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STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE DETERMINATION OF GEFITINIB IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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Abstract:

A simple, reproducible, and efficient reverse phase high performance liquid chromatographic method was developed for determination of Gefitinib in pure form and marketed pharmaceutical dosage forms. A column having Kromasil C_{18}, 250 mm x 4.6 mm i.d.5µm particle size in isocratic mode with mobile phase containing Methanol: Acetonitrile (65:35 v/v) was used. The flow rate was 1.0 ml/min, and the effluent was monitored at 245 nm. The retention time (min) and linearity range (ppm) for Gefitinib were (2.800 min) and (6-14µg/ml) respectively. The method has been validated for linearity, accuracy, and precision, robustness, and limit of detection, and limit of quantitation. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.507µg/ml and 1.539µg/ml for Gefitinib respectively. The developed method was found to be accurate, precise and selective for determination of Gefitinib in bulk and marketed pharmaceutical dosage form.

Key Words: *Gefitinib, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.*

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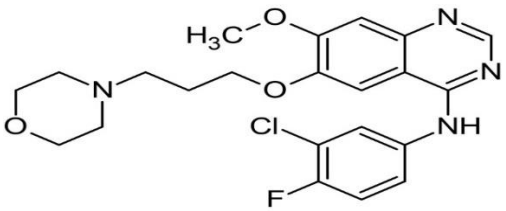
INTRODUCTION:

Gefitinib is a member of the class of quinazolines that is quinazoline which is substituted by a (3-chloro-4-fluorophenyl) nitrilo group, 3-(morpholin-4-yl) propoxy group and a methoxy group at positions 4, 6 and 7, respectively. An EGFR kinase inhibitor used for the treatment of non-small cell lung cancer. It has a role as an epidermal growth factor receptor antagonist and an antineoplastic agent. It is aromatic ether, a member of monochlorobenzenes, a member of monofluorobenzenes, a secondary amino compound, a tertiary amino compound, a member of quinazolines and a member of morpholines.

Synonyms: Gefitinib, 184475-35-2, Iressa, ZD1839, Irressat, Gefitinibum, CCRIS 9011, UNII-S65743JHBS, Gefitinib (GMP), NSC-759856.

Chemical Structure:



IUPAC Name: N-(3-chloro-4-fluoro phenyl)-7-methoxy-6-(3-morpholin-4-yl propoxy) quinazolin-4-amine

Molecular Formula: $C_{22}H_{24}ClFN_4O_3$

AIM & OBJECTIVE

Review of literature for Gefitinib gave information regarding its physical and chemical properties, various analytical methods that were conducted alone and in combination with other drugs.

Literature survey reveals that certain chromatographic methods were reported for simultaneous estimation of Gefitinib and single method is available for such estimation by RP-HPLC.

Validation is a necessary and important step in both framing and documenting the capabilities of the developed method.

The utility of the developed method to determine the content of drug in commercial formulation was also demonstrated. Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredient.

The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness and robustness, limit of detection and limit of quantification. This method provides means to quantify the component. This proposed method was suitable for the analysis of pharmaceutical dosage forms.

The Primary Objective of Proposed Work is:

To develop new simple, sensitive, accurate and economical analytical method for the estimation of Gefitinib in bulk and marketed pharmaceutical dosage form.

To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the Gefitinib in bulk and marketed pharmaceutical dosage form.

PLAN OF WORK

- To develop a new analytical method for the estimation of Gefitinib by RP-HPLC in bulk and marketed pharmaceutical dosage form.

MATERIALS AND METHODS:

Equipments:

Table-1: List of Equipments

S.No.	Instruments/ Equipments /Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyser (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Chemicals and Reagents:

Table-2: List of Chemicals used

S.No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Working Standard: Working Standard of Gefitinib: 10ppm

Solubility Study:

Table-3: Solubility Results

Solvents	Solubility
Methanol	Soluble
Ethanol	Soluble
Acetonitrile	Soluble
DMSO	Freely Soluble
Dimethyl Formamide	Soluble
Dichloromethane	Soluble
Water	Soluble

METHODOLOGY:

METHOD DEVELOPMENT

Standard Preparation for UV-Spectrophotometer Analysis:

The standard stock solutions – 10 mg of Gefitinib standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Gefitinib, so that the same wave number can be utilized in HPLC UV detector for estimating the Gefitinib.

