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VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR DETERMINATION OF ERLOTINIB RELATED SUBSTANCE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple HPLC method was developed and validated for detection & quantitation of Erlotinib related substances which may co-exist in solid pharmaceutical dosage forms. The HPLC separation was achieved on a YMC-Basic; 3µ, 150 × 4.6 mm column using mobile phase of Mobile phase A-Water :Tetrahydrofuran : Trifluroacetic acid (965:35:1.5) ; Mobile phase B-Water: Actonitrile: Tetrahydrofuran: Triflurocetic acid (460:460:80:1.5) at a flow rate of 1.5 ml/min. The UV detector was operated at 245 nm, and column temperature was adjusted at 50 °C. The method was validated for specificity, linearity, precision, accuracy, robustness, limit of detection andquantitation. The degree of linearity of the calibration curves, the percent recoveries of Erlotinibrelated substances, the limit of detection and quantitation, for the HPLC method were determined. The method was found to be simple, specific, precise, accurate, and reproducible. The method was applied for the quality control of commercial Erlotinib tablets to quantify its related substances.

KEYWORDS: Erlotinib, HPLC, Observation.

INTRODUCTION

Erlotinib (ERL), chemically known as N-(3-ethynylphenyl)-6, 7-bis (2-methoxyetho -xy) quinazolin-4- amine. Erlotinib is an epidermal growth factor receptor inhibitor (EGFR inhibitor) and used to treat nonsmall cell lung cancer (NSCLC)), the oral epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitor (TKI). Erlotinib is an established second-line treatment for advanced NSCLC.[1-2] The molecule structure is shown in Figure-1.

The route of synthesis of ERL and possible degradants resulted, seven known impurities which are not reported in any of the pharmacopeia. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.[3,4] As per Literature there is no single method available for the determination of all impurities in a single method.[5-7] It is felt necessary to develop a stability indicating method for ERL related impurities in API and tablet dosage formulation by ICH approach.

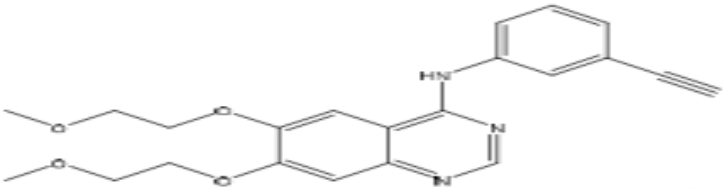


Fig. 1: Chemical structure of Erlotinib.

MATERIALS AND METHODS

Materials

Mobile phase A: Water: Tetrahydrofuran: Trifluroacetic acid (965:35:1.5).

Mobile phase B: Water: Actonitrile: Tetrahydrofuran: Triflurocetic acid (460:460:80:1.5).

Diluent: Water: Acetonitrile (50:50).

Balnk: Diluents.

Dimer Impurity stock solution: Weigh approximately 17.5 mg of dimer standard of Erlotinib in a 10 ml volumetric flask dissolve in chloroform and sonicate for 10 minute, dilute to volume with chloroform.

Resolution Solution: Weigh approximately 70.0 mg of Erlotinib Hydrochloride in a 25 ml volumetric flask; add 40 µl of dimer impurity stock solution. Dissolve and dilute to volume with diluents. Sonicate for 30 minutes.

Ref solution

Take 32.80 mg of Erlotinib Hydrochloride working standards into 50 ml volumetric flask and make volume up to the mark with diluents. Dilute 1 ml of reference stock solution to 200 ml volumetric flask with diluents.

LOQ (Disregard solution)

Dilute 4 ml ref solution to 10 ml with diluents. (0.04% of test solution).

Test solution

Take 1297.8 mg of crushed powder equivalent to 300 mg of Erlotinib into 100 ml volumetric flask and make volume up to the mark with diluents. (3.0 mg/ml).

