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Bioanalytical Method Development and Validation for the Estimation of Raloxifene Hydrochloride in Human Plasma by RP-HPLC Method

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KEYWORDS
Raloxifene, Bioanalytical, RP-HPLC, human plasma, validation

ABSTRACT:

A rapid, simple, sensitive, and selective HPLC method was developed and validated for the determination of raloxifene hydrochloride (RLF) in human plasma using a liquid-liquid extraction method. Separation of RLF was achieved using a Phenomenex RP-C8 column, with acetonitrile and phosphate buffer (pH 3.5, adjusted with orthophosphoric acid) as the mobile phase in a 40:60 ratio. Detection was carried out at 240 nm. The method was validated for specificity, selectivity, sensitivity, linearity, accuracy, precision, recovery, and stability parameters. The retention times of RLF and amlodipine (Internal standard) were found to be 4.1 and 6.3 min, respectively. Linearity was observed ($r > 0.999$) in the concentration range of 40-200 ng/ml. The inter-day and intra-day accuracy and precision were found to be within acceptable limits. The percentage recovery ranged from 99.4% to 100.4%.

INTRODUCTION

Raloxifene hydrochloride is [6-hydroxy-2-(4-hydroxypenyl) – benzothiophen – 3 – y1]-[4-[2-(1-piperidy1) ethoxy] pheny1] – methanone. RLF is indicated for the treatment and prevention of osteoporosis in postmenopausal women[1-2] . The biological actions of RLF are through binding to estrogen receptors, which results in activation of certain estrogenic pathways and blockade of others. RLF decreases resorption of bone and reduces biochemical markers of bone turnover to the premenopausal range. Several methods have been reported for quantitation of RLF in bulk and in pharmaceutical dosage forms[3-6] and in animal plasma[7-10]. Two LC-MS/MS[11-13] methods only reported for the analysis of RLF in human plasma. But the methods reported suffers from several limitations such as lack of sensitivity, long run time, less reproducibility, complicated sample preparation procedure, requirement of the high volume of plasma samples and high degree of interference. Based on the above facts, a novel bioanalytical method was developed for quantification of RLF in human plasma.

MATERIALS METHODS

RLF were obtained as gift sample from orchid health care Chennai. Amlodipine was obtained as gift samples from sir Krishna Pharmaceutical Pvt Ltd, Hyderabad. Acetonitrile, HPLC grade water and potassium dihydrogen phosphate was purchased from Sigma Scientific Ltd, India. All other chemicals used were of analytical reagent grade. Human plasma was collected from the KMCH and stored at – 40 °C until use.

Instrumentation:

The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 2080 Plus) with sampler programmed at 20 µl capacity per injection. The C8 column (150 X 4.6 mm X 5 µm) in isocratic mode was employed for the analysis with photo diode array detection. Mobile phase consisted of a mixture of acetonitrile: phosphate buffer (pH 3.5 adjusted with OPA) (40:60), was filtered through a 0.45 micron membrane filter and degassed before use. Flow rate

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was set at 1 ml/min and analyte was detected at 240 nm. The injection volume of analyte was 20 µl and analysis was performed at ambient temperature.

Preparation of standard stock solution:

Stock solutions of RLF and amlodipine was prepared at a concentration of 1000 µg/ml. From the standard stock solution, the working standard solutions were prepared using mobile phase to get 10 µg/ml of each drug. The stock solutions were stored at 2-8^0 C.

Optimization of HPLC method:

The analyte was extracted from the plasma samples using a liquid-liquid extraction procedure. In this method, ethyl acetate was employed as the extracting solvent to separate the analyte from the plasma matrix. Aliquot amount of blank rat plasma was spiked with appropriate amount of RLF and internal standard amlodipine. Adequate volume of ethyl acetate was added to the plasma solution. Then this mixture was then vortex mixed for about 40 sec and centrifuged for 15 min at 3000 rpm. The dried extract was then reconstituted with mobile phase, filtered with 0.20 µm membrane filter and injected into the HPLC system.

