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Development and validation of stability indicating method for the quantitative determination of Raloxifene hydrochloride and its related impurities using UPLC

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ABSTRACT

The present paper describes the development of a simple, economic and time efficient stability indicating UPLC method for Raloxifene hydrochloride (RLH) in the presence of its impurities and degradation products generated from forced degradation studies. The drug substance was subjected to stress conditions of acid hydrolysis, base hydrolysis, oxidative hydrolysis, photolysis and thermal degradation. The degradation of Raloxifene hydrochloride was observed under oxidative hydrolysis and base hydrolysis. The drug was found to be stable in all other stress conditions applied. Successful separation of the drug from synthetic impurities and degradation products formed under forced degradation was achieved on a Extended C18 column using a mixture of 5mM ammonium acetate and methanol (50:50,v/v) as mobile phase in an isocratic elution mode. The eluents were monitored at 280 nm. The developed UPLC method was validated with respect to linearity, accuracy, precision, specificity and robustness. It can be used to test the stability samples of Raloxifene HCl.

Key words: Raloxifene hydrochloride, UPLC, Validation, Stress conditions, Degradation products.

INTRODUCTION

Raloxifene hydrochloride (RLH), [6-hydroxy-2-(4-hydroxy phenyl) benzo[b]thien-3-yl]-[4-[2-(1-piperinyl) ethoxy]-phenyl] methanone, is an antiosteoporotic. It is a nonsteroidal benzothiophene, which is the first selective oestrogen receptor modulator (SERM) to be

approved for the prevention and treatment of osteoporosis in postmenopausal women [1].Clinically it is effective in the treatment of breast cancer[2,3] and reduction of fracture risk[4].It is an estrogenagonist in bone, where it exerts an anti –resoptive effect. The results of several large clinical trails have shown that raloxifene reduces the rate of bone loss at both distal sites and in the spinal column and may increase bone mass at certain sites[5]. HPLC, LC-MS-MS and Spectrophotometric methods were reported for determination of Raloxine in Pharmaceutical tablets [6 and 7], Pharmaceutical bulk [8], human urine [9], and rat plasma [10]. The concern with reported methods, they were not studied with respect to its stability indicating nature. The UPLC technique is a latest and the impurities were separated in a short time. There were no reported methods for Raloxifene Hydrochloride on UPLC as per the literature search.

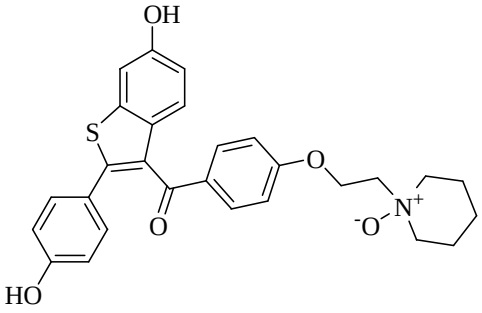
EXPERIMENTAL SECTION

2.1 Chemicals:

Samples of Raloxifene hydrochloride and its four impurities namely imp -A, imp-B, imp-C, and imp-D (Fig.1) was received from Dr.Reddy's laboratories Ltd., Hyderabad, India. HPLC grade Methanol, AR grade Ammonium Acetate was purchased from Merck, Darmstadt, Germany. High purity water was prepared by using a Millipore Milli Q plus purification system.

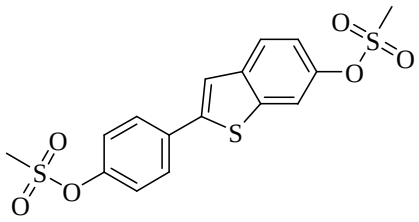
Fig.1: Chemical structure of Imp-A, Imp-B, Imp-C,Imp-D and Raloxifene HCl

Imp-A



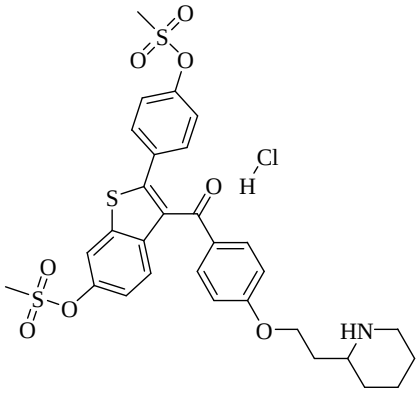
[2- (4-hydroxyphenyl)- 6-hydroxybenzo[b]thien-3-yl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone N-oxide.

