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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR THE DETERMINATION OF LAPATINIB IMPURITIES IN BULK AND FINISHED FORMULATIONS

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ABSTRACT: A simple, sensitive and stability indicating reverse phase high performance liquid chromatography method (RP-HPLC) was developed and validated for the estimation of Lapatinib and its related substances in bulk and finished dosage forms. The newly developed method was assessed using Zorbax Eclipse C18 (3.5 µm, 100 × 4.6 mm) HPLC column with pH- 4.5 ammonium formate buffer and acetonitrile as mobile phase in gradient elution mode with a run time of 35 minutes. UV detection was carried out at a wavelength of 261 nm with an injection volume of 5 µL. The developed method was validated as per the ICH guidelines with respect to precision, accuracy, linearity, robustness, specificity and system suitability. Lapatinib samples were exposed to thermal, photolytic, acidic, basic and oxidative stress conditions. The stressed samples were analyzed by the developed method to establish the stability indicating power of the method. The LOD values were 0.009, 0.012 and 0.011 µg/ml and the LOQ values were 0.027, 0.035 and 0.0304 µg/ml respectively for impurity-1, impurity-2 and impurity-3 of Lapatinib. The average recovery values of Lapatinib related substances were found to be in the range of 97.5-101.2 %. The developed method was linear over a range of 0.027- 1.5 µg/ml for Lapatinib impurities. The proposed method was found to be suitable and accurate for the determination of Lapatinib related substances in bulk and finished formulations.

INTRODUCTION: Lapatinib is a protein tyrosine kinase inhibitor that dually inhibits the growth of the tumor-causing breast cancer stem cells [1]. Breast cancer is the most common malignancy and the second most common cause of cancer-related death in Western Europe.

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Lapatinib functions as an inhibitor to EGFR and erbB-2 receptors. These specific receptors are part of the Type I receptor kinase family involved in cell proliferation, differentiation and anti-apoptotic signals produced by growth factors. Cells with inactive Type I receptors don't exhibit uncontrolled growth; it is believed that an inhibitor of erbB kinase [2] could be useful in inhibiting uncontrolled cell growth *via* stasis or cell death (QPS). It is a dual tyrosine kinase inhibitor and is also used as an adjuvant therapy when patients have progressed on Herceptin [3]. This blocks receptor phosphorylation and activation, preventing subsequent downstream

signalling events. It was marketed under the trade names Tykerb and Tyverb. Lapatinib is a synthetic compound with competitive inhibition of natural product that is naturally derived substrate. Based on the recent studies, GSK announced the approval of Lapatinib in first-line therapy in triple positive (hormone receptor, EGFR, HER2) breast cancer patients [4 - 5].

Literature survey reveals that HPLC, HPLC-UV, LC-MS/MS, UPLC/MS-MS methods have been reported for the estimation of lapatinib in bulk, finished formulations and in biological samples [6-14]. Lapatinib is not official in any of the pharmacopeia for its qualitative and quantitative determination. Recently stability indicating HPLC method for the determination of Lapatinib in bulk and its degraded products was reported [15]. In the reported methods, the peak shapes of the impurities were broad with poor peak shapes and there was no clear resolution between the impurities. Hence there is a requirement for the development of stability indicating method with proper peak shapes and resolution for the determination of impurities in drug substance and drug product. The peak shape and presence of impurities can have a significant impact on the product quality, safety and efficacy, hence proper peak shape and the amount of impurities need to control in the drug substance as well as drug product.

The objective of the present work is to develop a stability indicating HPLC method and validate for the routine use of estimation of impurities in quality control laboratories with respect to specificity, limit of detection and quantification, linearity, precision, ruggedness and robustness with proper peak shape. Stability indicating method indicates the variations in the drug under different environmental conditions like light, humidity and heat.

Physical Properties: Lapatinib ($C_{29}H_{26}ClFN_4O_4S$) used in the form of Lapatinib ditosylate mono hydrate with chemical name and chemical formula as N-[3-Chloro-4-[(3-fluorophenyl) methoxy] phenyl]- 6- [5- [[[2- (methylsulfonyl) ethyl] amino] methyl]-2-furanyl]-4-quinazolinamineBis(4-methyl benzenesulfonate) [$C_{29}H_{26}ClFN_4O_4S$ $(C_7H_8O_3S)_2$ H_2O] with molecular weight of 943.38. It is a non-hygroscopic yellow color crystalline solid. It has no chiral centre and exhibits no stereo or geometrical isomerism. It exists as monohydrate, anhydrate and solvate crystal forms. The active substance is in the form of monohydrate with single polymorphic form. Melting range of Lapatinib ditosylate is 240-242 °C. Molecule is slightly acidic with a pH 4.0 in saturated aqueous solution. The salt form (Lapatinib ditosylate) of the drug substance has limited solubility in water (~7 µg/ml). Partition coefficient in octanol-water system at 25 °C is 6.0. UV spectrum of Lapatinib in pH 4.5 buffers at 37 °C was determined by using UV-Visible spectro-photometer. Lapatinb ditosylate monohydrate is a BCS class-II drug and is formulated as an immediate release oral dosage forms containing 250mg of Lapatinib and marketed under the trade name Tykerb.

