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LC and LC–MS/MS study of forced decomposition behavior of anastrozole and establishment of validated stability-indicating analytical method for impurities estimation in low dose anastrozole tablets

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ABSTRACT

Anastrozole tablets were subjected to different ICH prescribed stress conditions of thermal, hydrolysis, humidity, photolysis and oxidation stress. The drug was found to be stable for all the stressed conditions except for oxidation. Separation of anastrozole from its potential impurities, degradation products and five anastrozole related compounds as main impurities were achieved on Inertsil ODS-3V, 250 mm × 4.6 mm i.d, 5 μm analytical column using reversed phase high performance liquid chromatography (RP-HPLC). The elution of impurities employed time dependent gradient programmed mobile phase consisting of water as mobile phase-A and acetonitrile as mobile phase-B at column flow rates of 1 ml/min and at 215 nm UV detection. The same method was also extended to LC–MS/MS studies which were carried out to identify the degradation product. The method developed was established to have sufficient intermediate precision as similar separation was achieved on another instrument handled by a different operator. The LOQ for anastrozole related compound-A (RC-A), related compound-B (RC-B), related compound-C (RC-C), related compound-D (RC-D), related compound-E (RC-E) and anastrozole were 0.05, 0.03, 0.03, 0.06, 0.06 and 0.06 μg ml^{-1} respectively. The linearity of the proposed method for all the above related compounds was investigated in the range of LOQ to 0.600 μg ml^{-1} respectively. The specificity was established through peak purity testing using a photo-diode array detector. Method was validated according to ICH guidelines and statistical analysis of the data proved to be suitable for stability testing at quality control.

1. Introduction

Anastrozole 2-[3(1-cyano-1-methyl-ethyl)-5-(1H-1,2,4-triazol-1-yl methyl) phenyl]-2-methyl-propinenitrile is a potent and selective non-steroidal aromatase inhibitor. It significantly lowers serum estradiol concentrations and has no detectable effect on formation of adrenal corticosteroids or aldosterone. Many breast cancers have estrogen receptors and growth of these tumors can be stimulated by estrogen. In postmenopausal women, the principal source of circulating estrogen (primarily estradiol) is conversion of adrenally generated androstenedione to estrone by aromatase in peripheral tissues, such as adipose tissue, with further conversion of estrone to estradiol. Armidex® [1] is the innovator reference listed drug (RLD) available as 1 mg tablets.

An official monograph of anastrozole tablets does not exist in any pharmacopoeia.

So far no analytical method on degradants and related compounds of anastrozole in anastrozole tablets are reported in the literature. A review of literature revealed two chromatographic methods. The reported method [2] focused only on the assay development while method [3] on isolation and characterization of only three process related impurities. Both these methods development were established on anastrozole active pharmaceutical ingredient (API). Also a number of reports [4–7] exist on procedures for its determination from biological fluids, such as plasma and urine. Therefore, it is very imperative to develop a suitable stability-indicating analytical method for anastrozole tablets such that the methods could be easily adapted for stability testing, routine and in-process quality control analysis or similar studies.

Now we present a method that establishes inherent stability of anastrozole in finished dosage form through forced decomposition

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studies under variety of conditions recommended by the International Conference on Harmonization (ICH) [8,9].

Anastrozole 1 mg tablet is a very low dose and potent drug product wherein the content of active ingredient in the dosage form is only 1%. Apart from the chromatographic separation, extraction of impurities at very micro-levels in the low dosage form as per ICH guidelines [10] was very much essential. The main hindrance during developmental studies was low sample concentration because of the low dose. However accurate and precise quantification at 0.1% level of 1 mg in the presence of 99% pharmaceutical excipients within the formulation matrix for all related compounds through rigorous validation process proved the extraction efficiency of the method.

2. Experimental

2.1. Reference substances, chemicals and reagents

Anastrozole API (99.7% pure) and its related compounds as main impurities, RC-A (88.1%), RC-B (98.5%), RC-C (88.8%), RC-D (99.7%) and RC-E (99.5%) used for the study were kindly supplied by Dr. Reddy's Laboratories Limited, Hyderabad, India (Fig. 1) and were used as such without any further purification. Anastrozole tablets 1 mg (98.8%) was obtained from Formulations Research Department of Dr. Reddy's Laboratories. HPLC grade acetonitrile were purchased from Merck, Germany. Sodium hydroxide was purchased from Ranbaxy Laboratories and hydrochloric acid was purchased from LOBA Chemie Pvt. Ltd. (India). Hydrogen peroxide was procured from s.d. Fine-chem Ltd. (India). All other reagents were of analytical reagent grade. High pure water was prepared by using Millipore Milli Q plus purification system.

