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Separation and Identification of Oxidative Degradation Products of Osimertinib Tablets by using HPLC, UPLC-QTOF-MS/MS and Evaluation of their in-silico Safety Assessment

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***Abstract*- The present study focused on identification of the Oxidative degradation products of Osimertinib (OSM) Tablets by using of high-resolution mass spectrometry. A Ultra Performance Liquid Chromatography (UPLC) coupled with high resolution time of flight (Q-TOF-MS) was used for identification of oxidative degradation products (DPs) of OSM. Three major degradation products (DPs) were formed and their separation was carried out on a non-polar stationary phase by high-performance liquid chromatography system (HPLC). Three oxidative degradants were identified with masses, DP-1 (532.2655), DP-2 (m/z 516.2802) and DP-3 (m/z 516.2657) in this study. Further, interpretation of high resolution spectral data of degradants was carried out and proposed the elemental composition, RDB (ring double bond), mass error in ppm and molecular structure. Additionally, the in-silico safety assessed through widely accepted and respected structure activity relation (QSAR) and statistical based software's DEREK nexus™, and MultiCASE , Case Ultra™ for all DPs and OSM**

***Index Terms*- Osimertinib Mesylate (OSM), Oxidative Degradation Products UPLC-QTOF-MS/MS, In-Silico Safety assessment.**

I. Introduction

Osimertinib (OSM) (Fig. 1; IUPAC name: "N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy -5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phen yl)acrylamide" molecular formula: $C_{28}H_{33}N_7O_2$; molar mass: 499.61 g mol−1 and CAS registry number: (1421373-65-0) occurs as a non-hygroscopic white to brown colored crystalline powder [1],[2] It is highly selective an oral, novel mono-anilino-pyrimidine third-generation EGFR TKI, has been developed to selectively target the T790M mutation EGFR in a covalent irreversible manner. The OSM therapy has been used to altered tumor growth [3], [4],[5],[6]. During the Therapy of any drug product the efficacy is not only depending on the toxicological properties of the active drug substance, but also on the impurities present in it. The Impurity profiling is an critical to ensure the safety of pharmaceutical products. [7]

To the best of our knowledge, there are very limited studies are available in the literature on the degradation products of OSM drug product and substance. Therefore, the goal of present study was identification of the oxidative degradation products of Osimertinib using UPLC-TOF-MS and MS/MS analysis. During this study three novel oxidative degradants (N-Oxides) with m/z 532.2655, m/z 516.2802, and m/z 516.2657 were identified Fig. 3, Based on high resolution mass spectra data elemental composition, molecular structures, RDB value, and mass error calculated.

A simple mass compatible high performance liquid chromatography (HPLC) method has been developed to monitor the Oxidative degradation profile. In addition, the HPLC method was transferred to UPLC for UPLC-Q-TOF-MS analysis to identify the degradants with accurate mass measurements.

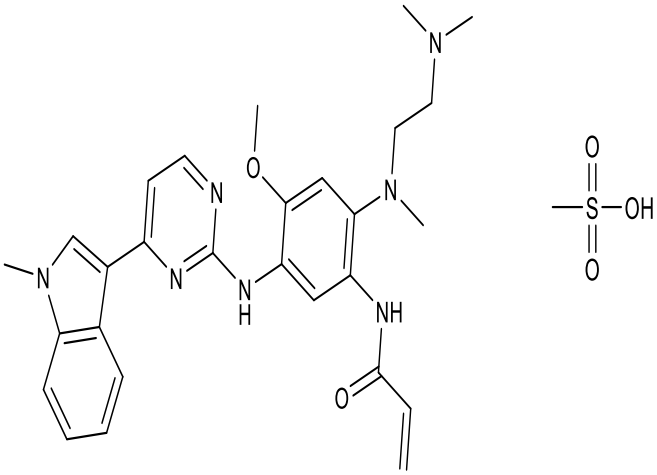

Fig. 1. Chemical structure of Osimertinib Mesylate (OSM)

II. Experimental and Instrumentation

A. Chemicals and reagents

Osimertinib Mesylate (OSM) Tablets and API were received as a gift sample from Cipla Laboratories Ltd. (Mumbai,

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India). HPLC grade methanol (MeOH) were obtained from Thermo fisher Scientific India, (Mumbai, India) and HPLC and LC-MS grade Acetonitrile (ACN) was obtained from Honeywell International India (New Delhi, India). Analytical reagent (AR) grade lab reagents such as formic acid were procured from S.D. Fine, ammonia solution (NH_4OH) and Hydrogen peroxide (H_2O_2, 30%, AR grade) 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).

B. High-performance liquid chromatography (HPLC)

The method development for degradation products done on Agilent 1220 HPLC system equipped with a quaternary pump, photodiode array detector, automatic liquid sampler injector, and degasser module. 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 C-18 column (25 cm x 4.6 mm internal diameter, 5 m 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 programme was set for separation of components as follows: time/solvent-B (%): 0.0/25, 5.0/25, 15/40, 30.0/80, 60.0/80, 70.0/25, 80.0/25 with flow rate 1.0 ml/min. The analytes were monitored at a wavelength of 268 nm. the column temperature was maintained at 40°C. 10 µL injection volume was used for sample analysis

C. Electrospray ionization mass spectrometry (ESI-Q-TOF-MS)

The HPLC separation method was transferred UPLC-Q-TOF-MS for identification of 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 +2.5 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 50–1000 Da.

D. Procedure for degradation studies

The oxidative stress conditions were performed as per ICH guidelines. Initially the sample was weighed equivalent to 5mg/ml of OSM and treated with 10% H_2O_2 at 80°C for 2hrs, However the OSM degrades completely thus the concentration of H_2O_2 was optimized to 10ml 3% H_2O_2 and heated the solution for 2.0 hrs at 80°C temperature and make up the volume with diluent and injected on HPLC-PDA and analyzed using analytical method illustrated in Section B. the degradation products and its amount found after oxidation is outlined in Table 1

The study insights the chemical behavior of the active which helps to develop the formulation and packaging material.

III. RESULTS AND DISCUSSION

A. Analytical Method development for DPs

The OSM Mesylate drug substance and drug products are a non-compendial product and no literatures were found on the public domain for Degradation products. Thus initially, samples were analyzed by available literature for assay [8] but the DPs were not separated out. Thus, new HPLC method developed as outlined in section II B, the isolation for all DPs was also done by transferring the same method on semipreparative HPLC However after lyophilization the isolated impurities get degraded. The HPLC method was transferred to UPLC-Q-TOF-MS for further identification.

