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Validated stability indicating liquid chromatographic determination of ebastine in pharmaceuticals after pre column derivatization: Application to tablets and content uniformity testing

Fawzia Ibrahim, Mohie Khaled Sharaf El- Din, Manal Ibrahim Eid and Mary Elias Kamel Wahba*

Abstract

An accurate, simple, sensitive and selective reversed phase liquid chromatographic method has been developed for the determination of ebastine in its pharmaceutical preparations. The proposed method depends on the complexation ability of the studied drug with Zn^{2+} ions. Reversed phase chromatography was conducted using an ODS C18 (150 × 4.6 mm id) stainless steel column at ambient temperature with UV-detection at 260 nm. A mobile phase containing 0.025%w/v Zn^{2+} in a mixture of (acetonitril/methanol; 1/4) and Britton Robinson buffer (65:35, v/v) adjusted to pH 4.2, has been used for the determination of ebastine at a flow rate of 1 ml/min. The calibration curve was rectilinear over the concentration range of 0.3 - 6.0 $\mu\text{g/ml}$ with a detection limit (LOD) of 0.13 $\mu\text{g/ml}$, and quantification limit (LOQ) of 0.26 $\mu\text{g/ml}$. The proposed method was successfully applied for the analysis of ebastine in its dosage forms, the obtained results were favorably compared with those obtained by a comparison method. Furthermore, content uniformity testing of the studied pharmaceutical formulations was also conducted. The composition of the complex as well as its stability constant was also investigated. Moreover, the proposed method was found to be a stability indicating one and was utilized to investigate the kinetics of alkaline and ultraviolet induced degradation of the drug. The first-order rate constant and half life of the degradation products were calculated.

Background

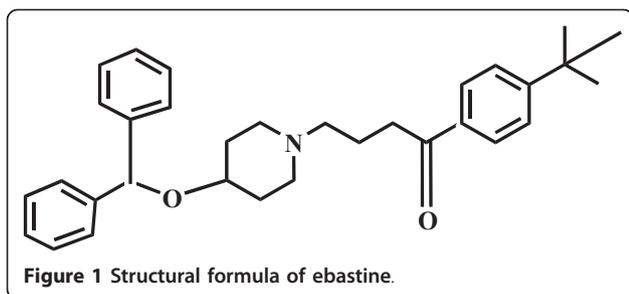
Ebastine; (4'-*tert.*-butyl-4-[4-(diphenylmethoxy)- piperidino]butyrophenone (Figure 1), is a potent and selective histamine H_1 -receptor antagonist in vivo, which has little anticholinergic and antiserotonergic properties [1]. This agent also has no effects on cardiovascular and psychomotor functions, which occurred during treatment with classical antihistamine agents such as chlorpheniramine and diphenhydramine [1]. Literature survey reveals that the drug was determined by spectrofluorimetry [2], and by high performance liquid chromatography either alone in its dosage forms [3,4] or in presence of its metabolites [5,6].

Derivatization is considered as an important tool for analysis, especially using chromatography, and great strides have been made in developing key reactions for several classes of compounds since it enhances analyte recovery, improves separation, detectability and compound identification [7].

Several liquid chromatographic techniques applying metal complex derivatization has been reported for the determination of many compounds of pharmaceutical interest such as dithiocarbamates [8], halogenated 8-hydroxyquinolines [9], glycosaminoglycans [10], β -lactams [11] and various secondary amino drugs (sympathomimetic, β -blocking, anti-arrhythmic agents) [12].

The aim of the present work is to develop an efficient novel liquid chromatographic method for the determination of ebastine after its complexation with Zn^{2+} in a short chromatographic run, and to prove the stability-

* Correspondence: marywahba2004@yahoo.com
Department of Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt



indicating property of the method, and its advantage over the previously reported HPLC methods [3,4] in terms of its high sensitivity. Moreover, the method illustrates a full detailed study for the kinetic degradation of the drug applying the proposed method, where different kinetic parameters have been calculated.

Experimental

Materials and reagents

- Ebastine (EBS); of purity 99.94% was kindly provided by Meivo Pharmaceutical Company, Cairo, Egypt.
 - Britton Robinson buffer was prepared [13] by mixing 0.03 M of each of acetic acid, o-phosphoric acid and boric acid. The pH was adjusted using 0.2 M sodium hydroxide.
 - Methanol (Sigma-Aldrich), HPLC grade
 - Acetonitrile (Sigma-Aldrich), HPLC grade.
 - Sodium hydroxide (2M solution), hydrochloric acid (2M solution), hydrogen peroxide (6% v/v solution); (BDH, Poole, UK).
 - Acetate buffer (pH 3.5 - 5.6) and borate buffers (pH 6 - 9) were prepared according to the British Pharmacopoeia [14].
 - Zinc (II) chloride (BDH, Poole, UK), 1×10^{-3} M aqueous solution, were prepared in distilled water.

Pharmaceutical preparations

- * Bastab[®] tablets (BN#112038), labeled to contain 20 mg ebastine/tablet, Meivo Pharmaceutical Company, Cairo, Egypt.
- * Evastine[®] syrup (BN# 94634), labeled to contain 5 mg ebastine/5 ml, Marcyrl Pharmaceutical Industries, El Obour City, Egypt.
- * Ebastel[®] tablets (BN# 916201), labeled to contain 10 mg ebastine/tablet, Global Napi Pharmaceutials, Cairo, Egypt.

All were obtained from commercial sources in the local market.

Apparatus

- Separation was performed with a Shimadzu C-R6A Chromatopac equipped with a Rheodyne injector valve with a 20 μ L loop and a UV/VIS detector.

- A Shimadzu UV 1601 PC Spectrophotometer equipped with a pair of 1 cm matched cells, recording range: 0-2; wavelength: 200-400 nm; factor:1; number of cells:1; cycle time:0.1 min was used.

- Mass spectroscopy was performed on DI Analysis Shimadzu QP-2010 Plus.
- Infra red spectroscopy was conducted using Mattson 5000 FITR Spectrometer.
- TLC aluminium sheets 20 \times 20 silica gel 60 F254 for TLC were used.

Columns and mobile phases

Separation was achieved on an EC nucleosil C18-SN: 4115568 column (150 mm \times 4.6 mm id (5 mm) combined with a guard column (Merck, Darmstadt, Germany). The columns were operated at ambient temperature. The analytical system was washed daily with 60 ml of 1:1 mixtures of water and methanol to eliminate the mobile phase; this did not cause any change in the column performance. The mobile phase was prepared by mixing (acetonitrile/methanol; 1/4) with 0.03 M Britton Robinson buffer in a ratio of 65:35 v/v at pH of 4.2. The mixture was then sonicated for 30 minutes. The resulting mobile phase was filtered through a 0.45 μ m membrane filter (Millipore, Ireland).

Standard solutions

A stock solution containing 10.0 mg/ml of EBS was prepared in methanol and further diluted with the same solvent to obtain the working concentration range for the spectrophotometric measurements, and diluted with the mobile phase for the HPLC measurements. This solution was found to be stable for at least two weeks when kept in the refrigerator.

A stock solution containing 1.0 mg/ml of cetirizine hydrochloride internal standard was prepared in methanol and further diluted with the mobile phase to obtain a final concentration of 15.0 μ g/ml.

