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Development and Validation of HPTLC Method for Estimation of Roxithromycin in Tablet Dosage Form

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ABSTRACT

A new, simple, precise, accurate, high-performance thin-layer chromatography (HPTLC) method for the determination of Roxithromycin in tablet dosage form has been developed and validated. Roxithromycin from the formulation was separated on Silica gel 60 F_{254} HPTLC plates with a mobile phase consisting of Hexane: Ethyl acetate: Methanol in the ratio 7:2:1% (*V/V/V*). Densitometric analysis of Roxithromycin was performed at the wavelength of 254nm. Well-resolved bands were obtained with R_F values of 0.18 for Roxithromycin, respectively. As per ICH guidelines, the method was validated for specificity, precision, robustness, and recovery. The calibration curve was found to be linear in the concentration range of 375-1050 ng/band for Roxithromycin, respectively. A correlation coefficient is 0.9998 for Roxithromycin, respectively. The method is selective and specific, with potential application in the pharmaceutical analysis of Roxithromycin in a tablet dosage form.

INTRODUCTION

Roxithromycin is chemically (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,10*E*,11*S*,12*R*,13*S*,14*R*)-6-[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6methyloxan-2yl]-oxy-14-ethy-l7,12,13-trihydroxy-4[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy-10-(2-methoxyethoxymethoxyimino)-3,5,7,9,11,13-hexamethyl-oxacyclotetradecan-2-one (Fig.1). Roxithromycin is a semi-synthetic derivative of the macrolide antibiotic erythromycin which comprised an N-oxime side chain on the lactone ring, with antibacterial and anti-malarial activities. It is given orally in a dose of 75 and 150 mg twice daily before meals, in the treatment of susceptible infections. The drug is officially recognized in the European as well as the British pharmacopeia [1-7].

A literature survey reveals several numbers of methods involving infrared reflectance spectroscopy, liquid chromatography-mass spectrometry, plasma analysis, HPLC-ECD, and spectrofluorimetric determination of Roxithromycin are also reported[8-12]. Liquid chromatographic methods for the detection of Ambroxol hydrochloride in combination with Roxithromycin are also been reported [13-15].

However, there was no HPTLC method reported so far for the estimation of roxithromycin in a pharmaceutical tablet dosage form. This work aims to develop a novel validated, simple, rapid, accurate, precise, and sensitive HPTLC method for the estimation of roxithromycin in a tablet dosage form.

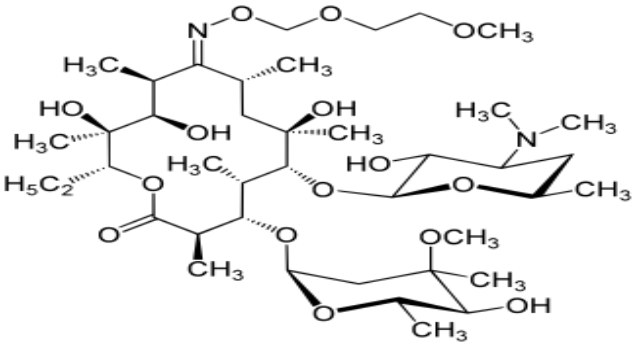

Fig. No. 1: Chemical Structure of Roxithromycin

MATERIALS AND METHODS

Instrumentation

The HPTLC system comprising of CamagLinomat V, sample applicator (CAMAG, Switzerland), coupled with Camag Hamilton Bonaduzmicrolitre syringe (100 µl), UV chamber with dual-wavelength UV lamps, and CAMAG TLC scanner 4 controlled by vision CATS software (CAMAG) was used for the application and detection of spots respectively. The Chromatographic separations of drugs were performed using pre-coated HPTLC plates (silica gel 60 F_{254}) 250 µm thickness; (Sigma-Aldrich) and a CAMAG twin-trough developing chamber was used for chromatographic development.

Chemicals and reagents

Reference standards of Roxithromycin and (Roxid 75 mg) formulation were obtained from CDTL (Mumbai, India). Analytical grade ethanol, 1-propanol, and 25% ammonia are from Finar Chemicals (Mumbai, India), and silica gel 60 F_{254} plates from Sigma-Aldrich (Mumbai, India) were used.

