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Isolation, Identification and Characterization of Afatinib Novel Degradation Products by NMR and HRMS:RP-UPLC Method Development and Validation

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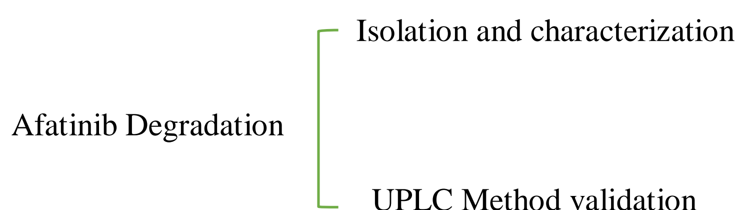
ABSTRACT

Afatinib (AFA) is an anilino quinazoline derivative and it 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. Two degradants were identified in acid hydrolysis referred as AFA-DP1, AFA-DP2 , one degradant was formed in base hydrolysis referred as AFA-DP3 and one degradant was formed in peroxide mediated hydrolysis referred as AFA-DP4. Out of four degradants two are novel and two are already published, here also one degradant structure was conformed by mass and another one by 1H and 13C NMR. In our study all the four degradation product structures were confirmed by HRMS and 1D (1H, 13C) and 2D (COSY, HSQC and HMBC) based on 1D and 2D NMR data proton and carbon chemical shift values assigned exactly for all DPs. A stability indicating RP-UPLC method was developed and validated with shorter run time and method was validated in terms of linearity, specificity, accuracy, LOD and LOQ.

Graphical Abstract



Keywords: Afatinib, Method validation, Degradation products, HRMS, NMR, ^{15}N HSQC, ^{15}N HMBC.

INTRODUCTION

Afatinib IUPAC nomenclature is ([N-[4-[(3-chloro-4-fluorophenyl) amino]-7-[[(3S)-tetrahydro-3-furanyl] oxy]-6-quinazolinyl]-4(dimethyl amino)-2-butenamide]). Afatinib is an anilino quinazoline derivative and irreversible tyrosine kinase receptor inhibitor. It is used in the therapy of metastatic non-small cell lung cancer (NSCLC) which is the common type of lung cancer [1]. Afatinib selectively and irreversibly binds and inhibits the epidermal growth factor receptors and certain epidermal growth factor receptor (EGFR) mutants which result in the inhibition of tumour growth and angiogenesis in tumour cells [2]. Recently, the Food and Drug Administration granted approval to AFT for a broadened indication in first-line treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumours have non-resistant EGFR mutations as detected by an FDA-approved test. The most common adverse reactions reported for AFT across clinical trials are diarrhea, rash/acne dermatitis which varies with individuals [3]. The Afatinib chemical structure is shown in figure 1.

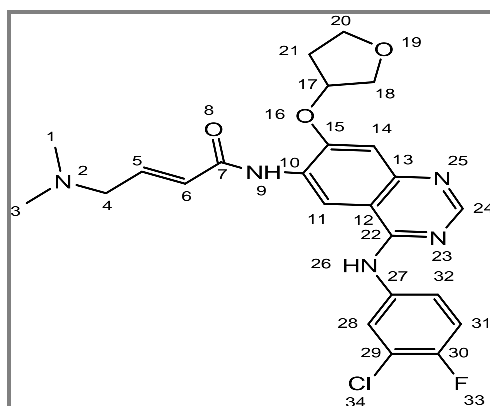


Figure 1. Chemical structure of Afatinib (AFA).

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 Harmonisation (ICH) and other regulatory authorities [4-6] 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 [7]. 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 on the stress stability studies of Afatinib in solid dosage forms and Active Pharmaceutical Ingredients (APIs) by HPLC and UPLC [8-10].there is few reports on the isolation and characterization of degradants by mass and NMR [11]. Few UPLC methods and validations were reported in major pharmacopoeias [12]. 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, method development and validation. 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 eliminate the significant time and cost, speed, resolution and sensitivity .UPLC flow rate range 0.01 to 2mL 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 Afatinib bulk drug and reduced analysis time with good efficiency.

MATERIALS AND METHODS

Chemicals and reagents: Afatinib 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 Milli-Q grade, Ammonium bicarbonate (Sigma-Aldrich).

