CHAPTER – 3

A simple and quick stability-indicating method for assay of anastrozole in low dose anastrozole tablets by high performance liquid chromatography (HPLC)
Introduction

The International Conference on Harmonisation (ICH) guidelines has been incorporated as law in EU, Japan, and in US, but in reality, besides these, other countries are also using them. As these guidelines reflect the current inspectional tendencies, they carry the de facto force of regulation. However in the absence of any guidance from regulatory agencies, practical steps need to be followed for establishment of stability-indicating assays. Assay test methods though (classically and primarily) have to be designed stability-indicating by nature, are not meant to be selective to each decomposition products and other constituents in the drug substance or formulations.

The objective of the present work was the development and validation of a simple, stability-indicating, shorter run time method for assay of anastrozole in the tablet dosage form. So far no literature references are available for determination of anastrozole in the tablet dosage form. As anastrozole is a low dose drug product, consisting of only 1% of active ingredient in the tablet, there exists a potential tendency for segregation of active substance from the excipients in the formulation matrix during manufacturing. Since manufacturing involves multiple steps, it is very much essential to assay for the active content at each step for the quality of the product before proceeding into further step. For this purpose the methods at quality control needs to be fast, accurate, precise, specific and robust. The longer run time analysis may cause delay at manufacturing process. In view of the above “Quality by design” concept in mind now we present a very simple and short run-time isocratic stability-indicating method in order to furnish “Quality by testing” for anastrozole tablets.
Previously an HPLC method has been reported for assay of anastrozole. But in the reported method [1], studies were established only on drug substance which comprises a triple combination of solvents as mobile phase with gradient elution and a longer run time. The sample concentration in the reported method was 100 μg/ml which is difficult to achieve for anastrozole in low dose tablets due of huge excipients interferences. Because of the longer run time, requirement of gradient elution for analysis, limitation for sample concentration and “Quality by design” concept the reported method [1], was found to be not suitable for its determination in the tablet dosage form. Also a number of reports [2 - 7] exists on procedures for its determination and in biological fluids, such as plasma and urine. One method was reported on some impurities isolation of anastrozole [8].

Anastrozole 2-[3(1-cyano-1-methyl-ethyl)-5-(1H-1, 2, 4-triazol-1-yl methyl) phenyl]-2-methyl-propinenitrile is a potent and selective non-steroidal aromatase inhibitor. It significantly lowers serum estradiol concentrations and has no detectable effect on formation of adrenal corticosteroids or aldosterone. Many breast cancers have estrogen receptors and growth of these tumors can be stimulated by estrogen. In postmenopausal women, the principal source of circulating estrogen (primarily estradiol) is conversion of adrenally-generated androstenedione to estrone by aromatase in peripheral tissues, such as adipose tissue, with further conversion of estrone to estradiol. Armidex [9], is the innovator reference listed drug (RLD) available as 1mg tablets.
Experimental

Reference substances, Chemicals and Reagents

Anastrozole API (purity, 99.7%), Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E were kindly supplied by Process Research Department of Dr. Reddy's Laboratories Limited, Hyderabad, India and the respective chemical structures are given in Fig 3.1. Anastrozole tablets and placebo tablets were obtained from Formulations Research Department of Dr. Reddy's Laboratories. HPLC grade acetonitrile were purchased from Merck, Germany. Sodium hydroxide was purchased from Ranbaxy Laboratories and hydrochloric acid was purchased from LOBA Chemie Pvt.Ltd (India). Hydrogen peroxide was procured from S.D Fine-Chem Ltd (India). All other reagents were of analytical reagent grade. High pure water was prepared by using Millipore Milli Q plus purification system.

