Arabian Journal of Chemistry (2016) 9, S1428–S1434

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Application of experimental design for the optimization of forced degradation and development of a validated stability-indicating LC method for luliconazole in bulk and cream formulation

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Received 31 August 2011; accepted 17 March 2012
Available online 25 March 2012

KEYWORDS

Luliconazole;
Experimental design;
Surface response curve;
Transformations;
Stability-indicating method

Abstract A stability-indicating LC method was developed and validated for the determination of luliconazole in bulk and cream formulation. Luliconazole was exposed to acid, alkali and water hydrolysis, oxidation effect by hydrogen peroxide, dry heat and photolytic conditions. Full factorial design was used during forced degradation experiments and the factors/combination of factors which were most likely to effect degradation of luliconazole under various conditions were identified and further were optimized using surface response curve. Drug was found to be stable under wet heat and dry heat conditions, but substantial degradation was observed under acid, alkali, oxidative and photolytic conditions. Drug and its degradation products were optimally resolved on HiQ sil C-18HS (250 × 4.6 mm, 5 μm) column with the mobile phase consisting of methanol and water (80:20, v/v) at a flow rate of 1 mL/min, detection was performed at 296 nm. The procedure was validated for specificity, linearity, accuracy and precision. There was no interference of excipients and degradation products in the determination of active pharmaceutical ingredient. The method was accurate and precise and the response was found to be linear in the range of 2–14 μg/mL. The method was found to be simple and fast by making use of experimental design.

1. Introduction

Forced degradation/stress testing, defined as the stability testing of drug substance and drug product under conditions exceeding those used for accelerated testing (Klick et al., 2005).

From a drug development and regulatory perspective, forced degradation studies provide data for the identification of possible degradation products, prediction of degradation pathway, validation of stability-indicating analytical

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2012.03.019
1878-5352

procedures, identification of conditions in which the drug is less
stable, the choice of packing material and selection of storage conditions (Alsante et al., 2007).

Although the regulatory guidance documents define the concept of stress testing, they do not provide detailed information about a stress testing strategy. The experimental conditions to conduct stress testing are described in a general way and the exact stress conditions to be applied are not described (Bakshi et al., 2002). The available guidance documents also do not state the extent to which stress tests should be carried out (that is how much stress should be applied or how much degradation should be aimed for).

To address the two issues – experimental design for forced degradation and extent of forced degradation to be attempted, researchers have suggested that degradation can be achieved by exposing the drug, for extended periods of time, to extremes of pH (aqueous, hydrochloric acid or sodium hydroxide solutions) at elevated temperatures, to hydrogen peroxide at room temperature or to UV light and to dry heat (in an oven) while adopting trial and error approach to select the strength, temperature and time of exposure to the stress conditions so as to achieve degradation to an extent of 10–20%. (Singh and Bakshi, 2000; Reynolds et al., 2000), actually, such trial and error approach are cost, time and labor intensive and should be substituted by more systemic approach.

One such systemic approach is to adopt statistical nested design like factorial design (Lundstedt et al., 1998; Morgan et al., 1989; Leardi, 2009) to reveal the variables (strength, temperature or time of exposure) which are most likely to influence degradation and modify only these parameters to effect the adequate degradation. The basic concept of factorial design also addresses the interaction between two variables. This can reveal the combination of variables (heat/pH, heat/time, etc.), which could result in increased susceptibility of the drug to degradation, thus giving valuable inputs toward the ultimate choice of storage conditions. The present work directed toward the use of factorial design to bring about forced degradation under various stress conditions.

Luliconaozole (LCZ) was selected as a model drug for this study. It is chemically, 4-(2,4-dichlorophenyl)-1,3-dithiolan-2-ylidene-1-imidazolylacetonitrile, a novel antifungal drug launched in India by Ranbaxy Laboratories Ltd. The compound was originally screened from active compounds related to lanoconazole, a potent antidermatophytic drug. LCZ possesses a wide spectrum of antifungal activity and is very potent against dermatophytes (Uchida et al., 2004). Till date no analytical method was reported for quantitative estimation of LCZ.

Thus, a simple and rapid stability-indicating liquid chromatographic method has been developed and validated for LCZ.