Calibration curve

Aliquots of EBS working standard solution (20 μ g/ml) covering the concentration range (0.3-6.0 μ g/ml) were transferred into a series of 10 ml volumetric flasks, 2.5 ml of acetate buffer pH 5 were added, followed by 1.5 ml of 1×10^{-3} M ZnCl₂, mixed with 15 μ g/ml aliquots of cetirizine hydrochloride and diluted with the mobile phase to the mark. Twenty μ L aliquots were injected (in triplicates) and eluted with the mobile phase under the reported chromatographic conditions. The calibration curve was constructed by plotting the peak area ratio against the final concentration of the drug (μ g/ml). Alternatively, the corresponding regression equation was derived.

Analysis of tablets

Twenty tablets were weighed and pulverized. An accurately weighed quantity of the powder equivalent to 20

mg of EBS was transferred into a small conical flask, and extracted three successive times each with 30 ml of methanol. The extract was filtered into 100 ml volumetric flask. The conical flask was washed with few millilitres of methanol and completed to the mark with the same solvent. The procedure was followed as described under "Calibration Curve". The nominal contents of the tablets were calculated using either the calibration graph or the corresponding regression equation.

Preparation of the degradation products

For the kinetic study, 1 ml aliquots of EBS standard solution were transferred into a series of 25 ml volumetric flasks to obtain a final concentration of 400 µg/ml where 2 M sodium hydroxide, 2 M hydrochloric acid, or 6% hydrogen peroxide were added to prepare the alkaline, acidic, or oxidative degradation products respectively. The solutions were left in a thermostatically controlled water bath at different temperature settings for a fixed time interval (15 minutes). Regarding the UV degradation, a methanolic solution of the studied drug was exposed to Deuterium lamp in a cabinet distance of 15 cm at room temperature, and aliquots of the hydrolyzed solutions were analyzed every 3 hours interval.

Aliquot volumes of the degraded solutions were transferred to a series of 10 ml volumetric flasks and neutralized with 2 M hydrochloric acid, or 2 M sodium hydroxide for alkaline and acidic degradation respectively, and the steps were completed as described under "calibration curve". The absorption spectra were recorded at 260 nm, $\log a/a-x$ versus time (minutes) was plotted to get the reaction rate constant and the half life time $t_{1/2}$.

For HPLC measurements, the above solutions were completed to the volume with the mobile phase, and the material was tested for degradation by the apparent decrease in the peak area ratio of the formed complex which appears at 5.3 minute.

Isolation of the formed complex

The formed complex was successively isolated and purified by preparative TLC using methylenechloride: methanol (90:10 v/v). The solvent was removed by evaporation under reduced pressure, and the purity of the complex was tested by TLC. The TLC was performed using chloroform: methanol (75:25 v/v) as a developing solvent, where the R_f of ebastine and EBS-Zn²⁺ complex were 0.74 and 0.55 respectively, which in turn confirms the completeness of the complex formation reaction.

After confirmation of the purity of the complex, infra red (IR) and mass spectroscopy (MS) were performed to elucidate the structure of the resultant product.

The IR spectrum of the studied complex shows the same bands of the main functional groups in ebastine; such as the stretching band of the C-N of the tertiary amine at 1361 cm⁻¹, the stretching band corresponding

to C = O at 1677 cm⁻¹, and also the ether C-O band at 1072 cm⁻¹ (Figure 2), which means that the aforementioned functional groups were not altered through the complexation reaction.

The MS spectrum of the complex (Figure 3) shows the molecular ion peak at m/z 1070, and putting in consideration that the molecular weight of ebastine is 470 [15]; the resultant spectrum confirms the reaction stoichiometry that two moles of EBS reacts with one mole of Zn²⁺. The molecular ion peak in turn is subjected to further fragmentation in the MS through breakage of the bonds formed between Zn²⁺ and each of the ether oxygen and the nitrogen atom of the tertiary amino group to yield a peak at m/z of 470; while the fragments appearing at m/z 161 and 678 suggest fragmentation at the carbonyl atom (Scheme 1).

fragmentation pattern of EBS-Zn²⁺ complex in the MS.

According to this interpretation, the reaction pathway was proposed to proceed as shown in scheme 2. Scheme 2

pathway of complex formation reaction between EBS and Zn²⁺ using the proposed method.

Validation of the method

The method was tested for linearity, sensitivity, accuracy, precision and robustness.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample [16]. Linearity of the proposed method was assessed by estimating the linear dependence of the obtained peak area ratios on the concentration of EBS.

Sensitivity

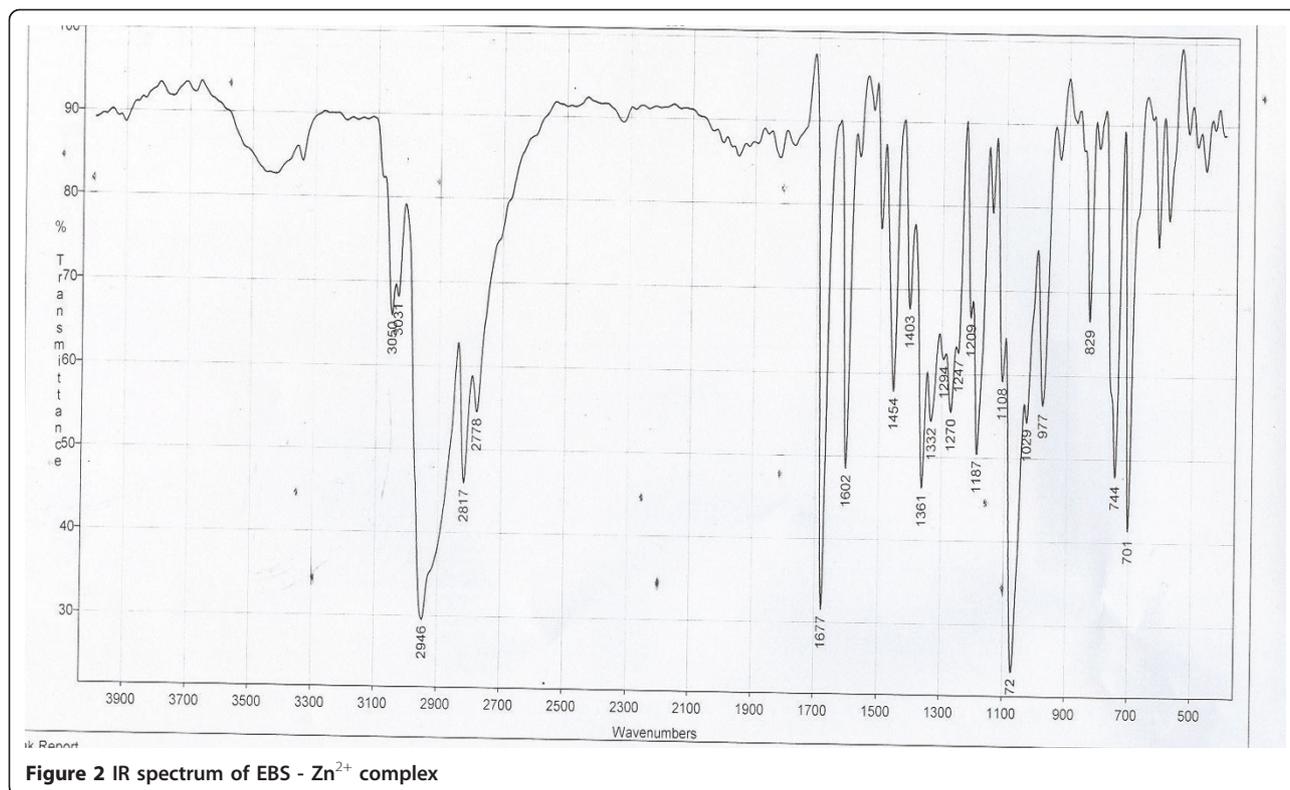
Detection limit (LOD) is the lowest concentration of the drug that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1 [17] and is confirmed by analyzing a number of samples near this value using the following equation:

$$\text{The signal - to - noise ratio } s = H/h$$

Where H = height of the spectrum corresponding to the drug

h = absolute value of the largest noise fluctuation from the baseline of the spectrum of a blank solution.

