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"LC, MS^n and LC–MS/MS studies for the identification and Characterization of degradation products of Atenolol"

Saurabh Sharad Gore

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ABSTRACT:
In the present study, comprehensive stress testing of Atenolol (ATL) was carried out according to International Conference on Harmonization (ICH) Q1A (R2) guideline. ATL was subjected to Neutral, oxidative, acidic, alkaline, photolytic and thermal stress conditions. The drug showed instability in Oxidative, Alkaline and Photolytic conditions, while it remained stable to neutral, Acidic, and thermal stress. A total of four degradation products (DPs) were formed from ATL, which could be separated by the developed gradient LC method on a C18 column. The products formed under various stress conditions were investigated by LC–MS/MS analysis. The previously developed LC method was adopted for LC–MS/MS studies. A complete fragmentation pathway of the drug was first established to characterize all the degradation products using LC–MS/MS and multi-stage mass (MSn) fragmentation studies. The obtained mass values were used to study elemental compositions, and the total information helped with the identification of DPs, along with its degradation pathway. All Characterized DPs were subjected for in-silico study for various toxicities and BBB penetration where all DPs were found non-carcinogenic, Non-hepatotoxic and non-mutagenic, where DP1, DP3 and DP4 was found to be BBB penetrating which may cause serious sleep disturbances when consumed.

Keywords: Atenolol, ICH, LCMS-MS, Degradation products, In-Silico

I. INTRODUCTION:

During the drug development process, stability testing of medicines under various stress situations is essential. Standards for stability testing released by the International Conference on harmonisation (ICH) and other international organizations calling for harmonisation demand the reporting, locating, and classifying degradation products (DPs). However, DPs produced during storage could be very low levels; Therefore, it is recommended that stress studies produce them in higher the sums. However, it might be exceedingly challenging to identify these DPs at times,from the resulting strained mixture because of their smaller quantities [1-4]. As a result, hyphenated methods like LC-MS are widely used right now.

Atenolol (ATL) is a cardio selective beta-blocker used in a variety of cardiovascular conditions.Atenolol is an ethanolamine compound having a (4-carbamoylmethylphenoxy) methyl group at the 1-position and an N-isopropyl substituent. It has a role as a beta-adrenergic antagonist, an anti-arrhythmia drug, an antihypertensive agent, a sympatholytic agent, a xenobiotic and an environmental contaminant. It is a member of ethanolamines, a monocarboxylic acid amide and a propanolamine.[5]

Fig 1.1: Structure of Atenolol[18-20]

A thorough search of the literature found that there are numerous papers on the pharmacokinetic and bio analytical method development for ATL. The estimate of the single drug amlodipine in pure bulk samples, dose forms, and in combination with different drugs like Amlodipine, Chlorthalidone, etc. is reported in a

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wide range of literature using a stability-indicating HPLC,UHPLC, LCMS-MS approach.[6-7]

Several articles on photo stability and thermal investigations of ATL are available. A report on the identification and characterisation of three ATL thermal degradation products that resulted from cyclization and intermolecular interactions is available. Additionally, there aren't many reports on expedited stability investigations and the isolation and characterisation of ATL impurities connected to processes. According to the aforementioned literature, all of the degradation products/impurities of ATL have reported masses and fragmentation patterns that are very different from those of our degradation products of ATL, which were generated under various stress settings. In fact, the masses of DPs proposed in this article were compared to the stated masses of impurities and their fragment ions, and they were all determined to be dissimilar to one another.[9-15]

II. EXPERIMENTAL:

1.1 Drugs and Reagents: Pure ATL was purchased from Aarti Pharmaceuticals Pvt. Ltd. (Mumbai, India). All the chemicals used were of MS grade and Analytical grade produced from Merk, Germany. Ultra-pure water obtained from Merk, Germany was used throughout the studies.

1.2 Equipment:

Instrument consist of BruckerImpact II UHR-TOF Mass Spectrometer System, Dionex, UHPLC Ultimate 3000 System.HR-MS, HR-MS/MS, U-HPLC-MS, U-HPLC-MS/MS with Ionization source ESI & APCI, Mass resolution of 50,000 FSR and having Mass range of 100 to 3500 m/z. DPs were separated on a STAR C18 column (250 X 4.6mm, 5µm) (Merck Lichro, Switzerland) with a mobile phase consisting of Phosphate buffer (1gm of Sodium dihydrogen phosphate and 2gm of Di Potassium hydrogen phosphate in 1000ml ml of water); pH 5(adjusted with Ortho phosphoric acid) Methanol and Acetonitrile in the ratio of 40: 30: 30(v/v) in the gradient elution mode with a flow rate of 0.350 ml/min with 40min gradient.Performed on Software LCMS: HighStar 3.2, UHPLC: Chromeleon, Mass: OTOF-Q-control

1.2.1 Degradation Studies:

Drug acid degradation study was carried out in 1N HCL. Weighed accurately 10 mg of ATL and transfer to 10 ml volumetric flask, add 10 ml of 1N HCL (1mg/ml), vortex it and kept at 50℃ for eight hours. Drug alkali degradation study was carried out in 1N NaOH. Weighed accurately 10 mg of ATL and transfer to 10 ml of volumetric flask, add 10 ml of 1NNaOH (1mg/ml), vortex it and kept 50℃ for eight hours. Oxidative degradation study was carried out in Hydrogen Peroxide (H_2O_2) at room temperature. Weighed accurately 10 mg of ATL and transfer to 10 ml of volumetric flask, add 10 ml of Hydrogen Peroxide (1mg/ml), vortex it and kept at room temperature for eighthours. Weighedaccurately 10mg of ATL and transfer to 10 ml of volumetric flask, add 10 ml of water (1mg/ml) and vortex it and it was kept in hot air oven at 80℃ for eighthours. Weighed accurately 10mg of ATL and transfer to 10 ml of volumetric flask, add 10 ml of water (1mg/ml) and vortex it, and it was kept under UV light(sunlight) for eight hours.

