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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AFATINIB IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Objective: A simple, rapid, precise and accurate reversed phase high performance liquid chromatographic method has been developed for the determination of Afatinib.
Methods: In This method uses a X-terra RP-18, 5µm, 4.6 x 250mm analytical column, a mobile phase of acetonitrile: potassium dihydrogen phosphate buffer; pH 3.0 is adjusted with orthophosphoric acid in a ratio of 60:40. The instrumental settings are a flow rate of 1.0 ml/min and PDA detector wavelength at 254 nm.
Results: The retention times for afatinib were 2.83 min. The method was validated and shown to be linear. The linearity range for afatinib was 60-180 µg/ml. The Percentage recoveries for Afatinib are ranged between 98 to 117 microgram/ml . The correlation coefficient of afatinib was 0.999. The relative standard deviation for six replicates is always less than 2%.
Conclusion: The Statistical analysis proves that the method is suitable for routine analysis of Afatinib as a bulk drug and in pharmaceutical formulation.

INTRODUCTION:

Monoclonal antibodies targeting vascular endothelial growth factor (VEGF) or the epidermal growth factor receptor (EGFR) represent well-established treatment options for colorectal cancer. The VEGF antibody bevacizumab enhances the efficacy of oxaliplatin-based and irinotecan-based chemotherapy[1-3], presumably by normalization of the tumour vasculature [4-7] . EGFR antibodies, such as cetuximab and panitumumab, may act on the tumour cells directly, inhibiting cellular growth, differentiation and proliferation, and inducing antibody dependent cell-mediated cytotoxicity [8-9]. Monoclonal antibodies against the EGFR have demonstrated activity as mono-therapy in pretreated patients [10-11]. Cetuximab in combination with chemotherapy also significantly prolongs progression-free survival (PFS) in the first-line treatment of patients with metastatic colorectal cancer compared with chemotherapy alone[12] . Simultaneous targeting of tumour cell receptors may also offer the potential for synthetic lethality of therapeutic agents that have little activity as mono-therapy [13-16], and cross-talk of pathways may involve mechanisms that can be used to overcome resistance[17] .Afatinib is a novel, potent and irreversible inhibitor of both the epidermal growth factor receptor (EGFR) / human epidermal growth factor receptor (HER)1 and HER2 kinases. Preclinical studies demonstrate that afatinib has effective anti-tumour activity in a variety of human xenograft models. Phase I studies have shown that afatinib is well tolerated across a range of different dosing schedules, the maximum tolerated dose (MTD) initially being defined as 70 mg once daily for non-continuous dosing of afatinib. Several

phase II trials yielded promising results in non-small cell lung cancer (NSCLC) patients with EGFR mutations. Phase III trials in NSCLC and breast cancer are currently ongoing[18].

1.1 Chemistry

This compound belongs to the class of organic compounds known as quinazolinamines. These are heterocyclic aromatic compounds containing a quianazoline moiety substituted by one or more amine groups. Afatinibhas the chemical name is given in table 1..Afatinib is an irreversible kinase inhibitor and binds to the kinase domains of EGFR (ErbB1), HER2 (ErbB2), and HER4 (ErbB4) to inhibit tyrosine kinase auto-phosphorylation. This results in a down regulation of ErbB signalling and subsequent inhibition of proliferation of cell lines expressing wild-type EGFR, selected EGFR exon 19 deletion mutations, or exon 21 L858R mutations. It also inhibited in vitro proliferation of cell lines overexpressing HER2. Overall, tumour growth was inhibited.

There are no analytical methods that have been reported for the estimation of Afatinib in bulk and in pharmaceutical formulations at the time of commencement of research work. The present HPLC method deals with new simple, accurate and reliable estimation of Afatinib in sterile powder for injections which have been not reported earlier. Complete validation parameters were not able to be found in any of the methods reported in the past. Studying the stability of a drug and being able to monitor degradation products aids in the clinical treatments/early product development and shelf life for the drug. They are not suitable for regular/routine analysis in pharmaceutical industry where sample size is more and also less sensitive when compared to HPLC methods. Hence, by considering all these factors, the author has made some humble attempts, hoping to fill this gap, and succeeded in developing analytical methods using HPLC methods.

