ISSN : 0974-7419 Volume 12 Issue 2

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 12(2) 2013 [76-82]

Development and validation of stability-indicating high-performance thin-layer formulation chromatography method for estimation of cilostazole in bulk and in pharmaceutical formulation

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ABSTRACT

A simple, selective, precise and Stability-indicating High-performance thin-layer chromatographic method for analysis of Cilostazole both in a bulk and in pharmaceutical formulation has been developed and validated. The method employed, HPTLC aluminium plates precoated with silica gel as the stationary phase. The solvent system consisted of toluene: ethyl acetate: methanol: ammonia (3.5:2:0.8:0.3 *v/v/v*). The system was found to give compact spot for Cilostazole (R_f value of 0.52±0.02). Densitometric analysis of Cilostazole was carried out in the absorbance mode at 258 nm. The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.992±0.0001 with respect to peak area in the concentration range 300 - 1800 ng per spot. The mean value ± S.D. of slope and intercept were 4.6809 ± 0.005 and 2284.4 ± 4.20 with respect to peak area. The method was validated for precision, recovery and robustness. The limits of detection and quantification were 15.69 and 45.07 ng per spot, respectively. Cilostazole was subjected to acid and alkali hydrolysis, oxidation and thermal degradation. The drug undergoes degradation under acidic and basic conditions. This indicates that the drug is susceptible to acid and base. The degraded product was well resolved from the pure drug with significantly different R_f value. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of investigated drug. The proposed developed HPTLC method can be applied for identification and quantitative determination of Cilostazole in bulk drug and pharmaceutical formulation.

KEYWORDS

Cilostazole;
HPTLC;
Validation;
Stability;
Degradation.

INTRODUCTION

The parent drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH)[1] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability-indicating and should be fully validated. Cilostazol, 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl) butoxyl]-3, 4-dihyro-

2(1*H*)-quinolinone is aphosphodiestrase inhibitor with antiplatelet and vasodilation activity[1] (Figure 1). Cilostazol is selective CAMP phosphodiestrase inhibitor. It inhibits platelet aggregation and is direct arterial vasodilator its main effects are dilation of arteries supplying blood to legs and decreasing platelet coagulation. Literature survey revealed one spectrophotometric and few chromatographic[2-8] methods for estimation of Cilostazol in bulk, pharmaceutical formulations and biological fluid. However to our knowledge no information related to the stability indicating high performance thin-layer chromatography (HPTLC) determination of cilostazol in pharmaceutical dosage forms has ever been mentioned in literature. HPTLC is a widely used analytical technique due to its advantages of low operating cost, high sample throughput, and minimum sample preparation requirement. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus reducing the analysis time and cost per analysis[9,10]. Hence, the objective of the present study was to develop a stability-indicating HPTLC method[11] for estimation of cilostazol as bulk drug and in formulations and to perform stress studies under a variety of ICH-recommended test conditions[1,12]. The proposed method was validated for linearity, ac-curacy (recovery studies), specificity, precision, robustness, ruggedness, LOD (limit of detection), LOQ (limit of quantitation), and repeatability according to the ICH guidelines[13,14] and its updated international convention[15].

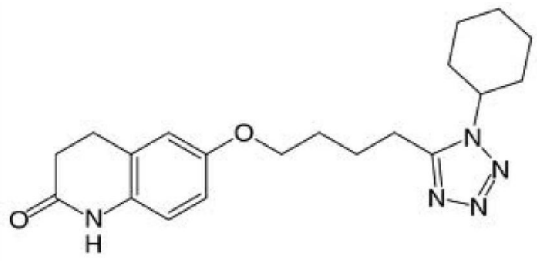

Figure 1 : Chemical structure of cilostazole

EXPERIMENTAL

Chemicals and reagents

Cilostazole was supplied as a gift sample from Glenmark pharmaceutical Ltd., Nasik, India. All chemicals and reagents used were of Analytical grade and were purchased from Merck Chemicals, India.

HPTLC instrumentation

The samples were spotted in the form of bands of 6 mm width with a Camag microlitre syringe on precoated silica gel aluminium plates 60 RP-18 F_{254} (10 × 10 cm with 250 mm thickness, E. Merck), using a Camag Linomat 5 applicator. The plates were prewashed with methanol and activated at 60 ^{0}C for 5 min prior to chromatography. The slit dimension was kept at 6.00 × 0.45 mm (micro) and 20 mm/s scanning speed was employed. The mobile phase consisted of toluene: ethyl acetate: methanol: ammonia (3.5:2:0.8:0.3 *v/v/v*), and 10 ml of mobile phase was used. Linear ascending development was carried out in a 10 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25^{0}C±2). The length of the chromatogram run was approximately 8 cm. Subsequent to development; the TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on a Camag TLC scanner 3 and was operated by WINCats software.