Imp-B



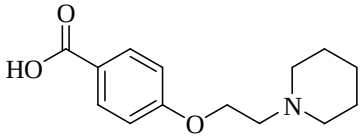
6-Methylsulfonyloxy-2-[(4-methylsulfonyloxy)phenyl] benzothiophene

Imp-C



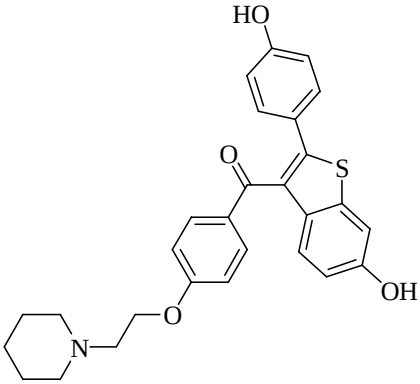
6-Methylsulfonyloxy-2[(4-methyl sulfonyloxy)phenyl]-3-[[4(2-piperidinyl)ethoxy]benzoyl]benzothiophene Hydrochloride

Imp-D



4-[2-(piperidinyl)ethoxy]benzoic acid

Raloxifene Hydrochloride



[6-hydroxy-2-(4-hydroxyphenyl)- benzothiophen-3-yl]- [4-[2-(1-piperidyl)ethoxy]phenyl] -methanone

2.2 Equipment:

The UPLC system used for the method development and validation consisted of gradient pumps from waters Corporation, Japan, photo diode array detector from Waters Crop., Japan, with auto sampler and auto injector. The UPLC system was equipped with data acquision and processing software “Mass Lynx software” Waters Crop., Japan.

2.3 Preparation of Standard solutions:

A stock solution of Raloxifene Hydrochloride was prepared by dissolving appropriate amount of substance in methanol. Working solutions of 300µg /mL were prepared from the above stock

solutions for the determination of related substance and assay. Stock solutions of impurities (mixture of Imp-A, Imp-B, Imp-C and Imp-D) at 0.3 mg/mL were also prepared in methanol.

2.4 Chromatographic Conditions:

The Chromatographic separation was achieved on an Extended C18, 50mm X 3.0 mm ID with 1.8 microns particles. Aqueous Ammonium Acetate (5mM) and Methanol (50:50 v/v) used as a mobile phase. The mobile phase was filtered through nylon membrane (pore size 0.45 µm) and degassed by using vacuum pump and sonicate for 15 minutes prior to use. The flow rate of mobile phase was 0.7 mL/min. The column temperature was maintained at 25°C and wave length was monitored at 280 nm.The injection volume was 2µL. The standard and the test dilutions were prepared in methanol.

2.5 Validation of the method:

2.5.1 Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed UPLC method for Raloxifene HCl was carried out in the presence of its impurities namely imp -A, imp-B, imp-C, and imp-D. Stress studies were performed for Raloxifene HCl bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254nm), heat (70 °C) , acid (0.5N HCl),base (0.5N NaOH),Oxidation (3.0 % H_2O_2) and water hydrolysis (60 °C) to evaluate the ability of the proposed method to separate Raloxifene hydrochloride from its degradation products. For heat and light studies, study period was 24 hours and for the acid, base oxidation, it was 12 hours. Peak purity test was carried out of Raloxifene peak by using PDA detector in stress samples. Assay studies were carried out for stress samples against qualified Raloxifene hydrochloride reference standard. Assay was also calculated for Raloxifene hydrochloride samples by spiking all for impurities at the specification level (i.e., 0.3%).

2.5.2 Precision:

The precision of the assay method was evaluated by carrying out six independent assays of Raloxifene hydrochloride test samples against a qualified reference standard and calculate the % RSD of assay. The precision of the related substances method was checked by injecting six individual preparations of Raloxifene hydrochloride (0.3mg/ mL) spiked with 0.3 % of imp -A, imp-B, imp-C and imp-D with respect to Raloxifene hydrochloride analyte concentration. % RSD of area for each imp -A, imp-B, imp-C and imp-D was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

2.5.3 Limit of detection (LOD) and Limit of Quantification (LOQ):

The limit of detection and limit of quantification were determined at a signal to noise of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations of imp -A, imp-B, imp-C, imp-D and then calculated the %RSD of the peak area.

