Journal of Saudi Chemical Society (2016) **20**, S313–S322

King Saud University

Journal of Saudi Chemical Society

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Quality by Design (QbD) approach to develop HPLC method for eberconazole nitrate: Application to hydrolytic, thermal, oxidative and photolytic degradation kinetics

M. Vamsi Krishna *, Rajendra N. Dash, B. Jalachandra Reddy, P. Venugopal, P. Sandeep, G. Madhavi

Alliance Institute of Advanced Pharmaceutical and Health Sciences, Ameerpet, Hyderabad 500038, Andhra Pradesh, India

Received 28 April 2012; accepted 2 December 2012
Available online 14 December 2012

KEYWORDS

Eberconazole nitrate;
QbD approach;
Stability indicating HPLC;
Degradation kinetics

Abstract Stability of eberconazole nitrate (EBZ) was investigated using a stability indicating HPLC method. Quality by Design (QbD) approach was used to facilitate method development. EBZ was exposed to different stress conditions, including hydrolytic (acid, base, neutral), oxidative, thermal and photolytic. Relevant degradation was found to take place in all the conditions. The degradation of EBZ followed (pseudo) first-order kinetics under experimental conditions. The kinetic parameters (rate constant, $t_{1/2}$, and t_{90}) of the degradation of EBZ were calculated.

1. Introduction

Eberconazole nitrate (EBZ) is an imidazole derivative (Sweetman, 2009), used topically as a 1% cream in the treatment of superficial fungal infections (Barbanoj et al., 2005). EBZ (Fig. 1), {1-(2,4-dichloro-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl)-1H-Imidazole nitrate}, acts by inhibition of fungal lanosterol 14α-demethylase (Torres-Rodríguez et al., 1999). It is a basic, white, amorphous powder which is readily soluble in methanol and dichloromethane. The absorption maximum in the ultraviolet range is 208 nm. Fig. 2 shows the absorption spectrum of EBZ (5 μg mL^{-1}; molar absorptivity: 7.06×10^4 L mol^{-1} cm^{-1}).

Literature review reveals that there is no HPLC method reported for analysis of EBZ, but there are several methods available for the estimation of other azole antifungal agents namely fluconazole, itraconazole, voriconazole, posaconazole, ravuconazole and isavuconazole. Chromatographic methods available for these antifungal agents were reviewed (Ekiert et al., 2010). In the modern analytical laboratory, there is always a need for significant stability-indicating methods (SIMs)

Abbreviations: Rt, retention time; K, capacity factor; T, tailing factor; K, rate constant.

* Corresponding author. Tel.: +91 9849563353; fax: +91 40 66663326.
E-mail address: marothu_vamsi@rediffmail.com (M.V. Krishna).

Peer review under responsibility of King Saud University.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.jscs.2012.12.001

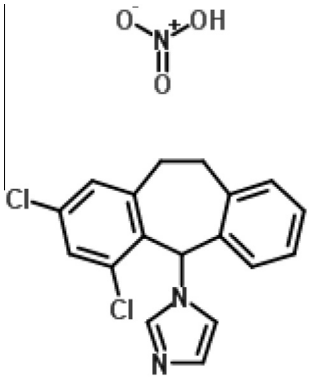


Figure 1 Chemical structure of Eberconazole nitrate (EBZ).

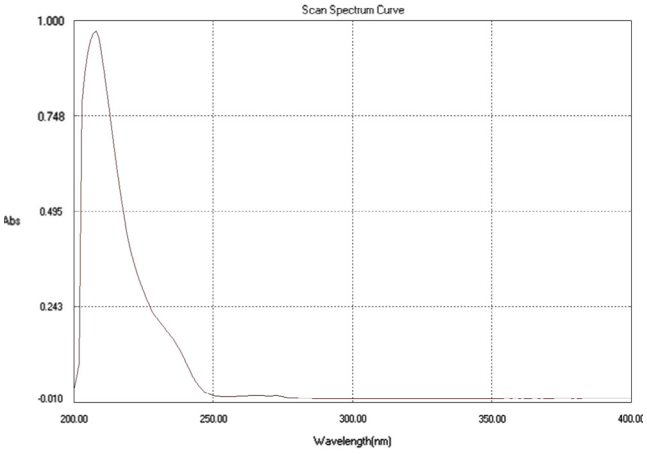


Figure 2 UV absorption spectrum of EBZ in methanol.

of analysis. Environmental factors, such as temperature, pH, buffer species, ionic strength, light, oxygen, moisture, additives and excipients, can play an important role in the stability of drug substances. Stress testing can help in identifying degradation products and provide important information about the intrinsic stability of drug substances (Singh and Bakshi, 2000). With the advent of the International Conference on Harmonization (ICH) guidelines (ICH guideline Q1A (R2), 2003), requirements for the establishment of SIMs have become more clearly mandated. The guidelines explicitly require the conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products. Moreover, kinetic studies on the decomposition of drugs using stability testing techniques are essential for their quality control and to predict the expiry date of pharmaceutical products. The scientific novelty of the present work is that the suggested method represents the first stability indicating HPLC method for the analysis of EBZ. Besides, it is the first kinetic study for EBZ degradation to calculate the strength of this azole molecule.

A response surface methodology (RSM) approach was used to identify the optimum conditions for analysis during method development. The iterative procedure used in these studies included performing experiments in the region of the best known solution, fitting a response model to the experimental data and then optimizing the estimated response model. The conventional practice of modification of a single factor at a time may result in poor optimization as other factors are maintained at constant levels that do not depict the combined effect of all the factors involved in a separation. This approach is also time consuming and requires a vast number of experiments to establish optimum levels. These limitations can be eliminated by collectively optimizing all parameters using RSM. Furthermore RSM was used to evaluate the relative significance of several other factors in the presence of complex interactions. Compared with the traditional optimization method, RSM has distinct advantages such as the use of minimum number of experiments, shorter time of operation and feasibility of generating data that may be analyzed statistically to provide valuable information on the interactions among experimental parameters. These designs are rotatable (or near rotatable) and require three levels for each factor. Diagrams of global optimum, which are more direct, were made (Iuliani et al., 2010).

