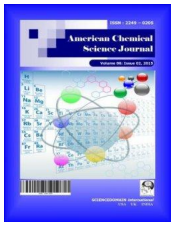

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Application of Multi-criteria Decision Making Approach and Experimental Design and Development of Stability-indicating UPLC Method for Simultaneous Determination of Losartan Potassium and Hydrochlorothiazide in Bulk and Tablet Dosage Form

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Authors' contributions

This work is a collective contribution of all authors. Author HHAS designed and supervised the research, author KAA drew the structures with Marvin Sketch software and carry out data analysis and desirability function calculations. Author MWN carried out the analyses and supplied some references, author AMA has designed the study, managed the literature searches, wrote the protocol and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

This paper describes a multi response optimization methodology in combination with experimental design as a powerful technique for development of a RP-UPLC method for the simultaneous determination of losartan potassium and hydrochlorothiazide in combined dosage forms. The response surface design by means of 2^4 full factorial design was used taking resolution, symmetry

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of two peaks, and two retention factors as the responses with four important factors, pH of the mobile phase, percentage of the organic modifier, buffer concentration and column temperature, were used to design mathematical models. Derringer's desirability function was used for reaching a suitable compromise among the responses. Optimal conditions included mobile phase consisting of acetonitrile–acetate buffer 38:62 v/v, pH 5.25 and buffer concentration of 28 mM as the mobile phase and at a flow rate of 0.3 ml/min and a column temperature of 37°C. The calibration plot was linear over the concentration ranges of 0.05–7 µg/mL for Losartan and 0.0125-4 µg/mL for hydrochlorothiazide having correlation coefficients not less than 0.999. Limits of detection and quantification were 0.287, 0.869 µg/mL and 0.487, 1.47 µg/mL, for Losartan and hydrochlorothiazide respectively. The specificity and stability-indicating capability of the method was proven through forced degradation studies, which showed no interference of the excipients. The robustness of the method was evaluated by Youden and Steiner's robustness test. The method is simple, rapid, and robust for simultaneous determination of losartan potassium and hydrochlorothiazide with minimum amount of solvent mobile phase and shortest run time about 4.5 min.

Keywords: Losartan potassium; hydrochlorothiazide; UPLC; stability indicating; multi-criteria decision making approach.

1. INTRODUCTION

Losartan potassium (LOP) (Fig. 1a), 2-n-butyl-4-chloro-5-hydroxymethyl-1-[2′-(1H-te-trazol-5-yl)(biphenyl-4-yl) methyl] imidazole, potassium salt, is an angiotensin II receptor antagonist acting mainly by selective blockade of AT1 receptors reducing the effect of angiotensin II [1]. Hydrochlorothiazide (HCTZ) (Fig. 1b), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, which is widely used in antihypertensive pharmaceutical preparations, reduces active sodium reabsorption and peripheral vascular resistance. A combination dosage form of 50 mg losartan potassium and 12.5 mg hydrochlorothiazide is widely used for treatment and management of edema and hypertension.

The literature reports many analytical methods for the quantitative determination of these compounds individually or in their combination with other drugs, like the use of HPLC [2-8], capillary electrophoresis (CE) [9-11], high performance thin layer chromatography (HPTLC) [12], voltammetry [13,14] spectrofluorimetry [15] and spectrophotometry [16-19]. Although a number of HPLC methods for simultaneous determination of losartan and hydrochlorothiazid have been reported [20-24], but there is only one chemometrics-assisted HPLC method have yet been reported [24].

In recent times, ultra-performance liquid chromatography (UPLC) appears very applicable in many fields of analysis [25,26]. However, to the best of our knowledge, there have been no publications or literature reporting UPLC methods for simultaneous determination of LOP and HCTZ. Optimization of UPLC conditions is a complicated process, because UPLC utilizes a wide selection of chromatographic factors as the type and concentration of organic modifier, pH, buffer molarity, temperature, and flow rate, etc. Therefore, a systematic approach such as experimental design to optimize chromatographic separations is more essential [27, 28].The best experimental design approach for the purpose of modeling and optimization is the response surface design [29].

Chemometrics can be used to accomplish a variety of goals in chromatography laboratory, such as: speeding methods development, make better use of chromatographic data, and explain the chromatographic process [30]. When one needs to optimize more than one response at a time the use of multicriteria decision making (MCDM), a chemometric technique is the best choice [31]. There are different approaches of MCDM [32] which include the path of steepest ascent, constrained optimization procedure, Pareto-optimality, utility function, and Derringer's desirability function. The Pareto-optimal method and the Derringer's approach have their own advantages and that the decision on which method to use depends on the problem and the availability of chromatographic expertise.

The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable, while for the utility functions, this is not the case. Further, Derringer's method offers the user flexibility in the definition of desirability functions.

In the present work, a UPLC method was developed, optimized and validated for simultaneous determination of LOP and HCTZ present in marketed tablet formulation and evaluate the stability of LOP and HCTZ in bulk and tablets after stress tests with minimum use of solvent and shortest run time. The robustness also was evaluated by Youden and Steiner's robustness test. In order to understand the sensitivity of the chromatographic factors on the separation of analytes and to simultaneous optimization of several chromatographic goals, chemometric protocols of response surface methodology and Derringer's desirability function were successfully employed.

2. EXPERIMENTAL

2.1 Apparatus

ACQUITY UPLC system (Waters, Milford, USA) used consisted of a binary solvent manager, a sample manager and ACQUITY UPLC Tunable UV (TUV) Detector. The output signal was monitored and processed using Empower2 software. The pH of the solutions was measured by a pH meter (Thermo Orion Model 420 A, USA). All solutions were degassed by ultra-sonication (Power Sonic 420, Labtech, Korea) and filtered through a 0.22-µm Nylon filter (PALL life sciences, USA).