Instrumentation

The chromatographic separation was performed on Agilent HPLC 1100 series, Agilent Technologies, USA. The HPLC system consisted of an on-line degasser (G1379A), low pressure quaternary system delivery module (G1311A), auto injector and auto sampler (G1313A), column oven (G1316A) UV-visible detector (G1314A). The output signal was monitored and processed using Empower software (Waters) on Pentium computer (Digital Equipment Co). Robustness and Peak purity testing was done on another HPLC system equipped with separation module (Waters 2695 model) and photo-diode array detector (Waters 2996 model).
Chromatographic Conditions

The chromatographic separation was carried out at constant mobile phase flow rate of 1.0 ml/min under isocratic elution with 60:40 (v/v) water and acetonitrile on Inertsil ODS-3V, 150x4.6mm i.d., 5µm stainless steel analytical column (GL Sciences Inc, Japan). UV-VIS detector was set at 215nm. All analyses were done at ambient room temperature and volume of solution injected on to the column was 20 µl.

Extraction solvent

Mixture of water and acetonitrile in the ratio of 50:50 v/v respectively was found to be the best solvent to extract anastrozole from the tablet dosage form. Selection of diluent was made based on anastrozole solubility [9], and minimal interferences due to excipients used in the formulation matrix. Also, addition of water in the diluent helps to disintegrate the tablets.

Samples

Anastrozole 1mg tablets and the corresponding placebo tablets (without API) were used throughout the development and validation. Other test samples used were accelerated stability samples as per ICH [10] with similar composition. All the samples were treated according to test solution preparation.

Solution preparation

Preparation of standard solution:

Anastrozole API was accurately weighed and dissolved in the diluent so as to obtain a concentration of 40 µg/ml.
Preparation of test solution

Ten intact tablets was taken in a 250mL volumetric flask, added about 200mL of the diluent. The contents of flask were kept on a rotary shaker for about 10 min (until the tablet disintegrates completely) and sonicated for 30 minutes with intermediate shaking (by maintaining the sonicator temperature at about 25°C). Finally, volume was completed with diluent and mixed well so as to obtain about 40 µg/ml of the concentration of the anastrozole in the test solution. The solution was centrifuged in a tight enclosure for about 5 min at 3500 rpm and 20µl of clear supernatant solution was injected directly on to the column.

Forced decomposition studies for establishment of stability-indicating:

Drug product (anastrozole tablets) and the placebo tablets were used in all decomposition studies. The pH of the buffered solutions was measured before and after the reaction and no change in the pH was observed. All the solutions for use in forced degradation studies were prepared by dissolving the drug product in small volumes of stressing reagents. After the completion of degradation, these solutions were diluted with diluent to yield stated concentration of 40 µg/ml. Conditions employed for performing stress studies were as follows:

Hydrolytic studies

Acid decomposition studies were performed by heating the drug product solution in 0.5 N HCl at 80°C on a water bath for 2 h. The studies in alkaline conditions were done in 0.5 N NaOH at 80°C for 2 h. For the study in neutral conditions, the drug product solution in water was heated at 80°C for 2 h prior to analysis.
Oxidation studies

Solutions for use in oxidation studies were prepared in 5% hydrogen peroxide and the resultant solution heated on a water bath at 80°C for 1 h was injected prior to analysis.

Thermal stress studies

Anastrozole tablets and placebo tablets were exposed to dry heat of 100°C in a convention oven for 7 days.

Photostability studies

Susceptibility of the drug product to light was studied [11]. Tablets for photostability testing were placed in a light cabinet and exposed to light resulting in an overall illumination of $\geq 200$ Wh/m² at 25°C with UV radiation at 320-400nm. Control samples which were protected from light with aluminum foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

Impurities spike study

Anastrozole solution was spiked with Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E each at 2 µg/ml level (Fig 3.2).

Results

Method Validation

The proposed test method was validated to include requirements of International conference on Harmonization (ICH) guidelines [12-13]. Parameters like specificity,
linearity, precision, accuracy, range, robustness, ruggedness and system suitability were examined.