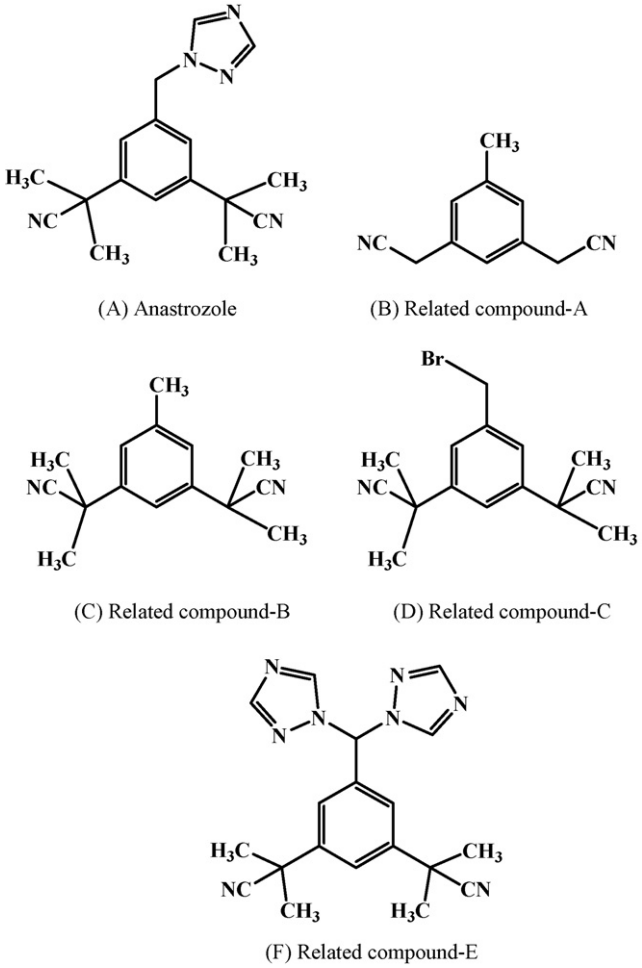

Fig. 1. Molecular structure of anastrozole and its related compounds (RC).

2.2. Instrumentation

2.2.1. HPLC conditions

The chromatographic separation was performed on Agilent HPLC 1100 series, Agilent Technologies, USA. The HPLC system consisted of an on-line degasser (G1379A), low pressure quaternary system delivery module (G1311A), auto injector and auto sampler (G1313A), column oven (G1316A) UV–vis detector (G1314A). The output signal was monitored and processed using Empower software (Waters) on Pentium computer (Digital Equipment Co.). Robustness and peak purity testing was done on another HPLC system equipped with separation module (Waters 2695 model) and photo-diode array detector (Waters 2996 model).

2.2.2. Mass-spectrometry conditions

Liquid chromatography–mass spectrometry (LC–MS/MS) analysis was carried out using MDS Sciex API, 4000 Q trap triple quadrupole mass spectrometer (Applied Biosystems, Foster city, CA, USA) coupled with Agilent HPLC 1100 series quaternary system delivery module. Analyst software (version 14.2) was used for data acquisition and data processing. LC–MS spectra were acquired from m/z 100 to 1000 in 0.1 amu steps with 2.0 s dwell time. Anastrozole degradation sample was subjected to LC–MS/MS analysis with similar chromatographic conditions as mentioned in Section 2.3.

2.3. Chromatographic conditions

Degassed water as solvent-A and acetonitrile as solvent-B was used as mobile phase. The impurities were eluted according to step gradient by changing the % of (solvent-B) at different times, T (min)/% solvent-B = 0/35, 20/35, 30/65, 50/65, 55/35 and 60/35. Inertsil ODS-3V, 250 mm × 4.6 mm i.d, 5 μm (GL Sciences Inc., Japan) stainless steel analytical column was used as stationary phase. Constant flow rate of 1.0 ml/min was employed throughout the analysis. UV–vis detector was set at 215 nm. All analyses were done at ambient room temperature and volume of solution injected on to the column was 50 μl. The chromatogram was collected up to 60 min.

2.4. Extraction solvent

Degassed mixture of water and acetonitrile in the ratio of 50:50 (v/v) respectively was found to be the best solvent to extract RC-A, RC-B, RC-C, RC-D, RC-E and anastrozole. Selection of diluent was made based on anastrozole solubility [1] and minimal interferences due to excipients used in the formulation matrix. Also, addition of water in the diluent helps to disintegrate the tablets.

2.5. Samples

Anastrozole 1 mg tablets (98.8% assay) and the corresponding placebo tablets (without API) were used throughout the development and validation. Other test samples used were accelerated stability samples as per ICH [11] with similar composition. All the samples were treated according to test solution preparation.

2.6. Solution preparation

2.6.1. Standard solution

Anastrozole API was accurately weighed and dissolved in the diluent so as to obtain a concentration of 0.40 μg ml^{-1} (0.2% of the concentration of the anastrozole in the test solution).

Table 1
Relative retention times (RRT); *RRF* values and LOD-LOQ.

RC	RRT	*RRF*	LOD		LOQ	
			Signal/noise = 3:1	($\mu g\,ml^{-1}$)	Signal/noise = 10:1	($\mu g\,ml^{-1}$)
RC-A	1.20	1.4	2.66	0.016	10.20	0.054
RC-B	2.04	1.1	3.21	0.010	9.86	0.032
RC-C	2.09	1.0	2.94	0.011	9.54	0.037
RC-D	0.57	0.9	2.70	0.019	10.33	0.063
RC-E	0.68	0.9	2.92	0.018	9.52	0.059
Anastrozole	1.00	1.0	2.61	0.020	9.51	0.065

2.6.2. Test solution

Ten intact tablets were taken in a 50 ml volumetric flask, added about 40 ml of diluent. The contents of flask were kept on a rotary shaker for about 10 min (until the tablet disintegrates completely) and sonicated for 30 min with intermediate shaking (by maintaining the sonicator temperature at about 25 °C). Finally, volume was completed with diluent and mixed well so as to obtain anastrozole concentration of about 200 $\mu g\,ml^{-1}$. The solution was centrifuged in a tight enclosure for about 5 min at 3500 rpm and 50 μl of clear supernatant solution was injected directly on to the column.