2.2 Software

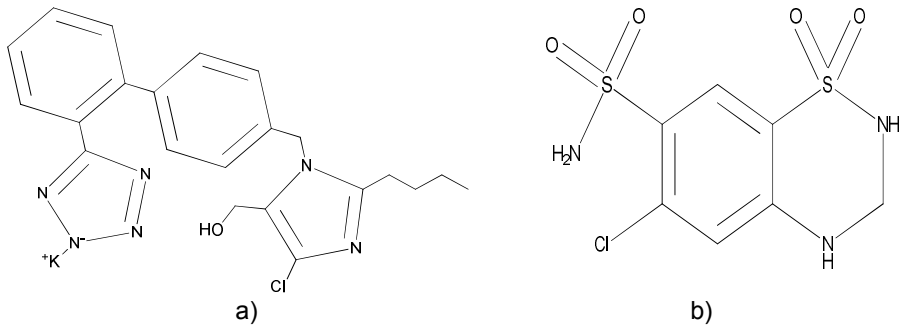
Experimental design, data analysis and desirability function calculations were performed by using Design-Expert® 8.0.7.1 (Stat-Ease Inc., Minneapolis) and MarvinSketch 5.8.2 (Chem Axon Ltd., Somerville, MA, USA and Budapest, Hungary).

2.3 Chemicals and Reagents

Working standards of LOP (99.9%) and HCTZ (100.02 %) and Sortiva H 50/12.5 tablets (containing 50 mg of LOP and 12.5 mg of HCTZ) were obtained from SPIMACO (Saudi Pharmaceutical Industries & Medical Appliances Corporation, Qassim, KSA). The HPLC grade acetonitrile, acetic acid and analytical grade sodium acetate were purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Milli Q Plus water purification system (Millipore, Milford, MA, USA).

2.4 Standard Solutions

Stock standard solutions of LOP (1 mg/ml) and HCTZ (0.25 mg/ml) were prepared individually in mobile phase. The prepared stock solution was stored at 4ºC protected from light. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the analysis day. The working solutions for linearity of 0.05–7.0 µg/ml for LOP and 0.0125–4.0 µg/ml for HCTZ were prepared by dilution of stock solutions with mobile phase. The working solutions for precision and accuracy, containing 0.05, 4.0 and 7.0 µg/ml for LOP and 0.0125, 2.0 and 4.0 µg/ml for HCTZ, were prepared as laboratory mixtures by adding the known amounts of LOP and HCTZ to the placebo mixture.



a) b)

Fig. 1. a. Structure of losartan potassium; b. structure of hydrochlorothiazide

2.5 Sample Preparation

Five tablets were weighed and finely powdered. An amount of tablet powder equivalent to 50 mg of LOP and 12.5 mg of HCTZ were accurately weighed and transferred in a 100 ml volumetric flask; 70 ml of mobile phase was added. This mixture was subjected to sonication for 10 min for complete extraction of drugs and the solution was made up to the mark with mobile phase to obtain a concentration of LOP and HCTZ as 0.5 and 0.125 mg/ml, respectively. The solution was centrifuged at 2504 g for 10 min; the clear supernatant was collected and filtered through a 0.22-µm (Nylon 66-membrane) filter. About 1 mL of supernatant solution was diluted to 100 mL with the mobile phase.

2.6 Chromatographic Procedure

Chromatographic separations were carried out on an ACQUITY BEH® C18 analytical column (50 mm×2.1 mm i.d., 1.7 µm). The mobile phase consisted of acetonitrile: acetate buffer (pH 5.25, 28 mM) 38:62 v/v pumped at a flow rate of 0.3 ml min^{-1} and a column temperature of 37°C, and the detection was monitored at a wavelength of 248 nm.

2.7 Forced Degradation Studies

Forced degradation studies were performed to provide an indication about stability of the studied drugs and specificity of the proposed method. The forced degradation studies were carried out by preparing several standard solutions of LOP and HCTZ at 10mg mL^{-1}, for each degradation study. Each sample was analyzed according to the previous procedures described under the chromatographic procedure. Forced degradation studies under different conditions were carried out according to the following procedure:

(a) Acidic and basic conditions: 1 ml of stock LOP and HCTZ solutions were treated with0.1 M HCl or 0.1 M NaOH. The solutions were placed in a water bath at 70°C for 4 hours in the dark (to exclude the possible degradative effect of light).
(b) Neutral degradations: LOP and HCTZ solutions were treated with water. The solutions were placed in a water bath at 70°C for 3 hrs.
(c) Oxidation with H_2O_2: 1 ml of stock LOP and HCTZ solutions were exposed to H_2O_2 5% solution. These solutions were kept at room temperature in dark for 4 hrs.
(d) Photolytic degradation: 10 mg bulk powder samples of LOP and HCTZ were exposed to UV light (365 nm) in a photostability chamber for 24 hrs.
(e) Thermal degradation: 10 mg of each drug powder was kept in an oven at 80°C for 24 hrs.

Once the stress conditions were completed, mobile phase was added to the samples in order to achieve the standard solution concentration of 4.0 µm mL^{-1}. Moreover, all the solutions and blanks were filtered with a 0.22 µm syringe filtration disk PVDF.

2.8 Experimental Design and Methodology

The investigation was carried out in several steps. First, to perform a screening of the factors that could potentially influence chromatographic retention and to establish a region over which each factor is to be studied, in this study, the independent variables were defined during the preliminary study. The second step is the choose design. The third step is to choose a response and the fourth, a mathematical model can then be produced relating the response to the factors.

In the present study, to optimize five responses with different targets, and in order to ensure the best chromatographic performance of the analytical procedure, the multicriteria decision making methodology was employed by means of Derringer's desirability function, concerning its applicability to both linear and non-linear models.

2.9 Method Validation

Method validation was performed according to ICH specifications [33] for selectivity, linearity, accuracy, precision, robustness, system suitability test, limit of detection and limit of quantitation.

3. RESULTS AND DISCUSSION

3.1 Development of a Chromatographic Method

The development of a chromatographic method is very complex due to the wide number of parameters that influence the separation, selectivity and all other performance criteria.

Experimental design enables a faster method development process by choosing the experiments efficiently and systematically to give reliable and coherent information. In that way, each chromatographic parameter can be examined by conducting a series of experiments for several parameters that are changed at the same time. Before specific parameter limits for individual factorial design were selected, preliminary experiments were performed.

The degree of ionization of the drug strongly affects solubility and retention. Additionally, the knowledge of dissociation constant of ionisable compounds at different pH values and the solvent composition is also significant to determine the optimal separation conditions in RP-LC. Considering the chemical structures, it is possible to consider a number of proton acceptor and donor groups (Fig. 2), to assume ionized structures of LOP and HCTZ (Figs. 3, 4).

