DEVELOPMENT OF STABILITY INDICATING METHOD OF ANAGLIPTIN

6.1. SELECTION OF DRUG

Anagliptin (ANA) belongs to the category of dipeptidyl peptidase-4-inhibitor or gliptins [1,2]. It is used the treatment of type II diabetes. It is approved in Japan in 2012. ANA produces its action by stimulating insulin secretion, inhibits the release of glucagon, reduce gastric emptying. It causes increase in production of incretin hormones and gastric inhibitory peptide. It is marketed as Suiny tablets 100 mg by Sanwa Kagaku Kenkyusho, Kowa. The recommended dose of Anagliptin is 100 mg twice daily. It is used either individually or in combination with other drugs. It is approved by CDSCO in India in 2017. It is not official in any pharmacopoeia. Literature review reveals that there are no reports on identification and characterization of degradation products of anagliptin. Aim of the present work was to develop stability indicating method, identify and characterize the degradation products, isolate the major degradation products and further characterize by mass, nmr and I.R. spectral techniques.

6.2. DRUG PROFILE [3]

General Properties

IUPAC name: N-[2-({2-[(2S)-2-cyanopyrrolidin-1-yl]-2-oxo-ethyl} amino)-2-methylpropyl]-2-methylpyrazolo [1, 5-a] pyrimidine-6-carboxamide

Molecular Formula: $C_{19}H_{25}N_7O_2$

Molecular Weight: 383.45g/mole

Log P: -0.77

pKa: acidic 13.3, basic 8.29

Solubility: water, methanol, acetonitrile

Drug category: Antidiabetic

Mechanism of action: ANA inhibit the plasma DPP-4 activity and increase the plasma active GLP-1 levels. By preventing GLP-1 inactivation, they are able to increase the

secretion of insulin and suppress the release of glucagon by the alpha cells of pancreas. This leads blood glucose level to normal.

Uses: in the treatment of type II diabetes

Marketed Formulation: ANA is marketed as Suiny tablets. As this formulation was not available in India at the time of study, synthetic mixture was prepared [4] (Table 6.1).

Table 6. 1- Composition of ANA synthetic mixture

Composition	Content
Anagliptin	100 mg
Cross povidone	15 mg
Microcrystalline cellulose	30 mg
Magnesium stearate	1.5 mg

6.3. LITERATURE REVIEW

The literature already reported is summarized here:

- *Review on analytical methods on metformin HCl and anagliptin by Bhatti PV et. al.[5]*

A detailed review on analytical methods for metformin and ANA is showing that many analytical methods have developed for Metformin HCl and ANA.

- *Development and validation of five simple UV-Spectrophotometry methods for estimation of Anagliptin in bulk and in-house tablets by Patil A et. al.[6]*

Five UV-spectrophotometric methods developed for determination of ANA in bulk and in-house tablets. Five methods are based on Zero order, First Order and Second Order derivative Spectrophotometry considering amplitude and Area under curve of spectrum. In all the methods linearity was observed in the concentration range of 2-8 µg/mL with correlation coefficient of 0.999. The methods were validated as per ICH guidelines.

- *First order derivative spectrophotometric method for simultaneous estimation of anagliptin and metformin HCl in bulk and synthetic mixture by Shah S et. al. [7]*

First order derivative spectrophotometric method for the simultaneous estimation of ANA and metformin hydrochloride in synthetic mixture. The first order derivative absorption at 233 nm (zero cross point for Metformin HCl) was used for ANA and 247 nm (zero order point for Anagliptin) was used for Metformin HCl. The linearity was obtained in the range of 5-25
µg/mL for Metformin HCl and 1-5µg/mL for ANA. Mean % recoveries were found to be in the range of 99.18-100.44% for Metformin HCl and 98.18-101.5% for ANA.

- *Spectrophotometric method development for determination of anagliptin in tablet dosage form by Shah J et. al. [8]*

Simple and cost effective spectrophotometric method was developed for estimation of ANA in pharmaceutical formulations. ANA showed maximum absorbance at 247 nm and obeyed beer's law in the concentration of 3-15 µg/mL. The method was validated as per ICH guidelines. Calibration curves showed linear relationship between absorbance and concentration with correlation coefficient of 0.999. The percentage recovery was found to be 98.18-99.98± SD.

- *Development and Validation of enantioselective analysis of anagliptin by using Chiral Lux Cellulose-3-Column by Gampa N et. al. [9]*

Stereo specific normal phase high performance liquid chromatographic was developed for the separation and estimation of enantiopurity of ANA using Lux cellulose -3[cellulose tris(4-benzoate)] chiral column with mobile phase of n-hexane, ethanol, triethylamine (80 : 20 : 0.5) with isocratic elution with 1mL/min of flow rate. Detection was performed at 254 nm. ANA (R) (desired form) and ANA (S) (undesired form) eluted at retention time of 9.11 min and 8.09 min. Enantio-selective method was validated as per ICH guidelines.

- *Application of Quality by Design in the development of HPTLC method for estimation of Anagliptin in bulk and in house tablets by Patil A et.al [10]*

Quality by Design based method was developed for Normal Phase High Performance Thin –Layer Chromatography method for qualitative and quantitative estimation of ANA in bulk and in-house tablets. Chromatographic separation was achieved on

aluminum backed silica gel F254 with mobile phase dichloromethane and methanol in the ratio of 9.2: 0.8. Detection was performed at 248 nm. Critical method parameters were mobile phase ratio and saturation time. The method was optimized using central composite design, responses were retention factor, peak area and peak height. Optimum solution was searched through numerical and graphical optimization through design space.

- *Pharmacokinetic and metabolism of [14 C] Anagliptin, a novel dipeptidyl peptidase-4- inhibitor in human by Furuta S et. al . [11]*

The disposition of ANA was investigated in six healthy men. ANA was rapidly absorbed with peak plasma concentration of unchanged drug achieved at mean time of 1.8 hour post dose. Unchanged drug and carboxylate metabolite (M1) were the major components in the plasma covering 66% and 23,4% of total plasma radioactivity area under the curve. .

- *Stability indicating chromatographic method development and validation for the simultaneous estimation of metformin HCL and anagliptin in its synthetic mixture by HPLC by Bhatti P et. al. [12]*

Stability indicating RP-HPLC method was developed for the simultaneous estimation of Metformin HCL and ANA in its synthetic mixture. Mobile phase was mixture of phosphate buffer pH 7 and acetonitrile (35: 65). Flow rate was 1mL/min and detection was performed at 247 nm. Metformin and ANA eluted at retention time of 4.227 min and 5.893 min. The drugs degraded under acidic, alkaline, oxidative, thermal and photolytic condition.

6.4. SECTION - A

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD

6.4.1. EXPERIMENTAL

6.4.1.1. Chemicals and Reagents

- ANA bulk was purchased from Shouguang Qihang International Trade Co. China.
- HPLC grade acetonitrile, methanol was purchased from Rankem Pvt. Ltd. Mumbai.

- Hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide H_2O_2) were purchased from S.D. Fine Chemical Ltd. Mumbai.
- 0.22 µm Nylon 6,6 membrane filter , Ultipore® N,66® for filtration of mobile phase was procured from Pall Life Sciences ,USA.
- 0.45 µm Nylon 6,6 syringe filter for sample filtration was procured from Pall Life Sciences ,USA.

6.4.1.2. Equipments and Instruments

Equipments and Instruments utilized in the present study are same as those mentioned in section 3.4.1.2.

6.4.1.3. Chromatographic conditions

Buffer used in mobile phase was 10 mM ammonium acetate buffer which was prepared by dissolving 770 mg of ammonium acetate in 1000 mL of double distilled water. pH of buffer was adjusted to 5 with acetic acid. Mobile phase A composed of ammonium acetate buffer pH5: methanol: acetonitrile in the ratio of 90:5:5. Mobile phase B composed of acetate buffer pH 5 : methanol: acetonitrile in the ratio of 50:25 :25. Before use, mobile phase was filtered with 0.2µ membrane filter and sonicated for 5 min. Analysis was performed with column oven temperature at 40ºC with detection wavelength of 247 nm and flow rate of 1mL/min. The injection volume was 20µL. Analysis was performed on Waters Symmetry C 18 column (150 x 4.6 mm i.d. x 3.5µm particle size).The separation was performed in gradient mode (Table 6.2).

Table 6. 2- Gradient programme for ANA

Time (min)	Mobile Phase A	Mobile Phase B
0.01	95	5
10-15	95	5
15-25	55	45
25-40	55	45
41-45	95	5

45	Stop	

6.4.1.4. Preparation of Standard solution

ANA standard solution (1mg/mL) - 25 mg of ANA was weighed accurately and transferred to 25 mL volumetric flask, dissolved in water and acetonitrile (50 :50) and volume was made up to the mark with water and acetonitrile (50 :50).

Working standard solutions were prepared in water and acetonitrile (50: 50) to produce concentration in the range of 10-120 µg/mL with respect to ANA.

6.4.1.5. Preparation of forced degradation sample

For forced degradation study, stock solution of ANA (1mg/mL) was prepared in water: acetonitrile (70:30).

6.4.1.5.1. Acid degradation

1 mL of ANA stock solution was transferred to 10 mL of volumetric flask, to this was added 1 mL of 1 M HCl. The solution was heated at 80ºC for 6 hrs. The solution was neutralized with 1 M NaOH and volume was made up to 10 mL with mobile phase to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

6.4.1.5.2. Alkaline degradation

1 mL of ANA stock solution was transferred to 10 mL of volumetric flask, to this was added 1 mL of 0.1 M NaOH. The solution was kept at room temperature for 1 hr. The solution was neutralized with 0.1 M HCl and volume was made up to 10 mL with mobile phase to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

6.4.1.5.3. Oxidative degradation

1 mL of ANA stock solution was transferred to 10 mL of volumetric flask, to this was added 1 mL of 0.3 % hydrogen peroxide. The solution was kept at room temperature for 2 hrs. The volume was made up to 10 mL with mobile phase to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

6.4.1.5.4. Neutral hydrolysis degradation

1 mL of ANA stock solution was transferred to 10 mL of volumetric flask, to this was added 1 mL of water. The solution was heated at 80ºC for 6 hrs. The volume was made up to 10 mL with mobile phase to make the concentration of 100µg/mL before injecting into HPLC system.

6.4.1.5.5. Dry heat degradation

For dry heat degradation, 50 mg of ANA was spread in petridish and kept in oven at 80ºC for 11 days. From this, 10 mg of ANA was transferred to 10 mL of volumetric flask, dissolved in water: acetonitrile (70:30) to make concentration of 1mg/mL. From this, concentration of 100 µg/mL of solution was prepared and injected into HPLC system.

6.4.1.5.6. Photolytic degradation (Dry)

For photolytic degradation, 50 mg of ANA was spread in 1 mm thickness and was exposed in photolytic chamber for 11 days. From this, 10 mg of ANA was transferred to 10 mL of volumetric flask, dissolved in water: acetonitrile (70:30) to make concentration of 1mg/mL. From this, concentration of 100 µg/mL of solution was prepared and injected into HPLC system

6.4.1.5.7. Photolytic degradation (Solution)

For photolytic degradation in solution form, 10 mg of ANA was transferred to 10 mL of volumetric flask , volume was made 10 mL with water: acetonitrile (70:30) and kept in photolytic chamber for 11 days. From this, concentration of 100 µg/mL of solution was prepared and injected into HPLC system.

6.4.1.6. HPLC method validation

The developed method was validated as per ICH Q2B guideline.

For linearity, standard dilutions of ANA were prepared in the concentration ranging from 10 to 120µg/mL from ANA stock solution and were injected in triplicate. Linearity was determined by plotting peak area and concentration of solution. From the graph regression equation and regression coefficient was determined.

For precision, intra-day and inter-day precision were evaluated at concentration levels ranging from 10-120µg/mL (in triplicates). Peak areas corresponding to the

concentration was calculated and % RSD was determined for intra-day and inter -day precision.

% Recovery was evaluated by standard addition method. Accuracy of the method was evaluated at concentration of 40µg/mL. Accuracy of method was confirmed by recovery study from a solution of synthetic mixture at 3 level of standard addition (80%, 100% and 120%). The final concentrations for accuracy were 40, 72, 80, 88µg/mL. The concentrations were analysed in triplicates. % recovery and % RSD were calculated.

Limit of detection and limit of quantitation were calculated on the basis of standard deviation of the intercept and slope of the calibration curve. LOD and LOQ were calculated using equation 3.3*(σ/S) and 10*(σ/S), where σ is the standard deviation of intercept and S is the slope of the calibration curve.

For robustness, pH of buffer (4.8, 5.0, 5.2), factors like initial gradient ratio (5, 7, 9), flow rate (0.9, 1.0, 1.1mL/min) and column oven temperature (38, 40, 42 ºC) were changed. Robustness of the method was evaluated at 40 µg/mL of concentration in triplicates.

6.4.2. RESULTS

6.4.2.1. Determination of suitable wavelength

ANA solution of 10µg/mL was prepared and was scanned in the UV region of 200-400 nm and the spectrum was recorded. ANA shows strong maximum absorbance at 247 nm which was selected as the analytical wavelength (Fig. 6.1).

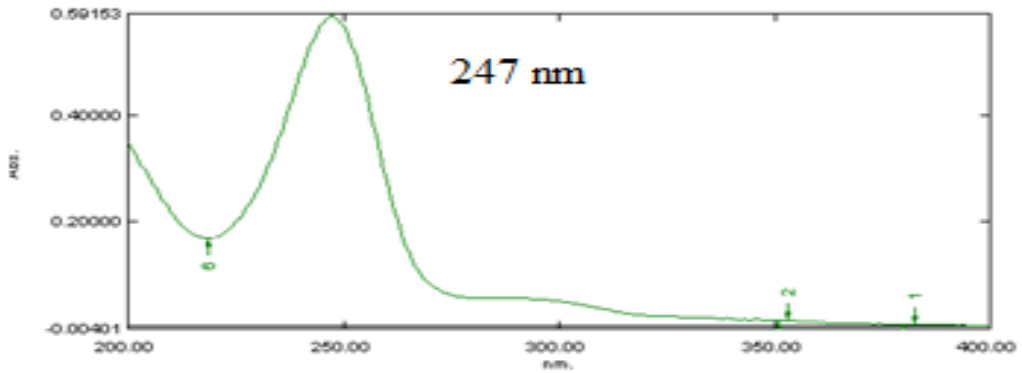


Fig. 6. 1- Zero order spectra of ANA (10µg/mL)

6.4.2.2. Method optimisation and development

During optimisation of method development, various trials were performed on Hypersil BDS C 18 column. Mobile phase like water: acetonitrile, water: methanol were tried. With the mobile phase water late elution was observed. Combination of acetate buffer with methanol or acetonitrile in the pH range 4-5 was tried. Broad peak shape was observed using methanol. Then trials were performed on Waters symmetry C 18 column method was developed with acetate buffer pH 6 and acetonitrile in the ratio of 80: 20 initially. During the development of stability indicating method, degradation peaks in alkaline conditions were merged and in oxidative condition degradation peaks were merged which was resolved by modification of gradient method and addition of methanol. One of the degradation peak DP3 was co-eluting with ANA. Resolution was achieved with acetate buffer pH 5 and column oven temperature of 40 ºC. Various trials for optimization are shown in Table 6.3.Chromatogram of ANA is shown in Fig.6.2 and optimised HPLC parameters are shown in Table 6.4.

Table 6. 3- Optimisation of HPLC conditions

Mobile Phase	**Ratio**	**Retention Time**	**Asymmetry**	**Theoretical Plates**	**Column**
Water : Methanol	50 :50	16.7 min (broad peak)	2.602	777.384	**Hypersil BDS C 18 (250 X 4.6, 5µ)**
Water : Acetonitrile	50 :50	16.0 min (broad peak)	2.809	3329	
Acetate buffer pH 6 : Methanol	50 :50	5.6 min	1.716	1355	
Acetate buffer pH 6 : Methanol	60 :40	8.8 min (broad peak)	2.1	943.8	

Acetate buffer pH 6 : Methanol: Acetonitrile	60 :30: 10	6.466 (broad peak)	1.344	2492	
Acetate buffer pH 6 : Acetonitrile	75: 25	4.66 min	1.266	3836	Waters Symmetry C 18 (150 X 4.6, 3.5 µ)
Acetate buffer pH 6 : Acetonitrile	80 : 20	6.87 min	1.234	6637	
Acetate buffer pH 5 : Acetonitrile : Methanol	**Gradient programme (Table 6.2)**	**22.43 min Sharp peak**			

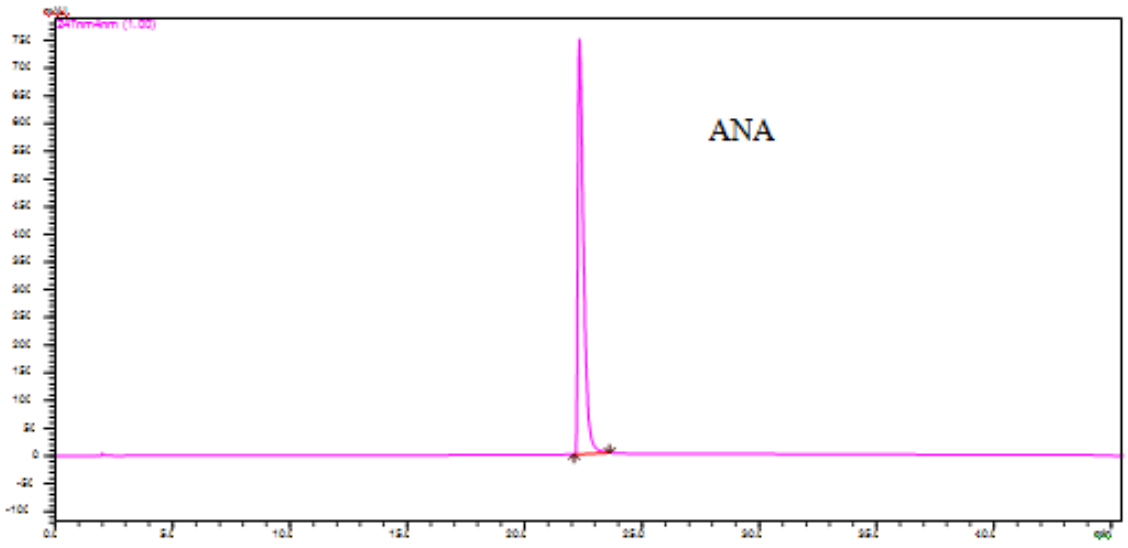


Fig. 6. 2- Chromatogram of ANA

Table 6. 4- Optimised HPLC parameters

Parameters	Optimised Value
Column	Waters symmetry C 18 (150 x 4.6mm i.d. , 3.5µ particle size)
Flow rate	1.0 mL/min
Retention time	22.43 min
Mobile phase	Gradient programme (Table 6.2)
Detection wavelength	247 nm
Needle wash	Water : acetonitrile (50 :50)
Column temperature	40ºC

6.4.2.3. Method validation using ICH Q2 (R1) guideline

6.4.2.3.1. *Linearity and range*

The calibration plotted for ANA was found to be linear in the range of 10-120 µg/mL. The regression equation was found to be y=11888x+38915 with regression coefficient (r^2) of 0.998.The linearity data is shown in Table 6.5 and calibration curve is shown in Fig. 6.3.

Table 6. 5- Linearity data of ANA

Conc.(µg/mL)	Peak Area (Mean ±%RSD)
10	1272158 ± 0.99
20	2344235 ± 0.77
40	5097335 ± 1.07
60	6869221 ± 0.62
80	9498973 ±0.89
100	11970325 ± 0.56
120	14393098 ± 0.76

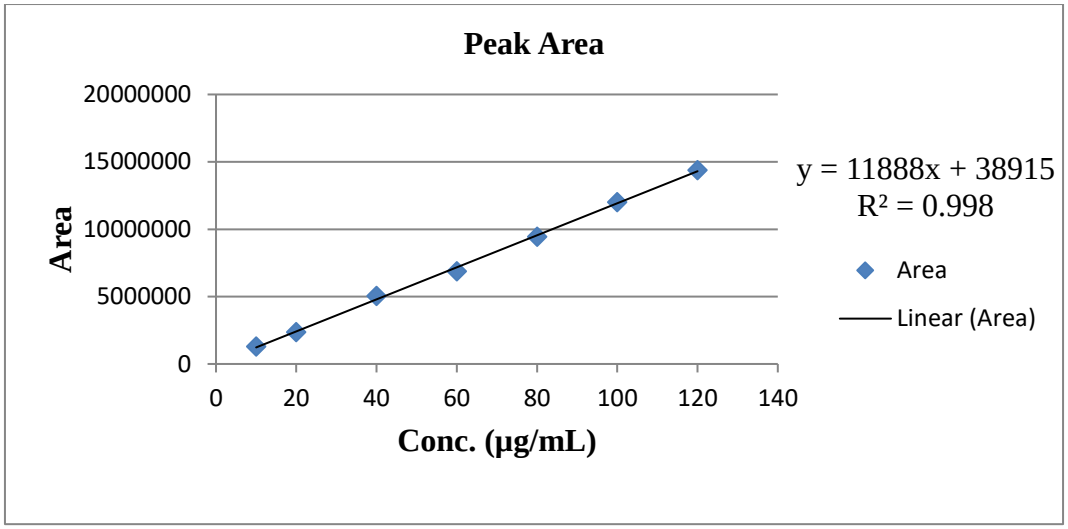


Fig. 6. 3- Calibration curve of ANA

6.4.2.3.2. *Precision*

Intra-day precision was performed by repeating the experiment three times in a day and inter-day precision was performed by repeating the experiments on three consecutive days. The average %RSD of intra-day and inter-day were found to be 1.05 and 1.17. The developed method was found to be precise (Table 6.6 and 6.7).

Table 6. 6- Intraday Precision of ANA

Conc. (µg/mL)	Peak Area				
	Set 1	Set 2	Set 3	Mean	%RSD
10	1284107	1248916	1273452	1268825	1.42
20	2360087	2398298	2344321	2367569	1.17
40	5035172	5092510	5154322	5090400	1.16
60	6872244	6920854	6804566	6865888	0.85
80	9483423	9306634	9476863	9422307	1.06
100	12004931	12113688	11892356	12003658	0.92
120	14380878	14508772	14289644	14393098	0.76
				%RSD	1.05

Table 6. 7 - Interday Precision of ANA

Conc. (µg/mL)	Peak Area				
	Set 1	**Set 2**	**Set 3**	**Mean**	**%RSD**
10	1284107	1269321	1248953	1267460	1.39
20	2360087	2387346	2316354	2354596	1.52
40	5035172	5027356	5125672	5062733	1.07
60	6872244	6949367	6821546	6881052	0.93
80	9483423	9387364	9561467	9477418	0.92
100	12004931	11294679	11632578	11477396	1.48
120	14380878	14396789	14167889	14315185	0.89
				%RSD	1.17

6.4.2.3.3. ***Accuracy***

Accuracy of method was determined by calculating % percent recovery of the analyte recovered. To the sample concentration of 40µg/mL, standard solution of ANA was added as 80%, 100% and 120% to give concentrations as 72, 80, 88 µg/mL. Recovery greater than 98% indicates the developed method was accurate (Table 6.8).

Table 6. 8- Accuracy data of ANA

Excess drug added to analyte (%)	**Theoretical Content (µg/mL)**	***Amount Found (µg/mL)**	**Recovery (%)± SD**
0	40	40.05	100.02 ± 0.62
80	72	71.57	98.70 ± 1.12
100	80	80.09	100.57 ± 1.27
120	88	87.9	99.31 ±0.59

***Average of three determinants**

6.4.2.3.4. ***Limit of detection and limit of quantification***

LOD and LOQ were found to be 3.81 and 11.54 µg/mL respectively.

6.4.2.3.5. *Robustness*

For robustness study, slight changes were made in pH of buffer, initial gradient ratio, flow rate, column oven temperature. The results were expressed as % RSD. % RSD less than 2 indicated that the developed method was robust (Table 6.9).

Table 6. 9- Robustness data of ANA

Factor	Levels	Retention Time		Peak Area		Tailing Factor		Theoretical Plates	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
pH of buffer	4.8	22.29	1.45	5062733	1.07	1.33	0.45	141717	0.01
	5.0	22.45	1.23	5103105	1.28	1.33	0.46	141707	0.01
	5.2	22.49	1.51	5117583	1.47	1.33	0.41	1417613	0.03
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Initial gradient ratio	3	23.5	0.28	5064189	1.03	1.33	0.62	1416343	0.02
	5	22.45	0.76	5103105	1.28	1.32	0.49	141712	0.03
	7	22.66	0.31	5080112	0.43	1.33	0.77	141722.7	0.01
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Flow Rate	0.9	23.75	0.89	4976377	1.01	1.32	0.38	141702	0.01
	1.0	22.45	1.23	5103105	1.28	1.32	0.54	141665	0.02
	1.1	21.36	0.56	5138255	0.80	1.32	0.51	141719.7	0.02
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD

Column Oven Temperature	38	23.12	1.13	5063693	1.03	1.34	0.50	141738	0.02
	40	22.45	1.23	5103105	1.28	1.32	0.39	141641.7	0.02
	42	22.12	1.14	5118218	1.11	1.353	0.604	141727.3	0.006

Flow rate is the critical factor in the robustness method

6.5.2.2.6. Specificity

The specificity was determined from the forced degradation studies as described in section 6.4.1.5. and 6.4.2.4. where Fig. 6.19 shows ANA peak is well separated from all degradation products formed during different stress conditions with sufficient resolution. In the forced degradation studies, for all degradation products peak purity index was greater than single point threshold, ensures degradation peaks are pure and peaks are not co-eluting. The specificity study ensures selectivity of the developed method which is able to separate and quantify ANA in presence of degradation products. Peak purity data of ANA and degradation products are shown in Table 6.10.

Table 6. 10 - Peak purity data of ANA and degradation products

S.No.	Peaks	Rt	Peak Purity Index	Single Point threshold
1	ANA	22.43	1.0000	0.9999
2	DP1	13.6 min	0.999970	0.999394
3	DP2	36.5 min	0.999959	0.999942
4	DP3	3.6 min	0.999677	0.993379
5	DP4	27.8 min	0.989968	0.810262
6	DP5	27.9min	0.972846	0.802845
7	DP6	24.9min	0.989968	0.810262
8	DP7	29.4min	0.99827	0.956448
9	DP8	6.2 min	0.97684	0.645985
10	DP9	6.9min	0.97375	0.673824
11	DP10	11.67 min	0.9996	0.9910
12	DP 11	8.9min	0.993774	0.866320
13	DP 12	18.7 min	0.984865	0.873845

6.4.2.3.7. *Stability in sample solutions*

Stock solution of ANA and stressed samples were prepared from standard stock solution and then stored at room temperature for 24 hrs. No additional peaks were observed which indicated stability of ANA sample solution.

6.4.2.3.8. *System Suitability Parameters*

System suitability tests were performed on freshly prepared solution with n=6 containing ANA. The results of system suitability parameters are shown in Table 6.11. Peak purity data of ANA is shown in Table 6.10 and peak purity curve is shown in Fig.6.4.

Table 6. 11- System suitability parameters of ANA

Parameters	Data Obtained
Retention Time (min ± SD)	22.43 ± 0.11
Tailing Factor ± SD	1.34± 0.01
Theoretical Plates ± SD	141712 ± 214.38

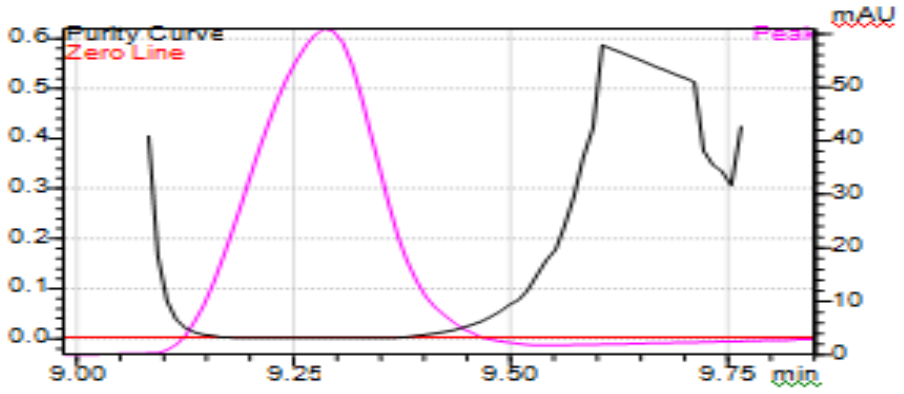


Fig. 6. 4- Peak purity of ANA

6.4.2.4. Stress Degradation studies

6.4.2.4.1. Alkaline degradation – significant degradation (26.49%) was observed when ANA was subjected to 0.1 M NaOH RT (40ºC) for 1 hr with the formation of

two degradation products DP3and DP1 at retention time of 3.6 and 13.6 min respectively (Fig. 6.5).

