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Development and Validation of Stability-Indicating Reverse Phase HPLC Method for the Determination of Related Substances in Neratinib Maleate Drug Substance

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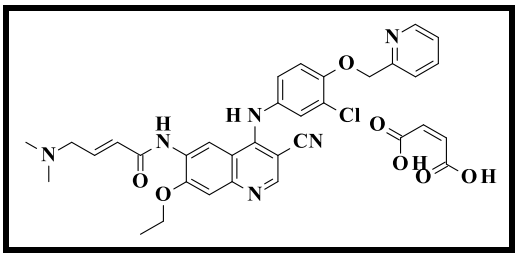
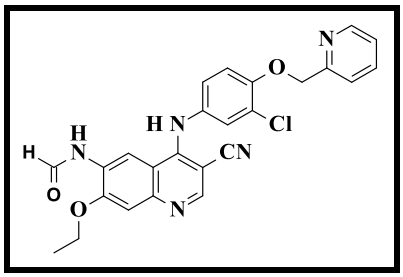
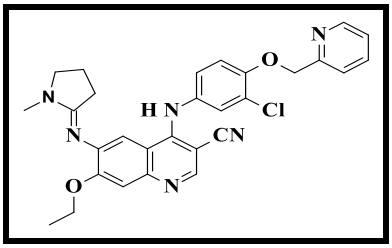
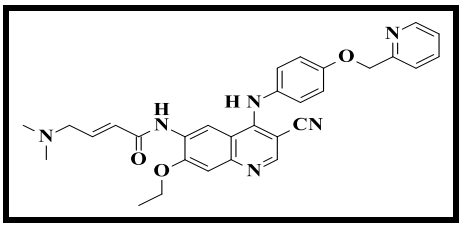
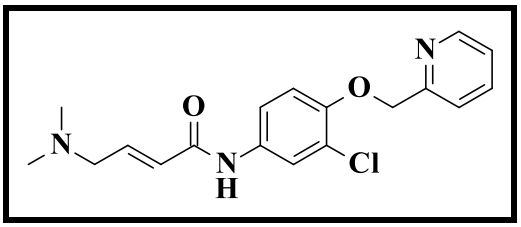
ABSTRACT

A gradient reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination of related substances of Neratinib maleate. The successful chromatographic separation of Neratinib maleate from its related substances was achieved on octadecyl silane chemically bonded to porous silica particles stationary phase i.e. X-Bridge C-18, 250mm x 4.6mm, i.d., 5µ column maintained at 55°C using phosphate buffer pH 2.5 and acetonitrile as mobile phases A & B respectively. Wavelength for UV detection: 265nm, flow rate: 0.9ml/min and Injection volume: 10µl. The performance of the method was validated according to the ICH guidelines for specificity, linearity, accuracy, precision, the limit of quantification and limit of detection. Neratinib was subjected to stress conditions of thermal, hydrolysis, humidity, peroxide and photolytic to observe the degradation products. Limit of detection of impurities was in the range of 0.007%–0.010% indicating the high sensitivity of the developed method. The experiment results are given in detail in this paper.

INTRODUCTION

Neratinib Maleate is a drug substance. Nerlynx is a brand *name* of Neratinib. Neratinib maleate is approved to treat: Breast cancer that is early-stage and HER2 positive. It is used as extended adjuvant therapy in patients who have already been treated with trastuzumab after surgery. A clinical trial demonstrated that Neratinib and Temsirolimus in combination produced responses in 19% of patients ranging from 2 to 18+ months in patients with HER2-mutant lung cancers. The empirical formula of Neratinib maleate is $C_{30}H_{29}ClN_6O_3.C_4H_4O_4$ and the molecular weight is 673.11 g/mol (Maleate salt), 557.0 (free base) and Neratinib is chemically known as (*E*)-*N*-[4-[3-Chloro-4-[(2-pyridinyl)methoxy]anilino]-3-cyano-7-ethoxy-6-quinolinyl]-4-(dimethylamino)-2-butenamide maleate. There are several processes and degradation impurities of Neratinib, which originated during the synthesis process and as well as degradation during stability studies or on storage. Neratinib is a very novel and recently synthesized drug. It is not listed in Pharmacopoeias. Best our knowledge, HPLC literature methods, and Pharmacopoeia methods are not available for the determination of Neratinib and its related substances. However, a few methods have been reported in the literature for the quantitation of the tyrosine kinase inhibitor Neratinib in human plasma by LC-MS/MS, published in 2016 [1]. UPLC-MS/MS Tandem mass spectrometric method Development and Validation for determination of Neratinib in Human Plasma. The method was performed on a C18Acquity UPLC BEH ™ column, published in 2015 [2]. The reported methods are related to Tandem mass spectrometric UPLC and LC-MS methods. Hence, the stability-indicating RP-HPLC method has been developed for the quantification of impurities related to Neratinib. The limit of each impurity is considered a 0.15% level by ICH guidelines based on a daily intake of 240 mg of Neratinib maleate [3-10]. The developed chromatographic method can resolve all these impurities with adequate resolution to achieve good chromatography and the optimized methodology has been validated to accomplish ICH guidelines on validations [11]. The chemical structures of Neratinib maleate and its free base (NET-I) related impurities [Impurity- A to E and APQ, APQ-Impurity-A] are shown in Fig.1.

