Development and Validation of Stability Indicating Method for the Determination of Exemestane by Reverse Phase High Performance Liquid Chromatography

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Abstract

Exemestane is an aromatase inhibitor used in the treatment of breast cancer. A selective stability-indicating reversed-phase high performance liquid chromatography (RP-HPLC) method has been developed which can separate and accurately quantitate low levels of exemestane. The stability-indicating capability of the method was demonstrated by adequate separation of exemestane and all the degradation product peaks from exemestane peak and also from each other in stability samples of exemestane. Chromatographic separation of exemestane and its degraded products were achieved by using isocratic elution at a flow rate of 1.0 mL/min on a C18 reverse phase column (Phenomenex, size: 250 × 4.60 mm, particle size 5 µm) at ambient temperature. The mobile phase used for the analysis was acetonitrile–water (60:40, %v/v) with UV visible detection at 242 nm. The proposed method was used to study the degradation behavior of drug under various stress conditions as per ICH recommended guidelines.

Introduction

Chemically exemestane is (8R,9S,10R,13S,14S)-10,13-dimethyl-6-methylidene-7,8,9,11,12,14,15,16-octahydrocyclopenta[a]phenanthrene-3,17-dione as shown in Figure 1. Aromatase is an enzyme that catalyzes various steps in the conversion of androgens to estrogens in peripheral tissues. It is the principle source of circulating estrogens in the post menopausal women (1). Exemestane is a potent and irreversible steroidal aromatase inactivator (2). It inhibits the conversion of adrenally generated androstenedione to estrone by aromatase in peripheral tissues, such as adipose tissues as well as tumours (3). Unlike non-steroidal inhibitors, exemestane acts as a false substrate for the aromatase enzyme and is processed to an intermediate that binds irreversibly to the active site of the enzyme, causing its inactivation (4).

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The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry and is expected to allow analysis of individual degradation products. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products (5).

In the literature, several LC methods were reported for determination of exemestane in biological samples (6–9). Drug is also reported for stability indicating LC method (10). During the extensive literature review it was observed that, no rapid and precise stability indicating LC method was reported for quantification of exemestane and its degradation products.

The main aim of this work was to develop a stability indicating LC method, which is selective for the quantification of all possible degradants and determination of exemestane. The developed method was validated as per ICH guidelines (11).

Experimental

Materials and reagents

Exemestane drug substance was obtained from Cadila Healthcare Ltd, Ahmedabad, India. HPLC grade acetonitrile, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, MA).

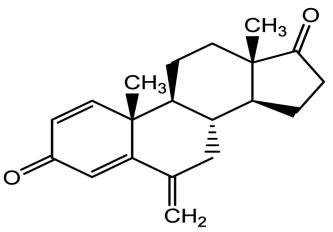

Figure 1. Chemical structure of exemestane.

HPLC

Instrumentation and software

A HPLC (PerkinElmer, Waltham, MA, Model: Lambda 25,) consisting of Binary LC Pump 200B/250 (PerkinElmer, Model: series 200), vacuum degasser, UV–vis detector (PerkinElmer, Model: series 200), C18 reverse phase column (Phenomenex, Torrance, CA, size: 250 × 4.60 mm, particle size 5 µm) and a sample injector system (Rheodyne, Oak Harbor, WA) with a 20 µL sample loop and Total Chrome Navigator software (version V 6.3.1) on computer (operated with Windows 2003 Professional).

Chromatographic conditions

The chromatographic column used was phenomenex, C18 (250 mm × 4.6 mm) column with 5 µm particles. The mobile phase consists of acetonitrile (solvent A), and water (solvent B). The flow rate of the mobile phase was kept at 1.0 mL/min and the column temperature was maintained at ambient and the chromatogram was monitored at a wavelength of 242 nm. The injection volume was 20 µL. A mixture of acetonitrile and water (60:40, v/v) was used as a diluent.

Preparation of standard solutions

Diluted standard solution of exemestane at a level of 10 µL/mL was prepared from reference standard (1000 µL/mL) to quantify the impurities in degradation sample analysis. A stock solution of degradation samples (acid hydrolysis, base hydrolysis, thermal, oxidation, and photolytic) at 10 µL/mL was also prepared in diluent.

Selection of detection wavelength

A 10 µL/mL solution of exemestane was prepared in acetonitrile–water (60:40) as a diluent. Solution was scanned using double beam UV–vis spectrophotometer between the range of 200 to 400 nm. The maximum absorbance wavelength of Exemestane was found to be 242 nm as illustrated in Figure 2.

