ORIGINAL ARTICLE

A New Stability Indicating RP-UFLC Method for the Estimation of Letrozole in Presence of Internal Standard

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Abstract

Introduction: Letrozole is an anti-cancer agent especially used for the treatment of estrogen-dependent breast cancers. A new stability indicating reversed-phase ultra-fast liquid chromatography (RP-UFLC) method has been proposed for the estimation of Letrozole in tablet dosage forms. **Materials and Methods:** Shimadzu Model UFLC system with PDA detector and Luna C8 column with mobile phase mixture methanol: Acetonitrile: 0.1% aq. acetic acid (10: 40: 50) with flow rate 1.0 mL/min was chosen on isocratic mode (Detection wavelength 255 nm). **Results and Discussion:** Letrozole obeys Beer–Lambert's law over the concentration range 0.05–100 µg/mL with linear regression equation y = 3679×–0.0002 (R^2 = 0.9999). The limit of quantification and limit of detection were found to be 0.0158 µg/mL and 0.0484 µg/mL. Stress degradation studies were performed such as acid hydrolysis, alkaline hydrolysis, thermal treatment, and oxidation and the method was validated as per ICH guidelines. The proposed RP-UFLC method was applied for the quantification of pharmaceutical formulations (Tablets) and found to be simple, precise, accurate, and robust. **Conclusion:** The proposed RP-UFLC method was applied for the quantification of pharmaceutical formulations (Tablets) and found to be simple, precise, accurate, and robust.

Key words: Internal standard, Letrozole, reversed-phase ultra-fast liquid chromatography, stability indicating, Voriconazole validation

INTRODUCTION

Letrozole (LZ) (CAS Number-112809-51-5) is an oral non-steroidal aromatase inhibitor used for the treatment of cancer.[1,2] It is chemically 4-[(4-cyanophenyl)-(1, 2, 4-triazol-1-yl) methyl] benzonitrile [Figure 1a] and it is an oral non-steroidal aromatase inhibitor used for the treatment of breast cancer. Letrozole acts by inhibiting the growth or inducing the regression of harmone responsive breast tumors. Voriconazole (VZ) (CAS no. 137234–62-9) [Figure 1b], an anti-fungal agent is chemically (2R, 3S)-2-(2, 4-Difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1, 2, 4-triazol-1-yl) butan-2-ol, is used as an internal standard (IS) for the present study.

In the literature survey, it was found that Letrozole was estimated by LC-ESI-MS/MS and LC-MS in human plasma, GC-MS in urine and RP-HPLC in Wistar rat serum, human plasma with florescence as well as UV detection, and pharmaceutical dosage forms.

Pallab *et al*., developed a bioanalytical method[3] for the quantification of Letrozole by LC-MS/MS in human plasma by liquid-liquid extraction technique using Phenomenex Kinetex 5 µ C18 column in the presence of an IS. Letrozole was eluted at 1.79 min and the IS was eluted at 1.82 min, respectively.

Joshi *et al*., developed a high-performance liquid chromatography-tandem mass spectrometry assay[4] for the determination of Letrozole in human plasma in the presence of an IS, Letrozole d4 (II). The drug was extracted by liquid extraction method with tert-butyl methyl ether using a Heidolph vibramax 110 shaker. The chromatographic

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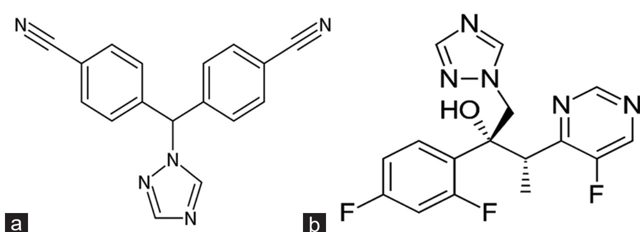

Figure 1: (a) Chemical structure of Letrozole and (b) Chemical structure of Voriconazole

separation was performed on a RP Hypersil BDS C18 column with a mobile phase mixture consisting of 10 mM ammonium acetate and acetonitrile (10: 90) (pH adjusted to 3.0 with acetic acid). The protonated analyte was quantitated in negative ionization by multiple reactions monitoring with a mass spectrometer and the assay has shown linearity over the concentration range 0.2–100 µg/mL.

