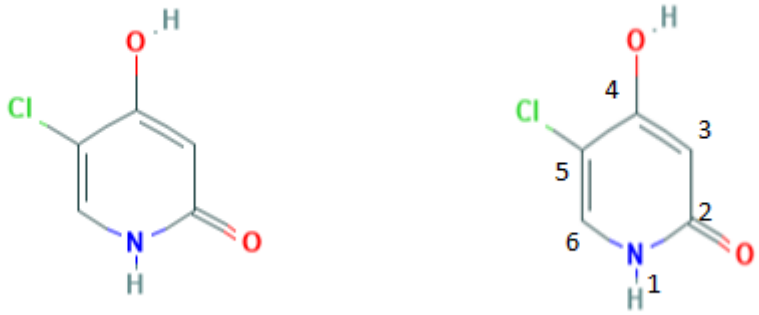
CHAPTER-5: IMPURITY PROFILING AND CHARACTERISATION OF IMPURITIES IN GIMERACIL

5.1 INTRODUCTION AND SELECTION OF DRUG:

Cancer is the second leading cause of death worldwide according to the World Health Organization data of the year 2018 [1]. Out of all types of cancer, gastric cancer standard second most frequent type of cancer which is responsible for the death of the patients [2]. There are various first- and second-line treatment strategies have been developed however recently oral fluoropyrimidines have been developed as inactive prodrugs of 5-fluorouracils which get absorbed intact through gastrointestinal mucosa and then converted to 5-fluorouracil by one or more enzymatic system [2]. One such development was combination of tegafur, gimeracil and oteracil which was approved by European Medicines Agency (EMA) in March-2011 and is commercially available as "Teysuno" [3]. The main active drug in this combination is tegafur, a prodrug of 5-fluorouracil which rapidly acts on cancer cells halting their growth by -inserting itself into the DNA and RNA strands and inhibiting their replication process [3]. The main role of gimeracil is to prevent the breakdown of 5-fluorouracil so that sufficient concentration of 5-fluorouracil can be maintained for sustained effect on cancer cells [4]. Gimeracil acts by selectively blocking the dihydropyrimidine dehydrogenase enzyme which is responsible for degradation of 5-fluorouracil [5]. The main action of oteracil in the combination is to block the conversion of tegafur prodrug into 5-fluorouracin in the gastrointestinal tract and thereby preventing the toxic effects of 5-fluorouracil in the gastrointestinal tract. Oteracil blocks the orotate phosphoribosyltransferase enzyme which is responsible for generation of 5-fluorouracil in the gastrointestinal tract [4]. Hence to enhance the efficacy and to reduce the unwanted toxicity, all the three components are vital in the combination therapy.

5.2 DRUG PROFILE [14,15]

Figure-5.1: Molecular structure of gimeracil with identification number.



IUPAC name: 5-chloro-4-hydroxy-1*H*-pyridin-2-one

Molecular formula: $C_5H_4ClNO_2$

Molecular weight: 145.54

Exact mass: 144.9930561

Monoisotopic mass: 144.9930561

pKa: 4.50

Commercial formulation: Teysuno (Combination of tegafur, gimeracil and oteracil) as capsule containing tegafur, gimeracil and oteracil (20mg, 5.8mg, 15.8mg respectively) from Taiho Pharmaceutical Co., Ltd and Nordic Group BV

5.3 LITERATURE REVIEW:

In the literature search, one LC-MS/MS method was found which is for the determination of tegafur, 5-fluorouracil, gimeracil and oteracil in human plasma [6]. However, this method involves the lengthy step of derivatization and use of internal standard. Moreover, method for estimation of related substances in capsule of combination drugs has been patented which contains the HPLC method [7]. Simultaneous estimation of tegafur and gimeracil has also been published with LC-MS/MS method [8]. However, to the best of our knowledge, there is no analytical method published anywhere which can estimate gimeracil without any derivatization procedure or can analyze gimeracil in presence of its

degradation products. Hence present study was initiated to develop HPLC method for estimation of gimeracil in presence of its degradation products. Moreover, the LC-MS compatible method was also developed and major forced degradation products were tried to be characterized by their mass values. International Conference on Harmonisation guidelines were followed to perform the forced degradation studies of the drug substance as well as for estimating the threshold of degradation products for further identification and characterization [9-12]. Both the methods were validated as per ICH guideline Q2 (R1) for the parameters of accuracy, method precision, linearity, limit of detection, limit of quantitation, robustness and ruggedness. [13]

The established methods would be definitely useful for the pharmaceutical industries to quantitatively estimate the content of gimeracil as well as their degradation products.

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

5.4.1 FORCED DEGRADATION STUDY AND ANALYTICAL METHOD DEVELOPMENT

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

5.4.1.1 EXPERIMENTAL

5.4.1.1.1 Chemicals, reagents and materials

The active pharmaceutical ingredient alectinib 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.

5.4.1.1.2 Instruments:

5.4.1.1.2.1 High performance liquid chromatography

The HPLC system (Waters Alliance 2695) equipped with PDA detector used with the Empower 3.0 software for data acquisition. 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.

5.4.1.1.2 Solutions preparation under various degradation conditions for forced degradation study

The sample solutions used for forced degradation study were having concentration of 0.25 mg/mL in diluent. Diluent was used as the mixture of mobile phase-A and acetonitrile in the ratio of 50:50

5.4.1.1.2.1 Acidic condition

Approximately 2.5 mg of gimeracil was accurately weighed and transferred into 10 mL of volumetric flask; 5mL of 5M HCl solution in water was added. The solution was heated at 80°C for 60 minutes and neutralized with 5M NaOH solution before injection. Finally, the solution was diluted up to the mark with diluent. A blank solution was also prepared under identical conditions omitting the sample in preparation.

5.4.1.1.2.2 Alkali condition

For alkali degradation, approximately 2.5 mg of gimeracil was accurately weighed and transferred into a 10 mL volumetric flask. 5mL of 5M NaOH solution in water was added. The solution was then kept in the water bath at 80 °C for 60 min. The solution was then neutralized with 5M HCl solution and finally diluted up to the mark with diluent before injection. A blank was also prepared under similar conditions omitting the sample in preparation.

5.4.1.1.2.3 Oxidation condition

Approximately 2.5 mg of gimeracil was accurately weighed and transferred into a 10 mL volumetric flask, followed by the addition of 5mL of 30 % H_2O_2 solution. The solution was heated in water bath for 60 minutes at 80°C temperature and diluted up to the mark with diluent. A blank was also prepared under the same condition omitting the sample in

preparation.

5.4.1.1.2.4 Photolytic condition

Approximately 2.5 mg of gimeracil was accurately weighed and transferred into a 10 mL volumetric flask and diluted up to the mark with diluent. Moreover, 50 mg of gimeracil was exposed directly to UV-visible light in the photo-stability cabinet for 4 hours duration. Similarly, the blank was also prepared in the same condition omitting the sample in preparation.

5.4.1.1.2.5 Thermal condition

Approximately 25 mg of gimeracil was accurately weighed and transferred to porcelain dish and spread well. The drug substance was exposed to heating at 105 °C for 2 hours.

Similarly, degradation was also performed with synthetic mixture solution by taking 43.32 mg weight of gimeracil synthetic mixture.

Unlike the first two drugs in the study (alectinib and nelarabine), analytical method for gimeracil by LC-UV-PDA does not contain MS compatible mobile phase. Hence, two methods were developed: one is for LC-UV-PDA and second is for LC-MS and HRMS study. Following sections are for experimental and results and discussion of method development trials for LC-UV-PDA method. The details of MS compatible method development have been discussed under Section 5.4.3 Identification of degradation products by HRMS as the method was used for identification in that study.
This following is the details about method development trial experiment details:

LC and LCMS methods were developed by starting 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:

5.4.1.1.3 Solution preparation for analytical method development:

250µg/mL solution of gimeracil was prepared by dissolving 2.5mg of gimeracil in diluent

of water as the drug is freely soluble in water. This solution was taken and initiated the development trials as shown in Section 5.4.2.1.4 to Section 5.4.2.4.6.

Since the commercial formulation of gimeracil capsules are not available, synthetic mixture of gimeracil with other excipients as per the literature [3] was prepared in laboratory and used in study wherever required. As per the label claim and information, following ingredients were mixed in the mentioned proportion for synthetic mixture preparation:

Table-5.1: List of ingredients added for preparation of synthetic mixture [3]

Sr No.	mg/Capsule	Ingredient
1	5.80	Gimeracil
2	93.60	Lactose Monohydrate
3	1.10	Magnesium Stearate
Total	100.5	NA

5.4.1.1.4 Selection of detection wavelength

Degradation sample of gimeracil was run in PDA detector of HPLC and found that the wavelength maxima of gimeracil peak at 287.8 nm and 230 nm. The peak purity of gimeracil is also appropriately high considering no other impurity peak got merged with it. However, when wavelength maximum of other degradation products was extracted, these were obtained at 230nm, 220.4nm, 273.5nm, 248.7, and 277.1 nm. (Figure-5.2-A, Figure-5.2-B) Based on this, the wavelength for determination in HPLC was decided to keep as 248 nm so that all the relevant peaks can have sufficient response in the chromatogram. With the wavelength of 248 nm, other experimental trials were performed for development of LC and MS compatible method as shown in Section 5.4.2.1.5 and Section 5.4.2.1.6 respectively.

Figure-5.2-A: Wavelength maximum of gimeracil showing two maxima at 287.8 nm and at around 230 nm.

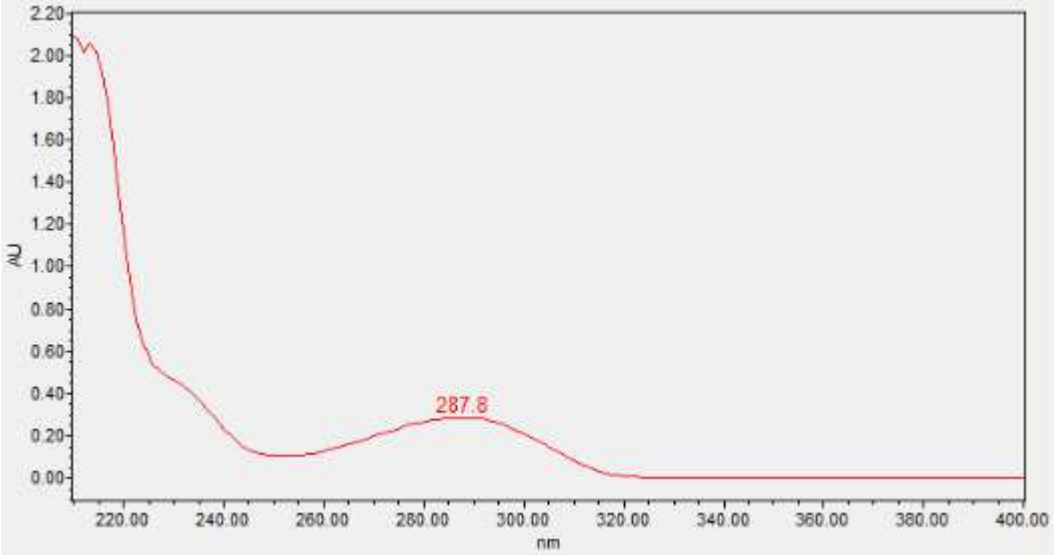
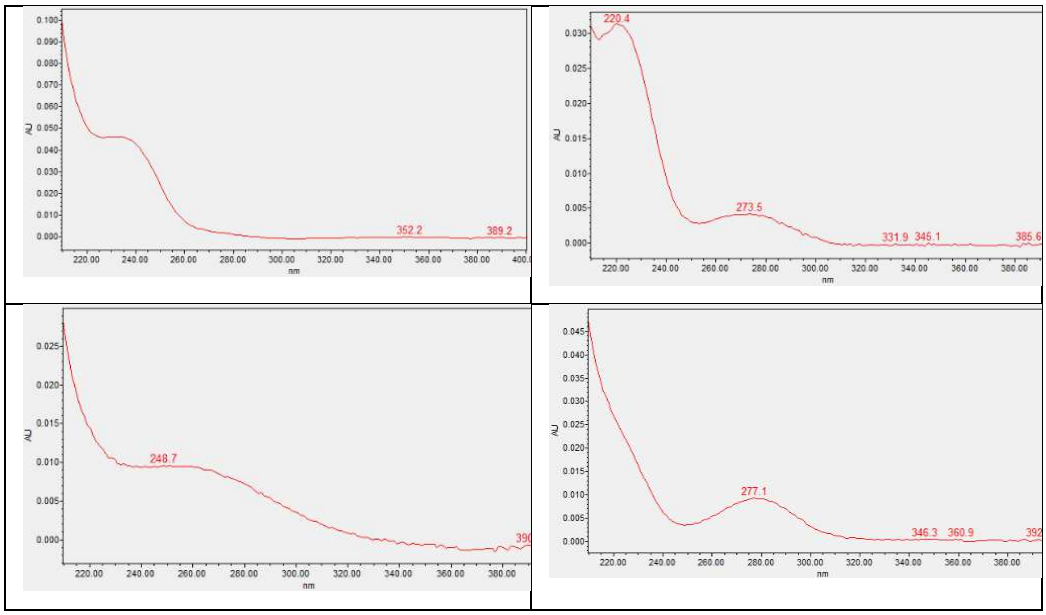


Figure-5.2-B: Wavelength maxima of other degradation products present in the chromatogram of degraded sample of gimeracil.



5.4.1.1.5 Mobile phase and column optimization

5.4.1.1.5.1 Trial-1

After decision on solution concentration and wavelength maximum determination as discussed in Section 5.4.2.1.3 and Section 5.4.2.1.4, trials for mobile phase selection were initiated. Based on pKa of gimeracil, the buffer for mobile phase preparation was selected as 10mM KH_2PO_4 buffer and pH kept at 2.50 with orthophosphoric acid. Initial chromatogram was run with 10µl injection volume and with isocratic flow (0.5ml/min) of mobile phase-A as buffer (10mM KH_2PO_4 buffer and pH kept to 2.50) and mobile phase-B as acetonitrile in the ratio of 50:50. The column is chosen as Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

5.4.1.1.5.2 Trial-2

From the results obtained in trial-1 experiments, it is required to retain the peak in the chromatogram with some peak retention reagents which can be used as mobile phase modifiers. To shift gimeracil peak further in the chromatogram, 1g per liter quantity of 1-octane sulphonic acid salt and 10mL of triethylamine were added in the buffer as ion pair reagent. Moreover, gradient was applied with mobile phase-B (acetonitrile) starting with 0.0% at 00 minutes and reaching to 25% at 25 minutes. Other analytical conditions were kept same as mentioned in trial-1 experiment.

5.4.1.1.5.3 Trial-3

Other than column selection and mobile phase chemistry, diluent selection also plays major role in eluting peak in good peak shape. Hence, due to no improvement in peak shape of gimeracil in trial-1 and trial-2 experiments, various diluents were tried such as water, methanol, mixture of water: methanol (50:50), and water: acetonitrile (50:50). Other analytical instrument parameters continued from trial-2 experiment.

5.4.1.1.5.4 Trial-4:

After taking efforts on trying different diluents, no major change in peak shape was observed which indicates the column chemistry might be playing role in elution of

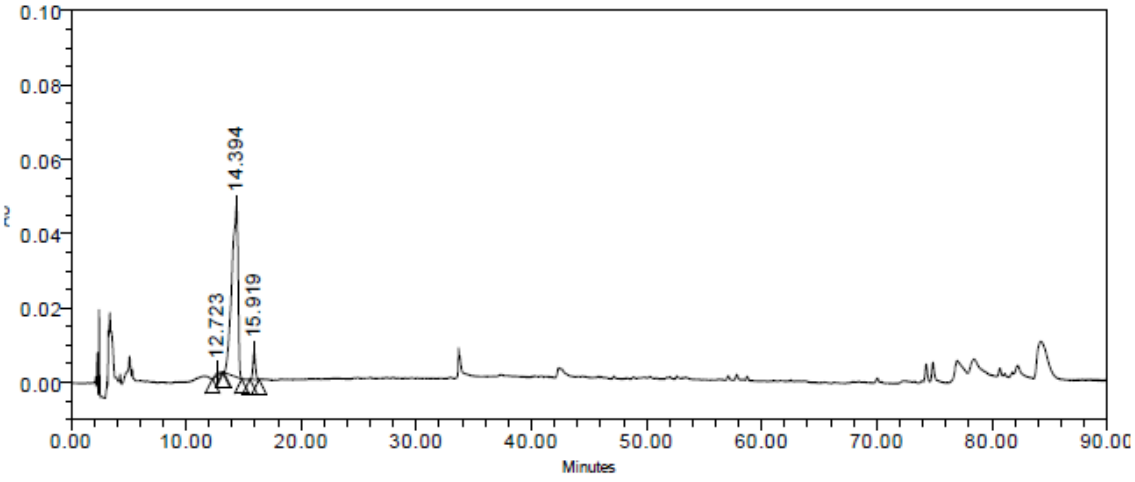
compound. Hence, considering this, it was tried to change the column. Initially, the chromatographic experiments were started with column Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ which was changed to similar composition but waters make and higher particle size column i.e., Waters X-Bridge C18 (250x4.6) mm, 3.5µ.

5.4.1.2 RESULTS AND DISCUSSION

5.4.1.2.1 Forced degradation study results

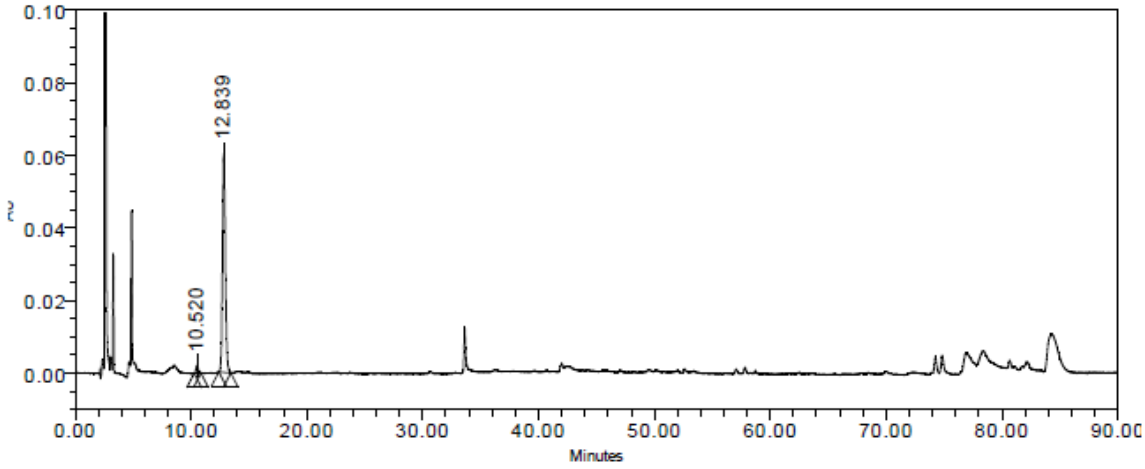
No degradation products were found to be generated except in oxidative degradation condition. In alkali condition, two impurities peaks were generated at RRT 0.88 and 1.11 at very high concentration of NaOH and heating at 80°C for 1 hour which is very harsh conditions. When 250µg/mL of gimeracil was exposed to 1mL of 1 M NaOH and heated for 70°C for 30 minutes, no major degradation products were observed. (Figure-5.3)

Figure-5.3: Chromatogram of gimeracil degraded in alkali degradation condition.



In oxidative degradation condition, the main peak of gimeracil observed to be reduced to almost half but again the degradation products were not detected under the chromatographic conditions i.e., UV/PDA detector even if the run time was extended to 90 minutes. It was therefore planned to develop a LC method with MS detector. Due to this limitation only, the degradation kinetic study was not performed for gimeracil and further study was extended to HRMS analysis.

Figure-5.4: Chromatogram of gimeracil degraded in oxidative degradation condition.



As shown in Figure-5.5, Figure-5.6 and Figure-5.7, no major degradation products were observed when gimeracil was degraded under acidic, thermal and photolytic degradation conditions. The unknown peak at low level at RT 4.838 minutes was observed in acidic degradation sample which is not observed when similar degradation sample injected in HRMS study. Since, thermal and photolytic degradation samples were run on different day sequence, the RT of gimeracil was observed to be shifted to 16.848 minutes from 12.848 minutes initially. However, other peaks pattern observed comparable, hence they were considered for study.

Figure-5.5: Chromatogram of gimeracil degraded in acidic degradation condition.

