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Research Paper

Analytical Quality by design (AQbD) approach: An advanced greener HPTLC method for Quantification of Lenvatinib in capsules

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ABSTRACT

An Advanced and greener novel HPTLC method was developed based on Analytical Quality by design (AQbD) for the quantification of Lenvatinib in capsules. Chromatographic estimation of Lenvatinib was carried out utilizing TLC plate precoated with silica gel 60 F254 using mobile phase containing Toluene: Methanol in ratio of 8:2 and scanning at 245 nm. The developed HPTLC method of Lenvatinib was successfully validated according to ICH Q2(R1) guidelines with respect to linearity, accuracy, precision, robustness, Limit of Detection and Limit of Quantitation. Method optimization was carried out using three critical method parameters(CMPs), that is, Volume of organic phase(A), Saturation time(B) and band width(C). Box- benken design was applied to study the response surface methodology for analyzing of retention factor and peak area was considered as critical analytical attributes (CAAs). Interaction effects of CMPs and CAAs are evaluated using Design expert software version 12. The confirmation of significance ($p<0.05$) of the method parameters are measured using analysis of variance (ANOVA). The linear calibration curve of Lenvatinib in the concentration range of 100-1000 ng/band was obtained. The mean recoveries for developed method were found to be more than 99%. The % RSD for both inter- day and intra- day precision was less than 2%. The assay was found to be 99.80 ± 0.37%. The greenness score of the developed method was calculated utilizing the "Analytical GREENness(AGREE)" and "Green Analytical Procedure Index(GAPI)" approach. AGREE and GAPI score showing an excellent greenness characteristic of the present method. The detailed quantitative results showed that this method is novel, sensitive, eco-friendly as well as cost effective.

Key- words: Lenvatinib, HPTLC, Analytical quality by design, Validation, AGREE, GAPI.

INTRODUCTION

Lenvatinib is a tyrosine kinase inhibitor mainly used for the treatment of thyroid cancer. Chemical name of Lenvatinib is 4-[3-Chloro-4-(cyclopropylcarbamoylamino)phenoxy]-7-methoxy-quinoline-6-carboxamide. The chemical formula and Mol. Wt. for Lenvatinib are $C_{21}H_{19}ClN_4O_4$ and 426.86 g/mol respectively. Lenvatinib is not official in any pharmacopoeia. Lenvatinib is freely soluble in Methanol and Acetonitrile. The chemical structure of Lenvatinib is shown in Fig.1.

Detailed Review of Literature survey reveals that very few analytical methods in bulk and pharmaceutical formulation have been reported for the analysis of lenvatinib includes, UV Spectrophotometric methods (Patve et. al., 2018), RP- HPLC, UPLC (Bandla & Ganapaty, 2018; Akula et. al., 2019), Bioanalytical (Veni et. al., 2020; Aghai et. al., 2020) and LC-MS (Mano & Kusano, 2015) but they were found to be more time consuming and expensive.

DOE is a tool for the optimization of analytical techniques which is utilized for the evaluation of principal effects along with their interactions. It is a systematic, risk-based and proactive approach to analytical method development which focuses on identifying and minimizing sources of variability and failure modes that may lead to establish robust method operable design space within meaningful system suitability criteria and ensuring that the method meets its intended performance requirements throughout the product and method lifecycle.

Now days, principles of green chemistry have inspired researcher's interest to minimize environmental health as well as issues related to human health. Recently various criteria are utilizing to assess the environmental impact of newly developed analytical methods. The analytical GREENness (AGREE) calculator is panoramic, adequate and user friendly approach to evaluate the greenness score of analytical procedures. It is based on twelve principles of green chemistry. The green analytical procedure index (GAPI) tool used to evaluate the green nature of developed procedures. The GAPI metric uses a pictogram to classify the greenness of each step of an analytical methodology, applying a colour scale, with two or three levels of evaluation for each stage.

The goal of the research paper is eco- friendly High performance thin layer chromatography method developed by Analytical quality by design (AQbD) approach and validated according to ICH Q2 (R1) regulations.

MATERIALS AND METHODS

Instrumentation and Chromatographic conditions:

The HPTLC CAMAG TLC system (CAMAG, Muttenz, Switzerland) was used for analysis. The sample were spotted in the form of bands with 100 mL sample syringe (Hamilton, Switzerland), on Merck TLC plates precoated with silica gel 60 F_{254} (20×10 cm with 0.2 mm layer thickness) at a constant rate of 15 nL/s using a nitrogen aspirator using a Camag Linomat V applicator. The plates were prewashed with methanol and activated at 110 ºC for 5 minutes prior to the sample application. The optimized chamber saturation time for mobile phase was 20 minutes at temperature of 25 ºC. The chromatogram run has 7 cm length Developed TLC plate was dried using an air-dryer. The slit dimension was 6.0 mm × 0.45 mm, and the scanning speed was 20 mm/s. Densitometric scanning was performed on Camag Densitogram HPTLC scanner III with WINCATS software (V 1.3.0) at 245 nm. Evaluation was performed using linear regression analysis via peak areas.

