VALIDATED STABILITY-INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF RALOXIFENE (ANTI-OSTEOPOROTIC AGENT) IN TABLETS

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ABSTRACT

Raloxifene hydrochloride is a new anti-osteoporotic agent, effective in the treatment of breast cancer. A stability-indicating high-performance liquid chromatographic method was developed and validated for the determination of Raloxifene Hydrochloride in tablet dosage forms. Reversed-phase chromatography was performed on Shimadzu Model LC-20AD Prominence with SPD M 20A Diode array detector using a Phenomenex Lichrosphere 100 C-18 (250 mm × 4.6 mm i.d., 5 µm particle size) column with sodium acetate: methanol (40:60, V/V) as mobile phase with a flow rate of 1 ml/min. UV detection was performed at 287 nm. Linearity was observed in the concentration range of 1.0–250 µg/mL with regression equation y = 95604 x – 26215 with correlation coefficient of 0.9999. The LOD and LOQ were found to be 0.267 µg/mL and 0.813 µg/mL respectively. The percentage relative standard deviation in precision and accuracy studies was found to be less than 2%. Raloxifene hydrochloride was subjected to stress conditions of degradation in aqueous solutions including acidic, alkaline, oxidation and photolysis. It was found that the drug is highly resistant towards all degradations as the decomposition was less than 3.5%. The developed method was validated with regard to linearity, accuracy, precision, selectivity and robustness and the method was found to be precise, accurate, linear and specific.

Keywords: Raloxifene hydrochloride, Isocratic elution, RP-HPLC, Validation, Stability-indicating, LOD, LOQ.

INTRODUCTION

Raloxifene hydrochloride [1] (Figure 1) belongs to the benzothiophene class of compound and it is currently used for prevention of osteoporosis in postmenopausal women. It was approved by Food and Drug Administration (FDA) in 1997. Chemically it is 6-Hydroxy-2- (4-hydroxy phenyl) benzo {b} thien-3-yl] [4-{2-(1-piperidinyl)-ethoxy}-phenyl] methanone with a molecular weight 510.05 g/mol. It binds to the nuclear estrogen receptors (ERα and ERß) and either activates or blocks ER-induced gene transmission, depending on the tissue involved. Raloxifene hydrochloride demonstrates estrogen agonist effects on bone and blood lipid levels while it is a competitive antagonist of estrogen at mammary and uterine estrogen receptors [2-3]. Raloxifene hydrochloride is cardio protective, in part due to its effects on plasma lipid distribution. Like estradiol, raloxifene hydrochloride reduces total and low-density lipoprotein (LDL) levels in plasma. However, unlike estradiol, it does not increase plasma high-density lipoprotein (HDL) and triglyceride levels in plasma [4]. There are also some adverse effects, namely increased incidences of hot flushes and in low percentage venous thromboembolic events [5].

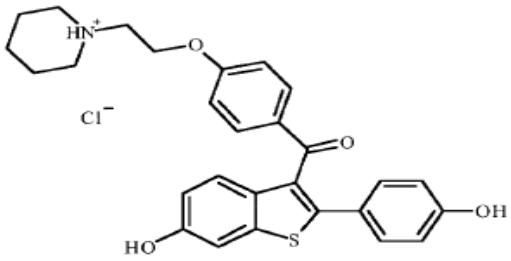

Figure 1: Chemical Structure of Raloxifene Hydrochloride

Analytical methods described in literature for the determination of Raloxifene hydrochloride in biological and other matrices involve spectrophotometry [6-13], liquid chromatography [14-30], capillary electrophoresis [31] and resonance rayleigh scattering [32].

Quality control of pharmaceutical products requires identification and quantification of the active ingredient and its impurities for safety and efficacy reasons. Impurities and potential degradation products that may exist in medicines can change the chemical, pharmacological and toxicological properties of the product. Since pharmacopoeias do not describe a suitable method for the determination of Raloxifene hydrochloride in pharmaceutical formulations, in the present work we developed simple, rapid and accurate reverse phase liquid chromatographic method for the determination of Raloxifene hydrochloride in tablets.

