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Study of forced degradation behavior of Eletriptan hydrobromide by LC and LC–MS and development of stability-indicating method

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ABSTRACT

The objective of the present study was to report the stability profile of novel antimigrain drug Eletriptan hydrobromide based on the information obtained from forced degradation studies. The drug was subjected to acid (0.1–1 mol L^{-1} HCl), neutral and base (0.1–1 mol L^{-1} NaOH) hydrolysis and to oxidative decomposition (3–15% (v/v) H_2O_2). Photolysis and thermo degradation at 75 °C were carried out in methanol solution and in solid state with both Eletriptan hydrobromide bulk drug and the tablet formulation. The products formed under different stress conditions were investigated by LC and LC–MS.

The experimental conditions for LC were chosen by employing experimental design and multicriteria decision making methodology. These powerful tools enabled the accomplishment of satisfactory resolution with the shortest possible analysis time. Analytes were separated on a C_{18} column (*XTerra*™, 150 mm × 3.9 mm, 5 μm) with the mobile phase composed of methanol–water solution of TEA (pH 6.52, 1%, v/v) (30:70, v/v) pumped at 1 mL min^{-1} flow rate. The column temperature was set at 50 °C and the detection at 225 nm using DAD detector. The LC method was suitably modified for LC–MS analysis which was further used to characterize the arisen degradation products. The possible degradation pathway was outlined based on the results.

The drug appeared to be instable towards every stress condition but oxidation. The stability was not jeopardized even under more exaggerated conditions such as increased temperature of the solutions to 75 °C, increased strength of acid/alkali solutions and prolonged testing period.

Validation of the LC-DAD method was carried out in accordance with ICH guideline. The method met all required criteria and was applied when testing the commercially available tablets.

1. Introduction

Forced degradation studies are usually part of the drug development strategy being undertaken to elucidate the intrinsic stability of the drug. Such studies are therefore conducted under more severe and exaggerated conditions than those usually used for long-term stability tests. The information gathered may help establishing the drug degradation pathway as well as development and validation of the suitable analytical procedures [1–3].

Eletriptan hydrobromide (EH) is a relatively novel serotonin 5-$HT_{1B/1D}$ receptor agonist used for the treatment of acute migraine headaches. Its pharmacological effects include the constriction of cerebral blood vessels and neuropeptides secretion blockade which eventually relieves the pain [4,5]. Chemically, EH is 3-{[(*R*)-1-methyl-2-pyrrolidinyl] methyl}-5-[2-(phenylsulfonyl) ethyl] indole hydrobromide (Fig. 1A). The literature revealed only one paper dealing with the determination of EH in biological matrixes [6] and a few studies of EH tablet formulations covering the assay of EH in the presence of related organic impurities [7,8]. Neither of the already published methods reports the physical and chemical stability of EH. Consequently, the main objective of this study was to carry out a comprehensive stress study on EH by subjecting it to various experimental conditions. The investigations involved acid, alkaline and neutral hydrolysis, oxidative and thermal decomposition and stability towards light. An integral aim of the study was to postulate possible degradation pathway of the drug.

The suitable stability-indicating LC-DAD method was developed for the analysis of stress samples. Since the chromatographic behavior of target substances may be influenced by various experimental parameters, the whole study was carried out by employing experimental design methodology. The investigations included the mutual changes of the mobile phase composition and the column temperature. The central composite design was used to obtain a predictive model which adequately represents changes in the chromatographic response within the zone of interest [9–13].

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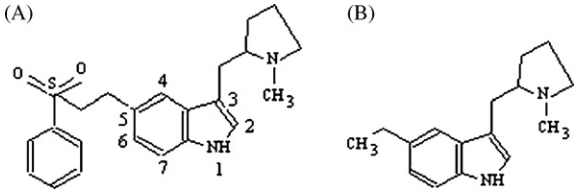

Fig. 1. Structures of Eletriptan (A) and UK 120.413 (B).

Furthermore, in order to achieve the best possible chromatographic performance of the method, it was necessary to evaluate several different target responses. In order to reach a compromise among them, the Derringer's desirability function was used. The desirability function was constructed for each individual response and afterwards the overall desirability function was established [13–17].

The developed stability-indicating LC-DAD method was validated and successfully applied for the analysis of the commercially available EH tablets.

2. Experimental

2.1. Drugs and reagents

The standard substances of Eletriptan hydrobromide, UK 120.413 and *Relpax*® tablets containing 20 mg of Eletriptan in a form of hydrobromide were obtained from *Pfizer H.C.P. Corporation*. Phenobarbiton USP reference standard, triethylamine (TEA) (*Merck*, Darmstadt, Germany), glacial acetic acid (*Zorka Pharma*, Šabac, Serbia), 85% formic acid (*Lach-ner*, Neratovice, Czech Republic), sodium hydroxide (*Centrohem*, Stara Pazova, Serbia), 36% hydrochloric acid (*Centrohem*, Stara Pazova, Serbia) and hydrogen peroxide (*Zorka Pharma*, Šabac, Serbia) were also used. All reagents were of analytical grade. Methanol-gradient grade (*Lab Scan*, Dublin, Ireland) and acetonitrile-HPLC grade (*Merck*, Darmstadt, Germany) were used for chromatography while water was obtained from *System Simplicity 185* purification systems (*Millipore*, Massachusetts, USA).