2.7. Quantitation

Peak areas were recorded for all the major peaks after blank (diluent) and placebo peaks correction. Respective peak areas were taken into account to quantitate [10,12] the amounts in percentage as follows:

$$\frac{A \times 50 \times C}{B \times 10 \times RRF}$$

where *A* is the peak area obtained for any individual peak apart from anastrozole in the test solution; *B* is the peak area obtained in the standard solution; *C* is the concentration of standard solution; 50 and 10 are the values obtained from the dilution factors of test solution; *RRF* is the relative response factor (Table 1).

2.8. Forced decomposition studies for establishment of stability-indicating

Drug product (anastrozole tablets) and the placebo tablets were used in all decomposition studies. The pH of the buffered solutions was measured before and after the reaction and no change in the pH was observed. All the solutions for use in forced degradation studies were prepared by dissolving the drug product in small volumes of stressing reagents. After the degradation these solutions were diluted with diluent to yield stated concentration of 200 $\mu g\,ml^{-1}$. Conditions employed for performing stress studies were as follows.

2.8.1. Hydrolytic studies

Acid decomposition studies were performed by heating the drug product solution in 0.5 M HCl at 80 °C on a water bath for 2 h. The studies in alkaline conditions were done in 0.5 M NaOH at 80 °C for 2 h. For the study in neutral conditions, the drug product solution in water was heated at 80 °C for 2 h prior to analysis.

2.8.2. Oxidation studies

Solutions for use in oxidation studies were prepared in 5% hydrogen peroxide and the resultant solution heated on a water bath at 80 °C for 1 h was injected prior to analysis.

2.8.3. Thermal stress studies

Anastrozole tablets and placebo tablets were exposed to dry heat of 100 °C in a convention oven for 7 days.

2.8.4. Humidity stress studies

A saturated solution of potassium sulphate was prepared and placed in a dry glass decicator at 25 °C which produced about 88–90% of relative humidity. Tablets of anastrozole and placebo were kept open on a petri dish in the above glass desiccator at 25 °C/90% RH, and the samples were analyzed after 15 days.

2.8.5. Photostability studies

Susceptibility of the drug product to light was studied [13]. Tablets for photo stability testing were placed in a light cabinet and exposed to light resulting in an overall illumination of ≥200 Wh/m^2 at 25 °C with UV radiation at 320–400 nm. Control samples which were protected from light with aluminum foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

3. Results and discussions

3.1. Degradation behavior of anastrozole

HPLC studies of samples obtained on forced decomposition studies of anastrozole under different conditions and by using the above experimental conditions suggested the following degradation behavior.

3.1.1. Hydrolytic, photostability, humidity and thermal stress studies

The drug was found to be highly stable under 0.5 M HCl, 0.5 M NaOH, and neutral (water) conditions at 80 °C for 2 h. No major degradants were observed in any of these conditions. Thermal and light stress also had no effect on anastrozole tablets and the tablets were found to be stable even at high humidity. The % impurities in all these samples were found to be <0.2%.

3.1.2. Oxidation studies

The drug was found to be highly labile to hydrogen peroxide at 80 °C for 1 h conditions. It decomposed to an extent of 15% in 1 h. The major degradation product had retention time of 5.7 min, and some small additional peaks were observed.

3.1.3. Identification and characterization of the major degradant by LC–MS/MS

From the above decomposition studies, an unknown degradant was observed at about retention time of 5.7 min under peroxide stress and was found to be the major degradant (Fig. 2). However this peak was not observed during any of the other decomposition studies and regular stability studies. The ESI spectrum of the unknown impurity at RT 5.7 min has displayed the molecular ion at *m*/*z* 311, the ESI spectra in positive ion mode further confirmed this with the presence of protonated molecular ion peak as base peak at *m*/*z* 312 (Q1 scan spectrum of unknown degradant is shown in Fig. 3A) which is 18 mass units higher than that of anastrozole (*m*/*z*: 293). The possibility of hydrolysis of cyano group

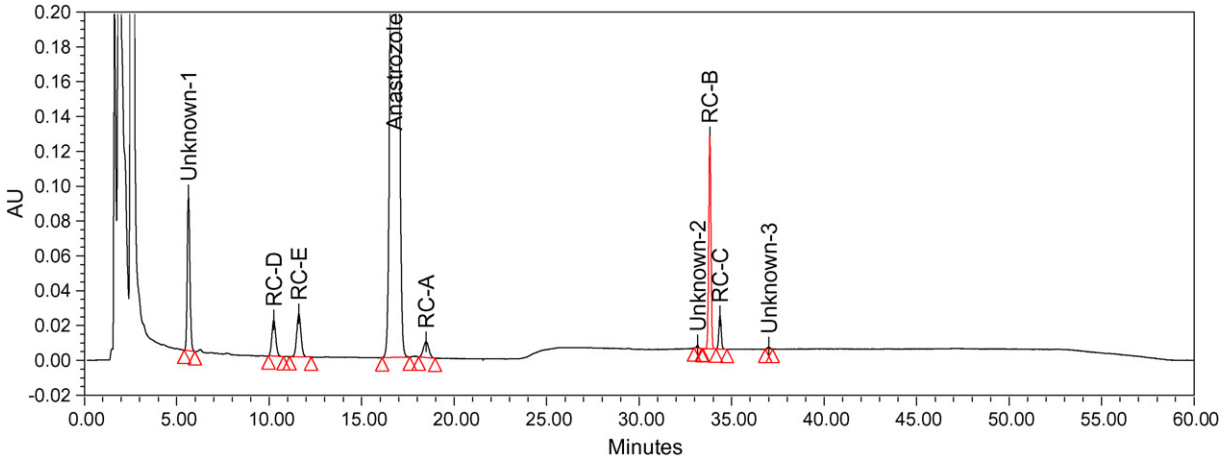

Fig. 2. Chromatographic separation of unknown degradant observed during peroxide stress, in the presence of spiked anastrozole related compounds.

