STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND METHOD VALIDATION FOR CONTENT ESTIMATION OF DOXEPIN IN DOXEPIN CAPSULES

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***ABSTRACT:** A simple, precise, accurate method was developed for the simultaneous estimation of Doxepin in bulk and marketed pharmaceutical dosage form by RP-HPLC technique.Phosphate buffer and HPLC Grade methanol in the ratio of (60:40%v/v) used as mobile phase run through Zorbax SB Phenyl (150mm × 4.6mm, 5µm) column with a flow rate of 1.2ml/min. The temperature of the column oven was maintained at 50 °C. The Auto sampler temperature was maintained at 25°C. Wavelength was selected 254 nm. Stock and working solutions were prepared by using the diluents buffer and methanol in the ratio of 65:35(v/v). Runtime was fixed to 20 min.Doxepin containing E-Isomer and Z-Isomer eluted at 6.989 and 7.936min respectively with good resolution the plate count, tailing factor and all system suitability parameters are within ICH range. Doxepin was found to be linear low in concentration range of 49.87-149.62µg/ ml in the linearity study, regression equation and coefficient of correlation for Doxepin was found to be within the limits. Percentage recovery for Doxepin was found in range of 98%-102% indicating accuracy of the proposed work. All the parameters were within the ICH guidelines and the method was economical and simple as retention times were less than in literature and decreased run time.*

***Key Words:** Doxepin, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.*

I. INTRODUCTION

Doxepin is a psychotropic agent used for the treatment of depression, anxiety, manic-depressive disorder, and insomnia. Doxepin[1] is a tricyclic antidepressant that widely used in the therapy of depression. Doxepin can cause mild and transient serum enzyme elevations but is a rare cause of clinically apparent acute cholestatic liver injury. A dibenzoxepin tricyclic compound. It displays a range of pharmacological actions including maintaining adrenergic innervation. Its mechanism of action is not fully understood, but it appears to block reuptake of monoaminergic neurotransmitters into presynaptic terminals. It also possesses anticholinergic activity and modulates antagonism of histamine H (1) - and H (2)-receptors. Doxepin[2] is a psychotropic agent with antidepressant and anxiolytic properties. It is a tertiary amine that can be presented as (E) and (Z) stereoisomers with the (Z) stereoisomer corresponding to cidoxepin. Doxepin commonly produces a 5:1 (E): (Z) racemic mixture. In a strict sense, doxepin is not a tricyclic antidepressant but it is commonly associated with the class since it shares a lot of properties with members of the drug family including amitriptyline, clomipramine, desipramine, imipramine, nortriptyline, protriptyline and trimipramine. Doxepin[3] exact mechanism of action is not very clear. However, doxepin is known to be a selective histamine H1 receptor blocker. This effect on histamine receptors indicates effectiveness in skin conditions. The IUPAC Name of Doxepin is 3-(6H-benzo[c] [1] benzoxepin-11-ylidene)-N, N-dimethyl propan-1-amine. The Chemical Structure of Doxepin is following

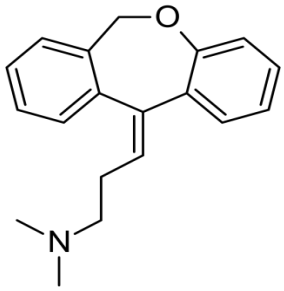


Fig.1. Chemical Structure of Doxepin

Though several methods are reported in literature for the estimation of Doxepin individually, no methods are reported for estimation of Doxepin in combination. The objective of the present study is to develop a novel, simple, accurate, precise, economic method for the estimation of Doxepin and validate the method with forced degradation studies according to ICH guidelines.

II. MATERIALS AND METHODS

Materials and Reagents

Water- (Milli Q)
Sodium Dihydrogen phosphate Anhydrous- HPLC Grade (Make: SRL)
Orthophosphoric acid, 88% - Emparta Grade (Make: Merck)
Methanol- HPLC Grade (Make: Merck)
Filter: 0.45µm membrane filter- for mobile phase filtration (Make: mdi)
0.45 PTFE syringe filter for sample solution (Make: Mdi)
Standard: Doxepin Hydrochloride Standard

Preparation of Mobile Phase A:

Weigh and transfer about 24.0g of sodium Dihydrogen phosphate Anhydrous in to 1000mL of water and mix well. Adjust pH to 2.5±0.05 with ortho phosphoric acid solution. Filter it through 0.45 µm membrane filter and degas.

Preparation of Mobile Phase B: Methanol (100%)

Preparation of Diluent:

Weigh and transfer about 24.0g of sodium Dihydrogen phosphate Anhydrous in to 1000mL of water and mix well. Adjust pH to 2.5±0.05 with ortho phosphoric acid solution. Filter it through 0.45 µm membrane filter and degas. Prepare a premixed and degassed mixture of buffer and methanol in the ratio of 65:35(v/v).

Preparation of Blank Solution: Use diluent as a blank

Preparation of Standard Solution:

Weigh and transfer about 56.5mg of Doxepin hydrochloride working standard (Equivalent to 50.0mg of Doxepin) into 100ml volumetric flask, add about 70ml of diluent and sonicate to dissolve and dilute to volume with diluent and mix well. Pipette and transfer 5ml of the solution into 25ml volumetric flask and dilute to volume with diluent and mix well. (Concentration of doxepin is about 100.0µg/mL)**.**

Preparation of Sample Solution for 100mg:

Weigh accurately and transfer 10 capsules in to 500mL of volumetric flask. Add about 250ml of diluent and sonicate for about 45 minutes with intermittent shaking by maintaining sonication bath temperature above 30°C. Take out the flask; allow it to come to room temperature. Dilute to volume with diluent and mix well. Centrifuge a portion of the sample solution at 5000 RPM for 10 minutes. Pipette and transfer 5ml of the supernatant solution into 100mL volumetric flask and dilute to volume with diluent and mix well. Filter the sample solution through 0.45µ PTFE filter after discarding first 3mL of filtrate. (Concentration of doxepin is about 100.0µg/mL).

III. RESULTS AND DISCUSSION

Method Development

Optimized Chromatographic Conditions:

HPLC Column: Zorbax SB Phenyl (150×4.6mm, 5µm)
Mobile Phase: (A: B): Buffer: Methanol (60:40% v/v)
Mode of Dilution: Isocratic
Column Oven Temperature: 50°C
Flow Rate: 1.2mL/min
Auto Sampler Temperature: 25°C
Injection Volume: 20µL
Wavelength: 254nm
Run time: 20 minutes

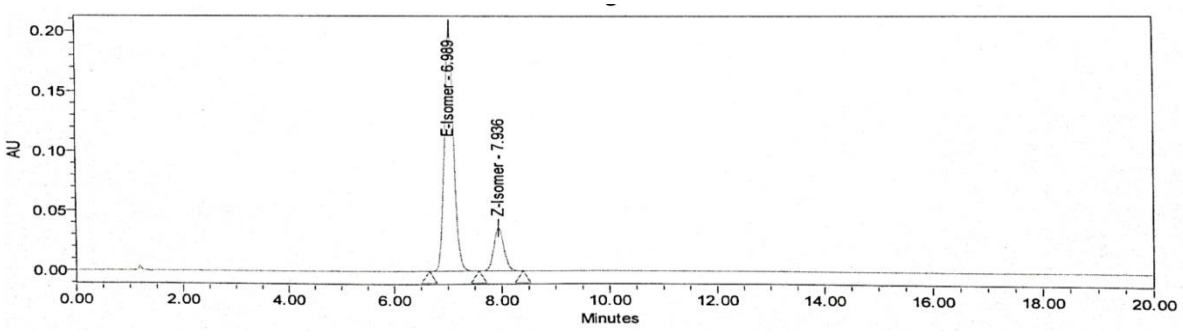


Fig.2. Optimized Chromatographic Condition

Validation of Method

System Suitability:

The System suitability[4] solution and Standard Solution were prepared and analysed as per test method to evaluate the system suitability parameters and the results were found to be within the limits. The standard solution was injected six times to evaluate system precision[5] and the result is found to be within the limits.