MATERIALS AND METHODS

Chemicals and Reagents

Raloxifene Hydrochloride standard (purity 99.80%) was obtained from M/S BAL Pharma, India. Methanol (HPLC grade), sodium acetate , glacial acetic acid , sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were obtained from Merck , India. HPLC grade water was obtained from Merck, India. The drug products of Raloxifene Hydrochloride, i.e. RALISTA® (Cipla Ltd. India), FIONA® (Dr. Reddy's Laboratories Ltd. India), RALOCIUM® (Bal Pharma Ltd., India) available as tablets (Label claim 60.0 mg) were procured from the pharmacy store and all other chemicals were of an analytical grade and used as received.

Instrumentation

Chromatographic separation was achieved by using a Shimadzu Model CBM-20A/20 A lite HPLC system, equipped with SPD M20A prominence photodiode array detector (250 mm × 4.6 mm, 5 µm particle size) maintained at 25 °C.

Chromatographic Conditions

Isocratic elution was performed using sodium acetate: methanol (40:60, V/V) as mobile phase with a flow rate of 1 mL/min. UV detection was performed at 287 nm.

Preparation of Raloxifene hydrochloride Stock Solution

Raloxifene hydrochloride stock solution (1000 µg/mL) was prepared by accurately weighing 25 mg of Raloxifene hydrochloride in a 25 mL amber volumetric flask and making up to volume with methanol. Working solutions for HPLC injections were prepared on a daily basis from the stock solution in a solvent mixture of sodium acetate: methanol (40:60, V/V) (mobile phase). Solutions were filtered through a 0.45 µm membrane filter prior to injection and 20 µL was injected into the HPLC system.

Preparation of Sample Solutions

Twenty tablets from each brand (RALISTA® and RALOCIUM®) were procured, weighed and crushed to a fine powder. Powder equivalent to 25 mg Raloxifene hydrochloride was accurately weighed into a 25 ml volumetric flask and made up to volume with mobile phase. The contents of the volumetric flask were sonicated for 30 min to enable complete dissolution of Raloxifene hydrochloride. The solution was filtered and the filtrate was diluted with mobile phase. 20 µL of these solutions were injected into the system and the peak area was recorded from the respective chromatogram.

Forced Degradation Studies/Specificity

The study was intended to ensure the effective separation of Raloxifene hydrochloride and its degradation peaks of formulation ingredients at the retention time of Raloxifene hydrochloride. Separate portions of drug product and ingredients were exposed to the following stress conditions to induce degradation. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method [33]. All solutions for use in stress studies were prepared at an initial concentration of 1 mg/mL of Raloxifene hydrochloride and kept at room temperature for 30 min. All samples were then diluted in mobile phase to give a final concentration of 50 µg/mL and filtered before injection.

Acidic and Alkaline Degradation

Raloxifene hydrochloride drug solution (1.0 mg/mL) was treated with 0.1 M HCl and kept at room temperature for 30 min and then the stressed sample was neutralized and diluted with mobile phase to give a final concentration of 50 µg/mL and filtered before injection.

Similarly stress studies in alkaline conditions were conducted by exposing the drug sample solution with 0.1 M NaOH for 30 min at room temperature and then the stressed sample was neutralized and diluted with mobile phase to give a final concentration of 50 µg/mL and filtered before injection.

Oxidative Degradation

Solutions for oxidative stress studies were conducted by treating the drug solution with 3% H_2O_2 for 30 min at room temperature and then diluted with mobile phase to give a final concentration of 50 µg/mL.

Photolytic Degradation

The drug solution (1 mg/mL) for photo stability testing was exposed to UV light for 4 hours in a UV light chamber (365 nm) and diluted with the mobile phase to give a final concentration of 50 µg/mL and filtered before injection.

Method Validation

The method was validated for the following parameters: system suitability, linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity and robustness [34].

Linearity

Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels of the analyte (1.0-250 µg/mL). 20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The solutions extracted from the marketed formulations were injected in to the HPLC system and the peak area of the chromatograms was noted. The analytical curve was evaluated on three different days. The peak area vs. concentration data was analyzed with least squares linear regression. The slope and y-intercept of the calibration curve was reported.

