CHAPTER-4: IMPURITY PROFILING, ISOLATION, CHARACTERISATION AND DEGRADATION KINETICS OF IMPURITIES IN NELARABINE

4.1 INTRODUCTION AND SELECTION OF DRUG:

Antimetabolites specifically nucleobase and nucleoside analogues are the anticancer agents that have revolutionized the clinical oncology and made the cancer into a curable disease[1]. Benchmark drugs in this category are cytarabine for acute myeloid leukemia and gemcitabine for pancreatic and lung cancer [2]. Nucleobase and nucleoside analogues act by mimicking endogenous nucleosides and thereby following phosphorylation and nucleotides. This can be mediated either by enzyme inhibition or by substitution of endogenous nucleosides leading to DNA and RNA damage and interference with DNA methylation [1]. Nelarabine is a guanosine nucleoside analogue, arabinosylguanine (ara-G) which is prodrug of arag-G and based on its activity was approved for the treatment of T-cell acute lymphoblastic leukemia (ALL) and lymphoma (LBL) [3].

4.2 DRUG PROFILE[20,21]:

IUPAC name: (2*R*,3*S*,4*S*,5*R*)-2-(2-amino-6-methoxypurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol

Molecular formula: $C_{11}H_{15}N_5O_5$

Solubility: Slightly soluble to soluble in water

Figure-4.1-A: Molecular structure of nelarabine

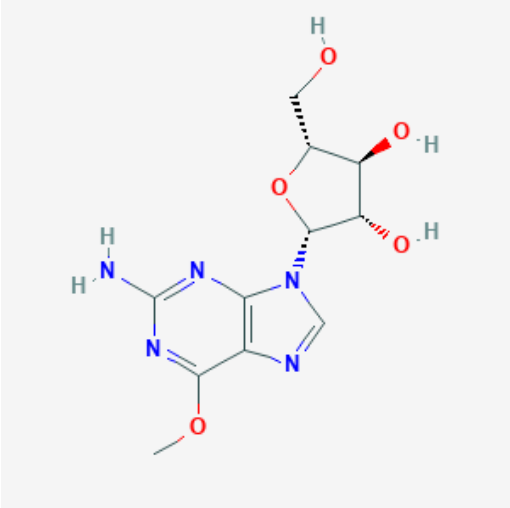
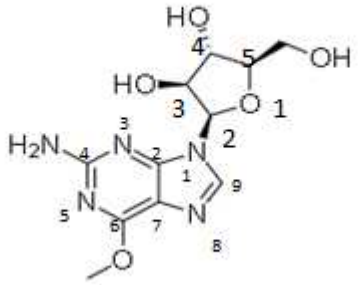


Figure-4.1-B: Molecular structure of nelarabine with designated numbers



(2R,3S,4S,5R)-2-(2-amino-6-methoxypurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol

Chemical name: 2-Amino-9-beta-D-arabinofuranosyl-6-methoxy-9H-purine

Molecular mass: 297.27

Commercial formulation: Arranon (US) and Atriance (EU) as 250 mg/50 mL (5 mg/mL) single-dose vial injection from Novartis Pharmaceuticals Corporation

4.3 LITERATURE REVIEW:

Nelarabine drug has been seed citations since 2005 as novel purine nucleoside analogues. During literature review, it was found that initially, T. Robak et al published article in 2005 showing the importance of purine nucleoside analogues such as fludrabine and pentostatin in the treatment of hematological malignancies [4]. Nelarabine and clofarabine drugs were mentioned as the under current clinical evaluations [4]. Until 2006, fludarabine, cladribine, chlorodeoxyadenosine and pentostatin were approved by FDA for hematological malignancies whereas clofarabine, nelarabine, 8-chloroadenosine and immucillin were considered as novel drugs in this category and were under clinical study [5-8]. However, there is no any literature found until 2014 for analytical method for estimation of nelarabine. One article was found to be published in 2014 where simultaneous determination of nelarabine and its active drug metabolite 9-b-D-Arabinofuranosylguanine (Ara-G) in human plasma [9]. Berg SL et. al, and Rodriguez CO Jr, et. al. reported HPLC and LC-MS method for quantification of nelarabine and other active moieties [10-11]. Form the years 2015 to 2020 various synthetic strategies were published for the novel drug nelarabine [12-13]. S Vidyadhara et. al reported HPLC method of nelarabine and als performed degradation of nelarabine but it was very

preliminary and did not identify any degradation products generated. However, there is not any published literature found which discuss about the degradation products of the nelarabine in various degradation condition and thereby characterization of its degradation products using sophisticated techniques of NMR, LC-MS and HRMS techniques.

4.4 SECTION-A: ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF NELARABINE IN PRESENCE OF ITS DEGRADATION PRODUCTS BY LC-MS

4.4.1 FORCED DEGRADATION STUDY AND ANALYTICAL METHOD DEVELOPMENT

The forced degradation experiments were performed according to ICH Q1B guidelines to test the stability of nelarabine under the following conditions: acidic and alkaline hydrolysis, oxidation, heat and light.

4.4.1.1 EXPERIMENTAL

4.4.1.1.1 Chemicals, reagents and materials

The active pharmaceutical ingredient nelarabine was generously provided by Sun Pharmaceuticals Industries Limited., Vadodara, India. Milli-Q water was obtained from the Milli-Q ®Integral water purification system. H_2O_2 30% (Perhydrol®) for analysis EMSURE® ISO, Merck), HCl 34-37% (Trace Metal grade, Fischer Scientific, UK), NaOH pellets (AR grade, Rankem, Mumbai, India) was utilized for stress degradation studies [21]. Formic acid 98-100% (analytical reagent grade, Rankem, Thane, India) was used for dilution. In addition, Trifluoroacetic acid was used of spectrochem (IR, NMR Grade).

4.4.1.1.2 Instruments:

4.4.1.1.2.1 High performance liquid chromatography

The HPLC system (Waters Alliance 2695) equipped with a PDA detector and with the Empower 3.0 software was used for chromatographic studies. The pH of the buffer solution was adjusted using Eutech (Model: PH-510) pH meter. Ultrasonic cleaner (Leelasonic-500) was used for degassing the mobile phase and other solutions.

4.4.1.1.3 Solutions preparation under various degradation conditions for forced degradation study

The sample solutions used for forced degradation study were having concentration of 0.1mg/mL in milli-Q water as a diluent. [15-19]

4.4.1.1.3.1 Acidic condition

Approximately 1 mg of nelarabine was accurately weighed and transferred into 10 mL of volumetric flask; 1mL of 1M hydrochloric acid solution in water was added. The solution was heated at 60°C for 1-2 minutes and neutralized with 1M sodium hydroxide solution before injection. Finally, the solution was diluted up to the mark with diluent. Similarly, the blank solution was also prepared in the same condition omitting the sample in preparation.

4.4.1.1.3.2 Alkali condition

For alkali degradation, approximately 1 mg of nelarabine was accurately weighed and transferred into a 10 mL volumetric flask. The solution was heated at 60°C for 15 minutes and then neutralized with 1M hydrochloric acid solution before injection and finally diluted up to the mark with diluent. Similarly, the blank was also prepared in the same condition omitting the sample in preparation.

4.4.1.1.3.3 Oxidation condition

Approximately 1 mg of nelarabine was accurately weighed and transferred into a 10 mL volumetric flask, followed by the addition of 1mL of 30 % hydrogen peroxide solution. The solution was heated in water bath for 15 minutes at 60°C temperature and diluted up to the mark with diluent. A blank was also prepared under the same condition omitting the sample in preparation.

4.4.1.1.3.4 Photolytic condition

Approximately 1 mg of nelarabine was accurately weighed and transferred into a 10 mL volumetric flask and diluted up to the mark with diluent. Moreover, 10 mg of nelarabine

was exposed directly to UV-visible light in the photo-stability cabinet for 24 hours duration. Similarly, the blank was also prepared in the same condition omitting the sample in preparation.

4.4.1.1.3.5 Thermal condition

Approximately 1 mg of nelarabine was accurately weighed and transferred into a 10 mL volumetric flask, diluted up to the mark with diluent, and exposed to heating at 60 °C for 60 minutes. Similarly, the blank was also prepared in the same condition omitting sample in preparation.

Similarly, degradation was also performed with synthetic mixture solution by taking 1.9 mg weight of nelarabine synthetic mixture in each possible degradation condition.
All the degradation samples were then injected in HPLC system after analytical method development trials and finalization of the analytical conditions and recorded the chromatograms with all the degradation products if generated. After confirmation of degradation in acidic, alkali, oxidative condition, those degradation samples were run later in LC-MS and HRMS instrument to obtain the exact mass and fragmentation pattern of degradation products.

For analytical method development, it was tried first to develop LC-UV-PDA detector method with mobile phase and diluent of MS compatible category so that it would be used for LC-MS and HRMS study later for structure elucidation purpose. In nelarabine, too, it was possible to develop LC-UV method with MS compatible mobile phase and diluent. Following sections are for method development trials:
MS compatible LC method was developed which was started with sample solution preparation, wavelength selection, stationary phase selection, followed by mobile phase and diluent optimization and peak symmetry optimization. The details are as mentioned below:

4.4.1.1.4 Solution preparation and diluent selection for analytical method development:

100µg/mL solution of nelarabine was prepared in milli-Q water. Since nelarabine observed to be freely soluble in water, initially the diluent was taken as milli-Q water only. During developmental trials of nelarabine, no major issues were noted for peak shape of nelarabine and its degradation products. Up to some extent, gradient program and other optimizations were required to be made but diluent as milli-Q water was not required to be changed. Hence, in final optimized condition also, milli-Q water was kept as diluent.

As the commercial formulation of arranon injection was not available, synthetic mixture solution was prepared in laboratory to be used in study wherever required [22]. As per the literature, arranon injection contains 5mg/mL nelarabine as active ingredient and 4.5mg/mL sodium chloride as inactive ingredient in 50mL water for injection. Considering this, synthetic mixture solution was prepared by dissolving mixture of nelarabine and sodium chloride in the ratio of 5:4.5 i.e., 1:0.9. Placebo solution was also prepared by dissolving only 4.5mg/mL sodium chloride as inactive ingredient in water.

4.4.1.1.5 Analytical wavelength selection:

Degradation sample of nelarabine was run in HPLC with PDA detector and the PDA chromatogram was recorded in the range of 200-400 nm to check the points of wavelength where sufficient response of nelarabine and its degradation products are observed maximum.

4.4.1.1.6 Mobile phase optimization:

4.4.1.1.6.1 Trial-1:

Method development initiated by taking water and acetonitrile as mobile phase-A and mobile phase-B respectively, however it was noted that retention time of nelarabine was observed very early and peak shape was not proper. The column selected was of Waters X-Bridge C18 (250X4.6), 3.5µ column was maintained at room temperature during analysis. The injection volume set as 20µl. The wavelength was selected as 248 nm based on wavelength maxima run. In this first trial, the run was performed using isocratic flow rate of 0.5mL/min with mobile phase-A and mobile phase-B in the ratio of 50:50 for 50

minutes duration.

4.4.1.1.6.2 Trial-2:

Hence to retain the nelarabine peak further in the chromatogram and to improve the peak shape, two changes were made: firstly, to reduce the percentage of organic mobile phase-B throughout the run and addition of trifluoroacetic acid in mobile phase-A at the concentration level of 0.01%. Based on this, the gradient program was changed from initial 50% acetonitrile to 0.0% acetonitrile at 0.0 minutes and reaching to 10% at 10 minutes. This 10% of mobile phase-B was again kept isocratic throughout the run before washing and equilibration step. Secondly, in case of trifluoroacetic acid, the target was to use minimum trifluoroacetic acid as it might suppress the response of compound in MS detector required for further analysis. Hence, initial concentration was chosen as 0.01% which was not required to be increased further based on the appropriate observations on retention time and other parameters. Other analytical conditions were kept same as trial-1.

4.4.1.1.6.3 Trial-3:

Further to this, to improve the baseline and baseline disturbance due to gradient programming, mobile phase-B composition was also slightly modified as the mixture of mobile phase-A and acetonitrile in the ratio of 10:90. All other conditions were kept same as trial-1 and trial-2

4.4.1.2 RESULTS AND DISCUSSION

4.4.1.2.1 Forced degradation results

Nelarabine drug was found to be degraded extensively in acidic and alkali condition and marginally in peroxide condition. The drug was found to be almost stable in thermal as well as UV exposure degradation condition (Figure-4.2-A to 4.2-E). The major degradation product generated in acidic degradation was found to be eluted at RRT 0.87 (21.91%) which observed to be easily generated when nelarabine exposed to acidic environment (Figure-4.2-B, Table-4.1). The major and significant degradation product in alkali degradation condition was found to be eluted at RRT 0.77 (Figure-4.2-C, Table-

4.2). Mass balance for acidic and alkali condition was also found to be achieved near to 100% suggesting all the degradation products generated are detected in the chromatograms. In oxidative degradation condition, nelarabine found to generate some degradation products but they are in very minor percentage as compared to nelarabine (Figure-4.2-D). These degradation products have been later on observed in HRMS study and characterized based on the spectrum obtained from HRMS study.

Table-4.1: Degradation products summary in acidic condition

Degradation products	**RT of Impurity**	**RRT**	**% observed**
DP-1 (Acid)	16.529	0.84	21.91
DP-2 (Alkali)	14.482	0.74	1.87
Nelarabine	19.663	1.00	75.72
Other degradation products	NA	NA	0.34
Mass balance			99.84

Table-4.2: Degradation products summary in alkali condition

Degradation products	**RT of Impurity**	**RRT**	**% observed**
DP-1 (Acid)	16.529	0.84	0
DP-2 (Alkali)	14.482	0.74	6.79
Nelarabine	19.663	1.00	93.19
Other degradation products	NA	NA	0
Mass balance			99.98

Figure-4.2-A: As such sample of nelarabine in water (100ppm)

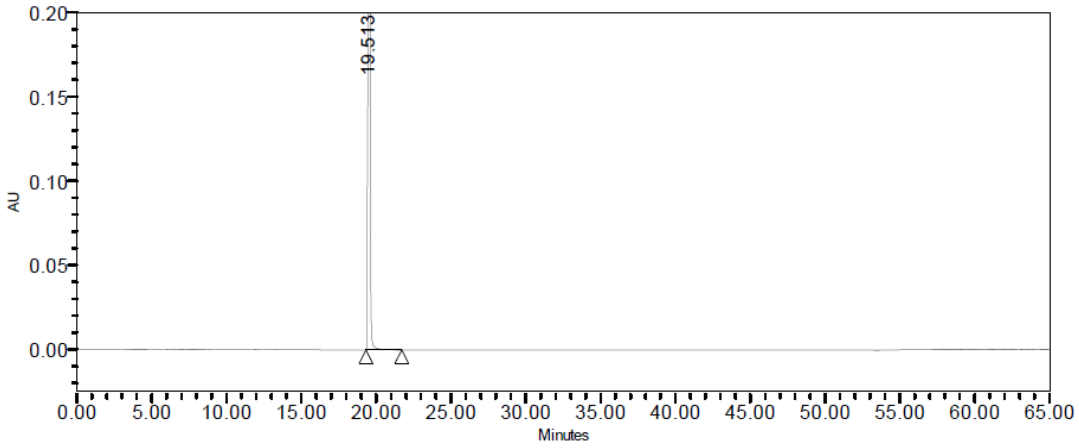


Figure-4.2-B: Degradation sample under acidic condition (1ml 1NHCl heating at 60°C for 02min)

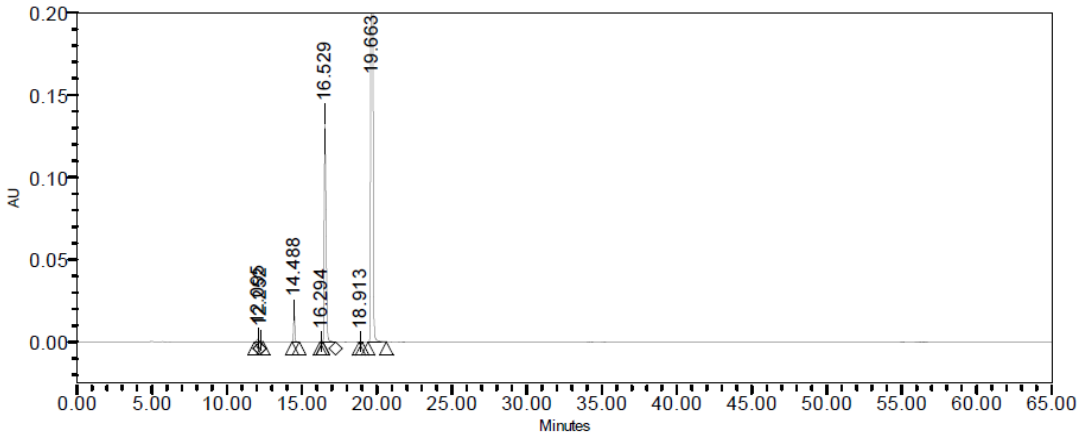


Figure-4.2-C: Degradation sample under alkali condition (1ml 1N NaOH heating at 60°C for 15min)

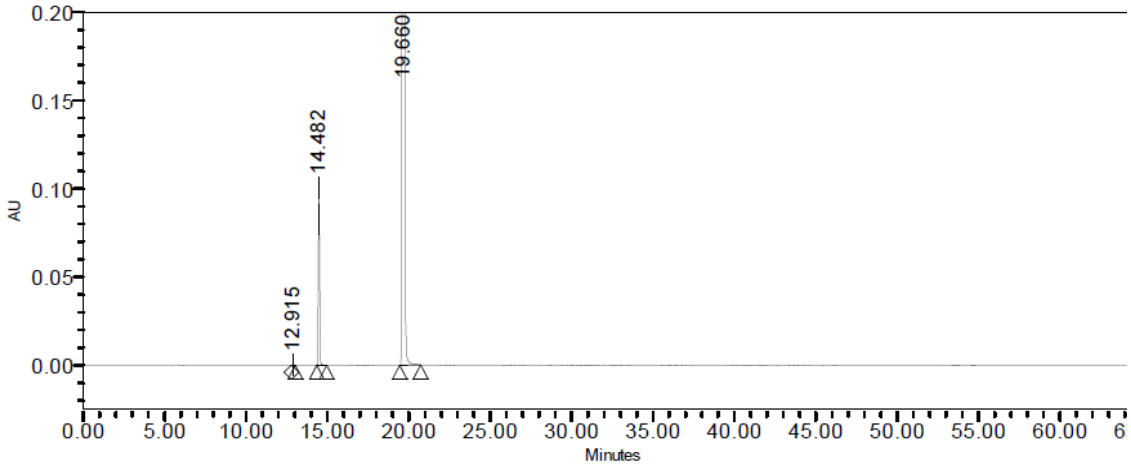


Figure-4.2-D: Degradation sample under oxidative condition (0.1ml 30% H_2O_2 heating at 60°C for 60min)

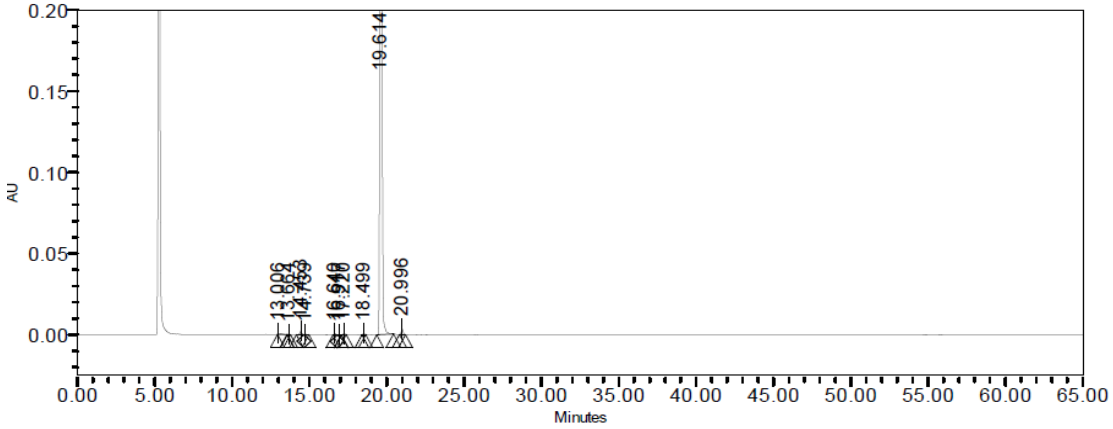


Figure-4.2-E: Degradation sample under thermal condition (Heating at 60°C for 60minutes)