2.1 METHOD DEVELOPMENT

2.1.1 Preparation of Mobile Phase

The contents of the mobile phase were 0.02 M Dipotassium hydrogen orthophosphate in water pH-3.0 with *ortho*-phosphoric acid and acetonitrile in the ratio of 40:60 v/v. They were filtered before use through a 0.45 µm membrane filter and degassed by sonication.

2.1.2 Preparation of Standard drug solution

A standard stock solution of the drug was prepared by dissolving 150 mg of Afatinib in 100 ml volumetric flask containing 30 ml of water, sonicated for about 15 min and then made up to 100 ml with water to get approximately 1500µg/mL.

2.1.3 Working Standard Solution

5ml of the primary standard stock solution of 1500µg/mL was taken in 50 ml volumetric flask and thereafter made up to 50 ml with mobile phase to get a concentration of 150µg/ml.

2.1.4 Preparation of Sample solution

20 film coated tablets of Afatinib (Gilotrif® 40 mg, Boehringer Ingelheim Pharma GmbH & Co. KG, Film coated tablets,) were and then powdered. A sample of the powdered tablets, equivalent to 150 mg of the active ingredient, was mixed with 70 ml of mobile phase in 100 ml volumetric flask. The mixture was allowed to stand for 1 hr with intermittent sonication for complete solubility of the drug, and then filtered through a 0.45 µm membrane filter, followed by addition of mobile phase up 100 ml to obtain a stock solution of 1500µg/mL. The resultant solution was further diluted by taking 5 ml of the stock solution with 50 ml of mobile phase to get the concentration of 150µg/mL.

2.1.5 Wave length selection

Using PDA detector, the wavelength selected to be 254 nm.

2.1.6 Procedure

Initially the mobile phase was pumped for about 30 min, to saturate the column thereby to set the baseline corrected. Then 20 µl of Afatinib standard and sample solution were injected separately. A quantitative determination of the active ingredient was made by compare the peak area of a sample injection to the corresponding peak area of a standard injection. The amount of Afatinib present in the sample was calculated through the standard calibration curve.

2.2. METHOD VALIDATION

The method was validated for the following parameters: system suitability, specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, and robustness.

2.2.1 System Suitability

The system suitability parameters were evaluated from standard chromatograms obtained by calculating the % RSD of Retention Time, tailing factor, theoretical plates, and peak areas frim six replicate

injection are within the range were shown in table

Acceptance Criteria

The no. of theatrical plates should not be less than 2000.

The tailing factor should not be more than 2.0

2.2.2 Linearity

2.2.2.1 Preparation of stock solution

A standard stock solution of the drug was prepared by dissolving 150 mg of Afatinib in 100 ml volumetric flask containing 30 ml of water, sonicated for about 15 min and then made up to 100 ml with water to get 1500 µg/ml standard stock solution. The resultant solution was further diluted by taking 5 ml of the stock solution with 50 ml of mobile phase to get the concentration of 150µg/mL (Working Standard solution). Further pipette 1, 2, 4, 6, 8 &10 ml of working standard solution as taken in 10ml volumetric flask Dilute up to the mark with diluents it contains the concentrations from 15, 30, 60, 90, 120 and 150µg/ml respectively. 120% dilution level containing 180µg/ml was prepared by taking 1.2 mL of primary standard solution was diluted upto 10 ml with diluent. Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels (15–180 µg/mL) of the assay analyte concentration and 20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The calibration curve was plotted by taking the concentration on the x-axis and the corresponding peak area on the y-axis. The data was treated with linear regression analysis method.

2.2.3 Accuracy

To determine the accuracy of the proposed method, different amounts of bulk sample of Afatinib within linearity limits were taken and analyzed by the proposed method. The results are representing.

2.2.3.1 Preparation Sample solution

Accurately weigh and transfer 150 mg equivalent of tablet powder into a 100ml clean dry volumetric flask add about 30ml of diluents and sonicated for 15 min and then made up to 100 ml with water to get 1500µg/ml standard stock solution. The resultant solution was further diluted by taking 5 ml of the stock solution with 50 ml of mobile phase to get the concentration of 150µg/mL. Further pipette 8 &10 ml of working standard solution as taken in 10ml volumetric flask Dilute up to the mark with diluents it contains the concentrations from 120 and 150µg/ml respectively. 120% dilution level containing 180µg/ml was prepared by taking 1.2 mL of primary standard solution was diluted upto 10 ml with diluent.