Chromatographic system

Mode : LC

Detector : UV 245 nm

Column : YMC-Basic; 3µ, 150 × 4.6 mm

Flow rate : 1.5 ml per minute

Sampler temperature : 25°C

Column temperature : 50°C

Inject volume : 10 µL

Gradient

Time	Mobile phase A	Mobile phase B	Remarks
0	100	0	Isocratic
5	100	0	Isocratic
35	75	25	Linear Gradiant
40	65	35	Linear Gradiant
55	20	80	Linear Gradiant
55.5	100	0	Linear Gradiant
65	100	0	Equilibration

Methods: Separately inject 10µl each blank, Resolution solution, LOQ (disregard) solution, reference solution, test solution and calculate the impurities found from the test solution disregarding any peak due to blank and the area of the principal peak in the chromatogram obtained with LOQ (disregard solution) (0.04 per cent of test solution).

Resolution

Resolution between Erlotinib dimer and Erlotinib Hydrochloride will be ≥ 1.5.

Calculation

$$\text{Isopropyl ether in \%} = \frac{\text{Peak area at RRT 0.46} \times 0.76 \times 0.1}{\text{Peak area in reference solution}}$$

$$\text{3-Br ErlotinibHCl \%} = \frac{\text{Peak area at RRT 1.26} \times 1.93 \times 0.1}{\text{Peak area in reference solution}}$$

$$\text{Any other single impurity \%} = \frac{\text{Peak area of any single unknown impurity} \times 0.1}{\text{Peak area in reference solution}}$$

$$\text{Total unknown impurities \%} = \frac{\text{Peak area total unknown impurities} \times 0.1}{\text{Peak area in reference solution}}$$

Total impurities in %= (Known impurities + total unknown impurities)

METHODS VALIDATION & OBSERVATIONS

Specificity

The specificity of the method for identification is tested by injecting following solutions into the chromatographic system:

- Diluent
- Placebo solution
- Resolution solution
- Reference solution
- Test solution

Preparation of Placebo solution: Weigh and take a quantity of powder about 812.4 mg of formulation placebo in a 100 ml volumetric flask. Add about 60 ml diluent, sonicate for 15 minutes and dilute up to mark with same solvent. Filter the solution through Whatmanfilter paper size# 41. Finally filter the solution with 0.45 micron disk filter.

Standard Preparation (Ref Solution): Take 32.80 mg of Erlotinib Hydrochloride working standards into 50 ml volumetric flask and make volume up to the mark with diluents. Dilute 1 ml of reference stock solution to 200 ml volumetric flask with diluents.

Sample Preparation (Test solution): Take 1133 mg of crushed powder equivalent to 300 mg of Erlotinib into 100 ml volumetric flask and make volume up to the mark with diluents. (3.0 mg/ml).

Procedure: Inject 10µl of blank solution, placebo solution, reference solutions, resolution sample & test solution one after another and obtain the chromatograms.

Observation.

Sl. No.	Name of solution	Name of Peak	Retention time
01.	Blank	Blank 01	48.883
		Blank 02	50.650
		Blank 03	52.642
02.	Placebo	Blank 01	48.850
		Placebo 01	19.183
		Blank02	50.617
		Placebo 02	51.695
		Blank 03	52.512
03.	Reference solution	Erlotinib	35.420
04.	Resolution Sample	Erlotinib	34.703
		Erlotinib dimer	36.472
05.	Test solution	Unknown 1	5.384
		Erlotinib	34.640
		Unknown 2	46.068
● **Remarks:** By retention time analysis of blank, placebo, reference and sample solution it is clear that there are no interfering peaks are observed from blank, placebo at the retention time of Erlotinib reference/ working standard and retention time of Erlotinib peak in resolution sample & sample are 34.703min & 34.640min for Erlotinib which is within ± 0.2 % minute of the Erlotinib peak in resolution samples's retention time.			

Acceptance Criteria & Results.