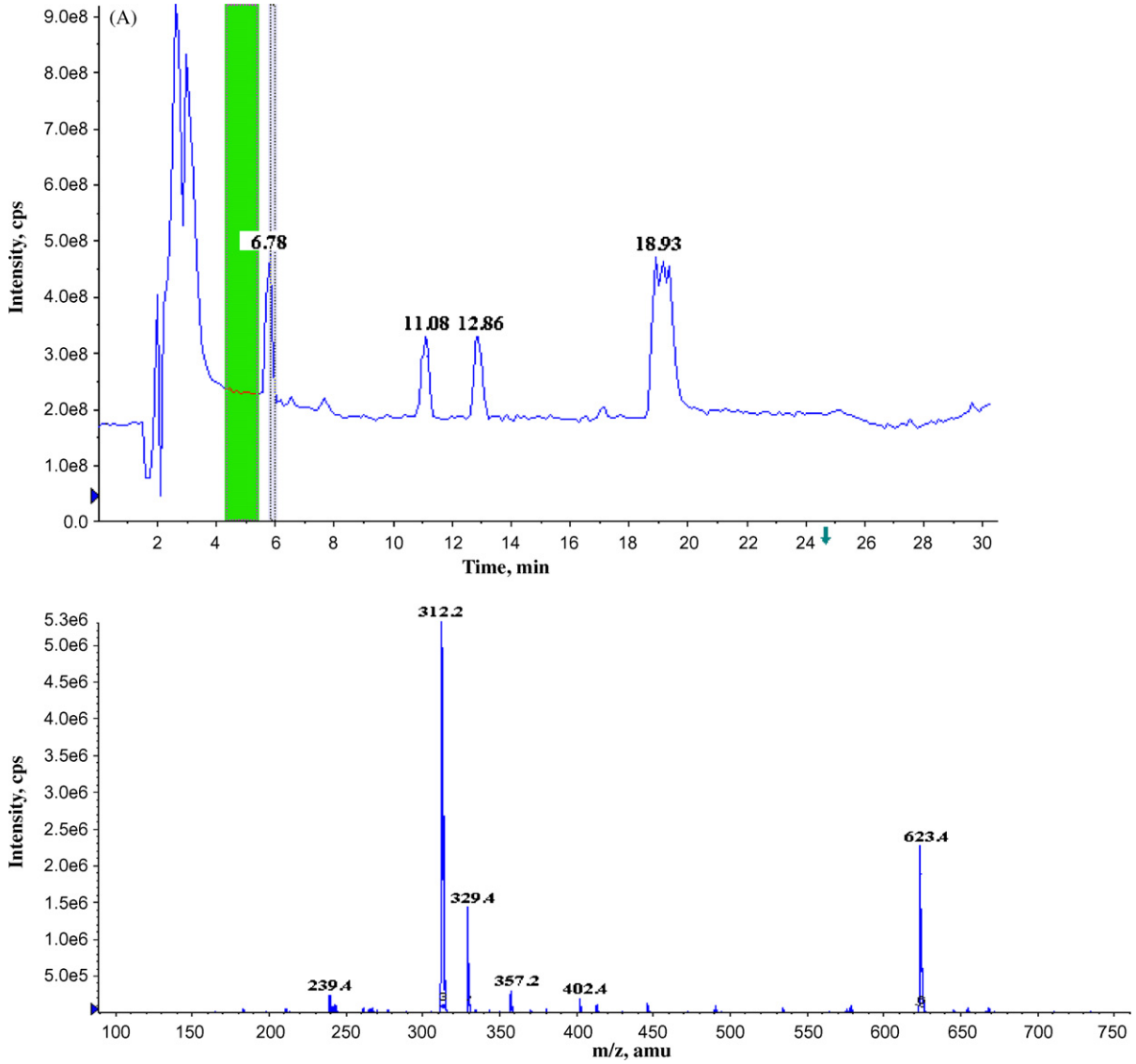

Fig. 3. (A) Q1 scan spectrum of unknown degradant peak observed at retention time of about 5.7 min under peroxide stress condition. (B) Fragmentation pattern (MS/MS spectra) of unknown degradant and anastrozole