In order to assess the system suitability parameters, the standard solution and system suitability solution were prepared and analysed in accordance with the test procedure. The finding was found to be with in the acceptable range. Five injections of the standard solution were made to test the system's precision, and the results were determined to be within acceptable limits.

Table-1: Results of System Suitability

System Suitability Parameters	Observed Value	Acceptance Criteria
The Tailing factor from the chromatogram of Standard solution		NMT 2.0
Doxepin (E)- isomer	1.34	
Doxepin (Z)- isomer	1.12	
% Relative standard deviation, determined from the sum of the peak areas of Doxepin(E)-isomer and Doxepin(Z)-isomer from five replicate injections of standard solution	0.14	NMT 2.0%
Theoretical plates from the chromatogram of standard solution		NLT 2000
Doxepin (E)- isomer	7870	
Doxepin (Z)- isomer	8395	
The resolution between the Doxepin (E)-isomer and Doxepin (Z)-isomer peaks from the chromatogram of standard solution	2.80	NLT 1.5

Method Precision (Repeatability):

The method precision[6] was performed by analysing the sample solution of Doxepin capsules at working concentration six times (six replicate sample preparations).

Table-2 shows percentage relative standard deviation of Doxepin assay[7] values of six replicate sample preparations.

Table-2: Results of Method Precision

Sample No.	%Assay
1	103.2
2	103.2
3	102.5
4	102.6
5	102.6
6	102.7
Mean	**102.8**
SD	**0.316228**
%RSD	**0.30**

Acceptance Criteria:

The % RSD[8] for Six assay results should not be more than 2.0. The assay value should be NLT 90% and NMT 110% of labelled amount of Doxepin.

Table-3: Results of System Precision

Injection Number	Peak Area	Acceptance Criteria
1	2875325	The % RSD for peak areas of

2	2884900	Doxepin (E)-isomer and Doxepin (Z)-isomer from six replicate Injections of standard solution should be not more than 2.0.
3	2881626	
4	2876143	
5	2880208	
Average	**2879640**	
SD	**3961.8**	
% RSD	**0.14**	

Intermediate Precision:

The ruggedness[9] of method was demonstrated by conducting the precision study by different analyst. Assay was performed for six individual test preparations of 100 mg strength as per test method. The % RSD for assay results from six individual test preparations is found to be with in the limit. The overall % RSD for the assay results obtained from both method precision[10] and Intermediate precision is found to be within the limit. The system suitability results were evaluated as per the test method and results are found to be within the limits.

Table-4: Results of Intermediate Precision:

Sample No.	%Assay
1	101.9
2	102
3	102.2
4	102.3
5	102.1
6	102.5
Average	**102.1**
SD	**0.216025**
%RSD	**0.21**

Acceptance Criteria:

The % RSD for six assay results should not be more than 2.0. The % RSD for assay results obtained from both method precision and intermediate precision[11] should not be more than 2.0.

Accuracy:

Doxepin recovery[12] was tested at levels ranging from 50% to 150% of the initial assay concentration. Sample solutions were prepared in triplicate for each level and analysed as per test method. According to the calculations, the individual % recovery, % average recovery[13] and % RSD for recovery at each level were calculated and the results are found to be within limit.

Table-5: Results of Accuracy

% Level Spiked	Sample No.	% Recovery	%Recovery Mean	%RSD
50%	1	100.3	100.6	0.26
	2	100.8		
	3	100.7		
80%	1	100.7	100.6	0.06
	2	100.6		
	3	100.6		
100%	1	100.3	100.4	0.36
	2	100.1		
	3	100.8		
120%	1	100.5	100.4	0.06
	2	100.4		
	3	100.4		
150%	1	100.6	100.7	0.11
	2	100.6		
	3	100.8		

Acceptance Criteria:

1) The Individual % recovery should be between 98.0 and 102.

2) The average % recovery of each level should be between 99.0 and 102.0 and % RSD for recovery at each level should be not more than 2.0.

Specificity:

A study to evaluate the interference from placebo was conducted. Samples were prepared in duplicate by taking placebo equivalent to the amount present in the test preparation[14] and analysed as per the test method. Chromatograms of placebo preparations are not showing any peak at the retention time of Doxepin.

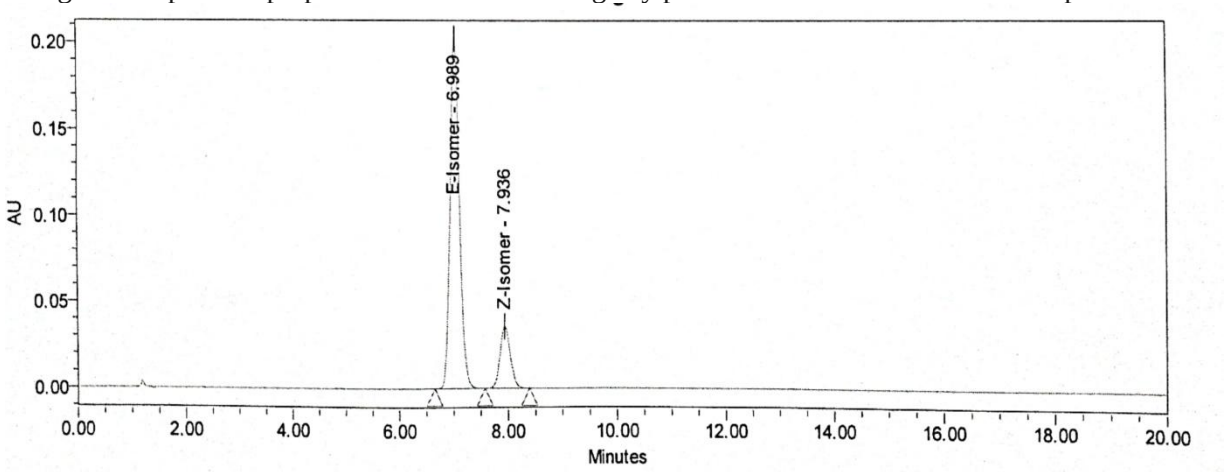


Fig-3: Chromatogram for Standard Solution

Table-6: Results of Placebo Interference

Sample No.	Peak Found at Rt of Analyte Peak (Yes/No)	Acceptance Criteria
1	No	Placebo should not show any peak at the retention time of Doxepin
2	No	

Linearity:

Linearity of detector response was established by plotting a graph between concentrations versus area. A series of solutions of Doxepin hydrochloride standard were prepared in the concentration ranging from 50µg/mL to 150µg/mL as Doxepin and analysed as per test method.

A graph was plotted with concentration in 49.87µg/mL-149 µg/mL on X- axis versus response (area) on Y- axis and determined the correlation coefficient[15]. The results are found to be within the limit Linearity[16] level preparations.

