A validated method without derivatization for the determination of Letrozole by high performance liquid chromatography-fluorimetric method for pharmaceutical preparation

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ABSTRACT
A high performance liquid chromatography method has been developed for determination of letrozole in tablets. The method has developed in reverse phase column with acetonitrile-50.0 mM phosphoric acid solution, pH: 7 (50:50, v/v) and 0.7 mL/min flow rate by using a fluorimetric detector. Letrozole has natural fluorescence properties, and there is no need for derivatization for this molecule. The detector was set at 256, 585 nm for excitation and emission wavelength respectively. The method was validated in accuracy, precision, specificity, linearity and system suitability parameters are studied. The linear range was found 50–700 ng/mL. The limit of detection and quantification for letrozole was found to be 14–50 ng/mL respectively. The recovery of drug is found to be 98.8%. The developed method has been validated and successfully applied to tablet analysis of drug substance. This method is simple, reproducible and can be used safely for routine analysis of letrozole in tablets.

Keywords: Letrozole, HPLC-FLD, pharmaceutical preparation

INTRODUCTION
Letrozole, 4,4’-((1H-1,2,4-triazol-1-yl)methylene) dibenzonitrile is a nonsteroidal competitive inhibitor of the aromatase enzyme system; it inhibits the conversion of androgens to estrogens (Figure 1). Letrozole (LTZ) is used for the treatment of breast tumor which is estrogen-dependent. Breast cancer is also responsible for 18% of deaths in cancer types (Lamb and Adkins, 1998; Annapurnaa et. al., 2012). In adult nontumor and tumor bearing female animals, letrozole is as good as ovariectomy in decreasing uterine weight, raising serum luteinizing hormone (LH), and resulting in the regression of estrogen dependent tumors. Unlike ovariectomy, treatment with letrozole does not cause an increase in serum follicle stimulating hormone (FSH). Letrozole inhibits gonadal steroidogenesis but has no important effect on adrenal mineralocorticoid or glucocorticoid synthesis.

Letrozole inhibits the aromatase enzyme by binding to the heme of the subunit of the cytochrome P450 enzyme competitively, concluding in a decrease of estrogen biosynthesis in all tissues. women suffering from breast cancer with treatment of letrozole effect by reducing serum estrone, estradiol and estrone sulfate and hasn’t been shown to importantly affect adrenal corticosterone, aldosterone synthesis, or synthesis of thyroid hormones (Annapurnaa et. al., 2012; Scott and Keam, 2006).

Numerous drug substances of interest cannot be detected because the lack of the structural properties essential for the production of signals compatible with general detectors, such as ultraviolet (UV) absorbance and fluorescence. Derivatization can be used as an effective modification method that can improve the overall specificity and sensitivity of trace analyses. Besides these advantages, derivatization includes disadvantages such as lower yields and time-consuming experimental procedures. Letrozole has natural fluorescence properties to detect lower concentrations from various matrices.

In the literature, limited method can be found on letrozole analysis in pharmaceutical preparations. Mondal et al. developed a spectrophotometric technique for the determination of letrozole in pharmaceutical formulation (Mondal and Pal, 2009). The RP-HPLC method (Mondal and Pal, 2009; Pallavi et al., 2012) and spectrophotometric derivative method (Acharjya et al., 2010)
have been reported for the analysis of letrozole from pharmaceuticals. Letrozole determination from biological materials were detected high performance liquid chromatography with fluorescence detector (HPLC-FLD) (Moussa et al., 2013; Rodríguez et al., 2013; Marfil et al., 1996; Zarghi et al., 2007) gas chromatography (Mareck et al., 2005), high performance liquid chromatography mass spectrometer (HPLC-MS) (Gomes and Bhosale 2013) and also stability studies of letrozole preparations were investigated (Mareck et al., 2005; Gomes and Bhosale 2013, Prasad and Govindrajulu, 2012). There is no publication of letrozole from pharmaceutical preparation by chromatographic method. The method developed can be applied to stability studies and also pharmacokinetic studies.

The aim of this study is to develop a simple and safe method based on fluorescence detection with a high performance liquid chromatography technique and an application of this method on pharmaceutical preparations.

MATERIALS AND METHODS

Apparatus

The HPLC system Shimadzu LC (liquid chromatography) 20 series (Shimadzu Analytical and Measuring Instruments, Kyoto, Japan) with fluorescence detector were used for the analysis of letrozole. The HPLC systems parts included a pump (Shimadzu LC-20AT) and an auto sampler (SIL-20HT). An instrument software (LC Solution) was also used.

