Preparation and characterization of two new forced degradation products of letrozole and development of a stability-indicating RP-LC method for its determination

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Abstract: Two new hydrolytic products of letrozole were identified and proved to be true degradation products obtained by alkaline and acidic degradation of the drug. The acid and amide forms of the nitrile groups of letrozole were prepared and identified by IR and mass spectroscopic techniques. Subsequently, a simple, precise and selective stability-indicating RPLC method was developed and validated for the determination of letrozole in the presence of its degradation products. Letrozole was subjected to alkali and acid hydrolysis, oxidation, thermal degradation and photo-degradation. The degradation products were well isolated from letrozole. The chromatographic method was achieved usinggradient elution of the drug and its degradation products on a reversed phase Zorbax Eclipse C18 column (100mm x 4.6mm, 3.5µm) using a mobile phase consisting of 0.01M KH_2PO_4 and methanol at a flow rate of 1mL min^{-1}. Quantitation was achieved with UV detection at 230nm. Linearity, accuracy and precision were found to be acceptable over the concentration range of 0.01-80µgmL^{-1}. The proposed method was successfully applied to the determination of letrozole in bulk, plasma and in its pharmaceutical preparation.

Keywords: Letrozole; reversed-phase liquid chromatography; stability-indicating assay; forced degradation; aromatase inhibitor.

INTRODUCTION

Letrozole (fig. 1a), chemically known as 4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl) methyl] benzonitrile (O'Neil, 2006), is used for the treatment of estrogen-dependent breast cancers (Lamb and Adkins, 1998). It is an oral non-steroidal aromatase inhibitor that has been introduced for the adjuvant treatment of hormonally-responsive breast cancer (Iveson *et al.*, 1993).

Letrozole has been determined in different pharmaceutical formulations by spectrophotometry (Zheng *et al.*, 2010; Acharjya *et al.*, 2010; Ganesh *et al.*, 2008; Mondal *et al.*, 2007; Ganesh *et al.*, 2007), TLC (Gu *et al.*, 2001) and HPLC using UV detection (Gu *et al.*, 2001; Shrivastava *et al.*, 2011; Mani *et al.*, 2010; Mondal *et al.*, 2009; Laha *et al.*, 2008; Wu *et al.*, 2007). Moreover, it has been determined in biological fluids by HPLC using UV detection Pfister *et al.*, 1994; Marfil *et al.*, 1996) and fluorescence detection (Zarghi *et al.*, 2007). Besides, LC-MS determination of letrozole has been described in human urine (Kang *et al.*, 2007) and human plasma (Joshi *et al.*, 2011; Beer *et al.*, 2010). Also, letrozole has been determined using UPLC-MS in sewage (Liu *et al.*, 2010) and in fish tissues (Xiao *et al.*, 2011). Its determination in biological fluids was also described in literature by micellar electro kinetic chromatography (Rodriguez-Flores *et al.*, 2008; 2009) and by an enzyme immunoassay (Pfister *et al.*, 1994). Two forced degradation studies of letrozole, in which the degradation products were not well resolved or investigated, have been recently published (Annapurna *et al.*, 2012; Ranganathan *et al.*, 2012).

In USP (2011) and BP pharmacopoeias (2009), the only reported related substances are shown in fig. 1b &c. No other impurities or degradation products are reported. It was, consequently, effort-worthy to predict any unreported degradation products and to separate their peaks from that of the drug. Two hydrolytic products of letrozole. The amide (fig. 1d) and the acid (fig. 1e) derivatives of the nitrile groups, were synthesized by partial and complete hydrolysis, respectively. They were then separated, crystallized and identified by IR and mass spectroscopic techniques. Then, to find out whether they are true degradation products of the drug, a forced degradation study of letrozole under acidic, basic, oxidative, thermal and photolytic conditions was carried out. The present manuscript also describes a new simple, sensitive and selective stability-indicating LC method for the determination of letrozole in the presence of its possible degradation products. The drug and all the degradation products were fully resolved from each other. The method was applied to the pharmaceutical formulation and to human plasma. Optimization of HPLC conditions to resolve letrozole from its degradation products on a reversed phase C18 column was fully discussed.

MATERIALS AND METHODS

Instrumentation

The HPLC (Agilent instrument 1260 series, Germany) system was equipped with vacuum degasser, mixer, autos

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ampler, gradient quaternary pump and UV/Vis detector. Separation and quantitation were made on Zorbax Eclipse C18 rapid resolution column (100mm x 4.6mm, 3.5µm). An Elma ultrasonic processor (Germany) was used.

Reagents and reference samples

Standard letrozole and Femara® tablets (labeled as a nominal dose of 2.5mg letrozole per tablet) were supplied by Novartis Pharma, Switzerland. HPLC grade acetonitrile and monobasic potassium phosphate were supplied by Sigma-Aldrich, Germany. HPLC grade methanol was supplied by Honeywell, USA. Bi-distilled water was produced in-house (Aquatron WaterStill, A4000D, UK). Membrane filters 0.45µm (Teknokroma, Barcelona, Spain) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise.

Chromatographic conditions

Chromatographic separation was achieved on a Zorbax Eclipse C18 rapid resolution column (100mm x 4.6mm, 3.5µm) by applying gradient elution based on a mobile phase consisting of (A) 0.01M aqueous monobasic potassium phosphate - (B) methanol. The separation was achieved with a gradient elution starting at 90% (A), decreasing to 70% in 8 min, holding for 1min, decreasing to 30% in 11min, holding for 5min then rising to 90% in 5 min. The mobile phase was pumped through the column at a flow rate of 1mL min^{-1}. Analyses were performed at 25°C and detection was carried out at 230nm. The injection volume was 20µL.

Standard stock solution preparation

Standard stock solution of letrozole (0.5mgmL^{-1}) was prepared by dissolving 50mg of the drug in acetonitrile: Water (1:1), sonicated and completed to volume in a 100 mL volumetric flask. The required concentrations were prepared by serial dilutions.