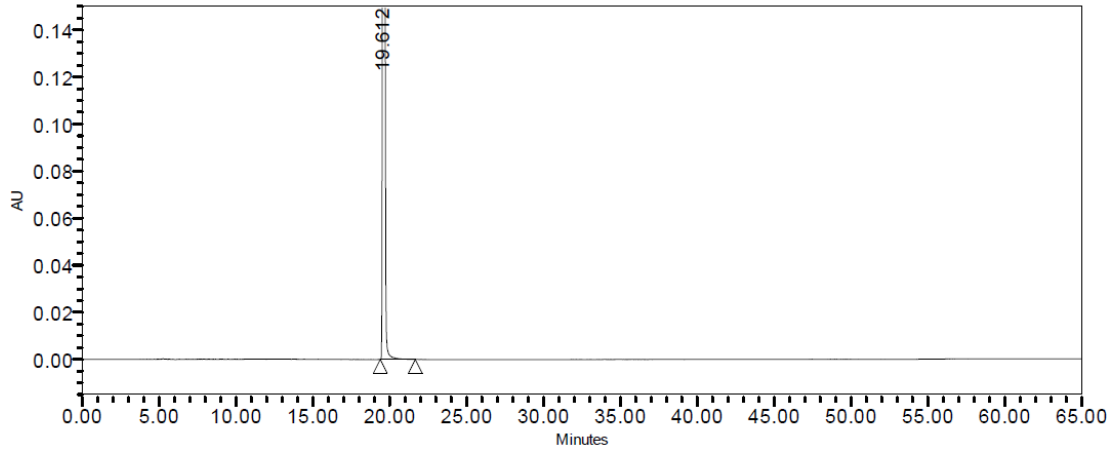
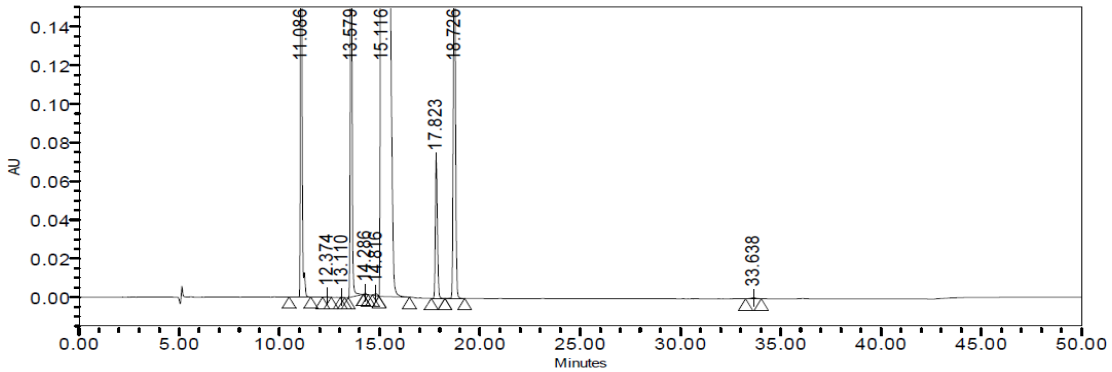


Figure-4.2-F: Degradation sample under acidic condition for preparative LC isolation (25ml 1M HCl heating at 60°C for 60min)



Following details covers the results and discussion obtained from method development trial experiments and then final analytical conditions for LC-MS, HRMS and NMR instrument methods.

4.4.1.2.2 Analytical wavelength selection:

For nelarabine, the wavelength maximum was observed at 246.6 nm and 280.9nm level whereas for other degradation products, it was ranging between 240 to 280nm region. But some degradation products do not have absorbance after 260 nm. Hence it was decided to keep 248nm as the detection wavelength in the analytical method to detect all possible impurities and nelarabine in the chromatogram. (Figure-4.3-A, 4.3-B)

Figure-4.3-A: Wavelength maxima of nelarabine

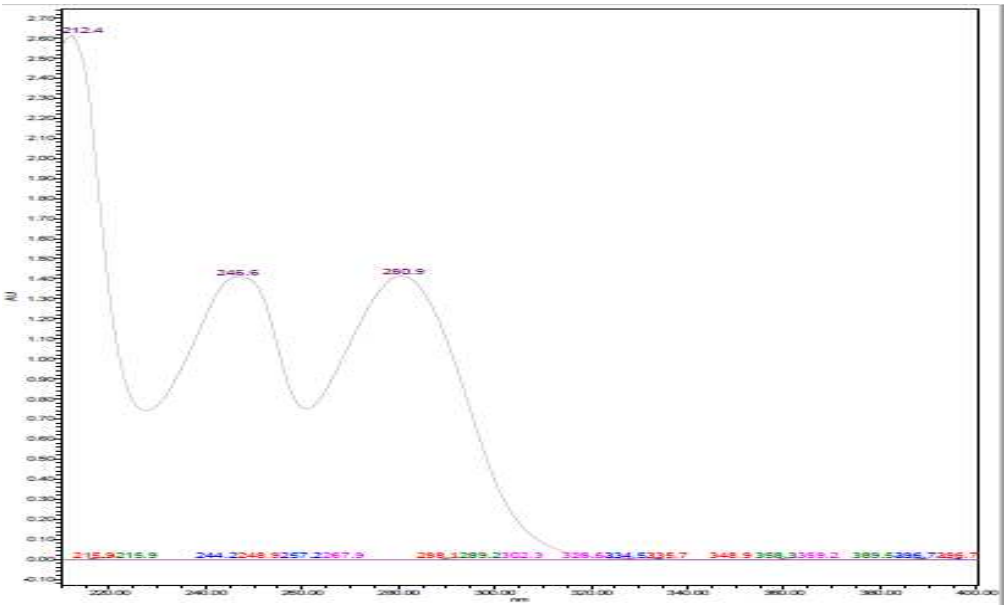
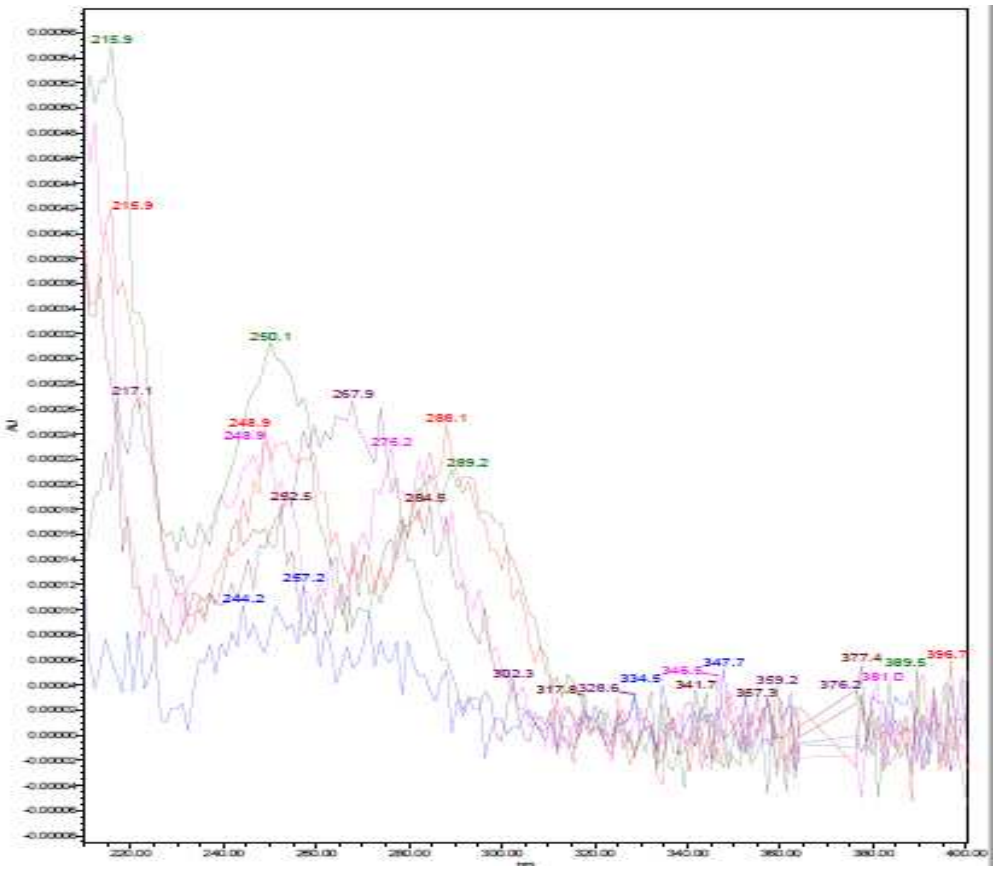


Figure-4.3-B: Wavelength maxima of nelarabine and its degradation products

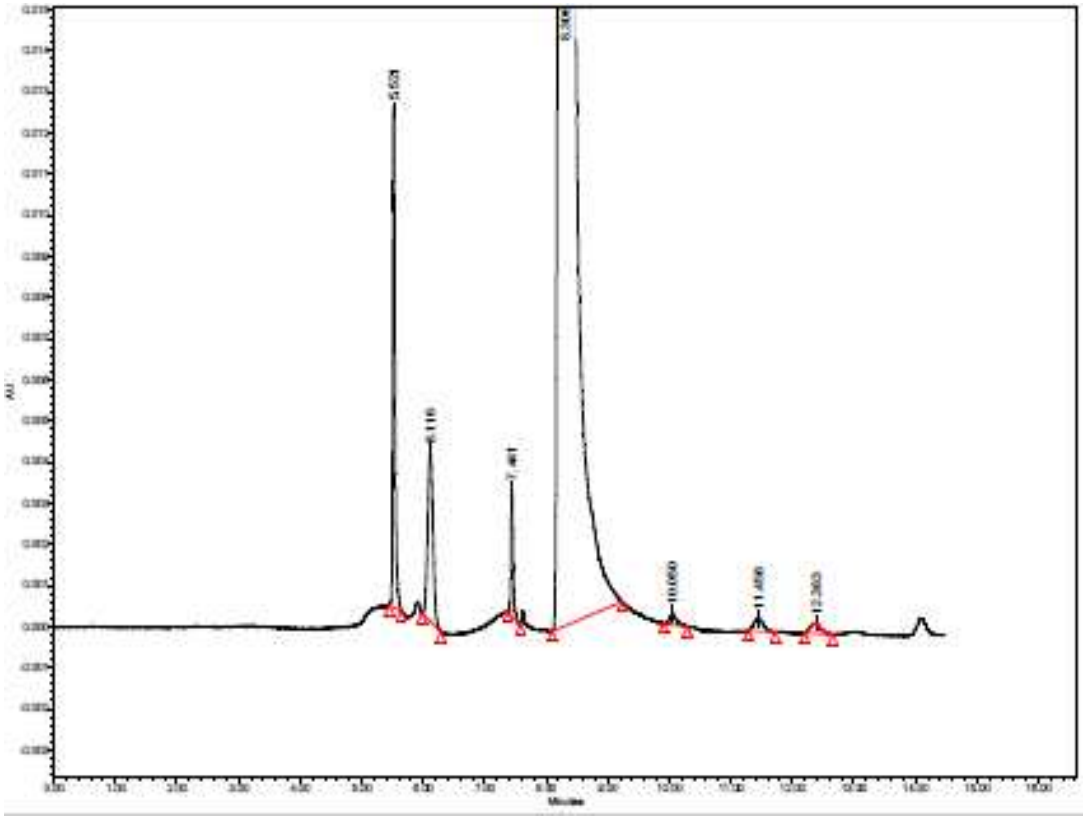


4.4.1.2.3 Mobile phase optimization:

4.4.1.2.3.1 Trial-1:

As shown in Figure-4.3-C, nelarabine peak eluted very earlier due to highly polar nature of the compound. Hence it was necessary to retain the compound peak further in the chromatogram by addition of some mobile phase modifiers. In nelarabine, it was observed that there are many polar impurities which elutes before nelarabine peak. Therefore, it was required to shift the retention time of nelarabine further late in the chromatogram in the region between 18-22 minutes so that proper resolution of all the impurities present before nelarabine peak can be achieved

Figure-4.3-C Chromatogram of nelarabine showing early elution



Chromatographic conditions:

Column: Waters X-Bridge C18 (250X4.6), 3.5µ

Mobile phase: Mobile phase-A as Water and mobile phase-B as acetonitrile

Detection wavelength: 248nm

Gradient and flow rate: Isocratic flow rate of 0.5mL/min with mobile phase-A and mobile phase-B in the ratio of 50:50 for 50 minutes duration.

4.4.1.2.3.2 Trial-2:

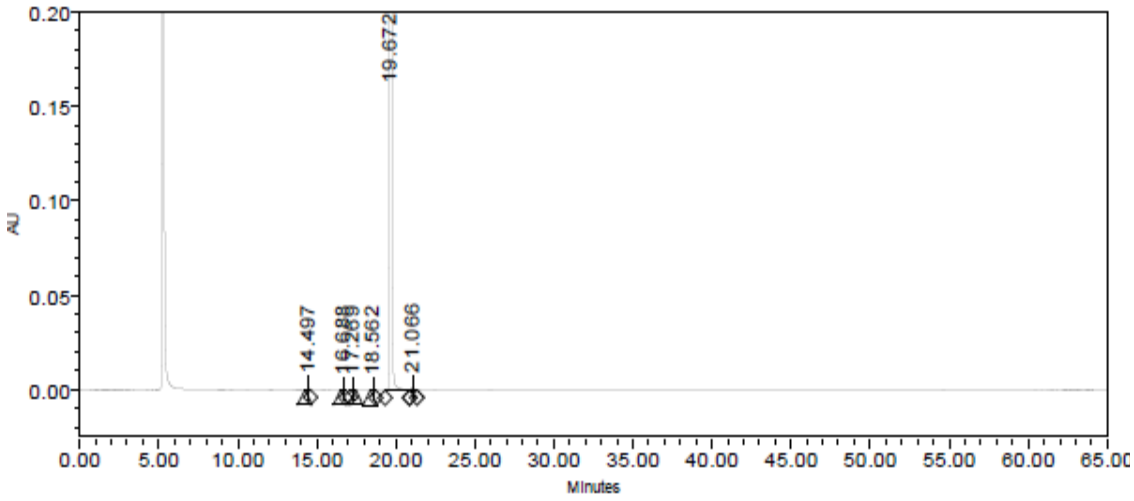
Since the focus was to try MS compatible method development, trifluoroacetic acid was thought to be added in mobile phase-A with first minimum concentration of 0.01%. With this change, nelarabine peak was found to be sufficiently retained in the region between 15-20 minutes (Figure-4.3-D). This low concentration of trifluoroacetic acid would help in MS analysis for baseline not getting disturbed as well as for sufficient response in TIC of degradation products.

4.4.1.2.3.3 Trial-3:

Moreover, the mixture of aqueous and organic composition in mobile phase is always recommended if possible. This would not only improve the gradient blank pattern of the run with improved baseline but also help to run the pump of the LC smoothly as compared to only organic components in mobile phase-B. Hence, mobile phase-B composition was modified to a mixture of mobile phase-A and acetonitrile in the ratio of 10:90 in addition to changes in gradient program, it showed significant improvement in baseline disturbance and peak symmetry parameters. (Figure-4.3-D)

Hence, after selection of appropriate diluent and sample concentration, wavelength maxima were decided. Then proper optimization of MS compatible mobile phase was satisfactorily achieved. Finally, MS compatible method was developed which can be used for LC, LC-MS and HRMS analysis in further study.

Figure-4.3-D: Chromatogram of nelarabine sufficiently retained at around 19.7 minutes.



Chromatographic conditions:

Column: Waters X-Bridge C18 (250X4.6), 3.5µ

Mobile phase: Mobile phase-A is composed of 0.01% trifluoroacetic acid in water whereas mobile phase-B is the mixture of mobile phase-A and acetonitrile in the ratio of 10:90.

Detection wavelength: 248nm

Gradient and flow rate: The gradient program was planned as linear gradient (Time/%B) which was set at 0.00/00, 10.0/10, 35.0/10, 35.5/00, 50/00.

4.4.1.2.4 Final analytical conditions for HPLC-UV-PDA method which is also MS compatible

Mobile phase-A is composed of 0.01% trifluoroacetic acid in water whereas mobile phase-B is the mixture of mobile phase-A and acetonitrile in the ratio of 10:90. Waters X-Bridge C18 (250X4.6), 3.5µ column was maintained at room temperature during analysis. The gradient program was planned as linear gradient (Time/%B) which was set at 0.00/00, 10.0/10, 35.0/10, 35.5/00, 50/00. The injection volume and flow rate were set as 20µl and 0.5mL/min respectively. The appropriate response of nelarabine and its degradation products were observed at a detection wavelength of 248nm. Mass spectrometric conditions were set as: capillary voltage: 3.5kV, cone voltage: 15V and 30V, extractor voltage: 1.00 V, RF lens: 0.4V, source temperature: 110°C, desolvation temperature: 350°C, cone gas flow: 25 L/Hr, desolvation gas flow: 650 L/Hr, collision energy: 2.0eV.

4.4.2 ANALYTICAL METHOD VALIDATION

Method validation experiments were conducted as per ICH guideline Q2 (R1) [15-19] and parameters such as linearity, sensitivity, accuracy, method precision, robustness and specificity were performed.

4.4.2.1 EXPERIMENTAL

The experimental details of each of the parameters are as per below texts:

4.4.2.1.1 Linearity

Linearity was performed by preparing the different concentration of nelarabine solution in the range of 25µg/mL to 150µg/mL. All the experiments were performed in triplicate. Calibration curve was plotted between the area response observed and the concentration. Correlation coefficient was calculated from the calibration curve. For good linear calibration curve, correlation coefficient should be more than 0.99.

4.4.2.1.2 Sensitivity

The LOD and LOQ was determined based on theoretical formula as per ICH guideline. The details would be as mentioned Chapter 3 Section 3.4.3.1.2.

4.4.2.1.3 Accuracy

The accuracy of the method was performed by the standard addition method whereby the 50%, 100% and 150% level concentration of 100µg/ml of nelarabine were spiked into the pre-quantified sample mixture of nelarabine. The spiked solutions were prepared in triplicate sets at each level, injected into the system and the %recovery was calculated at each of the three levels.

4.4.2.1.4 Precision

Repeatability was performed by injecting six replicate sample preparation of 100µg/mL. Method precision parameter was also performed by injecting three different levels of spiked samples at 50%, 100% and 150% levels of 100µg/mL in triplicate. The %RSD was calculated from the response observed at each level.

4.4.2.1.5 Robustness:

Robustness was performed by applying small but deliberate changes in flow rate. The flow rate was changed up to +/- 0.02mL/min and the effects on the results were monitored. The robustness of the method was estimated at 100µg/mL concentration and the retention time, peak shape, theoretical plates and tailing factor were compared.

4.4.2.1.6 Specificity

Specificity was ascertained by performing forced degradation study and identification of elution of the degradation products in the chromatograms. Forced degradation study was performed for acidic, basic, oxidative, thermal and UV light exposure.

4.4.2.1 RESULTS AND DISCUSSION

The results obtained for each of the parameters of linearity, sensitivity, accuracy, method precision, robustness and specificity were obtained satisfactorily within the specifications and are as mentioned in below details:

4.4.2.1.1 Linearity

The method was found to be linear in the range of 25µg/mL to 150µg/mL. The correlation coefficient was found to be 0.9973. The regression data are mentioned in Table-4.3 and calibration curve in Figure-4.4, 4.5 demonstrating good linear relationship between specified concentration and response observed.

