HPLC AND SPECTROPHOTOMETRIC STABILITY – INDICATING STUDY OF RALOXIFENE IN PURE FORM AND TABLETS

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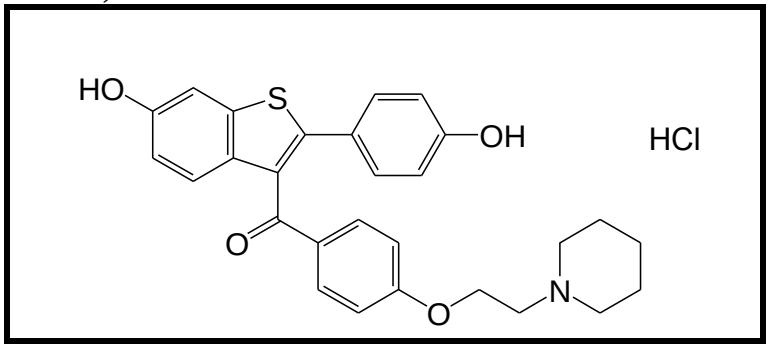
ABSTRACT:

Five simple, sensitive, accurate and precise methods were developed for determination of raloxifene (RLX) in pure form as well as in its pharmaceutical preparation. **Method [A]** is HPLC stability–indicating method, where the intact drug (RLX), the internal standard (methocarbamol) and RLX degradation products were separated using a YMC-pack ODS-AQ C_{18} column (150 mm X 4.6 mm ID, 3µm particle size) using acetonitrile–0.05 M KH_2PO_4 adjusted to pH 2.5 using H_3PO_4 (50 : 50 v/v) as a mobile phase at a flow rate 1 ml/min. and UV detection at 280 nm, where a good linearity was obtained in the range of 0.5 – 8 µgml^{-1}.The LOD was 0.077 µg ml^{-1} and the LOQ was 0.258 µgml^{-1}. **Method [B]** depended on measurement of the difference absorbance (ΔA) of the drug in the presence of its degradate between solutions in methanolic 0.1 M HCl and 0.1 M NaOH at 285 nm. Beer's low was obeyed in the range of 3 – 27 µgml^{-1}. LOD and LOQ were found to be 0.233 and 0.778 µg ml^{-1}, respectively. **Method [C]** is stability – indicating First-Derivative (^{1}D) for the determination of intact RLX in presence of its degradation product at 268 nm in the range of 3 – 18 µg ml^{-1} with LOD of 0.254 µg ml^{-} and LOQ of 0.849 µg ml^{-1}. **Method [D]** depended on ion pairing of RLX with eosin-Y dye at pH 3.5 to produce red coloured complex measured at 545 nm. Beer's low was obeyed in the range of 2 – 20 µgml^{-1}. LOD and LOQ were found to be 0.276 and 0.920 µg ml^{-1}, respectively. **Method [E]** depended on ion pairing of RLX with bromothymol blue (BTB) dye at pH 2.6 to form a chloroform-soluble coloured ion association complex. The formed complex could be extracted and measured at 420 nm. Good agreement with Beer's low was found in the range of 4 – 28 µgml^{-1}. LOD and LOQ were found to be 0.633 and 2.110 µg ml^{-1}, respectively. The percent recoveries ± RSD% of these methods were 100.83±0.920, 100.24±0.724, 100.15±0.586, 100.26±0.383 and 99.98±0.261, respectively. The obtained results were compared with those of the reported method and no significant difference was observed regarding accuracy and precision.

INTRODUCTION:

Raloxifene hydrochloride (RLX), 6-Hydroxy-2-(p-hydroxyphenyl) benzo[b]thien-3-yl-p-(2-piperidinoethoxy) phenyl ketone hydrochloride**,** is a second generation selective estrogen receptor modulator. It is a non-steroidal that belongs to the benzothiophene class of compounds. It is used in the treatment and prevention of osteoporosis and invasive breast cancer in postmenopausal women (**Martindale 35: The Complete Drug Reference CD and The Merck index 2006**).



Raloxifene Hydrochloride $C_{28}H_{27}NO_4S.HCl$ (M.wt. = 510.04)

RLX is not official in any pharmacopoeia, but several methods have been reported for its determination in pure sample, pharmaceutical preparation and in biological fluids including spectrophotometric **(Patel. *et al.*, 2007; Pavithra and Lakshmi 2006; Dharuman *et al.*, 2004; Annapurna *et al.* 2007; Basavaiah and Anil Kumar 2006; Basavaiah *et al.* 2008; Basavaiah *et al.*, 2009; Kalyanaramu and Raghubabu 2011; Attia *et al.*, 2009; Attia *et al.*, 2009; Kalyanaramu and Raghubabu 2011; Kalyanaramu and Raghubabu 2011 and Kalyanaramu *et al.*, 2011)**, spectrofluorimetric **(Turij *et al.*, 2007 and Turij *et al.*, 2005)**, HPLC **(Suneetha and Lakshmana Rao 2010; Jin 2004; Trontelj *et al.*, 2005; Wang Q. *et al.*, 2002; Nandini and Jayant 2001; Venkata *et al.*, 2006; Pavithra and Lakshmi 2006; Chandorkar *et al.*, 2006; Basavaiah *et al.*, 2008; Santhyaraj *et al.*, 2011; Madhu *et al.*, 2011 and Vijaykumar *et al.*, 2011),** LC MS-MS **(Trontelj e*t al.*, 2007 and Basavaiah K. *et al.*, 2009),** capillary electrophoresis **(Perez-Ruiz *et al.*, 2004)**, TLC densitometry **(Shirkhedkar *et al.*, 2012)** and resonance rayleigh scattering **(Li *et al.*, 2002 and Shao *et al.*, 2007)**.The main purpose of the present study is to stablish new simple, sensitive and validated methods for the determination of RLX in pure form and in its pharmaceutical dosage form.

EXPERIMENTAL

1.Material

1.1.Pure Sample:

Raloxifene hydrochloride was kindly supplied by South Egypt for Drugs and Industries Company (SEDICO), 6th October City, Egypt.

1.2.Pharmaceutical Preparation:

Evista® Tablets, product of Lilly Company, Spain, batch No. A470499, labeled to contain 60 mg RLX per tablet purchased from local market.

2.Chemicals and Reagents:

All chemicals and reagents used throughout the work were of analytical grade.Water used throughout the procedures was freshly distilled.

- Acetonitrile HPLC grade (Sigma;Aldrich, Germany; LabScan, Poland and Scharlau Chemie, Europian Union).
- Hydrochloric acid, anhydrous sodium acetate, Acetic acid, potassium chloride, potassium dihydrogen phosphate, sodium hydroxide, carbon tetrachloride, chloroform, ethylene chloride, methylene chloride, potassium acid phthalate and absolute ethanol (El-Nasr Company., Egypt).
- Methanol (Merck, Germany).
- Glacial acetic acid (Fisher Scientific, U.S.A).
- Phosphoric acid 85% (peypin-France).
- Eosin-Y (Sigma; Aldrich, Germany).
- Bromothymol blue (BTB) (Merck, Germany).
- 0.1 M methanolic HCl and 0.1 M methanolic NaOH.
- Eosin-Y (2×10^{-3}M), (3.13×10^{-4} M) and (1.25×10^{-4}M) aqueous solution.
- BTB 0.1% w/v and (9.8×10^{-4} M) prepared by dissolving in the least amount of ethanol and completing to volume with water.
- 0.05 M potassium dihydrogen phosphate.
- 0.2 M potassium chloride.
- 1 M and 0.2 M aqueous solution hydrochloric acid.
- 1 M aqueous solution sodium hydroxide.

- Acetate buffer (pH 2.5 – 4.4) (**British Pharmacopoeia, 2001)**.
- Phthalate buffer (pH 2.2 - 4) and HCl-buffer (pH 1.2 - 2)(**United States Pharmacopoeia XXIII, 1995)**.

