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High performance thin-layer chromatographic selective and stability indicating method for assay of ciprofloxacin in pharmaceuticals

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Abstract

A sensitive, precise, selective and stability indicating high performance thin-layer chromatographic (HPTLC) method was developed and validated for the quantitative analysis of ciprofloxacin hydrochloride. The stationary phase employed was precoated silica gel 60F_{254} HPTLC aluminium sheets and the mobile phase consisted of dichloromethane: methanol: ammonia (30:60:10, v/v/v). Densitometric measurement was performed in reflectance/ absorbance mode at 279 nm. In the chromatogram, a sharp and well-defined symmetrical peak was obtained with R_f value of 0.62 ± 0.03. With the mobile phase employed in the study complete separation of ciprofloxacin from other structurally similar fluoroquinolones was elucidated; the degradation products of ciprofloxacin, formed under accelerated stress conditions, are also well resolved. The linear regression analysis was deduced to be suitable for the calibration data in the concentration range 100-600ng spot^{-1}. The high throughput HPTLC method is convenient and precise for the quantitative analysis of ciprofloxacin hydrochloride in bulk drugs, tablets and counterfeit samples.

Key words: Ciprofloxacin, HPTLC, Stability indicating, Selective, Validation

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INTRODUCTION

Ciprofloxacin is a second-generation fluoroquinolone most commonly used as oral formulation that contain ciprofloxacin hydrochloride. The drug was approved for clinical use in 1987 [1] and since then it is an agent of choice for treatment of various infections for the reason that it is widely distributed to virtually all parts of the body. Apart from this, the high potency, broad spectrum of activity and reasonably acceptable safety profile have contributed to its wide usage [2].

The official method for the assay of ciprofloxacin is a RP-HPLC method (British Pharmacopoeia, 2004). Several methods have been reported in literature for the determination of

ciprofloxacin, in variety of matrices, including differential pulse polarography [3]; adsorptive stripping voltammetry [4]; HPLC with ultraviolet [5-7], fluorescence [8,9] and mass spectrometric detection [10,11]; spectrophotometry [12-15]; non-aqueous titrimetry [16]; ion-selective electrode [17]; capillary electrophoresis [18]; FT-Raman spectroscopy [19]; luminescence spectroscopy [20] and HPTLC [21]. The major advantage of HPTLC over all existing methods is that several samples can be run simultaneously using a small quantity of mobile phase. Thus, lowering the analysis time and cost per analysis, with high sample throughput [22]. The uniform particle size of precoated HPTLC plates enables achievement of a greater resolution and an easy reproducible separation. The reported HPTLC method [21] describes quantitative analysis of ciprofloxacin hydrochloride in coated tablets and includes limit test for its related compounds. The method although validated for assay and purity control of coated tablets does not display efficacy of mobile phase in bringing separation of ciprofloxacin from other structurally similar fluoroquinolones, especially norfloxacin.

The objective of this study was to develop an accurate, precise, selective and stability-indicating HPTLC densitometric method for the determination of ciprofloxacin in bulk drugs, tablets and counterfeit samples. The proposed method was validated as per ICH (International Conference on Harmonization) guidelines (ICH Q2 [R1], 2005).

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and aqueous solutions were prepared in doubly distilled water. The solvents used for preparing mobile phase were of chromatography grade. The standard of ciprofloxacin hydrochloride gifted by DRL (Hyderabad, India) was 99.72 % pure.

HPTLC Instrument & accessories

Camag HPTLC system (Switzerland) comprising of Automatic TLC sampler (ATS4), TLC Scanner 3, twin-trough chamber (20 × 10 cm), CATS 4 software and Reprostar 3 documentation system were used during this study.

Sample application and densitometric measurement

The sample was applied by spraying bands of 4 mm length, 10 mm apart and 15 mm away from border in all cases. The slit dimension was kept at 6 mm × 3 mm and 20 mm/s scanning speed was employed during densitometric measurement on Camag TLC scanner 3.