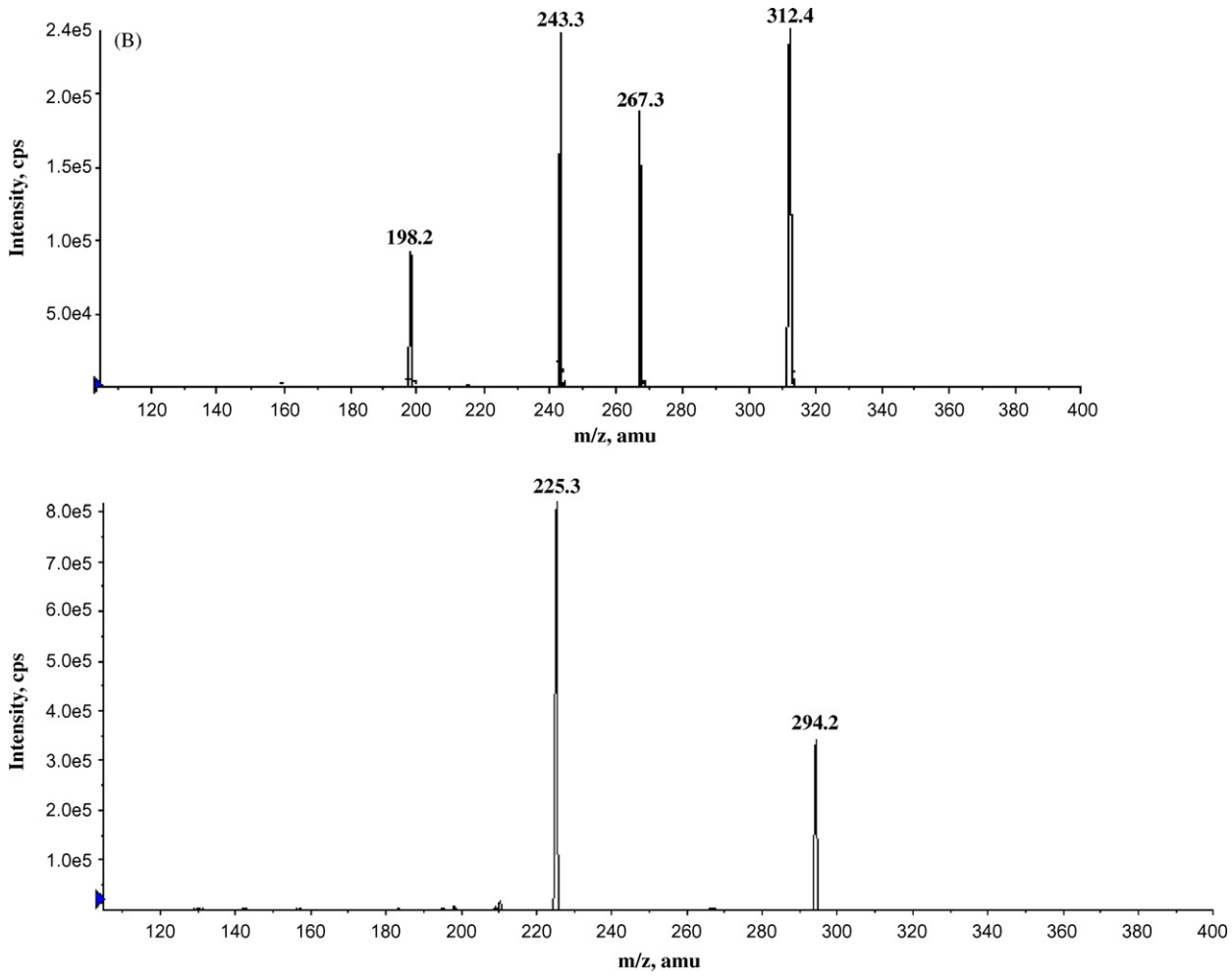

Fig. 3. (*Continued*).

fulfills the addition of 18 mass units to the molecular mass of anastrozole. Hence, the proposed impurity is a hydrolyzed product of anastrozole, an isobutyramide impurity, further fragmentation pattern (MS/MS spectra) of unknown degradant and anastrozole [5] is shown in Fig. 3B, which explains the hydrolysis of cyano group to carboxamide.

3.1.4. Separation studies

Initial separation studies were performed on the reported methods [2,3]. Lack of resolution between the peroxide degradant peak, related compounds and especially placebo peaks in the tablets, the above methods were found to be unsatisfactory. Also, 0.1% level of 1 mg dose for all RC's, were not detected in the above method. Hence the proposed method was developed. The mobile phase used in initial studies in this investigation was the same except methanol was used as solvent-B which resulted in longer retention times for RC-B, RC-C with poor detector response and less resolution. Alternatively acetonitrile as solvent-B was chosen and the gradient programme was modified accordingly so as to achieve good separation between RC-C and RC-B. Various brands of octadecyl (ODS) columns like waters, hypersil, develosil, inertsil and hichrome were used during development trials. However when the method in the present

Table 2
Weighted regression for RC's.

RC-A		RC-B		RC-C		RC-D		RC-E	
μg ml^{-1} (*X*)	Peak area (*Y*)	μg ml^{-1} (*X*)	Peak area (*Y*)	μg ml^{-1} (*X*)	Peak area (*Y*)	μg ml^{-1} (*X*)	Peak area (*Y*)	μg ml^{-1} (*X*)	Peak area (*Y*)
0.054	6962	0.032	3873	0.037	2799	0.063	5160	0.059	5426
0.135	17,405	0.155	18,217	0.145	10,505	0.158	12,942	0.137	13,452
0.213	31,008	0.272	32,073	0.202	15,324	0.238	19,395	0.221	21,121
0.271	34,939	0.328	37,088	0.270	20,742	0.317	25,966	0.295	28,823
0.406	52,344	0.474	56,377	0.404	30,148	0.475	41,099	0.442	40,243
0.541	69,749	0.619	68,240	0.539	40,157	0.633	51,850	0.589	55,297
[a]124,821		[a]108,278		[a]73,322		[a]78,561		[a]90,900	
[b]1069		[b]1019		[b]213		[b]731		[b]635	

[a] Slope (*b*)
[b] Intercept (*a*)

Table 3
Repeatability and Intermediate precision data evaluated through intra-day and inter-day studies.

