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VALIDATED RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ANTI-NEOPLASTIC AGENT ANASTROZOLE IN PURE FORM AND MARKETED PHARMACEUTICAL TABLET DOSAGE FORM

Koduri Swathi*, M. Roja, M. Sai Priyanka, T. Shravani, Sri Vidya, G.V.N.S. Sudeepti

Department of Pharmaceutical Analysis and Quality Assurance, Bojjam Narasimhulu Pharmacy College, Road Number 2, Vinayak Nagar, Vani Nagar, Saroor Nagar West, Sayeedabad, Hyderabad, Telangana 500059.

Author for correspondence: Koduri Swathi

ABSTRACT

A new, simple, rapid, precise, accurate, and reproducible RP-HPLC method for estimation of Anastrozole in bulk form and marketed formulation. Separation of Anastrozole was successfully achieved on a Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d. column in an isocratic mode of separation utilizing Methanol: Phosphate buffer (0.02M, pH-3.6) in the ratio of 45:55% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 255nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity, and robustness. The response was found to be linear in the drug concentration range of 12-28mcg/mL for Anastrozole. The correlation coefficient was found to be 0.9995 for Anastrozole. The LOD and LOQ for Anastrozole were found to be 5.004µg/mL and 15.164µg/mL respectively. The proposed method was found to be a good percentage recovery for Anastrozole, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of the standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords: Anastrozole, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.

INTRODUCTION

Anastrozole, sold under the brand name Arimidex among others, is a medication used in addition to other treatments for breast cancer. Specifically, it is used for hormone receptor-positive breast cancer. It has also been used to prevent breast cancer in those at high risk. It is taken by mouth. Anastrozole[1] is a nonsteroidal inhibitor of estrogen synthesis that resembles paclitaxel in chemical structure. As a third-generation aromatase inhibitor, Anastrozole selectively binds to and reversibly inhibits aromatase, a cytochrome P-450 enzyme complex found in many tissues including those of the premenopausal ovary, liver, and breast; aromatase catalyzes the aromatization of androstenedione and testosterone into estrone and estradiol, the final step in estrogen biosynthesis. In estrogen-dependent breast cancers, Anastrozole[2] may inhibit tumor growth. Anastrozole is a nonsteroidal inhibitor of aromatase that effectively blocks estrogen synthesis in postmenopausal women and is used as therapy of estrogen receptor-positive breast cancer. Anastrozole[3] has been associated with a low rate of serum enzyme elevations during therapy and rare instances of clinically apparent liver injury. The IUPAC Name of Anastrozole is 2-[3-(2-cyano propan-2-yl)-5-(1, 2, 4-triazol-1-yl methyl) phenyl]-2-methyl propanenitrile. The Chemical Structure of Anastrozole is

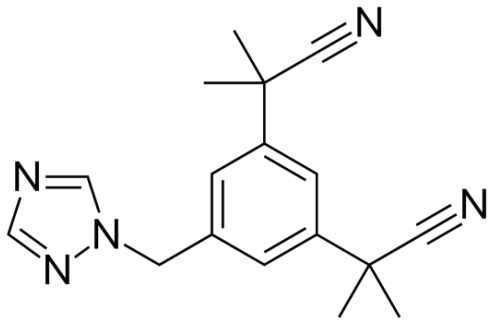


Fig 1: Chemical Structure of Anastrozole

MATERIALS AND METHODS

Table1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table 2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

Wavelength Detection (Or) Selection of Wavelength

The detection wavelength[4] was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm.[3]

Preparation of Standard Solution

10 mg of Anastrozole working standard[5] was accurately weighed and transferred into a 10 ml clean dry volumetric flask. Add about 7 ml of diluents and sonicate to dissolve it completely and volume was made up to the mark with the same solvent which gave stock solution of 1000 ppm. Further pipette 1 ml of the above stock solution into a 10 ml volumetric flask was diluted up to the mark with diluents (100 ppm solution). Further 1 ml of prepared 100 ppm solution was pipetted into a 10 ml volumetric flask and was diluted up to the mark with diluents which gave 10 ppm Anastrozole working standard solution. The solution was mixed well and filtered through 0.45µm filter.[4]

Preparation of Sample Solution

Twenty tablets were taken and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated well. A quantity of powder of Anastrozole equivalent to 10mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC grade methanol was added and the resulting solution was sonicated for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.1 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45µm) and finally sonicated to degas.[5]

Preparation of 0.02M Potassium dihydrogen orthophosphate Solution

About 2.72172grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water. The pH was adjusted to 3.60 with diluted orthophosphoric acid[6].

