Separation, Identification, Isolation and Characterization of Degradation Product of Osimertinib Tablets by UPLC-QTOF-MS/MS and NMR: Evaluation of *In-Silico* Safety Assessment for Osimertinib (OSM) and Degradation Product (DP)

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Abstract

The present work encompasses identification and characterization of major degradation product (DP) of OSM observed in base hydrolytic stress study. The separation of DP was carried out on a non-polar stationary phase by using high-performance liquid chromatography system (HPLC). Using waters X-bridge (250 mm × 4.6 mm, 5 μm) C18 column with gradient elution program. For the characterization study, stress samples were subjected to HPLC and UPLC-QTOF-MS/MS and based on mass fragmentation pattern, plausible structure was deduced. Further, the DP was isolated using semi-preparative liquid chromatography and concentrated the fractions using lyophilization. The isolated DP was subjected to extensive 1D (1H, 13C, and DEPT-135) and 2D (COSY, HSQC and HMBC) nuclear magnetic resonance (NMR) studies to authenticate the structure. The impurity was unambiguously named as N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-metho-xy-5-((4-(1-methyl -1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-3-methoxypropanamide. Additionally, the *In-Silico* structure activity relation (QSAR) assessed through statistical based software's DEREK Nexus[TM], and MultiCASE, Case Ultra[TM] widely accepted and respected software's for DP and OSM.

Keywords

Osimertinib Mesylate (OSM), Base Degradation, Semi-Preparative Isolation and Characterizations by HPLC, UPLC-QTOF-MS/MS , NMR Techniques

1. Introduction

Osimertinib Mesylate (OSM) (Figure 1; IUPAC name: "N-(2-{2 dimethylaminoethyl-methylamino}-4-methoxy-5-{[4-(1-methylindol-3-yl)pyrimidin-2yl]amino}phenyl)prop-2-enamide mesylate salt" molecular formula: $C_{28}H_{33}N_7O_2 \cdot CH_4O_3S$; molar mass: 596 g/mol [1]. Osimertinib is an oral, third-generation, irreversible EGFR-TKI that is proved to selectively inhibit both EGFR-TKI-sensitizing and EGFR T790M resistance mutations [2] [3] [4]. It was also approved to be one of the first-line treatment options for EGFR-mutant NSCLC patients owing to the positive results from the FLAURA study [5], which demonstrated significant survival benefits in both PFS and OS [6] [7]. In April 2014 based on phase I clinical trial results, the OSM was designated as a Breakthrough Therapy [8]. The tablet formulation of OSM Mesylate (Tagrisso™) developed by Astra-Zeneca has been granted accelerated approval by the United States Food and Drug Administration (US-FDA) in November 2015, for the therapy of patients with metastatic EGFR T790M mutation-positive NSCLC who have progressed on or after the first and second generation of EGFR TKI therapy [9].

Now a day's impurity profiling considers critically by all regulatory agencies to ensure the safety and efficacy of all pharmaceutical preparations. Certainly, out of specification (OOS) or out of trend (OOT) results are mostly observed when dealing with a relatively high number of degradants or impurities in finished product or drug substance during product storage [10]. The impurity profiling is the one of the critical quality attributes for the pharmaceutical preparations which ensures the pure and safe drug to the patient [11]. Therefore, chromatography plays a crucial role in separation of process/degradation impurities, based on the chemistry of molecule suitable column was used to develop a unique chromatography to ensure the purity of OSM drug. As per the ICH guidance, the stability studies of drug product or drug substance are one of the crucial quality control testing in the pharmaceutical industry [12], which assist to the drug manufacturer to maintain the quality of finished formulations or raw materials. The quality of the drug may alter with several environmental variables such as light, humidity, temperature, storage time, or physical parameters [13]. For the finished product or raw materials self-life would be assigned based on the stability studies and trend data of degradation products on various intervals of testing. Thus, degradation studies of drugs are paramount importance to forecast
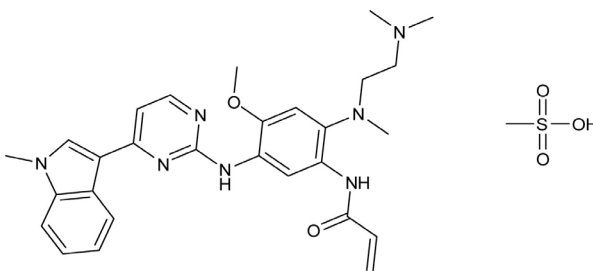

Figure 1. Chemical structure of Osimertinib Mesylate (OSM).

the manifestation of new degradant during the product storage, which provides further insight of stability of the active molecule and develop a stability-indicating chromatographic method to define shelf life as per ICH guidance [14].

Seldom the residual solvents present in the drug substance or drug products may reacts with the active drug in presence of acid, base or oxidative conditions which leads to the manifestation of new impurity. The degradation studies provide understandings to develop the formulations as well as rout of synthesis of active drug by ensuring the quality of the raw materials used for synthesis or formulation. Moreover, the degradation studies are also providing further insights to the drug manufacturer for to develop packaging components and storage of the drug products, which helps to increase self-life of drug substance or drug products.

To the best of our knowledge research on degradation products for OSM is not published elsewhere. Therefore, in the present work, we developed a novel chromatographic method by using C18 column chemistry by using HPLC-PDA and further transferred and scale up the same method on semi-preparative HPLC to isolate and characterize the major degradant observed in base hydrolysis of OSM using UPLC-Q-TOF-MS and NMR techniques. Thus, to understand the hydrolytic behavior of the drug, characterization of the isolated degradation is highly essential [15] [16].

To understand the degradation profile in the base hydrolysis, samples were subjected to UPLC-Q-TOF/MS after developing a simple mass compatible method for identification of accurate mass of parent and daughter ions.