Mareck *et al*., developed a GC-MS method[5] for the identification of Letrozole in urine samples collected on spot from women suffering with metastatic breast cancer and also for the detection of metabolites of Letrozole, mainly the metabolite, (bis-4-cyanophenylmethanol) by comparison of the mass spectrum and retention time with that of the reference, bis-4-cyano phenyl methanol, and the method was validated for the analysis of bis-4-cyanophenylmethanol.

Acharjya *et al*., developed a high-performance liquid chromatographic method for the determination of Letrozole in Wistar rat serum[6] using RP C18 column and mobile phase methanol:water (70:30) with flow rate 1.0 ml/min (Detection wavelength 239 nm). Letrozole was extracted by liquid-liquid extraction method using diethyl ether as the extracting solvent and the linearity was followed over the concentration range 0.15–100 µg mL.

Zarghi *et al*., developed a HPLC method for the determination of Letrozole in human plasma using fluorescence detection[7] and applied for the pharmacokinetic studies. The separation was achieved on a monolithic silica column using acetonitrile and phosphate buffer. A fluorescence detector was used for the quantitation with excitation and emission wavelengths at 230 and 295 nm and the linearity was observed over the concentration range 0.5–80 ng/mL.

Sekar *et al*., developed a bioanalytical method for the quantification of Letrozole by RP-HPLC in human plasma[8] using mobile phase mixture consisting of 0.02M phosphate buffer (pH 5.5) and acetonitrile (75: 25) and Phenomenex Luna C 18 column in the presence of an IS, Fluconazole, in which Letrozole was eluted at 4.29 min and the IS was eluted at 7.47 min. The linearity of Letrozole was found to be 50.55–120.00 ng/mL.

Annapurna *et al*., developed a stability-indicating high-performance liquid chromatographic method[9] for the determination of Letrozole using methanol: Tetra butyl ammonium hydrogen sulfate (80: 20) as mobile phase with a flow rate 1 mL/min (Detection wavelength 240 nm) and the linearity was observed over the concentration range 0.5–150 µg/mL.

Ganesh *et al*., developed a RP-HPLC method[10] for the determination of Letrozole in bulk and in tablet dosage forms using a mobile phase of acetonitrile: Water (50: 50) with UV detection at 265 nm and the linearity was shown over the concentration range 160–240 µg/mL.

Anand *et al*., developed a RP-HPLC method[11] for determination of Letrozole in bulk and its pharmaceutical dosage forms using Gemini C18 Phenomenex column and a mobile phase mixture of acetonitrile and water (50: 50) with UV detection at 265 nm and flow rate 1.1 ml/min. The linearity was shown over the concentration range 160–240 µg/mL and Letrozole was eluted at 4.53 min.

Annapurna *et al*., developed a RP-HPLC method[12] for the determination of Letrozole in bulk and in tablet dosage forms using a mobile phase of glacial acetic acid: Acetonitrile: Water (0.1: 50: 50) with UV detection at 240 nm and the linearity was shown over the concentration range 1–100 µg/mL.

Elkady and Fouad developed a stability indicating RP-HPLC method[13] for the determination of Letrozole and two of its forced degradation products were also prepared and characterized using IR ad Mass spectral studies. Mobile phase consisting of 0.01M KH_2PO_4 and methanol with flow rate 1 mL/I (UV detection at 230 nm) and Zorbax Eclipse C18 column was employed for the study and the linearity was shown over the concentration range 0.01–80 µg/mL.

Mondal *et al*., developed a RP-HPLC method[14] for determination of Letrozole in in raw material, pharmaceutical formulations such as tablets and nanoparticles and in release medium using FinePak C column and a mobile phase mixture of water, acetonitrile, and methanol (50: 30: 20) with UV detection at 240 nm and the linearity was shown over the concentration range 1–50 µg/mL and Letrozole was eluted at 9.8 min.

In the present study, a new stability indicating reversed-phase ultra-fast liquid chromatography (RP-UFLC) method has been developed for the estimation of Letrozole in the presence of and IS and VZ and the method was validated as per ICH guidelines.

