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"Method Development and Validation of Stability-Indicating RP-HPLC Method For Zonisamide and Cilostazol in Synthetic Mixture"

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ABSTRACT

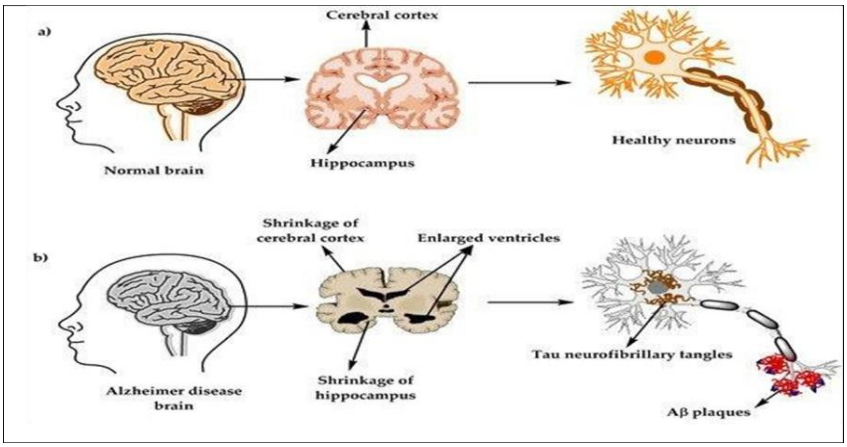
A simple, rapid, economical, precise, and accurate Stability indicating RP-HPLC method for simultaneous estimation of Zonisamide and Cilostazole synthetic mixture. Has been developed. The method has shown adequate separation for Zonisamide and Cilostazole from their degradation products. The separation was achieved by hyper sail BDS C 18 (250mm x 4.6 mm, 5 µm) column and Buffer (Potassium Phosphate, pH 4.0): Methanol (60:40) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 236 nm. These drugs were subjected to hydrolysis, oxidation, photolysis, and thermal to apply stress conditions.

Key Words: Zonisamide, Cilostazol, Stability indicating RP-HPLC Method, HPLC method, ICH Q2 (R1) guidelines.

INTRODUCTION

Definition of Alzheimer's disease. [01-03]

Alzheimer's disease is a progressive neurologic disorder that causes the brain to shrink (atrophy) and brain cells to die. Alzheimer's disease is the most common cause of dementia a continuous decline in thinking, behavioral and social skills that affect a person's ability to function independently. Approximately 5.8 million people in the United States age 65 and older live with Alzheimer's disease. Of those, 80% are 75 years old and older. Out of the approximately 50 million people worldwide with dementia, between 60% and 70% areestimated to have Alzheimer's disease. The early signs of the disease include forgetting recent events or conversations. As the disease progresses, a person with Alzheimer's disease will develop severe memory impairment and lose the ability to carry out everydaytasks. Medications may temporarily improve or slow the progression of symptoms. Thesetreatments can sometimes help people with Alzheimer's disease maximize function and maintain independence for a time.



Introduction To Stress Degradation Study[22]

According to an FDA guidance document, a stability-indicating method (SIM) is defined as "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substances and drug product". A stability-indicating method accurately measures the active ingredients, without interference from degradation products, process excipients, impurities, or other potential impurities. The ICH guidelines Q1A(R2) (2003) elaborate on stability testing of APIs "s and drug products to determine storage conditions, retest period, maximum expiring dating period of drug products, correctpackaging to protect the product, and transport conditions.

Forced degradation study:

The major routes of degradation of any drug substance include hydrolysis, oxidation,heat, and photolysis.

RESULT AND DISCUSSION

Drug Identification

The identification of drugs was carried out by performing melting point determination, solubility study, and taking IR and UV spectra as preliminary work which showed no following results.

Melting Point Determination

The melting point of Zonisamide and Cilostazole have been determined using Capillary Method.

Table : Melting Point of Drugs

Drug Name	Reported (°C)	Observed (°C)
Zonisamide	161-163^{o}C	163 ^{o}C
Cilostazol	159^{o}C	158^{o}C

Observation: The melting point of Zonisamide and Cilostazole was found to be inthe range of acceptance criteria as shown in the above table.

- **Solubility Study**

Table : Solubility Data of Zonisamide and Cilostazole

Solvent	Solubility	
	Zonisamide	Cilostazole
Water	Very slightly soluble	Slightly soluble
Acetonitrile	Soluble	Freely Soluble
Methanol	Freely soluble	Soluble

Identification by IR Spectroscopy

Zonisamide

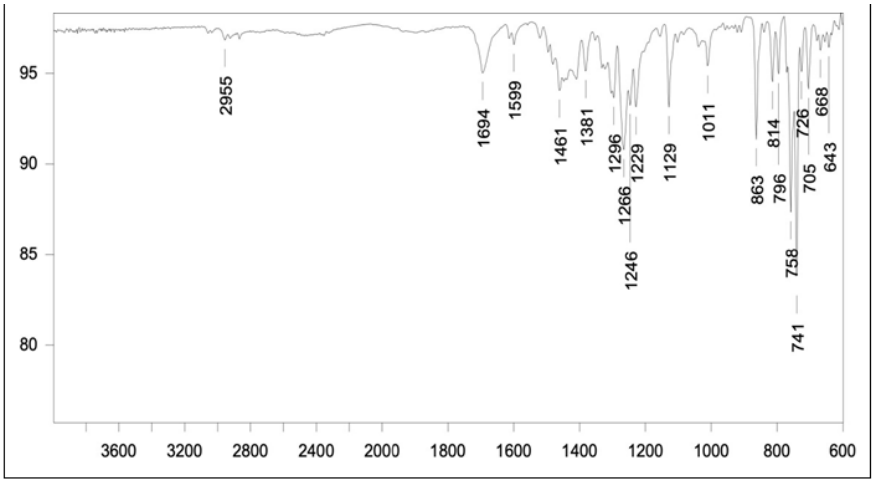


Table : IR Interpretation of Zonisamide

Functional Group	Frequency (cm^{-1})
N-H stretching	2955
C=C stretching	1461-1694
S=O stretching	1381

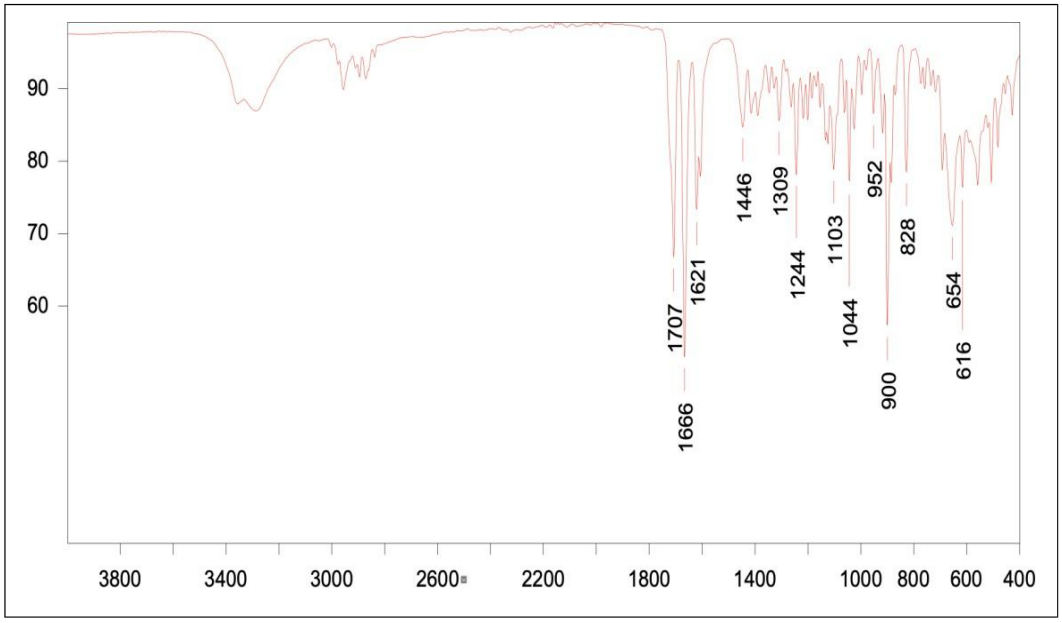


Fig : IR Spectra of Sample Cilostazole Table : IR Interpretation of Cilostazole

Functional Group	Frequency (cm^{-1})
N-H stretching	3200
C=C stretching	1446-1621
C-N stretching	1044=1244

METHOD DEVELOPMENT

Selection and Detection of Wavelength

The sensitivity of the HPLC method that uses UV detection depends upon the proper selection of detection wavelength. An ideal wavelength is the one that gives a good response to the drugs that are to be detected. At 236 nm both drugs give a good response and also degrade product detected at this wavelength. So 236 nm was selected for stability-indicating simultaneous estimation of Zonisamide and Cilostazole in synthetic mixture.