2. Experimental

2.1. Chemicals and Reagents

Osimertinib Mesylate (OSM) active and formulation samples obtained from Cipla Laboratories Ltd. (Mumbai, India). HPLC grade methanol (MeOH) was obtained from Thermo fisher Scientific India, (Mumbai, India) and HPLC and LC-MS grade Acetonitrile (ACN) obtained from Honeywell International India (New Delhi, India). Analytical reagent (AR) grade lab reagents such as formic acid was procured from S.D. Fine, ammonia solution (NH₄OH) and Hydrochloric acid (HCl) were procured from Rankem, (Avantor Thane, India) sodium hydroxide (NaOH) were procured from (Merck Life Science Pvt Ltd, Mumbai, India). To prepare all solutions and mobile phase HPLC grade water was obtained from a Milli-Q Gradient system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

2.2.1. High-Performance Liquid Chromatography (HPLC)
The method development for degradation product was done on Agilent 1220 HPLC system, photodiode array detector and automatic liquid sampler injector. Chromatographic data were recorded and processed using chromeleon software (Thermofisher Scientific ver. No 6.80). The test sample solutions were made

with diluent (Mobile phase A: B 50:50 v/v). The sample solutions for analysis were injected on a newly developed reverse-phase chromatographic method with Waters X-bridge C18 column (25 cm × 4.6 mm internal diameter, 5-micron particle size) used for the impurity separation and identification. Mobile phase A consists of buffer (0.1% formic acid with pH 5.5 with ammonia solution) was filtered through 0.45 m PTFE filter and degassed, Acetonitrile as mobile phase B. The analysis was carried out using the liner gradient program was set for separation of components as follows: time/solvent-B (%): 0.0/10, 5.0/15, 15/20, 30.0/30, 45.0/45, 50.0/70, 55.0/80, 60.0/80, 61.0/10, 65.0/10 with flow rate 1.0 ml/min. The analytes were monitored at a wavelength of 268 nm. The column temperature was maintained at ambient. 10 μL injection volume was used for sample analysis.

2.2.2. Semi-Preparative High-Performance Liquid Chromatography

The semipreparative HPLC separation and fraction collection of degradation product was carried out on Agilent HPLC 1200 with 900 μl loop size with semi preparative column Luna C18 column (250 cm × 10 mm internal diameter; 10 micron particle size) using Agilent quaternary pump, the sample concentration was optimized to 10 mg/ml, prepared in diluent. The mobile phase consists of A: Buffer (0.1% formic acid with pH 5.5 with ammonia solution) and B: Acetonitrile. The gradient program was set as follows: time (min)/solvent-B (%): 0.01/20, 20.0/30, 50.0/40, 80.0/50, 85.0/90, 86.0/20, 90.0/20 with flow rate of 2.3 ml/min. The analytes were monitored at 268 nm, with injection volume was used as 400 μL for faction collection. The fractions were, pooled together and lyophilized using lyophilizer (Make: BUCHI, Lyovapor, L-300).

2.2.3. Electrospray Ionization Mass Spectrometry (ESI-Q-TOF-MS)

The isolated and purified degradation product was further subjected for mass and its further fragmentation using an electrospray ionization-mass spectrometry system (Waters Xevo Q-tof Waters USA) with an electrospray ionization source at positive ion full scan mode mass spectrometry. Ion source temperature was set at 120°C, Desolvation Temperature was set to 350°C and the capillary cone voltage was +3 kV. Nitrogen gas (99.99% purity) was used as curtain gas and CAD gas was used as Helium at a pressure of 15 psi and 10 psi respectively with gas flow-rate of 6 mL·min^{-1} and nebulizer gas flow-rate was at 10 mL·min^{-1}. Zero air at a pressure of 45 psi was used as heater gas. The mass spectra were acquired in positive ion mode over the mass range of 50 - 4000 Da.

All the chromatographic conditions used for UPLC-Q-TOF-MS are same as chromatographic conditions of HPLC, outlined in section 2.2.1.

2.2.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

1D and 2D NMR spectra of the isolated DP and OSM Drug was recorded on varian^{TM} 500 MHz (Advance Neo) using best suitable solvent as methanol-d4 for OSM and DMSO-d6 for DP and Tetramethylsilane (TMS) as internal standard.

The NMR data for DP was generated in DMSO-d6 and active OSM in methanol-d4 for comparison. The 1H chemical shift values were reported on δ scale in ppm, relative to TMS (δ = 0.00 ppm) and in the 13C chemical shift values were reported relative to methanol-d4 (δ = ~48 ppm).

2.3. Procedure for Base Degradation Studies

Weigh equivalent to 500 mg of OSM and transferred in to 100 ml volumetric flask and added 20 ml methanol, sonicate to dissolve, further added 20 ml diluent, swirl the solution and added 10.0 mL 1.0 N NaOH solution and refluxed for 2 hrs at 100°C temperature then cooled the solution at room temperature and makeup the volume with diluent, then filter and injected on HPLC-PDA and analyzed using analytical method illustrated in Section 2.2.1

Note: Methanol is critical for solubilizing the Osimertinib Mesylate completely, in other solvents OSM is less soluble comparative to methanol.

