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RESEARCH ARTICLE

Development and Validation of an RP-HPLC Method for Gefitinib in Bulk and Pharmaceutical Dosage Forms Using Central-Composite Design

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Abstract

Objective: Development of an accurate, sensitive, precise, robust, economical and quick isocratic reverse-phase high-performance liquid chromatography (RP-HPLC) method complying quality by design (QbD) and authorize as per ICH guidelines for the quantitative evaluation of Gefitinib in bulk and pharmaceutical dosage form. Method: The simultaneous estimation of the Gefitinib with Sorafenib as an internal standard in bulk and pharmaceutical dosage forms by the assistance of chemo metrics, multi criteria decision-making approach. The separation was accomplished by utilizing/with Phenomenex Enable C_{18} column (15x4.6mm, 5µm particle size); and PDA-UV detection set at 250nm was developed and validated Gefitinib in pure form as well as pharmaceutical formulation, optimized by utilizing/with Derringer's desirability functions. The mobile phase used for the separation was a mixture of Acetonitrile and Phosphate buffer (30:70 %v/v) and the pH 3, which was adjusted with ortho-phosphoric acid, the flow rate was 1.6 ml/min. Result: Newly developed method resulted an elution time of the drug at 3.264 min. The regression coefficients (R^2) were observed to be 0.999 for all models. The detection (LOD) and quantification limits were 10.47 mg/ml, and 31.74 mg/ml, respectively. The relative standard deviation was calculated as 0.4412%. Conclusion: Method developed and validated by determining its precision; accuracy; and system stability. The results of the study demonstrated that the planned RP-HPLC method is simple; rapid; precise; and accurate, which is helpful for the analysis of Gefitinib in bulk as well as pharmaceutical dosage forms.

Keywords: *Chemo metrics, RP-HPLC, RSM, Tyrosine kinase inhibitors, Gefitinib, Sorafenib.*

Introduction

Gefitinib is an anti-neoplastic agent used in the treatment of carcinoma (breast, lung, and pancreatic) [1]. Gefitinib inhibits epidermal growth factor receptor (EGFR) tyrosine kinase by binding to ATP-binding site of the enzyme-like that of Erlotinib. The molecular formula Gefitinib is $C_{22}H_{24}ClFN_4O_3$, molecular weight is 446.902 g/mol, and the chemical name is N-93-chloro-4-fluorophenyl) 7 methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine(Fig.1). It is generally available as white or yellow coloured powder; soluble in organic solvents [12].

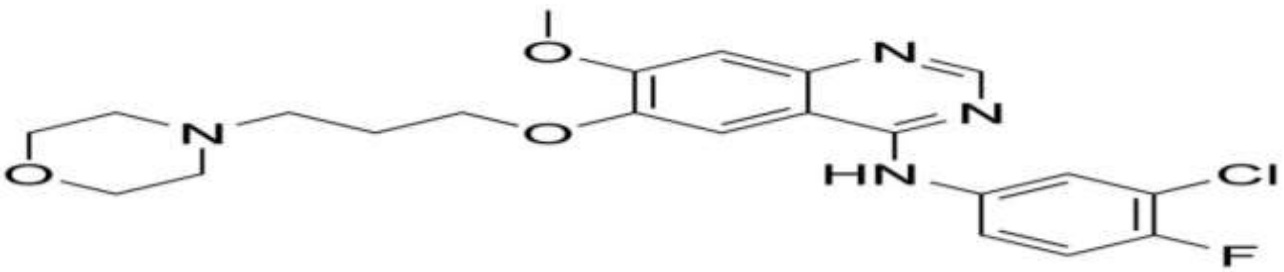

Figure 1: Structure of Gefitinib

Gefitinib is the specific inhibitor of epidermal growth factor receptor's tyrosine kinase domain [2, 3, & 4]. It belongs to a subfamily of Erb-B1; Erb-B2; Erb-B3; and Erb-B4 [2, 3, & 4]. An unseemly activity of the intracellular signaling prompts the uncontrolled cell division and leads to carcinoma [5].

Many cells, including malignant growth, have EGF receptors on their surfaces. The EGF is a protein that is expressed normally by the cells and that advances the development and multiplication of cells. At the point when EGF connects to EGFRs, it leads to the activation of tyrosine kinase to become active inside the cells [6].