2.2. Equipment and experimental conditions

HPLC-UV analyses were done by using *Hewlett-Packard* 1200 series (Palo Alto, CA, USA) chromatographic system equipped with on-line degasser, binary pump, column oven and diode array detector. Sample injection was made through *Rheodyne* injector valve with a 20 μL sample loop. The data was acquired with *HP ChemStation* software. The method used C_{18} *XTerra*™ (5 μm, 150 mm × 3.9 mm) column (*Waters*, Massachusetts, USA). After being loaded onto the column, the sample eluted at the temperature of 50 °C at a flow rate of 1 mL min^{-1} with the mobile phase consisted of methanol–water solution of TEA (1%, v/v) (30:70, v/v) while pH of the water phase was adjusted to 6.52 with glacial acetic acid. Before use, the mobile phase was degassed and vacuum filtered through 0.45 μm nylon membranes (*Alltech Associates*, Lokeren, Belgium). The detection of analytes was performed at 225 nm.

HPLC–MS analyses were carried out on a system in which the HPLC part consisted of *Agilent technologies* 1200 series chromatographic system (Waldron, Germany) comprising of on-line degasser, binary pump, auto injector, column oven and diode array detector. The MS system consisted of *Agilent technologies* 6210 Time-Of-Flight LC–MS system (Waldron, Germany). The whole system operated using *Agilent technologies Mass-Hunter workstation* software. The separations were achieved on C_{18} *Zorbax Eclipse plus* (150 mm × 4.6 mm, 1.8 μm,) column (*Agilent Technologies*, Wilmington, DE, USA). The mobile phase was pumped at 1.4 mL min^{-1} flow rate and consisted of acetonitrile–water solution of formic acid (0.2%, v/v) in a gradient elution program. The initial mobile phase consisted of acetonitrile–water phase in the ratio 5:95 (v/v) and after 1.5 min the amount of acetonitrile begun to increase linearly to reach 95:5 (v/v) level achieved in 26th minute. For the next 9 min the mobile phase composition did not change, and from 35th minute to 40th minute the amount of acetonitrile begun to decrease to reach 5:95 (v/v) level. The post time was 5 min. The HPLC analyses were performed at 40 °C and the samples were detected in the range 190–450 nm. The mass spectrometer operated in the positive electrospray ionization mode with mass/charge (m/z) ratio in the range of 100–2000 m/z. The desolvation gas was nitrogen set at 12 L min^{-1} flow rate and desolvation temperature was 350 °C. The nebulizer pressure was 45 psig. Capillary voltage was set at 4000 V and fragmentor voltage at 140 V.

2.3. Solutions

A 1.00 mg mL^{-1}, 0.20 mg mL^{-1} and 0.50 mg mL^{-1} stock solutions of EH, UK 120.413 and phenobarbiton as the internal standard were prepared in methanol.

Working solutions for stress decomposition studies were containing 500.00 μg mL^{-1} of EH and 5.00 μg mL^{-1} of UK 120.413. 50.00 μg mL^{-1} solution of phenobarbiton was prepared as well. The sample preparation for assay of EH in tablets was done in the following way: the quantity of twenty tablets was accurately weighed, finely powdered and the equivalent to 25.00 mg of EH was transferred with 25 mL of methanol into a 50-mL volumetric flask. After sonicating and shaking the mixture for 25–30 min, it was made up to volume with the same solvent. The working concentration of EH in the prepared sample was 500.00 μg mL^{-1}, same as in the case of EH standard substance.

The concentrations of EH in the six solutions used for construction of the standard curve were in the range of 25.00–250.00 μg mL^{-1}. The same six solutions of UK 120.413 were prepared in the concentration range of 0.05–0.50 μg mL^{-1}. The concentration of phenobarbiton in all these solutions was 5.00 μg mL^{-1}. The sample of EH tablet formulation was used to prepare the test solutions containing EH in the concentration 50.00 μg mL^{-1}. The concentration of phenobarbiton in the same solutions was also 5.00 μg mL^{-1}.

The known quantities of EH and UK 120.413 standard substances were added to the finely powdered EH tablets for the method accuracy testing. The working solutions were prepared afterwards according to the similar procedure already described with EH tablets to attain concentrations at 80%, 100% and 120% of EH label claim. Three solutions were prepared for each of the following concentrations: 40.00 μg mL^{-1}, 50.00 μg mL^{-1} and 60.00 μg mL^{-1} for EH and 0.08 μg mL^{-1}, 0.10 μg mL^{-1} and 0.12 μg mL^{-1} for UK 120.413. The concentration of phenobarbiton in each solution was 5.00 μg mL^{-1}.

2.4. Stress decomposition studies

Forced degradation studies of bulk drug and drug formulation included appropriate solid state and solution state stress conditions in accordance with the ICH regulatory guidance [3]. Some helpful practical aspects on conducting and development of stability-indicating assays of specific drugs were also found in literature [1–2,18–26].

