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Stability indicating High Performance Thin Layer Chromatographic determination of Raloxifene Hydrochloride

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Abstract

A simple, sensitive, selective, precise and stability indicating high-performance thin-layer chromatographic method was developed and validated for the determination of raloxifene hydrochloride as per the ICH guidelines. Precoated aluminium plates with silica gel $60F_{254}$ as the stationary phase and methanol: water: ammonia solution (9.4:0.5:0.1) was used as mobile phase this solvent system gave compact spot for raloxifene hydrochloride with R_f value of 0.60 ± 0.02 detected at 254 nm. Raloxifene was subjected to acid and alkali hydrolysis, oxidation and photodegradation. Peak area was in the concentration range of 100–500 ng $spot^{-1}$ with *r* value as 0.999. The mean value (±SD) of slope and intercept were 9.66 ± 0.05 and 956.33 ± 27.67 respectively. The method was validated for precision, accuracy, ruggedness and recovery. The LOD and LOQ were found to be 40.8 and 132.2 ng $spot^{-1}$. The peaks of the degradation products were well resolved from the pure drug and had significantly different Rf values. Since the degraded products under acidic, basic and oxidative conditions not interfered in the estimation of `pure drug it can be employed as stability-indicating one.

Key words: Raloxifene, HPTLC, stability indicating, stress degradation, Validation

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1. Introduction

Raloxifene (Fig 1) chemically called as [6-hydroxy-2-(4-hydroxyphenyl)-benzothiophen-3-yl]-[4-[2-(1-piperidyl) ethoxy] phenyl]-methanone is indicated for the treatment and prevention of osteoporosis in postmenopausal women[1]. Various methods have been reported in literature for the analysis of raloxifene Hydrochloride including visible spectrophotometric methods[2-4], high-performance liquid chromatography (HPLC)[5-14], liquid chromatography/electro spray tandem mass spectrometry [LC/ESI-MS)[15], No report is available related to the stability indicating HPTLC determination of raloxifene hydrochloride to elucidate the stability characteristics of the active substance. Hence a stability-indicating HPTLC densitometric method was proposed to develop an economic, accurate, specific, reproducible and for the determination of raloxifene hydrochloride in the presence of its degradation products. The proposed method was validated as per ICH guidelines.[16,17]

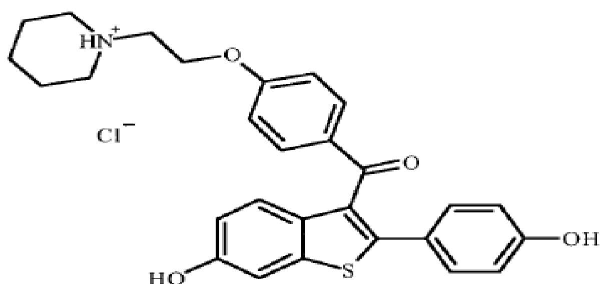

Figure 1. Raloxifene Hydrochloride

2. Materials and Methods

Materials

Raloxifene hydrochloride was received as a gift sample from orchid Laboratory Ltd., 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 width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm, 200 u-m thickness, E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nLs^{-1} was employed and space between two bands was 10 mm. The slit dimension was kept at 5 mm x 0.45 mm and 20 mms^{-1} scanning speed was employed. The mobile phase consisted of methanol:water:ammonia solution (25%) (9.4:0.5:0.1). Linear ascending development was carried out in twin-trough glass chamber saturated with the mobile phase. An optimized chamber saturation time 30 min provided to mobile phase at room temperature (25 ± 2 °C) at relative humidity of 55 ± 5%. The length of chromatogram run was 80 mm. Subsequent to the development, TLC plates were dried in a current of air with help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner in the absorbance mode at 254 nm. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum in the range of 190-400 nm. Evaluation was done using linear regression analysis via peak areas.

Calibration curve of Raloxifene hydrochloride

A stock solution of raloxifene hydrochloride (100 µg mL^{-1}) was prepared in methanol. Different volumes of stock solution 1, 2, 3, 4 and 5µL, were spotted on the TLC plate to obtain concentrations of 100, 200, 300,400 and 500 ng $spot^{-1}$ of raloxifene hydrochloride, respectively. Each concentration was spotted on the TLC plate using Linomat V applicator.. The data of peak areas plotted against the corresponding concentrations were treated by linear least-square regression analysis.