MATERIALS AND METHODS

Instrumentation and chromatographic conditions

Shimadzu Model UFLC system with Luna C8 column and PDA detector with mobile phase mixture methanol: Acetonitrile: 0.1% acetic acid (10: 40:50) was chosen on

Table 1: Literature survey

Mobile phase (v/v)	λ (nm)	Linearity (μg/mL)	Comment	Ref
Methanol: Tetra butyl ammonium hydrogen sulfate (80: 20)	240	0.5–150	RP-HPLC	9
Acetonitrile: Water (50: 50)	265	160–240	RP-HPLC	10
Acetonitrile: Water (50: 50)	265	160–240	RP-HPLC	11
Glacial acetic acid: Acetonitrile: Water (0.1: 50: 50)	240	1–100	RP-HPLC	12
0.01M KH_2PO_4: Methanol	230	0.01–80	RP-HPLC	13
Water: Acetonitrile: Methanol (50: 30: 20)	240	1–50	RP-HPLC	14
Methanol: Acetonitrile: 0.1% aq. Acetic acid (10: 40: 50)	255	0.05–100	RP-UFLC	Present work

isocratic mode with flow rate 1.0 mL/min and detection wavelength 255 nm.

Preparation of Letrozole solution

25 mg of Letrozole (API) was weighed accurately and transferred into a 25 mL volumetric flask and dissolved in HPLC grade acetonitrile (1000 μg/mL) and sonicated for 30 min and dilutions were made with the mobile phase. All the solutions were filtered before use through membrane filter.

Method validation[15]

Linearity, precision, accuracy, and robustness

A series of solutions of Letrozole (0.05–100 μg/mL) were prepared from the stock solution in 10 ml volumetric flasks with mobile phase, sonicated, and filtered through membrane filter. 20 μL of each of these filtered solutions were injected ($n = 3$) into the UFLC system and the peak area of each chromatogram was noted. Then, the mean peak area was calculated and a calibration graph was drawn by plotting the concentration of the drug solutions on the X-axis and the corresponding mean peak area of the chromatograms on the Y-axis.

The intraday precision studies were performed on the same day at different equal intervals of time by injecting the drug solutions (10, 20, and 40 μg/mL) and the interday precision studies were conducted by injecting the drug solution (10 μg/mL) on 3 successive days (Day 1, Day 2 and Day 3) and the % relative standard deviation (RSD) was calculated.

Accuracy studies were performed by spiking the formulation solution with 50, 100, and 150% of API of Letrozole and the resulting solutions were made up to volume in the volumetric flaks with the mobile phase and injected in to the UFLC system. The peak area of the chromatograms was noted, and thereby, the mean peak area, % RSD, and the percentage recovery were calculated from the calibration curve.

In robustness study, small changes such as mobile phase ratio, pH, flow rate, and detection wavelength were incorporated purposefully in the optimized chromatographic conditions and the method was studied.

Stress degradation studies[16]

Stress degradation studies were performed for studying the specificity of the proposed method. Letrozole was allowed to undergo acidic hydrolysis, alkaline hydrolysis, oxidation, and thermal degradation reactions.

Acidic hydrolysis was performed by treating Letrozole solution with 1 mL of 0.1 N HCl solution for 30 min at room temperature. The stressed sample was then neutralized with 1.0 mL 0.1N sodium hydroxide solution and made up to volume with the mobile phase. 20 μL of the resulting mixture was injected in to the UFLC system and the peak area of the chromatogram of Letrozole was noted and the percentage degradation was calculated.

Alkaline hydrolysis was performed by treating Letrozole solution with 1.0 mL 0.1N sodium hydroxide solution for 30 min at room temperature. The stressed sample was then neutralized with 1 mL of 0.1 N HCl solution and made up to volume with the mobile phase. 20 μL of the resulting mixture was injected in to the UFLC system and the peak area of the chromatogram of Letrozole was noted and the percentage degradation was calculated.

Oxidation reaction was performed by treating Letrozole solution with 1 mL 30% H_2O_2 solution for 30 min at room temperature and made up to volume with the mobile phase. Then, 20 μL of the stressed sample was injected in to the UFLC system and the peak area of the chromatogram of Letrozole was noted and the percentage degradation was calculated.

Thermal degradation was performed by heating Letrozole solution at 40°C for 30 min in water bath and made up to volume with the mobile phase. Then, 20 μL of the stressed sample was injected in to the UFLC system and the peak area of the chromatogram of Letrozole was noted and the percentage degradation was calculated.

Assay of Letrozole tablets

Letrozole was obtained as gift sample from Novartis India Ltd. Letrozole is available with label claim 2.5 mg with brand names Avenlet (Clarix Healthcare), Letroz (Niva Lifecare), Fempro (Cipla Ltd), Femara (Novartis), etc.