The typical chromatogram of oxidative degradation conditions is shown in Fig. 2. Under oxidation by using 3% H_2O_2 up to 10ml yielded three major degradants (DP-1, DP-2, DP-3) with additional multiple small degradants,

The typical chromatogram of oxidative degradation conditions is shown in Fig. 2

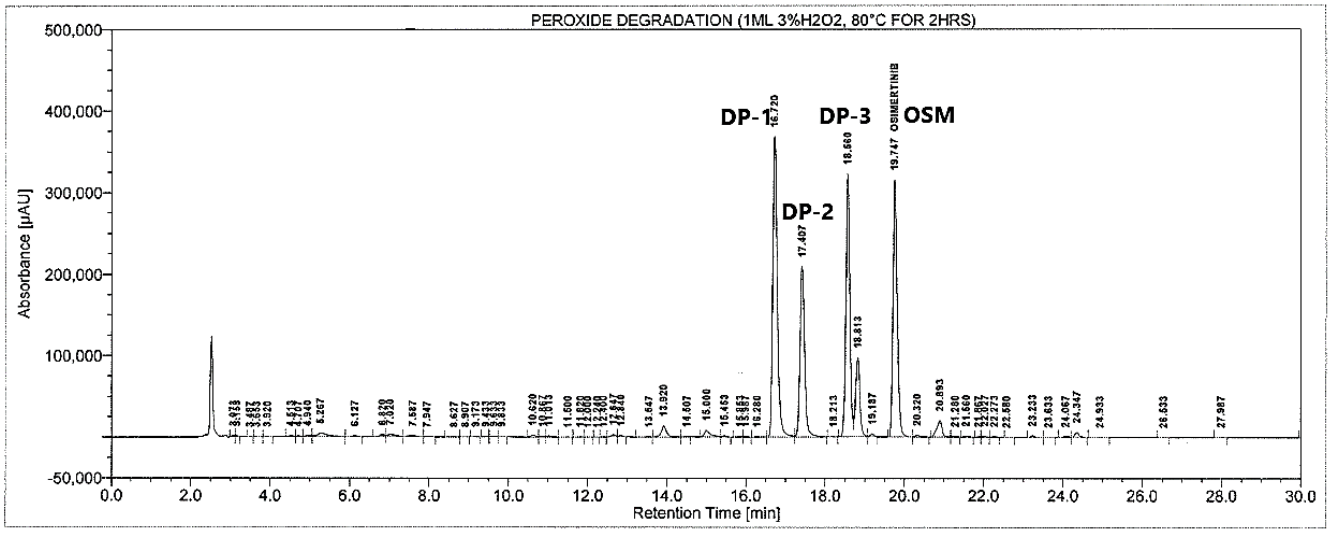


Fig. 2. 10ml 3% H_2O_2 degradation at 80°C for 2 Hrs,

B. Mass spectrometric characterization of novel degradation products

Waters Xevo Q-tof Mass spectrometric (MS) technique with mass lynx software (ver. No. 4.1) used for the identification of active drug and its novel degradation products using protonated molecule and fragmentation pathways. The obtained MS data were explored to elucidate the fragmentation pathways and structural arrangements of OSM
and its degradation products. The observed fragmentation pattern was then correlated with experimental fragmentation data. The ESI-Q-TOF-MS condition with ion spray voltage is optimized to +3.0 kV condition for better protonated and fragments ions. The three DPs were identified along with the target OSM drug outlined in Fig.3 and its RRTs were found at 0.84 (DP-1, m/z 532.2655), 0.88 (DP-2, m/z 516.2802), 0.95 (DP-34, m/z 5161.2657) which are showed in Fig.4-6

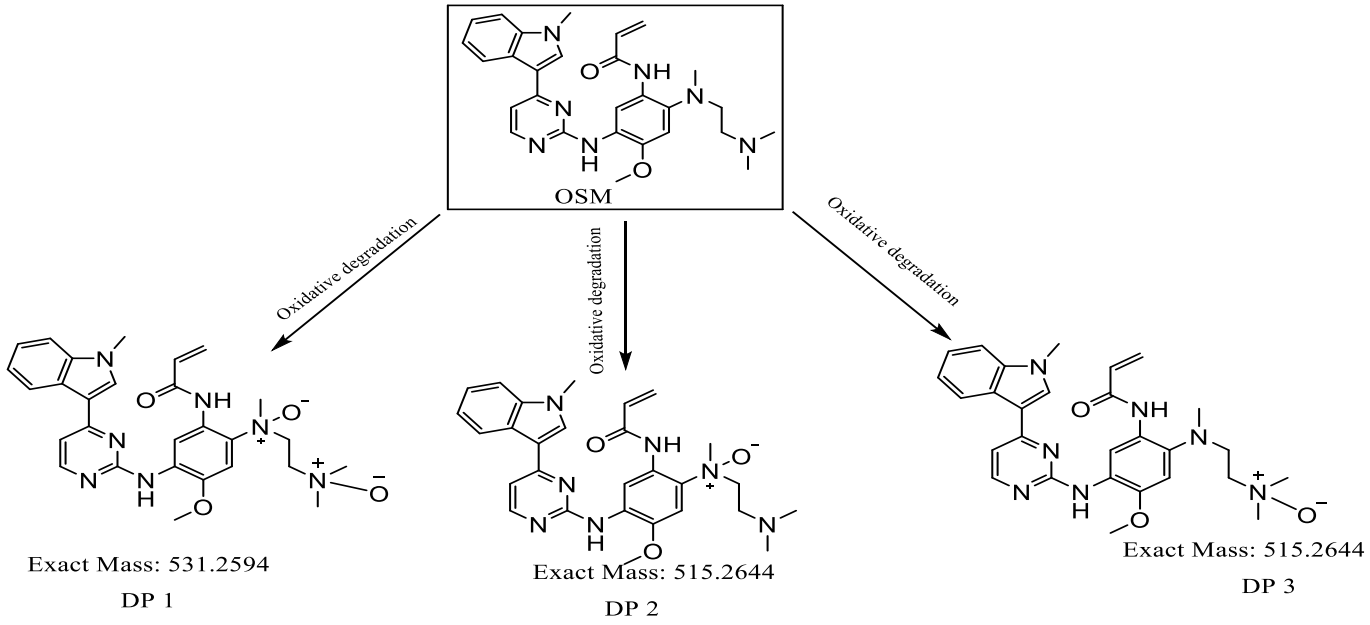


Fig 3: Chemical structures of Osimertinib (OSM) and its Oxidative Degradation Products (DPs)

C. DP-1 [(m/z 532.2655), M+H]

This degradation product was observed in the oxidation and eluted at the RRT about 0.84 (in HPLC) shows the mass m/z 532.2655Da (Fig. 4) which is 32 Da higher mass unit than OSM. Based on the RDB, nitrogen rule, the elemental composition $C_{28}H_{34}N_7O_4^+$ was proposed as the best probable molecular formula [table 2]. The RDB value for