Method validation

Precision

For system precision, repeatability of the sample application and measurement of peak areas were carried out in six replicates of the same spot (400 ng spot-1 of raloxifene hydrochloride) and were expressed in terms of percent relative standard deviation (% R.S.D.). For method precision, the intra- and inter-day variation for the determination of raloxifene hydrochloride was carried out at three different concentration levels of 300, 400 and 500 ng spot-1 and was expressed in terms of % R.S.D. and standard error (S.E.).

Robustness of the method

By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation,the effects on the results were examined. Mobile phases having different compositions of methanol-water-ammonia solution (25%) (9.2:1.1:0.9 and 9.4:0.5:0.1, v/v/v) were tried and chromatograms were obtained. Mobile phase volume and duration of saturation were varied at 20 ± 2 mL (18, 20 and 22 mL) and 30 ± 10 min (20, 30 and 40 min), respectively. Robustness of the method was studied in triplicate at a concentration level of 800 ng $spot^{-1}$.

Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), spotted and the signal-to-noise ratio was determined.

Recovery studies

Recovery studies were carried out by applying the method to drug samples to which known amount of raloxifene hydrochloride corresponding to 50, 100 and 150% of the raloxifene hydrochloride label claim had been added. At each level of the amount, six determinations were performed. This was done to check for the recovery of the drug at different levels in the formulations.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for raloxifene hydrochloride in sample was confirmed by comparing the Rf values and spectra of the spot with that of standard.

The peak purity of the raloxifene hydrochloride was assessed by comparing the two spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Ruggedness

A solution of concentration 500 ng $spot^{-1}$ was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated to calculate % R.S.D. to assess the ruggedness of the method.

Forced degradation study of Raloxifene Hydrochloride

A stock solution containing 50 mg of raloxifene hydrochloride in 50 mL methanol was prepared. This stock solution (1000 u.g mL^{-1}) was used for-forced degradation study to provide an indication of the stability indicating property and specificity of the proposed method.

Forced degradation studies

Preparation of acid- and base-induced degradation product

To five mL of methanolic stock solution of raloxifene hydrochloride, 5 mL of 0.1M HC1, 2M Hydrochloride and 10 mL of 0.1M NaOH,1M and 2M NaOH were added separately. These mixtures were refluxed separately for 3 h at 80◦C. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. Two microlitres (1000 ng spot-1) of the resultant solutions were applied on TLC plate and the chromatograms were recorded.

Preparation of hydrogen peroxide-induced degradation product

To five mL of methanolic stock solution of raloxifene hydrochloride, 5 mL of hydrogen peroxide is (3%, v/v was added. This solution was heated in boiling water bath for 10 min to remove completely the excess of hydrogen peroxide and then refluxed for 3 h at 80 °C. Two microlitres (1000 ng $spot^{-1}$) of the resultant solutions were applied on TLC plate and the chromatograms were recorded.

Photochemical and UV degradation product

Five millilitres of the methanolic stock solution of raloxifene hydrochloride was diluted to 20 mL with methanol (500 µg mL^{-1}) and the photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for 3 days (from 09:00 to 17:00 h at 30C, total 24 h) on a wooden plank and kept on terrace. The drug solution was also exposed to UV irradiation at 254 nm for 8 h in a UV-chamber. Two microlitres (1000 ng spot-1) of the resultant solutions were applied on TLC plate and the chromatograms were recorded. In all degradation studies, the average peak area of raloxifene hydrochloride after application (1000 ng spot-1) of six replicates was obtained.

3. Results and discussion

Method development and optimization

The TLC procedure was optimized with a view to develop a stability indicating assay method to quantify the raloxifene hydrochloride. Both the pure drug and the degraded products were spotted on the TLC plates and run in different solvent systems. Initially n-propanol-methanol-water in varying ratio was tried. The mobile phase n-propanol-methanol-water (4:1:2, v/v/v) gave good resolution with R_f value of 0.56 for raloxifene hydrochloride but typical peak nature was missing because of tailing. Also the spot of raloxifene hydrochloride was slightly diffused. Substitution of water with concentrated ammonia solution improved the spot characteristics and the quantity optimized was 0.9 mL. Finally, the mobile phase consisting of methanol: water: ammonia solution (9.4:0.5:0.1) gave a sharp and well-defined symmetrical peak at R_f value of 0.80±0.02. Well-defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature. The developed analytical procedure can be completed in about 1.5 h, which includes the pre-analysis steps viz. washing of HPTLC plates with methanol and activation at 60°C (30 min); preparation of mobile phase and saturation of development chamber (35 min).