Neratinib maleate (NET)	(*E*)-*N*-[4-[3-Chloro-4-[(2-pyridinyl)methoxy]anilino]-3-cyano-7-ethoxy-6-quinolinyl]-4-(dimethylamino)-2-butenamide maleate Mol. weight:673.11 Mol. formula: $C_{30}H_{29}ClN_6O_3.C_4H_4O_4$	
NET-I/Impurity-A	*N*-[4-[[3-chloro-4-(2-pyridinylmethoxy)phenyl]amino]-3-cyano-7-ethoxy-6-quinolinyl]formamide Mol. weight: 473.91 Mol. formula: $C_{25}H_{20}ClN_5O_3$	
NET-I/Impurity-B	4-[[3-chloro-4-(2-pyridinylmethoxy)phenyl]amino]-7-ethoxy-6-[(1-methyl-2-pyrrolidinylidene)amino]-3-quinolinecarbonitrile Mol. weight: 527.02 Mol. formula: $C_{29}H_{27}ClN_6O_2$	
NET-I/Impurity-C	(2*E*)-*N*-[3-cyano-7-ethoxy-4-[[4-(2-pyridinylmethoxy)phenyl]amino]-6-quinolinyl]-4-(dimethylamino)-2-butenamide Mol. weight: 522.60 Mol. formula: $C_{30}H_{30}N_6O_3$	
NET-I/Impurity-D	(*E*)-*N*-[3-chloro-4-(2-pyridylmethoxy)phenyl]-4-(dimethylamino)but-2-enamide Mol. weight: 345.82 Mol. formula: $C_{18}H_{20}ClN_3O_2$	

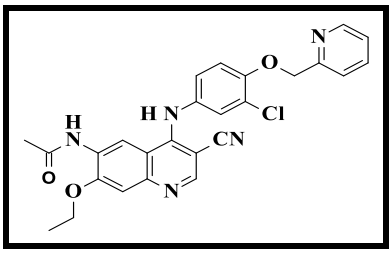
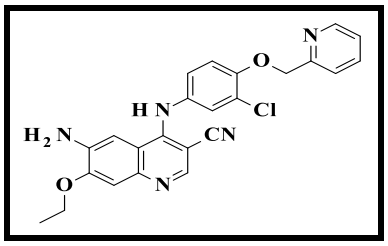
APQ/Impurity-A (Raw material impurity)	N-[4-[[3-chloro-4-(2-pyridinylmethoxy)phenyl]amino]-3-cyano-7-ethoxy-6-quinolinyl]acetamide Mol. weight: 487.94 Mol. formula : $C_{26}H_{22}ClN_5O_3$	
APQ (Raw material)	6-amino-4-[[3-chloro-4-(2-pyridinylmethoxy)phenyl]amino]-7-ethoxy-3-quinolinecarbonitrile Mol. weight : 445.90 Mol. formula: $C_{24}H_{20}ClN_5O_2$	

Figure No. 1: Chemical structures of Neratinib and its impurities

MATERIALS AND METHODS

Chemicals, reagents, standards, and samples

The investigated samples of Neratinib drug substance, its related impurities and Neratinib for system suitability were arranged from Natco Research Centre (A division of Natco Pharma Ltd., Hyderabad). HPLC grade of Potassium dihydrogen orthophosphate, Acetonitrile, and Methanol was procured from Merck, India and Orthophosphoric acid (~88%) was procured from Rankem and pure milli-Q water was used with the help of Millipore purification system (Millipore®, USA).

Instrumentation and methodology

The HPLC system used for method development, method validations as well as forced degradation studies on Waters Alliance 2695 separation module equipped with 2996 photodiode array detector and UV detector with Empower data handling system i.e. Empower 3 software, Build No: 2154 [Waters Corporation, MILFORD, MA 01757, USA] was used. HPLC column: X-Bridge C-18, 5µ (250mm × 4.6mm) (Make: Waters)], column oven temperature: 55°C, Sample cooler:5°C, Mobile phase A: Dissolve 2.72 g of Potassium dihydrogen orthophosphate in 1000 ml of water, adjust pH to 2.5±0.05 with orthophosphoric acid and filter this solution through 0.45µ membrane and degas it. Mobile phase B: Acetonitrile. Diluent: a mixture of water and acetonitrile in the ratio of 50:50%v/v. Flow rate:

0.9 ml/min, injection volume: 10µl, data acquisition time: 60 min and UV detection: 265 nm. The retention time of Neratinib: about 21 minutes. The pump is in gradient mode and the program is as follows: Time (min)/ A (v/v): B (v/v); T0.01/98:2,T3/98:2, T10/80:20, T20/78:22, T30/75:25, T40/60:40, T50/30:70, T52/98:2, T60/98:2.

