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Research Article

Stability Indicating Method Development and Validation of Ranolazine Hydrochloride in Bulk and Tablet Dosage Form by HPTLC.

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ABSTRACT
A novel stability indicating high performance thin layer chromatography HPTLC assay method was developed and validated for quantitative determination of Ranolazine Hydrochloride in bulk drugs and in pharmaceutical dosage form in the presence of degradation products generated from forced degradation studies. The present study is completed by using precoated silica gel aluminium plate 60 F – 254, (20 × 10 cm) with 250 µm thickness, as stationary phase and the mobile phase consisted of chloroform : methanol : toluene (5 : 1 : 1 v/v/v). The detection was carried out at the wavelength of 273nm. RAN was subjected to stress conditions of hydrolysis (acid, base, neutral), oxidation, photolysis, and thermal degradation. Degradation was observed for RAN in acid, base and in oxidation conditions. The drug was found to be stable in the other stress conditions attempted. The degradation products were well resolved from the main peak. The developed method was validated with respect to linearity, range, precision, repeatability, LOD and LOQ, robustness, specificity, and recovery. The analysis of the marketed product and the forced degradation studies prove the stability-indicating power of the method.
Keywords: Ranolazine Hydrochloride, HPTLC, Degradation, Validation.

INTRODUCTION

Ranolazine Hydrochloride (RAN) is an anti-anginal drug and chemically it is a piperazine derivative. Structurally it is N- (2, 6- dimethylphenyl) -2- [4- [2-hydroxy-3- (2-methoxyphenoxy) propyl] piperazin-1-yl] acetamide (Figure 1). RAN is believed to have its effects via altering the trans-cellular late sodium current. It is by altering the intracellular sodium level that RAN affects the sodium dependent calcium channels during myocardial ischemia. Thus, RAN indirectly prevents the calcium overload that causes cardiac ischemia. RAN is indicated for the treatment of chronic angina. RAN may be used with betablockers, nitrates, calcium channel blockers, antiplatelet therapy, lipid-lowering therapy, ACE inhibitors, and angiotensin receptor blockers.[1, 2] RAN has antianginal and anti-ischemic effects that does not depend on reduction in heart rate or blood pressure.[3] RAN has not appeared in any pharmacopoeia yet. Few spectrophotometric, [4] HPLC, [5, 6] LC-MS [7-10] and LCMS-MS [11-13] methods were reported in literature for determination of RAN. However, most of these reported methods were related to the quantitative assay of RAN in human or dog plasma. To our current knowledge, no article related to the stability indicating high performance thin layer chromatographic (HPTLC) determination of RAN and characterization of its degradation products has been reported yet as revealed by literature survey. Therefore the objective of this study was to develop a simple, economic, rapid, precise, and accurate stability indicating high performance thin layer chromatography (HPTLC) method for determination of RAN in presence of its degradation products formed under different stress conditions and to validate the method in accordance with ICH guidelines. [14-16]

MATERIALS AND METHOD

Solubility and stability studies : Solubility of RAN was checked in different organic solvents having wide range of polarity indices along with its stability in these solvents.

Selection of analytical wavelength: In order to determine the absorbance maxima, various concentrations of the drug from standard stock solution were spotted on the plate and developed. After developing, the plate was dried and then scanned for spectrum in the range of 190-400 nm (to get In situ spectra). Also by using UV spectrophotometer scanning of drug was carried out in the range of 200-400nm.

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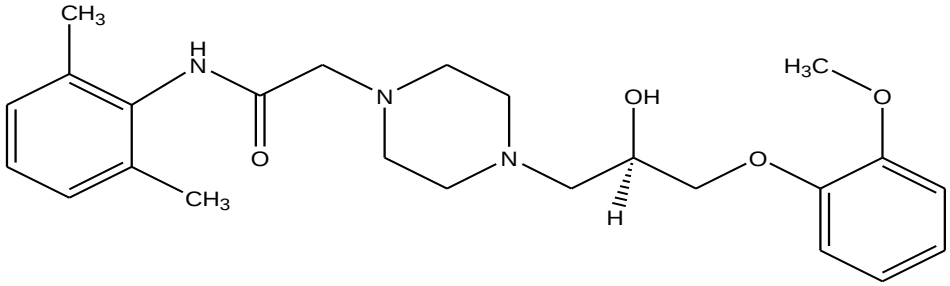

Figure 1: Chemical structure of RAN.