Tyrosine kinase triggers chemical processes that reason the cells and including tumor cells, develop, multiply, and spread. Gefitinib binds to EGFRs, and this way blocks the connection of EGF and the initiation of tyrosine kinase [7 & 8]. The mechanism by which arresting the growing and multiplying is overall different forms of the mechanisms of chemotherapy as well as hormonal treatment.

Response surface methodology (RSM) is a statistically designed experimental tool where enormous quantities of variables are at the same time considered [9 & 10]. The multivariate methodology has favorable circumstances included a decrease in the number of trial runs, improves statistical explanation outcomes, and indicates whether the parameters interact or not [11].

CCD is known as a multivariate investigational design which is utilized to optimize the chromatographic parameters and their associated impacts and quadratic effects on mobile phase composition, pH, and flow rate on the peak area [12]. The approval of the proposed method is done by the ICH guideline ICH Q2 (R1) [13].

Analytical Quality by Design (AQbD) is a precise way to deal with the development that begins with a predefined objective and emphasizes the method of comprehension and control dependent on sound science and quality hazard the executives.

AQbD plays a vital role in developing a robust method as an early risk assessment and distinguishes the basic analytical parameters and to concentrate on these factors in method development [14, 15, & 16]. Henceforth, the present investigation is aimed to develop a new rapid, sensitive, and validated RP-HPLC method for the examination of Gefitinib in bulk as well as pharmaceutical dosage forms.

Materials and Methods

Chemicals and Reagents

Gefitinib (GEF) references sample was a gift sample from Spectrum Labs Ltd, Hyderabad, India.

The chemicals and reagents incorporate Acetonitrile (Sd Fine-Chem Ltd), Pottasium dihydrogen phosphate Buffer (Sd Fine-Chem Ltd), ortho-phosphoric acid (AR grade) and Milli Q Water (Merk) were of HPLC grade. Membrane filter (Ultipor ®N_{66} Nylon 6,6 membrane, 0.45µm, was obtained from PALL Life Sciences). Gefitinib is commercially available as Geftinat marketed by Natco Rx India with a labeled claim of 250mg per tablet.

Instrumentation

The HPLC analysis was carried out on a Shimadzu HPLC system (Tokyo, Japan) with two LC-20AD separation modules, and SPD-m20A PDA detector, a Rheodyne injector (model 7125, USA). The chromatographic and integrated data were recorded using LC solution data acquisition software. An electronic weighing balance (0.1 mg) sensitivity, pH meter (DELUX), and Ultra-Sonicator (Sonica). Absorbance spectra were recorded using a UV-VIS spectrophotometer (Systronics, Japan) utilizing a quartz cell of 1 cm of path length.

Statistical Software

Experimental design, data analysis, and desirability function calculation were performed by utilizing version 11 of Design-Expert® software. The predictions of this study were achieved by using Micro-soft Excel 2007 software.

Preparation of Buffer

0.680gm of Phosphate Buffer (Pottasium dihydrogen orthophosphate) was weighed and transferred into a 500ml volumetric flask and dilute with 400ml of Milli Q water (HPLC grade), mix well and makeup to the final volume by using solvent. Adjusting the pH 5 (±0.5) by using ortho-phosphoric acid. The solution was filtered using a membrane filter.

Preparation of Standard Solution

Stock standard solution of GEF was prepared in the mobile phase. The stock solution was stored at 4^0C ± 0.05 and protected from light. Working standard solution of GEF was freshly prepared by diluting the stock solution with mobile phase before the

analysis. Calibration curves revealing peak area ratios of GEF were prepared at the range of 2, 4, 6, 8, and 10µg/ml.

Sample Preparation

Ten tablets (Geftinat) were weighed and then powdered by using mortar and pistil, which is equivalent to 100mg of GEF into a 10ml of volumetric flask and added 8ml of mobile phase and sonicated for 30min with occasional shaking. The volume made up to 10ml by using mobile phase and mixed. Filter the solution through the 0.45µm membrane filter. Transferred 1ml of above solution into a 10ml volumetric flask and makeup to final volume.