Preparation of standard solution and linearity study

An accurately weighed quantity of 10 mg Cilostazole was transferred to 10 ml volumetric flask, dissolved in methanol and volume was made up to mark with the same solvent to obtain concentration 1000 ng/µl. Aliquots of standard solutions 0.3, 0.6, 0.9, 1.2,1.5 and 1.8µl of Cilostazole was applied on TLC plate with the help of microlitre syringe, using Linomat 6 sample applicator to obtained the concentration of 300, 600, 900, 1200, 1500 and 1800 ng per spot. The standard curves were evaluated for within day and day-to-day reproducibility. Each experiment was repeated six times.

Method validation

Accuracy

The analysed samples were spiked with extra 80, 100 and 120% of the standard Cilostazole and the mixture were analysed by the proposed method. At each level of the amount, six determinations were performed. This was done to check the recovery of the drug at different levels in the formulations.

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Precision

Repeatability of sample application and measurement of peak area were carried out using six replicates of the same spot (1000 ng per spot of Cilostazole). The intra and inter-day variation for the determination of Cilostazole was carried out at three different concentration levels of 600, 900 and 1200 ng per spot.

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition of toluene-ethyl-acetate-methanol-ammonia (3:2:0.8:0.3 and 4.5:1.5.1:0.3, v/v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ±5%. The plates were prewashed by methanol and activated at 60±5^{0}C for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20 and 40 min.

Ruggedness

Ruggedness of the method was performed by spotting 500 ng of Cilostazole by two different analyst keeping same experimental and environmental conditions.

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for Cilostazole in sample was confirmed by comparing the R_f values and spectra of the spot with that of standard. The peak purity of Cilostazole was assessed by comparing the spectra at three different levels, i.e., peak start (*S*), peak apex (*M*) and peak end (*E*) positions of the spot.

Limit of detection (LOD) and limit of quantification (LOQ)

In order to determine detection and quantification limit, Cilostazole concentrations in the lower part of the linear range of the calibration curve were used. Cilostazole solutions of 900, 1000, 1100, 1200, 1300 and 1400 ng/spot were prepared and applied in triplicate. The LOQ and LOD were calculated using equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where, N is standard deviation of the peak areas of the drugs (n=3), taken as a measure of noise, and B is the slope of the corresponding calibration curve.

Application of proposed method to tablet formulation

To determine the concentration of Cilostazole in tablets (labeled claim: 50 mg per tablet), the contents of 10 tablets were weighed, their mean weight determined and they were finely powdered. The powder equivalent to 50 mg of Cilostazole was weighed. The drug from the powder was extracted with methanol. To ensure complete extraction of the drug, it was sonicated for 30 min and the volume was made up to 100 ml. The resulting solution was filtered using 0.41 µm filter (Millifilter, Milford, MA). The above solution (500ng per spot) was applied on TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate.

Forced degradation of cilostazole

Acid and base induced degradation

The 10 mg of Cilostazole was separately dissolved in 10 ml of methanolic solution of 1M HCl and 1M NaOH. These solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The 1ml of above solutions was taken and neutralized, then diluted up to 10 ml with methanol. The resultant solution were applied on TLC plate in triplicate (1.5 µl each, i.e. 1500 ng per spot). The chromatograms were run as described in Section 2.2.

Hydrogen peroxide-induced degradation

The 10 mg of Cilostazole was separately dissolved in 10 ml of methanolic solution of hydrogen peroxide (10.0%, v/v). The solution was kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The resultant solution was applied on TLC plate in triplicate (1 µl each, i.e. 1500 ng per spot). The chromatograms were run as described in Section 2.2.

Photochemical degradation

The photochemical stability of the drug was also studied by exposing the stock solution to direct sunlight for 8 h. The resultant solution (1 µL, i.e. 1,000 ng per spot) was applied on a TLC plate and chromatograms were run as described in section 2.2.

Dry heat degradation product

The powdered drug was stored at 55^{0}C for 3 h under dry heat condition showed no significant degra-

dation. In all degradation studies, the average peak areas of Cilostazole after application (1500 ng per spot) of three replicates were obtained.