Calibration curve

The linear regression data for the calibration curves (n = 3) as shown in Table 1 showed a good linear relationship over concentration range 100-500 ng $spot^{-1}$ (Fig 2) with respect to the peak area. Fig 3 displays the calibration curve for raloxifene hydrochloride at 254 nm. No significant difference was observed in the slopes of standard curves (ANOVA, P>0.05).

Table 1. Linear regression data for calibration curve (n= 3)

Concentration (ng/spot)	Raloxifene	
	Peak Area	Rf value
100	880	0.86
200	884	0.87
300	978	0.88
400	1033	0.88
500	1141	0.88

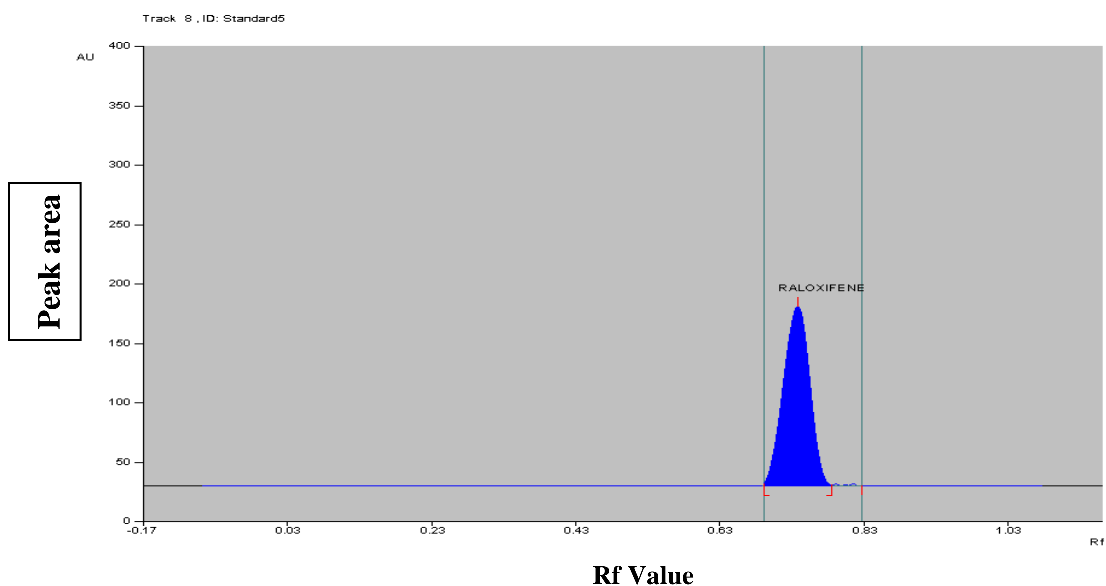

Figure 2. Chromatogram of standard raloxifene hydrochloride

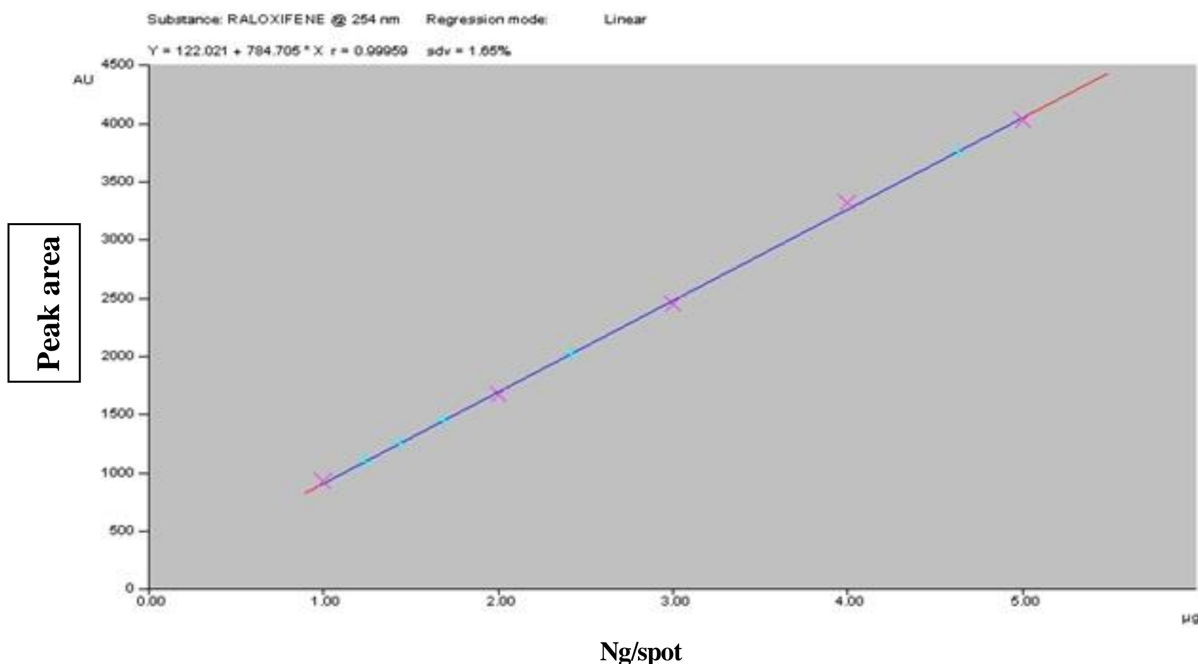

Figure 3. Calibration curve for raloxifene hydrochloride at 254 nm

Validation of the method

Precision

The % R.S.D. for repeatability of sample application (500 ng/spot) and measurement of peak areas were found to be 0.08 and 0.12%, respectively. The measurement of the peak area at three different concentration levels showed low values of S.E. and very low values of the % R.S.D (<2) for intra (Table 2) and inter-day (Table 3) variation, which suggested an excellent precision of the method.

Table 2. Intraday precision study

Drug	**Concentration (ng/spot)**	**Intraday precision**		
		Peak area	**SD**	**%RSD**
Raloxifene HCl (n=3)	300	1154	29.0057	1.5639
	400	1589	70.4651	1.8064
	500	1555	63.8513	1.1979

Table 3. Interday precision study