While the limit of quantification (LOQ); is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy. It is quoted as the concentration yielding a signal-to-noise ratio of 10:



1 and is confirmed by analyzing a number of samples near this value [17].

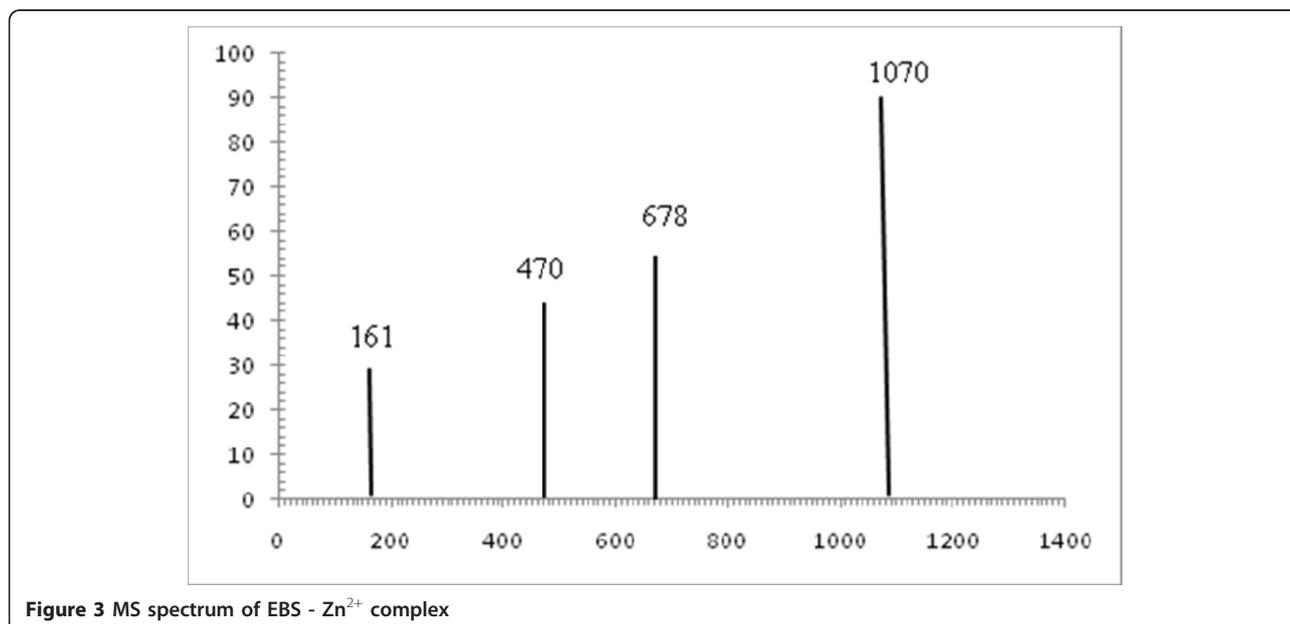
Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between an accepted reference value and the value found [16]. The accuracy of the proposed method was evaluated by analyzing standard

solutions of EBS. The results obtained by the proposed method were favorably compared with those obtained by the comparison method [4].

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same



homogeneous sample under the prescribed conditions. Precision may be considered through repeatability and intermediate precision [16].

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision [16]. The repeatability was evaluated through the replicate analysis of different concentrations of EBS samples, either in pure or in dosage forms.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. [16]. It was performed through replicate analysis of different concentrations of EBS samples, either in pure or dosage form on four successive days.

Robustness of the method

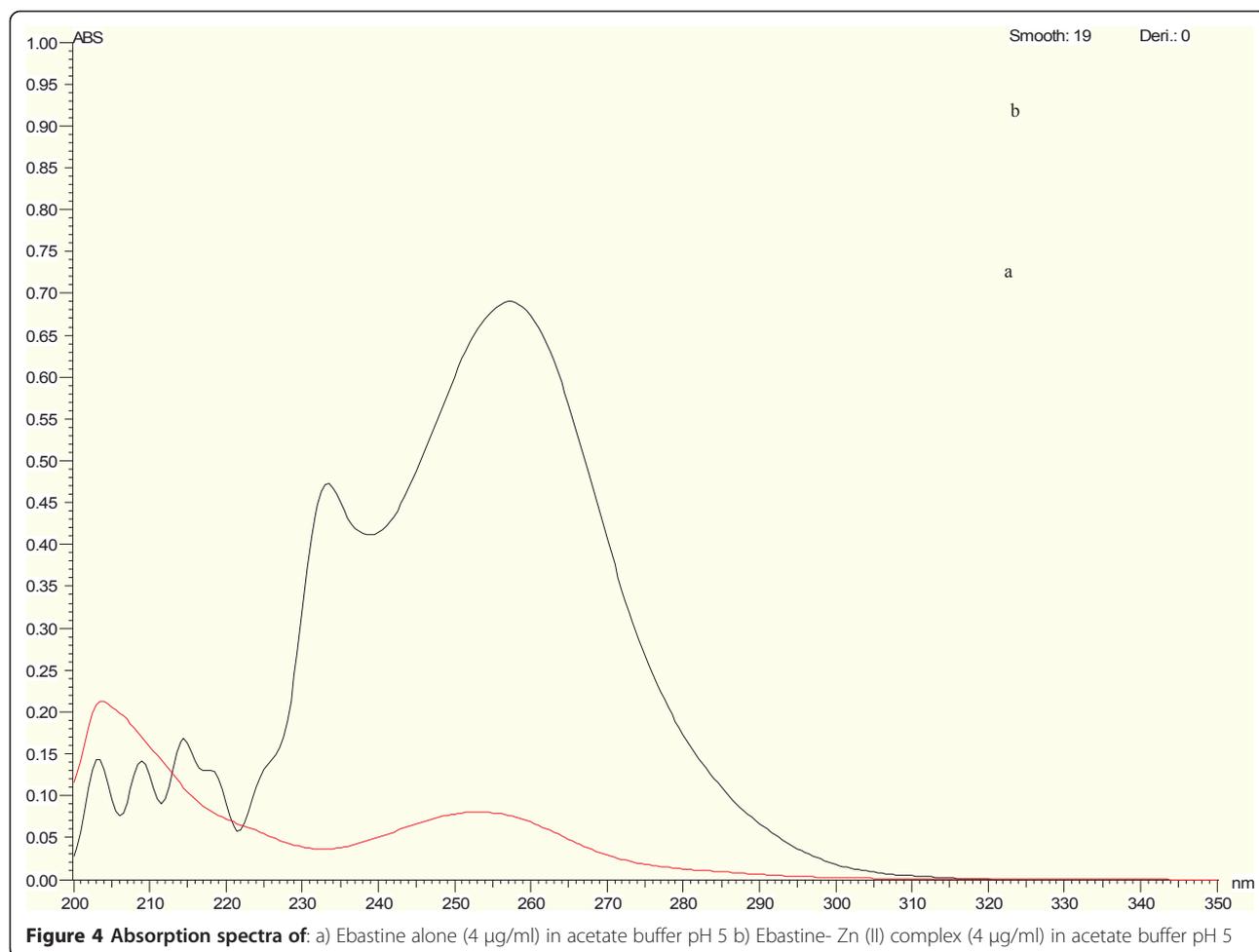
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but

