RP- HPLC Method Development, Validation and Forced Degradation Studies of Letrozole

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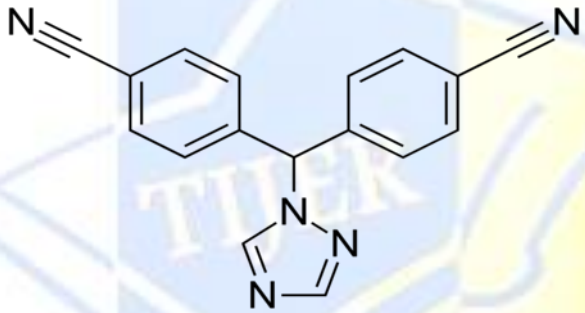
Abstract - The current research paper describes a highly specific, reproducible, and efficient stability- indicating HPLC method for estimation of Letrozole in bulk and Marketed Pharmaceutical Dosage form. The Chromatographic separation was carried out on a Waters C18, 5µm, 25cmx4.6mm i.d. with UV detection at 230 nm. The mobile phase contained Acetonitrile and Buffer (75:25% v/v at pH-2.9). The mobile phase was run isocratically. The flow rate of the mobile phase was maintained at 1.0 ml/min. The linearity of the calibration curve was obtained in the concentration range of 30 to 70 µg ml-1 and coefficient of determination (R2) was found to be 0.9985. The % RSD value for intraday and interday precision was below 1 which indicated that the method was precise. Limit of detection and limit of quantification were 0.001 and 0.003µg/ml respectively. Forced degradation studies were performed under different conditions. The drug was degraded in oxidative and thermal conditions. In the present research, a stability- indicating HPLC method has been developed for Letrozole. The developed method was validated as per ICHQ2R1 guidelines and was successfully applied for quantitative analysis of Letrozole in bulk and Marketed Pharmaceutical Dosage form.

Index Terms - Letrozole, RP-HPLC, Method Development, Validation, Accuracy, Precision.

I.Introduction

The Letrozole (LZ), denoted chemically as 4, 4'-[(1H-1,2,4-triazol-1-yl) methylene] bis-benzonitrile, is an oral non-steroidal aromatase inhibitor that has been introduced for the adjuvant treatment of hormonally-responsive breast cancer.[1].

Fig.1 Structure of Letrozole



II.Literature survey

Aswathi R. Hegde, et al. (2021) A simple bioanalytical liquid chromatographic method wasdeveloped and validated to quantify Letrozole (LTZ) in rat plasma. Protein precipitation using acidified chilled acetonitrile (containing 0.1% orthophosphoric acid) was used to extract LTZ from the plasma. Chromatographic separation was carried out on Kinetex C18 reverse phase (RP) column (250 mm × 4.6 mm i.d., 5 µm) using a mixture of 20 mM acetate buffer (pH 5.5) and acetonitrile (60:40 %v/v) eluting at 1.0 mL/min flow rate with the method responses measured at 240 nm. The optimized method was selective and established good linearity with recovery ranging between 91.16 and 99.44%. The validation experiments revealed that the method showed acceptable precision (2.61–7.48%) and accuracy (97.44– 102.70%) and was found to be stable. The sensitivity of the method was demonstrated by the lowest concentration (LLOQ) detected at 75 ng/mL. Using the developed method, single- dose oral pharmacokinetics in Sprague-Dawley rats was carried out to successfully confirm the applicability of the method for the quantification of LTZ in biological matrix[2]

III.Aim, Objectives and Plan of Work

AIM: The existing physicochemical methods are inadequate to meet the requirements; hence it is proposed to improve the existing methods and to develop new methods for the assay & stability studies of Letrozole in pharmaceutical dosage forms adapting different available analytical techniques like UV spectrophotometry and HPLC.

OBJECTIVES: According to the literature survey it was found that few analytical methods such as (HPLC analysis and LC-MS) were reported for the estimation of Me Letrozole. The objective of the proposed method is to develop simple and accurate methods for the determination of Letrozole by RP-HPLC method in pharmaceutical dosage forms & it's stability indicative studies.

The plan of the proposed work: -

a. To undertake solubility and analytical studies of Letrozole and to develop initial U.V. and chromatographic conditions.

b. Setting up of initial UV and chromatographic conditions for the method development in pure and pharmaceutical dosage forms.

c. Optimization of initial chromatographic and spectrophotometric conditions.

d. Analytical method validation of the developed RP- HPLC method.

e. Quantitative determination of Letrozole in pharmaceutical dosage form using the method developed and validated.