Experimental design approach has been applied to optimize HPLC experimental conditions, such as the resolution and time of analysis for pramipexole in tablets (Srinubabu et al., 2006) and for the determination and optimization of voriconazole in pharmaceutical formulations (Srinubabu et al., 2007). Experimental design was used to optimize a liquid chromatographic method for the separation of six compounds (Harang et al., 2001) and for the separation of the components of a cough syrup (De Beer et al., 1996). The liquid chromatographic separation of fosinopril sodium and its degradation product, fosinoprilat was optimized using an experimental design (Biljana et al., 2005). A capillary electrophoresis method was developed using the experimental design to separate trandolapril and verapamil (Capella-Peiroa et al., 2007). HPLC–ECD method was developed for the analysis of captopril using experimental design (Khamanga and Walker, 2011). HPLC method was developed using experimental design for the determination of tetranortriterpenoids in *Carapa guianensis* seed oil (Tappin et al., 2008).

The objective of this work was to develop a simple, sensitive, precise and accurate HPLC method for EBZ that could be applied to the stress degradation kinetics.

2. Materials and methods

2.1. Chemicals, reagents and solutions

Pharmaceutical grade eberconazole nitrate was gifted by Dr. Reddy's Laboratories Ltd. (Hyderabad, India). Methanol (HPLC grade) was purchased from Merck Chemical Company (India). Potassium dihydrogen orthophosphate, tetra butyl ammonium hydroxide (TBAH) and o-phosphoric acid, hydrochloric acid, sodium hydroxide, and 30% hydrogen peroxide used were of analytical grade and purchased from S D Fine Chem. Ltd. (Mumbai, India). Buffer was prepared by dissolving 1360 mg (10 mM) of potassium dihydrogen orthophosphate and 3330 mg of TBAH (10 mM) in 1 L of HPLC grade water.

Table 1 Hydrolytic, oxidative, thermal and photolytic stress testing conditions for EBZ.

Stress condition	Solvent	Temperature (°C)	Time (days)	Sampling time (days)
Hydrolytic				
Neutral	H_2O	60	25	1,2,3,4,6,7,11,14,25
Acidic	2 N HCl	60	30	1,2,3,4,6,7,11,14,25,30
	5 N HCl	60	30	1,2,3,4,6,7,11,14,25,30
Basic	2 N NaOH	60	30	1,2,3,4,6,7,11,14,25,30
	5 N NaOH	60	30	1,2,3,4,6,7,11,14,25,30
Oxidizing	3% H_2O_2	Room temperature	7	1,2,3,4,6,7
	10% H_2O_2	Room temperature	7	1,2,3,4,6,7
Thermal				
Moist heat	Methanol	60	14	1,2,3,4,6,7,11,14
Dry heat	Methanol	60	14	1,2,3,4,6,7,11,14
Photolytic				
Direct sunlight	Methanol	–	25	1,2,3,4,6,7,11,14,25

Table 2 Factors and levels used in the experimental design.

Factor	Level (−1)	Level (0)	Level (+1)
TBAH (mM)	5	7.5	10
pH	2.6	2.9	3.2
Organic phase (v/v)	20	25	30

2.2. HPLC instrumentation and chromatographic conditions

The HPLC system consisted of two pumps (Analytical Technologies P2230 HPLC pump), a manual injector with 20 µL capacity per injection, and a temperature-controlled column oven. The UV–vis detector (Analytical Technologies UV 2230) was operated at a wavelength of 220 nm. The software used was chromatography workstation A-2000, version 1.6. Columns used were Lichrospher C 18, 250 mm × 4.6 mm, 5.0 µm (Merck, Germany), Atlantis C 18, 250 mm × 4.6 mm, 5.0 µm (Waters Corporation, USA) and Alltima C-8, 250 mm × 4.6 mm, 5.0 µm (Grace, USA).

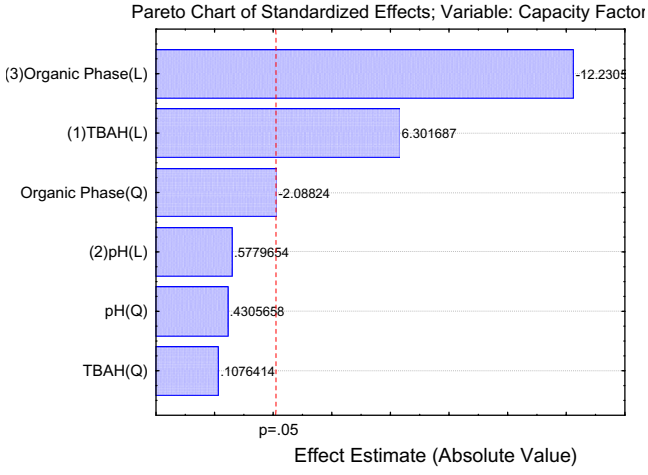


Figure 3 Pareto graph to show the influence of variables on the capacity factor of EBZ.

Chromatographic separation of EBZ was achieved at ambient temperature using a Lichrospher RP C18 (250 mm × 4.6 mm, 5 µm) analytical column; the mobile phase consisted of methanol–potassium dihydrogen orthophosphate (pH 2.8; 10 mM, tetra butyl ammonium hydroxide; 10 mM) (25:75, v/v) at a flow rate of 1.0 mL min^{-1}. pH of buffer was adjusted with o-phosphoric acid. Before use, the mobile phase was filtered through a 0.22 µm nylon membrane filter and sonicated for 15 min. Injection volume was 20 µL, and the optimum wavelength selected for quantification was 220 nm.

2.3. Construction of the calibration curve

Standard stock solution of EBZ was prepared in methanol at a concentration of 10 mg mL^{-1} and further diluted with the mobile phase to furnish the working standard stock solution of 100 µg mL^{-1}. The working standard stock solution was diluted with the mobile phase to prepare calibration samples in the concentration range of 0.5–100 µg mL^{-1}. Triplicate injections of 20 µL were made for each calibration sample and chromatographed under the specified HPLC conditions described previously. Peak areas were plotted against the corresponding concentration to obtain the calibration curve.

2.4. Forced degradation of EBZ

2.4.1. Hydrolytic conditions: acid, alkali and water induced degradation

Standard stock solution (1 mL) was transferred to each of five 10 mL volumetric flasks and the volume was made up to the mark with 2 N HCl, 5 N HCl, 2 N NaOH, 5 N NaOH and water separately. These were subjected to the conditions specified in Table 1.