RESULTS AND DISCUSSION

Development of optimum mobile phase

TLC procedure was optimized with a view to developing a stability-indicating assay method. Initially, Toluene: ethylacetate: methanol (3.5:2:0.8:*v/v/v*) gave good resolution with R_f value of 0.55 for Cilostazole but typical peak nature was missing. Finally, the mobile phase consisting of Toluene: ethylacetate: methanol: ammonia (3.5:2:0.8:0.3 *v/v/v/v*) gave a sharp and well defined peak at R_f value of 0.55 (Figure 2). Well-defined spots were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature.

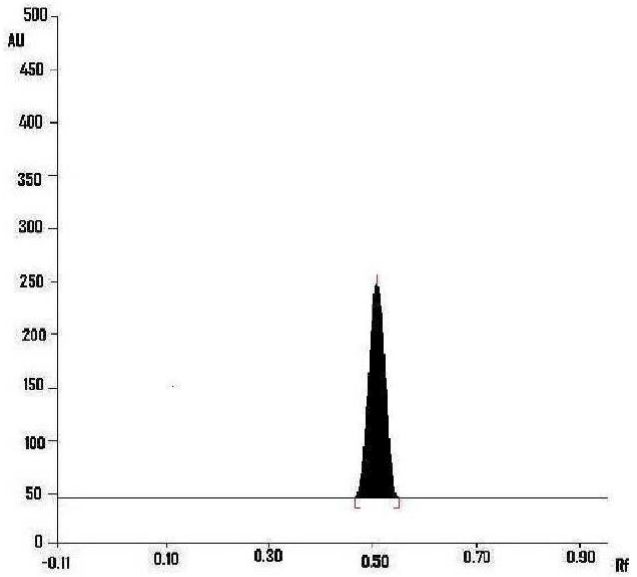

Figure 2 : Chromatogram of standard cilostazole (R_f: 0.55), UV detection at 258 nm, mobile phase- toluene: ethyl acetate: methanol: ammonia (3.5:2:0.8:0.3 *v/v/v*).

Calibration curve

The linear regression data for the calibration curves showed good linear relationship over the concentration range 300 – 1800 ng/spot (TABLE 1). Linear regression equation was found to be Y = 4.6809X + 2284.4 (r^2 = 0.9992).

Validation of method

Precision

The precision of the developed HPTLC method was expressed in terms of % relative standard deviation (% R.S.D.). The results depicted revealed high precision of the method is presented in TABLE 2.

TABLE 1 : Linear regression data for the calibration curve

Linearity range (ng per spot)	300 -1800
R^2 ± S.D.	0.9992 ± 0.0001
Slope ± S.D	4.6809 ± 0.005
Confidence limit of slope	2.526 – 2.540
Intercept ± S.D	2284.4 ± 4.20
Confidence limit of intercept	2275-2293

LOD and LOQ

Detection limit and quantification limit was calculated by the method as described in section 2.4.2. The LOQ and LOD were found to be 45.67 and 15.07ng respectively. This indicates that adequate sensitivity of the method.

TABLE 2 : Intra-day and inter-day precision of HPTLC method

Amount ng/spot	Mean Area	S.D.	%R.S.D.	S.E.
Intra-day precision*				
600	5088.5	0.4604	0.46	0.26
900	6488.26	0.354	0.35	0.16
1200	7913.06	0.2179	0.62	0.12
Inter-day precision*				
600	5088.62	0.4615	0.46	0.26
800	6485.93	0.354	0.35	0.28
1000	7924.11	0.3330	0.62	0.19

***mean of three determinatons**

Recovery studies

The proposed method when used for extraction and subsequent estimation of Cilostazole from the pharmaceutical dosage form after over spotting with 80, 100 and 120 % of additional drug; afforded good recovery of Cilostazole. The amounts of drug added and determined and the % recovery are listed in TABLE 3.

Specificity

The peak purity of Cilostazole was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., *r*2 (*S*, *M*) = 0.9998 and *r*2 (*M*, *E*) = 0.9988. Good correlation (r^2 = 0.9989) was also obtained between standard and sample spectra of Cilostazole (Figure 3).