3. Results and Discussion

3.1. Analytical Method Development for DP

The OSM Mesylate drug substance and drug products are a non-compendial product and no literature was found on the public domain for Degradation products. Thus, a novel analytical method developed to separate DP, and the same method adopted for further identification with the aid of UPLC-ESI-Q-TOF-MS. Initially, samples were analyzed by available literature [17] [18] by using the reported gradient as time/solvent-B (%): 0.0/25, 5.0/25, 15/40, 30.0/80, 60.0/80, 70.0/25, 80.0/25 however interference was observed with oxidative degradants for the base DP. Thus, a new method developed by optimizing the gradient as outlined in section 2.2.1 for OSM using XBridge BEH (Waters) HPLC column (dimension 25 cm × 4.6 mm *i.e.*, 5 μm particle size) with 1 ml of formic acid in 1000 ml water (adjusted to pH 5.5 with ammonia solution) was used as mobile phase A and acetonitrile was used as mobile phase B. The column temperature was maintained at ambient and the diluents used for the sample solution were 50:50 v/v of mobile phase A and B respectively. The DP was monitored at a wavelength of 268 nm. The analytical method development trails were conducted to optimize the mobile phase, column selection, and column temperature. The waters XBridge BEH column found suitable for stress samples. Therefore, waters XBridge BEH was chosen as the best suitable column for this analytical method and the analytical procedure as described in Section 2.2.1, The typical chromatogram of base degradation shown in Figure 2.

The newly developed chromatographic method for impurity has several advantages over the conventional HPLC methods such as this method can be directly applied for preparative/semi-preparative HPLC scale isolation for degradation products and as well as compatible with mass spectrometry characterization as the mobile phase of this method comprises mass compatible solvents, buffers (without phosphate, sulfate etc., buffers, which in turn could interfere
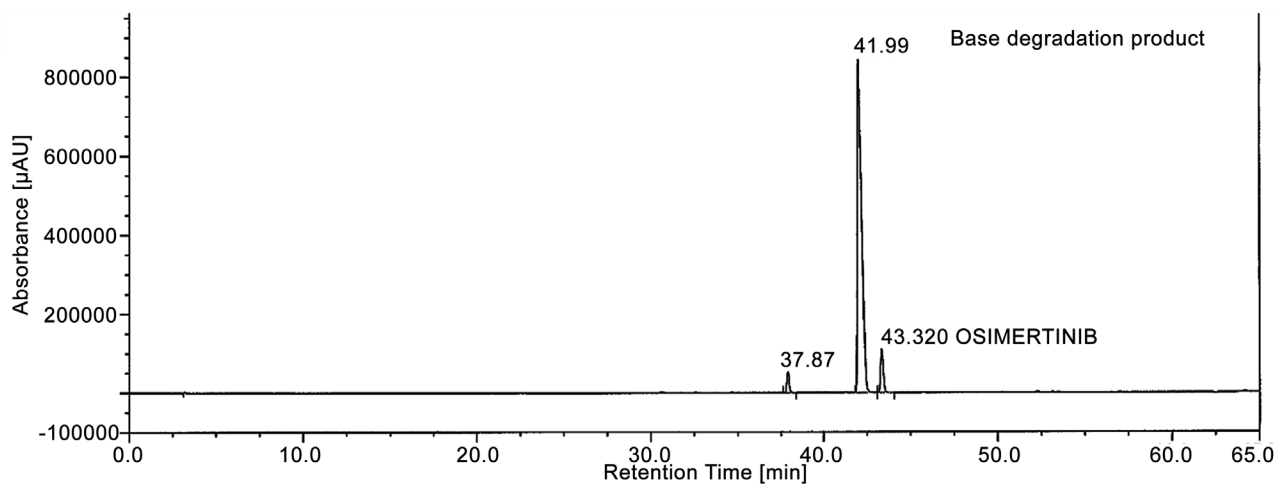
Figure 2. Base hydrolytic degradation 1 N NaOH at 100˚C for 2 hrs.

with mass ionization) with gradient elution. The retention time (RT), relative retention time (RRT), % DP, and peak purity are tabulated in Table 1.

3.2. Isolation of Novel Degradation Product

The developed HPLC method was directly transferred to semi-preparative HPLC and separation was achieved by optimizing the flow rate to accommodate more quantity of samples, to collect a higher amount of degradation product with less fractions using Phenomenex Luna PREP C18 (10.0 × 250 mm), 10 micron particle size mobile phase consists of A: Buffer (0.1% formic acid with pH 5.5 with ammonia solution) and B: Acetonitrile. The gradient program was set as follows: time (min)/solvent-B (%): 0.01/20, 20.0/30, 50.0/40, 80.0/50, 85.0/90, 86.0/20, 90.0/20 with flow a rate of 2.3 ml/min in order to collect the maximum amount of degradation product within the optimal time period, several portions of impurity fractions were collected on semi-prep HPLC. The collected degradant fractions were further lyophilized. The purity of the collected degradant was tested on HPLC as per method outline in Section 2.2.1, and was found about 90%. Further, the isolated degradant analyzed on NMR spectrometry and ascertain the structure which already deduced with the help of mass spectroscopic fragmentation pathways.

3.3. Mass Spectrometric Characterization of Novel Degradation Product

The Identification of active drug and its novel degradation product was studied on Waters Xevo Q-TOF of Mass spectrometric (MS) technique with mass lynx software (ver. No. 4.1). The protonated molecule and its further fragmentation pathways obtained from ESI-Q-TOF-MS further utilized to explore to elucidate structural arrangements of OSM and its degradation product. The ESI-Q-TOF-MS condition with ion spray voltage is optimized to +3.0 kV condition for better protonated and fragments ions. The base degradant was identified along with

Table 1. Retention time, relative retention time, % DP, and peak purity.