2.5.4 Linearity

Linearity test solutions for the assay method were prepared from Raloxifene Hydrochloride stock solutions at six concentration levels from 50% to 200% of assay analyte concentration (50%, 75%, 100%, 125%, 150%, and 200%). The peak area verses concentration data was treated by least squares linear regression analysis.

Linearity test solutions for the related substance method were prepared by dilution of stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% (1.8 µg/mL) of specification level of impurities namely imp -A, imp-B, imp-C and imp-D (LOQ, 50%, 75%, 100%, 150% and 200%). Above test were carried out of 3 consecutives days in the same concentration range for both assay and related substances method. The % RSD value for the Slope and Y-intercept of the calibration curve was calculated.

2.5.5 Accuracy:

The accuracy of the assay method was evaluated in triplicate at three concentration levels 50%,100% and 150 % of test concentration (0.3 mg/mL).The percentage of recoveries were calculated from the Slope and Y- intercept of the calibration curve obtained in the linearity study. The accuracy study of impurities was carried out in triplicate at 50%, 100% and 150 % of specification level (0.3%) to the Raloxifene Hydrochloride analyte concentration (300 µg /mL). The percentages of recoveries for impurities were calculated from the slope and Y- Intercept of the calibration curve.

2.5.6 Robustness:

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Raloxifene Hydrochloride, imp -A, imp-B, imp-C and imp-D was recorded.

The effect of the methanol ratio in mobile phase preparation studied on resolution by varying by -5 to + 5 %, while other mobile phase components were held constant as stated in Chromatographic conditions. The column temperature was varied by -5 to + 5°C and flow rate of the mobile phase varied from – 0.1 to +0.1 mL/min.

2.5.7 Solution stability and Mobile phase stability:

The solution stability of Raloxifene Hydrochloride in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 24 hrs.The same sample solutions were assayed for 6 hrs interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solution against freshly prepared reference standard solution for 6 hrs interval up to 48 hrs. Mobile phase prepared was kept constant during the study period. The % RSD for the assay of Raloxifene Hydrochloride was calculated during mobile phase and solution stability experiment.

The solution stability of Raloxifene Hydrochloride and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flasks at room temperature for 24 hrs. Content of imp -A, imp-B, imp-C, and imp-D were checked in the test solutions.

RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic conditions

The main objective of Chromatographic method is to separate Raloxifene Hydrochloride from Imp-A, Imp-B, Imp-C and Imp-D. Impurities were co-eluted using different stationary phases such as C8, Cyno and Phenyl as well as different mobile phases. The Chromatographic separation was achieved on an Extended C18, 50mm X 3.0 mm I.D with 1.8µ particles column using mixture of 5mM Ammonium Acetate and methanol(50:50v/v) as a mobile phase . The flow rate of the mobile phase was 0.7 mL / min, at 25 °C column temperature, the peak shape of the Raloxifene Hydrochloride was found to be symmetrical. In optimized chromatographic conditions of Raloxifene Hydrochloride, Imp -A, Imp-B, Imp-C and Imp-D were separated with resolution grater than 2, typical retention times were about 0.70, 1.59, 2.46, and 6.05, respectively (**Fig 2**).The system suitability results are given in **Table-1** and developed UPLC method was found to specific for Raloxifene Hydrochloride and its four impurities namely Imp -A, Imp-B, Imp-C and Imp-D (**Fig 2**).