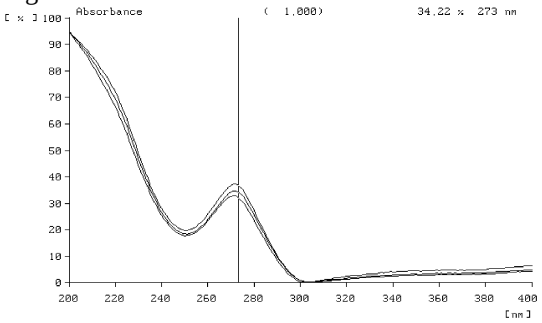

Figure 2: In situ HPTLC spectra of RAN standard drug (λ_{max}. 273 nm)

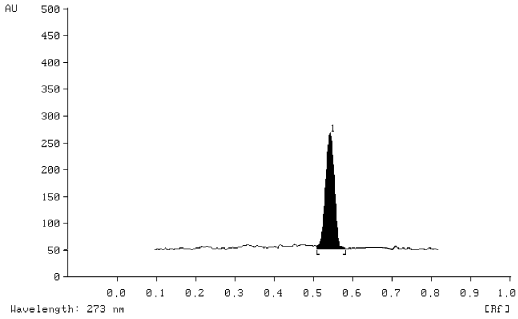

Figure 3: Densitogram of RAN standard drug 2000 ng spot^{-1} showing R_f 0.52 scanning at 273 nm.

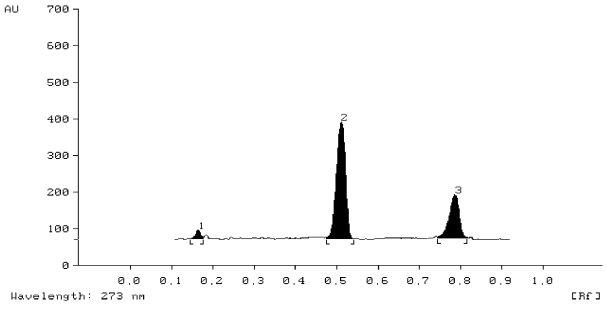

Figure 4: Densitogram of acid degradation product (Condition: 5 N hydrochloric acid at 80 °C for 6 hours)

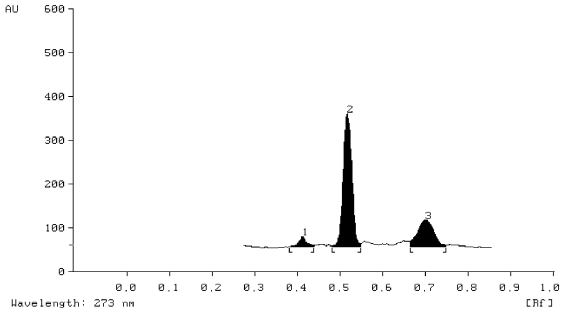

Figure 5: Densitogram of base degradation product (Condition: 3 N sodium hydroxide at 80 °C for 4 hours)

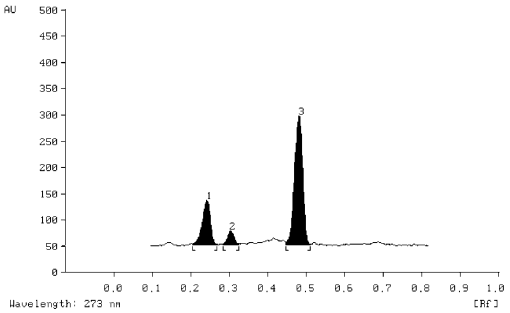

Figure 6: Densitogram of Oxidative degradation product (Condition: 20 % hydrogen peroxide at room temperature for 4 hours)

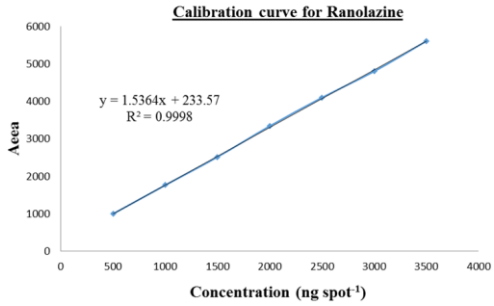

Figure 7 Calibration curve for RAN.