III. RESULT AND DISCUSSION:

1.3 Differential Scanning Calorimetry (DSC) Study of ATL:

The thermal behaviour of ATL previously to the decomposition temperature was investigated by DSC, in heat–cool–heat mode between 20 to 710℃, resulting in the three DSC profiles presented in Fig. During the first heating, an endothermic event was observed as a sharp peak at 156.461℃, and further shown sharp exothermic offset peak at 173.8℃ relating to the melting of the sample simultaneously calculated as peak maxima of 164.792℃ as an exact melting point of a compound I.e. ATL. The enthalpy associated to this process was DHfus = 499.387 J g-1. Since solid and liquid phases are in equilibrium. No crystallization event was observed in the cooling step, but a baseline change appeared in 30℃ regarding second order transition, suggesting that the drug has turned into an amorphous material.

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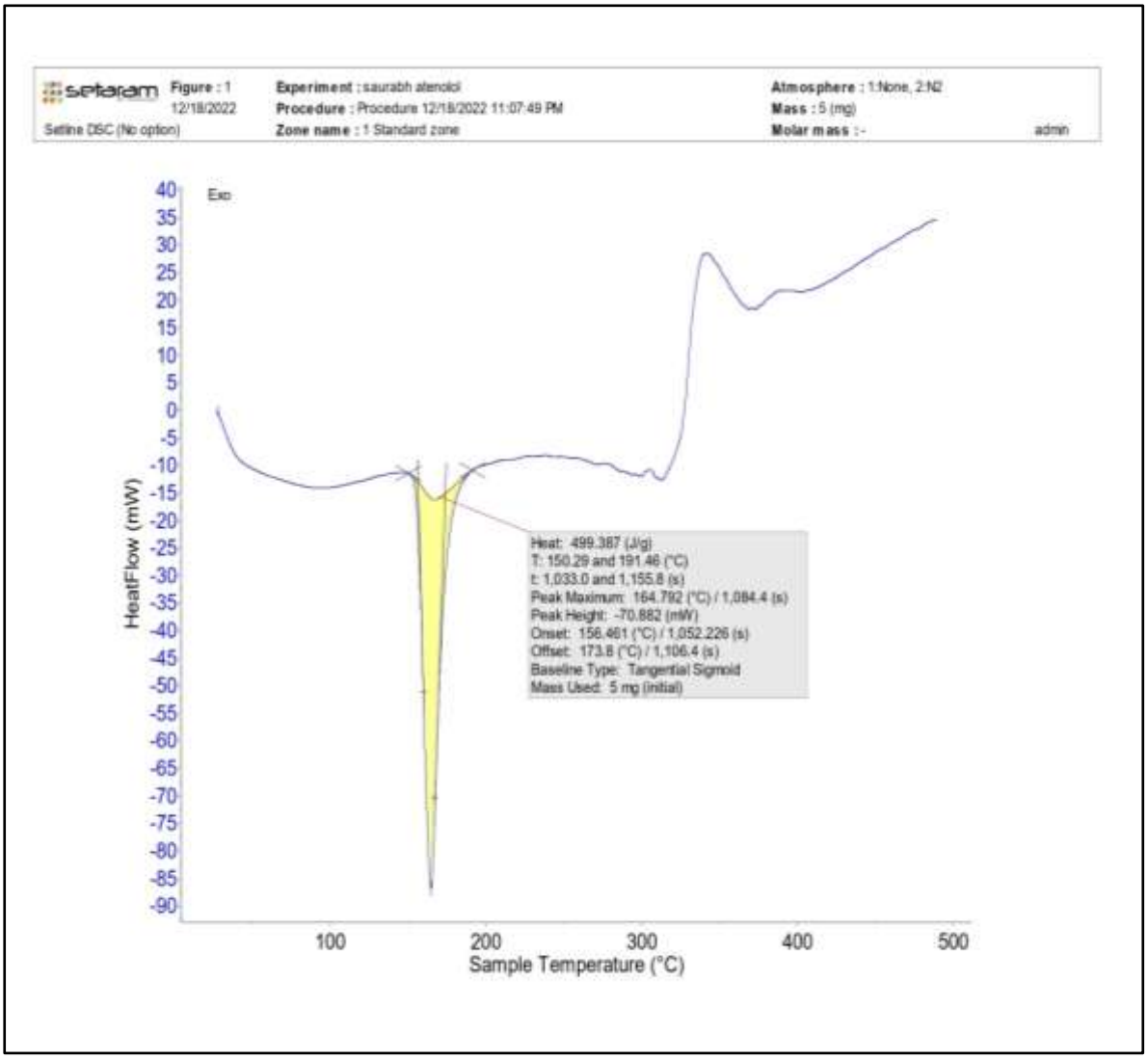

Fig4.1:Thermogram of ATL

Experimental Conditions:

Parameters	Conditions
Apparatus	Setline DSC
Crucible	30 µg
Molar Mass	5 g/mol
Safety Temperature	710 °C
Carrier Gas 1	None

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<table>
<tr><td>Carrier Gas 2</td><td>Nitrogen</td></tr>
<tr><td colspan="2">Zones</td></tr>
<tr><td>1 : Standard zone</td><td>Total duration: 2280 s
Acquisition Period: 0.2 s (Auto)
Number of Points: 11400
Sequences<table><tr><th>#</th><th>Type</th><th>Init T (°C)</th><th>Fin T (°C)</th><th>Time (s)</th><th>S.r.(K/m</th></tr><tr><td>1</td><td>→</td><td>20</td><td>20</td><td>600</td><td>0</td></tr><tr><td>2</td><td>↗</td><td>20</td><td>300</td><td>1680</td><td>10</td></tr></table></td></tr>
<tr><td>2 : Return to set temperature zone</td><td>Total duration: 168 s
Sequence<table><tr><th>#</th><th>Type</th><th>Init T (°C)</th><th>Fin T (°C)</th><th>Time (s)</th><th>S.r.(K/m</th></tr><tr><td>1</td><td>↘</td><td>300</td><td>20</td><td>168</td><td>99.99</td></tr></table></td></tr>
</table>

Table 4.1: Experimental conditions for DSC

4.2 UHPLC Analysis, Chromatographic Separation and Mass spectral Studies:

During the initial separation, acetonitrile and water were adopted as a mobile phase, separation of drugs and degraded products was optimum; since it showed satisfactory resolution and separation between drugs and degraded products, and hence it was kept unchanged.

