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Isocratic Reversed Phase Liquid Chromatographic Method Validation for the Determination of Cilostazol in Pure and Formulations

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ABSTRACT

A stability indicating isocratic reversed phase liquid chromatographic (RP-HPLC) method has been developed and subsequently validated for the determination of cilostazol in pure and formulations. Separation was achieved with a Inertsil C_{18} column, [250mm x 4.6mm I.D; particle size 5 µm] at ambient temperature at a flow rate of 1.0mL/min using the mobile phase containing 50mM sodium hydrogen phosphate dibasic dihydrate buffer (pH 3.0) and acetonitrile in the ratio of 50:50, v/v, at an wavelength of 257.40nm with an chromatographic runtime of 10 minutes respectively. The described method was linear over a range of 5.0 -17.5µg/mL for cilostazol. The accuracy of the method has been demonstrated at three concentration levels in the range of 50–150% and the recovery of cilostazol was found to be in the range of 99.84-100.78%. The developed method is simple, rapid, selective, accurate, stability indicating and useful for the assay of cilostazol in dosage forms and can be employed in the quality control analysis of bulk manufacturing and pharmaceutical formulations.

INTRODUCTION

Cilostazol [1] is a quinolinone derivative used for the treatment of intermittent claudication resulting from peripheral arterial disease.

Literature survey revealed that few HPLC methods[2-7] and only one stability indicating HPLC assay method[8] for the determination of cilostazol in pharmaceutical formulation were available and consequently this fact prompted the author to make an attempt to develop a new validated stability indicating RP-HPLC method for cilostazol in pure and pharmaceutical dosage forms, by degrading the drug under various stress conditions like acid hydrolysis, base hydrolysis, and oxidation, thermal and photolytic stress which in accordance with ICH guidelines.

MATERIALS AND METHODS

EXPERIMENTAL

a. INSTRUMENTATION: The chromatographic system used to perform development and validation of this assay method was comprised of a HPLC (Waters system) equipped with Waters 2695 separation module, Waters 2996 photodiode array and Inertsil C_{18} column, [250 mmx4.6 mm I.D; particle size 5 µm]. The volumes of standard and sample solutions were introduced to the column by means of a 20µL loop (Rheodyne) and connected to a multi-instrument data acquisition and data processing system using Waters Empower 2 software.

b. CHEMICALS & SOLVENTS: Cilostazol (99.9% Pure) supplied by Dr Reddys Labs. Hyderabad and its formulation Cilokem (strength: 50mg Cilostazol) from Alkem Labs, India Ltd were purchased from local pharmacy. Acetonitrile (HPLC Grade), orthophosphoric acid (AR Grade), sodium hydrogen phosphate dibasic dihydrate (AR Grade) were purchased from Qualigens Ltd., Mumbai, India. Purified water (Millipore system) was used for the preparation of buffer.

c. PREPARATION OF BUFFER: The mobile phase consisted of acetonitrile and 50 mM sodium hydrogen phosphate dibasic dihydrate buffer pH 3.0 in the ratio of 50:50, *v/v* was prepared. Buffer solution was prepared by dissolving 8.9 g sodium hydrogen phosphate dibasic dihydrate in 900 ml HPLC grade water and adjusted to pH 3.0 with orthophosphoric acid and final volume was made up to 1000ml with HPLC grade water. Priror to use this mobile phase, it

was filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd. Bengaluru, India) and degassed for 15 minutes in an ultrasonic bath.

d. DILUENT PREPARATION: Mobile phase is used as diluent in the present assay.

e. PREPARATION OF STANDARD DRUG SOLUTION (STOCK & WORKING): Standard stock solution of cilostazol (1.0mg/mL) was prepared by accurately weighing and transferring 100mg of cilostazol (99.9% pure) into a 100mL volumetric flask. To the above flask, add 30mL of diluent (water??) and sonicated to dissolve and later, diluted to volume with the same diluent. This stock solution was further diluted by transferring suitable aliquots into a separate 100mL volumetric flask and diluted to the volume with diluent to obtain final working standard solutions of cilostazol in the concentrations range 5.017.5µg/mL, respectively.

