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Original Article

VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF LETROZOLE IN BULK AND TABLET DOSAGE FORM

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Abstract
A new, simple and selective reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated for determination of Letrozole in bulk and tablet dosage form. Chromatographic analysis was performed on a Symmetry C18, 250 mm x 4.6 mm and 5µm Column with ambient temperature with a mobile phase containing mixture of Acetonitrile and water in the ratio of 60:40v/v, maintaining at a flow rate of 1.0 mL min-1. UV wavelength detection was performed at 238 nm. The proposed method was validated for accuracy, precision, specificity, linearity and sensitivity. The retention time of Letrozole was found to be 5.776minutes. Calibration plot was linear over the concentration ranges from 0–35 µg mL-1 for Letrozole. The Limit of detection and Limit of quantification of Letrozole was found to be 0.04 and 0.12 µg mL-1 respectively. The accuracy of the proposed method was determined by recovery studies and found to be 98.0% to 102%. Some major impurities and degradation products did not disturb the detection of Letrozole and the assay can thus be considered stability-indicating. The validated method was successfully used for quantitative analysis of marketed pharmaceutical preparations.

Key Words: Letrozole, Method Development, Validation, accuracy, precision and ICH Guidelines.

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1. INTRODUCTION

Letrozole (INN, trade name Femara®) is an oral non-steroidal aromatase[1,2,3] inhibitor that has been introduced for the adjuvant treatment of hormonally-responsive breast cancer. Estrogens are produced by the conversion of androgens through the activity of the aromatase enzyme. Letrozole blocks production of estrogens in this way by competitive, reversible binding to the heme of its cytochrome P450 unit. The action is specific, and letrozole does not reduce production of mineralo- or corticosteroids. In contrast, the antiestrogenic action[4] of tamoxifen, the major medical therapy prior to the arrival of aromatase inhibitors, is due to its interfering with the estrogen receptor, rather than inhibiting estrogen[5] production. Letrozole is approved by the United States Food and Drug Administration[6,7] (FDA) for the treatment of local or metastatic breast cancer that is hormone receptor positive or has an unknown receptor status in postmenopausal women. Side effects include signs and symptoms of hypoestrogenism. There is concern

that long term use may lead to osteoporosis, which is why prescriptions of Letrozole are often accompanied by prescriptions of osteoporosis-fighting medication such as Fosamax. Letrozole has shown to reduce estrogen levels by 98 percent while raising testosterone levels. The anti-estrogen action of letrozole is preferred by athletes and bodybuilders for use during a steroid cycle[8] to reduce bloating due to excess water retention and prevent the formation of gynecomastia related breast tissue that is a side effect of some anabolic steroids[9]. Usage above 2.5 mg/day is known to potentially temporarily kill sex drive. Above 5mg/day for extended periods may cause kidney problems. Letrozole has also been shown to delay the fusing of the growth plates in adolescents. This may boost the effectiveness of growth hormone, and thus Letrozole is used to treat adolescents and children with short stature. The IUPAC[10] Name of Letrozole is 4-[(4-cyanophenyl) (1H-1,2,4-triazol-1yl) methyl] benzonitrile. The molecular formula[11] is $C_{17}H_{11}N_5$.The Chemical[12] Structure of Letrozole is Figure No-1.

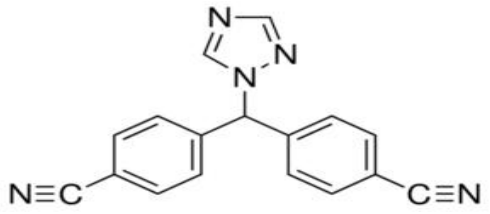

Fig-1: Chemical Structure of Letrozole

2. METHODOLOGY:

2.1 INSTRUMENTS USED

Table-1: Instruments used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC WATERS** with Empower2 Software with Isocratic with UV-Visible Detector.
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Vacuum Filtration kit (Labindia)
6.	Thermal Oven
7.	Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column
8.	P^H Analyzer **(ELICO)**

2.2 CHEMICALS / REAGENTS USED

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.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Potassium dihydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Dipotassium dihydrogen Peroxide	96%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Ortho phosphoric acid	96%	L.R.	Sd fine-Chem ltd; Mumbai

3. INSTRUMENTATION

The HPLC system[13] employed was HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.

