VALIDATED SPECTROPHOTOMETRIC METHODS FOR THE DETERMINATION OF RALOXIFENE HYDROCHLORIDE IN PHARMACEUTICALS

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ABSTRACT

Two new simple, precise, rapid and extraction-free spectrophotometric methods are proposed for the determination of raloxifene hydrochloride (RLX) using bromate-bromide mixture and two dyes, methylene blue and rhodamine B, as reagents. The methods entail the addition of a known excess of bromate-bromide mixture to RLX in hydrochloric acid medium followed by determination of residual bromine by reacting with a fixed amount of either methylene blue and measuring the absorbance at 665 nm (Method A) or rhodamine B and measuring the absorbance at 555 nm (Method B). The amount of bromate reacted corresponds to the amount of RLX. In both the methods, the absorbance is found to increase linearly with the concentration of RLX. Under the optimum conditions, RLX could be assayed in the concentration ranges 0.5-5.0 and 0.1-2.0 $\mu\text{g mL}^{-1}$ from method A and method B, respectively. The apparent molar absorptivities are calculated to be 7.0×10^4 and $1.1 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$ for method A and method B, respectively, and the corresponding Sandell sensitivity values are 0.0073 and 0.0048 $\mu\text{g cm}^{-2}$. The limits of detection and quantification have also been reported for both the spectrophotometric methods. The overall reproducibility of the methods was excellent and recoveries were 98.3-102.5% and 98.2-103.2% for method A and method B, respectively. The proposed methods can be readily utilized for bulk drug and in pharmaceutical formulations.

Key words: Raloxifene hydrochloride, Assay, Spectrophotometry, Bromate-bromide, Formulations.

INTRODUCTION

Raloxifene hydrochloride (RLX) is a selective estrogen receptor modulator that belongs to the benzothiophene class of compounds¹ (Fig.1). The chemical designation is methanone,[6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl]-[4-[2-(1-piperidinyl)ethoxy]phenyl]-, hydrochloride. RLX is used in the treatment and prevention of osteoporosis in post-menopausal women.

Literature on the methods for the determination of RLX in pharmaceuticals is scanty. High performance liquid chromatography (HPLC) has been used for the assay of RLX in bulk drug² and in dosage forms³⁻⁷. Chen *et al*⁸ have recently proposed an UV-spectrophotometric method for determination of RLX in capsules. A quantitative assay method for RLX in pharmaceutical preparations by capillary electrophoresis⁹ has recently been reported. A somewhat sensitive method based on the measurement of the intensity of resonance Rayleigh scattering (RRS)¹⁰ of the ion association complex of RLX with Evans Blue was developed by Fan *et al* but, the method requires an expensive instrumental set up. Visible spectrophotometry, in spite of its simplicity, reasonable accuracy and precision, and speed, has not been widely applied for the assay of RLX. There are only two reports on the visible spectrophotometric determination of RLX. Dharuman *et al*¹¹ have proposed two methods. One method is based on the formation of bluish green chromogen formed when the drug is treated with ferric-ferricyanide reagent. The other involves the reduction of Fehling's reagent by RLX followed by measurement of blue-green species at 430 nm. Very recently, Annapura *et al*¹² have reported three procedures based on redox and complexation reactions. Some of the reported methods, however, suffer from such disadvantages as poor selectivity, sensitivity, accuracy and precision (Table 1). The present investigation aims to develop sensitive and cost-effective methods for the determination of RLX in pure form and in dosage forms using spectrophotometric technique. The methods utilize bromate-bromide mixture as oxidimetric reagent, which has successfully been used for the sensitive spectrophotometric determination of many bioactive substances¹³⁻²¹. The proposed method has the advantages of speed sensitivity and simplicity besides being accurate and precise, and can be adopted by the pharmaceutical laboratories for industrial quality control.

Fig 1. Structure of raloxifene hydrochloride

EXPERIMENTAL

Apparatus

A Systronics model 106 digital spectrophotometer with 1-cm matched quartz cells was used for all absorbance measurements.

