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Analytical method validation and quantification of raloxifene hydrochloride and its related substance in API using reverse phase - liquid chromatographic method

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Abstract: The present paper describes the reverse phase-high performance liquid chromatographic method and was validated as per ICH guidelines for the determination of related substances in raloxifene hydrochloride. RP-Liquid chromatography technique was performed with pH 3.0 phosphate buffer and acetonitrile as mobile phase at a flow rate of 1.0 mL/minon Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower 3, with photodiode array detector using Inertsil BDS C_8 250 x 4.6 mm, 5µm column with UV detection at 280 nm. The method is specific and % RSD for system precision were 0.18 and 0.35 for raloxifene hydrochloride and Impurity- A respectively. For Method precision, % RSD for Raloxifene hydrochloride was 0.17 %. Linearity were observed for Raloxifene hydrochloride and Impurity- A, in the concentration range of 0.0006 and 0.0045 were linear (R^2 = 0.9999 and R^2 = 0.9999) Accuracy is calculated as % recovery. % Recovery for accuracy levels at 50, 100 and 150 % L for Raloxifene hydrochloride and Impurity- A were 103.56 ± 0.19, 114.23 ± 0.36, 107.09 ± 0.61 and 103.51 ± 0.30, 1143.41 ± 0.15, 103.62± 0.26, respectively. Rugedness was performed by different analyst on different days and % RSD between the areas is 0.84 Raloxifene hydrochloride. For Robustness, pH will shown the effect on retention time, hence better to maintain the pH at 3± 0.05. Signal to Noise ratio for Limit of detection and the limit of quantification were found to be in between 3-5 and > 10 for raloxifene hydrochloride and Impurity- A, respectively. The percent recovery was in good agreement; hence, the method is specific, simple, reproducible and accurate for the determination of Raloxifene hydrochloride.

Keywords: Raloxifene hydrochloride, estimation of related substances, liquid chromatography and percent recovery.

INTRODUCTION

Raloxifene hydrochloride is a nonsteroidal drug which comes under the classification of selective estrogen receptor modulator (SERM) belongs to the benzothiopene class of compounds [1,2]. It was approved by Food and Drug Adminstration (FDA) in 1997 [3]. Raloxifene hydrochloride is a generic name for 6-Hydroxy-2-(p-hydroxyphenyl) benzo[b]thien-3-yl-p-(2-piperidinoethoxy) phenyl ketone hydrochloride. The molecular formula of Raloxifene Hydrochloride is $C_{28}H_{27}NO_4S$. HCl and with molecular wight of 510.05 g/mol.

Raloxifene hydrochloride acts as an estrogen agonist on bone and on the liver thereby increasing bone mineral density and decreases LDL-cholesterol [4,5]. Currently, it is used for prevention of osteoporosis and to reduce the risk of invasive breast cancer in postmenopausal women who have osteoporosis or at high risk of invasive breast cancer [6-8]. It serves as a substitute for long-term female hormone replacement therapy. It is mostly supplied as 60 mg tablets for daily dose. However, the major obstacle for oral delivery of raloxifene is its poor systemic exposure, with only 2% absolute bioavailability, because of its poor solubility in aqueous fluids [9]. On September 14, 2007, the U.S Food and drug administration announced approval of raloxifene for reducing the risk of invasive breast cancer in postmenopausal women when compared with tamoxifen [10].

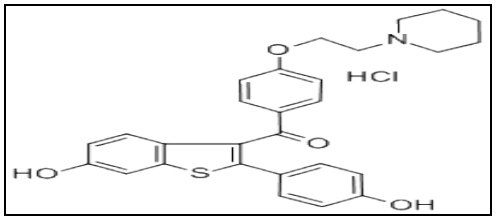

Fig-1: Chemical Structure of Raloxifene hydrochloride

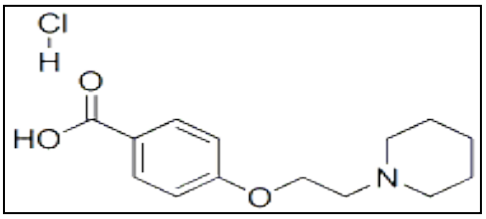

Fig-2: Chemical Structure of 4-[2-(1-Pipiridine) ethoxybenzoic acid hydrochloride (Impurity –A)

EXPERIMENTAL CONDITIONS

Instrumentation and chromatographic conditions

Chromatographic separation was achieved by using a Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower 3 photodiode array detector using Inertsil BDS C_8 (250mm× 4.6 mm, 5 µm particle size) column with eluent-A: phosphate buffer eluent-B: acetonitrile as mobile phase at a flow rate of 1.0 mL/min. with UV detection at 280 nm. Column maintained at temperature 35ºC. The overall run time was 50 min. 10 µL of sample was injected into the HPLC system.