Preparation of solutions:

Stock solution-1: Dissolve each 15mg of Neratinib impurity-A, impurity-B, impurity-C impurity-D, impurity-E, APQ, and APQ/ Impurity-A standards into a 50 mL volumetric flask, add 10 mL of diluent to dissolve and sonicate, then make up to volume with diluent.

Stock solution-2: Dissolve separately 15 mg of Neratinib maleate standard into a 50 mL volumetric flask, add 10 mL of diluent to dissolve and sonicate, then make up to volume with diluent.

Reference solution:

Prepare a solution by transferring each 0.15 mL of stock solution-1 and 0.1 mL of stock solution-2 into a 100mL volumetric flask, and add diluent and mix the solution, then make up to the mark with diluent.

System suitability solution:

Dissolve 15 mg of Neratinib maleate standard into a 50 mL volumetric flask, add 0.15 mL of stock solution-1 then make up to volume with diluent.

Evaluation of system suitability:

Inject blank followed by six replicate injections of the reference solution and one system suitability solution into the HPLC system and record the chromatograms. The system is suitable for analysis if and only if,

1. The relative standard deviation for each component and Neratinib should be not more than or equal to 5.0 % from the reference solution.

2. Theoretical plates for Neratinib peak in system suitability solution should be not less than 5000.

3. USP tailing for Neratinib peak in system suitability solution should be no more than 2.0.

4. Resolution between Neratinib and impurity-E peaks in system suitability solution should be not less than 1.5.

Sample solution:

Dissolve 30 mg of Neratinib maleate test sample into a 100 mL volumetric flask, dissolve with diluent and sonicate, then make up to volume with diluent.(0.3 mg/ml concentration).

RESULTS AND DISCUSSION

Method Validation

Specificity:

Specificity is the ability to assess unequivocally of the analyte in the presence of components which may be expected to be present. For the determination of specificity, injection of blank, all individual impurities solutions were prepared and injected to confirm the retention times. The solutions of Neratinib maleate drug substance (Control Sample) and Neratinib maleate spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. The specificity results are tabulated in Table 1. A typical representative HPLC chromatogram of Neratinib maleate drug substance spiked with all impurities is shown in Fig.2.

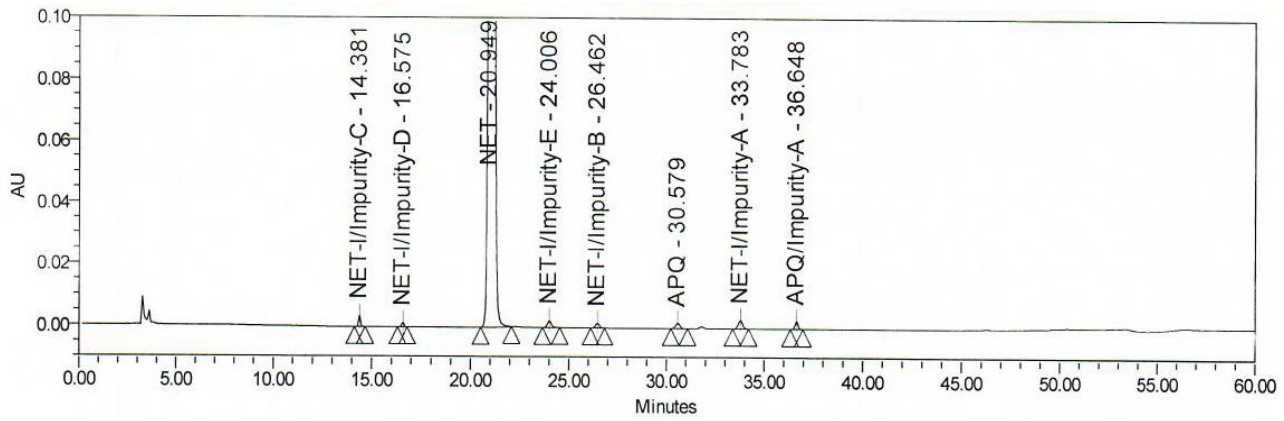


Figure No. 2: A typical representative HPLC chromatogram of Neratinib Maleate drug substance spiked with all Impurities.