DIFFERENT TRIALS FOR CHROMATOGRAPHIC CONDITIONS

Table-4: Different Chromatographic Conditions

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Develosil C_{18}, 250 mm x 4.6 mm and 5µm Column	Acetonitrile : Water = 65 : 35	0.8 ml/min	245nm	Base line noise is high	Method rejected
Develosil C_{18}, 250 mm x 4.6 mm and 5µm Column	Acetonitrile : Water = 55 : 45	0.8ml/min	245nm	Tailing is more	Method rejected
Zorbax C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Water = 30 : 70	0.9 ml/min	245nm	Extra peaks	Method rejected
Phenomenex C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Water = 60 : 40	1.0 ml/min	245nm	Good sharp peak	Method accepted
Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 45 : 55	1.0 ml/min	245nm	Improper peak separation	Method rejected
Kromasil C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 35 : 65	1.0 ml/min	245nm	Tailing peaks	Method rejected

Kromasil C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 70 : 30	1.0 ml/min	245nm	Tailing peaks	Method rejected
Kromasil C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 65 : 35	1.0 ml/min	245nm	Proper Peak	Method Accepted

Optimized Chromatographic Conditions:

Column : Kromasil C_{18}, 250 mm x 4.6 mm i.d.5µm particle size
Mobile Phase : Methanol: Acetonitrile (65: 35% v/v)
Flow Rate : 1.0ml/minute
Wave length : 245 nm
Injection volume : 10 µl
Run time : 7 minutes
Column temperature : Ambient

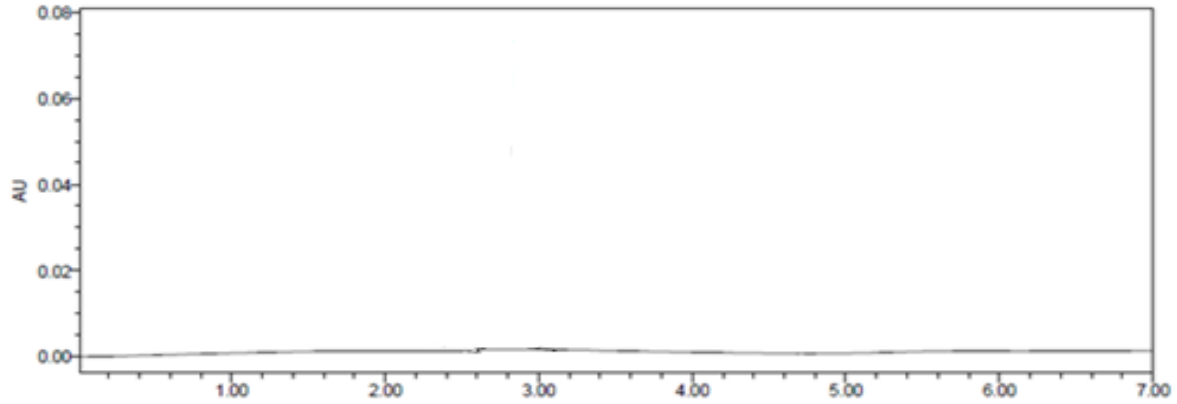


Fig-: Chromatogram for Blank Solution

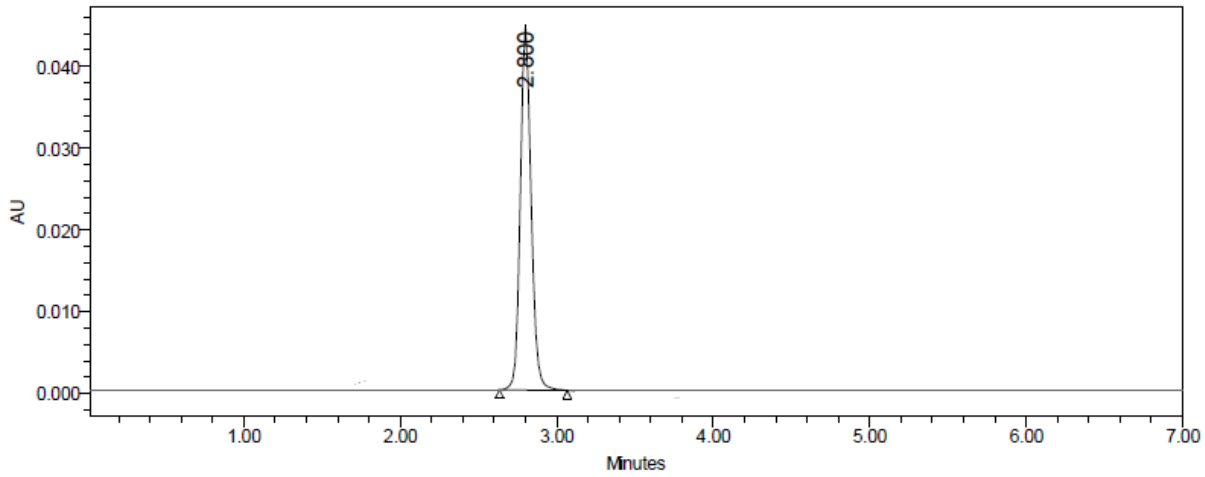


Fig-: Optimized Chromatogram for Gefitinib

Table 5-: Results of Optimized Chromatogram

S.No.	Peak Name	Rt	Peak Area	Height	USP Tailing	USP Plate Count
1	Gefitinib	2.800	716358	47457	1.38	5879

