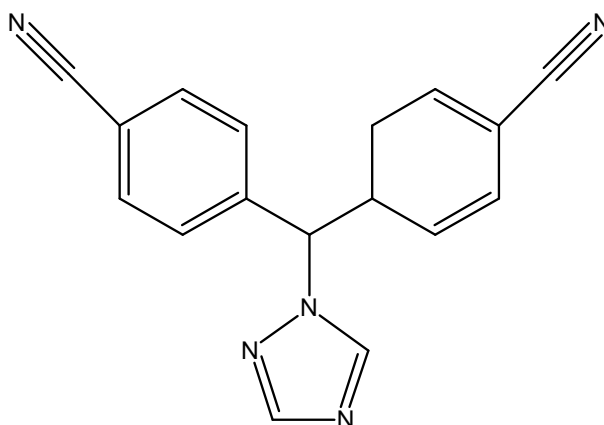


1. INTRODUCTION

Letrozole is used to prevent the occurrence of breast cancer specifically by reducing the amount of estrogen that the body makes. It is done by Letrozole that binds competitively and reversibly to the heme of the cytochrome P450 unit of the aromatase enzyme that actually produces estrogen. Thus Letrozole is an oral, third generation non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer. Letrozole has also proven to promote spermatogenesis in male patients suffering from non obstructive azoospermia. But in combination with misoprostol or mifepristone it is useful in pretreatment for termination of pregnancy. Hence it is generally not used during pregnancy in women. Letrozole can also be used as a treatment for gynecomastia and endometriosis. It is used to treat infertile women who have ovulation problems. They help the pituitary gland to improve the stimulation of developing follicles (eggs) in the ovaries. Letrozole has a half-life of 4 days and to reach a steady level in the blood it must be taken for 60 days. Letrozole is more sensitive towards alkaline conditions and very much resistant towards acidic, oxidative and photolytic degradations. The structure of Letrozole is given in Figure 1.

Figure 1: STRUCTURE OF LETROZOLE



Physical properties of Letrozole:

Molecular formula	:	C ₁₇ H ₁₁ N ₅
Molecular mass	:	285.3
IUPAC name	:	4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl) methyl]benzonitrile
Appearance	:	white to light yellow crystal
Nature	:	Free base
Pk _a	:	1.63
Solubility	:	Practically insoluble in water, freely soluble in methanol, dichloromethane, DMSO and slightly soluble in ethanol.

Available HPLC methods:

There are various analytical methods reported for the determination of Letrozole in pure drug, pharmaceutical dosage forms and in biological samples using HPLC as follows:

Mondal N et al¹ proposed a reversed phase high performance liquid chromatographic method for estimation of Letrozole in raw material, pharmaceutical formulations like tablets and nanoparticles and in release medium. The chromatographic system consisted of a FinePak C, column, an isocratic mobile phase composed of deionized water, acetonitrile and methanol (50:30:20 v/v/v) and UV detection at 240 nm. Letrozole was eluted at 9.8 min with no interfering peak of excipients used for the preparation of dosage forms.

TK Laha et al² proposed a rapid and sensitive reverse phase high performance liquid chromatographic method for the qualitative and quantitative assay of letrozole in pharmaceutical dosage forms. Letrozole was chromatographed on a reverse phase C18 column with a mobile phase consisting of acetonitrile and phosphate buffer (pH 7.8) in the ratio of 70:30 v/v. The mobile phase was pumped

at a flow rate of 1 ml/min. Acenaphthene was used as an internal standard and the eluents were monitored at 232 nm. The retention time of the drug was 3.385 min.

Afshin Zarghi et al³ proposed a simple, rapid and sensitive HPLC method for the analysis of letrozole in human plasma. The separation was achieved on a monolithic silica column using acetonitrile–phosphate buffer. A fluorescence detector was used for the quantification with excitation and emission wavelengths at 230 and 295 nm.

M. Mathrusri Annapurna et al⁴ proposed a stability-indicating high-performance liquid chromatographic method for the determination of Letrozole in tablet dosage forms. Reversed-phase chromatography was performed on Shimadzu Model LC-Class-Vp with Lichrocart / Lichrosphere 100 C-18 (250 mm × 4.6 mm, 5 µm particle size) column with methanol : tetra butyl ammonium hydrogen sulphate (80:20 V/V) as mobile phase at a flow rate of 1 ml/min. with UV detection at 240 nm.