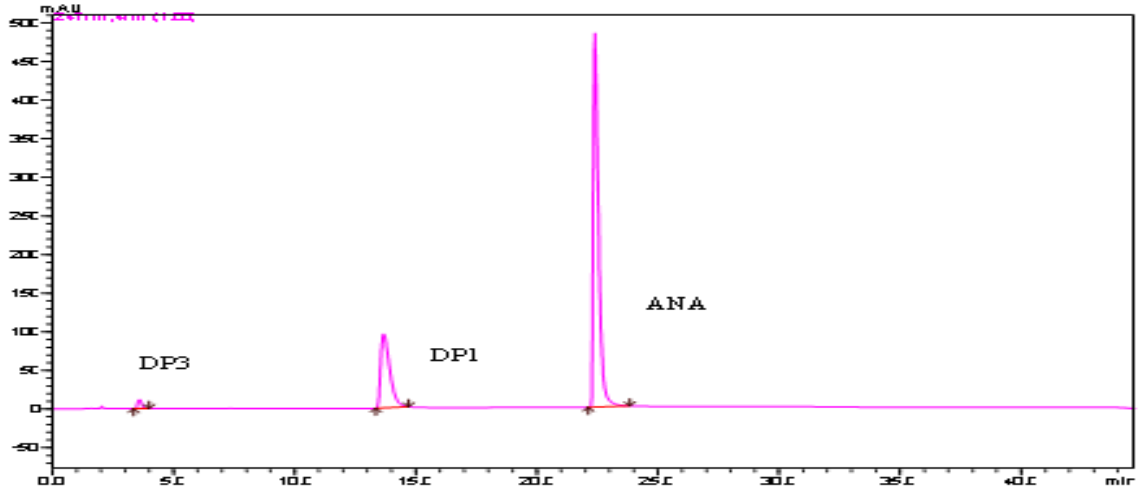


Fig. 6. 5 - Chromatogram of alkaline degradation (API)

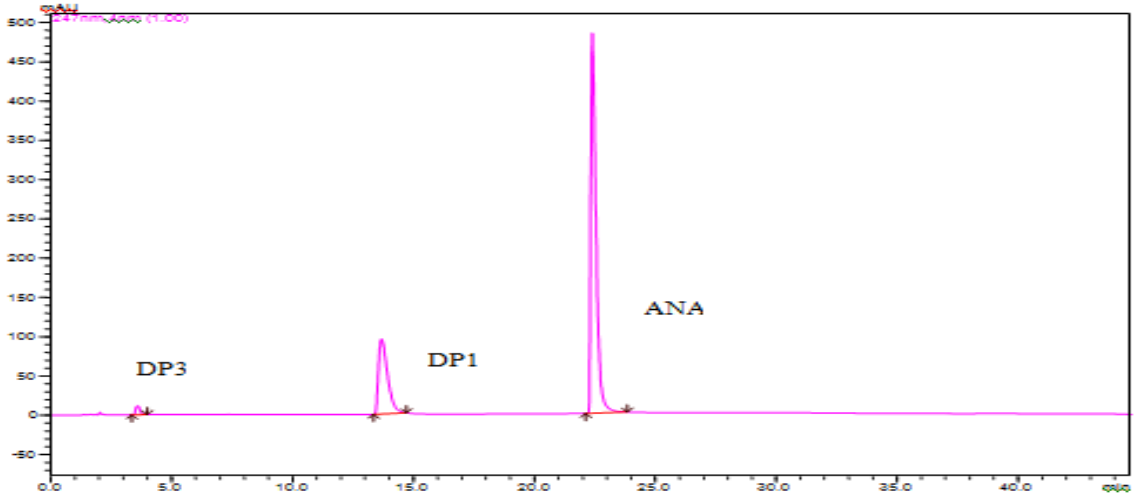


Fig. 6. 6- Chromatogram of alkaline degradation (synthetic mixture)

6.4.2.4.2. Oxidative degradation- significant degradation (17.5%) was observed when ANA was subjected to 0.3 % hydrogen peroxide at room temperature (40ºC) for 2 hrs with the formation of seven degradation products DP3, DP4, DP1, DP6, DP5, DP7 and DP2 at retention time of 3.59, 9.6, 13.8, 24.9, 27.9 , 29.4 and 36.5 minutes respectively. (Fig. 6.7).

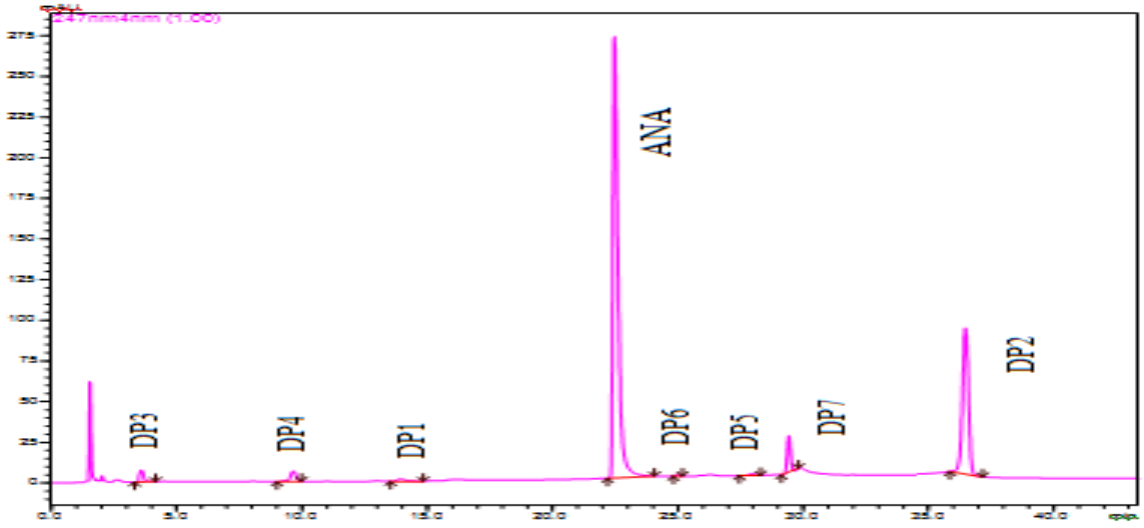


Fig. 6. 7-Chromatogram of oxidative degradation (API)

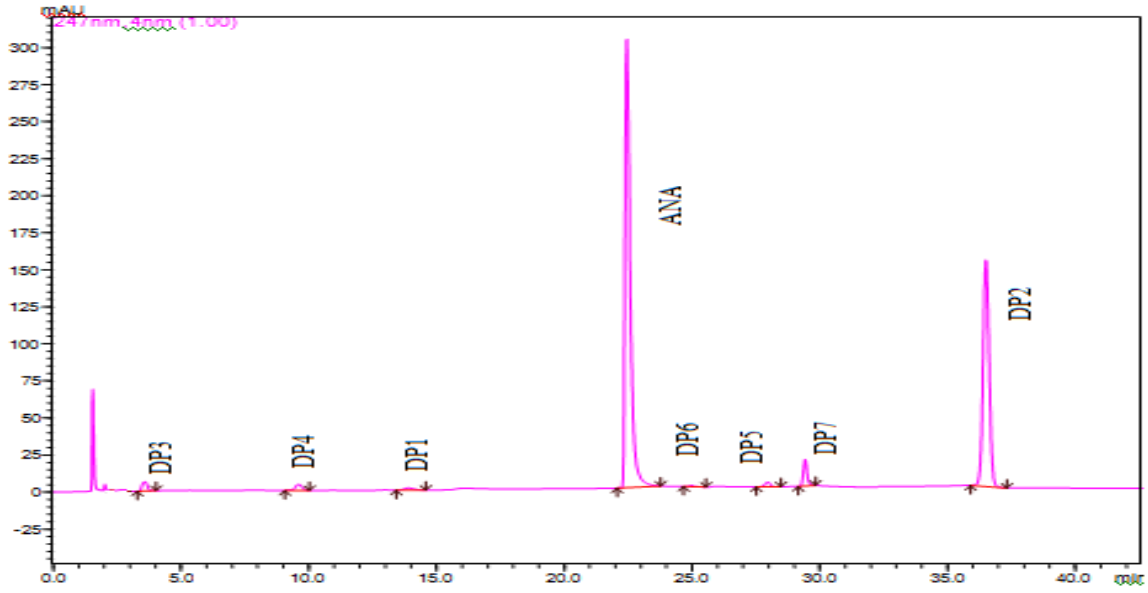


Fig. 6. 8- Chromatogram of oxidative degradation (synthetic mixture)

6.4.2.4.3. Acid degradation – Slight degradation (3.1%) was observed when ANA was subjected to 1 M HCl at 80ºC for 6 hrs with the formation of six degradation products DP3, DP8, DP9, DP11, DP1 and DP 12 at retention time 3.8, 6.2, 6.9, 8.9, 13.6 and 18.7 min respectively (Fig. 6.9).

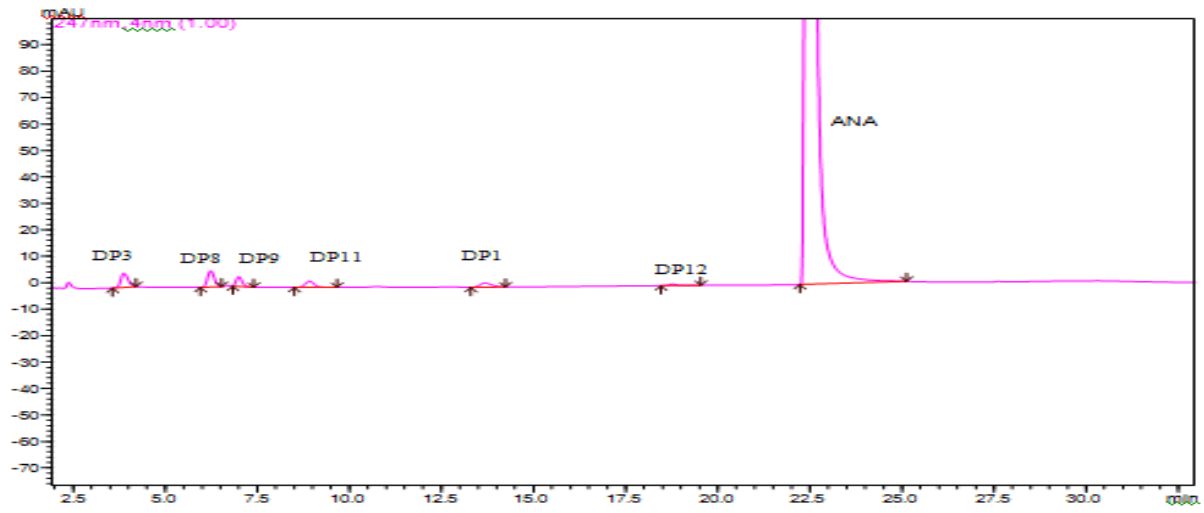


Fig. 6. 9- Chromatogram of acid degradation (API)

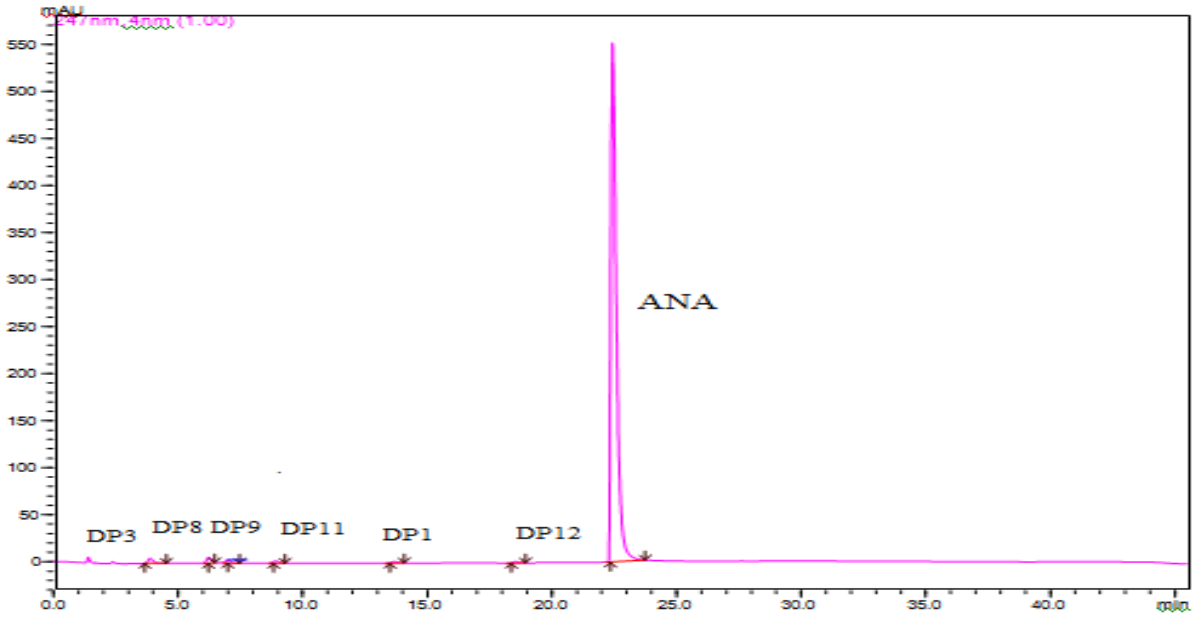


Fig. 6. 10- Chromatogram of acid degradation (synthetic mixture)

6.4.2.4.4. Neutral hydrolysis degradation- Slight degradation (4.2%) was observed when ANA was subjected to neutral hydrolysis at 80ºC for 6 hrs with the formation of five degradation products DP3, DP8, DP9 , DP1 and DP4 at retention time of 4.09, 6.04, 7.04, 13.9 and 27.8 min respectively (Fig. 6.11).

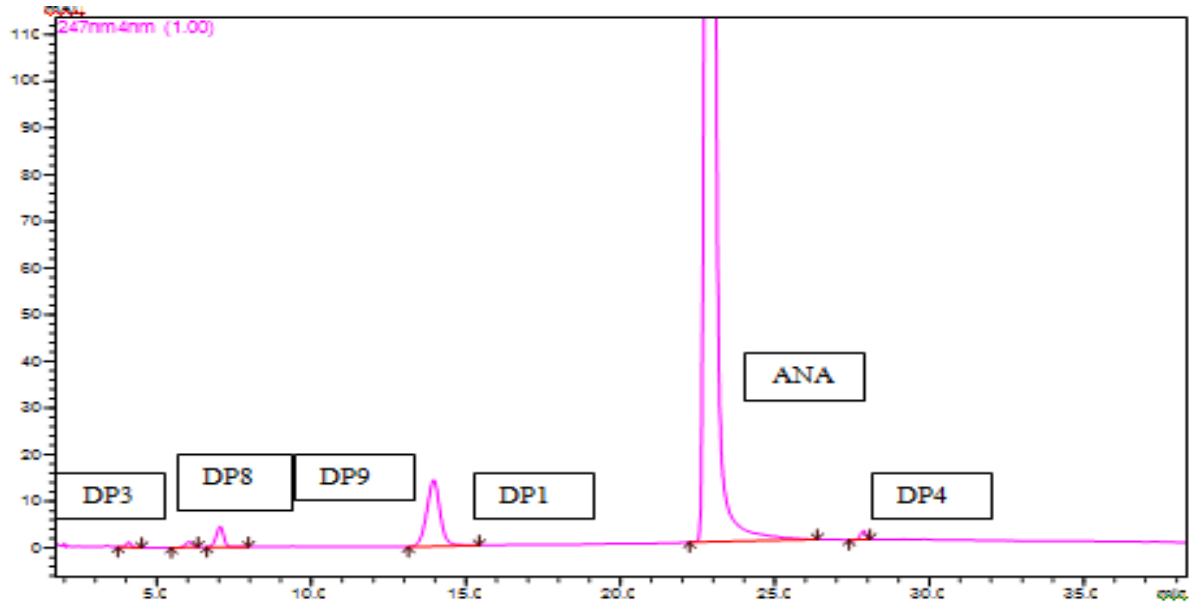


Fig. 6. 11-Chromatogram of neutral hydrolysis degradation (API)

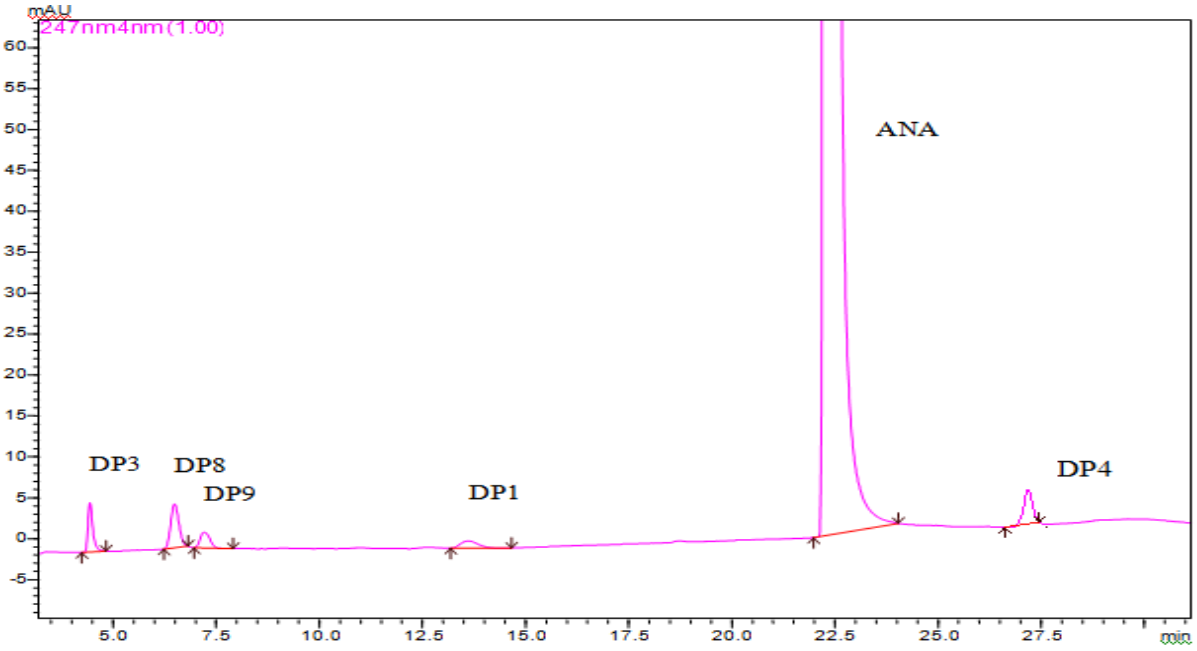


Fig. 6. 12- Chromatogram of neutral hydrolysis degradation (synthetic mixture)

6.4.2.4.5. Dry heat degradation - Slight degradation (1.45%) was observed when ANA was subjected to thermal degradation at 80ºC for 11 days with the formation of five degradation products DP10, DP3, DP9, DP4 and DP5 at retention time of 2.04, 4.07, 7.04, 9.93 and 27.4 minutes respectively. (Fig. 6.13).

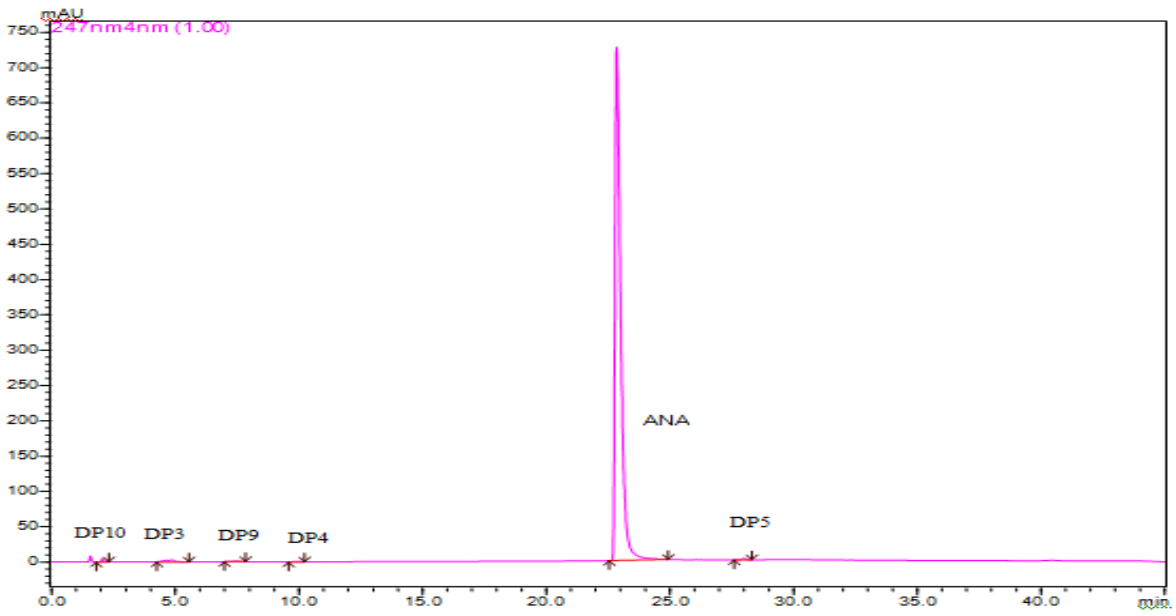


Fig. 6. 13- Chromatogram of thermal degradation (API)

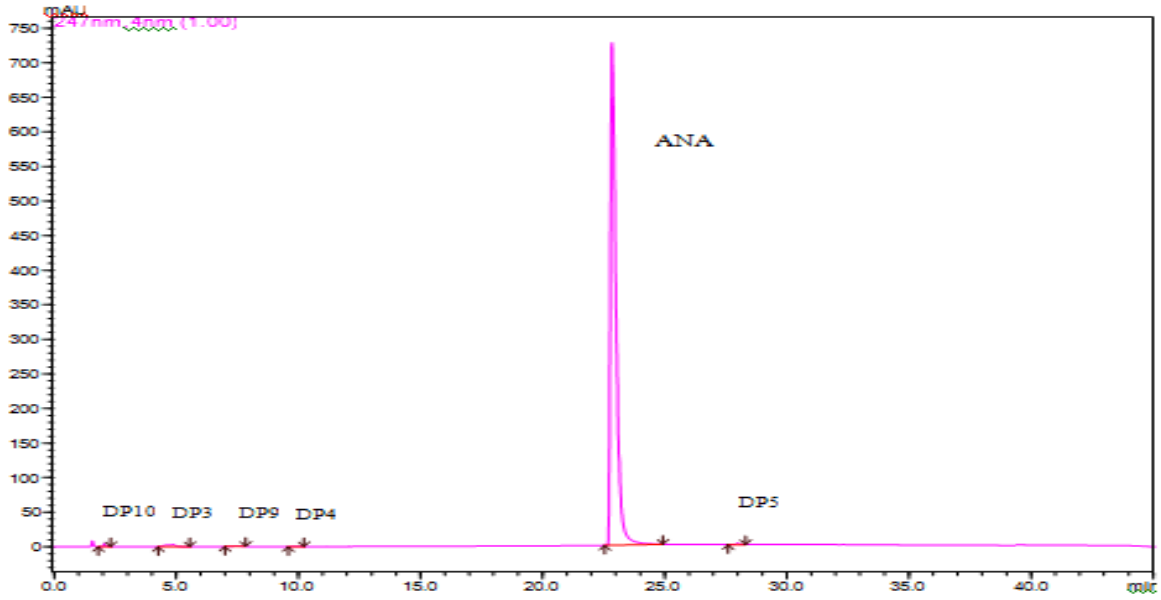


Fig. 6. 14-Chromatogram of thermal degradation (synthetic mixture)

6.4.2.4.6. Photolytic degradation - Slight degradation (0.8% and 3.78%) was observed when ANA was subjected to photolytic condition (dry and solution) for 11days (Fig. 6.15 and 6.17).

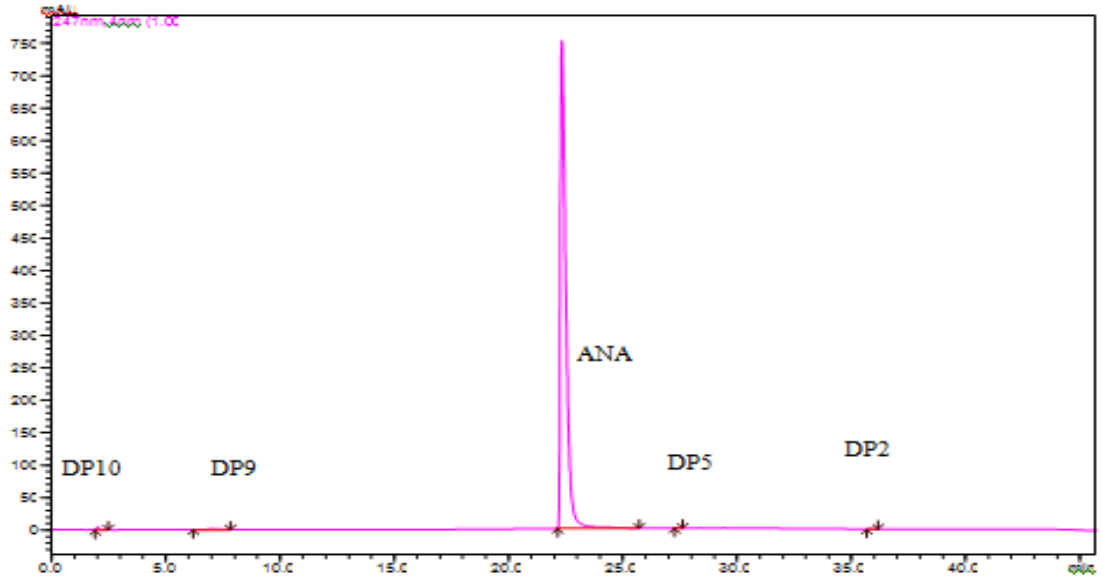


Fig. 6. 15- Chromatogram of Photolytic degradation (Dry) (API)

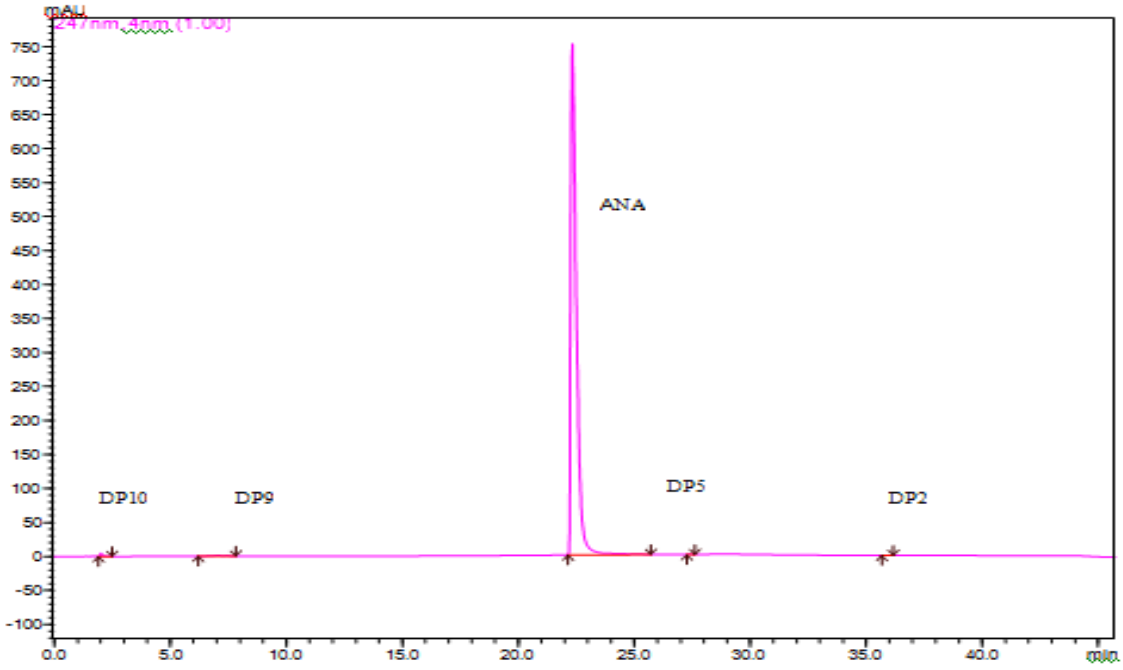


Fig. 6. 16- Chromatogram of photolytic degradation (Dry) (synthetic mixture)

