VALIDATED STABILITY- INDICATING HPTLC METHOD FOR *NINTEDANIB* & CHARACTERIZATION OF DEGRADANTS BY LC-MS^n

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ABSTRACT

A simple and rapid stability-indicating method for determination of nintedanib (NTB) in bulk drug using HPTLC and LC-MS^n was developed and validated. Stress degradation studies were carried out by hydrolysis, oxidation, thermal and photolytic. Drug was found to be stable in thermal whereas one degradant was found in acid hydrolysis, three in basic hydrolysis, five in oxidative and two in photolytic stress. The probable structures of the degradation products were predicted & the degradation pathway was also established. Chromatography was carried out using silica gel 60 F_{254} TLC plate and mobile phase of Chloroform : Methanol in the ratio 7:3 v/v. The densitometric determination was done at 386 nm. The degradants were not detectable when stressed as per ICH recommended conditions but on increasing the strength of acid, base and peroxide, the degradants were very much prominent and were easily detectable in HPTLC. The LC system consisted of a Zorbax Bonus C_{18} (150 mm×4.6 mm, 3.5 µ). A gradient mobile phase consisting of mobile phase A: 10mM Ammonium formate (0.05% formic acid): ACN (pH 3.9) (90:10) and mobile phase B: 10mM Ammonium formate (0.05% formic acid): ACN (pH 3.9) (10:90) with a flow rate of 0.7mL/min was used to separate the degradants up to a total retention time of 15 min. Mass spectrometric detection was performed using Thermo Scientific LCQ fleet Ion Trap LC/MS^n.

Keywords: Nintedanib, HPTLC, LC/MS^n, stress degradation, idiopathic pulmonary fibrosis

1. Introduction

Idiopathic Pulmonary fibrosis is a rare chronic lung disease identified by a progressive and irreversible loss of lung function, dyspnea, and cough. In this disease, lung tissues present deep inside becomes thick and stiff or scarred with time. With the thickening of lung tissues, exchange of oxygen in blood decreases and as a result brain and other organs start to fail due to the scarcity of oxygen. Symptoms include gradual onset of shortness of breath and a dry cough, repeated bouts of coughing that can't be controlled, tiredness and nail clubbing. Other symptoms include, gradual unintended weight loss, fatigue or malaise, aching muscles and joints. Many people live only about 3 to 5 years after diagnosis. The most common cause of death related to IPF is respiratory failure, others include pulmonary hypertension, heart failure, pulmonary embolism, pneumonia, and lung cancer. Treatments include oxygen therapy, pulmonary rehabilitation, lung transplant and some medicines just to stop the progression of disease.

NTB, chemically known as methyl (3z)-3-[[4-[methyl-[2-(4-methylpiperazine-1yl) acetyl] amino] anilino] phenyl methylidene]-2-oxo-1H-indole-6-carboxylate is a kinase inhibitor. It acts by selectively binding to the intracellular ATP binding pocket of fibroblast growth factor receptor (FGFRs), vascular endothelial growth factor receptor (VEGFRs) & platelet-derived growth factor receptor (PDGFRs) and thereby inhibiting them.

Literature review suggest that various methods involving UPLC, LC-MS, UV are already reported for the estimation of NTB in bulk drug, formulation, rat plasma & human plasma. The reported methods were limited to the estimation of NTB in formulation or in plasma, but none reported about the stability profile of the drug and there by establishment of the probable degradation pathway. But, till date a validated stability-indicating HPTLC method for the estimation of NTB in bulk drug and characterization of the degradants is not reported. The current manuscript is an attempt to report a validated HPTLC method as per ICH Q2(R1) guidelines for estimation of NTB. This study was designed to develop a simple, rapid, precise & accurate HPTLC method for determination of NTB in bulk drug and to validate such as per ICH guidelines.

2. Result and Discussion

2.1. Degradation Studies

Degradation studies were performed as per ICH suggested stress conditions and at a drug concentration of 1600ng/band. Different stress conditions like acid & base hydrolysis, oxidative degradation, photolytic and thermal degradation. Finally, the observed results were analyzed.

Acid hydrolysis

NTB was stressed with 0.1M HCl with reflux for 8 hrs. Simultaneously control was also performed. Readings were taken at an interval of 4 hrs. After 8 hrs. also the drug was found to be stable in 0.1M HCl. Then it was again performed with 1M HCl and enough degradation (>10%) was observed within 0 hrs., hence the study was stopped. (Table 1)

Base hydrolysis

NTB was stressed with 0.1M NaOH with reflux for 8 hrs. Simultaneously control was also performed. Readings were taken at an interval of 4 hrs. The drug was found to be stable till 4 hours and enough degradation (>10%) was observed at the 8^{th} hour. (Table 2)

Oxidative degradation

NTB was stressed with 3% H_2O_2 for 6hrs at room temperature. Simultaneously control was also performed. Readings were obtained at an interval of 3hrs. (Table 3)

Photolytic degradation

NTB was exposed to an illumination of 1.2×10^6 lux hours as per the ICH-recommended exposure limit and the reaction was monitored periodically. (Table 4)

Thermal Degradation

Negligible degradation was observed after subjecting the drug solution to temperatures 40ºC, 60ºC & 80ºC respectively. (Table 5)

Table 1. Acid hydrolysis of Nintedanib

0.1M HCl				1M HCl
Control	**0hr**	**4hr**	**8hr**	**0hr**
3141	3192	3100	3074	2781
3160	3131	3110	3041	2689
3138	3134	3102	3022	2840
% recovery	100.190	98.654	96.800	88.038

%recovery limit: 100 ± 10% (as per ICH Q1A (R2))

Table 2. Basic hydrolysis of Nintedanib

0.1M NaOH			
Control	**0hr**	**4hr**	**8hr**
3162	3116	3058	2801
3198	3118	3060	2790
3180	3117	3059	2786
% recovery	98.01887	96.19497	87.61006

%recovery limit: 100 ± 10% (as per ICH Q1A (R2))

Table 3. Oxidative degradation of Nintedanib

3% H_2O_2			
Control	**0hr**	**3hr**	**6hr**
3141	3105	2900	2832
3169	3137	2960	2832
3198	3176	2926	2830
% recovery	99.05343	92.40639	89.33529

%recovery limit: 100±10% (as per ICH Q1A (R2))

Table 4. Photolytic degradation of Nintedanib

Photolytic	
Control	**1.2×10^6 lux hr**
3131	3023
3151	3029
3171	3057
% recovery	96.36094

%recovery limit: 100±10% (as per ICH Q1A (R2))

Table 5. Thermal degradation of Nintedanib

Thermal			
Control	**40°C**	**60°C**	**80°C**
3176	3144	3130	3130
3147	3166	3122	3127
3132	3121	3158	3152
% recovery	99.74617	99.52406	99.51348

%recovery limit: 100±10% (as per ICH Q1A (R2))

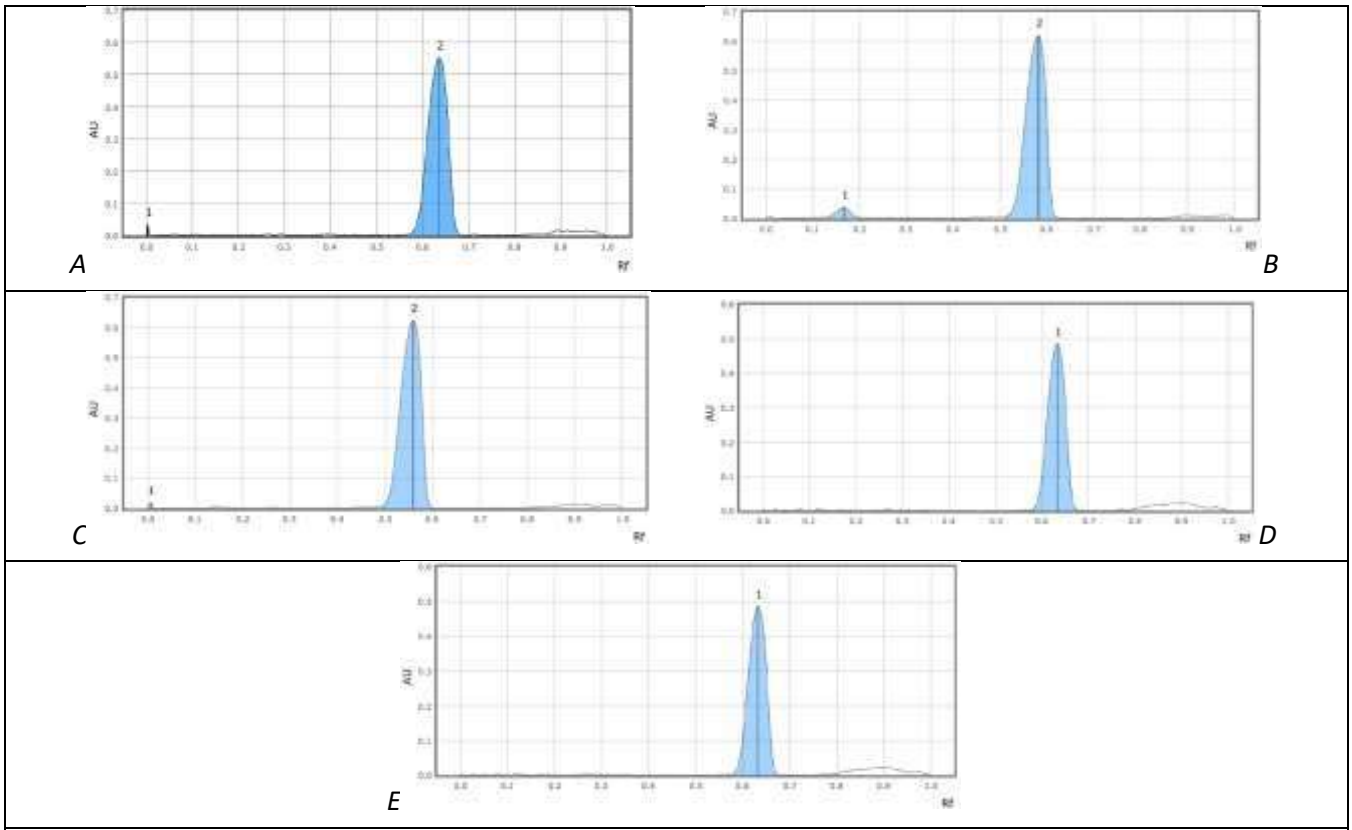


Fig 1. *A.* Densitogram of acid-stressed drug sample at 0.1M HCl, *B.* Densitogram of base stressed drug sample at 0.1M NaOH, *C.* Densitogram of oxidative stressed drug sample at 3% H_2O_2, *D.* Densitogram of photolytic degraded drug sample at 1.2×10^6 lux hours, *E.* Densitogram of thermally stressed drug sample.