3.1 Chromatographic Conditions

The determination was carried out on Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column with detection wavelength of 238 nm. The volume of injection is 20.0 µl and maintaining a flow rate[14] at 1ml/min. The

run time of the Letrozole is about 10.0 minutes and the column temperature is ambient.

3.2 Preparation of mobile phase

A mixture of above Acetonitrile 600ml (60%) and 400 ml of HPLC grade water (40%) were mixed and degassed[15] in ultrasonic water bath for 15 minutes and filtered through 0.45 µm filter under vacuum filtration.

3.3 Preparation of Diluent

Mobile phase[16] used as diluent.

3.4 Preparation of Standard Solution

Accurately weighed around 25mg of Letrozole working standard, taken into a 25 ml volumetric flask, then dissolved and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml.

Further dilutions[17] have been made to get the final concentration of 20µg/ml.

3.5 Preparation of Test Solution

Diluted quantitatively an accurately measured volume of label claim[18] solution with diluents to obtain a solution containing about a linear range.

4. METHOD VALIDATION[19]

1. Accuracy: *Recovery study:*

To determine the accuracy[20] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of LETROZOLE were taken and added to the pre-analyzed formulation of concentration 20µg/ml. From that percentage recovery[21] values were calculated. The results were shown in Table-3.

Table-3: Accuracy Readings

Sample ID	Concentration («g/ml)	Peak	% Recover	Statistical Analysis
	Amount Injected	**Amount Recovered**	**Area**	**y of Pure drug**	
S_1 : 80 %	16	16.263	96254	101.643	Mean=101.3827%
S_2 : 80 %	16	16.162	95682	101.012	S.D. = 0.329652
S_3 : 80 %	16	16.239	96121	101.493	%R.S.D.= 0.325156
S_4 : 100 %	20	19.834	116493	99.170	Mean= 99.9066%
S_5 : 100 %	20	20.082	117895	100.410	S.D. = 0.65209
S_6 : 100 %	20	20.028	117589	100.140	%R.S.D.= 0.652708
S_7 : 120 %	24	23.902	139542	99.591	Mean= 99.837%
S_8 : 120 %	24	23.961	139878	99.837	S.D. = 0.246
S_9 : 120 %	24	24.020	140213	100.083	%R.S.D.= 0.246402

2. Precision

The precision[22] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Letrozole (API). The percent relative standard deviation[23] was calculated for Letrozole are presented in the Table-4.

Table-4: Repeatability Readings

HPLC Injection Replicates of Letrozole	Peak Area
Replicate – 1	96993
Replicate – 2	96633
Replicate – 3	97515
Replicate – 4	98822

Replicate – 5	98731
Replicate – 6	97029
Average	**97620.5**
Standard Deviation	**938.8**
% RSD	**0.961683**

2.2 Intermediate Precision[24]:

2.2.1 Intra-assay & inter-assay:

The intra & inter day[25, 26] variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD[31] (% RSD < 2%) within a day & day to day variations for Letrozole revealed that the proposed method is precise.

Table-5: Results of intra-assay & inter-assay

Conc. of Letrozole (API) (µg/ml)	Observed Conc. Of Letrozole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
16	16.08	16	16.08	16
20	20.04	20	20.04	20
24	23.97	24	23.97	24

3. Linearity and Range

The calibration curve[27] showed good linearity[28] in the range of 0-35 µg/ml, for Letrozole (API) with correlation coefficient (r2) of 0.998 (Fig-2). A typical calibration curve has the regression equation of y = 5667.x + 4089 for Letrozole.