2.2.4 Precision: The precision of the method was ascertained separately from the peak area obtained by actual determination of 6 replicas of a fixed amount of drug and formulation. The HPLC systems was set up the described Chromatographic conditions, mentioned as above and follow the system to equilibrate, and then injected the 150 µg/ml concentration of Afatinib standard 6 times and recorded the response (peak area). The precision was repeated with the formulated sample of same concentration. The proposed method was extended by Injecting the 200 µg/ml of Afatinib sample 6 times recorded the response (peak area). The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated and presented

2.2.5 Sensitivity/Limit of quantification (LOQ) and limit of detection (LOD)

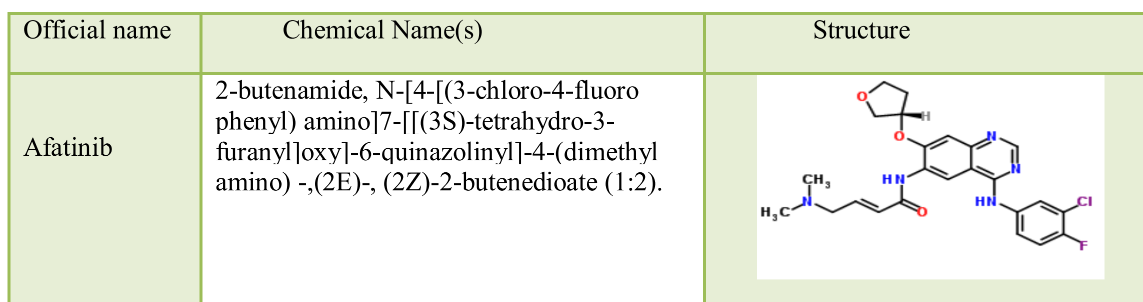
The limit of quantification (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1). Sensitivity of the method was established with respect to limit of detection (LOD) and LOQ for NLT. LOD and LOQ were established by S/N ratio method. LOD and LOQ were experimentally verified by injecting six replicate injections of each concentration obtained from dilution levels.

2.2.6. Robustness: To determine the robustness of this method, the experimental conditions were deliberately altered at two different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the mobile phase flow rate was varied by ±10%) and different column had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust.

3. RESULTS AND DISCUSSION

The following parameters were used to validate the method for the estimation of Afatinib in bulk sample and in powder for injections.

Table 1: Structural Features of Afatinib



Official name	Chemical Name(s)	Structure
Afatinib	2-butenamide, N-[4-[(3-chloro-4-fluoro phenyl) amino]7-[[(3S)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4-(dimethyl amino) -,(2E)-, (2Z)-2-butenedioate (1:2).	

2. MATERIALS AND METHODS

Table 2: Materials

S.NO	Materials	TYPE	BRAND
1	Acetonitrile	HPLC Grade	Merck
2	Water	HPLC Grade	Merck
3	Di-Potassium hydrogen Orthophosphate	AR GRADE	Rankem

Table 3: Instruments

S.NO	Instruments	Software	Model	Company
1	HPLC	Empower Software	Waters 515pump,Detector2487	AGILENT
2	UV-Spectrophotometer	UV Analyst	T-60	PG INSTRUMENT
3	Weighing balance	-	XEX 200	SHIMADZU
4	Sonicator	-	SE60US	ENERTECH
5	pH Meter	-	AD102U	ADWA

Fig.1: System suitability Chromatogram of Afatinib working stand solution contains – 150 micrograms/ml.