2.4.2. Oxidizing conditions: hydrogen peroxide-induced degradation

Standard stock solution (1 mL) was transferred to each of two 10 mL volumetric flasks and the volume was made up to the mark with 3% H_2O_2 and 10% H_2O_2 separately. These were subjected to the conditions specified in Table 1.

2.4.3. Thermal conditions: dry heat and moist heat induced degradation

Standard stock solution (1 mL) was transferred to each of two 10 mL volumetric flasks and the volume was made up to the mark with methanol. These were subjected to the conditions indicated in Table 1.

2.4.4. Photolytic degradation: exposure to sun light

Standard stock solution (1 mL) was transferred to a 10 mL volumetric flask and the volume was made up to the mark with methanol. This was subjected to the conditions mentioned in Table 1.

2.5. Sample collection, storage and preparation

Before collecting samples, the volume was made up to the mark with respective solvent. Sample (200 µL) was collected at specified sampling points as indicated in Table 1. The samples from acid and base induced degradation were neutralized by adding 200 µl of appropriate strength of NaOH and HCl. All samples were stored at 2–8 °C in the refrigerator. On the day of analysis samples were diluted with the mobile phase up to 10 mL, filtered with a 0.22 µm membrane syringe filter and injected three times for each sample into HPLC.

3. Results and discussion

3.1. Method development and optimization

Optimum wavelength of 220 nm was selected to reduce the base line noise at absorption maximum (208 nm) of EBZ. Based on EBZ solubility, methanol was selected as organic phase. Initially, reversed-phase analytical columns (C18 and C8) were tested with mobile phase composed of variable composition of methanol (80–20% v/v) and water. Then water was replaced with buffer (10 mM potassium dihydrogen orthophosphate) at different pH levels ranging from 2.5 to 6.0 with a flow rate of 1 mL min^{-1}. In the above employed conditions; EBZ did not get any capacity factor (k). EBZ was eluted along with the mobile phase, i.e. the retention volume was equal to

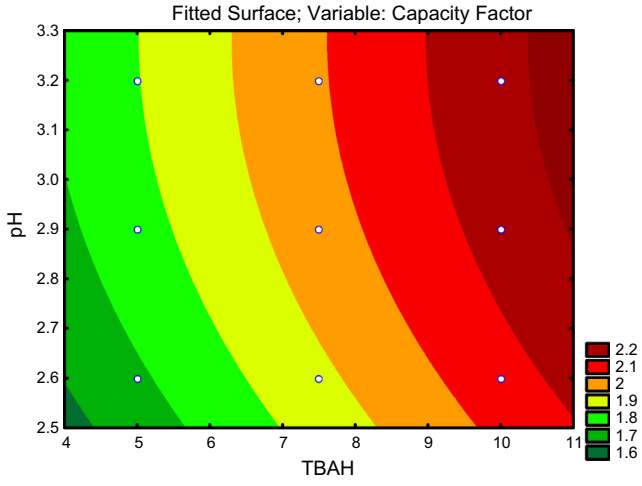


Figure 4 Contour plot for capacity factor as a function of TBAH concentration and buffer pH.

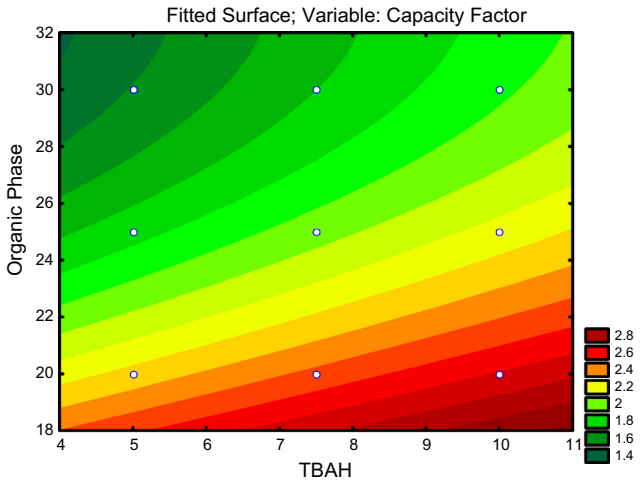


Figure 5 Contour plot for capacity factor as a function of TBAH concentration and organic phase.

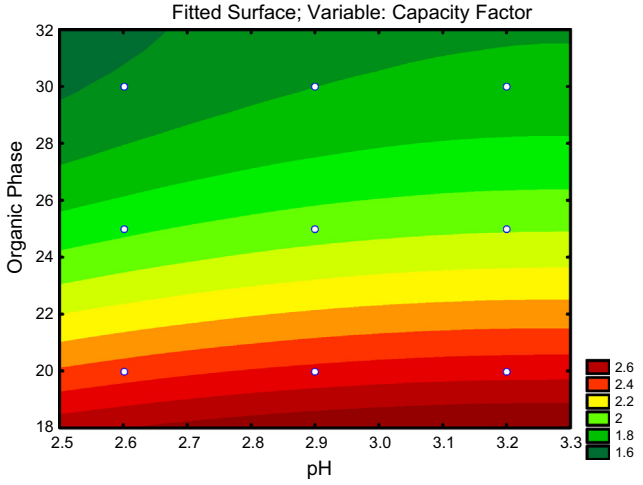


Figure 6 Contour plot for capacity factor as a function of pH and organic phase.

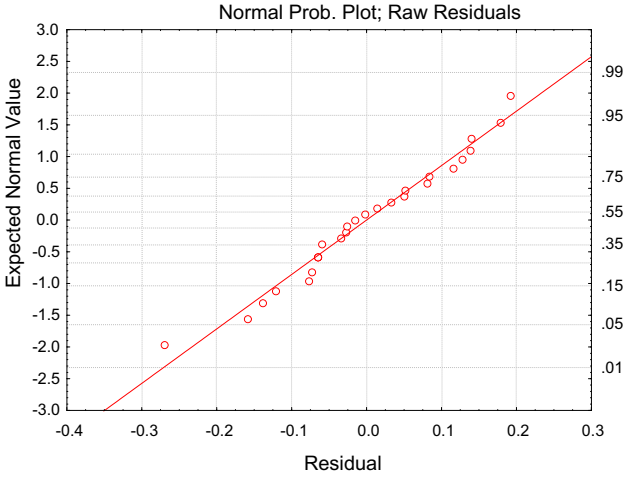


Figure 7 Normal probability plot of residuals for capacity factor.