**Specificity**

There were no interferences due to placebo and sample diluent at the retention times of anastrozole. All known impurities and unknown degradants were well separated. Specificity (peak homogeneity) was established by determination of purity of drug peak in forced decomposition and impurity spiked samples using a PDA detector. All known impurities, and unknown degradants were well separated and purity angle was found to be less than purity threshold for anastrozole. Purity plot for anastrozole peak in a mixture of impurity spiked solution proved that the method was specific to the drug (anastrozole) in anastrozole tablets as purity angle value was found to be less than purity threshold value.(Figs. 3.22, 3.33 and 3.4)

Apart from the peaks homogeneity, the DAD spectrum (Fig 3.5) for the all the impurities, and anastrozole were compared against their standard spectrums. Identity for all the impurities and anastrozole were performed by comparing their DAD spectrum and purity plots, with those of standards and, was found to be matching.

**System precision**

Instrumental precision for anastrozole at 40 μg/ml as system suitability was determined by analyzing five replicate injections on different systems and different days and the relative standard deviation was found to be less than 0.5% respectively.
Method precision (Intra-day) and intermediate precision (Inter-day)

Method precision or intra-day precision was performed by analyzing anastrozole tablets. Six replicates (n=6) solutions were prepared and each solution was injected in duplicate under the same conditions and mean value of peak area response for each solution were considered. Intermediate precision (inter-day precision) was performed by analyzing the study using different instrument, analyst, column and six different samples at the stated concentration. The results of repeatability and intermediate precision experiments are shown in Table 3.1. The developed method was found to be precise as the RSD values was <2.0% on both the variations respectively.

Accuracy (recovery test)

The accuracy of the proposed method was evaluated by the recovery studies (fig 6) which were carried out by extracting the tablets in the diluent at different concentrations in the range of 20-80 μg/ml. Three samples were prepared at each concentration and calculated the exact percentage recovery at each level for anastrozole. As shown in Table 3.2, the average recovery at each level was within 100±2% and the RSD at each level was ≤ 2%.

Accuracy curve

The accuracy curve (Linearity of test method) was established for anastrozole by plotting the values of actual concentration (μg/ml) on X-axis and measured concentration (μg/ml) on Y-axis as determined from accuracy section. The regression equation for the calibration curve was found to be y = 0.9993x - 0.0356, with correlation coefficient (r) 0.9996.
Response Linearity

The detector response linearity parameter of the curve for anastrozole was determined (Fig 3.7). The standard solutions containing anastrozole in the concentration range of 10-80 μg/ml were prepared and injected in duplicate into the chromatographic system. A weighted linear regression analysis calculation [14] was performed by weighting each value of (y) by a factor that is inversely proportional to the variance, 1/X^2 for weighted regression (w). The calculation of weighted regression, values of Slope (b) and Intercept (a) for anastrozole as a function of concentration μg/ml (X) and detector peak (area) response (y) is depicted in Table 3.3. In this simultaneous determination, the linear regression was found to be good over the concentrations range mentioned for anastrozole.

Stability of analytical solution

The stability of the standard and test solution was checked by analyzing these solutions at frequencies of initial, 24 h, 48 h and 72 h at room temperature, against freshly prepared standards. The result demonstrated that the standard solution, as well as the sample solution is stable for at least 72 h. During the stability studies no additional peaks developed and no changes in the chromatographic pattern were observed in either of the solutions.

Robustness

Robustness of proposed method was performed by keeping chromatographic conditions constant with following differences:

- Increasing the column oven temperature from 20°C to 30°C.
• Change in the flow rate of mobile phase from 0.8 ml to 1.2 ml/min

• Change in the composition of mobile phase from 55:45 to 65:35 v/v

• Using another column (Inertsil ODS-3V, 250x4.6mm i.d, 5μm, GL Sciences Inc).

For each absolute change, standard solution was injected five times. System suitability parameters like peak symmetry, theoretical plates and relative standard deviation were recorded and found to be within the acceptable limits. The observations of various parameter changes are tabulated in Table 3.4

Analysis of stability samples

Anastrozole tablets of three lab scale batches were subjected to the accelerated stability conditions of 60°C and 40°C/75% RH for 3 months. The test solutions subjected to HPLC analysis showed assay in the range 98.2% to 101% indicates the stability of the drug product.

