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Research Article

A degradation study of Aniracetam into its precursor including degradation pathway and identification with RP-HPLC method validation and LC/MS

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ABSTRACT

Aniracetam is smart drug known as N-anisoyl-2-pyrrolidinone. Its nootropic and neuroprotective drug. This drug is known for degrade in alkaline condition and produced two degradative by-products namely 4-MBA and N-anisoyl GABA. While it is stable in other stress condition such as acidic and oxidized. The degradation products are isolated, identified, characterized, and Mechanism explanation to the Origin of the degradation Products, to establish degradation Pathways of the drug and over degradation of degraded product by using newly developed and validated HPLC method, LC/MS, ^{1}H NMR, and IR.

Keywords: Alkali degradation, 4-MBA, N-anisoylGABA, Degradation pathway.

1. INTRODUCTION

Aniracetam (AN) is an ampakine drug a cyclic derivative of GABA. It is one from the groups of racetam and considerably more potent than piracetam. AN is prescribed by doctor in the treatment for some conditions, mainly myoclonus, however it is used off-label for a much wider range of like aging, emotional disturbances depressed mood, anxiety, agitation, sleep disorders, sickness, motion ,and behavior abnormalities (nocturnal delirium wandering) that are associated with cerebral infarction and Alzheimer"s and Parkinson's diseases[1-2]. It has been also reported to possess. Mechanisms for positivity modulation cholinergic and glutaminergic nervous systems as well as increasing synaptic efficacy and energy metabolism[1].

The International Conference on Harmonization (ICH) drug stability evaluation benchmark Q1A(R2) requires that analysis of stability samples should be done through the use of validated stability-indicating analytical methods. It also recommends carrying out of stress testing on this drug substance to determine its inherent stability characteristics and to ensure time suitability of the proposed analytic guideline[3-15]. Recently high performance liquid chromatography (HPLC) has proven to be one of the most promising developments in the area of rapid chromatographic separations. In the present study, reverse phase chromatographic stability indicating assay method is developed using LC/MS,NMR,IR and HPLC for AN drug . Here, we present a highly efficient way to recycle these two precursor and for the synthesis of AN[16-22] .

2. MATERIALS AND METHODS

2.1 Instrumentation and software.

High Performance liquid chromatography(HPLC) :The HPLC System of Agilent HPLC 1100 Series Variable Wavelength Detector (VWD) The Diole Array Detector (DAD),quaternary pump, Agilent Technologies international,1100 series, auto sample, micro auto sample, preparative auto sample, Thermos tatted column compartment ,used for this entire study and chromatographic separation was achieved on Eclipse XDB C_{18} (150 nm X 4.6 mm X 5µm)

column as stationary phase with binary gradient mode.

LC/Mass Spectrometry (LC/MS): HPLC-MS studies were carried out on a system in which HPLC was spectrometer manufactured by Waters .The accurate mass and composition for the precursor ions and the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument.

Nuclear Magnetic Resonance Spectroscopy: NMR was carried out using a Bruker Spectrospin 400MHz manufactured Bruker (Canton Massachusetts, USA). ^{1}H NMR Spectra of Degradation product was recorded using dimethyl sulfoxide (DMSO-d_6) as a dissolving solvent. TMS as an internal standard, Chemical shifts are reported as δ/ppm units.

Infrared spectroscopy: The IR spectra of degradation product were obtained using manufactured Bruker FT/IR-α ALPHA-T sr.no 200196 Plus FTIR spectrophotometer. The sample was prepared with KBr (Merck) which was dried in a hot air oven for 40 minutes before preparing samples. Melting ranges of the sample were determination on an VEEGO Model VMP-DS digital melting point instrument.

The other instruments used for data generation of degraded products were Analytical balance, digital pH meter and water bath.

2.2 Chemicals and reagents

The API of AN (99.9% pure) 1000mg was purchased from market. HPLC grade acetonitrile (SD fine limited). Analytical grade hydrochloric acid, sodium hydroxide flakes, hydrogen peroxide. Milli-Q Water purchased from market..