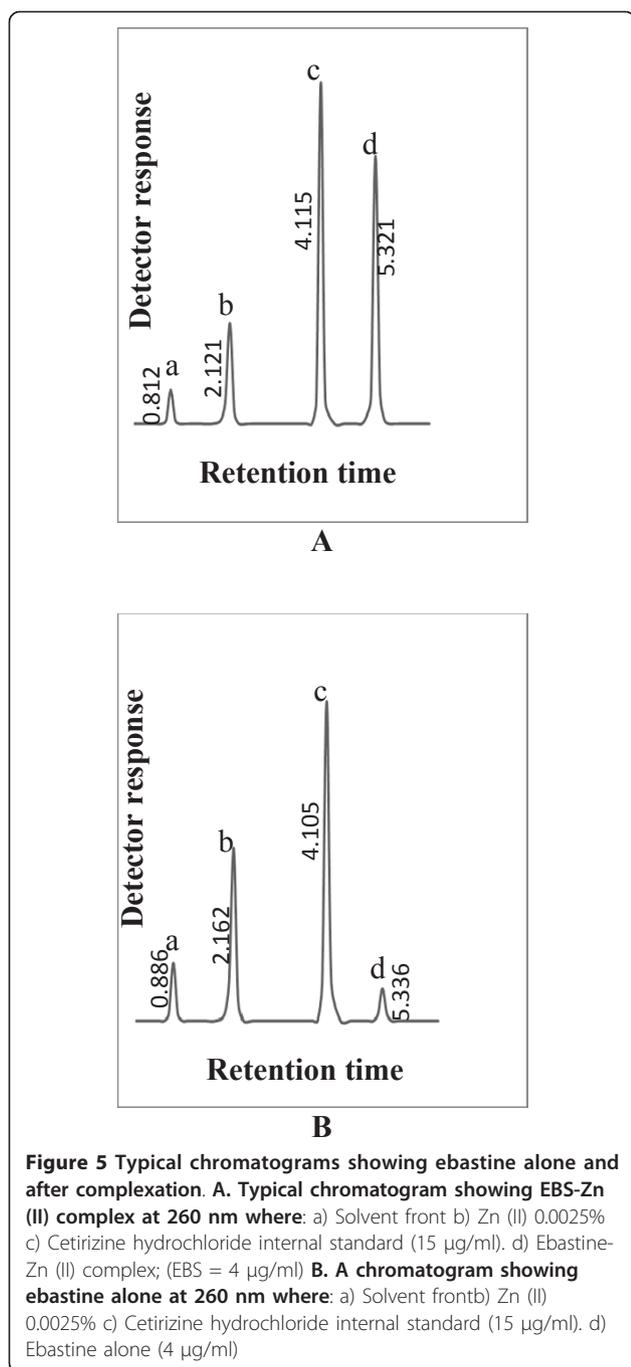
deliberate variations in method parameters and provides an indication of its reliability during normal usage [16]. The robustness of the adopted method was demonstrated by the consistency of the values of the peak area ratios with the deliberately minor changes in the experimental parameters such as, Britton Robinson buffer of pH 4-4.5 and (0.015 M - 0.05 M) molar strength, and organic phase: buffer ratio (65:35-70:30) v/v which did not greatly affect the peak area ratios.

Results and discussion

Ebastine is a weakly absorbing drug having a molar absorptivity of $9.4 \times 10^2 \text{ L. mole}^{-1} \text{ cm}^{-1}$. In the present study, EBS was found to form a highly stable complex with zinc (II); the complex was found to be formed instantaneously in the presence of acetate buffer of pH 5 and has molar absorptivity of $7.6 \times 10^4 \text{ L. mole}^{-1} \text{ cm}^{-1}$. The absorption spectrum of the complex showed a maximum absorbance at 260 nm (Figure 4).

Separation of the formed complex (Figure 5) was achieved using a mobile phase consisting of a mixture of (acetonitrile/methanol; 1/4) and 0.03 M Britton





Robinson buffer in a ratio of 65:35 v/v and adjusted to pH 4.2. The mobile phase was chosen after several trials with various proportions of the buffer and the organic phase at different pH values. Under the described chromatographic conditions complete base line separation with satisfactory resolution between the peaks was achieved in a short chromatographic run; less than 6 minutes. The proposed method was assessed for selectivity, linearity, precision, accuracy, and stability.

The different experimental parameters affecting the separation selectivity of the liquid chromatographic system have been investigated and optimized. Hence, the method was applied to the determination of EBS in its tablets, and further for content uniformity testing.

Optimization of the reaction conditions

The spectral properties of the formed complex as well as the different experimental parameters affecting its development and stability were carefully studied and optimized; such factors were changed individually while the others were kept constant. After which a detailed study for choosing the composition of the mobile phase was carried out for the optimum separation of the resultant complex.

Optimization of the reaction conditions required for complex formation

The effect of addition order on the absorbance value of the system was studied. The results showed that the addition order of EBS -acetate buffer - metal was the best regarding absorbance intensity reading.

Using different types of buffers such as phosphate or citrate having the same pH values selected for the proposed method gave the same results; however, acetate buffer was chosen throughout the study since no interference in the results was noticed as in the case of other buffers. The influence of pH on the absorbance value of the formed complex was investigated over the pH range 3.5 - 5.6 using acetate buffer and from 6 - 9 using borate buffers. Maximum and constant absorbance value was achieved at pH (4.5-5.5) using 2.5 ml of acetate buffer. Therefore, acetate buffer of pH 5 was used.

The effect of volume of acetate buffer of pH 5 was also studied keeping the concentrations of the drug and the metal constant. It was found that increasing the volume of acetate buffer (pH 5) resulted in a subsequent increase in the absorbance value of the formed complex up to 2 ml, after which the absorbance remained constant. Therefore 2.5 ± 0.5 ml of acetate buffer of pH 5 was chosen as the optimum volume of the buffer throughout this approach.

It was found that increasing the volume of Zn (II) (1×10^{-3} M) resulted in a gradual increase in the absorbance value of the complex up to 1 ml, after which it remained constant, therefore, 1.5 ± 0.5 ml of (1×10^{-3} M) was chosen.