Table No. 1: Specificity of impurities from system suitability solution

Peak Name	Retention Time (Minutes)	Relative retention time (RRT)	Peak Purity	
			Purity angle	Purity Threshold
NET-I/Impurity-C	14.703	0.671	0.729	0.982
NET-I/Impurity-D	16.966	0.774	0.841	1.370
NET-I/Impurity-E	25.190	1.149	0.732	1.226
NET-I/Impurity-B	27.777	1.267	1.029	1.743
APQ	31.719	1.447	0.729	1.327
NET-I/Impurity-A	35.335	1.612	0.649	0.946
APQ/Impurity-A	37.651	1.717	0.589	1.044
Neratinib Maleate	21.924	1.00	0.233	0.267

Forced degradation:

The degradation behavior of the Neratinib maleate has been studied by performing forced degradation studies. Neratinib maleate was subjected to different stress conditions [12] i.e acid/base hydrolysis [2N HCl /RT/6hrs/60°C /6 hrs & 0.1N NaOH/ 60°C /1 hr], peroxide degradation under oxidative stress [5%w/v hydrogen peroxide solution, 6hrs], thermal degradation [105°C/24Hours], UV Solid and solution/24 hours, Peak purity of Neratinib peak was established by using PDA detector in these stress samples. The forced degradation results are tabulated in Table 2. The typical representative HPLC chromatograms of forced degradation experiments are shown in Fig.3-9.

Table No. 2: Degradation study Results

Name of the impurity	RRTs at	The control sample (%)	2N HCl Solution RT 6 hrs	2N HCl solutions 60°C 6hrs	0.1N NaOH solutions 60°C 1hr	5% H_2O_2 solution RT 6hrs	UV solution 24 hrs	UV solid 24 hrs	Heat at 105°C 24 hrs
NET-I/Imp-C	0.671	ND	ND	0.13	0.01	ND	0.01	ND	ND
NET-I/Imp-D	0.774	ND	ND	ND	ND	ND	ND	ND	ND
NET-I/Imp-E	1.149	0.06	ND	0.03	10.74	0.07	0.08	0.06	0.18
NET-I/Imp- B	1.267	ND	ND	ND	0.01	ND	ND	ND	ND
APQ	1.447	ND	0.08	6.56	0.36	ND	ND	ND	ND
NET-I/Imp-A	1.612	0.07	ND	ND	0.07	0.07	0.07	0.07	0.07
APQ/Imp-A	1.717	0.02	0.02	ND	0.03	0.03	0.03	0.02	0.15
Unknown 1	1.525	0.05	0.05	0.05	ND	0.05	0.05	0.05	0.05
Unknown 2	1.063	ND	ND	0.25	5.22	0.16	ND	ND	ND
Unknown 3	0.911	ND	ND	0.12	ND	ND	ND	ND	ND
Unknown 4	0.957	ND	ND	ND	0.06	ND	ND	0.04	ND
Unknown 5	1.828	ND	0.07	0.07	ND	ND	ND	ND	ND
Purity	1.000	99.79	99.76	92.78	83.50	99.61	99.77	99.73	99.54
Mass balance	NA	100.00	100.00	95.47	96.64	98.64	100.04	101.07	101.72