Wavelength Determination

UV spectra of Zonisamide and Cilostazole were taken in Methanol and λmax was observed using Systronic 119

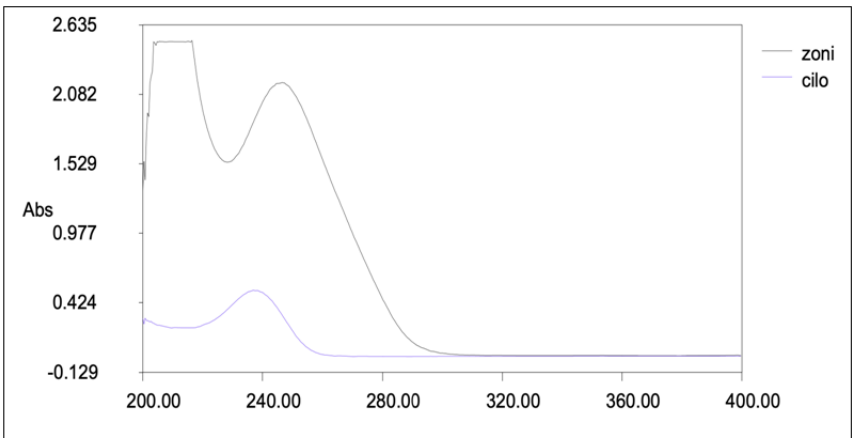


Fig Overlay UV Spectrum of Zonisamide and Cilostazole showing Selection of Wavelength Detection.

Observation

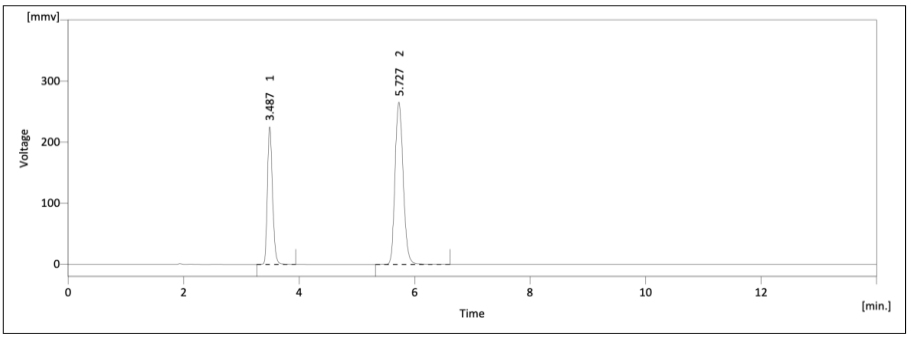
Zonisamide and Cilostazole both drug give higher absorbance at 236 nm.
So 236 nm has been selected as detection wavelength.
Note: All the chromatograms are shown at wavelength of 236 nm. So, 236 nm is shown in final optimized method.

FORCED DEGRADATION STUDY

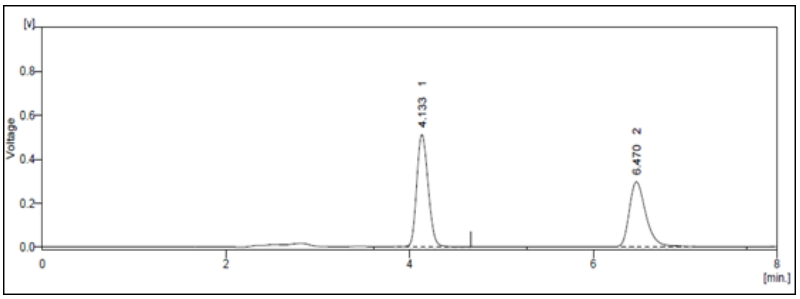
The sample was injected under various stress conditions. Here, chromatograms ofoptimized degrade conditionstion are shown.

Table : Different Degradation Conditions

Sr. No.	Stress Type	Stress Condition
1	Acid hydrolysis	0.1 N HCl at 70 °C for 4 hr.
2	Base hydrolysis	0.1 N NaOH 70 °C at for 3 hr.
3	Oxidative Degradation	3 % H_2O_2 at 70 °C for 2 hrs.
4	Thermal Degradation	105 °C for 3 hrs
5	Photolytic Degradation	UV for 8 hrs