Chemicals used: Orthophosphoric acid, Acetonitrile HPLC grade and water were obtained from Merck, India. All chemicals were of an analytical grade and used as received.

Preparation of Mobile Phase-A

Weigh accurately about 9.0 g of monobasic potassium hydrogen phosphate and transfer into 1000 mL of water, and mix. Add 0.6 mL of phosphoric acid, further adjust with phosphoric acid or potassium hydroxide solution to a pH of 3.0±0.1, and mix well.

Preparation of Mobile Phase-B Acetonitrile used as mobile phase-B.

Preparation of Diluent

700mL of Mobile Phase – A and 300 mL of Mobile Phase- B are mixed to prepare one litre of diluent and the solution are properly mixed.

Method validation

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. The following are the solution to be prepared for the study of Specificity for Related Substances by HPLC.

Prepare individual solution of the substance containing 0.003 mg/ml of Impurity-A, 0.003mg/ml of Raloxifene Hydrochloride and 0.12 mg/ml of N-oxide.

A spiked solution of impurity-A to the Raloxifene Hydrochloride drug substance and to check specificity study and to check for system suitability by injecting Blank, individual solutions and spiked solutions. Analysis was performed by PDA detector and peak purity was determined. Specificity chromatograms of blank, WSTD, IMP-A working standard and spiked solution were shown in Figure 3, 4.

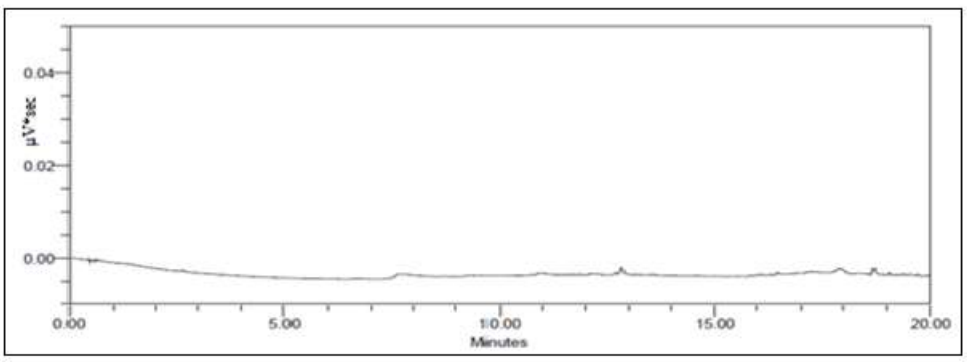

Fig-3: Specificity chromatogram of blank solution

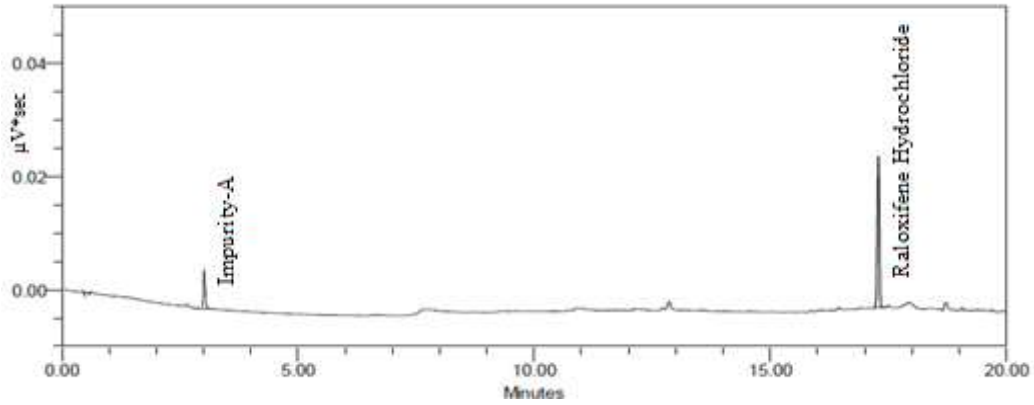

Fig-4: Specificity chromatogram of raloxifene hydrochloride and Impurity-A solution

Acceptance criteria

The tailing factor for Raloxifene Hydrochloride peak should be less than 2.0

The resolution for Raloxifene Hydrochloride and Impurity-A peak should be less than 3.0

The above study reveals that all the known impurities of Raloxifene Hydrochloride adequately resolved. Hence, the method is selective for the determination of related substances in Raloxifene Hydrochloride.