DP-1 is observed as 16 which is same as the 16 RDB value of OSM even though increase in molecular weight. It reveals that double bond and ring are same as OSM in DP-1. The fragmentation pattern confirms the structure with addition of

two oxygen atom. One Oxygen is inserted at N-Methylaniline position and second oxygen inserted at tertiary nitrogen at terminal forms di-N-oxide compound. The PDA spectra for DP-2 also changed due oxygen atom loan pair involved in resonance with phenyl ring (due to Nitro group) the third maxima of OSM 320nm [is shifted to red shift at 343nm [Fig. 10 a].

the characteristic fragment m/z 471.23 consist of oxygen atom on N-phenylaniline ring which formed due to loss trimethyl amine oxide in fragmentation study and the second fragment m/z 497.25 is due to loss of two water molecule and forms di-imine additionally, one more characteristic fragment m/z 119.08 corresponds to N,N-dioxide amine outlined in fig 7. Thus based on mass fragmentation pattern the probable structure of DP-1 hence could be postulated as N1-(2-acrylamido-5-methoxy-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-N1,N2,N2-trimethylethane-1,2-diamine dioxide

D. DP-2 [(m/z 516.2513), M+H]

This degradation product was observed in the oxidation and eluted at the RRT about 0.92 (in HPLC) shows the mass m/z 516.2513 Da (Fig. 5) which is 16 Da higher mass unit than OSM. Based on the RDB, nitrogen rule, the elemental composition $C_{28}H_{34}N_7O_3^+$ was proposed as the best probable molecular formula [table 2]. The RDB value for DP-2 is observed as 16 which is same as the 16 RDB value of OSM even though increase in molecular weight. It reveals that double bond and ring are same as OSM in DP 2. The fragmentation pattern confirms the structure with addition of 1 oxygen atom at N-Methylaniline position and forms N-oxide compound (N-methylaniline oxide) the PDA spectra for DP-2 also changed may due oxygen atom loan pair involved in resonance with phenyl ring (due to Nitro group) the third maxima of OSM 320nm [Fig. 10d] is shifted to red shift at 343nm [Fig. 10b]. the characteristic fragment m/z 473.23 consists of oxygen atom on N-phenylaniline ring which formed due to loss trimethyl amine in fragmentation study and the second fragment m/z 498.26 is due to loss of water molecule and forms imine [fig 8]. Thus based on mass fragmentation pattern the probable structure of DP-2 hence could be postulated as 2-acrylamido-N-(2-(dimethylamino)ethyl)-5-methoxy-N-methyl-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)aniline oxide.

E. DP-3 [(m/z 516.2513), M+H]

This degradation product was observed in the oxidation and eluted at the relative retention time (RRT) about 0.97 (in HPLC) which shows the mass m/z 516.2513 Da (Fig. 6) which is 16 Da higher mass unit than OSM. Based on the RDB, nitrogen rule, the elemental composition $C_{28}H_{34}N_7O_3^+$ was proposed as the best probable molecular formula [table 2]. The RDB value for DP-4 is observed as 16 which is same as the 16 RDB value of OSM API even though increase in molecular weight. It reveals that double bond and ring are same as OSM in DP 4. The fragmentation pattern confirms the structure with addition of 1 oxygen atom at tertiary nitrogen. the characteristics fragment m/z 455,25 is corresponding due to the loss of dimethyl amine ($(CH_3)_2$-NO), and fragments m/z 88 da is due to the loss of N,N-dimethylethanamine oxide C4H10NO which confirms the addition at terminal end. The Fragment m/z 499.27 corresponds to loss of water molecule and formed Imine at terminal end outlined in [Fig.9]. remaining some of the fragments are comparable with OSM . Additionally, the PDA spectral scan reveals no change in aromaticity of OSM [fig 10 c]. Hence The structure of DP 4 hence could be postulated as 2-((2-acrylamido-5-methoxy-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)(methyl)amino)-N,N-dimethylethan-1-amine oxide.

IV. In-silico safety assessment of degradation products

The safety assessment for Degradation products/process impurities is an paramount important for the pharmaceuticals, In this context, the application of promising computational methods (e.g. Quantitative Structure–Activity Relationships (QSARs), Structure–Activity Relationships (SARs) and/or expert systems) for the evaluation of genotoxicity is needed, especially when very limited information on impurities is available [9], Based on Derek Nexus: 6.1.0, Nexus: 2.3.0, and CASE Ultra v1.8.0.2 expert knowledge based SAR program which contains expert rules (derived from public and proprietary data) in toxicology and applies the rules to make predictions about the toxicity of chemicals, which is widely-respected and accepted for the assessment of mutagenicity has yield following outputs, Based on the safety assessment done on the above mentioned software's OSM is known Genotoxic negative, whereas DP-1, DP-2 and DP-3 observed positive for Genotoxicity by using Bacterial Mutagenicity by OECD 471 (GTI_BMUT) model, the positive alerts observed for 6 functional groups in DP-1 and DP-2 whereas for DP-3 is 7. The calculated probability of genotoxicity for DP-1 and DP-2 is 66.5% whereas for DP-3 is 77.8% in M-CASE, additionally by applying other model i.e GT_EXPERT, it shows Negative alert for Genotoxicity in the proposed structure. However, for further confirmation of above said alert, it is validated by in DEREK Nexus software and observed as negative for genotoxicity. Thus overall output for identified degradant's DP-1, DP-2 and DP-3 is Non-Genotoxic compounds.

V. Conclusions

The OSM was studied under various stress conditions as per ICH Guidelines by developing fast HPLC-PDA method. under oxidative degradation condition three DPs (DP-1, DP-2 and DP-3) were formed and it is well characterized by using UPLC-Q-TOF-MS studies. The probable structures of