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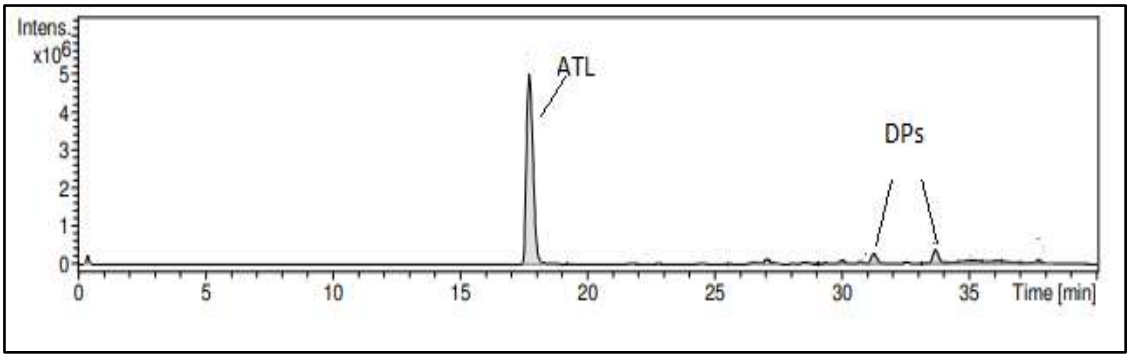

Fig4.2: Chromatogram showing separation of degradation products of ATL in the Photolytic stress sample

a) Mass fragmentation pattern of ATL:

A total of Four Degradation products (DPs) were found from ATL during its MS studies. A multi-stage (MS^n) mass fragmentation study was also carried out to determine the origin of each fragment, which could help propose the fragmentation pathway of ATL. Line spectra of ATL found in MS studies were compared with proposed fragmentation pattern of ATL.

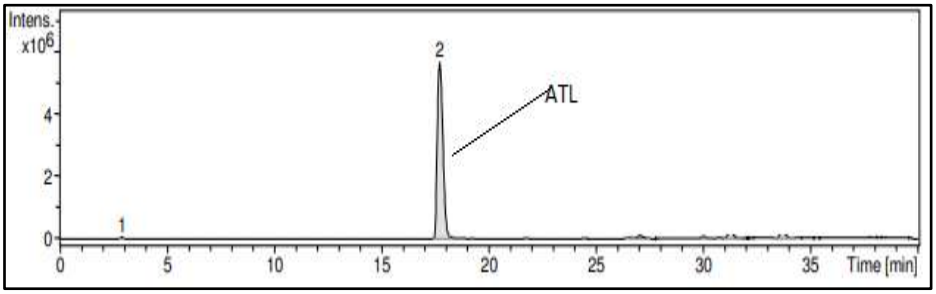

Fig4.3: LC of ATL without any Stress

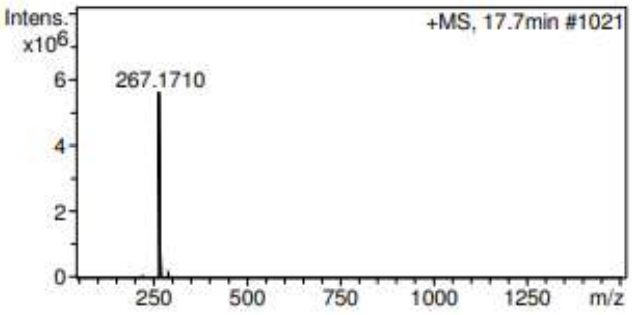

Fig4.4: MS spectra of ATL

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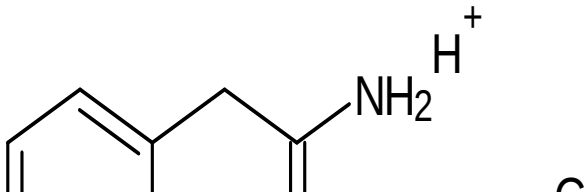

Fig 4.5: Proposed Fragmentation Pattern of ATL

Sr. NO.	Observed/Experimental Accurate Mass of ATL Fragments [M+H]:	Proposed molecular formula	Calculated Mass [M+H]:	Difference from parent ion
1	190.0859	$C_{12}H_{17}NO$	190.1220	77.0851
2	225.1234	$C_{11}H_{16}N_2O_3$	225.1233	42.0476
3	249.1597	$C_{14}H_{21}N_2O_2^+$	249.1592	18.0113
4	267.1710	$C_{14}H_{22}N_2O_3$	267.1703	0.0007
5	268.1740	$C_{14}H_{21}NO_3$	268.1703	-1.5393

Table4.2: Interpretation of MS data of fragments of ATL

b) Stress Degradation Behaviour of ATL:

A total of four significant DPs DP1-DP4 were formed from ATL during the stress degradation study. Among all the DPs, DP1 were found in oxidative stress DP2 and DP3 were formed in alkaline stress condition, while DP4 were formed in Photolytic Stress Condition. The chromatogram showing separation of ATL and all the DPs. The degradation products of ATL are denoted as DP1 to DP4 in accordance with the sequence.

i. Oxidative Stress:

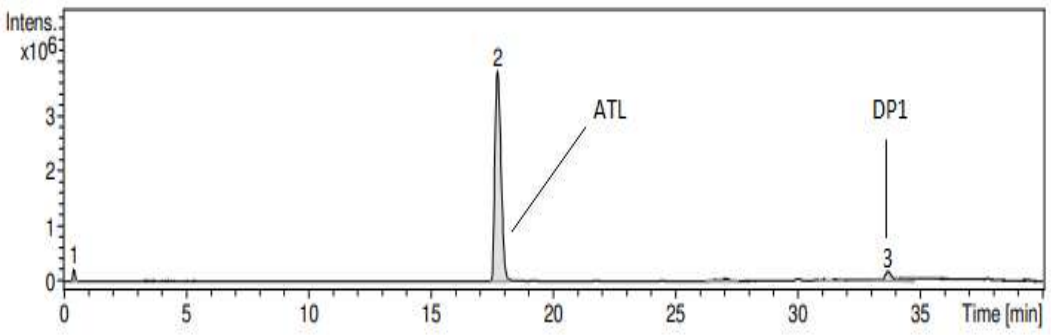

Fig 4.6

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ii. Thermal Stress:

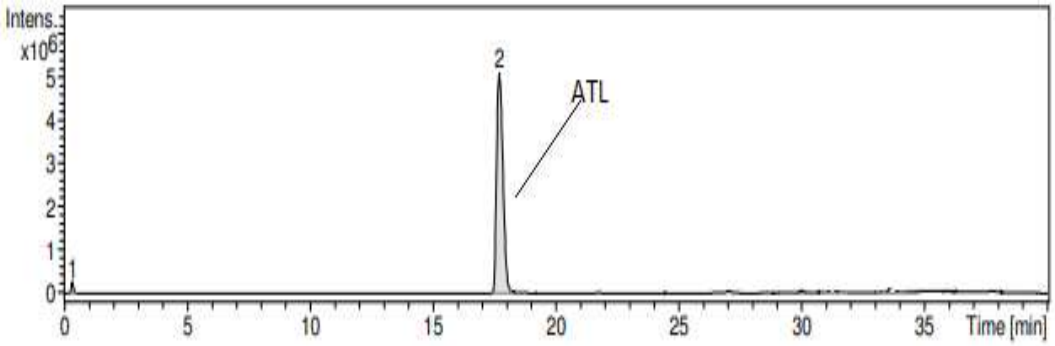

Fig 4.7

iii. Acidic Stress:

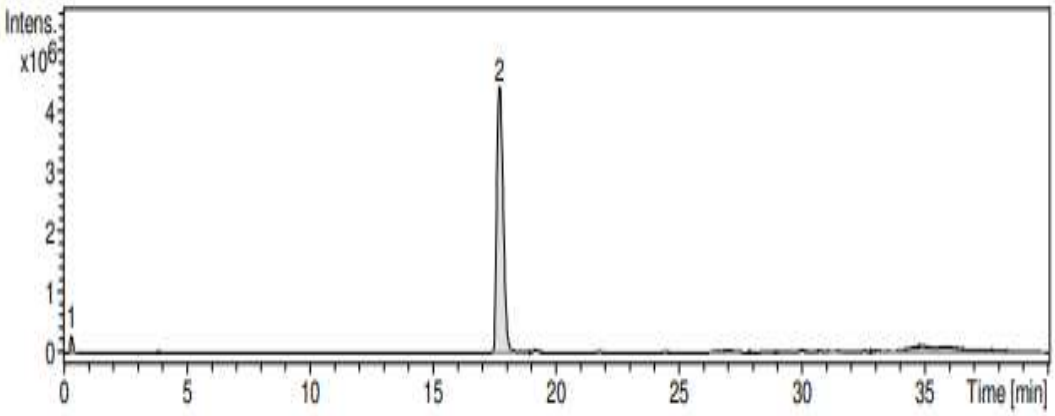

Fig 4.8

iv. Alkaline Stress:

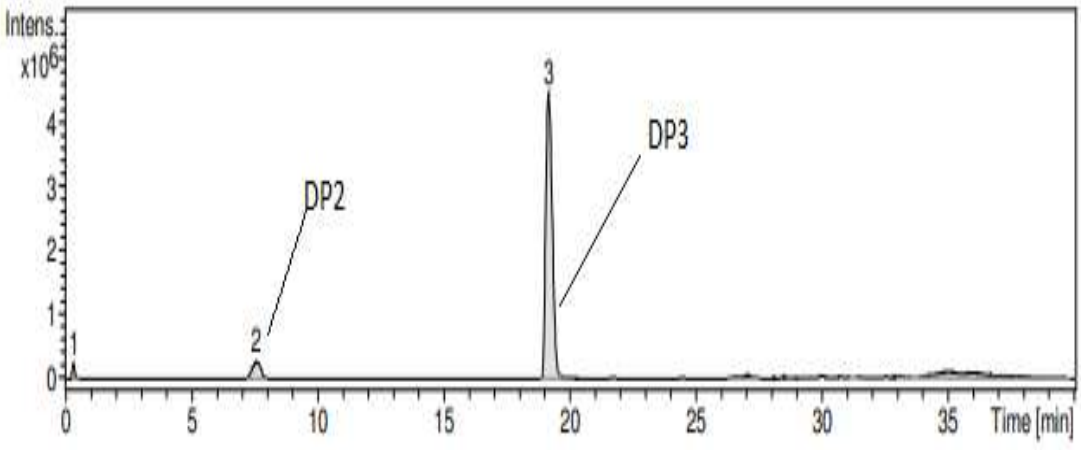

Fig 4.9

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V. Photolytic Stress:

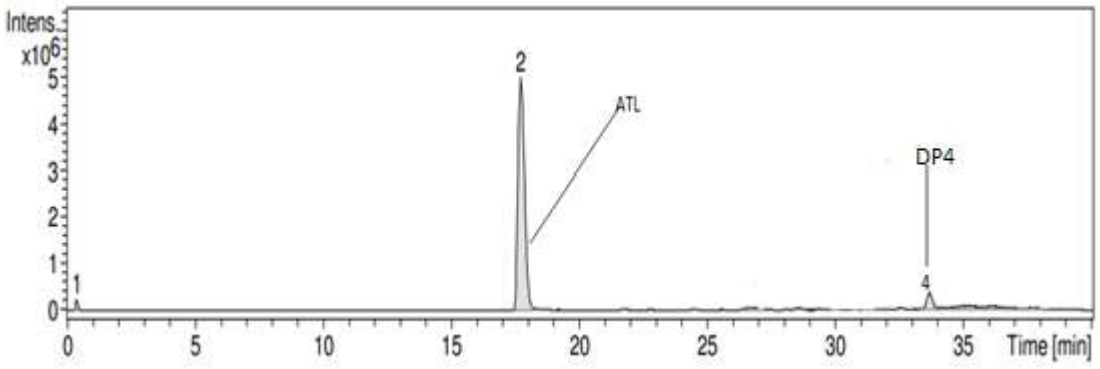

Fig 4.10

4.3 Characterization of degradation products:

The data obtained in MS, MS^n and LC–MS/MS studies were systematically utilized for the structure elucidation of degradation products of ATL, among these the fragment with m/z 267.1710 was the molecular ion peak as well as base Peak.