Table-4.3: Results of linearity of nelarabine

Linearity level	**Concentration** (µg/mL)	**Mean area response**
25%	25	345200
50%	50	710250
75%	75	1437984
100%	100	1928589
125%	125	2419230
150%	150	2902176
	Slope (S)	21006.13549
-	**Intercept**	-216337.66667
	Correlation Coefficient (r)	0.9973
	Standard deviation	986282.8701
	LOD (µg/mL)	1.55
	LOQ (µg/mL)	4.70

Figure-4.4: Linearity calibration curve of nelarabine

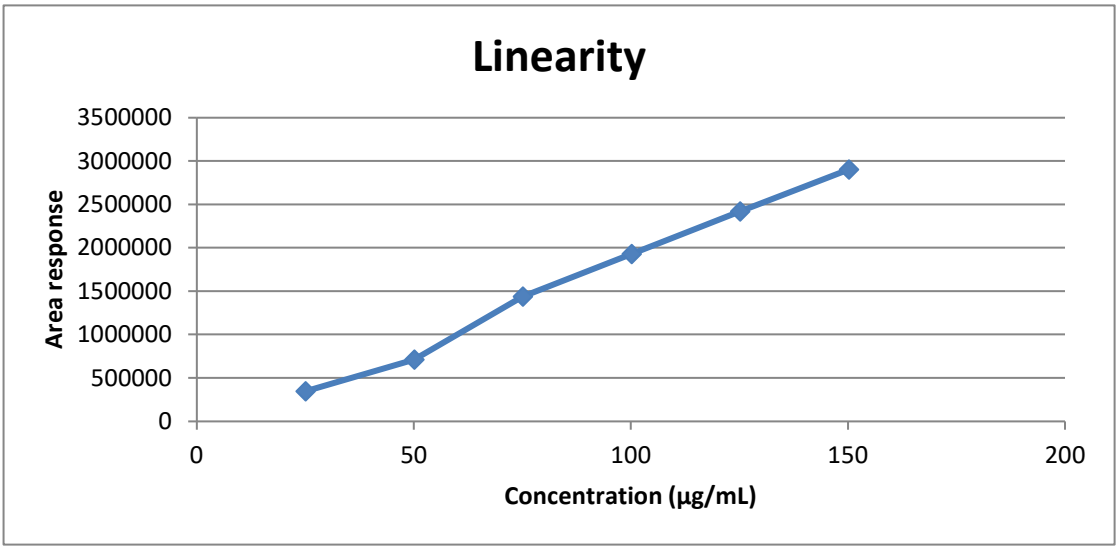
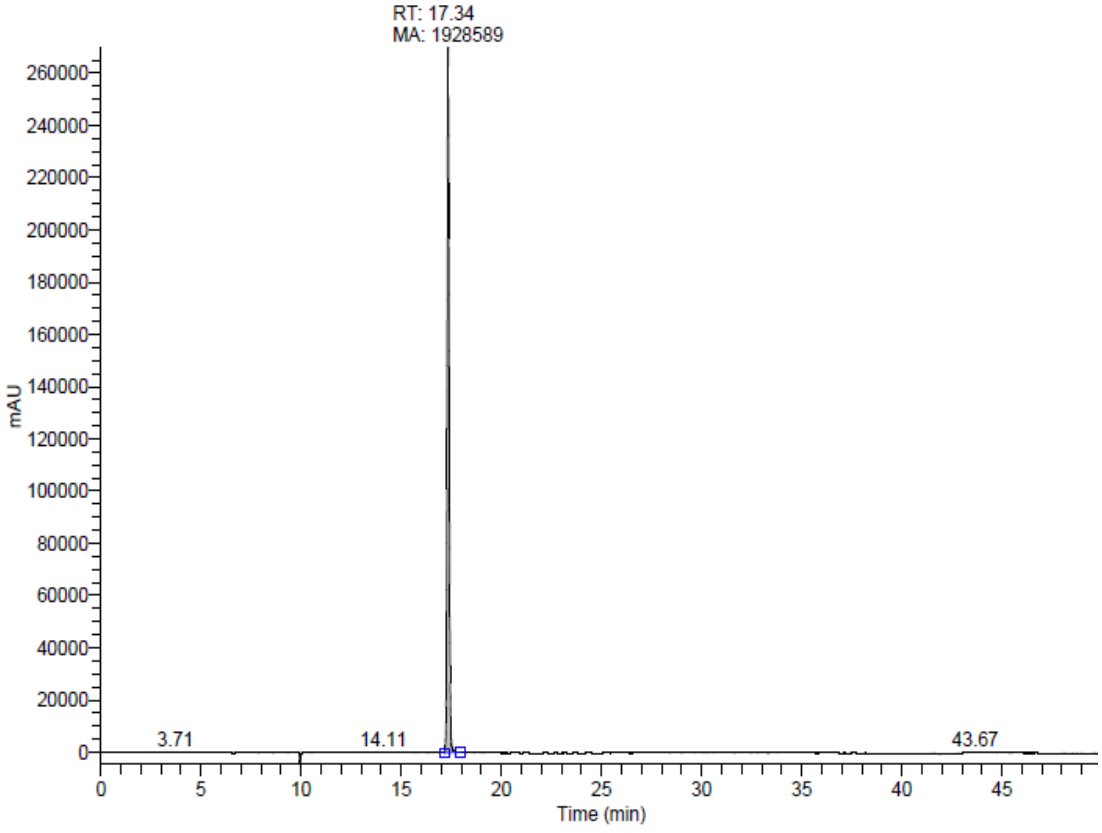


Figure-4.5 Chromatogram of 100µg/mL solution of nelarabine (100% linearity level)



4.4.2.1.2 Sensitivity (LOD and- LOQ)

Based on theoretical formula as per the ICH guideline, LOD was calculated as 1.55% level and LOQ was calculated as 4.70% level. Since the 100% level concentration is 100µg/mL, the LOD and LOQ in concentration (µg/mL) would remain same as 1.55µg/mL and 4.70µg/mL respectively. As per ICH guideline, the LOQ level at least below 50% level of target concentration is accepted for the method to be passed for method validation. The results obtained are well within the specifications as per ICH guideline.

The calculated values of LOD and LOQ suggest that the method can be sufficiently sensitive, but considering that the method was applied to study degraded samples, the linearity range was obtained at a higher level.

4.4.2.1.3 Accuracy

As such there is no any exact specifications of Recovery however; %recovery obtained between 75% to 125 which is considered reasonably acceptable with consideration of analytical errors and other matrix effects. As per the recovery experiment performed, the % recovery at the levels of 50%, 100% and 150% were obtained in the range of 92.54% to 109.67% which proves that the method is capable of estimating the content accurately near to true value. (Table-4.4 and Figure-4.6-A, 4.6-B, 4.6-C)

Table-4.4: %Recovery results at 50%, 100% and 150% levels

Accuracy level	Area of as such sample	%Amount spiked in as such sample	Observed area response	Area difference between recovered and as such sample	As such recovery %	%Recovery	Mean of area response	Standard deviation	%RSD
50% Set-1	710250	50.00	1652668	942418	48.87	97.73	935674.00	40408.31	4.32
50% Set-2	710250	50.00	1602568	892318	46.27	92.54			
50% Set-3	710250	50.00	1682536	972286	50.41	100.83			
100% Set-1	710250	100.00	2756242	2045992	106.09	106.09	2052039.67	60276.47	2.94
100% Set-2	710250	100.00	2825362	2115112	109.67	109.67			
100% Set-3	710250	100.00	2705265	1995015	103.44	103.44			
150% Set-1	710250	150.00	3652007	2941757	152.53	101.69	2949806.67	39080.3	1.32
150% Set-2	710250	150.00	3702535	2992285	155.15	103.44			
150% Set-3	710250	150.00	3625628	2915378	151.17	100.78			

Figure-4.6-A: Chromatogram of nelarabine at 50% level

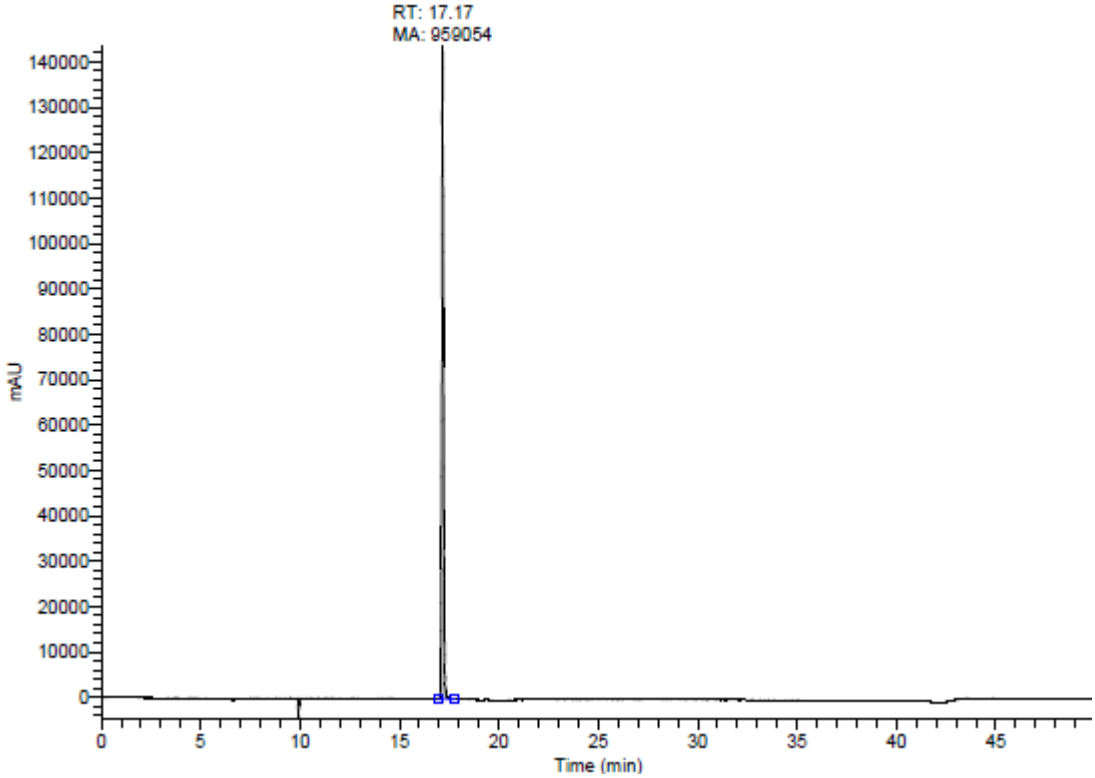


Figure-4.6-B: Chromatogram of nelarabine at 100% level

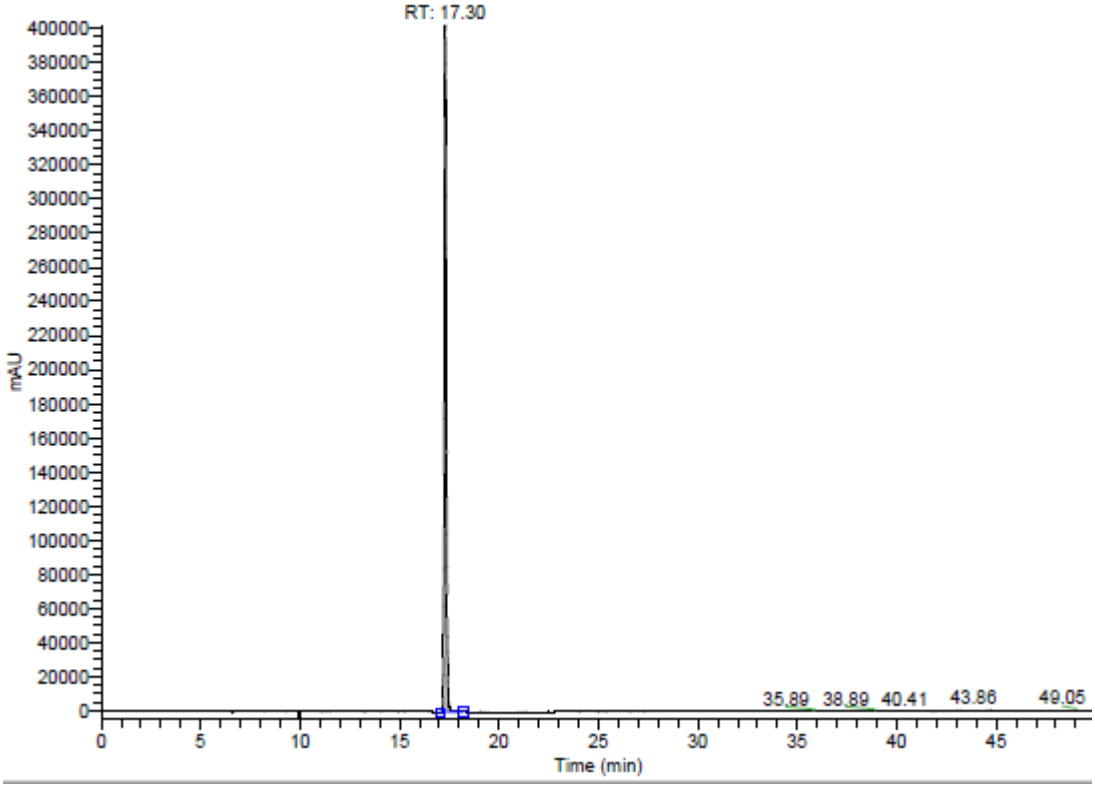
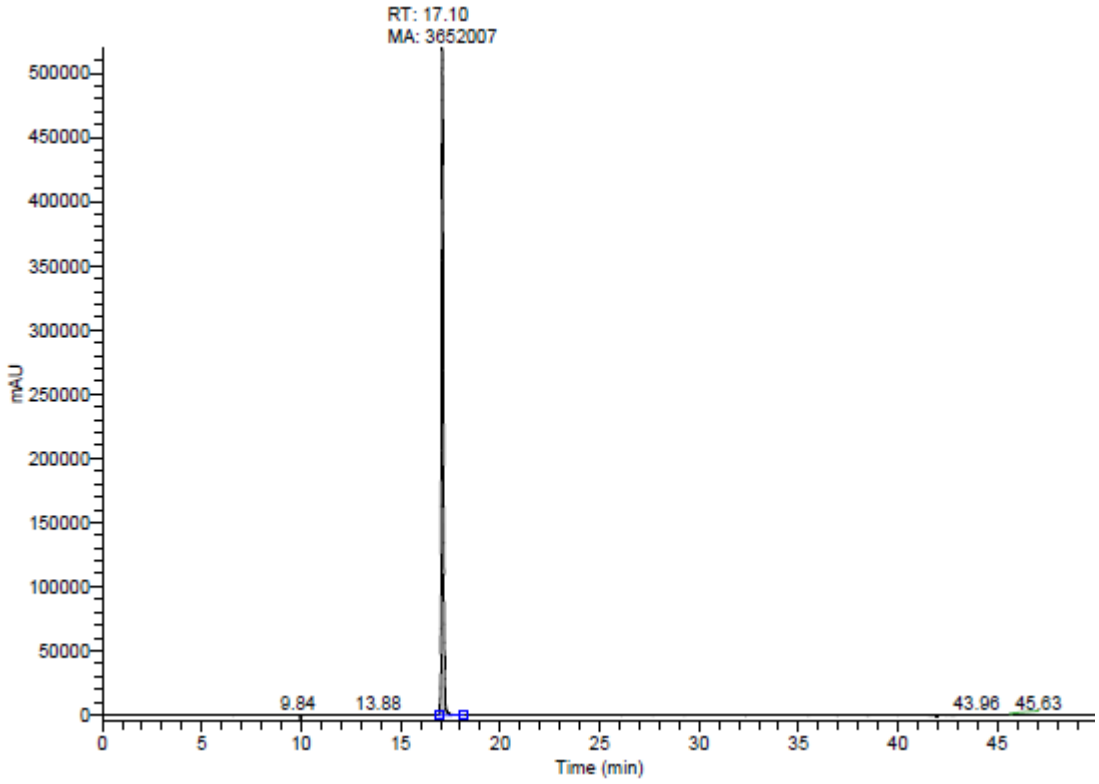


Figure-4.6-C: Chromatogram of nelarabine at 150% level



4.4.2.1.4 Precision

The %RSD of response observed for replicate preparation of 100µg/mL concentration suggest the variability of the method between the measurements. The % RSD of the six replicate- sample preparation at 100% level solutions was obtained as 0.16%. The %RSD of the response obtained at 50%, 100% and 150% level concentration was found in the range of 1.32% to 4.32%. Each kind of precision study results prove that the method is precise. (Table-4.5)

Table-4.5: Method precision results

Method Precision	
Method precision set	**Area response observed**
Set-1	2756242
Set-2	2755264
Set-3	2746582
Set-4	2756942
Set-5	2757564
Set-6	2758652
Mean	**2755207.67**
Standard deviation	**4379.47**
%RSD	**0.16**

4.4.2.1.5 Robustness

The chromatogram results were observed at flow rate of 0.48 mL/min and 0.52 mL/min. The peak shape obtained proper in each of the variations. The theoretical plates, tailing factor and retention times obtained were compared with that of actual method of analysis parameters. These results are not observed to be varied extensively. Hence proposed analytical method was proved to be robust. (Figure-4.7-A, 4.7-B)

Figure- 4.7-A: Chromatogram of nelarabine solution for robustness study at flow rate of 0.48 mL/min

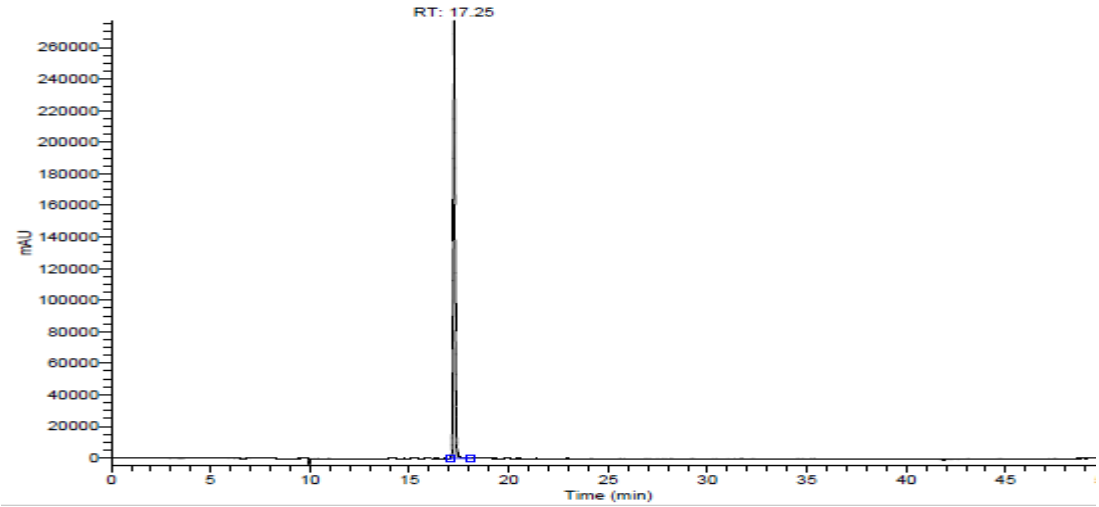
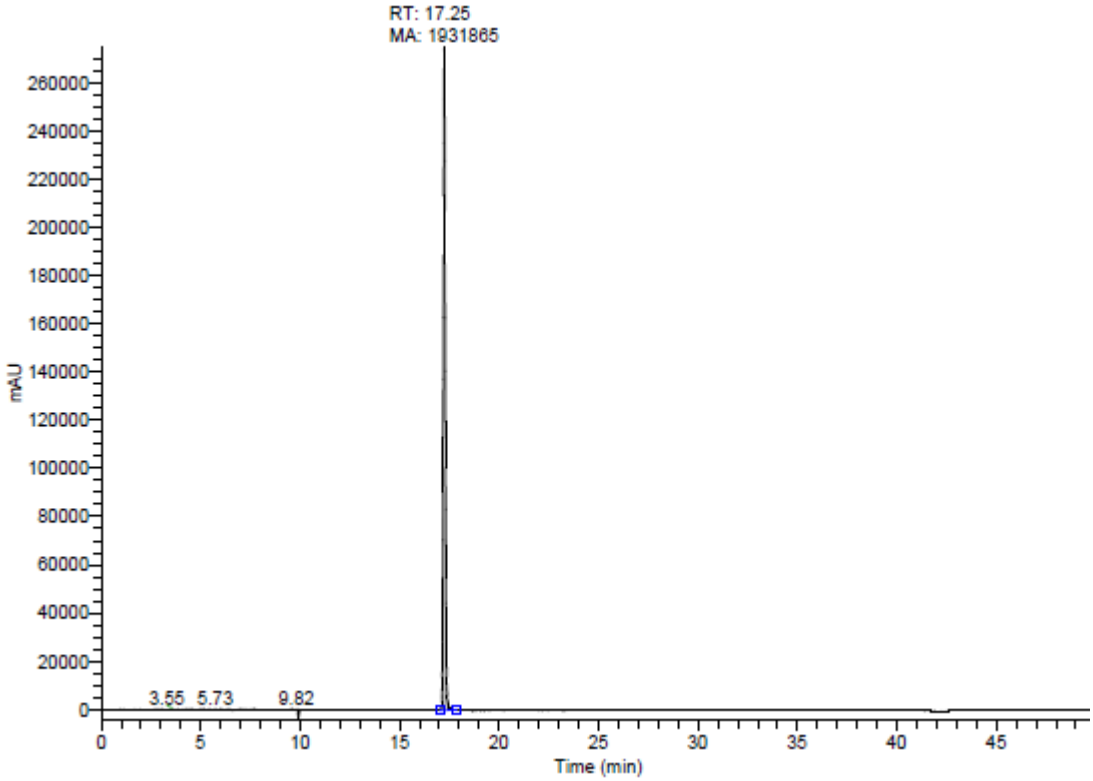


Figure-4.7-B: Chromatogram of nelarabine solution for robustness study at flow rate of 0.52 mL/min



4.4.3 IDENTIFICATION OF DEGRADATION PRODUCTS BY HRMS

After confirmation of degradation in oxidative condition, oxidative degradation sample was run in LC-MS and HRMS instrument to obtain the exact mass and fragmentation pattern of degradation products.