Linearity and range

Linearity of the method was studied by injecting six concentrations of the drug prepared in the mobile phase in the range of 40-200 ng/ml for RLF hydrochloride into the HPLC system keeping the injection volume constant. The peak area was plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

The precision of the method was verified by repeatability and intermediated precision studies. Repeatability studies were performed by analysis of three different concentrations. 40, 80, 120 ng/ml for RLF. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation

Limits of detection (LOD) and represent the concentration of the analyte that would yield signal-to-noise of LOD and 10 for LOQ, respectively. To determine the LOD and LOQ, serial dilutions of mixed standard solution of these drugs were made from the standard stock solution in the range of 0.1-1 µg/ml for RLF.

Specificity

The specificity of the method was confirmed from the chromatogram (Figure 2), which showed complete separation of RLF without any interference from the excipients. The peaks obtained were sharp, symmetrical and well-separated at the baseline.

Accuracy

Accuracy of the method was carried out by applying the method to preanalyzed drug sample to which known amount of RLF standard power corresponding to 80, 100 and 120 of label claim had been added (standard addition method), mixed and the powder extracted and analyzed by running chromatogram in optimized mobile phase.

RESULTS

Linearity

RLF showed good correlation coefficient (r^2 = 0.9990) in concentration range of 40 ng-200 ng/ml. The mean value of the slope and intercept were 51489 and 5844.4 respectively. The detector response over wide range of concentration of analyte were plotted to obtain the calibration curve (Figure 1).

Accuracy and Precision

The results of the accuracy and precision experiments are shown in Table 1 and Table 2. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, respectively as recommended by ICH guidelines.

LOD and LOQ

Signal-to-noise ratios of 3:1 and 10:1were obtained for the LOQ respectively. The LOD and LOQ were found to be 4 µg/ml and 8 µg/ml respectively.

Specificity studies

The peak purity of RLF was assessed by comparing their respective spectra at the peak start, apex, and end positions. The method was found to be specific, as there is no interference observed, and chromatogram showed good separation with low background noise.

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Recovery

As shown in Table 3, good recoveries of RLF, ranging from 97.30 % to 98.44 %, were obtained at various added concentrations.

DISCUSSION

Liquid-liquid extraction method was employed for the determination of RLF in human plasma, as the alternative solid-phase extraction (SPE) is complex, requires specialized instrumentation, and the SPE cartridges cannot be reused. The protein precipitation method was not adopted due to a higher possibility of interference during the entire chromatographic procedure and detection process. Additionally, this method may decrease the efficiency of column separation and increase the risk of contaminating the HPLC system, including the injector, column, and detector. Developed method is more economical because it avoids the use of SPE cartridges and does not require a costly LCMS instrument for the determination of RLF in human plasma. This method leverages the advantages of liquid-liquid extraction, offering increased recovery rates, enhanced specificity, and minimal interference, thereby ensuring accurate and reliable quantification.

Six different trails were performed to optimize the chromatographic condition's to quantify the drug by RP-HPLC. RLF was not eluted with initial separation attempted with 50: 50 methanol: water. Then organic phase acetonitrile with water in the ratio of 50:50 was tried but broadening of peak observed. Third attempt of separation was performed using phosphate buffer with pH adjusted to 4.5 (OPA) and acetonitrile in 50:50 ratio where peak tailing was observed. Hence in the 4th attempt phosphate buffer with pH adjusted to 4.5 (OPA) used along with acetonitrile where broadening of peak observed for internal standard. Fifth trail run was performed using phosphate buffer, pH adjusted to 4.5 (ortho phosphoric acid) along with acetonitrile where a broad peak at 9.3 min was observed for RLF. Finally, acetonitrile: phosphate buffer (pH- 3.5 adjusted with OPA) in the ratio of (40: 60) at flow rate of 1 ml/min eluted the analytes at a retention time of 4.1 and 6.3 min respectively and a good resolution for RLF and amlodipine without any interference from plasma matrix was obtained. None of the existing bioanalytical HPLC methods for the estimation of RLF have reported a retention time of less than 5 minutes. The developed method was rapid as it elutes RLX from biological matrix in just 4.1 min. The total run time of 10 min makes the method rapid and economical than the previously reported methods. The selection of amlodipine as the internal standard represents an innovative approach, as it has not been previously reported in the literature for the estimation of RLX in human plasma using HPLC methods.

