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Isolation, Identification and Characterization of Erlotinib Novel Degradation Products by NMR and Mass Spectrometry: RP-UPLC Method Development and Validation

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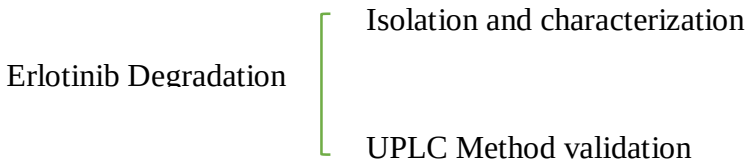
ABSTRACT

To assess the stability of Erlotinib under stress conditions, it was subjected to Acid, Base, peroxide, photolytic and thermal degradation according to the ICH guideline Q1A (R2). The drug showed degradation only in Acid and Peroxide mediate hydrolysis, it was stable in Basic, thermal and photolytic conditions. Four degradation products were formed, which were separated on an X-Bridge Prep C18 5µm, 19 mm × 250 mm Column employing GILSON Prep HPLC using gradient elution. The structures were established by extensive 1D and 2D NMR spectroscopic studies and mass spectrometry. The products were identified as 6,7-bis(2-methoxyethoxy)quinazolin-4(3H)-one(ERL-DP-1), 1-(3-((6,7-bis(2-methoxyethoxy)quinazolin-4-yl)amino)phenyl)ethan-1-one(ERL-DP-2), N-(3-(1-chlorovinyl)phenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine(ERL-DP-3),4-((3-ethynyl phenyl) amino)-6,7-bis(2-methoxyethoxy) quinazoline 1-oxide (ERL-DP-4). DP-4, the N-oxide derivative of Erlotinib was novel degradation products. A stability indicating RP-UPLC method was developed was validated with respect to specificity, linearity, accuracy, precision, limit of detection and limit of quantitation.

Graphical Abstract



Keywords: Erlotinib, Method validation, Degradation products, UPLC-MS, Nuclear magnetic Resonance Spectroscopy.

INTRODUCTION

Erlotinib (ERL) drug is a member of a class of targeted anticancer drug. It is used in treatment of non-small cell lung cancer (NSCLC), pancreatic cancer and ovarian, head, neck, breast, prostate, colorectal, hepatic, and renal cancers. It is a reversible tyrosine kinase inhibitor, which acts on the epidermal growth factor receptor (EGFR) [**1**]. Chemically it is *N*-(3-ethynylphenyl)-6, 7-bis (2-methoxyethoxy) quinazolin-4-amine and marketed in the United States by Genentech and OSI Pharmaceuticals and elsewhere by Roche (Figure 1).

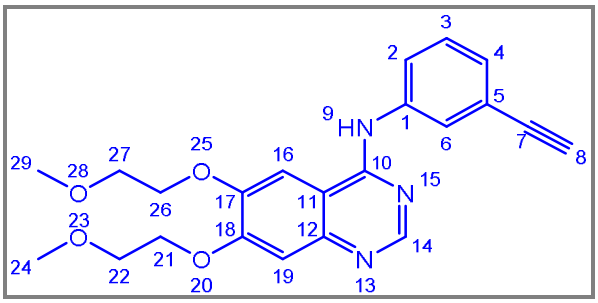


Figure 1. Chemical structure of Erlotinib (ERL).

It has been well documented that drugs undergo physicochemical 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 [**2-5**] require the reporting, identification and characterization of degradation products (DPs).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 [**1**]. 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 very few reports are available on the stress stability studies of Erlotinibin solid dosage forms and few reports on the isolation and characterization of degradants [**6, 7**], there are some reports on method development and validation of multi Active Pharmaceutical Ingredients (APIs) by HPLC and MS/MS [**8-11**], few reports of Erlotinib on therapeutic drug monitoring in human plasma [**12-13**].

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.No UPLC methods were reported in major pharmacopoeias.Ultra-Performance Liquid Chromatography (UPLC) is the alternate for the HPLC, UPLC technology has been adopted in laboratories around the world. The main advantage of the UPLC system eliminates the significant time and cost, speed, resolution and sensitivity. UPLC flow rate range 0.01 mL min^{-1}, back pressure up to 18000 psi and the detector high sensitive than the HPLC. In the present work UPLC technology has been applied to the method validation, assay determination of Erlotinib bulk drug and reduced analysis time with good efficiency.

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MATERIALS AND METHODS

Chemicals and reagents: Erlotinib drug substance was a kind gift sample from a manufacturing unit in Hyderabad. Solvents and buffers used for analysis were HPLC grade Acetonitrile (Merck), Formic acid (Merck), Dimethyl sulfoxide-d_6 containing 0.03% (v/v) TMS (Cambridge isotope limited) and water used was HPLC grade. Ammonium bicarbonate (Sigma-Aldrich)

Liquid Chromatography-Mass Spectrometry: Column: ACQUITY BEH C18, 2.1mm × 50 mm, 1.7µ; Mobile phase A: 0.05% formic acid in Milli-Q Water; 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 0.6mL min^{-1}, Temp: 50°C.

High Resolution Mass Spectrometry (HRMS): Accurate mass was measured with Q-TOF micro mass instrument equipped with micro Chanel plate detector and multimode ionization source (ES+APCI). The optimum conditions are desolvation gas flow 700 L h^{-1}, capillary voltage 3500v, cone voltage 30v, MCP voltage 2700v, positive ionization mode. Leucine Enkephalin (556.2771 Da) was used to calculate Lteff and Elemental compositions were calculated with Mass lynx (4.1) software.