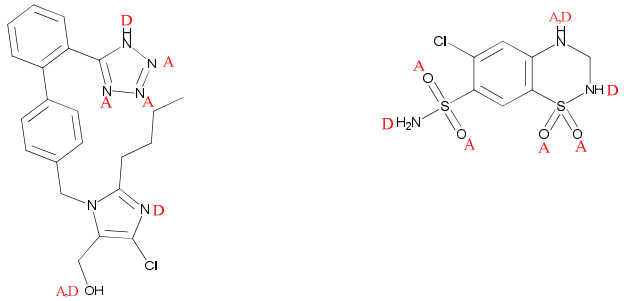


Fig. 2. Proton acceptor (A) and donor groups (D) of LOP and HCTZ

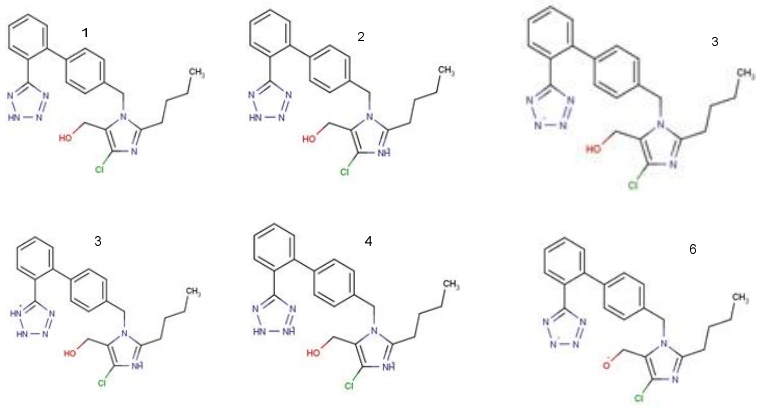


Fig. 3. Structural formula of ionized LOP

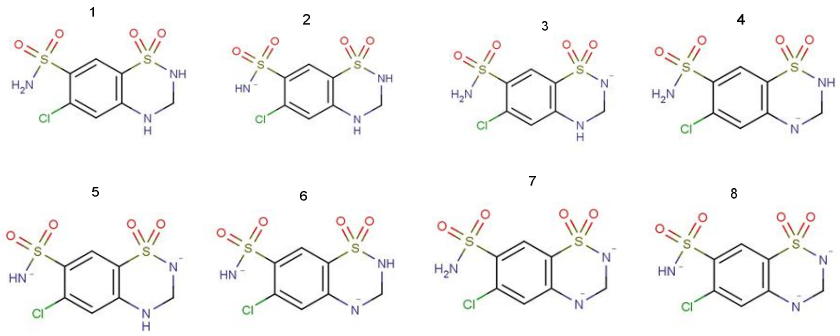


Fig. 4. Structural formula of ionized HCTZ

We also studied the molecules of LOP and HCTZ using MarvinSketch for calculation of pKa and degrees of ionization depending on the pH (Tables 1 and 2), it can be seen that LOP has two pKa values (4.10; 7.40) and at pH 4, LOP is present as a mixture of molecular (43.01%) and mono-protonated (56.97%) forms. The structure of HCTZ has three pKa values (9.09; 9.83; 11.31). At higher pH values, HCTZ is present as a mixture of different deprotonated species.

A pH interval from 4.0 to 6.0 was selected for further investigation. In this pH interval, LOP is partially ionized and HCTZ is completely unionized and under these conditions the following order of retention could be expected: HCTZ, then LOP [24].

Problems, such as, partial ionization of the analyte and strong interaction between analyte and residual silanoles or other active sites on the stationary phases can be overcome by proper mobile phase buffering and choosing the right ionic species and its concentration (ionic strength) in the mobile phase [34,35]. Thus, the concentration of buffer may also affect the separation. It was expected also, that the content of the organic modifier could govern the separation process. Acetonitrile was selected as organic modifier because it has lower viscosity, reduces back pressure and produce better peak shape.

It is well-known that an increase in column temperature by 1ºC will usually decrease the retention factor by1-2 % and in that manner column temperature could also affect baseline separation of analytes, thus the column temperature was subjected to investigation.

Depending on all these considerations, four factors which are pH of mobile phase, percentage of acetonitrile, buffer proportion and column temperature were selected as significant factors. In order to evaluate the effect of the selected factors, full factorial design was used to determine which parameters have a significant effect on the retention of investigated substances.

A 2^4 full factorial design (FFD) with three replicates at the zero level resulting in 19 experiments. The experimental data was coded in order to follow the significance of factors in an easier way. As a good separation is characterized by good resolution and short run time so, five responses were chosen as dependent variables: resolution (k_R), symmetry of the LOP peak (Sy L), symmetry of the HCTZ peak (Sy H), retention factor of LOP (R_t L) and retention factor of HCTZ (R_t H).The selected factors are shown in Table 3 along with their low (-1), medium (0) and high (+1) level settings, which were selected based on the results from preliminary test runs.

All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. A second order interaction was suggested for the relationship between input and output, and can be expressed as:

$$Y= \beta_0+ \beta_1x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4+ \beta_{1,2} x_1x_2 + \beta_{1,3} x_1x_3 + \beta_{1,4} x_1x_4+\beta_{2,3} x_2x_3 + \beta_{2,4} x_2x_4 + \beta_{3,4} x_3x_4+\beta_{1,2,3,4} x_1x_2x_3 x_4 +\varepsilon$$

Where Y represents the estimated response, x_1, x_2, x_3 and x_4 are the experimental factors in coded variables (x_1 = pH, x_2 = buffer concentration, x_3 = % ACN and x_4 = T), β_0 is the intercept, coefficients β_1, β_2, β_3 and β_4 are the estimated effects of the factors considered. The extent to which these terms affect the performance of the method is called the main effect. The coefficients $\beta_{1,2}$ to $\beta_{1,2,3,4}$ are called interaction terms. In this way, FFD provides information about the importance of interaction between the factors. The values of the obtained coefficients are listed in Table 5.