System precision

Raloxifene Hydrochloride working standard and Impurity-A working standard were analyzed for system precision study by injecting Blank and six injections of standard solution and the results of the study are shown in Table 2.

Acceptance criteria: The % RSD for peak areas of Raloxifene HCl and Impurity-A should not be more than 2.00.

Method precision

The precision of the method was determined by analyzing a sample solution at 100% of the specification limit (Six replicate sample preparations).

Preparation of test solution:

Weigh accurately about 30 mg of the sample into a 10 mL volumetric flask.. Dissolve and make up to the mark with diluent.

Acceptance criteria: The % RSD for peak areas of RLX Hcl and Unknown impurity should not be more than 2.00.

Linearity

The linearity of the HPLC method was demonstrated for raloxifene hydrochloride related substances solutions ranging from 20% to 150% of the specification limit.

Preparation of Impurity-A and Raloxifene hydrochloride Stock solution:

Weigh accurately about 30mg of each Impurity-A and raloxifene hydrochloride into a 100 mL volumetric flask. Dissolve and make up to the volume with diluent.

Preparation of 20% solution:

Take 0.1 ml of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50 mL with diluent.

Preparation of 40% solution:

Take 0.2 ml of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50 mL with diluent

Preparation of 80% solution:

Take 0.4 mL of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50 mL with diluent

Preparation of 90% solution:

Take 0.45 mL of above prepared Stock solution into a 50mLvolumetric flask, Dissolve and dilute to 50 mL with diluent

Preparation of 100% solution

Take 0.5mL of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50 mL with diluent

Preparation of 110% solution

Take 0.55mL of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50mL with diluent

Preparation of 120% solution:

Take 0.6mL of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50mL with diluent

Preparation of 150% solution:

Take 0.75mL of above prepared Stock solution into a 50mL volumetric flask, Dissolve and dilute to 50mL with diluents. The above concentrations were injected into HPLC System twice to determine the linearity.

Acceptance criteria: The plot of concentration versus average peak area of impurity-H and raloxifene hydrochloride should be linear with correlation coefficient not less than 0.99.

Accuracy

The accuracy of the method was determined using three solutions containing raloxifene hydrochloride and Impurity-A at approximately 50%, 100% and 150% of the strength of working standard concentration. Each solution was analyzed in triplicate and calculated the %Recovery with respect to standard.

Preparation of Recovery Stock solution:

Weigh accurately about each 15mg of Raloxifene Hydrochloride and Impurity-A into a 50mL volumetric flask, Dissolve and dilute to the volume with diluent.

Standard solution

Weigh accurately about each 30mg of raloxifene hydrochloride and Impurity-A into a 100mL volumetric flask, Dissolve and dilute to the volume with diluent.

Take 5.0 mL of above prepared standard solution into a 500mL volumetric flask and dilute to the mark with diluent.

Recovery @ 50% Level: Taken 0.5 mL of Recovery stock solution into a 100 mL volumetric flask and make up to the mark with standard solution.(0.0045mg/ml).

Recovery @ 100% Level: Taken 1.0 mL of Recovery stock solution into a 100 mL volumetric flask and maked up to the mark with standard solution.(0.006mg/ml)

Recovery @ 150% Level: Taken 1.5 mL of Recovery stock solution into a 100 mL volumetric flask and make up to the mark with standard solution.(0.0075mg/ml)
The above solutions were injected in to HPLC system twice along with bracketing standards to check recovery.

RUGEDNESS

Rugedness can be performed by different days by different analyst

Preparation of test solution:

Weigh accurately 30 mg of the sample into a 10 mL volumetric flask. Dissolve and dilute up to the mark with diluent. The same procedure was followed for six replication preparations.

Acceptance criteria

The %RSD for average peak areas of RLX HCl and known, single maximum unknown impurities should not be more than 2.00%

The %RSD for combined Day-1 and Day-2 average peak areas of RLX HCl and known, single maximum unknown impurities of should not be more than 5.00%

Robustness

The parameters of the method that was altered to test the robustness of the method. Flow rate was varied from actual from 1.0 to 0.9 and 1.1 mL/min. pH was varied ± 0.1 from actual i.e., 2.9 and 3.1. While changing the flow rate and pH, other parameters were unchanged to know the ruggedness of the method.

Preparation of standard solution:

Prepare individual solution of the substance containing 0.003 mg/ml of Impurity-H and 0.003 mg/ml of raloxifene hydrochloride standard solutions as per the method

Acceptance criteria:

The area difference between two injections of Raloxifene peak should not be more than 10.0%

Limit of detection (lod)

The limit of detection is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected.