Chemicals and Reagents:

Reference standard sample of Lenvatinib was procured from Ratnamani Healthcare pvt. Ltd., Chhatral. Marketed formulation of Lenvatinib capsules (Lenvima 4) purchased from Local Pharmacy store. Methanol and distilled water were HPLC grade purchased from Finar Ltd. Toluene, chloroform, ethyl acetate, hexane, formic acid, n- butanol, acetone and isopropyl alcohol was Analytical grade purchased from Ranbaxy chemicals.

Software(s):

For Experimental design, Data analysis (ANOVA) and desirability function estimate were performed by Design Expert® version 12.0. Method greenness assessment was performed by AGREE: The analytical greenness calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020) and ComplexGAPI (version 02-beta).

Selection of Wavelength:

The developed plate was scanned in spectrum mode by Densitogram CD -60 TLC scanner with software CAMAG at respected Rf value of the spot and the obtained spectrums were overlain. Multiple wavelengths densitogramof Lenvatinib (100 ng/µL) showed maximum considerable absorbance between 200-350 nm. Wavelength maxima 245 nm was selected for the analysis.

Selection of Mobile Phase:

Many solvent combinations were tried as mobile phase and finally Toluene: Methanol (8:2 v/v) was selected as it qualified all acceptance criteria of system suitability.

Preparation of standard Stock solution:

10 mg of Lenvatinib was weighed and transferred to a 10 mL volumetric flask. Volume was made up to the mark with methanol (conc. 1000 ng/µL). Different standard dilutions were prepared over the period of method development. The solution was stored at room temperature until it applied to the plate.

Preparation of working standard solution:

Pipette out 5 mL Lenvatinib stock solution and transferred it into 50 mL volumetric flask and volume was made upto mark with methanol (conc. 100 ng/µL).

HPTLC Method development by using the QbD approach:

The first step was to define the objectives of the method development, called ATP. The main objectives were to optimize chromatographic condition to improve the quality of chromatogram. Sly, successfully apply the developed method for estimation of Lenvatinib. Critical analytical attributes(CAAs) plays a recognizing role for identifying the variable that affect the chromatographic conditions. The retention factor (Rf) (R1) and peak area (R2) were identified as CAAs (Dependent variables) for proposed HPTLC method development. The critical method parameters (CMPs) also known as independent variables are the method parameters that are directly affect the CAAs. Three CMPs were considered for proposed HPTLC method development, volume of Toluene (A), Saturation time (B) and band width (C). Usually, the factors were examined at three levels (-1, 0, +1) or more in optimization experimental designs.

After defining the CAAs and CMPs, Box- behnken design (BBD) was applied to optimization. BBD could predict optimum condition with lowest runs and therefore saving time and cost, considered for study. Systemic statistical analysis of experimental results was carried out using Design Expert® (Version 12.0). Statistical tools like predicted vs. actual plot, ANOVA, lack of fit, prediction equations and contour plots were used to analyze each individual response parameter and design space was generated. Explains design matrix of BBD which accounts for 15 runs.

Validation of developed Chromatographic method

The method was validated as per ICH guidelines.

Specificity:

The specificity of the method was checking by the peak purity of analyte peak.

Linearity and Range:

Linearity was assessed by analysis of standard solution in a range of 100-1000 ng/band. From the Working Standard Solution (100 ng/µL) aliquots of 1, 2, 4, 6, 8 and 10 µL were spotted on TLC plate. Calibration curve was plotted and Correlation coefficient (r^2) was found.

System Precision (Repeatability):

For Repeatability studies, six replicate injections of Lenvatinib (400 ng/band) analyzed. Then average peak area and % RSD were calculated.

Intermediate Precision:

Intermediate Precision of analytical method demonstrates by Intraday and Interday Precision.

In Intraday Precision, Standard solution containing 200, 400 and 600 ng/band Lenvatinib was analyzed for three times on same day (0 hr, 3 hrs and 6 hrs) and then average peak area and % RSD were calculated.

In Interday Precision, Standard solution containing 200, 400 and 600 ng/band Lenvatinib was analyzed for three times on different day (Day- 1, 2 and 3) and then average peak area and % RSD were calculated.

Accuracy (Recovery study):

To measure accuracy of analytical method, recovery studies were carried out using standard addition method with different level 80 %, 100 % and 120 %.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

Limit of Detection is a lowest concentration in a sample that can be detected but not necessarily quantified under the optimized experimental conditions. The Limit of Quantitation is lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

Assay:

Ten Lenvatinib Capsules were accurately weighted and average weight was calculated. Weigh a quantity of mixed content of above capsules containing about 10 mg of Lenvatinib were transferred to 100 mL volumetric flask. Lenvatinib was extracted by addition of 80 mL Methanol. Above solution was sonicated for 20 mins and then filtered into 100 mL volumetric flask and fill upto mark with same solvent. Then the solution was analyzed using the proposed Chromatographic method.