Table-7: For Linearity Level Preparation

SXGT	Weight(mg)	14.21	M.W1	279.38
	Potency	99.2	M.W2	315.84
Level	**V1**	**V2**	**V3**	**Conc.(ppm)**
50%	25	2.5	25	49.87
80%	25	4	25	79.80
100%	25	5	25	99.75
120%	25	6	25	119.70
150%	25	1.5	5	149.62

Table-8: Results of Linearity

S. No.	Concentration(µg/mL)	Peak area
1	49.87	1385626

2	79.80	2301612
3	99.75	2874992
4	119.70	3423191
5	149.62	4272105

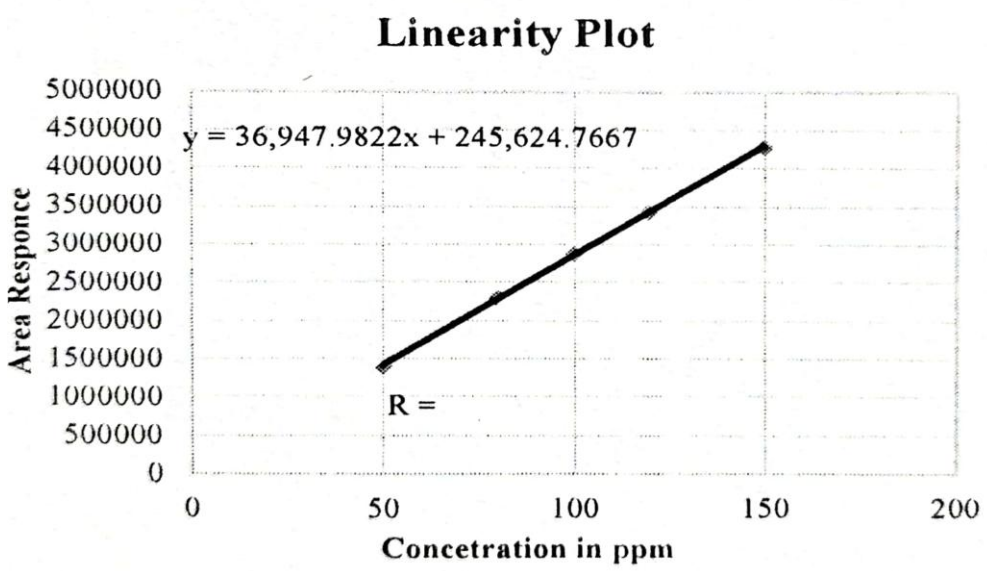


Fig.4. Calibration Curve of Doxepin

Slope (m) = 28822.2882

Co-efficient of Correlation = 0.9998

Acceptance Criteria: Coefficient of Correlation shall be not less than 0.999 and Linearity graph[17] of Doxepin at 254 nm.

Filter Validation:

Filter validation was conducted to establish the suitability of filters by using three different filters[18] namely, 0.45 µm PVDF filter (Mfg. by: M/s. Millipore), 0.45µm PTFE filter (Mfg. by: M/s. Millipore), and 0.45µm Nylon 66 filters (Mfg. by: M/s. Millipore).

Prepare standard solution (single preparation) and test solution of Doxepin capsules 100mg strength (triplicate preparations) as per the test method. Centrifuge some portion of the test solution and also filter the remaining portion of the test solution through 0.45 µm PVDF, PTFE, Nylon. Inject unfiltered standard solution[19], filtered standard solution, filtered test solution and centrifuged test solutions in duplicate.

Table-9: Filters used

Filter Description	Filters		
	PVDF	PTFE	Nylon 66
Manufacturers Name	Millipore	Millipore	Millipore
Size	0.45µm	0.45µm	0.45µm

Table-10: Results of Filter interference

Sample No	% Assay				Difference between centrifuged and		
	Centrifuged	PVDF filter	PTFE filter	Nylon 66 filter	PVDF filter	PTFE filter	Nylon 66 filter
1	101.80	101.80	101.21	101.22	0.00	0.58	0.57

Acceptance Criteria:

The difference between the response of filtered and unfiltered standard NMT ± 2.0%. The % assay of filtered test solutions should not deviate by ± 2.0 from that of centrifuged test solutions. The %RSD of each of the three centrifuged and three filtered test solution should be NMT 2.0.

Solution Stability:

Solution stability[20] was performed by analysing standard and sample preparation using doxepin capsules 100mg

periodically into HPLC system[21] at room temperature i.e.; 25°C.

Table-11: Results of Stability of Test Preparations

Time (Hours)	% Assay of test preparation	Difference from initial
Initial		NA
After 24 hours		0.05

Acceptance Criteria:

The test and standard solutions are considered stable with respect to the time interval if the % difference for peak areas is NMT 2.0.

Results: The standard and sample solution was stable upto 24hrs.

Conclusion:

From the above results standard and test preparation is concluded to be stable for a period of 24 hrs at room temperature.

Robustness:

1. Effect of Variation in Flow rate:

Robustness[22] was conducted to determine the effect of variation in flow rate. The system suitability parameters were evaluated at the flow rate of 1.0 mL/min. and 1.4 mL/min. The system suitability results were found to be within the limits for higher and lower flow rates. From this, it is concluded that the allowable variation in flow rate is from 1.0 mL/min to 1.4 mL/min.

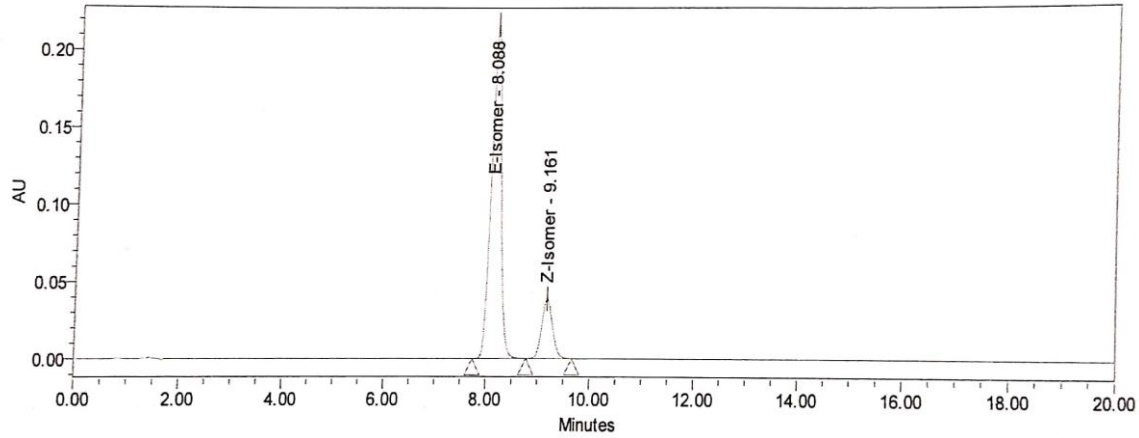


Fig.5. Chromatogram with Low Flow rate

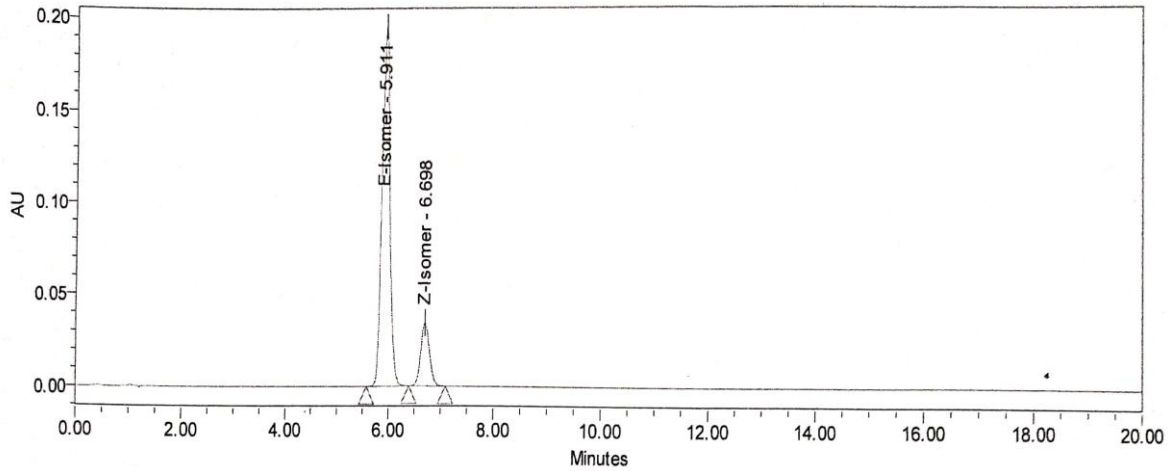


Fig.6. Chromatogram with High Flow rate

2. Effect of Variation in Column Oven Temperature:

Robustness was conducted to determine the effect of variation in column oven temperature. The system suitability parameters were evaluated at 45°C and 55°C column oven temperatures. The system suitability results were found to be within the limits at both column oven temperatures. From this, it is concluded that the allowable variation in column[23] oven temperature is from 45°C to 55°C.