CONCLUSIONS

In this study, a simple, sensitive, accurate, precise and stable HPLC method has been developed and validated for quantitation of RLF in human plasma using amlodipine as internal standard after liquid-liquid extraction technique. Increased recovery of the RLF in the human plasma resulted with liquid-liquid extraction method when compared with the reported methods. The developed and validated method can be routinely employed for the determination of Raloxifene in human plasma.

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Table 1: Accuracy and precision studies of Raloxifene hydrochloride (Interday)

Sl. No	Amount of drug (ng/ml)	Mean peak area	Accuracy	%RSD
1	40	21324	100.2	3.44
2	80	38432	99.4	6.94
3	160	57228	100.4	6.45

Table 2. Accuracy and precision studies of Raloxifene hydrochloride (Interday)

Sl. No	Amount of drug (ng/ml)	Mean peak area	Accuracy	%RSD
1	40	21990	101.07	7.88
2	80	38770	99.7	6.65
3	160	57228	100.4	6.85

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Table 3. Recovery study of Raloxifene hydrochloride

Level	Amount of drug added (ng/ml)	Amount of drug recovered from plasma (ng/ml)			% Recovery		
		ACN	ACN and methanol mixture	Ethy1 acetate	ACN	ACN and methanol mixture	Ethy1 acetate
I	50	40.1	45.4	49.2	80.2	90.8	98.4
II	100	81.5	89.6	97.2	81.5	89.6	97.2
III	150	125.1	136.4	146.9	83.4	90.9	97.3

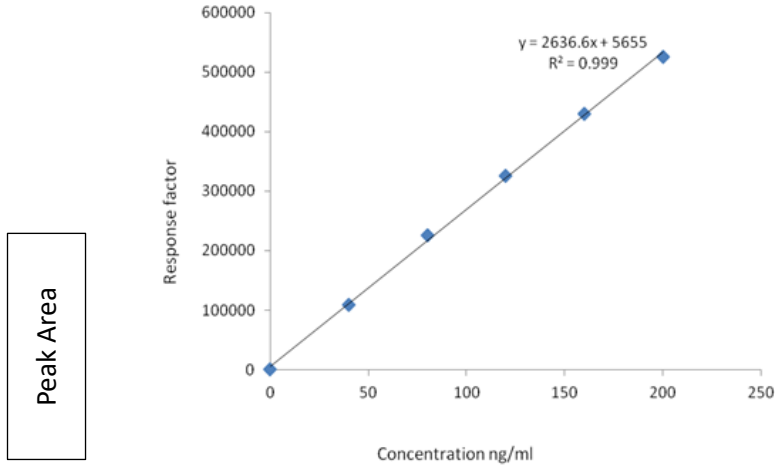
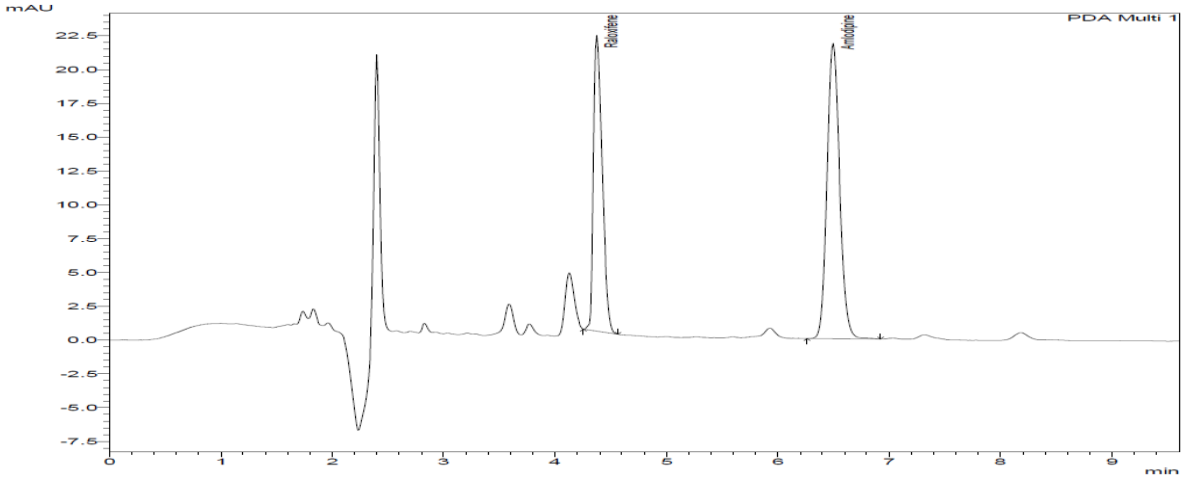