The reaction was carried out at different temperature settings (room temperature, 40, 60, 80, 100 °C) using a thermostatically controlled water bath. Maximum absorbance values were obtained at room temperature.

Different types of surfactants, such as sodium lauryl sulphate (anionic type), gelatin, and methyl cellulose

(non ionic types) were added to the reaction mixture to test their effect on the absorbance value of the formed complex hoping that they may enhance the absorbance readings, but it was found that all the studied surfactants had no significant effect on the absorbance value of the formed complex, hence the complex formation was carried out without their addition. Similarly different sensitizers were tested such as quinine, fluorescein and rhodamine-B. Addition of sensitizers to the reaction mixture was found to enhance the absorbance but with the lack of reproducibility. Therefore, the study was carried out without the addition of sensitizers.

The effect of ionic strength on the absorbance readings was followed by adding different concentrations of KCl (0.01- 0.1 M), which revealed that KCl did not have any significant effect on the formation of the studied complex.

The formation and stability of the formed complex was also studied by measuring the absorbance readings every 10 minutes interval, the consistency of the absorbance values indicated that EBS-Zn (II) complex was formed instantaneously and remained stable for at least 90 minutes.

Reaction stoichiometry

The stoichiometry of the reaction between EBS and Zn (II) was determined spectrophotometrically by applying Job's continuous variation method [18], where the plot reached a maximum value at a mole fraction of 0.6, which indicated the formation of a 2:1 EBS : Zn (II) complex.

The formation constant of the reaction product was calculated according to the following equation [19]:

$$K_f = \frac{A/A_m}{[(1 - A/A_m)^{n+1}]c^n n^n}$$

where A and A_m are the observed maximum absorbance and the absorbance obtained from the extrapolation of the two lines obtained from Job's continuous variation method, respectively; n is the number of moles of Zn^{2+} involved in the complex formation reaction ; n = 1; C is the molar concentration of the drug used in Job's continuous variation method.

Using the above equation K_f was found to be 5.2×10^5

Also, the Gibbs free energy changes (ΔG) of the reaction were calculated according to the following equation [19]:

$$\Delta G = -2.303 RT \log K_f$$

Where R is gas constant = 8.3 joule.degree⁻¹.mole⁻¹; T is temperature (K).

Using the above equation ΔG was found to be -2.4×10^3 Joule/Mole.

The high negative value of ΔG indicates that the reaction is spontaneous.

Chromatographic performances

A well-defined symmetrical peak was obtained upon measuring the response of the eluent under the performance parameters after thorough experimental trials that could be summarized as follows:

Ratio of aqueous: organic phase

The effect of Britton Robinson buffer: organic phase; (acetonitrile-methanol mixture) ratio on the separation of the formed complex was studied. Satisfactory separation was obtained with a mobile phase consisting of Britton Robinson buffer pH 4.2: organic phase (35:65-30:70 v/v) at ambient temperature. At lower organic phase concentrations (< 65%); decrease in the number of theoretical plates (NTP) and peak area ratios was observed.

Ratio of acetonitrile: methanol in the organic phase

The influence of the ratio of acetonitrile: methanol in the organic portion of the mobile phase was followed. Adequate resolution without peak tailing and maximum peak area ratios resulted when the organic phase was composed of acetonitrile : methanol in a ratio of 1:4.

Effect of pH

The pH of the mobile phase was investigated through pH from 3 to 6. It was found that proper resolution with highest NTP and peak area ratios was achieved at pH 4 - 4.5. When the pH was lower than 4, remarkable decrease in NTP with slight reduction in peak area ratios resulted, while at pH higher than 4.5, sharp decrease in the latter two parameters occurred.

Effect of buffer type

Different buffers of the same pH (4.2) were also studied. Phosphate and acetate buffer resulted in peaks lacking symmetry and in lower values of NTP and peak area ratios than those obtained by Britton Robinson buffer.

Effect of molar strength of Britton Robinson buffer

The effect of different molar strengths of Britton Robinson buffer (pH 4.2) over the range (0.0075-0.1 M) on the separation of the formed complex was investigated. It was found that (0.015-0.05 M) Britton Robinson buffer gave the highest NTP and peak area ratios. A gradual decrease in the previously mentioned parameters was observed when the molar strength was either lower than 0.015 M or higher than 0.05 M. Furthermore, alteration of the peak symmetry of the resultant complex was remarkably observed at the higher molar strength.

Effect of Zn (II) concentration

The presence of Zn (II) in the mobile phase was essential to prevent the hydrolysis of the formed complex. In

the absence of Zn (II) in mobile phase a very small peak area ratios was obtained, so a mobile phase containing 0.0025% of Zn (II) was chosen for good formation and separation of the formed complex (Figure 6).

Effect of different types of columns

Different types of columns were tried to perform the separation of EBS-Zn (II) complex. Successful separation process was achieved by the EC nucleosil C18-SN : 4115568 column; on the other hand, it was found that upon using Hibar prepacked column RT-250-4-L-100-RP8 distorted peaks were obtained, while Zorbax® SB-Phenyl column (250 mm × 4.6 mm id (5 mm) resulted in well separated peaks but with longer retention times.

Effect of detector wavelength

The UV detector response of the complex was studied from the range of 220-280 nm, and the best wavelength was found to be 260 nm showing highest sensitivity and appreciable absorbance of EBS-Zn (II) complex.

Effect of flow rate

The effect of different flow rates (0.6-1.5 ml/min) on the separation of the complex was tested, and it was found that 1 ml/min was the most suitable flow rate regarding the retention times and the symmetry of the peaks.

A collective study of experimental parameters affecting the chromatographic separation is summarized in Table 1.

Analytical performance and applications

Validation of the method

By using the above liquid chromatographic method, linear regression equation was obtained. The regression plot showed that, there was a linear dependence of the peak area ratio on the concentration of the drug over the working concentration range. The validity of the proposed method was evaluated by statistical analysis of the regression data (Table 2) regarding the standard deviation of the residuals ($S_{y/x}$), the standard deviation

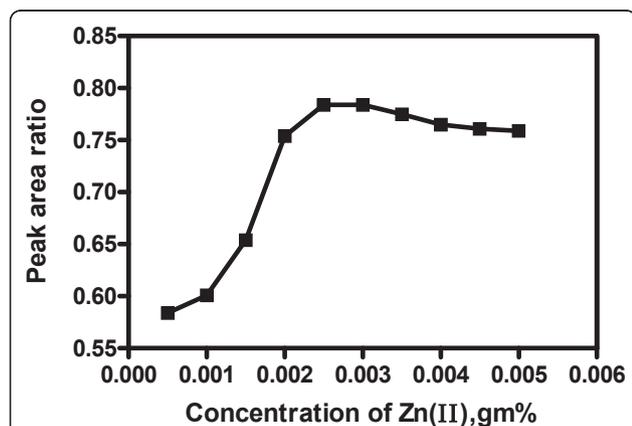


Figure 6 Effect of concentration of Zn (II) in the mobile phase on the peak area ratios of the formed complex.