Mobile phase optimization

The mobile phase composition was optimized to separate ciprofloxacin from structurally similar compounds of fluoroquinolone class and its own degradation products.

1. Separation of drug from structurally similar compounds

For wider application of this HPTLC method including analysis of counterfeit sample, the mobile phase system must display complete separation of ciprofloxacin from structurally similar compounds of the fluoroquinolone class.

2. Separation of degradation products: Stress study

To validate the stability indicating power of the analytical procedure, the separation of drug from its degradation products must also be displayed. According to the International Conference on Harmonization (ICH) guidelines (ICH Q1A [R2], 2003) entitled ‘Stability testing of new drug

substances and products, stress testing should include the effect of temperature, moisture, susceptibility to oxidation and photolysis to elucidate the inherent stability of the active substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension (ICH Q1A [R2], 2003). Examining degradation products under accelerated stress conditions is useful in developing and validating analytical procedures. A 1000µg mL^{-1} drug stock solution was subjected to various stress conditions as described below

Wet heat-induced degradation
To 5 mL of drug stock solution, 5 mL of distilled water was added and the solution was refluxed for 3 hr in boiling water bath (in dark). The final volume was adjusted with methanol to 25 mL.

Hydrogen peroxide-induced degradation
To 5 mL of drug stock solution, 5 mL of hydrogen peroxide (H_2O_2) (30%, v/v) was added and the solution was refluxed for 3 hr at 80 $^{\circ}$C in dark. The final volume was adjusted to 25 mL with methanol.

Acid- and base-hydrolysis
To 5 mL of drug stock solution, 5 mL of 5N HCl and 5 mL of 5N NaOH were added separately. These mixtures were refluxed for 3 hr at 80 $^{\circ}$C in dark. After cooling the solution was neutralized with alkali/acid and its final volume was adjusted to 25 mL with methanol.

UV degradation
The standard in solid state was exposed to 254 nm UV radiation for 8 hr in a UV-chamber. Later, from irradiated solid, solution of drug was prepared.

Photochemical
5 mL of drug stock solution was diluted with methanol to 25 mL. The photochemical stability of the drug was studied by exposing this solution to direct sunlight for 72 hr.

Dry heat-induced degradation
The solid drug standard was kept in oven at 100 $^{\circ}$C for 8 hr to study the inherent thermal stability of the drug. The solution of this dry heat exposed drug was prepared for further analysis.

These stressed samples were applied on HPTLC plate and the chromatograms were run along with the freshly prepared *"standard drug solution"*.

Method validation
Validation of the method was performed according to the International Conference on Harmonization guidelines 'Validation of Analytical Procedures' (ICH Q2 [R1], 2005) by evaluating following parameters

Precision
To evaluate repeatability of the sample application and measurement of peak area, system precision was determined by calculating deviation of six replicates at one concentration level (middle of linearity range). For method precision, the intra-day and inter-day variation for the determination of drug was estimated at three different concentration levels (n = 6) covering complete calibration range. The results are expressed in terms of relative standard deviation (RSD)

Robustness
Robustness is a measure of the capacity of a method to remain unaffected by small but deliberate variations in the method conditions. It is an indication of the reliability of the method. The robustness of proposed method was evaluated by studying effect of small changes in the mobile phase composition, mobile phase volume and chamber saturation time on chromatogram. Robustness of the method was studied in triplicate at one concentration.

Sensitivity
The detection and quantitation limits were determined by applying decreasing amounts of standard solution of drug to the plate. The densitometric measurements were made after development of the plate. The detection limit was the minimum concentration at which the peak area was reliably integrated. The quantitation limit was the minimum concentration at which the analyte was quantified with acceptable accuracy and precision.

Accuracy
The accuracy of the method was determined by use of standard additions at three different levels, i.e. multiple-level recovery studies. Recovery studies were carried out by analyzing drug samples to which known amount of standard corresponding to 50, 100 and 150% of the drug label claim had been added, prior to the extraction step. At each concentration level, six determinations were performed.