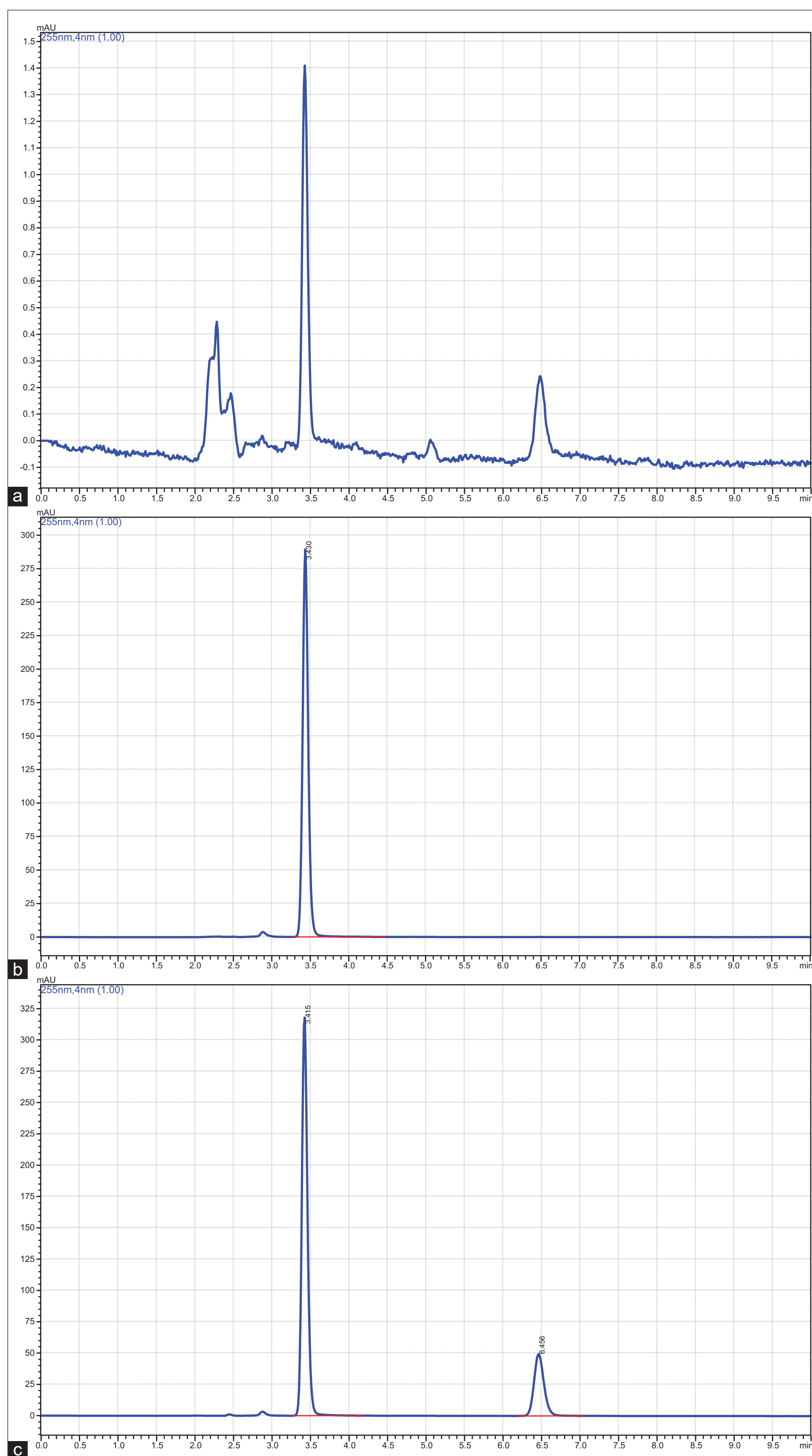

Figure 2: Typical chromatograms of a) Placebo. b) Letrozole (API) (Rt 3.430; theoretical plates 8013; and tailing factor: 1.208). c) Letrozole (API) with Voriconazole (IS) Rt 3.415 min; theoretical plates 8246; and tailing factor: 1.238) Voriconazole (IS) (Rt 6.456 min; theoretical plates 10763; tailing factor: 1.203; and resolution 15.228)