Discussions

Degradation behavior of Anastrozole:

HPLC studies of samples obtained from forced decomposition studies of anastrozole suggested the following degradation behavior. The drug was found to be highly stable under 0.5 N HCl, 0.5 N NaOH, and neutral (water) conditions at 80°C for 2 h. No major degradants were observed in any of these conditions. Thermal and light stress also had no effect on anastrozole tablets. The drug was found to be labile to hydrogen peroxide at 80°C for 1 h conditions. It decomposed to an extent of about 30% in 1 h. The major degradation product had retention time of about 2.6 min which was
well separated from all the impurities and anastrozole under selected conditions. The behavior of separation on Inertsil column is depicted in Figs. 3.2 – 3.4.

**Separation studies**

Stability-indicating studies by its virtue, not only involved separation of degradants observed under stress conditions, but also all known impurities. During development, apart from the separation of all impurities, sample extraction from formulation matrix was critical as the drug substance was only 1% in the tablet dosage form which comprises of 99% excipients. When methanol was used as extraction solvent, excipients also were extracted and this resulted in the baseline disturbance during the chromatographic run. Hence considering the nature excipients and anastrozole, acetonitrile was selected as compared to methanol which resulted in good baseline and addition of water in the diluent helps to disintegrate the tablets quickly. Accuracy studies under method validation proved the extraction efficiency of the diluent.

Acetonitrile was used in the mobile phase so as to match the diluent compatibility. After several combinations of the organic modifier in the mobile phase, eventually all the separations and good peak shape were obtained in the selected combination. The final selected mobile phase composition of water and acetonitrile, 60:40 (v/v) showed acceptable separation of anastrozole from all impurities within a very short run time which is the added advantage of this method. Inertsil ODS-3V, 150x4.6mm, 5μm column was used during the developmental studies as other brands of ODS was unable to separate all the impurities, 150mm length was selected to achieve shorter run times. The behavior of separation on Inertsil column is depicted in Fig 3.2.
Table 3.1: Results of Method Precision of Anastrozole in Anastrozole Tablets.

<table>
<thead>
<tr>
<th>Spiked (µg/ml)</th>
<th>Measured concentration (Intra-day)</th>
<th>Measured concentration (Inter-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>(µg/ml);±S.D; RSD%</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>40.09 ± 0.29; 0.72</td>
<td>39.97 ± 0.18; 0.45</td>
</tr>
</tbody>
</table>

*a* mean of six preparations at labeled amount of anastrozole 1mg/tablet;  
*b* mean of measured concentrations from six preparations

Table 3.2: Accuracy results of Anastrozole in Anastrozole tablets.

<table>
<thead>
<tr>
<th>Tablets taken</th>
<th>Level of addition</th>
<th>Spiked (µg/ml)</th>
<th>Measured concentration (µg/ml);±S.D; RSD%</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50%</td>
<td>20</td>
<td>20.03 ± 0.06; 0.30</td>
<td>100.2</td>
</tr>
<tr>
<td>7</td>
<td>70%</td>
<td>28</td>
<td>27.57 ± 0.12; 0.44</td>
<td>98.5</td>
</tr>
<tr>
<td>10</td>
<td>100%</td>
<td>40</td>
<td>39.77 ± 0.12; 0.31</td>
<td>99.4</td>
</tr>
<tr>
<td>15</td>
<td>150%</td>
<td>60</td>
<td>61.00 ± 0.66; 1.08</td>
<td>101.7</td>
</tr>
<tr>
<td>20</td>
<td>200%</td>
<td>80</td>
<td>79.30 ± 0.69; 0.87</td>
<td>99.1</td>
</tr>
</tbody>
</table>

Mean: 99.8  
SD: 1.22  
RSD (%): 1.23

*a* mean of three preparations at each level containing a labeled amount of anastrozole 1mg/tablet;  
*b* mean of measured concentrations from three preparations;  
*c* measurements of % recoveries of all the levels.
Table 3.3: Weighted Linear Regression of Anastrozole