Chromatographic development

The Plates were prewashed with ethanol and activated at 110°C for 30 min before chromatography. For saturation of the chamber, 30 ml of the mobile phase was transferred into the development tank closed with a lid. The assembly was aside for 30 min under room temperature. The samples were spotted in the form of narrow bands having a length of 8 mm. The application positions X and Y were kept at 8 mm and 20 mm, respectively, to avoid edge effects. The distance between the two bands was 20 mm. Bands were applied at a constant rate of 15 nL/s using a nitrogen aspirator.

Linear ascending development of chromatogram was carried out in a Camag twin trough glass chamber saturated with the mobile phase consisting Hexane: Ethyl acetate: Methanol in the ratio 7:2:1 % (*V/V/V*) for 30 min and chromatogram run was kept up to 80 mm. Following the development, the HPTLC plates were dried in a stream of air with the help of an air dryer in a wooden chamber with adequate ventilation.

Spectro densitometric analysis of the separated components was carried out using Camag TLC Scanner 4 in the reflectance–absorbance mode at 254 nm and the source of radiation utilized was a deuterium lamp. The slit dimension used was 6.0 mm × 0.3 mm and sensitivity at auto mode. The scanning speed was 100 nm/s. The evaluation was achieved by linear regression of the peak area response against the amount of drug by using vision CATS (CAMAG) software for peak area measurement and data processing.

Selection of wavelength of detection

UV spectra were detected by scanning the solution of Roxithromycin between 200 to 400 nm and 254 nm was selected as the wavelength for the determination of Roxithromycin because, at this wavelength, both drugs have shown maximum absorbance. Overlain peak purity spectra of Roxithromycin were depicted in **Fig. 7, 8**.

Preparation of solutions

Reference Standard Stock solution of Roxithromycin in methanol

The reference standard of Roxithromycin was accurately weighed to 10 mg and transferred into a 20 ml volumetric flask, and diluted with methanol up to the mark to get a stock solution having the strength of 500 µg/mL.

Working standard solution of Roxithromycin in methanol

Transferred 15ml from Roxithromycin standard stock solution to a 100 ml volumetric flask and diluted up to mark with methanol to get a working standard solution of Roxithromycin (75 µg/mL).

Analysis of marketed formulation

For the determination of Roxithromycin in tablet dosage form (brand name: Roxid 75mg Tablet DT, containing Roxithromycin 75 mg, marketed by Alembic Pharmaceutical Ltd.), was used for analysis. One tablet of Roxithromycin (75 mg) was dissolved in a 100 ml volumetric flask with diluent and sonicated for 5 min and mixed thoroughly then diluted with methanol up to the mark to get a stock solution containing 750 µg/mL of Roxithromycin. The given solution was filtered by Whatman filter paper No. 4. Transferred 1 ml from Roxithromycin stock olution to a 10 ml volumetric flask and diluted up to mark with

methanol to get a solution of Roxithromycin (75 µg/mL). From the resulting solution, a part of the 5 µL solution was applied to the HPTLC plate at six different positions. The plate was developed and analyzed as per the optimized chromatographic conditions.

Method Optimization of HPTLC

Considering the chemical nature of Roxithromycin silica gel F_{254} TLC plates were used as stationary phase. Different mobile phases containing solvents such as methanol, ethanol, ethyl acetate, hexane, and 1-propanol, in different proportions were tried. Initially, trials were done with a mobile phase comprising Hexane: 1-propanol: ethyl acetate (6:2:2 *V/V/V*) but low R_F values were found and the peak shape is not good. Further trials were made by modifying the mobile phase with methanol. Finally, the mobile phase comprising Hexane: Ethyl acetate: Methanol in the ratio of (7:2:1*V/V/V*) was found to be optimum with good peak shape, better separation, and acceptable R_F values. Trials are depicted in **Fig. 2-4**.

Method Validation

To fortify the suitability of the method for its intended purpose, it was validated according to the ICH guidelines Q2 (R1) [15] and validation of the optimized HPTLC method was carried out concerning the following parameters.