V Sekar et al⁵ developed a rapid high-performance liquid chromatography method for bioanalysis of letrozole in human plasma. Letrozole was found with symmetrical peak shapes on an analytical column Phenomenex Luna C₁₈ column using 75% 0.02M Phosphate buffer at pH 5.5 and 25% acetonitrile as the mobile phase. The retention times of Letrozole and fluconazole the internal standard were 4.29 and 7.47 min respectively.

Precht JC et al⁶ developed a rapid, sensitive, and specific method for the simultaneous quantification of letrozole and its metabolites 4,4'-(hydroxymethylene)dibenzonitrile (carbinol) and bis(4-cyanophenyl)methyl hexopyranosiduronic acid (carbinol-gluc) by UHPLC-ESI-MS/MS using in-house synthesized, stable isotope-labeled internal standards. Following solid-phase extraction in BondElut C18 96-well plates, the analytes were separated on a ZORBAX Eclipse XDB - C18 column (1.8 µm, 4.6×50 mm) with a gradient of acetonitrile in 0.1% acetic acid in water and detected on a triple quadrupole mass

spectrometer with electrospray ionization in the multiple reaction monitoring mode.

F. Marfil et al⁷ performed an automated liquid-solid extraction of compounds from plasma and urine was performed on disposable 100-mg C₈ columns using the ASPEC system. The separation was achieved on an ODS Hypersil C₁₈ column using acetonitrile-phosphate buffer, pH 7, as the mobile phase at a flow-rate of 1.5 ml/min. A fluorescence detector was used for the quantification. The excitation and emission wavelengths were 230 and 295 nm, respectively.

There are also some methods like Spectrophotometry⁸⁻¹⁰, the microarray approach¹¹, capillary gas chromatographic method with flame ionization detector¹², gas chromatography–mass spectrometry¹³ and TLC/HPLC¹⁴ methods.

There are a number of brands available with Letrozole as the chief and active constituent in the formulations given in Table 1.1.

Table 1.1: List of brand names of Letrozole

S.No.	Brand name	Formulation	Available strength	Manufacturer
1	FEMARA	Tablet	2.5 mg	NOVARTIS
2	FERTOLET	Tablet	2.5 mg	CIPLA
3	HERHOPE	Tablet	2.5 mg	TORRENT
4	LETOCOR	Tablet	2.5 mg	CHANDRA BHAGAT
5	LETOV	Tablet	2.5 mg	ZYDUS CADILA
6	LETOVAL	Tablet	2.5 mg	SUN PHARMA
7	LETROZ	Tablet	2.5 mg	SUN PHARMA
8	LETS	Tablet	2.5 mg	SAMARATH PHARMA
9	LETSHIL	Tablet	2.5 mg	RAICHEM

10	LUTROZOLE	Tablet	2.5 mg	LUPIN
11	ONCOLET	Tablet	2.5 mg	BIOCHEM
12	STIMUFOL	Tablet	5 mg	INTAS
13	ANOLET	Tablet	5 mg	SVIZERA
14	CELOFEM	Tablet	2.5 mg	CELON
15	ELLET	Tablet	2.5 mg	DEWCARE
16	EPILIT	Tablet	2.5 mg	EPITOME
17	FEMPRO	Tablet	2.5 mg	CIPLA
18	FEOFER	Tablet	2.5 mg	ALNA BIO
19	LETORIFE	Tablet	2.5 mg	MIRACALUS
20	LETOVA	Tablet	2.5 mg	ACEKINETIC
21	LETZOL	Tablet	2.5 mg	VHB
22	LETZUCIA	Tablet	2.5 mg	UNITED BIOTECH
23	LEXTRO	Tablet	2.5 mg	LEXUS
24	LTZ	Tablet	2.5 mg	BEWELL
25	MOMAZOL	Tablet	2.5 mg	KHANDELWAL
26	ORETA	Tablet	2.5 mg	DR REDDYS
27	ROZOLY	Tablet	2.5 mg	MALODY
28	SHANTROZ	Tablet	2.5 mg	SHANTHA BIOTECH
29	TROZET	Tablet	2.5 mg	DABUR
30	UBILIT	Tablet	2.5 mg	CUBIT
31	ZOLET	Tablet	2.5 mg	UNITED BIOTECH