Drug	Concentration (ng/spot)	Intraday precision		
		Peak area	SD	%RSD
Raloxifene HCl (n=3)	300	1956	30.6648	1.5891
	400	3692	71.5052	1.9747
	500	6492	71.1641	1.0932

Robustness of the method

The % RSD and standard error (S.E.) of the peak areas were calculated for change in mobile phase composition, mobile phase volume, duration of saturation and activation of prewashed TLC plates at a concentration level of 500 ng spot^{-1} in triplicate. The low values of % R.S.D. and S.E. (<2) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 4).

Table 4. Robustness study of Raloxifene HCl

Parameters	Modification	% Recovery
Mobile phase ratio	9:0.5:0.5	96.83
	8.5:1:0.5	92.21
Development distance	20 mm	95.65
Detection wavelength	254 nm	97.52

LOD and LOQ

The signal-to-noise ratio of 3:1 and 10:1 were considered as LOD and LOQ and were found to be 40.8 and 138.2 ng spot^{-1}, respectively, which indicated the sensitivity of the method.

Recovery studies

The proposed method when used for extraction and subsequent estimation of raloxifene hydrochloride after spiking with 50; 100 and 150% of additional drug afforded recovery of 99.19-101.93% of raloxifene hydrochloride.

Ruggedness

Low % R.S.D. value of 0.4384 between peak area values obtained for the same drug solution of raloxifene hydrochloride at a concentration of 500 ng spot^{-1} after 0, 6, 12, 24, 48 and 72 h proved the ruggedness of the method. This indicates that raloxifene is stable during analysis (Table 5).