Preparation of Internal Standard Solution

Internal standard (Sorafenib) was taken for accomplishing chromatographic differentiation for separation at particular retention time for working standard GEF. For this, 10mg of Sorafanib dissolved in 2ml of mobile phase and volume was adjusted to 10ml with mobile phase to form the internal standard solution. Further, the solution was suitably diluted to 5 mg/ml concentration, and precisely, a 1ml solution was added to each dilution of the standard solution of GEF [17].

Selection of Detection Wavelength

Gefitinib and Sorafenib showed significant absorbance at 250nm using a PDA detector.

Chromatographic Conditions

The composition of Mobile phase was Acetonitrile: Phosphate buffer (pH 3) at the ratio of 30:70 %v/v was used in isocratic mode at a flow rate of 1.6 ml/min. The mobile phase was filtered through the membrane filter and sonicated for 20 min before use. Injection volume was 20µl, and detection was performed at 250nm at ambient temperature.

Optimization of RP-HPLC-PDA Method

Initially, the trial and error method was applied to gain knowledge about the method execution and recognition of different essential independent variables and its effect on dependent variables. The Central-Composite design (CCD) with response surface was utilized for the optimization of experimental conditions of the method. In the present study, the experiments were planned and performed by the CCD [18].

In the proposed study, 20 trial runs were conducted and analyzed to obtain the results of retention time; capacity factor; resolution factor; and separation factors as per the CCD. Further investigation was performed using response surface methodology (RSM) to evaluate the relationship between the dependent and independent variables using obtained data (Tab. 1).

Method Validation

The optimized chromatographic method was totally validated as per ICH guidelines and Q2B. The calibration curves were tested using one-way ANOVA at a 5% significance level [19].

Table 1: Experimental design and results of a rotatable central composite design

Run	Factor A (ACN %v/v)	Factor B (pH)	Factor C (Flow rate)	Response 1 (tR)	Response 2 (K_1)	Response 3 (Rs)	Response 4 (α)
1	30	6	0.8	2.228	0.412	0.344	1.191
2	30	3	0.8	3.481	1.226	0.656	1.598
3	50	4.5	1.2	7.459	1.078	12.314	4.73
4	16.3641	4.5	1.2	1.894	3.263	0	0
5	50	4.5	1.2	7.478	1.046	12.166	4.83
6	70	3	0.8	3.543	0.934	6.111	0
7	50	4.5	1.2	7.417	1.064	12.306	4.774
8	70	6	0.8	4.081	1.1	1.649	1.202
9	30	3	1.6	3.599	0.504	2.866	4.201
10	50	4.5	1.2	7.408	1.014	11.988	4.732
11	50	7.02269	1.2	9.008	4.537	7.669	1.819
12	50	4.5	1.87272	4.836	1.031	11.415	4.993
13	30	6	1.6	2.172	2.879	0.319	0
14	70	6	1.6	2.052	1.994	1.351	1.214

15	70	3	1.6	1.786	0.932	5.262	2.958
16	50	4.5	1.2	7.458	1.076	12.332	4.728
17	83.6359	4.5	1.2	2.331	0.534	1.783	1.398
18	50	1.97731	1.2	2.476	0.33	0.925	2.856
19	50	4.5	0.527283	5.662	0.045	4.906	0
20	50	4.5	1.2	7.394	1.09	12.286	4.724

The model was also validated by ANOVA using design expert software, and the results are presented in Table 2. Based on *p*-value, a quadratic model was chosen for responses, for example, retention time, capacity, resolution, and separation factors of GEF. The significant effects observed with *p*-value under 0.05, whereas the low standard deviation (%CV), and adjusted *R*-square value showed an excellent relationship between the trail data and those of the fitted model. The predicted *R*-square value was low concordance with the adjusted *R*-square value for all responses [20].