RC	RRT	Spiked concentration[a] ($\mu g\,ml^{-1}$)	Measured concentration[a] ($\mu g\,ml^{-1}$) ± SD; RSD (%)	
			Intra-day	Inter-day
RC-A	1.20	0.302	0.282 ± 0.002; 0.70	0.288 ± 0.001; 0.35
RC-B	2.04	0.304	0.310 ± 0.002; 0.65	0.302 ± 0.001; 0.33
RC-C	2.09	0.297	0.290 ± 0.001; 0.34	0.298 ± 0.002; 0.67
RC-D	0.57	0.307	0.314 ± 0.003; 0.96	0.318 ± 0.003; 0.94
RC-E	0.68	0.300	0.294 ± 0.001; 0.34	0.288 ± 0.003; 1.04

[a] $n = 6$.

study was extended to mixtures of stressed samples with about 15–20% degradation (generated under peroxide stress conditions), the retention time of unknown degradant peak at about 5.7 min was not satisfactory on any of the above columns except for inertsil ODS column. The behavior of separation on Inertsil column is depicted in Fig. 2. Trials were also made by using different pH conditions in the mobile phase. But the buffered mobile phase had no effect either on separation or good peak response for impurities at low levels.

3.2. Method validation

The proposed test method was validated to include requirements of International Conference on Harmonization (ICH)

Table 4
Accuracy (recovery test).

SC[a] ($\mu g\,ml^{-1}$)	[b]Measured concentration ($\mu g\,ml^{-1}$) ±SD; RSD (%); recovery (%)		[c]Measured concentration ($\mu g\,ml^{-1}$) ±SD; RSD (%); recovery (%)	
(i) RC-A				
0.135	0.141 ± 0.004; 3.1	104.4	0.138 ± 0.004; 2.6	102.3
0.203	0.206 ± 0.002; 0.7	101.5	0.206 ± 0.001; 0.3	101.3
0.271	0.278 ± 0.001; 0.2	102.6	0.277 ± 0.003; 1.3	102.1
0.406	0.415 ± 0.002; 0.4	102.2	0.419 ± 0.003; 0.8	103.1
0.541	0.523 ± 0.002; 0.4	96.7	0.533 ± 0.004; 0.8	102.3
	Mean	101.5	Mean	102.2
	SD	2.9	SD	0.7
	RSD (%)	2.9	RSD (%)	0.6
(ii) RC-B				
0.168	0.165 ± 0.002; 1.2	98.2	0.171 ± 0.002; 1.2	101.8
0.240	0.237 ± 0.001; 0.5	98.8	0.237 ± 0.003; 1.3	98.6
0.339	0.335 ± 0.001; 0.2	98.8	0.332 ± 0.002; 0.6	97.9
0.511	0.503 ± 0.003; 0.6	98.4	0.508 ± 0.002; 0.4	99.4
0.659	0.667 ± 0.009; 1.3	101.2	0.670 ± 0.004; 0.7	101.7
	Mean	99.1	Mean	99.9
	SD	1.2	SD	1.8
	RSD (%)	1.2	RSD (%)	1.8
(iii) RC-C				
0.138	0.141 ± 0.001; 0.8	102.2	0.140 ± 0.004; 2.7	101.4
0.202	0.206 ± 0.004; 1.8	102.0	0.206 ± 0.002; 0.7	102.0
0.265	0.259 ± 0.001; 0.4	97.7	0.261 ± 0.002; 0.6	98.5
0.404	0.394 ± 0.008; 2.0	97.5	0.399 ± 0.004; 1.0	98.8
0.511	0.498 ± 0.001; 0.2	97.5	0.496 ± 0.003; 0.5	97.1
	Mean	99.4	Mean	99.6
	SD	2.5	SD	2.1
	RSD (%)	2.5	RSD (%)	2.1
(iv) RC-D				
0.158	0.163 ± 0.001; 0.7	103.2	0.157 ± 0.001; 0.6	99.2
0.238	0.243 ± 0.002; 0.6	102.1	0.233 ± 0.004; 1.6	97.8
0.321	0.330 ± 0.003; 0.9	102.8	0.324 ± 0.003; 1.0	100.9
0.512	0.502 ± 0.003; 0.5	98.0	0.522 ± 0.004; 0.8	102.0
0.671	0.660 ± 0.003; 0.5	98.4	0.677 ± 0.003; 0.4	100.7
	Mean	100.9	Mean	100.1
	SD	2.5	SD	1.7
	RSD (%)	2.5	RSD (%)	1.6
(v) RC-E				
0.147	0.152 ± 0.001; 0.7	103.4	0.150 ± 0.002; 1.4	102.3
0.221	0.218 ± 0.005; 2.1	98.6	0.221 ± 0.002; 0.9	100.0
0.295	0.307 ± 0.006; 1.9	104.1	0.305 ± 0.005; 1.7	103.4
0.442	0.452 ± 0.004; 0.9	102.3	0.447 ± 0.004; 0.9	101.0
0.589	0.581 ± 0.014; 2.5	98.6	0.578 ± 0.003; 0.6	98.1
	Mean	101.4	Mean	101.0
	SD	2.6	SD	2.1
	RSD (%)	2.6	RSD (%)	2.0

[a] Spiked concentration.
[b] $n = 3$ calculated using quantitation equation.
[c] Calculated using the weighted regression values of slope and intercept of the respective related compound.

Table 5
Accuracy curve.

RC	Regression equation	r^2	Range of intercept (p-value)	Range of slope (p-value)
RC-A	$Y = 0.9534X + 0.016$	0.9987	0.016 ± 0.031, 0.20	0.9534 ± 0.091, 5.87×10^{-5}
RC-B	$Y = 1.0151X - 0.008$	0.9997	-0.008 ± 0.020, 0.30	1.0151 ± 0.047, 6.75×10^{-6}
RC-C	$Y = 0.9527X + 0.010$	0.9998	0.010 ± 0.010, 0.05	0.9527 ± 0.031, 2.46×10^{-6}
RC-D	$Y = 0.9607X + 0.015$	0.9997	0.015 ± 0.017, 0.07	0.9607 ± 0.04, 4.87×10^{-6}
RC-E	$Y = 0.9833X + 0.009$	0.9990	0.009 ± 0.031, 0.43	0.9833 ± 0.083, 4.08×10^{-5}

p-Value probability value.

guidelines [14,15]. Parameters like specificity, linearity, precision, accuracy, range, robustness, ruggedness and system suitability were examined.