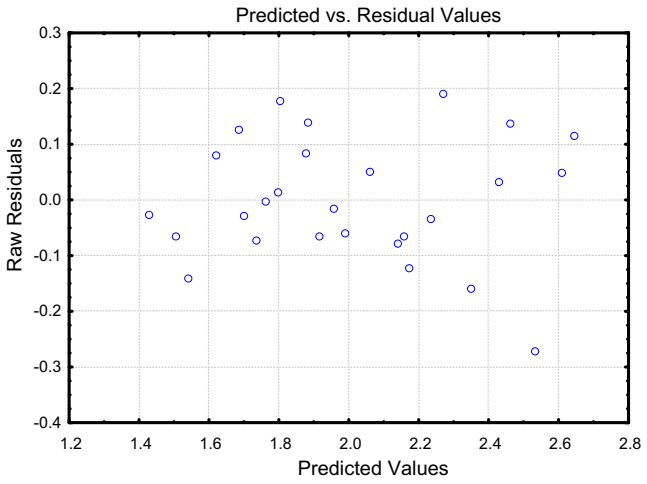


Figure 8 Plot of residuals versus predicted response for capacity factor.

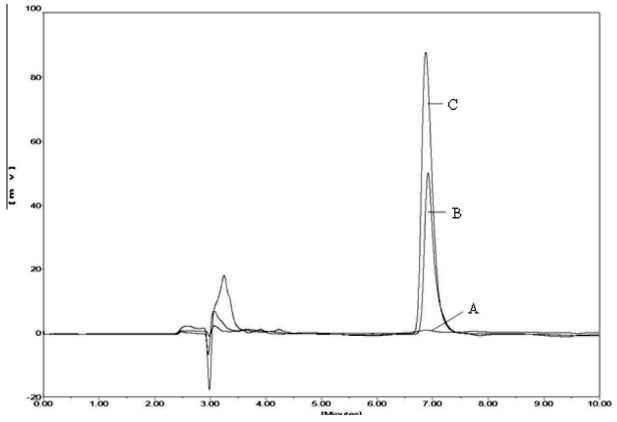


Figure 9 Representative chromatograms of EBZ blank (A), standard (B) and formulation (C).

Table 3 Stability of EBZ in the mobile phase.

Time (h)	Peak area (60 μgmL^{-1} of EBZ)
0	749
3	742
6	749
9	756
12	761
24	760

Mean ± SD, RSD (%), n = 6 752.83 ± 7.41, 0.98.

void volume. Seeing this elution behavior of EBZ, which is a basic drug having pK_a of 6.2, we employed ion pair methodology, using TBAH as an ion pair agent at the concentration of 10 mM. pH of the buffer (10 mM potassium dihydrogen orthophosphate) was adjusted to 3.2 with o-phosphoric acid, i.e., more than two units below the pK_a (6.2) to ionize EBZ by 100%. Based on the above conditions, the mobile phase composed of methanol: buffer at the 30:70 ratio eluted EBZ through the C18 stationary phase (Lichrospher, RP C18, 250 mm × 4.6 mm, 5 μ) having a k of 1.44. In order to get a satisfactory k between 2 and 3, pH of buffer was varied between 2.6 and 3.2, level of methanol was varied between 20% and 30% v/v and TBAH concentration was varied between 5 and 10 mM. Twenty-seven experiments were conducted using the full factorial design (3 factors, 3 levels, 27 runs), in order to rationally examine the effects of TBAH concentration, buffer pH and organic phase concentration on the capacity factor of EBZ. Experimental factors and levels used in the experimental design are shown in Table 2. The factors and ranges selected for consideration were based on previous univariate studies and chromatographic intuition. The data generated were analyzed using Statistica (Version 6.0).

Fig. 3 shows the influence of each factor on the capacity factor. Organic phase and TBAH were significant by linear regression; by quadratic regression only organic phase was significant and TBAH effect was smaller. Effect of pH was non-significant by both linear and quadratic regression. Two-dimensional contour plots are presented in Figs. 4–6 and are very useful for studying the interaction effects of the factors on the capacity factor. Capacity factor of EBZ increases as the TBAH concentration increases (Fig. 4). Fig. 5 shows that increase in TBAH concentration increases the capacity factor and increase in organic phase concentration decreases the capacity factor. The effect of pH on the capacity factor of EBZ was investigated in a pH range of 2.6–3.2; as can be seen from the contour plots (Figs. 4 and 6), it has no effect on capacity factor.

The model that has been developed can be used to predict the capacity factor of EBZ within the limits of the experiments. The normal probability plot of the residuals and the plot of the residuals versus the predicted response for capacity factor are shown in Figs. 7 and 8. Close inspection of Fig. 7 reveals that the residuals generally fall on a straight line which indicates that the errors are normally distributed, thus supporting the fact that the model fits the data adequately. These plots are very important and are required to check the normality assumption in a fitted model. This will ensure that the model provides an adequate approximation to the optimization process. It is clear that there is no obvious pattern followed in the residual versus predicted response as shown in Fig. 8. The plot reveals an almost equal scatter above and below the X-axis, implying that the proposed model is adequate and there is no reason to suspect any violation of the independence or constant variance assumption.

The optimized chromatographic conditions obtained from the design were mixture of 10 mM potassium dihydrogen orthophosphate (pH 2.8) containing 10 mM TBAH and methanol (25:75, v/v), at a flow rate of 1.0 mL min^{-1}. These chromatographic conditions achieved reasonable retention (k = 2.06) and symmetric peak shape for EBZ with a retention time of 7.05 min (Fig. 9). No interference from the blank and cream formulation excipients was observed at the retention time of EBZ (Fig. 9). Percentage of recovery (n = 6) obtained from the formulation was 100.5 ± 1.8.

3.2. Solution stability

The stability of EBZ in the mobile phase was investigated by analyzing the standard of EBZ (60 μg mL^{-1}) at 0, 3, 6, 9, 12 and 24 h. No significant variation in the peak area of standard

Table 4 System suitability data.

Property	Mean ± SD, $n = 6$	RSD (%)	Required limits
Retention time (*Rt*)	7.05 ± 0.04	0.56	RSD ⩽ 2%
Capacity factor (*k*)	2.06 ± 0.004	0.19	–
Theoretical plates (*N*)	32772 ± 182	0.55	N > 2000
Tailing factor (*T*)	1.31 ± 0.02	1.52	T ⩽ 2

Table 5 Recovery of the standard from stress degraded samples by standard addition method.