Table 5. Ruggedness study of Raloxifene HCl

Method Precision	Peak area	Area (average)	% Drug content	Average	SD	%RSD
Sample 1	3297	3262.4	99.15	98.86	0.4335	0.4384
Sample 2	3316		99.28			
Sample 3	3237		98.84			
Sample 4	3198		98.16			
Sample 5	5264		98.89			

Forced degradation study

The chromatograms of the samples treated with acid, base, hydrogen peroxide, dry and wet heat, photochemical and UV (254 nm) light, showed well separated peaks of pure raloxifene hydrochloride as well as some additional peaks for degradation products at different Rf values. The degradants identification was based on the comparison of the UV spectra of "stressed samples" with that of the "standard solution ". The spots of the degraded products were well resolved from the drug spot. The percentage degradation of raloxifene hydrochloride under different stress conditions was recorded in Table 6.

Table 6. Forced degradation of Raloxifene HCl

Stress condition/Duration/State	Degradation (%)
Acidic /1M HCl / 3 h / solution / 80ºC	97.42
Acidic/2M HCl/3 h/solution/80ºC	86.64
Basic / 0.1M NaOH / 3 h/solution / 80ºC	62.27
Basic / 1M NaOH / 3 h/solution / 80ºC	73.22
Basic / 2M NaOH / 3 h/solution / 80ºC	65.78
Oxidation / 3 % H_2O_2/ 3 h/solution / 80ºC	94.41
Photolysis / 10 µg/ml solution / direct sun	76.83