Chemicals

All the chemicals were analytical grade. Letrozole was obtained from Natco Pharma Limited (India). Methanol which is HPLC grade was purchased from Sigma-Aldrich (Taufkirchen, Germany) and Acetonitrile which is HPLC grade was purchased from VWR (Vienna, Austria). HPLC grade solvents were also used. Femera® was used as a tablet which contains 2.5 mg LTZ.

Chromatographic conditions

Chromatographic separation was carried out at 25 °C temperature using a reversed phase Intersil ODS (octadecyl silane) 5 μm, 250x4.6 mm column (Tokyo, Japan). The mobile phase consists of acetonitrile: phosphate buffer 50 mM pH:7 (50:50) (v/v). The flow rate of the mobile phase was 0.7 mL/min. Letrozole has natural fluorescence properties and the detector was set at 256, 585 nm for excitation and emission wavelengths respectively. The injection volume chosen was 20 µL.

Preparation of standard solutions and quality control samples

A stock solution of LTZ (letrozole) (0.1 mg/mL) was prepared and diluted to LTZ2 (1 µg/mL) with methanol. All solutions were stored at 4°C until the end of the study. The five calibration standards were prepared with methanol between the concentration ranges of 50 and 700 ng/mL. The quality control (QC) samples at a concentration of 50; 200; 400 and 700 ng/mL. All calibration standards and QC (quality control) samples were stored at 4°C until the end of the study.

Analytical method validation

In order to prove the acceptable nature of analytical method, the following protocol was performed during the method evaluation. There was no need for ethics committee approval for this study.

RESULTS

The chromatographic separation of analytes and other ingredients of drugs was optimized to provide an acceptable reso-
lution, a good peak shape and an intensity of the response. The mobile phase combination was changed consistently to establish chromatographic conditions giving an agreeable resolution. The retention time of letrozole is 9.9 minutes.

**System suitability test**

System suitability parameters summarized in Table 1a. were within acceptable limits. In Table 1b, system suitability acceptance limits are given according to FDA guidelines.

**Selectivity**

The selectivity of method was investigated by analyzing inactive ingredients of pharmaceutical preparations. Chromatograms were compared to the matrix for any interference or any of the method reagents (Figure 2).

**Sensitivity**

The lowest standard of concentration 50 ng/mL for LTZ on the calibration curve was identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20%. LOD (limit of detection) was found 14 ng/mL. The method developed is sensitive enough for the determination of LTZ from biological materials.

**Linearity**

The calibration curve contains five calibration samples within the range of 50-700 ng/mL including lower limit of quantification (LOQ) for LTZ. The acceptance criteria of back calculated concentration of standard batch was 15% deviation from the nominal value excluding the LOQ (for LOQ, 20% deviation was applied). The standard calibration curves was linear over the concentration range from 700 ng/mL with a mean of r² = 0.9998 for LTZ. Accuracy and precision results of calibration standard can be shown in Table 2. The LOQ was 50 ng/mL. The calibration curve had a regression equation of y = 223827 X + 56.3597.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity factor</td>
<td>k’ &gt; 2</td>
</tr>
<tr>
<td>Injection precision</td>
<td>RSD &lt; %1, n≥5</td>
</tr>
<tr>
<td>Resolution</td>
<td>Rs</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>T ≤ 2</td>
</tr>
<tr>
<td>Theoretical plate number</td>
<td>N &gt; 2000</td>
</tr>
</tbody>
</table>

**Table 1b. System suitability parameters in HPLC (According to FDA guideline)**

<table>
<thead>
<tr>
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<tr>
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</tr>
</tbody>
</table>