Level of standard added (%)	Amount of standard added (μg)	Sample	Mean peak area ± SD, RSD (%) ($n = 3$)		Amount of standard found (μg)	Recovery for standard (%)
			Standard + Sample	Standard		
80	40	1425.61 ± 15.24, 1.06	1930.53 ± 18.85, 0.90	504.92 ± 5.74, 1.13	40.02	100.05
100	50	1453.28 ± 16.31, 1.12	2079.12 ± 23.25, 1.11	625.84 ± 6.86, 1.09	50.05	100.1
120	60	1486.61 ± 16.56, 1.11	2240.32 ± 26.21, 1.16	753.71 ± 9.15, 1.21	60.01	100.02

Table 6 Results of intra-day and inter-day precision.

Concentration	Intra-day precision		Inter-day precision	
	Peak area		Peak area	
(μg mL^{-1})	Mean ± SD ($n = 6$)	RSD (%)	Mean ± SD ($n = 6$)	RSD (%)
10	140.50 ± 1.64	1.16	137.22 ± 2.28	1.66
40	528.16 ± 5.94	1.12	533.57 ± 6.93	1.30
60	713.16 ± 7.78	1.09	729.19 ± 13.02	1.78
80	938.33 ± 10.65	1.13	968.12 ± 16.69	1.72

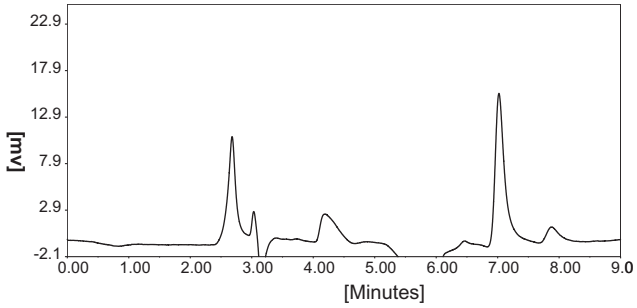


Figure 10 A model chromatogram of EBZ under acidic stress condition.

solution was observed (Table 3) and also no additional peaks were found in the chromatogram, indicating that EBZ was stable in the mobile phase.

3.3. Method validation

To confirm the suitability of the method for its intended purpose, the method was validated in accordance with the ICH guidelines (ICH guideline Q2 (R1), 2005) for system suitability, linearity, limits of detection and quantification, accuracy, intra-day and inter-day precision, specificity and robustness.

3.3.1. System suitability

System-suitability test was an integral part of method development and has been used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), capacity factor (*k*), number of theoretical plates (*N*) and tailing factor (*T*), were evaluated for six replicate injections of the drug at a concentration of 60 μg mL^{-1}. The results presented in Table 4 are within the acceptable limits.

3.3.2. Linearity

Linearity of the proposed method was evaluated according to the ICH guidelines. EBZ showed linearity in the concentration range of 10–80 μg mL^{-1}, ($r^2 = 0.999$). The regression equation obtained was $Y = 12.50X + 1.785$, where Y is peak area and X is concentration of EBZ (μg mL^{-1}). This equation was used to determine the amount of EBZ present in the stability samples.

3.3.3. Limits of detection and quantification

The limit of detection (LOD) was defined as the lowest concentration of EBZ resulting in a signal-to-noise ratio of 3:1 and limit of quantification (LOQ) was expressed as a signal-to-noise ratio of 10:1. Due to the difference in detector response, different concentrations ranging from 0.01 to 2 μg mL^{-1} were prepared and analyzed. The LOD and LOQ obtained were 0.3 and 0.9 μg mL^{-1}, respectively.

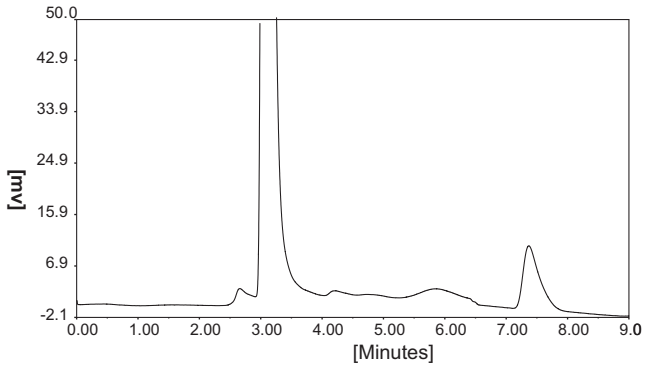


Figure 11 A model chromatogram of EBZ under oxidative stress condition.

3.3.4. Accuracy

Accuracy of the method was determined by performing the recovery experiments. Known amount of the standard at 80%, 100% and 120% levels was fortified to the degradation sample. Peak area of the standard was calculated by the difference of peak area between fortified and unfortified samples. Three replicate samples of each concentration level were prepared and the percentage recovery at each level ($n = 3$) was determined (Table 5). For EBZ, the results obtained are in good agreement with the added amounts.

3.3.5. Intra-day and inter-day precision

Intra-day and inter-day precision was evaluated by injecting four different concentrations (10, 40, 60, and 80 μg mL^{-1}) of EBZ. For intra-day variation, sets of six replicates of the four concentrations were analyzed on the same day; for inter-day variation, six replicates were analyzed on six different days. The intra-day and inter-day precision (%RSD) was found to be less than 2% (Table 6), indicating that the method was precise.

3.3.6. Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. The specificity of the HPLC method was illustrated in Figs. 10 and 11, where complete separation of EBZ was noticed in the presence of degradants. The average *Rt* ± standard deviation for EBZ was found to be 7.05 ± 0.04 min, for six replicates. The peaks obtained were sharp and had clear baseline separation.