IV.Experimental Work

Method Development of Letrozole

Standard & Sample Preparation for UV-Spectrophotometer Analysis:25 mg of Letrozole standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

The Standard & Sample Stock Solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Letrozole, so that the same wave number can be utilized in HPLC UV detector for estimating the Letrozole. While scanning the Letrozole solution we observed the maxima at 230 nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

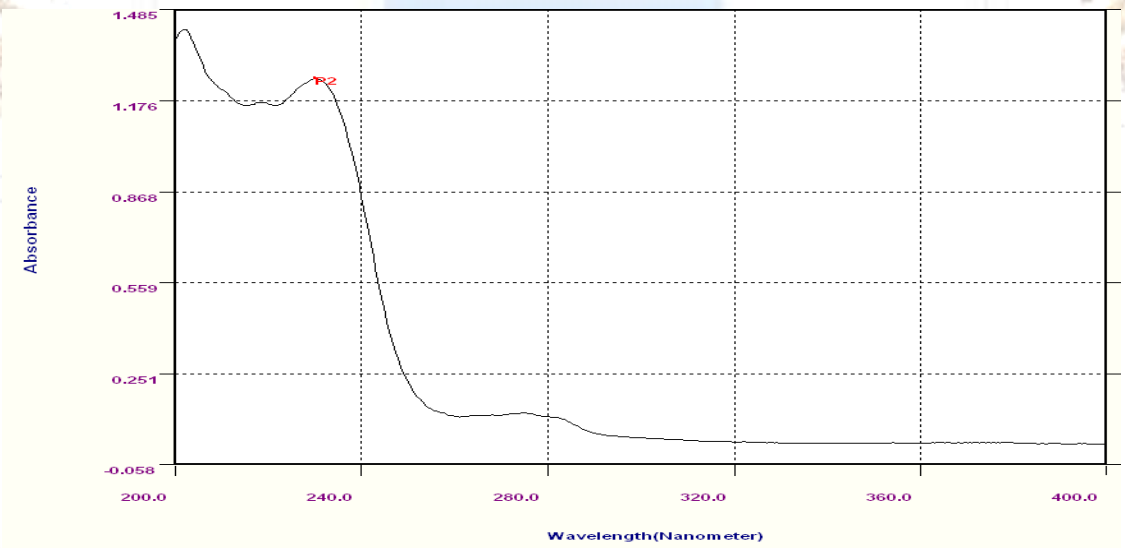


Fig 2: UV-Spectrum for Letrozole (230nm)

Mobile Phase Preparation: The mobile phase used in this analysis consists of a mixture of Buffer (0.01 M potassium dihydrogen phosphate & pH adjusted to 2.9 with ortho phosphoric acid) and Acetonitrile in a ratio of 25:75% v/v.

Preparation of Standard Solution: Working concentration should be around 10µg/ml.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.

Preparation of Test solution: Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range.

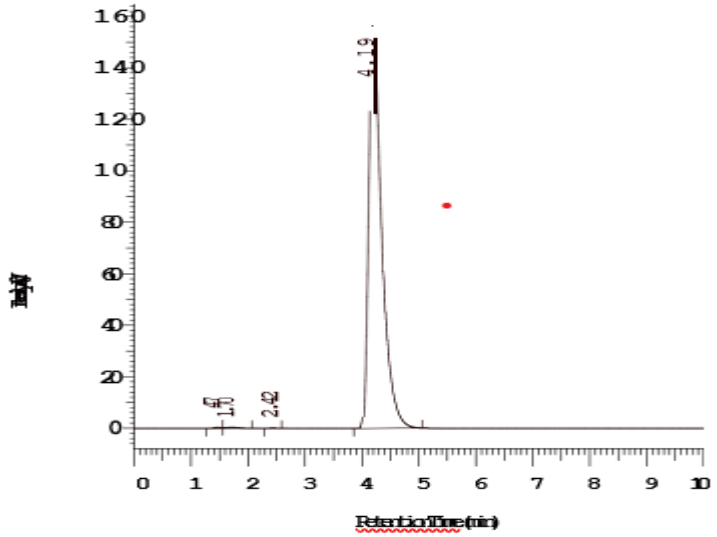


Fig 3: Optimized Chromatogram for Letrozole (Rt-4.19)

Table 1: Results of Optimized Chromatogram for Letrozole

S.NO.	DRUG	Rt	PEAK AREA
1	Letrozole	4.19	2654388

Method Validation:

Accuracy: Recovery Study: To determine the accuracy 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 10µg/ml. From that percentage recovery values were calculated. The results were shown in table-8.