Method greenness assessment:

The analytical GREENness (AGREE) calculator is panoramic, adequate, informative and user friendly approach to evaluate the greenness score of analytical procedures. It is based on twelve principles of green chemistry. Each one of 12 input variables is converted to a common scale ranging from 0 to 1. The sum of evaluation results for each principle is the final assessment result.

The GAPI represents total 15 pictograms, each representing a step within the main 5 pictograms; each pictograms shows the greenness of each step of an analytical methodology. Each step represented by three levels of colour scale as per greenness index. In GAPI, reagents, procedures, and instrumentation are evaluated.

RESULT AND DISCUSSION

Method development:

In this research work, multiple wavelength Densitogram showed maximum considerable absorbance between 200-350 nm. So wavelength selected for detection was 245 nm as shown in Fig.2.

Many solvent combinations with chloroform, methanol, ethyl acetate, hexane, formic acid, n-butanol, toluene, acetone, distilled water and isopropyl alcohol were tried as mobile phase and finally Toluene: Methanol (8:2 v/v) was selected as it qualified all acceptance criteria of system suitability which gave optimum separation of Lenvatinib.

Utilization of BBD method for optimization of chromatographic condition:

Box- behnken design (BBD) values of the 15 experimental runs and 3 centre points shown in Table 2. The collected data were subjected to statistical analyses using Design- Expert® software version 12.

A quadratic and linear model was selected. It was based on the PRESS value. The R^2 (Adjusted) value was found to be nearer to 1. The model validation was performed with ANOVA, and the results are shown in Table 3. Significance was found to be $P < 0.05$. The ratio obtained for the drugs showed an adequate signal. The % CV < 10% the R^2 (Adjusted) was found to be high. This shows an effective relationship between the obtained experimental data and the models.

A steepest slope or curvature indicates the sensitiveness of the response to a particular factor. 3D-response surface plots for each of volume of Toluene (A), Saturation time (B) and band width (C) in Fig.4 and 5 explain the effect of the CMPs on CAAs. The response surface analysis revealed significant interaction among the studied CMPs on the CAAs (retention factor and peak area). The curvature lines in the graphs indicate the significant effect of all the three CMPs over Rf; amount of toluene and band width has major influence on Rf while saturation time have negligible effect on Rf. Analysis was conducted on the model's response plots and perturbation plots, exposing that A and C affect responses more than B. 3D plots were shown in Fig. 3 and 4.

Method optimization:

Optimization was achieved by studying all responses in various experimental conditions using the Design expert® 12.0 software, and optimized HPTLC conditions and predicted responses are shown below. Overlay plot of optimized condition is shown in Fig.5.

The mobile phase containing Toluene: Methanol having ratio of 8:2 has excellent resolution with Rf value of 0.291. Under the chromatographic condition employed standard Lenvatinib and the formulation have shown sharp peak which shown in Fig.6.

Method validation of Chromatographic method

Linearity and Range:

The linearity for Lenvatinib was found in the range of 100-1000 ng/band. Linearity data are shown in table 4. Single lane chromatogram and 3D Overlain chromatogram are depicted in Fig.7 and 8 respectively. Photograph of TLC plate and calibration curve of Lenvatinib is presented in Fig.9 and 10 respectively.

System Precision (Repeatability):

Repeatability study carried out using Lenvatinib solution containing 400 ng/band and % RSD was found to be 1.039.

Intermediate Precision:

In Intraday Precision, % RSD was found to be 0.908-1.017 for Lenvatinib and in Interday Precision, % RSD was found to be 1.170-1.338 for Lenvatinib.

Accuracy:

The amount of Lenvatinib was calculated and % recovery was shown in table 7.

LOD and LOQ:

For Lenvatinib, LOD and LOQ are found to be 0.00408 ng/band and 0.01237 ng/band respectively.

Assay:

In Assay, Mean (n=5) mg of Lenvatinib found to be 9.98 mg. Assay of Lenvatinib capsules were found to be 99.80 ± 0.37%.

Method greenness assessment:

The results of AGREE metric is obtained as a graph which look like a clock showing the greenness score in the centre and a colour representation (dark green colour showing that the evaluated procedure is greener with values close to 1). The intuitive red-yellow-green colour scale reflects the procedure's performance in each principle. The scores corresponding to GAC principles 7, 9 and 10 are quite low, due to generation of large volume of analytical waste, energy used and type of reagents used during analysis, respectively While, in the case of principles 2 (minimum sample size), 4 (Integration of analytical processes) and 11 (Toxic reagents should be eliminated or replaced), excellent greenness score was achieved. Newly developed HPTLC method has an AGREE score of 0.55 and a middle colour of light green, Fig. 11(a) representing that it has a minimum environmental impact and can be consider a green method.