3.Apparatus:

- Shimadzu UV-Vis. 1650 Spectrophotometer, Japan.
- Perkin Elmir series 200 manual HPLC equipped with quaternary pump and total Chrom station software.
- UV lamp with short wavelength (254nm).
- Precoated TLC plates silica gel 60 GF254 (20 x20 cm), 0.22mm thickness (Fluka, Chemie, Switzerland).
- pH meter 3510, Jenway.
- Hot plate, torrey pines scientific, USA.
- FT-IR, Nicolet IR 200, Thermo electron corporation
- NMR, mercury-300 BB (NMR300).

4. Standard Solutions:

4.1. Standard solution of intact drug (RLX):

4.1.1. For method A (HPLC Technique):

Stock solution of the drug (0.1 mg ml^{-1}) was prepared by dissolving 10 mg powder in the least amount of methanol then completing the volume to 100 ml with mobile phase. Stock solution of methocarbamol as internal standard (0.05mg ml^{-1}) was prepared by dissolving 5 mg in 100 ml methanol.

4.1.2. For method B (ΔA Technique):

Two stock solutions of RLX (0.1 mg ml^{-1}) were prepared by dissolving 10 mg powder in 100 ml of 0.1 M methanolic HCl and 100 ml of 0.1 M methanolic NaOH.

4.1.3. For method C (First-Drivative Technique):

Standard solution of RLX (0.05 mg ml^{-1}) was prepared by dissolving 5 mg of the drug in methanol and completing the volume to 100 ml with the same solvent.

4.1.4. For methods D and E (Ion-Pair Technique):

10 mg of RLX powder was accurately weighed and transferred into 100 ml volumetric flask, then dissolved in the least amount of ethanol, the volume was completed to the mark with water to obtain (0.1 mg ml^{-1}). The working solution was prepared by diluting with water.

4.2. Standard solution for degradated sample:

50 mg of pure RLX powder were refluxed with 50 ml 1M HCl for 36 hours. After cooling the solution was neutralized by 1M NaOH, evaporated to dryness under vacuum, the residue was extracted three times with 25 ml methanol, filtered into 100 ml volumetric flask then the volume was adjusted by methanol. The obtained solution labeled to contain the degradation product derived from (0.5 mg ml^{-1}) of RLX.

5.Procedures:

5.1. Analysis of standard solution

5.1.1. For method A (HPLC Technique):

At ambient temperature, isocratic separation was carried out on YMC-Pack ODS-AQ C_{18} column (150 X 4.6 mm ID X 3µm) using a mobile phase consists of acetonitrile and 0.05

M KH_2PO_4 adjusted to pH 2.5 by using H_3PO_4 (50 : 50 v/v).The mobile phase was degassed by a degasser before pumped at flow rate 1 ml min^{-1}, before injecting the solutions, the column was equilibrated for at least 30 minutes with the mobile phase flowing through the system. The injected volume of the standard solution was 20µl and UV detection at 280 nm. Aliquots of drug solution (100 µg ml^{-1}) corresponding to (5 – 80 µg ml^{-1}) of RLX were transferred into series of 10 ml volumetric flasks containing (50 µg) of internal standard and adjusted to volume with the mobile phase. Calibration curve was constructed by plotting the peak area ratio versus the corresponding drug concentration in µg ml^{-1}.

5.1.2. For method B (ΔA Technique):

In a series of 50 ml volumetric flasks, aliquots of RLX (0.1 mg ml^{-1}) equivalent to (3 – 27 µg ml^{-1}) were transferred and completed to the mark with 0.1 M methanolic HCl solution. In another series of 50 ml volumetric flasks, the same volumes of drug solution were transferred and completed to the mark with 0.1 M methanolic NaOH solution. The acidic drug solutions were measured at 285 nm against the alkaline drug ones and the calibration graph was constructed.

5.1.3. For method C (First - Derivative Technique):

Aliquots of standard RLX solution in methanol (0.05 mg ml^{-1}) containing 0.03 – 0.18 mg of the drug were added to a series of 10 ml volumetric flasks and then diluted to the mark with methanol. First-derivative (^1D) spectra of the drug were recorded against methanol as a blank. The height of the peak at 268 nm was measured in cm for each drug concentration.

5.1.4. For method D (Ion-Pair Technique with Eosin-Y):

Aliquots of drug solution of (0.05 mg ml^{-1}) containing (0.02 – 0.2 mg) were transferred into a series of 10 ml volumetric flasks; the volumes were diluted to 8 ml with water. Eosin-Y 0.4 ml of (2 x 10^{-3} M) solution was then added and the mixture was mixed well before addition of 1.2 ml of acetate - buffer pH 3.5. The mixture was adjusted to the volume with water and the solution was allowed to stand for 10 minutes at room temperature, then the absorbance was measured at 545 nm against the reagent blank.

5.1.5. For method E (Ion-Pair Technique with BTB):

Into a series of 125 ml separating funnels, transfer aliquot portions of the standard drug solution (0.1 mg ml^{-1}) containing (0.1 – 0.7 mg), then add 4 ml of phthalate buffer pH 2.6 followed by 3 ml of **BTB (**0.1%**).**Adjust the total volume of the aqueous phase to 15 ml by the addition of water, then shake the contents for about one minute. Extract with two successive portions each of 10 ml chloroform, collecting the extract into a 25 ml volumetric flask. Complete to volume with chloroform and measure the absorbance at 420 nm against the reagent blank.

5.2. Analysis of pharmaceutical preparation:

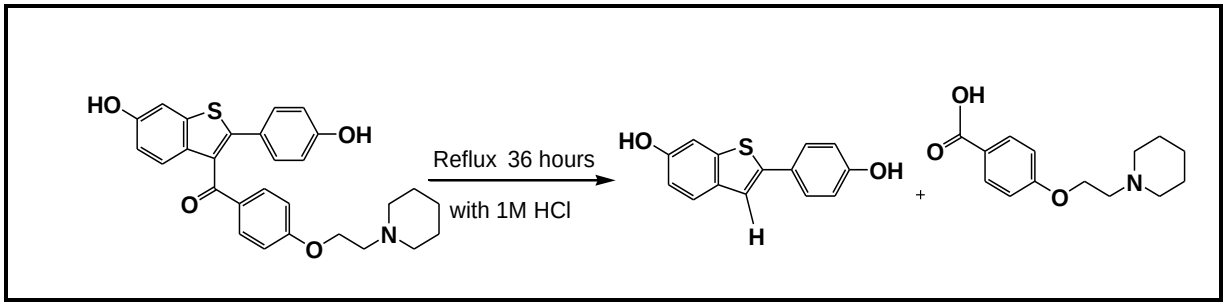
For method A, five tablets of Evista® 60 mg were weighed and finely powdered, then an accurately weighed amount of powder equivalents to 10 mg, dissolved in the least amount of methanol, then completing the volume to 50 ml with mobile phase, filtered into 100 ml volumetric flask and the volume was completed with the mobile phase. The obtained solution labeled to contain (0.1 mg ml^{-1}) of RLX. The solution was analyzed following the proposed procedure. **In method B**, two extractions were made, the first by transfering a quantity of the powder equivalent to 10 mg to 100 ml volumetric flask and shaked with 50 ml of 0.1 M methanolic HCl for about 15 minutes, filtered then the volume was completed to the mark with methanol. While the second one was done by transferring the same quantity of the powder to 100 ml volumetric flask and shaked with 50 ml of 0.1 M methanolic NaOH for

about 15 minutes, filtered then the volume was completed to the mark with methanol. Analyze aliquots of the clear filterate labeled to contain 0.1 mg ml^{-1}.**In method C,** an accurately weighed portion of Evista® 60 mg very fine powdered tablets equivalent to 5 mg of RLX was extracted three times with 25 ml methanol, filtered into 100 ml volumetric flask then the volume was adjusted with the same solvent. Aliquots of the obtained solution containing (0.03 – 0.18 mg) of RLX were analyzed by measuring the height of the peak in cm at 268 nm for each drug concentration by adopting the proposed ^{1}D procedure. Finally **for methods D & E,** an accurately weighed quantity of the well-mixed powdered Evista® 60 mg tablets equivalent to 10 mg of RLX was shaken with 50 ml ethanol for 15 minutes and filtered into 100 ml volumetric flask then the volume was adjusted with water. The obtained RLX solution (0.1 mg ml^{-1}) was subjected to colorimetric determination by the proposed methods.