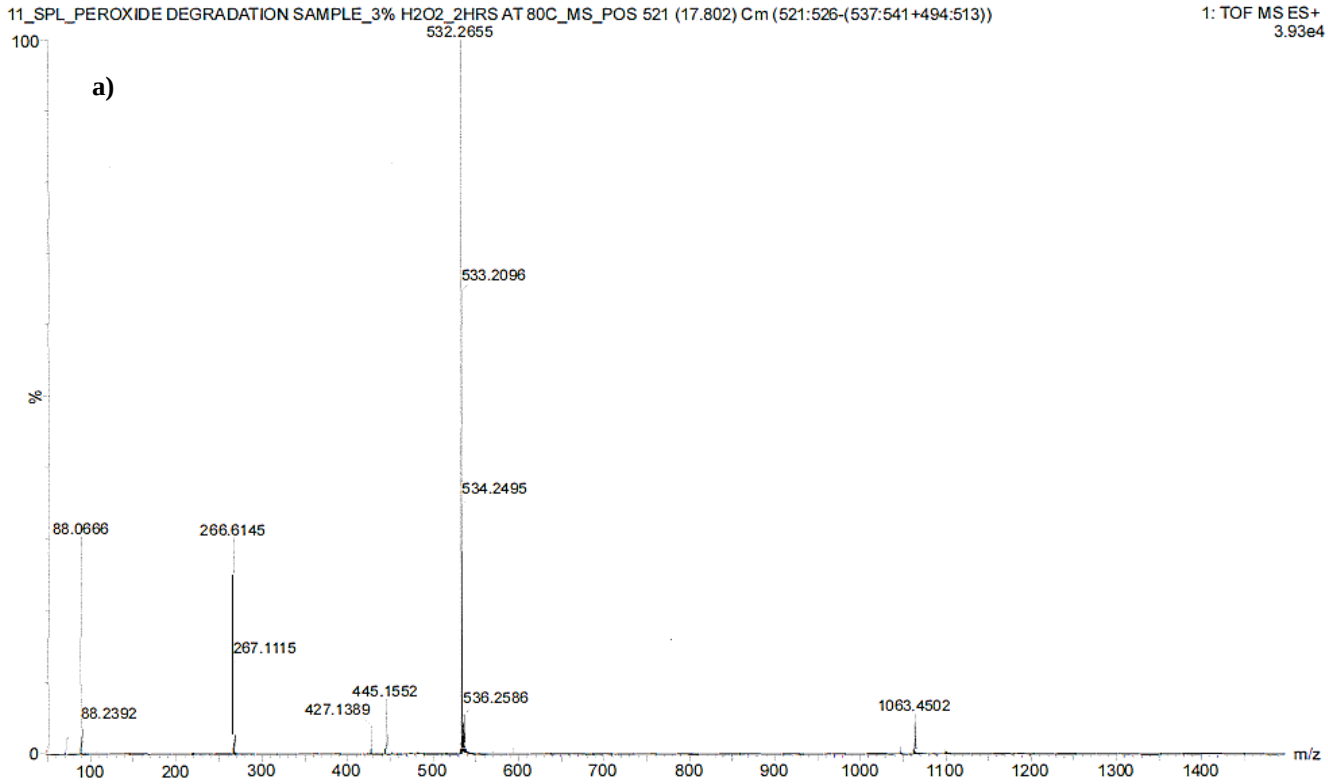
DP-1 is identified as N1-(2-acrylamido-5-methoxy-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-N1,N2,N2-trimethylethane-1,2-diamine dioxide, DP-2 is identified as 2-acrylamido-N-(2-(dimethylamino)ethyl)-5-methoxy-N-methyl-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)aniline oxide and
DP-3 as 2-((2-acrylamido-5-methoxy-4- ((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)(methyl)amino)-N,N-dimethylethan-1-amine oxide. Further, in silico safety assessment were predicted for DP-1 and DP-2 with OSM using widely accepted software's i.e., MCASE and DEREK Nexus. The results showed negative alerts for DP-1, DP-2 and DP-3 for genotoxicity.
The information provided in this study expected to be very useful for generic drug manufacturer during product development and monitoring of product quality of this important anti-Cancer agent

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-1	16.72	0.84	27.02	999.0
DP-2	17.40	0.88	15.9	999.15
DP-3	18.66	0.95	20.6	994.8
Drug (OSM)	19.74	1.00	21.6	951

Table 2. UPLC-Q-TOF-MS data of degradation products (DPs 1-3) along with their molecular formulae and major fragments in ESI positive mode

Identity	Accurate mass	Molecular formula	Exact mass	Error (ppm)	RDB	Nitrogen rule	Accurate masses of fragment Ions
$[DP\text{-}1+H]^+$	532.2655	$C_{28}H_{33}N_7O_4^+$	532.2666	2.06	16	odd	497.2300 453.1754, 441.2287, 427.1389, 399.1704, 384.1547, 356.1223, 343.0974 119.1072, 88.0726
$[DP\text{-}2+H]^+$	516.2802	$C_{28}H_{34}N_7O_3^+$	516.2717	16.4	16	odd	455.2536, 441.2287, 425.1982, 400.2297, 385.1930, 371.1889, 353.178
$[DP\text{-}3+H]^+$	516.2657	$C_{28}H_{34}N_7O_3^+$	516.2717	11.6	16	odd	499.3426 455.2566, 441.2700, 427.2536, 400.2545, 385.2415, 369.2353, 353.2112 343.1799 231.1457 88.0908 72.0947
OSM	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