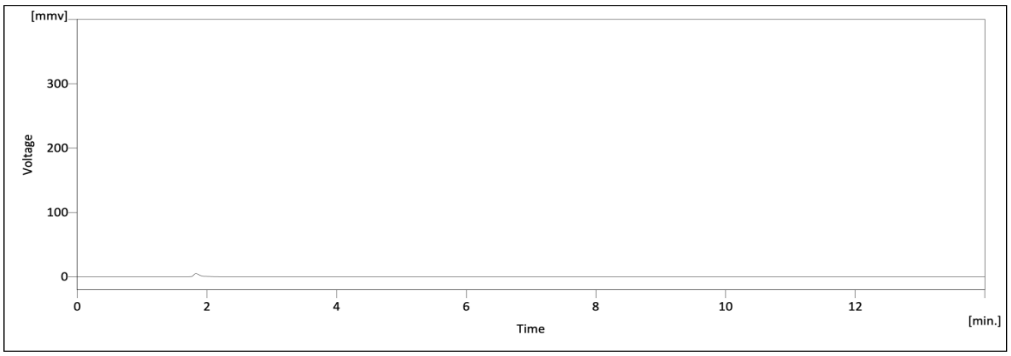


Zonisamide and Cilostazole Standard for stability

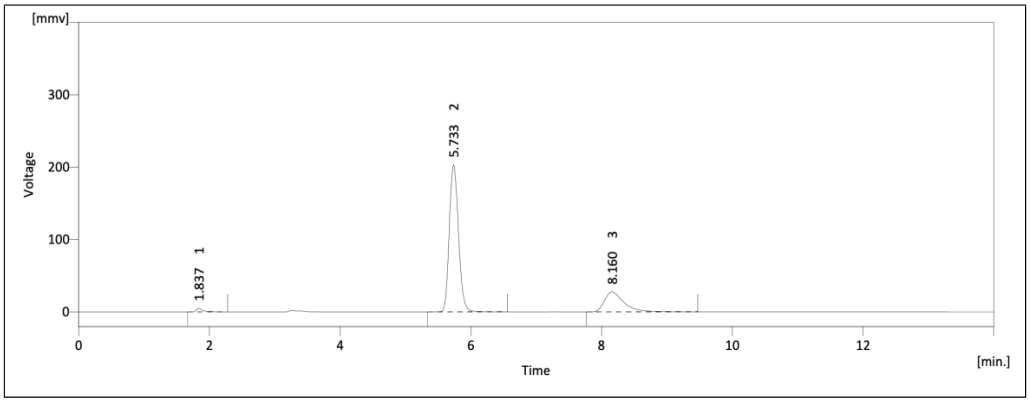


Zonisamide and Cilostazole Sample for Stability

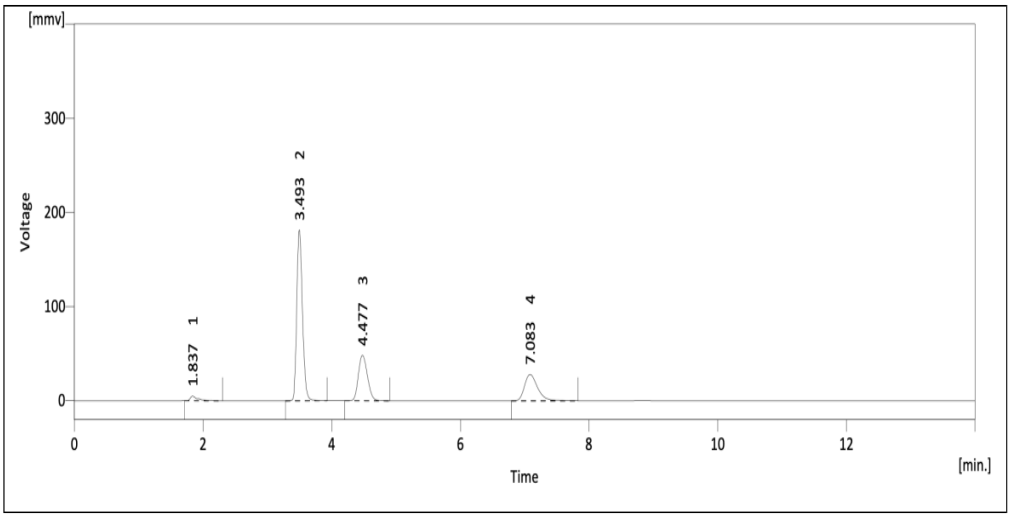
Chromatogram for Acid Degradation



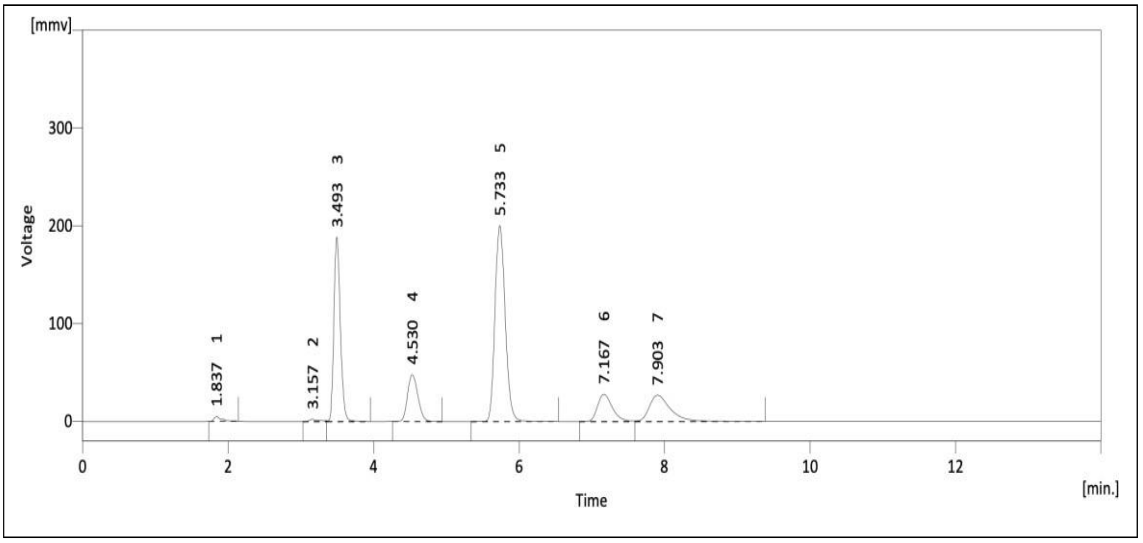
Zonisamide and Cilostazole Acid Degradation Blank



Zonisamide Acid Degradation Standard at 4 hrs.



Cilostazole Acid Degradation Standard at 4 hrs.

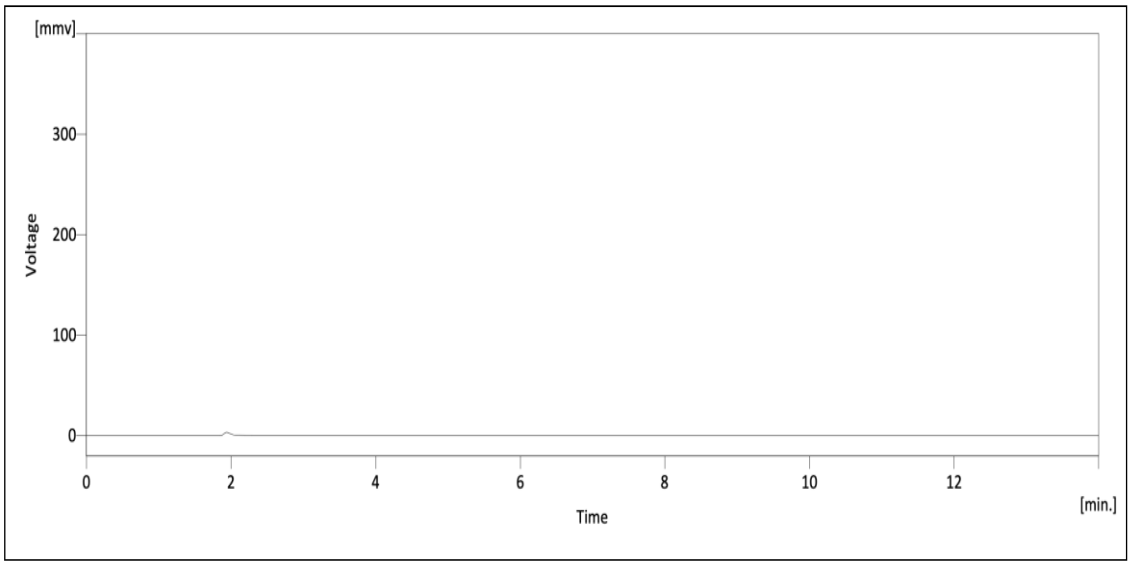


Zonisamide and Cilostazole Acid Degradation Sample at 4 hrs.

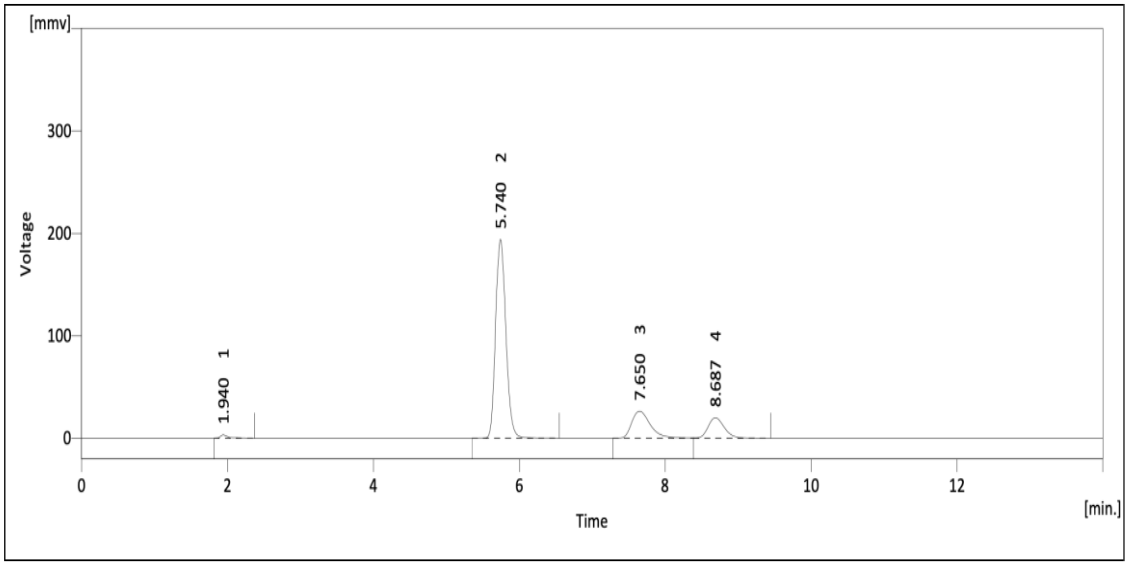
Observation

All solution has been reacted with 0.1 N HCl at 70 ^{o}C for 4 hours.
23.457% Zon and 19.28% Cilo have been degraded in a standard mixture.
24.563% Zon and 16.03% Cilo have been degraded in the sample mixture.