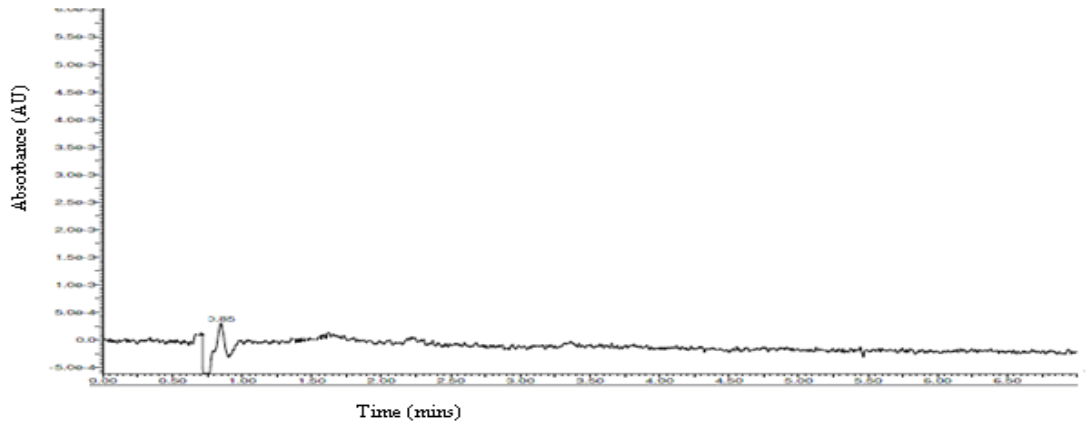
Table-1: System suitability data

System suitability	Raloxifene	Imp-A	Imp-B	Imp-C	Imp-D
R_t	1.41	0.7	1.62	2.5	6.04
RRt	1	0.49	1.14	1.77	4.28
R_s	2.7	-	2.4	4.5	6.2
T	1.0	1.0	1.0	1.0	1.0
N	4216	6487	10254	7541	8452

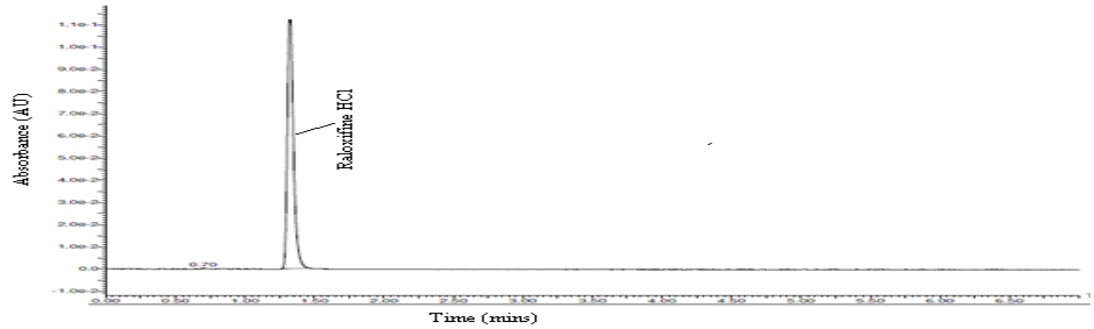
Rt,Retention time;RRt,Relative retention time, Rs,resolution;T,USP tailing factor;N, Theoretical plates

Fig.2: Chromatogram of (a) Blank (b) Raloxifene Hydrochloride bulk sample (c) Raloxifene sample spiked with all impurities.

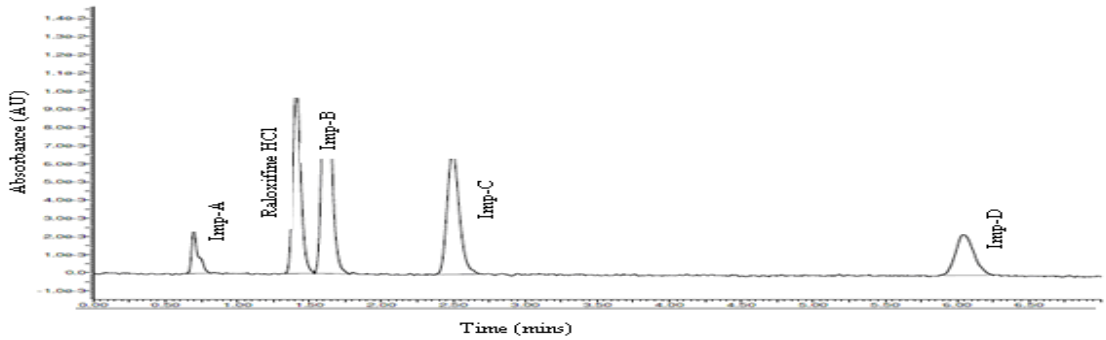
(a) Blank



(b) Raloxifene Hydrochloride bulk sample



c) Raloxifene sample spiked with all impurities.