Stress decomposition studies were performed initially with EH working concentration of 500 μg mL^{-1} in methanol. Acid hydrolysis was performed by mixing 1 mL of EH working solution in three separate 5-mL volumetric flasks with 1 mL of 0.1 mol L^{-1}, 0.5 mol L^{-1} and 1.0 mol L^{-1} HCl solutions, respectively, and the mixtures were kept at room temperature for 8 h. The study in alkaline condition was carried out in a similar manner with 0.1 mol L^{-1}, 0.5 mol L^{-1} and 1.0 mol L^{-1} NaOH for 8 h. These experiments were repeated at

higher temperature of 75 °C for 0.5 h while keeping all other conditions constant. For study in neutral condition, drug was held in methanol solution at room temperature for 24 h. Oxidative studies were carried out by mixing 1 mL of EH working solution in two separate 5-mL volumetric flasks with 1 mL of 3% (v/v) H_2O_2 and 15% (v/v) H_2O_2. The prepared mixtures were kept at room temperature for 12 and 5 h, respectively. Photolytic and thermo degradation studies were performed with bulk drug powder (1 mm thick layer in a Petri plate) and in methanol solution (1 mL of EH working solution) which were exposed to sunlight during 2 days and to 75 °C for 48 h, respectively. As a control, the parallel set of samples was kept in dark at refrigerator temperature. The same procedure concerning photolytic and thermal degradations was repeated with EH tablet formulation.

The specified stress conditions were selected in order to result in 5–20% degradation of EH. Prior to LC-DAD analysis, samples were withdrawn at appropriate time, neutralized (in case of acid and alkali hydrolysis), phenobarbiton was added as the internal standard (in order to properly estimate the degree of EH degradation) and the solutions were diluted with methanol to attain the predicted concentration of non-degradated EH of 100 μg mL^{-1}. The concentration of phenobarbiton was always 50 μg mL^{-1}.

Several control samples were prepared for comparison with the stressed samples. First of all, the drug solution stored under normal conditions was analyzed. The chromatograms of the blank solutions consisting of stress agents and without the drug and the zero time drug solutions together with stress agents were inspected in order to mark the peaks corresponding to stress agents and to distinguish them from the potential drug degradation products.

The stressed samples were detected under different wavelengths in order to ensure that no additional degradation products were formed with different extinction values than the parent drug. After recording UV spectrum (200–400 nm) of the drug and the representative samples from each stress condition, the detection wavelength of 225 nm was finally selected. The assay of EH was also investigated using the internal standard method. The total chromatographic run time was 2.5 times more than the retention of the drug peak.

2.5. Experimental design and multivariate optimization methodology

Response surface design by means of the central composite design (CCD) was used in chromatographic method development since the large number of experimental parameters had to be tested simultaneously. CCD for k factors consists of 2^k experiments corresponding to a full factorial design for the estimate of first-degree and interaction terms, $2k$ star experiments for the estimate of the square terms and n experiments at the centre of the domain to estimate of the experimental variance. Thus, for three factors a total of 18 experiments were required, including three-fold repetition of the centre point. The relationship between the inputs and the output in CCD may be presented in a form of a following second order polynomial equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

In the equation, y is the measured response associated with each factor level combination; b_0 is an intercept; b_1 to b_{33} are regression coefficients computed from observed experimental values of y; and x_1, x_2 and x_3 are coded levels of independent variables. The terms x_1x_2 and x_{i2} (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively.

The experimental data was coded in order to have a better insight into the significance of the factors influences. In such way, if the coefficient has larger value, this means that its significance was greater [9–13].

In order to ensure the best chromatographic performance of the method, the multicriteria methodology was employed by means of Derringer's desirability function. It is based on constructing a desirability function for each individual response and afterwards establishing the overall desirability function. The Derringer's desirability function D is defined as the geometric mean of individual desirability functions and may be expressed as:

$$D = (d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \ldots \times d_n^{pn})^{1/n}$$

In the equation, $p1$, $p2 \times pn$ refer to the weight of the responses, n the number of the responses and d_i is the individual desirability function of each response. Through the individual functions d_i, the analyst introduces the specifications that each response must fulfill and through the weighting the relative importance given to each of them. Weights pi may range from 0.1 to 10. With a weight of 1, d_i varies in a linear way. In the present study, we have chosen weights equal to 1 for all the responses.

The scale of desirability function is dimensionless. Therefore, it is possible to combine results obtained for properties measured on different scales. The scale of the individual desirability function ranges between $d_i = 0$, for a completely undesired response, to $d_i = 1$ for a fully desired response. A value of D different to zero implies that all the responses are in a desirable range simultaneously and consequently and for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values.

There are two ways of calculating the desirability function d_i based on the selected criterion, does the response has to be maximized or minimized:

$$d_i = \left(\frac{(y_i - y^-)}{(y^+ - y^-)}\right) \text{ or } d_i = \left(\frac{(y^+ - y_i)}{(y^+ - y^-)}\right)$$

In the equation, y_i is the predicted response using the fitted polynomial model from each experiment while y^+ and y^- represent the intended highest and the least possible limit set for that criterion, respectively [13–17].

For statistical analysis *Excel 2003* (version 11) included in *Microsoft Office 2003* and *StatSoft Statistica 5.0* software 1997 edition were used.