Sl. No.	Acceptance Criteria	Results
01.	No peak co-elutes with main peak	Complies
02.	No interfering peaks are observed from blank, placebo at the retention time of Erlotinib and impurity peaks.	Complies
03.	No interfering peaks are observed from diluent and placebo at the retention time of Erlotinib Hydrochloride in resolution sample and retention of sample will be within ± 0.2 minute of the Erlotinib peak in resolution sample's retention time.	Complies

Linearity: To check the Linearity prepares a dilution series of standard solution from 40 to 160% of the nominal concentration. Inject separately 3 times each concentration level & calculate correlation coefficient, r^2 from the calibration curve from average area.

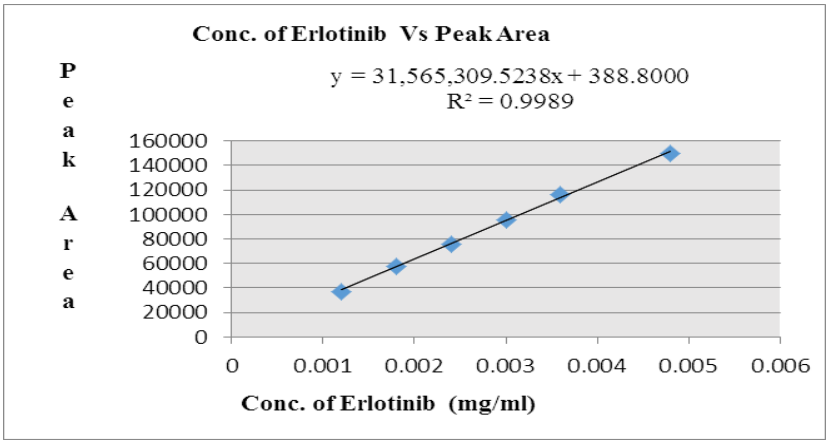
Linearity Stock Solution: Take 32.80 mg of Erlotinib Hydrochloride working standards into 50 ml volumetric flask and make volume up to the mark with diluents. Dilute 5 ml of reference stock solution to 100 ml volumetric flask with diluents.

Concentration level in (%) of the active ingredients concentration	Volume of stock solution added (ml) in 25 ml volumetric flask with Diluent	Approx. final concentration in (mg/ml)
		Erlotinib
40	1.0	0.0012
60	1.5	0.0018
80	2.0	0.0024
100	2.5	0.0030
120	3.0	0.0036
160	4.0	0.0048

Observation

Table: Different concentration of Erlotinib and respective peak area.

Concentration level in (%) of the active ingredients concentration	Approx. final concentration in (mg/ml)	Peak area for Erlotinib	
		Individual Area	Average Area
40	0.0012	36744	37132
		37362	
		37920	
60	0.0018	56239	57590
		58042	
		58490	
80	0.0024	75409	76099
		75981	
		76907	
100	0.0030	95504	95283
		94512	
		95831	
120	0.0036	115617	116266
		116699	
		1116482	
160	0.0048	148042	150260
		151127	
		151612	



Graph-1: Different concentration of Erlotinib VS Average Peak area.

From Graph-4: Regression equation,

$y = 31,565,309.5238x + 388.8000$, $R^2 = 0.9989$.

Correlation coefficient, R^2	0.9989
Intercept	388.8000
Slope of regression line	31565308.5238

Acceptance Criteria & Results

Sl. No.	Acceptance Criteria	Results
01.	Correlation coefficient : ≥ 0.990	0.9989
02.	Intercept	388.8000
03.	Slope regression line	31565308.5238

Precision

System precision/System suitability: System suitability testing is an integral part of many analytical procedures. System suitability test parameters depend on the type of procedure being validated. To check the system suitability of the system, inject the reference solution 6 times, immediate one after another, under conditions as similar as possible. Calculate the relative standard deviation for retention time and peak area.