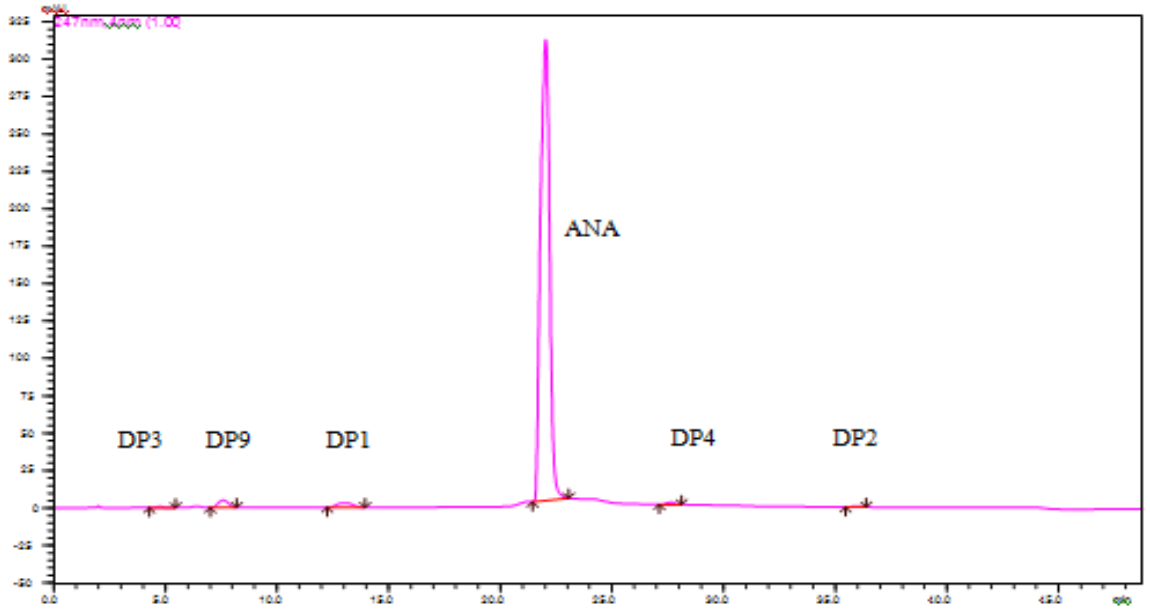


Fig. 6. 17 – Chromatogram of photolytic degradation (solution) (API)

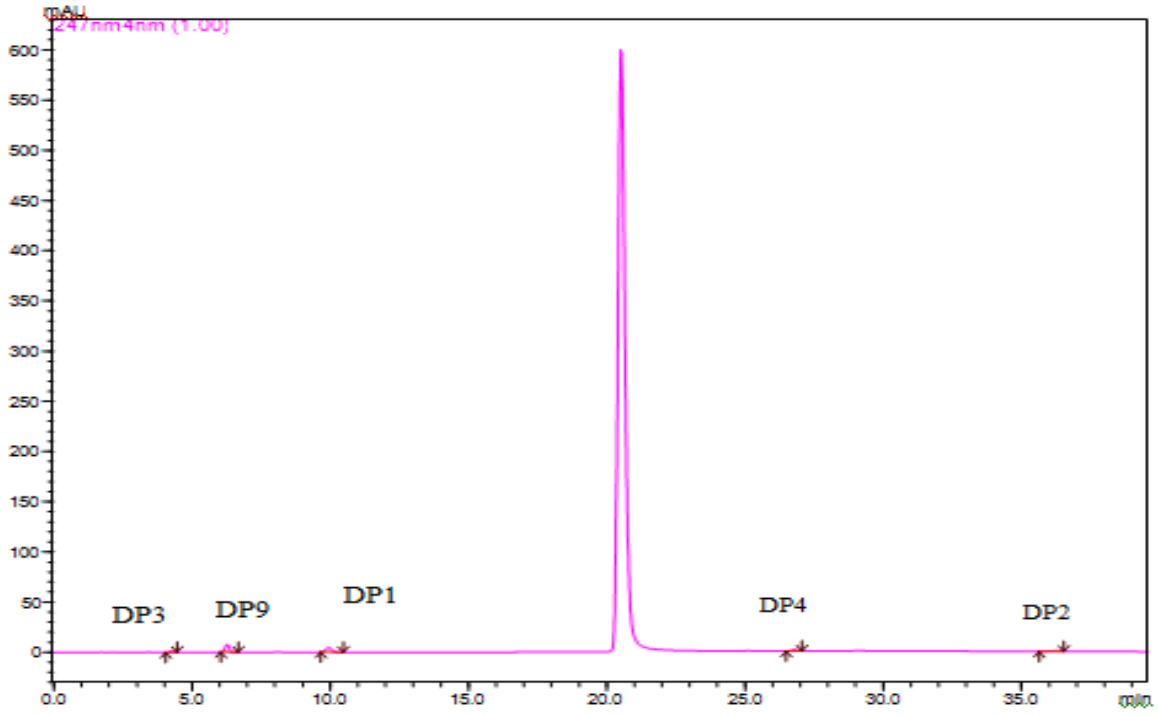


Fig. 6. 18 - Chromatogram of photolytic degradation (solution) (synthetic mixture)

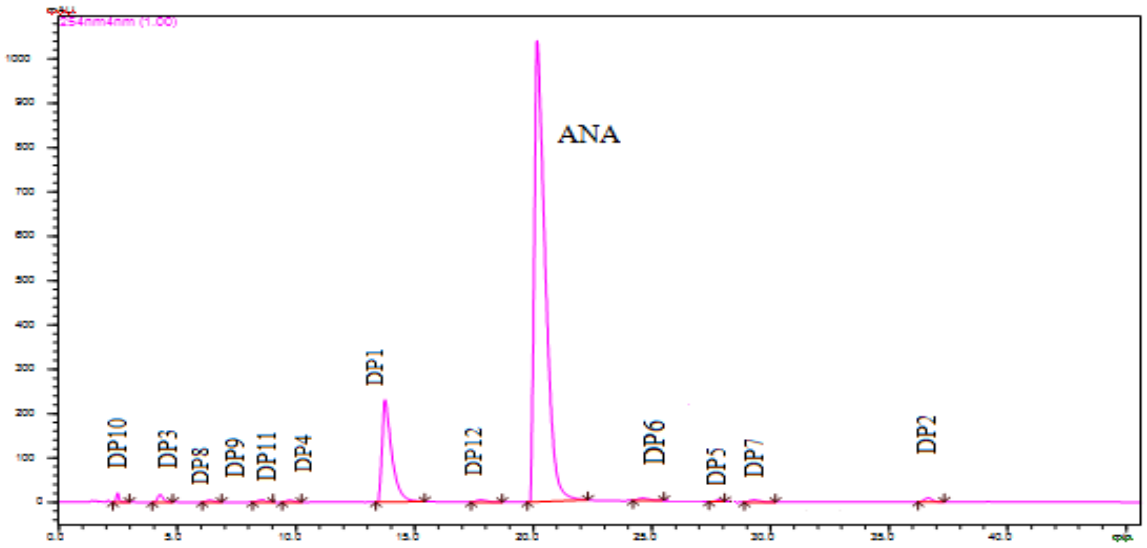


Fig. 6. 19- Chromatogram of combined degradation products of all stressors

Table 6. 12- Summary of forced degradation study of ANA

Stress Condition	Conditions	RT of Degradation Products	% of Degradation Products in API	% of Degradation products in synthetic mixture
Acid	1 M HCl 80ºC for 6 hrs	3.8 min(DP3) 6.2 min (DP8) 6.9 min (DP9) 8.9 min (DP11) 13.6 min(DP1) 18.7 min(DP12)	0.9% 0.9% 0.5% 0.4% 0.3% 0.09% **(3.1%)**	0.8% 0.8% 0.4% 0.4% 0.28% 0.08% **(2.97%)**
Alkaline	0.1 M NaOH RT for 1 hr	3.6 min(DP3) 13.6 min (DP1)	1.4% 25.09% **(26.49%)**	1.2% 23.98% **(25.18%)**
Neutral	80ºC for 6 hrs	4.09 min(DP3) 6.04 min(DP8) 7.04 min(DP 9) 13.9 min (DP1)	0.09% 0.14% 0.62% 3.2%	0.07% 0.12% 0.52% 3.0%

		27.8 min (DP4)	0.19% **(4.2 %)**	0.17% **(3.8 %)**
Oxidative	0.3% H_2O_2 RT for 2 hrs	3.59 min(DP3) 9.60 min(DP4) 13.89 min (DP1) 24.9 min (DP6) 27.9 min (DP5) 29.4 min (DP7) 36.5 min (DP2)	1.0% 1.0% 0.4% 0.15% 0.61% 2.46% 10.3% **(17.5 %)**	1.0% 0.9% 0.4% 0.15% 0.6% 2.4% 10.0% **(17.0%)**
Thermal	80˚C for 11 days	2.04 min (DP10) 4.07min (DP3) 7.04 min (DP9) 9.93 min(DP4) 27.93 min(DP5)	0.3% 0.68% 0.28% 0.02% 0.17% **(1.45%)**	0.3% 0.68% 0.3% 0.02 0.17% **(1.47%)**
Photolytic (Dry)	5382 Lux and 144UW/cm^2 for 11 days	2.0 min(DP10) 7.07 min (DP9) 27.4 min (DP5) 35.82 min (DP2)	0.2% 0.4% 0.02% 0.01% **(0.8 %)**	0.25% 0.45% 0.02% 0.01% **(1.1 %)**
Photolytic (Solution)		4.7min(DP3) 7.08 min (DP9) 13.6 min(DP1) 27.6 min(DP4) 36.5 min(DP2)	0.15% 1.0% 1.0% 0.9% 0.8% **(3.78%)**	0.1% 0.9% 0.7% 0.7% 0.8% **(3.24%)**

6.4.2.5. Applicability of the developed method for the analysis of synthetic mixture

Forced degradation study was performed on synthetic mixture. The conditions were same as mentioned for API and were analyzed in the same way as that of API. The degradation products were separated. Minor variation was observed in the degradation of API and formulation as shown in Table 6.12.

6.4.3. DISCUSSIONS

Maximum absorption wavelength of 247nm was selected as detection wavelength after scanning in the range from 200-400 nm. Various trials were taken for effective resolution of degradation products from ANA in alkaline and oxidative conditions. Degradation products were well separated from each other and from ANA using ammonium acetate buffer pH 5 : methanol and acetonitrile in gradient elution as mentioned in Table 6.2 with column oven temperature of 40ºC. Twelve different degradation products could be observed under various stress conditions.DP1 and DP3 main degradation products were observed under almost all the stress conditions but overall significant degradation was observed only under alkaline condition in alkaline (DP1) and oxidative conditions (DP2). As DP1 was 25.09% and DP2 was 10.3%, these two were considered as major degradation products and efforts were made to isolate them. The developed method can be utilized in presence of its degradation products so that ANA can be estimated in presence of its degradation products. Good correlation was obtained between peak area and concentration of ANA in the range of 10-120 µg/mL with regression coefficient r^2 0.998. % RSD for intra-day and inter-day precision was less than 2%. % Recovery was found to be in the range of 98.7-100.57%. % RSD for robustness studies was less than 2%.

6.5. SECTION - B

DEGRADATION KINETIC STUDY OF ANAGLIPTIN BY HPLC METHOD

The degradation kinetics was studied only for alkaline and oxidative degradation of ANA.

6.5.1. EXPERIMENTAL

6.5.1.1. Chemicals and Reagents

The chemicals and reagents used in the present section were same as those mentioned in section 6.4.1.1.

6.5.1.2. Equipments and Chromatographic Conditions

Equipments and chromatographic conditions were same as those mentioned in section 6.4.1.2.

6.5.1.3. Preparation of stock, sample and buffer solutions

Stock solution was same as those mentioned in section 6.4.1.5.

Alkaline degradation kinetics study - To the 1 ml of stock solution of in ANA in 10 mL of volumetric flask. , 1 mL of 0.05 /0.1/0.5 M sodium hydroxide was added. The solution were kept at RT (40º)/ 50˚C/60ºC from 30 minutes to 5 hrs. The solution was made up to volume with mobile phase to make the concentration 100µg/mL and injected in to the HPLC system.

Oxidative degradation kinetics study - To the 1 mL of stock solution of in ANA in 10 mL of volumetric flask. , 1 mL of 0.1%/0.3%/0.9% hydrogen peroxide was added. The solution were kept at RT (40ºC) from 30 minutes to 5 hrs. The solution was made up to volume with mobile phase to make the concentration 100µg/mL and injected in to the HPLC system.

6.5.2. RESULTS

6.5.2.1. Alkaline degradation kinetics study

Degradation rate kinetics was studied by % of drug remaining after degradation versus time (for zero order kinetics) using linear regression analysis, Log of % drug remaining after degradation (for first order process). Experiments were performed in triplicate and average values were taken for analysis. The rate constant (K), half-life ($t_{1/2}$) and activation energy (Ea) were calculated from slope of line at each temperature for alkaline degradation.

A regular decrease in concentration of ANA was observed with increasing time intervals and with increase in temperature. Regression equation and regression coefficient was obtained for zero order and first order kinetics for different concentration of sodium hydroxide and at different temperatures. On the basis of regression, degradation follows first-order kinetics since r^2 values are highest (close to 1) (Table 6.13).

On the basis of first-order kinetics, further study was performed to study the effect of temperature on the rate constant the Arrhenius plots were plotted (log of rate constant versus reciprocal of temperature). Arrhenius equation as

$$\log K = \log A - Ea/2.303\ RT$$

where K is the rate constant, A is the frequency factor, Ea is the activation energy, R is the gas constant(1.987 cal/deg/mol) and T is the absolute temperature. Arrhenius plot was obtained by plotting lnK versus 1/T. Graph was linear in the given temperature range. The first order kinetic plot and Arrhenius plot for alkaline degradation are shown in Fig 6.20-6.25. The values of degradation rate constant, half-life and activation energy are shown in Table 6.14.

Table 6. 13 - r^2 value and Regression Equation for zero order, first order reaction for alkaline degradation

S.No.	Conc. NaOH	Temp	r^2		Regression equation	
			Zero order	First Order	Zero order	First Order
1	0.05 M	40ºC	0.9783	0.9837	y=-7.00x+100.41	y= -0.03 x+1.99
		50˚C	0.8959	0.9478	y=-9.62x+75.54	y=-0.08x+1.89
		60˚C	0.8165	0.9765	y=-11.63x+51.44	y=-0.31x+1.89
2	0.1M	40ºC	0.9206	0.9511	y=-11.96x+79.59	y=-0.07x+1.82
		50˚C	0.9471	0.9957	y=-8.09x+43.61	y=-0.18x+1.76
		60˚C	0.6627	0.9903	y=-2.54x+10.30	y=-0.50x+1.69
3	0.5 M	40ºC	0.902	0.9848	y=-3.72x+19.33	y=-0.13x+1.67
		50˚C	0.8878	0.9826	y=-2.11x+10.66	y=-0.21x+1.14
		60˚C	0.6778	0.8355	y=-0.50x+2.044	y=-0.61x+0.55

Table 6. 14 - Degradation rate constant, half-life and Activation Energy Ea for first order kinetic of alkaline degradation

S.No.	Conc. NaOH	Temp	K	$t_{1/2}$ (hrs)	Activation energy
1	0.05 M	40ºC	0.06	9.93	83.9 KJ
		50˚C	0.18	3.76	
		60˚C	0.72	0.95	
2	0.1 M	40ºC	0.17	4.07	
		50˚C	0.43	1.60	
		60˚C	1.15	0.59	
3	0.5 M	40ºC	0.30	2.24	
		50˚C	0.48	1.43	
		60˚C	1.40	0.49	

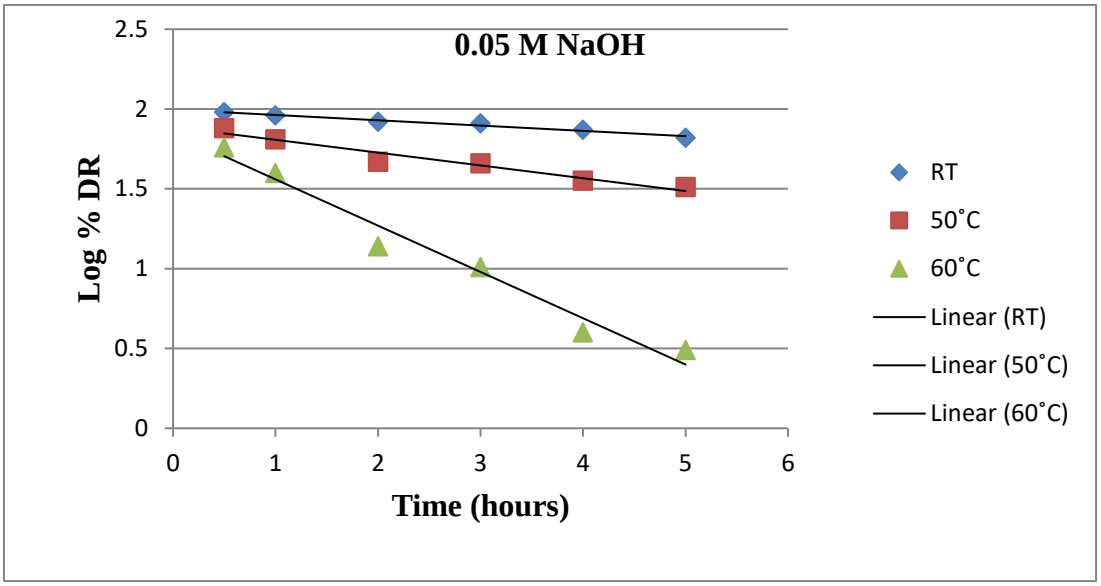


Fig. 6. 20 – First order reaction kinetics of 0.05 M NaOH

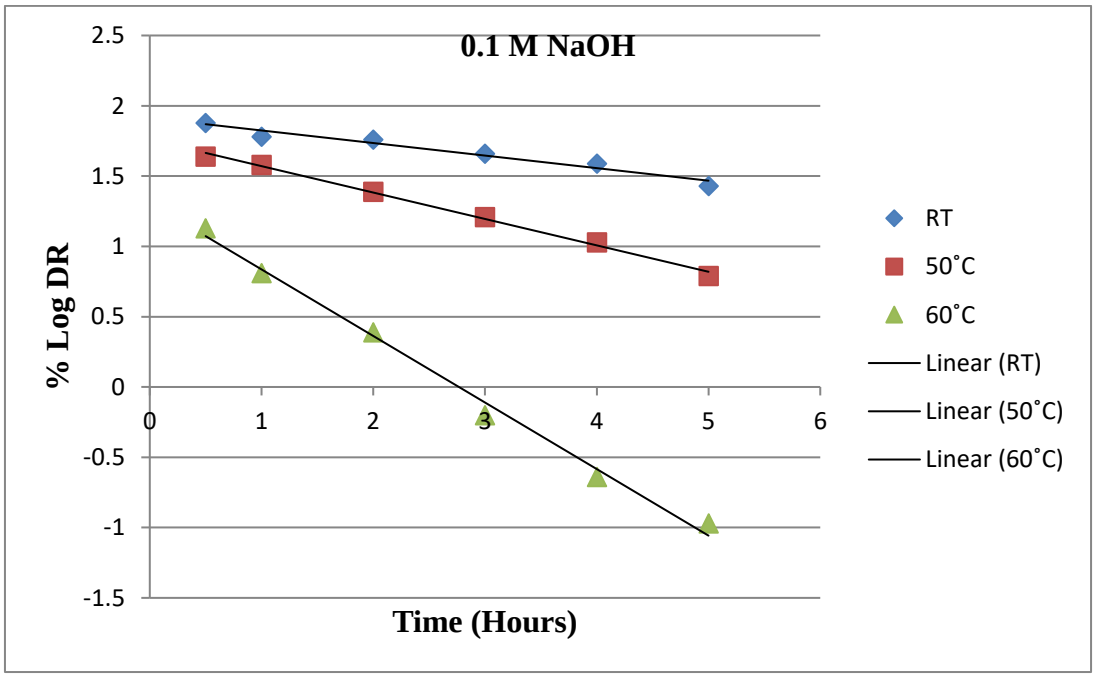


Fig. 6. 21– First order kinetics of 0.1 M NaOH

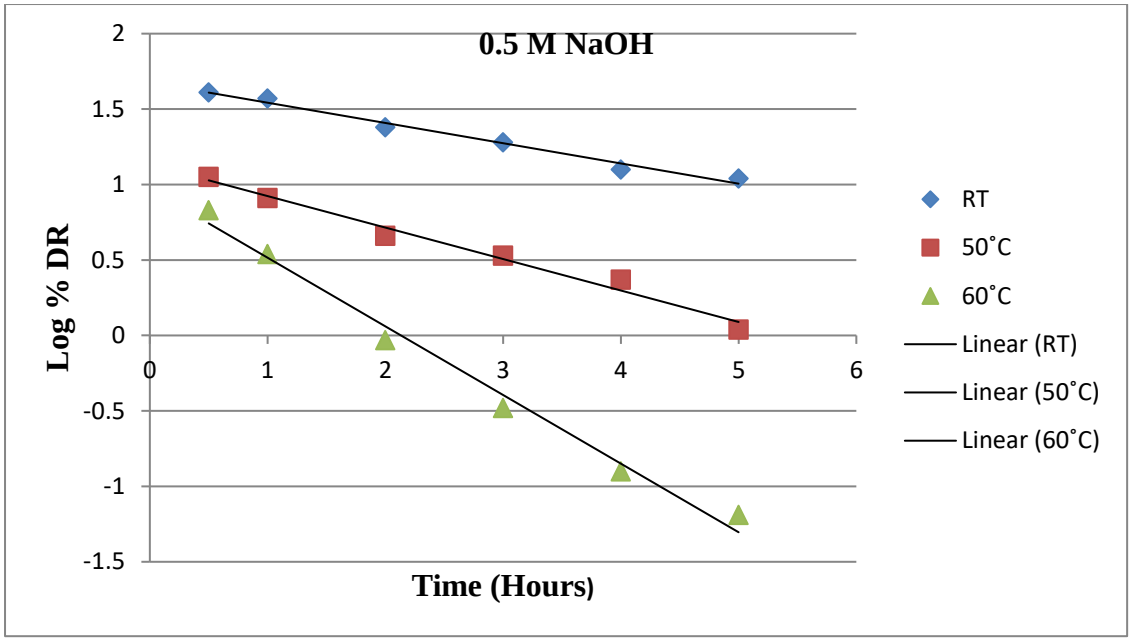


Fig. 6. 22– First order kinetics of 0.5 M NaOH

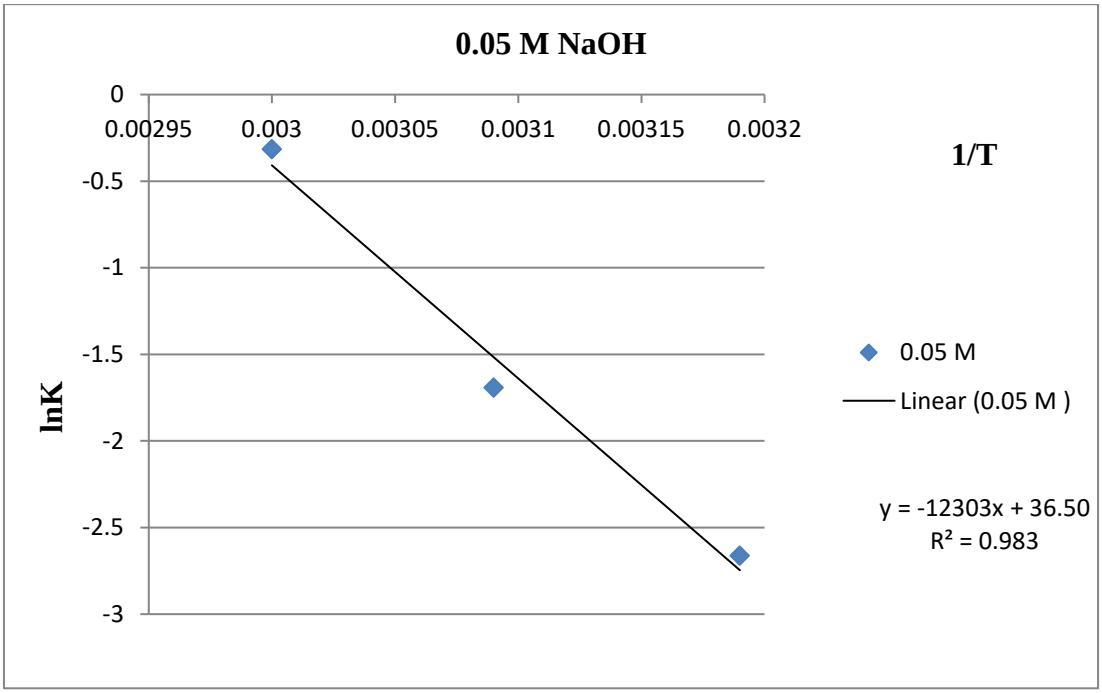


Fig. 6. 23 – Activation energy plot for 0.05 M NaOH

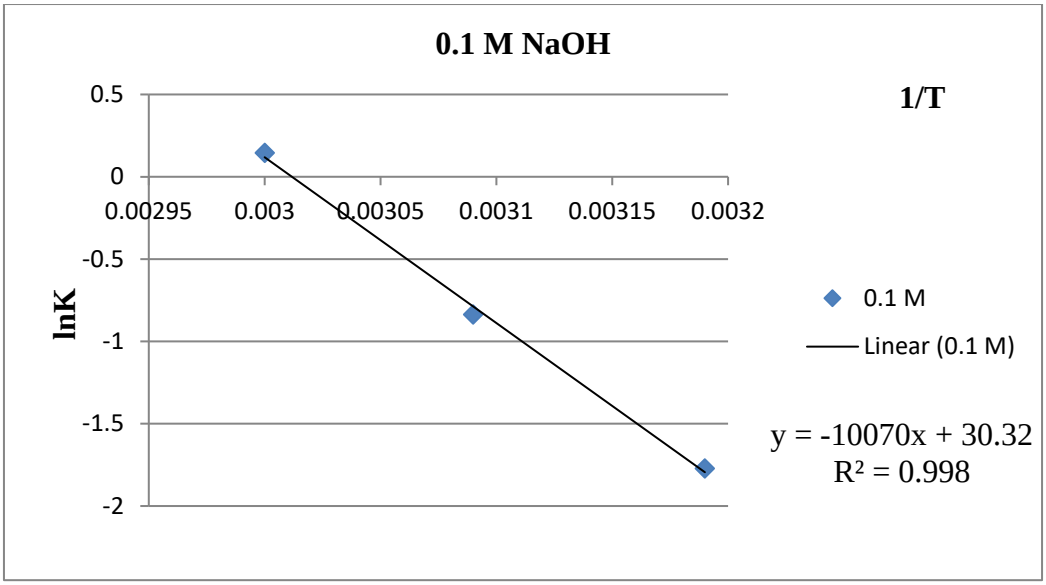


Fig. 6. 24 - Activation energy plot for 0.1 M NaOH

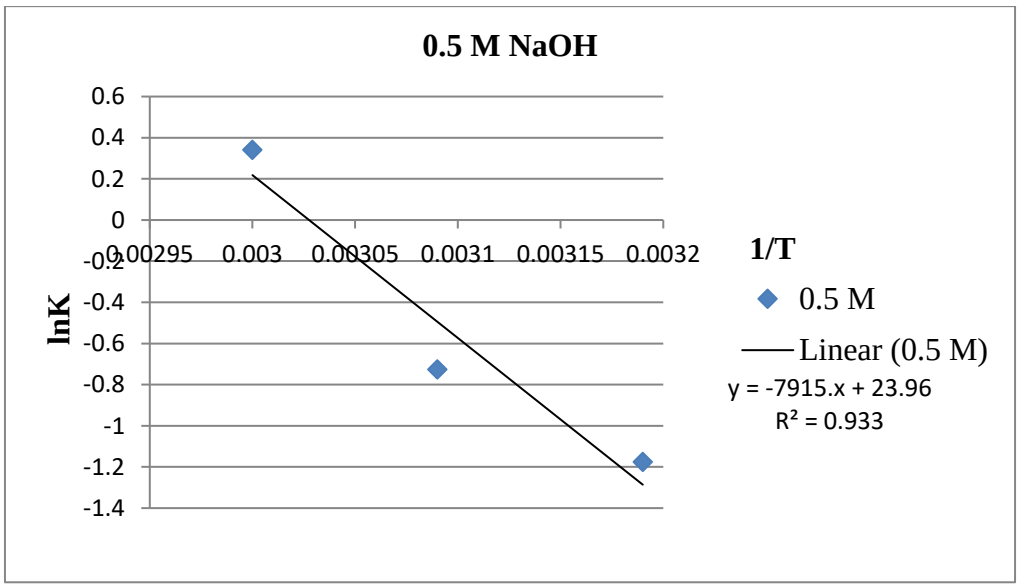


Fig. 6. 25 - Activation energy plot for 0.5 M NaOH

6.5.2.2. Oxidative degradation kinetics study

In oxidative kinetics study, order of reaction was estimated by plotting rate of reaction with the concentration of drug remaining. Rate of reaction was linear with concentration of drug remaining. Reaction followed first order kinetics. Degradation rate constant and half-life was calculated (Table 6.15). Plot of rate of reaction versus concentration of remaining drug for respective concentration of hydrogen peroxide is shown in Fig. 6.26-6.28.