Liquid Chromatography-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 30 v, MCP voltage 2700 v, positive ionisation mode. Leucine Enkephalin (556.2771 Da) was used to calculate Lteff and Elemental compositions were calculated with Mass lynx (4.1) software.

Ultra-performance liquid chromatography is used for reaction monitoring, Method conditions are Column: ACQUITY BEH C18, 2.1mm × 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^{-1}, Temp: 50°C.

Preparative HPLC: Gilson prep-HPLC (GX-271), DAD detector with column X Bridge C18 (250 × 19 mm) 5 µ with mobile phase A: 10mM Ammonium bicarbonate in Aquas 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/min at room temperature.

H-Class Ultra Performance Liquid Chromatography: H-Class 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 2.1X100 mm 1.7 µm, 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 0.3mL min^{-1}, Column temp 30°C.

Nuclear Magnetic Resonance spectroscopy: The ^1H, ^{13}C NMR 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 ^1H and ^{13}C chemical shifts are 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 ^1H 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, 2N HCl was used for acid hydrolysis and refluxed for 5 h and the formation of degradant percentage was very low and the reflection is 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, base peroxide hydrolysis.

RESULTS AND DISCUSSION

The degradants were formed after 5 h of stirring in the media. However, it was continued till 12 h to enrich their yields. For analytical study, 1 mL of the reaction mass was dissolved with mobile phase and 1 µL was injected into LC-MS system. Two degradants ware identified in acid hydrolysis, two degradants ware base hydrolysis and one degradant identified in peroxide mediate hydrolysis. AFA-DP-2 was obtained in both acid and basic mediated hydrolysis. However, no degradation products were formed in photolytic and thermal conditions. Acid, base and Peroxide treated solution was taken up for isolation of all the four degradants. The degradation chromatograms ware shown in figure 2.