2. Experimental

2.1. Chemicals and reagents

The LCZ reference standard (RS) was kindly supplied by Ranbaxy Laboratories Ltd. (Gurgaon, Haryana, India). The cream formulation, Lulifin™ (Ranbaxy Laboratories Ltd., Gurgaon, Haryana, India) composed of 10 mg of LCZ in each gram, was purchased in the market. All chemicals used were of analytical grade and all solvents were of LC grade. Methanol was purchased from SD Fine Chemicals (Mumbai, India). The 0.45 μm pore Nylon filter papers were purchased from Pall India Pvt. Ltd. (Mumbai, India).

2.2. HPLC instrumentation and conditions

HPLC system consisted of pump PU-2080 plus (JASCO Corporation, Tokyo, Japan), with Rheodyne Loop Injector (7725 i) fitted with 20 μL sample loop. Detection was carried out using UV-2075 detector (JASCO Corporation, Tokyo, Japan). The data acquisition was done using Borwin chromatography software version 1.50. The HPLC column was a HiQsil C18HS (250 mm × 4.6 mm i.d., 5 μm), Kya Technologies Corporation, Tokyo, Japan. The chromatographic separation was performed using mobile phase consisting of methanol and water (80:20, v/v), with flow rate of 1 mL/min. The detector was set at 296 nm.

2.3. Forced degradation study by factorial design

LCZ was subjected to stress under acidic, alkaline, oxidative, thermolytic and photolytic conditions. For acid, alkali, oxidative, dry heat and wet heat conditions, values of variables like time of exposure, temperature and strength were chosen so as to obtain 10–20% degradation. This choice was facilitated by the initial experiments as per the factorial design and performing multiple regression equation to identify conditions for desired 10–20% degradation.

(a) Acid degradation: 1 mg/mL mixture of LCZ in X_1 M HCl was heated under reflux at X_2 °C for X_3 min. Two levels were chosen for each of X_1, X_2 and X_3. The high level (+1) for X_1, X_2 and X_3 was 1 M, 75 min and 100 °C, respectively, and the low level (−1) for X_1, X_2 and X_3 was 0.1 M, 15 min and 60 °C, respectively. Since three variables were considered at two levels, a 2^3 factorial design was conducted to set up eight experiments.

(b) Alkali degradation: 1 mg/mL mixture of LCZ in X_1 M NaOH was heated under reflux at X_2 °C for X_3 min. Two levels were chosen for each of X_1, X_2 and X_3. The high level (+1) for X_1, X_2 and X_3 was 0.1 M NaOH, 30 min and 100 °C, respectively, and the low level (−1) for X_1, X_2 and X_3 was 0.01 M, 10 min and 60 °C, respectively. Since three variables were considered at two levels, a 2^3 factorial design was conducted to set up eight experiments.

(c) Oxidative degradation: 1 mg/mL mixture of LCZ was maintained in X_1% H_2O_2 in dark for X_2 min. Two levels were chosen for X_1 and X_2. The high level (+1) for X_1 and X_2 was 30% and 24 h, respectively, and the low level (−1) for X_1 and X_2 was 3% and 2 h, respectively. Since two variables were considered at two levels, a 2^2 factorial design was conducted to set up four experiments.

(d) Dry heat degradation: LCZ powder was spread as a thin film in petri plate and exposed to X_1 °C for X_2 min. Two levels were chosen for X_1 and X_2. The high level (+1) for X_1 and X_2 was 200 °C and 360 min, respectively, and the low level (−1) for X_1 and X_2 was 50 °C and 30 min,

respectively. Since two variables were considered at two levels, a 2^2 factorial design was conducted to set up four experiments.

(e) Wet heat degradation: 1 mg/mL of LCZ was heated under reflux at X_1 °C for X_2 min. Two levels were chosen for X_1 and X_2. The high level (+1) for X_1 and X_2 was 100 °C and 120 min, respectively, and the low level (−1) for X_1 and X_2 was 60 °C and 30 min, respectively. Since two variables were considered at two levels, a 2^2 factorial design was conducted to set up four experiments.

(f) Photolytic degradation: LCZ powder was spread as a thin film on petri plate and exposed to direct sunlight for 48 h. A control in dark was also run.