Selection of mobile phase

Different mobile phase systems like methanol–water, acetonitrile–water were tried in order to determine the best composition for separation of exemestane. It was found that acetonitrile–water (60:40, %v/v) gives good separation results and satisfactory peak symmetry as compared to others.

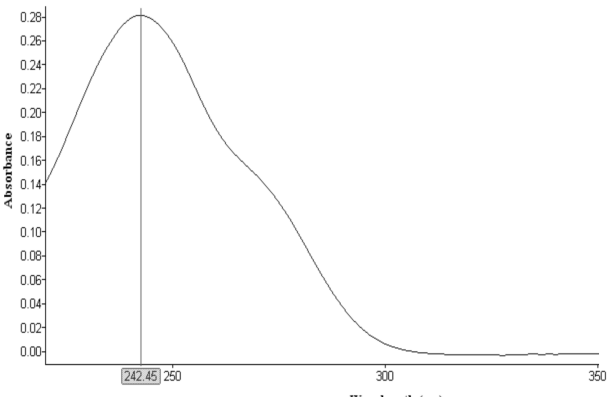

Figure 2. UV absorbance spectra of exemestane

Forced degradation studies of exemestane

Forced degradation of exemestane drug substance was carried out under acid/neutral/alkaline hydrolytic, oxidative, thermal and photolytic stress conditions. For hydrolytic and oxidative degradation, drug solutions were prepared with a concentration of 100 µg/mL. After degradation, aliquots were diluted to achieve a concentration of 10 µL/mL.

Hydrolytic degradation studies were carried out under acid (1 M HCl), neutral (water), and alkaline (1M NaOH) conditions by refluxing at a temperature of 80°C for 12 h. Oxidative degradation was carried out by exposing drug to 0.3% H_2O_2 solution at room temperature for 72 h.

Thermal degradation of exemestane drug substance was carried out in the solid state. For thermal degradation, the drug was spread in a borosilicate glass petri dish and placed in the hot-air oven maintained at 120°C for 24 h. Photo degradation studies were conducted by exposing the drug sample in photostability chamber (Thermolab 400G New Delhi India) at 1.2 million lux h for a total period of one week. After degradation, stock solutions were prepared by dissolving the samples in diluent to achieve a concentration of 1 mg/mL. From these solutions, aliquots were diluted to get the final concentration of 10 µL/mL of exemestane.

Preparation of calibration curve

Appropriate aliquots of standard stock solution (1000 µL/mL) was diluted to 100 µL/mL in 10 mL volumetric flask and resultant solution was diluted up to the mark with mobile phase to obtain final concentrations of 6, 8, 10, 12 and 14 µL/mL. These solutions were run into chromatographic system and the peak area was determined for each concentration of drug solution. Calibration curve of exemestane were constructed by plotting peak area ratio vs. concentration of exemestane and regression equation was computed and is shown in Table I.

Method development

Detection wavelength for the HPLC study was selected as 242 nm. The chromatographic conditions were optimized for resolution of the peak of the drug and degradation products under each forced degradation condition by varying the proportion of acetonitrile–water in the mobile phase. Subsequently, a mixture of samples of different stress conditions was used to optimize the chromatographic conditions for resolving exemestane and all the degradation products in a single run. An appropriate blank was injected before the analysis of all forced degradation samples. The method was then used to study the forced degradation behavior of exemestane and was also applied for the determination of exemestane.

Table I. Data for Calibration Curve

Sr. No.	Concentration (µg/mL)	Peak area
1	6	584173.86
2	8	792628.08
3	10	966578.04
4	12	1171116.28
5	14	1353103.47

Method Validation

As per the USP 30, NF 25 system suitability tests for HPLC were carried out on freshly prepared standard stock solution of exemestane. The method was validated for accuracy, precision, specificity, detection limit, quantitation limit and robustness by following procedures (12–16).