3.3.7. Robustness

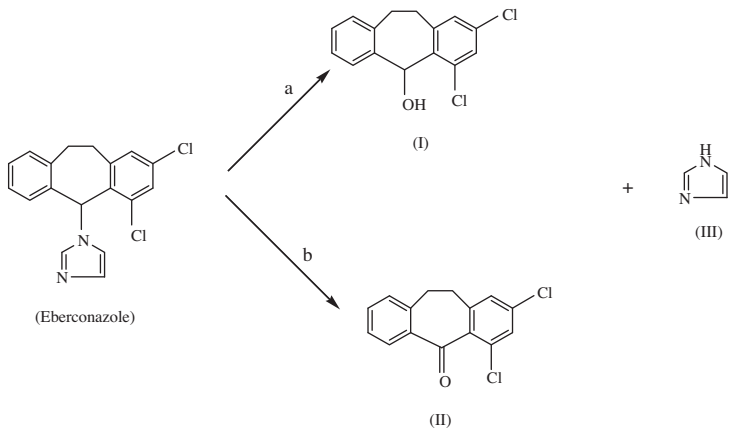
A method is robust if it is unaffected by small changes in operating conditions. To evaluate HPLC method's robustness few parameters were deliberately varied. The parameters included variation of C18 columns from different manufacturers, pH of the buffer, flow rate and percentage of methanol in the mobile phase. Two analytical columns were used during the experiment, one from Germany (Lichrospher C 18 column) and the other from USA (Atlantis C 18 column). Each of the three examined factors (pH, flow rate, and methanol percentage) selected was changed one at a time to estimate the effect. Replicate injections ($n = 6$) of standard solution (60 μg mL^{-1}) were performed under small changes of chromatographic parameters (factors). Flow rate was varied by 1 ± 0.1 mL min^{-1}; level of methanol in the mobile phase was varied by 25 ± 2% (v/v), while pH was varied by 2.8 ± 0.1. Results obtained are presented in Table 7, indicating that the results remained unaffected by small variations of these parameters. The results from the two columns indicated that there is no significant difference between the results from the two columns.

3.4. Stability-indicating property

An analytical method is stability-indicating if this method can separate all the process-related impurities and all the degrada-

Table 7 Results for the analysis of robustness.

Factors	Level	Retention time (min) Mean ± SD ($n = 6$)	Tailing factor Mean ± SD ($n = 6$)	Peak area Mean ± SD ($n = 6$)
A: Flow rate (mL min^{-1})				
0.9	−1	7.09 ± 0.02	1.35 ± 0.04	758.24 ± 8.45
1.0	0	7.05 ± 0.02	1.31 ± 0.03	751.45 ± 10.34
1.1	+1	6.99 ± 0.03	1.31 ± 0.03	749.12 ± 9.86
Mean		7.04 ± 0.03	1.32 ± 0 .03	752.6 ± 9.58
B: Percentage of methanol in the mobile phase (v/v)				
23	−1	7.11 ± 0.06	1.30 ± 0.02	756.65 ± 7.28
25	0	7.05 ± 0.07	1.32 ± 0.03	752.87 ± 6.57
27	+1	6.99 ± 0.06	1.31 ± 0.02	748.34 ± 8.43
Mean		7.05 ± 0.06	1.31 ± 0.02	752.62 ± 9.86
C: pH of buffer				
2.7	−1	7.05 ± 0.06	1.30 ± 0.02	752.45 ± 7.22
2.8	0	7.05 ± 0.04	1.33 ± 0.04	755.88 ± 6.16
2.9	+1	7.03 ± 0.03	1.36 ± 0.03	753.58 ± 8.65
Mean		7.04 ± 0.04	1.33 ± 0.03	753.95 ± 7.35
D: Columns from different manufacturers				
(I) Lichrospher C18 column		7.05 ± 0.08	1.34 ± 0.04	754.52 ± 7.24
(II) Atlantis C18 column		7.03 ± 0.04	1.32 ± 0.03	749.67 ± 8.68
Mean		7.04 ± 0.06	1.33 ± 0.03	752.09 ± 7.86



Scheme 1 Proposed degradation mechanism of EBZ under (a) hydrolytic/thermal/photolytic stress conditions (b) oxidative stress conditions.

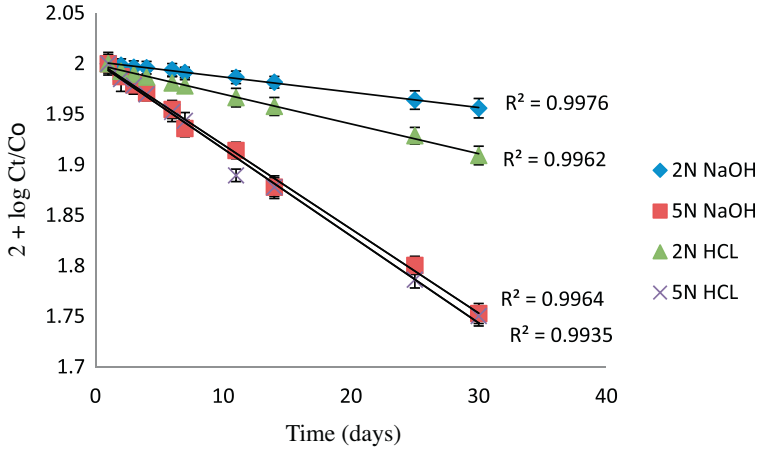


Figure 12 First order plots for the degradation of EBZ under acidic and basic stress conditions (each point represents the mean ± SD, $n = 3$).

tion products from the major peak of the sample. The model chromatograms of EBZ under acidic and oxidative stress conditions are presented in Figs. 10 and 11. EBZ under acidic and basic stress conditions showed same degradant peaks at the retention time of 2.8, 4.1 and 7.9 min. Stress samples under dry heat, moist heat, water hydrolysis and photolysis showed two degradant peaks at 2.8 and 4.1 min. Under oxidative stress conditions, EBZ showed two degradant peaks at 2.8 and 6.0 min; the peak observed at 3.1 min corresponds to the blank. This indicates that the drug is susceptible to hydrolytic (acid, base and water), oxidative, thermal and photolytic degradation. In all the above cases the degradant peaks did not interfere with the EBZ peak, suggesting that the method enabled specific analysis of EBZ in the presence of its degradation products. Scheme 1 presents the proposed degradation mechanism of EBZ in different stress conditions. 2,4-dichloro-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ol (I) and Imidazole (III) might be the major degradation products in hydrolytic/thermal/photolytic stress conditions; 2,4-dichloro-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-one (II) and Imidazole (III) might be the degradation products in oxidative stress conditions.