3.2.1. Specificity/selectivity

There were no interferences due to placebo and sample diluent at the retention times of anastrozole and its related compounds. Homogeneity of all the related compounds RC-A, RC-B, RC-C, RC-D, RC-E, anastrozole and degradants were established using a PDA detector. All known impurities (related compounds) and unknown degradants were well separated and for all the compounds, purity angle was found to be less than purity threshold. Apart from the peaks homogeneity, the DAD spectrum for the all the RC's (related compounds) and anastrozole were compared against their standard spectrums. Identity for all the RC's and anastrozole were performed by comparing their DAD spectrum, purity plots and their respective relative retention times (RRT), with those of standards and was found to be matching.

3.2.2. Relative response factor (RRF) for related compounds (RC)

Accurate mixed five known concentrations from 0.20 to 1.0 μg ml^{-1} (0.1–0.5% of the concentration of the anastrozole in test solution) for each related compound RC-A, RC-B, RC-C, RC-D, RC-E and anastrozole was prepared and injected in duplicate into the chromatographic system. Individual related compounds peak area as a function of exact concentration was compared with that of anastrozole. *RRF* for each individual related compound as depicted in Table 1, was established using the below equation. The slope of *RRF* vs. each concentration for RC-A, RC-B, RC-C, RC-D, and RC-E was found to be 0.007, 0.014, 0.050, 0.071 and 0.040, which shows that the slope of *RRF* vs. concentrations for all RCS is almost zero:

$$\frac{\text{slope of each individual related compound}}{\text{slope of anastrozole}}$$

3.2.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The limits of detection and quantitation were evaluated by serial dilutions of RC-A, RC-B, RC-C, RC-D, RC-E and anastrozole in presence of placebo in order to obtain signal to noise ratios of ~3:1 for LOD and ~10:1 for LOQ. Six replicates ($n = 6$) of placebo solutions consisting of RC-A, RC-B, RC-C, RC-D, RC-E and anastrozole at LOQ concentrations were prepared and injected for LOQ precision. The RSD for each RC and anastrozole was found to be <5%. The LOD, LOQ determinations are depicted in Table 1.

3.2.4. Response linearity

The detector response linearity parameter of the curve for the RC's (related compounds) of anastrozole was determined. The mixed six standard solutions containing RC-A, RC-B, RC-C, RC-D and RC-E in the concentration range of LOQ to about 0.60 μg ml^{-1} were prepared and injected in duplicate into the chromatographic system. A weighted regression analysis calculation [16] was performed by weighting each value of (Y) by a factor that is inversely proportional to the variance, $1/X^2$ for weighted regression (w). The calculation of weighted regression, values of Slope (b) and intercept (a) for each of the respective RC's as a function of concentration μg ml^{-1} (X) and detector peak (area) response (Y) is depicted in Table 2. In this simultaneous determination, the linear regression was found to be good over the concentrations range mentioned.

3.2.5. Precision

3.2.5.1. System precision. Instrumental precision for anastrozole at 0.4 μg ml^{-1} (0.2%) as system suitability was determined by analyzing six replicate injections on different systems and different days and the relative standard deviation was found to be 1.5% and 1.8% respectively.

3.2.5.2. Method precision (repeatability) and intermediate precision. Method precision or intra-day precision was performed by spiking RC-A, RC-B, RC-C, RC-D, and RC-E at 0.3 μg ml^{-1} level (0.15% level as specification limit) in anastrozole tablets. Six replicates ($n = 6$) solutions were prepared and each solution was injected in duplicate under the same conditions and mean value of peak area response for each solution were taken. Intermediate precision (inter-day precision) was performed by analyzing the study using different instrument, analyst, column and six different samples at the stated concentration. The results of repeatability and intermediate precision experiments are shown in Table 3. The developed method was found to be precise as the RSD values was <2.0% on both the variations respectively.

3.2.6. Accuracy (recovery test)

The accuracy of the method was evaluated [10,12] by the recovery studies which were carried out by spiking the five known concentrations of RC-A, RC-B, RC-C, RC-D, and RC-E in anastrozole tablets (range from 0.13 to 0.6 μg ml^{-1}). Three samples were prepared at each concentration. The results of the spiked concentrations for each RC in anastrozole tablets as depicted in Table 4, were calculated using (i) quantitation equation and (ii) by using the weighted regression values of slope and intercept for each related compound as obtained from Section 3.2.4 (Table 2). The results of accuracy as determined by both the calculation methods revealed that, the average recovery at each level and for each RC was within 100 ± 5% with RSD at each level was ≤5%. No significant difference was seen between the two calculations method.

3.2.7. Accuracy curve

The accuracy curve (linearity of test method) was established by plotting the values of spiked concentration (μg ml^{-1}) on X-axis and

Table 6
Peak areas of anastrozole and RC's observed during analytical solution stability.

RC	Peak areas			
	Initial	24 h	48 h	72 h
RC-A	39,718	39,286	39,105	38,972
RC-B	36,404	37,987	37,777	37,725
RC-C	27,669	26,429	26,006	26,016
RC-D	29,421	30,454	31,660	30,125
RC-E	29,903	29,177	29,643	29,011
Anastrozole	42,718	41,606	41,912	41,727

Table 7
System suitability parameters of anastrozole standard ($n = 6$) under robustness.