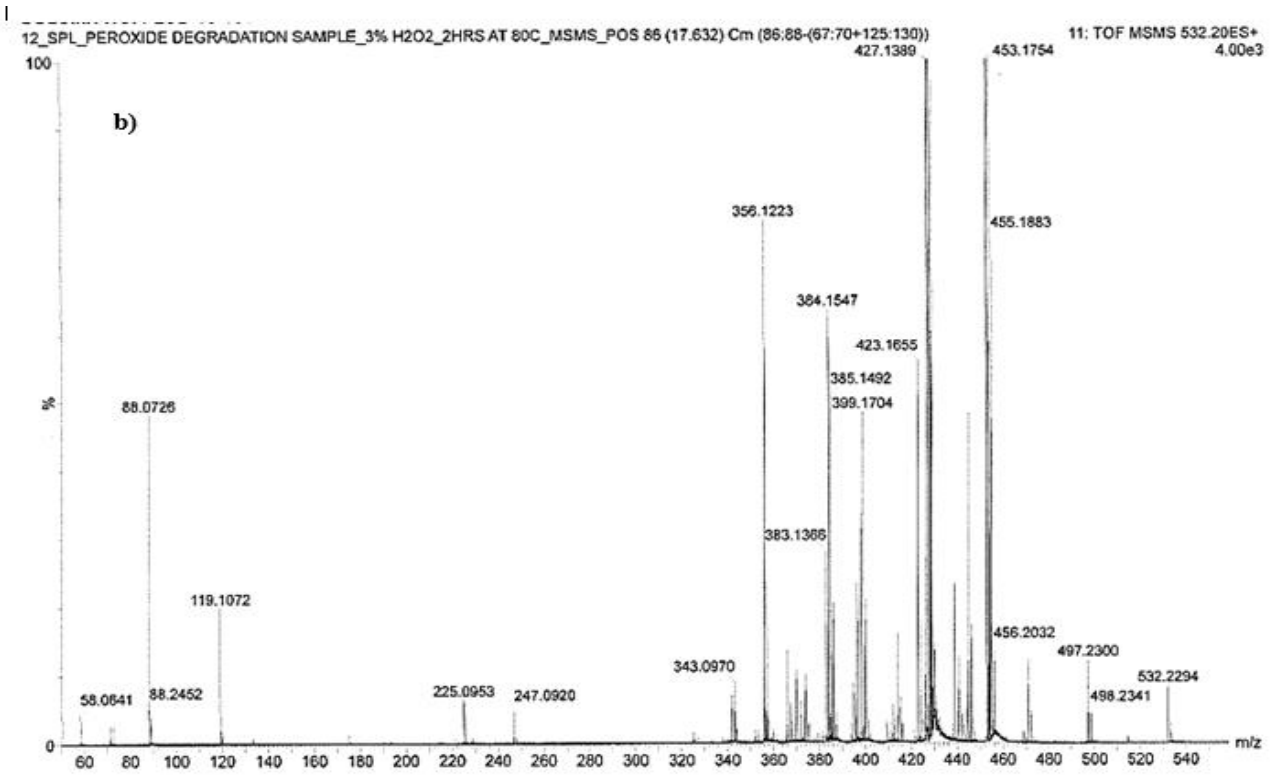
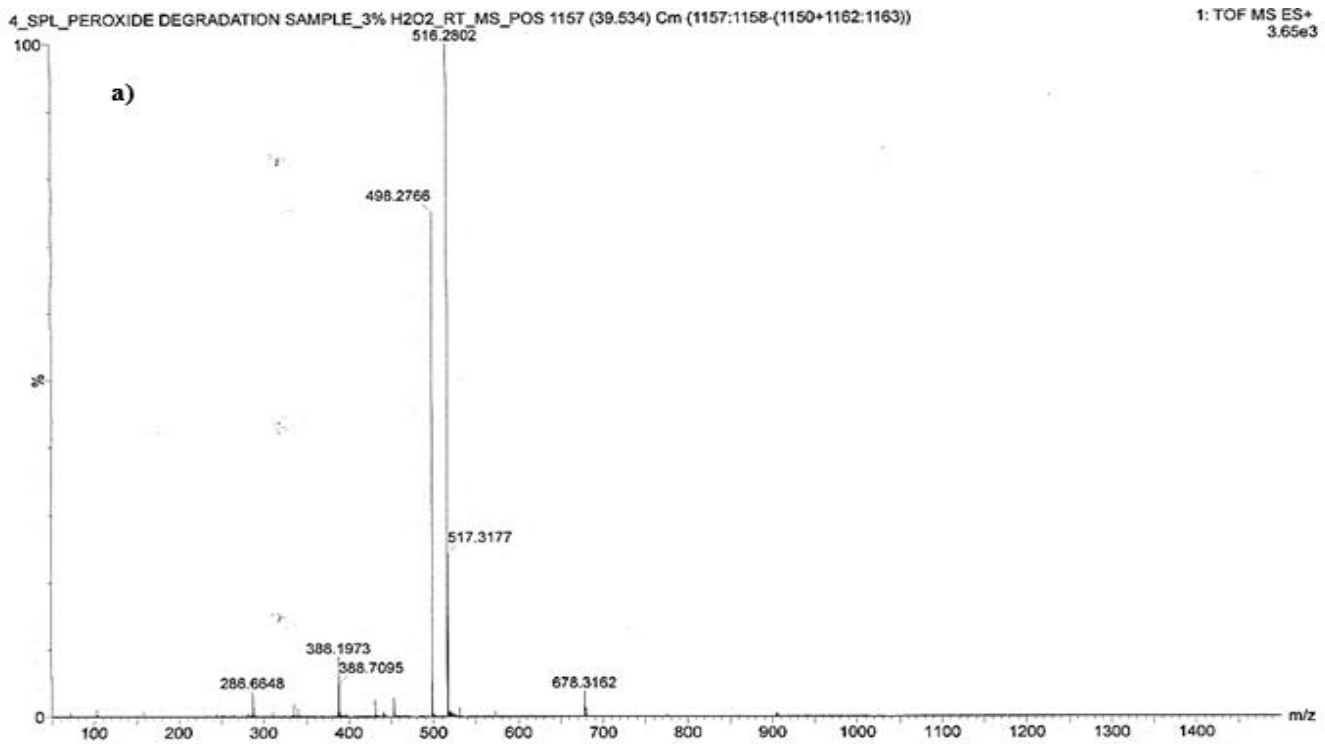


Fig.4 a) Q-TOF-MS spectrum of DP-1 under ESI positive conditions showing its molecular ion *m/z* 532.2655) and b) Several fragments along with their accurate masses