Table 6. 15 – Rate constant and half-lives of first order kinetics of oxidative degradation

Conc. of H_2O_2	Temperature	Rate constant K	$t_{1/2}$ hours
0.1%	RT (40°C)	0.009	75.2
0.3%		0.039	17.7
0.9%		0.115	6.02

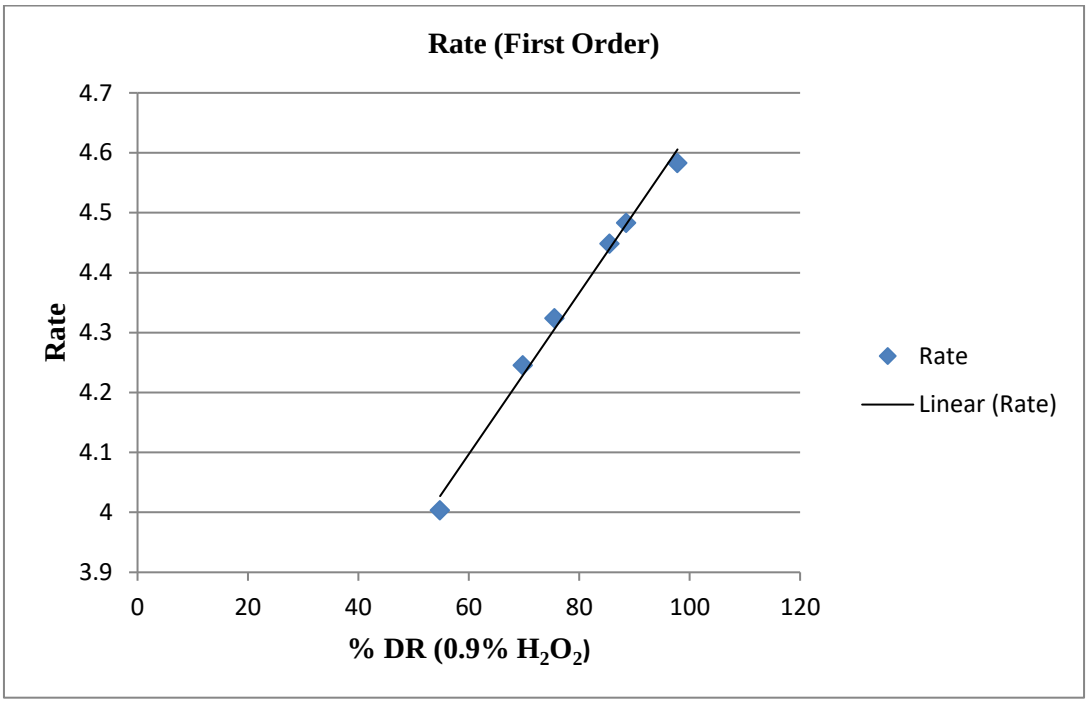


Fig. 6. 26 – First order kinetics of 0.9% hydrogen peroxide

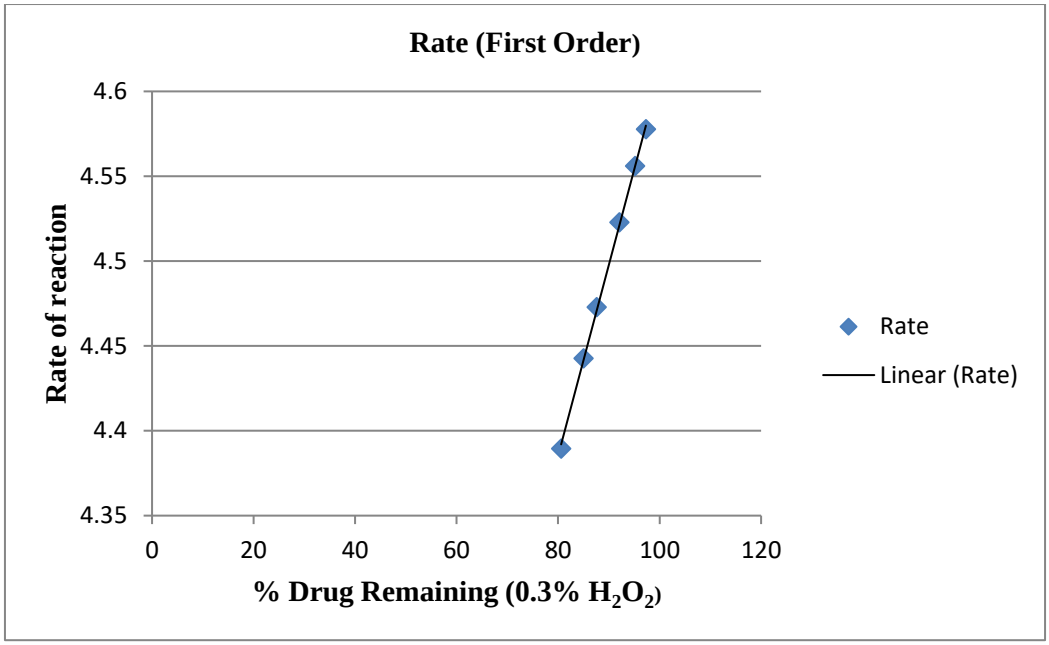


Fig. 6. 27 - First order kinetics of 0.3% hydrogen peroxide

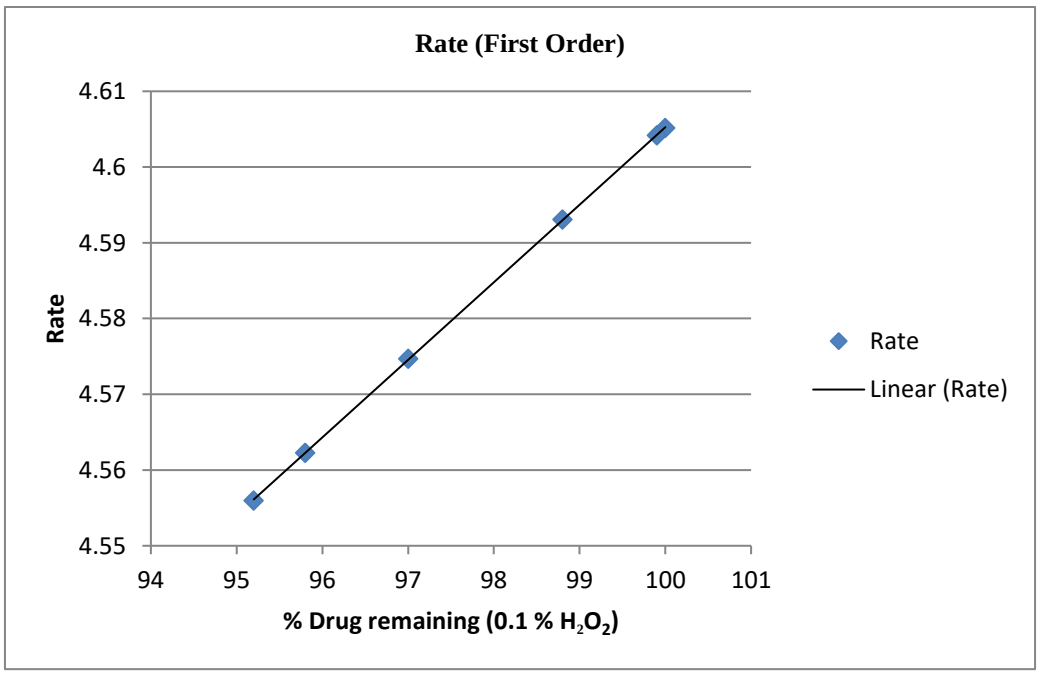


Fig. 6. 28 - First order kinetics of 0.1% hydrogen peroxide

6.5.3. DISCUSSIONS

Degradation kinetics was performed for alkaline and oxidative conditions.

For alkaline kinetics, factors taken for kinetics study were: concentration of sodium hydroxide (0.05 M, 0.1 M and 0.5 M), temperature (40º, 50ºand 60º C) and time (30 min to 5 hrs). Zero order kinetics study was performed by plotting graph between % drug remaining versus time and first order by plotting graph between log % Drug remaining versus time. Regression equation and regression coefficient were obtained for both zero and first order kinetics. Degradation follows first-order kinetics since regression coefficient r^2 *was* highest in first –order kinetics. Based on this degradation rate constant and half-life was calculated. On the basis of degradation rate constant by plotting lnk (rate constant) versus 1/T, activation energy was calculated which was found to be 83.9 KJ/mole.

For oxidative kinetics, factors taken for kinetics study were: concentration of hydrogen peroxide (0.1%, 0.3% and 0.9%), time (30 min to 5 hrs) at room temperature (40ºC). Zero order and first order were performed by plotting graph between rate of reaction versus % Drug remaining. Oxidative degradation followed first order kinetics since rate of reaction was linear with % drug remaining. Degradation rate constant was 0.009-0.115 and half –life were 75.2 -6.02 hrs respectively.

6.6. SECTION - C

ISOLATION AND CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS OF ANAGLIPTIN

6.6.1. EXPERIMENTAL

6.6.1.1. Chemicals and Reagents

Chemicals and reagents used in the present section are same as those mentioned in 6.4.1.1.

6.6.1.2. Equipments and chromatographic conditions

Preparative HPLC system composed of Shimadzu LC-20 AP pump and SPD 20A detector.

Separation was performed on Daisogel-SP-100-10-ODS-P (250X 20 mm i.d., 10µ particle size). Detection was performed at 247 nm. Flow rate was maintained at 60 mL/min. Sample was injected though Rheodyne 7725 injector valve. Data acquisition and integration was processed with Class VP software. Composition of mobile phase was same as mentioned in section 6.4.1.2.

Major degradation products were DP1 and DP2 obtained in alkaline hydrolysis and in oxidative condition respectively.

6.6.1.3. Enrichment of alkaline degradation sample (DP1)

500 mg of ANA was weighed accurately and transferred to 25 mL of volumetric flask. To this was added 10 mL of water and acetonitrile followed by 2 mL of 0.1 M NaOH. The solution for degradation was kept at room temperature for 24 hrs.

6.6.1.4. Enrichment of oxidative degradation sample (DP2)

500 mg of ANA was weighed accurately and transferred to 25 mL of volumetric flask. To this was added 10 mL of water and acetonitrile followed by 2 mL of 0.3% hydrogen peroxide. The solution for degradation was kept at room temperature for 24 hrs.

6.6.1.5. Analysis of degradation samples by analytical HPLC

The degradation samples in alkaline and oxidative conditions were diluted to respective concentration and were analysed as mentioned in section 6.4.1.5.2.and 6.4.1.5.3. In alkaline condition, DP1 is formed with complete degradation (Fig. 6.29) while in oxidative condition DP2 is formed with 33 % area by normalization (Fig. 6.30).

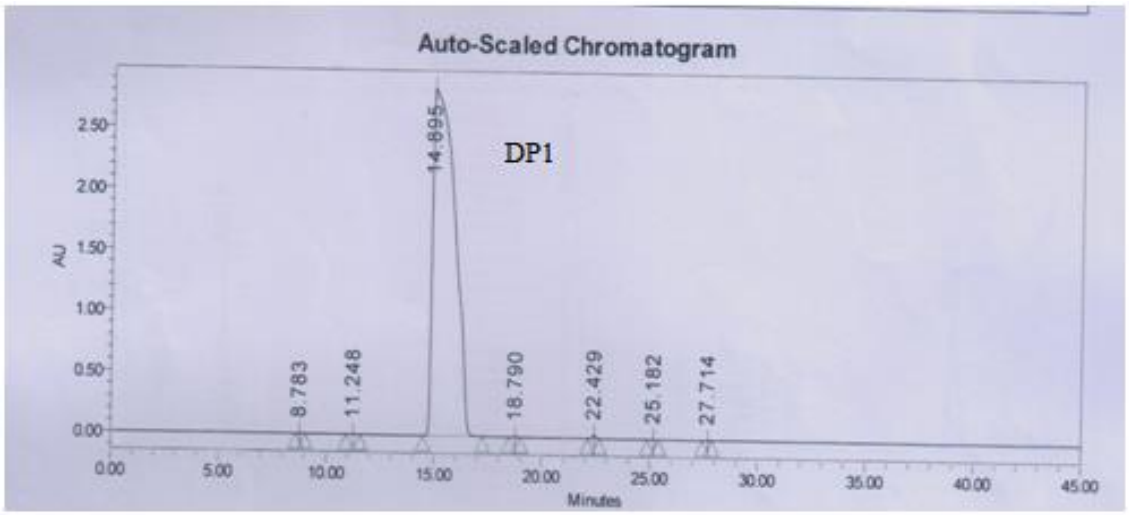


Fig. 6. 29- Chromatogram of isolated degradation products DP1 in alkaline condition

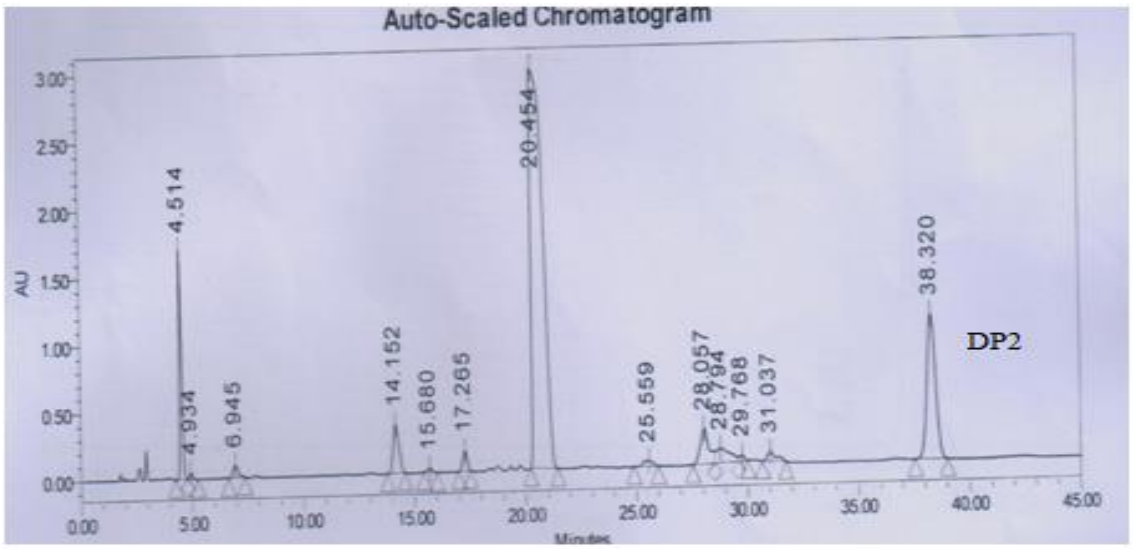


Fig. 6. 30- Chromatogram of oxidative condition

6.6. 1.6. Isolation of degradation products by preparative HPLC

DP1 and DP2 were purified by preparative HPLC. Fraction of DP1 and DP2 greater than 95% were collected together. Acetonitrile was removed by concentrating the solution on rota vapour. To confirm the retention time and purity of isolated fraction, it was analysed by analytical HPLC as mentioned in section 6.4.1.5.2.and 6.4.1.5.3. The solutions were kept in lyophilizer overnight. DP1 and DP2 were obtained as colorless solids. DP1 was obtained as 99.7 % purity and DP2 was obtained with 99.1 % purity (Fig. 6.31).

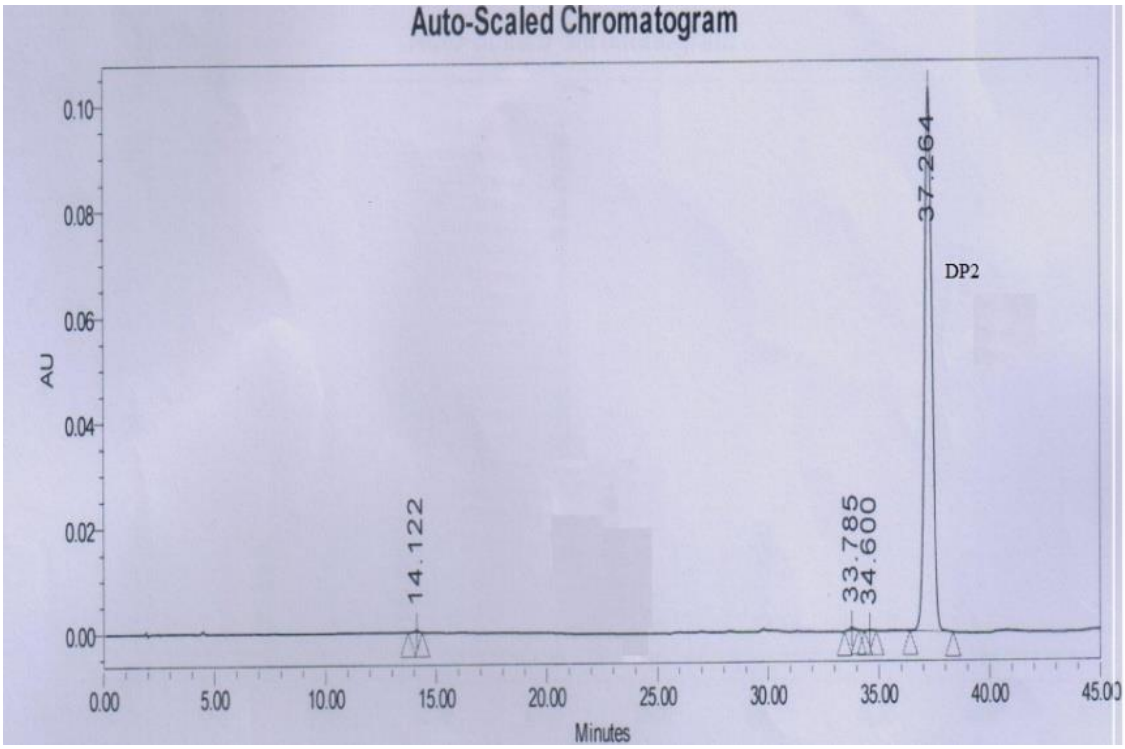


Fig. 6. 31 – Chromatogram of isolated DP2

6.6.2. RESULTS

6.6.2. 1. Structural characterization of ANA and degradation products

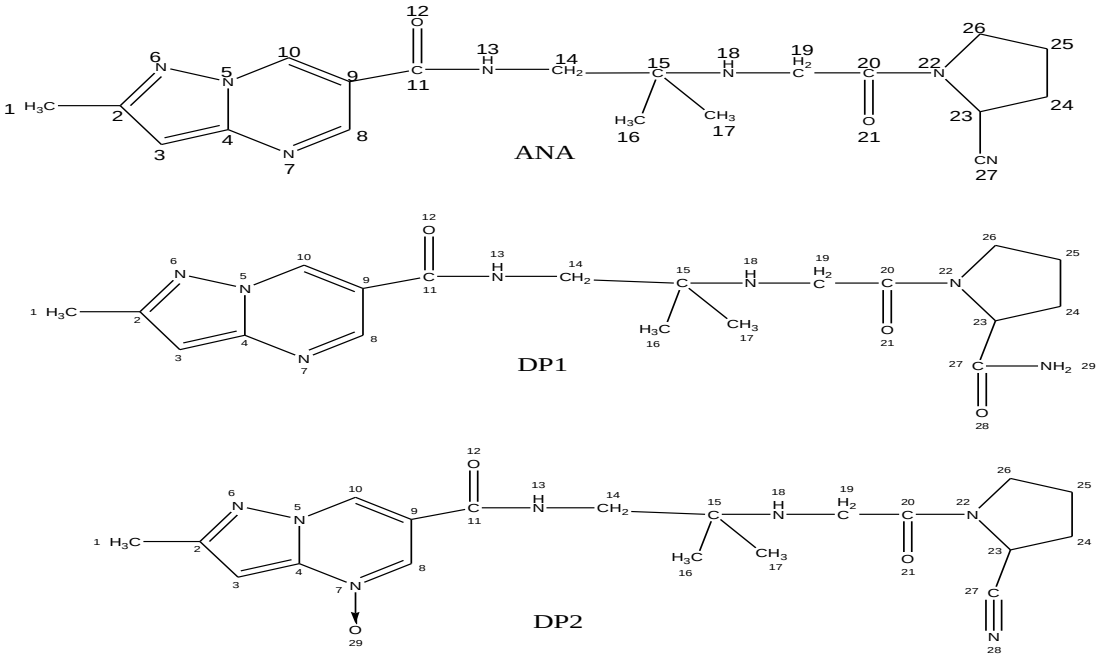


Fig. 6. 32 – Structure of ANA, DP1, DP2 with assigned numbers

6.6.2.1.1. Spectral data of ANA

Mass spectra

An ESI-MS/MS spectrum of ANA is provided in Fig 6.33. An ESI-MS spectrum of ANA shows protonated m/z 384. ANA shows product ions at m/z value of 231 with the loss of 1-(2-methylamino)acetylpyrrolidine-2-carbonitrile. Further fragmentation takes place at amide bond produces m/z value of 160 which is indicated by loss of isopropyl methyl amino cation (Fig. 6.34)

NMR spectra

^{1}H NMR spectra of ANA indicates presence of methyl group (position 1) at 2.50 ppm and two methyl groups (16, 17 position) at 1.05 ppm. Presence of two methylene groups at position 14 and 19 is indicated at 3.27 and 3.45 ppm. Methylene groups of pyrrolidine ring are present at 2.05, 2.14, 3.49, 3.63 ppm. Protons of pyrimidine ring are indicated at 9.43, 8.85 ppm. Amide proton is indicated at 8.45 ppm and secondary amine is indicated at 18 position is indicated at 3.34 ppm which are absent in D_2O exchange. ^{13}C NMR spectra of ANA show the presence of nitrile group at 114.9 ppm

and amide group at 162.95 ppm. Methyl groups in ^{13}C NMR are indicated as upwards peaks and methylene groups as downward peaks (Table 6.16).

IR spectra

IR spectra of ANA indicates presence of secondary amine at 3339 cm^{-1}, secondary amide –NH at 3281 cm^{-1}. Methyl stretching is present at 2940, 2926 cm^{-1}, methylene stretching at 2871, 2823 cm^{-1}. Presence carbonyl group is indicated at 1663 and 1611 cm^{-1}.Presence of nitrile group is indicated at 2249 cm^{-1} (Fig. 6.38), (Table 6.17).

6.6.2.1.2. Characterisation of DP1

Mass spectra

HR-MS spectra of DP1 indicates protonated 402.2105 which corresponds to molecular formula $C_{19}H_{27}N_7O_3$. An ESI-MS/MS spectrum of DP1 is provided in Fig. 6.39. An ESI-MS/MS spectrum of DP1 shows protonated m/z 402 which is 18 m/z more than ANA. ESI-MS/MS shows product ions at m/z values of 384, 270 and 242 which are formed by the loss of methyl group, pyrazolopyrimidine group and carbonyl group. Fragmentation pathway of DP1 is shown in Fig. 6.40.

NMR spectra

^{1}H NMR and ^{13}C NMR spectra of DP1 are compared with ANA. ^{1}H NMR spectra of DP1 shows additional two protons at position 29. Presence of these two protons are indicated at 7.3. and 6.9 ppm. These protons are absent in D_2O exchange which indicates the formation of $–NH_2$. In ^{13}C NMR spectra, nitrile peak which was present at 114.9 ppm in ANA is absent in DP1. There is formation of new peak at 173.76 ppm which indicates the presence of amide (Table 6.18).

IR spectra

In IR spectra of DP1, peak of nitrile group at 2249 cm^{-1} is disappeared. There is formation of peak at 1690cm^{-1} which indicates the presence of amide (Fig. 6.45 and Table 6.17).

On the spectral analysis by mass, NMR and IR it indicated that alkaline hydrolysis has taken place at nitrile group in ANA and nitrile group is converted to amide which has 18 m/z more than ANA. Formation of amide group is confirmed in ^{13}C

NMR spectra. Presence of two protons of amide is confirmed by ^1H NMR and D_2O exchange. On the basis of all above, DP1 is characterized as 1-[2-[1-(2-methylpyrazolo [1, 5-alpha] pyrimidine-6-carboxamido}-methyla-proan-2-yl-amino) acetyl) pyrollidine-2-carboxamide.

Mechanism of formation of DP1

DP1 is formed in alkaline condition. The mechanism of formation of DP1 is shown in Fig.6.46. ANA contains nitrile functional group. There is nucleophilic attack of hydroxide ion to nitrile group present in ANA as a result triple bond in nitrile is broken and there is formation of intermediate , this intermediate takes proton from water with the formation of imine which tautomerises to amide resulting in formation of DP1.

6.6.2.1.3. Characterisation of DP2

Mass spectra

HR-MS spectra of DP2 indicates molecular peak at m/z 398 which corresponds to molecular formula $C_{19}H_{25}N_7O_3$.DP2 is 16 m/z more than ANA. Mass spectrum of DP2 shows m/z of 398 which on further fragmentation gives at m/z 231 (Fig. 6.47 and Fig. 6.48).

NMR spectra

In NMR spectra of DP2, the number of protons and number of carbon are same as that of ANA (Table 6.19).

IR spectra

IR (Fig. 6.53) spectra of DP2 indicated formation of N-oxide at 1537cm^{-1} (Table 6.17).

On the basis of mass spectral analysis, it indicates that DP2 has 16 m/z more than ANA which indicates that there is formation of N-oxide. Position of N-oxide is confirmed by mass fragmentation pathway of DP2 which indicates that formation of N-oxide has taken place at pyrimidine ring. DP2 is characterized as N-[2-({2-[(2S)-2-cyanopyrrolidin-1-yl]-2-oxoethyl} amino)-2-methylpropyl]-2-methylpyrazolo-N-oxido-[1, 5-a] pyrimidine-6-carboxamide.

Mechanism of formation of DP2

DP2 is formed in oxidative condition. The mechanism of formation of DP2 is shown in Fig.6.54. Hydrogen peroxide gets broken to hydroxide ion. There is attack of hydroxide ion on pyrimidine nucleus of ANA which results in formation of N-hydroxide, from which proton is lost and there is formation of N-oxide of ANA, DP2.