2.4. Chromatographic analysis of stressed samples

Each of the stressed sample obtained was diluted with the mobile phase to get a final concentration of 10 μg/mL, 20 μL of the resulting solution was injected on HiQsil C18HS (250 mm × 4.6 mm i.d., 5 μm) at 1 mL/min and the eluent was monitored at 296 nm. The resulting chromatograms were studied for the appearance of secondary peaks and the% reduction in the area of drug peak with reference to standard LCZ solution (10 μg/mL). The % reduction in peak area was considered as % degradation.

2.5. Multiple regression analysis and selection of optimum conditions

Taking the % degradation as the dependent variable (Y), the results obtained for each type of forced degradation was subjected to multiple regression analysis to generate the following type of equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$$

where β_0 is the intercept, β_1, β_2 and β_3, β_{12}, β_{23}, β_{12} and β_{123} as regression coefficients for the variables and interaction between the variables.

Yates analysis was performed to retain only the significant coefficients. Thereafter, surface response curves were drawn to characterize the value of variables X_1, X_2 and X_3 required for adequate 10% degradation. Transformed values of variables were used to simplify the calculation. The actual values were obtained by using formula:

$$\text{Transformed value} = \frac{X - \text{the average of levels}}{\text{one} - \text{half the difference of the levels}}$$

Finally, degradation experiments were performed using theoretical indicated values and degradation samples chromatographed to confirm whether the experimental % degradation matched with the calculated one.

2.6. Calibration curve

One hundred milligrams of LCZ was accurately weighed and transferred into 100 mL volumetric flask and the volume was made up to the mark with methanol. The resulting solution (1 mg/mL) was diluted with the mobile phase to get concentration in the range of 2–14 μg/mL and each solution was subjected to chromatographic analysis in triplicate. The peak areas were plotted on x-axis and the respective concentrations on y-axis. Further, the linear regression was performed to generate least square line and regression equation of type $y = ax + b$.

2.7. Analysis of cream formulation

An appropriate portion of 1 g of Lulifin™ was weighed and transferred into a 100 mL beaker using weight by transfer method. Further, 20 mL of methanol was added and the mixture was heated on a water bath with occasional agitation, till the cream base gets melted. The melted mixture was transferred to 100 mL volumetric flask, maintained in water bath at temperature about 40 °C. Further, 5 mL of methanol was added to the same beaker and the washings were transferred into the volumetric flask. Finally the volume was adjusted to the mark with methanol. The mixture was filtered immediately with Whatman filter paper no. 1 to suction flask by applying vacuum. Suitable aliquots were removed and diluted with the mobile phase to get final concentration of 10 μg/mL and subjected to chromatographic analysis. The drug peak area was referred to the regression equation to get the sample concentration and % nominal label claimed.

2.8. Method validation

The method was validated as per the ICH guideline Q 2 (R1) (ICH, 2005). To evaluate the accuracy and precision, laboratory cream samples were prepared by spiking the cream base portions with appropriate known amounts of LCZ to reach concentrations of 80%, 100% and 120% of the expected amount of the analyte in real samples. The resulting mixtures were analyzed in triplicate over three days. The % recovery of added drug and % RSD were taken as measures of the accuracy and precision, respectively. Also, the results obtained were subjected to one-way analysis of variance and within-day mean square compared to between-day mean square by F-test. Further, the quantity added was plotted against quantity found and the slope and intercept of the resulting straight line was obtained. A slope of 1 and intercept of 0 were taken as indication of linearity of the method in the range of 80–120%.

3. Results and discussion

3.1. Multiple regression analysis and selection of optimum conditions

The forced degradation experiments set-up on the basis of factorial design were performed and the resulting samples were analyzed by LC. No degradation in drug peak area was observed in case of wet heat and dry heat conditions. Substantial degradation was observed in acidic, alkaline, oxidative and photolytic conditions (Fig. 1). The experimental conditions and degradation obtained for all degradation experiments performed as per the factorial design as summarized in Table 1. Further, when results obtained for each experiments performed under acid, alkali, oxidative degradation were subjected to multiple regression, the following equations resulted:

$$Y = 17.38 + 7.62X_1 + 6.38X_2 + 8.12X_3 + 0.62X_1X_2 - 0.88X_2X_3 + 3.38X_1X_3 - 1.62X_1X_2X_3 \text{ (acidic degradation)} \quad (1)$$

$$Y = 18.5 + 2.25X_1 + 6.5X_2 + 13X_3 - 0.25X_1X_2 + 2.5X_2X_3 + 0.75X_1X_3 - 0.25X_1X_2X_3 \text{ (alkaline degradation)} \quad (2)$$

$$Y = 49.75 + 97X_1 + 103X_2 + 1X_1X_2 \text{ (for oxidative degradation)} \quad (3)$$