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TABLE 3 : Recovery studies

Initial amount (ng)	Excess drug added to the analyte (%)	*Amount recovered (ng)	% Recovery	% R.S.D.	S.E.
500	0	500.18	100.02	0.52	0.13
500	400	900.98	100.10	0.28	0.16
500	500	998.31	99.83	0.55	0.32
500	600	1095.47	99.58	0.27	0.15

***mean of three determinations**

Robustness of the method

The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low values of %R.S.D. values as indicated are shown in (TABLE 4) indicated robustness of the method.

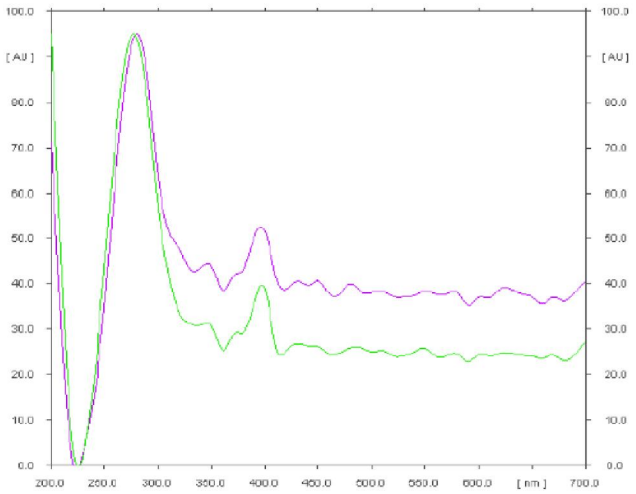

Figure 3 : Chromatogram of standard cilostazole (R_f: 0.55), UV detection at 258 nm, mobile phase- toluene: ethyl acetate: methanol: ammonia (3.5:2:0.8:0.3 *v/v/v*).

Analysis of the marketed formulation

A single spot at R_f 0.55 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients commonly present in the tablet. The % drug content and % RSD were calculated. The low % RSD value indicated the suitability of this method for the routine analysis of Cilostazole in pharmaceutical dosage forms.

Force degradation

The chromatogram of the acid degraded samples for Cilostazole showed additional peak at R_f value of 0.10, 0.44 and 0.48 (Figure 4) and base degraded drug shows at 0.09, 0.32 and 0.46 (Figure 5), and hydrogen peroxide shows at 0.09 and 0.19 (Figure

TABLE 4 : Robustness of the method*

Parameters	SD of peak area	%RSD
Mobile phase composition		
Toluene- ethylacetate-methano-ammonia (3.5:2:0.8:0.3, *v/vv/v*)	12.34	0.26
Toluene-Ethyl acetate- Methanol-ammonia (4.5:1.5:1:0.2, *v/vv/v*)	24.81	0.54
Mobile phase volume		
6.6 mL	16.96	0.36
13.2 mL	13.57	0.29
Development distance		
7 cm	12.34	0.26
7.5 cm	9.67	0.20
8 cm	4.54	0.098
Relative humidity		
55	9.88	0.21
65	5.67	0.12
Duration of saturation		
10 min	24.59	0.54
20 min	14.62	0.31
25 min	7.77	0.16
Activation of prewashed TLC plates		
8 min	10.3	0.22
10 min	5.74	0.12
12 min	3.06	0.06
Time from spotting to chromatography	9.89	0.21
Time from chromatography to scanning	15.70	0.37

*** mean of six determinations**

TABLE 5 : Forced degradation of cilostazol

Sample exposure condition	Number of degradation products (Rf values)	Cilostazol remained (1500 ng/spot)	S.D.	Recovery (%)
1 M HCl, 8h, RT	3 (0.14,0.23,0.39)	958.25	5.13	63.88
1 M NaOH, 8h, RT	3 (0.13, 0.36,0.44)	1062.29	7.84	70.81
10 % H2O2, 8h, RT	2 (0.12,0.19)	1119.31	4.93	74.61
Photo, 8 h	No degradation	1496.01	4.28	99.73
Heat, 3H, 55°C	No degradation	1487.34	6.43	99.15