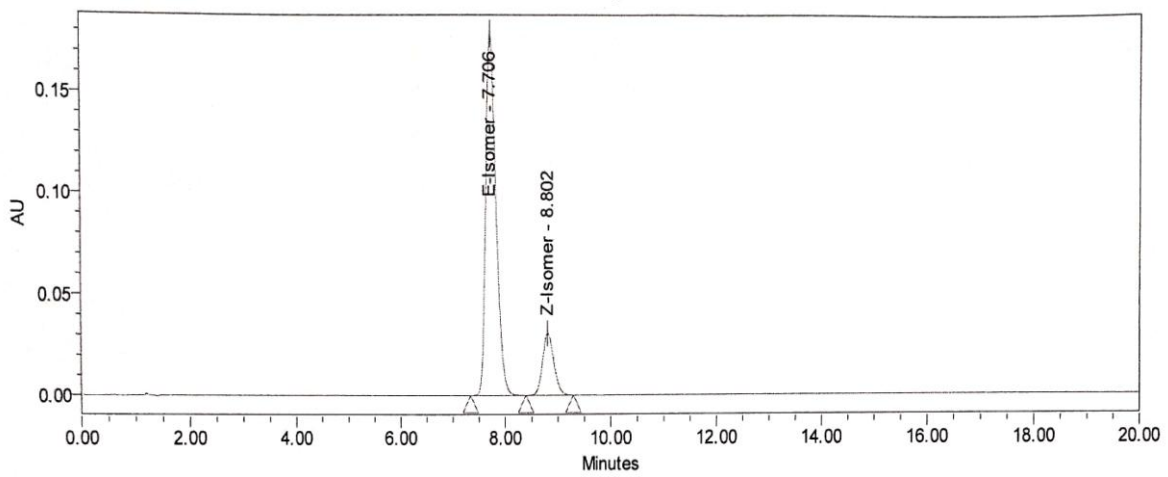


Fig.7. Chromatogram with Low Temperature

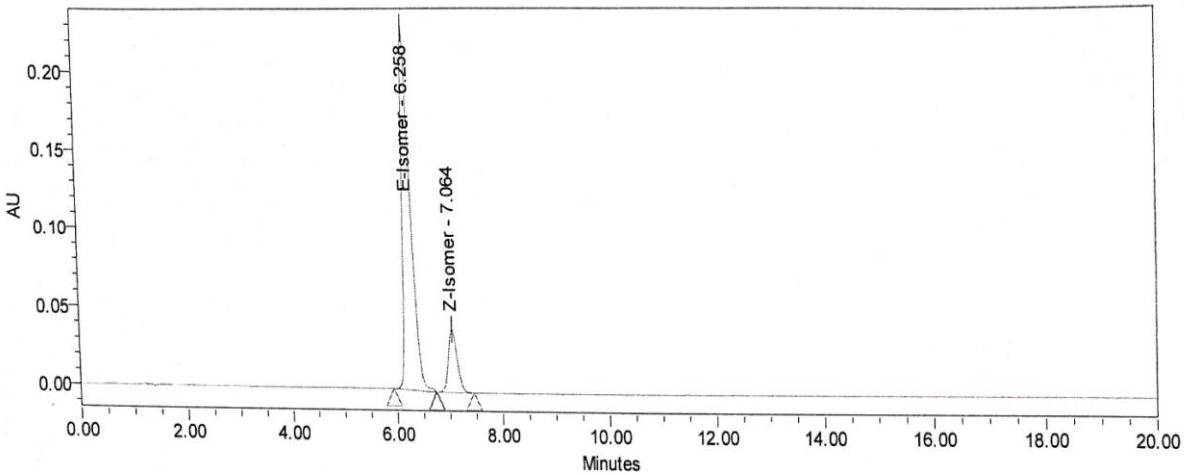


Fig.8. Chromatogram with High Temperature

3. Effect of Variation in Wavelength:

Robustness was conducted to determine the effect of variation in wavelength. The system suitability parameters were evaluated at 252nm and 256nm. The system suitability results were found to be within the limits for higher and lower wavelengths. From this, it is concluded that the allowable variation in wavelengths[24] is from 252nm to 256nm.

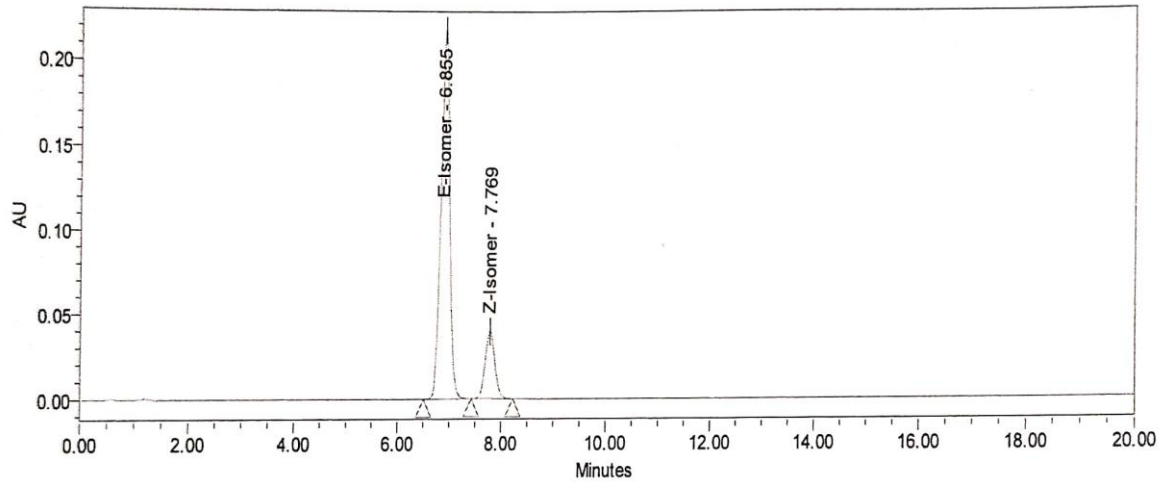


Fig.9. Chromatogram with Low Wavelength

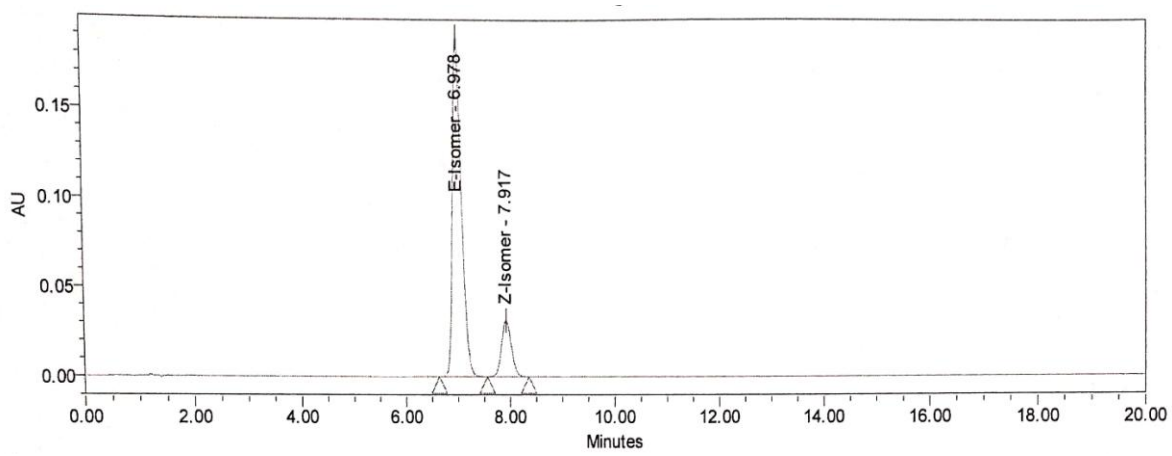


Fig.10. Chromatogram with High Wavelength

4. Effect of Variation in pH:

Robustness was conducted to determine the effect of variation in pH. The system suitability parameters were evaluated at the pH of 2.3 and 2.7. The system suitability results were found to be within the limits for higher and lower pH values. From this, it is concluded that the allowable variation in pH is from 2.3 to 2.7.