Table 2: Response models[a] and statistical parameters obtained from ANOVA for CCD

Responses	Regression model	Adjusted R^2	Model p-value	% C.V	Adequated precision
tR	+7.48+0.0525*A+0.6670*B-0.3744*C+0.4355*AB-0.4810*AC-0.0557*BC-2.16A^2-0.8761B^2-1.05C^2	0.6466	<0.0106	31.23	5.9845
K_1	+1.07-0.3405*A+0.7223*B+0.3145*C-0.0416*AB-0.1066*AC+0.5106*BC+0.2095*A^2+0.3987*B^2-0.2715*C^2	0.4915	<0.0490	59.57	6.6783
Rs	+12.29+0.9656*A+0.0081*B+0.8776*C-0.6893*AB-0.4165*AC-0.2105*BC-4.36*A^2-3.16*B^2-1.79*C^2	0.7634	<0.0017	41.63	7.9914
S	+4.76+0.0538*A-0.5048*B+0.9283*C+0.5082*AB+0.1947*AC-0.8425*BC-1.46*A^2-0.8776*B^2-0.8320*C^2	0.8793	<0.0001	26.07	10.6271

The perturbation, plots are presented for predicted models in order to gain an effect of an independent factor on a specific response with all other factor held constant at a reference point. A steepest slope or curvature indicates the sensitiveness of the response to an exact factor (Fig.2). The pH (factor B) had the most vital effect on a retention time tR_2, followed by factor A and C (Fig 2a). The factors pH and flow rate (B and C) had a significant effect on K_1 followed by factor A (Fig 2b). The flow rate (factor C) had the most critical effect on an $Rs_{(1, 2)}$ followed by factor A and B (Fig 2c). The factors pH and flow rate (B and C) had a significant effect on $S_{(1,2)}$ followed by factor A (Fig 2d).

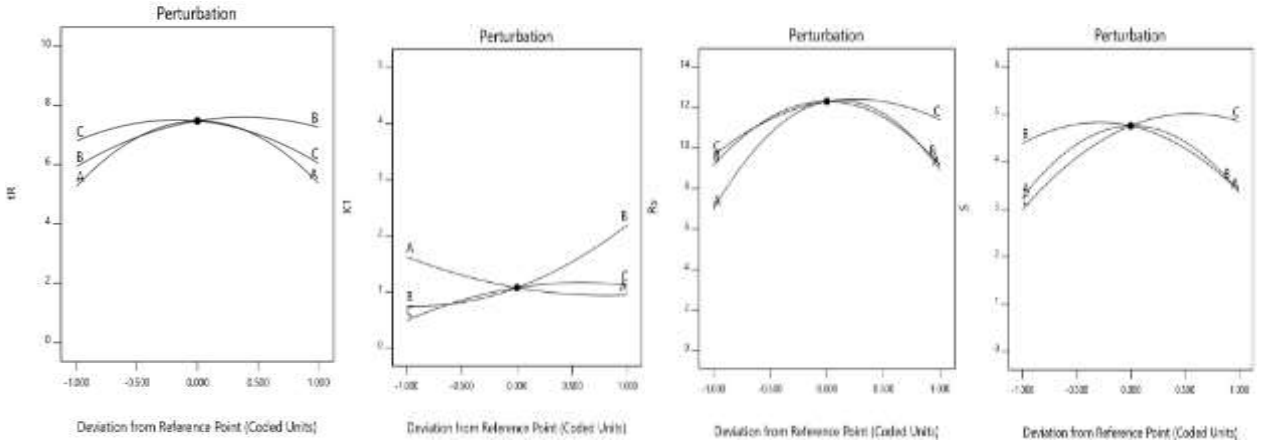

Figure 2(a) **Figure 2(b)** **Figure 2(c)** **Figure 2(d)**

Fig. 2: Perturbation plots showing the effect of each independent variables on (a) tR (b) K_1 (c) Rs, (d) S, where A is Acetonitrile concentration, B is the pH (buffer), C is the Flow rate

Response surfaces plots for K_1, $Rs_{(1,2)}$ and $S_{(1,2)}$ and tR_2 are illustrated in Fig. 3 (% ACN concentration was plotted against the pH. Flow rate held at constant at the center value). Analysis of perturbation plots and response plots of optimization models revealed that the factor A and B had the major effect on separation of the analytes, whereas the factor C, i.e. the flow rate, is of less significance.