Table 6. 16 – NMR assignments of ANA

ANA					
Position	**^{1}H (Fig.6.35)**	**Chemical Shift (ppm)**	**Position**	**^{13} C (Fig. 6.36)**	**DEPT (Fig. 6.37)**
3, 8, 10	1H	6.60, t, 8.85,d, 9.43,d	11	170.76	amide
13	1H	8.45,t, -NH, absent in D_2O exchange	20	162.95	amide
23,24, 25, 26	1H	4.75,m, 2.05,m , 2.14,m ,3.63,3.49,m	2, 4	148.69, 156.63	Quaternary carbon
19	2H	3.45,3.41,t	3	96.10	-CH-
18	1H	3.34,t, -NH absent in D_2O exchange	8,10	148.51, 135.09	-CH-
14	2H	3.28,3.27,d	9	148.69	Quaternary carbon
1	3H	2.50,d	27	114.9	-CN
16, 17	6H	1.05,s	15	53.17	Quaternary carbon
			14	46.93	$-CH_2-$
			23, 24,25, 26	46.13, 29.43,	-CH-, $-CH_2-$, -

		24.69, 45.18	CH_2-, -CH_2-
	19	44.30	-CH_2-
	16, 17	25.11	-CH_3- (two)
	1	14.35	-CH_3

Table 6. 17- I.R. spectral assignments of ANA, DP1 and DP2

ANA		**DP1**		**DP2**	
Wave number(cm-$^{1)}$	Assignments	Wave number (cm^{-1})	Assignments	Wave number (cm^{-1})	Assignments
3339	-NH	3260	Amide and amine merged	3339	Amide and amine merged
3281	Secondary amide	2974	CH_3 stretching	2974	CH_3 stretching
2926	CH_3 stretching	2966	CH_3 stretching	2875	CH_2 stretching
2940	CH_3 stretching	2873	CH_2 stretching	2242	Nitrile
2871	CH_2 stretching	2844	CH_2 stretching	1641	C=O secondary amide
2823	CH_2 stretching	1614	C=O secondary amide	1614	C=O tertiary amide
2249	Nitrile	1196	C-N Stretching amine	1537	N-oxide
1663	C=O secondary amide			1186	C-N Stretching amine

1611	C=O tertiary amide	
1196	C-N Stretching amine	

Table 6. 18- NMR assignments of DP1

DP1					
Position	**^{1}H (Fig.6.41)**	**Chemical Shift(ppm)**	**Position**	**^{13}C (Fig. 6.42)**	**DEPT (Fig. 6.43)**
1	3H	2.56,d	29	173.76	amide
3, 8, 10	3H	6.65,s, 8.90,d, 9.45,d	20	162.87	amide
13	1H	8.57,t.-NH absent in D_2O exchange	11	170.63	amide
29	2H	7.3,d,6.9,s	2,4	148.69, 156.66	Quaternary carbon
23,24,25, 26	7H	4.41,m, 2.04,3.35,m, 1.91,3.35m, 3.53,m	3	96.12	-CH-
19	2H	3.49,m	8, 10	148.44, 135.06	-CH-
14	2H	3.35,m	9	148.69	Quaternary carbon
18	1H	3.35,-NH,absent in	23, 24, 25, 26	59.60, 29.21, 24.05, 45.61	-CH-, -CH_2-, -CH_2-, -CH_2-

		D_2O exchange			
16	3H	1.11,s	15	53.25	Quaternary carbon
17	3H	1.06,s	14	46.82	$-CH_2-$
			19	44.05	$-CH_2-$
			16, 17	25.10	$-CH_3-$
			1	14.34	$-CH_3$

Table 6. 19 – NMR assignments of DP2

DP2					
Position	**^{1}H (Fig. 6.49)**	**Chemical Shift (ppm)**	**Position**	**^{13}C (Fig. 6.50)**	**DEPT (Fig. 6.51)**
1	3H	2.56,d	11	168.65	Amide
3, 8,10	3H	6.69,d, 8.906, 9.46,d	20	163.25	Amide
13	1H	8.46,t	27	114.7	-CN
23, 24, 25 , 26	7H	4.76,m, 1.92,m, 2.14,m, 3.78,7.8,m	2, 4	148.72, 156.90	Quaternary carbon
19	2H	3.67,m	3	93.30	-CH-
18	1H	3.55,d,-NH,absent in D_2O exchange	8, 10	148.16, 135.07	-CH-
14	2H	3.43,m	9	148.30	Quaternary

					carbon
16, 17	6H	1.10,s	15	61.63	Quaternary carbon
			14	55.60	$-CH_2-$
			16, 17	46.32	$-CH_3-$
			19	46.42	$-CH_2-$
			23, 24,25,26	43.06, 31.6, 29.32, 46	-CH-, $-CH_2-$, $-CH_2-$, $-CH_2-$
			1	14.37	$-CH_3$

