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Isolation, Identification and Characterization of Gefitinib Novel Degradation Products by NMR and HRMS, Method Development and Validation by UPLC

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Gefitinib (GFT) sold under the brand name Iressa, is a medication used to treat certain type of breast, lung and other cancers, Gefitinib was subject to stress degradation under acidic, basic, peroxide mediated oxidation, photolytic and thermal degradation. The stress degradation was performed according to ICH guidelines Q1A(R2) and the drug was inert under thermal and photolytic conditions. One degradant is identified in acid hydrolysis referred as 7-methoxy-6-(3-morpholinopropoxy) quinazolin-4(3*H*)-one (GFT-DP1) and two degradants were formed in peroxide mediated hydrolysis referred as 4-(3-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxy-1-oxidoquinazolin-6-yl)oxy)-propyl)morpholine-4-oxide (GFT-DP2) and 4-(3-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)-propyl)-morpholine-4-oxide (GFT-DP3). In present study, all the novel three degradation product structures were confirmed by HRMS and 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC and HMBC) based on 1D and 2D NMR data proton and carbon chemical shift values assigned exactly for all degradation products. A stability indicating RP-UPLC method was developed and validated with shorter run time and this method was validated in terms of linearity, specificity, accuracy, LOD and LOQ.

Keywords: Gefitinib, Degradation products.

INTRODUCTION

Gefitinib is an antineoplastic agent used to treat certain breast, lung and other cancers. Chemically it is, *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)-quinazolin-4-amine. Gefitinib is the first selective inhibitor of epidermal growth factor receptor's (EGFR)-tyrosine kinase inhibitor, which blocks signal transduction pathways implicated in the proliferation and survival of cancer cells. Many cells, including cancer cells, have receptors on their surfaces for epidermal growth factor (EGF), a protein which is normally produced by the body and promotes the growth and multiplication of cells. When EGF attaches to EGFRs, it causes an enzyme called tyrosine kinase to become active within the cells [1-3]. Tyrosine kinase triggers chemical processes that cause the cells, including cancer cells, to grow, multiply and spread. Gefitinib attaches to EGFRs and thereby blocks the attachment of EGF and the activation of tyrosine kinase. This mechanism for stopping cancer cells from growing and multiplying is very different from the mechanisms of chemotherapy and hormonal therapy [4].

It has been well documented that drugs undergo physico-chemical degradation during storage. Therefore, stability testing of an active pharmaceutical ingredient under various temperature and humidity conditions is indispensable during the drug development process. Stability testing guidelines issued by International Council for Harmonization (ICH) and other regulatory authorities [5-7] require the reporting, identification and characterization of degradation products.

Stress studies are performed to generate degradation products in higher amounts as they are formed in very low levels (0.1-0.5%, w/w) during storage [8]. Even then, many of the times, it is rather difficult to isolate these species from the stressed mixture due to their low amounts and subject them to spectral analyses for structural information. There are few reports on the stability indicating studies of gefitinib in solid dosage by

HPLC [9], in multi-drugs by HPLC [10,11], in human plasma [12,13]. Few spectrophotometric methods based on bulk drug for the determination of gefitinib [14]. The isolation and characterization of degradants by MS/MS technique is reported by Kallepalli & Annapurna [15], however, there is no reports on isolation and identification and characterization by HRMS and NMR (1D & 2D).

The present study is taken up to observe the degradation in milder conditions and to isolate, identify and fully characterize the degradants using various 2D NMR spectroscopic methods. In present work, UPLC technology has been applied to the method validation, assay determination of gefitinib bulk drug and reduced the analysis time with good efficiency.

EXPERIMENTAL

Gefitinib drug substance was received as a kind gift sample from one of the pharmaceutical manufacturing unit in Hyderabad, India. Solvents and buffers used for analysis were of HPLC grade *e.g.* acetonitrile (Merck), formic acid (Merck), ammonium bicarbonate (Sigma-Aldrich), DMSO-d_6 containing 0.03% (v/v) TMS (Cambridge isotope limited) and water used was Milli-Q grade.

High resolution mass spectrometry (HRMS): Accurate mass was measured with Thermo Q Exactive orbitrap HRMS instrument.