Linearity of the method was investigated by serially diluting the stock solution to give a concentration range of 6 to 14 µg/mL and injected 20 µL with universal injector (Rheodyne) (12–14). Calibration curve was constructed by plotting concentration against peak area as shown in Figure 3. The intra-day and inter-day precision study of exemestane was carried out by estimating the corresponding responses six times on the same day and on three different days (first, third, and fifth day) for three different concentrations of exemestane (8, 10, and 12 µg/mL) and the result are reported in terms of relative standard deviation (12–14). Accuracy/recovery was evaluated by spiking the mixture of degradation samples with three known drug concentrations and calculating the percent recovery from the differences between the peak areas obtained for concentrated and diluted solutions. The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities. Complete separation of exemestane in mobile phase was noticed. The average retention time was found to be 7.12 min respectively for six replicates. The peaks obtained were sharp and have clear baseline separation (14). The LOD and LOQ were separately determined based on the S/N ratio. For LOD the S/N ratio is 3:1 and for LOQ the ratio is 10:1. Robustness of the method was studied by changing the composition of organic phase by ±5% and also by changing the flow rate by ±0.1 mL/min and detection wavelength by 2 nm (14).

Results and Discusssion

The HPLC method developed was optimized with a view to develop a stability indicating method for exemestane. The mobile phase consisting of acetonitrile and water (60:40, %v/v) gave good resolution, sharp and symmetrical peak with a retention time of 7.12 min as shown in Figure 4A. The main target of the chromatographic method was to separate all the degradation products from exemestane standard. In the initial stages of the method development the ratio of the mobile phase was fixed as 80:20 (%v/v) of acetonitrile–water. In the later stages for the separation of degradants from the pure drug peak the mobile phase ratio was modified to 60:40 (%v/v) of acetonitrile–water and it was used as a final method for the analysis.

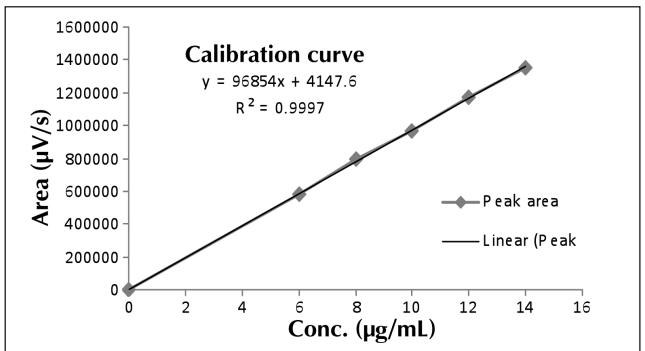

Figure 3. Calibration curve of exemestane.

Validation parameters

Method was validated as per ICH guideline with respect to linearity, accuracy, precision, specificity, robustness, limit of detection, and limit of quantification.

Linearity

Peak areas obtained with respective concentrations were subjected to the least square linear regression analysis to calculate the calibration equations and correlation coefficients. The calibration plot for exemestane assay was linear over the calibration range 6–14 µL/mL and the regression coefficient, slope, and intercept were 0.9997, 96854, and 4147.6 respectively. The linear regression data is computed in Table II.

Precision

Intra-day precision was expressed though relative standard deviation of seven repeated assays of samples at three concentration levels. Inter-day precision was determined by analyzing the same set of samples on five different days. Relative standard deviation in the precision study for the exemestane assay was less than 1.0 and confirmed that the method was highly precise as illustrated in Table III.

Recovery

Standard addition method was used to examine the recovery of the RP-HPLC method. Recovery of exemestane from bulk drug samples ranged from 99.1% to 100.5%. Results of recovery studies are shown in Table IV.

Limits of detection and quantification

LOD was 8 ng/mL for exemestane at a signal-to-noise ratio of 3:1 and the limit of quantification was determined as 15 ng/mL for Exemestane at a signal-to-noise ratio of 10:1.

Table II. Linear Regression Data for Calibration Curve

Parameters	Values
Range	6–14 µg/mL
r^2 value	0.9997
Slope	96854

Table III. Precision Studies

Actual conc. (µg/mL)	Calculated concentration ± S.D. (µg/mL), RSD (%)	
	Repeatability (n = 6)	Intermediate precision (n = 3)
8	7.908 ± 1.43,1.44	8.128 ± 1.49,1.46
10	10.176 ± 1.81,1.77	10.192 ± 1.82,1.78
12	12.130 ± 1.28,1.26	11.860 ± 1.42, 1.44