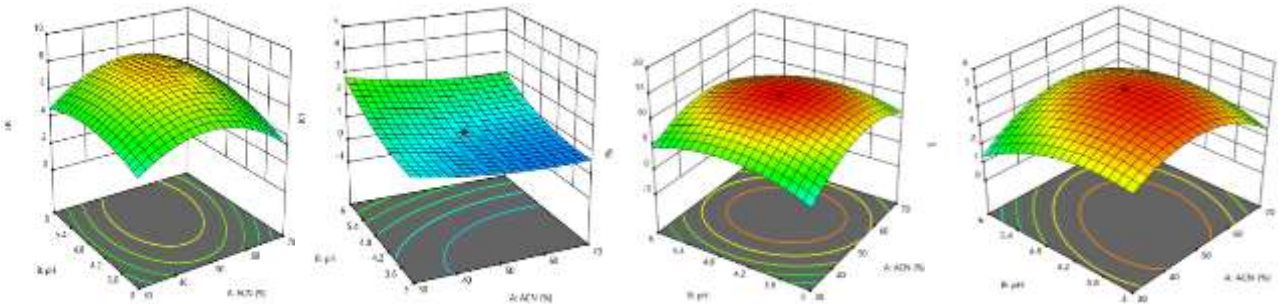

Figure 3(a) **Figure 3(b)** **Figure 3(c)** **Figure 3(d)**

Fig. 3: Response surfaces related to Acetonitrile (A), pH (B) and Flow rate (C): (a) retention time of the last peak (tR2), (b) capacity factor first peak (K_1), (c) resolution factor (Rs), and (d) separation factor (S)

Validation

The optimized chromatographic conditions were magnified applied to the validation of contention for the system suitability, linearity, accuracy, precision, sensitivity, selectivity, and robustness. The optimized RP-HPLC method was validated as per the guidelines of the (ICH) Q2 (R1) for various parameters [21].

System Suitability

System suitability tests are referred as the method of assessing chromatographic system prior to the sample analysis can start. The system suitability testing was evaluated, and percent relative standard deviation was commencing less than 2% confine demonstrating appropriateness of approach development.

Linearity

Linearity concentration ranges from 2 to 10 µg/ml of GEF was prepared. The calibration graph was plotted by taking the peak area versus concentration. The correlation coefficient, intercept, slope, and linear regression analysis were done [22]. The results are shown in Tab.3 & Fig. 4.

Sensitivity

With the formula $3.3\,\sigma/S$ and $10\,\sigma/S$, limit of detection (LOD) and limit of quantitation (LOQ) were calculated respectively, where "σ" is the standard deviation of the response (y-intercept) and "S" is the slope of the linearity plot [23].

Specificity

It was calculated by comparing the test results obtained from the analysis of the sample solution containing excipient through the results obtained from the standard drug [24].

Precision

It was calculated by different concentrations such as 2, 4, and 6 µg/ml of GEF samples analyzed triplicates [25] and the results are shown in Tab. 4.

Accuracy

It is the proximity in the accord between the accepted true value and the actual results obtained. Accuracy studies are generally evaluated by determining the sample of the analyte into the mixture of the samples to be analyzed. For accuracy studies, three different concentrations of solutions such as 8, 10, and 12 µg/ml were used. After injecting each concentration, mean, % recovery was calculated [26] and the results are shown in Tab.5.

Results and Discussion

Linearity

The results of method validation for linearity revealed that the above assay was linear over the concentration range between 2-10 µg/ml for GEF and the regression coefficients was 0.999 for GEF, and the equations used for this analysis is Y = 0.139x+0.036 for GEF (Tab. 3).