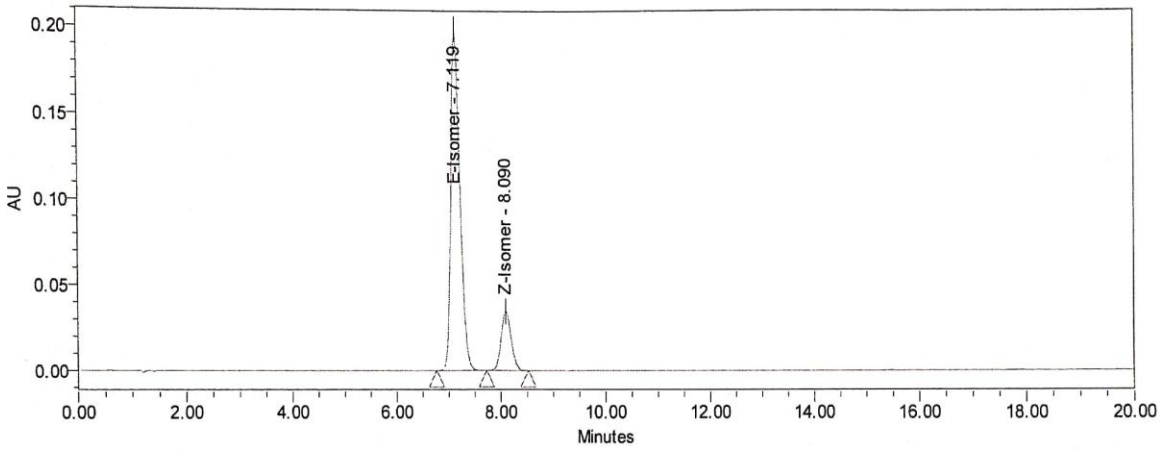


Fig.11. Chromatogram with Low pH

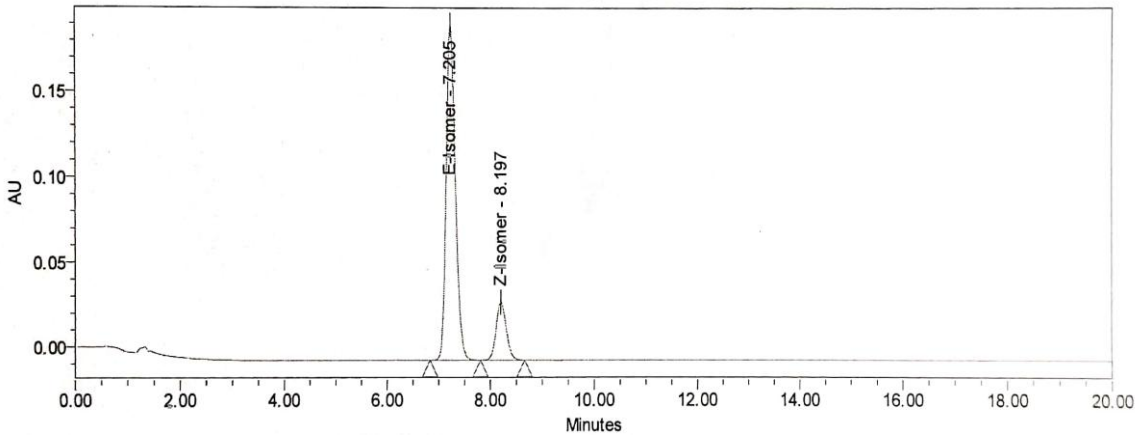


Fig.12. Chromatogram with High pH

Forced Degradation:

A study[25] was conducted to demonstrate the effective separation of degradants from Doxepin peaks in Assay method. Separate portions of Drug product and Placebo were exposed to the following stress conditions to induce degradation. Stressed samples were analysed as per test method with Photo diode array detector. The chromatograms of the stressed samples[26] were evaluated for peak purity of Doxepin peak using Waters Empower software. For all forced degradation samples, the purity angle is less than purity threshold with no purity flag for Doxepin peak. This indicates that there is no interference[27] from degradants in quantification of the Doxepin in Doxepin hydrochloride capsules.

Acid Degradation: Weighed accurately about 3499.88 mg of sample and transferred into 500 mL volumetric

flask. Optimum degradation has been found at a condition of 5 mL of 5 N HCl at 60°C for 2hrs. Added to it 5 mL of 5N HCl and kept at 60°C for 2hrs. Added 5 mL of 5N NaOH and shaken for the neutralization step to take place. Thereafter, added about 250mL of diluent and sonicated for about 45minutes with intermittent shaking by maintaining sonication bath temperature above 30°C.Then taken out the flask, allowed it to come to room temperature. Then diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 5000 RPM for 10 minutes. Then Pipetted and transferred 5ml of the supernatant solution into 100mL volumetric flask and diluted to volume with diluent and mixed well. Then filtered the sample solution through 0.45µ PTFE filter after discarding first 3mL of filtrate.