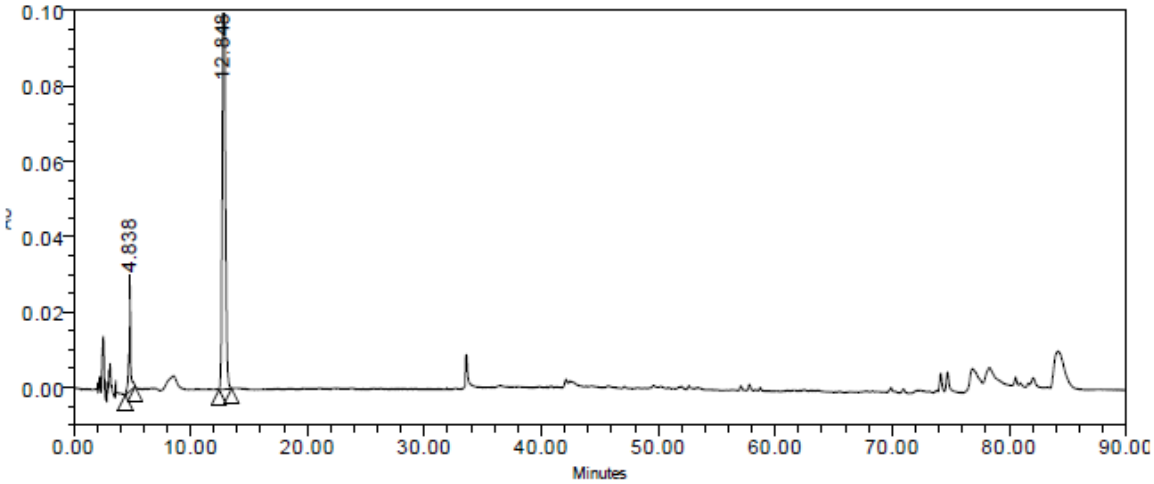


Figure-5.6: Chromatogram of gimeracil degraded in thermal degradation condition.

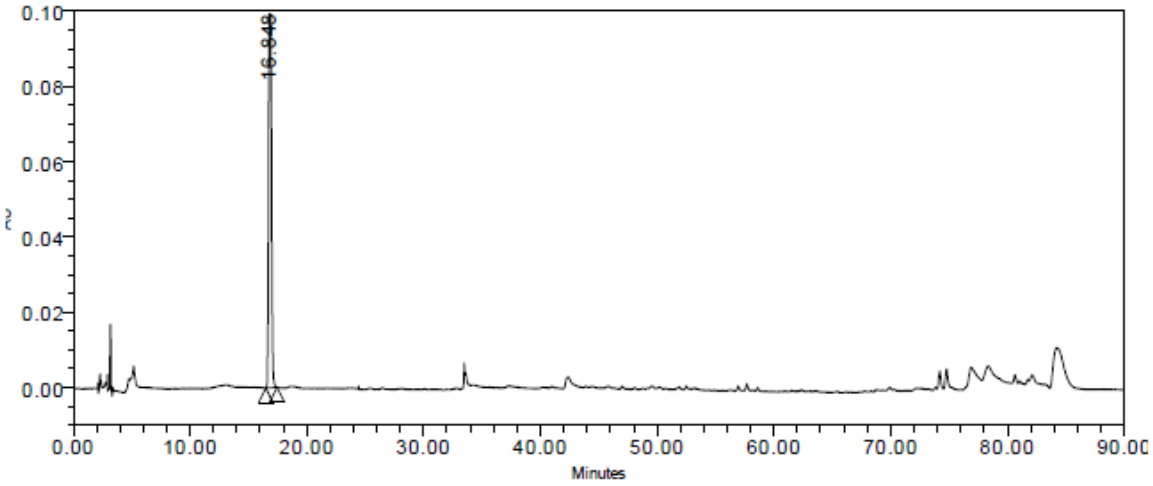
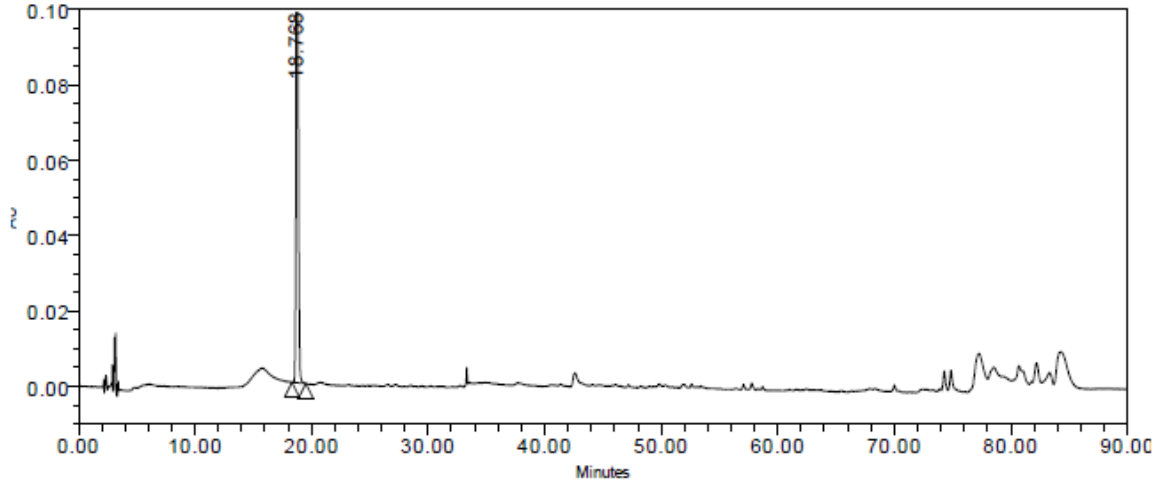


Figure-5.7: Chromatogram of gimeracil degraded in photolytic degradation condition.



This following is the details about the results and discussion obtained from method development trial experiments and then final analytical conditions for HPLC-UV-PDA instrument method.

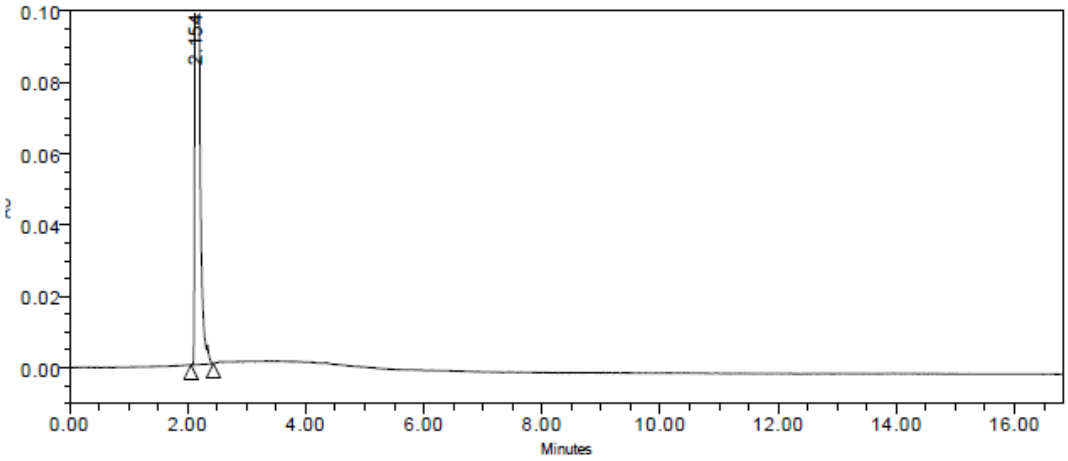
5.4.1.2.2 Mobile phase and column optimization

5.4.1.2.2.1 Trial-1

The peak shape of gimeracil was sharp but the retention time observed was very early, at around 2.154 minutes (Figure-5.8). Since gimeracil compound is polar in nature, it gets

easily eluted from the C18 column in aqueous buffer mobile phase system. In such cases, peak retention reagents are generally used to retain the peak and make their elution late in the chromatogram. In gimeracil retention, 1-octane sulphonic acid sodium salt is used as ion pair reagent as mentioned in experimental Section 5.4.2.1.5.2 Trial-2.

Figure-5.8: Chromatogram of gimeracil with pH 2.5 buffer and acetonitrile in isocratic flow rate in the ratio of 50:50.



Chromatographic conditions:

Column: Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

Mobile phase: mobile phase-A: 10mM KH2PO4 buffer and pH adjusted to 2.50 with orthophosphoric acid, mobile phase-B: acetonitrile

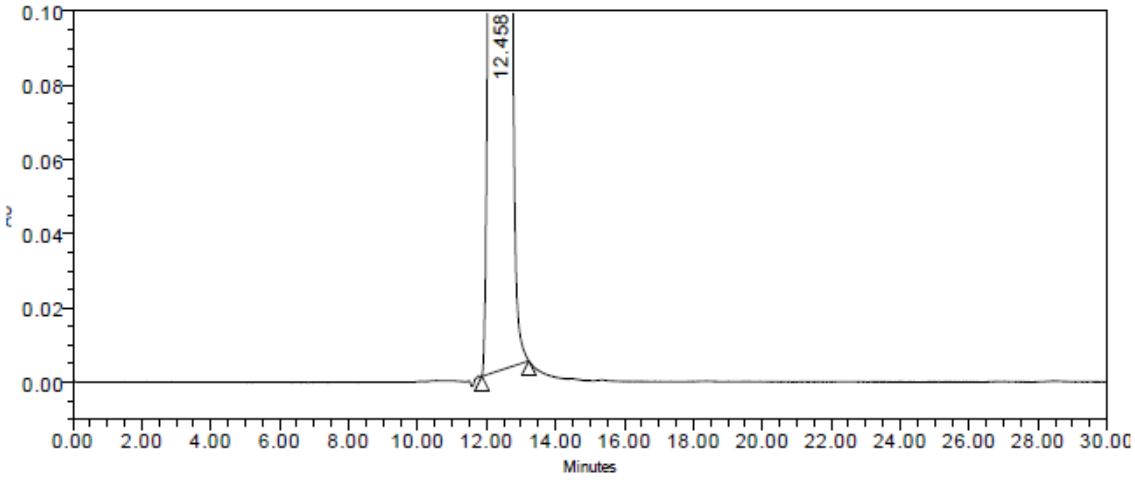
Detection wavelength: 248nm

Gradient and flow rate: isocratic flow (0.5ml/min) of mobile phase-A as buffer (10mM KH2PO4 buffer and pH kept at 2.50) and mobile phase-B as acetonitrile in the ratio of 50:50.

5.4.1.2.2.2 Trial-2

The retention time of gimeracil was further extended in the chromatogram at around 13 minutes due to ion pair reagent but the peak shape of gimeracil peak became broad. (Figure-5.9)

Figure-5.9: Chromatogram of gimeracil solution with mobile phase with ion pair reagent.



Chromatographic conditions:

Column: Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

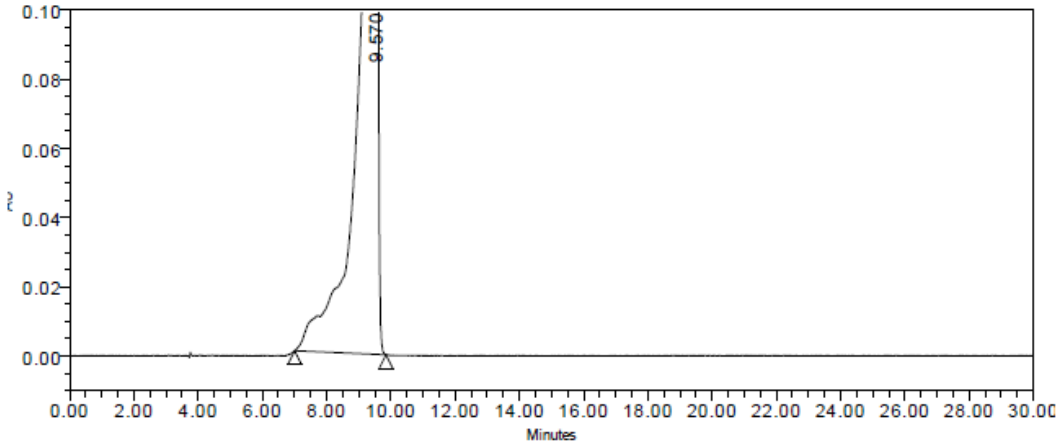
Detection wavelength: 248nm

Gradient and flow rate: mobile phase-B (acetonitrile) starting with 0.0% at 00 minutes and reaching to 25% at 25 minutes.

5.4.1.2.2.3 Trial-3

Out of all the four diluents taken, peak shape of gimeracil found bad in the diluent of methanol or mixture of water: methanol (50:50). In both the cases, the stringent fronting was noted. However, in water or mixture of water: acetonitrile (50:50) as diluent, the peak shape was found to be satisfactory hence this diluent was finalized for further experiments in the same method. (Figure-5.10, Figure-5.11 and Figure-5.12)

Figure-5.10: Chromatogram of gimeracil in the diluent of methanol in the same condition



Chromatographic conditions:

Column: Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

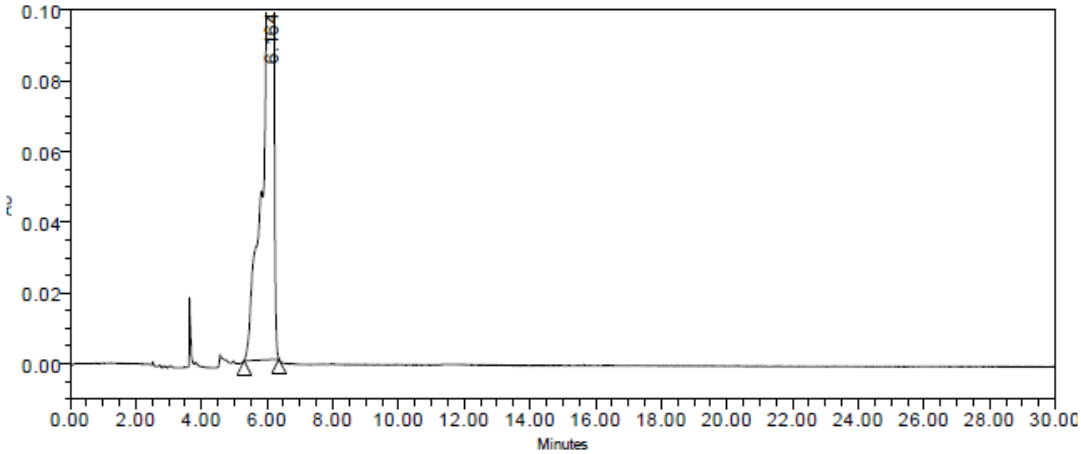
Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

Detection wavelength: 248nm

Gradient and flow rate: Mobile phase-B (acetonitrile) starting with 0.0% at 00 minutes and reaching to 25% at 25 minutes.

Diluent: Methanol

Figure-5.11: Chromatogram of gimeracil in the diluent of water: methanol (50:50) in the same condition



Chromatographic conditions:

Column: Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of

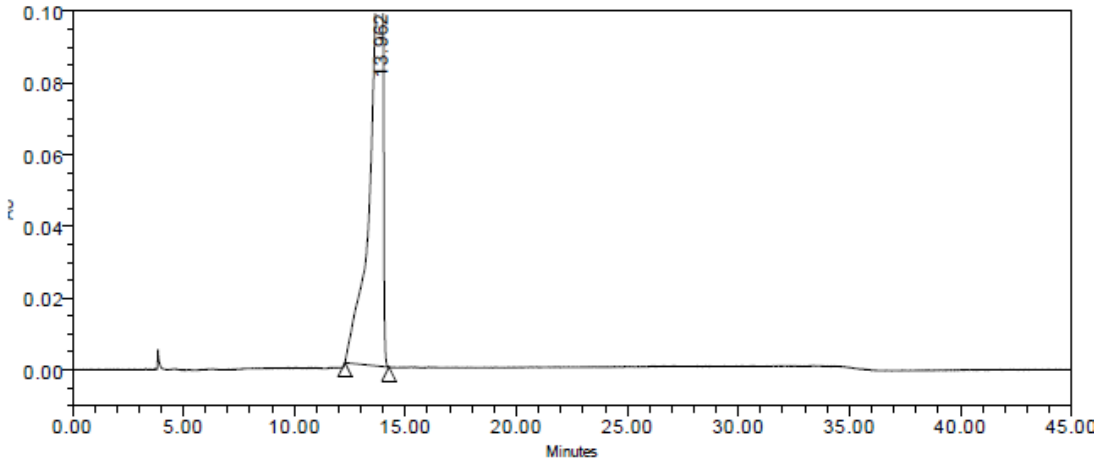
triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

Detection wavelength: 248nm

Gradient and flow rate: Mobile phase-B (acetonitrile) starting with 0.0% at 00 minutes and reaching to 25% at 25 minutes.

Diluent: Water:Methanol (50:50)

Figure-5.12: Chromatogram of gimeracil in the diluent of water: methanol (70:30) in the same condition



Chromatographic conditions:

Column: Poroshell 120 EC-C18 (250x4.6) mm, 1.7µ.

Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

Detection wavelength: 248nm

Gradient and flow rate: Mobile phase-B (acetonitrile) starting with 0.0% at 00 minutes and reaching to 25% at 25 minutes.

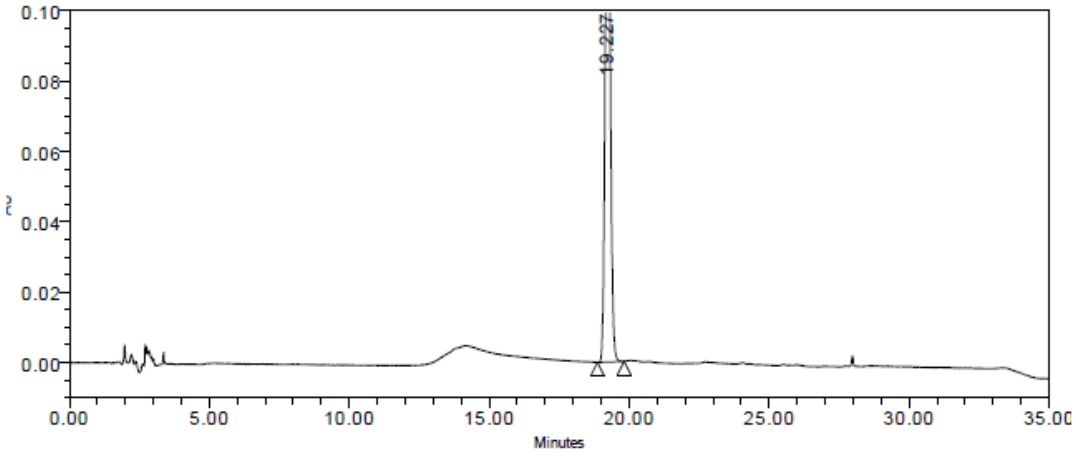
Diluent: Water:Methanol (70:30)

5.4.1.2.2.4 Trial-4

With X-Bridge C18 column, better peak parameters were observed, especially the number of theoretical plates was increased for gimeracil peak. This column was therefore used for other experiments (Figure-5.13). Here it is to be noted that the mobile phase and columns

have changed while development of MS-compatible method of gimeracil. So different columns and mobile phase conditions have been mentioned in MS compatible method section of gimeracil. Gimeracil synthetic mixture sample was injected in the same analytical conditions and identical chromatogram was obtained (Figure-5.14).

Figure-5.13: Chromatogram of gimeracil with X-Bridge column and other analytical conditions which have been finalized for LC method.