2.3 Details of Method Chromatographic conditions:

Reversed Phase High Performance liquid chromatography method with UV detection separation was achieved on zorbox Agilent Eclipsc XDB column c_{18}(150 nm× 4.6 mm×5µm) as stationary phase with binary gradient mode solvent phase A as composed of H_3PO_4(ortho phosphoric acid) buffer (pH ≈2, 0.02M) and phase B as acetonitrile, The flow rate of the mobile phase was 1.0 mL/min and the total elution time including the column re-equilibration was approximately 35 min. The UV detection wavelength was 215nm, injection volume was 10µL and experiments were conducted at 30 °C temperature, gradient elution of mobile phase (A%: B% to min.)80:20 to 0.01-10, 70:30 to 10-20, 30:70 to 20-30, has been used for HPLC analysis for forced degradation study in Acidic, Basic, and oxidative condition .

2.4 Preparation of solutions:

Specificity solution for t_R confirmation and System suitability solution :Accurately weighted 10mg of AN is transferred into a three different 100 mL volumetric flask, added about 50 mL of diluent and sonicate to dissolve the content. Makeup the volume up to the mark with diluent and mix. Concentration of AN (100µg/mL,100ppm) and same condition for preparation of test solution. The chromatogram show in figure 1.

2.5 Method Validation

Method Validation of the developed method for the determination of AN is performed according to the ICH guidelines "Validation of analytical procedures: text and Methodology Q2(R1)" [21-25] with standards API thus system suitability along with method selectivity, specificity , linearity, range, precision (repeatability) and intermediate precision, accuracy, limits of detection and quantification.

2.6 Forced Degradation Study:

Acid degradation: Accurately weighted 25mg of test sample AN is transferred into 50 mL volumetric flask, added about 5 mL of 5N hydrochloric acid solution 10mL diluent and sonicate to dissolve the content. Makeup the volume up to the mark with diluent and mix. Concentration of AN (500µg/mL,500ppm).Heat it at 60^{0}C on water bath for 1 hr, cool it at RT and adjust the pH 7.0 with of 5N sodium hydroxide solution. The chromatogram show in figure 1.

Alkali degradation: Accurately weighted 25mg of test sample AN is transferred into 50 mL volumetric flask, added about 5 mL of 5N sodium hydroxide solution 10mL diluent and sonicate to dissolve the content. Make the volume up to the mark with diluent and mix. Concentration of AN (500µg/mL,500ppm).Heat it at 60^{0}C on water bath for 1 hr, cool it at RT and adjust the pH 7.0 with of 5N hydrochloric acid solution. The chromatogram show in figure 1.

Oxidative degradation : Accurately weighted 25mg of test sample AN is transferred into 50 mL volumetric flask, added about 5 mL of 10% hydrogen peroxide solution and 10mL diluent and sonicate to dissolve the content. Makeup the volume up to the mark with diluent and mix. Concentration of AN (500µg/mL,500ppm).Heat it at 60^{0}C on water bath for 1 hr, cool it at RT . The chromatogram show in figure 1.

2.7 Isolation and Characterization of Degradation Product

As the drug degraded under alkaline condition to give two degradation product, it has been crucial to isolated and characterized . The amount of 500 mg of the drug was dissolved in 100ml of 1N NaoH to obtain a 5 mg/ml solution. Drug sample was at 60^0 C for 2 hrs under the stringent monitoring with thin layer chromatography. Degradation product was is generated (monitored by TLC data show in supporting material) appearing that turned prominent and completes the reaction later. The formed complete degradation product of the AN was confirmed by the TLC and cool the mass to RT the resultant solution was neutralized with HCL acid to give a precipitated product 01. The product 01 was filtrated washed with D.M Water and dried . Collect the precipitate of degradation Product 01 . Collected mother liquor is cooled to 0^0 to 5^0 to give a precipitated degradation product 02 . The degraded product 02 was filtrated washed with chilled D.M Water and dried . Collected the precipitate of degradation product 02 and product 01 appearance and physical state to crystalline powder and solid check melting point, subjected to HPLC, LC-MS/MS, IR, and ^{1}H NMR for structural identification. The structure elucidation of both the degradation product 01 and degradation product 02 was achieved with the systematic mass fragmention,^{1} H NMR spectra, FT-IR[23-27] .