f. SAMPLE PREPARATION: For the analysis of the tablet dosage form, ten tablets of market formulations (**Cilokem: 50mg**) were weighed individually and their average weight is determined. These tablets were then crushed to a fine powder and powder equivalent to the weight of 100mg cilostazol was transferred to a 100mLvolumetric flask containing 30mL of diluent, mixed thoroughly with intermittent shaking for 10 minutes and dilute to the volume with the same diluent. Further, filter the above solution by using 0.45µ filter. Later, transfer aliquots of this above filtrate into a series of different 100mL volumetric flasks and dilute to volume with the same diluent and proceeded with the above described procedure.

RESULTS AND DISCUSSION

I. METHOD DEVELOPMENT: Various development trials were conducted using different chromatographic parameters by considering the analytical conditions and different chemical nature of cilostazol.

Firstly, column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution of cilostazol peak. After evaluating all these factors, Inertsil C_{18} column (250mm x 4.6mm i. d., 5 µm particle size) was found to be giving satisfactory results and the resolution of cilostazol was eluted at 3.262mins with an run time of 10mins. Next preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did not give good peak shape.

By using 50 mM disodium hydrogen phosphate dihydrate buffer, adjusted to pH 3.0 with orthophosphoric acid and keeping mobile phase composition as acetonitrile and 50mM sodium hydrogen phosphate dibasic dehydrate buffer pH 3.0 in the ratio of 50:50, v/v, best peak shape was obtained. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce backpressure, the longer retention time and to achieve good peak shape **(Figure.2)**.

Finally, a flow rate of 1.0mL/min with an injection volume of 20µL with UV detection at 257.40nm was found to be best for the analysis of cilostazol. The chromatogram of cilostazol standard using the proposed method is represented in **(Figure.1)** and the system suitability results of the proposed method are presented in **Table.1.**

CHROMATOGRAPHIC CONDITIONS: The chromatographic separation of cilostazol was performed by injecting 20µL of standard and sample volumes into an Inertsil C_{18} column, [250mm x 4.6mm I.D; particle size 5 µm] at ambient temperature at a flow rate of 1.0mL/min using the mobile phase containing 50mM sodium hydrogen phosphate dibasic dihydrate buffer (pH 3.0) and acetonitrile in the ratio of 50:50, v/v, at an wavelength of 257.40nm with an chromatographic runtime of 10 minutes respectively.

II. FORCED DEGRADATION STUDIES: The stability indicating nature of the present developed RP-HPLC method was established by forced degradation of cilostazol under various stress conditions using the above said optimized chromatographic conditions. For this intentional degradation was attempted to stress conditions exposing it to acid (1N hydrochloric acid), alkali (1N NaOH), heat (100°C) and UV light (250nm wavelength) to evaluate the ability of the proposed method to separate cilostazol from its degradation products.

Acid hydrolysis: In this study the sample was treated with 5.0ml of acid (1N HCl) and kept for 1hr. After 1hr the solution was neutralized with 1N NaOH and analyzed using the proposed RP-HPLC method.

Alkali hydrolysis: In this study the sample was treated with 5.0ml of alkali (1N NaOH) and kept for 1hr. After 1hr the solution was neutralized with 1N HCl and analyzed using the proposed RP-HPLC method.

Photolysis: In this study the sample was kept under UV light for different time intervals (15mins – 7days) and analyzed by using the proposed RP-HPLC method.

Heat: In this study the sample was heated at 100^{o}C for 15mins and analyzed then by using the proposed HPLC method.