Pictogram created for GAPI utilizes a colour scale, with two or three levels of evaluation for each stage. The created pictogram can be used to evaluate and quantify from green to yellow to red colour respectively for the low, medium and high environmental impacts associated with each stage of the pre-analysis process and the analytical methodology. GAPI pictogram showing only three red zones which corresponding to Physico-chemical analysis, non- greener solvent used and no waste treatment. A representative diagram of pictogram for AGREE score and GAPI score of the proposed analytical assay is shown in Fig. 11(b).

CONCLUSIONS

Advanced, reliable, accurate, precise as well as eco- friendly HPTLC method for estimation of Lenvatinib in capsules has been developed and validated according to the ICH guidelines Q2(R1). The experimental design facilitates the evaluation of the selected factors simultaneously, including interactions between factors, in order to reach the optimum conditions. Selected CMPs were simultaneously optimized by applying $3(2^2)$ + 3 Box- behnken design. ANOVA indicate the significant effect of all the three CMPs over Rf; amount of toluene (A) and band width (C) has major influence on Rf while saturation time (B) have negligible effect on Rf. The analytical-QbD approach for method development has helped to better understand the method variables hence leading to less chance of failure during the method validation. An adequate separation and symmetrical band for Lenvatinib were obtained with a mobile phase containing a mixture of Toluene: Methanol (8:2 v/v) to get better repeatability and reproducibility. Retention factor of Lenvatinib was found to be 0.291 with selected wavelength was 245 nm. Proposed analytical method was successfully applied for greenness assessment using AGREE and GAPI tools and method was found to be greener. Hence, the developed HPTLC method is sensitive as well as

analysis with minimal risk to environment and it can be used for routine analysis of Lenvatinib in the capsule dosage form.

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CONFLICT OF INTERESTS

Declared no conflict of interest

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Table 1. Levels of independent variables

Factor	Coded value			Transformed value		
	Higher	Intermediate	Lower	Higher	Intermediate	Lower
Volume of organic phase (mL)	+1	0	-1	7.5	8	8.5
Saturation time (Minutes)	+1	0	-1	15	20	25
Band width (mm)	+1	0	-1	4	5	6

Table 2. Box- behnken design with measured response

Run	Pattern (X1X2X3)	A. Volume of organic phase (mL)	B. Saturation time (Minutes)	C. Band width (mm)	R1. Retention factor (Rf)	R2. Peak area
1	(-1,+1,0)	7.50	25.00	5.00	0.311	0.00241
2	(0,0,0)	8.00	20.00	5.00	0.291	0.00255
3	(+1, 0, -1)	8.50	20.00	4.00	0.230	0.00263
4	(+1,+1,0)	8.50	25.00	5.00	0.185	0.00259
5	(+1,0,+1)	8.50	20.00	6.00	0.181	0.00264
6	(0,+1,-1)	8.00	25.00	4.00	0.233	0.00258
7	(+1, -1, 0)	8.50	15.00	5.00	0.305	0.00261
8	(0,-1,+1)	8.00	15.00	6.00	0.225	0.00258
9	(0, 0, 0)	8.00	20.00	5.00	0.291	0.00255
10	(0,+1,+1)	8.00	25.00	6.00	0.235	0.00259
11	(0,-1,-1)	8.00	15.00	4.00	0.301	0.00245
12	(0,0,0)	8.00	20.00	5.00	0.291	0.00255
13	(-1,-1,0)	7.50	15.00	5.00	0.274	0.00251
14	(-1,0, +1)	7.50	20.00	6.00	0.325	0.00257
15	(-1,0,-1)	7.50	20.00	4.00	0.260	0.00248

Table 3. ANOVA of optimized model for selected CAAs

Response	Model	Y (Model Equation in terms of Actual factors)	R^2 (Adjusted)	P-value Prob > F	% CV	Precision (Adequate)
R1. Retention factor (Rf)	quadratic	- 7.21038 + 1.22775 (A) + 0.12077 (B) + 0.68200 (C) – 0.015700 (A*B) - 0.05700 (A*C) + 0.0039 (B *C) – 0.0435 (A^2) - 0.000455 (B^2) – 0.031125 (C^2)	0.9880	<0.0001	6.15	11.624
R2. Peak area	linear	+ 0.00379625 – 0.0005425 (A) - 0.000016 (B) + 0.00020875 (C) + 0.000011 (A*B) – 0.00004 (A*C) - 0.0000025 (B *C) + 0.00004 (A^2) -0.0000015 (B^2) + 0.00002 (C^2)	0.9159	0.0051	1.03	11.112

Table 4: Linearity data for Lenvatinib

Conc. (ng/band)	Mean Peakarea ± SD	% RSD
100	0.00306 ± 0.000037	1.218
200	0.00414 ± 0.000047	1.138
400	0.00536 ± 0.000057	1.065
600	0.00656 ± 0.000060	0.914
800	0.00797 ± 0.000082	1.024
1000	0.00965 ± 0.000092	0.956