Specificity

The specificity of the developed method was determined by analyzing reference standard drugs and test samples solutions containing Roxithromycin from marketed tablets to check interferences from formulation ingredients. The band for Roxithromycin in the sample was confirmed by comparing the R_F value and spectrum of the spot with that of a reference standard. The peak purity of Roxithromycin was determined by comparing the spectrum at three different regions of the spot, i.e., peak start(S), peak apex (M), and peak-end (E).

The results have shown that the purity exceeded 0.999 for all peaks, indicating the specificity of the method in the presence of various excipients.

Linearity

The linearity of the method was studied by injecting five concentrations of the drug and each concentration was applied three times to the HPTLC plates. The plate developed with the previously described mobile phase. The linearity of the response to Roxithromycin was assessed by plotting the mean peak area of Roxithromycin against concentration over the range of 375–1050 ng/band (n=3). The corresponding linear regression equation was y = 4E-06x - 0.0007for Roxithromycin. Calibration curves were found to be linear in the above concentration range with correlation coefficients of 0.9998 for Roxithromycin, respectively and the results are mentioned in **Table. 1**.

Precision

The precision of the method was verified by repeatability and intermediate precision studies and in terms of intraday and inter-day precisions. Intra-day and Inter-day were executed by carrying out the analysis of the reference standard drug at three different concentrations of 600, 750, and 900 ng/band for Roxithromycin. Intra-day precision (% RSD) was determined by the analysis of drugs three times on the same day. Inter-day precision (% RSD) was determined by the analysis of the same solution on three different days over 1 week. Repeatability was determined by applying the reference standard solution containing 750ng/band of Roxithromycin in six replicates. The developed method was found to be precise as the %RSD values for repeatability and intermediate precision studies were <2%, as recommended by ICH guidelines, and the results are mentioned in **Table 2,3.**

Sensitivity

The LOD and LOQ are calculated based on the standard deviation of the regression lines and slope of the calibration curves using the below equations:

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where σ is the standard deviation of the regression line and S is the slope of the calibration curve.

Using the trend line equations derived from the experiments, the sensitivity of the method in terms of LOD and LOQ was calculated based on the standard deviation of the regression lines and slope of calibration curves. The LOD and LOQ were found to be 20.49 and 62.08 ng/band, respectively for Roxithromycin.

Accuracy

The accuracy of the method was determined by [n=3] standard addition at three different levels, (110%, 120%, and 130% levels) and analyzed as per the proposed method. The proposed method showed good percentage recovery rates between 99.47-100.92 for Roxithromycin and the results are summarized in **Table 4.**

Robustness

To evaluate the robustness of the developed method, deliberate variations were made in the method parameters such as changing the mobile phase composition, the volume of the mobile phase, chamber saturation period and distance traveled and found no significant deviation in the results. The R_F and standard deviation of peak areas were calculated for each parameter and the %RSD was found to be <2%. The low values of the %RSD and no significant changes in the R_F, as the robustness of the method and the results are summarized in **Table 6**.

RESULTS AND DISCUSSION

A new, simple, accurate, fast, economic, and precise high-performance thin-layer chromatographic method was developed and validated for the estimation of Roxithromycin in a tablet dosage form.

The linearity by plotting the mean peak area of Roxithromycin against concentration over the range of 375–1050 ng/band. The corresponding linear regression equation was y = 4E-06x - 0.0007for Roxithromycin. Calibration curves were found to be linear in the correlation coefficients of 0.9998 for Roxithromycin, respectively, and the results are shown in **Fig. 6,9,** and **Table 1**.

The precision studies were executed by three different concentrations of 600, 750, and 1050 ng/band for Roxithromycin. Repeatability was determined by applying the reference standard

solution containing 750ng/band of Roxithromycin in six replicates. Results of the repeatability and intermediate precision experiments are shown in **Tables 2, and 3.**

The LOD and LOQ were found to be 20.49 and 62.08ng/band, respectively for Roxithromycin, indicating the sensitivity of the proposed method in the data mentioned in **Table 7**.

The proposed method showed good percentage recovery rates between 99.47-and 100.92 for Roxithromycin. The results of the recovery studies and their statistical validation are given in **Table 4**.