4.4.1.1.4 Analytical condition and instrument parameters for HPLC and LC-MS

The liquid chromatography-mass spectroscopy system (LC-MS/MS) of Waters Micro-mass with ZQ-Mass detector and electro-spray ionization mode was used.

As MS compatible method was developed as mentioned section 4.4.2, the same analytical conditions and instrument parameters were applied for HPLC and LC-MS instrument analysis.

4.4.1.1.5 Analytical conditions and instrument parameters for High resolution mass spectrometry

The high-resolution mass spectrometry (HRMS) Orbitrap Q-Exactive plus of Thermo system was used for identification of fraction masses of impurities observed in degradation studies.

The instrument and method parameters are same as mentioned in LC-MS. HRMS instrument related parameters are same as mentioned in chapter-3 Section 3.4.3.

These degradation products have been later on observed in HRMS study and characterized based on the spectrum obtained from HRMS study.

Figure-4.8 TIC spectra of degradation sample in oxidative condition by HRMS

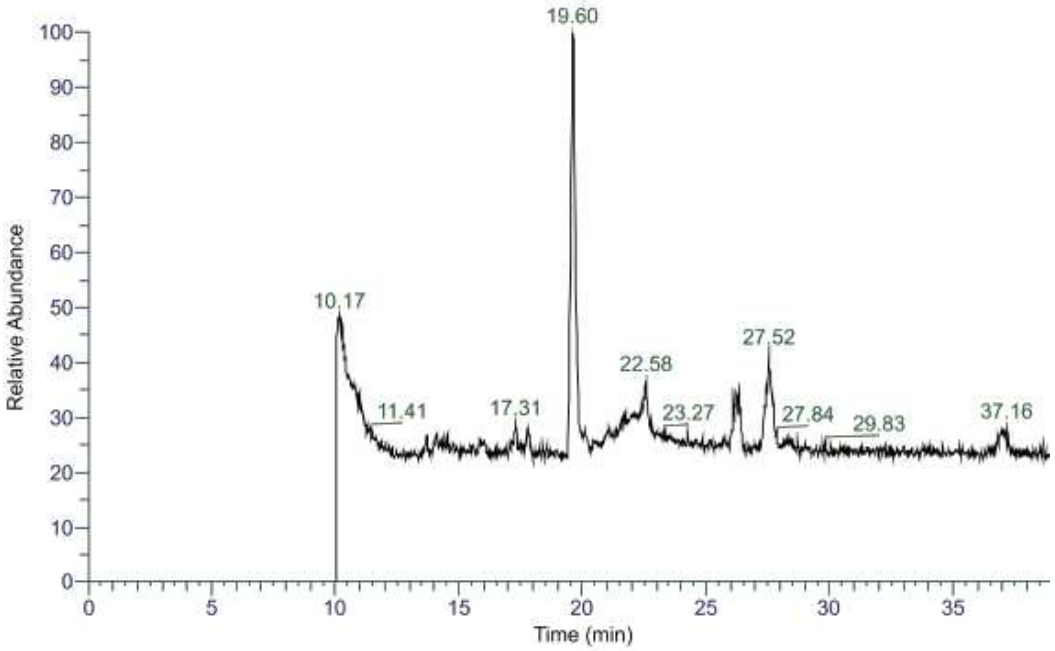


Table-4.6: Summary of degradation products observed by HRMS in various degradation conditions:

Degradation condition	**Degradation products**	**RT of Impurity**	**RRT**	**Observed molecular ion peak (m/z)**	**Further MS-MS fragmentation molecular ion peaks (m/z)**			
Acid degradation	**DP-1**	**17.06**	**0.87**	**166.072**	149.0456	142.9668	134.0462	
Alkali degradation	**DP-2**	**14.94**	**0.77**	**284.0986**	152.0565			
Peroxide degradation	**DP-3**	**13.66**	**0.70**	**118.0612**	119.0856	91.0546	86.0353	
	DP-4	**15.53**	**0.80**	**178.1438**	179.0636	177.1277	119.0856	88.0762, 161.0964, 146.1173 121.1015
	Same impurity as observed in acid degradation	**17.12**	**0.88**	**166.0723**	149.0233	98.9846	69.0706	
	DP-5	**17.5**	**0.90**	**194.1387**	135.0441	88.0761	74.0606	58.0659
	DP-6	**22.54**	**1.16**	**146.1541**	123.9646	74.097	73.0654	55.055
	DP-7	**26.32**	**1.35**	**162.1492**	146.1543	144.1387	126.1282	60.0453
	DP-8	**27.31**	**1.40**	**162.1492**	146.1543	144.1387		60.0453
	DP-9	**37.16**	**1.91**	**162.1492**	144.1387	116.1075	73.0655	60.0453

4.5 SECTION-B: DEGRADATION KINETIC STUDY:

The degradation kinetics study for nelarabine was performed in acidic, alkali and oxidative condition as the drug was found to be stable under all other stress conditions.

4.5.1 EXPERIMENTAL

4.5.1.1 Chemicals, reagents and Materials

Refer Section 4.4.1.1.1

4.5.1.2 Instrumentation and analytical conditions

Refer Section 4.4.1.1.2

4.5.1.3 Preparation of solutions:

To identify the degradation kinetic pathway of nelarabine, degradation study was carried out by exposing the samples to degradation from varying duration as well as varying concentration of solution. The sample solutions used were having a concentration of 0.1mg/mL in the milli-Q water. For acidic condition, an accurately weighed approximately 1 mg of nelarabine was exposed to 1mL of 0.1M hydrochloric acid and kept the solution at room temperature for 30minutes and 60 minutes duration respectively. Similarly, 1mL of 1M hydrochloric acid was taken and exposed the solution at room temperature for the duration of 15, 30, 45, 60minutes and in heating at 60°C for duration of 1, 2, 5, 15, 30, 60minutes. For alkali condition, an accurately weighed approximately 1 mg of nelarabine was exposed to 1mL 1N sodium hydroxide at 60°C for the duration of 15 minutes and 60minutes. In oxidative degradation also, 1 mg of nelarabine was exposed to 0.1mL 30% hydrogen peroxide at 60°c for 60 minutes and at room temperature for the duration of 24 hours. It was also exposed to 1mL of 30% hydrogen peroxide at 60°c for 15 minutes. In each varying condition, the percentage degradation as well as the increment of degradation product concentration were noted and summarized for degradation kinetic study.

4.5.2 RESULTS AND DISCUSSION

Based on the %degradation observed in each condition, the product is highly susceptible to acidic degradation condition. The drug seems to follow zero order

degradation kinetics as per the statistical values obtained from the calibration curves between %degradation and time duration (Table-4.7, 4.8, Figure-4.8-A, 4.8-B and 4.8-C). Based on order of reaction, rate constant was calculated as the negative value of slope of calibration curve. Moreover, temperature also plays a key role in degradation of nelarabine. For example, if the drug is exposed to acidic condition and kept at room temperature for 60 minutes, the drug found almost stable generating degradation products only within 11 percentage. However, if it is heated at 60°C in the same acidic condition for 60 minutes, it would get almost degraded generating more than 90% of degradation products (Table-4.9, Figure-4.9-A, 4.9-B, and 4.9-C).

Table-4.7: Conclusion of degradation kinetic study performed under varied conditions:

Condition	Concentration	Incubation conditions	Main peak	Total % Degradation observed	DP1 (%) (AT RRT 0.74)	DP2 (%) (AT RRT 0.84)	Conclusion
Acid	1mL 0.1N HCl	RT for 30 min	99.74	0.26	0.02	0.14	Below 1% degradation, No major degradation products generated
		RT for 60 min	99.61	0.39	0.05	0.29	Below 1% degradation, No major degradation products generated
	1mL 1N HCl	60°C for 1 min	86.08	13.92	0.95	12.38	Very fast degradation in presence of Acid + Heat
		60°C for 2 min	75.72	24.28	1.87	21.91	Very fast degradation in presence of Acid + Heat
		60°C for 5 min	47.67	52.33	3.85	46.33	Very fast degradation in presence of Acid + Heat
		60°C for 15 min	10.8	89.2	4.86	72.23	Condition to isolate DP-2 from Preparative LC
		60°C for 30 min	1.18	98.82	2.97	70.66	Another DP generated at RRT 0.61
		60°C for 60 min	0.38	99.62	0.77	48.56	Another DP generated at RRT 0.61
		RT for 15 min	96.3	3.7	0.62	3.05	Degradation increased linearly from 3.7% to 11.5 % at RT from 15, 30, 45 and 60 min
		RT for 30 min	93.97	6.03	0.81	4.94	
		RT for 45	91.42	8.58	1.19	7.35	

		min					
		RT for 60 min	88.5	11.5	1.72	9.49	Condition for FD study to have below 10% degradation
Alkali	1mL 1N NaOH	60°C for 15 min	93.19	6.81	6.79	0	Condition for FD study to have below 10% degradation
		60°C for 60 min	74.64	25.36	25.33	0	Condition to isolate DP-1 from Preparative LC
Peroxide	0.1mL 30% H_2O_2	60°C for 60 min	98.95	1.05	NA	NA	Below 1% degradation, No major degradation products generated
		RT for 24 hours	99.56	0.44	NA	NA	Below 1% degradation, No major degradation products generated
	1mL of 30% H_2O_2	60°C for 15 min	-	-	-	-	Some degradation products were clearly observed which masses were detected in HRMS instruments
Thermal	As such sample	60°C for 60 min	99.85	0	NA	NA	Below 1% degradation, No major degradation products generated

Table-4.8: Statistical results of plots between %degradation and time duration (minutes) after addition of 1mL of 1N HCl at 60°C heating

	Time duration (Minutes)	Volume of 1N HCl solution added and heated at 60°C	% Degradation	Log (% Degradation)	1/Log (% Degradation)
Set-1	1	1 mL	13.92	1.1436	0.8744
Set-2	2	1 mL	24.28	1.3852	0.7219
Set-3	5	1 mL	52.33	1.7188	0.5818
Set-4	15	1 mL	89.20	1.9504	0.5127
Set-5	30	1 mL	98.82	1.9948	0.5013
Set-6	60	1 mL	99.62	1.9983	0.5004
Slope (S)			1.32545	0.01126	-0.00440
Intercept			38.06575	1.48649	0.69831
Correlation Coefficient (r)			0.7933	0.7171	-0.6613
Standard deviation			38.2948	0.3599	0.1525

Figure-4.8-A: Calibration curve between % degradation vs. time

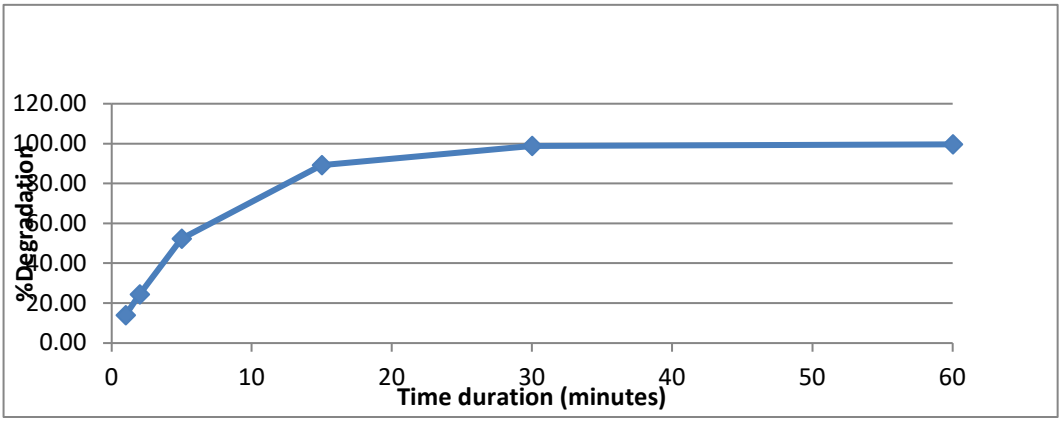


Figure-4.8-B: Calibration curve between Log %degradation vs. time

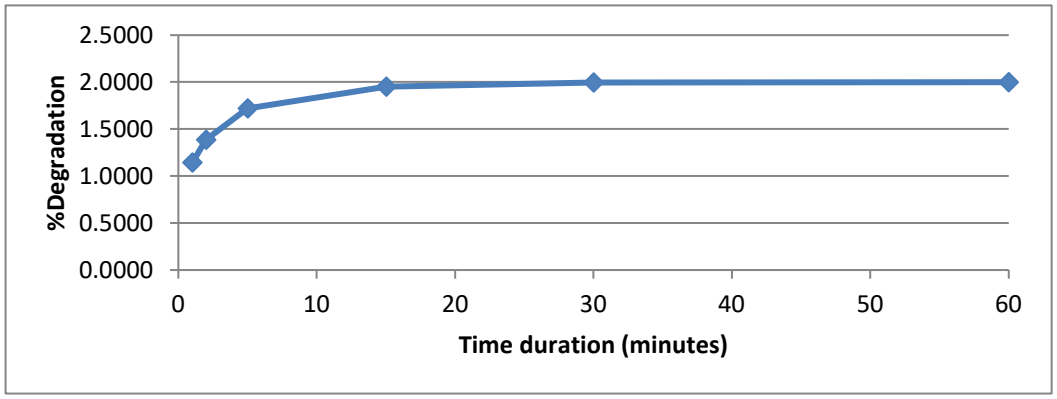


Figure-4.8-C: Calibration curve between 1/Log %degradation vs. time

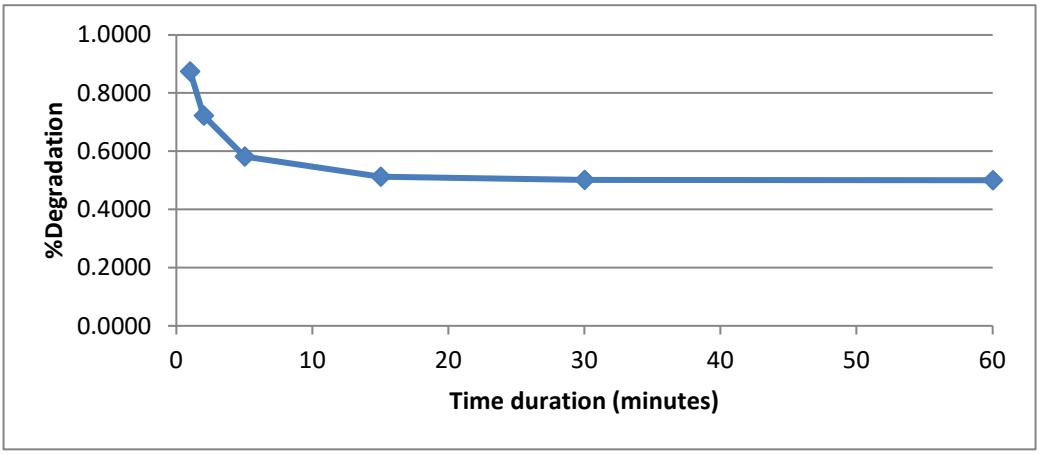


Table-4.9: Statistical results of plots between %degradation and time duration (minutes) after addition of 1mL of 1N HCl at room temperature

	Time duration (Minutes)	Volume of 1N HCl solution added and kept at RT	% Degradation	Log (% Degradation)	1/Log (% Degradation)
Set-1	15	1 mL	3.70	0.5682	1.7599
Set-2	30	1 mL	6.03	0.7803	1.2815
Set-3	45	1 mL	8.58	0.9335	1.0713
Set-4	60	1 mL	11.50	1.0607	0.9428
Slope (S)			0.17300	0.01087	-0.01775
Intercept			0.96500	0.42801	1.92932
Correlation Coefficient (r)			0.9987	0.9931	-0.9572
Standard deviation			3.3545	0.2120	0.3590

Figure-4.9-A: Calibration curve between % degradation vs. time

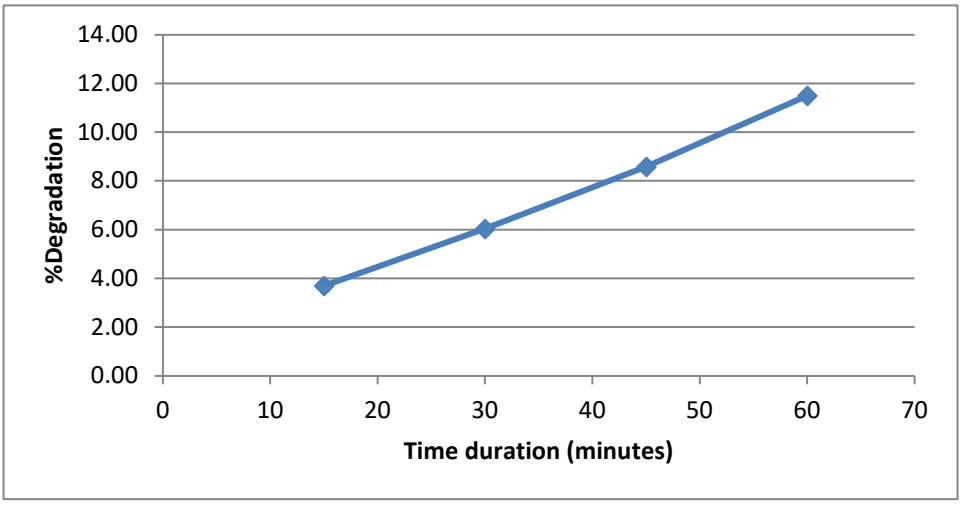


Figure-4.9-B: Calibration curve between Log %degradation vs. time

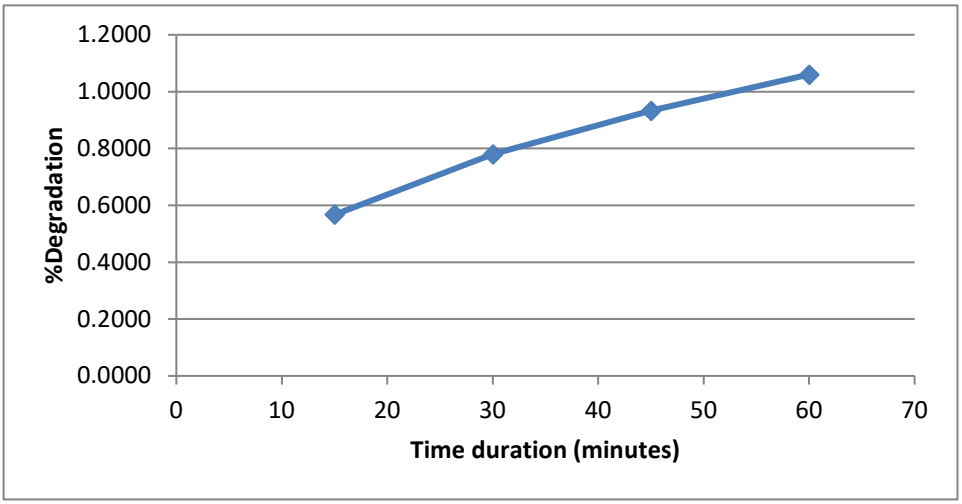
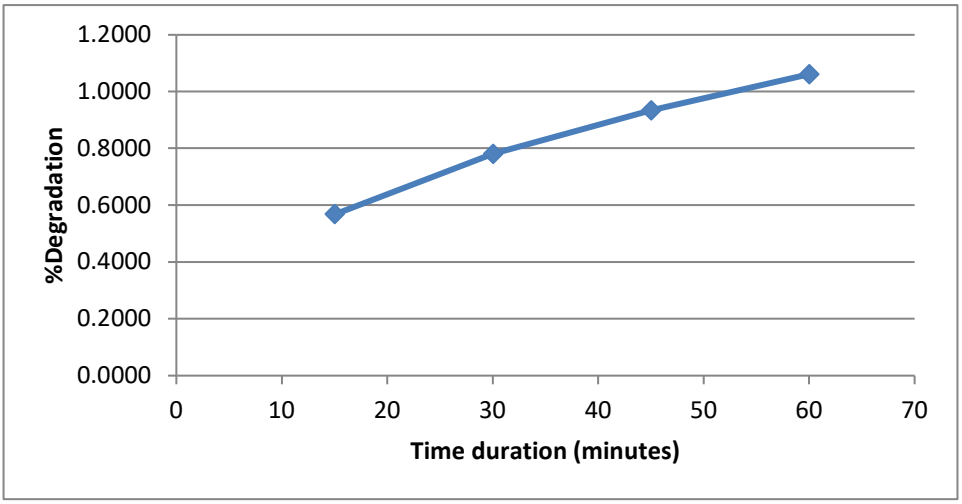


Figure-4.9-C: Calibration curve between 1/Log %degradation vs. time



4.6 SECTION-C: ISOLATION AND CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS OF NELARABINE

4.6.1 ISOLATION OF DEGRADATION PRODUCTS

4.6.1.1 Experimental:

4.6.1.1.1 Chemicals, reagents and Materials

Refer Section 4.4.1.1.1

4.6.1.1.2 Instrumentation and analytical conditions

4.6.1.1.2.1 Preparative high performance liquid chromatography

The column used was Phenomenex C18 (250X25) mm, 5µ. The mobile phase used was similar to that was used in analytical studies. The chromatographic separation was achieved with an injection volume of 5 ml and a flow rate of 25 mL/min for 35 minutes run time. The detection wavelength and gradient were similar to that used in the analytical method.