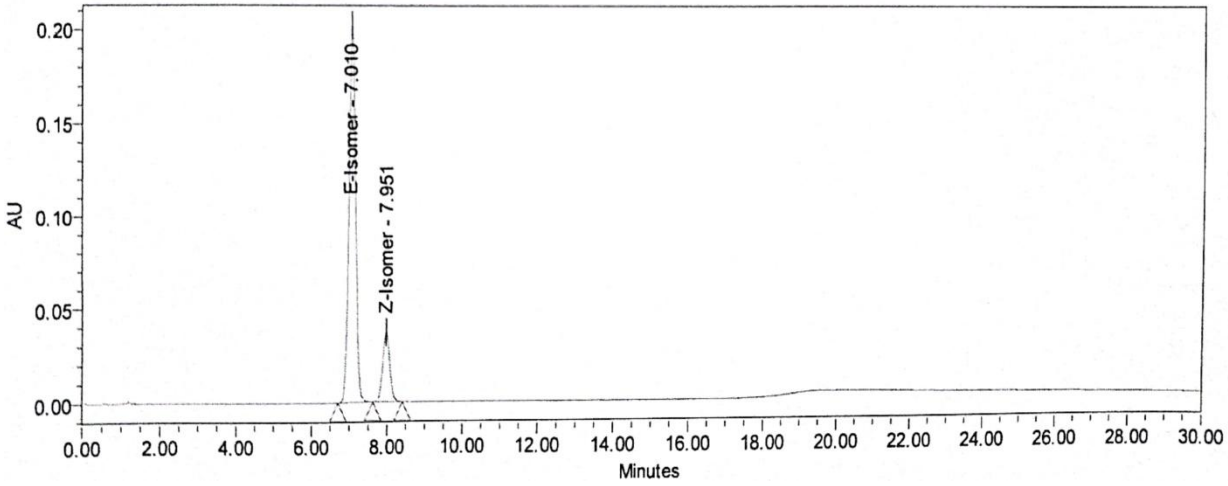
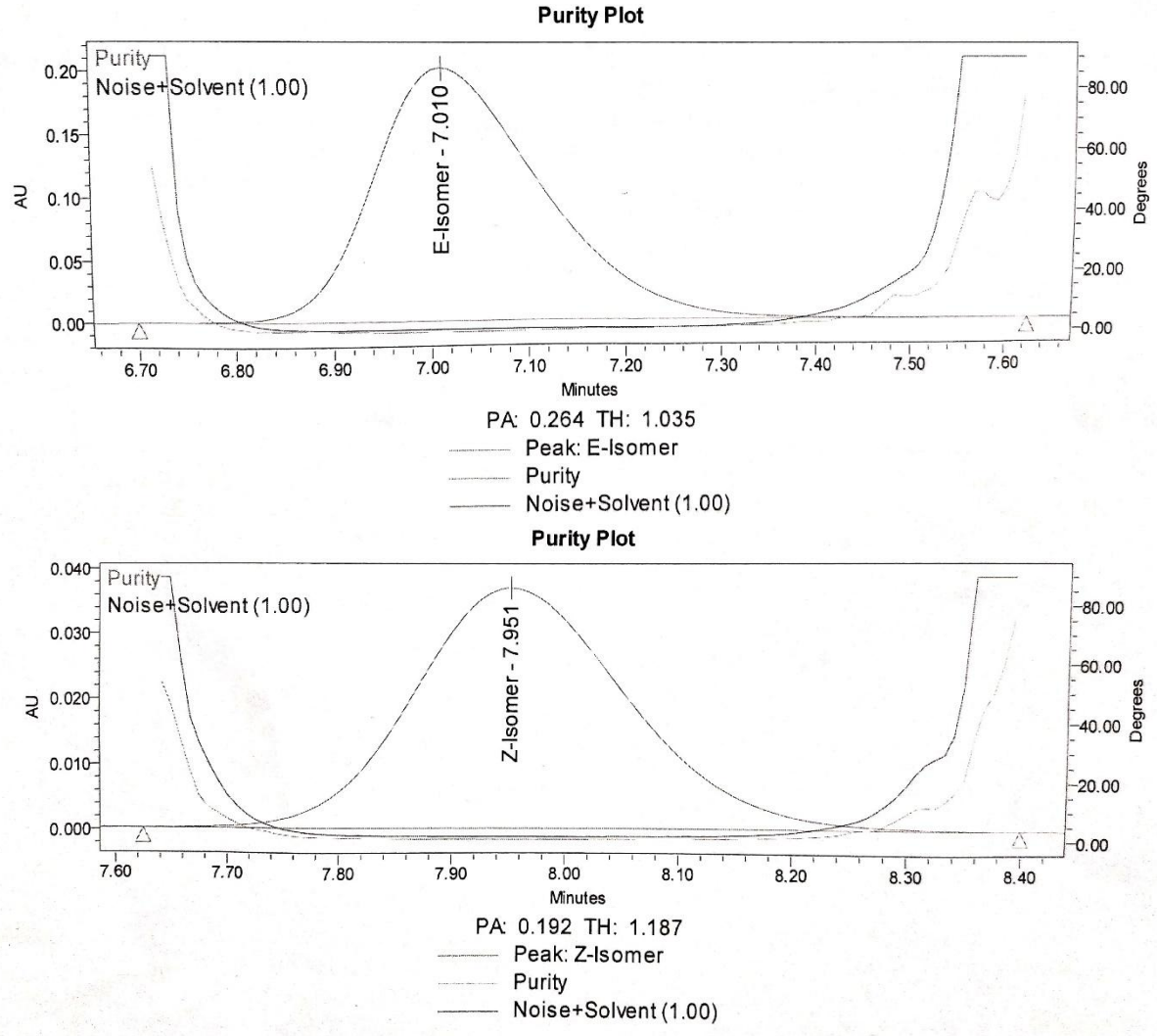


Fig.12. Peak Purity for Acid Stressed Sample



Basic Degradation:

Weighed accurately about 3499.68 mg of sample and transferred into 500 mL volumetric flask. Optimum degradation has been found at a condition of 5 mL of 5N NaOH on bench top for 12hrs. Added to it 5 mL of 5N NaOH and kept at 60°C for 2hrs. Added 5 mL of 5N HCl and shaken for the neutralization step to take place. Thereafter, added about 250mL of diluent and sonicated for about 45minutes with intermittent shaking by maintaining sonication bath temperature above 30°C.Then taken out the flask, allowed it to come to room temperature. Then diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 5000 RPM for 10 minutes. Then Pipetted and transferred 5ml of the supernatant solution into 100mL volumetric flask and diluted to volume with diluent and mixed well. Then filtered the sample solution through 0.45µ PTFE filter after discarding first 3mL of filtrate.

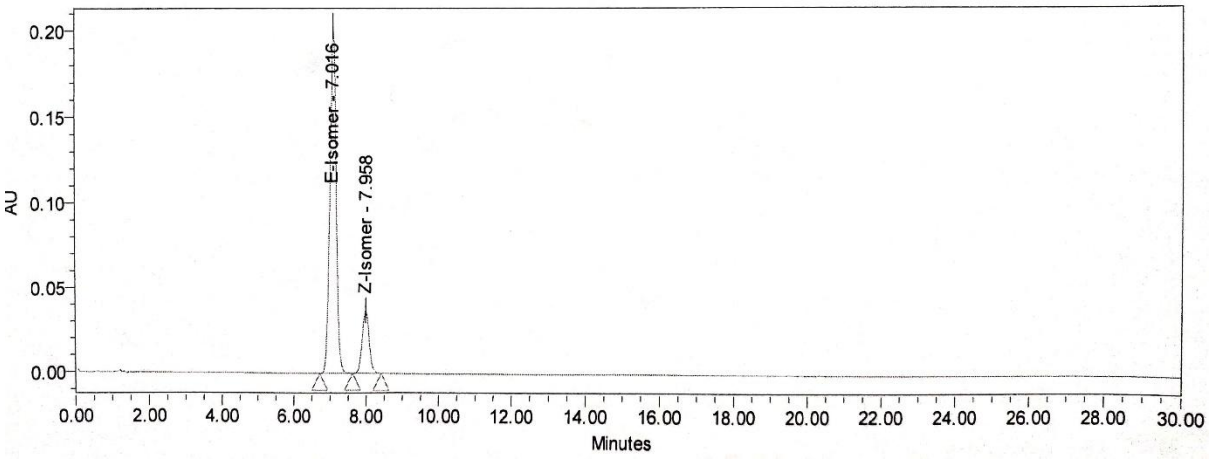
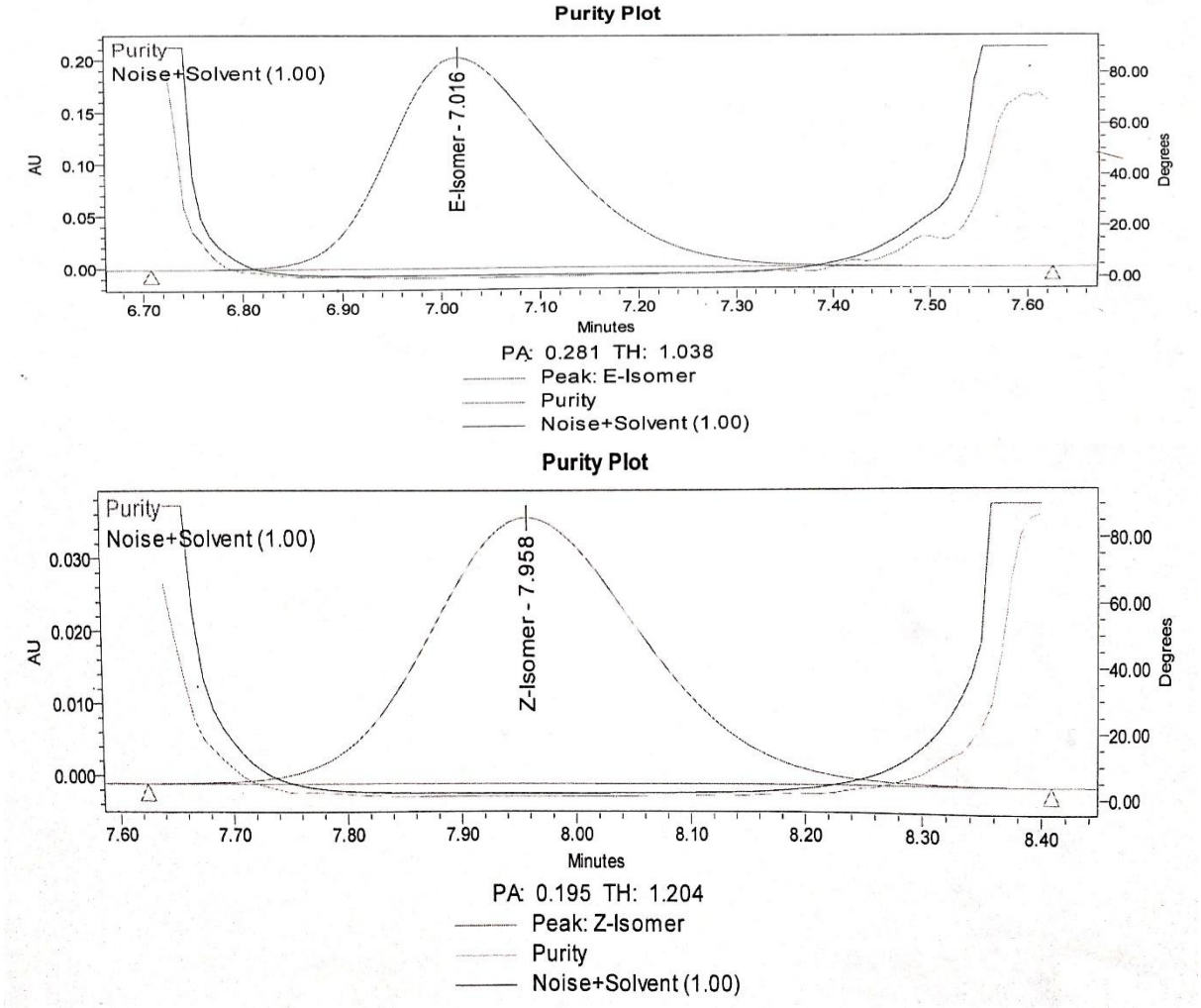