Standard Preparation

Take 32.80 mg of Erlotinib Hydrochloride working standards into 50 ml volumetric flask and make volume up to the mark with diluents. Dilute 1 ml of reference stock solution to 200 ml volumetric flask with diluents.

Procedure

Inject 10 µl of reference solution one after another in six replicates and obtain the chromatograms.

Observation

Table: Six replicates reading of standard solution

No. of Sample	Retention time for Erlotinib (min)	Average Retention time (min)	Relative standard deviation (%)	Peak area for Erlotinib	Average Area	Relative standard deviation (%)
01.	35.422	35.420	0.0	97971	99092	0.8
02.	35.431			99231		
03.	35.411			98421		
04.	35.416			99382		
05.	35.419			99213		
06.	35.423			100326		

Acceptance Criteria & Results

Sl. No.	Acceptance Criteria	Results	
		Retention time	Area
01.	Relative standard deviation is less than 10.0%.	0.0%	0.8%

Method precision

To check the repeatability of the method, prepared separately the test solution 6 times, immediately one after another, under conditions as similar as possible. % of each impurity in test preparation calculated with respect to the area of Lamivudine area in reference solution. Calculate the result for 6 determinations and calculate the relative standard deviation.

Observation

Table: Data for method precision.

No. of Sample	Peak area of Erlotinib in reference solution	Impurity		
		Unknown 01 in %	Unknown 02 in %	Total impurities in%
01.	99092	0.046	0.069	0.115
02.		0.048	0.072	0.120
03.		0.048	0.075	0.123
04.		0.048	0.072	0.120
05.		0.048	0.074	0.122
06.		0.048	0.073	0.121
Avg.	--	0.048	0.073	0.120
% RSD	--	1.71	2.86	----
Remarks: Relative standard deviation for Unknown-01 is 1.71% and Unknown-02 is 2.86%.				

Acceptance Criteria & Results

Sl. No.	Acceptance Criteria	Results
01.	The relative standard deviation of unspecified impurities for n ≥ 6 should be ≤ 20 %.	Complies

Intermediate precision

To check the repeatability of the method, prepare separately the sample solution 6 times, immediately one after another, under conditions as similar as possible. Calculate the result for 6 determinations and calculate the coefficient of variation.

Observation

Table: Data for method precision.

No. of Sample	Peak area of Erlotinib in reference solution	Impurity		
		Unknown 01 in %	Unknown 02 in %	Total impurities in%
01.	106796	0.044	0.062	0.106
02.		0.045	0.066	0.111
03.		0.045	0.066	0.111
04.		0.045	0.066	0.111
05.		0.045	0.067	0.112
06.		0.044	0.066	0.110
Avg.	--	0.45	0.66	0.110
% RSD	--	1.16	2.67	----
Remarks: Relative standard deviation for Unknown-01 is 1.16% and Unknown-02 is 2.67%.				

Acceptance Criteria & Results

Sl. No.	Acceptance Criteria	Results
01.	The relative standard deviation of unspecified impurities for n ≥ 6 should be ≤ 20 %.	Complies

Accuracy or Recovery: The accuracy of the method is evaluated by samples spiked with active ingredients. Data from triplicate determinations should be collected at 3 concentration levels i.e. 80%, 100% &120% of the label claim of the active ingredient. The accuracy is expressed in recovery rates.

Accuracy Standard stock solution: Take 32.80 mg of Erlotinib Hydrochloride working standards into 50 ml volumetric flask and make volume up to the mark with diluents. Dilute 5 ml of reference stock solution to 100 ml volumetric flask with diluents.