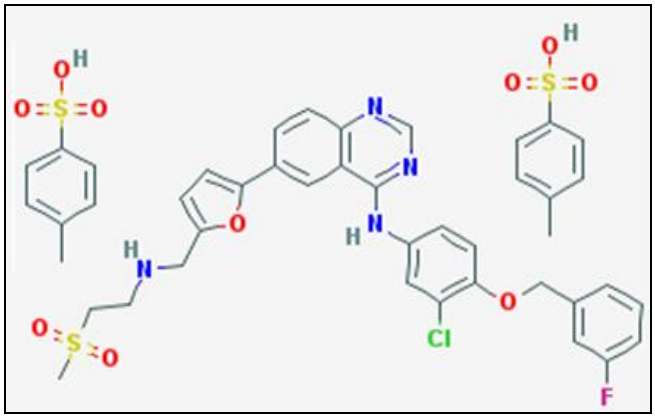

FIG. 1: LAPATINIB DITOSYLATE STRUCTURE

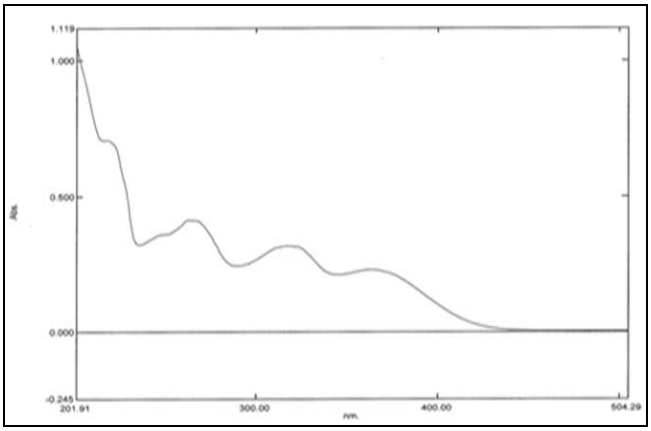

FIG. 2: UV SPECTRUM OF LAPATINIB IN pH-4.5 BUFFER

MATERIALS AND METHODS:

Chemicals, Reagents and Sample: Lapatinib ditosylate drug substance is gifted by synthetic division of Shilpa Medicare Ltd, Hyderabad, India. Tykerb 250mg marketed tablets of lapatitinib drug product were purchased from GSK. Acetonitrile of

HPLC grade was purchased from Rankem chemicals (Mumbai, India). Ammonium formate, sodium hydroxide, formic acid, hydrochloric acid and hydrogen peroxide were purchased from Merck chemicals (Darmstadt, Germany). HPLC grade water was obtained from milli-Q water purification system (Millipore, Milford, USA).

Equipments: The HPLC system used for the chromatographic method development, forced degradation and validation was Agilent-1260 quaternary pump separation module with a PDA detector. HPLC system consisted of quaternary pump G1311C and photodiode array detector G4212B. The signal output was monitored and processed using EZ chrome Elite software on a Lenovo computer. Chromatographic separation was achieved on zorbax eclipse C18 100×4.6 mm, with particle size of 3.5 µm was used. pH of the mobile phase was adjusted on a microprocessor water proof pH tester (pH tester 20, Eutech instruments, Oakton, USA). Thermal degradation study was carried out in a dry hot air oven (Ultra Biotech, Bangalore, India). Ultrasonic bath sonicator was purchased from Cole-parmer (Mumbai, India) and photolytic degradation was carried out on photo stability chamber purchased from Atlas suntester CPS + (Illinois, USA).

Chromatographic Conditions: The objective of the present study is to develop a rapid stability indicating HPLC method for the estimation of impurities of Lapatinib with proper peak shape and resolution. Chromatographic separation was performed on Agilent HPLC with Zorbax eclipse C18 100×4.6 mm, 3.5 µm column. Mobile phase A was 10 mM ammonium formate pH adjusted to 4.5 with formic acid and mobile phase B was acetonitrile. Diluent was prepared by mixing water and acetonitrile in the ratio of 40:60 (v/v). Injection volume was 5 µl, Flow rate was 1.0 ml/min, column oven temperature 40 °C, analysis was carried out at a wavelength of 261 nm with data acquisition time of 35 min.

Preparation of Buffer: Dissolved accurately 0.63 g of ammonium formate in 1000 ml of milli-q water and mixed well further adjusted the pH of the solution to 4.5 ± 0.05 with formic acid. The buffer solution was filtered through 0.22 µm nylon membrane filter.

Preparation of Standard Solution: A working standard stock solution of Lapatinib was prepared by dissolving standard equivalent to 50mg of Lapatinib into 50 ml volumetric flask, to this added 30 ml of diluent and sonicated for 10 minutes at a temperature not exceeding 20 °C. Allowed the solution to attain room temperature and then diluted to the volume with diluent to have a solution with concentration of 1000 ppm.

Preparation of Diluted Standard: Diluted 5 ml of the standard stock solution to 100 ml with diluent and mixed well, further diluted 1 ml of the resulting solution to 100 ml with diluent. The obtained solution is of concentration 0.5 ppm.

Preparation of Sample Solution: Weighed 20 tablets and determined the average weight of the tablets and crush them to a fine powder by using mortar and pestle. Transfer crushed powder equivalent to 50 mg of Lapatinib into 50 ml volumetric flask and added 30 ml of diluent and sonicated in ultrasonic bath for 20 minutes with intermediate shaking at a temperature not more than 20 °C. Allowed the flask to attain room temperature and diluted to the volume with diluent. Filter the solution through 0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution (1 mg/ml).

Preparation of Placebo Solution: Weighed accurately 100 mg of placebo powder into 50 ml volumetric flask added 30 ml of diluent and sonicated in ultrasonic bath for 20 minutes with intermediate shaking at a temperature not more than 20 °C. Allowed the flask to attain room temperature and diluted to the volume with diluent. Filter the solution through 0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution.