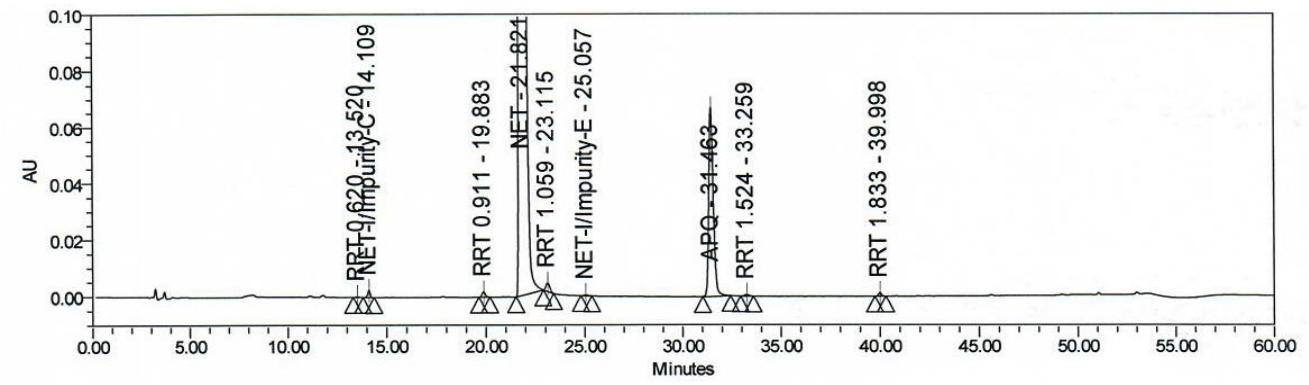


Figure No. 3: Typical chromatogram for 2.0 N HCl at 60°C 6 hrs

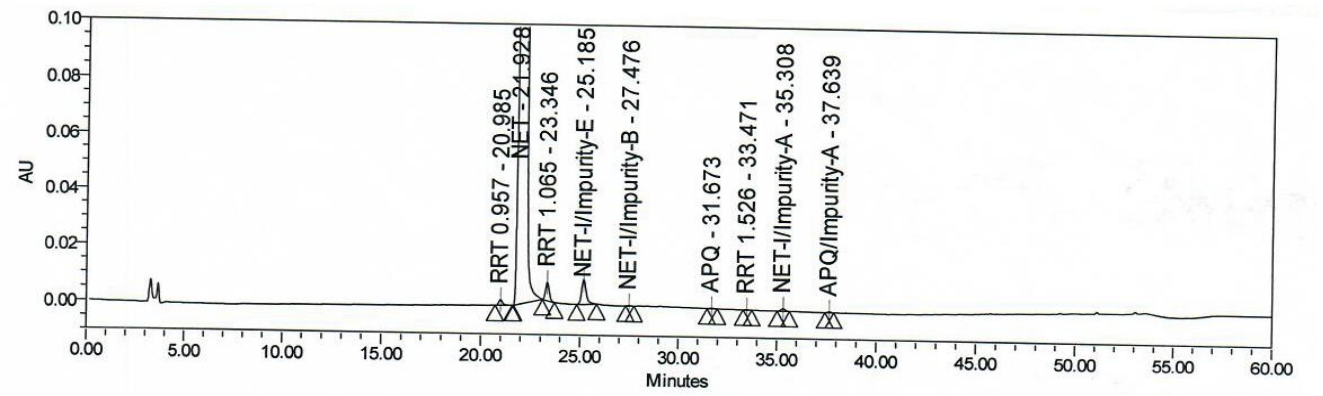


Figure No. 4: Typical chromatogram for 0.1 NaOH RT 6 hrs

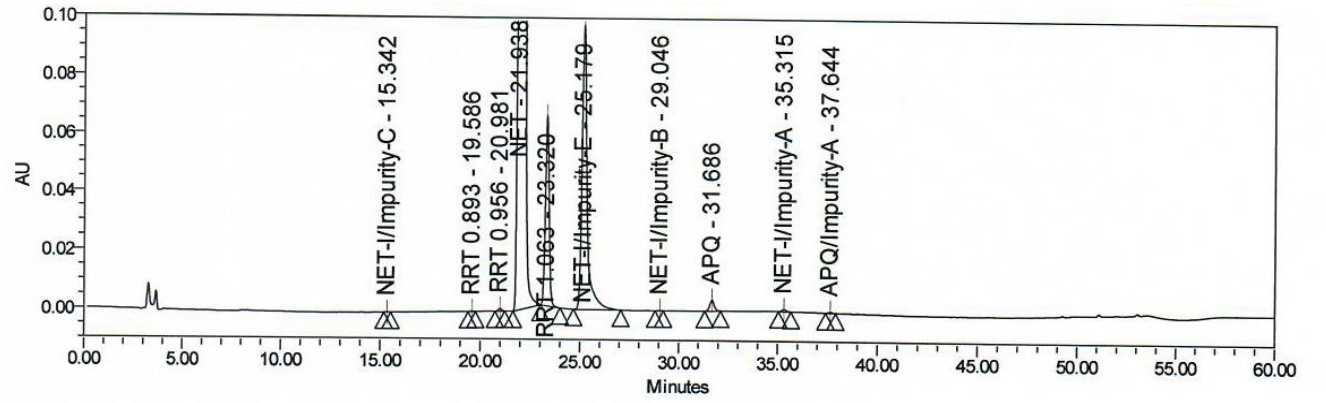


Figure No. 5: Typical chromatogram for 0.1 N NaOH at 60°C 1 hr

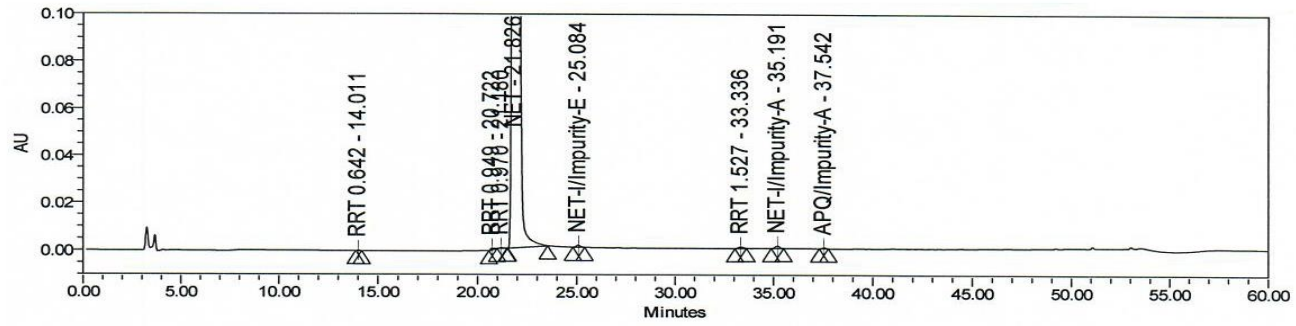


Figure No. 6: Typical chromatogram for Solid at UV 24 hrs

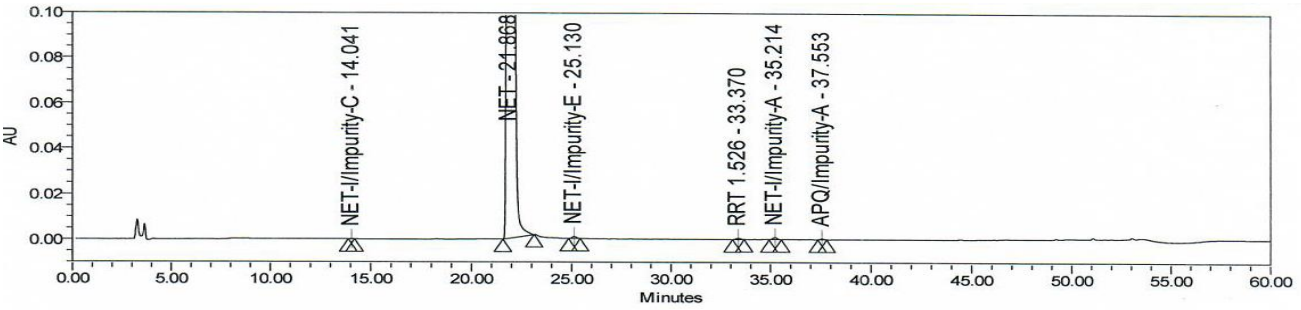


Figure No. 7: Typical chromatogram for Solution at UV 24 hrs

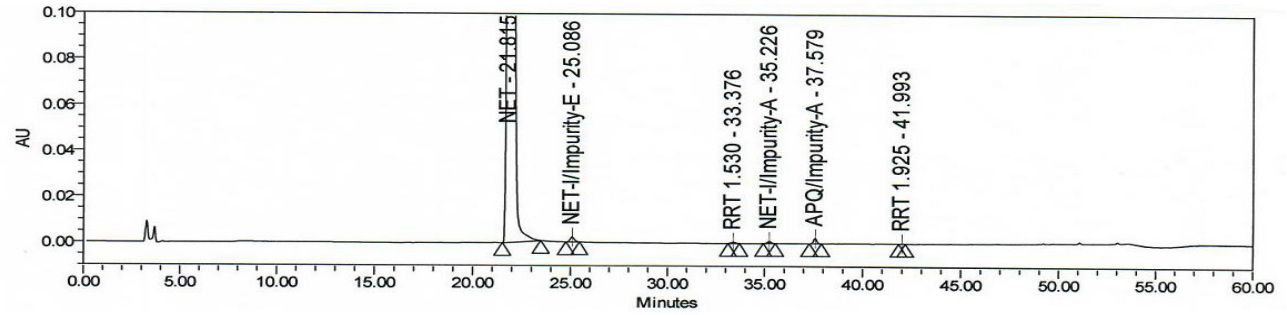


Figure No. 8: Typical chromatogram for Heat at 105 °C 24 hrs