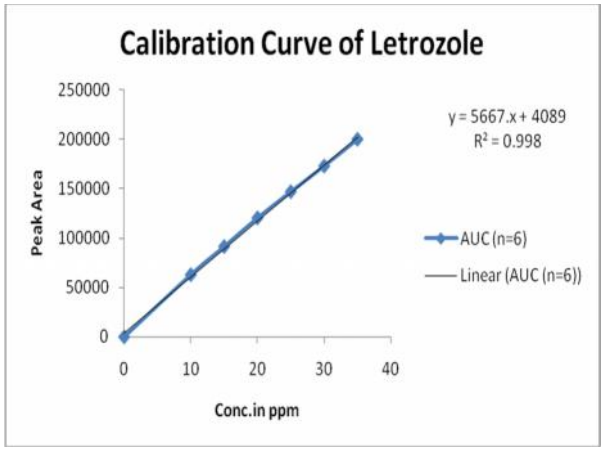

Fig-2: Calibration Curve for Letrozole

Table-6: Linearity Results for Letrozole

CONC.	AUC (n=6)
0	0
10	62895
15	91302
20	120283
25	146794
30	172745
35	199734

4. Method Robustness:

Influence of small changes in chromatographic conditions[29] such as change in flow rate (±0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) and Acetonitrile content in mobile phase (±2%) studied to determine the robustness[30] of the method are also in favour of (Table-7, % RSD < 2%) the developed RP-HPLC method for the analysis of Letrozole (API).

Table-7: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.04
Flow (0.9 ml/min)	0.09
Temperature (27^0C)	0.05
Temperature (23^0C)	0.17
Wavelength of Detection (240 nm)	0.08
Wavelength of detection (236 nm)	0.09

5. Limit of detection[31] (LOD) and Limit of quantification (LOQ):

The detection limit (LOD) and quantization limit (LOQ)[32] may be expressed as:

L.O.D. = 3.3 (SD/S).

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & Discussion

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.04 & 0.12 µg/ml respectively.

6. Assay[33] of Letrozole in Tablet dosage form:

Estimation of Letrozole in Tablet Dosage Form

Each Tablet contains: 2.5 mg

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent[34] to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system[35] and the observations were recorded.

A duplicate injection[36] of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-8.

% Assay = AT/AS × WS/DS × DT/WT × P/100 × AW/LC × 100

Where,

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Assay was performed as described in previous chapter. Results obtained are tabulated below:

Table-8: Assay of LETROZOLE Tablets

Brand name of tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Letronat (Natco Pharma Limited Company)	2.5	2.45 (±0.06)	99.6 (±0.48)

Result & Discussion: The assay of Letronat Tab tablets containing Letrozole was found to be 99.6 %.

8. FORCED DEGRADATION STUDIES

Following protocol was strictly adhered to for stability studies[37] of Letrozole Active Pharmaceutical Ingredient (API).The APIs of Letrozole was subjected to different stability conditions[38] in various ways to observe the rate and extent of degradation occur in the course of storage after administration to body.

This is one type of accelerated stability studies[41] that helps us determining the fate of the drug that is likely to happen after a long time storage, within a very short time as compare to the real time or long term stability testing.

The various degradation[39] pathways studied are acid hydrolysis, basic hydrolysis, and thermal degradation, photolytic and oxidative degradation.

Results of forced degradation studies:

The results of the stress studies indicated the specificity of the method that has been developed. Letrozole was stable in photolytic & thermal stress conditions. The results of forced degradation studies are given in the following Table-9.

Table-9: Results of forced degradation studies of Letrozole.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.89	12.11	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	85.35	14.65	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	90.63	9.37	100.00
UV (254nm)	24Hrs.	91.36	8.64	100.00
3% Hydrogen peroxide	24Hrs.	86.25	13.75	100.00

9. RESULTS & DISCUSSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Letrozole, different chromatographic conditions were applied & the results observed are presented in previous chapters.

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here Symmetry C18, 250 mm x 4.6 mm and 5µm particle size column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl).

The drug was found to be Letrozole is freely soluble in dichloromethane, methanol and Acetonitrile; slightly soluble in ethanol, insoluble in ether and chloroform and practically insoluble in water. Letrozole is soluble in organic solvents such as DMSO and dimethyl formamide. Using these solvents with appropriate composition newer methods can be developed and validated.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Letrozole it is evident that most of the HPLC work can be accomplished in the wavelength range of 200-250 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay, stability and purity which can help in the analysis of Letrozole in different formulations.

10. CONCLUSION

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Letrozole API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. This proposed method can be used for the further analysis of Letrozole in the future.

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