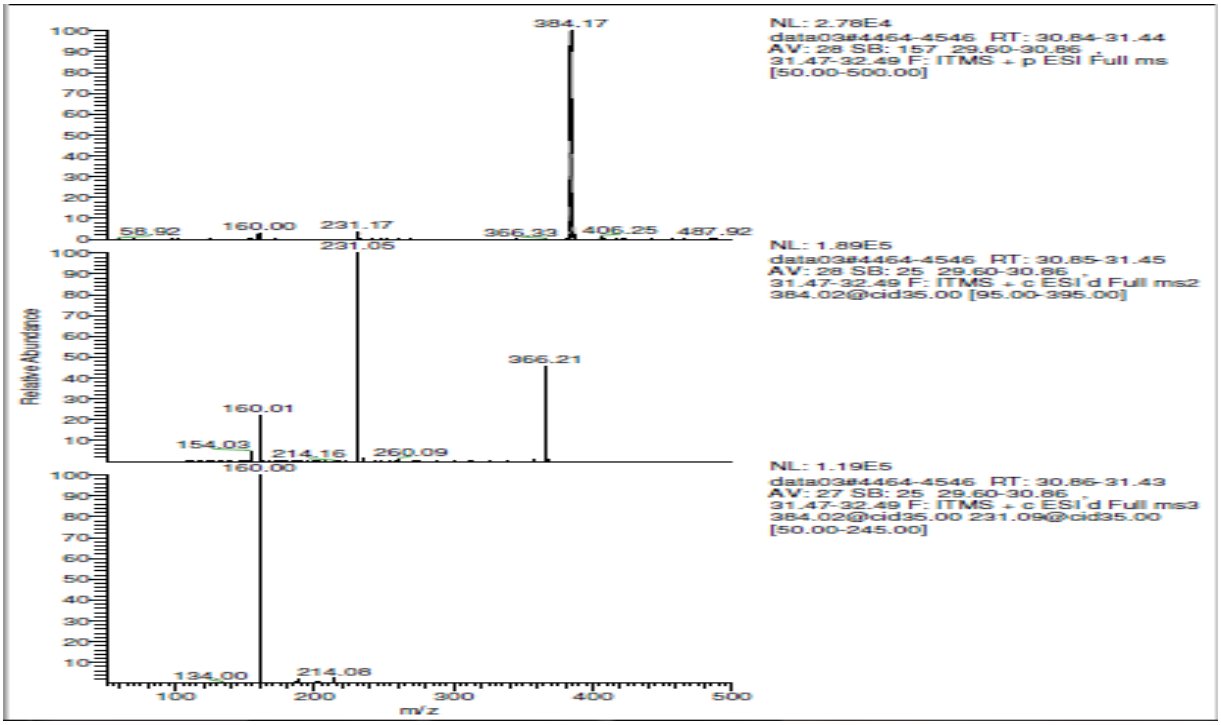


Fig. 6. 33- ESI-MS/MS spectrum of ANA

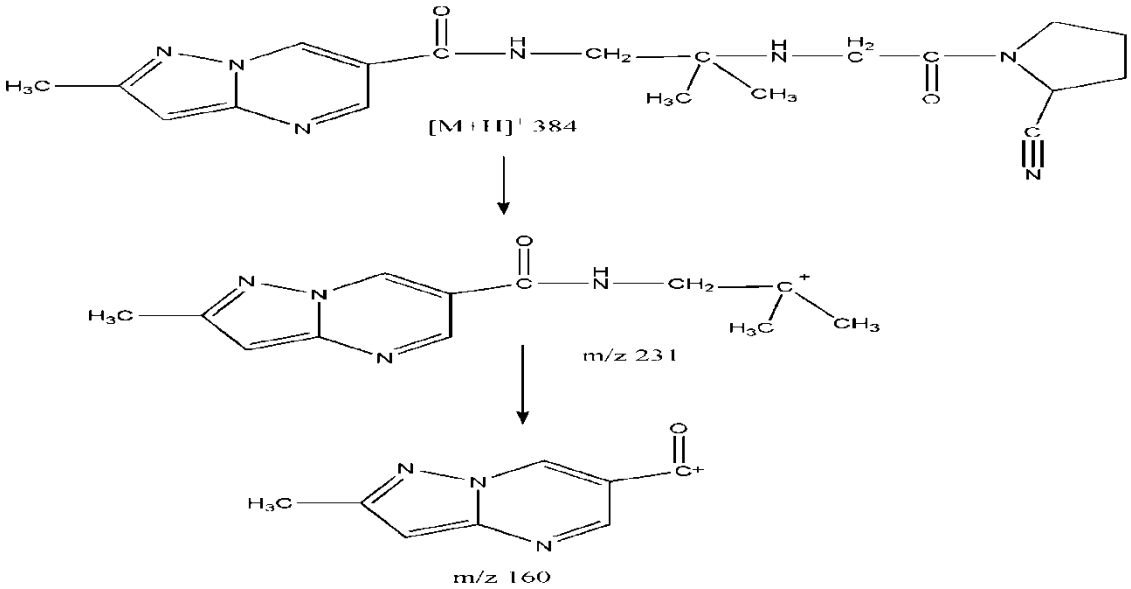


Fig. 6. 34- Fragmentation pathway of ANA

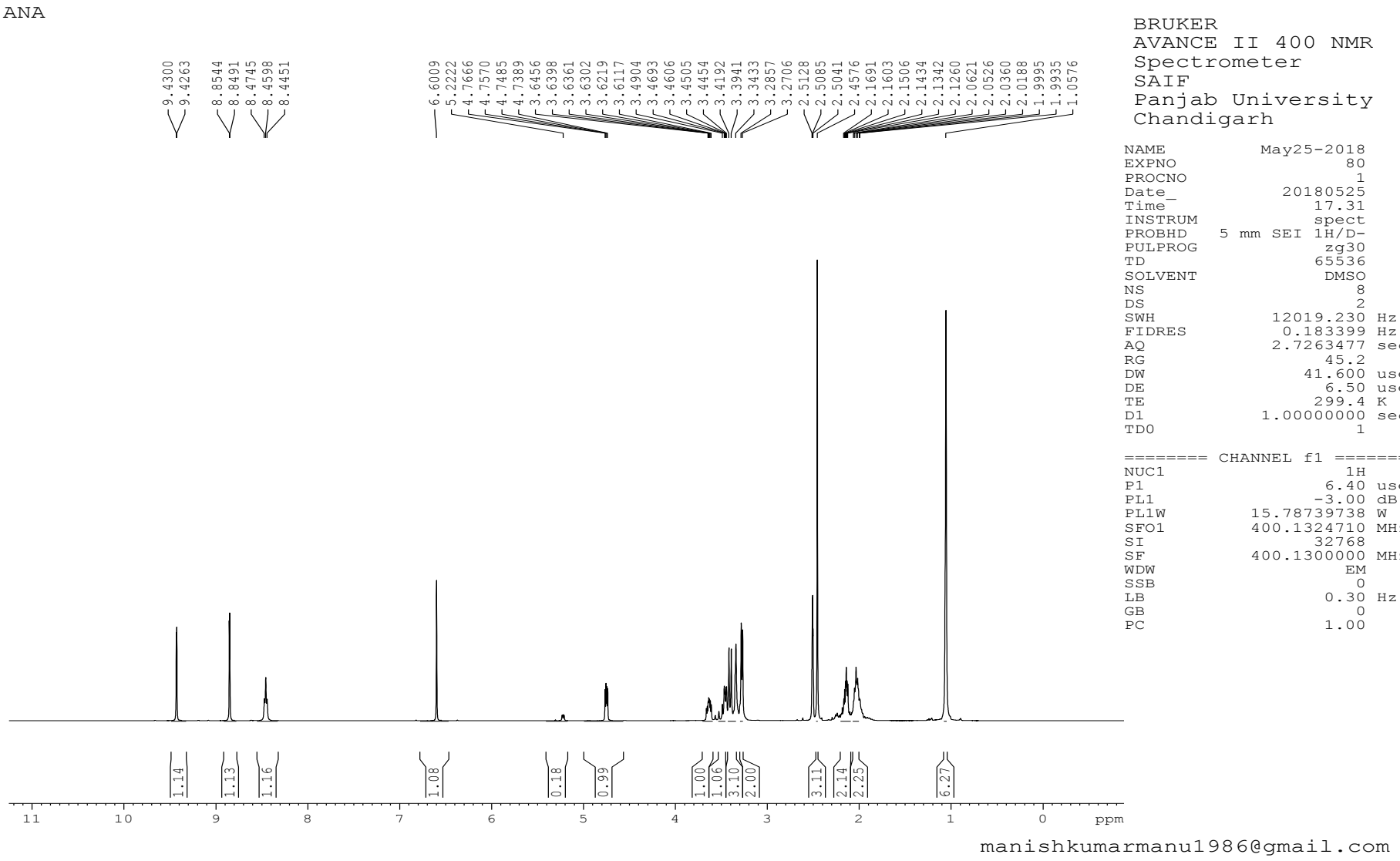


Fig. 6. 35 – ^{1}H NMR spectra of ANA

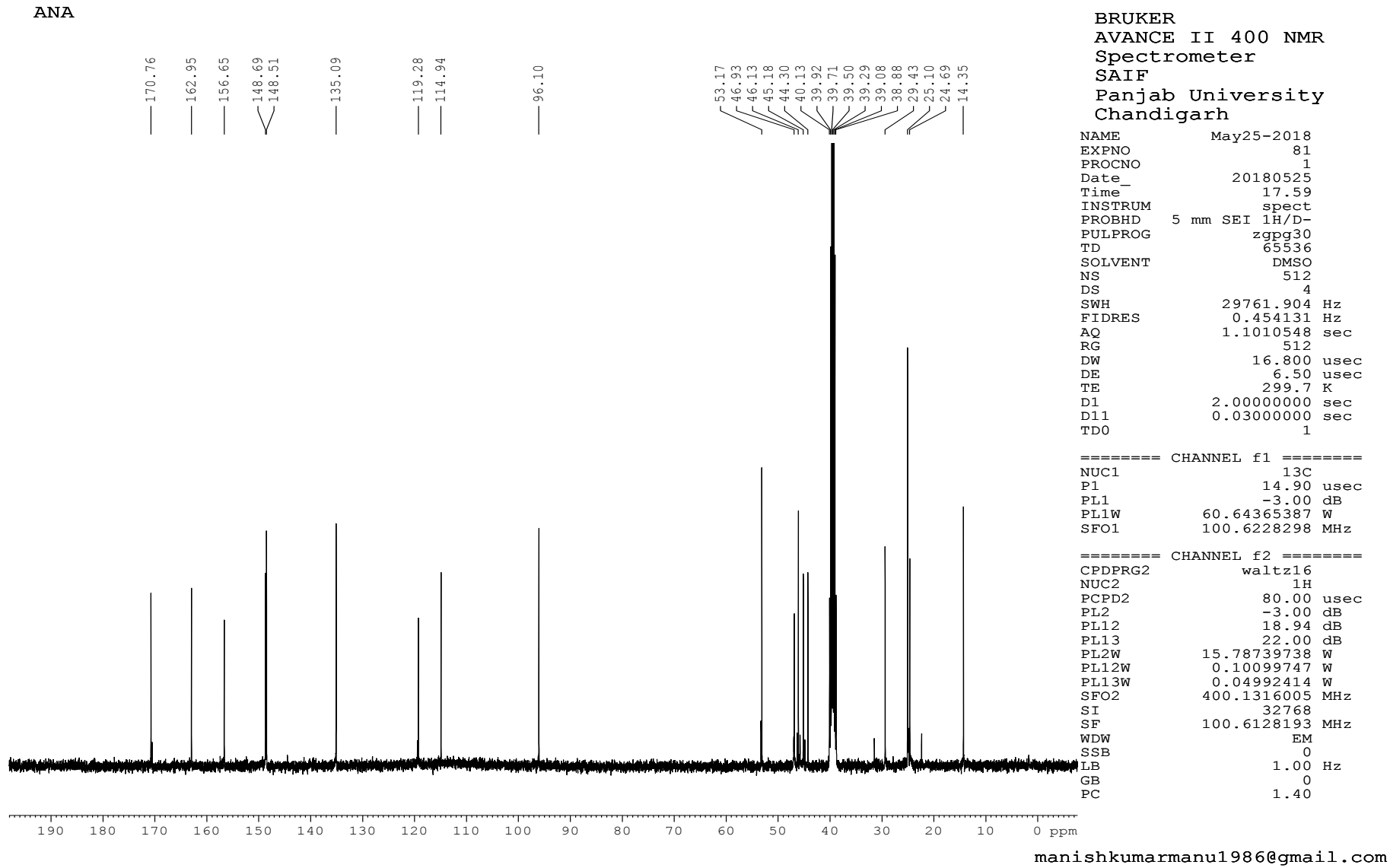


Fig. 6. 36 - ^{13}C NMR spectra of ANA

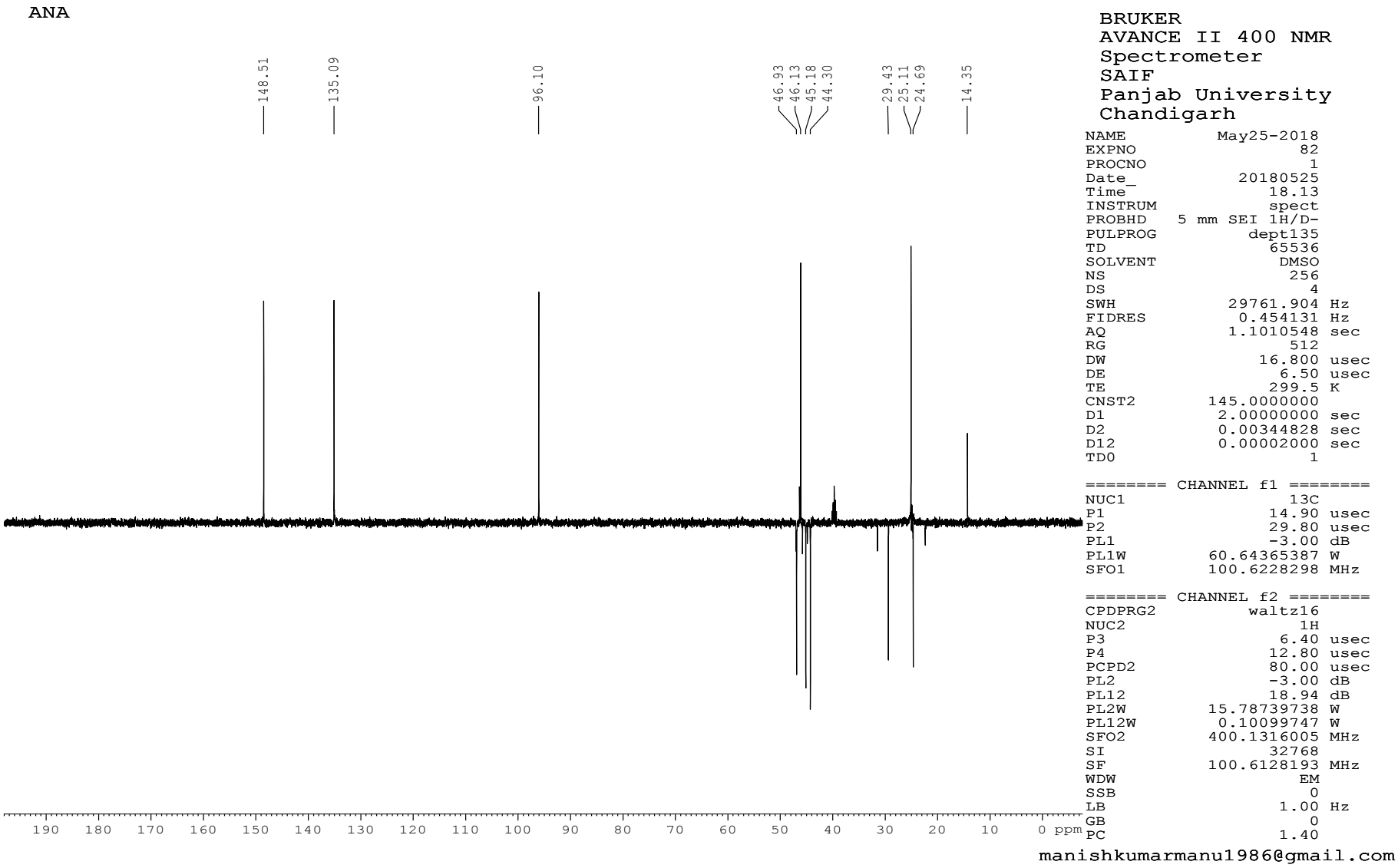


Fig. 6. 37 – DEPT spectra of ANA

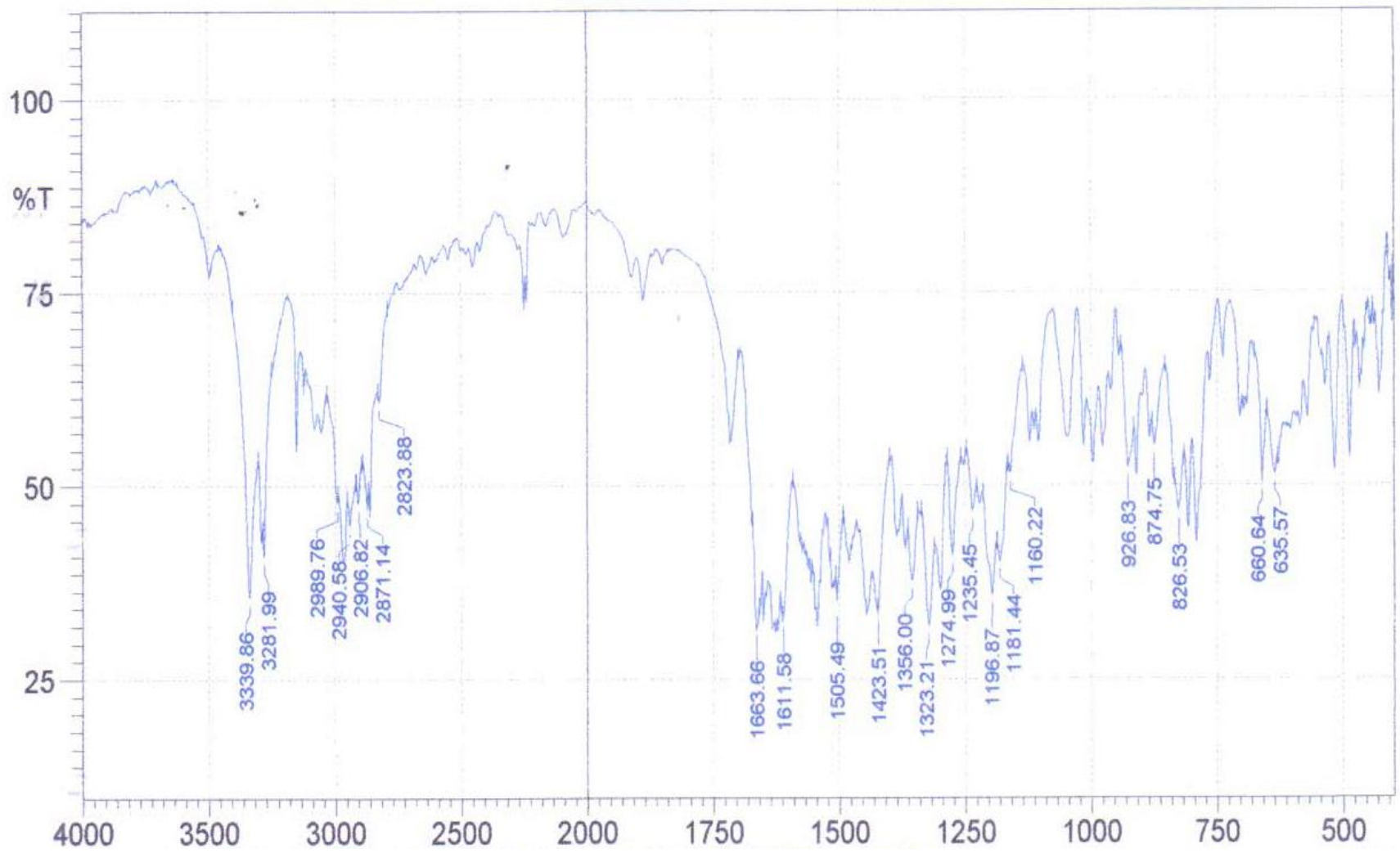


Fig. 6. 38- I.R. spectra of ANA

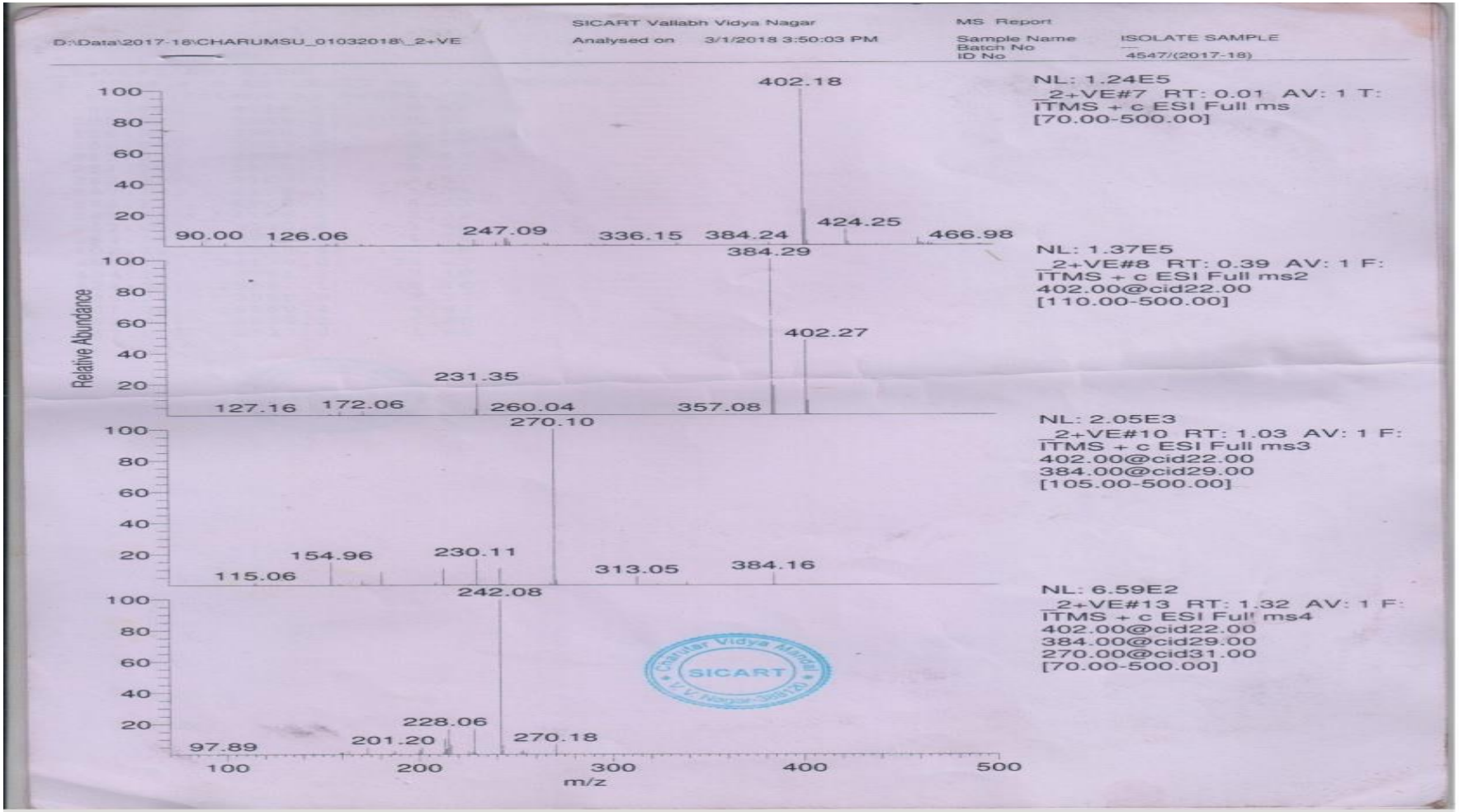


Fig. 6. 39- ESI-MS/MS spectra of DP1

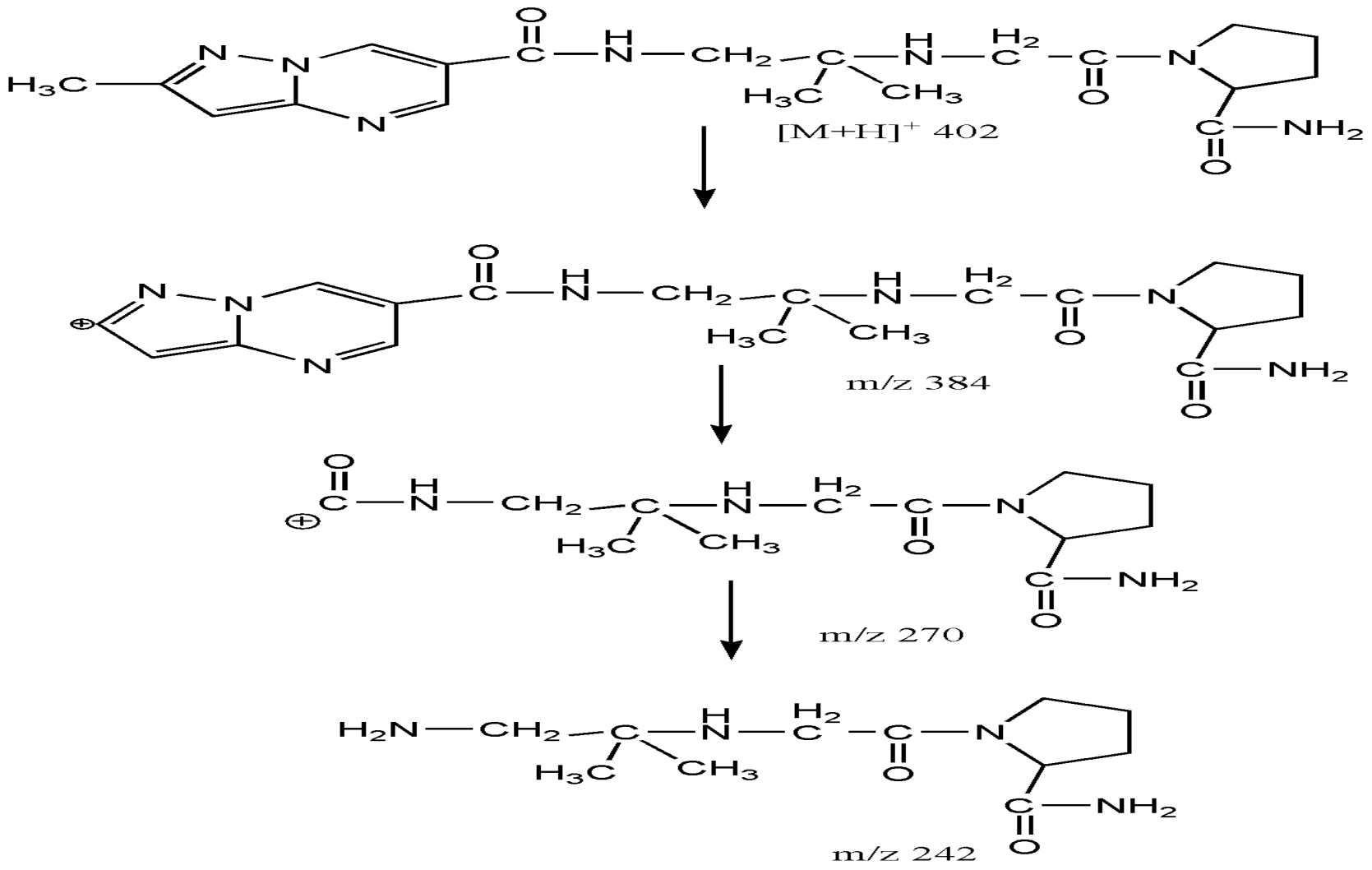


Fig. 6. 40- Fragmentation pathway of DP1

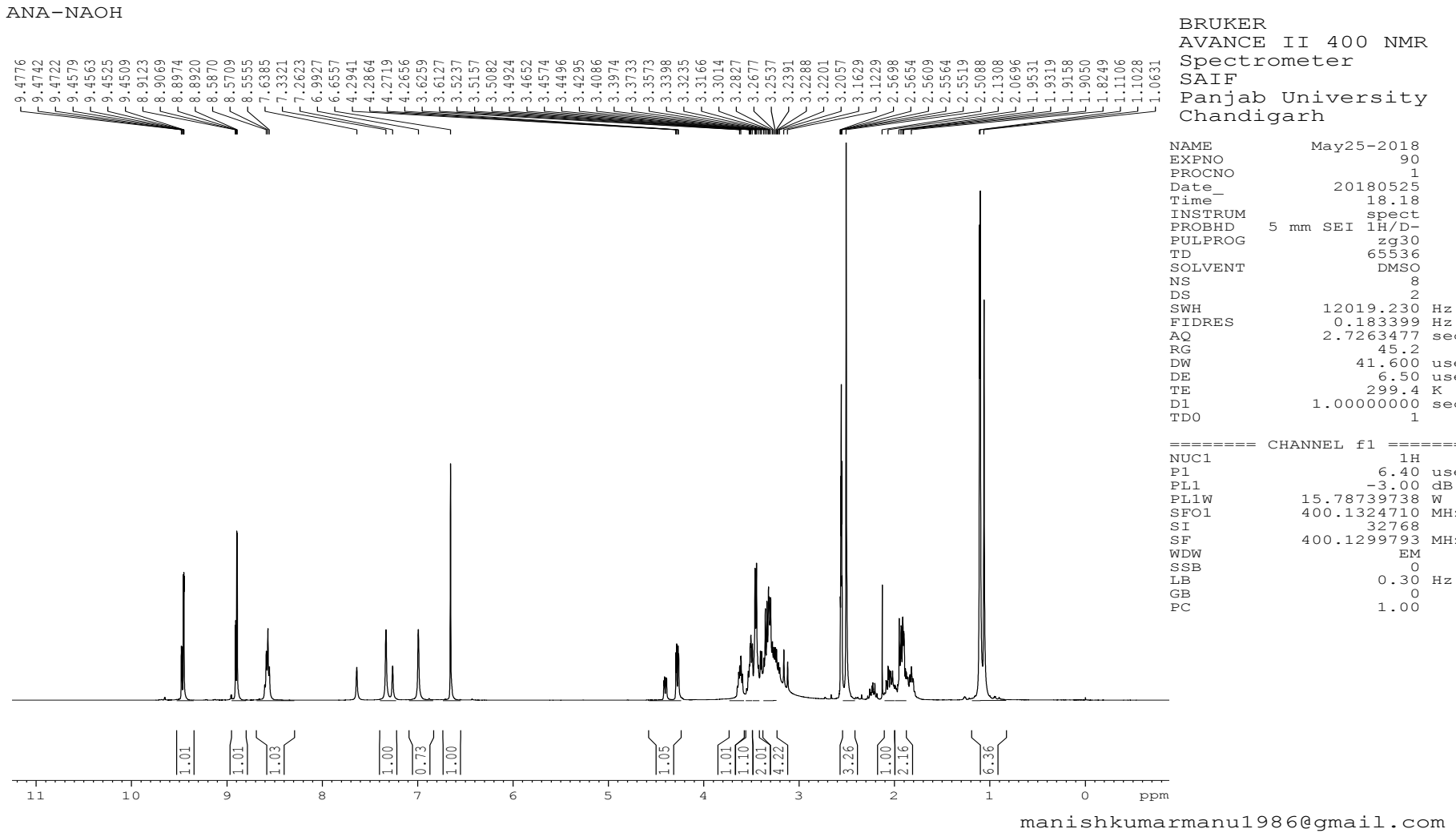


Fig. 6. 41 – ^{1}H NMR spectra of DP1

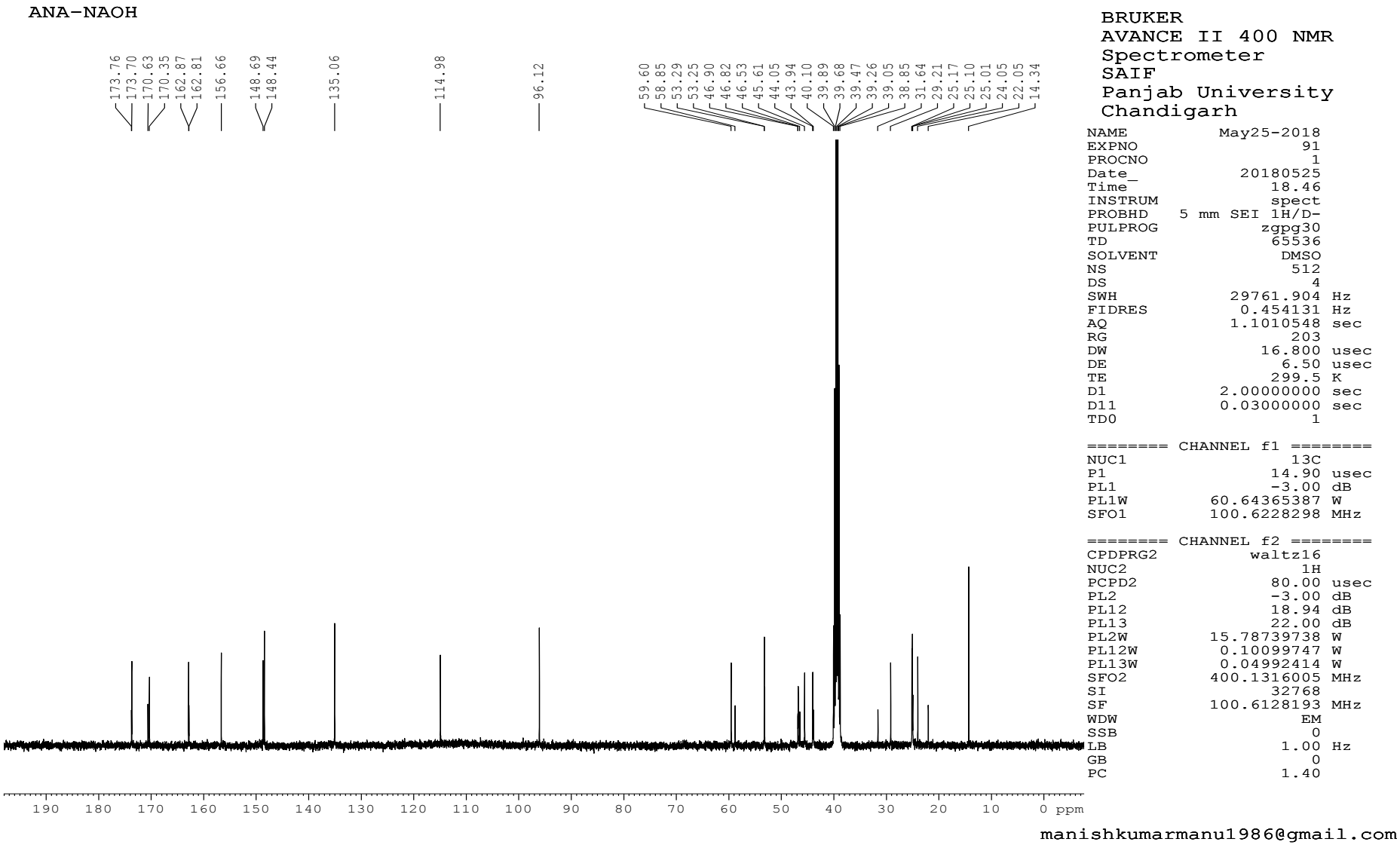


Fig. 6. 42 – ^{13}C NMR spectra of DP1

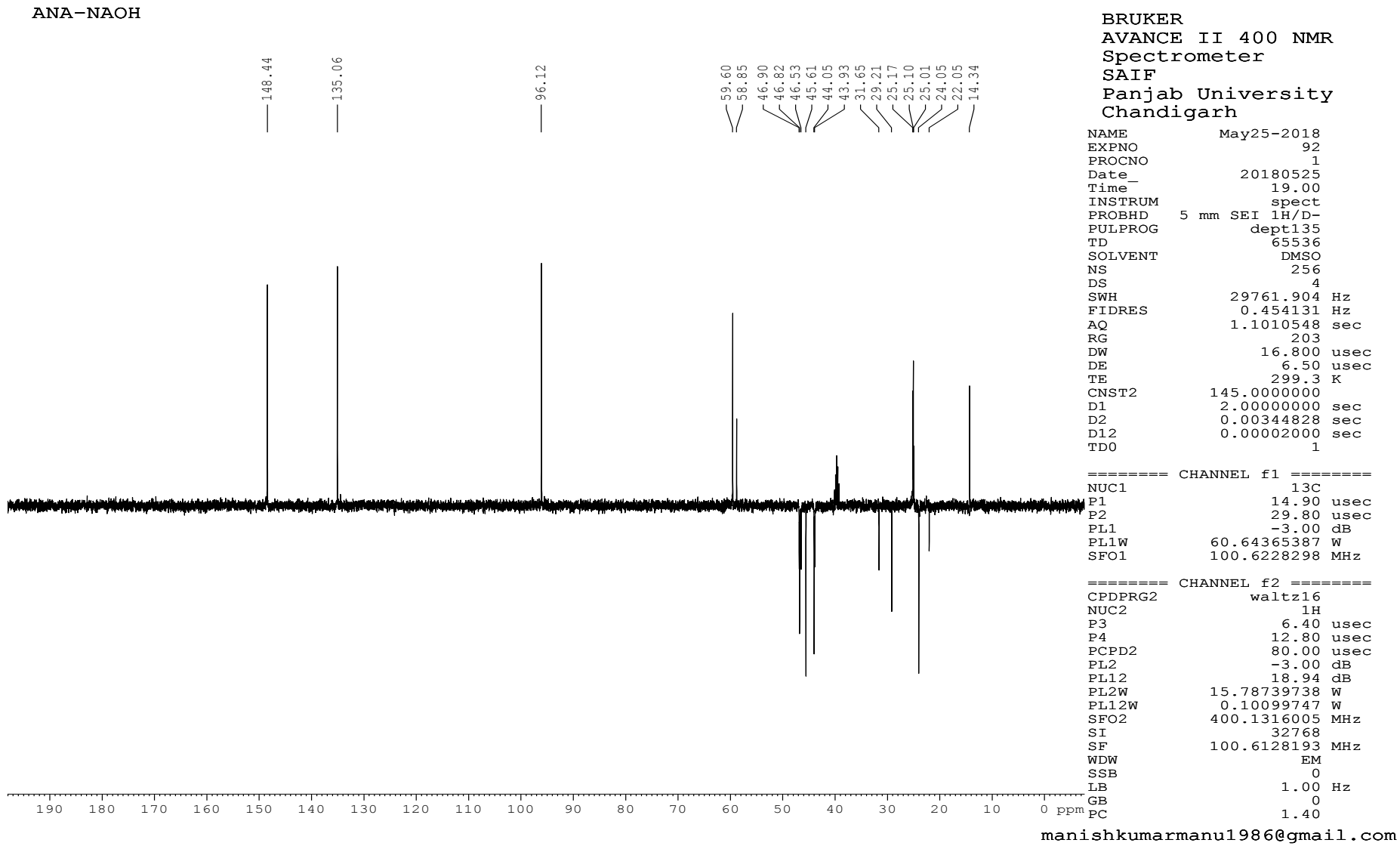


Fig. 6. 43- DEPT spectra of DP1

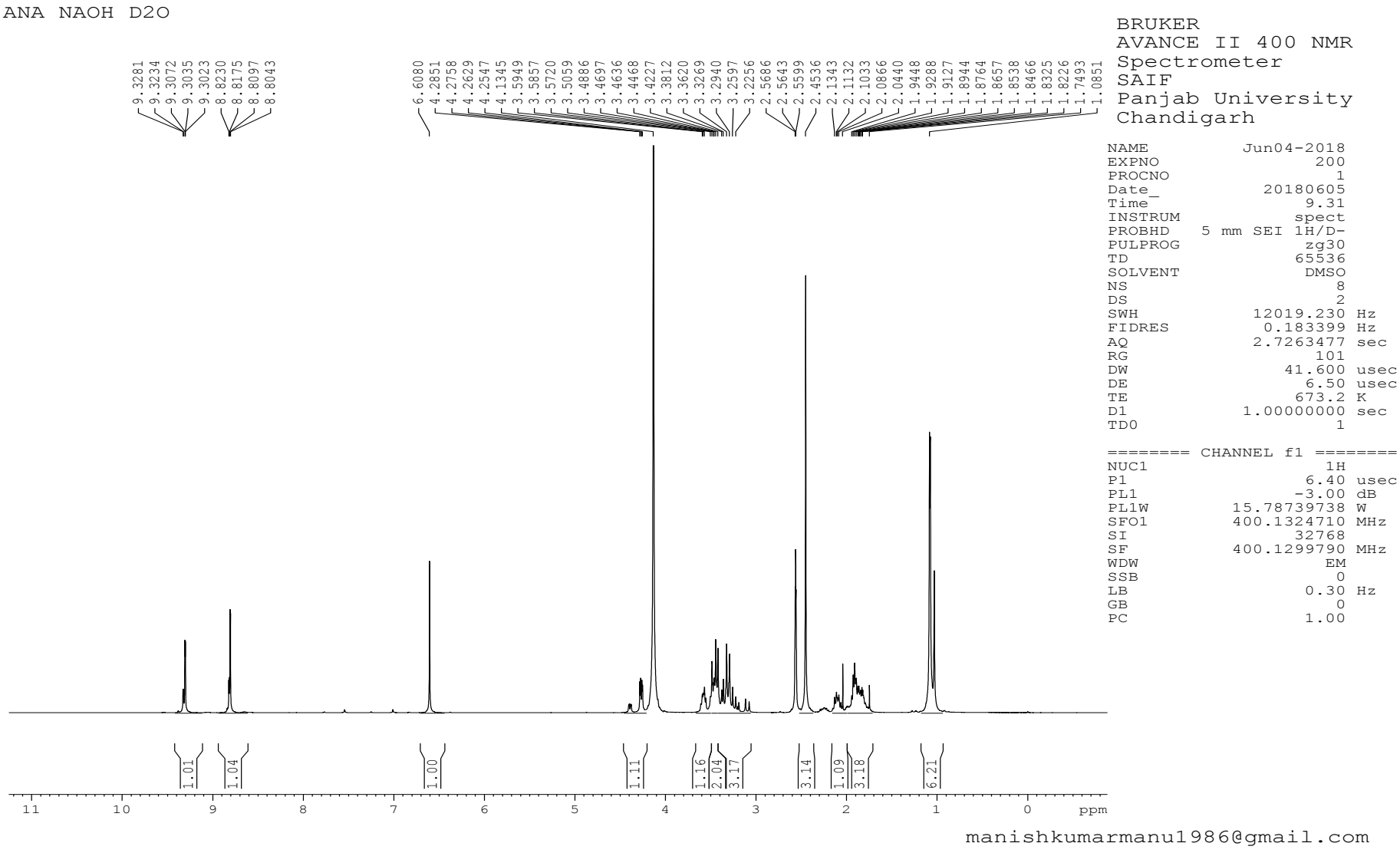


Fig. 6. 44 – D_2O exchange of DP1

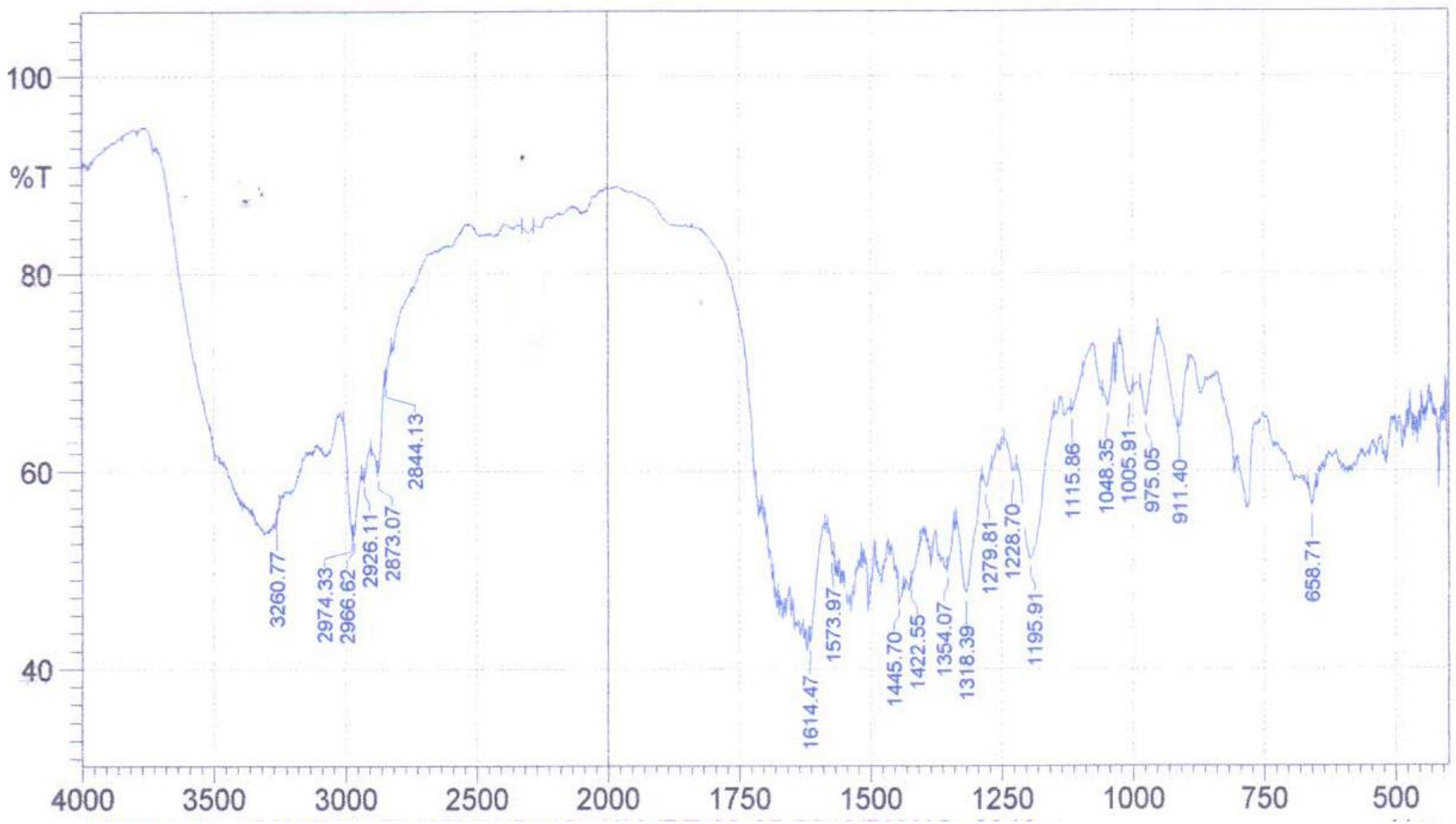


Fig. 6. 45 - I.R. spectra of DP1

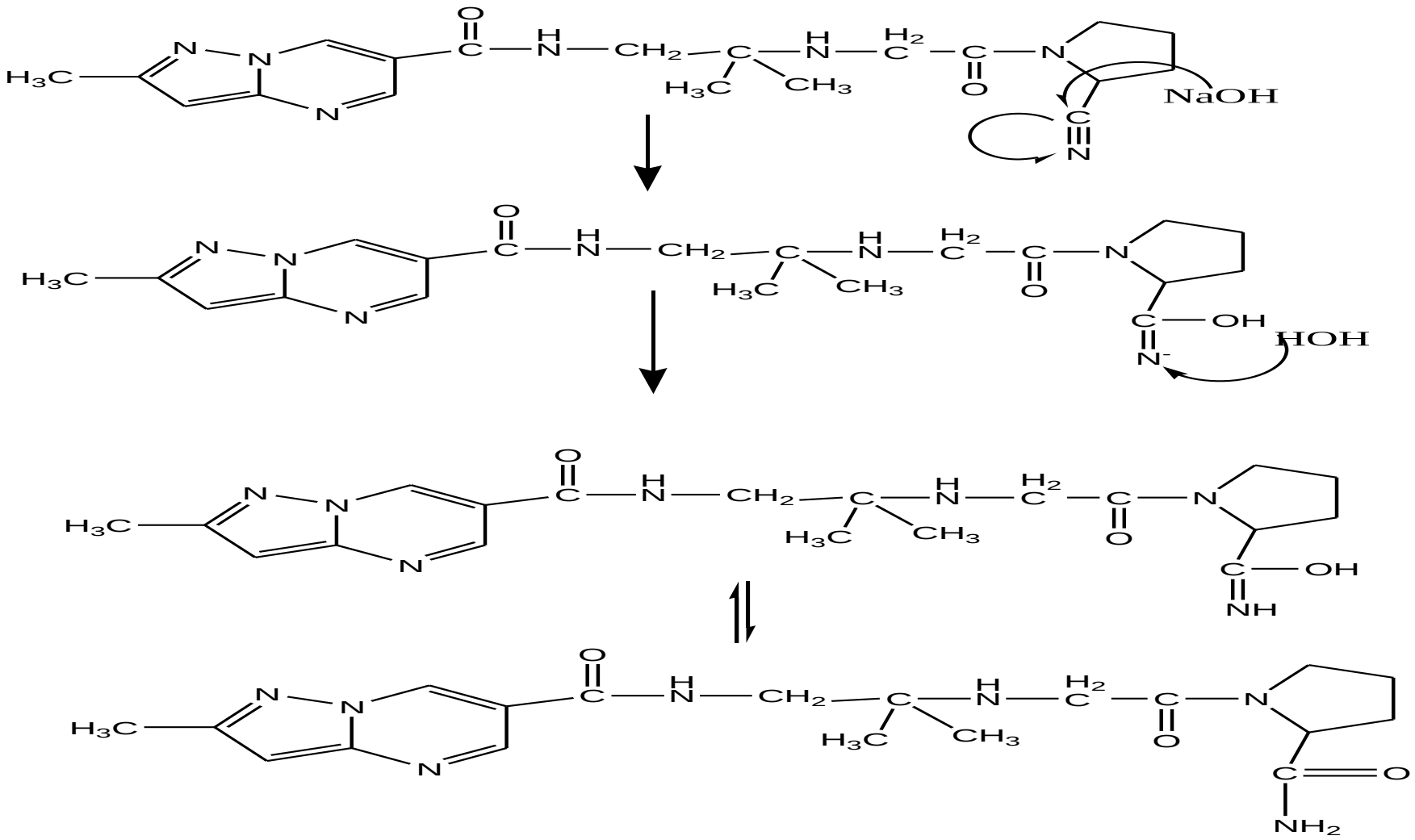


Fig. 6. 46- Mechanism of formation of DP1

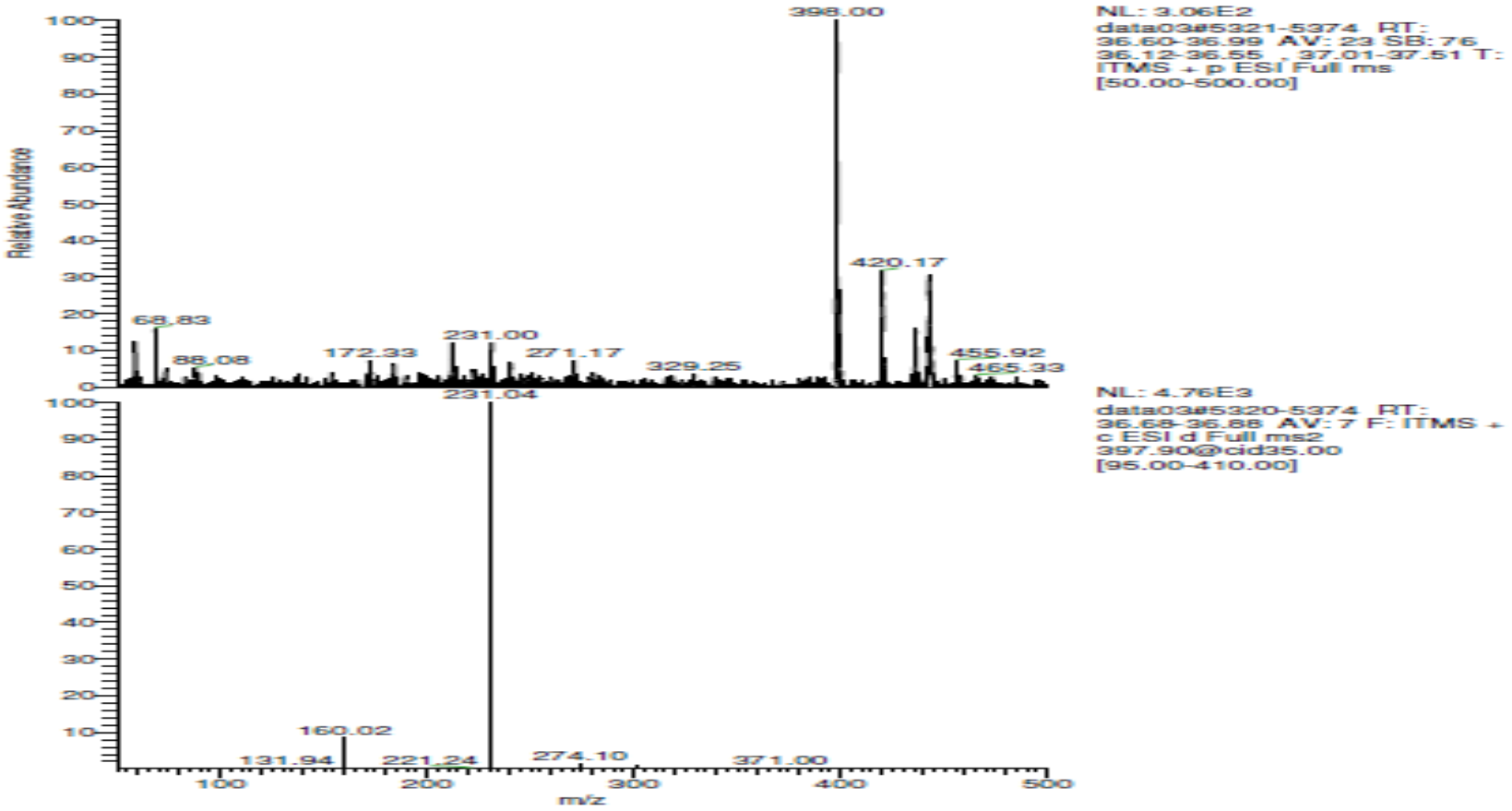


Fig. 6. 47- ESI-MS/MS spectra of DP2

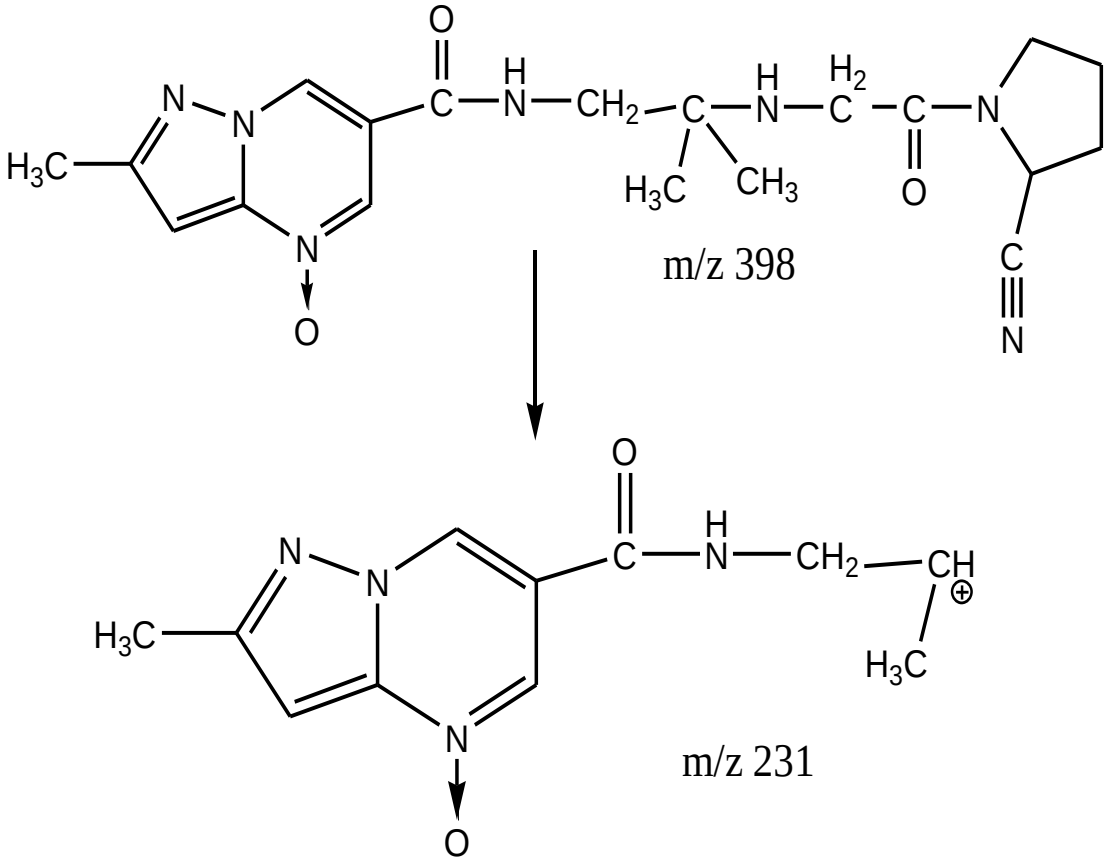


Fig. 6. 48 - Fragmentation pathway of DP2

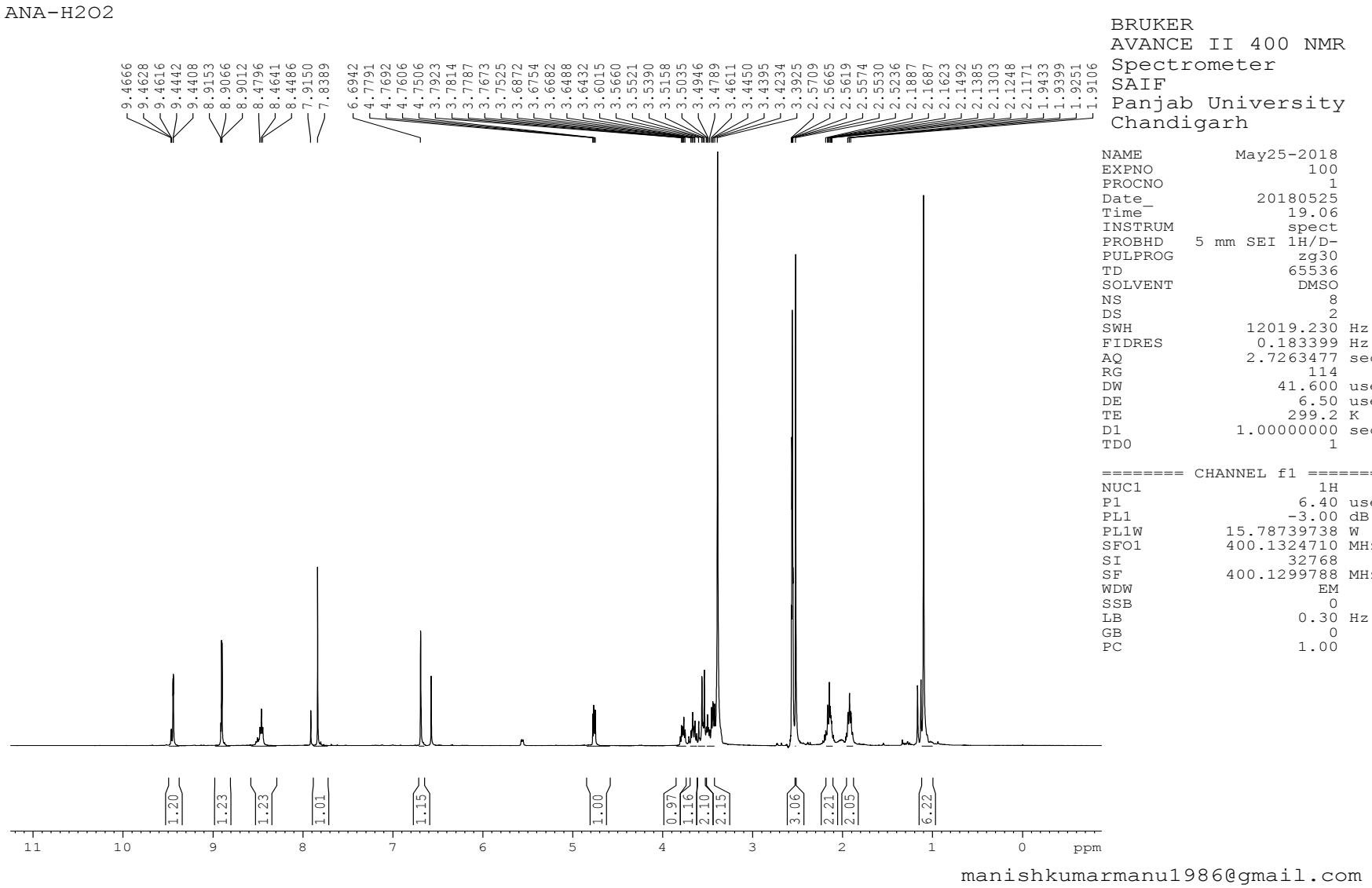


Fig. 6. 49- ^{1}H NMR spectra of DP2

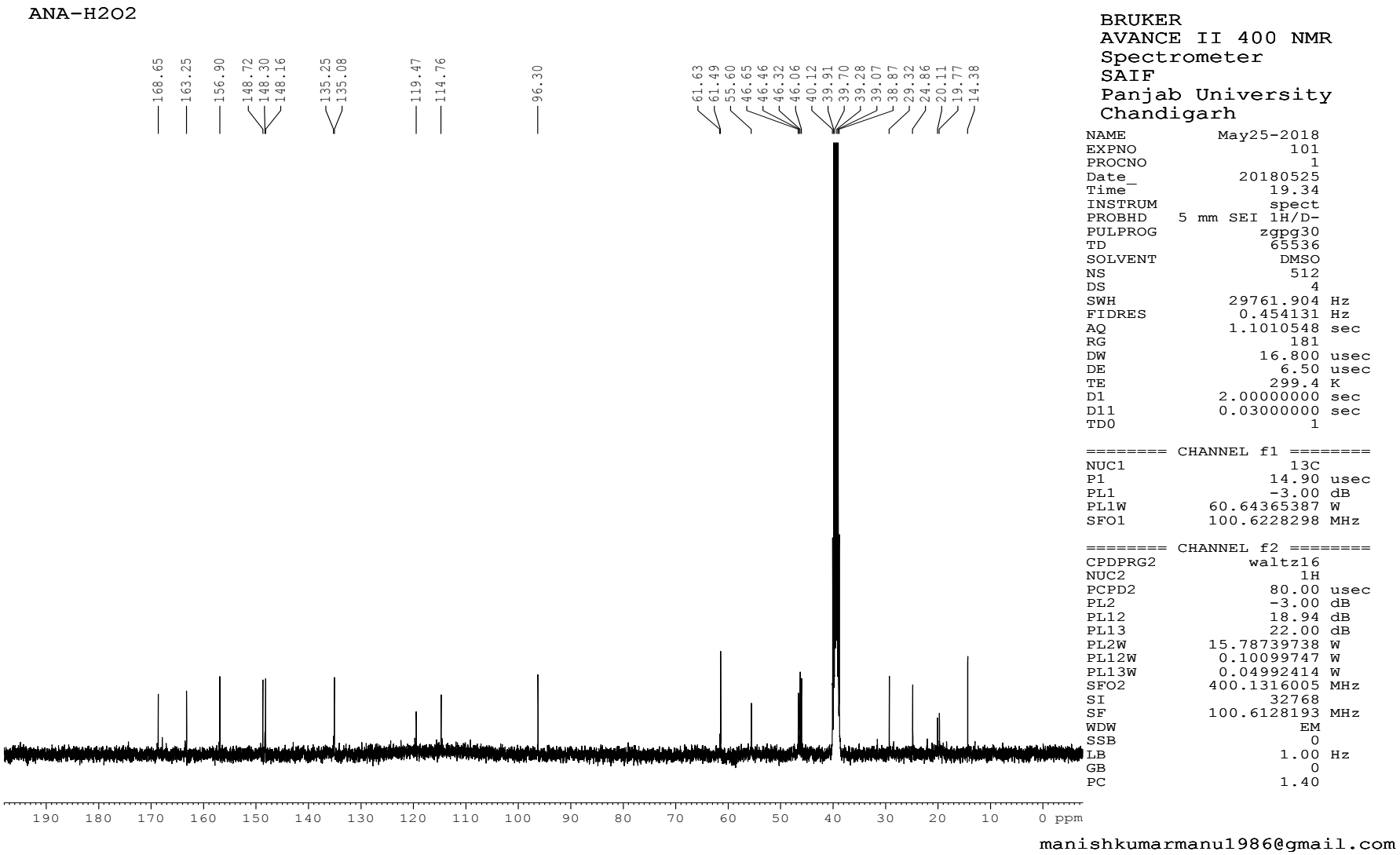


Fig. 6. 50– ^{13}C NMR spectra of DP2

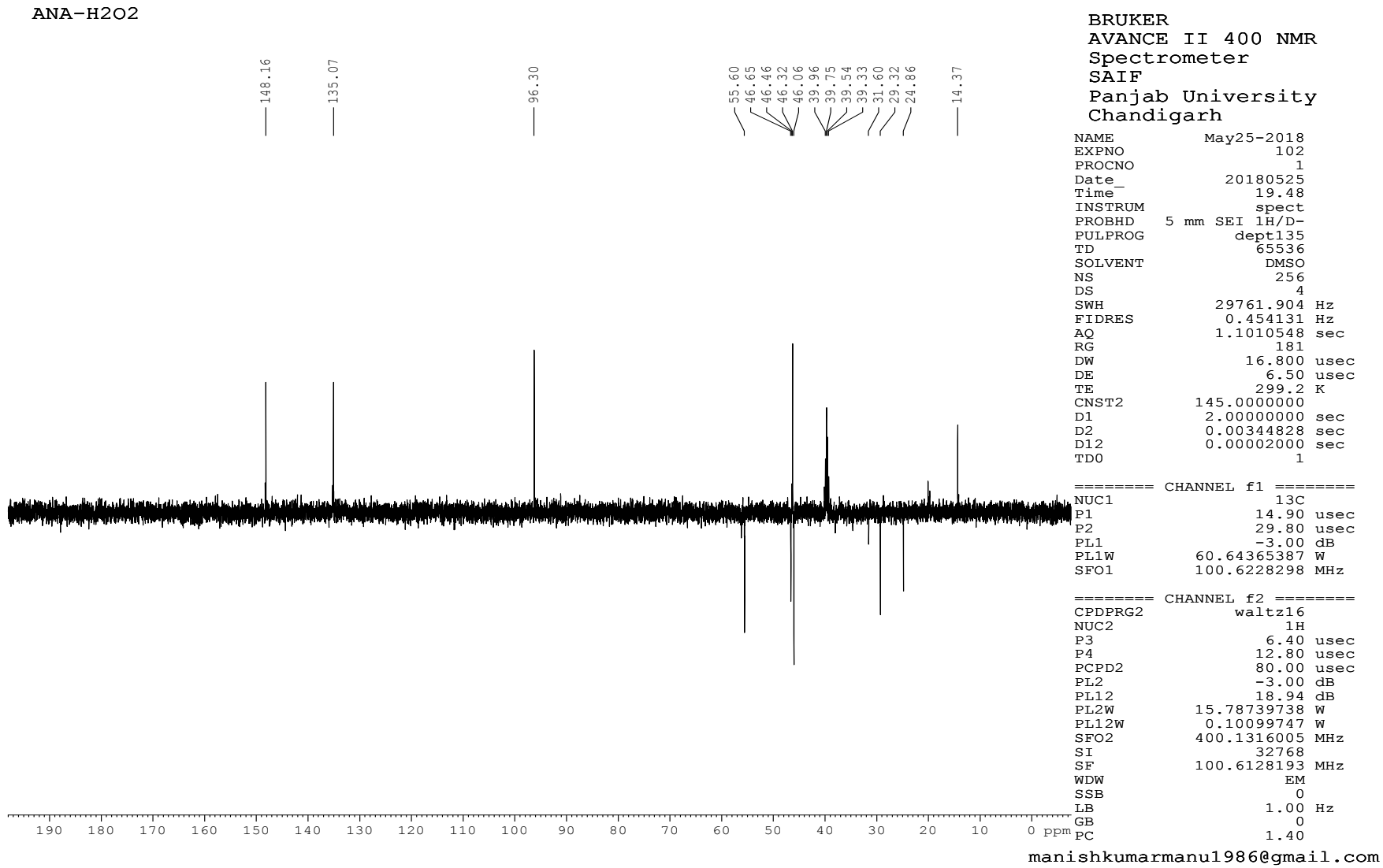


Fig. 6. 51 – DEPT spectra of DP2

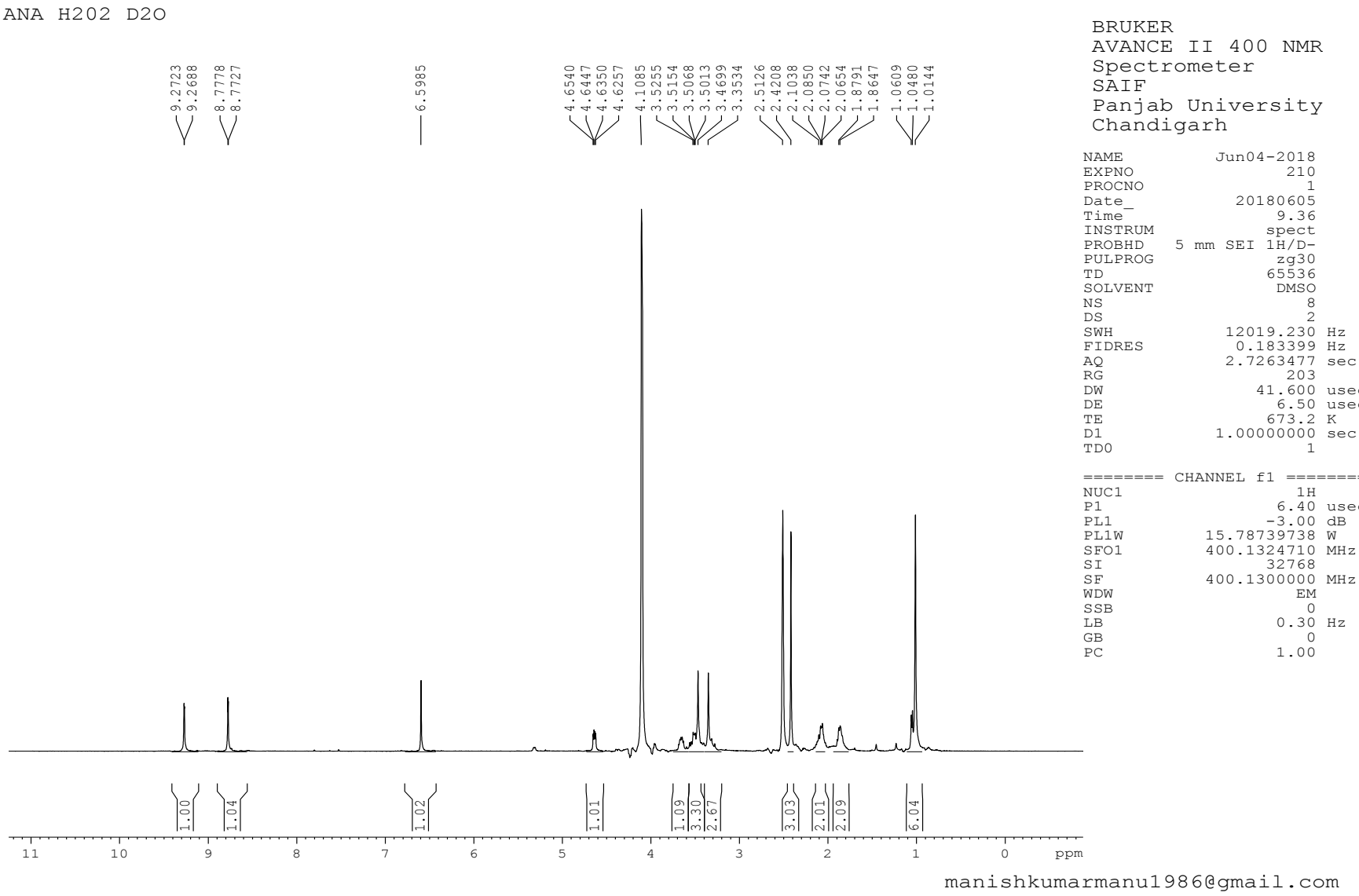


Fig. 6. 52 – D_2O exchange of DP2

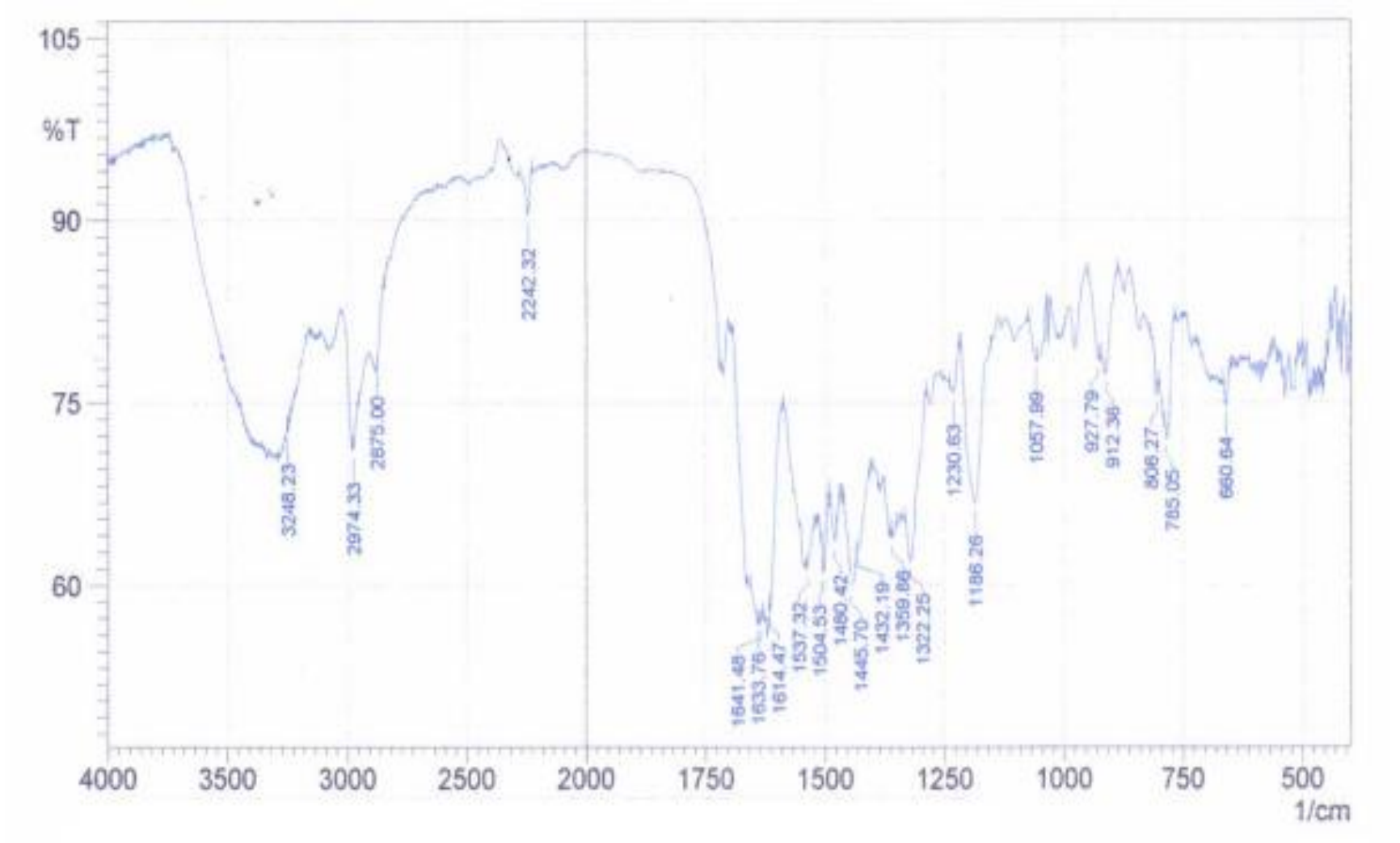


Fig. 6. 53 – I.R. spectra of DP2

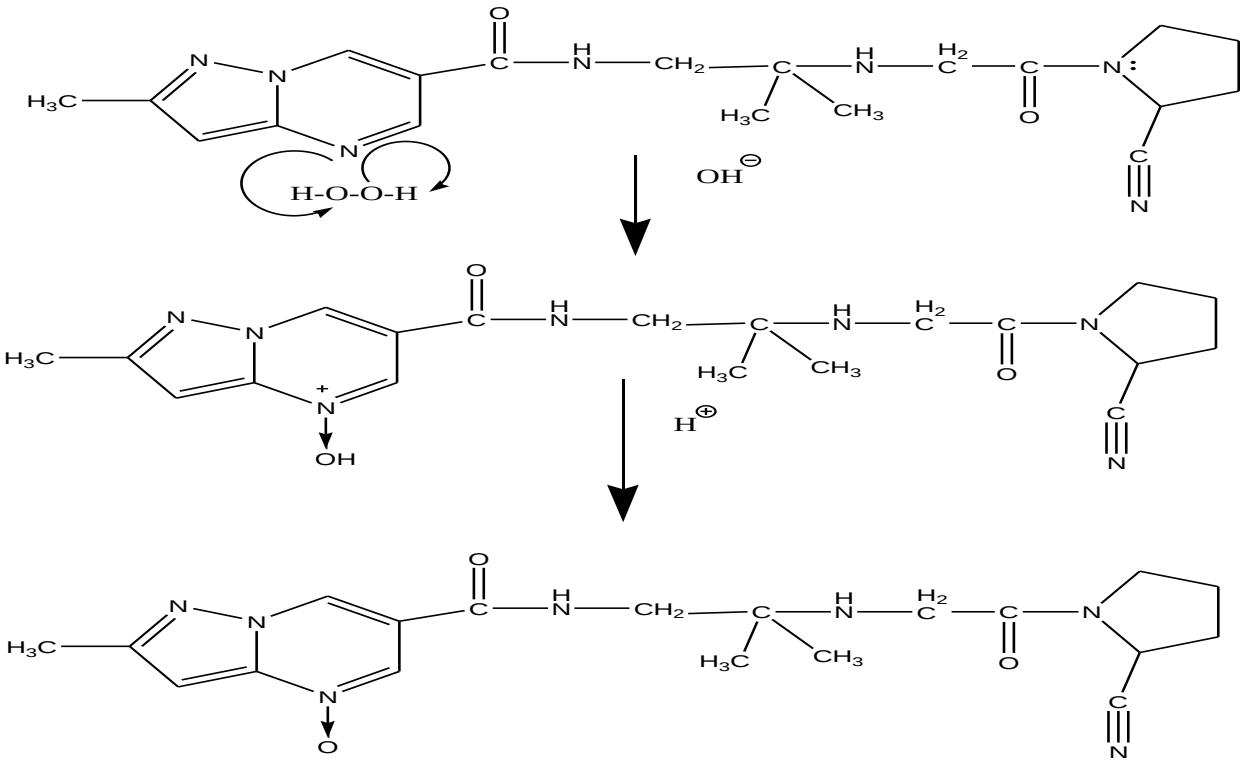


Fig. 6. 54- Mechanism of formation of DP2

6.6.3. DISCUSSIONS

Major degradation products DP1 in alkaline and DP2 in oxidative condition were isolated and purified by preparative HPLC. A mass spectrum of DP1 indicates that it has 18 m/z more than that of ANA. ^{1}H NMR spectra of DP1 indicates formation of amide protons and formation of carbonyl group and disappearance of nitrile peak in ^{13}C NMR spectra compared to ANA. This indicates that DP1 is formed from ANA by alkaline hydrolysis of nitrile group and converting to amide. DP1 is characterised as 1-[2-[1-(2-methylpyrazolo[1,5-alpha]pyrimidine-6-carboxamido}-methyla-propan-2-yl-amino)acetyl)pyrolidine-2-carboxamide. By mass spectral analysis DP2 has 16 m/z more than ANA. No. of protons in DP2 are same as that of ANA in ^{1}H NMR spectra. IR spectra of DP2 indicated formation of N-oxide at 1537cm^{-1}.DP2 is characterised as N-[2-({2-[(2S)-2-cyanopyrrolidin-1-yl]-2-oxoethyl} amino)-2-methylpropyl]-2-methylpyrazolo-N-oxido -[1,5-a]pyrimidine-6-carboxamide.

6.7. SECTION D

IMPURITY PROFILING AND DEGRADATION STUDY OF ANAGLIPTIN

6.7.1. EXPERIMENTAL

6.7.1.1. Chemicals and reagents

Chemicals and reagents used in the present section as same as those mentioned in 6.4.1.1.

6.7.1.2. Equipments and Chromatographic conditions

The equipments and chromatographic conditions used in impurity profiling and degradation study are same as those in mentioned in section 6.4.1.3.