3.2 Analysis of Variance (ANOVA)

The results were analyzed using Design Expert, and the results were summarized in Table6. Regression lack of fit was determined by performing an *F*-test in order to compare the variance due to the lack of fit to the variance due to purely experimental uncertainty. The lack of fit (SS_{lof}) and the pure error (SS_{pe}) sums of squares were calculated as well as SS_{lof}/SS_{pe} ratios which were compared with the tabled F_{crtic} at 95% confidence level (F-tab = 8.66).

$$SS_{lof} = \sum_{i}^{m}\sum_{j}^{n_i}(\hat{y}_i - \bar{y}_i)^2 \; SS_{pe} = \sum_{i}^{m}\sum_{j}^{n_i}(y_{ij} - \bar{y}_i)^2$$

Those ratios for the measured responses K_R, S_y L, S_y H, R_t L and R_t H were 0.117, 0.277, 0.167, 0.069 and 0.781, respectively. As the calculated quotients are lower than the tabled *F*-value, there is no model lack of fit and all models could be accepted. It could be concluded that models adequately represent the influence of the investigated variables on the responses.

Table 1. Ionization percentage of LOP depending on pH

pH	1	2	3	4	5	6	7	8	9	10	11	12	13	14
%-1	0.08	0.75	7.02	43.01	87.99	94.95	71.44	20.07	2.45	0.25	0.03	0	0	0
%-2	99.81	99.24	92.98	56.97	11.66	1.26	0.09	0	0	0	0	0	0	0
%-3	0	0	0	0.02	0.35	3.78	28.46	79.93	97.55	99.74	99.92	99.47	94.94	65.23
%-4	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0
%-5	0.08	0.01	0	0	0	0	0	0	0	0	0	0	0	0
%-6	0	0	0	0	0	0	0	0	0	0.01	0.05	0.53	5.06	34.77

Table 2. Ionization percentage of HCTZ depending on pH

pH	1	2	3	4	5	6	7	8	9	10	11	12	13	14
%-1	100	100	100	100	99.99	99.92	99.19	92.33	51.53	4.55	.05	0	0	0
%-2	0	0	0	0	0	0.04	0.41	3.78	21.11	18.65	2.16	0.06	0	0
%-3	0	0	0	0	0	0.04	0.39	3.62	20.21	17.85	2.07	0.05	0	0
%-4	0	0	0	0	0	0	0.02	0.15	0.84	0.74	0.09	0	0	0
%-5	0	0	0	0	0	0	0	0.1	5.78	51.04	59.03	15.56	1.84	0.19
%-6	0	0	0	0	0	0	0	0	0.08	0.74	0.86	0.23	0.03	0
%-7	0	0	0	0	0	0	0	0.01	0.42	3.7	4.28	1.13	0.13	0.01
%-8	0	0	0	0	0	0	0	0	0.03	2.72	31.47	82.97	98.00	99.80

Table 3. Factors and levels in coded format

Chromatographic factors		Levels used in experiments		
		Low-1	Medium0	High+1
x_1	pH	4	5	6
x_2	buffer concentration (mM)	20	30	40
x_3	% ACN	25	35	45
x_4	Temperature (◦C)	25	35	45

The matrix of experiments and results obtained as an average value of three runs are presented in Table 4

Table 4. Factorial design matrix and results of experiments

Experiment	Chromatographic factors				Results				
no.	x_1	x_2	x_3	x_4	K_R	Sy L	Sy H	Rt L	Rt H
1	-1	-1	-1	-1	32.63	1.11	1.62	9.29	0.829
2	1	-1	-1	-1	23.85	1.94	1.51	6.22	0.785
3	-1	1	-1	-1	31.17	0.922	1.59	9.02	0.773
4	1	1	-1	-1	22.32	1.95	1.45	6.83	0.711
5	-1	-1	1	-1	15.69	1.43	1.05	5.99	0.581
6	1	-1	1	-1	10.33	1.83	1.26	3.08	0.562
7	-1	1	1	-1	14.23	1.6	0.967	5.02	0.533
8	1	1	1	-1	5.45	1.72	1.16	2.67	0.45
9	-1	-1	-1	1	31.17	1.43	1.68	8.22	0.648
10	1	-1	-1	1	22.39	1.25	1.53	5.66	0.755
11	-1	1	-1	1	27.61	1.21	1.62	5.11	0.663
12	1	1	-1	1	21.95	1.51	1.55	4.88	0.567
13	-1	-1	1	1	12.03	0.922	1.39	2.99	0.545
14	1	-1	1	1	3.25	0.91	1.59	0.69	0.638
15	-1	1	1	1	10.57	0.611	1.35	4.33	0.511
16	1	1	1	1	1.79	1.18	1.49	0.503	0.572
17	0	0	0	0	17.55	1.35	1.42	5.1	0.632
18	0	0	0	0	17.77	1.34	1.43	4.95	0.637
19	0	0	0	0	18.12	1.36	1.42	4.99	0.635

Table 5. Model of coefficients

	β0	β1	β2	β3	β4	β1,2	β1,3	β1,4	β2,3	β2,4	β3,4	β1,2,3	β1,2,4	$\beta_{1,3,4}$	$\beta_{2,3,4}$	$\beta_{1,2,3,4}$
K_R	17.90	-3.99	-1.02	-8.73	-1.56	-0.023	0.023	-0.014	-0.14	0.15	-0.70	-0.40	0.41	-0.41	0.28	0.014
S_y L	1.35	0.19	-0.007	-0.07	-0.22	0.061	-0.056	-0.11	0.009	0.007	-0.15	-0.024	0.071	0.11	-0.02	0.036
S_y H	1.43	0.017	-0.028	-0.14	0.1	-0.001	0.076	-0.002	-0.012	-0.005	0.073	-0.007	-0.004	-0.005	0	-0.009
R_t L	5.03	-1.21	-0.24	-1.87	-0.98	0.14	-0.21	0.10	0.21	-0.11	-0.047	-0.26	-0.040	-0.21	0.42	-0.22
R_t H	0.63	-0.002	-0.035	-.084	-0.020	-0.020	0.009	0.023	0.002	0.001	0.038	0.007	-0.009	0.008	0.006	0.014