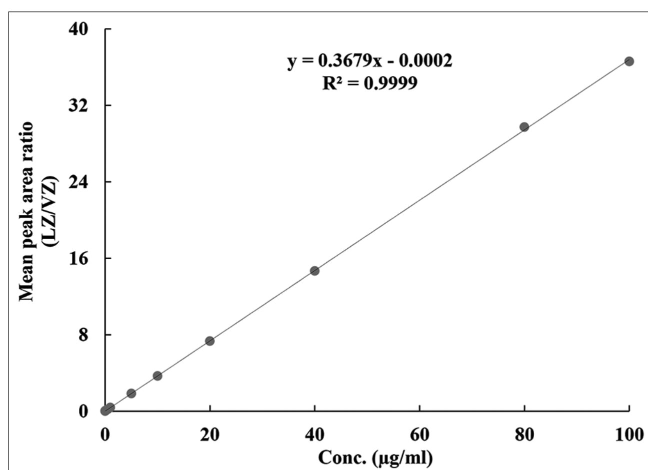

Figure 3: Calibration curve of letrozole in the presence of Voriconazole (IS)

Table 2: Linearity study

Conc. (µg/mL)		*Mean peak area		Peak area ratio (LZ/VZ)	% RSD
LZ	VZ	LZ	VZ		
0	0	-	-	-	-
0.05	10	8496	444315	0.0191	0.31
0.1	10	16413	444264	0.0369	0.54
0.5	10	82815	445002	0.1861	0.66
1	10	163249	444109	0.3676	0.81
5	10	815309	445063	1.8319	0.36
10	10	1631613	444212	3.6731	0.21
20	10	3263459	445121	7.3316	0.91
40	10	6521489	444601	14.6682	0.67
80	10	13229571	445032	29.7272	0.25
100	10	16254269	444327	36.5818	0.45

*Mean of three replicates

Two tablets of two different brands were collected from the local pharmacy store, weighed and tablet powder equivalent to 25 mg Letrozole was transferred to two different 25 mL volumetric flasks, and acetonitrile was added. The mixture was sonicated thoroughly and filtered through membrane filter and dilutions were made using the mobile phase as per requirement. 20 µL of each of these solutions were injected ($n = 3$) in to the UFLC system and the percentage recovery was calculated from the peak area of the chromatogram and the calibration curve.

RESULTS AND DISCUSSION

A new stability indicating RP-UFLC method has been proposed for the quantification of Letrozole in tablet dosage forms. Shimadzu Model UFLC system with PDA detector and Luna C8 column with mobile phase mixture Methanol: Acetonitrile: 0.1% Acetic acid (5: 45: 50) was chosen on isocratic mode with flow rate 1.0 mL/min and detection wavelength 255 nm after many trial runs with different mobile phases and their composition and flow rate. A detailed review of the literature is given in Table 1.

A typical chromatogram of placebo and that of Letrozole is shown in Figure 2, in which Letrozole was observed to be eluted at 3.430. A typical chromatogram of placebo and that of Letrozole API is shown in Figure 2a and b, in which the drug was observed to be eluted at 3.430 min. In Figure 2c, the chromatogram of Letrozole (API) in the presence of its IS and VZ was shown, in which both Letrozole and the IS were separated at different retention times 3.415 min and 6.456 min, respectively, with good resolution of about 15.228 indicating that the method is selective.

Linearity, precision, accuracy, and robustness

Letrozole obeys Beer–Lambert's law over the concentration range 0.05–100 µg/mL (% RSD 0.21–0.91) [Table 2] with

Table 3: Intraday precision study

Conc. (µg/mL)	*Mean peak area			*Mean peak area ratio±SD (% RSD)
	LZ	VZ	LZ/VZ	
10	1631514	444622	3.6731	3.6731±0.0081 (0.22)
20	3262982	445029	7.3321	7.3321±0.0447 (0.61)
40	6515621	444965	14.6430	14.6430±0.0761 (0.52)

*Mean of three replicates

Table 4: Interday precision study

Conc. (µg/mL)	*Mean peak area			*Mean peak area ratio±SD (% RSD)
	LZ	VZ	LZ/VZ	
Day 1	1631514	444622	3.6694	3.6694±0.0261 (0.71)
Day 2	1635487	445019	3.6751	3.6751±0.0199 (0.54)
Day 3	1641562	444842	3.6902	3.6902±0.0229 (0.62)

*Mean of three replicates

linear regression equation y = 3679x – 0.0002 (R^2 = 0.9999) [Figure 3]. The limit of quantification and limit of detection were found to be 0.0158 µg/mL and 0.0484 µg/mL. The % RSD in precision study was found to be 0.22–0.61 (Intraday) and 0.54–0.71 (Interday) [Table 3] indicating that the method is precise. The % RSD in accuracy study was found to be 0.47–1.02 [Table 4] with % recovery 98.18–99.53 and that of robustness study was 0.0463–0.0708 [Table 5] indicating that the method is accurate and robust [Table 6].