Table 2.: Accuracy Readings

Sample ID	Concentration (□g/ml)		%Recovery of Pure drug	Statistical Analysis
	Pure drug	Formulation		
S1 : 80 %	8	10	97.80195	Mean= 97.77365%
S2 : 80 %	8	10	98.40443	S.D. = 1.282992
S3 : 80 %	8	10	98.91457	% R.S.D.= 1.620294
S4 : 100 %	10	10	106.5315	Mean= 104.7917%
S5 : 100 %	10	10	105.3147	S.D. = 0.699603
S6 : 100 %	10	10	105.5288	% R.S.D.= 0.67404
S7 : 120 %	12	10	104.8831	Mean= 104.95%
S8 : 120 %	12	10	104.3077	S.D. = 0.866975
S9 : 120 %	12	10	104.6592	% R.S.D.= 0.785551

Precision:

Repeatability: The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of five replicates of a fixed amount of drug. Letrozole (API) the percent relative standard deviations were calculated for Letrozole is presented in the table.

Table 3: Results of Precision (Repeatability) for Letrozole

HPLC Injection Replicates of Letrozole	**Retention Time**	**Peak Area**
Replicate – 1	4.19	2685054
Replicate – 2	4.19	2654388
Replicate – 4	4.19	2593386
Replicate – 5	4.22	2680946
Replicate -6	4.21	2614531
Average	**4.191667**	**2648015**
Standard Deviation	**0.024014**	**36717.82**
% RSD	**0.572896**	**1.386617**

Intra-Assay & Inter-Assay: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Letrozole revealed that the proposed method is precise.

Table 4: 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**
8	7.864	0.567	8.074	0.472
10	10.056	0.859	9.689	0.852

Linearity & Range: The calibration curve showed good linearity in the range of 30-70µg/ml, for Letrozole (API) with correlation coefficient (r^2) of 0.998 (Fig.). A typical calibration curve has the regression equation of y = 50403x + 299.7 for Letrozole.

To evaluate the linearity, serial dilution of analyte was prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 30, 40, 50, 60 and 70µg/ml. The prepared solutions were filtered through Whatmann 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 was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis). The results which are given in Table below were within acceptable limits.

Preparations:

30µg/ml: 0.3ml of Letrozole stock solution in 10ml of volumetric flask diluted up to the mark with mobile phase.

40µg/ml: 0.4ml of Letrozole stock solution in 10ml of volumetric flask diluted up to the mark with mobile phase.

50µg/ml: 0.5ml of Letrozole stock solution in 10ml of volumetric flask diluted up to the mark with mobile phase.

60µg/ml: 0.6ml of Letrozole stock solution in 10ml of volumetric flask diluted up to the mark with mobile phase.

70µg/ml: 0.7ml of Letrozole stock solution in 10ml of volumetric flask diluted up to the mark with mobile phase.

Acceptance Criteria: correlation coefficient should not be less than 0.99.

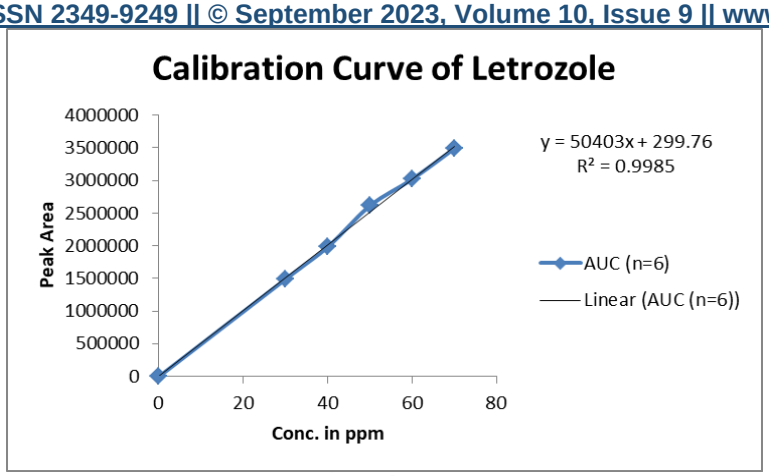


Fig 4 : Calibration Curve of Letrozole (API)

Table 5 : Linearity Readings of Letrozole

CONC.	AUC (n=6)
0	0
30	1490831
40	1984251
50	2614531
60	3021212

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate (0.1ml/min), Temperature (± 2^0C), Wavelength of detection (± 2nm) & acetonitrile content in mobile phase (± 2%) studied to determine the robustness of the method are also in favor of (Table- % RSD < 2%) the developed RP-HPLC method for the analysis of Letrozole (API).