Precision

The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of Raloxifene hydrochloride at three concentration levels (10, 50 and 100 µg/mL) (n=3) against a qualified reference standard. The %RSD of three obtained assay values at three different concentration levels was calculated. The interday precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (10, 50 and 100 µg/mL) and each value is the average of three determinations (n=3). The % RSD of three obtained assay values on three different days was calculated.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels (80, 100 and 120%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of Raloxifene hydrochloride in the drug product. The study was carried out in triplicate at 18, 20 and 22 µg/mL. The percentage recovery in each case was calculated.

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

The limit of quantification (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1) [34]. Sensitivity of the method was established with respect to limit of detection (LOD) and LOQ for Raloxifene hydrochloride. LOD and LOQ were established by slope method as mentioned below.

$$LOD = \frac{3.3 \times \text{standard deviation of y-intercept}}{\text{Slope of the calibration curve}}$$

$$LOQ = \frac{10 \times \text{standard deviation of y-intercept}}{\text{Slope of the calibration curve}}$$

LOD and LOQ were experimentally verified by injecting six replicate injections of each impurity at the concentration obtained from the above formula.

Solution Stability and Mobile Phase Stability

The solution stability of Raloxifene hydrochloride in the assay method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed at 12 h intervals over the study period. The mobile phase stability was also assessed by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 12 h intervals up to 48 h. The prepared mobile phase remained constant during the study period. The % RSD of the Raloxifene hydrochloride assay was calculated for the mobile phase and solution stability experiments. An additional study was carried out using the stock solution by storing it in a tightly capped volumetric flask at 4 ºC.

RESULTS AND DISCUSSION

The present proposed method is simple, precise, accurate and is applicable for a wide linearity range in comparison to the reported methods in the literature. A reversed-phase chromatographic technique was developed to quantitate Raloxifene hydrochloride at 287 nm. Methanol was chosen as an organic modifier in the mobile phase. Satisfactory resolution was achieved with use of a mixture of sodium acetate: methanol (40:60, V/V) as mobile phase with a flow rate of 1 ml/min as demonstrated in Figure 2. C18 column was adopted for the analysis because it provided a better separation of the analytes. The present method is a stability indicating RP-HPLC method which was not reported earlier.

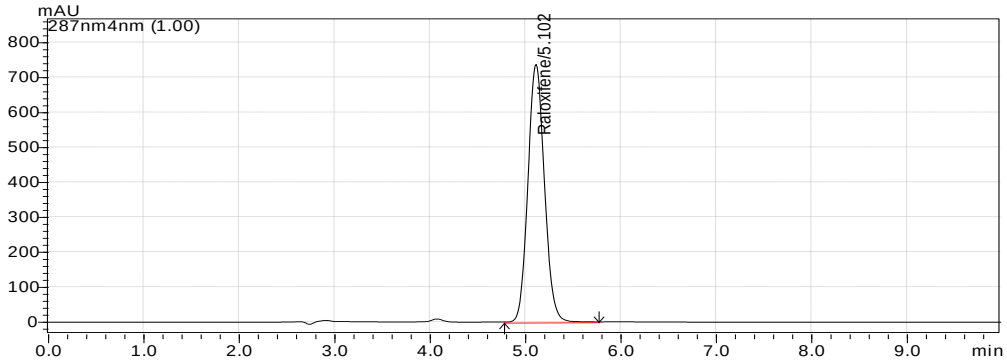

Figure 2: Representative chromatogram of Raloxifene hydrochloride (100 µg/mL)

The present stability-indicating method for the determination of Raloxifene hydrochloride in pharmaceutical formulations is specific because the drug peak was well separated even in the presence of degradation products. Overall, the data demonstrated that the excipients and the degradation products did not interfere with the Raloxifene hydrochloride peak, indicating the selectivity of the method. The complete separation of the analytes was accomplished in less than 10 min and the method can be successfully applicable to perform long-term and accelerated stability studies of Raloxifene hydrochloride formulations.