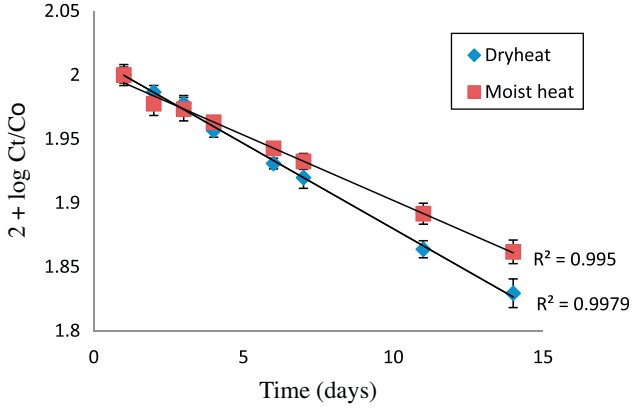


Figure 13 First order plots for the degradation of EBZ under thermal stress conditions (each point represents the mean ± SD, $n = 3$).

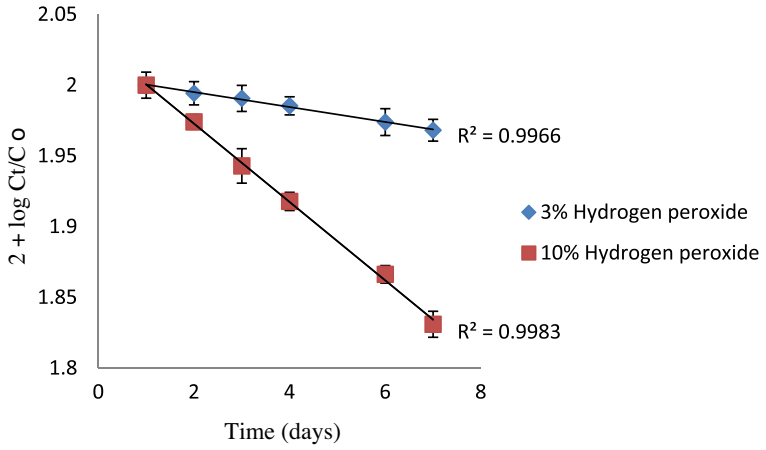


Figure 14 First order plots for the degradation of EBZ under oxidative stress conditions (each point represents the mean ± SD, $n = 3$).

3.5. Kinetic investigation

Treatment of EBZ under specified stress conditions resulted in a gradual decomposition of EBZ in all conditions. Since the degradation was performed with a large excess of solvent (9 mL) compared to drug solution (1 mL), the degradation of EBZ followed pseudo-first-order kinetics (Florence and Attwood, 1998) as a linear relationship between log percentage of EBZ remaining and time was established, having good correlation coefficients (Figs. 12–15). Pseudo-first-order is the term used when two reactants are involved in the reaction but one of them is in such a large excess that any change in its concentration is negligible compared with the change in concentration of the other reactant (drug). The kinetic parameters are presented in Table 8. Rate constant (K), time left for 50% potency ($t_{1/2}$) and time left for 90% potency (t_{90}) for each stress condition were calculated using Eqs. (1)–(3), respectively (Connors et al., 1986):

$$\log[C_t] = \log[C_0] - \frac{Kt}{2.303} \quad (1)$$

$$t_{1/2} = \frac{0.693}{K} \quad (2)$$

$$t_{90} = \frac{0.105}{K} \quad (3)$$

where K is the rate constant, $[C_0]$ is the concentration of EBZ at time $t = 0$ and $[C_t]$ is its concentration at time t.

The K values per day were found to be 3.48×10^{-3}, 19.18×10^{-3}, 6.78×10^{-3}, 19.84×10^{-3}, 12.14×10^{-3}, 63.79×10^{-3}, 30.69×10^{-3}, 23.53×10^{-3}, 20.20×10^{-3} and 21.72×10^{-3} for 2 N NaOH, 5 N NaOH, 2 N HCl, 5 N HCl, 3% H_2O_2, 10% H_2O_2, dry heat, moist heat, water hydrolysis and photolytic conditions, respectively. The rate constant values were increased as the strength of NaOH, HCl and H_2O_2 increased. K value was increased approx. six times as the strength of NaOH was increased from 2 N to 5 N, while the rate of degradation was increased up to approx. three times under the same conditions of acid (HCl) treatment, indicating more susceptibility of EBZ under basic media compared to acidic. The K value for water induced degradation was found to be similar to the degradation by 5 N HCl and 5 N NaOH, specifying the importance of water toward EBZ degradation. Between thermal treatments K value for dry heat was found to be higher than moist heat. Extensive degradation was observed in oxidative conditions, where K value was found to be highest among all the tested conditions. Hence the effect of oxygen needs to be considered for topical formulation of EBZ. Suitable antioxidants need to be a part of the topical formulation of EBZ. K value obtained for photolytic degradation was similar to water hydrolysis, 5 N NaOH and 5 N HCl. This illustrates the prominent effect of light toward the stability of EBZ. $t_{1/2}$ and t_{90} values for all the tested stress conditions are shown in Table 8, both $t_{1/2}$ and t_{90} were found to be lowest (10.86 and 1.65 days) for oxidative condition (10% H_2O_2)

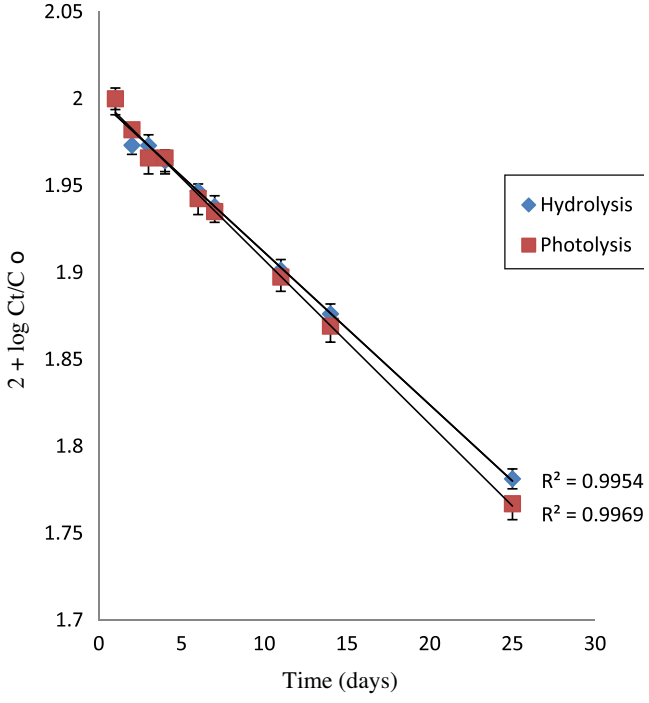


Figure 15 First order plots for the degradation of EBZ under hydrolytic and photolytic stress conditions (each point represents the mean ± SD, $n = 3$).