Preparative HPLC: Gilson prep-HPLC (GX-271), DAD detectorwith column X Bridge C18 (250×19 mm) 5µ with mobile phase A: 10 mM Ammonium bicarbonate inMilli-Q Water and B: Acetonitrile with gradient elution % B: 0/10, 1/10, 12/90, 12.5/98, 15/98,15.2/10,18/2 with a flow rate of 18 mL mi^{-1} at room temperature.

Ultra-Performance Liquid Chromatography: Ultra Performance Liquid Chromatography equipped with quaternary solvent manager and 2996 PDA detector was used for method validation. Method conditions are Column: ACQUITY UPLC BEH C-18 2.1X100mm 1.7µm, Mobile phase-(A) 0.05% Triflouroacetic acid in aqueous B-0.05% Triflouroacetic acid in Acetonitrile with gradient Time/percentage of B 0/30, 2.5/98, 5/98, 5.1/30, flow rate 0.3 mL min^{-1}, Column temp 30°C.

Nuclear Magnetic Resonance spectroscopy: The ^{1}H, ^{13}CNMR and 2D NMR spectra of base degradation impurities were recorded in DMSO-d_6 solvent on Bruker 500 MHz Avance-III HD NMR spectrometer equipped with Broad Band Observe Probe (BBO). The ^{1}H and ^{13}C chemical shifts were reported on δ scale in ppm, relative to tetra methyl 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).

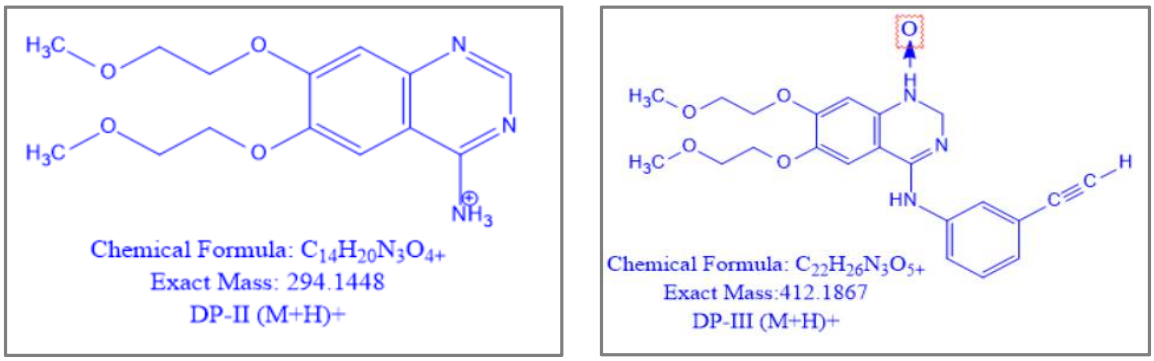
Stress methods: The stress conditions acid, base hydrolysis and oxidation were carried out as per ICH guideline, 0.2N HCl was used for acid hydrolysis and refluxed for 5 h and the formation of degradant percentage was very low and the reflux was extended to 12 h. 2N NaOH was used for base catalyzed hydrolysis and refluxed for 24 h. 30% hydrogen peroxide was used for peroxide mediated oxidation. The major degradants were identified in acid, Peroxide hydrolysis.

RESULTS AND DISCUSSION

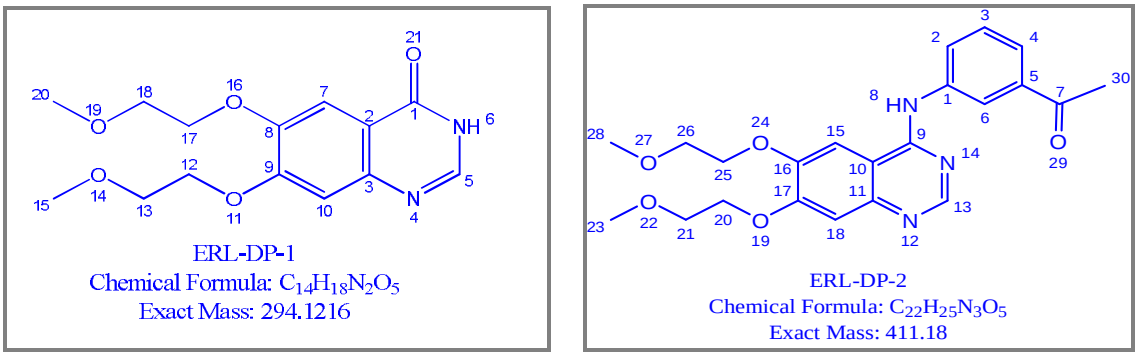
The degradants were formed after 12 h of refluxing in the media. However, it was continued till 24 h to enrich their yields. For analytical study, 1 mL of the reaction mixture Acid degradation solution was dissolved in Acetonitrile and diluted with mobile phase and 1 µL was injected into LC-MS system method. Acid drug solution showed three degradants, Peroxide showed two degradants (Figure 2).ERL-DP-1 was obtained in both acid and peroxide mediated degradation. However, no degradation products were formed in base treated drug solutions. Acid and Peroxide treated solution was taken up for the isolation and isolated all the four degradants.

DP1-294 and DP2-412 mass: Anand *et al* proposed the structure mass 294 and 412 with only mass data (Figure 1).