Preparation of Detection limit Solution:

Take 3.3 mL of above prepared Quantitation solutions into a 10 mL volumetric flask. Dissolve and dilute to 10 mL with mobile phase.

Acceptance criteria: For LOD solution Signal to Noise ratio should be about 3 to 5.

Limit of quantification (loq)

The limit of Quantitation is defined as the lowest concentration of an analyte in a Impurity-A and raloxifene hydrochloride that can be determined with acceptable precision under the stated operational conditions of the method. The limit of Quantitation is calculated from the signal to noise ratio.

Preparation of Quantificaiton limit Solution:

Prepare Quantitation limit solution in such a conc. for which the signal to noise ratio should be 10 to 15.

Acceptance criteria:

Signal to Noise ratio should be about 10 to 15 and the Quantitation limit should be less than the specification limit.

The relative standard deviation for the peak area of each Impurity-A and raloxifene hydrochloride should be less than 10.00%.

Results and discussion

Specificity

The study reveals that all the known impurities of raloxifene hydrochloride adequately resolved. Hence, the method is selective for the determination of related substances in Raloxifene Hydrochloride. Refer Table No.1

Table-1: Summary of Resolution and tailing factor for system suitability solution

S.No	RetentionTime(min)	Peak Name	Area	Resolution	Tailing factor
1	17.5	RLX HCl	10831647	-	1.36
2	3.0	Impurity-A	12	6.35	1.45

System PRECISION

The standard solution was injected for six times and % RSD was calculated and the results are well with in the acceptance limit. Refer Table No.2

Table-2: Summary of system suitability from standard solution

No. of inj'n	RLX HCl	Impurity-A
01	104876	12838
02	104525	12817
03	104497	12890
04	104439	12914
05	104580	12871
06	104303	12936
Average	104536.7	12877.67
%RSD	0.18%	0.35%

Method precision

Different sample preparation (six replications) was made with homogenous sample at 100% of the specification limit and results are well within the acceptance criteria. Refer Table No.3

Table 3. Method Precision

No.of prep'n	RLX Hcl
1	117235950
2	117098680
3	117024690
4	117189460
5	117446060
6	117531067
AVERAGE	117254317.8
STD DEV	197408.8
%RSD	0.17

Linearity

For linearity results obtained are shown in tables and figure show the line of best fit for average peak area versus concentration for each impurity and calculate Correlation Coefficient. Refer Table 4, Figure 7.

Table-4: Linearity for Raloxifene Hydrochloride and Impurity - A

Linearity level	Concentration(mg/ml)	Avg Peak Area of	
		Raloxifene Hydrochloride	Impurity-A
20% level	0.0006	24491	3384
40% level	0.0012	46745	6156
80% level	0.0024	89559	11559
90% level	0.0027	101929	12960
100% level	0.0030	112059	14367
110%level	0.0033	122645	15727
120%level	0.0036	135794	17291
150%level	0.0045	167303	21296

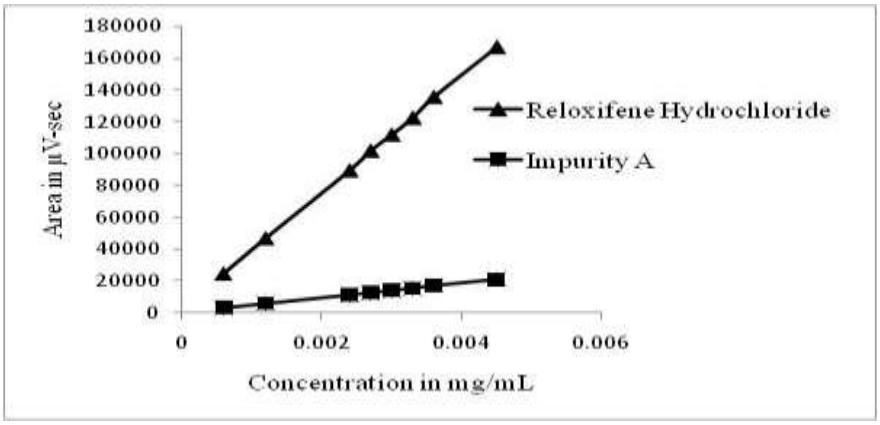

Fig-7: Linearity Curve of Raloxifene Hydrochloride and Impurity A

Accuracy

The reference standard solution was injected twice and the average was taken into consideration for calculation of % recovery (Table No.5) % Recovery was performed at three concentration levels i.e., 50, 100 and 150% and the results are given in Table No. 6