Table 3: Linearity values for Gefitinib

Conc	AVG Area
2	0.309
4	0.6034
6	0.8844
8	1.1301
10	1.4401

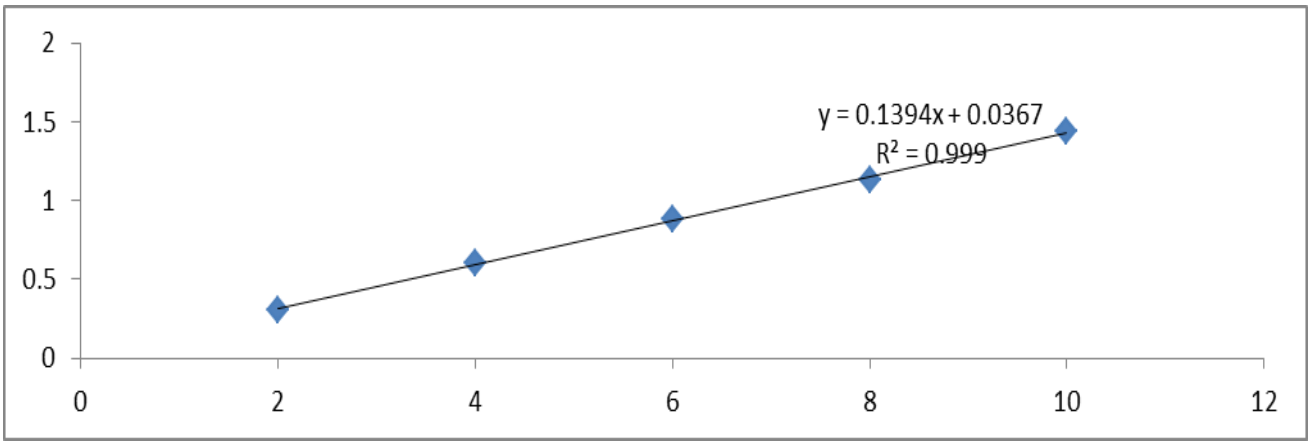

Fig.4: Calibration curve for Gefitinib

Precision

Precision was evaluated by the estimation of intraday precision by assay of three different concentrations of GEF such as 2, 4, and 6µg/ml at various time intervals. The RSD (%) for intraday precision for GEF were in the range of 0.32-0.68%, which was within the acceptable limit. The developed method exhibited good precision for the drug.

Table.4: Precision values for Gefitinib

Con	Drug area	Internal Standard	Drug/IS	AVG	Sdv	%Rsd
2	89616	291937	0.30697	0.309076	0.002106	0.681526
	90085	291468	0.309073			
	90554	290999	0.311183			
4	177001	292077	0.606008	0.603436	0.002571	0.42599
	176532	292546	0.603433			
	176063	293015	0.600867			
6	270852	306224	0.88449	0.884493	0.002886	0.326313
	270383	306693	0.881608			
	271321	305755	0.88738			

Accuracy

The accuracy of the samples has been calculated from the measured concentrations of samples extrapolated from calibration curve particularly generated for the determination of the accuracy and validation of the method. The results of accuracy studies for Gefitinib and Sorafenib are summarized in Tab. 5. It is clearly evident from the conclusion that % RSD of the compound less than 2; hence, the method can be considered as accurate.

Table 5: Accuracy study for Gefitinib

Percentage	Gefitinib	IS	Drug/IS	AVG	Sdv	%RSD	% Recovery
80%	839413	330917	2.536627	2.536699	0.000472	0.018602	99.92
	839682	330948	2.537202				
	839451	330979	2.536267				
100%	939947	332017	2.831021	2.811189	0.017283	0.614784	99.82
	930916	332548	2.799343				
	930885	332079	2.803203				
120%	998970	322579	3.096823	3.087708	0.01586	0.513643	99.77
	998901	322548	3.096907				
	989932	322517	3.069395				

Specificity and Selectivity

Specificity and selectivity were studied to find out the presence of interfering components in the working solution of GEF. The results indicate that the retention time of GEF is at 3.264 min. There is no variation in the retention time of the compound as compared to the standard drug. They are free from interference from formulation excipients as well as solvent from each other. This indicates that the method is a selective and specific for determination GEF.

Limit of Detection and Quantification

The LOD and LOQ of GEF were calculated as 10.46 and 31.74 µg/ ml, respectively. The values indicated that the method is susceptible to quantify for the drug.

Application of the Newly Developed Method

The developed RP-HPLC method is sensitive and specific for the quantitative assurance of GEF. The technique was approved for various parameters and consequently, has been applied for the estimation of the drug in pharmaceutical dosage forms, such as tablets. Each tablet was analyzed in triplicate after extracting the drug, as mentioned in the sample preparation of the experimental section. The recovered amount of GEF was 100.5% (Tab. 6). From the study is observed that none of the ingredients of tablet interfered with the analyte peak. The method was validated for precision, accuracy, linearity, system suitability, sensitivity, and robustness.

The study is proved that the method is a convenient and effective for quality control as well as simultaneous routine analysis of GEF in pharmaceutical dosage forms. The measured signal was shown to be precise, accurate, and linear over the concentration ranges tested with a retention time of 3.264 min. and made the method economical due to lower solvent consumption.