In our case study 294 and 412 mass data shown different structure, it was confirmed by NMR study, DP1 observed NH proton at 7.98 ppm, did not observe any NH_3^+ Protons. This data itself indicating our assigned structure is correct one compare to the previous article proposed structure.



Published structure 294 and 412(M+H) [**1**]



Present study confirmed structure by NMR

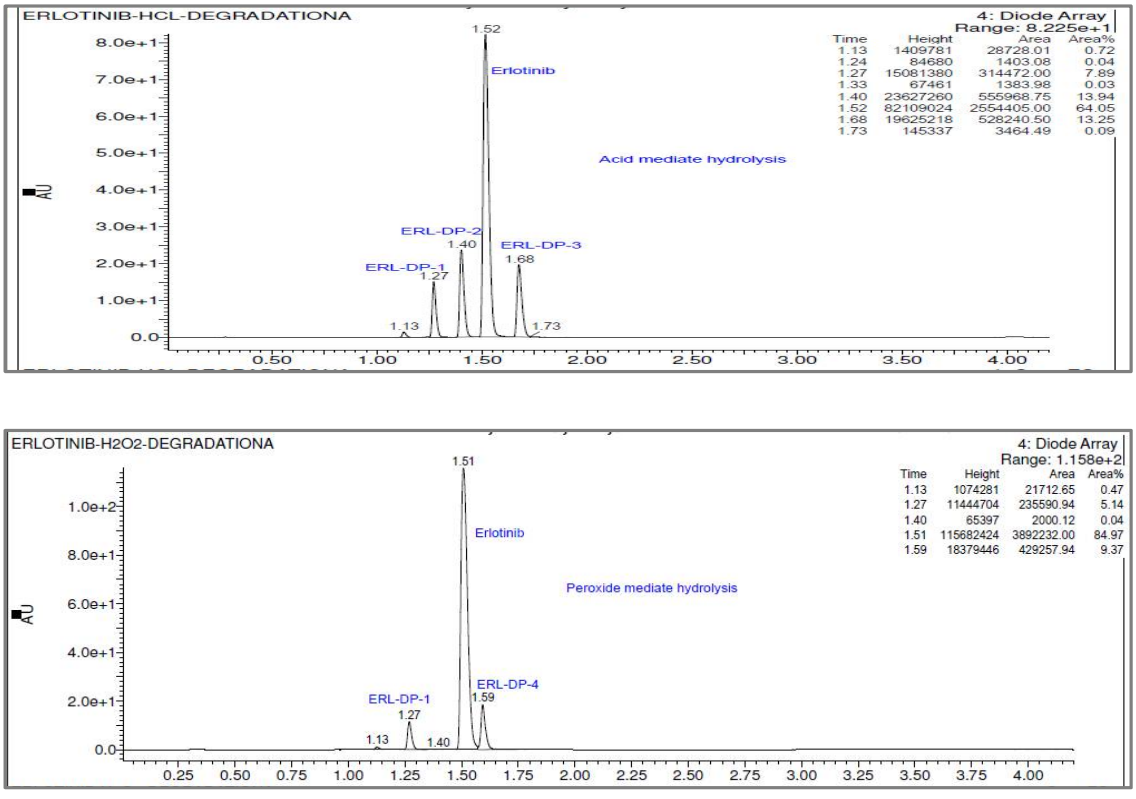


Figure 2. Acid, peroxide degrdation products of Erlotinib.

Isolation of Acid and Peroxide degradation products: The Acid and Peroxide degradation products were isolated by the above. The fractions corresponding to the four peaks were collected and distilled by rotavapour and lyophilized. Degradation products were labeled as ERL-DP-1, ERL-DP-2, ERL-DP-3 and ERL-DP-4. The structures of these degradation products were elucidated by mass spectrometry and 1D, 2D NMR data. ERL-DP-4 was found to be new (Figure 3).