Method Validation:

Specificity: Specificity is the ability of the method to measure the analyte response in presence of its potential impurities. Specificity of the developed HPLC method for Lapatinib was carried out in the presence of blank, placebo and its known impurities *i.e.* imp-1, imp-2 and imp-3 for the accurate measurement of amount of impurities present in the sample. As a part of specificity, stress studies [16] were carried out for Lapatinib

ditosylate drug substance, drug product and placebo under stress conditions like oxidation, acid, base, photolytic and thermal (105 °C).

These stress samples were analysed using the proposed method at a test concentration of 1000 ppm to separate all the three Lapatinib impurities along with its degradation impurities at a quantification level of 0.15 %. In these stress conditions the peak purity test was verified for the Lapatinib peak and its other known impurities by using diode array detector.

Precision: Precision of the analytical method is the closeness agreement for a series of measurement from multiple samplings as mentioned in ICH Q2 (R1). As per the guidelines [17-21], method precision and intermediate precision were analyzed on the homogeneous sample and the % RSD of individual impurity for precision and intermediate precision was calculated and reported.

LOD and LOQ: The detection limit (LOD) and quantification limit (LOQ) for all the three impurities were established by means of linearity method. The impurity solutions from concentration ranging from 0.05 ppm to 0.5 ppm with 5 different levels were prepared and injected. Based on the impurity response and STEYX value, the least concentration of each impurity up to which it can be identified and quantified were calculated and verified.

Linearity: Linearity of the detector response was established for all the known impurities and Lapatinib with concentration ranging from LOQ to 150% of the specification level (0.15%) with respect to test concentration. The samples were analyzed as per the described test method. A linearity graph was plotted between the responses of impurity (Y-axis) against actual concentration in ppm (X-axis) and determined the correlation co-efficient and Y-intercept at 100% response.

Accuracy: Accuracy of the analytical method is the closeness of agreement between the true value and experimental value. Accuracy of the three impurities was performed at 5 different levels ranging from LOQ to 150 % of the specification level of the impurity with respect to test concentration level. The % recovery was calculated by comparing the impurity level at each level of spiked sample with as such sample.

Robustness: The robustness of the method was evaluated to establish the capability of the method by changing the experimental conditions and studying its impact on the system suitability [22]. Robustness was performed by changing the method parameters like mobile phase flow rate and column temperature.

RESULTS AND DISCUSSION:

Method Development and Optimization: As there was no stability indicating HPLC method available for the determination of related substances in Lapatinib drug product with proper peak shapes, the objective of the current method was to separate all the potential impurity peaks arise during the forced degradation study and stability studies with proper peak shape and resolution. For the optimization of the HPLC method, forced degradation sample was taken as reference. There was spectral co-elution of impurities on different stationary phases like C8, C18 and phenyl with different buffer ratios and organic modifiers like methanol and acetonitrile.

Initial trials were taken on pH - 3.0 phosphate buffer with acetonitrile as mobile phase and test concentration of 1000 ppm in mobile phase was injected in which there was no clear separation between the impurities and Lapatinib. Further trials were taken by varying the pH value of the mobile phase buffer from 3.0 to 4.5. *i.e.*, ammonium formate buffer was selected as a mobile phase as it LC-MS compatible and also having maximum buffering capacity at its pKa (pKa of ammonium formate is 4.5).

Forced degradation samples were injected and found that all the three known impurities (Impurity-1, Impurity-2 and Impurity-3) were spectrally pure with longer run time and broader peak shapes in isocratic mode. In order to shorten the run time gradient separation mode was optimized with satisfactory separation. Optimal separation was achieved on Zorbax Eclipse C18 100×4.6 mm, 3.5 µm HPLC column maintained at 40 °C. Gradient elution was performed using the mixture of 10 mM ammonium formate buffer pH - 4.5 (pH was adjusted with formic acid) and acetonitrile as

organic modifier at a flow rate of 1.0 mL/min. HPLC detection was carried out at a wavelength of 261 nm. Sample compartment was maintained at a temperature of 5 °C. Gradient programme was mentioned in **Table 1**. Mobile phase A is 10 mM Ammonium formate in milli-q water. Mobile phase B is 100% Acetonitrile.

TABLE 1: GRADIENT PROGRAMME FOR HPLC METHOD

Time (minutes)	Flow rate (ml / min)	% of mobile phase-A	% of mobile phase-B
0	1	60	40
8	1	60	40
19	1	42	58
26	1	10	90
29	1	10	90
30	1	60	40
35	1	60	40

System Suitability: System suitability solution was prepared and injected to evaluate the system suitability of the method and found that Lapatinib and its 3 known impurities were separated with good resolution, with typical retention times of 15.21, 17.46, 21.11 and 23.76 respectively. Chromatogram with separation of impurities was mentioned in **Fig. 3**. The system suitability results were given in **Table 2**. The developed HPLC method was found to be specific for Lapatinib and its known impurities namely **Fig. 3** shows separation of all the three known impurities from Lapatinib in the proposed method.