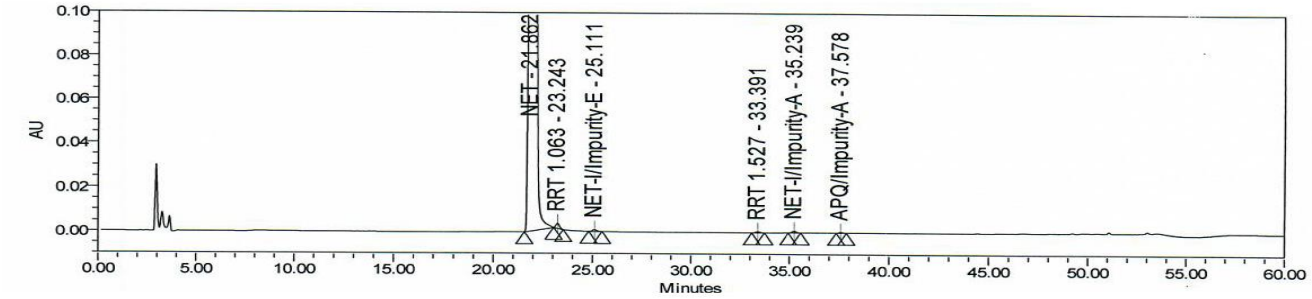


Figure No. 9: Typical chromatogram for 5% H_2O_2 RT 6hrs

Limit of Detection (LOD)/ Limit of Quantification (LOQ):

The limit of detection and limit of quantification is determined by calculating the signal to noise ratio method. By comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The results obtained for each impurity is listed in Table 3. These are determined from the formula S/N ratio is 3:1 for LOD and 10:1 for LOQ respectively. The LOD and LOQ results are tabulated in Table 3. The typical representative HPLC

chromatograms of Blank, LOD, and LOQ experiment are shown in Fig.10 and Fig.11 and Fig.12.