Table 5: Repeatability data for Lenvatinib

Conc. (ng/band)	Area	Mean (n= 6) ± SD, % RSD
400	0.00542	0.00538 ± 0.000056, 1.039
	0.00538	
	0.00548	
	0.00532	
	0.00538	
	0.00535	

Table 6: Intraday and Interday data for Lenvatinib

Conc. (ng/band)	Intraday	Interday
	Peak area (Mean, n= 3) ± SD, % RSD	Peak area (Mean, n= 3) ± SD, % RSD
200	0.00417 ± 0.00004, 0.908	0.00416 ± 0.00006, 1.338
400	0.00541 ± 0.00006, 1.017	0.00542 ± 0.00007, 1.227
600	0.00658 ± 0.00007, 0.989	0.00652 ± 0.00008, 1.170

Table 7: Accuracy data for Lenvatinib

Conc. Level	Amt. Taken (ng/band)	Amt. added (ng/band)	Total amount (ng/band)	Amt. Recovered (ng/band)	% Recovery ± SD	% RSD
80%	200	160	360	160.60	100.38 ± 1.075	1.071
100%	200	200	400	199.75	99.88 ± 0.843	0.845
120%	200	240	440	238.56	99.40 ± 0.397	0.400

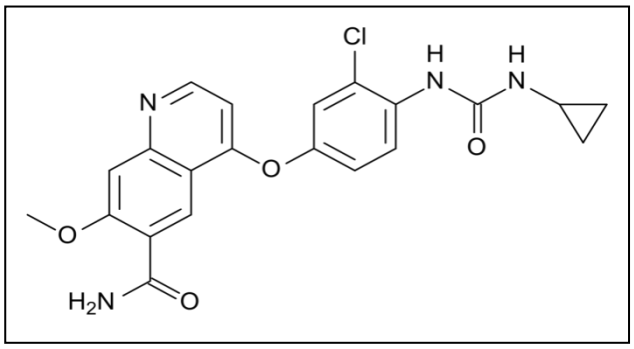


Fig. 1. Chemical structure of Lenvatinib

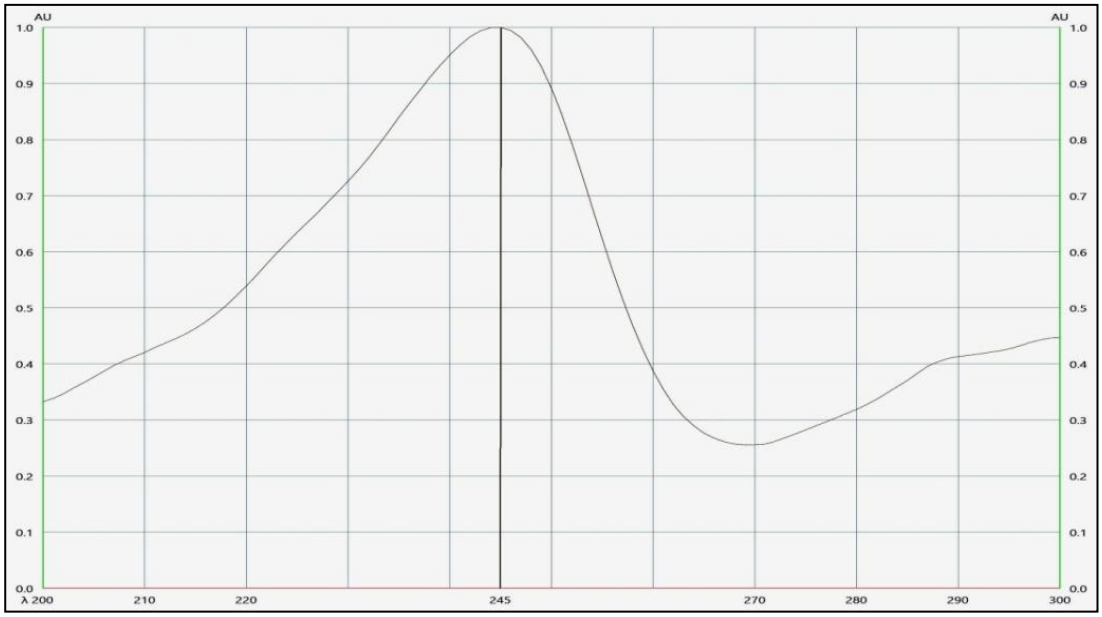
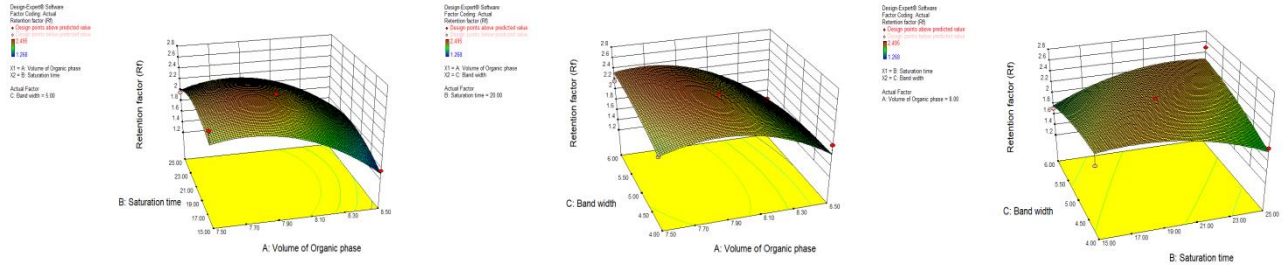