3.2 Results of forced degradation studies:

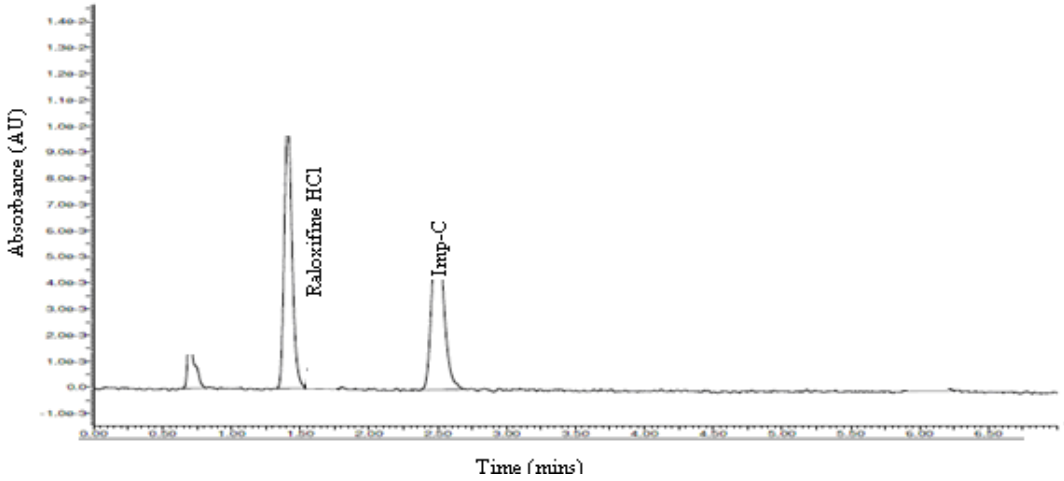
Degradation was not observed in Raloxifene HCl sample when subjected to stress conditions like light, heat and acid hydrolysis. Raloxifene HCl was degraded to Impurity C under Oxidative hydrolysis, in base hydrolysis Raloxifene HCl was degraded to Impurity D and Impurity A **(Fig.3).**Peak purity test results confirmed that the Raloxifene HCl Peak is homogenous and pure in all the analyzed stress samples. The assay of Raloxifene HCl is unaffected in the presence of all impurities and its degradation products confirms the stability indicating power of the method. The summary of forced degradation studies is given in **Table 2**.

Table-2: Summary of forced degradation

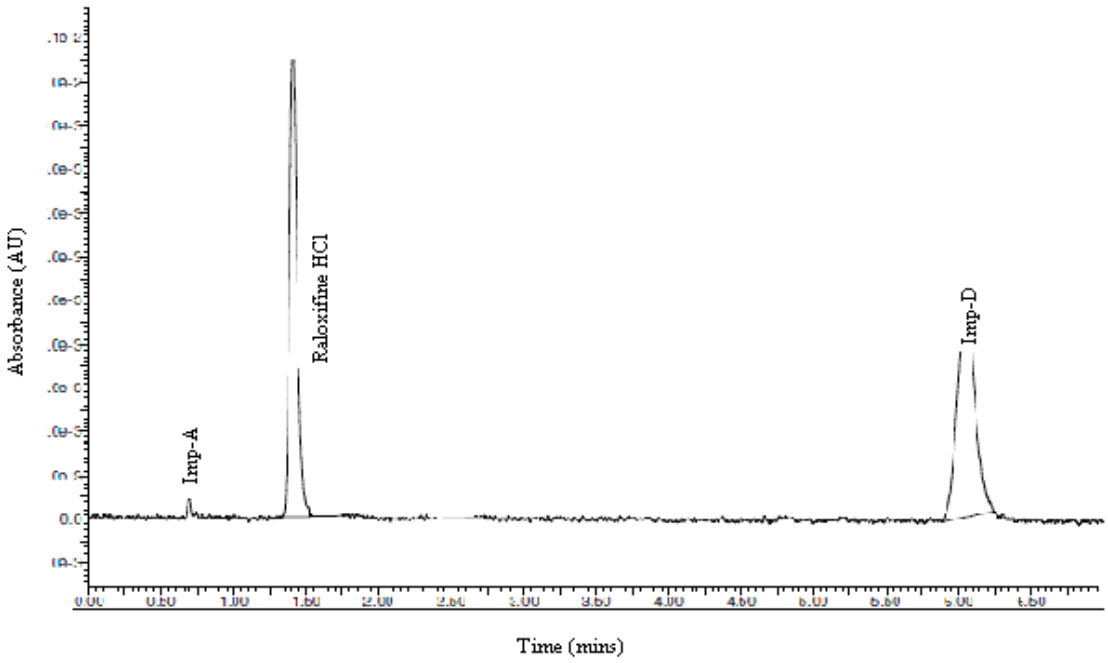
Stress conditions	Time(h)	Assay of Active substance	Total Impurities	Mass balance (Assay+Impurities)
Normal		99.7%	0.12%	99.8%
acid hydrolysis	12	99.2%	0.61%	99.8%
Base hydrolysis	12	70.2%	29.9%	100.1%
Oxidation(3%H_2O_2)	12	80.3%	21.4%	101.7%
water,70 ^oC	12	99.2%	0.4%	99.6%