Chromatographic conditions:

Column: Waters X-Bridge C18 (250 X 4.6) mm, i.d., 3.5µ

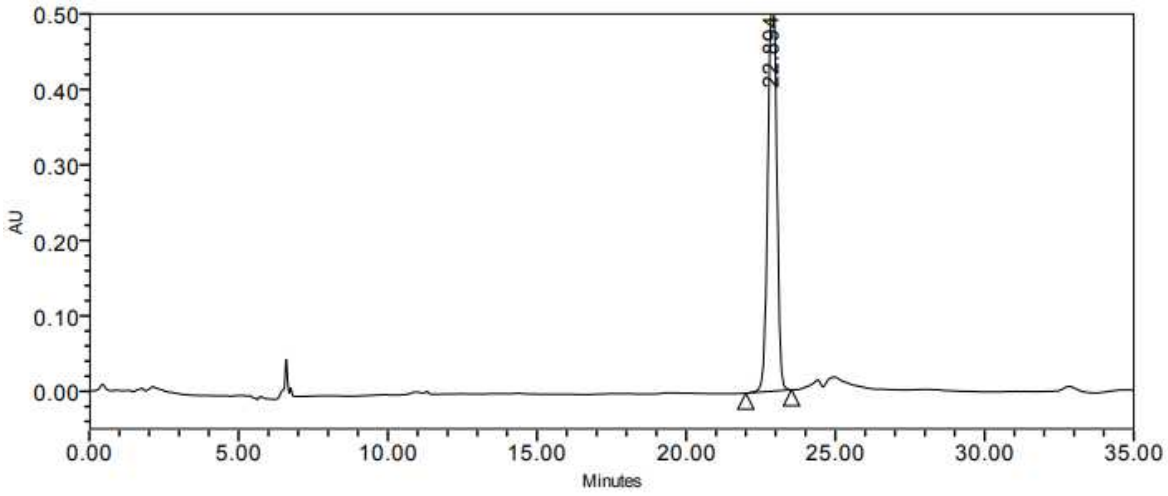
Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

Detection wavelength: 248nm

Gradient and flow rate: Mobile phase-B from 0% to 30% at 30 minutes. Last five minutes of gradient was kept at initial equilibration phase for mobile phase-A and mobile phase-B

Diluent: Mobile phase-A and acetonitrile in the ratio of 50:50

Figure-5.14: Chromatogram of gimeracil synthetic mixture at equivalent to gimeracil 250µg/mL



Chromatographic conditions:

Column: Waters X-Bridge C18 (250 X 4.6) mm, i.d., 3.5µ

Mobile phase: Mobile phase-A was 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only.

Detection wavelength: 248nm

Gradient and flow rate: Mobile phase-B from 0% to 30% at 30 minutes. Last five minutes of gradient was kept at initial equilibration phase for mobile phase-A and mobile phase-B

Diluent: Mobile phase-A and acetonitrile in the ratio of 50:50

5.4.1.2.3 Final analytical conditions:

5.4.1.2.3.1 High performance liquid chromatography

Waters X-Bridge C18 (250 X 4.6) mm, i.d., 3.5µ (Make: Waters) column was maintained at room temperature during analysis. Mobile phase-A was composed of 10mM solution of potassium dihydrogen orthophosphate with 10mL of triethylamine and 1.00 g of 1-octane sulphonic acid salt per liter of mobile phase and further adjusting the pH of this solution to 2.50 with orthophosphoric acid. Mobile phase-B was composed of acetonitrile only. Diluent was optimized as the mixture of mobile phase-A and acetonitrile in the ratio of 50:50. Wavelength for estimation of gimeracil was kept at 248nm and flow rate was maintained at 0.5mL/min. Injection volume was set at 10µl and the gradient was linear for 0 to 30 minutes starting %mobile phase-B from 0% to 30% at 30 minute. Last five minutes

of gradient was kept at initial equilibration phase for mobile phase-A and mobile phase-B

5.4.2 ANALYTICAL METHOD VALIDATION

Method validation experiments were conducted as per ICH guideline Q2 (R1) [13] and parameters such as linearity, sensitivity, accuracy, method precision, robustness and specificity were performed.

5.4.2.1 EXPERIMENTAL

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

5.4.2.1.1 Linearity

Linearity was performed by preparing the different concentration of gimeracil solutions in the range of 125µg/mL to 375µg/mL. All the experiments were performed in triplicate. Calibration curve was plotted between the area response observed and concentration. Correlation coefficient was calculated from the calibration curve. For good linear calibration curve, correlation coefficient should be more than 0.99.

5.4.2.1.2 Sensitivity

LOD and LOQ were practically determined by injecting series of concentration 125 µg/mL to 375 µg/mL of gimeracil solution and identifying the lowest possible level at which the %RSD of the triplicate injections is within 2% specifications. Similarly, the lowest possible concentration level was identified at which the detection of the peak response is possible.

Theoretically based on the linearity curve, the LOD and LOQ were also calculated by the formula as per ICH Q2 (R1) guideline. As per the guideline, the LOD = 3.3 x σ/S and LOQ = 10 x σ/S where σ is standard deviation of intercept from the calibration curve and S is mean slope of the calibration curve.

5.4.2.1.3 Accuracy

The accuracy of the method was performed by the standard addition method whereby the 50%, 100% and 150% level concentration of 250µg/mL was spiked into the pre-quantified sample mixture of gimeracil. The spiked solutions were prepared in triplicate and injected into the system and the %recovery was calculated at each of the three levels.

5.4.2.1.4 Precision

Repeatability was performed by injecting six replicate sample preparation of 250µg/mL concentration. Method precision parameter was performed by injecting three different levels of spiked samples at 50%, 100% and 150% levels of 250µg/mL in triplicate. The %RSD was calculated from the response observed at each level.

5.4.2.1.5 Robustness:

Robustness was performed by applying small but deliberate changes in flow rate modification. The flow rate was changed to +/- 0.2mL/min and the effects on the results were monitored. The robustness of the method was estimated at the level of 100% target concentration (250µg/mL) and the retention time, peak shape, theoretical plates and tailing factor were compared.

5.4.2.1.6 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.

5.4.2.2 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:

5.4.2.2.1 Linearity

The method was found to be linear in the range of 125µg/mL to 375µg/mL.. The correlation coefficient was found to be 0.9918. The regression data are mentioned in Table-5.2 and calibration curve in Figure-5.15 demonstrating good linear relationship between specified concentration and response observed.

Table-5.2: Linearity, LOD, and LOQ results

Level (%)	Concentration (µg/mL)	Area			Mean	Standard deviation	%RSD	
150	**375**	5526216	5532548	5526582	5528448.67	3554.8403	0.06	
125	**312**	4465278	4432421	4526829	4474842.67	47925.252	1.07	
100	**250**	4075839	4068705	4097739	4080761.00	15129.866	0.37	
75.2	**188**	2974623	2938796	2958164	2957194.33	17933.172	0.61	
50	**125**	1960043	1964125	1965690	1963286.00	2915.4919	0.15	
24.8	**62**	901486	895285	884592	893787.67	8545.9531	0.96	
12.4	**31**	456689	460031	461595	459438.33	2506.1224	0.55	
5	**12.5**	180955	184137	183522	182871.33	1687.8407	0.92	
2.5	**6.25**	86582	86035	86878	86498.33	427.68251	0.49	
1.248	**3.12**	29967	30797	30676	30480.00	448.3715	1.47	LOQ with 2% RSD limit
0.672	**1.68**	25799	24874	25841	25504.67	546.57692	2.14	
0.3	**0.75**	10822	11053	10654	10843.00	200.32723	1.85	
0.148	**0.37**	6161	5914	5789	5954.67	189.30487	3.18	LOD
0.072	**0.18**	0	0	0	0	0	NA	
Slope					**13861.51**			
Intercept					**335528.69**			
Correlation Coefficient (r)					**0.9918**	**NLT 0.99**		
Standard deviation					1378913.50			
LOD (µg/mL)					3.28			
LOQ (µg/mL)					9.95			

Figure-5.15: Linearity plot between Area response vs concentration of linearity level.

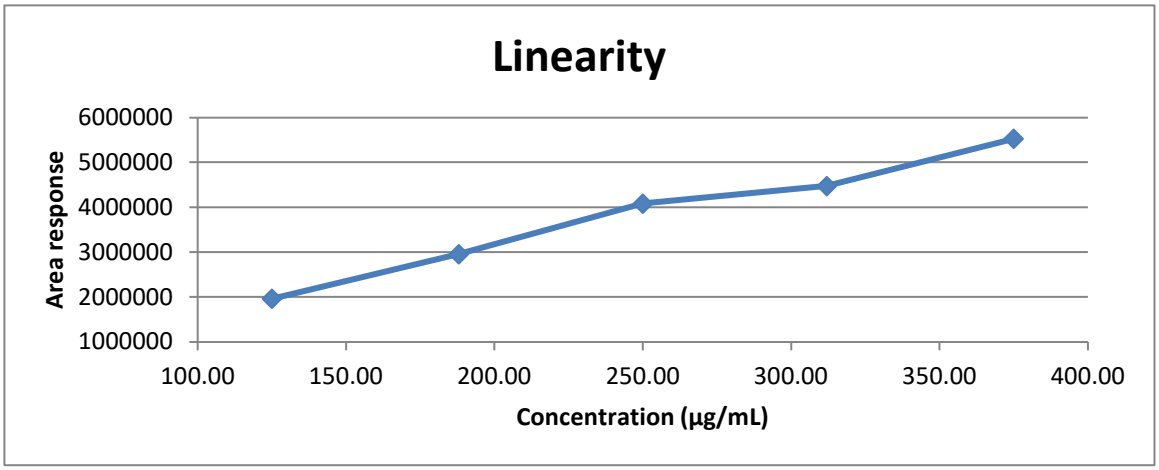
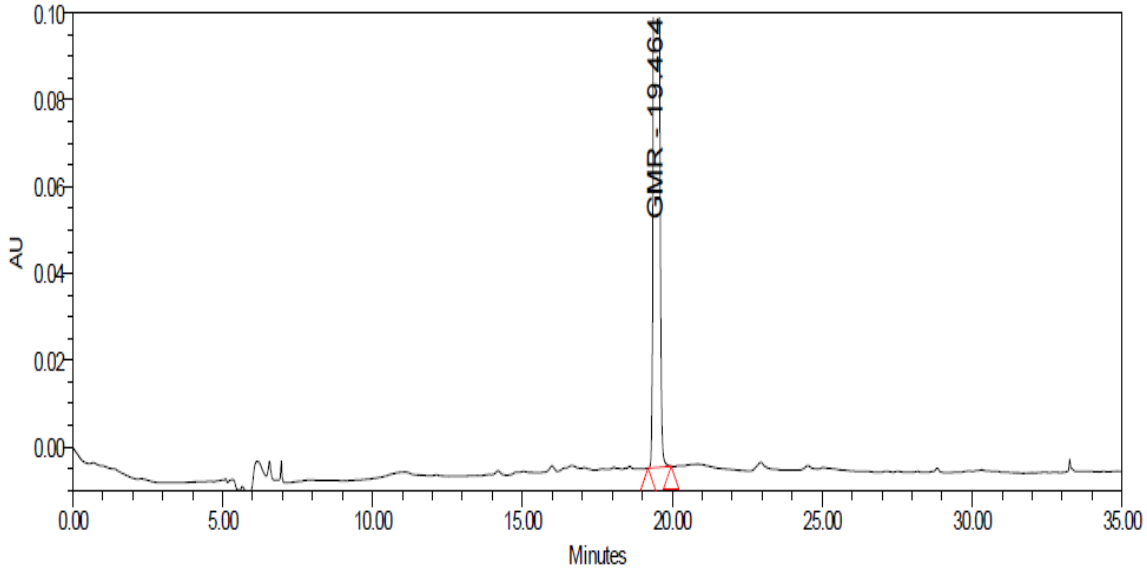


Figure-5.16: Chromatogram of gimeracil with concentration of 250µg/mL.



5.4.2.2.2 Sensitivity (LOD and LOQ)

As per the practical response observed over the entire range of concentration, the lowest concentration which is possible to quantify is 3.12µg/mL level where the %RSD of the three injections was obtained as 1.47%. The lowest concentration which is possible to detect by this analytical method was found to be 0.37µg/mL below which the response was not detected by the analytical method.

Based on theoretical formula as per the ICH guideline, LOD was calculated from calibration curve of linearity plot (Figure 5.15) as 3.28% level (1.312µg/mL) and LOQ was

calculated as 9.95% level (3.98µg/mL).

5.4.2.2.3 Accuracy

As per the recovery experiment performed, the % recovery at the levels of 50%, 100% and 150% of 250µg/mL concentrations were obtained in the range of 109.14% to 114.82% which proves that the method is capable of estimating the content accurately near to true value.

Table-5.3: Recovery results at the levels of 50%, 100% and 150%

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	1153711	50.00	2444526	1290815	52.88	105.75	1288693.00	15587.71	1.21
50% Set-2	1153711	50.00	2456822	1303111	53.38	106.76			
50% Set-3	1153711	50.00	2425864	1272153	52.11	104.22			
100% Set-1	1153711	100.00	3672041	2518330	103.16	103.16	2514973.67	12112.43	0.48
100% Set-2	1153711	100.00	3655248	2501537	102.47	102.47			
100% Set-3	1153711	100.00	3678765	2525054	103.43	103.43			
150% Set-1	1153711	150.00	5043544	3889833	159.34	106.23	3926257.33	43336.59	1.10
150% Set-2	1153711	150.00	5068465	3914754	160.36	106.91			
150% Set-3	1153711	150.00	5127896	3974185	162.79	108.53			

5.4.2.2.4 Precision

The % RSD of the six replicates of sample preparation at 250µg/mL was obtained as 0.20%. The %RSD of the response obtained at 50%, 100% and 150% levels of 250µg/mL concentration was found in the range of 0.48% to 1.21%. Each kind of precision study results prove that the method is precise.

5.4.2.2.5 Robustness

The chromatogram results were observed at flow rate of 0.48 mL/min, 0.52 mL/min. The

peak shape obtained proper in each of the variations. The slight RT variation was observed from 19.464 minutes to 20.072 minutes with lower flow rate and to 19.029 minutes with higher flow rate. In robustness experiments, two additional blank peaks were observed at RT 6.157 minutes and 7.106 minutes which are to be disregarded (Figure-5.17 and Figure-5.18). The theoretical plates, tailing factor and retention times obtained were compared with that of actual method of analysis parameters. Hence proposed analytical method was proved to be robust.

Figure5.17 Chromatogram of gimeracil sample 250µg/ml with flow rate 0.48 mL/min

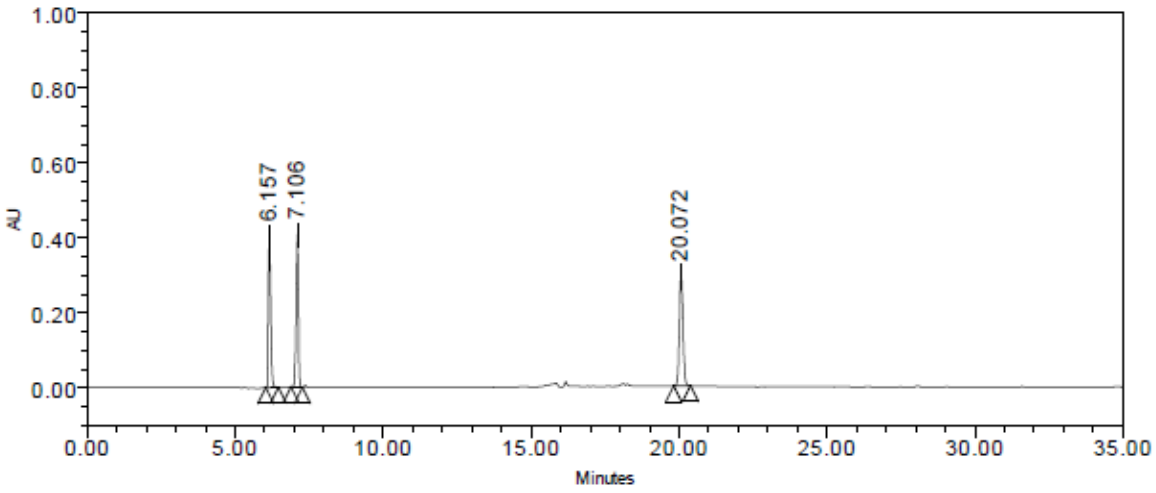
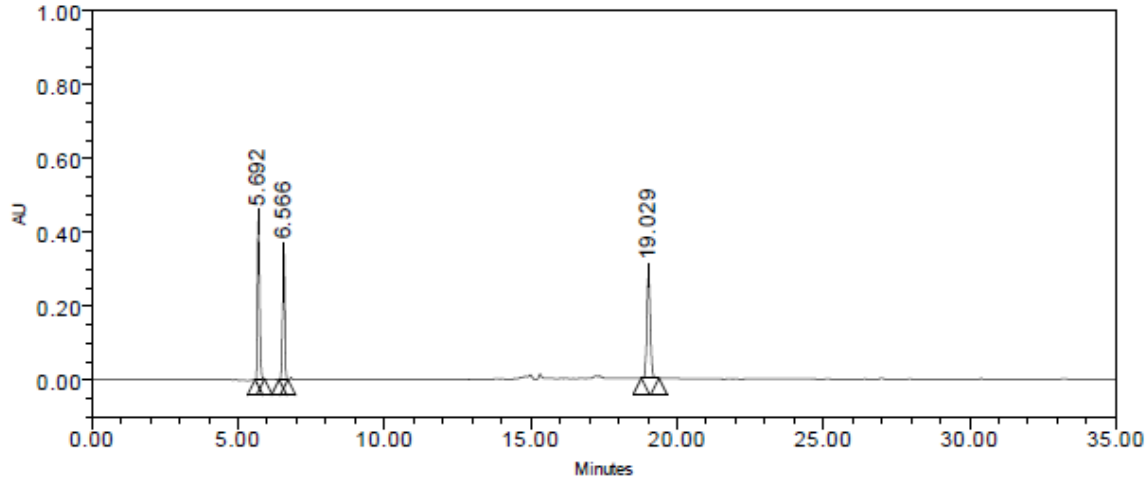


Figure-5.18 Chromatogram of gimeracil sample 250µg/ml with flow rate 0.52 mL/min



5.4.3 IDENTIFICATION OF DEGRADATION PRODUCTS BY HRMS

5.4.3.1 EXPERIMENTAL

5.4.3.1.1 Liquid chromatography mass spectrometry

The liquid chromatography mass spectroscopy system (LC-MS/MS) of Waters Micro-mass with ZQ-Mass detector and electro spray ionization mode was used. The LC-MS system of Thermo LCQ-Fleet with UHPLC Ultima-3000 was also used for development of MS compatible method

5.4.3.1.2 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.

5.4.3.1.3 Analytical method development for MS compatible LC method for LCMS and HRMS study

Since the LC-UV-PDA method of gimeracil does not contain MS compatible eluents, it was required to optimize the mobile phase and accordingly other analytical conditions for development of MS compatible method. Wavelength maximum and other analytical conditions kept same as LC-UV-PDA method. Only **Mobile phase and column modification** were made as mentioned in below experimental trials:

5.4.3.1.3.1 Trial-1:

The buffers and ion pair reagent used in LC method are not volatile and therefore this mobile phase cannot be utilized for MS detector analysis. Other than mobile phase, all the analytical conditions such as wavelength, gradient program, flow rate and injection volume and column were kept same as mentioned in LC method (Section-5.4.2.1.5). If chromatographic pattern is not observed, some parameters such as gradient program or flow rate can be modified later on. Hence, considering this, buffer for MS compatible method was tried to be used by taking water only and adjusting the pH of it to 2.50 with 98% formic acid solution.

5.4.3.1.3.2 Trial-2

To reduce the concentration of formic acid in the buffer, the buffer composition was slightly modified to 0.05% of formic acid in water instead of adjusting the pH to 2.50 with formic acid. All the other conditions kept same as trial-5.

5.4.3.1.3.3 Trial-3

Other than diluent and mobile phase, column is crucial for good peak shape of the compound. Hence, to improve peak symmetry further, the column was also changed from Waters X-Bridge C18 (250x4.6) mm, 3.5µ in LC method to YMC Pack Pro C18 (250x4.0) mm, 3µ in MS compatible method.

All the degradation samples were then injected in HPLC system with the analytical conditions mentioned in results and discussion of this section and recorded the chromatograms with all the degradation products if generated. 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.