Ultra-performance liquid chromatography conditions were as follows: column: ACQUITY BEH C18, 2.1 mm × 50 mm, 1.7 µ; Mobile phase A: 0.05% formic acid (Aq); mobile phase B: 0.05% formic acid acetonitrile; T/% of B: 0.0/3.0, 2.2/98, 3.2/98, 3.5/3, 4.2/3; flow rate 0.6 mL/min, temp.: 50 ºC.

Preparative HPLC: Shimadzu LC-20AP fully automatic preparative system consist of high-pressure gradient with a maximum flow rate of 150 mL/min, this system is capable of automatic continuous fractionation using preparative columns with 50 mm internal diameters. And SPD20A UV-VIS detector and Lab solution Software provides control within a complete prep workstation.

H-Class ultra performance liquid chromatography: H-Class ultra performance liquid chromatography equipped with quaternary solvent manager and 2996 PDA detector used for method validation. Method conditions were Column: ACQUITY UPLC BEH C18 2.1 × 100 mm 1.7 µm, Mobile phase-(A) 0.05% triflouroacetic acid in aqueous B-0.05% triflouroacetic acid in acetonitrile with gradient time/% of B: 0/30, 2.5/98, 5/98, 5.1/30, 8/30, flow rate 0.3 mL/min, column temp. 30 ºC.

Nuclear magnetic resonance spectroscopy: The ^{1}H, ^{13}C and 2D NMR spectra of base degradation impurities were recorded on Bruker 500 MHz Avance-III HD NMR spectrometer using DMSO-d_6 solvent equipped with broad band observe probe (BBO). The ^{1}H & ^{13}C chemical shifts were reported on δ scale in ppm , relative to tetramethyl silane (TMS) as internal standard. The spectra were set to δ 0.00 ppm in ^{1}H NMR (TMS) and δ 39.50 ppm in ^{13}C NMR (DMSO-d_6).

Stress methods: The stress conditions acid, base hydrolysis and oxidation were carried out as per ICH guideline, 0.5 N HCl is used for acid hydrolysis and refluxed for 5 h and the formation of degradant percentage was very low and the reflection was extended to 12 h. The NaOH (1 N) was used for base catalyzed hydrolysis and refluxed for 24 h, 30% hydrogen peroxide was used for peroxide mediated oxidation. The major degradants were identified in acid, base peroxide hydrolysis.

RESULTS AND DISCUSSION

The degradants were observed after 5 h of stirring in the media. However, it was continued till 12 h to enrich their yields. For analytical study, 1mL of the reaction mass was dissolved with mobile phase and 1 µL was injected into LC-MS system. one degradant was identified in acid hydrolysis, while two degradants were identified in peroxide mediate hydrolysis. However, no degradation products were formed in base, photolytic and thermal conditions. Acid and peroxide treated solution were taken up for isolation of all the three degradants. The degradation chromatograms are shown in Fig. 1.

Isolation of acid and peroxide degradation products: The fractions corresponding to the three peaks were collected, distilled and lyophilized. Degradation products were labeled as GFT-DP-1 (m.f.: $C_{16}H_{21}N_3O_4$, exact mass: 319.15); GFT-DP-2 (m.f.: $C_{22}H_{24}N_4O_5ClF$, exact mass: 478.14); and GFT-DP-3 (m.f.: $C_{22}H_{24}N_4O_4ClF$ exact mass: 462.15). All three degradation products were confirmed by NMR experiments *i.e.*, ^{1}H NMR, COSY, HSQC, HMBC. Both ^{1}H and ^{13}C NMR data of GFT-H_2O_2-DP1, GFT-H_2O_2-DP2 and GFT-HCl-DP1 are listed in Table-1.

Structure elucidation of gefitinib acid degradation product-1 (GFT-DP-1): The mass spectrum of GFT-DP-1 shows protonated molecular ion peak at 320.1609 $[M+H]^+$ and protonated molecular formula $C_{16}H_{22}O_4N_3$ was confirmed by HRMS experiment and ^{1}H NMR, COSY, HSQC, HMBC, the HRMS spectrum of GFT-DP-1 is shown in Fig. 2.