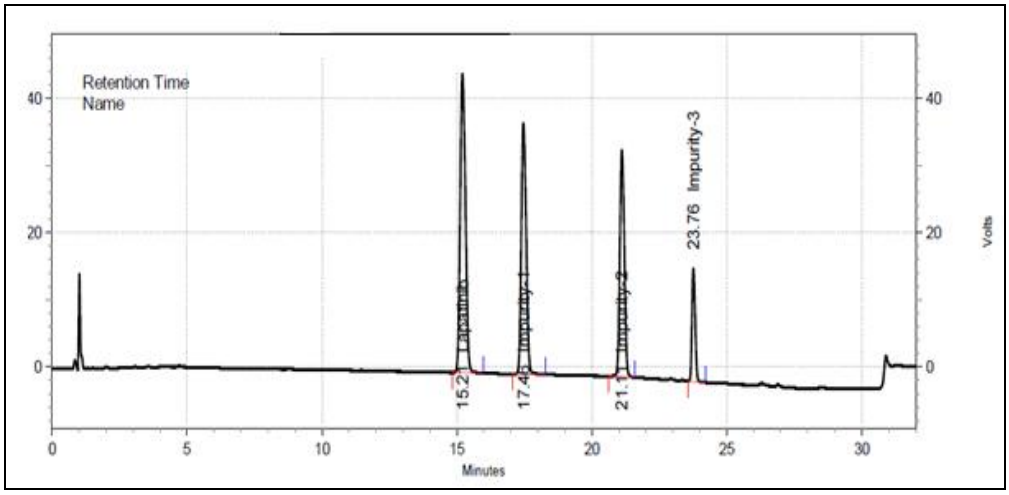

FIG. 3: SYSTEM SUITABILITY CHROMATOGRAM

TABLE 2: SYSTEM SUITABILITY RESULTS

System suitability					
Name	**Retention time (min)**	**RRT**	**Theoretical plates**	**Asymmetry**	**Resolution**
Lapatinib	15.21	1.00	8220	1.17	NA
Impurity-1	17.46	1.15	12645	1.18	6.4
Impurity-2	21.11	1.39	12164	1.14	11.2
Impurity-3	23.76	1.56	18064	1.17	9.9

RRT: Relative retention time

Precision: Method precision was performed by analysing the spiked sample with known impurities at the specification level (0.15%) and % impurity was calculated. The % relative standard deviation was found to be 1.02, 0.74 and 1.17 for impurity-1, impurity-2 and impurity-3 respectively. Intermediate precision was performed on a different day, different HPLC system using different HPLC column of the same manufacturer. The relative standard deviation for these spiked samples was also within 2.0% limit. The results were mentioned in **Table 3**. Hence the developed method is precise for its intended use.

TABLE 3: METHOD PRECISION AND INTERMEDIATE PRECISION RESULTS

Method precision				Intermediate precision			
	% impurity				% impurity		
Sl. No	Imp-1	Imp-2	Imp-3	Sl. No	Imp-1	Imp-2	Imp-3
1	0.149	0.151	0.148	1	0.152	0.148	0.152
2	0.148	0.150	0.152	2	0.151	0.149	0.153
3	0.147	0.152	0.152	3	0.152	0.149	0.151
4	0.151	0.151	0.149	4	0.15	0.151	0.151
5	0.147	0.149	0.152	5	0.149	0.150	0.148
6	0.149	0.150	0.15	6	0.148	0.151	0.151
Avg	0.149	0.150	0.151	Avg	0.150	0.150	0.151
SD	0.002	0.001	0.002	SD	0.002	0.001	0.002
% RSD	1.0	0.7	1.2	% RSD	1.1	0.8	1.1
		Cumulative average			0.149	0.150	0.151
		Cumulative SD			0.002	0.001	0.002
		Cumulative % RSD			1.2	0.8	1.1

LOD and LOQ: Limit of detection and limit of quantification were established by means of slope method. Five different concentration of Lapatinib and its impurity mixture (imp-1, imp-2 and imp-3) below the specification limit of 0.15% were prepared and injected. A calibration curve was plotted between concentration and response of the individual peaks. Slope and STEYX value were calculated for the calibration curve. The results were tabulated in **Table 4**. From these values limit of detection (LOD) and limit of quantification (LOQ) values were calculated. The solution mixture of Lapatinib and its impurities were prepared and injected to confirm the calculated LOQ values. The LOQ mixture chromatogram was mentioned in **Fig. 4**.

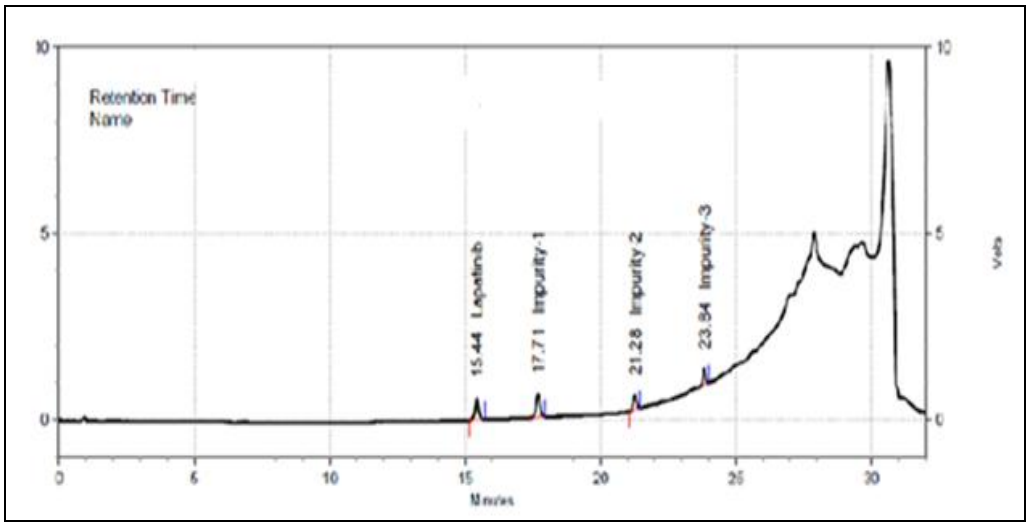

FIG. 4: LOQ MIXTURE SOLUTION

TABLE 5: ESTABLISHMENT OF LOD AND LOQ

		Area response			
Sl. No	Concentration (ppm)	Lapatinib	Impurity-1	Impurity-2	Impurity-3
1	0.05	125462	50245	76542	90215
2	0.1	252145	105610	151532	188491
3	0.2	509854	204581	311023	365698
4	0.3	767851	305498	451037	552389
5	0.5	1248752	502345	765008	900321
Correlation coefficient		0.9998	0.9999	0.9999	1.0000
Slope		2501992	1000837.3	1525916.7	1797190.9
STEYX		9177.5	2666.5	5318.7	6059.6
Limit of detection (LOD) in ppm		0.01	0.009	0.01	0.01
Limit of quantification (LOQ) in ppm		0.04	0.03	0.03	0.03