2. EXPERIMENTAL

2.1. Instrumentation

A Shimadzu electronic balance (AX-200) was used to weigh the drug and then for wavelength checking UV-2306 spectrophotometer was used. An isocratic Shimadzu HPLC model (VP series) instrument with Inertsil ODS C18 column (250 mm x 4.6 mm, 5 μ m) was used to develop a High Pressure Liquid Chromatographic method for the quantitative estimation. The instrument was equipped with a LC 20AT pump for solvent delivery and variable wavelength programmable SPD-10AVP detector. Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A 20 μ L Rheodyne inject port (7725i) was used for injecting the samples. Data was analyzed by using PEAK software.

2.2. Chemicals and Solvents

Pure sample of Letrozole was obtained from Alna Bio. Acetonitrile, methanol and ortho phosphoric acid (HPLC grade) was procured from E. Merck, Mumbai (India). Letrozole, as a pharmaceutical form, in the brand name of Feofer was purchased from the local market.

2.3. The Mobile phase

The mobile phase containing acetonitrile, methanol and ortho phosphoric acid (0.1%) in the ratio of 25:30:45 was used for the elution.

2.4. Standard solution of the drug

Initially a stock solution was prepared by dissolving 10 mg of the drug in the solvent, made upto 100 ml in a volumetric flask and appropriate dilutions were done using the solvent chosen. A standard solution of 50 ppm was obtained by this process for subsequent analysis.

2.5. Sample solution

The tablet forms of Letrozole (Feofer) were powdered to a fine form and then powder equivalent to 10 mg of the drug was dissolved in 5 ml of the mobile phase

taken in 10 ml volumetric flask. After dissolution the solution was filtered through Ultipor Nylon 6, 6 membrane sample filter paper and the filtrate was adjusted to the mark with the same solvent to obtain a concentration of 50 ppm.

3. METHOD DEVELOPMENT

Development of a suitable RP HPLC method involves selection of the appropriate wavelength, solvent, stationary and mobile phases. In order to establish these requirements, a systematic study on the effect of various factors involved was undertaken by varying each of them keeping all other conditions constant as follows:

3.1. Detection of wavelength

The UV absorption spectrum of the drug in its diluent was taken and the wavelength of maximum absorbance was found to be at 242 nm. Hence the detection of the drug was monitored at 242 nm.

3.2. Choice of stationary phase

A number of trials using different octadecyl columns of various types and configurations from different manufacturers were performed and finally an Inertsil ODS C-18 5 μ m column having 250 x 4.6mm internal diameter was chosen for the method development as it gave the expected separation with good peak shapes.

3.3. Selection of the Mobile phase

As selection of stationary and mobile phases depends upon the nature of the sample and properties of the molecule a number of solvents were analyzed, mixed in various proportions and tested under isocratic conditions with varied flow rates to separate the drug on the ODS C-18 column with various combinations. An ideal separation was achieved with mobile phase containing acetonitrile, methanol and ortho phosphoric acid (0.1%) in the ratio of 25:30:45 (v/v/v). This was finally selected as it gave a well defined chromatographic peak with better resolution, base line separation and low tailing factor.

3.4. Flow rate

An effective flow rate is one that is minimum with a short run time which can minimize the usage of solvents. The optimum flow rate of 1.0 ml/min was attained by varying it between 0.5–1.5 ml/min. This was ideal for the successful elution of the analyte.

3.5. Optimized chromatographic conditions

Optimization of mobile phase was performed based on chromatographic separation, peak shape and peak area obtained. The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions. Based on the above proceedings, the Chromatographic conditions thus optimized are shown in Table 1.2.

These optimized conditions were maintained for the determination of Letrozole in bulk and pharmaceutical forms. When blank solution containing only the mobile phase without the drug was injected, no peak was obtained. The chromatograms of standard, blank, tablet sample are shown in Figure 2, 3 and 4 respectively.