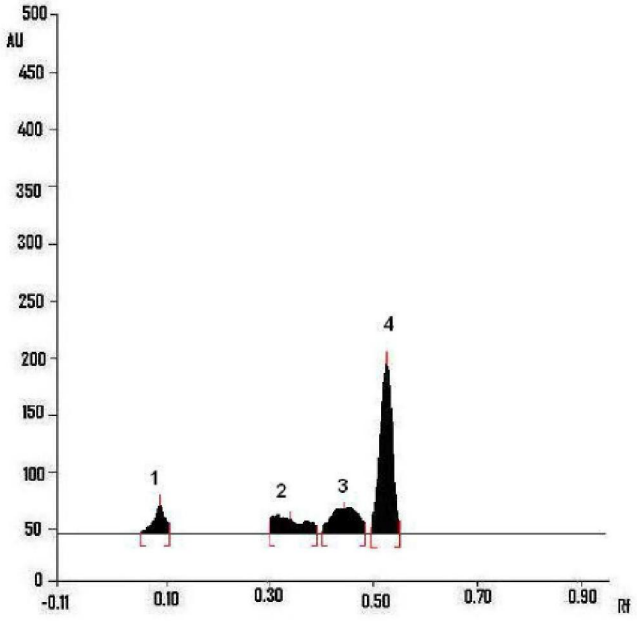

Figure 4 : HPTLC chromatogram of acid (1 N HCl, 8h, R.T.) treated cilostazole; peak1 (impurity) (R_f: 0.10), peak 2 (impurity) (R_f: 0.44), peak 3 (impurity) (R_f: 0.48), peak4 (Cilostazole) (R_f: 0.55)

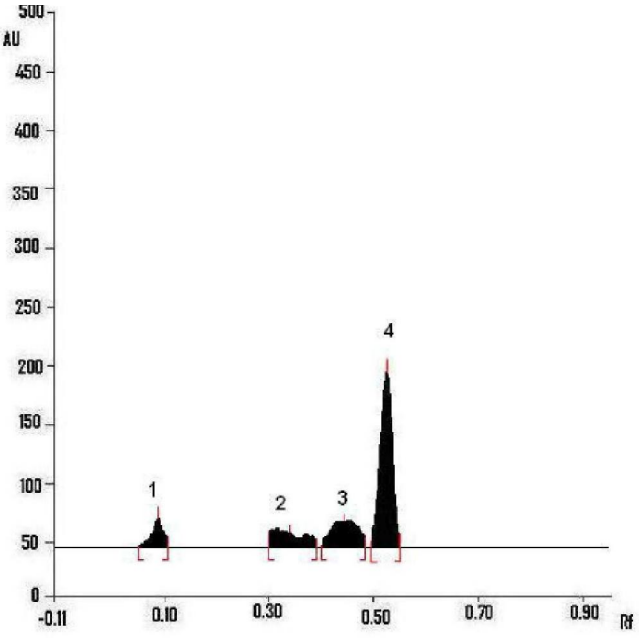

Figure 5 : HPTLC chromatogram of base (1N NaOH, 8h, R.T.) treated cilostazole; peak1 (impurity) (R_f: 0.09), peak 2 (impurity) (R_f: 0.32), peak3 (impurity) (R_f: 0.46), peak4 cilostazole (R_f: 0.55)

6) respectively. The spot of the degraded product was well resolved from the Cilostazole spot. In both cases, the concentration of the drug was changing from the initial concentration, indicating that Cilostazole undergoes degradation under acidic, basic and oxidative

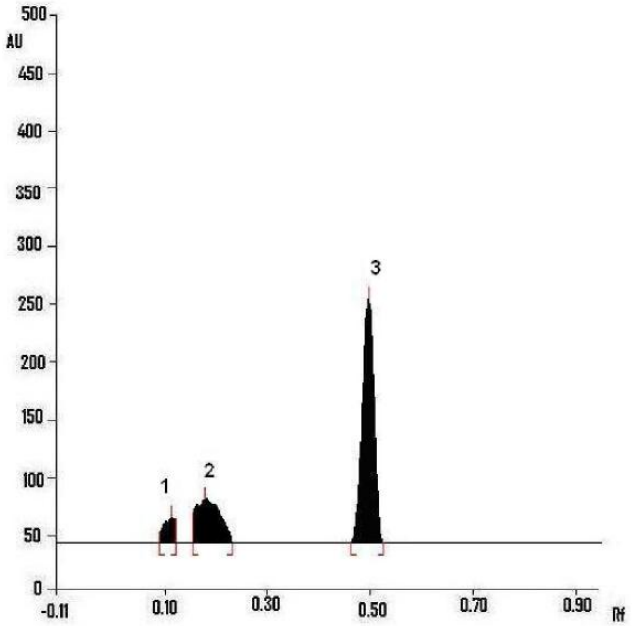

Figure 6 : HPTLC chromatogram of hydrogen peroxide (10% w/v, 8 h, R.T.) treated Cilostazole; peak1 (impurity) (R_f: 0.09), peak 2 (impurity) (R_f: 0.19), peak 3 (Cilostazole) (R_f: 0.55)