Instrumentation and Chromatographic Condition.: Precoated silica gel aluminium plate 60 F – 254, (20 × 10 cm) with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai are used through the study. The plates were prewashed with methanol and activated at 110ºC for 5 min prior to chromatography. The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on using a Camag Linomat IV applicator (Switzerland). A constant application rate of 0.1 µl/s was employed and space between two bands was 6 mm.

The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. The mobile phase consisted of chloroform : methanol : toluene (5 : 1 : 1 v/v/v) and 7 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 x 10 cm twin trough

glass chamber (Camag, Muttenz, Switzerland). It was saturated (lined on the two bigger sides with filter paper that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in dark with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25ºC ± 2) at relative humidity of 60 % ± 5. The length of chromatogram run was 8 cm and approximately 20 min. Subsequent to the development, TLC plates were dried in a current of air in wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 273 nm for all measurements and operated by CATS software (V 4.06, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

Table 1. Summary of degradation of RAN by HPTLC.

Stress Condition	Time (hours)	% Recovery	% Degradation	R_f of Degradants
Acid Degradation 5 N HCl, reflux (80ºC)	6	82.92	17.08	0.17, 0.79
Alkali Degradation 3 N NaOH, reflux (80ºC)	4	81.97	18.03	0.42, 0.70
Neutral Degradation reflux (80ºC)	48	98.38	1.62	--
Oxidative Degradation 20 % v/v of H_2O_2, at room temp.	4	81.04	18.96	0.24, 0.36
Thermal Degradation (60ºC)	48	97.95	--	--
Photo Degradation	10 Days	98.87	1.13	--

Note: Each sample was applied six times (N=6).

Stock solutions

Standard stock Solutions: A stock solution of RAN (1 mg/ml) was prepared by dissolving 10 mg of RAN accurately weighed in a 10 ml volumetric flask and was dissolved in 5 ml of HPLC grade methanol and volume was made up to the mark with HPLC grade methanol. The solution was further diluted as per requirement.

Sample stock Solutions: For preparing sample stock solution, twenty tablets were weighed (Rolazine of Macleods Pharmaceuticals Ltd. having label claim: 500 mg RAN per tablet) and their average weight was calculated (680.36 mg). The tablets were finely powdered and powder equivalent to 500 mg of RAN was accurately weighed and transferred into a 100 ml volumetric flask containing 80 ml methanol (HPLC grade) and sonicated for 30 mins then made the volume up to the mark with same. The solutions were filtered through a 0.45 µm nylon filter. The solution was further diluted as per requirement.

Table 2. Linearity study of proposed HPTLC method for RAN.

Sr. No.	Concentration (ng spot^{-1})	Average area	SD	%RSD
1.	500	998	15.25	1.528056
2.	1000	1765	25.35	1.436261
3.	1500	2519	31.46	1.248908
4.	2000	3342	39.95	1.195392
5.	2500	4102	57.80	1.409069
6.	3000	4806	59.67	1.241573
7.	3500	5613	34.25	0.610191

Note: Each sample was applied six times (N=6).

Forced degradation studies: In order to determine whether the analytical method and assay were stability-indicating, RAN standard stock solutions was stressed under various conditions to conduct forced degradation studies. This solution was used for forced degradation to provide an indication of the stability indicating property and specificity of proposed method.

Table 3. Precision studies of proposed HPTLC method for RAN.

Concentration (ng spot-1)	Measured concentration ± SD, RSD (%)	
	Intraday precision	Inter day precision
500	502.83 ± 7.17, 1.42	500.39 ± 4.51, 0.90
2000	2024.63 ± 37.36, 1.84	1989.06 ± 26.47, 1.33
3500	3570.49 ± 57.16, 1.62	3495.72 ± 43.78, 1.25

Note: Each sample was applied six times (N=6), and the result is expressed in Measured concentration ± SD, %RSD.

Acid degradation: To 5 ml of standard stock solution 5 ml of 5 N hydrochloric acid was added. The solution was kept for 6 hours at reflux of 80ºC. The resultant solutions were applied on TLC plate in such a way that final concentration achieved was 2500 ng spot^{-1} and the TLC plate was developed in optimized chromatographic conditions.

Alkali degradation: To 5 ml of standard stock solution 5 ml of 3N sodium hydroxide was added. The solution was

kept for 4 hours at reflux of 80ºC. The resultant solution was applied on TLC plate in such a way that final concentration achieved was 2500 ng spot^{-1} and the TLC plate was developed in optimized chromatographic conditions.