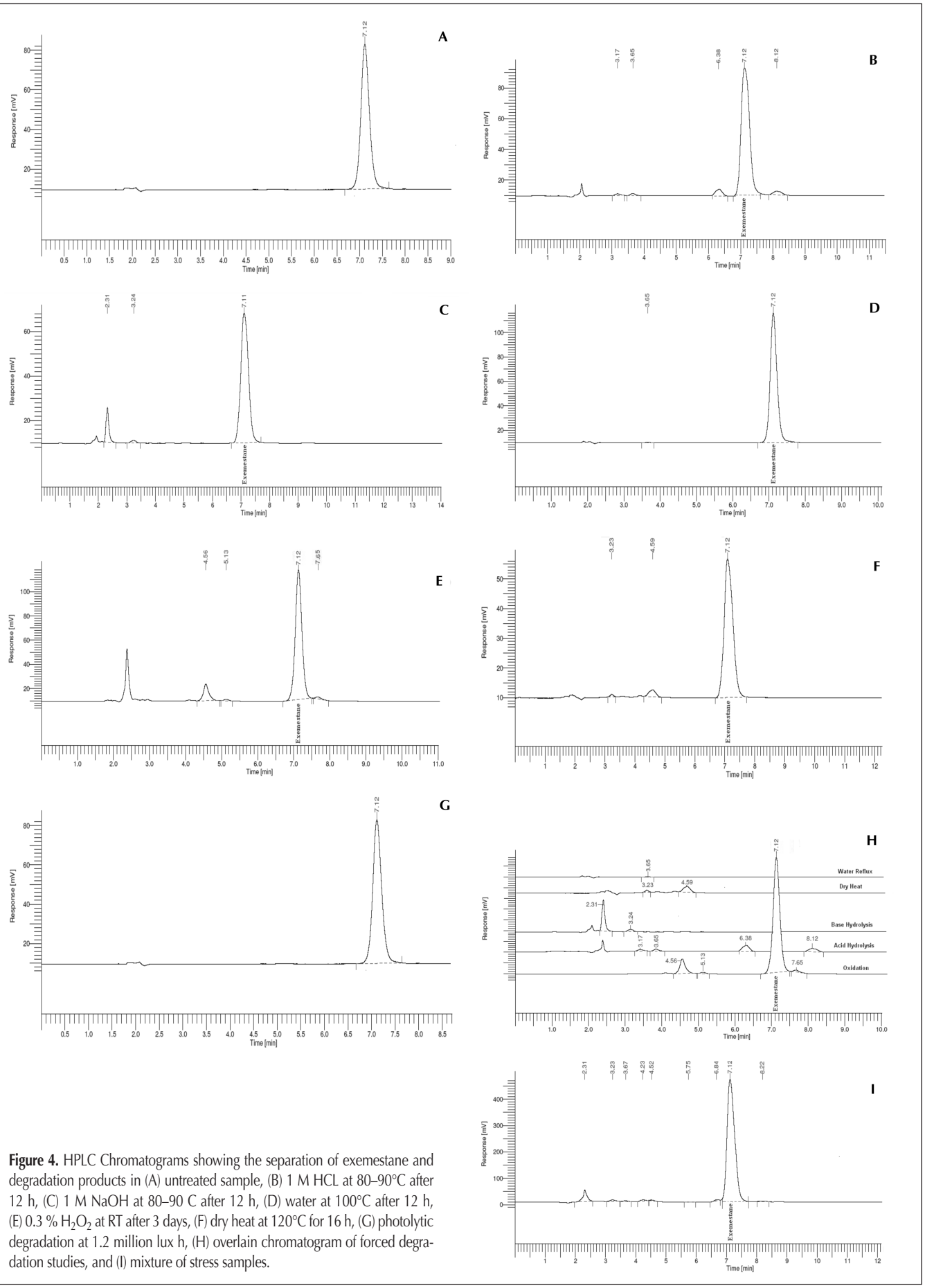

Figure 4. HPLC Chromatograms showing the separation of exemestane and degradation products in (A) untreated sample, (B) 1 M HCL at 80–90°C after 12 h, (C) 1 M NaOH at 80–90 C after 12 h, (D) water at 100°C after 12 h, (E) 0.3 % H_2O_2 at RT after 3 days, (F) dry heat at 120°C for 16 h, (G) photolytic degradation at 1.2 million lux h, (H) overlain chromatogram of forced degradation studies, and (I) mixture of stress samples.

Specificity

There was no interference due to sample diluents and degradation products. Resolution between closely eluting degradation products, and exemestane was greater than 2.0, indicating the stability indicating capability of the method.