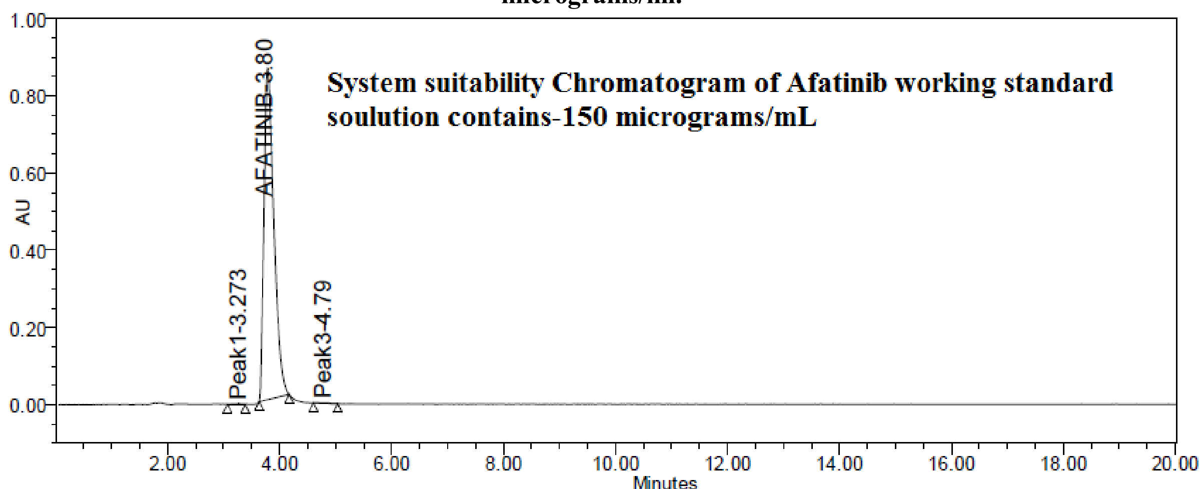


Table 4: System Suitability Studies of Afatinib

S. No	Peak Name	R.T	Peak Area	Peak %	RT-Ratio	Tailing	USP Plate count	USP Resolution
1.	Peak1	3.273	11109	0.11	0.86	0.78	2720.04	
2	AFATINIB	3.80	10473896	99.69		1.56	2006.16	1.72
3	Peak3	4.790	21089	0.20	1.26	1.14	4166.07	3.11

Fig .2: Chromatogram to illustrate Precision of Standard drug

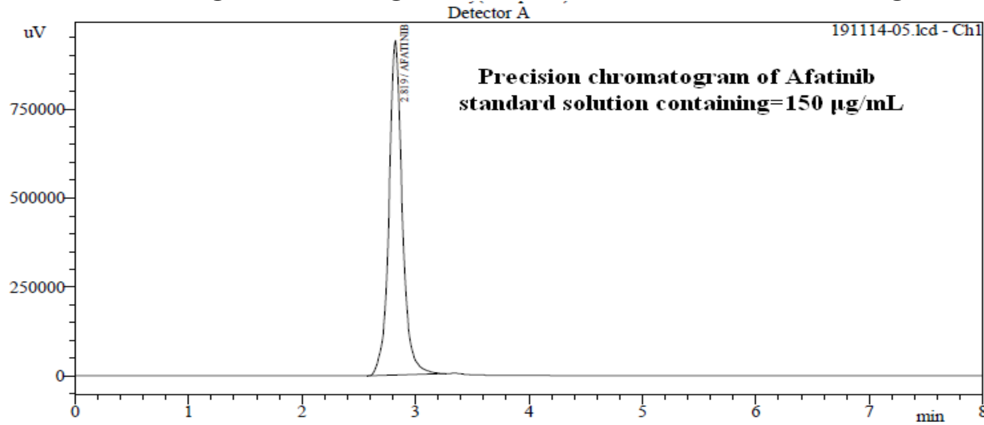


Table 5: Precision of Standard drug with statistics

Injection No.	Name of the drug & conc. (150 µg/ml)	Retention time in min.	Peak Area
1	Afatinibinjection-1	2.819	7698622
2	Afatinibinjection-2	2.818	7698577
3	Afatinibinjection-3	2.817	7693136
4	Afatinibinjection-4	2.813	7683915
5	Afatinibinjection-5	2.813	7670573
6	Afatinibinjection-6	2.812	7668883
Mean		2.815	7685618
% RSD.		0.108	0.175
Std. Deviation		**0.003**	**13438**

Fig .3: Chromatogram to illustrate Precision of AfatinibSample solution.