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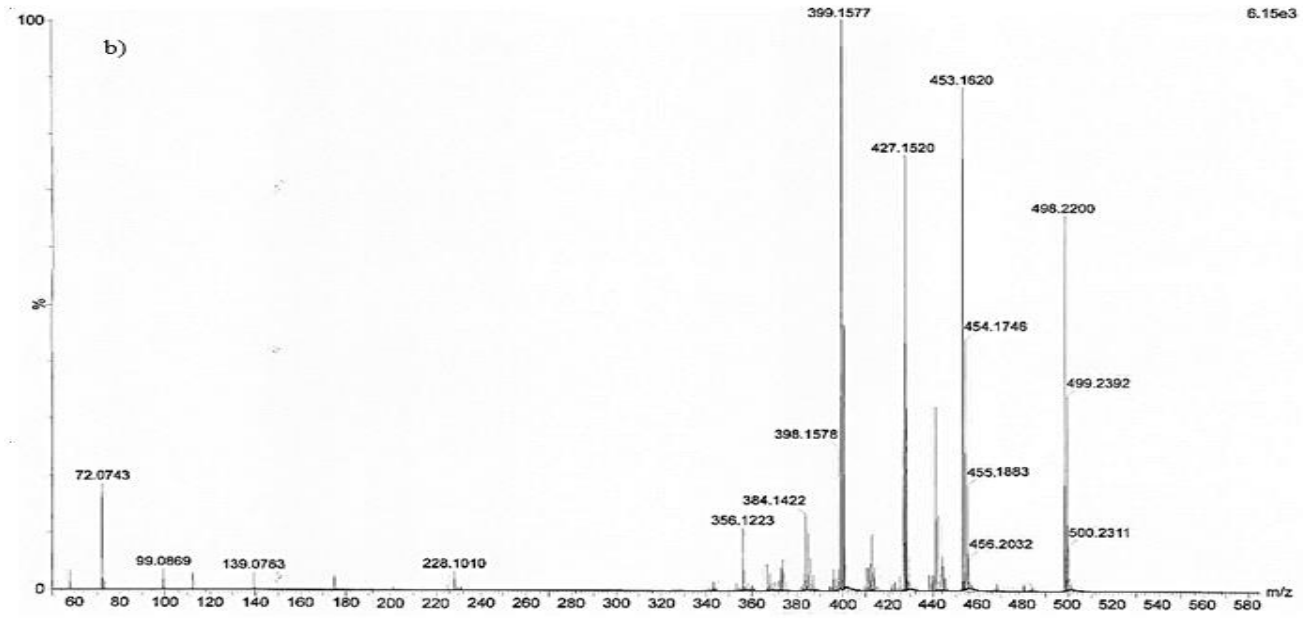
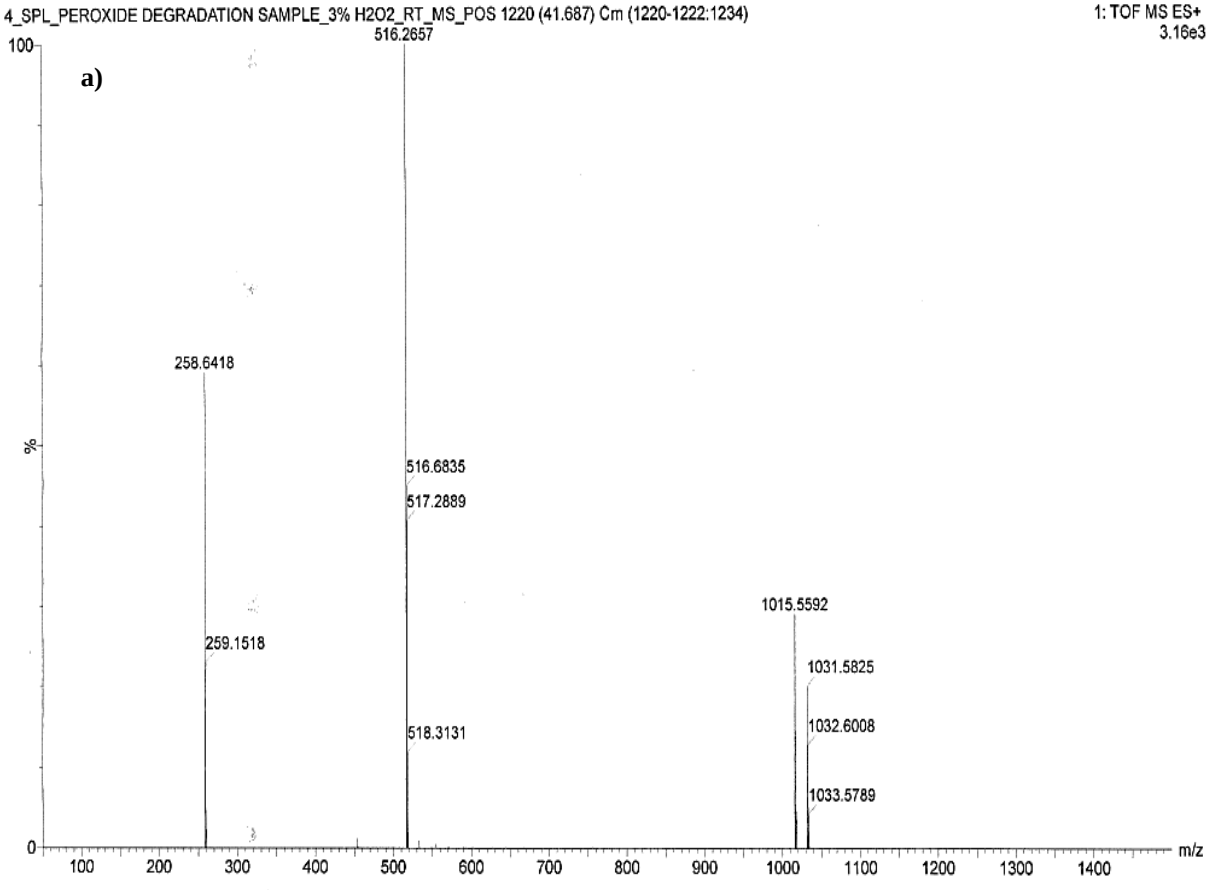


Fig. 5 a) Q-TOF-MS spectrum of DP-2 under ESI positive conditions showing its molecular ion *m/z* 516.2802) and b) Several fragments along with their accurate masses



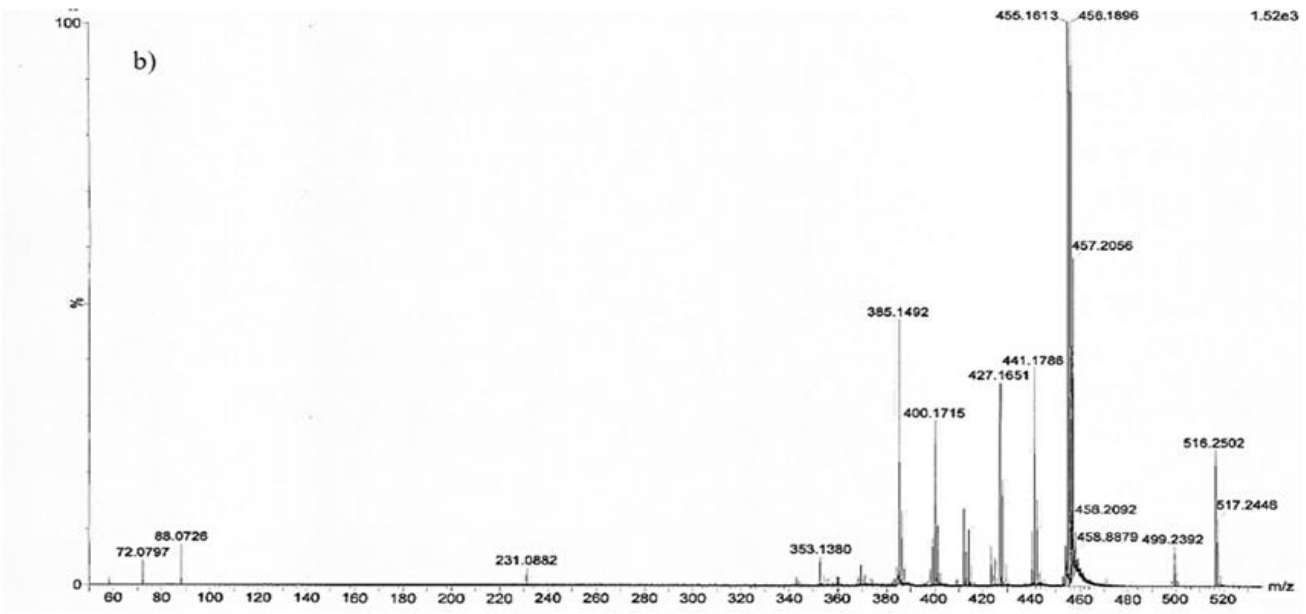


Fig. 6 a) Q-TOF-MS spectrum of DP-3 under ESI positive conditions showing its molecular ion (*m/z* 516.2657) and b) Several fragments along with their accurate masses,