Sample preparation

Tablet sample preparation

Twenty tablets were accurately weighed and powdered in a mortar. A quantity of the powdered tablets equivalent to (2.5mg) letrozole was sonicated with 50ml acetonitrile: Water (1:1), cooled and filtered into a 100mL volumetric flask. The solution was completed to volume with the same solvent mixture to obtain a concentration equivalent to 25µg mL^{-1}.

Plasma sample preparation

The spiked plasma samples (500µL) were extracted after precipitation of proteins using 1mL of acetonitrile. Sildenafil citrate was used as an internal standard and was added to acetonitrile used in precipitation of plasma proteins. Then, the mixture was vortex-mixed and centrifuged at 3500 rpm (2 and 5 min, respectively). The organic phase was separated and transferred to another tube and a 20µl volume was injected into the chromatograph.

Procedure

Linearity and repeatability

Accurately measured aliquots of stock standard solutions equivalent to 0.1-800µg letrozole were transferred into a series of 10mL volumetric flasks. The solutions were completed to volume with water. A volume of 20µL of each solution was injected in triplicates into the chromatograph. The conditions including the mobile phase at flow rate 1mL $min^{-1}$and detection at 230 nm were adjusted. A calibration curve in water was obtained by plotting area under the peak (AUP) against concentration (C). The repeatability of the method was assessed by analyzing a solution containing 25µg mL^{-1} of letrozole (n=6). The precision (% R.S.D.) was calculated.

The linearity of the method was also checked in plasma. Accurately measured aliquots of plasma samples spiked with letrozole producing concentrations equivalent to 0.01-80µgmL^{-1} were prepared after its extraction as mentioned in "plasma sample preparation".

Assay of letrozole in bulk, Femara® tablets and human plasma

The procedure mentioned in "Linearity and repeatability" was repeated using concentrations equivalent to 15- 45µg mL^{-1}letrozolein bulk and equivalent to 1-15µgmL^{-1} in plasma samples. For the determination of the examined drugs in Femara® tablets, the sample solution prepared in "sample preparation" was diluted to prepare a solution equivalent to 40µg mL^{-1} and 75µg mL^{-1} of letrozole; and then injected in triplicates. The concentrations of letrozole were calculated using calibration equations (in bulk and in plasma).

Partial hydrolysis of letrozole

Letrozole (350 mg) was dissolved in absolute ethanol (5 mL) and 2.5% NaOH (1mL) was added. The solution was stirred and 6% H_2O_2 (5mL) was steadily run in. The solution was cooled from outside by ice water bath to avoid warming over 40-50°C. After 1hr, the mixture was kept at 50°C for 3 hrs by external heating. While still warm, 5% H_2SO_4 was added until neutral to litmus paper. The ethanol was removed and the residue was concentrated under reduced pressure. The residue was cooled and the crystals were filtered off and washed with water. It was recrystallized from absolute ethanol.

Complete hydrolysis of letrozole

A mixture of concentrated H_2SO_4 (25mL) and distilled water (15mL) was refluxed for 30 min at 100-120°C. Letrozole (350mg) was added and the solution was refluxed and stirred for 3hrs. The reaction mixture was allowed to cool, poured into ice-cold water and filtered off the precipitated acid. The crude product was purified