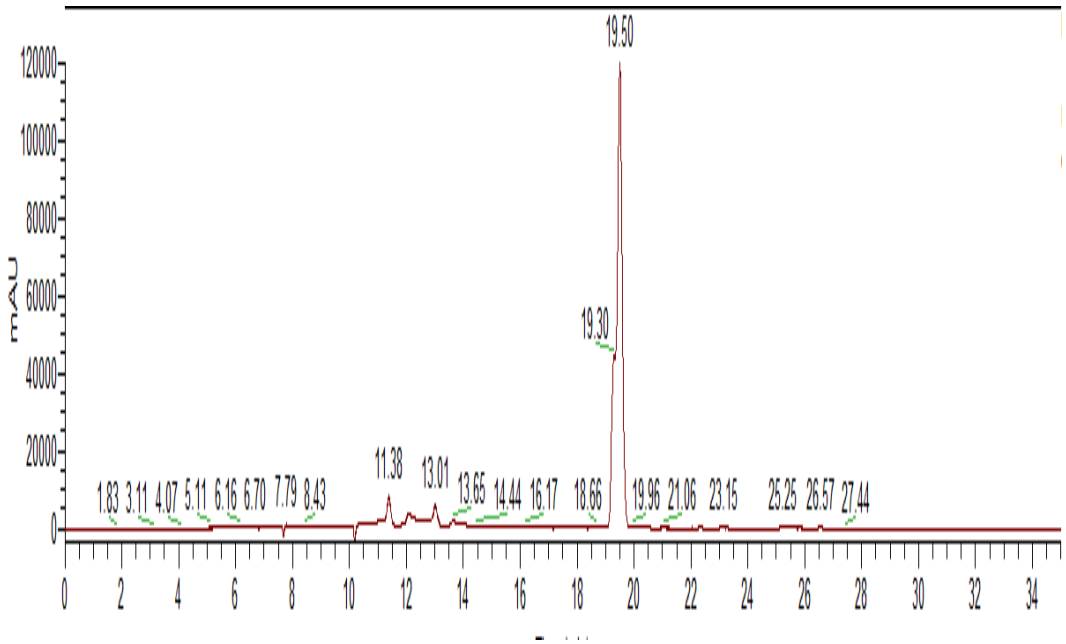
5.4.3.2 RESULTS AND DISCUSSION

5.4.3.2.1 Analytical method development results for MS compatible LC method for LCMS and HRMS study

5.4.3.2.1.1 Trial-1:

With pH 2.50 buffer in MS compatible method, the peak shape was not found sharp as well as retention time variation was observed (Figure-5.19). Due to disturbed baseline and improper peak shape, it is thought to reduce the concentration of formic acid. Therefore, next trial-6 was planned with 0.05% formic acid as buffer.

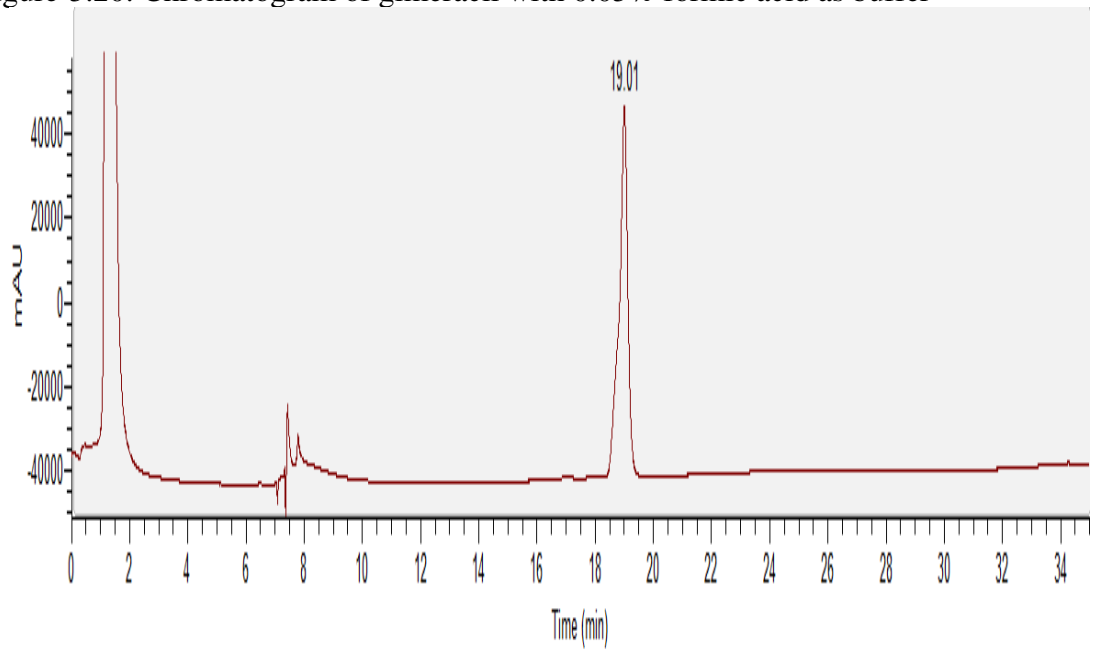
Figure-5.19: Chromatogram of gimeracil with MS compatible buffer at pH 2.50 showing improper peak shape.



5.4.3.2.1.2 Trial-2

With lower concentration of formic acid for ionization in MS detector, it was observed that the peak shape was improved but still it was expected to reach to good peak symmetry (Figure-5.20).

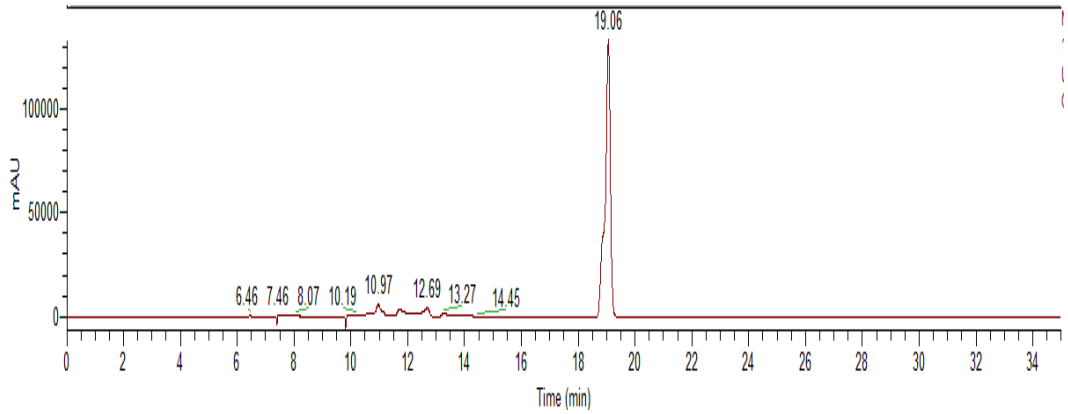
Figure-5.20: Chromatogram of gimeracil with 0.05% formic acid as buffer



5.4.3.2.1.3 Trial-3

As mentioned in Section 5.4.2.1.6.3 of trial-7, Further with change of column to similar YMC make in approximately same dimensions, it was observed to improve overall chromatography not only in terms of main peak but also in terms of its degradation products. This chromatography was again reproduced in HRMS study where all the degradation products peaks and its exact mass were obtained (Figure-5.21).

Figure-5.21: Chromatogram of gimeracil with YMC make column and 0.05% formic acid solution as buffer.



5.4.3.2.1.4 Analytical condition and instrument parameters for LC-MS

Mobile phase-A was composed of 0.05% formic acid solution in water and mobile phase-B was composed of acetonitrile only. Diluent was decided as water only. YMC Pack pro C18 (250x4.0) mm, 3µ column was taken for the LC-MS study for better peak shape. Wavelength, gradient, flow rate, injection volume and other parameters were kept same and LC method.

5.4.3.2.1.5 Analytical condition and instrument parameters for HRMS

The instrument and method parameters are same as mentioned in LC-MS. Instrument method parameters for HRMS instrument were set as per below listed table-5.4:

Table-5.4: HRMS Instrument method parameters:

Full MS / dd-MS² (TopN)	
General	**Full MS**
Runtime: 0 to 35 min	Microscans 1
Polarity: Positive and Negative	Resolution 70,000
In-source CID: 0.0 eV	AGC target 1e6
Default charge state: 1	Maximum IT 100 ms
Inclusion — NA	Number of scan ranges 1
Exclusion — NA	Scan range 100 to 1000 m/z
Tags — NA	Spectrum data type Profile
dd-MS² / dd-SIM	**dd Settings**
Microscans 1	Minimum AGC target 5.00e3
Resolution 17,500	Intensity threshold 5.0e4
AGC target 5e5	Apex trigger —
Maximum IT 100 ms	Charge exclusion —
Loop count 5	Multiple charge states all
MSX count 1	Peptide match preferred
TopN 5	Exclude isotopes on
Isolation window 2.0 m/z	Dynamic exclusion 30.0 s
Isolation offset 0.0 m/z	If idle .. do not pick others
Scan range 200 to 2000 m/z	
Fixed first mass —	
(N)CE / stepped (N)CE nce: 30	
Spectrum data type Profile	
Tune page parameters	
Scan type	Full MS
Source	HESI Source
Sheath gas flow rate	60
Aux gas flow rate	20
Sweep gas flow rate	0
Spray voltage (kV)	3.5
Spray current (µA)	0
Capillary temperature (°C)	320
S-lens RF level	55
Aux gas heater temp (°C)	450

When gimeracil drug substance was degraded in various degradation conditions, it was

not found to be degraded in conditions except oxidative degradation. Even, in oxidative degradation condition, the main peak was observed to be reduced but again the degradation products were not detected in UV detector and response of impurities were not observed in LC method. Hence, it was thought to develop MS compatible method and run in HRMS instrument so that any TIC peaks can be detected and further characterized based on MS and MS-MS fragments. In HRMS study, total fourteen degradation products were observed in TIC chromatogram of gimeracil in oxidative degradation condition (Figure-5.22). Out of fourteen, seven degradation products (DP-4, DP-5, DP-6, DP-8, DP-9, DP-10 and DP-12) were observed to be oxidative products of gimeracil where one, two or three oxygen atoms were incorporated in the molecular structure of gimeracil. Out of these seven DPs, three isomeric products were noted with addition of three oxygen molecules mass in gimeracil and three isomeric products were noted with addition of one oxygen molecules mass. Since, there are multiple positions in the structure of gimeracil, where these oxygen molecules can be attached and still the molecular mass of the compound shall be same. Hence multiple isomers were found in the TIC chromatograms. Other than these degradation products, remaining seven degradation products can also be clearly identified based on accurate HRMS and MS-MS data which have been discussed in individual section. (Figure-5.23, Table-5.5, Table-5.6).

Figure-5.22: TIC chromatogram of gimeracil in oxidative degradation condition

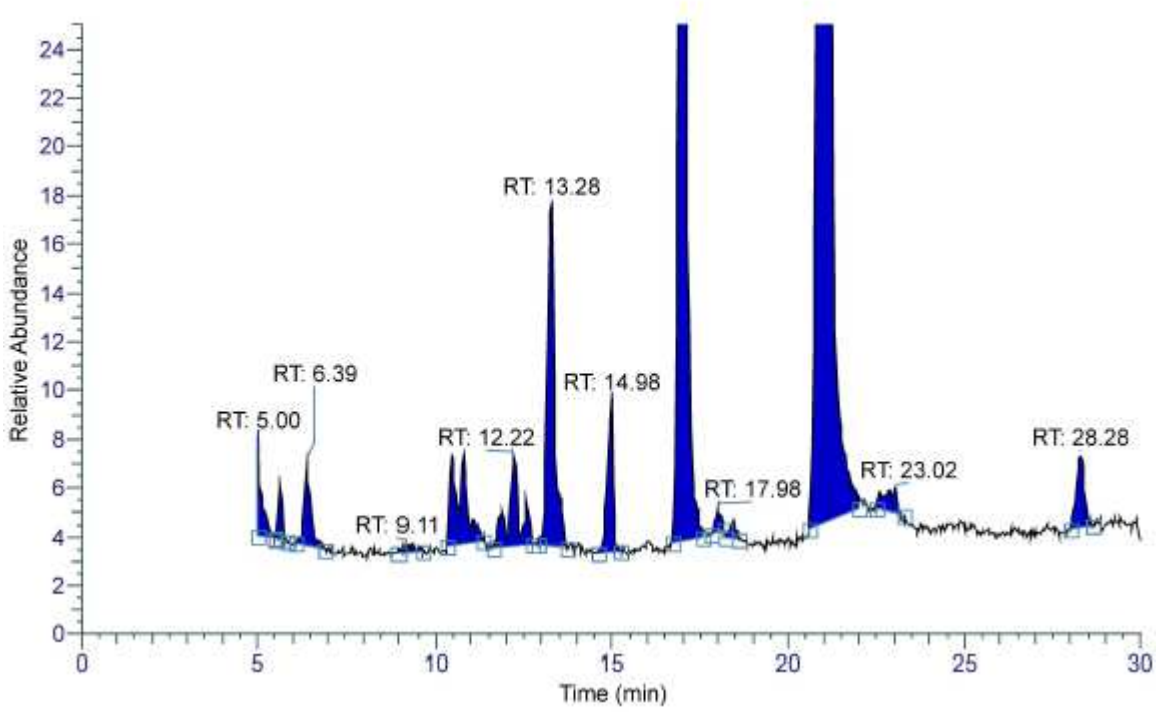


Table-5.5: Peak list of TIC chromatogram of gimeracil in oxidative degradation condition

Name	RT (Min)	Peak Area	% Area
	5.00	1820480023.52	0.76
	5.64	1363373724.92	0.57
	6.39	2964009581.80	1.24
	9.11	123418280.62	0.05
	9.22	100279002.29	0.04
	9.34	118231282.50	0.05
	10.44	3324780947.63	1.39
	10.78	2920983579.82	1.22
	11.05	1108850172.55	0.46
	11.88	1390362132.75	0.58
	12.22	2628697985.90	1.1
	12.56	995866463.31	0.42
	13.28	15425365599.70	6.44
	14.98	5163000275.08	2.16
	16.99	48533938053.88	20.27
	17.79	245962792.80	0.10
	17.98	1664004586.19	0.70
	18.43	579268890.58	0.24
	21.07	145507785570.59	60.77
	28.28	3443197369.83	1.44

Figure-5.23: HRMS spectrum of gimeracil peak

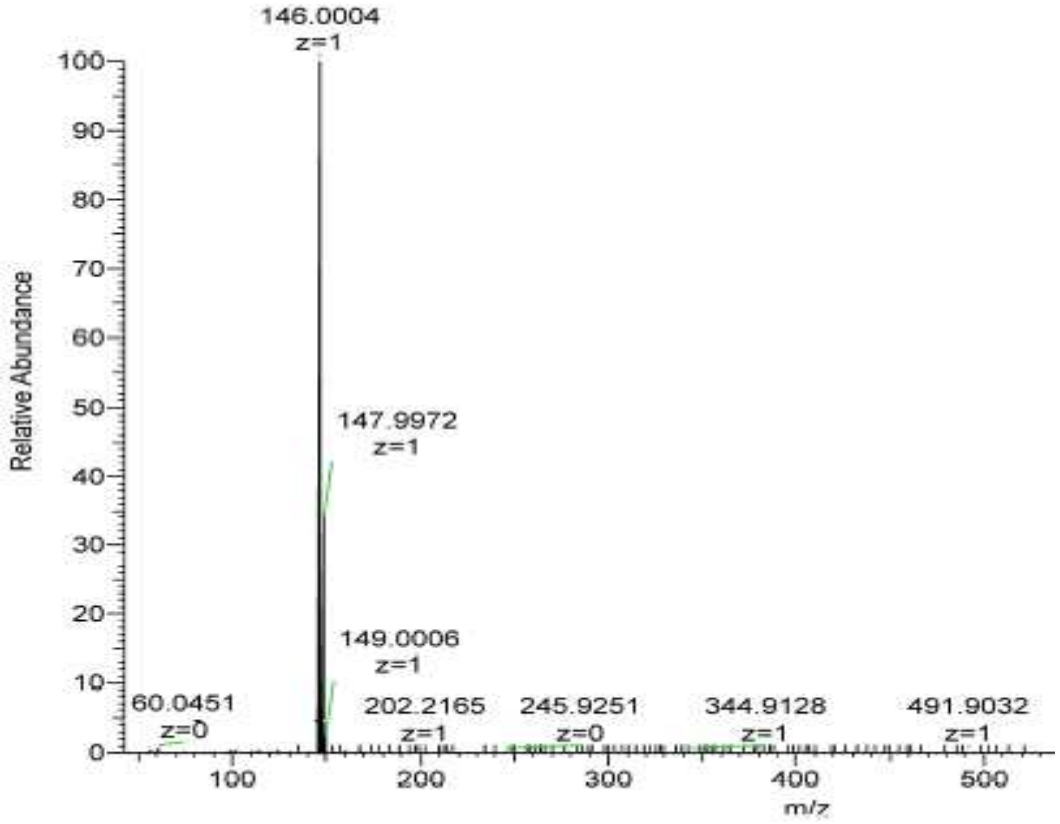


Table-5.6: Degradation products summary of gimeracil under oxidative degradation condition in HRMS study.

DP	RT of DP	RRT	Observed molecular ion peak (m/z)	Further MS-MS fragmentation molecular ion peaks (m/z)						Degradation products
DP-1	5.64	0.27	**74.097**	57.0706						Propionamide or (E)-3-aminoprop-1-en-1-ol
DP-2	6.39	0.30	**98.9845**	80.9607	78.9565					(Z)-penta-2,4-dienoic acid
DP-3	10.4	0.50	**102.128**	74.097	58.658					Pentanamide
DP-4	10.8	0.51	**194.1386**	176.1282	162.1124	133.1096	116.1072	100.1124	88.0762, 74.097, 58.0658	Gimeracil + 3O
DP-5	11.1	0.52	**178.1438**	102.128	74.097					Gimeracil + 2O
DP-6	11.9	0.56	**194.1387**	176.1281	162.1124	**148.0968**	135.0442	118.854	100.0761,74.097, 58.0658	Gimeracil + 3O
DP-7	12.2	0.58	**146.1539**	**128.1436**	86.097					Conversion of hydroxy of gimeracil to ketone
DP-8	12.6	0.60	**194.1388**	**86.097**	58.0058	211.9407				Gimeracil + 3O
DP-9	13.3	0.63	**162.1488**	144.1383	**130.1227**	101.1203. 102.0917	86.0969	72.0814	58.0658	Gimeracil+O
DP-10	15	0.71	**162.1488**	**116.1071**	139.9821	144				Gimeracil+O
DP-11	17	0.81	**130.159**	74.097	57.0706					5-chloropyridine-2(1H)-one or 3-chloropyridin-4-ol
DP-12	18	0.85	**161.9953**	**124.0009**						Gimeracil+O
DP-13	18.4	0.87	**144.1747**	121.9664	88.1125	57.0706				Double bond creation at N-H position of gimeracil
Gimeracil	21.1	1.00	**146.0004**	160.016	127.9894	103.9899	90.0113	78.0111	68.9977, 54.9529	Gimeracil main peak
DP-14	28.3	1.34	**186.2218**	130.1592	100.0758	80.0978	74.0971	57.0707		1-amino-4-chloropentane-1,2,3,5-tetraol

5.5 SECTION-B: CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS OF GIMERACIL

5.5.1 Structure elucidation of degradation product-1

The DP-1 eluted at RT 5.64 minutes with molecular ion peak of 74.0970 m/z (Figure-5.25). From the structure of gimeracil, if it is broken down then with carboxamide group and side chain of two carbons, it would possible to generate propionamide which has exact mass of 73.0950000006. The MS/MS fragmentation spectra suggest the presence of peak 57.0706 m/z which also correlated with the exact mass of propionaldehyde structure (58.0800000005) in ionized form with one proton lesser than 58.080000005. This seems possible to form when propionamide breaks down further with high collision energy (Figure-5.26). Hence, the DP-1 is propionamide with the structure as shown in Figure-5.24.