3. Results and discussion

3.1. Forced degradation studies

Degradation was not observed in EH sample when it was subjected to light, heat, acid and base hydrolysis even after the exposure to higher temperature, longer period of testing (up to 48 h) and increased strength of acid/alkali solutions. EH showed to be very unstable towards oxidation. Mild degradation was observed while the sample was exposed to 3% (v/v) H_2O_2. The degradation was also accelerated by increasing H_2O_2 concentration to 15% (v/v). The summary of results is presented in Table 1. According to the lit-

Table 1
Summary of results from forced degradation studies.

Stress condition	Drug assay (%)	Remarks
Acid hydrolysis (1 mol L^{-1} HCl, 8 h)	98.75	No significant degradation
Base hydrolysis (1 mol L^{-1} NaOH, 8 h)	97.09	No significant degradation
Neutral hydrolysis (methanol, 24 h)	99.30	No significant degradation
Oxidation (15% H_2O_2 (v/v), 5 h)	88.04	One major product formed
Photolysis (daylight exposure, 48 h)	99.45	No significant degradation
Thermal degradation (75 °C, 8 h)	98.12	No significant degradation

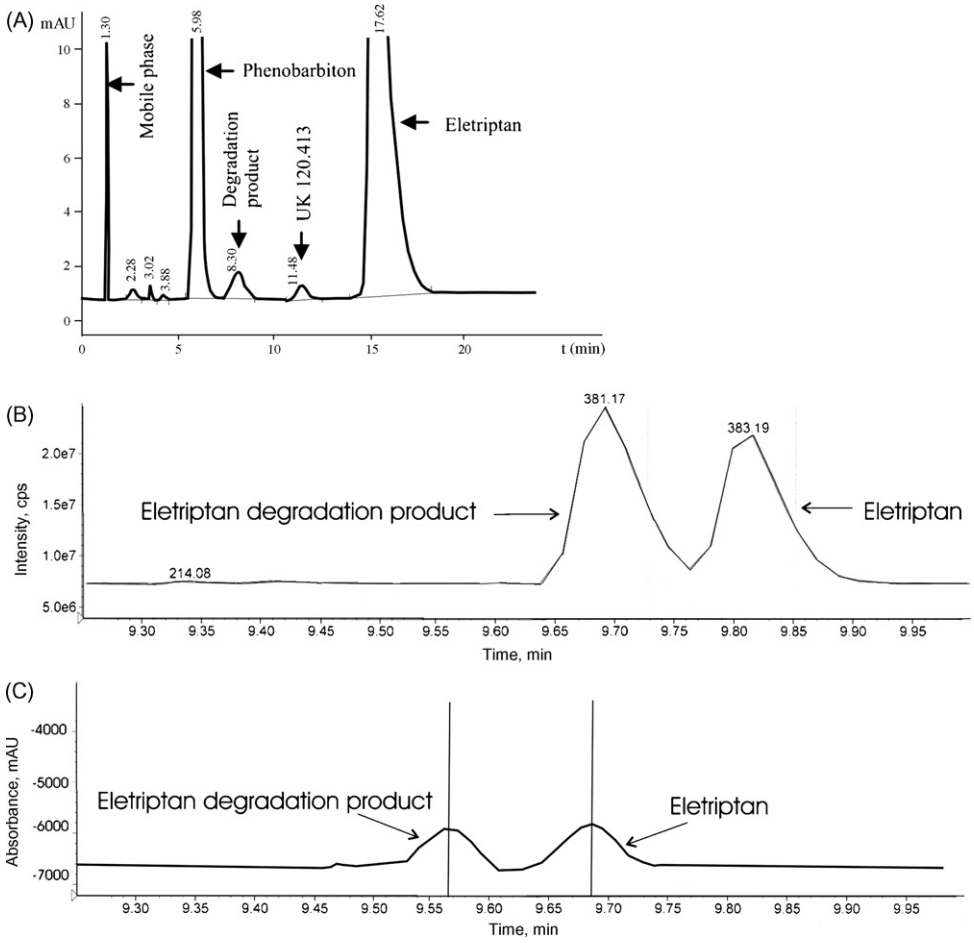

Fig. 2. The LC-DAD (isocratic elution program) (A), LC–MS total ion (B) and LC-UV (gradient elution program) (C) chromatograms of Eletriptan sample exposed to oxidation.

erature reports, UK 120.413 (Fig. 1B) could be found in EH tablet formulations as related organic impurity [7]. Therefore, LC-DAD studies were carried out with all stress solutions individually and afterwards in the presence of UK 120.413.

The LC-DAD analyses revealed the significant formation of one mayor degradation product. Its retention time was shorter compared to EH indicating different polarity. After the suitable optimization of experimental conditions, EH degradation product was finally well resolved from its parent drug as well as from UK 120.413 and internal standard (Fig. 2A). Chromatographic peak purity results were obtained from the spectral analysis report and the peak purity values greater than 990 confirmed that the EH peak is homogenous and pure in all analyzed samples. This finding led to the conclusion that the assay of EH could not be compromised in the presence of all possible accompanying substances. The forced degradation behavior of the drug in both bulk and tablet formulation appeared to be similar since no additional products were noticed suggesting thereby there was no significant interaction between the drug and the excipient.