HPLC Method Development and Optimization

Initially the stressed samples were analyzed using a mobile phase consisting of water: acetonitrile (60:40, v/v) at a flow rate of 1.0 mL/min. Under these conditions, the resolution and peak symmetry were not satisfactory, so the mobile phase was changed to sodium acetate: methanol (40:60, V/V) with a flow rate of 1 ml/min under which peaks were well resolved with good symmetry and sharpness. Therefore, mobile phase containing sodium acetate: methanol (40:60, V/V) ml/min was chosen as the best chromatographic response for the entire study.

Method Validation

System Suitability

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. The parameters measured were peak area, retention time, tailing factor, capacity factor and theoretical plates. In all measurements the peak area varied less than 2.0%, the average retention time was 5.1 ± 0.05 minutes. The capacity factor was more than 2, theoretical plates were 9976 (more than 2000) and tailing factor was 1.21 (less than 2) for the Raloxifene hydrochloride peak. The proposed method offers high sensitivity and Raloxifene hydrochloride can be detected accurately. In all

the cases, the Raloxifene hydrochloride peak was well separated from the degradation products.

Linearity

The calibration curve for Raloxifene hydrochloride was linear over the concentration range of 1.0–250 µg/mL. The linearity data was given in Table 1.

Table 1: Linearity of Raloxifene hydrochloride

S. No.	Conc. (µg/mL)	Mean peak area ± SD	%RSD
1	1	120607.3 ± 420.48	0.35
2	2	210763 ± 724.12	0.34
3	5	474532 ± 415.1	0.09
4	10	914800 ± 4107.18	0.45
5	20	1925154 ± 2000	0.1
6	50	4710544 ± 2252.07	0.05
7	100	9327404 ± 1239.79	0.01
8	150	14355444 ± 16977.73	0.12
9	200	19064698 ± 8899.48	0.05
10	220	20851161 ± 44404.39	0.21
11	250	24105638 ± 23100.65	0.1

A calibration curve was drawn by taking the concentration on the x-axis and the corresponding peak are on the y-axis. The regression equation was found to be y = 95604 x - 26215 with correlation coefficient 0.9999.

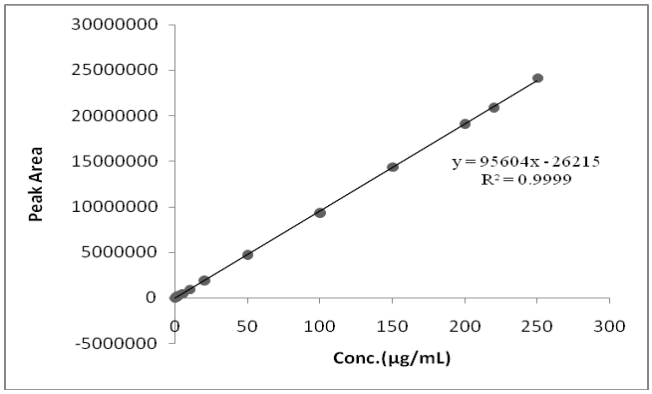

Figure 3: Calibration Curve of Raloxifene hydrochloride

Precision

The precision of the method was determined by repeatability (Intra-day precision) and intermediate precision (Inter-day precision) of the Raloxifene hydrochloride standard solutions. Repeatability was calculated by assaying three samples of each at three different concentration levels (10, 50 and 100 µg/mL) on the same day. The inter-day precision was calculated by assaying three samples of each at three different concentration levels ((10, 50 and 100 µg/mL) on three different days. The % RSD range was obtained as 0.16-0.54 and 0.17-0.53 for intra-day and inter-day precision studies respectively (Table 2).

Table 2: Intra-day and inter-day precision studies of Raloxifene hydrochloride

S No.	Conc. (µg/mL)	Intra-day precision		Inter-day precision	
		Mean* ± SD	% RSD	Mean* ± SD	% RSD
1	10	914689 ± 3496.7	0.38	917227.7 ± 3276.9	0.36
2	50	4740823 ± 25518.5	0.54	4720829 ± 24989.4	0.53
3	100	9344304 ± 15036.8	0.16	9329345 ± 15459.1	0.17

**Mean of three replicates*

Because the stability of standard solutions can also affect the robustness of analytical methods, the stability of standard solutions of the drug substance used in this method was tested over a long period of time. One portion of a standard solution was kept at room temperature and the other portion was stored under refrigeration at approximately 4ºC and the content of these solutions was regularly compared with that of freshly prepared solutions. No change in drug concentrations were observed for solutions stored under refrigeration. But it is recommended that the sample and standard solutions be freshly prepared in amber colored flasks to protect from light.