The peak purity of Roxithromycin was assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the band. The results shown in **Table 8** and depicted in **Fig. 6**, demonstrate that the purity exceeded 0.999 for all peaks, indicating the specificity of the method in the presence of various excipients.

The result of the robustness shows low RSD values and no significant change in R_f as in **Table 6.**

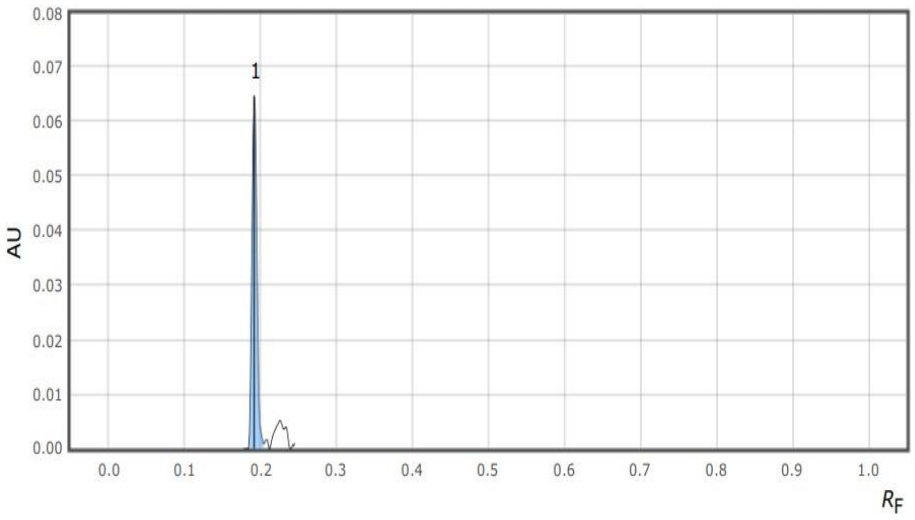

Fig.No. 2: Trial 1: Hexane: Ethyl acetate: 1-propanol (6:2:2*V/V/V*)

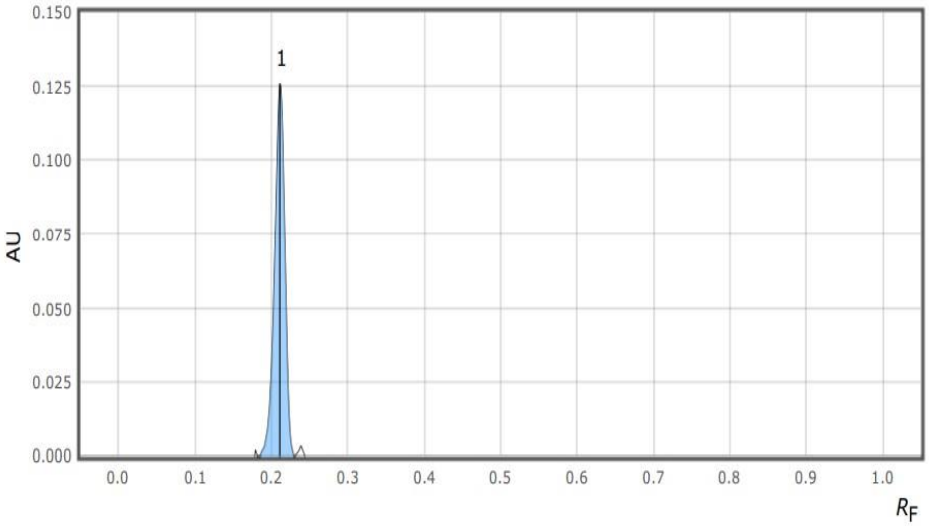

Fig.No. 3: Trial 2: Hexane: Ethyl acetate: Methanol (7:1:2*V/V/V*)