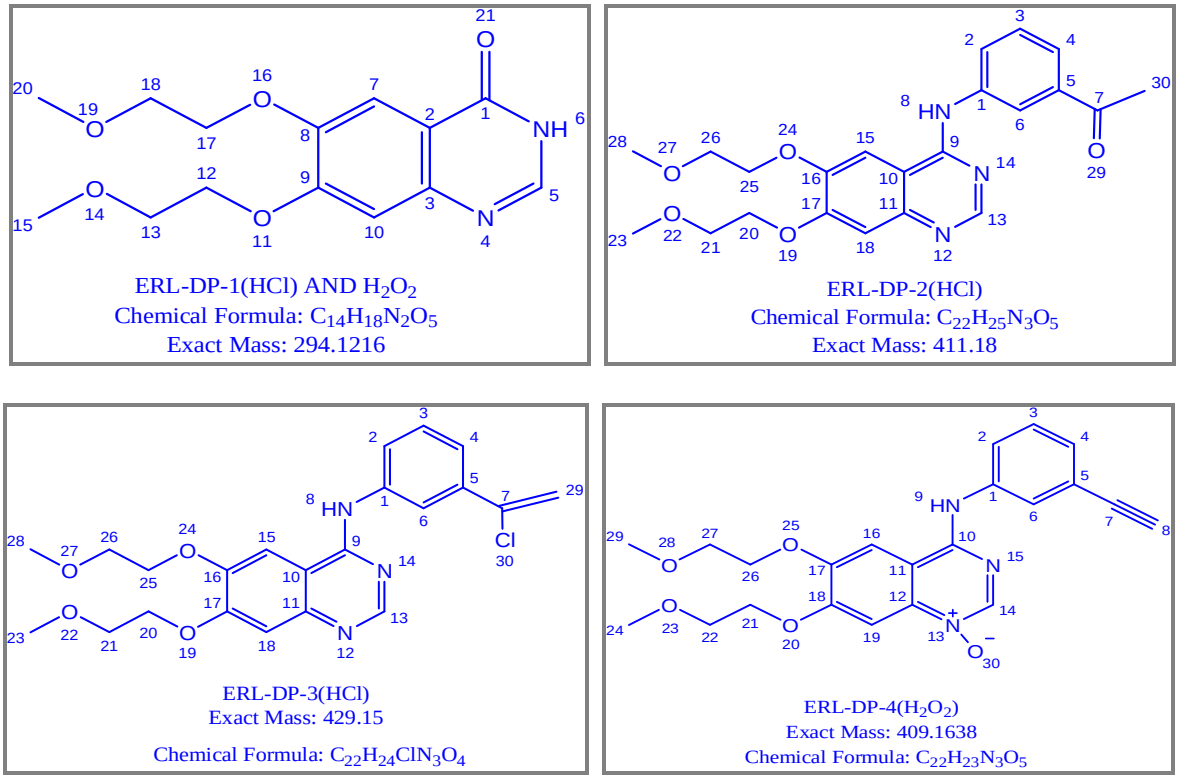


Figure 3. Chemical structures of Erlotinib degradation products.

LC-MS analysis: The LC- MS study of the Erlotinib drug substance along with the isolated degradants was performed and the fragmentation pathways were shown in supplementary data.

Structural elucidation of ERL-DP-1(HCl) and H_2O_2: The mass spectrum of ERL-DP-1 shows protonated molecular ion peak at 295.1294 $[M+H]^+$ and protonated molecular formula $C_{14}H_{19}N_2O_5$ was confirmed by LC-MS experiment, the HRMS spectrum of ERL-DP-1 was shown in figure 4.

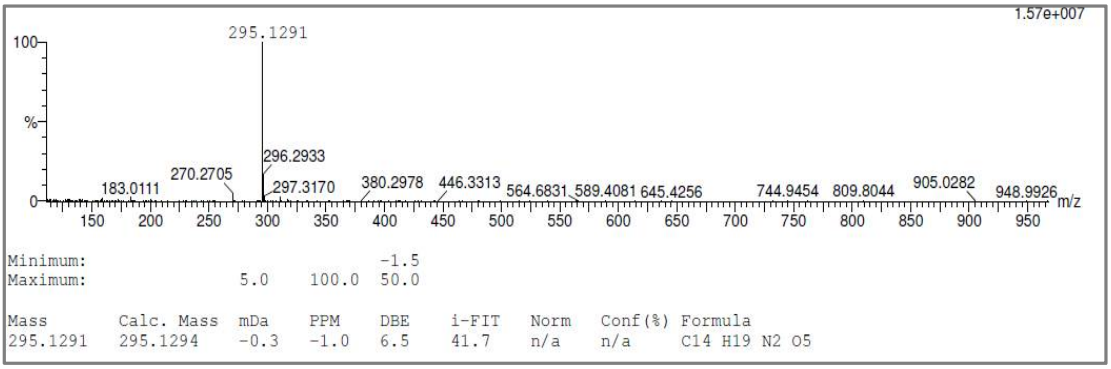


Figure 4. HRMS spectrum of DP-1.

DP-1 had 3 aromatic protons, 14 aliphatic protons and one exchangeable amide protons. ^{13}C NMR revealed that it had 8 aromatic carbons and 6 aliphatic carbons.^{13}C-HSQC analysis provided the

information that it had 2 methyl, 4 methylene and 3 methyne protons. In ^{15}N- HSQC, proton at 12.1 ppm showed one bond correlation with Nitrogen at 163.3 ppm figure 5.

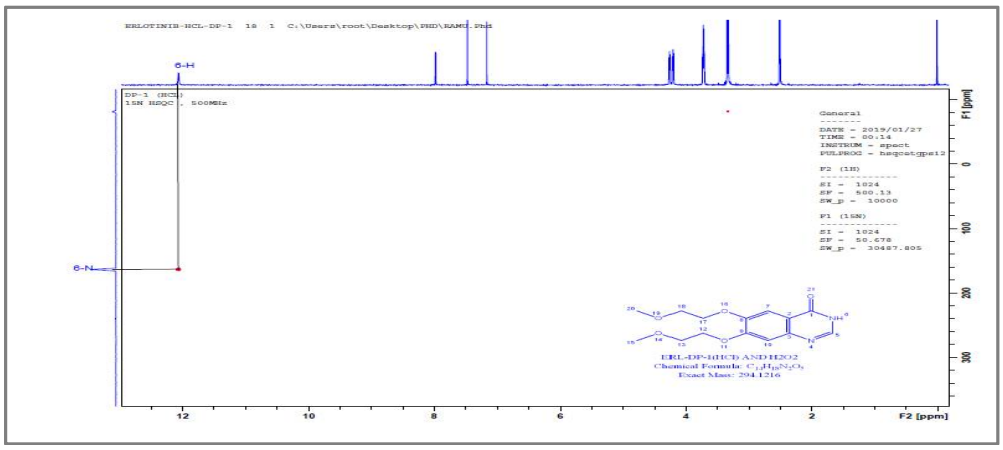


Figure 5. ^{15}N- HSQC spectrum of DP-1.