RESULTS AND DISCUSSION

Degradation of Raloxifene:

Accelerated degradation of RLX was achieved upon heating under reflux with 1 M hydrochloric acid for 36 hours. The degradate formation illustrated in the following scheme:



Confirmation of degradation product:

For isolation of RLX degradation product from the reaction medium, the solution after reflux with 1 M hydrochloric acid for 36 hours was cooled, neutralized with 1 M sodium hydroxide, evaporated under vacuum till dryness and extracted with methanol. The obtained solution was tested by TLC on silica gel 60 GF_{254} plates. Separation of the intact drug and its corresponding degradate was achieved by using mobile phase consists of methanol - chloroform (6.5 : 3.5 v/v) and UV detection at 254 nm. $\mathbf{R_f}$ values of intact RLX and its corresponding degradate were 0.603 and 0.776, respectively.

IR spectrum of the intact drug showed broad bands at 3140 cm^{-1} due to the OH groups. Also, a ketone group at 1641 cm^{-1} and at 1463 cm^{-1} for C=C aromatic. While the IR spectrum of the degradate showed a broad band at 3434 cm^{-1} due to OH group of a carboxylic acid. Also, a band at 1702 cm^{-1} of the C=O group, as shown in **Figures (1,2)**.

^{1}HNMR spectrum of the intact drug showed signals at 1.31 broad multiplet of 2 H, 1.62 multiplet of 4H and at 1.72 triplet of 4H for the piperidine ring. Then, at 2.91 triplet of 2H and 4.37 of 2H for ethoxy group. Also, signals between 6.64 : 7.67 multipet for 11H aromatic and at 9.8 for 1H of phenolic OH group. Finally a singlet signal at 10.2 for the phenolic OH group in benzothiophene moity. While the degradate showed signals for the piperidine ring as multiplet of 2H at 1.31, triplet of 4H at 1.42and 1.64. Also, a broad band of 2H overlapping with DMSO at 2.47 and at 4.03 of 2H in the ethoxy group. 4H aromatic appear as multiplet between 6.64 : 7.61, moreover, a hump at 9.9 indicating 1H of COOH group, as shown in **Figures (3, 4)**.

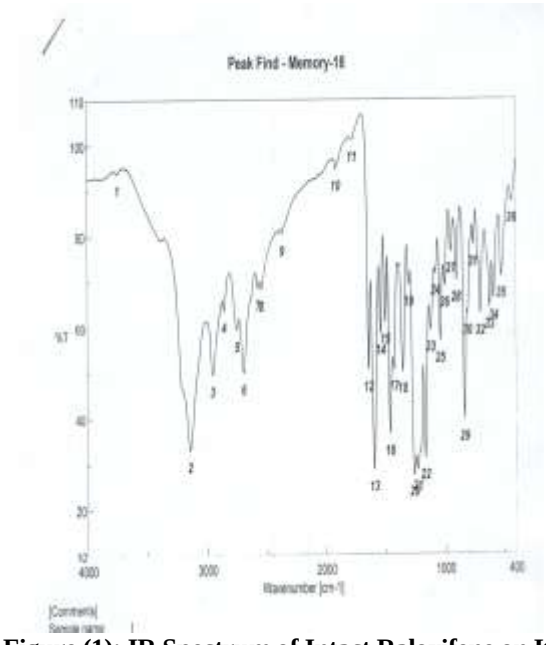


Figure (1): IR Spectrum of Intact Raloxifene on KBr Disc

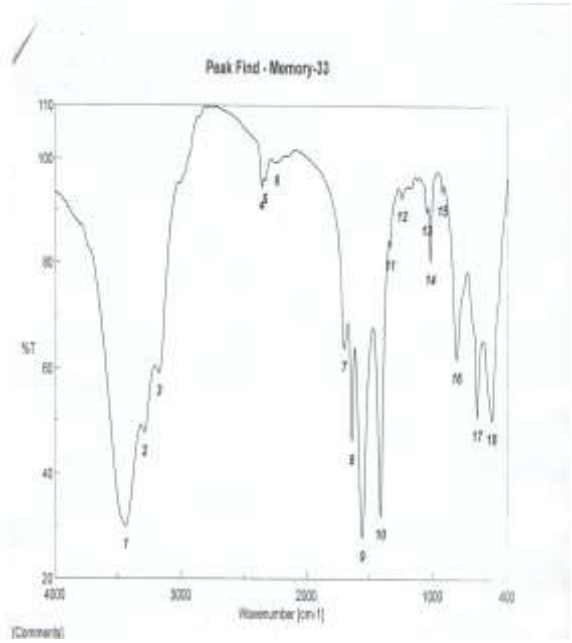


Figure (2): IR Spectrum of Degradated Raloxifene on KBr Disc.

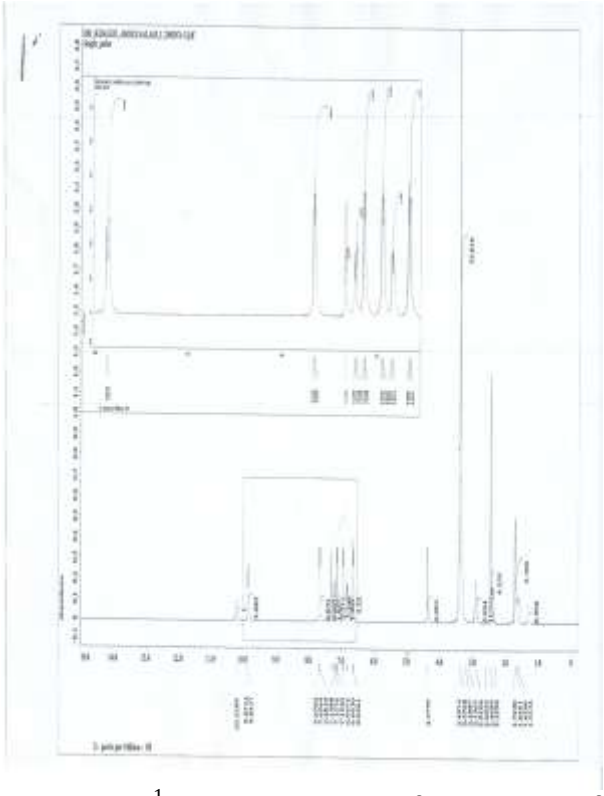


Figure (3): ^{1}HNMR Spectrum of intact Raloxifene in Deuterated DMSO.