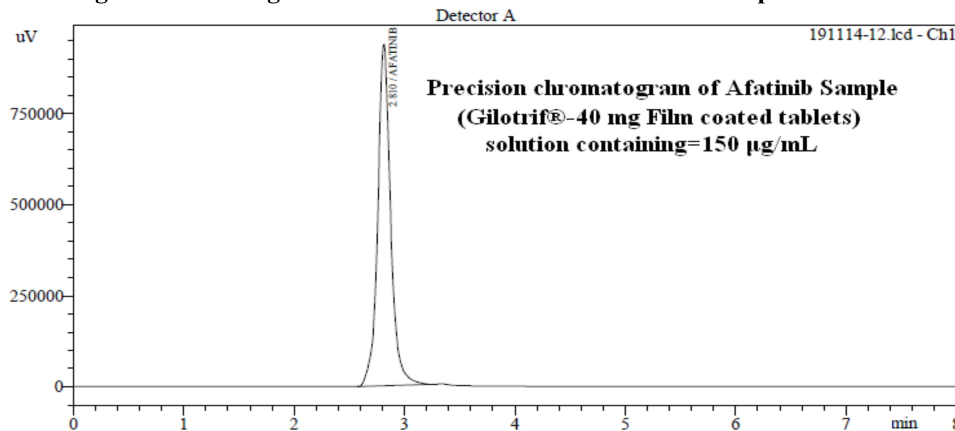


Table 6: Precision study of Sample Solution (Gilotrif® 40 mg, film coated tablets) with statistics

Injection No.	Name of the drug & conc. (150 µg/ml)	Retention time in min.	Peak Area
1	Gilotrif® injection-1	2.812	7661994
2	Gilotrif® injection-2	2.810	7655525
3	Gilotrif® injection-3	2.810	7636160
4	Gilotrif® injection-4	2.810	7624722
5	Gilotrif® injection-5	2.809	7641083
6	Gilotrif® injection-6	2.809	7644532
Mean		2.810	7644002
Std. Deviation		0.038	0.175
% RSD		**0.001**	**13405**

Fig.4: Standard Calibration Curve of Afatinib

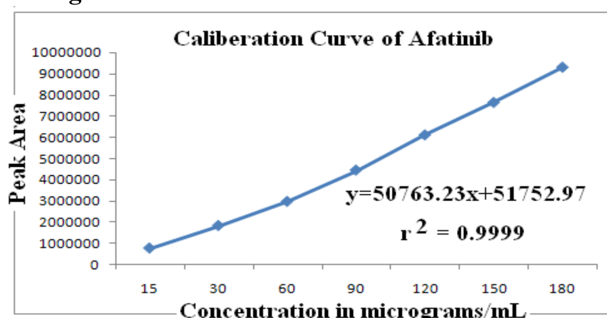


Table 7: Standard calibration values of Afatinib

Concentration of drug (µg/mL)	Retention time	Peak Area
15	2.807	770604
30	2.808	1836552
60	2.802	2974351
90	2.807	4452572
120	2.806	6113963
150	2.806	7646271
180	2.806	9310301

Table 8: Recovery Peak areas of Afatinib by Accuracy studies

S.No	Recovery at 80% dilution Level Peak areas		Recovery at 100% dilution Level Peak areas		Recovery at 120% dilution Level Peak areas	
	Standard	Spiked	Standard	Spiked	Standard	Spiked
1	5906572	6804782	7670239	8429009	9284253	10331455
2	5937767	6808106	7669595	8424708	9287667	10304172
3	5909404	6802275	7668384	8422411	9305486	10307276
Avg	5917914	6805054	7669406	8425376	9292468.7	10314301
Std.Dev	17251	2925	942	3349	11402	14937
%RSD	0.292	0.043	0.012	0.040	0.123	0.145
% Recovery	109.6%		109.17		117.00	

Table 9: Robustness study of Afatinib Standard solution at 100 % level (150 µg/mL)

Parameter	Peak areas of Afatinib in Flow increase study		Peak areas of Afatinib in Flow decrease study		Peak areas of Afatinib in Variable column Study	
	Retention time	**Peak Area**	**Retention time**	**Peak Area**	**Retention time**	**Peak Area**
Injection-1	2.560	7142182	3.122	8541351	2.810	7780044
Injection-2	2.560	7149981	3.121	8546792	2.809	7785883
Injection-3	2.559	7156931	3.120	8542528	2.809	7792276
Mean	2.560	7149698	3.121	8543557	2.809	7786068
% RSD	0.041	0.103	0.026	0.034	0.016	0.079
Std. Dev	0.001	7379	0.001	2863	0.000	6118