The LC method was suitably modified for LC–MS studies used to characterize the degradation product arisen under oxidative stress. The *m*/*z* ratios of molecular ions obtained in positive electrospray ionization mode were compared to the molecular weights of EH (mol. wt. 382.06) and the known related organic impurity UK 120.413 (mol. wt. 242.18). The LC–MS total-ion chromatogram (Fig. 2B) of the stressed EH sample confirmed the formation of only one degradation product, as it was previously concluded with LC-DAD method. The LC-UV chromatogram of the stressed EH sample was also recorded under the same chromatographic conditions as LC–MS total-ion chromatogram (Fig. 2C). The gradient elution program starting from a weak mobile phase also verified the absence of any other degradation product.

The studies of indole chemistry have shown that there are several preferred sites of oxidation in the ring [19,27]. The oxidation in C-3 position would normally be favored process, but in the case of EH that position already contains N-methyl-2-pyrrolidinylmethyl group. Therefore, the C-2 and C-4 positions became reactive towards hydroxyl radical attack. Following attack at C-4, the position C-7 would also be activated. The presence of oxidations at both C-4 and C-7 would allow the formation of a highly preferred quinone-type resonance structure. The formation of N-oxide at both indole and pyrrolidin rings would also be favored processes. Surprisingly, the appearance of mass spectra of EH (Fig. 3A), its degradation product (Fig. 3B) led to different conclusions. The degradation product eluted at about 9 min and gave the molecular ion at *m*/*z* ratio 381.16 while the molecular ion of EH was at *m*/*z* ratio 383.17. Mass spectra of UK 120.413 (Fig. 3C) showed the molecular ion at *m*/*z* ratio 243.18. Having in mind the molecular weight of EH, it seemed that no additional oxygen atom appeared in the structure of EH degradation product. At the same time, published paper concerning the metabolism of EH [28] revealed the formation of tetracyclic quaternary ammonium metabolite which chemical structure could be related to the formation of the above mentioned molecular ion. The actual structure of degradation product might be determined using NMR or LC-NMR methods, but this is not necessary if this degradation product does not appear during accelerated or long-term stability testing [1,3]. This kind of stability testing was not the integral part of the present study.

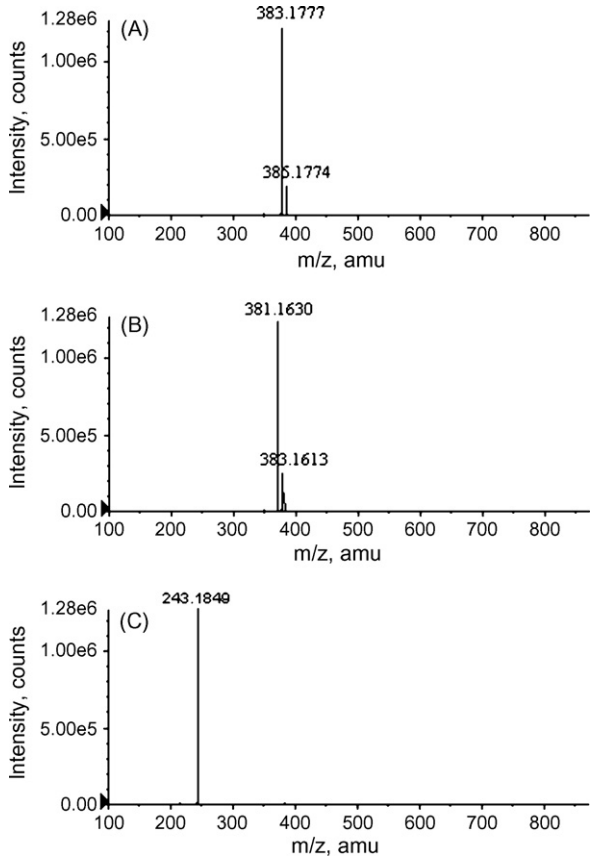

Fig. 3. The mass spectra of Eletriptan (A), its degradation product (B) and UK 120.413 (C).

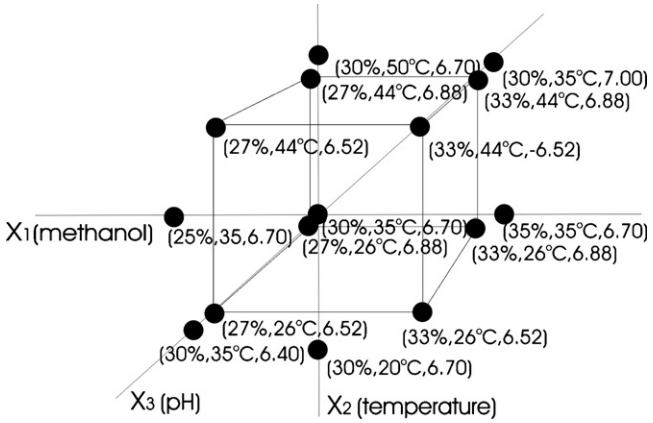

Fig. 4. Graphical representation of the experimental points together with the experimental domain investigated through central composite design.