Drug/Degradation products	Retention time (RT) (Minutes)	Relative retention time (RRT)	Degradation product (%)	Peak purity (Match Factor)
DP	41.99	0.97	89.5	985.65
Drug (OSM)	43.32	1.00	7.3	951

the target OSM drug and its RRT was found at 0.97 (DP, m/z 532.2606). The accurate mass data and its fragments are reported in Table 2 as below.
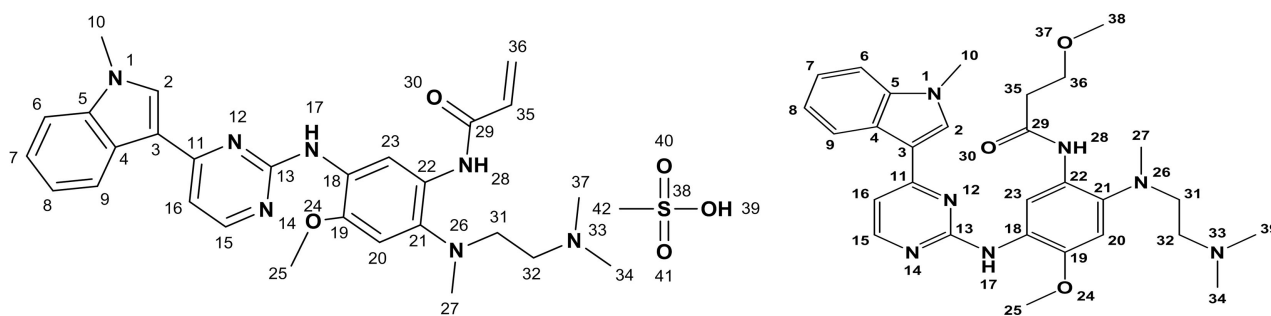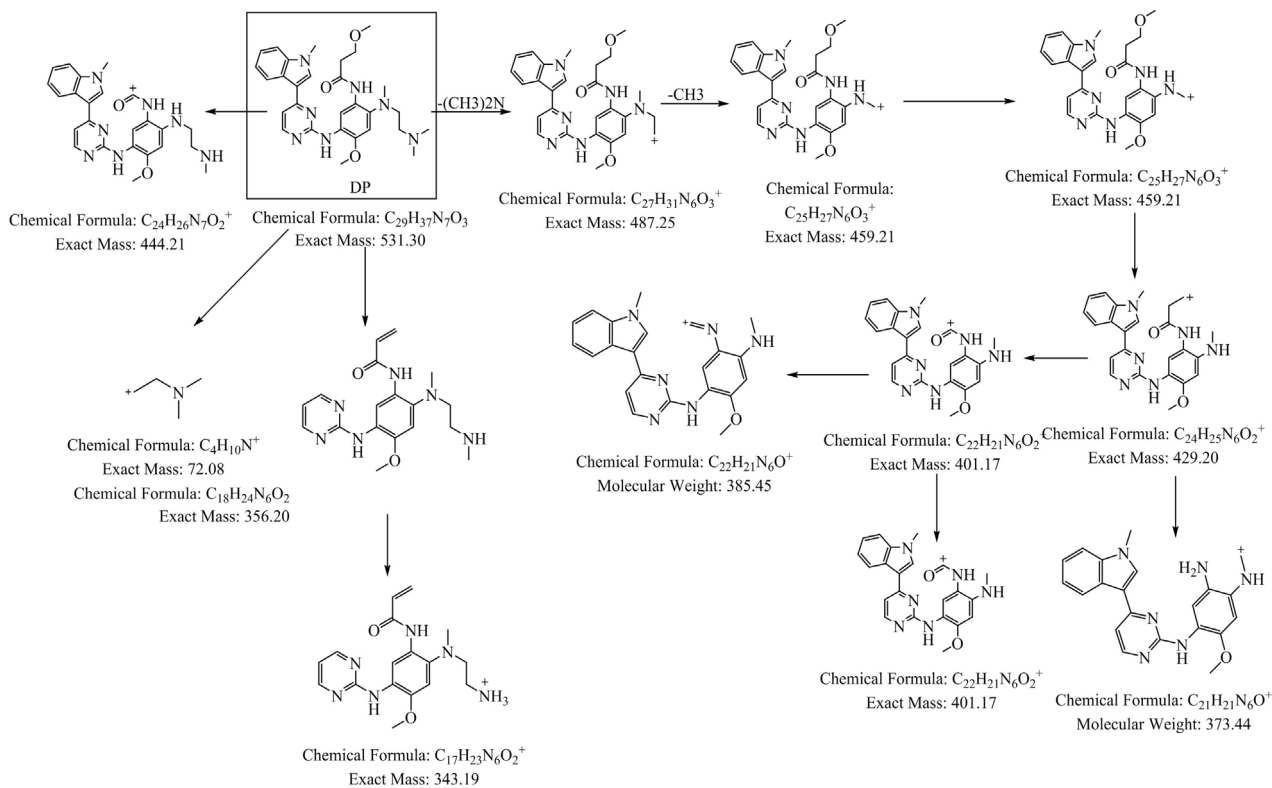
Based on molecular weight and fragmentation pattern, the proposed structure of degradation product is presented in Figure 3. The probable structure was proposed for the individual fragments are showed in Figure 4. The base degradant comprises C-C double bond of acrylamide converts to ether (by addition of methoxy group) the fragments m/z 487.2863 and m/z 459.2525 are the characteristic fragments which confirms the presence methoxy (-OCH$_3$).