3.RESULTS AND DISCUSSION

3.*1 HPLC Studies on the Stressed Solutions*

The forced -degradation study shows that AN is under stress condition. AN did not degrade under acid, oxidative , stress conditions a mixture of solution, which it degraded under Alkali stress condition and only two degradation product was formed. The specificity and selectivity of the method with the samples under these stresses were demonstrated through the evolution of retention times(t_R),retention factor (Rt_R), resolution(R_s), and purity data for all peaks in the chromatograms. The t_R ,R_s and Rt_R of this drug and degradation product show are tabled in Table.1

This drug and degradation products carry the notations degradation product 01, degradation product 02 and AN in accordance with the sequence in which the peaks appeared from left to right on HPLC chromatogram (mixture of stressed sample) (Figure1) High performance liquid chromatography very useful and easy available category of separation technique based upon well-established principles of liquid chromatography, which utilized Reversed Phase High Performance liquid chromatography method for mobile phase. These mobile phase gradient to affect dramatic increase in resolution, sensitivity and speed of analysis. Method validation includes several parameters have been performed systematically on HPLC instruments as per ICH guidelines Q2A[24-25]. HPLC results show are tabled in (Table 2, Table 3).

3.2 HPLC-MS/MS studies on forced decomposition samples of AN.

The degradation product in chromatogram were present in the total- ion chromatogram, recorded by using the method. the mass spectrum of the drug. The observed m/z values for ion peak $[M+H]^+$ and considerable fragments of the drug is 220.39 and its degradation product is 237.8,152.8,219.8. of these, peak I and peck II(degradation product 02, degradation product 01) was found to be degraded products and peak III is of AN . According to the m/z values and fragmentation pattern, the structures for degradation products could be proposed The spectrum of the above given data is shown in Figure 2.

3.3 IR Spectrum AND ^{1} H NMR spectra Data

Degradation Product 01

IR (KBr),v (cm^{-1}: 1684(C=O stretching) and 2982(COO-H stretching). The spectrum of the above given data is shown in figure 3,and supporting material .

Degradation Product 02

IR (KBr),v (cm^{-1}: CO-NH stretching),2927(COO-H stretching) and 3318(NH stretching). The spectrum of the above given data is shown in figure 3,and supporting material.

The obtained IR spectrum of degradation product are shown in figure 3, which also facilitate for the confirmation of degradant i.e , Degradation product 01 =4-MBA. Degradation product 02 =NAG. The melting point, IR spectrum, Mass data, appearance and physical state are show are tabled in Table 4.

^{1} H NMR spectra Data

Degradation Product 01

^{1}H NMR, 400 MHz, δ(ppm):3.829(s,3H of CH_3),7.03(d,2H of CH),7.98(d,2H of CH),12.627(s,1H of OH).The spectrum of the above given data is shown in figure 3,and supporting material .

Degradation Product 02

^{1} H NMR, 400 MHz, δ(ppm):1.74(t,2H of CH_2),2.50(p,2H of CH_2), 3.25(t,2H of CH_2), 3.805(s,3H of CH_3), 7.03(d,2H of CH),7.90(d,2H of CH),8.332(s,1H of NH),12.627(s,1H of OH).The spectrum of the above given data is shown in figure 3, show are tabled in Table 5 and supporting material.

We had also check the thermal and UV stress conditions also had been checked for degradation

study of AN. No degradation was observed by thermal and UV Stress condition data mentioned in supporting material.

3.4 Mechanism explanation to the origin of the degradation Products to establish degradation pathways of the drug and over degradation of degraded product.

The accurate mass of degradation product 01 product m/z 152.8(+ESI) was 67.0 lower than the drug 219.8 The accurate mass of degradation product 02 m/z 237.8 (+ESI) was 18.0 higher than the drug 219.8. This clearly indicated addition of H_2O molecule to the drug. As the application of nitrogen rule and proposed the presence of even nitrogen ie, $C_{12}H_{15}NO_4$ (Theoretical mass 237.10) as the most probable molecular formula. Degradation product 02 is generated from the drug by simple amide hydrolysis in basic condition. Since degradation product 02 was formed in basic. It was observed that AN undergoes base hydrolysis. Degradation study of product 01 using aqueous 1N NaOH solution .The mechanism is operated by hydrolysis. The hydroxyl group ($^-$OH) of NaOH attacks an electrophilic carbon of >C=O group which an removal of tertiary nitrogen gives 4-MBA and PD as by products. Degradation study of product 02 using aqueous 1N NaOH solution . The mechanism is operated by hydrolysis. The hydroxyl group ($^-$OH) of NaOH attacks an electrophilic carbon of >N-C=O which as rearrangement gives carbonial. This carbonial abstract proton from water to give NAG. The established over degradation of NAG to 4-MBA was also observed in alkali condition. Degradation pathway of AN is shown in figure 4. The isolated degradation products are subjected to Mass studies to obtain their accurate mass fragment patterns. Which help to design tentative structure of degradation products which are confirmed by ^{1}H NMR and IR on the basis of results complete degradation path for AN has been established. We have also performed.