Figure 2: Chromatogram of standard solution

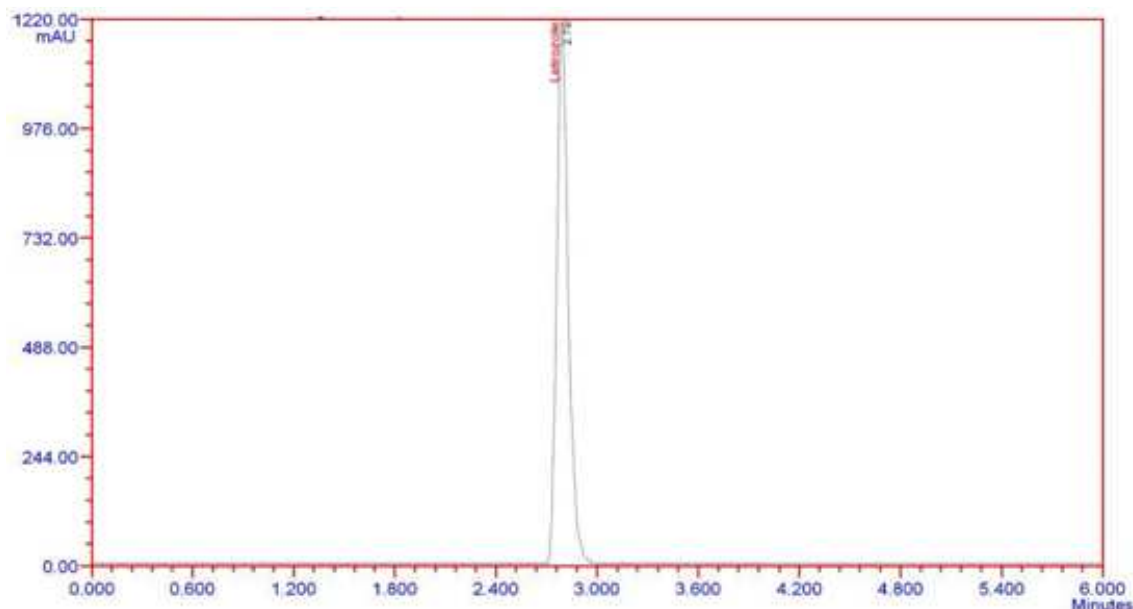


Figure 3: Chromatogram of blank (No Peak)