Fig.3: Stress study Chromatogram of Raloficine HCl a) in Hydrogen Peroxide b) in base

a) Stress study Chromatogram of Raloficine HCl in Hydrogen Peroxide



b) Stress study Chromatogram of Raloficine HCl in base



3.3 Precision

The %RSD of assay of Raloxifene Hydrochloride during the assay method precision study was within 0.22% and the %RSD for the area of Imp -A, Imp-B, Imp-C, and Imp-D in related substances method precision study was with in 4.4 %. The %RSD of the assay results obtained in the intermediate precision study was with in 1.5 % ,%RSD for the area of Imp -A, Imp-B, Imp-C, and Imp-D were well within 2.5% conforming good precision of the method.

3.4 Limit of detection (LOD) and Limit of Quantification (LOQ):

The limit of detection of all impurities namely Imp -A, Imp-B, Imp-C and Imp-D were achieved 0.17, 0.10, 0.08 and 0.18 µg/mL for 2 µL injection volume. The limit of quantification of all

impurities namely Imp -A, Imp-B, Imp-C and Imp-D are 0.54, 0.35, 0.26 and 0.62 µg/mL for 2 µL injection volume. The precision at the LOQ concentrations for Imp -A, Imp-B, Imp-C and Imp-D were below 7.5%.

Table-3 : Limit of Detection and Limit of Quantification

Name	Imp-A	Imp-B	Imp-C	Imp-D
LOD	0.02%	0.01%	0.01%	0.02%
LOQ	0.05%	0.03%	0.03%	0.05%

3.5 Linearity:

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 150 to 600 µg/|mL and correlation coefficient obtained was grater than 0.99.Linearity was checked for assay method over same concentration range for 3 consecutives days. The % RSD value of the Slope and Y-Intercept of calibration curve were 1.4 and 2.5 respectively. The result shows that an excellent correlation existed between the peak area and concentration of the analysis.

Linear calibration plot for the related substances method was obtained over the calibration ranges tested i.e. LOQ to 200 % for impurity Imp -A, Imp-B, Imp-C and Imp-D. The correlation coefficient obtained grater than 0.998. Linearity was checked for the related substances method over the same concentration ranges for 3 consecutives days. The %RSD values of the Slope and Y-intercept of calibration curve were 3.2 and 2.8 respectively. The above results shows that an excellent correlation existed between the peaks are and the concentrations of Imp -A, Imp-B, Imp-C and Imp-D.

Table-4: Linearity data

Name	Raloxifene	Imp-A	Imp-B	Imp-C	Imp-D
Linearity (n=3)					
Intercept	1022	845	452	341	102
Slope	412	1456	3214	5412	8745
r	0.9991	0.9994	0.9987	0.9989	0.9994

n, number of determinations

3.6 Accuracy

The percentage recovery of Raloxifene hydrochloride in bulk drug samples was ranged from 99.8 to 100.2 **% (Table-5)**.The percentage recoveries of all four impurities in Raloxifene Hydrochloride samples varied from 98.5-102.5%.

Table-5: Accuracy data

Name	Raloxifene	Imp-A	Imp-B	Imp-C	Imp-D
Accuracy (% Recovery)	99.8-100.2	98.5-102.5	99.1-101.9	98.9-101.4	99.2-100.9

3.7 Robustness

In all the deliberate varied chromatographic conditions (flow rate, composition of organic solvent & column temperature) the resolution between critical pair, i.e. Raloxifene HCl and imp-B was grater than 1.5, illustrating the robustness of the method.

3.8 Solution stability and Mobile phase stability

The % RSD of assay of Raloxifene Hydrochloride during solution stability experiments were within 0.3% .No significant change were observed in the content of impurities namely imp -A, imp-B, imp-C and imp-D during the solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phase used during the assay and the related substance determination were stable for 48 hrs.

CONCLUSION

The UPLC method developed for quantitative and related substance determination of Raloxifene hydrochloride is linear, accurate, precise, rapid and specific. The method was fully validated showing satisfactory data for all method validation parameters tested. The developed method is stability indicating and can be conveniently used by quality control department to determine the related substance and assay in regular Raloxifene Hydrochloride production samples and also stability samples. The UPLC technique is a latest and the impurities were separated in a short time.

Acknowledgment

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