4. CONCLUSION

It is concluded that AN degrade in to its precursor by alkali degradation condition. We had identified and characterized these degradation products by IR,LC-MS, ^{1}H NMR and also analyzed by newly developed and validated HPLC method. This HPLC method is useful for routine analysis of AN and its related substance. The alkali degradation of AN is described and to established degradation pathways of the drug. The degraded product can used for the preparation of AN and similar structure products. Indirectly, the study highlights the benefit of the use of ICH stress testing approach in the establishment of complete degradation pathways of drugs. It is hoped that this report on stability indicating method and degradation route of AN would be helpful for the multiple generic producers of the drug throughout the world by preserving them from repetition of same studies.

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ABBREVIATIONS

AN: Aniracetam;
4-MBA: 4-Methoxy benzoic acid (Degradation Product 01);
NAG: N-anisoyl GABA(Degradation Product 02);
PD: 2-pyrrolidinone;
API: Active pharmaceutical ingredient;
ICH: International conference of Harmonization;
LOQ: limit of Quantification;
LOD: limit of detection;
RT: Room temperature;
t_R : Retention time;
Rt_R: Relative time;
R_S : Resolution;
T_f: Tailing factor;
R^2: Coefficient of determination;
ppm: Parts per million
RSD: Relative Standard Deviation
SD: Standard Deviation
mg: milli gram
mL: millilitre
nm: nano- meter
µg : micro gram
mix.: Mixture
Hrs: hours
GABA **:** Gamma-Amino Butyric Acid

Table 1.
Summary of system suitability test with linearity, t_R(retention time), Coefficient of determination (R^2), Y-intercept, and LOQ data for proposed Method validation (% Purity) of Aniracetam

Substance	Range (µg/mL)	t_R,(retention time)	Rt_R, (relative time)	Coefficient of determination (R^2)	Y-intercept	LOQ	LOD	SLOPE
AN(%Purity)	1 to 300	6.51	1	0.999	2775.×+41.82	0.019	0.0065	34648.73
Degradation Product 01	-	8.44	0.84	Resolution (Rs) 10.89	-	-	-	-
Degradation Product 02	-	5.13	0.51	Resolution (Rs) 19.47	-	-	-	-

Table 2
Degradation of analyte applying forced degradation.

Stress condition	Acidic	Alkali	Oxidation
% Degradation	No Degradation	100%	No Degradation

Table 3
Summary of Accuracy, Recovery, Robustness and Stability test study data for proposed Method validation (% Purity) of AN

Parameter	Condition	Data	AN
Flow rate	0.9 mL/min	% RSD	1.82
	1.1 mL/min	% RSD	1.58
pH in Mobile Phase	pH (1.9)	% RSD	0.50
	pH (2.1)	% RSD	0.46
Column tempeature	25^{0}C	% RSD	1.41
	35^{0}C	% RSD	0.94
Stability at ^{0}C	at RT	% RSD	1.82
	24hrs		
	48 hrs		2.60
	At 5^{0}C		0.78
	24hrs		
	48 hrs		1.20
Accuracy on level	Data		
75 %	% Recovery		97.65
	% RSD		0.966
100 %	% Recovery		99.36
	% RSD		0.983
125 %	% Recovery		100.20
	% RSD		0.991
LOQ	% Recovery		96.05
	% RSD		0.951
Precision study	Method	% RSD	0.69 0.92

Table 4
Summary of forced degradation study data for proposed method of AN and purity, melting range FT-IR and Mass spectra data for degradation product 01(MBA), degradation product 02(NAG) Copies of FT-IR and Mass spectra chromatography are presented in sublimity figure.