Table 6. Statistical parameters obtained by ANOVA

Response	F	Model P-value	%C.V.	Adequate precision	R^2	Adjusted R^2
K_R	1248.85	0.0008	1.39	135.30	0.9999	0.9993
S_y L	1598.76	0.0006	0.69	158.10	0.9999	0.9994
S_y H	1377.87	0.0007	0.36	152.52	0.9996	0.9993
R_t L	1144.76	0.0009	1.30	146.05	0.9999	0.9993
R_t H	1949.22	0.0005	0.43	150.59	0.9999	0.9993

The adjusted R^2 were well within the acceptable limits [36] which revealed that the experimental data shows a good fit with the second-order polynomial equations.

$$adjR^2 = 1 - \left[\frac{\left(\frac{SS_{res}}{df_{res}}\right)}{\left(\frac{SS_{res}+SS_{mod}}{df_{res}+df_{mod}}\right)}\right]$$

Where $SS_{res} =$ summation squares of the residuals, $SS_{mod} =$ summation squares of the model, df_{res}, df_{mod} are degree of freedom of residuals and of model respectively. For all models, P value of <0.05 are obtained, implying these models are significant.

The adequate precision value is a measure of the "signal to noise ratio". It compares the range of the predicted values at the design points to the average prediction error.

$$Adequate\ precision = \left[\frac{\max(\hat{Y}) - min(\hat{Y})}{\sqrt{\bar{V}(\hat{Y})}}\right]$$

Where $(\hat{Y}) =$ Predicted response, $\bar{V}(\hat{Y})$ = Average standard deviation of all predicted responses.

A ratio greater than 4 is desirable [37]. In our work, the ratio was found to be in the range of 135.3–158.1, which indicates an adequate signal, and hence, model is significant for the separation process.

The coefficient of variation (C.V.) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10% [37].

$$CV = \frac{standard\ deviation}{mean} x\ 100\ (\%)$$

The C.V. for all the models was found to less than 10%, good reproducibility of the models.

A positive value of the coefficient means an increase in the response with an increase in the chromatographic parameter, while a negative value means a decrease in the response with an increase in the chromatographic parameter. Accordingly, could be concluded from the results that acetonitrile percentage in the mobile phase and pH have highest influence on K_R, R_t L and less influence on the other responses. The temperature of the column exerts positive influence on S_y H and negative influence on S_y L, respectively.

3.3 Multi-criteria Decision Making

In the present study, Derringer's desirability function was used to optimize five responses with different targets. In Derringer's desirability function approach, each response is transformed into a desirability value *di* and the total desirability function **D**, which is the geometric mean of the individual desirability values, is computed and optimized [38].

Subsequently the transformations of all individual desirability points for the predicted values are converted into overall desirability function, **D**, by computing their geometric mean by following equation.

$$\boldsymbol{D} = [d_1 \times d_2 \times d_3 \times \ldots\ldots \times d_n]^{1/n}$$

The above equation can be extended when the weight or importance has been considered:

$$\boldsymbol{D} = [d_1^{P1} \times d_2^{P2} \times d_3^{P3} \times \cdots \times d_n^{Pn}]^{1/n}$$

Where (*pn*) is the weight of the responses, (n) is the number of responses, (d_n) is the individual desirability function of each response. Weight of the response is the relative importance of each individual functions (*di*). The scale of the individual desirability function ranges between (*di* = 0), for a completely undesired response, to (*di* = 1) for a fully desired response. Weights can range from 0.1 to 10. Weights lower than 1 give less emphasis to the goal, whereas weights

greater than 1 give more emphasis to the goal. In this study, we select weights equal to 1 for all responses at which (*di*) varies in a linear way.

A value of ***D*** different from zero implies that all responses are in a desirable range simultaneously and consequently, for a value of ***D*** close to 1, the combination of the different criteria is globally optimal, so the response values are near target values. The desirability function ***D*** can be represented in a form of a three-dimensional plot for better visualization of the results [39].

In our study there were different goals and different responses, which made reaching of a suitable compromise among them is very difficult, but it was possible by using of Derringer's desirability function.

The first goal was to maximize the retention factor of the HCTZ peak to prevent its overlapping with the mobile phase peak. The second goal was to decrease the total analysis run time by decreasing the retention factor of the LOP peak which had the greatest affinity for the stationary phase. The third was to obtain better peak symmetries of LOP and HCTZ. The individual desirability function *di* for each goal was calculated then global desirability function ***D*** was estimated as geometrical mean of the individual desirability functions. The goals of multicriteria optimization for each response in this study are presented in Table7.

For the better visualization of the results, a three-dimensional plot of the overall desirability function was presented in Fig. 5.

The overall desirability function of 1 was obtained with 37.99% of acetonitrile, pH 5.24 of the mobile phase, 36.95ºC of column temperature and 28.08 mM buffer concentration. Some small changes in optimal conditions were made and the final conditions were 38% of acetonitrile, pH 5.25 of the mobile phase, 37ºC of column temperature and 28 mM buffer concentration.

Table 7. Criteria for optimization of individual responses and results

Response	Lower limit	Upper limit	Goal	Importance	Results	
					Predicted	Experimental
K_R	1.79	32.63	In range	3	14.1	15.6
S_y L	0.611	1.95	In range	3	1.3	1.28
S_y H	0.967	1.68	In range	3	1.41	1.34
R_t L	0.503	9.29	4	5	4.0	4.12
R_t H	0.45	0.829	Maximize	5	0.617	0.695

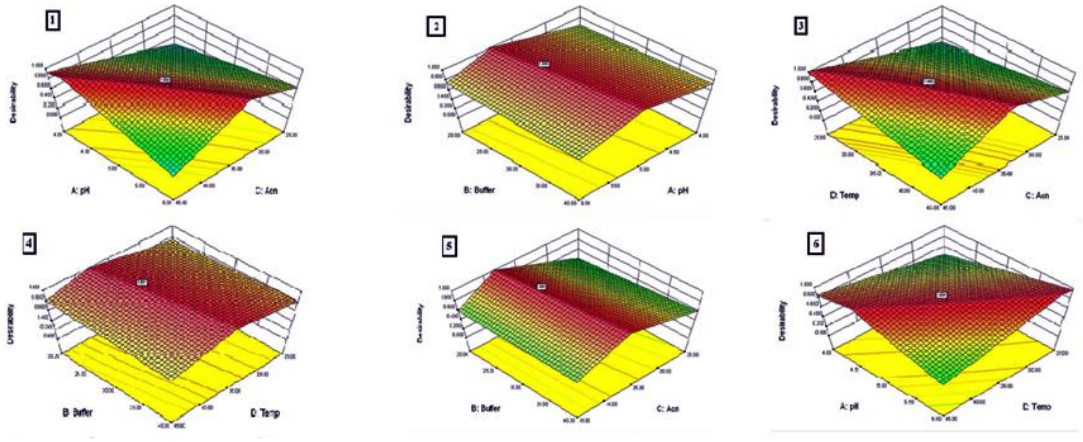


Fig. 5. 3-D plots of the Derringer's desirability function in correlation with a variation of % ACN and pH (1), buffer concentration and pH (2), temperature and % ACN (3), buffer concentration and temperature (4), buffer concentration and % ACN (5) and pH of mobile phase and temperature of column (6)

