The aim of the present study is to determination coefficient in octanol/water is absorptivity (a) of 34,000. Its partition coefficient in octanol/water is 627.4±132.0 µg/mL and it is non-volatile solid. Its solubility in water is 3.9 with glacial acetic acid, acetonitrile and triethylamine in the ratios of 65.75:34.0:25: v/v/v. was run isocratically through a C18 (250mmX4.6 mm, 5µm) reverse phase analytical column. The experiment was carried out at room temperature for raloxifene hydrochloride. Analytical run time was less than 10 min. Mean recovery was 98.2% for 0.5-50 µg/ ml concentrations. The assay exhibited good linear relationship. Quantification limit was at 50ng/ml of raloxifene hydrochloride and accuracy and precision were over the concentration range of 0.5-50 µg/ml. This method was found to be applicable for determination of the raloxifene hydrochloride in active pharmaceutical ingredient (API).

Key words: API, HPLC, Osteoporosis, Raloxifene hydrochloride, SERM, Triethylamine.

1. INTRODUCTION
Selective estrogen receptor modulators (SERMs) constitute a family of compounds that demonstrate tissue selective estrogenic and antiestrogenic activities. Raloxifene hydrochloride belongs to the benzothiophene class of compound and it is currently used for prevention of osteoporosis in postmenopausal women. It was approved by Food and Drug Administration (FDA) in 1997 [1]. It binds to the nuclear estrogen receptors (ERα and ERβ) and either activates or blocks ER-induced gene transmission, depending on the tissue involved. Raloxifene hydrochloride demonstrates estrogen agonist effects on bone and blood lipid levels while it is a competitive antagonist of estrogen at mammary and uterine estrogen receptors [2, 3].

Raloxifene hydrochloride is cardioprotective, in part due to its effects on plasma lipid distribution. Like estradiol, raloxifene hydrochloride reduces total and low-density lipoprotein (LDL) levels in plasma. However, unlike estradiol, it does not increase plasma high-density lipoprotein (HDL) and triglyceride levels in plasma [4]. There are also some adverse effects, namely increased incidences of hot flushes and in low percentage venous thromboembolic events [5].

Raloxifene hydrochloride is a generic name for [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl][4-[2- (1-piperidinyl)ethoxy]-phenyl]ethanone hydrochloride with a molecular weight of 510.05 g/mol and the structure shown in Fig. 1. It is off-white to pale yellow distilled water for analytical purpose was obtained from milli-Q R-O system.

2.2. Chromatographic conditions
The HPLC system consisted of a Shimadzu LC-20AT liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA, USA) with a 20 µl sample loop and UV-Visible detector (Shimadzu, Kyoto, Japan). Data collection, integration and calibration were accomplished using LC Solutions chromatography Data System. The chromatographic separation of raloxifene hydrochloride was accomplished using 250x4.6 mm Phenomenex C18, 5µm reverse phase analytical column. The mobile phase consisted of double distilled water (the pH was adjusted to 3.9 with glacial acetic acid), acetonitrile and triethylamine in the proportion of 65.75:34.0:25 v/v/v. Before use, the mobile phase was filtered by passing it through a 0.45µm filter and the filtrate is degassed by using bath sonicator. The mobile phase was pumped at an isocratic flow of 1 ml/min at room temperature. The UV detection wave length was set at 287 nm. All separations were performed at ambient temperature [5].

2.3. Preparation of stock and standard solutions
Stock solution of raloxifene hydrochloride was prepared in methanol at 1mg/ml and was kept at -20°C. This stock solution was diluted with methanol to obtain the concentrations required for preparation of standard working solutions. Raloxifene hydrochloride working solutions were in the range of 0.5-50 µg/ml. Samples for the determination of recovery, precision and accuracy were prepared by spiking quality control (QC) standard raloxifene hydrochloride concentrations (1, 2.5, 5, 10, 25 µg/ml) and stored at -20°C.

2.4. Validation parameters and procedures
The RP-HPLC assay validation was done as per ICH Q2A and Q2B guidelines [10, 11]. These tests included determination of accuracy, precision, linearity, sensitivity and limit of detection and quantification.