Fig 1. Calibration curve for Raloxifene hydrochloride



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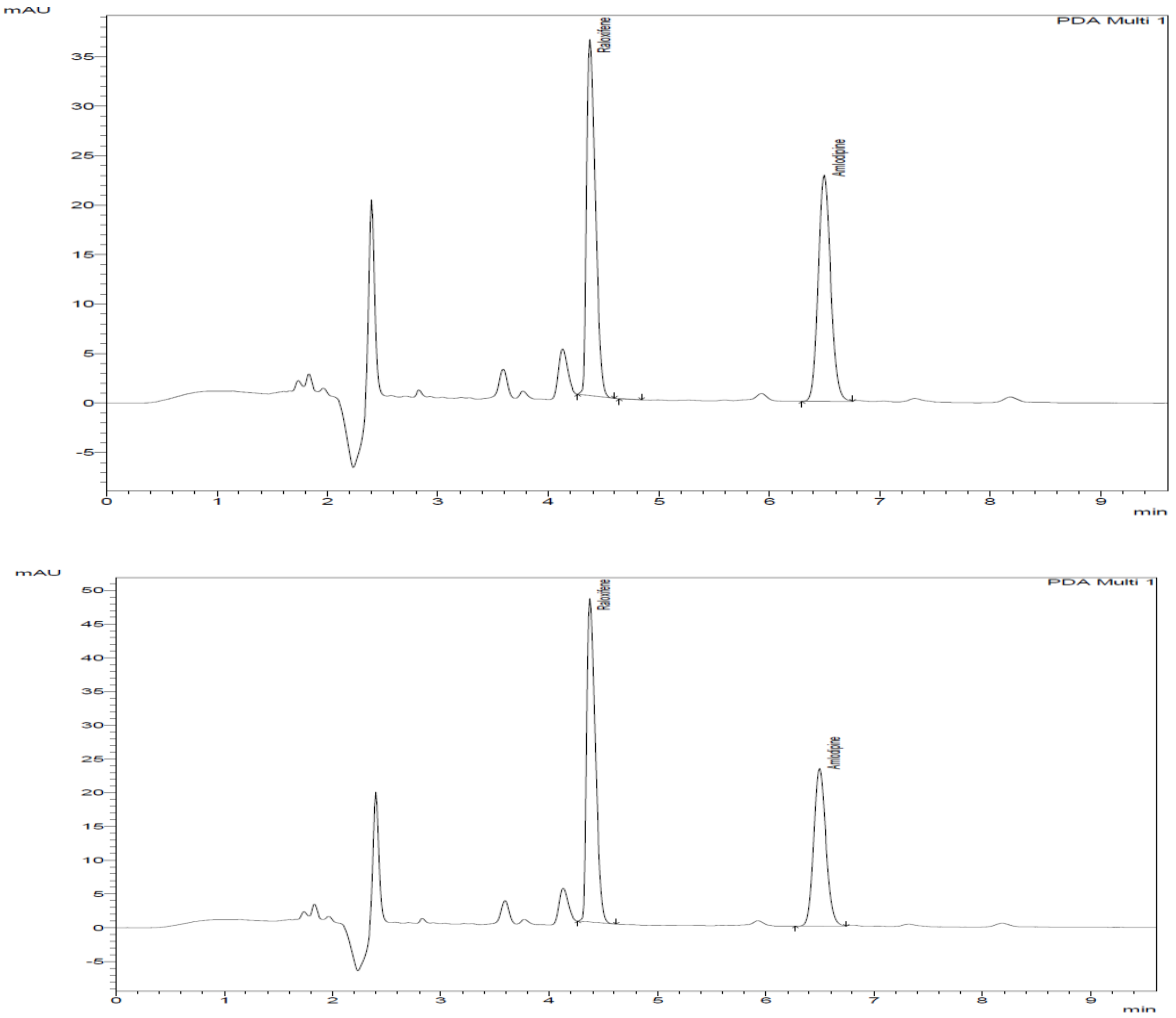

Fig 2. Representative chromatograms of Raloxifene extracted from human plasma along with internal standard Amlodipine