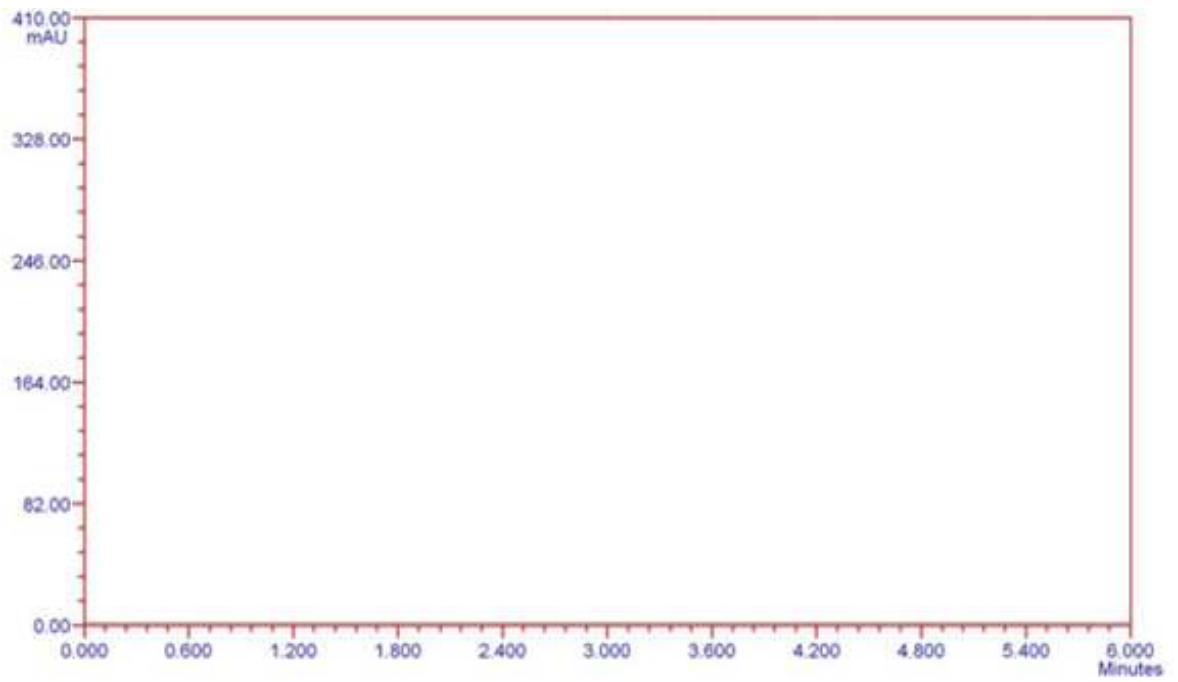


Figure 4: Chromatogram of formulation

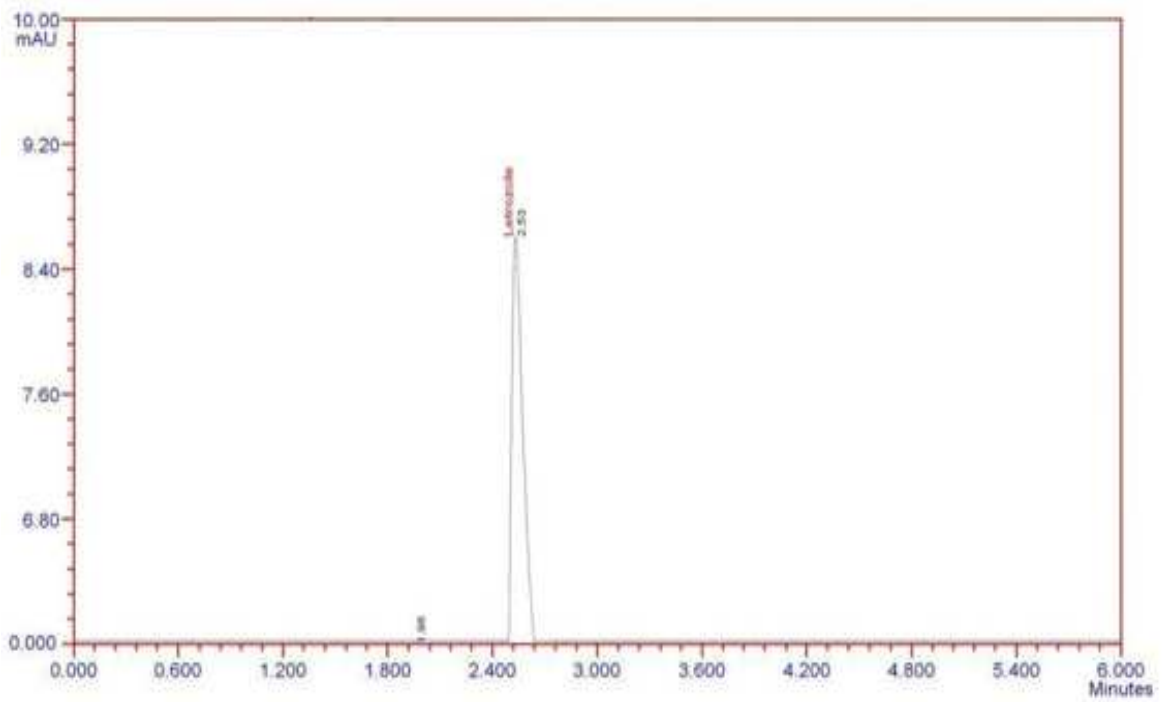


Table 1.2: Optimized chromatographic conditions for estimation of Letrozole

S.No.	Parameter	Condition
1	Mobile phase	Acetonitrile: Methanol: O.P.A (0.1%) (25:30:45)
2	Pump mode	Isocratic
3	Mobile phase pH	4.8
4	Diluent	Mobile phase
5	Column	Inertsil ODS C-18, 5 μ m, 250 x 4.6mm
6	Column Temp	Ambient
7	Wavelength	242 nm
8	Injection Volume	20 μ L
9	Flow rate	1.0 ml/min
10	Run time	6 min
11	Retention Time	2.79 min

4. RESULTS AND DISCUSSION

The experimental method developed above was employed for its subsequent validation and determination of Letrozole in bulk and pharmaceutical forms. The following results were obtained correspondingly.

Validation of a proposed analytical method to determine the assay should meet the requirements for the intended analytical application as per ICH guidelines. The typical analytical parameters used in validation of the assay include Precision, Accuracy, Linearity, Robustness, Limit of detection, Limit of Quantification, Selectivity or Specificity.