Accuracy

The method accuracy was proven by the recovery test. A known amount of Raloxifene hydrochloride standard (10 µg/mL) was added to aliquots of samples solutions and then diluted to yield total concentrations as 18, 20 and 22 µg/mL as described in Table 3. The assay was repeated over 3 consecutive days. The resultant % RSD was 0.27 (<2.0 %) with a recovery 99.40-99.9 %.

Table 3: Accuracy - recovery study of Raloxifene hydrochloride by standard-addition method

Sample No.	Spiked Conc. (µg/mL)	*Measured Conc. (µg/mL)	(%) Recovery*	(%) RSD *
1	18	17.9	99.4	0.27
2	20	19.9	99.5	
3	22	21.98	99.9	

**Mean of three replicates*

Limit of Detection and Limit of Quantification

The LOQ and LOD were determined based on the 10 and 3.3 times the standard deviation of the response, respectively, divided by the slope of the calibration curve. The LOD and LOQ were found to be 0.267 µg/mL and 0.813 µg/mL respectively.

Selectivity/Specificity

The specificity of the developed method was determined by injecting sample solutions (50 µg/mL) which were prepared by forcibly degrading under such stress conditions as heat, light, oxidative agent, acid and base under the proposed chromatographic conditions. The

stability indicating capability of the method was established from the separation of Raloxifene hydrochloride peak from the degraded samples derived from the software. The degradation of Raloxifene hydrochloride was found to be very similar for both the tablets and standard.

Solution Stability and Mobile Phase Stability

The %RSD of the assay of Raloxifene hydrochloride from the solution stability and mobile phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable up to 48 h at room temperature and up to 3 months at 4ºC.

Analysis of Commercial Formulations (Tablets)

The proposed method was applied to the determination of Raloxifene hydrochloride tablets RALISTA®, FIONA® and RALOCIUM® and the result of these assays yielded 99.96- 100.04 % respectively with RSD < 2.0 %. The result of the assay (Table 4) indicates that the method is selective for the assay of Raloxifene hydrochloride without interference from the excipients used in these tablets.

Table 4: Analysis of Raloxifene hydrochloride commercial formulation (Tablets)

S. No	Formulation	Labeled claim (mg)	*Amount found (mg)	% Label claim ± SD*
1	RALISTA®	60	59.98	99.96 ± 0.05
2	FIONA®	60	60.03	100.0±0.01
3	RALOCIUM®	60	60.02	100.04±0.01

**Mean of three replicates*

The typical chromatograms for Raloxifene hydrochloride obtained from the extracted marketed formulations were shown in Figure 4a and 4b.

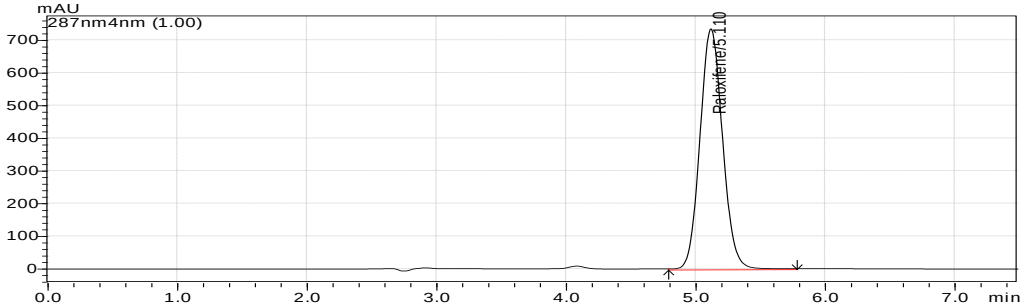

Figure 4(a): Representative Chromatogram of Raloxifene Hydrochloride (100 µg/mL) (RALOCIUM® 60 mg)