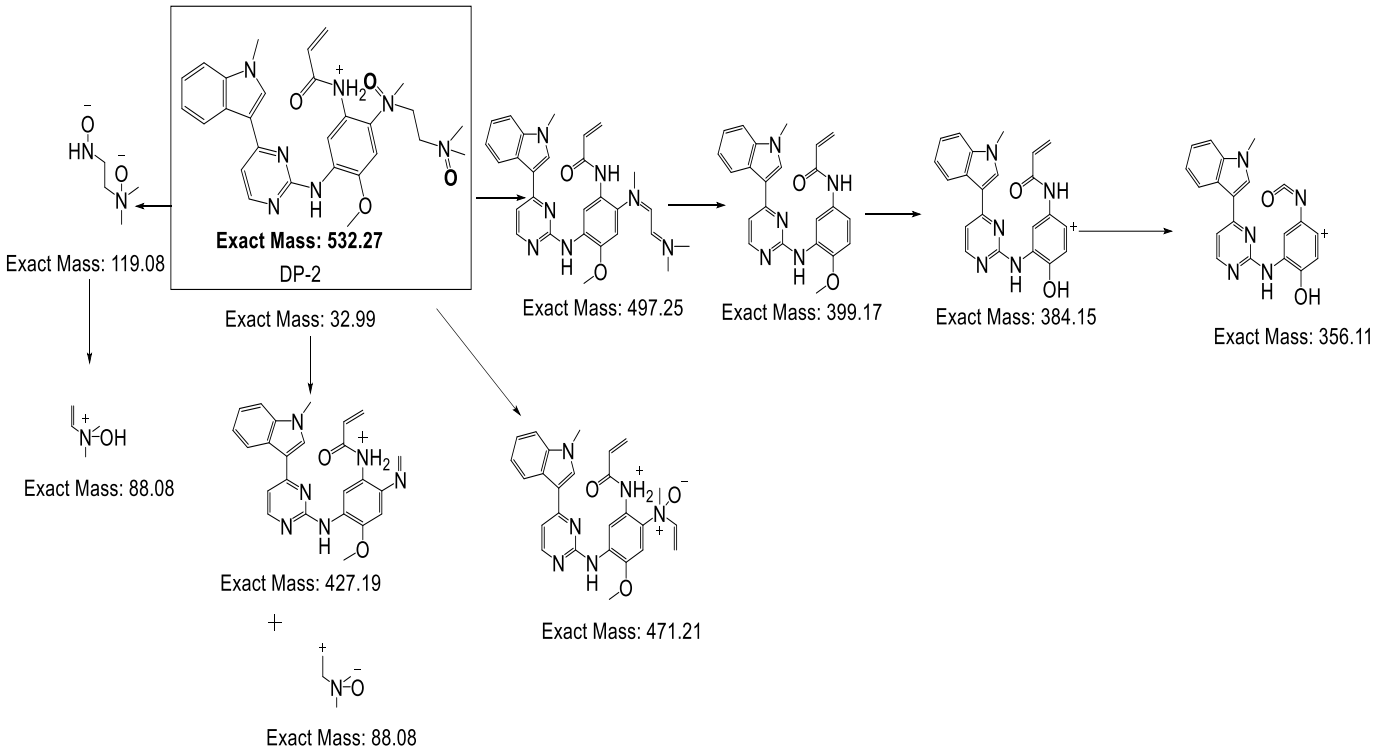


Fig.7 Plausible Fragmentation pathways of DP-1 in ESI positive mode with molecular weight m/z 516.2368. refer fig.4 for mass and fragments

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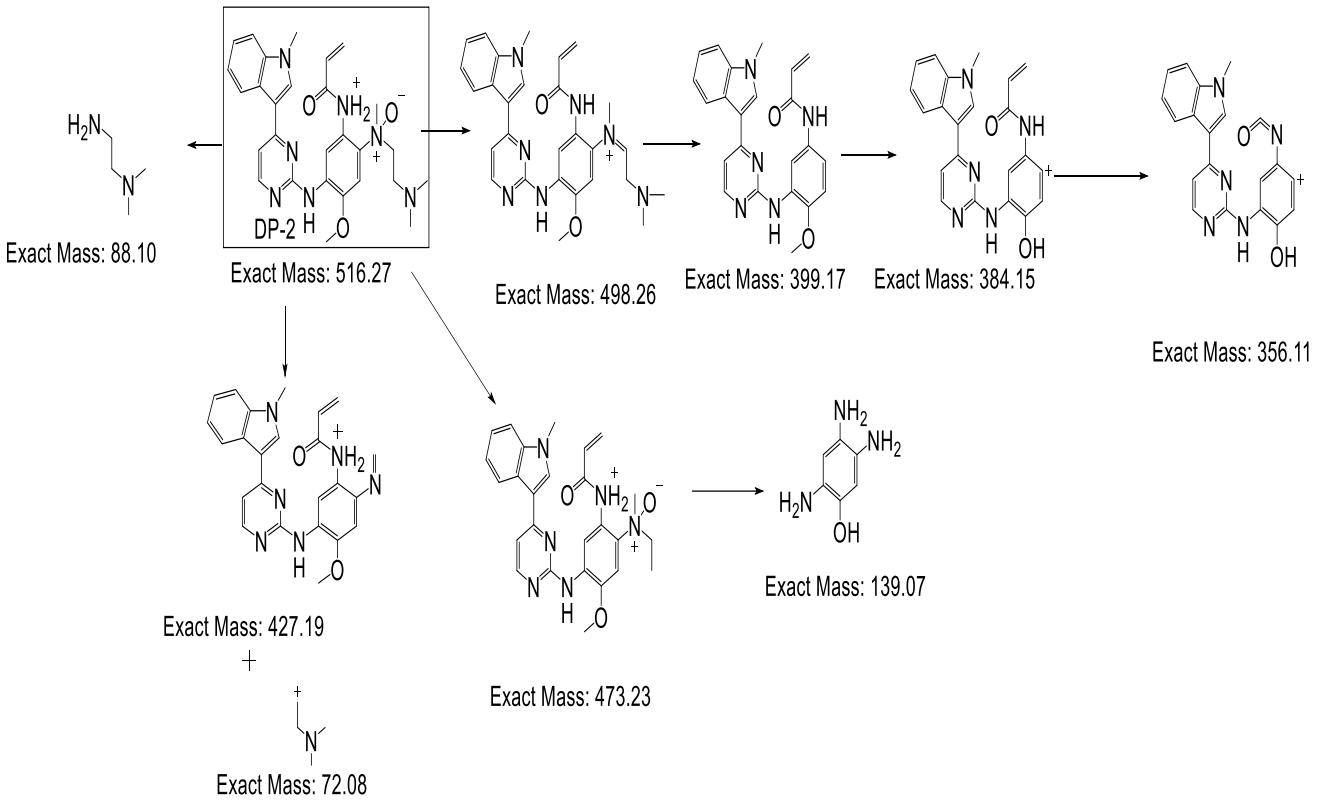