Yates analysis indicated that under acidic condition, strength of HCl (X_1) and the temperature (X_3) were most significant factors and for alkaline condition, time of exposure (X_2) and the temperature (X_3) were significant. Furthermore, surface response curves were generated for acid and alkali conditions, respectively. For acid condition, the surface response curve (Fig. 2) was generated by keeping the X_2 at a value of 0 and transformed values of X_1 and X_3 for y-axis value of 10% of degradation were calculated using Eq. (1). Similarly, the surface response curve was generated for alkali condition (Fig. 3) by keeping the X_1 at a value of 0 and transformed values of X_2 and X_3 for y-axis value of 10% of degradation were calculated using Eq. (2). Figs. 2 and 3 depict the surface response curves, predicting the optimum degradation lines for 10% degradation of LCZ under acid and alkali conditions, respectively. These lines suggested that for acidic stress, 10% degradation would result by using 0.15 M HCl and heating at 80 °C for 45 min. When these conditions were adopted in practice, the resulting degradation was 11%. Also, for alkaline degradation from surface response curve line, 10% degradation would result by using 0.05 M NaOH and heating at 65 °C for 20 min. These conditions when adopted in practice 13% degradation was achieved.

For oxidative degradation, the rough grids of predicted responses were determined from Eq. (3) by considering the values of X_1 and X_2 from −1 to +1, respectively. From this, it has been observed that 18.75% degradation can be achieved when $X_1 = 0.75$ and $X_2 = -1$. The actual values for X_1 and