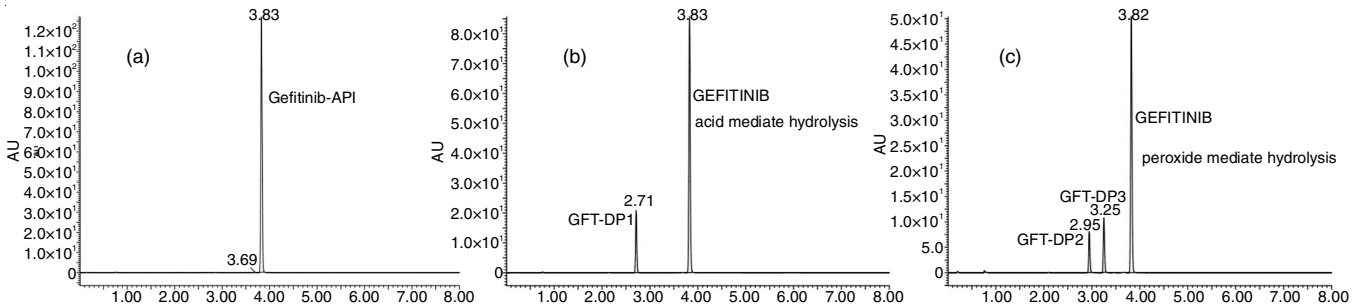

Fig. 1. The chromatograms of gefitinib-API (a), acid (b) and peroxide degrdation products (c)

TABLE-1
^{1}H AND ^{13}C CHEMICAL SHIFT VALUES OF GEFITINIB AND ITS DEGRADATION PRODUCTS

Assignment	GFT-API		GFT-DP-1		GFT-DP-2		GFT-DP-3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		136.8		160		136.7		136.9
2	7.78	122.4	12.06		7.84	121.8	7.96	122.1
3	7.45	116.6	7.98	143.8	7.44	116.6	7.46	116.5
4		153.2				153.1		153
5		118.8		144.8		118.9		118.7
6	8.12	123.5		115.6	8.08	123	8.23	123.3
7			7.13	108.1				
8				154.6				
9	9.56			147.8	9.84		9.69	
10		156	7.44	105.8		147.5		156.1
11								
12	8.5	152.6	3.9	55.9	8.64	139.2	8.51	152.7
13								
14		147	4.1	66.8		138.8		146.9
15		108.8	1.93	25.7		109.7		108.8
16	7.21	107.3	2.43	54.7	7.79	98.7	7.21	107.3
17		154.5				155.1		154.5
18		148.3	2.37	53.3		148.9		147.6
19	7.81	102.5	3.58	66.2	8.29	104.8	8.23	103.9
20								
21	3.94	55.9	3.58	66.2	4	56.2	3.95	55.8
22			2.37	53.3				
23	4.19	67.1			4.39	67.8	4.36	67.6
24	2	25.8			2.35	20.7	2.35	20.7
25	2.48	54.9			3.43	66.9	3.41	67.1
26								
27	2.39	53.4			3.00,3.42	63.7	2.95,3.39	63.8
28	3.58	66.2			3.69,4.11	61	3.66,4.13	61
29								
30	3.58	66.2			3.69,4.11	61	3.66,4.13	61
31	2.39	53.4			3.00,3.42	63.7	2.95,3.39	63.8

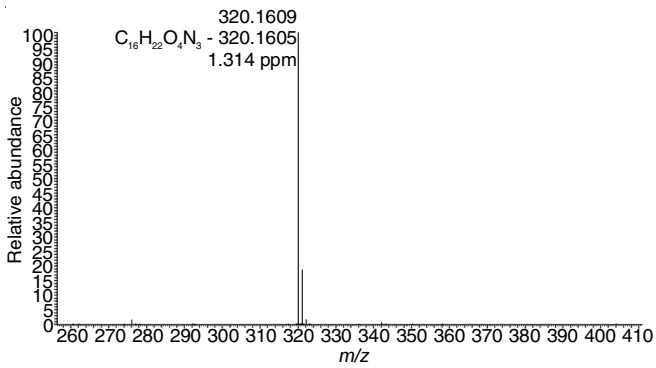

Fig. 2. HRMS spectrum of gefitinib acid degradation product (DP-1)