Figure-5.24: Molecular structure of DP-1

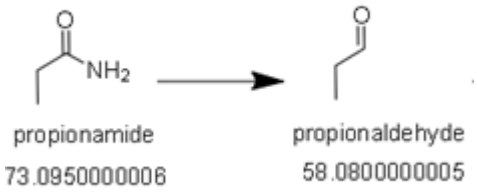


Figure-5.25: HRMS spectrum of DP-1

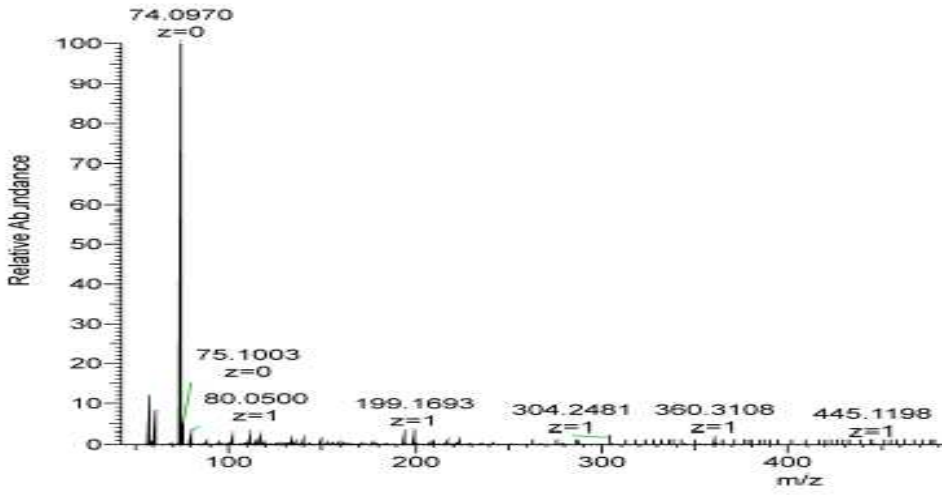
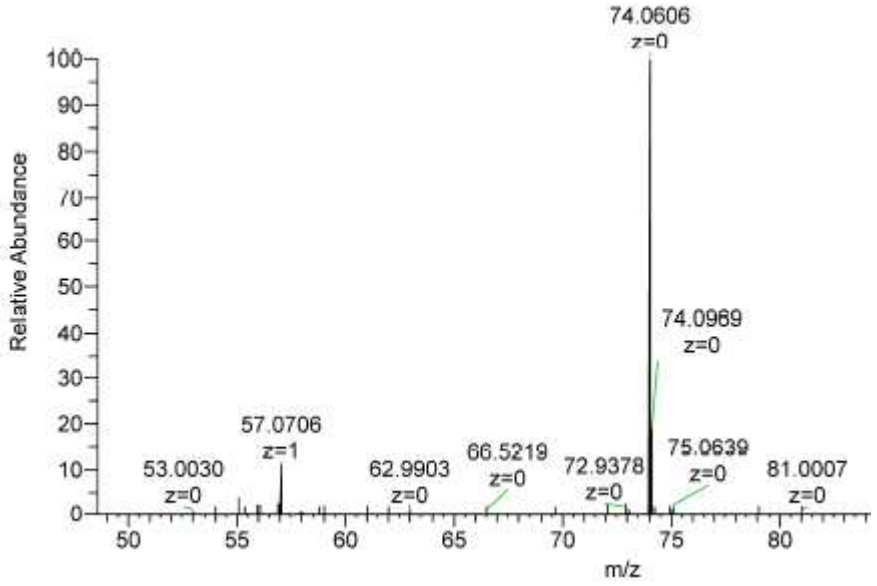


Figure-5.26: HRMS/MS spectrum of DP-1



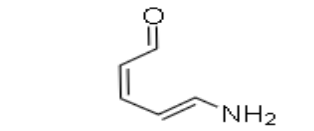
5.5.2 Structure elucidation of degradation product-2

Three possibilities were thought of for the molecular ion peak observed at 98.9845 from the structure of gimeracil (Figure-5.27). In all the three possibilities, common modification is removal of chloride functional group and breakage of bond between –NH and -C=O positions i.e., 1 and 2 positions respectively.

As shown in Figure-5.27-A, first possibility was thought of is no other modification other than chloride group removal and bond breakage between –NH and –C=O positions. However, this structure produces molecular mass 97.117000007 which is one proton mass lesser than as expected for molecular ion peak of 98.9845. Hence, this would not be possible.

Figure-5.27-A: First possibility of molecular mass near to 98.9845 m/z

a. 97.117000007

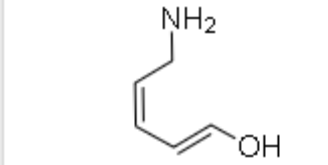


(2*Z*,4*E*)-5-aminopenta-2,4-dienal

Moving on to the second possibility is thought in the direction of one proton mass increment in the structure produced in first possibility. Hence it was assumed to convert –C=O to –C-OH (Figure-5.27-B). But this would again add two protons in the structure not one. The molecular mass of this structure would produce one proton mass higher than expected for DP-2 nullifying this probability also.

Figure-5.27-B: Second possibility of molecular mass near to 98.9845 m/z

b. 99.1330000008



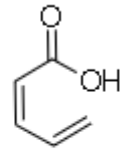
(1*E*,3*Z*)-5-aminopenta-1,3-dien-1-ol

Third probability is formation of carboxylic acid with from the structure of gimeracil in addition to removal of chloride group and breakage of bond between position 1 and 2. This structure would have exact mass of 98.10100000065 which exactly correlated with the molecular ion peak observed at 98.9845 m/z. Moreover, the MS/MS spectra molecular ion peak 80.9607 m/z also correlated with the removal of terminal –OH group and formation of one double bond between position 5 and 6 of gimeracil (Figure-5.28, 5.29). Other major MS/MS fragment molecular ion peak 78.0700000004 was also found correlated when one more similar triple bond is formed between position 1 and 2 further in the same structure. (Figure-5.27-C)

Figure-5.27-C: Third possibility of molecular mass near to 98.9845 m/z

c. 98.10100000065

(*Z*)-penta-2,4-dienoic acid



(*Z*)-penta-2,4-dienoic acid

98.10100000065

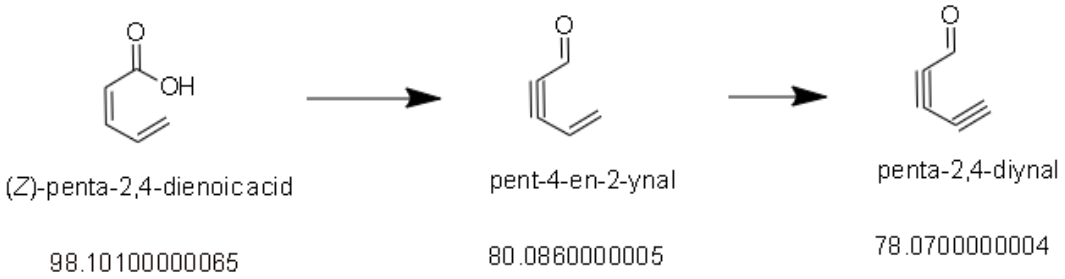


Figure-5.28: HRMS spectrum of DP-2

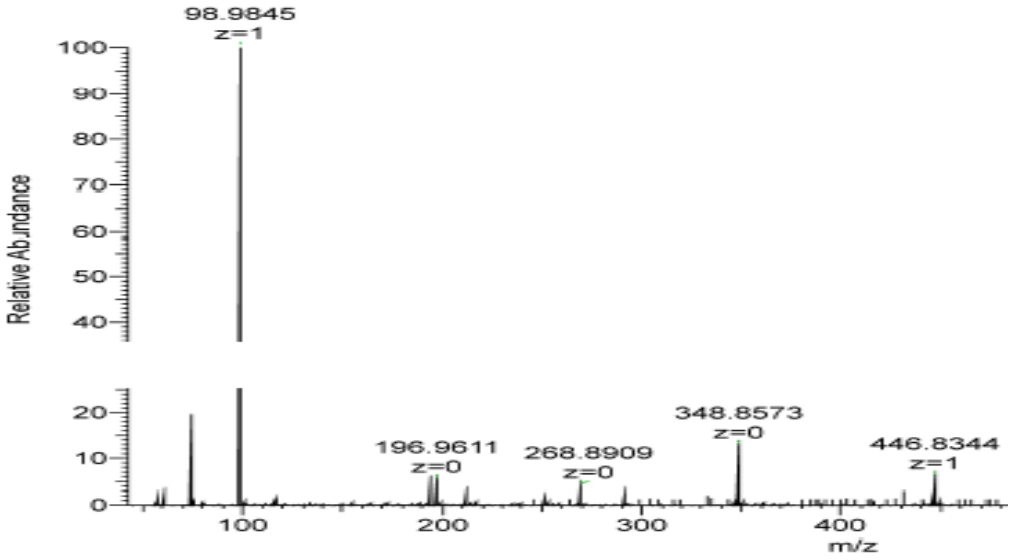
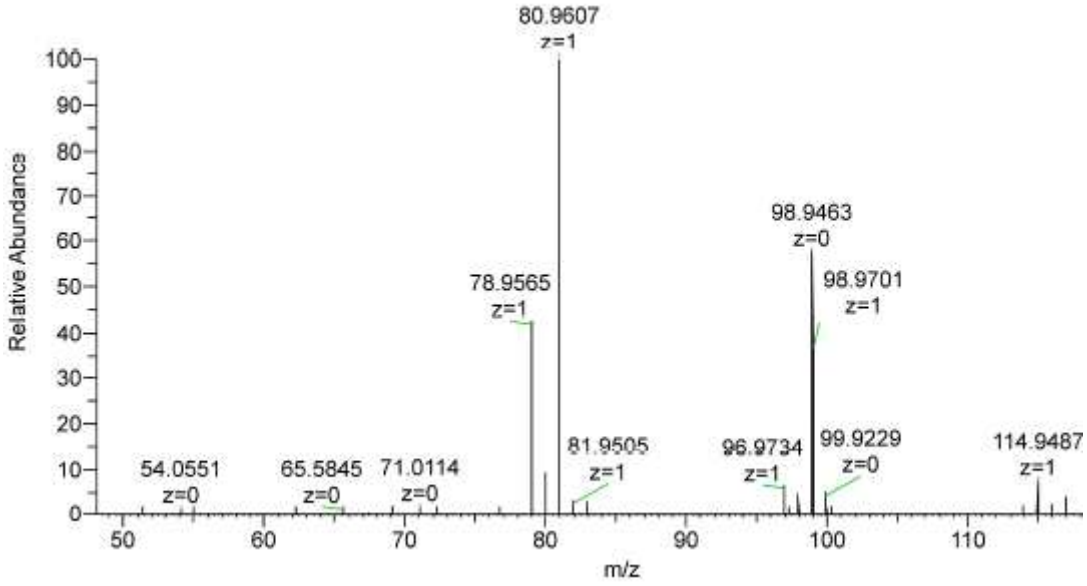


Figure-5.29: HRMS/MS spectrum of DP-2



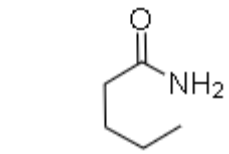
5.5.3 Structure elucidation of degradation product-3

DP-3 eluted at RRT 0.50 (RT 10.4 min) with molecular ion peak at 102.128 m/z. Based on the presence of two characteristic MS/MS fragmentation peaks at 74.097 m/z (propionamide) and 58.658 m/z (propionaldehyde), (Figure-5.31, Figure-5.32) as discussed in DP-1 section presence of -CONH2 group must be intact in the DP-3 structure. Considering this, the structure of DP-3 which is highly probable is of Pentanamide (Figure-5.30-A) with exact molecular mass as 101.1490000009.

Other than this, other probability with mass near to 101.128 was also thought with retention of chloride group in the structure with aliphatic carbon chain only. However, the mass of this structure anyway does not match exactly as it has one proton mass higher than the expected 101.128. There is no any scope in the structure to get reduced the mass of single proton hence this probability is nullified. (Figure-5.30-B)

Figure-5.30-A: Molecular structure of DP-3

101.1490000009



pentanamide

101.1490000009

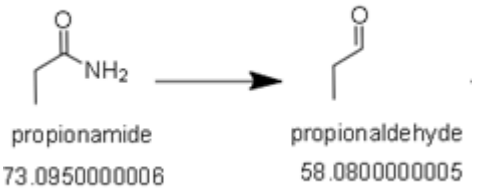
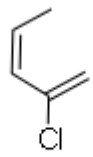


Figure-5.30-B: Other possibility which was thought but not possible due to mass does not match exactly.

102.56100000065



(*Z*)-2-chloropenta-1,3-diene

Figure-5.31: HRMS spectrum of DP-3

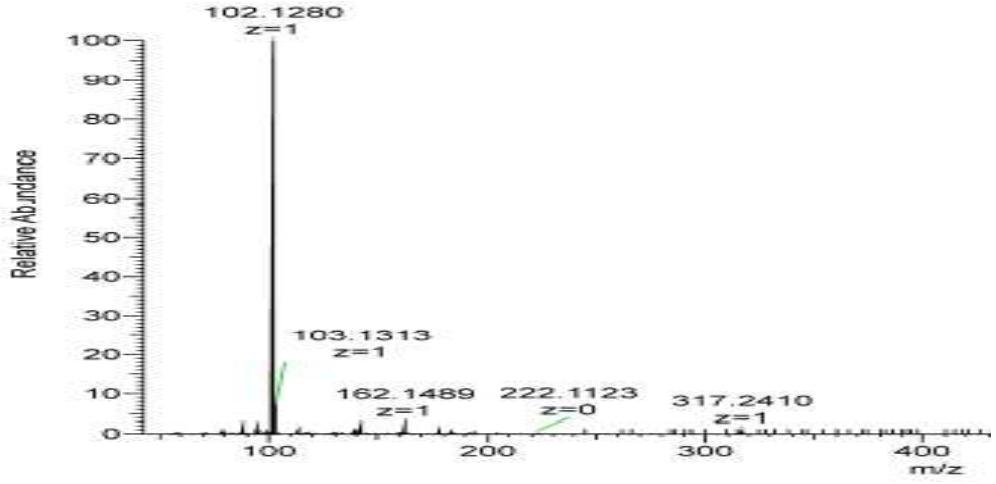
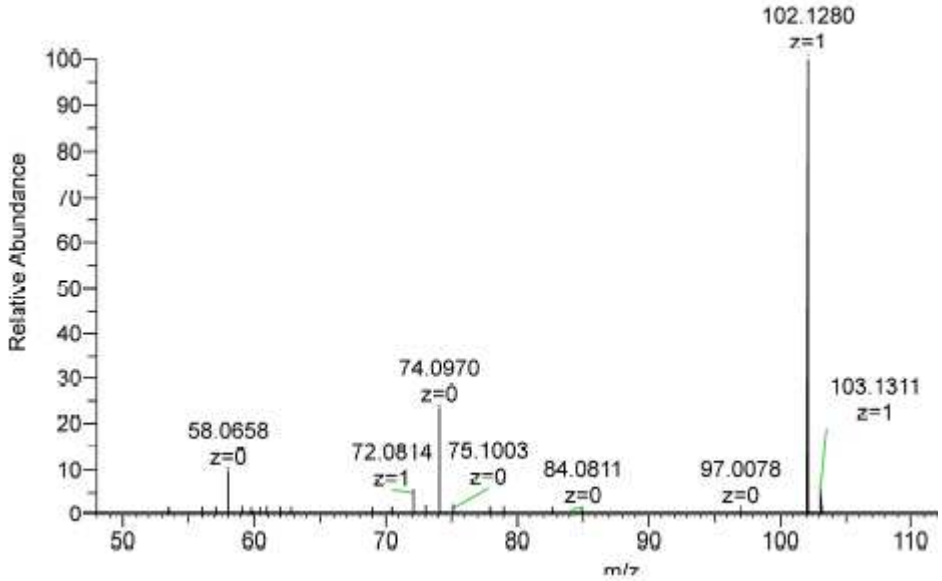


Figure-5.32: HRMS/MS spectrum of DP-3



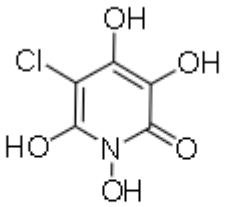
5.5.4 Structure elucidation of degradation product-4, 6 and 8

DP-4, DP-6 and DP-8 eluted at RRT 0.51, 0.56, and 0.60 (RT 10.8min, 11.9min, and 12.6min respectively) with same molecular ion peak at 194.1386 m/z, 194.1387 m/z and 194.1388 m/z respectively (Figure-5.34 to Figure-5.39). Since the molecular ion peak of gimeracil is 146.0004 m/z, the molecular ion of these DPs at ~194 clearly suggest the addition of three oxygen molecules mass (146.0004+48=194.0004). Apart from two oxygen molecules in the structure of gimeracil in the form of hydroxy group at 4th position and ketone group at 2nd position, there are positions of 3rd position -CH, 6th position –CH and 1st position –NH where oxygen molecules can be incorporated. The first probability of the structure is formation of hydroxy group (-OH) at these three positions which has the exact mass of 193.5390000008 which exactly correlated with the molecular ion peak observed at 194.1386 m/z. (Figure-5.33-A). Looking at the structure of DP-4, it is highly probable to form multiple isomers of the structures if the ketone functional group position is changed from 2th position to either 4th position or 6th position generating same molecular mass and they are thought of for the DP-6 and DP-8 respectively. Based on these, the structure and chemical name of DP-4, DP-6 and DP-8 can be depicted as shown in the Figure-5.33-A, Figure-5.33-B and Figure-5.33-C

respectively.