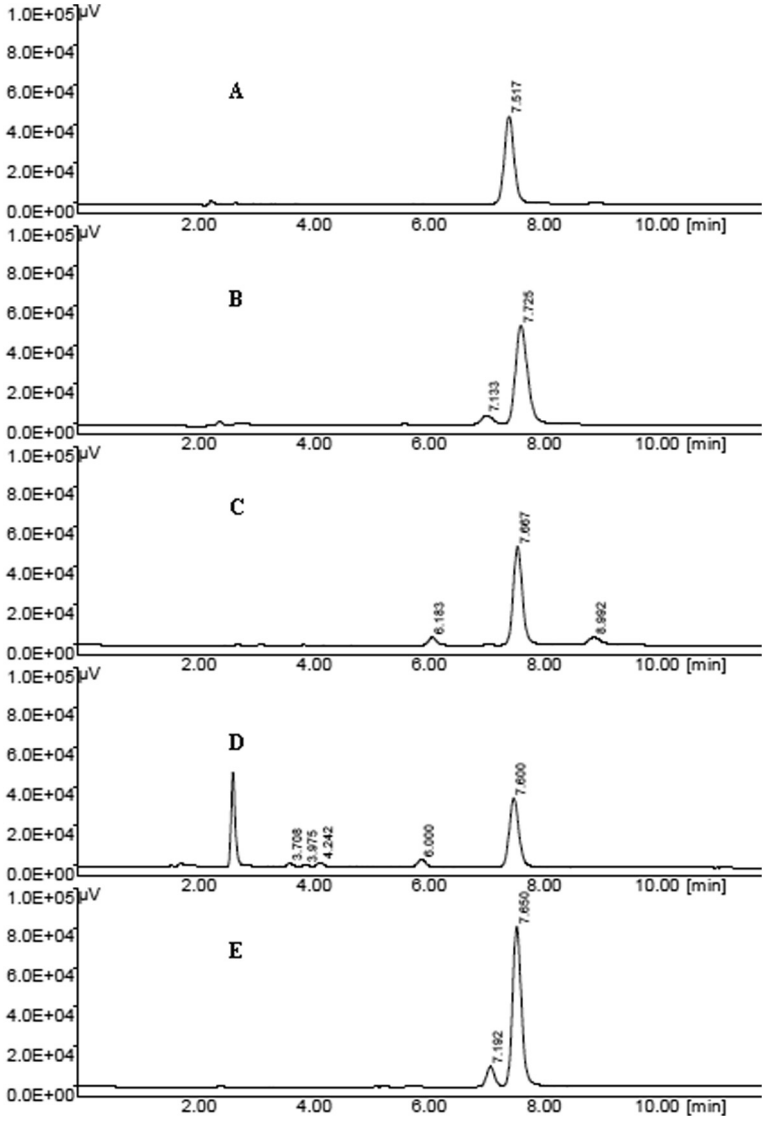

Figure 1 Chromatograms obtained for (A) reference substance, (B) acid hydrolysis, (C) alkali hydrolysis, (D) oxidative degradation and (E) photolytic degradation. Chromatographic condition: HiQSil C18HS (250 × 4.6 mm i.d., 5 μm); flow rate: 1 mL/min, mobile phase: methanol:water (80:20%, v/v); detection: 296 nm.

Table 1 Experimental design and their resulting % degradation under various conditions.

Expt. no.	2^3 Factorial design					Expt. no.	2^2 Factorial design				
	X_1	X_2	X_3	Acid hydrolysis	Alkali hydrolysis		X_1	X_2	Oxidative	Wet heat	Dry heat
1	−1	−1	−1	0	0	1	−1	−1	0	No degradation obtained	
2	+1	−1	−1	4	3	2	−1	+1	48		
3	−1	+1	−1	10	8	3	+1	−1	51		
4	+1	+1	−1	23	11	4	+1	+1	100		
5	−1	−1	+1	8	19						
6	+1	−1	+1	32	26						
7	−1	+1	+1	21	38						
8	+1	+1	+1	41	43						

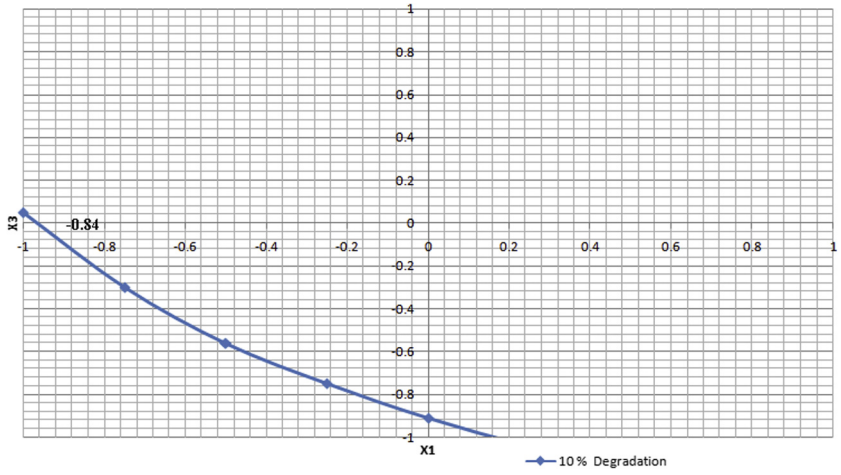

Figure 2 Surface response curve of transformed values for 10% acid degradation.

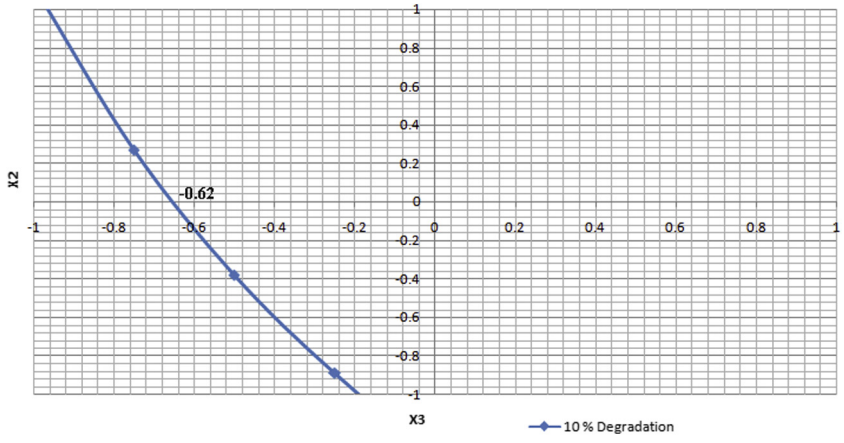

Figure 3 Surface response curve of transformed values for 10% alkali degradation.