Compound	Purity (%)	Melting range (^{0}C)	IR (cm^{-1})	MS m/z	Appearance and Physical State
Degradation product 01(MBA)	99.9	182-185	1022(CH bending),1257(C-O stretching), 1424(CH_2 bending),1684(C=O stretching),2982(COO-H stretching,very broad)	152.8(+ESI)	Crystalline powder and solid
Degradation product 02(NAG)	99.4	180-183	1179(CH bending),1256(C-O stretching), 1430(CH_2 bending),1706(CO-NH stretching),2927(COO-H stretching), 3318(NH stretching,spike)	237.8 (+ESI)	Crystalline powder and solid

Table 5
Summary of forced degradation study data for proposed method of AN and ^{1}H NMR spectra data for degradation product 01(MBA), degradation product 02(NAG)

Assignments: s:singlet,d:doublet,t:triplet,p:pentet
Numbering of All compounds Shown in figure 3.
Copies of ^{1}H NMR spectra chromatography are presented in sublimity figure.

Position	^{1}H NMR - δ(ppm)	
	degradation product 01 (MBA)	degradation product 02(NAG)
1	3.829(s,3H of CH_3)	3.805(s,3H of CH_3)
2	7.0(d,2H of CH, 7.041,7.035,7.029,7.017,7.012,7.005	7.0(d,2H of CH, 7.031,6.995,6.977)
3	7.9(d,2H of CH,7.915,7.908,7.903,7.891,7.886,7.879)	7.9(d,2H of CH, 7.907,7.887,7.830,7.812)
4	12.627(s,1H of OH)	8.332(s,1H of NH)
5	-	3.2(t,2H of CH_2, 3.340,3.254)
6	-	1.740(p,2H of CH_2)
7	-	2.5(t,2H of CH_2, 2.676,2.509,2.336,2.267)
8	-	12.093(s,1H of OH)