Concentration level in (%) of the active ingredients concentration	Volume of stock solution added (ml) in 25 ml volumetric flask with diluent	Approx.final concentration in (mg/ml)
		Erlotinib
80	2.0	0.0024
100	2.5	0.0030
120	3.0	0.0036

Preparation of Erlotin Tablet 150 mg accuracy test solutions: Take three 150 ml volumetric flask and labeled it as 80%, 100% & 120%.Weigh and transfer placebo equivalent to 1 tablet (406.2 mg) into the marked volumetric flask each. Weigh 131.12 mg, 163.90 mg and 196.68 mg of Erlotinib Hydrochloride API into the 80%, 100%, 120% marked volumetric flask respectively. Add 60 ml of diluent into the each volumetric flask and soniate for 15 minute to dissolve and make volume up to the mark at room temperature. Filter the

solution through Whatman filter paper size# 41. Dilute 3 ml each of this above solution to 100 ml with diluent. Further dilute 2 ml of this solution to 20 ml with diluents. Finally filter the solution through 0.45 micron disk filter.

Following table describe the concentration of sample at different level.

Concentration level in (%) of the active ingredients concentration	Approx. final concentration in (mg/ml)
	Erlotinib
80 × 3 sample	0.0024
100 × 3 sample	0.0030
120 × 3 sample	0.0036

Observation: The sample solution for evaluating the Accuracy / Recovery was prepared as 80% – 120% of nominal analyte of Erlotinib.

% of nominal concentration	Concentration of Erlotinibin Standard (mg/ml)	Average Peak area (Standard)	Average Peak area (Sample)	Recovery from sample in %
80	0.0024	81490	79832	**97.97**
100	0.0030	94525	92920	**98.30**
120	0.0036	117760	116787	**99.17**
			Average	**98.48**
			Minimum	**97.97**
			Maximum	**99.17**
Remarks: Individual recovery for Erlotinibis from 97.97 – 99.17% and mean recovery is 98.48%				

Acceptance Criteria & Results

Sl. No.	Acceptance Criteria	Results
01.	Individual recovery % must be between 97 - 103 %	97.97 – 99.17%
02.	Mean recovery % must be between 98 - 102%	98.48%

Limit of Quantification (LOQ)

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices. LOQ concentration of Erlotinibwas determined based on standard deviation of response and slope method. Linearity was performed in the range of 40%, 60%, 80%, 100%, 120% and 160% of the working concentration of reference solution (0.003 mg/ml of Erlotinib). Linearity graph of concentration in mg/ml (X-axis) versus peak response (Y-axis) was plotted. Correlation coefficient, slope of regression line and standard deviation of regression line was calculated. LOQ was determined on the basis of equation given below. Six replicate injections of LOQ concentrations were injected.

Limit of Quantification = (10 × σ) / S

Where,

σ = Residual standard deviation of regression line (STEYX)

S = Slope of calibration curve.

Theoretical LOQ concentration: (10 × 1516.68426 / 3165309.5238)

= 0.00048 (mg/ml)

Where,

1516.68426 = Residual standard deviation of regression line (STEYX)

3165309.5238= Slope of calibration curve

Table: Data for LOQ.

Sample ID	Sample Concentration (mg/ml) (Actual)	Retention time	Peak Area
LOQ-001	0.0010 mg/ml	35.445	30574
LOQ-002		35.443	30270
LOQ-003		35.465	31287
LOQ-004		35.470	29711
LOQ-005		35.472	30576
LOQ-006		35.473	31013
Average		35.461	30572
%RSD		0.0	1.8

Limit of Detection (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value. LOD concentration of Erlotinib determined based on standard deviation of response and slope method. Linearity to be perform in the range of 40%, 60%, 80%, 100%, 120% and 160% of the reference solution concentration (0.2 mg/ml of Erlotinib). Linearity graph of concentration in mg/ml (X-axis) versus peak response (Y-axis) plotted. Correlation coefficient, slope of regression line and standard deviation of regression line calculated. LOD determined on the basis of equation given below.