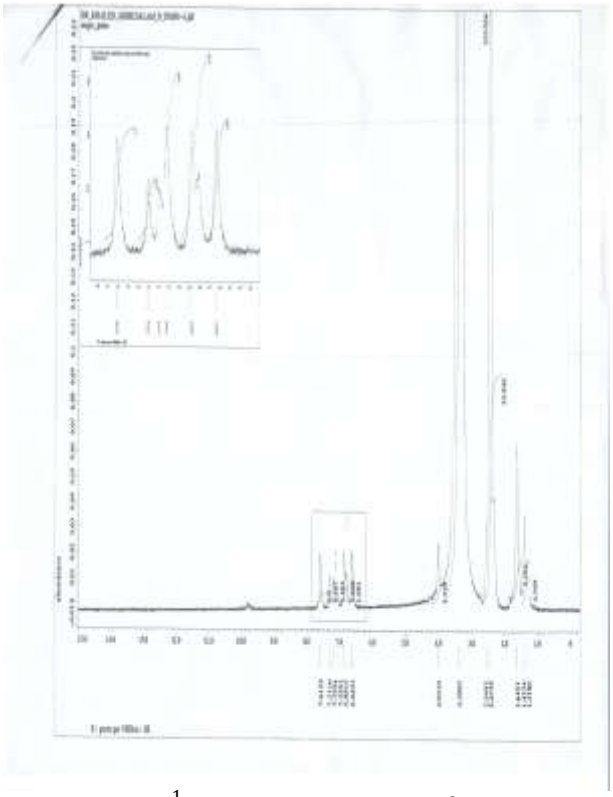
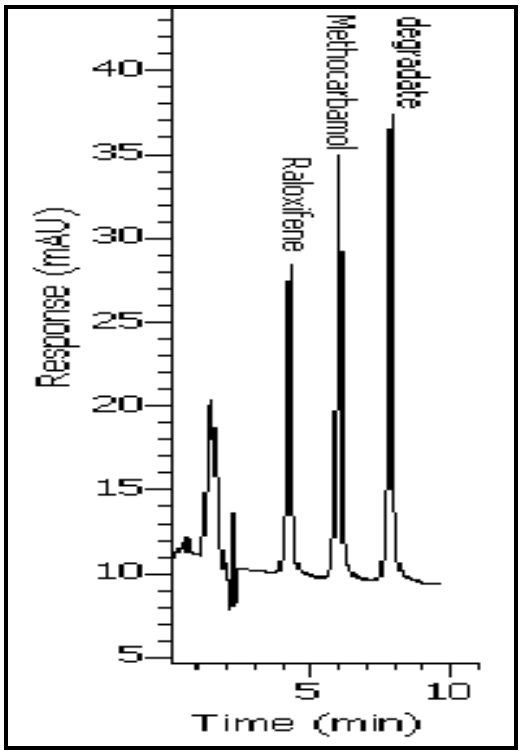


Figure (4): ^{1}HNMR Spectrum of Degradated Raloxifene in Deuterated DMSO.

In method A (HPLC Technique):

RLX was selectively determined in presence of its degradation product using HPLC procedure. A simple and sensitive reversed phase HPLC procedure was suggested for the selective quantitative determination of RLX in presence of its degradation product. Different chromatographic conditions affecting the chromatographic separation were optimized after taking in consideration the resolution between the drug, its degradation product and the internal standard. Several mobile phases were tried in order to separate the intact drug from

it's degradate and the internal standard including acetonitrile : 0.05 M KH_2PO_4 adjusted to pH 2.5 using orthophosphoric acid 85 % in different ratios. Good separation was carried out on YMC-Pack ODS-AQ C_{18} column (150 X 4.6 mm ID X 3µm) using a mobile phase consists of acetonitrile and 0.05 M KH_2PO_4 adjusted to pH 2.5 using H_3PO_4 (50 : 50 %) at flow rate 1 ml min^{-1} and UV detection at 280 nm, as shown in **figure (5).**



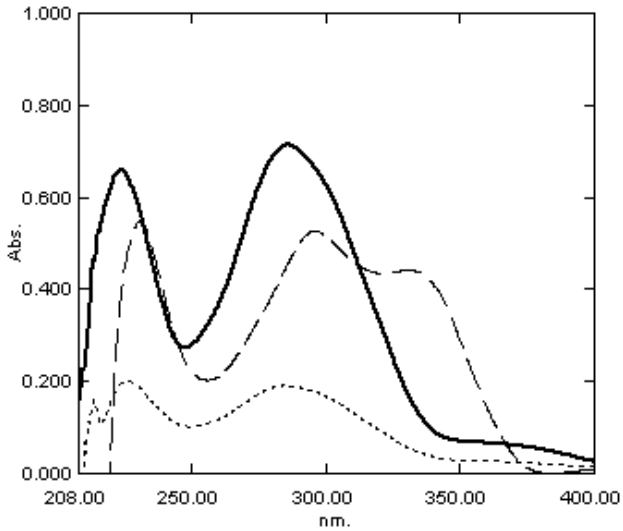
Figure(5): HPLC Chromatogram of Mixture of Intact Raloxifene (2µg ml^{-1}), Degraded Raloxifene (5µg ml^{-1}) and Methocarbamol as Internal Standard(°µg ml^{-1})

Specificity:

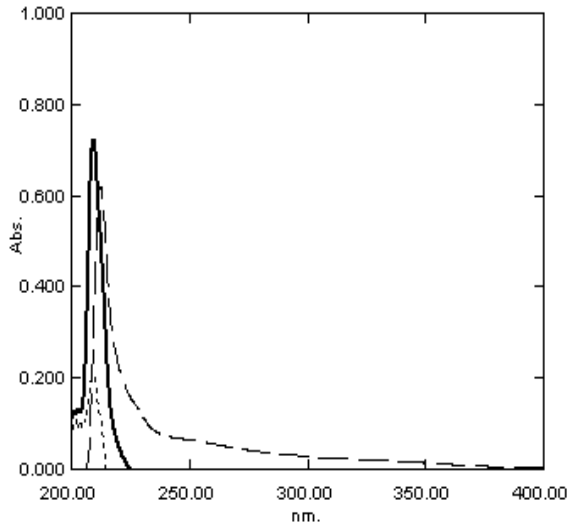
The specificity of the proposed method was assured by applying the laboratory prepared mixtures of the intact drug together with its degradation product. The proposed method was adopted for the specific determination of intact RLX in presence of up to 87.5% of its corresponding degradate. The percentage recovery ± SD % was 100.37 ± 1.260 %, **table (2)**.

For method B (ΔA Technique):

The change in the absorbance of only the intact RLX between the acid and alkaline media could be used for the determination of the intact drug in presence of its degradate. Absorbance difference measurements of both intact RLX and its degradate between 0.1M methanolic hydrochloric acid and 0.1 M methanolic sodium hydroxide at 285 nm was used for the determination of the intact drug in presence of its degradate, where the (ΔA) of the later equals to zero at this $\lambda_{max,}$ as shown in **figures (6, 7).**



Figure(6): UV- Spectra of Intact Raloxifene (6 µg ml^{-1}) in 0.1 N HCL (—), 0.1N NaOH (— —) and their Difference ΔA(…..).



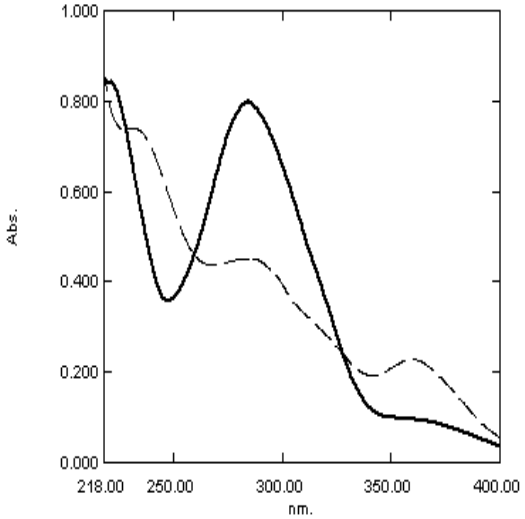
Figure(7): UV- Spectra of Degradated Raloxifene (6 µg ml^{-1}) in 0.1 N HCL (—), 0.1N NaOH (— —) and their Difference ΔA(…..).

Specificity:

The specificity of the proposed procedure was assured by applying it to laboratory prepared mixtures of the intact drug together with its degradation product. The proposed procedure was adopted for the selective determination of intact RLX in presence of up to 77.7% of its corresponding degradate. The percentage recovery ± SD % was 100.01±0.724%, **table (3)**.

For method C (^{1}D Technique):

The zero-order spectra of intact RLX and its degradation product show severe overlapping as shown in **figure (8).** However, this sever overlapping in zero-order spectra can be resolved by conversion of zero-order to higher first derivative spectra of RLX and its degradation product. **Figure (9)** showing that, the sever overlapping in zero-order spectra can be resolved at 268 nm, at this wavelength zero cross point of degradation product showing no interference to intact RLX. So that, the peak at this wavelength was chosen for selective determination of the intact drug in presence of its degradate. At the described wavelength linear relationship was obtained between the peak in cm and the RLX concentration in the range (3 - 18 µg ml^{-1}).



Figure(8): Zero-order Spectra of Intact Raloxifene (6µg ml^{-1}) (—) and its Degradation Product (6 µg ml^{-1}) (– –) in Methanol.