Table-5: Standard solution_Accuracy Test

Injection No.	Area counts of	
	Raloxifene Hydrochloride	Impurity-A
1	113215	14972
2	113801	14857
Average	113508	14915

Table-6: Standard solution_Accuracy Test

	Standard Area		Area counts		%Recovery		Average ± S.D (% RSD)	
	Raloxifene Hydro chloride	Impurity-A	Raloxifene Hydro chloride	Impurity-A	Raloxifene Hydroc hloride	Impurity-A	Raloxifene Hydroc hloride	Impurity-A
Recovery 50%-1	113508	14915	176718	23128	103.79	103.38	103.56 ± 0.20 (0.19)	103.05 ± 0.31 (0.30)
Recovery 50%-2			176108	23043	103.43	103.00		
Recovery 50%-3			176181	22989	103.47	102.76		
Recovery 100%-1	113508	14915	259746	34080	114.41	114.25	114.23 ± 0.41 (0.36)	114.41 ± 0.17 (0.15)
Recovery			258249	34121	113.76	114.39		

100%-2								
Recovery 100%-3			259980	34179	114.52	114.58		
Recovery 150%-1	113508	14915	302023	39664	106.43	106.38	107.09 ± 0.65 (0.61)	106.62 ± 0.28 (0.26)
Recovery 150%-2			303918	39734	107.10	106.56		
Recovery 150%-1			305703	39872	107.73	106.93		

Ruggedness

In the Part of ruggedness Day-1 study is considered from method precision results done by Analyst-1. On Day -2, Analyst-2 was performed as same procedure followed by Analyst-1.

Table-7: Summarize the results of the ruggedness study

No. of prep'n Day-I and Day-II	**RLX Area**	**Remarks**
01	117235950	**Ruggedness Day-I & Analyst- I Average areas (Taken from Method Percision)**
02	117098680	
03	117024690	
04	117189460	
05	117446060	
06	117531067	
01	115138254	**Ruggedness Day-II & Analyst- II areas**
02	115523903	
03	115268466	
04	115206447	
05	115858038	
06	115556174	
Average	**116339765.8**	
% RSD	**0.84**	

Robustness

The variation in flow rate and pH of Mobile Phase and the results are recorded in **Table No. 8**.

Table-8: Summarized the results of the robustness study obtained for each parameter altered

Parameter condition	**Avg RT of RLX and Imp-H**		**Area**		**Area difference**
			Inj-1	**Inj-2**	
Actual	RLX	20.677	114740	115464	0.63%
	Impurity-A	3.358	13499	13457	0.31%
0.9 ml/min (Low Flow)	RLX	21.765	126411	127754	1.06%
	Impurity-A	3.731	14948	14972	0.16%
1.1 ml/min (High Flow)	RLX	19.561	103092	102856	0.13%
	Impurity-A	3.041	12331	12301	0.24%
pH:2.9	RLX	20.593	115720	115751	0.03%
	Impurity-A	3.350	13511	13597	0.64%
pH:3.1	RLX	20.633	115769	115602	0.14%
	Impurity-A	3.333	13481	13399	0.61%

As shown in the study change in pH composition, effect the retention time of raloxifene hydrochloride and its known impurities. Therefore it is recommended to maintain the pH in the range of 3.00±0.05.

Limit of quantification (LOQ)

The limit of quantitation was determined by measuring the signal to noise for standard with respect to working concentration. The result obtained for each peak is listed in below table calculates the % RSD for quantitation Solution and the results are as follows:

Table-9: Limit of Quantification (S/N Ratio)

No.of inj	Area of RLX Hcl	Area of Impurity-A	S/N Ratio	
			RLX HCl	Impurity- A
1	972	468	10.846	14.476
2	967	452	10.938	14.266
3	940	412	10.868	14.489
4	957	463	10.986	14.686
5	907	442	10.977	15.230
6	1027	412	10.517	14.287
AVERAGE	961.667	441.500	-	-
STD DEV	**39.707**	**24.558**	-	-
%RSD	**4.13**	**5.56**	-	-

Limit of detection (LOD)

The limit of detection was determined by measuring the signal to noise for standard with respect to working concentration. The result are given in Table No.10.

Table-10: Limit of Detection (S/N Ratio)

No.of inj	RLX Hcl	Impurity-H	S/N Ratio	
			RLX HCl	Impurity- H
1	255	146	4.660	4.789
2	249	163	4.518	4.628

CONCLUSIONS

The results obtained in this study demonstrate that the HPLC method described in the method of analysis is selective, accurate, precise, linear, rugged and robust for the determination of related substances in raloxifene hydrochloride drug substance. Therefore the method is suitable for its intended use.

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