4.6.1.1.3 Sample preparation for Isolation of Impurities by Preparative-HPLC

A series of sample solutions (4.0 mg/mL), prepared in milli-Q water which were degraded in the degradation condition of acid and alkali as the major degradation products were observed in these conditions only. Then, isolation of degradation products was carried out through preparative-HPLC. An approximately 100 mg of nelarabine was transferred into a 50mL volumetric flask, added 25ml of 1M sodium hydroxide or 1M hydrochloric acid whichever applicable. The solution was further heated at 60 °C for 60 minutes to maximize the generation of degradation products. The solutions then injected in preparative LC and fractions were collected in test tubes.

4.6.1.2 Results and discussion:

As shown in Figure-4.9D, the degradation sample of nelarabine shows maximum generation of degradation impurities which were isolated in preparative test tubes. The collected fractions were confirmed in analytical HPLC method for elution at the same retention time. After confirmation, the collected fractions were evaporated on rotavapor to remove the organic portions and then freeze dried the aqueous medium contained desired degradation products to get in solid form. The isolated products were again injected in analytical method to confirm their purity. The purity of DP-1 and DP-2 were obtained as 94.99% and 91.60% respectively (Figure-4.9-E)

Figure-4.9D: Chromatogram of acid degradation sample prepared for preparative LC

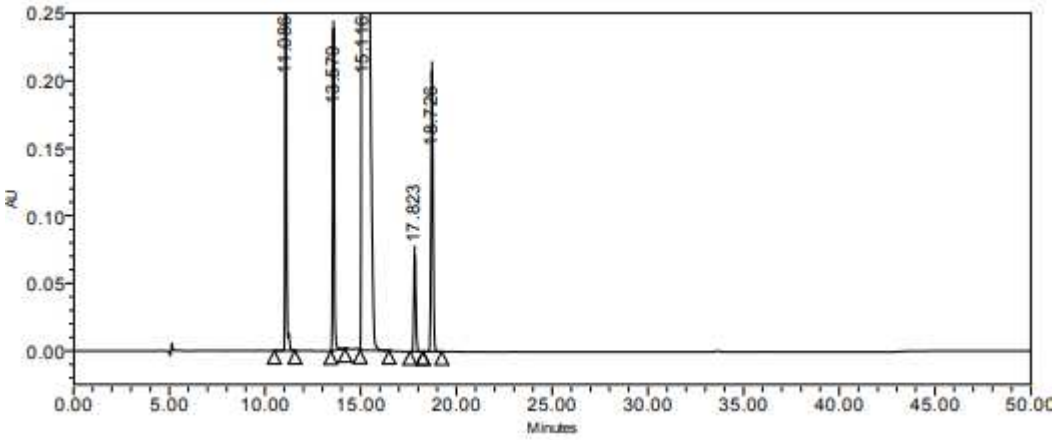
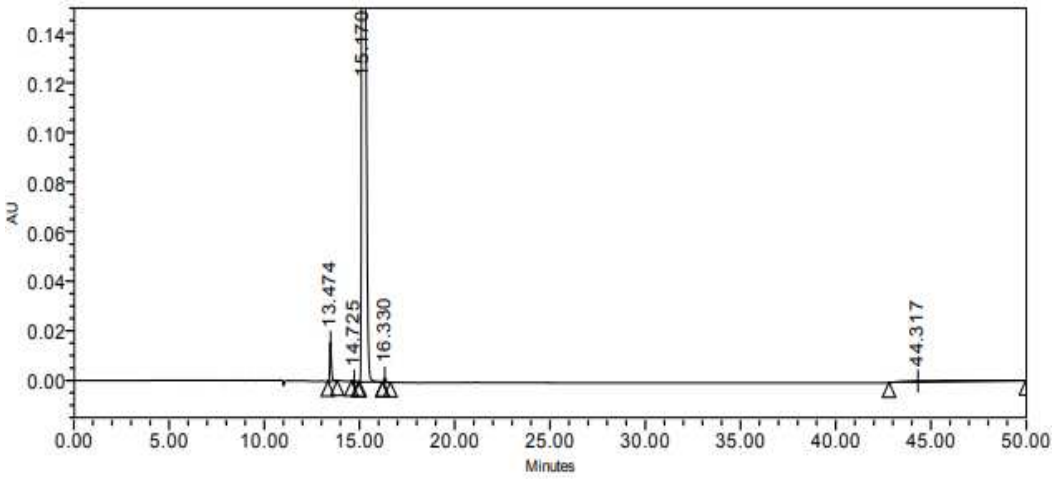


Figure-4.9E: Chromatogram of isolated DP-1



Peak Results

	RT	Area	% Area
1	13.474	93556	1.93
2	14.725	2335	0.05
3	15.170	4615176	94.99
4	16.330	10593	0.22
5	44.317	137075	2.82
Sum		4858734.3	100.0

4.6.2 CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS OF NELARABINE

4.6.2.1 Experimental

4.6.2.1.1 Chemicals, reagents and materials

Refer Section-4.4.1.1.1 under analytical method development.

4.6.2.1.2 Instruments

4.6.2.1.2.1 Liquid chromatography mass spectrometry

Refer section 4.4.1.1.2 Instrument used for forced degradation study.

4.6.2.1.2.2 High resolution mass spectrometry

Refer section of Instrument used for forced degradation study.

4.6.2.1.2.3 Nuclear magnetic resonance spectrometry

The NMR experiments for nelarabine and its degradation products were performed on Bruker AVANCE 400 MHz NMR instrument equipped with Broadband Observe (BBO) probe.

4.6.2.1.3 Sample solution preparation:

Refer Section 4.4.1.1.3, sample solution preparation under degradation condition of acidic, alkali and oxidation forced degradation study. As the degradation products were generated majorly in these conditions, samples of this conditions were run in LC-MS and HRMS for structure elucidation study.

4.6.2.1.4 Analytical condition and instrument parameters for HPLC and LC-MS

The conditions were set same as mentioned in Section 4.4.2.2.3 under results and discussion section of analytical method development study.

4.6.2.1.5 Analytical conditions and instrument parameters for High resolution mass spectrometry

The conditions were set same as mentioned in Section 4.4.1.1.5 under results and

discussion section of forced degradation study.

3.6.2.1.6 Analytical conditions and instrument parameters for Nuclear magnetic resonance spectrometry

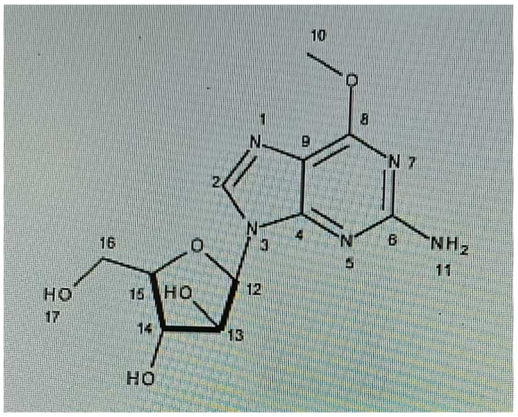
The probe temperature was set as 298K throughout experiment cycle. The chemical shifts of 1H and 13C spectra were recorded on a delta scale in ppm with reference to tetra methyl silane (TMS). The axis of the scale was calibrated as 2.56 ppm for dimethyl sulphoxide (DMSO-d6) peak in 1H spectra and at 39.5 ppm for DMSO-d6 peak in 13C NMR spectra.

4.6.2.2 Results and discussion:

4.6.2.2.1 Spectroscopic aspects of nelarabine:

Nelarabine drug was run in HRMS instrument to get its exact mass and its further fragmentation mass. The mass of nelarabine peak was obtained as 298.2253 m/z which further fragmented into 166.0720 as the main peak in MS-MS spectrum which clearly indicates that the weak bond between arabinosyl and guanine moiety breaks in high collision energy and produces the mass of 166.0720 i.e., of guanine moiety. Nelarabine drug was also run in NMR instrument to acquire its proton NMR spectra and 13C NMR spectra so that the NMR spectra of isolated degradation products can be compared with nelarabine spectra and effective conclusion can be made (Figure-4.11, 4.12). Nelarabine in the structural fusion of two moieties: 6-methoxy-9H-purin-2-amine having exact molecular mass as165.15600000095 and 2-(hydroxymethyl) tetrahydrofuran-3,4-diol having exact molecular mass as 134.13100000095 (Figure-4.10).

Figure-4.10: Molecular structure and mass of two moieties in nelarabine



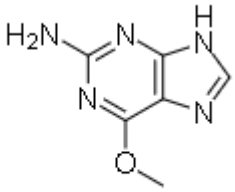
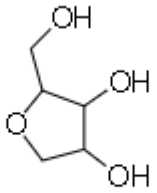
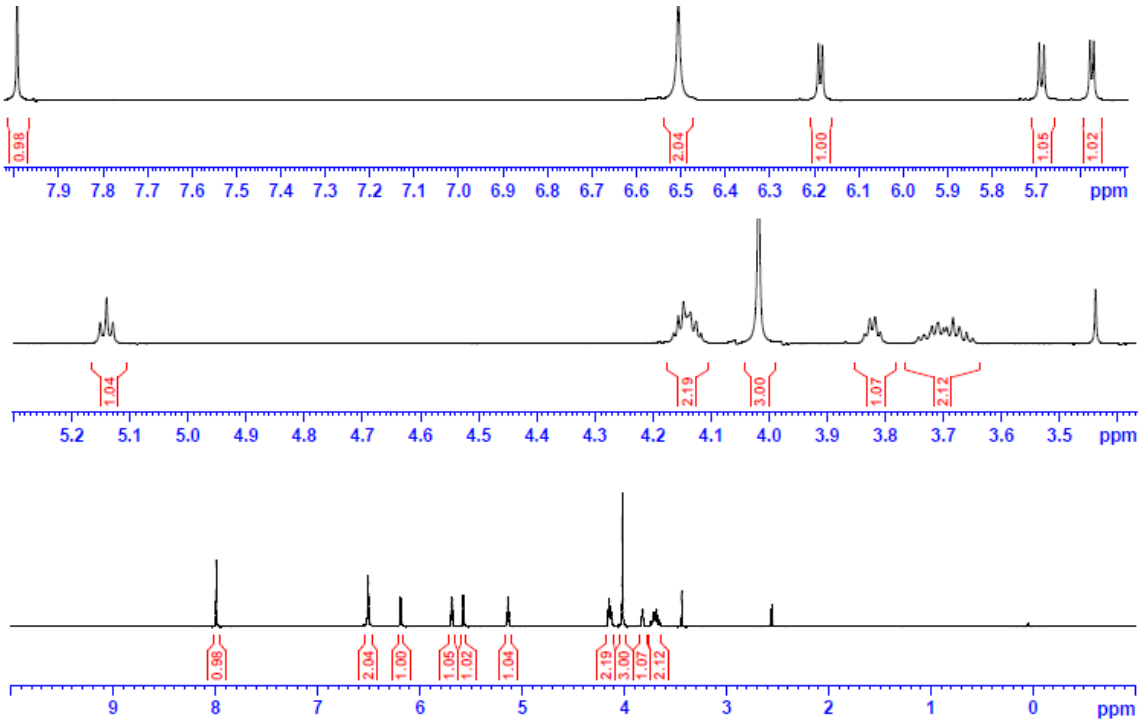
165.15600000095  6-methoxy-9*H*-purin-2-amine	134.13100000095  2-(hydroxymethyl)tetrahydrofuran-3,4-diol

Figure-4.11: 1H spectra of nelarabine



igure-4.12: 13C-BBD, DEPT-90 and DEPT-135 spectrum overlay of nelarabine

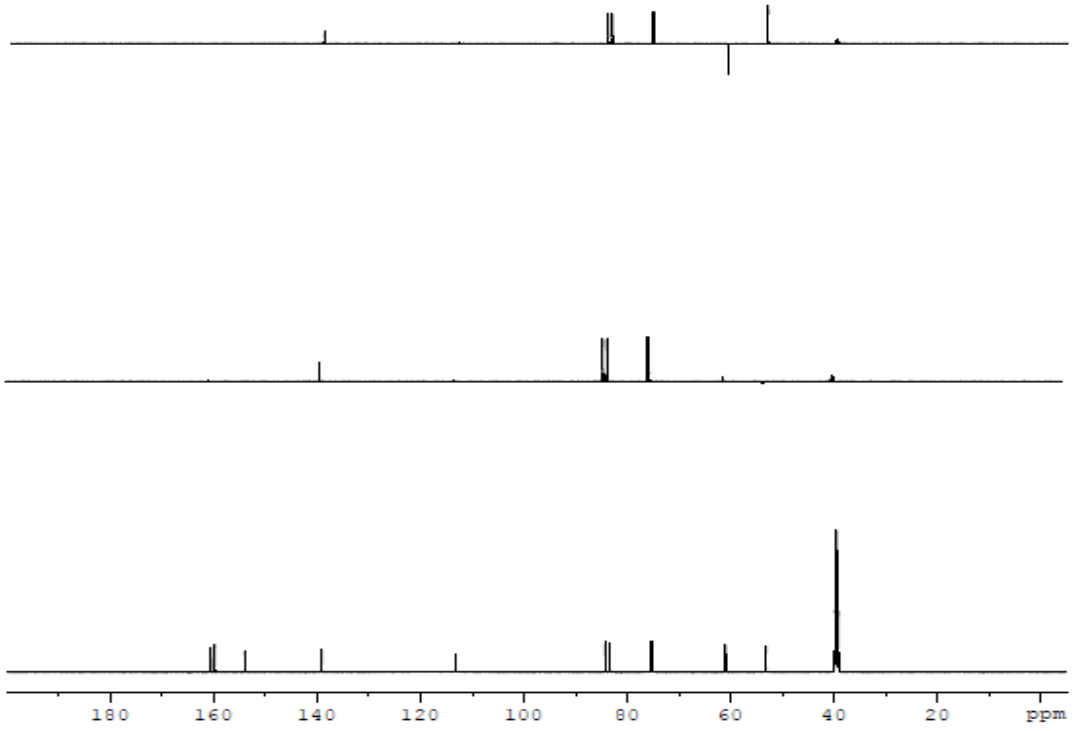


Table-4.10 Proton identification of Nelarabine through 1H spectra

Chemical shift of Nelarabine (ppm)	Multiplicity	No. of Protons	Assignment in structure
4.00	Singlet	3	10
3.63-3.73	Multiplet	2	13, 14, 15, 16
3.79-3.82	Multiplet	1	
4.10-4.15	Multiplet	2	
5.13	Triplet	1	17, 18, 19
5.56	Doublet	1	
5.67	Doublet	1	
6.49	Singlet	2	11
6.17	Doublet	1	12
7.98	Singlet	1	2
		15	

Table-4.11 Carbon identification of Nelarabine through 13C-BBD, DEPT-90 and DEPT-135 spectra

Chemical shift of Nelarabine (ppm)	No. of Carbons	Assignment in structure
53.17	1	10
60.95	1	16
75.26	1	13, 14
75.49	1	
83.37	1	12, 15
84.20	1	
113.17	1	9
139.10	1	2
153.99	1	4
159.78	1	6, 8
160.54	1	
	11	

4.6.2.2.2 Degradation product-1 (Impurity at RRT 0.84 RRT (RT 17.06 min) in acidic degradation)

This degradation product was isolated from preparative LC as shown in the degradation chromatogram. The isolated product was analyzed in LC-MS, HRMS and NMR spectroscopy. The molecular mass of DP-1 was obtained as 166.0720 m/z which further fragmented into 149.0456, 142.9668, 134.0462 and 98.9844. This mass value reflects to be of guanine moiety in nelarabine structure. The isolated degradation products were run in the NMR instrument to get the proton NMR spectra. The singlet peak at around 8.45 ppm is due to –CH proton of the five membered ring of the guanine moiety. Due to conjugated bond system and thereby more electronegative atmosphere, this peak has been observed in the aromatic region between 8 and 9 of the 1H proton spectrum. Moreover, the most intense single peak at around 4.10 ppm is due to three protons of -O-CH3 group of the guanine moiety. The protons of –CH3 are aliphatic and generally such protons are thought to be observed in between 1.0 to 2.5 ppm region of the spectra. But, due to presence of more electronegative oxygen atom, its chemical shift value has been observed to be shifted toward more electronegative region of the spectra that is near to 4.10. The protons of -N-H2 and -N-H was not observed due to its exchange with D2O of DMSO-d6 solvent. Overall, these proton peaks and its mass observations it clearly confirms the intactness of the guanine moiety of the structure. Hence the structure of DP-1 can be confirmed as shown in Figure-4.13 and its chemical name can be stated as 6-methoxy-9*H*-purin-2-amine.