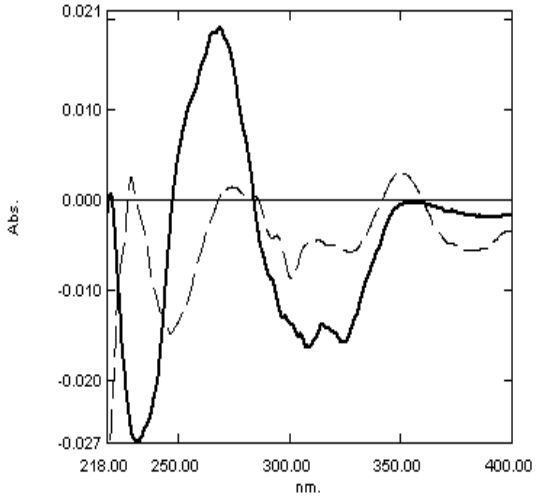


Figure (9): First-order Spectra of Intact Raloxifene (6 µg ml^{-1}) (—) and its Degradation Product (6 µg ml^{-1}) (– –) in Methanol.

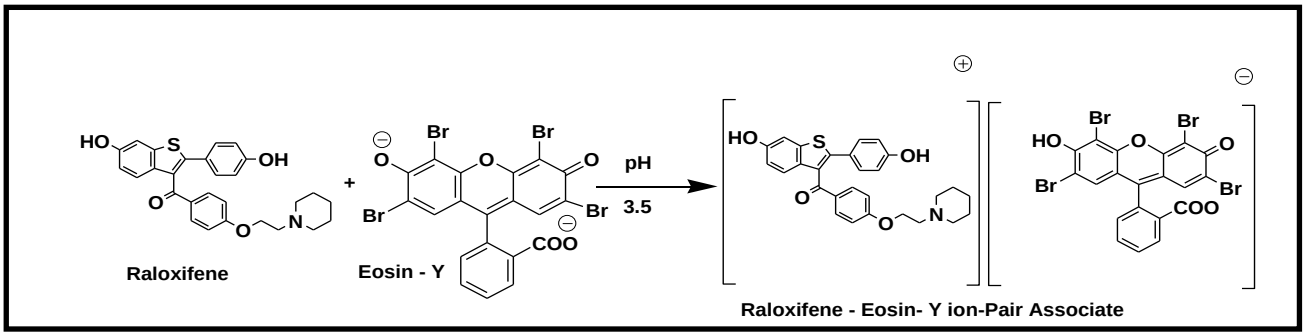
Specificity:

The specificity of the proposed method was assured by applying the laboratory prepared mixtures of the intact drug together with its degradation product. The proposed method was adopted for the specific determination of intact RLX in presence of up to 88.8% of its corresponding degradate. The percentage recovery ± SD % was 100.28±0.801%, **table (4)**.

For method D (Ion-Pair Technique with Eosin-Y):

Eosin-Y dye is an anionic halofluorescein derivative that strongly associated with raloxifene in acidic medium to form ion pair associate.

This ion-pair associate of the studied drug with the anion of the halofluorescein dye (eosin – Y) was slightly soluble in water, but under the optimized experimental conditions, it become freely soluble and did not need an extraction into organic solvents nor the addition of non-ionic surfactants. This complex was probably formed via the electrostatic interaction between the maximally protonated terminal amino group of the drug (the most basic center in the drug molecule) and the carboxylate anion of the dye. This might primarily occur in acidic solutions (pH 1.2–4.4)(**El-Brashy A M.** *et al.* 2004) as shown in the following scheme:



Raloxifene reacts with eosin–Y dye to form a highly red coloured ion-pair associate that exhibits an absorption maximum at 545 nm as shown in **figure (10).** Different parameters involved in the reaction were studied such as pH, buffer volume, concentration of the reagent, reaction time and colour stability. The effect of pH of the medium as well as the buffer volume were studied and revealed that 1.2 ml of pH 3.5 acetate buffer were sufficient to give maximum absorbance **figures (11, 12).** The effect of eosin – Y volume was also studied, **figure (13)** revealed that, 0.4 ml of (2×10^{-3} M) were sufficient to give maximum absorbance. The maximum color intensity for eosin-Y was attained after 10 minutes at room temperature and remained stable for further one hour. Heating at high temperatures had no effect on the rate of reaction; conversely, it caused complex breakdown and weakened the color intensity. Therefore, the room temperature was chosen as optimum for the assay procedure.

Molar ratio and continuous variation (Job's) methods were applied for determination of stoichiometry of the reaction of RLX with eosin–Y. **Drug : reagent ratio** was found to be 1:1, as shown in **figures (14, 15).**

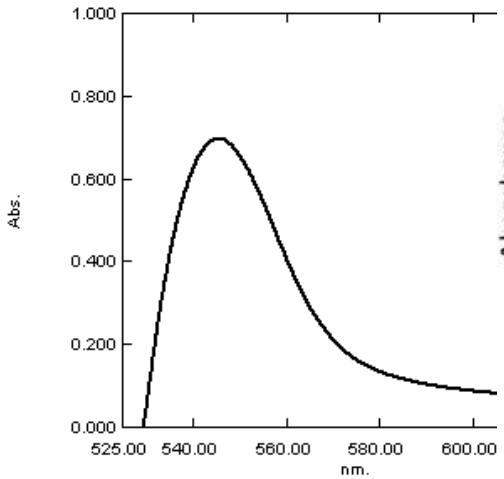


Figure (10): Absorption Spectrum of Raloxifene (16µgml^{-1}) Reaction Product with 0.4ml Eosin –Y Dye.

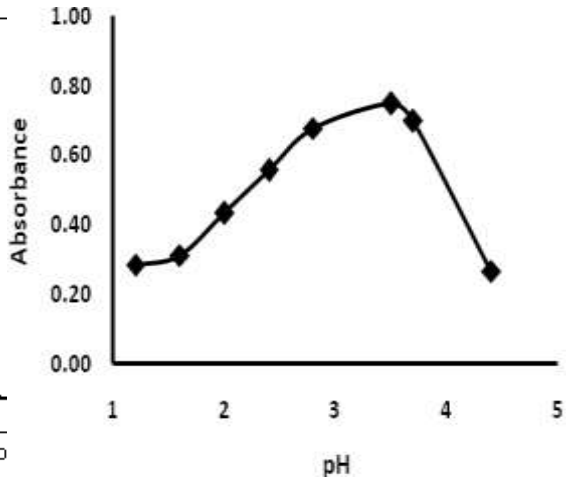


Figure (11): Effect of pH on The Absorbance of Raloxifene (16 µg ml^{-1}) Reaction Product with Eosin - Y at 5٤٠ nm.

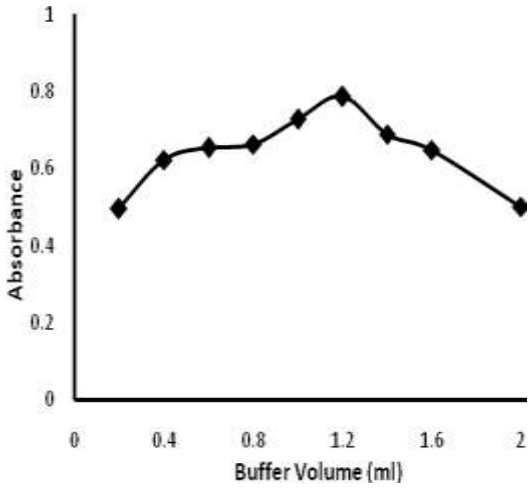


Figure (12): Effect of Buffer Volume on the absorbance of Raloxifene (16 µg ml^{-1}) Reaction Product with Eosin - Y at 545 nm.