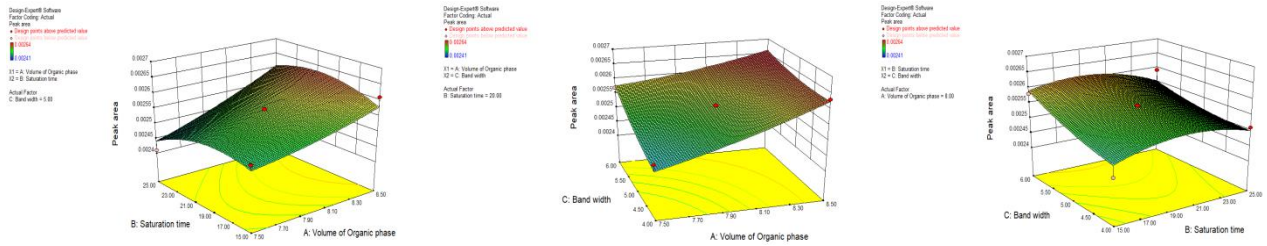
Fig. 2. Densitogram for Lenvatinib (100 ng/µL) at 245 nm



3(a) 3(b) 3(c)

Fig.3. 3D plots showing effect on Retention factor Rf of (a) Factor A and B; (b) Factor A and

C; (c) Factor B and C



4(a) 4(b) 4(c)

Fig.4. 3D plots showing effect on Peak area of (a) Factor A and B; (b) Factor A and C; (c) Factor B and C