Stability of drug solution and drug applied on plate
The drug solutions of 400 µg mL^{-1} were prepared at different time intervals prior to analysis *viz* 72, 48, 24, 12 ,6 and 0 hr and stored at 25°C in normal daylight condition. All of them were applied (1 µL) on a plate and developed simultaneously. The peak areas of spots were compared with zero hr spot, considering its intensity 100% and the deviation was calculated.

The stability of drug applied (1 µL) on the plate was studied by making measurement on a spot after time lapse of 0, 1, 2, 3, 6, 12 and 24 hr from the point of application.

Specificity
To verify the specificity of the method, the standard and sample spots were analyzed 1) by comparing their R_f values and 2) by comparing spectra of their spots. The peak purity of the sample was assessed by comparing the spectra at three different levels, *viz*. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Optimized chromatographic conditions
The experimental conditions for chromatographic development were: stationary phase precoated silica gel $60F_{254}$ HPTLC aluminium sheets (20 × 10 cm) (prewashed with methanol, and activated by heating at 60 °C for 10 min); mobile phase, dichloromethane – methanol - ammonia (30:60:10, v/v/v); chamber saturation time, 20 min; temperature, 25 ± 2 °C; migration distance, 60 mm; wavelength of detection, 279 nm.

Calibration curve
A stock solution containing 1.1102 mg mL^{-1} ciprofloxacin hydrochloride (~ 1 mg ciprofloxacin) was prepared in 1:1 methanol and water mixture. The stock solution was diluted to obtain 100 µg mL^{-1} standard solution. A series of spots was obtained by applying 1, 2, 3, 4, 5 and 6 µL standard solution on HPTLC plate, covering the concentration range 100–600 ng $spot^{-1}$ ciprofloxacin. For each concentration level, three spots were applied on the plate. The curve of peak area against ng

amount was treated by different regression models: linear least-square, second order polynomial and third order polynomial.

Sample Preparation

Twenty tablets were powdered, an amount of powder equivalent to 100 mg ciprofloxacin was transferred to a 100 mL calibrated volumetric flask and extracted with 1:1 methanol-water mixture for 10 min by shaking mechanically. The solution was diluted to volume with the same solvent and filtered. The sample solution is diluted ten times and 5 µL of diluted solution is applied on the plate (n=5).

RESULTS AND DISCUSSION

Mobile phase optimization

The TLC method was optimized in view to develop a selective and stability indicating procedure. While performing scouting runs it was observed that in order to elute fluoroquinolones presence of ammonia or acid was essential. Further, if they were in insufficient quantity double spots or long tail was observed, which is due to different ionic forms of drugs [25]. Therefore, most of the mobile phases tried contained about 10-20% ammonia. The selectivity of mobile phase system was adjudged by separation of ciprofloxacin from other structurally similar fluoroquinolones, especially norfloxacin. The mobile systems tried for bringing separation of ciprofloxacin (C) from norfloxacin (N) are given in Table 1.

Table 1. R_f values obtained for ciprofloxacin (C) and norfloxacin (N) using various mobile phases (A to E) on silica gel 60F_{254} HPTLC sheets

S. No.	Mobile system (v/v)	R_f	
		C	N
A	Methanol-ammonia (70:30)	0.54	0.51
B	Chloroform- methanol-ammonia (25:60:15)	0.58	0.52
C	Dichloromethane-methanol-ammonia (30:60:10)	0.62	0.51
D	Dichloromethane-isopropanol-methanol-ammonia (23:39:23:15)	0.40	0.36
E	Dichloromethane-isopropanol-methanol-ammonia (27:33:26:14)	0.45	0.38

Table 2. 'Overall data quality' of regression analyses of calibration curve of ciprofloxacin