Figure-5.33-A: Molecular structure of DP-4

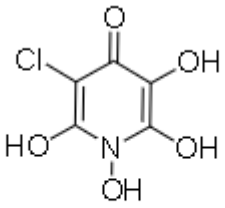
193.5390000008



5-chloro-1,3,4,6-tetrahydroxypyridin-2(1*H*)-one

Figure-5.33-B: Molecular structure of DP-6

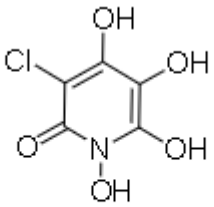
193.5390000008



3-chloro-1,2,5,6-tetrahydroxypyridin-4(1*H*)-one

Figure-5.33-C: Molecular structure of DP-8

193.5390000008



3-chloro-1,4,5,6-tetrahydroxypyridin-2(1*H*)-one

Figure-5.34: HRMS spectrum of DP-4

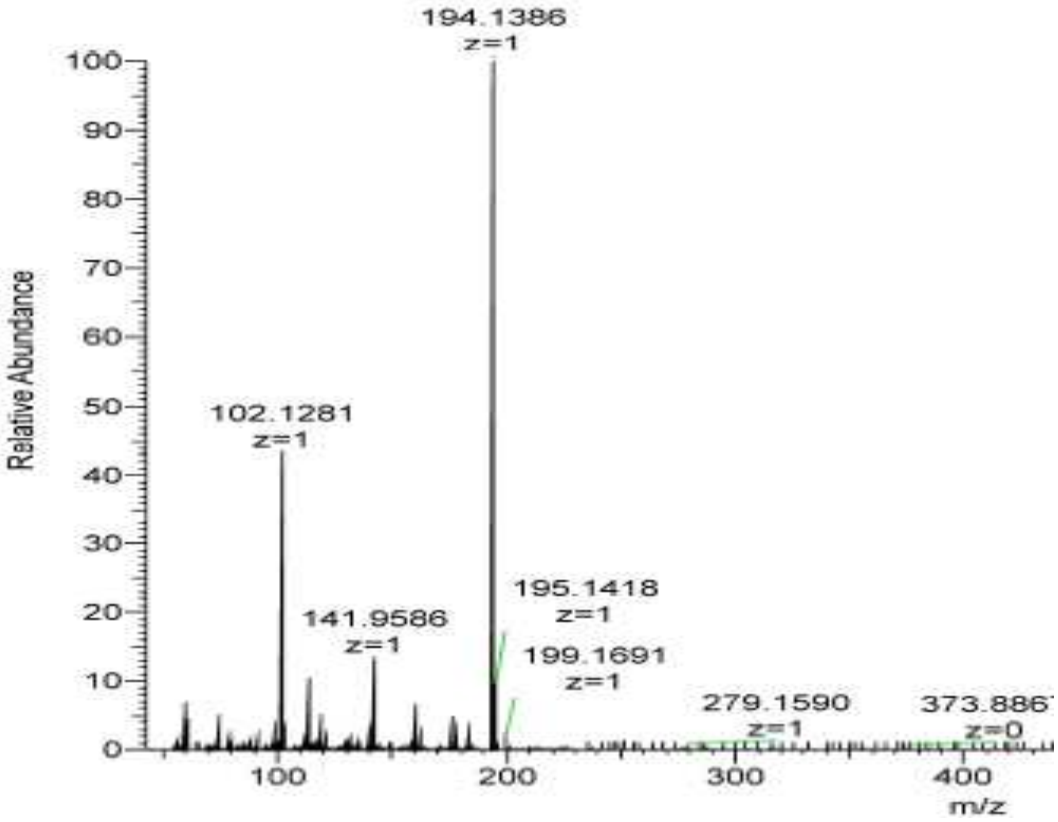


Figure-5.35: HRMS/MS spectrum of DP-4

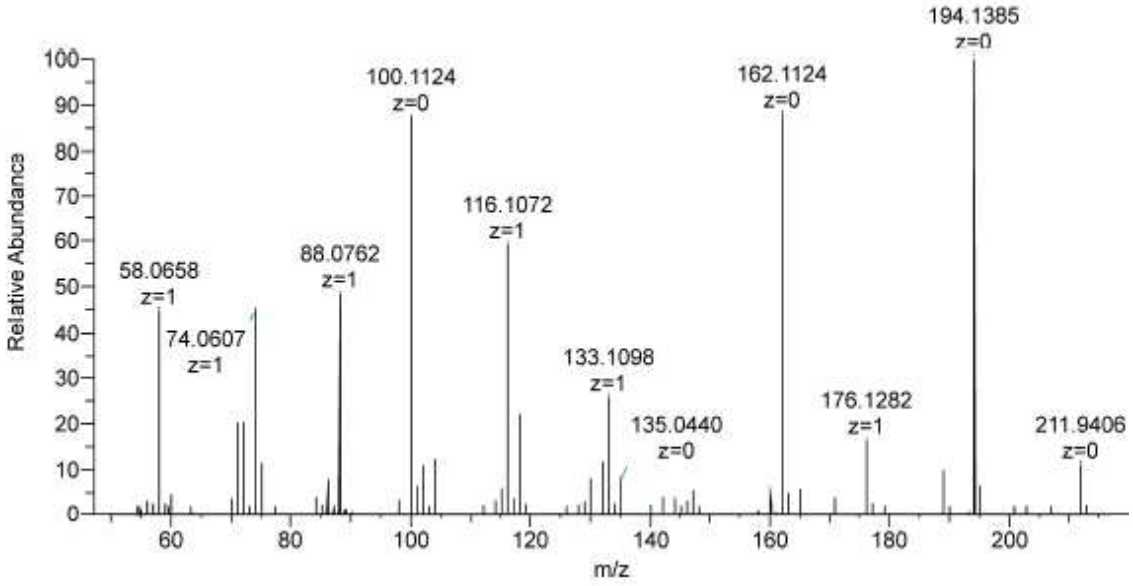


Figure-5.36: HRMS spectrum of DP-6

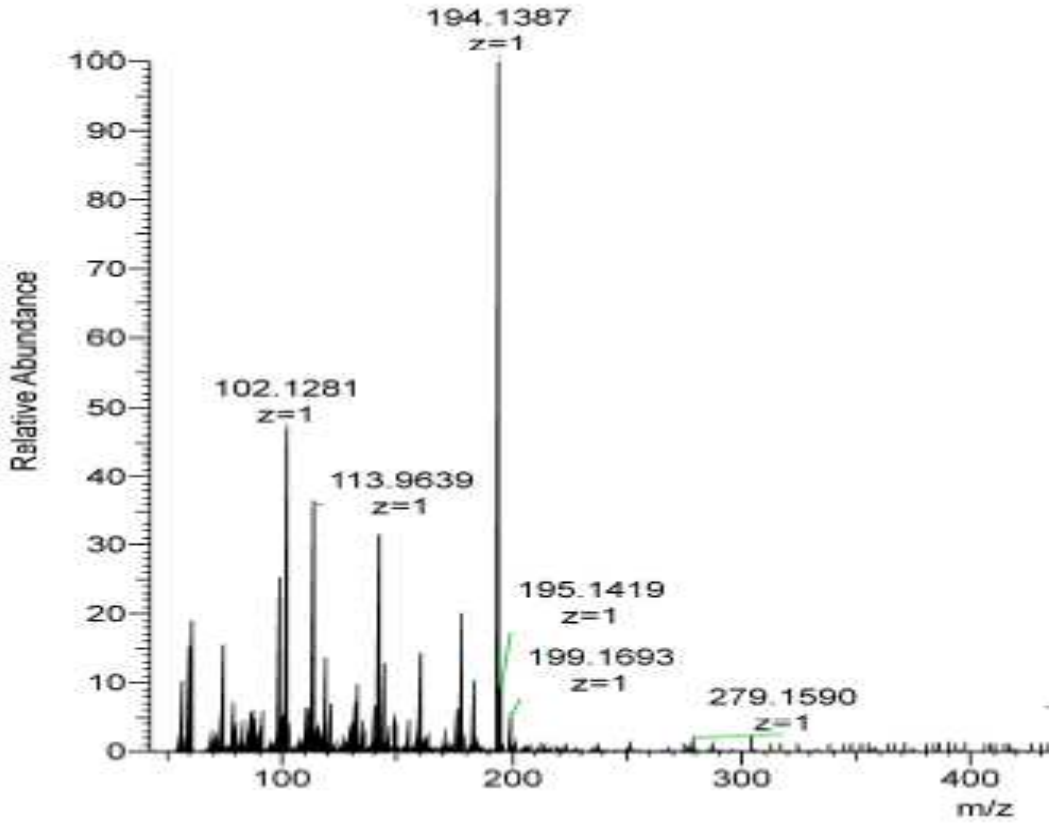


Figure-5.37: HRMS/MS spectrum of DP-6

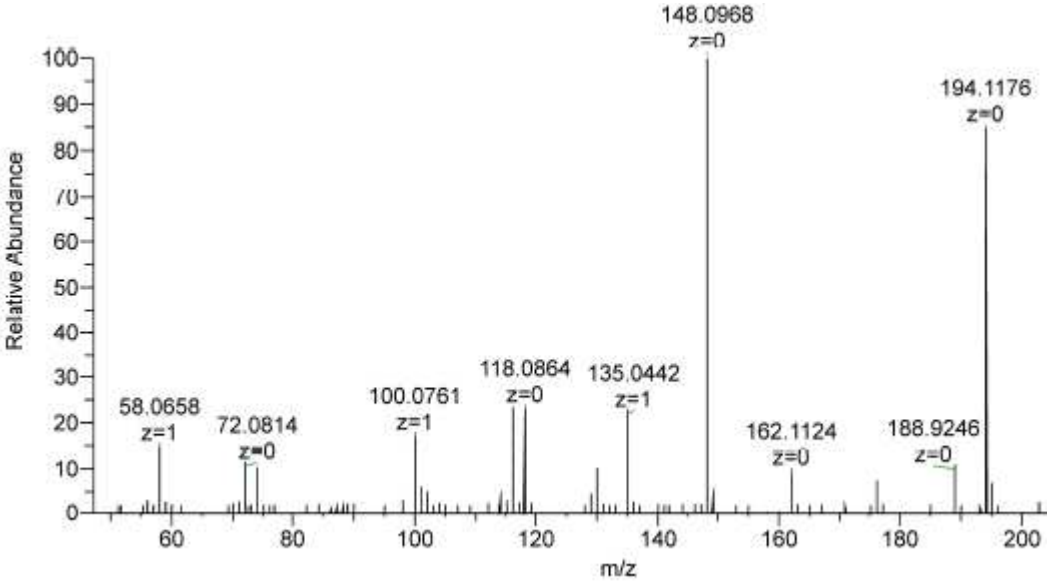


Figure-5.38: HRMS spectrum of DP-8

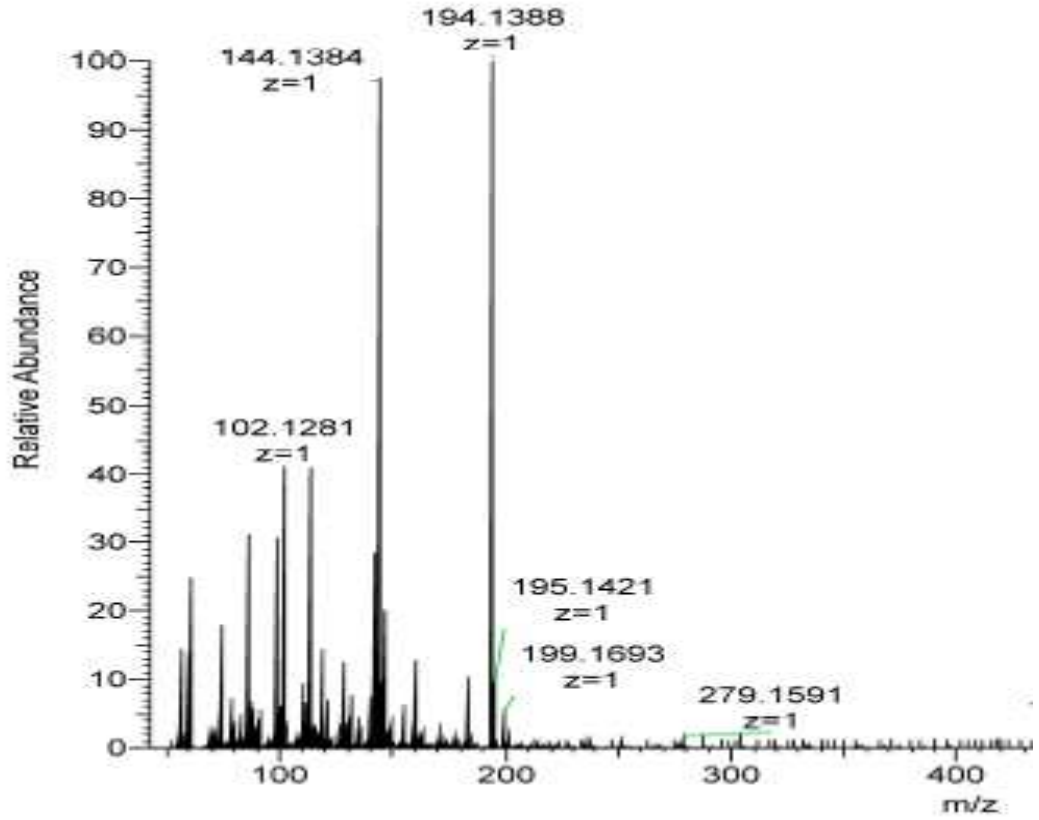
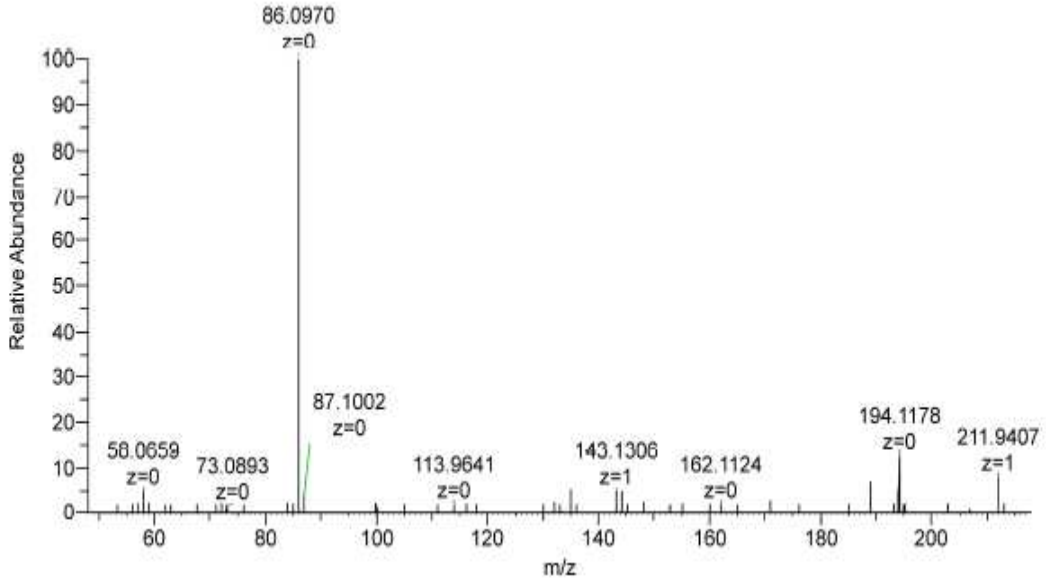


Figure-5.39: HRMS/MS spectrum of DP-8



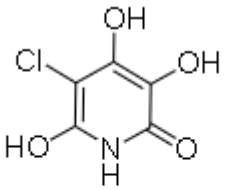
5.5.5 Structure elucidation of degradation product-5

This degradation product eluted at RRT 0.52 (RT 11.1 min) with molecular ion peak at 178.1438 m/z (Figure-5.41 and Figure-5.42). This molecular ion peak suggests the addition of two oxygen molecules mass in the structure of gimeracil 146.0004+32=178.0004). The most probable and obvious positions where oxygen molecular mass can be added in the structure of gimeracil are at 3rd and 6th positions. Addition of hydroxy group and not ketone group is also logical because addition of hydroxy group would only add 16 molecular mass for one position of the structure as the hydrogen of –CH group at 3rd or 6th position would be removed but one more hydrogen would come along with –OH hydroxy functional group. If ketone is to be formed at the 3rd or 6th position, it would add 15+15 molecular mass for each position because hydrogen of –CH would be removed. Hence this shall not match with the molecular ion peak where 16+16 molecular masses have been increased. The MS/MS spectrum of DP-5 shows the presence of 102.1281 m/z prominent peak which again thought to be formed by formation of pentanamide due to breakage of DP-5 at high collision energy in HRMS (Figure-5.42).

Considering these facts, the structure and chemical name of the DP-5 shall be depicted as shown in Figure-5.40.

Figure-5.40: Molecular structure of DP-5 and its fragmentation moieties

177.54000000075



5-chloro-3,4,6-trihydroxypyridin-2(1*H*)-one

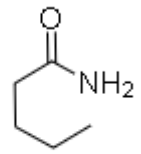
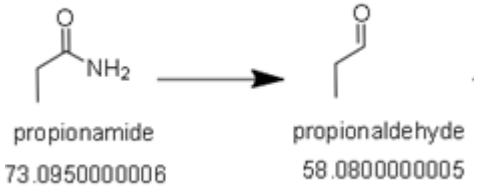
 pentanamide 101.1490000009	 propionamide 73.0950000006 propionaldehyde 58.0800000005

Figure-5.41: HRMS spectrum of DP-5

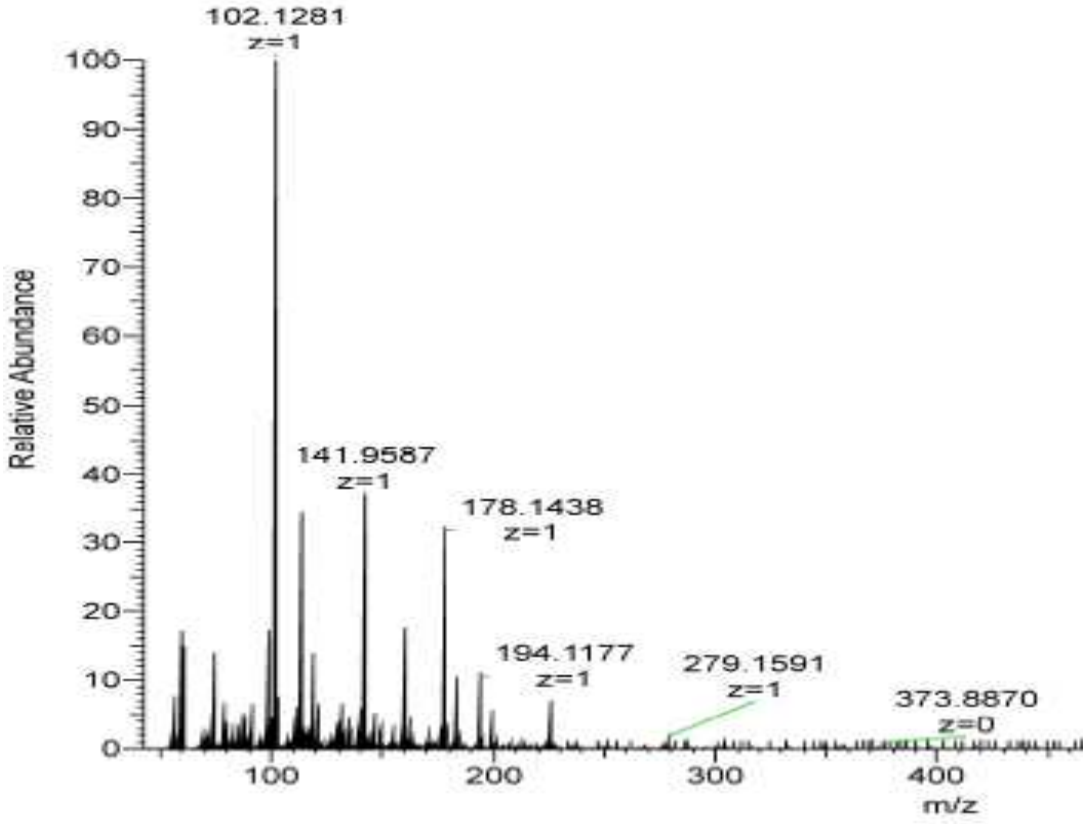
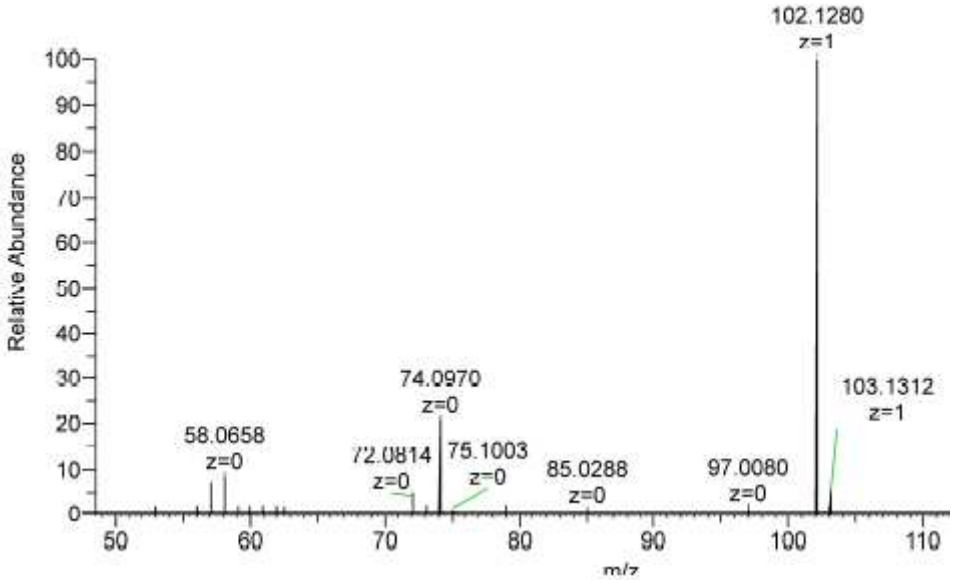


Figure-5.42: HRMS/MS spectrum of DP-5



5.5.5 Structure elucidation of degradation product-7

DP-7 eluted at RRT 0.58 (RT 12.2 min) in HRMS chromatogram with molecular ion peak at 146.1539 m/z which is almost same as gimeracil molecular ion peak of 146.0004 m/z (Figure-5.44, Figure-5.45). The isomeric degradation product of gimeracil which can be formed by oxidative degradation condition would be only possible hydroxy group of gimeracil is converted to ketone group. This is highly likely to be formed with slight modification of conversion in oxidative degradation condition. Other isomeric forms can also be thought of but they required more intense modification in the structure of gimeracil which is not possible to be happened during normal storage of drug. Hence, the structure and chemical name of DP-7 can be depicted as shown in Figure-5.43.