3.4. Characterization of the Degradation Product [(m/z 532.2606), M + H]

This degradant was observed in Base hydrolysis and eluted at RRT about 0.97 (in HPLC) and shows molecular weight m/z 532.2606 Da [Figure 5(a)] which is 32 mass unit higher than the OSM API (500.2738 [M + H]+). The RDB value for DP is observed as 15.5 which decreased form the 16 RDB value of OSM API even though increase in molecular weight. It reveals that a double bond reduction in the DP. The molecular weight m/z 532.2606 further subjected to fragmentation and confirmed the structure after fragmentation Figure 5(b). Based on fragmentation pathways it is evident that the DP contains methoxy group (-OCH$_3$) attached to reduced C-C double bond of acrylamide. Further the structure was ascertained by 1H, and 13C NMR spectrometry, The Ethylene proton signal of acrylamide (-CH=CH$_2$) group is absent in NMR which was seen in OSM refer Figure 6 and Figure 7. The proton H-35 (6.54 δ ppm) and H-36' and H-36" (5.9 and 6.52 δ ppm respectively) of OSM protons were found absent in DP. Whereas new -CH$_3$ and two -CH$_2$ proton signals observed at H-38 (3.16 δ ppm) and H-35, H-36 (at 2.92 δ ppm, 3.65 δ ppm) respectively. Additionally, DP contains -OCH$_3$ group is also further ascertained by 13C, DEPT-135 and 2DNMR including, HSQC, COSY and HMBC technique. The PDA spectral scan evident no change in the aromatic region. The detail interpretation of 1D and 2D NMR for DP and OSM explained in Table 3 in the HMBC experiments the main key correlations, C-38 of -OCH$_3$ (at 58.47 δ ppm) correlates with the proton of H-36 and C-36 of -CH$_2$ (at 68.68 δ ppm) correlates with proton of H-38 of -OCH$_3$. Additionally, the correlation of C-29 with H-36 evident there is reduction of carbon-carbon double bond of acrylamide refer Figure 8. Rest of the correlations are comparable with OSM. The explained correlation is seen in COSY experiment as well, The H-36 is correlated with H-35 Hence, The structure of base degradant unambiguously named as N-(2-((2-(dimethylamino)

UPLC-Q-TOF-MS data of degradation product DP along with their molecular formulae and major fragments in ESI positive mode.

Identity	Accurate mass	Molecular formula	Exact mass	Error (δ ppm)	RDB	Nitrogen rule	Accurate masses of fragment Ions
[OSM + H]	500.2738	$C_{28}H_{34}N_7O_2^+$	500.2768	5.99	16	odd	455.2536, 427.2267, 412.2011, 385.2055, 369.2130, 354.2035, 341.1895, 72.0825
[DP + H]	532.2606	$C_{28}H_{34}N_7O_4^+$	532.2666	11.27	15.5	odd	487.2863, 459.2525, 444.2433, 429.470, 412.0000, 401.1000, 385.2179, 373.2069, 354.2035, 343.1595, 72.0825

Figure 3. Chemical structures of Osimertinib Mesylate (OSM) and base Degradation Product (DP).

Figure 4. Plausible fragmentation pathways of DP in ESI positive mode with molecular weight m/z 532.2606.
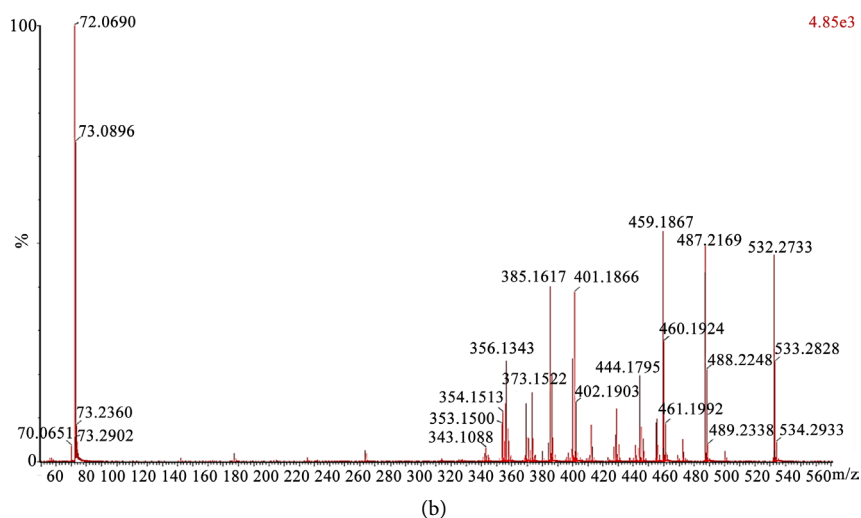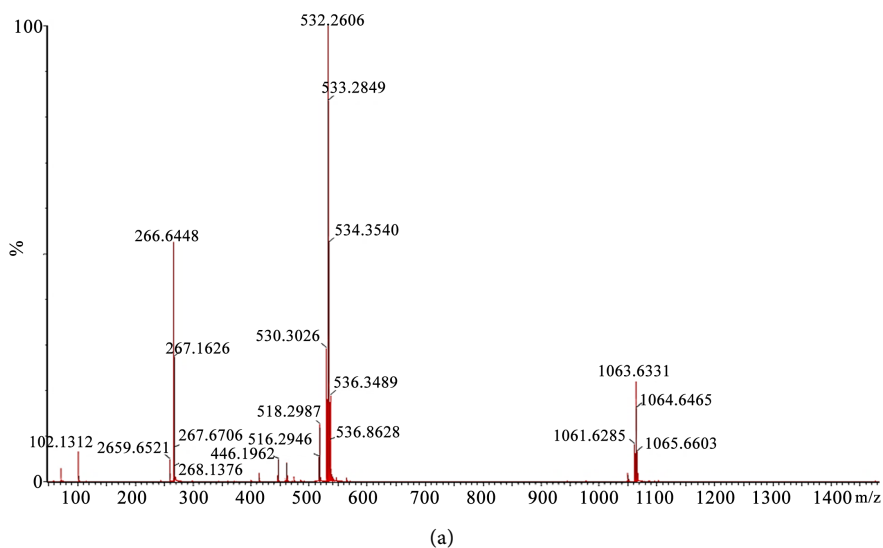
(a)