For LC-MS analysis , ANA degradation samples were analysed in same chromatographic conditions as mentioned in section 6.4.1.3.The m/z values were determined in both positive and negative ESI mode. On the basis of molecular weight, structures of DPs were proposed and degradation pathway was postulated.

6.7.1.3. Preparation of stock, sample and buffer solutions

Stock, sample and buffer solutions were prepared in the same was as mentioned in section 6.4.1.5.

6.7.2. RESULTS

6.7.2.1. LC-PDA Study

Forced degradation studies of ANA showed the formation of degradation products in LC-PDA and are summarized in Table 6.20. Significant degradation was observed in alkaline and oxidative conditions. LC-MS technique was used for identification alkaline and oxidative degradation products.

Table 6. 20 – Summary of forced degradation study of ANA analysed by LC-PDA

Stress Condition	Conditions	RT of Degradation Products	% of Degradation Products in API	% of Degradation products in synthetic mixture
Acid	1 M HCl 80ºC for 6 hrs	3.8 min(DP3) 6.2 min (DP8) 6.9 min (DP9) 8.9 min (DP11) 13.6 min(DP1) 18.7 min(DP12)	0.9% 0.9% 0.5% 0.4% 0.3% 0.09% **(3.1%)**	0.8% 0.8% 0.4% 0.4% 0.28% 0.08% **(2.97%)**
Base	0.1 M NaOH RT for 1 hr	3.6 min(DP3) 13.6 min (DP1)	1.4% 25.09% **(26.49%)**	1.2% 23.98% **(25.18%)**
Neutral hydrolysis	80ºC for 6 hrs	4.09 min(DP3) 6.04 min(DP8) 7.04 min(DP 9) 13.9 min (DP1) 27.8 min (DP4)	0.09% 0.14% 0.62% 3.2% 0.19% **(4.2 %)**	0.07% 0.12% 0.52% 3.0% 0.17% **(3.8 %)**
Oxidative	0.3 % H_2O_2RT for 2 hrs	3.59 min(DP3) 9.60 min(DP4) 13.89 min (DP1) 24.9 min (DP6) 27.9 min (DP5) 29.4 min (DP7) 36.5 min (DP2)	1.0% 1.0% 0.4% 0.15% 0.61% 2.46% 10.3% **(17.5 %)**	1.0% 0.9% 0.4% 0.15% 0.6% 2.4% 10.0% **(17.0%)**

Thermal	80˚C for 11 days	2.04 min (DP10) 4.07min (DP3) 7.04 min (DP9) 9.93 min(DP4) 27.93 min(DP5)	0.3% 0.68% 0.28% 0.02% 0.17% **(1.45%)**	0.3% 0.68% 0.3% 0.02 0.17% **(1.47%)**
Photolytic Dry	5382 Lux and 144UW/cm^2 11 days	2.0 min(DP10) 7.07 min (DP9) 27.4 min (DP5) 35.82 min (DP2)	0.2% 0.4% 0.02% 0.01% **(0.8 %)**	0.25% 0.45% 0.02% 0.01% **(1.1 %)**
Solution		4.7min(DP3) 7.08 min (DP9) 13.6 min(DP1) 27.6 min(DP4) 36.5 min(DP2)	0.15% 1.0% 1.0% 0.9% 0.8% **(3.78%)**	0.1% 0.9% 0.7% 0.7% 0.8% **(3.24%)**

6.7.2.2. LC-MS study and characterization of DPs

ANA (m/z 384)

An ESI-MS/MS spectrum is provided in Fig.6.33 and proposed fragmentation pathway is shown in Fig. 6.34. Mass spectral interpretation of ANA in mentioned in section 6.6.2.1.1.

DP1 (m/z 402)

An ESI-MS/MS spectrum is provided in Fig.6.39 and proposed fragmentation pathway is shown in Fig. 6.40. Mass spectral interpretation of ANA in mentioned in section 6.6.2.1.2.

DP2 (m/z 398)

An ESI-MS/MS spectrum is provided in Fig.6.47 and proposed fragmentation pathway is shown in Fig. 6.48. Mass spectral interpretation of ANA in mentioned in section 6.6.2.1.3.

DP3 (m/z 271)

An ESI-MS/MS spectrum of DP3 is provided in Fig.6.55. Mass spectrum of DP3 shows protonated m/z 271. Further takes place to m/z 126. DP3 may be formed from DP1 by loss of methyl pyrazolo pyrimidine group (Fig.6.56).