Preparation of Mobile Phase:
The mobile phase used in this analysis containing of a mixture of Methanol and Acetonitrile in the ratio of 65:35% v/v was prepared in the volume of 1000ml in which 350ml of Acetonitrile was mixed with 650ml of Methanol respectively.

Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Gefitinib working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette out 0.1ml of Gefitinib from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
Result: The selected and optimized mobile phase was Methanol: Acetonitrile (65: 35% v/v) and conditions optimized were flow rate (1.0 ml/minute), wavelength (245nm), Run time was 07 mins. Here the peak has shown better theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

METHOD VALIDATION
System Suitability Test
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 analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-6.1.

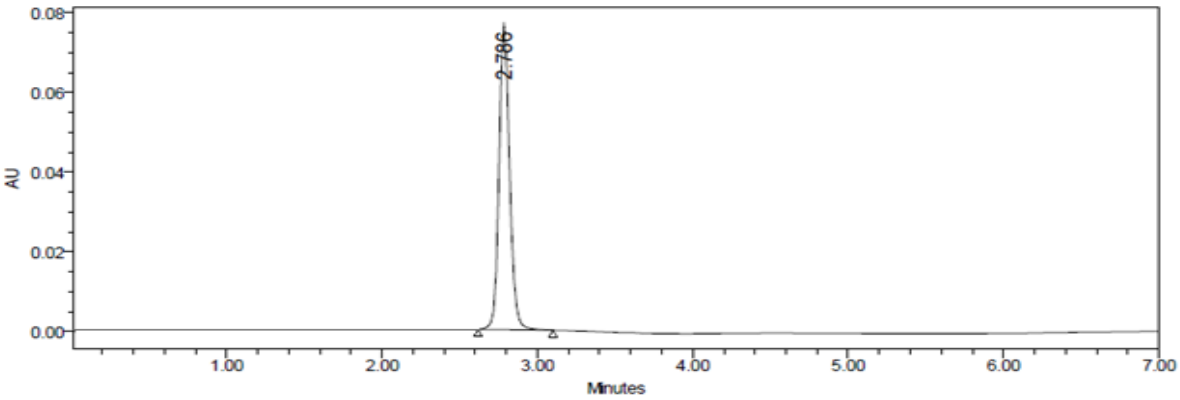


Fig-: Chromatogram for System Suitability Injection-1

Table-6: Data of System Suitability Test

S.No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			**716207.8**		**5821.5**	**1.36**
S.D			**1347.976**			
%RSD			**0.18821**			

Acceptance Criteria and Result:

S.No.	Parameter	Limit	Result
1	Tailing factor	T ≤ 2	1.36
2	Theoretical plate	N > 2000	5821.5

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 Gefitinib were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 74143x + 7294.9. The results were shown in table-6.1.