(b)

Figure 5. (a) Q-TOF-MS spectrum of DP under ESI positive conditions showing its molecular ion (m/z 532.2606); (b) MS/MS fragmentation pattern of DP.
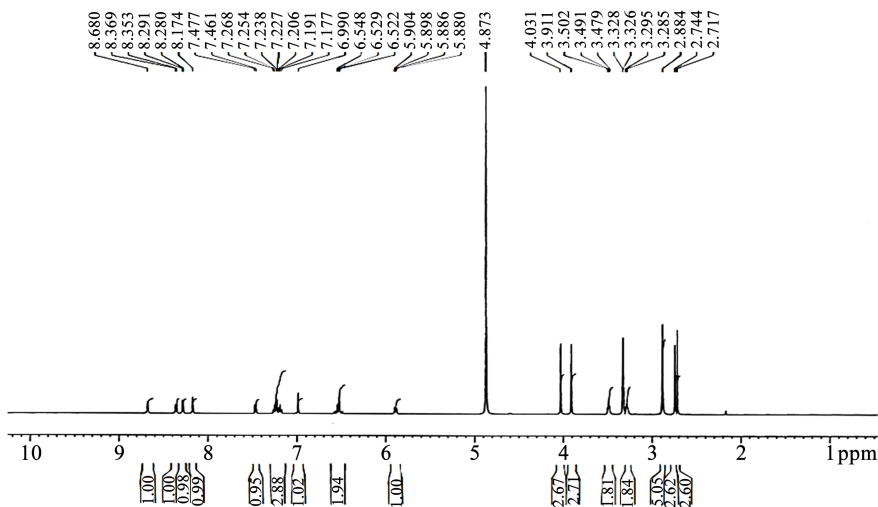

Figure 6. 1H NMR spectra of OSM.

Table 3. 1D and 2D NMR of OSM and DP.

		1D NMR (1H, 13C NMR, DEPT-132) and 2D NMR (COSY, HSQC, HMBC) for Osimertinib Mesylate in Methanol-d4 and DP in DMSO-D6 respectively data assignments[a]						
		Osimertinib Mesylate refer **Figure 3**			Degradation Product refer **Figure 3**			
Position	Types of Carbon (OSM-API) (multiplicity)	13C (δppm)/ 1H(δppm)	DEPT-135	13C(δppm)/ 1H(δppm)	DEPT-135	COSY (H-H)	HSQC (H-C)	HMBC (H-C)
1	N	-/-	-	-/-	-	-	-	-
2	CH (s)	132.6/8.17	CH	137.45/8.6	CH	H-2	C-2	C-3, C-4, C-10, C-5
3	C	113.16/-	-	112.89/-	-	-	-	-
4	C	125.80/-	-	125.81/-	-	-	-	-
5	C	138.67/-	-	121.8/-	-	-	-	-
6	CH (d)	109.62/7.46	CH	110.97/7.88	CH	H-7	C-6	C-4, C-8
7	CH (t)	121.07/7.25	CH	121.84/7.25	CH	H-6, H-8, H-9	C-7	C-5, C-9
8	CH (t)	122.17/7.17	CH	121.29/7.14	CH	H-7, H-6	C-8	C-4, C-6
9	CH (d)	121.37/8.35	CH	122.43/8.22	CH	H-8, H-7	C-9	C-3, C-5, C-7
10	CH$_3$ (s)	32/3.9	CH$_3$	33.39/3.92	CH$_3$	H-10	C-10	C-2, C-5
11	C	159.53/-	-	160.39/-	-	-	-	-
12	N	-/-	-	-/-	-	-	-	-
13	C	163.22/-	-	162.08/-	-	-	-	-
14	N	-/-	-	-/-	-	-	-	-
15	CH (d)	156.38/8.28	CH	138.18/8.31	CH	H-16	C-15	C-13, C-11, C-16
16	CH (d)	107.62/7.23	CH	107.51/7.22	CH	H-15	C-16	C-11, C-15, C-3
17	NH	-/*	-	-/7.01	-	-	-	-
18	C	124.17/-	-	160.396/-	-	-	-	-
19	C	148.07/-	-	146.23/-	-	-	-	-
20	CH (s)	103.07/6.99	CH	105.72/7.88	CH	H-20	C-20	C-32, C-21, C-19, C-18
21	C	138.19/-	-	134.28/-	-	-	-	-
22	C	126.23/-	-	164.25/-	-	-	-	-
23	CH (s)	117.74/8.86	CH	113.91/9.04	CH	H-23	C-23	C-22, C-21, C-19, C-18
24	O	-/-	-	-/-	-	-	-	-
25	CH$_3$ (s)	55.47/4.03	CH$_3$	56.47/3.84	CH$_3$	H-25	C-25	C-19
26	N	/-	-	-/-	-	-	-	-
27	CH$_3$ (s)	38.07/2.74	CH$_3$	43.33/2.69	CH$_3$	H-27	C-27	C-21, C-31
28	NH	-/*	-	-/9.88	-	H-28	-	C-21, C-23, C-29
29	C	165.75/-	-	168.84/-	-	-	-	-
30	O	-/-	-	-/-	-	-	-	-
31	CH$_2$ (t)	54.39/3.2	CH$_2$	57.28/2.92	CH$_2$	H-32	C-31	C-21, C-27, C-32
32	CH$_2$ (t)	47.31/3.50	CH$_2$	55.60/2.34	CH$_2$	H-31	C-32	C-34, C-37
33	N	-/-	-	-/-	-	-	-	-
34	CH$_3$ (s)	42.01/2.88	CH$_3$	45.62/2.24	CH$_3$	H-34	C-34	C-32