The recovery results from the above studies indicated that cilostazol did not undergo any significant degradation on acid, alkali, photolysis and heat conditions. The results of all specificity parameters of various degradation studies of cilostazol are listed in the respective chromatograms were represented in **Figure.2a - d.**

III. METHOD VALIDATION: After the development of suitable RP-HPLC method, it is subjected to validation as per ICH norms [i.e., system suitability, linearity, LOD and LOQ, accuracy, precision, robustness and ruggedness studies]. All the validation studies were carried out with the injection volumes of 20µL of standard and sample solutions of cilostazol into the column respectively.

a. SYSTEM SUITABILITY: System suitability tests for the developed RP-HPLC were performed as per the ICH Guidelines. This test was carried at optimized chromatographic conditions by injecting 20µL of standard solution of cilostazol into the column at the concentration level 15µg/ml and the results of system suitability parameters [Theoretical plates, resolution, tailing factor] were determined and are presented in **Table.1** respectively.

b. SPECIFICITY: The specificity of the developed RP-HPLC method for cilostazol was evaluated with the studies of diluent interference in the resolution of the present studied drug. From these studies it is revealed that the diluent has no interference on capecitibine resolution.

c. LINEARITY: The linearity of the present RP-HPLC method was evaluated in the concentration range of 5.0-17.5µg/mL, corresponding to 25-150% of the nominal content of cilostazol. The peak area of each concentration was determined and recorded. A calibration plot was plotted with the peak areas recorded versus the concentration and was found to be linear in the investigation concentration range (**Figure.3)**. The linear regression equation revealed y = 544623.073x-1331942.42 with correlation coefficient of 0.9999 respectively. The linearity results for were tabulated in **Table.2.**

d.LOD & **LOQ:** The Limit of detection and quantification for the proposed method of cilostazol were calculated using standard deviation of the response and slope of calibration curve. The LOD value for cilostazol was found to be 0.0481µg/mL and the LOQ value 0.160µg/mL(**Table.2)**.

e. METHOD PRECISION: Repeatability of the developed method was studied by carrying out by method precision. The method precision was determined from the results of six independent determinations at 100% level of standard concentration of cilostazol and the results were reported in **Table.3.** The developed method was found to be precise as the %RSD values for the repeatability was 0.36%.

f. ACCURACY [RECOVERY STUDIES]: The accuracy of the developed RP-HPLC method of cilostazol was evaluated in triplicate by addition of three different amounts of cilostazol, equivalent to 50, 100, and 150% of the amount originally present to a previously analyzed sample. The results of these studies showed the best recoveries (99.84-100.78%) of the spiked drug at each added concentration, indicating that the proposed method was accurate and suitable for intended use. The values are reported in **Table.4** respectively.

g. ROBUSTNESS: The evaluation of robustness of the developed RP-HPLC method was made by making slight changes in chromatographic conditions that include the change in flow rate and change in column temperature. Each of the above mentioned factor were changed at two levels (-2,+2) at one time with respect to optimized parameters. The results of these studies reported **Table. 5** negligible effect on the chromatographic parameters by slight variations in chromatographic conditions

h. RUGGEDNESS: The ruggedness of the present RP-HPLC method was evaluated by two different analysts (Analyst I & Analyst II) with different instrument in the same laboratory. The % RSD for peak areas of cilostazol was calculated and was reported in **Table.6** and these results revealed the ruggedness of the developed method

i. SOLUTION STABILITY STUDIES: The stability studies for cilostazol (30 $\mu g.mL^{-1}$) in mobile phase were carried out at various time intervals for 24hrs at 35ºC. From these studies it was found that the analyte (Cilostazol) was stable in mobile phase for 24hrs that indicating the reliability of analysis in the proposed procedure (**Table.7)**.

j. ASSAY OF CILOSTAZOL IN FORMULATIONS: The assay of cilostazol in formulations [**Cilokem; 50mg of Cilostazol**] was established by injecting solution of sample formulation and with the present chromatographic conditions developed within the concentration range of linearity previously determined for standard. All determinations were carried out in triplicate and the average drug content was found to be 99.92% of the labeled claim. The result of this study was shown in **Table. 8** respectively.