Reagents and materials

All chemicals used were of analytical reagent grade and distilled water was used to prepare all solutions. A standard solution equivalent to 2mM KBrO_3 - 20mM KBr was prepared by dissolving accurately weighed 3.34 g of KBrO_3 (Sarabhai M. chemicals, Baroda, India) and 15.0 g of KBr (S.d. Fine Chem. Ltd., Mumbai, India) in water and diluting to 1 liter in a volumetric flask. The above solution was diluted appropriately with water to get 25 and 10 $\mu\text{g mL}^{-1}$ concentrations for method A and method B, respectively. To prepare 40 $\mu\text{g mL}^{-1}$ methylene blue for method A, first, a 500 $\mu\text{g mL}^{-1}$ dye solution was prepared by dissolving 71.5 mg of dye (Qualigen fine-chem., Mumbai, assay 70%) in water and diluting to 100 mL in a calibrated flask, and filtered using glass wool. This was appropriately diluted to get the required concentration. For method B, first, a 500 $\mu\text{g mL}^{-1}$ rhodamine B solution was prepared by dissolving 62.5 mg of dye (s.d.fine-chem Ltd., Mumbai, 80 % assay) in water and diluting to 100 mL, and filtered. This was appropriately diluted with water to get 50 $\mu\text{g mL}^{-1}$. Hydrochloric acid (5M) was prepared by diluting 43 mL of concentrated acid (s.d.fine-chem Ltd., Mumbai, Sp gr 1.18) to 100 mL with water. Pharmaceutical grade RLX, certified to be 99.8% pure was procured from Cipla India Ltd, Mumbai, India, and was used as received. A 1 mg mL^{-1} solution of RLX was prepared by dissolving accurately weighed 250 mg of pure drug in 38 mL glacial acetic acid and warmed to get clear solution, followed by diluting to 250 mL with water in a calibrated flask. This stock solution (1000 $\mu\text{g mL}^{-1}$) was diluted with water to get working concentrations of 10 and 5 $\mu\text{g mL}^{-1}$ RLX for method A and method B, respectively.

Table 1. Comparison of the proposed methods with the existing spectrophotometric and other methods.

Sl. No.	Method	Experimental details.	Detection	Linear range, $\mu\text{g mL}^{-1}$	LOQ $\mu\text{g mL}^{-1}$	Remarks	Ref. No.
1.	HPLC	Octadecyl-bonded silica column; 3:7 acetonitrile: 0.05 M ammonium acetate as mobile phase.	UV at 286 nm	NA	NA	Application to related substances in RLX raw medicine.	2
2.	HPLC	NA	UV	0.5-10	0.610	Less accurate and precise; % e_R , -6.9-0.3; intra-day and inter-day RSD values were 2.38% and 7.93%, respectively.	3
3.	HPLC	Acetonitrile- 0.01 M sodium dodecylsulphate (55:45, pH 4.0) was the mobile phase.	UV at 286 nm	NA	NA	-	4
4.	HPLC	Merck RP-18 cartridge octadecylsilane was the stationary phase; acetonitrile:water (40:60, pH 3.0) was mobile phase (1 mL min^{-1})	UV at 225 nm	250-750	-	Less sensitive; narrow linear dynamic range; less precise (RSD > 0.8 %).	5
5.	HPLC	Intersil C_{18} column; acetonitrile-phosphate buffer (pH 2.0) was the mobile phase (1 mL min^{-1})	UV at 280 nm	0.5-200	NA	Less precise, intra-day and inter-day RSD >1%.	6
6.	RP-HPLC	Water symmetry C_{18} ; acetonitrile-phosphate buffer (pH 2.0) was the mobile phase (1 mL min^{-1})	UV-230 nm	10-60 mg mL^{-1}	4.04 mg mL^{-1}	Least sensitive	7
7.	UV-Spectrophotometry.	NA	NA	NA	NA	Less accurate and precise, recovery, 99.32% with RSD of 0.895.	8
8.	Capillary electrophoresis	pH 4.5 buffer consisting of 20 mM sodium acetate was used.	-	-	-	Migration behavior and hence results dependent on too many variables.	9
9.	Resonance Rayleigh scattering (RRS)	Intensity of RRS of the ion-association complex formed b/n RLX and Evans blue in NaAc-HCl buffer for (pH 1.8) measured.	-	0-8.3	18.9 ng mL^{-1} (LOD)	Not very selective	10
10.	Visible spectrophotometry	(i). FeCl_3 -ferricyanide (ii). Fehling reagent.	735 nm	NA	NA	Prone to interference by reducing substances.	11
11.	Visible spectrophotometry.	(i). 0.1 N-NaOH (ii). FeCl_3 -O-phen. (iii). FeCl_3 -bipyridyl	425 nm 510 nm 521 nm	5-150 ($\epsilon = 3.6 \times 10^3$) 1-10 ($\epsilon = 6.3 \times 10^4$) 2-25 ($\epsilon = 2.1 \times 10^4$)	- - -	Measurement at shorter wavelength where the interference from excipients is serious. Involves heating at 60 °C for 15 min.	12
12.	Vis- spectrophotometry.	a). BrO_3^- -Br/MB b). BrO_3^- -Br/Rh B	665 nm 555 nm	0.5-5.0 $\epsilon = 7.0 \times 10^4$ 0.1-2.0 $\epsilon = 1.1 \times 10^5$	0.18 0.13	No heating involved, measurement at longer wavelengths where interference from excipients is far less; highly sensitive.	Present work.