Degree of polynomial	Regression coefficient	% RSD of fit	N
1	0.9988	2.12	18
2	0.9977	1.81	18
3	0.9987	1.41	18

When methanol and ammonia mixture was used the spot was diffused. The spot became compact by using chloroform and dichloromethane as modifiers. Dichloromethane as a modifier provided better resolution as can be seen for system C. In literature, mobile phases D and E have been reported for separation of ciprofloxacin and norfloxacin [25]. In these systems by increasing proportion of dichloromethane, the separation improves (for E; ΔR_f = 0.07), but the best separation was achieved with system C (ΔR_f = 0.11). The efficacy of mobile phase C in bringing separation of ciprofloxacin from other fluoroquinolones is illustrated in Figure 1. This system has been selected for further study as it also brings separation of ciprofloxacin from its degradation products obtained under different stress conditions (Figure 2). By observing

intensity of the ciprofloxacin peak in the figure, it is apparent that with acid and base treatment the damage is highest; followed by oxidation treatment and dry heating; wet heat, UV and photochemical treated samples have suffered lesser damage. In all stressed samples, impurity peaks have emerged in the chromatogram, but they are all well resolved. The shape of ciprofloxacin peak has not deformed in any stressed sample. Further, spectra of principal peak of stressed samples are identical to the spectrum of untreated standard. Thus, selectivity and stability indicating attribute of method is elucidated.

The chromatogram of ciprofloxacin obtained after development with dichloromethane: methanol: ammonia (30:60:10) gave a sharp and well-defined symmetrical peak with $R_f = 0.62 \pm 0.03$ (Figure 3).

Figure 1. Separation of ciprofloxacin from other fluoroquinolones on silica gel 60F_{254} HPTLC sheets with dichloromethane – methanol - ammonia (30:60:10, v/v) as mobile phase. Plate image under 254 nm illumination