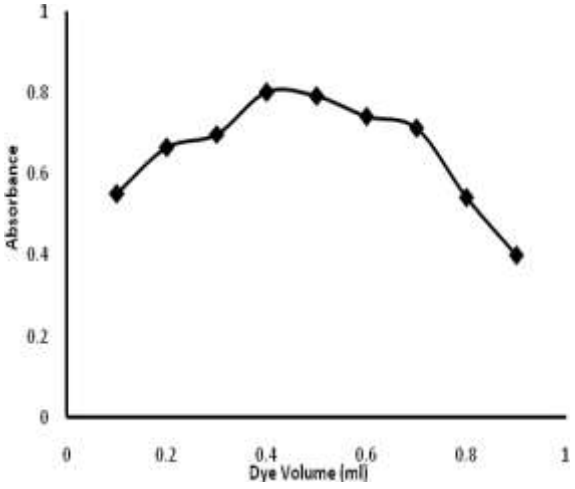


Figure (13): Effect of Eosin - Y Volume on The Absorbance of Raloxifene (16 µg ml^{-1}) Reaction Product at 54٠ nm.

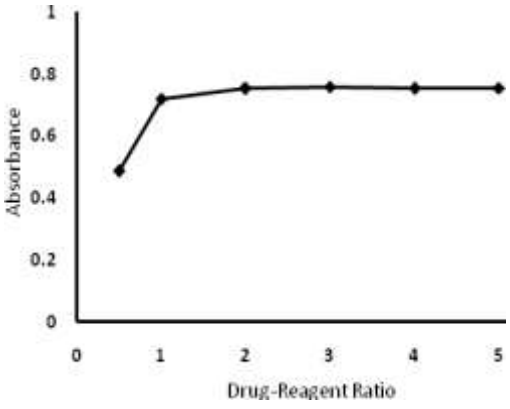


Figure (14): Stoichiometry of the Reaction of Raloxifene(3.13x10^{-4}M) with Eosin-Y(3.13x 10^{-4} M) by Molar Ratio Method at 54٠nm.

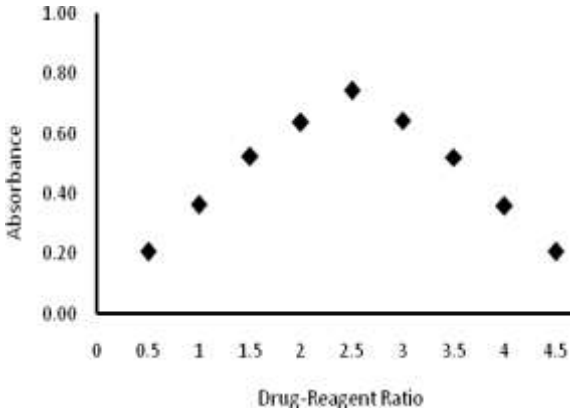
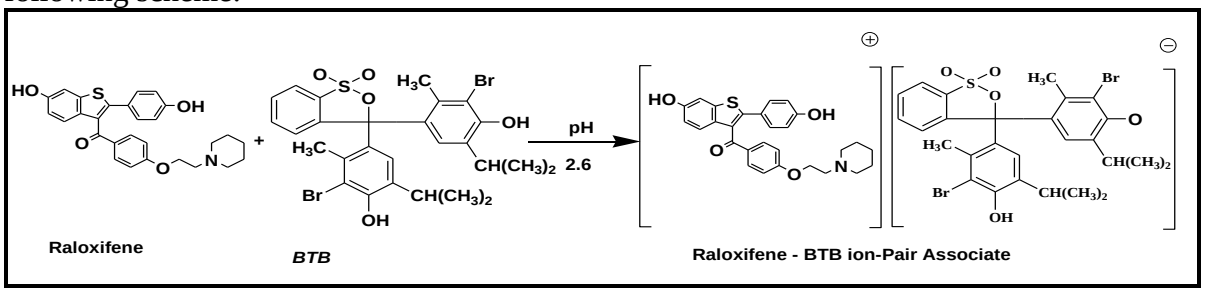


Figure (15): Stoichiometry of The Reaction of Raloxifene (١.2٠ x 10$^{-٤}$M) with eosin –Y (١.2٠ x 10$^{-٤}$M) by Continuous Variation (Job's) Method at 54٠ nm.

The last method E (Ion-Pair Technique with BTB):

At a selected pH value, some basic compounds form ion association complexes on treatment with acid dyes. These complexes are often coloured and extractable in organic solvents to be measured spectrophotometerically. This property is applied for determination of RLX through measurement of the absorbance of the formed yellow coloured complex at 420 nm using BTB (**figure 16).** The mechanism of the reaction was illustrated in the following scheme:



Various parameters affecting the reaction process were studied. The effect of time was studied by allowing the reactants to stand for different times up to one hour, the product was extracted with chloroform and the absorbance was measured at the suggested wavelength. The results reveal that the complex is formed at once producing maximal intensity. In order to establish the optimum pH, the drug was allowed to react with BTB in aqueous solutions buffered to different pH values, then the complex formed was extracted with chloroform and the absorbance was measured at the corresponding λ_{max}, maximum absorbance was obtained upon using potassium acid phthalate buffer solution of pH 2.6 (**figure 17).** Also the volume of the buffer was optimized to be 4 ml (**figure 18)**.

The drug was allowed to react with different volumes of the dye solution (0.1%) and it was found that 3 ml of BTB was sufficient to attain maximum absorbance (**figure 19)**.

Also, some organic solvents e.g. chloroform, methylene chloride, ethylene chloride and carbon tetrachloride were applied for extraction of the complex. It was found that chloroform is the ideal solvent for extracting the formed complex, yielding maximum absorbance intensity (**figure 20)**. Shaking time of about 1 minute, produced a constant absorbance. The absorbance of the separated extract was stable for more than one hour.

Molar ratio method was applied to determine of stoichiometry of the reaction between the drug and the acidic dye. Results reveal a 1:1 complexation ratio under the optimum conditions attained for the reaction between RLX and BTB (**figure 21).**

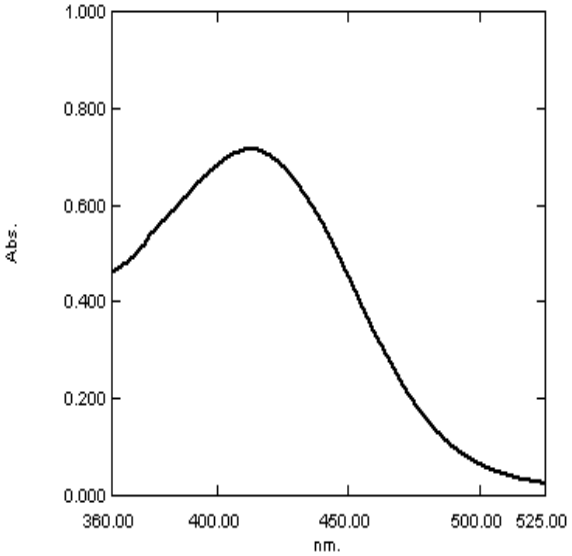
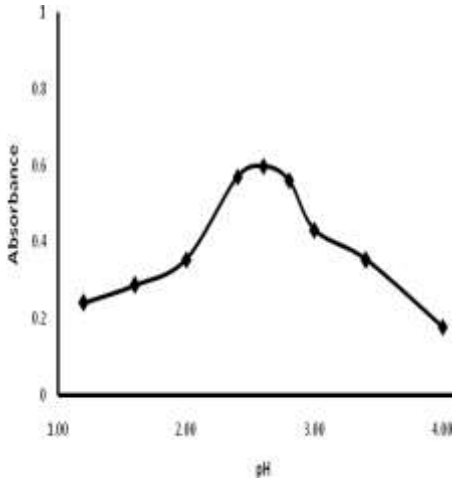


Figure (16): Absorption Spectrum of Raloxifene (20µgml^{-1}) Reaction Product with 3ml BTB Dye.



Figure(17): Effect of pH on The Absorbance of Raloxifene (20 µg ml^{-1}) Reaction Product with BTB Dye at 420 nm.