3.2. Development of the stability-indicating method

The basic character of EH indicated its possible reactions with free silanol groups on the stationary phase. These reactions often result in peak tailing and bad peak symmetry. C_{18} *XTerra*™ column has a specific packing which shields the free surface silanol groups in the packing from interactions with basic compounds and therefore was used in this study. The mentioned reactions of the analytes may be even more prevented with the addition of the modifiers such as TEA to the mobile phase. The preliminary experiments demonstrated that the combination of the stationary phase and 1% (v/v) TEA gave the satisfactory peak shapes and consequently assisted in the separation of structurally similar substances.

Due to the basic characteristics, the chromatographic behavior of EH appeared to be very pH-dependent. Small changes in pH level of the mobile phase solution in the range 6.40–7.00, consequently led to the changes in the retention of all the substance and even baseline separation was endangered. Methanol was chosen as the organic modifier on the bias of great solubility of all analytes. The increase of methanol (even in very small percent) and column temperature led to evident change of retention times and resolution. Therefore, it was necessary to examine the influence of these three chromatographic parameters in detail through CCD. The investigated experimental range and the position of experimental points are shown in Fig. 4. In order to gather as much information as possible, several responses were observed: the separation factor between the analytes and the retention factor for the analyte with the longest retention time. The obtained coefficients for the response surface models are presented in Table 2. The repetition of the centre experimental point provided a precise estimation of the experimental errors and a measure of the adequacy of the models (lack of fit). The results were analyzed with the assistance of ANOVA method and the statistics are presented in Table 2. The regression lack of fit was determined by performing an *F*-test in order to compare the variance due to the lack of fit with the variance due to purely experimental uncertainty. The *F*-ratios calculated as SS_{lof}/SS_{pe} were compared with the tabled *F*-value for 5 and 3 degrees of freedom at 95% confidence level ($F_{tab} = 9.01$). Since they were not greater than the F_{tab}, there was no evidence of models lack of fit and the models could be accepted as providing an adequate representation of the data. The fraction of explained variation represented as the coefficient of determination R^2 was also evaluated. The values

Table 2
The estimates of the coefficients for CCD regression models and statistical parameters obtained from ANOVA.

		$RS_{Eletriptan/UK\ 120.413}$	$RS_{Eletriptan/Degradation\ product}$	$RS_{UK\ 120.413/Degradation\ product}$	$k'_{Eletriptan}$
Coefficients of the regression model	b_o	4.925	5.060	0.077	10.950
	b_1	−0.937	−0.651	0.339	−4.353
	b_2	−1.193	0.287	0.149	−2.562
	b_3	0.170	0.137	−0.175	0.526
	b_{11}	−1.065	0.119	0.639	1.076
	b_{22}	0.525	−0.054	0.649	0.399
	b_{33}	0.034	0.536	1.073	0.272
	b_{12}	0.055	−0.058	1.369	1.089
	b_{13}	−0.482	−0.232	0.629	−0.218
	b_{23}	−0.362	−0.348	0.199	0.041
ANOVA	SS_{pe}	77.502	46.416	116.744	415.037
	SS_{lof}	60.818	17.048	102.137	413.622
	F-ratio	0.785	0.768	0.875	0.997
	R^2	0.885	0.868	0.875	0.997
	R^2 adjusted	0.743	0.723	0.735	0.993

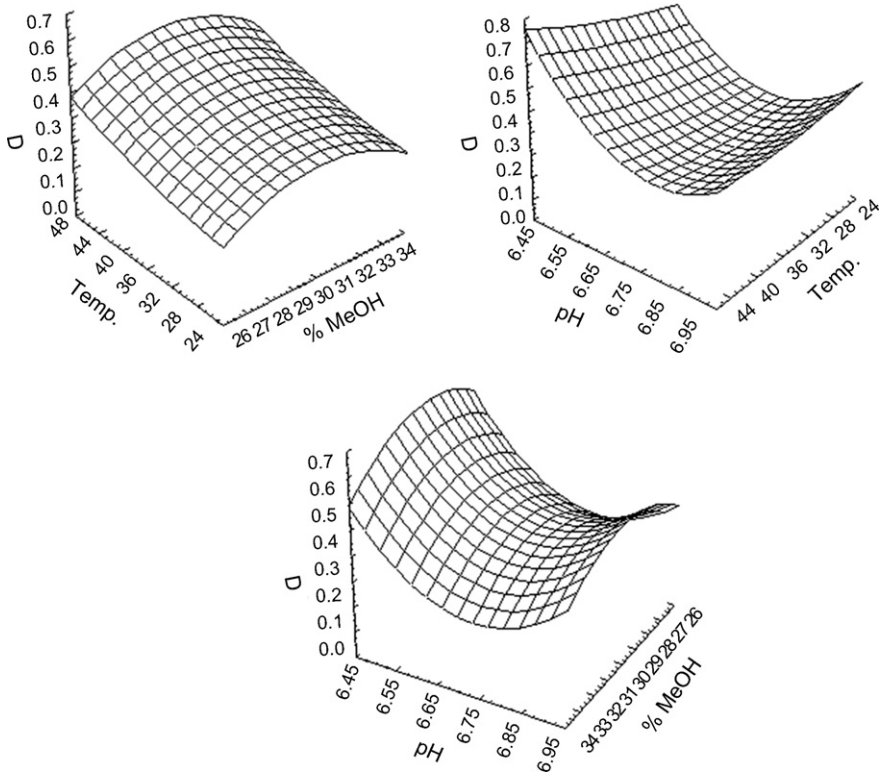

Fig. 5. 3-D plots of the Derringer's desirability function in correlation with the variation of methanol content, column's temperature and pH of the water phase.

of R^2 and R^2 adjusted taking into account the degrees of freedom indicated that the regression models fit the data well [29].