The stability profiling of the drug was carried out as per the ICH guidelines and the degradation was performed till 10%, but at this condition, the detection of the degradants was not possible due to the sensitivity of the instrument. Hence, to detect and quantify the degradants, the drug was completely

degraded and then analyzed. The procedure and results are as follows: *Acid Hydrolysis:* Drug (4000 ng/band) was stressed with 1M HCl with reflux for 12hrs, then the solution was assayed.

Base Hydrolysis: Drug(4000ng/band) was stressed with 1M NaOH with reflux for 12hrs, then the solution was assayed.

Oxidative Degradation: Drug(4000ng/band) was stressed with 30% H_2O_2 at RT for 24hrs, then the solution was assayed.

Photolytic degradation: Drug(4000ng/band) was exposed to 6×10^6 lux hours for 24hrs, then the solution was assayed.

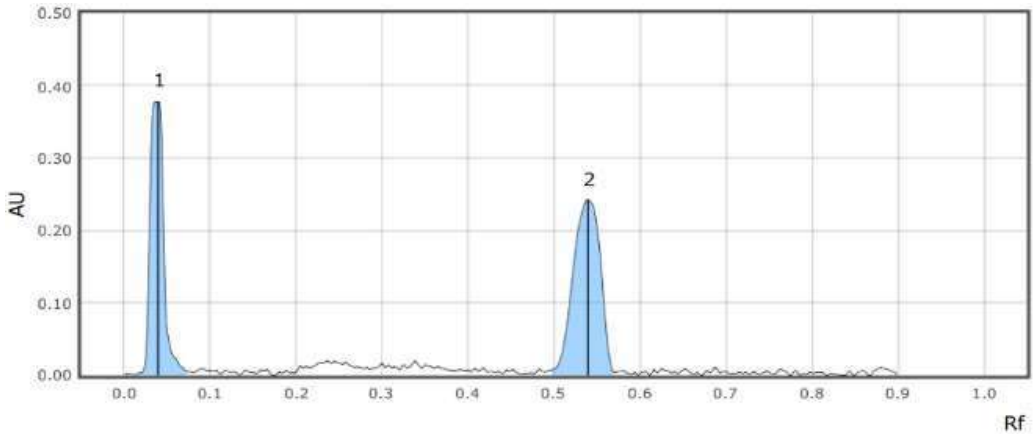


Fig 2. Densitogram of acid stressed drug sample at 1M HCl (No of DPs detected: 1)

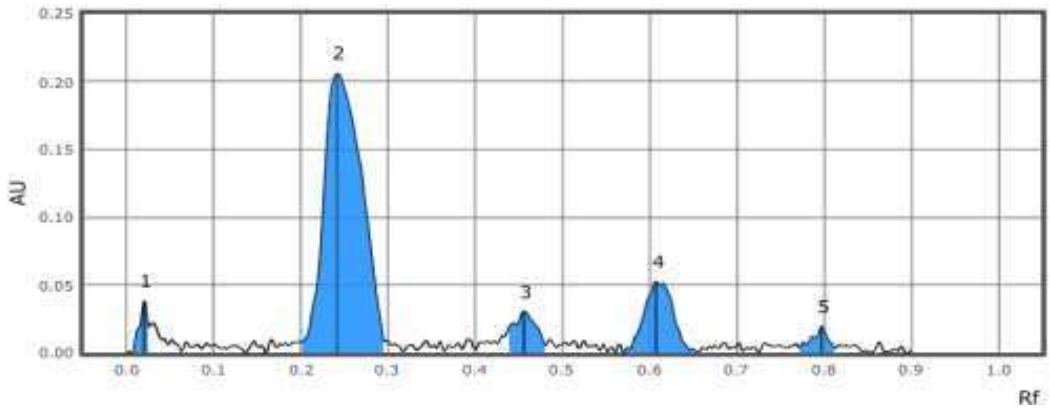


Fig 3. Densitogram of base stressed drug sample at 1M NaOH (No of DPs detected: 4)

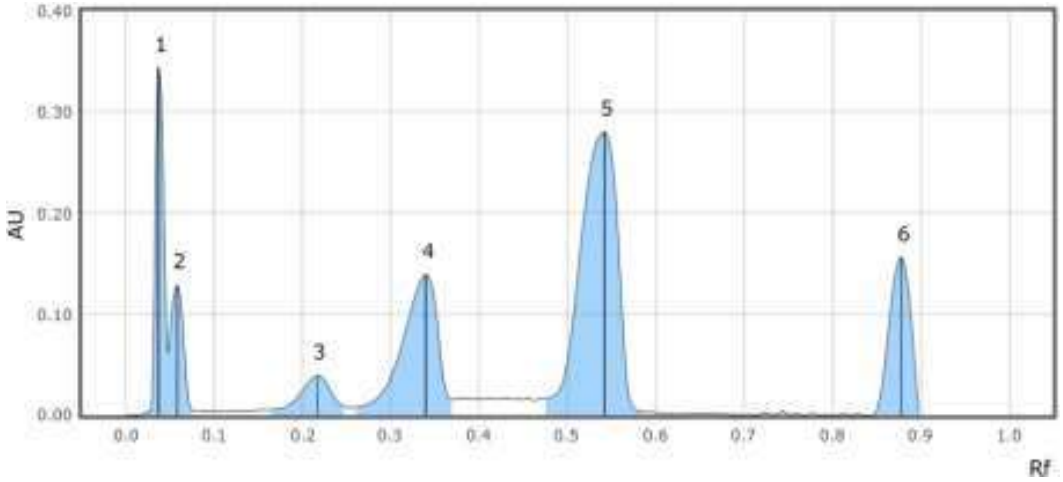


Fig 4. Densitogram of oxidative degraded drug sample at 30% H_2O_2 (No of DPs detected: 5)

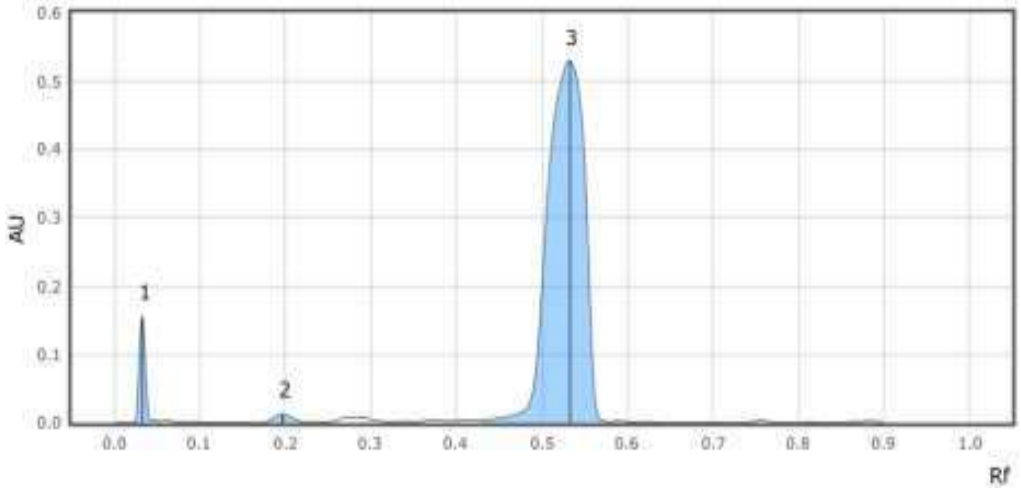


Fig 5. Densitogram of photolytic degraded drug sample at 6×10^6 lux hours (No of DPs detected: 2)

Identification of degradation products and establishment of the degradation pathway

Mass fragmentation pattern of the degradants was established with the help of Thermo Scientific LCQ fleet Ion Trap LC/MS^n**.** Mass parameters were optimized to the following values: cone gas flow: 5 l/min (N_2), nebulizer pressure: 35 psi**,** desolvation temperature: 250ºC**,** capillary voltage: 3500V**,** cone voltage: 15V**,** fragmentor voltage: 100V**.** In the subsequent step, the information on each individual fragment was obtained from LC-MS studies.

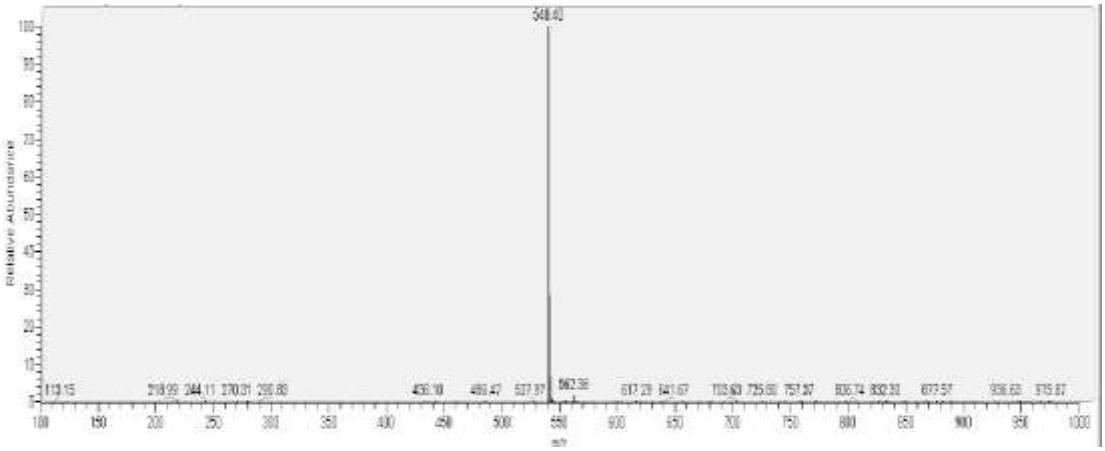
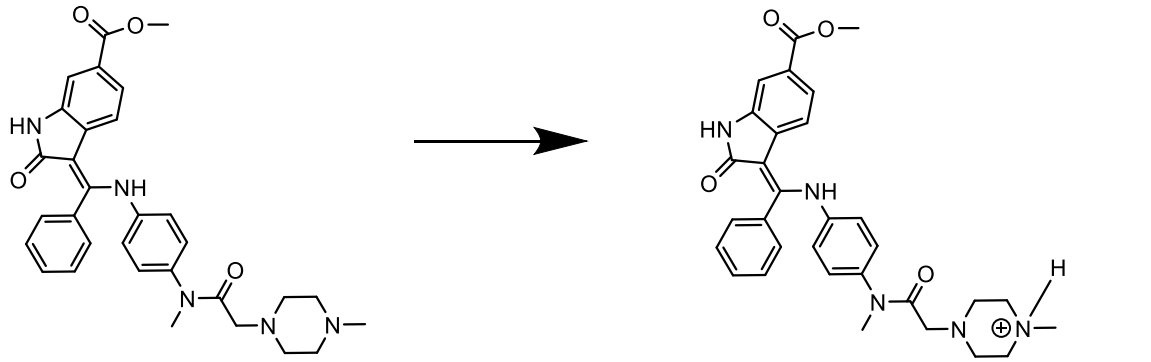