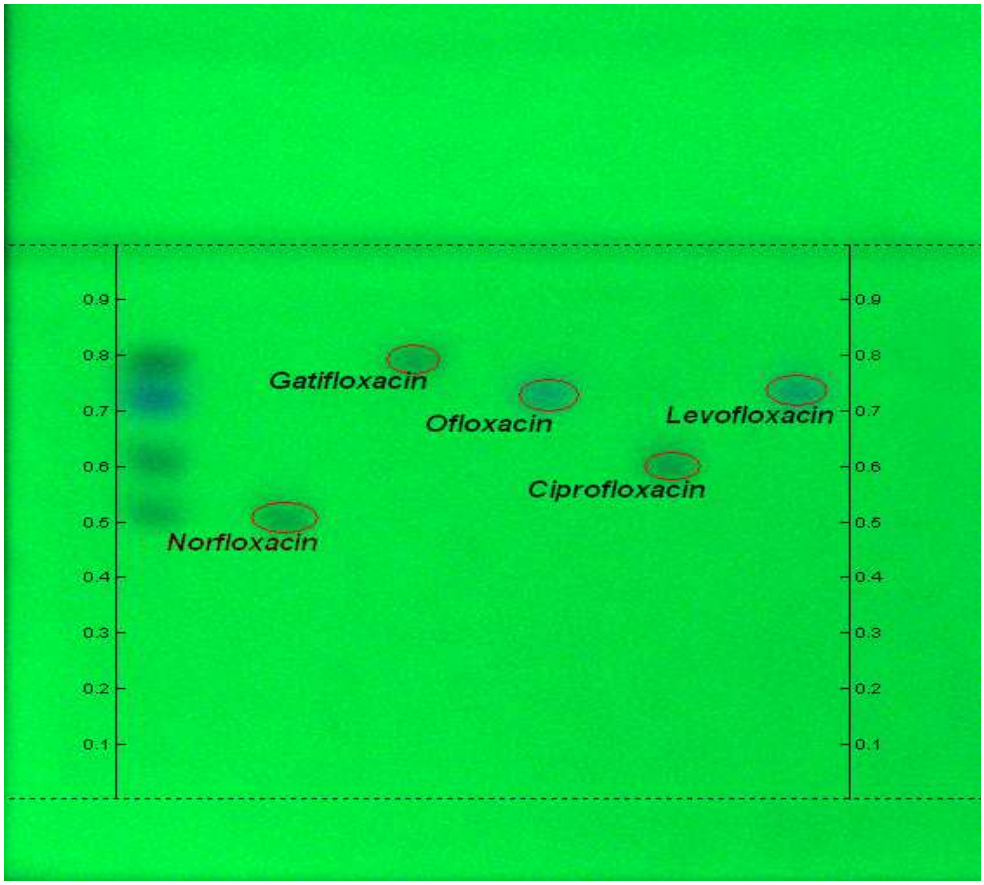


Figure 2. Chromatogram of standard ciprofloxacin and stressed samples recorded at 279 nm

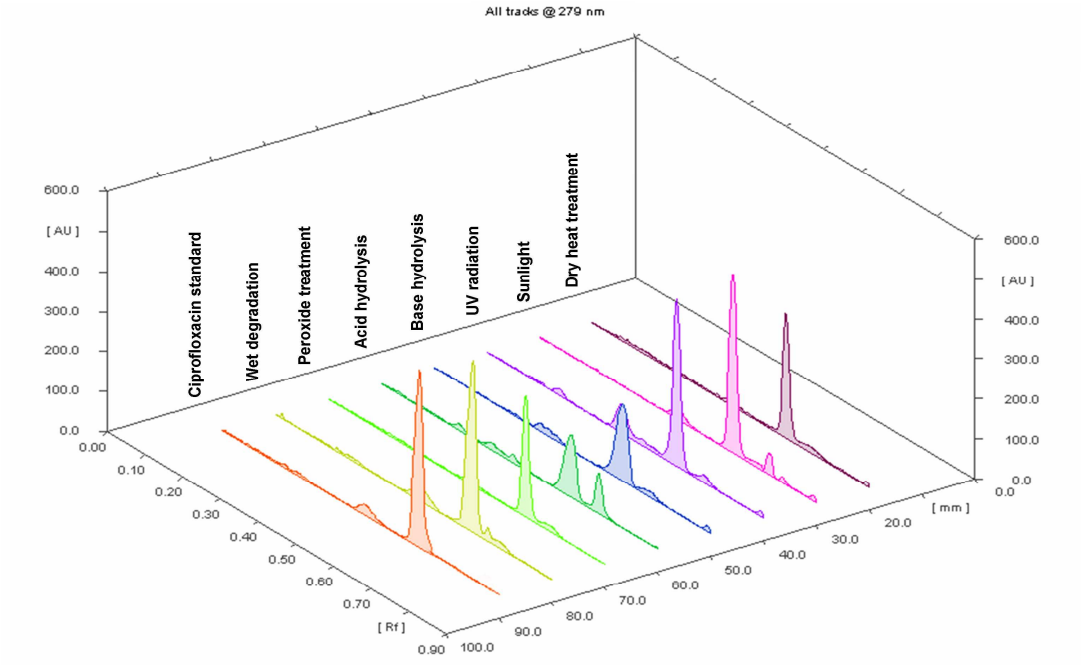


Figure 3. Chromatogram of standard ciprofloxacin (R_f = 0.62)