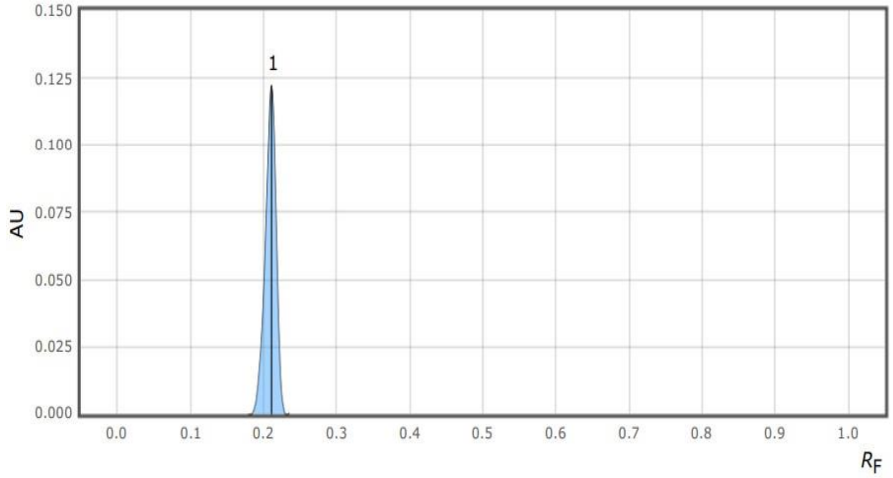

Fig. No. 4: Final HPTLC densitogram under optimized conditions showing R_f values of 0.18 for Roxithromycin (750 ng/band)

Hexane: Ethyl acetate: Methanol (7:2:1*V/V/V*)

Fig.No. 5: Image of HPTLC plate at 254 nm

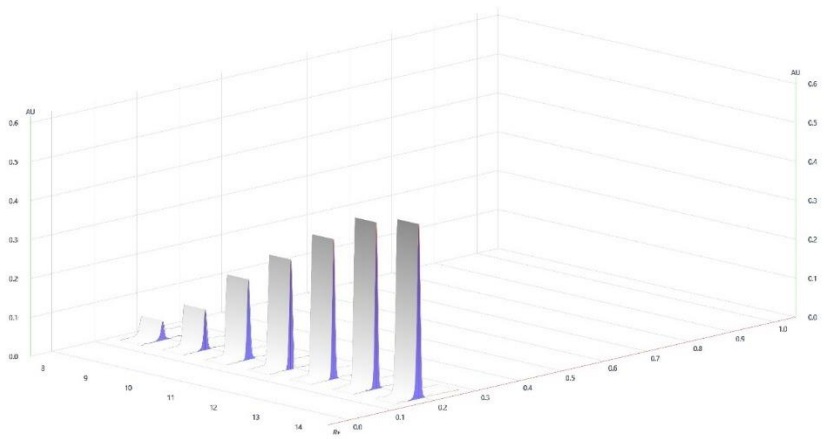

Fig. No. 6: Three-dimensional densitogram for the linearity of Roxithromycin at 254 nm