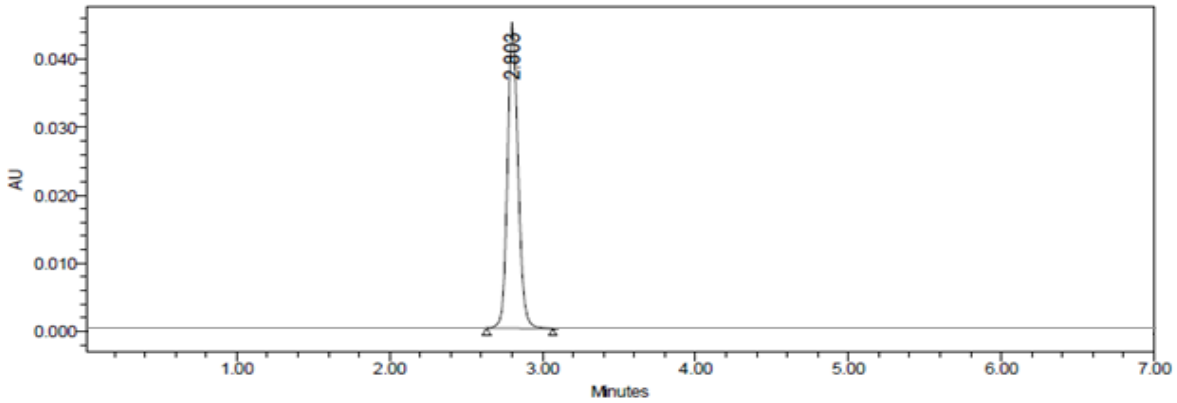


Fig-: Chromatogram for Accuracy-80%, Replicate -1

Table-7: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Mean % Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80 %	8	8.013	601425	100.162	Mean = 100.195%	% Mean Recovery = 100.364%
S_2 : 80 %	8	8.012	601396	100.150		
S_3 : 80 %	8	8.022	602123	100.275		
S_4 : 100 %	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100 %	10	10.039	751642	100.390		
S_6 : 100 %	10	10.030	750969	100.300		
S_7 : 120 %	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120 %	12	12.073	902431	100.608		
S_9 : 120 %	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug are 99.686 which is within the range of 98-102%.

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 Gefitinib (API). The percent relative standard deviation was calculated for Gefitinib.

Table-8: Results of Repeatability readings

HPLC Injection Replicates of Gefitinib	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		**716495**	**5930.333**	**1.37**
Standard Deviation		**1268.126**		
% RSD		**0.17699**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines.

Table 9-: Peak results for Intra-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate	Injection
1	Gefitinib	2.784	716587	48685	1.38	5954	1
2	Gefitinib	2.768	717845	48698	1.39	5935	2
3	Gefitinib	2.786	716857	46989	1.36	5798	3
4	**Average**		**717096.3**	**48124**	**1.376**	**5895.66**	
5	**S.D**		**662.2698**				
6	**% RSD**		**0.092354**				

Table-10: Peak results for Inter-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate	Injection
1	Gefitinib	2.780	716987	49867	1.34	5968	1
2	Gefitinib	2.794	718695	48574	1.33	5998	2
3	Gefitinib	2.775	718542	48569	1.39	5859	3
4	**Average**		**718074.7**	**49003.33**	**1.353333**	**5941.667**	
5	**S.D**		**945.0483**				
6	**% RSD**		**0.131609**				

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Gefitinib revealed that the proposed method is precise.

Linearity & Range:

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

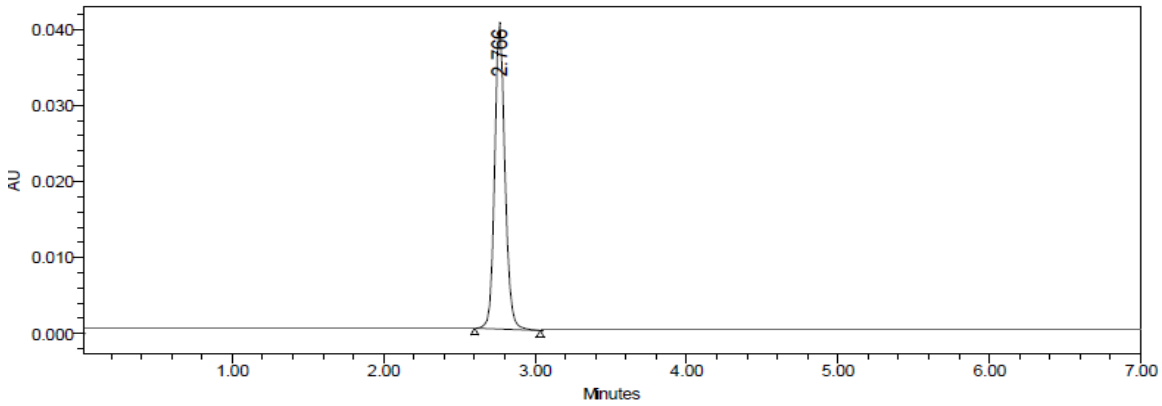


Fig-: Chromatogram for linearity-1

Table-11: Linearity Concentrations of Gefitinib

S.No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

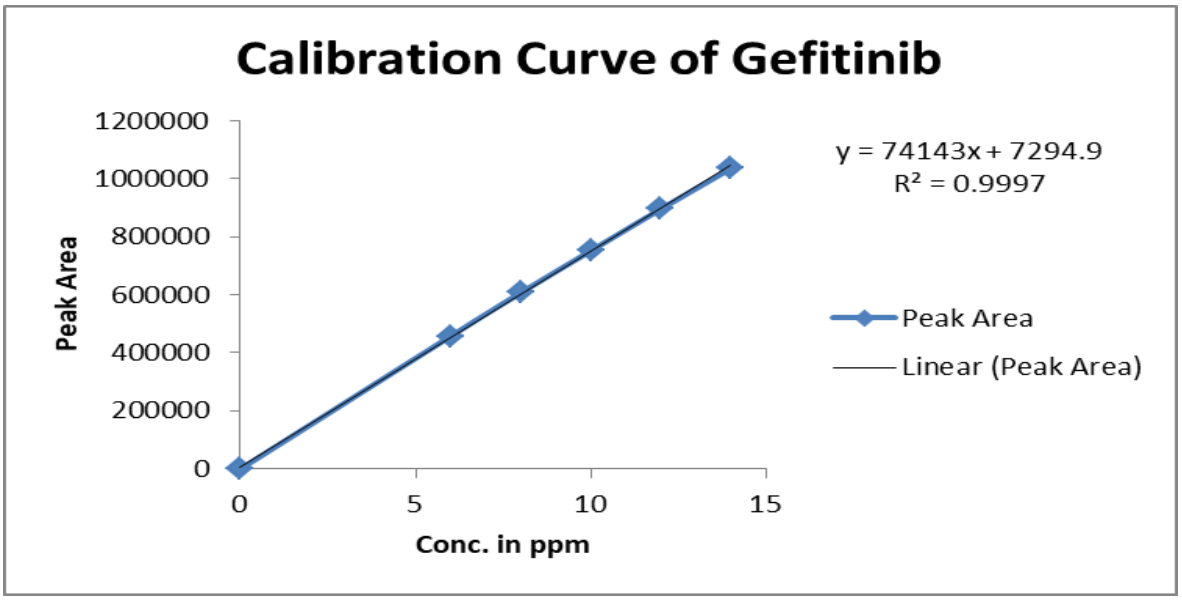


Fig-: Calibration Curve of Gefitinib

Observation: We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Gefitinib with correlation coefficient (R^2) of 0.9997. A typical calibration curve has the regression equation of y = 74143x + 7294.9 for Gefitinib.

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 245nm (±2nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness of the method are also in favour of (Table-, % RSD <2%) the developed RP-HPLC method for the analysis of Gefitinib (API).

Table-12: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39
More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240nm)	6247	1.47

LOD & LOQ: The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

Estimation of Gefitinib in Pharmaceutical TABLET Dosage Form

Gefitero Tablet 250mg

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 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.
A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-6.26.

ASSAY

$$\% \text{ Assay} = AT/AS \times WS/DS \times DT/WT \times P/100 \times AW/LC \times 100$$

Where:
AT = Peak Area of Gefitinib obtained with test preparation
AS = Peak Area of Gefitinib 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 obtained are tabulated below:

Table-13: Assay of Gefitinib Tablets

Brand name of Tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Gefitero Tablets	250	249.563 (± 0.536)	99.478% (± 0.368)

Result & Discussion: The %Purity of Gefitero Tablets containing Gefitinib was found to be 99.478% (± 0.368).

STABILITY STUDIES

Results of degradation studies: The results of the stress studies indicated the specificity of the method that has been developed. Gefitinib was stable in Acidic, Photolytic & Oxidative conditions. The result of forced degradation studies are given in the following table-7.6.

Table-14: Results of forced degradation studies of Gefitinib

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.635	12.365	100
Basic Hydrolysis (0.1N NaOH)	24Hrs.	94.154	5.846	100
Thermal Degradation (60^0C)	24Hrs.	90.311	9.689	100
UV (254nm)	24Hrs.	91.205	8.795	100
3% Hydrogen peroxide	24Hrs.	89.346	10.654	100

SUMMARY

The analytical method was developed by studying different parameters.

First of all, maximum absorbance was found to be at 245nm and the peak purity was excellent.

Injection volume was selected to be 10µl which gave a good peak area.

The column used for study was Kromasil C_{18}, 250 mm x 4.6 mm i.d.5µm particle size because it was giving good peak.

Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time.

Mobile phase is Methanol and Acetonitrile (65:35% v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.

Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery.

Run time was selected to be 7min because analyze gave peak around 2.800min and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.

The analytical method was found linearity over the range of 6-14ppm of the Gefitinib target concentration.

The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION:

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Gefitinib in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps.

Gefitinib is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF).

Methanol and Acetonitrile: Phosphate buffer (65:35% v/v) was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Gefitinib in bulk drug and in pharmaceutical dosage forms.

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