X_2 were determined. Thus, when LCZ was kept in 25% H_2O_2 for 2 h, resulted in 15% degradation. For photolytic conditions about 8% degradation has been obtained.

3.2. Calibration curve and analysis of marketed formulation

When calibration standards in the range of 2–14 µg/mL were analyzed in triplicate and plot of peak area vs concentration was subjected to least square regression. The respective linear equation was $y = 101218.73x - 13215.42$, where x is the concentration (µg/mL) and y is the peak area (µV). The correlation coefficient was 0.9997. Student's t-test was performed to verify the significance of experimental intercept and slope in the regression equation. According to the results, it was not significantly different from zero and one value, respectively, for $p > 0.05$. The analysis of variance was applied to verify

Table 2 Accuracy and precision studies.

Amount added (mg)	Amount found (mg)			Within mean square	Between mean square	*F*
	Days					
	1	2	3			
8	7.99	8.08	7.78	0.0065	0.0302	4.60
	8.03	7.89	7.83			
	8.11	8.01	7.94			
Mean	8.04	7.99	7.85			
% RSD	0.75	1.20	1.04			
10	9.89	10.13	9.89	0.0062	0.0170	2.72
	10.02	9.99	9.81			
	9.91	10.01	9.99			
Mean	9.94	10.04	9.89			
% RSD	0.70	0.75	0.91			
12	12.39	12.04	12.24	0.2306	0.1029	4.46
	12.43	12.34	12.12			
	12.72	12.11	12.34			
Mean	12.51	12.16	12.23			
% RSD	1.43	1.29	0.90			

the linearity of the method, from the result it has been observed that the calculated F (15,319) was greater than the tabulated F (6.61) at $\alpha = 0.05$, concluded a linear relationship exists between the peak area and concentration.

When cream formulation was analyzed using developed method, the results obtained in good agreement with the nominal amount of the drug. The drug content was found to be 100.46% ± 1.19 ($n = 3$) of the label claimed.

3.3. Method validation

The results obtained for accuracy and precision studies are shown in Table 2. The % recovery close to 100% and the low values of % RSD suggest an acceptable accuracy and precision of the method.

Furthermore, the intra- and inter-day results at each level were subjected to one-way analysis of variance and the F values for each level were determined as the ratio of between mean square (BMS) to within mean square (WMS). (F = BMS/WMS). The obtained values were found to be less than the tabulated $F_{(2,6)}$ at $\alpha = 0.05$ (tabulated F value = 5.14). These indicated that there was no significant difference between intra- and inter-day variability, suggesting good intermediate precision of the method.

A plot of quantity added to the quantity obtained resulted in a straight line with the slope of 1.009 and the intercept of 0.98, encompasses 1 and 0, respectively. This indicated the linearity of the method in the selected range of 80–120% of the label claimed.

The chromatograms of blank and placebo solutions showed no interfering peak at the retention time of the drug indicating specificity of the developed method.

4. Conclusions

Hence with regard to the method done, it can be concluded that

- LCZ is susceptible to degradation under acid, alkali, oxidative and photolytic conditions, but stable under wet heat and dry heat conditions.
- Use of factorial design, expedited the revolution of variables that are most likely to influence the extent of degradation. For acid condition, strength of HCl and the temperature had largest influence on % degradation of LCZ. While for alkali condition, time of exposure and temperature were significant. For oxidative degradation, it has been observed that there is no interaction between the strength of H_2O_2 and the time of exposure.
- The use of surface response curves to identify theoretical values of variables for optimum degradation was successful, because when these parameters were put in practice, the % degradation obtained matched the predicted degradation. This suggests that factorial design approach can replace the trial and error approach used to achieve optimum degradation in forced degradation studies.
- Validation experiments proved that the LC conditions used were able to impart sufficient specificity and stability-indicating capability to the method. Also, the method had desired accuracy, precision and linearity.
- Analysis of cream formulation, proved practical range of the method.
- Thus, factorial design approach was successfully used to achieve optimum degradation conditions for LCZ and an accurate, precise and specific stability-indicating LC method was developed for the drug in cream formulation.

Acknowledgements

Authors are highly thankful to the trustees of Bhujbal Knowledge City, Nashik, for providing necessary analytical facilities and financial support to carry out this work and to the Ranb-

axy Laboratories Ltd., Haryana, for providing gift sample of luliconazole.

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