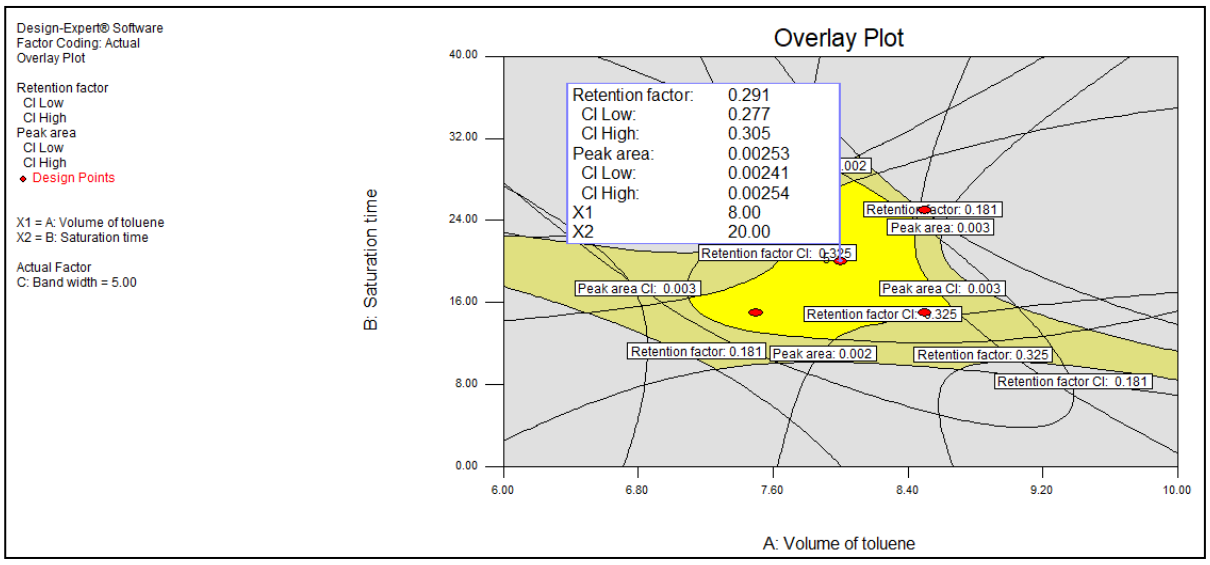


Fig.5. Overlay plot of optimized condition

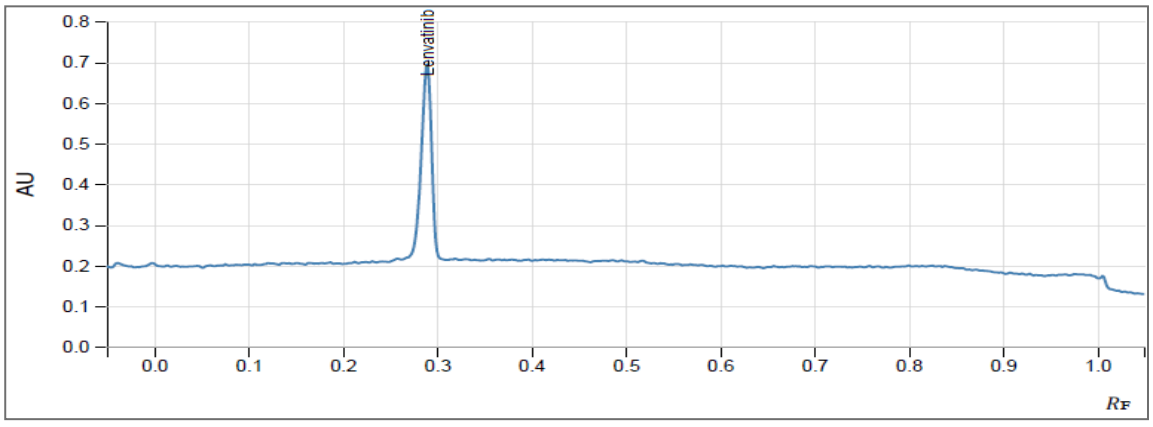


Fig.6. HPTLC chromatogram of standard Lenvatinib

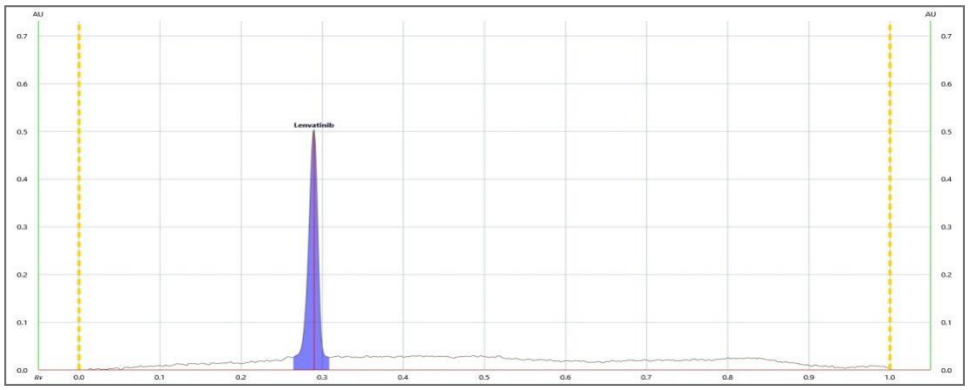


Fig.7. Chromatogram of Lenvatinib (100 ng/band)

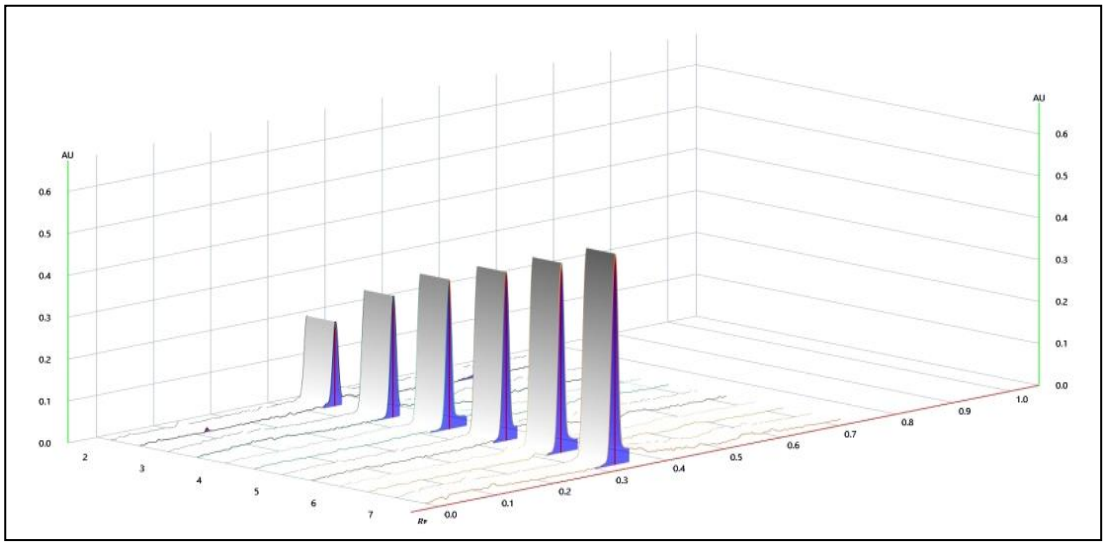


Fig.8. 3D Overlain Chromatogram of Lenvatinib Standards (100-1000 ng/band)