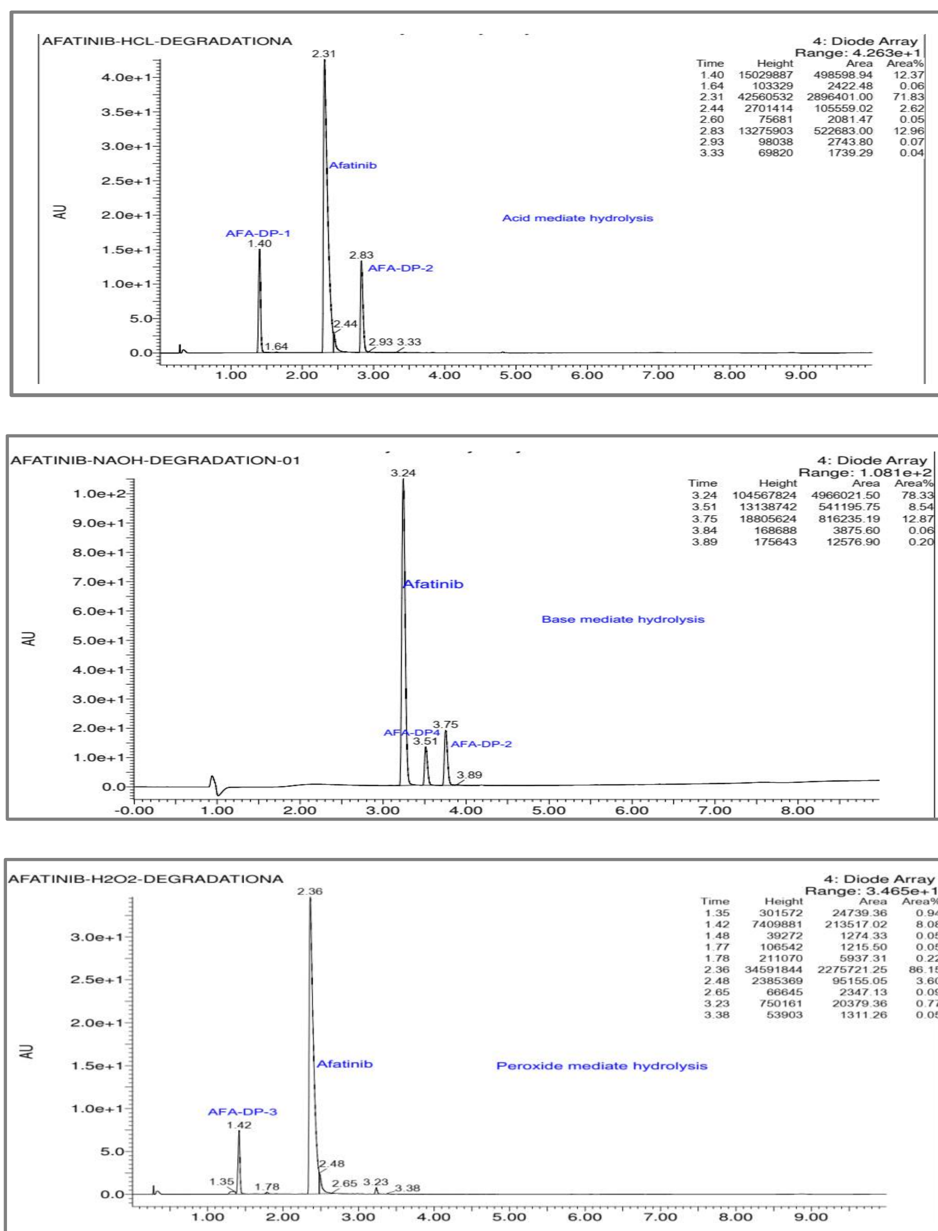


Figure 2. Acid, Base, peroxide degrdation products of Afatinib.

Isolation of Acid, base and Peroxide degradation products: The fractions corresponding to the four peaks were collected, distilled and lyophilized. The degradation products were labelled as AFA-DP-1,(6-amino-7-((tetrahydrofuran-3-yl)oxy)quinazolin-4(3H)-one). AFA-DP-2. (N4-(3-chloro-4-fluorophenyl)-7 ((tetrahydrofuran-3-yl)oxy)quinazoline-4,6-diamine). AFA-DP-3, ((E)-4-(dimethyl amino)-N-(4-oxo-7-((tetrahydrofuran-3-yl)oxy)-3,4-dihydroquinazolin-6-yl)but-2-namide. AFA-DP-4,(.1-(4-((3-chloro-4-fluorophenyl)amino)-7-((tetrahydrofuran-3-yl)oxy)quinazolin-6-yl)-5-hydroxy pyrrolidin-2-one). The structures of these degradation products were elucidated by the analysis of HRMS and 1D, 2D NMR data. Two novel degradation products ware formed and two were reported, on the basis of mass spectrometry. The chemical structures of degradation products were shown in figure 3.

Structure elucidation of AFA-DP-1: The mass spectrum of AFA-DP-1 shows protonated molecular ion peak at 248.1031 $[M+H]^+$ and protonated molecular formula $C_{12}H_{14}N_3O_3$ was confirmed by HRMS experiment, the HRMS spectrum of AFA-DP-1 was shown in figure 4.