2.4.1. Linearity, limit of detection (LOD) and limit of quantification (LOQ)
Standard calibration samples were prepared by making serial dilutions from the stock solution of raloxifene hydrochloride (1mg/ml). Calibration curve of concentration versus peak areas was plotted at concentration range of 0.5-50 µg/ml. The limit of detection (LOD) and the lower limit of quantification (LOQ) were measured according to the FDA’s guidance for bioanalytical method validation in 2001 [12]. The limit of detection was defined as the lowest concentration of raloxifene hydrochloride resulting in a peak height greater or equal to three times from background noise (S/N = 3). The LOQ was investigated in extracted samples from five different days. For the determination of LOQ, the percentage deviation and % RSD are to be less than 20%.

2.4.2. Precision and Accuracy
The precision and accuracy were determined by analyzing spiked standard and extracted samples at different concentrations ranging from 1, 2.5, 5, 10, 25 µg/ml. The precision of an HPLC method was determined as the coefficient of variation (%RSD) of intra- and inter-day. The intra-day precision was determined by analyzing the spiked standard and extracted samples prepared within a day. The inter-day precision was determined by analyzing the spiked standard and extracted samples analyzed on five different days. After concentrations were calculated by re-fitting peak areas obtained with different standard solutions into a derived regression equation from the set of these standard solutions,
%R.S.D. was determined at each concentration of the standard solutions from their average value and S.D.

The accuracy of the HPLC method was demonstrated by percentage deviation. The calculated concentrations (or conc. found) were obtained by re-fitting peak areas from standard solutions of known concentrations (or conc. added) into a derived regression equation. The conc. found and conc. added was then used to determine the absolute percentage deviation at each concentration of the standard solutions.

2.4.3. Recovery
The absolute recovery was calculated by comparing the peak areas of compounds after liquid-liquid extraction with those obtained on direct injection onto the column of the same amount of raloxifene hydrochloride dissolved in mobile phase. Each measurement was made in triplicates.

\[
\text{Recovery} (\%) = \frac{\text{peak area of extracted standard}}{\text{peak area of unextracted standard}} \times 100
\]

2.4.4. System suitability
The purpose of system suitability to define a set of parameters that are measured prior to each experiment that will tell the analyst if the system is performing adequately or not. The suitability parameters that are evaluated for HPLC method includes peak area reproducibility and retention time.

3. RESULTS

3.1. Chromatography
To facilitate quality control study of raloxifene hydrochloride, a sensitive, specific and reproducible HPLC method has been developed and validated for quantitative determination of raloxifene hydrochloride in-vitro samples. After the pretreatment with a rapid single liquid–liquid extraction step, the in-vitro samples containing raloxifene hydrochloride were separated by reverse phase HPLC with UV detection at 287nm.

The representative chromatograms of raloxifene hydrochloride spiked in unextracted standard concentration and blank are shown Fig. 2 and Fig. 3. The retention time of raloxifene hydrochloride was 5.51 min, and the peaks were sharp. There was good baseline separation of raloxifene hydrochloride.

3.2. Linearity, limit of detection (LOD) and limit of quantitation (LOQ)
Peak areas of raloxifene hydrochloride were measured. A representative calibration graph of peak area versus concentration in the range of 0.5-50µg/ml resulted in regression equation of the calibration curve was calculated as \( y = 68455x - 10868 \) (correlation coefficient, \( r^2 = 0.9994 \)), where \( y \) is the peak area of raloxifene hydrochloride and \( x \) is the concentration of raloxifene hydrochloride. These results demonstrated a good linearity between the peak areas versus concentrations. The limit of detection (LOD) and limit of quantitation (LOQ) was 10ng/ml (S/N=3) and 30ng/ml. This method used a rapid single-step liquid-liquid extraction with 1:4 ratios of methanol and acetonitrile.