Table 4. Robustness studies of proposed HPTLC method for RAN.

Parameter	SD of peak area	% RSD
Mobile phase composition (± 0.1 mL)	32.92	1.002
Amount of mobile phase (± 5%)	43.71	1.347
Time from spotting to chromatography (± 10 min.)	33.40	1.010
Time from chromatography to scanning (± 10 min.)	50.71	1.522

Note: Each sample was applied six times (N=6).

Neutral degradation: 10 ml of standard stock solution of RAN was refluxed for 48 hours, the resultant solution was applied on TLC plate in such a way that final concentration achieved was 2500 ng spot^{-1} and the TLC plate was developed in optimized chromatographic conditions.

Oxidative degradation: To 5 ml of standard stock solution 5 ml of 20% hydrogen peroxide was added separately. The solution was kept for 4 hours at room temperature. The resultant solutions was applied on TLC plate in such a way that final concentration achieved was 2500 ng spot^{-1} and the TLC plate was developed in optimized chromatographic conditions.

Thermal degradation: 50 mg of the drug was spread in a borosilicate glass petri dish and placed in a hot air oven maintained at 60^{0}C for 48 hours, then the solution was prepared to achieve a final concentration of 2500 ng spot^{-1} and the TLC plate was developed in optimized chromatographic conditions.

Photo degradation: 50 mg of the drug was spread in a borosilicate glass petri dish and placed in a light cabinet (Thermo lab, India) and exposed to ≥200 W h m–2 UV irradiation at 320–400 nm, at 25°C, for 10 days. After removal from the light cabinet, sample was analyzed by making final concentration 2500 ng spot^{-1} in optimized chromatographic conditions.

Validation of the stability indicating method

Linearity and range: Linearity of the method was determined with the standard stock solution. The standard stock solution was diluted in such that to obtain concentration of 500 ng/µl. This solution was applied on plate as 1 µl, 2 µl, 3 µl, 4 µl, 5 µl, 6 µl, 7 µl to get concentrations of 500 ng spot^{-1}, 1000 ng spot^{-1}, 1500 ng spot^{-1}, 2000 ng spot^{-1}, 2500 ng spot^{-1}, 3000 ng spot^{-1}, 3500 ng spot^{-1}respectively, each concentration is applied for six times for linearity study. The plates were developed using optimized mobile phase and applied optimized chromatographic conditions.

Precision: Precision of the method was determined with the standard stock solution. The standard stock solution was diluted in such that to obtain concentration of 500 ng/µl. This solution was applied on plate as one micro liter, four micro liter, & seven micro liter to get concentrations of 500 ng spot^{-1}, 2000 ng spot^{-1}, & 3500 ng spot^{-1} respectively, each concentration is applied for six times on same day for intra-day precision study & three consecutive days for inter day precision study. The plates were developed using optimized mobile phase and applied optimized chromatographic conditions. The measurement of peak areas for RAN were expressed in terms of % RSD (relative standard deviation).

Limit of Detection and Limit of Quantitation: Limits of detection (LOD) and Limits of quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ. The LOD and LOQ were determined by measuring the magnitude of analytical background by injecting a blank and calculating the signal-to-noise ratio for by injecting a series of solutions until the S/N ratio 3 was obtained for the LOD and 10 for the LOQ. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. To determine the LOD and LOQ, serial dilutions of mixed standard solution of RAN was made from the standard stock solution.

Robustness of the method: To evaluate the robustness of a HPTLC method, few parameters were deliberately varied. The robustness of the method was studied, during method development at three different concentration levels of 500 ng spot-1, 2000 ng spot-1, & 3500 ng spot-1, by determining the effects of small variation, mobile phase composition (±0.1 %), amount of mobile phase (±5 %), time from spotting to chromatography (±20 min) and

Table 5. Recovery studies of proposed HPTLC method for RAN.

Drug	Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)
RAN	500	50	750	750.7977	100.3191
		100	1000	1004.7862	100.9572
		150	1265	1252.3931	100.3099

Note: Each sample was applied six times (N=6).

scanning time (±20 min). Each concentration was applied in six times and % RSD was calculated.

Table 6. Determination of RAN in commercial formulations by HPTLC.