Continued

35	CH (s)	130.60/6.54	CH	37.63/2.92	CH$_2$	H-36	C-35	C-29
36	CH$_2$ (dd)	127.11/5.9 and 6.52	CH$_2$	68.68/3.65	CH$_2$	H-35	C-36	C-29
37	CH$_3$ (s)	42.01/2.88	CH$_3$	-/-	-	-	-	-
38	S	-/-	-	58.47/3.16	CH$_3$	H-38	C-38	C-36
39	OH	-/*	-	45.62/2.24	CH$_3$	H-39	C-39	C-32
40	O	-	-					
41	O	-/	-					
42	CH$_3$ (s)	43.94/2.7	CH$_3$					

[a]S, singlet; d, doublet; t, triplet; dd, doublet of doublet, refer the structural formula given in **Figure 3** for numbering. *protons exchanged due to solvents.
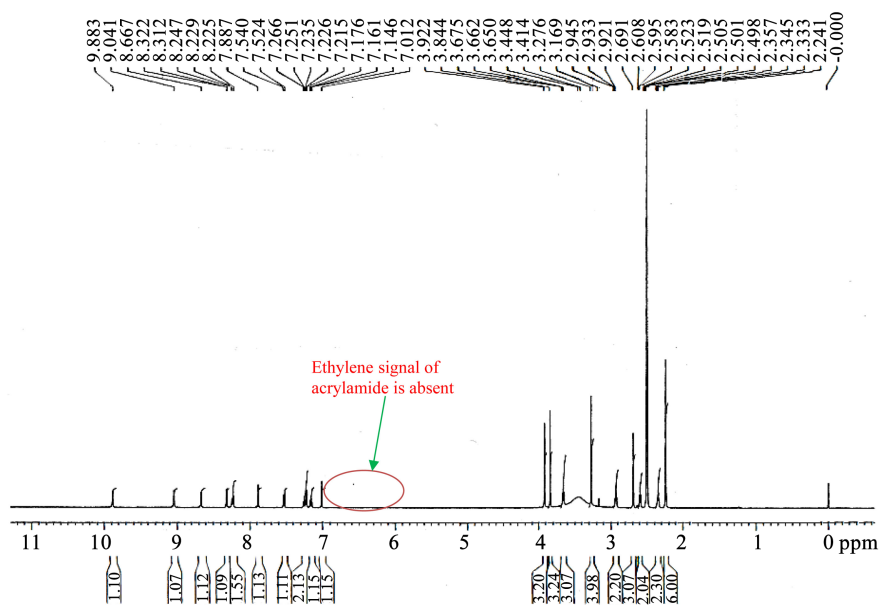

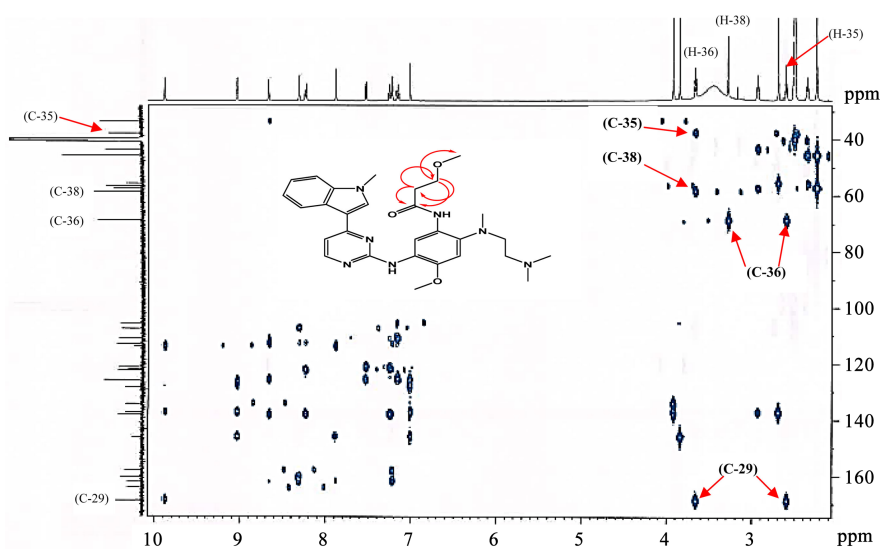**Figure 7.** 1H NMR spectra of base degradant (DP).

Figure 8. HMBC NMR spectra of base degradant.

ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)
amino)phenyl)-3-methoxypropanamide. The plausible synthesis scheme is outlined in Figure 9.

4. *In-Silico* Safety Assessment of Degradation Product

The safety assessment for impurities is crucial for pharmaceuticals, in this context the application of computational methods e.g., Quantitative Structure-Activity Relationships (QSARs), Structure-Activity Relationships (SARs), and/or expert systems for the evaluation of genotoxicity are needed, especially when very limited information on impurities is available [19]. The SAR was predicted through widely accepted and respected predication software tools e.g., DEREK, Lhasa ltd and MCASE, CASE Ultra, the endpoints of the predictions are based on molecular structure and reactivity. DEREK yields semi-quantitative assessments of a DNA reactive functional groups (e.g., Genotoxicity, or chromosome damage, Mutagenic,) of the input chemical structure. DEREK is a knowledge and rule-based semi-quantitative estimations tools, the learning set was created using both bacterial mutagenicity and all other available genotoxicity data [20].

MCASE dissociates the input molecule into multiple fragments and evaluates statistically for bacterial mutagenicity by using two models GT1_BMUT (OECD 471 Bacterial Mutagenicity) and GT_EXPERT (Expert Rules for Bacterial Mutagenicity) from the available database for mutagenicity and generates quantitative data from various reputed sources the outcomes are tabulated in Table 4.