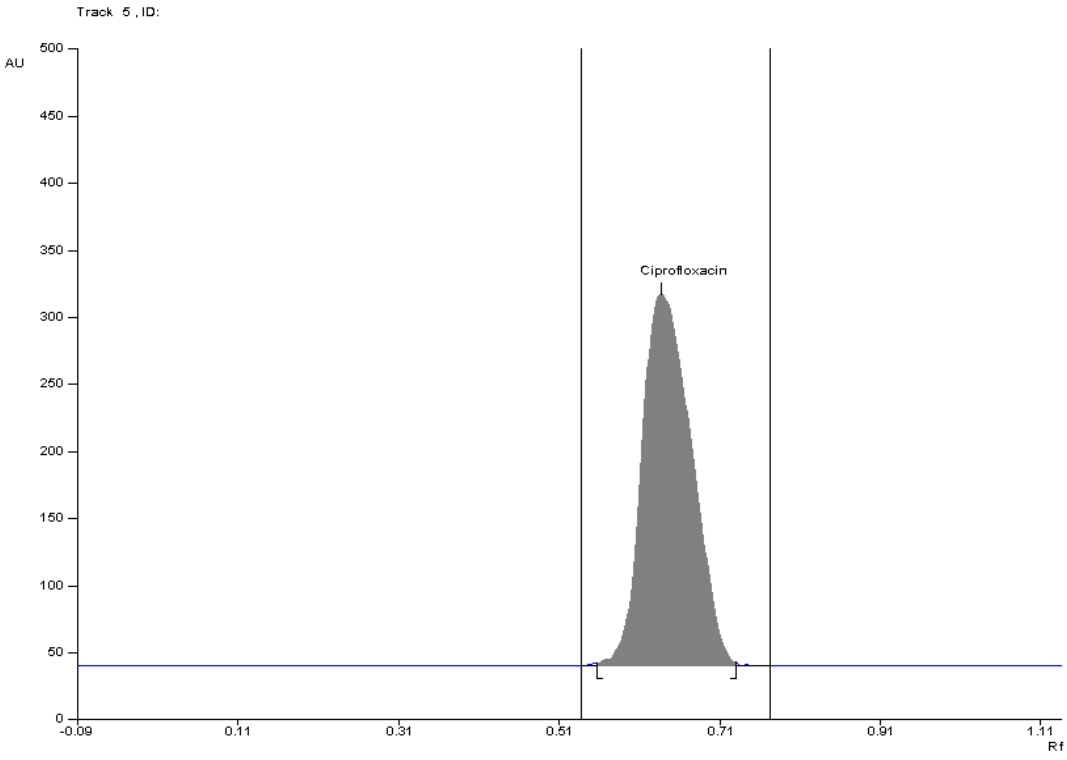
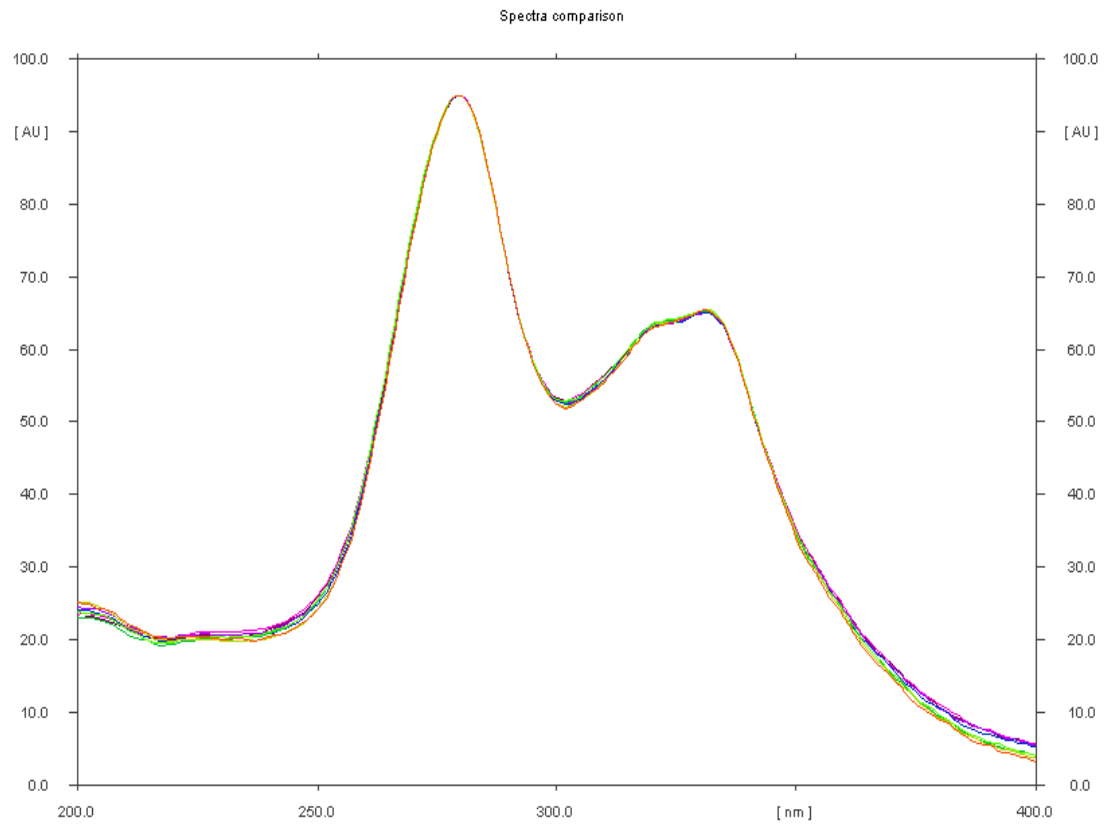