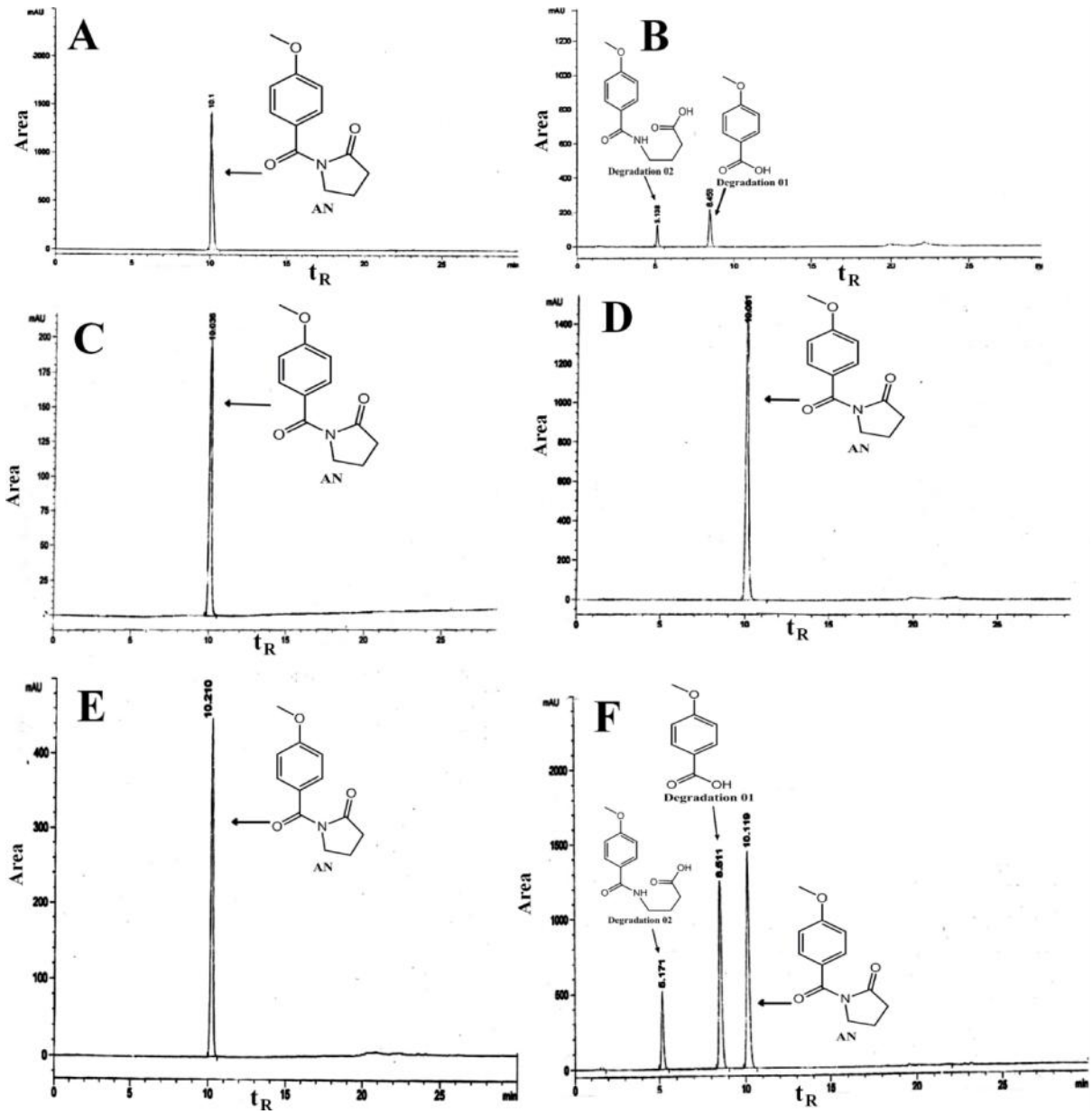

Figure 1

Chromatograph Forced acid, alkali, oxidative , system suitability solution , test solution spiked with degradation products for HPLC method development and validation. chromatograph of (A) acidic degradation,(B) alkali degradation,(C) oxidative degradation,(D) system suitability solution,(E) test solution(F) test solution spiked with degradation products.