Fig. 8 Plausible Fragmentation pathways of DP-2 in ESI positive mode with molecular weight m/z 516.2368 refer fig. 5 for mass and fragments

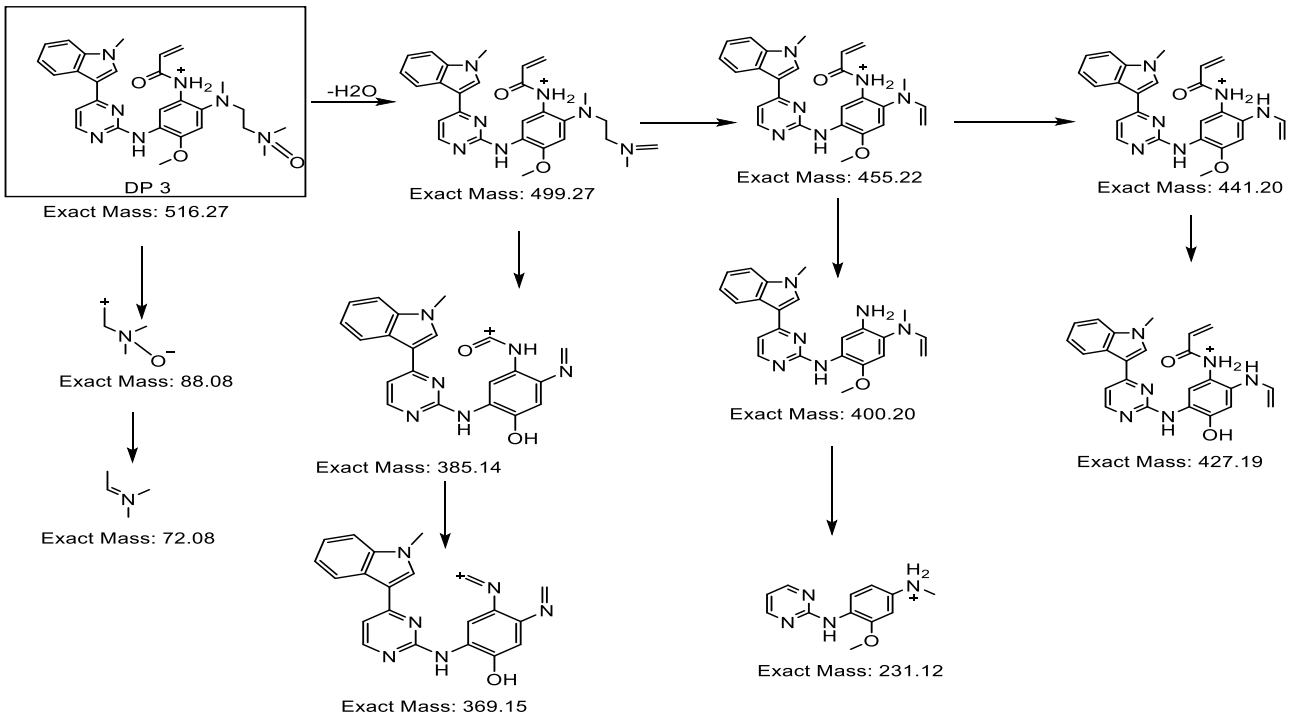
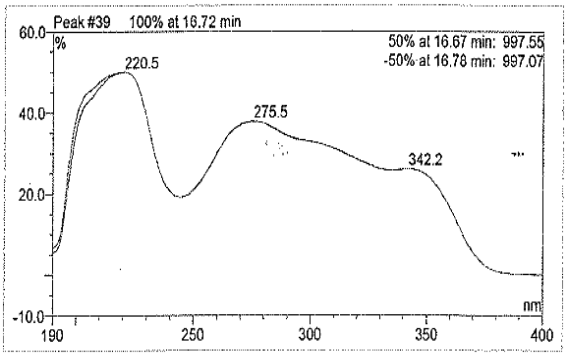
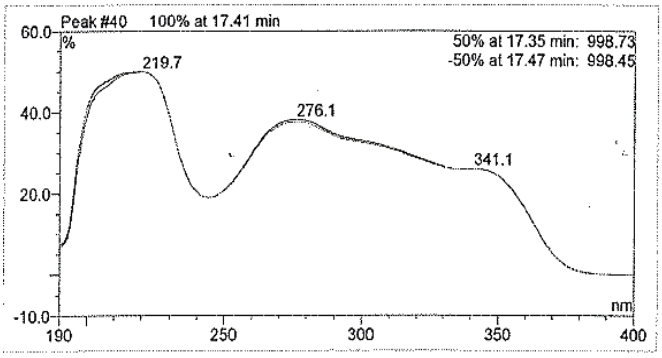


Fig. 9 Plausible Fragmentation pathways of DP-3 in ESI positive mode. Refer fig 6. for mass and fragmentation. The accurate mass data and its fragments are reported in Table No.3 as below

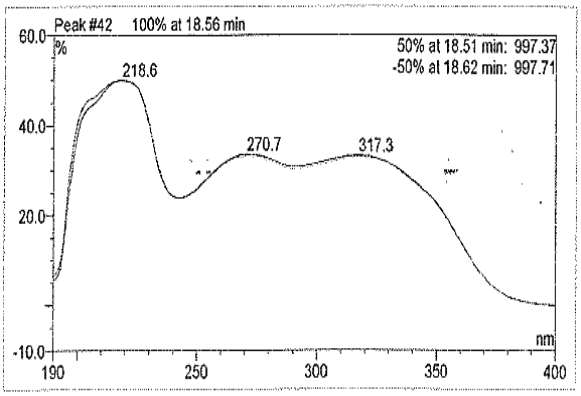
a) DP-1



b) DP-2



c) DP 3



d) OSM

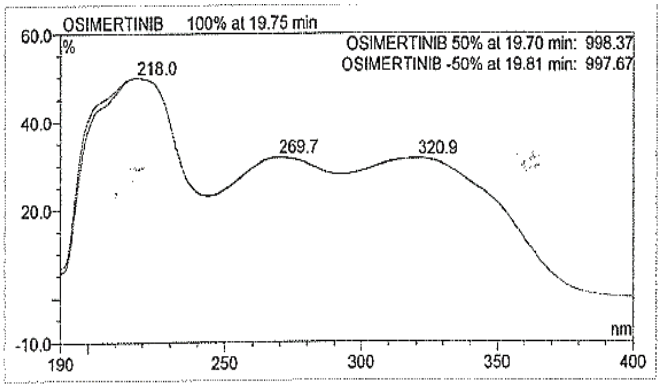


Fig. 10 PDA spectral scans for a) DP-1, b) DP-2, c) DP-3 d) OSM

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