Table 8 Summary of EBZ degradation kinetics.

Stress condition	K (day^{-1})[a]	$t_{1/2}$ (days)[b]	t_{90} (days)[c]	Degraded (%)
2 N NaOH	3.48×10^{-3}	198.99	30.24	9.64
5 N NaOH	19.18×10^{-3}	36.13	5.49	43.39
2 N HCl	6.78×10^{-3}	102.23	15.53	18.87
5 N HCl	19.84×10^{-3}	34.93	5.31	43.72
3% H_2O_2	12.14×10^{-3}	57.06	8.67	7.08
10% H_2O_2	63.79×10^{-3}	10.86	1.65	32.24
Dry heat	30.69×10^{-3}	22.58	3.43	32.49
Moist heat	23.53×10^{-3}	29.45	4.47	27.26
Hydrolysis	20.20×10^{-3}	34.31	5.21	39.57
Photolysis	21.72×10^{-3}	31.91	4.85	41.53

[a] Rate constant per day.
[b] Half-life.
[c] Time left for 90% potency.

and highest (198.99 and 30.24 days) for alkaline hydrolysis with 2 N NaOH.

4. Conclusion

The proposed HPLC method provides simple, accurate and reproducible quantitative analysis for the determination of EBZ in the presence of its degradants. It was found that EBZ was rapidly degraded under oxidative, hydrolytic (acid and alkali) and photolytic conditions. The degradation of EBZ was found to be of pseudo-first-order kinetics in analyte's concentration. The reaction rate increases with increase in strength of the acid/base/H_2O_2 solution. This study suggests that the formulation scientist needs to incorporate antioxidants in the topical formulation of EBZ and also care should be taken to prevent photolysis upon its exposure to sun light.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Barbanoj, M.J., Antonijoan, R., Garcia-Gea, C., Puntes, M., Gich, I., Jane, F., 2005. Eberconazole cream: topical and general tolerability, sensitisation potential and systemic availability. Methods Find. Exp. Clin. Pharmacol. 27, 227–234.

Biljana, J., Mirjana, M., Darko, I., Andelija, M., Slavko, M., 2005. Microemulsion liquid chromatographic method for characterisation of fosinopril sodium and fosinoprilat separation with chemometrical support. Anal. Bioanal. Chem. 383, 687–694.

Capella-Peiroa, M.E., Font-Ruberta, M., Álvarez-Rodrígueza, L., Esteve- Romeroa, J., Durgbanshib, A., Bose, D., 2007. Optimization using a factorial design for the separation of trandolapril and verapamil by capillary electrophoresis. J. Liq. Chromatogr. Relat. Technol. 30, 2975–2988.

Connors, K.A., Amidon, G.R., Stella, V.J., 1986. Chemical stability of pharmaceuticals. A Handbook for Pharmacists. Wiley, New York.

De Beer, J.O., Vandenbroucke, C.V., Massart, D.L., De Spiegeleer, B.M., 1996. Half-fraction and full factorial designs versus central composite design for retention modeling in reversed-phase ion-pair liquid chromatography. J. Pharm. Biomed. Anal. 14 (5), 525–541.

Ekiert, R.J., Krzek, J., Talik, P., 2010. Chromatographic and electrophoretic techniques used in the analysis of triazole antifungal agents – a review. Talanta 82, 1090–1100.

Florence, A.T., Attwood, D., 1998. Physicochemical Principles of Pharmacy. Macmillan Press, London.

Harang, V., Karlsson, A., Josefson, M., 2001. Liquid chromatography method development and optimization by statistical experimental design and chromatogram simulations. Chromatographia 54 (11–12), 703–709.

ICH guideline Q1A (R2), 2003. Stability testing of new drug substances and products. ICH [Online]. Available at: <http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step 4/Q1A_R2__Guideline.pdf> (accessed 12.03.11.).

ICH guideline Q2 (R1), 2005. Validation of analytical procedures: text and methodology. ICH [Online]. Available at: <http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf> (accessed 12.03.11.).

Iuliani, P., Carlucci, G., Marrone, A., 2010. Investigation of the HPLC response of NSAIDs by fractional experimental design and multivariate regression analysis. Response optimization and new retention parameters. J. Pharm. Biomed. Anal. 51 (1), 46–55.

Khamanga, S.M., Walker, R.B., 2011. The use of experimental design in the development of an HPLC–ECD method for the analysis of captopril. Talanta 83, 1037–1049.

Singh, S., Bakshi, M., 2000. Guidance on conduct of stress tests to determine inherent stability of drugs. Pharm. Tech. 24, 1–14.

Srinubabu, G., Jaganbabu, K., Sudharani, B., Venugopal, K., Girizasankar, G., Rao, J.V.L.N.S., 2006. Development and validation of LC method for the determination of pramipexole using an experimental design. Chromatographia 64, 95–100.

Srinubabu, G., Raju Ch, A.I., Sarath, N., Kiran Kumar, P., Seshagiri Rao, J.V.L.N., 2007. Development and validation of a HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design. Talanta 71, 1424–1429.

Sweetman, S.C., 2009. Martindale: The Complete Drug Reference. Pharmaceutical Press, London.

Tappin, M.R.R., Nakamura, M.J., Siani, A.C., Lucchetti, L., 2008. Development of an HPLC method for the determination of tetranortriterpenoids in *Carapa guianensis* seed oil by experimental design. J. Pharm. Biomed. Anal. 48, 1090–1095.

Torres-Rodríguez, J.M., Mendez, R., López-Jodra, O., Morera, Y., Espasa, M., Jimenez, T., Lagunas, C., 1999. In vitro susceptibilities of clinical yeast isolates to the new antifungal eberconazole compared with their susceptibilities to clotrimazole and ketoconazole. Antimicrob. Agents Chemother. 43 (5), 1258–1259.