Table No 3: Limit of detection and Quantification for Neratinib (NET) and its impurities

Component	LOQ		LOD		LOQ (%)	LOD (%)
	Concentration (mg/ml)	Signal to noise ratio (S/N)	Concentration (mg/ml)	Signal to noise ratio (S/N)		
NET- I/Imp-C	0.00007485	22.7:1	0.00002470	8.3:1	0.025	0.008
NET-I/Imp-D	0.00009852	11.3:1	0.00003251	3.7:1	0.032	0.010
NET-I/Imp-E	0.00009156	10.7:1	0.00003021	4.3:1	0.030	0.010
NET-I/Imp-B	0.00009396	12.0:1	0.00003101	3.9:1	0.031	0.010
APQ	0.0000768	11.1:1	0.00002534	3.7:1	0.026	0.008
NET-I/Imp-A	0.00007485	10.8:1	0.00002470	3.5:1	0.025	0.008
APQ/Imp-A	0.00006088	11.8:1	0.00002009	4.8:1	0.020	0.007
Neratinib maleate	0.0000769	12.4:1	0.0000254	4.8:1	0.026	0.008

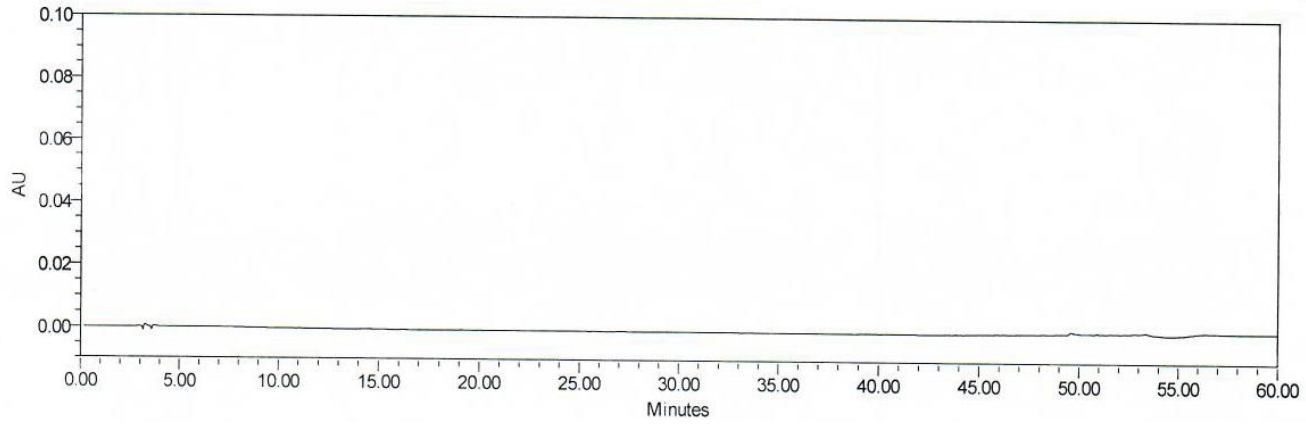


Figure No. 10: A typical representative HPLC chromatogram of Blank

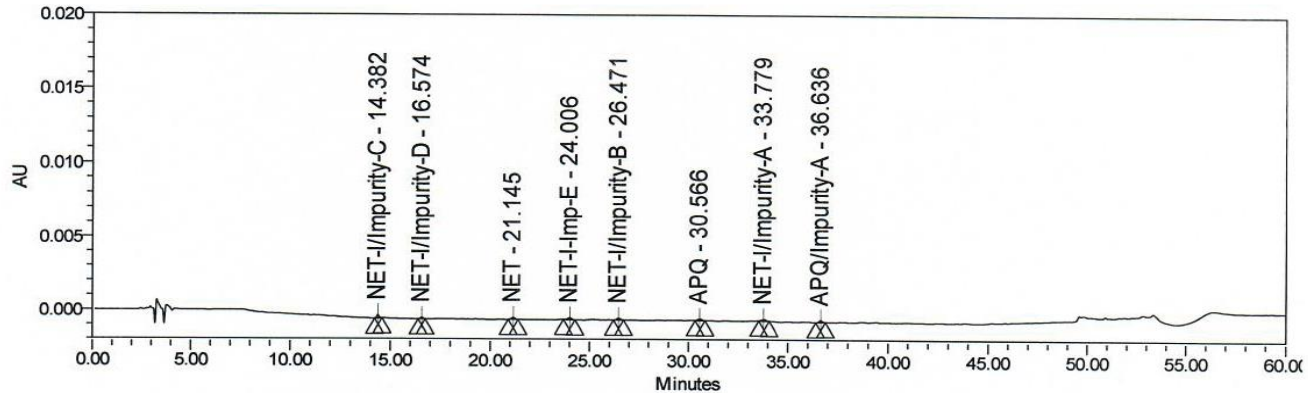


Figure No. 11: A typical representative HPLC chromatogram of LOD

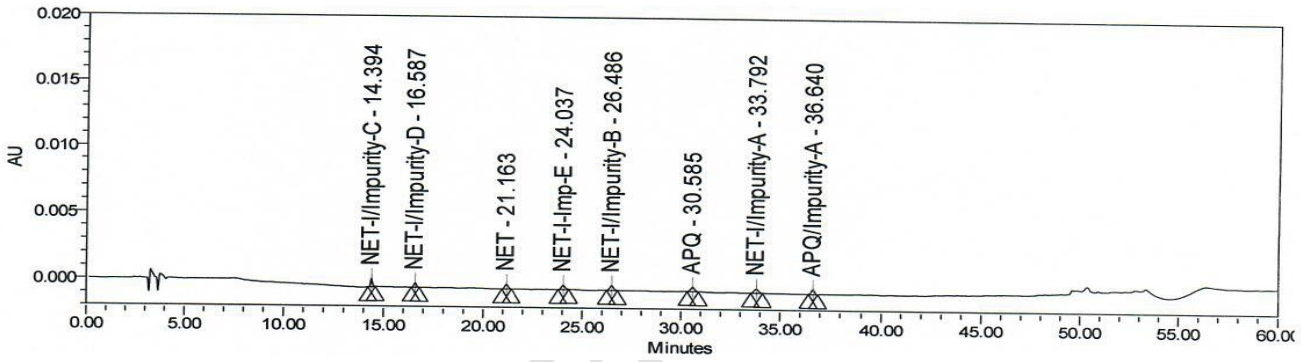


Figure No. 12: A typical representative HPLC chromatogram of LOQ

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Linearity

A series of solutions were prepared using Neratinib maleate and its impurities at concentration levels from LOQ to 150% of specification level and each solution was injected and calculated the statistical values like slope, intercept and correlation coefficient from linearity plot drawn for concentration versus area. The statistical values are presented in Table 4.

Table No. 4: Statistical evaluation of Linearity

Component	Slope	Intercept	The correlation coefficient (R)	R^2	Intercept value w.r.to 100% conc. std response
NET- I/Imp-C	60866530.5689	-283.2079	0.9996	0.9992	-1.05
NET-I/Imp-D	24680870.6455	112.5482	0.9996	0.9992	0.92
NET-I/Imp-E	52526156.8009	-1234.7720	0.9996	0.9993	-5.41
NET-I/Imp-B	49614531.5663	-110.7415	0.9998	0.9995	-0.5
APQ	58836126.1195	-154.2593	0.9996	0.9993	-0.6
NET-I/Imp-A	73458474.5816	-42.0350	0.9995	0.9990	-0.1
APQ/Imp-A	63334180.1790	-284.5971	0.9993	0.9986	-1.0
Neratinib maleate	53075498.3879	-43.4089	0.9993	0.9986	-0.3

Accuracy