CONCLUSION

In the present study, a new stability indicating RP-HPLC assay method for cilostazol was developed by following the ICH guidelines. Good reproducibility was obtained for the deduced calibration plot that was determined by calculating the slope, intercept and %RSD. This chromatographic assay fulfilled all the requirements for being a reliable and feasible method, including accuracy, linearity, recovery and precision. The method was found to be robust as there was no significant change in the peak area and retention time. Therefore, this validated RP-HPLC method can be reliably adopted for routine quality control analysis of cilostazol in tablets form.

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TABLE 1: SYSTEM SUITABILITY PARAMETERS FOR CILOSTAZOL BY THE PROPOSED METHOD

S.NO .	PARAMETERS	RESULTS
1.	THEORETICAL PLATES (N)	3329
2.	TAILING FACTOR	1.12
3.	RETENTION TIME	3.262
4.	PEAK AREA	5470042

TABLE 2: LINEARITY STUDIES FOR CILOSTAZOL BY THE PROPOSED METHOD

LINEARITY OF RESPONSE FOR CILOSTAZOL		
% LEVEL (APPROX.)	CONCENTRATION (µg/mL)	AVERAGE AREA
25	5.0	1383806
50	7.5	2761172
75	10.0	4118865
100	12.5	5468271
125	15.0	6841893
150	17.5	8196396
SLOPE		544623.073
INTERCEPT		-1331942.42
RSQ		0.9999
LOD (µg/mL)		0.0481
LOQ (µg/mL)		0.160

TABLE 3: RESULTS OF METHOD PRECISION BY THE PROPOSED METHOD

S.No	NAME	RT	AREA
1	**SOLUTION-1**	3.261	5516324
2	**SOLUTION-2**	3.264	5518520
3	**SOLUTION-3**	3.265	5511324
4	**SOLUTION-4**	3.268	5506523
5	**SOLUTION-5**	3.261	5475635
6	**SOLUTION-6**	3.258	5475166
AVG*		3.263	5500582
STD DEV*		0.004	19942.9
% RSD*		0.109	0.36

*Values are the averages of six determinations

TABLE 4: RECOVERY STUDIES (ACCURACY) OF CILOSTAZOL

S.No	50%	100%	150%
INJECTION-1	2760324	5467645	8195944
INJECTION-2	2758465	5462036	8193210
INJECTION-3	2749568	5460645	8190231
AVG*	2756119	5463442	8193128.333
AMT RECOVERED*	50.39	99.84	149.80
%RECOVERY*	100.78	99.84	99.87

*****Values are the averages of three determinations

TABLE 5: RESULTS OF ROBUSTNESS STUDY

ROBUST CONDITIONS		CILOSTAZOL		
		THEORETICAL PLATES	RT	PEAK AREA
FLOW RATE	0.8ml/min	3693	3.600	6030243
	1.2 ml/min	3650	3.583	6054128
TEMPERATURE	33°C	3346	3.264	5462354
	37°C	3367	3.252	5451236

TABLE 6: RESULTS OF RUGGEDNESS STUDIES OF CILOSTAZOL

		ANALYST-1		ANALYST -2	
S NO	NAME	RT	AREA	RT	AREA
1	INJECTION-1	3.261	5516324	3.259	5436458
2	INJECTION-2	3.264	5518520	3.261	5437025
3	INJECTION-3	3.265	5511324	3.263	5439630
4	INJECTION-4	3.268	5506523	3.260	5442315
5	INJECTION-5	3.261	5475635	3.265	5450245
6	INJECTION-6	3.258	5475166	3.265	5452154
AVG*		3.263	5500582	3.262	5442971
STD DEV*		0.004	19942.9	0.003	6732.1
% RSD*		0.109	0.36	0.079	0.124