The proton NMR spectrum revealed that GFT-HCl-DP1 had 17 aliphatic protons, 3 aromatic protons, one –NH proton observed. The ^{13}C Spectra of GFT-HCl-DP1 had 8 aliphatic carbons, 7 aromatic carbons and one carbonyl carbon. HSQC Analysis revealed that DP-2 had 3 methine, 7 methylene, one methyl in spectrum (Fig. 3). The compound named to be 7-methoxy-6-(3-morpholinopropoxy)quinazolin-4(3*H*)-one. All resonances values *i.e.*, ^{1}H and ^{13}C are reported in Table-1.

The protons and carbons of chloro fluoroaniline ring were missing in both ^{1}H and ^{13}C NMR analysis hence carbonyl group was observed in ^{13}C NMR. The proton at 7.98 ppm (H-3) was correlated with –NH(H-2) PROTON IN COSY experiment.

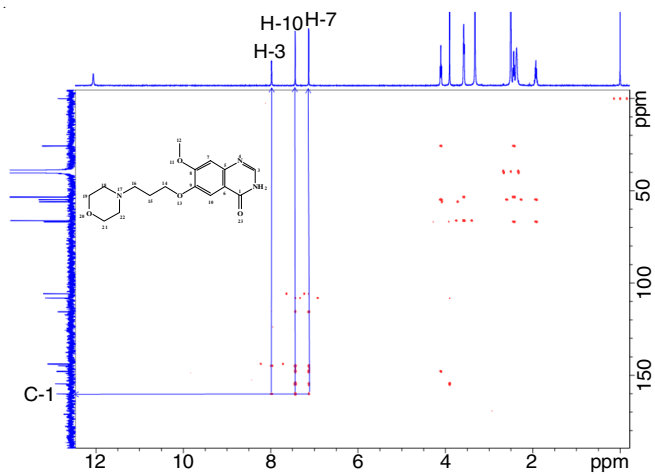

Fig. 3. HMBC spectrum of gefitinib acid degradation product (DP-1)

Structure was confirmed by proton and carbon correlations in HMBC some of the important correlations are stated here. The carbonyl carbon at 160.0 ppm (C-1) was correlated with three protons at 7.13 ppm (H-7) (weak intensity), 7.44 ppm (H-10) and 7.98 ppm (H-3). In the same way, carbon at 144.8 ppm (C-5) was correlated with three protons at 7.13 ppm (H-7), 7.44 ppm (H-10) and 7.98 ppm (H-3). All ^{1}H and ^{13}C chemical shift values were assigned by using NMR data as shown in Table-1.

Structure elucidation of gefitinib oxidative degradation product-2 (GFT-DP-2): The mass spectrum of GFT-DP-2 shows protonated molecular ion peak at 479.1488 $[M+H]^+$ and protonated molecular formula $C_{22}H_{25}O_5N_4ClF$ was confirmed by HRMS experiment and ^1H NMR, COSY, HSQC, HMBC, the HRMS spectrum of GFT-DP-2 is shown in Fig. 4.

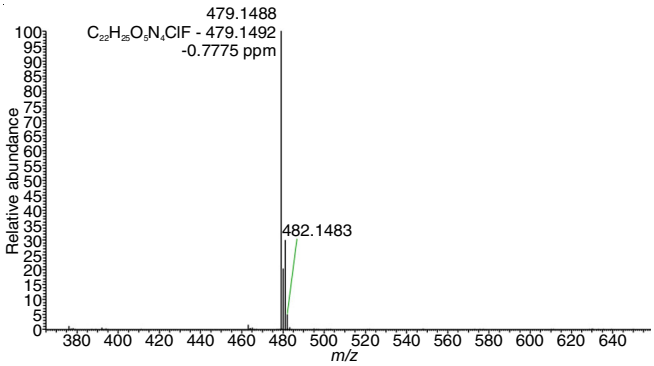

Fig. 4. HRMS spectrum of gefitinib degradation product degradation product (DP-2)