Table 6 : Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.06
Flow (0.9 ml/min)	0.04
Temperature (27^0C)	0.08
Temperature (23^0C)	0.11
Wavelength of Detection (231 nm)	0.03
Wavelength of detection (229 nm)	0.02

LOD & LOQ: The LOD and LOQ were calculated by the use of the equations LOD = 3.3 × σ / S and LOQ= 10 × σ / S where σ is the standard deviation of intercept of Calibration plot and S is the average of the slope of the corresponding Calibration plot. The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.001 & 0.003 µg/ml respectively.

Table 7: Parameters RP-HPLC

Calibration range (µg / ml)	30-70µg/ml
Detection Wavelength(nm)	230nm
Mobile phase (PHOSPHATE BUFFER: Acetonitrile)	25:75% v/v, pH-2.9
Regression equation (Y*)	y = 50403x +299.7 for Letrozole
RT	4.19± 0.057 min for Letrozole
Slope (m)	50403 for Letrozole
Intercept (c)	299.76 for Letrozole
Correlation Coefficient (r2)	R^2 = 0.9985 for Letrozole
RECOVERY	Letrozole 95-103%
System Suitability	Tailing factor:0.98 Theoretical plate: 6341 Resolution: 3.5

*Y = bx + a where x is the concentration Letrozole in µg / ml and Y is the absorbance at the respective λmax.

Forced Degradation Studies: Following protocol was strictly adhered to for forced degradation of Letrozole Active Pharmaceutical Ingredient (API). The API (Letrozole) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. This is one type of accelerated stability studies 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 pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

Acid Hydrolysis: An accurately weighed 25 mg. of pure drug was transferred to a clean & dry 25 ml volumetric flask. To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from that 0.1 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

Table 8: Peak Results of Acid Degradation

Sr no.	**Name**	**RT**	**Area**
1	Letrozole	3.71	709457

Basic Hydrolysis: An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 10 ml volumetric flask. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. From that 0.1 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of NaOH (after all optimized conditions)

Table 9 : Peak Results of Basic Degradation

Sr no.	**Name**	**RT**	**Area**
1	Letrozole	2.13	1813807

Thermal Degradation: An accurately weighed 1 mg. of pure drug was transferred to a clean & dry 100 ml volumetric flask, make up to the mark with mobile phase & was maintained at 50 ^{0}C. For 24 hrs. Then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Table 10 : Peak Results of Thermal Degradation

Sr no.	Name	RT	Area
1	Letrozole	3.63	2316440

Oxidation with (3%) H_2O_2: Accurately weighed 1 mg. of pure drug was taken in a clean & dry 100 ml. volumetric flask. 30 ml. of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100 ppm solution. The above sample was injected into the HPLC system.

Table 11: Peak Results of Oxidative Degradation

Sr no.	Name	RT	Area
1	Letrozole	3.67	2167972

Results of Degradation Studies: The results of the stress studies indicated the **Specificity** of the method that has been developed. Letrozole was stable in oxidation & temperature stress conditions. The result of forced degradation studies are given in the following table.

Table 12 : Results of Forced Degradation Studies of Letrozole API.

Stress Condition	Time	Assay of Active Substance	Assay of Degraded Products
Acid Hydrolysis (0.1 M HCl)	24Hrs.	26.79127	78.21
Basic Hydrolysis (0.I M NaOH)	24Hrs.	68.76934	45.53
Thermal Degradation (50 ^{0}C)	24Hrs.	81.8397	23.163

V. Conclusions:

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Letrozole different chromatographic conditions were applied. 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 waters C18, 5µm, 250 x4.6 mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluents for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, water, 0.1N NaOH, 0.1NHCl).The drug was found to be highly soluble in acetonitrile & dichloromethane and methanol. Drug was freely soluble in water. 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 287 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 and stability studies which can help in the analysis of Letrozole in different formulations.

VI.References

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