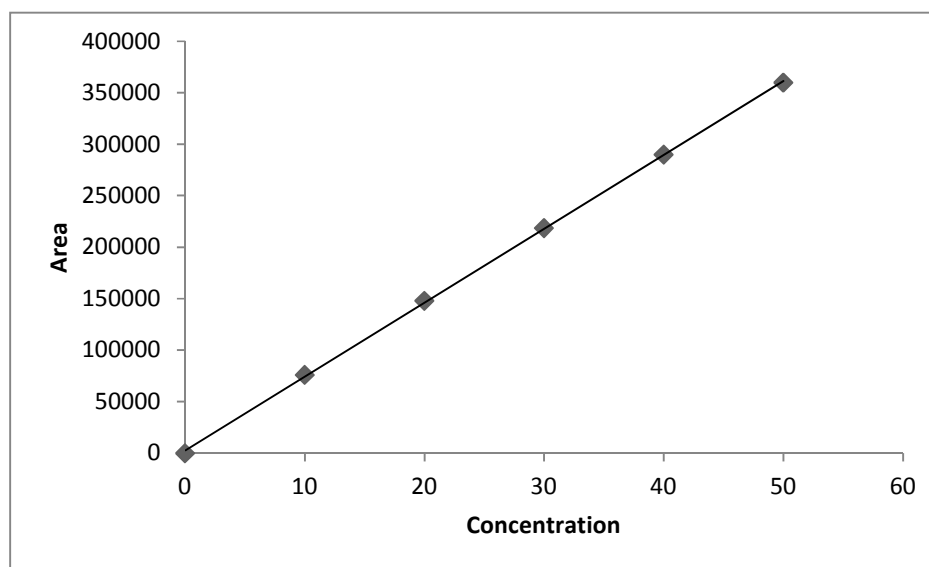
4.1 Linearity

Linearity is the method's ability to obtain peak area results that are proportional to the concentration of the analyte within a given range. Linearity was performed by preparing standard solutions of Letrozole at different concentration levels including working concentration mentioned in experimental condition i.e. 50 ppm. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. The peak responses were read at 242 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The calibration plot is shown in Figure 5. The regressions of the plots were computed by least square regression method. Linearity results obtained are presented in Table 1.3.

Table 1.3: Linearity studies of Letrozole

Level	Concentration of Letrozole (in ppm)	Mean peak area
1	10	76035.6
2	20	147988.8
3	30	218648.3
4	40	289953.4
5	50	359932.7
Range: 10 to 50 ppm	Slope Intercept Correlation coefficient	7097.588 5584.12 0.999

Figure 5: Calibration plot for Letrozole



The results obtained indicate a linear relationship between peak response and concentration of Letrozole in the range of 10-50 ppm.

4.2 Precision

Precision is the degree of reproducibility of an analytical method under normal operational conditions. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. Precision of the method was performed as Intraday precision and Inter day precision. The precision is then expressed as the relative standard deviation.

4.2.1. Intraday precision

The Intraday precision was studied by preparing and injecting six replicate standard solutions of Letrozole (50 ppm) using the proposed method. The percent relative standard deviation (% RSD) was calculated for the peak areas and it was found to be 0.771%, which is well within the acceptance criteria of not more than 2.0%. Results of intraday system precision studies are shown in Table 1.4.

Table 1.4: Intraday Precision Results for Letrozole

Sample	Conc. (in ppm)	Injection No.	Peak Area	% RSD
Letrozole	50	1	357687.2	0.771
		2	357057.8	
		3	353871.4	
		4	355822.7	
		5	350869.5	
		6	352173.3	

4.2.2. Interday precision

The interday precision was studied by preparing and injecting six replicates of standard solutions of Letrozole (50 ppm) on three different days over a period of

one week. The percent relative standard deviation (% RSD) was calculated and it was found to be 0.740%, which is well within the acceptance criteria of not more than 2.0%. Results of interday system precision studies are shown in Table 1.5. The precision results obtained above indicate a very good agreement of the method with respect to its reproducibility at any period of time.