Table 1 Effect of experimental parameters on the separation of EBS-Zn (II) complex

Parameter	K'	N	HETP	Rs	Peak area ratio
Buffer type and pH					
Britton Robinson buffer					
pH					
3	3.41	1036	0.0145	2.73	0.726
3.5	3.92	1034	0.0145	3.58	0.743
4	5.53	2706	0.0055	5.012	0.784
4.5	5.13	2698	0.0055	5.012	0.784
5	3.84	1507	0.0099	4.177	0.752
5.5	3.66	769	0.0195	3.166	0.688
6	3.76	258	0.0581	3.133	0.564
Phosphate buffer pH 4.2	4.26	1479	0.0101	2.72	0.366
Acetate buffer pH 4.2	4.06	1027	0.0146	1.271	0.521
Ratio of aqueous: organic phase(acetonitril: methanol; 1:4)					
45:55	4.02	837	0.0179	1.088	0.29
40:60	4.25	1034	0.0145	4.15	0.45
35:65	5.53	2706	0.0055	5.012	0.784
30:70	5.52	2687	0.0056	5.009	0.783
Ratio of acetonitril: methanol					
1:4	5.53	2706	0.0055	5.012	0.784
2:3	3.52	1210	0.0124	3.64	0.645
3:2	2.73	2205	0.0068	4.45	0.429
4:1	4.103	1393	0.0108	4.41	0.235
Acetonitril only	5.08	1347	0.0111	4.745	0.198
Methanol only	4.938	1817	0.0083	4.371	0.349
Ionic strength of BRb (Molar)					
0.0075	4.256	911	0.0165	3.714	0.48
0.01	5.23	1895	0.0079	4.015	0.69
0.015	5.53	2706	0.0055	5.012	0.784
0.02	5.52	2716	0.0053	5.009	0.782
0.05	5.55	2720	0.0052	5.011	0.783
0.07	4.62	1816	0.0083	4.127	0.642
0.1	4.67	1173	0.0128	4.143	0.513
Flow rate (mL/minute)					
0.6	4.348	466	0.0322	2.11	0.92
0.7	4.291	1013	0.0148	2.64	0.84
0.8	4.342	1070	0.0141	2.69	0.75
1	5.53	2706	0.0055	5.012	0.784
1.1	5.26	1982	0.0076	4.857	0.662
1.2	4.74	1486	0.0101	4.33	0.679
1.3	4.25	1057	0.0142	3.21	0.54
1.4	4.12	1013	0.0148	2.94	0.49
1.5	3.97	925	0.0162	2.76	0.33

Where:

K' is the capacity factor; $K' = t_R - t_M / t_M$

N is the number of theoretical plates; $N = \frac{5.55 t_R^2}{w_{1/2}^2}$

HETP is the height equivalent to a theoretical plate; HETP = L/N

R_s is the resolution factor; $R_s = \frac{2(t_R - t_M)}{w_R + w_M}$

(t_R is the retention time of the complex, t_M is the time from injection to the appearance of the solvent front, $w_{1/2}$ is the peak width at half height, L is the length of the column, w_R and w_M are the peak width at base line for the complex and the solvent front respectively)

Table 2 Performance data of the proposed method

Parameter	Value
Concentration range (µg/ml)	0.3-6.0
LOD(µg/ml)	0.13
LOQ(µg/ml)	0.26
Correlation coefficient (r)	0.9999
Slope	0.1975
Intercept	4.78×10^{-4}
$S_{y/x}$, Standard deviation of the residuals	8.93×10^{-4}
S_a , Standard deviation of the intercept of the regression line	7.49×10^{-5}
S_b , Standard deviation of the slope of the regression line	1.49×10^{-4}

of the intercept (S_a), and standard deviation of the slope (S_b) [20]. The small values of the figures point to the low scattering of the points around the calibration graph and high precision of the proposed method

Linearity

The peak area ratio of EBS varied linearly with the concentration over the range 0.3-6 mg/ml as mentioned in Table 2.

Linear regression analysis of the data gave the following equation:

$$P = 0.1975 C + 4.78 \times 10^{-4} (r = 0.9999)$$

Where C is the concentration in mg/ml and P is the peak area ratio.

Sensitivity

The calculated values of LOQ and LOD are listed in Table 2.

The linear dependence of the peak area ratio *versus* the concentration of the drug was shown by calculation of the regression equation previously mentioned

Accuracy

Statistical analysis [20] of the results obtained by the proposed and comparison methods using student's t-test and variance ratio F- test, showed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 3). The comparison method is based on an HPLC measurement of EBS using a mobile phase consisting of methanol: water (90:10, v/v/v) with UV detection at 262 nm.

Precision

Repeatability

The mean percentage recoveries based on the average of four separate determinations for pure and dosage forms are abridged in Table 4.

Intermediate precision

The percentage recoveries are based on the average of four separate determinations. The results are shown in Table 4.

Pharmaceutical applications

Dosage forms analysis

The proposed method was successfully applied to the determination of EBS in its dosage forms. The average percentages found of different concentrations were based on the average of three replicate determinations. The results shown in Table 5 are in good agreement with the comparison method [4].

Content uniformity testing

Due to the high precision of the method, and its ability to rapidly estimate the concentration of the drug in a single tablet extract with sufficient accuracy, the method is suited for content uniformity testing which is a time

Table 3 Application of the proposed method for the analysis of ebastine in pure form

Amount taken (µg/ml)	Proposed method		Comparison method [4]
	Amount found (µg/ml)	% Recovery	% Recovery
0.3	0.2976	99.21	100.62
0.5	0.5031	100.62	99.15
0.7	0.7030	100.43	99.34
1.0	0.9972	99.72	
2.0	2.0148	100.74	
3.0	3.0072	100.24	
4.0	3.9912	99.78	
5.0	4.981	99.62	
6.0	6.0264	100.44	
$\bar{X} \pm SD$		100.09 ± 0.52	99.70 ± 0.79
Student's t test		0.61(1.86)*	
Variance ratio F test		2.3(5.25)*	

Figures between parentheses are the tabulated t and F values at $p = 0.05$ [20].

Table 4 Validation of the proposed HPLC method for determination of ebastine in pure and dosage forms

Preparation	Repeatability, % Recovery	
	Ebastine (5.0 µg/ml)	Ebastine (6.0 µg/ml)
Ebastine pure form	99.95	100.95
	100.25	100.38
	100.54	99.65
	100.87	99.12
X̄ ± SD	100.41 ± 0.39	100.03 ± 0.81
Bastab [®] tablets	Ebastine (0.3 µg/ml)	
	99.65	99.35
	99.84	99.98
	100.58	100.87
Evastine [®] syrup	Ebastine (2.0 µg/ml)	
	100.14	99.32
	100.55	100.84
	100.92	100.99
Evastine [®] syrup	Ebastine (3.0 µg/ml)	
	99.64	100.06
	100.19 ± 0.52	100.28 ± 0.76
	X̄ ± SD	100.31 ± 0.55

Table 5 Determination of ebastine in its dosage forms by the proposed HPLC method

Preparation	Proposed method			Comparison method [4]
	Amount taken (µg/ml)	Amount found (µg/ml)	% Recovery	% Recovery
Bastab [®] tablets	0.3	0.2974	99.14	99.23
	0.7	0.6981	99.72	99.61
	1.0	1.0094	100.94	100.61
	3.0	3.0036	100.12	
	5.0	4.9835	99.67	
	6.0	5.9844	99.74	
X̄ ± SD			99.88 ± 0.61	99.82 ± 0.71
Student's t test			0.39(2.02)*	
Variance ratio F test			1.35(3.78)*	
Evastine [®] syrup	0.3	0.30216	100.72	100.12
	0.7	0.69615	99.45	99.61
	1.0	1.0011	100.11	99.51
	3.0	3.0144	100.48	
	5.0	5.031	100.62	
	6.0	5.9994	99.99	
X̄ ± SD			100.23 ± 0.48	99.75 ± 0.33
Student's t test			0.23(2.02)*	
Variance ratio F test			2.12(3.78)*	

Figures between parentheses are the tabulated t and F values at p = 0.05 [20].

consuming process when using conventional assay technique. The steps of the test were adopted according to the United States Pharmacopoeia [17] procedure. The acceptance value AV was calculated for each of the commercially available tablets and it was found to be smaller than the maximum allowed acceptance value L1. The results demonstrated reasonable drug uniformity as shown in Table 6.