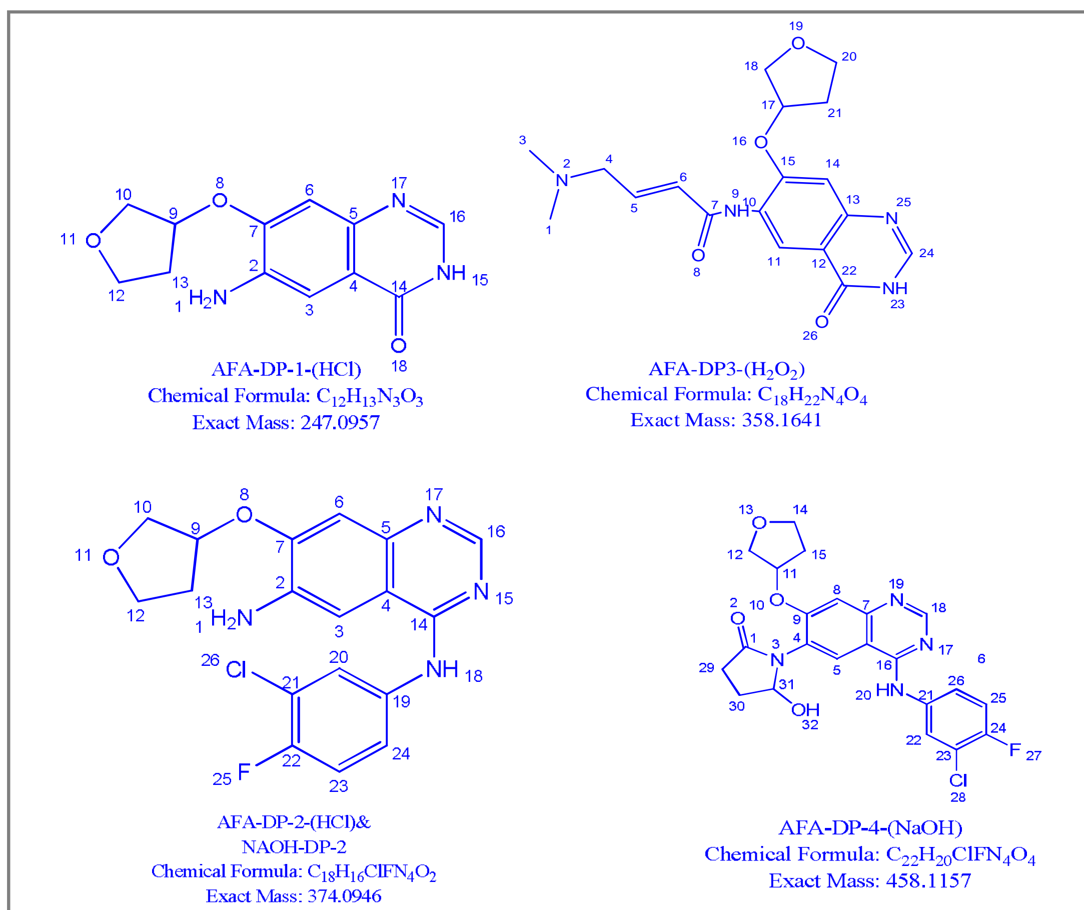


Figure 3. Chemical structures of degradation products.

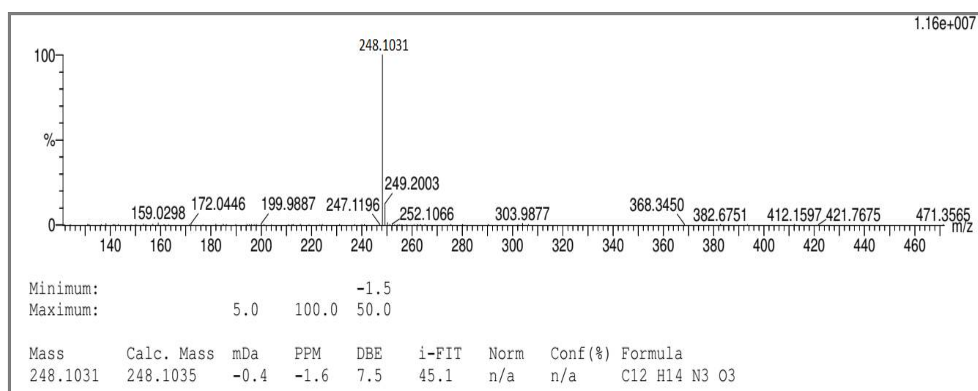


Figure 4. HRMS spectrum of AFA-DP-1.

It had 7 aliphatic protons, 3 aromatic protons, one Amine protons and one amide NH protons observed in ^{1}H NMR. Amine (-NH2) protons observed at 5.31 ppm and It don't have 3-chloro-4-fluorophenyl) amino and (dimethylamino) but-2-enamide group protons of Afatinib drug substance. It is due to fact that these groups were cleaved during acidic hydrolysis of drug substance with HCl. ^{13}C NMR revealed that it had 4 aliphatic carbons and 8 aromatic carbons. Amide carbonyl carbon (14th position) observed at 160.1 ppm in 13C NMR. In HMBC 16th position proton (7.79 ppm) showed correlation with 14th position carbonyl carbon at 160.1ppm. 1st position NH_2 protons (5.31ppm) showed correlation with C-3(106ppm) and C-7(149.8ppm) as shown in figure 5.