a) DP1: Among these the fragment with m/z 209.1535 was the molecular base peak, and 338.3422 were found to be molecular ion peak.The drug (AM) underwent loss of Acetamide group and resulted in the formation of DP1. There onwards DP1 followed a parallel fragmentation pathway involving loss of Phenol group ions with m/z 116 and m/z 98, respectively. A loss of another Hydroxyl moiety to from m/z 190 resulted in the formation of fragment with m/z 162 with loss of another methyl group

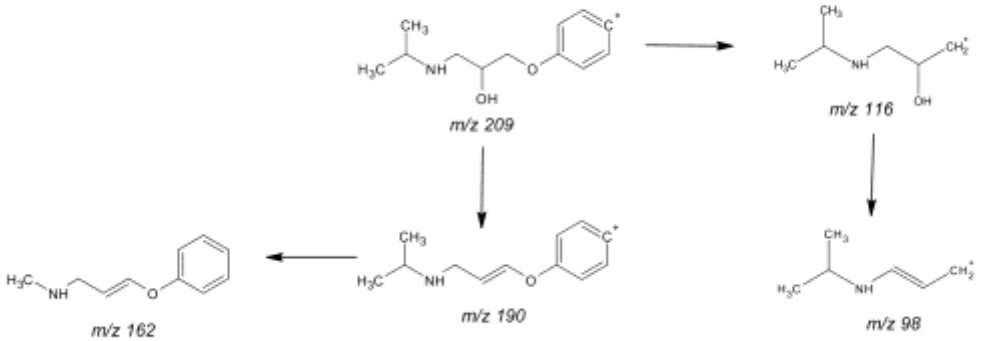

Fig 4.11: Proposed Fragmentation Pattern for DP1

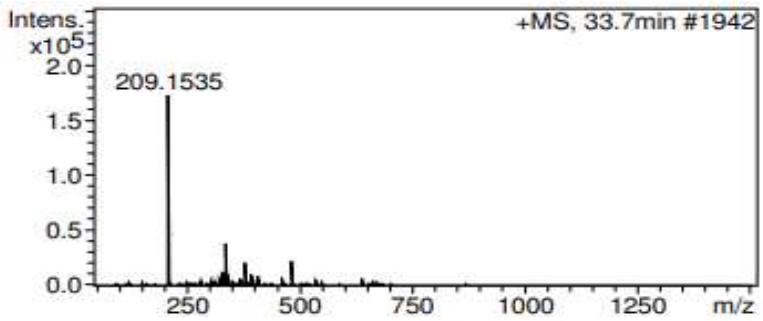

Fig 4.12: Mass Spectra of DP1

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Sr. NO.	Observed/Experimental Accurate Mass of DP4 Fragments [M+H]:	Proposed molecular formula	Calculated Mass [M+H]:	Difference from parent ion
1	209.1544	$C_{12}H_{18}NO^+$	209.1404	-0.014
2	190.1287	$C_{12}H_{16}NO^+$	191.12991	19.0117
3	162.1748	$C_{10}H_{12}NO^+$	163.0986	46.9626
4	116.1747	$C_6H_{14}NO^+$	117.1142	92.9657

Table No4.3:Interpretation of MS data of fragments of DP1

b) **DP2:** Among these the fragment with m/z 226.9515 was the molecular ion peak. The drug (AM) underwent loss of methyl group and resulted in the formation of DP2. There onwards DP2 followed a parallel fragmentation pathway involving loss of Acetamide group ions with m/z 167.09531 and m/z 149.082967, respectively. A loss of another Phenyl moiety to from m/z 71.072402 resulted in the formation of fragment with m/z 162 with loss of another methyl group, the proposed fragmentation for DP2 is given in fig.

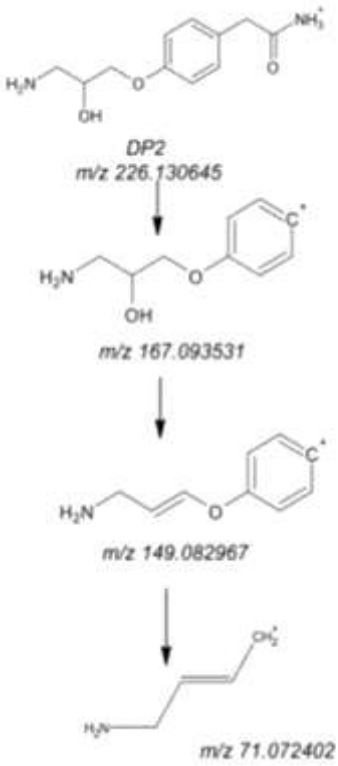

Fig 4.13: Proposed Fragmentation Pattern for DP2

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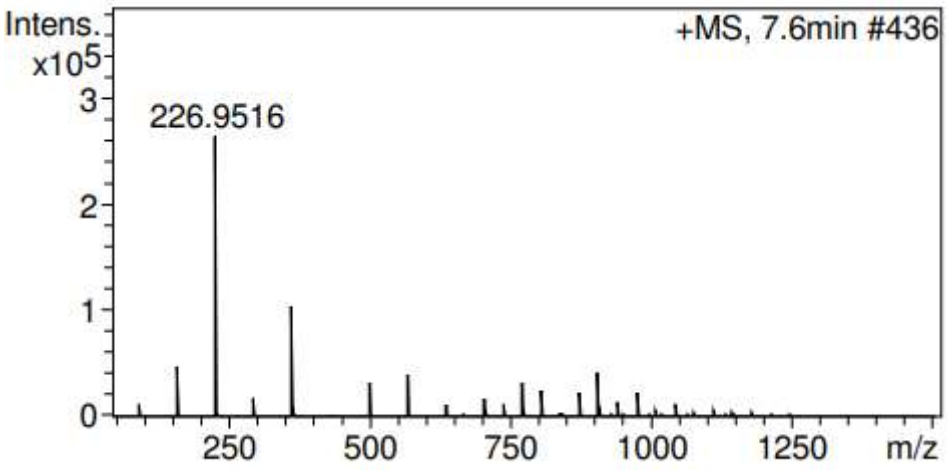