Procedures**Spectrophotometric method using methylene blue (Method A)**

Aliquots of pure RLX solution (0.5 to 5.0 mL; 10 $\mu\text{g mL}^{-1}$) were transferred into a series of 10 mL calibrated flasks and the total volume was adjusted to 5.0 mL with water. To each flask were added 1 mL of 5M hydrochloric acid followed by 1 mL of bromate-bromide mixture (25 $\mu\text{g mL}^{-1}$ w.r.t KBrO_3). The flasks were stoppered, content was mixed well and the flasks were set aside for 10 min with occasional shaking. Finally, 1 mL of 40 $\mu\text{g mL}^{-1}$ methylene blue solution was added to each flask, diluted to the mark with water and the

absorbance of solution was measured at 665 nm against reagent blank after 10 min.

3.2. Spectrophotometry with rhodamine B (Method B)

Varying aliquots (0.2-4.0 mL) of standard 5 $\mu\text{g mL}^{-1}$ RLX solution were measured accurately and delivered into a series of 10 mL calibrated flasks and the total volume was brought to 4.0 mL with water. To each flask were added 1 mL each of 5 M hydrochloric acid and bromate-bromide mixture (10 $\mu\text{g mL}^{-1}$ in KBrO_3) successively; the flasks were stoppered, and let stand for 10 min with occasional shaking. Then, 1 mL of 50 $\mu\text{g mL}^{-1}$ rhodamine B solution was added

to each flask, the volume was adjusted to the mark with water and mixed well. The absorbance of each solution was measured at 555 nm against a reagent blank after 10 min.

In either spectrophotometric method, the concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

Assay procedure for formulations

An amount of finely ground tablet powder equivalent to 100 mg of RLX was accurately weighed into a beaker, 15 mL of glacial acetic acid was added and stirred for 20 min and warmed. Then, the volume was made up 100 mL in calibrated flask with water, mixed well, and filtered using a Whatman No 42 filter paper. First 10 mL portion of the filtrate was discarded and a suitable aliquot of the subsequent portion (1 mg mL⁻¹ RLX) was diluted appropriately to get 10 and 5 µg mL⁻¹ concentrations for analysis by spectrophotometric method A and method B, respectively.

RESULTS AND DISCUSSION

Optimization of experimental conditions

The proposed spectrophotometric methods are indirect and are based on the determination of the residual bromine (*insitu* generated) after allowing the reaction between RLX and a measured amount of bromine to be complete. The surplus bromine was determined by reacting it with a fixed amount of either methylene blue or rhodamine -B dye. The amount of bromine reacted corresponds to the amount of RLX, which forms the basis for the assay.

RLX when added in increasing concentrations to a fixed concentration of *insitu* generated bromine, consumes the latter proportionately and there occurs a concomitant fall in the concentrations of bromine. When a fixed concentration of dye is added to decreasing concentration of bromine, a concomitant increase in the concentration of dye results. Consequently, a proportional increase in the absorbance at the respective $\lambda_{\max}$ is observed with increasing concentrations of RLX (fig.2 and 3).