Supporting that DP-1 had one amide NH proton. In ^{15}N HMBC analysis, H-5 (7.98ppm) correlated with N-4 (239.4 ppm) and NH-6 (163.3 ppm) as shown in figure 6.

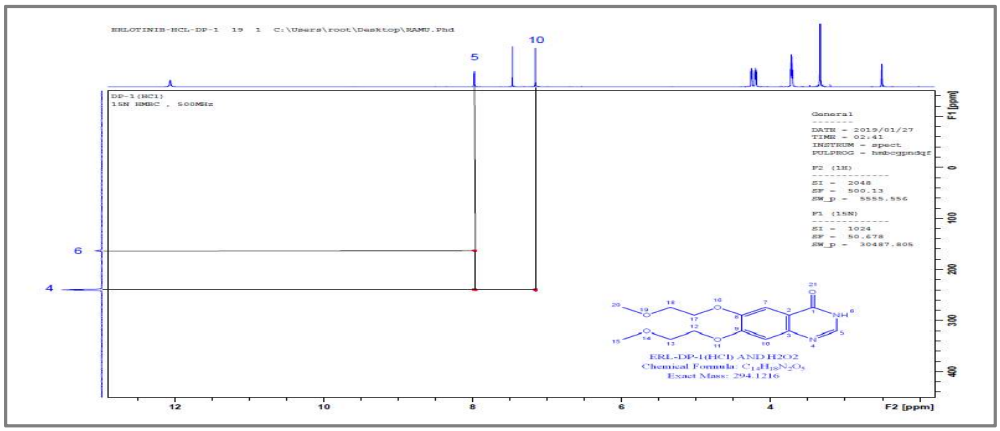


Figure 6. ^{15}N- HMBC spectrum of DP-1.

In ^{13}C-HMBC analysis H-5 (7.98 ppm) correlated with C-3 (144.7ppm) and 1st position carbonyl carbon at 160 ppm (Figure 7).

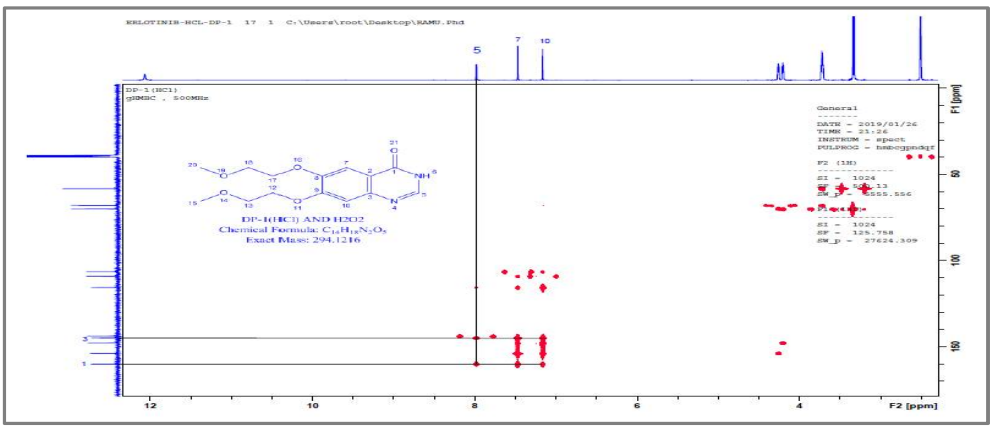


Figure.7. HMBC spectrum of DP-1.

In NOESY1D Experiment, Irradiation of NH at 12.1 ppm gives rise to signal enhancement of 5-H (7.98 ppm) (Figure 8).

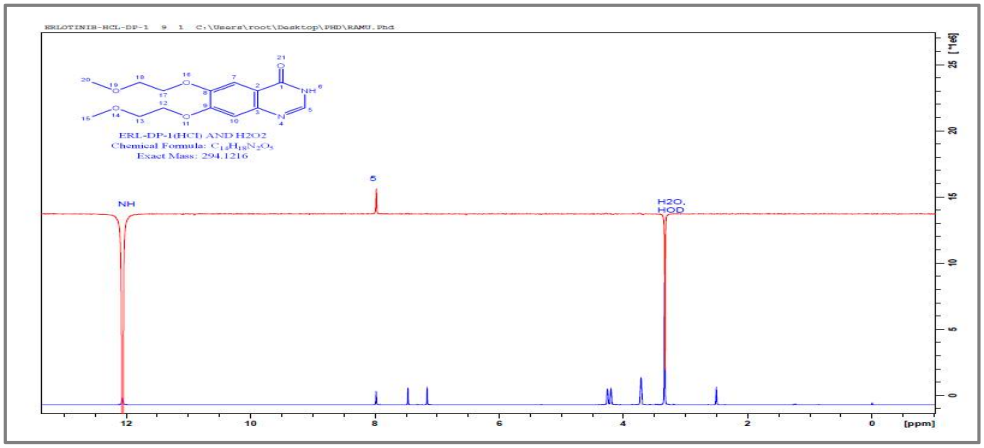


Figure 8. NOESY spectrum of DP-1.

All these analysis supporting to structure of ERL-DP-1 was shown in figure 3. Proton and carbon chemical shift values of ERL-DP-1 was assigned as shown in below table 1.