Interferences

Many drugs which are frequently co-administered with EBS such as erythromycin and ketoconazole were carefully tested. The studied drugs were found to be adequately separated from the formed complex (Figures 7 a, b).

Forced degradation studies of EBS

In order to establish whether the analytical method was stability indicating, EBS was stressed under various conditions to contact forced degradation studies [16]. Methanol was used as a co-solvent in all forced degradation studies.

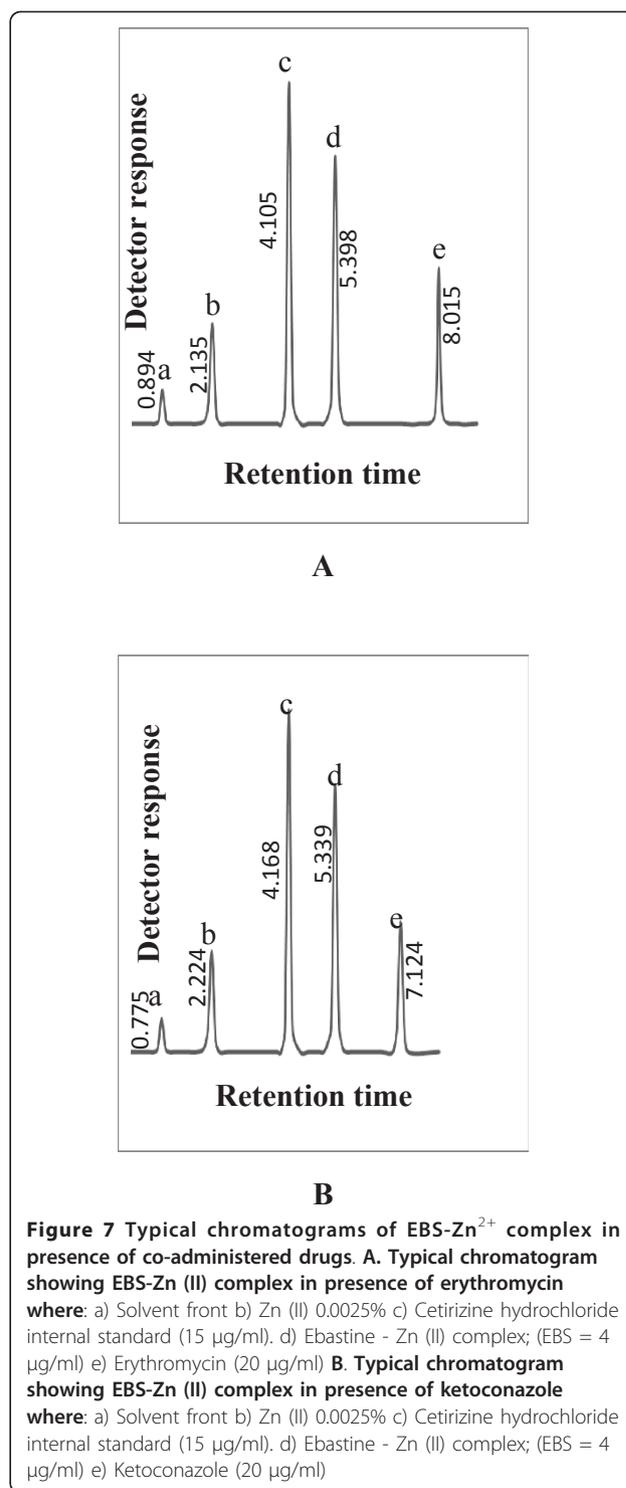
Degradation kinetics study

For the kinetic study, 2 M sodium hydroxide was used for alkaline degradation of the drug. Regarding the UV degradation, the methanolic solution of EBS was exposed to Deuterium lamp in a wooden cabinet distance of 15 cm for different time intervals.

The apparent decrease in the peak area ratios of EBS upon alkaline degradation with 2M sodium hydroxide in

Table 6 Content uniformity testing of ebastine in its dosage forms using the proposed method

Parameter	Percentage of the label claim	
	Bastab [®] tablets	Ebastel [®] tablets
Data		
	100.25	100.67
	101.12	99.64
	100.65	99.18
	99.81	100.66
	99.54	100.82
	100.91	101.11
	99.34	99.37
	100.55	99.42
	101.18	100.68
	99.45	99.35
Mean X ⁻	100.28	100.09
SD	0.704	0.754
%Error	0.22	0.24
Acceptance value (AV)	1.69	1.81
Maximum allowed value (L1)		15.00



a boiling water bath, and under UV lamp at different time intervals are demonstrated in Figures 8 (I-IV) and 9 (I-IV).

The degradation was found to be temperature dependent (Figures 10 and 11). Regarding the alkaline

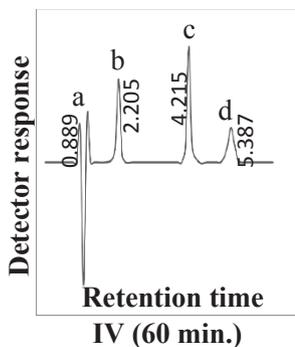
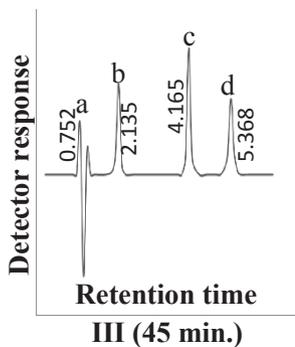
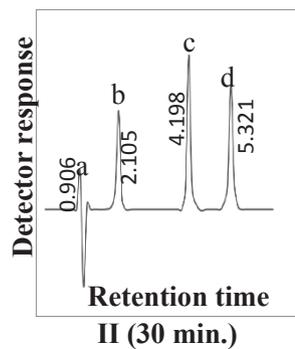
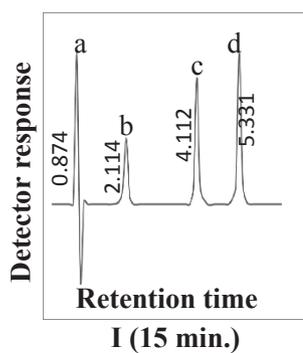


Figure 8 (I-IV) Typical chromatograms showing the decrease in peak area ratios of EBS- Zn (II) complex by alkaline degradation at different time intervals where: a) Solvent front b) Zn (II) 0.0025% c) Cetirizine hydrochloride internal standard (15 µg/ml), d) Ebastine- Zn (II) complex