Figure 4. Spectra comparison of standard ciprofloxacin and tablet sample



Calibration curve for quantitative analysis of ciprofloxacin

In the concentration range 100 to 600 ng spot^{-1}, the calibration plot exhibit perfect linear relationship between peak area and concentration of ciprofloxacin as indicated by very high correlation coefficient and low %RSD (Table 2). On using second or third order polynomial fitting models, the %RSD reduces to some extent, but as such there is no improvement in correlation coefficient value. Therefore, linear least square curve fitting was found to be suitable for calibration data of ciprofloxacin. The data of linear regression analysis *viz* slope, intercept and correlation coefficient, their standard deviations and confidence ranges are provided in Table 3. No significant difference was observed in the slopes of standard curves (ANOVA, $P > 0.05$).

Table 3. Linear regression data for calibration curve (n= 3) of ciprofloxacin

Linearity range	100 – 600 ng spot^{-1}
Curve fitting equation	Y = A + B*X
Regression coefficient (r ± S.D.)	0.9988 ± 0.0001
Intercept ± S.D.	1732.9 ± 117.1
Confidence limit intercept[a]	1441.9 to 2023.9
Slope ± S.D.	24.412 ± 0.301
Confidence limit of slope[a]	23.665 to 25.159

[a] *95% Confidence limit*

Table 4. Intra-day and inter-day precision of HPTLC method of ciprofloxacin

Concentration [ng/spot]	Intraday precision [%RSD, n = 5]	Concentration [ng/spot]	Interday precision [%RSD, n = 5]
125	0.56	150	2.03
300	0.71	400	0.66
450	1.11	525	0.69

Table 5. Robustness of HPTLC method (n=3; 350 ng spot^{-1}) of ciprofloxacin

Parameter varied	R_f	% RSD	SE (%)
Mobile phase composition (Dichloromethane-methanol-ammonia, v/v)			
31:60:9	0.60	0.93	1.88
29:60:11	0.65	0.62	1.26
25:65:10	0.65	0.26	0.53
35:55:10	0.63	1.01	2.04
Mobile phase volume (for 10 × 10 cm chamber)			
9 mL	0.61	0.26	0.52
11 mL	0.64	0.93	1.89
Duration of chamber saturation			
20 min	0.63	0.82	1.65
40 min	0.64	0.50	1.01

Method validation

Precision

The assessment of repeatability of sample application and measurement of peak area was performed at one concentration (250 ng spot^{-1}) using ciprofloxacin standard. The relative standard deviations were found to be 0.21 and 0.13% (n = 6) respectively.