Table 1. ^{1}H, ^{13}C Chemical shift values of Erlotinib and it's degradation products
Structural elucidation of ERL-DP-2

Assignment Number	Erlotinib		DP-1(HCl) and H_2O_2		DP-2(HCl)		DP-3(HCl)		DP-2(H_2O_2)	
	^{1}H (PPM)	^{13}C (PPM)	^{1}H (PPM)	^{13}C (PPM)	^{1}H (PPM)	^{13}C (PPM)	^{1}H (PPM)	^{13}C (PPM)	^{1}H (PPM)	^{13}C (PPM)
1	-	139.8	-	160	-	140	-	139.7	-	139.5
2	7.9	122.8	-	115.5	8.22	126.7	7.95	123.2	7.81	122.1
3	7.4	128.9	-	144.7	7.55	128.9	7.45	128.8	7.41	129.2
4	7.21	126.5	-		7.73	123.4	7.44	121.1	7.21	126.5
5	-	121.8	7.98	143.9	-	137.3	-	136.3	-	121.8
6	8.0	124.8	12.1	-	8.3	121.2	8.07	120	7.89	124.2
7	-	83.5	7.47	106.5	-	197.8	-	138.6	-	83.4
8	4.2	80.5	-	147.8	9.62	-	9.59		4.21	80.7
9	9.5	-	-	153.9	-	166.3	-	156.6	9.59	-
10	-	156.0	7.16	109.1	-	108.9	-	108.9	-	147.4
11	-	109.0	-	-	-	156.2	-	147	-	109.8
12	-	147.0	4.2	68.1	-	-	-	-	-	139
13	-	-	3.72	70.0	8.49	152.8	8.48	152.8	-	-
14	8.5	152.8	-	-	-	-	-	-	8.67	139.2
15	-	-	3.34	58.3	7.9	103.2	7.89	103.3	-	-
16	7.87	103.2	-			148.2	-	148.2	7.95	104.2
17	-	148.1	4.2	68.1		153.8	-	153.6	-	149.5
18	-	153.7	3.72	70.1	7.24	108.3	7.23	108.2	-	154.4
19	7.23	108.2	-	-	-	-	-	-	7.87	99.6
20	-	-	3.34	58.3	4.3	68	4.29	68	-	-
21	4.29	68.0	-	-	3.76	70	3.75	70	4.34	68.5
22	3.75	70.0	-	-	-	-	-	-	3.78	69.8
23	-	-	-	-	3.36	58.3	3.36	58.3	-	-
24	3.35	58.3	-	-	-	-	-	-	3.36	58.3
25	-	-	-	-	4.3	68.4	4.29	68.4		
26	4.29	68.4	-	-	3.79	70.1	3.79	70.1	4.34	68.6
27	3.78	70.1	-	-	-	-	-	-	3.8	69.9
28	-	-	-	-	3.38	58.4	3.38	58.4	-	-
29	3.37	58.4	-	-	-	-	5.66,6.08	114.3	3.38	58.4
30	-	-	-	-	2.62	26.8	-	-	-	-

The HRMS spectrum of ERL-DP-2 shows protonated molecular ion peak at 412.1874$[M+H]^+$ and protonated molecular formula $C_{22}H_{26}N_3O_5$ was confirmed by HRMS experiment, the HRMS spectrum of ERL-DP-2 was shown in figure 9.

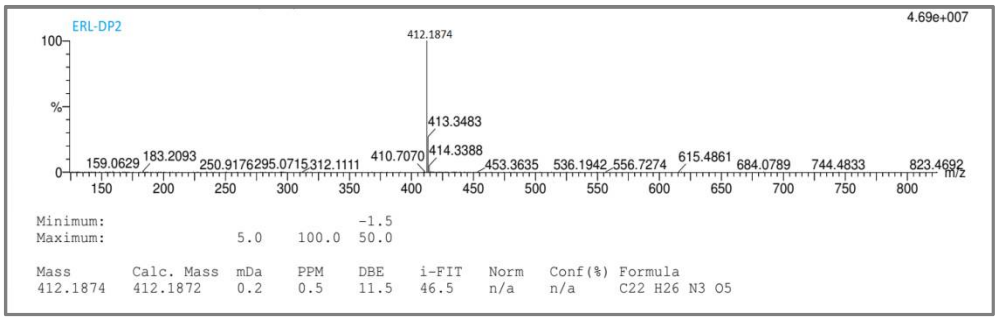


Figure 9. HRMS spectrum of DP-1.

DP-2 had all protons like Erlotinib drug substance except acetylene proton. It had acetyl protons instead of acetylene proton. It had 7 aliphatic carbons and 15 aromatic carbons in 13C NMR. Acetyl carbonyl carbon observed at 197.8 ppm in ^{13}C NMR. HSQC Experiment revealed that it had 3 methyl, 4 methylene and 7 methyne protons. In HMBC Experiment, H-30 (2.62 ppm) correlated with 7th position carbonyl carbon at 197.8 ppm and C-5 (137.3ppm) (figure 10).This main key proton versus carbon correlation in HMBC supporting to structure of DP-2 as shown in figure 3.