Figure-4.13: Molecular structure of DP-1

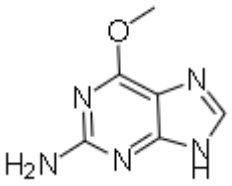


Figure-4.14: Mechanism of formation of DP-1 from nelarabine

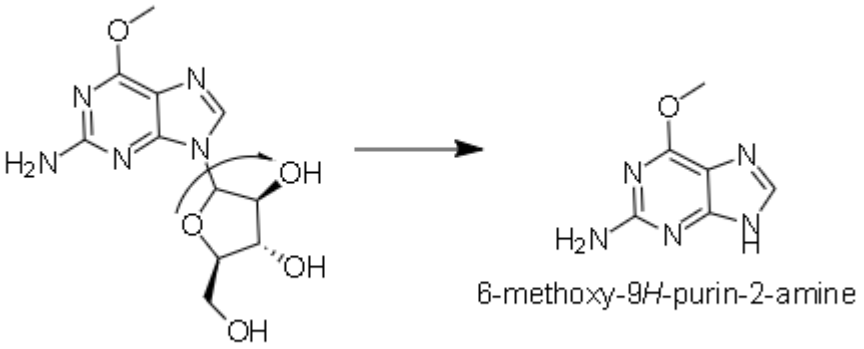


Figure-4.15: TIC spectra of degradation sample in acidic condition by HRMS

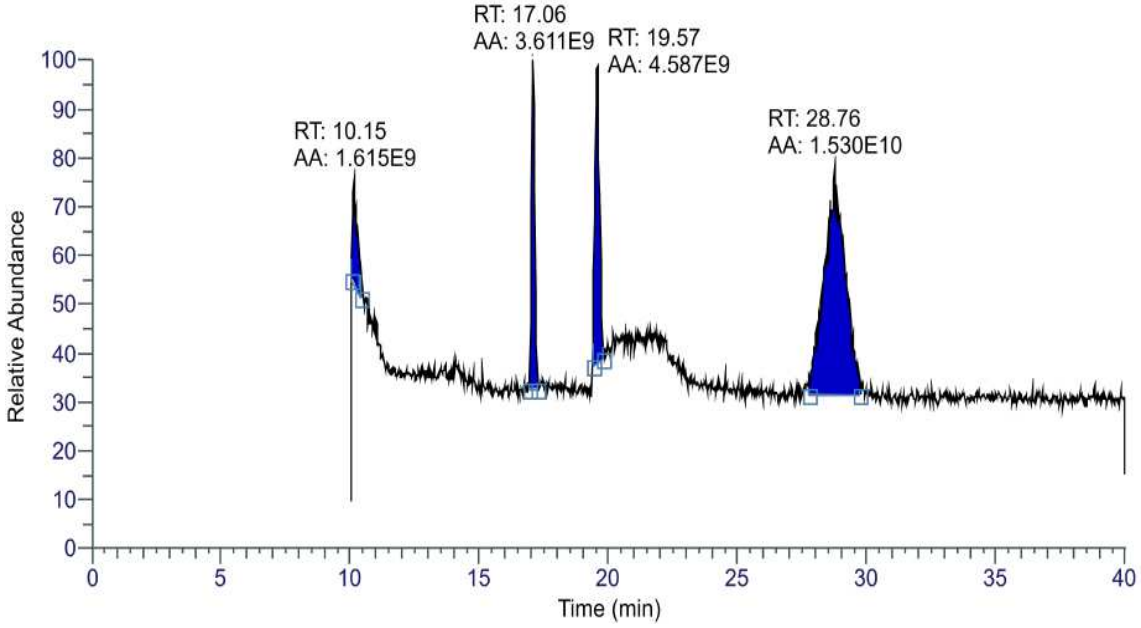


Figure-4.16: HRMS spectrum of DP-1 in acidic condition

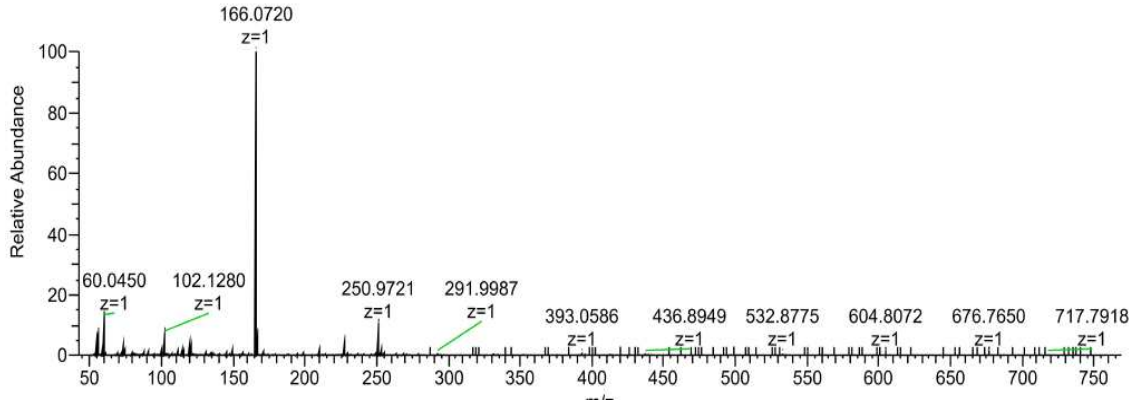


Figure-4.17: HRMS-MS spectrum of DP-1 m/z 166.0720

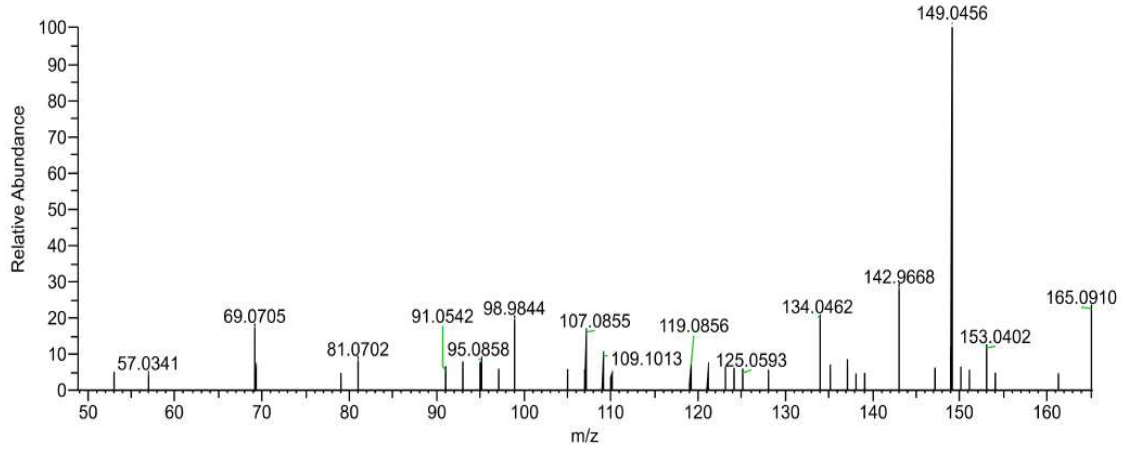
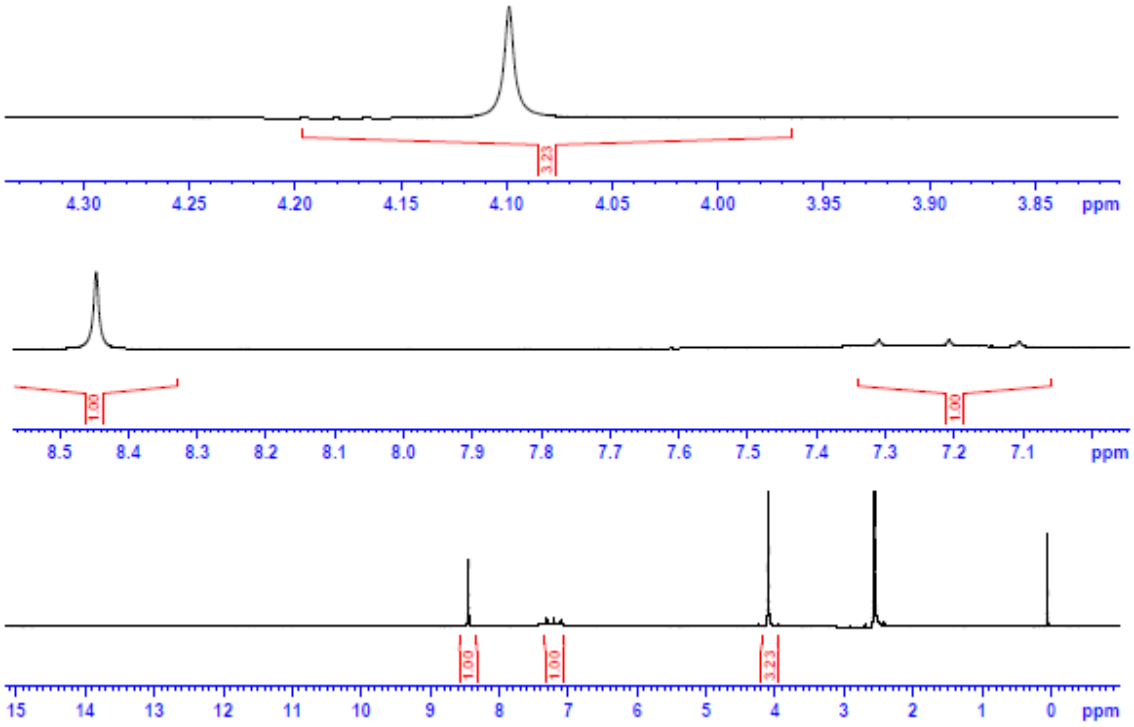


Figure-4.18: 1H proton spectra of DP-1:



4.6.2.2.3 Degradation product-2 (Impurity at RRT 0.74 RRT (RT 14.94 min) in alkali degradation)

This degradation product was isolated from preparative LC as shown in the degradation chromatogram. The isolated product was analyzed in LC-MS, HRMS and NMR spectroscopy. The molecular mass peaks were obtained as 284.0722 and 152.0566 m/z in DP-2 peak spectrum in HRMS which MS-MS spectrum giving only one major peak as 152.0565 indicating the stability of this structure portion at the given collision energy (Figure-4.22, 4.23). The difference between the nelarabine mass and the exact mass of DP-2 based on its molecular ion peak is 14.2778 (297.27-282.9922=13.1978). This is very much probable if the –CH3 group of alkali labile –O-CH3 group is removed in presence of alkali and heat forming –OH group instead of –O-CH3. This probability becomes also true if the MS-MS fragmentation of DP-2 is seen or the second major molecular ion peak of DP-2 spectrum is seen. The MS-MS fragmentation of DP-2 indicates the presence of clear molecular ion peak of 152.0565 which is the mass of guanine moiety of nelarabine after replacement of –OCH3 group with –OH group. Hence, the guanine moiety of nelarabine is having major and distinct m/z value of 166.0720 which is observed in MS-

MS fragmentation of nelarabine as well as in MS spectrum of DP-1. On the other hand, the guanine moiety of DP-2 is having major and distinct m/z value of 152.0565. If –OCH3 group is replaced with –OH group, this change should be reflected in 1H proton NMR spectra. When 1H NMR spectra of nelarabine and DP2 are compared, there is only one major difference which is the absence of single major peak of three protons of –CH3 group in the DP-2 1H NMR spectrum. Instead, only one proton peak is observed at that position (Figure-4.24). So, this concludes the structure of DP-2 as Figure-4.19 and the chemical name of the DP-2 can be stated as 2-Amino-6-hydroxypurine arabinoside. The mechanism of forming of this degradation product is shown in Figure-4.20.

Figure-4.19: Molecular structure of DP-2

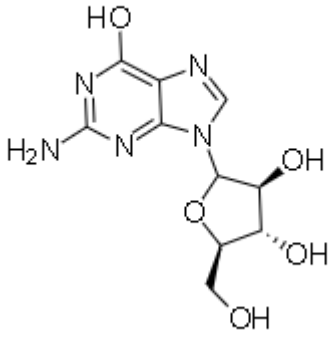


Figure-4.20: Mechanism of formation of DP-2 from nelarabine

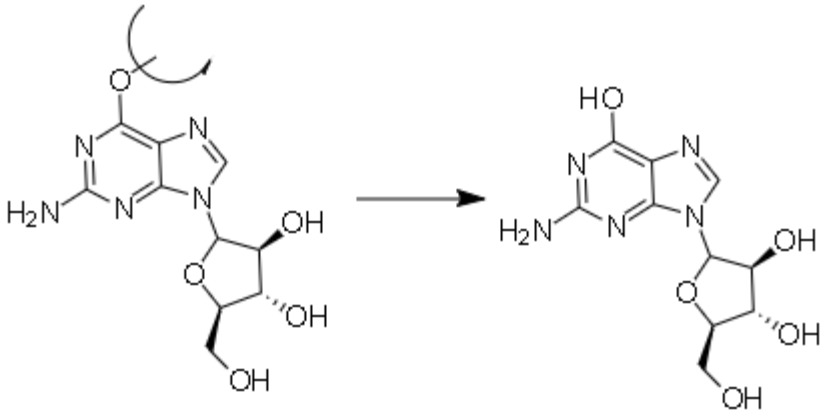


Figure-4.21: TIC spectra of degradation sample in alkali condition by HRMS

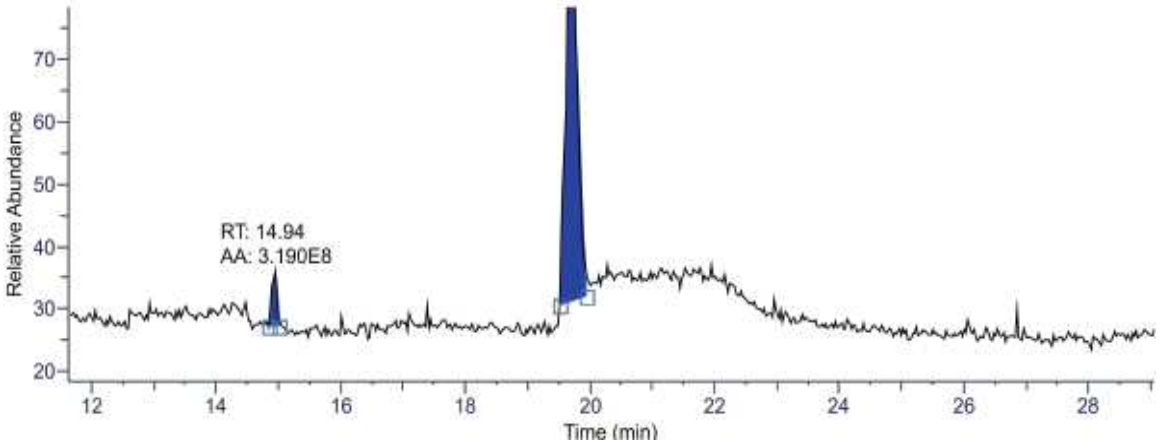


Figure-4.22: HRMS spectrum of DP-2 in alkali condition

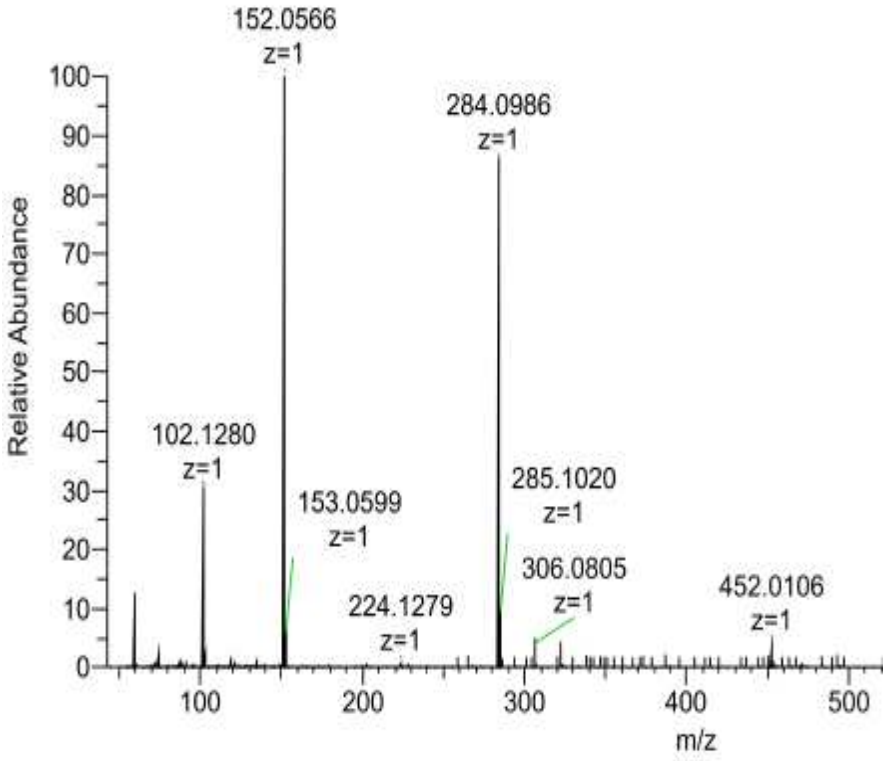


Figure-4.23: HRMS-MS spectrum of DP-2 m/z 284.0986

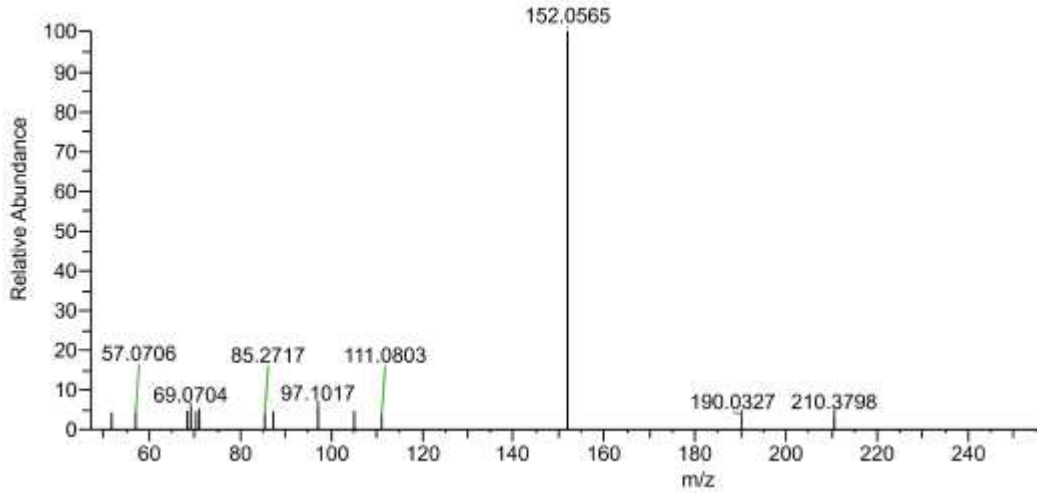
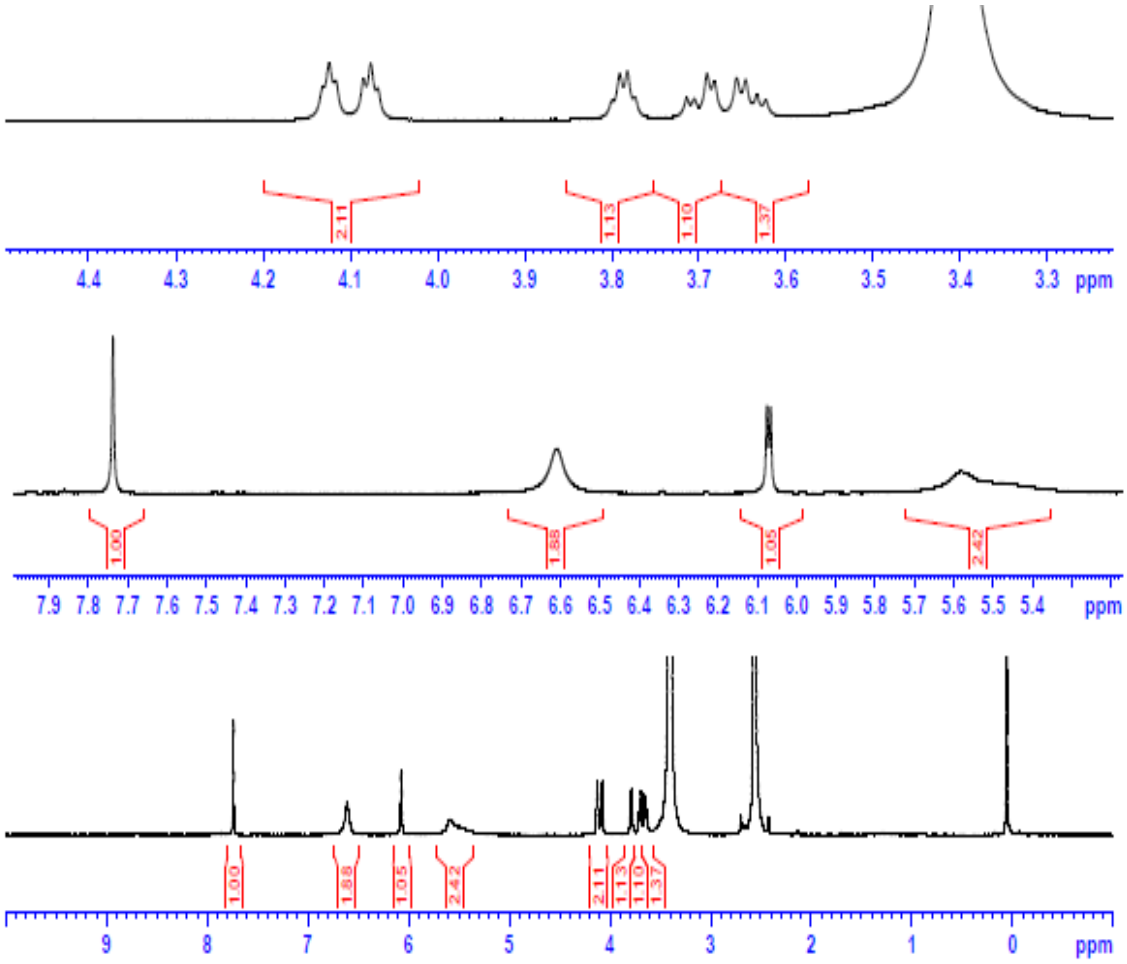


Figure-4.24: 1H proton spectra of DP-2:



4.6.2.2.4 Degradation product-3 (Impurity at RRT 0.70 (RT 13.66 min) in oxidative degradation)

The degradation product at RRT 0.70 (RT 13.66) was observed in the oxidative degradation run in HRMS analysis with the molecular mass of 118.0612 m/z (Figure-4.27). This is probable due to removal of hydroxy group at position 6 of arabanosyl moiety (134-16-1=117). However, its fragmentation shows the molecular ion peak at 119.0856 (i.e., 117+1=118) due to immediate conversion of –CH2 group into-CH3 group. Moreover, there are two more fragmentation peaks observed in MS-MS spectrum i.e., at 91.0546 and at 86.0353 m/z (Figure-4.28). The fragment ion with m/z value 91.0546 is thought to be generated by removal of terminal –CH2CH3 group plus ketone formation in any of the hydroxy group of the moiety (118-15-13+2-2=90). The fragment ion with m/z value 86.0353 is probable due to removal of two hydroxy group in the position 3 and 4 of 2-methyltetrahydrofuran-3,4-diol. Hence, based on this observation, the probable structure

of this degradation product is as per Figure-4.25 and the chemical name of the DP-3 can be stated as 2-methyltetrahydrofuran-3,4-diol.