Figure-5.43: Molecular structure of DP-7

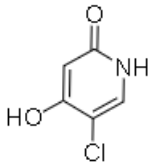
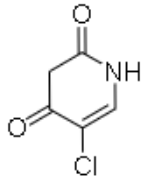
145.54200000065	**145.54200000065**
 5-chloro-4-hydroxypyridin-2(1*H*)-one GIMERACIL	 5-chloropyridine-2,4(1*H*,3*H*)-dione

Figure-5.44: HRMS spectrum of DP-7

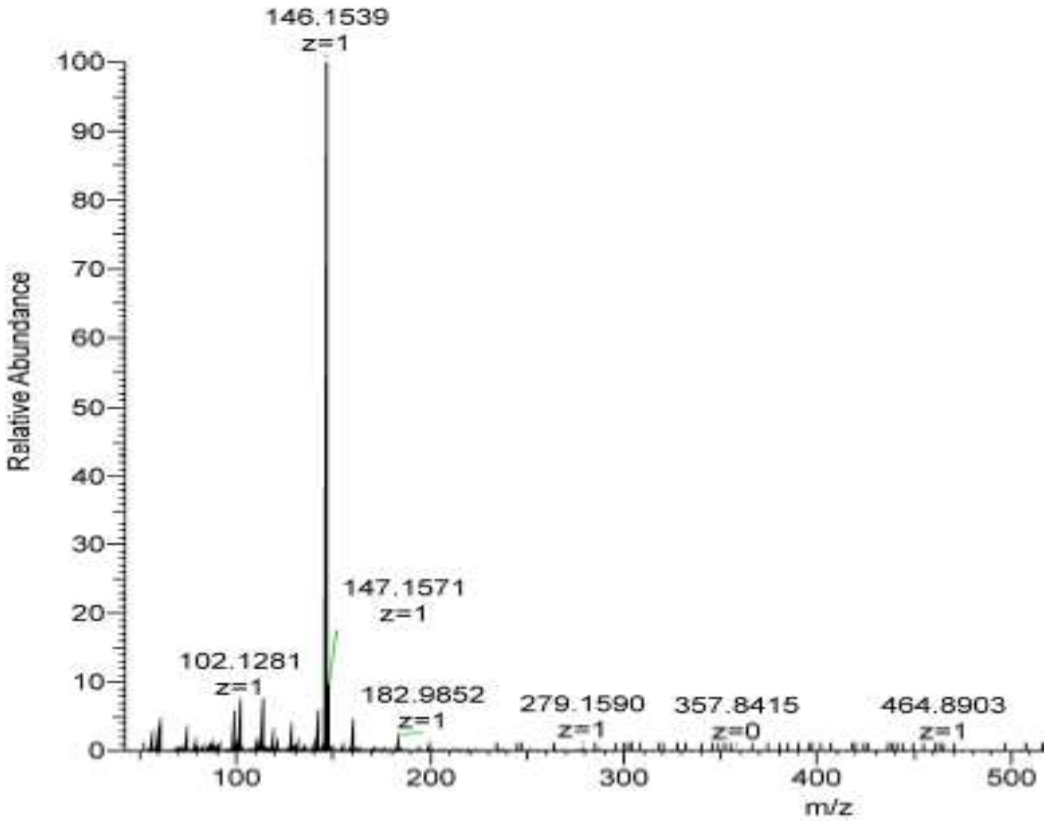
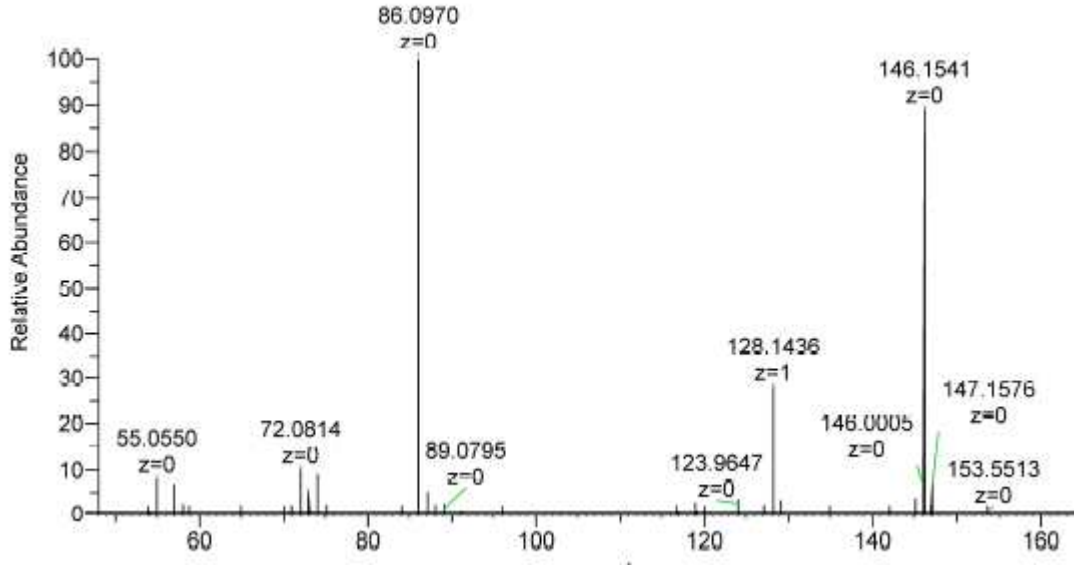


Figure-5.45: HRMS/MS spectrum of DP-7



5.5.6 Structure elucidation of degradation product-9, 10 and 12

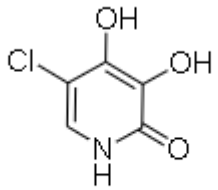
DP-9, DP-10 and DP-12 eluted at RRT 0.63, 0.71 and 0.85 (RT 13.3 min, 15.00 min, and 18.00 min respectively) with molecular ion peaks at 162.1488 m/z, 162.1488 m/z, and 161.9953 m/z respectively (Figure-5.47 to Figure-5.52). The molecular ion peaks suggest the addition of one oxygen molecular mass in the structure of gimeracil (146.0004+16=162.0004). As discussed in the sections of DP-4, DP-6 and DP-8, there are three positions in gimeracil structure where oxygen molecule can be incorporated with increment of only oxygen molecules mass (16). These positions are 1st, 3rd and 6th position of gimeracil structure. If all these positions were occupied and ketone functional groups change, three isomers of DP-4, DP-6 and DP-8 created, if two of these positions were filled up with hydroxy group, it created DP-5 but if only one of these three positions were occupied, isomeric degradation products i.e., DP-9, DP-10 and DP-12 products shall be generated with molecular mass as 161.5410000007. In conclusion, DP-9, DP-10 and DP-12 are isomeric degradation products with addition of hydroxy group at 1st, 3rd or 6th positions of the gimeracil structure. One more point about the difference in these isomeric degradation products is the polarity difference. The oxidative degradation products with 2 or 3 hydroxy groups incorporated in the structure seems to be more polar

than those with one hydroxy group. Hence DP-4, DP-5, DP-6, DP-8 eluted earlier in the chromatogram (i.e., at RRT 0.51, 0.52, 0.56 and 0.60 respectively) whereas DP-9, DP-10 and DP-12 eluted at later in the chromatogram (i.e., at RRT 0.63, 0.71, and 0.85 respectively). The MS/MS Spectra of these isomeric degradation products and gimeracil are showing characteristic peaks such as 74.097 m/z and 58.0658 m/z.

Considering these facts, the molecular structure and chemical names of DP-9, DP-10 and DP-12 can be depicted as shown in Figure-5.46-A, Figure-5.46-B and Figure-5.46-C respectively.

Figure-5.46-A: Molecular structure of DP-9

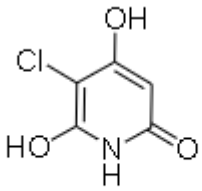
161.5410000007



5-chloro-3,4-dihydroxypyridin-2(1*H*)-one

Figure-5.46-B: Molecular structure of DP-10

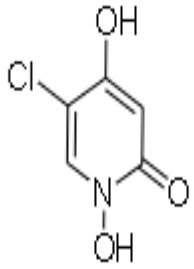
161.5410000007



5-chloro-4,6-dihydroxypyridin-2(1*H*)-one

Figure-5.46-C: Molecular structure of DP-12

161.5410000007



5-chloro-1,4-dihydroxypyridin-2(1*H*)-one

Figure-5.47: HRMS spectrum of DP-9

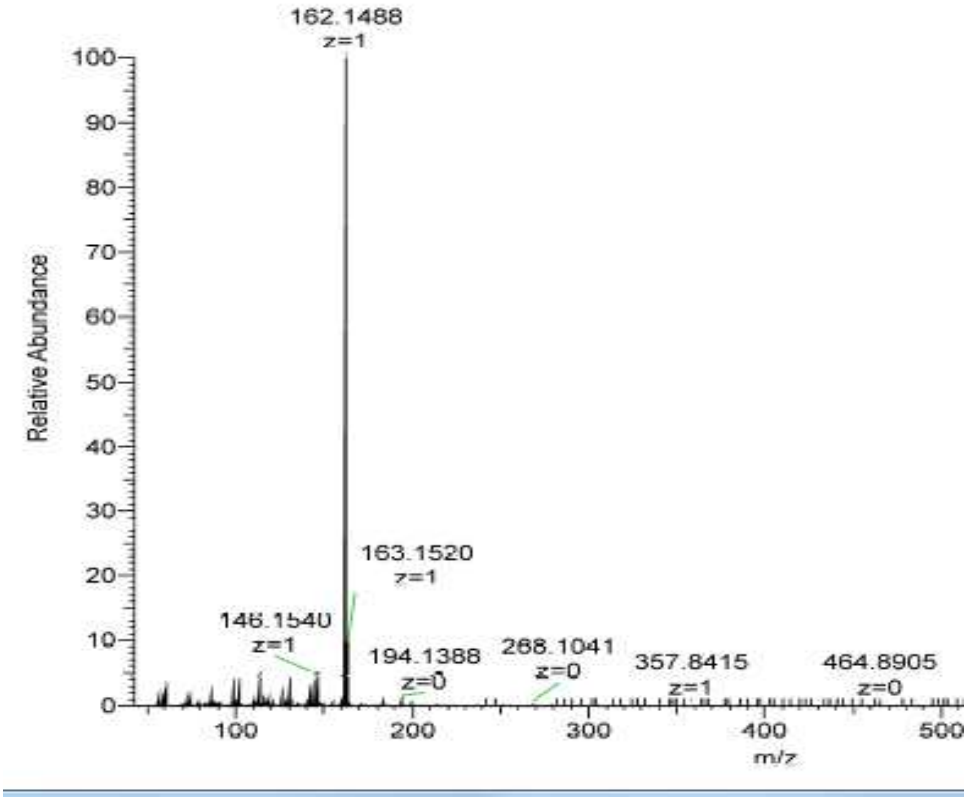


Figure-5.48: HRMS/MS spectrum of DP-9

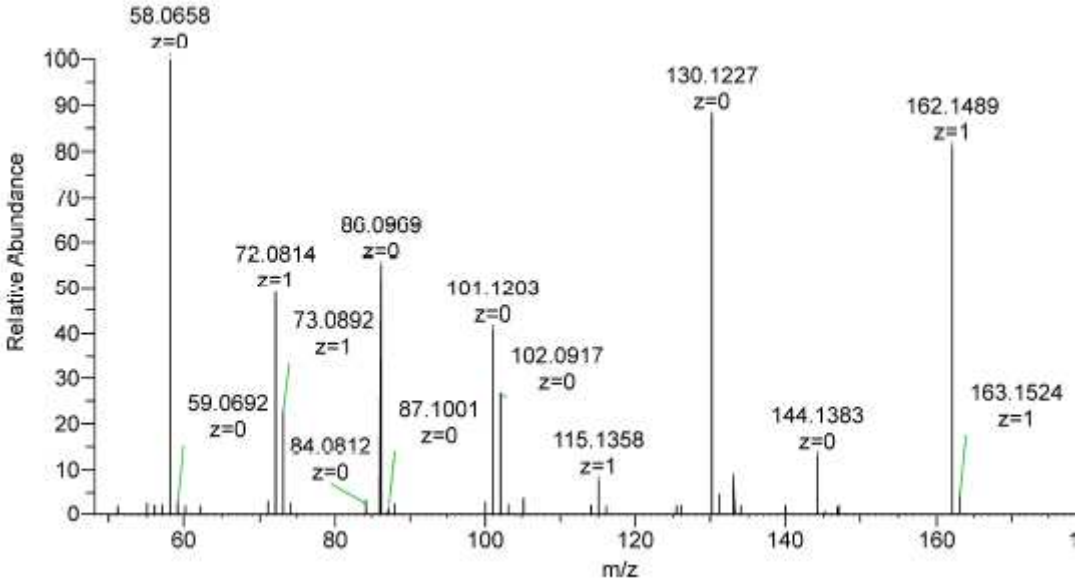


Figure-5.49: HRMS spectrum of DP-10

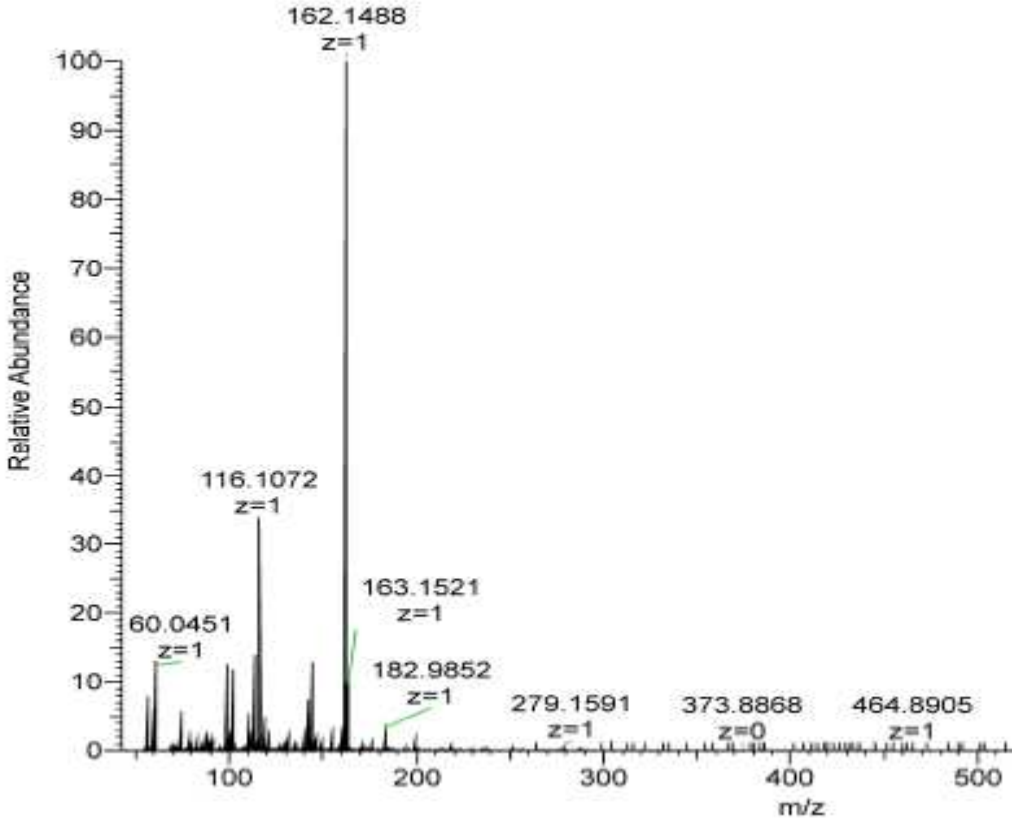


Figure-5.50: HRMS/MS spectrum of DP-10

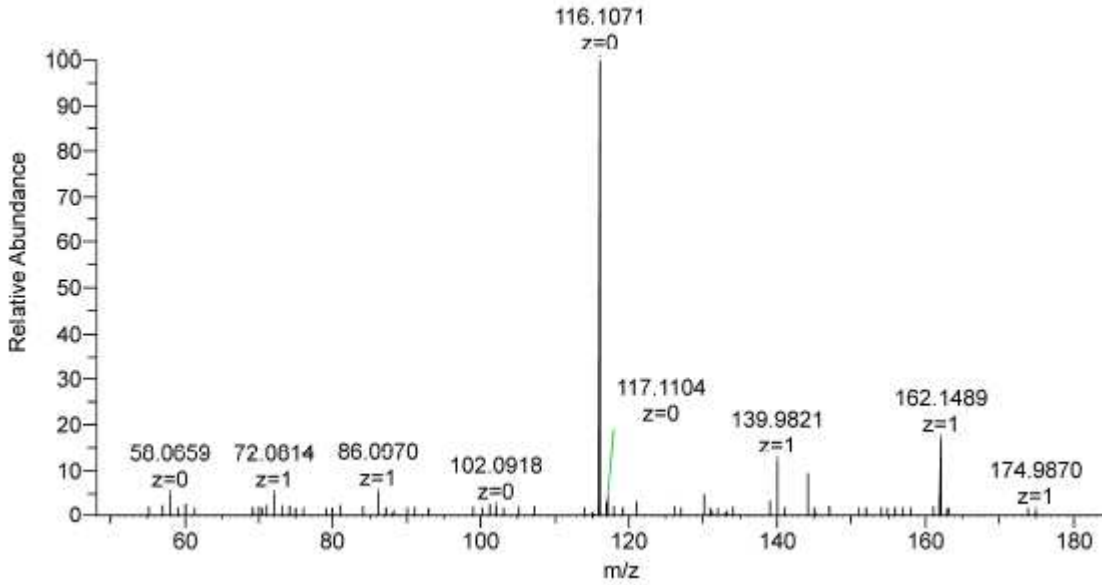


Figure-5.51: HRMS spectrum of DP-12

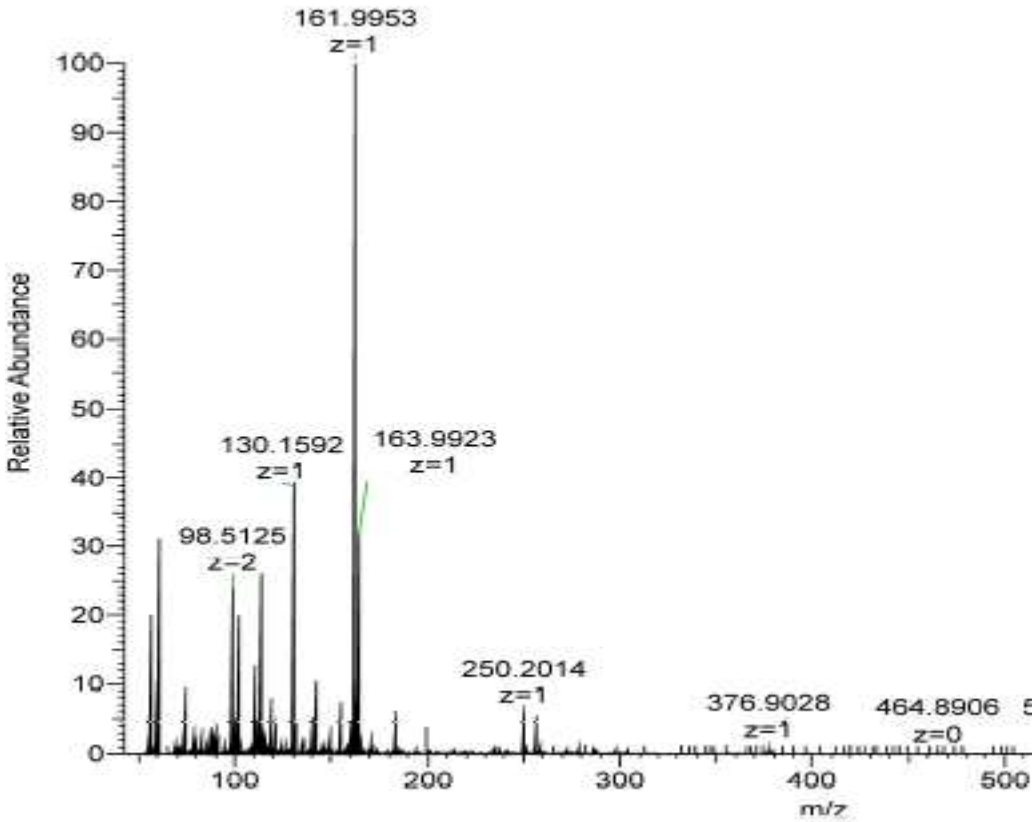
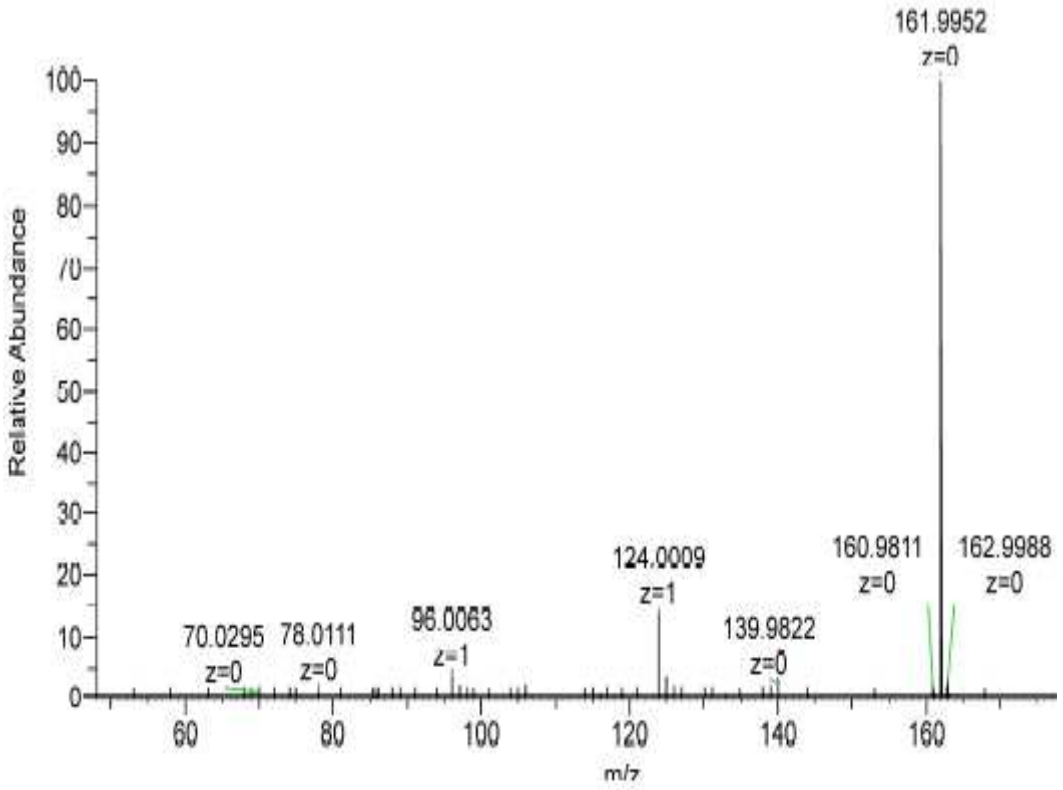


Figure-5.52: HRMS/MS spectrum of DP-12



5.5.7 Structure elucidation of degradation product-11

DP-11 eluted at RRT 0.81 (RT 17.00 min) with molecular ion peak at 130.159 m/z (Figure-5.54). This clearly indicates the removal of one oxygen molecule from gimeracil structure (146.0004-16= 130.0004). There are two oxygen molecules present in gimeracil structure, one is in keto functional group and second is in hydroxy functional group. Either of them can be removed generating two possibilities having same molecular mass (Figure-5.53-A and Figure-5.53-B). The interesting point is the further fragmentation pattern, which also reveals almost same molecular masses. The MS/MS fragmentation peaks of 73.095 m/z and 58.0800 m/z can be derived in any of these both the possibilities (Figure-5.55). Hence DP-11 molecular structure and chemical name can be depicted as shown in (Figure-5.53-A and Figure-5.53-B).