Robustness

The chromatograms obtained by varying the parameters like flow rate, mobile phase composition and maximum absorbance wavelength were found to be satisfactory.

Forced degradation

Acid induced-degradation

On refluxing the drug in acidic conditions (1 N HCl) for 12 h at 80°C, additional peaks were observed at 3.17, 3.65, 6.38, and 8.12 min of retention time, indicating the formation of four unknown degradation products. As shown in the chromatogram, almost 7.34% degradation was observed. The assay of the active substance in acid degraded sample was found to be 92.66 % as shown in Figure 4B.

Base induced-degradation

The chromatogram of the base induced degraded samples of exemestane showed additional peaks at 2.32 and 3.24 min of retention time. As compared to acidic conditions, exemestane was found to be more susceptible to alkaline conditions, while refluxing at 80°C for 12 h. Around 10% of the degradation was recorded. The assay of the active substance in base catalyzed degraded sample was found to be 90.12% as shown in Figure 4C.

Neutral catalyzed degradation

On exposing the drug to water reflux at 80°C for 12 h, a very negligible degradation was observed at 3.65 min of retention time. Around 0.30% degradation was observed. The assay of the active substance in water catalyzed degraded sample was found to be 99.70% as shown in Figure 4D.

Oxidative-degradation

On keeping the drug in 0.3% H_2O_2 at room temperature for 72 h it was revealed that exemestane peak area decreased significantly and additional peaks were observed at 4.56, 5.13, and 7.65 min of retention time, indicating that drug is highly susceptible to undergo oxidative degradation. Around 11.15% degradation was observed. The assay of the active substance in acid degraded sample was found to be 88.85% as shown in Figure 4E.

Thermal degradation

When the drug exposed to dry heat in oven at 120°C for 24 h, it was observed that there was rise in two degradation products at 3.23 and 4.59 min of retention time and also a significant change in peak area of the drug was observed. Almost 4.39% degradation was observed. The assay of the active substance in thermal degraded sample was found to be 95.61% as shown in Figure 4F.

Photolytic degradation

When drug was exposed to photolytic degradation at 1.2 million lux h for a period of one week, no degradation was observed as shown in the chromatogram. The assay of the active substance in photo degraded sample was found to be 100 % as shown in Figure 4G.

Summary of forced degradation studies

The results of the forced degradation studies i.e the assay of the active substance and the total degradants in the stability samples is given below in the Table V. The chromatogram showing the results of forced degradation studies and the mixture of stress samples is also shown in Figure 4H and Figure 4I, respectively.

Conclusion

The analytical method described in this paper is suitable for determination (assay) of exemestane and this method has been demonstrated to be accurate, linear, precise, specific and robust, which can be used for the routine analysis of exemestane during stability studies. This method is a stability indicating method and it can separate all degradation products from exemestane (API).

Table IV. Accuracy

Conc. of drug taken (µg/mL)	Conc. of standard added (µg/mL)	Conc. found ± S.D. (µg/mL), (n = 3)	% Recovery
10	8	17.912 ± 0.200	99.5
10	10	20.101 ± 0.324	100.5
10	12	21.812 ± 0.410	99.1

Table V. Results of Forced Degradation Studies

Stress Condition	Time	Assay of active substance (%w/w)	Total degradants (%w/w)	Remarks
Acid degradation	12 h	92.66	7.34	Three unknown degradation products formed
Base degradation	12 h	90.12	9.88	Two unknown degradation products formed
Neutral degradation	12 h	99.70	0.30	One unknown degradation product formed
Oxidation (0.3%H2O2,RT)	3 days	88.85	11.15	Two unknown degradation products formed
Heat (120°C)	24 h	95.61	4.39	Two unknown degradation products formed
Photo Degradation	1 week	100%	0.00	No degradation was observed

Additional findings in this study are, the method which is already reported uses 100% organic modifier as the mobile phase and retention time of drug was more than 20 min which is costly and time consuming. Our method was found to be cost effective and rapid, because the mobile phase is composed of acetonitrile:water (60:40, %v/v) with the retention time of drug at 7.12 min. From the results of the forced degradation studies it can be concluded that drug was found to degrade rapidly by oxidation, followed by base hydrolysis, acid hydrolysis, dry heat ,and neutral catalyzed degradation. As the method is successfully validated using ICH guidelines, it can be readily implemented in quality control laboratories for the purpose of lot release and stability testing of exemestane API.

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