*Values are the averages of six determinations

TABLE 7: RESULTS OF STABILITY STUDIES OF CILOSTAZOL IN MOBILE PHASE AT CONCENTRATION OF 15mcg/ mL

MEAN±SD CONCENTRATION OF CILOSTAZOL IN MOBILE PHASE AT AMBIENT TEMPERATURE(N=6)					
At 0hr	**At 2hr**	**At 6hr**	**At 12hrs**	**At 20hrs**	**At 24hrs**
99.98±0.16	99.96±0.23	99.92±0.85	99.98±0.67	99.98±0.56	99.99±0.73

TABLE 8: RESULTS OF ANALYSIS OF CILOSTAZOL IN FORMULATIONS

PHARMACEUTICAL FORMULATION	AMOUNT OF CILOSTAZOL		% RECOVERY
	LABELLED	FOUND*	
CILOKEM	**50 mg**	49.96	99.92 %

*Values are the averages of three determinations.

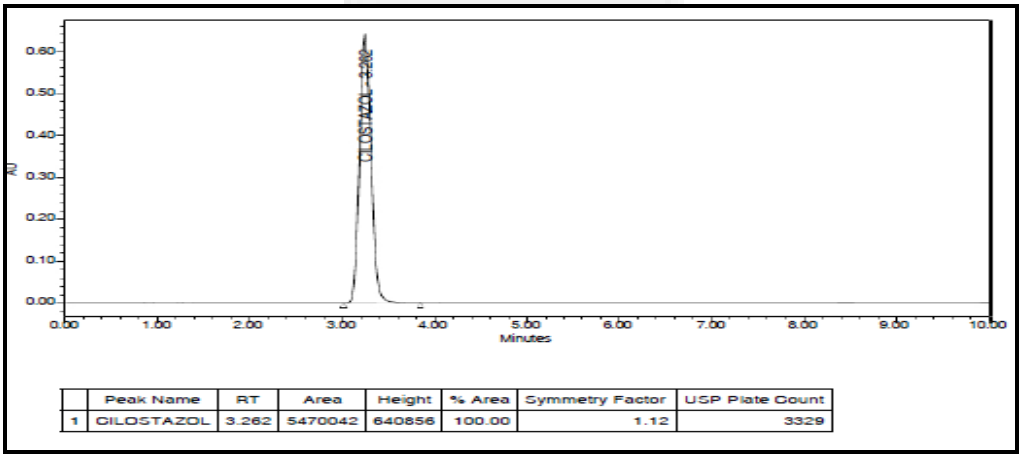
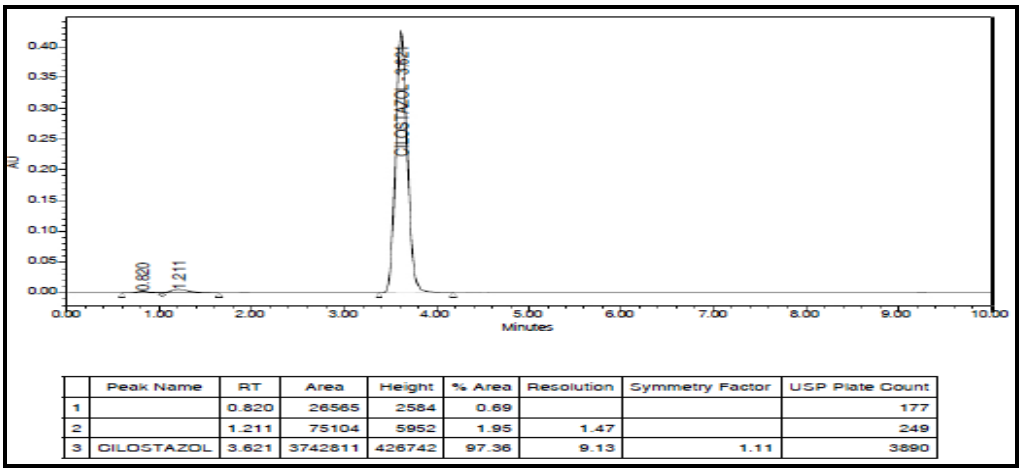
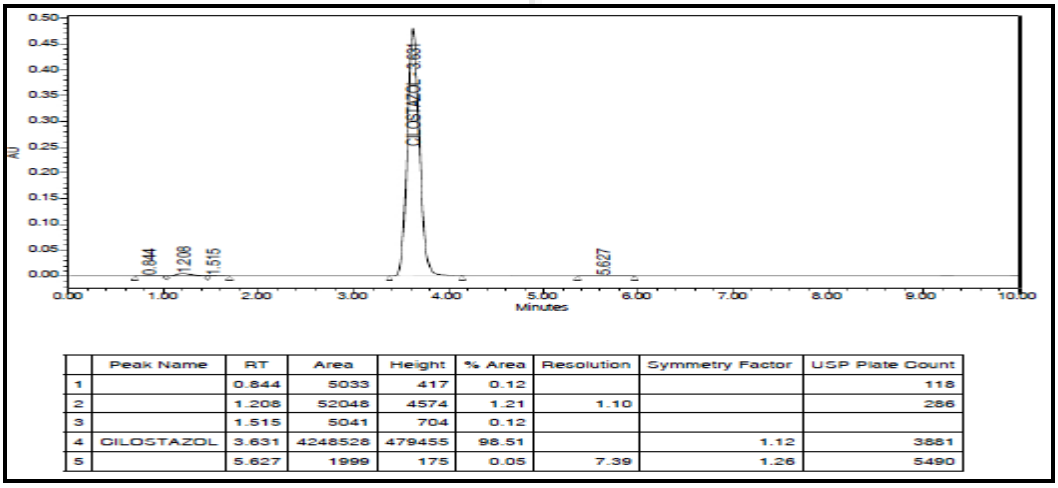