Figure-5.53-A One probability of molecular structure and fragmentation pattern of DP-11

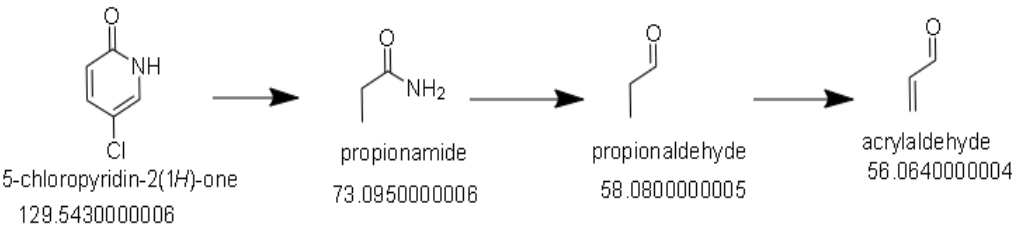


Figure-5.53-B Second probability of molecular structure and fragmentation pattern of DP-11

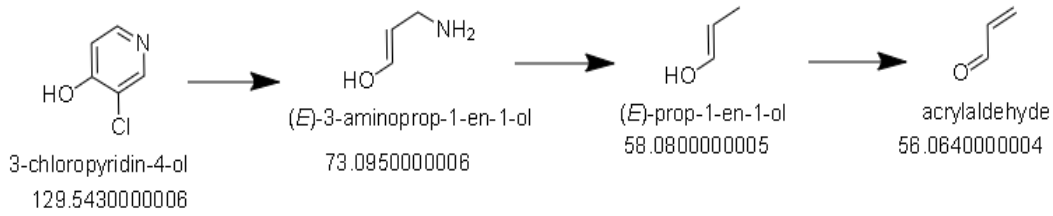


Figure-5.54: HRMS spectrum of DP-11

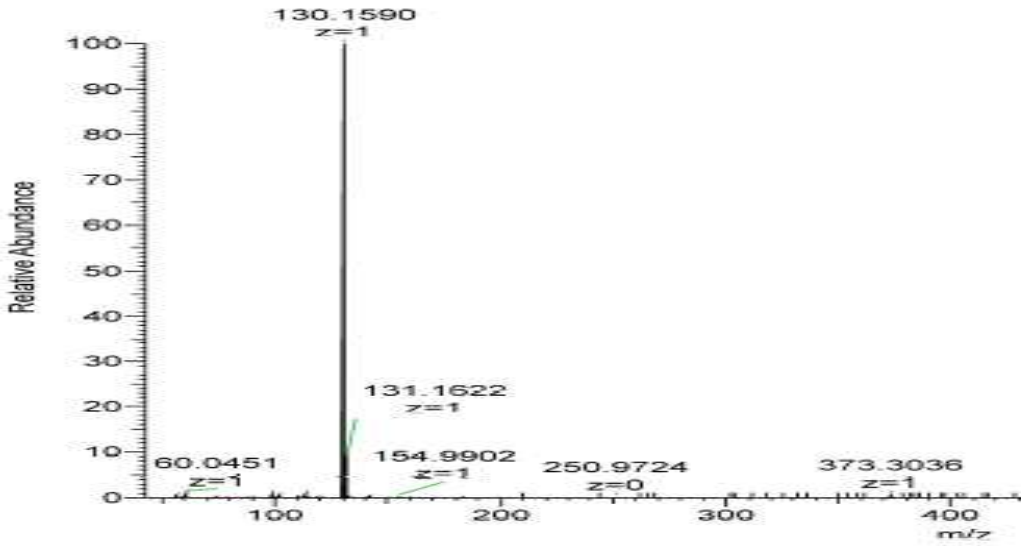
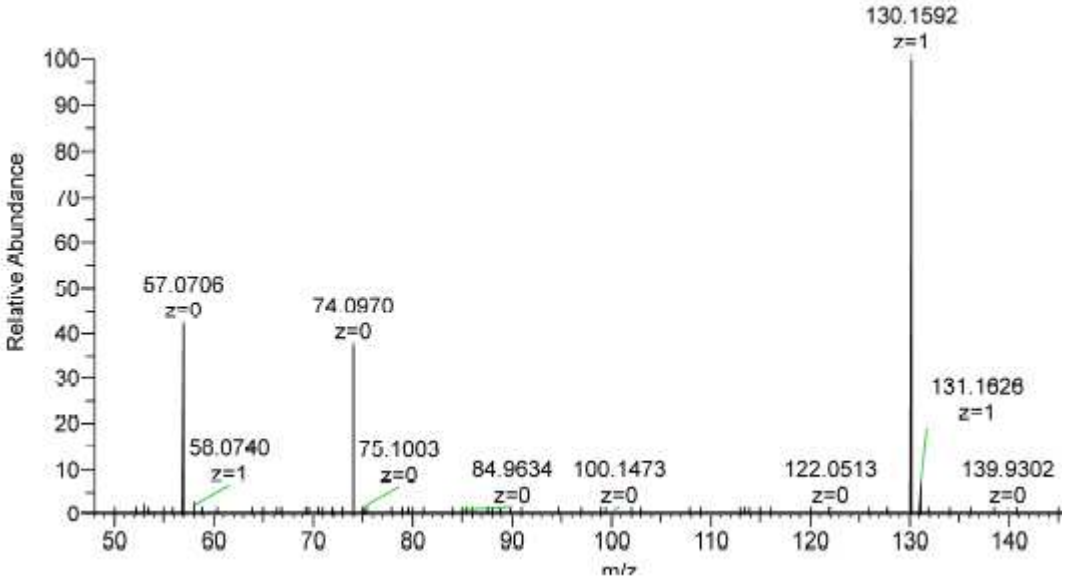


Figure-5.55: HRMS/MS spectrum of DP-11



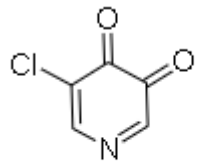
5.5.8 Structure elucidation of degradation product-13

DP-13 eluted at RRT 0.87 with molecular ion peak at 144.1747 m/z (Figure-5.57, Figure-5.58). The difference between the molecular ion peak of DP-13 and gimeracil is nearly of two protons (146.0004-144.1747= 1.8257). This is possible if two protons of gimeracil structure can be removed by generation of double bond at some positions in gimeracil. One probability in the direction of removal of hydrogen from gimeracil structure was thought as conversion of hydroxy group at 4th position to ketone functional group. However, this would remove one hydrogen molecule of hydroxy group but again add two hydrogen molecules to the adjacent 3rd position carbon due to double bond breakage between 3rd and 4th positions. Hence this probability was nullified. Second probability that was assumed was creation of double bond between 1st and 2nd positions with conversion of ketone functional group to hydroxy functional group due to double bond creation. But this modification again would not reduce any hydrogen molecular mass as the hydrogen molecule removed from –NH from 1st position was compensated by the addition of one hydrogen molecule in the form of hydroxy group at 2nd position. Hence, overall molecular mass would be same as gimeracil. Third possibility that can remove the two hydrogen molecules mass from gimeracil is double bond creation between 1st and 2nd

position along with shifting of ketone functional group from 2nd to 3rd position carbon. And since the ketone functional group is present at 3rd position, double bond between 3rd and 4th position would not be exists converting hydroxy functional group at 4th position to ketone functional group. Hence based on this, the molecular structure and chemical name of the DP-13 can be depicted as shown in Figure-5.56.

Figure-5.56: Molecular structure of DP-13

143.52600000055



5-chloropyridine-3,4-dione

Figure-5.57: HRMS spectrum of DP-13

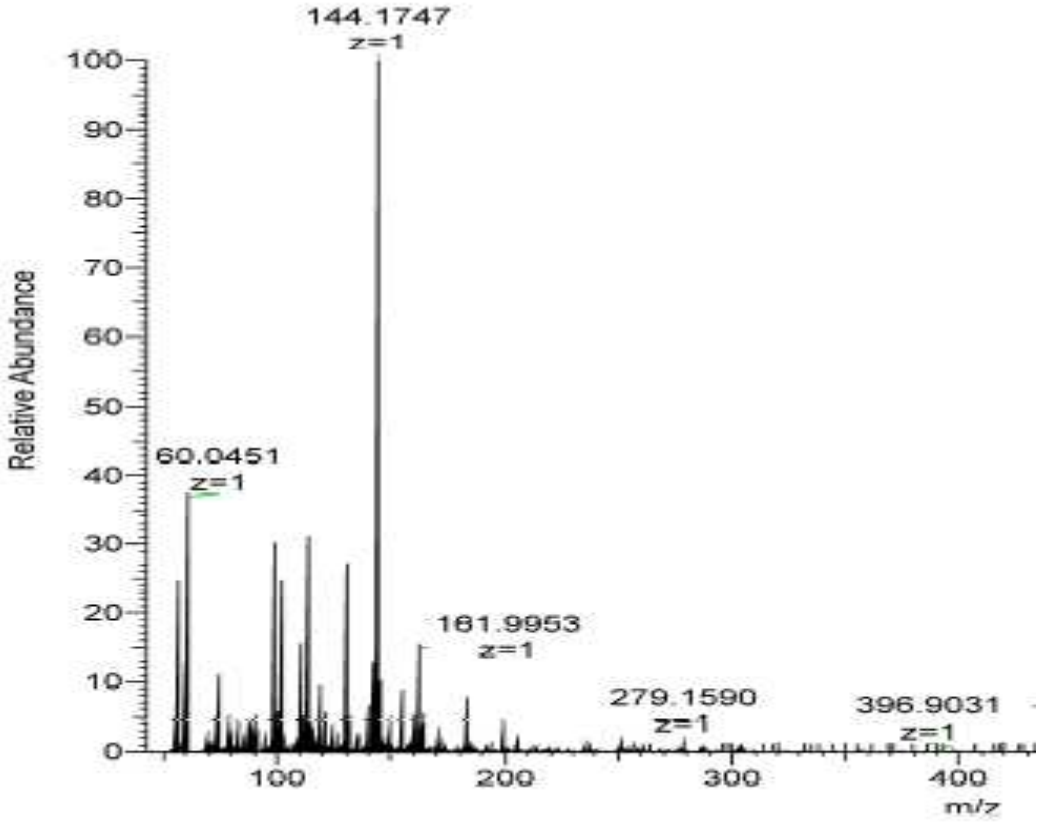
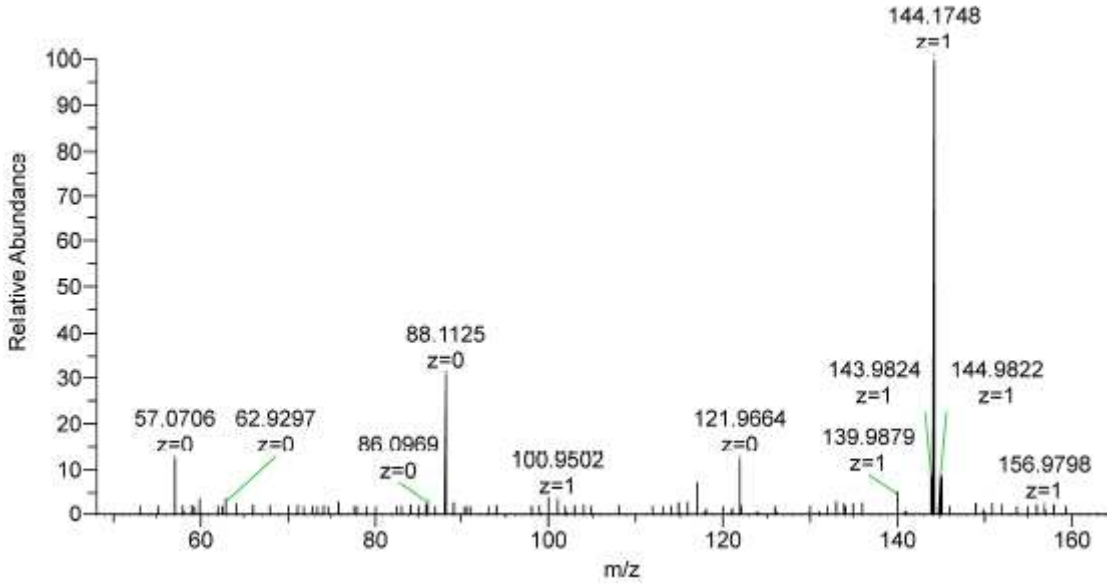


Figure-5.58: HRMS/MS spectrum of DP-13

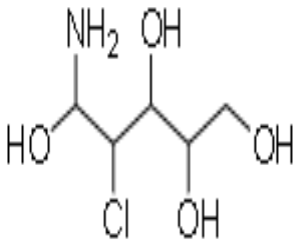


5.5.9 Structure elucidation of degradation product-14

DP-14 eluted at RRT 1.34 (28.3 min) with molecular ion peak at 186.2218 (Figure-5.60, Figure-5.61). The difference between the molecular ion peak of DP-14 and gimeracil is 40.2214 (186.2218-146.0004=40.2214) which indicates that either three molecules of oxygen (48) are incorporated in the structure of gimeracil and further removal of 8 hydrogen by inclusion of four double bonds in the same structure or by forming ketone group from hydroxy. However, there are already two double bonds in the gimeracil structure, and no any major scope of creation of more double bonds in the structure. Hence the other way to increase 40 molecular mass in the structure is to incorporate two oxygen molecules mass (32) and further 8 hydrogen molecules mass can be added in the structure by incorporating 8 more hydrogen in the structure by breakage of existing two double bonds plus breakage of ring system from 1st and 2nd position. Thereby, it creates aliphatic structure as shown in Figure-5.59 which has exact mass of 185.60400000115 which can clearly confirm the molecular ion peak obtained at 186.2218 m/z. There is no other way identified for creation of this molecular mass degradation product. Hence, the structure and chemical name of the DP-14 can be depicted as shown in Figure-5.59.

Figure-5.59: Molecular structure of DP-14

185.60400000115



5-amino-4-chloropentane-1,2,3,5-tetraol

Figure-5.60: HRMS spectrum of DP-14

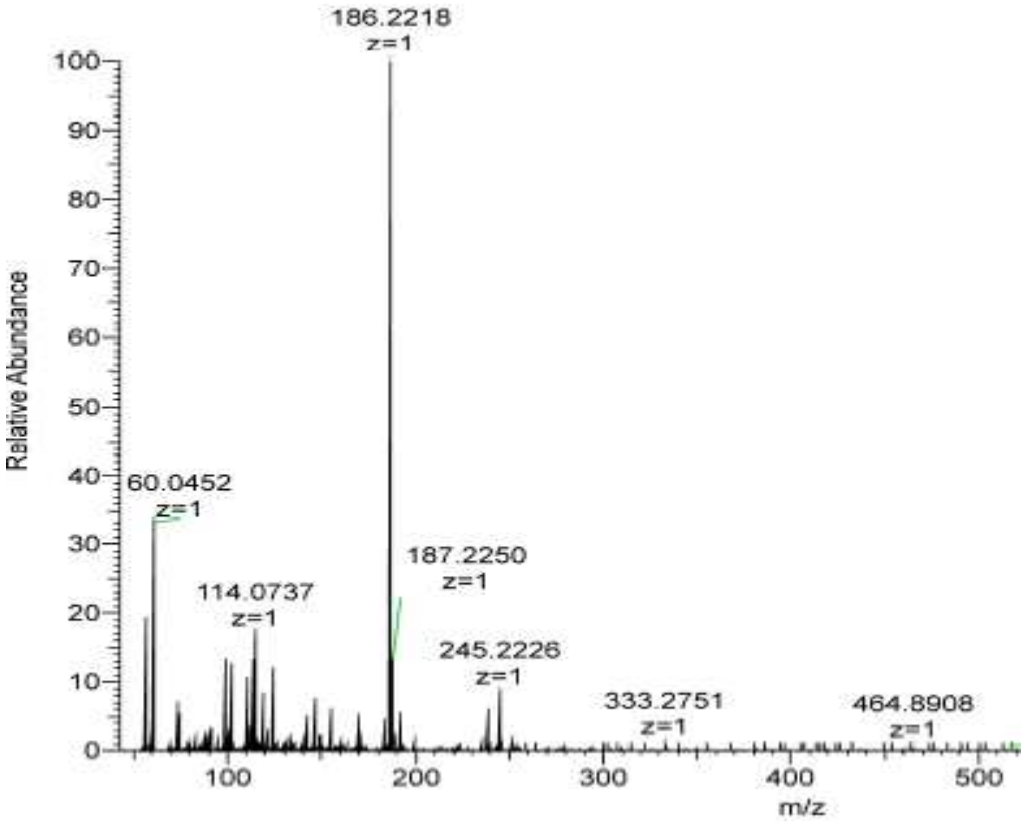
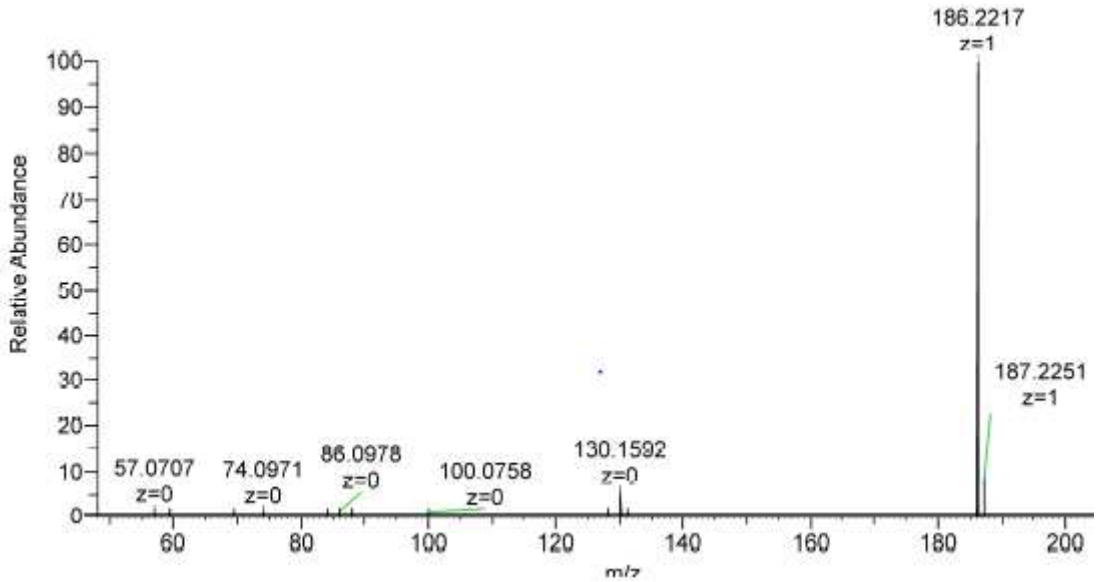


Figure-5.61: HRMS/MS spectrum of DP-14



5.6 CONCLUSION:

This chapter covered two sections. In first section, analytical method development, forced degradation study and method validation study were covered in detail. The method developed for LC as well as MS compatible. The method was validated as per ICH guidelines and all the parameters' results were found satisfactory. The degradation products were difficult initially to identify in HPLC method due to very low amount of degradation products and detector limitations. Later on, study was performed in HRMS instrument and total 14 degradation products were detected in TIC chromatogram with details of MS and MS/MS fragmentation pattern. All the degradation products were characterized based on their exact mass and further fragmentation patterns.

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