were recorded.

Table 10. Results of forced degradation studies

Parameters	Degradation time	Peak area*	% Degradation	% of Active drug present after degradation
Control sample	-	45649673	-	-
Neutral sample	30 minutes	45378295	0.55	99.40
Acidic degradation	30 minutes	44726617	1.99	97.97
Alkaline degradation	30 minutes	44387624	2.71	97.23
Oxidative degradation	30 minutes	43572830	4.49	95.45
Thermal degradation	48 hours	44548875	2.36	97.58
Photolytic degradation	7 days	44865984	1.66	98.28

4.1.4. Preparation of sample stock solution

Tablet powder equivalent to 40 mg of REG was weighed and transferred in to 100 mL volumetric flask, 30 mL of mobile phase was added and sonicated for 15 min and the volume was made up to the mark with mobile phase. From this solution further dilution was made to get the final concentration of 160 µg/mL, 10 µL of the final solution were injected into the system and the chromatograms were recorded.

4.2. Forced Degradation Studies of REG

4.2.1. Control Sample

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase. Further pipette 4 mL of the above stock solution and transferred to 10 mL volumetric flask and made up to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and 10 µL of the solutions were injected in to the system and the chromatograms were recorded.

4.2.2. Neutral Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase. Further pipette 4 mL of the above stock solution and transferred to 10 mL volumetric flask and made up to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and the solution was refluxed in water bath for 30 minutes at 80 ^{0}C and 10 µL of the refluxed solutions were injected in to the system and the chromatograms were recorded.

4.2.3. Acid Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase, 4 mL of the above stock solution was transferred to 10 mL volumetric flask to that 4 mL of 2 N hydrochloric acid was added and refluxed for 30 minutes at 80^{0}C. The resulting solution was diluted to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and 10 µL of the refluxed solutions were injected in to the system and the chromatograms were recorded.

4.2.4. Alkaline Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase, 4 mL of the above stock solution was transferred to 10 mL volumetric flask to that 4 mL of 2 N sodium hydroxide was added and refluxed for 30 minutes at 80^{0}C. The resulting solution was diluted to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and 10 µL of the refluxed solutions were injected in to the system and the chromatograms were recorded.

4.2.5. Oxidative Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase, 4 mL of the above stock solution was transferred to 10 mL volumetric flask to that 4 mL of 3 % hydrogen peroxide [H_2O_2] was added and refluxed for 30 minutes at 80^{0}C. The resulting solution was diluted to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and 10 µL of the refluxed solutions were injected in to the system and the chromatograms were recorded.

4.2.6. Thermal Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase. Further pipette 4 mL of the above stock solution and transferred to 10 mL volumetric flask and made up to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and the solution was placed in oven at 80^{0}C for 48 hours, 10 µL of the solutions were injected in to the system and the chromatograms were recorded.

4.2.7. Photolytic Degradation Studies

A quantity tablet powder equivalent to 40 mg of REG was accurately weighed and transferred to 100 mL

volumetric flask and it is dissolved in 10 mL of the mobile phase. The solution was sonicated for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume was made up to 100 mL with mobile phase. Further pipette 4 mL of the above stock solution and transferred to 10 mL volumetric flask and made up to 10 mL with mobile phase to get the final concentration of 160 µg/mL of REG and the solution was exposed to UV light by keeping the volumetric flask in UV chamber for 7 days, 10 µL of the solutions were injected in to the system and the chromatograms were recorded.

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