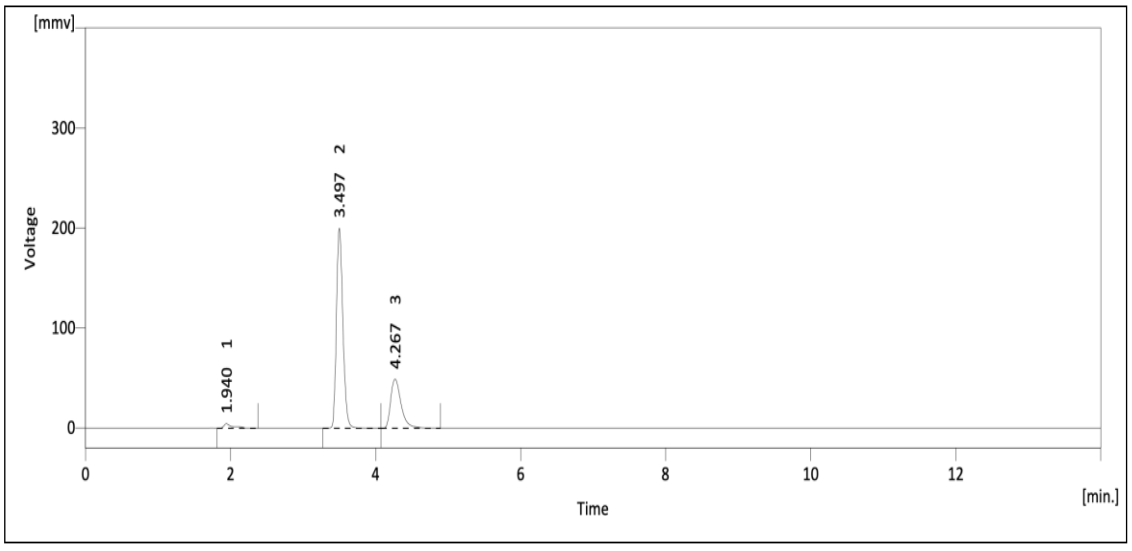
Chromatogram for Base Degradation



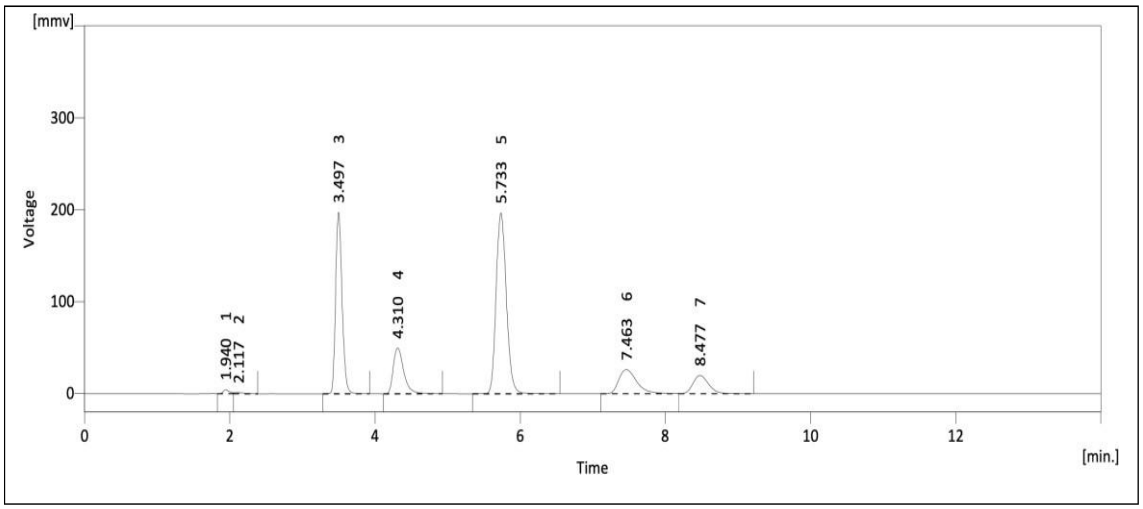
Zonisamide and Cilostazole Base Degradation Blank



Zonisamide Base Degradation at 3 hrs.



Cilostazole Base Degradation at 3 hrs.

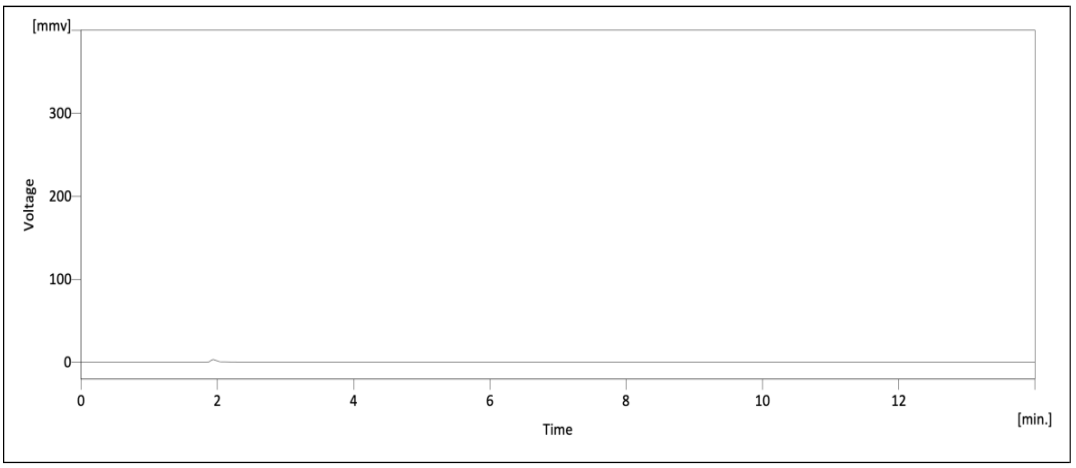


Zonisamide and Cilostazole Base Degradation Sample at 3 hrs.

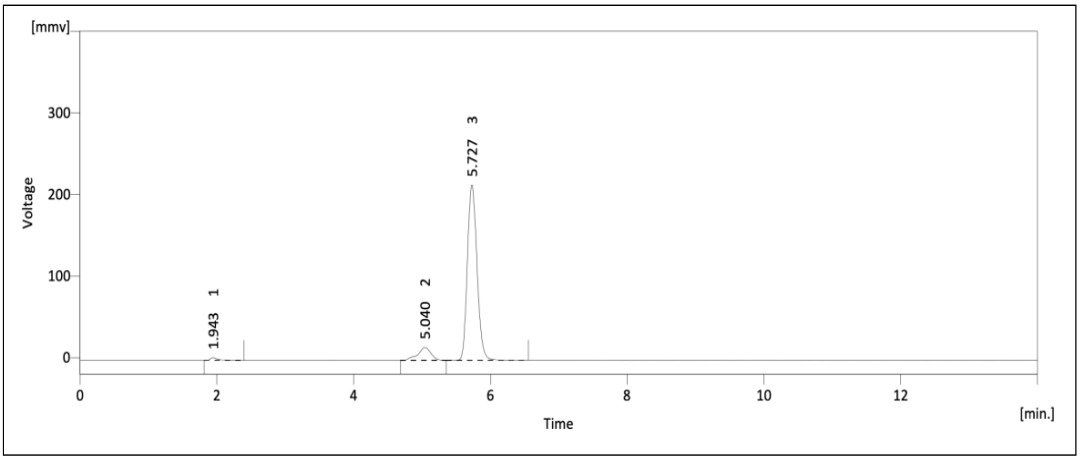
Observation

All solution has been reacted with 0.1N NaOH at 70 ^{o}C for 3 hours.
26.818% Zon and 11.03% Cilo have been degraded in the standard mixture.
25.955% Zon and 12.25% Cilo have been degraded in the sample mixture.

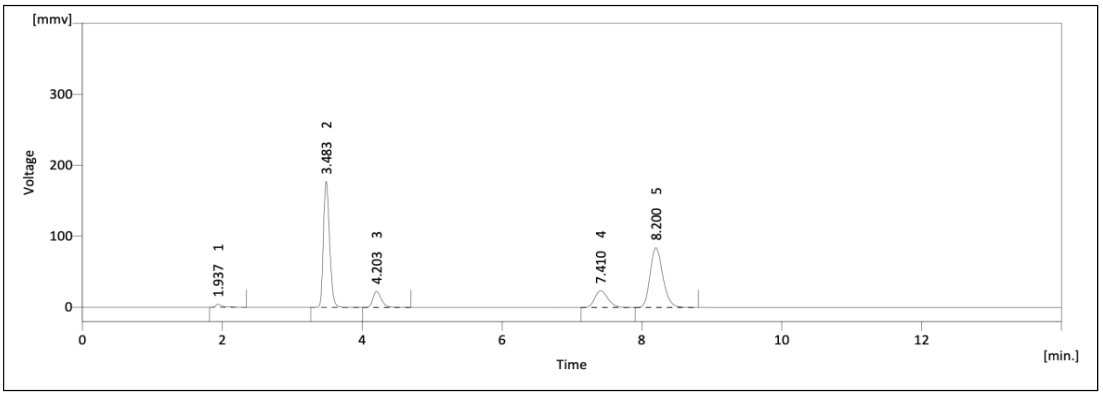
Chromatogram for Oxidative Degradation



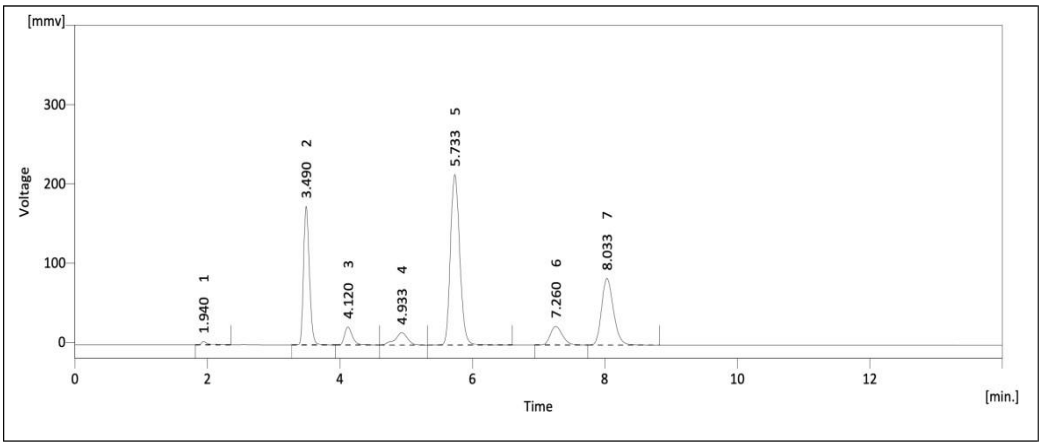
Zonisamide and Cilostazole Oxidation Degradation Blank



Zonisamide Oxidation Degradation at 4 hrs.