Table 10: Robustness study of Gilotrif®-40 mg tablets solution at 100 % level (150 µg/mL)

Parameter	Peak areas of Afatinib in Flow increase study		Peak areas of Afatinib in Flow decrease study		Peak areas of Afatinib in Variable column Study	
	Retention time	**Peak Area**	**Retention time**	**Peak Area**	**Retention time**	**Peak Area**
Injection-1	2.561	7171521	3.122	8676086	2.809	7767794
Injection-2	2.560	7199787	3.118	8675465	2.808	7728248
Injection-3	2.560	7193828	3.123	8674522	2.808	7755779
Mean	2.560	7188379	3.121	8675358	2.808	7750607
% RSD	0.020	0.207	0.082	0.009	0.029	0.262
Std.Dev	0.001	14900	0.003	788	0.001	20274

Fig 5: LOD & LOQ Chromatogram of Afatinib standard solution at 0.01 % dilution level containing 0.015 µg/ml

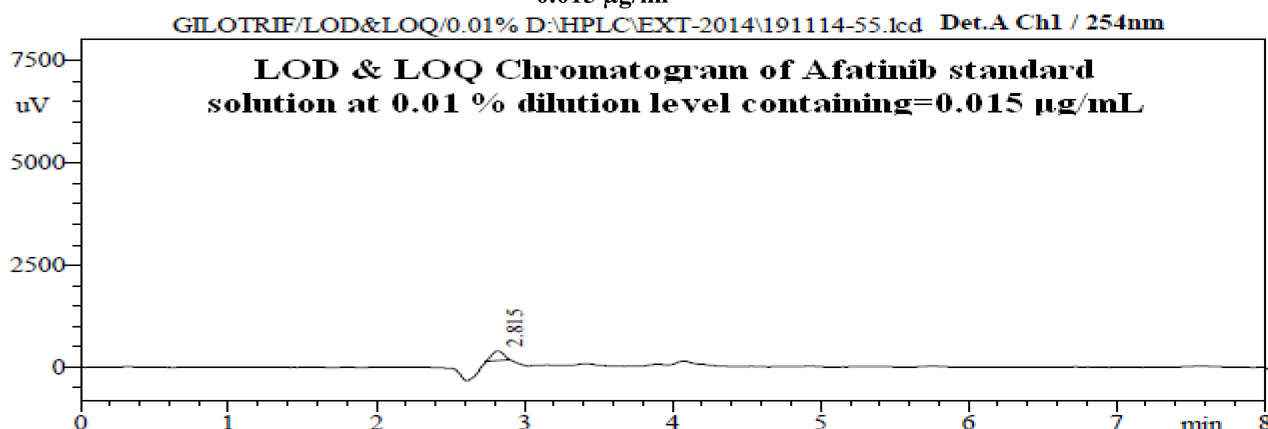


Table 11: Optical & Regression Characteristics of HPLC method

Parameter	Results of HPLC Method
Detection wavelength (nm)	254
Linearity range (µg/mL)	15-180
Regression Equation (y=mx + c)	Y=50763.23x+51752.97
Slope (m)	50763.23
Intercept (c)	51752.97
Correlation coefficient	0.9999
Relative Standard deviation*	Standard solution-0.108 Sample solution-0.01
% error in bulk samples	**0.054**

* Average of six determinations ** Average of three determination

Table12: Performance & Detection Characteristics of HPLC method

Parameter	Results of the proposed HPLC method	
	Afatinib Standard solution	**Afatinb Sample (Gilotrif®-40 mg tablets) Solution**
Retention time (min)	2.835	2.830
Theoretical plates (n)	2804.151	2804.439
Plates per meter (N)	11216.6	11217.75
HETP	$8.915\text{x}10^{-5}$	$8.9144\text{x}10^{-5}$
Peak asymmetry (T)	1.088	1.092
Linearity range (µg/mL)	15-180	
Limit of Detection (µg/mL)	0.015	
Limit of Quantification (µg/mL)	0.045	

3.1 System Suitability

The system suitability tests were carried out on freshly prepared standard stock solution of Afatinib. The system was suitable for use, the tailing factors for Afatinib were 1.094 and USP theoretical plates were found to be significantly high around 2790.658.