Fig 6. NTB Fragmentation Pattern (m/z: 540.40)

Table 6. Mass fragmentation details of NTB

Peak no.	MS data	Best Molecular Formulae	Exact mass of most probable structure
$(M+H)^+$ (Parent)	540.40	$C_{31}H_{34}N_5O_4^+$	540.26



Chemical Formula: $C_{31}H_{33}N_5O_4$
Exact Mass: 539.25

Chemical Formula: $C_{31}H_{34}N_5O_4^+$
Exact Mass: 540.26
m/z: 540.26 (100.0%), 541.26 (33.5%), 542.27 (5.4%), 541.26 (1.8%)

Acid Fragmentation: DP_1

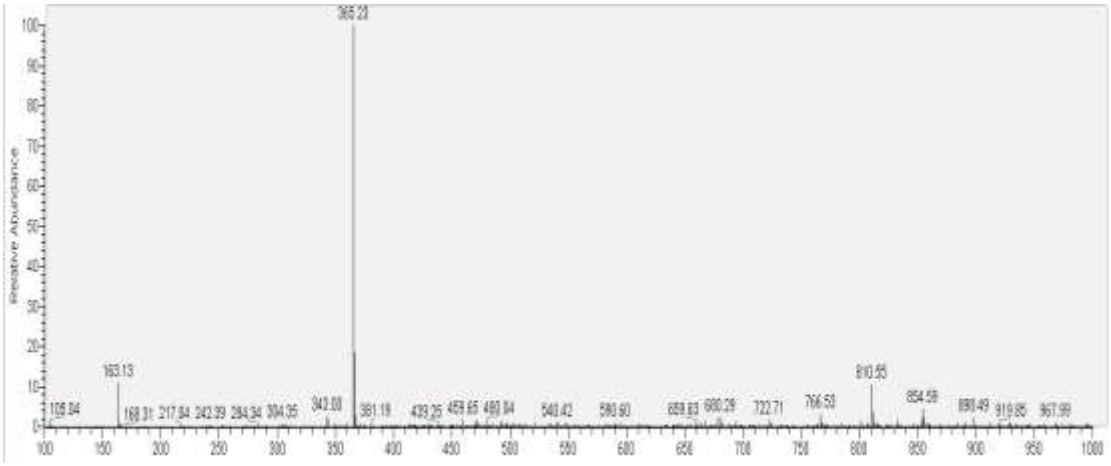
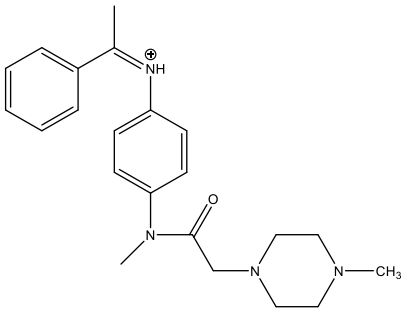


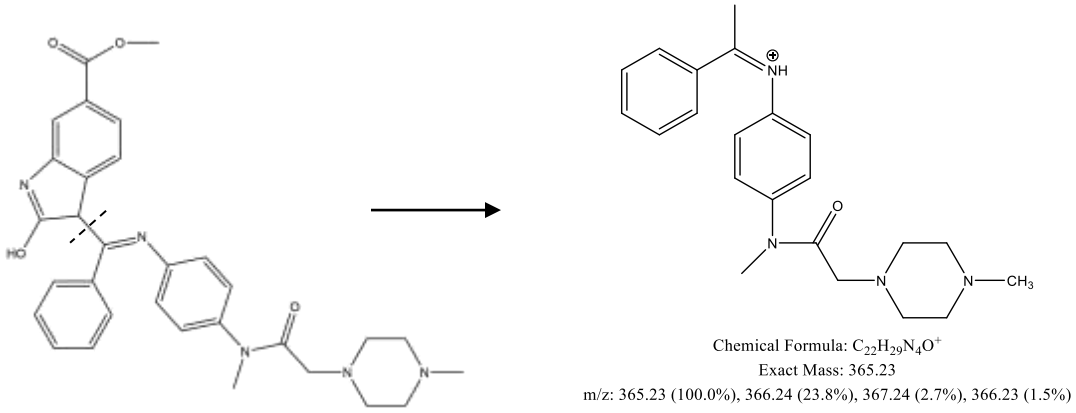
Fig 7. Mass spectra of DP_1 (base peak m/z: 365.23)

Based on our idea and organic synthetic disconnection approach the proposed structure and its fragmentation pattern is given below.



Chemical Formula: $C_{22}H_{29}N_4O^+$
Exact Mass: 365.23

<u>Proposed degradation pathway of 365.23</u>



Chemical Formula: $C_{22}H_{29}N_4O^+$
Exact Mass: 365.23
m/z: 365.23 (100.0%), 366.24 (23.8%), 367.24 (2.7%), 366.23 (1.5%)

Base Fragmentation: DP_2

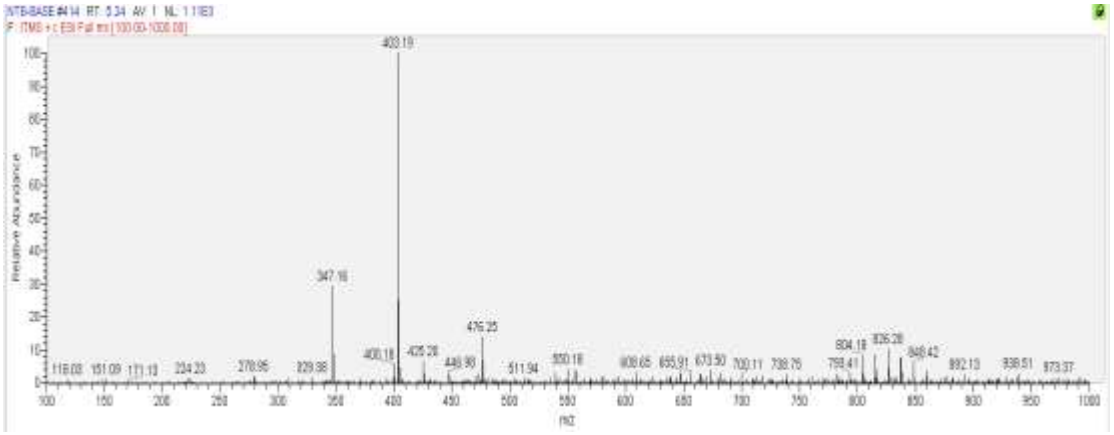
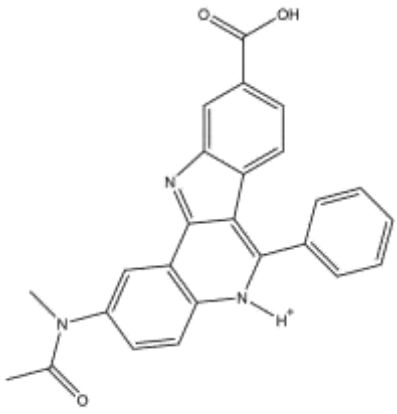
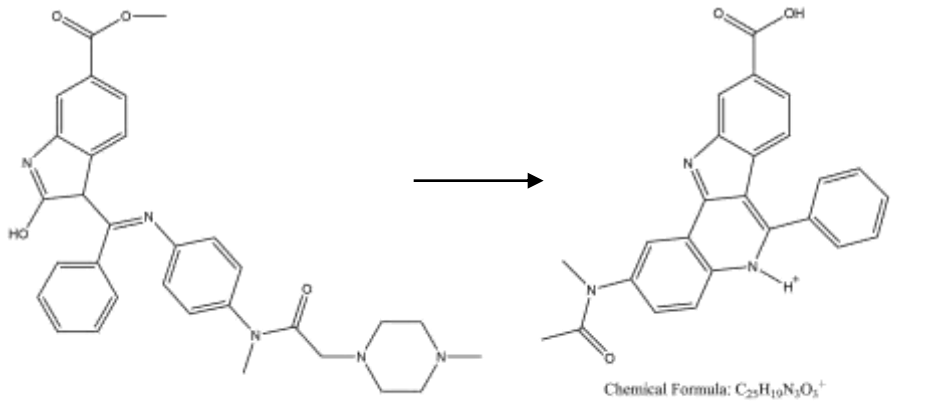


Fig 8. Mass spectra of DP_2 (base peak m/z: 409.14)



Chemical Formula: $C_{25}H_{19}N_3O_3^+$
Exact Mass: 409.14

<u>Possible degradation pathway of 409.14</u>



Chemical Formula: $C_{31}H_{33}N_5O_6$
Exact Mass: 539.25

Chemical Formula: $C_{25}H_{19}N_3O_3^+$
Exact Mass: 409.14
m/z: 409.14 (100.0%), 410.15 (27.0%), 411.15 (2.7%), 410.14 (1.1%)