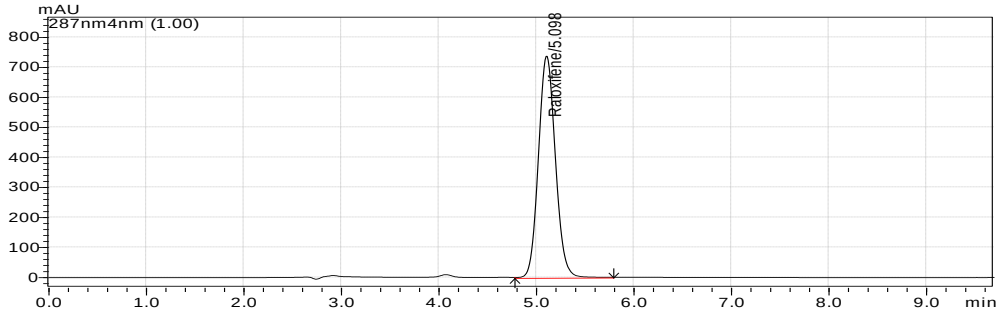

Figure 4(b): Representative Chromatogram of Raloxifene Hydrochloride (100 µg/mL) (RALISTA® 60 mg)

Forced Degradation Studies

Raloxifene hydrochloride standard and tablet powder was found to be quite stable under dry heat conditions. Typical chromatograms obtained following the assay of stressed samples are shown in Figure 5a-5d.

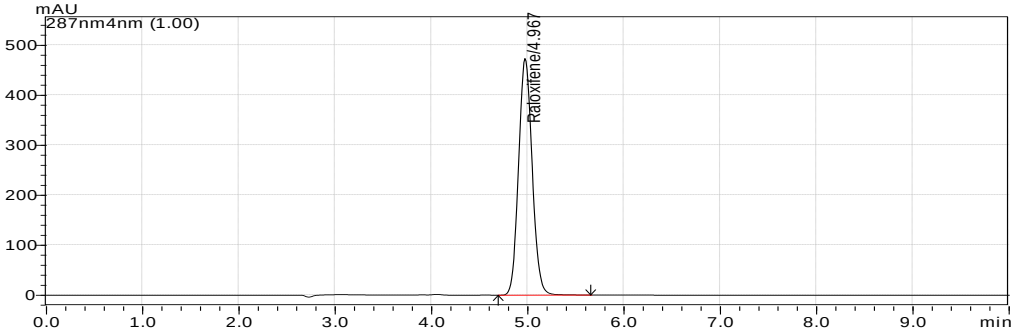

Figure 5a: Typical Chromatogram of Raloxifene hydrochloride (50 µg/mL) on Acidic degradation

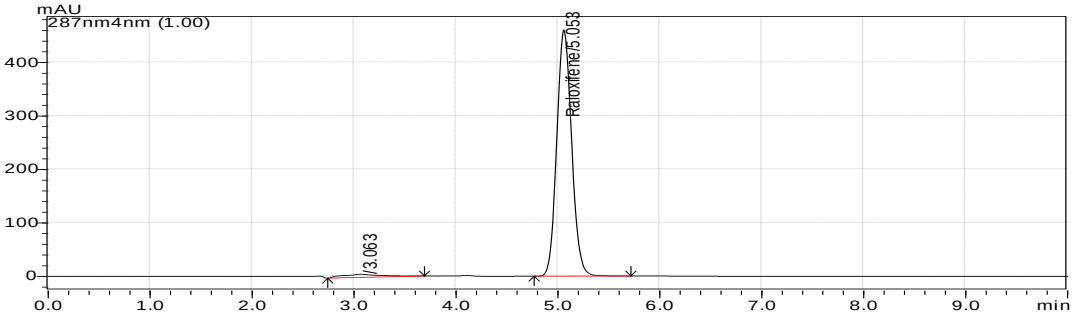

Figure 5a: Typical Chromatogram of Raloxifene hydrochloride (50 µg/mL) on Alkaline degradation