Fig.14. Peak Purity for base Stressed Sample



Oxidative Degradation:

Weighed accurately about 3502.56 mg of sample and transferred into 500 mL volumetric flask. Optimum degradation[28] has been found at a condition of 5 mL of 30% H2O2 on bench top for 3hrs. Added to it 5 mL of 30%H2O2 and kept at 60°C for 2hrs. Thereafter, added about 250mL of diluent and sonicated for about 45minutes with intermittent shaking by maintaining sonication bath temperature above 30°C.Then taken out the flask, allowed it to come to room temperature. Then diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 5000 RPM for 10 minutes. Then Pipetted and transferred 5ml of the supernatant solution into 100mL volumetric flask and diluted to volume with diluent and mixed well. Then filtered the sample solution through 0.45µ PTFE filter after discarding first 3mL of filtrate.

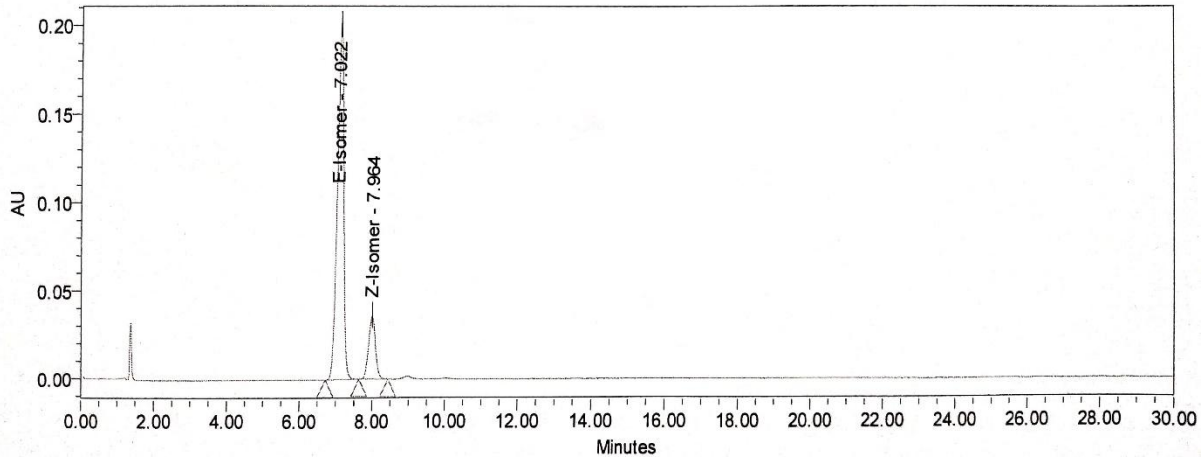
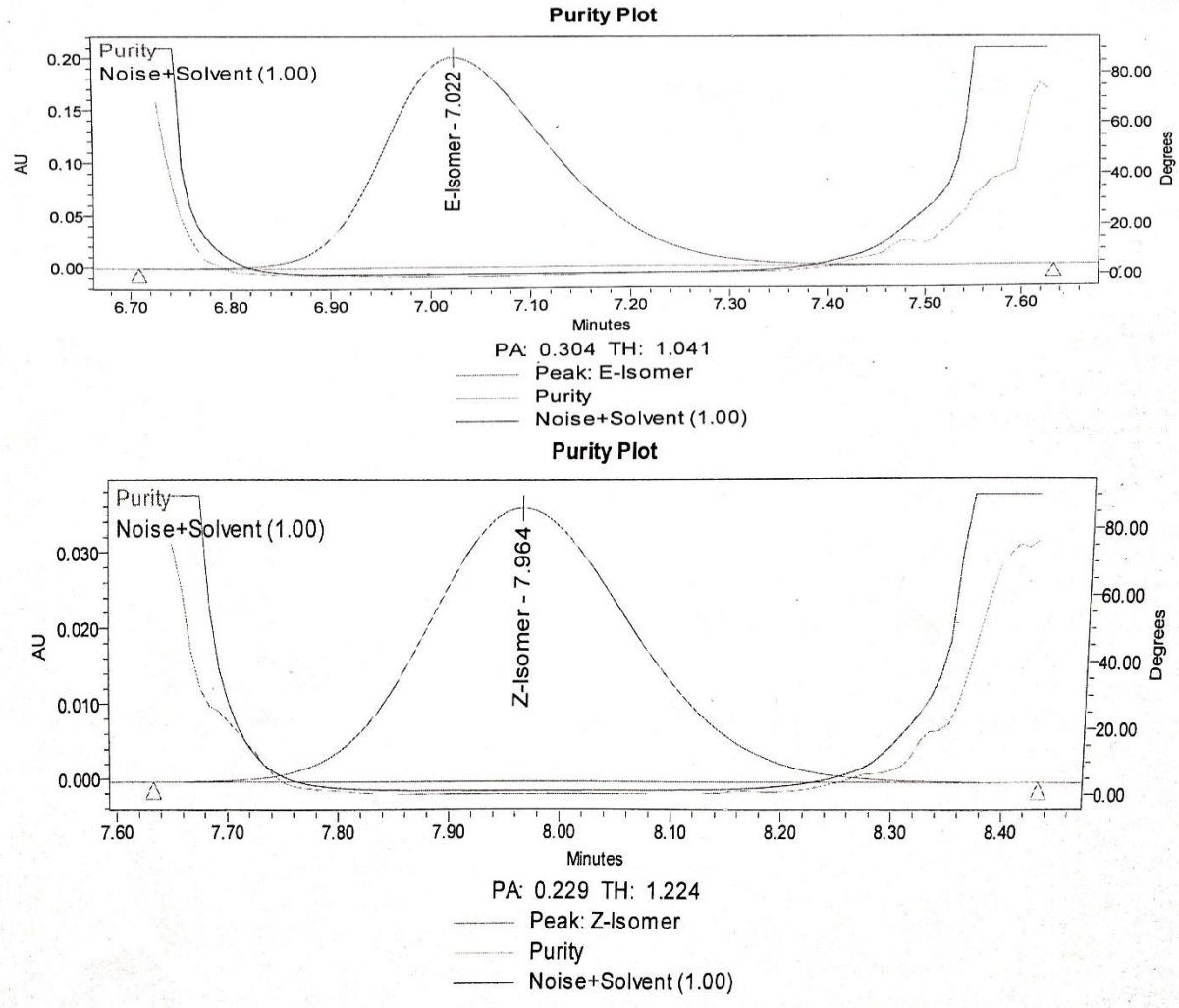


Fig. 15. Peak Purity for Oxidative Stressed Sample



Thermal Degradation:

Weighed accurately about 3501.25 mg of sample and exposed to 105°C for 24hrs. Then transferred into 500mL volumetric flask. Thereafter, added about 250mL of diluent and sonicated for about 45minutes with intermittent shaking by maintaining sonication bath temperature above 30°C.Then taken out the flask, allowed it to come to room temperature. Then diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 5000 RPM for 10 minutes. Then Pipetted and transferred 5ml of the supernatant solution into 100mL volumetric flask and diluted to volume with diluent and mixed well. Then filtered the sample solution through 0.45µ PTFE filter after discarding first 3mL of filtrate.