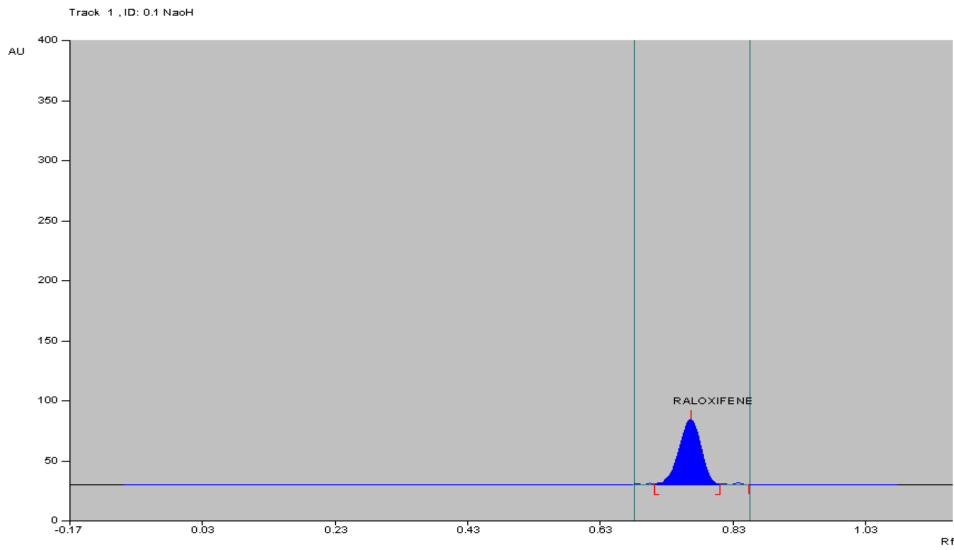

Figure 4. Densitogram of RH subjected to base degradation in 0.1M NaOH

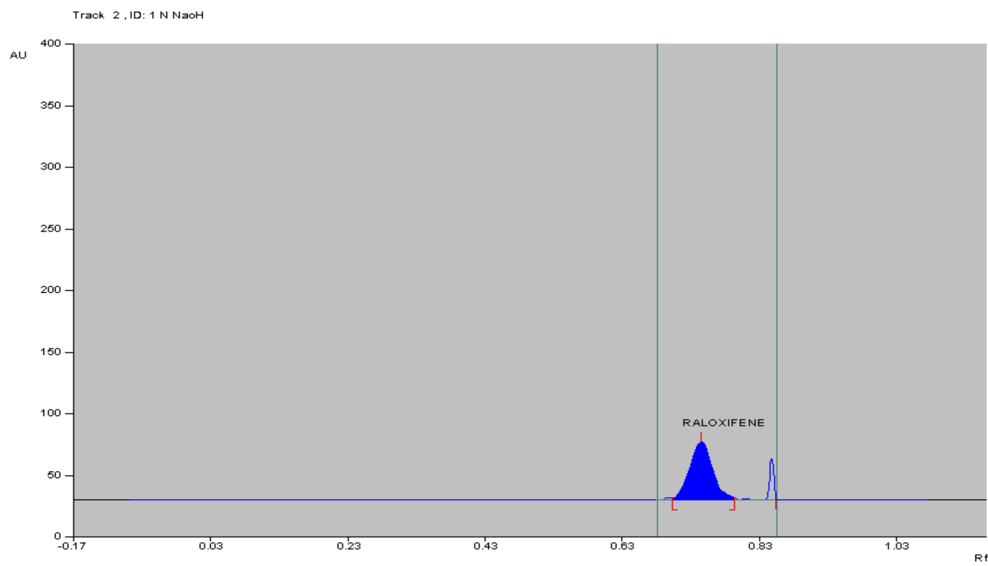

Figure 5 Densitogram of RH subjected to base degradation 1 M NaOH

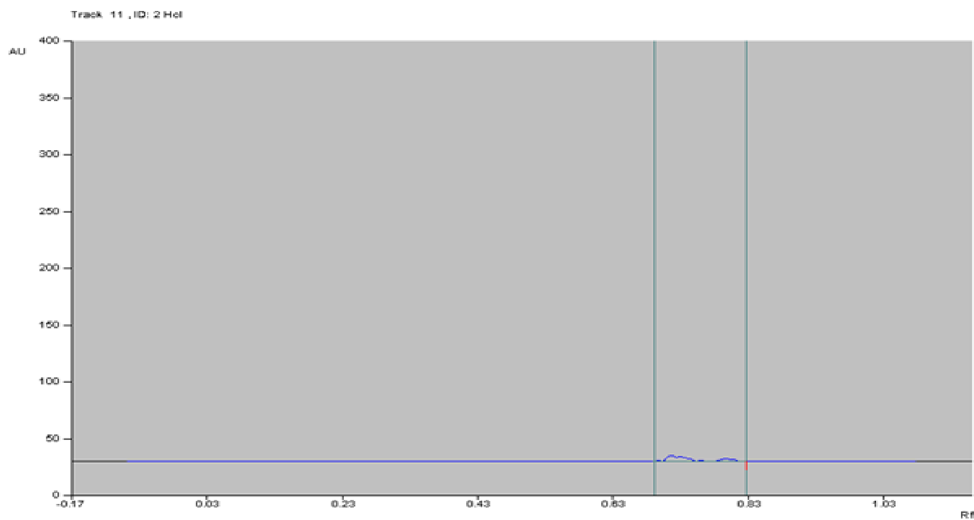

Figure 6 Densitogram of RH subjected to acid degradation 2M HCl

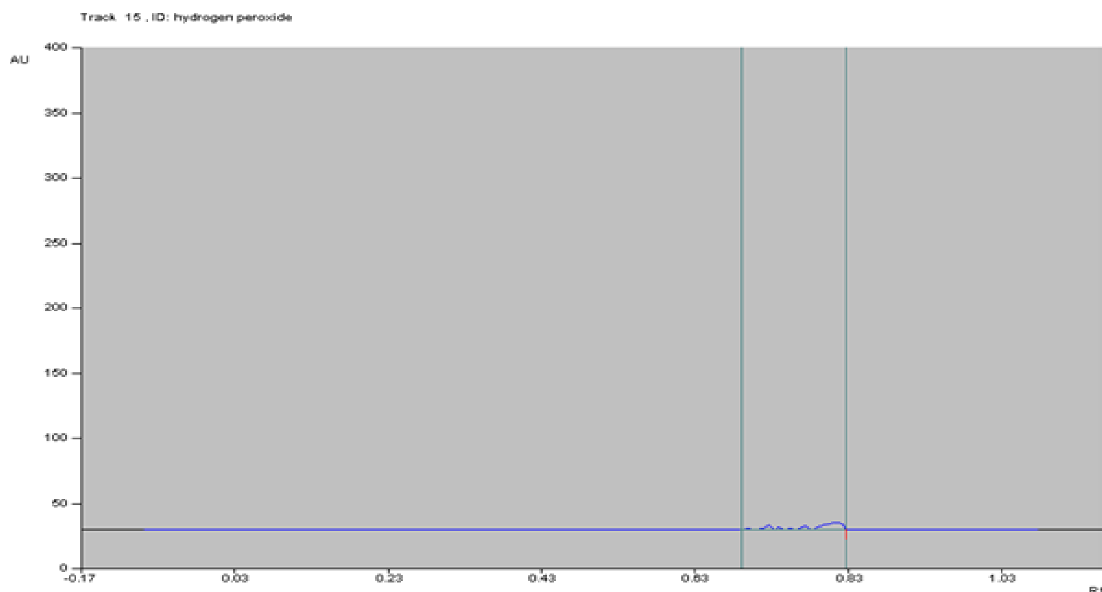

Figure 7. Densitogram of RH subjected to degradation of 3 % Hydrogen peroxide

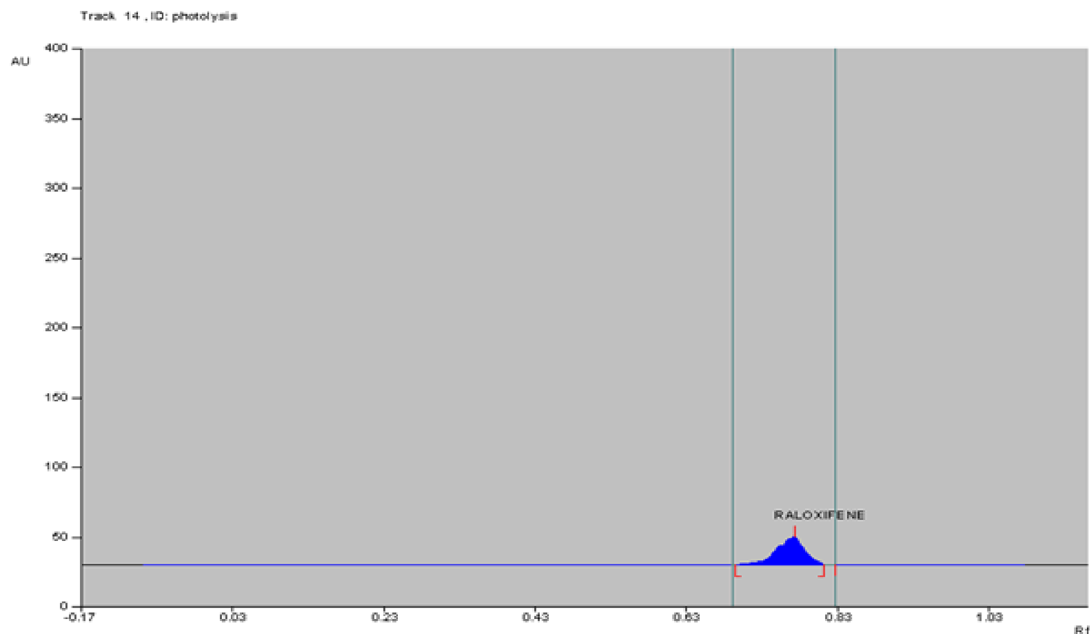

Figure 8. Densitogram of RH subjected to degradation of photolysis

The chromatograms of stress studies clearly indicated the complete degradation of raloxifene hydrochloride in acid hydrolysis with 2M Hydrochloride. The chromatograms of the base degraded sample showed three peaks (Fig. 4 and 5). The areas of the base-degraded product peaks were found to be extremely small than the area of standard drug concentration (1000 ng spot^{-1}) indicating that the raloxifene hydrochloride undergoes mild degradation under basic conditions. The chromatogram of raloxifene hydrochloride treated with 3% (v/v) hydrogen peroxide showed complete degradation, suggesting the raloxifene hydrochloride highly unstable towards oxidation. The chromatogram of the sample exposed to photochemical degradation or ultraviolet (UV) light at 254 nm showed no additional peaks other than the standard raloxifene hydrochloride peak at R_f=0.60 ±0.02 (Fig 6). This indicates that the drug is stable towards the photochemical for the exposure period under study.

4. Conclusion

The developed HPTLC technique is precise, specific, accurate and stability indicating for the determination of raloxifene hydrochloride. Statistical analysis proves that the method is reproducible and selective for the analysis of raloxifene hydrochloride. The method can be used to determine the purity of the drug available from various sources by detecting the

related impurities. It may further be extended to study the degradation kinetics of raloxifene hydrochloride and also for its determination in plasma and other biological fluids. As the method separated the drug from its degradation products, it can be employed as a stability indicating one.

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