The % RSD for all parameters was observed under 2, which shows the validity of technique and assay results obtained by this method are in reasonable agreement. Chromatogram of GEF is shown in Fig. 5.

Table 6: Assay for Geftinat in tablet formulation

Cons	Gefi	IS	Drug/IS	OBT AMT	Mean	SD	%RSD	Labeled amt	% Recovery
10	449639	313917	1.43235	10.043	10.05	0.007	0.069652	250 mg	100.5
	448208	312548	1.434045	10.057					
	450077	313979	1.433462	10.05					

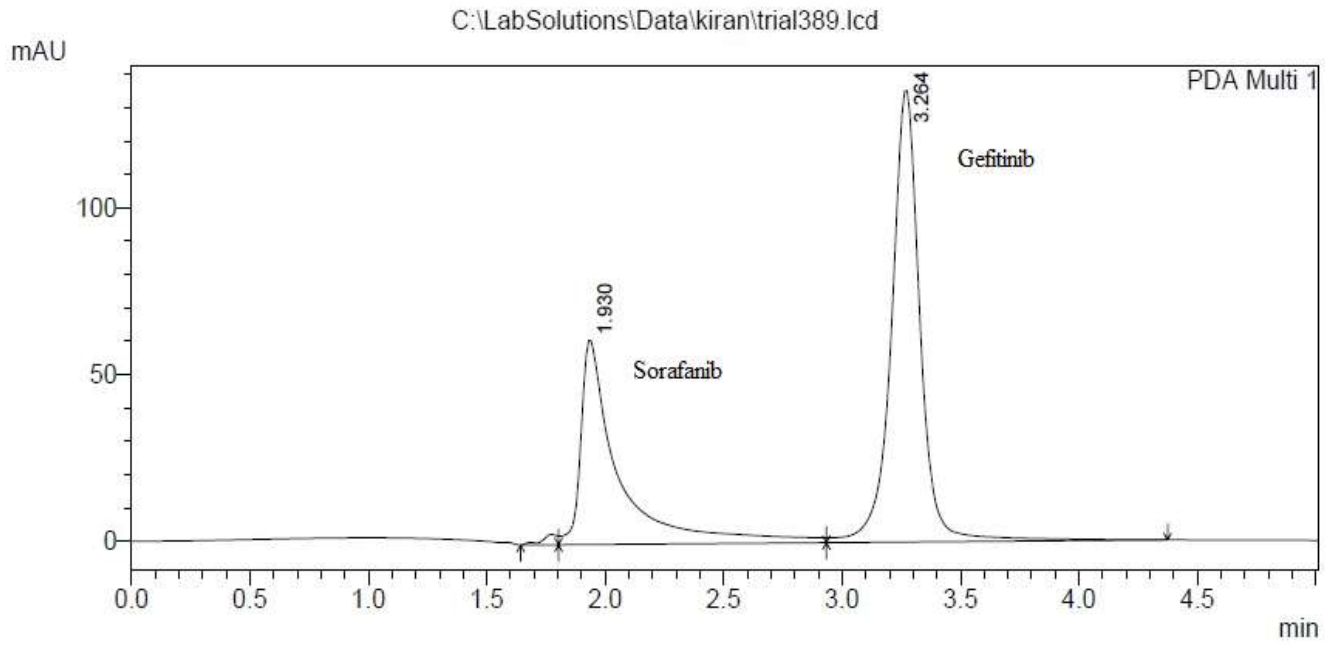

Fig.5: Optimized chromatogram of Gefitinib

Conclusion

An efficient isocratic reversed-phase high-performance liquid chromatography method was developed, which was optimized and validated for the simultaneous estimation of TKI's namely Gefitinib in bulk and pharmaceutical formulations using Chemometrics Multi-Criteria Decision Making-Approach.

This process reduces overall assay development time and gives essential information, for example, the sensitivity of various chromatographic factors and their interaction effects on the attributes of separation. Time of analysis; resolution; and quality of the peaks were simultaneously optimized by applying useful tools of Chemometrics: Central Composite Design and Derringer's desirability function. The validation study supported the selection of the assay conditions by confirming that the assay was specific, linear, accurate, precise, and robust. Therefore, the developed RP-HPLC method can be used for the quality control analysis of Gefitinib.

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