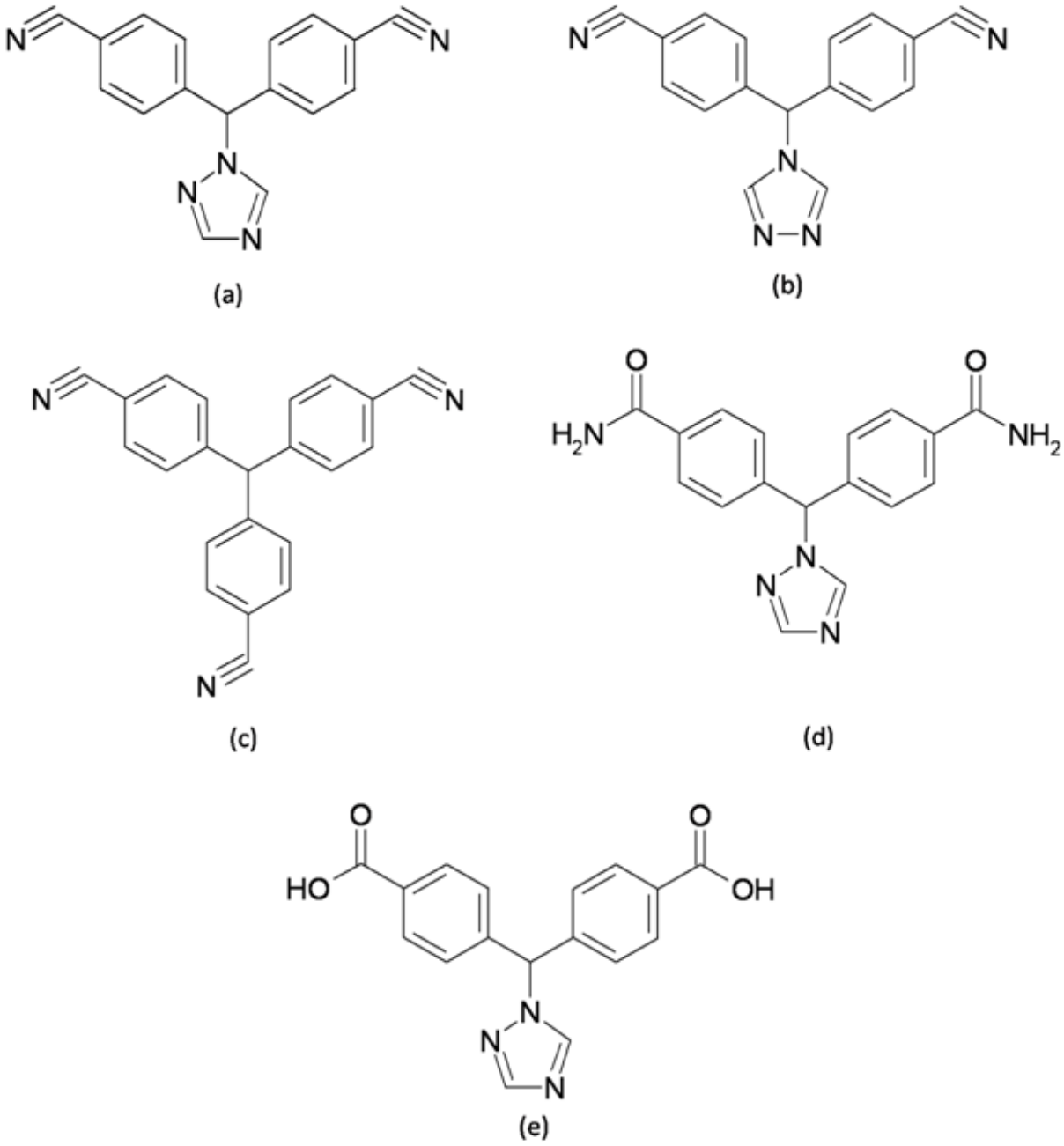


Fig. 1: Chemical structure of (a) letrozole, (b) 4,4'-(4H-1,2,4-triazol-4-ylmethylene) dibenzonitrile, (c) 4,4',4"-methanetriyltribenzonitrile, (d) amide degradation product, (e) acid degradation product.

by dissolving in excess 10% NaOH, filtered and then acidified with dilute H_2SO_4. Finally, it was recrystallized from absolute ethanol.

Forced degradation of letrozole

Forced degradation of bulk drug was studied either in solid state or in solution state stress Studies were carried out according to the ICH regulatory guidance (Q1A (R2), 2003).

Prior to injection, samples were withdrawn at appropriate time, neutralized (in case of acid and alkali hydrolysis) and then the solutions were diluted with water. The total chromatographic run time was about two times the retention of the drug peak.

Acid- and base-induced degradation

To prepare drug solutions in molarity of 0.1M, 0.5M, 1M HCl separately, an appropriate volume of 2M HCl was

added to 5ml of stock drug solution and the mixture was diluted with water to 10ml. The mixtures were kept at RT for48 hr. Alkaline degradation studies were carried out similarly using molarities of 0.1M, 0.5M and 1M NaOH for 48 hr. This was repeated but using molarities 0.1M, 0.5M, 1M and 2M at higher temperature of 75°C for 0.5 hr while all other conditions were kept constant. All these experiments were carried out in the dark to prevent photo-degradation. Twenty microliters of the resultant solutions were injected onto column and the chromatographs were run as described in "Chromatographic conditions".

Table 1: System suitability tests for the proposed LC method for the determination of letrozole in bulk

Parameter	Data
N	189986
T	1.05
%R.S.D. of 6 injections of	
Peak area	0.103
t_R(min)	0.012

(N: Number of theoretical plates; T: Tailing factor; % R.S.D.: %Relative standard deviation; t_R: retention time)

Table 2: Results obtained by the proposed LC method for the determination of letrozole

Parameter	Data
Retention time (min)	16.67
Wavelength of detection (nm)	230
Range of linearity (µg mL^{-1})	0.01-80
Regression equation	Area =0.1224$C_{\mu g\ mL^{-1}}$+48.219
Regression coefficient (r^2)	0.9999
LOD (µg mL^{-1})	0.003
LOQ (µg mL^{-1})	0.010
S_b	0.0004
S_a	15.70
Confidence limit of the slope	0.1224±0.001
Confidence limit of the intercept	48.219±40.36
Standard error of the estimation	32.04
Inter-day (%R.S.D.)	0.320-0.940
Intra-day (%R.S.D.)	0.09-0.340
Drug in bulk	100.55± 0.53
Drug in dosage form	100.25± 1.09

Oxidative degradation

Appropriate volumes of 30% (v/v) H_2O_2were separately added to 5 ml of stock solution, reach final concentrations of 3% and 15% (v/v) H_2O_2. The prepared mixtures were kept at RT for 7 hr. Twenty microliters of the resultant solutions were injected onto column and the chromatographs were run as described in Chromatographic conditions.

Thermal and photolytic degradation

To study dry heat degradation, the dry powder of the drug was heated in an oven at 55°C for 72hr. The photochemical stability of the drug was also studied by exposing the dry powder to sunlight for 24 hr. Finally, powder was dissolved in water. Twenty microliters of the resultant solutions were injected onto column and the chromatographs were run as described in "Chromatographic conditions".

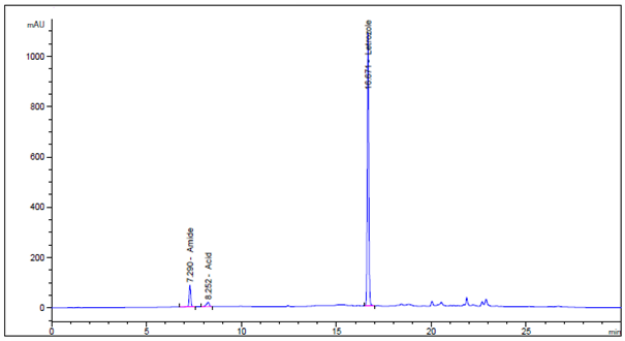


Fig. 2: HPLC chromatogram of letrozole, amide and acid degradation products.

RESULTS

System suitability tests

System suitability tests are important tests of liquid chromatographic methods in order to reach optimized conditions of the proposed method (USP, 2011). They are mainly used to test the resolution and reproducibility and to verify that they are suitable for the analysis performed. The parameters of these tests include column efficiency (number of theoretical plates), tailing of chromatographic peak, repeatability as %R.S.D of peak area for six injections of a solution of a 25µg $mL^{-1}$and reproducibility of retention as %R.S.D of retention time. The results of these tests for the proposed method are listed in table 1.

Method validation

Linearity

In this study, eight concentrations were chosen. Each concentration was analyzed three times. Good linearity of the calibration curve was verified by the high correlation coefficient. The analytical data of the calibration curve including standard deviations for the slope and intercept (S_b, S_a) are summarized in table 2.