Base Fragmentation: DP_3

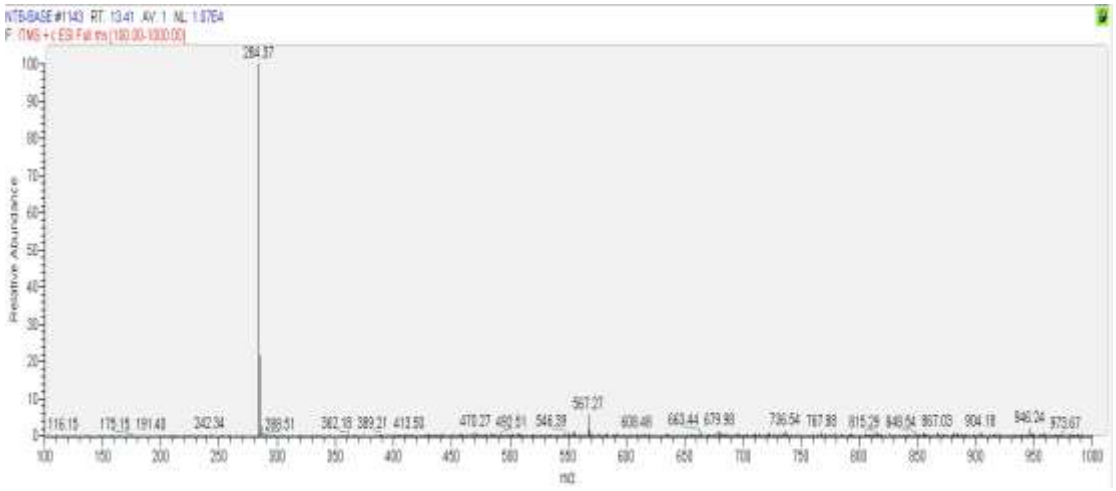
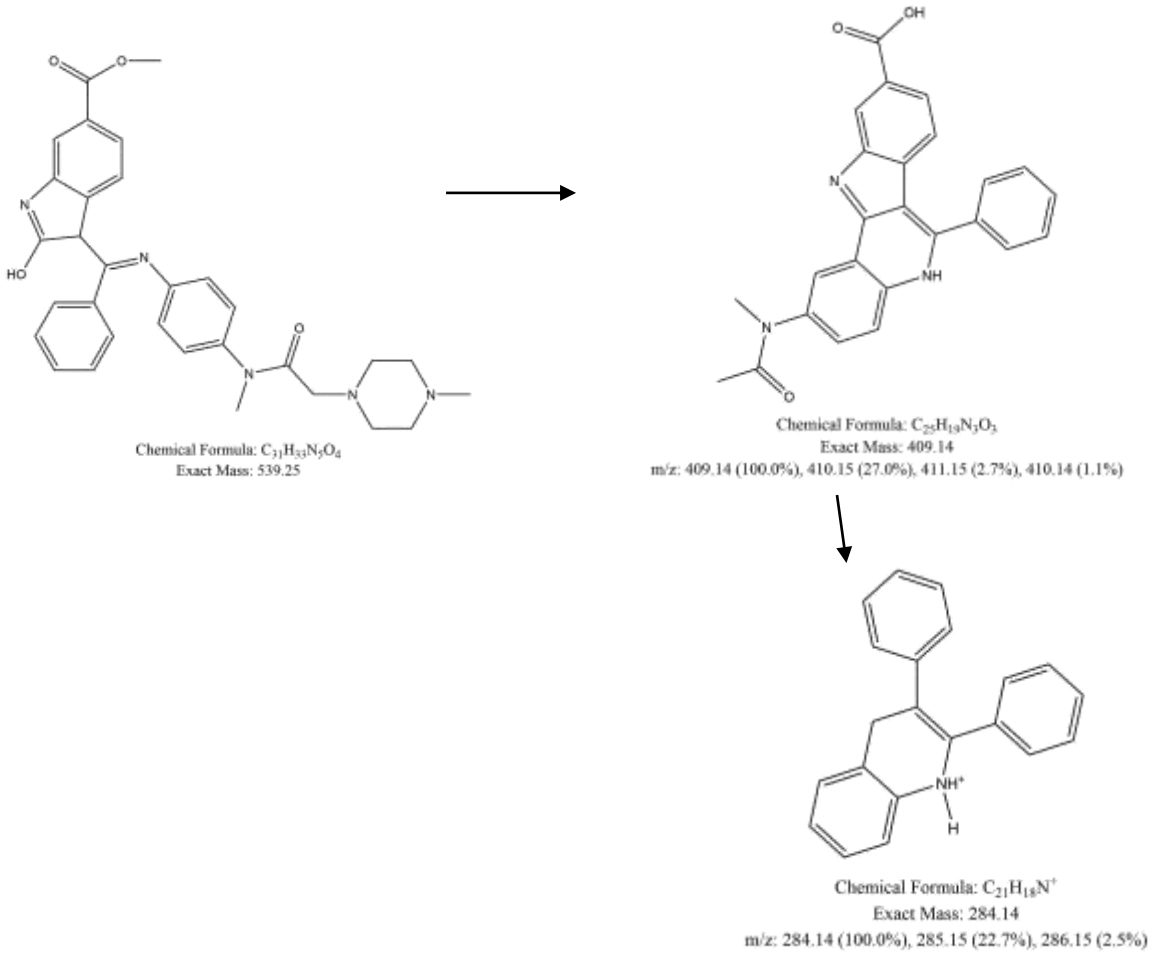


Fig 9. Mass spectra of DP_3 (base peak m/z: 284.14)

Possible degradation pathway of 284.14



Base Fragmentation: DP_4

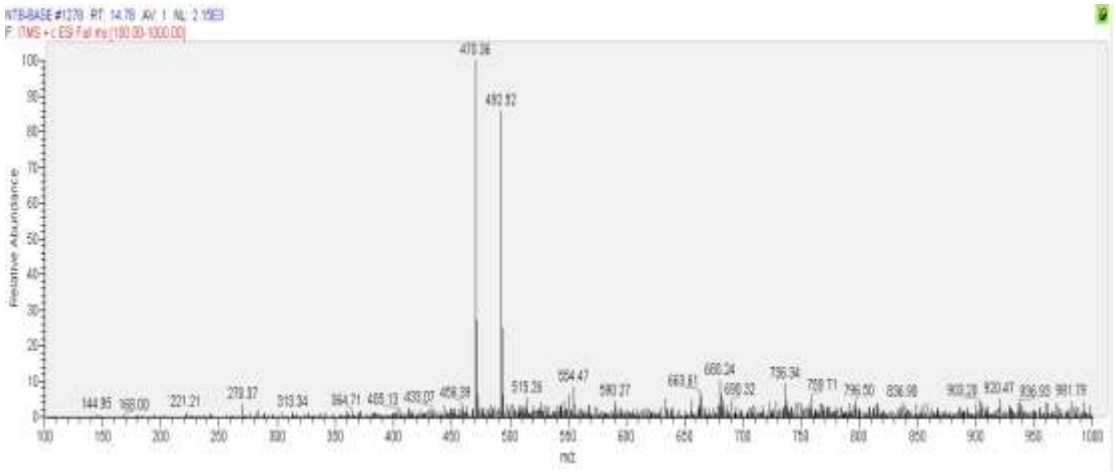
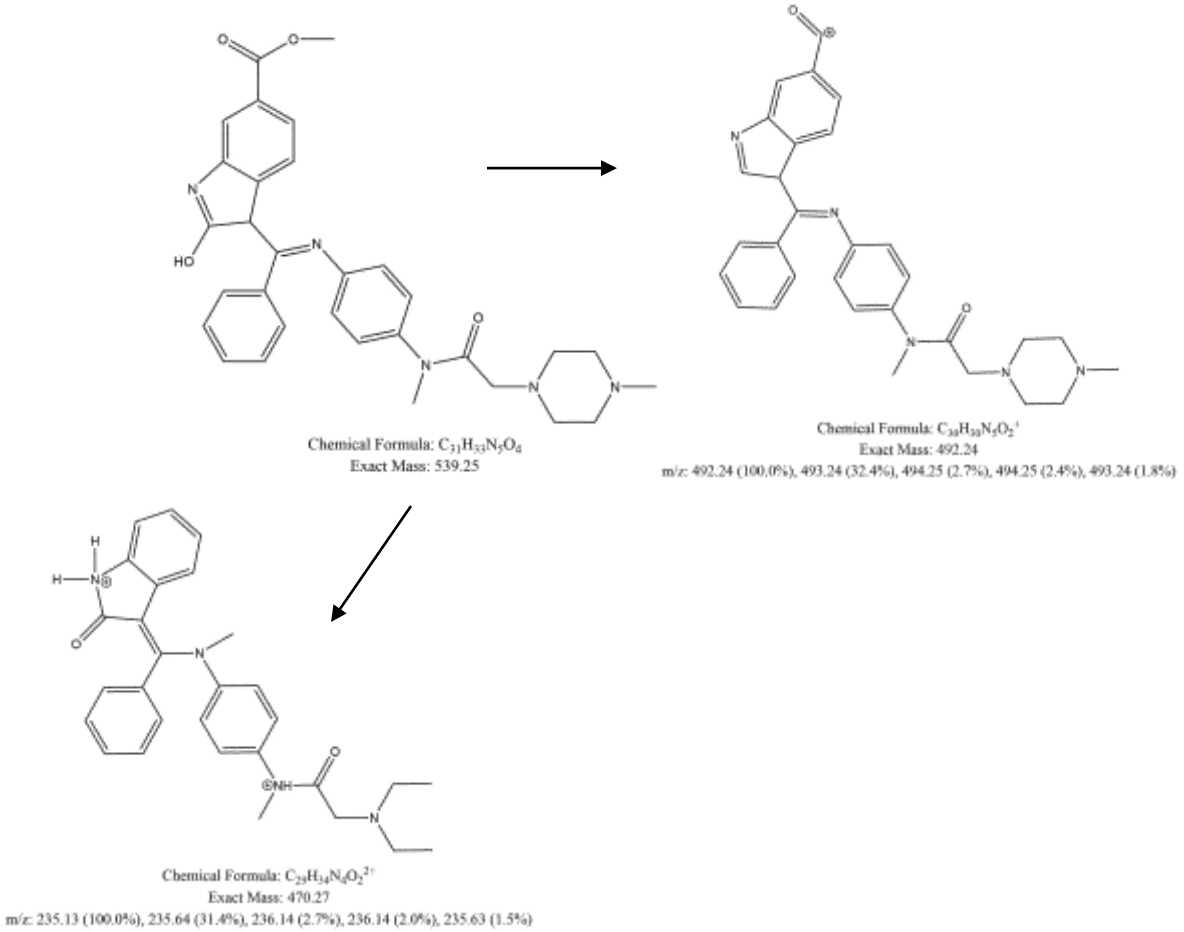


Fig 10. Mass spectra of DP_4 (base peak m/z: 470.27, 492.24)