Figure-4.25: Molecular structure and mass of DP-3 and its MS-MS fragments

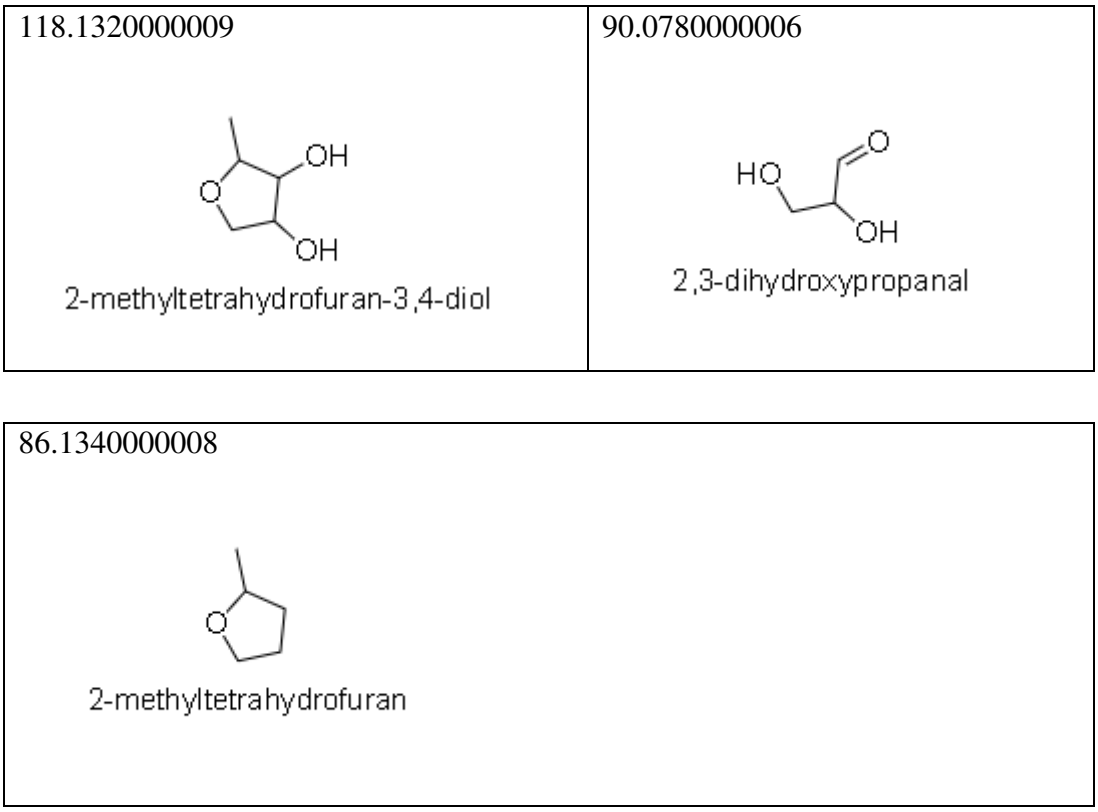


Figure-4.26: TIC spectra of degradation sample in oxidative condition by HRMS

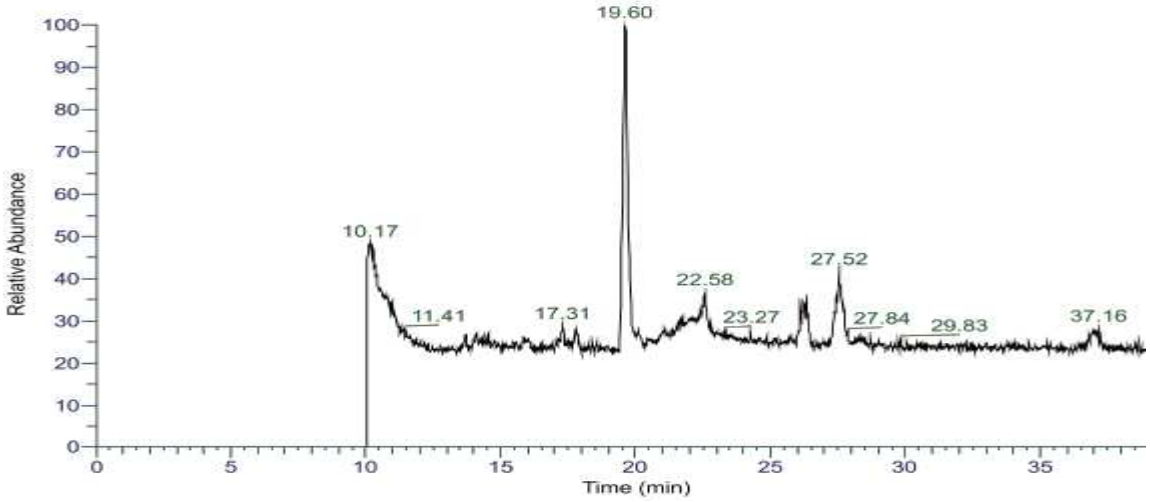


Figure-4.27: HRMS spectrum of DP-3 in oxidative condition

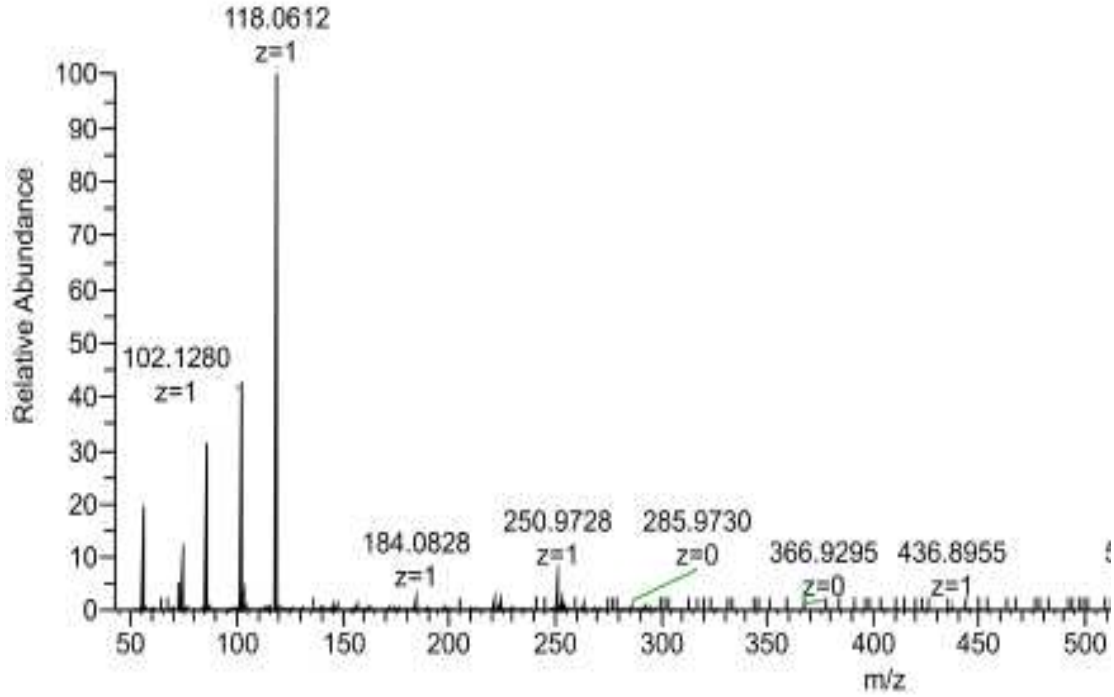
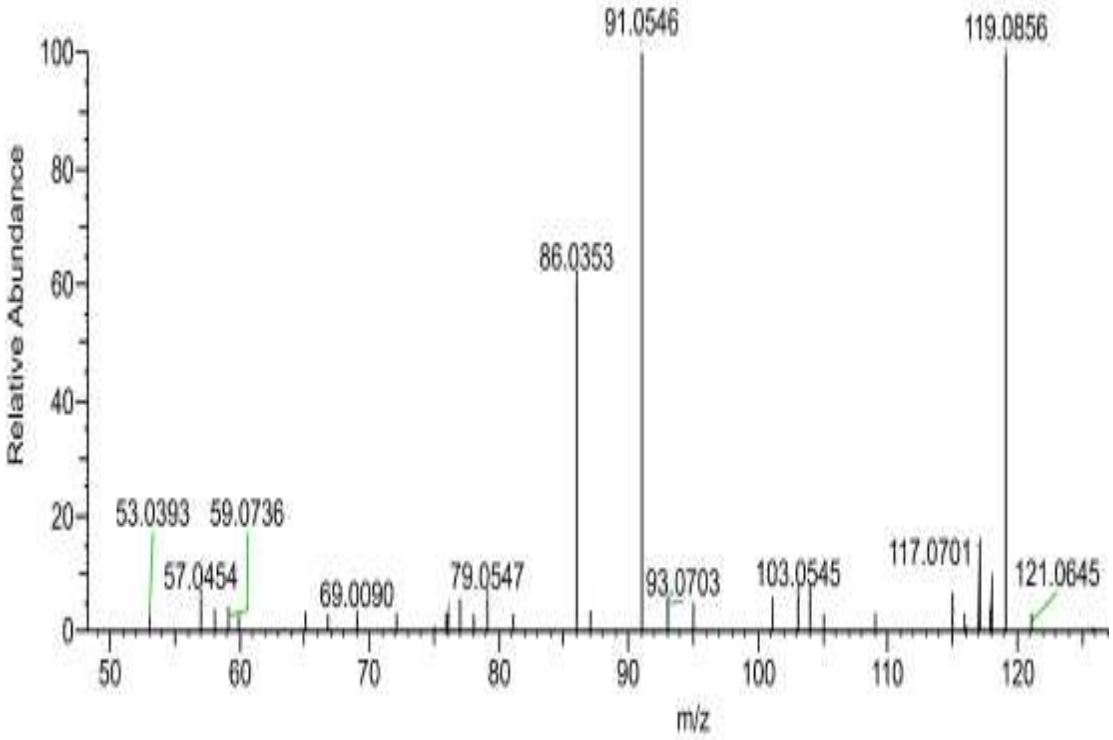


Figure-4.28: HRMS-MS spectrum of DP-3 m/z 118.0612

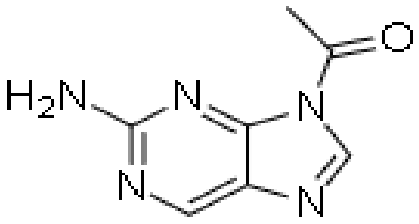


4.6.2.2.5 Degradation product-4 (Impurity at RRT 0.80 RRT (RT 15.53 min) in oxidative degradation)

Based on the molecular ion peak observed for this peak was observed at 178.1438 which is possible in two ways modification of nelarabine structure in oxidative degradation as shown in Figure-4.29 having exact mass of 177.167000001 and 177.15600000115 respectively. However, when MS-MS fragmentation of this peak was reviewed (Figure-4.30, 4.31), it shows clear major peak at 119.0856 which is only possible in the arabinosyl moiety modification not with the guanine moiety modification. (Refer Figure-4.29)

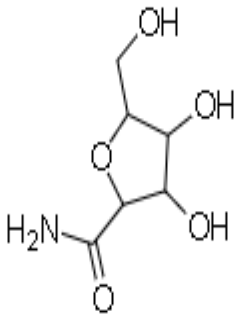
Figure-4.29: Molecular structure and mass of DP-4 and its fragments

Possibility-A for 177.167000001



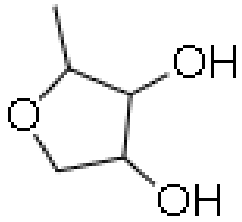
1-(2-amino-9*H*-purin-9-yl)ethan-1-one

Possibility-B for 177.15600000115



3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-carboxamide

118.1320000009



2-methyltetrahydrofuran-3,4-diol

Figure-4.30: HRMS spectrum of DP-4 in oxidative condition

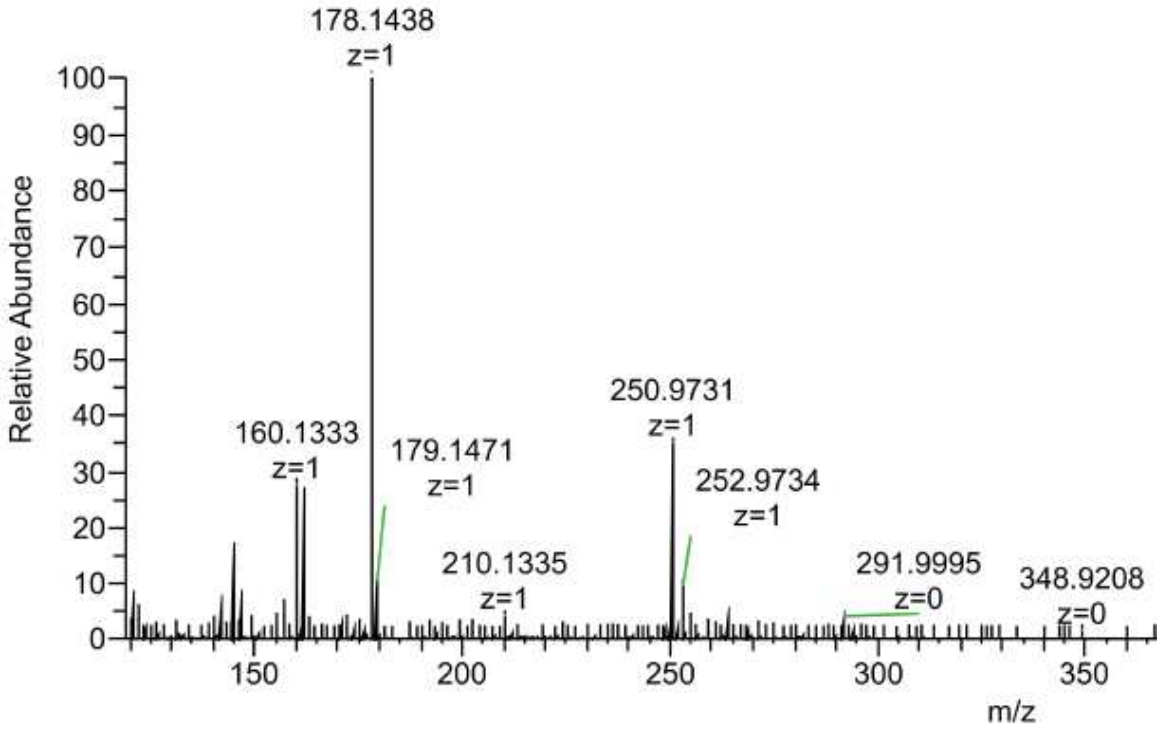
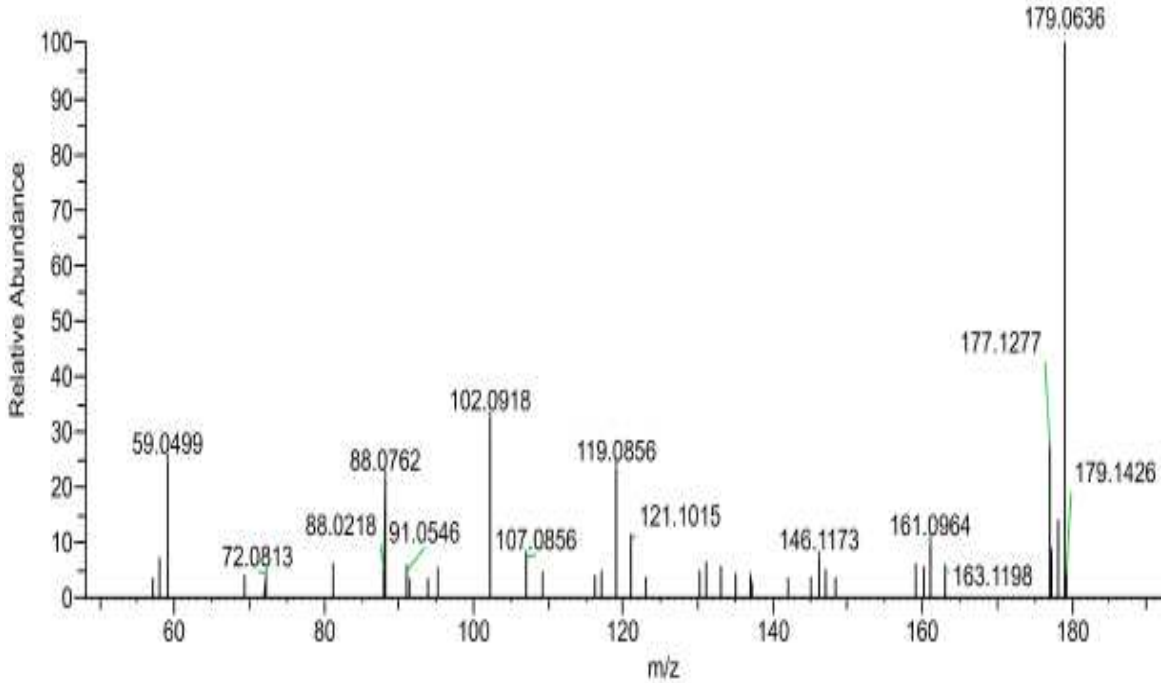


Figure-4.31: HRMS-MS spectrum of DP-4 m/z 178.1438

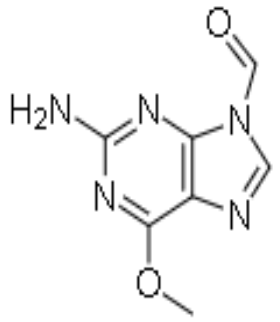


4.6.2.2.6 Degradation product-5 (Impurity at RRT 0.90 RRT (RT 17.5 min) in oxidative degradation)

This degradation product is formed by formation of aldehyde of purine moiety due to oxidative degradation. The exact mass of after formation of aldehyde at the position-2 of guanine moiety of nelarabine structure is 193.1600000105 which clearly matches with the primary molecular ion peak observed in HRMS spectrum as 194.1387 m/z (Figure-4.33). Further MS-MS spectra generated the molecular ion peak at 135.0441 which also confirms with the removal of aldehyde group and –OCH3 group from the structure due to high collision energy generating the fragment with exact mass of 135.000000075 (Figure-4.34). Hence based on above observations, the structure of this degradation product can be stated as mentioned in Figure-4.32 and the chemical name of the DP-4 can be stated as 2-amino-6-methoxy-9*H*-purine-9-carbaldehyde.