The proton NMR spectrum revealed that GFT-DP-2 had 17 aliphatic protons, 6 aromatic protons, one –NH proton. The ^{13}C spectrum of GFT-DP-2 had 8 aliphatic carbons and 14 aromatic carbons. HSQC Analysis revealed that GFT-DP-2 had 6 methine, 7 methylene, one methyl in spectrum. Analysis and interpretation from HMBC, compound named to be 4-(3-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxy-1-oxidoquinazolin-6-yl)oxy)propyl)morpholine-4-oxide. The compound has higher chance to exist in the form of *N*-oxides, in this case the compound contains two *N*-oxide groups because there was a shift in ^1H and ^{13}C values in NMR analysis when compared to gefitinib-API. One *N*-oxide formed on nitrogen of morpholine ring because there was a change in ^1H and ^{13}C ppm values at 3.43 ppm (H-25), 66.9 ppm (C-25), 3.42 ppm (H-27), 63.7 ppm (C-27), 3.42 ppm (H-31), 63.7 ppm (C-31) positions and another *N*-oxide formed on nitrogen of pyrimidine ring because there was a change in ^1H and ^{13}C ppm values at 8.64 ppm (H-12), 139.2 (C-12), 138.8 ppm (C-14) positions. ^1H and ^{13}C values are reported in Table-1. Fig. 5 confirmed the changes through proton and carbon correlations in ^{13}C HMBC.

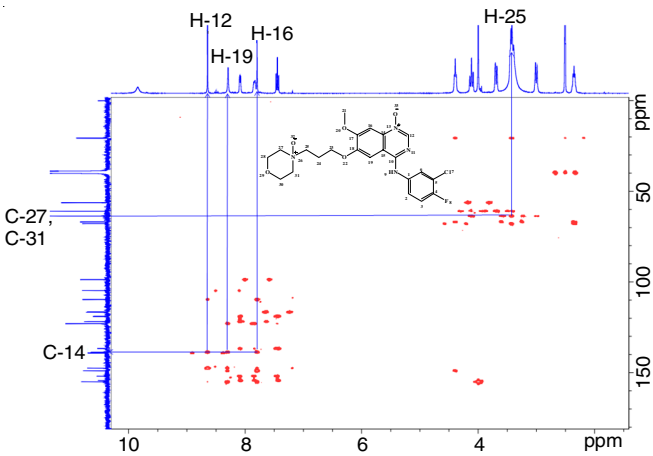

Fig. 5. HMBC spectrum of gefitinib oxidative degradation product (DP-2)

In HMBC, methylene protons at 3.43 ppm (H-25) are correlated with carbons at 63.7 ppm (C-27), 63.7 ppm (C-31). The methine protons of 7.79 ppm (H-16), 8.29 ppm (H-19) and 8.64 ppm (H-12) are correlated with carbon at 138.8 ppm (C-14). All ^1H and ^{13}C chemical shift values were assigned by using NMR data as shown in Table-1.

Structure elucidation of gefitinib oxidative degradation product-3 (GFT-DP-3): The mass spectrum of GFT-DP-3 shows protonated molecular ion peak at 463.1549 $[M+H]^+$ and protonated molecular formula $C_{22}H_{25}O_4N_4ClF$ was confirmed by HRMS experiment and ^1H NMR, COSY, HSQC, HMBC, the HRMS spectrum of GFT-DP-3 is shown in Fig. 6.

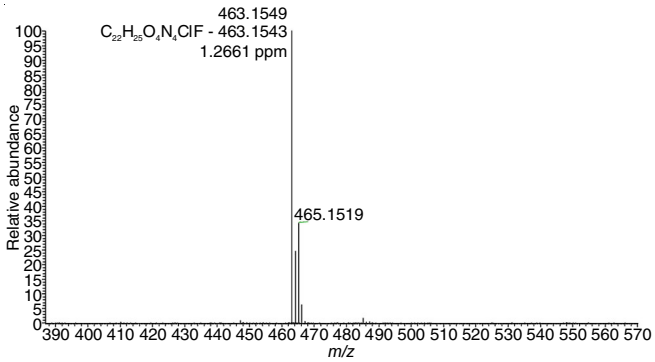

Fig. 6. HRMS spectrum of gefitinib oxidative degradation product (DP-3)