3.2 Precision

The precision of the method was ascertained separately from the peak area obtained by actual determination of 6 replicas of a fixed amount of drug and formulation. The HPLC systems was set up the described Chromatographic conditions, mentioned as above and follow the system to equilibrate, and then injected the 150 µg/ml concentration of Afatinib standard 6 times and recorded the response (peak area). The proposed method was extended to the pharmaceutical dosage forms by injecting the 150 µg/ml of Afatinib sample with the formulated sample from (Gilotrif®-40mg, Boehringer Ingelheim Pharma GmbH & Co. KG, film coated tablets) contains Afatinib of same concentration 6 times and recorded the response (peak area). The percent relative standard deviation and percent range of error (at 0.05 and 0.01

confidence limits) were calculated and presented.

3.3 Linearity

Aliquots of standard Afatinibstock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of Afatinib are in the range of 15-180 µg/ml. Each of these drug solutions (20 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 254 nm and a Calibration graph was obtained by plotting peak area versus concentration of Afatinib. The linearity Chromatograms is presented

3.4 Recovery Studies

Recovery studies were conducted by analyzing pharmaceutical formulation in the first instance for the active ingredient in the concentration of 80% of the working standard (contains 120 µg/mL of Afatinib); 100% of the working standard solution (contains 150 µg/mL of Afatinib) and 120% of the working standard solution (contains 180 µg/mL of Afatinib) by the proposed method. Each concentration was injected 3 times and the peak area was recorded. Known amounts of pure drug [10% of the working standard solution contains 15 µg/mL of Afatinib for 80% of the working standard, for 100% of the working standard, for 120% of the working standard] was then added to each 3 previously analyzed formulation and the total amount of the drug was once again determined by the proposed method (each concentration was again injected 3 times) after keeping the active ingredient concentration within the linearity limits. The Recovery chromatograms are shown in Table 9.

3.5 Robustness

A method is robust if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at two different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the mobile phase flow rate was varied by ±10%) and different column had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust. When the chromatographic conditions were deliberately altered, system suitability results remained within acceptance limits and selectivity for individual substance was not affected. The results of the study prove the robust nature of the method.

3.6 Limit of Detection [LOD] and Limit of Quantification [LOQ]

The detection limit of the method was investigated by injecting standard solutions Afatinib into the HPLC column. By using the signal-to-noise method the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating LOD and signal-to-noise ratio of 10 is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise. Chromatograms illustrating the LOD are shown in figure 2.10. The limit of detection (LOD) and limit of quantification (LOQ) for Afatinib were found to be 0.015µg/ml and 0.045 µg/ml respectively.

4. CONCLUSION

There are few reports on the HPLC determination of Afatinib in pharmaceutical formulations in the literature prior to commencement of this work. The author has developed a sensitive, accurate and precise HPLC for the estimation of Afatinib in bulk drug. From the typical chromatogram of Afatinib as shown in fig 3.1.2, it was it found that the retention time was 2.835 min. The contents of the mobile phase were Buffer: Acetonitrile 40: 60 (v/v). Solvent-A (Buffer) is 3.48 gms of Di Potassium hydrogen *ortho*-phosphate (0.03M) in 1000 ml of water and by adjusting the pH to 3.0 with dilute *ortho*-phosphoric acid. Solvent-B: Acetonitrile in a isocratic mode of separation was used to resolute the Afatinib at a flow rate of 1.0 ml/min and eluents were monitored at 254 nm, was found to be most suitable to obtain a peak well defined and free from tailing. In the present developed HPLC method, the standard and sample preparation required less time and no tedious extraction were involved. A good linear relationship (r^2=0.9998) was observed between the concentration range of 15-180 µg/mL. The assay of Afatinib in bulk was found to be 99.37%. From the recovery studies it was found that about 109.17 % on average of Afatinib was recovered which indicates

high accuracy of the method. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the film coated tablets. This demonstrates that the developed HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of bulk and sterile powder for injection dosage form of Afatinib within a short analysis time. It can be seen from the results presented that the proposed procedure has good precision and accuracy. Results of the analysis of pharmaceutical formulations revealed that proposed methods are suitable for their analysis with virtually no interference of the usual additives present in the pharmaceutical formulations. The above proposed method obviates the need for any preliminary treatment and is the method could be of use for process development as well as quality assurance of Afatinib in bulk drugs**.**