Fig 2. Beer's law curve for method A

Fig 3. Beer's law curve for method B

Preliminary experiments were performed to fix the upper limits of the dyes that could be determined spectrophotometrically, and these were found to be 4 and 5 µg mL⁻¹ for methylene blue and rhodamine B, respectively. A bromate concentration of 2.5 µg mL⁻¹ was found to irreversibly destroy the blue colour of

4 µg mL⁻¹ methylene blue whereas 1.0 µg mL⁻¹ bromate was required to bleach red colour due to 5 µg mL⁻¹ rhodamine B. Hence, different concentrations of RLX were reacted with 1 mL of 25 µg mL⁻¹ bromate in method A and 1 mL of 10 µg mL⁻¹ bromate in method B followed by determination of the residual bromine as described under the respective procedures.

For both the steps, i.e. for the reaction between RLX and bromine, and for bleaching of dye by bromine, hydrochloric acid medium was found to be ideal. One mL of 5 M hydrochloric acid in a total volume of ~4-5 mL was adequate for the bromination step which was complete in 10 min in both methods and the same quantity of acid was employed for the estimation of the dye. Contact time of 10 min is not critical and any delay upto 20 min for method A, and 30 min for method B, will have no effect on the absorbance.

Table 2. Analytical and regression parameters of spectrophotometric Methods

Parameter	Method A	Method B
$\lambda_{\max}$, nm	665	555
Beer's law limits, µg mL ⁻¹	0.5 – 5.0	0.1 – 2.0
Molar absorptivity, L mol ⁻¹ cm ⁻¹	7.0×10 ⁴	1.1×10 ⁵
Sandell sensitivity, µg cm ⁻²	0.0073	0.0048
Limit of detection, µg mL ⁻¹	0.06	0.04
Limit of quantification, µg mL ⁻¹	0.18	0.13
Regression equation, Y*		
Intercept (a)	-0.0090	-0.0050
Slope (b)	0.1410	0.2140
Correlation coefficient, (r)	0.9997	0.9997
S _a	0.0061	0.0039
S _b	0.0019	0.0035

*Y = a+bX, where Y is the absorbance and X concentration in µg mL⁻¹

S_a = Standard deviation of intercept.

S_b = Standard deviation of slope.

Method Validation

Accuracy and precision

To evaluate the accuracy and precision of the methods, pure drug solution at three different levels (within the working limits) was analysed, each determination being repeated seven times. The relative error (%) and relative standard deviation (%) were less than 2.0 and indicate the high accuracy and precision for the methods (Table 3). For a better picture of reproducibility on a day-to-day basis, a series of experiments were performed in which standard drug solution at three different levels was determined each day for five days with all solutions being prepared afresh each day. The day-to-day relative standard deviation values were in the range of 2.2-2.7% and represent the best appraisal of the methods in routine use. These results are also given in Table 3.

Linear range, and regression and sensitivity parameters.

A linear correlation was found between absorbance at $\lambda_{\max}$ and concentration of RLX.

The graphs showed negligible intercept and are described by the equation:

$$Y = a + bX$$

(Where Y = absorbance of 1-cm layer of solution; a = intercept; b = slope and X = concentration in µg mL⁻¹). Regression analysis of the Beer's law data using the method of least squares was made to evaluate the slope (b), intercept (a) and correlation coefficient (r) for each system and the values are presented in Table 1. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell sensitivity values of both methods are also given in Table 1. The limits of detection (LOD) and quantitation (LOQ) calculated according to ICH guidelines²² are also presented in Table. 1 and reveal the very high sensitivity of the proposed methods.

Table 3. Evaluation of accuracy and precision

Method	OLZ Taken, $\mu\text{g mL}^{-1}$	Intra-day accuracy and precision			Intra-day accuracy and precision		
		RLX found*, $\mu\text{g mL}^{-1}$	RE %	RSD %	RLX found**, $\mu\text{g mL}^{-1}$	RE %	RSD %
	1.5	1.47	2.00	0.58	1.46	2.67	2.46
A	2.5	2.49	0.4	0.62	2.43	2.80	2.20
	3.5	3.45	1.43	0.74	3.43	2.00	2.64
	0.5	0.49	2.00	1.04	0.48	2.00	2.58
B	1.5	1.48	1.33	0.75	1.46	2.67	2.34
	2.0	1.98	1.00	0.62	1.93	3.50	2.70

RE relative error; RSD Relative standard deviation.