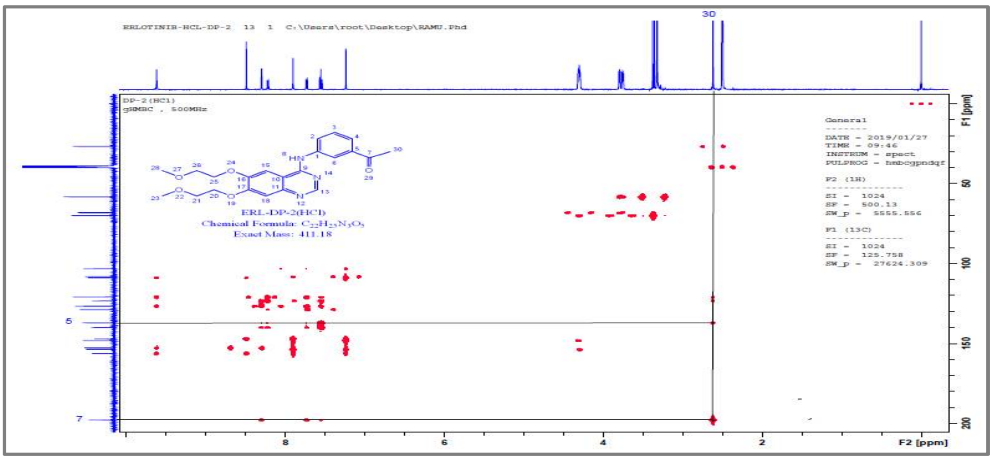


Figure 10. HMBC spectrum of DP-2**.**

Structural elucidation of ERL-DP-3: The HRMS spectrum of ERL-DP-3 shows protonated molecular ion peak at 430.1531 $[M+H]^+$ and protonated molecular formula $C_{22}H_{25}N_3O_4Cl$ was confirmed by HRMS experiment, the HRMS spectrum of ERL-DP-3 was shown in figure 11.

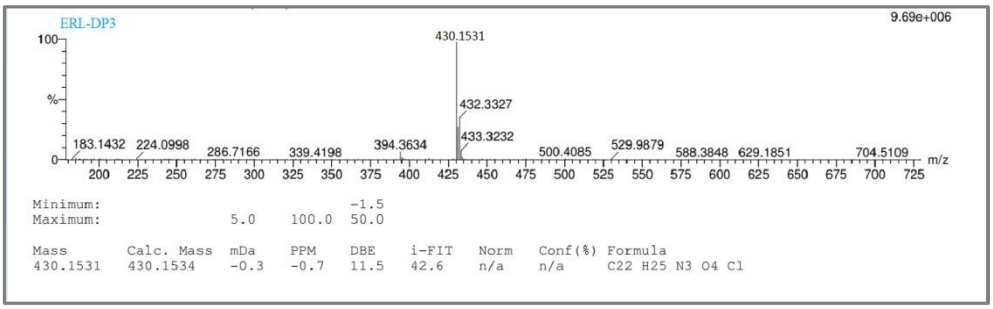


Figure 11. HRMS spectrum of DP-3.

ERL-DP-3 had all protons like Erlotinib drug substance except acetylene proton. It had alkene protons instead of acetylene proton. It had 6 aliphatic carbons and 16 aromatic carbons in ^{13}C NMR. Alkene carbons observed at 138.6 ppm and 114.3 ppm. Alkene protons observed at 5.66 ppm and 6.08 ppm. It was confirmed by HSQC Experiment. In HSQC experiment, H-29 (5.66, 6.08 ppm) correlated with C-29 (114.3 ppm) figure 12.

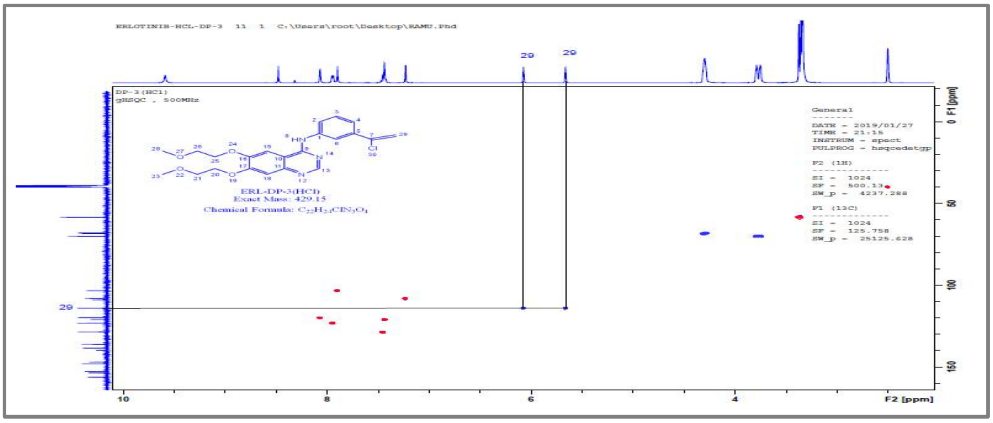


Figure 12. HSQC spectrum of DP-3.

In HMBC Experiment, H-29(5.66, 6.08 ppm) correlated with C-7(138.6 ppm) and C-5(136.3 ppm). This proton versus carbon correlation supporting to structure of DP-3 was shown in figure 13.

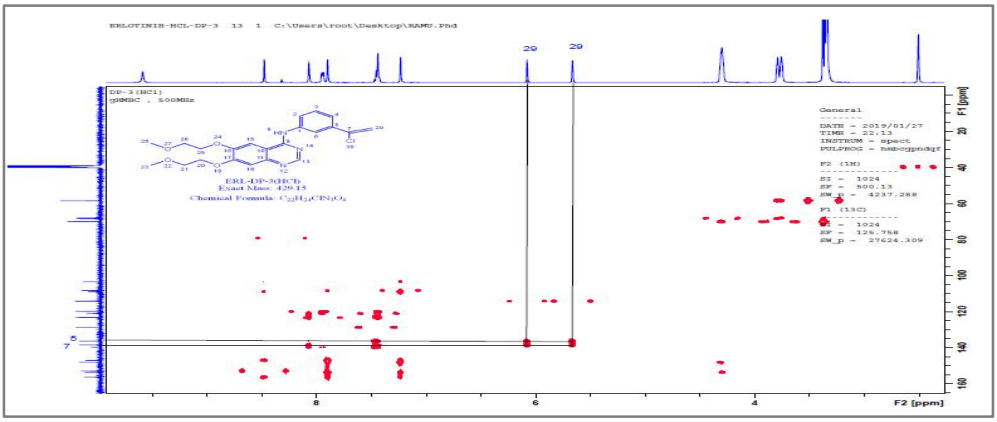


Figure 13. HMBC spectrum of DP-3.