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5. REFERENCES:

1. Kabbinavar FF, Hambleton et al. *Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer.* Journal of clinical oncology. 2005; 23 (6): 3706- 3712.
2. Saltz LB, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S et al. *Bevacizumab in combination with oxaliplatin based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study* Journal of clinical oncology. 2008; 26 (12): 2013-2019.
3. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W et al. *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* NewEngland Journal of Medicine. 2004; 350 (23): 2335-2342.
4. Crino L, Mezger J, Griesinger F, Zhou C, Reck MM. *MO19390 (SAiL): safety and efficacy of first-line bevacizumab (Bv)-based therapy in advanced non-small cell lung cancer (NSCLC)*. Journal of Clinical Oncology. 2009; 20; 27(15_suppl):8043.
5. Nicholson RI, Gee JM and Harper M2. *EGFR and cancer prognosis*. European Journal of Cancer. 2001; 37(Suppl 4): 9-15.
6. Cappuzzo F. *EGFR FISH versus mutation: different tests, different end-points*. Lung Cancer. 2008; 60(2): 160-165.
7. Bailey R, Kris M, Wolf M, Kay A, Averbuch S, Askaa J et al. *Gefitinib ('Iressa', ZD1839) monotherapy for pretreated advanced non-small-cell lung cancer in IDEAL 1 and 2: tumor response is not clinically relevantly predictable from tumor EGFR membrane staining alone*. Lung Cancer. 2003; 41:S71,
8. Hoda D, Simon GR and Garrett CR: *Targeting colorectal cancer with anti-epidermal growth factor receptor antibodies: focus on panitumumab*. TherClin Risk Manag. 2008; 4 (6): 1221-1227.
9. Cunningham et al. *Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer*. N Engl J Med. 2004; 351 (4): 337-345.
10. Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, Au HJ et al. *Cetuximab for the treatment of colorectal cancer*. New England Journal of Medicine. 2007; 357(20): 2040- 2048.
11. Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B et al. O*pen-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer.* Journal of Clinical Oncology. 2007; 25 (13): 1658-1664.
12. Van Cutsem E, Kohne CH, et al. *Cetuximab and chemotherapy as initial treatment for metastatic*

colorectal cancer. New England Journal of Medcine. 2009; 360 (14): 1408-1417.

13. Kawada I, Soejima K et al. *An alternative method for screening EGFR mutation using RFLP in non-small cell lung cancer patients*. Journal of Thoracic Oncology. 2008; 3 (10): 1096- 1103.
14. Schiller JH. *Small cell lung cancer: defining a role for emerging platinum drugs*. Oncology. 2002; 63 (2): 105-114.
15. Herbst RS, O'Neill et al. *Phase II study of efficacy and safety of bevacizumab in combination with chemotherapy or erlotinib compared with chemotherapy alone for treatment of recurrent or refractory non small-cell lung cancer*. Journal of Clinical Oncology. 2007; 25(30): 4743-4750.
16. Herbst R, Sun Y, Eberhardt W, Germonpre P, Saijo N, Zhou C et al. *Vandetanib plus docetaxel versus docetaxel as second-line treatment for patients with advanced non-small-cell lung cancer (ZODIAC): a double-blind, randomised, phase 3 trial*. The Lancet Oncology. 2010; 11 (7): 619-626.
17. John T, Liu G and Tsao MS. *Overview of molecular testing in non-small-cell lung cancer: mutational analysis, gene copy number, protein expression and other biomarkers of EGFR for the prediction of response to tyrosine kinase inhibitors*. Oncogene. 2009; 28(Suppl 1): S14-23.
18. Bouche O, Maindrault-Goebel F, Ducreux M, Lledo G, Andre T, Stopfer P et al. *Phase II Trial of Weekly Alternating Sequential BIBF 1120 and Afatinib for Advanced Colorectal Cancer*. Anticancer Research. 2011; 31(6): 2271-2281.