Fig 4.14: MS Spectra of DP2

Sr. NO.	Observed/Experimental Accurate Mass of ATL Fragments [M+H]:	Proposed Molecular formula	Calculated Mass [M+H]:	Difference from parent ion
1	226.9515	$C_{11}H_{17}N_2O_3$	226.130645	0.8209
2	116.0935	$C_9H_{12}NO_2$	116. 9262	109.4253
3	149.0822	$C_9H_{11}NO$	149.4829	76.4829
4	71.0724	$C_4H_{10}N$	70.4785	154.8730

Table No4.4: Interpretation of MS data of fragments of DP2

c) DP3:

DP3 was found as most significant DP among all of other DPs, as a result it shows sharp peak in LC and also showed significant change in RT of LC. After MS study of DP3 we obtained m/z 268.1556, so as per proposed fragmentation pattern of ATL it was concluded that found degradation product of Atenolol acid in which it produced due to loss of one amine group and attachment other new hydroxyl group which occurred due to alkaline stress of 0.1N NaOH which results in substitution of amine group by hydroxyl group. Proposed Fragmentation pattern for DP3 is given in above fig.

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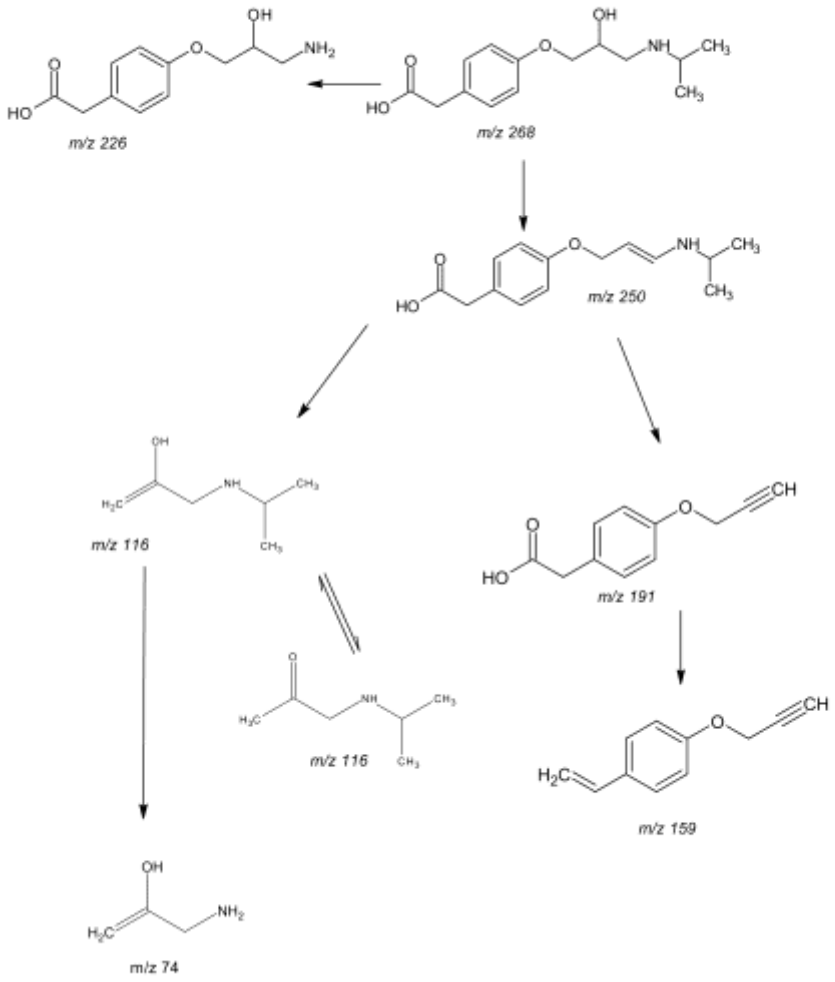

Fig4.15: Proposed Fragmentation Pattern for DP3

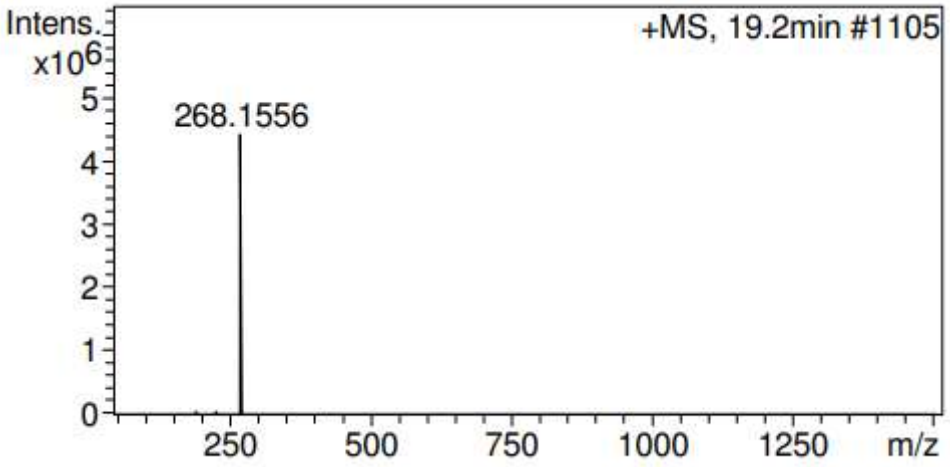

Fig 4.16: Mass Spectra of DP3

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Table4.5: Interpretation of MS data of fragments of DP3

1	116.1066	$C_6H_{13}NO$	116.10699	152.0474
2	191.0703	$C_{11}H_{10}O_3$	191.070271	77.0841
3	226.1073	$C_{11}H_{15}NO_4$	226.1073	42.047
4	250.1443	$C_{14}H_{19}NO_3$	250.14377	18.0105
5	268.1556	$C_{14}H_{21}NO_4$	268.15433	-0.0013

d) DP4:

DP4 was found in the sample injection of photolytic stress, as we see we found the same DP as we earlier found in Oxidative Stress Among these the fragment with m/z 209.1544 was the molecular base peak.The drug (AM) underwent loss of Acetamide group and resulted also in the formation of DP4. There onwards DP4 followed a parallel fragmentation pathway involving loss of Phenol group ions with m/z 116 and m/z 98, respectively. A loss of another Hydroxyl moiety to from m/z 190 resulted in the formation of fragment with m/z 162 with loss of another methyl group.As we concluded than presence of DP in above stress sample produces significant change in the MS and also gives significant fragments. Since we found same DP in two stress conditions but there is slight chance in their experimental masses.The proposed fragmentation for DP4 is given in above figure which shows the possible fragments of DP4.