Table 5: Accuracy study

Spiked Conc. ((µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Mean Conc. (µg/mL) ±SD (%RSD)	% Recovery
10 (50%)	20	30	29.86±0.1403 (0.47)	99.53
	20	30		
	20	30		
20 (100%)	20	40	39.27±0.3495 (0.89)	98.18
	20	40		
	20	40		
30 (150%)	20	50	49.69±0.5068 (1.02)	99.38
	20	50		
	20	50		

*Mean of three replicates

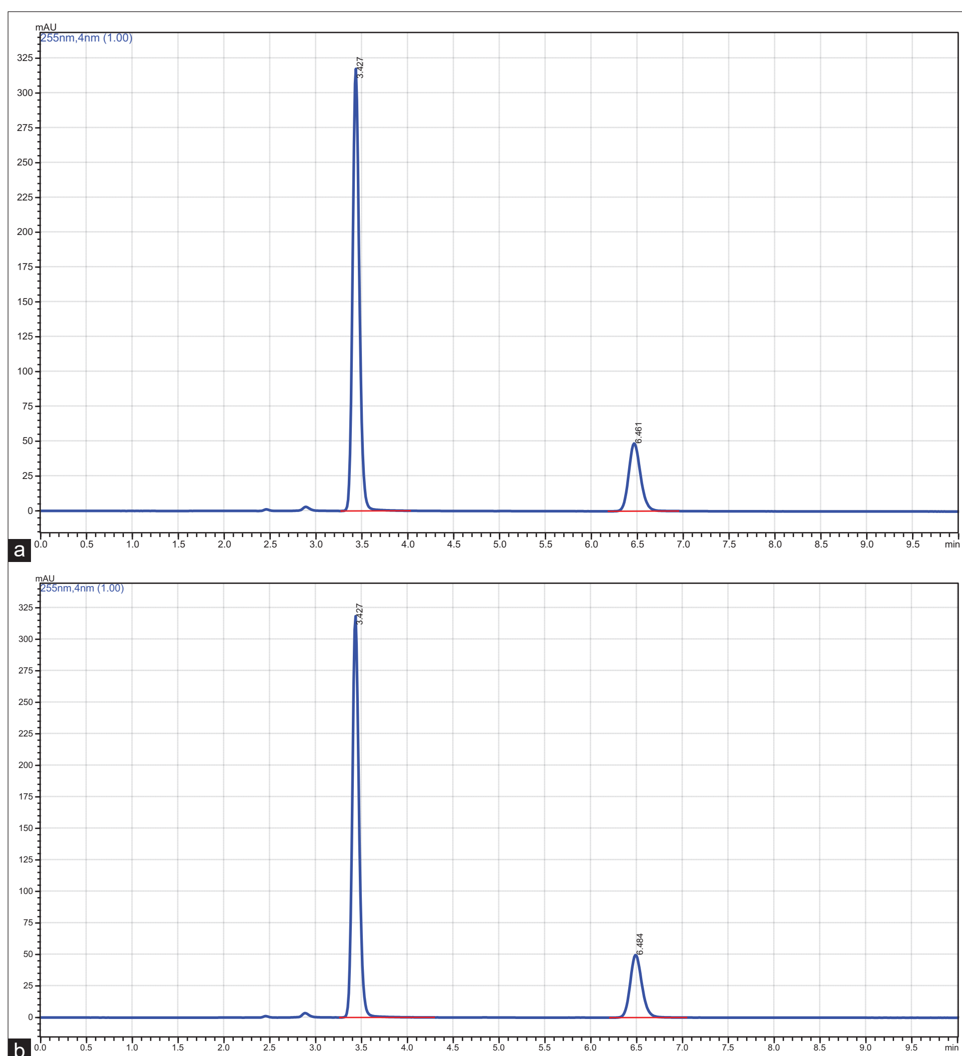

Figure 4: Typical chromatograms of Letrozole tablets in the presence of Voriconazole (IS). a) Letrozole tablet (Brand I) (Rt 3.427 min; theoretical plates 8274; tailing factor; 1.235) IS; (Rt 6.461 min; theoretical plates 10674; tailing factor; 1.204; resolution 15.135). b) Letrozole tablet (Brand II) (Rt 3.427 min; theoretical plates 8275; tailing factor; 1.237) IS; (Rt 6.484 min; theoretical plates 10914; tailing factor; 1.201; and resolution 15.320).