Accuracy

Accuracy of the results was calculated by % recovery of 3 different concentrations of letrozole (15-35-45µg mL^{-1}) in bulk. The results obtained including the mean of the recovery and standard deviation are displayed in table 2.

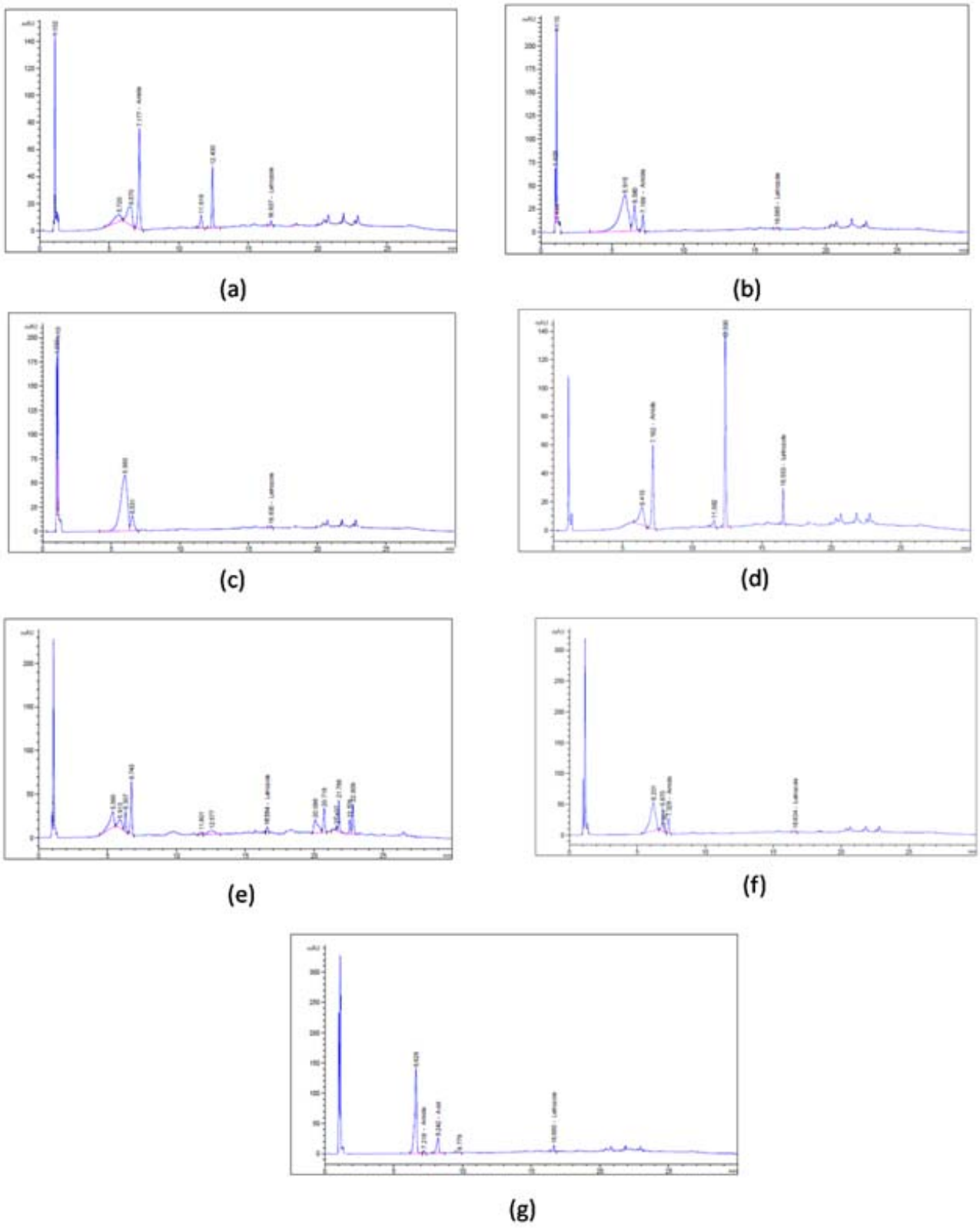


Fig. 3: HPLC chromatogram of degraded letrozole in (a) 0.1N NaOH at RT; (b) 0.5N NaOH at RT; (c) 1N NaOH at RT; (d) 0.1N NaOH at 75°C; (e) 0.5N NaOH at 75°C; (f) 1N NaOH at 75°C; (g) 2N NaOH at 75°C.