Formulation (500 mg)	RAN found (mg per tablet)	
	Mean ± SD n= 6	Recovery (%)
1st Lot	493.77 ± 1.03	98.75
2nd Lot	496.17 ± 0.94	99.23

Note: Each sample was applied six times (N=6).

Specificity: To confirm the specificity of the proposed method, sample stock solution of marketed formulation of RAN in a concentration of 500 ng $spot^{-1}$, 2000 ng $spot^{-1}$, & 3500 ng $spot^{-1}$ were spotted on TLC plate, developed and scanned as described earlier. The peak purity of RAN was assessed by comparing the spectra at three different levels i.e., peak start, peak apex and peak end positions of the spot.

Recovery: Recovery studies were carried out by applying the method to drug sample to which known amount of RAN corresponding to 50, 100 and 150 % of label claim had been added (standard addition method). At each level of the amount, six determinations were performed and the results obtained were compared with expected results.

Stability in sample solution : The sample stock solution stored at room temperature for 24h and three concentrations 500 ng $spot^{-1}$, 2000 ng $spot^{-1}$, & 3500 ng $spot^{-1}$ for RAN were applied on HPTLC plate, after development the densitogram there was no additional peak found.

Analysis of the marketed formulation (ASSAY): The sample stock solution was further diluted to obtain three different concentration levels i.e. 500 ng $spot^{-1}$, 2000 ng $spot^{-1}$, & 3500 ng $spot^{-1}$. One microliter of each sample solution was applied six times to the HPTLC plate to give concentration 500 ng $spot^{-1}$, 2000 ng $spot^{-1}$, and 3500 ng $spot^{-1}$ for RAN. The plate was developed in the previously described chromatographic conditions. The peak areas of the spots were measured at 273 nm and concentrations in the samples were determined using multilevel calibration developed on the same plate under the same conditions using linear regression equation.

RESULTS AND DISCUSSION

Solubility and stability studies: Solubility of RAN was checked in different organic solvents having wide range of polarity indices along with its stability in these solvents. It was found that RAN was freely soluble as well as stable in methanol & slightly soluble in water.

Selection of analytical wavelength: The UV spectra of RAN scanned in UV spectrophotometer and in situ HPTLC (Figure 2), both shows λmax at 273 nm. Therefore 273 nm was selected as scanning wavelength for all study of RAN.

Optimization of the HPTLC method: Initially many different combinations of mobile phases were tried for the method development. tolune: ethyl acetate and Toluene: Ethyl acetate: methanol in different proportions were tried. It was found that in the toluene: ethyl acetate: methanol the movement of the drug was very less and poor resolution was observed. The mobile phase of chloroform: methanol: toluene (5: 1: 1 v/v/v) gave compact spot of RAN with good peak shape. The R_f of RAN was 0.52 ± 0.02 (Figure 3). The mobile phase was also able to resolve all the degradent products at 273 nm wavelength. The R_f of all the degradation components were between 0.15 to 0.79. Therefore this mobile phase combination was chosen for the validation studies. The optimized saturation time for mobile phase was 30 min at room temperature (25°C ± 2) at relative humidity of 60 % ± 5.

Forced degradation studies of RAN: HPTLC studies of samples obtained on stress testing of RAN under different forced conditions, was carried out using optimized mobile phase and scanning conditions.

Acid degradation: The rate of degradation in acid was slower as compared with that in alkali. The drug was quite stable in acidic conditions. Initially 0.1 N hydrochloric acid at reflux of 80°C was used but no degradation was found, so that the strength of acid was increased gradually up to 5 N, 17.08 % degradation was observed with 5 N hydrochloric acid at reflux of 80°C for 6 hours. Whereas a densitogram of acid-degraded RAN showed two degradation products, at R_f 0.17, and R_f 0.79 (Figure 4).

Alkali degradation: The drug was found to undergo alkaline degradation faster as compared to acid degradation. Initially 0.1 N sodium hydroxide at room temperature was used but the no degradation was found, so by gradual hardening the conditions at 3 N NaOH at reflux of 80°C for 4 h, 18.03 % degradation was observed. Whereas a densitogram of base-degraded RAN showed two degradation products at R_f 0.41, and $R_{f\ 0.70}$ (Figure 5).