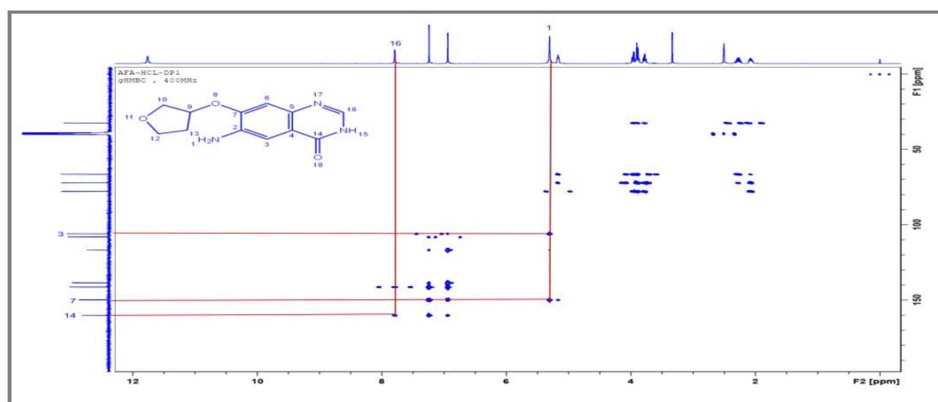


Figure 5. HMBC spectrum of AFA-DP-1.

This key proton versus carbon correlations in HMBC supporting to structure of AFA-DP-1 as shown in figure 3. All 1H and 13C chemical shift values were assigned by using NMR data as shown in table 1.

Table 1. 1H, 13C Chemical shift values of Afatinib and its degradation products.

Assig-nment	Afatinib		AFA-DP-1(HCl)		AFA-DP-2(HCl) and -AFADP-2(NaOH)		AFA-DP-1(H2O2)		AFA-DP-1(NaOH)	
	1H	13C	1H	13C	1H	13C	1H	13C	1H	13C
1	2.19	45.1	5.31		5.39		2.26	44.8		173.7
2				138.5		138.9				
3	2.19	45.1	7.24	106	7.4	101	2.26	44.8		
4	3.1	59.7		116.7		110.5	3.19	59.3		126.6
5	6.81	142.2		141.3		144.5	6.78	140.8	8.38	125.3
6	6.6	125.7	6.94	108.1	7.05	107.3	6.64	126.6		109
7		163.6		149.8		150.4		163.4		151
8									7.29	108.8
9	9.45		5.17	77.9	5.24	78	9.27			156.8
10		127.5	3.90, 3.96	72.2	3.95,4.00	72.1		127.4		
11	8.96	116					8.88	117.2	5.3	78.2
12		108.9	3.77, 3.88	66.5	3.79,3.92	66.5		115.6	3.83, 3.98	72.2
13		148.7	2.08, 2.27	32.5	2.12,2.32	32.5		146.5		
14	7.24	108		160.1		155	7.15	108.6	3.8	66.5
15		153.2	11.76					152.4	2.03, 2.28	32.5
16			7.79	141.2	8.37	150.2				156.9
17	5.3	78.7					5.27	78.9		
18	4	72			9.4		4	71.9	8.59	154.9
19						137.5				
20	3.79,3.93	66.6			8.2	122.4	3.77, 3.93	66.6	9.9	
21	2.16,2.34	32.4				118.6	2.16,2.32	32.3		136.7
22		156.7				152.6		160.1	8.18	123.4
23					7.39	116.4	12.08			118.7
24	8.53	153.8			7.81	121.4	8.01	144.9		153.2
25									7.43	116.5
26	9.81								7.83	122.3
27		136.8								
28	8.13	123.5								
29		118.6							2.41, 2.59	28.9
30		153							1.89, 2.43	28.5
31	7.42	116.4							5.5	83.4
32	7.8	122.4							6.36	

Structure elucidation of AFA-DP-2: The mass spectrum of AFA-DP-2 shows protonated molecular ion peak at 375.1021 $[M+H]^+$ and protonated molecular formula $C_{18}H_{17}N_4O_2ClF$ was confirmed by HRMS experiment, the HRMS spectrum of AFA-DP-2 was shown in figure 6.