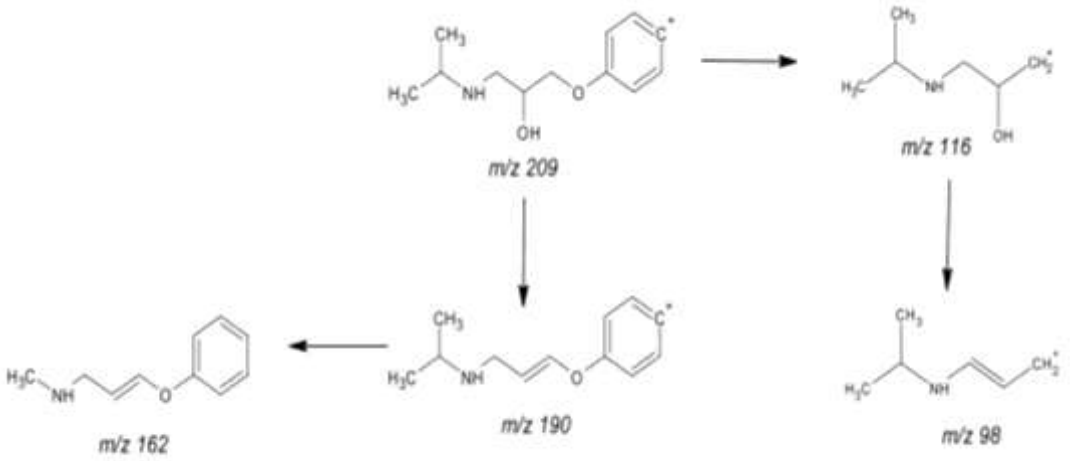

Fig 4.17: Proposed Fragmentation pathway for DP4

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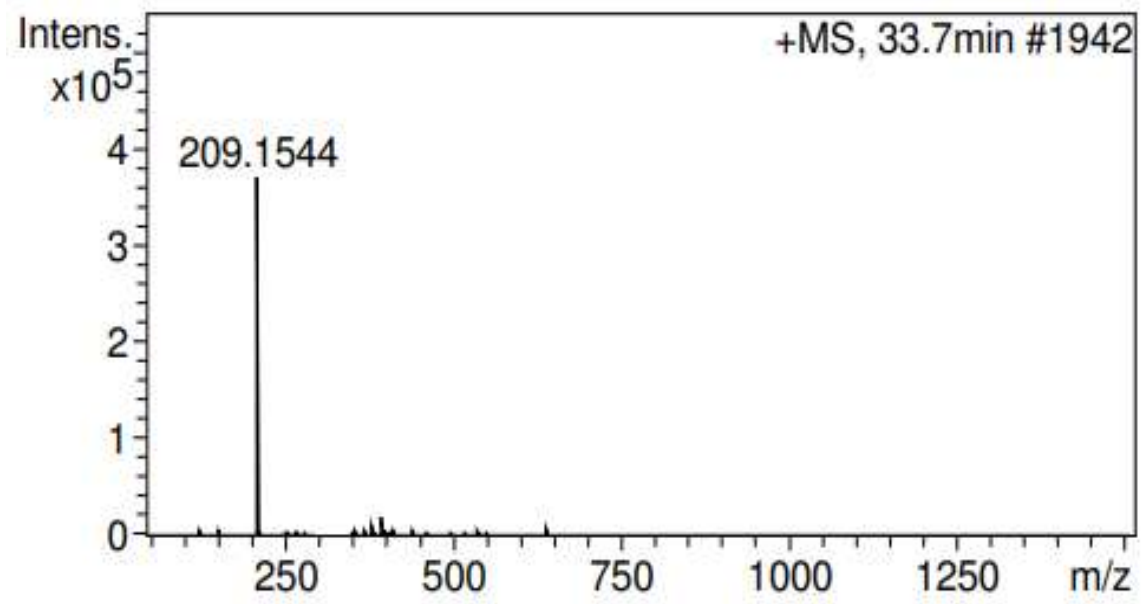

Fig4.18: Mass Spectra of DP4

Sr. NO.	Observed/Experimental Accurate Mass of DP4 Fragments [M+H]:	Proposed molecular formula	Calculated Mass [M+H]:	Difference from parent ion
1	209.1544	$C_{12}H_{18}NO^+$	209.1404	-0.014
2	190.1287	$C_{12}H_{16}NO^+$	191.12991	19.0117
3	162.1748	$C_{10}H_{12}NO^+$	163.0986	46.9626
4	116.1747	$C_6H_{14}NO^+$	117.1142	92.9657

Table No4.6:Interpretation of MS data of fragments of DP4

4.4 Degradation pathway of ATL:

Based on the data obtained from the line spectra of LC–MS/MS studies of each degradation product, a final fragmentation pathway of ATL was established. The degradation pathway of ATL is shown in conclusion along with the proposed structures and masses of all the DPs.