The method precision was expressed in terms of intra-day variation and inter-day variation. Intra-day precision was determined by analyzing ciprofloxacin standard solution at three concentrations 125, 300 and 450 ng spot^{-1} on the same day. Inter-day precision was assessed by analyzing ciprofloxacin standard solution at three concentrations 150, 400 and 525 ng spot^{-1} on different days over a period of one week. The results of the precision studies are shown in Table 4. The precision of the method was satisfactory.

Robustness

The reliability of method was assessed by varying mobile phase composition, volume and chamber saturation time; and studying its impact on R_f value and precision in determination of 350 ng spot. The method is found to be sufficiently reliable as the R_f (0.62 ± 0.03) do not change significantly and the precision is good (Table 5)

Table 6. Recovery studies performed on HPTLC method of ciprofloxacin (n = 5)

Standard added to analyte (%)	Theoretical amount (ng)	Amount found (ng)	Recovery (%)	%RSD (n=5)	SE
0	200	199.35	99.68	0.82	0.73
50	300	301.76	100.59	1.27	1.71
100	400	399.16	99.79	0.79	1.42
150	500	501.30	100.26	0.65	1.47

Table 7. Assay of ciprofloxacin tablets by HPTLC method

Trade name	Label claim mg)	Average (mg)	Recovery %	%RSD	SE (%)
Ciprobiotic[a]	500	506.2	101.24	0.55	1.24
Maflo[b]	500	494.14	98.83	0.98	2.16
Dummy substandard (~200mg) [c]	500	199.20	39.84	0.70	0.63
Strox[d]	200	171.43	85.72	1.05	0.81
Maflo[e]	500	463.11	92.62	0.58	1.20

[a] Emcure, India (B.No. EDB06001); [b]Mapra, India (B.No. 03J01MS); [c]Admixture of standard with starch; [d]Dabur, India (B.No. 1033) - Exp. dt. Sep 04; [e] Mapra, India (B.No. 03J01M5) - Exp. dt. Feb 06

Sensitivity
The LOD and LOQ were found to be 4.5 ng spot^{-1} and 20 ng spot^{-1} indicating that the method is sufficiently sensitive for the content assay of drug.

Accuracy
The proposed method when used for estimation of ciprofloxacin from tablets, after spiking with 50, 100 and 150% of standard, afforded recovery between 99.68 and 100.59% (Table 6).

Stability of drug solution and drug applied on plate
The high stability of drug solution stored in dark at room temperature is evident from the analysis of a 100 µg mL^{-1} drug solution stored for 0, 6, 12, 24, 48 and 72 hr prior to measurement. The relative standard deviation for peak area determination was 0.88%. The stability of drug on silica was estimated by spotting drug solution (300 ng spot^{-1}) on a plate, storing the plate for 1, 2, 3, 6, 12, 24 and 48 hr in dark and then performing densitometric measurement at 279 nm. In this case, the relative standard deviation for peak area determination was 0.56%. Thus, it is inferred that ciprofloxacin has very high stability in solution and on plates, when stored in dark.

Specificity
The assessment of peak purity of ciprofloxacin by comparing the spectra of a tablet sample and the standard at peak start, peak apex and peak end positions indicated good correlation (r = 0.9984) between the standard and the tablet sample spectra (Figure 4).

Tablet analysis
The results of assay by the HPTLC method of two different marketed products, a dummy substandard sample (40%) which is an admixture of standard and starch, and two expired drug samples is shown in Table 7. A well resolved spot at R_f = 0.62 was observed in the chromatogram of ciprofloxacin samples extracted from tablets. There was no interference from the excipients present in the tablets. In case of two tablet brands A and B, the label claim matches with the observed drug content. Even for the dummy substandard sample C the content matches with the theoretical value. The content of ciprofloxacin in expired drug was less than the prescribed pharmacopoeia limit. The method was found to be accurate and precise for the quantitative analysis of bulk drugs, tablets and counterfeit samples.

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