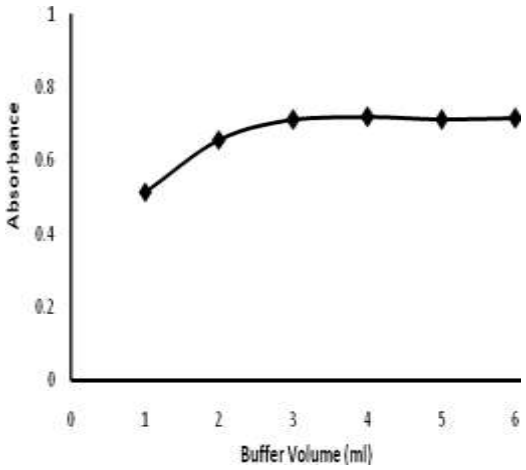


Figure (18): Effect of Buffer Volume on the absorbance of Raloxifene (20 µg ml^{-1}) Reaction Product with BTB Dye at 420 nm.

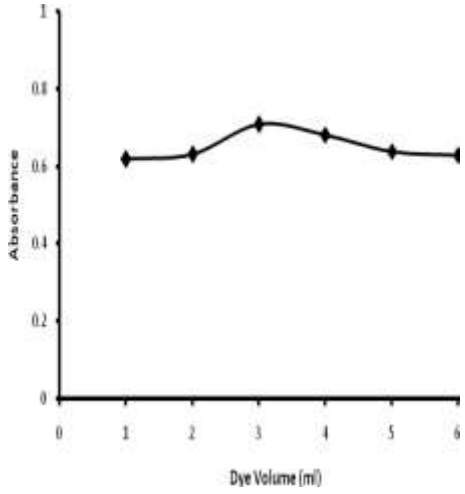


Figure (19): Effect of BTB Dye Volume on The Absorbance of Raloxifene (20 µg ml^{-1}) Reaction Product at 420nm.

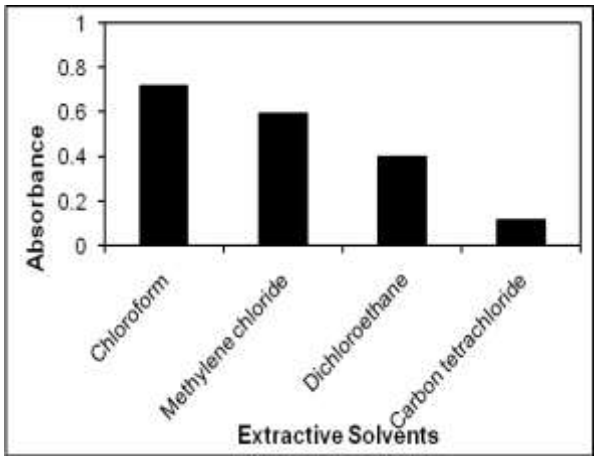


Figure (20): Effect of Extractive Solvenst on The Absorbance of Raloxifene (20 µg ml^{-1}) Reaction Product with BTB Dye at 420 nm.

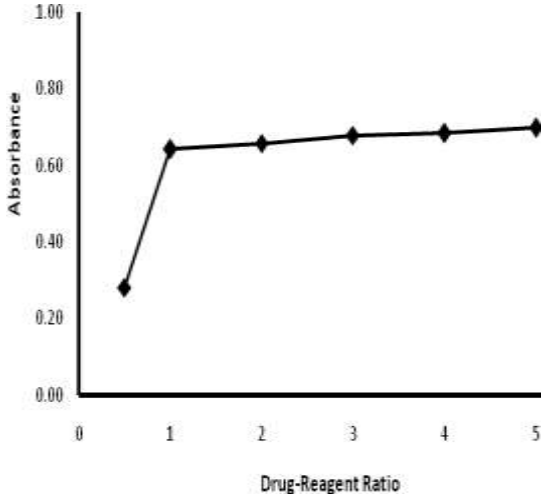


Figure (21): Stoichiometry of the Reaction of Raloxifene ($9.8x\ 10^{-4}M$) with BTB Dye ($9.8x\ 10^{-4}$ M) by Molar Ratio Method at420nm.

Validation of the procedures:

Linearity:

The linearity range for **method A**, under the optimized conditions, a linear relationship between the peak area ratio and the corresponding drug concentrations was obtained over the range of 0.5 – 8 µg ml^{-1}.**While in methods B, D** and **E** the linearity ranges of the absorbances and the corresponding concentrations were in the range of 3 – 27, 2– 20 and 4–28 µg ml^{-1}, respectively. Also for **method C**, the linearity range of peak height in cm and corresponding drug concentrations was in the range of 3–18 µg ml^{-1}.

LOD and LOQ:

The LOD and LOQ were assessed using the slope of the calibration curve and the standard deviation of the blank, **table (1)**.

Accuracy and precision:

Intraday and interday of the proposed procedures were calculated, **table (5)** revealed the results of the developed methods.

Stability of standard drug solution

The stability of RLX solutions were evaluated by analysis of the stock solution for each procedure. for **method A,** it was found to be stable for 5 days at room temperature and 8 days if stored in refrigerator. **While** in **method B,** it was found to be stable for 4 days either at room temperature or in refrigerator. Also, for **methods C, D and E**, it was found to be stable for more than 7 days at room temperature and in refrigerator.

System suitability:

System suitability test for **method A** was applied to a representative chromatogram to check various parameters such as the number of theoretical plates (N), resolution factor (R), capacity factor (K^-), tailing factor (T) and selectivity factor(α). The results obtained revealed that the chromatographic conditions described here allow complete base line separation between drug, its degradate and the internal standard peaks with minimum tailing.

Ruggedness:

The proposed HPLC procedure was evaluated using different sources of acetonitrile; Sigma; Aldrich, LabScan, Scharlau. The **RSD%** was found to be 1.46 proving ruggedness of the procedure.

Robustness:

The robustness of the proposed HPLC procedure was evaluated by the introduction of intentional variation in the mobile phase contents ratios. It was found that, using 49 - 51 ml of 0.05 M KH_2PO_4 did not affect the system suitability parameters, confirming robustness of the procedure.

Analysis of pharmaceutical preparation

The proposed procedures were also adopted for the determination of RLX in Evista® 60 mg tablets. It should be pointed out that no interference by excipients and additives in Evista® 60 mg tablets. The recovery of the proposed procedures was determined by applying standard addition technique **table (6)**.

The results obtained by the proposed procedures were statistically compared with those obtained by the reported one depending on the UV absorbance in 0.1 M NaOH (**Pavithra DC** and **Lakshmi SS.** 2006). The calculated " t" and " F " values are less than tabulated ones confirming accuracy and precision at 95 % confidence limits **table (7).**

CONCLUSION

Simple, accurate and precise methods were developed for the analysis of raloxifene hydrochloride in pure form and its tablets. The sensitivity, reproducibility and simplicity of the proposed methods makes it valuable in routine analysis of raloxifene hydrochloride. In addition, some of the proposed methods mainly, HPLC, ΔA and first derivative methods were found to be stability - indicating methods. This offer an advantage over the published methods.

Table (1): Selected spectral data for the determination of (RLX) by the proposed procedures:

Method	*A*	*B*	*C*	*D*	*E*
λ_{max} nm	280	285	268	545	420
Linearity range ($\mu g ml^{-1}$)	0.5 ─ 8	3 - 27	3 — 18	2 — 20	4 — 28
LOD ($\mu g ml^{-1}$)	0.077	0.233	0.254	0.276	0.633
LOQ ($\mu g ml^{-1}$)	0.258	0.778	0.849	0.920	2.110
Working range ($\mu g ml^{-1}$)	0.5 ─ 8	3 - 27	3 — 18	2 — 20	4 — 28
Response factor ± SD	0.353 ± 0.013	0.036 ± 0.073	0.084 ± 0.008	0.053 ± 0.006	0.038 ± 0.014
A (1%, 1 cm)	4533	353	2133	526	376
Regression Parameters - *Slope* ± *S.D*	$0.353\pm9.8x10^{-3}$	$0.035\pm7.4x10^{-2}$	$0.052\pm1.6x10^{-3}$	$0.050\pm1.15\ x10^{-4}$	$0.039\pm2.3x10^{-3}$
-Intercept ± *S.D*	$0.081\pm8.9x10^{-2}$	$0.009\pm 3.7x10^{-2}$	$0.095 \pm 4.4x10^{-3}$	$0.014\pm4.5x10^{-3}$	$-0.017\pm2.6x10^{-3}$
Correlation Coefficient (r^2)	0.9998	0.9998	0.9998	0.9999	0.9999