* Mean value of seven determinations

**Mean value of five determinations

Application to analysis of commercial samples

In order to check the validity of the proposed methods, RLX was determined in some commercial formulations. Table 4 gives the results of the determination from which it is clear that there is a close agreement between the results obtained by the proposed methods and the label claim. The results were also compared with those of the reference method ¹² statistically by a Student's t- test for accuracy and variance ratio F- test for precision at 95 % confidence level. The calculated t- and F-values (Table III) did not exceed the tabulated values ($t=2.77$, $F=6.39$) four degrees of freedom and this would indicate that there was no significant difference between the proposed methods and the reference method with respect to accuracy and precision.

The accuracy and validity of the proposed methods were further ascertained by performing recovery studies. Pre-analysed tablet or capsule powder was spiked with pure RLX at three different levels and the total was found by the proposed methods. Each determination was repeated three times. The recovery of the pure drug added was quantitative and revealed that co-formulated substances such as crospovidone, FD and C Blue No.2 aluminum lake, hypromellose, lactose monohydrate, magnesium stearate, modified pharmaceutical glaze, polyethylene glycol, polysorbate 80, povidone, propylene glycol, and titanium dioxide did not interfere in the determination. The results of recovery study are compiled in Table 5.

Table 4. Results of determination of raloxifene hydrochloride in formulations and statistical comparison with the reference method

Formulation Brand name [#]	Nominal amount, mg	% Found* $\pm$ SD		
		Reference method	Method A	Method B
FIONA ^a	60	100.3 $\pm$ 0.46	99.8 $\pm$ 1.05 $t=1.05$ $F=5.21$	101.1 $\pm$ 1.03 $t=1.70$ $F=5.01$
GYNISTA ^b	60	102.3 $\pm$ 0.51	101.95 $\pm$ 1.02 $t=0.72$ $F=4.00$	100.6 $\pm$ 1.16 $t=3.22$ $F=5.17$
RONAL ^c	60	101.2 $\pm$ 0.65	99.95 $\pm$ 1.02 $t=2.37$ $F=2.46$	101.9 $\pm$ 1.33 $t=1.12$ $F=4.19$

*Mean value of five determinations

[#]Marketed by: a. Reddy's Ltd. ; b. Micro(Nova) Ltd.; c. Blue cross Ltd.

Tabulated t-value at 95% confidence level is 2.77

Tabulated F-value at 95% confidence level is 6.39.

Table 5. Results of recovery experiments by standard addition method

Formulation studied	Method A				Method B			
	Amount of drug in tablet, µg	Amount of pure drug added, µg	Total found, µg	Pure drug recovered*, %	Amount of drug in tablet, µg	Amount of pure drug added, µg	Total found, µg	Pure drug recovered*, %
FIONA 60	4.990	5	10.07	101.5	5.055	5	10.19	102.6
	4.990	20	25.49	102.5	5.055	10	15.01	99.5
RONAL 60	4.990	45	50.40	100.9	5.055	15	20.10	100.3
	4.998	5	9.98	99.7	5.095	5	10.26	103.2
	4.998	20	24.66	98.3	5.095	10	14.92	98.2
	4.998	45	50.27	100.6	5.095	15	20.17	100.5

*Mean value of three determinations

5.0. CONCLUSIONS

Two useful micro methods for the determination of RLX have been developed and validated. The methods are simple and rapid taking not more than 15-20 min for the assay. Both spectrophotometric methods are more sensitive than the existing UV and HPLC methods, and are free from such experimental variables as heating or extraction step. The methods rely on the use of simple and cheap chemicals and techniques but provide a sensitivity comparable to that achieved by sophisticated and expensive technique like HPLC. Thus, they can be used as alternatives for rapid and routine determination of bulk sample and tablets.

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