<u>Possible degradation pathway of 470.27 & 492.24</u>



Oxidative fragmentation: DP_5

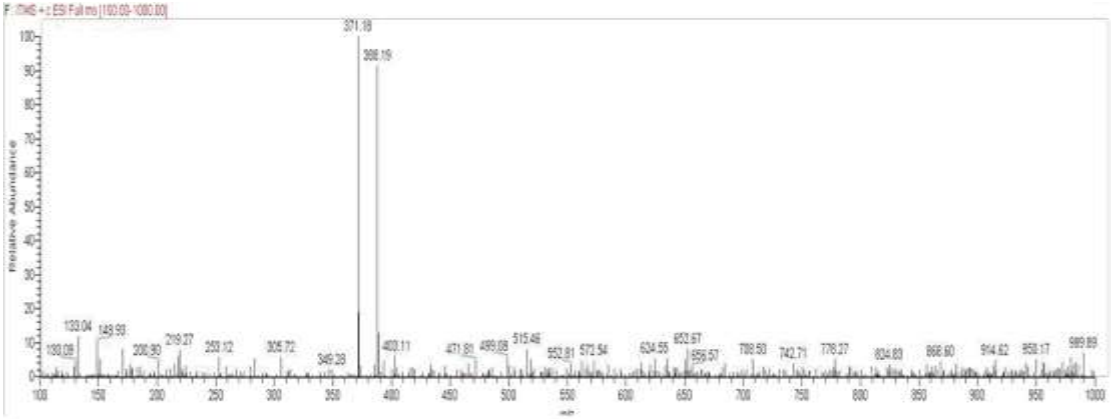
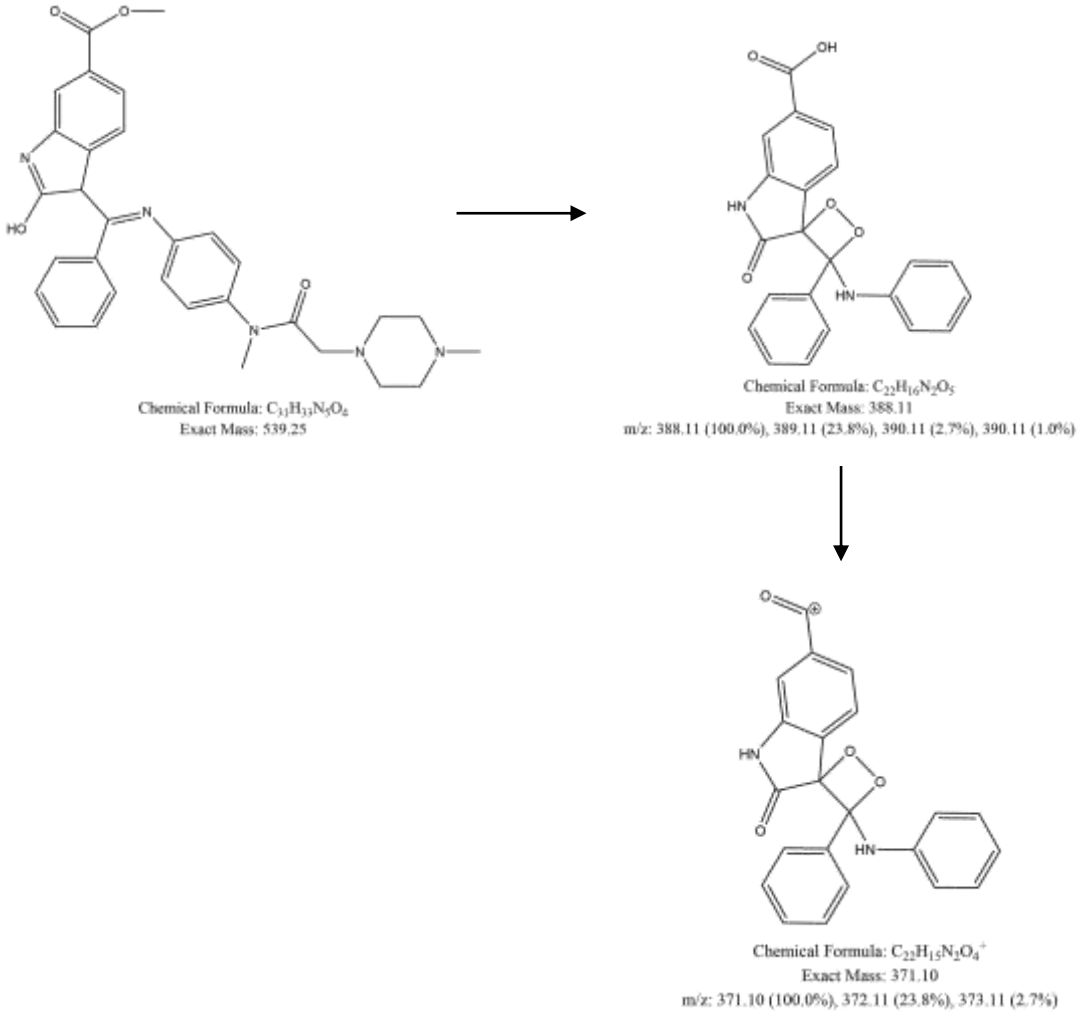


Fig 11. Mass spectra of DP_5 (base peak m/z: 371.10, 388.11)

Possible degradation pathway of 371.10 & 388.11



Oxidative fragmentation: DP_6

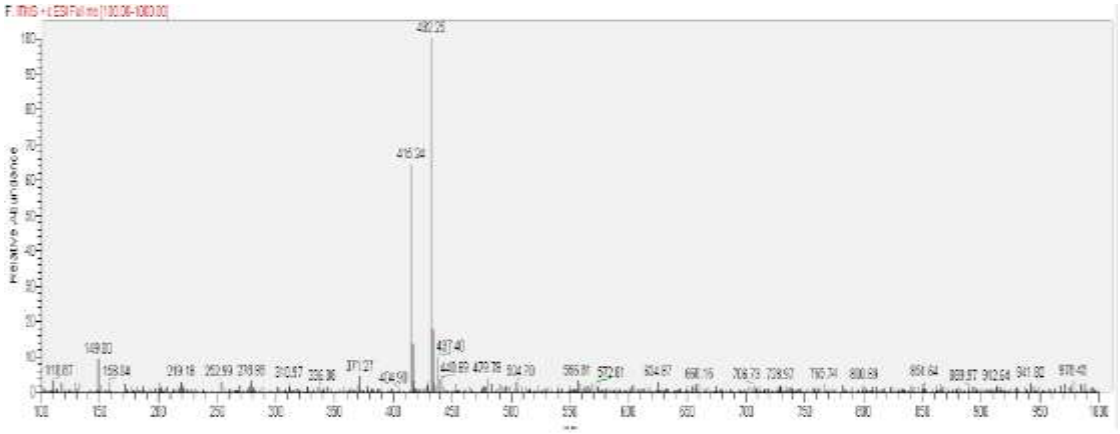
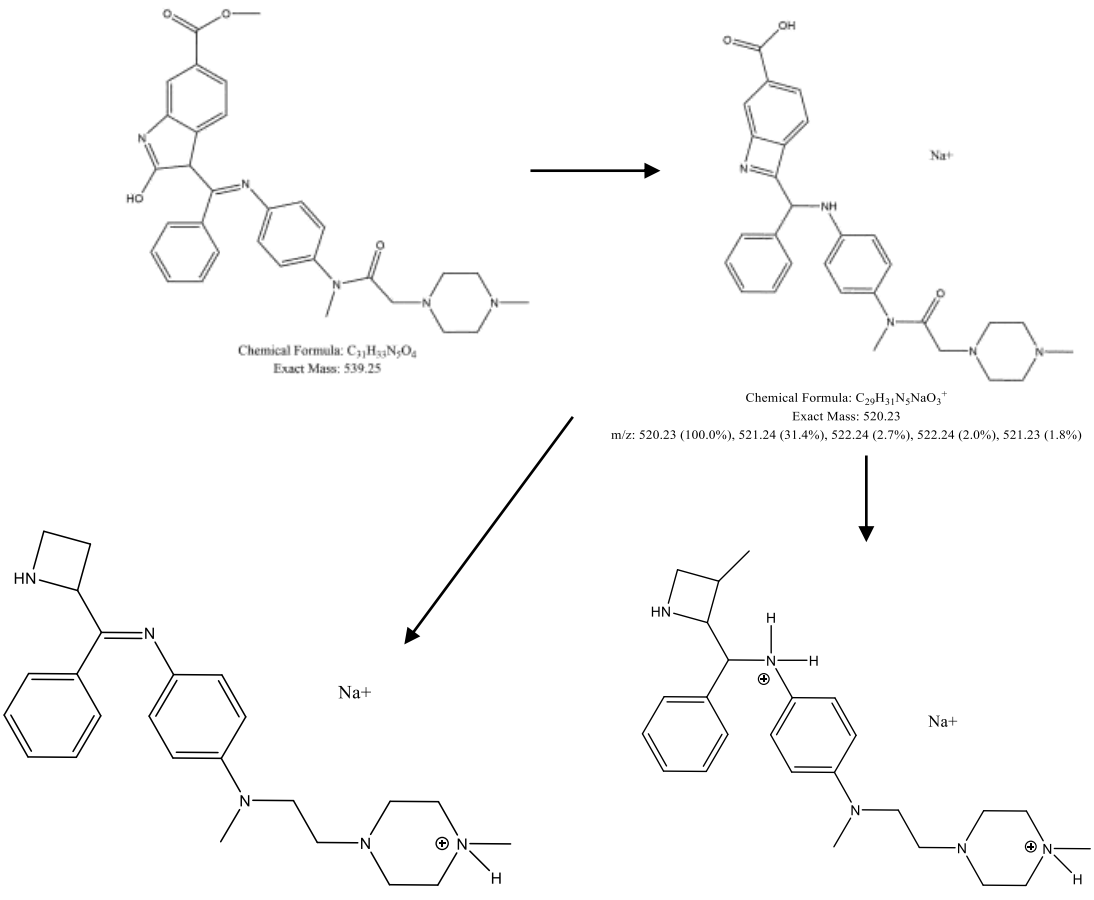


Fig 12. Mass spectra of DP_6 (base peak m/z: 415.27, 432.31)