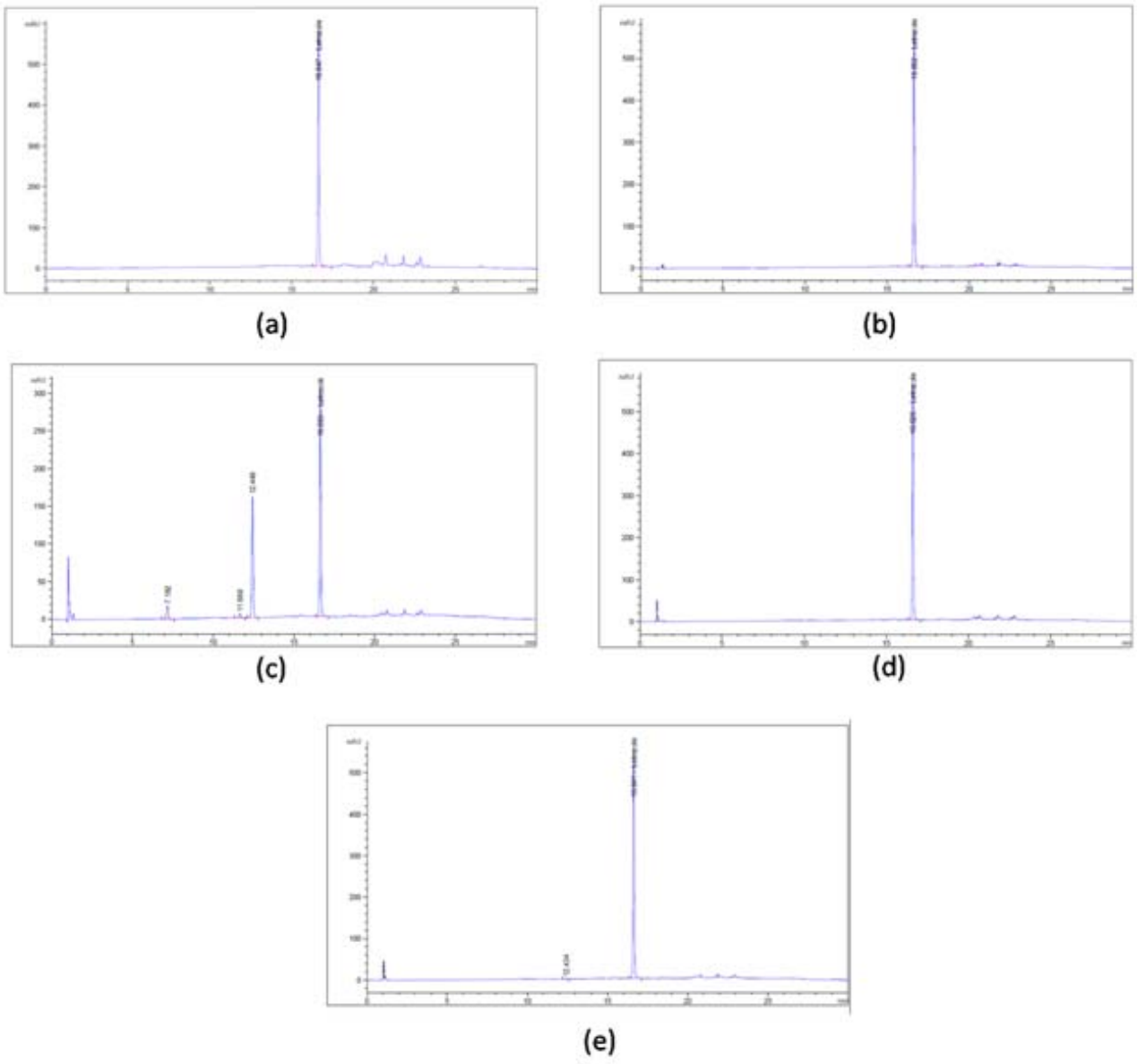


Fig. 4: HPLC chromatogram of degraded letrozole in (a) 0.1N HCl at RT; (b) 0.5N HCl at RT; (c) 1N HCl at RT; (d) 1N HCl at 75°C; (e) 2N HCl at 75°C.

Precision

The intra-day precision of the method was assessed by six determinations for each of the three concentrations of letrozole (20-25-30µgmL^{-1}). The repeatability of sample and measurement of peak area for active compound were expressed in terms of percentage relative standard deviation (%R.S.D.) and found to be less than 1% in three concentrations. Besides, inter-day precision (using the same three concentrations in triplicates for three consecutive days) was carried out for letrozole and results are displayed in table 2.