FIGURE 1: TYPICALCHROMATOGRAM OF CILOSTAZOL STANDARD



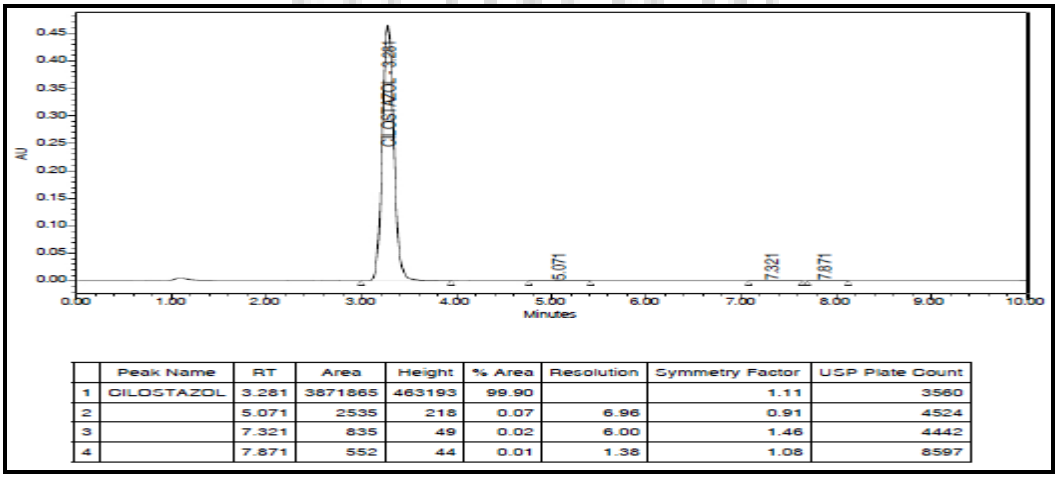
	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		0.820	26565	2584	0.69			177
2		1.211	75104	5952	1.95	1.47		249
3	CILOSTAZOL	3.621	3742811	426742	97.36	9.13	1.11	3890