The accuracy of the method is determined by using the solutions containing Neratinib maleate samples spiked with the respective impurities at approximately LOQ to 0.60 % level or LOQ to 400% level (w.r.t test concentration). The percentage recovery calculated should be in the range of 80 to 120, and at LOQ level the % recovery calculated should be in the range of 70-130. The percentage recovery values for all the impurities are calculated and tabulated in Table 5.

Table No. 5: Statistical evaluation of Recovery/Accuracy

Spiked levels	% Recovery						
	NET-I/ IMP-C	**NET-I/ IMP-D**	**NET-I/ IMP-E**	**NET-I/ IMP-B**	**APQ**	**NET-I/ IMP-A**	**APQ/ IMP-A**
LOQ	108.2	97.4	125.6	93.7	106.8	120.2	123.2
50 % (0.075%)	107.3	107.6	115.8	102.6	105.5	107.3	113.4
100 % (0.15%)	106.2	107.0	112.1	103.7	105.0	106.2	108.0
150% (0.225%)	110.3	107.9	111.3	109.6	107.2	104.8	111.0
200% (0.3%)	105.4	102.6	112.8	104.4	101.5	105.7	103.3
250% (0.375%)	106.7	103.9	112.6	107.4	103.6	102.6	106.0
300% (0.45%)	108.5	103.1	113.2	106.6	104.9	102.4	104.5
400% (0.60%)	109.6	106.7	112.3	107.0	106.4	104.0	111.8

Stability of solutions:

Standard solution and sample solution spiked with impurities were prepared and analyzed initially and at different time intervals for 24hrs by keeping the solutions at room temperature (~ 25°C) and refrigerator condition (~2-8°C) and found the solutions were stable.

CONCLUSION

Reverse phase stability-indicating HPLC method was developed and validated for the quantitative determination of the process and degradation impurities of Neratinib maleate. The results obtained from validation experiments proved that the chromatographic method is well separated from all impurities from drug substance. The present study will help the manufacturers and suppliers of Neratinib maleate to quantify and qualify the purity based on degradation data. Thus, it can be used for routine analysis, quality control and for determining quality during the stability studies of pharmaceutical analysis.

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