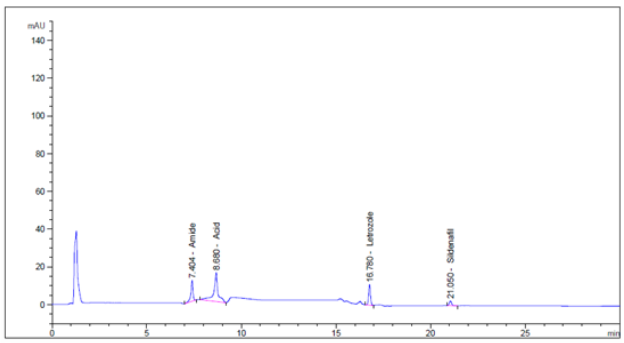
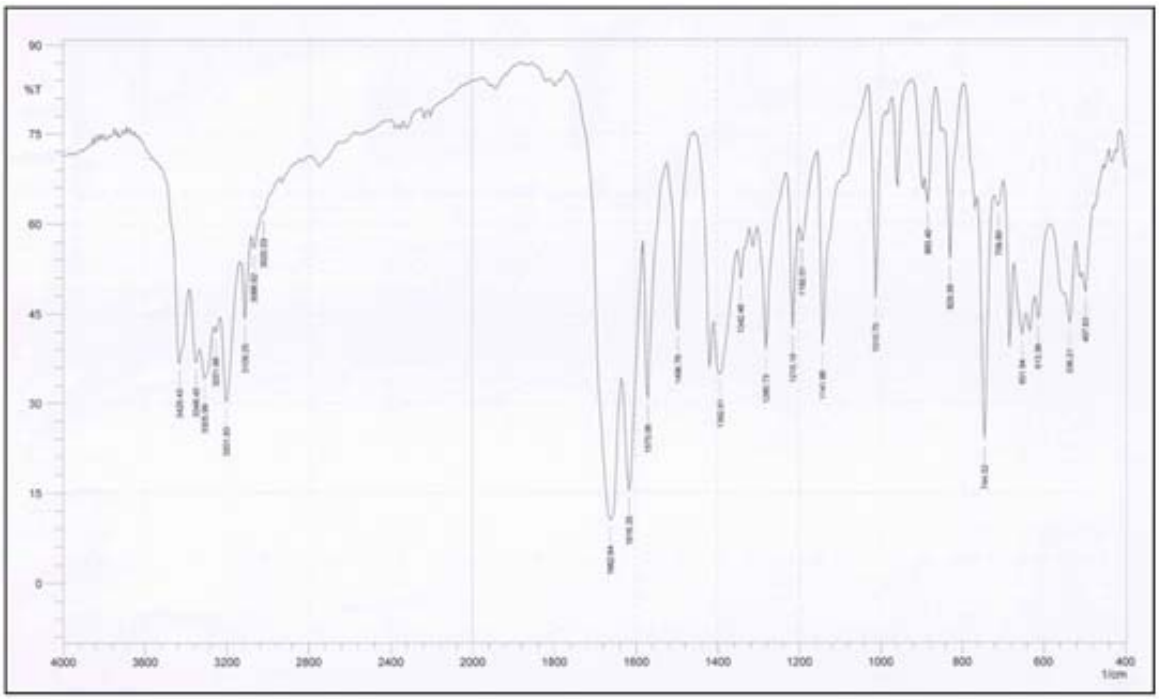
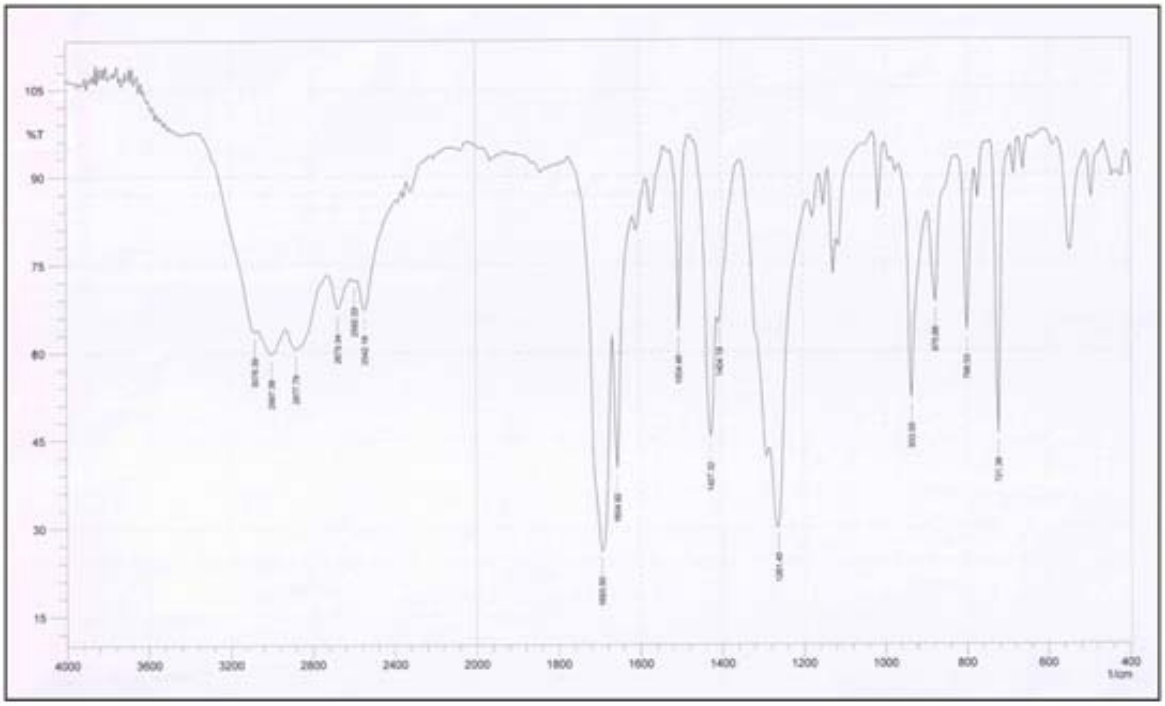


Fig. 5: HPLC chromatogram of letrozole in human plasma.

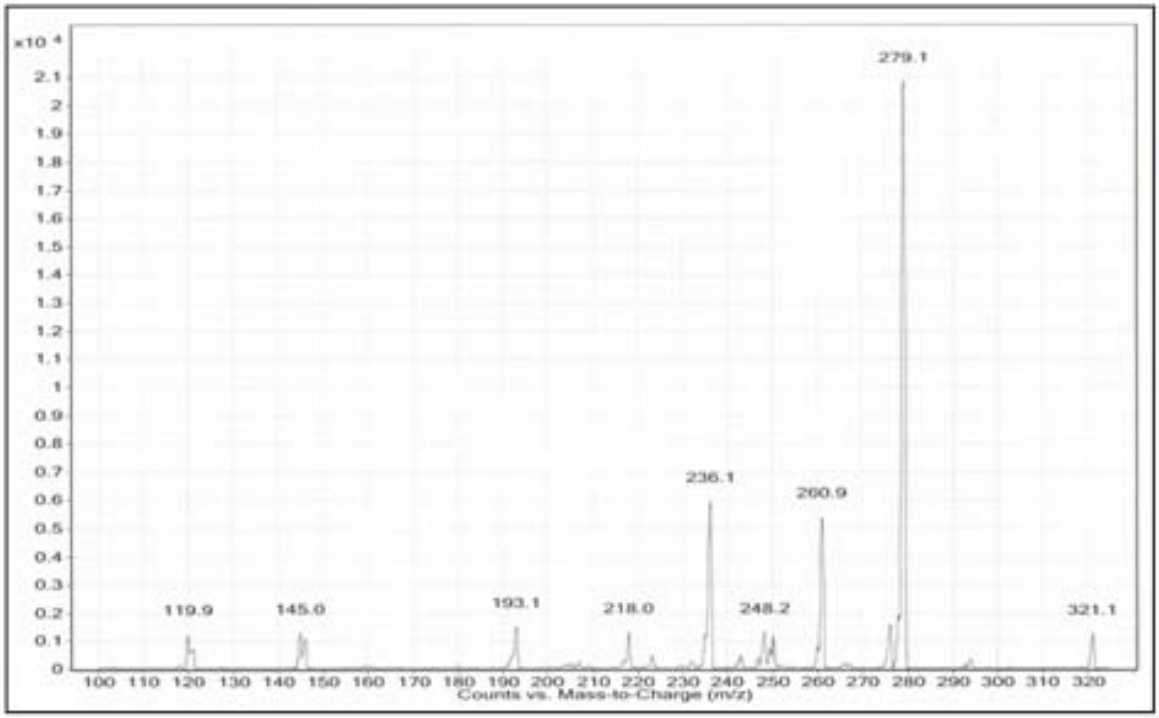


(a)