Cilostazole Oxidation Degradation at 4 hrs.



Zonisamide and Cilostazole Oxidation Degradation sample at 2 hrs.

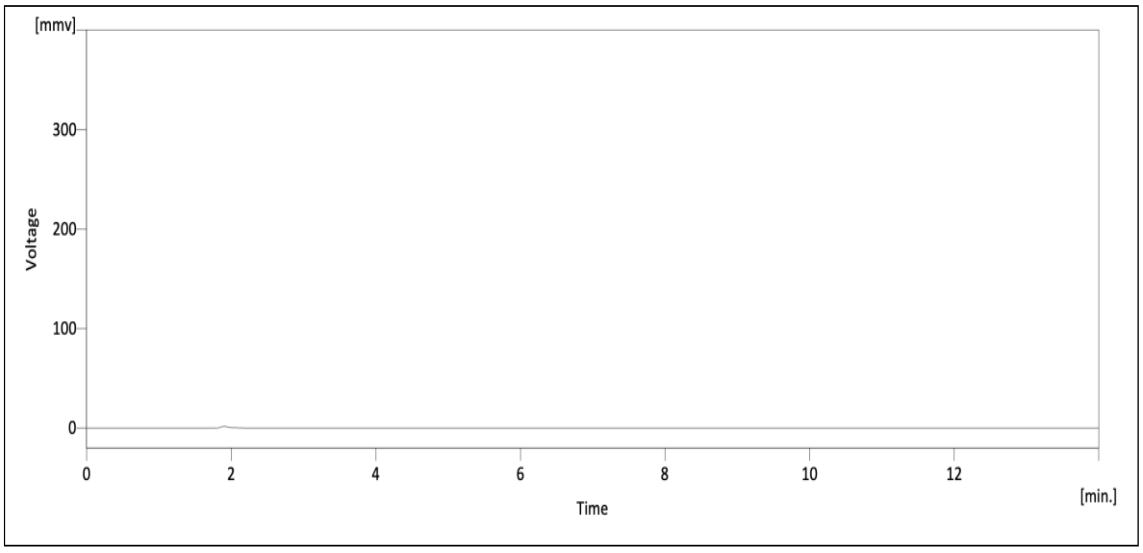
Observation

All solution has been reacted with 3% H_2O_2 for 3 hours at 70^oC

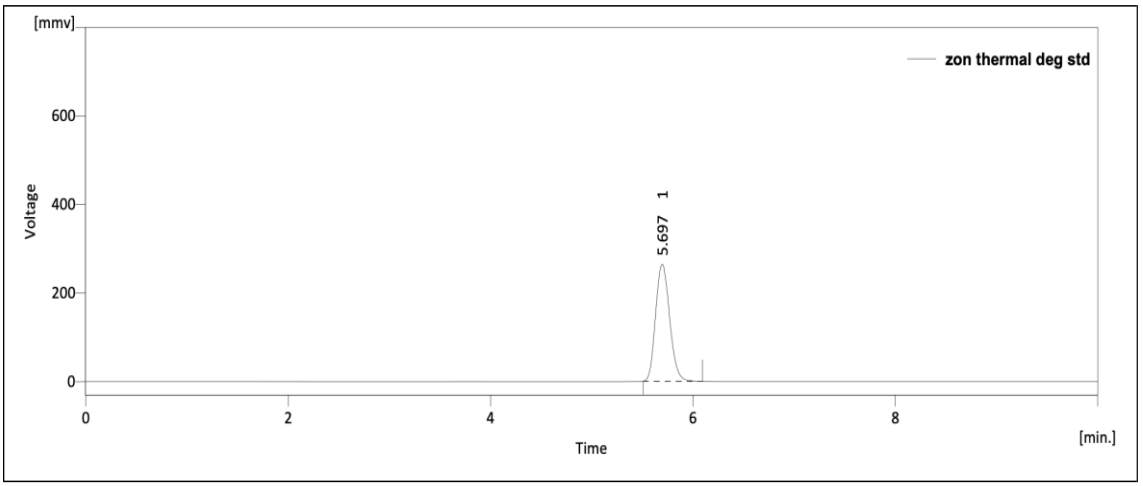
19.176% Zon and 21.38% Cilo have been degraded in the standard mixture.

19.055% Zon and 22.32% Cilo have been degraded in the sample mixture.

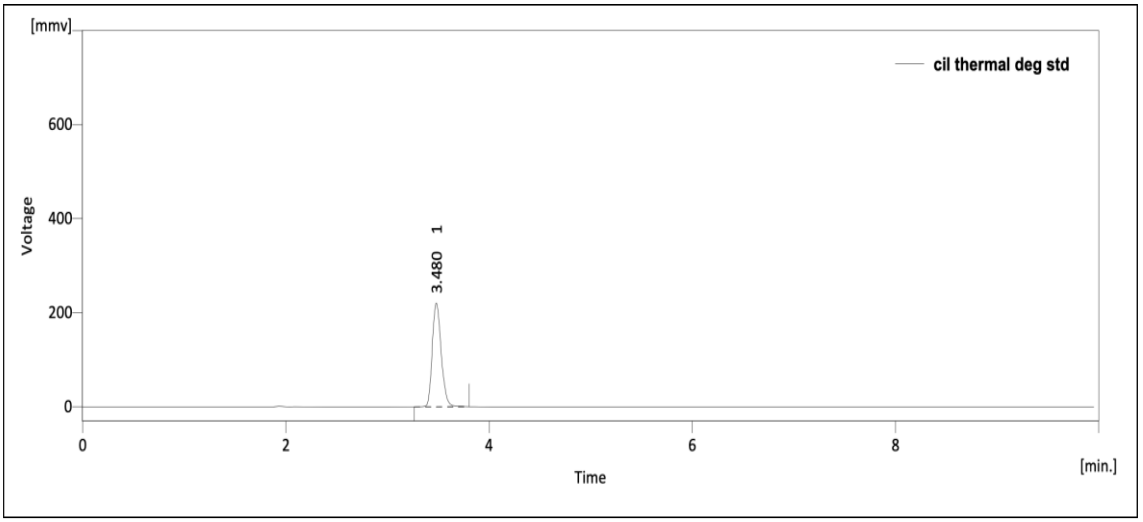
Chromatogram for Thermal degradation



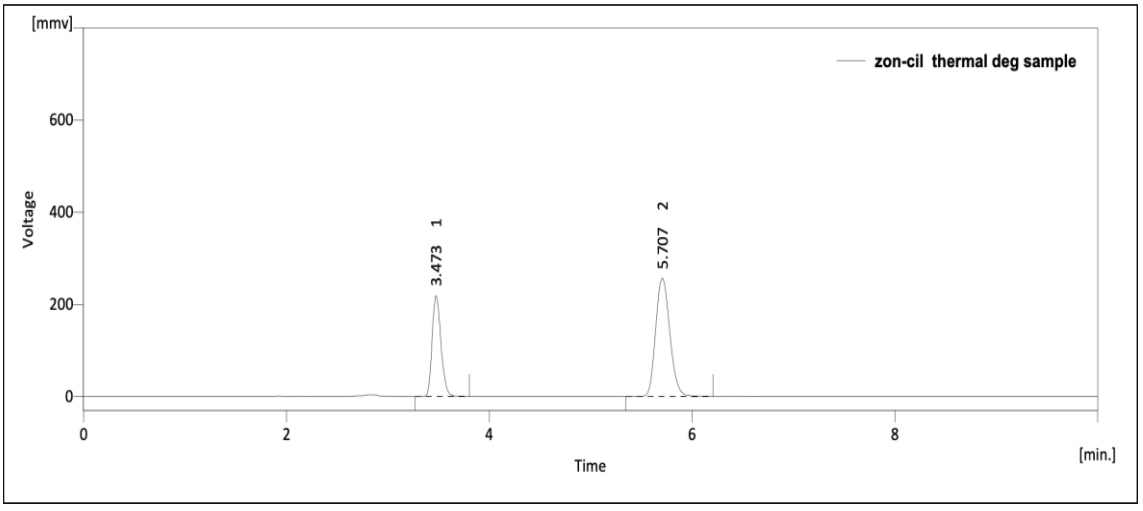
Zonisamide and Cilostazole Thermal Degradation Blank



Zonisamide Thermal Degradation at 3 hrs.