Preparation of Mobile Phase

550ml of Phosphate buffer (0.02M) pH 3.60 and 450ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration[7].

Method Validation

Accuracy

The accuracy[8] of the method was determined by calculating % recovery. A known amount of Anastrozole was added to a placebo and the amounts were estimated by measuring the peak area. These studies were carried out in triplicate over the specified concentration range and the amount of Anastrozole was estimated by measuring the peak area ratios. The percentage recovery[9] and standard deviation of percentage recovery were calculated.

Precision

The precision[10] of the method was determined in terms of Intra-day[11] and inter-day precision. For intra-day precision studies, a standard solution of 10 ppm was injected at various time intervals and percent related standard deviation (%RSD) was estimated. The inter-day precision[12] was studied by injecting the same concentration of standard solution on consecutive days and the % RSD of the signal was calculated. The repeatability, intermediate precision and reproducibility of the developed method were determined.

Specificity

Specificity[13] is the ability to assess unequivocally the analyte in the presence of components etc. The blank (diluent), placebo, standard (10 ppm), sample (10 ppm) were prepared and injected to prove that the method developed was specific to Anastrozole.

Linearity and range

The linearity of the method was determined at six concentration levels ranging from 12-28 ppm of

Anastrozole. A regression line was plotted of peak area v/s concentration. The correlation coefficient and equation of the regression line[14] were calculated. The interval of lowest assessed concentration to the highest is the linearity[15] range of the procedure.

LOD and LOQ

The detection limit[16] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit[17] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Where, = the standard deviation of the response, Slope = slope of the calibration curve.

Robustness

Robustness[18] of the developed method was studied by changing the flow rate and column temperature. The effect of flow rate was studied by keeping all chromatographic conditions same except the flow rate, i.e. 0.9ml/min and in the next run 1.1ml/min respectively.

Similarly, the effect of temperature was studied by keeping all chromatographic conditions[19] same except the temperature, i.e. 35 °C and in the next run with 25 °C respectively.

System Suitability

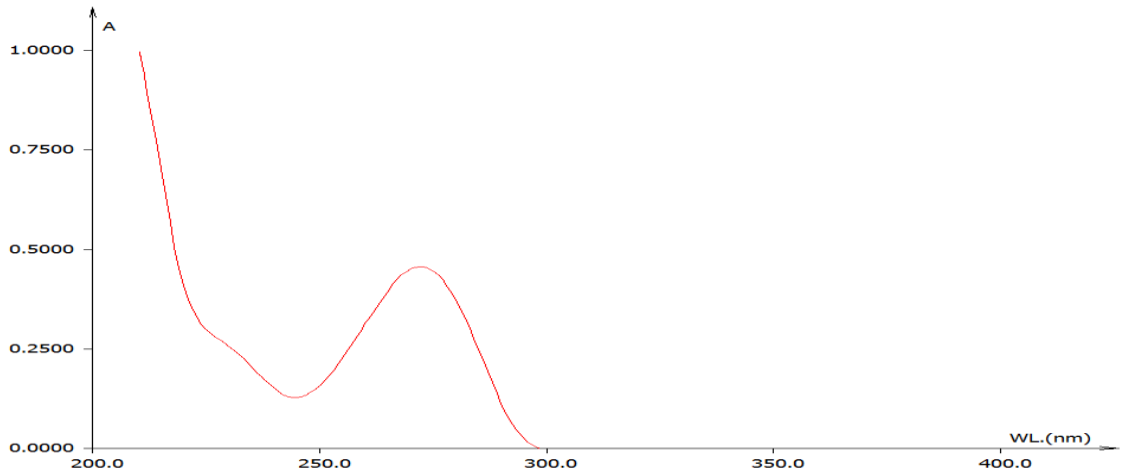
The system suitability[20,21] parameters like retention time, the number of USP theoretical plates, USP tailing, and peak area and peak height were evaluated.