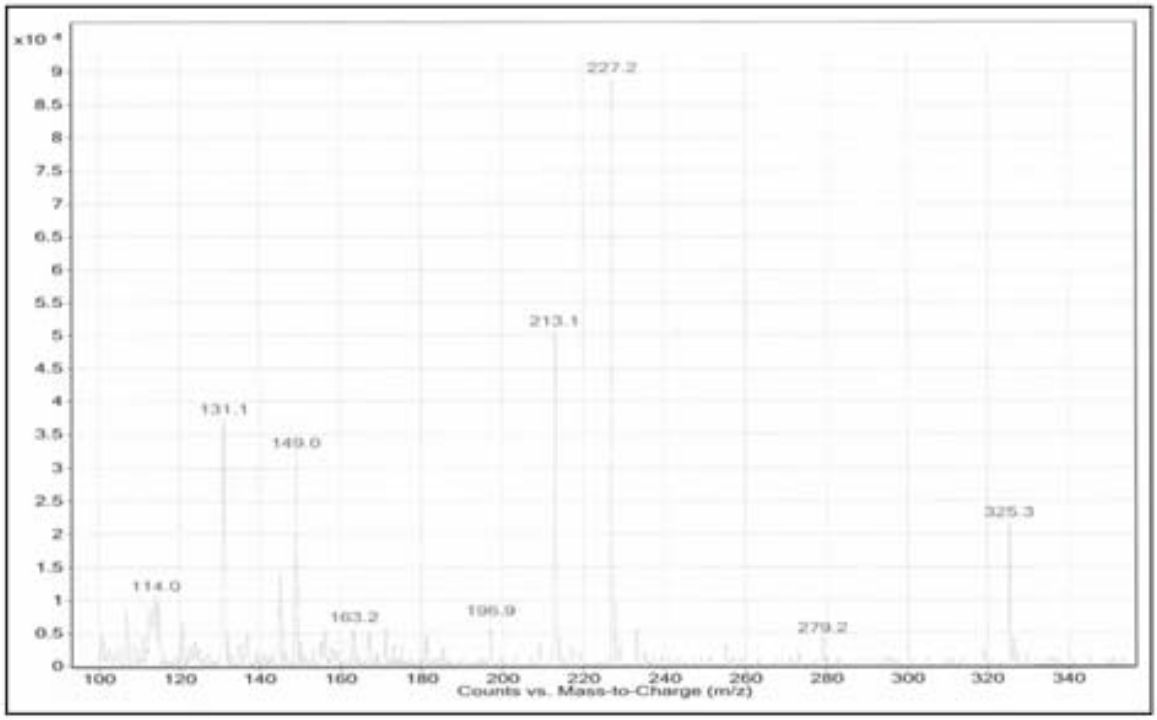


(b)

Fig. 6: IR spectra of (a) amide degradation product, (b) acid degradation product.



(a)



(b)

Fig. 7: Mass spectra of (a) amide degradation product, (b) acid degradation product.

Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. Good resolution and absence of interference from any of the degradation products are shown in figs. (2-4). Besides, the chromatogram of the pharmaceutical formulation samples was checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention time of the examined drug after extraction of the active ingredient. Moreover, our method was capable of detecting spiked letrozole and its synthesized degradation products in human plasma without any interference, fig. 5. In addition, there was no difference between the chromatogram of letrozole in the sample solution and that obtained by the standard solution. Moreover, results close to 100% were obtained for the determination of letrozole in dosage form, table 2. These results confirm the specificity of the proposed method.

Limit of detection and limit of quantitation

Limit of detection (LOD) which represent the concentration of the analyte at S/N ratio of 3 and limit of quantification (LOQ) representing the concentration of analyte at S/N ratio of 10 were determined experimentally for the proposed methods and results are given in table 2.

DISCUSSION

Method development

During the development phase, various mobile phase compositions like methanol with water, or acetonitrile with water, in different proportions, were attempted in an isocratic mode using Zorbax Eclipse C18 column (100 mm x 4.6 mm, 3.5µm). All attempts with isocratic mode could not succeed to resolve the peak of letrozole from peaks of its acid or amide degradation products. Hence, an attempt has been made to develop gradient elution instead of isocratic elution. Based on these investigations, good chromatographic separation between letrozole and its degradation products was achieved by the use of a gradient mobile phase system consisting of (A) 0.01M aqueous monobasic potassium phosphate- (B) methanol with a gradient elution starting with90% (A), decreasing to 70% in 8 min, holding for 1 min, decreasing to 30% in 11 min, holding for 5 min then rising to 90% in 5 min. Detection was carried out at 230 nm to obtain sufficient peak intensity for both drugs and degradants. In literature, two stability-indicating HPLC methods were reported for the determination of letrozole in the presence of its degradation products but none of them could identify any of the obtained degradants (Annapurna *et al.*, 2012; Ranganathan *et al.*, 2012). In the present work, optimization was carried out in order to reach better resolution between peaks of letrozole and degradants using simple mobile phase composition. The retention time of letrozole was found to be 16.67 min, fig. 2.Complete method validation was checked for the determination of letrozole in bulk but in plasma, only linearity was checked, suggesting a good method of extraction and an internal standard for further bioanalytical method validation.

Partial and complete hydrolysis of letrozole

Amide and acid degradation products were prepared by carrying out partial and complete hydrolyses of letrozole, respectively, using reported methods for the hydrolysis of aromatic nitriles (Furniss *et al.*, 1994). The structures of the degradation products were confirmed by IR spectroscopic investigation (fig. 6) in which the characteristic peak of nitrile groups at 2200cm^{-1} disappeared and the peaks of the carbonyl groups of the amide and the acid product appeared at 1662.64 and 1693.50cm^{-1}, respectively. For the amide, new peaks of the two primary amino groups appeared at 3305.99-3429.43cm^{-1}confirming partial hydrolysis of the nitrile groups, while for the acid degradation product, the characteristic peak of the carboxylic OH group appeared at 2542.18-2673.34cm^{-1}. Mass spectra (fig. 7) also confirmed the structures of the amide and acidic degradants by showing the molecular ion peak at 321.1 (M+) and 323.3 (M+2) respectively.