The proton NMR spectrum revealed that DP-3 had 17 aliphatic protons, 6 aromatic protons, one –NH proton observed. The ^{13}C spectrum of DP-2 had 8 aliphatic carbons and 14 aromatic carbons. HSQC Analysis revealed that DP-2 had 6 methine, 7 methylene, one methyl in spectrum. Analysis and interpretation from HMBC, the compound named to be 4-(3-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)-oxy)propyl)morpholine-4-oxide. All resonances *i.e.*, ^1H and ^{13}C are reported in Table-1. It has one N-oxide group on the nitrogen of morpholine ring, which was confirmed through HMBC by observing changes in ^1H and ^{13}C values of GFT-DP-3. Degradation product when compared with API molecule. In HMBC experiment, the protons at 3.41 ppm (H-25), 4.13 ppm (H-28), (H-30) are correlated with carbon at 63.8 ppm (C-27), (C-31). The protons at 2.35 ppm (H-24), 4.36 (H-23) ppm are correlated with carbon at 67.1 ppm (C-25). All the correlation are complies to the structure shown in Fig. 7. All ^1H and ^{13}C chemical shift values were assigned by using NMR data as shown in Table-1.

Method development and validation: UPLC method for the gefitinib drug was developed within 5 min run time and the methods were validated according to regulatory guidelines in precession, accuracy, LOD, LOQ. Linearity was performed with different concentration levels of the sample, the recovery experiments were performed to determine the accuracy of the method and the accuracy was proved by spiking 10% of known sample solution to the different concentration (50%, 100% and 150%) of the sample.

Gefitinib standard solution (0.4 mg mL^{-1}) was injected for system suitability test, the retention time of gefitinib was 2.25 min and plate count, USP tailing values are 47788, 1.28. The chromatogram of gefitinib standard is shown in Fig. 8.

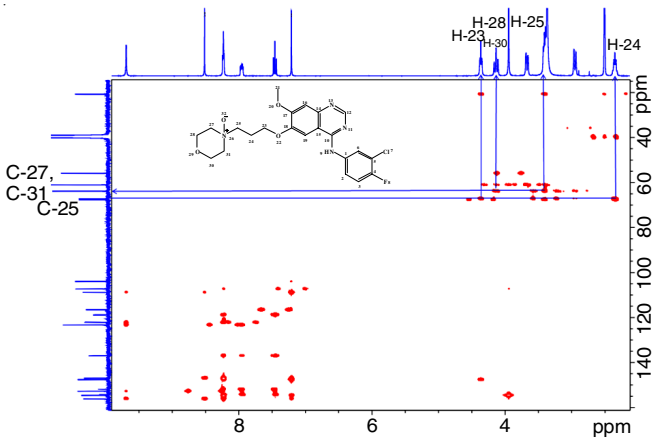

Fig. 7. HMBC spectrum of gefitinib oxidative degradation product (DP-3)

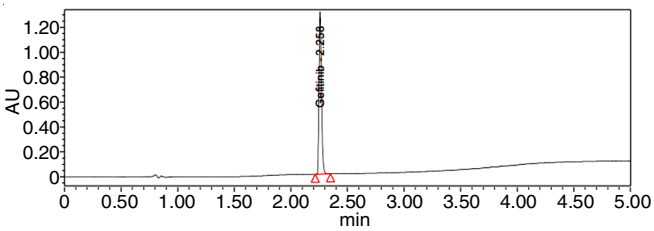

Fig. 8. UPLC chromatogram of gefitinib

Gefitinib intraday method precession was checked with six repeated concentration preparations, the % of RSD value is 1.1 and inter day method precession % of RSD is 0.6, the results are shown in below Fig. 9.