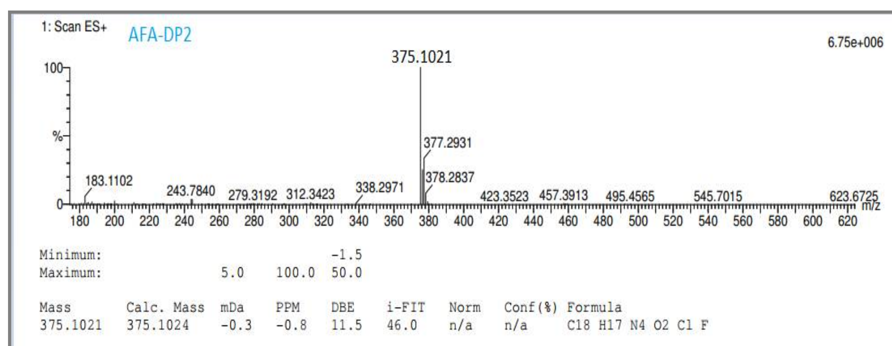


Figure 6. HRMS spectrum of AFA-DP-2.

It had 7 aliphatic protons, 6 aromatic protons, one Primary Amine protons and one secondary amine NH protons observed in ^{1}H NMR. Amine ($-NH_2$) protons observed at 5.39 ppm. It don't have (dimethylamino) but-2-enamide group protons of Afatinib drug substance. It is due to fact that this group was cleaved during acidic hydrolysis of drug substance with HCl as well as basic hydrolysis of drug substance with NaOH. ^{13}C NMR revealed that it had 4 aliphatic carbons and 14 aromatic carbons. In HMBC, 1st position NH_2 protons (5.39 ppm) showed correlation with C-3(101 ppm) and C-7(150.4 ppm) as shown in figure 7.

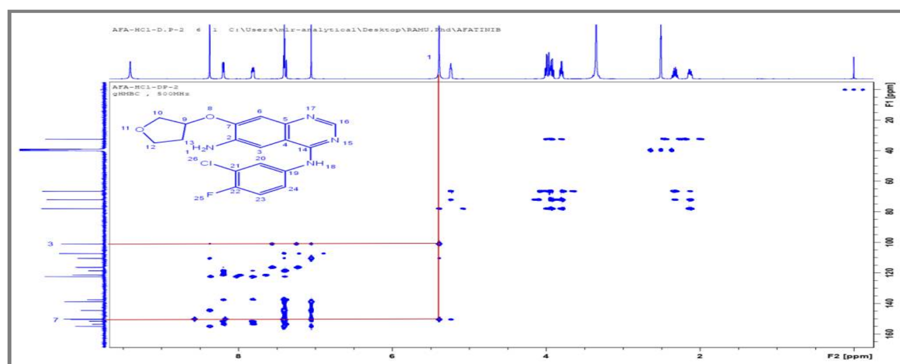


Figure 7. HMBC spectrum of AFA-DP-2

This key proton versus carbon correlations in HMBC supporting to structure of AFA-DP-2 as shown in figure.3. All 1H and 13C chemical shift values were assigned by using 2D NMR data as shown in table 1.

Structure elucidation of AFA-DP-3: The mass spectrum of AFA-DP-3 shows protonated molecular ion peak at 359.1716 $[M+H]^+$ and protonated molecular formula $C_{18}H_{23}N_4O_4$ was confirmed by HRMS experiment, the HRMS spectrum of AFA-DP-3 was shown in figure 8.

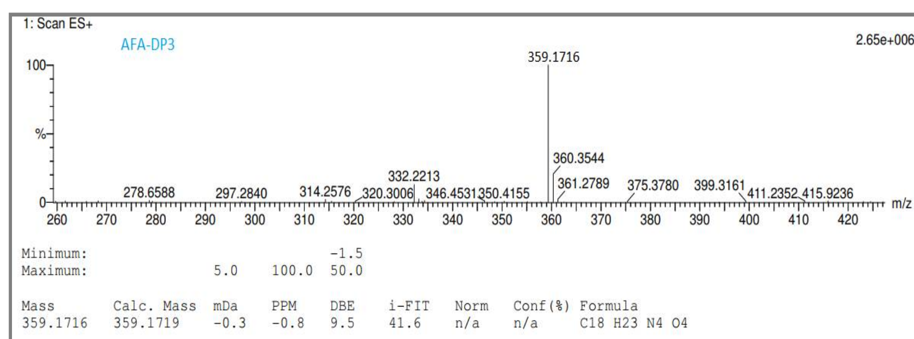


Figure 8. HRMS spectrum of AFA-DP-3.