Possible degradation Pathway of 415.27 & 432.31



Chemical Formula: $C_{24}H_{34}N_5Na^{2+}$
Exact Mass: 415.27

Chemical Formula: $C_{25}H_{39}N_5Na^{3+}$
Exact Mass: 432.31

Oxidative fragmentation: DP_7

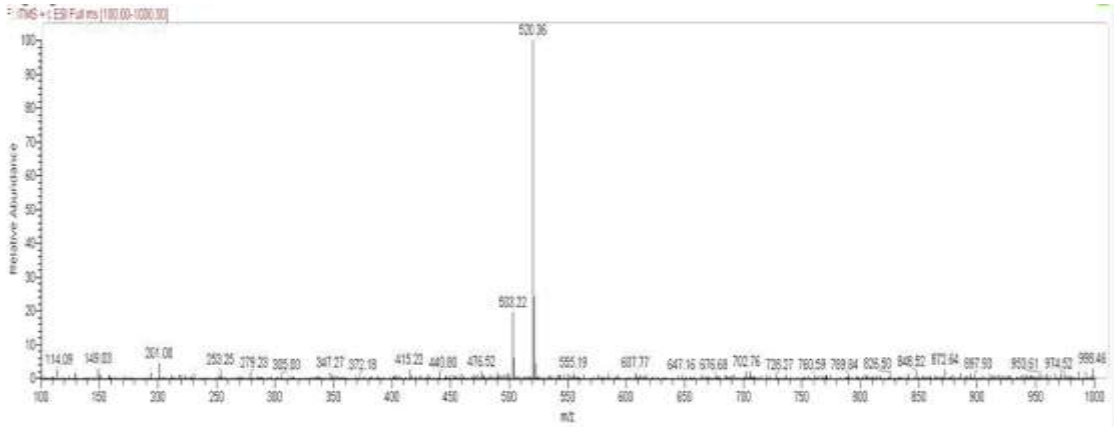
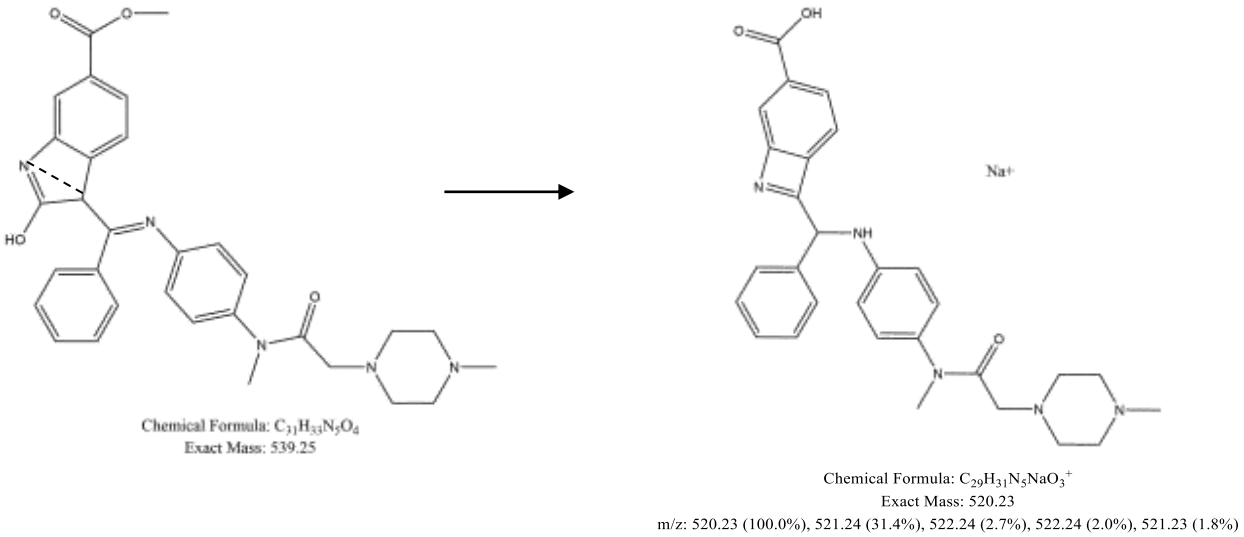


Fig 13. Mass spectra of DP_7 (base peak m/z: 520.23)

Possible degradation Pathway of 520.23



Chemical Formula: $C_{31}H_{33}N_5O_4$
Exact Mass: 539.25

Chemical Formula: $C_{29}H_{31}N_5NaO_3^+$
Exact Mass: 520.23
m/z: 520.23 (100.0%), 521.24 (31.4%), 522.24 (2.7%), 522.24 (2.0%), 521.23 (1.8%)

Oxidative fragmentation: DP_8

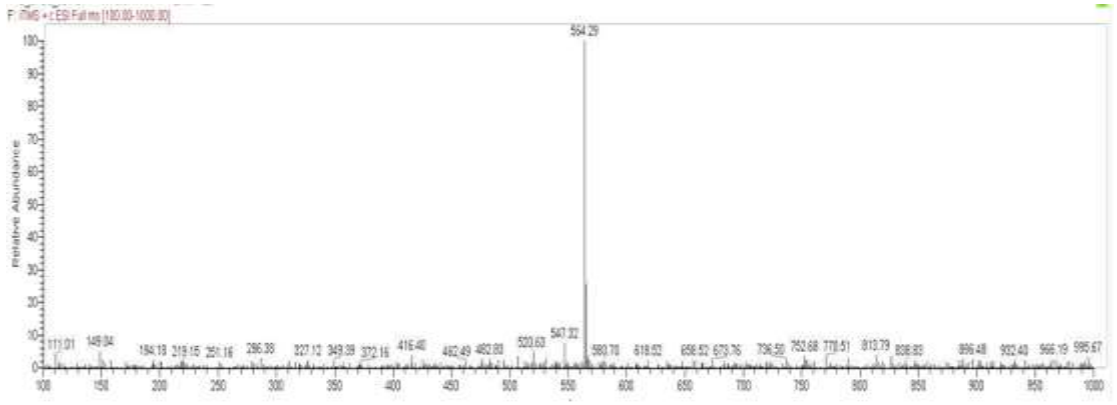
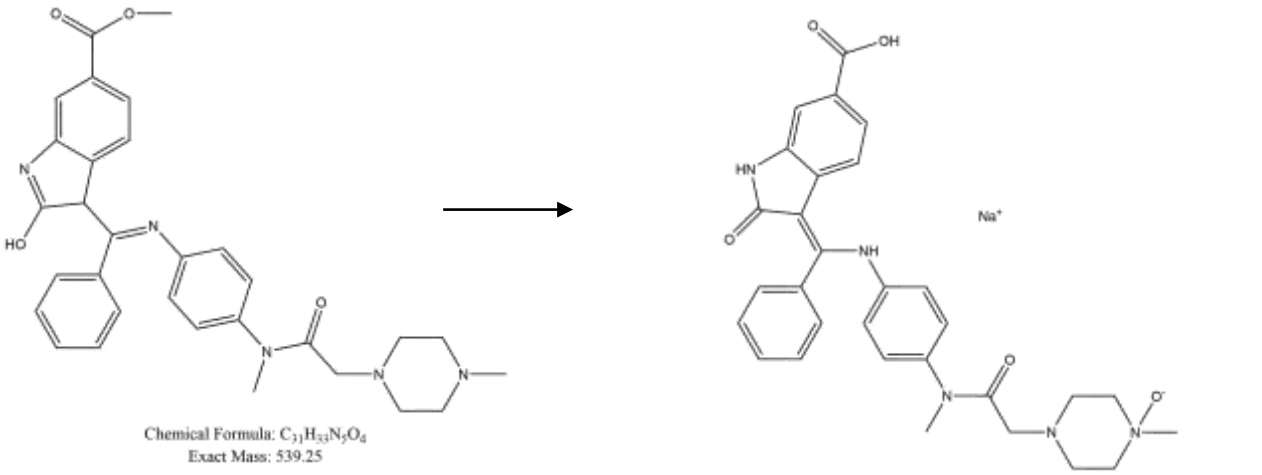


Fig 14. Mass spectra of DP_8 (base peak m/z: 564.22)

Possible degradation pathway of 564.22



Chemical Formula: $C_{31}H_{33}N_5O_4$
Exact Mass: 539.25

Chemical Formula: $C_{30}H_{31}N_5NaO_5$
Exact Mass: 564.22
m/z: 564.22 (100.0%), 565.23 (32.4%), 566.23 (5.1%), 565.22 (1.8%), 566.23 (1.0%)

Oxidative fragmentation: DP_9

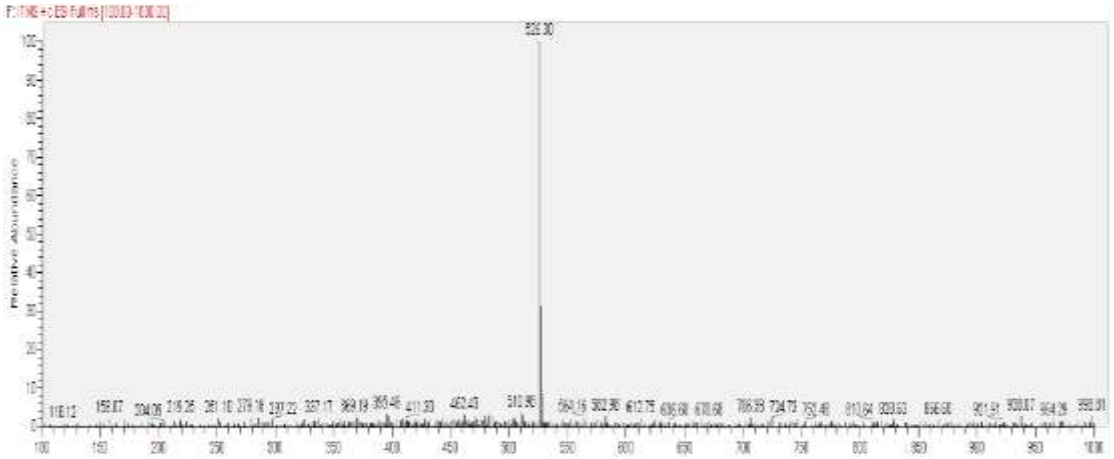
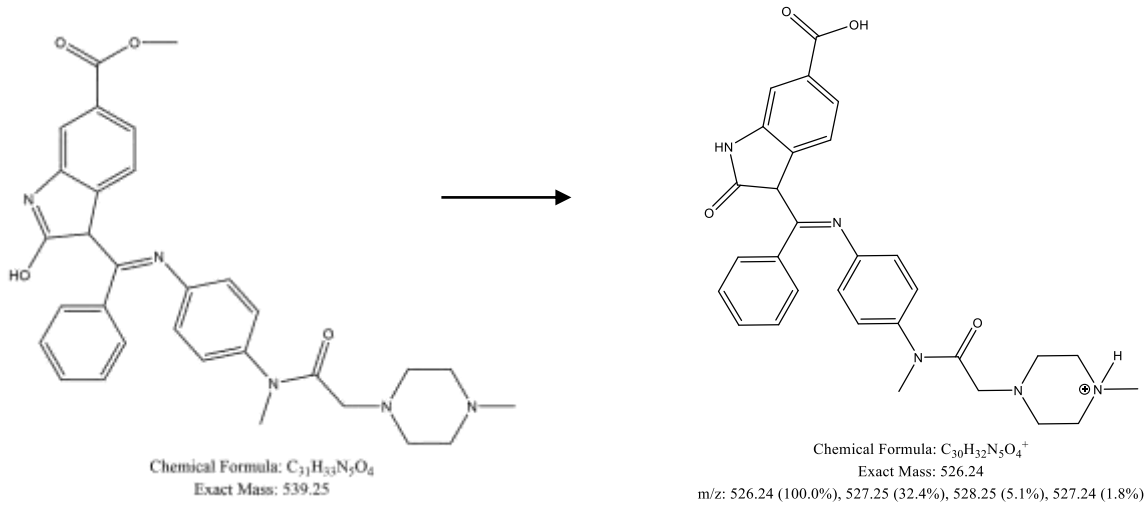