Cilostazole Thermal Degradation at 3 hrs.

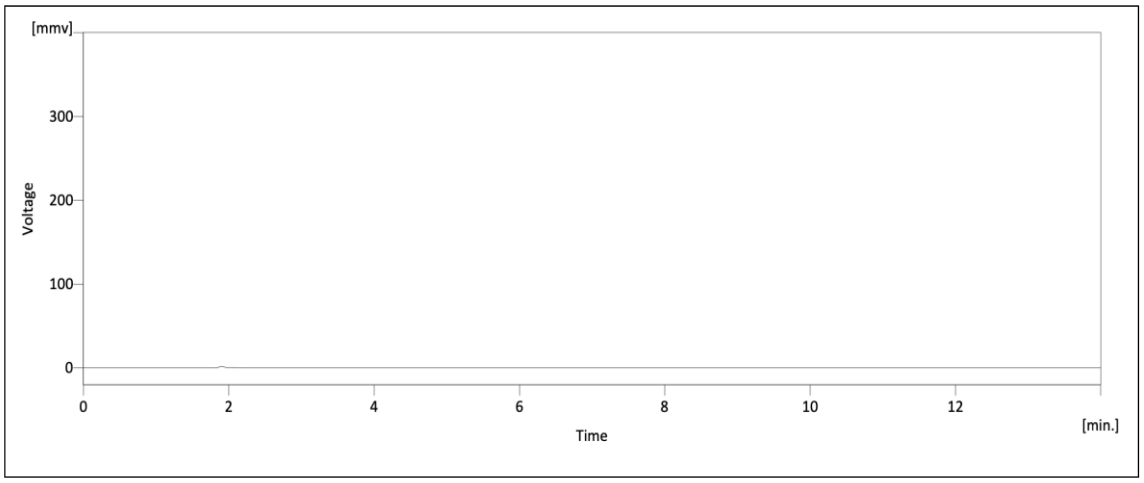


Zonisamide and Cilostazole Thermal Degradation sample at 3 hrs.

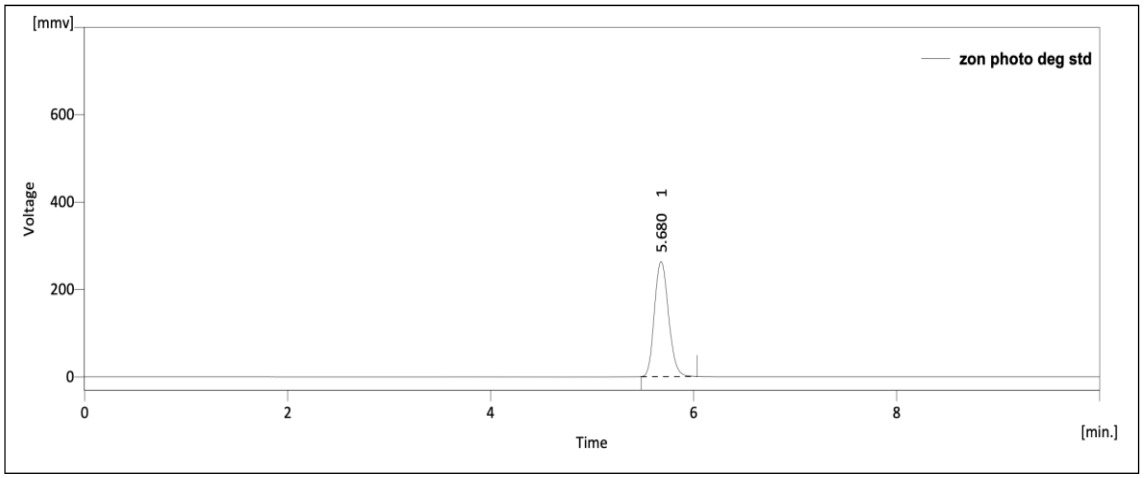
Observation

All solutions have been kept at 105^0C for 3 hours in a constant temperaturewater bath
1.957% Zon and 2.55% Cilo have been degraded in the standard mixture.
3.851% Zon and 3.17% Cilo have been degraded in the sample mixture.

Chromatogram for Photolytic Degradation

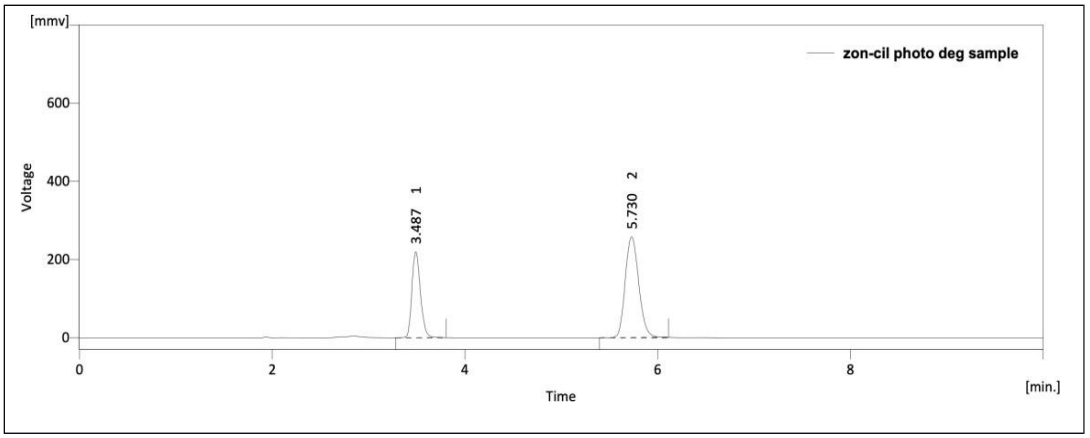


Zonisamide and Cilostazole Photo Degradation Blank



Zonisamide Photo Degradation at 8 hrs.

Fig 6. Cilostazole Photo Degradation at 8 hrs.



Zonisamide and Cilostazole Photo Degradation sample at 8 hrs.

Observation

All solutions have been kept under UV light for 8 hours
2.556% Zon and 1.31 % Cilo have been degraded in the standard mixture.
3.467% Zon and 2.45% Cilo have been degraded in the sample mixture.

Calculation for Stability

Table Zonisamide and Cilostazole Standard for Stability

Drugs	Area
Zonisamide	2609.246
Cilostazol	1387.644

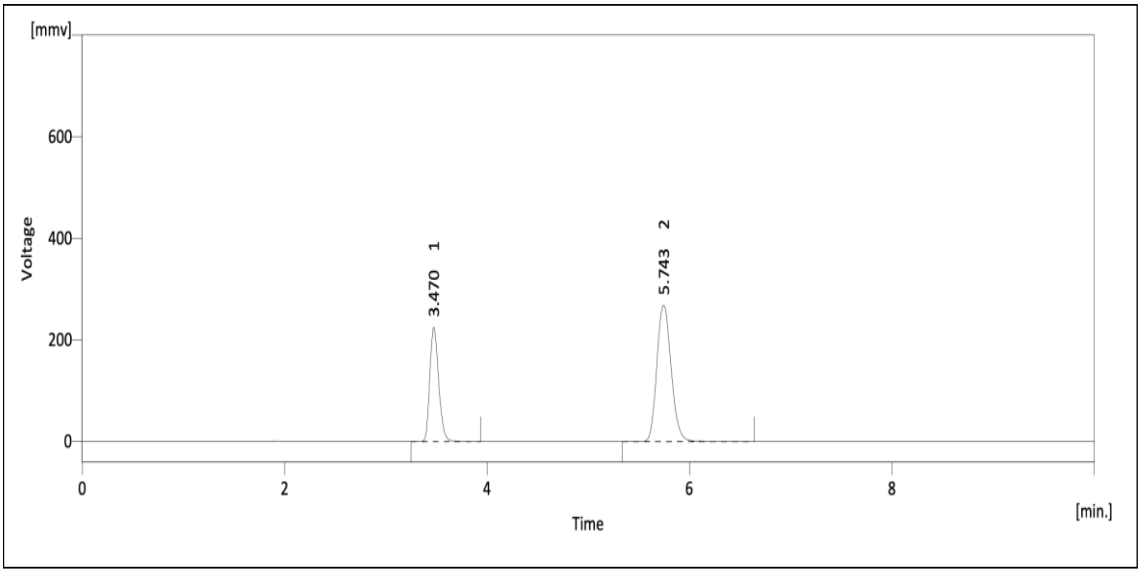
Zonisamide % Degradation

Zonisamide				
Parameter	**Standard**		**Sample**	
	Area	**%Degradation**	**Area**	**%Degradation**
Acid	1997.196	23.457	1968.338	24.563
Base	1909.487	26.818	1932.007	25.955
Thermal	2558.191	1.957	2508.756	3.851
Oxidation	2108.892	19.176	2112.049	19.055
Photo	2542.562	2.556	2518.784	3.467