Table (2): Determination of (RLX) in Mixtures with its Degradation Product by the Proposed HPLC Procedure:

Intact in ($\mu g\ ml^{-1}$)	Degradate in ($\mu g\ ml^{-1}$)	Percent of degradate	Intact found in ($\mu g\ ml^{-1}$)	Recovery % of intact
7	1	12.5	7.12	101.71
6	2	25	6.1	101.67
5	3	37.5	5.08	101.60
4	4	50	3.95	98.75
3	5	62.5	2.98	99.33
2	6	75	1.99	99.50
1	7	87.5	1	100.00
Mean ± SD				100.37±1.260

Table (3): Determination of (RLX) in Mixtures with its Degradation Product by the Proposed ΔA Procedure:

Intact in ($\mu g\ ml^{-1}$)	Degradate in ($\mu g\ ml^{-1}$)	Percent of degradate	Intact found in ($\mu g\ ml^{-1}$)	Recovery % of intact
24	3	11.1	24.13	100.54
21	6	22.2	21.16	100.76
18	9	33.3	18.1	100.56
15	12	44.4	15.06	100.40
12	15	55.5	11.96	99.67
9	18	66.6	8.91	99.00
6	21	77.7	5.95	99.17
Mean ± SD				100.01±0.724

Table (4): Determination of (RLX) in Mixtures with its Degradation Product by the Proposed First Derivative (^{1}D) procedure:

Intact (µg ml^{-1})	**Degradate (µg ml^{-1})**	**Degradate %**	**Intact found (µg ml^{-1})**	**Recovery % of Intact**
16	2	11.1	16.16	101.00
14	4	22.2	14.10	100.71
12	6	33.3	12.03	100.25
10	8	44.4	10.15	101.50
8	10	55.5	7.97	99.63
6	12	66.6	5.95	99.17
4	14	77.7	3.98	99.50
2	16	88.8	2.01	100.50
Mean ± SD				100.28±0.801

Table (5): Intraday and interday accuracy and precision for the determination of RLX by the proposed procedures:

Method	***Conc. µgml^{-1}***	***Intraday***			***Interday***		
		Found Conc. ± SD	***Accuracy (R%)***	**Precision *(RSD%)***	***Found Conc. ± SD***	***Accuracy (R%)***	***Precision (RSD%)***
Method A	٢	2.02±0.018	101.00	0.891	2.00±0.060	100.00	0.500
	٤	4.05±0.039	101.25	0.963	3.96±0.062	99.00	1.566
	٦	5.98±0.070	99.67	1.171	5.96±0.054	99.33	0.906
Method B	6	5.98±0.021	99.67	0.351	6.07±0.031	101.17	0.511
	12	12.15±0.025	101.25	0.206	11.98±0.019	99.83	0.159
	24	24.00±0.031	100.00	0.129	23.99±0.018	99.96	0.075
Method C	٦	6.10±0.012	101.67	0.197	5.99±0.020	99.83	0.334
	٩	9.10±0.070	101.11	0.769	8.98±0.036	99.78	0.401
	١٢	11.97±0.690	99.75	0.576	12.05±0.065	100.42	0.539
Method D	٦	6.05±0.015	100.83	0.248	5.94±0.016	99.00	0.269
	١٠	10.15±0.018	101.50	0.177	9.90±0.030	99.00	0.303
	١٤	13.91±0.026	99.36	0.187	14.10±0.038	100.71	0.270
Method E	١٢	12.17±0.027	101.42	0.222	11.98±0.019	99.83	0.159
	١٦	16.22±0.041	101.38	0.253	16.20±0.012	101.25	0.074
	٢٠	19.92±0.034	99.60	0.171	19.99±0.029	99.95	0.145

***Table (6)*:** Application of standard addition technique for the determination of RLX in its pharmaceutical preparation (Evista® tablets) by the proposed producers:

	Claimed taken (μgml^{-1})	Pure added (μgml^{-1})	Pure found (μgml^{-1})	Recovery % of pure found
Method A	2	6	5.98	99.67
	3	5	4.94	98.80
	4	4	4.05	101.25
	5	3	3.05	101.67
	6	2	2.00	100.00
Mean				100.28
RSD%				1.17
Method B	3	6	6.10	101.67
		12	12.17	101.42
		18	17.90	99.44
		24	23.98	99.92
Mean				100.61
RSD%				1.09
Method C	6	3	3.01	100.33
		6	6.04	100.67
		9	8.96	99.56
		12	12.15	101.25
Mean				100.45
RSD%				0.70
Method D	4	4	4.04	101.00
		8	8.07	100.88
		12	12.13	101.08
		16	15.94	99.63
Mean				100.65
RSD%				0.68
Method E	8	8	7.93	99.13
		12	12.13	101.08
		16	16.15	100.94
		20	19.89	99.45
Mean				100.15
RSD%				1.00

Table (7): Statistical analysis of results obtained by the proposed and reported methods for the determination of RLX in its pharmaceutical preparation:

	Evista® Tablets					
Method	**A**	**B**	**C**	**D**	**E**	Reported method
N**	7	9	8	7	8	5
Mean	100.٨٣	100.٢4	100.١٥	100.٢٦	٩٩.٩٨	99.٨9
SD	0.٩20	0.٧٢4	0.٥٨6	0.٣٨3	٠.٢٦1	0.٦50
RSD%	0.٩20	0.٧٢4	0.٥86	0.٣٨3	٠.٢٦1	0.٦50
t***	1.٥١٢ (2.228)	0.٨٦٥ (2.179)	1.١٤٦ (2.201)	0.٩٨٧ (2.228)	1.١٩٩ (2.201)	ـــــ
F***	1.٣٨ (6.16)	4.٩7 (6.04)	1.٩0 (6.09)	٢.2٥ (6.16)	٣.١٩٠ (6.09)	ـــــ

* UV absorbance in acidic medium **(Pavithra DC and Lakshmi SS.2006)**.
** Number of experimental.
*** The values in parenthesis are tabulated values for "t" and "F" at $P < 0.05$

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تقييم عقار رالوكسيفين بواسطة دلالية الثبات باستخدام كروماتوجرافيا السوائل المضغوطة و طرق طيف ضوئية في صورته النقية والأقراص.

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تم تعيين عقار رالوكسيفين فى صورته النقية وكذلك فى الأقراص الصيدلية باستخدام خمس طرق تحليلية جديدة وتتلخص هذه الطرق فى الأتى (١) استخدام كروماتوجرافيا السوائل ذات الضغط العالى فى الحالة المعكوسة لفصل العقار فى صورته السليمة عن نواتج التكسير وتم تعينه عند طول موجى قدره ٢٨٠ ن.م (٢) استخدام الفرق فى الإمتصاص الطيف ضوئى للعقار بين الوسط الحامضى والقاعدى لتعين العقار فى صورته السليمة فى وجود نواتج التكسير عند طول موجى ٢٨٥ ن.م.(٣) تتناول الطريقة الثالثة استخدام المقياس الطيفى للمشتق الأول لتعيين رالوكسيفين فى وجود نواتج تكسيره عند طول موجى ٢٦٨ ن.م. (٤)هذا وقد تم استخدام الإزدواج الأيونى مع صبغة الإيوسين الأصفر لتعين العقار عند طول موجى قدرة ٥٤٥ ن.م. (٥) وفى الجزء الأخير من هذا البحث قد تم استخدام الإزدواج الأيونى مع صبغة البروموثيمول الأزرق لتعين العقار فى وجود محلول منظم عياريته ٢.٦ عند طول موجى قدرة ٤٢٠ ن.م. هذا وقد تم تطبيق هذة الطرق فى تحليل الأقراص وبمقارنة هذة الطرق إحصائيا بالطريقة المنشورة لم يكن هناك فرق بينهم.