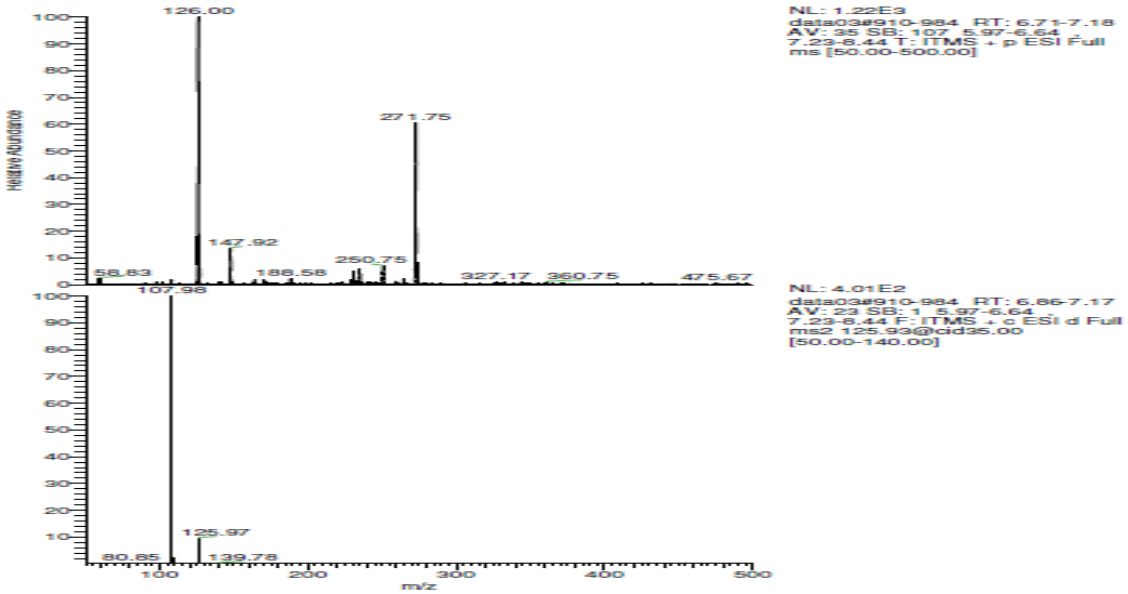


Fig. 6. 55- ESI-MS/MS spectra of DP3

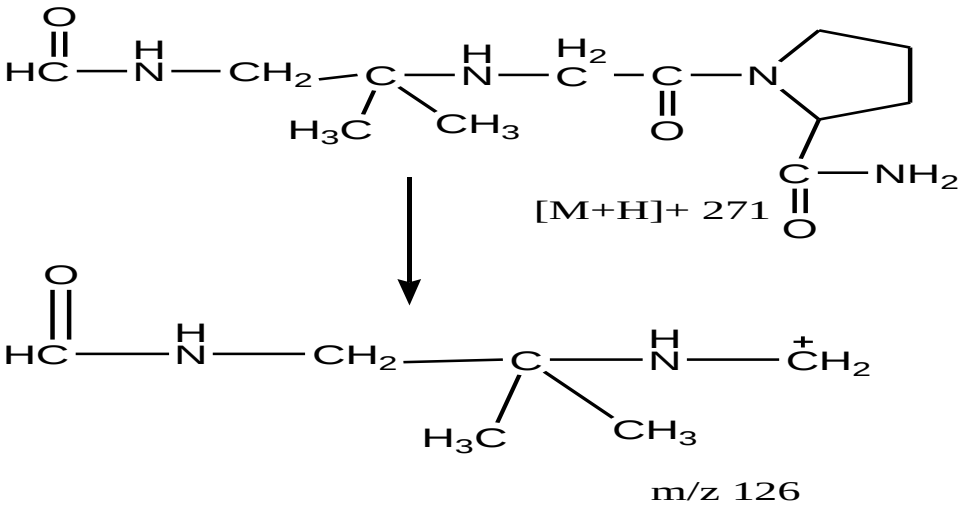


Fig. 6. 56 - Fragmentation pathway of DP3

DP4 (m/z 150)

An ESI-MS/MS spectrum of DP4 is provided in Fig 6.57. Mass spectrum of DP4 shows protonated m/z of 150 which on further fragmentation gives at m/z 126 (Fig. 6.58). DP4 may be formed from DP5 by fragmentation.

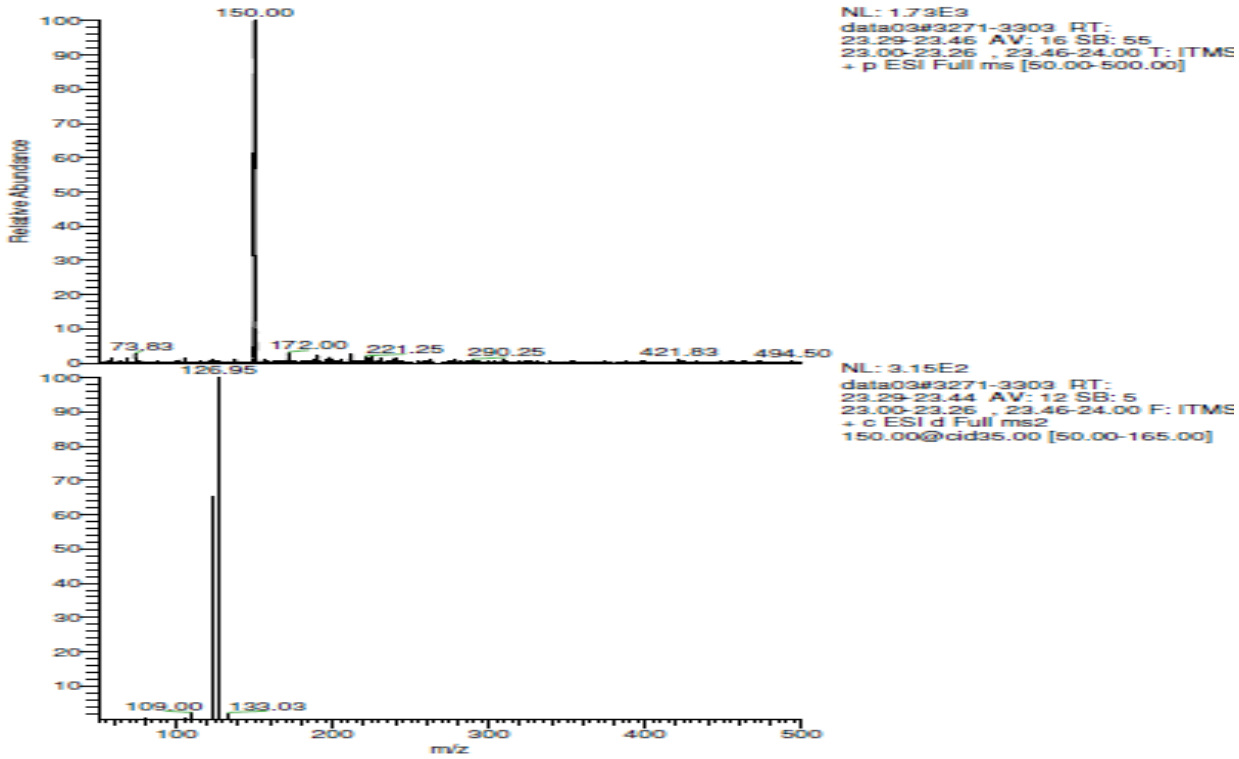


Fig. 6. 57- ESI-MS/MS spectra of DP4

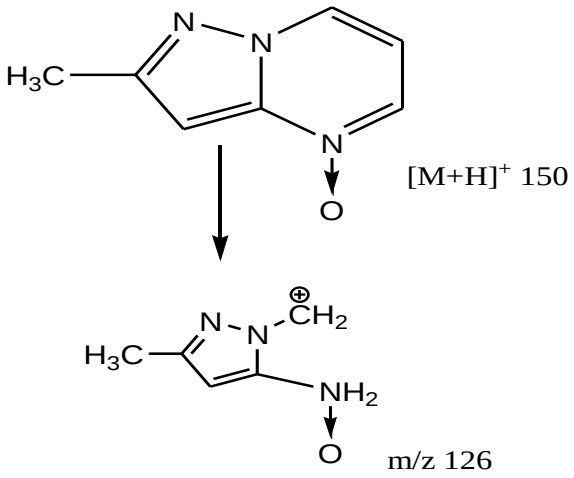


Fig. 6. 58- Fragmentation pathway of DP4

DP5 (m/z 418)

An ESI-MS/MS spectrum of DP5 is provided in Fig.6.59. Mass spectrum of DP5 shows positive ESI-MS of DP5 $[M+H]^+$ ion at *m/z* value of 418 which undergoes further fragmentation at *m/z* value of 231 (Fig. 6.60). DP5 may be obtained from DP1 by formation of N-oxide at pyrazolo-pyrimidine ring.

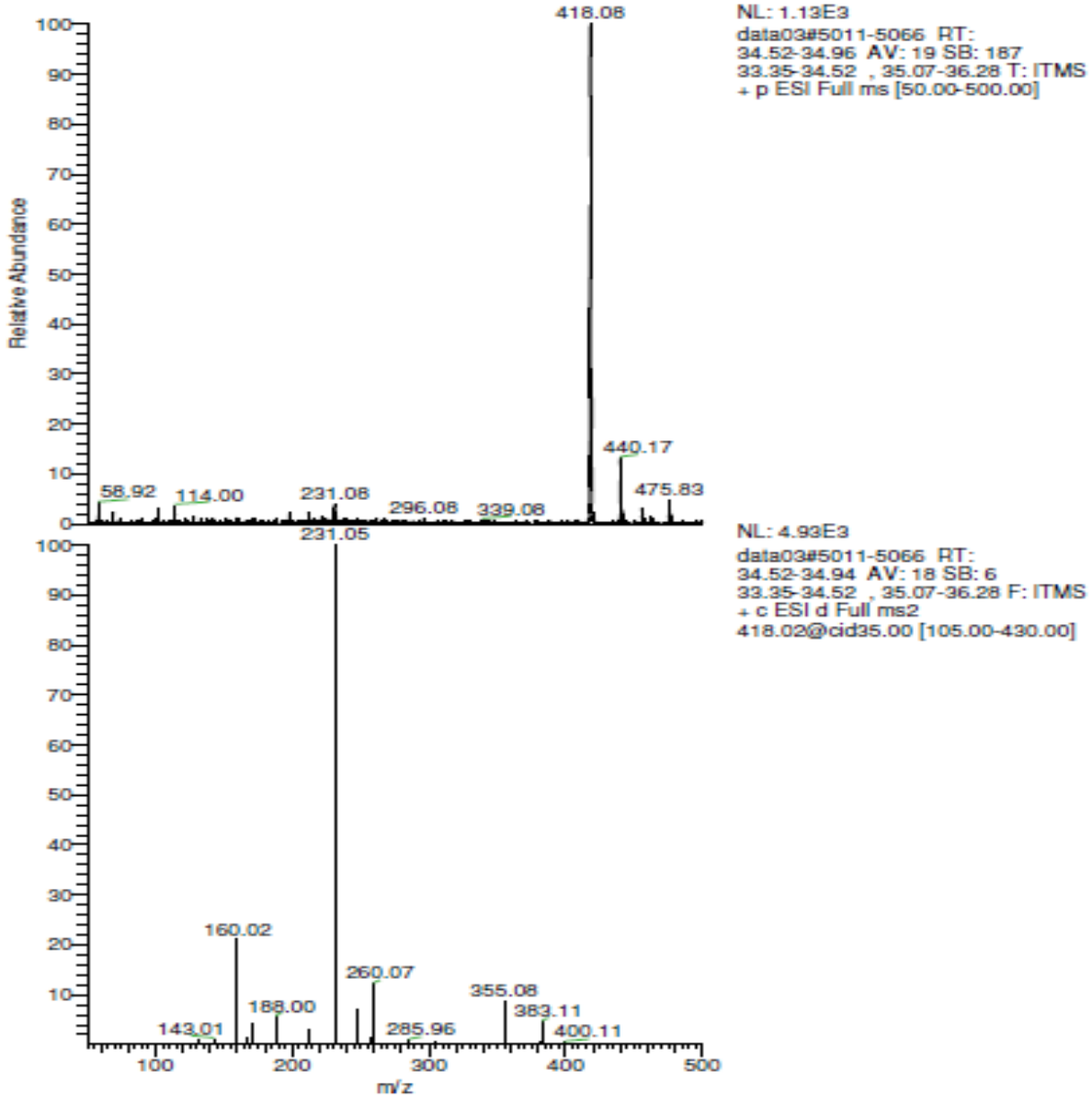


Fig. 6. 59 – ESI-MS/MS spectra of DP5

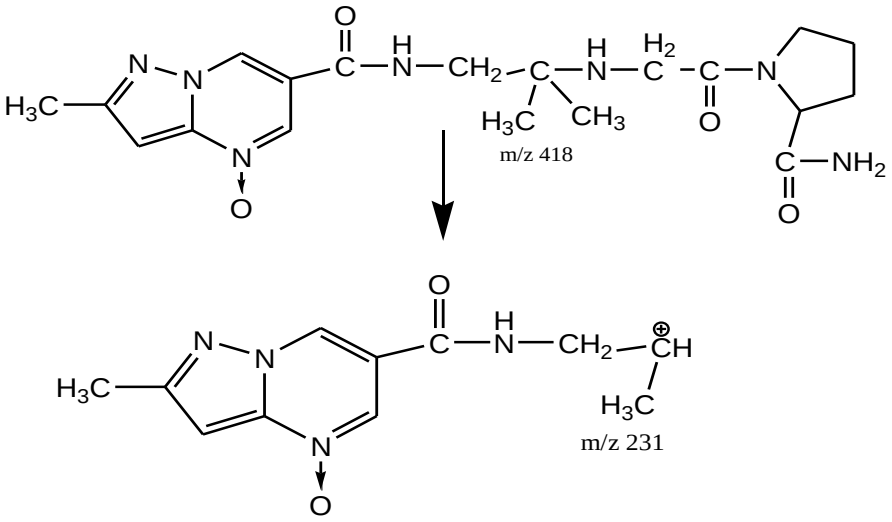


Fig. 6. 60 - Fragmentation pathway of DP5

6.7.2.3. Degradation pathway of ANA

In alkaline condition, ANA having nitrile functional group undergoes alkaline hydrolysis and converts to amide thereby forming DP1. Further fragmentation of DP1 takes place and DP3 is formed (Fig. 6.61).

In oxidative condition, DP1 is formed from ANA by oxidation of nitrile group to amide. DP1 is converted to DP3 due to fragmentation of pyrazolo-pyrimidine ring. DP5 is formed from DP1 by hydroxylation of DP1. Oxidation takes place at pyrazolo-pyrimidine ring as a result DP2 is formed. Fragmentation of DP5 takes place and DP4 is formed (Fig. 6.62).

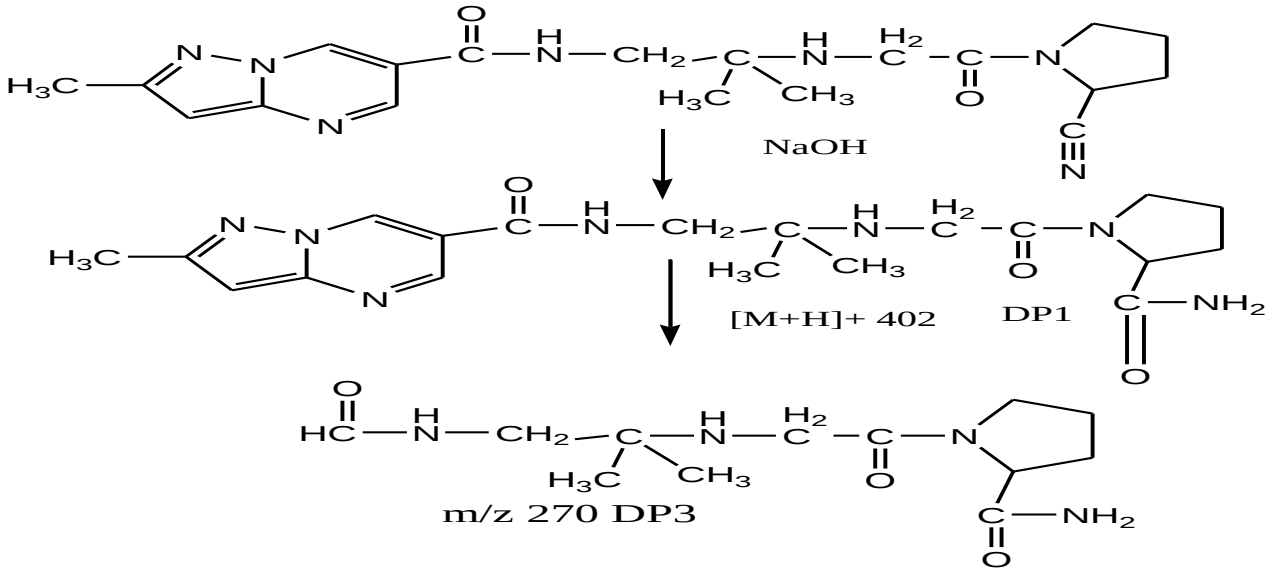


Fig. 6. 61 – Degradation pathway in alkaline condition

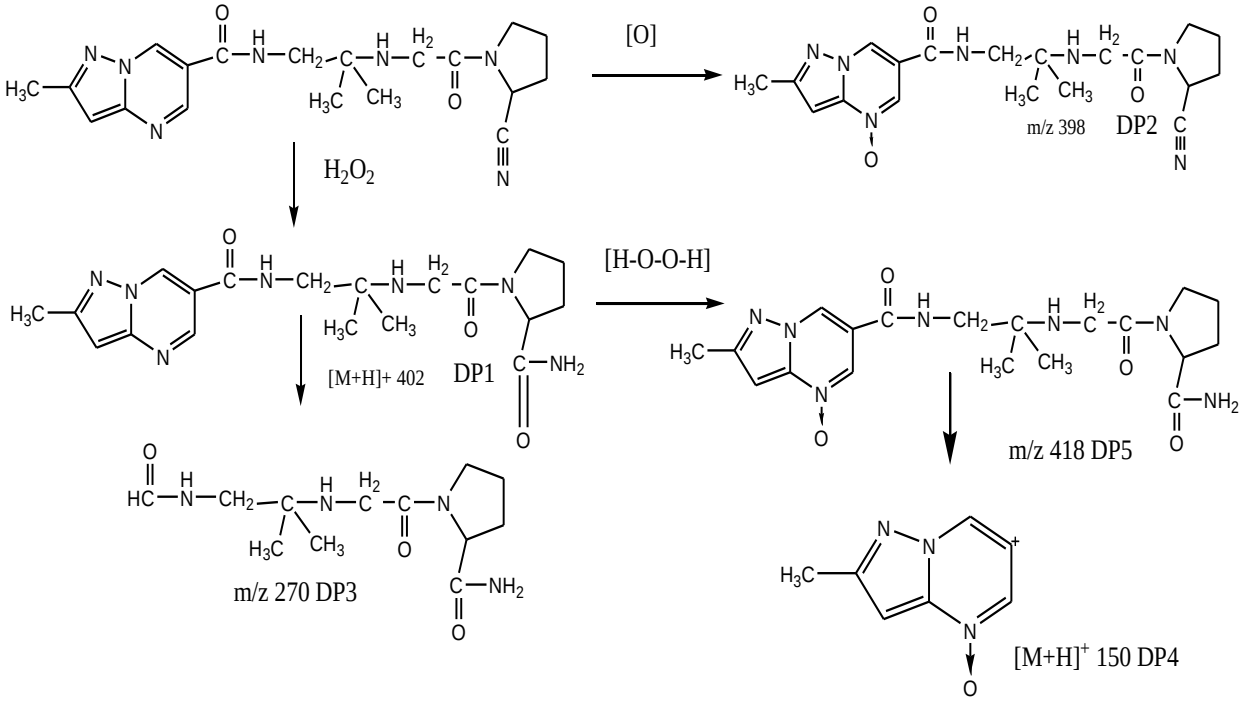
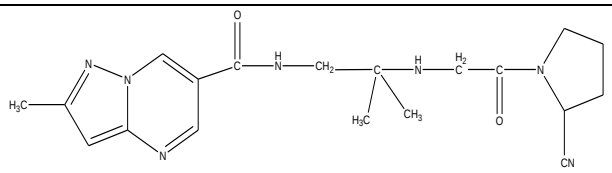
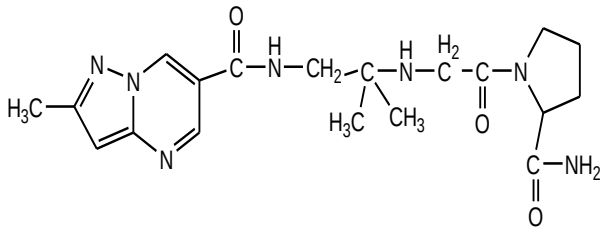
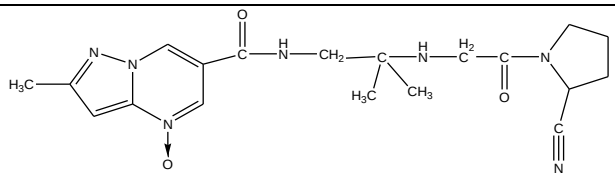
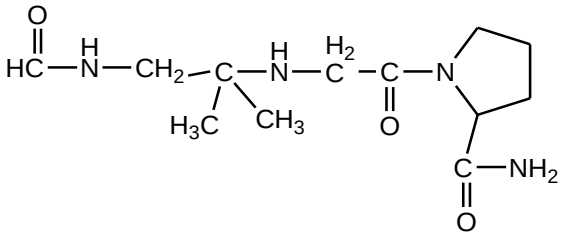
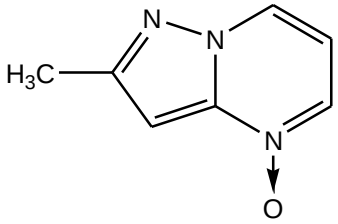
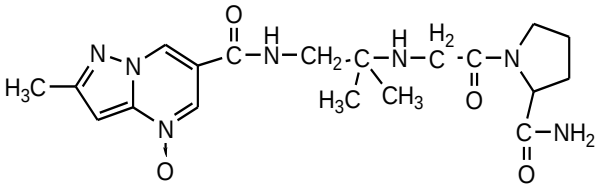


Fig. 6. 62- Degradation pathway in oxidative condition

Table 6. 21 - Chemical structures of ANA and its degradation products

Analyte	Structure	Molecular Formula Molecular Weight Fragments(m/z)	Degradation Route	Rt (LC-PDA)
ANA		383.45g, Fragments - 231, 159		22.43 min
DP1	 1-[2-[1-(2-methylpyrazolo[1,5-alpha]pyrimidine-6-carboxamido}-methyla-propan-2-yl-amino)acetyl)pyrolidine-2-carboxamide	401.21g, $C_{19}H_{27}N_7O_3$, Fragments – 384, 270, 242.	Alkaline, oxidative	13.6 min
DP2	 N-[2-({2-[(2S)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2-methylpyrazolo-[1,5-a]pyrimidine-N-oxide-6-carboxamide	$C_{19}H_{25}N_7O_3$, 399g , Fragments-231	Oxidative	36.5 min

DP3	 1-(2-(1-foramido-2-methylpropan-2-ylamino)acetyl)pyrrolidine-2-carboxamide	$C_{12}H_{22}N_4O_3$, 270.16 g, Fragment - 126	Alkaline , Oxidative	3.59 min
DP4	 2-methylpyrazolo[1,5-a]pyrimine-N-oxide	$C_7H_7N_3O$, 149.05 g, Fragment - 126	Oxidative	9.60 min
DP5	 1-[2-[1-(2-methylpyrazolo[1,5-alpha]pyrimidine-6-carboxamido}-methyla-propan-2-yl-amino)acetyl)pyrolidine-N-oxide-2-carboxamide	$C_{19}H_{27}N_7O_4$, 417.21g, Fragment - 231	Oxidative	27.9 min

6.7.3. DISCUSSIONS

Two degradation products in alkaline conditions and five degradation products in oxidative conditions were identified by LC-MS. DP1 and DP3 are formed in both alkaline and oxidative conditions. DP1 is formed from ANA by alkaline hydrolysis/oxidation of ANA. DP3 is formed from DP1. DP2 is formed from ANA by formation of N-oxide. DP5 is formed from DP1 by formation of N-oxide which on further fragmentation forms DP4.Details of ANA and its degradation products are given in Table 6.21. The reported HPLC [12] method shows significant degradation in acidic, alkaline, oxidative, thermal and photolytic conditions. However number of

degradation products in each condition and degradation products are not identified. Comparison of reported method and our developed HPLC method is shown in Table 6.22.

Table 6. 22 - Comparison of reported method [12] and developed HPLC method

Degradation conditions	**Reported Method [12]**	**Developed method**
Acid	0.1 M HCl RT for 5 hrs 23.03% degradation	1 M HCl 80˚C for 6 hrs, 3.1% degradation, 6 DPs
Alkaline	0.1 M NaOH RT for 4 hrs, 29.5% degradation	0.1 M NaOH RT for 1 hr, 26.49 % degradation, 2 DPs
Neutral hydrolytic	---	Water 80˚c for 6 hrs, 4.2% degradation , 5 DPs
Oxidative	3% H_2O_2 RT 3 hrs, 32.5% degradation	0.3% H_2O_2 RT for 2 hrs, 17.5% degradation , 7 DPs
Thermal	70˚C for 4 hrs, 20.2% degradation	Dry at 80℃ for 11 days, 1.45% degradation, 5 DPs
Photolytic (Dry)	---	11 days , 0.8% , 4 DPs
Photolytic (Solution)	UV chamber for 36 hrs,11.7% degradation	11 days, 3.78%, 5 DPs
Other information	Number of Degradation products are not reported and not identified	Degradation products in alkaline and oxidative conditions are identified by LC-MS, one degradation products each in alkaline and oxidative condition are isolated and characterised by IR, NMR and Mass

6.8. CONCLUSION

Stability indicating method was developed for determination of Anagliptin by HPLC. Significant degradation was observed in alkaline and oxidative condition and slight degradation was observed in acidic, neutral hydrolytic and photolytic condition. The method developed was validated as per guidelines by ICH. Degradation products in oxidative and alkaline conditions were identified by LC-MS. Both alkaline and oxidative degradation follows first-order kinetics. One degradation product each in alkaline and oxidative condition was isolated and characterized by mass, NMR and IR techniques. The degradation pathway in alkaline and oxidative condition was postulated. Structures of all these degradation products are not reported hitherto.

6.9 REFERENCES

1. Nishio S, Abe M, Ito H. Anagliptin in the treatment of type 2 diabetes : safety, efficacy and patient acceptability. Diabetes, Metabolic syndrome and Obesity: Targets and Therapy 8 : 163-171.
2. Kato N, Oka M, Murase T, Yoshida M, Sakiri S, Yakufu. Synthesis and pharmacological characterization of potent, selective and orally bio-available isoindoline class dipeptidylpeptidase IV inhibitors. Organic and Medicinal Chemistry Letters . 2011: 1-7.
3. www.drugbank.ca/drugs/DB12417. Accessed on 4th June 2019.
4. Patents.google.com.patent/WO2014051023A1/en.
5. Bhatti P. Review on analytical methods of metformin HCl and anagliptin. World Journal of Pharmacy and Pharmaceutical Sciences. 2017; 10(6) : 396-403.
6. Patil A, Shirkedkar A. Development and validation of five simple UV-spectrophotometry methods for estimation of anagliptin in bulk and in house tablets. Pharmaceutical Methods. 2016; 7(2) : 127-131.
7. Shah S, Maheshwari D. First order derivative spectrophotometric method for simultaneous estimation of anagliptin and metformin HCl in bulk and synthetic mixture. Journal of Global Trends in Pharmaceutical Sciences. 2015; 6(4) : 2925-2929.

8. Shah J, Majithia R, Maheshwari D. Development and validation of analytical method for estimation of anagliptin tablet dosage form by UV spectrophotometric method. International Journal of Pharmacy and Technology.2015; 6(4) : 7765-7771.
9. Gampa N, Chevala N, Pinamanneni P, Arulswamy P, Venkata P, Rama Rao N. Development and Validation of enantioselective analysis of anagliptin by using Chiral Lux Cellulose-3-Column. International Journal of Chemical and Pharmaceutical Analysis. 2017; 4(2) : 1-8.
10. Patil A, Shirkhedkar A. Application of quality-by-design in the development of HPTLC method for estimation of anagliptin in bulk and in-house tablets. Eurasian Journal of Analytical Chemistry. 2017; 12(5) : 443-458.
11. Furuta S, Smart C, Hackett A, Benning R, Warrington S. Pharmacokinetic and metabolism of [14 C] Anagliptin, a novel dipeptidyl peptidase-4- inhibitor in humans. Xenobiotica. 2013; 43(5) : 432-442.
12. Bhatti P, Panchal H. Stability indicating chromatographic method development and validation for the simultaneous estimation of metformin HCl and anagliptin in its synthetic mixture by HPLC. World Journal of Pharmaceutical Research. 2017; 6(11) : 956-973.
13. Guideline ICH. Validation of analytical procedure : Methodology Q2B, in: International Conference on Harmonisation, IFMPA, Geneva (Switzerland), 1996.
14. Guideline ICH. Stability testing of new drug substances and products Q1A(R2), in : International Conference on Harmonisation , IFMPA, Geneva (Switzerland), 2006.
15. Guideline ICH. Stability testing: Photostability testing of new drug substances and products Q1B, in: International Conference on Harmonisation , IFMPA, Geneva (Switzerland), 1996.
16. FDA, Guidance for Industry: Stability Testing of Drug substances and Drug Products (Draft Guidance), Food and Drug administration, Rockville, MD, 1998.