It had 15 aliphatic protons, 5 aromatic protons and 2 amide NH protons observed in ^{1}H NMR. It don't have 3-chloro-4-fluorophenyl) amino group protons of Afatinib drug substance. It is due to fact that this group was cleaved during oxidation of drug substance with H_2O_2. ^{13}C NMR revealed that it had 7 aliphatic carbons and 11 aromatic carbons. Amide carbonyl carbon (22nd position) observed at 160.1 ppm in 13C NMR. In HMBC, 24th position proton (8.01ppm) showed correlation with 22nd position carbonyl carbon at 160.1ppm and 13th position carbon at 146.5ppm as shown in figure 9. This HMBC data matched with structure of AFA-DP-1 as shown in figure 3.

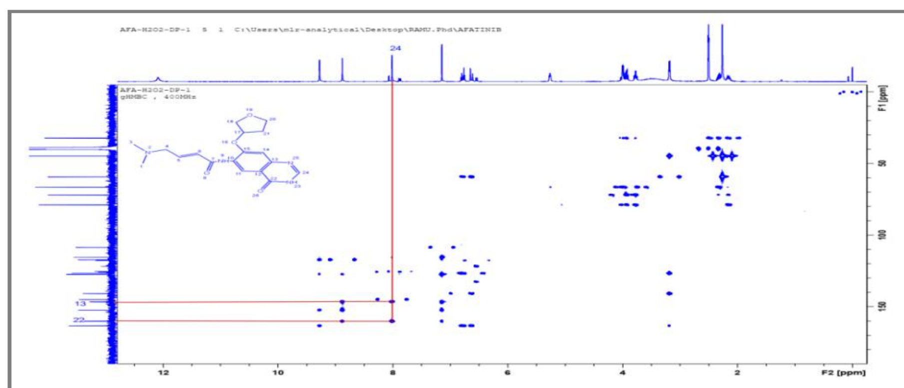
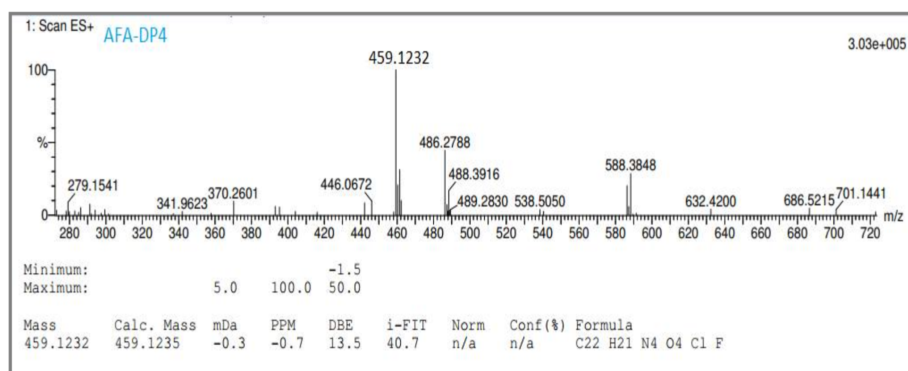


Figure 9. HMBC spectrum of AFA-DP-3(HCl).

Structure elucidation of AFA-DP4: The mass spectrum of AFA-DP-4 shows protonated molecular ion peak at 459.1232 $[M+H]^+$ and protonated molecular formula $C_{22}H_{21}N_4O_4ClF$ was confirmed by HRMS experiment, the HRMS spectrum of AFA-DP-4 was shown in figure 10.



Minimum:				-1.5				
Maximum:		5.0	100.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
459.1232	459.1235	-0.3	-0.7	13.5	40.7	n/a	n/a	C22 H21 N4 O4 Cl F

Figure.10. HRMS spectrum of AFA-DP-4.