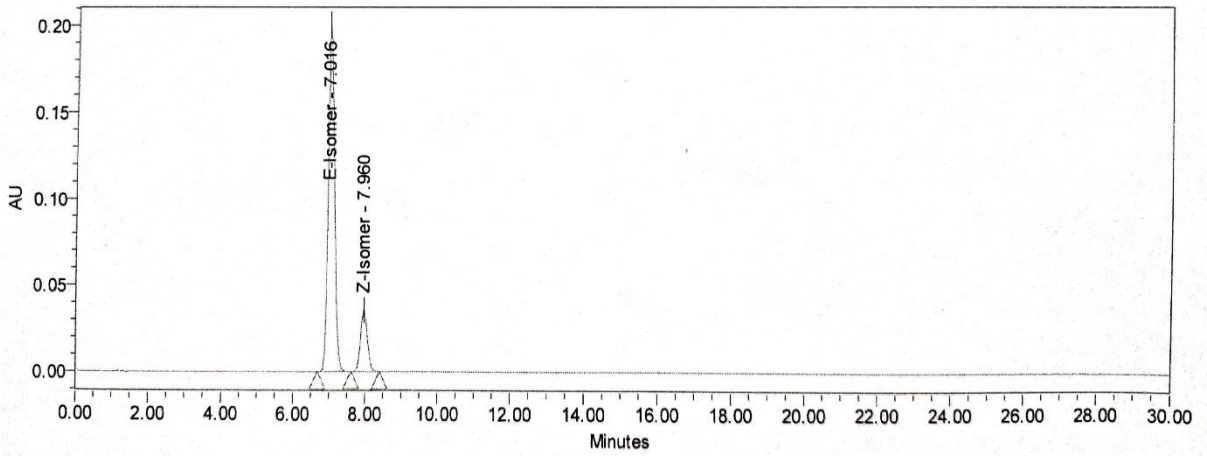
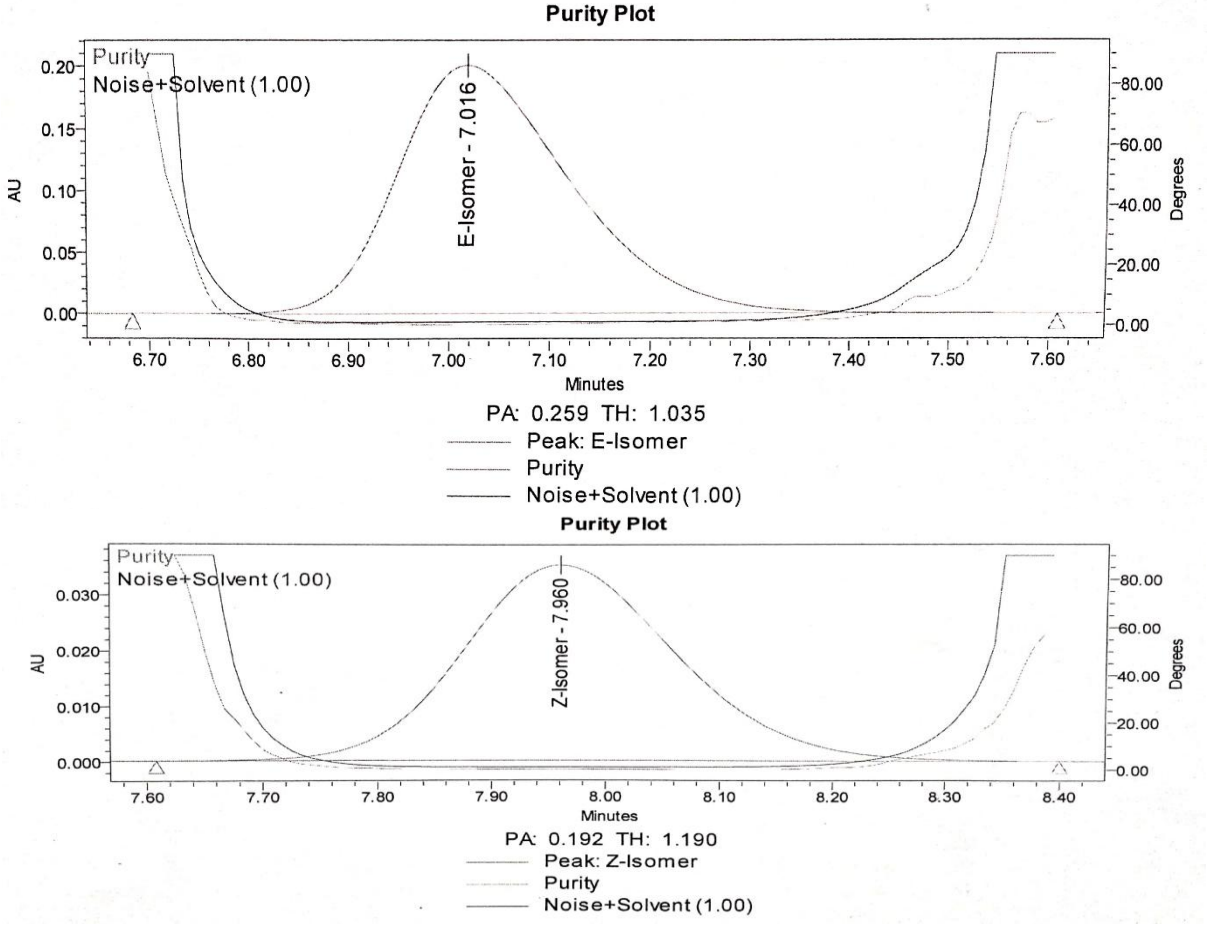


Fig.16. Peak Purity for Thermal Stressed Sample



Acceptance Criteria:

No blank and placebo interference at the retention time of Doxepin peak. Peak purity for the Doxepin peak should be pass.

Table-12: Peak Purity Results From Forced Degradation Studies

S.No.	Condition	%Assay	%Degradation	Purity Angle		Purity Threshold		Purity Flag
				(E)-isomer	(Z)-isomer	(E)-isomer	(Z)-isomer	
1	As such	100.99	-	0.207	0.156	1.039	1.206	No
2	Acid	99.30	1.67	0.264	0.192	1.035	1.187	No
3	Base	99.47	1.51	0.281	0.195	1.038	1.204	No
4	Oxidative	98.53	2.44	0.304	0.229	1.041	1.224	No
5	Thermal	97.64	3.32	0.259	0.192	1.035	1.190	No

IV. CONCLUSION

A simple, fast, accurate and precise RP-HPLC analytical method has been developed and validated for the quantitative analysis of Doxepin in bulk and marketed pharmaceutical dosage form. The results obtained show the developed method to be cost effective, rapid (shorter retention time), simple, accurate (the value of %RSD less than 2), precise and can be successfully employed in the routine analysis of the drug in bulk and marketed pharmaceutical dosage form. This study was a typical example of the development of a stability-indicating assay established following the recommendations of ICH guidelines. The simplicity and reproducibility of the proposed method fulfils the objective of this research work.

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