3.3. Precision and accuracy
The precision of the assay method was validated by the determination of the intra- and inter-day coefficient of variation (%R.S.D.) and percentage deviation. The intra-day precision data over the concentration range of 0.5-50 µg/ml for raloxifene hydrochloride and all %R.S.Ds were less than 5% and the average %R.S.D was 0.92% for raloxifene hydrochloride. The inter-day precision data over the concentration range of 0.5-50 µg/ml for raloxifene hydrochloride and all %R.S.Ds were less than 5% and the average %R.S.D was 2% for raloxifene hydrochloride. The accuracy of the method was verified by comparing the concentrations measured from extracted sample with actual added concentrations. The intra- and inter-day accuracy data expressed as percentage deviation of raloxifene hydrochloride assay and the data was shown in Table 1.

<table>
<thead>
<tr>
<th>Spiked concentrations (µg/ml)</th>
<th>Calculated concentration (µg/ml, mean± S.D,)</th>
<th>R.S.D (%)</th>
<th>Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.11±0.04</td>
<td>4.44</td>
<td>11.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.43±0.10</td>
<td>4.11</td>
<td>-2.8</td>
</tr>
<tr>
<td>5</td>
<td>4.95±0.13</td>
<td>2.62</td>
<td>-1.0</td>
</tr>
<tr>
<td>10</td>
<td>9.52±0.20</td>
<td>2.10</td>
<td>-4.8</td>
</tr>
<tr>
<td>25</td>
<td>24.42±0.18</td>
<td>1.25</td>
<td>-1.4</td>
</tr>
<tr>
<td>Inter-day (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.06±0.01</td>
<td>0.94</td>
<td>6.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.47±0.08</td>
<td>3.23</td>
<td>-1.2</td>
</tr>
<tr>
<td>5</td>
<td>4.94±0.13</td>
<td>2.63</td>
<td>-1.2</td>
</tr>
<tr>
<td>10</td>
<td>10.07±0.16</td>
<td>1.58</td>
<td>0.7</td>
</tr>
<tr>
<td>25</td>
<td>24.42±0.18</td>
<td>0.73</td>
<td>-2.3</td>
</tr>
</tbody>
</table>

3.4. Recovery
The recovery of raloxifene hydrochloride after liquid-liquid extraction with 1:4 ratios of methanol and acetonitrile was evaluated at five concentrations of 1, 2.5, 5, 10, 25 µg/ml. Absolute recovery was calculated by comparing the peak areas of extracted and unextracted raloxifene hydrochloride samples. Table 2 shows the recovery efficiency of raloxifene hydrochloride from in-vitro samples and the average extraction efficiency of were found to be 98.2%.

<table>
<thead>
<tr>
<th>QC samples (µg/ml)</th>
<th>Concentration of QC samples after extraction</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.98</td>
<td>98.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.44</td>
<td>97.6</td>
</tr>
<tr>
<td>5</td>
<td>4.91</td>
<td>98.2</td>
</tr>
<tr>
<td>10</td>
<td>9.87</td>
<td>98.7</td>
</tr>
<tr>
<td>25</td>
<td>24.63</td>
<td>98.5</td>
</tr>
</tbody>
</table>

3.5. System suitability
The %CV for area response for the drug was 1.5%, which is within the acceptance value ±2%. The %CV for retention time for the drug was 0.5% respectively, which is within the acceptance range of ±2%.

4. DISCUSSION
The present method for the determination of raloxifene hydrochloride from in-vitro samples is sensitive, specific accurate, and reproducible. The excellent separation is demonstrated in the chromatograms and no interfering peaks were observed. The calibration curve was linear and the method was suitable for the analysis of in-vitro samples over the range of 0.5µg to 50µg/ml.

The accuracy of the method was in compliance with the proposed limits and the precision of the method was satisfactory. The system suitability of the method shows that the performance of the chromatographic system is not significantly influenced by variations of the operational parameters inside an accepted domain. This method shows the system suitability parameters are within the limits only.

A rapid single-step liquid-liquid extraction with 1:4 ratios of methanol and acetonitrile shows good recovery. This method was used for the analysis of in-vitro samples collected during a pharmacokinetic study. In conclusion, the HPLC method has been successfully applied to analysis of raloxifene hydrochloride during pharmacokinetic and elution kinetic studies.

REFERENCES
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