**Table 2. Accuracy and precision results of calibration standards**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration ng/mL</th>
<th>Mean</th>
<th>Recovered amount</th>
<th>Deviation (SD)</th>
<th>%CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST1</td>
<td>0.05</td>
<td>0.0496</td>
<td>99.2</td>
<td>0.002191</td>
<td>4.417117</td>
<td>6</td>
</tr>
<tr>
<td>ST2</td>
<td>0.1</td>
<td>0.099167</td>
<td>99.2</td>
<td>0.003251</td>
<td>3.277957</td>
<td>6</td>
</tr>
<tr>
<td>ST3</td>
<td>0.3</td>
<td>0.295167</td>
<td>98.4</td>
<td>0.006765</td>
<td>2.291962</td>
<td>6</td>
</tr>
<tr>
<td>ST4</td>
<td>0.5</td>
<td>0.497</td>
<td>99.4</td>
<td>0.011367</td>
<td>2.287046</td>
<td>6</td>
</tr>
<tr>
<td>ST5</td>
<td>0.7</td>
<td>0.689333</td>
<td>98.5</td>
<td>0.020363</td>
<td>2.954067</td>
<td>6</td>
</tr>
</tbody>
</table>

Accuracy is evaluated by mean and the recovered amount and precision is evaluated by standard deviation (SD) and coefficient of variation (%CV).

%CV: Coefficient of variation
SD: Standard deviation
n: Number of samples

**Figure 2. a, b. Placebo and letrozole (300 ng/mL) Chromatogram**
Accuracy and precision

Precision and accuracy of intra-day were settled by analysis of six replicates of 5 concentrations including low, medium and high concentrations of quality control samples. Inter-day precision and accuracy were examined by the analysis of these quality control samples on three separate batches. The precision of the method was showed as the percentage of coefficient of variation and the accuracy of the method was showed in terms of relative errors. The precision was showed as the percentage of coefficient of variation. Table 3 presents a summary of the accuracy and precision.

Table 3. Results of accuracy and precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration ng/mL</th>
<th>Mean</th>
<th>Recovered amount</th>
<th>Deviation(SD)</th>
<th>%CV</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>QC1</td>
<td>50</td>
<td>0.048188</td>
<td>96.36</td>
<td>0.004665</td>
<td>9.68</td>
<td>18</td>
</tr>
<tr>
<td>QC2</td>
<td>200</td>
<td>0.195722</td>
<td>97.85</td>
<td>0.006676</td>
<td>3.41</td>
<td>18</td>
</tr>
<tr>
<td>QC3</td>
<td>400</td>
<td>0.402313</td>
<td>100.57</td>
<td>0.017843</td>
<td>4.43</td>
<td>18</td>
</tr>
<tr>
<td>QC4</td>
<td>700</td>
<td>0.689056</td>
<td>98.43</td>
<td>0.013584</td>
<td>1.97</td>
<td>18</td>
</tr>
</tbody>
</table>

Accuracy is evaluated by mean and the recovered amount and precision is evaluated by standard deviation (SD) and coefficient of variation (%CV).

%CV: Coefficient of variation

SD: Standard deviation

n: Number of samples

Sample preparation

Tablet formulation containing 2.5 mg LTZ, ten tablets were powdered and one tablet weight was diluted to 100 mL flask to volume with methanol, 6 replicates were prepared in this way. Further dilution was carried out by transferring an appropriate amount for 250 ng/mL final concentration (Figure 3). Recovery from pharmaceutical preparation was found to be 98.8 %.

RESULTS AND DISCUSSION

Letrozole has natural fluorescence properties which allows it to be analyzed without derivatization. System suitability parameters were within acceptable limits. The method developed has been validated. The method was found to be selective as seen in Figure 2. Chromatographic separation can differentiate the peak of analytes. The method was sensitive enough for the determination of letrozole from pharmaceutical preparations. Because of minority of papers on letrozole for pharmaceutical preparations, it is aimed to develop more sensitive and lower detection limits. Based on experiments and statistical analyses of letrozole, our data show that the proposed method demonstrates greater sensitivity to relevant studies including Mondal’s (Mondal and Pal, 2009) and Narataj’s (Nataraj et al., 2012). Considering the calibration curve and LOD and LOQ values, the proposed method is more sensitive than reported studies (Mondal and Pal, 2009; Pallavi et al., 2012). As a result, the correlation coefficient and the linear range of our method are more convenient and effective than the other studies (Prasad et al., 2012), (Acharjya et al., 2010). This method can be applied to pharmaceutical analysis of letrozole for quality control during the process as well as cleaning swap analysis due to lower detection limit of the method. For further studies this method can be used for biological fluids such as plasma.

The developed method for the determination of LTZ from pharmaceutical preparation has been found accurate, precise, selective, and suitable for the quality control analysis.
Acknowledgements
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Conflict of Interest: The authors have no conflict of interest to declare.

REFERENCES