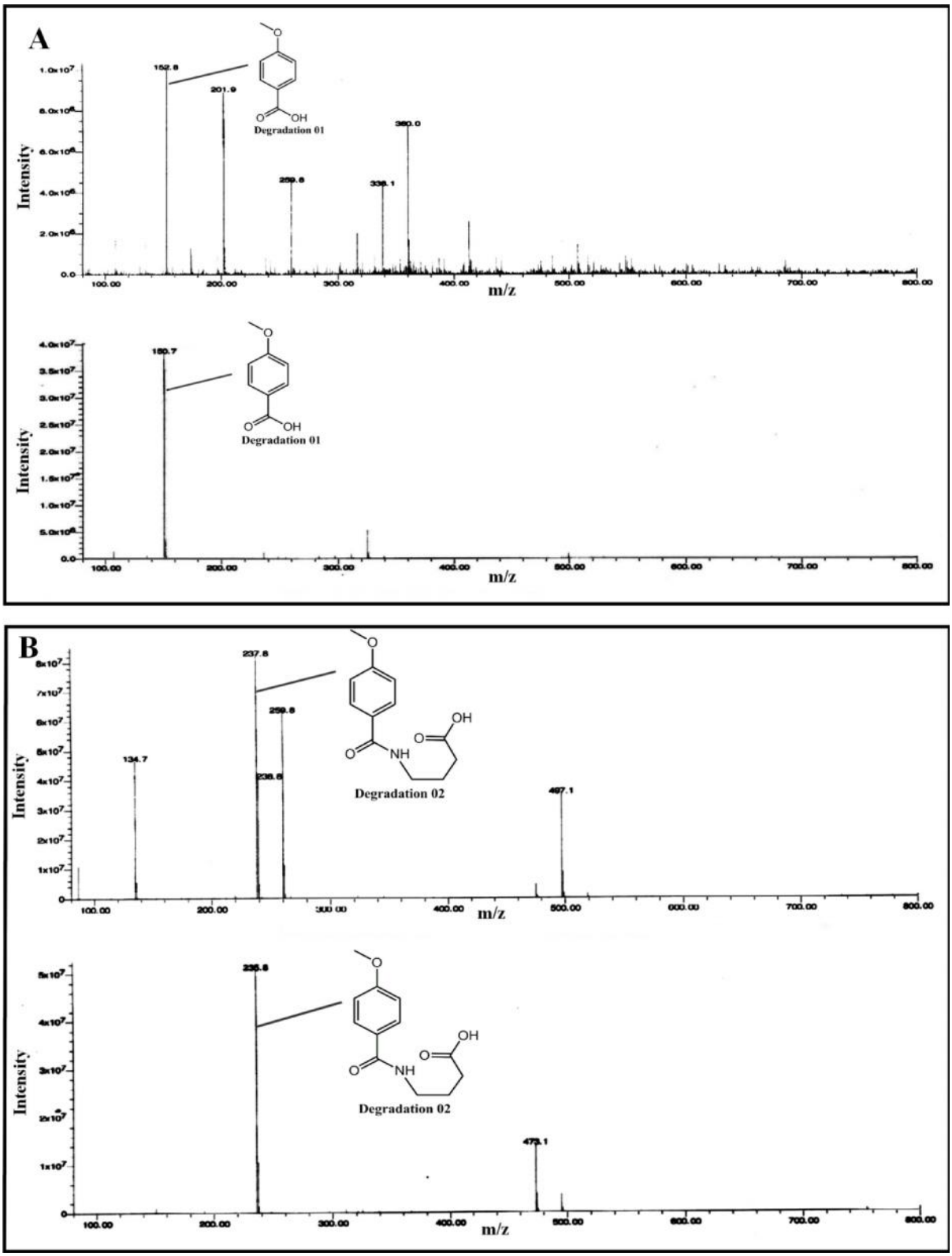

Figure 2
Comparison of LC/MS spectra structures of (A)Degradation Product 01(MBA), (B) Degradation Product 02(NAG).

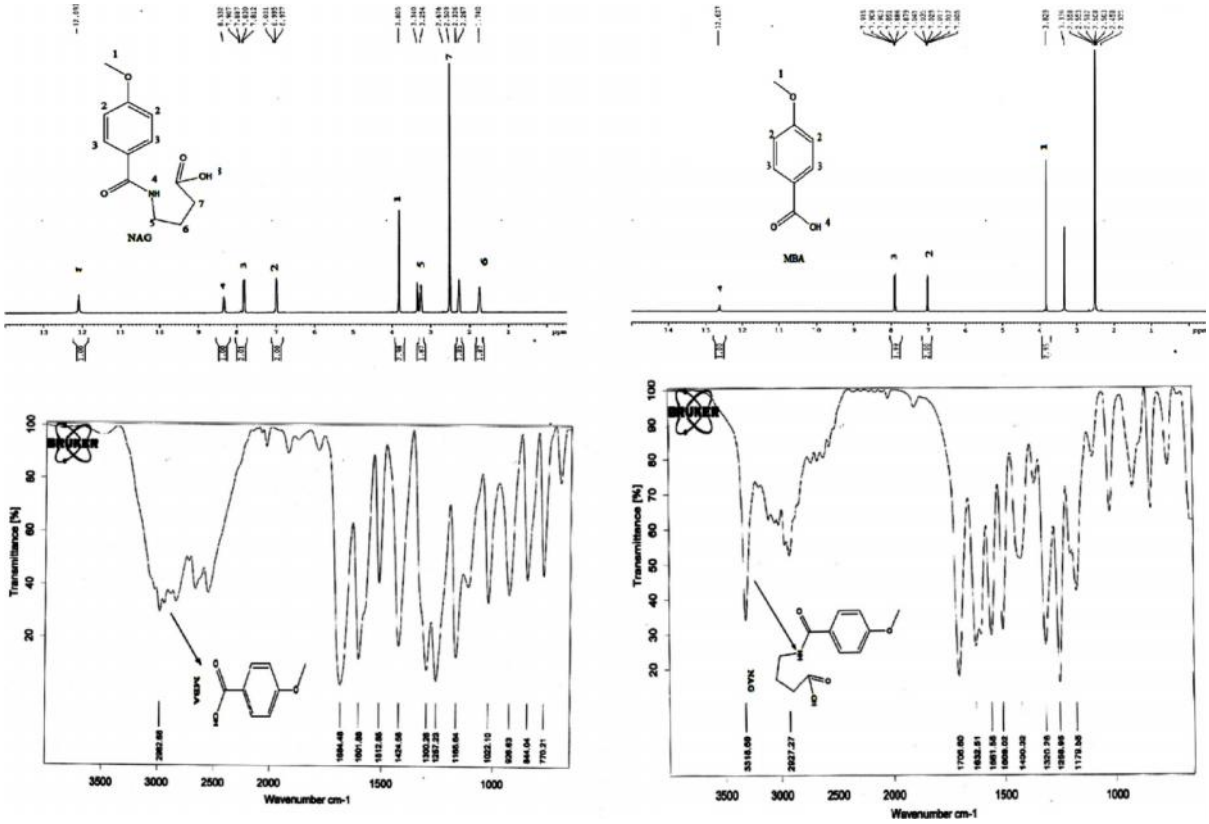

Figure 3
Comparison of ^{1}H NMR and IR Spectra Structures of degradation product 01(MBA), degradation product 02(NAG).