Limit of Detection = (3.3 × σ) / S

Where,

σ = Residual standard deviation of regression line (STEYX)

S = Slope of calibration curve

Theoretical LOD concentration: (3.3 × 1516.68426 / 3165309.5238)

= 0.00016 (mg/ml)

Where,

1516.68426 = Residual standard deviation of regression line (STEYX)

3165309.5238= Slope of calibration curve

Table: Data for LOD.

Sample ID	Sample Concentration (mg/ml) (Actual)	Retention time	Peak Area
LOD (Actual)	0.0005 mg/ml	35.991	14722

Robustness

Stability of the analytical solutions

The stability of analytical solution is demonstrated by carrying out the analysis on the Reference and Test solution immediately after they are prepared and then at suitable intervals at room temperature.

The test solution to be kept on bench top under normal laboratory conditions and to be analyzed at suitable time intervals to establish bench top solution stability up to 8 hrs.

Time program: Initial, After 4hours & After 8 hours

In a table summarize the % change between the initial results and the results at each time point calculated with respect to the fresh standard where appropriate.

Acceptance Criteria

Standard solution: ± 2.0% with regard to initial

Sample solution: ± 2.0% with regard to initial

Standard Solution				Sample solution		
Time in Hours	Area	% Results	% Change	Area	% Results	% Change
Initial	97412	-----	-----	97172	99.73	-----
4th Hour	96390	100.49	0.49	96910	101.01	1.28
8th Hour	97577	99.32	0.68	97577	100.62	0.89

Remarks: From the above study, there is no significant change in % result of standard & sample solution a suitable interval after 4 hours & 8 hours.

Sl. No.	Acceptance Criteria		Results (%)	
01.	Standard solution: ± 2.0% with regard to initial	Erlotinib	4 Hr	0.49
			8 Hr	0.68
02.	Sample solution: ± 2.0% with regard to initial	Erlotinib	4 Hr	1.28
			8 Hr	0.89

Acceptance Criteria & Result

Acceptance Criteria	Result
Must be Robust	Complies

Sl. No.	Validation Parameters	Acceptance Criteria	Results	
			Erlotinib	
1.0	Specificity	No peak co-elutes with main peak	Complies	
		No interfering peaks are observed from blank, placebo at the retention time of Erlotinib and impurity peaks.	Complies	
		No interfering peaks are observed from diluent and placebo at the retention time of Erlotinib Hydrochloride in resolution sample and retention of sample will be within ± 0.2 minute of the Erlotinib peak in resolution sample's retention time.	Complies	
2.0	Linearity	Correlation coefficient : ≥ 0.990	0.9989	
		Intercept: To be reported	388.8000	
		Slope regression line : To be reported	31565308.5238	
3.0	Precision			
	3.1 System precision	Relative standard deviation is less than 2.0%.	Rt. Tm.	Area
			0.0%	0.8%
	3.2 Method precision (Repeatability)	The relative standard deviation of unspecified impurities for n ≥ 6 should be ≤ 20 %.	Complies	
	3.3 Intermediate precision	The relative standard deviation of unspecified impurities for n ≥ 6 should be ≤ 20 %.	Complies	
4.0	Accuracy or Recovery	Individual recovery % must be between 97 -103%	97.97 – 99.17%	
		Mean recovery % must be between 98 -102 %	98.48%	
5.0	LOQ	To be reported	0.0010 mg/ml	
6.0	LOD	To be reported	0.0005 mg/ml	
7.0	Robustness			
	Stability of analytical solution	Standard solution: ± 2.0% with regard to initial	4 Hr	0.49
			8 Hr	0.68
		Sample solution: ± 2.0% with regard to initial	4 Hr	1.28
			8 Hr	0.89

CONCLUSION

A simple, sensitive, specific, accurate and precise stability indicating HPLC method was validated for the routine analysis of tablet dosage form of Erlotinib related substance. The method is sensitive enough for the detection of analysis in pharmaceutical formulation when compared to the research works found in the literature. The method can be employed for the routine analysis of Erlotinib related substance.

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