Neutral degradation: No degradation was observed by refluxing the drug solution in water for 48 hours. Whereas a densitogram of neutral degraded RAN does not showed any degradation product only the peak area of RAN (98.38%) was reduced slightly.

Oxidative degradation: The drug was found to undergo oxidative degradation in 20% H_2O_2. The reaction was carried out at room temperature for 4 hours. The drug showed a degradation of around 18.96 %, whereas a densitogram of oxidative-degraded RAN showed two degradation products, at R_f 0.24, and R_f 0.30 (Figure 6).

Thermal Degradation: No degradation was observed in the drug treated at 60°C for 48 hours. Whereas a densitogram of thermal degraded RAN does not showed any degradation product only the peak area of RAN (97.95%) was reduced slightly.

Photo Degradation: RAN was found to be stable to photo degradation as no degradation was seen after exposing drug to UV light for 10 days. Peak area was found to be 98.87%.

Validation of the stability indicating method: The validation studies of the stability indicating method for RAN was carried out using optimized mobile phase and scanning conditions by HPTLC.

Linearity and range: For linearity and range of RAN by HPTLC, seven concentrations 500 ng μl^{-1}, 1000 ng μl^{-1}, 1500 ng μl^{-1}, 2000 ng μl^{-1}, 2500 ng μl^{-1}, 3000 ng μl^{-1}, 3500 ng μl^{-1} were prepared from standard stock solution in optimized mobile phase. Each concentration was injected in six times. The RAN showed linear increase in area by increasing concentration in a range of 500 ng μl^{-1} to 3500 ng μl^{-1} (Figure 7) with good correlation coefficient of (r^2=0.999). The average % RSD was in the acceptable limit (Table 2).

Precision: The Intraday precision and Inter day precision experiments are shown in Table 3. The developed method was found to be precise as the RSD (%) values for Intraday precision and Inter day precision studies were < 2%, as recommended by ICH guideline.

LOD and LOQ : The signal to noise ratios of 3:1 and 10:1 were considered as LOD and LOQ respectively. For HPTLC method the LOD and LOQ were found to be 250 ng $spot^{-1}$ and 440 ng $spot^{-1}$ respectively for RAN.

Robustness of the method: Robustness of the method was tested by small changes in parameters and the effects on the results were examined. The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2%. The values of %RSD are (as shown in Table 4) indicates robustness of the HPTLC method.

Specificity: The peak purity of RAN was assessed by comparing their respective spectra at peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9994 and r (M,E) =0.9998. Good correlation (r=0.999) was also obtained between standard and sample spectra of RAN.

Recovery Studies: Recovery studies of the drugs were carried out for the accuracy parameter. These studies were carried out at three levels i.e. multiple level recovery studies. Sample stock solutions in three 10 ml volumetric flasks in that 50 %, 100 % and 150 % of the standard drug solutions were added. Dilutions were made and recovery studies were performed. The average recovery of three levels was found that 100.53%, i.e. within the limits (Table 5)

Stability in sample solution: Solution of three concentrations 500, 2000, and 3500 ng $spot^{-1}$ for RAN were prepared from sample solution and stored at room temperature for 24h applied HPTLC plate, after development the densitogram there was no additional peak found.

3.6 Estimation of RAN from Pharmaceutical dosage form (Assay)

Experimental results of the amount of RAN in tablets (using three concentrations i.e. 500 ng $spot^{-1}$, 2000 ng $spot^{-1}$, and 3500 ng $spot^{-1}$), expressed as percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any excipients, which are present in tablets. The drug content was found to be 98.99 % ± 0.33. Two different lots of commercially available RAN tablet were analyzed using the proposed procedures and the results are tabulated in Table 6.

CONCLUSION

The developed HPTLC method provide simple, accurate, reproducible and stability indicating for quantitative analysis for determination of RAN in pharmaceutical dosage form, without any interference from the excipients and in the presence of its acidic, alkaline, neutral, oxidative, thermal and photolytic degradation products. The method was validated as per ICH guidelines. Statistical tests indicate that the proposed HPTLC method reduce the duration of analysis and appear to be equally suitable for routine determination of RAN in pharmaceutical dosage form in quality control laboratories, where economy and time are essential. This study is a typical example of development of a stability indicating assay, it is one of the rare studies where forced decomposition was done under all different suggested conditions and the degradation products were resolved. Hence it is proposed for the analysis of the drug and degradation products in stability samples in industry. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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