Linearity and Range: Linearity of the developed method was evaluated for seven different levels of Lapatinib and its 3 known impurities. The concentrations ranged from LOQ to 200 % of impurity specification limit (0.15% with respect to test concentration). The respective peak area was recorded and plotted against standard (impurity) concentration and the graph resulted in straight line. The correlation coefficient, slope, intercept and % Y-intercept values were calculated and tabulated for Lapatinib and its known impurities. The compiled results were tabulated below in **Table 5**.

TABLE 5: LINEARITY ESTABLISHMENT OF IMPURITIES AND LAPATINIB

	Lapatinib		Impurity-1		Impurity-2		Impurity-3	
Sl. No	Conc. (ppm)	Area	Conc. (ppm)	Area	Conc. (ppm)	Area	Conc. (ppm)	Area
1	0.04	41821	0.03	22015	0.03	50116	0.03	76683
2	0.25	264651	0.3	221908	0.1	150348	0.1	230049
3	0.5	564764	0.5	393200	0.5	754820	0.5	1138743
4	1	1106611	1	785426	1	1471899	1	2277584
5	1.5	1555544	1.5	1163553	1.5	2262312	1.5	3386992
6	2	2211426	2	1534565	2	2943862	2	4555100
7	3	3204003	3	2321542	3	4412697	3	6632752
Correlation coefficient		0.9994		0.9999		0.9999		0.9998
Slope		1071249		772980		1470541		2222470
Y- Intercept		9737		469		12936		32746
% Y-Intercept		0.88		0.06		0.88		1.44

Accuracy: Recovery studies were performed to judge the accuracy of the test method. The study was evaluated by spiking the known quantity of impurities at various levels on the placebo. From the amount of impurity found the % recovery was calculated. Recovery was performed at five different levels ranging from LOQ to 150 % of the specification level. The % recovery of each impurity was found to be within the acceptance criteria of 95 % to 105 %. So the method is accurate for the determination of Lapatinib and its impurities. The mean recovery values for the impurities were tabulated in **Table 6**.

TABLE 6: AVERAGE RECOVERY VALUES OF IMPURITIES AT DIFFERENT LEVELS

		% Mean recovery ± SD		
Sl. No	Concentration level	Impurity-1	Impurity-2	Impurity-3
1	LOQ	97.42 ± 0.52	99.87 ± 0.33	100.42 ± 0.24
2	25%	98.63 ± 0.48	98.70 ± 0.29	101.90 ± 0.33
3	50%	100.02 ± 0.34	99.16 ± 0.15	100.62 ± 0.62
4	100%	99.26 ± 0.34	100.14 ± 0.05	98.06 ± 0.44
5	150%	101.20± 0.16	101.11± 0.12	102.35± 0.28

Solution Stability: There was no change in the area counts of the Lapatinib and its respective impurities, when both the standard and sample solutions were monitored periodically for a period of 48 hrs at room temperature (not more than 27 °C) and at a temperature of 5 ± 3 °C using related substances method.

Specificity: The specificity of the method was evaluated by verifying the peak purity of the sample. The method was found to be specific as there was no interference from blank and placebo at the retention time of main peak. No degradant peaks were observed at the retention time of Lapatinib during the degradation and stability study indicates that the method is stability indicating and also peak purity index for all the impurities and Lapatinib was >999 indicates that there was no spectral co-elution for any of the peaks in this method and also the resolution between the neighbouring peak was greater than 2.0. Peak near to dead volume in sample chromatogram was observed as Tosylate peak in the sample. Typical chromatograms of (a) blank (b) placebo (c) standard and (d) sample were mentioned in **Fig. 5**.

Forced Degradation Studies: Forced degradation studies were performed to establish the stability indicating power of the method. In this study Lapatinib raw material, finished product and

placebo were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 1 mg/ml in diluent. Sample equivalent to 50mg of Lapatinib was placed into 50 ml volumetric flask added 30 ml of diluent and sonicated for 20 min with intermediate shaking at a temperature not more than 20 °C and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralised after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress conditions under which the study was performed, the amount of Lapatinib remains, % impurities generated and mass balance results were tabulated in **Table 7**. The chromatograms for forced degradation study were summarized **Fig. 6**.