The data collected from the performed CCD experiments enabled us to extract some important conclusions about the behavior of the analytes. It was clearly noticed that the small decrease of methanol content in the mobile phase led to evident increase in the retention of EH. The other substances appeared to be less sensitive to that kind of influence. The increase of the water phase pH also resulted in prolonged retention time of EH. This influence was less dramatic, while the influence of the column temperature had the

Table 3
Criteria for the multivariate optimization of the individual responses.

Response	Goal	Lower limit	Upper limit
$Rs_{Eletriptan/UK\ 120.413}$	To maximize	1.200	6.734
$Rs_{Eletriptan/Degradation\ product}$	To maximize	1.200	7.617
$Rs_{UK\ 120.413/Degradation\ product}$	To maximize	1.200	4.945
$k'_{Eletriptan}$	To minimize	6.523	21.405

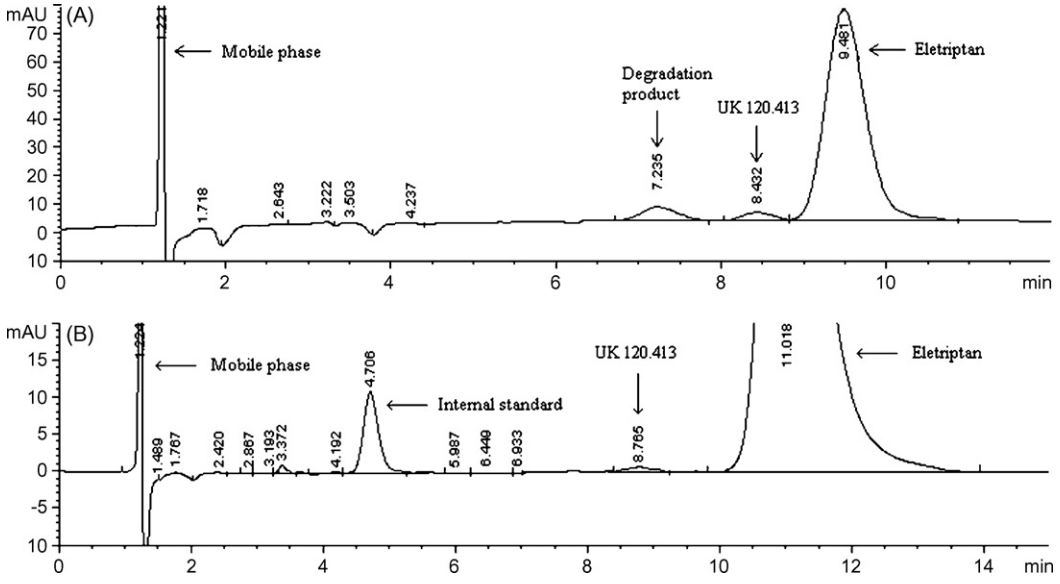

Fig. 6. LC-DAD chromatogram of Eletriptan together with its degradation product and UK 120.413 (A) and test solution prepared with the sample of Eletriptan tablets together with phenobarbiton as the internal standard (B) taken under optimized experimental conditions.

Table 4
System suitability parameters.

Parameter	Eletriptan	UK 120.413	Degradation product
System precision	0.22%*	0.68%*	0.55%*
Retention factor	6.765	5.906	4.925
Resolution *Rs*	2.272 ($Rs_{Elet./UK\ 120.413}$)	2.835 ($Rs_{UK\ 120.413/Degr.\ prod.}$)	/
Peak symmetry	0.916	0.951	0.934
N**	5011.507	7559.955	4019.053
HETP***	0.003	0.002	0.004

* Relative standard deviation calculated using the data from six replicate injections.
** Number of theoretical plates.
*** Height equivalent to a theoretical plate.

opposite direction. The significance of the experimental parameters' interactions was of minor importance in all cases. At the same time, the resolution between all analytes was mostly affected with the variations of the column temperature.

Since the selected responses were not affected in the same manner with the changes in experimental parameters, it was necessary to find a suitable compromise. The goals were marked for each response as shown in Table 3. For each response, the individual desirability function d_i was calculated using the predicted values from the fitted models, and afterwards the global desirability function D was estimated. For better visualization of the results, the global desirability function D was represented in a form of a three-dimensional plot and presented in Fig. 5. Finally, the coordinates producing the function maximum were easily selected as the best operating conditions. It was noticed that the chromatographic conditions should include the mobile phase consisting of methanol - water solution of TEA (pH 6.52, 1%, v/v) (30:70, v/v) and column's temperature 50 °C. The representative chromatogram taken under these conditions was presented in Fig. 6A.