The representative chromatogram taken under these conditions is represented in Fig. 6 which showed complete resolution of the analytes in a short analysis time and approximately the same experimental values as predicted values.

3.4 Method Validation

3.4.1 System suitability test

System suitability testing was carried out by sex of replicate injections of standard LOP and HCTZ (4.0 μgmL^{-1}). The acceptance criteria were less than 2% relative standard deviation (RSD) for peak areas, USP tailing factor (T) less than 2.0, USP plate count (N) more than 5000, for LOP and HCTZ peaks from standard solutions. The results (Mean ± % RSD of six replicates) of the chromatographic parameters (Table 8) indicate the good performance of the system.

3.4.2 Linearity and range

The linearity of the relationship between peak area and concentration was determined by analyzing standard solutions over the concentration ranges 0.05 – 7.0 and 0.0125 – 4.0 μgmL^{-1} for LOP and HCTZ respectively. Table 9 presents the performance data and statistical parameters including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept (S_a) and slope (S_b). Regression analysis shows good linearity as indicated from the correlation coefficient values (>0.9995).

3.4.3 Accuracy

Accuracy of the proposed method was determined by measuring the reference standard of LOP and HCTZ recovery in triplicate at three different concentration levels (0.05, 4.0, 7.0 μgmL^{-1} and 0.0125, 2.0, 4.0 μgmL^{-1} for LOP and HCTZ, respectively). The percentage recoveries obtained for LOP and HCTZ (Table 10) were within 0.47-1.28% for LOP and 0.64-1.63% for HCTZ, indicating that the method is accurate and also found that there was no interference due to the presence of excipients in the tablet formulations.

3.4.4 Precision

The intra-day precision was determined by preparing three working standard solutions which covered entire established calibration curves of each analyte in concentrations of 0.05, 4.0 and 7.0 μgmL^{-1} for LOP and 0.0125, 2.0 and 4.0 μgmL^{-1} for HCTZ on the same day and subsequently injecting each solution into the chromatography system three times. The intermediate precision of the method was also evaluated on different days with different analyst using the same prescribed conditions. The values of % RSD for intra-day and inter-day variation were found very well and within 2%limit, indicating that the current method is precise (Table 11).

Table 8. Chromatographic characteristics of system suitability study

Parameters	Value (Mean ± RSD)	
	LOP	HCTZ
Peak area	29320583.76±0.16	7463340.36±0.16
Tailing factor	1.4±1.06	1.12±0.45
Retention time	4.12±0.02	0.696±0.04
Theoretical plates	8127±0.08	5198±0.10

Table 9. Statistical parameters for individual calibration curves

Parameter	LOP	HCTZ
Range µg/ml	0.05-7	0.0125-4
slope ($X10^6$)	7.321330	1.833051
Intercept	56470	189145
r	0.9999	0.9995
LOD	0.020	0.042
LOQ	0.061	0.129

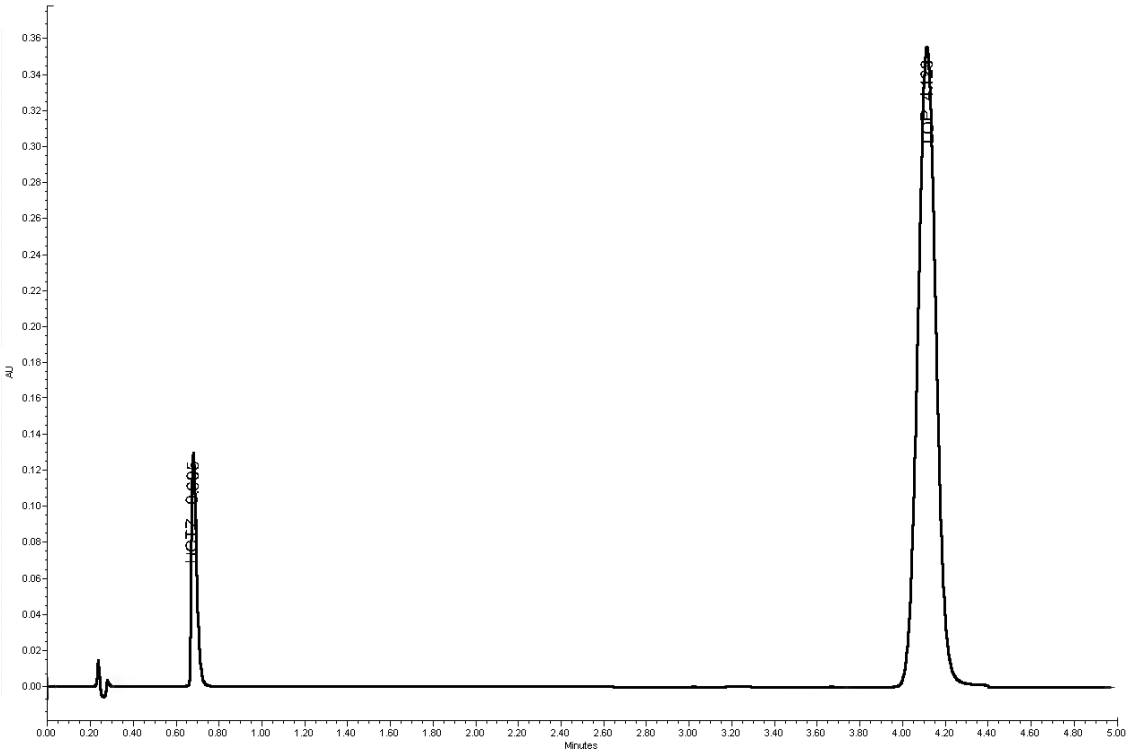


Fig. 6. LC chromatogram of the working standard mixture of LOP and HCTZ taken under optimized optimum conditions