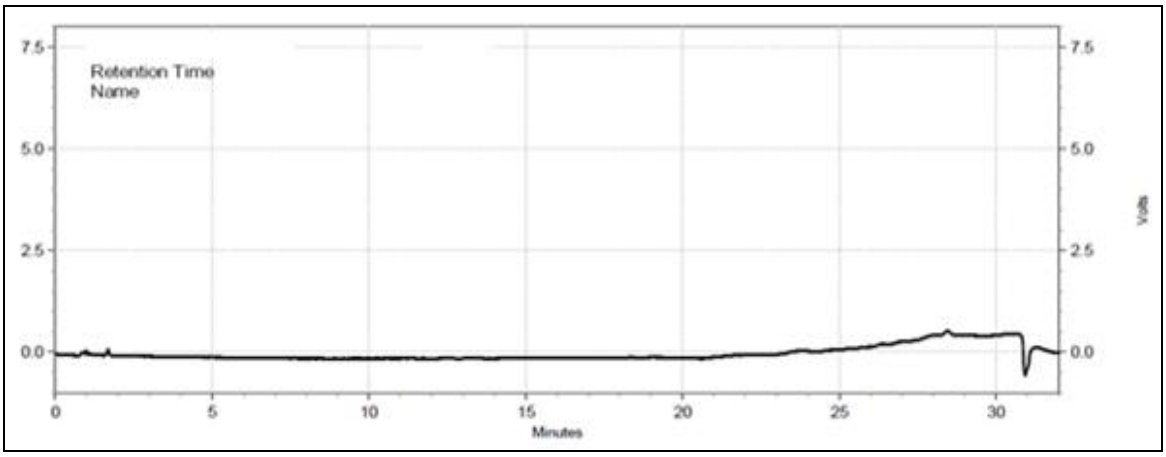

FIG. 5A: TYPICAL CHROMATOGRAM OF BLANK

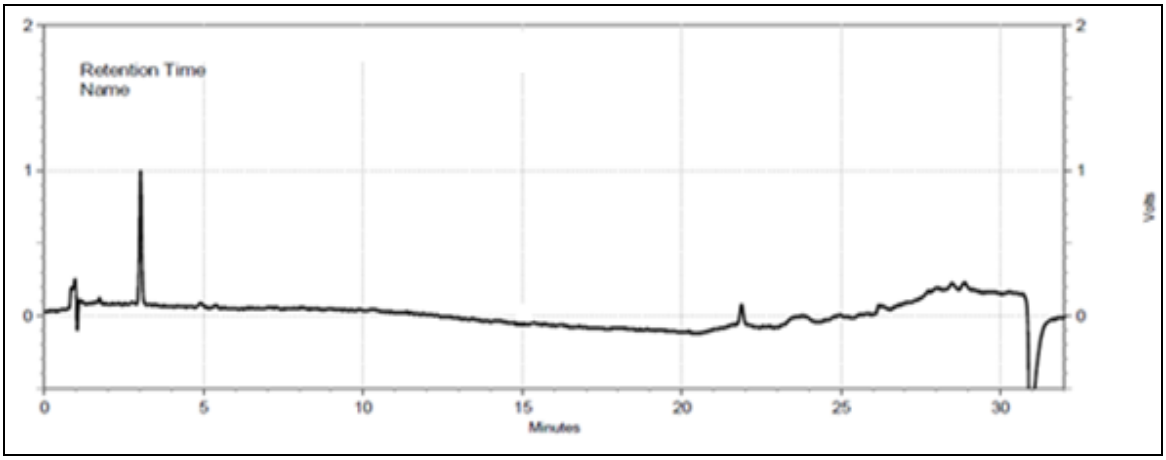

FIG. 5B: TYPICAL CHROMATOGRAM OF PLACEBO

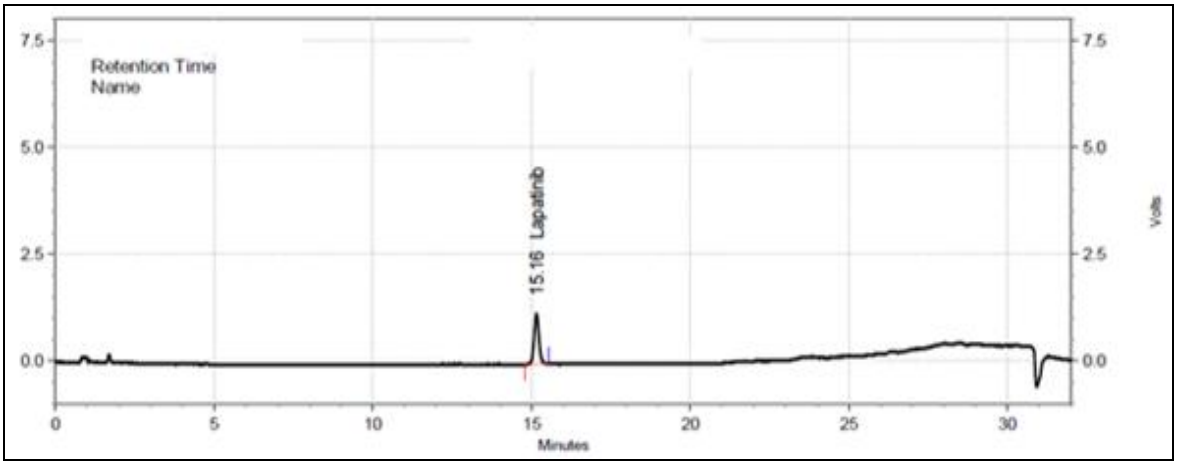

FIG. 5C: TYPICAL CHROMATOGRAM OF STANDARD

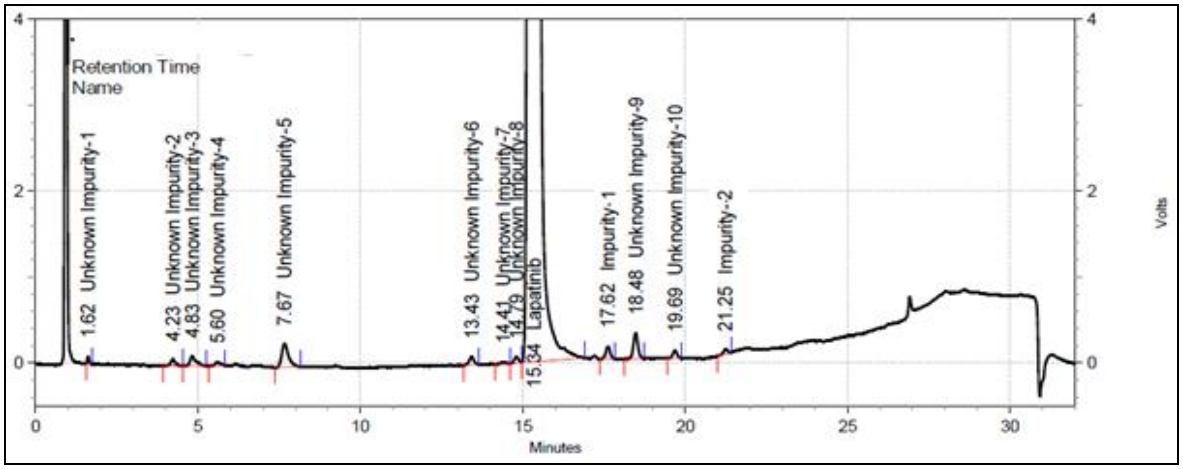

FIG. 5D: TYPICAL CHROMATOGRAM OF SAMPLE

TABLE 7: STRESS CONDITIONS AND ITS RESULTS

Sl. No	Stress condition	% Drug remained	% impurities	Mass Balance
1	2N HCl_60 °C_8 Hrs	82.4	17.6	97.6
2	1 N NaOH_60 °C_4 Hrs	78.6	21.4	96.8
3	10 % H2O2_60 °C_24 hrs	81.9	18.1	95.2
4	105 °C_48 Hrs	97.4	2.6	98.9
5	Photolytic stability	98.2	1.8	99.4