3.3. Quantitative determination

After the optimal chromatographic conditions were chosen, the developed LC method was validated and method selectivity, linearity, precision, accuracy, robustness, limit of quantification and limit of detection were tested [30].

System suitability parameters were measured in order to verify the system performance. All results are reported in Table 4.

The selectivity of the method was investigated by observing potential interferences between EH, its potential degradation products and UK 120.413 with tablet excipient and no interfering peaks were present in the chromatograms. In order to check and to ensure the homogeneity of peaks in the test solution prepared with the sample of EH tablets, peak purity was examined and satisfactory results were achieved for all the substances in the study.

Table 5
Linear regression analysis for Eletriptan hydrobromide and UK 120.413.

	Eletriptan hydrobromide	UK 120.413
Concentration range	25.00–250.00 μg mL^{-1}	0.05–0.50 μg mL^{-1}
$Y = ax + b$	0.7279 × −1.3280	0.7192 × −0.0025
Correlation coefficient	0.9999	0.9992
Standard deviation of slope	0.005	0.043
Standard deviation of intercept	0.715	0.021
Deviation value for intercept ($t_{0.05}$ = 2.571)	1.857	1.188

The linearity of the method was tested for both EH and UK 120.413 standard solutions. The internal standard method was used for quantification. The reported correlation coefficients indicated that the relationship between peak area ratio of the drug to the internal standard and the concentration was highly linear over the entire investigated concentration range (Table 5). The statistical significance of the intercept of the obtained calibration curves was tested using the Student's *t*-test methodology and the results confirmed the absence of interferences.

The precision of the method was considered as inter-assay and intermediate precision and was expressed as relative standard deviation values. The accuracy study was performed in three concentrations at 80%, 100% and 120% of the label claims for EH tablets and was expressed as the Recovery value. The obtained results for both method precision and accuracy are presented in Table 6.

The method robustness was inspected during the method development procedure. The statistically significant influences of the main experimental parameters considering the chromatographic behavior of the analytes under study were clearly marked after the performed CCD experiments. Hence, it was evident that it would be necessary to suitably control the experimental parameter settings in order to maintain the reliability of the results.

The limit of detection (LOD) and the limit of quantification (LOQ) values were calculated based on the slope of the calibration curve

Table 6
Precision and accuracy of the proposed LC-DAD method.

	Compound	Injected (μg mL^{-1})	Found (μg mL^{-1})	RSD (%)	Recovery (%)
Intra-assay precision	Eletriptan hydrobromide	50.000	50.681 ± 0.452	0.89[a]	101.36
	UK 120.413	0.100	0.102 ± 0.001	1.42[a]	101.87
Intermediate precision	Eletriptan hydrobromide	50.000	51.046 ± 0.626	1.23[b]	102.09
	UK 120.413	0.100	0.102 ± 0.002	1.66[b]	102.03
Accuracy	Eletriptan hydrobromide	40.000	40.862 ± 0.262	0.64[c]	102.16
		50.000	49.735 ± 0.201	0.40[c]	99.47
		60.000	61.125 ± 0.574	0.94[c]	101.87
	UK 120.413	0.080	0.079 ± 0.001	0.78[c]	99.01
		0.100	0.099 ± 0.001	0.81[c]	98.90
		0.120	0.120 ± 0.001	0.88[c]	99.99

[a] Relative standard deviation calculated after the analysis of 9 samples analyzed in 1 day.
[b] Relative standard deviation calculated after the analysis of 9 samples analyzed on 3 separate days by 3 different analysts.
[c] Relative standard deviation calculated after the analysis of 3 samples.

Table 7
Determination of the compounds under study in the commercially available tablets.

Compound	Taken ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Found (mg/tbl.)	RSD* (%)	Recovery (%)
Eletriptan hydrobromide	50.000	47.755 ± 0.470	19.102 ± 0.188	0.98	95.51
Compound	Max. allowed ($\mu g\,mL^{-1}$)	Found* ($\mu g\,mL^{-1}$)	Found (%)	RSD* (%)	
UK 120.413	0.100	0.078 ± 0.001	0.16	1.69	

* Relative standard deviation calculated after the analysis of 10 prepared solutions.

and the standard deviation of the response. LOD values for EH and UK 120.413 were 0.02 $\mu g\,mL^{-1}$ and 0.01 $\mu g\,mL^{-1}$ and LOQ values were 0.07 $\mu g\,mL^{-1}$ and 0.04 $\mu g\,mL^{-1}$, respectively.

The applicability of the proposed method was examined by analyzing commercially available EH tablets. The obtained results and the representative LC-DAD chromatogram of the test solution is shown in Table 7 and Fig. 6B, respectively.

All the obtained data fully met the criteria from the ICH regulative in every observed segment of the method validation.

4. Conclusions

The data generated from the performed forced degradation studies enabled the evaluation of EH stability under a variety of ICH recommended conditions. Such data is valuable for the safety and potency assessment of a drug product. A simple and rapid isocratic RP-HPLC method proved to be able to measure the drug in the presence of degradation products and related organic impurities expected to be present in the formulation. In that manner, the proposed chromatographic procedure confirmed its applicability as a stability-indicating method.

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