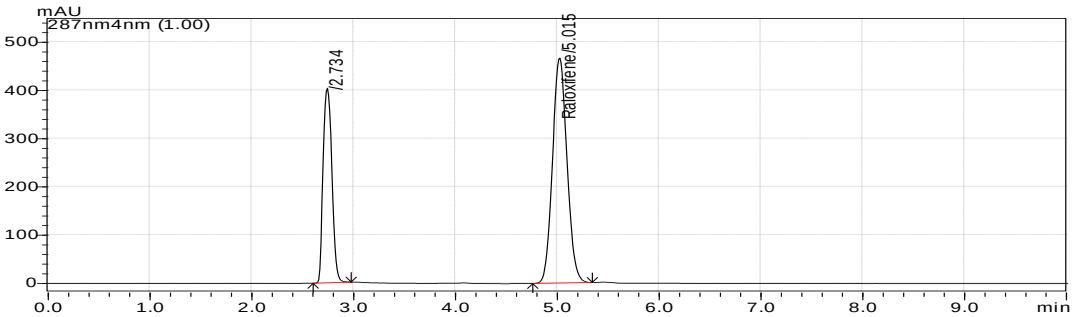

Figure 5c: Typical Chromatogram of Raloxifene hydrochloride (50 µg/ mL) on Oxidative degradation

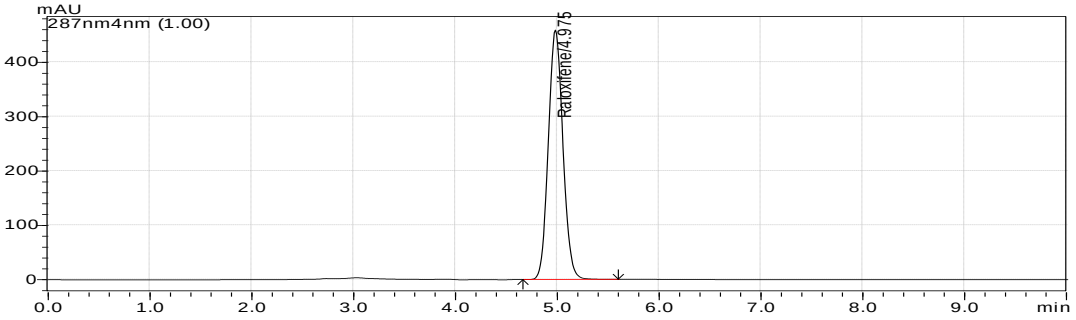

Figure 5d: Typical Chromatogram of Raloxifene hydrochloride (50 µg/mL) on Photolytic degradation

Table 5: Forced degradation studies of Raloxifene hydrochloride

Stress Conditions	% Drug Recovered	% Drug Decomposed
Standard Drug	100	-
Acidic Hydrolysis	97.48	2.52
Alkaline Hydrolysis	96.65	3.35
Oxidative degradation	97.47	2.53
Photolytic degradation	98.48	1.52

Mean of three replicates

A very slight decomposition was seen on exposure of Raloxifene hydrochloride drug solution to acidic (2.52), alkaline (3.35) and oxidation (2.53). During the oxidative degradation one major degradant was observed at 2.734 mins without interfering the elution of drug peak (5.015 mins) and the percentage of drug decomposition was found to be 2.53 % indicating that the drug is highly resistant towards oxidation. Raloxifene hydrochloride has undergone photo degradation (1.52) very slightly i.e. less than 2.0 %. Table 5 summarizes the data of degradation studies.

CONCLUSION

The proposed stability-indicating HPLC method was validated as per ICH guidelines and applied for the determination of Raloxifene hydrochloride in pharmaceutical dosage forms. The method was found to be accurate, precise, robust and specific as the drug peak did not interfere with the extra peaks aroused during the forced degradation studies. At the same time the chromatographic elution step is undertaken in a short time (< 6 min). No interference from any components of pharmaceutical dosage form or degradation products and therefore the method can be successfully applied to perform long-term and accelerated stability studies of Raloxifene hydrochloride formulations. In conclusion, the high sensitivity, good selectivity, accuracy and reproducibility of the proposed method is suitable for quality control analysis of complex pharmaceutical preparation containing Raloxifene hydrochloride. The reduction of acetonitrile consumption is one of the best solutions to the current global acetonitrile shortage and will safeguard against future risk.

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