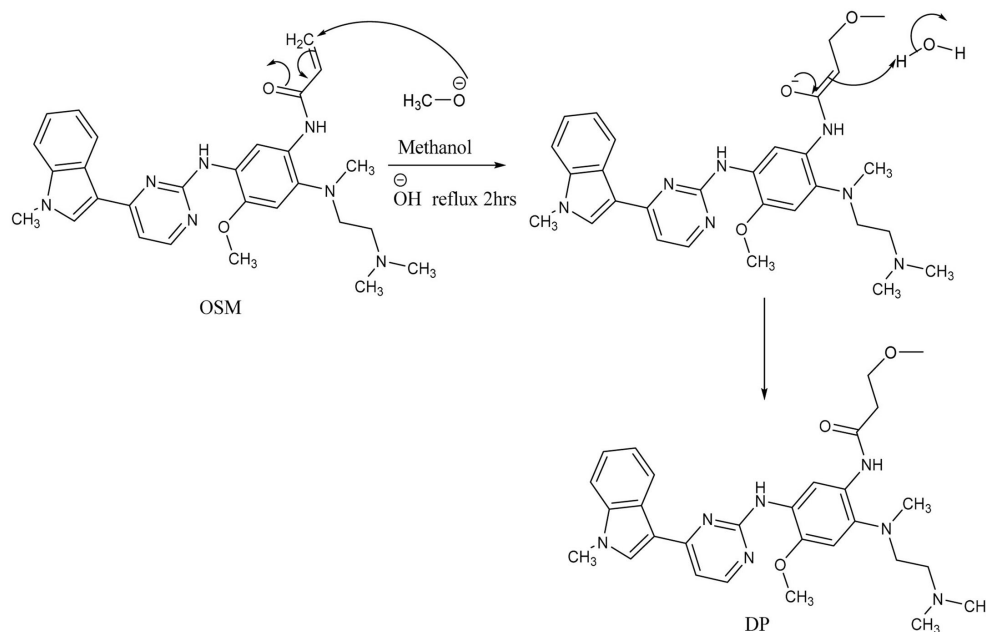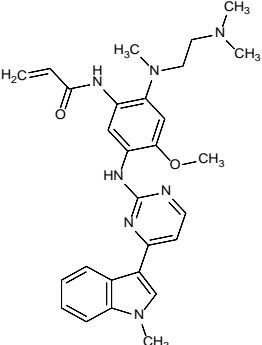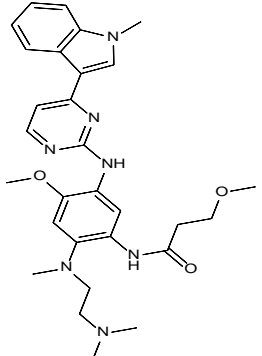Based on the safety assessment done on the above-mentioned software's, the OSM and DP are observed as Non-genotoxic.

Figure 9. Scheme for the synthesis of DP in presence of methanol and base.

Table 4. Statistical outcome for OSM and DP.

Name of the molecules	M-CASE Output	DEREK Output
Osimertinib [OSM]	Suggested Outcome: known Negative The query chemical is a known negative compound. Call Confidence: High, GT1_BMUT: Bacterial Mutagenicity by OECD 471 Test: Known Negative Calculated Probability: 77.8%, Positive Alerts: 7 Deactivating Features: 1, Fragment ID #2002: cH:[c.]1:[c.]:c:cH:n1-C_3H_3 This the deactivating/mitigating feature. Usually inhibits the effects of positive alerts. GT_EXPERT: Expert Rules for Bacterial Mutagenicity: Known Negative	Mutagenicity *in vitro* is INACTIVE (No misclassified or unclassified features) in • bacterium, • Escherichia coli, • Salmonella typhimurium.
Degradation Product[DP]	Suggested Outcome: Negative Call is based on analog analysis of alerts. All identified alerts/features were found to be irrelevant to activity. Call Confidence: Moderate, GT1_BMUT: Bacterial Mutagenicity by OECD 471 Test: Inconclusive Calculated Probability: 46.9%, Positive Alerts: 7 Deactivating features: 4 Fragment ID #623: C_3H_3-O-C_3H_2, Fragment ID # 1017: C_3H_2-C_3H_2-O-C_3H_3, Fragment ID # 385: C_3H_2-C_3H_2-C_2(=O)-N_3H-c, Fragment ID # 2002: cH:[c.]1:[c.]:c:cH:n1-C_3H_3 These are the deactivating/mitigating feature. Usually inhibits the effects of positive alerts. Fragment ID #2002: cH:[c.]1:[c.]:c:cH:n1-C_3H_3 GT_EXPERT: Expert Rules for Bacterial Mutagenicity: Negative	Mutagenicity *in vitro* is INACTIVE (No misclassified or unclassified features) in • bacterium, • Escherichia coli, • Salmonella typhimurium.

5. Conclusions

In contrast to our previously developed method for oxidative degradants, the newly developed method is considered to be more appropriate to separate all the plausible OSM degradants. The OSM was studied under base hydrolytic conditions and yielded one major degradant. Further, it is well characterized by using UPLC-Q-TOF-MS and NMR studies (1H, 13C, DEPT-135, COSY, HMBC and HSQC) and the structure is proposed as N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-3-methoxypropanamide. This impurity can be controlled in finished formulations or drug substance by adequate control of residual methanol and diluent for sample preparation.

Further, in silico safety assessment was predicted for DP with OSM using widely-respected and accepted software's *i.e.*, MCASE and DEREK Nexus. The results showed that the DP was observed as non-genotoxic.

The shreds of evidence in this study are expected to be useful for generic drug manufacturer during product development.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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