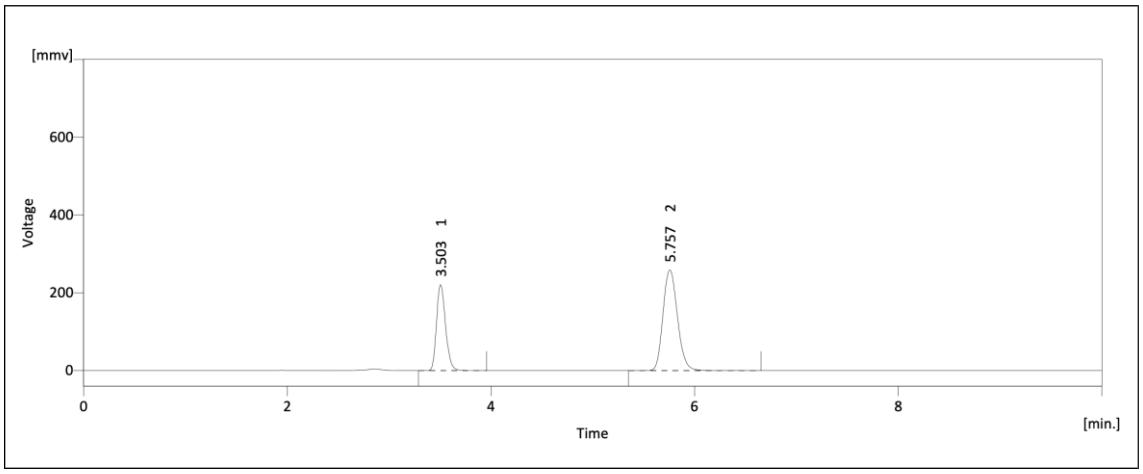
Cilostazole % Degradation

Cilostazole				
Parameter	**Standard**		**Sample**	
	Area	**%Degradation**	**Area**	**%Degradation**
Acid	1120.042	19.28	1165.267	16.03
Base	1234.564	11.03	1217.595	12.25
Thermal	1352.217	2.55	1343.593	3.17
Oxidation	1091.004	21.38	1077.94	22.32
Photo	1369.465	1.31	1353.703	2.45

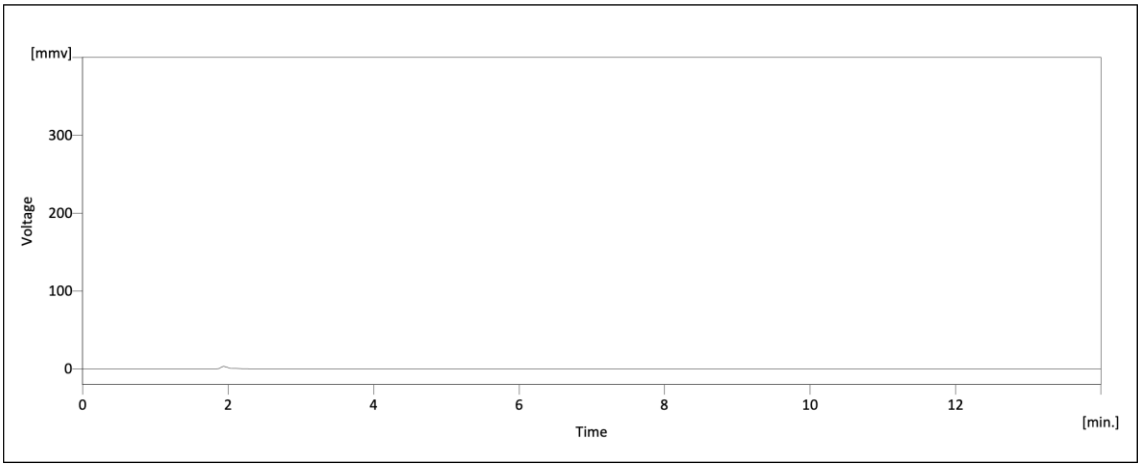
Method Validation
Specificity



Chromatogram of Zonisamide and Cilostazole Standard



Chromatogram of Zonisamide and Cilostazole Sample



Chromatogram of Zonisamide and Cilostazole Blank

Linearity and Range

Table Linearity Data for Zonisamide.

Sr. No	Concentration (µg/ml)	Area
1	12.5	1306.184
2	18.75	1929.307
3	25	2638.816
4	31.25	3150.319
5	37.5	3955.194
6	62.5	6595.817
7	75.0	7862.973

Linearity Data for Cilostazole

Sr. No	Concentration (µg/ml)	Area
1	12.5	681.427
2	18.75	1006.184
3	25	1375.925
4	31.25	1594.389
5	37.5	2061.696
6	62.5	3451.063
7	75.0	4114.731

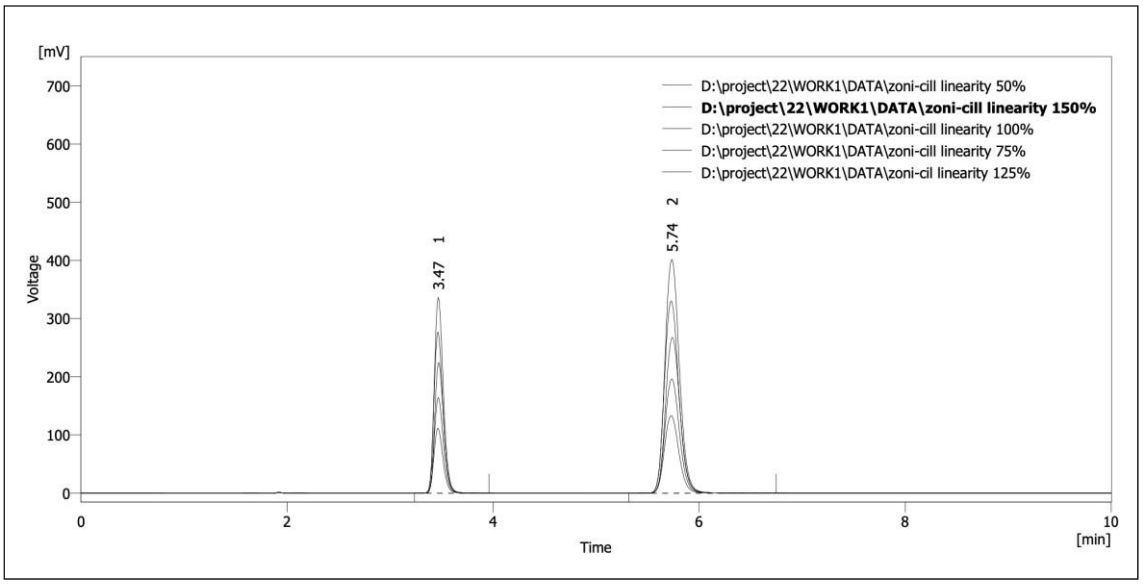
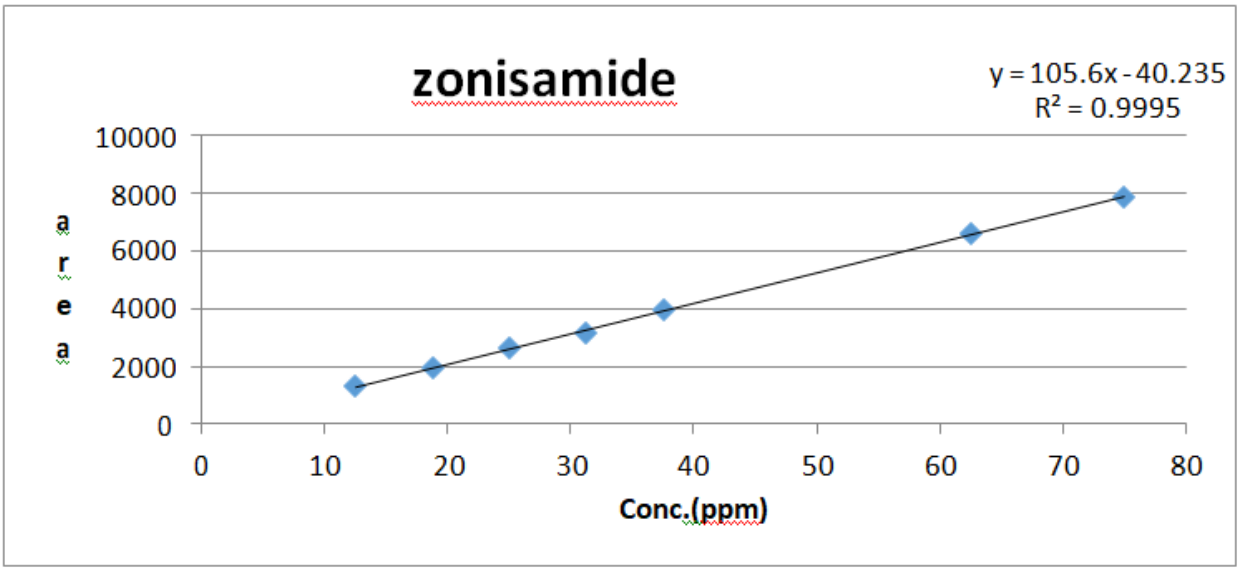
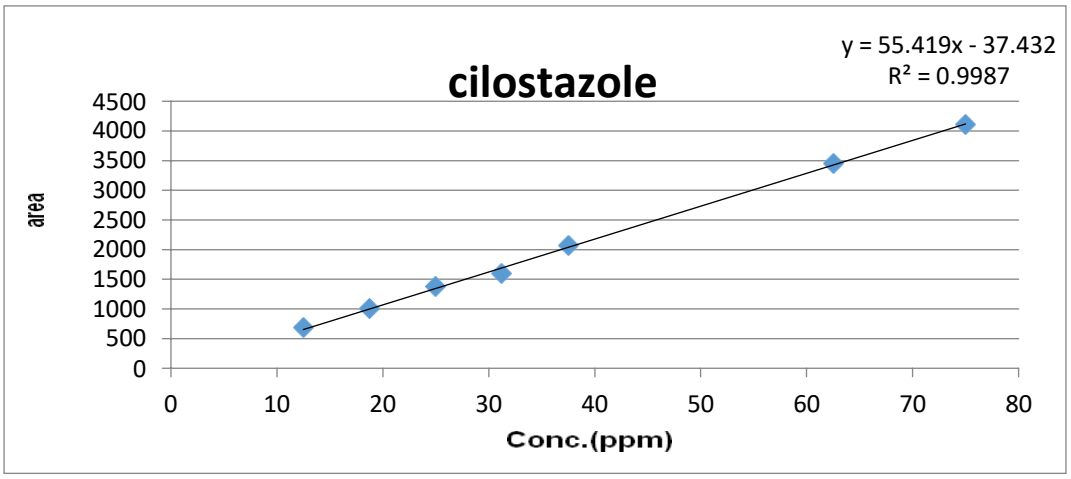