Table 1.5: Interday Precision Results for Letrozole

Sample	Conc. (in ppm)	Injection No.	Peak Area	% RSD
Letrozole	50	1	353255.1	0.740
		2	354071.2	
		3	352806.4	
		4	353442.0	
		5	355032.7	
		6	359871.6	

4.3. Selectivity

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of possible interference creatable substances such as synthetic precursors, excipients, etc. The selectivity of method was confirmed by comparing the chromatograms of blank, standard and tablet sample. It was found that there is no interference due to excipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The results are shown in Table 1.6.

Table 1.6: Selectivity Study

Name of the solution	Retention Time (in min)
Blank	No peak
Standard	2.79
Sample	2.53

4.4. Accuracy

Accuracy of an analytical method is the extent to which test results are close to their true value. It is measured from the result of a quantitative determination of a well characterized known sample. The amount measured is compared to the known amount. The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Peak area was compared before and after the addition of the drug. The standard addition method was performed at 50%, 100%, 150% level of 20 ppm. The solutions were analyzed at each level as per the proposed method. The percent recovery and % RSD was calculated and results are presented in Table 1.7. This indicates that the proposed method was accurate.

Table 1.7: Accuracy results

% Level	Conc. (ppm)	Area	% Recovery	% RSD
50	30	217427	99.44157	1.28
100	40	295486	101.9082	
150	50	359995	100.0175	

4.5. Robustness

Robustness is a measure of the method's capacity to remain unaffected by slight variations in the parameters of the method which consequently indicates its reliability during normal usage. This was carried out by varying two parameters from the optimized chromatographic conditions. The results are shown in Table 1.8.

Table 1.8: Robustness results

Parameter changed	Change	Area	% Recovery
Standard	-	359932	-
Mobile phase	15:40:45	365968	101.677
	35:20:45	363750	101.0608
Wavelength	240	365907	101.66
	244	359995	100.0175
pH	5	366684	101.8759
	4.6	366922	101.942

4.6. Limit of detection and Limit of quantification

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. In chromatography the detection limit is the injected amount that results in a peak height of at least twice or three times as high as the baseline noise level.

Limit of quantification (LOQ) is the minimum injected amount that gives precise measurements. In chromatography, it typically requires peak heights of 10 to 20 times higher than baseline noise at precision of <10-15% RSD between results. The sample was dissolved by using the mobile phase and injected until the peak disappeared. After 0.1 ppm dilution, peak was not observed clearly. So it confirms

that 0.1 ppm is the Limit of Detection and Limit of Quantification was found to be 0.36 ppm. The LOD and LOQ of Letrozole are given in Table 1.9.

Table 1.9: Limit of Detection and Limit of Quantification for Letrozole

Parameter	Measured volume
Limit of Quantification	0.36 ppm
Limit of Detection	0.1 ppm

4.7. Formulation:

The validated method was applied for the assay of commercial tablets containing Letrozole. The formulation tablets of Letrozole were crushed to give finely powdered material. Powder equivalent to 10 mg of drug was taken in 10 ml of volumetric flask containing 5 ml of mobile phase and was shaken to dissolve the drug and then filtered through Ultipor N₆₆ Nylon 6,6 membrane sample filter paper. Volume of the filtrate was adjusted to the mark with the same solvent to obtain concentration of 50 ppm. An aliquot of this solution was injected into HPLC system. Peak area of Letrozole was measured and compared against the peak area of the standard solution. The proposed method was able to estimate Letrozole in the tablet formulation with an accuracy of 93%. The results presented good agreement with the labeled content as shown in Table 1.10.

Table 1.10: Formulation results

Brand	Dose (mg)	Sample Conc.	Standard area	Sample area	Amount found	% assay
Feofer	2.5 mg	50 ppm	76035.6	1027.7	46.5 ppm	93

5. CONCLUSION

The rapid analysis within 6 min and a low flow rate allows for a short assay runtime and also reduces solvent costs with simultaneous minimizing of environmental impact of toxic solvents allowing us to analyze a large number of samples in short period of time, making it suitable for the routine analysis of Letrozole and also quantification of Letrozole in pharmaceutical dosage forms when compared with other available methods.. The evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters made us to the conclude that the current RP-HPLC method can successfully used for rapid and reliable determination of Letrozole in tablet formulation and also in bulk drugs. This method does not employ any buffer mixtures in its mobile phase and hence can be considered advantageous over other existing simple analytical HPLC methods.

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