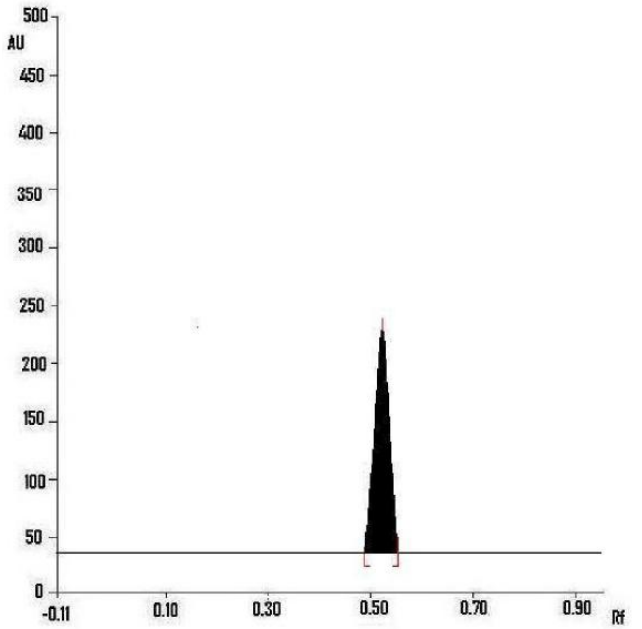

Figure 7 : HPTLC chromatogram of photo degraded (8 h) treated Cilostazole; peak 1 (Cilostazole) (R_f: 0.55)

conditions. The chromatograms of photo-degraded (Figure 7) and dry heat (Figure 8) samples of Cilostazole showed only the spots of the pure drug. The lower R_f values of degraded components indicated that they were less polar than the analyte itself. The results are listed in TABLE 5.

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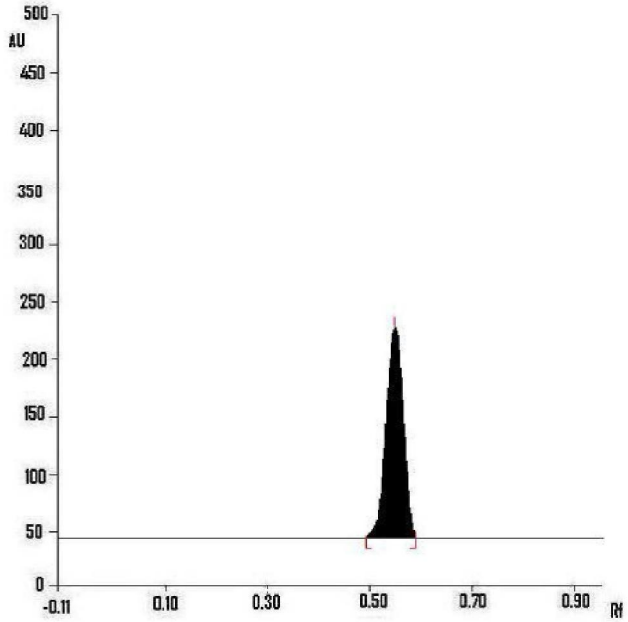

Figure 8 : HPTLC chromatogram of heat treated cilostazole (3 h, R.T); peak 1 (Cilostazole) (R_f: 0.55)

TABLE 6 : Summary of validation parameter

Parameter	Data
Linearity range (ng spot^{-1})	300 – 1800
Correlation coefficient	0.9992
Limit of detection (ng spot^{-1})	15.69
Limit of quantitation (ng spot^{-1})	45.07
Recovery (n = 9)	99.83
Ruggedness (% R.S.D.)	
Analyst I	0.42
Analyst II	0.45
Precision (%R.S.D.)	
Repeatability of application (n = 6)	0.52
Inter-day (n = 6)	0.47
Intra-day (n = 6)	0.47
Robustness	Robust
Specificity	Specific

CONCLUSION

The developed HPTLC method was precise, specific, accurate and stability indicating and validated based on ICH guidelines. Statistical analysis proves that the method is repeatable and selective for the analysis of Cilostazole as bulk drug and in pharmaceutical formulations. The method can be used to determine the purity of the drug available from the various sources by detecting the related impurities. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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