Fig 15. Mass spectra of DP_9 (base peak m/z: 526.24)

Possible degradation pathway of 526.24



Photolytic Fragmentation: DP_{10}

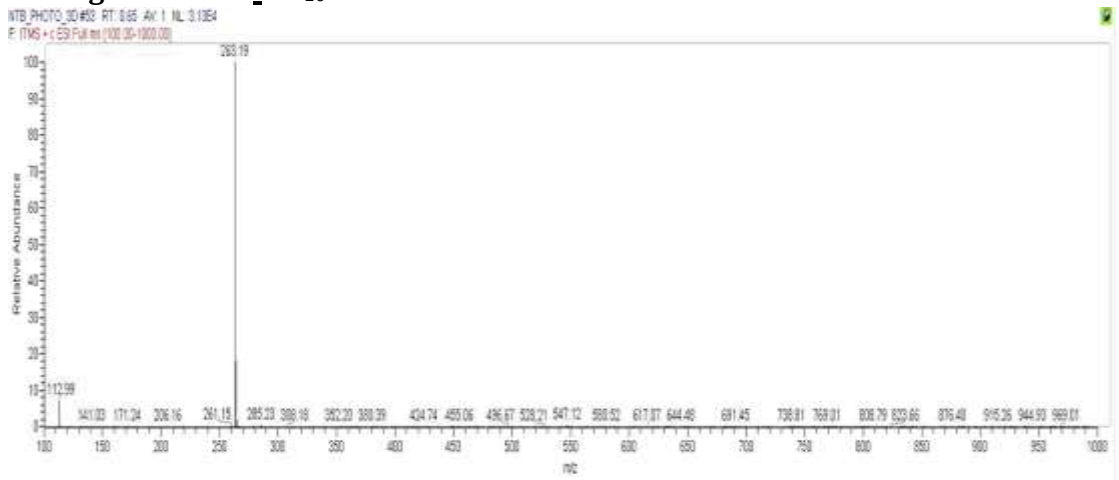
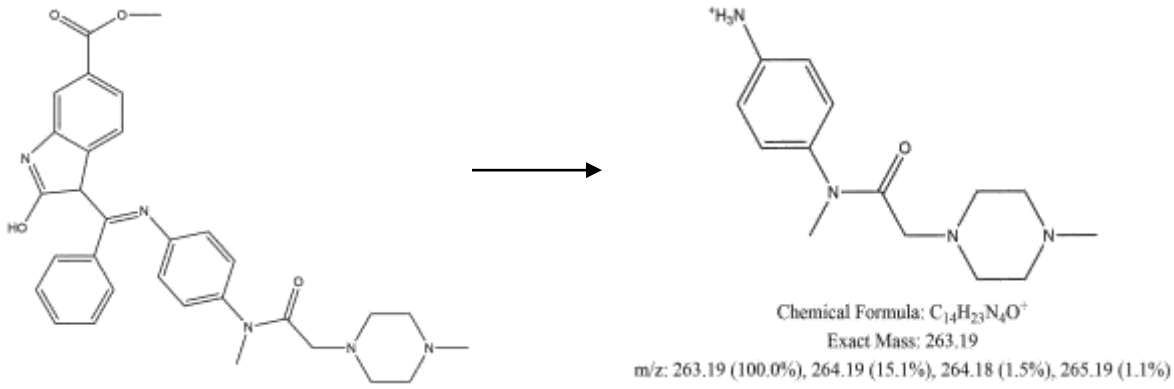


Fig 16. Mass spectra of DP_{10} (base peak m/z: 263.19)

<u>Possible degradation pathway of 263.19</u>



Photolytic Fragmentation: DP_{11}

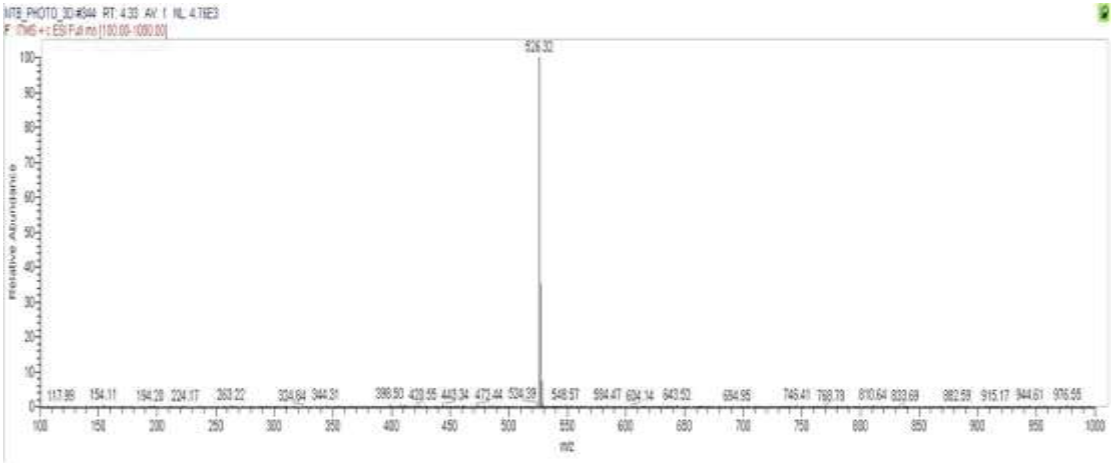
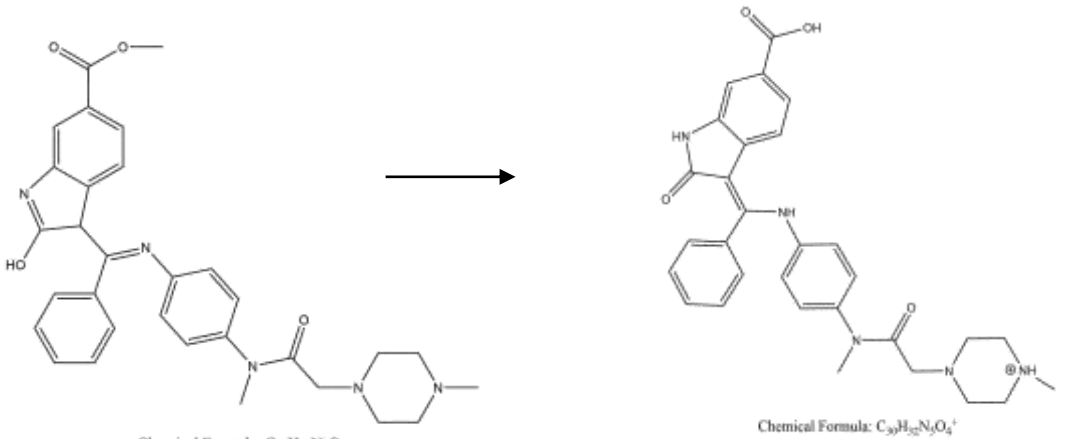


Fig 17. Mass spectra of DP_{11} (base peak m/z: 526.24)

Possible degradation pathway of 526.24



3. Summary

Summary of Validation Parameters- Summary of analytical validation parameters of NTB by HPTLC is as follows:

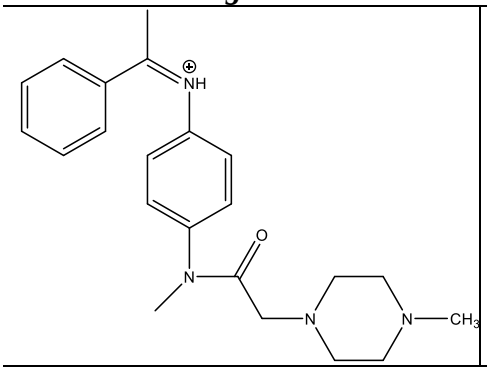
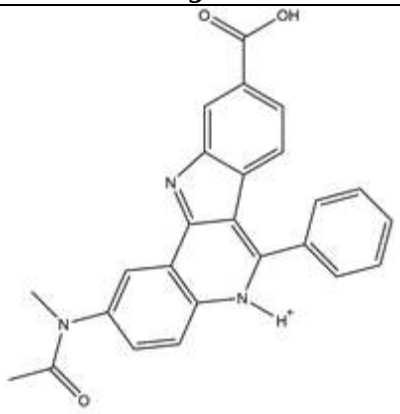
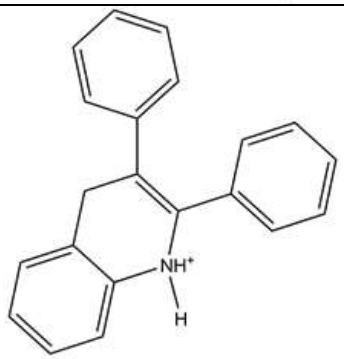
Table 7. Summary of Validation Parameters of Analytical methods