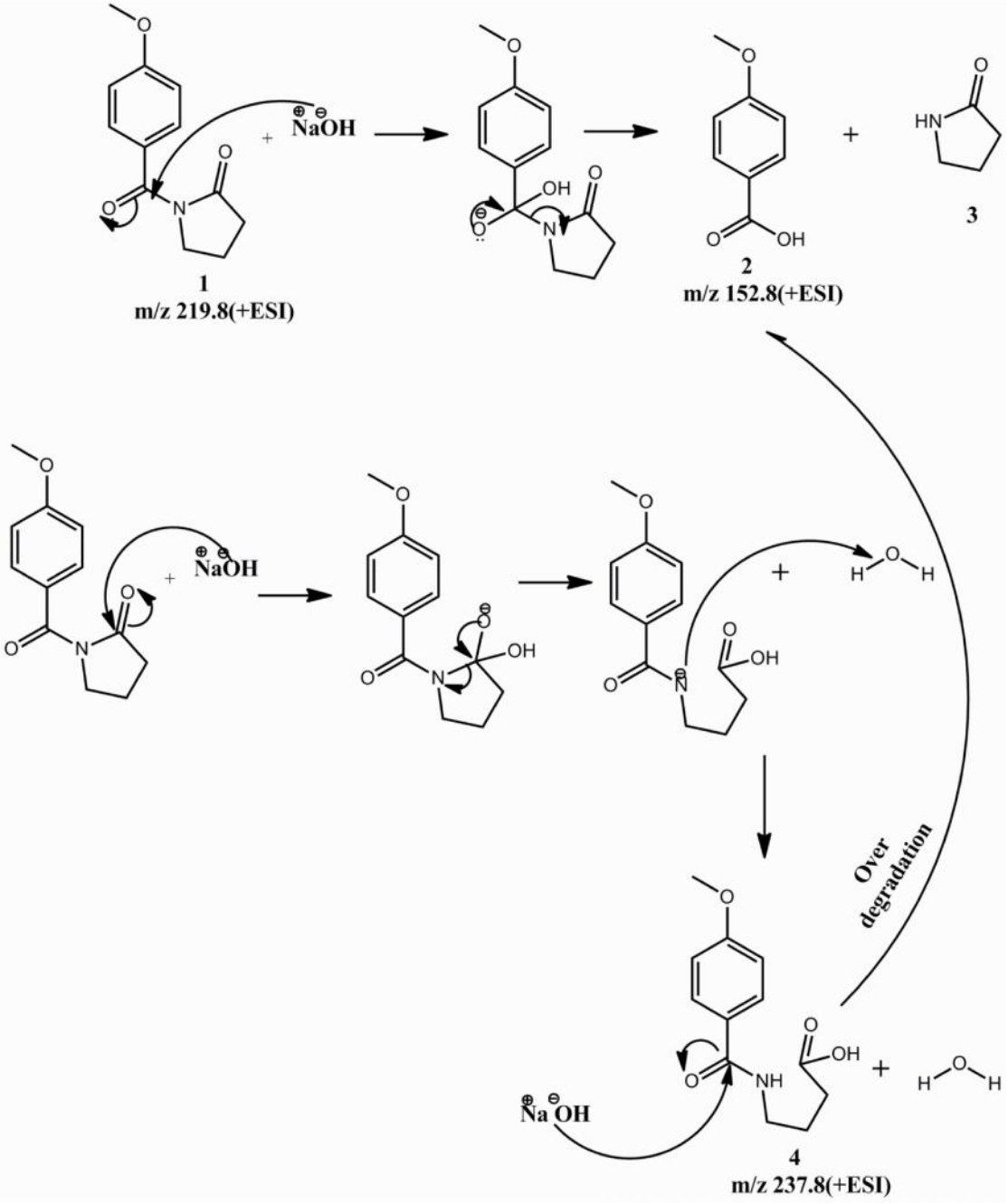

Figure 4
Mechanism explanation to the origin of the degradation products, to establish degradation pathways of the drug and over degradation of degraded product. pathways reactions scheme structured numbering. (1)AN, (2) 4-MBA, (3)PD, (4) NAG.

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