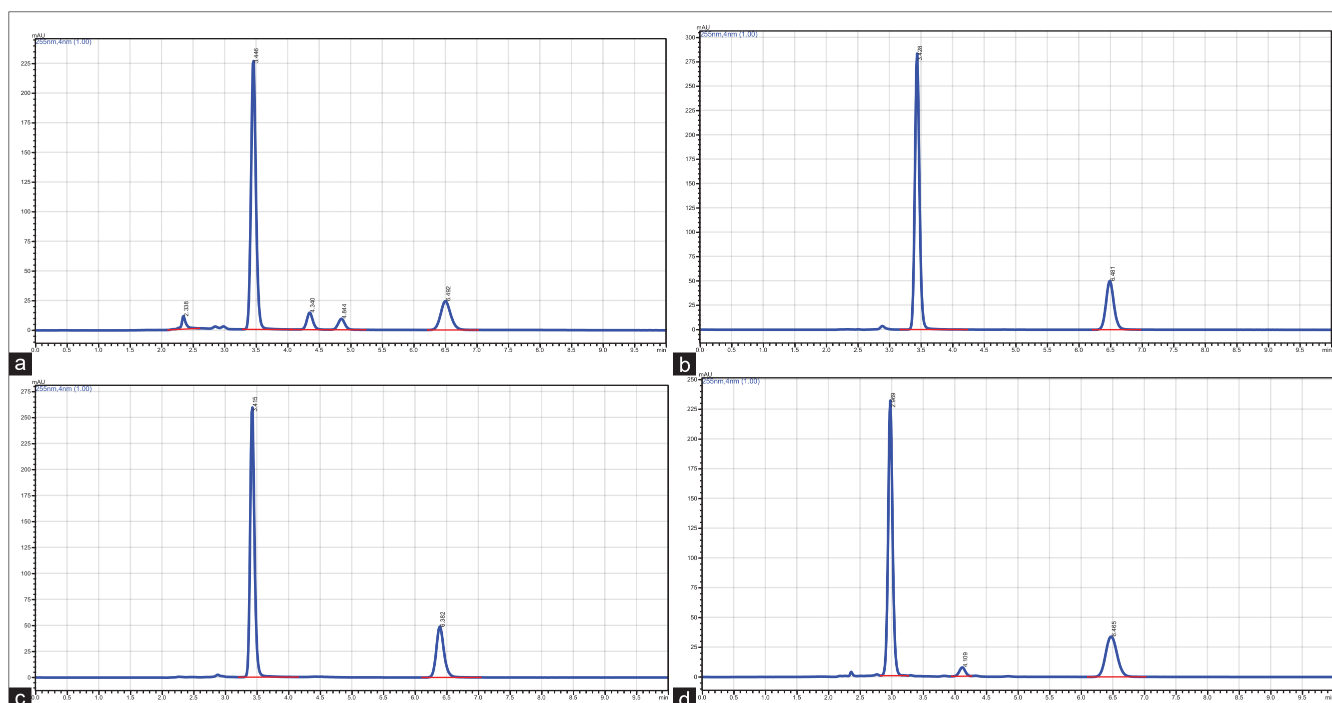

Figure 5: Typical chromatograms of letrozole in the presence of IS during stress degradation studies. (a) Acidic hydrolysis, (b) thermal degradation, (c) oxidative degradation, and (d) Alkaline degradation

Table 6: Robustness study

Parameter	Condition	*Mean peak area		*Mean peak area ratio LZ/VZ	*Mean peak area ratio±SD (% RSD)
		LZ	VZ		
Flow rate (±0.1 ml/min)	0.9	1632287	444268	3.6741	3.6724±0.0026 (0.0708)
	1.0	1631514	444622	3.6694	
	1.1	1633125	444552	3.6736	
Detection wavelength (±2 nm)	253	1632169	444411	3.6727	3.6708±0.0017 (0.0463)
	255	1631514	444622	3.6694	
	257	1631648	444569	3.6702	
Mobile phase composition Methanol: Acetonitrile: 0.1% aq. Acetic acid (10: 40: 50) (±2%, v/v)	10: 42: 48	1632013	444427	3.6722	3.6714±0.0017 (0.0463)
	10: 40: 50	1631514	444574	3.6694	
	10: 38: 52	1631845	444327	3.6726	