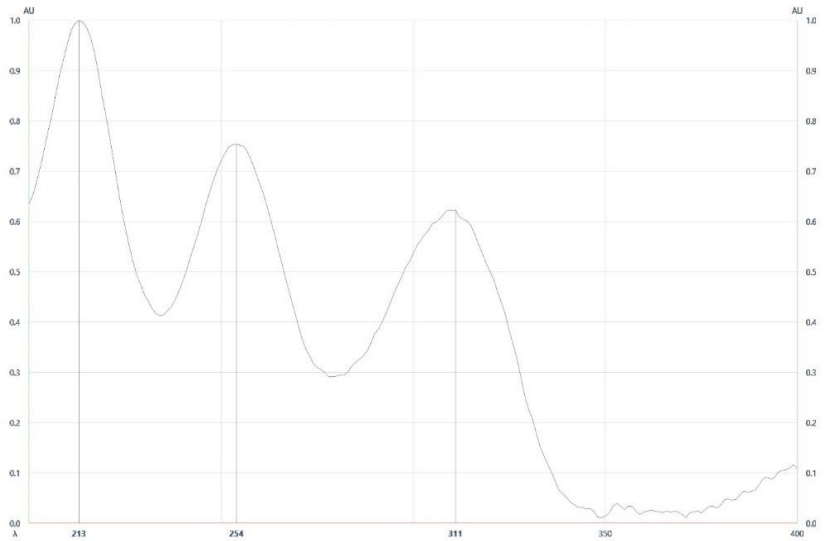

Fig. No.7: UV spectra of Roxithromycin

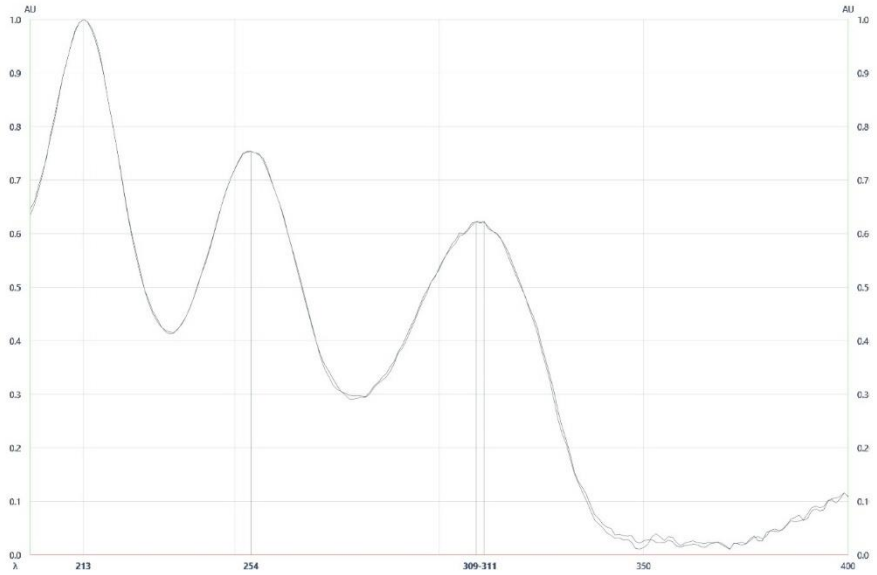

Fig.No. 8: Peak purity spectra of Roxithromycin

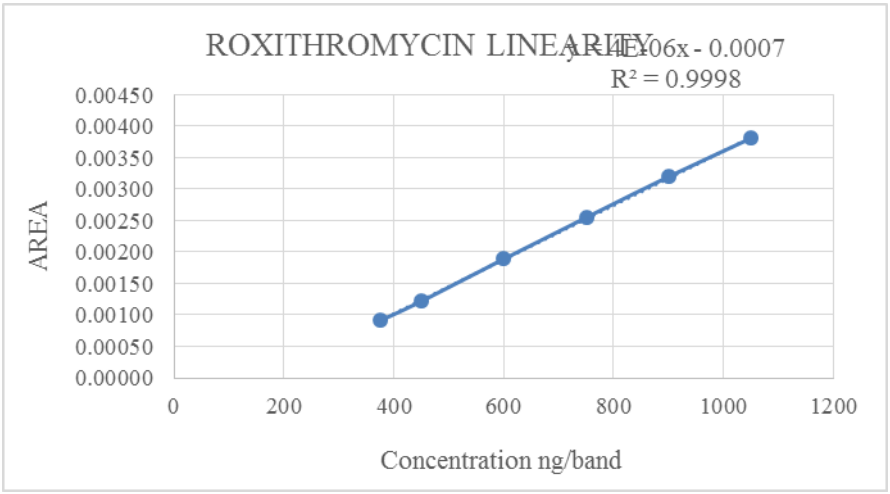

Fig.No. 9: Calibration curve of Roxithromycin (750-1050 ng/spot)

Table No. 1: Linearity data of Roxithromycin

Concentration (ng/band)	Peak Area
375	0.00091
450	0.00122
600	0.00189
750	0.00255
900	0.00320
1050	0.00381

Table No. 2: The result from the determination of precision Roxithromycin as repeatability

Concentration (ng/band)	Peak Area
750	0.00259
	0.00252
	0.00255
	0.00256
	0.00255
	0.00258
Average (n=6)	0.002558333
SD	0.0000248
RSD (%)	0.970662178

Table No. 3: The result from the determination of precision Roxithromycin

Roxithromycin Concentration (ng/band)	Intra-day precision		Inter-day precision	
	Peak area SD (n=3)	%RSD	Peak area SD (n=3)	%RSD
600	0.00187 ±0.0000231	1.23	0.00162 ±0.0000153	0.93
750	0.00255 ±0.0000252	0.98	0.00233 ±0.0000306	1.03
900	0.00323 ±0.0000251	1.08	0.00288 ±0.0000200	0.69