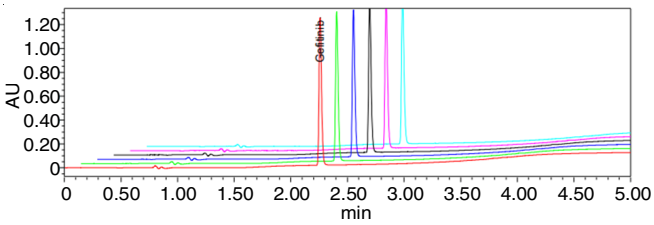

Fig. 9. Gefitinib interday method precession chromatogram

The UPLC method, LOD, LOQ values were 0.0013 mg mL^{-1} (S/N 3.59), 0.001 mg mL^{-1} (S/N 19.57), gefitinib linearity was demonstrated with the concentration ranging 0.075-0.450 mg mL^{-1} and the correlation coefficient was greater than 0.999. The accuracy of the method was checked with different concentration levels (50%,100% 150%) with spiking of known concentration gefitinib standard solution and recovery of the UPLC method was proved, the % of recovery was 99.16 for the assay of gefitinib and the results are shown in Fig. 10.

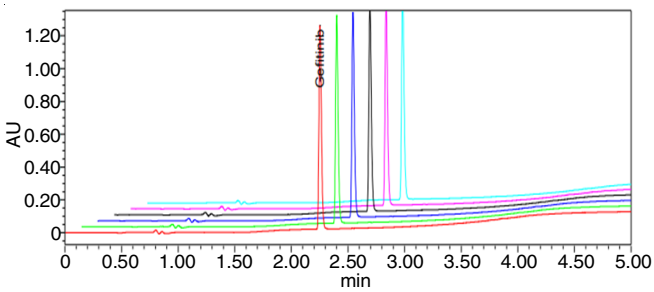

Fig. 10. Gefitinib intraday method precession chromatogram

Method robustness of the UPLC method was checked by changing the percentage of acetonitrile organic solvent (± 0.2 mL min^{-1}), pH of the mobile phase (± 0.2), column oven temp. (± 5 °C), different systems, there is no recognized changes were observed. The stability of gefitinib drug solution was checked at accurate temperature (2-8 ºC) for the period of 40 days, the mobile phase stability was checked (2, 4, 7 days) with gefitinib drug solution and no important changes observed. The validation parameters are shown in Tables 2 and 3.

TABLE-3
ASSAY RECOVERY OF GEFITINIB

Level (%)	Amount added (μg mL^{-1})	Amount recovered (μg mL^{-1})	Recovery (%)
50	160.07	164.72	102.9
100	199.05	200.64	100.8
150	299.09	301.51	100.8

TABLE-2
ACCURACY OF GEFITINIB

50% Accuracy	Neat 50% as such area	Observed 50% + 10% spike area	Observed-Neat	Recovery (%)
Preparation-1	993642	1187535	193893.00	99.60
Preparation-2	983478	1180940	197462.00	101.44
Preparation-3	992958	1177438	184480.00	94.77
Average	990026.0		191945.0	98.6
100% Accuracy	Neat 100% as such area	Observed 100% + 10% spike area	Observed-Neat	Recovery (%)
Preparation-1	1913834	2116948.00	203114.00	104.34
Preparation-2	1918328	2105290.00	186962.00	96.04
Preparation-3	1921458	2115207.00	193749.00	99.53
Average	1917873.3		194608.3	100.0
150% Accuracy	Neat 150% as such area	Observed 150% + 10% spike area	Observed-Neat	Recovery (%)
Preparation-1	2862656	3061044	198388.00	101.91
Preparation-2	2865220	3059482	194262.00	99.79
Preparation-3	2882593	3076818	194225.00	99.77
Preparation-4	2871168	3059537	188369.00	96.77
Preparation-5	2878638	3067628	188990.00	97.08
Preparation-6	2871536	3062921	191385.00	98.32
Average	2871968.5		192603.2	98.9

Application: Gefitinib stress degradation studies provide degradation pathway, chemical properties, which helps in the development of formulation and package, The UPLC method is faster than tradition method of analysis and UPLC system eliminate the significant time and cost

Conclusion

During the acid and peroxide degradation of gefitinib, the degradation products were formed. The degradation products were unambiguously characterized by HRMS, FT-IR and NMR 1D and 2D techniques. This UPLC method described the validation for gefitinib drug with shorter runtime.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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