<table>
<thead>
<tr>
<th>µg/ml (X)</th>
<th>Mean Peak area (y)</th>
<th>SD</th>
<th>CV</th>
<th>Weight (w)</th>
<th>$\sum wXy$</th>
<th>$\sum wX$</th>
<th>$\sum wy$</th>
<th>$\sum wX^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.9</td>
<td>406545</td>
<td>63.6</td>
<td>0.0002</td>
<td>0.0102</td>
<td>82130</td>
<td>0.202</td>
<td>8296</td>
<td>2</td>
</tr>
<tr>
<td>18.8</td>
<td>833211</td>
<td>15.6</td>
<td>0.0000</td>
<td>0.0028</td>
<td>88639</td>
<td>0.106</td>
<td>4715</td>
<td>2</td>
</tr>
<tr>
<td>40.5</td>
<td>1641506</td>
<td>715.6</td>
<td>0.0004</td>
<td>0.0006</td>
<td>81062</td>
<td>0.049</td>
<td>2002</td>
<td>2</td>
</tr>
<tr>
<td>59.2</td>
<td>2511540</td>
<td>650.5</td>
<td>0.0003</td>
<td>0.0003</td>
<td>84849</td>
<td>0.034</td>
<td>1433</td>
<td>2</td>
</tr>
<tr>
<td>78.9</td>
<td>3278000</td>
<td>509.1</td>
<td>0.0002</td>
<td>0.0002</td>
<td>83102</td>
<td>0.025</td>
<td>1053</td>
<td>2</td>
</tr>
</tbody>
</table>

Slope of the calibration curve, $(b) = 41945$; Intercept $(a) = 790$, $(w) = 1/X^2$
### Table 3.4: System Suitability of Anastrozole under Robustness

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variation</th>
<th>RSD (%)</th>
<th>USP Theoretical plates</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20°C</td>
<td>0.2</td>
<td>6673</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>0.1</td>
<td>6624</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>0.2</td>
<td>6853</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 ml/ min</td>
<td>0.1</td>
<td>7336</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>1.0 ml/ min</td>
<td>0.1</td>
<td>6624</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>1.2 ml/ min</td>
<td>0.2</td>
<td>6013</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Mobilephase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55:45 (v/v)</td>
<td>0.1</td>
<td>7776</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>60:40 (v/v)</td>
<td>0.1</td>
<td>6888</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>65:35 (v/v)</td>
<td>0.1</td>
<td>6500</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Column/Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>6987</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>6612</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>6723</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.1: Molecular structure of anastrozole and its related compounds (RC).
Fig. 3.2.1: HPLC chromatogram showing the separation of Anastrozole from its impurities.

Fig. 3.2.2: Purity plot of Anastrozole in the presence of impurities spiked solution.
Fig. 3.3: HPLC chromatogram showing the separation of Anastrozole from the degradation product observed under peroxide condition.
Fig. 3.4: Overlay of HPLC chromatograms of forced decomposition samples except for peroxide condition.
Fig. 3.5: DAD spectrum of Anastrozole, its impurities and degradant (in the peroxide condition).
Fig. 3.6: Overlay of HPLC chromatogram showing the peaks due to Anastrozole in tablets at various levels as obtained from accuracy experiment.

Fig. 3.7: Overlay of HPLC chromatogram showing the peaks due to Anastrozole at various levels as obtained from linearity experiment.
Conclusions

This chapter presents a simple analytical method on determination of anastrozole in finished dosage form by HPLC. The proposed method is a simple isocratic with short run time, stability indicating method when compared to long run time, gradient reported method\(^1\). The proposed HPLC method has the ability to separate anastrozole from all its impurities and excipients found in the tablet dosage form and therefore can be applied to the analysis of samples at quality control. The shorter run time analysis is particularly important in quality control to support the In-process samples during the manufacturing, as each step in production is dependent on the result awaited from quality control before proceeding to further step. The proposed method is rapid, direct, specific, accurate, precise, stability-indicating and validated for the routine analysis in the finished dosage form. The method also may be extended to the bulk drug substances.
References


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