Table No. 4: Result from recovery data of Roxithromycin

% LEVEL	Amount Spiked (ppm)	Amount Recovered (mg/tab)	%Recovery	Mean % Recovery	SD	%RSD
100	750	754	100.38	99.47	0.7567	0.7580
	750	747	99.27			
	750	751	98.78			
110	915	916	100.43	99.97	0.6638	0.6638
	915	917	100.35			
	915	909	99.15			
120	930	937	100.59	100.43	0.3507	0.3484
	930	933	100.07			
	930	935	100.63			
130	945	957	101.18	100.92	0.2743	0.2729
	945	953	100.89			
	945	956	100.69			

Table No. 5. Analysis of marketed formulation

Drug	Label claim (mg/tablet)	Amount found (mg)	Label claim Estimated (%)	(%) RSD
Roxithromycin	75	74.95	99.47	0.75

Table No. 6: Robustness results of proposed HPTLC method

Change in the mobile phase ratio (7:2:1 ± 0.2 in methanol content)				
Drug	Ratio	R_F	Area ± SD (ng/band)	%RSD
Roxithromycin	7:2:0.8	0.182±0.02	0.00253 ±0.0000248	0.85
	7:2:1	0.184±0.02	0.00258 ±0.0000255	0.97
	7:2:1.2	0.186±0.02	0.00261 ±0.0000258	1.1
Change in chamber saturation time (40 min ± 5)				
Drug	Saturation time (min)	R_F	Area ± SD (ng/band)	%RSD
Roxithromycin	35	0.183±0.02	0.00253 ±0.0000243	0.89
	40	0.184±0.02	0.00255 ±0.0000247	0.92
	45	0.185±0.02	0.00257 ±0.0000249	0.95
Change in mobile phase volume (20 ± 5)				
Drug	Volume (ml)	R_F	Area ± SD (ng/band)	%RSD
Roxithromycin	15	0.184±0.02	0.00256 ±0.0000245	0.86
	20	0.185±0.02	0.00257 ±0.0000248	0.93
	25	0.187±0.02	0.00259 ±0.0000251	0.97
Change in distance traveled (80mm ± 5)				
Drug	Distance traveled (mm)	R_F	Area ±SD (ng/band)	%RSD
Roxithromycin	75	0.183±0.02	0.00252 ±0.0000243	0.92
	80	0.184±0.02	0.00255 ±0.0000245	0.97
	85	0.186±0.02	0.00258 ±0.0000247	0.99

Table No. 7: LOD and LOQ results of Roxithromycin

Sr. No.	Area
1	0.00259
2	0.00252
3	0.00255
4	0.00256
5	0.00255
6	0.00258
Average	0.0025583
SD	0.0000248
% RSD (Limit NMT 2%)	0.97
Regression equation	y = 4E-06x - 0.0007
slope	0.000004
LOD	20.49
LOQ	62.08

Table No. 8: Analytical Validation parameters for Roxithromycin using the HPTLC method

Parameters	Roxithromycin
Linearity	
Linearity range (ng/band)	500-1050
Correlation coefficient (r2)	0.9998
Precision (%RSD)	
Repeatability	0.97
Intra-day precision	0.98-1.23
Inter-day precision	0.69-1.03
Sensitivity	
LOD (ng/band)	20.49
LOQ (ng/band)	62.08
Specificity	
r (S, M)	0.9994
r (M, E)	0.9997
Accuracy	
100	99.47 ±0.75
110	99.97 ±0.66
120	100.43 ±0.34
130	100.92 ±0.27

CONCLUSION

The present method was found to be simple, specific, precise, accurate, reliable, and selective, saving both cost and time. This study reports a simple, fully validated HPTLC protocol for the quantification of Roxithromycin in pharmaceutical formulation. It demonstrates that the method can accurately quantify the drug content of the tablet formulation without excipient interference or the necessity of a drug extraction step before analysis. Thus, the developed method can be implemented in quality control laboratories for the routine analysis of the drug in their pharmaceutical formulation.

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