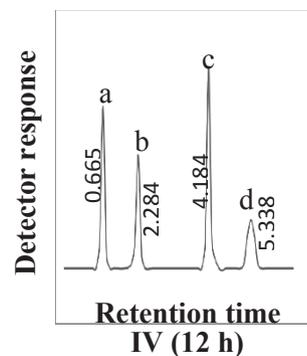
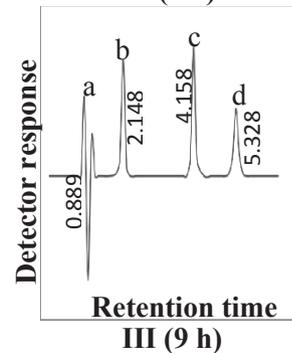
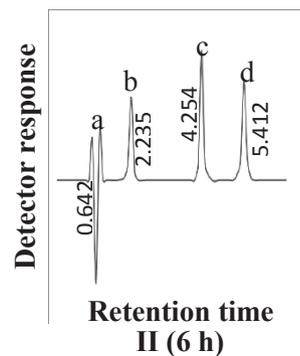
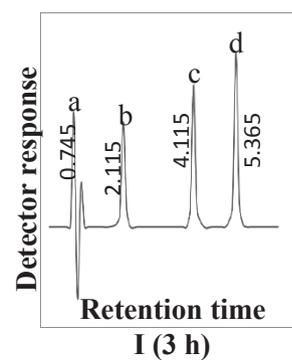
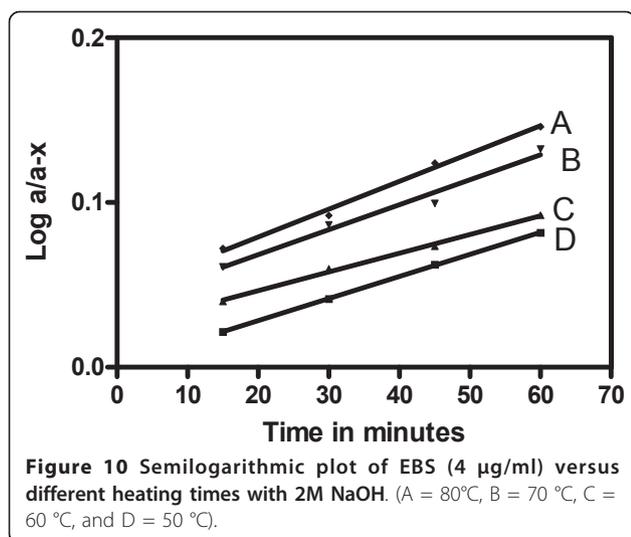


Figure 9 (I-IV) Typical chromatograms showing the decrease in peak area ratios of EBS- Zn (II) complex by UVdegradation at different time intervals where: a) Solvent front b) Zn (II) 0.0025% c) Cetirizine hydrochloride internal standard (15 µg/ml), d) Ebastine- Zn (II) complex



degradation, the first order degradation rate constant and the half life time at each temperature were calculated (Table 7) according to the following equations:

In $a/a-x = Kt$ where a is the initial concentration of the drug, x is the concentration of the resulting degraded solution after time t , and K is the reaction rate constant

While the half life time could be calculated as follows:
 $t_{1/2} = 0.693/K$.

Plotting $\log K_{obs}$ values versus $1/T$, the Arrhenius plot was obtained (Figure 12). The activation energy was also calculated by the equation:

In $K = -Ea/RT + \ln A$ where: Ea is the activation energy, K is first order reaction rate constant, R is the gas constant, and T is the temperature in kelvin.

On the other hand, the proposed method did not indicate either the acidic or the oxidative degradation of ebastine. In both cases, the peak corresponding for the

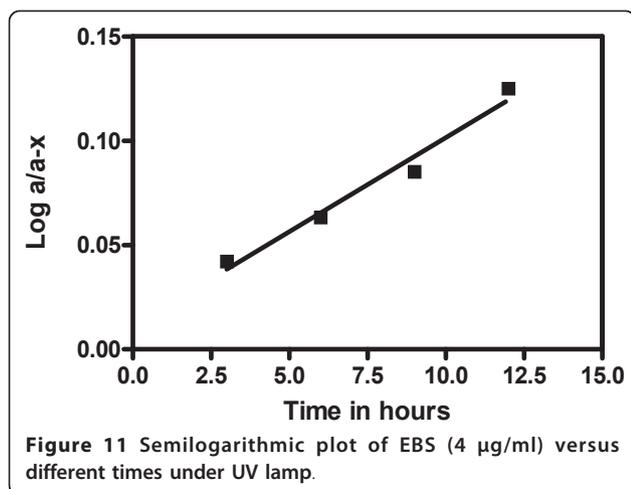


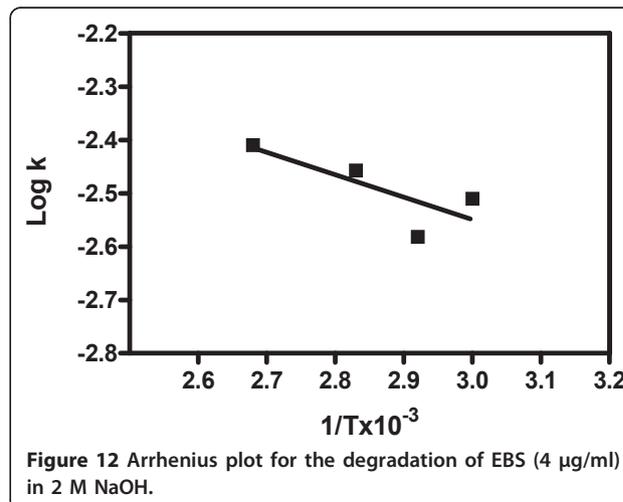
Table 7 Effect of temperature on the kinetic parameters of EBS

Temperature (°C)	K (min. ⁻¹)	t _{1/2} (min.)
50	0.001137	609
60	0.00134	517
70	0.001515	421
80	0.001691	410
Ea = (K.Cal.mol ⁻¹)		7.3

complex did not appear in the chromatogram, pointing out that the degradation step altered the chemical moiety responsible for the complexation between EBS and Zn (II). The acidic degradation is supposed to act on the basic centre of the studied drug; tertiary amine, which is involved in the complex formation, and hence, the complex will be no further be formed. While, the oxidative degradation carried out with 6% hydrogen peroxide may cause the oxidation of the tertiary amino group to the N-oxide derivative, and subsequently alter the complex formation.

Conclusion

A simple, sensitive and rapid HPLC method has been developed for the determination of ebastine in its pharmaceuticals. The sensitivity of the proposed method allows its application for content uniformity testing. Extensive study concerning the composition and stability of the complex was also conducted. Another advantage of the studied method is its stability indicating property which was utilized to investigate the kinetics of alkaline and ultraviolet induced degradation of the drug. Validation was carefully studied to elicit an assay which can be used in routine quality control laboratories.



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Authors' contributions

FI designed the proposed method and analyzed the data statistically. MKSE proposed, planned and supervised the whole work. MIE coordinated the study and modified the text. MEKW carried out the experimental work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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