Fig. Overlay chromatogram of different concentrations of binary mixturesof Zonisamide and Cilostazol



Calibration Curve of Zonisamide (12.5-75.0 µg/ml).



Calibration Curve of Cilostazole (12.5-75.0 µg/ml).

Precision
Repeatability

Repeatability Data for Zonisamide.

Zonisamide				
Sr. No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
		2628.227		
1.	25.0	2633.585	2626.660±20.219	0.770
		2586.921		
		2644.136		
		2630.867		
		2636.221		

Repeatability data for Cilostazole

Cilostazole				
Sr No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	25.0	1365.556	1370.361 ±8.470	0.618
		1355.670		
		1375.926		
		1378.688		
		1371.784		
		1374.543		

Intraday precision

Intraday precision data for Estimation of Zonisamide and Cilostazol.

Sr. No.	Zonisamide			Cilostazole		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	12.5	1289.071 ± 8.346	0.647	12.75	675.412± 6.343	0.939
2	25.0	2609.733± 20.069	0.769	25.5	1366.585± 15.698	1.149
3	37.5	3914.149± 41.572	1.062	38.25	2043.460±19.374	0.948

Interday precision

Interday Precision data for Estimation of Zonisamide and Cilostazol.

Sr. No.	Zonisamide			Cilostazole		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	12.5	1291.889±11.293	0.874	12.5	672.262±9.972	1.483
2	25.0	2616.637.±33.942	1.297	25.0	1372.525±23.099	1.683
3	37.5	3917.968±23.514	0.600	37.5	2041.072±18.026	0.883

6.1.1. Accuracy

Recovery Data for Zonisamide.

Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	12.5	6.25	6.359	101.741	100.264 ± 1.655
2		12.5	6.25	6.155	98.475	
3		12.5	6.25	6.286	100.577	
4	100 %	12.5	12.5	12.677	101.414	100.508± 1.101
5		12.5	12.5	12.603	100.827	
6		12.5	12.5	12.410	99.283	
7	120 %	12.5	18.75	18.858	100.577	99.994 ± 0.644
8		12.5	18.75	18.619	99.303	
9		12.5	18.75	18.769	100.102	

Recovery Data for Cilostazol.

Sr. No.	Conc. Level (%)	Sample Amount	Amount Added	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	12.5	6.25	6.362	101.785	100.156 ± 1.635
2		12.5	6.25	6.157	98.516	
3		12.5	6.25	6.260	100.167	
4	100 %	12.5	12.5	12.600	100.798	101.036 ± 0.584
5		12.5	12.5	12.713	101.701	
6		12.5	12.5	12.576	100.610	
7	120 %	12.5	18.75	18.785	100.189	99.464 ± 0.812
8		12.5	18.75	18.485	98.586	
9		12.5	18.75	18.679	99.619	

LOD and LOQ

Limit of Detection

Limit of Detection Data for Zonisamide and Cilostazol.

Zonisamide	Cilostazole
LOD = 3.3 x (SD / Slope) = 3.3 x (58.562/260.700) = 0.741 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (50.111/133.900) = 1.235 µg/ml

Limit of Quantitation

Limit of Quantitation Data for Zonisamide and Cilostazole.

Zonisamide	Cilostazole
LOQ = 10 x (SD / Slope) = 10 x (58.562/260.700) = 2.246 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (50.111/133.900) = 3.742 µg/ml

Robustness

Robustness data for Zonisamide.

Sr No.	Area at Flow rate(- 0.2 ml/min)	Area at Flow rate(+ 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	2726.090	2562.392	2694.242	2514.690	2696.866	2557.256
2	2693.081	2536.563	2655.831	2482.026	2657.852	2540.851
3	2746.918	2593.915	2726.090	2536.020	2717.819	2591.321

% R.S.D	0.997	1.120	1.307	1.083	1.131	1.004

Robustness data for Cilostazole

Sr No.	Area at Flow rate(- 0.2 ml/min)	Area at Flow rate(+ 0.2 ml/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	1393.284	1310.780	1372.748	1293.065	1369.052	1304.322
2	1426.891	1344.275	1413.130	1316.762	1408.879	1341.581
3	1432.351	1352.503	1421.411	1322.290	1417.146	1351.151
% R.S.D	1.492	1.654	1.857	1.184	1.839	1.857

Analysis of marketed formulation by the developed method.

The applicability of the proposed method was tested by analyzing the commerciallyavailable Tablet formulation Entresto. The results are show.

Analysis of Marketed Formulation.

Synthetic mixture	Label claim		Assay (% of label claim*) Mean ± S. D.	
	Zonisamide	Cilostazole	% Zonisamide	% Cilostazole
	40mg	25mg	95.94 ± 1.07	98.31 1.28

METHOD VALIDATION SUMMARY

Summary of Ciloidation Parameters of Stability IndicatingRP-HPLC Method for Zonisamide and Cilostazol

Sr. No.	Parameter		Zonisamide	Cilostazol
1	Specificity		Specific	
2	Linearity & Range		12.5-75.0	12.5-75.0
3	Regression equation		y = 105.6x-40.235	y =55.419x-37.432
4	Correlation coefficient (r^2)		0.9995	0.9987
5	Precision (% RSD)	Repeatability	0.770	0.618

		Interday	0.600-1.297	0.883-1.683
		Intraday	0.647-1.062	0.939-1.149
6	Accuracy (% recovery)		99.994-100.508	99.464-101.036
7	Limit of Detection(LOD)		0.741 µg/ml	1.235 µg/ml
8	Limit of Quantification(LOQ)		2.246 µg/ml	3.742 µg/ml
9	Robustness (% RSD)		The system suitability parameters were found well within the acceptance criteriaas per system suitability	

CONCLUSION

- Stability Indicating RP-HPLC methods have been developed and validated for the determination of Zonisamide and Cilostazole in a synthetic mixture.
- The methods are found to be specific as there was no interference of any co- eluting impurities after the stress degradation study.
- The degraded products are well resolved, indicating the method can also be useful for the determination of degraded products. The proposed method is found to be simple, accurate, precise, and robust.
- Hence, it can be used successfully for the routine analysis of Zonisamide and Cilostazole in a synthetic mixture. and for analysis of stability samples obtained during accelerated stability study

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