RESULTS AND DISCUSSION

Method Development

Wavelength Detection (Or) Selection of Wavelength

The UV spectrum of Anastrozole was obtained and the Anastrozole showed absorbance's maxima at 255nm. The UV spectra of drug are follows:



While scanning the Anastrozole solution we observed the maxima at 255nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Fig 2: UV Spectrum of Anastrozole

Method Optimization

Optimized Chromatographic Method

Table3: Optimized Chromatographic Method

Mobile phase	Methanol : Phosphate buffer (0.02M, pH-3.6) = 45:55
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic

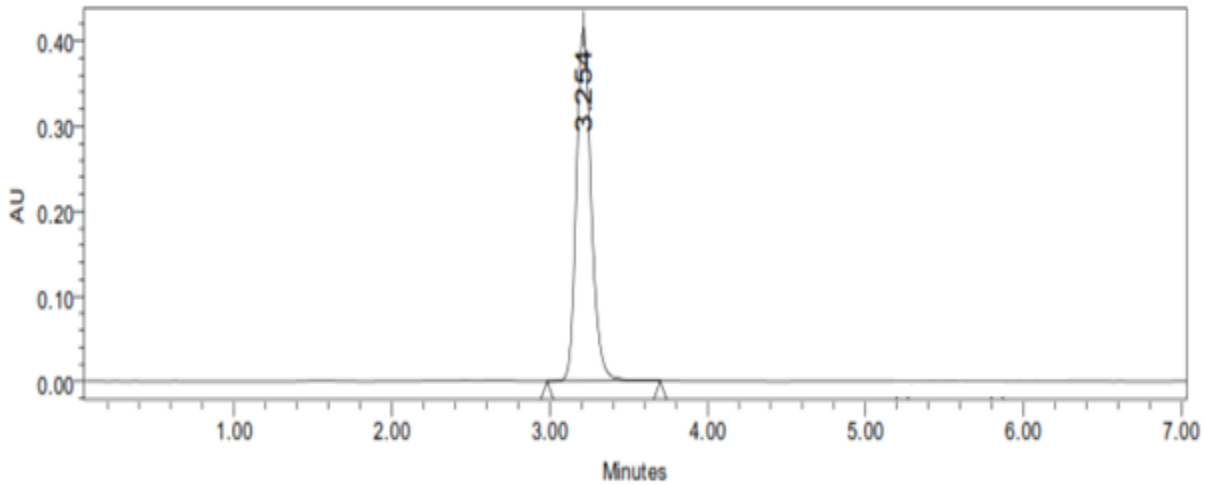


Fig 3: Chromatogram of Anastrozole in Optimized Chromatographic Condition

Method Validation

System Suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations[22] and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-4.

Table 4: System suitability results for Anastrozole (Flow rate)

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Anastrozole = 0.12
2	Theoretical plate	N > 2000	Anastrozole = 7258
3	Tailing Factor	(Tf) < 2	Anastrozole = 1.25

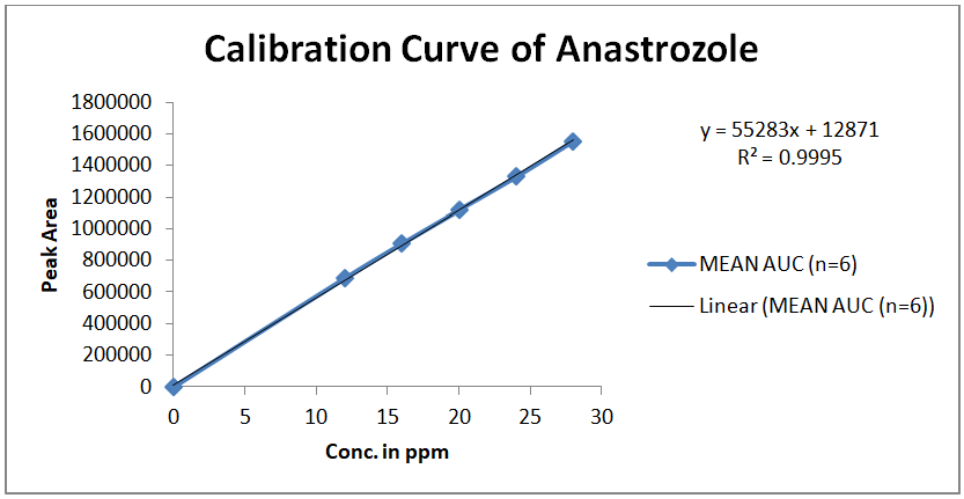
Linearity

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentrations ranging from 0-28µg/ml for Anastrozole. The prepared solutions were filtered

through Whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[23] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs

The resultant areas of linearity peaks are plotted against Concentration.



Linearity range[24] was found to be 0-28µg/ml for Anastrozole. The correlation coefficient was found to be 0.9995, the slope was found to be 55283 and intercept was found to be 12871 for Anastrozole.