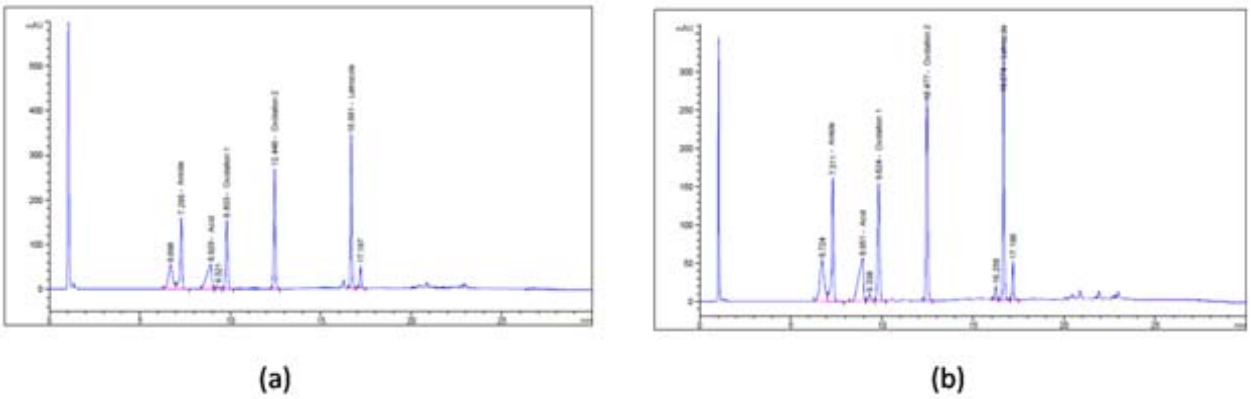
Degradation behavior

Acid- and base-induced degradation

Studying the chromatograms of the alkaline degraded samples for letrozole using different molarities, either on cold or on hot, it showed nearly complete disappearance of the intact drug and appearance of additional peaks at retention times of 5.8, 6.5min whose areas increase by increasing molarity (fig. 3). Also, additional peaks appeared at 7.2 (amide peak), 11.62, 12.43min only when using lower molarities, which are suggested to be due to intermediate products, which could not be identified. This reflects that letrozole is alkali-labile leading to partial hydrolysis of cyano to amide groups. Molarity of NaOH while heating was increased to 2M to test for the sensitivity of letrozole to alkaline medium. In 2M NaOH, further hydrolysis of amide to carboxylic acid occurs, fig. 2. Acidic degradation studies showed that the drug is more stable in acidic media. Only in high molarities as 1M HCl, additional peaks of intermediate products and that of the amide appeared, as shown in fig. 4. The peaks of degraded products were well resolved from the drug peak.

Hydrogen peroxide-induced degradation

The sample degraded with 3% and 15% v/v hydrogen peroxide showed additional peak at 9.79min. This may be attributed to the formation of N-oxide derivative of the triazole ring. Method was validated for the resolution ability of intact drug and all degradants peaks was confirmed by spiking the oxidized solution with amide and acid degradation products (fig. 8)



(a) (b)

Fig. 8: HPLC chromatogram of degraded letrozole in (a) 3% H_2O_2spiked with amide and acid degradation products; (b) 15% H_2O_2spiked with amide and acid degradation products

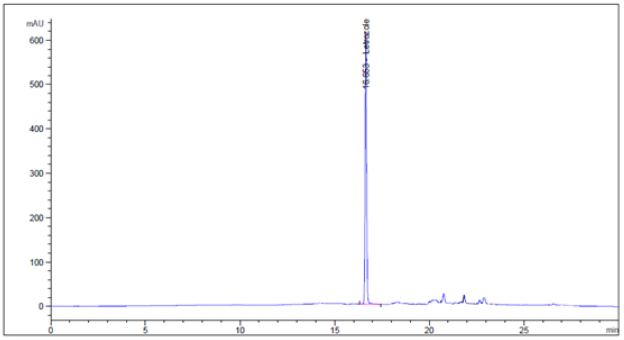


Fig. 9: HPLC chromatogram of dry heat degraded letrozole.

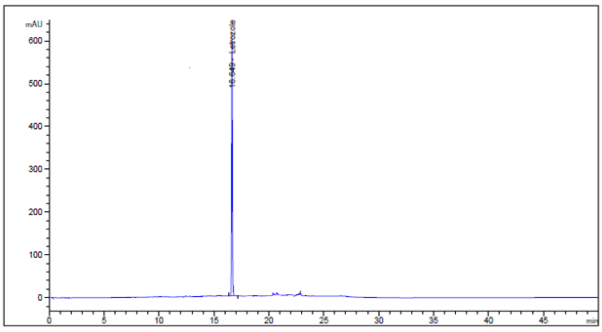


Fig. 10: HPLC chromatogram of sunlight-degraded letrozole.

Thermal and photolytic degradation

The samples degraded under dry heat conditions (fig. 9) and the photo degraded sample (fig. 10) showed no additional peaks.

From the aforementioned data, the drug was found to be sensitive to acid and base hydrolysis and oxidation while resistant to dry heat degradation, and photo degradation.

CONCLUSION

The degradation behavior of letrozole under acid, alkali, oxidation, thermal and photolysis conditions was studied by developing a validated stability-indicating LC method. The drug was found to be degraded in alkaline and acidic conditions due to the presence of cyano groups, which is susceptible to partial or complete hydrolysis, and also it was found to be susceptible to be degraded in oxidation conditions but stable to thermal and photolysis conditions. The amide and acidic hydrolytic products were found to be true degradation products of letrozole. The proposed LC method is simple, precise, accurate and convenient for the separation and quantification of letrozole either alone or in the presence of its degradants. Therefore, the proposed LC method can be used for the quality control of the cited drug.

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