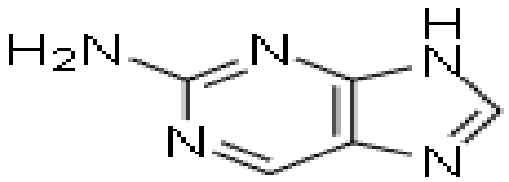
Figure-4.32: Molecular structure and mass of DP-5 and its fragments

193.16600000105



2-amino-6-methoxy-9*H*-purine-9-carbaldehyde

135.13000000075



9*H*-purin-2-amine

Figure-4.33: HRMS spectrum of DP-5 in oxidative condition

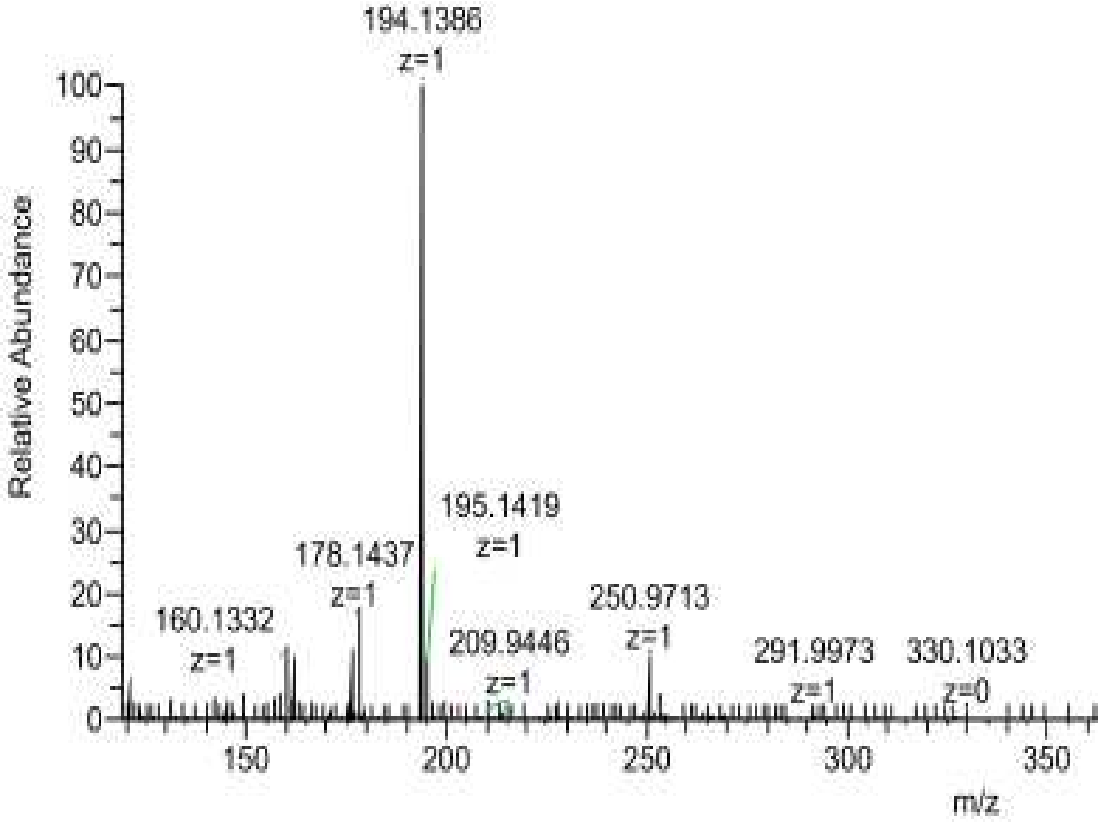
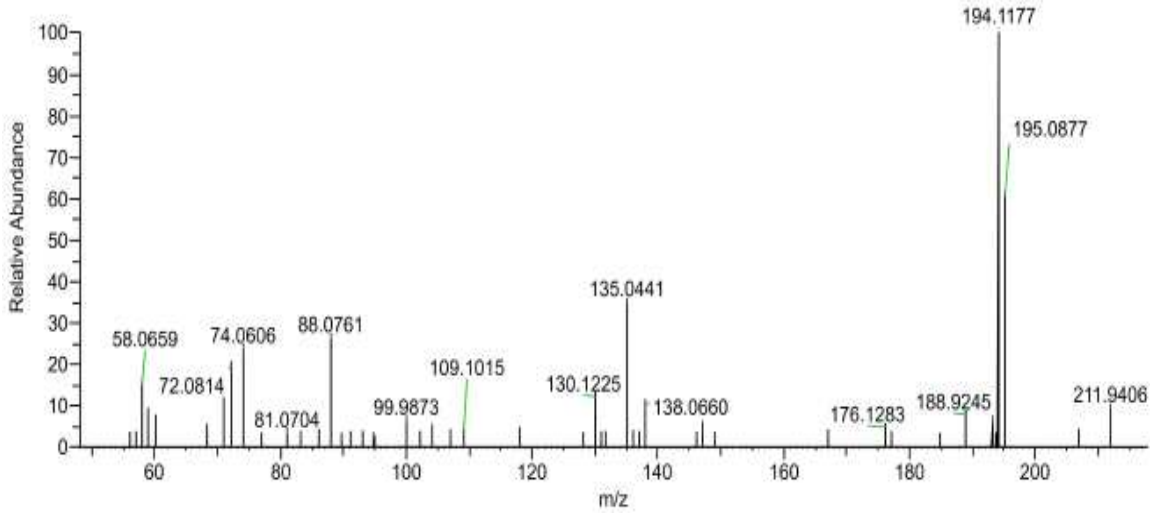


Figure-4.34: HRMS-MS spectrum of DP-5 m/z 194.1386

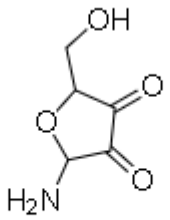


4.6.2.2.7 Degradation product-6 (Impurity at RRT 1.16 RRT (RT 22.54 min) in oxidative degradation)

This degradation product has the main molecular ion peak at 146.1541 m/z (Figure-4.36) which resembles to the molecular structure as per Figure-4.35 which has exact mass of 145.11400000085. Here, two hydroxy group of the arabinosyl moiety has been converted into ketone functional groups forming dione product. Based on this, the chemical structure of this degradation product can be depicted as shown in Figure-4.35 whereas the chemical name of the structure can be stated as 2-amino-5-(hydroxymethyl) furan-3,4(2H,5H)-dione.

Figure-4.35: Molecular structure and mass of DP-6

145.11400000085



2-amino-5-(hydroxymethyl)furan-3,4(2*H*,5*H*)-dione

Figure-4.36: HRMS spectrum of DP-6 in oxidative condition

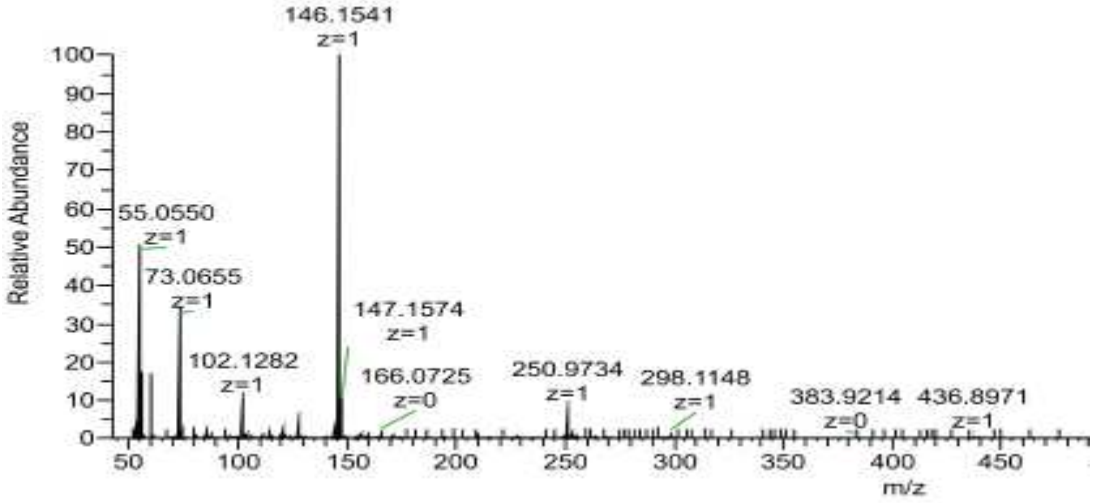
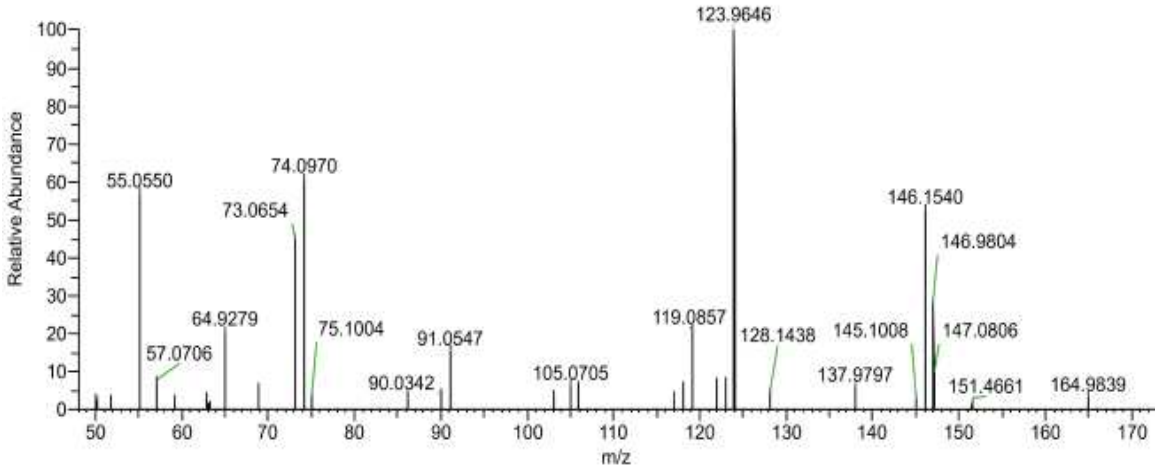


Figure-4.37: HRMS-MS spectrum of DP-6 m/z 146.1541



4.6.2.2.8 Degradation product-7 (Impurity at RRT 1.35 RRT (RT 26.32 min) in oxidative degradation),

Degradation product-8 (Impurity at RRT 1.40 RRT (RT 27.31 min) in oxidative degradation),

Degradation product-9 (Impurity at RRT 1.91 RRT (RT 37.16 min) in oxidative degradation)

These degradation products have been observed to be eluted at RRT 1.35, 1.40 and 1.91 with respect to nelarabine in oxidative degradation condition's chromatographic run in

HRMS. However, their molecular ion peak values are same i.e., 162.1492 which suggest that they are in some way having same molecular formula but different molecular structure. Hence, they are positional isomers having different molecular structures and thereby eluting at different retention times in chromatography but having same molecular mass. From the structure of nelarabine, it is clear that if –NHCH3 chain of guanine moiety is retained as functional group in the arabinosyl moiety and one out of any three hydroxy group of arabinosyl moiety is converted into carbonyl group, then the molecular mass of the generated fragment structure would be as 161.1570000011 which exactly matches with the molecular ion peak generated in each of these three degradation products i.e., at RRT 1.35, 1.40 and 1.91 respectively. Now, since there are three hydroxy functional groups present in the arabinosyl moiety of the structure, there are three possibilities of generating carbonyl group from hydroxy group giving rise to three positional isomers having same molecular mass (Refer Figure-4.38 to Figure-4.43).

Figure-4.38: Molecular structure and mass of DP-7

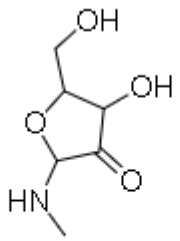
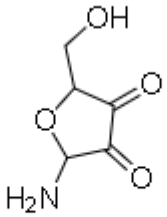
161.1570000011    4-hydroxy-5-(hydroxymethyl)-2-(methylamino)dihydrofuran-3(2*H*)-one
145.11400000085    2-amino-5-(hydroxymethyl)furan-3,4(2*H*,5*H*)-dione

Figure-4.39: Molecular structure and mass of DP-8

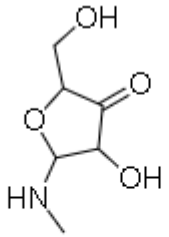
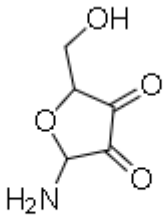
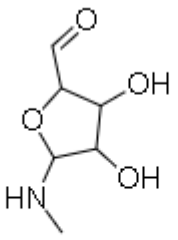
161.1570000011  4-hydroxy-2-(hydroxymethyl)-5-(methylamino)dihydrofuran-3(2*H*)-one
145.11400000085  2-amino-5-(hydroxymethyl)furan-3,4(2*H*,5*H*)-dione

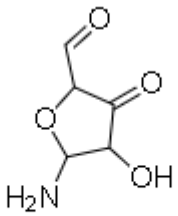
Figure-4.40: Molecular structure and mass of DP-9

161.1570000011



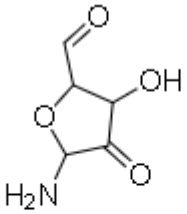
3,4-dihydroxy-5-(methylamino)tetrahydrofuran-2-carbaldehyde

145.11400000085



5-amino-4-hydroxy-3-oxotetrahydrofuran-2-carbaldehyde

145.11400000085



5-amino-3-hydroxy-4-oxotetrahydrofuran-2-carbaldehyde

143.09800000075

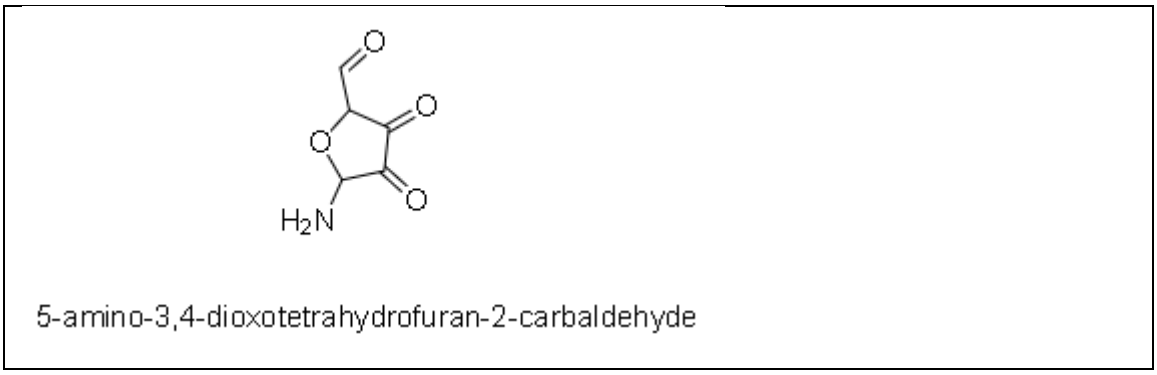


Figure-4.41: HRMS spectrum of DP-7 in oxidative condition

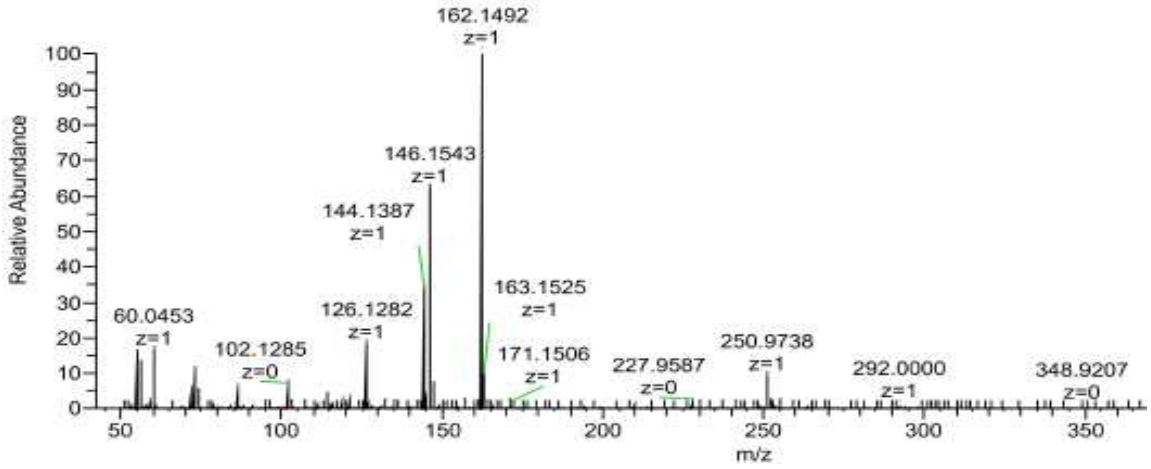


Figure-4.42: HRMS spectrum of DP-8 in oxidative condition

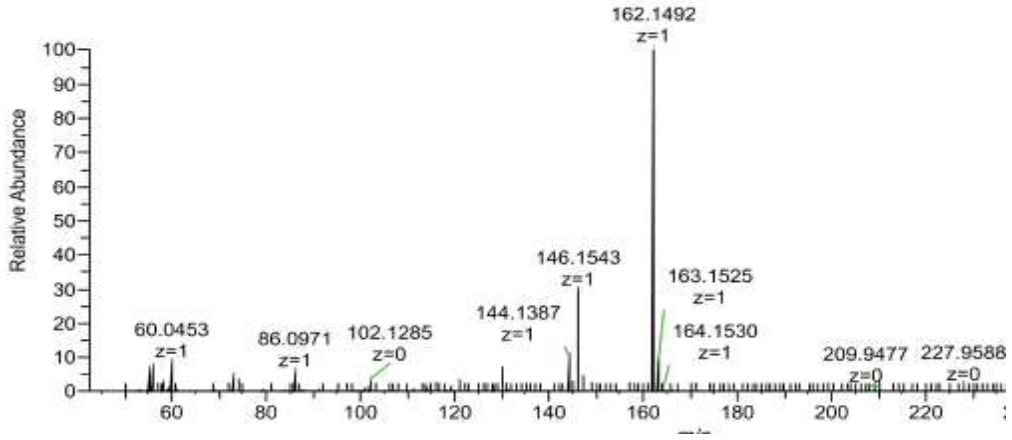
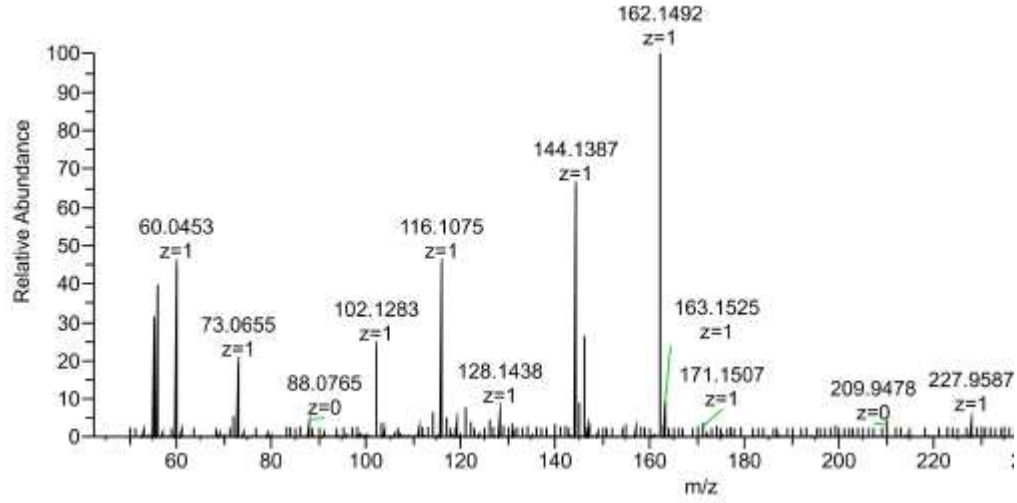


Figure-4.43: HRMS spectrum of DP-9 in oxidative condition



4.7 CONCLUSION:

This chapter covered three main sections i.e., analytical method development and validation for estimation of nelarabine and its degradation products, degradation kinetic study and structure elucidation of observed degradation products of nelarabine. Analytical method which was developed for estimation of nelarabine and its degradation products found to be validated as per all the parameters of ICH guideline's acceptance criteria. In section-B, the drug nelarabine was found to be very susceptible to acidic degradation condition and found to follow zero order degradation kinetics. In section-C, total 9 degradation products were characterized based on their LC, LC-MS, HRMS, and NMR data. Out of this, DP-1 was observed in acidic and peroxide degradation condition, DP-2

was observed in alkaline degradation condition and remaining all the degradation products were observed in HRMS TIC spectra in oxidative degradation condition. All the nine degradation products were satisfactorily characterized and their probable structures with chemical names were presented in individual sections.

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