Structural elucidation of ERL- DP-4(H_2O_2): The HRMS spectrum of ERL-DP- 4 shows protonated molecular ion peak at 410.1712 $[M+H]^+$ and protonated molecular formula $C_{22}H_{24}N_3O_5$ was confirmed by HRMS experiment, the HRMS spectrum of ERL-DP-4 was shown in figure 14.

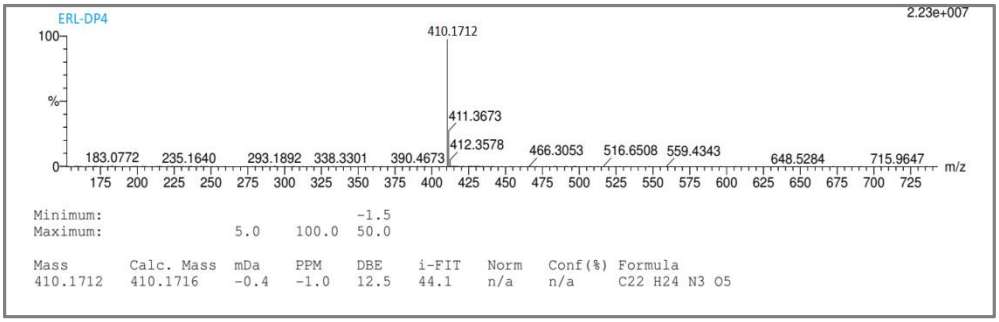


Figure 14. HRMS spectrum of DP-4.

ERL-DP-4 had 16 mass units higher than Erlotinib drug substance indicating that it had one oxygen atom. During oxidation one oxygen atom attacked on drug substance. Now challenge is identification of position of oxygen on which nitrogen. Based on ^{1}H and ^{13}C chemical shift differences between drug substance and DP-2(H_2O_2) as shown in table 1.Concluded that oxygen atom attacked on 13^{th} position nitrogen as shown in figure 3.

Method development and validation: UPLC method with 5 min run time method was developed as mentioned in section 2.5 and the UPLC method was validates as per regulatory guidelines in terms of precession (intra, inter day), limit of detection and quantitation. Linearity was performed with 25%, 50%, 75%, 100%, 125%, 150% of the sample, accuracy and the recovery experiments were conducted to determine the accuracy of the method. Accuracy was proved by spiking 10% of standard solution to the 50%, 100% and 150% of the sample.

Erlotinib standard solution (0.4 mg mL^{-1}l) was injected in the UPLC system for system suitability test, the retention time of the Erlotinib was 2.21 min and USP Tailing, plate count values are 1.31, 31672. Intraday method precession, inter day method precession was checked with six repeated concentration preparations and the % of RSD values are 0.1, 0.8 and the results are shown in table 2.

Table.2. Validation parameters of Erlotinib

Validation parameter	Erlotinib drug
Intraday method precision(n=6, % of RSD)	0.1
Interday method precision(n=6, % of RSD)	0.8
LOD-LOQ	
Limit of detection (mg mL^{-1})	0.0012
Limit of quantification (mg mL^{-1})	0.004
Linearity	
Calibration range (mg mL^{-1})	0.075-0.450
Calibration points	6
Correlation coefficient	0.9998

The detection limit and quantitation were 0.0012 mg mL^{-1} (S/N 4.73), 0.004 mg mL^{-1} (S/N 10.92) and Erlotinib linearity was demonstrated with the concentration ranging 0.075-0.450 mg mL^{-1} and the correlation coefficient was greater than 0.999 and the accuracy and recovery of the method was proved, the % of recovery was 101.5 for the assay of Erlotinib and the results were shown in table 3.

Table 3. Assay recovery of Erlotinib

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

Method robustness was checked by changing the organic solvent composition (± 0.2 mL min^{-1}) pH(±0.2), column temp (±5°C), different systems, there is no illustrious changes were observed and the stability of the Erlotinib drug solution was checked at precise temperature (2-8°C) for the period of 40 days, the mobile phase stability was checked (2, 4, 7 days) with Erlotinib drug solution and there was no significant changes were observed.

APPLICATION

Erlotinib stress degradation studies provides degradation pathway, chemical behaviour of the molecule which in 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

Four degradation products were formed during the acid and peroxide degradation of Erlotinib. All of them were unambiguously characterized by HRMS and NMR techniques. While two of the degradants were reported previously by Anand *et al.* group. The reported structures based on mass data only and these structures completely wrong. Based on our NMR study confirmed structure was explained in detailed. Another two degradation products were newly reporting. Also, it described the validation of UPLC method for Erlotinib drug with shorter runtime.

ACKNOWLEDGEMENT

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Supplementary data: All the HRMS, LC-MS and NMR spectra are provided as supplementary data.

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