Parameter	Variation	RSD (%)	USP theoretical plates	USP Tailing
Temperature	20 °C	0.8	13,730	1.1
	25 °C	1.1	12,498	1.1
	30 °C	0.6	15,655	1.1
Flow rate	0.8 ml/min	1.5	15,489	1.2
	1.0 ml/min	1.1	12,498	1.1
	1.2 ml/min	0.7	12,672	1.1
Column/day	1	1.6	12,315	1.0
	2	1.1	12,498	1.1
	3	0.7	14,320	1.1

measured concentration ($\mu g\,ml^{-1}$) on *Y*-axis as determined from accuracy section. The correlation coefficient (r^2), regression equation for the slope and *Y*-intercept values and the confidence range for the slope and *Y*-intercept values are depicted in Table 5, which shows that the accuracy curve is very much linear with minimum *Y*-intercept in the concentrations range mentioned.

3.2.8. Stability of analytical solution

The stability of the standard and test solution was checked by analyzing these solutions at frequencies of initial 24, 48 and 72 h at room temperature, against freshly prepared standard. The result demonstrated that the standard solution, as well as the sample solution is stable for at least 72 h. During the stability studies no additional peaks developed and no changes in the chromatographic pattern were observed in either of the solutions. The respective peak areas during the study as depicted in Table 6 shows almost negligible variation with respect to the initial time. Also at the end of the study (72 h) the purity angle were found to be less than threshold and the DAD spectrums matched with those of the standards for all the RC's and anastrozole. This proved the peak homogeneity and identity for the peaks during the study period.

3.2.9. Robustness

Robustness of proposed method was performed by keeping chromatographic conditions constant with following differences:

(i) Change in the flow rate of mobile phase from 0.8 to 1.2 ml/min.
(ii) Increasing the column oven temperature from 20 to 30 °C.
(iii) Using another column of the same brand of different Lot (Inertsil ODS-3V, 250 mm × 4.6 mm i.d, 5 μm). For each change, standard solution was injected six times. System suitability parameters like peak symmetry, theoretical plates and relative standard deviation of peak areas of six injections were recorded for anastrozole and the data were found to be within the acceptable limits (Table 7). Test samples spiked with RC-A, RC-B, RC-C, RC-D, and RC-E at 0.3 $\mu g\,ml^{-1}$ level in anastrozole tablets were prepared in triplicate and analyzed in duplicate for each change. Recoveries and RSD were calculated for each component during each change and found to be within 100 ± 5% and <5.0% respectively.

3.2.10. Analysis of stressed samples

Anastrozole tablets of three lab scale batches were subjected to the accelerated stability conditions of 60 °C for 3 months. The test solutions subjected to HPLC analysis showed no major degradants with % of total impurities <0.2% indicates the stability of the drug product. Peak identity was found to be matching with those of the standard DAD spectrum and peak homogeneity was proven through PDA detector.

4. Conclusions

This paper reports for the first time a novel method on anastrozole to quantitate related compounds (impurities) and degradants in finished dosage form by RP-HPLC. The results of forced decomposition studies undertaken according to the ICH guidelines reveal that the method is selective and stability-indicating. The proposed HPLC method has the ability to separate anastrozole from their degradation products, impurities, related compounds; excipients found in the tablet dosage form and therefore can be applied to the analysis of samples at quality control. The method is rapid, direct, specific, accurate, precise, stability-indicating and validated for the routine analysis in the finished dosage form. The method may also be extended to evaluate active drug substance.

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References

[1] Drugs at FDA, Armidex (assessed on 15.01.2008).
[2] G. Saravanan, M.V. Suryanarayana, M.J. Jadhav, N. Someswararao, P.V.R. Acharyulu, Chromatographia 66 (2007) 435–438.
[3] S.G. Hiriyanna, K. Basavaiah, J. Braz. Chem. Soc. 19 (2008).
[4] G.D. Mendes, D. Hamamoto, J. Ilha, A. dos Santos Pereira, G. De Nucci, J. Chromatogr. B 850 (2007) 553–559.
[5] Rapid Communications in Mass Spectrometry, vol. 20 (2006) pp. 1954–1962.
[6] K.P. Adam, K. Sowell, A. Austin-Petersen, C.J. Buggé, CEDRA Corporation, Austin, TX.
[7] L. Repetto, O. Vannozzi, A. Hazini, A. Sestini, M. Pietropaolo, R. Rosso, Ann. Oncol. 14 (2003) 1587–1588.
[8] M. Bakshi, S. Singh, J. Pharm. Biomed. Anal. 28 (2002) 1011–1040.
[9] M. Bakshi, B. Singh, A. Singh, S. Singh, J. Pharm. Biomed. Anal. 26 (2001) 891–897.
[10] ICH, Guidance for Industry: Q3B (R2) Impurities in New Drug Products, Centre for Drug Evaluation and Research (CDER), Rockville, 2006.
[11] ICH, International Conference on Harmonisation, Q1A (R2) IFPMA, Geneva, 2003.
[12] European pharmacopoeia (Ph. Eur, 2.2.46).
[13] ICH, International Conference on Harmonisation, Q1B, 1996.
[14] ICH, International Conference on Harmonization Q2 (R1) IFPMA, Geneva, 2005.
[15] FDA, Guidance for Industry: Analytical Procedures and Methods Validation (Draft Guidance), Food and Drug Administration, Rockville, 2000.
[16] S. Bolton, Pharmaceutical Statistics, third ed., Marcel Dekker Inc., New York, 1997.