*Mean of three replicates

Table 7: Assay of letrozole tablets

Brand	Label claim (mg)	Amount found (mg)	% Assay	Figure
Brand I	2.5	2.48	99.20	4A
Brand II	2.5	2.47	98.80	4B

*Mean of three replicates

Assay of Letrozole tablets

The proposed RP-UFLC method was applied to two brands of Letrozole tablets procured from different manufacturers and the assay was performed. In Figure 4a, the representative chromatogram of Letrozole tablets (Brand I) in the presence of IS was shown, in which both letrozole and the IS were separated at different retention times 3.427 min and 6.461 min, respectively, with good resolution of about 15.135 indicating that the method is selective.

In Figure 4b, the representative chromatogram of Letrozole tablets (Brand II) in the presence of IS was shown, in which both Letrozole and the IS were separated at different retention times 3.427 min and 6.484 min, respectively, with good resolution of about 15.320 indicating that the method is selective. The percentage of purity was found to be 98.80–99.20 for Letrozole [Table 7].

Stress degradation studies

Letrozole was eluted at 3.415 min with theoretical plates 8246 and tailing factor 1.238, whereas the IS was eluted at

Table 8: Stress degradation studies of letrozole in the presence of Voriconazole (IS)

Stress condition	Rt (min)		*%Recovery	*% Drug degradation	Theoretical plates (>2000)		Tailing factor (<2.0)		Resolution (>2.0)
	LZ	VZ			LZ	VZ	LZ	VZ	
Standard drug	3.415	6.456	100	------	8246	10763	1.238	1.203	15.228
Acidic degradation 0.1N HCl/80°C/30 min	3.446	6.492	76.47	23.53	7436	7177	1.210	1.159	7.803, 5.030 2.458, 6.329 (Extra peak 2.338, 4.340, 4.844)
Alkaline degradation 0.1N NaOH/80°C/30 min	2.969	6.465	70.09	29.91	7092	5684	1.169	1.134	6.661 8.754 (Extra peak 4.109)
Oxidative degradation 30%H_2O_2/80°C/30 min	3.415	6.382	76.76	23.24	9230	11771	1.290	1.240	15.720
Thermal degradation Water/80°C/30 min	3.428	6.481	88.33	11.67	8348	11256	1.199	1.154	15.477

*Mean of three replicates

6.456 min with theoretical plates 10763 and tailing factor 1.203 with resolution 15.228.

During the acidic degradation, Letrozole was at 3.446 min with theoretical plates 7436 and tailing factor 1.210, whereas the IS was eluted at 6.492 min with theoretical plates 7177 and tailing factor 1.159 [Figure 5a]. Extra peaks were observed at 2.338 min, 4.340 min, and 4.844 min with resolution values 7.803, 5.030, 2.458, and 6.329 indicating that all the peaks were well separated without interference and the method is specific. The percentage recovery was found to be 76.47.

During the thermal degradation, Letrozole was eluted at 3.428 min with theoretical plates 8348 and tailing factor 1.199, whereas the IS was eluted at 6.481 min with theoretical plates 11256 and tailing factor 1.154 with resolution 15.477 [Figure 5b]. The percentage recovery was found to be 88.33.

During oxidation Letrozole was eluted at 3.415 min with theoretical plates 9230 and tailing factor 1.290, whereas the IS was eluted at 6.382 min with theoretical plates 11771 and tailing factor 1.240 with resolution 15.720 [Figure 5c]. The percentage recovery was found to be 76.76.

During the alkaline degradation, Letrozole was at 2.969 min with theoretical plates 7092 and tailing factor 1.169, whereas the IS was eluted at 6.465 min with theoretical plates 5684 and tailing factor 1.134 [Figure 5d]. Extra peak was observed at 4.109 min with resolution values 6.661 and 8.754 indicating that all the peaks were well separated without interference and the method is specific. The percentage recovery was found to be 70.09.

The details of the stress degradation studies are shown in Table 8 with the acceptable criteria for the system suitability parameters and the corresponding chromatograms are shown in Figure 5.

CONCLUSION

A new stability indicating RP-UFLC method has been proposed for the estimation of Letrozole in tablet formulations and the proposed method was validated as per ICH guidelines. The method is simple, specific, selective, precise, accurate, and robust and can be used for the routine analysis of Letrozole and there is no interference of excipients.

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