It had 12 aliphatic protons, 6 aromatic protons, one aromatic NH(9.9 ppm) proton and aliphatic OH proton(6.36 ppm) observed in ^{1}H NMR. It don't have (dimethylamino)but-2-enamide group protons of Afatinib drug substance. It is due to fact this group was converted to 5-hydroxypyrrolidin-2-one during basic hydrolysis of drug substance with NaOH. ^{13}C NMR revealed that it had 7 aliphatic carbons and 15 aromatic carbons. Amide carbonyl carbon (1st position) observed at 173.7 ppm in 13C NMR. It contains 5-hydroxypyrrolidin-2-one group and it was confirmed by COSY. In COSY, H-31(5.5ppm) correlated with H-30(1.89, 2.43 ppm). H-30(1.89, 2.43 ppm) correlated with H-31(5.5ppm) and H-29(2.41,2.59 ppm) as shown in figure 11. All proton versus proton correlations in COSY and HSQC, HMBC data matched with structure of AFA-DP1(NaOH) as shown in figure 3.

Method development and validation: UPLC method with 5 minutes run time method was developed as mentioned in section 2.4 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.

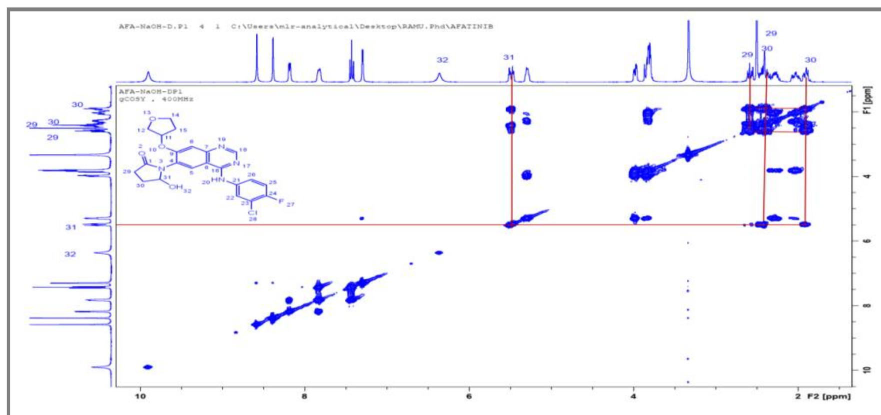


Figure.11. COSY spectrum of AFA-DP-4.

Afatinib standard solution (0.4 mg mL^{-1}) was injected in the UPLC system for system suitability test, the retention time of the Afatinib was 2.21 min and USP Tailing, plate count values are 1.29, 46467.69. Intraday method precession, inter day method precession was checked with six repeated concentration preparations and the % of RSD values are 0.3, 0.2 and the results are shown in table 2.

Table 2. Validation parameters of Afatinib

Validation parameter	Afatinib drug
Intraday method precision(n=6, % of RSD)	0.5
Interday method precision(n=6, % of RSD)	0.6
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 4.94), 0.004 mg mL^{-1}(S/N 70.05) and Afatinib 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 99.16 for the assay of Afatinib and the results were shown in table 3.

Table 3. Assay recovery of Afatinib

Level (%)	Amount added (µg mL^{-1})	Amount recovered (µg mL^{-1})	Recovery (%)
50	160.01	157.94	98.70
100	200.05	198.85	99.40
150	299.19	297.40	99.40

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 Afatinib 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 Afatinib drug solution and there was no significant changes were observed.

APPLICATION

Afatinib stress degradation provides degradation pathway, chemical behaviour of the molecule which in helps in the development of formulation and package, UPLC method validation eliminates the significant time and cost.

CONCLUSION

Four degradation products were identified during the Acid, Base, Peroxide degradation of Afatinib out of four two is novel and remaining two were already published in JPBA, but here also one degradant structure was confirmed by mass and another one by 1H and 13C NMR. In our study All the degradants were unambiguously characterized by HRMS,LC-MS and 1D(1H,13C) and 2D(COSY, HSQC and HMBC) based on this 1D and 2D NMR data proton and carbon chemical shift values assigned exactly for all degradant products. UPLC method validation was performed with shorter run time, good efficiency, it eliminate the significant time and cast.

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

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