Fig 4: Standard Curve for Anastrozole

Table 5: Linearity Readings for Anastrozole

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Accuracy

Recovery Study

To determine the accuracy of the proposed method, recovery studies[25] were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Anastrozole were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 55283x + 12871. The results were shown in table-6.

Table 6: Accuracy results of Anastrozole

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1: 80 %	8	8.064107	458679	99.867	Mean= 100.4113%
S_2: 80 %	8	7.843532	446485	100.637	S.D. = 0.473694346

S_3: 80 %	8	8.19449	465887	100.73	% R.S.D.= 0.471753
S_4: 100 %	10	9.892661	559767	99.41	Mean= 100.6646667%
S_5: 100 %	10	9.978655	564521	100.868	S.D. = 1.166369295
S_6: 100 %	10	10.19623	576549	101.716	R.S.D.= 1.158667
S_7: 120 %	12	11.85907	668476	99.878	Mean= 100.4637%
S_8: 120 %	12	12.16785	685546	100.69	S.D. = 0.51154309
S_9: 120 %	12	12.18644	686574	100.823	% R.S.D. = 0.509181

The mean recoveries were found to be 100.411, 100.664, and 100.463% for Anastrozole. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision

The precision of each method was ascertained separately from the peak areas obtained by the actual determination of six replicates of a fixed amount of drug Anastrozole. The percent relative standard deviations were calculated for Anastrozole are presented in Table-7.

Table 7: Repeatability Results of Anastrozole

HPLC Injection Replicates	AUC for Anastrozole
Replicate – 1	285479
Replicate – 2	284571
Replicate – 3	286954
Replicate – 4	283261
Replicate – 5	285964
Replicate – 6	284259
Average	**285081.3**
Standard Deviation	**1318.666**
% RSD	**0.462558**

The repeatability study which was conducted on the solution having the concentration of about 10µg/ml for Anastrozole (n =6) showed a RSD of 0.462558% for Anastrozole. It was concluded that the analytical technique showed good repeatability[26].

Intermediate Precision / Ruggedness

Table 8: Ruggedness Results for Anastrozole

Conc. of Anastrozole (API) (µg/ml)	Observed Conc. of Anastrozole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.21	0.76	8.23	0.46
10	10.37	0.33	10.36	0.57
12	12.56	0.23	12.56	0.75

Intraday and interday studies show that the mean RSD (%) was found to be within the acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within a day and between days[27]. Hence, the method at the selected wavelength was found to be precise.

Robustness

Robustness is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness[28] of a method is done by varying the chromatographic

parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength, etc., and determining any possible effect on the results obtained by that method.

Table9: Result of Method Robustness Test for Anastrozole

Change in parameter	% RSD
Flow (0.8 ml/min)	0.554
Flow (1.2 ml/min)	0.867
More Organic	0.886
Less Organic	0.817
The wavelength of Detection (257 nm)	0.813
The wavelength of detection (253 nm)	0.794

Influence of small changes in chromatographic conditions[29] such as a change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-9, % RSD < 2%) the developed RP-HPLC method for the analysis[30] of Anastrozole (API).

LOD

The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.[31]

L.O.D. = 3.3 (SD/S).
Where, SD = Standard deviation of the response
S = Slope of the calibration curve

Observation: The LOD was found to be 5.004µg/ml for Anastrozole.

LOQ

The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ.[32]

L.O.Q. = 10 (SD/S)
Where, SD = Standard deviation of the response
S = Slope of the calibration curve
Observation: The LOQ was found to be 15.164µg/ml for Anastrozole.

Assay

Assay refers to chromatography-based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity.[33]

$$\text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area
AS = Standard preparation Peak Area

WS = Working standard weight taken in mg
WT = Sample weight taken in mg
DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

The assay was performed as explained in the previous chapter. The results which are obtained are following:

Table 10: Recovery Data for estimation Anastrozole in Armotraz

Brand name of Anastrozole	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Armotraz (Cipla Pharmaceuticals Pvt Ltd)	1mg	0.893 (± 0.368)	99.698 (±0.476)

The amount of drug in Armotraz Tablet was found to be 0.893 (±0.368) mg/tab for Anastrozole & % Purity was 99.698 (±0.476) %.

CONCLUSION

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Anastrozole API. Further, the proposed RP-HPLC method has excellent sensitivity, precision, and reproducibility. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Anastrozole in different formulations.

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