FIGURE 2.a. CHROMATOGRAM OF ACIDIC FORCED DEGRADATION STUDY



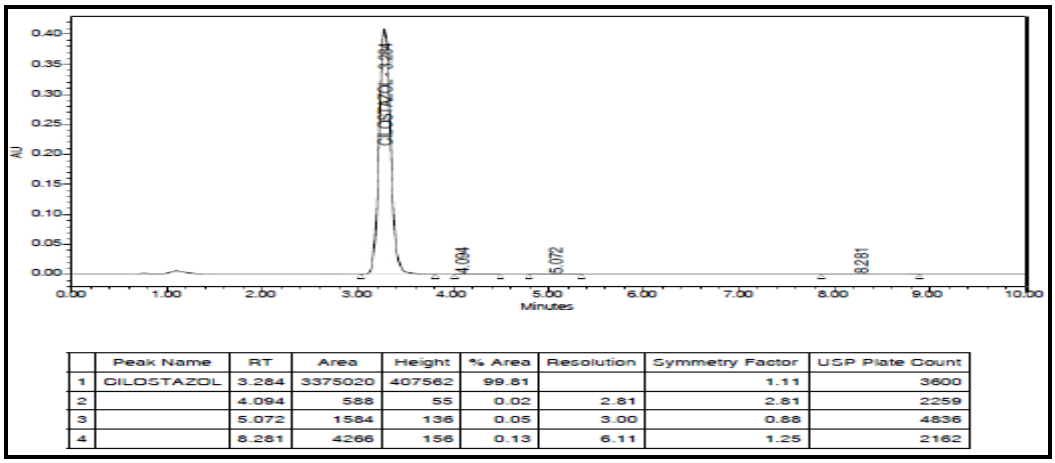
	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		0.844	5033	417	0.12			118
2		1.208	52048	4574	1.21	1.10		286
3		1.515	5041	704	0.12			
4	CILOSTAZOL	3.631	4248528	479455	98.51		1.12	3881
5		5.627	1999	175	0.05	7.39	1.26	5490

FIGURE 2.b. CHROMATOGRAM OF ALKALI FORCED DEGRADATION STUDY



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1	CILOSTAZOL	3.281	3871865	463193	99.90		1.11	3560
2		5.071	2535	218	0.07	6.96	0.91	4524
3		7.321	835	49	0.02	6.00	1.46	4442
4		7.871	552	44	0.01	1.38	1.08	8597

FIGURE 2c. CHROMATOGRAM OF UV- LIGHT DEGRADATION STUDY



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1	CILOSTAZOL	3.284	3375020	407562	99.81		1.11	3600
2		4.094	588	55	0.02	2.81	2.81	2259
3		5.072	1584	136	0.05	3.00	0.88	4836
4		8.281	4266	156	0.13	6.11	1.25	2162

FIGURE 2d. CHROMATOGRAM OF THERMAL DEGRADATION STUDY

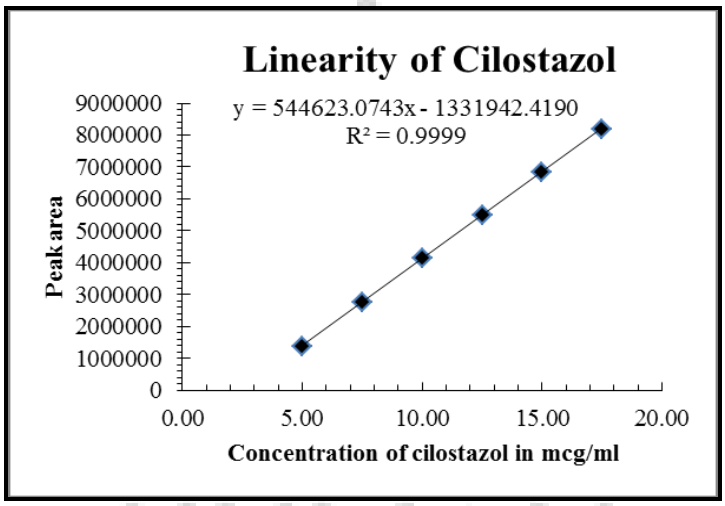


FIGURE 3: LINEARITY CURVE FOR CILOSTAZOL