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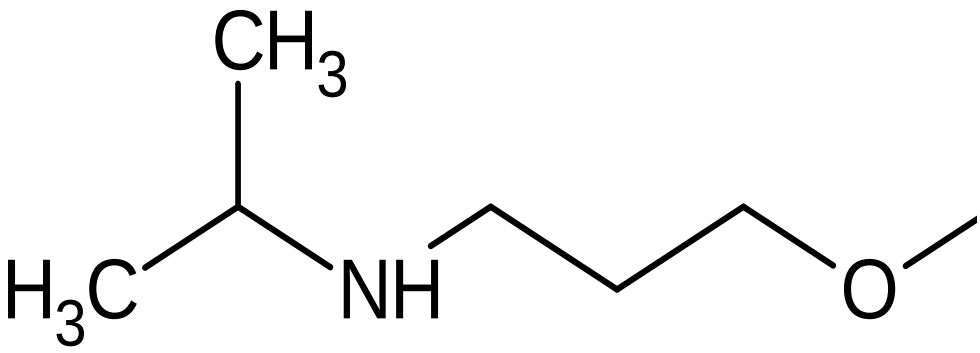

Fig 4.19 Degradation Pathway of ATL

5 In-silico Toxicity and carcinogenicity predictions of characterized DPs with the help of various Insilco toxicity prediction tools:

For Insilco toxicity predictions we used various kinds of web tools such as LAZAR Toxicity predictions, Protox II, Discovery Studio, etc.

- **DP1:**

Fig 5.1: DP1

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Molecular Weight: 209
IUPAC Name: 4-{3-[(propan-2-yl)amino]propoxy}phen-1-ylium

In-silico toxicity predictions:

1. Blood Brain Barrier Penetration (Human): Penetrating
2. Maximum Recommended Daily Dose (Human):

Predictions:
1.06 (mg/kg_bw/day)

3. Hepatotoxicity: Inactive
Hepatotoxic Probability: 0.82%
4. Carcinogenicity(Humans): Inactive
Carcinogenicity Probability: 0.84%
5. Mutagenicity: Inactive
Mutagenicity Probability: 0.81%

➢ **DP2**

Fig 5.2: DP2

Molecular Weight: 225
IUPAC Name: [4-(3-amino-2-hydroxypropoxy)phenyl]ethanamidium

In-silico toxicity predictions:

1. Blood Brain Barrier Penetration (Human): Non-Penetrating
2. Maximum Recommended Daily Dose (Human):

Predictions:
8.2(mg/kg_bw/day)

3. Hepatotoxicity: Inactive
Hepatotoxic Probability: 0.94%
4. Carcinogenicity(Humans): Inactive
Carcinogenicity Probability: 0.80%
5. Mutagenicity: Inactive
Mutagenicity Probability: 0.71%

➢ **DP3:**

Fig 5.3:DP3

Molecular Weight: 267
IUPAC Name: (4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetic acid

In-silico toxicity predictions:

1. Blood Brain Barrier Penetration (Human): Penetrating
2. Maximum Recommended Daily Dose (Human):

Predictions:
0.861 mg/kg_bw/day)

3. Hepatotoxicity: Inactive
Hepatotoxic Probability: 0.98%
4. Carcinogenicity(Humans): Inactive
Carcinogenicity Probability: 0.87%
5. Mutagenicity: Inactive
Mutagenicity Probability: 0.88%

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➢ **DP4:**

Fig 5.4: DP4

Molecular Weight: 209
IUPAC Name: 4-{3-[(propan-2-yl) amino]propoxy}phen-1-ylium

In-silico toxicity predictions:

1. Blood Brain Barrier Penetration (Human): Penetrating
2. Maximum Recommended Daily Dose (Human):
Predictions:
1.06 (mg/kg_bw/day)
3. Hepatotoxicity: Inactive
Hepatotoxic Probability: 0.82%
4. Carcinogenicity(Humans): Inactive
Carcinogenicity Probability: 0.84%
5. Mutagenicity: Inactive
Mutagenicity Probability: 0.81%

IV. CONCLUSION AND SUMMARY:

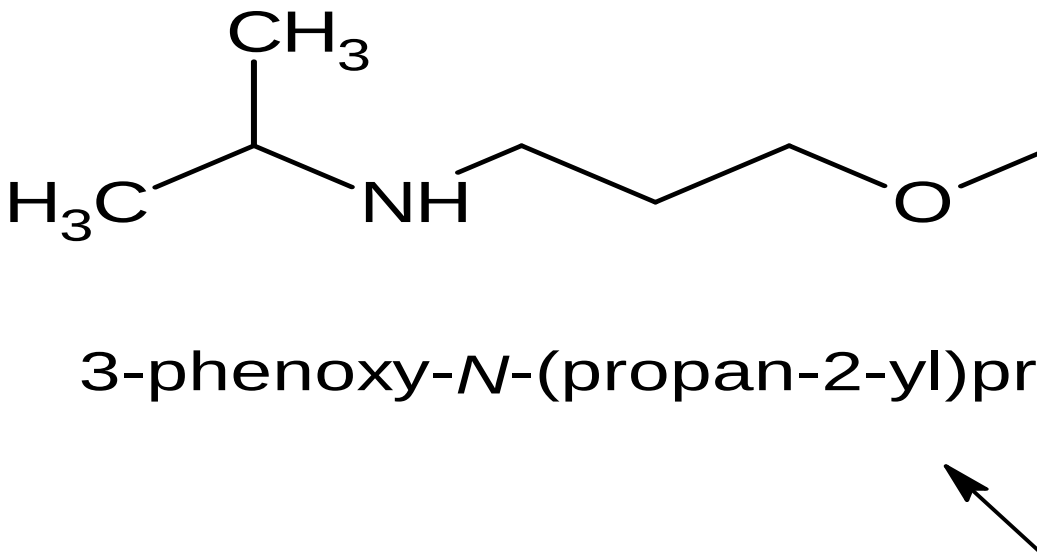

Fig 6.1: Proposed Degradation Pathway of ATL along with its respective DPs

A forced degradation study on Atenolol was performed to determine its labile behaviour under respective stress condition. The drug was labile to oxidative, alkaline and photolytic stresses, while it was stable in other neutral, acidic and thermal conditions. The LC separation studies revealed the formation of four degradation products from the drug. DP-I was formed in Oxidative, DP2 and DP3 in alkaline conditions and DP-IV was generated in photolytic condition. The structures of

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all these degradants were resolved with the help of MS, MS^n, and LC–MS/MS analysis. The complete degradation pathway of the drug was established. The in-silico carcinogenicity study explored the carcinogenic potential of the drug and DPs, while the values obtained by LAZAR Toxicity and Protox software for the drug and DPs revealed a high probability of BBB Penetration which can readily produce sleep disturbances on the sleep of consumer.

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