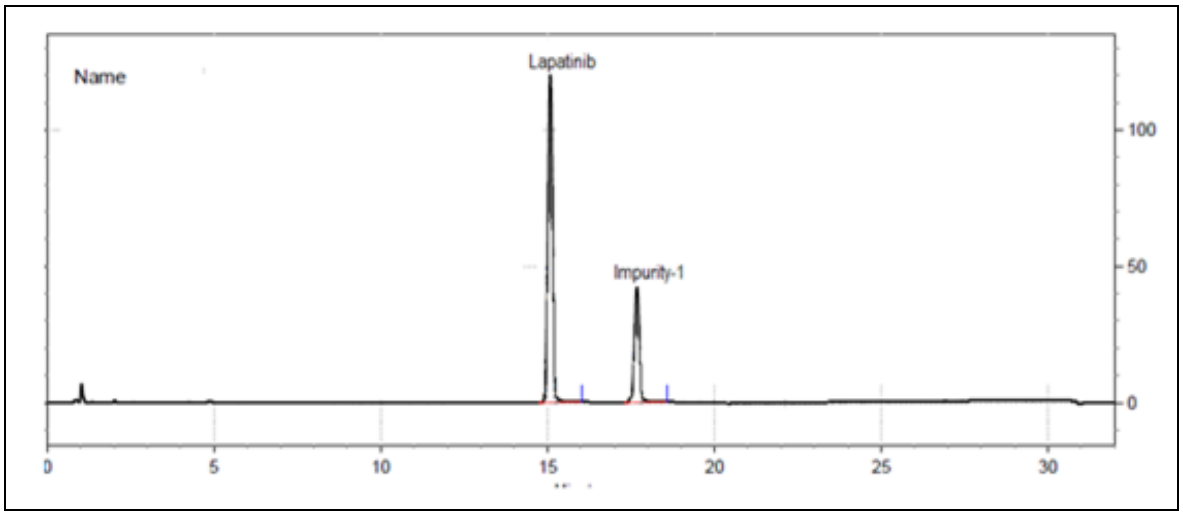

FIG. 6A: TYPICAL CHROMATOGRAM OF ACID DEGRADATION

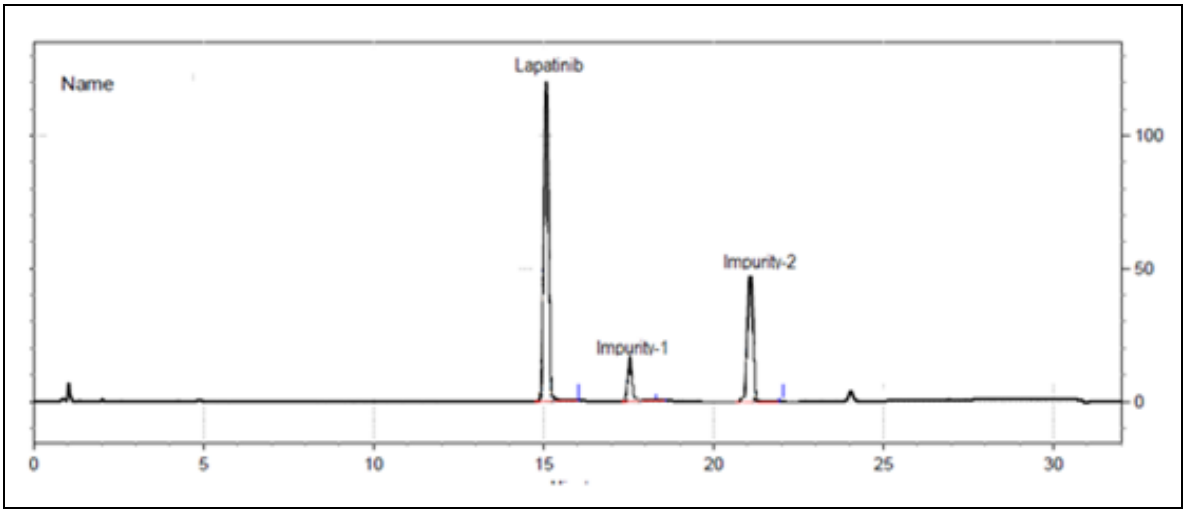

FIG. 6B: TYPICAL CHROMATOGRAM OF BASE DEGRADATION

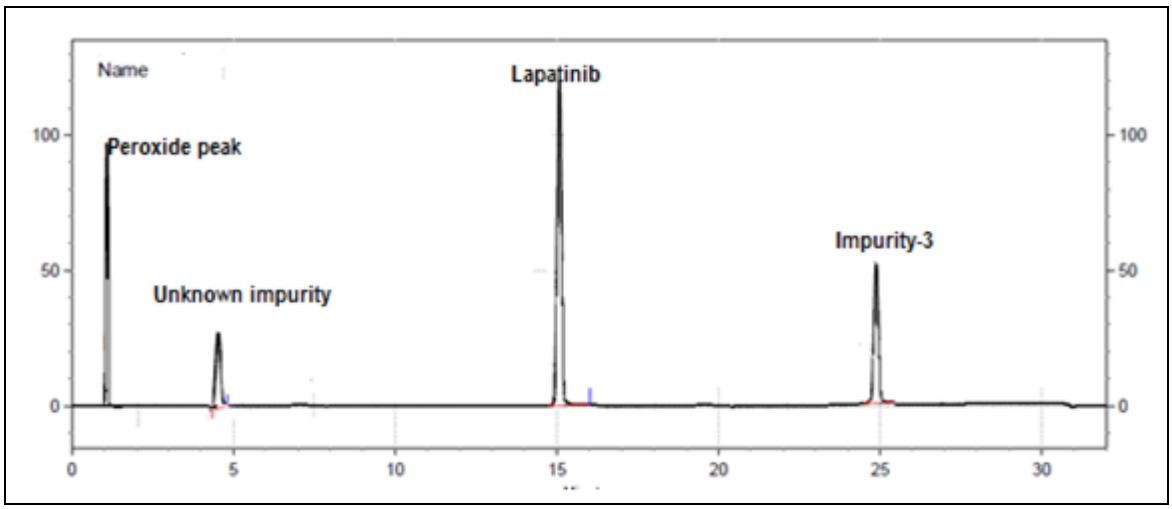

FIG. 6C: TYPICAL CHROMATOGRAM OF PEROXIDE DEGRADATION

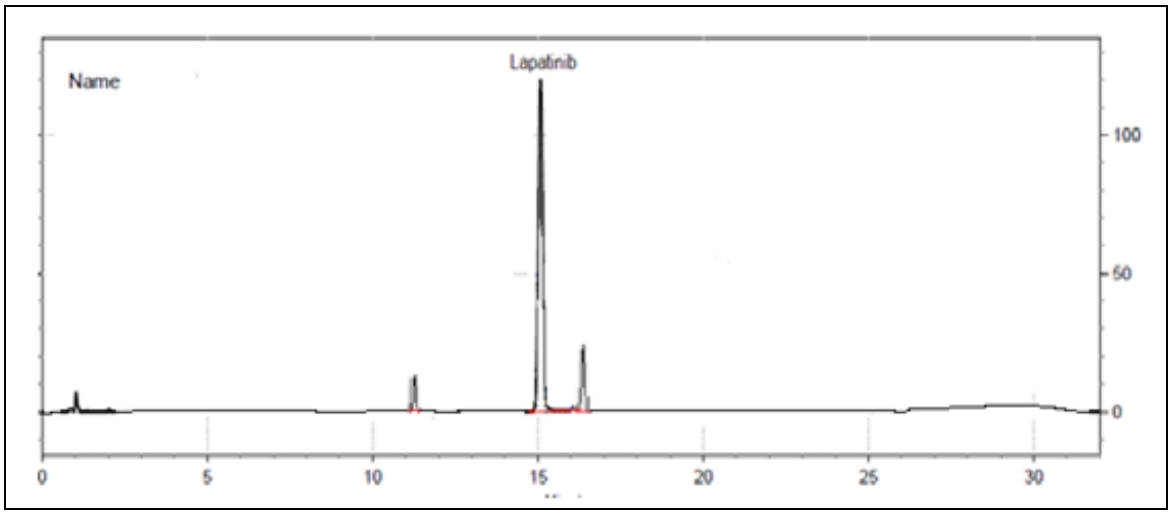

FIG. 6D: TYPICAL CHROMATOGRAM OF THERMAL DEGRADATION

DISCUSSION: The proposed HPLC method obeys linearity within the concentration range of 0.03-3.0ppm for impurity-1, impurity-2 and impurity-3 with correlation coefficient of 0.999 for all the three impurities. The detection limit and quantification limits as LOD values for the three impurities are 0.009, 0.01 and 0.01 ppm respectively and LOQ values are 0.03, 0.03 and 0.03 ppm respectively. Inter and intraday precision with cumulative % RSD for the impurities were found to be 1.2, 0.8 and 1.1 respectively for each impurity. % Recovery values for the three impurities were found to be between 97.4 and 102.4 for the concentration range between LOQ and 150% of the test concentration (1 mg/ml).

CONCLUSION: A novel, reverse phase liquid chromatographic method has been developed and validated for the estimation of Lapatinib and its known impurities namely impurity-1, impurity-2 and impurity-3 using LC-MS compatible HPLC method. The proposed method is found to be simple, accurate, precise, linear, specific and robust. Hence it can be used for the determination of Lapatinib and its related substances in bulk and in finished formulations.

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