Table 10. Results for method accuracy

Analyte	Injected (µg/mL)	Peak area	Found (µg/mL)	% Recovery	% Recovery (mean ±RSD)
		415215	0.049	99.25	
	0.05	428393	0.050	101.65	100.18 ±1.28
		421072	0.049	99.65	
		29546787	4.02	100.71	
LOP	4	29400361	4.00	100.2	100.22±0.47
		29268577	3.99	99.77	
		50500434	6.89	98.48	
	7	51562027	7.03	100.51	99.93±1.27
		51715774	7.05	100.81	
		211997	0.0124	99.2	
	0.0125	211863	0.0123	98.4	99.73±1.63
		212519	0.0127	101.6	
		3904742	2.02	101.38	
HCTZ	2	3818596	1.98	99.22	100.05±1.16
		3836925	1.99	99.55	
		7558016	4.02	100.57	
	4	7503020	3.99	99.81	99.89±0.64
		7466362	3.97	99.31	

Table 11. Precision of the proposed RP-UPLC method

Analyte	Injected (µg/mL)	Intra-day precision		Inter-day precision		Overall (mean ±RSD)
		Found (µg/mL)	% Recovery	Found (µg/mL)	% Recovery	
		0.049	99.19	0.0496	99.29	
	0.05	0.049	99.35	0.0502	100.41	0.049±0.61
		0.049	99.78	0.0498	99.67	
		3.931	98.29	3.98	99.72	
LOP	4	3.98	99.65	3.94	98.59	3.97±0.67
		3.99	99.77	3.99	99.88	
		7.02	100.31	6.92	98.97	
	7	7.03	100.45	6.94	99.18	6.98±1.41
		7.07	101.1	7.10	101.51	
		0.0124	99.38	0.0124	99.48	
	0.0125	0.0125	100.54	0.0124	99.72	0.0123±0.47
		0.0124	99.49	0.0123	98.49	
		1.99	99.52	2.00	100.31	
HCTZ	2	1.97	98.89	1.97	98.96	1.98±0.87
		1.98	99.11	1.97	98.99	
		3.95	98.96	3.97	99.43	
	4	3.98	99.52	3.93	98.45	3.95±0.53
		3.98	99.64	3.96	99.04	

3.4.5 Specificity and selectivity

Specificity is defined as the ability to access unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components, and this is well demonstrated in detail through the analysis of pharmaceutical dosage form and forced degradation studies. The chromatograms of standards and that of sample solution showed that there is no difference between retention times of standard drugs and retention times of drugs in sample solution, also, there is no extra peaks were observed from any of the inactive ingredients in the dosage form, indicating that the developed method was specific and selective. Selectivity was also demonstrated by separation of the LOP and HCTZ from their relevant degradation products under different stress conditions.

3.4.6 LOD and LOQ

In the present study, the LOD and LOQ were calculated as 3.3 σ/s for LOD and 10 σ/s for LOQ, where σ is the standard deviation of the response and sis the slope, determined from the corresponding calibration curve. From Table9, LOD was 0.020 µg mL^{-1} and 0.042 µg mL^{-1} for LOP and HCTZ respectively, and LOQ were 0.061 µg mL^{-1} and 0.129 µg $mL^{-1}$for LOP and HCTZ respectively. These values are better than the literature values [24] and are adequate for determination in pharmaceutical samples.

3.4.7 Robustness

The robustness of an analytical method is the measurement of its capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

Although the robustness of the method was partially evaluated during the optimization process, The robustness evaluation was also performed during the validation process using the method proposed by Youden and Steiner [40], in which, Robustness was determined by analyzing the same sample (4.0 µg/ml) under variations of seven analytical parameters of the method.

The mobile phase related factors including mobile phase pH, buffer proportion, % of acetonitrile, along with the column temperature, acetonitrile supplier, detector wavelength and column age were carefully chosen as the seven variables for Youden and Steiner's robustness test. The effect of each variable was investigated at two levels as indicated by the upper and lower case letters. The analytical conditions at the nominal values are represented by capital letters and the conditions with the small variation are represented by lowercase letters (Table 12).

The observed results to be evaluated were the recoveries of LOP and HCTZ from the tablet placebo matrix at the work concentration. In each combination, three injections of sample solutions were carried out and the analyses results are shown by letters from (s) to (z). From the results, to determine the influence of variations of each parameter, the mean of the four values corresponding to the capital letters (nominal conditions) was compared to the mean of the four values corresponding to the lowercase letters (altered conditions). For example, to evaluate the effect of the buffer proportion in the final result of the analyses, the following equation was employed:

$$Effect\ C/_C = \frac{s+u+w+y}{4} - \frac{t+v+x+z}{4}$$

Considering the standard deviation of the eight results, the following criterion was applied: If the effect values higher than the $SD\sqrt{2}$ (SD is the standard deviation), the factor has a significant effect and the method is sensitive to changes in the factor concerned.

The results and the experimental range of the selected variables evaluated in the robustness assessment are given in Table 13. The difference (X–x), mean values, standard deviations and criterions (SD√2) were calculated and used to evaluate the results (Table 14). The results meet the acceptance criterion, with no significant changes in the content results when the modifications were made in the experimental conditions, thus showing the method to be robust.

3.5 Forced Degradation Studies

Forced-degradation studies were carried out on each of the two drugs in this combination in order to produce the possible relevant degradants and test their chromatographic behavior using the developed UPLC method (Fig. 7).