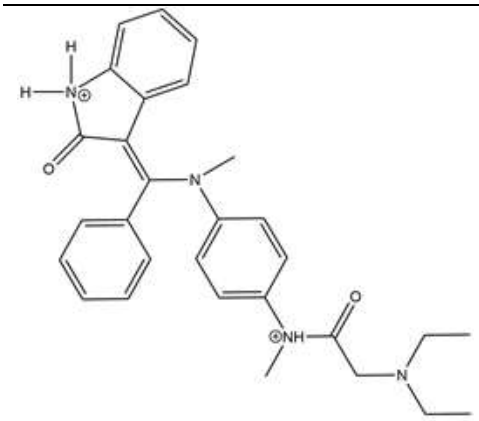
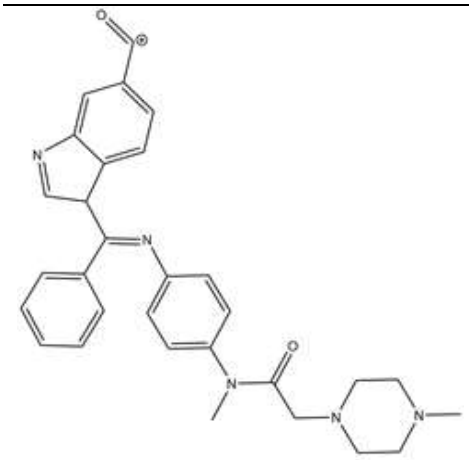
S. No.	Parameter	HPTLC
1	**Linearity range**	800-3200 ng/band
2	**Correlation coefficient**	0.999
3	**LOD**	83.357 ng/band
4	**LOQ**	252.599 ng/band
5	**Precision (% RSD)**	Less than 2%
6	**Accuracy (% Recovery)**	99.65% – 101.43%
7	**Robustness**	Less than 1%

Table 8. Comparison of stressed degradation products in HPTLC (as per modified method) & LC-MS (as per ICH Q2(R1))

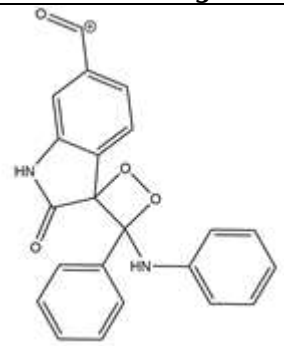
Condition in HPTLC	No. of DPs detected	Rf in HPTLC	Condition in LC-MS	No. of DPs detected	Rt in LCMS	Comments if any
Acid (1M) with reflux for 12hrs	1	0.04	Acid (0.1M) with reflux for 8hrs	1	9.98	Labile
Base (1M) with reflux for 12hrs	4	0.01	Base (0.1M) with reflux for 8hrs	3	5.24	Very Labile
		0.22			13.41	
		0.46			14.78	
		0.80				
H_2O_2 (30%v/v) at RT for 24hrs	5	0.03	H_2O_2 (3%v/v) at RT for 6hrs	5	1.37	Extremely labile
		0.05			1.88	
		0.21			3.49	
		0.37			3.97	
		0.89			6.06	
Light (6×10^6 lux h) for 24hrs	2	0.01	Light (1.2×10^6 lux h) for 24hrs	2	0.65	Labile
		0.19			4.33	

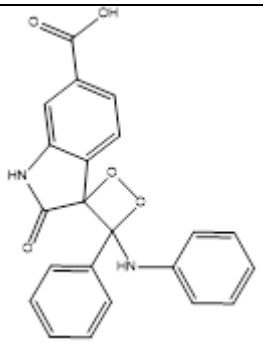
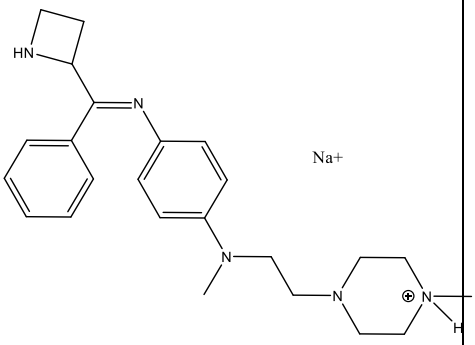
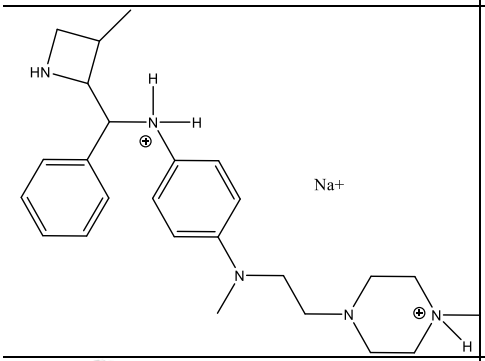
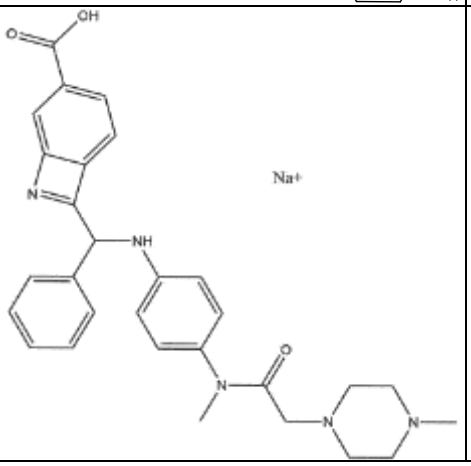
Summary of degradation products, probable structure, m/z & IUPAC name

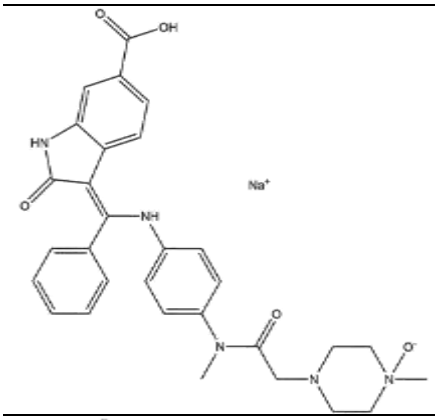
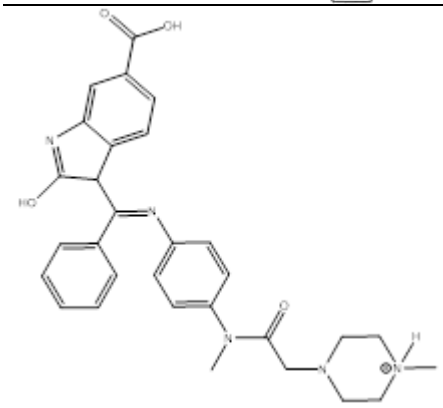
Degradation products	m/z	IUPAC Name
Acid stressed degradants		
	365.3	(Z)-4-(N-methyl-2-(4-methylpiperazin-1-yl)acetamido)-N-(1-phenylethylidene)benzenaminium
Base stressed degradants		
	409.14	2-(N-methylacetamido)-6-phenyl-5H-indolo[3,2-c]quinoline-9-carboxylic acid
	284.14	2,3-diphenyl-1,4-dihydroquinolin-1-ium

	470.27	2-(diethylamino)-N-methyl-N-(4-(methyl((2-oxoindolin-3-yl)(phenyl)methyl)amino)phenyl)acetamide cation
	492.24	(Z)-(3-(((4-(N-methyl-2-(piperidin-1-yl)acetamido)phenyl)imino)(phenyl)methyl)-3H-indol-6-yl)(oxo)methylium

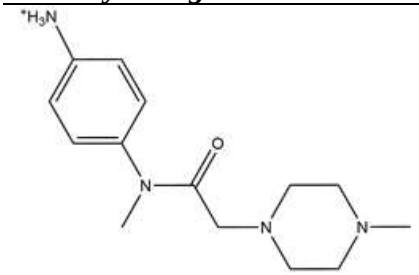
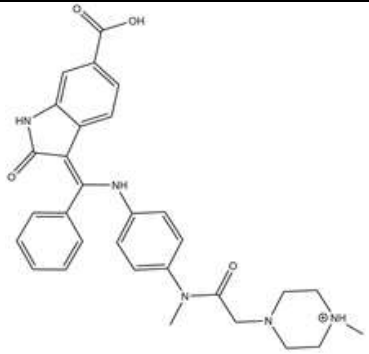
Oxidative stressed degradants

	371.10	oxo(2-oxo-4'-phenyl-4'-(phenylamino)spiro[indoline-3,3'-[1,2]dioxetan]-6-yl)methylium

	388.11	2-oxo-4'-phenyl-4'-(phenylamino)spiro[indoline-3,3'-[1,2]dioxetane]-6-carboxylic acid
	415.27	Sodium salt of (E)-4-(2-((4-((azetidin-2-yl(phenyl)methylene)amino)phenyl)(methyl)amino)ethyl) -1-methylpiperazin-1-ium
	432.31	Sodium salt of 1-methyl-4-(2-(methyl(4-(((3-methylazetidin-2-yl)(phenyl)methyl)ammonio)phenyl)amino)ethyl)piperazin-1-ium
	520.23	Sodium salt of 8-(((4-(N-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methyl)-7-azabicyclo[4.2.0]octa-1(6),2,4,7-tetraene-4-carboxylic acid

	564.22	Sodium salt of 4-(2-((4-(((6-carboxy-2-oxoindolin-3-yl)(phenyl)methyl)amino)phenyl)(methyl)amino)-2-oxoethyl)-1-methyl-1l4-piperazin-1-olate
	526.24	(E)-4-(2-((4-(((6-carboxy-2-hydroxy-3H-indol-3-ylidene) (phenyl)methyl)amino)phenyl)(methyl)amino)-2-oxoethyl)-1-methylpiperazin-1-ium

Photolytic degradants

	263.19	4-(N-methyl-2-(4-methylpiperazin-1-yl) acetamido)benzenaminium
	526.24	(E)-3-(1-((4-(N-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)ethylidene)-2-oxoindoline-6-carboxylic acid

4. Conclusion

In summary, for the first time, a simple, rapid, precise and robust stability-indicating HPTLC method has been developed and validated for the determination of Nintedanib (NTB) in bulk drug.

Furthermore, NTB was subjected to stress studies under various ICH recommended conditions. The drug was found to degrade in acidic, alkaline, oxidative and photolytic conditions. The additional findings in this study are that the drug undergoes extensive degradation under alkaline and oxidative stress, degrades to a mild extent in acidic and photolytic conditions and is stable to thermal stress. The method was validated for parameters like linearity, precision, accuracy, robustness. The degradants were not detectable when stressed as per ICH recommended conditions but on increasing the strength of acid, base and peroxide, the degradants were very much prominent and were easily detectable in HPTLC. However, through characterization based on only Mass Spectroscopy, structure of the degradant products was predicted, and their probable fragmentation pattern was also proposed. For further analyses and more accurate prediction, high-grade analytical tools like high resolution ^{1}H NMR, ^{13}C NMR, can be employed for the establishment of fragmentation pattern and degradation pathway.

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