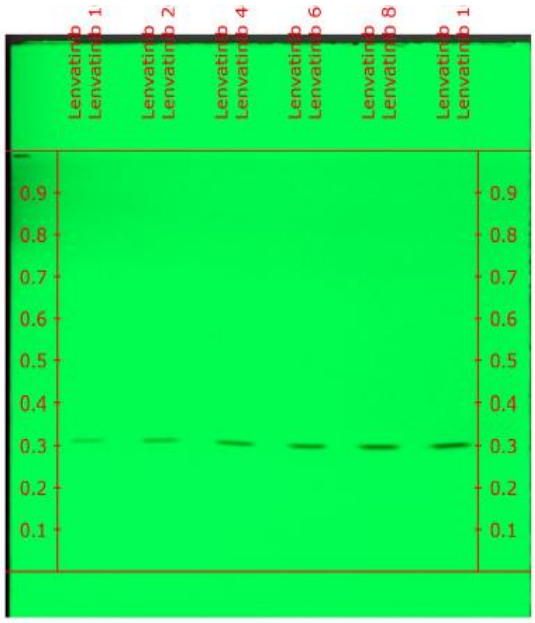


Fig.9. TLC plate photograph

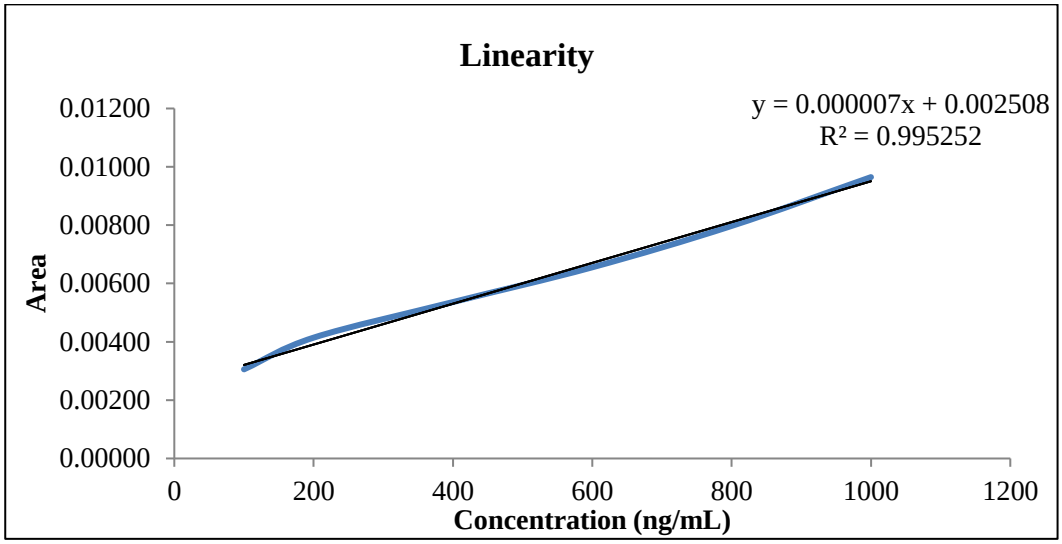
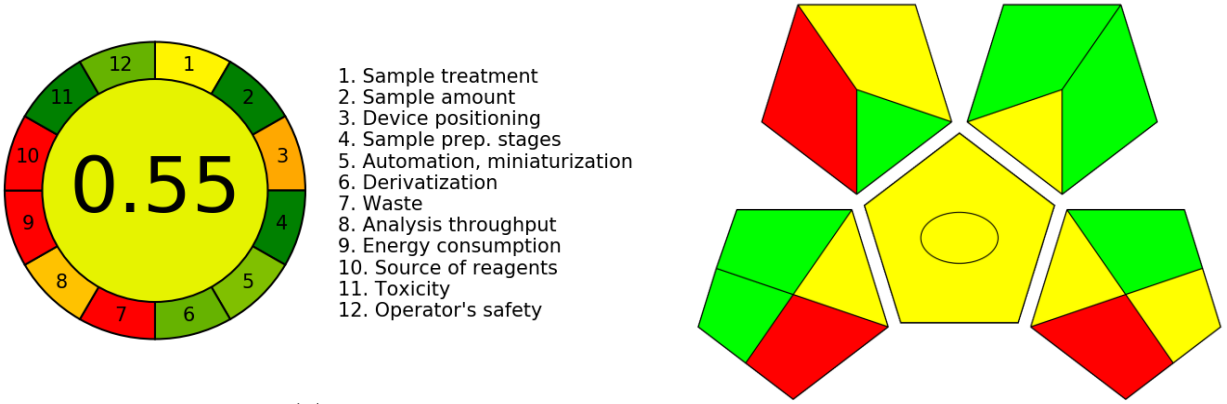


Fig.10. Calibration curve of Lenvatinib



(a) **(b)**

Fig. 11: Results of (a) AGREE and (b) GAPI analysis for the developed HPTLC method