Table 12. Analytical parameters and variations for the robustness evaluation of the chromatographic method for LOP and HCTZ quantitation

Selected variable	Unites	Abbreviation[a]	High level	Low level
Acetonitrile concentration (%)	-	A,a	38	35
Acetonitrile supplier	-	B,b	Merck	Fluka
Buffer concentration (%)	mM	C,c	28	30
Wavelength (nm)	nm	D,d	248	250
Buffer pH	%	E,e	5.25	5.35
Column temperature (°C)	°C	F,f	37	35
Column age	-	G,g	New	Old

Table 13. Results obtained in eight runs performed for robustness evaluation, for LOP and HCTZ solutions

Experiment variables	Experimental condition							
	1	2	3	4	5	6	7	8
Acetonitrile concentration (%)	A	A	A	A	a	a	a	a
Acetonitrile supplier	B	B	b	b	B	B	b	b
Buffer concentration (%)	C	c	C	c	C	c	C	c
Wavelength (nm)	D	D	d	d	d	d	D	D
Buffer pH	E	e	E	e	e	E	e	E
Column temperature (°C)	F	f	f	F	F	f	f	F
Column age	G	g	g	G	g	G	G	g
results	s	t	u	v	w	x	y	z
LOP	100.11	99.8	99.78	98.82	99.03	99.78	99.21	99.18
HCTZ	100.26	100.26	100.26	100.26	100.26	100.26	100.26	100.26

Table 14. Effects of the analytical parameters in content of the chromatographic method for LOP and HCTZ quantitation

Effect	Content (%) LOP*	Content (%) HCTZ*
Acetonitrile concentration (%) (A=38, a=35)	99.62- 99.3=0.327	99.96-99.86=0.275
Acetonitrile supplier (B=Merck, b=Fluka)	99.68-99.24=0.432	99.86-99.78=0.085
Buffer concentration (%) (C=28, c=30)	99.53-99.39=0.137	100.04-99.6=0.44
Wavelength (nm) (D=248, d=250)	99.57-99.35=0.222	100.06-99.58=0.475
Buffer pH (E=5.25, e=5.35)	99.71-99.21=0.479	99.85-99.78=0.07
Column temperature(°C) (F=37, f=35)	99.28-99.64=-0.357	99.75-99.87=-0.11
Column age (G=New, g=Old)	99.48-99.44=0.0325	99.64-100.0=-0.36
SD√2	0.649	0.608

**Average of the values obtained at nominal conditions – average of the values obtained at altered conditions*

3.5.1 Acid-Induced degradation study

In acidic hydrolysis, HCTZ degrades as observed by the decreased area of the same concentration of the intact drug by 14%, with giving of additional degradation peak at t_R 0.63 min. Significant degradations of LOP was observed in acidic condition (8.16%) leading to the formation two degradations products were detected at t_R 6.60 and 8.87 min. The degradations products of LOP are isomer resulting from dimerization of two molecules of LOP by formation of a bond between nitrogen atom in tetrazole ring and carbon atom in 5- methanol in imidazole ring producing dimer and water [41].

3.5.2 Base-induced degradation study

In basic medium, degradation of HCTZ was noticed from the decrease of its peak area which reached 79% of the expected area, this product showed at t_R 0.63 min. No signs of degradation of LOP could be observed under basic conditions. LOP peak appeared at its specific retention time with area almost identical.

3.5.3 Neutral degradation study

Neutral conditions enhance HCTZ degradation to 31% decrease in original one, and an additional peak was observed at t_R 0.63 min. while no signs of degradation of LOP could be observed.

3.5.4 Oxidation with H_2O_2degradation study

Oxidative H_2O_2 increase degradation of HCTZ to 13.58% from the peak area compared to a standard of the same concentration, with formation of two degradations products were detected at t_R0.32 and 0.63 min. No signs of degradation of LOP could be observed under oxidative conditions.

3.5.5 Photolytic degradation

Under UV irradiation, the UPLC chromatogram showed non-significant degradation of HCTZ and LOP. Finally, No signs of degradation of HCTZ, LOP could be observed under dry heat conditions. The chromatograms did not show any extra peaks.

3.6 Analysis of Pharmaceutical Preparations

The validated proposed method was used for the analysis of LOP and HCTZ in tablets. Five replicate determination were made and satisfactory results were obtained in agreement with the label claim, where no interference from excipients and additives was observed as shown in Table 15. The amount of LOP was found to be 50.125 mg and amount of HCTZ was found to be 12.445 mg for Sortiva H tablets (50 mg LOP+ 12.5 mg HCTZ).

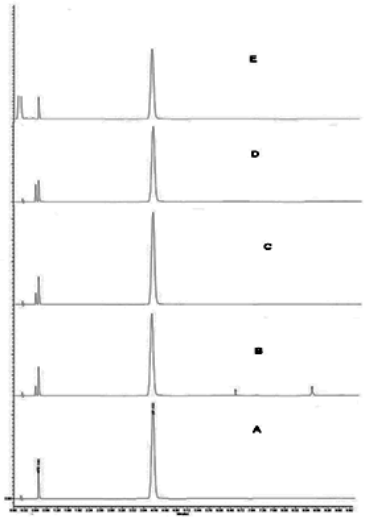


Fig. 7. Chromatograms corresponding to (A) a real sample of LOP and HCTZ; (B) a real sample of acid degradation; (C) base degradation; (D) neutral degradation and (E) oxidation degradation

Table 15. Application of the proposed UPLC method to the analysis of LOP-HCTZ mixture in pharmaceutical tablet

Drug	Pharmaceutical dosage forms	% Recovery** ± S.D.		*t*-value*	F-value*
		Proposed method (n=5)	Reported method (n=5)		
LOP	Sortiva-H®	100.25±0.582	100.51±0.829	0.5735	2.031
HCTZ		99.56±0.419	99.5±0.821	0.1310	3.832

**Tabulated values at 95 % confidence limit: t= 2.78 and F = 6.39, ** Average of 3 determinations*

4. CONCLUSION

A novel, simple and sensitive method for simultaneous determination of LOP and HCTZ has been developed and validated. To our knowledge, this is the first UPLC method for the analysis of LOP and HCTZ in pharmaceutical tablets with minimum volume of mobile phase (1.2 mL) and shortest time (about 4.5 minutes). The results of the validation studies show that the RP-UPLC method is sensitive, accurate, specific and stability-indicating. The method possesses significant linearity (R^2 not less than 0.9991) and precision, with a mean RSD of 0.47% with no interference from the excipient or degradation products. The use of experimental design and Derringer's desirability function enable fast optimization of the chromatographic parameters with minimum number of runs and short time. In this way, the presented paper is valuable for further investigation and establishment of other chromatographic methods.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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